REGULATION OF INTERFERON REGULATORY FACTOR 1 [IRF1] ANTIVIRAL FUNCTIONS BY ITS UBIQUITINATION

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Abstract

Interferon regulatory factor 1 (IRF1) is a transcriptional factor that regulates the expression of antiviral genes. IRF1 expression is downregulated in cancer cells, which supports efficient replication of oncolytic viruses. Posttranslational modifications are one of the major cellular mechanisms that regulate IRF1 expression and functions. To understand roles of IRF1 ubiquitination on innate antiviral immunity, we determined the antiviral activity of IRF1 ubiquitin resistant mutants. IRF1 ubiquitin resistant mutants (78K, 275K, and 299K) were generated and transfected into DLD-1, DU145 or MDA-MB-468 cells. The cells were then challenged with vesicular stomatitis virus (VSV) at MOI 1. Virus infection was evaluated by Western blotting against vesicular stomatitis virus glycoprotein (VSV-G), and progeny virus production. The 275K or 299K mutants showed higher antiviral activity when compared to wild type IRF1. Conversely, cells transfected with the 78K mutant were more susceptible to VSV infection than those transfected with wild type IRF1. In conclusion, 78K, 275K, and 299K sites are IRF1 ubiquitination sites which regulate IRF1 antiviral functions.

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List of Abbreviations and Symbols

6xHis: 6 poly-histidine

°C: degrees Celsius

µg: micrograms

µL: microliter

µM: micromolar

aa: amino acid

AMP: Adenosine monophosphate

ATP: Adenosine trisphosphate

CHIP: C-terminus of Hsc70-interacting protein

cIAP2: Baculoviral IAP repeat-containing protein 3

DAMP: Damage-associated molecular patterns

DBD: DNA binding domain

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DUBs: Deubiquitination enzymes

E1: Ubiquitin-activating enzyme E1

E2: Ubiquitin-conjugating enzyme E2

E3: Ubiquitin protein-ligase enzyme E3

FBS: Fetal bovine serum

g: Gravitational force

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GBP2: Guanylate-binding protein 2

HDM2: Human double minute 2 protein

HIV: Human immunodeficiency virus

IFN: Interferon

IFN-α: Interferon alpha

IFN-β: Interferon beta

IFNγ: Interferon gamma

IFNε: Interferon epsilon

IFNκ: Interferon kappa

IFNλ: Interferon lambda

IFNω: Interferon omega

IFNAR1: Interferon alpha receptor 1

IL-1: Interleukin-1

IRF1: Interferon regulatory factor 1

IRF9: Interferon regulatory factor 9

IRF-E: IFN regulatory factor element

ISG: Interferon-stimulating genes

ISRE: IFN-stimulated response element

JAK-STAT: Janus kinase-signal transducer and activator of transcription

K/Lys: Lysine

MDM2: Mouse double minute 2 protein

MEK: Mitogen activated protein kinase kinase

mL: milliliter

MOI: Multiplicity of infection

ng: nanogram

OAS2: 2'-5'-Oligoadenylate synthetase 2

PAMP: Pathogen-associated molecular patterns

PBS: Phosphate-Buffered Saline

p-ERK: phospho-ERK (phospho-extracellular receptor kinase)

pfu: plaque-forming unit

PKR: Protein kinase R

PRR: Pattern recognition receptors

R: Arginine

SDS: Sodium dodecyl sulfate

STAT1: Signal transducer and activator of transcription 1

STAT2: Signal transducer and activator of transcription 2

Tat: Transactivator of Transcription

TLR7: Toll-like Receptor 7

Ub: Ubiquitin

VSV: Vesicular stomatitis virus

Chapter 1: Introduction

1.0 Thesis overview

Living cells are a marvelous work of evolution and they are also known as the building blocks of life. Cells are made of biomolecules and among them are proteins which have a wide variety of functions such as acting as enzymes, receptors, transport molecules, and regulate gene expression (Aharoni et al., 2005; Lodish et al., 2000; and Marcotte et al., 1999). Moreover, proteins can be modified by post-translational modifications, which are biochemical modifications to one or more amino acids on a protein (Walsh et al., 2005). These modifications occur after mRNA translation and play an essential role in cellular functions (Deribe et al., 2010; and Zhao et al., 2010). The most common post-translational modifications include phosphorylation, glycosylation, acetylation, ubiquitination, and ubiquitin-like modifications (Ciechanover et al., 1980; Kouzarides et al., 2000; Ponder et al., 2007; and Rubien et al., 1975). Protein ubiquitination is known to be a key regulator of both protein stability and activity, and is involved in the regulation of several cellular pathways (Chen et al., 2003; Ciechanover et al., 1980; and Hicke et al., 2001). As a result, it is not surprising that alterations in the ubiquitination of proteins are an important factor in the development of illnesses such as cancers and neurodegenerative diseases (Alves-Rodrigues et al., 1998; and Nakayama et al., 2005). Thus, the ubiquitination pathway is an excellent target for drug development to regulate the function of specific proteins.

The focus of this thesis will be the ubiquitination of a protein known as

interferon regulatory factor 1 (IRF1). IRF1 is known to have antiviral, antitumor and immunoregulatory functions which makes it a key candidate for the development of immunotherapies (Fujita et al., 1988; Harada et al., 1993; and Tanaka et al., 1994). Specifically, I will focus on the antiviral activities of IRF1 and how they are affected by its ubiquitination. Also, since it was discovered that Ras/MEK activation downregulates IRF1 functions and subsequent expression of crucial interferon-inducible antiviral genes, it will be essential to determine if there is a connection between Ras/MEK activation and ubiquitination of IRF1 (Christian et al., 2012).

1.1 Ubiquitination

Ubiquitination of proteins was originally discovered as a reversible post-translational modification that initiates protein degradation (Ciechanover et al., 1980). Ubiquitin is a 76 amino acid (aa) protein that binds to target proteins via an isopeptide linkage between the C-terminal carboxyl group of ubiquitin and a lysine residue of the target protein (Hochstrasser et al., 1995).

Ubiquitin is activated by an enzyme called ubiquitin-activating enzyme E1 in an ATP-dependent manner. Ubiquitin then binds with the E2 ubiquitin-conjugating enzyme, which sometimes permits formation of an isopeptide bond between the target protein and the ubiquitin molecule. An E3 ubiquitin protein-ligase enzyme is required in order to form the isopeptide bond with the target protein. The E3 enzyme facilitates binding between the ubiquitin conjugated E2 enzyme and the lysine residue in the target protein (See Figure 1) (Pickart et al.,

1988; and Wilkinson K.D., 1987). For degradation of target proteins, formation of an ubiquitin oligomer is required (at least 4 ubiquitin molecules), which can be recognized by the proteasome to initiate protein degradation (Ciechanover et al., 1980; and Hochstrasser et al., 1995). Furthermore, ubiquitin molecules are removed from the target proteins via deubiquitination enzymes (DUBs). (Amerik et al., 2004; Gan-Erdene et al., 2003; Love et al., 2007; and Reyes-Turcu et al., 2009).

Figure 1. Ubiquitination pathway

Ubiquitin is activated by the E1 enzyme (Ubiquitin-activating enzyme E1) in an ATP-dependent manner. Ubiquitin binds with the E2 ubiquitin-conjugating enzyme. The E3 ubiquitin protein-ligase enzyme transfers the ubiquitin conjugated with E2 enzyme to the lysine residue of a target protein. After several rounds of this process, the target protein is conjugated with an ubiquitin oligomer which is a signal for degradation. The target protein can also be deubiquitinated by deubiquitinating enzymes. Adapted from Hershko, 1983; and Amerik, 2004; with permission.

Ubiquitination Pathway

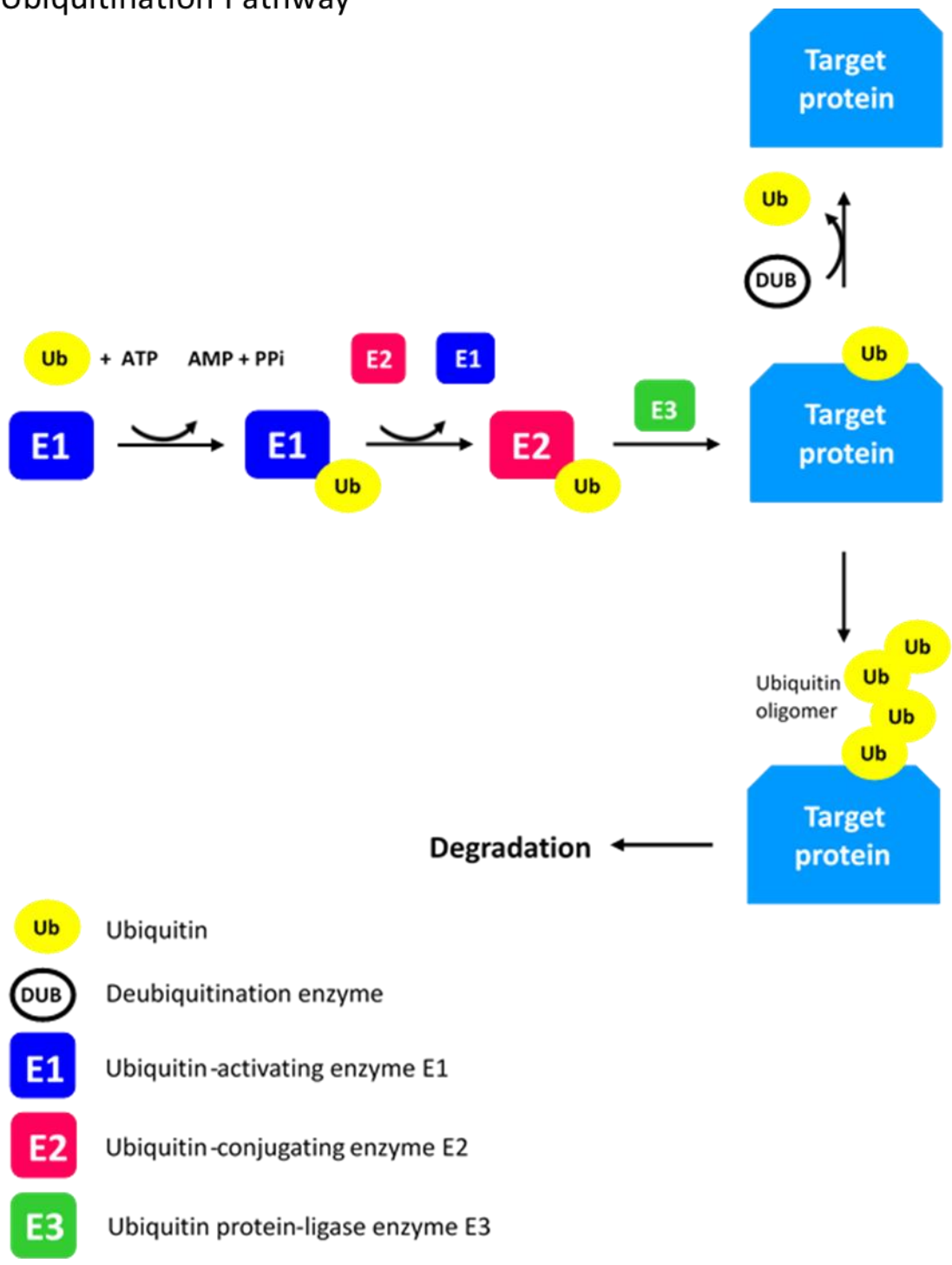

Figure 1.

There are 3 types of ubiquitination: mono-ubiquitination, poly-ubiquitination, and multi-mono-ubiquitination (See Figure 2B) (Komander et al., 2009). Mono-ubiquitination, characterized by the attachment of a single ubiquitin molecule to a lysine residue in the target protein, is known to regulate DNA repair, protein localization and functions, viral budding, and gene transcription (Chen et al., 2003; Hicke et al., 2001; Hoege et al., 2002; and Smogorzewska et al., 2007). Poly-ubiquitination, where ubiquitin molecules form an ubiquitin chain from a lysine residue in the target protein, is involved in regulating protein degradation and signal transduction (See Figure 2C) (Chau et al., 1989; Jin et al., 2001; and Sun et al., 2004). Multi-mono-ubiquitination, is the attachment of a single ubiquitin molecule to several lysine residues in a target protein, and is known to regulate receptor endocytosis (Haglund et al., 2003).

Figure 2. Different forms of ubiquitination

(A) Graphic description of in silico-predicted ubiquitination sites on IRF1 protein, made by analyzing the complete amino acid sequence of IRF1 protein in the GPS SUMO website (Zhao et al., 2014).

(B) Graphic representation of mono-ubiquitination, multi-mono-ubiquitination, and poly-ubiquitination (Komander et al., 2009).

(C) Examples of 48K and 63K linked poly-ubiquitination chain formation (Chau et al., 1989; and Jin et al., 2001)

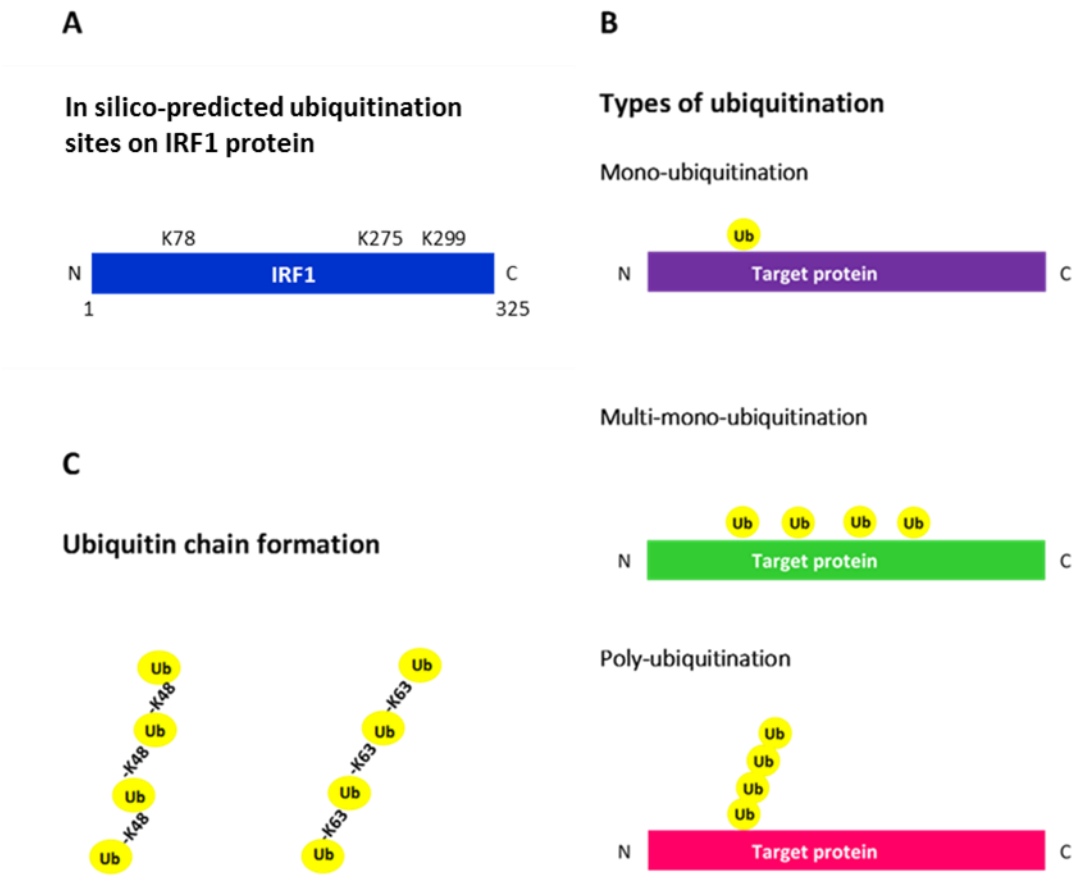
A
In silico-predicted ubiquitination sites on IRF1 protein
K78
K275
K299
N
IRF1
C
1
325
B
Types of ubiquitination
Mono-ubiquitination
Ub
N
Target protein
C
Multi-mono-ubiquitination
Ub
Ub
Ub
Ub
N
Target protein
C
Poly-ubiquitination
Ub
Ub
Ub
Ub
N
Target protein
C
C
Ubiquitin chain formation
Ub
K48
Ub
K48
Ub
K48
Ub
Ub
K63
Ub
K63
Ub
K63
Ub

Figure 2.

1.2 Ras/Raf/MEK/ERK pathway and ubiquitination

The Ras/Raf/MEK/ERK cascade reaction is an important signaling pathway, which is composed of the Rapidly accelerated fibrosarcoma (Raf) kinase, Proto-oncogene protein P21 (Ras), Mitogen-activated protein kinase/ERK kinase (MEK) 1/2, and Extracellular-signal-regulated kinases (ERK) 1/2 (Alessi et al., 1994; Hekman et al., 2002; Huang et al., 1995; and Newton et al., 2003). This pathway plays an important role in cells such as regulating cell proliferation, differentiation and apoptosis, and is activated by a variety of growth factors, chemokines, hormones, and neurotransmitters (Tamborini et al., 2010; and Zhang et al., 2002). Activating mutations of Ras isoforms (K-Ras, N-Ras or H-Ras) or its downstream elements are present in approximately 40% of all colorectal cancer tumours, 20 to 30% of all non-small cell lung cancer tumours, and 90% of pancreatic ductal adenocarcinoma (Bos, 1989; Khan et al., 2019; Mellema et al., 2015; and Prior et al., 2012).

The cascade starts by Ras recruiting and activating the serine/threonine protein kinase Raf that will activate MEK1/2. Then, MEK 1/2 will activate ERK1/2 and its activation will cause the phosphorylation of several substrates to regulate different transcription factors, and gene expression (Santarpia et al., 2012). Specifically, one of these substrates is known as Mitogen and Stress-Activated Protein Kinase 1 (MSK1). Recently, it was discovered that activation of MSK1 by the Ras/Raf/MEK/ERK cascade lead to the phosphorylation and activation of the Tripartite motif-containing 7 (Trim7) ubiquitin ligase (Chakraborty et al., 2015).

Activation of Trim7 by MSK1 induced the ubiquitination of RING domain AP-1 co-activator 1 (RACO-1), which is a transcriptional co-activator of c-Jun and stimulates the expression of genes required for cellular proliferation (Chakraborty et al., 2015; Lamph et al., 1988; Ryseck et al., 1988; and Shaulian et al., 2001). The results showed that RACO-1 was stabilized, and transcription of c-Jun was increased consequently (Chakraborty et al., 2015). To date the relationship between the Ras/Raf/MEK/ERK pathway and ubiquitination of IRF1 has not been studied, thus is an interesting mechanism to study due to the varied array of functions that IRF1 has.

1.3 Role of Interferons in innate immunity

Innate immunity is present from birth and is defined as the first line of defense against pathogens. It consists of many factors such as physical barriers, humoral and cellular components that fight against foreign microbes. Physical barriers include the skin, the gastrointestinal tract, the respiratory tract, cilia, and body hair (Abbas et al., 1994; and Matsui et al., 2015). Furthermore, humoral components such as the complement system, chemokines, and cytokines contribute to the nonspecific defense against pathogens (Dunkelberger et al., 2010; Irwin et al., 2011; and O'Shea et al., 2002). Cellular defenses involves phagocytosis mediated by macrophages, neutrophils, basophils and dendritic cells and direct cell lysis mediated by Natural-Killer cells (Lanier et al., 2005; Li et al., 2006; Medzhitov et al., 2000; and Renshaw et al., 2012). Moreover, the innate immune response is based on the detection of molecular structures that

are uniquely present on microorganisms known as pathogen-associated molecular patterns (PAMPs) which are recognized by pattern recognition receptors (PRRs) on immune cells (Akira et al., 2006; Janeway et al., 1989; and Schatz et al., 1992). In addition, PRRs recognize host molecules containing damage-associated molecular patterns (DAMPs), which are molecules that are released from dying cells damaged by pathogens (Eppensteiner et al., 2002).

The recognitions of PAMPs and DAMPs by PRRs lead to induction of inflammatory cytokines and subsequently to destruction of infected cells or microbes (Gordon et al., 2002). For example, macrophages have a variety of surface receptors, which allow them to recognize several endogenous and exogenous ligands (Kraal et al., 2000; and Taylor et al., 2005). These receptors include the toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I family, and lectins (Kraal et al., 2000; Taylor et al., 2005; and Zhang et al., 2014). PRRs stimulations induce nuclear factor-κB (NF-κB)-dependent expression of cytokines or chemokines, such as Interleukin-6 (IL-6), tumor necrosis factor (TNF), and the interferon regulatory factor (IRF)-dependent expression of Type I interferons (IFNs), which further activates adaptive immune response (Gabriele et al., 2007; Gadina et al.,2017; Kaisho et al., 2001; Prieto et al., 2019; Satoh et al., 2010; and Wakefield et al., 2010).

IFNs are one of major humoral components of innate immunity. IFNs are a family of cytokines involved in antiviral defense and are classified in three different types: Type I IFNs (IFNα, IFNβ, IFNω, IFNκ and IFNε), Type II IFNs

(IFNγ) and Type III IFN (IFNλ) (Ank et al., 2006; Benoit et al., 1993; Chen et al., 2013; Grayfer et al., 2014; Kleemann et al., 2008; Lillehoj et al., 2004; Miyamoto et al., 1988; O'Shea et al., 2002; Taguchi et al., 1991; and Xi et al., 2012). IFNs activate macrophages, induce an antiviral state in uninfected cells, and promote apoptosis of virally infected cells (Darnell et al., 1994; and Luft et al., 1998).

Type I IFNs can be produced by almost any cell upon viral infection. Their main function is to limit viral reproduction during the first days of a viral infection (Muller et al., 1994; and Miyamoto et al., 1988). Upon viral infection, cells rapidly produce IFNα and IFNβ, leading to the production of hundreds of interferon-stimulated genes (ISGs) such as guanylate binding protein 2 (GBP2), interferon stimulated gene 15 (ISG15), and interferon regulatory factor 1 (IRF1) by the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Decker et al., 1991; Smith et al., 1999; and Zuniga et al., 2007). The importance of Type I IFNs towards antiviral defense was demonstrated by deleting the type I IFN receptor (IFNAR1) in mice. The IFNAR1 deficient mice were unable to mount normal innate immune responses making them extremely susceptible to infection by several viruses such as vesicular stomatitis virus (VSV), newcastle disease virus (NDV) and vaccinia virus (VV) (Gresser et al., 1976; and Muller et al., 1994). As a result, Type I IFNs and ISGs have been established as the major components for the establishment of a host antiviral state (Decker et al., 1991; and Smith et al., 1999).

Type II IFNs are secreted only by natural-killer cells and T lymphocytes. Their main functions include antiviral defense, regulation of Major Histocompatibility Complex (MHC) expression, T cell regulation, inhibition of cell growth and apoptosis (Kleemann et al., 2008; Muller et al., 1994; and O'Shea et al., 2002). Moreover, Type III IFN is a key component of the innate immune response to intestinal or respiratory mucosal viral infections (Kotenko et al., 2003; and Robek et al., 2005). Even though Type I and Type III IFNs bind to different receptors, they both activate transcription factors signal transducer and activator of transcription 1 (STAT1), signal transducer and activator of transcription 2 (STAT2), and interferon regulatory factor 9 (IRF9), which leads to the upregulation of a large overlapping set of ISGs (Doyle et al., 2006; and Zhou et al., 2007). Furthermore, interferon regulatory factor 3 (IRF3), interferon regulatory factor 7 (IRF7), and NF-κB are essential components of type III IFN pathway as in for type I IFNs (Odendall; 2014; and Zhou et al., 2007). Since IRF1 controls the expression of antiviral, antitumor, and immunoregulatory genes, it is vital to understand the different mechanisms that regulate its expression.

1.4 Interferon regulatory factor 1

Interferon regulatory factor 1 (IRF1) was initially discovered as a transcriptional activator of the Interferon-β gene (IFN-β) (Miyamoto et al., 1988). Furthermore, IRF1 is part of a large family of transcriptional factor proteins with a variety of different functions such as transcriptional regulation of the IFN pathways, DNA repair, differentiation and regulation of immune cells, apoptosis,

and oncogene/tumor suppressors (Frontini et al., 2009; Harada et al., 1993; Heylbroeck et al., 2000; Nehyba et al., 2002; Pamment et al., 2002; Shaffer et al., 2009; Tanaka et al., 1994; and Taniguchi et al., 2001).

1.4.1 IRF1 structure and function

The IRF1 protein consists of 3 domains; the DNA binding domain (DBD), the transactivation domain, and the enhancer domain. The DBD consists of the first 120 aa of the IRF1 protein. The DBD core recognition sequence of the helix-turn-helix motif of IRF1 is 5'-GAAA-3' (Escalante et al., 1998; Fujii et al., 1999; and Harada et al., 1989). As well, the DBD is known to bind to other motifs, such as the IFN-stimulated response element (ISRE) A/GNGAAANNGAAACT and the IFN regulatory factor element (IRF-E) G(A)AAAG/CT/CGAAAG/CT/C, to activate antiviral transcription (Darnell et al., 1994; and Fujii et al., 1999). The transactivation domain of IRF1 consists of 71 aa (185 to 256 aa). This region is known to form the helix-loop-helix necessary for IRF1 transcriptional activity (Kim et al., 2003). The enhancer domain of IRF1 is found in the last 68 aa (257 to 325 aa), which stabilize the IRF1 protein (Nakagawa et al., 2000). IRF1 is known to play a key role in induction of the IFN-β gene during virus infection and, regulates expression of IFN-α and IFN-β inducible genes (Fujita et al., 1988). For antitumor functions, studies have demonstrated that IRF1 overexpression reverted transformation induced by oncogenic Ras in NIH3T3 cells (Harada et al., 1993). Furthermore, while Mouse Embryonic Fibroblasts (MEFs) cells require activation of at least 2 oncogenes for their transformation, the introduction of a single

oncogene was sufficient to transform IRF1 depleted MEFs (Tanaka et al., 1994), suggesting antitumor roles of IRF1. For immunoregulatory functions, IRF1 is known to either upregulate or downregulate certain immunoregulatory genes such as Interleukin-15 (IL-15), programed death-ligand 1 (PD-L1), Class II major histocompatibility complex transactivator (CIITA), Class I MHC, and the TNF-related apoptosis-inducing ligand (TRAIL) (Giroux et al., 2003; Lorenzi et al., 2012; Moon et al., 2017; Park et al., 2004; Ogasawara et al., 1998 and Rahat et al., 2001).

1.4.2 IRF1 ubiquitination

To date, there are 3 ubiquitin E3 conjugating ligases identified that initiate IRF1 ubiquitination; C-terminus of Hsc70-interacting protein (CHIP), human double minute 2 protein (HDM2)/ mouse double minute 2 protein (MDM2), and baculoviral IAP repeat-containing protein 3 (cIAP2) (Harikumar et al., 2014; Landre et al., 2013; Narayan et al., 2011; and Remeli et al., 2016). Poly-ubiquitination of IRF1 C-terminal region (291 to 325 a.a residues) causes degradation of IRF1 by the proteasome (Nakagawa et al., 2000). Conversely, mono-ubiquitination of IRF1 DBD (1 to 120 a.a residues) increases its transcriptional activity by enhancing the ability of IRF1 to bind to DNA and preventing degradation (Landre et al., 2013; Landre et al., 2017; and Narayan et al., 2011). IRF1 ubiquitination is a target of viral immune evasion. For example, 48K linked poly-ubiquitination induced by viral transactivator Tat targets IRF1 for degradation in order to inhibit antiviral functions of IRF1 during HIV infection

(Remoli et al., 2016). Moreover, IRF1 ubiquitination plays a role in regulation of the innate immune system. The production of chemokine ligand 5 (CCL5) and C-X-C motif chemokine 10 (CXCL10) induced by Interleukin-1 (IL-1) is mediated by 63K linked poly-ubiquitination of IRF1 (Harikumar et al., 2014). Finally, toll-like receptor 7 (TLR7) activation is known to induce 63K linked poly-ubiquitination of IRF1, which in turn halts degradation of IRF1 (Tulli et al., 2018).

1.5 Oncolytic virus therapy

Oncolytic viruses specifically infect cancer cells without damaging normal cells (Parato et al., 2005). Dr. Alice Moore was the first to demonstrate the concept of viral oncolysis using mice that were infected with Russian encephalitis virus (Moore, A, 1950). Similarly, the first clinical case of cancer remission following infection with chicken pox (varicella zoster) was reported in 1953 (Bierman et al., 1953). However, it was only in 1996 that the first clinical trial for viral therapy of cancer was approved in North America.

As cancer progresses, cancer cells accumulate several mutations that allow them to grow uncontrollably. In some cases these oncogenic mutations are the target of viral oncolysis (Hanahan et al., 2011). Oncolytic viruses can be classified into 2 different types; wild type viruses or genetically engineered viruses (Russell et al., 2012). Wild type viruses include vaccinia virus, vesicular stomatitis virus, reovirus, and senecavirus (Kirn et al., 2007; Lal et al., 2009; Reddy et al., 2007). Previously, reovirus were known to only replicate in cancer cells with an activated Ras signaling pathway, thus, specifically targeting Ras-

activated cancer cells (Coffey et al., 1998; and Hashiro et al., 1977). However, recently it was elucidated that reovirus-induced cell death in Ras-activated cancer cells is not absolute, but rather enhanced or more efficient relative to untransformed cells (Marcato et al., 2007; and Shmulevitz et al., 2005). Also, Vesicular stomatitis virus is known to exploit disruptions in the interferon response in order to establish infection in cancer cells, such as defects with protein kinase R (PKR), 2'-5'-Oligoadenylate synthetase 2 (OAS2), and interferon regulatory factors (Jha et al., 2013; Moerdyk-Schauwecker et al., 2012; Stodjl et al., 2000; and Noser et al., 2007). Moreover, genetically engineered oncolytic viruses are created by deleting or adding genes to reduce viral pathogenesis or to boost the antitumor immune response (Elsedawy et al., 2013; and Kaur et al., and 2009). One example of a genetically engineered oncolytic virus is the modified Herpes simplex Virus 1, known as Talimogene Laherparepvec (T-VEC). This virus is the first oncolytic virus approved by the U.S Food and Drug Administration (FDA) for the treatment of melanoma (Andtbacka et al., 2015; Harrington et al., 2015; and Johnson et al 2015).

1.6 Previous research in Kensuke Hirasawa lab

The Hirasawa lab has been studying how suppression of the IFN system via Ras/Raf/MEK/ERK pathway promotes viral infection and oncolysis in cancer cells. They showed that the IFN-induced antiviral response was interrupted by Ras through activation of Raf/MEK2, allowing oncolytic VSV viral replication (Battcock et al., 2006). Subsequently, they showed that in certain cancer cell lines treated with U0126 (MEK inhibitor), a specific subset of IFN-inducible genes

were upregulated (Christian et al., 2012). Moreover, these upregulated genes were mostly involved in regulation of apoptosis, antiviral response and cell differentiation. These results led to the conclusion that an activated Ras/MEK pathway suppresses transcription of these IFN inducible genes disrupting the IFN system in cancer cells (Christian et al., 2012). Furthermore, the Hirasawa lab was the first to identify IRF1 as a downstream target of the Ras/MEK pathway for the downregulation of antiviral effects induced by IFN in cancer cells (Komatsu et al., 2016). IRF1 downregulation by Ras/MEK decreases expression of a variety of IFN-stimulated genes, including the retinoic acid-inducible gene-I-like (RIG-I) receptors and GBP2, which are essential for the defense against viruses (Christian et al., 2015; and Komatsu et al., 2016). However, it still remains to be seen how Ras/MEK downregulates antiviral functions induced by IRF1.

1.7 Hypothesis and study objectives

Hypothesis: IRF1 ubiquitination regulates its antiviral functions.

Study Objectives:

1. Generate IRF1 mutants resistant to ubiquitination.
2. Determine antiviral activity of the IRF1 ubiquitination mutants.
3. Determine whether Ras/MEK modulates IRF1 ubiquitination.

Chapter 2: Materials and methods

2.1 Cell culture

Human cancer cell lines (DLD-1, DU145, and MDA-MB-468) were obtained from the American Type Culture Collection (ATCC) (see Table 2.1), and maintained with high-glucose Dulbecco's modified Eagle's medium (DMEM) (Wisent Inc.) that was supplemented with 10% Fetal Bovine Serum (FBS) (Wisent Inc.), sodium pyruvate (ThermoFisher Scientific)(100mM), and an antibiotic-antimycotic mixture (ThermoFisher Scientific) (100 units/mL penicillin G sodium). The murine cell line (L929) was also obtained from ATCC (see Table 2.1), and maintained with high-glucose DMEM (Wisent Inc.) that was supplemented with 5% FBS (Wisent Inc.) and sodium pyruvate (ThermoFisher Scientific)(100mM), and an antibiotic-antimycotic mixture (ThermoFisher Scientific) (100 units/mL penicillin G sodium).

2.2 Site-directed mutagenesis

IRF1 mRNA coding sequence. Codon sequence for Lysines 78, 275 and 299 are highlighted in red:

atgcccatcactcggatgcgcatgagaccctggctagagatgcagattaattccaaccaaatcccggggctcat
ctggattaataaagaggagatgatcttccagatcccatggaagcatgctgccaagcatggctgggacatcaaca
aggatgcctgtttgttccggagctgggccattcacacaggccgatacaaagcaggggaaaaggagccagatc
ccaagacgtggaaggccaactttcgctgtgccatgaactccctgccagatatcgaggaggtgaaagaccaga

gcaggaacaagggcagctcagctgtgcgagtgtaccggatgcttccacctctcaccaagaaccagagaaaa

gaaagaaagtcgaagtccagccgagatgctaagagcaaggccaagaggaagtcatgtggggattccagcc

ctgataccttctctgatggactcagcagctccactctgcctgatgaccacagcagctacacagttccaggctacat

gcaggacttggaggtggagcaggccctgactccagcactgtcgccatgtgctgtcagcagcactctccccgact

ggcacatcccagtggaagttgtgccggacagcaccagtgatctgtacaacttccaggtgtcacccatgccctcc

acctctgaagctacaacagatgaggatgaggaagggaaattacctgaggacatcatgaagctcttggagcagt

cggagtggcagccaacaaacgtggatgggaaggggtacctactcaatgaacctggagtccagcccacctctg

tctatggagactttagctgtaaggaggagccagaaattgacagcccaggggggggatattgggctgagtctacag

cgtgtcttcacagatctgaagaacatggatgccacctggctggacagcctgctgaccccagtccggttgccctcc

atccaggccattccctgtgcaccgtag

Site-directed mutagenesis was conducted using the QuickChange Lightning Mutagenesis Kit (Agilent), following the manufacturer's instructions. Primers used for site-directed mutagenesis are listed in Table 2.2.1. PCR reaction mix for site-directed mutagenesis was as described in Table 2.2.2, and thermal cycling conditions as described in Table 2.2.3.

Table 2.1: Sources and origins of human and murine cancer cell lines

Cell line	ATTC identifier	Tissue	Cell type	Cells/well (24 Wells plate)
DLD-1	CCL-221	Colon	Adenocarcinoma	$1x10^5$
DU145	HTB-81	Prostate	Carcinoma	$1x10^5$
L929	CCL-1	Subcutaneous connective tissue, areolar and adipose	Fibroblast	$2x10^6$
MDA-MB-468	HTB-132	Breast	Adenocarcinoma	$2x10^6$

Table 2.2.1: Primers for site-directed mutagenesis (with the site of mutation in red)

Mutation	Primers
Human IRF1 K78R	F: 5'-gccagatcccaagacgtggagggccaactttc-3'
	R: 3'-cggtctagggttctgcacctcccggttgaaag-5'
Human IRF1 K275R	F: 5'-ctatggagactttagctgggaggagccagaaattg-3'
	R: 3'-gatacctctgaaatcgacatccctcctcggtctttaac-5'
Human IRF1 K299R	F: 5'-cagcgtgtcttcacagatctgaggaacatggatg-3'
	R: 3'-gtcgcacagaagtgtctagactccttgtacctac-5'

Table 2.2.2: PCR reaction mix for site-directed mutagenesis

Reagent	Volume
10x QuickChange Lightning buffer	5 µl
dNTP mix	1 µl
Quick solution reagent	1.5 µl
Forward Primer	1.038 µl (125ng)
Reverse Primer	1.081 µl (125ng)
UltraPure DNase/RNase-free dH_2O	39.91 µl
dsDNA template	0.47 µl (100ng)
QuickChange Lightning Enzyme	1 µl

Table 2.2.3: Thermal cycling conditions for site-directed mutagenesis

Temperature	Time	Cycles
95°C	2 minutes	1
95°C	20 seconds	18
60°C	10 seconds	
68°C	4 minutes	
68°C	5 minutes	1
4°C	Hold	N/A

2.3 His-tag purification assay

2.3.1 Cell culture and transfection

DU145 cells ($3x10^6$ cells per dish) were grown in 10 cm dishes to 90% confluency, then co-transfected with either 5 µg of pcDNA3.1 hIRF1 wild type, 5 µg of pcDNA3.1 hIRF1 K78R, 5 µg of pcDNA3.1 hIRF1 K275R, or 5 µg of pcDNA3.1 hIRF1 K299R, and 5 µg of pcDNA3.1 His ubiquitin using 15 µl of lipofectamine 3000 reagent (Invitrogen). At 4 hours post transfection, the media was removed, and 10 ml of complete DMEM was added. At 24 hours after transfection, cells were treated with 20 µM U0126 or DMSO (Sigma Life Science) for 6 hrs as well as the addition of 25 µM MG132 (proteasome inhibitor) 2 hours prior lysis. Cells were washed twice with ice-cold Phosphate-Buffered Saline (PBS), then scraped from plates and transferred to 15 ml centrifuge tubes. The tubes were centrifuged at 289*g* for 5 minutes at 4 °C. The supernatant was removed using a Pasteur pipette, and cells were re-suspended in 10 ml of ice-cold PBS. Then 1 ml of the cell suspension was transferred to a 1.5 ml microcentrifuge tube. While the 15 ml centrifuge tubes were on ice, the microcentrifuge tubes were centrifuged at 835*g* for 2 minutes at 4 °C, the supernatant was discarded and 100 ul of 2x Laemli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris-Cl pH 6.8, 10% 2-mercaptoethanol) was added to each tube and then frozen at - 80°C (Total cell lysate samples). Then the 15 ml tubes were centrifuged at 289*g* for 5 minutes at 4 °C, after which the supernatant was removed, and cells were re-suspended in

3.5 ml of buffer 1 (pH8.0, 6M Guanidinium Chloride, tris, 0.5M Na2HPO4, 0.5M NaH2PO4, 20μM N-ethylmaleimide) and frozen at - 80°C (His pull-down samples).

2.3.2 His-tag pull-down

Day 1: After the His pull-down samples were defrosted, 1.25 μl of β-mercaptoethanol (Sigma Life Science) and 0.5 M of imidazole (Sigma Life Science) were added to each sample. Then 4 ml of buffer 1 (see Table 2.3 for buffer content) were added in a clean 15 ml centrifuge tube with 50 μl of Ni-NTA beads (QIAGEN) and incubated at 4°C in a shaker for 1 hour. While the beads were in the shaker, the samples were sonicated at an amplitude of 30 for 30 seconds (10 seconds on, and 20 seconds off). Subsequently, samples were centrifuged at 1485*g* for 15 minutes at room temperature. The tubes with the beads were centrifuged at 2320*g* for 2 minutes at room temperature, after which the supernatant was discarded, and then samples were poured in the tubes with beads and incubated at 4°C overnight in a shaker.

Day 2: The next day the samples were spun down at 2320*g* for 2 minutes, the supernatant was discarded and 4 ml of buffer 1 was added, then the centrifugation step was repeated. The supernatant was discarded and 4 ml of buffer 2 (pH8.0) was added (see Table 2.3 for buffer content), then the centrifugation step was repeated. The supernatant was discarded and 4 ml of buffer 3 (pH6.3) was added (see Table 2.3 for buffer content), and the centrifugation step was repeated 2 more times. The supernatant was discarded and the samples were transferred to a 1.5 ml microcentrifuge tube with 1 ml of

fresh buffer 3. Tubes were then centrifuged at 5939*g* for 1 minute at room temperature, the supernatant was discarded and 1 ml of buffer 3 was added this and step was repeated 3 times. The supernatant was discarded and 60 µl of elution buffer was added and samples were incubated at room temperature for 20 minutes. Finally, samples were boiled for 2 minutes and stored at -20°C.

Table 2.3: Buffer components for Pull-down.

Buffer	Reagent	Grams/Volume
Buffer 1	Guanidinium-HCL	17.19 g
	Tris	0.036 g
	0.5M Na_2HPO_4	5.59 ml
	0.5M NaH_2PO_4	0.4 ml
Buffer 2	Urea	240.21 g
	Tris	0.61 g
	0.5M Na_2HPO_4	93.2 ml
	0.5M NaH_2PO_4	6.8 ml
	14.3M β-mercaptoethanol	175 µl
Buffer 3	Urea	240.21 g
	Tris	0.61 g
	0.5M Na_2HPO_4	25.5 ml
	0.5M NaH_2PO_4	74.5 ml
	14.3M β-mercaptoethanol	175 µl

2.4 Virus and infection

Vesicular stomatitis virus (VSV) was used to infect cells at MOI 1. DU145, DLD1, and MDA-MB-468 cells were grown in 24 well plates until 90% confluency, and transfected with 0.5 µg of either pcDNA3.1 hIRF1 wild type, pcDNA3.1 hIRF1 K78R, pcDNA3.1 hIRF1 K275R, or pcDNA3.1 hIRF1 K299R. At 24 hours after transfection cells were split (1:2 dilution). When cells reached 80% to 90% confluency cells were infected with VSV and lysed 24 hours later.

2.5 Plaque Assay

L929 cells ($2x10^5$ cells per well) were plated in 12 well plates. Then 2 days later, cells were infected in duplicate with different virus concentrations from the supernatant of infected cells ($1x10^{-1}$, $1x10^{-2}$, $1x10^{-3}$, $1x10^{-4}$, $1x10^{-5}$, and $1x10^{-6}$) for 30 minutes at 37°C in a 5% CO_2 humidified atmosphere. The plate was shaken three times during the incubation period. Then 1 ml of agar (pre-heated at 42°C) was added to each well, and the plates were incubated at 37°C in a 5% CO_2 humidified atmosphere overnight. The next day 1 ml of Neutral red (Sigma Life Science) in agar was added to each well and plaques were counted 8 hours after incubation at 37°C. After optimal dilution concentration was verified, new cells were infected in triplicates, and viral titer was calculated in plaque-forming units per ml (pfu/ml).

2.6 Transient transfection of DNA

DNA was transfected using 0.5 µg or 5 µg of DNA when indicated, and Lipofectamine 3000 (Invitrogen) reagent following the manufacturer's instructions.

2.7 Western blot analysis

A 10% SDS gel was prepared using 3 ml of autoclaved dH_2O, 1.5 M Tris-HCl (Invitrogen), 30% Acrylamide (Bio-Rad), 70 µl 10% SDS (Invitrogen), 70µl 10% APS (Invitrogen), and 3µl Temed (Invitrogen). 15 µl of samples were loaded, and electrophoresis was performed at 120V for 1 hour and 20 minutes (or 20 mA for 50 minutes). The gel was then transferred to a nitrocellulose membrane (Bio-Rad) using the Trans-Blot Turbo Transfer system (Bio-Rad) (7 min, 25 V and 2.5 A) or in a tank at 100 V for 1 hour. After the transfer, membrane was blocked in 5% skim milk for 2 hours at room temperature and incubated overnight with primary antibody at 4°C in a shaker. The next day the membrane was washed 3 times for 10 mins with TBS-T, and incubated with the secondary antibody (peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Santa Cruz)). After 1 hour at room temperature the membrane was washed again, 3 times for 10 mins with TBS-T.

ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Baie d'Urfe, QC) was used to image the membrane. Antibody to human IRF1 (#612046) was purchased from BD Transduction Laboratories (Mississauga, ON), VSV-G (VSVII-M) from Alpha Diagnostic (San Antonio, TX), GAPDH (6C5) from Abcam (Toronto, ON), phospho-ERK-1/2 (#9101) from Calbiochem, and 6xHis (sc-8036) from Santa Cruz Biotechnology (Dallas, TX).Specific conditions for the antibodies are as denoted in Table 2.7.

2.8 Statistical analysis

Results were analyzed by one-way ANOVA with Tukey's post-hoc test, $p<0.01$, using Graph Pad Prism 4.0c software (GraphPad Software, La Jolla, California).

Table 2.7: Primary and secondary antibody conditions for western blot.

Antibody	1ry Antibody condition	2ry Antibody condition	Band weight
IRF-1	1:2000 in TBS-T	1:5000 Anti-mouse IgG in 5% TBS-T milk	48 kDa
VSV-G	1:10000 in 5% TBS-T milk	1:5000 Anti-mouse IgG in 5% TBS-T milk	60 kDa
GAPDH	1:60000 in TBS-T	1:5000 Anti-mouse IgG in TBS-T	37 kDa
p-ERK	1:1000 in TBS-T	1:5000 Anti-rabbit IgG in 5% TBS-T milk	42, 44 kDa
6xHis	1:5000 in 5% TBS-T milk	1:5000 Anti-mouse IgG in 5% TBS-T milk	N/A[1]

1 Not applicable

Chapter 3: Results

3.1 Effects of IRF1 ubiquitination on antiviral functions.

Ubiquitination regulates different cellular processes by modulating protein stabilities and transcriptional activities. In order to determine how ubiquitination affects antiviral functions of IRF1, IRF1 mutants resistant to ubiquitination were generated. First, I conducted a literature search and *in silico* analysis. I identified IRF1 ubiquitination sites (Lysines 78, 275 and 299) potentially involved in the regulation of antiviral functions. Second, the predicted Lysine sites were converted to Arginine in order to prevent the binding of ubiquitin molecules from generated IRF1 mutants (K78R, K275R, and K299R). To determine if these mutations affect IRF1 antiviral functions, they were transfected in different cancer cell lines (DLD-1, DU145 or MDA-MB-468).The cells were then infected for 24 hours with VSV at MOI of 1. At time 0 (before VSV infection), IRF1 transfection was confirmed by Western blot analysis. IRF1 expression in cells transfected with wild type IRF1 or IRF1 mutants was higher than those transfected with control vector, suggesting that all IRF1 constructs were successfully transfected (Figure 3). At 24 hours post VSV infection, I found that the cells transfected with K78R mutant were more susceptible to viral infection, compared to wild type IRF1, in all three cell lines tested. Conversely, the cells transfected with K275R or K299R mutant were more resistant to viral infection, compared to those transfected with wild type IRF1, when tested in DLD-1 cells (Figure 3A). It should be noted however that DU145 and MDA-MB-468 cells transfected with K275R or K299R

mutant showed similar levels of susceptibility to VSV infection to those transfected with wild type IRF1 (Figure 3B, C)

Figure 3. Antiviral functions of IRF1 mutants: K78R, K275R, and K299R. (A) DLD-1, **(B)** DU145 and **(C)** MDA-MB-468 human cancer cells were transfected with pcDNA3.1 (Control vector), pcDNA3.1 WT IRF1, pcDNA3.1 IRF1 K78R, pcDNA3.1 IRF1 K275R, or pcDNA3.1 IRF1 K299R, and then infected with vesicular stomatitis virus (VSV) for 24 hours at MOI=1. The expression levels of IRF1, VSV-G and GAPDH were determined by Western blot analyses (n=3, 3 independent experiments).

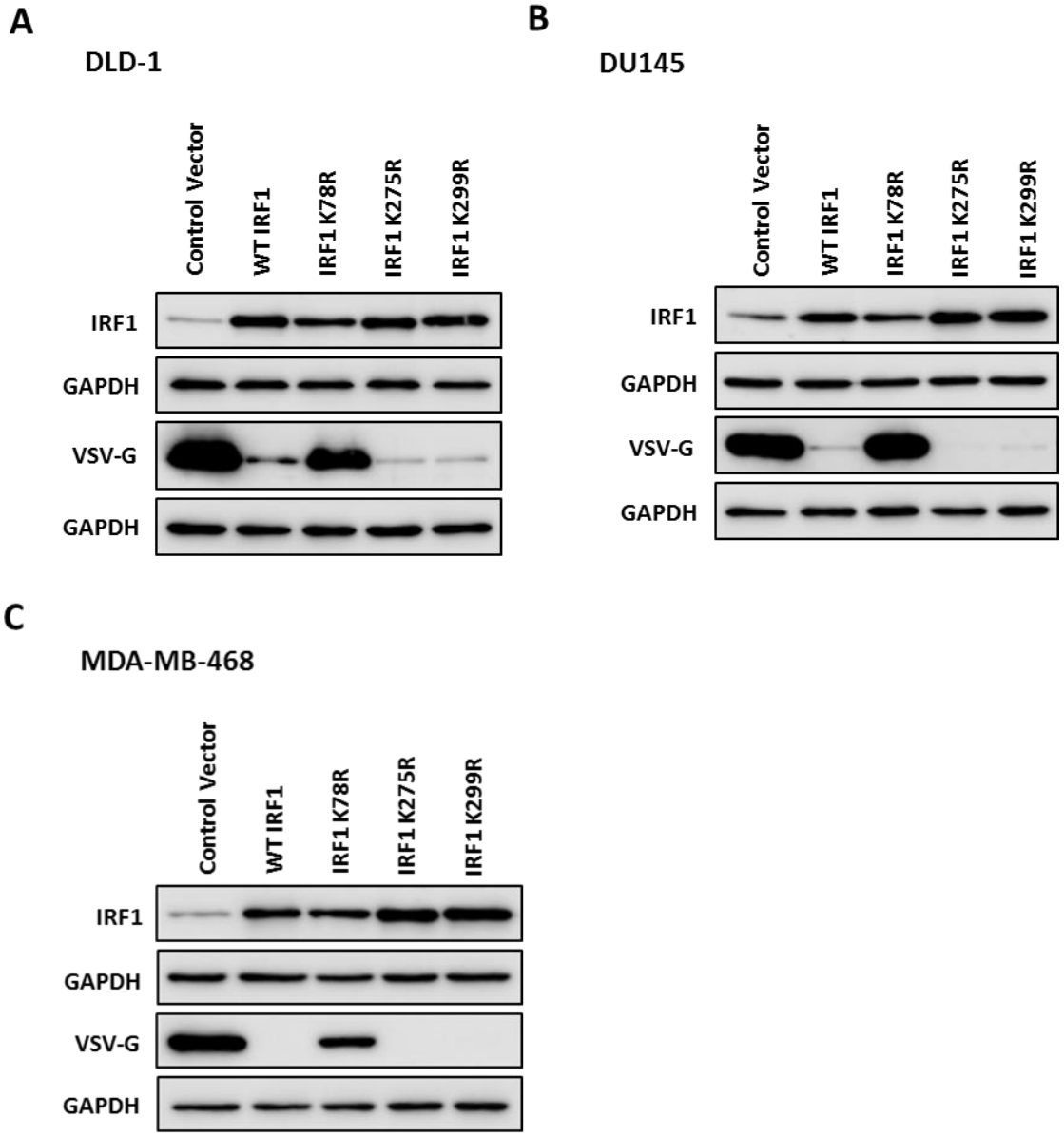
A
DLD-1
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
IRF1
GAPDH
VSV-G
GAPDH
B
DU145
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
IRF1
GAPDH
VSV-G
GAPDH
C
MDA-MB-468
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
IRF1
GAPDH
VSV-G
GAPDH

Figure 3.

3.2 K78R, K275R, and K299R sites affect IRF1 antiviral functions

In order to verify results of IRF1 mutant antiviral activities as shown by Western blot analyses, the progeny virus assay was performed. DLD-1, DU145 and MDA-MB-468 cells were transfected with control vector, wild type IRF1, IRF1 K78R, IRF1 K275R or IRF1 K299R mutant, and then challenged with VSV. The amounts of progeny viruses in the supernatant were determined at 24 hours post infection. In all cell lines, the IRF1 K78R mutant showed a significantly higher viral titer compared to wild type IRF1 (Figure 4. A, B, and C). In addition, the production of progeny viruses was significantly lower in DLD-1 cells transfected with K229R (Figure 4. A), and DU145 cells transfected with K275R mutant (Figure 4. B) compared to the counterparts transfected with wild type IRF1, respectively. However, in MDA-MB-468 cells, there was no significant difference in antiviral activities between wild type IRF1 and mutants K275R or K299R (Figure 4. C). These results demonstrate that K78 ubiquitination is essential for antiviral functions of IRF1 while K275 or K299 ubiquitination of IRF1 possibly reduces host antiviral defense.

Figure 4. K78R, K275R, and K299R sites affect IRF1 antiviral functions. (A) DLD-1, (B) DU145, and (C) MDA-MB-468 were transfected with pcDNA3.1 (Control vector), pcDNA3.1 WT IRF1, pcDNA3.1 IRF1 K78R, pcDNA3.1 IRF1 K275R, or pcDNA3.1 IRF1 K299R construct, and then challenged with VSV for 24 hours at MOI=1. The amount of progeny virus in the supernatant was measured by plaque assay. (n=3, triplicates of 3 independent experiments * $P < 0.01$ by one-way ANOVA with Tukey's post-hoc test).

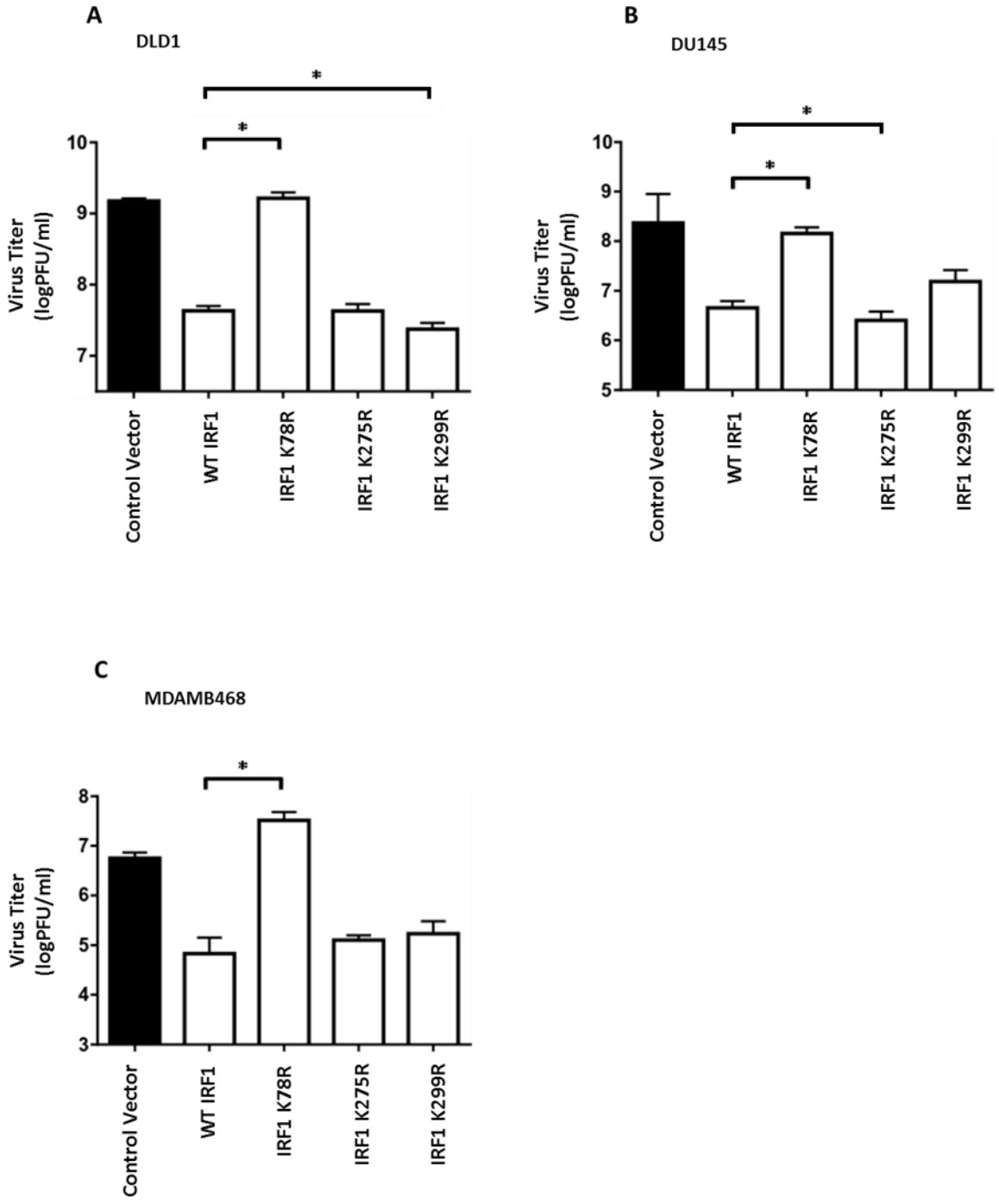
A
DLD1
Virus Titer (logPFU/ml)
10
9
8
7
*
*
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
B
DU145
Virus Titer (logPFU/ml)
10
9
8
7
6
5
*
*
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
C
MDAMB468
Virus Titer (logPFU/ml)
8
7
6
5
4
3
*
Control Vector
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R

Figure 4.

3.3 Ubiquitination status of wild type IRF1 and IRF1 mutants

Pull-down assays of ubiquitinated proteins was conducted to confirm if mutations did indeed block the binding of ubiquitin to IRF1. Wild type IRF1, or mutants (K78R, K275R, or K299R) were transfected with His tag ubiquitin into DU145 cells, and 24 hours later, the His-tag proteins were subjected to pull-down using Ni-NTA agarose beads. The pull-down samples were analyzed by Western blot using an antibody against IRF1. The results showed that IRF1 ubiquitination (both poly- and mono-ubiquitination) was reduced in cells transfected with IRF1 K275R or K299R mutant. Furthermore, IRF1 ubiquitination was reduced in those transfected with K78R mutant, compared to those transfected with wild type IRF1. Total cell lysate (TCL) was blotted with anti-IRF1 antibody to confirm that IRF1 constructs were successfully transfected (Figure 5). These results confirm that K78R, K275R, and K299R are ubiquitination sites of IRF1, and their mutations reduce IRF1 ubiquitination.

Figure 5. Confirmation of IRF1 ubiquitination sites. DU145 human cancer cells co-transfected with wild type IRF1, IRF1 mutants (K78R, K275R, or K299R), and/or a His-tagged ubiquitin protein. Cells were treated with MG132 for 2 hours before collection. His-tagged proteins were subjected to pull-down using Ni-NTA beads. Protein levels of IRF1 were determined by IRF1 Western blot analysis (n=3, 3 independent experiments).

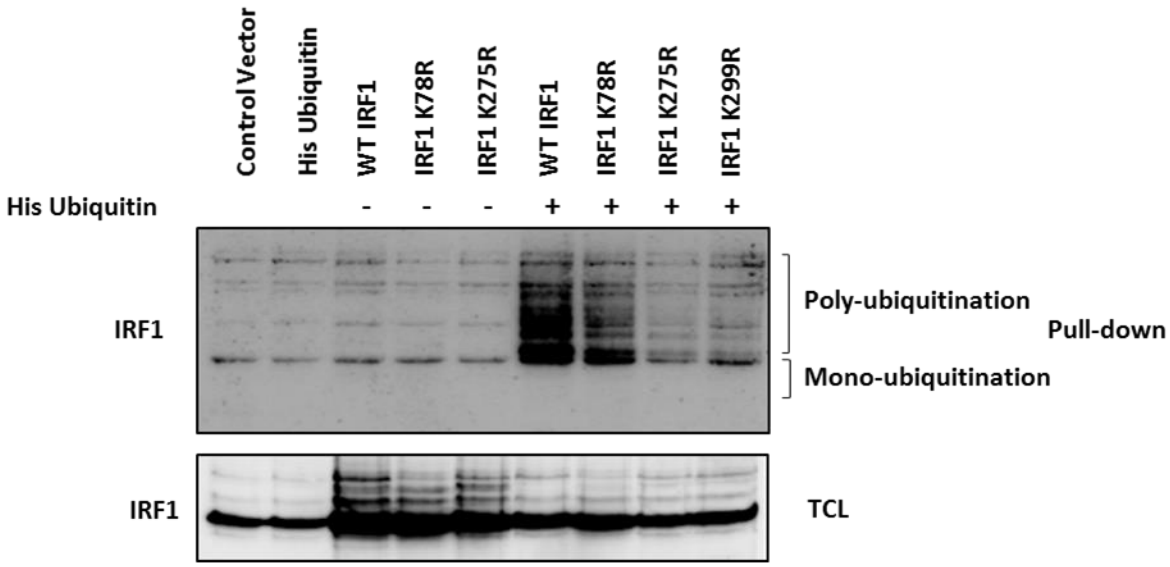
Control Vector
His Ubiquitin
WT IRF1
IRF1 K78R
IRF1 K275R
WT IRF1
IRF1 K78R
IRF1 K275R
IRF1 K299R
His Ubiquitin
- - - + + + +
IRF1
Poly-ubiquitination
Pull-down
Mono-ubiquitination
IRF1
TCL

Figure 5.

3.4 Downregulation of RAS/MEK pathway affects IRF1 ubiquitination

In order to determine if the MEK pathway regulates IRF1 ubiquitination, DU145 cells were transfected with wild type IRF1, IRF1 K78R, and/or His-tagged ubiquitin plasmid, and then treated with U0126 (MEK inhibitor) for 6 hours. IRF1 ubiquitination was determined by His-pull down assay as described above. When the cells transfected with wild type IRF1 and His-ubiquitin plasmid were treated with U0126, IRF1 ubiquitination was reduced compared to those treated with control vehicle (DMSO) (Figure 6). However, effects of MEK inhibition were not evident in cells transfected with mutant K78R and His-ubiquitin plasmid, as the ubiquitination of K78R IRF1 was low. Total cell lysates were blotted with the antibody against phosphorylated-ERK to verify that the inhibitor suppresses the MEK pathway. Furthermore, Western blotting analysis against His or IRF1 indicates that the plasmids were equally transfected into the cells. These results suggest that activated Ras/MEK pathway promotes IRF1 ubiquitination in cancer cells.

Figure 6. Downregulation of the RAS/MEK pathway reduces IRF1 ubiquitination. DU145 cells transfected with wild type IRF1, K78R IRF1 mutant, and/or His-tagged ubiquitin plasmid were treated with the MEK inhibitor (U0126 (20 μM)), or vehicle control (DMSO) for 6 hours. His-tagged proteins were purified using Ni-NTA Agarose and blotted with anti-IRF1 antibody. The levels of IRF1, 6xHis, and phosphorylated-ERK (p-ERK) in total cell lysates (TCL) were determined by Western blot analysis (n=3, 3 independent experiments).

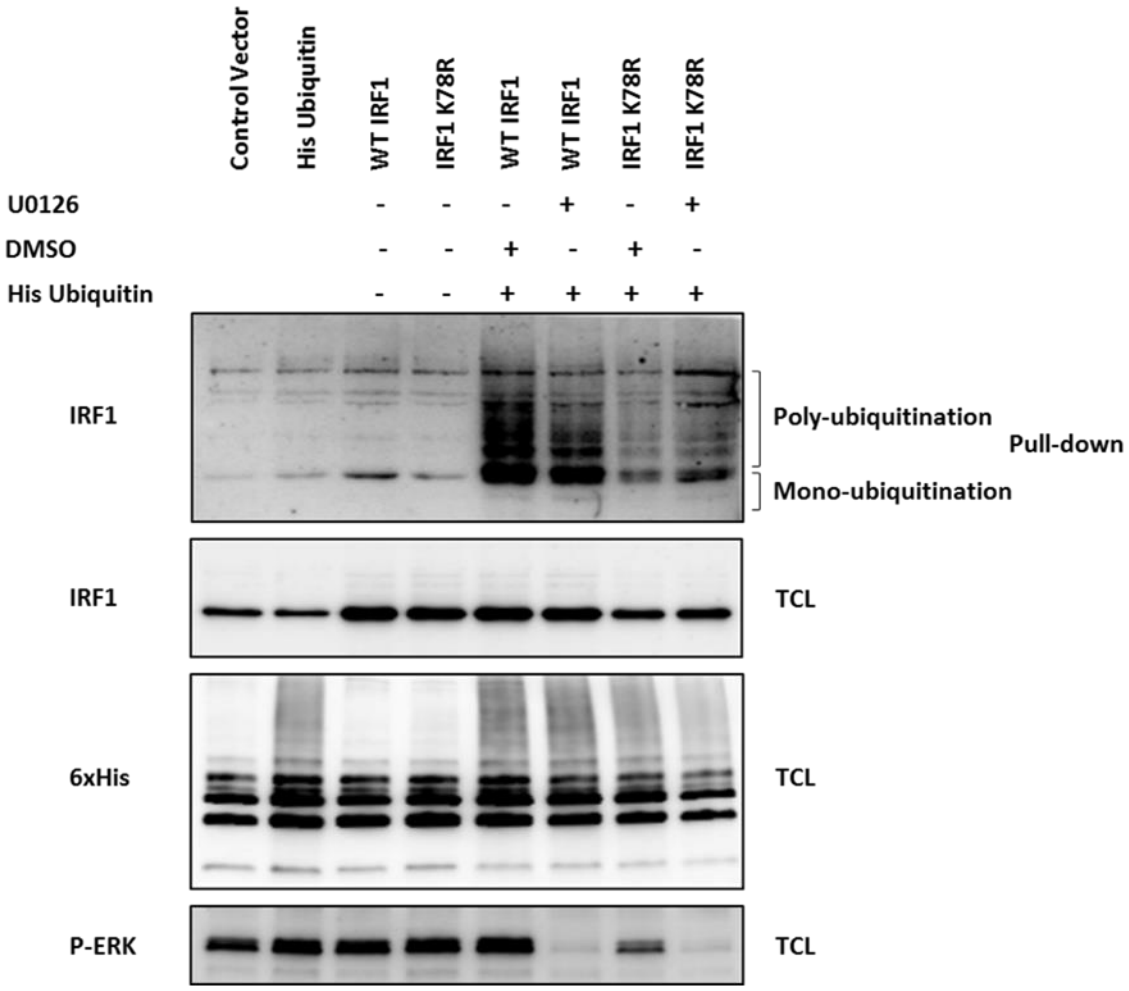
Control Vector
His Ubiquitin
WT IRF1
IRF1 K78R
WT IRF1
WT IRF1
IRF1 K78R
IRF1 K78R
U0126 - - - + - +
DMSO - - + - + -
His Ubiquitin - - + + + +
IRF1
Poly-ubiquitination
Pull-down
Mono-ubiquitination
IRF1
TCL
6xHis
TCL
P-ERK
TCL

Figure 6.

Chapter 4: Discussion

IRF1 is a transcriptional regulator that controls expression of antiviral genes. IRF1 is induced during viral infection through IFN responses. While there are innate antiviral mechanisms to protect hosts from virus infection in normal cells, some of these mechanisms malfunction in cancer cells (Stojdl, et al., 2000). Oncolytic viruses, which replicate only in cancer cells but not in normal cells, exploit these defects of host innate responses specific to cancer cells, and infect and lyse them (Farassati et al., 2001; Stojdl et al., 2003; and Strong et al., 1998). Low expression of IRF1 is one of the cancer-specific defects, which was identified in the Hirasawa laboratory (Komatsu, et al., 2015). It is essential to understand how IRF1 is downregulated in cancer cells, as it will help to further improve efficacy of oncolytic therapies. In the present study, I sought to determine if ubiquitination of IRF1 affects its antiviral functions. Ubiquitination has been known to target proteins for degradation, but recent studies show that it is involved in regulating gene transcription, DNA repair, interaction, and localization of proteins (Chen et al., 2003; Hicke et al., 2001; Hochstrasser et al., 2009; Kattah et al., 2017; Kirkin et al., 2011; Martín-Vicente et al., 2017; and Popovic et al., 2014). To determine how ubiquitination regulates antiviral functions of IRF1, IRF1 mutants were generated by targeting predicted ubiquitination sites (K78, K275, and K299). As a result, I found that the K78 ubiquitination site plays critical roles in promoting antiviral activities of IRF1. The IRF1 K78 is a mono-ubiquitination site that facilitates binding of IRF1 to DNA (Landre et al., 2017).

Moreover, in a DNA bound state, IRF1 is protected from protein degradation. Here we demonstrate that ubiquitination of the K78 site is essential for transcriptional activation of antiviral genes, as its mutation reduces antiviral ability of IRF1. On the contrary, mutations to K275 and K299 sites led to higher resistance against the viral infection when compared to wild type IRF1 in certain cancer cell lines. The C-terminal region of IRF1, which includes residues from the 291 to the 325 a.a, has been indicated to be the target of poly-ubiquitination, leading to IRF1 degradation (Nakagawa et al., 2000). To support this, the expression levels of IRF1 K275R and K299R mutants were higher than that of wild type IRF1 (Figure 3 B and C), indicating that the mutations protect IRF1 from degradation and increase its stability. The protection of IRF1 from K275 or K299-mediated ubiquitination resulted in promotion of antiviral responses in DU145 and MDAMB468 cells, respectively (Figure 4 B and C). Overall, these results demonstrate that ubiquitination on K78 IRF1 promotes host defense against virus infection while ubiquitination on K275 or K299 reduces IRF1 stability and antiviral activities.

In order to determine if the mutations change ubiquitination status of IRF1, the pull-down assay was carried out (Figure 5). The results showed that ubiquitination of IRF1 K275 and K299 mutants was significantly reduced compared to wild type IRF1 when they were mutated to Arginine. Similarly, mutation of IRF1 K78 site showed a reduction in ubiquitination, which was less compared to the mutations of IRF1 K275 and K299 sites. This could be due to the fact that IRF1 K78 is a mono-ubiquitination site, while IRF1 K275 and K299

are poly-ubiquitination sites. This should be further confirmed by using pulldown assay specific to mono- or poly-ubiquitination antibodies. In summary, IRF1 ubiquitination was inhibited in the mutant IRF1 constructs, confirming that K78, K275 and K299 are IRF1 ubiquitination sites.

Our laboratory previously found that Ras/MEK activation downregulated antiviral functions of IRF1 (Komatsu et al., 2015). To further investigate whether Ras/MEK targets IRF1 ubiquitination for regulation, I analyzed whether MEK inhibitor treatment modulates IRF1 ubiquitination. I found that ubiquitination of wild type IRF1 was considerably reduced in cells treated with U0126, compared to those treated with vehicle control (DMSO). However, the opposite results were observed in experiments using the IRF1 K78 mutant, where U0126 treatment increased the ubiquitination.

Although results may be difficult to interpret, they at least indicate that Ras/MEK activity modulates ubiquitination status of IRF1. It should be noted that Ras/MEK may regulate mono-ubiquitination mediated by K78 and poly-ubiquitination mediated by K275 and K299 differently. Therefore, for future studies to make conclusions pertaining to the interaction of IRF1 ubiquitination and Ras/MEK, it is necessary to establish an assay system which could distinguish mono- and poly-ubiquitination of IRF1.

Chapter 5: Future directions

In this research, I determined that IRF1 lysine K78 is an ubiquitination site modulated by Ras/MEK. However, the type of ubiquitination linkage remains to be determined. It will be essential to understand their ubiquitination linkage-type since a K48 linkage targets proteins for degradation, but a K63 linkage stabilizes the proteins. I propose to use ubiquitin lysine mutants. First, residues K48 or K63 on the ubiquitin molecule should be mutated to arginine residues. Then if the K48 mutant does not form a chain, it will indicate that there is a K48 linkage happening.

The IRF1 protein is known for its antiviral, antitumor and immunoregulatory functions. The focus of this thesis was on the antiviral activities of IRF1; nevertheless, it can be speculated that similar results could be obtained in regards to its antitumor and immunoregulatory functions. Using techniques such as cell-growth assay and qRT-PCR analyses could provide further understanding of the effects of Ras/MEK induced ubiquitination on IRF1 antitumor and immunoregulatory functions.

Chapter 6: Conclusion

Ubiquitination of IRF1 K78 is essential for transcriptional activation of antiviral genes, as its mutation showed to reduce the antiviral ability of IRF1. K275 and K299 mutations led to higher resistance against the viral infection in certain cell lines which suggest that ubiquitination of these sites leads to a reduction in antiviral transcription. In conclusion, K78 and K275/K299 are ubiquitination sites responsible for regulating IRF1 antiviral functions by respectively increasing or decreasing its antiviral functions. Ras/MEK is also involved in the regulation of IRF1 ubiquitination. These results suggest that oncogenic Ras/MEK may increase cellular sensitivity to oncolytic virus through IRF1 ubiquitination in cancer cells.

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