REGULATION OF INTERFERON REGULATORY FACTOR 1 BY PROTEIN INHIBITOR OF ACTIVATED STAT3

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A thesis submitted to the School of Graduate Studies in partial fulfillment of

the requirements for the degree of

Master of Science in Medicine

(Cancer and Development)

BioMedical Sciences

Faculty of Medicine

October 2020

Memorial University of Newfoundland

St. John's Newfoundland and Labrador

Abstract

Oncolytic viruses exploit tumor-specific cellular changes for selective replication, inducing cancer cell death. Our previous research showed that Ras/mitogen-activated protein kinase kinase (MEK) downregulation of interferon regulatory factor 1 (IRF1) is a major mechanism underlying viral oncolysis. Protein inhibitor of activated STAT 3 (PIAS3) is known as the E3 ligase of IRF1 sumoylation. The objective of this study was to identify if, and how, PIAS3 modulates cellular sensitivity to oncolytic viruses via regulation of IRF1. By conducting co-immunoprecipitation, I found that IRF1 has no direct interaction with PIAS3 found within HT1080 cells. However, CRISPR knockdown of PIAS3 increases IRF1 expression as well as transcription of IRF1-responsive anti-viral genes. Furthermore, PIAS3 knockdown HT1080 cells were equally sensitive to viral infection as their parent HT1080 cells. Together, these results demonstrate that PIAS3 does not directly interact with IRF1 but regulates expression and transcriptional activity of IRF1. Moreover, as CRISPR knockdown of PIAS3 did not change cellular sensitivity to viral infection, IRF1 modulation via PIAS3 does not play very critical roles in host innate antiviral responses.

General Summary

Oncolytic viruses selectively kill cancer cells but not normal cells. Our previous research has shown that the expression of antiviral protein, interferon regulatory factor 1 (IRF1), is suppressed in cancer cells, which increases their susceptibility to oncolytic virus infection. The objective of my MSc project was to determine if and how protein inhibitor of activated STAT 3 (PIAS3), a known modulator of IRF1, suppresses antiviral functions of IRF1 in cancer cells. I found that PIAS3 does not directly interact with IRF1 but regulates the expression and transcriptional activity of IRF1 in cancer cells. However, IRF1 modulation via PIAS3 is not sufficient to change cellular susceptibility to virus infection. These results suggest that PIAS3 is not involved in cellular mechanisms of viral oncolysis while PIAS3 modulates IRF1 to a certain extent.

Co-authorship Statement

In this thesis, most work was done by the author of the thesis Danyang Xu. The CRISPR knockdown clones were generated by Ysabel Meneses, another graduate student of the Hirasawa lab, which is described in Chapter 3 part 3. All the other experiments in the thesis were conducted by Danyang Xu.

Acknowledgements

First and foremost, I would like to show the deepest gratitude to my supervisor Dr. Kensuke Hirasawa for his patient guidance and valuable suggestions. I am grateful for all the positive energy that he delivered me while training me to be a future researcher. This experience has been precious to me not just for my research endeavors but my future life. He is always eager to exchange ideas with students, as well as to provide me all the assistance he could offer while I completed this thesis. He has always worked hard to make our laboratory a harmonious and cozy place to study and do research.

I would also like to express sincere gratitude towards my committee members, Dr. Michael Grant and Dr. Sheila Drover. They have always been keen to help with my research and offer me many insightful suggestions; I learned a lot through the comments and questions they posed during journal clubs.

I would like to thank all the professors and students in the cancer and development department for their guidance and help.

I want to give a special thanks to the lab seniors, Dr. Maria Licursi and Dr. Vipin Shankar, who offered me a helping hand on countless occasions and provided me with crucial hands-on training. My sincere appreciation and thank you to all members of the Hirasawa laboratory; I have learned a lot from these brilliant and earnest people. Special thanks to Ysabel Meneses for making the PIAS3 knockdown cells.

I would like to acknowledge Canadian Institutes of Health Research for the financial support of my research.

Finally, I would like to express my gratitude to my family for their constant love and support while I study on the other side of the world. They have been incredibly supportive of me chasing after my dreams, which I would not be able to accomplish otherwise.

Table of Contents

List of Figures

List of Tables

List of Abbreviations and Symbols

AD acidic region

ANOVA analysis of variance

BPB bromophenol blue

CBP cAMP-response element binding protein-binding protein

co-IP co-immunoprecipitation

CRISPR clustered regularly interspaced short palindromic repeats

DBD DNA binding domains

DMSO dimethyl sulfoxide

ERK extracellular signal-regulated kinase

FACS fluorescence-activated cell sorting

FDA Food and Drug Administration

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GBP2 guanylate binding protein 2

GFP green fluorescent protein

IFIT2 interferon induced protein with tetratricopeptide repeats 2

IFN interferon

iNOS inducible nitric oxide synthase

IP immunoprecipitation

IRF interferon regulatory factor

ISGF3 interferon stimulated gene factor 3

ISRE interferon-sensitive response element

JAK1 Janus kinase 1

LTD limited

MAP2 microtubule-associated protein 2

MEK mitogen-activated protein kinase kinase

miR micro RNA

MITF microphthalmia-associated transcription factor

MOI multiplicity of infection

NDV Newcastle disease virus

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NK natural killer

NO nitric oxide

Nr2e3 nuclear receptor subfamily 2, group E, member 3

OV oncolytic virus

PBS phosphate-buffered saline

PCR polymerase chain reaction

PIAS protein inhibitor of activated STAT

PINIT Pro-Ile-Asn-Ile-Thr

PMSF phenylmethylsulfonyl fluoride

PTM post-translational modification

qPCR quantitative PCR

RanBP2 Ran Binding Protein 2

RING Really Interesting New Gene

RIPA radioimmunoprecipitation assay

RLD RING-finger-like zinc-binding domain

RT-PCR reverse transcription-polymerase chain reaction

RQ relative quantification

SAE SUMO-activating enzyme subunit

SAP N-terminal scaffold attachment factor-A/B, ACINUS and PIAS

SD standard deviation

SIM SUMO interaction motif

STAT signal transducers and activators of transcription

SUMO small ubiquitin-like modifier

TBS tris-buffered saline

TGF-β transforming growth factor-β

TLR9 Toll-like receptor 9

TNF-α tumor necrosis factor-α

TYK2 tyrosine kinase 2

Ubc9 ubiquitin carrier protein 9

VSV Vesicular stomatitis virus

ZNF451 Zinc Finger Protein 451

Chapter 1 Introduction

1.1 Oncolytic viruses

1.1.1 History of oncolytic viruses

Oncolytic viruses (OVs) are a class of virus that preferentially infect cancer cells, inducing cancer cell lysis via immune stimulation. The idea of using OVs as an anti-cancer agent originated in the mid-19th century, when several cases reports revealed viral infection coincidentally led to tumor remission.[1-4] Later on, similar incidents were observed as tumors on patients regressed coincident with viral infections such as hepatitis, glandular fever, and measles. These caused researchers to note the potential oncolytic nature of viruses. In most cases, however, naturally developed viral infections weakened tumor growth very briefly and insufficiently.

The beginning of the 20th century brought sporadic attempts to utilize viruses therapeutically.[4] Hepatitis B virus was one of the first used in clinical cancer therapy. In 1949, a clinical trial was conducted in which 22 patients with Hodgkin's disease were treated with tissue extracts, or injected with unpurified human serum containing hepatitis virus.[5] Results showed that 14 out of 22 patients developed hepatitis. Seven out of 22 patients showed improvement in clinical aspects of disease and 4 showed a reduction in tumor size.[5] Although many of the patients suffered from side effects of viral infection, with one patient death confirmed, the results were still encouraging at the time, as the fate of patients afflicted with cancer was desperate at that time. A few years later, clinical

trials using glandular fever serum as a treatment for acute leukemia were conducted, which achieved more encouraging results.[6] Many other human pathogens were then administered to treat cancer patients in the following decades.[4]

In the meantime, by building on established *ex vivo* tissue and cell culture systems, animal models of cancer were gradually developed through the mid-20th century. As a result, oncolytic viruses have since been tested using laboratory animals (mice and rodents in most cases) implanted with cancer. Many of these experiments were highly encouraging, with complete tumor remission achieved,[7,8] and researchers noted that oncolytic viruses tended to have higher efficacy in animal models compared to the patients.[9-11]

The idea of using non-human viruses to treat cancer patients arose in the early 1950s, as said viruses produced little pathology in humans while retaining their oncolytic potency towards cancer cells.[8] Related studies have since been conducted, and several animal viruses were screened for oncolytic potential against human cancer cells.[12,13] Later clinical trials showed that some of animal viruses did not have enough therapeutic effect on human cancer.[14] Some viruses failed in animal experiments, with the infection causing death to animals.[7,9,15,16] Until today, several animal viruses such as Vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV [which showed efficacy in human cancer therapy, without human pathogenicity]) are still investigated intensively as

oncolytic agents.[8]

Since the 1950s, researchers have been investigating ways to manipulate viruses in order to make them more oncolytic or to increase their tumor-specificity. Despite these efforts, there was little success in the field until the 1990s, when development of recombinant DNA technology made virus engineering possible. Researchers' focus was then cast upon virus manipulation and viral genome engineering in order to build viruses which are safer, yet more aggressive and specific to cancer cells. In 2005, the first oncolytic virus H101, a genetically altered adenovirus, in combination with chemotherapy, was approved to treat nasopharyngeal carcinoma in China.[17]

In 2015, talimogene laherparepvec (T-VEC), a genetically modified herpes simplex virus type 1 (HSV-1), became the first OV approved by the Food and Drug Administration (FDA) in the United States, which is a landmark in the field.[18] Today, a wide range of viruses are under clinical investigation, including viruses with nonhuman hosts such as VSV, and NDV; many well understood or easily engineered human viruses like Adenovirus, Herpes simplex virus, Measles virus, Poliovirus, Vaccinia virus and Reovirus (human virus with low pathology).[18]

1.1.2 Molecular mechanism of oncolytic viruses

OVs take advantage of aberrant molecular changes in cancer cells to facilitate their replication. In normal cells, different molecular signaling pathways are exploited in antiviral defense. When infected by viruses, cells release interferons (IFNs) which triggers the activation of the JAK-STAT (Janus kinase–signal transducer and activator of transcription) pathway and the anti-viral machinery by inducing transcription of hundreds of anti-viral genes.[19] Briefly, IFNs bind to their cognate receptors and this further activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). These two kinases phosphorylate the IFN receptor chains, which recruits the STAT proteins and induce their phosphorylation and dimerization. The dimerized STAT proteins together with IFN regulatory factor 9 (IRF9) form interferon stimulated gene factor 3 (ISGF3) complex. Next, the complex translocates into nucleus and binds to IFN-stimulated response element (ISRE) to stimulate antiviral gene expression. It should be noted, however, cancer cells are generally insensitive to IFNs, thus anti-viral defense via IFN stimulation is impeded,[20] allowing viruses to proliferate in cancer cells.

Ras is a small GTPase that plays an important role in regulating cell proliferation.[21] The aberrant regulation of Ras is a common trait in many types of cancer. It has been widely noted that the activated Ras signaling pathway is one of the major mechanisms for viral oncolysis.[22-26] Several mechanisms underlying Ras-dependent viral

oncolysis have been identified.[27-29] Ras signaling pathway activation in cancer cells causes the cells to undergo increased proliferation, subsequently leading to more protein production, which is beneficial for more efficient viral replication.[30] In addition, activation of Ras can inhibit the function of anti-viral protein kinase R, further supporting production of viral particles.[27] Ras signaling is also considered to be a negative regulator of IFN signaling.[31,32] The Hirasawa lab demonstrated that activation of the Ras-Raf-mitogen-activated protein kinase kinase (MEK)-mitogen-activated protein kinase kinase (ERK) pathway can inhibit host anti-viral response induced by type I IFNs.[33,34] The inhibition has been further illustrated to be caused by Ras/MEK downregulation of a group of IFN-mediated antiviral genes, through negatively regulating interferon regulatory factor 1 (IRF1), a transcription factor for these genes (Figure 1).[35-37] Thus, IRF1 is considered a key protein that links Ras activation with IFN insensitivity in molecular mechanisms underlying viral oncolysis.

P53 deficiency is also a feature of cancer cells exploited by oncolytic viruses. The tumor suppressor protein p53 has been named the 'guardian of the genome' as it can activate the transcription of its responsive genes to induce cell apoptosis, cell cycle arrest or DNA repair upon genomic stress.[38] Certain viruses are prone to infect cancer cells with mutant p53, as intact p53 mediates abortive apoptosis to protect cells post infection.[39,40]

Figure 1. Connection between Ras activation and IFN insensitivity in viral oncolysis

Ras-MEK-ERK pathway inhibits type I IFNs-induced anti-viral genes through downregulation of IRF1.

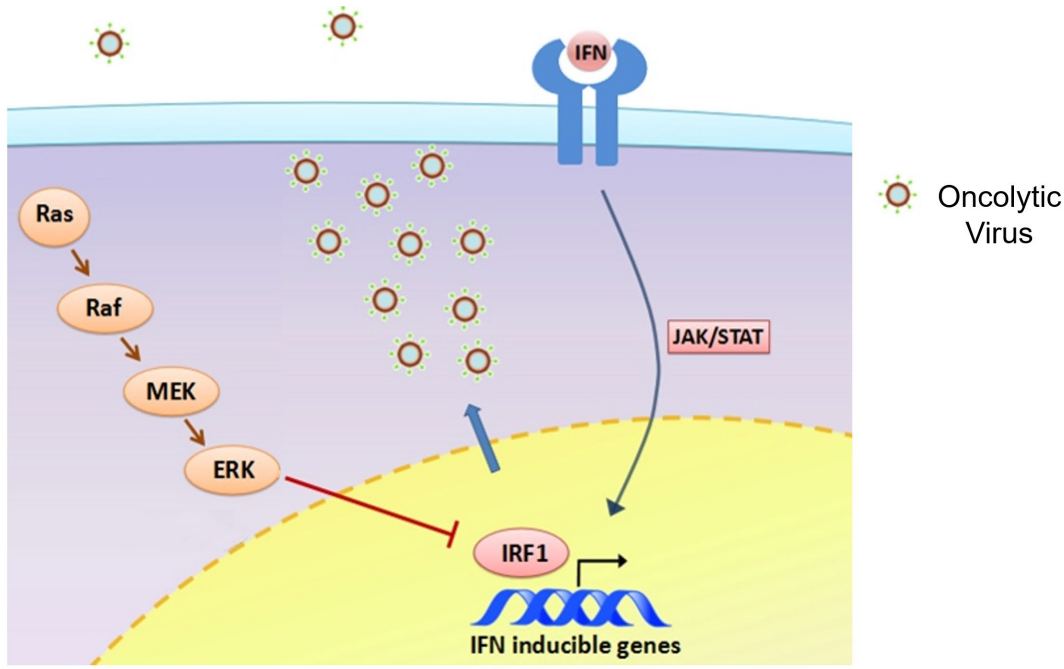
IFN
Ras
Raf
MEK
ERK
JAK/STAT
IRF1
IFN inducible genes
Oncolytic Virus

Figure 1

1.2 IRF1

Interferon regulatory factor 1 (IRF1) was the first member of the interferon regulatory factor (IRF) family identified as a transcription activator for type I interferon.[41] The IRF family is a group of transcription regulators, of which nine members were found in humans. They have highly conserved DNA-binding domains (DBDs) in their N-terminus, forming a helix-turn-helix structure.[42] IRFs bind to promoter regions of target genes to regulate gene transcription,[43] either to the IRF-binding element (IRF-E), or to the interferon-sensitive response element (ISRE).

IRF1 plays a crucial role in anti-viral immune response. Viral infections, including Newcastle disease virus, Encephalomyocarditid virus and VSV, induce type I IFN genes in most cell types.[44,45] IRF1 is involved in activating type I IFN genes during viral infection, although their induction is not dependent only on IRF1.[46] The Toll-like receptor 9 (TLR9)/MyD88/IRF1/IFN-β pathway is one of the IRF1-mediated signaling cascades, which has been shown to have potent anti-viral properties *in vitro* and *in vivo*.[47] In addition, IFN-γ, which is the only member of the type II class of interferons, is involved in the TLR9/MyD88/IRF1/IFN-β pathway as it induces activation and translocation of IRF1 from cytoplasm to nucleus.[48] IRF1 interacts with nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) upon stimulation with IFN-γ and tumor necrosis factor-α (TNF-α) induced by herpes simplex virus infection, mediating

production of nitric oxide (NO) by promoting the transcription of inducible nitric oxide synthase (iNOS).[49] This innate immunity is important to the host, as the production of NO is essential for defending against bacteria, viruses, tumor cells and other pathogens. IRF1 also plays essential roles in the development of immune cells, and influences differentiation of natural killer (NK) cells.[50] Moreover, IRF1 is required for the differentiation of T helper 1 cells, as shown by absence of T helper 1 differentiation in *in vivo* IRF1-/- mouse system.[51]

Furthermore, IRF1 demonstrates anti-tumor functions. IRF1 alleles are often singly or completely lost in different types of cancers, such as leukemia, esophageal cancer and gastric cancer.[52-54] IRF1 inactivation can also be caused by aberrant splicing of the gene or the binding of nucleophosmin to IRF1, which prohibits its DNA binding ability.[55,56] IRF1 has also been shown to have anti-tumor growth effects in *in vivo* experimental system.[57] Notably, IRF1 activates transcription of tumor suppressor genes, genes regulating cell cycle arrest and/or programmed cell death (which cause cancer cell death).[58-63] IRF1 also induces transcription of anti-proliferative genes such as p21WAF1/CIP1 and p53.[58,62] IRF1 can be considered a potential biomarker due to its anti-tumor properties as well as its ability to elevate neoadjuvant radio/chemotherapy responses in cervical cancer.[64]

IRF1 function is not only regulated at the transcriptional level but also at

posttranscriptional and/or posttranslational levels. Several micro RNAs (miRs) have been shown to regulate IRF1 expression, such as miR-23a, miR-383 and miR-132a-3p.[65-67] Posttranslational modifications (PTMs) have also been demonstrated to be crucial for IRF1 regulation.[68] Commonly found types of PTMs for IRF1 are acetylation, phosphorylation, ubiquitination and sumoylation. P300 regulates IRF1 acetylation, increasing DNA binding ability and transcriptional activity of IRF1.[69] IRF1 phosphorylation by Casein kinase II is also important in maintaining its transactivation function.[70] In contrast, inhibitor of NF-κB kinase ε was reported to phosphorylate IRF-1 in primary $CD4^+$ T cells, which inhibits its transcriptional activity.[71] IRF1 can be degraded by ubiquitin-mediated proteasome,[72-74] an essential method for modulating IRF1 protein level. Furthermore, recent studies showed IRF1 phosphorylation initiates ubiquitination-mediated degradation, as well as transcriptional activation, which is a key component for its anti-proliferative activity in cancer cells.[75] Sumoylation of IRF1 has generally been reported to inhibit its transcriptional activity.[76,77] Interestingly, increased IRF1 sumoylation not only hinders its anti-cancer function, but also converts it into an oncogenic protein.[78]

1.3 PIAS3

Protein inhibitors of activated signal transducers and activators of transcription 3 (PIAS3), which belongs to the protein inhibitor of activated STAT (PIAS) family, is a major cellular inhibitor of STAT3. The family consists of four members in mammalian cells, which include PIAS1, PIAS2, PIAS3 and PIAS4, sharing more than 40% sequence identity.[79] PIAS orthologs were also identified in yeasts, plants and other nonvertebrate animals such as *Drosophila*.[80-82] PIAS3 was first found as a homologue of PIAS1, as well as being a novel chaperone of specific K(+) channels.[83,84] Considerable research has been conducted on PIAS proteins, elucidating that they play important roles in immune function, cytokine signaling and other forms of cellular regulation.[79,85,86]

Five key structures of human PIAS proteins have been identified: the N-terminal scaffold attachment factor-A/B, ACINUS and PIAS (SAP) domain, the Pro-Ile-Asn-Ile-Thr (PINIT) motif, the Really Interesting New Gene (RING)-finger-like zinc-binding domain (RLD), the highly acidic (AD) region, and the C-terminal serine/threonine-rich (S/T) region (Figure 2).[87] The RLD and SAP domain are the most conserved among PIAS proteins, and the SAP domain is usually found in chromatin binding proteins.[88] Nuclear magnetic resonance analysis revealed that the SAP domain of PIAS1 has DNA binding capability.[89] The featured LXXLL motif (X represents any amino acid) was characterized within the SAP domain of PIAS proteins, and is a motif

responsible for the interplay of nuclear receptors and their co-regulators.[90] RLDs of PIAS proteins are required for their small ubiquitin-like modifier (SUMO)-E3-ligase activity,[91] while the genuine RING domains are often present in ubiquitin E3 ligases.[92] The PINIT motif is highly conserved within the PIAS family, being responsible for the subcellular localization of PIAS3.[93] A region with 51 amino acids in PINIT motif was shown to be an inhibitory domain for both microphthalmia-associated transcription factor (MITF) and STAT3.[94-97] AD located close to the C-terminus is also conserved among PIAS proteins. A putative SUMO interaction motif (SIM) was found within AD for most PIAS proteins including PIAS3.[86] SIMs interact non-covalently with SUMO proteins, and are also found in many nuclear proteins reportedly involved in sumoylation.[85] The S/T region of the C-terminus is highly variant among PIAS proteins but was still found in all PIAS proteins except for PIAS4. The functional feature of S/T region remains to be defined. Two PIAS3 isoforms, PIAS3 and PIAS3β, have been identified. PIAS3β has an insertion of 39 amino-acid residues in between the SAP domain and PINIT domain.[79]

PIAS proteins are known for their roles as SUMO E3 ligases. In the process of sumoylation, inactive SUMO precursors of either SUMO1, SUMO2, or SUMO3 are cleaved and activated in an ATP-dependent manner by SUMO E1 activating enzyme, which is a heterodimer composed of SUMO-activating enzyme subunit (SAE) 1 and SAE2 in humans.[98] Next, SUMO molecules are transferred from the E1 enzyme to the

SUMO E2 conjugating enzyme called ubiquitin carrier protein 9 (Ubc9). Finally, with assistance of a SUMO E3 ligase, SUMO is transferred from the E2 enzyme to the lysine residue of the substrate. It should be noted that E3 ligases are not mandatory for sumoylation, as Ubc9 itself can mediate transfer of SUMO to substrate target proteins without E3 ligase interaction in some cases.[99] Nevertheless, SUMO E3 ligases help stabilize the substrate-E2-SUMO complex, which promotes the efficiency of SUMO transfer.[100] So far, ten different proteins have been identified to have SUMO-E3-ligase activity in humans, including the PIAS family, Ran binding protein 2 (RanBP2), and the zinc finger protein 451 (ZNF451) family.[99] While it was originally believed that each E3 enzyme was specific to some substrates, considering there are thousands of sumoylation substrates and only ten identified E3 ligases, the notion that E3 ligases determine substrate specificity is controversial at best.[99] More studies should be conducted to identify how SUMO E3 ligases conduct substrate specific sumoylation.

PIAS3 and other PIAS proteins can facilitate a variety of functions in cellular regulation. PIAS proteins interact with more than 60 proteins, a large portion of which are transcription factors.[87] These interactions can either repress or promote their transcription functions. The regulatory mechanisms vary, but are highly dependent on the SUMO-E3-ligase activity within PIAS, or at least on their ability to facilitate SUMO modification.[85] One example of how PIAS3 regulates cellular functions is its direct

interaction and promotion of sumoylation within nuclear receptor subfamily 2, group E, member 3 (Nr2e3), resulting in conversion of Nr2e3 to a transcriptional repressor of the photoreceptor-specific genes.[101] Moreover, PIAS3 promotes transcriptional responses of transforming growth factor-β (TGF-β)/Smad signalling by interacting with p300/cAMP-response element binding protein-binding protein (CBP), the transcriptional coactivator. The RLD of PIAS3 is essential for the interaction with p300/CBP.[102] PIAS3 also directly associates with the p65 subunit of NF-κB and suppresses its transcriptional activity. For this interaction, the N-terminal LXXLL motif of PIAS3 is required.[103] PIAS1 has also been reported to negatively regulate NF-κB signaling by blocking DNA binding activity towards the downstream genes.[104] Although PIAS3 and PIAS1 perform as SUMO E3 ligases for the estrogen receptor α (ERα), PIAS1 regulates ERα transcriptional function independently of its SUMO E3 ligase function, as the PIAS1 mutant that lacks E3 ligase activity could still regulate the transcriptional function of ERα.[105] Furthermore, the binding of PIAS3 to STAT3 physically inhibits the DNA binding activity of STAT3 and stops transcriptional activation of STAT3-mediated genes.[83] Through interacting with STAT3 (a key regulator of the immune response) PIAS3 is involved in immune regulation.[87] Similarly, PIAS1 impairs transcriptional activity of STAT1 in the same way.[83] Apart from these, PIAS4 functions as SUMO E3 ligase to stimulate the sumoylation and transcriptional activity of p53.[106] PIAS1 and

PIASxα are reported to interact functionally with the glucocorticoid receptor-interacting protein 1 in order to regulate its transcriptional activity.[107]

Figure 2. Schematic domain structure of PIAS3

Five key domains of human PIAS3 protein and their functional features are illustrated.

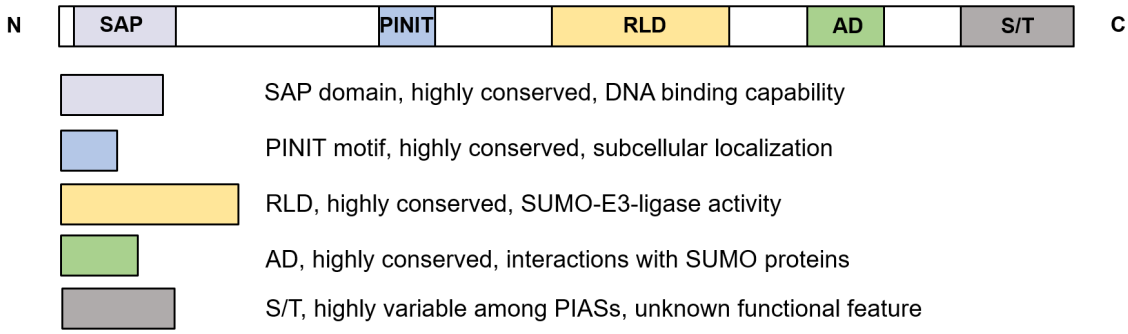
N
SAP
PINIT
RLD
AD
S/T
C
SAP domain, highly conserved, DNA binding capability
PINIT motif, highly conserved, subcellular localization
RLD, highly conserved, SUMO-E3-ligase activity
AD, highly conserved, interactions with SUMO proteins
S/T, highly variable among PIASs, unknown functional feature

Figure 2

Chapter 2 Rationale

The regulation of Ras/MEK on IRF1 is an important cellular mechanism for viral oncolysis. In a previous work, we demonstrated that Ras/MEK does not down-regulate IRF1 at the transcriptional level.[108] Considering that IRF1 is dramatically modulated by posttranslational modifications, I hypothesized that PTMs of IRF1 by Ras/MEK could be essential to determine cellular sensitivity to oncolytic viruses in cancer cells. PIAS3 is a key regulator as a SUMO E3 ligase for many transcription factors including IRF1.[77] The objectives of my MSc project were as follows:

1) Determine if PIAS3 directly interacts with IRF1.
2) If this is the case, determine if the Ras/MEK pathway regulates the interaction between IRF1 and PIAS3.
3) Determine if PIAS3 modulates transcriptional and antiviral functions of IRF1.

Chapter 3 Material and Methods

3.1 Cells and Viruses

Human fibrosarcoma cells (HT1080) were obtained from the American Type Culture Collection (Manassas, VA, USA). VSV (Indiana strain) was provided by Dr. John C. Bell (Centre for Innovative Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, Canada). The VSVs were amplified and titrated by plaque assay using the mouse fibroblast cell line L929 cells.

3.2 Cell culture

The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Multicell, Wisent Inc., QC, Canada) with 10% fetal bovine serum (GE Healthcare Life Sciences, Illinois), 1 mM sodium pyruvate (Life Technologies LTD, UK) and antibiotic-antimycotic (Thermo Fisher Scientific, Massachusetts). Cells were maintained in 10-cm culture dishes at 37°C with 5% CO_2 for the use of experiments in this project. Cells were subcultured three times a week on average. For subculture, 0.25% trypsin-EDTA (Life Technologies LTD) was used to detach cells.

3.3 Generation of CRISPR (clustered regularly interspaced short palindromic repeats) knockdown clones

The PIAS3 CRISPR clones were generated by Ysabel Meneses, another

graduate student in the Hirasawa lab. PSpCas9-2A-GFP plasmid (purchased from Addgene, Massachusetts) was digested and ligated with sgRNA targeting PIAS3 gene (sense 5'-CAC CGA GTG CGC TTC CTC AAA CCG C - 3', anti-sense 5'- AAA CGC GGT TTG AGG AAG CGC ACT C- 3'). After validation, the newly-generated plasmid was transfected into HT1080 cells. Twenty-four hours post transfection, cells were subjected to fluorescence-activated cell sorting (FACS) with the Beckman Coulter MoFlo Astrios EQ Cell Sorter (Beckman Coulter, CA) to select green fluorescent protein (GFP) positive cells. To obtain CRISPR single cell clones, GFP-positive cells were collected, and a limiting dilution series was conducted in a 96-well plate. For the dilution, 4,000 cells were plated in the first well of the first column (8 rows and 12 columns in a 96-well plate). Then, half of the cells in the first well were transferred into the second well of the same column. The 1/2 dilution was repeated down the entire 8 rows of the first column. Next, half of the cells in the wells of the first column were transferred into the corresponding wells in the second column of the plate. The 1/2 dilution was repeated down the entire 12 columns. Formation of colonies from single cells was monitored daily and appearance of GFP-positive cells was checked under fluorescent microscope regularly. The wells with GFP-positive single colonies were marked. The cells were grown to full confluence, and then transferred into 24-well plates. When the cloned cells grew to full confluency in the 24-well plates, they were collected and the PIAS3

expression levels of these clones were assessed by western blot analysis conducted by Danyang Xu. The CRISPR clone 1, clone 3 and clone 6 were also amplified and frozen for later use.

3.4 Western Blot Analysis

Cells were washed with phosphate-buffered saline (PBS) buffer. Then total cell protein in each well of a 24-well plate was collected with 100 µL radioimmunoprecipitation assay (RIPA) buffer with phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Missouri), aprotinin (Sigma-Aldrich) and phosphatase inhibitors (halt phosphatase inhibitor cocktail, Thermo Fisher Scientific). Fifty µL sample buffer (188 mM Tris–HCl (pH 6.8), 6% sodium dodecyl sulfate, 0.3% bromophenol blue, 30% glycerol, 15% β-mercaptoethanol) was added into each sample and they were boiled for 5 minutes. Ten µL of each sample was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was run with constant current of 20 mA. Proteins were then transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad, ON, Canada) with Tran-blot Turbo Transfer System (Bio-Rad). The membrane was blocked with 5% skim milk in tris-buffered saline (TBS) with Tween 20 (TBS-T) for 1 hour, and then blotted with primary antibodies in conditions shown in table 1 for overnight at 4 °C. The following day, membranes were washed three times with TBS-T, 5

minutes for each. Membranes were blotted with corresponding secondary antibody (conditions shown in table 1) for 1 hour at room temperature followed by three washes in TBS-T. Specific bands were detected with ImageQuant LAS 4000 (GE Healthcare Life Sciences, QC, Canada) using chemiluminescence substrate (Bio-Rad), and analyzed with ImageQuant Software (GE Healthcare Life Sciences).

3.5 Immunoprecipitation

HT1080 cells were plated into 10-cm culture dishes. When cells were about 70 % confluent, they were co-transfected with 5 μg pcDNA3.1 empty vector plasmid, pcDNA3.1-IRF1 plasmid and pcDNA3.1-FLAG-PIAS3 plasmid using Superfect Transfection Reagent (Qiagen, ON, Canada) according to manufacturer's instruction. 24 hours after transfection, cells co-transfected with pcDNA3.1-IRF1 and pcDNA3.1-FLAG-PIAS3 plasmid were treated with 20 μM U0126 (Cell Signalling Technology, Danvers, MA) or the same volume of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for 6 hours. U0126 is a highly selective inhibitor for both MEK1 and MEK2, that blocks the phosphorylation activity of MEKs towards ERKs.[109,110] Four hours after U0126 treatment, 25 μM MG132 (Sigma-Aldrich) was added to prevent protein degradation. After treatment, cells were washed twice with ice-cold PBS, then lysed with 1 mL 1 % TritonX-100 lysis buffer (20mM TrisHCl pH8, 1% TritonX-100, 10%

glycerol, 2mM EDTA, 137 mM NaCl) which was supplemented with protease inhibitors PMSF (Sigma-Aldrich, Missouri), aprotinin (Sigma-Aldrich), halt phosphatase inhibitor cocktail (Thermo Fisher Scientific), and 20 μM desumoylation inhibitor N-ethylmaleimide (Sigma-Aldrich). The cell lysate was centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatant was pre-cleared for 1 hour using 30 μl of pre-equilibrated protein A agarose beads (Thermo Fisher Scientific), then immunoprecipitated with 2 μg mouse anti-IRF1 antibody (BD biosciences), or mouse anti-Flag M2 antibody (Sigma-Aldrich) overnight at 4 °C. The next day, cell lysate with antibody was incubated with 30 μl pre-blocked protein A agarose beads for 2 hours at 4 °C. After incubation, the complex was centrifuged at 5000 rpm for 1 minute at 4 °C. The supernatant was discarded, and the precipitated beads were washed three times with 1 mL 1 % TritonX-100 lysis buffer. Proteins were eluted by boiling the beads in 1x sample buffer as described previously for 10 minutes. Samples were subjected to SDS-PAGE, followed by western blot analysis using the IRF1, PIAS3 and β-Actin antibodies listed in table 1.

3.6 Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from HT1080 parental cells and HT1080 PIAS3 knockdown clone 1, clone 3 and clone 6 cells. cDNA was synthesized from the RNA using the

RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts). Quantitative PCR (qPCR) was performed in triplicate using *power*SYBR®Green PCR Master Mix (Life Technologies LTD, UK) to detect Guanylate Binding Protein 2 (GBP2), Interferon Induced Protein with Tetratricopeptide Repeats 2 (IFIT2), Microtubule-associated Protein (MAP2), signal transducers and activators of transcription 2 (STAT2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and analyzed with StepOnePlus qPCR system (Applied Biosystems, CA). The polymerase chain reaction (PCR) procedure was as manufacturer's instructions: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and then followed by melt-curve analysis. For data analysis and statistical analysis, the GBP2, IFIT2, MAP2 and STAT2 mRNA levels of each sample was normalized to GAPDH. Then fold change of each sample toward the parental cells sample was calculated. The One-way analysis of variance (ANOVA) with Tukey's post-hoc test was performed using GraphPad Prism 6.0 software (GraphPad Software, CA). Sequence for all primers used is listed in table 2.

3.7 Virus Infection

HT1080 cells and PIAS3 CRISPR clone 1, 3 and 6 cells were plated the day before infection. When cells reached about 80 % confluency, they were subjected to VSV infection (multiplicity of infection (MOI) = 1/4, 1/8, 1/16, 1/32 and 1/64) for 16 hours.

Cells were then lysed, followed by western blot analysis for detection of virus protein.

Table 1. Primary and secondary antibody conditions for western blot

Antibody	**Primary antibody condition**	**Secondary antibody condition**
IRF1 monoclonal antibody catlog:612047 (BD biosciences)	1:1000 in 5 % milk	1:5000 Anti-mouse IgG in 5 % milk
PIAS3 (D5F9) XP monoclonal antibody (Cell signaling technology)	1:1000 in TBS-T	1:5000 Anti-rabbit IgG in TBS-T
VSV-G monoclonal antibody (VSV11-M)	1:10,000 in 5 % milk	1:5000 Anti-mouse IgG in 5 % milk
β-Actin A5441 monoclonal antibody (Sigma-Aldrich)	1 in 60,000 in TBS-T	1:5000 Anti-mouse IgG in TBS-T

Table 2. qPCR primers

Gene Name	5’ Primer	3’ Primer
GAPDH	ATCTTCTTTTGCGTCGCCAG	ACGACCAAATCCGTTGACTCC
GBP2	TTTCACCCTGGAACTGGAAG	TGCACAACCGAGGATCATTA
IFIT2	ATTGCACTGCAACCATGAGTG	TCCCTCCATCAAGTTCCAGGT
MAP2	CCATTTGCAACAGGAAGACAC	CAGCTCAAATGCTTTGCAACTAT
STAT2	GGAATCAGGCATGTGTCCCTT	TTCACCTCTCACCCCAATGGA

Chapter 4 Results

4.1 IRF1 does not have direct interaction with PIAS3

To determine if IRF1 interacts with PIAS3, a co-immunoprecipitation (co-IP) experiment was conducted using HT080 cells. I also determined if MEK inhibition modulates the interaction between IRF1 and PIAS3. Cells were transfected with pcDNA3.1-IRF1 plasmid and/or pcDNA3.1-Flag-PIAS3 plasmid. At 18 hours after transfection, the cells were treated with U0126 (20 μM) or the same volume of DMSO for 6 hours. At 22 hours after transfection, the proteasome inhibitor, MG132 treatment (25 μM) was added for 2 hours to prevent protein degradation. At 24 hours after transfection, cells lysates were prepared for immunoprecipitation with either anti-Flag antibodies or anti-IRF1 antibodies, followed by western blot analysis. As shown in Figure 3**,** the cells effectively express IRF1 or PIAS3 (input) indicating that the transfection was successfully performed. I also detected PIAS3 protein in samples immunoprecipitated with anti-PIAS3 antibody (Figure 3A) as well as IRF1 protein in immunoprecipitation with anti-IRF1 antibody (Figure 3B). These results suggest that the immunoprecipitation successfully pull-downed the target proteins. However, IRF1 was not detected in the PIAS3-pulldown samples. Similarly, PIAS3 was not detected with the IRF1-pulldown samples, indicating that IRF1 and PIAS3 do not have close interaction in HT1080 cells. Thus, we could not determine if MEK inhibition modifies their interaction.

Figure 3. Identification of interaction between IRF1 and PIAS3 in HT1080 cells

Co-immunoprecipitation (Co-IP) assay was conducted with HT1080 cells overexpressed with IRF1 and/or Flag tagged PIAS3 treated with or without U0126. Total cell lysates were subjected to immunoprecipitation (IP) with antibodies against Flag (A) or antibodies against IRF1 (B), followed by western blot analysis with anti-IFR1 antibodies as well as anti-PIAS3 antibodies to detect IRF1 and Flag tagged PIAS3. Data here are 2 representative results of 4 independent experiments.

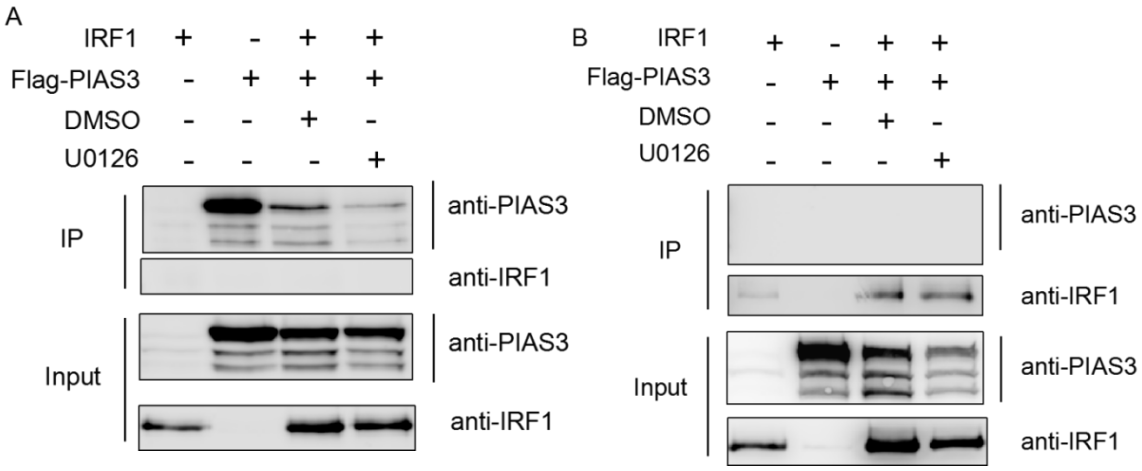
A
IRF1 + - + +
Flag-PIAS3 - + + +
DMSO - - + -
U0126 - - - +
IP
anti-PIAS3
anti-IRF1
Input
anti-PIAS3
anti-IRF1
B
IRF1 + - + +
Flag-PIAS3 - + + +
DMSO - - + -
U0126 - - - +
IP
anti-PIAS3
anti-IRF1
Input
anti-PIAS3
anti-IRF1

Figure 3

4.2 IRF1 expression is increased in response to PIAS3 knowckdown

To further determine the role of PIAS3 on IRF1 regulation, we decided to establish PIAS3 knockdown HT1080 cells. As mentioned in chapter 3, PIAS3 was knocked down via CRISPR-Cas9 technique. After screening more than 20 clones, three clones showed less PIAS3 expression compared with parental cells (Figure 4). When the levels of IRF1 expression were examined in the PIAS3 knockdown HT1080 cells, I found that PIAS3 CRISPR knockdown increased IRF1 levels compared to that in the parental control. The increase of IRF1 in PIAS3 clone 6 was not as clear as that in the other two clones. Nonetheless, considering less amount of the loading control (β-actin) in PIAS clone 6 compared to in control, the IRF1 level is higher. This result suggests that PIAS3 regulates IRF1 expression (Figure 4) while I did not confirm their direct interaction by immunoprecipitation (Figure 3).

Figure 4. IRF1 expression levels in PIAS3 CRISPRR knockdown clones

Expression levels of PIAS3 and IRF1 in PIAS CRISPR-knockdown clones (PIAS3 clone 1, clone 3 and clone 6) were determined by western blot, with β-Actin as a loading control. HT1080 parental cells were used as the control. Data here are representative of 2 independent experiments.

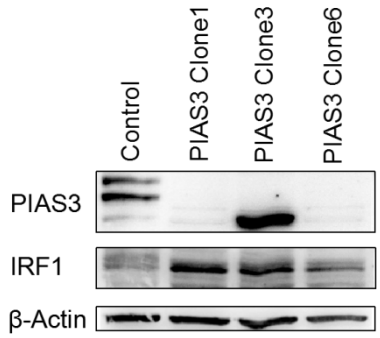
Control
PIAS3 Clone1
PIAS3 Clone3
PIAS3 Clone6
PIAS3
IRF1
β-Actin

Figure 4

4.3 PIAS3 knockdown increases transcription levels of some IRF1-mediated anti-viral genes

As PIAS3 knockdown increases IRF1 expression level, we wanted to identify whether this could further modulate the function of IRF1 as a transcriptional regulator. Therefore, we conducted qRT-PCR to detect transcriptional levels of IRF1-mediated antiviral genes including GBP2, IFIT2, MAP2 and STAT2 in HT1080 parental and PIAS3 CRISPR knockdown cells. As is shown in Figure 5**,** all three PIAS3 CRISPR clones display significantly increased transcription of GBP2 compared to control cells. PIAS3 clone 1 and clone 6 showed significantly increased expression of MAP2 while PIAS clone 3 demonstrated a significant increase in IFIT2 expression level. There was no difference in STAT2 transcription level among parental cells and the PIAS CRISPR clones. The results of qRT-PCR demonstrate PIAS3 knockdown increases transcription levels of some but not all IRF1-mediated anti-viral genes.

Figure 5. Changes in transcriptional level of certain IRF1-mediated antiviral genes by PIAS3 knockout

The gene transcriptional level of GBP2, IFIT2, MAP2 and STAT2 in HT1080 parental cells, PIAS3 CRISPR-knockout clone1, clone 3 or clone 6 were determined by quantitative RT-PCR. The relative quantification (RQ) of transcriptional level was calculated by normalizing to GAPDH expression levels and then divided by that of the parental control (*P< 0.01). Plot shows mean +/- SD (standard deviation) of three biological replicates.

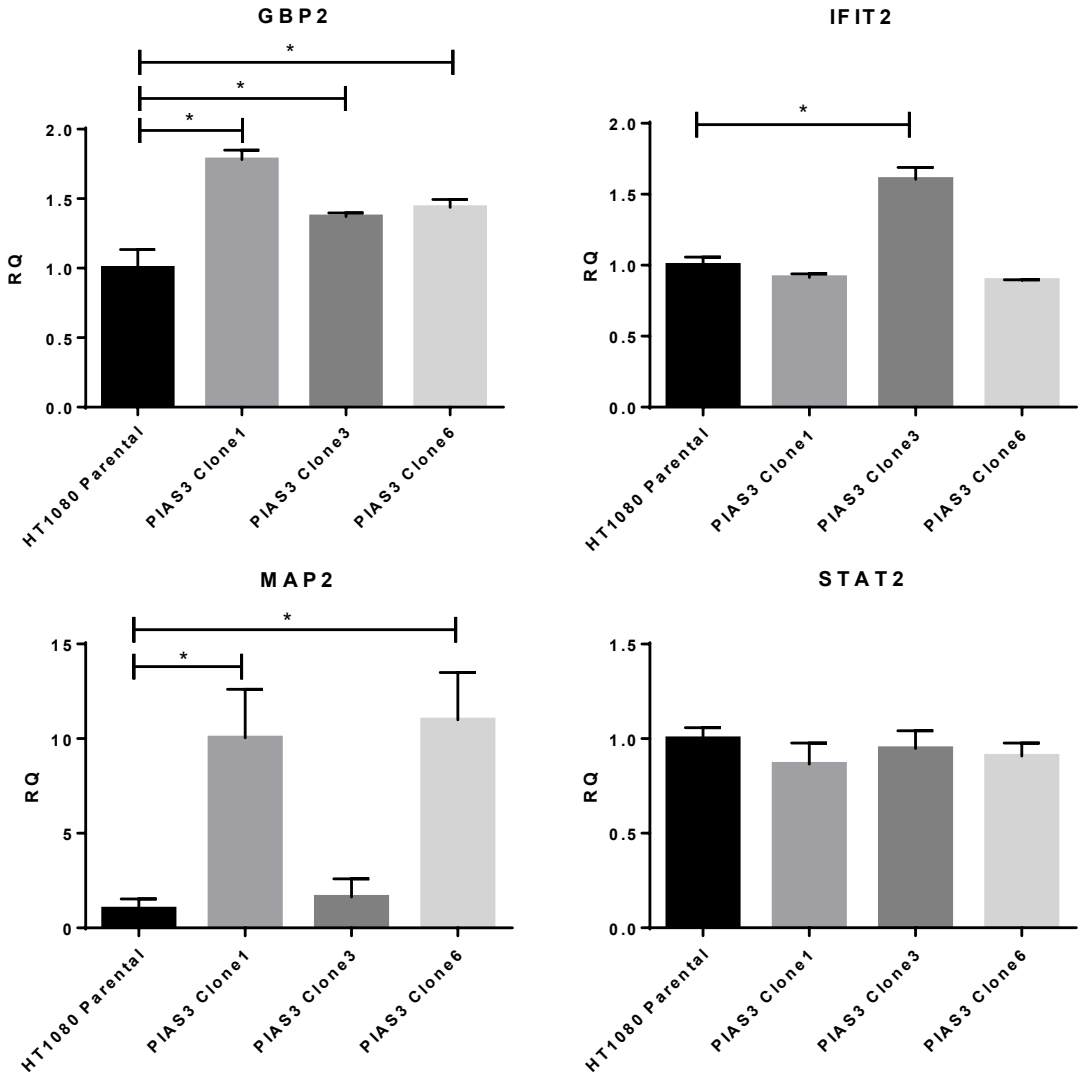
GBP2
IFIT2
MAP2
STAT2
RQ
HT1080 Parental
PIAS3 Clone1
PIAS3 Clone3
PIAS3 Clone6
*

Figure 5

4.4 PIAS3 knockdown does not increase cellular resistance to virus infection

As IRF1 expression increased in response to PIAS3 knockdown, resulting in increased expression of the antiviral proteins, we wanted to identify if this could have any effects on cell susceptibility towards virus infection. To test this possibility, the PIAS3 CRISPR clones along with HT1080 parental cells were subjected to VSV infection with different MOIs (MOI=1/4, 1/8, 1/16, 1/32 and 1/64) for 16 hours. Cells were lysed for western blot analysis to detect intracellular viral proteins. PIAS3 CRISPR clone 3 cells were resistant to virus infection compared with the parental control throughout different MOIs, as less virus protein was detected (Figure 6). However, PIAS3 CRISPR clone 1 and clone 6 cells were more susceptible to virus infection compared with the control cells with higher MOI (MOI = 1/4, 1/8, 1/16, 1/32) as more virus protein was detected in these two clones than in the parental cells. As the sensitivity to VSV infection was not consistent amongst the three PIAS3 CRISPR clones, the results are not conclusive. However, the results suggest that PIAS3 knockdown is not sufficient to increase cellular resistance to virus infection because two of the three PIAS3 CRISPR clones showed higher susceptibility to VSV.

Figure 6. Determination of cell susceptibility of PIAS3 CRISPR knockout clones towards VSV infection

HT1080 parental cells as control and PIAS3 knockout cells (PIAS3 clone 1, clone3 and clone 6) were infected with VSV (MOI=0, 1/4, 1/8, 1/16, 1/32 or 1/64) for 16 hours. Western blot analysis was conducted to detect virus protein (VSV-G). β-Actin was used as a loading control here. Data here are representative of 2 independent experiments.

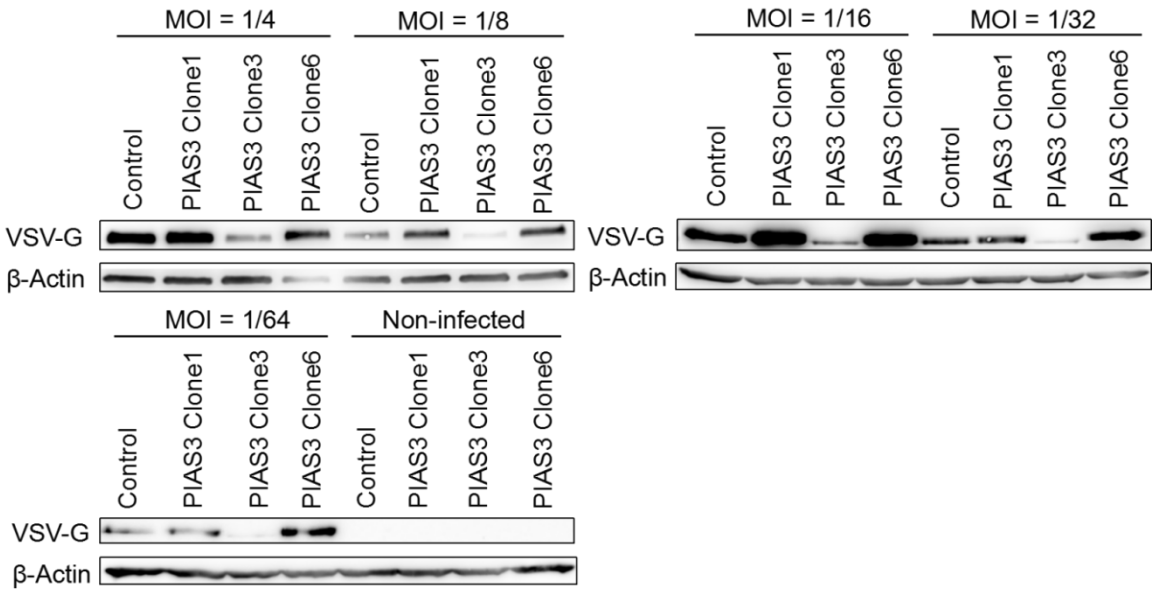
MOI = 1/4
MOI = 1/8
MOI = 1/16
MOI = 1/32
Control
PIAS3 Clone1
PIAS3 Clone3
PIAS3 Clone6
VSV-G
β-Actin
MOI = 1/64
Non-infected

Figure 6

Chapter 5 Discussion

In summary of the results, IRF1 does not have direct interaction with PIAS3 in HT1080 cells while PIAS3 regulates IRF1 expression. Moreover, knockdown of PIAS3 increases transcription levels of IRF1-mediated anti-viral genes but is not sufficient to increase cellular resistance to virus infection.

A direct interaction between PIAS3 and IRF1 was expected as PIAS3 has been reported to be a SUMO E3 ligase of IRF1 by Nakagawa and Yokosawa.[77] In their study, they were able to detect the binding of the two proteins using immunoprecipitation in 293T cells where FLAG-tagged IRF1 and myc-tagged PIAS3 were co-transfected. The cellular signalling environment varies for different cell lines, which could be the reason for the inconsistency with the previous study as we used HT1080 cells. In addition, more than one PIAS proteins could bind to IRF1 and promote its sumoylation.[111] Therefore, it is possible that other proteins than PIAS3 serve as SUMO E3 ligase(s) for IRF1 in HT1080 cells, which could be why we did not detect the direct interaction between PIAS3 and IRF1 in our system. PIAS3 binds to IRF1 to promote SUMO1-mediated sumoylation of IRF1 in 293T cells. In HT1080, we were not able to detect high sumoylation level of IRF1 without overexpressing SUMO proteins together with IRF1 (data not shown). This indicates that the SUMO pathway mediated by PIAS3 is not very active in HT1080 cells. This could be another possible explanation why we did not detect the direct interaction. To answer this, we would overexpress other elements of the SUMO

pathway such as the SUMO E2 conjugating enzyme Ubc9 to confirm the interaction of IRF1 and PIAS3 in HT1080.

Two bands of IRF1 were observed in HT1080 control cells (Figure 4 lane 1). However, when PIAS3 was knocked down by CRISPR, the lower band increased its intensity while the upper band was not detected (Figure 4 lane 2 and 3). This may suggest that PIAS3 knockdown blocked posttranslational modifications of IRF1 (upper band) and increased expression of non-modified IRF1 (lower band). However, the upper band of IRF1 in HT1080 control cells was not always shown or consistent in experiments we conducted. We assume that this could be due to the lot-to-lot difference of antibodies or sample preparation. To further study this, we will need to test different IRF1 antibodies and different lysis buffers specially containing proteasome inhibitors.

Although we were not able to detect direct interaction between IRF1 and PIAS3, PIAS3 modulates IRF1 expression and its transcriptional functions. Increased expression of IRF1 was observed in all the three PIAS3 CRISPR knockdown clones of HT1080 cells. However, these clones showed different expression profiles of the IRF1 mediated anti-viral genes. For GBP2, all three clones have increased expression compared with the control cell. Clone 3, but not clone 1 or 6, showed significantly higher expression of IFIT2 than the parental cells while this was completely opposite for MAP2. The CRISPR clones were derived from single positive cell colonies or very few cells

after FACS sorting following transfection of the CRISPR plasmid into HT1080 cells. We would expect molecular variations in different clones as the gene expression background of each single cell varies even though they originated from the same cell line. To reach a clear conclusion, we will need to conduct RNA sequencing to obtain global changes in transcription of IRF1-mediated antiviral genes caused by PIAS3 knockdown.

There are weakness and limitations in the methodology and experimental design of my thesis. When I conducted the immunoprecipitation analysis with cell lysates treated with the MEK inhibitor (Figure 3), I did not conduct western blot analysis for phosphorylated ERKs to confirm if the MEK inhibitor inhibits the pathway. Although this did not change our conclusion as there was no direct interaction detected between IRF1 and PIAS3, it should have been included in Figure 3. Second, it was not optimal to use HT1080 parental cells as a control of the PIAS3 CRISPR clones because the parental cells are pooled cells while the CRISPR clones are from single cells. However, it was not technically possible to have the same single cell to generate both control and CRISPR knockout clones. Alternatively, we could have all the GFP positive cells collected after FACS sorting following transfection of the PIAS3 CRISPR plasmid. In this case, the effects of PIAS3 knockdown on the antiviral transcription as pooled cells, which are comparative to parental HT1080 cells. We assume that this approach is more feasible than the approaches we used. At last, even though efforts were made to get single cell

when generating PIAS3 knockdown clones including doing limiting dilution and checking under microscope (Chapter 3 part 3), it is still not guaranteed that the three clones were absolutely generated from single cells. Whereas, PIAS3 knockdown was consistent in the three clones while these experiments were being conducted, which shows that this technical limitation would hardly affect the results of my experiments regarding the three PIAS3 clones.

There was an intensive band below endogenous PIAS3 protein on anti-PIAS3 western blot analysis of PIAS3 CRISPR clone 3, which was not observed in PIAS3 CRISPR clone 1 and clone 6 (Figure 4). Total human PIAS3 protein is comprised of 619 amino acids, which should be around 68 kDa. The lower band of the clone 3 can either be a nonspecific band or aberrant protein isoform of PIAS3. As I repeated the experiment several times and always found the lower band in clone 3, but not in clone 1 or 6, it is not likely that it is a non-specific band. Interestingly, the expression profiles of IRF1-mediated antiviral genes (IFIT2 and MAP2) were different in clone 3 from clone 1 and clone 6 (Figure 5), which suggests possible involvement of the protein in regulating the transcription of IRF1-mediated antiviral genes. If it is an aberrant isoform of PIAS3, it would be a novel regulator of IRF1 which functions in the absence of PIAS3. Then this could further shed light on the interplay between PIAS3 and IRF1.

The PIAS3 knockdown did not increase cellular resistance to virus infection

(Figure 6). This might be because the changes of the IRF1-mediated antiviral transcription were not enough to increase cellular defense towards virus infection. While the expression of GBP2 (less than 2 folds) and MAP2 (10 folds) was significantly increased in PIAS3 CRISPR clone 1 and clone 6, the other antiviral genes, IFIT2 and STAT2, showed no difference. Here again, global gene expression analysis using RNA sequencing will help us to better understand how much antiviral transcription is modulated by the PIAS3 knockout.

Altogether, we showed PIAS3 indirectly regulates IRF1 expression and transcriptional activities. Whereas, we did not elucidate cellular mechanisms of how PIAS3 regulates IRF1. We sought to detect the direct interaction between the two, yet, we did not find it in the cell line we tested. Although direct interactions are essential for protein sumoylation, it is still reasonable to consider that PIAS3 regulates IRF1 through promoting its sumoylation, presumably in an indirect way. To confirm this, I suggest conducting an *in vitro* sumoylation assay using IRF1 and PIAS3 in the future. Otherwise, it is also possible that PIAS3 indirectly modulates IRF1 through signaling pathways, as they both play parts in the JAK/STAT pathway.[112,113] There might be an unidentified crosstalk between IRF1 and PIAS3 within JAK/STAT pathway. To identify the unknown connection, we will further conduct gain- and loss- of function experiments based on further literature research.

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