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Los Angeles

Systematic Identification of Cellular Regulators of Gammaherpesvirus Lytic Replication

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular and Medical Pharmacology

by

Jun Feng

2012

ABSTRACT OF THE DISSERTATION

Systematic Identification of Cellular Regulators of

Gammaherpesvirus Lytic Replication

by

Jun Feng

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2012

Professor Ren Sun, Chair

Gammaherpesviruses are clinically important human pathogens associated with multiple types of tumors. The life cycle of gammaherpesviruses consists of two distinct phases, latency and lytic replication, both of which are important for the viral-associated pathogenesis. Murine gammaherpesvirus 68 (MHV-68) has been used as a model to study virus-host interactions of human gammaherpesviruses because of its genomic and biological similarities.

During the course of MHV-68 lytic infection, the virus is highly dependent on cellular machinery to facilitate its replication. Host cells, on the other hand, develop mechanisms to sense and restrict virus replication. Here, we present an integrative systems approach to elucidate the interactions between MHV-68 and its host. Our previous yeast-two-hybrid analysis and genome-wide expression profiling implicated hundreds of cellular factors mediating virus-

host interactions. Among the cellular factors identified, those involved in kinase- and transcription factor- regulated signaling are the most highly enriched. Therefore, high-throughput screens of cellular kinome and transcription factors cDNA and siRNA libraries were carried out, which uncovered 125 MHV-68 dependency factors and 213 restriction factors. We found that many cellular factors affect MHV-68 replication by regulating RTA transcription. Characterization of this subset of genes showed they also play roles in KSHV latency maintenance and reactivation. Furthermore, cellular factors regulating later steps of viral replication, such as viral late gene expression and egress, were also identified and validated. Finally we compared the viral restriction factors to the results of a kinome siRNA screen designed to identify cellular regulators of innate immune response to cytosolic DNA. Remarkably, 23 genes were found among the top 10% of the hits of both screens. The MHV-68 restriction factor RIOK3 was confirmed to play a role in IRF3 activation and type I interferon induction mediated by both cytosolic DNA and RNA. RIOK3 is required for cellular antiviral response to both DNA virus (MHV-68) and RNA virus (Influenza A), suggesting a central role in innate immunity. Further analysis revealed that RIOK3 complexes with TBK1 and IRF3, bridging the activity between the two. Collectively, this multilayered approach unfolded a comprehensive map of gammaherpesvirus-host interactions which will provide basis for developing new anti-viral therapies.

The dissertation of Jun Feng is approved.

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2012

This dissertation is dedicated to my parents, Guanxing Feng and Jianying Yang,

for their love, support and encouragement.

TABLE OF CONTENTS

ABSTRACT ii

DEDICATIONS v

TABLE OF CONTENTS vi

LIST OF FIGURES viii

ACKNOWLEDGEMENTS x

VITA xii

PUBLICATIONS AND PRESENTATIONS xii

CHAPTER 1. INTRODUCTION 1

1.1 Herpesviruses 2

1.2 Herpesvirus life cycle 2

1.3 Classification of herpesviruses 3

1.4 Tumor-associated gammaherpesvirus 4

1.5 Murine gammaherpesvirus 68 serves as a small animal model system 6

1.6 Functions of host cellular factors in gammaherpesvirus replication 7

1.7 Bibliography 9

Chapter 2. PTK7 REGULATES HERPESVIRUS EGRESS 14

2.1 Abstract 15

2.2 Introduction 16

2.3 Materials and Methods 18

2.4 Results 22

2.5 Discussion 39

2.6 Bibliography 46

CHAPTER 3. SYSTEMACTIC IDENTIFICATION OF VIRUS-HOST INTERACTIONS THAT REGULATE MHV-68 LYTIC REPLICATION 52
3.1 Abstract 53
3.2 Introduction 54
3.3 Materials and methods 55
3.4 Results 59
3.5 Discussion 76
3.6 Bibliography 79
CHAPTER 4. RIOK3 IS A NOVEL REGULATOR OF TYPE I INTERFERON PATHWAY 82
4.1 Abstract 83
4.2 Introduction 84
4.3 Materials and Methods 86
4.4 Results 90
4.5 Discussion 101
4.6 Bibliography 110
CHAPTER 5. SUMMARY AND PERSPECTIVES 114

LIST OF FIGURES

Figure 2-1. TPL2 enhances MHV-68 lytic replication. 23

Figure 2-2. TPL2 upregulates viral lytic gene expression. 25

Figure 2-3. TPL2 activates MHV-68 Rta promoter via an AP-1 responsive element. 28

Figure 2-4. PTK7 enhances MHV-68 lytic replication through a distinct pathway. 30

Figure 2-5. PTK7 overexpression does not affect MHV-68 viral gene transcription, genome replication and protein expression. 32

Figure 2-6. PTK7 helps MHV-68 release to the supernatant..... 34

Figure 2-7. Kinase domain of PTK7 is dispensable for its role in regulating MHV-68 egress... 36

Figure 2-8. Knockdown of PTK7 increases the cell-associated virus. 38

Figure 2-9. PTK7 regulates HSV-1 egress. 40

Figure 3-1. Gene Ontology (GO) analyses of cellular factor identified from gene expression profiling and Y2H. 60

Figure 3-2. Bioinformatics analyses of cDNA screen result. 63

Figure 3-3. Human kinome siRNA screen for cellular regulator of MHV-68 replication. 66

Figure 3-4. Kinome siRNA screen results with bioinformatics analysis and validation. 67

Figure 3-5. Effect of knocking down MHV-68 restriction factors on viral lytic protein expression. 69

Figure 3-6. To determine the step(s) of MHV-68 replication affected by identified restriction factors..... 71

Figure 3-7. FASTK is required for KSHV latency maintenance. 73

Figure 3-8. Identification of MHV-68 restriction factors involved in the type I IFN production or signaling. 75

Figure 4-1. RIOK3 is required for IFN-β induction by BDNA. 91

Figure 4-2. RIOK3 is required for IFN-β induction by Sendai virus..... 93

Figure 4-3. RIOK3 is required for IFN-β induction by poly(I:C)..... 94

Figure 4-4. Loss of RIOK3 affects host defense..... 96

Figure 4-5. RIOK3 is required for IRF3 activation induced by BDNA or Sendai virus. 97

Figure 4-6. Overexpression of RIOK3 activates IRF3. 98

Figure 4-7. RIOK3 functions downstream of TBK1. 100

Figure 4-8. RIOK3 interacts with IRF3. 102

Figure 4-9. RIOK3 interacts with phosphorylated TBK1, and RIOK3 is an adaptor protein that bridges TBK1 and IRF3 upon stimulation..... 103

Figure 4-10. Proposed models. 106

Figure 4-11. RIOK3 is required for BDNA-mediated IFN-β induction in THP-1 cells..... 108

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CHAPTER 1

INTRODUCTION

1.1 Herpesviruses

Herpesviruses are large, double-stranded DNA viruses that are ancient and widely disseminated in nature [1]. The herpesvirus virion is composed of an enveloped icosadeltahedral capsid encompassing a linear viral genome [1]. The envelope is derived from cellular membranes and contains numerous protrusion of glycoproteins [2]. Tegument is an amorphous, sometimes asymmetric structure existing between the capsid and the envelope [1]. The herpesvirus genome ranges from 120 to 250 kilobases (kb) encoding between 70 to 200 genes, and the size of a mature herpesvirus particle varies from 120 nm to 300 nm in diameter with a capsid around 100 nm [1].

All members of the herpesvirus family share four significant biologic properties: encoding a large array of enzymes homologous to cellular counterparts; viral DNA synthesis and capsid assembly in the nucleus, production of infectious progeny virus accompanied by the destruction of infected cells, and establishing latent infection in a specific type of cells [1].

1.2 Herpesvirus life cycle

All herpesviruses have two distinct phases in their life cycle, lytic replication and latency, as characterized by different gene expression profiles. During latency, the viral genome remains as a non-integrated episome in the host cell, and only a subset of genes (latent genes) are expressed [1]. When the host cell replicates, the circular viral genome can also replicate to maintain its latent load in the host. Latency is a central feature of all herpesviruses, since efficient establishment of latency allows the viral genome to persist despite host immune responses to many viral antigens and other potentially adverse signals in the microenvironment [3]. Periodically latent viruses can reenter the lytic replication phase, a phenomenon termed

reactivation. Upon reactivation, viral genes are extensively expressed in a highly regulated cascade (involving immediate-early, early and late genes), leading to the production of infectious viral particles and ultimately resulting in the lysis of infected cells. Viruses that reactivate from latency back into the lytic replication cycle can generate new infections and ultimately replenish the pool of latently-infected cells, further ensuring the chronic maintenance of the herpesvirus infection. With these two phases of life cycle, herpesviruses can establish a life-long persistent infection in the host [4-6].

1.3 Classification of herpesviruses

Members of the herpesvirus family are classified into three subfamilies: Alphaherpesviruses, Betaherpesviruses and Gammaherpesviruses [1]. The classification was made originally based on the biologic properties of the viruses, however, with the advent of genome sequencing, the assigned classes of each known herpesvirus are now known to correlate well with their genome sequence arrangement [1]. Eight human herpesviruses have been identified so far, which are subdivided into alpha-, beta-, and gamma-herpesviruses. Alphaherpesviruses (Herpes simplex 1 and 2 (HSV-1, HSV-2) and Varicella zoster virus/VZV) in general have a variable host range and relatively short reproductive cycle. They spread rapidly in culture, destruct infected cells efficiently, and establish latent infections primarily in neuronal cells [1]. Betaherpesviruses (Cytomegalovirus/HCMV, HHV-6 and HHV-7) have restricted a host range, long reproductive cycle, and they establish latency in secretory glands, lymphoreticular cells and several other tissues [1]. Gammaherpesviruses (Epstein Barr virus/EBV/HHV-4 and Kaposi's sarcoma-associated herpesvirus/KSHV/HHV-8) have the narrowest host range, establish latency in lymphoid tissues and have specific preference for either T or B lymphocytes [7].

Gammaherpesviruses are uniquely associated with lymphoproliferative diseases and lymphomas [7].

1.4 Tumor-associated gammaherpesvirus

Members of the gammaherpesvirus subfamily are distinct in their ability to establish long-term latency in lymphocytes and to cause benign or malignant tumors in latently-infected hosts [5]. Gammaherpesviruses can be further divided into four genera – the well-established Lymphocryptovirus and Rhadinovirus and the more recently defined Macavirus and Percavirus [8]. Of relevance to the human gammaherpesviruses: EBV is a lymphocryptovirus, while KSHV is a rhadinovirus.

EBV is one of the most common human viruses. Most people become infected with EBV sometime during their lives[9]. In the United States, about half of all five-year-old children and 90 to 95 percent of adults between 35 and 40 years of age have been infected [9, 10]. Many children become infected with EBV, and these infections usually cause no symptoms or are indistinguishable from the other mild, brief illnesses of childhood [10, 11]. In the United States and in other developed countries, many persons are not infected with EBV in their childhood years [11, 12]. When infection with EBV occurs during adolescence or young adulthood, it causes infectious mononucleosis 35% to 50% of the time [11, 12]. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma and Hodgkin's disease [13-17].

KSHV was recently identified in 1994 from a KS lesion in an AIDS patient [18]. The seroprevalence of KSHV is low (<5%) in most Western populations but the infection is quite common throughout sub-Saharan Africa [19]. KSHV is the etiologic agent of three types of

human tumors: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) [18-23].

Unlike alphaherpesviruses and betaherpesviruses, the pathogenesis of gammaherpesviruses is closely linked to latency. This is evidenced by the fact that PEL cells are predominantly infected with a latent form of KSHV, expressing only very few viral genes, such as LANA, v-cyclin, vFLIP, Kaposin, vIRF2, vIRF3 and viral miroRNAs [23-30]. Nonetheless these latent viral genes can promote cell survival and increase transformation potential [31-39]. Although latency and latent gene expression are essential for the survival of cells latently infected with gammaherpesviruses, more and more evidence suggests that lytic reactivation also plays critical roles in tumorigenesis. KSHV RTA transactivates the promoters of many viral genes that might have important functions in pathogenesis, including K12/Kaposin, vIL-6, vMIP-I, vIRF-1, vGPCR, and K1 [40-44]. The upregulation of these viral lytic genes is capable of inducing signaling through a paracrine fashion to promote cell proliferation, protect cells from apoptosis, and facilitate angiogenesis. Alternatively, lytic replication with slow kinetics or abortive replication can also enhance cell growth in a cell autonomous manner [45, 46]. Consistent with this notion, spontaneous reactivation in a small percentage of cells was observed in both KS specimen and infected PEL and endothelial cells [47]. In addition, many cytokines expressed in KS lesions are known to reactivate KSHV, further supporting a role for lytic reactivation in pathogenesis [48, 49].

In spite of their medical importance, the studies of human gammaherpesvirus infection have been limited mostly to *in vitro* experiments because of their restricted host range [19, 50]. Although EBV and KSHV are able to infect human B lymphocytes and some epithelial and endothelial cell lines, they do not undergo robust *de novo* lytic replication but rather enter latency, and very few

viral progeny are produced. Therefore, the analysis of lytic gene functions in the context of natural infection has been largely limited. This then limits the study of virus-host interactions controlling pathogenesis.

1.5 Murine gammaherpesvirus 68 serves as a small animal model system

The identification of gammaherpesviruses in murid rodents offered the possibility of developing a mouse model of gammaherpesvirus pathogenesis. Murine gammaherpesvirus 68 (MHV-68) was isolated from bank voles (*Myodes glareolus*) and yellow-necked field mice (*Apodemus flavicollis*) captured in Slovakia, and it is infectious to laboratory mice [51, 52]. Like KSHV, MHV-68 is a rhadinovirus and is genetically and biologically closely related to primate gammaherpesviruses, including human viruses KSHV and EBV [53]. MHV-68 encodes about 80 genes [53]. A number of genes are involved in latency and reactivation, such as LANA, v-cyclin, v-Bcl-2, and RTA, that are well conserved with primate rhadinoviruses [7]. Several MHV-68 unique genes are not required for replication, and they likely play a role in chronic infection *in vivo* [7]. Following intranasal infection, MHV-68 establishes acute productive infection in the lung epithelial cells and subsequent latent infection primarily in B cells [7, 54]. Because MHV-68 infects mice, the study of viral gene functions in chronic infection and host response to viral infection *in vivo* is made possible. MHV-68 infection, like that of its human virus counterparts, leads to splenomegaly and lymphoproliferative diseases [55, 56]. The infection also promotes the development of lymphomas and increases the frequency of tumor development in immune-deficient mice [57]. Collectively, the characteristics of *in vivo* pathogenesis make MHV-68 an appropriate model to study KSHV and EBV infection.

In addition, MHV-68 is able to establish a productive infection in fibroblast and epithelial cell lines derived from several mammalian species, including humans, allowing the analysis of productive viral lytic cycle *in vitro* and genetic manipulation of MHV-68 [51, 58, 59].

Taken together, MHV-68 has provided us an excellent model to study virus-host interactions and to develop therapeutic strategies against gammaherpesvirus associated diseases.

1.6 Functions of host cellular factors in gammaherpesvirus replication

Like all other herpesviruses, the gammaherpesvirus can establish life-long persistent infection in the host. In the past, tremendous efforts have been made to identify the viral determinants of chronic infection; however, viruses cannot reproduce on their own but instead are only able to replicate by infecting a host cell [60, 61]. Due to the limited genome size, successful completion of each step of viral replication not only requires the coordinated interactions among the viral proteins, but also highly depends on the assistance of a large number of cellular factors. While the viruses exploit host cell functions, host cells are capable of using complex surveillance mechanisms to sense the invading virus and restrict its replication. The innate immune response, for example, plays a critical role in controlling the lytic MHV-68 replication. However, not surprisingly, the virus has also evolved the ability to effectively evade and/or sabotage cellular defense mechanisms. Several studies have identified KSHV and MHV-68 viral proteins involved in the inhibition of the host innate and adaptive immune responses. In particular, KSHV and MHV-68 ORF36 bind to phosphorylated IRF3, thus inhibiting the production of interferon-β (IFNβ) [62]. KSHV ORF45 interacts with and inhibits IRF7 from entering the cell nucleus [63]. KSHV and MHV-68 ORF54 inhibit type I IFN signaling by inducing the degradation of the type I IFN receptor IFNAR1 [64]. The virus-host interaction is not one-way

but rather dynamic and complex, and a delicate balance between virus and host ensures the survival of both. Identifying additional cellular or viral factors contributing to the maintenance of this balance would add tremendous understanding to gammaherpesvirus persistent infection and pathogenesis.

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Chapter 2

PTK7 REGULATES HERPESVIRUS EGRESS

2.1 Abstract

Like all other viruses, the herpesvirus utilizes various cellular proteins for successful infection and replication. To elucidate the roles cellular factors play in regulating gammaherpesvirus lytic replication, we conducted a functional screen of a cellular kinase and transcription factor library and identified multiple kinases as positive regulators of murine gammaherpesvirus 68 (MHV-68) replication. More extensive analysis was carried out on two of the most potent enhancers, TPL2 and PTK7. TPL2 and PTK7 regulate MHV-68 replication through two distinct pathways. TPL2 enhances MHV-68 lytic replication by upregulating lytic gene expression and promoter activities of viral lytic genes including RTA and ORF57. We showed that TPL2 stimulates the promoter activities of key viral lytic genes including RTA and ORF57 in an AP-1-dependent manner. We further identified an AP-1 responsive element on the MHV-68 RTA promoter as the cis element mediating the upregulation of RTA promoter activity by TPL2. PTK7, on the other hand, enhances virus production without affecting viral transcript or intracellular protein levels of immediate-early, early, and late genes. We found that PTK7 overexpression increases extracellular virus production but not cell-associated virus. In contrast, knocking down endogenous PTK7 using shRNA increased cell-associated virus level. Furthermore, we also showed that ectopic expression of PTK7 promotes herpes simplex virus-1 (HSV-1) egress in 293T cells. Thus we have identified a tyrosine kinase PTK7, which is conserved from frog to human, as a novel cellular facilitator of herpesvirus egress. Through the functional screen, we were able to identify novel cellular signaling pathways that mediated MHV-68 lytic replication at various stages.

2.2 Introduction

Gammaherpesviruses are a family of large, membrane-enveloped, double-stranded DNA viruses, including Epstein-Barr (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), herpesvirus saimiri, and murine gammaherpesvirus 68 (MHV-68, or γHV-68). Human gammaherpesviruses EBV and KSHV are associated with a number of malignancies. EBV is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and Hodgkin's disease [1-5]. KSHV is the etiological agent of three types of human tumors: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and a plasmablastic variant of multicentric Castleman disease (MCD) [6-11]. Gammaherpesviruses, like other herpesviruses, have two phases in their life cycle: latency and lytic replication [12]. Although latent infection is essential for gammaherpesvirus-associated tumorigenesis, lytic reactivation and lytic replication are also believed to play important roles in the persistent infection of gammaherpesviruses and the associated pathogenesis [13]. However, the study of lytic replication of the human gammaherpesviruses is largely hampered by the lack of a cell culture system that can support robust lytic replication of EBV and KSHV. MHV-68, a rodent gammaherpesvirus, shares sequence homology with EBV and KSHV and is able to undergo efficient lytic replication in a number of common cell lines, including those of human origins. It therefore provides a system to effectively study gammaherpesvirus lytic replication *in vitro* and *in vivo* [14-16]. Upon *de novo* lytic infection of MHV-68, the virus capsid presumably utilizes the cellular cytoskeleton to navigate to the host nucleus where it injects the viral genome [17, 18]. Viral immediate early (IE) and early genes (E) are expressed prior to DNA synthesis, after which viral DNA replicates in a rolling circle mechanism in the nucleus. Finally viral late genes (L) are expressed. The mRNA transcripts of all transcribed viral genes are still exported to the cytoplasm for translation and processing, and some viral proteins re-enter the nucleus. Viral

capsid assembly occurs in the nucleus, and the envelope from the nuclear membrane is acquired as the viral progeny exit the nucleus. Finally, the host cell is destroyed upon completion of viral progeny production and exit from the infected cell [12, 19, 20]. IE transcription factor RTA is the critical viral gene initiating the whole lytic cascade, and it is highly conserved between rodent and human gammaherpesviruses [21-25]. Early genes encode proteins important for viral genomic DNA replication, which is required for the expression of late genes, many of which encode structural proteins [26, 27].

Many questions still remain regarding the regulation of gammaherpesvirus lytic replication, one critical aspect of which is the roles that cellular genes play. As with all other viruses, gammaherpesviruses rely on cellular machineries for replication and propagation. For example, several cellular genes have been shown to mediate the KSHV entry in different types of cells [28-30]. Other cellular genes such as Topoisomerases (Topo) I, II, and Poly(ADP-ribose) polymerase 1 (PARP-1) were shown to function during KSHV lytic DNA replication [31]. While viruses exploit host cell functions, host cells in turn are capable of evolving mechanisms, such as type I interferon response, to sense and restrict the invading virus.

Despite the progress made, most of the identified cellular factors affect gammaherpesvirus lytic replication by regulating the expression or function of RTA due to its the essential role in activation of the lytic gene expression cascade. Less is known about cellular factors' functions during later steps of gammaherpesvirus lytic replication such as viral DNA replication, late gene expression, virus assembly, and egress.

Kinases and transcription factors are two of the largest families of genes in eukaryotes. These critical cellular proteins regulate many aspects of cell homeostasis, including cell survival, proliferation, differentiation, and metabolism. Therefore, gammaherpesviruses are likely to be

regulated by kinases and transcription factors. Here, we present a kinome and transcription factor cDNA library screen to unbiasedly identify cellular genes regulating MHV-68 lytic replication. Through the screen, we identified multiple kinases that can positively regulate MHV-68 replication. We then conducted more extensive analyses on two of the most potent enhancers, TPL2 and PTK7. TPL2 and PTK7 regulate MHV-68 replication through two distinct pathways: TPL2 enhances MHV-68 lytic replication by upregulating viral lytic gene expression, and PTK7 enhances MHV-68 lytic replication by regulating viral egress.

2.3 Materials and Methods

Cells

HEK293T cells and Vero cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. All cell lines were cultured in a humidified 5% CO_2 atmosphere at 37°C.

Full-length cDNA expression library and plasmids

MHV-68 RTA promoter reporter construct pBLRP was derived by cloning a 1127 bp region upstream of the RTA start codon into the pBL plasmid. Serial 5' deletion clones of the reporter construct were derived from pBLRP. RP-M1 and RP-M2 were constructed by mutating the AP-1 element on the RTA promoter using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). pGL3/M3promoter-LUC was described earlier [19]. TPL2 and PTK7 expression plasmids contain cDNA of each human gene. shRNA lentiviral vectors were constructed by annealing 2 DNA oligos containing target sequences and cloning into the pLKO.1-TRC vector (Addgene) following the instructions from the manufacture. The sequences of the oligos are 5'-

CCG GAA GCA GCC GTC CTC CCA GGA TCT CGA GAT CCT GGG AGG ACG GCT GCT TTT TTT G -3' (shPTK7-1 forward), 5'- AAT TCA AAA AAA GCA GCC GTC CTC CCA GGA TCT CGA GAT CCT GGG AGG ACG GCT GCT T -3' (shPTK7-1, reverse), 5'- CCG GAA GAA TGG GAC CTT GCG CAT CCT CGA GGA TGC GCA AGG TCC CAT TCT TTT TTT G -3' (shPTK7-2, forward), 5'- AAT TCA AAA AAA GAA TGG GAC CTT GCG CAT CCT CGA GGA TGC GCA AGG TCC CAT TCT T -3' (shPTK7-2, reverse), 5'- CCG GCC TGA GGA TTT CCA AGA GCA ACT CGA GTT GCT CTT GGA AAT CCT CAG GTT TTT G -3' (shPTK7-3, forward), 5'-AAT TCA AAA ACC TGA GGA TTT CCA AGA GCA ACT CGA GTT GCT CTT GGA AAT CCT CAG G -3' (shPTK7-3, reverse), 5'- CCG GCA ACA AGA TGA AGA GCA CCA ACT CGA GTT GGT GCT CTT CAT CTT GTT GTT TTT G -3' (shCtrl forward), 5'- AAT TCA AAA ACA ACA AGA TGA AGA GCA CCA ACT CGA GTT GGT GCT CTT CAT CTT GTT G -3' (shCtrl reverse). Full-length kinase and transcription factor cDNA expression library was constructed by OriGene Technologies. Full length PTK7 expression plasmid was generated by PCR using 5'- CAC CAT GGG AGC TGC GCG GGG AT -3' (forward) and 5'- CGG CTT GCT GTC CAC GGT GC -3' (reverse) primers, cloned into pENTR/D-TOPO vector and transferred to different expression plasmids. PTK7 mutants were generated similarly to the full-length PTK7 using the following primers: PTK7-IN forward 5'- CAC CAT GAA GAA GCG CTG CAA AGC CAA -3', reverse 5'- CGG CTT GCT GTC CAC GGT GC -3'; PTK7-DIN forward 5'- CAC CAT GGG AGC TGC GCG GGG AT -3', reverse 5'- CTT GCA GTA GAA CAT GAG GCC C -3'; PTK7-DKD forward 5'- CAC CAT GGG AGC TGC GCG GGG AT -3', reverse 5'- CTT ATC ACT TGT GCT GTG GCG -3'.

Cell transfection

An appropriate number of cells were seeded onto each well of a 48-well plate so that cells were 80-90% confluent at the time of transfection. Cells were transfected with expression plasmids, reporter plasmids, or shRNA vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations.

Viruses and plaque assays

MHV-68 was originally obtained from the American Type Culture Collection (VR1465). The recombinant MHV-68-M3/FL virus was constructed by S. Hwang by integrating to the left side of the wild type MHV-68 viral genome a firefly luciferase gene driven by the viral M3 promoter [83]. Virus stocks were grown by infecting Vero cells at a multiplicity of infection (MOI) of 0.1. The titers of the produced viruses were determined by plaque assays, using a monolayer of Vero cells overlaid with 1% methylcellulose. After 6 days of infection, the cells were fixed and stained with 2% crystal violet in 20% ethanol and plaques were counted to determine the titers.

Quantitating MHV-68-M3/FL virus titer with Firefly luciferase assay

1.5×10^3 293T cells were seeded in 80 μl of medium into each well of a 96-well plate one day prior to infection. 40 μl of different dilutions of samples containing MHV-68-M3FL virus were added to each well. Luciferase activities were determined using the Bright-Glo Luciferase Assay System (Promega).

Luciferase reporter assay

HEK293T cells were seeded onto 48-well plates 24 hours prior to transfection so that cells were ~90% confluent by the time of transfection. Cells were then transfected with a DNA mixture

containing 1 ng MHV-68 RTA firefly luciferase reporter plasmid together with 0.1 ng pRL-SV40 Renilla luciferase plasmid and cDNA expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The total amount of DNA transfected for each well was adjusted to 0.4 μg using appropriate control plasmids. Cells were cultured for an additional 24 hours and dual-luciferase assays were performed using a kit provided by Promega.

RT-Q-PCR analysis

HEK293T cells were transfected with expression plasmids as previously described. At 24 hours post-transfection, cells were infected with MHV-68 at an MOI of 0.5 or 1. Cells were collected at different time points post-infection and RNA was purified using PureLink RNA Mini Kit (Invitrogen) and reverse transcribed into cDNA with qScript cDNA Synthesis Kit (Quantas). RT-PCR was then done with the following primers to quantify MHV-68 lytic gene transcripts: ORF50 forward 5'- GAT TCC CCT TCA GCC GAT AAG -3', reverse 5'- CAG ACA TTG TAG AAG TTC AGG TC -3'; ORF6 forward 5'- ATG TCT CCC CAT ATT CTT GC -3', reverse 5'- ACA TGG AAG TGT TGG CTG T -3'; ORF29 forward 5'- TCC AGC ACC TTG ATG TGT GC -3', reverse 5'- TTG CTG GAA GTG CTT CCT TCT -3'. GAPDH transcript was measured using primers, forward 5'- TGC ACC ACC AAC TGC TTA GC -3' and reverse 5'- GGC ATG GAC TGT GGT CAT GAG -3', as an internal control

Quantitation of viral genome copies

HEK293T cells were transfected with expression plasmids as previously described. At 24 hours post-transfection, cells were infected with MHV-68 at an MOI of 0.1. Cell lysates or cell culture supernatants were collected at different time points post-infection and total DNA was extracted

by the phenol-chloroform protocol. Viral genome copies were determined by Q-PCR using primers: 5'- ACC TTG AAA CCC GTG AAG G -3' and 5'- CAT CTG CCA CGA CCT GAG AT -3'. MHV-68 BAC DNA (10^0 -10^7 copies) was included as a standard for copy number determination.

Immunoblotting and antibodies

For immunoblotting, cells were directly lysed on the plate with 1x protein sample buffer (0.625 M Tris pH 6.8, 2% SDS, 10% glycerin, 5% β-mercaptoethanol, 0.002% Bromophenol blue), transferred to Eppendorf tubes, and boiled for 10 minutes prior to SDS-PAGE. The antibodies used in this study were as follows: mouse anti-V5 (Invitrogen, R960-25), mouse anti-actin (Sigma A5316), and mouse anti-PTK7 (Sigma WH0005754M2). Rabbit hyperimmune serum against MHV-68-infected rabbit cells [84], ORF26 and ORF65 were derived in our lab. Secondary antibodies conjugated to HRP were donkey anti-rabbit IgG (GE Healthcare NA934V) and sheep anti-mouse IgG (GE Healthcare NXA931).

2.4 Results

2.4.1 Kinome and transcription factor cDNA library screen for cellular regulator of MHV-68 replication

To systematically study the effects of cellular gene expression on MHV-68 lytic replication, we screened a library of 353 kinase and 700 transcription factors for those that could enhance or inhibit MHV-68 lytic replication. The overall scheme of the screen is illustrated in Figure 2-1A. Briefly, we first reverse-transfected 60 ng of individual kinase or transcription factor expression plasmid pre-dotted in wells of a 384-well plate into HEK293T cells. Since plasmid DNA had

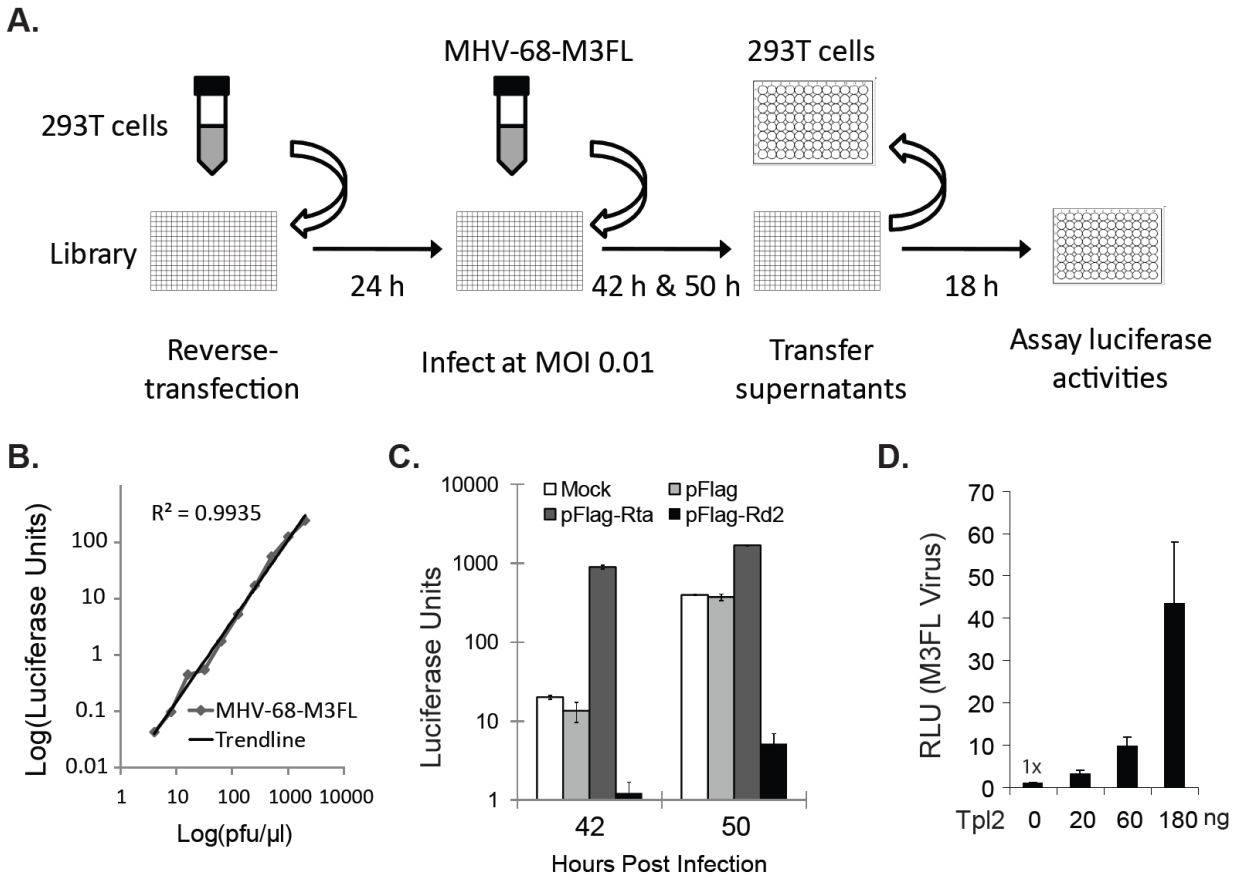

Figure 2-1. TPL2 enhances MHV-68 lytic replication. **(A)** Overall scheme of the screen. Individual kinase expression plasmid pre-dotted on a 384-well plate was reverse-transfected into HEK293T cells. Cells were infected with MHV-68-M3/FL at an MOI of 0.01 at 24 hr post transfection. At 42 and 50 hr post infection, 50 µl of supernatants were transferred to naive HEK293T cells that were seeded on 96-well plates (hereafter referred to as destination plates) one day prior to transfer. Luciferase activities of HEK293T cells on the destination plates were measured at 18 hr post transfer. **(B)** Luciferase activities as indicators of MHV-68-M3/FL titers. HEK293T cells were infected with serially diluted MHV-68-M3/FL viruses. Luciferase activities were determined at 18 hr post infection and plotted against titers determined by plaque assays. Representative results are presented as the means for triplicates. **(C)** Effects of RTA and a dominant-negative mutant of RTA on MHV-68-M3/FL lytic replication. HEK293T cells were mock transfected or transfected with indicated plasmids, followed by MHV-68-M3/FL infection, supernatant transfer, and luciferase assays of cells on the destination plate as described in **(A)**. **(D)** Dose response of MHV-68-M3/FL lytic replication to TPL2 transfection. Virus production at 42 hr post infection from HEK293T cells transfected with indicated amounts of the TPL2 plasmid was determined as in **(C)**.

been pre-dotted into wells of the 384-well plate, reverse-transfection was more suitable for our screen than conventional transfection due to the reverse order of addition of DNA and adherent cells. We used HEK293T cells for the screen because HEK293T cells can be transfected at high efficiency using typical liposomal transfection reagents (>90% cells as determined by EGFP fluorescence microscopy after cells were transfected with a plasmid encoding EGFP, pEGFP-N1, data not shown). More importantly, HEK293T cells support robust replication of MHV-68. We then infected cells with a recombinant MHV-68 reporter virus, MHV-68-M3/FL, that encodes firefly luciferase under the viral lytic gene M3 promoter. Cells were then infected at an MOI of 0.01. 42 and 50 hours later, supernatants containing viruses produced and released by transfected HEK293T cells were transferred to fresh HEK293T cells. The relative amounts of viruses in the transferred supernatants were then determined by assaying luciferase activities 18 hours after supernatants transfer. The luciferase activities have a near linear relationship with virus titers determined by plaque assays across a wide range of virus titers and were therefore used to indicate the relative amounts of virus (Figure 2-1B). The use of MHV-68-M3/FL in combination with luciferase assays enabled us to quickly (1 day instead of 6 days required for conventional plaque assays) determine the relative amounts of viruses in the supernatants in a much less laborious way and is therefore suitable for our medium-throughput screen. We used RTA and Rd2, a dominant negative version of RTA, as positive and negative regulators of MHV-68 lytic replication, respectively, to optimize the screen conditions. Under the optimized conditions, RTA and Rd2 significantly increased and decreased the luciferase readings respectively, indicating that RTA enhanced, whereas Rd2 inhibited, virus lytic replication and production (Figure 2-1C). RTA and Rd2 were subsequently used as controls in our screen.

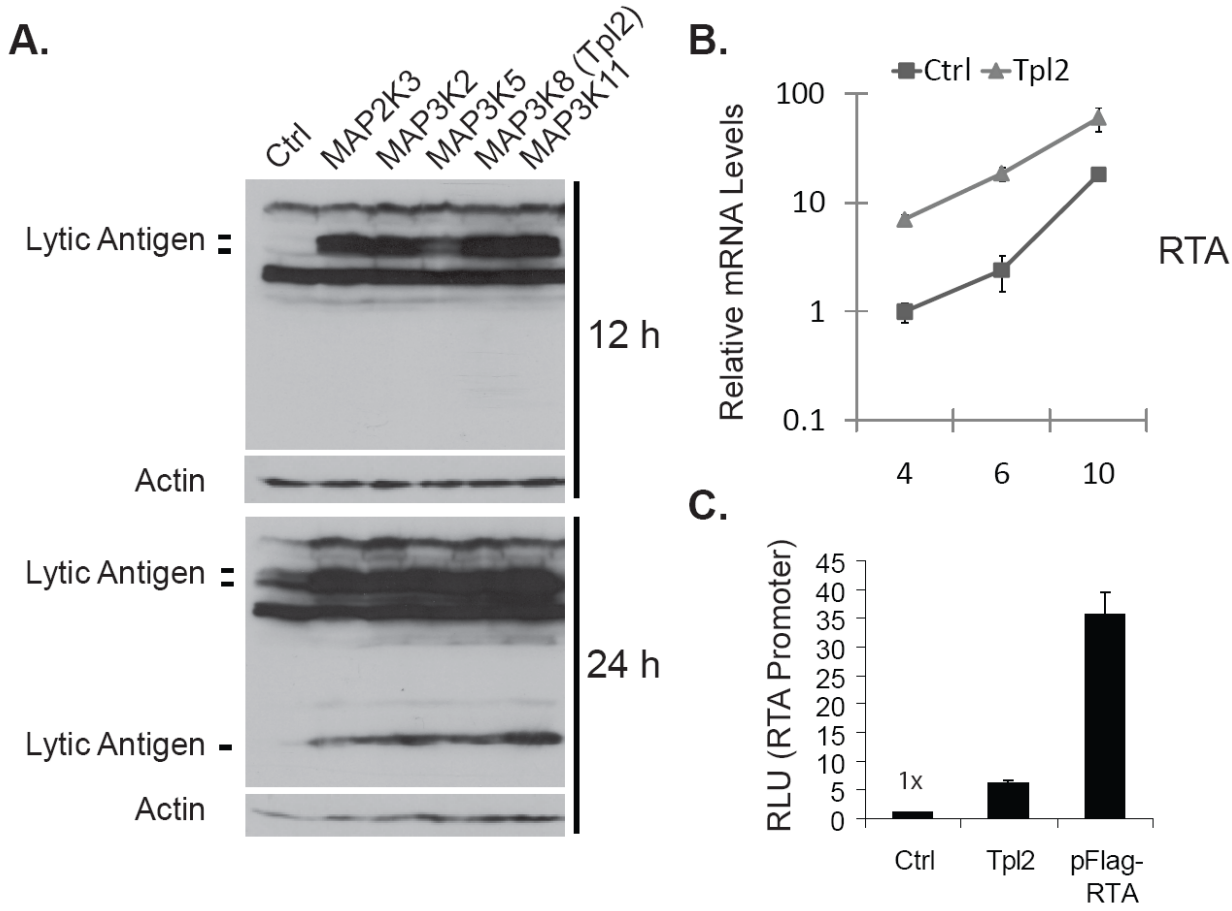

Figure 2-2. TPL2 upregulates viral lytic gene expression. (A) Western blotting analysis of MHV-68 lytic protein expression in cells expressing various kinases. HEK293T cells were transfected with individual kinase expression plasmids and infected with MHV-68 at an MOI of 1. At 12 or 24 hr post infection, whole-cell lysates were analyzed by western blotting for MHV-68 lytic antigen expression using rabbit hyperimmune serum against MHV-68-infected rabbit cells. Actin immunoblot is shown as a loading control. **(B)** TPL2 increased the mRNA levels of RTA. HEK293T cells were transfected with TPL2 or the backbone control plasmid and infected with MHV-68 at an MOI of 1 at 24 hr post transfection. The relative mRNA levels of RTA normalized to actin were determined by reverse-transcription quantitative PCR at indicated time points post infection. **(C)** TPL2 activates MHV-68 RTA promoter. HEK293T cells were cotransfected with pBLRP, a luciferase reporter construct containing approximately 1.1-kb upstream sequence of RTA, the control plasmid SV40-RL (Renilla luciferase), and TPL2, pFlag-Rta, or the control backbone plasmid. Luciferase activity was determined at 24 hr post transfection. Results are presented as the means for triplicates with their respective standard deviations.

2.4.2 TPL2 enhances MHV-68 lytic replication

Through this screen, we found that several members of the mitogen-activated protein kinase (MAPK) family including MAP2K3, MAP3K2, MAP3K5, MAP3K8/TPL2, and MAP3K11 enhanced MHV-68 lytic replication when overexpressed (Figure 2-2A and data not shown). We focused on one of the strongest stimulators, MAP3K8/TPL2 (hereafter referred to as TPL2), to study the mechanism by which it enhances MHV-68 lytic replication. We confirmed the ability of TPL2 expression to promote viral lytic replication in a secondary screen of the hits from our primary screen, and we showed that TPL2 expression enhanced MHV-68 lytic replication in a dose-dependent manner (Figure 2-1D).

2.4.3 TPL2 upregulates viral lytic gene expression

To determine which step(s) of virus replication is affected by TPL2 expression, we first examined the effect of TPL2 expression on MHV-68 lytic protein expression during virus replication (Figure 2-2A). Cells were transfected with plasmids encoding kinases or the control backbone plasmid. At 24 hours post-transfection, cells were infected with MHV-68 at an MOI of 1. At both 12 hours and 24 hours post-infection, the expression of TPL2 as well as other kinases examined enhanced MHV-68 lytic protein expression (Figure 2-2A). These results suggest that TPL2 may promote virus replication through increasing the levels of viral lytic proteins. We then asked if TPL2 enhanced virus lytic gene expression at the mRNA level. RTA, a transcription factor encoded by MHV-68, is necessary and sufficient to initiate MHV-68 lytic replication during both de novo infection and lytic reactivation. We therefore examined the effects of TPL2 expression on the mRNA levels of RTA during MHV-68 lytic replication (Figure 2-2B). Cells transfected with a TPL2 expression plasmid or the control plasmid were

infected with MHV-68 at an MOI of 1. Cells were collected at 4, 6, and 10 hours post-infection. The mRNA levels of RTA were then measured with reverse transcription quantitative PCR (RT-Q-PCR). TPL2 increased the mRNA levels of RTA up to 7-fold at as early as 4 hours post-infection (Figure 2-2B). The mRNA levels of RTA in TPL2-transfected cells remained significantly higher than in those from the control transfection at 6 and 10 hours post-infection. RTA transactivates the expression of other viral genes, including viral early genes, which are required for viral DNA replication and the expression of late genes. Not surprisingly, the mRNA levels of open reading frame 6 (ORF6), a viral early gene, and M9 (or ORF65), a viral late gene also increased in cells transfected with TPL2 than in those transfected with the control plasmid (data not shown).

We then asked if TPL2 enhanced RTA mRNA levels through upregulating RTA transcription. We used luciferase reporter assays to determine if TPL2 expression affects RTA promoter activity (Figure 2-2C). Cells were cotransfected with TPL2 or the control plasmid together with a RTA promoter luciferase reporter plasmid. Luciferase activities were determined at 24 hours post-transfection. TPL2, as well as RTA, significantly enhanced MHV-68 RTA promoter activity (Figure 2-2C), indicating that TPL2 enhances RTA mRNA levels through activating RTA transcription. We concluded that TPL2 enhances MHV-68 lytic gene expression and enhances viral lytic replication.

2.4.4 TPL2 activates the MHV-68 RTA promoter via an AP-1 responsive element

Because of the critical role of RTA in controlling the whole cascade of viral lytic gene expression, we asked if a cis-element in the RTA promoter region mediates the enhancement of RTA promoter activity by TPL2. We measured the effects of TPL2 expression on a series of

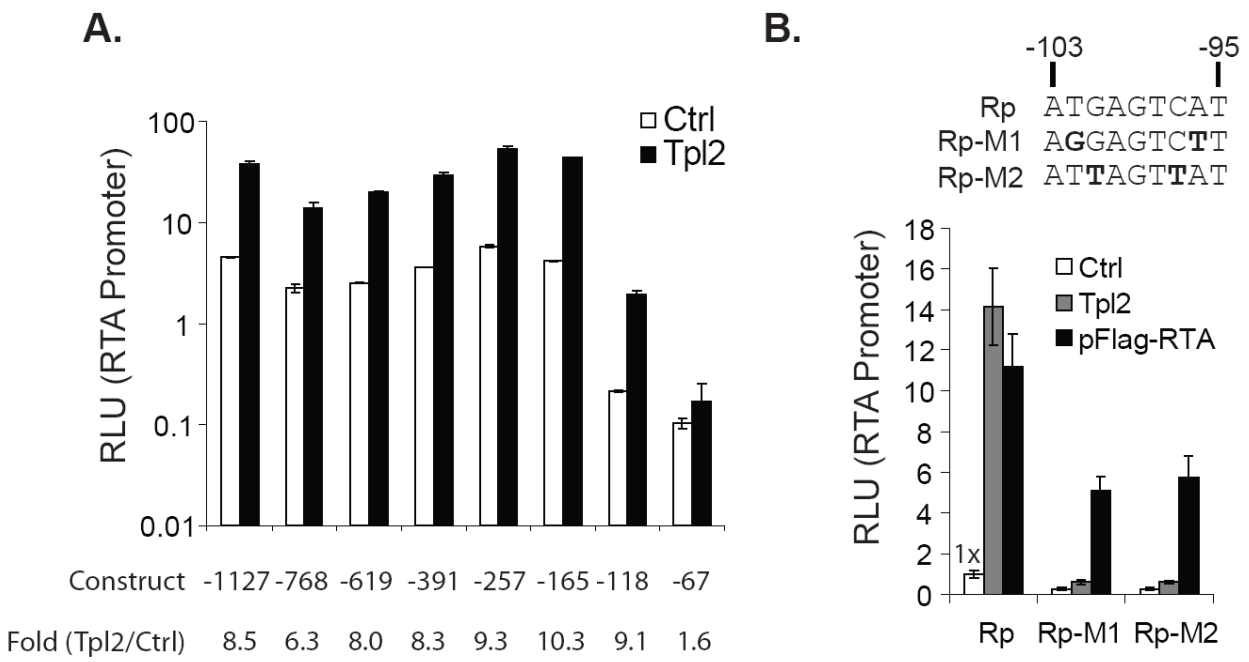

Figure 2-3. TPL2 activates MHV-68 Rta promoter via an AP-1 responsive element. (A) HEK293T cells were cotransfected with RTA promoter luciferase constructs pBLRP with serial 5' deletions and SV40-RL, a control Renilla luciferase plasmid and TPL2 or the control backbone plasmid. Luciferase activity was determined at 24 hr post transfection. **(B)** HEK293T cells were cotransfected with mutant pBLRP with nucleotides in the AP-1 elements mutated and SV40-RL, a control Renilla luciferase plasmid and TPL2, pFlag-Rta, or the control backbone plasmid. Luciferase activity was determined at 24 hr post transfection. Upper panel shows the mutations introduced to pBLRP. Results are presented as the means for triplicates with their respective standard deviations.

RTA promoter luciferase reporter constructs with increasing lengths of deletions from the 5' end (Figure 2-3A). We found that the deletion of a region encompassing 67 bp to 118 bp upstream of the RTA ATG start codon greatly reduced the activation of RTA promoter by TPL2 from 9.1 fold to 1.6-fold (Figure 2-3A). Examination of this region revealed an AP-1 motif (ATGAGTCAT) spanning the -103 bp to -95 bp region (Figure 2-3B, upper panel). We previously showed that TPL2 expression enhanced AP-1 activity and activated the RTA promoter in an AP-1 dependent manner [32]. Therefore this AP-1 motif may mediate the activation of RTA promoter by TPL2. To test this, we constructed two RTA promoter reporter constructs which each had two nucleotides within the AP-1 motif mutated (Figure 2-3B, upper panel). Both sets of double mutations largely abolished the ability of TPL2 to activate the RTA promoter (Figure 2-3B, lower panel), indicating that this AP-1 motif mediates the activation of RTA promoter by TPL2. We also noted that the mutant promoters had lower activities than the wild type promoter when cells were transfected with either the control plasmid or the RTA expression plasmid (Figure 2-3B, lower panel). However the relative folds of promoter activation by RTA did not decrease when the AP-1 motif was mutated, indicating that the AP-1 motif specifically mediates the activation of the RTA promoter by TPL2, not RTA (Figure 2-3B, lower panel).

2.4.5 PTK7 enhances MHV-68 lytic replication through a distinct pathway

From the same screen, we identified another kinase, PTK7, as a positive regulator for MHV-68 replication. We confirmed the ability of PTK7 expression to promote viral lytic replication in the secondary screen and showed that PTK7 overexpression can also enhance MHV-68 lytic replication in a dose-dependent manner (Figure 2-4A). Interestingly, when we expressed other

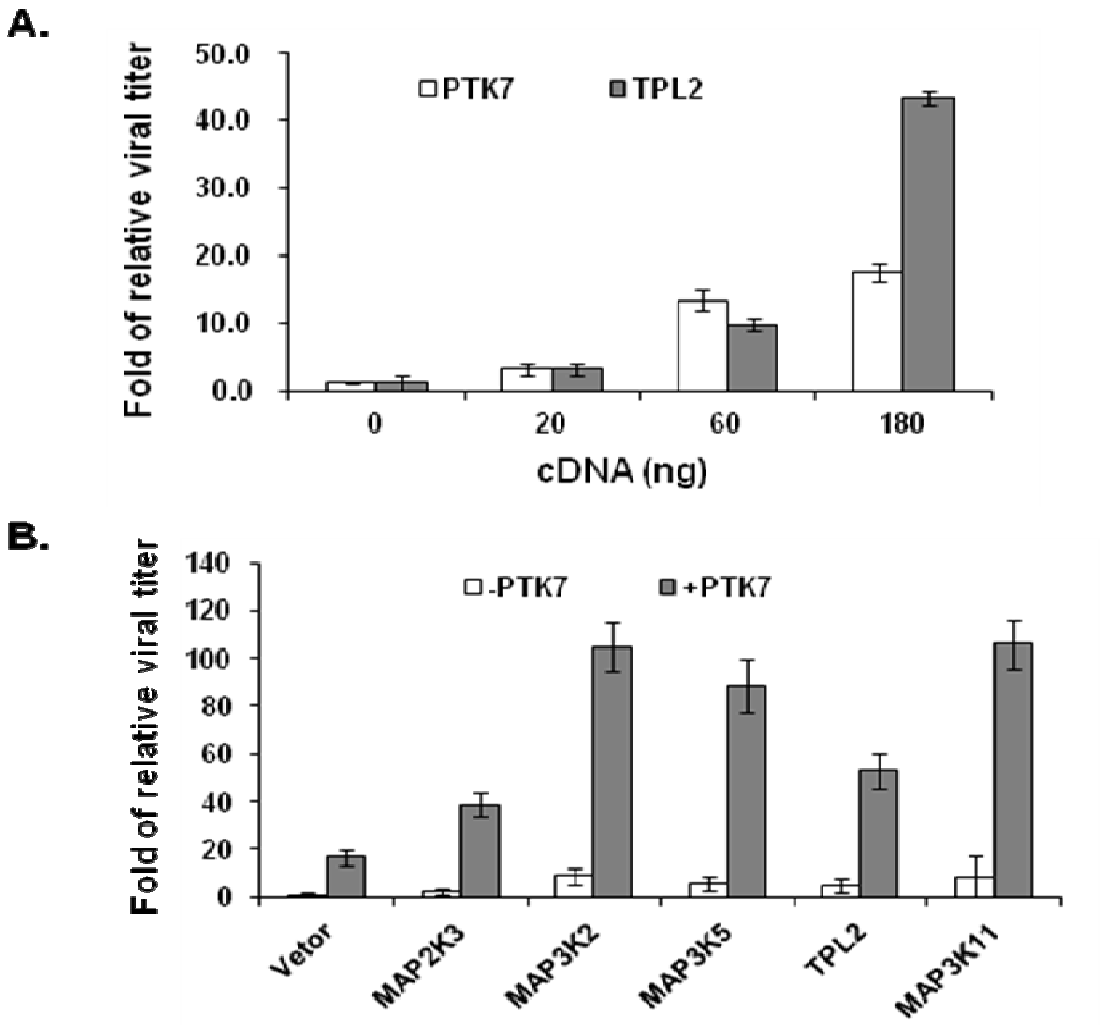

Figure 2-4. PTK7 enhances MHV-68 lytic replication through a distinct pathway. (A) HEK293T cells were transfected with the indicated amount of plasmids, and were infected with MHV-68-M3/FL virus at an MOI of 0.05 24 hr later. Viral supernatants were collected at 48 hr post-infection and were titered by luciferase assay. **(B)** HEK293T cells were cotransfected with the indicated plasmids in combinaion with the PTK7 plasmid or empty vector. 24 hr later, cells were infected with MVH-68-M3/FL virus at an MOI of 0.05. Viral supernatants were collected at 48 hr post-infection and were titered by luciferase assay. Results are presented as the means for triplicates with their standard deviations.

individual MHV-68 replication enhancers such as MAP kinases together with PTK7, PTK7 further enhanced MHV-68 replication by up to 10-fold compared to expressing the individual enhancers alone (Figure 2-4B), suggesting that PTK7 enhances MHV-68 lytic replication through a distinct pathway.

2.4.6 PTK7 does not affect MHV-68 gene transcription, genome replication and protein expression

To understand which step(s) of MHV-68 viral replication is/are regulated by PTK7 overexpression, we first looked at viral lytic gene transcription. During MHV-68 *de novo* infection, the viral lytic gene expression follows the strict order of immediate-early, early and late genes, allowing us to examine each individual step of viral gene expression. HEK293T cells were transfected with PTK7 plasmid or the control plasmids then infected with MHV-68 at an MOI of 0.5 at 24 hours post-transfection. Total RNA was collected at 4, 7, and 10 hours post-infection. The mRNA levels of the representative immediate early (ORF50), early (ORF6), and late (ORF29) genes were then measured by reverse transcription-quantitative PCR (RT-Q-PCR). As shown in Figure 2-5A, at all three time points examined the transcription of the three viral gene was unaffected by PTK7 overexpression compared to vector control; however, the positive control TPL2 enhanced viral IE gene (ORF50) transcription as early as 4 hours post-infection.

We then asked if the viral genome replication was affected by PTK7 overexpression. Cells transfected with PTK7 plasmid or the control plasmids were infected with MHV-68 at an MOI of 0.1 at 24 hours post-transfection. Total genomic DNA was collected at 10, 24 and 34 hours post-infection, and the viral genome copy number was then measured by Q-PCR. Consistent with

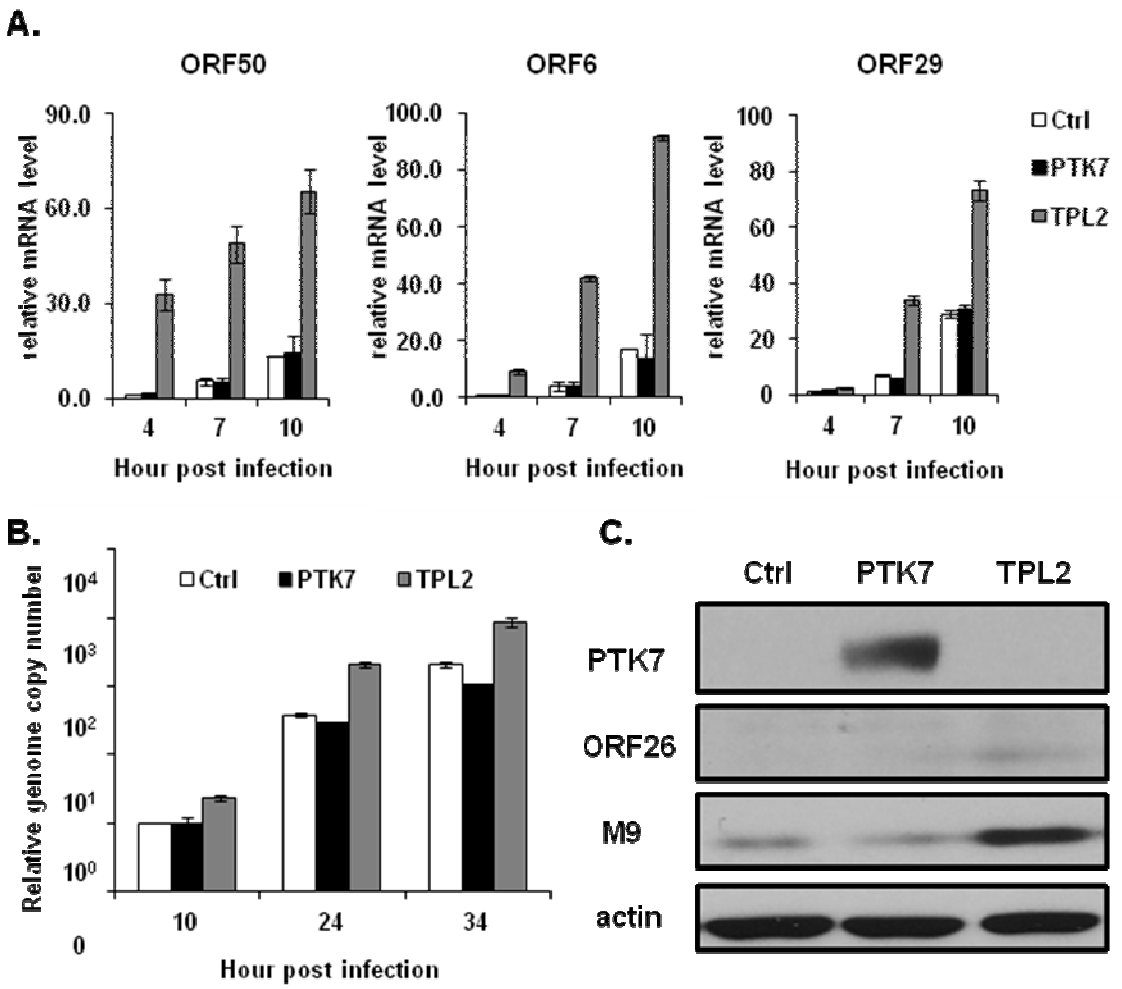

Figure 2-5. PTK7 overexpression does not affect MHV-68 viral gene transcription, genome replication and protein expression. HEK293T cells were transfected with the indicated plasmids and 24 hr later were infected with WT MHV-68 virus at an MOI of 0.1. **(A)** Total mRNA was collected at the indicated time points and QPCR was performed to analyze the indicated viral transcripts. Results are presented as the means for triplicates with their respective standard deviations. **(B)** Genomic DNA was harvested at the indicated time points and QPCR was performed to analyze the viral genome copy number. Results are presented as the means for triplicates with their respective standard deviations. **(C)** Cell lysates were collected at 24 hr post-infection and the western blot was performed to analyze the expression of the indicated viral proteins.

previous results, the positive control TPL2 enhanced viral genome replication (Figure 2-5B). However, PTK7 overexpression did not affect viral genome replication (Figure 2-5B).

We also examined the effect of PTK7 expression on MHV-68 lytic protein expression during virus lytic replication. Cells transfected with PTK7 plasmid or the control plasmids were infected with MHV-68 at an MOI of 0.5 at 24 hours post-transfection. At 24 hours post-infection, cell lysates were collected and subjected to western blot analysis. Antibodies against the two major capsid proteins ORF26 and M9 were used to assess viral protein expression. Again PTK7 overexpression did not affect the two viral lytic protein expression (Figure 2-5C). Thus we concluded that early steps of viral replication up to viral lytic protein/late gene expression are not affected by PTK7 expression.

2.4.7 PTK7 regulates MHV-68 egress

To further investigate the mechanism how PTK7 regulates MHV-68 egress, we performed a multi-step growth curve. HEK293T cells transfected with PTK7 or control plasmids were infected with MHV-68-M3/FL virus at an MOI of 0.05, and cell culture supernatant containing MHV-68-M3/FL virus produced by transfected 293T cells were collected at various time points. The MHV-68-M3/FL virus in the supernatants was then titered by infecting naïve 293T cells, and the relative amounts of viruses were determined by measuring luciferase activities at 18 hours post-infection.

We observed faster viral growth kinetics in PTK7-transfected cells than in those transfected with the control vector, but the virus from the control vector transfected cells eventually caught up. Since we observed an over 10-fold increase of viral titer in PTK7-overexpressing cells at 48 hours post-infection, we conducted more detailed analysis at the 48-hour time point (Figure 2-

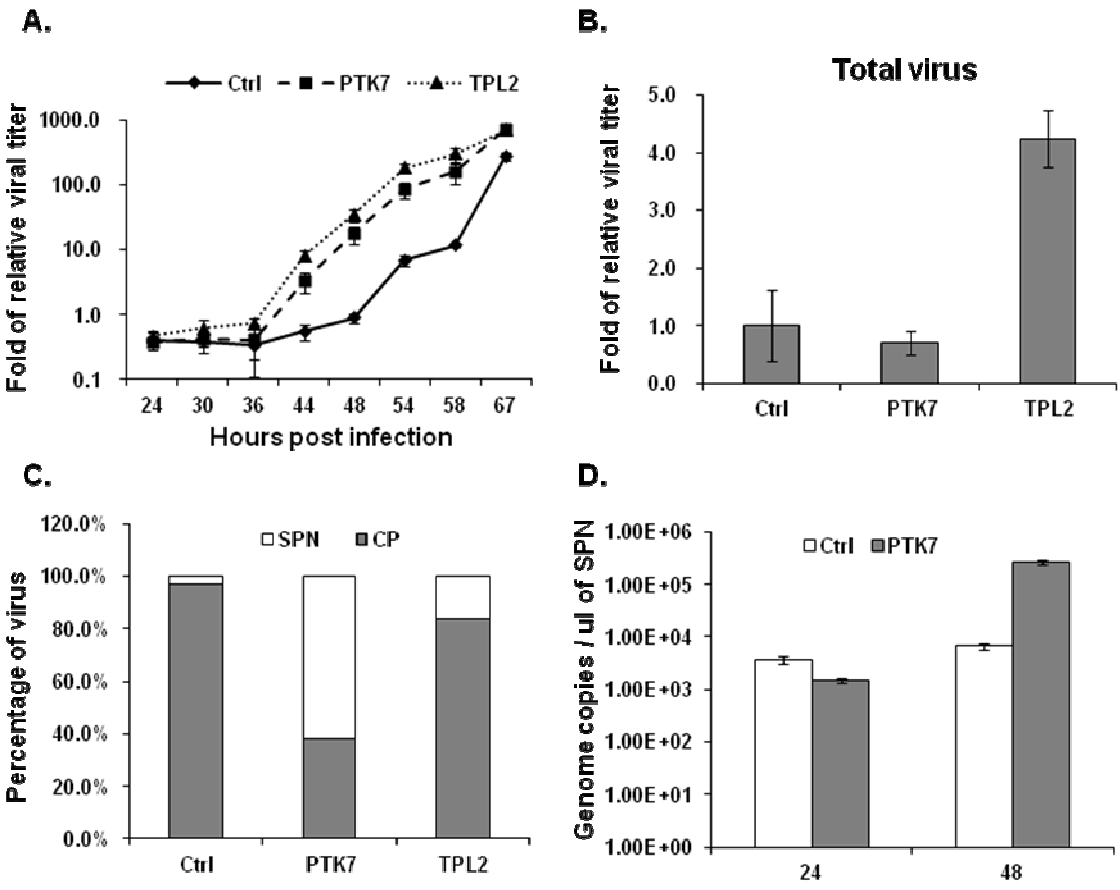

Figure 2-6. PTK7 helps MHV-68 release to the supernatant. **(A)** HEK293T cells were transfected with the indicated plasmids and were infected with MHV-68-M3/FL virus 24 hr later at an MOI of 0.05. Viral supernatants were collected at the indicated time points and were titered by luciferase assay. **(B)** and **(C)** HEK293T cells were transfected with the indicated plasmids and were infected with MHV-68-M3/FL virus 24 hr later at an MOI of 0.05. **(B)** Total virus was collected at 48 hr post-infection and titered by luciferase assay. **(C)** Virus in the supernatant and in the cell pellet was collected and titered separately. **(D)** HEK293T cells were transfected with the indicated plasmids and 24 hr later were infected with MHV-68-M3/FL virus at an MOI of 0.05. Viral supernatants were collected at the indicated timepoints and viral DNA was extracted by phenol-chloroform protocol. Viral genome copy number was measure by QPCR. Results are presented as the means for triplicates with their respective standard deviations.

6A). At 48 hours post-infection, infected cells and supernatant were harvested together, subjected to three freeze and thaw cycles to release any intracellular infectious viral particles, and titered by luciferase assay. Interestingly and surprisingly, the total viral production between PTK7-overexpressing cells was similar to that of control vector transfected cells, although the positive control TPL2 increased the total viral production (Figure 2-6B). In a parallel experiment, we titered the virus in the supernatant and cell-associated virus separately, and we found that at the same time point, more than 80% of total infectious virus was released to the supernatant in the PTK7 overexpressing cells, while in the control vector transfected cells the majority of the virus still remained in the cell pellet (Figure 2-6C). In addition, we analyzed the viral genome copies in the viral supernatant. As shown in Figure 2-6D, virus genome copies from 1 ul of PTK7 overexpressing cell culture supernatant were about 30-fold higher than that from control cells, which is proportional to the difference seen in the viral titer. This indicates that the increase of viral titer in the supernatant of PTK7 overexpressing cell is not due to the increase of the viral infectivity per se. Because the production of total infectious viral particles were the same between PTK7 overexpressing cells and control cells, and the only difference was the distribution of the virus, this strongly suggests that PTK7 regulates MHV-68 replication after the virus assembly, possibly at the egress step.

2.4.8 Kinase domain of PTK7 is dispensable for its role in regulating MHV-68 egress

To gain more insight into the mechanism underlying the role of PTK7 in the MHV-68 egress, we set out to determine the functional domain of PTK7 that is responsible for mediating MHV-68

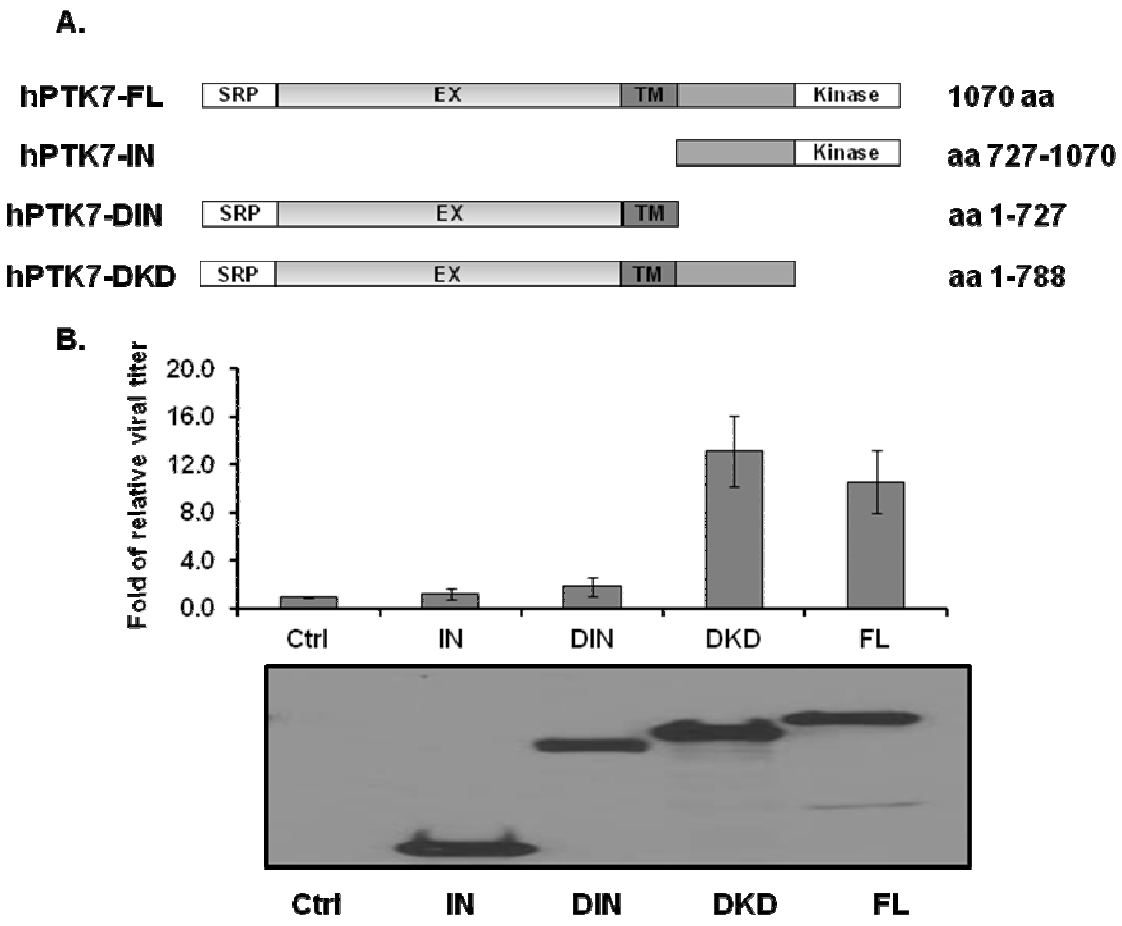

Figure 2-7. Kinase domain of PTK7 is dispensable for its role in regulating MHV-68 egress. (A) Schematic representation of PTK7 mutants. **(B)** HEK293T cells were transfected with the indicated PTK7 mutants. The expression of individual mutant was analyzed by western blot (lower panel) at 24 hr post-transfection. Transfected cells were then infected with MHV-68/M3/FL virus at an MOI of 0.05. Viral supernatants were collected at 48 hr post-infection and titered by luciferase assay. Results are presented as the means for triplicates with their respective standard deviations.

egress. Sequence analysis showed that PTK7 consists of an extracellular domain containing seven immunoglobulin-like loops, a single transmembrane domain, and an intracellular domain with a C-terminus homologous to tyrosine kinases. We constructed three PTK7 truncation mutants accordingly: an extracellular domain deletion mutant (PTK7-IN), an intracellular domain deletion mutant (PTK7-DIN) and a kinase domain deletion mutant (PTK7-DKD) (Figure 2-7A). The expression of all mutants were confirmed by western blot analysis (lower panel in Figure 2-7B). To determine which domain is required for PTK7's function in MHV-68 egress, HEK293T cells were transfected with individual PTK7 mutants and a similar viral replication assay as described earlier was performed. As shown in Figure 2-7B upper panel, PTK7-DKD was the only mutant that was able to enhance MHV-68 egress to a similar level of full-length PTK7. Thus, we concluded that the kinase domain of PTK7 is dispensable for its role in regulating MHV-68 egress.

2.4.9 Knockdown of PTK7 blocks MHV-68 egress

To independently confirm the role of PTK7 in MVH-68 egress, an RNA interference approach was used. We constructed several shRNAs to deplete PTK7. shPTK7-3 was able to substantially knockdown the expression of transfected PTK7 in HEK293T cells at day 2 post-transfection (Figure 2-8A). We then tested whether knockdown of PTK7 could inhibit MHV-68 egress. HEK293T cells were transfected with individual shPTK7 or control shRNA and were infected with MHV-68-M3/FL virus at 48 hours post-transfection. Cell-associated virus was separated from the virus in the supernatant and was titered by luciferase assay. As predicted, knocking down PTK7 using shPTK7-3 increased the cell-associated MHV-68 titer (Figure 2-8B). The increase of cell-associated viral titer indicated that the virus got trapped inside of the cells

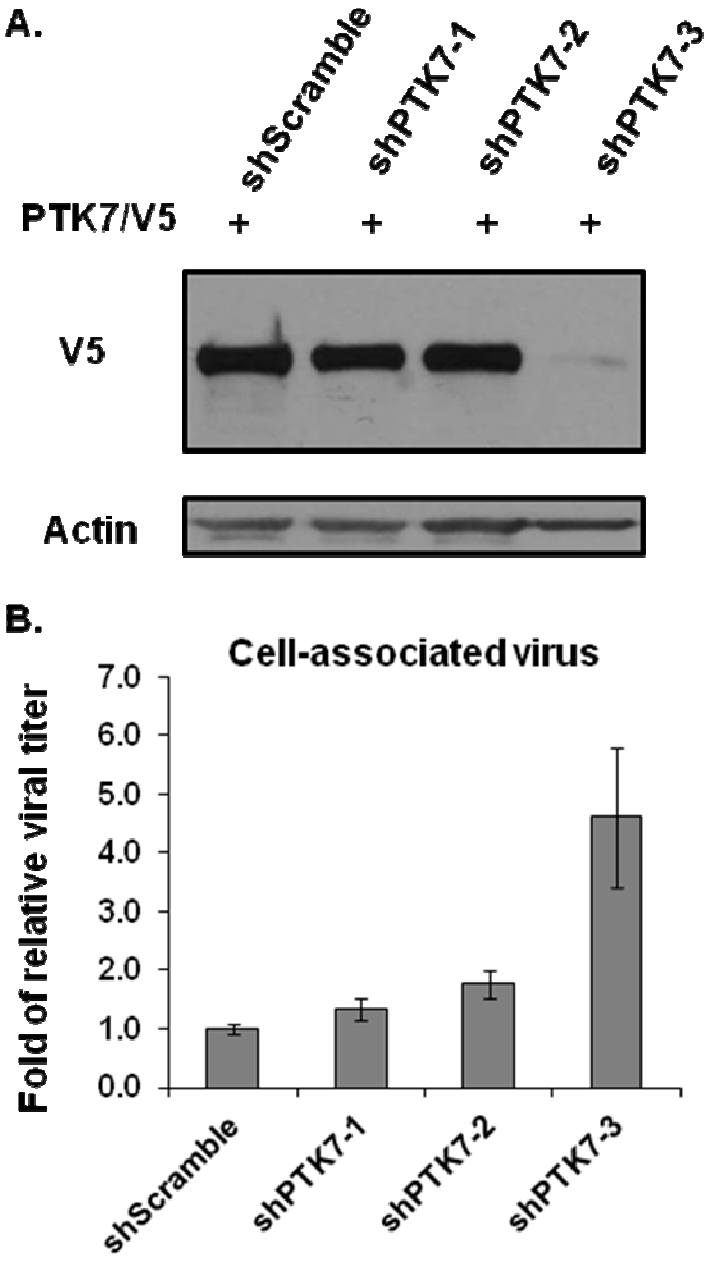

Figure 2-8. Knockdown of PTK7 increases the cell-associated virus. (A) HEK293T cells were transfected with PTK7/V5 expression plasmid and the indicated shRNAs. At 48 hr post transfection, lysates were analyzed by the indicated antibodies. **(B)** HEK293T cells were transfected with the indicated shRNAs and 48 hr later were infected with MHV-68-M3/FL virus at an MOI of 0.05. Cell-associated virus was collected and titered by luciferase assay. Results are presented as the means for triplicates with their standard deviations.

when PTK7 was knocked down, which further corroborate the role of PTK7 in regulating MHV-68 egress.

2.4.10 PTK7 regulates HSV-1 egress.

Lastly, we asked if the regulation of viral egress by PTK7 is MHV-68 specific. To address this question, we tested the role of PTK7 in the egress of HSV-1, which belongs to the alphaherpesvirus subfamily. We observed a similar phenotype to that of MHV-68. In brief, overexpression of PTK7 did not affect total HSV-1 viral production (Figure 2-9A). But when separating the virus in the supernatant and in the cell pellet, we did see an increase of viral titer in the supernatant (Figure 2-9B). These results indicated that PTK7 could also regulate HSV-1 egress. Thus, we concluded that PTK7 regulates a conserved viral egress mechanism shared by herpesviruses.

2.5 Discussion

In this study, through screening a cellular kinase cDNA library, we identified multiple cellular kinases as positive regulators of MHV-68 replication. We demonstrated that TPL2 promotes MHV-68 lytic replication by enhancing viral lytic gene expression, notably the expression of RTA in an AP-1 dependent manner. AP-1 activity plays an important role during EBV and KSHV reactivation [33-35]. Our results demonstrated the essential role of AP-1 activity in the lytic gene expression of a gammaherpesvirus during de novo infection. TPL2 has been previously shown to be required for EGF-induced activation of Fos promoter and AP-1 activity [36]. Yet the exact mechanism of the activation of Fos promoter downstream of TPL2 is not clear. Overexpression of TPL2 is known to activate MAP kinase pathways through MEK1,

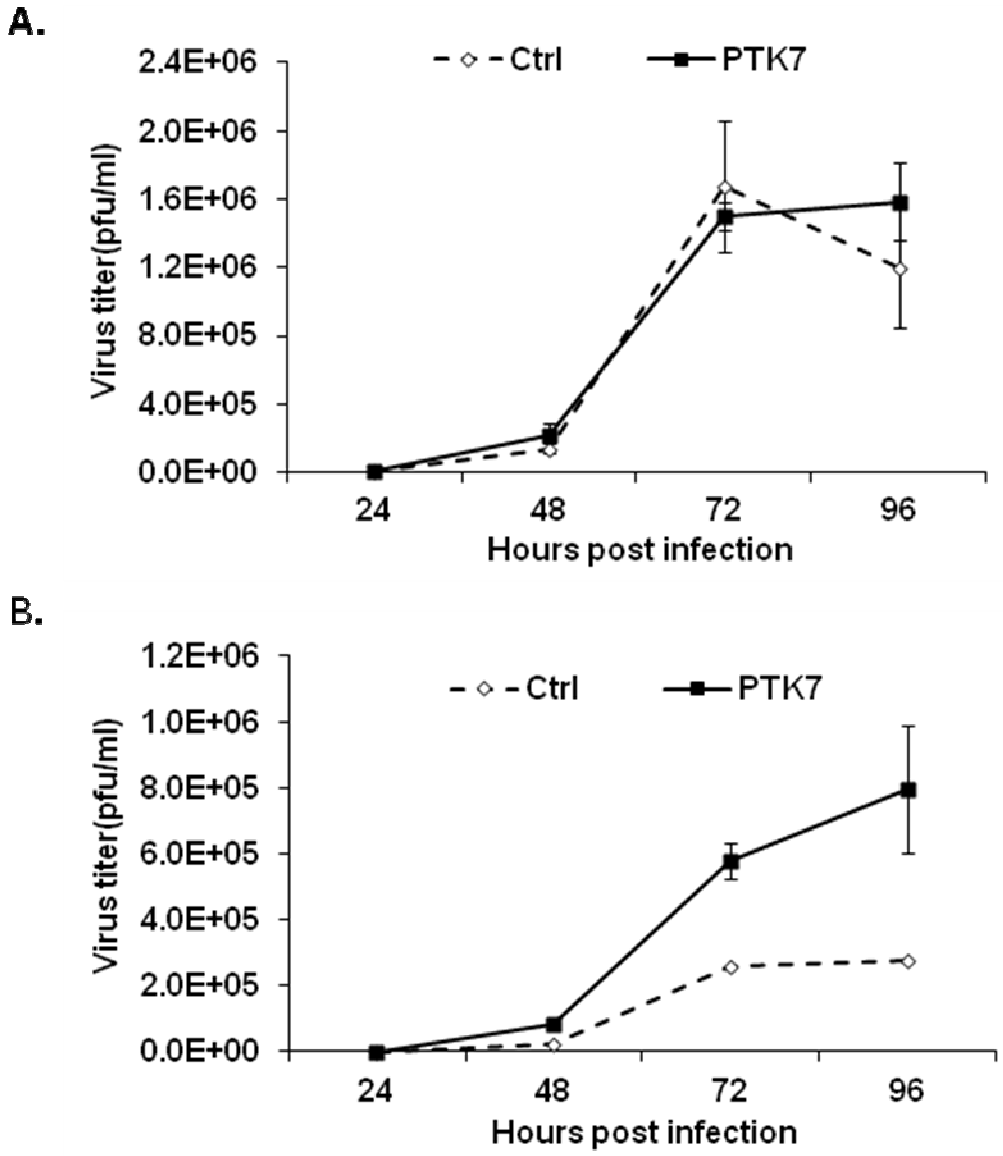

Figure 2-9. PTK7 regulates HSV-1 egress. **(A)** and **(B)** HEK293T cells were transfected with the indicated plasmids and 24 hr later were infected with HSV-1 at an MOI of 0.01. **(A)** Total virus was collected and titered by plaque assay. **(B)** Viral supernatants were collected and titered by plaque assay. Results are presented as the means for triplicates with their standard deviations.

MEK2, MEK4, MEK5, and MEK6, leading to the activation of downstream extracellular signal-regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs) and p38 kinases [37-39]. The ERK pathway can enhance Fos transcriptional activity through multiple mechanisms [40-44]. Therefore, it is possible that TPL2 upregulates Fos expression and AP-1 activity by activating the ERK pathway. Alternatively, TPL2 has been shown to be able to phosphorylate histone H3 and cooperate with phosphorylated H3 to activate Fos promoter [45]. Further studies probing these two possibilities may help us to further delineate the pathway linking TPL2 and Fos.

Although we showed that the enhancement of viral replication by TPL2 is in large part mediated by AP-1 dependent activation of the RTA promoter, it is entirely possible that TPL2 also contributes to viral replication through RTA-independent upregulation of other viral lytic genes. For example, we showed that overexpression of TPL2 or Fos activated the ORF57 promoter, whereas overexpression of a dominant negative version of Jun, Tam-67, inhibited the ORF57 promoter [32]. As noted previously, ORF57 is essential for infectious virion production of KSHV [46-48]. Therefore, RTA-independent upregulation of ORF57 expression may further promote the enhancement of viral lytic replication by TPL2.

The importance of AP-1 transcription factors in viral gene expression and lytic replication has been demonstrated in other members of gammaherpesvirus, such as KSHV and EBV [34, 47, 49, 50]. Notably, the BZLF1 gene of EBV encodes a viral transcription factor that is related to Fos and is able to induce virus productive replication [34, 49]. Although a previous study has demonstrated that downstream MAPK members such as Erk1/2, p38, and Jnk1/2 play important roles in promoting KSHV lytic gene expression and virus replication by upregulating AP-1 activities [50], it is not absolutely clear which of the several upstream MAP3K members is required for efficient virus replication. Our study supports a role for TPL2 as an upstream

mediator of the AP-1 dependent activation of RTA, ORF57, and possibly other viral lytic genes during the lytic replication of MHV-68 and possibly other members of gammaherpesviruses.

As TPL2 regulates MHV-68 lytic replication by enhancing viral IE gene expression, another cellular kinase PTK7 regulates MHV-68 replication at a much later step, namely viral egress. Herpesvirus assembly and egress are complex processes. After viral gene expression and DNA synthesis, capsid proteins are translocated from the cytoplasm to the nucleus and assembled antocatalytically into preformed capsids. Viral DNA is then pumped into the preformed capsids, resulting in mature nucleocapsids. Nucleocapsids then proceeds to the egress pathway, in which capsids move from the nucleus to extracellular spaces [51-53]. All herpesviruses appear to share similar egress pathways, although certain distinctions are emerging. There are several proposed mechanisms of herpesvirus envelopment, and the best-accepted one is described as follows: nucleocaspids undergo envelopment at the inner nuclear membrane, de-envelopment at the outer nuclear membrane, tegumentation in the cytoplasm, and finally reenvelopment by budding into cytoplasmic membranes, such as trans-golgi network (TGN), producing mature, infectious virions inside a cellular vesicle. Enveloped virions produced in the TGN are then transported to the plasma membrane through exocytosis. Fusion between transport vesicle and the plasma membrane releases viruses to the extracellular spaces [51-53]. In the past, most studies on herpesvirus egress have been focused on the steps prior to the transportation of mature virions from TGN to the extracellular spaces, and many viral and cellular proteins have been identified to play roles in the various steps. However, little is known about viral or cellular proteins involved in the final release of the virus. Here we demonstrated the role of PTK7 in promoting the final release of the herpesvirus to the extracellular space. We found that: (i) PTK7 overexpression does not affect MHV-68 viral gene transcription, genome replication, or lytic

protein expression; (ii) ectopic expression of PTK7 does not affect the production of total infectious virus particles; (iii) virus from PTK7-overexpressing cells releases faster to the supernatant; and (iv) knockdown of PTK7 inhibits the release of MHV-68.

The virus egress process discussed above does not take into consideration the fact that many herpesviruses replicate in polarized cells at some stages. When herpesviruses infect polarized cells such as neurons and epithelial cells, enveloped virions packaged into transport vesicles are sorted to specific cell surfaces in these polarized cells, and this can promote cell-to-cell spread across epithelial cell junctions or transport into neuronal axons for spread to peripheral tissues [51, 52, 54]. These processes are highly dependent on the dynamic rearrangement of the host cytoskeleton and cellular organelles [51, 55-58]. PTK7, also known as colon carcinoma kinase-4 (CCK4), is an evolutionarily conserved transmembrane receptor containing a catalytically inactive kinase domain [59, 60]. PTK7 has been shown to play a role in noncanonical Wnt signaling or the planar cell polarity (PCP) pathway since loss of PTK7 in both mouse and Xenopus leads to profound defects in embryonic morphogenesis (e.g., gastrulation, neural tube closure, neural crest migration, etc) [61-66]. Activation of the non-canonical Wnt/PCP pathway by Wnt ligands involves binding to Frizzled (Fz) receptors and co-receptors, and PTK7 has been proposed to be one of the co-receptors to recruit downstream signaling molecules [61, 63, 67, 68]. Multiple downstream branches can be activated through the small GTPases Rho/Rac/Cdc42 and JNK, which control remodeling of the actin cytoskeleton and gene expression [65, 67-69]. In addition to being a co-receptor in the non-canonical Wnt/PCP, PTK7 has been shown to form a complex with Plexin proteins, which were discovered as regulators of axon guidance [66, 70]. The signaling downstream of Plexins could also lead to cytoskeleton reorganization, affecting cell shape, motility and cell-cell interaction [71-73]. When we examined the cellular localization

of PTK7, it was interesting to see an asymmetric localization pattern (data not shown). PTK7 is localized to the cell membrane but preferentially to the surface protrusions. Though the mechanism of action of PTK7 is not clear, it is possible that PTK7 modulates herpesvirus egress by mediating the cytoskeleton reorganization.

Recently several studies have also shown that PTK7 could affect canonical Wnt signaling, though the role of PTK7 remains controversial [74, 75]. The canonical Wnt signaling pathway is β-catenin dependent and important in the regulation of cell proliferation and differentiation. Gammaherpesviruses are known to exploit Wnt signaling to advance their life cycle mainly by stabilizing β-catenin and in turn activating downstream gene expression for cell proliferation [76]. Our results shown here also raised the possibility that the canonical Wnt signaling pathway might play a role in gammaherpesvirus egress.

One advantage of functional screens with cDNA expression or RNAi gene knockdown is the ability to identify multiple genes that regulate virus replication in a relatively unbiased fashion [77-81]. In addition, multiple components of a known cellular pathway or machinery can be identified through such screens, helping to link a specific pathway or machinery to virus replication [77, 79, 81]. Although we employed two similar functional screens in our study to identify cellular pathways regulating virus replication, it is reasonable to believe that a combination of screens of different natures will also be able to identify novel cellular pathways or machineries functioning in not only virus replication, but also other cellular processes. Indeed, a recent study combined a genome-wide RNAi functional screen with interactome data to identify multiple cellular pathways regulating early-stage HIV-1 replication [82]. Thus, the employment of various combinations of functional screens and other high-throughput approaches

will likely help us identify additional cellular pathways and machineries regulating virus replication with unprecedented speed in future studies.

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CHAPTER 3

SYSTEMACTIC IDENTIFICATION OF VIRUS-HOST INTERACTIONS THAT REGULATE MHV-68 LYTIC REPLICATION

3.1 Abstract

During the course of viral infection, the virus is highly dependent on the cellular machinery to facilitate its replication. Host cells, on the other hand, develop mechanisms to sense and restrict virus replication. Here, we present an integrative systems approach to elucidate the interactions between MHV-68 and its host. Our previous yeast-two-hybrid analysis and genome-wide expression profiling implicated hundreds of cellular factors in mediating virus-host interaction. Among the cellular factors identified, kinase- and transcription factor- regulated signaling are the most highly enriched. Therefore, high-throughput screens of cellular kinome and transcription factors cDNA and siRNA libraries were carried out, which uncovered 144 MHV-68 dependency factors and 213 restriction factors. We found that many of identified cellular genes affect MHV-68 replication by regulating RTA transcription. Characterization of this subset of genes showed they also play roles in KSHV latency maintenance and reactivation. Cellular factors regulating later steps of viral replication, such as viral late gene expression and egress, were also identified and validated. Finally we compared the viral restriction factors to the results of a kinome siRNA screen performed to identify cellular regulators of innate immune response to cytosolic DNA. Remarkably, 23 genes were found among the top 10% of the hits of both screens. Collectively, this multilayered and unbiased approach provides a comprehensive network of gammaherpesvirus-host interactions.

3.2 Introduction

Human gammaherpesvirus, including Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), are distinct in their oncogenic potential and are etiologically associated with various types of tumors, especially in immunosuppressed patients such as those with AIDS [1-4]. Although the introduction of highly active antiretroviral therapy (HAART) has significantly lowered the incidence of gammaherpesvirus-associated malignancies, certain malignancies remain the leading cancers in some resource-poor developing countries [5]. Thus, there is still a need for therapies specific to gammaherpesvirus-associated malignancies.

Gammaherpesviruses have two distinct phases in their life cycles: latency and lytic replication [6]. Though tumorigenesis induced by gammaherpesviruses requires multiple genes expressed during latent infection, it has been suggested that lytic viral gene products expressed during sporadic reactivation in tumor lesions also promote cell growth [7-9]. During gammaherpesvirus lytic replication, the virus is highly dependent on the cellular machinery to facilitate its replication. Host cells, on the other hand, develop complex systems to detect and eliminate virus replication. A better understanding of the interplay between gammaherpesviruses and the cells they replicate in will provide insight into the tumor-promoting properties of the viruses and may lead to the development of potential therapeutic strategies.

The study of virus-host interaction during lytic replication of human gammaherpesviruses is limited by the lack of a cell culture system that can support robust lytic replication of human gammaherpesviruses. Murine gammaherpesvirus-68 (MHV-68), a rodent gammaherpesvirus, shares significant similarities in genome organization and biological properties to its human counterparts [10-12]. Moreover, MHV-68 undergoes efficient lytic replication in a number of common cell lines, including those of human origins [10-12]. Thus MHV-68 provides a useful

experimental system to study the biological significance of virus-host cell interactions in the course of virus lytic replication.

In recent years genome-scale screens have been widely used to address many biological questions because of their unbiased nature. Several recent studies utilized an integrative functional genomics strategy to elucidate host factors involved in HIV and Influenza A virus replication [13, 14]. This systems approach has enormous potential for the discovery of both new therapeutic targets, and a better understanding of virus-host interactions.

Here, we present an integrative systems approach to elucidate the interactions between MHV-68 and its host. Our previous yeast-two-hybrid analysis and genome-wide expression profiling revealed that cellular genes involved in kinase- and transcription factor- regulated signaling were over-represented among the host factors identified from the two studies. High-throughput screens of cellular kinome and transcription factors cDNA and siRNA libraries were then carried out, uncovering 144 MHV-68 dependency factors and 213 restriction factors, regulating virus replication at various steps. Cellular genes shown to affect MHV-68 RTA transcription also played roles in KSHV latency maintenance and reactivation. Finally we found that the MHV-68 restriction factors identified from our screen greatly overlapped with the results of an siRNA library screen designed to identify cellular regulators of innate immune response to cytosolic DNA.

3.3 Materials and methods

Cells and virus

HEK293T, NIH3T3, Vero, BC-3 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. All cell

lines were cultured in a humidified 5% CO_2 atmosphere at 37°C. MHV-68 was originally obtained from the American Type Culture Collection (VR1465). The recombinant MHV-68-M3/FL virus was constructed by S. Hwang by integrating to the left side of the wild type MHV-68 viral genome a firefly luciferase gene driven by the viral M3 promoter [15]. Virus stocks were grown by infecting Vero cells at an MOI of 0.1. The titers of the produced viruses were determined by plaque assays, using a monolayer of Vero cells overlaid with 1% methylcellulose. After 6 days of infection, the cells were fixed and stained with 2% crystal violet in 20% ethanol and plaques were counted to determine the titers.

Gene expression profiling

NIH3T3 cells were mock infected or infected with MHV-68 at an MOI of 3. RNA was harvested at 0, 1, 2, 4, 8, 12, 16 hours post-infection and subjected to microarray analysis as previously described [16].

Yeast-two-hybrid screens and analysis

Yeast-two-hybrid screens and analysis were performed as previously described [17].

cDNA library screen

A cDNA library screen was performed as described in Chapter 2 and [18].

siRNA library screen and data analysis

Kinome libraries comprising 3152 siRNAs targeting 788 human kinases, kinase regulatory subunits, and hypothetical kinases were arrayed in 384-well plates (14 ng per siRNA per well).

The library matrix was introduced into HEK293T cells by a high-throughput robotic system, and 40 hours later the cells were infected with MHV-68-M3/FL virus at an MOI of 0.01. 10 ul of cell culture supernatant from each well was transferred to naïve HEK293T cells seeded in the destination plates at 52 and 58 hours post-infection and luciferase assay were performed 18 hour later. The screen was run in duplicate and analyzed by Redundant siRNA Activity (RSA) as previously described [13, 19].

Cell transfection

An appropriate number of cells were seeded onto each well of a 48-well plate so that cells were 80-90% confluent at the time of transfection. Cells were transfected with expression plasmids, reporter plasmids or shRNA vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations.

Plasmids

shRNA lentiviral vectors were constructed by annealing 2 DNA oligos containing target sequences and cloning into the pLKO.1-TRC vector (Addgene) following the instructions from the manufacture. KSHV RTA_ and PAN_firefly luciferase reporter constructs were described in promoter [20] and [21].

Reporter analysis

HEK293T or BC-3 cells were seeded onto 48-well plates 24 hours prior to transfection so that cells were ~90% confluent by the time of transfection. Cells were then transfected with a DNA mixture containing 50 ng of KSHV RTA_firefly luciferase reporter plasmid or PAN_firefly

luciferase reporter plasmid together with 5 ng pPGK_renilla luciferase plasmid and shRNA constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The total amount of DNA transfected for each well was adjusted to 0.4 μg using appropriate control plasmids. Cells were cultured for an additional 48 hours followed by dual-Luciferase assays using a kit provided by Promega.

RT-Q-PCR analysis

HEK293T cells were transfected with various shRNA constructs as previously described. At 48 hours post-transfection, cells were infected with MHV-68 at an MOI of 0.2. Cells were collected at different time points post-infection and RNA was purified using PureLink RNA Mini Kit (Invitrogen) and reverse transcribed into cDNA with qScript cDNA Synthesis Kit (Quantas). RT-PCR was then done with the primers for ORF50, ORF6, ORF29, and GAPDH as described in Chapter 2.

Quantitation of viral genome copies

HEK293T cells were transfected with various shRNA constructs as previously described. At 48 hours post-transfection, cells were infected with MHV-68 at an MOI of 0.1. Cell lysates were collected at different time points post-infection and total DNA was extracted by phenol-chloroform protocol. Viral genome copies were quantitated as described in Chapter 2.

Immunoblotting and antibodies

Immunoblotting was performed as described in Chapter 2. The antibodies used in this study were as follows: mouse Anti-KSHV ORF59 (Advanced Biotechnologies 13-211-100), mouse

anti-actin (Sigma A5316) donkey anti-rabbit IgG (GE Healthcare NA934V) and sheep anti-mouse IgG (GE Healthcare NXA931). The rabbit polyclonal antibodies against MHV-68 ORF26 and ORF65 were derived in our lab. The rabbit polyclonal antibody to KSHV K8 was a kind gift from Dr Jae Jung.

3.4 Results

3.4.1 Identification of host factors involved in MHV-68 replication through genome-wide expression profiling and yeast-two-hybrid analysis

As an initial step towards understanding the role of host genes potentially involved in MVH-68 lytic infection, we undertook a tudy to analyze the cellular transcriptional responses to *de novo* MHV-68 infection. To provide the best snapshots of MHV-68 induced genome-wide changes during infection, we examined the gene expression profiles of cells at 0, 1, 2, 4, 8, 12, and 16 hours after infection in duplicate experiments. Using a 2-fold increase or decrease as the threshold, we found that 429 transcripts were upregulated and 694 transcripts were downregulated after MHV-68 infection. To determine if particular pathways were over-represented among these genes, we performed Gene Ontology (GO) analysis (Figure 3-1A and 3-1B). In the ug-regulated group, GO terms such as ion transport, neuron differentiation, chemical homeostasis, and apoptosis are high enriched, whereas in the downregulated group, genes involved in intracellular transport, cell cycle, protein localization and cell proliferation are over-represented.

Next, we identified host factors that were directly manipulated through physical associations with viral proteins. Using a yeast-two-hybrid (Y2H) approach, we systematically investigated direct binary contacts among the viral proteins, as well as each of the viral protein and each of

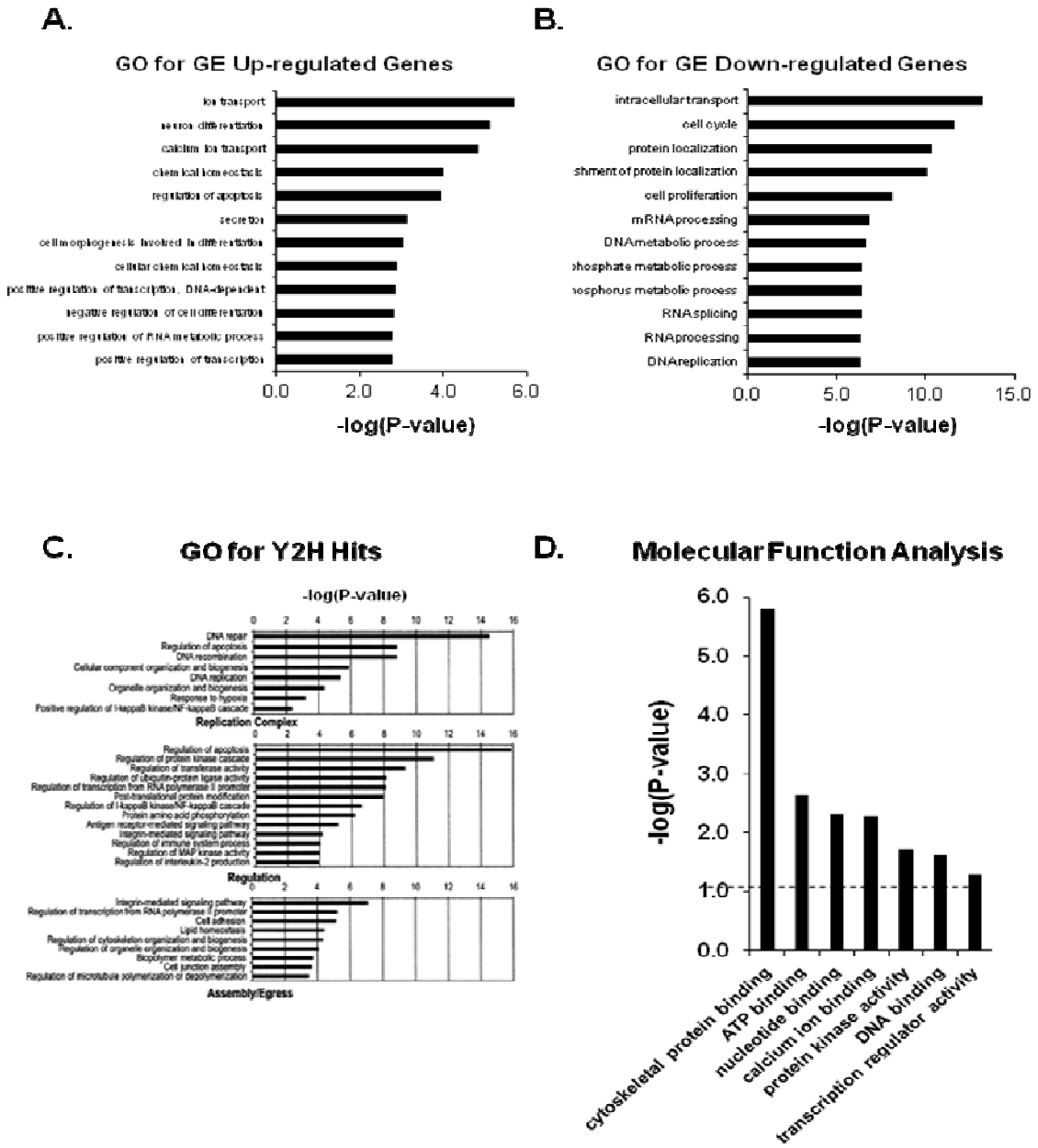

Figure 3-1. Gene Ontology (GO) analyses of cellular factor identified from gene expression profiling and Y2H. (A) and **(B)** Gene expression profiling of MHV-68 infected cells identified 429 up-regulated genes and 694 down-regulated genes. GO analyses of enriched cellular pathways in these identified cellular genes. **(C)** Y2H of viral-host PPI identified 197 distinct cellular genes. These genes were grouped based on the known or predicted functions of their viral partners, and subjected to GO analysis. **(D)** Molecular function analysis was performed on the 1292 distinct genes identified from gene expression profiling and Y2H.

the human proteins available in the human liver cDNA library [17]. We discovered 23 intra-viral interactions among the 84 viral protein constructs, and 243 pairwise interactions between the 84 MHV-68 constructs and 197 distinct cellular proteins. Interestingly, some of the viral-interacting cellular factors were also affected at the transcriptional level by MHV-68 infection. We found that the transcript level of 16 out 197 viral-interacting cellular proteins changed significantly during MHV-68 infection, suggesting an important role of these cellular factor in MHV-68 replication. To further characterize the cellular factors identified in the Y2H screen, we grouped them according to known or predicted functions of their viral partners (replication complex, regulatory, assembly/egress, and capsid or envelope structural proteins) and analyzed the functional annotations of all cellular proteins and their immediate neighbors in the cellular PPI network (Figure 3-1C). Interestingly, the cellular factors identified in the screen were enriched in GO terms related to the function of their viral partners. For example, the targets of the viral DNA replication proteins participated in DNA replication, recombination, and repair. Similarly, viral regulatory proteins tended to interact with cellular proteins involved in the regulation of ubiquitin-ligase/protein kinase activities, protein amino acid phosphorylation, and the antigen receptor and integrin-mediated signaling pathways. Viral assembly/egress proteins, on the other hand, tended to interact with cellular proteins involved in cell adhesion, lipid homeostasis, and regulation of cytoskeleton organization/biogenesis.

Furthermore, we determined the enriched molecular functions of the cellular factors identified from gene expression profiling and Y2H (Figure 3-1D). Seventy-three molecular function terms, assigned to 1292 genes, were significantly enriched. Notably, 78 genes were associated with the term transcription regulator activity and 44 genes were linked to term protein kinase activity, underscoring the role of transcription factors and kinases in MHV-68 lytic replication.

3.4.2 Functional interrogation of cellular kinases and transcription factors

The transcriptional responses and physical interactions identified 1292 cellular factors that could impact MHV-68 replication. Since the cellular factors involved in kinase- and transcription factor- regulated signaling are the most highly enriched, we measured the effect of perturbing individual cellular kinases or transcriptions factor by overexpressing or by small interfering RNA (siRNA) on MHV-68 replication.

3.4.3 Kinome and transcription factor cDNA library screen

To systematically identify cellular kinases or transcription factors that regulate MHV-68 replication, we screened a cDNA library of 353 kinases and 700 transcription factors [18]. As described in Chapter II, the screen was optimized in a 384-well plate format using RTA and Rd2 as positive and negative regulators of MHV-68 lytic replication, respectively. The empty vector was used as the negative control which does not have effect on MHV-68 replication. The replication-competent MHV-68-M3/FL virus was used in combination with luciferase assay to determine the relative viral titers among different cDNA-transfected cells. The luciferase activity in each well of a 384-well plate was normalized to the wells transfected with empty vector. The MHV-68 viral dependency factor was defined as the cDNA that increased viral titer over 2-fold compared to the empty vector, and the MHV-68 viral restriction factor was defined as the cDNA that decreased viral titer over 66.7%. Using these criteria, we identified 144 MHV-68 dependency factors (13.7% of the total genes screened) and 125 restriction factors (11.9% of the total genes screened). The percentage of genes discovered here is much higher than several

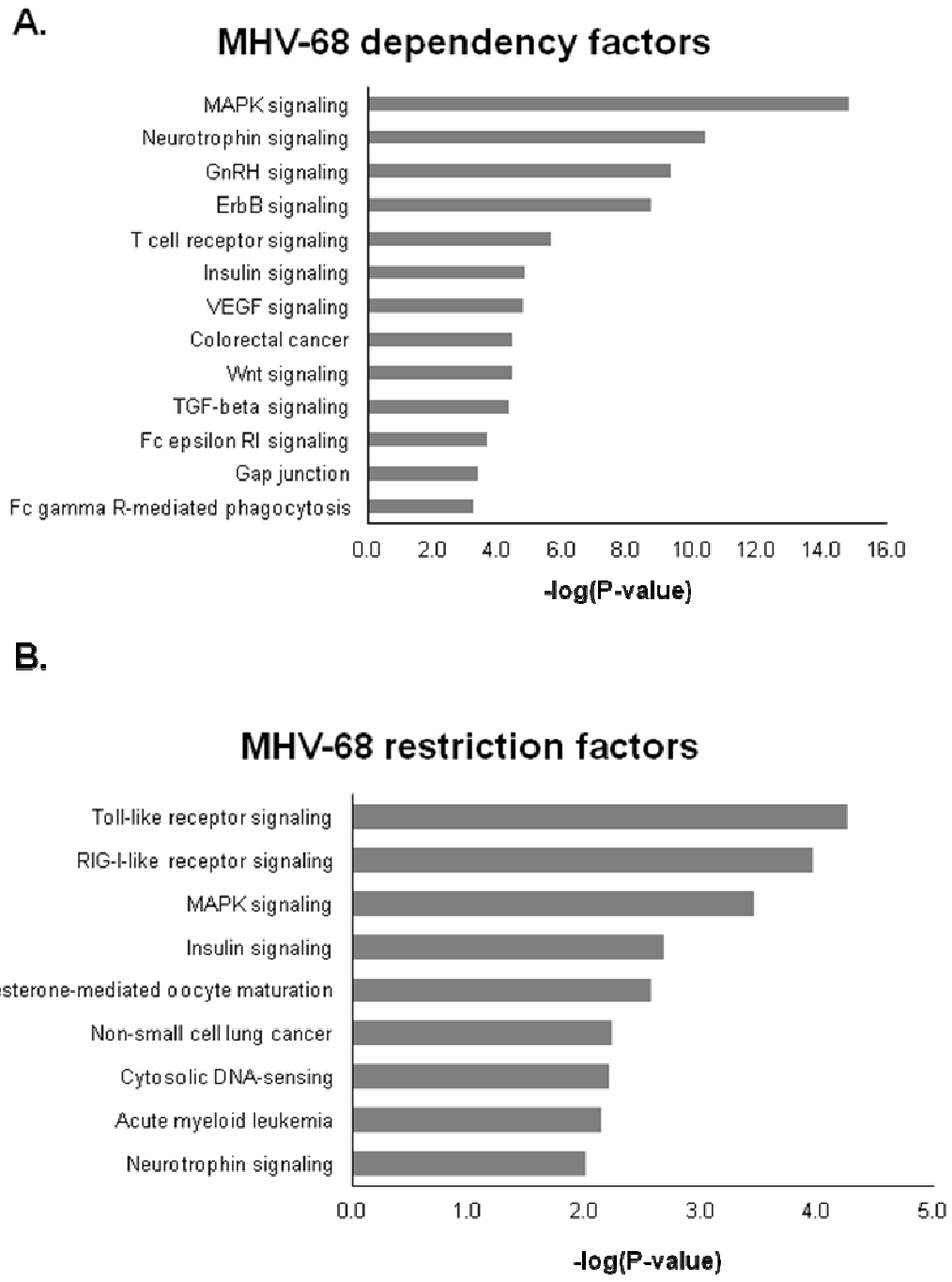

Figure 3-2. Bioinformatics analyses of cDNA screen result. (A) Cellular pathways that were enriched in the 144 identified MHV-68 dependency factors. **(B)** Cellular pathways that were enriched in the 125 identified MHV-68 restriction factors.

previous genome-wide screens which usually identified up to 2% of the total genes as hits, demonstrating that kinases and transcription factors play more important roles in viral replication. We then employed bioinformatics to identify enriched pathways and networks within these gene sets (Figure 3-2). Forty-one pathways assigned to 133 MHV-68 dependency factors and twenty-three pathways assigned to 110 MHV-68 restriction factors were significantly enriched. The most significant and non-redundant terms, assigned to MHV-68 dependency factors, include MAPK signaling pathway (24 genes, p=1.54e-15), Wnt signaling pathway (10 genes, p=3.75e-5) and Gap junction (7 genes, p=4.3e-4). As for the MHV-68 restriction factors, the most significant terms are Toll-like receptor signaling pathway (7 genes, p=5.38e-5), MAPK signaling pathway (24 genes, p=3.54e-4), and Cytosolic DNA-sensing pathway (4 genes, p=6.14e-3). Importantly, multiple pathways identified in our screen are consistent with the known pathways involved in gammaherpesvirus lytic replication. For example, Toll-like receptor signaling pathway plays a key role in the innate immune system and TLR9 was shown to contribute to the antiviral immunity to MHV-68 infection [22]. Our screen confirmed the functional role of several Toll-like receptor downstream effectors, TBK1, IRF3 and IKBKB, in MHV-68 replication.

It is noteworthy that several pathways were enriched in both MHV-68 dependency factor and restriction factor datasets, most likely due to the highly connected cellular signaling network. MAPK signaling pathway, for example, has multiple downstream branches and different degree of activation can possibly lead to various cellular functions such as cell proliferation, cell cycle arrest or apoptosis. Further characterization of these factors will provide more insight on how the virus utilizes or fine-tunes these pathways to facilitate its replication or how host cells respond to incoming viral infection.

To address how these identified cellular factors regulate MHV-68 lytic replication, we analyzed viral gene transcription, DNA replication, protein expression and virion production to determine the step(s) of viral replication affected. Two examples have been shown in Chapter 2, demonstrating that cellular factors affecting different steps of MHV-68 replication could be identified from the same screen.

3.4.4 Kinome siRNA library screen

As a complementary approach, a siRNA library screen of human kinome was performed to search for genes regulating MHV-68 lytic replication (Figure 3-3A). Prior to infection, HEK293T cells were reverse-transfected with an arrayed kinome-wide siRNA library, which targets 788 human kinases, kinase regulatory subunits, and hypothetical kinases. Four unique siRNAs were used to interrogate each gene and were arrayed in separate wells. Cells were then infected with MHV-68-M3/FL at an MOI of 0.01 at 40 hours post-transfection. At 52 and 58 hours post-infection, supernatants containing viruses produced and released by transfected HEK293T cells were transferred to naïve HEK293T cells that were seeded on 384-well plates (referred to as destination plates) 1 day prior to transfer. Luciferase activities of HEK293T cells on the destination plates were measured at 20 hour post-transfer. The screen was optimized using siRNA against GL3, RTA and the host factor MYC as controls: siRNA against GL3 had no effect on MHV-68 replication, siRNA against RTA resulted in inhibition of MHV-68 replication, and siRNA targeting MYC led to an increase in MHV-68 replication (Figure 3-3B). The screen was run in duplicate to ensure the reproducibility of the results. Since the screen was executed in an arrayed format, we employed the Redundant siRNA Activity (RSA) Analysis to

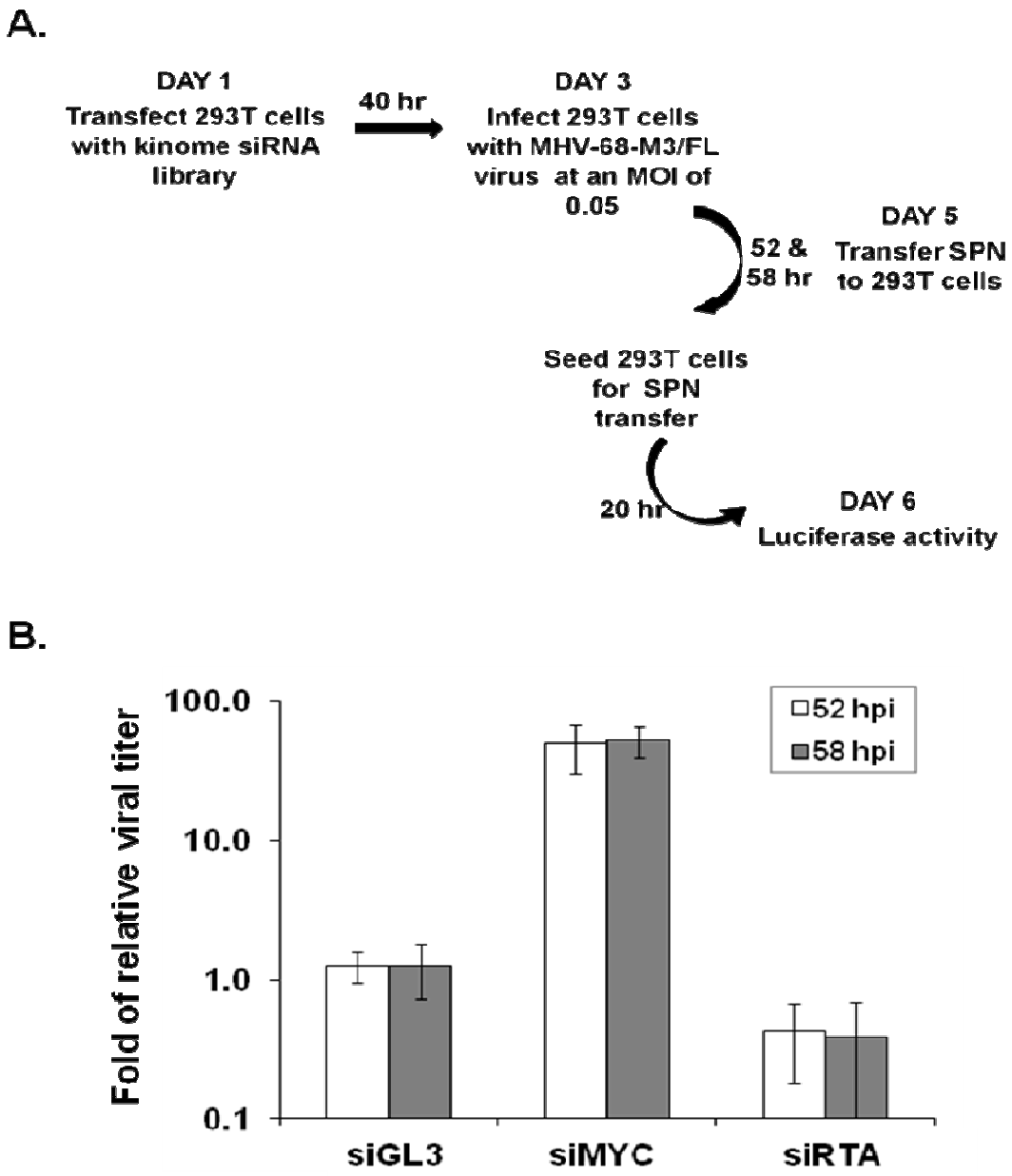

Figure 3-3. Human kinome siRNA screen for cellular regulator of MHV-68 replication. (A) Overall scheme of the screen. Individual siRNA targeting kinase pre-dotted on a 384-well plate was reverse-transfected into HEK293T cells. Cells were infected with MHV-68-M3/FL at an MOI of 0.01 at 40 hr post-transfection. At 52 and 58 hr post infection, 10 μl of supernatants were transferred to naive HEK293T cells that were seeded on 384-well plates (hereafter referred to as destination plates) one day prior to transfer. Luciferase activities of HEK293T cells on the destination plates were measured at 18 hr post-transfer. **(B)** Effects of siRNA targeting GL3, MYC and RTA on MHV-68-M3/FL lytic replication. HEK293T cells were transfected with indicated siRNA, followed by MHV-68-M3/FL infection, supernatant transfer, and luciferase assays of cells on the destination plate as described in **(A)**.

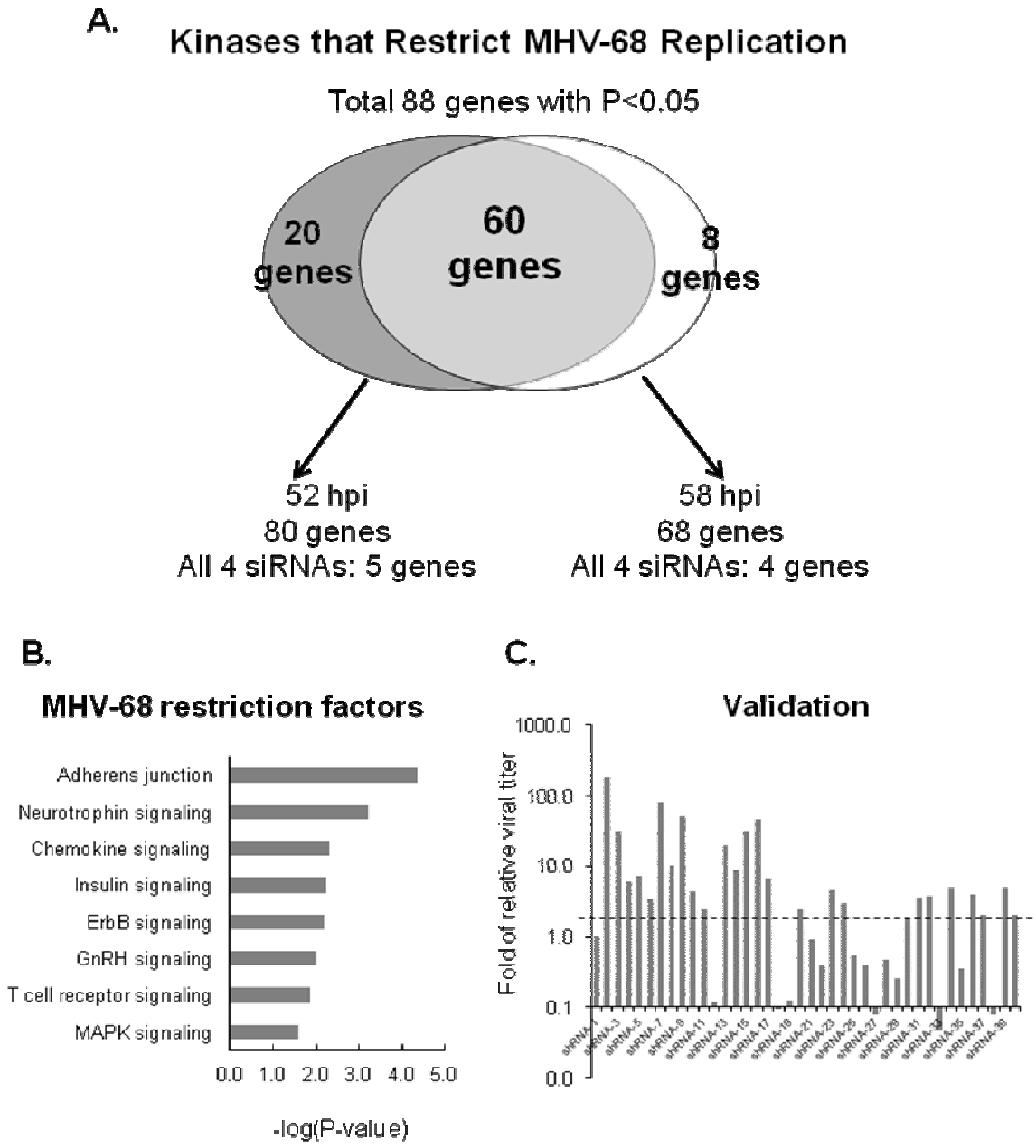

Figure 3-4. Kinome siRNA screen results with bioinformatics analysis and validation. (A) Screen results were analyzed by the Redundant siRNA Analysis (RSA) to identify MHV-68 restriction factors for which at least two independent siRNAs could enhance virus replication significantly (2-fold or greater enhancement of MHV-68 replication). **(B)** Bioinformatics analysis of cellular pathways that were enriched in the identified MHV-68 restriction factors. **(C)** Validation of primary screen. 16 top hits were picked for the follow-up study. Two shRNAs for each gene were constructed and tested in viral replication assay.

identify MHV-68 restriction factors for which at least two independent siRNAs could enhance virus replication significantly (2-fold or greater enhancement of MHV-68 replication) [13, 19]. Using this method, we were able to identify a total of 88 MHV-68 restriction factors, among which 80 genes were from the first time point and 68 genes were from the second time point (Figure 3-4A). This analysis revealed a significant overlap between the two time points (60 overlapping hits), demonstrating the robustness of the assay condition. More significantly, the hits included eight kinases for which all four siRNAs tested increased on MHV-68 replication: ERBB2, GMFG, NEK4, PTPRJ, PYCS, RAF1, RIOK3 and TAO1.

Analysis of over-represented biological annotations identified more than 200 statistically enriched categories (Figure 3-4B). Signaling molecules, including those involved in Adherens junction, Neurotrophin signaling pathway, Chemokine signaling pathway and ErbB signaling pathway were over-represented among the 88 factors, underscoring the importance of these cellular functions during MHV-68 infection. Again, it is interesting that the pathways enriched for MHV-68 restriction factors share significant similarity with those of MHV-68 dependency factors, although we did not observe any overlapping hits between the two lists.

3.4.5 Identification of cellular factors that regulate MHV-68 replication at different steps

To gain more insight into the mechanisms of these restriction factors in MHV-68 lytic replication, we picked 16 top hits for the follow-up study. We constructed two shRNAs for each gene, and performed the virus replication assay. 12 genes were confirmed to be MHV-68 restriction factors for which at least one shRNA could enhance MHV-68 replication over 2-fold to have both shRNAs (Figure 3-4C). As another method of validation, western blot analysis was

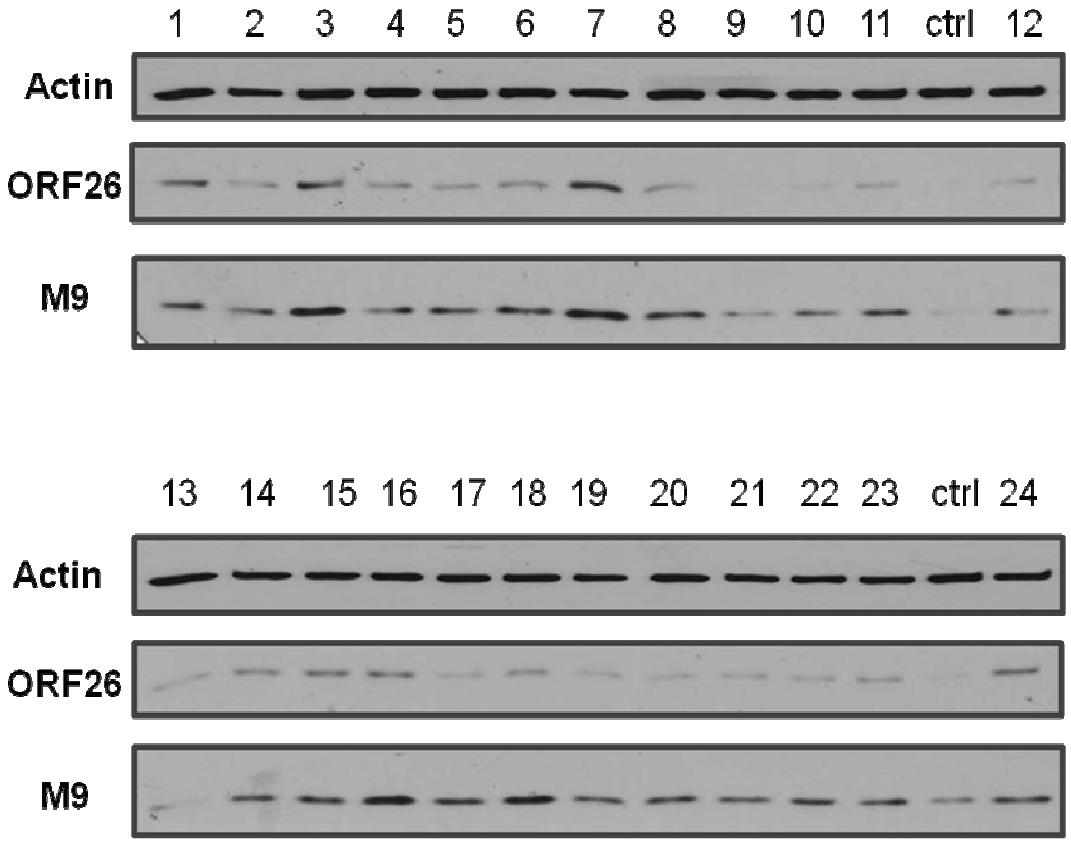

Figure 3-5. Effect of knocking down MHV-68 restriction factors on viral lytic protein expression. HEK293T cells were transfected with various shRNAs and 24 hr later were infected with WT MHV-68 virus at an MOI of 0.1. Cell lysates were collected at 24 hr post-infection and western blot was performed to analyze the expression of the indicated viral proteins.

performed to examine the effect of shRNA on viral lytic antigen expression. Most of the shRNAs lead to the increase of viral lytic antigen expression (Figure 3-5). Among these 12 genes, 7 genes have both shRNA enhancing MHV-68 replication. Therefore, we chose these 7 genes for further study.

To determine the role of the selected cellular proteins in MHV-68 replication, we measured the effect of knocking down specific cellular gene(s) on MHV-68 lytic gene transcription and viral DNA replication. For lytic gene transcription, RT-Q-PCR analysis was carried out. HEK293T cells were transfected with various shRNAs, and at 48 hours post-transfection, cells were infected with WT MHV-68 virus at an MOI of 0.2. Total RNA was extracted at various time points post-infection and cDNA was synthesized. Q-PCR was performed using primers specific for MHV-68 RTA (Immediate Early), ORF6 (Early), and ORF29 (Late) genes (Figure 3-6A, 3-6B and 3-6D). For viral DNA replication, Q-PCR analysis was carried out. HEK293T cells were transfected with various shRNAs, and at 48hours post-transfection, cells were infected with WT MHV-68 virus at an MOI of 0.2. Genomic DNA was extracted at 16 hours post-infection. Q-PCR was performed using primers specific for MHV-68 ORF57 and the human β-actin gene (Figure 3-6C). From the above analysis, we found genes involved in regulating different steps of virus replication.

3.4.6 Characterization of cellular genes involved KSHV latency maintenance and reactivation

One characteristic of herpesvirus is the two-phase life cycle: lytic replication and latency. Both lytic replication and latency play important roles in disease development. It is therefore critical to understand the mechanism(s) by which the switch between latency and lytic regulation is

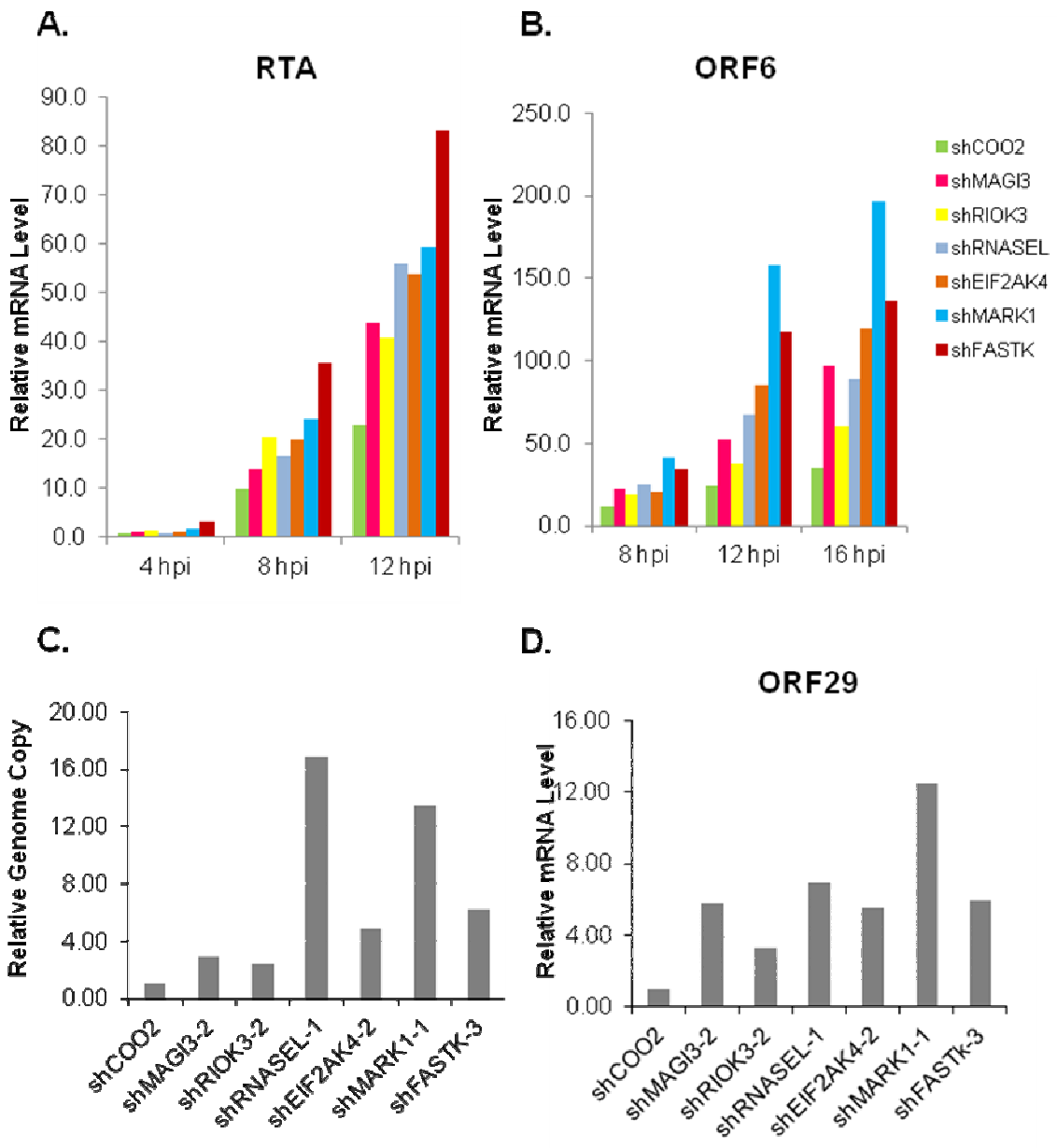

Figure 3-6. To determine the step(s) of MHV-68 replication affected by identified restriction factors. (A), (B) and (D) HEK293T cells were transfected with the indicated shRNAs, and 48 hr later were infected with WT MHV-68 virus at an MOI of 0.2. Total RNA was extracted at the indicated time points and cDNA was synthesized. Q-PCR was performed using primers specific for MHV-68 RTA, ORF6 and ORF29 genes. **(C)** HEK293T cells were transfected with the indicated shRNAs, and 48 hr later were infected with WT MHV-68 virus at an MOI of 0.2. Genomic DNA was extracted at 16 hr post-infection. Q-PCR was performed using primers specific for MHV-68 ORF57 and the human β-actin gene.

regulated. Previously when we examined the step(s) of MHV-68 replication regulated by these selected cellular genes, we found a group of cellular genes that regulate MHV-68 replication through regulating RTA transcription. Since RTA is a conserved gene between MHV-68 and KSHV, we set out to test if KSHV RTA transcription is also regulated by these cellular genes. We focused on FASTK because its siRNA showed the strongest phenotype on MHV-68 RTA transcription. To test this, we first performed a KSHV RTA promoter reporter assay. The result showed that knocking down FASTK can also enhance KSHV RTA promoter activity (Figure 3-7A). RTA is the master regulator of gamma-herpesvirus lytic replication and is necessary and sufficient to induce virus reactivation from latency [23, 24]. We then tested if FASTK plays roles in gamma-herpesvirus reactivation. We used the PEL cell line BC-3, which is latently infected with KSHV, to monitor KSHV reactivation. BC-3 cells were transfected with pPAN-luc (PAN promoter driven luciferase reporter), control plasmids PGK-RL, and shRNA. Luciferase activity was determined 48 hours post-transfection. PAN is a lytic transcript, and its promoter is highly responsive to RTA expression. The induction of the PAN promoter can be used as the indicator of reactivation. We found that depletion of FASTK could increase the PAN promoter activity to a similar level of MYC depletion in BC-3 cells (Figure 3-7B). To further verify the role of FASTK in KSHV reactivation, flow cytometry was performed to monitor the percentage of reactivated cells. BC-3 cells were transfected with individual shRNAs and selected with 1 ug/ml of puromycin. After 2 days of puromycin selection, cells were stained with anti-ORF59 which is a marker for lytic replication and subjected to flow cytometry analysis. As shown in Figure 3-7C, FASTK knockdown led to an increase of ORF59-positive cells. Furthermore, western blot analysis confirmed that the expression of viral lytic protein K8 was significantly increased by shFASTK, indicating increased KSHV reactivation (Figure 3-7D).

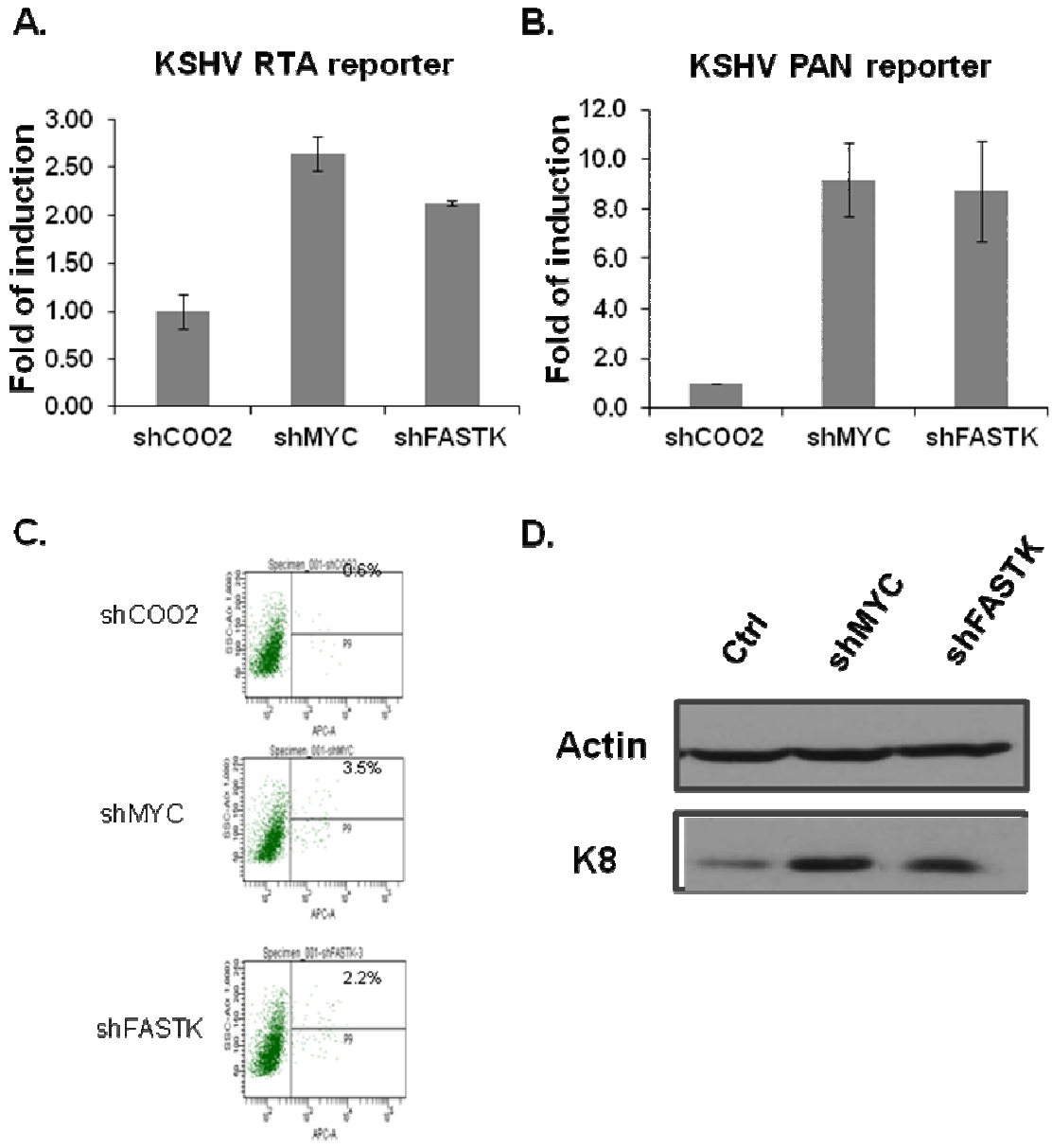

Figure 3-7. FASTK is required for KSHV latency maintenance. **(A)** HEK293T cells were cotransfected with RTA_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated shRNAs. Luciferase assays were performed 48 hr post-transfection. **(B)** BC-3 cells were cotransfected with PAN_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated shRNAs. Luciferase assays were performed 48 hr post-transfection. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to control shRNA. **(C) and (D)** BC-3 cells were transfected with the indicated shRNAs and selected with puromycin for 2 days. Cells were stained with anti-ORF59 and subjected to flow cytometry analysis **(C)**. Cell lysates were harvested for western blot analysis with the indicated antibodies **(D)**.

Taken together, our data showed that depletion of FASTK could induce viral lytic genes expression by modulating RTA transcription in KSHV-latently-infected cells and could also lead to viral reactivation; thus we established that FASTK plays an essential role in maintaining KSHV latency in PEL cells.

3.4.7 Test the possible role of selected cellular genes play in type I interferon (IFN) signaling

Although we have identified hundreds of MHV-68 restriction factors from the two functional screens, our understanding of the mechanisms by which restriction factors interfere with MHV-68 replication, and how their effects may be avoided by MHV-68 in host cells, is far from complete. One highly effective antiviral response is the production of type I IFNs, such as interferon-α (IFN-α) and interferon-β (IFN-β) [25, 26]. These IFNs activate the JAK-STAT pathway to stimulate the expression of interferon-stimulated genes (ISGs), which collectively inhibit viral replication and assembly [27-29]. Therefore it is possible that these MHV-68 restriction factors play roles in the type I IFN pathway.

We compared our siRNA screen results with another independent kinome siRNA screen that was designed to identify cellular regulators of innate immune response to cytosolic DNA. The comparison of siRNA screens of kinases that restrict MHV-68 replication (siRNA activities enhance replication) and kinases required for ISRE-luc induction by B-DNA (siRNAs inhibit ISRE induction) showed significant overlaps in genes (23 overlapping genes, $p=1.5x10^{-9}$).

To further test the functional contribution of these genes to the type I IFN pathway, we performed reporter assays to examine the effect of knocking down these genes on the type I IFN production or signaling. For the type I IFN production, we cotransfected HEK293T cells with

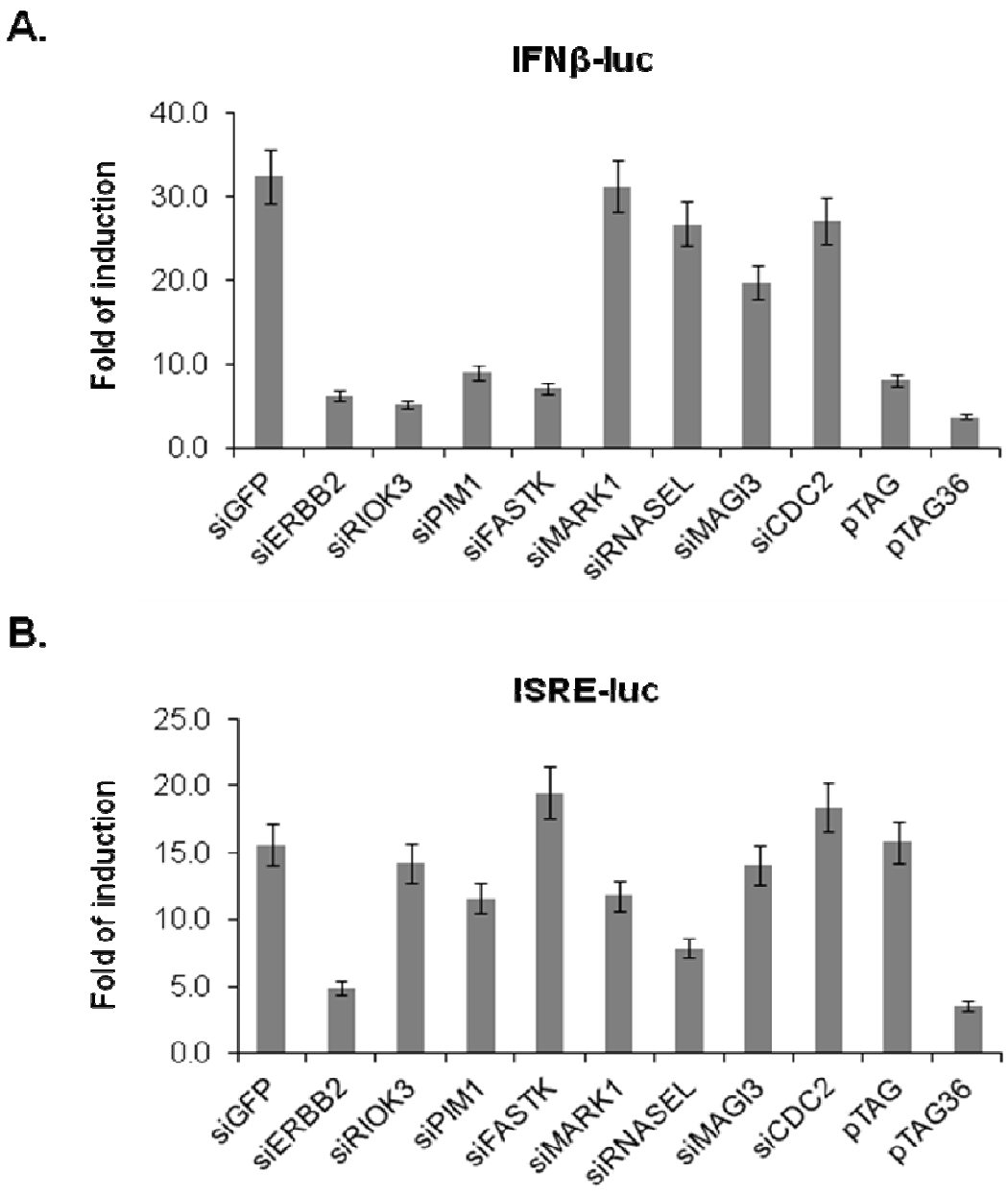

Figure 3-8. Identification of MHV-68 restriction factors involved in the type I IFN production or signaling. **(A)** HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left untreated or transfected with BDNA for 20 hr before luciferase assays were performed. **(B)** HEK293T cells were cotransfected with ISRE_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left untreated or treated with IFNα for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control siRNA targeting GFP.

IFNB-luc (interferon-beta promoter driven luciferase reporter), control plasmid PGK-RL, and siRNAs; 24 hours later cells were left untreated or treated with BDNA. Luciferase assay was performed 16 hours post-treatment. For the type I IFN signaling, HEK293T cells were cotransfected with ISRE-luc (Interferon-sensitive response element driven luciferase reporter), control plasmid PGK-RL, and siRNAs; and cells were left untreated or treated with IFN-α 24 hours post-transfection. Luciferase assay was performed 16 hours later. As shown in Figure 3-9, knocking down of ERBB2 inhibited both BDNA-induced IFNB reporter activity and IFN-α induced ISRE reporter activity. On the other hand, depletion of PIM1, RIOK3, or FASTK only inhibited BDNA-induced IFNB reporter activity and RNASEL knockdown only led to the inhibition of IFN-α-induced ISRE reporter activity, suggesting the specific roles of these MHV-68 restriction factors in the type I IFN pathway (Figure 3-8). Further characterization of these factors will not only extend our understanding of virus-host interaction but also of the inner workings of the cells. As will be described in Chapter 4, we continued to study one of the MHV-68 restriction factors and addressed its function in the type I IFN pathway.

3.5 Discussion

Herpesviruses have co-evolved with their hosts, adopting diverse strategies to evade the host immune system and to hijack existing cellular machinery. To capture this complex relationship, we applied multiple unbiased systems biology approaches to explore the interactions between MHV-68 and its host cells. First, we defined the regulatory responses of the host using genome-wide mRNA profiling of cells exposed to MHV-68 infection. Second, we assembled a physical map of binary associations between viral and cellular proteins. GO analyses of these two studies led us to acknowledge the importance of cellular kinases and transcription factors in MHV-68

lytic replication. To validate the function of cellular kinases and transcription factors in MHV-68 lytic replication, we assessed the gain-of-function effect of individual kinases and transcription factors on viral replication. This allowed us to identify 144 MHV-68 dependency factors and 125 restriction factors. As a complementary approach, we further functionally interrogated the human kinome with siRNA, which led to the identification of 88 additional MHV-68 restriction factors. By integrating experimental results with systematic data mining, this study presented a comprehensive map of physical and regulatory interactions between MHV-68 and its host cells.

Gene enrichment analysis of host factors identified in multiple screens both supports and extends the previous knowledge on cellular signaling on gammaherpesvirus lytic replication. We found that the virus targets diverse signaling pathways by affecting multiple components in each pathway through both physical and regulatory interactions. Among the pathways identified from our screens are the MAPK signaling, Toll-like receptor signaling, NF-kappa B cascade and regulation of apoptosis previously reported to be important for gammaherpesvirus lytic replication [18, 22, 30-32]. In addition to these known regulatory pathways, we identified novel pathways, such ErbB signaling, Wnt signaling and TGF-beta signaling, that may be required for efficient gammaherpesvirus lytic replication. Further investigation of the mechanisms of how these signalings affect gammaherpesvirus replication will provide more insight to the virus-host interactions.

In both functional screens, we employed the replication-competent MHV-68-M3/FL reporter virus. By exposing naïve HEK293T cells to culture supernatant from transfected and infected HEK293T cells at least 42 hours after infection, then detecting luciferase activity 18 hours later,

we were able to identify cellular factors that regulate any step(s) of the complete viral lifecycle, from virus-host receptor binding to the completion of a second round of viral infection.

Gammaherpesvirus is known for its two-phase life cycle, and cellular genes are thought to not only play roles in the course of lytic replication, but also the switch between latency and lytic replication. Many studies have shown that many cellular pathways regulate the balance between latency and lytic replication via expression of an viral immediate early gene product RTA which is highly conserved among gammaherpesviruses. As a group of cellular genes were shown to regulate MHV-68 replication by affecting RTA transcription, we also tested their role in gammaherpesvirus reactivation. We showed that knocking down cellular gene FASTK could induce KSHV spontaneous reactivation by increasing RTA expression.

Upon MHV-68 infection, host cells detect viral components through pattern recognition receptors (PPRs) like Toll-like receptors, and they induce type I IFNs and downstream antiviral programs. At the same time, viral encoded proteins, such as ORF36, ORF45, and ORF54, subvert host defenses through several mechanisms [33-35]. The two functional genetic screens led us to discover many MHV-68 restriction factors that may play a role in the type I IFN pathway. How these viral restriction factors regulate type I IFN pathway and whether they are specific to MHV-68 or respond to a broader class of viruses remains to be determined.

Taken together, this integrative approach toward genome-wide virus-host interaction analysis has revealed cellular factors that coordinately regulate different steps of MHV-68 lytic replication. The cellular factors identified by these efforts represent a starting point for further defining a more detailed virus-host interaction network on the molecular basis. Such a comprehensive network will provide new opportunities for the development of host-factor-directed anti-viral therapies.

3.6 Bibliography

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CHAPTER 4

RIOK3 IS A NOVEL REGULATOR OF TYPE I INTERFERON PATHWAY

4.1 Abstract

Detection of cytosolic nucleic acids by pattern recognition receptors (PPR) activates downstream transcription factors such as NF-κB and IRF3, which leads to the induction of type I interferons (IFNs) and elicits the innate immune response. Here we identified RIOK3 as a novel adaptor protein that is essential for the cytosolic nucleic acid induced type I IFN production through a kinome-wide RNAi screen. Knockdown of RIOK3 blocks both BDNA and Sendai virus induced IRF3 activation, IFNβ production, and ectopic expression of RIOK3 activates IRF3. Knockdown of RIOK3 also inhibits cellular antiviral response. Epistasis experiments show that RIOK3 functions upstream of IRF3 phosphorylation and downstream of TBK1. RIOK3 interacts with IRF3 and also interacts with the active form of TBK1. Thus, our data suggests that RIOK3 plays a critical role in the type I IFN pathway by bridging TBK1 and IRF3.

4.2 Introduction

The innate immune response acts as the first line of defense, limiting infectious pathogens directly and helping to shape the ensuing adaptive immune response [1, 2]. The production of Type I interferons (IFNs), such as IFNα and IFNβ, is the most immediate response upon infection [1-4]. These secreted IFNs then bind to their receptors on the cell surface and activate the Jak/STAT pathway to induce the expression of a wide range of interferon-stimulated genes (ISGs), which collectively mediate the inhibition of pathogens [5-8].

Type I IFNs can be stimulated upon sensing certain highly conserved molecular components in bacteria or viruses, termed pathogen-associated molecular patterns (PAMPs), by several classes of germline-encoded pattern recognition receptors (PRRs) [1, 9-11]. Nucleic acids are among the most potent and broadly recognized PAMPs [12]. Previous studies have led to the discovery of several different classes of PRRs that recognize nucleic acids [1, 11, 13, 14]. One of the major classes is the membrane-associated Toll-like receptors (TLRs), the leucine rich repeat (LRR) domains of which can recognize endosomal dsRNA (TLR3), single stranded (ss)RNA (TLR7/8), and unmethylated CpG DNA (TLR9) [15]. TLRs dimerize upon ligand stimulation and differentially recruit adaptor proteins MyD88 or TRIF. The activation of adaptor proteins initiates downstream signaling cascade culminating in the activation of NF-κB, mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IRFs) [16]. TLRs are only expressed in a subset of specialized cells, such as macrophages and dendritic cells, while almost all nucleated cells are able to recognize foreign nucleic acids and induce the production of type I IFNs [17]. RIG-I like receptors (RLRs), including two RNA helicase proteins (retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), represent a class of ubiquitously expressed PRRs [11, 18, 19]. Both RIG-I and MDA5 contain caspase

recruitment domains (CARDs) and DExD/H-box helicase domains and they can respond to cytosolic dsRNA. Upon ligand recognition, RIG-I and MDA5 activate the adaptor protein mitochondrial antiviral signaling protein MAVS (also known as IPS-1, CARDIF and VISA) via CARD domain interaction [20-23]. MAVS then triggers the activation of TBK1/IKKε and NF-κB, which in turn induces the production of type I IFN [20-23]. Recent studies have also identified a group of cytosolic DNA sensors, such as DAI, IFI16, DDX41, which induces type I IFNs independent of TLR signaling [24-26]. Similar to cytosolic RNA sensing pathway, recognition of cytosolic DNA also leads to activation of TBK1 and IRF3 and production of type I IFNs. The recently identified adaptor protein stimulator of interferon genes (STING, also known as MITA, MPYS, ERIS, and TMEM173) plays a critical role in the signaling pathway upstream of TBK1 in the cytosolic DNA recognition pathway [27-30].

Although a variety of receptors present in the cytosol can induce type I IFNs, the majority converge at the level of TBK1/IKKε [3, 31]. These kinases phosphorylate and activate the transcription factors IRF3 and/or IRF7 which reside in the cytoplasm in unstimulated cells [3, 31-33]. Phosphorylation of IRF3 and/or IRF7 leads to their dimerization, nuclear translocation, and association with CREB binding protein (CBP)/p300 [34-36]. Activated IRF3 and/or IRF7 then assemble into a transcriptional enhanceosome with the transcription factors NF-κB, and activating transcription factor 2 (ATF-2)/c-Jun, all of which function cooperatively to drive IFNα/β gene transcription [4, 35, 37].

Upstream of TBK1/IKKε, adaptor molecules integrate signals between different classes of PRRs and TBK1/IKKε kinase activity. To date, several distinct adapter molecules have been identified, including: MyD88 and TRIF, Toll/Interleukin- 1 receptor (TIR) domain containing adapter molecules that are recruited either directly or indirectly to TIR domains of TLRs; MAVS, a

CARD domain containing adapter molecule which is localized to the mitochondrion and relays signals from the RLRs; and STING, an endoplasmic reticulum resident protein that transduces signals from the cytosolic DNA sensors.

Despite the tremendous advances in understanding cytosolic nucleic acid sensors and signaling components, key questions such as ligand specificity and spacial and temporal activation of different signaling molecules remain to be solved. To more comprehensively understand the molecular basis of the innate immune response, we conducted a kinome-wide RNAi analysis to systemically identify the essential molecular components for cellular response to cytosolic DNA. In this study, we identified RIOK3 as a novel regulator of type I IFN pathway, the knockdown of which abolished both BDNA and Sendai virus induced IRF3 activation and IFNβ production. Ectopic expression of RIOK3 activated IRF3. Knockdown of RIOK3 also inhibited cellular antiviral response to both DNA and RNA viruses. RIOK3 functioned downstream of TBK1, and constitutively interacted with IRF3. RIOK3 also interacted with the active form of TBK1, which is required for IRF3 activation. Collectively, our data suggested that RIOK3 plays a critical role in the type I IFN pathway by bridging TBK1 and IRF3.

4.3 Materials and Methods

High-throughput screening

A 384-well plate-based assay was optimized to identify siRNAs that influence cellular response to cytosolic BDNA. A human kinome library comprised of 2068 synthetic siRNAs targeting 517 unique human genes in total was arrayed in 384-well plates such that each well contained two unique and identifiable siRNAs per gene (7 ng siRNA per gene, per well). There were on average 2 wells per gene or 4 siRNAs per gene. Each plate also contained positive controls

(RIG-I siRNAs), negative controls (GL2-luciferase siRNA), and scramble siRNA controls. The library was introduced into the HEK293T_ISRE luciferase reporter line by a high-throughput transfection process (Konig et al., 2008). 5 ug/ml of poly(dA:dT) was added to the wells 30 hours after siRNA transfection. 16 hours post-stimulation, Bright-Glo (Promega) was added in equal volumes to each well and the luminescence associated with each sample was analyzed. The screen was run in duplicates and statistically analyzed. All steps were performed using a fully integrated high-throughput cellular genomics robotic system (GNF Systems).

Cells and reagents

HEK293T, THP-1 and MDCK cells were obtained from the American Type Culture Collection (ATCC) and were maintained in DMEM medium supplemented with 10% FBS and RPMI medium supplemented with 10% FBS respectively. Transient transfections in HEK293T and THP-1 cells were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Poly(dA:dT) was obtained from Invivogen. For stimulation of cells, Poly(dA:dT) was mixed with Lipofectamine 2000 (Invitrogen) at a ratio of 1:1 (w/v), and then added to cells at a final concentration of 5 μg/ml. Sendai virus was purchased from Charles River. For stimulation, cells were infected with 500 HAU/ml of Sendai virus.

Plasmids

Expression constructs for human RIG-I, MAVS, TBK1, IRF3 and RIOK3 were constructed by PCR amplification and standard recombinant DNA techniques.

Reporter analysis

HEK293T cells were seeded at $5x10^4$ cells per well in a 48-well plate 12 hours prior transfection. Cells in each well were cotransfected with 50 ng of the luciferase reporter plasmid together with 400 ng of expression plasmids or 10 nmole of various siRNA constructs using Lipofectamine 2000 (Invitrogen). As an internal control, 5□ng pPGK-RL was transfected simultaneously. Cells were then stimulated with poly(dA:dT) or Sendai virus or left untreated. 20 hour post-stimulation, luciferase activity was measured by the dual-luciferase reporter assay system (Promega).

Real-time PCR

For real-time PCR analysis, total RNA was extracted from cells using PureLink RNA Mini Kit (Invitrogen) and reverse transcribed into cDNA with qScript cDNA Synthesis Kit (Quantas). RT-PCR was then done with the following primers to quantify cellular transcripts: *Gapdh* (NM_002046.4): 5′- TGC ACC ACC AAC TGC TTA GC -3′ and 5′- GGC ATG GAC TGT GGT CAT GAG -3′; human *ifnb1* (NM_002176.2) : 5′- GCT TGG ATT CCT ACA AAG AAG CA -3′ and 5′- ATA GAT GGT CAA TGC GGC GTC -3′; human *isg15* (NM_005101.3): 5′- ACT CAT CTT TGC CAG TAC AGG -3′ and 5′- CAG CTC TGA CAC CGA CAT G -3′.

Enzyme-linked immunosorbent assay (ELISA)

HEK293T cells were transfected with various siRNA constructs and stimulated with poly(dA:dT) or Sendai virus. Supernatants were collected 10 hr post-stimulation. IFNβ ELISA were performed according to the manufacturer's instructions (PBL).

Immunoblotting and immunoprecipitation.

For immunoblotting, cells were directly lysed on the plate with 1x protein sample buffer (0.625 M Tris pH 6.8, 2% SDS, 10% glycerin, 5% β-mercaptoethanol, 0.002% Bromophenol blue), transferred to Eppendorf tubes and boiled for 10 minutes prior to SDS-PAGE. For all immunoprecipitation assays, HEK293T cells were washed once with PBS and then lysed on ice with lysis buffer (50 mM Tris pH 7.5, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mM PMSF, 1 mM Na_3VO_4, and 1 mM NaF. The cell lysates were then incubated with a specific antibody (2 ug antibody per sample) or control IgG conjugated Sepharose protein G beads overnight at 4˚C. The immunoprecipitates were washed 3 times with lysis buffer then boiled and analyzed by western blot. The antibodies used in this study were as follows: rabbit anti-IRF3 (Santa Cruz, sc-9082), rabbit anti-phospho-IRF3 (Epitomics, 2562-1), rabbit anti-phospho-TBK1 (Cell Signaling, 5483), mouse anti-FLAG (Sigma, F3165), HA (Sigma, H3663), and V5 (Invitrogen, R960-25).

RNA interference

HEK293T cells were transfected with a final concentration of 30 nM of siRNA using Lipofectamine 2000 (Invitrogen). The siRNAs used in this study were purchased from Qiagen. The sequences of the siRNAs are as follows: GFP: GCA AGC UGA CCC UGA AGU UCA U; RIOK3_5, GCG GCA GUU AUA UCA UGA ATT; RIOK3_6, GGC UGU AUU AGU ACA GGA ATT.

Virus propagation and quantitation.

MHV-68-M3/FL virus was propagated in Vero cells and the viral titer was measured by firefly luciferase assay. HEK293T cells were seeded in 80 μl of medium in each well of a 96-well plate 1 day prior to infection, and 40 μl of different dilutions of samples containing MHV-68-M3FL virus was added to each well. Luciferase activities were determined using the Bright-Glo luciferase assay system. Influenza A virus was propagated by infecting MDCK cells and viral titer was measured by limiting dilution.

4.4 Results

4.4.1 Identification of RIOK3 as an essential molecule of cytosolic DNA sensing pathway

To systematically identify essential molecular components for cellular response to cytosolic DNA, we conducted a kinome siRNA library screen. In this screen, we transfected individual siRNA targeting kinases into HEK293T_ISRE cells harboring a stable luciferase reporter under the control of an interferon-sensitive response element (ISRE), which is responsive to transfected double-stranded BDNA. Subsequently transfected cells were stimulated with 5 ug/ml of BDNA to induce ISRE reporter. The activation of ISRE reporter was measured by relative luciferase activities, and the fold induction was calculated in comparison to the untreated sample. Since HEK293T cells do not express TLR9 or other nucleic acid TLRs, it is likely that the observed activities are primarily a result of a cytoplasmic DNA recognition response pathway. RIOK3 was one of the top hits whose siRNAs led to the significant inhibition of the BDNA-induced ISRE reporter activity.

To validate the screen results, two RIOK3 siRNA constructs, which can knockdown RIOK3 expression efficiently (Figure 4-1B, siRIOK3_6 was used for all the experiments described below. Similar results were obtained with siRIOK3_5), were subsequently confirmed to block

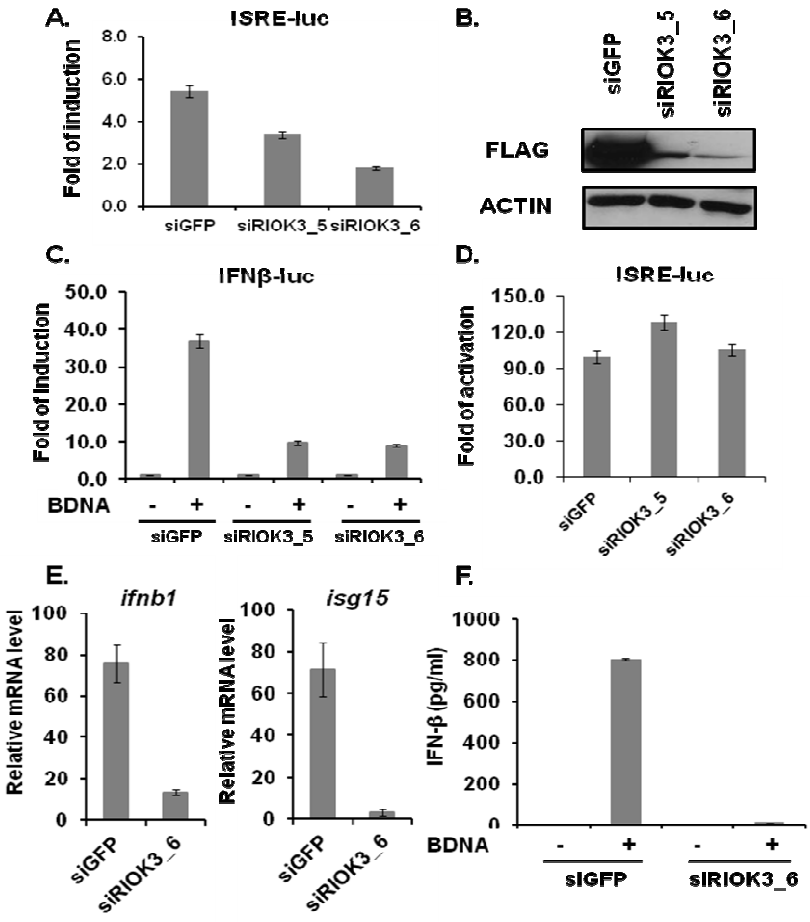

Figure 4-1. RIOK3 is required for IFN-β induction by BDNA. (A) Effect of RIOK3 RNAi on BDNA induced ISRE promoter activation. HEK293T_ISRE cells were transfected with the indicated siRNAs, and 24 hr later, cells were left untreated or transfected with BDNA for 20 hr before luciferase assays were performed. Fold of activation was calculated in comparison to untreated control siRNA. **(B)** Effect of RIOK3 RNAi on the expression of transfected RIOK3. HEK293T cells were transfected with FLAG-RIOK3 expression plasmid and the indicated siRNAs. At 48 hr post transfection, lysates were analyzed by the indicated antibodies. **(C)** and **(D)** Effect of RIOK3 RNAi on BDNA induced IFNβ promoter activation **(C)** and IFNα induced ISRE promoter activation **(D)**. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter **(C)** or ISRE_firefly-luciferase reporter **(D)**, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left untreated or transfected with BDNA **(C)** or treated with 3×10^4 units/ml of IFNα **(D)** for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control siRNA. **(E)** and **(F)** Effect of RIOK3 RNAi on BDNA induced *ifnb1* and *isg15* transcription and IFNβ expression. HEK293T cells were transfected with the indicated siRNAs, and were left untreated or treated with BDNA 40 hr later. RNA was extracted 8 hr post-treatment, and RT-PCR was performed to examine the relative mRNA level of *ifnb1* and *isg15* **(E)**. The concentration of IFNβ in the supernatant was measured by ELISA 12 hr post-treatment **(F)**.

the BDNA-induced ISRE promoter activity (Figure 4-1A). To examine whether RIOK3 is required for type I IFN production or signaling, we first performed IFNβ promoter reporter assay as a measurement of IFNβ production. As shown in Figure 4-1C, knockdown of RIOK3 significantly inhibited BDNA induced IFNβ promoter activity. In contrast, the IFNα induced ISRE promoter activity was not affected by depletion of RIOK3 (Figure 4-1D), which indicates that RIOK3 is required for type IFN production but not for signaling. Consistent with reporter assay results, knocking down of RIOK3 was sufficient to block the induction of endogenous interferon β1 (*ifnb1*) and interferon stimulated gene 15 (*isg15*) transcription and IFNβ protein by BDNA, as measured by QPCR and ELISA respectively (Figure 4-1E, F).

4.4.2 RIOK3 is required for cytosolic dsRNA induced type I IFN induction

Because RIOK3 is essential for type I IFN induction upon cytosolic DNA recognition, we then examined whether RIOK3 is also required for type I IFN induction by Sendai virus, which is an RNA virus of the paramyxoviridae family and can be sensed through RIG-I pathway [19]. As shown in Figure 4-2A, both RIOK3 siRNAs dramatically reduced the activation of IFNβ promoter. QPCR analysis showed that Sendai virus induction of *ifnb1* and *isg15* RNA was strongly blocked in the absence of RIOK3 (Figure 4-2B). Similarly, RNAi of RIOK3 abolished the production of endogenous IFNβ protein by Sendai virus, as measured by ELISA (Figure 4-2C). In addition, We also tested whether knockdown of RIOK3 could affect poly(I:C)-mediated type I IFN production, which is largely governed by MDA5 [38]. Knockdown of RIOK3 resulted in a reduction in the IFNβ promoter activity and *ifnb1* expression (Figure 4-3). Thus we conclude RIOK3 is required for both RIG-I- and MDA5- dependent signaling. Together with the

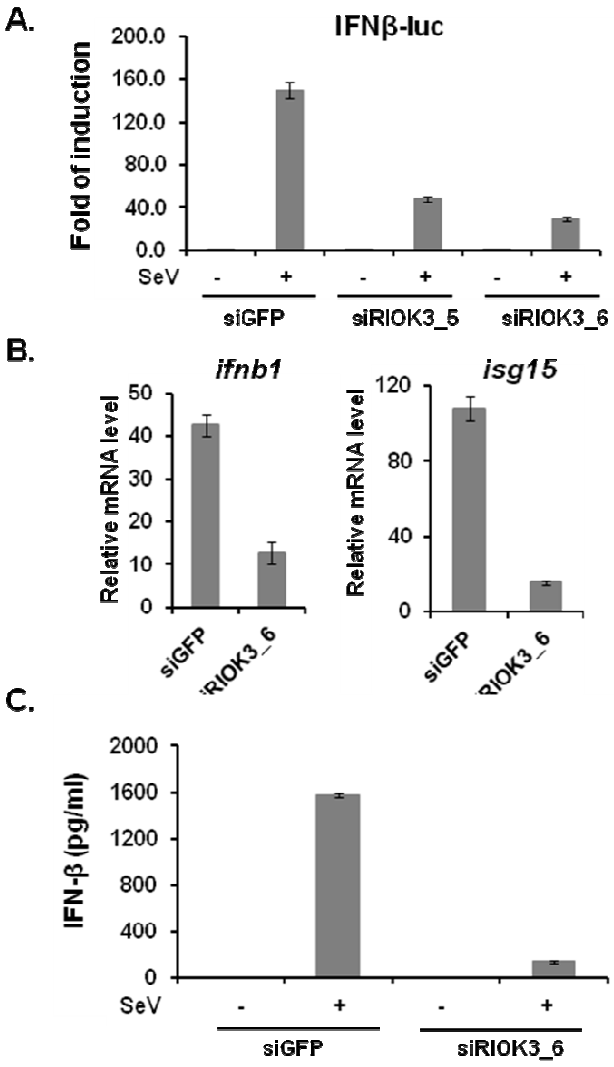

Figure 4-2. RIOK3 is required for IFN-β induction by Sendai virus. (A) Effect of RIOK3 RNAi on Sendai virus induced IFNβ promoter activation. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left uninfected or infected with 100 HAU/ml of Sendai virus for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control siRNA. **(B)** Effect of RIOK3 RNAi on Sendai virus induced ifnb1 and isg15 transcription. HEK293T cells were transfected with the indicated siRNAs, and were left uninfected or infected with 100 HAU/ml of Sendai virus 40 hr later. RNA was extracted 8 hr post-infection, and RT-PCR was performed to examine the relative mRNA level of *ifnb1* and *isg15*. **(C)** Effect of RIOK3 RNAi on Sendai virus induced IFNβ protein expression. HEK293T cells were transfected with the indicated siRNAs, and were left uninfected or infected with 100 HAU/ml of Sendai virus 40 hr later. The concentration of IFNβ in the supernatant was measured by ELISA 12 hr post-infection.

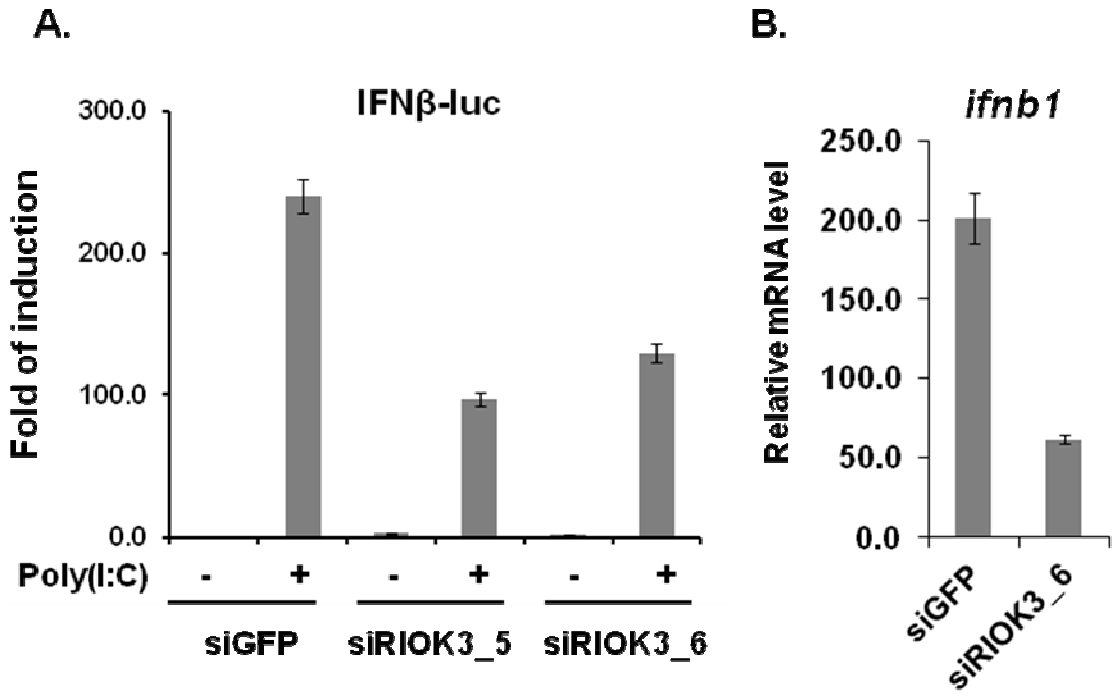

Figure 4-3. RIOK3 is required for IFN-β induction by poly(I:C). (A) Effect of RIOK3 RNAi on poly(I:C) induced IFNβ promoter activation. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left untreated or transfected with poly(I:C) for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control siRNA. **(B)** Effect of RIOK3 RNAi on poly(I:C) induced ifnb1 transcription. HEK293T cells were transfected with the indicated siRNAs, and were left untreated or treated with poly(I:C) 40 hr later. RNA was extracted 8 hr post-treatment, and RT-PCR was performed to examine the relative mRNA level of *ifnb1*.

data presented in Figure 4-1, these results suggested that RIOK3 functions as a shared node of both intracellular DNA and RNA recognition pathways.

4.4.3 Loss of RIOK3 affects host defense against DNA and RNA viruses

Since RIOK3 is critically involved in induction of type I IFN by cytosolic nucleic acids, we next evaluated the importance of this protein in mediating immune defense against selected viruses. Here murine gammaherpesvirus 68 (MHV-68), a DNA virus of the herpesvirus family, and Influenza A virus, an RNA virus of the orthomyxovirus family, were used. HEK293T cells were transfected with control or RIOK3 siRNA, and were infected with replication-competent MHV-68-M3/FL virus or Influenza A virus at MOI of 0.05. Viral titer was measured by luciferase assay or limiting dilution 48 hr post-infection. Knockdown of RIOK3 led to an increase of both MHV-68-M3/FL and Influenza A viral titer by 5-fold and 15-fold, respectively, compared to control cells (Figure 4-4). These data indicated that depletion of RIOK3 rendered cells more susceptible to virus infection and replication, possibly due to the impaired production of type I IFN. Thus, we concluded that RIOK3 plays a pivotal role in cellular antiviral response.

4.4.4 RIOK3 functions downstream of TBK1 and upstream of IRF3 phosphorylation

As cytosolic dsDNA and dsRNA recognition pathways were thought to converge at the level of TBK1, which is involved in the activation of IRF3, we hypothesized that RIOK3 may function at the level of, or downstream from TBK1 activity in the pathway. To test this, we first examined whether IRF3 activation was affected by RIOK3 knockdown, since both BDNA and Sendai virus induced IFNβ activity is IRF3 dependent (Figure 4-5A).

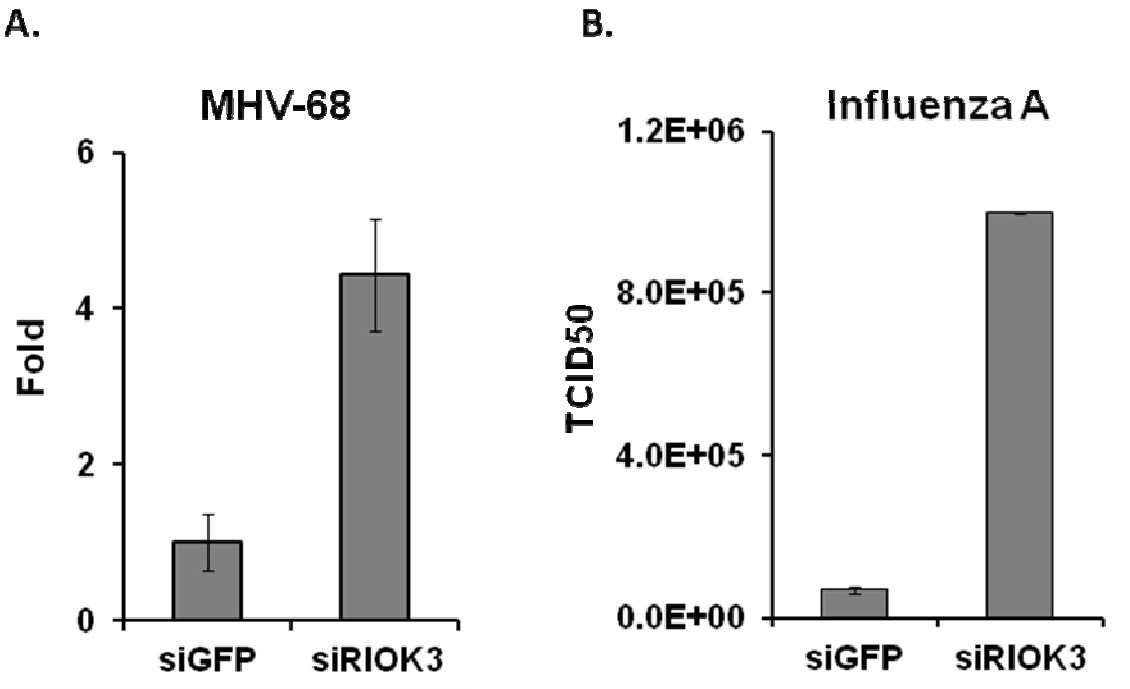

Figure 4-4. Loss of RIOK3 affects host defense. (A) Effect of RIOK3 RNAi on MHV-68 replication. HEK293T cells were transfected with the indicated siRNAs. 48 hr later, cells were infected with replication competent MHV-68-M3/FL luciferase reporter virus at an MOI of 0.05 for 48 hr. The culture supernatant was transferred to naïve 293T cells and relative viral titer was measured by luciferase assay 24 hr later. **(B)** Effect of RIOK3 RNAi on Influenza A replication. HEK293T cells were transfected with the indicated siRNAs. 48 hr later, cells were infected with Influenza A virus at an MOI of 0.01 for 48 hr. The culture supernatant was collected and the viral titer was measured by limiting dilution.

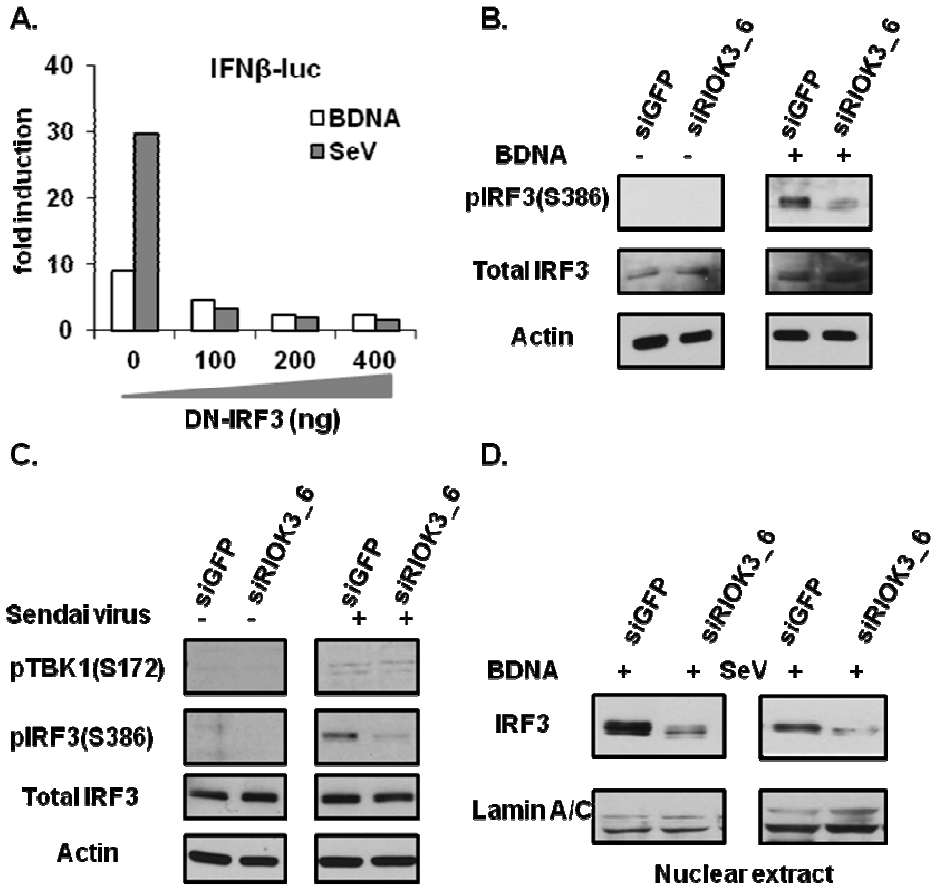

Figure 4-5. RIOK3 is required for IRF3 activation induced by BDNA or Sendai virus. (A) Both BDNA and Sendai virus induced IFNβ responses are IRF3-dependent. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated amount of dominant-negative IRF3. At 24 hr post-transfection, cells were left untreated or treated with BDNA or Sendai virus for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control vector. **(B)** Effect of RIOK3 RNAi on BDNA induced IRF3 phosphorylation. HEK293T cells were transfected with the indicated siRNAs, and were left untreated or treated with BDNA 40 hr later. Cell lysates were collected 4 hr post-treatment, and immunoblot was performed with the indicated antibodies. **(C)** Effect of RIOK3 RNAi on Sendai virus induced IRF3 phosphorylation. HEK293T cells were transfected with the indicated siRNAs, and were left uninfected or infected with 100 HAU/ml of Sendai virus 40 hr later. Cell lysates were collected 4 hr post-infection, and immunoblot was performed with the indicated antibodies. **(D)** Effect of RIOK3 RNAi on BDNA and Sendai virus induced IRF3 nuclear translocation. HEK293T cells were transfected with the indicated siRNAs, and were left untreated or treated with BDNA or Sendai virus 24 hr later. Nuclear extracts were prepared 6 hr post-treatment and analyze by immunoblot with the indicated antibodies.

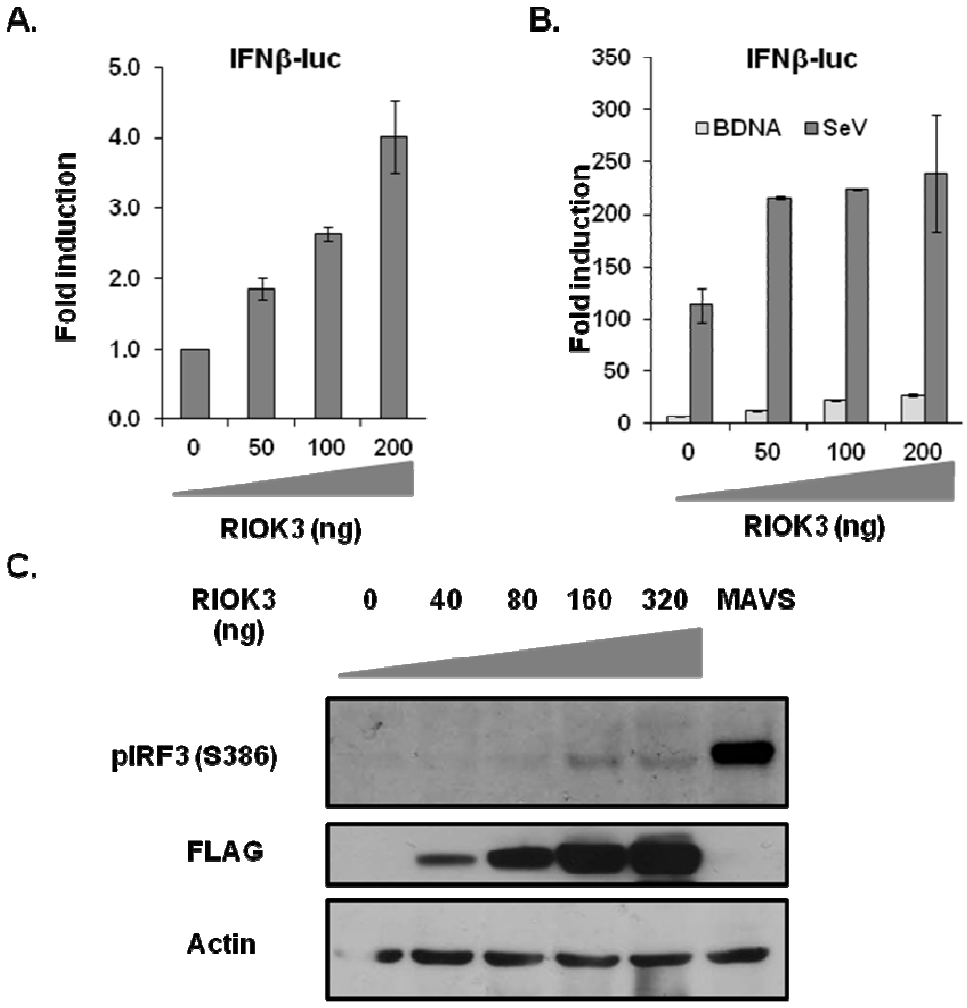

Figure 4-6. Overexpression of RIOK3 activates IRF3. (A) RIOK3 activates IFNβ promoter in a dose-dependent manner in HEK293T cells. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as internal control, and together with the indicated amount of RIOK3. Luciferase assay was performed 48 hr post-transfection. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to control vector. **(B)** Overexpression of RIOK3 boosts the BDNA and SeV induced IFNβ promoter activity. HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated amount of RIOK3. At 24 hr post-transfection, cells were left untreated or treated with BDNA or Sendai virus for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control vector. **(C)** Overexpression of RIOK3 results in IRF3 phosphorylation. HEK293T cells were transfected with the indicated amount of RIOK3. 24 hr later, cell lysates were collected and analyzed by immunoblot with the indicated antibodies.

As shown in Figure 4-5B and 4-5D, knockdown of RIOK3 inhibited BDNA-induced IRF3 phosphorylation and nuclear translocation, which are hallmarks of IRF3 activation. Similarly, knockdown of RIOK3 also reduced Sendai virus induced IRF3 phosphorylation and nuclear translocation (Figure 4-5C and 4-5D). To further analyze RIOK3 function, we constructed RIOK3 expression plasmid. Ectopic expression of FLAG-tagged RIOK3 in HEK293T cells activated IFNβ promoter activity in a dose-dependent manner (Figure 4-6A). Consistently, overexpression of RIOK3 induced IRF3 phosphorylation in a dose-dependent manner (Figure 4-6C). Furthermore, RIOK3 overexpression potentiated IFNβ promoter activity upon BDNA or Sendai virus stimulation (Figure 4-6B). These results suggested that RIOK3 functions upstream of IRF3 activation.

Previous studies have demonstrated that various proteins are directly or indirectly involved in IRF3 activation, including RIG-I, MAVS, TRIF, STING, TBK1, and IKKε etc. Thus we sought to determine the epistatic relationship between RIOK3 and these signaling molecules upstream of IRF3. We observed that overexpression of RIG-I, MAVS or TBK1 could induce IFNβ promoter activity, and this induction was markedly impaired upon depletion of RIOK3 (Figure 4-7A, 4-7B and 4-7C). Moreover, RNAi of RIOK3 did not affect TBK1 phosphorylation induction by Sendai virus (Figure 4-5C). Collectively, the results shown in Figure 4-6, 4-7 and 4-8 delineated a pathway in which RIOK3 functions downstream of TBK1 and upstream of IRF3.

4.4.5 RIOK3 interacts with phosphorylated TBK1and IRF3.

As mentioned previously, RIOK3 functions downstream of TBK1 and upstream of IRF3 in both cytoplasmic dsDNA- and dsRNA-triggered intracellular signaling pathways. To further examine the mechanisms of RIOK3's function in these pathways, we attempted to determine if RIOK3

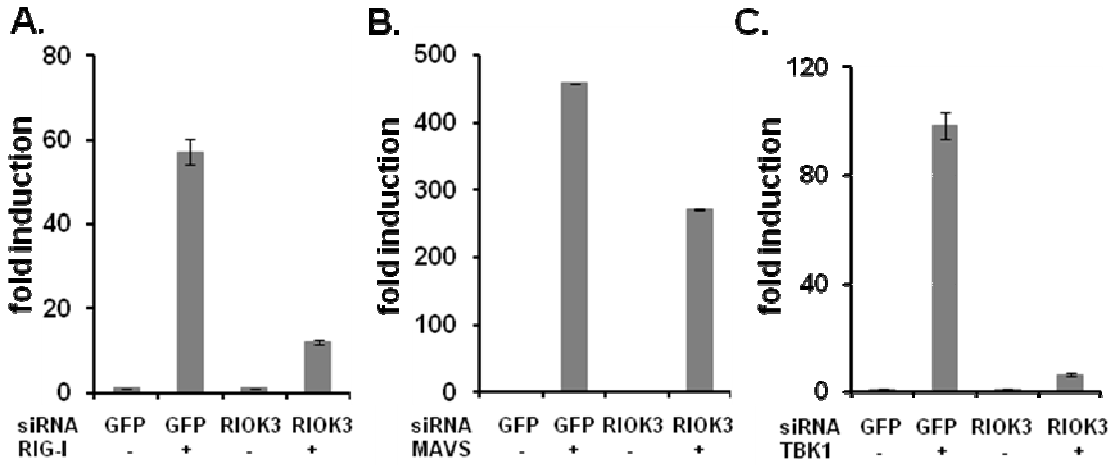

Figure 4-7. RIOK3 functions downstream of TBK1. (A) (B) (C) HEK293T cells were cotransfected with IFNβ_firefly-luciferase reporter, PGK_renilla-luciferase reporter as an internal control, and together with the indicated siRNAs. At 24 hr post-transfection, cells were left untreated or transfected with BDNA for 20 hr before luciferase assays were performed. Firefly luciferase values were normalized to renilla luciferase. Fold of activation was calculated in comparison to untreated control siRNA.

could interact with TBK1 and IRF3. TBK1 or IRF3 was cotransfected with RIOK3 into HEK293T cells. Coimmunoprecipitation results showed that RIOK3 could interact strongly with IRF3. In addition, RIOK3 could constitutively interact with endogenous IRF3 (Figure 4-8A and 4-8B). To map the interaction domain on IRF3 and RIOK3, we constructed several IRF3 and RIOK3 truncation mutants (Figure 4-8D). It was observed that the IRF association domain (IAD) of IRF3 is necessary and sufficient for its interaction with RIOK3 (Figure 4-8C). On the other hand, the RIO domain and C-terminus of RIOK3 is critical for its interaction with IRF3 (Figure 4-8D).

In contrast, RIOK3 could coimmunoprecipitate only marginally with TBK1. It should be noted, though, that the TBK1 detected in the pull down sample migrated slightly slower than that in the input, suggesting the phosphorylation of the protein (Figure 4-9A). We then probed the blot with phospho-TBK1 specific antibody, and indeed the slower-migrating band corresponded to phospho-TBK1 (Figure 4-9B). These data indicated that TBK1 interacts with RIOK3 in a phosphorylation-dependent manner. Since it has been shown that TBK1 is responsible for IRF3 phosphorylation and activation, it is possible that RIOK3 serves as an essential adaptor bridging TBK1 and IRF3.

4.5 Discussion

Both cytosolic foreign dsDNA and dsRNA can be recognized by different PRRs in the cytosol and induce the production of type I IFNs. Recent studies have identified and characterized two important adaptor molecules MAVS and STING downstream of PRRs [20-23, 27-30, 39]. The major converging point of the two cytosolic nucleic acids recognition pathways is TBK1/IKKε and IRF3, which could ultimately induce the robust expression of type I IFNs. Type I IFNs are

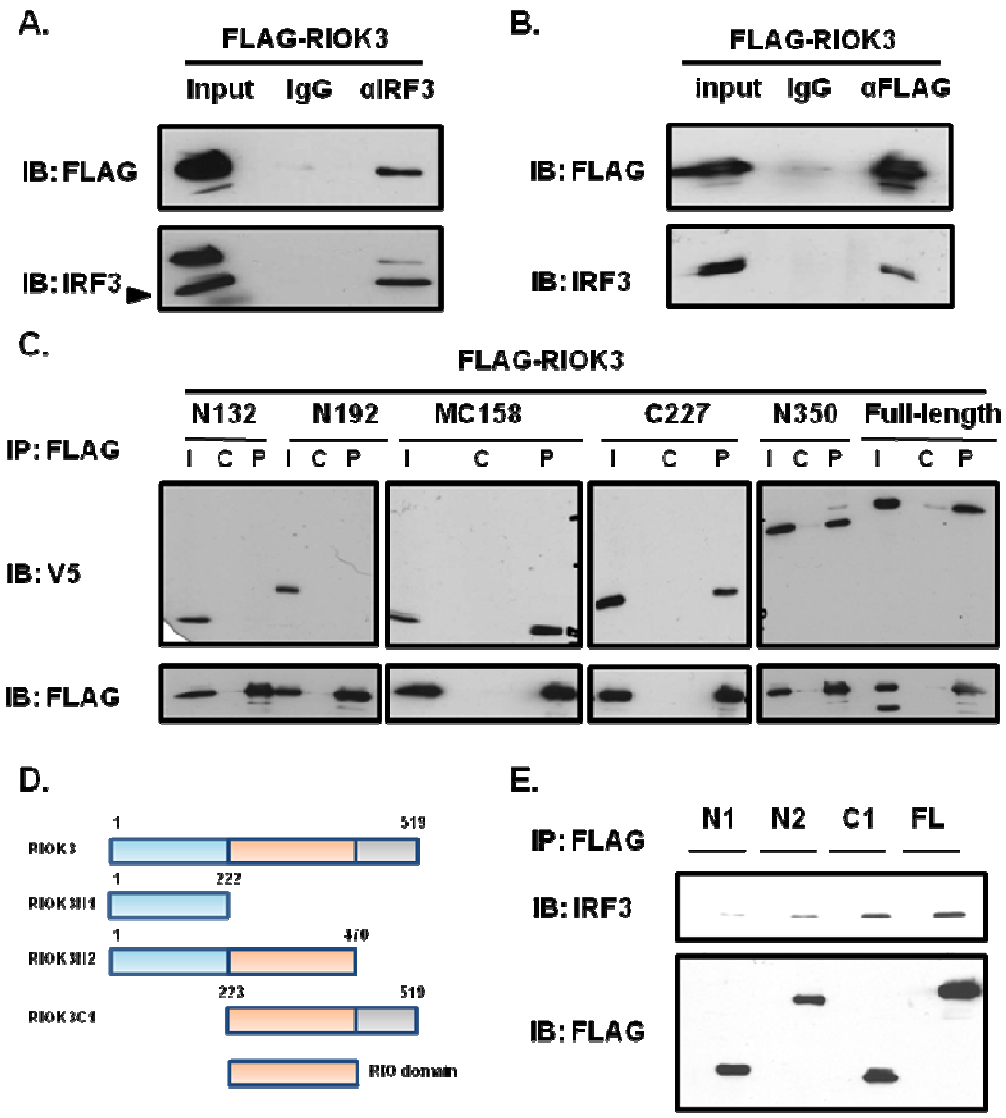

Figure 4-8. RIOK3 interacts with IRF3. (A) (B) RIOK3 interacts with IRF3. HEK293T cells were transfected with FLAG-RIOK3. Cell lysates were immunoprecipitated with the indicated antibodies. **(C) (E)** Domain mapping of the RIOK3-IRF3 interaction. HEK293T cells were transfected with the indicated plasmids and immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. **(D)** Schematic illustration of RIOK3 deletion mutants.

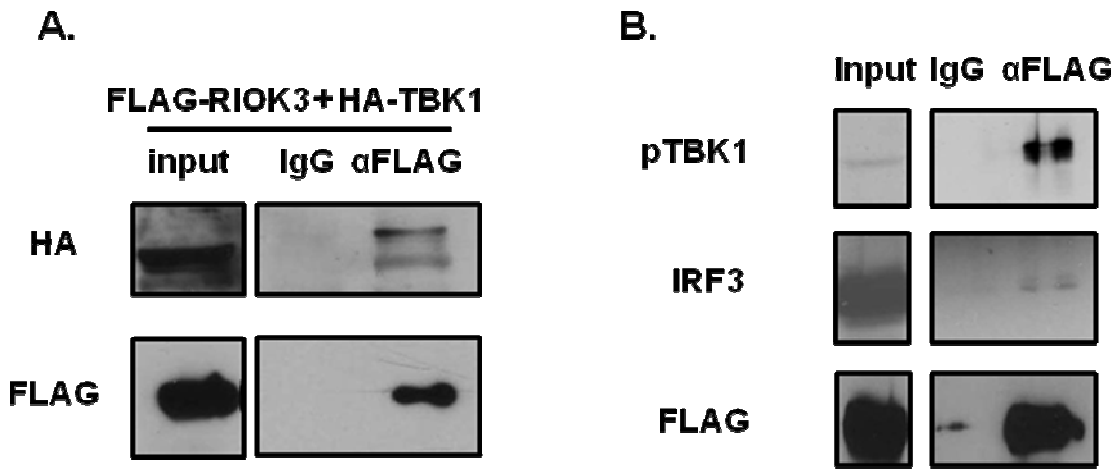

Figure 4-9. RIOK3 interacts with phosphorylated TBK1, and RIOK3 is an adaptor protein that bridges TBK1 and IRF3 upon stimulation. (A) RIOK3 interacts with phosphorylated TBK1. **(B)** RIOK3 interaction with TBK1 in a stimulation-dependent manner.

then secreted, after which they bind to IFN receptors in an autocrine or paracrine fashion, and activates Jak/STAT pathway to initiate the production of ISGs which are the potent effectors of antiviral innate immune responses. In this study, we report the identification of an adaptor protein, RIOK3, that is critically involved in both cytosolic DNA and RNA mediated type I IFN pathway. More specifically, RIOK3 is required for type IFN production rather than downstream signaling. Knockdown of endogenous RIOK3 abolished BDNA and Sendai virus induced IRF3 activation, and therefore IFNβ production. Conversely, ectopic expression of RIOK3 resulted in enhanced activation of IRF3. Consistently, overexpression of RIOK3 activated basal IFNβ promoter activity and also boosted BDNA or Sendai virus induced IFNβ promoter activity. In addition, knockdown of RIOK3 enhanced both DNA and RNA viruses' replication. These data demonstrated that RIOK3 is a critical component in the cytosolic nucleic acids induced type I IFN pathway and in the innate antiviral responses.

Several lines of evidence suggest that RIOK3 acts downstream of TBK1. First, RIOK3 is required for both cytosolic dsDNA- and dsRNA- mediated type I induction. Second, overexpression of RIOK3 activated only IRF3, whereas TBK1 activated both NF-κB and IRF3. Third, knockdown of RIOK3 inhibited RIG-I-, MAVS- and TBK1- mediated IFNβ activation. Lastly, RIOK3 interacted with IRF3 constitutively, but associated with TBK1 in a TBK1 phosphorylation-dependent manner.

IRF3 is a critical transcription factor that is essential for type I IFN response. Upon stimulation, cytoplasmic IRF3 is activated by phosphorylation of amino acids at the C-terminus, resulting in homodimerization, nuclear translocation, and interaction with co-activator CBP/p300. To date, TBK1 and Ikkε have been characterized and shown to be responsible for phosphorylation of IRF3 at the C-terminal regulatory domain (between residues 385 and 405), which contains 7

potential phosphorylation sites at serine and threonine residues [33, 34, 40-42]. However, the exact phosphorylation sites and the contribution of each phosphorylated serine and threonine residue to IRF3 activation remains controversial [33, 34, 40-42]. Two distinct groups of serine/threonine residues have been implicated in the activation of IRF3. Group 1 includes serines 385 and 386, whereas group 2 includes serines 396, 398, 402, and 405 and threonine 404. Previously Sharma and McWhirter *et al*.showed that the group 2 serine/threonine residues are the primary targets of phosphorylation by TBK1/IKKε [31]. On the other hand, Mori *et al.* proposed that serine 386 is the critical target for inducible phosphorylation which determines activation [42]. Here, we showed that RIOK3 could interact with IRF3 and that ectopic expression of RIOK3 could induce IRF3 phosphorylation at serine 386. Though yet to be proven, these observations raise an attractive model: RIOK3 is another kinase which directly phosphorylates IRF3. Although the IRF3 activation by RIOK3 overexpression is not as strong as that by TBK1/IKKε, it is possible that RIOK3 phosphorylates IRF3 at a specific site preparing IRF3 into an more accessible conformation for the recruitment of TBK1/IKKε, which leads to the sequential phosphorylation on other serine/threonine residues (Figure 4-10A). Like IRF3, IRF7 needs to be specifically phosphorylated to become active, presumably by the same kinases [43, 44]. Although IRF7 is not required for the first wave of induction of type I IFNs but rather the amplification of type I IFNs after viral infection in most cell types, it will still be interesting to see if RIOK3 also plays a role in IRF7 activation [36, 45, 46].

The mechanism of RIOK3 activation is currently unknown. RIOK3 belongs to the RIO family of atypical kinases, which is conserved from Archaea to human [47, 48]. There are 3 RIO kinases: RIO1, RIO2 and RIO3. All organisms contain at least two RIO proteins, which are homologous to yeast RIO1 and RIO2. Previous studies using yeast have shown that RIO1 and

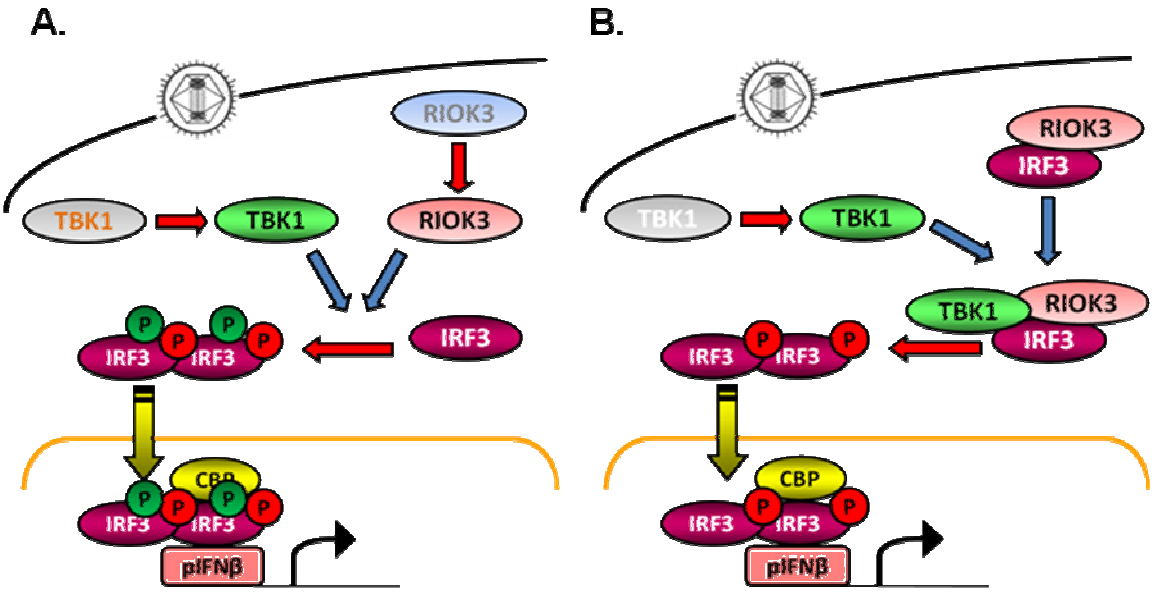

Figure 4-10. Proposed models. **(A)** RIOK3 directly phosphorylates IRF3 upon BDNA or SeV stimulation, and this phosphorylation is required for the full-activation of IRF3. **(B)** RIOK3 functions as an adaptor protein to bridge TBK1 and IRF3 during BDNA or SeV stimulation.

RIO2 are important for ribosome biogenesis and cell cycle progression [49, 50]. However, RIO3 was only discovered in the multicellular eukaryotes and the biological function of human RIOK3 remains largely unknown [47, 51]. To determine whether the tissue distribution of RIOK3 is relevant to its function in innate immune response, we extracted mRNA expression profiles from the GNF Tissue Atlas [52]. The expression of RIOK3 could be detected in various human tissues, and it was predominantly expressed in hematopoietic cell types which are expected to play major roles in immune surveillance (data not shown). The function of RIOK3 in type I IFN pathway was further tested in a more physiologically relevant cell type, THP1 cells (Figure 4-11). Knockdown of RIOK3 in the monocytic cell line THP1 also blocked the IFNβ expression induced by BDNA, further supporting the role of RIOK3 in the type I IFN pathway. RIOK3 has previously been shown to possess a functional kinase domain and be able to autophosphorylate itself [53]. Thus, it is possible that RIOK3 is activated by autophosphorylation upon stimulation and that the active form of RIOK3 can further activate downstream IRF3.

Coimmunoprecipitation experiments indicated that RIOK3 constitutively interacted with IRF3, whereas TBK1 was recruited to RIOK3 upon activation. The interaction between RIOK3 and TBK1 is reminiscent of the interaction between IRF3 and TBK1. In the mammalian overexpression system, IRF3 interacts with TBK1 weakly, most likely only interacting with the active form of TBK1 for its activation. Hence, it is entirely possible that at a certain point after stimulation all three proteins are present in the same complex, which is required for the full activation of IRF3. Even if IRF3 is not the direct substrate of RIOK3, our data still suggested that RIOK3 could act as an adaptor protein for the recruitment of TBK1 to IRF3 (Figure 4-10B). It is of interest to examine whether the interaction between TBK1 and IRF3 can be enhanced or abolished by RIOK3 overexpression or knockdown, respectively. Unlike TBK1, the other IRF3

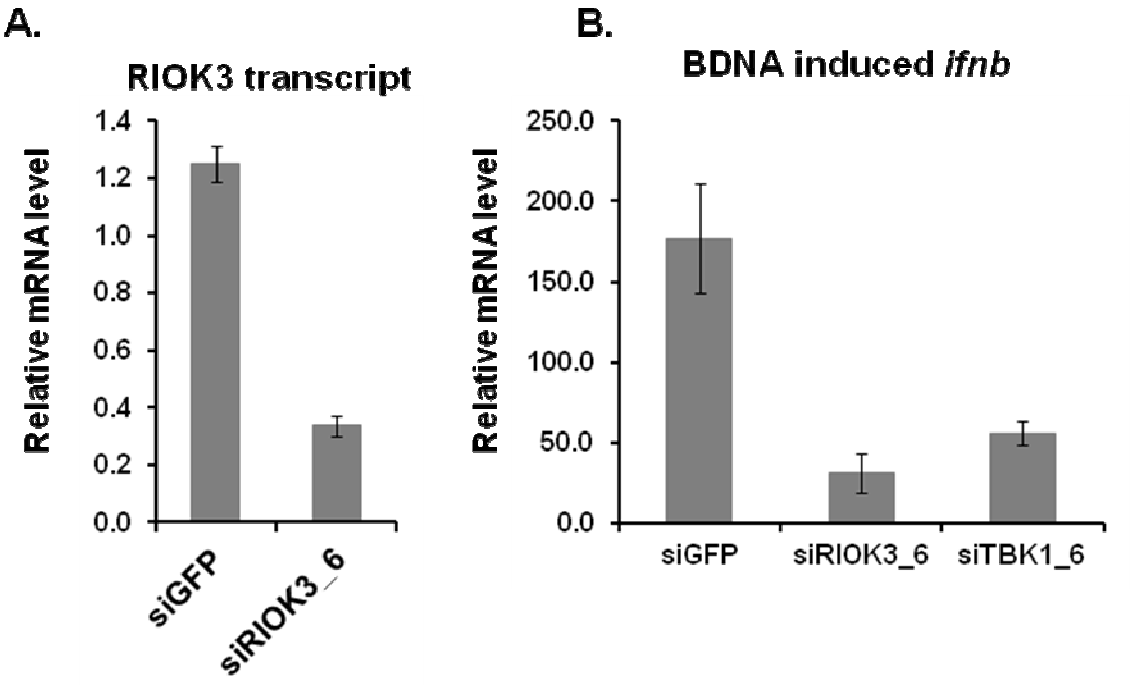

Figure 4-11. RIOK3 is required for BDNA-mediated IFN-β induction in THP-1 cells. (A) Effect of RIOK3 RNAi on the expression of endogenous RIOK3. THP-1 cells were transfected with the indicated siRNAs. At 48 hr post transfection, RNA was extracted and RT-PCR was performed to measure the transcripts of *riok3*. **(B)** Effect of RIOK3 RNAi on BDNA induced *ifnb1* transcription. THP-1cells were transfected with the indicated siRNAs, and were left untreated or treated with BDNA 40 hr later. RNA was extracted 8 hr post-treatment, and RT-PCR was performed to examine the relative mRNA level of *ifnb1*.

kinase IKKε is only expressed in certain immune cells [54]. It has been demonstrated that Ikkε is also required for the amplification of type I IFNs like IRF7 [54]. Further studies on the interaction between RIOK3 and other components of type I IFN pathway, such as IRF7 and Ikkε, will provide us with more insight into the functions and mechanisms of RIOK3.

It has been shown that many viruses encode specific proteins that function to antagonize IFN induction and signaling. For example, NS3/4A protease of hepatitis C virus interferes with IRF3 phosphorylation and thus blocks viral RNA-induced IFN production [55]. ORF36 of KSHV and MHV-68 can bind to the active form of IRF3 in the nucleus, prevent its association with other transcription coactivator, and thereby block viral induced IFN production [56]. It is therefore possible that many viral proteins might antagonize the RIOK3-mediated signaling pathway by targeting it for degradation or preventing its activation and interaction with TBK1 or IRF3.

Although experiments using RIOK3-deficient mice are needed to clarify the *in vivo* significance of this protein in antiviral immune responses, our study has demonstrated that RIOK3 can act as an adaptor molecule that is required for the induction of antiviral innate immune responses downstream of TBK1. Furthermore, the discovery of drugs that modulate RIOK3 functions may be therapeutically useful for limiting viral infection.

4.6 Bibliography

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CHAPTER 5

SUMMARY AND PERSPECTIVES

Persistent infections of gammaherpesviruses, such as EBV and KSHV, are directly associated with cellular transformation and malignancies, especially in the immunocompromised patients. The two-phases life cycle of gammaherpesviruses is essential for the persistent infections and viral-associated pathogenesis. Also, the cellular environment plays a significant role in the outcome of virus infection. To better understand the contribution of cellular factors to the regulation of gammaherpesvirus replication and pathogenesis, we undertook the study of the virus-host interactions that regulate gammaherpesvirus lytic replication in a systematic way. The study of the medically and clinically important human gammaherpesviruses, especially the regulation of lytic replication and reactivation, is difficult due to their inability to replicate in cell culture and the lack of a small-animal model. In this study, MHV-68, a genetically and biologically similar virus, was utilized as a model to study the virus-host interactions of human gammaherpesviruses, because it offers such advantages as the ability to replicate in cell culture, a manipulatable genome, and infection of mice.

Chapter 2 describes the results of a genetic screen conducted to identify kinases and transcription factors that regulate MHV-68 lytic replication. Multiple kinases as positive regulators of MHV-68 replication were found from the screen. We then focused on two of these kinases, TPL2 and PTK7, to studying the mechanisms by which they enhanced virus replication. We found that TPL2 and PTK7 regulate MHV-68 replication through two distinct pathways. TPL2 enhances MHV-68 lytic replication by transcriptionally upregulating viral lytic gene expression including RTA and ORF57. TPL2 stimulates the promoter activities of key viral lytic genes including RTA and ORF57 in an AP-1-dependent manner. We further identified an AP-1 responsive element on the MHV-68 RTA promoter as the cis element mediating the upregulation of RTA promoter activity by TPL2. PTK7, on the other hand, enhances virus production without

affecting viral transcript or intracellular protein levels of immediate-early, early, and late genes. Moreover, PTK7 exhibits synergistic effect on MHV-68 replication with other positive regulators, indicating a different mechanism of regulation. We found that PTK7 overexpression increases extracellular virus production but not cell-associated virus. In contrast, knocking down endogenous PTK7 using shRNA increases cell-associated virus level. Finally, we also showed that ectopic expression of PTK7 promotes herpes simplex virus-1 (HSV-1) egress in 293T cells, suggesting PTK7 regulates a conserved viral egress mechanism shared by herpesviruses. Although we have established a role for PTK7 in regulating herpesvirus egress, the exact mechanism of regulation is unclear. In future studies, as a first step more definitive experiments such as IFA and EM studies can be carried out to investigate the localization of PTK7 and virus particles during the course of viral infection. In addition, Y2H or IP/MS experiments can be performed to identify the PTK7 interacting cellular proteins and viral proteins, which will provide more molecular basis for the mechanistic study.

Chapter 3 presents an integrative systems approach to elucidate the interactions between MHV-68 and its host. We have previously performed Y2H to analyze MHV-68 viral-viral and viral-cellular protein-protein interactions and genome-wide expression profiling to measure cellular transcriptional responses to MHV-68 infection, which confirmed and extended the current understanding of cellular pathways involved in MHV-68 lytic replication. These two studies implicated hundreds of cellular factors in mediating virus-host interaction. Among the cellular factors identified, those involved in kinase- and transcription factor- regulated signaling are the most highly enriched. Therefore, we conducted high-throughput screens of cellular kinome and transcription factors cDNA and siRNA libraries, which uncovered 144 MHV-68 dependency factors and 213 restriction factors. We found that many of identified cellular genes affect MHV-

68 replication by regulating RTA transcription. Characterization of this subset of genes showed they also play roles in KSHV latency maintenance and reactivation. Furthermore, cellular factors regulating later steps of viral replication, such as viral late gene expression and egress, were also identified and validated. Finally we compared the viral restriction factors to the results of a kinome siRNA screen that was performed to identify cellular regulators of innate immune response to cytosolic DNA, and found 23 overlapping hits between the two independent screen. Further analysis of these overlapping genes identified novel regulators of type I IFN pathway. Thus, this study presents a comprehensive map of physical and regulatory interactions between MHV-68 and its host cells by integrating experimental results with systematic data mining. The cellular factors identified by these efforts represent a starting point for further defining a more detailed virus-host interaction network on the molecular basis.

Chapter 4 reports the identification of RIOK3 as a novel adaptor protein in type I IFN pathway. RIOK3 was one of the overlapping hits from RNAi screens for MHV-68 restriction factors and regulators of innate response to cytosolic DNA. Further characterization showed that knockdown of RIOK3 blocks both cytosolic DNA and RNA induced IRF3 activation, IFNβ production, and ectopic expression of RIOK3 activates IRF3. Knockdown of RIOK3 significantly increases both DNA virus (MHV-68) and RNA virus (Influenza A) replication, suggesting a central role in the cellular antiviral response. Moreover, epistasis experiments suggested that RIOK3 functions upstream of IRF3 phosphorylation and downstream of TBK1. RIOK3 interacts with IRF3 and also interacts with active form of TBK1. Therefore, our data suggested that RIOK3 plays a critical role in the type I IFN pathway by bridging TBK1 and IRF3. To extend this part of the study, we would like to investigate how RIOK3 gets activated and whether RIOK3 also interacts with other components in the type I IFN pathways, such as MAVS,

STING and IRF7, which will further illuminate the relationship between RIOK3 and innate immunity. We are also interested to examine the *in vivo* significance of RIOK3 in antiviral immune responses by generating RIOK3 knockout mice. Moreover, our validation study also suggested the roles of several other MHV-68 restriction factors in type I IFN pathway, and further characterization of these cellular genes will not only contribute to our knowledge of virus-host interactions but also the cellular function.

In summary, we have generated a comprehensive map of gammaherpesvirus-host interactions using multilayered systems biology approaches, and defined the roles of multiple cellular genes in MHV-68 replication as well as in the intracellular signaling pathway. Based on the comprehensive knowledge of virus-host interactions, new therapeutic strategies can be developed to combat the gammaherpesvirus associated diseases.