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Interactions between the Unfolded Protein Response and  
Murine Gammaherpesvirus-68 Infection

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

by

Jiaying Feng

2013



# ABSTRACT OF THE DISSERTATION

Interactions between the Unfolded Protein Response and  
Murine Gammaherpesvirus-68 infection

By

Jiaying Feng

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2013

Professor Ren Sun, Chair

The gammaherpesviruses are unique in their oncogenic potential and their ability to establish lifelong infection. Their success results from a variety of delicate interactions with the natural hosts. In this thesis, we study the interaction between a host response termed the unfolded protein response (UPR) and infection of murine gammaherpesvirus 68 (MHV-68). MHV-68 is a model virus widely used to study the human gammaherpesviruses. UPR is triggered by accumulated unfolded proteins in the endoplasmic reticulum (ER) and activates a collection of signaling pathways (IRE1, ATF6, PERK) that can be beneficial or deleterious to viral replication. We found that cellular UPR is important for MHV-68 replication. The infection cycle of MHV-68 leads to a changing UPR in the host cells. Cellular UPR is inhibited at the early stages of infection and becomes upregulated later on.

To identify viral modulators of cellular UPR, an unbiased screen was conducted, uncovering M1 as a strong UPR inducer and ORF40 as an inhibitor. M1 is found to localize to the ER and markedly induces the production of ER chaperones. It selectively induces the chaperon-producing branches (IRE1, ATF6) while sparing the translation-blocking arm (PERK). On the other hand, the viral helicase-primase component ORF40 is capable of downregulating each UPR pathway. We provide evidence that ORF40 may function through binding to and stabilizing the inactive complexes of the transmembrane stress sensors and chaperones to prevent UPR initiation. Despite the seemingly contradictory effects of the two viral proteins, further investigation revealed distinct expression patterns of ORF40 and M1 genes. ORF40 is expressed at the early stages of virus infection while M1 peaks at later stages. Intriguingly, M1 is found to be able to interfere with the anti-UPR function of ORF40. These findings explain the dynamic modulation of cellular UPR by MHV-68 during infection with ORF40 functioning at the early stage and M1 at the late stages of viral life cycle.

Collectively, the delicate regulation of UPR by MHV-68 suggests this cellular response is crucial in controlling gammaherpesvirus infection. Further investigation on the molecular interaction between the virus and UPR may yield important information for developing new anti-viral therapies.

This dissertation of Jiaying Feng is approved.

Heather Christofk

Steven Dubinett

Jing Huang

Yibin Wang

Ren Sun, Committee Chair

University of California, Los Angeles

2013

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## VITA

- June 2007  
B.S., Bioengineering  
School of Material Science and  
Chemical Engineering  
Zhejiang University  
Hangzhou, P.R. China
- 2007-2012  
Graduate Student Researcher  
Laboratory of Dr. Ren Sun  
Department of Molecular and Medical  
Pharmacology  
University of California, Los Angeles  
Los Angeles, California, USA
- 2008-2009  
Teaching Assistant  
Department of Molecular, Cell and  
Developmental Biology  
University of California, Los Angeles  
Los Angeles, California, USA
- 2009  
Advancement to Candidacy for Ph.D.  
University of California, Los Angeles  
Los Angeles, California, USA

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Xudong Li, **Jiaying Feng** and Ren Sun. *Oxidative Stress Induces Reactivation of Kaposi's Sarcoma-Associated Herpesvirus and Death of Primary Effusion Lymphoma Cells*. J Virol. 2011 Jan;85(2):715-24

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CHAPTER 1  
INTRODUCTION

## **Herpesvirus**

Herpesviruses are a family of large DNA viruses with a wide host range in nature. From bivalves to humans, most animal species yield one or several herpesviruses during their lifetime [1]. The herpesvirus virion is composed of an icosahedral capsid, encompassing a linear double-stranded viral DNA. The capsid is surrounded by an amorphous tegument, and is wrapped in a cell membrane-derived envelope with viral glycoprotein spikes on the surface [2]. The size of a herpesvirus particle varies from 120 nm to 260 nm in diameter, with a capsid size around 125nm. The herpesvirus genome ranges from 124 to 230 kilobases (kb) in length, and encodes about 100-200 genes [3].

All herpesviruses share four biological properties: 1> encode a panel of enzymes involved in nucleotide metabolism, DNA synthesis, and protein processing; 2> viral DNA synthesis and viral capsid assembly take place in the nucleus; 3> production of infectious virions leads to the destruction of host cells; 4> ability to establishing long-term latent infection in their natural host [3].

## **Persistent infection of herpesvirus**

The herpesviruses establish a life-long persistent infection by engaging two distinct genetic programs: latency and lytic replication [4]. During latency, the viral genome persists in the cell nucleus as a circular episome, and a limited number of viral genes are expressed [3]. However, when exposed to certain stimuli, latent virus enters the lytic replication, a phenomenon termed reactivation. During lytic replication, the viral genome become linear and viral genes are extensively expressed in a highly regulated manner. In addition, new infectious virions are produced which ultimately leads to the destruction of the infected cells. The lytic phase generates

*de novo* infections and enlarges the pool of latently infected cells, and therefore is believed to play a central role in viral pathogenesis in the host [3].

### **Classification of herpesvirus**

The members of herpesviruses were classified into the alpha, beta, and gamma subfamilies based on their biological properties [3]. The alphaherpesviruses have a wide host range, a relatively short life cycle, and primarily establish latent infection in the sensory ganglia. This subfamily contains the herpes simplex virus 1 (HSV-1) and Varicella zoster virus. The betaherpesviruses have a restricted host range, a long reproductive cycle, and can maintain latent form in secretory glands and lymphoreticular cells. Human cytomegalovirus belongs to this the betaherpesvirus. The gammaherpesvirus also has a limited host range. Most of the viruses in this group specifically infect either T or B lymphocytes and establish latent infection in lymphoid tissue. This subfamily includes the Epstein–Barr virus (EBV) and the Kaposi's sarcoma-associated herpesvirus (KSHV) [3]. The gammaherpesviruses are distinctively associated with lymphomas and other lymphoproliferative diseases [5].

### **Tumor-associated gammaherpesvirus**

The gammaherpesviruses are unique in their ability to establish long-term dormant infection in lymphocytes and in their oncogenic potential [6]. Gammaherpesviruses are subdivided into four genera: Lymphocryptovirus, Rhadinovirus and the newly-defined Macavirus and Percavirus [7, 8]. Of relevance to the human gammaherpesviruses, EBV is a lymphocryptovirus, while KSHV is a rhadinovirus.

EBV (formally, human herpesvirus 4, HHV-4) was first documented in 1963 [9]. It is one of the most common human viruses and occurs worldwide. In the United States, 90-95% of the adults become infected with EBV sometime during their life [10]; about 50% of all 5-year-old children have been infected with EBV [11]. Infection by EBV early in life typically causes no symptoms, however infection in adolescence or early adulthood causes infectious mononucleosis [11, 12]. Although rarely found in the United States, EBV is associated with nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease and other types of malignancies [9, 13-16].

KSHV (formally, human herpesvirus 8, HHV-8) was first discovered in 1994 and is the most recent human herpesvirus identified [17]. The infection rate of KSHV in North America is less than 10% of the general population [18]. However, in some portions of Africa, more than 50% of the population have been infected with the virus. KSHV is associated with Kapos's sarcoma, a cancer commonly found in AIDS patients, and primary effusion lymphoma and some forms of multicentric Castleman's disease [17-22].

### **Necessity of a model for human gammaherpesvirus infection**

Although both EBV and KSHV are medically important, studies of the two human gammaherpesviruses have been limited both *in vitro* and *in vivo* due to the restricted host range of the human gammaherpesviruses [18, 23]. *In vitro*, both EBV and KSHV are capable of infecting human B-lymphocytes and a number of epithelial and endothelial cells [24], but the infection typically results in latency rather than lytic replication [8]. Therefore, studies of the viral lytic gene functions have been mostly relying on single viral protein expression, which markedly limit the analysis in the context of natural infection. Additionally, lack of a robust small animal model has also hindered the research of virus-host interactions in a biologically

significant system. Therefore, it has become crucial to develop new experimental systems to gain a better understanding of gammaherpesvirus pathogenesis, establishment of persistent infection, and disease progression.

### **Murine gammaherpesvirus 68 as a small animal model system**

To help elucidate the pathogenesis of human gammaherpesvirus infection, a mouse model for gammaherpesvirus infection has been developed and widely studied. Murine gammaherpesvirus 68 (MHV-68; also referred to as  $\gamma$ HV68) was initially isolated in 1980 from bank voles (*Myodes glareolus*) and yellow-necked field mice (*Apodemus flavicollis*) captured in Slovakia. The virus is capable of infecting both outbred and inbred mice [24-29].

MHV-68 is genetically and biologically related to primate gammaherpesvirus, including EBV and KSHV [30]. Upon intranasal infection of the mice, MHV-68 lytically infects lung epithelial cells and subsequently establishes latent infection in the B cells of spleen [24, 31]. Infection of MHV-68 leads to splenomegaly and lymphoproliferative diseases similar to the symptoms seen in human gammaherpesvirus infection [32, 33]. In addition, MHV-68 infection in immune-deficient mice results in increased frequency of tumor development in the host mice [34].

The MHV-68 genome is approximately 120 kb and encodes about 80 genes [30]. Like all herpesviruses, the genes expressed during MHV-68 lytic replication cycle are divided into three groups: immediate early, early, and late open reading frames (ORFs) [35]. Immediate early genes encode transcription factors (e.g. RTA), and the early genes encode enzymes required for DNA replication before viral genome duplicates. The late genes are expressed post viral gene replication and encoded proteins required for virion assembly [36]. The viral genome also

contains a number of MHV-68 specific genes that are not required for virus replication, but are believed to play important roles in *in vivo* infection [5].

Unlike KSHV and EBV, which tend to establish latent infections in the cell culture system, MHV-68 is able to establish robust lytic infection in various cell types, including those of human, primate, rodent, and murine origins [25, 37, 38]; In addition, generation of recombinant viruses and mutagenesis studies are made feasible with the complete genome sequence of the virus [30] and with the establishment of the MHV-68 BAC system [39-41]; Finally, the MHV-68 mouse infection provides a tractable and cost effective small animal system. Collectively, these advantages of MHV-68 have made it an excellent model system to study virus-host interaction in human gammaherpesvirus infection, which would facilitate the development of new therapeutics against the gammaherpesvirus associated malignancies.

### **Interactions between virus infection and host responses**

Viruses are intracellular parasites. Viral proliferation depends on the successful recruitment of host apparatuses and cellular components to support productive infection. Virus infection of mammalian cells comprises a series of events including viral entry, RNA expression, protein synthesis and processing, genome replication, virion assembly and egress. Each step of the infection cycle requires coordinated interactions among the viral proteins and more importantly, the utilization of numerous cellular machineries and resources [42, 43]. In the meantime, upon sensing the invasion of viruses, host cells often launch elaborate defense mechanisms to restrict viral replication. Therefore, in order to create a favorable environment for their replication, it is not surprising that most viruses have evolved mechanisms to overcome the obstacles they

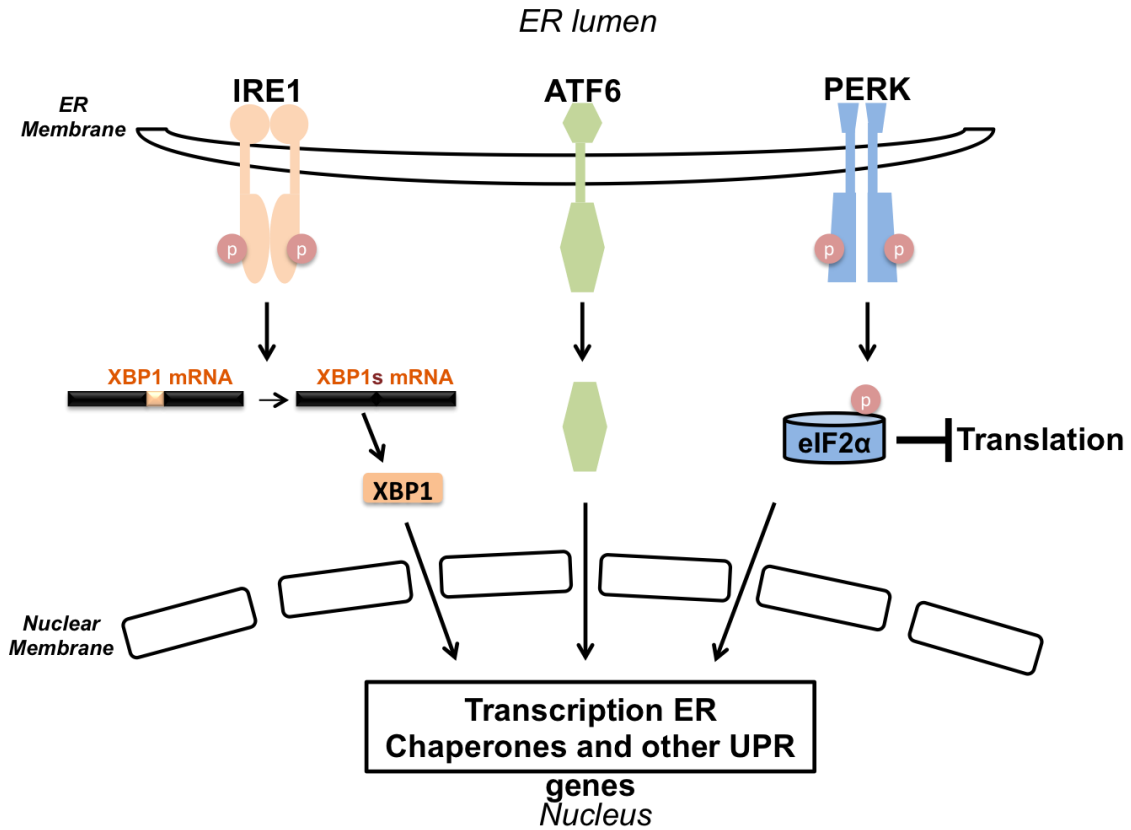
confronted with, and developed strategies to properly manage and adapt to the rapidly changing environment in their hosts.

One of the obstacles involves the protein-folding processes. During a productive infection cycle, viruses produce a large amount of viral proteins in a short period. Such burden pushes the cellular folding capacity to its upper limit and leads to endoplasmic reticulum (ER) stress. As discussed below that the ER stress response presents both threats and opportunities to viral replication. It thus becomes important for the virus to sabotage and/or modulate the stress response in order to create a suitable environment for viral infection.

### **Endoplasmic reticulum stress and unfolded protein response**

The endoplasmic reticulum (ER) plays a central role in protein synthesis, folding, and transportation with the help of a large set of ER-resident chaperones [44]. Multiple disturbances that alter ER homeostasis, such as calcium dysregulation, glucose deprivation, and viral infection can cause accumulation of misfolded/unfolded proteins that exceeds the folding capacity of the ER, and elicits the evolutionarily conserved unfolded protein response (UPR) [43, 45, 46]. Through a collection of ER-to-nucleus signaling pathways that control specific gene expression, the UPR is designed to re-establish homeostasis in the ER lumen. Notably, if UPR prolongs and cells are unrecovered, apoptosis will be triggered.

Up to date, three distinct UPR signaling pathways have been identified. Each signaling arm is specifically mediated by one of three ER membrane-bound stress sensors: inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Figure 1-1). The three UPR transducers are constitutively expressed in all known metazoan cells. However, it remains controversial on how the three



**Figure 1-1.** A scheme illustrating the unfolded protein response signaling pathways. A fully executed UPR comprises at least three signaling pathways that initiate from the three transmembrane proteins IRE1, ATF6 and PERK.



signaling proteins sense the stress in the ER lumen [47]. The prevailing theory is that the inactive transducers are bound by ER resident chaperones in un-stressed conditions, and become activated when the excess unfolded proteins compete away the associated chaperones [43]. More recently, however, one study found that the IRE1 protein itself possesses unique properties in sensing the stress. The state of chaperone association is indispensable, but is not sufficient to determine sensor's activation status [48]. This finding suggests that the stress-sensing mechanism of each transducer could be more sophisticated than previously speculated. Although the initiation of UPR remains obscure, the three UPR signal-transduction pathways have been well documented:

*PERK* - As UPR initiates, the most immediate response is a transient, reversible shutoff of protein synthesis, which reduces the influx of newly synthesized polypeptides into the stressed ER lumen [49]. This translational attenuation is signaled through the PERK transducer. PERK undergoes oligomerization upon stress, induces autophosphorylation and activates its kinase domain. Active PERK phosphorylates and inactivates the eukaryotic translation factor-2 on the alpha subunit (eIF2 $\alpha$ ) at Ser 51 [50]. EIF2 $\alpha$  phosphorylation inhibits the guanine nucleotide exchange factor eIF2B, a pentameric that recycles eIF2 complex to its active GTP-bound form. Less active eIF2 result in less translation initiation [51, 52]. In addition to reducing global protein synthesis, activation of PERK also contributes to the transcription of approximately one third of the UPR genes via upregulation of transcription factor ATF4 [52-55].

*IRE1* - IRE1 was the first stress transducer identified in yeast. The IRE1 gene is conserved in all eukaryotic cells. It encodes a type I ER transmembrane protein kinase that is required for UPR [56, 57]. It was subsequently discovered that IRE1 is a bifunctional enzyme. The protein also possesses a site-specific endoribonuclease (RNase) activity regulated by its

intrinsic kinase module [58]. Under non-stress condition, IRE1 remains inactive in a monomeric form. In response to ER stress, similar to PERK, IRE1 undergoes oligomerization and trans-autophosphorylation in the plane of the ER membrane and activate its RNase activity [59, 60]. The RNase activity of IRE1 induces a precise endonucleolytic cleavage of its only known substrate: an mRNA that encodes a transcription factor termed XBP1 (X-box binding protein-1) in metazoans [61, 62]. IRE1 cuts the precursor XBP1 mRNA twice, removing a 26-nt intron. The 5' and 3' mRNA fragments are then ligated. This splicing reaction generates a translational frame shift and the spliced mRNA encodes a larger form of XBP1. The mature XBP-1 is stable and functions as a potent transcriptional activator of a wide array of UPR target genes (e.g. ERdj4) [61, 62]. Whereas the protein encoded by the precursor XBP1 mRNA is unstable and inhibits UPR target gene expression.

*ATF6* - The bZIP-containing activating transcription factor (ATF6) was identified as a regulatory protein that directly binds UPR-activated promoter elements [63], and a member of a novel class of metazoan-specific ER stress transducer [64]. ATF6 localizes at the ER membrane with a stress-sensing portion projecting into the ER lumen. Under conditions of ER stress, ER-localized ATF6 (full-length, 90kD) is transported from ER to the Golgi complex, where it is sequentially cleaved by two Golgi-resident proteases: first by S1P (site 1 protease) in the luminal domain, and then in the intra-membrane region by S2P (site 2 protease) [64-67]. The cytosolic DNA-binding domain of ATF6 (50kD) is subsequently released and translocates to the nucleus. From there, ATF6 activates UPR gene expression. Its target genes including the precursor XBP1 and ER chaperone genes glucose regulated protein 78 and 94 (GRP78 and GRP94) [66, 68, 69].

Although PERK, IRE1 and ATF6 signaling proceeds independently in ER-stressed cells, the three branches of the UPR extensively communicate with each other. For example, previous

studies in *C. elegans* have suggested a functional redundancy between the ATF6 and IRE1 signaling arms of UPR: mutations that block either arm were well tolerated; however compromising both branches impeded worm development [70]. Additionally, the transcriptional effects of the three axes of the UPR overlap considerably, which is achieved by part through mutual positive reinforcement. For instance, XBP1, the central player of IRE1, is transcriptionally activated by ATF6 [61].

### **The key players of UPR: molecular chaperones**

Molecular chaperones were originally defined as “structurally unrelated classes of proteins that mediate the correct assembly of other proteins, but themselves not part of the final product” [71]. Through controlled binding and release of target substrates, the molecular chaperones possess the ability to transiently assist in the folding, assembly and function of other macromolecules. They play essential roles in maintaining cellular homeostasis through multiple biological processes such as disassembling polypeptide aggregates, transporting proteins across membranes and escorting proteins for degradation by the ubiquitin-proteasome system [44, 71, 72]. Malfunction of molecular chaperones has been associated with a number of human diseases including neurodegenerative diseases, cardiovascular diseases and cancer [73, 74].

At least 20 different families of proteins have been identified with chaperone activity [75]. Most of the intracellular chaperones function as housekeeping proteins and are constitutively expressed in non-stressful situations. The various families of chaperones are highly conserved throughout evolution and are present in the cytosol and ER in almost every cell type. The ER contains a large set of molecular chaperones that play key roles in ER protein quality control. They recognize and interact with a variety of newly synthesized polypeptides or

misfolded proteins to promote their refolding to the native state [74, 76]. The major ER chaperones are the glucose-regulated proteins (GRPs) that include GRP78 and GRP94 [44, 73]. GRP78 functions through specifically interacting with the hydrophobic residues in the misfolded/unfolded proteins. The function of GRP94 has been fully understood. Recent studies have suggested that GRP94 may provide a platform for the assembly of large ER chaperone complex during UPR [44, 73].

The ER chaperones are drastically induced in response to ER stress to provide cytoprotection against the stress conditions [77, 78]. During UPR, a large amount of GRPs and other chaperones are produced downstream of the UPR pathways, and are transported back to the ER lumen to restore the protein folding capacity. It is important to note that the production of various ER chaperones is coordinated by the crosstalk between the three signaling branches. It has suggested that under ER stress, the production of GRP78 and GRP94 is principally induced by the ATF6 pathway but is also partially controlled via the IRE1 pathways; likewise, although the IRE1 branch has a dominant impact on the induction of the ERdj4 gene, the ATF6 pathway is also believed to play a role. [69, 79, 80].

Virus infection often leads to elevated expression of cellular chaperones. In fact, increased expression of a number of chaperones has been suggested to be a biomarker for some viral infection. For instance, a higher level of chaperone GRP94 significantly correlates with the progression of hepatitis B virus infection and thus can be used as a diagnostic and/or prognostic biomarker of HBV-related diseases [81]. On the other hand, accumulating evidences have indicated that actually viruses themselves require a variety of molecular chaperones to support productive infection. For example, the simian virus 40 utilizes chaperones for viron uncoating and entry into the host cells [82]; the hepatitis C virus (HCV) requires the GRP94 chaperone for

protease maturation [83]; both HCV and HBV are reported to involve a number of ER chaperones for viral protein folding [84, 85]; and the human immunodeficiency virus type 1 (HIV-1) relies on chaperone cyclophilin A for virion assembly [86].

### **Interactions between cellular UPR and virus infection**

Host UPR presents both opportunities and threats to virus infection. Some UPR functions can be deleterious to viral infection, while others can be beneficial. For examples, the viruses would not benefit from translational attenuation caused by activation of PERK, induction of apoptosis, and by an increased production of degradative factors. As such, different viruses have evolved a variety of mechanisms to manipulate the UPR in order to create a more favorable environment for productive infection.

*Interaction with ER Chaperones* – Infection of cells with RNA viruses often stimulates the expression of ER chaperone proteins. Examples include paramyxoviruses, such as simian virus 5 and respiratory syncytial virus [87, 88], and the flavivirus family. Works on hepatitis C virus (HCV) have demonstrated that both infection and expression of a viral non-structural protein NS4B can stimulate the UPR signaling [89, 90]; the dengue virus (DENV) and Japanese encephalitis virus (JEV) infection increase UPR molecules GRP78 and ERdj4 [91]. In addition, studies with hemagglutinin of Influenza virus revealed that the virus could induce production of GRP78 and GRP94 [92]. Recent studies have also demonstrated that the replication of the severe acute respiratory syndrome coronavirus (SARS-coV) markedly induces the production of GRP78 and GRP94 proteins, and the key player is the SARS-coV spike protein [93].

*Modulation of PERK signaling* – Several lines of evidence have indicated a relationship between viral replication and the PERK pathway. It has been reported that in cells infected with

HSV-1, viral protein  $\gamma_134.5$  plays an important role in dephosphorylating eIF2 $\alpha$  [94]; more interestingly, it was later discovered that the glycoprotein B of HSV-1 inhibits cellular PERK through direct binding to the stress sensor [95]. Both mechanisms lead to the resumption of protein synthesis in the infected cells. In addition, investigation in HCMV has shown that virus infection causes a wave of upregulation of UPR factors [96]; however it prevents eIF2 phosphorylation via inducing two viral genes TRS1 and IRS1 [97]. Finally, the African swine fever virus, which uses ER as a site for virion assembly, also blocks PERK signaling through unknown mechanism [98].

*Regulation of ATF6 and IRE1 signaling* – ATF6 and IRE1 function in relatively late stages of UPR as compared to PERK. Replication of HCV has been shown to stimulate the ATF6 signaling but inhibit the IRE1-XBP1 branch [89, 99]. A different pattern of the ATF6 and IRE1 signaling is observed in HCMV-infected cells [96]: the ATF6 pathway is suppressed, while the IRE1 signaling pathway is upregulated. It was later discovered that the cytomegalovirus US11 gene is sufficient to trigger such effects [100]. Another example is the infection by arenavirus. Acute infection of arenavirus selectively activates the ATF6 branch of the UPR to boost virus replication and host cell viability, while avoiding induction of both IRE1 and PERK signaling [101].

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CHAPTER 2  
MATERIALS AND METHODS

## **Cell culture**

Human embryonic kidney 293T, 293FT, BHK-21, and Vero cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml of penicillin and 100mg/ml of streptomycin (P/S). 293FT-IRE1 and 293FT-K599A cell lines were maintained in the same medium as above plus Hygromycin (100ug/ml) and Blasticidin (10ug/ml). NIH3T3 cells were maintained in DMEM containing 10% bovine calf serum (BCS) and P/S. All cells were cultured in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C.

## **Chemicals**

Thapsigargin (Tg) was purchased from Sigma-aldrich and was dissolved in DMSO (stock concentration 10mM). Tetracycline (Tet) was purchased from and was dissolved in water (stock concentration: 1mg/ml). Phosphonoacetic acid (PAA) was purchased from Sigma-aldrich and was dissolved in DMSO (stock concentration 200mg/ml).

## **Plasmids**

GRP78-fluc, GRP78mut-fluc and GRP94-fluc reporter constructs were kindly provided by Dr. Kazu Mori, Kyoto University, Kyoto, Japan [1]. The ERdj4-fluc plasmid was a gift from Dr. Laurie Glimcher, Harvard Medical School, Boston, MA [2]. The 5xATF6-fluc and ATF6 expression plasmid was provided by Dr. Ron Prywes, Columbia University, New York, NY [3] and obtained via online purchase (Addgene plasmid 11976). The XBP1 splicing reporter plasmid (XBP1u-fluc) was provided by Dr. Yi-Ling Lin, Academia Sinica, Taipei, Taiwan, Republic of China [4]. The PERK and IRE1 expression plasmids were kindly provided by Dr. Yibin Wang, University of California, Los Angeles, CA.



The wild type ORF40, ORF44, ORF56, M1 and M3 coding sequences (GenBank U97553) were PCR amplified from MHV-68 BAC DNA with an EcoRI site and Kozac sequence immediately upstream of the start codon and a c-terminal hemagglutinin (HA) tag before the stop codon and a BglII site downstream. The PCR fragments were cloned into a pCMV mammalian expression vector (Clontech). All the mutant plasmids were similarly constructed using primer sequences listed in Table 2-1. Prediction of signal peptide locations in M1 and M3 proteins was performed on the SignalP 4.0 Server [5] (<http://www.cbs.dtu.dk/services/SignalP/>).

### **Reporter assays**

293T Cells were grown to 60-75% confluency in 48-well plates and transfection was performed using BioT (Bioland Scientific LLC) according to manufacturer's instructions. Cells were lysed 24 hours post-transfection for dual-luciferase assays (Promega). For each assay, the firefly luciferase activity was normalized to the renilla luciferase reading in the same well, and the ratio was calculated based on the vector control (the value of which was set as 1).

### **MHV-68 and plaque assay**

MHV-68 was originally obtained from the American Type Culture Collection (VE1465). Plaque assay was used to determine the titer the concentrated MHV-68 virus. 10-fold serial dilutions of each virus were prepared to infect a monolayer of Vero cells. After 1-hour incubation, the infected cells were overlaid with 5% methylcellulose-DMEM. After 6 days post infection, cell were fixed and stained with 2% crystal violet in 20% ethanol. Plaques were counted at the optimum dilution to calculate virus titer.

### **MHV-68-M3FL and quantification using firefly luciferase assay**

The recombinant MHV-68-M3FL virus was previously constructed in the lab by inserting to the left side of MHV-68 genome a firefly luciferase gene driven by the promoter of M3 open reading frame [6]. Firefly luciferase assay was performed to quantify infectious MHV-68-M3FL particle. One day prior to infection,  $1.5 \times 10^3$  293T cells were seeded in 70ul of medium into each well of a 96-well plate. 30ul of the original or diluted MHV-68-M3FL samples were added to the wells in triplicates. Luciferase activities were measured using the Bright-Glo Luciferase Assay System (Promega).

### **Construction of recombinant MHV-68**

Recombinant MHV-68 was constructed using a two-step Red-mediated recombination method reported previously [7]. Briefly, the target sequence was divided into two fragments with an overlapping region of 100-200bp. The two fragments were inserted upstream and downstream of a kanamycin-resistance cassette that contains an adjoining I-SceI site in a transfer plasmid with the backbone of pGEM-7zf(+). Using the resulting plasmid as a template, PCR was performed with primers bracketing the two sequence fragments and kanamycin-resistance cassette. The PCR product was subsequently digested with DpnI to eliminate the template plasmid, followed by gel extraction and electroporation into SHG68 competent cells harboring MHV-68 BAC, at 1.8kV, 200 $\Omega$ , 25  $\mu$ F (1 mm cuvette). Positive transformed clones were then made subject to second round Red recombination that removes the kanamycin-resistant cassette. The resulting Kan-sensitive clones were confirmed by sequencing and expanded for BAC DNA purification. The viral BAC DNA was then transfected into 293T cells with an equal amount of plasmids that express the Cre recombinase to remove the BAC sequence. Three days post transfection, single

viral clones were isolated through limiting dilution, validated by PCR, and propagated. The genomic integrity of the recombinant viruses was verified by EcoRI, BglII and KpnI restriction enzyme digestion. The primers used for constructing the recombinant MHV68 are listed in Table 2-2.

### **Immunofluorescence assay**

NIH3T3 cells were grown to 60%-75% confluency in 24-well plates and were either transfected with designated plasmids or infected with the M1-cHA MHV-68 at a multiplicity of infection (MOI) of 10. Transfected cells were fixed 24 hours post-transfection, and infected cells were fixed at indicated time points using 4% formaldehyde. Cells were then permeabilized and blocked in PBS containing 10% FBS, 0.5% BSA and 0.5% Triton-X for 1 hour at room temperature, followed by incubation of primary antibodies overnight. Mouse anti-HA (Sigma) was used at 1:1000 dilution and the Alexa Fluor 488 conjugated anti-Concanavalin A (Invitrogen) at 1:100 dilution. Cells were washed and incubated with Alexa Fluor 594 goat-anti mouse IgG (Invitrogen) (1:1000) for 1 hour. The Hoechst dye was added for 5 minutes prior to analysis under a fluorescence microscope.

### **Electron microscopy**

Electron Microscopy Analysis was performed as previously described [8]. Cells were rinsed with PBS and fixed for 1 hr in 2% glutaraldehyde in PBS (pH 7.4) on ice. Cell pellets were collected and subjected to osmium post-fixation (1% OsO<sub>4</sub> in PBS) for 1 hr (on ice), 2% uranyl acetate en bloc staining for 1 hr (on ice), followed by dehydration in an ascending ethanol series. The sample was infiltrated and embedded in Spurr's resin and then sectioned about 75 nm using an

UCT ultratome (LEICA). Sections were collected on naked grids (100 mesh, copper) and stained with saturated aqueous uranyl acetate and lead citrate from both sides. TEM imaging was performed using an FEI Tecnai TF20.

### **Western blot analysis**

Cells were harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl and 1 mM EDTA, 1% NP-40 and 0.25% sodium deoxycholate) supplemented with protease inhibitors and phosphatase inhibitors where applicable. The protein lysates were then centrifuged, combined with 4 × protein sample buffer (0.25 M Tris pH 6.8, 40% glycerin, 20% β-mercaptoethanol, 8% SDS, 0.008% Bromophenol blue) and boiled for 5 minutes. The denatured proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% non-fat, and incubated with one of the following primary antibodies: monoclonal mouse anti-KDEL (Assay Designs), monoclonal rabbit anti-phospho-eIF2a and mouse anti-total eIF2a (Cell Signaling), monoclonal mouse anti-HA and mouse anti-FLAG (Sigma), mouse anti-myc tag (Millipore), mouse polyclonal anti-ORF 26 and M9 (generated in our lab). The membranes were extensively washed and incubated with appropriate secondary antibodies conjugated to HRP (donkey anti-rabbit or anti-mouse IgG, GE Healthcare) and developed by the Western Lighting system (Perkin-Elmer).

### **Immunoprecipitation**

293T cells were rinsed with PBS and lysed on ice with lysis buffer (as described in Western blot analysis). Cell lysates were then incubated with Sepharose protein G beads that are conjugated with specific antibodies or control IgG overnight at 4 °C. The immunoprecipitates were washed

three times with lysis buffer then boiled in protein sample buffer (0.625M Tris pH6.8, 2% SDS, 10% glycerin, 5% -mercaptoethanol, 0.002% Bromophenol blue), and analyzed by western blot.

### **Real-time PCR**

Total RNA from cultured cells was isolated using RNA Mini Kit (Invitrogen) and reverse transcribed into cDNA using qScript cDNA synthesis Kit (Quantas). 5ul of the diluted cDNA synthesis reaction was combined with 10x PCR buffer, Taq, Fluorescein, SYBR green and dNTPs, and specific primer sets listed in Table 2-3,. The reactions were run at 95 °C for 3 minutes, followed by 40 cycles at 95 °C for 15 seconds, 56-60 °C for 15 seconds, and 72 °C for 15 seconds. The results were analyzed with software Opticon Monitor (MJ research),

### **XBP-1 splicing assay**

XBP-1 splicing assays, the XBP-1 cDNA was PCR-amplified using the indicated primers and were then made subject to PstI digestion. Digestion of the unspliced XBP1 produces two DNA fragments (291bp and 307bp). The final DNA products were resolved on a 2.5% agarose gel, stained with ethidium bromide and visualized by UV.

**Table 2-1: Primer sequences for plasmid construction**

<b>Name</b>	<b>Direction</b>	<b>Primer Sequence (5' to 3')</b>
<b>M1</b>	Forward	GAATTCAAATGCAGCTGGCCACCTTAT
<b>M1_FLAG</b>	Reverse	GAAGATCTTTACTTGTTCATCGTCATCCTTGTAATCGG ACTGCTGCCAGG
<b>M1ΔSP</b>	Forward	GAATTCAAATGTCCTGCATACTGGGCCAAAG
<b>M1_F2</b>	Forward	GAATTCAAATGGACCCTGACTGCCAGT
<b>M1SP+F2</b>	Forward	GAATTCAAATGCAGCTGGCCACCTTATGCCTTCTTTC CTGCATACTGGGCCAAAGCATAGCTCACTGGGACCC TGATCTGCCAGT
<b>M3</b>	Forward	GAATTCAAATGGCCTTCCTATCCACATCTG
<b>M3_FLAG</b>	Reverse	GAAGATCTTTACTTGTTCATCGTCATCCTTGTAATCAT GATCCCCAAAATACTCCAGC
<b>M1SP+M3</b>	Forward	GAATTCAAATGCAGCTGGCCACCTTATGCCTTCTTTC CTGCATACTGGGCCAAAGCATAGCTCACTGGCTAGG TTTGGCACCTGCTCT
<b>M3SP+M1_F1</b>	Forward	GAATTCAAATGGCCTTCCTATCCACATCTGTGCTCAT TAAATGCTGCATCCTCCTGTT
<b>M3SP+M1_F2</b>	Forward	AAATGCTGCATCCTCCTGTTGGCAGGAGGATTGGCT GAGAGCCACTGGCCATCTGTGGTC
<b>ORF40</b>	Forward	GAATTCAAATGTGCAGACTTCAATGG
	Reverse	GAAGATCTTTATGATAAATTTAATAAAGTTTCATAA TTTGACAATAT
<b>40_423</b>	Forward	GAATTCAAATGGTAAAAACCAGCTGTGTCCA
<b>40_846</b>	Forward	GAATTCAAATGACATCATGGAAAGAACACTTACTTA AG
<b>40_1272</b>	Forward	GAATTCAAATGGTGGGACCCGACCT
<b>ORF44</b>	Forward	ACGCGTCGACCATGGCACTCTCTGACAGATT
	Reverse	GAAGATCTTTAATATACAAGTAATGTTTTTGGGTTGT G
<b>ORF56</b>	Forward	GAATTCGGATGGCCAGATACCACAGC
	Reverse	GTCGACCTACTCGAGCATGCACG

**Table 2-2: Primer sequences used for recombinant MHV-68 construction**

<b>Name</b>	<b>Direction</b>	<b>Primer Sequence (5' to 3')</b>
EcoRI_M1cHA_LF	Forward	GGAATTCAAATTATGGCTTCTTTAGAACCCGAC
M1cHA_LF_R	Reverse	TTAAGCGTAGTCTGGGACGTCGTATGGGTATCCTCCTCCTCCGGACTGCTGCCCAGG
M1cHA_LF_F	Forward	GGAGGAGGAGGATACCCATACGACGTCCCAGACTACGCTTAAGCCTGAATACATGTTTACTGGG
BgIII_M1cHA_LF	Reverse	GGAAGATCTTTAATGGACTGAAGCCCGTCA
SalI_M1cHA_RF	Forward	ACGCGTCGACGAACAAAGTTCTTTTACTGCAGAAAGC
BamHI_M1cHA_RF	Reverse	CGCGGATCCGCTTACAACCTACGCGGC
EcoRI_M1s_LF	Forward	GGAATTCAGATGCGTAAGGAGAAAATACCGC
M1S_LF1_NheI	Reverse	GCTTTGGCCCAGCTAGCAGGAAAGAAGGC
M1S_LF1_NheI	Forward	GCCTTCTTTCCTGCTAGCTGGGCCAAAGC
M1S_LF2_SpeI	Reverse	GAGGTGGCAGACTAGTTGGTGGGTAG
M1S_LF2_SpeI	Forward	CTACCCACCAACTAGTCTGCCACCTC
BgIII_M1s_LF	Reverse	GGAAGATCTATCCAGCAAAGGTGAGATCCAG
SalI_M1s_RF	Forward	ACGCGTCGACCCACCTCAGGAAGGGTCA
BamHI_M1s_RF	Reverse	CGCGGATCCTGGTGGCACCCATCTTGATG
EcoRI_40FLAG_LF	Forward	GGAATTCGAACAGTTGAACTTGTCACAAACAGTAG
40FLAG	Reverse	CTTGTCATCGTCATCCTTGTAATCCATTTTACCAGCGGCAGAAGC
40FLAG	Forward	ATGGATTACAAGGATGACGATGACAAGTGCA GACTTCAATGGCACG
BgIII_40FLAG_LF	Reverse	GGAAGATCTGGCAGATATTTTCATTGTGCATA AACGG
SalI_40FLAG_RF	Forward	ACGCGTCGACTGTGCGTTTTTTCAGATTGGAAC AAC
BamHI_40FLAG_RF	Reverse	CGCGGATCCGTATGTCTCGGGAATCATATACA TAGACG

**Table 2-3. Primer sequences for quantitative RT-PCR**

<b>Target Gene</b>	<b>Direction</b>	<b>Primer Sequence (5' to 3')</b>
GRP78 (Human)	Forward	CGGGCAAAGATGTCAGGAAAAG
	Reverse	TTCTGGACGGGCTTCATAGTAGAC
GRP78 (Mouse)	Forward	CATGGTTCTCACTAAAATGAAAGG
	Reverse	GCTGGTACAGTAACAACCTG
GRP94 (Human)	Forward	GAATGCTTCGCCTCAGTTTG
	Reverse	TCATCTTCGTCTTGCTCTGTG
GRP94 (Mouse)	Forward	AATAGAAAGAATGCTTCGCC
	Reverse	TCTTCAGGCTCTTCTTCTGG
ERdj4 (Human)	Forward	AAAATAAGAGCCCGGATGCT
	Reverse	CGCTTCTTGGATCCAGTGTT
ERdj4 (Mouse)	Forward	TTAGAAATGGCTACTCCACAGTCA
	Reverse	TTGTCCTGAACAATCAGTGTATGTAG
XBP1 (Human)	Forward	CTGGAACAGCAAGTGGTAGA
	Reverse	CTGGATCCTTCTGGGTAGAC
XBP1 (Mouse)	Forward	GAGTCCGCAGCAGGTG
	Reverse	GTGTCAGAGTCCATGGGA
GAPDH (Human)	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG
Actin (Mouse)	Forward	CACCCACACTGTGCCCATCTAC
	Reverse	GTGAGGATCTTCATGAGGTAGTC
ORF50	Forward	GATTCCCCTTCAGCCGATAAG
	Reverse	CAGAC TTGTAGAAGTTCAGGTC
ORF57	Forward	GACCAAATGATGGAAGGAC
	Reverse	GCAGAGGAGAGTTGTGGAC
ORF29	Forward	CCACCAAAAACATGTGTCCT
	Reverse	TTGCTGGAAGTGCTTCCTTCT
M1	Forward	GCCCTTGTCACITTTCTTTGG
	Reverse	TCCTGTGTTCTTGCCCATC



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CHAPTER 3  
CELLULAR UPR IS IMPORTANT FOR  
MHV-68 REPLICATION

## ABSTRACT

Endoplasmic reticulum (ER) stress triggers the homeostatic unfolded protein responses (UPR) in mammalian cells to ensure efficient folding, sorting and processing of client proteins. In this study, we explored the effects of activated UPR pathways on MHV-68 replication. We found that in MHV-68 infected cells, chemical-induced UPR significantly upregulates infectious virion production. To determine the role of individual UPR signaling pathways in modulating virus replication, we measure the virion production in the presence active IRE1 $\alpha$  or PERK signaling in the host cell. Enhanced IRE1 $\alpha$  and ATF6 signaling result in a markedly increased production of infectious MHV-68; whereas, activated PERK signaling has no obvious effect on virus multiplication. We also examined how MHV-68 replication influences host UPR. Interestingly, infection of MHV-68 at the early stage results in an inhibited UPR; however, as the virus matures at the late stage of infection, UPR becomes significantly upregulated. Our findings suggest the chaperone producing pathway(s) of cellular UPR plays an important role at the late stage of MHV-68 replication.

## INTRODUCTION

Accumulating evidence has suggested the importance of cellular unfolded proteins response (UPR) in virus replication [1-6]. ER stress/UPR caused by virus infection has been observed to modulate the three signaling branches that lead to cell survival or cell death [7-11]. The fact that viruses have developed strategies to modulate the UPR pathways suggests that these cellular signaling cascades play crucial roles in controlling viral replication, and understanding the underlying mechanism of this viral-host connection may provide insights in the development of novel therapeutics.

In current study, we investigated how UPR influences the infection cycle of MHV-68 in the host cells. We examined the effects of chemical-stimulated UPR on MHV-68 production. We further assessed the role of individual UPR pathways on virus production by specifically activating the IRE1 $\alpha$ , ATF6, and PERK signaling arms. Finally, we show the impact of infection on host UPR during the MHV-68 infection cycle.

## RESULTS

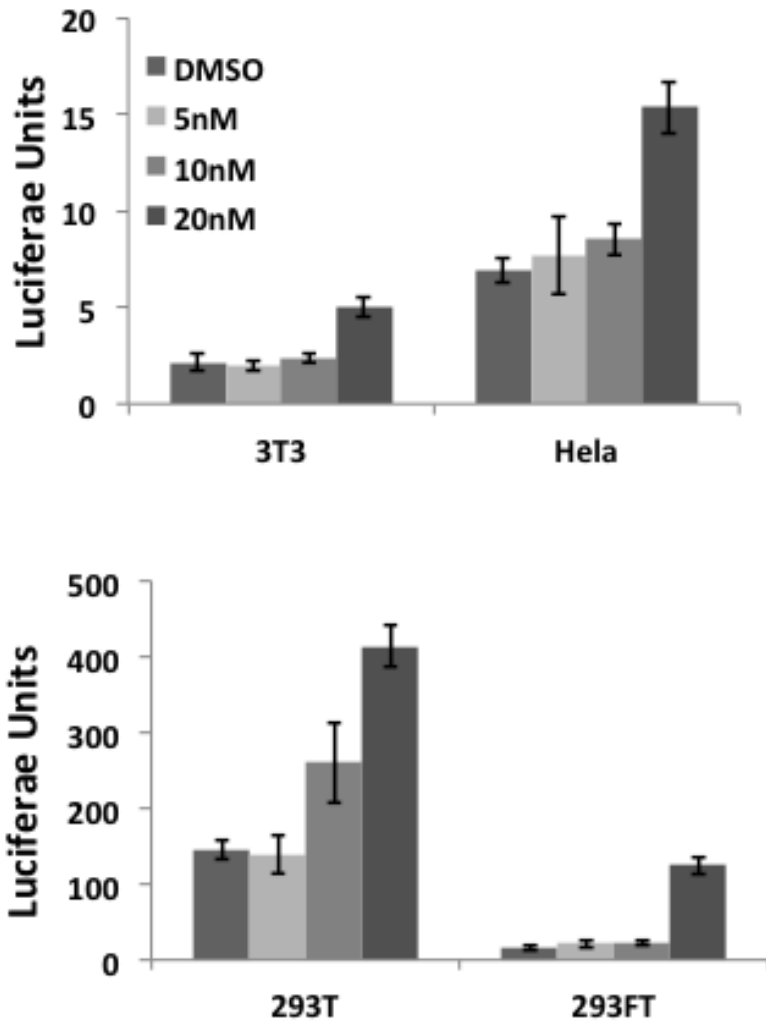
### **Chemical-induced UPR enhances the production of infectious MHV-68**

In order to determine the role of cellular UPR in controlling MHV-68 infection, we assessed the effect of a pharmacological ER stress inducer on virion production. Thapsigargin (Tg) is an irreversible inhibitor of the ER Ca<sup>2+</sup>-ATPase. It depletes calcium storage in the ER lumen and induces ER stress [12]. NIH3T3 cells are pretreated with increasing doses of Tg for 1 hour. Tg was then washed off with growth medium, and cells were infected with a recombinant MHV-68 reporter virus MHV-68-M3FL, which encodes firefly luciferase under the viral lytic gene M3 promoter [13]. Viruses released from the NIH3T3 cells were transferred onto naïve 293T cells.

The relative titers of the virus from each sample were determined by luciferase analysis. The luciferase activity has a wide-range, linear relationship with virus titers measured by plaque assay and therefore has been used to determine the relative amounts of infectious virion production [14]. We measured an increased luciferase reading at the highest dose of Tg treatment (Figure 3-1). Similar analyses were performed with HeLa, 293T and 293FT cells, and the observations were similar to that of NIH3T3 with 293T displaying a stronger response. These findings suggest that chemical induced ER stress can enhance infectious virion production of MHV-68.

### **Enhanced IRE1 $\alpha$ signaling of UPR promotes the production of infectious MHV-68**

Next, we investigated whether by enhancing any of the UPR signaling branches can positively influence MHV-68 virion production. We started from the IRE1 $\alpha$  signaling arm. We utilized a tetracycline inducible 293FT cell line 293FT-IRE1 $\alpha$  with the parental 293FT as control. We also included the 293FT-IRE1 $\alpha$ K599A cell line as a negative control, which expresses a kinase-dead IRE1 $\alpha$  protein when induced with tetracycline. 293FT, 293FT-IRE1 $\alpha$ , and IRE1 $\alpha$ K599A were pre-treated with increasing doses of Tetracycline. Western blot analysis showed a dose-dependent expression of the IRE1 $\alpha$  protein treated with tetracycline (Figure 3-2A). Importantly, we observed a significant increase in XBP-1 mRNA splicing in tetracycline-treated 293FT-IRE1 $\alpha$  cells (data not shown), but not in the other two cell lines, indicating that IRE1 $\alpha$  overexpression specifically leads to enhanced signaling of the pathway. The induced cell lines were then infected with MHV-68-M3FL at MOI 3 for 24 hours (one-step infection) and infectious virion production was similarly analyzed as in Figure 3-1. Significantly higher luciferase activities were observed with 293FT-IRE1 $\alpha$  cells treated with increasing



**Figure 3-1. Thapsigargin enhances production of Infectious MHV-68.** NIH3T3, HeLa, 293T and 293FT cells were infected with MHV-68-M3FL at MOI 3. 24 hours post infection, viruses released from the cells were transferred to naïve 293T cells. The relative titers of the transferred virus were determined by luciferase analysis 18 hours post transfer.

concentrations of tetracycline (Figure 3-2B); In contrast, 293FT and 293FT-IRE1 $\alpha$ K599A cells treated with tetracycline had no obvious effect on MHV-68 virion production.

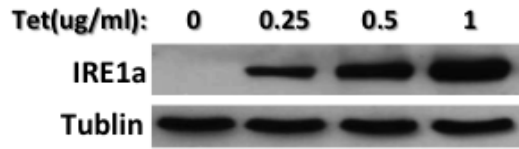
To assess the effect of IRE1 $\alpha$  signaling on a long-term, multi-step infection, water- or tetracycline-treated 293FT-IRE1 $\alpha$  cells were infected with MHV-68-M3FL at MOI 0.01. Viruses produced from the cells were collected at 24, 36, 48, 72, and 96 hours post infection and titered by luciferase assay. We observed an accelerated production of infectious virion particles in IRE1 $\alpha$ -induced cells as compare with that of uninduced cells (Figure 3-2C). In addition, we also observed viral gene expression at a faster kinetics in cells treated with tetracycline (data not shown).

In summary, these findings suggest that the enhanced IRE1 $\alpha$  signaling can promote MHV-68 production.

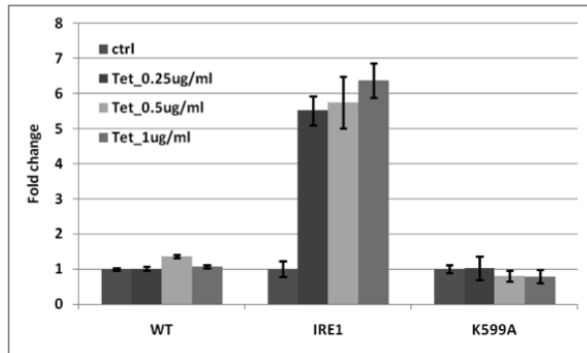
### **Activated ATF6 signaling enhances MHV-68 virion production**

We next assessed whether the ATF6 pathway play a role in MHV-68 replication. 293T cells transfected with an increasing dose of ATF6 plasmids were infected with MHV-68-M3FL. Notably, increased expression of the 90kD full-length ATF6 led to a dose-dependent production of the cleaved ATF6 (50kD) (Figure 3-3A), indicating a stronger signaling of the pathway. 24 hours post transfection, virus production from the cells was measure by luciferase analysis as described above. Activation of ATF6 signaling resulted in a higher level of luciferase activity as shown in Figure 3-3B, suggesting that the ATF6 arm of UPR also plays a positive role in enhancing the virion production of MHV-68.

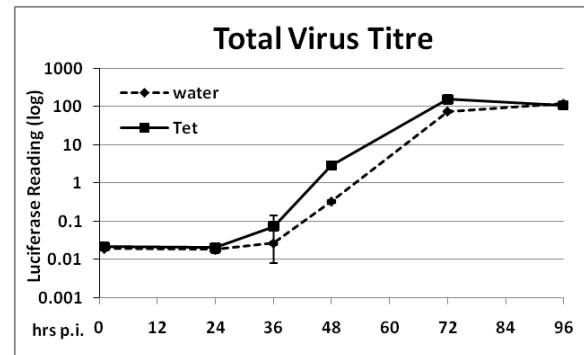
**A**



**B**



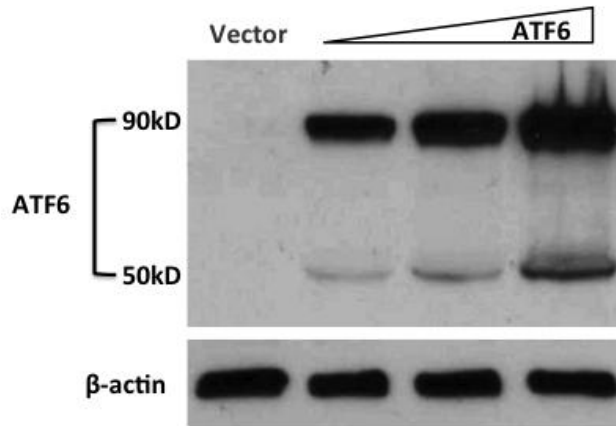
**C**



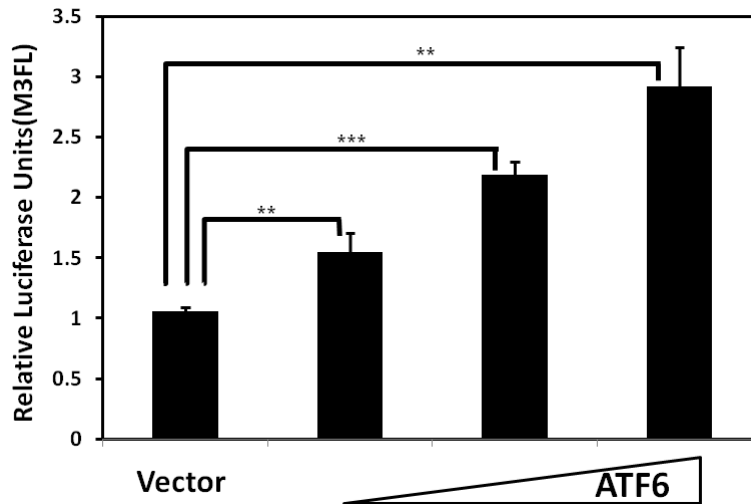
**Figure 3-2. IRE1 $\alpha$  signaling promotes the production of infectious MHV-68.** (A) 293FT, 293FT-IRE1 $\alpha$ , and IRE1 $\alpha$ K599A were pre-treated with indicated doses of tetracycline for 24 hours and were then infected with MHV-68-M3FL at MOI 3. Cell lysates were collected 24 hours post tetracycline treatment and analyzed by western blot using antibody against IRE1 $\alpha$  and tublin. (B) Luciferase assay was performed as in Figure 3-1 to determine relative virus titer. (C) 293FT-IRE1 $\alpha$  cells were treated with water or tetracycline (1 $\mu$ g/ml) and were infected with MHV-68-M3FL at MOI 0.01. Viruses produced from the cells were collected at 24, 36, 48, 72, and 96 hours post infection and titered by luciferase assay.



**A**



**B**



**Figure 3-3. ATF6 signaling enhances MHV-68 virion production.** (A) 293T cells were transfected with increasing amount of ATF6 expression plasmids or the vector control. Cell lysates were collected 24 hours post transfection and analyzed by western blot using antibodies against the HA tag and  $\beta$ -actin. (B) 293T cells were transfected as in (A) and 24 hours post transfection, cells were infected with MHV-68-M3FL at MOI 3. Luciferase assay was conducted to determine relative virion production as described in Figure 3-1.

### **PERK signaling does not affect MHV-68 virion production**

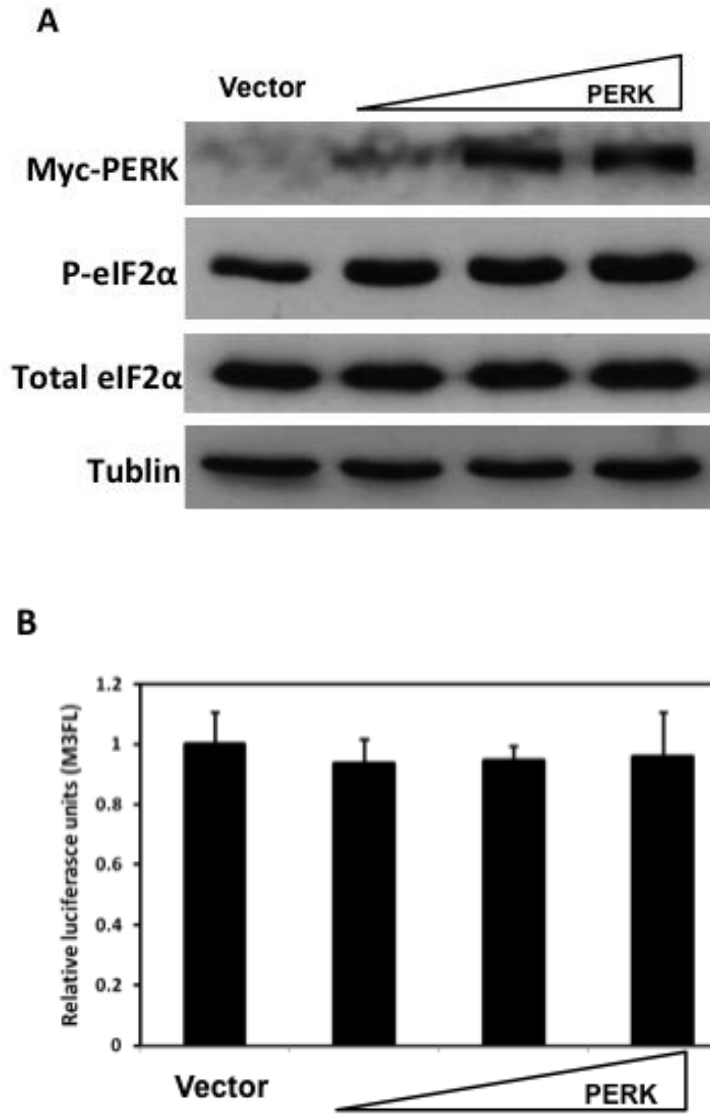
To determine whether the PERK pathway also has an effect on virus replication, we transfected 293T cells with an increasing amount of PERK expression plasmids, and infected the cells with MHV-68-M3FL. Production of infectious virions was determined by luciferase assay as described in Figure 3-1. The PERK signaling was measured by the phosphorylation of eIF2 $\alpha$ . As depicted in Figure 3-4A, expression of PERK protein leads to increased level of eIF2 $\alpha$  phosphorylation even at the lowest dose examined, suggesting that the signaling had become activated. However, MHV-68-M3FL virion production was not affected by induced PERK signaling (Figure 3-4B).

These results indicate that unlike the IRE1 $\alpha$  pathway, the translation-blocking PERK signaling arm of UPR does not influence MHV-68 virion production.

### **MHV-68 differentially regulates UPR at different stages of the infection cycle**

Since by stimulating cellular UPR can markedly upregulate MHV-68 replication and production, we hypothesize that MHV-68 may require and utilize the cellular UPR at certain stages of its infection cycle. To determine whether MHV-68 is indeed capable of manipulating host UPR during infection, 293T cells infected with MHV-68 were collected every three hours post infection for RNA extraction, and were analyzed by quantitative RT-PCR to measure the mRNA levels of GRP78. GRP78 is a UPR gene that is highly stress-inducible and its expression level serves as a UPR marker [15, 16]. MHV-68 significantly inhibited GRP78 transcription at the early stage of infection (3 to 12 hours post infection). However, at the late stage of infection (16 to 24 hours post infection), GRP78 expression was markedly upregulated (Figure 3-5).

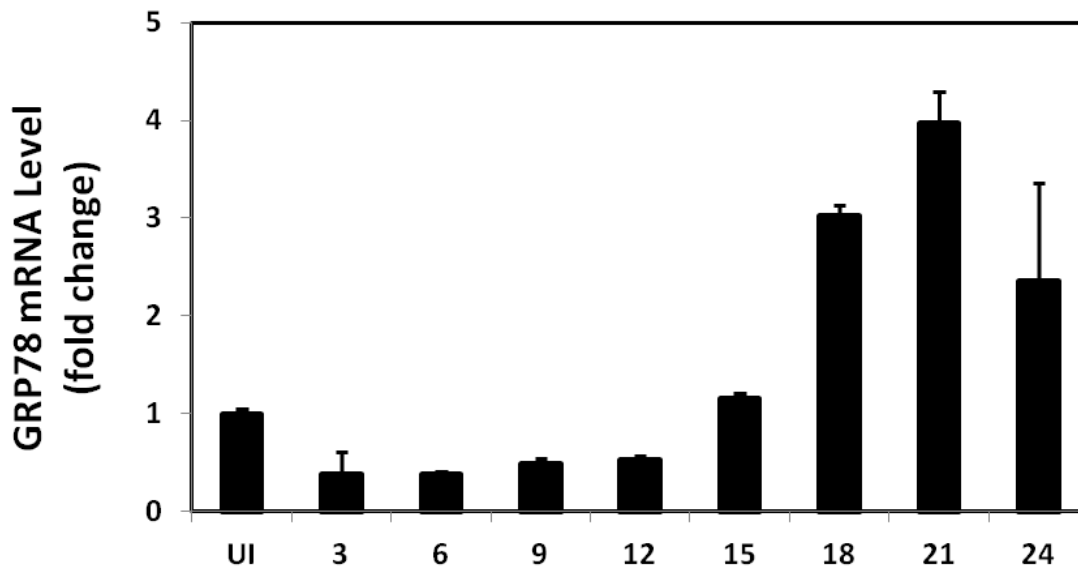
The differential modulation of cellular UPR during the course of MHV-68 infection indicates that this host response may play important roles at the late stage of viral life cycle.



**Figure 3-4. PERK signaling has no effect on MHV-68 production.** (A) 293T cells were transfected with increasing amount of PERK expression plasmids or the vector control. Cell lysates were collected 24 hours post transfection and analyzed by western blot using antibodies against Myc tag, phosphorylated-eIF2 $\alpha$ , total eIF2 $\alpha$ , and tublin. (B) 293T cells were transfected as in (A) and 24 hours post transfection, cells were infected with MHV-68-M3FL at MOI 3.

Luciferase assay was conducted to determine relative virion production as described in Figure 3-

1.



**Figure 3-5. MHV-68 differentially regulates UPR at different stages of infection cycle.** 293T cells were infected with MHV-68 at MOI 3. Cells were collected every 3 hours for 24 hours post infection and subject to RNA extraction. Quantitative RT-PCR was performed using a primer set specific for GRP78. GRP78 mRNA levels were normalized to that of GAPDH mRNA. The fold changes shown were calculated relative to the values obtained in vector-transfected cells (set as 1).

## DISCUSSION

In present study, we investigated the role of cellular UPR in modulating MHV-68 infection and how MHV-68 infection cycle influences host UPR.

We provided evidence that induction of UPR by thapsigargin treatment leads to elevated level of infectious MHV-68 production. This finding is in agreement with previous work on the human gammaherpesviruses: treatment with thapsigargin induces the expression of KSHV lytic cycle-associated proteins and leads to increased virus production in latently infected PEL cells [17]; in EBV infected cells, exposure to thapsigargin followed by recovery resulted in the activation of the EBV gene expression and virus release [18]. These studies along with our study suggest that the positive effect of the cellular UPR on virus replication is conserved within the gammaherpesvirus family.

In addition, we showed that by specifically enhancing the IRE1 $\alpha$  signaling branch of UPR can markedly upregulate the production of infectious virion during both one-step and multi-step infection cycles. We previously found that XBP1, the key player of the IRE1 $\alpha$  signaling pathway, is capable of inducing MHV-68 RTA gene expression (data not shown); consistently, the earlier studies in our lab showed that XBP1 efficiently initiates KSHV reactivation and lytic gene expression by activating the KSHV RTA promoter [19]; furthermore, XBP-1 has also been found to plays a critical role in EBV reactivation [20]. Taken together, these findings suggest a likely conserved mechanism in the IRE1 $\alpha$ -XBP1-mediated upregulation of viral replication among the gammaherpesvirus family members.

We demonstrated, for the first time, that ATF6 pathway positively modulates the gammaherpesvirus replication. The ATF6 pathway is the pro-survival axis of UPR, and is responsible for the production of majority of the chaperone. Induction of ATF6 signaling

augments the folding capacity in the ER. This adaptive response of the host cell may facilitate the biosynthesis of a large number of viral proteins required for optimal virion assembly during acute infection, meanwhile protect cell against virus-inflicted damage. Therefore, an activated ATF6 signaling would create an environment for virus multiplication.

On the other hand, unlike IRE1 $\alpha$  and ATF6, the activated PERK signaling arm does not positively affect, if not negatively, on MHV-68 replication. Induced PERK pathway leads to a global translational arrest that ceases new protein synthesis. Therefore, this UPR pathway has been perceived as the deleterious aspect of UPR to virus infection and is often negatively regulated during virus replication [2, 21-23]. Thus upregulation of PERK may also be unfavorable to MHV-68 infection.

Finally, we demonstrated that MHV-68 infection could differentially mediate the cellular UPR. Through measuring the transcript level of GRP78, we observed reduced GRP78 expression at the early stage of viral replication. The hypothesis is that in order to initiate the infection cycle, it is critical for the virus to attenuate the UPR that would otherwise become detrimental to initial replication through, for instance, translation attenuation and increase protein degradation. Whereas, we found that at the late stage of infection, GRP78 expression became highly induced. As discussed in more details in later chapters, the elevated expression of GRP78 temporally correlates with MHV-68 late gene production and virion assembly. This indicates that at the late stage of viral life cycle, when abundant viral structure proteins are being produced and processed, assistant from chaperones become necessary.

In summary, our findings in chapter 2 suggest that cellular UPR plays a crucial role in MHV-68 infection cycle; and most likely, it effects at the late stage of viral replication.

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## CHAPTER 4

M1 OF MHV-68 INDUCES ENDOPLASMIC RETICULUM

CHAPERONE PRODUCTION

## ABSTRACT

Viruses rely on host chaperone network to support their infection cycles. In particular, the endoplasmic reticulum (ER) resident chaperones play key roles in synthesizing and processing viral proteins. Influx of a large amount of foreign proteins exhausts the folding capacity in the ER and triggers the unfolded protein response (UPR). A fully-executed UPR comprises a collection of signaling pathways that induce ER folding chaperones, increase protein degradation, block new protein synthesis and may eventually activate apoptosis, presenting both opportunities and threats to the virus. Here, we define a role of the MHV-68 M1 gene in differential modulation of the UPR pathways to enhance ER chaperone production. Ectopic expression of M1 markedly induces the ER chaperone genes and expansion of the ER. The M1 protein accumulates in the ER during virus infection and this localization is indispensable for its function indicating that M1 acts from the ER. Indeed, we found that M1 protein selectively induces the chaperon-producing branches (IRE1, ATF6) and, interestingly, sparing the translation-blocking arm (PERK). We identified, for the first time, a viral factor capable of selectively intervening the initiation of ER stress signaling to induce chaperon production. This finding provides a unique opportunity of using viral protein as a tool to define the activation mechanisms of individual UPR pathways.

## INTRODUCTION

The M1 gene is encoded within a locus of a number of open reading frames (ORFs) that are exclusive to the MHV-68 genome. Each of these ORFs is dispensable for *in vitro* growth, but essential for regulating aspects of *in vivo* infection [1-9]. M1 exhibits a 25% identity and 45% similarity to M3, another MHV-68 specific ORF. Previous studies have demonstrated that when mutated individually, M1 and M3 are nonessential genes for MHV-68 infection *in vitro* and *in vivo* [1, 10, 11]. Only one studies has reported on the extracellular function of the M1 protein as an activator of V4 $\beta$ <sup>+</sup> CD8<sup>+</sup> T cells [12], however, the intracellular activity of M1 remains unknown. In this study, we defined a role of M1 in controlling ER chaperone production through analysis with protein ectopic expression and usage of a recombinant MHV-68. Our findings on M1 gene in interacting with the host chaperone system provide new implications in how virus selectively deregulates cellular pathways amongst the complex virus-host interaction.

## RESULTS

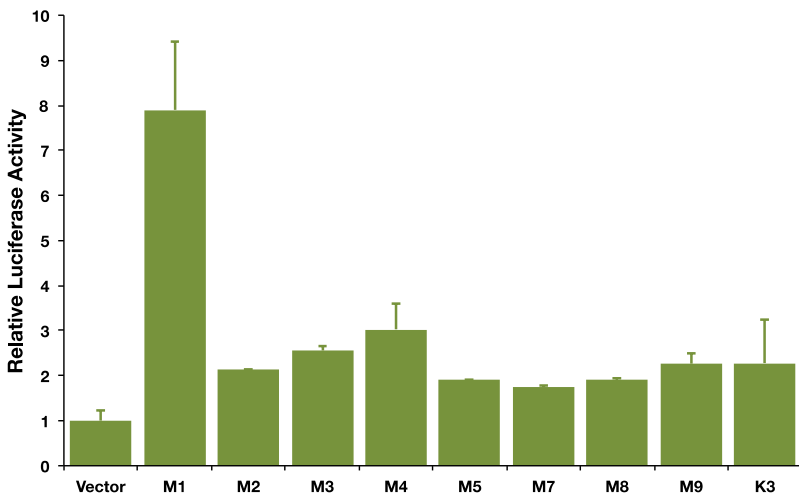
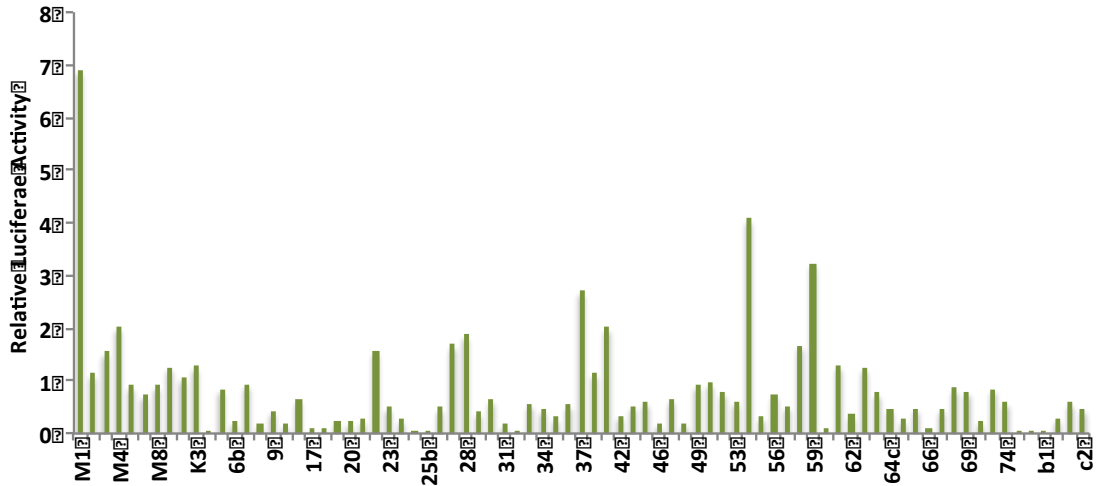
### **M1 induces ER chaperone genes**

In order to systematically identify viral components of MHV-68 that modulate the expression of ER chaperone genes, we conducted a genomic viral ORF screen using a reporter system based on GRP78 expression. GRP78 is the most abundant ER resident chaperone and is highly stress-inducible [13]. The reporter construct (GRP78-fluc) used in the study contains the promoter region of GRP78 driving the coding sequence of firefly luciferase gene [14, 15]. GRP78-fluc was co-transfected into the 293T cells with PGK\_renilla-luciferase (an internal control plasmid in which the renilla luciferase expression is driven by the constitutively active PGK promoter), and either individual ORFs of MHV-68 or a vector control. The reporter activity was measured by

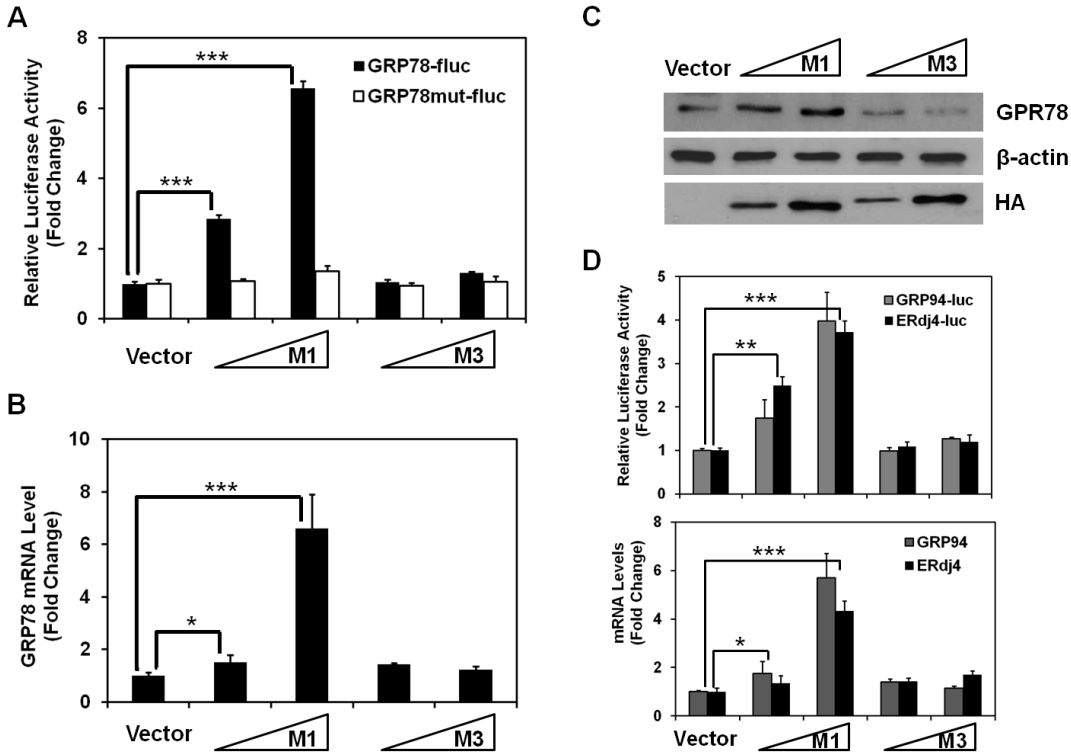
dual-luciferase assay 24 hours post transfection (Figure 4-1). From the screen, a strong induction (>6-fold) on the reporter was consistently noted with multiple clones of M1 (Figure 4-1 & 4-2); in contrast, transfection with the sequence-related M3 showed basal-level activity comparable to that of vector control. The observed changes in reporter activity were specific for the stress response elements within the promoter region of GRP78, because M1 had no effect on the mutant reporter plasmid (GRP78mut-fluc) in which the response elements were eliminated [14]. In line with the reporter assays, the endogenous mRNA and protein levels of GRP78 were also upregulated in a dose-dependent manner with M1 expression as shown, respectively, by real time RT-PCR (Figure 4-2B) and western blot analyses (Figure 4-2C).

Since M1 protein expresses to a high level in transfected 293T cells, it may be argued that the induction of the chaperone was simply due to protein overload in the ER. However, the equally high expression of M3 protein (Figure 4-2C) did not elicit the same response rendering this possibility unlikely. It should also be noted that when performing a titrated transfection of the plasmid encoding M1, strong activation of the chaperones genes was observed even with a small amount of the plasmid (0.06ug/60mm-dish) however no effect was seen in cells transfected with maximal amount of the M3-encoding plasmid (2.4ug/60mm-dish) (Figure 4-2A and data not shown).

In addition to increased production of GRP78, we also observed significantly enhanced gene expression of the other two major UPR chaperones GRP94 and ERdj4 in the presence of M1 (Figure 4-2D). These findings directed our attention to the changes in the ER morphology. We speculated that in order to accommodate the newly-synthesized folding machineries, the amount or the size of the ER must have changed dramatically as described previously [16]. Indeed, electron microscopy analysis revealed a massive expansion of the ER in the M1-



**Figure 4-1. Screen for viral mediators of host unfolded protein response.** 293T cells were transfected with GRP78-fluc, PGK\_RL, and either individual ORFs of MHV-68 or a vector control. The reporter activity was measured by dual-luciferase assay 24 hours post transfection. The ratio of firefly luciferase activity to renilla luciferase activity was calculated based on the value of the vector control (set as 1)The upper panel is the readout from individual viral genes, and the lower panel is a zoom-in toward the left end of upper chart.



**Figure 4-2. M1 induces expression of ER chaperone genes.** (A) 293T cells were transfected with GRP78-fluc or GRP78mut-fluc reporter plasmids, PGK-RL and increasing amounts of M1 or M3 plasmids. Cell lysates were collected 24 hours post-transfection and analyzed by dual-luciferase assay. (B) 293 T Cells were transfected with HA-tagged M1 or M3 expression plasmids for 24 hours and harvested for RNA extraction. Quantitative RT-PCR was performed using primers specific for GRP78. GRP78 mRNA levels were normalized to that of GAPDH . The fold changes shown were based on the values obtained in vector-transfected cells (set as 1). (C) 293T cells were transfected as in (B) and harvested for western blot analysis using antibodies specific for GRP78 and  $\beta$ -actin. (D) The promoter activities of GRP94 and ERdj4 were determined by reporter assay as described in (A) using the GRP78-fluc and ERdj4-fluc reporter plasmids, and mRNA levels were determined by quantitative RT-PCR as in (B) using specific primer sets for indicated genes. \* $P < 0.05$ . \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by Student's *t* test.

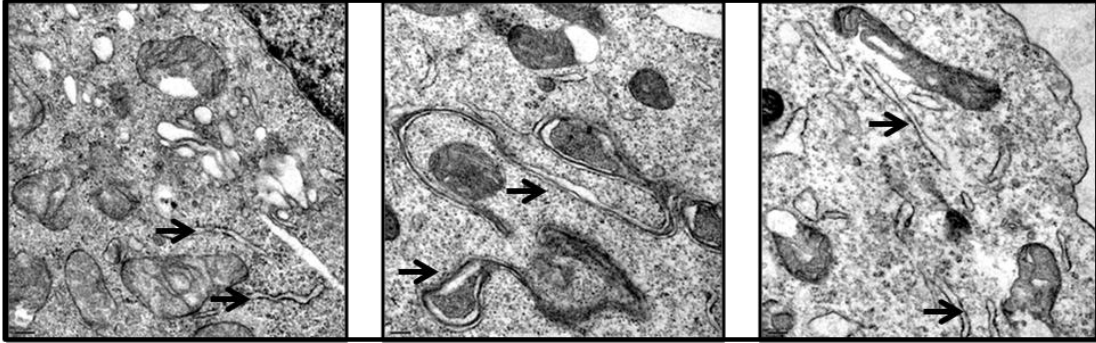
expressing cells as compared to cells transfected with the M3 plasmid or the control vector (Figure 4-3).

### **ER localization of M1 is indispensable for ER chaperone induction**

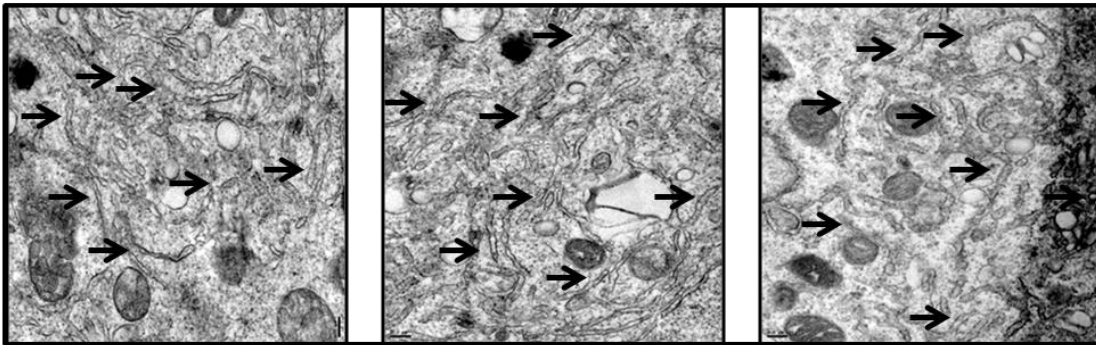
In order to gain insights into the mechanisms by which M1 induces ER chaperone expression, we examined the cellular localization of M1 protein. Sequence analysis of M1 and M3 showed that both proteins contain signal peptides and cleavage sites located close to the N-terminus. The results also indicated that both M1 and M3 are secreted proteins, an observation confirmed in our study (Figure 4-4A) and by other groups [10, 12]. It has been well established that in eukaryotic cells, nascent proteins destined for secretion first enter the ER, where they are modified and assembled prior to transit into the secretory pathway. Indeed, both M1 and M3 proteins localize to the ER, as evidenced by significant co-distribution of the HA (M1/M3) and concanavalin A (Con A) signals (Figure 4-4B). Conc A is a probe reported to recognize the residues of  $\alpha$ -mannopyranosyl and  $\alpha$ -glucopyranosyl commonly found in the ER and Golgi apparatus. To determine whether in the context of viral infection that M1 protein also enters ER, we constructed a recombinant MHV-68 (M1cHA) with an HA tag added to the C-terminus of the M1 ORF. The tagged virus is replication competent (Figure 4-5) and enables detection of the M1 protein during infection. M1 expression was induced at 12 hours post infection in the NIH3T3 cells, in accordance with the production of viral capsid proteins ORF26 and M9 (Figure 4-6A). In addition, we observed significant a co-localization of M1 with Conc A in the infected NIH3T3 cells (Figure 4-6B), suggesting the presence of M1 protein in the ER lumen.



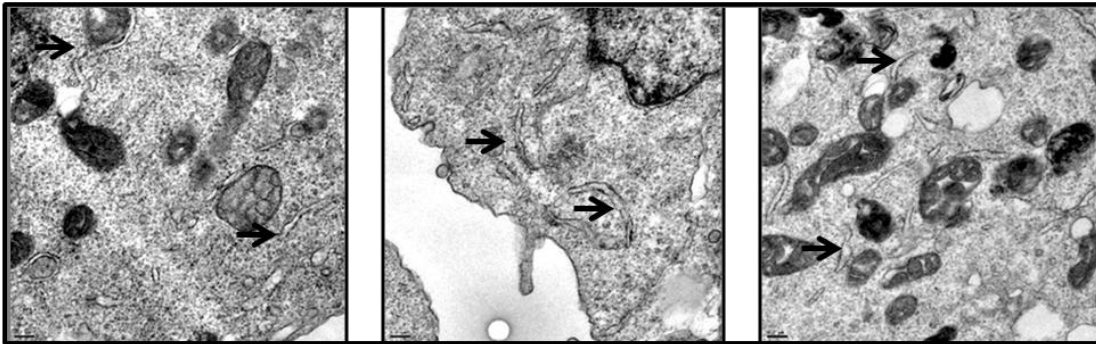
**A. Vector**



**B. M1**

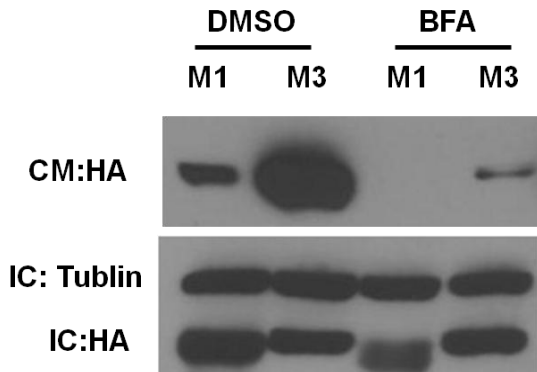


**C. M3**

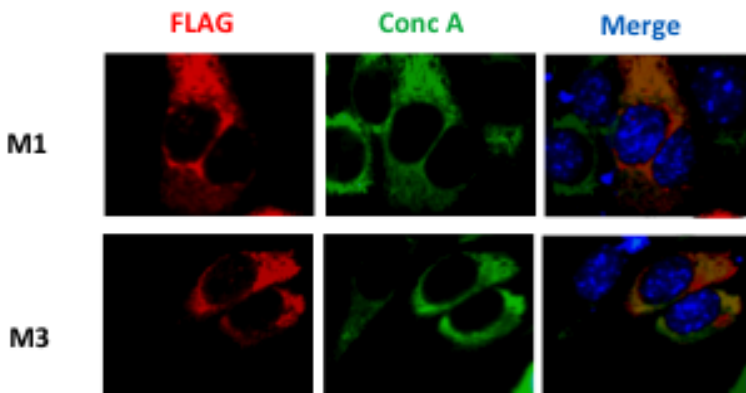


**Figure 4-3. M1 induces ER expansion.** 293T cells were transfected with vector control (A) or plasmids encoding M1 (B) or M3 (C) for 24 hours, and were prepared for electron microscopy analysis. Arrows point to representative ER

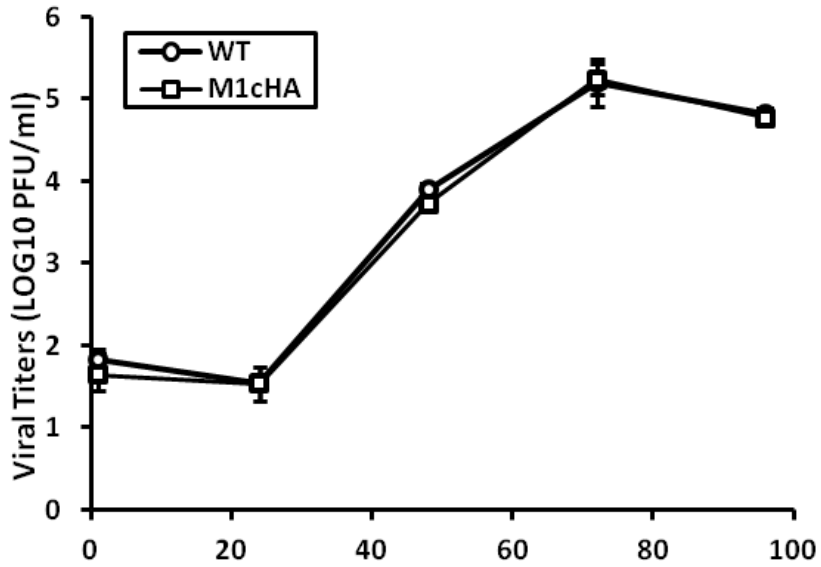
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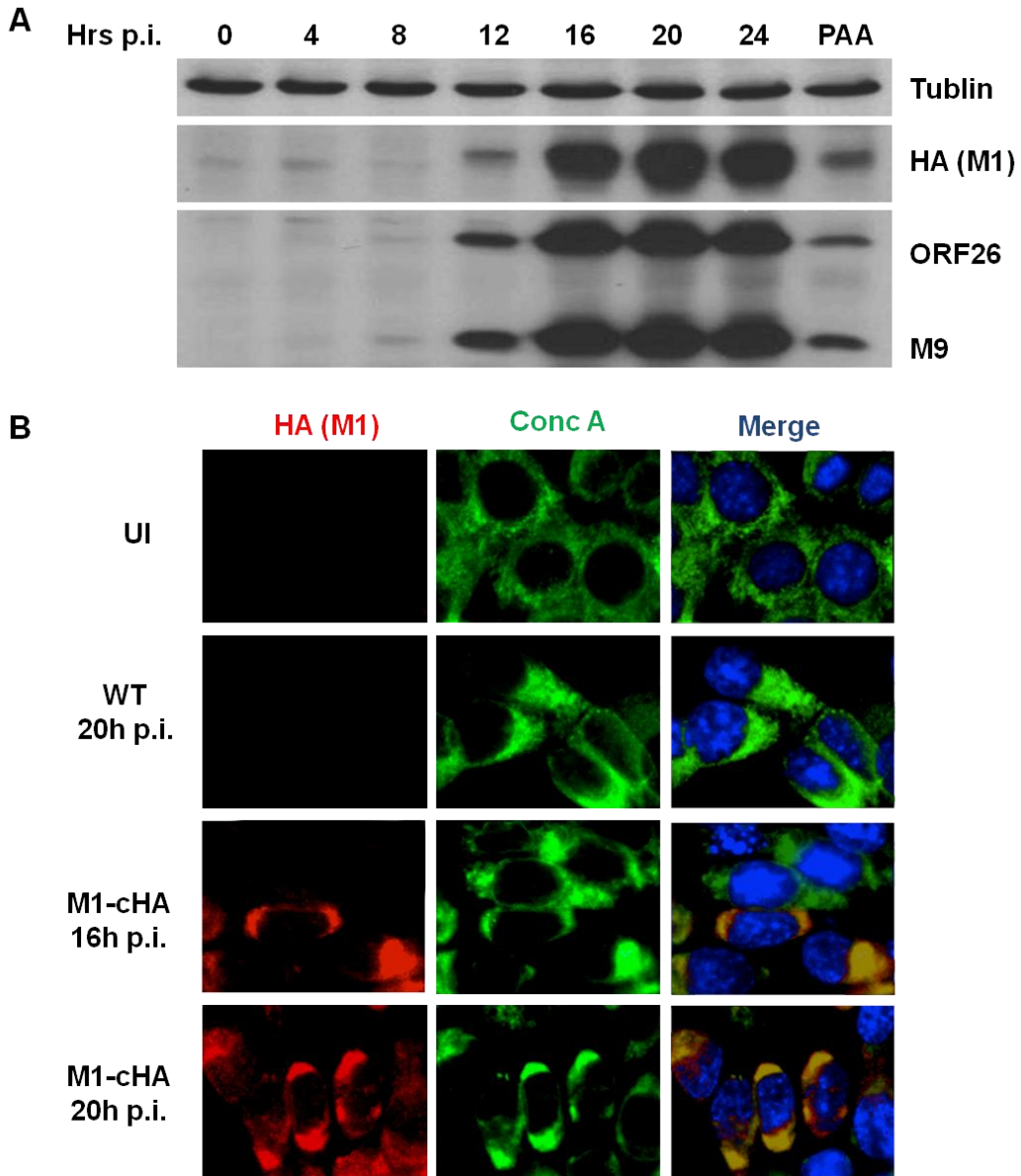
B



**Figure 4-4. M1 and M3 are secreted proteins.** (A) 293T cells were transiently transfected with the M1 or M3 expression plasmids and were treated with 10ug/ml of brefeldin A (BFA) (Sigma) or DMSO as solvent control. BFA is a strong inhibitor of protein secretion. 24 hours post treatment, proteins were collected from the culture medium (CM) or cells (IC: intracellular) respectively, and were analyzed by western blot using anti-HA antibody to probe for M1 and M3 proteins and anti-tublin for an internal control. (B) NIH3T3 cells transfected with M1 or M3 expression plasmids for 48 hours. Cells were fixed and analyzed by IFA using HA (red), conc A (green) and Hoechst (Blue) antibodies.



**Figure 4-5: The multi-step growth curve of M1cHA MHV-68.** NIH3T3 cells were infected with wild-type (WT) and M1cHA MHV-68 at MOI 0.05 and harvested at 1, 24, 48, 72 and 96 hours post infection. Following three freeze-thaw cycles of the entire cell and supernatant lysate, total viral titers were determined by plaque assay.



**Figure 4-6. M1 localizes to cellular ER during MHV-68 infection.** (A) NIH3T3 cells were infected with M1-cHA MHV-68 at MOI 10 and harvested at indicated time points for western blot analysis using antibodies specific for tublin (top), HA (middle), ORF26 and M9 (bottom). (B) NIH3T3 cells were mock-infected or infected with wild-type (WT) or M1-cHA MHV-68 at MOI 10 and fixed for IFA analysis as described in Materials and Methods using antibodies against HA (red), concanavaline A (conc A) (green) and Hoechst (Blue).

We then asked whether the ER localization is required for M1 to induce chaperone genes. To test this hypothesis, we generated an M1 mutant (M1 $\Delta$ SP) by removing the 18-aa signal peptide from the protein (Figure 4-7A). M1 without the peptide lost its ER-specific distribution (Figure 4-7C). Importantly, expression of M1 $\Delta$ SP did not activate luciferase expression from the GRP78 promoter, indicating that the ER localization of M1 is required for its function (Figure 4-7D). These results also rule out the possibility that M1 acts as a transcriptional activator of the chaperone genes, because even though M1 $\Delta$ SP displayed an increased nuclear localization, it did not activate the chaperone gene.

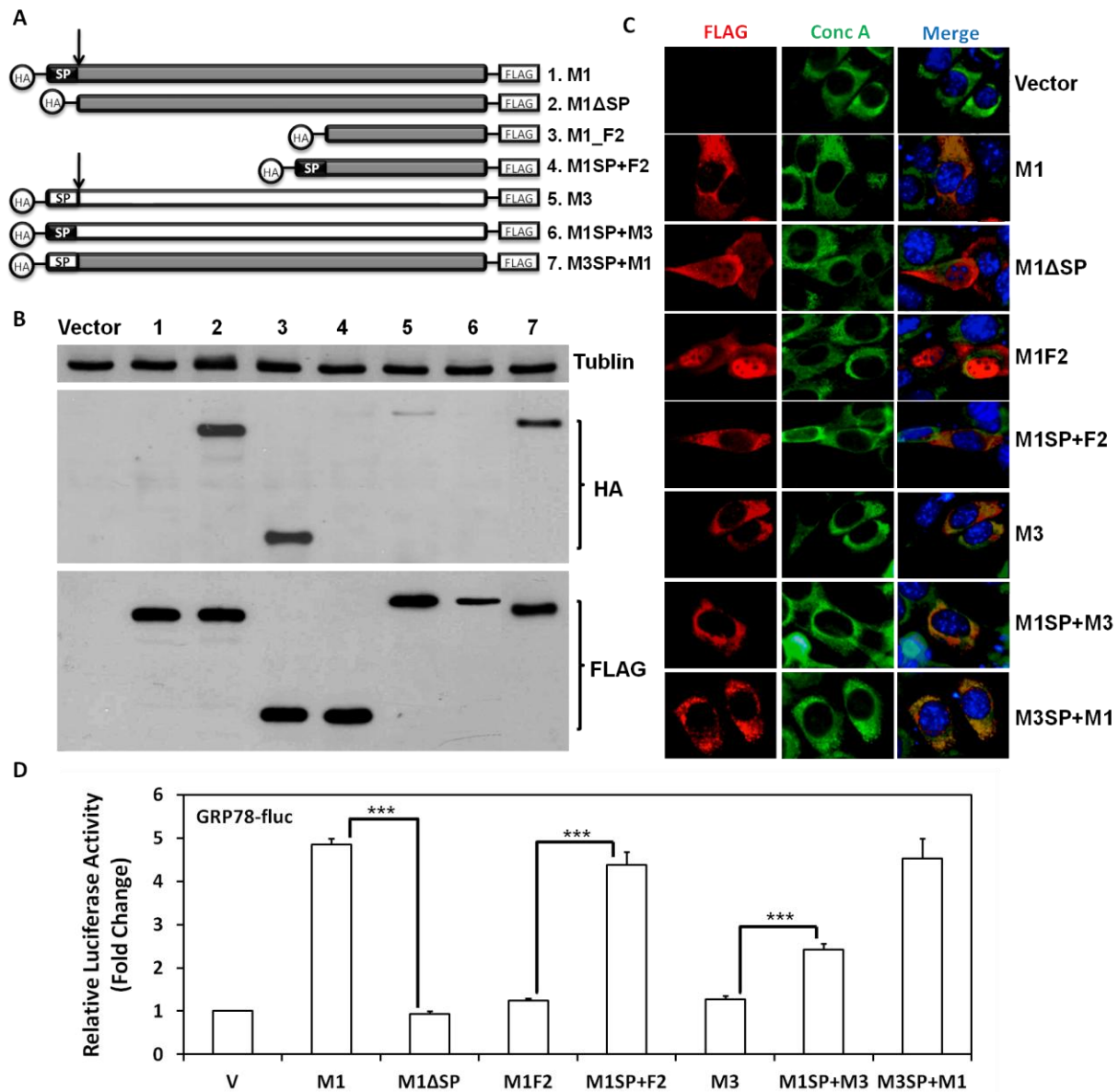
To further assess the importance of the ER localization of M1, we constructed and analyzed the following mutants: a C-terminal fragment of M1 (M1\_F2); an M1\_F2 fused with the M1 signal peptide at its N-terminus (M1SP+F2); an M3 mutant containing the M1 signal peptide (M1SP+M3) and an M1 mutant containing the M3 signal peptide (M3SP+M1) (Figure 4-7A). All four clones were also engineered with an N-terminal HA tag and a C-terminal FLAG tag. This arrangement allowed us to determine whether the N-terminal cleavage took place due to the presence of the signal peptide. Cells were transfected with individual clones and subject to western blot analysis using HA and FLAG antibodies respectively (Figure 4-7B). As expected, proteins bearing the M1 signal peptide sequence (i.e.: M1, M1SP+F2, M1SP+M3) could not be detected by the HA antibody (upper panel: Lane 1, 4, 6) indicating a cleavage at the N-terminus. In addition, IFA analysis revealed that all three proteins located to the ER (Figure 4-7C). In contrast, clones without the signal peptide (M1 $\Delta$ SP, M1\_F2) were detectable by both HA and FLAG antibodies (upper and lower panels: Lane 2, 3), and IFA analysis showed that these proteins are distributed throughout the cells with no specific ER localization (Figure 4-7C). It is noteworthy that the HA antibody picked up a small amount of M3 protein suggesting that

cleavage of M3 was incomplete when the protein is translated and processed (Lane 5). And the retarded cleavage became more evident in M3SP+M1 expression (Lane 7) indicating that the M3 signal peptide causes slower cleavage than does that of M1.

Next, we performed reporter assays to determine how the mutations affected ER chaperone induction. 293T cells were transfected with each mutant construct and the GRP78-fluc reporter plasmid. Similar to what we observed with M1 $\Delta$ SP, the M1\_F2 had no effect on the reporter activity. However, with the addition of M1 signal peptide, the mutant protein (M1SP+F2) induced reporter activity to the same degree as wild type M1. More interestingly, while M3 had no effect on GRP78 expression (Figure 4-2), by replacing its own signal peptide with that of M1, the M3 protein became a modest inducer of the GRP78 reporter (~a 2-fold increase). On the other hand, not surprisingly, even with a swapped signal peptide, the ER-localized M3SP+M1 was still capable of stimulating the GRP78 promoter (Figure 4-7D). Collectively, the above findings indicate that M1 acts through ER-to-nucleus signaling pathways and that the ER localization of M1 is indispensable for its function in ER chaperones induction. In addition, mutagenesis studies revealed that the M1 protein requires both its signal peptide and at least portion of its intraluminal fragment to reach the full potential of function.

### **M1 activates the IRE1 and ATF6 axes of UPR to induce ER chaperone genes**

ER chaperones are constantly expressed under all growth conditions. However a dramatic increase in their synthesis can be caused by activation of UPR in stressful conditions. Induction of different ER chaperone genes requires the cooperation between the three UPR signaling pathways [17-19]. Thereby, to specify the signaling branch(es) affected by M1, we set out to examine the status of each UPR signaling axis in M1-expressing cells.



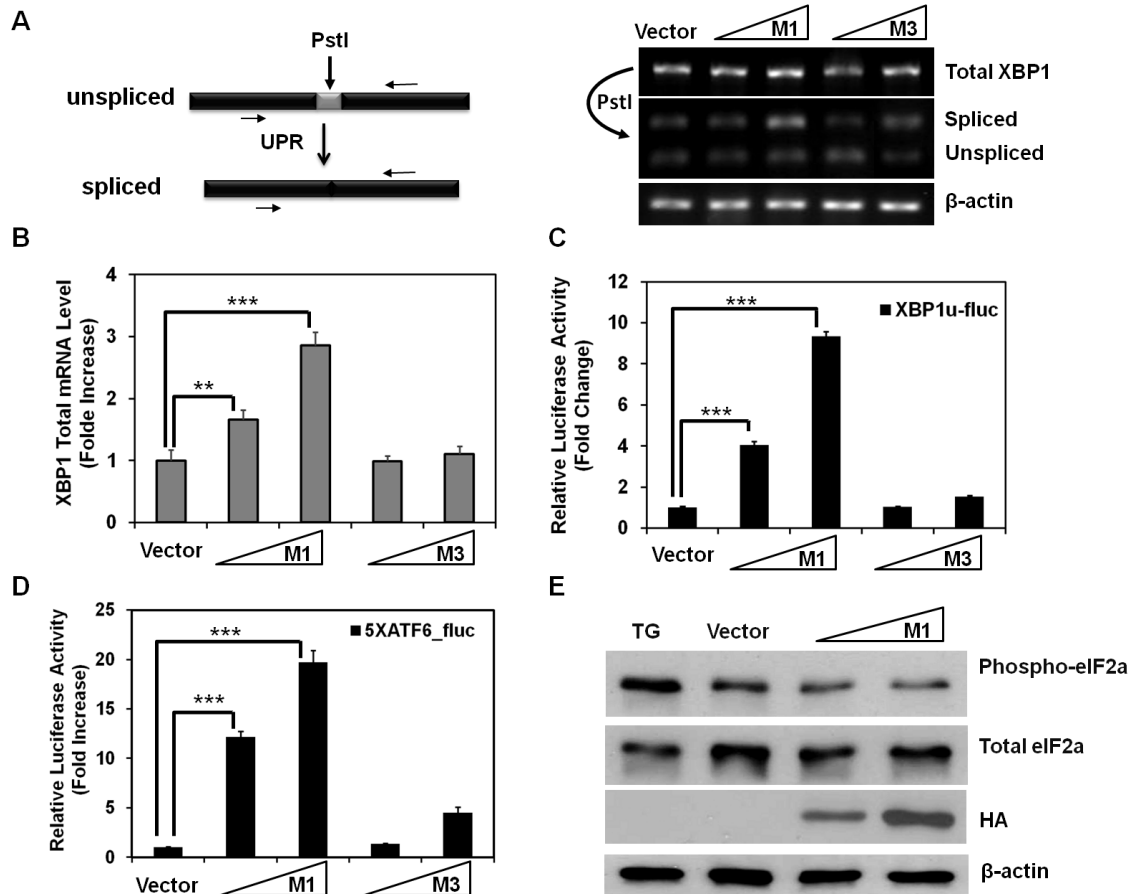
**Figure 4-7. M1 requires ER localization for ER chaperone induction.** (A) Schematic diagram of the M1 mutant constructs. (B) 293T cells were transfected for 24 hours with M1 mutant plasmids and harvested for western blot analysis using antibodies against tublin (top), HA (middle) or FLAG (bottom). (C) NIH3T3 cells transfected with the indicated M1 mutant constructs were fixed and analyzed by IFA using FLAG (red), conc A (green) and Hoechst (Blue) antibodies. (D) 293T cells were transfected with GRP78-fluc, PGK-RL, and M1 mutant plasmids. Cell lysates were collected 24 hours posttransfection and analyzed by dual-luciferase assays.

To test whether M1 affects the IRE1 pathway, we probed for the unconventional splicing of XBP1 mRNA using RT-PCR and reporter assay. XBP1 cDNA was amplified using primers flanking the splicing sites and the PCR products were made subject to PstI digestion. PstI cuts a site within the 26-nt intron in the unspliced XBP1 (XBP1u) but leaves the spliced XBP1 (XBP1s) intact. In cells expressing M1, an elevated level of XBP1s was observed, manifested by an increased amount of spliced products that are resistant to PstI digestion (Figure 4-8A). To quantitatively measure the splicing of XBP1, we employed a splicing-specific reporter system (pXBP1u-fluc). pXBP1u-fluc consists the coding sequence of firefly luciferase conjugated to the second ORF of XBP1u. Therefore, the luciferase is expressed only after IRE1-induced splicing removes the 26-nt intron [20]. Consistent with the PstI digestion results, M1 expression markedly increased the reporter activity to about 9-fold that of vector control (Figure 4-8C).

When examining XBP1 splicing, we noticed that cells transfected with M1 expressed a higher level of total XBP1 mRNA than did vector or M3-transfected cells (Figure 4-8B). Since XBP1 is produced downstream of activated ATF6, we suspected that the ATF6 pathway may also be stimulated by M1 expression. To determine if the ATF6 pathway is activated in response to M1, we used a reporter plasmid (p5XATF6-fluc) that contains five copies of ATF6 consensus binding site upstream of the firefly luciferase coding sequence [21]. M1 strongly upregulated the luciferase activity driven from the reporter (Figure 4-8D), suggesting that the ATF6 pathway was also activated by M1.

Finally, to investigate the PERK-mediated signaling in response to M1 expression, we examined the phosphorylation of eIF2 $\alpha$  (p-eIF2 $\alpha$ ) induced by PERK upon UPR. To our surprise, p-eIF2 $\alpha$  was not influenced by M1 expression (Figure 4-8E). As a positive control, treatment





**Figure 4-8. M1 activates the IRE1 and ATF6, but not the PERK pathways of UPR. (A)**

Left: analysis scheme for XBP1 mRNA splicing: the approximate location of the 26-nt intron, PstI digestion site, and PCR primers are shown. Right: the reverse transcripts of total XBP1 mRNA were analyzed by PstI digestion (described in Chapter 2).  $\beta$ -actin transcripts were included as loading controls. **(B)** Total XBP1 mRNA level was determined by quantitative RT-PCR. **(C)** Reporter assay was performed using the XBP1u-fluc and was analyzed as described in Figure 1A. **(D)** Reporter assay was performed using the 5XATF6-fluc plasmid. **(E)** 293T cells were transfected with M1 plasmid or the control vector for 24 hours, treated with 20nM Thapsigargin (TG) (lane 1) or DMSO (lane 2,3,4) for 30 minutes, and harvested for western blot analysis using antibodies specific for phosphorylated-eIF2a, total eIF2a and  $\beta$ -actin.

of Thapsigargin (TG), an ER stress inducer, led to a significant increase in the ratio of p-eIF2 $\alpha$  to total eIF2 $\alpha$ , indicating the PERK-eIF2 $\alpha$  pathway was functionally intact and our assay is valid.

### **Infection by M1-deficient virus leads to reduced ER chaperone production.**

To extend the study on the importance of M1 in virus-mediate ER chaperone production, we constructed two recombinant MHV-68: An M1-stop virus (M1S) that contains two stop codons close to the N-terminal of the coding sequence (Figure 4-9A) and a revertant virus of the M1S (M1R) in which the two stop codons were reverted back to wild type sequence to ensure what we observed with M1S viruses can be attributed to M1 deficiency rather than other unintentional mutations in the viral genome. Removal of the M1 expression from the MHV-68 genome had no effect on viral growth kinetics in vitro (Figure 4-10) consistent with previous observations [1, 12].

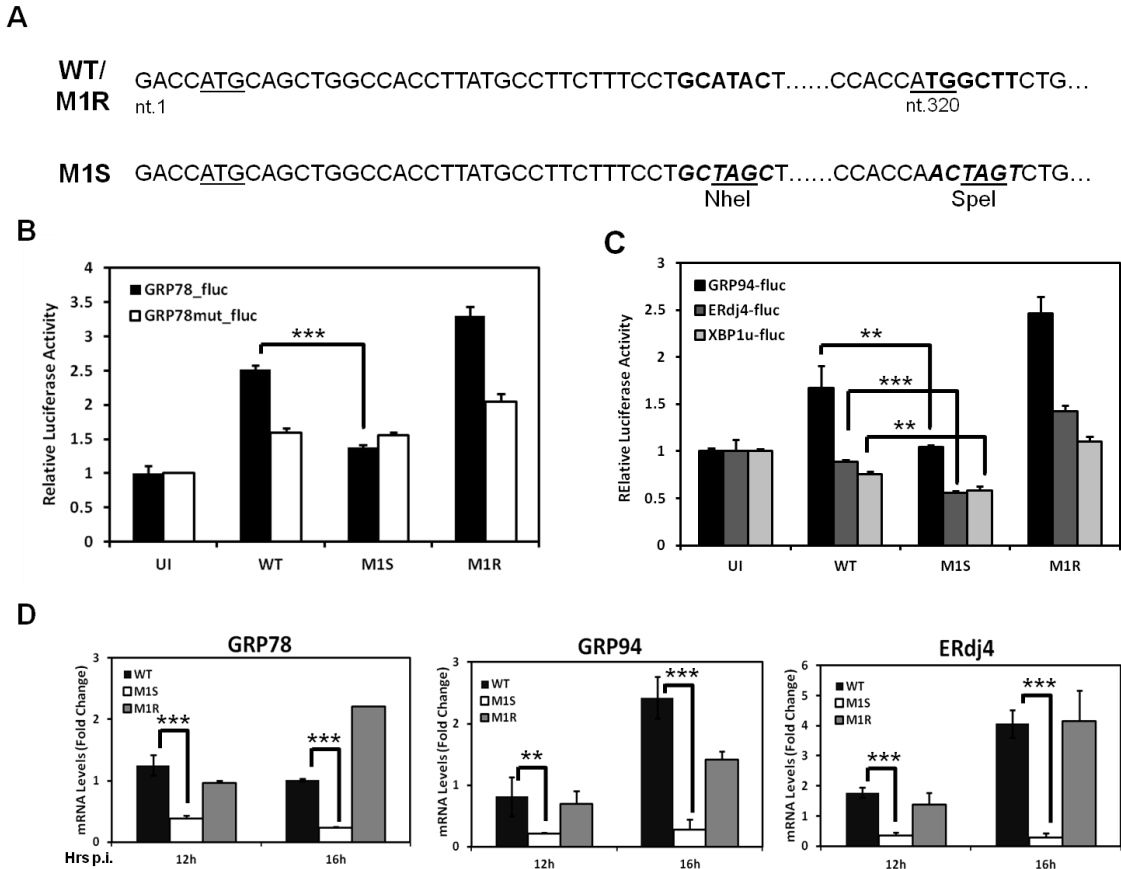
293T cells transfected with GRP78-fluc reporter construct were either mock infected or infected with the wild type (WT), M1S or M1R MHV-68 at MOI 5. Cells were collected 18 hours post infection for dual-luciferase assays. Infection with WT and M1R virus led to a moderate increase in luciferase production, whereas M1S was not able to induce the reporter activity to an equivalent level (Figure 4-9B). To further demonstrate that the observed phenotype is specific to the GRP78 promoter, cells were transfected with the GRP78mut-fluc plasmids and were identically infected. None of the viruses had a significant effect on the mutant reporter construct. We next performed similar tests with the other available reporter constructs including GRP94-fluc, ERdj4-fluc and XBP1u-fluc. In all cases, attenuated reporter activities were observed in cells infected with the M1S virus as compared to cells infected with WT and M1R MHV-68 (Figure 4-9C).

To precisely define the kinetics of chaperone gene expression influenced by M1 during infection, the total RNA was harvested at indicated time points from NIH3T3 cells that were infected with WT, M1S or M1R viruses. The GRP78 mRNA level was determined by RT-PCR. Interestingly, prior to 8 hours post infection, the transcript levels of GRP78 remained comparable among all infected cells (Data not shown). However, at 12 and 16 hours post infection, times at which M1 protein becomes abundantly expressed (Figure 4-6A), the cells infected with M1S showed a major reduction of GRP78 transcript in comparison to cells infected with WT or M1R viruses (Figure 4-9D).

It should be noted that infection by WT or M1R MHV-68 led to a lower induction of chaperone genes (Figure 4-9B) in comparison to cells transfected with M1 gene coding plasmids (Figure 4-2A). One possible explanation is the different protein expression levels of M1 during infection versus transient over-expression; on the other hand, based on the screen results, we speculated that there are one or more viral factors that actually function to limit the signaling pathways involved in regulation of both the chaperone genes and other cellular factors unfavored in viral replication. It would be of interest to uncover how virus manipulates the chaperone network and its associated cellular machineries in order to achieve a balanced and beneficial outcome.

## DISCUSSION

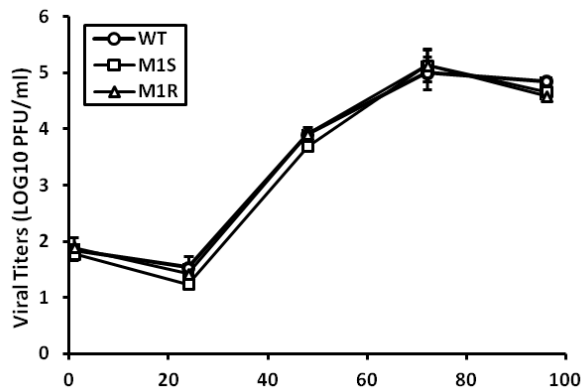
In present work, we identified M1 of MHV-68 that can efficiently induce the ER chaperone gene expression. In particular, we found that the ER-localized M1 functions through selective activation of the chaperone-inducing branches (IRE1 and ATF6) of UPR pathways while sparing the translation-inhibiting cascade (PERK).



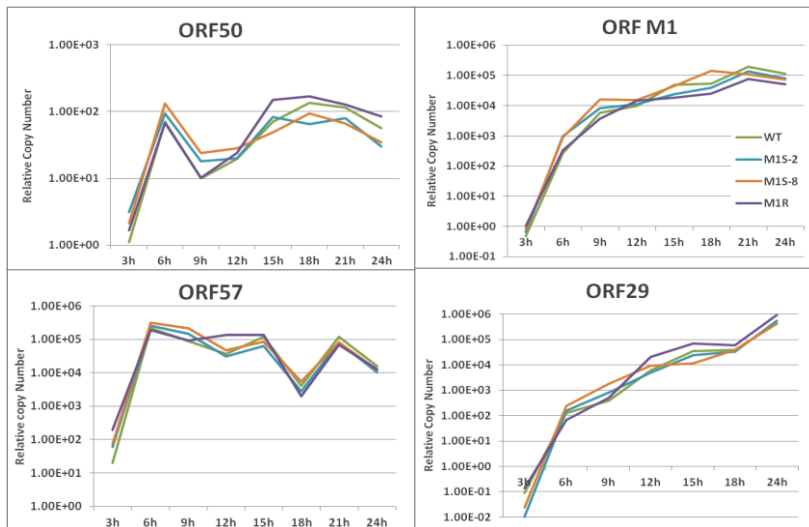
**Figure 4-9. Infection by M1-deficient MHV-68 leads to reduced ER chaperon production.**

(A) Schematic diagram showing the construction of the M1stop MHV68 by introducing two stop codons (TAG) and two digestion sites (NheI, SpeI) into the M1 gene. (B) 293T cells were transfected for 24 hours with GRP78\_fluc or GRP78mut\_fluc and PGK\_RL plasmids, and were mock infected or infected with wild-type (WT), M1stop (M1S) or M1 revertent (M1R) MHV-68 at MOI 5 for 18 hours. Cells were lysed and analyzed by dual-luciferase assay as in Figure 1A. The ratio was calculated based on the uninfected control. (C) Reporter assays were performed using the GRP94-fluc, ERdj4-fluc and XBP1u-fluc constructs. (D) NIH3T3 cells were mock infected or infected with WT, M1S or M1R MHV-68 at MOI 10. Cells were harvested at indicated time points for RNA extraction and analyzed by Quantitative RT-PCR using primer sets specific for indicated genes. The fold change is calculated based on the uninfected cells.

**A**



**B**



**Figure 4-10: Multi-growth curve and viral gene transcription of WT, M1-stop (M1S) and M1-revertant (M1R) MHV-68.** NIH3T3 cells were infected with indicated MHV-68 at MOI 0.05 and harvested at 1, 24, 48, 72 and 96 hours post infection. Following three freeze-thaw cycles of the entire cell and supernatant lysate, viral titers were determined by plaque assay. (B) Comparison between the WT, M1-stop (M1S) and M1-revertant (M1R) MHV-68.

M1 is a viral gene unique to MHV-68. Previous study has reported that disruption of the M1 gene led to enhanced reactivation of the virus in *in vivo* infection [1]. It was later reported by the same group that secreted M1 protein act as a viral superantigen and is responsible for V $\beta$ 4<sup>+</sup> CD8<sup>+</sup> T cell stimulation during MHV-68 infection in mice [12]. However, it has not been fully elucidated whether M1 plays a role inside the host cell. Here we discovered a novel function for the intracellular M1 protein. We found that M1 differentially modulate the UPR signaling cascades and preferentially induces the two chaperone-producing branches to activate ER chaperone expression. Specifically, the M1 protein stimulates the IRE1 and the ATF6 axes but spares the PERK pathway of UPR signaling. It is of interest that the virus selectively acts on the beneficial aspects of the UPR program while avoiding the detrimental features. One possible explanation is that the virus takes advantage of different stress sensing or activation mechanisms deployed by the three ER transmembrane sensors (i.e. IRE1, ATF6 and PERK). Although it is still unclear how these signaling proteins sense the ER stress, a recent study used the three-dimensional structure analysis to demonstrate that IRE1 activation can be caused by direct binding to the unfolded proteins rather than by chaperone association as suggested earlier on [22]. This finding implicates that the three branches of UPR signaling network may be distinctly modulated at the initiation stage. We propose that certain undefined properties in the M1 protein leads to the differentiated modulation on the UPR sensors. Therefore, using M1 as a tool may offer a unique opportunity to dissect the mechanism underlying UPR sensing and regulation.

It is worth mentioning that we also demonstrated that M1 requires ER localization for inducing chaperones, because the induction was lost by removal of the signal peptide, concomitantly with the loss of ER localization. More interestingly, the signal peptide of M1 enables non-inducing protein fragments to become activators of ER chaperone genes (Figure 4-

7). We suspect that the 18-aa signal peptide of M1 protein plays an important role in switching on the IRE1 and ATF6 signaling axes through mechanisms that worth future investigation. Still, the presence of the intraluminal portion of M1 is critical to reach the full potential of its function in activating ER chaperone genes. Our hypothesis is that M1 requires the signal peptide to translocate to the action point, where the protein carries out its function.

It has been extensively reported that during a wide array of virus infections, cellular chaperones are elicited and play essential roles at various stages of the viral life cycle. Accumulating studies have shown that viruses are able to engage the host chaperone machinery to support effective cell entry and nuclear import [23-28], viral genome replication [29-32], viral protein expression and folding [33-36], and virion assembly [37-40]. In addition, examples have been found wherein viruses pack the host chaperons into the virion core before egress and that the incorporated proteins are necessary for invading new cells [41]. More surprisingly, some viruses can even encode proteins that exhibit chaperone-like activities to facilitate their infection (e.g.: TAg of SV40) [42]. Furthermore, host chaperons are also utilized by the viruses to manipulate other cellular processes. Earlier studies have demonstrated that elevated levels of chaperone proteins can protect cells against apoptosis and confer resistance to cytotoxic and antimicrobial drugs [43-45]. Several reports further proposed that specific inhibition of the infection-induced chaperones may provide a solution to the appearance of drug resistant pathogens [46, 47]. One recent study found that the inhibition of the Hsp90 chaperone *in vitro* and *in vivo* can prevent the outgrowth of EBV-transformed lymphoblastoid [48]. Moreover, a growing body of evidence suggests that improper activation of the UPR pathways and uncontrolled production of chaperones can adversely affect the immune response, a phenomenon exploited by certain viruses for an adaptive advantage [49, 50]. To sum up, by augmenting the

expression of chaperone proteins can create an environment favorable to virulence and may have become a survival tactic among different viruses. In response to such high demand of cellular chaperones during infection, viruses have the urge to evolve mechanisms to boost the chaperone production.

The fact that gamma-herpesviruses are capable of establishing lifetime persistence suggests that the viruses were evolved with ingenious skills to interact with their hosts. It is natural that different viruses adopt distinct approaches to tackle similar environmental challenges. For instance, previous studies have found that the M2 protein unique to MHV-68 enhances the cellular interleukin-10 (IL-10) expression to promote B cell growth and differentiation [51] while EBV encodes a viral IL-10 homolog to achieve the same goal [52]. Another example is the regulation of cell cycle through cyclin D: MHV-68 and KSHV depend on encoding the conserved viral cyclins [53, 54] whereas EBV utilizes a viral gene (EBNA3C) to enhance the functional activity of cellular cyclin D1 for cell cycle progression [55]. Though relevant examples are lacking in regulation of UPR, previous work on human cytomegalovirus (beta-herpesvirus) has shown that viral infection can upregulate GRP78 expression without affecting the UPR pathways [56, 57], while studies on the herpes simplex virus type 2 (alpha-herpesvirus) revealed that the virus can encode a protein with chaperone-like activity. Therefore, although M1 is unique to MHV-68, we strongly believe that the human gamma-herpesviruses possess distinct chaperone-regulating approaches to achieve similar goals.

Our findings defined a new role for the M1 gene of MHV-68 in manipulating the host chaperone machinery. The ability of M1 to selectively activate the UPR signaling presents a unique opportunity for defining the sensing and activation mechanism of individual UPR pathways.



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CHAPTER 5  
THE HELICAE-PRIMASE COMPONENTS OF MHV-68  
INHIBIT CELLULAR UPR

## ABSTRACT

The unfolded protein response (UPR) poses both opportunities and threats to virus infection. An active UPR leads to universal translation attenuation, and transcriptional upregulation of genes encoding protein degradation factors. In addition, prolonged UPR triggers apoptosis. Therefore, it is crucial for the viruses to cope with the detrimental aspects of UPR in order to establish productive infection. In this study, we identified that one of the MHV-68 helicase-primase component ORF40 is capable of inhibiting host UPR at the early stage of virus infection. ORF40 downregulates each UPR signaling arms and the production of ER chaperone GRP78/GRP94 in both non-stressed and stressed environments. We provide evidence that ORF40 may function through binding to and stabilizing the interactions between GRP78 and the transmembrane stress sensors and thus prevent UPR initiation. We further show that the M1 can interfere with the anti-UPR function of ORF40. Considering the different expression pattern of ORF40 and M1 at the early and late stages of virus replication, it explains the dynamic modulation of MHV-68 infection on UPR during the infection cycle as depicted in the previous chapters. Finally, we show that such inhibitory effect of ORF40 is conserved in the other two helicase-primase components of MHV-68 (ORF44 and ORF56) and among other herpesviruses. These findings suggest that herpesviral helicase-primase factors may have evolved additional functions to protect virus from the deleterious effects of cellular UPR at the early stage of infection.

## INTRODUCTION

As depicted in previous chapters, the details of the UPR signaling mechanisms have been well established. However, the question of how ER stress is “sensed” remains obscure. One theory states that in non-stress cells, the luminal domains (N termini) of PERK, IRE1 and ATF6 transducers are held by the ER chaperone GRP78, which prevents their aggregation and activation [1-3]. Whereas in response to ER stress, unfolded/misfolded proteins accumulate and compete away GRP78 from the transmembrane signaling transducers, allowing oligomerization and autophosphorylation of IRE1 and PERK, mobilization of ATF6 to the Golgi for activation, and finally launching the UPR program. Although the precise activation mechanisms of these proximal ER-stress sensors are not fully understood, dissociation from GRP78 seems to be a prerequisite. Still, other mechanisms are believed to contribute the initiation of the UPR, including direct activation of the UPR signaling proteins by unfolded proteins as discussed in Chapter 4 and in previous studies [4-7].

Many enveloped DNA and RNA viruses can induce and benefit from the production of ER chaperones downstream of UPR in the mammalian cells [8-12]. However, viruses that induce the stress program must face the consequences that can be detrimental to viral replication. Most notably, the general arrest of protein synthesis caused by the eIF2 $\alpha$  phosphorylation must be dealt with at the early stage of infection to allow the expression of viral and cellular proteins crucial for the proceeding of infection cycle; additionally, the induced expression of protein degradation factors could also considerably damage various viral replication machineries; finally, induction of apoptosis of the host cell can be deleterious and must be properly modulated. Therefore, it has been proposed that in order to survive the stress response, viruses developed



mechanisms that mediate the UPR, keeping the beneficial aspects while suppressing the harmful ones.

In the present work we show that ORF40 of MHV-68 can significantly inhibit host UPR through modulating individual UPR signaling pathways. We also investigated possible mechanisms on how ORF40 function as an inhibitor, and how ORF40 and M1 coordinate their opposite effects on UPR during MHV-68 replication.

ORF40 is recognized as one of the helicase-primase components of MHV-68 that function in the viral DNA replication complex [13]. MHV-68 also contains two other helicase-primase components: ORF44 and ORF56. All three ORFs are essential for MHV-68 replication [14]. Interestingly, in this study, we demonstrated that ORF44 and ORF56 can also efficiently downregulate cellular UPR. Further investigation revealed that the inhibitory effect of viral helicase-primase factor is conserved in other members of the herpesvirus family.

## RESULTS

### **ORF40 inhibits cellular UPR**

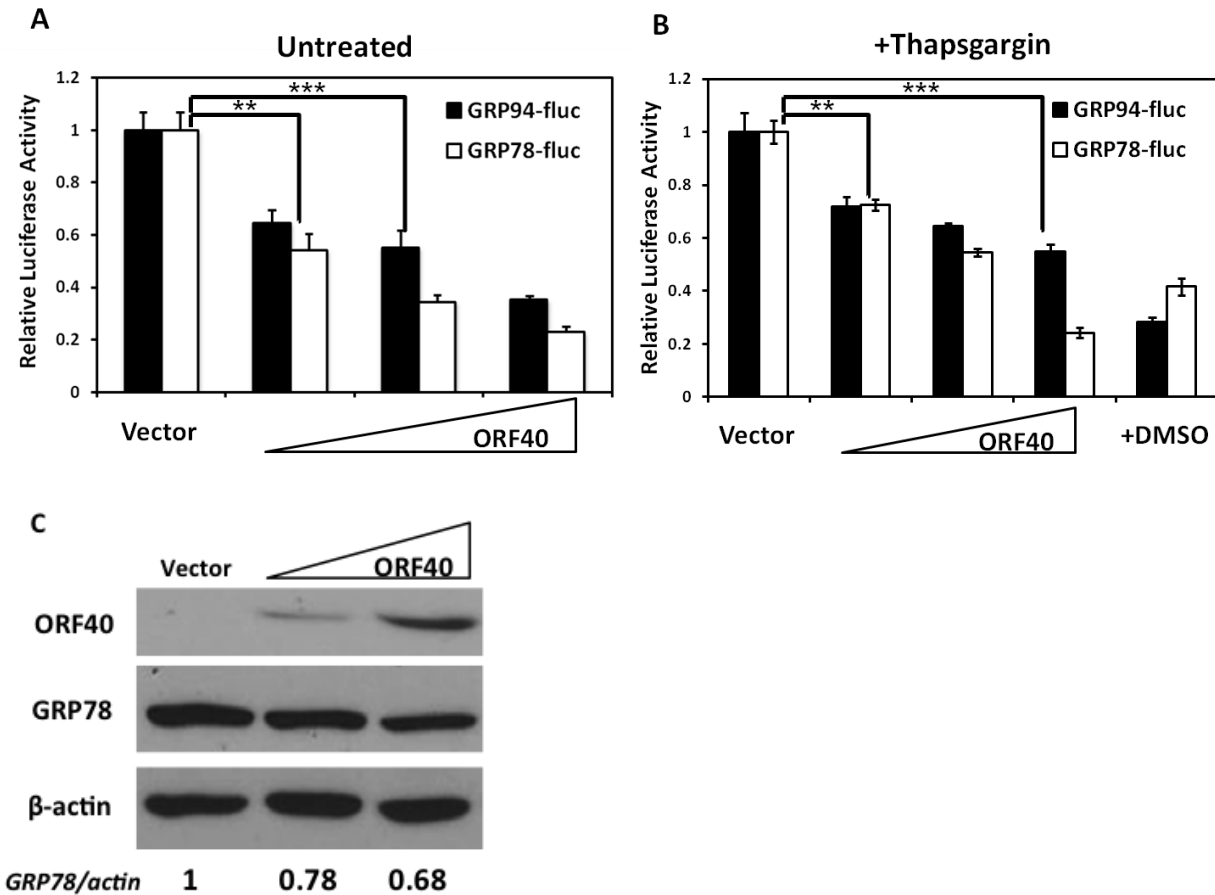
In an unbiased screen conducted to identify viral modulators of cellular unfolded protein response (UPR) (described in Chapter 4, Figure 4-1), one of the viral open reading frames (ORFs), ORF40, showed strong anti-UPR activity. Co-transfection of ORF40-expressing plasmid with the GRP78-fluc reporter constructs in 293T cells produced a dose-dependent decrease in the reporter activity from the basal level (Figure 5-1A). Similar negative impact was observed in cells co-transfected with the GRP94-fluc reporter construct. To further assess whether ORF40 is capable of inhibiting an induced UPR, the identically transfected 293T were treated the ER stress inducer thapsigargin (Tg) at 10ng/ml for 24 hours. ORF40 expression

significantly lowered the reporter activity from an elevated level (Figure 5-1B). Notably, at the highest dose, ORF40 neutralized the positive effects of Tg on GRP78 promoter activity to a level comparable to, or lower than the DMSO-treated control (Figure 5-1B, upper panel). Consistent with reporter assay, increased expression of ORF40 led to a decreased expression level of GRP78 protein as depicted in Figure 5-1C.

Next, in order to pinpoint the signaling branch(es) through which ORF40 places the effect, we examined the activation status of each UPR signaling arm in ORF40-expressing cells. To assess whether ORF40 affects IRE1 pathway, we used the XBP1 splicing reporter construct (pXBP1u-fluc) to quantitatively measure the activity of the IRE1-XBP1 axis (as described in Chapter 4, Figure 4-8). ORF40 markedly reduced the reporter activity to more than 50% that of the vector control (Figure 5-2A). The activation state of the ATF6 branch was determined using an ATF6-specific reporter plasmid (p5XATF6-fluc) also described in Chapter 4, Figure 4-8. The reporter activity was also downregulated by ORF40 (Figure 5-2B). Finally, to investigate the PERK-mediated signaling in response to ORF40, we examined the phosphorylation of eIF2 $\alpha$  (p-eIF2 $\alpha$ ) downstream of PERK. P-eIF2 $\alpha$  was strongly inhibited in cells expressing ORF40 as indicated by ratio of p-eIF2 $\alpha$  signal to that of total eIF2 $\alpha$  (Figure 5-2C). These results suggest that ORF40 is capable of downregulating each UPR signaling arms and inhibiting the overall cellular UPR.

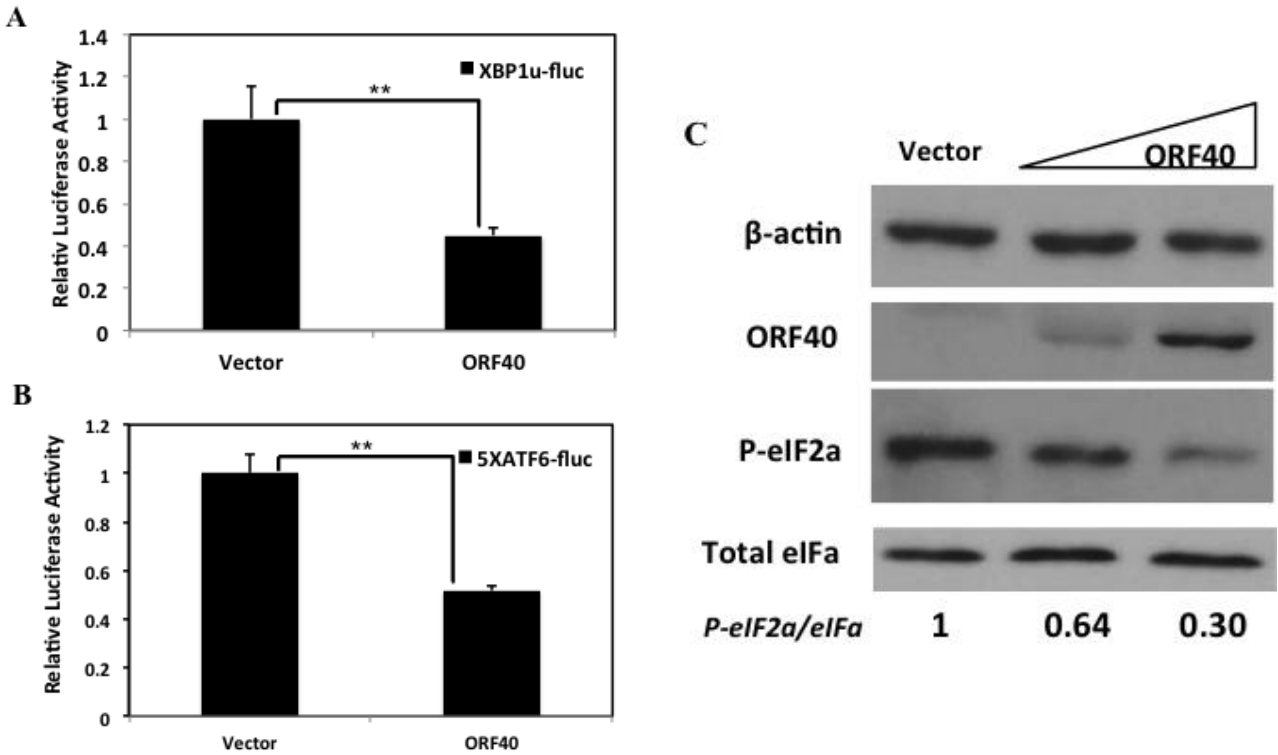
### **ORF40 interacts with GRP78 and the stress transducers**

We next sought to define the mechanisms of how ORF40 inhibit cellular UPR. From the results of a Yeast-Two-Hybrid screen, which was performed to characterize viral-cellular protein interactions of MHV-68 infection [15], we found that ORF40 interacts with the ER chaperone



**Figure 5-1. ORF40 inhibits cellular UPR.** (A) 293T cells were transfected with GRP78-fluc or GRP94-fluc, PGK\_RL, and either ORF40 expression plasmid or a vector control. The reporter activity was measured by dual-luciferase assay 24 hours post transfection. The ratio of firefly luciferase activity to renilla luciferase activity was calculated based on the value of the vector control (set as 1). (B) 293T cells were transfected as in (A). 8 hours post transfection, cells were treated with DMSO or Thapsigargin at 10ng/ml and analyzed 24 hour post transfection as described in (A). (C) Cells were transfected with ORF40 expression plasmids for 24 hours and were harvested for western blot analysis using antibodies specific for FLAG (ORF40), GRP78 and  $\beta$ -actin. The signal intensity of the bands was determined by ImageJ (NIH). \* $P < 0.05$ .

\*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by Student's *t* test.



**Figure 5-2. ORF40 inhibits the UPR signaling pathways.** (A) Reporter assay was performed as described in Figure 5-1A using the XBP1u-fluc (XBP-1 splicing reporter). (B) Reporter assay was performed using the 5XATF6-fluc plasmid. (C) 293T cells were transfected with ORF40 expression plasmid or the control vector for 24 hours, treated with 20nM Thapsigargin (TG) for 30 minutes and harvested for western blot analysis using antibodies specific for phosphorylated-eIF2a, total eIF2a, FLAG (ORF40) and  $\beta$ -actin.

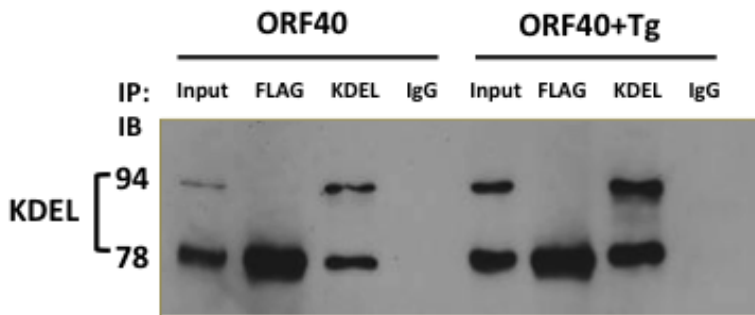
GRP78. We were able to confirm the interaction by immunoprecipitation analysis in non-stress cells and cells induced with Thapsigargin (Figure 5-3A). GRP78 was detected using the antibody that recognizes the KDEL peptide. The tetrapeptide KDEL is a retention signal that prevents ER luminal protein, such as GRP78 and GRP94, from entering the secretory pathway [16-18]. Thus, the KDEL antibody recognizes both GRP78 and GRP94 chaperones, whereas as depicted in Figure 5-3A, ORF40 specifically interacts with GRP78, not GRP94.

Since GRP78 plays a crucial role in the UPR initiation through binding to the inactive stress sensors, we hypothesized that ORF40 inhibits individual UPR pathways and the global UPR through interacting with, and stabilizing the GRP78/sensor proteins complex in the plane of the ER membrane. To test this hypothesis, we co-expressed FLAG-tagged ORF40 plasmid with Myc-tagged PERK or IRE1 $\alpha$  plasmids respectively in 293T cells and immunoprecipitated the cell lysates using FLAG- or Myc-antibody conjugated beads. Intriguingly, ORF40 interacts with IRE1 $\alpha$  (Figure 5-3B, upper panel) and with PERK (lower panel). Also as expected, ORF40, IRE1 $\alpha$ , and PERK interact with GRP78. With these results, we propose that ORF40 binds to and sustains the inactive form of GRP78/ IRE1 $\alpha$ , GRP78/PERK complexes inside the ER lumen, and consequently suppresses the induction of cellular UPR.

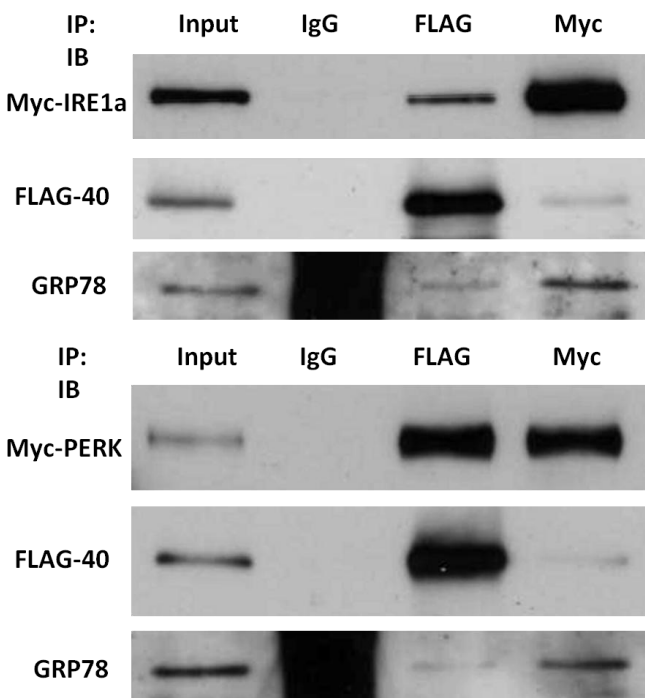
### **M1 interferes with the anti-UPR function of ORF40 at late stages of infection**

In Figure 3-3, we showed that cellular UPR was differentially regulated during MHV-68 infection: the UPR marker GRP78 was initially downregulated from 3 to 12 hours post infection and became upregulated at later time points. We hypothesize that the viral suppressor (ORF40) of UPR play the inhibitory role at early stages of infection, whereas at the late stages of infection,

A



B



**Figure 5-3. ORF40 interacts with GRP78, IRE1 and PERK.** (A) 293T cells were transfected with FLAG-ORF40 expression plasmid and were harvest 24 hours post transfection for immunoprecipitation analysis using beads conjugated with FLAG, KDEL or non-specific IgG. Western blot was performed using antibodies specific for KDEL. (B) 293T cells were transfected with FLAG-ORF40 and Myc-IRE1a (upper panel) or Myc-PERK (lower panel) expression plasmids. Immunoprecipitation was performed using FLAG, Myc or IgG conjugated beads and western blot was performed using antibodies against Myc and FLAG epitope tags, and GRP78.

the inducer (M1) takes over and acts to enhance chaperone production as demonstrated in Chapter 4.

To investigate this possibility, we set out to determine the expression kinetics of ORF40 and M1 protein during MHV-68 infection. We constructed a recombinant MHV-68 with an HA tag on the C terminus of the M1 gene and a FLAG tag on the N terminus of ORF40. This arrangement allows simultaneous detection of the protein expression of the two genes. As shown in Figure 5-4A, ORF40 was induced as early as 4 hours post infection and its expression was maintained at a moderately high level thereafter. Whereas M1 was induced at later hours, and became detectable around 8 hours post infection. The expression of M1 peaked between 12- to 24-hour time points. Similar to what has been shown in Figure 4-5, the expression kinetics of M1 approximated that of the capsid proteins ORF26 and M9, both of which have been identified as the late genes of MHV-68. Phosphonoacetic Acid (PAA) specifically inhibits viral DNA polymerase [20], and has been routinely used to distinguish the early and late genes in the herpesviruses [21]. Treatment of PPA significantly reduced the expression levels of M1 at 24-hour post infection but had minimal impact on ORF40 expression, suggesting that M1 is a late gene of MHV-68 while ORF40 is an early gene.

In Chapter 4, we demonstrated M1 a robust inducer of the host UPR. We observed strong induction of the UPR reporter even with a small amount of M1 protein. However, despite the fact that M1 expression was induced at 8 hours post infection (Figure 5-4A), the GRP78 expression level was not affected (Figure 3-3). This led to our assumption that at the early stage of virus infection, when M1 protein is not abundantly expressed, ORF40 can negatively modulate the effect of M1 in inducing cellular UPR. To test this hypothesis, we co-transfected ORF40 with increasing amount of M1 in 293T cells containing the GRP78-fluc reporter construct. As shown

in Figure 5-4B, a low dose of M1 had minimal effect on the anti-UPR function of ORF40; whereas an increased dose of M1 overrides the inhibition and significantly upregulated the reporter activity.

The above findings suggest that during MHV-68 infection, the helicase-primase components are expressed at earlier time and function to inhibit UPR induction; whereas M1 is induced at later stage of infection, and functions as a UPR inducer to upregulate chaperone gene expression.

### **The inhibitory effect of the helicase-primase components of MHV-68 is conserved**

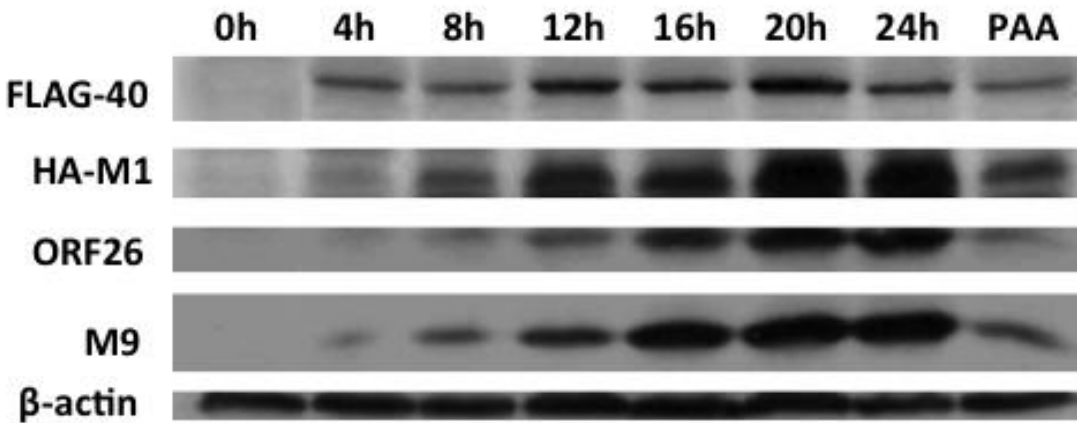
The MHV-68 possesses three helicase-primase components: ORF40, ORF44 and ORF56 [13].

We also examined the effect of ORF44 and ORF56 on cellular UPR. In the GRP78-fluc reporter system, both ORF44 and ORF56 significantly inhibited the reporter activity to more than 50% that of the vector control (Figure 5-5A), indicating that the three helicase-primase components of MHV-68 have similar effects on host UPR.

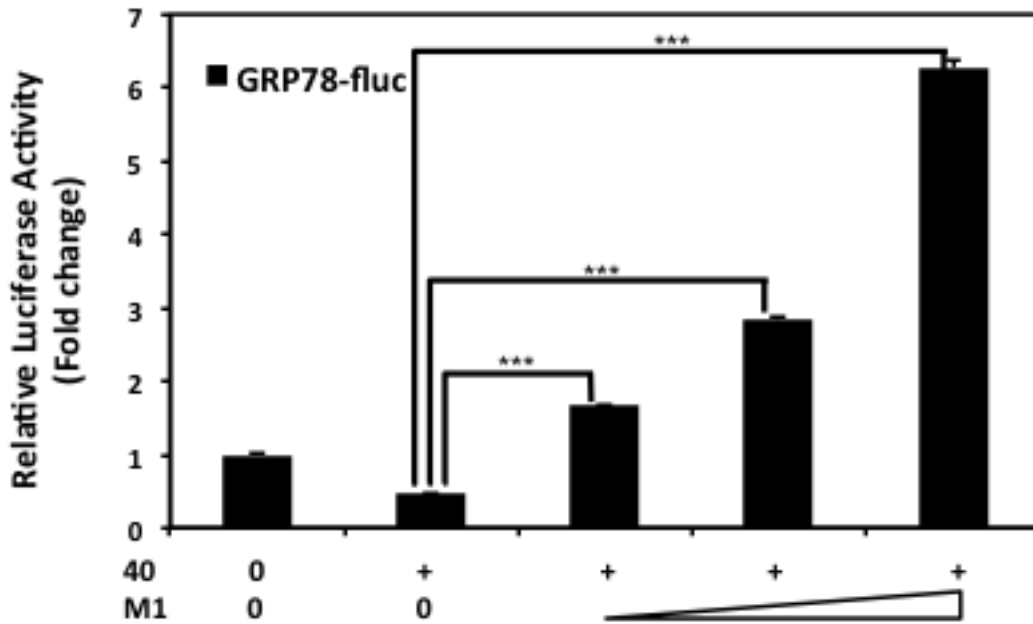
Counterparts of each of the three helicase-primase components have been identified in other herpesviruses. Sequence analysis has shown that MHV-68 ORF44 bears the highest sequence identity with the corresponding genes in other herpesviral genomes [22]. With that in mind, we assessed whether the ORF44 equivalents in KSHV and HCMV also have an impact on cellular UPR. Interestingly, ORF44 of KSHV and UL105 of HCMV ( $\beta$ -herpesvirus) strongly downregulated the GRP78 promoter activity (Figure 5-5B). This finding suggests that the anti-UPR function of the helicase-primase components may be a conservative function among the different herpesviruses.



A

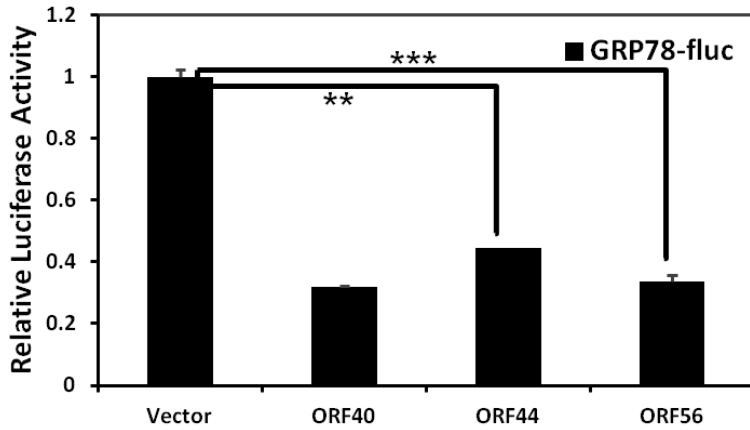


B

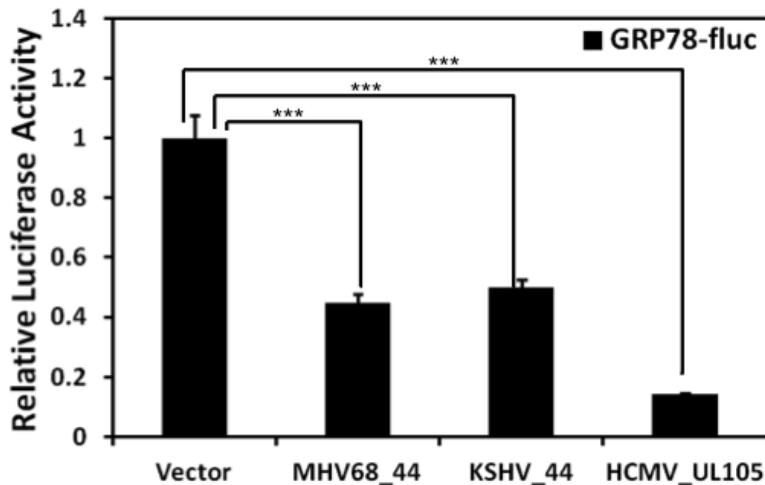


**Figure 5-4. M1 interferes with the anti-UPR function of ORF40.** (A) 3T3 cells were infected with the recombinant M1cHA40FLAG-MHV-68 at MOI 10, and harvested at indicated time points for western blot analysis using antibodies specific for FLAG (ORF40), HA (M1), ORF26, M9 and  $\beta$ -actin. (B) 293T cells were transfected with GRP78-fluc, PGK-RL and indicated combination of ORF40 and M1-expressing plasmids. Cell lysates were harvested for dual luciferase analysis 24 hours post transfection.

**A**



**B**



**Figure 5-5. The function of MHV-68 helicase-primase components in inhibiting UPR is conserved in other herpesviruses. (A)** 293T cells were transfected with GRP78-fluc, PGK\_RL, and ORF40, ORF44, ORF56 expression plasmids or a vector control. The reporter activity was measured by dual-luciferase assay 24 hours post transfection. **(B)** 293T cells were transfected with GRP78-fluc, MHV-68 ORF44, KSHV ORF44, HCMV UL105 expression plasmids or a vector control and analyzed by dual-luciferase assay 24 hours post transfection.

## DISCUSSION

Manipulation of the host UPR by different viruses has been well documented in the literature. The non-beneficial downstream aspects of UPR, such as inhibition of translation initiation, induction of apoptotic cascades and ER-associated degradation, have been proposed to be mediated by the virus at the initiation stage of infection to allow the proceeding of viral replication cycle [9, 23-25]. In this study, we demonstrated that ORF40 of MHV-68 is capable of inhibiting cellular UPR at the early stages of the infection cycle. In both the normal and ER-stressed cells, ORF40 negatively modulates UPR through downregulating each UPR signaling pathway. As one of the essential ORFs of the viral DNA replication complex, ORF40 is expected to play the role inside the nucleus. However, previous studies have shown that in KSHV and EBV, the components of the viral DNA replication complex mostly reside in the cytoplasm before nuclear translocation at a specific time [26, 27]. With this in mind, we hypothesize that the cytoplasmic ORF40 has evolved an additional function as a modulator of cellular responses outside the nucleus.

The preliminary mechanistic studies showed that ORF40 interacts with GRP78 and the stress sensors IRE1 and PERK. We propose the ORF40 controls the initiation of UPR signaling through binding to and stabilizing the inactive complexes of GRP78/IRE1 and GRP78/PERK (maybe GRP78/ATF6 as well) at the plane of ER membrane (Figure 5-3). In order to prove the hypothesis in the context of virus replication and in *in vivo* assays, it would be critical to determine the putative binding site(s) in the ORF40 protein. Because ORF40 is an essential gene of MHV-68, identifying the binding sites would allow the disruption of the anti-UPR function of ORF40 while preserving the helicase-primase activity through introducing point mutations into the viral genome.

In Chapter 4, we showed that MHV-68 possesses a strong UPR inducer: M1. It seems contradictory for the virus to carry both an inducer and an inhibitor to modulate host UPR. However, as shown in Figure 5-4A, ORF40 and M1 have very different expression kinetics during MHV-68 replication. ORF40, a member of the viral DNA replication complex, is induced at the very early stage of the viral life cycle, and its moderate expression level is sustained thereafter; whereas M1 is recognized as a late gene whose protein expression peaks along with the other late genes, including genes that encode most viral package proteins whose expression requires an increased protein folding capacity of the host cell. Considering the different expression patterns of ORF40 and M1 at different stages of virus replication, it explains the dynamic modulation of MHV-68 infection on UPR during its infection cycle depicted in Chapter 3. Although ORF40 functions as a strong inhibitor of cellular UPR and its expression is sustained throughout the viral life cycle, M1 is capable of overriding the anti-UPR activity of ORF40 later on when it becomes abundantly expressed and selectively activate the chaperone-producing pathways (Figure 5-4B). It would be interesting to reveal the mechanisms of how M1 takes over the negative control of ORF40 on cellular UPR; for instance, does M1 function through disrupting the interactions between ORF40 and the chaperone/sensor complexes?

Finally, we show that the anti-UPR function of ORF40 is conserved in the other two helicase-primase components of MHV-68 (ORF44 and ORF56). We also show that some of the helicase-primase counterparts in the other herpesviruses carry similar inhibitory function (Figure 5-5). These findings suggest that the examined herpesviral helicase-primase factors also have evolved additional functions to protect viruses from the deleterious effects of cellular. It is not surprising that many of the viral genes possess multiple functions, because with a compact genome, it is necessary for the virus to maximize the functionality of each viral gene available.

Still, whether the viral factors of KSHV and HCMV place the effect through a similar mechanism remains to be determined.

In summary, our data demonstrate that one of the helicase-primase components of MHV-68 can function as a UPR inhibitor at the early stage of virus replication, and it functions through stabilizing the inactive complexes of GRP78 and the stress sensors. This finding and the findings by other groups [8, 9, 11, 23-25, 28-36] suggest that proper modulation of the cellular UPR and is an important determinant for viral life cycle. This study also implicates that it is crucial to characterize the regulation of cellular responses at different stages of viral replication in order to gain a better understanding of the virus-host interactions during infection.

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CHAPTER 6  
SUMMARY AND PERSPECTIVES

## SUMMARY

Gammaherpesviruses persist in life-long infections through establishing a harmonious relationship with their natural hosts. A delicate balance between the viral replication and host responses must be achieved for successful infection. This dissertation examines host-pathogen interactions that ensure efficient infection of MHV-68, a model gammaherpesvirus for the human gammaherpesviruses KSHV and EBV. Specifically, we studied the interaction between MHV-68 and a cellular response termed the unfolded protein response (UPR). The first part (Chapter 3) of the thesis investigates the interaction between MHV-68 infection and host UPR, while the second part (Chapter 4 and 5) characterized two viral genes that play distinct roles in regulating the host UPR at different stages of MHV-68 replication. These studies have significant implications regarding our understanding of virus and host interactions during gammaherpesvirus infection.

Chapter 3 describes the mutual influences between the cellular UPR and MHV-68 replication. We found that chemical-induced UPR can significantly boost the production of infectious MHV-68. Further investigation revealed that enhancing the IRE1 $\alpha$  or ATF6 signaling alone can lead to a vastly increased production of infectious virions; on the other hand, when activating the PERK signaling, which attenuates global protein synthesis, has no obvious effect on viral replication. We also investigated the impact of MHV-68 replication on host UPR. Interestingly, infection of MHV-68 at the early stage results in an inhibited UPR, whereas during the time of viral assembly, UPR becomes strongly upregulated. These findings suggest that the cellular UPR plays different roles and is differentially regulated during the course of MHV-68 infection.

Chapter 4 presents the results of an unbiased screen performed to identify viral mediators of the cellular UPR. Further characterization of one such viral protein, M1, was conducted to understand its molecular mechanism in regulating the UPR. M1, a non-essential gene specific to MHV-68, strongly enhances the ER chaperone production and induces ER expansion. Further investigation demonstrated that the M1 protein localizes to the ER during MHV-68 infection, and it functions through selective activating of the IRE1 $\alpha$  and ATF6 pathways to promote chaperone production, while sparing PERK pathway which blocks translation. M1 is the first viral factor identified to date that is capable of selectively intervening the initiation of ER stress and skewing the signaling toward the beneficial aspects of UPR. This finding also provides a unique opportunity of using viral protein as a tool to define the activation mechanisms of individual UPR pathways.

Chapter 5 reports the characterization of viral genes with anti-UPR activity. ORF40, one of the helicase-primase components of MHV-68, was identified to inhibit host UPR at the early stage of virus infection. ORF40 strongly downregulates each UPR signaling arms and limit the production of ER chaperone GRP78/GRP94 in both normal and stressed cells. Evidence was provided that ORF40 may function through interacting with and stabilizing the inactive complexes of GRP78 and UPR initiators. Further investigation shows that M1 can overcome the anti-UPR function of ORF40 at the late stages of viral replication. Lastly, we show that anti-UPR function of ORF40 is conserved in the other two helicase-primase components of MHV-68 (ORF44 and ORF56) and among a few other herpesviruses assessed, suggesting a conserved effect in the herpesviral family.

## PERSPECTIVES

The above work led to interesting observations about the dynamic and ever-changing virus-host interactions at different stages of infection. It was unexpected that MHV-68 installs multiple genes in its genome that play opposite roles towards the same host response. Despite further studies demonstrated that these genes functions at different stages of the viral life cycle, it is surprising that how delicate the virus operates and coordinates the activity of different genes to satisfy its needs. These results once again underscore the importance and complexity of virus-host interactions during gammaherpesvirus infection, a lot of which remain to be decoded.

To elucidate the many uncovered interactions can be a daunting task if taking the traditional approach, i.e. studying one or two cellular/viral factors at a time. In such case, our group along with other groups has conducted various large-scale screens, including yeast-two-hybrid [1], cDNA [2], siRNA, kinase [3], and transcription factor library screens, to identify cellular and viral regulators that are involved in the infection cycle of gammaherpesvirus. These high-throughput methods are extremely powerful and have provided valuable insights into the virus-host interactions during gammaherpesvirus replication. However, one major concern of these screens is that all of the analyses measure the “averaged” readouts across the infected and uninfected cell populations. Considering the dynamics and variability of the viral infection cycle, significant biology could be masked by such averaging. We thus sought to employ a rapid, multiparametric approach to study virus-host interaction at the single-cell level.

One applicable technique is the phospho-specific flow cytometry (or Phospho-flow) that has emerged recently as a novel approach to track signaling events in individual cells [4-6]. Phospho-flow measures phosphorylation events by flow cytometry using fluorophore-labeled antibodies that target specific phospho-epitopes. This form of 'single-cell proteomics' can

identify cellular signaling pathways that become activated or inhibited in the infected cells; it can also quantitatively measure the changes of the viral protein expression in altered cellular environments at different stages of infection; more importantly, results from these analyses can be integrated together to create a network map of signaling events that are important for viral infection, and further to link specific signaling profiles with the outcomes of viral infection. We have applied this technique in a number of biological systems including the KSHV reactivation system. We used 5-drug combinations (Bortezomib, db-cAMP, Prostratin, Valproate, and Dexamethasone) as stimuli to induce virus reactivation and performed Phospho-flow analysis to quantitatively measure the activation status of various signaling proteins, and correlate that to the levels of KSHV reactivation that was also quantified by flow cytometry. Significant correlation was observed between signaling pathways and viral reactivation that are previously known, suggesting that the approach is accurate and applicable.

Although still at the exploratory stage of this new technology, we are confident that with the increasing reservoir of phospho-specific antibodies, advancing of the technology (e.g. Cytof, single-cell mass cytometry [7]) and computational analysis, we will be able to accelerate the process of discovering new virus-host interactions. The enrichment of the interaction network that connects various cellular and viral machineries would add tremendous understanding to the gammaherpesvirus persistent infection and help developing therapeutic strategies against the virus-associated diseases.

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