

Characterizing Cellular Responses During Oncolytic Maraba Virus Infection

by

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Abstract

The rising demand for powerful oncolytic virotherapy agents has led to the identification of Maraba virus, one of the most potent oncolytic viruses from *Rhabdoviridae* family which displays high selectivity for killing malignant cells and low cytotoxicity in normal cells. Although the virus is readied to be used for clinical trials, the interactions between the virus and the host cells is still unclear. Using a newly developed interferon-sensitive mutant Maraba virus (MG1), we have identified two key regulators of global translation (4E-BP1 and eIF2 α) responsible for the inhibition of protein synthesis in the infected cells. Despite the translational arrest upon viral stress, we showed an up-regulation of anti-apoptotic Bcl-xL protein that provides a survival benefit for the host cell, yet facilitates effective viral propagation. Given the fact that eIF5B canonically regulates 60S ribosome subunit end joining, and is able to replace the role of eIF2 in delivering initiator tRNA to the 40S ribosome subunit upon the phosphorylation of eIF2 α , we have tested whether eIF5B mediates the translation of target mRNAs during MG1 infection. Our results show that the inhibition of eIF5B significantly down-regulates the level of Bcl-xL steady-state mRNA, thus indirectly attenuates viral propagation.

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To Mom, Dad, and Behnoush whom without their incredible sacrifice and unconditional love, I would never be able to pursue my dreams.

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Table of Contents

List of Abbreviations	vii
List of Figures	xi
Chapter 1: Introduction	1
1.1 Maraba virus	1
1.1.1 Clinical implications	4
1.2 Virus and Translation	5
1.2.1 Regulation of translation initiation	6
1.3 Eukaryotic Initiation Factor 4E (eIF4E)	7
1.3.1 4E-binding proteins (4E-BPs)	7
1.4 Eukaryotic Initiation Factor 2 (eIF2)	8
1.4.1 Regulation of eIF2 α phosphorylation	9
1.5 Stress granule formation	9
1.6 Selective translation mechanism	11
1.6.1 B-cell lymphoma-extra Large (Bcl-xL)	11
1.6.2 X-linked inhibitor of apoptosis (XIAP)	14
1.7 Eukaryotic Initiation Factor 5B (eIF5B)	16
1.8 Rationale and hypothesis	17
Chapter 2: Materials and Methods	19
2.1 Cell culture, expression constructs and transfection	19
2.2 Protein extraction and Western blot analysis	20
2.3 Pulse [³⁵ S]-Methionine labeling	21
2.4 Immunofluorescence and Confocal microscopy	21
2.5 RNA extraction and RT-qPCR	22
2.6 Kinetic cell imaging	24
2.7 Statistical analysis	24
Chapter 3: Results	25
3.1 Host protein synthesis is inhibited during MG1 infection.	25
3.2 MG1 modulates the host translation machinery through eIF2 and 4E-BP1 pathways.	27
3.3 Phosphorylation of eIF2 α plays a significant role in the inhibition of host and MG1 translation.	31
3.4 Phosphorylation status of eIF2 α only affects MG1 propagation but not Bcl-xL and XIAP protein expression.	34
3.5 Stress granule formation does not regulate MG1 propagation in the host cells.	37
3.6 eIF5B is involved in the expression of Bcl-xL, XIAP and MG1 at different levels.	40

3.7 eIF5B knock-down is not involved in MG1 mRNA translation but significantly affect the rate of infection.	44
Chapter 4: Discussion	47
Conclusion	56
References	57
Licenses	69

List of Abbreviations

4E-BP	eIF4E binding protein
Akt	also called, Protein kinase B (PKB)
ATF4	Activating Transcription Factor 4
AUG	Adenine/Uridine/Guanine (start codon)
Bak	BCL2 antagonist/killer
Bax	BCL2 associated X protein
BCA	Bicinchoninic acid assay
Bcl-2	B-cell CLL/ lymphoma 2
Bcl-xL	B-cell lymphoma extra large
Bcl-xS	B-cell lymphoma extra short
BH	Bcl-2 homology domain
BHK	Baby hamster kidney fibroblasts
BiP	Binding immunoglobulin protein
BIR	Baculovirus IAP repeat
cDNA	Complementary deoxyribonucleic acid
CHOP	CCAAT/enhancer binding protein homologous protein
cIAP1	cellular inhibitor of apoptosis 1
cIAP2	cellular inhibitor of apoptosis 2
c-Myc	Cellular-myelocytomatosis oncogene
CSFV	Classical swine fever virus
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dsRBD	double-stranded RNA binding domain
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G3BP	GTPase-activating protein-binding protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN2	General control non-derepressible-2
GFP	Green fluorescence protein
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
HCV	Hepatitis C virus
hnRNP A1	Heterogeneous nuclear ribonucleoprotein A1
Hoechst	Trihydrochloride trihydrate
Hpi	Hour-post infection
HRI	Heme-regulated inhibitor
HRP	Horseradish peroxidase
HuR	Human antigen R
IRES	Internal ribosome entry site
IRF1	Interferon regulatory factor 1
ITAF	IRES trans-acting factor
JNK	c-Jun N-terminal kinase
kDa	kilodalton
MEF	Mouse embryonic fibroblast
Met	Methionine
MG1	Interferon-sensitive mutant strain of Maraba virus
MNK1/2	MAPK interacting Ser/Thr kinase 1/2
MOI	Multiplicity of infection
MOMP	Mitochondrial Outer Membrane Permeability
mRNA	Messenger ribonucleic acid

mTOR	mammalian target of rapamycin
NF- κ B	Nuclear factor- κ B
Ns	Non-significant
ORF	Open reading frame
OV	Oncolytic viruses/vaccine
PABP	PolyA-binding protein
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PCBP2	Poly(rC)-binding protein 2
PDCD4	Programmed Death Cell Protein 4
PERK	PKR-like endoplasmic reticulum kinase
P _i	inorganic Phosphate
PI3K	Phosphatidylinositide 3-kinase
PKR	Protein kinase RNA-activated
PPIA	Peptidylprolyl Isomerase A
PVDF	Polyvinylidene difluoride
RBP	RNA binding protein
RdRp	RNA-dependent RNA polymerase
RIPA	Radioimmunoprecipitation Assay
RING	Really interesting new gene
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPL13A	Ribosomal protein large subunit 13A
RPL36A	Ribosomal protein large subunit 36A
RT-qPCR	Reverse Transcription Quantitative PCR
S51A	Phosphor-null eIF2 α mutant at Serine 51
S6K1	Ribosomal protein S6 kinase 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Stress granule
siRNA	small interfering RNA

Smac	Second mitochondria-derived activator of caspase
TIA-1	T-Cell-Restricted Intracellular Antigen-1
TIAR	TIA1 cytotoxic granule-associated RBP-like 1
Tris-Cl	Tris-base/hydrogen chloride
TRITC	Tetramethylrhodamine
tRNA	transfer RNA
TEMED	Tetramethylethylenediamine
TRITC	Tetramethylrhodamine isothiocyanate
U2OS	human osteosarcoma cell line
U343	human glioma cell line
UPR	Unfolded protein response
UTR	Untranslated region
UV	Ultraviolet
VSV	Vesicular stomatitis virus
V/V%	Volume/Volume%
W/V%	Weight/Volume%
WT	Wild type
XIAP	X-chromosome linked inhibitor of apoptosis protein

List of Figures

Figure 1: The phylogenetic tree of <i>Rhabdoviridae</i> family	2
Figure 2: The mechanisms of cap-dependent and IRES-mediated translation initiation	12
Figure 3: The effect of MG1 infection on the rate of host protein synthesis	26
Figure 4: MG1 uses eIF2 α and 4EBP1 to control the translation mechanism of their host	29
Figure 5: Impact of eIF2 α phosphorylation on the rate of host and MG1 protein synthesis	32
Figure 6: The effect of eIF2 α phosphorylation on host and viral protein synthesis during MG1 infection	35
Figure 7: The induction of stress granules by MG1 virus does not noticeably repress viral propagation	38
Figure 8: eIF5B knock-down negatively controls the expression of Bcl-xL and MG1	42
Figure 9: eIF5B knock-down does not regulate MG1 mRNA translation but affect the rate of infection and cytotoxicity	45

Chapter 1: Introduction

1.1 Maraba virus

Maraba virus is a negative single-stranded RNA virus from *Rhabdoviridae* family, primarily collected from Phlebotamin sand flies (*Lutzomyia* spp.) in the Amazon basin of Brazil (Travassos da Rosa, AP., et al., 1984). *Rhabdoviridae* family consists of more than 250 viruses which are divided into six large genera. Although these viruses are highly similar, they are very different in terms of pathogenicity, life cycle, and host range (Fauquet, CM., et al., 2005; Brun, J., et al., 2010). According to the standard serological tests such as complement-fixation, Maraba virus was expected to be a vesiculovirus (Travassos da Rosa, AP., et al., 1984). Indeed, further studies using “shot gun” sequencing technique and phylogenetic analysis by amino acid alignment of Maraba L protein to the members of *Rhabdoviridae* family categorized Maraba virus as a vesiculovirus and confirmed its close relativity to Vesicular Stomatitis Virus, VSV (Brun, J., et al., 2010).

Based on the genomic structure of Maraba virus available at National Centre for Biotechnology Information, its genome sequence contains five distinct cistrons with specific transcriptional stop/start codons for each gene (N, P, M, G and L gene) (Brun, J., et al., 2010; Travassos da Rosa, AP., et al., 1984). The L polymerase protein and Phospho-protein, as the co-factor, form the viral RNA-dependent RNA polymerase (RdRp). These two proteins in association with the Nucleocapsid protein which encapsidates the viral RNA genome, assemble the viral ribonucleoprotein (RNP) complex. The transmembrane Glycoprotein residing in the viral envelope is involved in the receptor binding and cell entry (Li, J., et al., 2013).

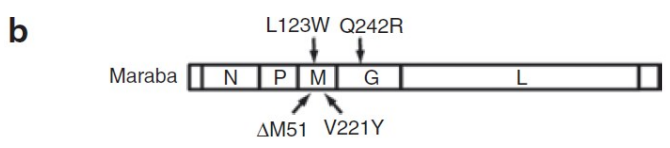
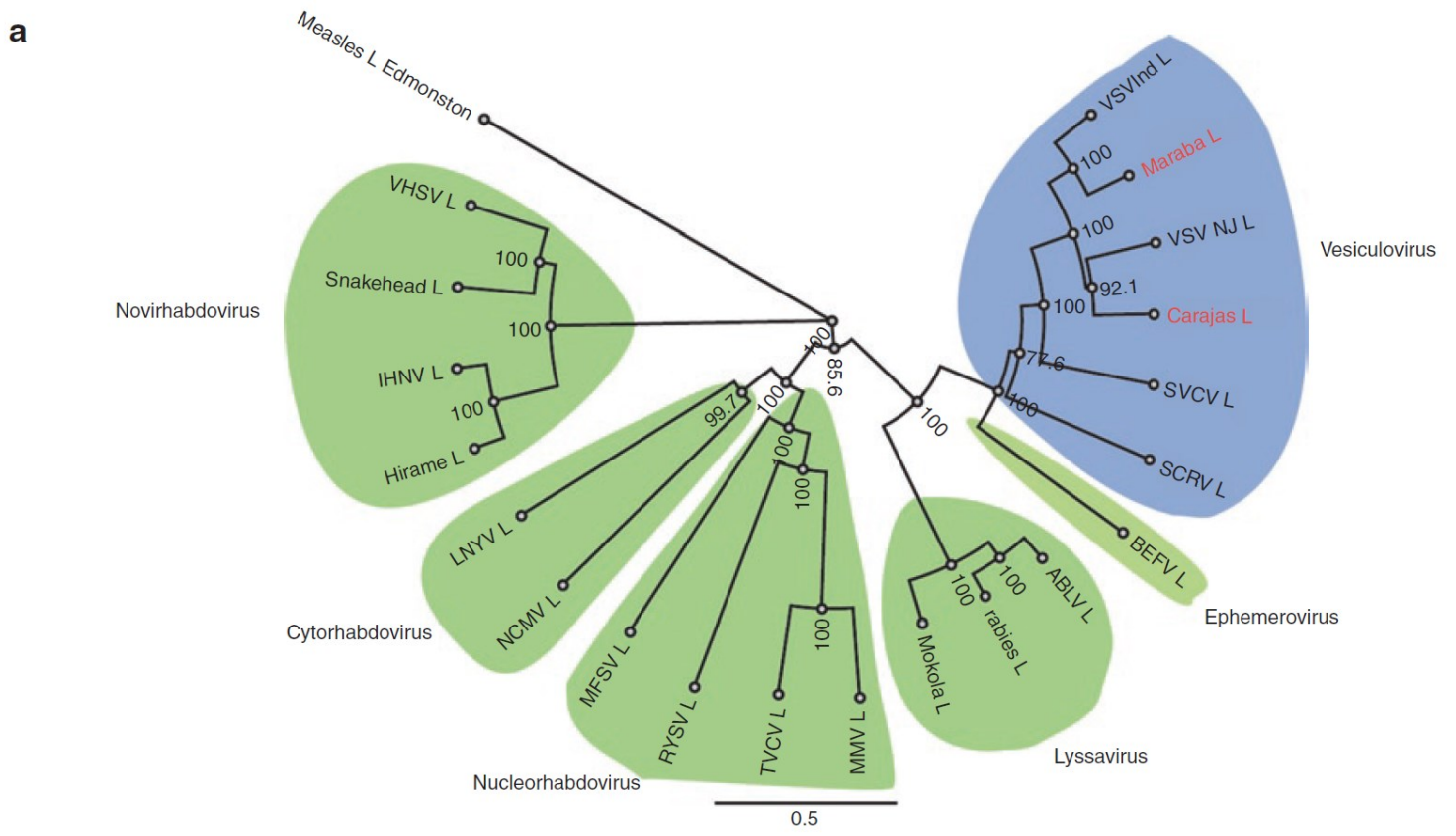


Figure 1: The phylogenetic tree of *Rhabdoviridae* family (a) Rhabdovirus family is divided into six genera and includes more than 250 identified viruses. Following the amino acid sequencing of Maraba Large polymerase protein, Maraba virus is classified as a species in the genus of vesiculovirus which confirms its close similarity to Vesicular Stomatitis virus (VSV). The length of the horizontal lines represents the amount of genetic changes over time. (b) Among several mutations introduced into the backbone of wild-type Maraba virus genome, L123W and Q242R point mutations in M and G proteins, termed as MG1, were found to significantly enhance the replication rate and tumor-targeting ability of the virus. Reprinted with permission from Nature Publishing Group Ltd: Identification of Genetically Modified Maraba Virus as an Oncolytic Rhabdovirus, *Molecular Therapy*, 2010, volume 18, no. 8, page 1440-1449 (Brun, J., et al., 2010).

Similar to other Rhabdoviruses, Maraba virus has a small bullet-shaped virion and rapid replication in the mammalian cells. Their replication cycle also occurs within the cytoplasm of their host cells, thus eliminates the possibility of genotoxicity (Brun, J., et al., 2010). Nonetheless, the exact replication mechanism of Maraba virus is still unclear and requires further investigation.

1.1.1 Clinical Implications

The promising therapeutic properties of Oncolytic Viruses (OV) for killing different types of cancer cells have turned into a higher demand for more potent OV platforms. Due to the rare contribution of Rhabdoviruses to any diseases in humans, as well as the lack of pre-existing antibodies and humoral immunity against them in human population, they have been characterized as suitable candidates for Oncolytic vaccines (Power, AT., et al., 2007; Brun, J., et al., 2010). Therefore, the great safety profiles of this family led to the identification of new vesiculoviruses with higher tumor selectivity (Mahoney, DJ., et al., 2011; Pol, JG., et al., 2014).

Previous studies on the administration of VSV as an oncolytic vaccine for cancer immunotherapy showed promising outcomes. Due to the antigenic similarity between Maraba virus and VSV (Zhang, J., et al., 2014; Koppers-Lalic, D., et al., 2011), the oncolytic potency and the immune response to the virus was evaluated in several recent studies. The findings claimed that Maraba virus displays effective cytolytic activity against human tumor lines and low virulence in normal cells (Brun, J., et al., 2010; Pol, JG., et al., 2014). In the next step, the backbone of wild-type Maraba virus was genetically modified by introducing the same mutations which has improved the replication and oncolytic potency of VSV. The equivalent mutations on VSV were found to be L123W and Q242R in the sequence of M and G proteins of Maraba virus (Brun, J., et al., 2010). It has been reported that the genetically modified Maraba virus, called MG1, demonstrated superior ability to enhance anti-tumor immunity and tumor-targeting properties than

attenuated VSV (Zhang, J., et al., 2014; Brun, J., et al., 2010). Taken together, the significant therapeutic efficacy of MG1 determined in pre-clinical studies has led to the administration of the virus in future clinical trials for the treatment of different forms of neoplasms. Of note, the world's first Maraba trial was conducted at The Ottawa Hospital.

1.2 Virus and Translation

Viruses rely on the host cellular machinery to complete their replication cycle. Although some viruses have sufficient components for the transcription of their genome, all of them are dependent on the protein synthesis machinery of their hosts for mRNA translation (Trask, SD., et al., 2012). Therefore, the regulation of various cellular processes particularly translation are challenged and modified during viral infection. These cellular responses are essential for both the virus to synthesize their own proteins and the host to focus on the production of anti-viral as well as pro- or anti-apoptotic proteins (Lyles, DS., et al., 2000).

On one hand, the shutdown of host's global translation is known as an anti-viral strategy for the infected cells to reduce the viral propagation. On the other hand, many viruses selectively repress the translation mechanism of the infected cells in order to employ an alternate mode of translation for the synthesis of their own proteins (Connor, JH., et al, 2005; Williams, BR., et al., 1999). Studies on the translational control during VSV infection demonstrated that the global translation of the host cells is rapidly repressed early after infection, thereby leading to the inhibition of viral protein synthesis in the later phase of infection (Connor, JH., et al., 2005).

In following section, the importance of translation regulation during the cellular stress is explained in more details.

1.2.1 Regulation of translation initiation

Global translation also known as cap-dependent translation is a complex cellular process which is mediated at three distinct steps: initiation, elongation, and termination (Kapp, LD., et al., 2004). The initiation of cap-dependent translation, as a rate-determining step, is of particular importance for maintaining cellular function under physiological and pathophysiological conditions. During cellular stress, key control points of translation initiation, components of eIF4F complex and the ternary complex, are highly modified to fine-tune the stress response (Raught, B., et al., 1999; Montero, H., et al., 2015; Kapp, LD., et al., 2004).

eIF4F complex consists of cap-binding protein eIF4E, helicase eIF4A and eIF4G which acts as a bridge between eIF4A and Poly(A)-binding protein (PABP) (Pestova, TV., et al., 2001; Gingras, AC., et al., 1999). The other complex mediating the initiation phase of cap-dependent translation is 43S pre-initiation complex assembled by 40S small ribosomal subunit, eIF3 and the ternary complex (Preiss, T., et al., 2003; Pestova, T.V., et al., 2000b).

It has been proposed that the formation of eIF4F complex is altered during viral infection to promote viral mRNA translation, one of the major viral targets regulating this process is cap-binding protein, eIF4E. Studies have shown some viruses modulate the function of this initiation factor through de-phosphorylation at Serine 209. This effect causes conformational changes in the protein leading to the translation inhibition (Pyronnet, S., et al., 2001; Mohr, I., 2006). One example of translation regulation via eIF4E is reported in cells infected with VSV where global translation is shut-down by the de-phosphorylation of eIF4E and 4E-binding protein 1 (Connor, JH., et al., 2002). Additionally, some viruses repress global translation of the host cell by targeting the ternary complex which consists of eIF2, GTP and Met-tRNA_i. It is known that inactivation of

eIF2 through phosphorylation of its α subunit leads to the inhibition of protein synthesis in host cells (Donnelly, N., et al., 2012).

1.3 Eukaryotic Initiation Factor 4E (eIF4E)

The proper formation of eIF4F complex is considered as one of the main check points for the translational control. It consists of the scaffolding protein eIF4G, the RNA helicase eIF4A, eIF4E which binds to the 5' m⁷GpppN cap structure of mRNAs and mediates the recruitment of 40S ribosomal subunit along with eIF4G (Preiss, T., et al., 2003; Gary, NK., et al., 1998). Therefore, the crucial role of eIF4E in mediating the initiation of global translation makes it a key target of several mechanisms affecting protein synthesis within the cell. There are three proposed mechanisms regulating the translation efficiency of mRNAs via eIF4E; phosphorylation of eIF4E at Serine 209 by the mitogen-activated kinases (Mnk1 and Mnk2) (Pyronnet, S., et al., 2001); the interactions between 4E-binding proteins and eIF4E (Gingras, AC., et al., 2001; Poulin, F., et al., 1998); and lastly overexpression of eIF4E by c-Myc (Raught, B., et al., 1999; Schmidt, EV., 2004; Carroll, M., et al., 2013).

1.3.1 4E-binding proteins (4E-BPs)

The interaction between eIF4E and eIF4G is regulated by a family of translational repressors, called 4E-binding proteins. The three members of this family are 4E-BP1, 4E-BP2, and 4E-BP3 with numerous phosphorylation sites mediating their activities within the cell. Studies showed that hypo-phosphorylation of these proteins enhances their affinity to bind to eIF4E, thus preventing its interaction with eIF4G (Poulin, F., et al., 1998; Nehdi, A., et al., 2014).

Among the three members of 4E-BP family, the function of 4E-BP1 has been characterized the best. It has been proposed that the activity of 4E-BP1 is directly modulated by an essential

regulator of translation, mTOR1. Mammalian target of rapamycin (mTOR) along with some other regulatory proteins act upstream of 4E-BP1, and control its phosphorylation status in response to various cellular stimuli. Therefore, mTOR-4E-BP-eIF4E axis is a central pathway for translational regulation, targeted by many viruses in the infected cells (Le Sage, V., et al., 2016).

1.4 Eukaryotic Initiation Factor 2 (eIF2)

One of the essential factors for the formation of the ternary complex is eIF2 consisting of three subunits α , β , γ (Koromilas, A., et al., 2015). It is responsible for the delivery of the initiator tRNA, Met-tRNA_i, to the P site of small ribosomal subunit in a GTP-dependent manner. In an effort to find the start codon of an mRNA, 43S pre-initiation complex binds near the 5' cap structure and moves in 3' direction to reach the first AUG codon, GTP bound to eIF2 is hydrolyzed to GDP and P_i, and the newly formed GDP.eIF2 complex is released from the 40S subunit respectively (Lorsch, JR., et al., 2010; Lorsch, JR., et al., 2010; Proud, CG., 2001). At this state eIF2 becomes inactive, thus the next round of initiation requires the replacement of GDP to GTP which is catalyzed by the guanine exchange activity of eIF2B (Proud, CG., 2001a; Koromilas, A., et al., 2015).

A substantial body of evidence shows that phosphorylation of α subunit of eIF2 at Serine 51 inactivates its function and turns the protein into a competitive inhibitor of eIF2B. Through this process, eIF2.GDP binds to eIF2B and forms an inactive eIF2-eIF2B complex which inhibits GDP recycling. Therefore, insufficient number of active ternary complexes becomes a limiting factor for the formation of 43S and results in the repression of translation initiation (Ron, D., et al., 2007, Holcik, M., 2015). Interestingly, some studies suggested despite the inhibitory effect of eIF2 α phosphorylation on cap-dependent translation, it can promote selective translation of certain mRNAs which are inadequately translated under normal physiological conditions (Holcik, M., et al., 2005; Blais, JD, et al., 2004).

1.4.1 Regulation of eIF2 α phosphorylation

Four serine-threonine kinases have been identified responsible for the phosphorylation of eIF2 α in response to distinct type of stresses. The activation of Heme-regulated inhibitor (HRI) is triggered by iron deficiency, heavy metals, cytoplasmic stresses, and heat shock (Ranu, RS., et al., 1979; Matts, RL., et al., 1991; Lu, L., et al., 2001; Holcik, M., 2015). The general control non-derepressible-2 (GCN2) is activated by uncharged tRNA following amino acid starvation, and exposure to UV radiation (Roussou, I., et al., 1988; Jiang, HY., et al., 2005). The RNA-dependent protein kinase PKR is activated following the recognition of double-stranded RNA which then induces interferon-mediated apoptosis during viral infection (Taylor, DR., et al., 1996; Koromilas, A., et al., 2015). Finally, PKR-like endoplasmic reticulum kinase (PERK) is activated upon the accumulation of misfolded proteins in the ER in order to restore ER homeostasis (Harding, HP., et al., 1999).

1.5 Stress granule formation

Stress granules are cytoplasmic foci composed of translationally-stalled mRNAs. Various environmental stresses have been observed to trigger the formation of these non-membranous structures in different cell lines. Even though the composition of stress granules is dependent on the nature and duration of the stress in a way to promote the cell survival, they primarily contain housekeeping genes from disassembled polysomes, initiation factors such as eIF4E, eIF3, eIF4A, eIF4G and small ribosomal subunits (Anderson, P., et al., 2006; Anderson, P., et al, 2002b).

Furthermore, it is speculated that stress granules are the site of mRNA-sorting for the purpose of storage, degradation and translation of the stalled mRNAs based on their integrity and composition (Anderson, P., et al, 2002a). Besides, the assembly of stress granules is an energy-

saving approach for the cell to reprogram the translation machinery and switch to the selective translation of specific mRNAs encoding heat-shock proteins and other stress-induced transcripts to better cope with the stress (Anderson, P., et al, 2002b; Harding, HP., et al., 2000).

There are many suggested pathways associated with the formation of stress granules in response to the environmental stresses (such as heat shock, viral infection, oxidative and hypertonic shock). In most cases, stress granules are formed when the translation initiation complex is formed in the absence of the ternary complex, eIF2-GTP-tRNA_i^{Met}. Subsequently, the eIF2/eIF5-deficient 48S pre-initiation complex co-localizes with RNA-binding proteins TIA-1 and TIAR, the key SG markers, to form the core component of stress granules (Kedersha et al., 2002; Anderson, P., et al, 2002b). Apart from the formation of SGs as a result of the eIF2 inactivation and subsequent depletion of small ribosomal subunit from the ternary complex, eIF4E is also able to regulate the assembly of stress granules through the de-phosphorylation of 4E-BP1 (Fournier, M., et al., 2012).

Formation of stress granules is considered as a cellular stress response to the infection with many viruses. In most cases, the transient inhibition of global translation as a result of SG induction can restrict viral propagation and promote selective translation of anti-viral transcripts. Many viruses have evolved mechanisms to prevent their mRNAs sequestered in the SGs by hindering their formation. For example, Herpes simplex virus blocks the formation of SGs (Dauber, B., et al., 2011), Poliovirus prevents SG formation by encoding 3C protease which cleaves a SG-nucleating protein called G3BP (White, JP., et al., 2007), West Nile virus traps TIA-1 and TIAR in their replication cycle and therefore represses SG assembly (Emara, M., et al., 2007; Lloyd, RE., 2012).

1.6 Selective translation mechanism

It is becoming obvious that targeting the translation machinery, as the final step of gene expression, is the most time-efficient approach for the cells to manage the stress and enhance their chance of survival (Holcik, M., et al., 2005). One strategy taken by cells is to limit their translation products to those protein having determinant roles in cell proliferation and/or apoptosis pathways. Many of these mRNAs can be translated in a cap-independent mechanism by harbouring an internal ribosome ending site (IRES) at their 5' UTR. The high GC content at their long 5' ends provides a complex secondary structure facilitating the selective recruitment of ribosome when the cap-dependent translation is disrupted (Holcik, M., et al., 2005; Hellen, CU., et al., 2001). Among the IRES containing mRNAs, Bcl-xL and XIAP are known to have key functions in cell recovery and inhibition of apoptosis (Holcik, M., 2015) which are further discussed in the following sections.

1.6.1 B-cell lymphoma-extra Large (Bcl-xL)

B-cell lymphoma-extra Large (Bcl-xL) is an anti-apoptotic protein from Bcl-2 family. Similar to other proteins of this family, Bcl-xL has four conserved α -helical motifs termed, Bcl-2 homology domains (BH1-BH4) (Oltvai, ZN., et al, 1993; Zhou, F., et al., 2010). It resides in the external membrane of mitochondria, peri-nuclear and ER membrane through its hydrophobic carboxy terminal domain (Sorenson, CM., 2003; Zhou, F., et al., 2010). Bcl-xL mRNA is encoded by *bcl-x* gene which has multiple transcription initiation sites and consensus motifs for different transcription factors. These characteristics facilitate differential expression of the gene according to different signal transductions, and provide a higher level of transcriptional control (Grad, JM., et al 2000).

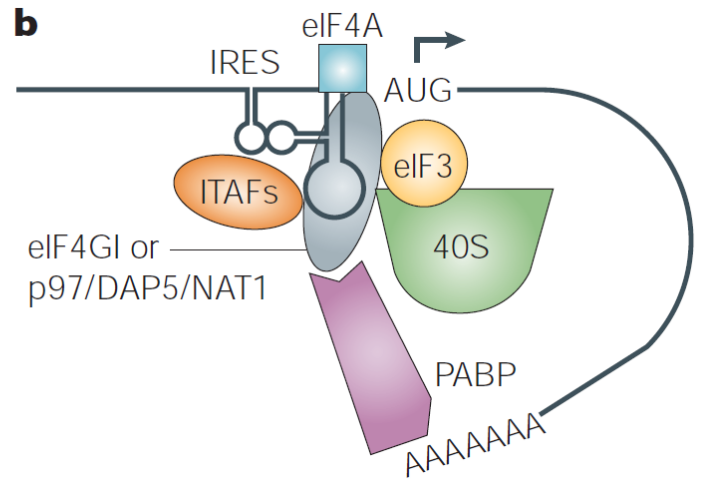
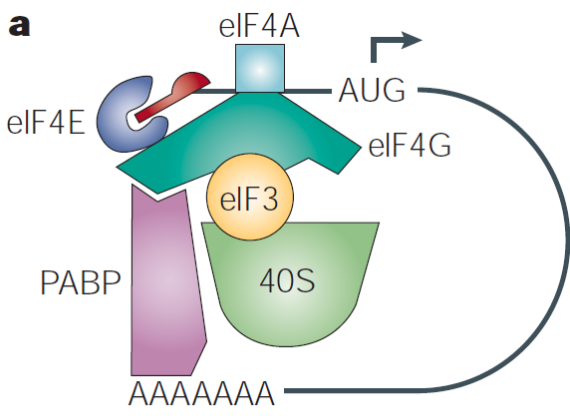


Figure 2: The mechanisms of cap-dependent and IRES-mediated translation initiation. (a) Under normal physiological condition, the selection of mRNAs undergoing translation is mediated via cap-dependent translation mechanism. The cap-binding protein eIF4E is responsible for the recognition of 5'GpppN- cap structure of mRNAs, which along with RNA helicase eIF4A and the scaffolding protein eIF4G form eIF4F complex. 40S ribosomal subunit is then recruited to mRNA through its interaction with eIF3 and eIF4G, and scans 5' UTR of mRNAs in the 5' to 3' direction in order to find AUG initiation codon. (b) During the course of cellular stress, the translation of certain mRNAs harboring IRES elements at their 5'UTR are promoted. This mode of protein synthesis, termed IRES-mediated translation does not rely on the binding of eIF4E to the 5' cap structure. In fact, the presence of IRES elements with the assistance of eIF4G facilitate the recruitment of 40S ribosome. Also, many ITAFs have been identified to regulate IRES activity by functioning as RNA chaperone. Reprinted with permission from Nature Publishing Group Ltd: Translational Control in Stress and Apoptosis, Nature Reviews Molecular Cell Biology, 2005, volume 6, page 318-327 (Holcik, M., and Sonenberg, N., 2005).

Two of the most distinct variants of *bcl-x* gene, Bcl-xL (233 amino acids) and Bcl-xS (170 amino acids) are the products of alternative splicing with opposing functions. It has been proposed that the deletion of BH1 and BH2 domains accounts for the pro-apoptotic function of Bcl-xS (Sorenson, CM., 2003). Besides, studies demonstrated that the regulation of Bcl-xL expression is not restricted only to the level of transcription and alternative splicing. In fact, Bcl-xL mRNA contains an IRES element at 5' UTR which controls its expression in the interactions with a number of RNA-binding proteins such as hnRNP A1, PDCD4 and HuR during stress conditions (Bevilacqua, E., et al., 2010; Durie, D., et al., 2013; Yoon, A., et al., 2006).

The main functions attributed to Bcl-xL is maintaining mitochondrial membrane integrity and preventing the activation of intrinsic pathway of apoptosis. In response to stress stimuli, two pro-apoptotic proteins from Bcl-2 family, Bak and Bax, translocate to the mitochondrial outer membrane from the cytosol, and increase the mitochondrial outer membrane permeability (MOMP) through their oligomerization. Consequently, Cytochrome C is released into the cytosol, and this event leads to the activation of apoptosis pathway. It has been reported that Bcl-xL represses this process by antagonizing Bak, and preventing its oligomerization in the mitochondrial membrane (Yi, CH., et al., 2011; Zhou, F., et al., 2010). Furthermore, recent studies revealed that Bcl-xL is also involved in the inhibition of autophagy by sequestering Beclin 1 which is an essential protein for the formation of autophagosome (Zhou, F., et al., 2010; Pattingre, S., et al., 2005). Therefore, Bcl-xL prevents autophagic-mediated death in cells under prolonged nutrient deprivation (Zhou, F., et al., 2010; Uchiyama, Y., et al., 2008).

1.6.2 X-linked inhibitor of apoptosis (XIAP)

The X-linked inhibitor of apoptosis protein is the key member of IAP family encoded by the xq25 region of the X-chromosome. Similar to the other seven members of this family characterized

by the presence of BIR (Baculoviral IAP Repeat) motifs (ranging from 1-3) in their structure, XIAP contains all three BIR domains at its N-terminus through which it directly binds to caspases. Also, the protein contains a C-terminal RING (Really Interesting New Gene) zinc finger domain with E3 ubiquitin ligase activity for the catalysis of caspases and ubiquitination (Holcik, M., 2003; Holcik, M., et al., 2001; Chaudhary, AK., et al., 2016).

XIAP is known to be ubiquitously expressed in all human tissues except in peripheral blood leukocytes. Therefore, the main functions of the protein are not tissue-specific including its roles in apoptosis, innate immunity, cell migration, invasion, metastasis, differentiation, and etc. (Liston, P., et al., 1996; Galban, S., et al., 2010; Prakash, H., et al., 2010). Originally, the role of XIAP was identified as the inhibitor of caspase 3- and 7- through BIR2 domain, and caspase 9 through BIR3 domain, thus it is able to block both the intrinsic and extrinsic caspase-dependent apoptotic cell death (Deveraux, QL., et al., 1998; Shiozaki, EN., et al., 2003; Chaudhary, AK., et al., 2016). XIAP also mediates the ubiquitination of caspase activator, Smac, as well as the initiator caspase 9, and executioner caspase 3 (Suzuki, Y., et al., 2001; Morizane, Y., et al., 2005). Besides, XIAP takes part in the innate immunity and inflammatory pathways by activating mitosis, c-Jun N-terminal kinase (JNK) and (NF)- κ B in response to Cytokines, bacterial or viral infection (Levkau, B., et al., 2001; Estornes, Y., et al., 2015; Bauler, LD., et al., 2008). On the contrary, during early mitochondrial depolarization the translocation of XIAP from the cytosol to the mitochondrial outer membrane enhances the release of Cytochrome C and Smac in a Bax/Bak-mediated way. This pro-apoptotic role of XIAP was found to be suppressed by Bcl-xL protein (Owens, TW., et al., 2010).

Due to the critical role of XIAP in mediating apoptotic cell death, its mRNA structure has evolved in a way to ensure a continuous or up-regulated translation upon the exposure of cells to stress stimuli when the cap-dependent translation is inhibited. Genomic organization analysis of

XIAP loci revealed a long 5' untranslated region containing an IRES element at the upstream of the AUG codon within a 162 nt region (Holcik, M., 2003; Holcik, M., et al., 1999). In addition, several RNA-binding proteins such as autoantigen La and hnRNP C1,2 are found to assemble on the IRES element of XIAP and mediate its cap-independent translation during cellular stresses (Holcik, M., et al., 2000; Holcik, M., et al., 2003).

1.7 Eukaryotic Initiation Factor 5B (eIF5B)

Eukaryotic Initiation Factor 5B is the ortholog of bacterial IF2 belonging to the family of guanine nucleotide-binding (G) protein (Bourne, HR., et al., 1991; Kuhle, B., et al., 2014). The protein is found to have a conserved function throughout the evolution which is its crucial role in the 60S subunit end joining and mediating the translation elongation step. The assembly of an elongation-competent 80S ribosome requires the GTPase activity of eIF5B to catalyze the 60S subunit joining (Roll-Mecak, A., et al., 2000; Pestova, TV., et al., 2000a; Kuhle, B., et al., 2014). The structure of eIF5B is composed of a GTP-binding domain at N-terminus, a β -barrel domain II along with domain III and IV, as well as an N-domain with sequence and length variability between different species (Eiler, D., et al., 2013; Roll-Mecak, A., et al., 2000; Simonetti, A., et al., 2013; Shin, BS., et al., 2002). In addition to its canonical function, eIF5B has been found to replace the role of eIF2 upon the phosphorylation of its α subunit during cellular stresses. It is reportedly responsible for the delivery of the initiator Met-tRNA to mediate the translation initiation on some viral and cellular IRES such as HCV, CSFV and XIAP (Terenin, IM., et al., 2008; Pestova, TV., et al., 2008; Thakor, N., et al., 2011).

1.8 Rationale and hypothesis

The newly-discovered Maraba virus has been characterized as a powerful oncolytic agent exhibiting efficient tumor-destructive abilities. Although the attenuated mutant Maraba, MG1, is prepared for clinical trials, the mechanisms of viral propagation and host anti-viral response need further elucidation. Throughout this study, we addressed the role of various host factors and the mechanisms of cellular responses during VSV infection to understand the interplay between Maraba virus and their host cells.

In previous studies, two phases for the translational control during VSV infection were proposed. First, 4E-BP1 is being dephosphorylated and its interaction with eIF4E leads to the shut-down of the host global translation (Montero, H., et al., 2015, Connor, JH., et al, 2005). However, eIF2 is yet unaffected, so that viral transcript is efficiently translated. During late phase of infection, viral protein synthesis is inhibited by the phosphorylation of α subunit of eIF2 (Connor, JH., et al, 2005). Since Maraba virus is a vesiculovirus and close relative of VSV, I speculate that it may employ similar mechanisms to take over the translation machinery of their host cells.

Performing Ribosome profiling on U343 cells infected with WT and MG1 Maraba virus, our collaborators in Dr. Alain's lab have identified a cohort of IRES harbouring mRNAs under selective translation and transcription during Maraba infection. Among the candidate genes, their data suggest selective translation of anti-apoptotic protein, Bcl-xL during the viral stress which had been previously shown to be regulated by cap-independent translation during other types of cellular stress conditions (Thakor, N., et al., 2012, Durie, D., et al., 2013). I therefore hypothesize that ***“MG1 uses eIF2 α and/or 4E-BP1 pathways to control the translation mechanism of their hosts, and recruits an alternative translation strategy to promote its propagation”*** My goal is

to validate that the alternation in Bcl-xL expression is regulated at translational level, and dissect the underlying mechanism(s) of translation inhibition in response to MG1 infection.

Objectives:

1. Investigate the control of global translation during Maraba virus infection
2. Determine the mechanism of selective translation of anti-apoptotic protein Bcl-xL during viral infection
3. Characterize the mechanism of MG1 propagation within the host cells

Chapter 2: Materials and Methods

2.1 Cell culture, expression constructs and transfection

U2OS cells were grown at 37°C in 5% CO₂ in HyClone™ High-Glucose Dulbecco's Modified Eagle's Medium (Thermo Scientific) supplemented with 1% L-Glutamine, 100,000 U/L Penicillin, 100 µg/L Streptomycin and 10% Heat-inactivated Fetal Bovine Serum. Mouse embryonic fibroblasts (MEFs), phosphor-null eIF2α (S51A) and WT MEFs, were a gift from Dr. Maria Hatzoglou (Departments of Nutrition, Pathology, and Biochemistry, Case Western University School of Medicine, Cleveland, Ohio) and were also cultured in standard condition in serum-, antibiotic- and L-Glutamine- supplemented DMEM at 37°C in 5% CO₂.

Transient knock-down experiments were performed using RNAiMax reagent (Invitrogen, Burlington, ON, Canada) as per manufacturer's protocol, siRNA targeting EIF5B (Stealth RNAi™ siRNAs, Cat#1299001, Invitrogen), and negative control siRNA (Qiagen, Cat #102720). 1.0E05 U2OS cells were seeded in 6-well plates and grown in antibiotic-free DMEM for 24 hrs. Cells were transfected with 30nM siEIF5B, and non-targeting control siRNA for 72 hrs.

For MG1 infection experiments, GFP-fused interferon-sensitive mutant Maraba virus (MG1) was provided by Dr. David Stojdl (Children's Hospital of Eastern Ontario Research Institute, Ottawa). After 48 to 60 hrs of siRNA transfection, the media was removed from the cells and replaced by 2 mL complete DMEM with or without MG1 virus at MOI of 0.1 for at least 12 hrs. For the time-course experiments, 2.5E05 cells were seeded in 6-well plates, culture medium was replaced by complete DMEM containing MG1 virus at MOI=0.1, and plates were incubated at 37°C in 5% CO₂ for 0-18 hour infection. Cells were harvested for protein and RNA extractions every 3-hour.

2.2 Protein extraction and Western blot analysis

For protein extraction, cells were washed twice with cold Phosphate-buffered saline (PBS), then lysed in 70-80 μ L 1X RIPA buffer (50mM Tris-CL pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5% Sodium deoxycholate, 1% (v/v) NP-40, 0.5% (w/v) SDS) supplemented with HaltTM Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific #78440) and incubated on ice for 20 min. Lysates were scraped using Corning[®] cell lifter and centrifuged at 4°C at 14,000 RPM for 15 min. Supernatant was collected for subsequent protein analysis experiments and stored at -20°C. Bradford protein assay (Bio-Rad) was performed to measure protein concentration. Equal amount of proteins were diluted in 2X Laemmli buffer with the addition of 5% Dithiothreitol (DTT) and loaded into 10% SDS-PAGE. Protein samples were separated at the constant voltage of 150V for 1.5 hrs and transferred onto the 0.2 μ m PVDF membrane by wet transfer using 1X Transfer buffer (25 mM Tris, 192 mM glycine, 15% MeOH) at 100V for 1-1.5 hrs. The membranes were blocked with 5% skim milk or BSA in TBST buffer for 1 hr. The levels of following proteins were determined: Phospho-eIF2 α (Anti-Rabbit, 1:2000 in TBST with 1% BSA, Cell Signaling Technology #9721), eIF2 α (Anti-Rabbit, 1:5000 in TBST with 1% BSA, Abcam# ab26197), XIAP (Anti-Rabbit, 1:2000 in TBST with 1% BSA, Cell Signaling Technology #2045), RIAP3 (Anti-Rabbit, 1:2000 in TBST with 1% BSA, provided by Dr. Robert Korneluk, CHEO, Ottawa), Bcl-xL (Anti-Rabbit, 1:2000 in TBST with 1% BSA, Cell Signaling Technology, Cat#2762S), 4EBP1 (Anti-Rabbit, 1:4000 in TBST with 1% BSA, Cell Signaling Technology #9644, a gift from Dr. Tommy Alain), EIF5B (Anti-Mouse, 1:1000 in TBST with 1% BSA, Santa-Cruz #sc-393564), Maraba viral proteins (Anti-Rabbit, 1:2000 in TBST with 1% BSA, provided by Dr. David Stojdl, CHEO, Ottawa), α -Tubulin (Anti-Mouse, 1:10,000 in TBST with 1% skim milk, Abcam #ab7291), GAPDH (Anti-Mouse, 1:10,000 in TBST with 1% BSA, a gift from Dr. Robert Korneluk, CHEO,

Ottawa). Probed membranes were incubated with HRP-linked α -mouse (Cell Signalling Technology, Cat#7076S) or α -rabbit (Cell Signalling Technology, Cat#7074S) secondary antibodies for 1 hr. HRP signals were detected on film (GE Healthcare) using ECL reagent (GE Healthcare). The densitometry analysis was performed using Image Studio version 5.2 software (LI-COR Biosciences), and the measured signals of the target proteins were normalized to the Tubulin (for human antibodies) or GAPDH (for mouse antibodies).

2.3 Pulse [³⁵S]-Methionine labeling

Following the treatment of cells for the related experiments, cells were washed twice in 1 mL methionine and cysteine free DMEM supplemented with 10% FBS and 2mM L-Glutamine, then incubated in 1 mL DMEM-Met,Cys for 15 min at 37°C and 5% CO₂. For radiolabeling newly synthesized proteins, cells were pulse-labeled with 1 mL of 100 μ Ci ³⁵S-Met, Cys (Perkin Elmer, NEG772) for 20 min at 37°C at varying time, then washed twice with pre-chilled 1X PBS buffer and lysed in RIPA buffer (as described above). Total protein content were separated on a 10% SDS-PAGE gel and stained with Coomassie blue dye. The signals of radiolabeled proteins were visualized on X-ray hyper-sensitive film.

2.4 Immunofluorescence and Confocal microscopy

Immunofluorescence was performed to visualize stress-granule marker, TIAR in S51A and WT MEFs. 2.0E05 S51A and WT MEFs were seeded on cover slips in 6-well plates for 24 hrs. Time course MG1 infection was carried out after removing the culture medium from cells and replacing 2 mL of complete DMEM containing GFP-MG1 virus at MOI of 0.1 for 12 hrs. Cells were washed 3X in 1X PBS at 0,3,6,9 and 12 hpi, and fixed with 3.7% Paraformaldehyde for 20 min. Cells were permeabilized with 0.2% v/v Triton X-100 solution for 5 minutes, followed by blocking with FCS

blocking buffer (1% FCS, 0.2% BSA, 0.4% Triton X-100 in 1X PBS buffer). Coverslips were incubated overnight at 4°C with TIAR antibody (Anti-Rabbit, 1:400 in 0.2% BSA, 0.4% Triton X-100, dissolved in 1X PBS buffer, Cell Signaling Technology #D32D3). Coverslips were washed 3X for 5 min with Triton X-100/BSA buffer, and incubated with secondary antibody for 1 hr (Alexa Fluor anti-rabbit 594, 1:1000 in Triton X-100/BSA buffer) on shaker at room temperature. Nuclei of the cells were stained using 1 µg/mL Hoechst 33342 solution (Trihydrochloride Trihydrate, Invitrogen) in 1X PBS for 5 min, and washes 3X for 5 min with 1X PBS buffer. Coverslips were mounted onto glass slides using Dako Fluorescent Mounting Medium (Dako North America, Inc.). Cells were visualized using the 60X objective with water (Olympus Fluoview FV-1000 Laser Confocal Microscope, Richmond Hill, Ontario, Canada).

2.5 RNA extraction and RT-qPCR

Total RNA was extracted using RNeasy[®] RT following the manufacturer's protocol. Media was removed from the plates and cells were lysed using 0.7 mL of RNeasy into the wells of 6-well plates. Next, the extracts were transferred to the RNase-free tubes and homogenized by adding 0.28 mL of RNase-free water. The samples were then shaken for 15 seconds and incubated at RT for 15 min. Following, the samples were centrifuged at 12,000 x g for 15 min at RT, then the supernatant were transferred to the new RNase-free tubes. An equal amount of 100% isopropanol was added to the supernatant, the mix was incubated for 10 min at RT. For the precipitation of the RNA, samples were centrifuged at 12,000 x g for 10 min at RT, the pellet was washed with 75% ethanol and centrifuged at 6,000 x g for 3 min. The RNA yield was solubilized in RNase-free water and the concentration was measured using Nano-drop1000.

Reverse transcription was performed by using qScript[™] cDNA SuperMix (Quanta Bioscience) and incubation of the mix at the PCR machine with the following thermo-cycling condition: 25°C

for 5 min, 42°C for 30 min, 85°C for 5 min. 1 µL of cDNA products, 10 µL of SYBR green Master Mix (Qiagen), 2 µL of primers, 7 µL of H₂O were used to perform quantitative Real-Time PCR on the Eppendorf qPCR machine. The Ct values were generated by the standard curve method, and the obtained data were represented relative to the geometric mean of RPL13A and RPL36A as human control genes, and RPL13A and PPIA as mouse control genes.

mRNA target	Catalogue number	Company
BCL2L1 Specific (Human)	QT00997423	Qiagen (QuantiTect primers)
XIAP(Human)	QT00042854	Qiagen (QuantiTect primers)
RPL36A (Human)	QT01668030	Qiagen (QuantiTect primers)
RPL13A (Human)	QT02321333	Qiagen (QuantiTect primers)
PPIA (Mouse)	MHK-1	RealTime Primers
RPL13A (Mouse)	MHK-1	RealTime Primers

Table 1: RT-qPCR primers used in this project.

MG1 RT-qPCR primers:

Forward: 5'-GGTGATGGGCAGACTATGAAA-3'

Reverse: 5'-CCTAAGGCCAAGAAACAAAAGAG-3'

Standard curves:

Bcl-xL (Human): $y = -4.364x + 17.446$

XIAP (Human): $y = -3.6221x + 21.715$

RPL36A (Human): $y = -3.6867x + 19.598$

RPL13A (Human): $y = -3.5938x + 22.237$

PPIA (Mouse): $y = -3.7536x + 23.394$

RPL13A (Mouse): $y = -4.0611x + 17.577$

MG1: $y = -3.8027x + 14.248$

2.6 Kinetic cell imaging

To determine the cytotoxicity and rate of viral infection, cells were seeded into 6-well plates a day prior to the treatment. Upon the treatment of the cell with MG1 virus at MOI of 0.1 and 250 nM of Cytotox Red reagent (Essen Bioscience), they were imaged every two hours for the duration of the experiment using IncuCyte™ ZOOM Content Kinetic Imaging System (Essen Bioscience). Fluorescent signal from Cytotox Red dye and GFP-MG1, as well as cell confluence were used for the analysis with IncuCyte™ ZOOM and GraphPad Prism 5 software. The rate of Cytotoxicity was determined by normalizing the number of Cytotox Red positive cells to the phase confluence, and the rate of MG1 infection was calculated as the percentage of GFP-expressing cells to the phase confluence.

2.7 Statistical analysis

Data are represented as a mean \pm standard deviation of at least three independent biological replicates. Collected data from the repeated experiments were exported to GraphPad Prism 5 software for graph and statistical analysis. Student's T-test and Two-way Anova were performed to determine the p-value and statistical significance.

Chapter 3: Results

3.1 Host protein synthesis is inhibited during MG1 infection.

Previous studies proposed a rapid repression of host translation mechanism during VSV infection which subsequently leads to the inhibition of viral protein synthesis (Rose, JK., et al., 2001, Conner, JH., et al., 2005). To explore if similar events take place during MG1 infection, Pulse [³⁵S]-methionine labeling was performed to observe the effect of viral infection on the regulation of global translation machinery. U2OS cells were infected with MG1 virus at MOI of 0.1 in a time course with 3-hour time interval. To radiolabel newly-synthesized proteins, cells were incubated with [³⁵S]-methionine at 37°C, 5% CO₂ for 20 min. Then, the extracted protein lysates were separated on 10% SDS-PAGE, and exposed to hyper-sensitive film to visualize the rate of global translation during MG1 infection. As illustrated in Figure 3, we have observed the synthesis of MG1 proteins L, G, N, P and M starting at 6 hpi, followed by a marked inhibition of cytoplasmic translation at about 9 hpi in the pulse [³⁵S]-labeled cells.

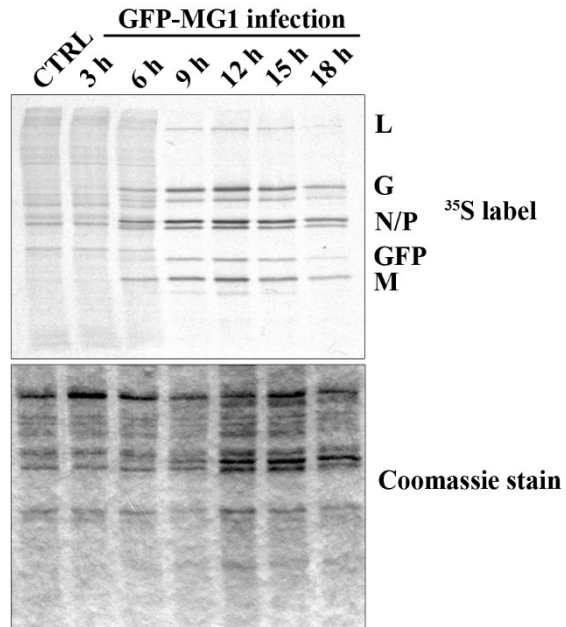


Figure 3: The effect of MG1 infection on the rate of host protein synthesis. U2OS cell were mock-infected or infected with MG1 (MOI=0.1) for 18-hour time course as indicated. Then, they were pulse labeled with [³⁵S]-methionine for 20 min, and separated by 10% SDS-PAGE electrophoresis. The synthesis of host protein were compared to the control lane (uninfected cells) appeared as a ladder of bands. Viral proteins are specified to the right of the image and Coomassie blue stain of autoradiograph gel was used to demonstrate the protein loading.

3.2 MG1 modulates the host translation machinery through eIF2 and 4E-BP1 pathways.

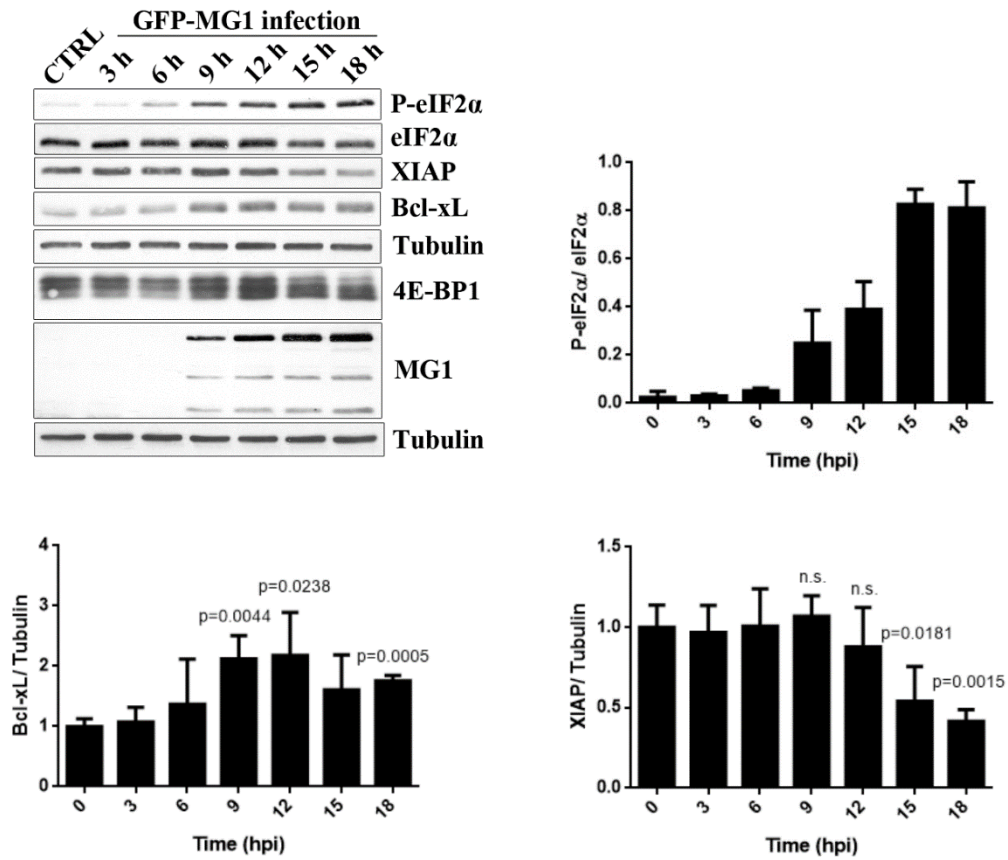
In recent studies, two phases for the translational control during VSV infection have been described. Firstly, de-phosphorylation of 4E-BP1 prevents the formation of eIF4F complex through its interaction with eIF4E, thereby inhibiting the host global translation. However, eIF2 is still active, and viral transcript is being efficiently translated. During late phase of infection, viral protein synthesis is also inhibited by the phosphorylation of α subunit of eIF2 (Conner, JH., et al., 2005). To determine whether MG1 uses similar mechanism to take over the translation machinery of their host cells, we monitored the phosphorylation status of eIF2 α and 4E-BP1 by western blotting. As illustrated in Figure 4.A, eIF2 α was gradually phosphorylated and 4E-BP1 was dephosphorylated during the time course of MG1 infection in U2OS cells. However, it seemed that the phosphorylation of eIF2 α had started before de-phosphorylation of 4E-BP1 occurred, suggesting the leading contribution of eIF2 α inactivation to the repression of host global translation initiation.

Our collaborators at Dr. Alain's lab performed ribosome profiling on U343 cells infected with WT Maraba and MG1 viruses, and have identified a cohort of IRES-containing mRNAs under selective translation and transcription during the viral infection. Their data suggested that Bcl-xL mRNA is selectively translated during the infection with either viruses (Graber, TE., and Alain, T., unpublished data). To validate the preliminary results from the ribosomal profiling experiment, we performed western blot analysis to examine expression levels of Bcl-xL in the protein lysates from the experiment described above. Our findings confirmed the up-regulation of Bcl-xL protein during MG1 infection despite the cessation of global protein synthesis. In attempt to determine whether other IRES-containing anti-apoptotic candidates are also up-regulated at translational level during the infection, we selected to monitor the levels of XIAP protein during the time course

of MG1 infection since they are reportedly co-regulated by specific ITAFs during cellular stress conditions (Yoon, A., et al., 2006; Lewis, SM., et al., 2007; Bevilacqua, E., et al., 2010). The result demonstrated that the levels of XIAP protein remained unaltered during the 12 hpi, but were noticeably decreased to about 50% of its maximum level at 15 hpi (Figure 4.A). This down-regulation can likely be attributed to the complete shut-down of global translation seen after 12hpi (Figure 1), and the degradation of XIAP protein.

Since the increase observed in Bcl-xL protein during MG1 infection could have been associated with the increase in the abundance of Bcl-xL mRNA. RT-qPCR was therefore performed to measure steady-state Bcl-xL and XIAP mRNAs at 12 hpi, when the maximum level of Bcl-xL protein had been detected. Given the fact that there are two isoforms identified for Bcl-x gene, Bcl-xL and Bcl-xS, we used Bcl-xL-specific primers to ensure the accurate measurement of Bcl-xL mRNA. The result of this experiment reinforced the notion that the increase in Bcl-xL protein was regulated only at translational level, as the level of Bcl-xL mRNA remained unchanged under the conditions tested. Interestingly, we detected a significant elevation in the level of steady-state XIAP mRNA in the infected cells compared to the negative control group which can be associated with the increase in the rate of transcription and/or mRNA stability of XIAP (Figure 4.B). Together with the findings from western blot analysis, it is likely that the global translation inhibition during MG1 infection consequently down-regulates XIAP protein synthesis despite the increase in levels of steady-state XIAP mRNA.

A



B

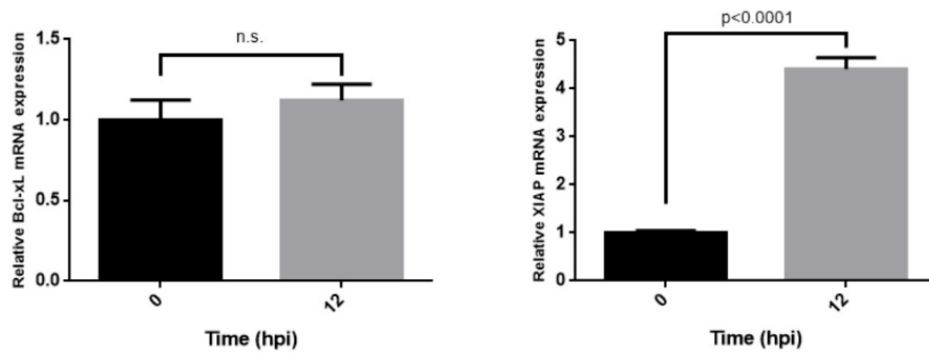


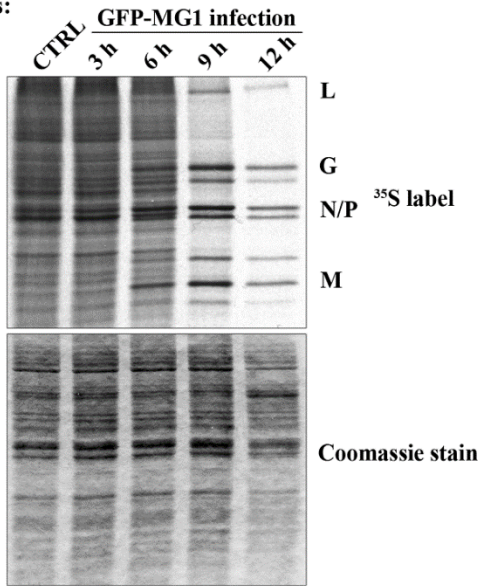
Figure 4: MG1 uses eIF2 α and 4E-BP1 to control the translation mechanism of their host. (A) Top left panel: western blot analysis demonstrating phosphorylation of eIF2 α , dephosphorylation of 4E-BP1 and up-regulation of Bcl-xL proteins during a time course of MG1 infection at MOI=0.1. Top right panel: the alternation of p-eIF2 α was quantified relative to the total eIF2 α protein. Bottom left panel: Quantification of Bcl-xL protein level relative to tubulin showed a significant increase at the indicated time points. Bottom right panel: measuring XIAP protein level relative to tubulin revealed XIAP mRNA did not undergo selective translation during MG1 infection. (B) Steady-state mRNAs of Bcl-xL, XIAP, RPL13A and RPL36A were measured in the negative control group, and infected cells at 12 hpi. Ct values were normalized by the standard curve of each primer, and represented relative to the geometric mean of RPL13A and RPL36A. Left panel: RT-qPCR using Bcl-xL-specific primers showed MG1 infection does not have any significant impact on steady-state Bcl-xL mRNA. Right panel: Elevated expression of XIAP mRNA was detected after MG1 infection at 12 hpi.

3.3 Phosphorylation of eIF2 α plays a significant role in the inhibition of host and MG1 translation.

Early studies on VSV model of infection proposed that host global translation is more sensitive to the phosphorylation of eIF2 α than viral protein synthesis. It has been shown that even low level of eIF2 α early after infection is sufficient to significantly repress cytoplasmic translation, whereas higher level of inactivated eIF2 α is required to block viral translation (Centrella, M., et al., 1982). On the contrary, Conner, JH., et al., 2005, claimed that phosphorylation of eIF2 α does not have any impact on the rate of host protein synthesis in the context of VSV infection since the host translation inhibition happens before the inactivation of eIF2 α (Conner, JH., et al., 2005).

We decided to investigate whether the translation of MG1 is similar to either of these proposed models. To determine the impact of eIF2 α phosphorylation on global translation and MG1 protein synthesis, we performed pulse [³⁵S]-methionine labeling in a phosphor-null eIF2 α (S51A) MEFs and the genetically-paired wild type MEFs after a time course infection with MG1 at MOI of 0.1. Protein lysates were run on 10% SDS/PAGE and the radioactive signal were visualized on hyper-sensitive film. Comparing the relative rate of cytoplasmic translation between the two cell lines confirmed the critical role of the eIF2 α phosphorylation in the shutdown of global translation. Additionally, a higher rate of MG1 translation was detected in S51A MEFs than WT-MEF at 12 hpi (Figure 5).

WT MEFs:



S51A MEFs:

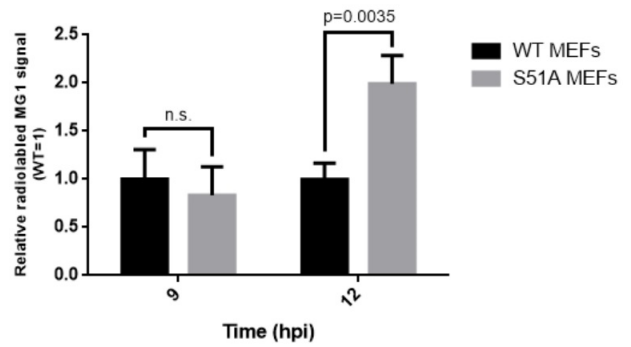
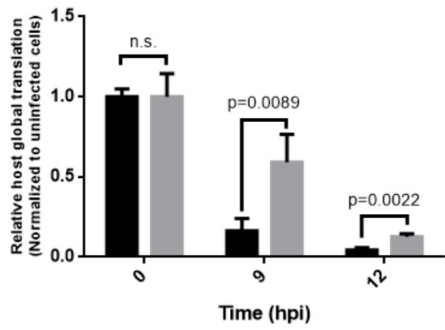
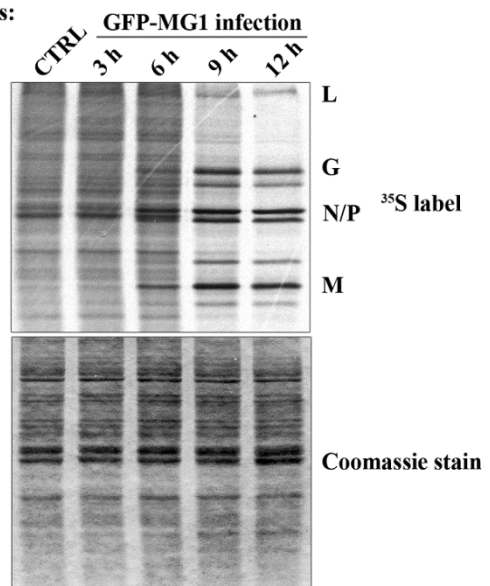


Figure 5: Impact of eIF2 α phosphorylation on the rate of host and MG1 protein synthesis. Top panels: Both WT and S51A MEFs were infected with MG1 at MOI=0.1 for the indicated time points, followed by pulse [³⁵S]-methionine labeling for 20 min. Protein lysates were electrophoresed on 10% SDS-PAGE gel and visualized on hyper-sensitive film. Bottom left panel: The rate of host protein synthesis was determined by densitometry of the relative radioactive signal on the area between the viral L and G bands, normalized to the entire related lane on Coomassie gel at 0, 9 and 12 hpi. Bottom right panel: The rate of viral translation was compared between the two cell lines by measuring the relative radioactive signal from the viral M protein to Coomassie stain, and normalized to the signal measured from the WT MEFs at each time points. Data represented are the mean \pm S.D. of three independent experiments.

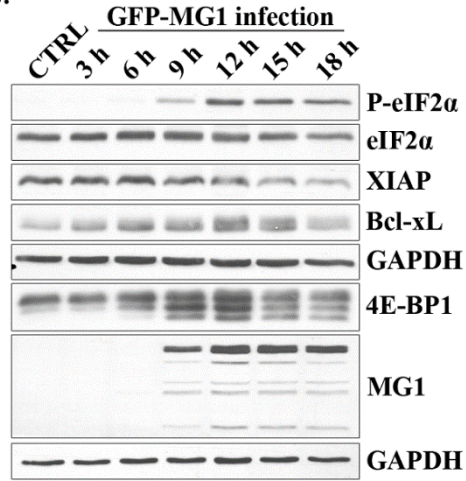
3.4 Phosphorylation status of eIF2 α only affects MG1 propagation but not Bcl-xL and XIAP protein expression.

Based on our previous observation, MG1 mRNA is translated more efficiently when eIF2 α is not phosphorylated. To understand whether eIF2 α phosphorylation has any effect on the expression profile of Bcl-xL, XIAP and MG1 proteins, western blotting technique were used to monitor any alternation in these proteins during the time course of MG1 infection in phosphor-null eIF2 α (S51A) and wild type MEFs. In both cell lines, elevated level of Bcl-xL protein at 12 hpi were observed, yet XIAP protein level remained unaffected (Figure 6.A). Moreover, RT-qPCR was performed to assess the rate of MG1 replication between the two cell lines by amplifying MG1 genomic RNA at 12 and 15 hpi. Consistent with the finding from the translational assay, the result showed approximately 2 fold increase in the level of MG1 RNA in S51A MEFs when compared to WT MEFs at 15 hpi, suggesting the inactivation of eIF2 α may indirectly affect the ability of the virus to replicate its genome within the host cells (Figure 6.B).

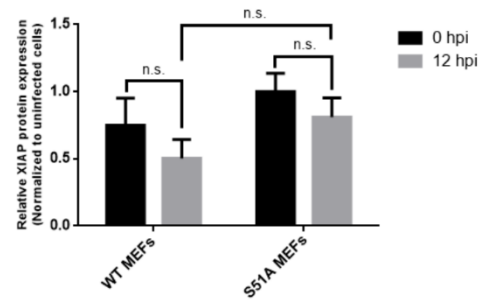
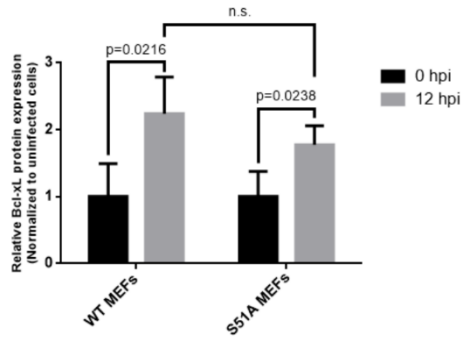
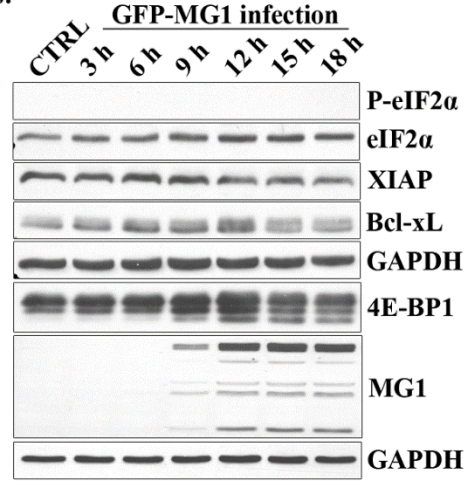
Next, we decided to address the physiological consequences of eIF2 α phosphorylation on viral propagation and cytotoxicity of the virus in the MEF cell lines. For this purpose, we performed a live-cell imaging technique to monitor the rate of MG1 infection, and count the number of dead cells using a fluorescent DNA-binding dye that only enters the dying cell when the plasma membrane integrity is reduced during the cellular stress. We observed that MG1 virus propagated more effectively and appeared to be less cytotoxic in the S51A MEFs than in the WT MEFs (Figure 6.C).

A

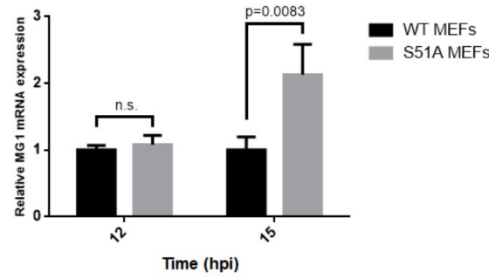
WT MEFs:



S51A MEFs:



B



C

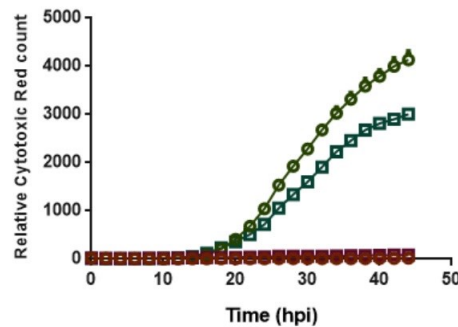
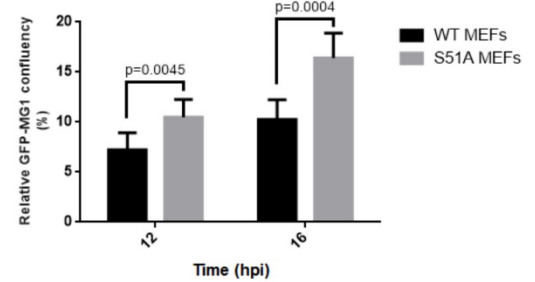
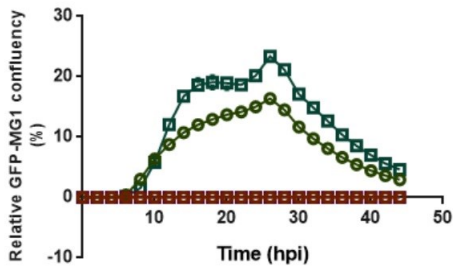


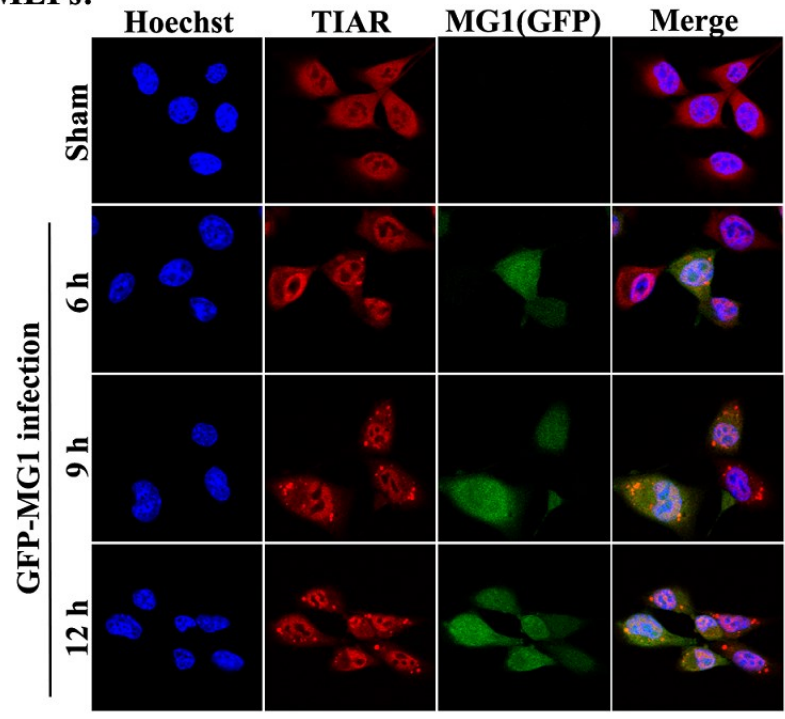
Figure 6: The effect of eIF2 α phosphorylation on host and viral protein synthesis during MG1 infection. (A) Western blot of MG1 time course in WT and S51A MEFs demonstrates the level of Bcl-xL and XIAP proteins as well as de-phosphorylation of 4E-BP1. GAPDH is shown as the loading control. Bottom left panel: Quantification of Bcl-xL protein relative to GAPDH at indicated time points after MG1 infection at MOI=0.1. Bottom right panel: Quantification of XIAP protein level relative to GAPDH. (B) RT-qPCR was performed to measure the level of MG1 genomic RNA relative to the geometric mean of RPL13A and PPIA mRNAs in WT and S51A MEFs at 12 and 15 hpi. (C) Top left panel: the confluency of GFP signal from WT and S51A MEFs were monitored in real time using IncuCyte live cell imaging. Y axis represents the percentage of GFP-MG1 to cell confluency. Top right panel: Fold GFP-MG1 confluency between the two cell lines at 12 and 16 hpi. Bottom panel: Cells mock-infected or infected with MG1 virus were treated with Cytotox Red reagent, and the activity of the fluorescent red dye was monitored over a time course. Y axis represents the number of cells emitting fluorescent red normalized to the cell confluency.

3.5 Stress granule formation does not regulate MG1 propagation in the host cells.

In a study by Dinh, PX., et al., 2013, the induction of stress granules by VSV has been investigated. Their results suggest that VSV triggers the formation of SG-like structures containing the viral replicative proteins, RNAs and cellular RNA-binding proteins such as TIA-1, TIAR and PCBP2, but lacking eIF3, eIF4A and processing bodies. Although, it is believed that the formation of VSV-induced granules is an anti-viral response from the infected cells to interfere with the viral propagation, they found this phenomenon which occurred simultaneously with eIF2 α phosphorylation did not have any noticeable inhibitory impact on VSV mRNA translation (Dinh, PX., et al., 2013).

We have addressed the effect of stress granules on MG1 propagation by performing immunofluorescence staining of S51A and wild type MEFs using TIAR protein, as a marker of stress granule formation. MEF cells were grown onto coverslips in 6-well plates for 24 hrs, followed by a time course of infection with GFP-expressing MG1 at MOI of 0.1 for 12 hrs. Immunofluorescence was performed using primary anti-rabbit TIAR antibody for visualizing SGs and Hoechst stain for detecting the nuclei. We have found that MG1 infection stimulates the formation of stress granules early after infection in the WT MEFs, however, although largely absent a few SG granules were also detected in the later time points in some of S51A MEFs (Figure 7). This observation further confirmed that the phosphorylation of eIF2 α is the main contributor in the assembly of stress granules during some viral infections. Consistent with VSV infection, stress granule formation did not inhibit MG1 propagation in the WT MEFs compared to S51A mutant pair.

WT MEFs:



S51A MEFs:

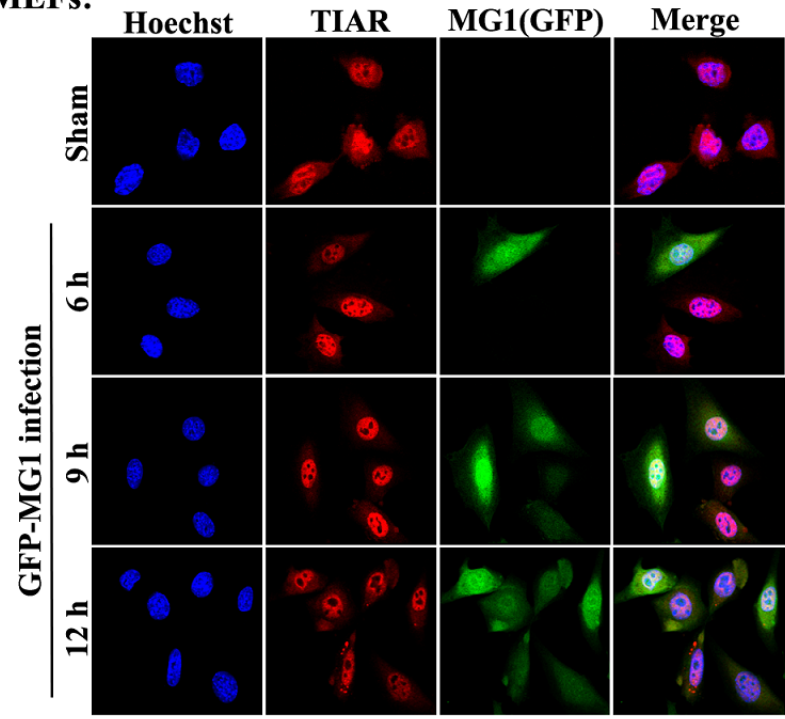


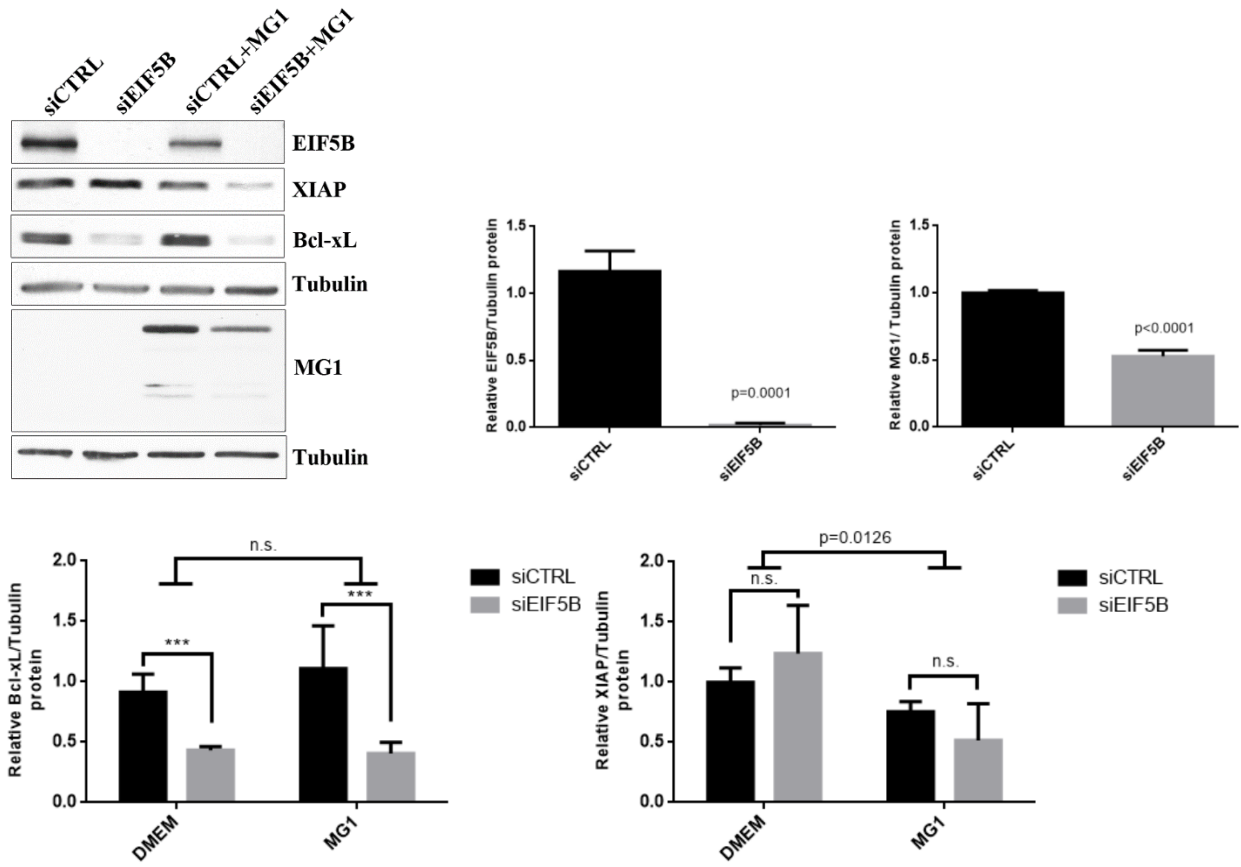
Figure 7: The induction of stress granules by MG1 virus does not noticeably repress viral propagation. WT and S51A MEF cell lines were infected with MG1 virus at MOI=0.1 for a time course of 12 hrs. Then, the formation of stress granules using TIAR anti-rabbit antibody was determined by immunofluorescence at the indicated time points. Cells were imaged by confocal microscopy using 60X water objective. Nuclei were visualized by Hoechst staining through DAPI filter, infected-cells with GFP-expressing MG1 were visualized through FITC, and TIAR was visualized through TRITC.

3.6 eIF5B is involved in the expression of Bcl-xL, XIAP and MG1 at different levels.

It is known eIF2 is responsible for the delivery of initiator-Met tRNA in a GTP-dependent manner. However, during MG1 infection the inactivation of eIF2 α blocks this process, so that both host cell and the virus should utilize an alternative strategy(ies) to selectively translate the mRNAs required for the cell survival and viral protein synthesis. Recent studies suggested an alternative role for eIF5B in delivering initiator tRNA to the 40S ribosome subunit upon the inactivation of eIF2 α . It has been shown eIF5B mediates the translation of HCV mRNA as well as selective translation of XIAP mRNA during serum starvation (Yamamoto, H., et al., 2014; Thakor, N., et al., 2012). We have therefore decided to investigate the role of eIF5B in the regulation of Bcl-xL, XIAP and MG1 expression both at the translational and transcriptional levels. Initially, we transiently transfected U2OS cells with siRNA targeting eIF5B and non-targeting siRNA, as the negative control group. After 60 hrs of knock-down, cells were infected with MG1 at MOI of 0.1 for 12 hrs. Western blotting and RT-qPCR analysis were performed to examine the expression profile of Bcl-xL, XIAP and MG1 at protein and RNA levels. Our data showed that eIF5B does not have any role in the selective translation of XIAP during MG1 infection, as we have observed XIAP protein was significantly down-regulated in both siCTRL and siEIF5B transfected cells after MG1 infection (Figure 8.A). Interestingly, the reduction of eIF5B expression was found to negatively modulate the expression of Bcl-xL and MG1 at both mRNA and protein levels (Figure 8.A and B). These observations eliminated the possibility that eIF5B has any direct translational role in the expression of Bcl-xL and MG1 proteins. In fact, based on the canonical role of eIF5B in 60S ribosomal subunit end joining, it is possible that the impairment of ribosome assembly after knocking down eIF5B would lead to the faster degradation of the target mRNAs. Nonetheless, since MG1 replication is mediated by its L polymerase protein, we assume that even a slight down-

regulation of this protein may profoundly affect the rate of viral propagation, and could explain how this event indirectly has a significant impact on the level of MG1 proteins. Furthermore, it is likely that there is a correlation between the level of Bcl-xL protein and the rate of MG1 infection, so that the reduction of Bcl-xL expression in the absence of eIF5B would indirectly lead to the lower chance of MG1 virus to efficiently propagate in the host cell.

A



B

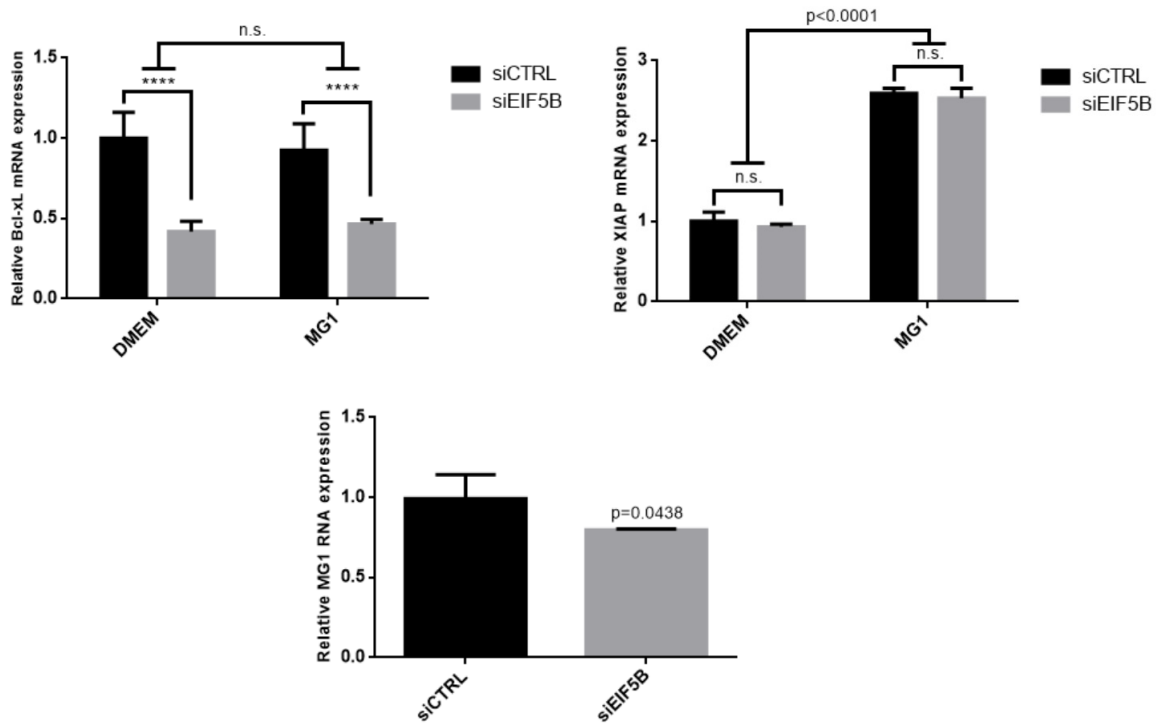
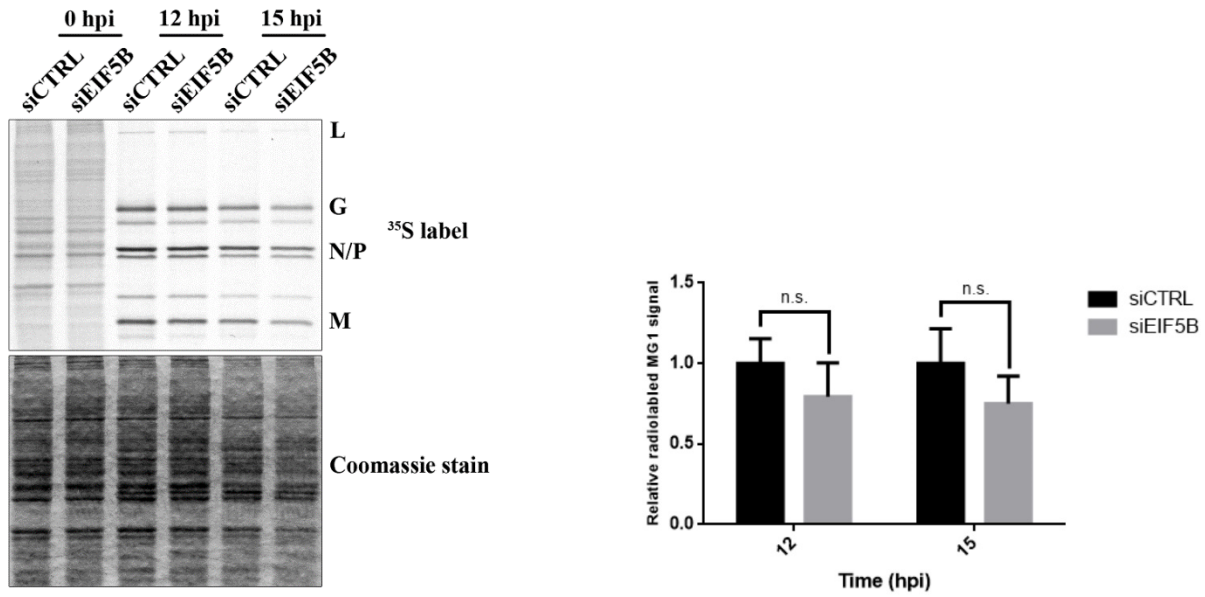


Figure 8: eIF5B knock-down negatively controls the expression of Bcl-xL and MG1. (A) U2OS cells were transfected with eIF5B-targeting or non-targeting control siRNAs followed by 12 hrs of mock- or infection with MG1 at MOI=0.1. Western blot analysis was used to determine the expression of eIF5B, Bcl-xL, XIAP, MG1 and Tubulin (loading control). The expression of each protein was quantified relative to Tubulin by densitometry, and the error bars represent the mean of 3 independent experiments \pm standard deviation. Bottom panels: Quantification of Bcl-xL (***, $P= 0.0009$) and XIAP proteins. (B) Steady-state mRNA levels of Bcl-xL, XIAP, RPL36A, RPL13A and MG1 RNA were measured by RT-qPCR in siCTRL or siEIF5B transfected cells after 12 hpi with MG1 or DMEM treatment (as the negative control). Ct values were normalized by the standard curve method and represented relative to the geometric mean of RPL13 A and RPL36A. Top left panel: Quantification of Bcl-xL mRNA (****, $P < 0.0001$).

3.7 eIF5B knock-down is not involved in MG1 mRNA translation but significantly affect the rate of infection.

To further investigate whether eIF5B regulates the translation of MG1 mRNA, Pulse [³⁵S]-methionine labeling was performed after U2OS cells were transfected with si-EIF5B and siCTRL for 60 hrs, and subsequently infected with MG1 virus for 12 and 15 hrs. The relative signals from radiolabeled viral M protein were compared between eIF5B-depleted cells and the negative control group. The results demonstrated that eIF5B had not been involved in the translation of MG1 mRNA, and the slight decrease observed in radiolabeled MG1 proteins might be attributed to the lower abundance of available MG1 mRNA in the absence of eIF5B (Figure 9.A). Furthermore, we have monitored the rate of MG1 infection and cell viability in eIF5B-depleted cells compared to the negative control group. For this purpose, we performed a live-cell imaging technique and treated the cell with Cytotox Red reagent at the time of MG1 infection at MOI of 0.1. Our results showed that reducing the level of eIF5B protein significantly down-regulated the rate of MG1 infection and enhanced the cell viability during the viral stress (Figure 9.B). The latter finding was surprising, since knocking down eIF5B was observed to notably reduce the level of Bcl-xL mRNA and protein as well as diminish the level of XIAP by approximately 30%.

A



B

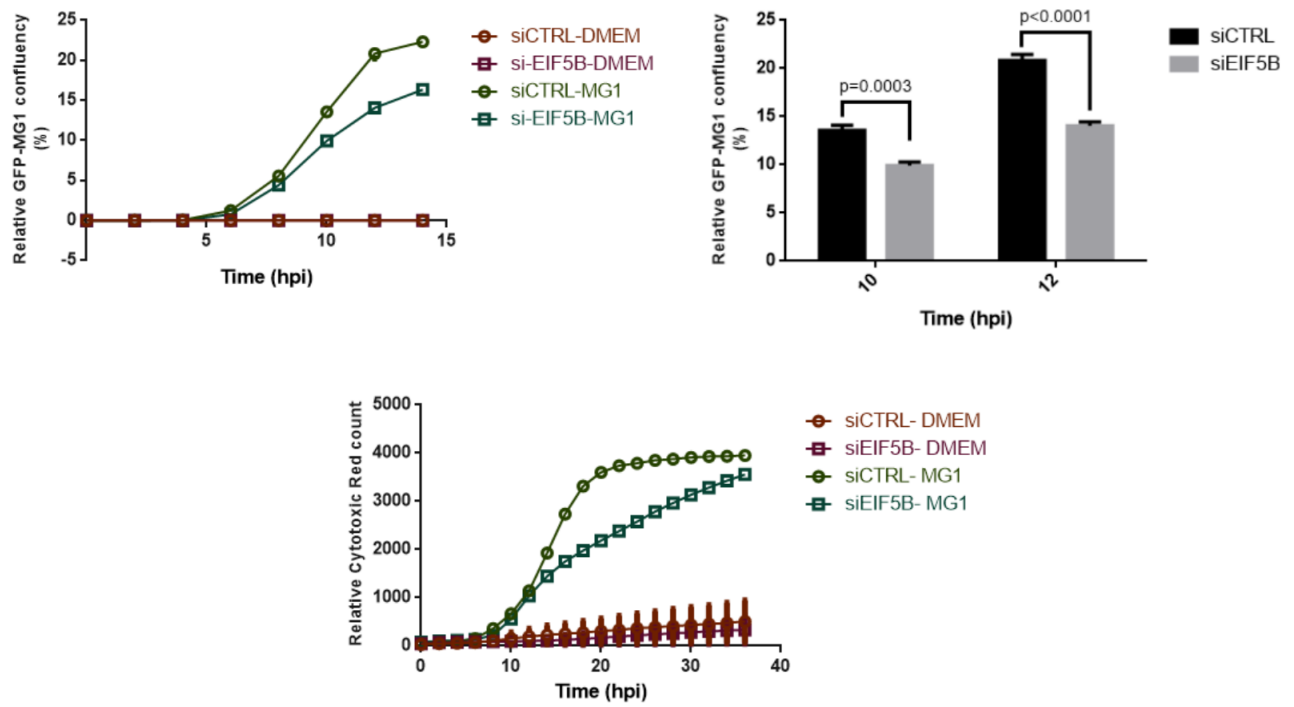


Figure 9: eIF5B knock-down does not regulate MG1 mRNA translation but affect the rate of infection and cytotoxicity. (A) Top left panel: A representative blot from [³⁵S]-methionine labeling translational assay, U2OS cells were transfected with siCTRL or siEIF5B, followed by MG1 infection at MOI=0.1 for 12 and 15 hrs. Top right panel: The rate of MG1 mRNA translation was quantified by densitometry of M protein signal relative to Coomassie blue stain at 12 and 15 hpi. (B) Top left panel: U2OS cells were treated as in (A) and the rate of MG1 infection was assessed using IncuCyte Zoom live cell imaging. Y axis represents the percentage of GFP-MG1 to cell confluency. Top right panel: Fold confluency of GFP-MG1 signal at 10 and 12 hpi. Bottom panel: The rate of MG1 cytotoxicity was compared between the indicated conditions using Cytotox Red reagent and IncuCyte Zoom. Y axis represents the number of cells emitting fluorescent red normalized to the cell confluency.

Chapter 4: Discussion

The rhabdovirus Maraba has been described as a compelling oncolytic vaccine due to its ability to boost adaptive immunity and tumor-cell specificity (Pol, JG., et al., 2014). Given the close relatedness of Maraba virus with VSV based on their amino acid sequences and antigenic similarity, it is expected that both viruses share comparable OV properties (Zhang, J., et al., 2014; Brun, J., et al., 2010). Recent administration of an attenuated strain of Maraba virus, MG1, with higher tumor-targeting ability demonstrated promising results in prolonging the survival from breast cancer, controlling epithelial ovarian cancer metastasis, and the treatment of glioblastoma (Bourgeois-Daigneault, M., et al., 2016; Tong, JG., et al., 2015; Wollmann, G., et al., 2012). Therefore, we speculated that Maraba virus may employ similar strategy(ies) as VSV in order to propagate within the tumor cell lines.

Previous studies on the mechanisms of translational control during VSV infection proposed two different models. According to the earlier model, phosphorylation of eIF2 α is the main contributor to the shut-down of global translation mechanism in the infected cell (Centrella, M., et al., 1982). However, the recent study conducted by Connor, JH., et al. 2005, suggested that the inhibition of host translation machinery is regulated through de-phosphorylation of eIF4E and 4E-BP1, and the inactivation of eIF2 α only represses the translation of VSV mRNA during the late phase of infection (Connor, JH., et al., 2005). We have investigated whether MG1 virus follows any of these models in order to take over the host protein synthesis machinery. Our data from the time course experiment in U2OS cells demonstrated phosphorylation of eIF2 α as well as de-phosphorylation of 4E-BP1 during MG1 infection. Consistent with the first proposed model for VSV, we observed that inactivation of eIF2 α preceded the de-phosphorylation of 4E-BP1. Our follow-up western blot and [³⁵S]-Methionine labeling experiments in WT and S51A MEFs further

reinforced the dominant role of eIF2 α in the repression of cytoplasmic translation in response to MG1 infection. It is important to note that we also observed down-regulation in the rate of MG1 translation during the later time point in WT MEFs compared to the S51A MEFs. Although this event may be associated with the direct impact of eIF2 α inactivation on MG1 translation efficiency, the data from survival assay using Cytotox Red suggested the lower rate of infection at 12 hpi in WT could be due to the higher rate of cell death and the less opportunity for the virus to propagate within the host cells.

The role of PKR kinase as a key regulator of eIF2 α phosphorylation during viral stress has been extensively studied, it is believed that the transcription of PKR (or eIF2AK2) is triggered by the interferon response during early phase of infection with RNA viruses (Galluzzi, L., et al., 2008). The activation of PKR is induced upon the recognition and binding of dsRNA by the N-terminus double-stranded RNA binding domain (dsRBD), which subsequently leads to the dimerization and auto-phosphorylation of the protein. The formation of PKR-PKR complex preserves the protein in an open state for carrying out the phosphorylation of eIF2 α through its C-terminal kinase domain (Meurs, E., et al, 1990; Donnelly, N., et al., 2013). Also, previous studies on PKR knock-out mice revealed that the phosphorylation of eIF2 α is primarily caused by PKR activity during VSV infection (Balachandran, S., et al., 2000; Stojdl, DF., et al., 2000). Therefore, it is likely that the inactivation of eIF2 α during Maraba virus infection is similarly mediated through the PKR pathway. Importantly, activated PKR is involved in the eIF2 α -mediated apoptosis (Srivastava, SP., et al., 1998), an event that is also linked with NF- κ B signaling (Gil, J., et al., 1999) and the expression of pro-apoptotic transcription factors such as CHOP (Lozon, TI., et al., 2011), therefore, the higher rate of survival observed in the S51A MEFs compared to the WT MEFs may be related to the blockade of PKR-eIF2 α pathway during MG1 infection.

In order to further confirm our findings, a follow-up experiment could be performed by treating the cells with Thapsigargin, a drug that induces eIF2 α phosphorylation by causing ER stress, to determine the rate of MG1 and host protein synthesis while eIF2 α is already phosphorylated. Similar study on VSV-infected cells showed that the transient treatment of BHK cells with Thapsigargin at 0, 2, and 4 hpi resulted in the inhibition of both VSV and host translation mechanism, and both of which were similarly sensitive to the effect of the drug on eIF2 α phosphorylation (Connor, JH., et al., 2005).

Similar to the regulatory role of activated-PKR kinase during viral infection, recent studies proposed a significant contribution of pancreatic endoplasmic reticulum kinase (PERK) in mediating eIF2 α phosphorylation during VSV infection. It has been shown that the glycosylation of VSV G protein imposes ER stress which subsequently causes the activation of PERK and eIF2 α inactivation. It is suggested that the inhibition of protein synthesis through eIF2 α phosphorylation would prevent further entry of nascent polypeptides into the ER, therefore restore its homeostasis (Baltzis, D., et al., 2007). Under normal condition the N-terminus of PERK resides inside the ER lumen, bound to BiP protein, while the C-terminus kinase domain of PERK is in the cytosol (Harding, HP., et al., 1999). Upon the accumulation of misfolded proteins in the ER, BiP dissociates from PERK and this event facilitates PERK dimerization from their N terminal regions, which then leads to the activation of the protein (Bertolotti, A., et al., 2000). Since Maraba virus also encodes G protein, it would be interesting to investigate the role of PERK in mediating eIF2 α phosphorylation in Maraba-infected cells in the future studies.

Although we have observed a significantly higher rate of translation inhibition in WT MEFs compared to the S51A MEFs at 9 and 12 hpi from the [³⁵S]-methionine labeling experiment, still a noticeable repression of protein synthesis was detected after 9 hpi in the mutant MEFs,

suggesting the involvement of other mechanism(s) possibly eIF4E-mediated pathways in the inhibition of translation initiation. One of the central pathways targeted by many viruses is the serine/threonine kinase mTOR complex 1 and 2. While mTORC2 is mainly involved in the cell survival and actin organization (Sarbasov, DD., et al., 2004; Jacinto, E., et al., 2004), mTORC1 regulates the cap-dependent translation through the activation of its downstream proteins such as 4E-binding proteins and p70 ribosomal S6 kinase 1 (S6K1) (Le Sage, V., et al., 2016). Once mTORC1 becomes activated, it phosphorylates Thr 37/46 and then Serine 65, Thr 70 of 4E-BP1. This regulatory effect leads to the dissociation of 4E-BPs from eIF4E, and promotes the translation initiation (Gingras, AC., et al., 1999; Gingras, AC., et al., 2001). In our MG1 time course experiment in U2OS and MEF cell lines, we have demonstrated gradual de-phosphorylation of 4E-BP1, the downstream effector of mTORC1, indicating that MG1 modulates the function of eIF4E by inactivating mTORC1 nexus. Moreover, de-phosphorylation of eIF4E and other 4E-BPs may also contribute to the regulation of protein synthesis in MG1-infected cell which should be determined in the follow-up experiments.

Based on the unpublished data from Dr. Alain's lab where they performed Ribosome profiling on WT and MG1 infected-U343 glioblastoma cells line, a cohort of IRES containing mRNA candidates have been identified which were differentially translated. One of the hits from the translome analysis was anti-apoptotic Bcl-xL mRNA exhibiting enhanced translational efficiency (TE) during the infection with both viruses (Graber, TE., and Alain, T., unpublished data). According to previous studies, many anti-apoptotic proteins are up-regulated via IRES-mediated translation during cellular stress. Among those, Bcl-xL and XIAP have been previously reported to be co-regulated (Holcik, M., et al., 1999; Yoon, A., et al., 2006) in order to control both intrinsic and extrinsic pathways of apoptosis. Studies have found specific RNA-binding

proteins, known as IRES-trans acting factors (ITAFs) such as PDCD4 and hnRNP A1 regulate IRES-mediated translation of XIAP and Bcl-xL mRNAs during stress stimuli (Bevilacqua, E., et al., 2010; Lewis, SM., et al., 2007; Liwak, U., et al., 2012). We therefore investigated the translation regulation of both transcripts during MG1 infection. Our results from the time course experiment in U2OS cells confirmed the elevated expression of Bcl-xL at translational level, however, the level of XIAP protein was diminished at later time points during MG1 infection. We next performed RT-qPCR to determine whether MG1 infection has any impact on the transcription of Bcl-xL and XIAP. As we expected the expression of Bcl-xL mRNA was unaffected, yet we have observed a significant increase in XIAP steady-state mRNA level at 12 hpi. These observations suggest that Bcl-xL and XIAP are in fact differentially regulated during MG1 infection. Some studies demonstrated that the activation of PERK during prolonged ER stress leads to the unfolded protein response (UPR)-induced expression of IAPs (cIAP1, cIAP2 and XIAP) in order to protect the cells against the apoptotic stimuli (Hamanaka, RB., et al., 2009; Hu, P., et al., 2004; Warnakulasuriyarachchi, D., et al., 2004). It is suggested that PERK mediates the transcriptional up-regulation of IAPs via the activation of PI3K-Akt pathway which then leads to the increased nuclear translocation and transcriptional activity of NF- κ B protein (Hamanaka, RB., et al., 2009; Hu, P., et al., 2004). Interestingly, study performed by Hamanaka, RB., et al., 2009 showed that inducing ER stress by treating NIH-3T3 cells with Thapsigargin led to the accumulation of cIAP1 and 2 but not XIAP protein, suggesting that XIAP mRNA translation was down-regulated in a mechanism independent of other IAPs. One possible explanation may lie in the phosphorylation of eIF2 α through PERK kinase activity which on one hand reduces the rate of XIAP protein synthesis, and on the other hand enhances the translation of ATF4, thus promotes the degradation of XIAP protein (Hiramatsu, N., et al., 2014).

On the contrary to the rescue effect of global translation repression on the cells under ER stress, some observations claimed that cells expressing PERK-null or phosphor-null eIF2 α mutants are more prone to cell death in response to ER stress (Harding, HP., et al., 2000b; Scheuner, D., et al., 2001). This result is in parallel with the pro-apoptotic role of PERK which can negatively influence cell survival through CCAAT/enhancer binding protein homologous protein (CHOP) acting downstream of ATF4 protein (Harding, HP., et al., 2000a; Rutkowski, DT., et al., 2006). Paradoxically in our time course experiment in MEF cell lines, we demonstrated the higher rate of cell survival in S51A-infected MEFs than in WT-infected MEFs. Based on our observation, MG1 regulates the inhibition of translation through both eIF2 α and eIF4E pathways, thereby impairing one of these pathways does not completely block the protein synthesis inhibition, yet the effect may be sufficient to rescue the cells from the detrimental effect of ER stress.

As mentioned above, we have validated the up-regulation of Bcl-xL protein synthesis during MG1 infection. It has been previously reported that selective translation of Bcl-xL and/or Bcl-2 mRNAs not only prevents the premature death of infected cells, but can ensure the persistent virus production. Therefore, many DNA viruses such as herpesviruses and poxviruses have evolved mechanisms to either encode proteins that mimic the anti-apoptotic function of Bcl-2 or Bcl-xL, or recruit an alternate strategy to up-regulate the expression of these proteins (Cuconati, A., et al., 2002).

Besides, the induction of Type I interferon expression such as IFN α and IFN β after viral infection trigger the kinase activity of PKR which then promote the activation of NF- κ B and IRF1 (Neumann, S., et al., 2015). The induction of NF- κ B reportedly enhances Bcl-xL transcription, and as a result can effectively block the intrinsic pathway of apoptosis during the infection (Sun, SC., et al., 2011; DiDonato, JA. et al., 2012).

Of note, studies on VSV Matrix protein revealed the impact of this protein on inhibiting host gene expression by interfering with all three RNA polymerases of the host, blocking the nucleocytoplasmic shuttling of RNAs and inhibiting the host translation mechanism through eIF2 and eIF4E pathways (Gaddy, DF., et al., 2005). However, it has been reported that Interferon-inducing strain of VSV expressing mutant M protein (M51R) is unable to inhibit host RNA synthesis and block mRNA transport between the nucleus and cytoplasm, in fact the induced expression of some proteins opposing the function of eIF2 α kinases such as GADD-34 can delay the inhibition of both host and viral translation (Stojdl, DF., et al., 2003; Connor, JH., et al., 2005). Since it was claimed that the mutant matrix protein of MG1 was also unable to prevent the nuclear/cytoplasmic mRNA transport (Brun, J., et al., 2010), it would be valuable to explore whether GADD-34 or other identified proteins from VSV studies mediate de-phosphorylation of eIF2 α during the later phase of MG1 infection.

The abundance of available ternary complex is a critical factor for the regulation of translation initiation. Although the translation of certain mRNAs containing short upstream ORF (uORF) at their 5' UTR, such as ATF4, are enhanced when the formation of ternary complex is impaired (Harding, HP., et al., 1999; Holcik, M., et al., 2005; Blais, JD, et al., 2004), most mRNAs require an alternative mechanism for Met-tRNA delivery to circumvent the translation initiation inhibition as a result of eIF2 α phosphorylation. Intriguingly, previous reports demonstrated that during some forms of cellular stress the translation of IRES-harboring mRNAs such as Bel-xL (during hypertonic stress), c-Src and c-Myc (during ER stress) are up-regulated during eIF2 α inactivation (Bevilacqua, E., et al., 2010; Allam, H., et al., 2010; Shi, Y., et al., 2015). One suggested model of eIF2-independent translation initiation has been observed during HCV and CSFV infection. Both of these viruses are reported to utilize eIF5B for the delivery of Met-tRNA into the P site of

the 43S ribosome when eIF2 is inactivated in the infected-cells (Pestova, TV., et al., 2008; Yamamoto, H., et al., 2014). Similar mechanism of eIF5B-mediated translation was also reported for XIAP mRNA through the IRES-mediated translation of its long splice variant (Thakor, N., et al., 2012; Holcik, M., 2015).

In addition to the canonical roles of eIF5B in global translation by mediating the ribosome subunit joining and inhibiting cell cycle arrest, under many stress conditions eIF5B was proposed to replace the function of eIF2 to facilitate the formation of the ternary complex (Holcik, M., 2015). Therefore, we have tested whether MG1 and host translation machinery switches to eIF5B-dependent mode to bypass the eIF2 α phosphorylation. Our data showed that eIF5B does not regulate the expression of XIAP protein, although we observed an approximately 30% reduction (none-significant) of XIAP protein level in eIF5B-depleted cells during MG1 infection. Furthermore, we found a significant down-regulation of Bcl-xL mRNA and consequently protein levels after knocking-down eIF5B under both normal and viral stress conditions. Due to the translational-specific role of eIF5B, we speculate that reducing the level of eIF5B negatively affects the amount of Bcl-xL mRNAs incorporated in the translation machinery, thereby leading to their rapid degradation. To test this hypothesis, future experiments could monitor the mRNA stability of Bcl-xL after knocking-down eIF5B by treating the cells with ActinomycinD, a suppressor of eukaryotic transcription system, in a time course of 8 hrs, with regard to 4.6 hrs half-life of Bcl-xL mRNA. Furthermore, we addressed the role of eIF5B in mediating the translation of MG1 virus, since eIF2 inactivation does not completely abolish the translation of the virus during the infection. Our results revealed that knocking-down eIF5B significantly reduced the level of MG1 RNA by 20%, however, this effect was found to be more significant at protein level by approximately 50% reduction. After performing pulse [³⁵S]-methionine labeling in siCTRL and

siEIF5B treated cell during a time course of MG1 infection, we found eIF5B did not mediate the translation of MG1 virus, and possibly would play an indirect regulatory role in both translation and replication of the virus. Also, our results from IncuCyte live cell imaging showed that despite the down-regulation of Bcl-xL expression, eIF5B-depleted cells exhibited higher survival rate in response to MG1 infection which may be associated with their lower rate of infection. Further experiments would be needed to determine the precise underlying mechanism(s) of eIF5B controlling MG1 propagation and unraveling the possible correlation between the level of available Bcl-xL protein and the rate of MG1 infection.

In addition to eIF5B, other proposed candidates substituting the function of eIF2 during its inactivation are eIF2D and DENR/MCT-1 complex (Holcik, M., 2015). It is worth investigating the possible role of eIF2D in mediating the expression of Bcl-xL and MG1 proteins with regard to the canonical function of eIF2D in delivering the initiator tRNA in a GTP-independent manner (Dmitriev, SE., et al., 2010). Besides, Multiple copies in T-cell lymphoma-1 (MCT-1) in association with DENR (density regulated protein) promotes the translation of certain mRNAs controlling cellular cycle and apoptosis through their interaction with the cap structure of mRNAs and the translation initiation complex (Holcik, M., 2015). It has been also observed that DENR/MCT-1 complex could enhance HCV IRES-mediated translation in an eIF2-independent mechanism (Skabkin, MA., et al., 2010), thus it may also play a similar role during MG1 infection.

Conclusion:

At the outset, we hypothesized that MG1 primarily controls the host global translation initiation by modulating the phosphorylation of eIF2 α through the kinase activity of PKR and/or PERK. By monitoring the phosphorylation status of 4E-BP1, we found that mTOR1-4EBP1-eIF4E axis may also contribute to the inhibition of protein synthesis in MG1-infected cell, even though eIF2 α inactivation had occurred prior to the de-phosphorylation of 4E-BP1 in our time course experiments. In addition, we showed a selective up-regulation of Bcl-xL mRNA translation during MG1 infection which was found to be regulated independently of XIAP protein expression. Due to the rapid shut-down of global translation, our data suggests that the infected cells switch to an alternate translation initiation pathway(s) to regulate selective translation of MG1 and distinct mRNAs such as Bcl-xL required by the virus or the host for the stress adaptation. Finally, we have identified a potential role for eIF5B in modulating MG1 propagation and Bcl-xL expression. In contrast to the translational function of eIF5B, we found down-regulation of Bcl-xL and MG1 protein expression are in fact mediated at transcriptional or post-transcriptional levels in the eIF5B-depleted cells. Overall, this study was the initial step to understand the strategies by which MG1 reprograms the host translation machinery, and uncover the mechanism of its propagation.

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