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Characterization of mammalian orthoreovirus (MRV) induced stress granules (SGs) and implications of eIF2α phosphorylation on viral translation

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Characterization of mammalian orthoreovirus (MRV) induced stress granules (SGs) and implications of eIF2α phosphorylation on viral translation

by

Qingsong Qin

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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2010

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ABSTRACT

Mammalian orthoreoviruses (MRV) are non-fusogenic, nonenveloped, icosahedral, RNA viruses, containing a 10-segmented double-stranded RNA genome, belonging to the family Reoviridae. Infection with many mammalian orthoreovirus (MRV) strains results in shutoff of host, but not viral, protein synthesis via protein kinase R (PKR) activation and phosphorylation of translation initiation factor eIF2α. When cells are under stressful environments, such as heat shock, oxidative stress, nutritional starvation, and viral infection, several kinases (PKR, PERK, HRI, or GCN) are activated, which phosphorylate eIF2α (S51), resulting in the formation of stress granules (SGs), discrete areas in the cytoplasm where cellular mRNAs are held in a translationally inactive state. We examined MRV-infected cells to characterize SG formation in response to MRV infection. We found SGs formed at early times following infection (2-6 h p.i.) in a manner dependent on phosphorylation of eIF2α. MRV induced SG formation in all four eIF2α kinase knockout cell lines, suggesting at least two kinases are involved in induction of SGs. Inhibitors of MRV disassembly prevented MRV-induced SG formation, indicating that viral uncoating is a required step for SG formation. Inactivation of MRV virions by ultraviolet (UV) light, or treatment of MRV-infected cells with the translational inhibitor, puromycin, did not prevent SG formation, suggesting that viral transcription and translation are not required for SG formation. Viral cores were found to colocalize with SGs, however, cores from UV-inactivated virions did not associate with SGs, suggesting viral core particles are recruited into SGs in a process that requires the synthesis of viral mRNA. These results demonstrate that MRV particles induce SGs in a step following viral disassembly but preceding viral mRNA transcription, and that core particles are themselves recruited to SGs, suggesting the
cellular stress response may play an inhibitory role in viral translation.

As infection proceeds, MRV disrupts SGs despite sustained levels of phosphorylated eIF2α, and further, interferes with the induction of SGs by other stress inducers. MRV interference with SG formation occurs downstream of eIF2α phosphorylation suggesting the virus uncouples the cellular stress signaling machinery from SG formation. We additionally examined mRNA translation in the presence of SGs induced by eIF2α phosphorylation dependent and independent mechanisms. We found that irrespective of eIF2α phosphorylation status, the presence of SGs in cells correlated with inhibition of viral and cellular translation. In contrast, MRV disruption of SGs correlated with release of viral mRNAs from translational inhibition, even in the presence of phosphorylated eIF2α. Viral mRNAs were also translated in the presence of phosphorylated eIF2α in PKR-/- cells. These results suggest that MRV escape from host cell translational shutoff correlates with virus-induced SG disruption, and occurs in the presence of phosphorylated eIF2α in a PKR independent manner.

In order to escape from host cell translational shutoff induced by eIF2α phosphorylation, MRV must use a translational strategy different from that used by most cellular mRNA. We have recapitulated a 10-plasmid-based reverse genetics system in our lab and have established inducible viral protein-expressing cell lines to examine whether unique genomic sequences in the viral mRNA, or alternatively, virally encoded proteins, play a critical role in viral translation at late times in MRV infection. Taken together, these studies have added significantly to our knowledge on viral-host interactions and regulation of viral translation in members of the Reoviridae family, and further, have laid the groundwork for important studies examining the mechanisms of tumor oncolysis by MRV.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Mammalian orthoreovirus (MRV) is a genus of *reoviridae* family that can infect all mammalian species, but is generally non-pathogenic to immune-competent humans or other animals that it infects (5). However, MRV is a potent and specific oncolytic agent that selectively kills ras-activated tumor cells over normal cells (9). MRV is also an ideal virus for the study of injury-induced encephalitis in the mouse model because the viral replication cycle has been thoroughly studied and can be dissected with different inhibitors (3). Infection of cells with MRV activates protein kinase R (PKR) and induces phosphorylation of the eukaryotic translation initiation factor eIF2 alpha subunit at the active site serine at amino-acid position 51 (6). eIF2α is a major component of ternary complexes (eIF2-GTP-tRNAiMet) which bind to the cellular 40S ribosome for the purpose of delivering the initiating methionine of nascent protein synthesis in the canonical model of eukaryotic translation. When phosphorylated, eIF2α has about 40 times higher affinity to eIF2B, a nucleotide exchange factor that normally serves to exchange GDP for GTP to reload ternary complex for a new round of translation. This increased affinity prevents nucleotide exchange and results in inhibition of ternary complex formation and subsequent translation initiation [in the review (1)]. Though most cellular translation is shutoff as a result of MRV-induced eIF2α phosphorylation, viral mRNA translation is unaffected (8). In recent work, eIF2α phosphorylation was also found to be sufficient to trigger the formation of distinct structures in stressed cells called stress granules (4). Stress granules are dynamic non-membranous cytoplasmic matrices, which sequester stalled 43S translation initiation
complexes containing cellular mRNA and associated translation initiation factors and ribosomal proteins together with RNA binding proteins TIAR, TIA-1, and G3BP (2, 10). Stress granules are thought to reprogram cellular translation and only allow stress response proteins to be translated when cells are under stress (11). A previous study found that MRV infection induces SGs at late times post-infection, suggesting that these structures might be related to host cellular translational shutoff following infection (7).

In our studies, we further examined the relationship between MRV infection and SG formation throughout infection by performing time-course experiments. We found that MRV infection induces SGs at early times post-infection in an eIF2α phosphorylation-dependent manner, at a step following virus uncoating and preceding viral transcription and translation. We additionally found that as infection proceeds and viral proteins accumulate, MRV interferes with SG formation at a step downstream of eIF2α phosphorylation. In order to understand the physiological impact of SGs on viral translation, we developed a new protein labeling technology to visualize protein synthesis in the presence and absence of SGs. We found viral translation correlates with SG disruption, regardless of the status of eIF2α phosphorylation and independent of PKR. These findings show, for the first time, that MRV induction of and escape from cellular translational shutoff are independent mechanisms. They also show that while MRV induction of host cell translational shutoff is PKR dependent, MRV escape from shutoff is PKR independent, and in fact, occurs in the presence of high levels of phosphorylated eIF2α. The question of how viral translation occurs in the global cellular environment of limited ternary complex that occurs in the presence of phosphorylated eIF2α has yet to be determined. In order to begin to answer this question, we have established a recently reported 10-plasmid-based reverse genetics system for MRV, as
well as created inducible cell lines expressing each MRV protein. These important reagents will allow us to test our hypotheses that specific viral RNA sequences or viral proteins play a role in MRV escape from host cell translational shutoff. This research has expanded our knowledge of the function of SGs in viral infection, the viral translation strategies in the host shutoff environment, and has laid important groundwork for future studies aimed at identifying mechanisms behind the oncolytic properties of MRV.

Dissertation organization

This dissertation includes an abstract and six chapters. The abstract describes findings in my research and my thoughts towards future study. Chapter 1 is a general introduction for the projects I worked on during my graduate study toward the Degree of Philosophy. Chapter 2 contains a literature review that describes some aspects related to my research projects. Chapter 3 was published in the Journal of Virology in 2009. Chapter 4 is prepared for publication. Chapter 5 describes a new strategy I have developed for future studies on my research project and will be included in a future publication. Chapter 6 is a general conclusion that describes where this research is leading and how it impacts other fields. At the end of chapter 2, 3, 4, and 5 are references, tables, figures and legends.

References


CHAPTER 2. A REVIEW OF MAMMALIAN ORTHOREOVIRUS PROPERTIES, VIRAL INFECTION, CELLULAR STRESS RESPONSE, AND VIRAL ONCOLYSIS

Introduction and significance

The non-fusogenic mammalian orthoreoviruses (MRV) are members of the family Reoviridae, which contains 9 genera that are classified according to structural similarity (spherical icosahedrons), nucleic acid type and composition (10-12 segmented double-stranded RNA), and replicative strategies. Viruses from this family infect a wide range of hosts including human, animals, plants, insects, and fish (49). Some are very important human, animal, and plant pathogens, such as rotaviruses which are the leading worldwide pathogen causing acute gastroenteritis in infants and young children, bluetongue viruses which cause serious hemorrhagic disease in livestock (sheep, goats, and cattle), and fijiviruses which cause significant crop failure and economic loss in rice and maize.

Mammalian orthoreovirus encloses a 10-segmented double-stranded RNA (dsRNA) genome within a non-enveloped, multilayered, icosahedral protein capsid (97). A growing amount of evidence suggests that MRV is a potent and specific oncolytic agent which preferentially kills ras-activated tumor cells over normal cells (124). Ras is member of a family of genes encoding small GTPases that are involved in cellular signal transduction. Deregulation of Ras signaling can ultimately lead to oncogenesis and cancer (86, 124). Studies have shown promising data suggesting reovirus selectively kills human colon cancer, ovarian cancer (56), breast cancer (99), pancreatic cancer (48), metastatic tumors (3), and glioma (117, 134). Reovirus has the potential to replicate in 80% of cancer cell types, as most tumor cells possess an activated ras-pathway (33, 86). Viral uncoating, translation of viral
mRNA, and virus-induced apoptosis is enhanced in ras-activated tumor cells (87). However, the precise mechanism of oncolysis by MRV is not clearly established. Research on virus-cell interactions, viral RNA replication, and translation will enhance our understanding of the mechanism by which reovirus selectively replicates in these tumor cells, and will further expand our knowledge on the basic transcription, translation, and replication strategies of this important viral family.

**Mammalian orthoreovirus properties**

**Structure of virions, subviral particles (ISVPs), and viral cores**

Mature reovirus particles, or virions, are environmentally stable, enclosing the ten-segmented dsRNA genome within an icosohedral structure consisting of eight proteins (45). The dsRNAs are protected not only by a densely packed core shell primarily formed by λ1 and σ2, but also by an outer capsid formed by tight σ3-μ1, λ2-σ3, and λ2-μ1 interactions. Virions (850 Å in diameter) can be converted into two distinct types of subviral particles, intermediate subviral particles (ISVPs) (800 Å in diameter), or cores (600 Å in diameter) (Fig. 1A) through the cleavage of outer capsid proteins by trypsin or chymotrypsin in vitro (23, 45) and cellular proteases (cathepsin L, and B) in vivo (9, 46). During natural infection, virions attach to cell surface receptors, enter cells through receptor-mediated endocytosis into the endosome where virions are transformed into ISVPs through the cleavage of σ3 and μ1 by proteases (cathepsin L and B) (Fig 1A) (46). ISVPs can then further penetrate endosomal membranes through pores formed by the cleaved N terminal domain of μ1 (1, 137), and further transform into core particles which do not have outermost capsid proteins (σ3 and μ1) (Fig 1A). Core particles are released into the cytoplasm, and do not further disassemble. Core particles are able to transcribe viral RNA in vivo and in vitro (12, 79).
ISVPs have lost the outermost capsid protein $\sigma_3$, but maintain a cleaved version of outer capsid protein $\mu_1$ ($\mu_1\delta/\delta + \phi$) and retain infectivity (24). Cores lose all outer capsid proteins, ($\sigma_3$, $\mu_1$, and receptor binding protein $\sigma_1$), and also lose the ability to penetrate cell membranes and infect cells. Transfecting cores directly into cells with tranfection reagents (eg. Lipofectamine) can generate new infectious virus (60). All three reovirus particle types have distinct morphologies that can be visualized by negative-stain electron microscopy and cryoelectron micrographs. Virions appear roughly spheroidal, with smooth perimeters; ISVPs appear even more spheroidal than virions, with long fibers ($\sigma_1$) extending from surfaces; and cores have prominent spikes ($\lambda_2$) protruding from their surfaces at axes of fivefold symmetry (98).

**Components of viral particles**

**Outermost capsid proteins**

Structural proteins $\mu_1$ and $\sigma_3$ (at a ratio of 1:1) form the lattice of the outer capsid of virions. $\sigma_3$ and $\mu_1$ bind to each other to stabilize the capsid structure (98). The $\mu_1$ protein is organized in trimeric complexes within virions and forms heterohexamers with $\sigma_3$ (Fig. 1B). $\sigma_3$ covers $\mu_1$ to prevent it from interacting with the cellular membrane (Fig 1A, B). Adhesin $\sigma_1$, in the form of trimers seen as a long fiber (40 nm) protruding from the 12 vertices of the icosahedral virion (Fig 1A), plays a role in cell attachment. The crystal structure of $\sigma_1$ reveals an elongated trimer with a compact head consisting of a beta-barrel fold which binds to the junction adhesion molecule 1 (JAM1) receptor on the cell surface, and a fibrous tail containing a triple beta-spiral which in turn plays a role in forming a trimer (Fig 1C) (26). Four positions between residues 415 and 447 contribute to forming the receptor-binding head
domain across the interface between two subunits. Antigen-specific immunoglobulin A or G binding to this receptor-binding domain prevents infection (55).

Following viral entry through receptor mediated-endocytosis, σ3 is cleaved by a variety of proteases including cathepsin L, which is an important step in viral disassembly. Two regions are hypersensitive to proteases at early steps of this processing. Carboxyl-proximal residues within σ3 are primary determinants of strain difference in viral entry, determining the rate at which early cleavage occurs. This indicates that proteolytic processing of σ3 during viral disassembly is a multi-step pathway (59). Cleavage of σ3 exposes the membrane penetration protein μ1 to endosomal membranes. Autolytic cleavage of μ1 divides it into myristoylated μ1N and μ1C. Dissociation of μ1N triggers a major conformational change of the entire μ1 trimer (24, 80). The myristoylated μ1N inserts and generates holes in the endosomal membrane to release viral core particles into cytoplasm (1, 57).

**Inner capsid proteins**

Inner capsid (core) proteins λ1 and σ2 form the primary icosahedral lattice of the inner capsid. λ2 (mRNA capping guanylytransferase and methyltransferase) protrudes and forms turrets from the surfaces of the core shell around each fivefold axis (20, 84). λ3 and μ2 (RNA-dependent RNA polymerase and putative polymerase cofactor) are located within the cage formed by λ1 and σ2 near the five axes (66, 125). λ1 and σ2 are expected to contact genomic RNA since both proteins can bind to double stranded RNA (43, 77). The 10 dsRNA genomic segments serve as templates for mRNA synthesis by the viral transcriptase (λ3) within the core. Each copy of λ3 anchors to the inner surface of the icosahedral core shell,
and primarily contacts three molecules of shell protein $\lambda_1$ and overlaps with a five-fold axis. Thus one copy of $\lambda_3$ is bound per vertex, transcribes viral RNA, and orients nascent RNA to pass into the large external cavity of the pentameric capping enzyme complex formed by the protein $\lambda_2$ (125, 138). $\mu_2$ is a divalent cation-dependent nucleoside triphosphatase that can remove the 5'-\gamma-phosphate from RNA. $\mu_2$ interacts with the RNA-dependent RNA polymerase ($\lambda_3$) \textit{in vitro}, and the presence of $\lambda_3$ mildly stimulates the triphosphatase activities of $\mu_2$ (66). $\lambda_2$ forms a hollow cylinder which sequesters mRNA to ensure completion of the capping reaction (108) which is performed by the methyl- and guanylyl-transferase activities of the $\lambda_2$ protein (20, 31, 85).

Once cap structures ($m^7GpppGpC$) are added at the 5' termini of the newly synthesized positive (+) RNA, the new viral mRNAs are released into the cytoplasm for translation of viral proteins. The newly synthesized inner core proteins ($\lambda_1, \lambda_2, \lambda_3, \sigma_2, \mu_2$) assemble into progeny core particles, and during this process, viral mRNAs serve as templates for the synthesis of progeny dsRNA. The newly assembled core particles can also synthesize mRNA for later translation. At some point, the core particles are further coated with three remaining viral outer capsid proteins ($\sigma_1, \mu_1$ and $\sigma_3$) to produce infectious progeny virions (98). The regulation of virion assembly is not well understood.

\textbf{Viral entry}

MRV infection begins with the attachment to a cell surface receptor junction adhesion molecule 1 (JAM-1) by the $\sigma_1$ protein that extends as a homotrimer from the 12 five-fold axes of the virion (14). $\alpha$-2-3-linked sialic acid conjugated to glycoproteins on the surface of M cells serves as a coreceptor for $\sigma_1$ attachment (13, 25). $\sigma_1$ is one of the determinants of
viral infectivity and tissue tropism (50). Following attachment, virions are internalized via receptor-mediated endocytosis (Fig 2). While the virions are within the acidic environment of the endocytic vesicles, the outer capsid protein σ3, in a heterohexameric complex with μ1, is proteolytically cleaved from the virion to generate ISVPs. Ammonium chloride (22) and E-64 (8) can prevent this transformation by either increasing the pH of endosomes to prevent protease digestion or directly inhibiting proteases. ISVPs, which contain cleaved μ1 as the primary outer surface protein, are then transformed into an additional particle type, the ISVP*, which results in the production of a hydrophobic conformer of μ1. During this transformation, μ1 undergoes a conformational change, the σ1 protein is released from the particle, and core particles are released into the cytoplasm (54). The ISVP to ISVP* transition is a necessary and transient step for membrane penetration and release of the transcriptionally active core particle into the cytoplasm (24).

**Viral transcription and translation**

After core particles are released into the cytosol, positive-sense RNA [(+) RNA] is transcribed from negative-sense genomic RNA [(−) RNA] in a 5’ to 3’ direction by the λ3 RNA-dependent RNA polymerase and transcriptase cofactor μ2 from within the parental core particles (125). μ2 has nucleoside triphosphatase activity that can remove the 5’ gamma-phosphate from RNA (66). Newly synthesized 5’ termini are moved through the turret-like structures formed by λ2 located near the five-fold axes of cores where newly transcribed RNAs are capped by enzymatic activities of λ2 (123, 138) and released into the cytoplasm. Reovirus mRNAs are not polyadenylated (11). Eukaryotic mRNAs have 5’ terminal cap structures and 3’ terminal poly (A) tails which bind to cap binding protein eIF4E (translational
initiation factor 4E) and poly (A) binding protein (PABP). Translation initiation factor eIF4G binds to eIF4E and PABP to form eIF4F complex, which circularizes mRNA. eIF4G acts as a scaffold to stabilize eukaryotic mRNA and recruit other translational initiation factors and ribosomal proteins for forming translation initiation complex in the cytoplasm [reviewed (132)]. The related *Reoviridae* family virus, rotavirus encodes a protein, NSP3, that can bind to 3’ termini of rotavirus mRNA and interact with eIF4G, probably playing a role in circularizing mRNA and stabilizing viral mRNA (42, 53, 106). NSP3 binding to eIF4G evicts polyA binding protein from cellular mRNA, suggesting NSP3 plays a role in host translational shutoff (96, 101, 105). However, knockout of NSP3 does not reduce viral translation (95), suggesting NSP3 binding to viral mRNA probably is not important for viral translation. So far no MRV counterpart of NSP3 has been reported, and how reovirus mRNA is stabilized and translated in the cytoplasm has not been determined.

Secondary, or “late” viral mRNA transcription, represents transcription by progeny subviral particles. Zarbl et al proposed that mRNAs synthesized at late time post-infection are uncapped, and hypothesized that the capping enzyme (λ.2) on progeny core particles is blocked by outer capsid proteins (136). Because reovirus infection induces the cell to shutoff translation, this hypothesis was used to explain how viral mRNAs, by virtue of being uncapped, are preferentially translated in infected cells during host translation shutoff (78). However, because structural data suggest genomic RNAs are capped (44), it is unlikely that uncapped mRNAs are packaged into progeny particles to serve as a template for negative-sense RNA synthesis. For this reason, whether all reovirus mRNAs are capped, or whether some mechanism exists to produce uncapped mRNAs during reovirus infection remains controversial.
The translation of individual reovirus transcripts is variable; nucleotide sequences of reovirus mRNAs at the -3 and +4 positions flanking the initiator AUG (Kozak sequence) and the length of 5’ terminal untranslational regions (UTR) contribute to differing translation efficiencies in vitro (71-74) so that viral protein synthesized is proportional to the needs for the assembly of virions.

**Viral genomic RNA replication and viral assembly**

Neither dsRNA or negative-sense RNA is found free in the cytoplasm, suggesting that the synthesis of complementary minus-strand RNA occurs either during the core assembly process or within assembled core particles (98). Some nucleotides in the 5’ and 3’ termini of the 10 genomic segments are conserved among strains of different serotypes (T1L, T2J, and T3D) as follows, 5’-GCTA………TCATC-3’, suggesting they serve as signals for genomic RNA assembly (103). The presence of three nucleotides, A-U-U, at positions 79-81 of the S2 gene are essential for the incorporation of *in vitro*-generated ssRNAs into new reovirus progeny viral particles (110). A minimum of 98 nucleotides at the 3’ terminus is required for S2 genome packaging (111). The 5’ and 3’ termini of M1 and L1 genes were also demonstrated to be important for genome packaging (112, 113). The assembly of the positive-sense RNA complements of MRV genome segments into cores (RNA assortment) occurs at the level of positive-sense RNA, and is thought to be a selective process for a number of reasons: i) the central cavity of the viral core cannot accommodate much more than the 10 unique genome segments, ii) all 10 of the segments must be present for the virus to be infectious, and iii) a particle to PFU (plaque forming unit) ratio as low as 1 has been reported. The mechanism by which viral (+) RNAs are selected for packaging is only beginning to be understood, but likely involves RNA-protein and RNA-RNA interactions. At
early times in infection, (+) RNAs representing each of the 10 viral genome segments have been isolated from infected cells in complex with the viral proteins σNS, μNS, and σ3, suggesting that these proteins may be involved in forming early RNA assortment complexes. At later times in infection, core particles that contain viral (+) RNAs can be isolated from infected cells, suggesting that one or more of the five core proteins also binds viral (+) RNAs. It is unclear at what step in the RNA assortment and core assembly process that the ssRNA is replicated to dsRNA.

**Viral factories**

Viral factories, also called inclusion bodies, were thought to be the places for the assembly of reovirus virions as electronic micrographs showed different sizes of viral particles (cores, incomplete particles, and complete virions) embedded in these viral factories (98). Early during reovirus infection, inclusion structures begin to form throughout the cytoplasm. As infection proceeds, these structures grow in size and migrate to the perinuclear region of the cell. In recent years, a number of studies have found that viral factories play important roles in regulating viral replication and translation. A key player in forming reovirus factories is the non-structural protein μNS encoded by the MRV M3 gene. When expressed in transfected cells, nonstructural protein μNS forms viral factory-like cytoplasmic phase-dense globular matrices (19). The carboxyl-proximal domains (amino-acids 471-721) including two predicted coiled-coil domains are necessary and sufficient for forming the viral factory-like matrix (16). N-terminal regions of μNS recruit other viral proteins (structural proteins λ2, λ3, λ1, μ2, σ2, and nonstructural protein σNS), as well as intact viral core
particles to viral factories (17, 90, 91). μNS binding to core particles does not inhibit the transcription and capping activities of cores (18).

μNS-formed viral factories colocalize with the cellular microtubule network (β-tubulin) and viral microtubule associated protein μ2. Inhibition of microtubule-formation with nocodazole prevents small cytoplasmic factories from coalescing into large perinuclear structures (7, 19, 102). Rare reovirus strains form globular viral factories, while most strains form filamentous structures. The globular or filamentous morphology of viral factories was mapped to the reovirus μ2 protein (19, 102). μ2 from globular strains is more prone to temperature-dependent mis-folding and therefore displays increased aggregation, increased levels of ubiquitinated μ2, and decreased association with microtubules (92).

Knockdown of μNS diminishes viral replication, viral translation, and inclusion formation. Complementation of μNS can restore viral replication, however, if the complementing μNS protein cannot form viral factories, replication is not restored (69). This strongly suggests viral factories play a crucial role in reovirus infection. For rotavirus, knockdown of NSP2, a component of viral factories, causes the decrease of positive (+) and double stranded RNA synthesis and viral assembly, while knockdown of structural protein VP7 has no effect on the synthesis of dsRNA. Immunofluorescence assays show dsRNAs are synthesized in viral factories (120). Some cellular proteins, like heat shock protein 70 (hsp70) and ubiquitin, are also located in viral factories (92). Because of the localization of all of the above viral components and cellular proteins within factories, it has long been suggested that many of the processes that are key to the successful replication of reovirus occur within these structures.
**MRV infection, the cellular stress response, and shutoff of host protein synthesis**

**stress granule formation**

Many types of external stress trigger phosphorylation of the alpha subunit of the translation initiation factor eIF2 through the activation of four individual kinases (PKR, PERK, HRI, or GCN). eIF2α phosphorylation prevents the formation of ternary complex, eIF2-GTP-tRNA\textsubscript{Met}, leading to the inhibition of translation of most cellular proteins, and a buildup of stalled ribosomal complexes (51). A consequence of this buildup is the formation of structures in the cytoplasm called stress granules (SGs), which sequester mRNAs and translation initiation factors in a translationally inactive state until the cells recover from stress or undergo apoptosis (5, 63). In the absence of cellular stress, eIF2α binds to GTP and initiator methionyl-tRNA (met-tRNA\textsubscript{i}), to form a ternary complex which subsequently binds to the 40S ribosomal complex to form the 43S preinitiation complex. mRNA bound to an additional initiation complex (eIF4F) then binds to the 43S complex to become the 48S initiation complex. At a later point in initiation, the eIF2-bound GTP is hydrolyzed to release all of the initiation factors from the ribosome that then continues with translation elongation. Following this release, the GDP bound to eIF2 must be exchanged for GTP in a reaction that is catalyzed by the guanine nucleotide exchange factor, eIF2B. Upon this exchange, eIF2-GTP can again bind Met-tRNA\textsubscript{i} to initiate a new round of translation. When the alpha subunit of eIF2 is phosphorylated, it changes from a substrate of eIF2B to a competitive inhibitor that binds eIF2B with high affinity, inhibiting the exchange of GDP for GTP. There is an excess of eIF2 relative to eIF2B in the cell, therefore, very little eIF2α phosphorylation results in the lack of initiator methionyl-tRNA (met-tRNA\textsubscript{i}) and reduced levels of translation initiation.
Another consequence of eIF2α phosphorylation is the induction of SGs (Fig 3) [reviewed in (4, 62)]. SGs also can be induced through an eIF2α phosphorylation independent pathway. For example, drugs that inhibit ribosome recruitment to initiation complexes (NSC119893) (94), hippouristinol, pateamine A (82), 15D-PGJ2 (68)) downstream of ternary complex recruitment or depletion of translational initiation factors [4B, 4H, or poly (A) binding protein] have been shown to induce stress granule formation independent of eIF2α phosphorylation (94). On the other hand, stabilizing the polysome with emetine can prevent SG formation (64).

SGs appear to serve as sites of mRNA triage that monitor the cellular environment to determine if a cell will undergo a path of repair or apoptosis. A number of proteins have been implicated as being important for directing mRNAs and proteins to and from SGs. The related proteins TIA-1 and TIAR, which play important roles in splicing and translational regulation (76), have both been implicated in SG formation (65). These proteins consist of three N-terminal RNA recognition motifs (RRMs), and a C-terminal prion-related domain (PRD). The N-terminal domain binds to several identified motifs, including poly-A, and is likely involved in binding the mRNAs that are recruited to SGs under times of stress. The C-terminal domain is capable of undergoing self-aggregation, and it is this characteristic that is thought to modulate SG assembly (52). Increasing the expression of chaperone heat shock protein hsp70 expression reduces the aggregation of PRD (65). Genetic knockout of TIA-1 leads to significantly increased levels of TIAR protein, and vice-versa, suggesting that the expression levels of each of these proteins is regulated by the other. Knockout of TIA-1 impairs, but does not prevent the formation of SGs, suggesting that TIAR plays some role in SG formation, but the increase in TIAR protein that results from TIA-1 knockout does not
completely compensate for the loss of TIA-1 in this capacity. A third protein, the RasGAP-associated endoribonuclease, G3BP, a protein that binds Ras and modulates its activity, has also been implicated in SG formation in a manner that is regulated by phosphorylation. Cellular stresses result in the dephosphorylation of serine 149 in G3BP, allowing this protein to assemble in SGs (127). A recent study showed that G3BP interacts with the tubulin deacetylase and ubiquitin binding protein, HDAC6, in a manner that is dependent on dephosphorylation of G3BP serine 149. Knockout of HDAC6 severely impairs SG formation, suggesting that HDAC6 association with G3BP may be required for G3BP and TIA-1/R accumulation in SGs (75). The inhibition of the ubiquitin-dependent proteasome system (UPS) also induces SG assembly (88). Furthermore, the deacetylase and ubiquitin binding domains of HDAC6 were both required for SG formation, supporting previous evidence that both the microtubule network and the ubiquitin:proteasome system play some role in SG formation and/or regulation.

**Virus infection, protein kinase R, and eIF2α phosphorylation**

Early studies showed that infection with most reovirus strains inhibits the translation of cellular mRNA while maintaining the translation of viral RNA. The innate immune response has been implicated in MRV-induced host translational shutoff via activation of the dsRNA kinase, PKR. Viral infection can stimulate the secretion of interferon which then signals the activation of PKR (10). PKR can also be activated by binding dsRNA, at which point it homodimerizes and undergoes autophosphorylation (83). Activated PKR phosphorylates serine 51 on the alpha subunit of the cellular translation initiation factor eIF2 (21), which prevents the ternary complex eIF2-GTP-tRNA_{Met} and results in translational inhibition. PKR plays an important role in interferon response to viral infection as mice lacking PKR
surrender to the lethal infection of vesicular stomatitis virus, and display increased susceptibility to influenza virus. Under environmental stress, phosphorylation of eIF2α inhibits most cellular translation; mRNAs whose expression is required for the response to cellular stress escape this inhibition in a variety of ways (118). The highly structured IRES (internal ribosome entry site) of Hepatitis C virus has more than 50% based-pair helices which leads to significant increase of autophosphorylation of PKR and phosphorylation of eIF2α. The activation of PKR inhibits cap-dependent translation but not viral translation (135). Internal ribosome-entry sites (IRESs) allow these mRNAs to escape the inhibitory effects of phospho-eIF2α in stressed cells, possibly by several mechanisms. For example, simian picornavirus recruits tRNAi^{Met} in an eIF2 independent manner (39). Hepatitis C virus recruits eIF2 and tRNAi^{Met} separately without forming ternary complex (eIF2-GTP-tRNAi^{Met}), which contributes to evasion of the interferon-induced antiviral response (109, 126). Third, alphavirus mRNA can also be efficiently translated in the presence of phosphorylated eIF2α through GTP-independent recruitment of Met-tRNAi^{Met} (131).

At late times post-infection, cellular translation is shut off in cells infected by most reovirus strains and viral proteins are the primary translation products (58). Although reassortment studies have suggested that the viral structural protein σ3 is the determinant of this phenotype, the functional differences between σ3 from strains that can prevent shutoff of host cell translation and those that cannot are not well-defined (15, 81). σ3 from all reovirus strains is a sequence-independent dsRNA binding protein which can functionally replace both vaccinia virus VAI RNA and adenovirus E3L protein, both known to be PKR inhibitors (115). It has been suggested that σ3 inhibits PKR by binding dsRNA. In reovirus strains that
do not induce host shutoff, it has been suggested that \( \sigma 3 \) is localized throughout the cell, preventing activation of PKR and subsequent shutoff. In strains of reovirus that cannot prevent host shutoff, it has been suggested that \( \sigma 3 \) is primarily localized in or near viral factories, therefore translation is predicted to be inhibited by cellular PKR activation in all places in the cell, except viral factories (115). How reovirus translation continues in an environment of limited ternary complex when eIF2\( \alpha \) is phosphorylated is otherwise poorly defined.

**Viral infection and stress granule formation**

Recently, a number of viruses have been shown to have some impact on SG formation and disruption, suggesting that SG perturbation may be a common mechanism for a wide variety of viruses to combat this aspect of the cellular stress response. Additionally, several viruses appear to benefit from their association with SG effector proteins. The West Nile virus 3’ stem loop of negative sense RNA is bound specifically by both TIA-1 and TIAR, and this association appears to be beneficial for viral genome synthesis (47). Although the exact function of TIA-1 and TIAR in WNV infection remains unknown, viral dsRNA and the non-structural protein NS3 have both been found to associate with the SG effector protein TIA-1/R and prevent SG formation. Further, WNV-infection prevents SG formation and also progressively prevents SG formation induced by sodium arsenite (89). A member of the alphavirus genus, Semliki Forest virus (SFV), has been shown to induce the phosphorylation of eIF2\( \alpha \) and transient SG formation (89). In the case of SFV, it has been reported that viral replication begins adjacent to SGs, and that SGs near replicating virus are disassembled, a process that appears to be concomitant with a switch from cellular to viral RNA translation (131). Another member of the alphavirus family, Sindbis virus (SV), also induces eIF2\( \alpha \)
phosphorylation, however, SV 26S subgenomic mRNA can be translated in the presence of phosphorylated eIF2α, using a cellular homologue eIF2A instead of eIF2α for translational initiation (114). A further study showed that 26S subgenomic mRNA can also be translated using canonical translation initiation out of the context of viral infection, while initiation can occur without some initiation factors (eg. eIF4G, eIF2) in the context of viral infection (104). Interestingly, Sindbis virus non-structural protein nsp3 has been shown to associate with G3BP, an important effector protein for SG formation, suggesting that this virus may additionally interfere with SG formation (35). Poliovirus has also recently been found to induce, then disrupt cellular SGs (133). G3BP was shown to be a target for the poliovirus 3C protease, which cleaves this effector protein, preventing the formation of SGs (133). Rotavirus, a Reoviridae family member, was also recently found to induce eIF2α phosphorylation, but disrupt SGs by a mechanism that remains to be determined (96). A previous study suggested reovirus induces SGs at late time post-infection in response to high levels of eIF2α phosphorylation, suggesting SGs are a possible mechanism for host shutoff at late times post-infection (122).

**Reovirus-induced apoptosis**

Reovirus-induced apoptosis has been thoroughly studied because the presence of apoptosis is strongly correlated with virus-induced injury in mouse models. MRV disseminates systemically in newborn mice following infection, and causes injury to a variety of organs (128, 129). The determinants of reovirus-induced apoptosis have been mapped to S1 and M2 gene segments by reassortant genetics. S1 and M2 encode cellular receptor binding protein σ1 and membrane penetration protein μ1 (130). Incubation of infected cells with anti-σ1, anti-μ1, or anti-σ3 monoclonal antibodies blocks viral uncoating and therefore prevents
apoptosis (34). The presence of protease inhibitor E-64 and ammonium chloride (NH₄Cl), which prevent viral uncoating, abolishes reovirus induced-apoptosis suggesting viral uncoating is essential for reovirus induced apoptosis (41). UV-inactivated reoviruses, which can bind and enter cells, but cannot replicate also induce apoptosis, indicating that viral binding and/or disassembly but not viral replication is required for apoptosis (27-30).

Several pathways are reported to be involved in reovirus-induced apoptosis, depending on the cell type or tissue that is infected. In epithelial cells, reovirus-induced apoptosis is initiated through activation of cellular transcription factors NF-κB and c-JUN and release of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (29). In neurons, reovirus induced apoptosis is mainly initiated through cell death receptors (DR4 and DR5) (40, 41). Calpain, a cytosolic cysteine protease, was reported to play an important role in induction of apoptosis, and inhibition of calpain protects against reovirus-induced apoptotic myocardial injury (70). Furthermore, reovirus-induced apoptosis has also been demonstrated to occur through mitochondrial-mediated, caspase-dependent pathways and death receptor pathways (37).

Some studies have shown JAM-1-independent, antibody-mediated entry of reovirus into cells or direct ISVP entry into cells leads to apoptosis, suggesting that attachment protein σ1 is dispensable for reovirus-induced apoptosis (32). Additional studies showed residues 582-611 within the C-terminal cleavage fragment of expressed μ1, or φ domain, are sufficient to induce apoptosis (36, 38). Mutations at Lys 594 and Ile595 within the φ domain of μ1 diminish apoptosis-inducing capacity of ectopically expressed μ1, while mutant viruses display no growth defect, suggesting μ1 plays an essential role in apoptosis. Furthermore,
mutant viruses containing these mutations in \( \phi \) produce less histopathologic injury than wild-type viruses (36).

**Reovirus oncolysis**

Reovirus has been studied as an oncolytic agent for some time for at least two reasons: (1) in otherwise isogenic cells, ras-transformation leads to susceptibility to reovirus infection where non-transformed normal cells are resistant, and (2) reovirus is considered an “ophan” virus because it is nonpathogenic to humans (98). However, the mechanism of MRV oncolysis is not clearly understood. Inhibiting the Ras/RalGEF/p38 pathway changes host cell permissiveness to reovirus, suggesting the Ras/Ral/p38 pathway benefits reovirus replication (100). Murine C26 colorectal carcinoma cells contain a mutant KrasD12 gene. Knockout of Kras D12 does not inhibit viral translation and virus yield, but reovirus-induced tumor cell apoptosis is completely abrogated as a result of K-ras knockdown, suggesting reovirus-induced apoptosis underlies the ras dependency of reovirus oncolysis (121). Marcato et al (2007) showed Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release (87). Many cancer cells also have increased expression of beta-catenin. Inducing beta-catenin expression by inhibiting glycogen synthase kinase (GSK)-3-beta sensitizes reovirus-induced apoptosis of colon cancer (93). In a clinical trial, reovirus was shown to kill small lung cancer cells when combined with chemotherapy drug taxane and other cancer therapeutic agents, and killing of cells was found to be due to accelerated apoptosis (116). These findings again suggest that reovirus-induced apoptosis is a possible mechanism for reovirus oncolysis. It has also been proposed that in untransformed NIH3T3 fibroblast cells activated PKR inhibits viral replication by phosphorylating eIF2\( \alpha \), resulting in blockage of viral protein synthesis. In ras-transformed
NIH3T3 cells, PKR phosphorylation and activity are impaired by signaling through activated ras, allowing viral translation (119, 124). This suggests viral translation is also important for viral oncolysis.

A recent study showed that cellular ischemia or hypoxia promotes eIF2α phosphorylation and SG formation (61). Tumor hypoxia is a major obstacle for radiation and chemotherapy because hypoxic tumor cells are resistant to apoptosis. The RACK1 protein plays a key role in this resistance by functioning as a mediator between the SG-assembly survival pathway and the stress-responsive MAPK cell death pathway. Hypoxia induced SGs sequester RACK1, which prevents apoptosis induction by X-rays and genotoxic drugs through the stress activated p38 and JNK MAPKKK (SAPK) pathways (6). Our data, which shows reovirus disrupts SGs late in infection, may suggest that reovirus-induced release of RACK1 from SGs may contribute to the mechanism of reovirus-induced oncolysis and synergistic killing of tumor cells when coupled with chemotherapy.

Recently, Prestwich et al (107) found reovirus failed to reduce tumor burden in severe combined immunodeficient mice bearing either B16ova or reovirus-sensitive B16tk metastases; however, both active reovirus and UV-inactivated reovirus purged the lymph node and splenic metastases in C57BL/6 mice by priming the specific antitumor cytotoxic lymphocytes, suggesting reovirus mediated oncolysis is not totally dependent on direct viral oncolysis or viral replication and immune-mediated anti-tumor activity is also involved in oncolysis. Some cell lines (eg. human HT1080 fibrosarcoma cells) are highly resistant to reovirus infection and reovirus persistently exists in some cells. Acquisition of resistance to reovirus raises a concern for cancer therapy (67), however, persistently infected cells can be killed within tumours upon rechallenge with reovirus (2).
References


reovirus strains to induce apoptosis are determined by the viral attachment protein σ1. J. Virol. 69:6972-6979.


Fig 1. Structure cartoons for viral proteins. (A) Reconstructed images from Cryo-EM pictures. Reovirus virion, intermediate subvirion particle (ISVP), core. Bar=20nm. This is adapted from Chandran et al (23). (B) Cartoon representations of crystal structures: σ3 monomer and dimer, μ1 monomer and trimer, and σ3–μ1 hexamer, alpha helices in hot pink, beta sheets in yellow, zinc ions in black, and beta-octylglucoside molecule in violet.
Images are from Max Nibert Lab at Harvard University medical school (http://nibertlab.med.harvard.edu/gallery). (C) Cartoon representation of crystal structures: σ1 trimer, each monomer is shown as blue, yellow, and red. This is adapted from Chappell et al (26).
Fig 2. The replication cycle of MRV. Following viral attachment, viruses enter cells through receptor-mediated endocytosis. Viral core particles are released into the cytoplasm without further disassembly. Cores transcribe viral mRNA for translation. New viral proteins are synthesized in the cytoplasm and form new viral core particles and possibly concurrently enclose viral mRNA into the cores for progeny genomic dsRNA synthesis. Newly assembled core particles launch secondary round of mRNA synthesis. Progeny virions are assembled in viral factories and released from by cell lysis.
Fig 3. The cellular stress response and stress granule formation. Many types of external stress trigger phosphorylation of the alpha subunit of the translation initiation factor eIF2 through the activation of four individual kinases (PKR, PERK, HRI, or GCN). eIF2α phosphorylation prevents the formation of ternary complex (eIF2-GTP-tRNA\textsubscript{Met}), leading to the inhibition of translation of most cellular proteins, and a buildup of stalled ribosomal
complexes. A consequence of this buildup is the formation of structures in the cytoplasm called stress granules (SGs), which further inhibit protein translation by sequestering mRNAs and translation initiation factors in a translationally inactive state until the cells recover from stress or undergo apoptosis (2). Three RNA binding proteins (TIAR, TIA-1, and G3BP) are thought to play key roles in stress granule formation.
CHAPTER 3. MAMMALIAN ORTHOREOVIRUS PARTICLES INDUCE AND ARE RECRUITED INTO STRESS GRANULES AT EARLY TIMES POST INFECTION

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Abstract

Infection with many mammalian orthoreovirus (MRV) strains results in shutoff of host, but not viral, protein synthesis via protein kinase R (PKR) activation and phosphorylation of translation initiation factor eIF2α. Following inhibition of protein synthesis, cellular mRNAs localize to discrete structures in the cytoplasm called stress granules (SGs), where they are held in a translationally inactive state. We examined MRV-infected cells to characterize SG formation in response to MRV infection. We found SGs formed at early times following infection (2-6 h p.i.) in a manner dependent on phosphorylation of eIF2α. MRV induced SG formation in all four eIF2α kinase knockout cell lines, suggesting at least two kinases are involved in induction of SGs. Inhibitors of MRV disassembly prevented MRV-induced SG formation, indicating that viral uncoating is a required step for SG formation. Inactivation of MRV virions by ultraviolet (UV) light, or treatment of MRV-infected cells with the translational inhibitor, puromycin, did not prevent SG formation, suggesting that viral transcription and translation are not required for SG formation. Viral cores were found to
colocalize with SGs, however, cores from UV-inactivated virions did not associate with SGs, suggesting viral core particles are recruited into SGs in a process that requires the synthesis of viral mRNA. These results demonstrate that MRV particles induce SGs in a step following viral disassembly but preceding viral mRNA transcription, and that core particles are themselves recruited to SGs, suggesting the cellular stress response may play a role in the MRV replication cycle.

**Introduction**

The non-fusogenic mammalian orthoreovirus (MRV) is a member of the *Reoviridae* family of segmented double-stranded RNA (dsRNA) viruses. The genome of MRV consists of 10 segments of dsRNA contained within a non-enveloped, multi-layered, protein capsid. During entry into cells, the outermost MRV capsid layer is removed by endosomal proteases, creating intermediate subvirion particles (ISVPs). ISVPs undergo an additional conformational change resulting in a particle (ISVP*) that is capable of penetration of the endosomal membrane. Coincident with cellular membrane disruption, the inner capsid, or core, is released into the cytoplasm (11). The core particle, which contains the viral polymerase, guanylyltransferase, and methyltransferase enzyme activities, transcribes mRNAs corresponding to each of the 10 viral genes in the cytoplasm (14, 20). MRV mRNAs are unique from cellular mRNAs in that they do not contain a 3′ polyA tail, but do have an m7GpppN cap structure on their 5′ end (6). As infection proceeds, distinct viral structures, termed viral factories (VFs), form in the cytoplasm primarily through the action of the non-structural protein, μNS, which constitutes the structural matrix of the factories (8, 10). Core particles, viral proteins, newly synthesized viral mRNA, and dsRNA are localized within VFs, suggesting that transcription, replication, and assembly of progeny viral core particles
occur within these structures (7, 8, 10, 35, 36).

Infection with MRV has been shown to induce phosphorylation of the α subunit of the translation initiation factor eIF2 (39, 45, 47, 55), a modification that inhibits host cell translation initiation by preventing the formation of ternary complex (eIF2/GTP/tRNA\textsubscript{Met}) [reviewed in (42, 44)]. In the case of MRV infection, phosphorylation of eIF2\textsubscript{α} is associated with the activation of protein kinase R (PKR) (55). PKR activation and subsequent eIF2\textsubscript{α} phosphorylation, together with the interferon (IFN) regulated 2’,5’-oligoadenylate synthetase RNAse L system, are necessary for MRV-induced host cell translation shutoff (48). MRV mRNA continues to be translated following shutoff of host cell translation, although the mechanism for escape is not well understood. Moreover, studies have indicated that MRV replication may benefit from this aspect of the cellular response to infection by demonstrating that MRV replication is more efficient in the presence of PKR and phosphorylatable eIF2\textsubscript{α} (47).

An additional level of cellular translation regulation has recently been identified. Treatment of cells with drugs that inhibit various aspects of protein translation (32, 38), or other external stress (such as nutrient starvation, heat shock, oxidative stress, or viral infection), that lead to the phosphorylation of eIF2\textsubscript{α} by specific kinases [PKR, PKR-like endoplasmic reticulum kinase (PERK), general control non-derepressible kinase (GCN), Heme-regulated inhibitor kinase (HRI)], is sufficient to trigger the formation of distinct structures in the cytoplasm termed stress granules (SGs) [reviewed in (3, 24)]. SGs sequester stalled 43S pre-initiation complexes (including 40S ribosomes, mRNAs, and many translation initiation factors) in a translationally inactive complex to hold non-stress related cellular translation in check. Once the stress is removed, SGs are disassembled and the
translational material is released for reuse [reviewed in (24)]. The translational silencing proteins, T cell intracellular antigen 1 (TIA-1) and TIA-1 related protein (TIAR) play a role in SG aggregation. These proteins contain three N-terminal RNA recognition motifs (RRM), and a C-terminal domain that resembles the aggregation domain of prion proteins (prion-related domain, or PRD) (21). The N-terminus of TIA-1/TIAR presumably binds to mRNA through its RNA recognition domains and sequesters the mRNAs and associated translation initiation factors and small ribosomal subunits into SGs by auto-aggregation of the prion-like C-terminal PRD of TIA-1/TIAR [reviewed in (3)]. Additional proteins, including Ras-GAP SH3 domain binding protein (G3BP) (51), tristetraprolin (TTP), Fragile X mental retardation-related protein (FMRP), as well as many others, have been implicated in SG formation [reviewed in (1)].

Many viruses alter the normal course of SG induction during their replication cycle. For poliovirus, viral infection induces transient SG formation at an early phase of infection (2-6 h p.i.), and later disrupts SGs by cleaving SG component G3BP with poliovirus 3C proteinase (54). Semliki Forest virus (SFV) infection results in the shutoff of host protein synthesis largely due to activation of the cellular stress response via phosphorylation of eIF2α. SFV infection also induces transient SG formation during the early phase of infection (2-5 h p.i.). Later, SGs are dispersed by an unknown mechanism (34). West Nile Virus disrupts SG formation by utilizing SG-effector proteins TIA-1 and TIAR during RNA replication within viral replication complexes (17). A previous study reported that MRV infection induces SGs in a strain-specific manner at late times post infection (19.5 h p.i.) that correlates with the extent of host translation shutoff and eIF2α phosphorylation (47). Whether SGs are hurdles that must be overcome during viral translation has not yet been
fully determined.

In this study we found cells infected with MRV transiently form SGs at an early phase of infection (2-6h.p.i.), which dissociate as infection progresses. eIF2α phosphorylation was found to be necessary for MRV induction of SGs, however, a single eIF2α kinase necessary for this phosphorylation was not identified. Utilizing known pharmaceutical inhibitors of virus disassembly and translation, as well as UV-inactivated virus, we identified the steps in the viral life cycle that are necessary or dispensable for SG induction. Finally, we show that MRV core particles are recruited to SGs in a manner that depends on synthesis of viral mRNA.

Materials and methods

Cells and reagents. CV-1 (African Green Monkey kidney fibroblast), HeLa (human cervical cancer cell), MEF (mouse embryonic fibroblast), PKR−/−, PERK−/−, GCN−/−, HRI−/−, MEFS51S/S51S and MEFS51A/S51A cells were maintained in DMEM (Dulbecco's modified essential medium, Invitrogen) containing 10% fetal calf serum (Atlanta Biologicals) and penicillin/streptomycin (100 IU/mL) (Mediatech). DU-145 cells were maintained in EMEM (Eagle’s modified essential media, Invitrogen) containing 10% fetal calf serum (Atlanta Biologicals), and penicillin/streptomycin (100 IU/mL). Spinner adapted L929 cells were maintained in c-MEM (Joklik's minimal essential medium, Sigma-Aldrich) containing 2% fetal calf serum, 2% fetal bovine serum (HyClone Laboratories), 2 mM L-Glutamine (Mediatech) and penicillin/streptomycin (100 IU/mL) (Mediatech). Primary antibodies used in immunofluorescence and immunoblotting assays are as follows: goat polyclonal α-TIA-1 antibodies (sc-1751), goat polyclonal α-TIAR antibodies (sc-1749) (Santa Cruz Biotechnology, Inc.), rabbit polyclonal α-eIF2α antibodies (A300-721A), rabbit
polyclonal α-eIF3 antibodies (A300-376A) (Bethyl Laboratories), rabbit polyclonal α-eIF4G antibodies (#2498), rabbit polyclonal α-eIF4E antibodies (#9742), and rabbit polyclonal α-phospho-eIF2α (Ser51) antibodies (#9721) (Cell Signaling Technologies). Mouse monoclonal antibody (7F4) against MRV structural protein λ2, rabbit polyclonal α-μNS antiserum, and rabbit polyclonal α-MRV core antiserum have been previously described (9, 53). Rabbit antiserum against DCP1a has been described (26). Secondary antibodies used in immunofluorescence and immunoblotting experiments are as follows: Alexa 488- or Alexa 594-conjugated donkey α-mouse IgG antibodies, Alexa 488-conjugated donkey α-goat IgG antibodies, Alexa 350- or Alexa 594-conjugated donkey α-rabbit IgG antibodies (Invitrogen), and HRP-conjugated goat α-rabbit IgG antibody (Invitrogen). Sodium Arsenite (SA) (Sigma-Aldrich) was used at a final concentration of 0.5 mM. E-64 (Roche) was used at a final concentration of 2 mM. Ammonium chloride (NH₄Cl) was used at a final concentration of 2 mM. Puromycin (Invivogen) was used at final concentration of 0.1 mg/mL.

**Virions and Intermediate Subvirion Particles (ISVPs).** MRV stocks (T1L, T2J, T3D⁺) were obtained from Dr. Max Nibert (Harvard Medical School, Boston, MA). The superscript C in T3D⁺ is used to differentiate the T3D strain used in this study from another T3D strain (T3D⁻) that has previously been shown to differ in M1 gene sequence, factory morphology, and μ2 ubiquitination phenotype (37). Purified virions were prepared as described (12) and stored in virion buffer (150 mM NaCl, 10 mM Tris pH 7.4, 10 mM MgCl₂) at 4 °C. ISVPs were prepared as previously described (12, 41). Titers of purified virus were determined by standard MRV plaque assay in L929 cells (19). UV light-inactivated virions were prepared as
follows: MRV virions were diluted in virion buffer to a final titer (5.0 × 10⁹ pfu/ml), 0.9 mL/well, in 6-well cell culture plates. The viral solution was exposed to UV light with the intensity of 1.0 Joules/cm². The resulting virions were shown to retain viral entry by immunofluorescence assay, and additionally confirmed to be completely deficient in virus replication by plaque assay.

**Infection.** 1.0 × 10⁵ or 2.0 × 10⁵ cells were seeded onto 12-well cell culture plates or 35 mm cell culture dishes the day before infection. Cells were infected with MRV virions, ISVPs or UV-inactivated virions at 10, 100 or 1000 pfu/cell as indicated in each experiment. Viral particles were diluted in phosphate buffered saline (PBS) [137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ (pH 7.5)] containing 2 mM MgCl₂, and adsorbed to cells for 1 h, at which point, cells were overlayed with DMEM and incubated at 37°C until harvested.

**Immunofluorescence (IF) assay.** Cells were seeded on six-well (9.6-cm²) dishes containing 18-mm-diameter coverslips at a density of 2 × 10⁵ cells/well, then incubated overnight at 37°C. At indicated times p.i., cells were fixed at room temperature for 10 min with 2% paraformaldehyde in PBS and then washed three times with PBS. Fixed cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min and then washed three times with PBS. Samples were blocked for 10 min with 2% bovine serum albumin (BSA) in PBS. Primary and secondary antibodies were diluted in 2% BSA in PBS. After blocking, cells were incubated for 1 hour with primary antibody, washed three times with PBS, and then incubated for an additional hour with secondary antibody. Immunostained cells were washed a final three times with PBS, and mounted on slides with ProLong™ reagent with or without DAPI (4’,6-diamidino-2-phenylindole dihydrochloride) (Invitrogen). Immunostained samples were examined with a Zeiss Axiovert 200 inverted microscope equipped with
fluorescence optics. Confocal images were taken on a Leica SP5 X confocal microscope. For each field selected, a total of 35 0.1 µm serial sections were taken horizontally. Images were prepared using Photoshop and Illustrator software (Adobe Systems).

**Immunoblotting assay.** 2.0 × 10^5 cells were seeded on 35 mm dishes the day before infection and then infected with viable and UV light-inactivated MRV virions as indicated. Infected cells were harvested at different times p.i., and lysed with 100 µl lysis buffer (200 mM Tris pH 8.0, 137 mM NaCl, 10% Glycerol, 1% NP-40). Protein concentrations for each sample were measured by EZQ® protein quantitation reagent (Invitrogen), and equivalent amounts of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose by electroblotting in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol [pH 8.3]). Nitrocellulose containing transferred proteins was blocked for 15 min with 5% non-fat skim milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl [pH 7.6]) containing 0.1% Tween-20 (TBS-T) and then incubated overnight with primary antibodies in TBS-T containing 1% milk. Blots were washed three times for 15 min each with TBS-T, followed by a 4 h incubation with HRP-conjugated secondary antibodies in TBS-T containing 1% milk. Blots were washed a final three times and exposed to Western Lightning ™ plus–ECL enhanced chemiluminescence substrate (Perkin Elmer). Images were collected using a ChemiDoc™ XRS camera (Bio-Rad), and protein bands were quantified using Quantity-One software (Bio-Rad). At least two independent experiments were performed, and a representative experiment is shown.

**SG quantification.** Infected cells were immunostained at 2 or 4 hours p.i. as indicated in each experiment. More than 10 fields were randomly chosen and counted, with at least 200
cells counted for each sample slide. MRV core positive cells were recorded as infected cells, and SG-containing cells were counted from within the infected cell samples. The percentage of SG-containing cells in infected and uninfected cells was calculated and Pearson’s Chi-squared test ($X^2$) was applied to examine if there was a significant difference in SG formation between infected cells and non-infected cells. At least two independent experiments were performed, and a representative experiment is shown.

Results

**MRV infection induces cytoplasmic structures containing SG marker proteins at early times p.i.** A previous study found that infection with MRV strains c87 and c8, and T3D induces SGs to differing degrees in the DU-145 prostate cancer cell line at late times p.i. (19.5 h p.i.) (47). To further explore the ability of MRV to induce SGs, we infected HeLa, CV-1, MEF and DU-145 cells with purified virions from MRV strains T1L, T2J, and T3D$^C$. Mock infected cells, and cells treated with the known SG inducer, sodium arsenite (SA), served as negative and positive controls (data not shown). SA induces SGs in 98% of treated cells through activation of the heme-regulated inhibitor kinase (HRI) and phosphorylation of eIF2$\alpha$ (33). Cells were infected with virus at 100 pfu/cell or 1000 pfu/cell, then examined throughout infection for cytoplasmic granules containing SG marker proteins using immunofluorescence microscopy. At early times p.i. (2-6 h), we found an increased number of T2J- and T3D$^C$-infected cells which contained cytoplasmic granules positive for SG-marker proteins G3BP, TIA-1, or TIAR compared to mock infected cells (Fig 1, and data not shown). We quantified the number of SG-containing cells following T3D$^C$ virion infection (1000 pfu/cell, 4 h p.i.). We found that 34.2% of infected HeLa cells, 21.0% of infected CV-1 cells, 35.6% of infected MEF cells, and 34.0% of DU-145 cells contained putative SGs,
whereas no uninfected HeLa, CV-1, or DU-145 cells and only 0.4% of uninfected MEF cells contained putative SGs. Pearson’s Chi-squared test ($X^2$) ($p<0.005$) showed that infection of these four cell types with MRV T3D$^C$ virions at 1000 pfu/cell induces SG formation in a significantly increased number of cells compared to non-infected cells at early times p.i.

Unlike T2J and T3D$^C$ virion-infected cells, we did not observe putative SGs in T1L virion-infected cells, even when the pfu/cell was increased to 10,000 (data not shown). We hypothesized that this was due to delayed entry of T1L virions into these cells based on a large decrease in cells containing viral core particles at early times p.i. as measured by immunofluorescence assay using antibodies specific for MRV core particles (data not shown). In order to clarify this, we repeated these experiments using T1L, T2J, and T3D$^C$ ISVPs, which are predicted to directly enter the cell through membrane penetration. Similar to our findings with T2J and T3D$^C$ virions, all T1L, T2J, and T3D$^C$ ISVPs induced cytoplasmic structures that stained with SG-marker proteins in HeLa, CV-1, and MEF cells at early times p.i. (Fig. 2A and data not shown). This suggests that the inability of T1L virions to induce SGs is based on a deficiency in virus entry in these cell types. Furthermore, MRV ISVPs induce SGs in a dose-dependent manner (Fig. 2B), with the number of SG-positive cells increasing with viral particle number used to infect the cells. We found that at later times in virus infection (8-24 h p.i.), as viral protein translation increased and viral factories became the prominent structures within the cytoplasm, SGs were no longer present in any tested cell type infected with 1, 10, 100, or 1000 pfu/cell with T1L, T2J, or T3D$^C$, unlike the previous report that MRV induces SGs at late times p.i. (Fig. 5B, Fig. 6, top right panel, and data not shown) (47).

**MRV-induced SGs are structurally similar to SGs induced by eIF2α**
phosphorylation. Because there are a growing number of cytoplasmic structures that form in cells in response to different stresses [reviewed in (3)], we further characterized SGs induced by MRV infection to determine if they contained components, such as translation initiation factors (eIF4G, eIF4E, and eIF3), that are characteristic of SGs induced by phosphorylation of eIF2α, and other types of protein synthesis inhibition (25). HeLa cells were infected with ISVPs (T1L, T2J, and T3D<sup>C</sup>) or MRV virions (T2J, T3D<sup>C</sup>) with 1000 pfu/cell. At 2 h p.i. or 4 h p.i., cells were fixed for three-color immunofluorescence using mouse antibody (7F4) against viral core protein λ2 to visualize infected cells, goat antibodies against TIAR or TIA-1 to visualize SGs, and rabbit antibodies against eIF4G, eIF4E, or eIF3 to visualize translation initiation factors. Mock cells, and sodium arsenite (SA)-treated cells were used as negative and positive controls for SG formation (data not shown). SG effector proteins TIAR (or TIA-1) colocalized in independent experiments with eIF3 (Fig. 3, rows 1 and 2), eIF4G (Fig. 3, row 3), and eIF4E (Fig. 3, row 4) in the granules that formed in MRV-infected cells. As a negative control for SGs, we examined the localization of DCP1a, a marker for processing bodies (PBs) (26), and found that this protein did not localize in the structures induced by MRV infection and was found instead in small separate structures that are likely PBs (Fig. 3, row 5). These results indicate MRV-induced SGs are structurally similar to those induced by inhibition of protein translation initiation and eIF2α phosphorylation.

Viral uncoating is required for MRV induction of SGs. To unravel the steps in MRV infection that are necessary for induction of SGs, we examined the effects of previously described pharmaceutical inhibitors of viral uncoating (ammonium chloride and E-64) on the ability of MRV infection to induce SGs. It has previously been shown that these drugs are able to block viral uncoating by changing the pH of endosomes (ammonium
chloride) or blocking endosomal protease activity (E-64) (5, 49). HeLa cells were pretreated with 2 mM ammonium chloride or 2 mM E-64 for 4 h preceding infection. Following this incubation, cells were infected with T3D<sup>C</sup> virions (1000 pfu/cell), and incubated for 1 h at room temperature, then re-treated with ammonium chloride or E-64 at the above concentrations. At 4 h p.i. and 8 h p.i., cells were fixed and stained with antibodies to detect viral cores and SGs. In contrast to untreated, infected cells that contain both core particle staining and SGs (Fig. 4, row 3), neither core particle staining or SGs were observed in the ammonium chloride- or E-64-treated, infected cells at 4 h p.i. (Fig. 4, rows 1 and 2). Control experiments indicate that ammonium chloride or E-64 do not induce SGs or prevent SG formation induced by SA (Fig. 4, rows 4 and 5). At 8 h p.i., MRV virions were still unable to uncoat and induce SGs (data not shown). These results suggest viral uncoating is required for SG induction.

**UV-inactivated MRV virions induce SGs.** In order to examine whether gene expression is involved in SG induction, we examined the ability of UV-inactivated virions to induce SGs. T3D<sup>C</sup> virions were inactivated by UV light treatment as described in Materials and Methods. HeLa cells were infected with untreated T3D<sup>C</sup> virions, or UV-treated T3D<sup>C</sup> virions and at 0, 2, 4, 6, 12, and 24 h p.i., cells were fixed and immunostained to detect viral core particles and SGs. We found that similar to untreated virions (Fig. 5A, row 1), UV-inactivated virions were able to enter cells and to induce SGs (Fig. 5A, row 2). Additionally, we quantified the number of SGs that were induced by active versus inactive viruses over time (Fig. 5B). Interestingly, UV-inactivated virions induced higher percentages of SG-containing cells throughout MRV infection in comparison to viable MRV infected cells (Fig. 5B). In fact, by 12 h p.i., less than 5% of cells infected with active MRV contained SGs,
whereas, nearly 40% of cells infected with UV-inactivated virus still contained SGs at this time. By 24 h p.i., no actively infected cells contained SGs, while 20% of cells infected with UV-inactivated MRV still contained SGs. These results suggest that SG induction is mainly due to viral entry events. Moreover, because cells containing SGs appear to rapidly decrease as viral mRNA is transcribed and translated in actively infected cells, but remain present in a large number of cells infected with virus that is unable to express protein, these results also suggest that viral gene expression may be involved with SG disruption as viral infection progresses. The reason for the decline over time of SG-containing cells following infection with UV-inactivated virus remains to be determined.

Translation is dispensable for SG induction, but required for SG disassembly in MRV infected cells. To further examine the role of viral mRNA transcription and translation in MRV induction of SGs, we examined MRV-induction of SGs in the presence of a known pharmaceutical inhibitor of translation (puromycin). Puromycin is a general inhibitor of cellular and viral protein translation that inhibits translation elongation by forming methionyl-puromycin (31). HeLa cells were treated with puromycin for 1 h preceding infection. Following this incubation, cells were infected with T3DC virions at 1000 pfu/cell, and incubated for 1 h at room temperature, at which point cells were re-treated with puromycin at the above concentration. At 10 h p.i., cells were fixed and stained with antibodies to detect viral cores and SGs. In untreated, infected cells, at 10 h p.i., rabbit α-core antiserum stained viral factories, and only 2.2% of infected cells formed SGs (Fig. 6A, row 1), confirming our previous observations that SGs dissipate as MRV infection proceeds. In puromycin-treated, infected cells, no viral factories were present as a result of puromycin translation inhibition, however, 88.3% of cells contained SGs (Fig. 6A, row 2) at this time. In
uninfected, puromycin-treated cells, only 2.5% of cells formed SGs (Fig. 6A, row 3, left). Similar to previously reported data (50), puromycin treatment did not prevent SA-induction of SG, and 99.0% of SA-treated cells contained SGs (Fig. 6A, row 3, right). Immunoblots against MRV non-structural protein \( \mu \text{NS} \) confirmed no MRV protein synthesis in the puromycin treated, infected cells at 10 h p.i., while untreated cells contained significant levels of \( \mu \text{NS} \) synthesis (Fig. 6B). These results suggest that viral translation is not required for MRV-induction of SGs. They further suggest, similar to our results with UV-treated virions, that when viral protein synthesis is inhibited, the SGs that form in response to MRV infection are not disrupted. This again implicates a role for MRV protein expression in SG disruption as viral infection proceeds from early to late times.

**eIF2\( \alpha \) phosphorylation correlates with, and is required, for MRV-induction of SGs.** Many lines of evidence suggest that eIF2\( \alpha \) phosphorylation is sufficient to induce SGs (29), however, a number of drugs and small molecules have been described that can induce SGs independently of eIF2\( \alpha \) phosphorylation (38). These include the small molecule NSC119893, which inhibits the interaction between eIF2\( \alpha \) and Met-tRNA (38), and the drugs pateamine (15) and 15D-PGJ2 (28), which bind and inhibit translation initiation factor eIF4A. To determine if MRV induction of SGs correlates with phosphorylation of eIF2\( \alpha \), we examined the relative levels of phosphorylated eIF2\( \alpha \) compared to total cellular eIF2\( \alpha \) during MRV infection at times in which SGs were present. HeLa cells were infected with untreated or UV-treated T3D\(^C\) virions, and at 0, 2, 4, and 6 h p.i. samples were collected. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against MRV non-structural protein \( \mu \text{NS} \), total eIF2\( \alpha \), and phosphorylated eIF2\( \alpha \) (Fig. 7A). These
experiments show that the level of phosphorylated eIF2α increases in both active virus-infected cells (Fig. 7A, left panel) and UV-inactivated virus-infected cells (Fig. 7A, right panel) at times when SGs are routinely found in MRV-infected cells. This suggests MRV-induced SG formation may occur as a result of eIF2α phosphorylation.

In order to examine whether phosphorylation of eIF2α is required for MRV induction of SGs, we utilized a cell line, MEF^{S51A/S51A}, in which the eIF2α gene is genetically altered such that the serine residue at amino acid 51 is changed to an alanine. This results in loss of the phosphorylation event that is necessary to inhibit ternary complex formation (46). MEF^{S51A/S51A} are unable to form SGs following sodium arsenite treatment, which activates eIF2α phosphorylation through the HRI kinase, but are able to form SGs following treatment with NSC11893 (38), pateamine A (15), or 15D-pGJ2 (28). Wildtype MEF^{S51S/S51S} and mutant MEF^{S51A/S51A} cells were infected with ISVPs (T1L, T2J, or T3D^C) or virions (T2J, T3D^C) at 1000 pfu/cell. At 2 h p.i. or 4 h p.i., cells were fixed and stained for immunofluorescence with antiserum against MRV cores to visualize infected cells, and antibodies against TIAR to visualize SGs. In these experiments, although we counted varying numbers of SG-containing cells in infected wildtype MEF^{S51S/S51S} cells (ranging from 13%-54% depending on virus strain, and particle type used), we did not detect any infected cells containing SGs in the mutant MEF^{S51A/S51A} cells (Fig. 7B and data not shown). These findings suggest that SG induction by MRV occurs through a pathway that requires eIF2α phosphorylation.

At least two eIF2α kinases are involved in MRV induction of SGs. Because our data suggested that eIF2α phosphorylation is required for SG formation at early times in
infection, we were interested in further identifying the cellular pathway involved in MRV induction of SGs. Four kinases have been identified that phosphorylate eIF2α in response to different cellular stresses. These include PKR, which is activated by viral infection, PERK, which is activated by protein misfolding in the endoplasmic reticulum (ER), GCN, which is activated by nutrient deprivation, and HRI, which is activated by oxidative stress [reviewed in (3)]. We utilized a knockout cell line for each of the four eIF2α kinases (PKR-/-, PERK-/-, HRI-/-, GCN-/-) to determine if knockout of any individual stress kinase results in loss of SG formation following MRV infection. Each cell line was infected with T3D<sup>C</sup> at 1000 pfu/cell, and at 4 h p.i., cells were fixed and immunostained to visualize viral cores and SGs. We found that each individual knockout cell line was able to form SGs in response to MRV infection with 36.5 % GCN-/- cells, 40.1 % PERK-/- cells, 19.1 % PKR-/- cells, and 13.2 % HRI-/- cells containing SGs (Fig. 8, left and middle columns). Only rare cells contained SGs in these cell lines when they were mock-infected (Fig. 8, right column). These results indicate that at least two eIF2α kinases can be activated to phosphorylate eIF2α by MRV infection at early times p.i., and suggest that the signaling pathway leading to SG induction by MRV infection is more complex than activation of a single eIF2α kinase.

**Viral core particles are recruited to SGs in a transcription-dependent manner.** In our study, we found that in MRV-infected, SG-containing cells, a portion of viral core particles localized to SGs at early times p.i. (Fig. 1, 2, 3, 4, 5, 6, 7, 8). This colocalization was variable depending on cell type and experiment, however in each experiment, core particles were visualized in SGs. In order to confirm these findings, we examined MRV core and SG localization using confocal microscopy. HeLa cells were infected with T1L ISVPs, and at 2 h p.i., cells were fixed and stained with antibodies against MRV cores and TIAR to
visualize core particle localization relative to SGs. Confocal images confirmed that many, but not all, viral core particles are intensely localized in SGs induced by MRV (Fig. 9A). To determine whether gene expression is necessary for the localization of viral cores to SGs, we examined the localization of viral core particles in UV-inactivated infected cells, and puromycin-treated, infected cells. UV irradiation is expected to prevent viral transcription (and in our studies, we found no evidence of protein translation or replication following UV-treatment of particles; see Fig. 7A, and data not shown). Puromycin is not expected to prevent viral transcription but is expected to prevent viral translation (and in our studies we did not detect viral protein translation following puromycin-treatment, see Fig. 6B) (31). In these experiments, we found that in the cells infected with UV-inactivated viruses, no viral core particles colocalized in SGs (Fig. 9B). However, in the puromycin-treated, infected cells, although not all viral core particles localized to SGs, by 10 h p.i, almost all cells formed SGs which contained viral core particles (Fig. 9C). These findings suggest that the localization of viral core particles to SGs may be mediated by newly synthesized viral mRNA. The absence of complete colocalization of core particles with SGs is likely a result of two issues. First, virion preparations always contain a portion of viral particles defective in viral transcription, and second, some cores are likely not actively synthesizing viral mRNA at the moment when cells were fixed for immunostaining. While viral core particles may be targeted to SGs via viral mRNA, it has yet to be determined whether viral mRNA, like cellular mRNA, is translationally silent when cores are sequestered in SGs.

Discussion

**Viral entry is required for MRV-induction of SGs.** SG formation is a defensive mechanism used by host cells in response to many types of external or internal stresses (2).
The size and number of SGs within cells are dependent on the intensity of different stresses. For example, SA induces SGs in a dose-dependent manner, with a lower dose of SA inducing fewer and smaller stress granules, and vice versa (2). In these studies, we also found that the number of cells containing MRV-induced SGs is dependent on the levels of core particle entry into the cytoplasm. ISVPs from all prototype viral strains were able to induce SG formation in a dose-dependent manner, with increasing pfu/cell resulting in increasing numbers of SG-containing cells. In fact, using ISVPs, a significant amount of SGs could be induced at 10 pfu/cell (Fig. 2). It is important to note that our experiments quantifying numbers of SG-containing, infected cells are complicated by the fact that viral infection and SG-formation are both dynamic processes. Because cells and viral infections are not synchronized in these experiments, fixation for immunofluorescence represents only a snapshot of a population of cells at one moment in time. It is likely that infected cells will be at different stages of these dynamic processes at this moment, and that the percentage of cells in which MRV induces SGs is much higher than what is indicated in these experiments. In fact, we found that when MRV gene expression was inhibited by UV inactivation (Fig. 5) or by puromycin treatment (Fig. 6), much higher percentages of SG-containing infected cells were observed in these assays. MRV produces 200-3000 plaque forming units in each infected cell (40). All of these viruses are likely released simultaneously into the surrounding cellular matrix by cell lysis, making it likely that the conditions used in our experiments are biologically relevant.

**SG induction by MRV requires viral uncoating but not viral gene transcription and translation.** Previous studies showed that MRV infection induces phosphorylation of eIF2α at late times p.i., likely through activation of PKR kinase during viral replication,
resulting in SG formation (47). However, in our study, we tested several cell lines (HeLa, L929, MEF, CV-1, Cos-7, DU-145) and found that no SGs formed in cells containing the large, perinuclear VFs that form in infected cells at late times in infection (12-24 h p.i.). We consistently visualized a small percentage of SG-containing, infected cells expressing very low levels of viral protein when we examined SGs at late times p.i. (Fig. 5B, Fig. 6A and Qin and Miller, unpublished data). These findings suggest that as MRV infection continues, MRV-induced SGs are dispersed. We cannot fully explain the difference in our data to the published report indicating that MRV-infected cells contain SGs at late times p.i. We have tested more than ten cell lines (in this study, and data not shown), and the presence of SGs at early, but not late, times in MRV infection does not appear to be cell-type specific. Moreover, while we have demonstrated that the induction of SGs following MRV infection is dependent on pfu/cell, the absence of SGs in cells at late times in infection is not dependent on pfu/cell, as cells expressing high levels of MRV protein following infection with 1, 10, 100, or 1000 pfu/cell also do not contain SGs at later times p.i. (Fig. 5B, Fig. 6A, and data not shown). It is also interesting that a recent report has shown that the highly related rotavirus also does not contain SGs at late times in infection, even though eIF2α is phosphorylated at these times (39).

In order to understand the mechanism of SG induction by MRV, we chose some well defined pharmaceutical inhibitors to block specific steps of viral infection to identify which step is required for SG induction. We found viral uncoating is required for SG formation based on the fact that treatment of cells with ammonium chloride and E-64, which inhibit the cleavage and subsequent conformation changes of outer capsid proteins (σ3 and μ1) in the endosome, prevents the formation of SGs following MRV infection (Fig. 4). We also found
that viral transcription and translation are not required for SG formation based on the fact that UV-inactivated MRV virions and ISVPs still retain their ability to induce SGs (Fig 5A), and cells treated with puromycin are still able to form SGs (Fig 6A) even though viral translation is completely inhibited. These findings suggest MRV-induction of SGs is mainly dependent on viral entry and precedes viral protein synthesis and replication.

**Phosphorylation of eIF2α is a key factor for SG formation in response to MRV infection during viral entry.** SG formation is a complicated process, and the mechanism involved in the formation and dispersal of these structures is poorly understood. Formation of SGs can be induced by phosphorylation of eIF2α (3), inhibiting the formation of ternary complex (eIF2-GTP-tRNA_Met), or interfering with translation initiation factors [eIF4A (15), eIF4B, eIF4H, or PABP (38)]. Phosphorylation of eIF2α appears to play an important role in SG-induction by some, but not all viruses (18, 34). This is not surprising given the fact that many viruses activate PKR, including alphavirus (52), reovirus (47), rotavirus (39), and influenza viruses (22). Herpes simplex virus 1 activates PERK as well as PKR (13). Activated PKR or PERK phosphorylate eIF2α on Ser 51, which leads to the failure of ternary complex formation (eIF2-GTP-tRNA_Met), leading to SG formation (25). Our data shows that phosphorylation of eIF2α is required for SG formation following MRV infection (Fig. 7). However, PKR does not appear to be the only kinase that phosphorylates eIF2α during MRV infection. We found MRV infection induces SGs in a PKR-/− cell line as well as other eIF2α kinase knockout cell lines (GCN-/−, PERK-/−, and HRI-/−) (Fig. 8). We speculate, based on this data, that MRV infection can induce eIF2α phosphorylation through a number of pathways. It has previously been shown that MRV infection activates the unfolded protein
response, which involves the PERK kinase (47), raising the possibility that both PKR and PERK kinases may play a role in MRV induction of SGs.

The recruitment of viral cores in SGs may be a consequence of pathogen-host co-evolution. During MRV-induced SG formation, we observed many viral core particles localized in SGs (Fig. 9A). Our data suggests viral mRNA transcription is necessary for core localization to SGs. How newly synthesized viral mRNA might mediate core particle localization to SGs is unknown. Following the release of core particles into the cytoplasm, they do not further disassemble (56). Viral core particles directly transcribe viral mRNA from within the core, and newly synthesized viral mRNA is released from λ2-formed turrets as mRNA synthesis proceeds (56). We propose the process of SG formation is triggered as a result of phosphorylation of eIF2α during viral entry. It is possible that during SG assembly, some major component(s) of SGs such as RNA binding proteins TIAR, TIA-1 (27), G3BP (51), FMRP (16), or Stau 1 (50), play a role in sequestering viral mRNA and its associated core particles into SGs. SGs sequester and translationally silence mRNA. Some stress-induced transcripts, such as ATF4, GCN4, and Hsp70 are selectively translated when SGs are present in cells (24), although the molecular features that distinguish between constitutive and stress-induced transcripts are not well understood. For West Nile virus, TIAR and TIA-1 interact with the 3’ stem-loop of the complementary minus-strand RNA and facilitate virus replication. This finding suggests that viruses can either escape SG arrest or take advantage of SGs. Whether MRV translation is inhibited by SGs is undetermined, although, in every case in this study, MRV infection continued following SG-formation. Our data suggests that SGs are diminished as viral proteins accumulate (Fig. 5B, Fig. 6A, panel 1, and data not shown), suggesting that SGs are disassembled as infection proceeds. It is unlikely that the
dissolution of SGs seen in MRV infected cells relieves the host cell translation shutoff seen following infection with some strains of MRV, because in our experiments SGs were absent at late times p.i. in cells infected with both viral strains that induce host translational shutoff (T2J), and those that do not induce host cell translational shutoff (T3D). However, SG disruption may be necessary for the synthesis of viral protein in the translational shutoff environment. MRV induction and disruption of SGs, and subsequent escape from the translation inhibition that is a consequence of this induction could be the consequence of co-evolution between MRV and the host cell.

**SGs and MRV factories.** SG formation depends on the microtubule network (23). The deacetylase HDAC6 was proposed to coordinate the formation of SGs by mediating the motor-protein-driven movement of SG components along microtubules (30). During MRV infection, the viral core protein μ2, and the VF structural matrix protein, μNS, are associated with the microtubule network. While the association of μ2 with microtubules plays a role in VF morphology, the association of μNS with microtubules appears to be important for the development of MRV factories from small structures scattered throughout the cytoplasm, to larger perinuclear structures (10, 43). MRV core particles are embedded in microtubule-associated VFs (4, 8) and associate with μNS, both in vitro and in vivo (9, 10). The fact that μNS binds to viral cores, and uses the microtubule network to facilitate VF formation, coupled with our findings that viral core particles colocalize with microtubule associated-SGs at very early times p.i. suggests there may be a link between SGs and VF formation. The possibility that MRV VFs may nucleate around core-containing MRV-induced SGs through interactions between μNS, viral cores, and the microtubule network will be interesting to
examine in future studies.

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Figure 1. Infection with MRV induces SG formation at early times p.i. HeLa (first row), MEF (second row), CV-1 (third row), and DU-145 (fourth row) cells were infected with MRV T3D<sup>C</sup> virions (1000 pfu/cell). At 4 h p.i., cells were fixed and immunostained with rabbit α-MRV core polyclonal antiserum (left column) and mouse monoclonal antibody against G3BP (first row, right column), goat α-TIA-1 (second row, right column), or goat α-TIAR (third and fourth rows, right column) polyclonal antibodies, followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-mouse IgG or donkey α-goat IgG. More than 200 infected cells were counted from each slide and percentage of infected cells containing SG-like granules at the time of fixation is indicated. Scale bars=10 μm.
Figure 2. Infection with MRV ISVPs induces SG formation in a dose dependent manner

(A) HeLa cells were infected with T1L ISVPs (top row), T2J ISVPs (middle row) or T3D\textsuperscript{C} ISVPs (bottom row) (1000 pfu/cell). At 2 h p.i., cells were fixed and immunostained with rabbit $\alpha$-MRV core antiserum (left column) and goat polyclonal $\alpha$-TIAR antibodies (right column) followed by Alexa 594-conjugated donkey $\alpha$-rabbit IgG and Alexa 488-conjugated donkey $\alpha$-goat IgG. Scale bars=10 $\mu$m. (B) Cells were infected with MRV ISVPs (T1L, T2J,
T3D\textsuperscript{C}) at 10, 100, or 1000 pfu/cell, and fixed and immunostained as in (A). More than 200 infected cells were counted in each treatment to calculate the percentage of infected cells containing SG-like granules at the time of fixation.
Figure 3. MRV-induced SGs contain translation initiation factors eIF3, eIF4G, eIF4E but not P body component DCP1a. HeLa cells were infected with T1L ISVPs (1000 pfu/cell) for 2 h, then fixed and immunostained with mouse α-λ.2 monoclonal antibody (7F4) to visualize MRV cores (left column), goat α-TIA-1 or goat α-TIAR antibodies (second column), and rabbit α-eIF3, eIF4G, eIF4E antibodies, or DCP1a antisera (third column) followed by Alexa 594-conjugated donkey α-mouse IgG, Alexa 488-conjugated donkey α-goat IgG, and Alexa 350-conjugated donkey α-rabbit IgG. Merged images are shown in right.
Figure 4. Ammonium chloride and E-64 prevent MRV induction of SGs. HeLa cells were pretreated with 2 mM ammonium chloride (NH₄Cl) (row 1), 2 mM E-64 (row 2), or left untreated (row 3) for 4 hours, then infected with MRV T3D⁰ virions (1000 pfu/cell). At 4 h p.i., cells were fixed and immunostained with rabbit α-MRV core polyclonal antisera (rows 1, 2, 3; left columns) and goat α-TIAR polyclonal antibodies (rows 1, 2, 3; right column),
followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Uninfected HeLa cells were treated with 2 mM ammonium chloride (row 4), or 2 mM E-64 (row 5), and incubated (rows 4,5; left column), or additionally treated for 1 h with 0.5 mM SA (rows 4, 5; right column), then fixed and stained with goat α-TIAR polyclonal antibodies followed by Alexa 594-conjugated donkey α-rabbit IgG. Scale bars=10 μm.
Figure 5. **UV-inactivated MRV virions induce SGs.** (A) HeLa cells were infected with untreated (top row) or UV-inactivated (bottom row) T3D^C virions (1000 pfu/cell). Cells were fixed at 4 h p.i., and immunostained with rabbit α-MRV core polyclonal antiserum (left column) and goat α-TIAR antibodies (right column), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. (B) HeLa cells were infected, fixed and immunostained as in (A) at 2, 4, 6, 12, and 24 h p.i. The percentage of infected cells containing SGs was calculated at each time point as described in Materials and Methods.
Fig 6. Puromycin inhibits viral translation, but does not prevent MRV induction of SGs. (A) HeLa cells were untreated (row 1) or pretreated with 0.1 mg/ml puromycin (row 2) for 1 h, then infected with MRV T3Dvirions (1000 pfu/cell) for 1 h. New medium containing 0.1 mg/ml puromycin was added to cells following infection (row 2). At 10 h p.i., cells were fixed and immunostained with rabbit α-MRV core polyclonal antisera (rows 1, 2; left column) and goat α-TIAR polyclonal antibodies (rows 1, 2: right column), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Uninfected HeLa cells were treated with 0.1 mg/ml puromycin (row 3; left and right column), and incubated (left column) or treated with 0.5 mM SA for 1 h (right column) after 9 h puromycin treatment, then fixed and stained with goat α-TIAR polyclonal antibodies.
followed by Alexa 488-conjugated donkey α-goat IgG. Following immunostaining, the percentage of infected cells containing SGs (rows 1, 2) or total cells containing SGs (row 3) was quantified as described in Materials and Methods. Percentages of SG-containing cells are indicated. Scale bars=10 μm. (B) HeLa cells were infected with MRV T3D C virions (1000 pfu/cell) with or without 0.1 mg/ml puromycin (Pm) as indicated, and at 4 and 10 h p.i., cells were lysed, and proteins were separated by SDS-PAGE followed by immunoblotting using α-μNS polyclonal antiserum, or α-β-actin polyclonal antibodies. Proteins were detected using HRP-conjugated goat α-rabbit IgG, followed by chemiluminescence imaging.
Fig 7. eIF2α phosphorylation correlates with and is required for MRV induction of SGs. (A) HeLa cells were infected with untreated or UV-inactivated T3D<sup>C</sup> virions (1000 pfu/cell), and cells were harvested at 0, 2, 4, and 6 h p.i. Following cell lysis, proteins were separated by SDS-PAGE and immunoblotted using rabbit α-μNS polyclonal antiserum, rabbit α-eIF2α polyclonal antibodies, and rabbit α-phospho-eIF2α polyclonal antibodies. Proteins were detected and quantified using HRP-conjugated goat α-rabbit antibodies, followed by chemiluminescence imaging. Levels of phosphorylated eIF2α relative to total eIF2α at each time point were calculated, normalized to the level seen at t=0 and are indicated below each time point. (B) MEF<sup>S51S/S51S</sup> (S/S) (row 1) and MEF<sup>S51A/S51A</sup> (A/A) (row 2) cells were infected with MRV T3D<sup>C</sup> virions (1000 pfu/cell). At 4 h p.i., cells were fixed and immunostained with rabbit α-MRV core antiserum (left column) and goat α-TIAR polyclonal antibodies (right column), followed by Alexa 594-conjugated donkey α-rabbit
IgG and Alexa 488-conjugated donkey α-goat IgG. Scale bars=10 µm.

**Fig 8. MRV induces SG formation in eIF2α kinase knockout cell lines.** GCN/- (row 1), PERK/- (row 2), PKR/- (row 3), or HRI/- (row 4) cells were infected with T3D^C virions (1000 pfu/cell) (left and middle columns), or mock infected (right column). At 4 h p.i., cells were fixed and immunostained with rabbit α-MRV core antiseum (left column) and goat α-TIAR polyclonal antibodies (middle, and right columns), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. More than 200 infected cells were counted from each slide and percentage of infected cells containing SG-like granules at the time of fixation is indicated. Scale bars=10 µm.
Fig 9. MRV core particles colocalize with SGs in a manner dependent on viral gene expression. (A) HeLa cells were infected with MRV T1L ISVPs (1000 pfu/cell). At 2 h p.i., cells were fixed and stained with rabbit α-MRV core antiserum (left) and goat α-TIAR antibodies (middle), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Merged image is shown (right). Confocal images were taken at 0.1 μm slice intervals using a Leica SP5 X confocal microscope. The boxed regions in each image were amplified and are shown in insets in the merged image. (B) HeLa cells were infected with UV-inactivated T3D^C virions (1000 pfu/cell). At 4 h p.i., cells were fixed and stained with rabbit α-MRV core antiserum (left), and goat α-TIAR antibodies (middle). Merged image is shown (right). The boxed region in the merged image was amplified and is shown in inset. (C) HeLa cells were pretreated with 0.1 mg/ml puromycin for 1 h, incubated with T3D^C virions (1000 pfu/cell) for 1 h, then retreated with puromycin for an additional 10
h, at which point, cells were fixed and stained with rabbit \( \alpha \)-MRV core antiserum (left), and goat \( \alpha \)-TIAR polyclonal antibodies (middle). Merged image is shown (right). The boxed region in the merged image was amplified and is shown in inset.
CHAPTER 4. MAMMALIAN ORTHOREOVIRUS ESCAPE FROM HOST

TRANSLATIONAL SHUTOFF CORRELATES WITH STRESS GRANULE DISRUPTION AND IS INDEPENDENT OF EIF2α PHOSPHORYLATION AND PKR

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**Abstract**

In response to mammalian orthoreovirus (MRV) infection, cells initiate a stress response, which includes eIF2α phosphorylation and protein synthesis inhibition. We have previously shown that early in infection MRV activation of eIF2α phosphorylation results in the formation of cellular stress granules (SGs). In this work we show that as infection proceeds, MRV disrupts SGs despite sustained levels of phosphorylated eIF2α, and further, interferes with the induction of SGs by other stress inducers. MRV interference with SG formation occurs downstream of eIF2α phosphorylation suggesting the virus uncouples the cellular stress signaling machinery from SG formation. We additionally examined mRNA translation in the presence of SGs induced by eIF2α phosphorylation dependent and independent mechanisms. We found that irrespective of eIF2α phosphorylation status, the presence of SGs in cells correlated with inhibition of viral and cellular translation. In contrast, MRV disruption of SGs correlated with release of viral mRNAs from translational inhibition, even
in the presence of phosphorylated eIF2α. Viral mRNAs were also translated in the presence of phosphorylated eIF2α in PKR-/− cells. These results suggest that MRV escape from host cell translational shutoff correlates with virus-induced SG disruption, and occurs in the presence of phosphorylated eIF2α in a PKR independent manner.

**Introduction**

The non-fusogenic mammalian orthoreoviruses (MRV) are a member of a large family of animal and plant viruses (*Reoviridae*), which includes many members that are of considerable importance in human, animal and plant disease (24). Members of this virus family share a number of strategies for viral invasion and growth within host cells. Following infection with many of the viruses from this family, including MRV, the host cell initiates a stress response that culminates in shutoff of protein translation (5, 26, 37). Remarkably, viral RNAs are able to escape this inhibition and continue to be translated in the shutoff environment. (34, 36, 47). In the case of MRV, the innate immune response has been implicated in host translational shutoff via activation of the dsRNA kinase, PKR (6, 15, 31, 34, 39). PKR is activated by binding dsRNA, at which point it homodimerizes and undergoes autophosphorylation (46). Activated PKR phosphorylates serine 51 on the alpha subunit of the cellular translation initiation factor eIF2 (17). In the absence of cellular stress, eIF2α binds to GTP and initiator methionyl-tRNA (met-tRNA$_i$), to form a ternary complex which subsequently binds to the 40S ribosomal complex to form the 43S pre-initiation complex. As translational initiation proceeds, eIF2-bound GTP is hydrolyzed to release initiation factors from the ribosome that then continues with translation elongation. Following release, the GDP bound to eIF2 must be exchanged for GTP in a reaction that is catalyzed by the guanine nucleotide exchange factor, eIF2B. Upon this exchange, eIF2-GTP can again bind met-
tRNA<sub>i</sub> to initiate a new round of translation. When eIF2α is phosphorylated, it changes from a substrate of eIF2B to a competitive inhibitor that binds eIF2B with high affinity, inhibiting the exchange of GDP for GTP. This binding results in global inhibition of protein synthesis [reviewed in (25, 27)]. There is an excess of eIF2α relative to eIF2B in cells, therefore, very little eIF2α phosphorylation can result in inhibition of protein synthesis (13). mRNAs whose expression is required for response to cellular stress, as well as RNAs of other viruses that induce host translational shutoff, escape this inhibition in a variety of ways (44).

It has previously been shown that some strains of MRV prevent host cell translational shutoff (4). The MRV structural protein, σ3, was found to play a key role in MRV interference with the host cell translational inhibition response in these strains (6, 15, 33, 35). MRV reassortants containing the gene for encoding σ3 proteins from non-host-shutoff strains were shown to prevent host shutoff in the otherwise genetic background of host-shutoff strains (36). σ3 is a sequence-independent dsRNA binding protein which can functionally replace both vaccinia virus VAI RNA and adenovirus E3L protein, both known to be PKR inhibitors (6, 15). Based on this, it was proposed that σ3 from MRV strains that prevent host shutoff bind dsRNA and interferes with PKR activation following MRV infection, thereby preventing eIF2α phosphorylation and subsequent translational inhibition. Previous studies also suggest that differences in host translational shutoff induced by MRV strains may result from differences in the intracellular localization of σ3 in infected cells (34). In these studies, MRV strains that prevent host translational shutoff were found to have σ3 diffusely distributed throughout the cytoplasm, and MRV strains that do not prevent host cell translation shut-off were shown to contain σ3 localized primarily around MRV factories.
These authors proposed a mechanism for MRV prevention of host cell translational shutoff in which the diffuse localization of σ3 in some strains results in inhibition of PKR activation throughout the cell, and subsequent release of both cellular and viral translation from PKR inhibition. Alternatively, these authors suggested that the localization of σ3 predominantly in viral factories in strains that cannot prevent shutoff results in inhibition of PKR activation in these areas only (where MRV translation may take place), resulting in the shutoff of cellular translation by active PKR elsewhere in the cytoplasm (34). While strong evidence supports a role for σ3 in modulating the ability of MRV to prevent host cells from shutting off protein translation in response to infection, the mechanism behind the ability of viral mRNAs to escape translational shutoff and continue translating mRNA in MRV strains that cannot prevent shutoff remains poorly understood.

A number of recent studies have illustrated the downstream consequences of eIF2α phosphorylation on mRNA and the cellular translation machinery. As described above, phosphorylation of eIF2α results in a reduction in the availability of ternary complex, leading to an increase in 48S pre-initiation complexes that are unable to recruit 60S ribosomal complexes for translation initiation. This destabilization of polysomes leads to the rapid localization of mRNAs, translation initiation factors, and small, but not large, ribosomal subunits to discrete structures in the cytoplasm called stress granules (SGs) (8, 9, 12). A number of proteins, such as TIAR/TIA-1 and G3BP, all of which have RNA-binding and self-aggregation domains, have been identified that play key roles in the formation and recruitment of protein and RNA components to SGs. Importantly, SGs are not seen in cells growing in favorable conditions, but form during times of cellular stress including heat shock, oxidative stress, UV irradiation, and upon infection by many viruses (3, 9, 20, 21, 29,
eIF2α phosphorylation is sufficient to induce SGs, however, drugs that inhibit ribosome recruitment to initiation complexes (hippuristinol, pateamine A, 15D-PGJ2) downstream of ternary complex recruitment, have also been shown to induce SG formation independent of eIF2α phosphorylation (2, 11, 18). SGs appear to serve as sites of mRNA triage that monitor the cellular environment to determine if a cell will undergo repair or apoptosis (8).

We have recently shown that MRV infection induces formation of SGs in an eIF2α phosphorylation dependent manner at a step following virus uncoating, but preceding viral gene expression (28). In the previous study, we found that as MRV infection proceeds, SGs are disrupted in a manner that is dependent on viral translation, suggesting that a viral protein, or protein complex may be involved in SG disruption. Initial induction and subsequent disruption of SGs during viral infection has been reported in a number of viral systems. Some mechanisms for dispersing SGs have been identified. For example, SG formation following West Nile virus infection occurs by TIAR/TIA-1 binding to the 3′ stem loop of negative strand viral RNA (3). Alternatively, poliovirus prevents SG formation by cleaving G3BP with the viral 3C proteinase (43). A number of other viruses, including Semliki Forest Virus (20) and rotavirus (22) also interfere with SG formation, although the mechanisms for this interference remain to be identified. In this study, we examined the step at which MRV is able to disrupt SGs and found that the disruption occurs downstream of eIF2α phosphorylation. We further examined the impact of SGs on viral and cellular translation in the absence and presence of eIF2α phosphorylation in uninfected and infected cells, and found that SG disruption correlates with release of viral, but not cellular mRNA from host translational inhibition, and that MRV translation occurs in the presence of high
levels of phosphorylated eIF2α that are inhibitory to cellular translation in a manner independent of PKR inhibition.

Materials and methods

Cells and reagents. HeLa, Cos-7, PKR+/+, and PKR-/- cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium, Invitrogen Life Technologies) containing 10% fetal calf serum (Atlanta Biologicals) and penicillin/streptomycin (100 IU/mL, Mediatech). Spinner adapted L929 cells were maintained in c-MEM (Joklik's minimal essential medium, Irvine Scientific) containing 2% fetal calf serum and 2% fetal bovine serum (HyClone Laboratories, Atlanta Biologicals), 2 mM L-Glutamine (Mediatech) and penicillin/streptomycin (100 IU/mL, Mediatech). Primary antibodies used in immunofluorescence and immunoblotting assays are as follows: goat polyclonal α-TIAR antibody (Santa Cruz Biotechnology), rabbit polyclonal α-eIF2α antibody (Bethyl Laboratories), rabbit polyclonal α-phospho-eIF2α (Ser51) antibody and rabbit anti-β-actin (Cell Signaling Technologies). Rabbit polyclonal antiserum against μNS was made by the Iowa State University Hybridoma facility by injection of rabbits with peptides corresponding to μNS AA 1-20, and μNS AA 21-40 synthesized on a Multiple Antigen Peptide System (MAPS). Secondary antibodies used in immunofluorescence and immunoblotting experiments are as follows: Alexa 488-, Alexa 594-, or Alexa 350-conjugated donkey α-mouse, α-rabbit or α-goat IgG antibodies (Invitrogen), and alkaline phosphotase-conjugated goat α-rabbit IgG antibodies (Bio-Rad Laboratories). Sodium arsenite (Sigma-Aldrich) was used where indicated at a final concentration of 0.5 mM. Cycloheximide (Sigma-Aldrich) was used where indicated at a final concentration of 10 μg/ml. 15D-PGJ2 (Sigma-Aldrich)
was used where indicated at a final concentration of 50 μM. Alkaline phosphatase-conjugated streptavidin (Invitrogen) and Alexa 488-conjugated streptavidin (Invitrogen) were used at 1:1000 and 1:100 dilutions respectively.

**Virions.** Purified MRV virions (T1L, T3D<sup>C</sup>, T3D<sup>N</sup>, T2J strains) are our laboratory stocks. Purified virions were prepared as described (23) and stored in dialysis buffer (150 mM NaCl; 10 mM Tris pH 7.4; 10 mM MgCl<sub>2</sub>) at 4 °C.

**Viral infection.** 2.0 × 10<sup>5</sup> (4.0 × 10<sup>5</sup>) cells were seeded onto 35 (60) mm dishes the day before transfection or infection. Cells were infected with MRV strains T1L, T2J or T3D<sup>C</sup> at 100 PFU/cell or 1000 PFU/cell as indicated in each experiment. Virus was diluted in PBS + 2 mM MgCl<sub>2</sub>, and adsorbed to cells for 1 h, at which point, cells were refed with DMEM and incubated at 37°C until harvested.

**L-azidohomoalanine (AHA) protein labeling.** For in situ translation, Cos-7 cells were seeded on 12-well dishes containing 12-mm-diameter round coverslips at a density of 1x10<sup>5</sup> cells/well, incubated overnight at 37°C, then infected with MRV or mock infected. At indicated times p.i., medium was replaced with prewarmed methionine-minus medium containing sodium arsenite, cycloheximide, or 15D-PGJ2 at indicated concentration for 30 min at which point AHA was added at a final concentration of 50 μM, and cells were incubated for an additional 30 min. Cells were fixed with 100% ice cold methanol at -20°C for three min, then washed three times with PBS [137 mM NaCl, 3 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5)]. Fixed cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min, and washed three times with PBS. Slides were transferred, cell side up to parafilm, and incubated with reagents provided for biotin labeling from Invitrogen. Alterations to the protocol provided by the manufacturer are as follows. For each reaction,
25 µl of Component A+B was added to 15 µl PBS, and 40 µl of this solution was added to coverslips. 2.5 µl of Component C was added to coverslips, followed by 2.5 µl of Component D, and 5 µl of Component E. Coverslips were incubated in this solution for 20 min, then rinsed and processed for immunofluorescence. For Western blot assays, Cos-7 cells were seeded on 6 well dishes at a density of 2 × 10^5 cells/well, then incubated overnight at 37°C, and infected with MRV or mock infected. At indicated times p.i., old medium was replaced with pre-warmed methionine-minus medium containing sodium arsenite, cycloheximide, or 15D-PGJ2 at indicated concentrations for 45 min at which point AHA was added at a final concentration of 50 µM, and cells were incubated an additional 1 h. Cells were harvested and lysed with 1% SDS in 50 mM Tris-HCl pH 8.0 and then AHA labeled nascent proteins were further conjugated with biotin as described in the manual with the following alterations. For each reaction, 30 µl of Component A+B was added to 50 µl of cell lysate previously label with AHA, followed by 5 µl of Component C, 5 µl Component D, and 10 µl of Component E. Cells were then incubated, rotating end over end for 20 min. Following labeling, proteins were precipitated, resuspended in protein loading buffer, and separated on SDS-PAGE.

**Immunofluorescence (IF) assay.** At indicated times p.i., cells were fixed at room temperature for 10 min with 2% paraformaldehyde in PBS or 100% methanol for 3 min and then washed three times with PBS. Fixed cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min and then washed for three times with PBS. Samples were blocked by a 10 min incubation with 2% BSA in PBS. Primary and secondary antibodies were diluted in 2% BSA in PBS. After blocking, cells were incubated for 1 hour with
primary antibodies and Streptavidin conjugated Alexa 488, washed three times with PBS, and then incubated for an additional hour with secondary antibodies. Immunostained cells were washed for a final three times with PBS, and mounted on slides with Prolong reagent with or without DAPI (Invitrogen). Immunostained samples were examined with a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics. Images were prepared by using Photoshop and Illustrator software (Adobe Systems).

**Immunoblotting.** Infected cells were harvested at different time points p.i., and lysed with 100 µl lysis buffer (50 mM Tris pH 8.0, 1% SDS). Protein concentrations for each sample were measured by EZQ® protein quantitation Kit (Invitrogen), and equivalent amounts of protein from each sample were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane by electroblotting in transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3)]. The nitrocellulose containing transferred proteins was blocked for 15 min with 5% BSA in Tris-buffered saline (20 mM Tris, 137 mM NaCl [pH 7.6]) containing 0.1% Tween (TBS-T) and then incubated overnight with primary antibodies in TBS-T containing 1% milk or AP-conjugated streptavidin. Blots incubated with primary antibodies were washed three times for 15 min with TBS-T, followed by a 4 h incubation with AP-conjugated secondary antibodies in TBS-T containing 1% milk. Blots were washed for a final three times and AP conjugate substrates (Biorad) were applied to develop color.

**Results**

**Despite high levels of eIF2α phosphorylation, SGs are not present in MRV infected cells at late times post-infection.** We have previously shown that MRV infection induces SGs at early times (2-4 h) post-infection in an eIF2α phosphorylation-dependent manner. We
additionally found that as infection proceeds, SGs are disrupted in infected cells in a manner dependent on viral gene expression. To begin to examine the step at which MRV disrupts SGs, we first examined SG formation and the phosphorylation status of eIF2α at late times in MRV infected cells. L929, Cos-7, and Hela cells were infected with MRV (strains T1L, T2J, and T3DC) and at 24 h p.i., were fixed, permeabilized, and immunostained with α-μNS and α-TIAR to visualize infected cells and SGs, respectively. Consistent with our earlier studies, we found that in L929 (Fig. 1A), Cos-7 (Fig. 1B), or HeLa (Fig. 1C) cells there are no SGs at late times in either uninfected cells, or in T1L (second row), T2J (third row), or T3DC (bottom row) infected cells that are expressing high levels of viral protein. On the contrary, in control cells treated with sodium arsenite (SA), an oxidative substance that induces high levels of eIF2α phosphorylation via activation of the HRI kinase, 99% of cells contained SGs (Figs. 1A, 1B, and 1C, top rows).

eIF2α phosphorylation is required for MRV induction of SGs at early times in infection, and has been found to be sufficient for SG induction in cells. Although it has been reported previously that MRV infected cells contain high levels of phosphorylated eIF2α, it was possible that SGs are disrupted in MRV infected cells as viral infection continues because eIF2α phosphorylation is diminished at later times in infection. To examine this possibility, we measured the relative levels of eIF2α phosphorylation versus total eIF2α by performing immunoblots on cell lysates harvested from untreated- and SA-treated mock infected cells, and MRV infected cells at 24 h p.i. We found that infection with all three strains of MRV led to an increase in induction of eIF2α phosphorylation compared with mock infected cells, with T2J inducing levels of eIF2α phosphorylation similar to SA-treatment in all cell types, and T1L and T3DC inducing varying increases in eIF2α
phosphorylation (Fig. 1D). MRV strain- and host cell- specific differences in eIF2α phosphorylation induction are consistent with previously published results (16). As we did not detect SGs in any tested cell lines with the three prototype MRV strains at these times p.i., this data suggests that at late times in infection, MRV disrupts the pathway between eIF2α phosphorylation and SG formation.

**MRV infection renders cells unable to form SGs in response to strong external stress signals.** The absence of SGs in MRV infected cells suggested that MRV is able to uncouple eIF2α phosphorylation from SG induction. However, it was also possible that levels of phosphorylated eIF2α induced by MRV were not sufficient to induce SGs in infected cells. To further examine the capacity of MRV to prevent SG formation in response to stress signals, we examined uninfected and infected cells for SG formation following sodium arsenite treatment. Cos-7 cells were infected with MRV strains T2J or T3DC, then at 19 h p.i., cells were treated with sodium arsenite for 1 h, then fixed and stained for immunofluorescence microscopy using antibodies against non-structural protein μNS to visualize infected cells, and antibodies against TIAR to visualize SGs. Remarkably, while SGs were apparent in all uninfected cells, infected cells did not contain SGs (Fig. 2A, B, C). This experiment was repeated using other known SG localized protein markers, including TIA-1, eIF3, eIF4E, and eIF4G, with identical results (data not shown). This finding was both viral strain and cell type independent as all three prototype strains (T1L, T2J, and T3D) interfered with SG formation in a number of cell types including Cos-7, CV-1, HeLa, L929, and MEFs (Fig. 2 A, B, and C, and data not shown). These findings confirm that MRV actively interferes with the cellular stress response by disrupting or preventing SG formation.
MRV SG interference is downstream of eIF2α phosphorylation. Because both MRV infection and sodium arsenite treatment induce eIF2α phosphorylation, our data suggested that MRV interference with SG formation at late times in infection occurs in the presence of phosphorylated eIF2α, however, it remained possible that in MRV-infected cells, SGs are prevented by viral interference with phosphorylation of eIF2α. We used two additional assays to rule out this possibility. First, we examined the levels of phosphorylated eIF2α in MRV-infected versus uninfected cells upon addition of SA. At 19 h p.i., MRV (T1L, T2J, T3D<sup>C</sup>) and mock infected cells were subjected to SA for 1 h, then cells were harvested. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, then immunoblotted with antibodies against viral non-structural protein μNS, total eIF2α and phosphorylated eIF2α. Relative fold-increases in the level of eIF2α phosphorylation in the absence and presence of SA treatment were calculated using chemiluminescence. We found that SA induced eIF2α phosphorylation to similar levels in infected and uninfected cells (Fig. 3A). Because SGs do not form in response to SA treatment in MRV-infected cells at this time p.i. (Fig. 2), this suggests MRV interference with SGs is downstream of eIF2α phosphorylation.

We also examined the ability of MRV to interfere with SGs induced by NSC119893, and 15D-PGJ2, both of which induce SGs independently of eIF2α phosphorylation. NSC11983 binds to eIF2α and inhibits ternary complex formation, and 15D-PGJ2 binds to eIF4A, and inhibits translation initiation. Cos-7 cells were infected, and at 19 h p.i., cells were treated with NSC11983 or 15D-PGJ2 for 1 h, then fixed, permeabilized, and stained with antibodies against mNS and TIAR to visualize infected cells and SGs. We found that MRV infection was able to completely interfere with SGs induced by these drugs, confirming
that the prevention of SG-formation by MRV does not occur as a result of interference with eIF2α phosphorylation (Fig. 3B). These data confirm that MRV interference with SG formation occurs at some point downstream of eIF2α phosphorylation. Taken together with our previously published data showing that SGs are disrupted in a manner requiring the expression of viral proteins, these data support a hypothesis that a viral protein or nucleoprotein complex is involved in disruption/prevention of SGs in the presence of eIF2α phosphorylation in MRV-infected cells.

Development of a non-radioactive protein labeling strategy for \textit{in situ} detection of newly translated proteins. In order to examine the role of SG disruption in MRV escape from host cell translational shutoff, we next developed a method to examine individual cells to determine if the presence of SGs interfered with new protein translation, as well as to examine total viral and cellular protein translation. We took advantage of a new commercially available technology, Click-iT AHA (Invitrogen), that utilizes click chemistry to label proteins as they are being synthesized. This technology utilizes a methionine analogue (L-azidohomoalanine) that is added to cells and incorporated into proteins as they are translated. Proteins are then subjected to biotin-labeled alkyne and a copper catalyst, which results in biotin-labeling of proteins that were synthesized during the L-AHA labeling period (Fig. 4A). We manipulated this system so that labeled proteins could be detected using immunofluorescence assays (Fig. 4B), or Western blot (Fig. 4C) using Alexa 488-, or alkaline phosphatase-conjugated streptavidin respectively.

\textbf{MRV and cellular translation are inhibited when SGs are present in sodium arsenite-treated cells at early times post-infection.} In order to examine the effect of SGs on viral translation, we needed to create an environment in which MRV mRNAs were being actively
translated to levels that we could measure in our assays, but where amounts of MRV protein were not yet high enough to prevent SG formation. We reasoned that if we treated cells with sodium arsenite at 5-6 h p.i., MRV-induced SGs would already be disrupted, and viral protein synthesis would be initiated, but that the limited amounts of viral protein present at these times would not be sufficient to interfere with SG formation induced by sodium arsenite.

To visualize protein synthesis on an individual cell basis, Cos-7 cells were infected with MRV and at 5 h.p.i., cells were left untreated, or were treated with sodium arsenite or cycloheximide, then labeled with AHA. After labeling, cells were fixed, permeabilized, and subjected to a biotin alkyne click reaction. Cells were stained with virus and SG specific antibodies, or Alexa 488-conjugated streptavidin, then examined by immunofluorescence microscopy for MRV infection, SG formation, and new protein synthesis. In this assay, both uninfected and infected untreated cells were found to synthesize new proteins during the labeling period (Fig. 5A, top row). Cycloheximide, which inhibits translation at the elongation step, inhibited translation of new proteins in both infected and uninfected cells (Fig. 5A, middle row). At this early time point in infection, sodium arsenite induced SGs in both uninfected and infected cells (Fig. 5A, bottom row). Upon examination of individual cells, we found that both uninfected and infected cells contained SGs, and that in the presence of SGs, neither uninfected nor infected cells were able to support new protein synthesis (Fig. 5A, bottom row).

To examine the entire population of cells, these experiments were repeated. However, instead of performing immunofluorescence after AHA-labeling, proteins were harvested and labeled with biotin. Labeled proteins were separated on SDS-PAGE, transferred to
nitrocellulose, then blotted with alkaline phosphatase-conjugated streptavidin to visualize new protein synthesis. Similar to what was seen on an individual cell basis, new cellular and viral protein synthesis were detected in both uninfected and infected untreated cells, but cycloheximide and SA strongly inhibited both cellular and viral translation (Fig. 5B). These results confirmed that SA induction of eIF2α phosphorylation results in the inhibition of both cellular and viral translation at early times in MRV-infected cells in a manner that correlates with the presence of SGs in cells.

**Viral, but not cellular, mRNAs escape eIF2α phosphorylation induced translational shutoff when SGs are disrupted at late times post-infection.** SGs were not observed at late times in MRV infection irrespective of high levels of eIF2α phosphorylation induced by the virus or by SA. To determine whether viral or cellular proteins are synthesized at late times when SGs are disrupted by the virus, at 20 h p.i. Cos-7 cells were left untreated, or were treated with cycloheximide or sodium arsenite. Cells were labeled with AHA, then fixed, permeabilized, and subjected to a biotin click reaction. Labeled cells were stained with virus- and SG-specific antibodies, or Alexa 488-conjugated streptavidin, then examined by immunofluorescence microscopy for MRV infection, SG formation, and new protein synthesis. In the absence of drugs, both uninfected and infected cells were found to synthesize new proteins during the labeling period (Fig. 6A, top row). Cycloheximide inhibited translation of new proteins in both infected and uninfected cells (Fig. 6A, middle row). Similar to what was seen at early times p.i., in the presence of SA, uninfected cells contain SGs and cannot support new protein synthesis. In contrast, MRV infected cells do not contain SGs, and only these cells are capable of supporting protein synthesis in the presence of the high levels of eIF2α phosphorylation induced by SA (Fig. 6A, bottom row).
While this result shows that protein synthesis is active only in MRV infected cells that do not contain SGs, it does not determine if MRV disruption of SGs correlates with release of all protein translation inhibition, or if just viral mRNA is translated under these conditions. To answer this question, we repeated these experiments. However, instead of visualizing proteins by fluorescence microscopy, we separated labeled proteins on SDS-PAGE, transferred them to nitrocellulose, and blotted with alkaline phosphatase-conjugated streptavidin. Examination of the proteins synthesized in the absence and presence of drugs on the Western blot confirmed that SA induced near complete shutoff of cellular protein synthesis in the absence of MRV infection (Fig. 6B, left panel), and further, that only proteins that migrated at the size of MRV proteins (λ, μ, σ) were translated in the presence of SA in MRV infected cells (Fig. 6B, right panel). We confirmed that proteins translated in the presence of SA in MRV infected cells were viral by immunoblotting the same membrane with virus protein-specific antibodies (data not shown). Taken together with data obtained at early times p.i., these data suggest that MRV and cellular translation are inhibited in the presence of high levels of phosphorylated eIF2α when SGs are present, but that at late times in infection, even though high levels of eIF2α remain phosphorylated, disruption of SGs by MRV correlates with escape of viral, but not cellular mRNA, from host cell translational shutoff.

**MRV translation is inhibited by 15D-PGJ2 at early times post-infection when SGs are present in cells.** The addition of SA to cells induces the phosphorylation of eIF2α and SG formation (10). Although somewhat controversial, it has previously been reported that MRV mRNAs that are synthesized at early times in infection contain a 5’ cap structure, whereas those synthesized at late times are uncapped (45). Therefore, it was possible that our above
data suggested that at early times in infection, MRV mRNAs are capped, and cannot escape translational inhibition that occurs as a result of phosphorylation of eIF2α, but that at late times in infection MRV mRNAs are not capped and can escape this shut off, irrespective of SG presence in the cell. To uncouple eIF2α phosphorylation from SG formation in cells, we next examined whether the eIF4A inhibitor, 15D-PGJ2, which induces SGs, but does not induce eIF2α phosphorylation (11), inhibits viral translation at early times p.i. Cos-7 cells were infected, and at 5 h p.i., cells were either left untreated, or treated with cycloheximide or 15D-PGJ2. Cells were then labeled with AHA in the presence of drugs, subjected to the biotin alkyne click reaction, stained with virus- and SG-specific antibodies, or Alexa 488-conjugated streptavidin, and examined by immunofluorescence microscopy. In the absence of drugs, both uninfected and infected cells were translationally active (Fig. 7A, top row), and in the presence of cycloheximide, neither uninfected nor infected cells were translationally active (Fig. 7A, middle row). In the presence of 15D-PGJ2, uninfected cells contained SGs, and did not contain newly synthesized proteins (Fig. 7A, bottom row). 15D-PGJ2 also induced SGs in many, but not all, MRV infected cells at this time p.i., and in infected SG-containing cells, no new protein synthesis was observed (Fig. 7A, bottom row). Some MRV infected cells did not contain SGs in response to 15D-PGJ2, likely because these cells were expressing sufficient levels of viral protein to prevent SG formation when the drug was added. In infected cells that did not contain SGs, new protein synthesis was observed (data not shown).

To visualize the impact of 15D-PGJ2 treatment on translation of the entire cell population, we labeled cells in the absence and presence of drugs, and harvested total proteins. Proteins were subjected to a biotin alkyne click reaction, separated on SDS-PAGE,
and transferred to nitrocellulose. AP-conjugated streptavidin was used to visualize new protein synthesis. In these experiments, 15D-PGJ2 prevented host cell protein translation in uninfected cells, and inhibited viral protein translation in infected cells (Fig. 7B). As we noted in immunofluorescence assays, 15D-PGJ2 did not completely inhibit viral translation in these experiments. Similar to our immunofluorescence data, we suggest that viral translation that escapes 15D-PGJ2 inhibition in these experiments occurs in MRV infected cells that are expressing sufficient viral protein such that SG formation is prevented following 15D-PGJ2 treatment at this time p.i. These data suggest SG interference with MRV translation is independent of eIF2α phosphorylation.

**MRV but not cellular mRNAs escape 15D-PGJ2-induced translational shutoff when SGs are disrupted at late times in infected cells.** To further separate the effect of SGs from the effect of eIF2α phosphorylation on viral translation, we examined the impact of 15D-PGJ2 on cellular and viral translation at late times in MRV infection using the same experiments that was done at 5 h p.i. In the absence of drugs, both uninfected and infected cells were found to synthesize new proteins during the labeling period (Fig. 8A, top row). Cycloheximide inhibited translation of new proteins in both infected and uninfected cells (Fig. 8A, middle row). Similar to what was seen at early times p.i., in the presence of 15D-PGJ2, uninfected cells contained SGs and were not synthesizing new proteins (Fig. 8A, bottom row). In contrast, MRV infection interfered with 15D-PGJ2 induction of SGs, and infected cells were capable of supporting new protein synthesis (Fig. 8A, bottom row).

In order to confirm these findings, we repeated these experiments and harvested total proteins. Proteins were labeled with biotin alkyne, separated on SDS-PAGE, and transferred to a nitrocellulose membrane. When the membrane was blotted with alkaline phosphatase-
conjugated streptavidin, we found that 15D-PGJ2 completely inhibited cellular protein synthesis in uninfected cells, but did not inhibit viral protein synthesis in infected cells (Fig. 8B). We also confirmed that 15D-PGJ2 treatment did not increase the levels of phosphorylated eIF2α in infected and uninfected cells, compared with untreated samples (Fig. 8B). These results indicate that MRV interference with SG formation following 15D-PGJ2 treatment correlates with virus escape from host cell translational shutoff, and further, suggest that it is the presence of SGs and not eIF2α phosphorylation that inhibits MRV translation at early times in infection.

**MRV escape from host translation inhibition is independent of PKR.** It was previously reported that there is a strain specific difference in the ability of MRV to induce host cell shutoff, with infection by most MRV strains inducing eIF2α phosphorylation and subsequent shutoff of host cell translation while infection with some MRV strains does not (39). Using reassortant genetics, this difference in induction of host cell shutoff was mapped to the MRV dsRNA binding protein σ3, supporting a hypothesis where σ3 binds dsRNA, inhibiting PKR phosphorylation of eIF2α, and preventing host cell shutoff (15). While it is clear that σ3 plays an important role in prevention of host translational shutoff, it remains unclear if σ3 inhibition of PKR plays a role in MRV mRNA escape from cellular translational shutoff in viral strains that do not interfere with shutoff. Because we consistently found that MRV translation was able to escape SA-induced translational shutoff, which occurs through HRI kinase phosphorylation of eIF2α, and not PKR phosphorylation of eIF2α, we suspected that MRV escape from virus induced translational shutoff may be independent of σ3 inhibition of PKR. To determine if PKR was necessary for MRV translational escape from SA induced eIF2α phosphorylation, we infected PKR+/+ and PKR-/- cells with MRV and at 20 h p.i.,
cells were treated with cycloheximide or SA, and labeled with AHA. Proteins were harvested and subjected to a biotin alkyne click reaction, separated on SDS-PAGE then transferred to nitrocellulose, which was blotted with AP-conjugated streptavidin. In PKR+/+ cells, untreated uninfected and infected cells were translationally active (Fig. 9). In cycloheximide treated uninfected and infected cells, both viral and cellular translation were inhibited. In SA treated cells, cellular, but not viral, translation was inhibited (Fig. 9). In PKR-/- cells we found essentially identical results, with viral translation escaping SA-induced translational shutoff (Fig. 9), strongly suggesting that MRV translation can escape phospho-eIF2α-induced host cell translational inhibition independent of σ3 inhibition of PKR.

**Discussion**

**MRV disrupts SGs in infected and drug-treated cells downstream of eIF2α phosphorylation.** We recently reported cells form SGs in response to MRV infection at early times post-infection in an eIF2α-phosphorylation dependent manner, and that SGs are disrupted as viral proteins accumulate in a manner that is dependent on viral protein synthesis (28). In this work, we show that in addition to disrupting SGs over time in infected cells, MRV also prevents SG formation following treatment with SG-inducing drugs SA and 15D-PGJ2. The disruption/prevention of SGs in infected and SA-treated cells was found to occur in the presence of high levels of eIF2α phosphorylation. Disruption of SGs induced by 15D-PGJ2 clearly is independent of interference with eIF2α phosphorylation, because this drug induces SGs independent of eIF2α phosphorylation. These data support a hypothesis where MRV encodes a protein or nucleoprotein complex that actively disrupts or interferes with SG
formation, perhaps by direct or indirect interference with SG effector protein aggregation. Other viruses have been shown to encode viral factors that actively disrupt SGs by interfering with SG effector proteins. Poliovirus protease 3C specifically cleaves G3BP resulting in SG disruption (43), and West Nile virus RNA binds TIAR/TIA-1 proteins which are recruited to viral replication centers, ultimately resulting in SG disruption (3). Other viruses such as Sindbis virus and rotavirus also disrupt SGs in the presence of eIF2α phosphorylation (42), although the mechanism has not been determined. The identification of an MRV component involved in SG disruption is currently underway.

**SG disruption correlates with MRV escape from host translational shutoff.** Because SGs participate in sequestration of translation initiation factors following cellular stress (12), they are likely to take part in the inhibition of translation that occurs following infection with many viruses. It therefore seems likely that viruses have developed ways to disperse SGs to release translation initiation factors that are necessary to carry out viral protein synthesis. Although many viruses have been shown to disrupt SGs, most studies have not yet delineated the importance of SG disruption on successful viral infection. It has been shown that some viruses, such as VSV, Sindbis virus, and HSV, replicate better in TIA-1/- cells, which are deficient in the ability to form SGs, suggesting that the function of SGs in translation inhibition may be detrimental to successful virus infection (14). In the case of poliovirus, it was found that the ability of the virus protease to disrupt SGs via G3BP cleavage was important for successful virus infection, as expression of a non-cleavable G3BP inhibited poliovirus replication (43). Additionally, the binding of TIAR/TIA-1 to West Nile virus RNA disrupts SGs and also appears to promote viral RNA replication, though it has yet to be shown that SG disruption per se plays an important role in this process (14). In this work, we
provide evidence that in the case of MRV, SG disruption is an important step in virus circumvention of the host immune response by demonstrating that MRV translation is inhibited when SGs are present, but not inhibited when SGs are disrupted by the viral infection. Correlation between the release of MRV mRNAs from translation inhibition and SG disruption occurred in the presence of phosphorylated eIF2α and when eIF4A was inhibited, bolstering the argument that SGs themselves impact viral translation.

**MRV translation occurs in the presence of phosphorylated eIF2α and when eIF4A is inhibited.** Although our data supports a hypothesis that SGs play some role in regulation of MRV translation, and SG disruption appears to coincide with viral RNA escape from cellular translational inhibition, it is not likely that SG disruption is sufficient for viral mRNA translation in the stressed environment. An obvious question that arises, is how MRV mRNAs are able to compete for the limited ternary complex that is available in the cell when eIF2α is phosphorylated following infection, or SA treatment. A possibility that we are considering is that MRV translation is refractory to a high concentration of phosphorylated eIF2α as a result of an unidentified alternative translation initiation pathway. Many viral and cellular mRNAs contain specialized structures or elements within their sequence that allow them to be preferentially translated in an environment of high levels of phosphorylated eIF2α. These can include certain IRESs, such as that found in Hepatitis C virus RNA which recruits eIF2α and tRNA^{Met} independently of ternary complex (30, 40), upstream ORFs (uORFs), which induce ribosome stalling that leads to preferential translation of some stress-related mRNAs such as ATF4 (41), and mRNAs that can recruit tRNA^{Met} independent of eIF2α, such as Sindbis virus 26S RNA (32). It would not be completely surprising to learn
that MRV may have developed a non-canonical pathway for translation initiation because although MRV mRNAs do possess an m7pppG cap structure like cellular mRNAs, they do not possess a polyA tail, and therefore, may have differing mechanisms for initiation. Moreover, the fact that MRV protein synthesis was not inhibited by 15D-PGJ2 when SGs were disrupted also suggests that MRV mRNA translation occurs via an alternative pathway that is independent of eIF4A. A detailed examination of MRV translation, including identification of cellular translation initiation factors that are required, identification of viral proteins that are involved, and examination of viral RNA sequences to identify important sequences or structures is warranted in the future.

**MRV translation: role of σ3 inhibition of PKR.** Previous data implicated the MRV σ3 dsRNA binding protein in prevention of cellular translational shutoff following infection with some MRV strains (7). The MRV σ3 protein has also been shown to functionally replace other viral PKR inhibitors when expressed in cells infected with these viruses (1). For these reasons, the PKR inhibition activity of σ3 was suspected to also be involved in the ability of MRV mRNAs to escape translational shutoff in strains that could not prevent this cellular response to infection (34). Two lines of evidence from this study suggest that this is not the case. First, irrespective of their host cell shutoff phenotype, all strains of MRV were able to continue translating mRNA in the presence of sodium arsenite at late times in MRV infection. Because sodium arsenite induces eIF2α phosphorylation via the HRI kinase (19), and not PKR, any σ3 mediated PKR inhibition would not impact eIF2α phosphorylation under these conditions. Moreover, we also show that sodium arsenite treatment of infected cells induces levels of eIF2α phosphorylation similar to those in mock infected cells, which
suggests that even if PKR is involved in sodium arsenite induction of eIF2α phosphorylation, σ3 is not significantly inhibiting PKR under these circumstances. Finally, we directly showed that MRV mRNA translation is not inhibited in the presence of sodium arsenite in PKR-/- cells, strongly suggesting that MRV inhibition of PKR is not involved in MRV escape from host translational shutoff. Taken together, these data suggest that the ability of MRV to prevent host cell translational shutoff, and the ability of MRV mRNAs to escape host cell translational shutoff likely occur through independent mechanisms.

A Model for the role of SGs in MRV infection. Based on previous data and this study, we have developed a working model for the role of SGs and SG disruption during MRV infection. In this model, when MRV infects cells, the cell attempts to inhibit viral replication by activation of PKR, phosphorylation of eIF2α, and formation of SGs. The presence of SGs inhibits viral translation, as is artificially shown by treatment of infected cells at this early time with sodium arsenite or 15D-PGJ2. However, there must be a delicate balance between SG formation and viral translation, as ultimately, the virus is able to disperse SGs as infection proceeds, and this ability is dependent on the accumulation of viral proteins in the cell. At later times in infection, even though eIF2α is phosphorylated, SGs are no longer able to form in MRV infected cells. This ability to disrupt or prevent SG formation correlates with virus escape from translational inhibition, as is shown artificially by the ability of MRV, but not cellular mRNAs to be translated in cells following treatment with sodium arsenite or 15D-PGJ2 only when the virus is capable of disrupting SGs.
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Figure 1. Cells infected with MRV do not contain SGs at late times post-infection despite eIF2α phosphorylation. (A) L929 (B) Cos-7 and (C) HeLa cells were mock infected (top row, left), mock infected, then treated with sodium arsenite for 1 h prior to fixation (top row, right) or infected with MRV T1L (second row), T2J (third row), or T3Dänder C (bottom row). At 20 h p.i., cells were fixed and immunostained with rabbit α-μNS polyclonal antiserum (second, third, and bottom rows, left column) and goat α-TIAR polyclonal antibody (top row, left and right columns, second, third and bottom rows, right column), followed by Alexa 594- conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Bar=10 μM. (D) L929, Cos-7, and HeLa cells were mock infected, mock infected and treated for 1 h with sodium arsenite prior to harvest, or infected with MRV T1L, T2J, or T3Dänder C. At 20 h p.i. cells were harvested and proteins were separated on SDS-PAGE, and transferred to nitrocellulose. Membranes were blotted with rabbit α-μNS, rabbit α-phosphorylated eIF2α, or rabbit α-eIF2α polyclonal antibody, followed by goat α-rabbit IgG conjugated with HRP. Bound HRP conjugates were detected by chemiluminescence staining, quantified by Quantity-One software, and levels of eIF2α phosphorylation in the sodium arsenite-treated mock, and infected cells relative to untreated mock cells were calculated and plotted on the graph.
Figure 2. MRV infection renders cells unable to form SGs in response to Sodium Arsenite. (A) L929 (B) Cos-7 or (C) HeLa cells were infected with MRV T2J (top rows) or T3D<sup>C</sup> (bottom rows). At 20 h.p.i., cells were treated with sodium arsenite for 1 hour, then
fixed and immunostained with rabbit α-μNS polyclonal antiserum (left columns) and goat α-TIAR polyclonal antibody (middle columns), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Merged images containing DAPI-stained nuclei (blue) are shown (right columns). Bar=10 μM.
Figure 3. MRV interference of SGs is downstream of eIF2α phosphorylation. (A) Cos-7 Cells were infected with MRV T1L, T2J or T3D<sup>C</sup>. At 20 h.p.i., cells were left untreated or treated with sodium arsenite for 1 hour. Samples were harvested and proteins were separated on SDS-PAGE and electroblotted to nitrocellulose. Nitrocellulose membranes were then immunoblotted with rabbit α-μNS, rabbit α-phosphorylated eIF2α, or rabbit α-eIF2α polyclonal antibodies as indicated, followed by goat α-rabbit IgG conjugated with HRP. Bound HRP conjugates were detected by chemiluminescence and quantified with Quantity-One software. Fold-increase in levels of phosphorylated eIF2α in sodium arsenite treated uninfected and infected cells relative to untreated cells were calculated and are shown. (B) Cos-7 Cells were infected with MRV T3D<sup>C</sup> and at 20 h.p.i., cells were treated with either NSC11983 (top row) or 15D-PGJ2 (bottom row) for 1 hour, then fixed and immunostained with rabbit α-μNS polyclonal antiserum (left column) and goat α-TIAR polyclonal antibody (right column), followed by Alexa 594-conjugated donkey α-rabbit IgG and Alexa 488-conjugated donkey α-goat IgG. Bar=10 μM.
Figure 4. A non-radioactive protein labeling strategy for detection of newly translated proteins. (A) L-azidohomoalanine (L-AHA) is a methionine analog that is metabolically incorporated into nascent proteins as they are translated. Once L-AHA is incorporated, azide-modified nascent proteins can be covalently linked to biotin-conjugated alkyne in a “click” reaction (Invitrogen Click-iT® AHA) (B) Cells grown on coverslips are infected with virus, incubated overnight, then treated with drugs as indicated. L-AHA is added to cells and is incorporated into proteins that are being synthesized during the labeling period. Nascent proteins are labeled with biotin in a click reaction and detected by Alexa 488-conjugated streptavidin.
streptavidin. (C) Cells grown on plastic dishes are infected with virus, incubated overnight, then treated with drugs as indicated. L-AHA is added to cells and is incorporated into proteins that are being synthesized during the labeling period. Nascent proteins are labeled with biotin in a click reaction, separated on SDS-PAGE and transferred to nitrocellulose, then detected by alkaline phosphatase (AP)-conjugated streptavidin.
Figure 5. Viral and cellular translation are inhibited when SGs are present in sodium arsenite-treated cells at early times post-infection. (A) Cos-7 cells were infected with T3D and at 5 h p.i., cells were left untreated, (top row) or treated with cycloheximide (middle row) or SA (bottom row) for 30 min, then L-AHA was added in the presence of the drugs for an additional 30 min. Cells were fixed and permeabilized, labeled with biotin in a click reaction, then stained with Alexa 488-conjugated streptavidin to visualize nascent protein synthesis (middle column). Cells were additionally stained with antibodies against \( \mu \)NS (left column) and TIAR (right column) followed by Alexa 350-conjugated donkey \( \alpha \)-rabbit IgG or Alexa 594 conjugated donkey \( \alpha \)-goat IgG antibody to visualize infected cells and stress granules. Bar=10 \( \mu \)M. (B) Cos-7 cells were infected with T3D and were treated with drugs in A. 60 min following drug-treatment, L-AHA was added and cells were incubated for an additional 60 min. Cells were harvested and proteins were labeled with biotin in a click reaction, then separated on SDS-PAGE and transferred to nitrocellulose by electroblotting.
AHA-labeled proteins were detected by incubation of blots with AP-conjugated streptavidin. Identical sample volumes were examined in parallel using β-actin-specific antibodies, followed by AP-conjugated goat α-rabbit IgG as a protein loading control.
Figure 6. MRV, but not cellular, mRNAs escape eIF2α phosphorylation induced translational shutoff when SGs are disrupted at late times post-infection. (A) Cos-7 cells were infected with T3D and at 5 h p.i., cells were left untreated, (top row) or treated with cycloheximide (middle row) or SA (bottom row) for 30 min, then L-AHA was added in the presence of the drugs for an additional 30 min. Cells were fixed and permeabilized, labeled with biotin in a click reaction, then stained with Alexa 488-conjugated streptavidin to visualize nascent protein synthesis (middle column). Cells were additionally stained with antibodies against μNS (left column) and TIAR (right column) followed by Alexa 350-conjugated donkey α-rabbit IgG or Alexa 594 conjugated donkey α-goat IgG antibody to visualize infected cells and stress granules. Bar=10 μM. (B) Cos-7 cells were infected with T3D and were treated with drugs in A. 60 min following drug treatment, L-AHA was added and cells were incubated for an additional 60 min. Cells were harvested and proteins were labeled with biotin in a click reaction, then separated on SDS-PAGE and transferred to...
nitrocellulose by electroblotting. AHA-labeled proteins were detected by incubation of blots with AP-conjugated streptavidin. Identical sample volumes were examined in parallel using β-actin-specific antibodies, followed by AP-conjugated goat α-rabbit IgG as a protein loading control.
Figure 7. 15D-PGJ2 induces SGs and inhibits cellular and viral translation at early times post-infection. (A) Cos-7 cells were infected with T3D and at 5 h p.i., cells were left untreated, (top row) or treated with cycloheximide (middle row) or 15D-PGJ2 (bottom row) for 30 min, then L-AHA was added in the presence of the drugs for an additional 30 min. Cells were fixed and permeabilized, labeled with biotin in a click reaction, then stained with Alexa 488-conjugated streptavidin to visualize nascent protein synthesis (middle column). Cells were additionally stained with antibodies against μNS (left column) and TIAR (right column) followed by Alexa 350-conjugated donkey α-rabbit IgG or Alexa 594 conjugated donkey α-goat IgG antibody to visualize infected cells and stress granules. Bar=10 μM. (B) Cos-7 cells were infected with T3D and were treated with drugs in A. 60 min following drug treatment, L-AHA was added and cells were incubated for an additional 60 min. Cells were harvested and proteins were labeled with biotin in a click reaction, then separated on SDS-PAGE and transferred to nitrocellulose by electroblotting. AHA-labeled proteins were detected by incubation of blots with AP-conjugated streptavidin. Identical sample volumes...
were examined in parallel using β-actin-specific antibodies, followed by AP-conjugated goat α-rabbit IgG as a protein loading control.
Figure 8. Viral but not cellular mRNAs escape 15D-PGJ2-induced translational shutoff when SGs are disrupted at late times in infected cells. (A) Cos-7 cells were infected with T3D and at 20 h p.i., cells were left untreated, (top row) or treated with cycloheximide (middle row) or 15D-PGJ2 (bottom row) for 30 min, then L-AHA was added in the presence of the drugs for an additional 30 min. Cells were fixed and permeabilized, labeled with biotin in a click reaction, then stained with Alexa 488-conjugated streptavidin to visualize nascent protein synthesis (middle column). Cells were additionally stained with antibodies against μNS (left column) and TIAR (right column) followed by Alexa 350-conjugated donkey α-rabbit IgG or Alexa 594 conjugated donkey α-goat IgG antibody to visualize infected cells and stress granules. Bar=10 μM. (B) Cos-7 cells were infected with T3D and were treated with drugs in A. 60 min following drug treatment, L-AHA was added and cells were incubated for an additional 60 min. Cells were harvested and proteins were labeled with biotin in a click reaction, then separated on SDS-PAGE and transferred to nitrocellulose.
by electroblotting. AHA-labeled proteins were detected by incubation of blots with AP-conjugated streptavidin. Identical sample volumes were examined in parallel using total eIF2α- and phosphorylated eIF2α-specific antibodies, followed by AP-conjugated goat α-rabbit IgG as a protein loading control, and to examine the impact of 15D-PGJ2 on eIF2α-phosphorylation.
Figure 9. SA does not inhibit MRV translation in PKR-/- cells at late times post-infection. (A) PKR+/+ and PKR-/- cells were infected with T3D and at 20 h p.i. cells were left untreated or treated with cycloheximide or SA for 60 min at which point L-AHA was added and cells were incubated an additional 60 min. Cells were harvested and proteins were labeled with biotin in a click reaction, then separated on SDS-PAGE and transferred to nitrocellulose by electroblotting. AHA-labeled proteins were detected by incubation of blots with AP-conjugated streptavidin. Identical sample volumes were examined in parallel using β-actin-specific antibodies, followed by AP-conjugated goat α-rabbit IgG as a protein loading control.
Figure 10. A model for the role of SGs in MRV infected cells. Illustration diagramming the interplay between MRV and SGs throughout viral infection and in the presence of sodium arsenite and 15D-PGJ2.
CHAPTER 5. ESTABLISHMENT OF A 10 PLASMID-BASED REVERSE GENETICS SYSTEM AND INDUCIBLE CELL LINES FOR FUTURE STUDY OF MRV TRANSLATION

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Abstract

Mammalian orthoreoviruses escape from host translational shutoff in presence of increased levels of phosphorylated eIF2α. In order to understand the mechanism behind viral escape from host shutoff, we want to examine what factors (viral proteins, modifications of viral mRNA, or unique sequences of viral mRNA) are involved in viral translational escape from host shutoff by eIF2α phosphorylation. Here we recapitulated a 10-plasmid based reverse genetics system for mammalian orthoreovirus strains (T1L and T3D) and established a platform to generate inducible viral protein-expressing cell lines in the background of L929 cell line. Using this reverse genetics system and inducible cell lines, we expect to generate viral protein knockout mutants to test if viral proteins play a role in viral escape from host shutoff. We also expect to examine whether viral mRNA is involved in viral escape from host shutoff using several plasmid constructs which can be used to generate different mRNA sequences and L-AHA protein labeling technique as we described in the Chapter 4. Taken together, we laid an important groundwork for future study in viral escape from host
translational shutoff caused by eIF2α phosphorylation, which can potentially enhance our understanding of the oncolytic role of mammalian orthoreovirus in cancer therapy.

**Introduction**

Reverse genetics is an approach for studying the functional role of specific gene sequences by directed deletion, point mutation, and gene silencing. Plasmid-based reverse genetic systems were developed for nearly all major groups of RNA- and DNA-containing viruses to study the functional roles of each gene in the viral genome. Reverse genetics systems allow artificial manipulation of viral genomes at the cDNA level by site-directed mutagenesis, deletion/insertion and rearrangement, and have led to the accumulation of a significant amount of new knowledge relating to the replication, biological characteristics and pathogenesis of these viruses. Members of the Reoviridae family contain a genome with 10-12 individual segments of dsRNA. Viral mRNA is capped, but has no poly (A) tail (32). Reassortment experiments, which rely on the natural ability of genomic segments from different parental viruses to be packaged in progeny virus, have been utilized for the past three decades to “map” identified virus phenotypes to specific genes in the reovirus genome (10). This genetic approach has contributed significantly to our understanding of viral entry and replication mechanism for members of the Reoviridae family (e.g., reovirus, rotavirus). However, applications using this method are limited by the lack of ability to directly manipulate the gene of interest.

Roner et al (1990) showed transfection of dsRNA or ssRNA from reovirus serotype 3 into L929 cells with the help of deficient reovirus serotype 2 infection was able to rescue serotype 3 viruses (36). However, this method readily introduced unwanted reassortants, and was not well accepted in the field. Mundt et al (1996) reported that transfection of cells with
synthetic transcripts of a two‐segmented double‐stranded RNA virus, Birnavirus (Infectious Bursal Disease Virus, IBDV) was able to rescue virus without the use of helper viruses. Viral cDNA of IBDV was cloned into a plasmid downstream of bacteriophage T7 polymerase, viral RNAs were transcribed from linearized plasmids in vitro, and purified transcripts were used to rescue viruses from transcript‐transfected cells (30). This reverse genetics system allowed manipulation of specific genes for two‐segmented double stranded RNA viruses. Boot and Colleagues (1999) devised the first plasmid based reverse genetics system for two segmented dsRNA viruses (Birnaviruses) with the help of fowlpox virus expressing T7 polymerase. They prepared plasmids carrying the full‐length IBDV sequences of segment A and B, flanked by a bacteriophage T7 polymerase promoter and hepatitis delta virus (HDV) ribozyme and followed by a T7 RNA polymerase terminator. The HDV ribozyme gives the advantage that allows complete transcribed viral RNA sequence upstream of the cleavage site to be cut off by the ribozyme (3). Qi et al (2008) then reported rescue of IBDV from cDNA plasmids using a cellular polymerase II system (34), in which complete viral RNAs are synthesized by polymerase II, then cleaved by the hammerhead ribozyme at the 5′ termini and the hepatitis delta virus ribozyme at the 3′ termini to generate an identical copy of the virus RNAs. This approach does not need the introduction of a helper virus expressing T7 polymerase which was used in previous strategies. A simplified version of the polymerase II based reverse genetics strategy was then described for the rescue of viruses with high efficiency (10^{11} TCID_{50} / mL) (2). In this system, viral cDNAs were fused at the 5′ termini to the transcription start site of the immediate early cytomegalovirus promoter instead of using hammerhead ribozyme.

Utilizing a system similar to that described for two‐segmented viruses, a plasmid
based reverse genetics system was recently developed for mammalian orthoreovirus (19). Recombinant reovirus was rescued by transfecting 10 plasmids into murine L929 cells which were also co-infected with replication deficient vaccinia viruses expressing T7 RNA polymerase. In these plasmids, viral cDNAs are flanked by T7 polymerase promoter at 5’ termini, and by hepatitis delta ribozyme at 3’ termini. Recently, this method was further improved through the development of a four-plasmid reverse genetics system with much higher efficiency compared with his previous 10-plasmid based reverse genetics system (22). In this method, each of four plasmids contains more than two cDNAs from viral genomic segments, and are transfected into BHK (Baby Hamster Kidney) cells which constitutively expresses T7 RNA polymerase rather than using vaccinia viruses (22). Using this system, Kobayashi and his colleagues generated viable mutants and confirmed a number of previously published findings (11, 12, 19). However, this 10 plasmid-based reverse genetics system is not sufficient to rescue virus mutants containing lethal defects.

In order to study the functional roles of essential genes, cell lines expressing functional corresponding viral proteins have to be established to complement the current plasmid-based reverse genetics system for reovirus. Since overexpression of some viral proteins, such as μ1, is lethal for cells, inducible cell lines expressing these viral proteins are required to complement the plasmid-based reverse genetics system. In this work, we have established an inducible L929 cell line for the expression of individual reovirus proteins. We have performed proof of concept experiments that this cell line is capable of inducible expression of several reovirus proteins. In addition, we have successfully recapitulated the ten-plasmid reverse genetics system for reovirus using T7 expressing BHK cells.

This work has laid the foundation for a number of important studies aimed at
identifying the role of individual reovirus proteins in SG regulation, and in identifying viral protein and RNA sequences or structures involved in reovirus escape from host translational shutoff following virus induced eIF2α phosphorylation.

Materials and methods

Cells and reagents. Cos-7, cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium from Invitrogen Life Technologies) containing 10% fetal calf serum (HyClone Laboratories) and penicillin/streptomycin (100 IU/mL) (Mediatech). Spinner adapted L929 cells were maintained in c-MEM (Joklik's minimal essential medium, Irvine Scientific) containing 2% fetal calf serum and 2% fetal bovine serum (HyClone Laboratories), 2 mM L-Glutamine and penicillin/streptomycin (100 IU/mL). BHK-T7 (Baby Hamster kidney cells constitutively expressing T7 polymerase)(7) were maintained in Glasgow’s MEM (Lonza) containing non-essential amino acids (Invitrogen), selected with G418 (100 μg/ml) at every other passage. Primary antibodies used in immunofluorescence are as follows: Rabbit polyclonal antiserum against μNS was made by the Iowa State University Hybridoma facility by injection of rabbits with peptides corresponding to μNS AA 1-20, and μNS AA 21-40 synthesized on a Multiple Antigen Peptide System (MAPS), monoclonal antibody 4F2 against reovirus T3D σ3. Secondary antibodies used in immunofluorescence experiments are as follows: Alexa 594-conjugated goat α-mouse IgG antibodies, Alexa 488-conjugated goat α-rabbit IgG antibodies (Invitrogen),

Virions and plasmids. Purified MRV stocks (T1L, T3D) in our laboratory were originally obtained from Dr. Max Nibert at Harvard University. Purified virions were prepared as described (8) and stored in dialysis buffer (150 mM NaCl; 10 mM Tris pH 7.4; 10 mM
MgCl$_2$) at 4 °C. 10 plasmids containing each gene from MRV T1L strain (pT7L1T1L, pT7L2T1L, pT7L3T1L, pT7M1T1L, pT7M2T1L, pT7M3T1L, pT7S1T1L, pT7S2T1L, pT7S3T1L, pT7S4T1L) were made by Dr. Cathy Miller in Dr. Max Nibert’s lab. The backbone of these plasmids is pCI-neo (promega) which was removed of CMV promoter and replaced with bacteriophage T7 promotor. Viral genes from MRV T1L strain were cloned into this modified pCI-neo plasmid, flanked by T7 promoter at 5’ terminus of viral gene and by Hepatitis delta ribozyme functional domain at 3’ terminus of the viral sequence, which make sure intact viral RNA can be generated by T7 polymerase. 10 plasmids for each gene from MRV T3D strain (pT7L1T3D, pT7L2T3D, pT7L3T3D, pT7M1T3D, pT7M2T3D, pT7M3T3D, pT7S1T3D, pT7S2T3D, pT7S3T3D, pT7S4T3D) were obtained from Dr. Terry Dermody’s lab (19). Plasmids used for making inducible L929 cell line were made as follows: Plasmids (pFRT/LacZ, pcDNA6/TR, pOD44, pcDNA5/FRT/TO) were purchased from Invitrogen. The M3 gene (from MRV strain T1L) was PCR amplified from plasmid pCI-neo-M3 with primers containing Hind III and BamHI sites, and was cloned into HindIII/BamHI digested pcDNA5/FRT/TO to make a μNS inducible expression plasmid (pcDNA5/FRT/TO-M3). Each of the remaining MRV genes were also PCR amplified with restriction enzyme-containing primers and individually cloned into pcDNA5/FRT/TO.

**Plasmid transfection and viral infection.** 4.0 × 10$^5$ BHK-T7 cells were seeded onto 35mm 6-well plates the day before transfection. 10 plasmids were mixed at different concentrations [L genes (2μg/each), M genes (1.75 μg/each), and S genes (1.5 μg/each)]. BHK-T7 cells were co-transfected with 10 plasmids using 2 μL of TransIT-LT1 transfection reagent (Mirus) per microgram of plasmid DNA. Four days later, transfected cells and media were harvested and recombinant virus was isolated using plaque assay as previously described.
Plaques were picked and virus was propagated by growth in L929 cells. Cos-7 cells were overlayed with rescued MRV viruses from infected L929 cell lysates, virus was adsorbed to cells for 1 h, at which point, cells were refed with DMEM and incubated at 37°C for 24 h for immunostaining to test viral phenotypes.

**RT-PCR.** Total RNA extracted from infected L929 cells with wildtype (T1L, T3D) or rescued viruses were used as templates, and sequences of primers were used for PCR amplification of the M1 gene as shown in the table 1. RT-PCR was performed as described in the manual of Superscript III transcriptase® with platinum® Taq HiFi kit (Invitrogen). RT-PCR products were sequenced by the Iowa State University DNA facility.

**Extraction of viral genomic dsRNA and electropherotyping of MRV dsRNA segments.** 3×10⁵ L929 cells were infected with rescued viruses or wildtype T1L and T3D strains. At 24 h p.i., total RNA was extracted from infected cells with TRIZOL® reagent (Invitrogen) per the manual instructions. RNA mixed with 5 × loading buffer (50% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol) was preheated at 60°C for 5 min, and separated on an 8% TBE polyacrylamide gel (15 ml H₂O, 5 mL 5 × TBE, 5 mL 40% acrylamide (37.5 : 1), 30μL TEMED, and 300 μL 10% APS) at 60 V for 14 h in 1 × TBE (10.8 g Tris, 5.5g boric acid, 40 mL 0.5M EDTA, pH8.0). Gels were stained in ethidium bromide (EB) buffer for 2 h and dsRNA segments were visualized by UV transillumination. Images were obtained using a Chemi-doc XRS imaging system (Bio-rad).

**Immunofluorescence (IF) assay.** Cells were fixed at room temperature for 10 min with 2% paraformaldehyde in PBS buffer (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ (pH 7.5)) and then washed three times with PBS. Fixed cells were permeabilized by incubation with 0.2% Triton X-100 in PBS for 5 min and then washed three times with PBS. Samples were blocked
by a 10 min incubation with 2% bovine serum albumin in PBS. Primary and secondary antibodies were diluted in 2% bovine serum albumin in PBS. After blocking, cells were incubated for 1 hour with primary antibodies, washed three times with PBS, and then incubated for another hour with secondary antibodies. Immunostained cells were washed for a final three times with PBS, and mounted on slides with Prolong reagent with or without DAPI (Invitrogen). Immunostained samples were examined with a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics. Images were prepared by using Photoshop and Illustrator software (Adobe Systems).

**Establishment of inducible L929 cell lines expressing viral proteins.** The Flp-In™-TRex™ Core Kit (Cat No. K6500-01, Invitrogen) was utilized to generate cell lines expressing reovirus proteins. Four plasmids (pFRT/LacZ, pcDNA6/TR, pOD44, pcDNA5/FRT/TO-M3) were used to generate an inducible L929 cell line expressing viral protein µNS (encoded by M3). First, L929 cells were transfected with linearized plasmid pFRT/LacZ and incubated for 48 hours, then split into 9-cm cell culture petri dishes. This plasmid is expected to randomly integrate into genomic DNA, providing the Flip Recombinase Target integration site for the M3 expressing plasmid (pcDNA5/FRT/TO-M3). Zeocin (400 µg/mL) was added to dishes to select for zeocin-resistant cell colonies for two weeks until cells containing the pFRT/LacZ plasmid formed visible cell colonies. These colonies were isolated with sterile cloning cylinders and grown in individual 3.5 cm 6-well cell culture plates. Harvested zeocin-resistant colonies were individually tested to confirm integration of the plasmid pFRT/LacZ into an active transcription site with a β-Gal staining kit (Cat No. K1465-01, Invitrogen). PCR was also used to amplify the LacZ and Zeocin-resistant genes to further confirm that isolated cell lines contained this plasmid. pFRT/LacZ
containing cell lines were then transfected with the pcDNA6/TR plasmid and grown in the presence of blasticidin (10 μg/mL) to select blasticidin-resistant cell colonies. This plasmid randomly integrates into genomic DNA and constitutively expresses the tetracycline repressor, which can bind to the tetracycline operator and inhibit the expression of M3 in pcDNA5/FRT/TO-M3. PCR was used to amplify the blasticid resistant gene to confirm blasticidin-resistant cell colonies contain plasmid pcDNA6/TR. Finally, pcDNA5/FRT/TO-M3 and pOD44 were co-transfected into the confirmed zeocin and blasticidin-resistant cell line (L929-FRT/LacZ-DNA6/TR). Hygromycin B (500 μg/mL) was added to select cell colonies containing pcDNA5/FRT/TO-M3. To confirm integration of pcDNA5/FRT/TO-M3 into the FRT site in the cellular genomic DNA, a β-Gal staining kit was used to assess the loss of LacZ. Additionally, final cell lines were examined to see if they express NS in response to doxycycline induction by immunofluorescence microscopy.

Results

Recapitulation of a plasmid based reverse genetics system for MRV. The 10 plasmid-based reverse genetics system was originally reported by Kaboyashi et al (19, 22). The T3D viral cDNA segments were cloned into a vector as previously described (Figure 1A) and the T1L viral cDNA segments were cloned into the pCI-neo vector using a similar strategy (data not shown). Recombinant virus was rescued from cell transfected with each of the 10 T3D or T1L plasmids as shown in Figure 1B. 10 plasmids from strain T1L, 10 plasmids from strain T3D, or 2 plasmids from T1L (pT7S1T1L, pT7M1T1L) and 8 plasmids from T3D (pT7L1T3D, pT7L2T3D, pT7L3T3D, pT7M2T3D, pT7M3T3D, pT7S2T3D, pT7S3T3D, pT7S4T3D) were co-transfected into BHK-T7 cells respectively. At 96-hour post-transfection, samples were harvested and recombinant virus was selected following plaque
assay on L929 cells. Isolated plaques containing rescued virus were harvested and grown in L929 cells. To confirm that we had isolated recombinant T1L virus, RNAs from rescued and wildtype T1L- and T3D-infected cells were used as templates to amplify the M1 gene segment (from nucleotides 1767-2304) with RT-PCR (Fig 2A). A mutation (T to C, top strand; A to G, bottom strand) at position 2173 was previously introduced in the M1 cDNA of the plasmid pT7M1T1L to allow discrimination between recombinant and wild-type virus. Sequencing results confirmed that the M1 gene from the rescued recombinant T1L virus contained the introduced mutation at nucleotide 2173, while the wildtype T1L M1 gene did not contain this mutation (Fig 2B). To confirm that we had rescued the T3D/8,T1L S1/M1 recombinant reassortant virus, we additionally separated viral dsRNA segments extracted from rescued recombinant and wildtype viruses on a TBE polyacrylamide gel. These electropherotyping results showed that the band patterns of dsRNA segments of rescued viruses from T1L plasmids, T3D plasmids, or from T3D/8, T1L S1/M1 matched up with the patterns of wildtype T1L and T3D viruses as expected (Fig 2C). We further examined the σ3 phenotype of recombinant virus (T3D/8, T1LS1/M1) by immunofluorescence assay utilizing a monoclonal antibody (4F2) that specifically recognizes T3D σ3, but does not cross-react with T1L σ3. These results further confirmed that the rescued virus (T3D/8, T1LS1/M1) contained T3D σ3 (Fig 2D). Taken together, these assays ruled out the possibility that the rescued viruses came from contamination of cells from wildtype lab strains of virus and confirmed that we were able to repeat this complex assay using our plasmids and cell lines.

**Establishment of inducible cell lines expressing reovirus proteins.** In order to rescue viruses that contain mutations in the genome that impede viral replication, cell lines expressing the corresponding proteins must be created to complement the effects of the lethal
mutations. In addition, because the constitutive expression of some viral proteins may be lethal to cells, the expression of viral proteins in these cell lines need to be inducible. To create cell lines that inducibly express reovirus proteins, we took advantage of the Flp-In™-T-REx™ system from Invitrogen. The overall strategy for creation of these cell lines is outlined in Figure 3. First, L929 cells were transfected with plasmid pFRT/LacZ, which integrates into the genome of the cell resulting in a genomic copy of the Flip Recombinant target site fused in frame with lacZ in the cells. Cell colonies that were resistant to zeocin were selected and amplified. To confirm that the isolated zeocin-resistant cell colonies contained the pFRT/LacZ plasmid integrated in the genome, cells were fixed with 2% paraformaldehyde and incubated with the substrate for β-Gal, and examined for blue color (Fig 4A). These results suggest pFRT/LacZ is integrated into a transcriptionally active site in the genome. In addition, we performed RT-PCR on RNA isolated from these cell lines using primers specific for genes encoding LacZ and zeocin-resistance. PCR results indicated that isolated zeocin-resistant cell colonies (7, 8, 10, and 20) contained LacZ and zeocin-resistance genes while L929 cells did not (Fig 4B). Cell colonies containing plasmid pFRT/LacZ were named Flp-in L929 cells. In a second step, Flp-in L929 cell line number 7 was randomly chosen and transfected with plasmid pcDNA6/TR, which contains the blastacidin resistance gene, and constitutively expresses the tetracycline repressor. RNA from twenty-three blastacidin-resistant cell colonies was isolated and examined by PCR for the presence of the blastacidin-resistance gene. Fifteen of the tested cell colonies were positive for the blastacidin-resistance gene (1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 23), however, surprisingly, 8 of them were negative for this gene (3, 19, 20, 21, 22, 24, 25, 26) (Fig 4C). This suggests that some cells are able to gain resistance to blastacidin without receiving the
pcDNA6/TR plasmid. The blasticidin-resistant cell colonies containing pcDNA6/TR and pFRT/LacZ were named L929-FRT-DNA6. In a final step, L929-FRT-DNA6 cell line number 2 was randomly chosen and co-transfected with µNS expression plasmid pcDNA5/FRT/TO-M3 and the FRT recombinase expressing plasmid pOD44. Hygromycin B was used to screen for cell colonies in which pcDNA5/FRT/TO-M3 integrated within the previously introduced FRT site in the cellular genome. Hygromycin B-resistant cell colonies were then tested for their ability to inducibly express µNS in the presence, but not the absence of doxycycline (an analog of tetracycline) by immunofluorescence microscopy (Fig 4D). In these experiments, cell lines were identified that inducibly express µNS, suggesting that we had successfully established an inducible µNS expressing cell line. In addition, because the L929-FRT-DNA6 cell line has an integrated FRT site, this line can easily serve as a platform to construct isogenic inducible cell lines that express each of the other nine viral proteins. To this end, we have also cloned each of the reovirus genes into the pcDNA5/FRT/TO plasmid for the construction of each of these cell lines.

Discussion

Future applications of the reverse genetics system and inducible cell lines. The 10 plasmid-based reverse genetics system for reovirus is a very powerful tool to study the functional role of viral genes (19). In order to study the functional role of critical proteins, such as µNS, or to make otherwise potentially lethal mutations in the virus, inducible cell lines are required to complement the function of deficient proteins generated by mutations/deletion with this reverse genetics system. For example, the viral nonstructural protein µNS plays an essential role in the viral replication cycle and knockout of µNS results
in the failure of viral replication (20). In reovirus-infected cells, the μNS C-terminal domain forms non-membranous structures in the cytoplasm (5) and the N-terminal region plays roles in recruitment other viral proteins (σ2, σ3, σNS, μ2, λ1, λ2, λ3, and core particles) (6, 21, 27-29). With the plasmid-based reverse genetics system and inducible μNS expressing cell line, we can generate virions containing mutation or deletions in the M3 gene (encoding μNS) to further examine functional roles of the cytoplasmic matrix formed by the C-terminus of μNS, and how interactions between μNS and other viral proteins affect viral replication, translation and assembly. Additionally, we recently found that MRV infection disrupts stress granules (SGs) and prevents SG formation induced by sodium arsenite at late times post-infection. Based on colocalization of μNS with SGs (data not shown) and the fact that both μNS formed viral factories and SGs are associated with microtubule network (18, 24, 33), we have hypothesized that μNS plays a role in the disruption or prevention of SG formation. Using the reverse genetics system and the μNS inducible cell line, we can rescue viral particles that contain deletions or mutations in the microtubule associated region of μNS, or the SG-associated region of μNS to test if microtubule or SG association is a factor in SG disruption.

The reverse genetics system and its implication in viral translation regulation. So far, the efficiency of the plasmid-based reverse genetic systems is not very high. The efficiency of the 10-plasmid reverse genetics system is about 100 pfu/ml at 96 hours post transfection (19), and the improved 4 plasmid reverse genetics system only increases the efficiency to $10^{4.5}$ pfu/ml after 96 hours post-transfection (22). Both biochemical and structural analysis has shown that reovirus genomic plus-RNAs and mRNAs generated by core particles are
capped (13, 16, 17, 38). Ribosome binding to reovirus mRNA in protein synthesis requires 5' terminal 7-methylguanosine (m7G) in an in vitro translation system in wheat germ extracts (4). Some studies have suggested viral mRNAs may not be capped at late times post-infection (39, 42), however, this idea is not widely accepted within the field. The clarification of whether capping is required for the translation of viral mRNA at late time infection is a critical step towards understanding the mechanism behind viral translational escape from host cellular shut off by eIF2α phosphorylation for all members of the Reoviridae family. The 10-plasmid based reverse genetics system and improved 4-plasmid reverse genetics developed by Kobayashi and his colleagues did not address the question of whether the founder viral mRNAs transcribed from cDNA plasmids by T7 polymerase are capped (19, 22). In this reverse genetics system, presumably, T7 polymerase transcribes viral mRNA from cDNA plasmids in the cytoplasm, transcripts are translated into viral proteins by cellular translational machinery in the cytoplasm, and then translated viral proteins including viral transcriptase (λ3), triphosphatase (μ2), methyltransferase and guanylytransferase (λ,2) may launch another round of viral mRNA synthesis in the cytoplasm. If first round founder viral mRNAs are not capped, uncapped transcripts must be translated at a low efficiency through some unknown mechanism different from the mechanism used by capped eukaryotic mRNA. If capping is required for the translation of viral mRNA, there are two possibilities. First, uncapped founder mRNAs transcribed by T7 polymerase are transported into the nucleus and capped by cellular capping enzymes in the nucleus at a very low efficiency. It is extremely unlikely that the T7 polymerase C-terminal domain (CTD) can recruit capping enzymes in the nucleus, because unlike eukaryotic polymerase II, a T7 polymerase fused with a eukaryotic CTD cannot recruit capping enzymes (14, 31). Second, a small amount of
cDNA plasmids may enter the nucleus during transfection and capped viral mRNAs may be generated by the cellular polymerase II and cellular capping enzymes at a very low efficiency, at which point, capped viral mRNAs are then transported to the cytoplasm for viral protein synthesis.

Like the Reoviridae, in Birnaviruses such as IBDV, dsRNAs are protected within the particles throughout the virus cycle. Viral particles carry out several enzymatic activities to synthesize viral mRNAs with cap structures at 5’ termini but no poly (A) tail (9). A recent study showed that use of a polymerase II reverse genetics system significantly improved the efficiency of rescuing viruses compared with previously reported T7 polymerase-based reverse genetics system because the polymerase II based system increases the expression of viral proteins which pave the way for second round of viral replication (2). Pre-transfection of a plasmid encoding the viral polyprotein also significantly increased the efficiency of T7 polymerase-based reverse genetics system (2). This suggested that mRNA capping is required for the viral translation of IBDV. In order to answer the question of whether the founder viral mRNAs are capped during viral transcription in the reovirus reverse genetics system, we can potentially co-transfect several polymerase II-based plasmids encoding the viral transcription apparatus to see if this strategy improves the efficiency of rescuing viruses.

In addition, because Lys-190 and Asp-191 are the only amino acids necessary for λ2 guanylyltransferase activity and the methyltransferase domains of λ2 are known (25, 26), we can mutate the methyltransferase and guanylyltransferase domains of the reovirus λ2 to determine if capping activity is required for reverse genetics. If mRNA capping is required for viral translation, we could use a polymerase II based reverse genetics system to rescue viruses, which may be more efficient than the current T7 polymerase based reverse genetics
system.

**Future plans to determine the mechanism of viral translation in the presence of increased levels of eIF2α phosphorylation with the plasmid-based reverse genetics system.** We have recently found that reovirus translation escape from cellular shutoff occurs in the presence of eIF2α phosphorylation at late times post infection as described in the chapter 4. Our results suggest that viral translation escape from cellular shutoff correlates with the disruption of SGs, and reovirus likely utilizes a unique translation mechanism to translate viral proteins in the presence of a limited amount of ternary complex (eIF2-GTP-tRNA\textsubscript{iMet}). Different viruses have evolved different strategies in order to translate viral proteins in the presence of high levels of eIF2α phosphorylation (37, 40, 41). For example, the translation initiation of subgenomic mRNA (26S) from Sindbis viruses is not affected when eIF2α is phosphorylated in the presence of sodium arsenite. Genetic data suggest that a highly stable hairpin loop downstream of start codon (AUG) is necessary to provide resistance to eIF2α phosphorylation (37, 41). In addition to the conventional eukaryotic eIF2- and eIF5-dependent pathway of 80S complex assembly, the hepatitis C virus (HCV) internal ribosome entry site (IRES) can direct the assembly of 80S translation initiation complexes without eIF2 and eIF5 when eIF2α is inactivated by phosphorylation under stress conditions or treatment of IFNα (35, 40). Bacteria have three translation initiation factors: IF1, IF2 and IF3; their counterparts in eukaryotic cells are eIF1A, eIF5B and eIF1 (1). Like bacteria, HCV only uses the minimum of translation factors under certain conditions. Only eIF3 and eIF5B (the analog of bacterial IF2), are required for the assembly of HCV translation initiation complex (40), suggesting the origin of eIF2 in evolution is relevant to
the appearance of ribosomal scanning in eukaryotes as HCV translation initiation does not need ribosomal scanning. Reovirus genomic RNA segments have very short 5' UTRs of about 12-32 nucleotides (nts) in length (32). 40S ribosomes protect the entire stretch from the cap to the initiation codon, while the 80S complexes protect ~30 nts which are closer to the initiation codon (23). Our studies have shown that the helicase eIF4A is not required for MRV translation. These data strongly suggest that the assembly of the 80S translation initiation complex for reovirus mRNA may not require ribosomal scanning, and possibly does not require eIF2. If this is the case, reovirus must use an eIF2 independent-pathway for viral translation at late times post-infection when host cellular translation is shut off by eIF2α phosphorylation.

We intend to use plasmid-based reverse genetics to examine whether a unique sequence in the reovirus genome or any viral proteins are required for viral translation in the presence of high levels of phosphorylated eIF2α. First, we will test if a unique 5' terminal sequence is required for viral protein expression in the presence of phosphorylated eIF2α. A number of plasmids have been created or will be created (Fig 5), including: A) CMV-26S-GFP, a positive control GFP RNA that contains the 26S enhancer region from Semliki Forest viruses as previously described (41); B) CMVGFP, a negative control, representing a cellular mRNA, which expresses a GFP mRNA that contains a cap structure and poly (A) tail; C) CMV-5'-S3-GFP-S3/3', an S3GFPS3 fusion that has GFP inserted into a full length S3 gene and will express an S3/GFP/S3 fusion RNA containing a cap structure and poly (A) tail; D) CMV-GFP-HD, a control that expresses GFP RNA containing a cap structure, but not a poly (A) tail; E) CMV-5'S3GFPS3/3’HD, which expresses S3GFPS3 RNA containing a cap structure, but not a poly (A) tail; F) CMV-HR-5’-GFP-3’-HD, which express GFP without a
cap structure or poly (A) tail. G) CMV HRS3GFPS3HD, which expresses S3GFPS3 RNA without a cap structure or poly (A) tail. HR represents the hammerhead ribozyme which cleaves newly synthesized mRNA at its 5’ terminus, HD represents hepatitis delta virus ribozyme which cleaves newly synthesized mRNA at its 3’ terminus. We will first examine the expression of the proteins encoded by these constructs in transfected cells in the presence of phosphorylated eIF2α. Once we identify a sequence or structure necessary for translation of reovirus mRNA, we will then examine the importance of that region in viral mRNA translation by creating viruses in which these regions are mutated using the reverse genetics system. In addition, we will also examine the role played by individual virus proteins in translation of viral mRNA in the presence of phosphorylated eIF2α by generating reverse genetics viruses that do not express individual viral proteins. Each of these approaches using the reverse genetics systems should reveal important information on how reovirus translation escapes phospho-eIF2α induced host translational shutoff.

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29. **Miller, C. L., M. M. Arnold, J. Kim, and M. L. Nibert.** Reovirus λ3 polymerase is recruited to the location of viral transcription within reovirus factories via an association with non-structural protein μNS. Manuscript in preparation.


Fig 1. The MRV 10-plasmid-based reverse genetics system. A. Construction of reverse genetics plasmids. Viral cDNAs are fused at their native 5’ termini to the bacteriophage T7 RNA polymerase promoter, and fused at their native 3’ termini to the Hepatitis Delta virus (HDV) ribozyme sequence. B. Schematic of approach. The ten reovirus cDNA contracts are transfected into BHK cells which constitutively express T7 polymerase. Nascent viral
mRNAs generated by T7 polymerase correspond to the native 5’ termini. The native 3’ termini are generated by self-cleavage by the HDV ribozyme. At 4 days post-transfection, transfected cells are lysed, subjected to three cycles of freeze/thawing, and viable virus is rescued from the cloned cDNA containing plasmids by plaque assay using L929 cells.
Fig 2. Characterization of reovirus rescued from 10-plasmid based reverse genetics system. **A.** RT-PCR for partial M1 gene sequences (1767-2304) from rescued viruses (T1L RG), wildtype T1L viruses (wt T1L) and plasmid pT7M1(T1L). **B.** Sequencing of the partial M1 sequences (1767-2304) from wt T1L, rescued viruses T1L RG, and plasmid pT7M1 (T1L). Both forward and reverse sequencing indicate the M1 gene from wt T1L viruses has
an A (T) at position 2173, while the M1 gene from rescued T1L viruses has a G (C) at this position similar to that seen in plasmid pT7M1(T1L). C. Electropherotypes of dsRNA from wildtype T1L, T1L recombinant, recombinant virus (T3D/8, T1L S1/M1), T3D recombinant, and wildtype T3D. Viral dsRNA was extracted from infected Cos-7 cells with TRIzol and separated on an SDS-polyacrylamide gel, followed by ethidium bromide staining to visualize viral gene segments. Gene segments are indicated. D. Immunofluorescence staining of wildtype T3D, wildtype T1L, and rescued recombinant virus (T3D/8, T1L S1/M1). Infected Cos-7 cells were immunostained with mouse monoclonal antibody 4F2 against viral protein σ3 and rabbit anti-μNS polyclonal antibodies, followed by secondary goat anti-mouse IgG conjugated with Alexa 594 and goat anti-rabbit IgG conjugated with Alexa 488. Scale bar = 10μm.
Fig 3. Schematic for establishing an inducible L929 cell line expressing viral
nonstructural protein μNS. (1) L929 cells were transfected with plasmid pFRT/LacZ. L929 cells with plasmid pFRT/LacZ integrated into genomic DNA at a transcriptionally-active site were selected with Zeocin (400 μg/mL) and confirmed with a β-Gal staining kit and RT-PCR. This cell line is named Flp-in-L929. (2) Flp-in-L929 line was transfected with plasmid pcDNA6/TR. Cell lines containing this plasmid were selected with blasticidin (10 μg/mL), and constitutively express the tetracycline repressor. This cell line remains resistant to zeocin and is named L929-FRT-DNA6/TR. (3) L929-FRT-DNA6/TR were cotransfected with plasmids pcDNA5/FRT/TO-M3 and pOD44. Cell lines resistant to hygromycin B were selected. These lines selectively express μNS in the presence of doxycycline (2 μg/mL). The selected cell lines were named L929-FRT-DNA6-M3. The FLP recombinase expressed by plasmid pOD44 facilitates recombination between plasmid pcDNA5/FRT/TO-M3 and cellular genomic DNA at the previously integrated FRT site. In the absence of doxycycline, an analog of tetracycline, expression of μNS is inhibited by the tetracycline repressor protein that is constitutively expressed by the integrated plasmid pcDNA6/TR. When doxycycline is present, it binds to tetracycline repressor proteins and therefore prevents binding to the tetracycline Operator, resulting in the release of expression of μNS.
Fig 4. Characterization of an inducible L929 cell line expressing viral protein μNS. A. β-Gal staining for zeocin-selected Flp-in-L929 cell line. Zeocin-selected Flp-in-L929 cells were fixed with 2% paraformaldehyde and then stained with a commercially available β-Gal
Kit (Invitrogen). β-Gal positive cells are stained blue. **B.** Genomic DNA from selected β-Gal positive cells and L929 cells were extracted and used as templates for PCR using primers specific for the amplified genes. **C.** Twenty-four blasticidin-resistant cell colonies were selected, and genomic DNAs were extracted from those cell lines as templates for amplifying the blasticidin resistant gene. The pcDNA6/TR plasmid was used as a positive template control (+). **D.** L929-FRT-DNA6-M3/2 cell cells were treated with or without doxycycline (2μg/mL) for 48 hours and fixed with 2% paraformaldehyde. Cells were immunostained with rabbit anti-μNS antibody, followed with Alexa 488 conjugated to goat anti-rabbit IgG.
Fig 5. Plasmid constructs for identification of MRV sequence or structure important for translation in the presence of eIF2α phosphorylation. A) CMV-S26-GFP, a positive control GFP RNA that contains the 26S enhancer region from Semliki Forest Virus that has previously been shown to escape host cell translational shutoff. B) CMVGFP, a negative control representing cellular mRNA, which expresses a GFP mRNA that contains a Cap structure and poly A tail C) CMV-5’S3-GFP-S3/3’, an MRV S3/GFP/S3 fusion that has the GFP gene inserted into the full length MRV S3 gene and will express an S3/GFP/S3 fusion RNA containing a Cap and poly A tail, D) CMVGFP-HD, a control which expresses the GFP gene containing a Cap, but no polyA tail E) CMV-5’S3-GFP-S3/3’-HD, an MRV S3/GFP/S3 fusion that has the GFP gene inserted into the full length MRV S3 gene and will express an
S3/GFP/S3 fusion RNA with a Cap, but no poly A tail. F) CMV-HR-5’GFP-3’-HD, a control GFP mRNA that does not contain a Cap or a polyA tail, or G) CMV-5’-HR-S3-GFP-S3/3’-HD, an MRV S3/GFP/S3 fusion that has the GFP gene inserted into the full length MRV S3 fusion RNA that does not contain a Cap or poly A tail. Constructs D-G each contain a Hepatitis d ribozyme sequence positioned to cleave RNAs at the exact 3’ end of the MRV or GFP gene. Constructs F and G contain a hammerhead ribozyme positioned to cleave RNAs at the exact 5’ end of the MRV or GFP gene. Each of these RNAs will be examined for their ability to be translated in the presence of SA.
Table 1. Primers used in the reverse genetics system and the study of establishing an inducible cell line expressing viral protein μNS.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>T1L M1 (1767-2304)</td>
<td>Forward 5'-ATGCTACTAGCAGCAGCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GATGAAGCGCGTACGTAGTCTTAG-3'</td>
</tr>
<tr>
<td>Zeocin resistant gene</td>
<td>Forward 5'-CCAAGTTGACCAGTGCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCAGTCTGCCTCTCGGC-3'</td>
</tr>
<tr>
<td>LacZ gene</td>
<td>Forward 5'-GCCGTGTTTTACAACGT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACAAACCGTCGGATTCTC-3'</td>
</tr>
<tr>
<td>Blasticidin resistant gene</td>
<td>Forward 5'-AACCATGGCCAAGCCTTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTAGCCCTCCCACACATATAA-3'</td>
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CHAPTER 6. GENERAL CONCLUSIONS

In this project, we first described that infection of mammalian orthoreovirus leads to an stress granule formation, following viral uncoating, but preceding viral transcription or translation at early times post-infection (2-6h. p.i.). MRV induction of SGs was found to be triggered by increased levels of phosphorylated eIF2\(\alpha\) activated by at least two kinases (PKR, PERK, GCN, HRI). However, though eIF2\(\alpha\) phosphorylation was maintained throughout infection, SGs were not observed in MRV-infected cells at late times post-infection, suggesting that SG formation is unlikely to be involved in shutting off cellular translation following MRV infection at late times post-infection as previously proposed. We further found that viral gene expression renders cells unable to form SGs in response to external stress signaling, and that this interference is downstream of eIF2\(\alpha\) phosphorylation.
The mechanism for MRV disruption of SGs is not yet determined and will be an important avenue of future study.

SGs hold cellular translation in check and only allow the expression of stress response genes, determining whether cells will be destined for apoptosis or recovery from stress. In our study, we showed viral particles are recruited into SGs following virus entry into the cytoplasm in a manner dependent on newly synthesized viral mRNAs. Viral translation correlates with the disruption of SGs. Cellular, but not viral, translation is inhibited by increased levels of eIF2\(\alpha\) phosphorylation seen following MRV infection or treatment with sodium arsenite, as well as inhibition of eIF4A, suggesting MRV uses a unique translational initiation mechanism which does not require, or is at least less dependent on ternary complex (eIF2-GTP-tRNA\(^{Met}\)) and eIF4A for initiation. We further showed MRV translation is not inhibited in the presence of eIF2\(\alpha\) phosphorylation in the PKR-/- MEF cell line, which
directly demonstrated that viral translation escape from host translational shutoff is not through viral protein σ3 inhibition of PKR in the vicinity of viral translation as previously proposed. This represents the first evidence that MRV induction of host cell translational shutoff, and MRV escape from translational shutoff are independent mechanisms. This has laid important groundwork for future studies identifying the mechanism of MRV translation initiation in the presence of limited ternary complex.

Hypoxia is a common feature of malignant tumors where tumors rapidly outgrow their vascular supply and develop hypoxic microenvironments. Hypoxia induces phosphorylation of eIF2α leading to the formation of stress granules (SGs), which likely contributes to the resistance of hypoxic cells to apoptosis induced by cancer therapeutics. Our studies found that MRV infection actively disrupts SGs and successfully translate viral proteins in the presence of phosphorylated eIF2α. These findings enhance our understanding of the mechanisms behind MRV escape from cellular translational inhibition in the presence of eIF2α phosphorylation when cells are treated with IFN, X-ray, genotoxic drugs, and will lead to important future studies impacting our understanding of the mechanisms behind MRV oncolysis of tumor cells.
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