Examination of viral infection and host response to define therapeutic potential of oncolytic mammalian orthoreovirus

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Examination of viral infection and host response to define therapeutic potential of oncolytic mammalian orthoreovirus

by

Luke D. Bussiere

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Microbiology

Program of Study Committee:
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
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The International Agency for Research on Cancer estimates that 9.6 million people died of cancer in 2018. Prostate cancer (PCa) is the fourth most commonly diagnosed cancer worldwide, and is the most common in men within the United States. Development of new cancer therapies has helped many PCa patients experience remission, but far too many die due to tumor resistance toward therapy. Tumors are heterogeneous collections of aberrant cells growing uncontrollably, and as they progress, they evolve becoming more genetically and metabolically diverse throughout the tissue. Within the tumor, microenvironments form that differ dramatically from one region to another. One tumor microenvironment of particular interest is tumor hypoxia, an area of low oxygen that is increasingly resistant to cancer therapy. Within these hypoxic regions, the hypoxia-inducible factor 1 alpha subunit (HIF-1α) accumulates, enters the nucleus, and enhances transcription of genes involved in growth, survival, angiogenesis, and metastasis, resulting in increased tumor aggressiveness. When patients have high amounts of HIF-1α accumulation within the blood serum, they typically have much poorer prognosis as compared to those without elevated HIF-1α. Novel HIF-1α inhibitors are being investigated for safety and efficacy to inhibit these effects, as well as to sensitize hypoxic tumors to current cancer therapies. While viruses are not often thought of as therapies, mammalian orthoreovirus (MRV) has been shown to inhibit HIF-1α in vitro and in vivo. MRV is a cancer killing, oncolytic, virus that specifically targets, infects, replicates, and lyses cancerous tissue while leaving healthy tissue undamaged. The virus has undergone various Phase I-III clinical trials and has been shown to be safe for the treatment of humans, but it has only been shown to be effective in some patients while others receive little to no benefit. Therefore, research is needed to investigate the
viral impact on the cell to better understand which tumor types or tumor environments are most amenable to MRV therapy.

This work began investigating the basic biology of MRV factories utilizing a modified virus so that infection could be observed in live-cell microscopy. By adding a tetraeysteine (TC) tag within the viral μNS protein, the viral factories could be observed using the TC labeling FIAsh-EDT2 reagent. This study provided data about factory dynamics and presented a potential tool for further investigation of MRV oncolytics. At the same time systematic investigation into the stage of viral replication necessary to inhibit HIF-1α was investigated. Using a transcriptional deficient virus along with endosomal breakdown inhibitors, a step between viral capsid cleavage and transcription was determined to be sufficient for HIF-1α inhibition. Subsequent work looked into the role of viral components, protein or dsRNA, and cellular stress granule (SG) formation caused by virus infection to further elucidate the mechanism of MRV-induced HIF-1α inhibition. Evidence suggests that the viral capsid proteins and dsRNA are not required to decrease HIF-1α accumulation, but early during infection SG formation is.

Altogether this work has produced valuable information into the mechanism of MRV-induced inhibition of HIF-1α and provided a new technique for further study of MRV replication and oncolysis.
CHAPTER 1. GENERAL INTRODUCTION

Millions of people die each year as a direct result of viral infection (1-5), but viruses are also being used therapeutically to save lives (6, 7). One area of great interest is the use of oncolytic, or cancer killing, viruses in cancer patients. These viruses can be naturally oncolytic, like mammalian orthoreovirus (MRV), or they can be engineered to specifically kill cancerous cells while leaving the healthy tissue unharmed (8). The engineered herpes simplex virus is the first oncolytic virus to be approved by the FDA, and is being used in melanoma patients currently (9). With many other viruses undergoing clinical trials, in the future there will likely be a handful of viruses that can be used in various cancer situations to help alleviate pain and suffering. One of the front-runners is MRV, which has been examined in Phase I-III clinical trials, and has seven active or recruiting trials currently (10). MRV has been shown to be an effective cancer killing therapy that is safe, with little side effects observed in over thirty clinical trials. While safety is important and many patients observe some benefit, unfortunately many patients have experienced little to no benefit and it is unclear why. Therefore, further research into MRV replication and response of the host is necessary to determine which patients can benefit most from MRV therapy.

Previous data has suggested that MRV inhibits the hypoxia-inducible factor 1 alpha subunit (HIF-1α) (11-15), which regulates over 2% of all genes within some cells (16). HIF-1α has been shown to upregulate genes involved in survival, proliferation, angiogenesis, and metastasis resulting in cancers that are more aggressive, more resistant to therapy, and inhibit the body’s natural immune response against the tumor (17-20). Within regions of low oxygen, called hypoxia, the lack of oxygen allows for the accumulation of HIF-1α. In normal tissue this is important for survival of the cells that lack oxygen, but when HIF-1α accumulates within a
tumor, clinicians find that these patients have a poorer prognosis (21). Therefore, new HIF-1α inhibitors are needed in conjunction with standard cancer therapeutics to kill resistant tumors due to the detrimental effects of tumor hypoxia. To determine if MRV could be a good therapy for hypoxic tumors, it is necessary to understand the mechanisms of HIF-1α inhibition. Thus far, inhibition of HIF-1α has been shown to be dependent on RACK1-mediated proteasomal degradation and translational inhibition at early and late times during infection, respectively (11, 12). Apart from this little is known about how viral infection inhibits HIF-1α, therefore additional research into MRV infection and the host response is required to better understand this inhibition.

During MRV infection two different inclusions are formed within the cell. First, stress granules (SGs) are formed as a result of stress induced by viral infection. SGs are non-membranous inclusions that are an aggregation of ribonucleoprotein (RNP) complexes, composed of stalled initiation factors and ribosomal subunits bound to the 5’ cap of mRNA (22). These stress granules form due to stress induced inhibition of translation, and they work to sequester translational factors into the granule (23). This occurs for two reasons, first it inhibits RNPs from moving throughout the cell potentially activating additional responses; and second, in the case of viral replication, it can be used to halt infection through inhibition of translation (24). Interestingly, SGs have been suspected of regulating HIF-1α accumulation (25, 26), but since the virus induces and quickly disrupts SGs the effect this has on HIF-1α accumulation is unknown (27). The second inclusion observed within the cell, the viral factories (VFs), begin to form while the SGs are being disrupted. VFs are the site of viral transcription, translation, assembly/packaging, and replication (28-32), and recent work has shown that VFs sequester various SG proteins to the periphery of the factory (33). This sequestration may disrupt SG
formation and potentially provides translational machinery, which is necessary for translation of viral transcripts, available for viral replication (32, 33). Clearly the VF are important during viral infection, but our study of them has been limited due in part to our inability to observe them in live-cell microscopy.

Within this work MRV was genetically modified for the first time so that VF could be observed in live-cell microscopy. This has provided valuable data into the behavior of VF and has produced a novel tool for future study of MRV replication. Additionally, work was conducted to investigate the stage of viral replication that is necessary to inhibit HIF-1α accumulation. A step between viral capsid cleavage and transcription was shown to be sufficient to inhibit HIF-1α, and removal or mutation of capsid proteins or dsRNA resulted in little to no rescue of HIF-1α accumulation. This suggests the capsid proteins and dsRNA are not necessary to inhibit HIF-1α. Furthermore, SG formation in response to MRV infection was investigated and suggested to be necessary at early times during infection for decreased HIF-1α accumulation. Together this work defined the therapeutic potential of MRV in hypoxic tumors, through investigation into virus and host mechanisms of HIF-1α inhibition.

**Dissertation organization**

This dissertation is broken into six chapters. Chapter 1 is a general introduction into the rational for the research conducted. Chapter 2 is a literature review of MRV, tumor hypoxia, viral factories, stress granules, and additional relevant material for the work presented in this dissertation. Chapter 3 is a modified manuscript published in the *Journal of Virology* detailing our early work modifying MRV with a tetracysteine tag to allow observation of VFs, which play an essential role in viral infection, in live-cell microscopy. This work was conducted at the same time we were investigating the mechanisms of MRV induced HIF-1α inhibition shown in
chapters 4 and 5. Chapter 4 is work prepared for submission to the Journal of Virology looking into the stage of viral replication necessary for HIF-1α inhibition in hypoxic prostate cancer cells. Chapter 5 is a manuscript prepared for submission to Virology that continues on the work from chapter 4 by looking specifically at the host response to viral infection and its role in HIF-1α inhibition. Chapter 6 is a general conclusion summarizing the contribution of this work.

References


CHAPTER 2. LITERATURE REVIEW

Introduction

In 2019 the World Health Organization recognized 14 different viruses that caused outbreaks across the world including: Ebola virus, Zika virus, dengue virus, yellow fever virus, chikungunya virus, measles causing rubeola virus, and MERS-coronavirus (1). Apart from these viruses that rapidly spread throughout a region causing disease, and receive much news attention, there are many silent viruses responsible for the deaths of millions of people. Since 2016 it is estimated that each year across the globe 128,500 children five years of age or younger die as a result of rotavirus, 291,000 to 646,000 people die from influenza infection, and 770,000 HIV patients die due to AIDS-related illness (2-4). In 2018 it was estimated that 9.6 million cancer patients died, and of those 20% or 1.92 million are estimated to be due to cancer causing oncoviruses (5, 6). Clearly infectious agents have plagued human health, as well as the health of our crops and animals, but over a century of research has taught us that viruses can also benefit humans. For instance, in the late 1990s to early 2000s researchers proposed that infection with the flavivirus GB virus C prolongs onset of AIDs in HIV patients (7), and in 2008 researchers proposed that plant viruses could help provide resistance to stressors in various plants, including drought and freezing conditions (8). Currently virotherapy, or the use of viruses to alleviate disease in humans, has become an area of great interest. Viruses are being investigated as gene editing tools with the expectation that they can be engineered to integrate a functioning gene in place of a nonfunctioning gene in ailing patients. Adeno-associated viruses (AAVs) have been particularly intriguing in gene editing and have been shown to be effective in various clinical trials. One example where AAV has been an effective gene editing tool was in a clinical trial inserting factor IX into factor IX defective hemophilia patients, thereby restoring blood clotting
(9). Virotherapy has also proven to be beneficial in cancer patients as several modified or unmodified viruses can kill tumor cells while leaving the host unscathed (10). These cancer killing or oncolytic viruses are going through numerous preclinical and clinical trials in conjunction with standard cancer therapeutics, and currently mammalian orthoreovirus is leading the pack.

**Mammalian orthoreovirus**

Mammalian orthoreovirus (MRV) is a benign virus that does not cause disease in healthy individuals but can result in mild malaise, fever, or diarrhea in immunocompromised individuals (11). Children are especially susceptible since MRV is transmitted via the fecal oral route. In fact, a study found that 50% of healthy children five or six years old had antibodies against MRV, suggesting most people have been exposed early in life (12). There are three serotypes of MRV classified as type 1, 2, or 3, and within each serotype there is one or two prototypic strains: type 1 Lang (T1L), type 2 Jones (T2J), type 3 Dearing (T3D), and type 3 Abney (T3A) (11). T1L was recovered from a healthy child, while T2J and T3D were isolated from children with diarrhea, and T3A was isolated from a child with upper respiratory illness (11). The T3D virus is commonly referred to as T3D\textsuperscript{C} or T3D\textsuperscript{F} with the superscript “C” or “F” denoting the Cashdollar or Fields lab from which it originates. The T3D\textsuperscript{F} strain was originally called T3D\textsuperscript{N} for the Nibert lab (13, 14), but is now recognized as being from the Fields lab and likely a lab derived mutant from the original T3D\textsuperscript{C} (15, 16). T3D\textsuperscript{F} has been found to harbor several mutations including the highly researched proline to serine mutation at amino acid 208 in the \(\mu_2\) protein (14, 17). This mutation yields differences in viral factory (VF) morphology which helped identify T3D\textsuperscript{F} as a mutant of T3D\textsuperscript{C} (14, 17). In addition to these three serotypes, in the 1980’s the unclassified Ndelle virus was characterized and found to be indistinguishable from viruses in the *Reoviridae*
family (18, 19). At that time, it was classified as an Orbivirus, however this has since been called into question (18, 19). Ndelle virus is now considered to be a putative fourth serotype of MRV called type 4 Ndelle (T4N). Apart from these strains collected from humans, MRV has been shown to naturally infect and cause disease in other mammals including diarrhea in pigs, and can infected and spread in the bat population (20-23).

MRV is a segmented double-stranded RNA (dsRNA) virus, from the Reoviridae family, containing 10 segments which encode for 8 structural proteins and 4 non-structural proteins (11). The MRV genome segments have been classified by size according to banding pattern on agarose gel electrophoresis with 3 Large (3.8-3.9 kb) segments, L1-3, 3 Medium (2.1-2.3 kb) segments, M1-3, and 4 Small (1.2-1.3 kb) segments, S1-4. The structural proteins $\sigma_1$, $\sigma_2$, $\sigma_3$, $\mu_1$, $\mu_2$, $\lambda_1$, $\lambda_2$, and $\lambda_3$ are encoded by S1, S2, S4, M2, M1, L3, L2, and L1, respectively, while the nonstructural $\sigma_{NS}$ is encoded by S3 and $\mu_{NS}$ is encoded by M3 (Fig. 1). The S1 and M3 segments also encode $\sigma_{1s}$ and $\mu_{NSC}$, respectively, that result from leaky scanning in which the ribosome ternary complex skips the first AUG and initiates downstream, resulting in a total of 12 proteins encoded by the 10 MRV genome segments (11).

The 10 dsRNA genome segments are located in the core of the virus and may organize in layers of parallel striations based on evidence from cytoplasmic polyhedrosis virus, another member of the Reoviridae family (24). Each of the 10 dsRNA segments are believed to bind a protein complex of $\lambda_3$, the RNA-dependent RNA-polymerase, and $\mu_2$, a cofactor with NTPase, RTPase, and potential helicase activity (25-27), which reside inside the viral core. The viral core is composed of repeating units of $\lambda_1$ and $\sigma_2$ capsomeres with $\lambda_2$ turrets equally spaced on the vertices of the core (28). These turrets are composed of a $\lambda_2$ pentamer that align in a way to form a barrel that goes into the core and allows the escape of the mRNA from inside the core.
Figure 1. MRV structure, segments, and proteins. The 10 MRV gene segments (left), and the encoded viral protein (middle), and the structural proteins (right) and how they assemble to form the complete virion (bottom) are shown. Within each gene segment the open reading frame/s is/are dark or light gray, and the 5′ and 3′ untranslated regions are shown in white.

Each turret was believed to associate with one $\lambda.3/\mu.2$ complex and one dsRNA, but since MRV only has 10 genome segments and 12 turrets, scientists were unsure what two of the turrets were used for. Now it is believed that two of the turrets are open and do not associate with the $\lambda.3/\mu.2$
complex, which suggests that each turret is responsible for one dsRNA segment only (24). The capsid proteins $\sigma_3$ and $\mu_1$ associate with the $\lambda_1/\sigma_2$ capsomeres to cover the core, but do not cover the turrets which protrude further from the core than the capsid proteins (28). The attachment protein, $\sigma_1$, forms a trimer and slides inside the barrel of each turret and is locked in position when the $\lambda_2$ pentamer tightens around the $\sigma_1$ trimer, and is not released until cleavage of the outer capsid proteins during viral infection (29).

**Viral life cycle**

Viral infection begins when the attachment protein $\sigma_1$ binds to sialic acid and/or JAM-A on the surface of the cell (30-32). Attachment results in clathrin-mediated endocytosis of the virus into the cell in an early endosome (33). The endosome can progress into a late endosome, in which infection proceeds, or a recycling endosome, in which the virus is shuttled back to the membrane and infection does not proceed (34). If the virus enters into the early and late endosome then infection can occur, during this time the pH of the late endosome drops and results in the activation of proteases including cathepsin-L and -B which cleave the outer capsid protein $\sigma_3$, resulting in $\sigma_3$ clearance from the virion (35, 36). This can be recapitulated *in vitro* by treating MRV with $\alpha$-chymotrypsin for a short amount of time which also cleaves $\sigma_3$ and $\mu_1$ resulting in the formation of an infectious subviral particle (ISVP) (37-39). The ISVP within the late endosome no longer has $\sigma_3$ bound to the $\mu_1$ protein which frees $\mu_1$ to be cleaved by endosomal proteases into $\mu_1\delta$ and $\phi$ fragments (40). The $\mu_1\delta$ fragment further autocleaves into the $\mu_1N$ and $\delta$ fragments (41, 42), and both $\mu_1N$ and $\phi$ are released from the viral core resulting in the formation of an ISVP* (Fig. 2) (43). The myristoylated $\mu_1N$ fragment, and to some degree the $\phi$ fragment, once cleaved are responsible for poking holes in the endosomal membrane (41, 44). Since $\mu_1$ is in high copy number, 600 copies per virion, many holes are produced in the
Figure 2. Virus, intermediate particles, and the core structure. The virus is composed of all 8 structural proteins, and transitions into the ISVP once the outer capsid protein σ3 (blue) and μ1 (red) are cleaved within the endosome and σ3 is released. The μ1N and φ fragment of μ1 are released and the δ fragment changes conformation attached to the core. This results in the λ2 (green) pentamer conformational change that release σ1 (black) from the turrets forming the ISVP*. Finally, the rest of μ1 is released and the core is able to escape the endosome.

endosomal membrane, but these holes are suggested to be too small for the virus to move through to escape the endosome (45). Therefore, it has been hypothesized that the large number of holes allows for the movement of water between the cytoplasm and the endosome due to osmotic differences, this influx of water is suggested to result in bursting of the endosomal membrane to release the viral core (45). During the release of μ1N and φ, the attachment protein σ1 is released from the turret as the λ2 pentamer changes confirmation and releases the attachment protein resulting in a transition from an ISVP to an ISVP* (29). The δ fragment of μ1 is released and once the virion has lost σ3, μ1, and σ1 it is described as the viral core.

The virus core escapes the endosome and is able to begin transcribing mRNA from the dsRNA within the core (46). Interestingly, the core does not disassemble and therefore it is believed that the cell is not exposed to the dsRNA genome segments except for a portion of virions in which abnormal disassembly occurs (47, 48). The dsRNA is transcribed by the λ3 polymerase and the mRNA is shuttled through the barrel of the λ2 turrets where 5’ capping occurs, and is released into the cytoplasm to be translated (28). MRV mRNA has a short 5’
untranslated region (UTR) that may aid in viral mRNA translation during cellular translational inhibition. During viral infection cellular translation is halted to inhibit viral replication, but certain cellular stress genes are still translated. These genes often possess an upstream open reading frame (uORF) that is closer to the 5′ end of the mRNA aiding in translation initiation, which is otherwise stopped during stress (49). It has been shown that MRV inhibits cellular translation but the viral proteins are still translated (50), and it has been speculated that the short 5′ UTR may allow translation initiation similar to mechanism of uORFs within cellular genes. As the viral proteins are translated, the nonstructural protein μNS aggregates to form inclusions called viral factories (VFs) (51). The VF matrix protein, μNS, has been shown to be sufficient to form these inclusions and binds directly with σ2, σ3, μ2, λ1, λ2, and λ3, which make up the viral core, as well as the core itself and the nonstructural σNS protein (17, 52-55). As VFs mature from small into large structures, the viral cores are observed embedded within the factory where transcription, translation, assortment, viral core assembly/packaging, virion capsid assembly, and dsRNA replication occur (53, 55-58). While we know that these processes occur at the VF we currently do not understand the mechanisms of MRV assortment or assembly/packaging. Replication of the RNA is predicted to occur within the core, where λ3 produces dsRNA from the packaged +ssRNA forming an infectious virus (11). The virus life cycle is completed when MRV lyses the host cell and releases into the environment (Fig. 3). Additionally, work with the Reoviridae member, rotavirus, has shown that rotavirus escapes in membrane bound vesicles called exosomes prior to cell lysis (59). MRV has been suggested to lyse cells around 24 h post-infection (p.i.), but in standard plaque assays there is significant cell clearage as a result of multiple rounds of viral replication and lysis by 24 h p.i. Therefore, if cell lysis is the only mechanism of MRV escape, 24 h does not provide enough time for MRV to
infect enough cells to form a plaque. Together this suggests that MRV may, like rotavirus, utilize exosomes to escape from the cell prior to lysis to spread from cell to cell.

Figure 3. Viral replication. The virus attaches to sialic acid and/or JAM-A and enters the cell via clathrin-mediated endocytosis. Within the endosome pH-dependent proteases cleave the outer capsid proteins releasing µ1 to pokes holes within the endosome to allow escape of the viral core into the cytoplasm. The core transcribes mRNA which is translated during primary transcription and translation, which results in accumulation of the VF protein µNS. VFs form within the cell where secondary rounds of transcription and translation, along with assortment, assembly/packaging, and viral replication occur. MRV finishes its life cycle when it is released from the cell.

Viral factories

Since most of the stages of viral replication occur within the viral factory it has been a focus of much research. VFs have been shown to be essential for viral replication as mutations in the carboxyl-terminus of the matrix protein µNS, which inhibit aggregation and factory
formation, result in loss of viral recovery (60, 61). Although VFs are essential, they are not identical between MRV strains. The T3DF strain has VFs that are globular in nature while the other strains have varying degrees of filamentous VFs (Fig. 4) (14). A single amino acid difference within the µ2 structural protein of T3DF is responsible for a difference in VF morphology and function between T3DF and the other strains (14, 17, 62). T3DF has a serine at amino acid 208 of µ2 which inhibits binding and acetylation of the microtubules observed in the other strains that have a proline at amino acid 208 (14), inhibiting µ2 recruitment of µNS and VFs to the microtubules (17). Microtubules are still necessary in T3DF infected cells as µNS also binds to the microtubules which allows VFs to fuse together, but µNS association is not sufficient to observe filamentous VF formation (14, 17). Apart from differences in morphology, VF function is altered when tight microtubule association is lost. It has been shown that during assembly of the viral core or virion, the viral cores organize in straight lines along the microtubules in T1L infected cells, but the cores arrange in no discernable pattern in T3DF infected cells that lack µ2/microtubule binding (62). These straight lines of cores associating with the microtubules have been further suggested to play a role in efficient packaging of the dsRNA into the core. In T1L infected cells a higher percentage of the total virions have the complete dsRNA genomes, while in T3DF infected cells more virions lack dsRNA. Furthermore, when T1L infected cells are treated with the microtubule destabilizing drug nocodazole, the percentage of virions lacking the dsRNA segments increases (62). While we are unsure how microtubules aid in successful packaging of dsRNA, it is clear VF association with microtubules is beneficial for viral propagation.
Figure 4. Viral factory morphology between MRV strains. Adapted from Miller et al. (52). Cells infected with T1L or T3DF present different VF morphology. In the T1L infected cells the VFs are filamentous and form along the microtubules due to μ2 binding to microtubules. T3DF VFs are globular throughout infection due to a mutation within μ2 (P208S) which inhibits μ2/microtubule binding.

The VF is made up of the matrix protein μNS which is suggested to be sufficient to form the VF (17). In cells transfected with μNS alone viral factory-like (VFL) structures form that are globular inclusions that look similar to VFs in T3DF infected cells, further suggesting μ2’s involvement in filamentous morphology in other MRV infected cells (17). The carboxyl-terminal third of μNS is responsible for factory formation and λ3 binding, while the amino-terminal third is responsible for binding the core proteins σ2, μ2, λ1, and λ2, and the nonstructural σNS protein (52-55). Therefore, during infection, μNS forms the VF and recruits or retains the viral proteins needed to assemble the viral core within the VF, aiding virus assembly. Since the transcriptionally active viral cores are assembled at the VF, these cores increase transcription at the VF producing large amounts of viral mRNA (53). Interestingly, the VF has also been shown to sequester translational initiation machinery around the periphery of the factory including
eIF3a, eIF3b, and the ribosomal protein S6 (58, 63). During times of cellular stress, these proteins are found at stress granules (SGs) as a mechanism to inhibit translation. Therefore, recruitment of these proteins around the VF potentially acts as a mechanism to increase translation of the viral mRNA. MRV is not the only virus shown to recruit translational machinery proteins around replication complexes. Hepatitis C virus has also been shown to recruit SG associated proteins to replication factories (64), and in West Nile virus and dengue virus infected cells SG nucleating proteins TIA-1 and TIAR colocalize to viral replication complexes (65). Alternatively, MRV has been shown to initiate cellular translational inhibition and this recruitment to the VF could provide an environment that is protected from cellular inhibition allowing translation of the viral mRNA. Either way VFs are essential for viral replication, and they play a role in many stages of viral infection.

**Stress granules**

Viral factories play a large role in viral infection and replication, but it is also clear that the VF disrupts MRV induced SG during infection (63). Stress granules are membrane-less inclusion bodies that form as a result of eIF2α phosphorylation from cellular stressors including infection, heat shock, protein misfolding, or salt or pH imbalance (66). eIF2α can be phosphorylated by four known kinases that recognize cellular stress. These proteins are heme-regulated inhibitor (HRI), general control non-depressible 2 (GCN2), protein kinase R (PKR), and PKR-like endoplasmic reticulum kinase (PERK), and in stress conditions one or more of these proteins is activated resulting in eIF2α phosphorylation (67). eIF2α is one of three subunits that make up eIF2 which is part of the ternary complex composed of eIF2, GTP, and Met-tRNAi (68, 69). The ternary complex binds to the 40S ribosomal subunit with other initiation factors to form the 43S preinitiation complex (PIC), which is primed to bind mRNA to begin translation.
Once 43S PIC binds to the mRNA, this 48S PIC is ready to begin ribosomal scanning prior to translation (71). During ribosomal scanning Met-tRNA\textsubscript{i} bound to eIF2 aids in identifying the start codon for initiation of protein translation. Once the Met-tRNA\textsubscript{i} binds to the start codon, AUG, the GTP bound by the eIF2\textsuperscript{α} is hydrolyzed to GDP by eIF5 to provide energy needed to release eIF2 from the preinitiation complex (71). Once eIF2-GDP is released the 60S ribosomal subunit binds the 40S subunit and translation begins (71). The inactive eIF2 needs to replace GDP for GTP to become active and to bind Met-tRNA\textsubscript{i}. Therefore, the eIF2\textsuperscript{α} subunit binds to eIF2B which aids in release of GDP from the protein (72, 73). eIF2\textsuperscript{α} when it is non-phosphorylated binds eIF2B within the catalytic site allowing GDP release, but when eIF2\textsuperscript{α} is phosphorylated it binds the catalytic and regulatory site of eIF2B preventing GDP release (73). This inactive eIF2-GDP is unable to bind Met-tRNA\textsubscript{i} and therefore, ternary complex cannot form to assist in ribosomal scanning. The 48S* PIC, which is the 48S PIC that is lacking ternary complex, stalls at the 5′ end of the mRNA forming a ribonucleoprotein (RNP) complex (Fig. 5) (70). Under stress and eIF2\textsuperscript{α} phosphorylation many RNPs accumulate signaling the SG nucleating proteins, G3BP1, G3BP2, TIA-1, and TIAR to bind and aggregate to form SGs (74, 75). These nucleating proteins also recruit additional SG effector proteins to the SG and result in cytoplasmic inclusions within the cell of translationally inept RNP complexes.

MRV infection induces phosphorylation of eIF2\textsuperscript{α} and SG formation early during infection through at least two of the eIF2\textsuperscript{α} kinases: HRI, GCN2, PKR, or PERK (76). While eIF2\textsuperscript{α} remains phosphorylated, SGs are quickly disrupted once the virus undergoes transcription
Figure 5. Phosphorylation of eIF2α results in translational inhibition. Under normal conditions (bottom) the 40S ribosomal subunit and multiple initiation factors come together along with the ternary complex on the 5′ end of the mRNA. The ternary complex composed of eIF2, GTP, and Met-tRNAi binds to the 43S PIC allowing ribosomal scanning to occur. Once AUG is found GTP is hydrolyzed and eIF2-GDP is released along with the other initiation factors and translation continues. eIF2-GDP binds eIF2B, which exchanges GDP for GTP, and eIF2-GTP can bind Met-tRNAi, form the 43S PIC, and initiate translation again. During stress eIF2α is phosphorylated triggering eIF2-GDP to get stuck binding eIF2B without exchange of GDP for GTP. The 43S* PIC missing ternary complex cannot initiate translation and becomes stuck at the 5′ end (top). This results in RNP accumulation and SG formation.

and translation (76). Similarly, rotavirus also induces eIF2α phosphorylation but SGs do not form during infection suggesting rotavirus may be more efficient at quenching SG formation (77). As mentioned previously, MRV factories are suggested to inhibit SG formation, which require transcription and translation to form (63, 76). In cells infected with transcription
deficient, UV-inactivated MRV, there are significantly more SGs that remain within the cell for an extended duration, further suggesting the role the viral proteins and VF formation play in disrupting SGs (76). Within the VF, the nonstructural σNS protein, associates with and sequesters G3BP1 to the periphery of VFs (63). G3BP1 is constitutively expressed within the cell and when eIF2α is phosphorylated resulting in RNP accumulation, G3BP1 binds to the RNA and nucleates the SG. Additionally, the G3BP1 homolog, G3BP2, has been shown to nucleate SG, but when both proteins are knocked down, SG formation is inhibited (75). Since σNS recruits G3BP1 to VF it is hypothesized that this functions to disrupt current SG and inhibit future SG formation.

Inhibition of SG formation can be beneficial for viral infection since SGs not only inhibit translation, but also function as part of the innate immunity within the cell. For instance, SGs are suggested to play a role in inducing antiviral cytokines. In cells lacking the SG nucleating proteins G3BP1 and G3BP2, researchers found that influenza A viral infection resulted in 6-fold less IFN-β mRNA compared with cells expressing G3BP1 and G3BP2 (78). Therefore, for successful replication, viruses have evolved to inhibit SG formation and signaling. Apart from MRV inhibition of SGs, influenza A virus also inhibits SG formation through reduced PKR activation (79). Researchers mapped this phenotype to the NS1 protein which when mutated to inhibit RNA binding lost its ability to inhibit PKR activation and SG formation (80). In addition, poliovirus and encephalomyocarditis virus induce SGs but also utilize proteinases to cleave G3BP1. This cleavage breaks down current SGs and inhibits future SG formation, but interestingly, when G3BP1 cannot be cleaved, SGs are rescued (81, 82). Along with MRV, these viruses have evolved to inhibit SGs and their detrimental effects to viral replication.
Stress granules have also been implicated in cancer aggressiveness, including decreasing cell death and increasing cell metastasis. Receptor for activated C kinase 1 (RACK1) is sequestered to SGs during stress, and in cancer cells researchers showed that this sequestration inhibits apoptosis signaling within the cell (83). Furthermore, in KRAS mutated colon cancer cells SG formation was found to be enhanced as a result of stress compared to non-mutated KRAS cells (84). When these cells were treated with the chemotherapeutic oxaliplatin, the KRAS mutants had more SGs and were more resistant to cell killing, but when SG formation was inhibited with COX1/2 inhibitors, these mutants were resensitized to oxaliplatin treatment (84). These studies exemplify how SGs can negatively impact or desensitize tumors to standard cancer therapy. As for SG impact on cell metastasis, in mouse cells lacking G3BP1, researchers observed less growth and metastasis of orthotopic tumors (85). Moreover, osteosarcoma cells with G3BP1 knockdown, when implanted in mice showed significant decrease in SGs formation and metastasis compared to wt osteosarcoma cells, while tumor proliferation rate remained the same (86). Altogether this suggests that SG knockdown can benefit cancer patients, and since MRV inhibits SGs and kills tumors, MRV is a natural candidate for cancer therapy.

**MRV oncolytics**

The utilization of viruses to kill cancer is a growing idea that has shown promise. Currently within the United States talimogene laherparepvec (T-VEC) is the only FDA approved oncolytic virus for treatment of cancer (87). T-VEC is a modified herpes simplex virus containing GM-CSF, which enhances inflammation, is being used to treat melanoma (88). Being the first oncolytic virus approved by the FDA, it has hopefully opened the door for other oncolytic viruses to be approved for use within the clinics, including MRV. As previously described, MRV does not cause significant disease in healthy individuals, but that does not mean it does not have clinical significance. MRV is a cancer killing virus that targets, replicates in, and
kills tumor cells in infected cancer patients. The virus was first described as a potential oncolytic virus in 1998 and has shown efficacy in killing tumors in preclinical trials in mice, has been shown to be safe in Phase I-II clinical trials, and has progressed thus far to one Phase III clinical trial in head and neck cancer patients (89, 90). Oncolytics Biotech Inc is a Canadian company that has patented and is currently organizing clinical trials utilizing Pelareorep, a proprietary unmodified MRV, in conjunction with common cancer therapeutics against several cancer types. MRV has been used in clinical trials to treat over 1100 patients. Patients were originally treated intratumorally but shortly after beginning clinical trials it was shown that MRV can be delivered intravenously to reach the cancerous tissue, and currently 900+ patients have been treated intravenously with MRV. Recent data from 13 clinical trials from Oncolytics Biotech suggest that 81% of all cancerous tissue becomes infected with replicating virus (91). Even difficult to reach primary and secondary tumors within the brain have been shown to be infected with intravenous delivery of MRV (92).

It was unclear how MRV was able to evade the immune response within the blood since MRV is prevalent within humans and results in neutralizing antibody production. In healthy children ages 5-6 in Tennessee researchers found that 50% had neutralizing antibodies against MRV (12). Furthermore, in an attempt to link liver disease with MRV infection, researchers found that 38 of 43 patients, with or without liver disease, had antibodies against serotype 3 MRV (93). This suggests that most patients that receive MRV therapy in clinical trials will have an immediate immune response against the virus. While it is possible that some treated with MRV therapy did not have previous antibodies against the live virus, clinicians should expect them to develop an immune response after the first treatment potentially nullifying future MRV treatments. Yet throughout numerous clinical trials the virus evades the immune system and
reaches primary and secondary tumors with ease (91). This has puzzled virologists until recent
data has suggested that MRV utilizes monocytes to spread throughout the body. Upon entry into
the blood, MRV is bound by neutralizing antibodies and phagocytosed by monocytes, which
then deliver the virus into cancerous tissue (94). Once within the tumor microenvironment, the
virus escapes the monocyte, via a mechanism that we do not currently understand, infects and
kills the malignant cells. This hijacking of the monocyte may explain how MRV gains access to
various areas of the body, including the brain, and provides a mechanism in which MRV evades
immune surveillance.

While originally believed to exclusively lyse tumor cells, it has become clear that MRV
not only kills tumors but also induces antitumor immunity to aid in cancer killing and to provide
lasting immunity against cancer relapse. In mouse models, researchers took splenocytes from
mice with prostate tumors treated with MRV, and then injected them into naïve mice that were
subsequently challenged with prostate cancer tissue to investigate MRV-induced antitumor
immunity. Mice treated with splenocytes from MRV-treated mice survived significantly longer
than those treated with splenocytes from inactivated MRV- or PBS-treated mice (95). This is
exciting as MRV induced antitumor immunity provides a mechanism by which the virus can
prevent future relapse in cancer patients. Not only can MRV induce an immune response against
cancer antigens, it has also been shown to make cold tumors that suppress the immune response,
into hot tumors that are actively penetrated by the innate and adaptive immune system. MRV
activates innate immunity by transforming immature into mature dendritic cells that produce
inflammatory cytokines (96, 97). Furthermore, MRV treated dendritic cells activate natural killer
cell cytolytic activity and increase migration of immune cells into tumor microenvironments (96,
97). Infection with MRV also activates the adaptive immune response. In eight out of nine
Table 1. Several clinical trials with Pelareorep. Adapted from Clements et al. (103). IT, intratumoral; IV, intravenous; BR, OR, best or overall response; CR, PR, MjR, MnR, complete, partial, major, or minor response; SD, PD, stable or progressive disease; NR, not reported.

<table>
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<th>Phase</th>
<th>No of patients</th>
<th>Cancer type(s)</th>
<th>Mode of administration</th>
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<th>Dose of combination therapeutic</th>
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<td>IT</td>
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<td></td>
<td></td>
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<td>N/A</td>
<td>(BR) 1 PR 10 PD</td>
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<td>33</td>
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<td>(OR) 8 SD 2 PD</td>
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tumors extracted from patients with brain cancer treated with intravenous MRV treatment, CD8+ T cells were found in and around blood vessels (92). This compared to three out of six patient tumors not treated with MRV with CD8+ T cells around blood vessels, and overall the levels of T cells were lower compared to those treated with MRV. CD8+ T cells are responsible for detection and destruction of cancerous cells, suggesting that MRV infects tumors and recruits the immune system to an otherwise immunosuppressive tumor. Apart from this, MRV increases T cell cytokine secretion and cytotoxicity through dendritic cell activation (96, 98). These features of MRV infection aid in immune system penetration into the tumor microenvironment and provide evidence for the use of MRV with cancer immunotherapies that rely on the ability of the T cells to reach the tumor.

Currently there are seven active or recruiting clinical trials utilizing MRV therapy, and three are investigating the use of MRV with an antibody immunotherapy targeting either PD-1 or PD-L1 (90). PD-1 stands for programmed death 1 receptor and it can be bound by PD-1 ligand (PD-L1) or PD-L2 (99). When the T cell receptor binds to an MHC on a cell presenting an aberrant antigen the T cell can be activated to induce cytokines and secrete granzymes to kill the cell, but binding to the MHC is not sufficient to signal T cell activation. Instead numerous coreceptors bind between the two cells and these coreceptors send signals that activate or inhibit T cell function (100). PD-1 is one of these coreceptors and upon binding to PD-L1 the T cell activity is suppressed (101). In normal tissue these coreceptors work to inhibit T cell activation against self, inhibiting autoimmune diseases, but many tumors evolve to express high levels of PD-L1 to suppress T cell activation. Most normal tissue expresses little to no PD-L1, but in one study researchers found that all collected samples of melanoma, lung, ovary, and colon cancer tested had high levels of PD-L1 (102). While PD-L1 is responsible for suppressing cytotoxic T
cells it can also induce T regulatory cells and anti-inflammatory IL-10 production (102, 104). T regulatory cells inhibit the immune response and are also necessary to inhibit autoimmune diseases in healthy tissue. Unfortunately, tumors not only inhibit cytotoxic T cell activation but can increase T regulatory cells around the tumor resulting in immunosuppressive tumor environments that are ignored by the immune system (105). Therefore, since PD-1/PD-L1 binding results in T cell inhibition and T regulatory cell induced immunosuppression, antibodies against PD-1 and PD-L1 have been utilized to reverse this immunosuppression. These treatments work by binding PD-1 or PD-L1 to inhibit PD-1/PD-L1 binding resulting in increased CD8+ T cell activation and killing. Currently MRV is being investigated in clinical trials with three FDA approved antibody immunotherapies against PD-1 or PD-L1: atezolizumab, pembrolizumab, or nivolumab (90). Clinicians hope that MRV recruitment of the innate and adaptive immune response to the tumor in addition to antibody dependent PD-1/PD-L1 binding inhibition, will reverse tumor suppression of T cells and result in a robust immune response against the tumor. Additionally, some evidence suggests that MRV infection increases PD-L1 on the tumor and thus PD-1 or PD-L1 antibodies may benefit MRV therapy by boosting virus induced antitumor immunity and cell killing (92).

Tumor hypoxia

Cancer is a collection of aberrant cells that grow chaotically, without correct regulation. As cancer amasses into a large solid tumor, there are various distinct regions or environments which vary genetically and metabolically. Increased heterogeneity within solid tumors results in increased cancer resistance and worse clinical outcome (106). One microenvironment that has garnered much attention is tumor hypoxia, or regions of low oxygen, due to outgrowth from the blood vessels (Fig. 6). These hypoxic microenvironments upregulate many genes, including pathways aimed at restoring oxygen levels, and are associated with aggressive cancers (107).
Figure 6. Diagram of tumor hypoxia. As a solid tumor grows rapidly regions of the tumor become distant from the nearest blood vessels (dark red). Close to the blood vessels O₂ levels vary between 3-8% and these cells (pink) receive sufficient O₂, but as cells get further away, areas of hypoxia (0.5-3% O₂) and anoxia (< 0.5% O₂) form. When oxygen concentration get too low (anoxia) the cells (black) begin to die, but within hypoxic regions the cells (grey and blue) can survive for long periods of time.

Since cancerous cells divide rapidly within a solid tumor, there are areas that move too far away from the nearest blood vessel resulting in decreased oxygen and nutrient supply. If cells grow too far away from the blood supply the oxygen levels can become so low that the cells die, these areas are call anoxic, or without oxygen. In between the normoxic, or normal oxygen level, regions and the anoxic regions there are regions called hypoxic. Hypoxia describes a region that has low oxygen, usually 3% oxygen and lower, and these regions are common within solid tumors (107). Tumor hypoxia usually results in more aggressive forms of cancer that are more
resistant to cancer therapies, tend to metastasize through the body, and grow and replicate faster than normoxic regions (107, 108). These regions are also more difficult to treat in part because the most common cancer therapies, chemotherapy and radiation, are both inhibited in hypoxic regions. First, radiation requires the production of radical oxygen species that cause damage to cellular DNA resulting in cell death. Radiation has diminished effect in cells that lack oxygen present to produce these radicals, and therefore strategies to decrease tumor hypoxia have increased radiation therapy success (109). Second, chemotherapeutics are delivered to the tumor via the blood supply, and since hypoxic regions have a lack of a blood supply the delivery of these therapies is hindered (110). Apart from deficient delivery, hypoxia alters cell metabolism and upregulates various genes, including the multidrug resistance 1 protein, resulting in increased resistance to chemotherapies (110, 111).

Under hypoxic conditions hypoxia-inducible factors (HIF) accumulate within the cell and become active. HIF-1 was the first identified in 1991 (112), there are now three known HIF subtypes, HIF-1, -2, and -3 with most research focused on understanding the first two. Both HIF-1 and HIF-2 proteins are composed of an alpha (HIF-α) and beta (HIF-β) subunit which are constitutively expressed, but only HIF-α is regulated according to oxygen prevalence (113). When HIF-1α and -2α accumulate, they enter the nucleus and bind to HIF-β to form HIF-1 or -2 along with p300/CBP, the transcriptional coactivator, on hypoxia response elements resulting in transcriptional upregulation of target genes (113). HIF-1 and -2 have been shown to upregulate genes involved with survival, proliferation, immortalization, metastasis, and angiogenesis, and therefore, have been intensively studied in cancer (114, 115). If oxygen levels are brought back to normoxic levels, the HIF-α subunits are targeted for degradation inhibiting HIF dependent transcriptional activation. HIF-α subunits are constitutively made but under normoxic conditions
are quickly bound by propyl hydroxylase domain (PHD) proteins resulting in oxygen dependent hydroxylation of HIF-α, which is further recognized by Von Hippel–Lindau tumor suppressor (VHL) protein which ubiquitinates HIF-α targeting the protein for proteasome-mediated degradation (113). Factor inhibiting HIF (FIH), which is also regulated by oxygen, hydroxylates HIF-α resulting in the inability of HIF-α to bind p300/CBP thus suppressing HIF transcriptional upregulation (116, 117). All these pathways are part of the oxygen dependent pathway and if oxygen is low HIF-α accumulates within the cell, but HIF-α is also regulated by the oxygen independent pathway. HIF-1α has a PAS-A domain that is a competitive binding site of both heat shock protein 90 (HSP90) and RACK1 (118), and HIF-2α also has a PAS-A domain but RACK1 binding has not formally been shown. RACK1 binding to HIF-1α results in ubiquitination and targeted degradation of the protein, while HSP90 protects HIF-1α accumulation (Fig. 7). The balance between HSP90 and RACK1 binding to HIF-1α can be altered in cells resulting in various HIF-1α basal levels of accumulation between cells, and may provide a mechanism for increased HIF-1α accumulation observed in many cancers (118, 119).

Together HIF-1α and -2α increase cellular survival, proliferation, immortalization, metastasis, and angiogenesis, but they are active at varying stages of hypoxia. When cells undergo extreme hypoxia, when oxygen levels are below 0.5%, HIF-1α is active first for around 24 h (120). If the cells remain under chronic hypoxia, HIF-2α will take over and HIF-1α will no longer accumulate (120). In a tumor it is common to have dips in oxygen concentration, but then oxygen levels can rise to a more normal state. Additionally, HIF-2α is primarily found in endothelial, hepatic, renal, lung, and myeloid cells while HIF-1α is expressed in all cells (120, 121). While both are responsible for upregulating genes involved with cancer aggressiveness,
Figure 7. HIF-α regulation under normoxia and hypoxia. HIF-α is constitutively expressed but is quickly degraded under normoxic conditions. When oxygen is present the oxygen dependent pathway (right) is active in which the PHD protein hydroxylates HIF-α, targeting it for VHL binding, ubiquitination, and proteasome-mediated degradation. Additionally, the FIH protein can hydroxylate HIF-α and inhibit translation. Under hypoxic conditions (left) these pathways are inactive and HIF-α accumulates. The oxygen independent pathway (middle) is active under normal and hypoxic conditions and involves competitive binding of HSP90 with HIF-1α or -2α, and RACK1 with HIF-1α which results in proteasome-mediated degradation (RACK1 binding), or protection (HSP90 binding). RACK1 is suggested to bind HIF-2α as well, but has not formally been shown. HIF-α is responsible for the upregulation of numerous genes involved in growth, survival, angiogenesis, and metastasis.

HIF-1α has been suggested to control over 2% of all genes directly or indirectly in endothelial cells under hypoxic conditions, and therefore has been more heavily examined (122). HIF-1α accumulation is a significant problem in cancer patients as patients with increased HIF-1α within the blood serum are diagnosed with a worse prognosis compared to patients without high HIF-1α levels (123). Increases in HIF-1α and the downstream hypoxic response have also been shown to create an immunosuppressive environment that inhibits inflammation and increases T regulatory cells and tumor-associated macrophages that induce immune tolerance (124). Since HIF-1α is associated with more aggressive cancer, a large amount of research has focused on looking for HIF-1α inhibitors (125). Various drugs have been investigated that inhibit HIF-1α in preclinical...
and clinical trials. In mice, researchers have suggested that PX-478, a HIF-1α inhibitor, works to sensitize resistant pancreatic ductal adenocarcinoma to gemcitabine chemotherapy and helps increase antitumor immunity (126). While further research is necessary to investigate these and new HIF-1α inhibitors mechanism of action and safety, one potential HIF-1α inhibitor is MRV which has already been shown to be safe in humans. MRV has been shown in vitro and in vivo to inhibit HIF-1α in various tumors (127-130). This inhibition has also proven sufficient to inhibit various HIF-1 and HIF-2 target genes (130, 131). Additionally, studies have demonstrated that MRV replicates efficiently under hypoxic conditions in vitro, and in vivo MRV penetrates and replicates in hypoxic regions within solid tumors (128, 130). Further research into the mechanism of MRV induced inhibition of HIF-1α has revealed that RACK1-mediated proteasomal degradation and translational inhibition are utilized in MRV infected cells (128). Altogether, this data suggests that MRV could be a useful therapeutic in hypoxic tumors to inhibit the hypoxic response and kill cancerous cells.

**Prostate cancer**

Prostate cancer (PCa) is estimated to develop in 1 in 7 men during their lifetime with 60% of diagnosed cases in men 65 years of age or older (132). Furthermore, in 2019 the American Cancer Society estimated that 174,650 men would be newly diagnosed with PCa, making it the number one most diagnosed cancer in the United States (133). While advancing cancer therapeutics and prevention have been gradually lowering the number of PCa caused deaths each year, PCa is still the second leading cause of cancer related death, behind lung cancer, in men (133). Those that get PCa have a 5-year survival rate of 90%, which is higher than many other cancers like pancreas and lung cancer that have a 5-year survival rate of 9% and
19%, respectively (133). Yet despite a higher survival rate PCa claims the lives of many patients, and therefore new therapies are needed to combat this malignancy.

As mentioned, the 5-year survival rate of PCa patients is 90%, and this number goes up to 99% if the PCa is localized within the prostate (133). In patients with localized PCa, clinicians often begin treatment by surgically removing part or the whole prostate (134). During surgery they often implant radioactive iodine-125 or palladium discs to kill any additional parts of the tumor that were not resected (134, 135). External beam radiation is also a potential early treatment option that specifically destroys tumor cells within the prostate (135). Furthermore, some men with early stage PCa or with advanced age can take a wait and watch approach, where clinicians actively monitor the tumor instead of treating these individuals (135). The wait and watch approach is possible since early PCa grows slowly and many men will die of natural causes before the tumor causes significant disease. This does not mean that these men are unaffected by the tumor as patients often describe difficulty or sometimes pain urinating, increased frequency of urination, erectile dysfunction, and discomfort when sitting (136, 137). Therefore, when localized to the prostate, the tumor is a nuisance but does not usually result in death of the patient.

On the other hand, the 5-year survival rate drops to 29% in men with advanced metastatic PCa as treatment of these tumors is increasingly more difficult (133). Once the tumor has metastasized throughout the body, local radiation and surgery are not sufficient and therefore clinicians use hormone therapy and chemotherapy to treat the cancer. The prostate requires free testosterone binding to the androgen receptor (AR) on the cell to signal growth, and depletion of testosterone can inhibit growth of PCa (138, 139). Therefore, in the clinic PCa patients can be medically castrated to deplete free testosterone and inhibit cell growth (140). Therapy usually
will inhibit growth of the tumor a few years, but the tumor becomes castration-resistant due to increases in AR on the surface of the cell or the receptor can send a signal without testosterone binding (141). Clinicians then turn to AR antagonists to inhibit signaling of the AR which again inhibits growth for a few years until the tumor again mutates during treatment. Unfortunately, the AR often mutates so that AR antagonists can actually enhance receptor signaling (142). These tumors are considered androgen-independent PCa and begin growing uncontrollably and spreading throughout the body. At this point patients are usually treated with chemotherapy, but an immunotherapy called Sipuleucel-T has also been shown to be effective in some patients. Sipuleucel-T is an immunotherapy that stimulates patient T cells to target the prostatic acid phosphatase that is expressed in most PCa cells using the patient’s immune system to kill the tumor (143). If immunotherapy or chemotherapy does not kill the tumor, the patient is placed under palliative care.

Like many cancers, PCa tumors often become resistant to hormone therapy, radiation, and chemotherapy, as well as metastasize due to tumor hypoxia, but these tumors also confer resistance from SG formation. Researchers have shown that if the hypoxic response is repressed using HIF-1α inhibitors, current androgen receptor inhibitors work better in decreasing PCa growth (144). In addition, up to 15% of all PCa patients experience increased SG formation resulting from a mutated SPOP gene (145). These SPOP-mutated cancers are more resistant to docetaxel treatment, the most common PCa drug administered, by allowing excess caprin1 accumulation and SG formation (145). Together these studies suggest that the HIF-1α inhibiting and SG disrupting MRV would be a good therapy for PCa in conjunction with other cancer therapies. Interestingly MRV has already undergone Phase II clinical trials for treatment of men with metastatic castration-resistant PCa (90). Men were treated with docetaxel or MRV and
docetaxel, but unfortunately patients treated with MRV and docetaxel were not shown to benefit more than those treated with docetaxel alone. While MRV therapy was shown to be safe and tolerable, similar to other clinical trials, it did not improve overall survival (146). This suggests that the patients selected for this clinical trial were not a good fit for MRV therapy, and future research into MRV and PCa can provide insight into what kind of tumor environments or tumor genetic profiles might respond favorably to MRV therapy in PCa.

Conclusion

The use of viruses to benefit humans is not a new idea, but it is a concept that is rapidly being adopted by scientists and clinicians. Mammalian orthoreovirus is a great example of how a virus that does not cause disease in healthy people can be used to kill tumors where a weakened immune response provides an ideal location for MRV to replicate. The virus has already been shown to be safe in Phase I-III clinical trials, but has shown limited benefit to many patients. Therefore, additional research is necessary to determine the tumor types, tumor genetics, or microenvironments that MRV is most beneficial. MRV has many qualities that a beneficial cancer therapeutic should have, including the ability to reach primary and secondary tumors through intravenous administration, induction of antitumor immunity to kill the tumor and inhibit relapse, inhibiting pathways and proteins that are associated with negative patient prognosis, and it presents minimal side-effects. Oncolytic MRV is going to benefit many cancer patients, and continued examination of the virus will equip clinicians with the needed information to determine which cancer patients will be most amenable for this therapy.

References


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CHAPTER 3. CHARACTERIZATION OF A REPLICATING MAMMALIAN ORTHOREOVIRUS WITH TETRACYSSTEINE-TAGGED μNS FOR LIVE-CELL VISUALIZATION OF VIRAL FACTORIES

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**Abstract**

Within infected host cells, mammalian orthoreovirus (MRV) forms viral factories (VFs), which are sites of viral transcription, translation, assembly, and replication. The MRV nonstructural protein μNS comprises the structural matrix of VFs and is involved in recruiting other viral proteins to VF structures. Previous attempts have been made to visualize VF dynamics in live cells, but due to current limitations in recovery of replicating reoviruses carrying large fluorescent protein tags, researchers have been unable to directly assess VF dynamics from virus-produced μNS. We set out to develop a method to overcome this obstacle by utilizing the 6-amino-acid (CCPGCC) tetracysteine (TC) tag and FlAsH-EDT2 reagent. The TC tag was introduced into eight sites throughout μNS, and the capacity of the TC-μNS fusion proteins to form virus factory-like (VFL) structures and colocalize with virus proteins was characterized. Insertion of the TC tag interfered with recombinant virus rescue in six of the eight mutants, likely as a result of loss of VF formation or important virus protein interactions. However, two recombinant (r)TC-μNS viruses were rescued and VF formation, colocalization with associating virus proteins, and characterization of virus replication were subsequently examined. Furthermore, the rTC-μNS viruses were utilized to infect cells and examine VF dynamics using live-cell microscopy. These experiments demonstrate active VF movement with
fusion events as well as transient interactions between individual VFs and demonstrate the importance of microtubule stability for VF fusion during MRV infection. This work provides important groundwork for future in-depth studies of VF dynamics and host cell interactions.

**Importance**

MRV has historically been used as a model to study the double-stranded RNA (dsRNA) *Reoviridae* family, the members of which infect and cause disease in humans, animals, and plants. During infection, MRV forms VFs that play a critical role in virus infection but remain to be fully characterized. To study VFs, researchers have focused on visualizing the nonstructural protein μNS, which forms the VF matrix. This work provides the first evidence of recovery of replicating reoviruses in which VFs can be labeled in live cells via introduction of a TC tag into the μNS open reading frame. Characterization of each recombinant reovirus sheds light on μNS interactions with viral proteins. Moreover, utilizing the TC-labeling FlAsH-EDT2 biarsenical reagent to visualize VFs, evidence is provided of dynamic VF movement and interactions at least partially dependent on intact microtubules.

**Introduction**

Mammalian orthoreovirus (MRV) is a segmented, double-stranded RNA (dsRNA) virus that has been utilized as a tool to study the life cycle of the virus family Reoviridae. The virus is also of particular interest because it is classified as an oncolytic virus and is currently being studied in various preclinical and clinical trials to treat multiple tumor types (1). During MRV infection, inclusion bodies called viral factories (VFs) form, which were first identified in 1962 and later described as small particles that coalesced to form a reticulum-like structure around the nucleus (2, 3). VFs have historically been hypothesized to be the location of viral replication and assembly of viral core particles (4, 5), and recent data showing core particle recruitment and
positive-strand RNA synthesis at VFs (6, 7) and translation of viral RNA within and around VFs (8) suggest that they are also the site of viral transcription and translation.

VFs are largely comprised of the viral nonstructural protein μNS, which is suggested to be the only virus protein required to form the matrix of VFs (9). When expressed in transfected cells from a plasmid, μNS forms inclusions similar to VFs that are termed viral factory-like (VFL) structures (9). Moreover, μNS formation of VFLs results in specific VFL localization of each of the five viral structural proteins that make up the core particle (λ1, λ2, λ3, σ2, and μ2), the nonstructural σNS protein that is involved in virus translation and replication, as well as the intact core particle itself (6, 9,–11). The mechanism of VFL formation by μNS is not fully understood; however, several regions of the protein, including the carboxyl (C)-terminal 7 amino acids (aa) and a putative metal chelating structure formed by amino acids His570 and Cys572, have been shown to be necessary for VFL formation in transfected cells and replication in infected cells (12,–14). Additionally, the C-terminal 250 amino acids (aa 471 to 721) of μNS, which comprise a predicted coiled-coil domain, are sufficient to form VFL structures (12, 15). Deletion of aa 471 to 561 disrupts VFL formation, suggesting that this region also plays a role in the assembly of these structures (12).

Much of the work defining VFs has been done using transient transfection of plasmids expressing μNS and other virus proteins, primarily because it has thus far been difficult to visualize these proteins during infection in live cells. While researchers have been able to add fluorescent tags to viral proteins in other viruses and recover recombinant virus, the MRV genome has only recently been made amenable to reverse-genetics technologies (16,–19). Utilizing this technology, a virus in which the coding region within the S4 gene segment was replaced by the enhanced green fluorescent protein (EGFP) gene has been recovered; however,
this virus replicates only when propagated in cells expressing the S4 gene product σ3 (16). Both replicating and nonreplicating recombinant viruses have also been recovered with fluorescent proteins iLOV and UnaG independently expressed downstream of the N-terminal half of viral spike protein σ1 (σ1-N), as well as a fusion of σ1-N with UnaG, which could infect cells but was unable to replicate in the absence of wild-type MRV (19, 20). In addition, recombinant reoviruses in which small protein-coding sequences (6His, hemagglutinin [HA] tag, and 3HA tag) were added to the σ1 protein have been recovered (21). However, there is currently no other published evidence of a replicating, recombinant virus in which μNS or other viral proteins have been successfully tagged with fluorescent or other tags. To overcome this difficulty in visualizing VFs in infected live cultures, cells transiently expressing EGFP-μNS have been infected with intermediate subvirion particles (ISVPs) (8), and VFs formed by the dual expression of virus-expressed μNS and EGFP-μNS have been examined. This has led to important findings, including demonstrating the interaction of the cellular vesicular network with VFs during infection; however, the addition of the non-wild-type EGFP-μNS may alter VF function and virus replication, complicating the interpretation of results. The ability to rescue a replicating recombinant virus expressing a fluorescent or fluorescence-competent tagged μNS would allow for direct visualization of VF dynamics in MRV-infected cells.

One possible solution for tagging the μNS protein within the viral genome is to exploit the 4,5-bis(1,3,2-dithioarsolan-2-yl)fluorescein, also known as the fluorescein arsenical helix binder, bis-EDT adduct (FlAsH-EDT2), which fluoresces green when bound to a small 6-aa tag (CCXXCC) (22). FlAsH-EDT2 was first described in 1998 and was shown to fluoresce when bound to two pairs of cysteines separated by two amino acids (CCXXCC), referred to as a tetracysteine (TC) tag (22). This technique has been utilized in several recombinant viruses,
including HIV-1, to visualize TC-Gag protein during infection, and in bluetongue virus, a member of the *Reoviridae* family, to label viral protein VP2 to visualize virus particle entry (23, 24). Once the TC tag is added to a protein, FlAsH-EDT2, which contains two 1,2-ethanediithiol motifs, creates covalent bonds with the TC motif, resulting in fluorescence. FlAsH-EDT2 can be added at any time postinfection (p.i.), allowing for visualization of TC-tagged proteins throughout the virus life cycle. As CCPGCC is rare to find within natural proteins and is the preferred FlAsH-EDT2 binding sequence (25), it is a good amino acid sequence to use for the TC tag, resulting in highly specific FlAsH-EDT2 binding with little background fluorescence.

In this study, we introduced the CCPGCC TC tag in frame into eight sites throughout the μNS protein and examined the impact of the insertion on known μNS functions. We further demonstrated that we could rescue recombinant viruses expressing the TC tag at two sites within μNS. Recombinant (r) TC-μNS viruses were characterized with regard to replication, VF formation, and stability of the TC tag within the M3 genome segment over multiple viral passages. Finally, rTC-μNS viruses were used to examine μNS and VF dynamics in live cells and to observe the role of microtubules in VF fusion and movement throughout the cell.

**Results**

**Construction of plasmids expressing the TC tag from within the μNS protein.** Specific regions within the μNS protein that are required for recruitment of the viral core and six other MRV proteins to VFs and for forming VFs have previously been identified (6, 7, 9, 10, 12) (Fig. 1A). As our goal was to rescue a type 1 Lang (T1L) MRV expressing the TC tag, CCPGCC, from within the μNS protein, we reasoned that there would be sites of insertion that would not allow rescue of virus as a result of interference with μNS functions during infection. For this reason, to locate a position within the protein that would be tolerated during virus infection, we
Figure 1. FlAsH-EDT2 labeling and VFL formation in μNS CCPGCC mutants. (A) Diagram of μNS depicting the location and sequence of TC-μNS mutations and previously described functional regions of μNS necessary for protein localization. (B) BHK-T7 cells were transfected with the indicated plasmids, and at 18 h p.t., cells were labeled with FlAsH-EDT2 (middle columns) for 90 min and then fixed and immunostained with α-μNS antibody (left columns), followed by Alexa 594-conjugated donkey α-rabbit IgG. A merged image is also shown with DAPI staining (right columns). Images are representative of the observed phenotype. Bars, 10 μm.

inserted the nucleotide sequence encoding CCPGCC at eight positions throughout a μNS-encoding M3 gene expression plasmid. We initially reasoned that utilization of existing amino acids of the TC tag from within the μNS protein may prevent disruption of μNS function and constructed clones in which we incorporated the remainder of the tag into an existing PG, by flanking the PG residues with two CC residues at aa 28 [TC-μNS(#1)], 132 [TC-μNS(#2)], and
334 [TC-μNS(#4)], or an existing CP, by flanking the CP with a C and GCC at aa 491 [TC-
μNS(#5)]. Although μNS is highly conserved between strains, to improve our chance of success
in rescuing a recombinant virus expressing a tagged μNS, we performed a sequence comparison
between the μNS protein from the three major MRV serotypes (T1L, T2J, and T3D) and
identified two areas of 6 aa that were not highly conserved. We replaced these regions with
CCPGCC in the T1L μNS [TC-μNS(#6), aa 614 EAAAKC to CCPGCC; and TC-μNS(#7), aa
545 QSLNAQ to CCPGCC]. Finally, we reasoned that we may be able to rescue a virus where
the μNS protein sequence was not disrupted and the CCPGCC was added as a fusion to either the
μNS amino (N) or carboxyl (C) terminus to make TC-μNS(N-term) and TC-μNS(C-term) (Fig.
1A).

**VFL formation and biarsenical labeling of TC-μNS in transfected cells.** Insertion of the TC
tag does not ensure that the protein will be labeled upon exposure to biarsenical compounds, as
folding of the tagged protein may prevent or interfere with compound binding. It is also plausible
that the TC tag would prevent VFL formation due to a conformational change in μNS resulting
in loss of μNS-μNS association. In order to examine FlAsH-EDT2 labeling and VFL formation
of the TC-μNS fusion proteins, we transfected BHK-T7 cells with plasmids expressing each of
the eight TC-tagged proteins. At 18 h posttransfection (p.t.), FlAsH-EDT2 reagent was added.
We began to observe fluorescence within 15 min in most samples (data not shown), which
increased in intensity throughout the labeling period of 90 min, at which point the FlAsH-EDT2
reagent was removed and cells were fixed and subjected to immunofluorescence assays with
antibodies against μNS, followed by Alexa 594-conjugated secondary antibodies (Fig. 1B). Upon
microscopic observation, we found colocalization of the FlAsH-EDT2 and μNS staining (Fig.
1B), indicating that the FlAsH-EDT2 was binding TC-μNS in six of the eight proteins, including
TC-μNS(#1), TC-μNS(#4), TC-μNS(#6), TC-μNS(#7), TC-μNS(N-term), and TC-μNS(C-term). TC-μNS(#5) exhibited weak FlAsH-EDT2 labeling, and TC-μNS(#2) did not exhibit detectable labeling, although there were high levels of μNS protein expressed in these cells. We next examined the effect of the TC tag on mutant VFL formation, and we found that TC-μNS(#1), TC-μNS(#4), TC-μNS(#6), TC-μNS(#7), TC-μNS(N-term), and TC-μNS(C-term) formed VFLs. TC-μNS(#1), TC-μNS(#4), and TC-μNS(N-term) had little to no diffuse μNS staining and formed subjectively tighter VFLs than TC-μNS(#6), TC-μNS(#7), and TC-μNS(C-term), which formed less distinct VFLs with various levels of diffuse μNS staining. TC-μNS(#2) and TC-μNS(#5) did not form VFLs and instead were entirely diffuse in all transfected cells. These results suggest that the TC tag can be added at multiple sites throughout the μNS protein without disrupting VFL formation and that the expressed TC-μNS protein was label competent utilizing FlAsH-EDT2.

Insertion of the TC tag within the N-terminal two-thirds of μNS leads to diminished recruitment of viral proteins to VFLs. We hypothesized that we could also utilize these mutants to learn more about μNS localization with MRV proteins by investigating the interaction of each mutant with individual virus proteins that were previously identified as μNS-associating partners. We separated the TC-μNS mutants based on previously identified functions of μNS to include the N-terminal third (aa 1 to 221), the central third (aa 222 to 470), and the C-terminal third (aa 471 to 721). Previous studies have found that the μNS N-terminal third is both necessary and sufficient to bind virus proteins σNS, μ2, λ1, λ2, and σ2, the C-terminal third is necessary and sufficient to bind λ3, form VFs, and bind cellular clathrin, and the central third has been implicated in recruiting cellular protein Hsc70 to VFs (6, 7, 9, 10, 12, 26, 27). To examine the impact of the introduced TC-μNS mutations, we cotransfected cells with each pTC-μNS
mutant or wild-type pT7-M3 along with plasmids expressing each of the other six virus proteins individually. As TC-μNS(#2) and TC-μNS(#5) did not form VFLs, we did not examine the impact of these mutations on μNS localization with other viral proteins. At 18 h p.t., cells were fixed and stained with antibodies against μNS and each respective protein or associated protein tag followed by Alexa 594- and 488-conjugated secondary antibodies. Three representative pictures of each condition were acquired, and μNS recruitment of λ1, λ2, λ3, σ2, and σNS to VFLs or μ2 recruitment of μNS to microtubules was quantified by comparing the pixel intensities of μNS and associating proteins at VFLs or microtubules. Each quantified interaction of TC-μNS and associating protein was made relative to wild-type μNS, and a Student t test was used to determine if each TC-μNS mutant was significantly decreased (with significance set at P values of <0.05), denoted by a minus sign (−), in comparison with the wild type, in Table 1. We found that each TC-μNS mutant had a statistically significant diminished colocalization with one or more viral proteins, with the exception of the C-terminal mutant, which maintained wild-type levels of colocalization of all proteins. Since the N-terminal third of μNS is necessary for multiple interaction with μNS-associating proteins, it is not surprising that the insertion within

Table 1. Summary of TC-μNS mutant properties. (-), no VFL formation or no FlAsH-EDT2 labeling or significantly diminished (P < 0.05) colocalization of TC-μNS and interacting proteins compared to wild-type μNS; (+), VFL formation or FlAsH-EDT2 labeling or no significant decrease in colocalization of μNS and interacting proteins compared to wild-type μNS. ND, no data collected.
this coding region resulted in the disruption of colocalization of the most proteins. As the central and C-terminal thirds of μNS are not implicated in viral protein recruitment to VFLs aside from λ3, it unclear why TC insertions in these regions result in decreased colocalization, but this effect may suggest loss of proper μNS folding in these mutants. Altogether, these findings suggest that the C terminus may be the most amenable region of μNS for TC-tag insertion.

Rescue of MRV expressing TC-μNS. All of the TC-μNS mutants were tested in a plasmid-based reverse-genetics approach to attempt to rescue viruses containing the CCPGCC motif in the μNS protein (17). Briefly, a modified version of the previously described T7-driven, 4- and 10-plasmid systems was used (17). Three plasmids from the four-plasmid system were used to provide 8 of the MRV genes (pT7-M1-S1-S2-S4-RZ, pT7-L3-S3-RZ, pT7-L1-M2-RZ). The L2 and M3 wild-type and M3 TC mutant genes were each provided from individual plasmids (pT7-L2, pT7-M3/TC-M3 mutants). Plasmids were cotransfected into BHK-T7 cells and incubated for 6 days, at which point cells and media were harvested and subjected to three freeze/thaw cycles, followed by standard MRV plaque assay on L929 cells (28). Three experiments were done for each mutant, and wild-type M3 and no-M3 plasmid positive and negative controls were included in each experiment. To allow for slow-growing viruses, plaque assays were incubated for 5 days and monitored each day for plaques. In each experiment, there were greater than 10⁴ plaques formed in the positive control and no plaques formed in the negative control. There were also no plaques recorded in any experiment with pTC-μNS(N-term), pTC-μNS(#1), pTC-μNS(#2), pTC-μNS(#4), pTC-μNS(#5), or pTC-μNS(#6). However, we observed plaques forming from days 3 to 5 on a single experiment with pTC-μNS(#7) and pTC-μNS(C-term), suggesting that we had recovered rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L) viruses with TC-tagged μNS. Plaques were picked, and the viruses were passaged 10 times on Vero cells to amplify the viruses. After
Figure 2. VFs from recombinant viruses label with FlAsH-EDT2. CV-1 cells were infected with T1L, rTC-μNS(C-term-T1L)/P2, or rTC-μNS(#7-T1L)/P2, and at 18 h p.i., cells were labeled with FlAsH-EDT2 (middle column) for 45 min and then fixed and immunostained with α-μNS antibody (left column), followed by Alexa 594-conjugated donkey α-rabbit IgG. A merged image is also shown with DAPI staining (right column). Images are representative of the observed phenotype. Bars, 10 μm.

two passages (P2), CV-1 cells were infected with recombinant viruses and were labeled with FlAsH-EDT2 at 18 h p.i. and subsequently prepared for immunofluorescence to label μNS. We observed FlAsH-EDT2 labeling of μNS and VFs in cells infected with each virus (Fig. 2), indicating that we had recovered two fluorescence-competent, recombinant viruses. While both
viruses exhibited F1AsH-EDT2 labeling, rTC-μNS(#7-T1L) factories were qualitatively less fluorescent than rTC-μNS(C-term-T1L) factories.

**TC-μNS containing viruses exhibit growth deficiencies.** After two passages of both rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L), the titers of viruses were determined on L929 cells, and replication assays were performed to determine the fitness of the recombinant viruses relative to wild-type T1L. Following infection with a multiplicity of infection (MOI) of 0.2, samples were taken at 0, 12, 24, 36, and 48 h p.i., freeze/thawed three times, and subjected to plaque assays on L929 cells (Fig. 3A). While both recombinant viruses replicated in a manner comparable to that of the wild type up to 12 h p.i., we observed a statistically significant attenuation of virus replication at 24, 36, and 48 h p.i. in the recombinant viruses relative to the wild type. Overall, the wild-type virus replicated to between 10- and 100-fold higher than rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L) after 24 h p.i. Despite this attenuation, recombinant viruses were able to replicate to about 10^1-fold relative to time zero at 48 h p.i., suggesting that they were capable of completing the entire virus life cycle, albeit slower than wild-type T1L (Fig. 3A). We additionally measured the plaque size of each virus and found that the sizes of the rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L) plaques were 55% and 45%, respectively, of the wild-type plaque size (Fig. 3B). These findings suggest that addition of the TC tag in the C-terminal third of μNS inhibits viral infection relative to wild-type virus; however, both rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L) replicated to titers that should be sufficient for live cell imaging.

**Recombinant viruses retain recruitment of μNS-associating proteins to VFs.** In an attempt to explain the attenuated nature of rTC-μNS(#7-T1L) and rTC-μNS(C-term-T1L), we examined μNS recruitment of viral proteins to VFs in cells infected with each recombinant virus. CV-1
Figure 3. Recombinant TC-μNS virus replication. L929 cells were infected with T1L, rTC-μNS(C-term-T1L)/P2, or rTC-μNS(#7-T1L)/P2 and at 0, 12, 24, 36, and 48 h p.i. cells were harvested. Harvested cells were subjected to standard MRV plaque assay. (A) Plaques from each time point were counted, and the relative viral titer increase from time zero is plotted. The means and standard deviations were calculated from two experimental replicates within two different biological replicates. A two-tailed Student t test was used to calculate P values for significant
differences between recombinant virus and wild-type virus in Microsoft Excel: *, $P < 0.05$; **, $P < 0.01$. (B) Cells were fixed and stained with crystal violet and imaged to visualize plaque size relative to wild-type T1L. Five plaques were measured using ImageJ in each panel to determine the average plaque diameter (bottom left).

cells were infected with T1L, rTC-μNS(C-term-T1L)/P5, or rTC-μNS(#7-T1L)/P5 at an MOI of 1, and at 18 h p.i. cells were fixed and stained with antibodies against μNS and σNS, λ2, μ2, or the core particle followed by Alexa 594- and 488-conjugated secondary antibodies (Fig. 4A to C). The core antibody has been shown to bind λ1, λ2, and σ2 (6), and we do not have access to an antibody that specifically detects λ3. We observed the recruitment of σNS, λ2, and the core proteins to VFs and the recruitment of VFs to microtubules in both recombinant viruses, with rTC-μNS(C-term-T1L) displaying a phenotype qualitatively more similar to that of wild-type virus than that of rTC-μNS(#7-T1L) (Fig. 4C), which consistently possessed somewhat smaller VFs than T1L. These data suggest that rTC-μNS(C-term-T1L) may better depict natural VFs in live cell imaging.

**Recombinant virus growth deficiency occurs after viral entry, transcription, and translation.** As each recombinant virus was capable of forming VFs and recruiting known μNS-associating proteins to VFs similar to the wild type, we further investigated their growth deficiency to identify at which stage of growth the defect occurs. We began by performing plaque assays to determine the PFU of specific stocks of wild-type and rTC-μNS viruses. L929 cells were infected with wild-type T1L, rTC-μNS(C-term-T1L)/P5, or rTC-μNS(#7-T1L)/P5, and samples were collected at 0, 12, 24, and 48 h p.i. and subjected to immunoblot analysis with antibodies (i) against μNS to examine specific impacts of the TC-tag insertion on μNS expression and (ii) against σNS to examine expression of a viral protein other than μNS. Antibodies against α-tubulin were also used as a protein loading control (Fig. 5A). We observed that both recombinant viruses expressed substantially larger amounts of μNS and σNS than did
Figure 4. Colocalization of MRV proteins with recombinant virus-expressed μNS. CV-1 cells were infected with T1L (A), rTC-μNS(C-term-T1L)/P2 (B), or rTC-μNS(#7-T1L)/P2 (C) and at 18 h p.i. were immunostained with mouse (second and third rows) or rabbit (first and fourth rows) antibodies against μNS and mouse σNS antibodies (first row), rabbit μ2 antibodies (second row), MRV core rabbit antibodies (third row), λ2 mouse antibodies (fourth row), followed by Alexa 594-conjugated donkey α-rabbit or α-mouse IgG (first column) and Alexa 488-conjugated donkey α-mouse or α-rabbit IgG (second column). Merged images with DAPI-stained nuclei are also shown (third column). Bars, 10 μm.
wild-type T1L at each time point, suggesting that the TC tag within μNS does not disrupt μNS protein expression and that the recombinant viruses were not defective at early stages of infection, including virus entry, RNA transcription, and protein translation. Importantly, these data also potentially suggest that more recombinant virions than wild-type virions may be required to achieve the same PFU.

To explore this hypothesis, we examined the virus preparations directly by subjecting identical PFU of the same wild-type and recombinant virus stocks to immunoblot analysis with antibodies against the virion, which detected substantially more protein/PFU in the recombinant viruses than in the wild type (Fig. 5B). We additionally examined genomic RNA associated with the same PFU of wild-type and recombinant virus and again found that there were substantially higher levels of genome associated with the same PFU of recombinant viruses than of wild-type viruses (Fig. 5C). Since the recombinant viruses were not purified, we cannot confirm that the extra protein (Fig. 5B) or the extra RNA (Fig. 5C) is not a result of incomplete virion production, but we believe that these results point to the likelihood that the recombinant viruses require more virions to produce the same amount of plaques as wild-type virus. Finally, a repeated plaque assay of the same stocks showed that the PFU between the viruses remained essentially the same (Fig. 5D). Together these findings suggest that the growth defect in the recombinant viruses is downstream of early events such as entry, transcription, and translation, likely at the level of assortment, replication, or assembly. These findings further suggest that the recombinant viruses are capable of producing infectious virions, and the diminished titers relative to wild-type virus over time (Fig. 3) are likely not a result of a catastrophic replication deficiency but are instead a result of diminished efficiency in one or more of the later steps in the replication cycle.
Figure 5. Recombinant virus replication characterization. (A) L929 cells were infected with 5.0 × 10^5 PFU of T1L, rTC-μNS(C-term-T1L)/P5, or rTC-μNS(#7-T1L)/P5, and samples were collected at 0, 12, 24, and 48 h p.i. and were run on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with α-μNS (first row), α-σNS (second row), and anti-α-tubulin antibodies (third row). (B) T1L, rTC-μNS(C-term-T1L)/P5, and rTC-μNS(#7-T1L)/P5 (5.0 × 10^5 PFU each) and 5.0 × 10^7 PFU of T1L (T1L 100×) were run on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with T1L α-virion antibody (μ1). (C) RNA extracted from 1 × 10^8 PFU of T1L, rTC-μNS(C-term-T1L)/P5, and rTC-μNS(#7-T1L)/P5 or 1 × 10^9 PFU of T1L (T1L 10×) was run on 10% SDS-PAGE for 12 h to visualize dsRNA genomic segments. (D) T1L, rTC-μNS(C-term-T1L)/P5, and rTC-μNS(#7-T1L)/P5 (5.0 × 10^5 PFU) were subjected to a plaque assay on L929 cells.
A single recombinant virus retains the TC tag over 10 passages. We next focused our attention on the stability of the TC tag within the recombinant viruses. Viral RNA was extracted from the second and fifth passages of each recombinant virus using TRIzol LS followed by reverse transcription (RT)-PCR of the M3 genome segment and sequencing. At passage 2, the rTC-μNS(#7-T1L) M3 genome segment showed a mixture of viruses in which the dominant

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**Figure 6. Sequencing of recombinant virus passages.** rTC-μNS(C-term-T1L) and rTC-μNS(#7-T1L) viruses were passaged 10 times and sequenced to determine the stability of the TC tag. (A) The pTC-μNS(#7) and rTC-μNS(#7-T1L) viruses from passages 2 and 5 were sequenced, and the TC tag and acquired second-site mutation, portrayed with an asterisk (*), are shown. All other regions of M3 were the same as in the wild type. (B) CV-1 cells were infected with the rTC-μNS(#7-T1L)/P5 at an MOI of 1, and at 18 h p.i., cells were labeled with FlAsH-EDT2 (middle column) for 45 min and then fixed and immunostained with rabbit α-μNS antibody (left column), followed by Alexa 594-conjugated donkey α-rabbit IgG. A merged image is also shown with DAPI staining (right column). Images are representative of the observed phenotype. Bar, 10 μm. (C) The pTC-μNS(C-term) and rTC-μNS(C-term-T1L) viruses from passages 2, 5, 7, and 10 were sequenced, and the TC tag and acquired second-site mutation, portrayed with an asterisk, are shown. All other regions of M3 were the same as in the wild type.
phenotype had a 1649G→A mutation that resulted in a C550Y mutation producing a CCPGCY instead of CCPGCC TC tag while a smaller portion of the recombinant virus population contained the CCPGCC (Fig. 6A). By passage 5, the virus had stabilized and contained exclusively the C550Y mutation. These data showed that our earlier FlAsH-EDT2-labeling experiments using μNS(#7-T1L)/P2 contained a mixed population of viruses (Fig. 2). As this labeling was diminished relative to rTC-μNS(C-term-T1L), we were curious as to whether the labeling defect was a result of the C550Y mutation or whether this mutation would lead to a complete loss of labeling. Therefore, we infected CV-1 cells with rTC-μNS(#7-T1L)/P5, which contained the CCPGCY mutation exclusively to determine if μNS would label with FlAsH-EDT2. We found that VF labeling was similarly diminished using this passage, suggesting that the FlAsH-EDT2 reagent was able to maintain binding, albeit to a lesser extent, to the CCPGCY sequence (Fig. 6B).

Sequencing the M3 gene of passage 2 of the rTC-μNS(C-term-T1L) virus also showed a mixed population of viruses in which the CCPGCC TC tag itself remained stable but the virus had acquired a second-site mutation at 2153C→T, which resulted in a T718I mutation within the protein upstream of the TC tag (Fig. 6C). This mutation became dominant by passage 5. To determine if this was a stable mutation, we passaged the rTC-μNS(C-term-T1L) virus five further passages and again sequenced the M3 genome segment at passages 7 and 10. We found that the seventh-passage virus exclusively contained the T718I mutation and this mutation remained stable at passage 10 (Fig. 6C) while both passages retained the original CCPGCC TC tag. We performed replication assays on wild-type T1L, rTC-μNS(C-term-T1L)/P2, and rTC-μNS(C-term-T1L)/P7 followed by plaque assays to determine if the T718I mutation restored the virus to wild-type T1L replication kinetics or impacted replication in any way, but we observed
no significant difference between the two passages (data not shown). Because the rTC-μNS(C-term-T1L) TC tag remained stable over 10 passages and labeled with FlAsH-EDT2 to higher levels than rTC-μNS(#7-T1L), we proceeded forward using the rTC-μNS(C-term-T1L) virus for live-cell imaging.

**VF dynamics during MRV infection.** To provide a proof of principle that rTC-μNS(C-term-T1L) will be a useful tool to study VF dynamics in infected cells, we performed a short time-lapse live-cell experiment in which we examined VF movement and interaction at a single time in MRV infection. BHK-T7 cells were infected with rTC-μNS(C-term-T1L)/P2, and at 18 h p.i., FlAsH-EDT2 was added to the cells and the mixture was incubated for 45 min. Still images of the infected cells were then captured every 20 s for 2 h. Mock-infected cells without FlAsH-EDT2, as well as FlAsH-EDT2-labeled cells infected with wild-type T1L at an MOI of 100 were included as controls to demonstrate specificity of the FlAsH-EDT2 reagent (Fig. 7A). At this time point in infection, rTC-μNS(C-term-T1L) was found to form small VFs in the periphery of

![Image](image_url)

**Figure 7. TC-μNS-labeled VF dynamics in infected cells.** BHK-T7 cells were mock infected or infected with T1L or rTC-μNS(C-term-T1L)/P2, and at 18 h p.i., cells were imaged using live-cell microscopy. (A) Mock-infected (top left) or T1L-infected cells with FlAsH-EDT2 labeling (top right) controls are shown. Bar, 40 μm. (B) rTC-μNS(C-term-T1L)/P2-infected BHK-T7 cell images shown 0 to 60 min following FlAsH-EDT2 incubation. Red arrows indicate VF fusion, and yellow arrows indicate the kissing motion of VFs (at a magnification of ×4). Bar, 10 μm.
infected cells that move in short stochastic motions in the cell, while larger, more stationary VFs were found at the nuclear periphery (Fig. 7B; see also Movie S1 in the supplemental material). Small VFs were highly mobile and could be seen fusing with larger VFs (Fig. 7B, red arrows). In addition, small VFs could be seen moving toward one another or toward larger VFs, touching briefly, and then moving away in a kissing motion (Fig. 7B, yellow arrow), which may be a result of incomplete VF fusion. These data suggest that rTC-μNS(C-term-T1L) will be an extremely useful tool for future in-depth studies examining VF dynamics and function during MRV infection.

VF dynamics in the presence of nocodazole. In addition to facilitating our understanding of VF formation and movement throughout infection, we postulated that rTC-μNS(C-term-T1L) will also be indispensable for understanding the role of VF interactions with cellular proteins throughout infection. One such interaction that has been previously defined is that of μNS and the μNS-interacting protein, μ2, with cellular microtubules (MTs). Destabilization of MTs leads to the formation of small VFs/VFLs concentrated at the periphery of the cell in infected cells or cells transfected with a plasmid expressing μNS, suggesting that MTs play a critical role in VF/VFL movement or fusion (9, 29). In addition, the μ2 protein of most MRV strains induces hyperacetylation and stabilization of MTs and also influences the morphology of VFs to a filamentous phenotype, rather than the globular VFL phenotype seen when μNS is expressed alone (9, 29). Until recently, most evidence suggested that tight association of VFs with MTs (14) or MT stability (30) in MRV-infected cells does not significantly alter viral replication. However, a recent paper has suggested that MT disruption significantly decreases MRV replication by 50%, and further, substantially decreases the crystalline-like array of genome-containing virus particles seen in VFs in MRV-infected cells with intact MTs, suggesting that
MTs play a critical role in genome packaging (31). We utilized our rTC-μNS(C-term-T1L) virus to further explore the role of MTs in VF movement and/or fusion within the cell. Vero cells were mock infected or infected with T1L or rTC-μNS(C-term-T1L)/P5 at an MOI of 100, and treated at 6 h p.i. with and without 10 μM nocodazole, which destabilizes MTs (Fig. 8A). At 10 h p.i., cells were labeled with FlAsH-EDT2, and at 12 h p.i., FlAsH-EDT2 was removed, and still images were taken of VFs every 2 min for 2 h. In cells not treated with nocodazole, we observed stable filamentous VFs that were presumably MT associated as well as globular VFs, which exhibited fluid movement around the cell, resulting in some VF fusion events (Fig. 8B, red arrows; see also Movie S2 in the supplemental material) similar to those observed in BHK-T7 cells. We also identified a new fusion event in which a small VF moves from the microtubule toward a large VF, fuses and then pulls the large VF toward the microtubule, at which point the large VF fuses with VF material already on the microtubules (Fig. 8B, blue arrows; Movie S2). While this interaction has not previously been identified, it might suggest that smaller, more dynamic VFs may be instrumental in assisting and/or facilitating VF fusion. We also recorded the disruption of two VFs in close proximity by movement of other VFs directly between them (Movie S2, green arrow). In cells treated with nocodazole, there was no obvious association of VFs with MTs, and all VFs were globular and not filamentous in nature, as expected. Somewhat surprisingly, the VFs appeared to be very dynamic, exhibiting short stochastic movements throughout the cell (Fig. 8C, green arrows; see also Movie S3 in the supplemental material). While we observed VF movement, we did not see the accumulation of VFs at the nuclear periphery that we observed in cells without nocodazole treatment. We were also unable to detect any VF fusion events throughout multiple experiments and instead repeatedly imaged multiple clusters of small VFs that appeared unable to fuse (Fig. 8C, yellow arrow; Movie S3).
together, these observations suggest that the small VF phenotype previously observed following nocodazole treatment in fixed cells (9, 29) is likely a result of an inhibition or inability of VFs to fuse with one another and not a result of total inhibition of VF movement within the cell. In addition to defining a role for MTs in VF fusion and migration, this result further suggests that VFs are able to utilize cellular components apart from microtubules to move within cells.

Figure 8. TC-μNS-labeled VF dynamics in infected cells with and without nocodazole. Vero cells were infected or mock infected with T1L or rTC-μNS(C-term-T1L)/P5, and at 6 h p.i. cells were treated or untreated with 10 μM nocodazole. (A) At 12 h posttreatment, mock-infected cells were immunostained with rabbit anti-α-tubulin antibody (left and middle columns), followed by Alexa 594-conjugated donkey α-rabbit IgG. A merged image is also shown with DAPI staining (right column). (B) At 10 h p.i., T1L- and rTC-μNS(C-term-T1L)/P5-infected Vero cells without 10 μM nocodazole were labeled with FlAsH-EDT2 for 2 h, at which point rTC-μNS(C-term-T1L)/P5-infected cells were imaged using live-cell microscopy. Red and blue arrows indicate VF fusion (at a magnification of ×3). (C) At 10 h p.i., T1L- and rTC-μNS(C-term-T1L)/P5-infected Vero cells with 10 μM nocodazole were labeled with FlAsH-EDT2 for 2 h, at which point rTC-μNS(C-term-T1L)/P5-infected cells were imaged using live-cell microscopy. Yellow arrows indicate a VF cluster unable to fuse, and green arrows indicate VF movement (at a magnification of ×2). Bars, 10 μm.
Discussion

In this paper, we have demonstrated recovery of T1L recombinant MRV with a TC tag introduced into the nonstructural VF matrix protein, μNS. This is the first time a replicating recombinant MRV has been created in which VFs encoded from the genome can be fluorescently labeled during infection. Adding the TC tag to μNS in a recombinant virus allows for several improvements to existing technologies. Foremost in these improvements is the ability to visualize VFs as they form from a modestly modified μNS protein expressed from virus transcripts. As our rTC-μNS virus undergoes the full MRV replication cycle, findings from future studies using our virus should accurately reflect VF dynamics and interactions throughout MRV infection. In this study, we limited our observation of VFs over short time-lapses; however, there is obvious potential to increase the observation period to a full replication cycle to observe the initial formation of VFs and the interactions between VFs throughout the MRV life cycle. The detection limit of FlAsH-EDT2 labeling of TC-μNS is under investigation; however, as we observed very small VFs and VFLs in our studies, we expect this approach to be quite useful in delineating early versus late events in VF formation and maturation.

There are several other questions that can be explored using this virus. Because the FlAsH-EDT2 reagent can be removed from cells at any time, pulse-chase studies can be performed to examine TC-μNS stability within VFs over time. Moreover, a second reagent, ReAsH-EDT2 (25), which fluoresces in the red spectrum (608 nm), can be used subsequent to FlAsH-EDT2 labeling to determine differences in the behaviors of existing TC-μNS versus newly translated TC-μNS. In addition, as we demonstrate with MTs and nocodazole, FlAsH-EDT2 labeling can also be used in combination with inhibitory drugs and other fluorescent labeling techniques to examine VF interactions with cellular proteins that may modulate or be modulated by VFs, μNS, or other VF-localized virus proteins. Finally, although it will likely be
dependent on the location of the TC tag within individual virus proteins, demonstration of recovery of recombinant viruses containing the small TC tag in μNS suggests that other MRV proteins may be amenable to TC tagging, allowing their visualization during infection.

Apart from VF dynamics, the examination of the TC-μNS mutants and recombinant viruses presented in this study also produced some new insight into requirements for VFL and VF formation and μNS binding with other MRV proteins. One result that we found particularly interesting was that we were unable to recover the TC-μNS(#4) mutant, which contains the TC tag within a region of μNS known only to be the site of Hsc70 binding, which is not required for normal VF formation (26), but we were able to recover two mutants containing the TC tag within the C-terminal third, which is known to be important for VF formation. Our inability to rescue TC-μNS(#4) as well as the other mutants may be due to protein misfolding, but it is also possible that misfolding of the M3 RNA resulted in improper assortment or packaging, thus prohibiting viral recovery. We also found it interesting that the TC-μNS(#7) VFLs had a significantly diminished localization with μ2, λ1, and λ3 compared to wild-type μNS, but visually the rTC-μNS(#7-T1L) VFs localized to associating proteins in a fashion similar to that of the wild type. This may suggest that μNS-associating protein localization to VFs during infection are facilitated by interactions between multiple proteins and RNA; therefore, a defect in association between μNS and each individual protein is magnified in the absence of the full complement of VF-localized interactions in transfected cells.

During the characterization of the recombinant viruses, we concluded that both recombinant viruses were likely attenuated downstream of viral entry, transcription, or translation. Further investigation into recombinant VF formation and localization with μNS-associating proteins compared to wild-type VFs demonstrated that factory formation also was not
considerably altered, suggesting that the recombinant VFs closely mimic natural VFs at least to the stage of infection when prominent VFs are constructed. This points to assortment, assembly, or replication as being responsible for the recombinant virus growth attenuation. This could simply be a result of the TC tag inhibiting a function of the μNS protein downstream of VF formation or alternatively could be a consequence of the nucleotides encoding the TC tag disrupting proper M3 mRNA folding, resulting in inhibition of assortment, packaging, or replication of the viral genome. Presumably as a result of this attenuation, the rTC-μNS(#7-T1L) lost the inserted TC tag sequence within two passages. The rTC-μNS(C-term-T1L) virus maintained the TC tag over 10 passages but instead developed a T718I mutation upstream of the TC tag, which has not been previously documented in the M3 gene of MRV strains. We initially believed that this might be a compensatory mutation; however, when rTC-μNS(C-term-T1L)/P7 was directly tested against rTC-μNS(C-term-T1L)/P2 and wild-type T1L, viral replication remained similar to that of the earlier passage relative to the wild type (data not shown).

Nonetheless, since rTC-μNS(C-term-T1L) maintains the TC tag, produces VFs that appear identical to wild-type VFs, and has not acquired additional second-site mutations over five subsequent passages, we believe that rTC-μNS(C-term-T1L) has already enhanced in this study, and will continue to enhance, our ability to understand VF formation and function during MRV infection.

While it has already been shown that MRV required MT stability to form large, perinuclear VFs (9, 29), whether MTs are necessary for VFs to move into close proximity in order to fuse or strictly for fusion of VFs was unknown. Our data suggest that even in the presence of the MT-destabilizing drug nocodazole, VFs demonstrate movement within the cell (Fig. 8C; Movie S3). However, without stable MTs, VF fusion is a rare or nonexistent event,
suggesting that while MT stability is dispensable for VF movement, it is necessary for VF fusion. This also suggests that VFs may utilize other cellular components to move throughout the cell. It has previously been shown that nocodazole treatment results in many small VFs in the periphery of cells, as opposed to the perinuclear space (9, 29). Although our studies suggest that VFs are able to move in the absence of MT stabilization, this movement did not appear to result in a directed migration of the nonfused VF clusters toward the nucleus. Instead, VF movement was limited to small changes in location independent of direction, indicating that while VFs can move and appear to associate with each other in cells without MTs, directed overall migration of VFs to the perinuclear space appears to require MT stabilization. Finally, our data also show that once globular VFs associate with MTs to form filamentous VFs, they appear to remain associated with them and become relatively static and stable compared to globular VFs. Taken together with published data, our findings suggest that the path to mature VF formation includes steps whereby (i) μNS first self-associates and/or associates with other viral and cellular proteins to form small globular VFs, (ii) these small globular VFs move independent of MTs in the cell and encounter each other, (iii) following this encounter, smaller VFs fuse into larger VFs in an MT-dependent manner or fuse to filamentous VFs already associated with MTs, and (iv) the MT-associated VFs then either migrate toward, or accumulate in, the perinuclear space within the cell, where they remain relatively stable over time.

**Material and methods**

**Cells, viruses, antibodies, and reagents.** CV-1, Vero, and BHK-T7 cells (32) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies) supplemented with 10% (or 2% for Vero cells during virus amplification) fetal bovine serum (Atlanta Biologicals), penicillin (100 IU/ml)–streptomycin (100 μg/ml) solution (Mediatech), and 1% MEM nonessential amino acids solution (HyClone). To maintain the T7 RNA polymerase, 1
mg/ml of G418 (Alexis Biochemical) was added every fourth passage to BHK-T7 cells. L929 cells were maintained in Joklik modified minimum essential medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 2% bovine calf serum (HyClone), 2 mM L-glutamine (Mediatech), and penicillin (100 IU/ml)–streptomycin (100 μg/ml) solution. Our laboratory stock of MRV strain type 1 Lang (T1L) originated from the laboratory of B. N. Fields. The virus was propagated and purified as previously described (33). Primary antibodies used were as follows: monoclonal mouse anti-FLAG (α-FLAG) antibody (F1804; Sigma-Aldrich), monoclonal mouse α-HA antibody (26183; ThermoFisher), polyclonal mouse α-μNS, polyclonal rabbit α-μNS, α-μ2, and T1L α-virion antibodies (9, 33–35), monoclonal mouse α-σNS (3E10) and α-λ2 (7F4) antibodies deposited in the Developmental Studies Hybridoma Bank (DSHB) by T. S. Dermody (36, 37), and rabbit α-MRV core (38). Secondary antibodies were Alexa 594- and 488-conjugated donkey α-mouse or α-rabbit IgG antibodies (Invitrogen Life Technologies). FlAsH-EDT2 (ThermoFisher, Cayman Chemical) was used at a final concentration of 2.5 μM. Nocodazole (Acros Organics) was used at a final concentration of 10 μM.

**Plasmid construction.** pCI-σNS, pCI-λ1, pCI-λ2, pCI-λ3/HA, and pCI-σ2 were previously described (6, 10, 11). The T1L MRV reverse-genetics plasmids pT7-M1-S1-S2-S4-RZ, pT7-L3-S3-RZ, pT7-L1-M2-RZ, pT7-L2, and pT7-M3 were previously described (16, 17). The Flag-tagged μ2 plasmid (pCI-Flag/M1 T3D+) was made by PCR amplification of a plasmid encoding the Flag-tag and the M1 5’ end (nucleotides [nt] 1 to 564) using forward and reverse primers (Integrated DNA Technologies) containing an XhoI site and an EcoRV site, respectively, flanking Flag-M1 gene sequence homology. The PCR product and pCI-μ2 (29) were digested with XhoI and EcoRV and ligated. The TC-tagged μNS plasmids were constructed utilizing synthetic dsDNA gBlocks (Integrated DNA Technologies) containing each of the described
mutations and the surrounding μNS sequence flanked on each end by restriction sites as follows: TC-μNS(#1) (SacI-BbvCI), TC-μNS(#2) (BbvCI-XmaI), TC-μNS(#4) and TC-μNS(#5) (BclI-BstEII), TC-μNS(#6) and TC-μNS(#7) (BstEII-SalI), TC-μNS(N-term) (SacI-PciI), and TC-μNS(C-term) (SalI-NotI). Each gBlock and pT7-M3 were digested with the indicated restriction enzymes, and vector and insert fragments were ligated.

**Transfection, infection, and reverse genetics.** For transfections and infections, BHK-T7 and CV-1 cells were seeded at concentrations of $2.0 \times 10^5$ and $1.0 \times 10^5$ cells per well, respectively, in 12-well plates containing 18-mm glass coverslips the day before transfection or infection. For transfection, 1 μg of plasmid DNA and 3 μl of TransIT-LT1 reagent (Mirus Bio) were added to 100 μl of Opti-MEM reduced serum medium (Invitrogen Life Technologies), and the mixture was incubated at room temperature for 30 min, added dropwise to cells, and incubated at 37°C overnight. CV-1 cells were infected with T1L, rTC-μNS(C-term-T1L)/P5 or rTC-μNS(#7-T1L)/P5 in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) with 2 mM MgCl2 at an MOI of 1 for 1 h with shaking and then replenished with medium and incubated at 37°C overnight. For reverse genetics, $5.0 \times 10^5$ BHK-T7 cells were plated on a 6-well plate (9.5 cm²; Corning Inc.) and transfected as previously described (33). The cells were incubated for 6 days, at which point cells and media were subjected to three freeze/thaw cycles, and standard L929 cell plaque assays were performed (28). Recovered plaques were passaged 10 times on Vero cells for 7 to 28 days to allow for replication of slow-growing virus.

**Immunofluorescence assay.** At 18 h p.t. or p.i., cells were treated or not with FLAsH-EDT2 diluted to 2.5 μM in Opti-MEM (Invitrogen Life Technologies) for 45 to 90 min and then fixed with 4% paraformaldehyde for 20 min and washed twice with PBS. Cells were permeabilized
with 0.2% Triton X-100 in PBS for 5 min, washed twice with PBS, and blocked with 1% bovine serum albumin in PBS (PBSA) for 10 min. Cells were then incubated for 45 min at room temperature with primary antibodies diluted in PBSA and washed two times with PBS, followed by incubation with secondary antibody diluted in PBSA for 30 min and two additional PBS washes. Labeled cells were mounted with the ProLong Gold antifade reagent with DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Invitrogen Life Technologies) on slides. Each coverslip was then examined on a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics. Representative pictures were taken by a Zeiss AxioCam MR color camera using AxioVision software (4.8.2). Plot profiles were generated using ImageJ (2.0.0-rc-49/1.51d) to determine pixel intensities of viral protein localization relative to μNS-labeled VFLs to compare wild-type μNS to TC-μNS. A significant difference ($P < 0.05$), denoted as a minus sign (−) in Table 1, was determined using JMP Pro (12.0.1). Images were prepared using Adobe Photoshop and Illustrator software (Adobe Systems).

**Replication assay.** L929 cells were seeded at a concentration of $2.5 \times 10^6$ cells per 60-mm dish (Corning Inc.). Twenty-four hours postseeding, cells were infected with wild-type T1L, rTC-μNS(C-term-T1L)/P2 or /P7, or rTC-μNS(#7-T1L)/P2 virus at an MOI of 0.2. At 0, 12, 24, 36, and 48 h p.i. cells were harvested and subjected to three freeze/thaw cycles, and then standard MRV plaque assays were performed on L929 cells to determine viral titers (28). The means and standard deviations were determined from two experimental replicates in two different biological replicates. Student's $t$ test was used to determine the $P$ value using Microsoft Excel (Microsoft Office). After the viral titer was determined, 8% paraformaldehyde was added to each well, which was left to incubate overnight. The next day, the paraformaldehyde and overlay were removed, cells were washed with PBS twice, 1% crystal violet in 20% ethanol was added, and
the mixture was incubated at room temperature for 15 min. Cells were washed twice with water, and the plaques were imaged using a ChemiDoc XRS Imaging System (Bio-Rad). Plaque size was then determined by measuring five plaques from each virus using ImageJ (2.0.0-rc-49/1.51d), and the average was calculated. Images were prepared using Adobe Photoshop and Illustrator (Adobe Systems).

**Immunoblotting.** L929 cells were plated at a concentration of $1 \times 10^6$ cells per well in a 6-well plate. Twenty-four hours postseeding, cells were mock infected or infected with T1L, rTC-μNS(C-term-T1L)/P5, or rTC-μNS(#7-T1L)/P5 at an MOI of 0.25. At 0, 12, 24, and 48 h p.i., cells were washed twice with PBS and collected in 2× protein loading buffer (125 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol). Alternatively, $5 \times 10^6$ PFU of each virus was diluted to the same volume using PBS with 2 mM MgCl₂, and then 2× protein loading buffer was added to each sample. Cell lysates/virions were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose by electroblotting. Nitrocellulose membranes were incubated with primary and secondary antibodies in Tris-buffered saline (20 mM Tris, 137 mM NaCl [pH 7.6]) with 0.25% Tween 20 (TBST) for 18 h and 4 h, respectively, followed by addition of PhosphaGLO AP (SeraCare) substrate and imaging on a ChemiDoc XRS Imaging System (Bio-Rad).

**MRV genome analysis and reverse transcription.** T1L, rTC-μNS(C-term-T1L)/P2, /P5, /P7, or /P10, or rTC-μNS(#7-T1L)/P2 or /P5 ($1.0 \times 10^8$ PFU each) and $1.0 \times 10^9$ PFU of T1L (T1L 10×) were subjected to TRIzol LS (Life Technologies) extraction via the manufacturer's instructions. Briefly, viruses were homogenized in TRIzol LS, and the addition of chloroform separated each sample into protein, DNA, and RNA phases. The RNA phase was collected, and
isopropanol and ethanol were added to precipitate and wash RNA. Extracted RNA was separated on 10% SDS-PAGE at a constant 20 mA for 12 h, and the gel was incubated in water with 3× gel red (Phenix Research Products) for 1 h and imaged on a ChemiDoc XRS imaging system (Bio-Rad). Extracted RNA was also subjected to RT-PCR using SuperScript IV (Invitrogen Life Technologies) as per the manufacturer's instruction for sequencing.

**Live-cell imaging of VFs.** BHK-T7 and Vero cells were seeded on a 12-well, 14-mm glass bottom plate (MatTek Corporation) at concentrations of $2 \times 10^5$ and $7.5 \times 10^4$ cells per well, respectively, and then infected the following day with T1L at an MOI of 100, rTC-μNS(C-term-T1L)/P2 at an MOI of 5 (BHK-T7 cells), or rTC-μNS(C-term-T1L)/P5 at an MOI of 100 (Vero cells). For BHK-T7 cells, at 18 h p.i., medium was removed and cells were washed twice with DMEM without phenol red (HyClone) supplemented with a penicillin (100 IU/ml)–streptomycin (100 μg/ml) solution, 1% MEM nonessential amino acid solution, and 25 mM HEPES, followed by addition of FlAsH-EDT2 diluted to 2.5 μM in DMEM and incubated at 37°C with shaking every 15 min for a total of 45 min, at which point 800 μl of DMEM was added to each well and imaging was initiated. For Vero cells, at 10 h p.i. cells were washed and treated with FlAsH-EDT2 until 12 h p.i., at which point FlAsH-EDT2 was removed, medium was replenished, and imaging was initiated. In experiments involving nocodazole, at 6 h p.i. 10 μM nocodazole was added and maintained throughout the experiment. All cells were examined using an Olympus IX071 inverted fluorescence microscope on a vibration table, equipped with an environmental control chamber heated to 37°C. Still images and video were captured through a 40× apochromatic objective by a high-resolution Hamamatsu charge-coupled-device (CCD) camera using MetaMorph for Olympus MetaMorph Advanced (V 7.7.7.0). All image exposure conditions were maintained throughout the experiment, and background levels were set using
FLAsH-EDT2 expression in wild-type T1L-infected cells. Still images were processed using ImageJ (2.0.0-rc-49/1.51d) and assembled for publication using Adobe Photoshop and Illustrator (Adobe Systems).

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Footnote

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References


CHAPTER 4. INHIBITION OF HIF-1α IN PC3 CELLS IS ENHANCED IN UV-INACTIVATED VERSUS WILDTYPE MAMMALIAN ORTHOREOVIRUS INFECTION

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Abstract

Solid tumors are composed of a variety of distinct microenvironments and genetic subtypes, making treatment of the entire tumor difficult in cancer patients. Of these microenvironments tumor hypoxia, or regions of low oxygen, are particularly aggressive and resistant to standard therapeutics. Tumor hypoxia results from an outgrowth of cancer cells from blood vessels resulting in accumulation of the alpha subunit of hypoxia-inducible factor 1 (HIF-1α). HIF-1α enters the nucleus and upregulates target genes involved in proliferation, angiogenesis, and metastasis, prompting research into discovery of HIF-1α inhibitors. Interestingly, the cancer killing mammalian orthoreovirus (MRV) inhibits HIF-1α in various cancer cells under hypoxia. MRV is currently being investigated in numerous Phase I-II clinical trials, and therefore it could be utilized in inhibiting the downstream effects of HIF-1α while killing tumors in cancer patients. In this work we systematically investigated the stage of viral replication sufficient to inhibit HIF-1α, and found a step between viral capsid cleavage and transcription is sufficient. By removing or mutating the viral capsid proteins σ3 or µ1, or viral double-stranded RNA (dsRNA), we determined that each of these viral components play little to no role in HIF-1α inhibition in the context of normal infection. However, UV-inactivated MRV was found to inhibit HIF-1α via a similar mechanism, but to a greater extent compared to
wildtype (wt) MRV. This work has unveiled new data regarding the mechanisms of MRV-induced HIF-1α inhibition, and provided further evidence for the use of UV-inactivated or wt MRV for treatment of hypoxic tumors.

**Importance**

MRV has been shown to be safe in Phase I clinical trials, but the effectiveness of MRV has been questioned as it has failed to reach certain set points in previous Phase II-III trials necessary for FDA approval. These clinical trials show that MRV therapy will not be effective in all cancer patients, and therefore it is important to study MRV biology, the effect of viral therapy on different tumor types, and how various tumor microenvironments are impacted by MRV treatment. In this work we analyzed the stage of viral infection sufficient to inhibit HIF-1α. During this investigation we determined that UV-inactivated MRV inhibits HIF-1α to a greater extent than wt virus, providing evidence for its therapeutic use in cancer patients. Altogether this work has strengthened the assertion of using MRV to inhibit the devastating effects of tumor hypoxia.

**Introduction**

Viruses are generally considered disease causing agents which scientists work diligently to eliminate by vaccination, but evidence is mounting that a handful of viruses can be exploited to help improve human lives. Cancer killing, oncolytic viruses, are wildtype (wt) or engineered viruses that can be used therapeutically to locate, infect, and kill tumor cells while leaving the host unharmed (1). These oncolytic viruses have been shown to be effective in killing many tumor types in humans and other mammals by direct lysis of the cancerous cells, and have also been shown to be excellent activators of antitumor immunity (2, 3). One of the front-runners in oncolytic virotherapy is mammalian orthoreovirus (MRV) which has been shown to be safe in
Phase I clinical trials and has progressed to Phase II and III clinical trials against multiple tumor types (4). MRV is a particularly interesting oncolytic virus because it is naturally benign, and therefore it does not require engineering prior to use as a cancer therapeutic (5).

MRV is a segmented double-stranded RNA (dsRNA) virus that belongs to the Reoviridae family, which also includes pathogenic mammalian and plant viruses such as rotavirus, bluetongue virus, African horse sickness virus, and rice dwarf virus (6). Unlike these pathogenic family members, which cause life-threatening diarrhea and hemorrhagic disease, MRV infection typically results in mild diarrhea or respiratory disease in immunocompromised individuals. Children are particularly vulnerable because natural infection is primarily spread through the fecal-oral route. Viral infection begins when the virus binds to JAM-A and/or sialic acid on the cell surface and is endocytosed via clathrin-mediated endocytosis (7-9). The early endosome acidifies as it progresses into a late endosome, which is required for efficient infection (10, 11). During this process pH dependent proteases cleave the outer capsid protein σ3, allowing further cleavage and release of μ1, the endosomal pore forming protein (12-14). The μ1N fragment of μ1 is myristoylated and penetrates the membrane forming small pores that are suggested to be too small for viral escape (13, 15). The endosome may disintegrate due to the sheer number of holes produced by μ1, or differences in osmotic pressure between the cytosol and the endosome may result in a large influx of water and bursting of the endosome (15). Nevertheless, the virus escapes and resides in the cytoplasm where transcription, translation, assortment, assembly and replication occur at virus formed factories (16-20). Intact virions are then released when the cell bursts and are potentially released in exosomes, as seen in rotavirus infection (21).

In clinical trials when high titers of MRV are delivered intravenously the virus locates, infects, and lyses tumor cells, while evading the immune system. By the age of six, up to 50% of
children will express neutralizing antibodies against MRV (22). Moreover, in a study looking for
a connection between MRV infection and liver disease, researchers found 38 of 43 participants,
with or without liver disease, had antibodies against MRV (23). Yet despite anti-MRV immune
surveillance in patients receiving MRV therapy, data from 13 clinical trials demonstrated that
81% of all tumors contain replication competent MRV (24). How MRV locates tumors with high
efficiency is not well defined, particularly since most individuals have antibodies against the
virus. However, recent data suggests that the virus highjacks and is delivered to the tumor by
monocytes, then escapes the monocytes by an unknown mechanism (25). Once MRV locates the
tumor, the virus has been shown to induce antitumor immunity, causing immunosuppressive
tumor environments to become immunopermissive (26-28). One of the strongest evidences for
this was demonstrated in brain cancer patients that were treated intravenously with MRV and
then had their tumor resected. Patients receiving MRV therapy saw increased CD8+ T cells
around blood vessels compared to those not treated (29). In addition, in mouse models the
antitumor immune response induced by MRV infection was shown to be sufficient to inhibit
cancer relapse. Since many cancer therapeutics have off-target effects that are detrimental to the
patient, and do not protect against tumor relapse, novel therapies like MRV show promise.

While MRV is a promising therapeutic it has become clear in clinical trials that not all
tumors decrease in size or dissipate following MRV therapy (28). The virus has progressed
through Phase I-III clinical trials, but it has not reached primary set points such as progression
free survival necessary for FDA approval (4). Therefore, in order to improve the efficacy of
MRV as a cancer therapeutic, additional pre-clinical and clinical studies are necessary to better
understand 1) the basic biology of the virus, 2) the precise effects of the virus on different tumor
cell types, and 3) how different tumor environments are impacted by MRV therapy. Previously,
we and others have shown that MRV inhibits the accumulation of the alpha subunit of hypoxia-inducible factor-1 (HIF-1α) under hypoxic, or low oxygen conditions in vitro, and recently it has also been shown that MRV inhibits HIF-1α in tumors in vivo (30-33). HIF-1α is a master transcriptional regulator of cells growing in hypoxic conditions and modulates genes involved in cell growth, survival, angiogenesis, and metastasis (34, 35). Within cells growing in hypoxic environments the pathways regulating HIF-1α accumulation are inhibited, leading to high levels of HIF-1α (34). HIF-1α localizes to the nucleus, where it binds to HIF-1β and p300/CBP on the hypoxia response element (HRE), and activates transcription of downstream target genes (36). In non-tumor cells many of these genes are necessary for normal growth regulation, but within a tumor, the hypoxic environment, and specifically HIF-1α upregulation of downstream genes results in increasingly aggressive cancer growth (37). Hypoxic microenvironments are common in solid tumors, resulting in high HIF-1α accumulation leading to poorer prognosis in cancer patients compared to individuals with low HIF-1α accumulation (38). Furthermore, HIF-1α and tumor hypoxia have been shown to inhibit the immune response as well as various cancer therapeutics, therefore therapeutics that target HIF-1α are needed (39, 40).

Since MRV has already been shown to be safe through numerous clinical trials, emerging data from our lab and others suggests that MRV therapy could be used in hypoxic tumors to specifically inhibit HIF-1α accumulation and transcriptional regulation. Researchers have shown several benefits of HIF-1α inhibition in tumors including resensitizing resistant tumors to chemotherapeutics and rescuing natural killer cell lysis susceptibility (41, 42). Current literature suggests that MRV inhibition of HIF-1α is independent of the oxygen dependent VHL pathway, and instead utilizes RACK1-mediated proteasomal degradation and translational inhibition to prevent HIF-1α accumulation in infected cells (30, 33). Moreover, it has also been demonstrated
that both UV-inactivated MRV, and direct introduction of MRV dsRNA segments to cells is sufficient to inhibit HIF-1α accumulation, in a manner independent of dsRNA recognition by the RIG-I/IPS1 pathway (31). Apart from this, little is known about the mechanism of MRV induced inhibition of HIF-1α, and therefore careful study into the mechanism of inhibition is necessary to understand the extent by which MRV can be used in clinical trials to alter the hypoxic response.

In this work we systematically tested each step of the viral replication cycle to determine the stage at which MRV induces the inhibition of HIF-1α accumulation. Utilizing UV-inactivation and viral endosome breakdown inhibitors, we identified the step during infection that was sufficient to inhibit HIF-1α, and then examined different active pathways at this stage of infection. We removed or mutated viral proteins, or dsRNA, to further investigate the mechanism and necessary viral components. Finally, we compared the mechanism of UV-inactivated inhibition of HIF-1α to that of wt MRV to discern if UV-inactivated MRV mimicked what occurs during normal infection. This work has provided new insights into the mechanisms of HIF-1α inhibition by MRV, and will guide clinicians in the utilization of MRV therapy in hypoxic tumors.

Results

Replication defective T3DC virus inhibits HIF-1α accumulation in PC3 cells. Previous investigation into MRV-induced inhibition of HIF-1α has suggested that UV-inactivated MRV, which is transcription deficient, inhibits HIF-1α to wt MRV levels in various tumor cells in vitro (31). We have previously shown that MRV inhibits HIF-1α in prostate cancer (PCa) cell lines and reasoned UV-inactivated MRV would additionally inhibit HIF-1α (30). Therefore, to
Figure 1. UV-inactivated T3D<sup>C</sup> inhibits HIF-1α significantly more than wt T3D<sup>C</sup>. PC3 cells were mock infected or infected at an MOI = 20 with T3D<sup>C</sup> or an equal number of viral particles of T3D<sup>C</sup> exposed to 1 J/cm<sup>2</sup> of UV-irradiation (UVT3D<sup>C</sup>). At 20 h p.i. cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot or immunofluorescence analysis. (A) Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus structural protein λ1, the nonstructural μNS, HIF-1α, and β-actin. (B) Immunoblots were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. (C) Cells were fixed for immunofluorescence analysis with primary α-HIF-1α antibody, α-T3D antisera, and secondary Alexa 594-conjugated
donkey α-rabbit or Alexa 488-conjugated donkey α-mouse IgG. A merged image is also shown with DAPI staining. Images are representative of the observed phenotype. Bars = 10 µm. (D) Ten fields of views of cells were counted and the number of cells expressing nuclear HIF-1α is shown. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. \( P \) values were determined using a two tailed Student \( t \) test comparison in Microsoft Excel.

determine the extent by which UV-inactivation impacts MRV inhibition of HIF-1α accumulation in PCa cells, we mock infected or infected PC3 cells at an MOI = 20 with wt MRV strain T3DC\(^C\) and UV-inactivated T3DC\(^C\) (UVT3DC\(^C\)). At 20 h post-infection (p.i.) we exposed a subset of cells to hypoxic conditions (1% O\(_2\), 5% CO\(_2\) at 37°C) for 4 h before harvesting and subjecting to immunoblot assay. To confirm that our UV-inactivation was sufficient to interfere with viral transcription and translation, we immunostained with antibodies against both viral structural (\( \lambda_1 \)) and non-structural (\( \mu_{NS} \)) proteins. We found that while both T3DC\(^C\) and UVT3DC\(^C\) infected cells expressed \( \lambda_1 \), only T3DC\(^C\) expressed \( \mu_{NS} \), suggesting that the UVT3DC\(^C\) was unable to transcribe and translate protein (Fig. 1A). Immunostaining against HIF-1α revealed that both wildtype T3DC\(^C\) and UVT3DC\(^C\) inhibited accumulation of HIF-1α under hypoxic conditions. Quantification of immunoblot replicates demonstrated that HIF-1α accumulation was significantly less (\( P > 0.05 \)) in T3DC\(^C\) and UVT3DC\(^C\) infected cells compared to mock-infected cells (Fig. 1B). To strengthen this finding, we also performed immunofluorescence assays of cells infected with T3DC\(^C\) and UVT3DC\(^C\). Immunofluorescence showed that in cells infected with either T3DC\(^C\) or UVT3DC\(^C\), there was little to no HIF-1α accumulation (Fig. 1C-D). Interestingly, in both the immunoblot and immunofluorescence analysis, there was significantly less HIF-1α accumulation in UVT3DC\(^C\) infected cells compared to T3DC\(^C\) infected cells. Taken together, this data suggests that UV-inactivated MRV has the capacity to inhibit HIF-1α accumulation in PCa cells and does so with increased efficiency compared to wt virus.
Viral capsid cleavage and endosomal escape or endosomal breakdown is necessary for MRV-induced inhibition of HIF-1α accumulation. Since our previous data suggested that viral transcription and subsequent steps are not required for HIF-1α inhibition, we next looked at events directly upstream of viral transcription in the MRV life cycle inclusive of viral cleavage and escape from the endosome. PC3 cells were subjected to NH₄Cl or E64, which block viral capsid cleavage and virus escape from the endosome by preventing pH change within the endosome necessary for activation of proteases (NH₄Cl) or directly inhibiting cysteine proteases necessary for capsid protein cleavage (E64) (10). PC3 cells were mock infected or infected with T3DC 4 h following the addition of drugs. At 20 h p.i., cells were placed under hypoxic conditions for 4 h, and then harvested and subjected to immunoblot analysis (Fig. 2A). Similar to prior experiments, blots were immunostained with antibodies against the viral proteins μNS and λ1. In infected cells λ1 was present but not the nonstructural protein μNS, suggesting that the virus could infect the cells but was unable to escape the endosome to begin transcribing and translating protein. As expected, we observed significant HIF-1α inhibition in T3DC infected cells compared to mock-infected cells under hypoxia, however, there was not a significant difference in accumulated HIF-1α levels in infected cells treated with NH₄Cl or E64 compared to mock-infected cells (Fig. 2B). To determine if HIF-1α inhibition mediated by UVT3DC was also sensitive to NH₄Cl and E64 treatment, we repeated the previous experiment with cells infected with UVT3D³ and observed similar results (Fig. 2C-D). Lastly, our results were confirmed via immunofluorescence, and we found that T3D³ and UVT3D³ infected cells inhibited HIF-1α in the absence of drug treatment, and failed to inhibit HIF-1α when NH₄Cl or E64 were added (Fig. 2E). Together our data suggests that a step between viral capsid cleavage
**Figure 2. Viral capsid cleavage is necessary for HIF-1α inhibition.** PC3 cells were treated with NH₄Cl (200 mM) or E64 (20 µM) 4 h post infection and were then mock infected or infected at an MOI = 20 with T3DC or an equal number of viral particles of T3DC exposed to 1 J/cm² of UV-irradiation (UVT3DC). At 20 h p.i. cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot or immunofluorescence analysis. Cell lysates from T3DC infected PC3 cells (A) or UVT3DC infected PC3 cells (C) were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus structural protein λ1, the nonstructural µNS, HIF-1α and β-actin. Immunoblots of T3DC (B) or UVT3DC (D) infected cells were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. P values were determined using a two tailed Student t test comparison in Microsoft Excel. (E) Cells were fixed for immunofluorescence analysis with primary α-HIF-1α antibody, α-T3D antisera, and secondary Alexa 594-conjugated donkey α-rabbit or Alexa 488-conjugated donkey α-mouse IgG. A merged image is also shown with DAPI staining. Images are representative of the observed phenotype. Bars = 10 µm.

In the endosome and transcription in replication competent or deficient T3DC is sufficient to inhibit HIF-1α accumulation in PCa cells.

**Outer capsid protein σ3 is not necessary for T3DC-induced HIF-1α inhibition.** Data supports a hypothesis where MRV escapes the acidified late endosome following protease cleavage and removal of the σ3 protein from the virion, and membrane destabilization by the newly exposed hydrophobic portions of µ1 (10-15). Therefore, in addition to the viral core, there are other viral components that may be released into the cytoplasm during endosomal destabilization including σ1, σ3 (or σ3 cleavage fragments), µ1 (or µ1 cleavage fragments), and dsRNA. Since there are 600 copies of σ3 and µ1 per virion we began investigating the role of these two proteins on HIF-1α inhibition beginning with σ3 (43). While the outermost capsid protein, σ3, provides stability to the viral capsid it is not required for infection (44-47). Therefore, immediately prior to PC3 infection, T3DC was incubated with α-chymotrypsin for 5 min to cleave σ3 to produce replication competent infectious subviral particles (ISVPs). A portion of the ISVPs were then exposed to UV light to produce UVISVPs as done previously to make UVT3DC. T3DC,
Figure 3. Virus stripped of σ3 inhibits HIF-1α similar to wt virus. 1.6 × 10^7 CIUs of T3D<sup>C</sup> virus was treated with 200 µg/ml α-chymotrypsin for 5 min to cleave the outer capsid proteins σ3 and μ1 to form ISVPs. T3D<sup>C</sup> and ISVP were further subjected to 1 J/cm<sup>2</sup> UV-irradiation (UVT3D<sup>C</sup> and UVISVP). (A) Equal viral particles of each T3D<sup>C</sup>, UVT3D<sup>C</sup>, ISVP, and UVISVP were run on SDS-PAGE and stained with Coomassie to observe MRV structural proteins. ISVP production is confirmed by the cleavage of σ3 and μ1 to δ as shown. PC3 cells were mock infected or infected at an MOI = 20 with T3D<sup>C</sup> and an equal number of viral particles of UVT3D<sup>C</sup>, ISVP or UVISVP. At 20 h p.i. cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot or immunofluorescence analysis. (B) Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus nonstructural protein μNS, HIF-1α, and β-actin. (C) Immunoblots were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. P values were determined using a two tailed Student t test comparison in Microsoft Excel. (D) Cells were fixed for immunofluorescence analysis with primary a-HIF-1α antibody, a-T3D...
antisera, and secondary Alexa 594-conjugated donkey α-rabbit or Alexa 488-conjugated donkey α-mouse IgG. A merged image is also shown with DAPI staining. Images are representative of the observed phenotype. Bars = 10 µm.

UVT3DC, ISVP and UVISVP samples were separated on SDS-PAGE and stained with Coomassie to confirm successful ISVP production through demonstration of σ3 degradation and cleavage of μ1 into the δ fragment (Fig. 3A). PC3 cells were then infected for 20 h, followed by 4 h hypoxia treatment. These experiments demonstrated that both ISVP and UVISVP were able to significantly inhibit HIF-1α accumulation (Fig. 3B-C). ISVPs are expected to inhibit HIF-1α as they are replication competent, and therefore should provide the same phenotype as wt T3DC following transcription and translation of all viral mRNAs. On the other hand, UVISVPs lack σ3 and the means to transcribe and translate σ3 and were still able to inhibit HIF-1α accumulation. This data suggests that σ3 is dispensable for HIF-1α inhibition. Immunofluorescence assays of T3DC, ISVP and UVISVP infected cells confirmed that HIF-1α was inhibited in cells infected with ISVPs and UVISVPs (Fig. 3D).

**Apoptosis induced by μ1 contributes to HIF-1α inhibition.** We next turned our attention to the μ1 protein, which plays multiple known roles during MRV infection. Once μ1 is cleaved it pokes holes in the endosomal membrane and φ induces cellular apoptosis (13, 15, 48). While it is not possible to mutate sites involved in the membrane penetrating function of μ1, because these would result in a noninfectious virus, there are previously described mutants in the φ region of μ1 (I595K) of the T3DF strain of MRV that have decreased MRV’s capacity to induce apoptosis (49). T3DF and T3DC viruses are genotypically similar, however, to prove that differences within the two strains do not result in differences in HIF-1α inhibition we compared HIF-1α accumulation in T3DF and T3DC infected cells. Furthermore, T3DC, T3DF and I595K were UV-
inactivated prior to infection in PC3 cells, to determine the extent by which apoptosis inhibits HIF-1α at early stages preceding viral transcription. At 20 h p.i. cells were placed under hypoxia for 4 h, then harvested, lysed, and subjected to immunoblot analysis using antibodies against λ1, HIF-1α, μNS, and β-actin (Fig. 4A). Immunoblot data was quantified and UVT3D^C, UVT3D^F and UVI595K showed significant inhibition of HIF-1α accumulation compared to mock-infected cells under hypoxia (Fig. 4B). This suggests that mutation of a residue important in μ1 apoptosis induction does not fully prevent HIF-1α inhibition. However, there was a significant difference in the accumulation of HIF-1α between UVT3D^F and UVI595K infected cells, with the apoptosis deficient UVI595K inhibiting HIF-1α to a lesser extent than UVT3D^F. Differences between the viruses suggest the apoptosis function of μ1 may play a minor role in inhibition of HIF-1α accumulation during UV-inactivated virus infection.

To strengthen this hypothesis, we utilized the pan caspase inhibitor Z-VAD-FMK, which binds and inhibits caspase protease needed for apoptosis induction, in combination with T3D^C to observe the impact of virus-induced apoptosis on HIF-1α accumulation. To confirm that Z-VAD-FMK at 20 μM inhibits apoptosis we mock infected or infected PC3 cells with T3D^C or UVT3D^C, or treated cells with the apoptosis inducing docetaxel, and then treated with or without Z-VAD-FMK. After 20 h p.i. and treatment cells were exposed to hypoxia for 4 h and then caspase 3/7 activity was quantified (Fig. 4C). In T3DC, UVT3DC, and docetaxel infected/treated cells, caspase 3/7 activity, indicative of apoptosis induction, was increased compared to mock-infected cells in the absence of Z-VAD-FMK. When cells were exposed to Z-VAD-FMK, caspase 3/7 activity was decreased suggesting that Z-VAD-FMK at 20 μM is sufficient to inhibit viral induced apoptosis in PC3 cells. Next, PC3 cells were mock infected or infected, treated with or without Z-VAD-FMK, and placed under hypoxia as described for caspase 3/7 activity.
**Figure 4. µ1 induced apoptosis is not necessary for HIF-1α inhibition.** T3D\(^C\), T3D\(^F\), and I595K viruses at \(1.6 \times 10^7\) CIUs were UV-irradiated at 1 J/cm\(^2\). PC3 cells were mock infected or infected with UV1595K, or UV1595K, and at 20 h p.i. cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot analysis. (A) Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus structural protein \(\lambda_1\), the nonstructural \(\mu\)NS, HIF-1\(\alpha\), and \(\beta\)-actin. (B) Immunoblots were analyzed on Quantity One software to quantify HIF-1\(\alpha\)/\(\beta\)-actin protein levels. PC3 cells were mock infected or infected with T3D\(^C\) or UVT3D\(^C\), with or without treatment of the pan caspase inhibitor Z-VAD-FMK. At 20 h p.i. and treatment cells were exposed to normoxic or hypoxic conditions for 4 h and (C) were subject to caspase 3/7 activity assay along with treatment of the apoptosis inducing docetaxel (3 \(\mu\)M), or (D) were collected for immunoblot analysis as described in (A). (E) Immunoblots were analyzed as describe in (B). Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. \(P\) values were determined using a two tailed Student \(t\) test comparison in Microsoft Excel.

Cells were harvested and lysed for immunoblot analysis, and in cells treated with Z-VAD-FMK, T3D\(^C\) and UVT3D\(^C\) were able to inhibit HIF-1\(\alpha\) similar to untreated cells (Fig. 4D).

Quantification of immunoblot replicates demonstrated that there were no significant differences between cells treated with Z-VAD-FMK and those without (Fig. 4E). Together these
experiments suggest that μ1- or MRV-induced apoptosis does not fully inhibit HIF-1α accumulation, but may contribute minimally.

**Reovirus dsRNA contributes to HIF-1α inhibition in UV-inactivated but not wt infection.**

After investigating σ3 and μ1 we turned our attention to the extent by which the viral dsRNA within the viral core inhibited HIF-1α accumulation. A previous report suggested transfected MRV dsRNA as well as polyI:C can induce HIF-1α inhibition (31). However, delivery of dsRNA via transfection differs significantly from virion delivery, therefore we used an experimental approach that more closely mimics MRV infection. Upon cesium chloride (CsCl) gradient purification of MRV, a top and bottom component band form, where the bottom component consists primarily of virus containing a full complement of the ten viral genome segments, while the top component is considered genome deficient (50). For these experiments, we collected both the bottom component (T3D<sup>C</sup>) and the top component (TC) from the same CsCl gradient. We first extracted dsRNA from the same number of viral particles, calculated from absorbance at 260 nm (A<sub>260</sub>) = 2.1 × 10<sup>12</sup> particles, of T3D<sup>C</sup> and TC, and separated the dsRNA on SDS-PAGE (50). These experiments demonstrated that when the same number of particles were examined there was a substantial decrease in dsRNA within the TC compared to T3D<sup>C</sup> (Fig. 5A). Next, 1.88 × 10<sup>12</sup> viral particles of each T3D<sup>C</sup>, TC, UVT3D<sup>C</sup>, and UVTC were lysed and separated on SDS-PAGE and stained with Coomassie to examine virus structural proteins (Fig. 5B). The amount of each observed viral protein on Coomassie was similar between T3D<sup>C</sup> and TC, and UVT3D<sup>C</sup> and UVTC, suggesting that a similar number of TC and T3D<sup>C</sup> virions (Fig. 5B) result in decreased dsRNA in TC compared to T3D<sup>C</sup> (Fig. 5A). To determine the effect of dsRNA on HIF-1α accumulation during MRV infection, PC3 cells were infected with 1.88 × 10<sup>12</sup> particles, equivalent to an MOI = 20 in T3D<sup>C</sup> infected cells, of T3D<sup>C</sup>, TC,
Figure 5. dsRNA deficient reovirus inhibits HIF-1α accumulation compared to wt virus. T3D^C virus was purified on a CsCl2 gradient and two bands were collected, a bottom highly infectious bottom component (T3D^C) and a dsRNA deficient top component (TC). (A) 1.88 × 10^{12} particles of T3D^C and TC were subject to TRIzol RNA extraction and run on SDS-PAGE at 20 mA for 10 h to separate the 10 MRV genome segments. T3D^C and TC were UV-irradiated at 1 J/cm^2 (UVT3D^C and UVTC), and (B) 1.88 × 10^{12} particles of each virus was run on SDS-PAGE followed by Coomassie staining to observe total structural proteins. PC3 cells were mock infected or infected with T3D^C, TC, UVT3D^C, or UVTC, and at 20 h p.i. cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot analysis. (C) Cell
lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus structural protein λ1, the nonstructural μNS, HIF-1α and β-actin. (D) Immunoblots were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. $P$ values were determined using a two tailed Student $t$ test comparison in Microsoft Excel.

UVT3D$^C$, and UVTC. At 20 h p.i., cells were grown under hypoxia for 4 h, then harvested, lysed, and immunoblotted with antibodies against λ1, HIF-1α, μNS, and β-actin. The immunoblot and quantified data (Fig. 5C-D) show that the dsRNA deficient TC was able to inhibit HIF-1α to similar levels to wt T3D$^C$, suggesting dsRNA does not play a role in inhibition of HIF-1α accumulation during normal infection. Interestingly, when the viruses were UV-inactivated we observed a repeatable loss of HIF-1α inhibition in UVTC relative to UVT3D$^C$ infected cells, however, this decrease did not reach significance over five biological replicates. Altogether this suggests that dsRNA may play a minor role in inhibiting HIF-1α when the virus is unable to progress into its replication cycle, but not during active infection.

**UVT3D$^C$-induced inhibition of HIF-1α is dependent on the proteasome.** Throughout this work we observed significant differences between the impact of wt T3D$^C$ and UVT3D$^C$ on HIF-1α accumulation during infection, such as increasing the inhibition of HIF-1α accumulation. We reasoned that it is possible that UV-inactivation may result in a virus that utilizes different mechanisms to inhibit HIF-1α compared to wt virus. Using an approach similar to that published for T3D$^C$, we investigated the role the proteasome plays in UVT3D$^C$ inhibition of HIF-1α (30). PC3 cells were infected with T3D$^C$ or UVT3D$^C$, and at 8 h p.i. cells were treated with the proteasome inhibitor, MG132, and placed under hypoxia for 4 h. At 12 h p.i. the cells were harvested and subjected to immunoblot analysis. In the absence of MG132, T3D$^C$ and UVT3D$^C$ inhibited HIF-1α as expected, however, in the presence of MG132, both viruses were prevented
from inhibiting HIF-1α accumulation (Fig. 6A). While there was a significant decrease in HIF-1α in UVT3DC infected cells compared to mock-infected cells under hypoxia there was no significant difference when MG132 was added. (Fig. 6B). This data suggests that despite the differences that we measured in efficiency of HIF-1α inhibition between T3DC and UVT3DC, that like T3DC, UVT3DC inhibits HIF-1α accumulation at least in part by inducing proteasome-mediated degradation of HIF-1α.

**Figure 6.** UVT3DC inhibits HIF-1α through proteasome-mediated degradation early during infection. PC3 cells were mock infected or infected at an MOI = 20 with T3DC or an equal number of viral particles of T3DC exposed to 1 J/cm² of UV-irradiation (UVT3DC). At 8 h p.i. cells were treated with the proteasome inhibitor MG132 (50 μM) and exposed to normoxic or hypoxic conditions for 4 h at which point the cells were collected for immunoblot analysis. (A) Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus structural protein λ1, the nonstructural μNS, HIF-1α and β-actin. (B) Immunoblots were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. *P* values were determined using a two tailed Student *t* test comparison in Microsoft Excel.

**Discussion**

Mammalian orthoreovirus inhibition of HIF-1α is an intriguing phenotype of the virus that can be exploited to help inhibit the devastating effects of hypoxia and HIF-1α accumulation in tumors. In this work, the stages of viral infection were dissected to better understand how and
when during the virus life cycle MRV inhibits HIF-1α. Experiments with NH₄Cl, E64, and UV-inactivated virus suggest a step between viral capsid cleavage and transcription is sufficient for this phenotype. Upon cleavage of outermost capsid protein σ3, the viral protein μ1 undergoes cleavage and pokes holes in the endosomal membrane resulting in disruption and release of the viral core, along with endosomal contents into the cytoplasm. There is a large amount of cleaved σ3 and μ1 in the endosome, as well as a smaller amount of σ1 that are likely released from the endosome along with the core (43). Additionally, it has been proposed that UV-inactivation may destabilize the viral core and result in increased dsRNA release (51, 52). Therefore, in our work we focused on these viral components. It is evident that loss of the σ3 protein had no impact on HIF-1α accumulation, as UVISPs were comparable to UV-inactivated MRV at inducing decreased HIF-1α. Mutation resulting in loss of the apoptosis function of μ1 likewise did not substantially alter MRV inhibition of HIF-1α as compared to wt virus. We next turned our attention to the dsRNA, which has been suggested to play a role in HIF-1α inhibition in cells following transfection. Using virions substantially deficient of dsRNA genome segments, we observed inhibition of HIF-1α similar to wt virus. Taken together, this data suggests that a consequence of endosomal membrane disruption is primarily responsible for inhibition of HIF-1α protein accumulation, independent of viral outer capsid proteins, σ3 and μ1, or dsRNA.

While there is a possibility that σ1 may inhibit HIF-1α, we find this possibility to be unlikely. σ1 is a viral attachment protein that binds JAM-A and/or sialic acid needed for viral entry (7-9). Following removal of σ3, and subsequent μ1 cleavages, the MRV capsid changes confirmation to release σ1. There are 36 or less copies of σ1 per virion, compared to 600 copies of σ3 and μ1, therefore we find it unlikely that σ1 plays a role in HIF-1α inhibition within the
cytoplasm (53). Moreover, while it is possible that σ1/receptor binding could result in a downstream signal that inhibits HIF-1α, σ1-receptor engagement would still occur and downstream signals would presumably still be present in experiments where endosomal protease inhibitors are present. In infected cells treated with NH₄Cl or E64 we observed rescue of HIF-1α accumulation, suggesting any signaling that occurs as a result of σ1/receptor binding does not inhibit HIF-1α. It is also possible that release of the viral core into the cytoplasm may be sufficient to inhibit HIF-1α, and future work will investigate the extent by which the core and σ1 contribute to HIF-1α inhibition.

While we have not formally ruled out the possibility that σ1 or MRV core release is involved in MRV-induced HIF-1α inhibition, we find it more likely that the breakdown of the endosome may contribute to HIF-1α inhibition. It is interesting that the ssDNA, non-enveloped human parvovirus (H-1 parvovirus), which is also endocytosed and requires low pH for escape from the late endosome, has been shown to inhibit HIF-1α accumulation (54). When H-1 parvovirus is treated with NH₄Cl, HIF-1α is rescued from viral inhibition similar to what we have observed with MRV (55). The similarities between the requirements for HIF-1α inhibition between these two viruses may suggest that breakdown of the endosome and downstream signaling may result in HIF-1α inhibition. Additionally, disruption of the late endosome results in release of the endosomal contents into the cytoplasm, and while we focused on the viral components, the vesicle also contains other proteins and enzymes (56). These enzymes are not designed to be in the cytoplasm, and we reason that this release could result in various stress signals. In addition, late endosomes and lysosomes are targets for misfolded protein degradation, and the release of increased amounts of misfolded proteins can results in PERK activation (57). PERK phosphorylates eIF2α resulting in translation inhibition, stress granule (SG) formation,
and stress signaling within the cell (58). In cells treated with the drug thapsigargin, PERK is activated resulting in SG formation and inhibition of HIF-1α (59). Therefore, it is conceivable that endosomal breakdown and release of the core increases SG formation and signaling resulting in decreased HIF-1α.

It is interesting that UV-inactivated virus inhibits HIF-1α significantly more than wt virus, because UV-inactivated virus also induces substantially higher numbers of SGs that are maintained for a prolonged period of time compared to wt virus (60). SGs are aggregates of stalled mRNA-ribosome complexes, RNA-binding proteins, and SG effector proteins that form when cells experience stress (61). MRV-induction of SGs is dependent on eIF2α phosphorylation, and correlates with inhibition of cellular, but not viral translation (60, 62). Therefore, it is possible that the observed increase in HIF-1α inhibition induced by UVT3D<sup>C</sup> may occur, at least in part, as a result of increased translational shutoff. It has also been shown that SGs can directly impact HIF-1α accumulation. As mentioned previously PERK activated SGs can inhibit HIF-1α, and under extreme hypoxia, the SG nucleating proteins TIAR and TIA-1 can form SGs that also suppress HIF-1α expression (63). Therefore, future research investigating the role of SGs in HIF-1α inhibition is needed.

Apart from analyzing the stage of viral replication sufficient to inhibit HIF-1α, our data also demonstrated that UVT3D<sup>C</sup> induced inhibition of HIF-1α is dependent on proteasome-mediated degradation pathway early during infection, as seen in T3D<sup>C</sup> infected cells (30). Furthermore, this suggests that while UVT3D<sup>C</sup> may employ different mechanisms of HIF-1α inhibition, there are some commonalities between T3D<sup>C</sup> and UVT3D<sup>C</sup> infection. This is important since our data suggests that UVT3D<sup>C</sup> may be a better candidate for inhibiting HIF-1α in hypoxic tumors. While research has shown that MRV inhibits HIF-1α activity in vivo, the
UV-inactivated virus was unable to inhibit HIF-1α (32). It is curious that UV-inactivation works significantly better in vitro but does not show any inhibition in vivo. Since UV-inactivated virus cannot replicate and infect new cells after initial infection like wt MRV, this could explain why no inhibition was observed. Potentially for UV-inactivated virus to be useful in a clinical setting much higher titers of the virus would be necessary. Apart from UV-inactivation our data suggests the apoptosis inducing φ fragment may contribute minimally to HIF-1α inhibition, and therefore could be used therapeutically in hypoxic tumors. While the wt virus should be considered as the treatment of choice in most cancer patients with hypoxic tumors to lyse cells and inhibit the hypoxic response, it is important to consider that active viral therapy may not be amenable to all patients. Therefore, our work has provided further evidence for the use of MRV, UV-inactivated MRV or introduction of the φ fragment into the tumor to mitigate the detrimental effects associated with tumor hypoxia.

Material and methods

Cells, viruses, antibodies, and reagents. PC3 cells were maintained in F-12K nutrient mixture Kaighn’s modification medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals), and penicillin (100 I.U./ml) streptomycin (100 μg/ml) solution (Mediatech). L929 cells were maintained in Joklik modified minimum essential medium (Sigma-Aldrich) supplemented with: 2% fetal bovine serum, 2% bovine calf serum (HyClone), 2 mM L-glutamine (Mediatech), and penicillin (100 I.U./ml) streptomycin (100 μg/ml) solution. Our laboratory stock of MRV strain type 3 Dearing Cashdollar (T3DC) and type 3 Dearing Fields (T3DF) originated from the laboratory of B. N. Fields. The virus was propagated and purified as previously described using Vertrel XF (DuPont) instead of Freon (64, 65). Primary antibodies used were as follows: monoclonal rabbit α-HIF-1α antibody (BD Biosciences; #610958),
polyclonal rabbit α-β-actin antibody (Cell Signaling; #4967), polyclonal rabbit α-λ1 antibody (66), monoclonal mouse α-µ1 (4A3) antibody deposited to the DSHB by Dermody, T.S. (DSHB Hybridoma Product 4A3), and rabbit α-T3D antisera (67). Secondary antibodies used are Alexa 488- and 594-conjugated donkey α-mouse or α-rabbit IgG antibodies (Invitrogen Life Technologies; #A-21202, #A-21207), and goat α-mouse or α-rabbit IgG-AP conjugate antibodies (Bio-Rad Laboratories, #1706520, #1706518). MG132 (Enzo Life Sciences) was used at a concentration of 50 µM, NH₄Cl (Thermo Fisher Scientific) at a concentration of 20 mM, E64 (ApexBio Technology) at a concentration of 200 µM, α-chymotrypsin (Worthington Biochemical) at a concentration of 200 µg/ml, phenylmethanesulfonyl fluoride (PMSF) (MP Biomedicals) at a concentration of 5 µM, Z-VAD-FMK (Enzo Life Sciences) at a concentration of 20 µM, and docetaxel (Acros Organics) at a concentration of 3 µM.

**Hypoxia.** Cells were exposed to hypoxic conditions in a Galaxy 48R CO₂ Incubator (New Brunswick Scientific) equipped with 1-19% O₂ control set at 1% O₂, 5% CO₂ and 37°C. PC3 cells were exposed to hypoxia for 4 h (68).

**Infection, UV-inactivation, and ISVP production.** PC3 cells were infected with T3DC, T3DF or I595K at an MOI = 20 based on cell infectious units (CIU) as described (62). Virus was UV-inactivated by exposing wt virus to 1J/cm² of UV-irradiation (60). Since CIU is not possible in non-transcribing UV-irradiated virus we utilized the same number of viral particles, based on the calculation that one optical density unit at 260 nm = 2.1 x 10^{12} virions for each virus (50). ISVPs were produced by subjecting wt virus to α-chymotrypsin for 5 min followed by PMSF treatment to inhibit further cleavage.

**Immunoblotting and Coomassie.** PC3 cells or 1.6 x 10⁶ PFU of virus were lysed and harvested in 2x protein loading buffer (125 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4%
sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol), and subject to sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. Viral
proteins separated by SDS-PAGE were stained (0.25% Coomassie Brilliant Blue R-250 [Bio-
Rad Laboratories], 30% isopropanol, 20% methanol) and destained (40% methanol, 10% acetic
acid). Cellular proteins separated by SDS-PAGE were transferred to nitrocellulose by
electroblotting and nitrocellulose membranes were incubated in Tris-buffered saline (20 mM
Tris, 137 mM NaCl [pH 7.6]) with 0.25% Tween 20 (TBST) with primary and secondary
antibodies for 18 h and 4 h, respectively. Membranes were rinsed with TBST, treated with
NovaLume Atto Chemiluminescent Substrate AP (Novus Biologicals) and imaged on a
ChemiDoc XRS Imaging System (Bio-Rad Laboratories). Quantity One imaging software (Bio-
Rad Laboratories) was utilized to examine and quantify the intensity of protein bands.

**RNA extraction and electrophoresis.** 1.88 x 10^{11} viral particles of T3DC, TC, UVT3DC, and
UVTC were subjected to TRIzol LS (Life Technologies) extraction via the manufacturer’s
instructions. Briefly, viruses were homogenized in TRIzol LS, and the addition of chloroform
separated each sample into protein, DNA, and RNA phases. The RNA phase was collected, and
isopropanol and ethanol were added to precipitate and wash RNA. Extracted RNA was separated
on 10% SDS-PAGE at a constant 20 mA for 10 h, and the gel was incubated in water with 3X
gel red (Phenix Research Products) for 1 h and imaged on a ChemiDoc XRS imaging system
(Bio-Rad).

**Immunofluorescence.** At 24 h p.i., PC3 cells were fixed with 4% paraformaldehyde for 20 min,
permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum
albumin in PBS (PB5A) for 10 min with PBS washes between each step. Cells were then
incubated for 45 min at room temperature with primary antibodies diluted in PB5A and washed
two times with PBS, followed by incubation with secondary antibody diluted in PBSA for 45 min and two additional PBS washes. Coverslips with labeled cells were mounted with ProLong Gold antifade reagent with DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Invitrogen Life Technologies) on slides. Each coverslip was then examined on a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics to determine the number of cells expressing nuclear HIF-1α. Representative pictures were taken by a Zeiss AxioCam MR color camera using AxioVision software (4.8.2). Images were prepared using Adobe Photoshop and Illustrator software (Adobe Systems).

**Apoptosis detection.** Apoptosis induction by T3D^c^ and inhibition by Z-VAD-FMK was determined using the Caspase-Glo 3/7 Assay System (Promega). PC3 cells were plated at 1 x 10^4 cells per 96 well and 24 h later were mock-infected or infected with T3D^c^ or UVT3D^c^ and treated with or without Z-VAD-FMK. At 24 h p.i. media was removed and 50 µl fresh media and 50 µl Caspase-Glo Reagent was added to each well. After 30 min incubation at room temperature the 96 well plate was read on a Glomax Multi Detection Plate Reader (Promega).

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**References**


CHAPTER 5. MAMMALIAN ORTHOREOVIRUS INDUCED STRESS GRANULES PROVIDE A PLATFORM FOR HIF-1α AND RACK1 ASSOCIATION RESULTING IN DECREASED HIF-1α ACCUMULATION UNDER HYPOXIA

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Abstract

Mammalian orthoreovirus (MRV) is a cancer killing virus undergoing numerous clinical trials in cancer patients. The virus has been shown to be safe but has not been shown effective at killing all tumors. Therefore, research investigating the tumor type or tumor environments that MRV therapy is most amenable is necessary. Previous work has shown that MRV inhibits HIF-1α, the master transcriptional regulator under hypoxia that upregulates genes involved in growth, angiogenesis, and metastasis. In this work we investigated the role stress granules (SGs) play in HIF-1α inhibition. Stress granules are induced during MRV infection and were shown to recruit HIF-1α and RACK1 to the granule. RACK1 binds HIF-1α targeting it for degradation, suggesting that MRV induced SGs inhibit HIF-1α accumulation early during infection. This work provides further insight into the mechanism of MRV-induced HIF-1α inhibition, and evidence for the use of MRV in hypoxic tumor patients.

Introduction

It is common for mammalian cells to experience various stressors that result in cellular damage if not resolved. These stressors can include nutrient deprivation, oxidative stress, osmotic stress, heat shock, or viral infection and result in phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (1). eIF2α is one of three subunits that make up eIF2 which
is part of the ternary complex, comprising eIF2, GTP, and Met-tRNAi (2, 3). The ternary complex is responsible for recognizing the AUG start codon during ribosomal scanning. Ternary complex binds to the 40S ribosomal subunit along with additional initiation factors to form the 43S preinitiation complex (PIC) which is primed to bind mRNA (4). Once the 43S PIC binds to mRNA, now called the 48S PIC, it begins scanning for the start codon with the assistance of Met-tRNAi bound to eIF2 (3). Upon Met-tRNAi/AUG binding, the eIF2 is released leaving Met-tRNAi bound to the AUG. This release requires GTP to GDP hydrolysis by eIF5, and once eIF2 is released the 60S ribosomal subunit joins the 40S subunit and translation of the mRNA into protein can proceed (5). GTP is bound specifically by the eIF2α subunit and once it is hydrolyzed to GDP, eIF2α binds eIF2B within the catalytic site which helps facilitate GDP release so a new GTP can bind and return eIF2 into an active state (6, 7). When eIF2α is phosphorylated under conditions of stress the protein binds eIF2B within the catalytic site and the regulatory site inhibiting removal of GDP (7). Therefore, phosphorylated eIF2α results in an inactive eIF2 protein that cannot bind Met-tRNAi to form the ternary complex within the 43S PIC. When this occurs the 48S* PIC, which is a ternary deficient 48S PIC, is unable to begin scanning for the start codon and the proteins and ribosome remain stuck at the 5′ end of the mRNA, inhibiting translation (4). These stalled ribonucleoprotein (RNP) complexes accumulate within the cytoplasm and signal stress granule (SG) formation (8).

Stress granules are non-membrane bound inclusions that are nucleated by proteins G3BP1, G3BP2, TIA-1, or TIAR (9, 10). These proteins contain RNA recognition motifs that bind to the free RNA within the RNP and form the SGs to sequester the stalled RNP complexes (9, 11, 12). During viral infection these SGs form as a defense mechanism against infection. Through sequestration of translation factors and the ribosomal subunits into granules the cell
limits translation of the cell and of the infecting virus. Viruses have evolved mechanisms to disrupt these SGs to overcome cellular inhibition of translation. For instance, poliovirus and encephalomyocarditis virus utilize viral proteinases to cleave the SG nucleating protein G3BP1 to inhibit SG formation (13, 14). Additionally, mammalian orthoreovirus (MRV) disrupts SG early during infection via viral factory (VF) formation, which may benefit viral infection (15). This disruption results in SG associated proteins localizing around VFs, but the effect of this sequestration to the periphery is not currently understood (15, 16).

MRV is a clinically benign double-stranded RNA (dsRNA) virus that belongs to the Reoviridae family that contain pathogenic viruses including rotavirus, bluetongue virus, and African horse sickness virus (17). Study of MRV has been important to enhance our knowledge of these pathogenic viruses, but MRV is also being investigated as an oncolytic or cancer killing virus (18). Since MRV does not cause disease in healthy individuals, it is safe to administer to cancer patients, and in clinical trials intravenous delivery of MRV has been shown to efficiently reach and lyse tumor cells (19). Some patients have benefitted greatly from MRV therapy while others experience little benefit. These discrepancies between MRV therapy success and failure within various tumors are being investigated to determine which tumor types or tumor microenvironments are best suited for MRV therapy. One microenvironment of particular interest is tumor hypoxia, an area of low oxygen, which is common in solid tumors and results in more aggressive cancers that resist cancer therapeutics (20). Under tumor hypoxia the alpha subunit of hypoxia-inducible factor 1 (HIF-1α) accumulates, enters the nucleus, and increases the transcription of target genes involved in growth, angiogenesis, and metastasis (21, 22). Increases in HIF-1α in serum results in a more aggressive and worst prognosis for cancer patients, driving much research toward discovery of HIF-1α inhibitors (23, 24). Interestingly,
MRV has been shown to inhibit HIF-1α via RACK1-mediated proteasomal degradation and translational inhibition at early and late times during infection, respectively, under hypoxic conditions (25). This phenotype has been observed in vitro and in vivo, and has been shown to inhibit several downstream targets of HIF-1α transcriptional activation (25-29). Since MRV inhibits HIF-1α we have proposed that MRV oncolytic therapy should be extended to patients with high hypoxic tumor load, to kill the tumor as well as to inhibit the negative downstream effects of HIF-1α accumulation. Since hypoxia and HIF-1α inhibit many current therapeutics, the addition of MRV may also resensitize hypoxic solid tumors to these therapeutics. Therefore, to use MRV efficiently in hypoxic tumors it is important to understand the mechanism of viral inhibition of HIF-1α accumulation.

Previously we have identified that a step between viral capsid cleavage and transcription is sufficient to inhibit HIF-1α. Using virus mutants, and protein or dsRNA deficient viruses we further showed that the capsid proteins σ3, the apoptosis inducing μ1 protein, and viral dsRNA are not necessary for inhibition of HIF-1α during normal infection. In addition, UV-inactivated virus was shown to inhibit HIF-1α to a greater extent than wildtype (wt) virus, potentially due to increases in SG production in UV-inactivated MRV infected cells (30). Stress granules have been shown to sequester RACK1 and we reasoned since RACK1-mediated proteasomal degradation is necessary for HIF-1α inhibition in wt MRV infection, the sequestration of RACK1 to the SG or the release when SGs are disrupted could play a role in the inhibition of HIF-1α (31). In addition, there have been reports that SGs may increase or decrease HIF-1α accumulation further suggesting a role these granules may play in HIF-1α regulation. Therefore, in this work we investigated if SGs produced from viral infection were sufficient to inhibit HIF-1α at early times in infection. We identified that UV-inactivated virus and the T1L viral strain,
which induce more and/or prolong SG formation compared to the T3D\textsuperscript{C} strain, were able to inhibit HIF-1\textgreek{a} to a greater extent than T3D\textsuperscript{C}. Virus induced SGs were shown to sequester HIF-1\textgreek{a} and its binding partner RACK1 to the granule providing a mechanism for increased association and subsequent degradation of HIF-1\textgreek{a}. Lastly, we provided evidence that in cells unable to form canonical SGs, HIF-1\textgreek{a} was rescued in MRV infected cells early during infection. Altogether our data suggests that SGs are necessary for HIF-1\textgreek{a} inhibition at early times during infection and may provide a platform for RACK1/HIF-1\textgreek{a} binding and degradation of HIF-1\textgreek{a}.

Results

Increases in the number or duration of stress granules results in increased HIF-1\textgreek{a} inhibition. In previous work we reported that UV-inactivated MRV strain T3D\textsuperscript{C} (UVT3D\textsuperscript{C}) inhibits HIF-1\textgreek{a} accumulation significantly ($P < 0.05$) more than wt T3D\textsuperscript{C} in the prostate cancer cell line, PC3. Furthermore, we determined that UVT3D\textsuperscript{C} utilized proteasome-mediated degradation to inhibit HIF-1\textgreek{a} and did not require the outer capsid proteins $\sigma$3 or $\mu$1, or the dsRNA genome. To better understand the mechanism of T3D\textsuperscript{C} inhibition of HIF-1\textgreek{a} we looked to see if UVT3D\textsuperscript{C} was also able to inhibit HIF-1\textgreek{a} in additional cell lines. We utilized PC3s, the human osteosarcoma cell line U2OS, and the cervical cancer cell line HeLa. Each cell line was mock infected or infected with T3D\textsuperscript{C} at an MOI = 10 (U2OS) or 20 (PC3 and HeLa), or an equivalent number of UVT3D\textsuperscript{C} particles. After 20 h the cells were incubated under hypoxic (1% $O_2$, 5% $CO_2$, and 37°C) or normoxic conditions for 4 h at which time the cells were collected and subject to immunoblot analysis with antibodies against HIF-1\textgreek{a}, $\mu$NS, and $\alpha$-tubulin or $\beta$-actin. We observed that in each cell type the UVT3D\textsuperscript{C} was able to inhibit HIF-1\textgreek{a} accumulation suggesting this was not a cell specific response (Fig. 1A). UV-inactivated virus has been shown
Figure 1. UVT3DC and T1L inhibit HIF-1α to a greater extent than T3DC. (A) U2OS, PC3, or HeLa cells were mock infected or infected at an MOI = 10 (U2OS), or 20 (PC3 and HeLa) with T3DC or an equal number of viral particles of T3DC exposed to 1 J/cm² of UV-irradiation (UVT3DC). (B) PC3 cells were mock infected or infected at an MOI = 1 with T1L or T3DC. At 20 h p.i. all cells were exposed to normoxic or hypoxic conditions for 4 h and were collected for immunoblot analysis. Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus protein µ1 or µNS, HIF-1α, and β-actin or α-tubulin. (C) Immunoblots from (B) were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black and grey dots represent mock-infected replicates, red and green dots represent T1L or T3DC replicates, respectively, the black line represents the average and the blue lines represent the standard deviation. P values were determined using a two tailed Student t test comparison in Microsoft Excel.
to result in increased number and duration of SG formation in infected cells compared to wt virus, and since UVT3DC inhibits HIF-1α more efficiently we wondered if induction of SGs could play a role in HIF-1α inhibition (30). Work surrounding SGs has shown that various strains of MRV induce differing degrees of SGs, therefore we decided to investigate the extent by which the viral strains T1L, which induces more SGs than T3DC, inhibits HIF-1α compared to T3DC (30). PC3 cells were infected with T3DC or T1L at an MOI = 1, at 20 h p.i. the cells were incubated under hypoxia, CoCl2 treatment which mimics hypoxia, or normoxia and harvested at 24 h p.i. to be analyzed via immunoblot analysis (Fig. 1B). Immunoblot replicates were quantified and made relative to mock normoxic for each viral strain. We observed that T1L inhibited HIF-1α significantly more than T3DC infected cells while mock-infected cells at normoxic, hypoxic, or CoCl2 conditions showed no significant difference between immunoblots (Fig. 1C). In T1L infected cells more μNS was observed, indicative of more viral replication, but since cells were infected at the same MOI based on CIU the data suggests at the same MOI T1L inhibits HIF-1α to a greater extent. Together this data suggests that an increase in SG number or duration, as seen in UVT3DC or T1L infected cells compared to T3DC, early in infection results in greater HIF-1α inhibition by the virus.

HIF-1α and RACK1 localize to MRV induced stress granules. Stress granules are induced as early as 2 h p.i., but the virus disrupts and inhibits the formation of new SGs starting around 4-6 h p.i. (15, 30). Interestingly, certain SGs have been suggested to inhibit HIF-1α accumulation. During times of extreme hypoxia SGs nucleated by TIA-1 and TIAR have been shown to specifically inhibit HIF-1α, and therefore we reasoned that MRV-induced SGs may contribute to decreased HIF-1α accumulation (32). To investigate this possibility, we utilized U2OSΔΔG3BP1/2 cells which lack the SG nucleating proteins G3BP1 and G3BP2 and have
been shown to be unable to form canonical stress granules (33). U2OSΔΔG3BP1/2 cells were transfected with a plasmid expressing G3BP1 (pCI-G3BP1), to induce the formation of SGs, and then 20 h post-transfection (p.t.) the cells were exposed to hypoxia for 4 h. Cells were fixed and stained for immunofluorescence with antibodies against HIF-1α and G3BP1 to detect SG formation and HIF-1α accumulation and localization (Fig. 2A). Additionally, a subset of cells were transfected with pCI-G3BP1ΔRRMΔRGG, which is a G3BP1 mutant that cannot form stress granules, and in cells mock-transfected or with G3BP1ΔRRMΔRGG expression, HIF-1α accumulated under hypoxic conditions within the nucleus. In cells expressing G3BP1 and SGs, HIF-1α localized with the SG and little nuclear localization was observed. This data suggests that stress granules formed by overexpression of G3BP1 result in the recruitment and sequestration of HIF-1α to the SG. To determine if MRV-induced SGs also recruit HIF-1α, we infected U2OS cells with T3DC for 2 h with 4 h of hypoxia treatment. Antibodies against the SG marker TIA-1, the viral protein μNS, and HIF-1α were used and similar to SG induced by exogenous expression of G3BP1 we observed HIF-1α and μNS localized to viral induced SGs in infected cells (Fig. 2B, white arrows). Mock-infected cells on the other hand had no SGs and HIF-1α localized to the nucleus. Since SGs are induced and quickly disrupted during MRV infection we also infected cells with UVT3DC in hopes we would find more SGs with localization of HIF-1α, but instead found that HIF-1α did not accumulate anywhere within infected cells (data not shown). This is not surprising since UVT3DC inhibits HIF-1α significantly better than wt T3DC, and may suggest that at 6 h p.i. increased SG in UVT3DC infected cells inhibit HIF-1α accumulation more efficiently.
Figure 2. HIF-1α and RACK1 localize to T3D<sup>C</sup> induced SGs. (A) U2OSΔΔG3BP/2 cells were transfected with pCI-G3BP1 or pCI-G3BP1ΔRRMΔRGG, and at 20 h p.t. cells were exposed to normoxic or hypoxic conditions for 4 h and were fixed with paraformaldehyde. (B) U2OS cells were mock infected or infected at an MOI = 2 with T3D<sup>C</sup>, and at 20 h p.t. cells were exposed to normoxic or hypoxic conditions for 4 h and were fixed with paraformaldehyde. (C) U2OS cells were treated with SA (500 µM) for 1 h and were fixed with paraformaldehyde. (D) U2OS cells were mock infected or infected at an MOI = 2 with T3D<sup>C</sup> or an equal number of viral particles of T3D<sup>C</sup> exposed to 1 J/cm<sup>2</sup> of UV-irradiation (UVT3D<sup>C</sup>). All fixed cells were subject to immunofluorescence analysis with primary: α-HIF-1α, α-RACK1, α-TIA-1, α-G3BP1, or α-μNS antibodies, or α-core antisera followed by secondary: Alexa 594-conjugated donkey α-rabbit or α-goat, Alexa 488-conjugated donkey α-mouse, or Alexa 350-conjugated donkey α-rabbit IgG antibodies. A merged image is also shown with (A and C) and without (B and D) DAPI staining. Images are representative of the observed phenotype. Bars = 10 µm.

It was interesting to see HIF-1α localized at SGs since the HIF-1α binding protein, RACK1, has also been shown to localize to SGs (31). RACK1 binds to and ubiquitinates HIF-1α under normoxic and hypoxic conditions, targeting HIF-1α for proteasome-mediated degradation.
To demonstrate that RACK1 localizes to SGs in our system, we treated U2OS cells with the SG inducing drug sodium arsenite (SA) for one hour and then fixed and stained cells for immunofluorescence. We observed localization of RACK1 to the SGs, as shown by G3BP1 and RACK1 staining, in cells treated with SA while in mock-treated cells RACK1 and G3BP1 were diffuse throughout the cell (Fig. 2C). Since we observed strong localization of RACK1 to SA induced SGs we next looked to see if RACK1 localized to T3DC induced SGs. We infected U2OS cells with T3DC and UVT3DC, and 2 h p.i. the cells were placed under hypoxia for 4 h and then fixed and stained for immunofluorescence with antibodies against the viral core, RACK1, and TIA-1. In T3DC and UVT3DC infected cells we identified SGs that had RACK1 localization (Fig. 2D). We were unable to prove that cells treated with UVT3DC were infected due to limitations of detection of minimal viral core proteins, but SG formation seen in UVT3DC infected cells but not mock-infected cells (data not shown) gave us confidence that these cells were infected. Together these results suggest that HIF-1α and RACK1 localize to SGs induced by the virus early during infection and provide a mechanism by which MRV infection brings HIF-1α and RACK1 into close proximity to increase binding, ubiquitination, and degradation of HIF-1α.

**Early inhibition of HIF-1α is dependent on MRV induced stress granules.** If SG induction by MRV results in HIF-1α and RACK1 localization, binding, and degradation of HIF-1α, it stands to reason that without SGs the virus would be less efficient in inhibiting HIF-1α early during infection. Therefore, we decided to compare HIF-1α accumulation in U2OS and U2OSΔΔG3BP1/2 cells. We infected U2OS and U2OSΔΔG3BP1/2 cells with T3DC at an MOI = 2 or an equivalent number of UVT3DC particles, and 2 h p.i. cells were placed under hypoxia for 4-6 h at which point they were harvested for immunoblot analysis (Fig. 3A-B). Two biological
replicates demonstrate that T3DC and UVT3DC inhibited HIF-1α accumulation early during infection in U2OS cells, but both viruses were unable to inhibit HIF-1α in the U2OSΔΔG3BP1/2 knockout cells that lacked SG formation (Fig. 3A-B). To confirm this observation, we performed immunofluorescence assay of U2OS and U2OSΔΔG3BP1/2 cells infected with T3DC to count the number of infected cells in which HIF-1α was inhibited. Thirty infected cells were sampled from each cell type and the number of cells with and without SGs present and HIF-1α inhibition was collected (Fig. 3C). In infected U2OS cells lacking SG formation and U2OSΔΔG3BP1/2 cells, 7/7 and 29/30 of the cells, respectively, had no discernable inhibition in HIF-1α. In U2OS cells that did have stress granules 16/23 cells had no discernable inhibition of HIF-1α, but 7/23 observed a decrease in HIF-1α accumulation. Taken together this data suggests that MRV induced SGs facilitate the inhibition of HIF-1α accumulation in a population of cells, and in the absence of SGs HIF-1α cannot be inhibited early during infection.

**Figure 3. Stress granule formation is essential for T3DC induced HIF-1α inhibition early during infection.** (A) U2OS or U2OSΔΔG3BP1/2 cells were mock infected or infected at an
MOI = 2 with T3D\textsuperscript{C} or an equal number of viral particles of T3D\textsuperscript{C} exposed to 1 J/cm\textsuperscript{2} of UV-irradiation (UVT3D\textsuperscript{C}). At 2 h p.i. all cells were exposed to normoxic or hypoxic conditions for 4-6 h and were collected for immunoblot analysis. Cell lysates were run on SDS-PAGE, transferred to nitrocellulose and stained with antibodies against the virus protein μ1, HIF-1α, and β-actin. (B) Immunoblots were analyzed on Quantity One software to quantify HIF-1α/β-actin protein levels. Black dots represent each replicate, the black line represents the average and the blue lines represent the standard deviation. (C) U2OS or U2OS\textsuperscript{∆∆G3BP1/2} cells were infected at an MOI = 2 with T3D\textsuperscript{C}, and at 2 h p.i. cells were exposed to hypoxic conditions for 4 h. Cells were fixed with paraformaldehyde for immunofluorescence analysis with primary α-μNS and α-HIF-1α, and secondary Alexa 594-conjugated donkey α-rabbit and Alexa 488-conjugated donkey α-mouse IgG antibodies. The diagram represents 30 infected cells and whether there were SGs present and if HIF-1α was inhibited. SG (+) represents the number of cells out of thirty with SG formation, and SG (-) represents those without. An arrow pointing right (→) represents the number of cells with or without SGs that saw no inhibition of HIF-1α, and an arrow pointing down (↓) represents those with HIF-1α inhibition.

**Discussion**

HIF-1α accumulation and the hypoxic response result in tumors that are aggressive and resistant to many current therapies prompting researchers to find novel therapeutics that target HIF-1α (20, 24). MRV has already been shown to be a safe and effective oncolytic virus, and a potent inhibitor of HIF-1α and downstream gene activation, providing evidence for use of MRV as a specific therapy for patients with high hypoxic tumor load (25-29). In this work we provide further evidence for the mechanism of MRV induced HIF-1α inhibition. We have provided evidence that greater numbers and/or prolonged duration of SGs results in increased capacity to inhibit HIF-1α (Fig. 1). Through induction of exogenous and MRV induced SGs, we observed localization of HIF-1α and RACK1 to the granule (Fig. 2). Previous reports have shown that MRV inhibits HIF-1α via RACK1-mediated proteasomal degradation early during infection (25). Therefore, our data suggests SG formation and recruitment of HIF-1α and RACK1 brings these proteins in close proximity resulting in increased binding and subsequent decrease in HIF-1α accumulation. Lastly, we have shown that in the absence of SGs in U2OS\textsuperscript{∆∆G3BP1/2} cells
T3DC and UVT3DC infection is unable to inhibit HIF-1α early during infection (Fig. 3).

Together these results suggest that upon viral entry MRV induces SGs that bring HIF-1α and RACK1 into close proximity aiding in early inhibition of HIF-1α, and without SGs this early inhibition of HIF-1α is not possible.

While it is plausible that SGs recruiting HIF-1α and RACK1 into close proximity may result in HIF-1α ubiquitination and degradation, this observation does not explain differences observed later in infection at 12 or 24 h p.i. We have previously shown that in T3DC infected cells SGs are induced early during infection, but by 12 h p.i. less than 5% of T3DC infected cells still have SGs (30). Furthermore, at 12 h p.i. T3DC requires RACK1-mediated proteasomal degradation suggesting that RACK1/HIF-1α binding is not facilitated by SGs at this time.

Conversely in UVT3DC infected cells SGs remain in up to 40% of cells at 12 h p.i. suggesting that UVT3DC's inability to disrupt SGs like wt T3DC aids in HIF-1α and RACK1 binding and degradation further into the viral life cycle. It was interesting that in our experiments UVT3DC infected cells produced SGs that showed strong localization of RACK1 to the SG (Fig. 2E) while HIF-1α was not found accumulating in any UVT3DC infected cells at 6 h p.i. (data not shown). On the other hand, T3DC induced SGs were sparser, but they had HIF-1α and RACK1 localization to the granule. T3DC infected cells had RACK1 localization to a lesser degree compared to UVT3DC infected cells. This seems to suggest that UVT3DC induced stress granules may work more efficiently at recruiting RACK1 and HIF-1α resulting in enhanced decrease of HIF-1α accumulation early during infection. In addition, prolonged SG formation may lead to SG-induced inhibition of HIF-1α for a longer time during infection.

Still this does not address how SGs induced by T3DC contribute to HIF-1α inhibition early during infection (Fig. 3). MRV has been shown to disrupt SGs and within the past several
years it has become clear that the VFs assist in this disruption (15). VFs are made up of the viral matrix protein µNS which binds to most of the viral proteins resulting in large, cytoplasmic inclusions that localize around the nucleus. The VF is the site of viral transcription, translation, RNA assortment, core assembly/packaging, virion assembly, and replication, and without the VF viral replication is suppressed (16, 35-39). Recently it has been shown that the VF is able to disrupt MRV induced SGs and recruit SG associated proteins to the periphery of the factory (15, 16). This phenotype was mapped to an interaction between the viral σNS protein and G3BP1, which is required for peripheral protein localization of USP10, caprin1, TIA-1, and TIAR to the VF (15). We find this interesting for two reasons: first this suggests that VFs may recruit other SG localized proteins to the periphery, and second that VF recruitment of these proteins may mimic some SG functions in the cell. Since the VF sequesters G3BP1 and other SG associated proteins to the periphery, it stands to reason that HIF-1α and RACK1 may also be recruited to the VF. We may have observed such a phenotype when we noticed that at 6 h p.i. µNS, HIF-1α, and TIA-1 all localize within PC3 infected cells (Fig. 2B). At this stage of infection it looks like these are SGs that have recruited µNS and HIF-1α to the SG, but since this is only a brief snapshot it is also possible that the VFs are in the process of disrupting and sequestering SG associated proteins to the factory. This observation may suggest that the VF in T3DC infected cells may take the place of the SG in bringing HIF-1α and RACK1 into close proximity resulting in degradation of HIF-1α. Furthermore, in U2OSΔΔG3BP1/2 cells, the absence of G3BP1 inhibits SG formation as well as the recruitment of this and other SG associated proteins to the VF periphery (15). This could indicate that T3DC was unable to inhibit HIF-1α in these cells because the SG or VF did not increase RACK1/HIF-1α binding.
Second, even if HIF-1α and RACK1 are not localized to the VF periphery the VF may mimic SG function. Researchers have shown that SGs that have TIA-1 and TIAR aggregation under hypoxic conditions result in inhibited HIF-1α expression, and when TIA-1 and TIAR are knocked down with siRNA, HIF-1α accumulation is rescued (32). Furthermore, HIF-1α is suggested to possess a 3′ adenylate-uridylate-rich element (ARE) sequence which can be targeted by TIA-1 and TIAR-induced translational inhibition (32, 40). Since TIA-1 and TIAR are recruited to the periphery of VFs it seems plausible that they may still be able to inhibit translation of HIF-1α mRNA. Additionally, the SG inducing drug, thapsigargin, has been shown to inhibit HIF-1α through activation of PERK and SG formation that sequesters YB-1 to the granule. YB-1 has been shown to bind the 5′ end of HIF-1α mRNA to enhance translation and when it is localized at the SG, HIF-1α accumulation is inhibited (41). Altogether it is plausible that MRV disruption of SG and recruitment of SG associating proteins to the periphery of VFs acts as a mechanism for HIF-1α inhibition. Regardless of the role SGs play at later times during infection, our data suggests that SGs are essential for early inhibition of HIF-1α in MRV infected cells. This work and future research investigating the mechanism of SG inhibition of HIF-1α will provide further evidence for the use of MRV as a therapy against hypoxic tumors.

Materials and methods

**Cells, viruses, antibodies, and reagents.** HeLa, U2OS, and U2OSΔΔG3BP1/2 cells were maintained in Dulbecco’s modified Eagles’s medium (DMEM) (Invitrogen Life Technologies) supplemented with 1% MEM non-essential amino acids solution (HyClone) and PC3 cells were maintained in F-12K nutrient mixture Kaighn’s modification medium (Invitrogen Life Technologies). Additionally, each media was supplemented with 10% fetal bovine serum (Atlanta Biologicals), and penicillin (100 I.U./ml) streptomycin (100 μg/ml) solution
(Mediatech). Our laboratory stock of MRV strain type 3 Dearing Cashdollar (T3D<sup>C</sup>) and type 1 Lang (T1L) originated from the laboratory of B. N. Fields. The virus was propagated and purified as previously described using Vertrel XF (DuPont) instead of Freon (42, 43). Primary antibodies used were as follows: polyclonal goat α-TIA-1 (C-20) antibody (Santa Cruz; #sc-1751), polyclonal rabbit α-G3BP1 antibody (Novus; #NBP1-18922), monoclonal mouse α-RACK1 antibody (BD Biosciences; #610178), monoclonal rabbit α-HIF-1α antibody (BD Biosciences; #610958), polyclonal rabbit α-β-actin antibody (Cell Signaling; #4967), monoclonal rabbit α-α-tubulin (11H10) antibody (Cell Signaling; #2125), polyclonal rabbit α-μNS (4041) (30), monoclonal mouse α-μ1 (4A3) antibody deposited to the DSHB by Dermody, T.S. (DSHB Hybridoma Product 4A3), and rabbit α-core antisera (44). Secondary antibodies used are Alexa 350-, 488-, and 594-conjugated donkey α-mouse, α-rabbit, or α-goat IgG antibodies (Invitrogen Life Technologies; #A-21202, #A-21207, #A-10039, #A-11032), and goat α-mouse or α-rabbit IgG-AP conjugate antibodies (Bio-Rad Laboratories, #1706520, #1706518).

Cobalt chloride (CoCl<sub>2</sub>) was used at a final concentration of 500 μM, and sodium arsenite (SA) (Honeywell Fluka) was added to cells for 1 h at a final concentration of 500 μM before immunofluorescence assay.

**Hypoxia.** Cells were exposed to hypoxic conditions in a Galaxy 48R CO<sub>2</sub> Incubator (New Brunswick Scientific) equipped with 1-19% O<sub>2</sub> control set at 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C. Cells were exposed to hypoxia or the hypoxic mimicking drug CoCl<sub>2</sub> for 4-6 h (45).

**Infection.** Cells were infected with T3D<sup>C</sup> or T1L at various MOIs based on cell infectious units (CIU) as described (46). T3D<sup>C</sup> was exposed to 1 J/cm<sup>2</sup> of UV-irradiation to produce UVT3D<sup>C</sup>. Since CIU is not possible in non-transcribing UV-inactivated viruses, we utilized the same number of viral particles to compare between T3D<sup>C</sup> and UVT3D<sup>C</sup>.
**Immunoblotting.** PC3, HeLa, U2OS, or U2OSΔΔG3BP1/2 were lysed and harvested in 2× protein loading buffer (125 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol [DTT], 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol), and subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. Protein run on SDS-PAGE was transferred to nitrocellulose by electroblotting and the nitrocellulose membranes were incubated in Tris-buffered saline (20 mM Tris, 137 mM NaCl [pH 7.6]) with 0.25% Tween 20 (TBST) with primary and secondary antibodies for 18 h and 4 h, respectively. Membranes were rinsed with TBST, treated with PhosphaGLO AP (SeraCare), and imaged on a ChemiDoc XRS Imaging System (Bio-Rad Laboratories). Quantity One imaging software (Bio-Rad Laboratories) was utilized to examine and quantify the intensity of protein bands.

**Immunofluorescence.** At 6 h p.i., U2OS and U2OSΔΔG3BP1/2 cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) for 5 min, and blocked with 1% bovine serum albumin in PBS (PBSA) for 10 min with PBS washes between each step. Cells were then incubated for 45 min at room temperature with primary antibodies diluted in PBSA, and washed two times with PBS followed by incubation with secondary antibody diluted in PBSA for 45 min and two additional PBS washes. Coverslips with labeled cells were mounted with the ProLong Gold antifade reagent with or without DAPI (4,6-diamidino-2-phenylindole dihydrochloride; Invitrogen Life Technologies) on slides. Each coverslip was then examined on a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics and representative pictures were taken by a Zeiss AxioCam MR color camera using AxioVision software (4.8.2). Images were prepared using Adobe Photoshop and Illustrator software (Adobe Systems).
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References


CHAPTER 6. GENERAL CONCLUSION

Mammalian orthoreovirus has already been shown to be a cancer killing, oncolytic virus, but where it fits within a clinician’s tool belt of cancer therapies is yet to be determined. What tumor types, genetic profile, or microenvironments that MRV therapy will be most beneficial is still being studied. Within this dissertation we have worked to help clinicians answer these questions through investigation into the viral life cycle and the response of the cell, while focusing on tumor hypoxia.

We began this work investigating the VF which is the location of most of viral replication. Genetically modified MRV that expresses a tetracysteine tagged μNS protein was recovered to observe VF.s in live-cell microscopy. This recombinant virus was used to observe VF dynamics in the context of a live cell, which was previously not possible. VF.s are essential for viral infection, and in this work, we observed the role of microtubules in VF fusion and movement. While VF.s are able to move throughout the cell in the presence of the microtubule destabilizing drug nocodazole, they cannot fuse to one another to form larger VF.s. Additionally, the movement of VF.s in nocodazole treated cells was erratic, as opposed to directed and nuclear movement in cells not treated. Apart from improving our understanding of MRV infection, in the future this virus can also be used to follow viral infection within a tumor.

At the same time we were investigating VF.s we began examining the stage of viral infection that is necessary for HIF-1α inhibition. Using UV-inactivated virus that is transcription deficient, as well as endosomal protease inhibitors, we determined that a step between viral capsid cleavage and transcription is necessary. Removal or mutation of the outer capsid proteins σ3 and μ1, or the dsRNA, showed little to no rescue of HIF-1α, suggesting another component of the virus or breakdown of the endosome results in HIF-1α inhibition signaling. Furthermore,
UV-inactivated MRV inhibited HIF-1α to a significantly greater extent than wt MRV, but both viruses utilized proteasome-mediated degradation of HIF-1α. It was interesting to find that UV-inactivated virus inhibits MRV better, since UV-inactivated virus induces high amounts of SGs during viral infection. SGs have been suggested to regulate HIF-1α accumulation, suggesting that these MRV induced granules may play a role in HIF-1α inhibition within the cell.

Therefore, in the last study we examined the host response to MRV infection, through examination into SGs that are induced by the virus early during infection. When SGs were formed as a result of MRV infection, HIF-1α and RACK1 were found localized to the SG. Since RACK1 is a binding partner of HIF-1α this may increase RACK1/HIF-1α binding and subsequent degradation of HIF-1α. To further investigate this possibility, we looked to see if SGs were necessary at early times during infection for MRV-induced inhibition of HIF-1α.

When infection in the SG deficient cell line U2OSΔΔG3BP1/2 was compared to infection in the wt U2OS cells the data showed that MRV or UV-inactivated MRV was unable to inhibit HIF-1α in the absence of SG early during infection. This suggests that SG induction is necessary for early inhibition of HIF-1α, and may explain how UV-inactivated virus is able to inhibit HIF-1α more efficiently than wt virus.

Overall the data presented within this dissertation helps define the therapeutic potential of oncolytic mammalian orthoreovirus through examination of MRV infection and the host response. Future research is needed to fully elucidate the role the SG plays in HIF-1α inhibition, and to determine additional mechanisms of MRV-induced HIF-1α inhibition. This data provides further evidence for the use of MRV therapy in clinical trials that focus on patients with high hypoxic load.