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Uncovering the Role of Global mRNA Degradation during the Gammaherpesvirus Lifecycle

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Uncovering the Role of Global mRNA Degradation during the Gammaherpesvirus Lifecycle

By

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Abstract

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The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's Sarcoma-associated herpesvirus (KSHV) are very common in the human population and cause a number of diseases. During a lytic infection, these viruses block host gene expression through the global mRNA degradation activity of the viral proteins BGLF5 and SOX, in EBV and KSHV respectively. Despite significant advancements in understanding the mechanism of this process, termed host shutoff, little is known regarding the contribution of this activity towards the viral lifecycle, predominately due to experimental difficulties in studying these viruses. In this study, we aimed to uncover the role of host shutoff during a gammaherpesviral infection using the related murine gammaherpesvirus 68 (MHV68) as a model. Unlike EBV or KSHV, MHV68 has a tractable genetic system and readily available animal model. We first verified that the properties of host shutoff are conserved across KSHV, EBV, and MHV68. We then confirmed that host shutoff during MHV68 infection is executed by its SOX homolog, here termed muSOX. Deletion of muSOX from the viral genome resulted in a virus unable to replicate, likely due to the multi-functional nature of muSOX and its homologs. We therefore performed site-directed and random mutagenesis screens to identify mutants of muSOX which lacked host shutoff activity, but still retained its other known functions. We identified a single amino acid point mutant with the desired characteristics and introduced this mutant into the viral genome. The resulting virus caused significantly less mRNA degradation and host shutoff, yet was still viable for replication. By investigating the properties of infections with the mutant virus, we found host shutoff to be dispensable for lytic replication yet critical for latency establishment. These findings, for the first time, identify host shutoff as an important factor in the gammaherpesvirus lifecycle and link two distinct stages of the viral lifecycle, lytic replication and latency. Furthermore these findings suggest that the global manipulation of gene expression promotes a successfully lifelong gammaherpesvirus infection.

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Chapter 1:

Introduction

Host Shutoff

Viruses from diverse lineages often evolve common strategies to successfully overtake a host cell or organism. One such example is the widespread inhibition of host gene expression, also known as host shutoff, and multiple viruses carry out this phenomenon utilizing different strategies. For example, picornaviruses drive host shutoff by the proteolytic cleavage of the eukaryotic translation factors eIF4G and the cytoplasmic poly(A) binding protein. Viral messages escape this shutoff by using an alternative mechanism of translation in which the 5' untranslated region of the viral mRNA contains an internal ribosomal entry site, or IRES, which recruits the translation factors leading to efficient translation of the viral messages (120). Coronaviruses instigate host shutoff via a two pronged strategy in which the NSP1 protein binds to the 40S ribosomal subunit preventing translation of host messages and also degrades host mRNA (52). Influenzavirus also induces host shutoff (53). In the nucleus the viral polymerase clips the 5'cap of the host mRNAs and uses it to initiate viral mRNA synthesis, thus inhibiting host translation and inducing viral translation in one stroke (87). Thus host shutoff can be mediated through a wide variety of pathways.

The downregulation of gene expression is not specific to invading viruses. The intracellular bacterial pathogen *L. pneumophila* induces host shutoff by the activity of effectors injected into the host cell which inhibit protein synthesis. The effector proteins are believed to inhibit translation by modifying the elongation factor eEF1A in addition to other unknown mechanisms (29).

Host cells also stimulate downregulation of their own gene expression as a defense strategy to block invading pathogens. Innate immune signals upregulate type I interferon which induces the expression of protein kinase R (PKR). Recognition of double-stranded RNA (dsRNA), a common RNA virus replication intermediate, activates PKR which then phosphorylates the eukaryotic translation initiation factor 2-alpha (eIF2α) thereby inhibiting the translation of host and viral mRNAs (96). Not surprisingly viruses have evolved mechanisms to circumvent this host defense strategy. Most viruses avoid PKR induction by expressing products preventing PKR activation, such as inhibitory dsRNA, proteins which sequester dsRNA, or proteins which directly and indirectly block the PKR signaling cascade (96). In other cases, viruses have evolved to use this host defense response to their own advantage. Alphaviruses induce strongly activate PKR thereby inhibiting the translation of cellular messages. Viral messages escape PKR-mediated inhibition by a RNA structural element which recruits translation machinery to the viral messages thus bypassing the necessity of eIF2α (124).
For some viruses host shutoff is a consequence of viral gene expression strategies, such as influenzavirus cap snatching (87) or picornavirus translation factor cleavage to facilitate alternate mechanisms of ribosome engagement (120). For others, host shutoff can be a mechanism of immune evasion, such as coronaviruses where host shutoff serves to downregulate type I interferon (80). But often, the contribution of host shutoff towards the viral lifecycle is not apparent, in part due to the multifunctional nature of viral proteins and the lack of appropriate model systems. For example, the mechanism of host shutoff during a rhabdovirus infection has

been well characterized and is mediated by the alteration of translation initiation factors which viral mRNA overcomes through the direct recruitment of translation machinery via structural elements (13, 131); however, the contribution of host shutoff towards viral replication and pathogenesis is not well understood.

Herpesviridae

The Herpesviridae are a family of large double stranded DNA viruses infecting a wide range of organisms, from oysters to elephants (23, 91). Viral particles consist of a nucleocapsid core containing the genomic material surrounded by an enveloped membrane. The area between the envelope and the nucleocapsid core is the tegument and contains viral RNA and proteins which enter into a newly infected cell upon de novo infection (85). One of the defining features of herpesviruses is their ability to persist in a passive, latent state or undergo lytic replication upon infecting a host cell. During latency few, if any, viral genes are expressed, and the viral genome is maintained as an epichromosomal nuclear element which can remain in the nucleus throughout the life of the host cell or until external factors induce lytic cycle entry. On the contrary, during the lytic cycle herpesviruses express most of their genes in a tightly regulated progression to provoke viral replication and egress. The genes contributing to viral replication are broadly conserved across herpesviruses; however, those contributing towards latency establishment are more unique to specific viral clades (85).

The family Herpesviridae is divided into three subfamilies based on infectious characteristics and genomic conservation: the alpha-, beta-, and gammaherpesvirinae. Alphaherpesviruses were the first characterized and include the most well studied herpesvirus, herpes simplex virus type 1 (HSV-1). Evidence of herpes simplex infections date back to early civilizations, and indeed the herpesvirus family name originates from the greek word "herpes" meaning "to creep or crawl" in description of a herpetic skin lesion. Today, the majority of knowledge on the basics of herpesviral biology has come from research in HSV-1. This family also includes the most infamous herpesvirus, herpes simplex virus type 2 (HSV-2), commonly known as genital herpes, and varicella-zoster virus, the causative agent of chicken pox. The betaherpesviruses include the cytomegaloviruses, which are linked to several types of birth defects (74), and the ubiquitous human herpesviruses 6 and 7, common causes of rashes in infants (133). The gammaherpesviruses include Epstein-Barr virus (EBV/HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) which are linked to a number of lymphoproliferative disorders (30, 93).

Human Gammaherpesviruses

The gammaherpesviruses include the human pathogens EBV and KSHV. EBV is nearly ubiquitous in the human population with more than 90% of individuals testing positive for viral antibodies in their blood serum (45). EBV is the primary cause of infectious mononucleosis in young adults and is also linked to various lymphoproliferative diseases (18, 22). In most cases, individuals encounter EBV during their infancy or childhood, and the resulting infection is usually benign or manifests with cold-like symptoms. The virus then establishes latency in memory B-cells and is maintained in a latent state throughout the life of the host organism often causing no clinical symptoms. The virus constantly undergoes low levels of reactivation on the mucosal surfaces by which the virus spreads from host-to-host (41). Reactivating virus can reseed B-cell latency, but viral levels remain in equilibrium throughout the life of an infected host due to rapid clearance of lytically infected cells through a CD8+ T-cell response.

When contracted in young adulthood or later in life, infectious mononucleosis can ensue. Infectious mononucleosis, more commonly known as "mono" in the United States, develops predominantly due to a hyperactive immune response leading to fever, nausea, malaise, and swollen lymph nodes. These clinical symptoms stem from multiple pathways, such as cellular damage from replicating virus, high levels of circulating cytokines, the expansion of activated CD8+ T-cells which are required to clear replicating virus, and the expansion of B-cells harboring latent virus. Four to seven weeks post infection, replicating virus has been cleared and the immune response subsides (125).

In addition to infectious mononucleosis, EBV also is associated with several types lymphoblastoid cancers, such as Hodgkin's lymphoma, Burkitt's lymphoma, and post-transplant lymphoproliferative disease (18). These disorders result from the transformation of latent EBV infected B-cells. *In vitro* transformation of primary human B-cells occurs at a high frequency and is mediated by the latently associated EBV proteins along with the small non-coding EBER RNAs (54). *In vitro* derived lymphoblastoid cell lines express what is known as type 3 latency, where all nine of the latently associated genes can be detected, EBNA-1, -2, -3a, -3b, -3c, and –LP and LMP-1, -2a, and -2b, along with the several noncoding RNAs (106). Of particular interest is the LMP-1 protein, which upregulates NF-κB activity, thereby inducing B-cell survival and inhibiting lytic gene expression (8, 50). The EBNA-1 protein also plays a vital role in ensuring that the viral genome replicates and segregates with the host cells during multiple rounds of replication (99). *In vivo* the number of EBV gene products detected in cancer biopsies can be much less and transcriptional patterns generally fall into the following categories. During type I latency, only EBNA-1 can be detected, as exemplified by Brukitt's lymphoma cases. During type 2 latency, EBNA-1 is expressed along with LMP-1 and/or LMP-2, typical of Hodgkin's lymphoma biopsies. While *in vitro* experiments have provided much information as to the mechanism of B-cell transformation, the *in vivo* processes leading to disease remain poorly understood.

KSHV is found predominately in immunocompromised patients and thus is not as common as EBV. KSHV was first identified in HIV-AIDS patients during the middle of the AIDS epidemic in 1994 (10) and is the causative agent of Kaposi's sarcoma (KS), one of the most common AIDS-associated diseases. KS tumors contain a number of different cell types, but infected endothelial cells drive the tumor progression likely through the angiogeneic properties of the virus (30). KSHV also is associated with primary effusion lymphomas and multicentric Castleman's disease, lymphoproliferative disorders driven by transformation of latently infected B-cells, similar to some EBV diseases (30)

KSHV does not readily transform primary human B-cells *in vitro* as does EBV, thus the mechanisms of cellular transformation are difficult to compare. KSHV will establish latency in immortalized fibroblasts and endothelial cells (5). The latently expressed genes are not well conserved amongst EBV and KSHV and these two viruses have clearly evolved separate mechanisms by which to establish and maintain latency. In KSHV the dominant latent antigen is LANA, which is critical for episomal maintenance in the nucleus of latently infected cells (3), similar to the function of EBNA-1 in EBV although sharing no sequence homology (65). V-cyclin (ORF72) is a viral homolog to cellular cyclin D and can transform cells by overcoming cell-cycle arrest following *in vitro* transfection (116). Another latent gene v-FLIP (ORF71) also transforms cells upon over-expression (112), and similar to LMP-1 in EBV, potently activates NF-κB which induces B-cell survival and the inhibition of lytic gene transcription (8, 112)

Model Gammaherpesviruses

The gammaherpesviruses display strict host specificity, therefore research into the pathogenesis of EBV and KSHV has been hindered by lack of an animal model. Humanized mice have been developed and used to some extent, but these models are limited. Also, the human gammaherpesviruses default to the latent pathway upon de novo infection of cells in tissue culture, thereby inhibiting the robust, rapid expansion of large quantities of virus and generation of mutant viruses, which requires lytic cycle entry upon transfecting the viral-BAC genome into a permissive cell. The use of drugs to induce lytic cycle entry has overcome many of the problems outlined above (73), however drugs do not completely inhibit the latent pathway and the generation of viral mutants is still a developing field. Due to these deficiencies research in the related non-human viruses, such as herpesvirus saimiri, rhesus monkey rhadinovirus, and murine gammaherpesvirus 68, has greatly enhanced our knowledge of the gammaherpesvirus family.

Herpesvirus saimiri (HVS) was originally isolated from squirrel monkeys in 1968 and is closely related to KSHV (71). Following infection of primates HVS establishes latency predominantly within T-cells, and lymphoproliferative disorders of T-cell origin can develop (26). HVS shares some common latently associated ORFs such as v-cyclin and LANA (Orf73). However the primary driver of T-cell transformation is the unique STP gene which induces NF-κB expression, similar to the function of EBV LMP-1 and KSHV v-FLIP. Upon infection of permissive cells *in vitro* HVS predominately enters the lytic cycle unlike KSHV or EBV (26). HVS has limited use as a model system for human gammaherpesviruses due to its T-cell tropism; however, this property is commonly used to transform primary human T-cells *in vitro* (6).

Rhesus monkey rhadinovirus (RRV) was first isolated from rhesus macaques in 1997 shortly after the discovery of KSHV (19). Following an infection of immunocompromised rhesus monkeys, a proliferative B-cell lymphoma develops similar to multicentric Castleman's disease, making RRV an attractive model for this diseases (81). Similar to HVS, RRV enters the lytic pathway upon infection of cells *in vitro*. RRV is very closely related to KSHV with nearly all ORFs homologous between the two viruses, and only four genes from KSHV are absent from RRV, K3, K5, K7, and K12. Thus RRV has become a valuable tool in which to study KSHV pathogenesis; however, difficulties native to primate research still limits its widespread use.

Murine gammaherpesvirus 68 (MHV68) was originally isolated from bank voles in Czechoslovakia (7), but also infects other small rodents including laboratory mice (*Mus musculus)* (88). *In vitro* MHV68 enters the lytic cycle upon infection of permissive cells, similar to HVS and RRV. Infection of mice with MHV68 can lead to diseases similar to KSHV or EBV human diseases. Acute MHV68 infection is characterized by splenomegaly reminiscent to EBV-induced infectious mononucleosis in humans, but MHV68 splenomegaly differs in that a viral superantigen induces a unique Vβ4 $CD8^+$ T-cell proliferation not seen in EBV splenomegaly (109). Chronic MHV68 infection can cause lymphoproliferative disorders in BALB/C which resemble EBV and KSHV diseases in humans (113). In mice genetically deficient for gamma interferon signaling, infection can lead to arteritis (127), fibrosis (77), lymphoproliferation and malignancies (62, 113), underscoring the importance of this pathway in regulating persistent viral infection. Similar lymphoproliferative disorders have been observed in mice lacking the MHC class I molecule suggesting persistent viral replication may contribute to these diseases (118). MHV68 is well established as a model to investigate pathogenesis of gammaherpesviruses due to its tractable genetic system and common animal model with lots of available immunodeficient mutants (109).

Gammaherpesvirus Lifecycle

The lifecycle of MHV68 has been tracked following an intranasal infection through the use of a fluorescently-tagged virus (48, 109). This lifecycle, illustrated in Figure 1.1, is thought to mimic that of other gammaherpesviruses, although adherence to this model has not been experimentally proven. Upon intranasal infection the virus undergoes lytic replication in mucosal epithelial cells in the upper respiratory tract. From this point the virus travels to the lymphatic system where it establishes latency in resident cells inhabiting lymph organs, predominately B-cells within the spleen and cervical lymph nodes. Subsequently these cells undergo proliferation, thus expanding the pool of latently infected cells. The virus remains in this latent state in memory B-cells throughout the life of the host organism, undergoing constant low levels of lytic reactivation at the mucosal surface by which the virus spreads from host to host (109). The induction of the lytic cycle can be triggered by TLR signaling or other innate immune effectors (31).

The cellular fate following gammaherpesvirus infections are quite divergent, with human gammaherpesviruses predominately entering the latent pathway and other model viruses trending towards lytic cycle entry. The mechanisms which decide this fate are unknown, but recent work in KSHV suggests that a short bursts of genome-wide viral transcription occurs immediately after infection. Shortly thereafter transcription is silenced and latency is set (57). Likely the gene products of this short burst of transcription help decide the latent or lytic cellular fate.

Upon infecting a host cell *in vitro*, MHV68 defaults to the lytic pathway. The immediate early genes are first expressed, including RTA (Orf50) which is the primary gene responsible for lytic cycle induction. RTA drives the expression of many other viral genes and is often referred to as the "master regulator" (108). RTA is conserved amongst all gammaherpesviruses, but EBV contains an additional immediate early lytic promoter, ZTA (BZLF1). The immediate early genes induce the expression of the delayed early genes which prompt viral genome replication. Finally the late genes are expressed with most contributing towards the production of viral particles (20).

The mechanisms by which gammaherpesviruses establish latency are not completely understood, but certain pathways are conserved. Each of the gammaherpesviruses described above induces NF-κB through the activity of a latency-associated gene, although the genes responsible are not conserved. NF-κB enhances B-cell survival and inhibits the expression of lytic genes (106). In MHV68, the gene or genes responsible for NF-κB upregulation are not known, although the inhibition of this pathway in infected mice leads to inefficient latency establishment and persistence (58). Another commonality observed during latency establishment is the epigenetic silencing of the viral genome in which the methylation and histone deacetylation of viral genomes near lytic gene promoters transitions these genomic regions into a heterochromatic state and inhibits the expression of the lytic genes (40, 119). The mechanism by which this occurs remains unknown but the importance of this pathway is demonstrated by the altered latency establishment in mice genetically deficient for the methyltransferases DNMT3a and DNMT3b (38).

Herpesvirus-induced Host Shutoff

Both alpha- and gammaherpesviruses induce a prominent host shutoff phenotype during a lytic infection through the global degradation of mRNA (35), but while the strategies of host shutoff are similar, the viral host shutoff factors are distinct. The alphaherpesviruses encode a

ribonuclease termed VHS (U_L41) that degrades cytoplasmic mRNA (102, 135). VHS is the predominant effector of host shutoff during an infection, but a second factor, ICP27 (U_L54), also contributes by inhibiting the splicing of cellular messages (42, 43, 86, 98). VHS is under tight temporal regulation which helps drive the progression of the lytic cycle. VHS enters into an infected cell from the tegument in its active unbound form. In this state VHS quickly acts to degrade host mRNA. The mechanism by which viral messages escape activity of VHS is not clear, but evidence suggests that VHS can both degrade some viral messages and induce the translation of others (16). During later stages of the viral lifecycle, the virus encodes for an inhibitor of VHS, the late protein VP16, which binds to VHS (61).

HSV-1 and HSV-2 mutants lacking vhs replicate to near wild-type levels *in vitro*, but are severely attenuated following *in vivo* infection (56, 111). The severe attenuation appears to be a consequence of ineffective immune evasion. *In vitro* VHS has been linked to the downregulation of MHC class I and class II molecules (55, 121), and can inactivate dendritic cells (97). *In vivo* the attenuation of vhs-deficient HSV-2 is rescued during infection of mice lacking the interferon α/β receptor, suggesting that host shutoff plays an important role in inhibiting type I interferon signaling (79).

In the gammaherpesviruses, global mRNA degradation also causes host shutoff, but no VHS homolog exists (34, 95). Instead mRNA degradation is mediated by the DNA exonuclease protein; SOX (Orf37) in KSHV and BGLF5 in EBV. Similar to VHS, SOX and BGLF5 both possess *in vitro* ribonuclease activity (2, 9), although undiscovered cellular cofactors likely target the recruitment of these proteins to the messages to be degraded. In addition to degrading host mRNA, SOX, BGLF5, and VHS all induce the hyperadenylation of messages and the nuclear import of the cytoplasmic poly(A) binding protein, both of which are thought to enhance host shutoff (59, 64). Thus, different members of the herpesvirus family have independently evolved similar mechanisms to deplete the cellular transcriptome.

The SOX homolog is conserved across all families of herpesviruses where it plays an important role in viral genome maturation during lytic replication (69). Although the exact role of a DNase during replication is unknown, it is believed that the DNase is required to resolve branched replication intermediates which arise through recombination-dependent replication (132). In HSV-1, the SOX homolog is U_L12 and mutations in this gene lead to severe attenuation due to unresolved DNA intermediates and empty capsids (100). In the betaherpesvirus HCMV, the SOX homolog UL98 has a similar role, and a transposon mutagenesis screen has identified the gene as essential (101, 134). Trasposon mutagenesis screens in the gammaherpesvirus MHV68 have yielded varying results. Song et al. found the SOX homolog to be dispensable for viral replication (105), whereas Moorman et al. found the opposite (75). Upon further examination the mutant virus from Song et al. proved to be contaminated with wild-type virus (R. Sun, *personal communication*), therefore the SOX homolog appears to be important for viral replication in all herpesviral families.

Outline

Although host shutoff has been extensively characterized during human gammaherpesvirus infections, the contribution of this phenomenon towards viral fitness has not been elucidated, due largely to the difficulty in mutating these viruses as well as their restricted host range and lack of a common animal model. Based on observations in other viruses, it has been hypothesized that host shutoff plays a role in immune evasion or allocating cellular resources towards viral replication (64, 104). Here we have experimentally addressed these

proposed hypotheses and uncovered an important role of host shutoff during a gammaherpesvirus infection.

In this study we have developed MHV68 as a model for host shutoff in the human gammaherpesviruses and uncovered the mediator of host shutoff to be the SOX and BGLF5 homolog, here termed muSOX. We have generated a mutant version of MHV68 which lacks host shutoff activity and with this mutant have discovered an important role for this phenomenon during the viral lifecycle where host shutoff is dispensable for lytic replication, but contributes to efficient latency establishment within a host organism. These findings link two distinct phases of the viral lifecycle and indicate that the global manipulation of gene expression contributes to a lifelong gammaherpesvirus infection.

Figure
Figure 1.1

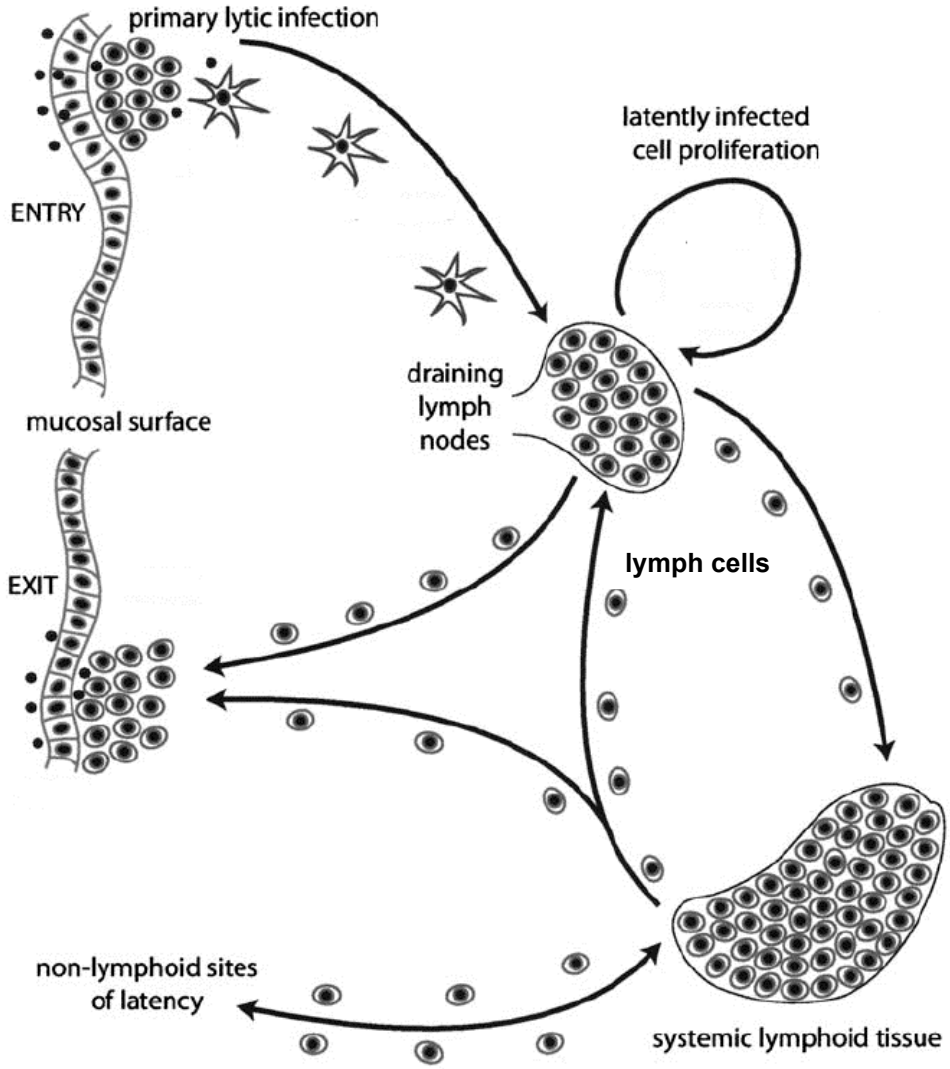

Figure 1.1 Current model of the MHV68 infection cycle. Following an intranasal infection lytic replication occurs in the lung and upper respiratory tract. The virus spreads to the lymph nodes where it establishes latency in memory B cells. The virus persists throughout the life of the host in latently infected B cells, with low level lytic reactivation occurring at mucosal surfaces. Adapted from Stevenson and Efstathiou, 2005 (109).

Chapter 2:

The Murine Gammaherpesvirus 68 Protein, muSOX, Induces Host Shutoff by Degrading mRNA in Addition to its DNA Exonuclease Activity

Background

The human gammaherpesviruses KSHV and EBV both cause host shutoff during a lytic infection through the degradation of cellular mRNA via the activity of the viral DNA exonuclease, termed SOX in KSHV and BGLF5 in EBV (34, 95). These genes are widely conserved across all herpesviruses and serve to resolve complex branched structures that arise during maturation of the viral DNA genome during lytic replication. Deletion of this gene from the alpha-, beta-, and gammaherpesviruses results in viruses severely attenuated, presumably due to the DNase activity role during replication (75, 100, 134). In addition to DNase activity, this protein has also evolved the separate mRNA degradation activity in KSHV and EBV which drives host shutoff. Although the mechanism of host shutoff has been characterized, the role of this activity during the viral lifecycle remains unknown. Both KSHV and EBV are difficult to genetically manipulate and the potential for *in vivo* studies is limited due to strict species specificity. Another gammaherpesvirus, MHV68, possesses a significantly more tractable genetic system and has proven to be a robust tool for the study of gammaherpesviruses, in both cell culture and murine models (107). MHV68 is therefore a potentially valuable model in which to assess the role of host shutoff in gammaherpesvirus replication and pathogenesis.

Here we demonstrate that MHV68 infection induces a global block to cellular gene expression which initiates early in infection, and is caused by the MHV68 SOX homolog, termed muSOX. All known activities of SOX and BGLF5 are mimicked by muSOX, underscoring a high level of functional conservation within the gammaherpesvirus subfamily. These results establish MHV68 as a new model to study the contributions of global inhibition of cellular gene expression towards viral replication and pathogenesis, and to analyze the mechanisms underlying gammaherpesvirus induced host shutoff.

Results

MHV68 Lytic Replication Induces a Shutoff of Host Gene Expression

Lytic infection with KSHV and EBV leads to a profound reduction in host gene expression driven by enhanced cellular mRNA turnover (34, 95), suggesting that host shutoff may be a phenotype conserved amongst gammaherpesviruses. We therefore sought to determine whether infection with the related murine herpesvirus 68 (MHV68) results in a similar reduction in host gene expression. Global protein synthesis was monitored by $^{[35]}$S-Cys/Met metabolic labeling of 3T3 murine fibroblasts either mock infected or infected with MHV68 (Figure 2.1A). At 21 h post infection we observed a clear reduction in overall cellular protein synthesis, indicative of host shutoff. Proteins accumulating at late times post infection are presumably viral, as many of these are not present in the uninfected sample. Both KSHV and EBV promote host shutoff by degrading mRNA (34, 95), therefore we assayed the levels of select host transcripts during a timecourse of MHV68 infection by Northern blotting (Figure 2.1B). By 12 h post infection, the level of GAPDH mRNA decreased 3-fold, and by 20 h the magnitude of its reduction increased to 5-fold relative to mock infected samples. To determine whether MHV68-induced host shutoff manifested as a result of early or late viral gene expression, we also infected cells in the presence of phosphonoacetic acid (PAA), which blocks viral genome replication thereby prohibiting the expression of late viral genes (82). PAA treatment had no effect on host shutoff, indicating that this phenotype is caused by an early viral gene(s).

In other gammaherpesviruses, the host shutoff function maps to the DNA exonuclease homolog. This protein, termed muSOX and encoded by ORF37 in MHV68, represents the most likely candidate for the MHV68 host shutoff factor. MuSOX shares significant homology with SOX and BGLF5 as demonstrated by alignment of the proteins (Figure 2.2). We generated and affinity purified polyclonal antibodies against muSOX to monitor its expression during an infection. Western blotting revealed muSOX expression to initiate approximately 8 h post infection and to peak by 12 h post infection (Figure 2.1C). In agreement with viral microarray data (20, 68), muSOX expression is uninhibited by PAA classifying it as an early gene (Figure 2.1C). Thus, muSOX expression coincides with the reduction in cellular mRNAs.

muSOX Induces mRNA Degradation

We hypothesized that, similar to SOX and BGLF5, muSOX could promote mRNA turnover. To test this hypothesis directly, we measured by northern blotting the mRNA levels of a GFP reporter in 293T cells co-transfected with empty vector or a muSOX expression plasmid. As predicted, northern blotting revealed a significant muSOX-induced reduction in reporter mRNA levels (Figure 2.3A). RNA half-life measurements performed in the presence of actinomycin D (ActD) subsequently confirmed that the decreased GFP mRNA levels in muSOX-expressing cells were a result of enhanced mRNA degradation, as the message half-life decreased from 55 h to 3 h in the presence of muSOX (Figure 2.3B). Given that muSOX possesses DNA exonuclease activity like its homologs (Figure 2.3C), we wanted to ensure these results were not an indirect effect of muSOX degrading GFP plasmid DNA. We therefore simultaneously measured the levels of the GFP reporter plasmid DNA and mRNA in the presence or absence of muSOX by qPCR (Figure 2.3D). Cells were treated with micrococcal nuclease just prior to lysis to remove any extracellular nucleic acid. Importantly, muSOX expression resulted in a significant decrease in GFP mRNA but not DNA levels, in agreement

with prior observations indicating that the analogous DNase activity of SOX does not contribute measurably to its host shutoff function (34).

To verify that muSOX-induced mRNA turnover was not just an artifact specific to the exogenously expressed GFP reporter, we also measured the half-life of endogenous GAPDH and β-actin mRNAs in cells transfected with muSOX or empty vector (Figures 2.3E & 2.3F). Indeed, both messages were degraded 4-8 fold more rapidly in muSOX-expressing cells. We anticipate this to be a conservative estimation of the rate of endogenous mRNA turnover by muSOX, because the ~20-30% of untransfected cells present in the muSOX samples will inflate the estimated mRNA half-life. We conclude that muSOX expression induces host shutoff via enhanced mRNA degradation and, thus, is a true functional homolog of SOX and BGLF5.

muSOX Is Necessary For Viral Replication and Host Shutoff

We next examined the contribution of muSOX towards MHV68-induced host shutoff. To this end, we sought to generate a MHV68 muSOX deletion mutant virus (MHV68.ΔmuSOX) and the corresponding marker rescue virus (MHV68.ΔmuSOX-MR) using a MHV68 bacterial artificial chromosome-based infectious clone (Figure 2.4A) (1). We were unable to recover any virus following transfection of the deletion mutant BAC genome into 3T3 cells, whereas the wild type and the marker rescue BAC yielded high viral titers (Figure 2.4B). This result was not unexpected, as the muSOX homologs HSV-1 U_L12 and EBV BGLF5 have been shown to be important for viral replication due to their role in viral genome processing (24, 36, 69). Indeed, we observed *in vitro* DNase activity for muSOX, as has been reported for its homologs (Figure 2.3C). This genome processing activity is presumably required for MHV68 replication, although host shutoff activity may also contribute to efficient virus production.

Failure of the MHV68.ΔmuSOX BAC to produce virus, coupled with low transfection efficiency of the BAC DNA, prevented measurement of endogenous cellular mRNA stability in the presence of this mutant. We therefore compared the half-life of the BAC-derived GFP message in BHK-21 cells transfected with MHV68.WT, MHV68.ΔmuSOX and MHV68.ΔmuSOX-MR BAC DNAs. To eliminate any complicating effects of viral DNA replication, experiments were conducted in the presence of PAA. Cells were treated with ActD at 18 h post-transfection and RNA was isolated at the indicated timepoints thereafter. Significantly, we observed a marked increase in the half-life of the GFP transcript in cells transfected with the ΔmuSOX BAC relative to WT and MR BAC-transfected cells (Figure 2.4C). Collectively, these data indicate that muSOX plays a prominent role in MHV68-induced host shutoff.

SOX Homologs Localize to Both the Nucleus and Cytoplasm

All herpesvirus DNase homologs examined to date localize predominantly or exclusively to the nucleus, in agreement with their roles in processing and packaging newly replicated viral DNA (37, 69, 89, 90). However, several lines of evidence suggest that cytoplasmic localization of the gammaherpesvirus SOX homologs plays an important role in host shutoff. First, KSHV SOX and EBV BGLF5 localize both to the nucleus and cytoplasm of cells, whereas the HSV-1 homolog, which lacks host shutoff activity, is constrained to the nucleus (34, 95). Second, a SOX nuclear localization signal mutant retains wild-type mRNA turnover activity in transfected cells (33). Finally, mRNA half-life studies demonstrated that cytoplasmic messages are destabilized in SOX-expressing cells (63).

We therefore predicted that MHV68 muSOX would also exhibit partial cytoplasmic localization, which might be integral to its host shutoff function. Given that plasmid-based over-expression does not always mimic natural protein localization, we monitored the localization of muSOX throughout the course of a viral infection with anti-muSOX polyclonal antibodies. The localization and kinetics of expression of muSOX were examined by immunofluorescence microscopy in 3T3 cells lytically infected with MHV68 (Figure 2.5). Expression of muSOX was detected beginning at 8 h post infection, in agreement with previous results for KSHV SOX (34), and was maintained throughout the lytic cycle. Importantly, muSOX localized to both the nucleus and the cytoplasm during viral infection, although the majority of muSOX exists in the nucleus (a magnified view of representative muSOX-expressing cells is shown in the bottom panels of Figure 2.5).

Discussion

In this report we have developed MHV68 as a model for gammaherpesvirus host shutoff. Previous studies have observed the down regulation of host gene expression following a lytic MHV68 infection yet did not explore the underlying mechanism. While measuring the transcriptional profile of the MHV68 genome, Ebrahimi et al. discovered severe downregulation of the host house-keeping gene transcripts (20). Stevenson et al. observed the downregulation of protein synthesis following MHV68 infection while searching for viral effectors which contribute to immune evasion (110). The authors identified the K3 gene as responsible for degrading the MHC Class I molecule, but did not link K3 to the global downregulation observed during an infection. In this study we identified the effecter of host shutoff to be muSOX.

The mechanism of host shutoff has been characterized in KSHV and EBV, and insights into the structure of SOX and BGLF5 indicated that the protein likely binds to DNA or mRNA and subsequently cleaves the substrate (2, 9, 15). *In vitro* these proteins indiscriminately cleave DNA and RNA, but the mechanisms determining specificity within a host cell remain unknown. From our studies SOX, BGLF5, and muSOX behave similarly in all assays performed (14), suggesting muSOX likely folds into a similar structure and functions in a similar manner as its homologs. Future experiments will uncover the degree of mechanistic conservation between these proteins and the cofactors which help mediate mRNA degradation.

In addition to mRNA degradation, muSOX also possesses DNA exonuclease activity which is important for viral genome maturation (69, 130). Because we observe no viral output following transfection of MHV68.ΔmuSOX-BAC, we surmise that DNase activity is important for viral replication, in agreement with the conclusions from the alphaherpesviruses experiments (100). Counter to our results, replication has been detected in EBV mutants with the BGLF5 gene disrupted, albeit at reduced levels compared to the wild type virus (24, 25). The results of these two experiments are difficult to compare due to different detection methods. To detect replicating MHV68 we quantified viral titers by plaque assays, in which a virus must carryout multiple rounds of replication. Feederle et al. detected EBV replication by methods independent of multiple replication cycles, such as qPCR, electron microscopy, and the primary infection of cells (24). Taken together these studies suggest that muSOX and its homologs are not necessary for viral genome replication yet they contribute towards efficient viral replication, likely through genome maturation carried out by the DNase activity. Fully assessing the contribution of DNase activity towards viral replication independent of any effect by host shutoff will require the generation of a mutant virus which lacks DNase activity yet retains mRNA turnover.

A notable difference between the muSOX homologs in alphaherpesviruses versus gammaherpesviruses is their subcellular localization; gammaherpesvirus SOX homologs are present in both the nucleus and the cytoplasm whereas the alphaherpesvirus homolog is exclusively nuclear. The gammaherpesvirus muSOX homologs likely interact with cellular components within the cytoplasm to target mRNA for degradation, but thus far no cellular proteins have been identified that physically interact with either SOX, BGLF5, or muSOX during infection.

The ability to severely restrict cellular gene expression is a conserved phenotype within the gammaherpesvirus subfamily, and therefore presumably plays an important role in viral replication and/or fitness. Analysis of the role of host shutoff during human gammaherpesvirus infections has been hampered by difficulties in generating and propagating KSHV and EBV mutants as well as viral host specificity. Our demonstration that host shutoff is phenotypically mimicked in MHV68 indicates that this virus presents a robust model to dissect the contribution

of host shutoff towards gammaherpesvirus replication and maintenance both in cultured cells and *in vivo*.

Materials & Methods

MuSOX PCR Mutagenesis

A hemagglutinin (HA) tag was introduced at the 5' end of muSOX by PCR using the primers 5'-CGGAATTCATGGCTTACCCATACGATGTACCTGACTATGCGATGGAAGGG TCGATTATTC-3' and 5'-ATAGTTTAGCGGCCGCTTAGGGGGTTATGGGTTTTCT-3'. HA-muSOX was then cloned into the EcoRI/NotI sites of pCDEF3 containing a T7 promoter to generate pCDEF3-T7-HA-muSOX.

Cells, transfections and viruses

293T, NIH 3T3, NIH 3T12, and human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BHK-21 cells (clone 15) were propagated in RPMI 1640 (Invitrogen) supplemented with 5% FBS. 293T cells were transfected with Effectene (Qiagen) following the manufacturer's protocol. BHK and 3T3 cells were transfected with SuperFect (Qiagen). BAC-derived MHV68 was generated by transfecting 2μg of BAC DNA per well of a 6-well plate of NIH 3T3 using SuperFect (Qiagen), then propagated in NIH 3T12 cells and titered by plaque assay on NIH 3T3 cells. To harvest virus, cells were passed through a dounce homogenizer and cellular debris was removed by pelleting and passing supernatant through a 0.45μm filter.

The green fluorescent protein (GFP)-expressing MHV68 bacterial artificial chromosome (BAC) infectious clone has been described elsewhere (1). Mutants were generated by allelic exchange as described previously (103). To generate the ΔmuSOX BAC, a targeting region consisting of a DsRed coding sequence flanked upstream by the terminal 979 nt of the ORF36 coding region (including the first 33 nt of the ORF37 coding region) and downstream by 756 nt of the 3' end of the ORF37 coding region followed by 178 nt of the ORF38 coding region was generated by Splicing by Overlap Extension PCR (SOE-PCR) (5'-GGAAGGGTCGATTATTCT GGATTTTTTTGAAGCCTCCTCCGAGAACGTCATC-3' and 5'-CGCCACCACCTGTTCCT GCCAGATAGTATTGTGTATGAAATAAAGTCCAGATATAAG-3'). The product was ligated into pGS284 between BglII and NotI sites and electroporated into the S17λpir strain of *E. coli.* The targeting vector for the rescue BAC was generated by ligating the WT region of the BAC into pGS284. Targeting vector-containing cells were cross-streaked with BAC-containing GS500 cells and successful recombinants were identified by colony PCR. BAC DNA was isolated from positive clones using the Qiagen Large-Construct kit (Qiagen). BAC variants were verified by diagnostic restriction digest with EcoRI and PvuII and sequencing of the region surrounding the recombination site.

Quantitative PCR

RNA was isolated from transfected cells using RNA-Bee reagent (Tel-Test) or the Zymo Mini RNA II Isolation Kit (Zymo Research). RNA samples were DNase-treated and reverse transcribed using AMV RT (Promega) and an oligo dT or 18S-specific primer. GFP mRNA copies were quantified by quantitative PCR on the resulting cDNA as described elsewhere (63). GFP cDNA copies were normalized to GAPDH cDNA copies using Rodent GAPDH Endogenous Controls or to 18S cDNA copies using Ribosomal Control Reagents (Applied Biosystems).

Plasmid DNA was analyzed for degradation by transfecting 293T cells with pCDEF3-GFP and pCDEF3 or pCDEF3-HAmuSOX. At 24 h post-transfection, cells were washed in PBS

and treated with micrococcal nuclease for 30 min at 37ºC (15 U/µl in 50 mM NaCl, 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$). The reaction was stopped by adding EDTA to 5 mM. DNA was isolated by lysing the cells in 20mM Tris, pH 8.0, 50mM EDTA, 200mM NaCl, and 1.2% SDS, followed by phenol/choloroform extraction. Samples were diluted to 0.15 µg/µl and quantitated by qPCR as above.

DNAse Assays

Proteins analyzed for DNase activity were in vitro transcribed and translated (IVT) using the Rabbit Reticulocyte Lysate system (Promega). From each reaction, 8 µl of protein was incubated with 200 ng of NotI-linearized pCDEF3 plasmid DNA in 42 µl of degradation assay buffer (0.1M $MgCl_2$, 0.5M Tris, pH 9.0, 100 µg/ml bovine serum albumin, 5mM β-mercaptoethanol) at 37ºC for the indicated time period and then phenol/chloroform extracted. Pellets were resuspended in 20 µl of water, resolved on a 1% agarose gel, and visualized by ethidium bromide staining. One-third of each IVT reaction was also resolved by SDS-PAGE, and gels were fixed, dried, and visualized by autoradiography to verify equivalent protein expression.

Cell Extracts, Western Blots, Northern Blots, and Immunofluorescence

For metabolic labeling experiments, infected 3T3 cells were incubated in methionine- and cysteine-free DMEM containing 10% FBS for one hour, then pulse-labeled in the same medium containing 0.1mCi/ml [^{35}S] EasyTag Express Protein Labeling Mix (PerkinElmer) for 30 min. For this and other experiments, cell lysates were prepared in RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 2mM EDTA, 1% Nonidet P-40, 0.1% SDS) containing Protease Inhibitor Cocktail (Roche) and quantified by Bradford assay (Bio-Rad). Equivalent masses of each sample were resolved by SDS-PAGE and either dried and exposed to film, or transferred to a PVDF membrane and Western blotted with either HA 12CA5 monoclonal antibodies (Invitrogen; 1:4,000) or muSOX polyclonal antibodies and either HRP-conjugated goat-anti-rabbit or goat-anti-mouse secondary antibodies (Southern Biotechnology Associates; 1:5,000). Rabbit polyclonal antisera (pAb) were raised against a maltose binding protein (MBP)-tagged full-length muSOX by standard methods (44). Serum was then affinity purified over MBP-muSOX and MBP columns.

For Northern blotting, total RNA was harvested using RNA-BEE (Tel-Test) and analyzed by agarose-formaldehyde gel electrophoresis. RNAs were transferred to a 0.45µm nylon membrane and probed with ^{32}P-labeled DNA probes generated using the Rediprime II random prime labeling system (GE Healthcare).

MuSOX localization was analyzed as described previously (59). Briefly, NIH 3T3 cells were grown on coverslips, and infected with MHV68. Cells were then stained with rabbit polyclonal anti-muSOX and AlexaFluor 546-conjugated goat anti-rabbit secondary antibody (1:1500). Coverslips were mounted in DAPI-containing Vectashield mounting medium (Vector Labs) to stain cell nuclei.

Figures

Figure 2.1

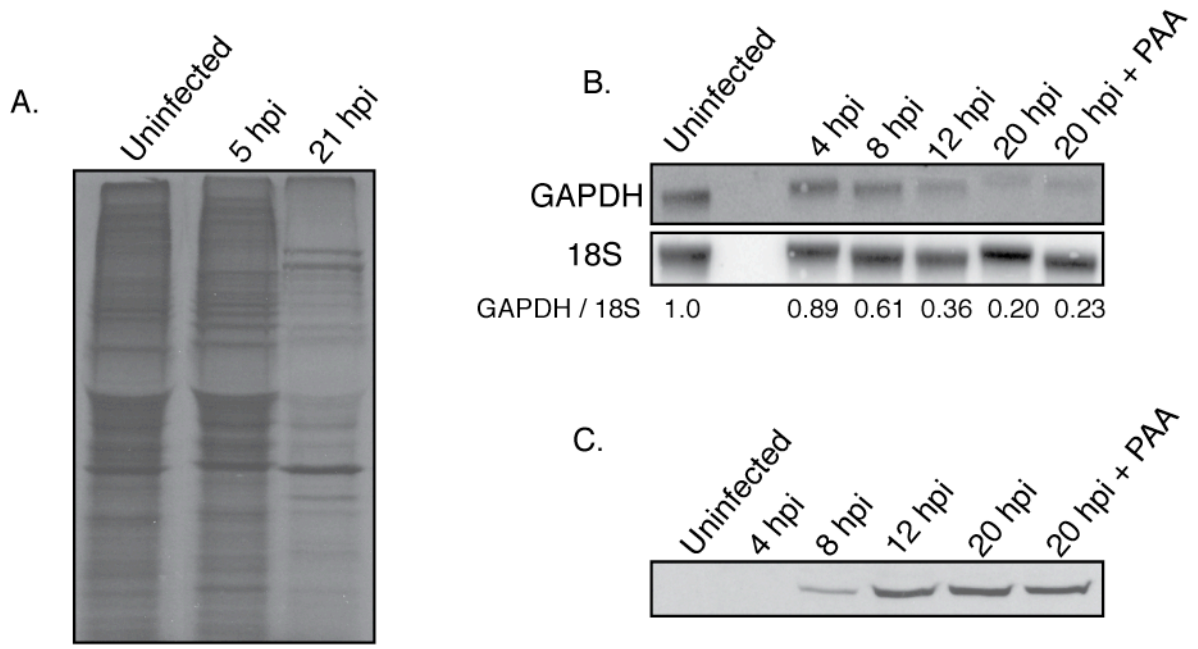

Figure 2.1 MHV68 promotes host shutoff. 3T3 cells were either mock infected or infected with MHV68 at an MOI of 10. (A) At the indicated times post-infection, cells were pulse labeled with $^{[35]}$S-Cys/Met and lysates were resolved by SDS-PAGE. The gel was then dried and visualized by autoradiography. (B) RNA was harvested from uninfected cells or cells infected for the indicated times and northern blotted with GAPDH and 18S (loading control) probes. Quantification (normalized to 18S levels) is shown, with the level of GAPDH mRNA in the uninfected sample set to 1.0. PAA indicates sample infected and cultured in the presence of 200 µg/ml phosphonoacetic acid. (C) Lysates were harvested from either uninfected cells or cells infected with MHV68 for the indicated times, and Western blotted with anti-muSOX antibodies. All figures are representative of three independent experiments.

Figure 2.2

Figure 2.2 SOX, muSOX, and BGLF5 protein alignment. KSHV SOX, MHV68 muSOX, and EBV BGLF5 proteins were aligned via ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical sequences are signified by an "*", and homologous sequences are signified by a "**.**" or "**:**".

Figure 2.3

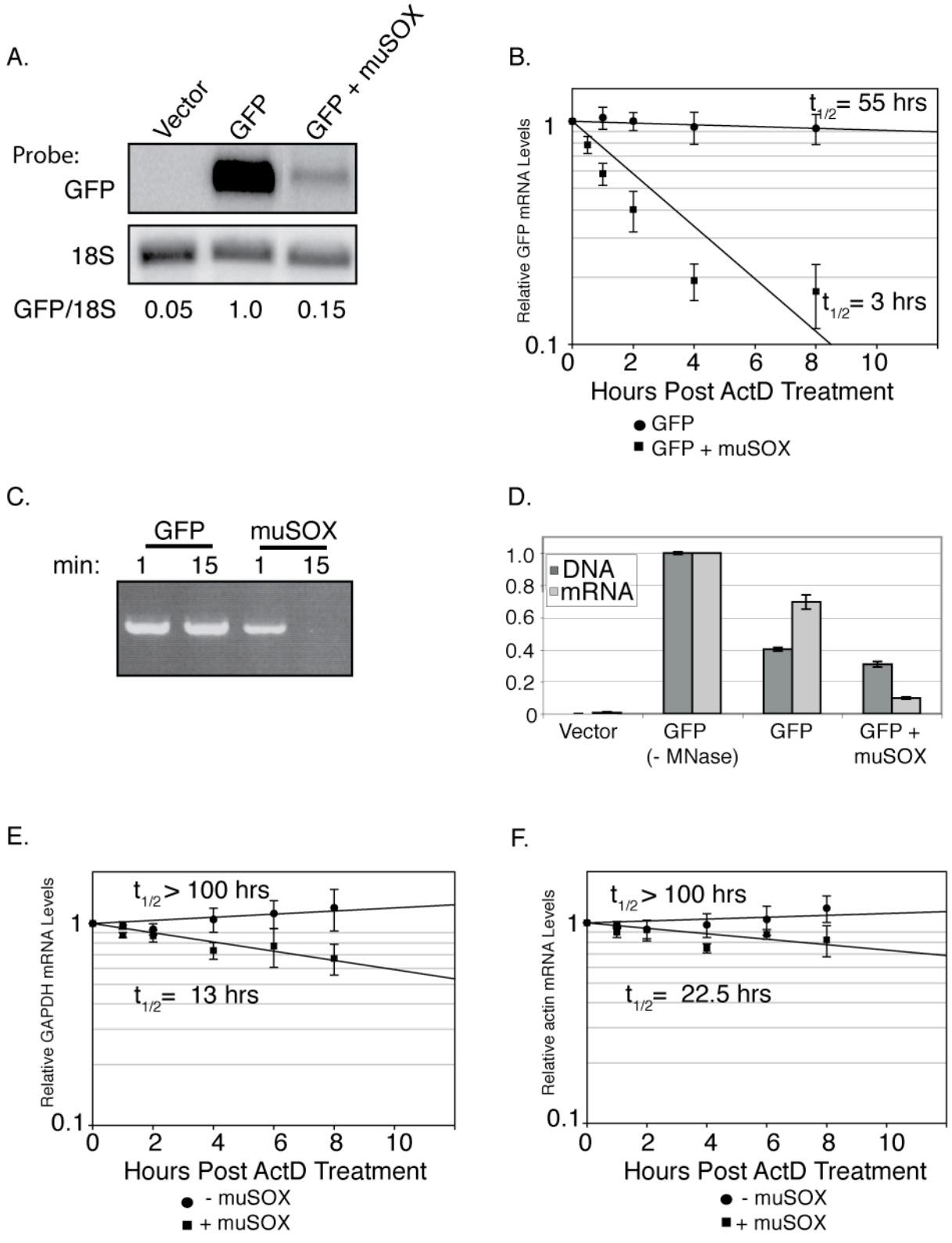
A.
Vector
GFP
GFP + muSOX
Probe:
GFP
18S
GFP/18S 0.05 1.0 0.15
B.
t1/2= 55 hrs
t1/2= 3 hrs
Relative GFP mRNA Levels
1
0.1
0 2 4 6 8 10
Hours Post ActD Treatment
GFP
GFP + muSOX
C.
GFP
muSOX
min: 1 15 1 15
D.
DNA
mRNA
1.0
0.8
0.6
0.4
0.2
0
Vector
GFP (- MNase)
GFP
GFP + muSOX
E.
t1/2> 100 hrs
t1/2= 13 hrs
Relative GAPDH mRNA Levels
1
0.1
0 2 4 6 8 10
Hours Post ActD Treatment
- muSOX
+ muSOX
F.
t1/2> 100 hrs
t1/2= 22.5 hrs
Relative actin mRNA Levels
1
0.1
0 2 4 6 8 10
Hours Post ActD Treatment
- muSOX
+ muSOX

Figure 2.3 MuSOX enhances mRNA degradation. (A) 293T cells were transfected with the indicated plasmids for 24 h. Total RNA was then isolated and analyzed by Northern blot with GFP and 18S probes. Quantification (normalized to 18S levels) is shown, with the level of GFP mRNA in the absence of muSOX set to 1.0. (B) Cells were transfected as described above, and GFP mRNA half-life was calculated via qPCR at different time points post actinomycin D (ActD; 2 μg/ml) treatment. (C) In vitro transcribed and translated muSOX or GFP (as a negative control) was incubated for 1 or 15 min with linear plasmid DNA in degradation assay buffer at 37°C. The DNA was then extracted, resolved by agarose gel electrophoresis, and visualized by ethidium bromide staining. (D) 293T cells were transfected with empty vector, or pCDEF3-GFP alone or together with pCDEF3-muSOX. With the exception of the –MNase control, all samples were treated with micrococcal nuclease prior to cell lysis to remove extracellular nucleic acid. Samples were then divided in half and either harvested for total cellular DNA or RNA. GFP DNA and mRNA levels were then calculated via qPCR. (E, F) Cells were transfected as described in A. Endogenous GAPDH (E) or β-actin (F) mRNA half-life was then calculated via quantification of Northern blots (normalized to 18S) at different time points post ActD treatment. Errors bars show the standard error between samples. All graphs represent a compilation of at least 3 independent experiments.

Figure 2.4

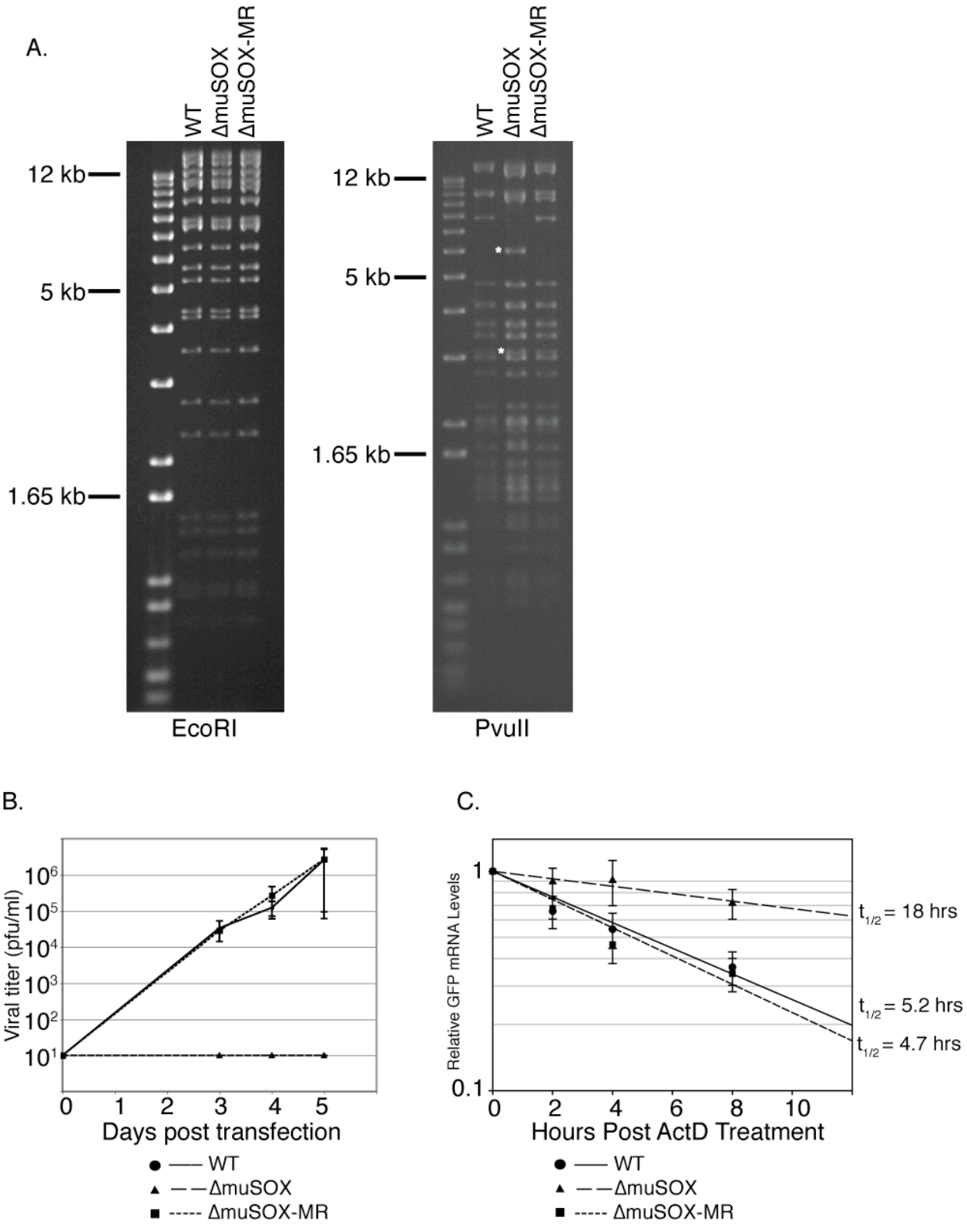
A.
WT
ΔmuSOX
ΔmuSOX-MR
12 kb
5 kb
1.65 kb
EcoRI
PvuII
B.
Viral titer (pfu/ml)
Days post transfection
WT
ΔmuSOX
ΔmuSOX-MR
C.
Relative GFP mRNA Levels
Hours Post ActD Treatment
t1/2 = 18 hrs
t1/2 = 5.2 hrs
t1/2 = 4.7 hrs

Figure 2.4 MuSOX is necessary for viral replication and host shutoff during infection. (A) EcoRI and PvuII digests of wild-type MHV68, MHV68.ΔmuSOX, and MHV68.ΔmuSOX-MR. In the PvuII digest, stars represent cleavage products generated as a result of introducing an additional PvuII site upon deletion of the muSOX gene. (B) Growth curve comparing viral titer following transfection of 3T3 cells with wild-type, MHV68.ΔmuSOX, and MHV68.ΔmuSOX-MR BAC DNA. Limit of detection is 10 pfu/ml. Data is a compilation of 3 independent replicates with the WT and deletion BACs, and 2 independent replicates with the marker rescue BAC. (C) The indicated BAC DNAs were transfected into BHK cells in the presence of 200 μg/ml PAA. BAC-derived GFP mRNA abundance was determined by qPCR at different times post-treatment with 5 μg/ml ActD. Errors bars show the standard error between samples from 3 independent experiments.

Figure 2.5

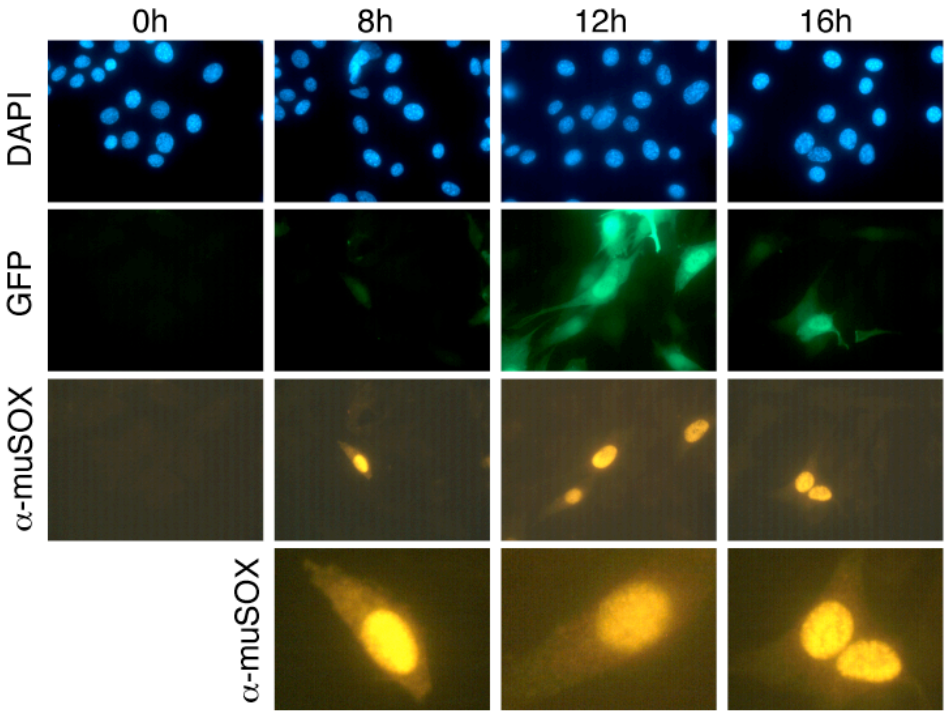

Figure 2.5 MuSOX localizes predominately to the nucleus but also the cytoplasm.
3T3 cells were infected with BAC-derived MHV68. At the indicated times, samples were subjected to immunofluorescence microscopy with anti-muSOX antibodies. GFP is encoded in the BAC, and serves as a marker of infection. Bottom panels show a magnified view of representative muSOX expressing cells at each timepoint. All samples were co-stained with DAPI to visualize nuclei.

Chapter 3:

The DNase and mRNase muSOX Activities are Genetically Separable and Differentially Expendable for Viral Replication *in vitro*

Background

Gammaherpesviruses elicit global mRNA degradation during a lytic infection, mediated by muSOX in MHV68 and its homolog in other viruses, leading to the shutoff of host gene expression. Removing the cytoplasmic mRNA pool induces nuclear import of the cytoplasmic poly(A) binding protein and hyperadenylation of nuclear messages, both of which are thought to increase the magnitude of host shutoff. MuSOX is a multi-functional protein, and in addition to its mRNA degradation activity it also possesses DNase activity, which is thought play an important role in viral genome maturation during lytic replication in all herpesvirus families. The DNase activity was originally characterized in the alphaherpesviruses, where seven highly conserved protein domains presumably come together during the three-dimensional folding of the protein and catalytically cleave DNA (36). Figure 3.1 shows an alignment of homologs from each family of herpesviruses with the seven domains highlighted, U_L12 from HSV-1, U_L98 from HCMV, and muSOX from MHV68. Except for motif V highlighted in blue, all of the domains share a high degree of conservation between the homologs from different herpesviral families. Although MuSOX homologs are found in all herpesviruses, only in the gammaherpesviruses do these proteins have the additional mRNA degradation activity. Single amino acid point mutations have been identified in SOX and BGLF5 which specifically remove mRNA degradation activity yet do not alter DNase activity (32, 136).

Despite advances in understanding the mechanism of host shutoff, the role of this activity in the gammaherpesvirus lifecycle remains unknown, mainly due to difficulties in genetic manipulation and lack of a small animal model for the human gammaherpesviruses. By generating a mutant version of MHV68 lacking mRNA degradation activity, we hope to uncover the role of host shutoff during a viral infection. Here we first identified a mutant version of muSOX lacking mRNA degradation activity but retaining the essential DNase activity. We then generated a virus with the mutant muSOX gene incorporated into the viral genome, and demonstrate that the resulting virus is defective for host shutoff. This virus replicated with similar titers as the wild-type virus, but unexpectedly displayed different plaque morphology. The results of these experiments for the first time assess the role of host shutoff and demonstrate that this activity is dispensable for gammaherpesvirus replication *in vitro*.

Results

Identification of a Host Shutoff Defective muSOX Mutant

We sought to evaluate the contribution of muSOX-induced mRNA turnover towards the gammaherpesvirus lifecycle through the generation of a muSOX mutant lacking mRNA turnover activity but retaining the conserved DNase function. We engineered mutations in the sites necessary for host shutoff activity in the muSOX homologs BGLF5 (136) and SOX (32) where possible, but surprisingly many of these amino acids are not conserved in muSOX (Figure 3.2). To search for other possible amino acid targets which could potentially contribute towards mRNA degradation, we identified amino acids conserved amongst all gammaherpesviruses but which lay outside the putative DNase domains (36). Multiple amino acids were identified, and we mutated those in regions of conserved stretches (Figure 3.2). Generation of muSOX Q127H yielded a mutant which was defective for DNase activity yet retained the host shutoff activity, as was reported for a similar mutant in SOX, Q129H (32). Unfortunately, none of the targeted mutations produced a muSOX mutant which lacked host shutoff activity, yet retained the essential DNase activity (Table 3.1).

We therefore designed an unbiased screen to investigate a large number of mutants following the methodology used to identify single-function SOX variants (Figure 3.3A) (32). Briefly, the muSOX gene was amplified by error-prone PCR under conditions designed to introduce 1-4 mutations per gene. The PCR product was then cloned into a mammalian expression vector, and clones were screened for the ability to repress expression of a GFP reporter in a fluorescence-based assay in 293T cells. Mutants that did not deplete GFP fluorescence were categorized as host shutoff defective and further analyzed for wild-type protein expression levels by western blotting, as even minor changes to the muSOX primary amino acid sequence often significantly reduced its protein expression. Finally, we tested candidate mutants for retention of DNase activity using an *in vitro* assay established previously for SOX and BGLF5 (32, 136). After screening approximately 150 candidates, we identified a clone that lacked mRNA degradation activity of the GFP reporter, but retained wild-type protein expression levels and DNase activity. Sequencing revealed this clone to have only a single non-silent mutation, the amino acid substitution R443M, which we modified to R443I for purposes of screening by restriction digest. MuSOX R443I failed to suppress GFP protein expression in the fluorescent-based assay for host shutoff (Figure 3.3B), which was a consequence of its inability to degrade GFP mRNA (Figure 3.3C). R443I is expressed at slightly higher levels than wild-type (WT) muSOX (Figure 3.3D), as expected of a mutant that cannot promote turnover of its own mRNA. Importantly, WT and R443I muSOX both degrade linear DNA with very similar kinetics (Figure 3.3E). We conclude that the amino acid substitution R443I produces a single-function muSOX mutant selectively defective for host shutoff activity, demonstrating that similar to BGLF5 and SOX, the DNA and RNA turnover activities of muSOX are genetically separable.

Generation of MHV68.ΔHS

To investigate the role of muSOX-induced host shutoff during viral infection, we engineered a host shutoff defective MHV68 virus by replacing WT muSOX with muSOX R443I using bacterial artificial chromosome (BAC)-based homologous recombination (1). The R443I mutation introduced an additional PsiI restriction site within the muSOX gene, allowing us to screen for successful recombination via restriction digest (Figure 3.4A). We also generated a mutant rescue (MR) virus by replacing the R443I mutant with WT muSOX to ensure that any

observed phenotypes could be attributed to the R443I mutation rather than to unexpected secondary mutations elsewhere in the viral genome. Restriction digests with PsiI and EcoRI confirmed that both recombinant viruses were successfully isolated and did not have any unexpected recombination events (Figures 3.4B & 3.4C). These viruses shall henceforth be referred to as MHV68.ΔHS (host shutoff defective virus) and MHV68.MR (mutant rescue virus). Sequencing of muSOX and the surrounding genomic regions in both recombinants confirmed that only the desired changes were introduced.

In vitro Characterization of MHV68.ΔHS

We next verified that the R443I mutation in MHV68.ΔHS conferred a host shutoff defect during viral infection. We assayed levels of several endogenous mRNAs by real-time quantitative PCR (RT-qPCR) following infection of NIH 3T3 fibroblasts with wild-type MHV68 (MHV68.WT), MHV68.ΔHS, or MHV68.MR. WT and MR infections reduced endogenous β-actin (actB), tubulin-β (tubb5), rplp2, and gapdh mRNA levels to 20-40% of the mock infected sample. In contrast, mRNA levels remained significantly higher in MHV68.ΔHS infected cells (Figure 3.5A-3.5D). Thus, the single amino acid change R443I in muSOX is sufficient to suppress virus-induced endogenous mRNA turnover. The somewhat lower endogenous mRNA levels in MHV68.ΔHS compared to mock infected cells could be due either to incomplete inactivation of the muSOX host shutoff function, or to the contribution of one or more additional viral genes towards host shutoff.

An additional phenotype linked to host shutoff caused by muSOX and its homologs is the relocalization of cytoplasmic poly(A) binding protein (PABPC) into the nuclei of infected cells (64). We observed clear nuclear relocalization of PABPC in cells infected with MHV68.WT, whereas in mock infected or cells infected with MHV68.ΔHS, PABPC remained cytoplasmic (Figure 3.5E). Thus, MHV68.ΔHS is defective for two hallmarks of host shutoff, mRNA depletion and PABPC relocalization. These results also demonstrate for the first time that muSOX-induced host shutoff is necessary to drive PABPC relocalization during a viral infection.

Previous studies with MHV68 mutants have shown that muSOX is critical for replication in cultured cells (14, 75). To determine the contribution of host shutoff activity towards this defect, we performed a multi-step growth curve in murine fibroblasts. 3T3 cells were infected at a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) per cell and MHV68.WT, MHV68.ΔHS, and MHV68.MR all replicated to equivalent titers and with similar kinetics (Figure 3.6). We sequenced the muSOX gene from MHV68.ΔHS samples at 5 days post-infection (dpi) and found no evidence for reversion or secondary mutations. Therefore, muSOX-dependent mRNA degradation had little effect on viral titers in 3T3 cells through multiple replication cycles.

MHV68.ΔHS Causes Larger Plaques to Develop and Increased Expression of Lytic Cycle Proteins

Interestingly, we noticed that plaques derived from MHV68.ΔHS infections differed morphologically from those obtained upon MHV68.WT or MHV68.MR infections (Figure 3.7A). To quantify these differences, we measured 75 plaques from 5 independent MHV68.ΔHS and MHV68.MR infections and found that, indeed, MHV68.ΔHS plaques were generally larger (p-value < 0.01) and had a broader frequency distribution (Figure 3.7B). This seemed unlikely to be a consequence of more rapid lytic replication, given the above growth curve results. In addition, the altered plaque size does not appear to be caused by enhanced cell-to-cell spread of

the mutant virus, as we observed no significant differences in the ratio of extracellular to intracellular virus produced in 3T3 cells infected with MHV68.WT, MHV68.ΔHS, and MHV68.MR (data not shown).

We and others have consistently observed that plaques formed upon infection with wild-type MHV68 are often quite heterogeneous in size. One possible explanation for this is asynchronous entry into the lytic cycle, in which smaller plaques arise from later entry into the lytic cycle. In this regard, we sought to determine whether the large plaque phenotype of MHV68.ΔHS might be due to enhanced entry into the lytic cycle, signified by an increased percentage of infected cells expressing early lytic proteins relative to the wild-type virus. To test this hypothesis, we acquired a version of MHV68 which constitutively expresses YFP from a CMV promoter (MHV68-YFP), regardless of whether infected cells are in the lytic or latent phase (12). YFP expression thus serves as a general marker of infection, whereas lytically infected cells are identified by immunofluorescence-based detection of the early lytic protein muSOX. 3T3 cells were infected at an MOI of 1 with either MHV68-YFP or a version of MHV68-YFP bearing the muSOX R443I mutation (MHV68-YFP.ΔHS), and the percentage of cells co-expressing muSOX and YFP was calculated at 18 hours post infection. A similar number of YFP-expressing cells were detected in cultures infected with MHV68-YFP and MHV68-YFP.ΔHS, indicating equivalent efficiency of initial infection. However, there was an approximately three-fold increase in the percent of cells co-expressing both YFP and muSOX following infection with MHV68-YFP.ΔHS compared to MHV68-YFP (30.1% versus 11.0%; Figure 3.7C). A similar trend was observed when the YFP- and muSOX-expressing cell populations were analyzed by flow cytometry following infection with these viruses (Figure 3.7D). Also in agreement with these findings were increased levels of the viral ORF54 transcript during a MHV68.ΔHS infection relative to MHV68.WT or MHV68.MR (Figure 3.8). Thus, enhanced and/or more rapid expression of lytic genes occurs in cells infected with MHV68.ΔHS, perhaps escalating entry into the lytic cycle and leading to larger plaques.

We also evaluated whether increased cell death might contribute to the larger plaques observed following a MHV68.ΔHS infection. We first measured necrosis by quantifying levels of lactate dehydrogenase released into the media at 16 hpi, but detected no measurable release following infection with any of the viruses (Figure 3.9A). We next monitored apoptosis by several assays, including measuring caspase activity, loss of plasma membrane symmetry, and increased membrane permeability. While there was a slight, but statistically significant, increase in caspase 3/7 activity following MHV68.ΔHS infection relative to MHV68.WT or MHV68.MR infection, in all three cases the level was below that observed in mock infected cells (Figure 3.9B), making it unlikely that this is a primary cause of the increased plaque size. Plasma membrane asymmetry was monitored using PE-conjugated Annexin V, which binds to a component of the plasma membrane normally found on the cytoplasmic surface, and membrane permeability was quantified by the nucleotide binding dye 7-Amino-Actinomycin (7-AAD). By flow cytometry we detected no significant increase in Annexin V binding or 7-AAD staining following infection with either MHV68.ΔHS or MHV8.MR relative to the uninfected control (Figure 3.9C). Collectively, these findings indicate that although a higher percentage of MHV68.ΔHS infected cells express viral lytic antigens, this does not appear to cause enhanced cell death, nor does it culminate in higher viral titers.

Discussion

Here we have identified a mutant of muSOX which lacks host shutoff activity and retains the DNA exonuclease activity. Modeling the muSOX three-dimensional structure from the KSHV SOX or EBV BGLF5 crystal structure (9, 15) positions amino acid R443 on the outer surface, away from the catalytic core where the cleavage of nucleic acids presumably takes place (data not shown). Although SOX, BGLF5, and muSOX are functionally homologous and likely induce mRNA degradation through a similar mechanism, the identified amino acid mutations that selectively remove host shutoff activity are not conserved, nor are they positioned in the same three-dimensional region. Likely these different mutations alter the local structure of each protein thereby inhibiting or weakening the mRNA or co-factor interactions which contribute to overall mRNA degradation.

Incorporation of the muSOX R443I mutant into the viral genome has allowed us for the first time to assess the role of host shutoff during a gammaherpesvirus infection. Host shutoff has been hypothesized to play a role in the diversion of gene expression resources and machinery towards the virus during lytic replication (14, 104). Our failure to detect a significant replication defect for MHV68.ΔHS during a multi-step growth curve in cultured murine fibroblasts implies that resource reallocation is unlikely to be the primary role for muSOX-induced mRNA depletion. However, because MHV68.ΔHS still retains residual mRNA degradation activity, it is possible that some degree of host shutoff could be important for viral replication. The results of these studies have shown that properties of host shutoff are conserved across all gammaherpesviruses studied to date. Furthermore using MHV68 as a model for dissecting the role of host shutoff, we have demonstrated that host shutoff is dispensable for viral replication in the gammaherpesviruses.

The mechanism of host shutoff has been well characterized in the gammaherpesviruses, yet how viral messages escape degradation remains an unanswered question. Recent experiments suggest that viral messages in fact do not escape muSOX-mediated degradation. We observed increased levels of Orf54 transcript following a MHV68.ΔHS infection relative to a wild-type infection (Figure 3.8), and similar results have been observed for nearly all of the viral transcripts. Higher levels of viral proteins correspond to increased transcript abundance (K. Clyde *personnel communication*). We hypothesize that increased lytic cycle entry could be linked to this phenomenon and host shutoff may be functioning to balance the level of viral protein expression in a cell in order to allow latency establishment.

Materials & Methods

PCR mutagenesis

MuSOX was randomly mutagenized by PCR with the Genemorph kit (Strategene) according to the manufacturer's protocol using 35 ng of pCDEF3-T7-HA-muSOX as template, the above primers, and 30 PCR cycles to generate a pool of random mutants. The mutants were then cloned into the EcoRI/NotI sites of pCDEF3-T7. R443I was generated by QuickChange (Stratagene) using the primers 5'-GCTCATCATCACTCCTGT TATAATTCCATCTACTGTGCTGC-3' and 5'-GCAGCACAGTAGATGGAATTATAACAGG AGTGATGATGAGC-3'.

Cells, Transfections, and Viruses

293T, NIH 3T3, NIH 3T12, and human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). 293T cells were transfected with Effectene (Qiagen) following the manufacturer's protocol. BHK and 3T3 cells were transfected with SuperFect (Qiagen). BAC-derived MHV68 was generated by transfecting 2μg of BAC DNA per well of a 6-well plate of NIH 3T3 using SuperFect (Qiagen), then propagated in NIH 3T12 cells and titered by plaque assay on NIH 3T3 cells. To harvest virus, cells were passed through a dounce homogenizer and cellular debris was removed by pelleting and passing supernatant through a 0.45μm filter.

The green fluorescent protein (GFP)-expressing MHV68 bacterial artificial chromosome (BAC) infectious clone has been described elsewhere (RγHV68A98.01 (1)), and mutants were generated by allelic exchange as previously described (103). To generate the MHV68.ΔHS BAC, a targeting region consisting of 566 nt upstream and 568 nt downstream of the mutation site was ligated into pGS284 between BglII and NotI restriction sites and electroporated into the S17λpir strain of *E. coli*. The MHV68-YFP BAC infectious clone was a generous gift from Dr. Samuel Speck (Emory University) (12), and MHV68-YFP.ΔHS was generated using the same methodology as MHV68.ΔHS. The targeting vector for the mutant rescue BAC (MHV68.MR) was generated by ligating the region around wild-type muSOX into pGS284. Targeting vector-containing cells were cross-streaked with BAC-containing GS500 cells and successful recombinants were identified by colony PCR and subsequent digest with PsiI. BAC DNA was isolated from positive clones using the Qiagen Large-Construct kit (Qiagen). BAC variants were verified by restriction digests with EcoRI and PsiI and sequencing of the region surrounding the recombination site. BAC-derived MHV68 virus was produced by transfecting 2 μg of BAC DNA into NIH 3T3 cells using SuperFect (Qiagen). Virus was then amplified in NIH 3T12 cells and titered by plaque assays on NIH 3T3 cells. Before infecting mice, the loxP-flanked BAC vector sequence was removed from the recombinant viruses by passaging the virus over Vero cells expressing Cre recombinase (kindly provided by Dr. Samuel Speck, Emory University) (76), and BAC removal was confirmed by PCR analysis.

Immunofluorescence Assays

MuSOX expression and PABPC or HA-muSOX localization were analyzed as described previously (14, 59). Briefly, NIH 3T3, HEK293T, or COS7 cells were grown on coverslips, and infected with MHV68 variants or transfected with HA-muSOX variants. Cells were then stained with rabbit polyclonal anti-muSOX (1:25 dilution), mouse monoclonal anti-PABPC 10e10 (1:25 dilution) (Santa Cruz Biotechnology), or mouse monoclonal anti-HA (1:500 dilution) (Abcam)

and AlexaFluor 546- or 488-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:1500). Coverslips were mounted in DAPI-containing Vectashield mounting medium (Vector Labs) to stain cell nuclei.

For flow cytometry analysis, NIH 3T3 cells were infected with the indicated virus and, 18 hpi, cells were washed with PBS and harvested via trypsin digestion. Cells were fixed in a 4% formaldehyde solution, permeabilized in 1% Triton X-100 and 0.1% sodium citrate in PBS, and incubated with anti-muSOX antibodies at a 1:12.5 dilution in 1% Triton X-100, 0.5% Tween, and 3% BSA in PBS. Stained cells were then washed in PBS and incubated with goat anti-rabbit antibodies conjugated to PE-Cy5.5 (Invitrogen) at a dilution of 1:150. Data were collected on an EPICS XL cytometer (Beckman-Coulter) and analyzed using FlowJo software (Tree Star).

Western Blots and Northern Blots

For Western blotting, cell lysates were prepared in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (vol/vol), 0.1% SDS (w/vol)] containing Protease Inhibitor Cocktail (Roche) and quantified by Bradford assay (Bio-Rad). Equivalent amounts of each sample were resolved by SDS-PAGE, transferred to a PVDF membrane, and Western blotted with anti-HA 12CA5 monoclonal antibodies (Invitrogen; 1:5,000) and HRP-conjugated goat anti-mouse secondary antibodies (Southern Biotechnology; 1:5,000).

For Northern blotting, total RNA was harvested using RNA-BEE (Tel-Test) and resolved by agarose-formaldehyde gel electrophoresis. RNAs were transferred to a 0.45 μm nylon membrane and probed with ^{32}P-labeled GFP DNA probes generated using the Rediprime II random prime labeling system (GE Healthcare).

DNase Assays

DNase activity of the muSOX variants was performed as described previously (14). Briefly, muSOX variants were *in vitro* transcribed with the mMessage mMachine T7 Kit (Ambion) and translated using rabbit reticulocyte lysates (Promega). Translational product was incubated with linear DNA at 37ºC for the indicated time period and then the DNA was phenol/chloroform extracted, and resolved by agarose gel electrophoresis. One-sixth each IVT reaction was also separated by SDS-PAGE. The gels were then fixed, dried, and visualized by autoradiography to verify equivalent protein expression.

Cell Death Assays

To measure levels of lactate dehydrogenase (LDH) released into the media during an infection, NIH 3T3 cells were infected at an MOI of 5 for 16.5 h. Infection supernatant (60μl) was mixed with 60μl LDH detection reagent in triplicate in a 96-well plate as described previously (17). Absorbance was read on an Elisa-reader at 490 nm wavelength. To detect caspase activity, NIH 3T3 cells were infected at an MOI of 5 for 24 h, whereupon caspase activity was measured using the Caspase-Glo 3/7 Assay System (Promega) following the manufacturer's protocol. To monitor Annexin V-PE and 7-AAD staining by flow cytometry, NIH 3T3 cells were infected at an MOI of 10 for 18 h. Cells were then washed with PBS and harvested via trypsin digestion. Infection supernatant, PBS wash, and cells were combined and pelleted by centrifugation. Cells were washed in PBS and then stained for Annexin V-PE (BD Biosciences, Material # 556422) and 7-AAD (BD Biosciences, Material # 559925) via the manufacturer's protocol using 3 μl of each dye. Data were collected on an EPICS XL cytometer (Beckman-Coulter) and analyzed using FlowJo software (Tree Star).

Figures
Figure 3.1

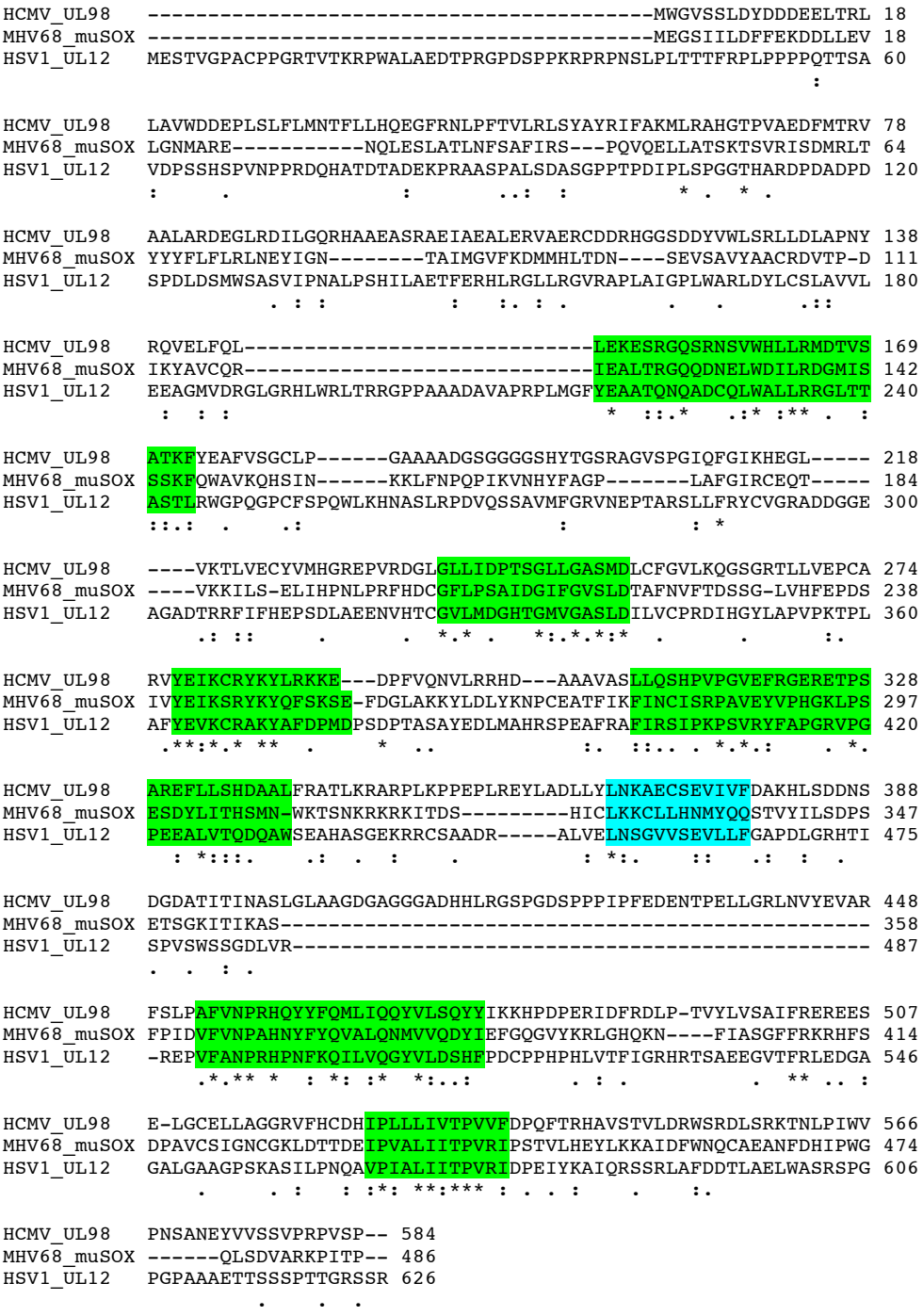

Figure 3.1 Protein alignment of the DNase homologs from HCMV, MHV68, and HSV-1. HCMV U_L98, HSV-1 U_L12, and MHV68 muSOX proteins were aligned via ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical sequences are signified by an "*", and homologous sequences are signified by a "**.**" or "**:**". Highlighted are the regions originally

designated as important for DNase activity in HSV-1 U_L12 (36).

Figure 3.2

Figure 3.2 Targeted mutational analysis of muSOX. Shown is the amino acid sequence of muSOX. Highlighted in green are amino acids which are conserved amongst the gammaherpesviruses but lay outside the putative DNase domain (36). In bold, capital letters are amino acids that were identified as important for host shutoff but not DNase activity in the muSOX homologs EBV BGLF5 (136) or KSHV SOX (32). Many of these sites are not conserved in muSOX. Asterisks (*) below an amino acid indicate sites which we targeted for site-directed mutagenesis. Q127 is within the conserved DNase domain and was targeted to generate a mutant lacking DNase activity yet retaining mRNA turnover.

Figure 3.3

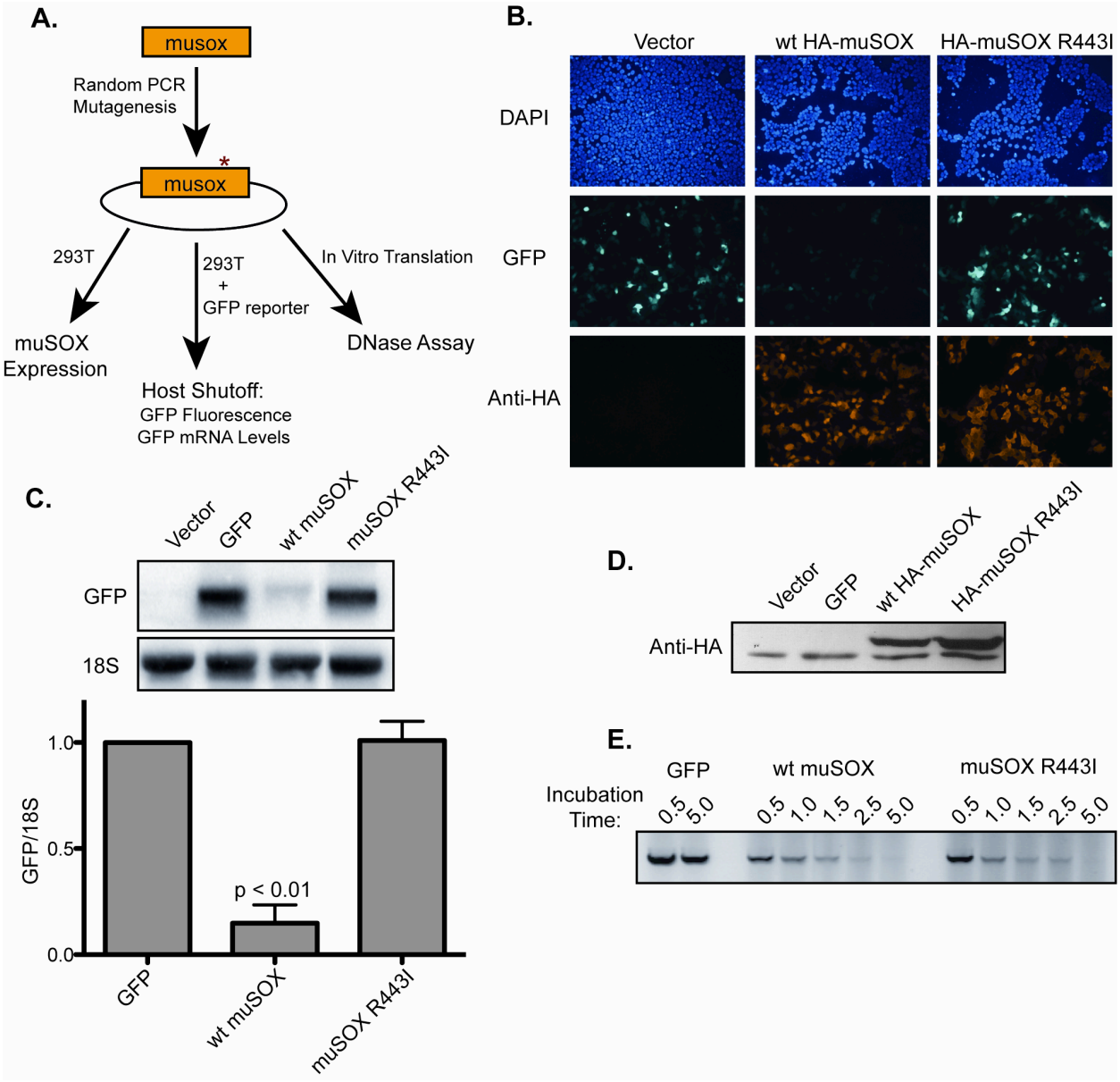
A.
musox
Random PCR Mutagenesis
musox
293T
293T + GFP reporter
In Vitro Translation
muSOX Expression
Host Shutoff: GFP Fluorescence GFP mRNA Levels
DNase Assay
B.
Vector
wt HA-muSOX
HA-muSOX R443I
DAPI
GFP
Anti-HA
C.
Vector
GFP
wt muSOX
muSOX R443I
GFP
18S
GFP/18S
1.0
0.5
0.0
p < 0.01
GFP
wt muSOX
muSOX R443I
D.
Vector
GFP
wt HA-muSOX
HA-muSOX R443I
Anti-HA
E.
GFP
wt muSOX
muSOX R443I
Incubation Time:
0.5
5.0
0.5
1.0
1.5
2.5
5.0
0.5
1.0
1.5
2.5
5.0

Figure 3.3 Isolation of the single-function muSOX mutant R443I. (A) Diagram of the random PCR mutagenesis screening strategy. (B) HEK293T cells were transfected with a plasmid expressing GFP alone or together with a plasmid expressing HA-tagged wild-type (wt) muSOX or muSOX R443I. GFP fluorescence was monitored 24 h post transfection, along with HA-muSOX, which was detected by immunofluorescence with anti-HA antibodies. Samples were co-stained with DAPI to visualize nuclei. (C) Cells were transfected for 24 h as described above, whereupon GFP mRNA and 18S rRNA levels were analyzed by northern blot. Shown is a representative northern blot for GFP mRNA and 18S rRNA, and below is the normalized data from five independent experiments with means and standard deviations shown. (D) Total protein was harvested 24 h post transfection with the indicated plasmids, and western blotted with anti-HA antibodies. The bottom band present in all samples is due to non-specific binding of the antibody. (E) *In vitro* translated GFP, wild-type muSOX, or muSOX-R443I was incubated with linear DNA for the indicated times. DNA was then extracted and separated by agarose gel electrophoresis. A representative figure from three independent experiments is shown.

Figure 3.4

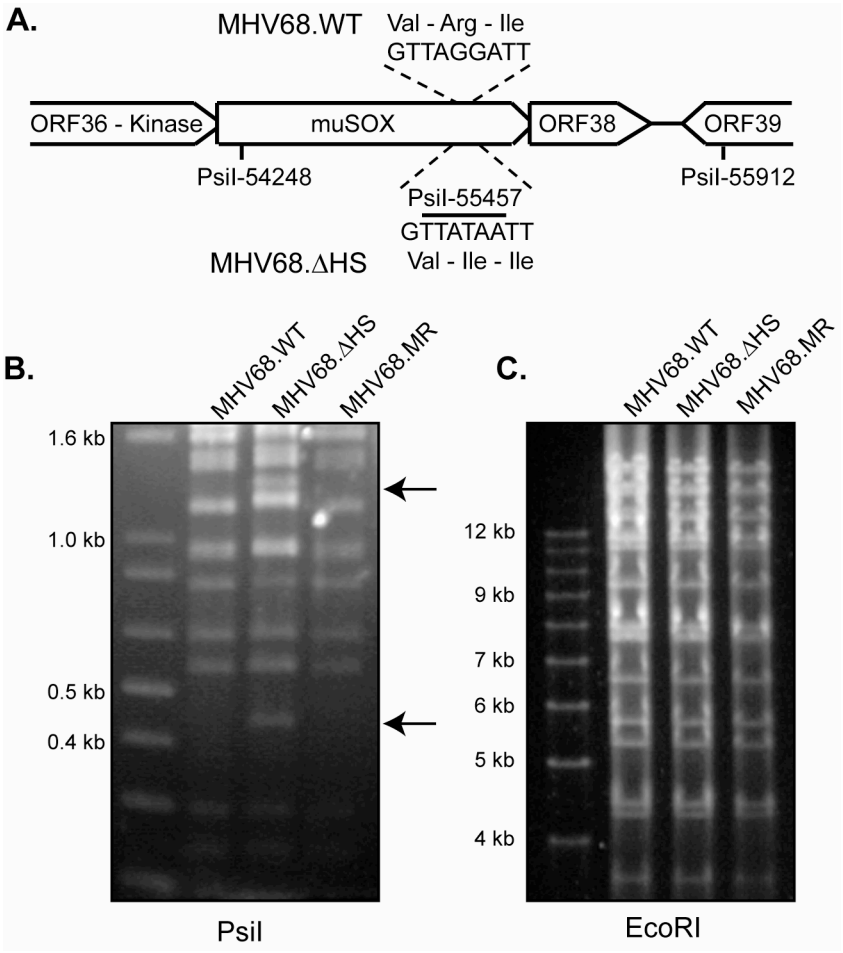

Figure 3.4 Generation of the MHV68.ΔHS virus. (A) Outline of the strategy for generating MHV68-muSOX-R443I (MHV68.ΔHS), in which the muSOX amino acid arginine at position 443 was mutated to isoleucine, creating an additional PsiI site. The number is derived from the sequenced MHV68 genome (126) (Refseq: NC_001826). (B) MHV68.WT, MHV68.ΔHS, and MHV68.MR BAC DNA were digested with the enzyme PsiI and subsequently resolved on an agarose gel to confirm successful generation of the desired mutants. Arrows indicate the new 1200 bp and 450 bp bands generated after introduction of the PsiI restriction site in muSOX R443I. (C) MHV68.WT, MHV68.ΔHS, and MHV68.MR BAC DNA were digested with the enzyme EcoRI and subsequently resolved on an agarose gel to confirm no unexpected recombination had occurred.

Figure 3.5

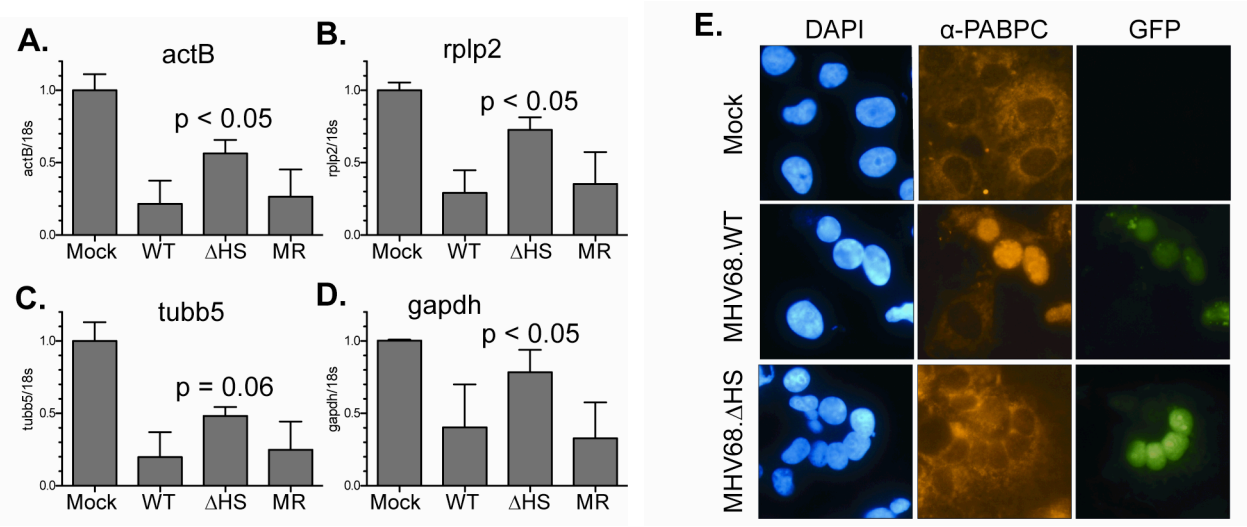

Figure 3.5 MHV68.ΔHS is defective for host shutoff. RNA was isolated from 3T3 cells infected with MHV68.WT, MHV68.ΔHS, or MHV68.MR at an MOI of 10 at 20 h post infection. ActB (A), Rplp2 (B), Tubb5 (C), GAPDH (D) and 18S RNA levels were quantified via RT-qPCR. The mean and standard deviation of the normalized mRNA/18S ratio is plotted for at least four independent experiments. The p-values comparing MHV68.ΔHS and MHV68.MR are indicated. (E) COS7 cells were infected with MHV68.WT or MHV68.ΔHS for 24 h. Both viruses express GFP from the BAC vector sequence, which serves as a marker for infection. PABPC localization was monitored by immunofluorescence with anti-PABPC antibodies, and cells were stained with DAPI to visualize nuclei.

Figure 3.6

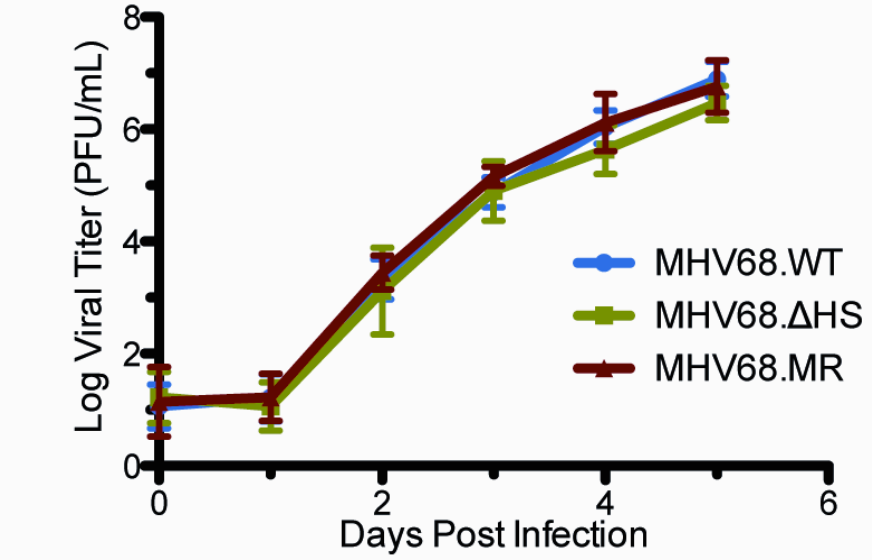

Figure 3.6 MHV68.ΔHS replicates with wild-type kinetics. Viral replication kinetics were determined by multi-step growth curves in mouse fibroblasts cells following an infection at a MOI of 0.1 with MHV68.WT, MHV68.ΔHS, or MHV68.MR. At the indicated times post infection virus was harvested, and the titer was determined by plaque assay. The mean and standard deviation from at least three independent experiments is graphed.

Figure 3.7

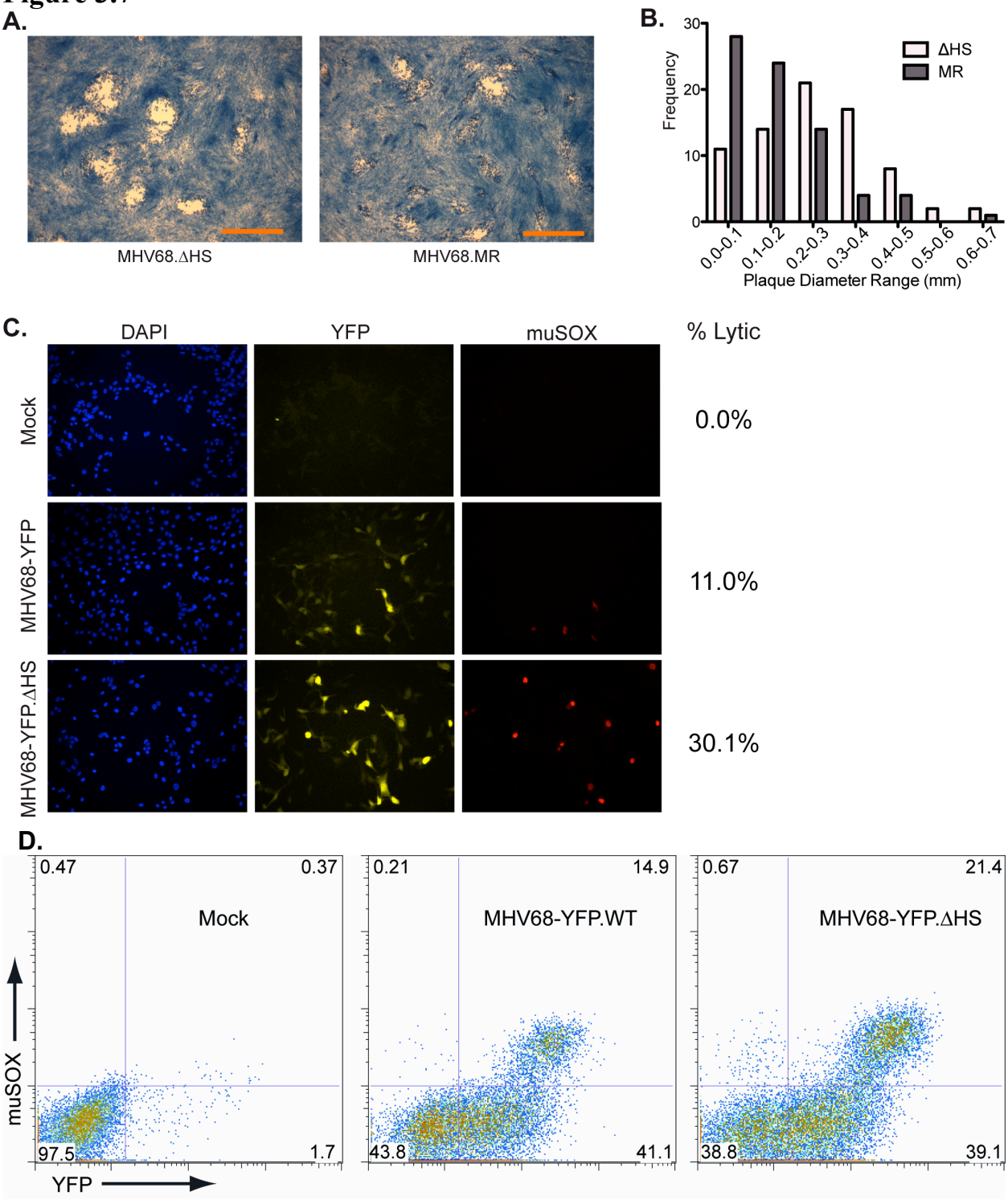
A.
MHV68.ΔHS
MHV68.MR
B.
Frequency
30
20
10
0
ΔHS
MR
0.0-0.1
0.1-0.2
0.2-0.3
0.3-0.4
0.4-0.5
0.5-0.6
0.6-0.7
Plaque Diameter Range (mm)
C.
DAPI
YFP
muSOX
% Lytic
Mock
0.0%
MHV68-YFP
11.0%
MHV68-YFP.ΔHS
30.1%
D.
0.47
0.37
Mock
97.5
1.7
0.21
14.9
MHV68-YFP.WT
43.8
41.1
0.67
21.4
MHV68-YFP.ΔHS
38.8
39.1
muSOX
YFP

Figure 3.7 MHV68.ΔHS generates larger plaques and increases the percentage of lytic antigen-expressing cells. (A) Shown are representative images of plaques generated 4 dpi in 3T3 cells infected with MHV68.ΔHS or MHV68.MR. The scale bar in the lower right corner of the images represents 1 mm. (B) Plaque diameters of 75 plaques from 5 independent experiments were measured at 4 dpi from cells infected with MHV68.ΔHS or MHV68.MR, and the frequency distribution was graphed. (C) 3T3 cells infected at an MOI of 1 with either MHV68-YFP or MHV68-YFP.ΔHS were analyzed at 18 hpi for YFP and muSOX expression by immunofluorescence using anti-muSOX antibodies. Samples were co-stained with DAPI to visualize nuclei. Shown are representative images from four independent experiments. The numbers of lytically infected cells (YFP- and muSOX-positive) were determined by counting multiple fields of view from four independent experiments, and the percentage of infected cells expressing muSOX is shown. (D) 3T3 cells infected at an MOI of 1 with either MHV68-YFP or MHV68-YFP.ΔHS were analyzed at 18 hpi for YFP and muSOX expression by flow cytometry using anti-muSOX antibodies. The percentage of cells within each quadrant is indicated. Shown is a representative graph of four independent experiments.

Figure 3.8

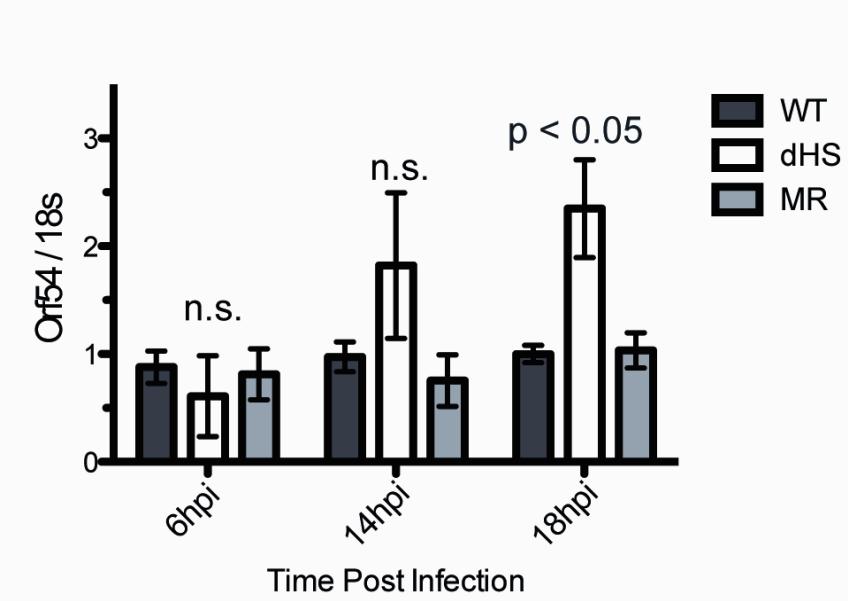

Figure 3.8 ORF54 transcript accumulates to higher levels during a MHV68.ΔHS infection. 3T3 cells were infected at an MOI of 5 with MHV68.WT, MHV68.ΔHS, or MHV68.MR. At the indicated times post infection total RNA was isolated and levels of ORF54 mRNA and 18S rRNA were quantified by RT-qPCR. Shown are the ORF54/18S ratios normalized to wild-type infection at 18 hpi along with the mean and standard deviation from at least five independent experiments. The p-values comparing MHV68.ΔHS to MHV68.MR are indicated. "n.s." indicates that the p-value is greater than 0.05.

Figure 3.9

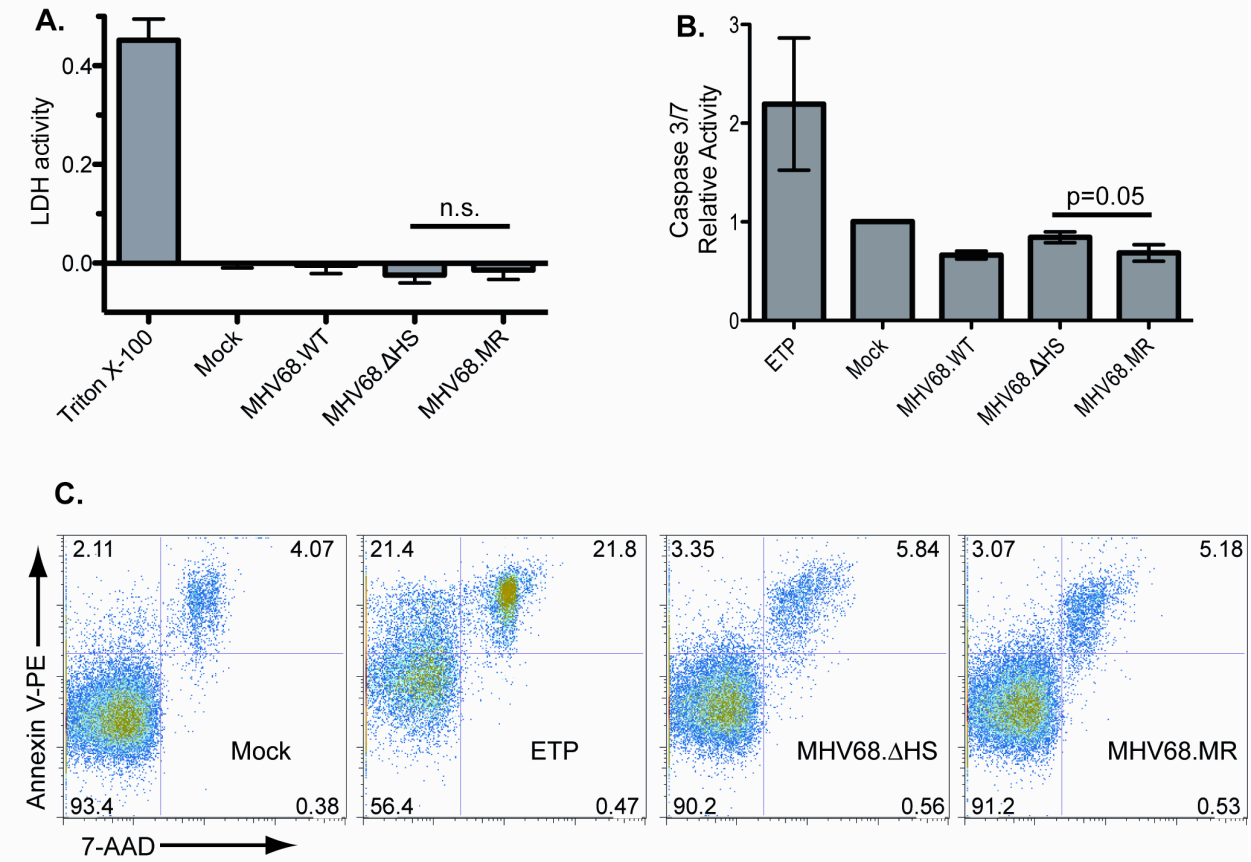

Figure 3.9 MHV68.ΔHS does not induce cell death. (A) 3T3 cells were infected for 16.5 h with MHV68.WT, MHV68.ΔHS, or MHV68.MR at an MOI of 5, whereupon levels of lactate dehydrogenase released into the media were quantified by an enzymatic color change assay. Triton X-100 was added at 1% to lyse cells as a positive control. Shown are the mean and standard deviation from three experiments. (B) 3T3 cells were infected as described above but for 24 h, then levels of caspase3/7 activity were quantified via a luciferase-based luminescent assay. Etoposide (ETP) was included at 25 μM as a positive control. Shown is the luciferase signal strength normalized to the mock infected control along with the mean and standard deviation from three independent experiments. The p-value comparing MHV68.ΔHS to MHV68.MR is indicated. "n.s." indicates that the p-value is greater than 0.05. (C) 3T3 cells were infected at an MOI of 10 and analyzed for Annexin V-PE and 7-AAD staining 18 hpi by flow cytometry. ETP was included at 25 μM as a positive control. The percentage of cells within each quadrant is indicated.

Table 3.1 Phenotype of muSOX mutants.

	Expression	Host Shutoff	DNase	Source
P174S	+	+	N.D.	SOX P176S Homolog [25]
V185A	+	+	N.D.	EBV V169A Homolog [24]
Q127H	+	+	-	SOX Q129H Homolog [25]
R62G	-	-	N.D.	Conserved amongst γ-HV[a]
F160V	-	-	N.D.	Conserved amongst γ-HV
H169L	+	+	N.D.	Conserved amongst γ-HV
R409S	+	-	-	Conserved amongst γ-HV
Δ314-318	+	-	-	Putative NLS[b]
R443I	+	-	+	Screen

N.D. = Not Determined

a. γ-HV = gammaherpesviruses

b. NLS = Nuclear Localization Signal

Chapter 4:

Global mRNA Degradation during Lytic Gammaherpesvirus Infection Contributes to Establishment of Viral Latency

Background

All herpesviruses exhibit a biphasic lifecycle. In the latent state few viral genes are expressed, and the viral genome is maintained in the nucleus of the host cell until stimulating signals induce lytic reactivation (39, 49, 78). During the lytic cycle the vast majority of viral genes are expressed, and the virus undergoes active replication to produce progeny virions. At this stage during productive replication, gammaherpesviruses induce host shutoff through global mRNA degradation via the muSOX (ORF37) protein in MHV68 and its homolog in other gammaherpesviruses (14, 34, 95). Following an intranasal infection with MHV68, the virus replicates in the upper respiratory tract, then traffics to the lymph system. Here the virus establishes latency predominately in memory B-cells where the virus can remain throughout the life of the host organism (48). The contribution of host shutoff towards this viral lifecycle is unknown.

We generated a mutant virus lacking host shutoff activity and demonstrated that such activity is dispensable for viral replication *in vitro*. Here we have analyzed the role of host shutoff during an *in vivo* infection. The host shutoff defective virus exhibited little to no defect during acute replication in the mouse lung following intranasal inoculation. Unexpectedly, the virus accumulated to significantly reduced levels in the lymph nodes at 10 days post infection and was highly attenuated at the downstream stage of latency establishment. Once trafficking was bypassed via an intraperitoneal infection, the mutant virus still was attenuated in establishing latency in the spleen. Our results identify for the first time a key role for the lytic mRNA turnover activity in establishing viral latency, emphasizing the important interplay between these seemingly disparate stages of the viral lifecycle.

Results

Host Shutoff is Dispensable for Acute Replication *in vivo*

The presumed natural route of MHV68 infection is through the upper respiratory tract (Figure 1.1) (72), whereupon the virus undergoes lytic replication in the mucosal epithelial cells lining the mouse lung and nasal cavity. Following intranasal inoculation, viral loads in the lung peak 5-8 dpi before subsiding as the virus traffics to the draining lymph nodes and ultimately to the spleen (114). A burst of lytic replication occurs in these sites of latency establishment (48, 72) which seeds subsequent latency, primarily in germinal center B cells, although also in macrophages and dendritic cells (106). By 14-18 dpi splenomegaly develops, as many as one out of 100 splenocytes harbor the latent viral genome (115).

Host shutoff manifests with delayed early kinetics during lytic replication, consistent with the onset of muSOX expression (14). We therefore hypothesized that MHV68.ΔHS would be attenuated during lytic viral replication in the lung. We infected C57BL/6 mice intranasally with $5x10^4$ pfu of MHV68.WT, MHV68.ΔHS, or MHV68.MR, and monitored viral titers in the lung at 3, 5, and 7 dpi by plaque assay. Viral titers were equivalent at 3 dpi for all three viruses, and at 5 and 7 dpi we observed only a very modest 2-3 fold defect in MHV68.ΔHS relative to MHV68.MR accumulation (Figure 4.1). Although MHV68.WT reached higher titers than MHV68.MR at 5 dpi, the two viruses accumulated to equivalent levels at all earlier and later timepoints (see subsequent sections). To ensure MHV68.ΔHS had not reverted back to WT or incorporated any compensatory mutations, we sequenced the muSOX gene from infected lung homogenate but found no changes. Thus, host shutoff appears largely dispensable for acute replication of gammaherpesviruses *in vivo*.

MHV68.ΔHS is Attenuated in Latency Establishment

To evaluate the role of host shutoff during later stages of the viral lifecycle, mice were infected intranasally with $5x10^4$ pfu of MHV68.WT, MHV68.ΔHS, or MHV68.MR, and the spleens were harvested at 17 dpi, during the peak of latency establishment. Interestingly, mice infected with MHV68.ΔHS did not display the characteristic splenomegaly and had spleens three to four times smaller than MHV68.WT and MHV68.MR infected mice (Figure 4.2A), indicating a role for host shutoff in viral pathogenesis.

To evaluate the cause of MHV68.ΔHS attenuation, we first performed an infectious center assay to test whether the splenocytes from infected mice harbored latent virus capable of lytic reactivation. In this assay, equivalent numbers of splenocytes from infected mice are overlayed onto cultured mouse fibroblasts. Spontaneous lytic reactivation of the splenocytes leads to infection of the fibroblasts, which can be quantified by counting the resulting plaques (115). Splenocytes from MHV68.ΔHS infected mice reactivated at a significantly lower frequency than those from MHV68.WT or MHV68.MR infections (Figure 4.2B). Plaque assays on spleen homogenates confirmed that no preformed virus was present, indicating that the virus detected in these experiments originated from latently infected cells.

The reduction in lytic reactivation frequency could either be a consequence of a decreased number of latently infected splenocytes or, alternatively, a failure of latently infected cells to undergo lytic reactivation. To distinguish these possibilities, we first monitored viral DNA load in the spleens of infected mice using a previously described qPCR assay for the glycoprotein B (gB) gene (129) and found that MHV68.ΔHS infected splenocytes harbor fewer viral genomes than splenocytes infected with MHV68.WT or MHV68.MR (Figure 4.2C). We

also analyzed the frequency of splenocytes harboring the viral genome by limiting dilution PCR. Briefly, serial dilutions of splenocytes were plated in a 96 well plate and subjected to nested PCR for the viral ORF50 gene (128). Spiked template controls confirmed that in this assay we were achieving near single copy sensitivity for the viral genome, with a very low rate of false positive. While approximately one out of 200 splenocytes latently harbor the MHV68.WT or MHV68.MR genome, the frequency of MHV68.ΔHS genome harboring splenocytes was less than one out of 10,000 splenocytes (Figure 4.2D). Thus, while host shutoff appears dispensable for acute replication in the lung, it plays an important role in the downstream events leading to efficient latency establishment in the spleen.

MHV68.ΔHS Exhibits Defects in Both Trafficking and Latency Establishment

During the normal course of an infection, the virus traffics from the site of lytic replication in the lung to the draining lymph nodes and spleen where latency is established. We reasoned that the decreased frequency of latently infected splenocytes during MHV68.ΔHS infection could be a consequence of a defect in trafficking, maintenance at these sites, or a latency establishment defect. We quantified levels of virus in the cervical lymph node at 10 dpi, an intermediate time point after peak virus replication in the lung but before peak latency establishment in the spleen. Following an intranasal inoculation, high levels of MHV68 have been found in the cervical lymph node at this time point (48, 72). Interestingly, we detected significantly lower levels of MHV68.ΔHS at this site relative to MHV68.MR (Figure 4.3A), suggesting that muSOX-induced mRNA degradation contributes to the ability of the virus to traffic to or be maintained at the sites of latency establishment.

To determine whether this was the root cause of the defect in latency establishment upon MHV68.ΔHS infection, we next infected mice via the intraperitoneal route, which bypasses the requirement for the virus to undergo acute replication and traffic through the lymph before reaching the spleen (28). At 19 dpi, we found significantly less MHV68.ΔHS virus in the spleen relative to MHV68.MR by qPCR (Figure 4.3B), indicating that a defect in viral trafficking cannot fully account for the latency establishment defect observed during an intranasal infection. Collectively, these data indicate that muSOX-induced host shutoff plays important roles both in the ability of MHV68 to traffic to the lymph nodes, as well as establish latency in the spleen.

Discussion

In this study we assesed the role of mRNA degradation during the viral lifecycle and found that host shutoff is dispensable for acute replication in the lung but important for down stream latency establishment. The role for muSOX-induced mRNA turnover clearly diverges from that of the alphaherpesvirus host shutoff factor vhs, which similarly induces global mRNA degradation but plays a critical role during acute replication *in vivo* (56, 60, 111). The severe attenuation of HSV-1 and HSV-2 vhs mutants is hypothesized to be a consequence of ineffective innate immune evasion, as vhs blocks dendritic cell activation and down-regulates the type I interferon response (79, 84, 97). In contrast, we find little difference between MHV68.MR and MHV68.ΔHS during acute replication in mice, suggesting that while alpha- and gammaherpesviruses block host gene expression via analogous mechanisms, the functional ramifications of this activity are distinct, and may relate to the unique *in vivo* biology of each class of virus.

In the gammaherpesvirus EBV host shutoff has been implicated in immune evasion, and *in vitro* studies with BGLF5 have identified immunological effectors which are downregulated in a host shutoff dependent manner (123, 136). Van Gent et al. observed downregulation of TLR9, an arm of the innate immune response (123). On the contrary, our results signify that host shutoff is dispensable for innate immune avoidance as we detected we detected no difference during acute replication in the lung between the MHV68.MR and MHV68.ΔHS (Figure 4.1). The apparent differences between these studies could be due to the unique biology of EBV and MHV68; however, we hypothesize that the downregulation of innate immune effectors is more likely a by-product of the expression of a general mRNA degradation protein and does not represent a physiological mechanism of immune avoidance. Zuo et al. found that expression of BGLF5 decreased killing by $CD8^+$ T-cells thus inhibiting the adaptive immune response (136). In the current study we have not analyzed the contribution of host shutoff towards adaptive immune avoidance, and indeed the MHV68.ΔHS defect observed during latency establishment could be due to enhanced $CD8^+$ T-cell dependent clearance. Future work will investigate how the adaptive immune response contributes towards MHV68.ΔHS attenuation.

The first major defect observed in an *in vivo* MHV68.ΔHS infection is significantly lower levels of the virus in the lymph nodes at 10 dpi. Following an intranasal infection, wild-type MHV68 undergoes lytic replication in the lung and upper respiratory track, after which the virus drains to the lymph nodes and spleen where a variety of cell types are infected, including macrophages, dendritic cells, and B-cells (27, 28, 94, 109). A burst of lytic replication occurs at these sites (48, 72), after which replicating virus is cleared and long-term latency is established. Lower levels of MHV68.ΔHS in the lymph node could be caused by a cell-type specific role for muSOX-dependent mRNA degradation in viral replication or immune evasion during viral transport to, and maintenance in, the lymphatic tissue. The means by which the virus traffics to the sites of latency establishment remain unclear, yet evidence suggests that latently infected B cells carry the virus given that viremia is undetectable during an infection, and latently infected B cells are present in the lung very early after infection (4, 28, 88). A failure of MHV68.ΔHS to establish latency in these cells might also cause their selective immune-based eradication and decreased accumulation in the lymph nodes. Alternatively, muSOX could influence the ability of the virus to reactivate from latency, as this process has been linked to efficient latency establishment. Mutations in a number of genes alter the frequency of lytic reactivation and lead to lower levels of latency establishment following an intranasal infection, such as v-cyclin (ORF72) (122), M1 (21), M2 (46, 66), and LANA (ORF73) (76, 83), but unlike muSOX these

genes are all expressed during latency as well as during lytic replication (67). Mutations in the lytic ORF36 gene encoding a protein kinase also lead to defects in latency establishment and reactivation (47, 117), which have been tracked to the requirement for ORF36 to inhibit the IRF-3 mediated type I interferon response (47) and its modification of the DNA-damage response protein H2AX (117).

The second major defect observed upon MHV68.ΔHS infection is a marked reduction in the number of infected splenocytes present during peak latency establishment relative to mice infected with wild-type or mutant rescue viruses. This defect could arise simply as a downstream consequence of the aforementioned impairment in trafficking. However, we detected significantly reduced levels of viral DNA in the spleen even after an intraperitoneal infection, which bypasses the need for acute replication in the lung and subsequent trafficking through the lymph nodes (28). Thus, MHV68.ΔHS appears defective in both trafficking and latency establishment in the spleen. Such a phenotype might occur if MHV68.ΔHS-infected cells preferentially entered the lytic cycle, perhaps enabling more efficient clearance by the immune system which is supported by our *in vitro* findings that MHV68.ΔHS enhances lytic cycle entry (Chapter 3).

Given the broad effects of host shutoff, the mechanism of MHV68.ΔHS attenuation presumably deviates from the gene-for-gene interactions associated with many virulence factors. Further research in this area will uncover the likely means by which host shutoff influences latency establishment, as well as reveal novel connections between the lytic and latent stages of the gammaherpesviruses lifecycle.

Materials & Methods

Cells, Transfections, and Viruses

HEK293T, COS7, NIH 3T3, NIH 3T12, and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). 293T cells were transfected with Effectene (Qiagen) following the manufacturer's protocol.

Quantitative PCR

To quantify RNA, we isolated RNA from transfected cells using RNA-Bee (Tel-Test) or the Zymo Mini RNA II Isolation Kit (Zymo Research). Samples were treated with Turbo DNase (Ambion) according to the manufacturer's protocol to remove genomic DNA contamination. To generate cDNA, RNA samples were reverse transcribed using AMV RT (Promega) and an oligo dT or 18S-specific primer. 18S rRNA or GAPDH mRNA levels were quantified using TaqMan ribosomal RNA control reagents or TaqMan rodent GAPDH control reagents (Applied Biosystems). Other endogenous mRNA levels were quantified using Applied Biosystems Taqman Gene Expression Assays (actB-Mm01205647_g1, rplp2-Mm03059047_gH, tubb5-Mm00495806_g1). To quantify viral genomes, DNA was first isolated from spleen cells or cervical lymph node cells by the Qiagen QIAamp DNA Mini Kit following the manufacturer's protocol. Viral DNA levels were then measured using a previously described assay targeting the MHV68 ORF8 gene (129). All qPCR reactions were performed with TaqMan Universal PCR Master Mix.

***In vivo* Infections and Experiments**

Female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and infected when 4-6 weeks old. Mice were anesthetized with isoflourane and inoculated intranasally with $5x10^4$ plaque forming units (pfu) in 20 µl DMEM (Invitrogen). For intraperitoneal infections, $1x10^3$ pfu in 0.2 ml PBS were injected into the peritoneal cavity of mice. Lungs were harvested at 3, 5, or 7 dpi, homogenized with a tissue homogenizer for 1 minute at 24,000 rpm in 10 ml DMEM with 10 % FBS, 100U of penicillin per ml, and 100mg of streptomycin per ml (Pen/Strep, Invitrogen). Spleens were harvested at 17 or 19 dpi. To identify any preformed infectious particles, half of each spleen was homogenized as above in 5 ml DMEM with 10% FBS, and Pen/Strep. The tissue homogenate was then assayed for viral particles by plaque assay on monolayers of NIH 3T3 cells overlaid with 1 % agarose for 4 days. The cells were then fixed and stained with 0.04% methylene blue. Splenocytes were isolated from the other half of the spleen by dissociating the spleen and passing through a 40 µm cell strainer (BD-Falcon). Cells were then pelleted, resuspended in red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA), and incubated at room temperature for 5 minutes. Cells were again pelleted and resuspended in RPMI medium with 10%FBS and Pen/Strep before counting. Cervical lymph nodes were harvested at 10 dpi and isolated in the same manner as splenocytes, but without lysing the red blood cells.

Infectious Center Assay

The number of reactivating splenocytes was determined as described previously (115). Briefly, 21,000 NIH 3T3 cells were plated per well in a 24-well plate the day before infection. $2.5x10^6$, $5x10^5$, or $1x10^5$ splenocytes were overlayed onto the NIH 3T3 cells and incubated for 4

days. Cells were then fixed with 10% formaldehyde and stained with 0.04% methylene blue to visualize plaques.

Limiting Dilution PCR

The frequency with which cells latently harbor the viral genome was determined as described previously (128). Briefly, 4-fold serial dilutions of splenocytes were plated in 96-well plates with 16 wells per dilution. Nested PCR for the ORF50 gene was performed and the resulting PCR product was run on an agarose gel. Wells positive for a PCR product by ethidium bromide staining contain at least one copy of the viral genome. Single-copy sensitivity was confirmed using serial dilutions of MHV68 BAC DNA.

Graph Design and Stastical Analysis

All graphs were designed and statistical analysis performed using the GraphPad Prism Software version 4 or 5. Data were analyzed for statistical significance using the Student's t-test or, for *in vivo* data, the Mann-Whitney nonparametric test. For limiting dilution PCR analysis, data were subject to nonlinear regression using the log(agonist) vs. response curve with a nonvariable slope.

Figures

Figure 4.1

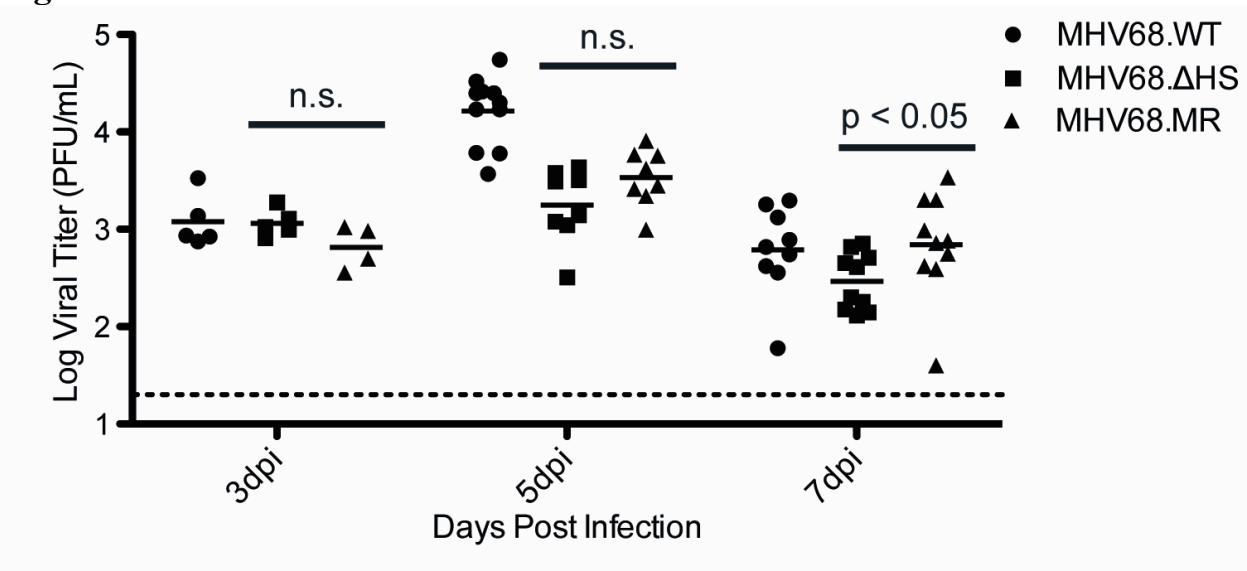

Figure 4.1 MHV68.ΔHS replicates to near MHV68.MR levels during the acute phase of infection in the mouse lung. C57BL/6 mice were infected intranasally with $5x10^4$ pfu MHV68.WT, MHV68.ΔHS, or MHV68.MR. At 3, 5, or 7 dpi lungs were harvested and homogenized, and viral titers were determined by plaque assay. Each point on the graph represents the viral titer from a single lung, and the bar indicates the mean titer for each virus. The dotted line represents the limit of detection at 20 pfu/lung. The p-values comparing MHV68.ΔHS and MHV68.MR are indicated, and "n.s." indicates a p-value greater than 0.05.

Figure 4.2

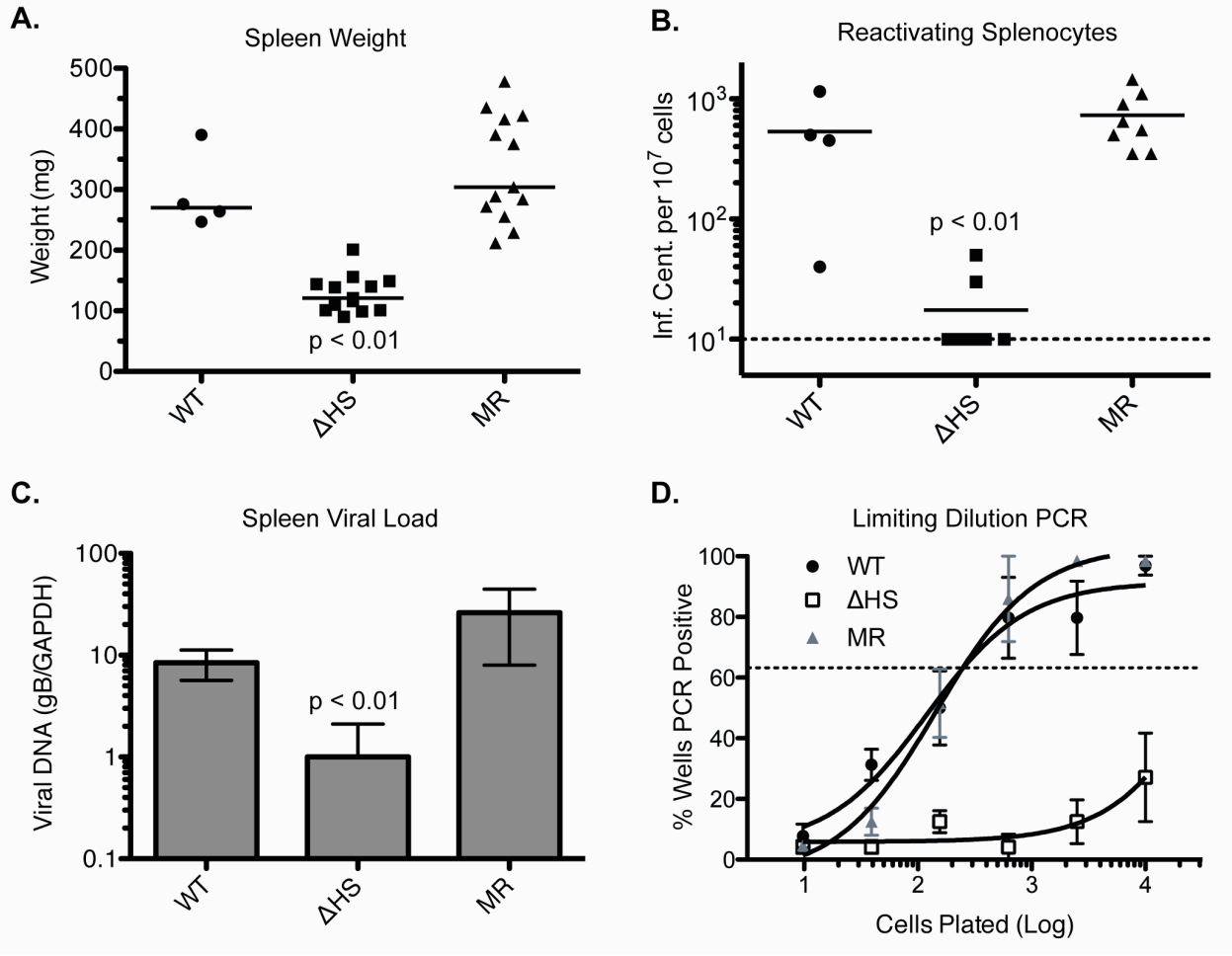
A.
Spleen Weight
Weight (mg)
500
400
300
200
100
0
p < 0.01
WT
ΔHS
MR
B.
Reactivating Splenocytes
Inf. Cent. per 10^7 cells
10^3
10^2
10^1
p < 0.01
WT
ΔHS
MR
C.
Spleen Viral Load
Viral DNA (gB/GAPDH)
100
10
1
0.1
p < 0.01
WT
ΔHS
MR
D.
Limiting Dilution PCR
% Wells PCR Positive
100
80
60
40
20
0
WT
ΔHS
MR
1
2
3
4
Cells Plated (Log)

Figure 4.2 MHV68.ΔHS is attenuated for latency establishment. In two independent experiments, C57BL/6 mice were infected intranasally with $5x10^4$ pfu MHV68.WT, MHV68.ΔHS, or MHV68.MR, and spleens were harvested at 17 dpi. (A) The weight of each spleen is plotted with the mean weight for each virus variant indicated by the bar. (B) Spleen cells were harvested and analyzed for lytic reactivation frequency via infectious center assay. The number of reactivating splenocytes per 10^7 cells is plotted with the mean for each virus indicated by the bar. The dashed line denotes the limit of detection. (C) DNA was isolated from spleen cells and analyzed by qPCR for the viral glycoprotein B (gB, ORF8) gene and endogenous GAPDH. The normalized gB/GAPDH ratio is plotted along with the mean and standard deviation for each viral infection. The p-values comparing MHV68.ΔHS to MHV68.MR are indicated. (D) The frequency of latently infected cells was determined by limiting dilution-PCR. The percent of wells positive for PCR product is plotted for each dilution. Each point represents the average and standard deviation of three or four mice. The dotted line at 63.2% is used to calculate the frequency of genome harboring cells according to the Poisson distribution.

Figure 4.3

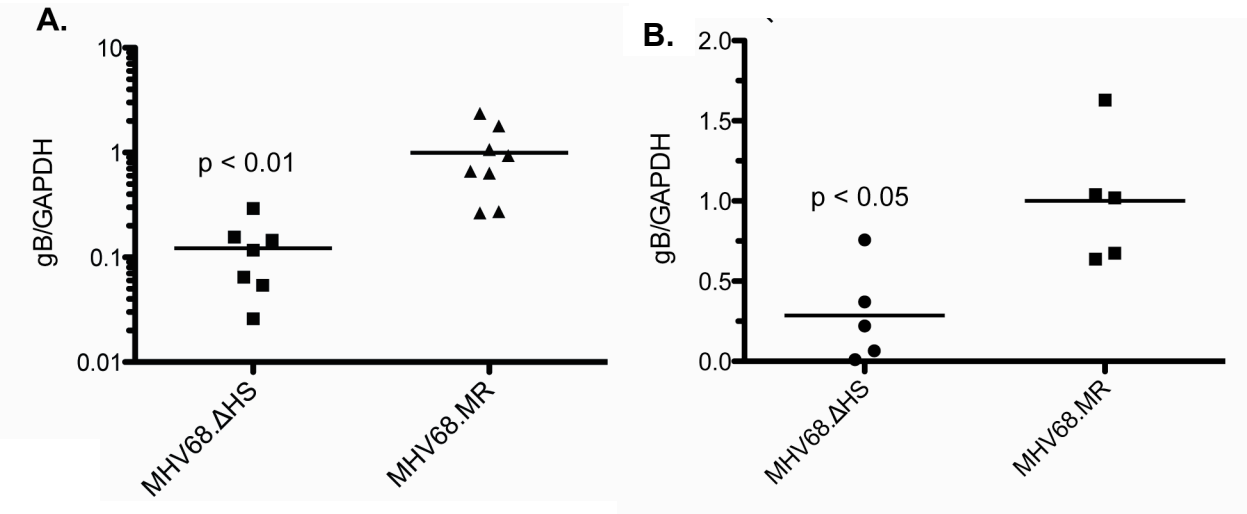

Figure 4.3 MHV68.ΔHS fails to traffic to the lymph system and establish latency. (A) C57BL/6 mice were infected intranasally with $5x10^4$ pfu MHV68.WT, MHV68.ΔHS, or MHV68.MR, and cervical lymph nodes were harvested at 10 dpi. DNA was isolated from the cells and analyzed by qPCR for the viral glycoprotein B (gB, ORF8) gene and endogenous GAPDH. The normalized gB/GAPDH ratio is plotted along with the mean for each viral infection. (B) Mice were infected intraperitoneally with $1x10^3$ pfu of each of the indicated viruses. DNA was isolated and quantified as above and the normalized gB/GAPDH ratio is plotted along with the mean for each viral infection. Each point on the graph represents the ratio from a single mouse. Data for each figure are compilations of two independent experiments and the p-values are indicated.

Chapter 5:

Perspectives

Further Discussion

Host shutoff in the human gammaherpesviruses has been well characterized, yet the contribution of this function towards the viral lifecycle remained unknown. Here we analyzed the impact of host shutoff both in tissue culture and *in vivo* infections with the murine gammaherpesvirus MHV68 and found this activity to be largely dispensable for acute lytic replication yet critical for the downstream viral accumulation in the lymph nodes and subsequent establishment of latency in the spleen.

The results of these studies were unexpected given that MHV68-induced host shutoff is executed by muSOX, a viral protein whose expression has only been detected during the lytic cycle (20, 51, 68), and yet we find host shutoff important for latency establishment. Gammaherpesviruses latency establishment involves the coordinated effort of multiple signaling pathways, both host and viral. The exact mechanisms of this activity are not well understood, but recurring themes have emerged (106). Upregulation of NF-κB signaling enhances B-cell survival and proliferation and inhibits the expression of viral lytic genes (8, 50). The KSHV protein v-FLIP and the EBV protein LMP-1 both induce the expression of NF-κB (11, 50). Although the homolog of either v-FLIP or LMP-1 is absent from the MHV68 genome, the upregulation of NF-κB is critical for latency in MHV68 as the expression of a constitutive inhibitor of NF-κB prevents the establishment of latency following an intranasal infection (58). A recent study in the intracellular bacteria *L. pneumophila* found host shutoff plays an important role in prolonging the NF-κB pathway by blocking the synthesis of the inhibitor IκB (29). It is interesting to speculate that a similar strategy may be at play in MHV68 to induce prolonged NF-κB signaling.

Another recurring theme during gammaherpesviral latency establishment is the methylation and histone deacetylation of viral genomes near lytic gene promoters, thus trasitioning these genomic regions into a heterochromatic state and inhibiting the expression of the lytic genes (40, 119). Mice lacking the histone deacetylases Dnmt3a and Dnmt3b have delayed latency establishment in the spleen relative to wild-type mice following an intranasal MHV68 infection (38). Genomes were isolated from these mice and found to lack methylation around the Orf50 promoter, thus showing the importance of epigenetic silencing on latency establishment. Host shutoff may be modifying the above pathways either directly or through the downregulation of contributing factors, and further studies with host shutoff deficient viruses will lead to a better understanding of how this process occurs.

MHV68 variants lacking the ability to establish latency have previously been generated by constitutively expressing (or over-expressing) the lytic transcription factor RTA. RTA over-expressing viruses generate larger plaques, replicate to near wild-type levels in the lung, but establish latency at lower levels in the spleen (70, 92), reminiscent of the findings presented in this study for MHV68.ΔHS. Furthermore, the constitutive RTA-expressing viruses have been found to efficiently traffic to the spleen but fail to be maintained at this site (70). This attenuation has been ascribed to the viruses producing excessive amounts of the RTA-inducible

lytic genes, creating an intracellular environment favoring immediate entry into the lytic cycle rather than entering latency. Similarly, we find higher levels of viral mRNA in a MHV68.ΔHS infection. Thus, during an infection, an important role for muSOX may be to ensure that factors driving lytic replication are not overrepresented. The commonalities observed thus far between an RTA over-expressing virus and MHV68.ΔHS are quite interesting, and future experiments should compare both of these mutants to wild-type virus in order to delineate the phenotypic effects caused by a general upregulation of viral lytic genes and those which are specific to host shutoff.

Based on our observation that an increased percentage of cultured 3T3 cells infected with MHV68.ΔHS express lytic markers relative to those infected with MHV68.WT, we speculate that attenuation of MHV68.ΔHS leads to decreased latency establishment thus supporting enhanced entry into the lytic cycle. However, if this represents enhanced entry into the lytic cycle, there must be a downstream defect that tempers subsequent viral output, as the host shutoff mutant virus does not replicate to higher titers in 3T3 cells than the wild-type virus. In this regard, host shutoff could be important for optimizing the balance of host or viral proteins in an infected cell, as has been reported in an EBV mutant lacking BGLF5, where altered levels of proteins inhibited viral maturation and egress (24). One mechanism that could shift the balance of infection away from latency is if muSOX down-regulates factors that inhibit latency establishment. Such negative regulators would then accumulate in the presence of the R443I mutant, driving cells into lytic replication. MuSOX activity may also be required for the virus to achieve the appropriate level of host and or viral factors packaged into the viral particle in order to efficiently establish latency in newly infected cells rather than enter the lytic cycle. While it is tempting to speculate a connection between the increased percentage of lytically infected 3T3 cells and the latency establishment defect *in vivo* with MHV68.ΔHS, at present the precise relationship between these observations remains unclear.

While we conclude host shutoff is dispensable for lytic replication yet important for latency establishment from our studies with the MHV68.ΔHS mutant, we hypothesize that the degree of host shutoff activity encoded by a gammaherpesvirus will influence the resulting phenotype of both *in vitro* and *in vivo* infections. We clearly observe reduced mRNA turnover during a MHV68.ΔHS infection, but because a low level of residual activity still remains we cannot exclude the possibility that some degree of host shutoff could be important for viral replication. Indeed a mutant completely devoid of any mRNA turnover could be more attenuated during replication. Similarly, a mutant virus with increased host shutoff activity relative to the MHV68.ΔHS mutant might be less attenuated during latency establishment. Other labs have independently identified different muSOX mutants with reduced host shutoff and are currently analyzing the phenotypes of these viral mutants (M. Song, *personal communication*). The results of such studies will indicate how the magnitude of host shutoff could shape the phenotype of an infection.

We hypothesize that the results from this study will be applicable to all gammaherpesviruses given the similar properties between muSOX, SOX, and BGLF5. A single function mutant of BGLF5 lacking host shutoff has been identified (136), and a mutant EBV virus with this mutation is under development (M. Rowe, *personal communication*). This virus will allow the comparison of the host shutoff role across different gammaherpesviruses at the *in vitro* level, although lack of a true animal model for EBV will limit *in vivo* comparisons.

Future Directions

In this study we have uncovered an important role for gammerherpesviral host shutoff and a link between the lytic phenomenon of global mRNA degradation and latency establishment. However understanding the mechanism of this link at the molecular and cellular levels and how this influences overall latency establishment at the systemic level will require further work. Because we find less MHV68.ΔHS in the spleen after an intraperitoneal infection, a defect in either viral maintenance within lymph tissues or a failure to establish latency likely leads to the observed attenuation of MHV68.ΔHS. These factors are certainly interdependent during an infection, and separation of these elements will require novel infection parameters with new mutant viruses. For example, by quantifying virus in the spleen over time following an intraperitoneal infection, we will be able to detect defects in viral maintenance in lymph tissue. To determine the contribution of host shutoff towards latency establishment devoid of any lytic replication effects, we can compare latency following an intraperitoneal infection of wild-type and host shutoff deficient viruses in a replication deficient genetic background. A mutant virus with the orf31 gene disrupted is unable to replicate yet establishes latency at wild-type levels following an intraperitoneal infection and introduction of muSOX R443I into this background would be relatively simple (28).

As discussed in Chapter 4, the attenuation of a host shutoff deficient virus could be due to an enhanced adaptive immune response. Given the large number of genetically deficient mice available, we can determine the overall contribution of the adaptive immune response towards the observed attenuation as well as the individual components responsible. We hypothesize that more lytically infected cells are present during a MHV68.ΔHS infection leading to increased CD8+ T-cell-mediated cell death, although other scenarios are possible.

At the cellular level, host shutoff appears to inhibit the ability of infected cells to enter the lytic cycle. Our *in vitro* studies were performed in immortalized fibroblasts, but future studies will compare viral replication and lytic cycle entry in cells more relevant to a natural infection, such as B-cells, macrophages, lung epithelial cells, and dendritic cells. Following an infection MHV68 has been found in all of these cell types, particularly B-cells where long-term latency is established (115), but unfortunately B-cells are inefficiently infected *in vitro*, thereby limiting the most informative studies. Never the less, investigations in these cell types will strengthen our hypothesis that enhanced lytic cycle entry inhibits latency establishment *in vivo*.

The molecular mechanisms leading to enhanced lytic cycle entry will be difficult to divulge given the broad effects of global mRNA degradation; however, known cellular and viral factors modify the ability of the virus to enter the latent or lytic cycle and the analysis of such factors could be informative. Upregulation of the NF-κB pathway and epigenetic silencing are common traits in all gammaherpesvirus latent infections and comparing the regulation of these pathways between a wild-type and MHV68.ΔHS infections will be informative. Furthermore overexpression of the NF-κB or methyltransferases could overcome enhanced lytic cycle entry and latency establishment attenuation observed following *in vitro* and *in vivo* infections with MHV68.ΔHS. However, determining the causal relationship of these pathways to the latency establishment attenuation and their intermediates will be difficult.

Concluding Remarks

All gammaherpesviruses studied to date block host gene expression through widespread mRNA degradation, yet the contribution of this function towards the viral lifecycle remained unknown. In this report we have found host shutoff to be dispensable for viral replication, yet

important for the virus to establish latency. These findings link the two distinct phases of the viral lifecycle, lytic replication and latency, and indicate that the global manipulation of gene expression contributes to a successful lifelong gammaherpesvirus infection. Future work in this area will provide insight into the mechanism by which host shutoff influences latency establishment and reveal novel connections between the lytic and latent stages of the gammaherpesviruses lifecycle**.**

References

1. **Adler, H., M. Messerle, M. Wagner, and U. H. Koszinowski.** 2000. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J Virol **74:**6964-74.
2. **Bagneris, C., L. C. Briggs, R. Savva, B. Ebrahimi, and T. E. Barrett.** 2011. Crystal structure of a KSHV-SOX-DNA complex: insights into the molecular mechanisms underlying DNase activity and host shutoff. Nucleic Acids Res**:**doi# 10.1093/nar/gkr111.
3. **Barbera, A. J., J. V. Chodaparambil, B. Kelley-Clarke, K. Luger, and K. M. Kaye.** 2006. Kaposi's sarcoma-associated herpesvirus LANA hitches a ride on the chromosome. Cell Cycle **5:**1048-52.
4. **Barton, E., P. Mandal, and S. H. Speck.** 2011. Pathogenesis and Host Control of Gammaherpesviruses: Lessons from the Mouse. Annu Rev Immunol.
5. **Bechtel, J. T., Y. Liang, J. Hvidding, and D. Ganem.** 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. J Virol **77:**6474-81.
6. **Biesinger, B., I. Muller-Fleckenstein, B. Simmer, G. Lang, S. Wittmann, E. Platzer, R. C. Desrosiers, and B. Fleckenstein.** 1992. Stable growth transformation of human T lymphocytes by herpesvirus saimiri. Proc Natl Acad Sci U S A **89:**3116-9.
7. **Blaskovic, D., M. Stancekova, J. Svobodova, and J. Mistrikova.** 1980. Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol **24:**468.
8. **Brown, H. J., M. J. Song, H. Deng, T. T. Wu, G. Cheng, and R. Sun.** 2003. NF-kappaB inhibits gammaherpesvirus lytic replication. J Virol **77:**8532-40.
9. **Buisson, M., T. Geoui, D. Flot, N. Tarbouriech, M. E. Ressing, E. J. Wiertz, and W. P. Burmeister.** 2009. A bridge crosses the active-site canyon of the Epstein-Barr virus nuclease with DNase and RNase activities. J Mol Biol **391:**717-28.
10. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865-9.
11. **Chaudhary, P. M., A. Jasmin, M. T. Eby, and L. Hood.** 1999. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene **18:**5738-46.
12. **Collins, C. M., J. M. Boss, and S. H. Speck.** 2009. Identification of infected B-cell populations by using a recombinant murine gammaherpesvirus 68 expressing a fluorescent protein. J Virol **83:**6484-93.
13. **Connor, J. H., and D. S. Lyles.** 2002. Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1. J Virol **76:**10177-87.
14. **Covarrubias, S., J. M. Richner, K. Clyde, Y. J. Lee, and B. A. Glaunsinger.** 2009. Host shutoff is a conserved phenotype of gammaherpesvirus infection and is orchestrated exclusively from the cytoplasm. J Virol **83:**9554-66.
15. **Dahlroth, S. L., D. Gurmu, J. Haas, H. Erlandsen, and P. Nordlund.** 2009. Crystal structure of the shutoff and exonuclease protein from the oncogenic Kaposi's sarcoma-associated herpesvirus. FEBS J **276:**6636-45.

16. **Dauber, B., J. Pelletier, and J. R. Smiley.** 2011. The herpes simplex virus 1 vhs protein enhances translation of viral true late mRNAs and virus production in a cell type-dependent manner. J Virol **85:**5363-73.
17. **Decker, T., and M. L. Lohmann-Matthes.** 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods **115:**61-9.
18. **Delecluse, H. J., R. Feederle, B. O'Sullivan, and P. Taniere.** 2007. Epstein Barr virus-associated tumours: an update for the attention of the working pathologist. J Clin Pathol **60:**1358-64.
19. **Desrosiers, R. C., V. G. Sasseville, S. C. Czajak, X. Zhang, K. G. Mansfield, A. Kaur, R. P. Johnson, A. A. Lackner, and J. U. Jung.** 1997. A herpesvirus of rhesus monkeys related to the human Kaposi's sarcoma-associated herpesvirus. J Virol **71:**9764-9.
20. **Ebrahimi, B., B. M. Dutia, K. L. Roberts, J. J. Garcia-Ramirez, P. Dickinson, J. P. Stewart, P. Ghazal, D. J. Roy, and A. A. Nash.** 2003. Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J Gen Virol **84:**99-109.
21. **Evans, A. G., J. M. Moser, L. T. Krug, V. Pozharskaya, A. L. Mora, and S. H. Speck.** 2008. A gammaherpesvirus-secreted activator of Vbeta4+ CD8+ T cells regulates chronic infection and immunopathology. J Exp Med **205:**669-84.
22. **Evans, A. S., J. C. Niederman, and R. W. McCollum.** 1968. Seroepidemiologic studies of infectious mononucleosis with EB virus. N Engl J Med **279:**1121-7.
23. **Farley, C. A., W. G. Banfield, G. Kasnic, Jr., and W. S. Foster.** 1972. Oyster herpes-type virus. Science **178:**759-60.
24. **Feederle, R., H. Bannert, H. Lips, N. Muller-Lantzsch, and H. J. Delecluse.** 2009. The Epstein-Barr virus alkaline exonuclease BGLF5 serves pleiotropic functions in virus replication. J Virol **83:**4952-62.
25. **Feederle, R., A. M. Mehl-Lautscham, H. Bannert, and H. J. Delecluse.** 2009. The Epstein-Barr virus protein kinase BGLF4 and the exonuclease BGLF5 have opposite effects on the regulation of viral protein production. J Virol **83:**10877-91.
26. **Fickenscher, H., and B. Fleckenstein.** 2001. Herpesvirus saimiri. Philos Trans R Soc Lond B Biol Sci **356:**545-67.
27. **Flano, E., S. M. Husain, J. T. Sample, D. L. Woodland, and M. A. Blackman.** 2000. Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J Immunol **165:**1074-81.
28. **Flano, E., Q. Jia, J. Moore, D. L. Woodland, R. Sun, and M. A. Blackman.** 2005. Early establishment of gamma-herpesvirus latency: implications for immune control. J Immunol **174:**4972-8.
29. **Fontana, M. F., S. Banga, K. C. Barry, X. Shen, Y. Tan, Z. Q. Luo, and R. E. Vance.** 2011. Secreted Bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent Legionella pneumophila. PLoS Pathog **7:**e1001289.
30. **Ganem, D.** 2007. Kaposi's Sarcoma-Associated Herpesvirus, p. 2847 - 2888. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.
31. **Gargano, L. M., J. C. Forrest, and S. H. Speck.** 2009. Signaling through Toll-like receptors induces murine gammaherpesvirus 68 reactivation in vivo. J Virol **83:**1474-82.

32. **Glaunsinger, B., L. Chavez, and D. Ganem.** 2005. The exonuclease and host shutoff functions of the SOX protein of Kaposi's sarcoma-associated herpesvirus are genetically separable. J Virol **79:**7396-7401.
33. **Glaunsinger, B., L. Chavez, and D. Ganem.** 2005. The exonuclease and host shutoff functions of the SOX protein of Kaposi's sarcoma-associated herpesvirus are genetically separable. J Virol **79:**7396-401.
34. **Glaunsinger, B., and D. Ganem.** 2004. Lytic KSHV infection inhibits host gene expression by accelerating global mRNA turnover. Mol Cell **13:**713-23.
35. **Glaunsinger, B. A., and D. E. Ganem.** 2006. Messenger RNA turnover and its regulation in herpesviral infection. Adv Virus Res **66:**337-94.
36. **Goldstein, J. N., and S. K. Weller.** 1998. The exonuclease activity of HSV-1 UL12 is required for in vivo function. Virology **244:**442-57.
37. **Goldstein, J. N., and S. K. Weller.** 1998. In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. J Virol **72:**8772-81.
38. **Gray, K. S., J. C. Forrest, and S. H. Speck.** 2010. The de novo methyltransferases DNMT3a and DNMT3b target the murine gammaherpesvirus immediate-early gene 50 promoter during establishment of latency. J Virol **84:**4946-59.
39. **Gregory, S. M., J. A. West, P. J. Dillon, C. Hilscher, D. P. Dittmer, and B. Damania.** 2009. Toll-like receptor signaling controls reactivation of KSHV from latency. Proc Natl Acad Sci U S A **106:**11725-30.
40. **Gunther, T., and A. Grundhoff.** 2010. The epigenetic landscape of latent Kaposi sarcoma-associated herpesvirus genomes. PLoS Pathog **6:**e1000935.
41. **Hadinoto, V., M. Shapiro, C. C. Sun, and D. A. Thorley-Lawson.** 2009. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. PLoS Pathog **5:**e1000496.
42. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68:**4797-810.
43. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol **68:**7790-9.
44. **Harlow, E., and D. Lane.** 1999. Using antibodies : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Henle, G., W. Henle, P. Clifford, V. Diehl, G. W. Kafuko, B. G. Kirya, G. Klein, R. H. Morrow, G. M. Munube, P. Pike, P. M. Tukei, and J. L. Ziegler.** 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J Natl Cancer Inst **43:**1147-57.
46. **Herskowitz, J., M. A. Jacoby, and S. H. Speck.** 2005. The murine gammaherpesvirus 68 M2 gene is required for efficient reactivation from latently infected B cells. J Virol **79:**2261-73.
47. **Hwang, S., K. S. Kim, E. Flano, T. T. Wu, L. M. Tong, A. N. Park, M. J. Song, D. J. Sanchez, R. M. O'Connell, G. Cheng, and R. Sun.** 2009. Conserved herpesviral kinase promotes viral persistence by inhibiting the IRF-3-mediated type I interferon response. Cell Host Microbe **5:**166-78.

48. **Hwang, S., T. T. Wu, L. M. Tong, K. S. Kim, D. Martinez-Guzman, A. D. Colantonio, C. H. Uittenbogaart, and R. Sun.** 2008. Persistent gammaherpesvirus replication and dynamic interaction with the host in vivo. J Virol **82:**12498-509.
49. **Iskra, S., M. Kalla, H. J. Delecluse, W. Hammerschmidt, and A. Moosmann.** 2010. Toll-like receptor agonists synergistically increase proliferation and activation of B cells by epstein-barr virus. J Virol **84:**3612-23.
50. **Izumi, K. M., and E. D. Kieff.** 1997. The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappaB. Proc Natl Acad Sci U S A **94:**12592-7.
51. **Johnson, L. S., E. K. Willert, and H. W. Virgin.** 2010. Redefining the genetics of murine gammaherpesvirus 68 via transcriptome-based annotation. Cell Host Microbe **7:**516-26.
52. **Kamitani, W., C. Huang, K. Narayanan, K. G. Lokugamage, and S. Makino.** 2009. A two-pronged strategy to suppress host protein synthesis by SARS coronavirus Nsp1 protein. Nat Struct Mol Biol **16:**1134-40.
53. **Kash, J. C., A. G. Goodman, M. J. Korth, and M. G. Katze.** 2006. Hijacking of the host-cell response and translational control during influenza virus infection. Virus Res **119:**111-20.
54. **Kieff, E. D. a. A. B. R.** 2007. Epstein-Barr Virus and Its Replication, p. 2603-2654. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia.
55. **Koppers-Lalic, D., F. A. Rijsewijk, S. B. Verschuren, J. A. van Gaans-Van den Brink, A. Neisig, M. E. Ressing, J. Neefjes, and E. J. Wiertz.** 2001. The UL41-encoded virion host shutoff (vhs) protein and vhs-independent mechanisms are responsible for down-regulation of MHC class I molecules by bovine herpesvirus 1. J Gen Virol **82:**2071-81.
56. **Korom, M., K. M. Wylie, and L. A. Morrison.** 2008. Selective ablation of virion host shutoff protein RNase activity attenuates herpes simplex virus 2 in mice. J Virol **82:**3642-53.
57. **Krishnan, H. H., P. P. Naranatt, M. S. Smith, L. Zeng, C. Bloomer, and B. Chandran.** 2004. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. J Virol **78:**3601-20.
58. **Krug, L. T., J. M. Moser, S. M. Dickerson, and S. H. Speck.** 2007. Inhibition of NF-kappaB activation in vivo impairs establishment of gammaherpesvirus latency. PLoS Pathog **3:**e11.
59. **Kumar, G. R., and B. A. Glaunsinger.** 2010. Nuclear import of cytoplasmic poly(A) binding protein restricts gene expression via hyperadenylation and nuclear retention of mRNA. Mol Cell Biol **30:**4996-5008.
60. **Kwong, A. D., and N. Frenkel.** 1989. The herpes simplex virus virion host shutoff function. J Virol **63:**4834-9.
61. **Lam, Q., C. A. Smibert, K. E. Koop, C. Lavery, J. P. Capone, S. P. Weinheimer, and J. R. Smiley.** 1996. Herpes simplex virus VP16 rescues viral mRNA from destruction by the virion host shutoff function. EMBO J **15:**2575-81.

62. **Lee, K. S., S. D. Groshong, C. D. Cool, B. K. Kleinschmidt-DeMasters, and L. F. van Dyk.** 2009. Murine gammaherpesvirus 68 infection of IFNgamma unresponsive mice: a small animal model for gammaherpesvirus-associated B-cell lymphoproliferative disease. Cancer Res **69:**5481-9.
63. **Lee, Y., and B. Glaunsinger.** 2009. Aberrant herpesvirus-induced polyadenylation correlates with cellular messenger RNA destruction. PLoS Biology **7:**e1000107.
64. **Lee, Y. J., and B. A. Glaunsinger.** 2009. Aberrant herpesvirus-induced polyadenylation correlates with cellular messenger RNA destruction. PLoS Biol **7:**e1000107.
65. **Leight, E. R., and B. Sugden.** 2000. EBNA-1: a protein pivotal to latent infection by Epstein-Barr virus. Rev Med Virol **10:**83-100.
66. **Macrae, A. I., E. J. Usherwood, S. M. Husain, E. Flano, I. J. Kim, D. L. Woodland, A. A. Nash, M. A. Blackman, J. T. Sample, and J. P. Stewart.** 2003. Murid herpesvirus 4 strain 68 M2 protein is a B-cell-associated antigen important for latency but not lymphocytosis. J Virol **77:**9700-9.
67. **Marques, S., S. Efstathiou, K. G. Smith, M. Haury, and J. P. Simas.** 2003. Selective gene expression of latent murine gammaherpesvirus 68 in B lymphocytes. J Virol **77:**7308-18.
68. **Martinez-Guzman, D., T. Rickabaugh, T. T. Wu, H. Brown, S. Cole, M. J. Song, L. Tong, and R. Sun.** 2003. Transcription program of murine gammaherpesvirus 68. J Virol **77:**10488-503.
69. **Martinez, R., R. T. Sarisky, P. C. Weber, and S. K. Weller.** 1996. Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. J Virol **70:**2075-85.
70. **May, J. S., H. M. Coleman, B. Smillie, S. Efstathiou, and P. G. Stevenson.** 2004. Forced lytic replication impairs host colonization by a latency-deficient mutant of murine gammaherpesvirus-68. J Gen Virol **85:**137-46.
71. **Melendez, L. V., M. D. Daniel, R. D. Hunt, and F. G. Garcia.** 1968. An apparently new herpesvirus from primary kidney cultures of the squirrel monkey (Saimiri sciureus). Lab Anim Care **18:**374-81.
72. **Milho, R., C. M. Smith, S. Marques, M. Alenquer, J. S. May, L. Gillet, M. Gaspar, S. Efstathiou, J. P. Simas, and P. G. Stevenson.** 2009. In vivo imaging of murid herpesvirus-4 infection. J Gen Virol **90:**21-32.
73. **Miller, G., L. Heston, E. Grogan, L. Gradoville, M. Rigsby, R. Sun, D. Shedd, V. M. Kushnaryov, S. Grossberg, and Y. Chang.** 1997. Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. J Virol **71:**314-24.
74. **Mocarski, E. S., T. Shenk, and R.F. Pass.** 2007. Cytomegaloviruses, p. 2701-2772. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia.
75. **Moorman, N. J., C. Y. Lin, and S. H. Speck.** 2004. Identification of candidate gammaherpesvirus 68 genes required for virus replication by signature-tagged transposon mutagenesis. J Virol **78:**10282-90.
76. **Moorman, N. J., D. O. Willer, and S. H. Speck.** 2003. The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency. J Virol **77:**10295-303.

77. **Mora, A. L., C. R. Woods, A. Garcia, J. Xu, M. Rojas, S. H. Speck, J. Roman, K. L. Brigham, and A. A. Stecenko.** 2005. Lung infection with gamma-herpesvirus induces progressive pulmonary fibrosis in Th2-biased mice. Am J Physiol Lung Cell Mol Physiol **289:**L711-21.
78. **Moser, J. M., J. W. Upton, K. S. Gray, and S. H. Speck.** 2005. Ex vivo stimulation of B cells latently infected with gammaherpesvirus 68 triggers reactivation from latency. J Virol **79:**5227-31.
79. **Murphy, J. A., R. J. Duerst, T. J. Smith, and L. A. Morrison.** 2003. Herpes simplex virus type 2 virion host shutoff protein regulates alpha/beta interferon but not adaptive immune responses during primary infection in vivo. J Virol **77:**9337-45.
80. **Narayanan, K., C. Huang, K. Lokugamage, W. Kamitani, T. Ikegami, C. T. Tseng, and S. Makino.** 2008. Severe acute respiratory syndrome coronavirus nsp1 suppresses host gene expression, including that of type I interferon, in infected cells. J Virol **82:**4471-9.
81. **O'Connor, C. M., and D. H. Kedes.** 2007. Rhesus monkey rhadinovirus: a model for the study of KSHV. Curr Top Microbiol Immunol **312:**43-69.
82. **Overby, L. R., E. E. Robishaw, J. B. Schleicher, A. Rueter, N. L. Shipkowitz, and J. C. Mao.** 1974. Inhibition of herpes simplex virus replication by phosphonoacetic acid. Antimicrob Agents Chemother **6:**360-5.
83. **Paden, C. R., J. C. Forrest, N. J. Moorman, and S. H. Speck.** 2010. Murine gammaherpesvirus 68 LANA is essential for virus reactivation from splenocytes but not long-term carriage of viral genome. J Virol **84:**7214-24.
84. **Pasieka, T. J., B. Lu, S. D. Crosby, K. M. Wylie, L. A. Morrison, D. E. Alexander, V. D. Menachery, and D. A. Leib.** 2008. Herpes simplex virus virion host shutoff attenuates establishment of the antiviral state. J Virol **82:**5527-35.
85. **Pellet, P. E., and B. Roizman.** 2007. The Family *Herpesviridae*: A Brief Introduction, p. 2479 - 2499. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia.
86. **Phelan, A., M. Carmo-Fonseca, J. McLaughlan, A. I. Lamond, and J. B. Clements.** 1993. A herpes simplex virus type 1 immediate-early gene product, IE63, regulates small nuclear ribonucleoprotein distribution. Proc Natl Acad Sci U S A **90:**9056-60.
87. **Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug.** 1981. A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23:**847-58.
88. **Rajcani, J., D. Blaskovic, J. Svobodova, F. Ciampor, D. Huckova, and D. Stanekova.** 1985. Pathogenesis of acute and persistent murine herpesvirus infection in mice. Acta Virol **29:**51-60.
89. **Reuven, N. B., S. Antoku, and S. K. Weller.** 2004. The UL12.5 gene product of herpes simplex virus type 1 exhibits nuclease and strand exchange activities but does not localize to the nucleus. J Virol **78:**4599-608.
90. **Reuven, N. B., A. E. Staire, R. S. Myers, and S. K. Weller.** 2003. The herpes simplex virus type 1 alkaline nuclease and single-stranded DNA binding protein mediate strand exchange in vitro. J Virol **77:**7425-33.
91. **Richman, L. K., R. J. Montali, R. L. Garber, M. A. Kennedy, J. Lehnhardt, T. Hildebrandt, D. Schmitt, D. Hardy, D. J. Alcendor, and G. S. Hayward.** 1999. Novel

endotheliotropic herpesviruses fatal for Asian and African elephants. Science **283:**1171-6.

92. **Rickabaugh, T. M., H. J. Brown, D. Martinez-Guzman, T. T. Wu, L. Tong, F. Yu, S. Cole, and R. Sun.** 2004. Generation of a latency-deficient gammaherpesvirus that is protective against secondary infection. J Virol **78:**9215-23.
93. **Rickinson, A. B., and E. Kieff.** 2007. Epstein-Barr Virus, p. 2655 - 2700. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.
94. **Rosa, G. T., L. Gillet, C. M. Smith, B. D. de Lima, and P. G. Stevenson.** 2007. IgG fc receptors provide an alternative infection route for murine gamma-herpesvirus-68. PLoS ONE **2:**e560.
95. **Rowe, M., B. Glaunsinger, D. van Leeuwen, J. Zuo, D. Sweetman, D. Ganem, J. Middeldorp, E. J. Wiertz, and M. E. Ressing.** 2007. Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proc Natl Acad Sci U S A **104:**3366-71.
96. **Sadler, A. J., and B. R. Williams.** 2007. Structure and function of the protein kinase R. Curr Top Microbiol Immunol **316:**253-92.
97. **Samady, L., E. Costigliola, L. MacCormac, Y. McGrath, S. Cleverley, C. E. Lilley, J. Smith, D. S. Latchman, B. Chain, and R. S. Coffin.** 2003. Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs- HSV vectors for dendritic cell-mediated immunotherapy. J Virol **77:**3768-76.
98. **Sandri-Goldin, R. M., M. K. Hibbard, and M. A. Hardwicke.** 1995. The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. J Virol **69:**6063-76.
99. **Sears, J., M. Ujihara, S. Wong, C. Ott, J. Middeldorp, and A. Aiyar.** 2004. The amino terminus of Epstein-Barr Virus (EBV) nuclear antigen 1 contains AT hooks that facilitate the replication and partitioning of latent EBV genomes by tethering them to cellular chromosomes. J Virol **78:**11487-505.
100. **Shao, L., L. M. Rapp, and S. K. Weller.** 1993. Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. Virology **196:**146-62.
101. **Sheaffer, A. K., S. P. Weinheimer, and D. J. Tenney.** 1997. The human cytomegalovirus UL98 gene encodes the conserved herpesvirus alkaline nuclease. J Gen Virol **78 (Pt 11):**2953-61.
102. **Smiley, J. R., M. M. Elgadi, and H. A. Saffran.** 2001. Herpes simplex virus vhs protein. Methods Enzymol **342:**440-51.
103. **Smith, G. A., and L. W. Enquist.** 1999. Construction and transposon mutagenesis in Escherichia coli of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. J Virol **73:**6405-14.
104. **Sokoloski, K. J., E. L. Chaskey, and J. Wilusz.** 2009. Virus-mediated mRNA decay by hyperadenylation. Genome Biol **10:**234.
105. **Song, M. J., S. Hwang, W. H. Wong, T. T. Wu, S. Lee, H. I. Liao, and R. Sun.** 2005. Identification of viral genes essential for replication of murine gamma-herpesvirus 68 using signature-tagged mutagenesis. Proc Natl Acad Sci U S A **102:**3805-10.

106. **Speck, S. H., and D. Ganem.** 2010. Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe **8:**100-15.
107. **Speck, S. H., and H. W. Virgin.** 1999. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. Curr Opin Microbiol **2:**403-9.
108. **Staudt, M. R., and D. P. Dittmer.** 2007. The Rta/Orf50 transactivator proteins of the gamma-herpesviridae. Curr Top Microbiol Immunol **312:**71-100.
109. **Stevenson, P. G., and S. Efstathiou.** 2005. Immune mechanisms in murine gammaherpesvirus-68 infection. Viral Immunol **18:**445-56.
110. **Stevenson, P. G., S. Efstathiou, P. C. Doherty, and P. J. Lehner.** 2000. Inhibition of MHC class I-restricted antigen presentation by gamma 2-herpesviruses. Proc Natl Acad Sci U S A **97:**8455-60.
111. **Strelow, L. I., and D. A. Leib.** 1995. Role of the virion host shutoff (vhs) of herpes simplex virus type 1 in latency and pathogenesis. J Virol **69:**6779-86.
112. **Sun, Q., S. Zachariah, and P. M. Chaudhary.** 2003. The human herpes virus 8-encoded viral FLICE-inhibitory protein induces cellular transformation via NF-kappaB activation. J Biol Chem **278:**52437-45.
113. **Sunil-Chandra, N. P., J. Arno, J. Fazakerley, and A. A. Nash.** 1994. Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am J Pathol **145:**818-26.
114. **Sunil-Chandra, N. P., S. Efstathiou, J. Arno, and A. A. Nash.** 1992. Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J Gen Virol **73 (Pt 9):**2347-56.
115. **Sunil-Chandra, N. P., S. Efstathiou, and A. A. Nash.** 1992. Murine gammaherpesvirus 68 establishes a latent infection in mouse B lymphocytes in vivo. J Gen Virol **73 (Pt 12):**3275-9.
116. **Swanton, C., D. J. Mann, B. Fleckenstein, F. Neipel, G. Peters, and N. Jones.** 1997. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature **390:**184-7.
117. **Tarakanova, V. L., E. Stanitsa, S. M. Leonardo, T. M. Bigley, and S. B. Gauld.** 2010. Conserved gammaherpesvirus kinase and histone variant H2AX facilitate gammaherpesvirus latency in vivo. Virology **405:**50-61.
118. **Tarakanova, V. L., F. Suarez, S. A. Tibbetts, M. A. Jacoby, K. E. Weck, J. L. Hess, S. H. Speck, and H. W. t. Virgin.** 2005. Murine gammaherpesvirus 68 infection is associated with lymphoproliferative disease and lymphoma in BALB beta2 microglobulin-deficient mice. J Virol **79:**14668-79.
119. **Tempera, I., and P. M. Lieberman.** 2009. Chromatin organization of gammaherpesvirus latent genomes. Biochim Biophys Acta **1799:**236-45.
120. **Thompson, S. R., and P. Sarnow.** 2000. Regulation of host cell translation by viruses and effects on cell function. Curr Opin Microbiol **3:**366-70.
121. **Trgovcich, J., D. Johnson, and B. Roizman.** 2002. Cell surface major histocompatibility complex class II proteins are regulated by the products of the gamma(1)34.5 and U(L)41 genes of herpes simplex virus 1. J Virol **76:**6974-86.
122. **van Dyk, L. F., H. W. t. Virgin, and S. H. Speck.** 2000. The murine gammaherpesvirus 68 v-cyclin is a critical regulator of reactivation from latency. J Virol **74:**7451-61.
123. **van Gent, M., B. D. Griffin, E. G. Berkhoff, D. van Leeuwen, I. G. Boer, M. Buisson, F. C. Hartgers, W. P. Burmeister, E. J. Wiertz, and M. E. Ressing.** 2011. EBV lytic-

phase protein BGLF5 contributes to TLR9 downregulation during productive infection. J Immunol **186:**1694-702.

124. **Ventoso, I., M. A. Sanz, S. Molina, J. J. Berlanga, L. Carrasco, and M. Esteban.** 2006. Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. Genes Dev **20:**87-100.
125. **Vetsika, E. K., and M. Callan.** 2004. Infectious mononucleosis and Epstein-Barr virus. Expert Rev Mol Med **6:**1-16.
126. **Virgin, H. W., P. Latreille, P. Wamsley, K. Hallsworth, K. E. Weck, A. J. Dal Canto, and S. H. Speck.** 1997. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol **71:**5894-904.
127. **Weck, K. E., A. J. Dal Canto, J. D. Gould, A. K. O'Guin, K. A. Roth, J. E. Saffitz, S. H. Speck, and H. W. Virgin.** 1997. Murine gamma-herpesvirus 68 causes severe large-vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus-induced vascular disease. Nat Med **3:**1346-53.
128. **Weck, K. E., S. S. Kim, H. I. Virgin, and S. H. Speck.** 1999. Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells. J Virol **73:**3273-83.
129. **Weinberg, J. B., M. L. Lutzke, R. Alfinito, and R. Rochford.** 2004. Mouse strain differences in the chemokine response to acute lung infection with a murine gammaherpesvirus. Viral Immunol **17:**69-77.
130. **Weller, S. K., M. R. Seghatoleslami, L. Shao, D. Rowse, and E. P. Carmichael.** 1990. The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. J Gen Virol **71 (Pt 12):**2941-52.
131. **Whitlow, Z. W., J. H. Connor, and D. S. Lyles.** 2006. Preferential translation of vesicular stomatitis virus mRNAs is conferred by transcription from the viral genome. J Virol **80:**11733-42.
132. **Wilkinson, D. E., and S. K. Weller.** 2003. The role of DNA recombination in herpes simplex virus DNA replication. IUBMB Life **55:**451-8.
133. **Yamanishi, K., Y. Mori, and P.E. Pellett.** 2007. Human Herpesviruses 6 and 7, p. 2819-2845. *In* D. M. Knipe, and P.M. Howley (ed.), Fields Virology, 5th ed, vol. 2. Lippincott Williams & Wilkins, Philadelphia.
134. **Yu, D., M. C. Silva, and T. Shenk.** 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc Natl Acad Sci U S A **100:**12396-401.
135. **Zelus, B. D., R. S. Stewart, and J. Ross.** 1996. The virion host shutoff protein of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. J Virol **70:**2411-9.
136. **Zuo, J., W. Thomas, D. van Leeuwen, J. M. Middeldorp, E. J. Wiertz, M. E. Ressing, and M. Rowe.** 2008. The DNase of gammaherpesviruses impairs recognition by virus-specific CD8+ T cells through an additional host shutoff function. J Virol **82:**2385-93.