A Gammaherpesvirus MicroRNA Targets EWSR1 (Ewing Sarcoma Breakpoint Region 1) In Vivo To Promote Latent Infection of Germinal Center B Cells

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ABSTRACT  Gammaherpesviruses, including the human pathogens Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), directly contribute to the genesis of multiple types of malignancies, including B cell lymphomas. In vivo, these viruses infect B cells and manipulate B cell biology to establish lifelong latent infection. To accomplish this, gammaherpesviruses employ an array of gene products, including microRNAs (miRNAs). Although numerous host mRNA targets of gammaherpesvirus miRNAs have been identified, the in vivo relevance of repression of these targets remains elusive due to species restriction. Murine gammaherpesvirus 68 (MHV68) provides a robust virus-host system to dissect the in vivo function of conserved gammaherpesvirus genetic elements. We identified here MHV68 mghv-miR-M1-7-5p as critical for in vivo infection and then validated host EWSR1 (Ewing sarcoma breakpoint region 1) as the predominant target for this miRNA. Using novel, target-specific shRNA-expressing viruses, we determined that EWSR1 repression in vivo was essential for germinal center B cell infection. These findings provide the first in vivo demonstration of the biological significance of repression of a specific host mRNA by a gammaherpesvirus miRNA.

IMPORTANCE  Gammaherpesviruses, including the human pathogens Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), directly contribute to the genesis of multiple types of malignancies. In vivo, these viruses infect B cells and manipulate B cell biology to establish lifelong infection. To accomplish this, gammaherpesviruses employ an array of gene products, including miRNAs, short noncoding RNAs that bind to and repress protein synthesis from specific target mRNAs. The in vivo relevance of repression of targets of gammaherpesvirus miRNAs remains highly elusive. Here, we identified a murine gammaherpesvirus miRNA as critical for in vivo infection and validated the host mRNA EWSR1 (Ewing sarcoma breakpoint region 1) as the predominant target for this miRNA. Using a novel technology, we demonstrated that repression of EWSR1 was essential for in vivo infection of the critical B cell reservoir. These findings provide the first in vivo demonstration of the significance of repression of a specific host mRNA by a gammaherpesvirus miRNA.

KEYWORDS  EWSR1, MHV68, gammaherpesvirus, in vivo, miRNA

Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) are ubiquitous human pathogens of the gammaherpesvirus family. Gammaherpesvirus infections are asymptomatic in immunocompetent individuals; however, in the setting of immunocompromise, these viruses are highly associated with the development of multiple types of malignances, including Burkitt’s B cell lymphoma, Hodgkin’s B cell lymphoma, nasopharyngeal carcinoma, gastric carcinoma, and Kaposi’s sarcoma (1–3).

A hallmark of gammaherpesvirus infections is their ability to switch between two distinct phases: lytic infection and latency. While lytic infection is a process defined by...
robust replication of the viral DNA genome, abundant expression of a majority of viral
genes, and the production of infectious viral particles, latency is characterized by the
restricted expression of a small subset of viral genes and the stable maintenance of viral
genome as an episome (4–6). During chronic infection, gammaherpesviruses establish
latency in the circulating reservoir of resting memory B cells (3, 7). The specific
mechanisms by which these viruses establish infection in the memory B cell population
in vivo remains poorly understood; however, several key findings have led to a
well-accepted model of gammaherpesvirus-driven B cell maturation in which the virus
initially infects naive B cells and then, independent of antigen, drives infected cells
through germinal center reactions into the memory B cell compartment (3, 7). As such,
germinal center B cells represent an essential stage of B cell differentiation and are a
major target for transformation in gammaherpesvirus-associated lymphomagenesis.

Gammaherpesviruses employ multiple molecular mechanisms to restrict viral gene
expression and evade the host immune system, thereby ensuring efficient establish-
ment of latency for the life of the host. One of these strategies is the transcription of
noncoding RNAs (ncRNAs) such as microRNAs (miRNAs). miRNAs are a class of small,
evolutionarily conserved regulatory RNA molecules approximately 22 nucleotides (nt)
in length. Typically, miRNAs posttranscriptionally regulate gene expression by binding
with imperfect complementarity to cognate sequences within mRNA target transcripts
(8). As a result of miRNA binding within the context of the RNA-induced silencing
complex (RISC), miRNA targets are silenced through mechanisms involving mRNA
degradation, destabilization, and translational repression (8, 9). Since their discovery, a
wide range of studies have revealed that mammalian miRNAs are involved in multiple
host biological processes, including development and differentiation, cell survival,
apoptosis, and immune system regulation (10). Thus, it is not surprising that many
viruses, and in particular herpesviruses, have evolved to utilize miRNAs to fine-tune
host and viral gene expression, contributing to immune evasion and promoting
persistent virus infection (11–14).

The human gammaherpesviruses EBV and KSHV encode 25 and 12 pre-miRNAs,
which generate at least 44 and 25 mature miRNAs, respectively (12, 13, 15–18).
Numerous putative targets for EBV and KSHV miRNAs have been identified through
bioinformatic analyses of high-throughput sequencing data from RNA isolated by
cross-linking immunoprecipitation assays, and more recently, through cross-linking,
ligation, and sequencing of hybrids (CLASH) (19–21). Repression of some of these
putative targets during virus infection has been shown to modulate essential cellular
pathways in vitro, promoting critical functions such as cell survival and proliferation and
dampening host innate immune responses (reviewed in references 14, 22, 23, and 24).
Despite this growing knowledge base, the specific in vivo functions of these miRNAs
and their targets in the natural host are largely unknown due to the strict species
specificity of these viruses.

Murine gammaherpesvirus 68 (MHV68) is a natural pathogen of rodents that is
genetically and pathogenically related to EBV and KSHV (25, 26). Thus, MHV68 infection
of mice provides a robust small animal model for investigating specific molecular and
cellular aspects of gammaherpesvirus latency and pathogenesis in vivo (25). Like the
human gammaherpesviruses, MHV68 establishes a stable and lifelong latency in B cells
and is highly associated with the development of lymphoproliferative disorders and B
cell lymphomas (27–29). MHV68 encodes a cluster of 14 pre-miRNAs, which generate
up to 28 mature miRNAs (13, 30–33). Interestingly, the MHV68 pre-miRNAs are all
generated from unique transcripts that encode tRNA-like elements (vtRNAs) with
embedded RNA polymerase III promoters upstream of one or two pre-miRNA stem-
loops (34, 35). MHV68 encodes eight of these tRNA-miRNA-encoding RNA (TMER)
transcripts (30, 36), which range in size from 200 to 250 nt and differ significantly in
sequence despite their similar structural features. The TMER transcripts are processed
using a noncanonical miRNA biogenesis pathway that utilizes tRNase Z instead of
Dicer to yield pre-miRNAs that are subsequently processed to mature miRNAs by
Dicer (34, 35).
Notably, the MHV68 TMERs, like the EBV EBERs, are among the only transcripts expressed in vivo in latently infected B cells and in infected B cells associated with lymphoproliferative disease and lymphoma (37–39). Likewise, we and others have demonstrated that MHV68 mature miRNAs are extensively expressed during latent infection (13, 30–35), suggesting that these TMER-encoded miRNAs may hold essential biological functions during chronic infection. Consistent with this possibility, we have previously demonstrated that an MHV68 mutant deficient in all 14 pre-miRNAs is attenuated for latency establishment in vivo (30). However, which miRNAs and miRNA targets mediate this phenotype remain unclear.

We describe here our efforts to use the MHV68 system to define for the first time the in vivo significance of miRNA-mediated repression of a specific host mRNA target. Using a panel of MHV68 TMER mutants, we determined that TMERS-encoded MHV68 miR-7 is required for efficient establishment of MHV68 latency in vivo and sought to determine the target that mediates this requirement. Using our recently defined set of qCLASH-identified host mRNA targets of MHV68 miRNAs (72) as a starting point, we validated molecular repression of the top miR-7 targets. Among these, the most highly repressed host mRNA transcript was EWSR1, which encodes the RNA binding protein and transcriptional repressor/activator EWSR1/EWS (40–42). Notably, shRNA-mediated selective repression of EWSR1 in miR-7-deficient viruses fully restored latent infection in vivo, demonstrating a key biological role for miR-7-5p repression of EWSR1. Moreover, miR-7-5p repression of EWSR1 specifically promoted latent infection of germinal center B cells, suggesting that EWSR1 repression may be a key regulatory step for gammaherpesvirus-driven B cell differentiation. This is to our knowledge the first identification of a biological role for a specific gammaherpesvirus miRNA-host mRNA interaction in vivo.

RESULTS

MHV68 miR-7 is required for the establishment of splenic latency in vivo. We have previously determined that the TMERS-derived miRNA mghv-miR-M1-7-5p is expressed in vivo during latency (30); however, whether this or any other TMERS-derived miRNA play a biologically significant role during infection is unknown. To assess this possibility, we generated a mutant virus, MHV68.ΔmiR7.12, which is deficient in both miR-7 and miR-12 pre-miRNA stem-loops (Fig. 1A; see Tables S1 and S2 in the supplemental material). Mutants were generated on the backbone of MHV68.ORF73βla, a β-lactamase-expressing marker virus that displays wild-type (WT) phenotypes during both lytic and latent infection (43).

![FIG 1](MBI034036-0011.png)  
**MHV68 TMER5 is required for efficient establishment of splenic latency in vivo.** (A) Schematic diagram of viruses carrying wild-type TMER5 (MHV68.WT) or mutated TMER5 carrying deletions of both pre-miRNA stem-loops (MHV68.ΔmiR7.12). (B) Virus titers during multistep lytic replication in vitro. NIH 3T12 fibroblasts were infected with MHV68.WT or MHV68.ΔmiR7.12 at an MOI of 0.05 and then overlaid with methylcellulose. At the indicated time points, cells and supernatants were harvested, and viral titers were determined by plaque assay. Values represent the means ± the standard errors of the mean (SEM). Each growth curve was performed in duplicate in each of two independent experiments. Significance was determined using a two-tailed, unpaired t test (*, P < 0.05). (C) Presence of viral genome in latently infected splenocytes harvested from in vivo samples. Three wild-type B6 mice per sample group per experiment were infected i.n. with 10^4 PFU of MHV68.WT or MHV68.ΔmiR7.12. After 16 days, splenocytes were harvested, pooled, and then subjected to limiting-dilution nested PCR to detect the presence of viral genome. The frequencies of cells harboring viral genome were determined using a Poisson distribution, as indicated by the line at 63.2%. Values represent means ± the SEM of three independent experiments. Significance was determined using a two-tailed, unpaired t test (*, P < 0.05). Notably, the MHV68 TMERs, like the EBV EBERs, are among the only transcripts expressed in vivo in latently infected B cells and in infected B cells associated with lymphoproliferative disease and lymphoma (37–39). Likewise, we and others have demonstrated that MHV68 mature miRNAs are extensively expressed during latent infection (13, 30–35), suggesting that these TMER-encoded miRNAs may hold essential biological functions during chronic infection. Consistent with this possibility, we have previously demonstrated that an MHV68 mutant deficient in all 14 pre-miRNAs is attenuated for latency establishment in vivo (30). However, which miRNAs and miRNA targets mediate this phenotype remain unclear.

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To first determine whether miR-7 or miR-12 contributed to lytic virus infection, we quantified the replication capacity of MHV68.ΔmiR7.12 versus parental WT MHV68.ORF73/H9252 (MHV68.WT) virus in both in vitro and in vivo assays. For in vitro multistep growth curve assays, NIH 3T12 fibroblasts were infected at a multiplicity of infection (MOI) of 0.05. Samples were then harvested at time points from 0 to 120h, and virus titers were determined by plaque assay (Fig. 1B). At 48h postinfection, MHV68.ΔmiR7.12 titers were significantly reduced compared to WT virus. However, titers recovered to nearly WT by later time points, indicating a slight delay in MHV68.ΔmiR7.12 replication. To determine whether this mutant virus displayed a similar replication delay in vivo, wild-type B6 mice per sample group per experiment were infected i.n. with 10^6 PFU of MHV68.WT, MHV68.ΔmiR7, or MHV68.ΔmiR12. After 16 days, splenocytes were harvested, pooled, and then subjected to limiting-dilution nested PCR to detect the presence of viral genome. The frequencies of cells harboring viral genome were determined using a Poisson distribution, as indicated by the line at 63.2%. Values represent means ± the SEM of three independent experiments. Significance was determined using a two-tailed, unpaired t test (*, P < 0.05).

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To define whether the critical function of TMERS-derived miRNAs in latency was mediated by miRNAs derived from pre-miR-7 or pre-miR-12, we generated two other mutant viruses, MHV68.ΔmiR7 and MHV68.ΔmiR12, which carry deletions of single pre-miR-7 or pre-miR-12 stem-loops, respectively (Fig. 2A; see Tables S1 and S2). Interestingly, in multistep lytic growth curves, both single stem-loop mutant viruses displayed slightly delayed replication kinetics (Fig. 2B) similar to the double stem-loop mutant MHV68.ΔmiR7.12 (Fig. 1B). However, any replication defects were again relatively minor as the delay was quickly overcome at later times postinfection.

In contrast to the comparable replication delays observed for both mutant viruses during lytic infection, the two mutant viruses demonstrated disparate phenotypes...
during latency. MHV68ΔmiR7 displayed a reproducible 6.1-fold attenuation to a frequency of 1 in 2,300 (Fig. 2C; see Table S3), which is similar to the defect observed during MHV68ΔmiR12 infection. Conversely, the ability of MHV68ΔmiR12 to establish latency (1 in 270) was similar to that of wild-type virus (1 in 380), demonstrating that miRNAs derived from miR-12 are completely dispensable for latent infection. Taken together, these data demonstrate that MHV68 pre-miR-7 is the critical determinant of TMER5 required for the efficient establishment of splenic latency in vivo.

mghv-mir-M1-7-5p targets and represses EWSR1 in vitro and in vivo. The regulatory function of miRNAs is typically mediated through sequence-specific binding to cognate mRNA targets (8). Because the pre-miR-7-deficient virus was attenuated for latency in vivo, we hypothesized that repression of at least one specific mRNA target of a pre-miR-7-derived miRNA must promote latent infection. To molecularly define specific targets of all MHV68 miRNAs, we have previously performed qCLASH on B cells latently infected with MHV68 (72). Within this study, we identified 10 and 15 targets of miR-7-5p and miR-7-3p, respectively (Table S4). However, because miRNA binding to mRNA targets does not necessarily equate to repression of the target transcript, we sought to molecularly validate these miR-7 targets in work described here. To focus these additional studies, we selected the top four most prevalent targets for each miRNA for molecular validation: miR-7-5p targets RGS16 (regulator of G-protein signaling 16), EWSR1, ARHGEF18 (rho/rac guanine nucleotide exchange factor 18), and BIRC5 (baculoviral IAP repeat containing 5), whereas miR-7-3p targets TMEM38B (transmembrane protein 38B), LARS2 (leucyl-tRNA synthetase 2 mitochondrial), RNF138 (ring finger protein 138), and ANAPC7 (anaphase promoting complex subunit 7).

To determine whether the binding of these specific miRNAs to their cognate qCLASH-identified target transcripts resulted in target repression, we quantified endogenous mRNA levels in the presence of individual miRNAs. NIH 3T12 cells were mock transfected or were transfected with miR-7-5p mimic (Fig. 3A) or miR-7-3p mimic (Fig. 3B); 24 h later, the levels of specific host transcripts were quantified by quantitative reverse transcription-PCR (qRT-PCR). Endogenous EWSR1 and ARHGEF18 transcripts were significantly reduced (61 and 40% reductions, respectively) in the presence of miR-7-5p, whereas the expression of BIRC5 transcript was not affected. Unexpectedly, RGS16 expression was increased 145% in the presence of miR-7-5p, suggesting that miR-7-5p may stabilize rather than repress this host transcript. In contrast, of the four miR-7-3p targets tested, only TMEM38B expression was significantly repressed (36% reduction). Thus, of the top miRNA-mRNA target interactions tested, miR-7-5p binding to EWSR1 resulted in the highest degree of target repression.

These results indicated that EWSR1 may be an important target for repression by miR-7-derived miRNAs. This level of repression was mediated by strong sequence complementarity between miR-7-5p and EWSR1, as defined by alignment of the short Ago-associated hybrid sequences recovered in qCLASH experiments (Fig. 3C). Interestingly, as opposed to miRNA binding interactions which frequently occur within the 3′ untranslated region (3′UTR) of the target transcript, miR-7-5p bound to the coding sequence of EWSR1. Supporting our observations of strong EWSR1 repression by miR-7-5p, this interaction displayed a high degree of complementarity throughout, including 6 of 7 nt within the seed sequence (nt 2 to 8) of the miRNA. Consistent with the strong repression of endogenous EWSR1 mRNA (Fig. 3A) mediated by this miRNA, miR-7-5p also resulted in a striking repression of both luciferase target transcripts carrying the EWSR1 miRNA binding sequence (Fig. 3D) and endogenous levels of EWSR1 protein (Fig. 3E).

Like EBV and KSHV, MHV68 predominantly establishes latent infection in circulating mature B cells (27, 43, 46–51). To determine whether EWSR1 is specifically repressed in latently infected B cells in vivo, we infected mice with MHV68.H2bYFP, an enhanced yellow fluorescent protein (eYFP)-marked recombinant MHV68 that is phenotypically wild type (46). At 16 dpi, splenocytes were harvested and sorted into infected (CD4+ CD8− CD14− CD19+ YFP+) versus noninfected (CD4− CD8− CD14− CD19+ YFP−) B cells
After RNA extraction, \(EWSR1\) and control \(BIRC5\) mRNA expression levels were determined by qRT-PCR (Fig. 4B). Compared to YFP– noninfected B cells, the level of \(EWSR1\) transcript was significantly decreased in YFP+/H11001 latently infected B cells. In contrast, the expression of control \(BIRC5\) transcript, which was not altered by \(miR-7-5p\) in vitro (Fig. 3A), was not inhibited in vivo and instead was increased during in vivo virus infection (Fig. 4B). Together, these data demonstrated that \(EWSR1\) is strongly repressed by \(miR-7-5p\) both in vitro and in vivo.

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**Specific repression of EWSR1 promotes MHV68 splenic latency in vivo.** The reduced *EWSR1* transcripts in infected B cells *in vivo* suggested that repression of EWSR1 protein may play an important role in regulating MHV68 latency. Because *miR-7* was required for efficient latency establishment *in vivo* (Fig. 2C), we hypothesized that specific repression of *EWSR1* by *miR-7-5p* promotes MHV68 latency. To test this hypothesis, we sought to provide a mechanistic link between specific targeting of *EWSR1* and *in vivo* latency. Fortunately, the MHV68 **TMER5**s offer a unique means to selectively target individual transcripts in infected cells: it has been previously demonstrated (i) that non-MHV68 miRNAs are processed normally and are fully functional in the context of a **TMER** unit (34) and (ii) that short hairpin RNAs (shRNAs), which resemble pre-miRNA stem-loops, can generate functional siRNAs when fused downstream of a tRNA (52). Thus, we speculated that direct replacement of the MHV68 *pre-miR-7* stem-loop with a sequence-specific shRNA directed against *EWSR1* should generate a functional and highly specific anti-*EWSR1* siRNA.

To test this possibility, we first designed four *EWSR1*-specific small interfering RNAs (siRNAs) (EW-siR-1, -2, -3, and -4), and four control siRNAs with scrambled sequences (SC-siR-1, -2, -3, and -4) (Fig. S2A) and then tested their ability to suppress *EWSR1*. NIH 3T12 cells were transfected with *EWSR1*-specific or scrambled siRNAs, and Western blotting for EWSR1 protein was performed 48 h after transfection (Fig. S2B). Compared to their respective scrambled siRNAs, *EWSR1*-specific siR-2, -3, and -4 exhibited potent inhibitory effects on target gene expression. Based on these results, *EWSR1*-specific siR-2 and -3, and their respective scrambled siRNAs, were chosen for generation of shRNAs for replacement of the *pre-miR-7* stem-loop. shRNAs were generated from the siRNA sequence, using the loop sequence “TCAAGAG” to link the siRNA with complementary base pairs on the stem-loop (Table S2 and Fig. S3).

To test whether the *EWSR1*-specific shRNAs could functionally restore *miR-7* activity, we generated viruses carrying the anti-*EWSR1* or scrambled shRNAs in place of the **TMER5**-derived miRNA stem-loops in the MHV68.ΔmiR7.12 virus. As described above (Fig. 2C), **TMER5**-encoded *pre-miR-7* was crucial for the efficient establishment of latency *in vivo*, but **TMER5**-encoded *pre-miR-12* was completely dispensable for infection. Likewise, the **TMER5** double stem-loop mutant virus MHV68.ΔmiR7.12, lacking both *pre-miR-7* and *pre-miR-12*, displayed an attenuated phenotype equivalent to that of the single *pre-miR-7* stem-loop mutant virus (Fig. 1C). Thus, to maximize shRNA efficacy in the context of virus infection, we inserted anti-*EWSR1* or scrambled shRNAs in place of both **TMER5**-derived miRNA stem-loops (Table S2). Importantly, *in silico* Mfold (53) predictions of **TMER5** folding indicated that the secondary structure of the **TMER5** vRNA was not affected by shRNA insertions (Fig. 5A).

To verify that **TMER5**-derived shRNAs retained functional activity, we examined repression of EWSR1 in luciferase assays and in assays for endogenous mRNA and endogenous protein. NIH 3T12 cells were transfected with recombinant pUC57 vectors carrying recombinant shRNA-expressing **TMER5**s. Notably, **TMER5**-encoded anti-*EWSR1* shRNAs significantly repressed the activity of luciferase transcripts containing a portion of the *EWSR1* coding region (nt 1000 to 1539) (Fig. 5B), demonstrating that the shRNAs are functionally active when encoded within **TMER5** transcriptional units. Likewise, **TMER5**-derived anti-*EWSR1* shRNAs suppressed endogenous *EWSR1* transcripts (Fig. 5C) and endogenous *EWSR1* protein (Fig. 5D). As expected, the repression of endogenous EWSR1 protein levels was not as robust from plasmid-expressed, **TMER5**-derived transcripts, which require expression and processing, as it was for direct transfection of siRNAs. Cumulatively, these findings demonstrated that anti-*EWSR1* shRNAs maintained functionality when expressed downstream of vRNA5 in the **TMER5** transcript.

Based on these results, we incorporated the anti-*EWSR1* shRNAs or scrambled shRNAs into the MHV68 genome in place of the *pre-miR-7* and *pre-miR-12* stem-loops to generate the recombinant viruses MHV68.EW.shR and MHV68.SC.shR, respectively (Fig. 5E). To verify that **TMER5**-derived shRNAs could be functionally expressed in the context of virus infection, NIH 3T12 cells were infected with MHV68.EW.shR or MHV68.SC.shR, and the levels of
endogenous EWSR1 mRNA and EWSR1 protein were quantified using qRT-PCR and Western blotting. Importantly, recombinant virus expressing anti-EWSR1 shRNAs significantly repressed endogenous mRNA (Fig. 5F) and protein (Fig. 5G) levels, demonstrating functionality of shRNAs expressed from the virus itself.
To determine whether recombinant virus expressing anti-
EWSR1 shRNAs specifically repressed EWSR1 transcript in latently infected B cells in vivo, we infected mice with wild-type MHV68, MHV68.EW.shR, or MHV68.SC.shR for 16 days and quantified EWSR1 expression in infected B cells. The phenotypically wild-type parental virus used in these studies (MHV68.ORF73/H9252) expresses /H9252-lactamase as a C-terminal fusion to the latency-associated nuclear antigen (mLANA); the use of this marker in combination with the fluorescent /H9252-lactamase substrate CCF4-AM facilitates the identification of infected cells from in vivo samples (43). Thus, following spleen harvest, infected B cells were flow cytometrically sorted based on CCF4-AM /H11001CD19 /H11001 gating (Fig. 5H). After RNA extraction from sorted cells, EWSR1 mRNA expression was determined by qRT-PCR (Fig. 5I). Notably, B cells infected with mutant virus carrying control scrambled shRNAs in place of pre-miR-7 displayed increased levels of EWSR1 transcript, providing additional evidence that TMERS-derived miRNAs suppress EWSR1 in vivo. In contrast, the level of EWSR1 in B cells infected with mutant virus carrying anti-EWSR1 shRNAs was reduced to a level below that of wild-type virus, demonstrating effective shRNA-mediated repression of EWSR1 in vivo. Together, these results demonstrated that anti-EWSR1 shRNAs expressed downstream of vtRNA5 in the TMERS transcriptional unit are fully functional during MHV68 infection.

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pre-miR-12 (Fig. 1B), which was not rescued by insertion of anti-EWSR1 shRNAs (Fig. S4).

Cumulatively, these data demonstrated that repression of the single miRNA target EWSR1 could functionally restore the attenuated latency phenotype of viruses deficient in pre-miR-7. Moreover, these findings demonstrate an essential role for viral miRNA-mediated repression of EWSR1 in promoting the establishment of MHV68 latency.

**Repression of EWSR1 modulates latent infection of germinal center B cells.**

Gammaherpesviruses have evolved multiple mechanisms to manipulate infected B cells, allowing the virus to drive naive B cells, independent of antigen, through the germinal center B cell stage into the memory B cell compartment that serves as the long-term latency reservoir (3, 7, 25, 55). To determine whether EWSR1 repression was required for any specific stage of mature B cell differentiation, we performed multiparametric flow cytometric analysis using staining for virus-positive cells in combination with staining for specific B cell surface markers. Thus, 16 days after infection of wild-type B6 mice with control wild-type virus or viruses expressing scrambled or anti-EWSR1 shRNAs, splenocytes were harvested for B cell subset staining with CCF4-AM and with antibodies directed against the B cell surface markers CD19, GL7, and IgM to identify naive, germinal center, and memory B cells and with β-lactamase substrate CCF4-AM to identify virus-infected cells. (B) Percent of virus-positive splenocytes. The percentage of total splenocytes staining positive for CCF4-AM cleavage is indicated for each infection group. (C) Percent of virus-positive CD19+ B cells displaying surface markers consistent with naive B cells (GL7– IgM–). (D) Percent of virus-positive CD19+ B cells displaying surface markers consistent with germinal center B cells (GL7+ IgM+). (E) Percent of virus-positive CD19+ B cells displaying surface markers consistent with memory B cells (GL7– IgM–). The values represent the means ± the SEM of five independent experiments. Significance was determined using a two-tailed, unpaired t test (**, P < 0.01; *, P < 0.05; ns, not significant).

**FIG 7** In vivo repression of EWSR1 promotes the infection of germinal center B cells. (A) Schematic of gating strategy used for multiparameter flow cytometric analysis of virus-positive B cell subsets, including representative flow plots. Wild-type B6 mice were infected i.n. with 10⁴ PFU of viruses carrying wild-type TMERS (MHV68.WT) or TMERS carrying anti-EWSR1 shRNAs (MHV68.EW.shR) or scrambled shRNAs (MHV68.SC.shR) in place of pre-miR-7 and pre-miR-12 stem-loops. After 16 days, the splenocytes were stained with antibodies directed against CD19, GL7, and IgM to identify naive, germinal center, and memory B cells and with β-lactamase substrate CCF4-AM to identify virus-infected cells. (B) Percent of virus-positive splenocytes. The percentage of total splenocytes staining positive for CCF4-AM cleavage is indicated for each infection group. (C) Percent of virus-positive CD19+ B cells displaying surface markers consistent with naive B cells (GL7– IgM–). (D) Percent of virus-positive CD19+ B cells displaying surface markers consistent with germinal center B cells (GL7+ IgM+). (E) Percent of virus-positive CD19+ B cells displaying surface markers consistent with memory B cells (GL7– IgM–). The values represent the means ± the SEM of five independent experiments. Significance was determined using a two-tailed, unpaired t test (**, P < 0.01; *, P < 0.05; ns, not significant).
nal center B cells accounted for 79% of B cells carrying wild-type virus, infection of germinal center B cells (Fig. 7D) with miR-7-5p-deficient virus expressing scrambled shRNAs was reduced to 57% of the total. Interestingly, the percentage of naive B cells (Fig. 7C) infected with the scrambled shRNA virus was significantly increased compared to wild-type virus (9% WT versus 23% SC.shR), suggesting a compensatory increase in infected naive B cells as a result of decreased germinal center B cell infection. Although infection of memory B cells with the scrambled shRNA virus was also slightly increased, these results were not statistically significant. Together, these data suggested that miR-7-5p may directly contribute to the virus-mediated differentiation of naive B cells into germinal center B cells. Consistent with this possibility, incorporation of anti-EWSR1 shRNAs into the miR-7-5p-deficient virus fully restored both naive B cell (9% WT versus 8% EW.shR) and germinal center B cell (79% WT versus 83% EW.shR) infection to levels equivalent to that of wild-type virus. Collectively, these data demonstrate that the TMERS-encoded mghv-miR-7 promotes the infection of germinal center B cells through targeted repression of the host transcript EWSR1.

DISCUSSION

With the development of new methodologies such as qCLASH, an increasing number of bona fide host mRNA targets for virus miRNAs have been recently defined. However, to date, the biological functions of these miRNA-mRNA interactions in in vivo host-virus infection systems remain almost completely unknown. We present here, for the first time, clear evidence of an in vivo role for a specific gammaherpesvirus miRNA-host mRNA interaction in the context of chronic infection. Through the systematic application of increasingly refined MHV68 mutant viruses, we determined that MHV68 TMERS-encoded pre-miR-7 was essential for establishing latency in vivo. Through in vitro validation of qCLASH-identified targets, we defined EWSR1 as a likely important host mRNA target of the pre-miR-7-derived miRNAs mghv-miR-M1-7-5p or mghv-miR-M1-7-3p. Subsequently, through the use of recombinant viruses expressing scrambled or target-specific shRNAs in place of TMERS-encoded miRNAs, we demonstrated that in vivo repression of EWSR1 completely restored the attenuation of MHV68 TMERS mutant viruses. Notably, the use of a unique marker for identification of infected cells in vivo revealed a specific defect of miR-7-deficient viruses in germinal center B cells, and repression of EWSR1 completely rescued this defect. Cumulatively, these findings directly implicate mghv-miR-M1-7-5p-mediated repression of the host mRNA target EWSR1 as a key regulatory step in promoting MHV68 germinal center B cell latency in vivo.

Establishment of a system for in vivo validation of gammaherpesvirus miRNA-mediated repression of host mRNA targets. miRNAs function via sequence-specific but nonperfect binding to complementary sequences on RNA targets. The critical nucleotides for target binding typically lie within the miRNA seed sequence (nt 2 to 7 of the miRNA) (8); however, CLASH-based studies have recently revealed a wide range of other types of complementation between Ago-associated miRNAs and their cognate binding partners (19–21, 72). Moreover, while single miRNAs can bind to multiple target RNAs, the in vivo consequence of repression of individual target RNAs is a function of both the biological relevance of the target and the in situ stoichiometry of the interactions between the miRNA and its particular target RNAs. Thus, defining the biologically relevant targets of individual miRNAs in an in vivo system a priori remains a difficult problem.

Here, we have combined the unique properties of the MHV68 TMERS with highly specific shRNAs to define the biological function of viral miRNA-mediated repression of a host target transcript. Based on previous reports demonstrating effective processing of heterologous miRNAs from the MHV68 TMERS (34) and functional expression and processing of shRNAs placed downstream of tRNAs (52), we speculated that direct replacement of an MHV68 pre-miRNA stem-loop with an shRNA would generate a functional and highly specific siRNA directed against the target transcript of interest. Indeed, substitution of the TMERS-encoded pre-miRNA stem-loops with shRNAs di-
rected against EWSR1, the most highly repressed target of a TMER5-encoded miRNA, fully restored attenuation of the TMER5 pre-miRNA mutant virus. These findings demonstrate the utility of this approach, and demonstrate for the first time the in vivo biological relevance of miRNA-mediated repression of a specific host mRNA target.

A biological function for EWSR1 in germinal center B cell latency. EWSR1/EWS is a multifunctional protein that is mostly known for its central role as a fusion protein in the genesis of Ewing’s sarcoma. In that scenario, fusion of the N-terminal transcription-activating domain of EWSR1 to the C-terminal DNA-binding domain of the transcription factor FLI-1 (Friend Leukemia Integration 1) generates an oncogenic transcriptional activator, EWS/FLI-1, that drives cell growth, proliferation, and transformation (59, 60).

In contrast, the functions of full-length EWSR1 are less fully understood. Native EWSR1 is a ubiquitous transcriptional repressor and RNA binding protein that is expressed in numerous tissues and cell types (61), including pro-/pre-B cells from the bone marrow and mature B cells from the spleen (41). In the context of cellular stress responses, EWSR1 is involved in the regulation of gene expression and alternative splicing of DNA damage response-associated genes (40, 42, 62). Although its function during virus infections remains unknown, a role is likely dependent upon cell type and virus genera. For example, transcriptomic analyses revealed EWSR1 repression in brain tissue of grouper persistently infected with nervous necrosis virus (63), but EWSR1 expression is required for efficient replication of hepatitis C virus (64).

It is noteworthy that previous work has demonstrated that EWS-deficient mice display a marked defect in pre-B cell development (41); however, a specific function for EWSR1 in circulating mature B cells has not been previously reported. We identified here a particularly prominent defect in germinal center B cell infection in the absence of pre-miR-7, which was rescued by expression of anti-EWSR1 shRNAs, demonstrating the biological significance of EWSR1 repression in this cell type during virus infection. Notably, germinal center B cells are central players in chronic gammaherpesvirus infection, likely playing a critical role in the transition between initial infection of naive B cells and the establishment of long-term infection in the circulating memory B cell compartment. While the transition from naive B cells to memory B cells normally requires specific antigen stimulation of the B cell receptor plus T cell-mediated activation of the costimulatory molecule CD40, gammaherpesviruses have evolved highly regulated mechanisms to drive this process independent of antigen. For example, it is thought that the expression of a restricted subset of EBV genes in naive B cells drives these cells through the germinal center stage before a more restricted program propels their ultimate differentiation to memory B cells (3, 7, 55). In support of this concept, MHV68 is preferentially detected in germinal center B cells during the establishment phase of latency (43, 46–48, 51, 65). Consistent with the importance of germinal center B cells during gammaherpesvirus infection, Hodgkin’s lymphoma arises from EBV-infected B cells that are blocked at the germinal center stage, and Burkitt’s lymphoma arises from a germinal center B cell that is entering the memory compartment but is stuck at the point of proliferation (3, 66).

The findings described here strongly suggest that repression of EWSR1 is critical for MHV68 infection of, or transition through, the germinal center B cell stage. Whether the restriction of germinal center B cell infection by EWSR1 relates to its activity as an RNA binding protein and/or its function as a transcriptional repressor remains to be tested. It is also plausible that EWSR1 functions to directly restrict germinal center B cell infection in some way. In support of this possibility, it is noteworthy that the EWSR1 family protein FUS has recently been demonstrated to regulate KSHV infection through suppression of lytic gene expression (67). Thus, the function of EWSR1 family proteins in gammaherpesvirus infection warrants significant further investigation. Moreover, the finding of a specific function for EWSR1 in germinal center B cells suggests that this protein may play a previously unappreciated role in B cell biology.
Conclusions and future directions. The findings presented here demonstrate for the first time the in vivo biological relevance of repression of a specific host mRNA by a gammaherpesvirus miRNA. In defining a specific function for mghv-miR-M1-7-5p repression of EWSR1 in germinal center B cells, these findings have revealed new insight into a functional role for EWSR1 in both gammaherpesvirus infection and B cell biology. Importantly, the approach used here also provides a guidepost for defining the biological relevance of gammaherpesvirus miRNA-mediated repression of host targets in vivo.

MATERIALS AND METHODS

Cell culture. NIH 3T12 murine fibroblasts (American Type Culture Collection, TCC CCL-164) were maintained in Dulbecco modified Eagle medium (DMEM; Corning, 10-013-CM) supplemented with 10% fetal bovine serum (FBS; Omega Scientific, FB-11) and 1× penicillin-streptomycin solution (Corning, 30-002-CI) at 37°C and 5% CO₂.

Generation of recombinant viruses. Wild-type bacterial artificial chromosome (BAC)-derived MHV68 (68), parental wild-type marker virus MHV68.ΔORF73 (a, a recombinant virus that expresses β-lactamase as a fusion to mLANA (43), and MHV68ΔmirR7.12, a recombinant virus that is deficient in the expression of the pre-miR-7 and pre-miR-12 double step-loops (69), have been previously described. All mutant viruses described here were generated on the BAC-derived wild-type MHV68.ΔORF73 (designated MHV68.WT in this study) backbone by en passant mutagenesis, as previously described (70). Briefly, forward and reverse primers (Table S5) containing the desired mutation, short sequences complementary to the viral genome that flank the mutation, and sequences complementary to the kanamycin (Kan) resistance gene were used to amplify the I-SceI site and Kan selection marker from the Kan plasmid template. The resulting PCR products were gel purified and electroporated into GS1783 Escherichia coli harboring the MHV68.WT BAC. After recovery, the transformed cells were grown at 30°C under Kan selection for 36 to 48 h. BAC DNA was extracted from the resulting colonies and the genomic integrity was confirmed by restriction fragment length polymorphism (RFLP) analysis. Sequences containing the desired mutations and Kan selection marker were then PCR amplified and verified by sequencing.

Subsequently, the Kan selection marker was removed after induction of I-SceI-mediated recombination by 1% arabinose, leaving behind the desired mutations. Mutations were validated by RFLP, PCR, and sequencing analyses. Virus stocks of the resulting mutant were generated by transfecting NIH 3T12 fibroblast cells with BAC DNA from a single positive clone by using the TransIT-X2 dynamic delivery system (Mirus Bio, MIR 6004). The Cre recombinase-based removal of the BAC cassette, virus passage, generation of virus stocks, and titer determination were carried out as previously described (30).

Virus growth curve assays. NIH 3T12 cells were seeded at 2 × 10⁵ per well in six-well plates (Corning, catalog no. 3506). One day later, cells were infected with viruses at an MOI of 0.05 in duplicate wells. At the indicated time points postinfection, the cells and supernatants were harvested in 2 ml of 1-mm zirconia/silica beads (BioSpec Products) and stored at –80°C. Samples were homogenized using a Mini-Beadbeater (BioSpec Products) prior to the determination of viral titers by plaque assay.

Mouse infections and organ harvests. Seven- to eight-week-old wild-type C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed at the University of Florida (Gainesville, FL) in accordance with all federal and university guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida. Mice were inoculated i.n. with 10⁴ (for acute infection) or 10⁵ (for latent infection) PFU of virus in 30 μl of serum-free DMEM under isoflurane anesthesia. Three mice were used per group, per time point, per experiment, for all studies. For acute replication assay, lungs were harvested in 1 ml of serum-free DMEM containing 500 μl of 0.5-mm zirconia/silica beads (BioSpec Products, 11079105Z) and stored at –80°C. Samples were homogenized using a Mini-Beadbeater prior to the determination of viral titers by plaque assay. For latency assays, spleens were harvested at 16 dpi in DMEM containing 10% FBS and then homogenized with rubber pists from 10-ml Soft-Ject Luer Lock syringes (Henke Sass Wolf, 5100-X00V0). The resulting single cell suspensions were treated with red cell lysis buffer (0.144 M ammonium chloride, 0.017 M Tris [pH 7.2]) to remove red blood cells, and subsequently filtered through a 100-μm-pore-size nylon cell strainer (Corning, catalog no. 352360).

Plaque assays. Plaque assays were performed as previously described (30, 69). Briefly, NIH 3T12 cells were seeded at 2 × 10⁵ per well in six-well plates 1 day before infection. Samples from lungs (for acute infection in vivo) or infected NIH 3T12 cells (for virus growth curve in vitro) were serially 10-fold diluted in serum-free DMEM, added to six-well plates in duplicate, and then overlaid with a 1:1 mixture of methyl cellulose (Acros Organics, catalog no. 258115000) and 2 × modified Eagle medium (Gibco, 11935-046) containing 10% FBS and 1× penicillin-streptomycin solution. After 7 days, neutral red stain was added, and the plaques were counted.

Latency assays. Limiting dilution nested PCR was performed to determine the frequency of splenocytes harboring the MHV68 viral genome, as previously described (30, 43–45). Briefly, single cell suspensions were serially 3-fold diluted in a background of uninfected RAW 264.7 murine macrophages to maintain a total of 10⁵ cells per PCR. Dilutions were then plated in 96-well PCR plates (Eppendorf, catalog no. 951020460) at 12 replicates per dilution. Positive-control plasmid carrying MHV68 ORF72 gene were plated at 10, 1, or 0.1 copies per reaction on a background of 10⁴ RAW 264.7 cells. RAW 264.7 cells with no plasmid DNA were also plated as a negative control. After plating, the cells were lysed with...
proteinase K at 56°C for 8 h, followed by inactivation at 95°C for 15 min. Two rounds of nested PCR were then performed using primers (Table S5) specific for MHV69 ORF72, as previously described (30, 45). The resulting 195-bp amplions were visualized on a 3% agarose gel. The data are expressed as the means and standard errors of the percentages of PCR products that were positive for viral genome at each cell dilution.

**Cell transfections.** For all transfections, NIH 3T12 cells were seeded at 1 × 10^5 per well in six-well plates 1 day prior to use. For the transfection of miRNAs and siRNAs, cells were transfected with 100 pmol of miRNA or siRNA per well using Lipofectamine RNAiMAX reagent (Invitrogen, 13778-075). The mirVana miRNA mimic Negative Control #1 (4464058), the mgyh-mir-M1-7p mimic (MC10992), and the mgyh-mir-M1-7p mimic (MC10723) were purchased from Ambion. EWSR1-specific siRNAs (Ew-siR-1, -2, -3, and -4) and their respective scrambled sequence siRNAs (SC-siR-1, -2, -3, and -4) were purchased from Invitrogen (Fig. S2). For the transfection of plasmids, cells were transfected with 2.5 μg of plasmid per well using Lipofectamine 2000 reagent (Invitrogen, 11668-027). Cells were then harvested for qRT-PCR assays at 24 h posttransfection or for Western blotting at 48 h posttransfection.

**Plasmid construction.** The pUC57-Amp plasmid-based TMERS mutants T5ΔmiR7.12-EW.shR and T5ΔmiR7.12-SC.shR, which carry anti-EWSR1 shRNAs (EW-shR-3 and EW-shR-2) or scrambled sequence shRNAs (SC-shR-3 and SC-shR-2) in place of pre-miR-7 and pre-miR-12 stem-loops, were synthesized and cloned at Geneviz. A 40-bp sequence upstream of TMERS and a 20-bp sequence downstream of TMERS were included in these constructs in order to ensure efficient expression of TMERS-encoded shRNAs in the context of the plasmids. The resulting recombinant plasmids pUC57-EW.shR and pUC57-SC.shR, expressing anti-EWSR1 shRNAs or scrambled shRNAs downstream of vRNA5, were verified by Sanger sequencing. For luciferase assays, the murine EWSR1 coding region (nt 303 to 848 or nt 1000 to 1539) was cloned into the Pmel site of the pmirGLO dual-luciferase miRNA target expression vector (Promega, E2940), and the resulting recombinant plasmids were verified by Sanger sequencing.

**Quantitative reverse transcription PCR.** At the indicated time points, the cells were harvested, and the total cellular RNA was extracted using the TRIzol reagent (Ambion, catalog no. 15596018). After Dnase I (Ambion, AM1907) treatment, RNA was transcribed into cDNA using ProtoScript II reverse transcriptase (New England Biolabs, M0368S) with random primer mix (New England Biolabs, S1330S). All DNaseI(Ambion,AM1907)treatment,RNAwasreversetranscribedintocDNAusingProtoScriptIIreverse transcription for the context of the plasmids. The resulting recombinant plasmids pUC57-EW.shR and pUC57-SC.shR, expressing anti-EWSR1 shRNAs or scrambled shRNAs downstream of vRNA5, were verified by Sanger sequencing. For luciferase assays, the murine EWSR1 coding region (nt 303 to 848 or nt 1000 to 1539) was cloned into the Pmel site of the pmirGLO dual-luciferase miRNA target expression vector (Promega, E2940), and the resulting recombinant plasmids were verified by Sanger sequencing.

**Luciferase reporter assays.** NIH 3T12 cells were seeded at 1 × 10^6 per well in 96-well plates (Corning, 3855S) 1 day before transfection. Cells were treated with Lipofectamine 2000 reagent (Invitrogen, 11668-027) and cotransfected with (a) luciferase expression plasmid (50 ng/well) carrying the EWSR1 miRNA target sequence and flanking region (nt 303 to 848) plus mir-7-5p mimic (2.5 pmol/well), or (b) luciferase expression plasmid (50 ng/well) carrying EWSR1 sequences (nt 1000 to 1539) plus either pUC57-EW.shR or pUC57-SC.shR (50 ng/well). Firefly and renilla luciferase activities were determined on the Promega GloMax Multi+ detection system using a Dual-Glo luciferase assay system (Promega, E2940) at 24 h posttransfection.

**Flow cytometry.** For flow cytometry-based quantification of infected B cell subsets in in vivo, harvested cells were stained using the β-lactamase substrate CCF4-AM (Thermo Fisher Scientific, K1096) and a combination of antibodies directed against specific B cell surface markers, as previously described (43). Briefly, isolated splenocytes were blocked in PBS containing 2% FBS, 0.5% bovine serum albumin, 0.1% sodium azide, and purified rat anti-mouse CD16/CD32 at 1:50 (BD Biosciences, catalog no. 553141). Cells were then stained with Alexa Fluor 700 rat anti-mouse CD19 at 1:200 (BD Biosciences, catalog no. 557958), Alexa Fluor 647 rat anti-mouse T- and B-cell activation antigen GL7 at 1:200 (BD Biosciences, catalog no. 561529), and APC-eFluor 780 rat anti-mouse IgM at 1:200 (Thermo Fisher Scientific, 47-5790-82). After cell surface marker staining, the cells were resuspended in freshly prepared staining buffer containing CCF4-AM substrate. Unstained, fluorescence minus one and isotype stained controls were included for all experiments. Fluorescence-activated cell sorting (FACS) acquisition was performed on a BD LSR II flow cytometer (BD Biosciences), and mature B cell subsets were gated as naive B cells (CD19^hi GL7^-IgM^-), germinal center B cells (CD19^hi GL7^-), and memory B cells (CD19^hi GL7^-IgM^-) based on...
well-established surface marker combinations (30, 56–58). Data were analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR).

For flow cytometry-based sorting of infected cells expressing YFP, mice were infected i.n. with 10^4 PFU of MHV68.H2bYFP, a phenotypically wild-type virus that expresses eYFP under the control of the H2b promoter (46). At 16 dpi, splenocytes were prepared and blocked as described above. Cells were then stained with allophycocyanin (APC)-conjugated rat anti-mouse CD4 at 1:200 (BD Biosciences, catalog no. 553051), APC-conjugated rat anti-mouse CD8 at 1:200 (BD Biosciences, catalog no. 553035), APC-conjugated rat anti-mouse CD14 at 1:100 (BD Biosciences, catalog no. 560634), and APC-Cy7-conjugated rat anti-mouse CD19 at 1:200 (BD Biosciences, catalog no. 557655). Infected B cells (CD4^+ CD8^+ CD14^+ CD19^+ YFP^+) and noninfected B cells (CD4^+ CD8^+ CD14^+ CD19^+ YFP^+) were sorted using a BD FACS Aria II flow cytometer (BD Biosciences). Sorted cells were immediately subjected to RNA extraction using an RNAqueous-Micro kit (Ambion, AM1931) prior to qRT-PCR analyses.

For sorting latently infected B cells expressing anti-EWSR1 or scrambled shRNAs, mice were infected i.n. with 10^4 PFU of MHV68.EW.shR or MHV68.SC.shR, or with wild-type control virus. At 16 dpi, splenocytes were harvested, and B cells were isolated by immunomagnetic negative selection using an EasySep mouse B cell isolation kit (Stemcell Technologies, catalog no. 19854). The cells were blocked and then stained with APC-Cy7-conjugated rat anti-mouse CD19 (BD Biosciences, catalog no. 557655) and with CCF4-AM as described above. Infected B cells (CCF4-AM^+ CD19^+) were sorted using a BD FACS Aria II flow cytometer (BD Biosciences). RNAs from sorted cells were immediately extracted by using an RNAqueous-Micro kit (Ambion, AM1931) prior to qRT-PCR analyses.

Statistical analyses. All data were analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA). Statistical significance was determined using a two-tailed, unpaired Student t test. For limiting-dilution nested PCR assays, the frequencies of cells positive for the MHV68 viral genome were determined by the intersection of nonlinear regression curves with the line at 63.2% using Poisson distribution analysis, which indicates the point at which one viral genome positive cell is present in a given population.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00996-19.

FIG S1, PDF file, 0.3 MB.

FIG S2, PDF file, 0.9 MB.

FIG S3, PDF file, 0.5 MB.

FIG S4, PDF file, 0.5 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, PDF file, 0.1 MB.

TABLE S3, PDF file, 0.1 MB.

TABLE S4, PDF file, 0.2 MB.

TABLE S5, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

S.A.T. was supported by National Institutes of Health grants R01AI108407 and P01CA214091.

We thank Rolf Renne, Erik Flemington, Stephanie Karst, and members of the Renne, Flemington, and Karst labs for helpful discussions and suggestions.

Y.W. and S.A.T. conceived and designed the experiments. Y.W. and E.R.F. performed the experiments. Y.W., E.R.F., and S.A.T. analyzed the data. W.L.B. contributed reagents and materials. Y.W. and S.A.T. wrote and edited the manuscript.

The authors declare that they have no conflicts of interest.

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