Lytic infection with murine gammaherpesvirus 68 activates host and viral RNA polymerase III-dependent promoters to enhance non-coding RNA expression

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ABSTRACT

RNA polymerase III (pol III) transcribes multiple non-coding (nc) RNAs that are essential for cellular function. Pol III-dependent transcription is also engaged during certain viral infections, including the gammaherpesviruses (γHVs), where pol III-dependent viral ncRNAs promote pathogenesis. Additionally, several host ncRNAs are upregulated during γHV infection and play integral roles in pathogenesis by facilitating viral establishment and gene expression. Here we sought to investigate how pol III-dependent transcriptional activity was regulated during gammaherpesvirus infection, using the murine gammaherpesvirus 68 (γHV68) system. To compare the transcriptional regulation of host and viral pol III-dependent ncRNAs, we analyzed a series of pol III-dependent promoters using a newly-generated luciferase reporter optimized to measure pol III activity. We measured promoter activity from these constructs at the translation level via luciferase activity and at the transcription level via RT-qPCR. We further measured endogenous ncRNA expression at single cell-resolution by flow cytometry. These studies demonstrated that lytic infection with γHV68 increased the transcriptional activity of multiple host and viral pol III-dependent promoters, and further identified the ability of accessory sequences to influence both baseline and inducible promoter activity after infection. These studies highlight how lytic gammaherpesvirus infection alters the transcriptional landscape of host cells to increase pol III-derived transcription, a process that may further modify cellular function and enhance viral gene expression and pathogenesis.

IMPORTANCE

Gammaherpesviruses are a prime example of how viruses can alter the host transcriptional landscape to establish infection. Despite major insights into how these viruses modify RNA polymerase II-dependent generation of messenger RNAs, how these viruses influence the activity of host RNA polymerase III remains much less clear. Small non-coding RNAs produced by RNA polymerase III are increasingly recognized to play critical regulatory roles in cell biology and virus infection. Studies of RNA polymerase III dependent transcription are complicated by its use of multiple promoter types and diverse RNAs with variable stability and processing requirements. Here, we established a reporter system to directly study RNA polymerase III-dependent promoter responses during gammaherpesvirus infection and utilized single-cell flow cytometry-based methods to reveal that gammaherpesvirus lytic replication broadly induces pol III activity to enhance host and viral non-coding RNA expression within the infected cell.

INTRODUCTION

Gammaherpesviruses (γHVs) are large, lymphotropic viruses that establish a life-long infection in their hosts, with long-term latency in lymphocytes (1, 2). The γHVs include the human pathogens, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), and murine gammaherpesvirus 68 (γHV68 or MHV-68; ICTV nomenclature *Murid herpesvirus 4*, MuHV-4) (3). These viruses establish a primary lytic infection in their host that is followed by a prolonged quiescent infection termed latency. Latency is maintained in healthy individuals by a homeostatic relationship between the virus and the host immune response; if this balance is disrupted (e.g., by immunosuppression), γHVs can reactivate from latency and actively replicate. Disruption between the balance of γHV infection and host immune control is associated with γHV multiple pathologies, including a range of malignancies (4).

The γHVs contain several types of non-coding (nc) RNAs, including nuclear ncRNAs and functional miRNAs; these diverse RNAs including ncRNAs transcribed by RNA polymerase II (e.g. the KSHV PAN RNA and the KSHV and EBV miRNAs) or by RNA pol III (e.g. the EBV-encoded small RNAs (EBERs) and the γHV68 tRNA-miRNA-encoded RNAs (TMERs)) (5-13). Viral ncRNAs are considered to have important host-modulatory functions, interacting with host proteins and regulating host and viral gene expression. For example, the EBV EBERs are highly expressed during latency, and were discovered through their interaction with the host lupus-associated antigen (La) protein, which putatively mediates EBER interaction with TLR3 (14-17).The EBERs have further been shown to interact with several host proteins including ribosomal protein L22, protein kinase R (PKR), and retinoic-acid inducible gene I (RIG-I) (18). These interactions can trigger sustained host innate immune responses that are implicated in the development of EBV-associated malignancies (16, 19-21). γHV68, a highly tractable small animal model of γHV infection, also encodes several pol III-transcribed ncRNAs known as the tRNA-miRNA-encoded RNAs (TMERs) (22, 23). The TMERs are dispensable for lytic replication and establishment of latency; however, these transcripts are required for pathogenesis during acute infection of an immunocompromised host (7, 24-26). The TMERs contain bi-functional elements with a tRNA-like structure on the 5' end and hairpin loops that are processed into biologically-active miRNAs (7), capable of targeting a number of RNAs for post-transcriptional regulation (27). Our lab has previously shown that the tRNA-like structure is sufficient to rescue pathogenesis of a TMER deficient viral recombinant, suggesting that like the EBERs, the TMERs may contribute to pathogenesis through their interactions with host proteins (26). Though TMER-host protein interactions have yet to be fully explored, it is notable that several characteristics of the EBERs, such as a 5'-triphosphate and 3'-polyU, are imparted by RNA polymerase III (pol III) transcription (28).These motifs can be recognized by host RNA-binding proteins, such as RIG-I or La, to trigger an innate immune response (16, 20, 28, 29).

Pol III is often considered to perform "house-keeping" functions, as it transcribes host genes required for cell growth and maintenance (e.g. U6 snRNA, tRNAs, and 5S rRNA) (30). Despite this, it is clear that the γHVs

can usurp pol III-dependent transcription mechanisms for their own purposes. Latent EBV infection has been shown to upregulate components of pol III and ultimately increase the expression of host pol III transcripts – particularly vault RNAs – that allow increased establishment of viral infection and gene expression (31-33). Similarly, γHV68 infection drives upregulation of host pol III-dependent short-interspersed nuclear element (SINE) RNAs, which in turn, mediate increased viral gene expression (34, 35). Additionally, our lab has reported that reactivation of a latently-infected γHV68 cell line results in increased expression of TMERs within a rare subset of the population (36). Notably, dysregulation of pol III is a common feature of many cancer cells, implicating γHV infection-driven alteration of pol III activity as one potential contributor to γHV-associated malignancies (37). Therefore, understanding how γHV infection alters pol III activity is integral to elucidating mechanisms of γHV pathogenesis.

Due to the importance of γHV ncRNAs during infection and the unique transcriptional regulation afforded by RNA pol III, the overall objective of this study was to characterize the activity of host and viral pol III-dependent promoters during lytic γHV68 infection. To this end, we used a luciferase-reporter system optimized to report pol III-dependent transcriptional activity to investigate how virus infection regulated pol III promoter activity *in vitro*. We found that γHV68 infection upregulates the activity of multiple viral and host pol III promoters, a process further associated with the induction of pol III-dependent targets. These studies indicate that lytic γHV infection can broadly enhance RNA pol III promoter activity to modify the ncRNA landscape of infected cells.

RESULTS

RNA polymerase III can transcribe RNA from a variety of gene-internal (type 1 and 2) and gene-external (type 3) promoters (Fig 1A). These promoters contain distinct motifs that determine which transcription factors bind to the promoter to recruit pol III (38). To understand how γHV lytic replication influenced RNA pol III promoter activity without the confounding factors of RNA sequence or stability, we sought to study a series of viral and host pol III promoters using a luciferase assay system previously used to study pol III promoter activity (39). Several host and viral pol III promoters encompassing a variety of promoter types and motifs were selected for analysis (Supplemental Figure 1). Pol III promoters included host (U6, tRNA-Tyr, and vault RNA 1-1) and viral promoters (γHV68 TMER1, TMER4, TMER5; EBV EBER1 and EBER2; and adenovirus VA1). A subset of studies further compared promoter variants, encompassing either the minimal promoter elements ("minimal") or including additional accessory sequences ("full"). See Supplemental Figure 1 for promoter schematics, and Table 1 for a list of all promoters examined.

To analyze the activity of pol III promoters by luciferase assay, we selected the reporter plasmid pNL1.1 (Promega), as the NanoLuc luciferase in this reporter creates a brighter signal and the protein is smaller than its Renilla and Firefly counterparts; the smaller protein size is more consistent with pol III processivity of small ncRNAs. Our analysis of the pNL1.1 sequence revealed a pol III termination signal within the luciferase coding gene (TTTT). Therefore, to examine the activity of pol III promoters without the potential for early termination, we introduced silent mutations into the NanoLuc reporter construct to remove the termination signal. This new vector was named "pNLP3" to reflect that it is a *NanoLuc* reporter optimized for *p*ol *III* (Fig 1B). The human U6 promoter was cloned into both pNL1.1 and pNLP3 to compare the effects of removing the pol III termination signal, with promoter activity measured 24 hours post-infection. We found that removal of the termination signal increased the luciferase output, indicating that there was more read-through of the full NanoLuc gene from pNLP3 (Fig 1B). Furthermore, RT-PCR analysis of the NanoLuc transcript transcribed from the U6 promoter in either the pNL1.1 or pNLP3 vector revealed more full-length NanoLuc transcript from the pNLP3 vector (Fig 1C). This indicates that the pNLP3 vector allows for optimal pol III transcription of the reporter gene. We therefore used the pNLP3 vector as the backbone for analysis of all other pol III promoters included in this study.

With an optimized pol III reporter construct, we assessed how different pol III promoters respond to γHV68 infection over time. We first compared the activity of the human U6 (type 3) and γHV68 TMER1 (type 2) promoters. HEK 293 cells were transfected with pNLP3 vectors containing either the U6 or TMER1 promoter, co-transfected with an SV40 (pol II promoter)-driven Firefly luciferase vector, then infected with γHV68 (Fig 2A). Cell lysates were collected every 4 hours for 24 hours post-infection to quantify promoter activity as defined by NanoLuc luciferase activity. This analysis revealed that γHV68 infection resulted in a time-dependent increase in NanoLuc activity for both the U6 and TMER1 promoters (left panel, Fig 2B-2C) relative

to mock-infected samples. γHV68 infection also resulted in a time-dependent increase in expression of the control, Firefly luciferase reporter (second panel from left, Fig 2B-2C). The results for dual luciferase assays are typically reported as relative luminescence units (RLUs), where the reporter luminescence units (LUs) are normalized to the luminescence units of the control luciferase (i.e., NanoLuc LUs / Firefly LUs). However, since γHV68 infection simultaneously increased luminescence from both the NanoLuc reporter, and from the control Firefly reporter, this normalization errantly implied decreased relative U6 promoter activity with infection (Fig 2B). Given the limitation of standardizing pol III promoter activity relative to a pol II promoter control (i.e. SV40 promoter), all subsequent analyses report promoter activity as a fold change in NanoLuc luminescence comparing mock and γHV68 infected samples. We found that the U6 promoter drives high basal luciferase activity under mock conditions (left panel, Fig 2B), with a further increase in raw and normalized U6-expressed NanoLuc LUs throughout infection (Fig 2B). In contrast, the TMER1 promoter was characterized by extremely low basal luciferase activity (left panel, Fig 2C) in mock conditions; however, this promoter was strongly induced by infection (Fig 2C). These data suggest that γHV68 lytic infection increases the activity of multiple pol III promoter types, with a more robust induction of the type 2 promoter of TMER1 compared to the U6 promoter.

We further analyzed the activity of several other promoter types to assess how they are regulated during γHV68 infection. Experiments indicated that γHV68 infection induced activity from multiple pol III promoters, including the human U6 and tRNA-Tyr promoters, the EBER1 and EBER2 promoters, and TMER1, 4, and 5 promoters (Fig 3). Though the vaultRNA1-1 promoter was cloned into the reporter, there was no detectable activity from this construct (unpublished data). These promoters have different levels of baseline NanoLuc luciferase activity under mock conditions, with the U6 and EBER promoters showing high activity, and the TMER promoter displaying minimal detectable activity under mock conditions (unpublished data). Of the promoters tested, the TMER promoters consistently showed the greatest induction of luciferase activity during infection.

We compared the sequences of TMER promoters to identify which features of these promoters could potentially contribute to the strong induction of activity during infection. The TMER promoters initially analyzed contained the TMER promoter, as well as extended sequence around the minimal promoter elements (see Supplementary Figure 1 for diagrams). Considering that the extra sequence included in these “full” promoters may contribute to infection-induced activity, we created a panel of “minimal” TMER promoters that contain only the sequence canonically identified as the minimal promoter elements necessary for transcription, i.e. the sequence beginning from the A box to the end of the B box (Supplemental Figure 1). Additionally, we removed the upstream sequence from the EBER promoters; these EBER minimal promoters were used to further analyze how extended sequences can alter promoter induction during infection. As previously described, these constructs were transfected into HEK 293 cells, then infected with γHV68. The fold change in NanoLuc activity

relative to mock-treated samples was compared after 24 h of infection (Fig 4). When we compared the relative inducibility of EBER “full” versus “minimal” promoters, “minimal” promoters showed greater virus-inducibility. This enhanced inducibility of the “minimal” EBER promoters likely reflects the reduced baseline luminescence from these promoters (unpublished data). Conversely, TMER minimal promoters displayed a weaker induction during infection than their “full” counterparts, suggesting the sequence surrounded the TMER minimal promoters drives stronger expression during infection. These results indicate that the sequence surrounding minimal pol III promoter elements impacts both the baseline activity and inducibility of these promoters during infection.

Luciferase readouts of pol III promoter activity allowed us to uniformly analyze pol III promoter activity. This assay does not directly measure the level of RNAs, however, instead relying on an enzymatic readout of luciferase protein activity. To ensure that γHV68 infection was inducing pol III activity transcriptionally, we used the same NanoLuc constructs to measure promoter activity at the RNA level by performing RT-qPCR for the NanoLuc transcript. Following the same protocol as used for the luciferase assays, HEK 293 cells were transfected with pGL3 and the pNLP3 vector expressed by pol III promoters of interest (as outlined in Fig 2). Cells were then infected with γHV68 and RNA was purified from cells 16 or 24 h post-infection. Primers targeting the NanoLuc gene were used for qPCR following reverse transcription of the RNA. Infection increased the NanoLuc RNA expression from the U6 and TMER1 promoters, with more modest induction from the EBER promoters (Fig 5A). These results indicate that γHV68 infection stimulates pol III-promoter activity from multiple host and viral promoters, measured at both the transcriptional and translational level. To extend these findings, we further measured NanoLuc RNA expression from “minimal” or “full” TMER promoters. These studies demonstrated the γHV68 infection increased NanoLuc RNA from the “minimal” promoter relative to mock infected samples, with further RNA induction from the “full” TMER promoter. These results strongly suggest that the NanoLuc reporter assay serves as a faithful readout for pol III-dependent transcription, quantified at both the RNA and protein level. These findings also emphasize that sequences outside of the minimal TMER promoters contribute to increased expression during infection.

γHV lytic replication critically depends on viral DNA replication and late gene transcription, processes that are inhibited by phosphonoacetic acid (PAA) (40, 41). We therefore tested the impact of PAA on virus-induced pol III induction. To do this, HEK 293 cells were transfected with pNLP3-TMER1 and infected as before, with one set of samples receiving PAA treatment (200μg/mL) after 1 h of viral inoculation. RNA was isolated 16 h post-infection and RT-qPCR was performed to detect the NanoLuc transcript. Notably, treatment with PAA during infection had no impact on the induction of NanoLuc RNA compared to γHV68 infected cells, indicating that viral DNA replication and late gene synthesis was not required for pol III induction (Fig 6A). Though PAA treatment had no effect on the level of NanoLuc RNA levels (i.e. transcription), PAA treatment was consistently associated with increase luciferase enzymatic activity, with PAA-treated γHV68-infected

cultures characterized by a greater apparent induction of luciferase activity compared to γHV68-infected cultures alone. This PAA-driven enhancement of luciferase activity was observed for multiple pol III promoters, including U6, TMER1, 4 and 5 and EBER 1 and 2 (Fig 6B). The increase in luciferase activity following PAA treatment, with minimal impact on NanoLuc RNA, strongly suggests that PAA treatment enhanced the translational output from the promoters tested. These data suggest that viral late gene expression plays an additional role in translation that is not seen at the transcriptional level, a phenomenon independent of pol III promoter activity.

Given the reported relationship between the NF-κB pathway and the expression of pol III-dependent transcripts (31), we analyzed the effect of NF-κB activation or inhibition on the activity of the U6 and TMER1 promoters via luciferase activity. First, we measured induction of an NF-κB reporter plasmid following treatment with either TNFα, a known inducer of the NF-κB pathway, or following γHV68 infection. Whereas TNFα induced NF-κB reporter activity at 4 and 24 hours post-treatment, γHV68 infection had no measurable impact on expression from the NF-κB reporter (Supplemental Figure 2A). Next, we analyzed the impact of NF-κB manipulation on pol III promoter activity. Treating cells with TNFα modestly increased U6 promoter activity, albeit to a lesser extent than γHV68 infection (Supplemental Figure 2B). TMER1 promoter activity was not affected by TNFα treatment. Inhibition of NF-κB with the BAY 11-7082 (BAY 11) compound increased U6-expressed luciferase activity in virus infected conditions, yet had no significant impact on TMER1 promoter activity after infection (Supplemental Figure 2C). This indicates a potential role of NF-κB in inhibiting pol III promoter activity during infection; however, this effect is only observed in the case of a gene-external (i.e. type 3) promoter. Ultimately, these data do not support a significant role of the NF-κB pathway in the observed induction of pol III promoter activity after γHV68 infection.

While our analysis of pol III promoter activity suggested a general induction during infection, a previous report suggested that only a subset of host pol III-transcribed genes – the SINE RNAs – are increased during lytic γHV68 infection (34). These studies were largely based on analysis of total RNA of infected cells. To gain further insight into endogenous host ncRNA expression during lytic γHV68 infection, we made use of the PrimeFlow Assay kit, which allows for flow cytometry-based quantitation of RNA expression at the single-cell level. Murine fibroblast cells (3T12) were mock-treated, infected with wild-type (WT) γHV68, or infected with an EBER-knock in (EBER-KI.γHV68) recombinant γHV68 that lacks the TMERs and instead contains insertion of the EBV EBERs into the TMER locus. EBER-KI.γHV68 was competent for viral replication (manuscript in progress). 3T12 cells were infected with an MOI of 1 and harvested at 16 h post-infection, conditions that result in a mixed population of virally infected and uninfected cells. Cells were then queried with fluorescent probes to detect viral gene expression as defined by either the γHV68 TMERs or the EBV EBERs, expressed from the context of the γHV68 genome. Host ncRNA for U6 snRNA or 4.5 rRNA (the murine equivalent of human 5S

rRNA) expression was then assessed, comparing expression between cells with virus expression (i.e. TMER+ or EBER+) and cells lacking virus expression (i.e. TMER- or EBER-). Gating schemes and probe specificity are shown in Supplemental Figure 3. Notably, probes for the TMERs and EBERs were specific for their intended targets, with no detectable TMER or EBER expression in mock-infected samples, TMER+ cells only present in WT γHV68 infected samples, and EBER+ cells only present in EBER-KI.γHV68 infected samples. We next compared the relative expression of the U6 (Fig 7A-B) and 4.5S ncRNAs (Fig 7C-D) between cells with active viral RNA expression versus those cells that did not express these viral ncRNAs. Analysis of the geometric mean fluorescence intensity (gMFI) for the U6 snRNA and 4.5 rRNA probes revealed increased expression of U6 and 4.5 rRNA ncRNAs in virally infected cells (i.e. TMER+ or EBER+) compared to uninfected cells (TMER- or EBER-) (Fig 7E-F). These data demonstrate an increase in host pol III-transcribed ncRNAs in γHV68-infected cells and emphasize the benefit of single cell analysis of endogenous RNA expression to quantify ncRNA expression during virus infection.

DISCUSSION

While γHV infection is known to alter expression of host genes, and viral ncRNAs are integral for pathogenesis, the transcriptional regulation of these ncRNAs has remained unclear. Here, we propose that different pol III promoter types allow for distinct means for transcriptional regulation during infection. This study focused on the impact of γHV68 lytic replication on pol III promoter activity, identifying that lytic infection drives a general upregulation of promoter activity across multiple host and viral pol III-dependent transcripts. This upregulation was measured by a luciferase assay optimized for pol III transcription, with increased pol III-dependent promoter activity quantified by luciferase activity and RNA expression, with complementary findings revealed through the use of flow cytometric analysis of ncRNA expression. These findings emphasize the utility of the modified NanoLuc luciferase system to analyze pol III promoter activity, and provide clear evidence for pol III promoters with large differences in basal and inducible promoter activity. They further emphasize the capacity of γHV lytic infection to modify pol III-dependent transcriptional machinery in infected cells, a process that likely facilitates productive virus replication (34). At this time, it remains unknown whether pol III machinery or transcription is altered during γHV68 latent infection or reactivation from latency.

Our use of a luciferase reporter to measure the activity of pol III promoters allowed us to directly compare the functional activity of multiple pol III promoters, while avoiding potential differences that may arise due to ncRNA sequence, structure, or stability. Though pol III promoters conventionally drive expression of non-coding RNAs, there is clear precedent that pol III can transcribe translation-competent RNA (42, 43), and luciferase reporters have been used for high throughput and unbiased analysis of pol III promoter activity (44, 45). Inspired by these studies, we cloned several host and viral ncRNA promoters into a NanoLuc luciferase reporter to measure their activity during lytic γHV68 infection. We chose the NanoLuc luciferase as our To further enhance the robustness of this reporter, we identified and removed a pol III termination sequence within the NanoLuc gene which approximately doubled luciferase reporter activity. Though pol III transcription should theoretically be terminated in the original NanoLuc reporter, pol III read-through of termination signals has been reported (46). In total, use of the modified NanoLuc reporter construct afforded a sensitive and robust readout for assessing pol III promoter activity.

Through use of this pol III reporter assay, we found that γHV68 infection increased promoter activity across a range of host and viral pol III promoters. This suggests that γHV68 infection generally upregulates pol III activity. Interestingly, the consequence/magnitude of induction elicited by infection varied between promoters. For example, the U6 promoter conveyed high basal activity with infection resulting in a modest induction of U6 promoter activity. Conversely, the TMER promoters exhibited extremely low basal activity in mock-infected conditions, with dramatic induction after γHV68 infection. The inducibility of the TMERs was further enhanced by accessory sequences outside of the minimal A and B box elements. One explanation for this enhanced induction is that these extended sequences may contain additional transcriptional elements that

are integral to the promoter itself. While formally possible, it is notable that accessory sequences across the TMERs are not conserved (Supplemental Fig 4A). An alternate explanation for the enhanced activity of the full TMER promoters is that inclusion of the extended sequence includes the full tRNA-like structure of the TMER genes (Supplemental Fig 4B). It is interesting to speculate that this tRNA-like structure could either lend greater stability to transcripts, or protect the transcripts from degradation by host exonucleases or the viral endonuclease, muSOX (47). Although pol III transcription is frequently associated with the transcription of housekeeping ncRNAs, there is clear precedent that pol III can also participate in inducible gene expression (e.g. γHV68-induced expression of SINEs) (34, 35). Whether the TMERs have conserved regulatory mechanisms with host inducible ncRNAs is currently unknown, however, the TMERs share more promoter similarity to the SINEs (type 2, gene internal) than to the vault RNAs (type 3, gene external).

Our studies demonstrated that infection increased NanoLuc expression at both the RNA and protein level, indicating that virus infection increased pol III promoter activity and not some secondary measurement. This induction was further associated with increased expression of the host ncRNAs U6 and 4.5 rRNA. While these findings emphasize the impact that infection has on pol III-dependent transcription, our studies did reveal one context in which infection impacted a post-transcriptional measurement. Unexpectedly, inhibition of viral late gene expression with PAA resulted in increased luciferase activity from nearly all of the promoters examined – this phenomenon was only seen at the level of NanoLuc protein activity, not at the level of NanoLuc RNA. The ability of PAA to enhance NanoLuc protein activity, with no commensurate change in RNA expression, suggests that viral late genes may have a possible role in tempering translation. Manipulation of host translational machinery by the herpesviruses is a common strategy that is required for optimal virus replication and the production of virus progeny (48).

Previous reports show that γHV infection can have diverse effects on pol III transcription, ranging from a general induction of pol III machinery (e.g. in the context of EBV and EBNA1 (33, 49), to the selective induction of pol III-transcribed RNAs (e.g. induction of specific host vaultRNAs in EBV latently infected cells (31, 32), and the host SINE RNAs in γHV68-infected cells (34)). One challenge in interpreting these different findings is that these studies have been done in different states of infection (latent versus lytic), in different cell types, and in different states of cellular transformation. In many cases, the mechanistic insights gained from these studies could only be gained through the use of in vitro studies. Future studies will benefit from direct comparisons of primary virus infection, when possible, coupled with the analysis of endogenous ncRNA expression. In keeping with this, we anticipate that the γHV68 system will afford unique insights in how the γHVs regulate pol III-dependent ncRNA expression, allowing the analysis of primary infection coupled with technologies to measure endogenous ncRNA expression. For example, our single-cell analysis of pol III-derived transcripts – U6 snRNA and 4.5 rRNA – supported that γHV68-infection not only increases pol III-dependent promoter activity, but also increases the endogenous expression of these transcripts. How host and

viral ncRNAs are regulated as a function of cell type and virus stage of infection remains an important unanswered question.

In total, our studies revealed a γHV68-dependent induction in the activity of host and viral pol III promoters. This induction was seen in the expression of a reporter gene, as well as in the endogenous expression of pol III-dependent transcripts. Though previous reports have focused on the virus-mediated upregulation of specific host ncRNAs, these experiments suggest a broader effect of lytic γHV68 infection on pol III activity. This suggests that γHV68 modulation of the host transcriptional landscape goes beyond mRNA regulation, and that pol III-dependent transcripts are likely to play a wider role in γHV68 pathogenesis than previously appreciated.

METHODS

Viruses and tissue culture. All viruses were derived from a BAC-derived WT γHV68 (50 Koszinowski UH. 2000) For some experiments, the TMER total knock-out (TMER-TKO) virus was used; this virus was generated as previously described (26). Viruses were propagated and titered as previously described (26). EBER-KI virus contains EBERs 1 and 2 in the TMER-TKO virus backbone. Its generation and characterization are described in a manuscript in preparation.

Human endothelial kidney (HEK 293) and murine fibroblast (3T12) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals), 2 mM L-glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin sulfate (complete DMEM). Cells were cultured at 37ºC with 5% CO_2.

Mutagenesis of pNL1.1 to create the pNLP3 NanoLuc luciferase reporter. The promoterless NanoLuc luciferase reporter vector pNL1.1[*Nluc*] was obtained from Promega, and primers were designed to introduce silent mutations to remove the pol III termination signal in the NanoLuc coding sequence; these primers are listed in Supplementary Table 1. Mutagenesis PCR was performed with the following cycles: (i) 95° for 30s, (ii) 12 cycles of 95°C for 30s, 55°C for 1 min, 68°C for 3 m. The resulting DNA was digested with DpnI (New England Biolabs Inc) and transformed into XL1-Blue super-competent cells (Agilent). Bacterial colonies were sequenced to confirm the correct mutations. The resulting plasmid was named "pNLP3" to indicate that it is a NanoLuc plasmid optimized for pol III.

Generating a pol III promoter-driven NanoLuc reporter panel. All promoters were generated to include XhoI and HindIII overhang sequences on the 5' and 3' ends respectively. Several promoters were constructed using ligated oligonucleotides. PCR-amplified promoters and pNLP3 were digested with XhoI and HindIII, then promoters were ligated into pNLP3 using T4 DNA ligase (New England BioLabs Inc). See Supplementary Table 1 for primers used to PCR-amplify promoters. Ligated constructs were transformed into One Shot electro- or chemically competent TOP10 *E. coli* cells (Thermo Fisher Scientific, #C404052 or #C404010), which were then plated at several dilutions on LB agar containing ampicillin. Resulting colonies were expanded in LB broth with ampicillin and plasmid was isolated using the QIAprep Spin Miniprep Kit (Qiagen). All constructs were confirmed by sequencing.

Transfecting cells. For transfections, HEK 293 cells were cultured in 5% FBS DMEM without penicillin or streptomycin for approximately 24 hours. Transfection solutions contained Opti-MEM (Thermo Fisher Scientific), NanoLuc plasmid (pNLP3 with inserted pol III promoters), and the Firefly control plasmid (pGL3-Control; Promega). After plasmids were added to the Opti-MEM, solutions were incubated with X-tremeGENE HP DNA Transfection Reagent (Sigma-Aldrich) for at least 15 minutes at room temperature. Transfections were performed in several plate formats, depending on the downstream use; transfection solutions were added dropwise to the appropriate wells (10uL of solution for 96-well plate, 100uL of solution in 12-well plates, 200uL

of solution in 6-well plates). For all transfections, the molar ratio of NanoLuc plasmid to Firefly plasmid was kept at 10:1; the total amount of DNA transfected per well was adjusted depending on the plate size (approximately 10ng for 96-well, 100ng for 12-well, and 200ng for 6-well). Cells were incubated with transfection solution for 24 hours prior to downstream applications, unless otherwise stated.

To analyze how promoter activity was affected by γHV68 infection, transfected HEK 293 cells were infected with WT, TMER-TKO, or EBER-KI γHV68 at a multiplicity of infection (MOI) of 1 plaque forming unit per cell. Cells were cultured for approximately 24 h prior to infection. Cell counts were determined by treating with 0.05% Trypsin-EDTA (Life Tech, #25300-054) to remove and cells. These cells were mixed with Trypan Blue dye (Bio-Rad, #145-0021) to obtain a live cell count using the TC20 Automated Cell Counter (Bio-Rad). Virus stocks were mixed with 5% complete DMEM, then added to cells and incubated with virus for 1 hour. Viral inoculum was then removed and replaced with complete 5% DMEM. For samples treated with phosphonoacetic acid (200 µg/mL, Sigma-Aldrich #284270), inoculum was removed and replaced with 5% complete DMEM containing the drug.

Dual luciferase assays. Following transfection and infection, HEK 293 cell lysates were collected for analysis of luciferase activity. All luciferase assays were performed using the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega). For experiments performed in the 12-well format, supernatant was removed and cells were scraped, collected into 1.5mL tubes, then pelleted. Cell pellets were resuspended in 250 µL of Passive Lysis Buffer, per the manufacturer's protocol. 80 µL of the cell suspensions were used for assays. For luciferase assays performed in the 96-well format, 80 µL of ONE-Glo™ EX Reagent was added directly to the cells and supernatant, as recommended by the manufacturer. Samples were incubated at room temperature while on a shaker for three minutes. This solution was then transferred from a transparent 96-well culture plate to a white-wall luminescence plate prior to reading Firefly luciferase activity on the Tecan Infinite® 200 PRO plate reader. Then, 80 uL of the NanoDLR™ Stop & Glo® Reagent was added to the solution and incubated at room temperature on a shaker for at least 10 minutes. Samples were read on the Infinite® 200 PRO again for the NanoLuc luminescence measurement.

RT-qPCR of NanoLuc RNA. RNA was isolated from transfected HEK 293 cells with TRIzol® Reagent (Thermo Fisher Scientific, #15596026) and DNase-treated with TURBO™ DNase (Invitrogen, #AM2238) following the manufacturer's protocols. RNA amplification and removal of DNA was confirmed by RT-PCR or PCR amplification of a control host gene, 18S. RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen, #210212), with the following conditions: (i) 50ºC for 30 m, (ii) 95ºC for 15 m, (iii) 40 cycles of 94ºC for 30 s, 52ºC for 30 s, and 72ºC for 30 s, (iv) 72ºC for 10 m, (v) hold at 4ºC. PCR was performed using Taq DNA polymerase (Qiagen, #201205) with the following conditions: (i) 95ºC for 5 min, (ii) 40 cycles of 94ºC for 30 s, 52ºC for 30 s, 72ºC for 30 s, (iii) 72ºC for 10 m, (iv) hold at 4ºC. RNA samples that showed no product following PCR amplification were deemed DNA-free, and converted to cDNA using SuperScript III Reverse Transcriptase

(Invitrogen, #18080093) following the manufacturer's protocol. 100ng of the cDNA was then used for qPCR analysis of the NanoLuc and 18S genes using the iQ™ SYBER® Green Supermix (Bio-Rad, #1708880) with the following conditions: i) 95°C for 3 m, ii) 40 cycles of 95°C for 15 s, 60°C for 1 m, iii) 95°C for 15 s, 60°C for 1 m, 95°C for 15 s. Amplification of NanoLuc was normalized to 18S expression to calculate the relative difference of NanoLuc expression using the Pfaffl method: $\frac{NanoLuc\ Primer\ Efficiency^{Target\ \Delta Ct}}{18S\ Primer\ Efficiency^{18S\ \Delta Ct}}$ (51).

PrimeFlow RNA assay. The PrimeFlow RNA assay kit (Thermo Fisher Scientific, # 88-18005-210) was used to analyze the endogenous expression of non-coding RNAs in mock and γHV68-infected 3T12 cells. Cells were infected at an MOI of 1 for 16 h, then processed with the PrimeFlow kit following the manufacturer's protocol. Cells were treated with probes for TMERs (Type 4/AF488), EBERs (Type 1/AF647), and U6 snRNA or 4.5 rRNA (Type 6/AF750). Samples were collected on an LSR II Flow Cytometer (BD Biosciences), and included single stain and "full minus one" controls. Flow cytometry data were analyzed using FlowJo software (version 10.6.1), with compensation based on single stained beads and cells. Compensated flow cytometry data were subsequently analyzed for singlet events (based on doublet discrimination), with positive and negative populations based on control samples.

Software and statistical analysis. Statistical analysis and graphing were done in GraphPad Prism (Version 8.0d). Statistical significance was tested by unpaired t test (comparing two conditions) or one-way ANOVA (comparing three or more conditions) and subjected to multiple corrections tests using recommended settings in Prism. All flow cytometry data were analyzed in FlowJo (version 10.6.1) with flow cytometry data shown as pseudo-color dot plots.

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FIGURE LEGENDS

Figure 1. RNA Polymerase III promoters contain distinct motifs. Pol III transcribes RNA from gene-internal (type 1 and type 2) or gene-external (type 3) promoters. Each promoter type contains distinct motifs, which are in turn bound by specific transcription factors (TF) that recruit pol III to the promoter. Viral promoters can have canonical type 2 promoters, such as adenovirus; however, the γHV68 TMERs contain a triplicated, overlapping A box motif, and the EBV EBER promoters include upstream elements. Gray boxes indicate the gene, while the "TTTT" marks the end of the gene where pol III transcription is terminated. Diagrams are not to scale. *A* = A box, *B* = B box, *C* = C box, *IE* = intermediate element, *"TTTT"* = pol III termination signal, *TF* = transcription factor, *TATA* = TATA box, *PSE* = proximal sequence element, *DSE* = distal sequence elements, *ETAB* = EBER TATA-like box, *ATF* = activating transcription factor binding sequence, *Sp1* = Sp1-like binding sequence. B) Optimization of the NanoLuc reporter vector. Mutations were introduced into the pNL1.1 vector to remove the pol III termination signal (TTTT) from the Nanoluc coding region, resulting in the pNLP3 vector. The human U6 promoter was cloned into each of these reporters, and dual luciferase assays were performed to compare luciferase output. Data shown is representative of two independent experiments with biological triplicates. Additionally, RNA was isolated from cells transfected with these two constructs. Cells were mock or WT γHV68-infected for 16 h, then cellular RNA was used as a template for primers targeting the entire NanoLuc gene (top gel, 534 nt), or targeting just the NanoLuc sequence upstream of the termination sequence (bottom gel, 234 nt). Data shown is from one experiment with biological triplicates. NTC = Non-template control, black triangles = primers, * = location of termination sequence in pNL1.1.

Figure 2. γHV68 infection increases luciferase activity when expressed from two different pol III promoter types. A) Experimental design. HEK293 cells are transfected with a control Firefly reporter (pGL3) and a pNLP3 reporter expressed from a pol III promoter of interest. 24 h post-transfection, cells are infected with wild-type (WT) γHV68 at an MOI of 1, or mock treated. Cell lysates are collected 24 h post-transfection for the dual luciferase assay. Promoter activity was measured for the (B) U6 and (C) TMER1 promoters using a dual luciferase assay. Cells were harvested every 4 hours post-infection up to 24 h. Each time-point was repeated for biological triplicates. Raw values for the Firefly and NanoLuc activity for each promoter are shown as luminescence units (LUs). Promoter activity was analyzed as average relative luminescence units (RLUs = NanoLuc LUs / Firefly LUs), and as the fold change in NanoLuc luminescence units compared to the 4 h uninfected samples (NanoLuc fold change = Infected Sample NanoLuc LU / Mock Sample NanoLuc LU). Error bars = SEM.

Figure 3. γHV68 infection induces activity of multiple pol III promoter types. Promoter activity was measured for the U6 (n = 8), tRNA (n = 3), EBER1 (n = 3), EBER2 (n = 4), TMER1 (n = 18), TMER4 (n = 4), and

TMER5 (n = 6) promoters during infection. Luciferase assays were performed as previously described, with cell lysates collected 24 h post-infection. Each independent experiment (n) contained biological triplicates or duplicates. All promoter activity changes are analyzed as the fold change in NanoLuc activity normalized to uninfected samples (Infected NanoLuc LU / Mock NanoLuc LU). Error bars = SEM. Significant differences analyzed by t-test and indicated as asterisks. P-values are indicated as follows: * = P ≤ 0.05, ** = P ≤ 0.01, *** = P≤ 0.001, **** = P ≤ 0.0001.

Figure 4. γHV68 infection differentially induces activity of promoters containing external sequences. Promoter activity was measured for the "minimal" or "full" promoters for EBER1, EBER2, TMER1, TMER4, and TMER5. Minimal promoters contained only the sequence constituting the internal promoter elements. Full promoters included sequence outside of the internal promoter elements; the full EBER promoters also contained upstream promoter elements, while full TMER promoters contained the entire tRNA-like section of the gene (see supplementary figure 1). Luciferase assays were performed as previously described, with cell lysates collected 24 h post-infection. All promoter activity changes are analyzed as the fold change in NanoLuc activity normalized to uninfected samples. Each experiment (n) included biological triplicates. Error bars = SEM. Significant differences analyzed by t-test and indicated as asterisks. P-values are indicated as follows: * = P ≤ 0.05, ** = P ≤ 0.01, *** = P≤ 0.001, **** = P ≤ 0.0001.

Figure 5. Effect of γHV68 infection on NanoLuc transcript levels. Promoter activity was measured for A) the U6, TMER1, and EBER full promoters, or B) the TMER1, TMER4, and TMER5 minimal vs. full promoters. Cells were treated as previously described and RNA was purified from cells at 16 h post-infection (U6 n = 2, TMER1, n = 5) or 24hpi (EBER1 n = 1, EBER2 n = 1, TMER4 n = 2, and TMER5, n = 1). RNA was converted to cDNA, then LightCycler real-time PCR using Syber Green was performed with primers targeting the NanoLuc gene and a host control gene (18S). The relative difference of NanoLuc was calculated using the Pfaffl method (2001, *Nucleic Acids Research*), where the ratio = $(E_{target})^{\Delta CP\ target\ (control\text{-}sample)}$ / $(E_{ref})^{\Delta CP\ ref\ (control\text{-}sample)}$. Each experiment (n) included biological triplicates or duplicates. Error bars = SEM. Significant differences analyzed by t-test and indicated as asterisks. P-values are indicated as follows: * = P ≤ 0.05, ** = P ≤ 0.01, *** = P≤ 0.001, **** = P ≤ 0.0001.

Figure 6. γHV68 infection coupled with PAA treatment further increases luciferase activity, but not transcript level, from pol III promoters. A) Promoter activity was measured via RT-qPCR of the NanoLuc transcript expressed from the TMER1 promoter during infection and concurrent phosphonoacetic acid (PAA) treatment. RT-qPCR was performed as previously described. Data is shown as NanoLuc relative difference. N = 1 with biological triplpicates.

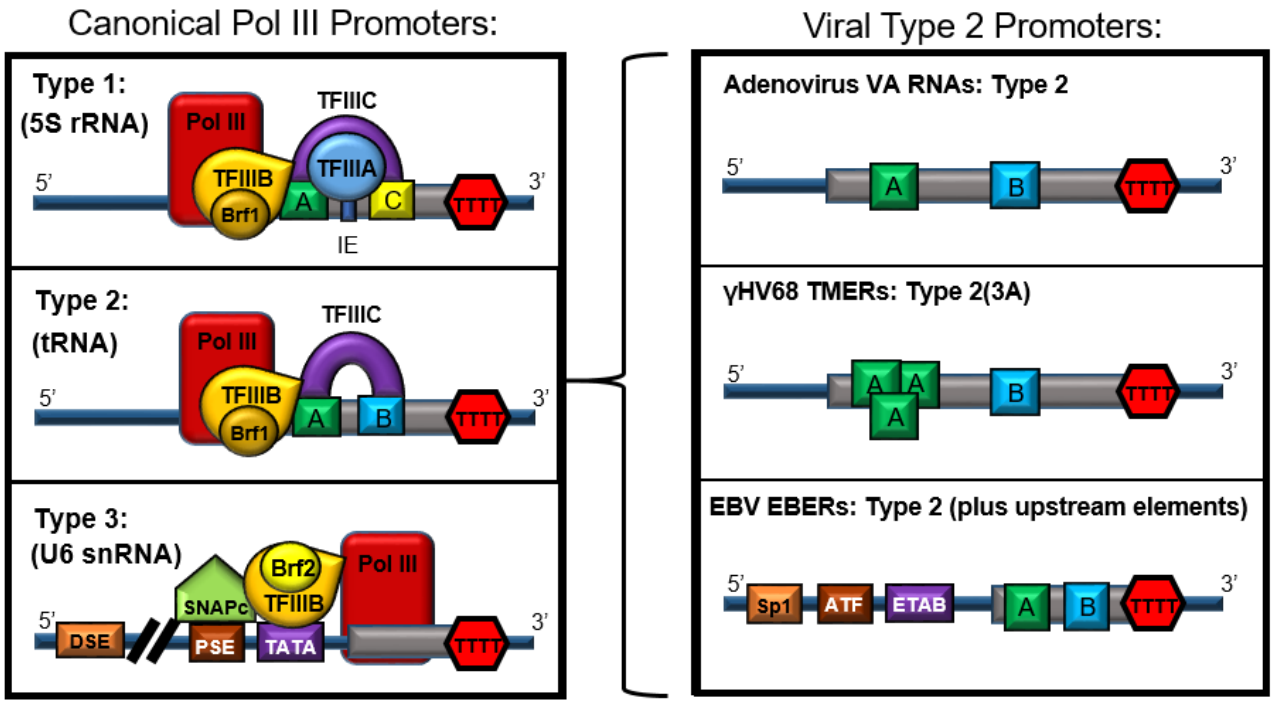
B) Promoter activity was measured via luciferase activity for the following promoters during infection, including with concurrent PAA treatment: TMER1 (n = 5), TMER4 (n = 2), TMER5 (n = 2), EBER1 (n = 2), EBER2 (n = 3), U6 (n = 2), and tRNA (n = 1). Each experiment contained biological duplicates or triplicates. Luciferase assays were performed as previously described, with cell lysates collected 24 h post-infection. NanoLuc fold change for infected samples is from previous figures for comparison to PAA-treated samples. All promoter activity changes are analyzed as the fold change in NanoLuc activity normalized to uninfected samples. Error bars = SEM. Significant differences analyzed by one-way ANOVA and indicated as asterisks. P-values are indicated as follows: * = P ≤ 0.05, ** = P ≤ 0.01, *** = P≤ 0.001, **** = P ≤ 0.0001.

Figure 7. Endogenous expression of murine U6 snRNA and 4.5S rRNA during γHV68 infection. Murine fibroblast cells (3T12) were infected with mock, WT γHV68, or an EBER knock-in (EBER-KI) γHV68 at an MOI=1, harvested at 16 hpi and subjected to the PrimeFlow RNA Assay and flow cytometric analysis. **(A,B)** Analysis of U6 snRNA expression comparing background fluorescence (”No probe”, left) versus U6 probe hybridization in Mock (middle), or WT gHV68 or EBER-KI.gHV68 infected cells (right). **(C,D)** Analysis of 4.5S rRNA expression comparing background fluorescence (”No probe”, left) versus 4.5S rRNA probe hybridization in Mock (middle), or WT gHV68 or EBER-KI.gHV68 infected cells (right). **(E,F)** The geometric mean fluorescence intensity (gMFI) was calculated for **(E)** U6 snRNA or **(F)** 4.5S rRNA in mock infected, or virus infected samples, comparing cells that differed in expression of either the TMERs or the EBERs in WT or EBER-KI infections respectively. Data depict flow cytometric analysis analyzing singlets, defined by sequential discrimination using SSC and FSC parameters. Data are from 3 biological replicates, with statistical significance assessed using an unpaired t-test. Statistical significance as follows: **, p<0.01 and ***, p<0.001.

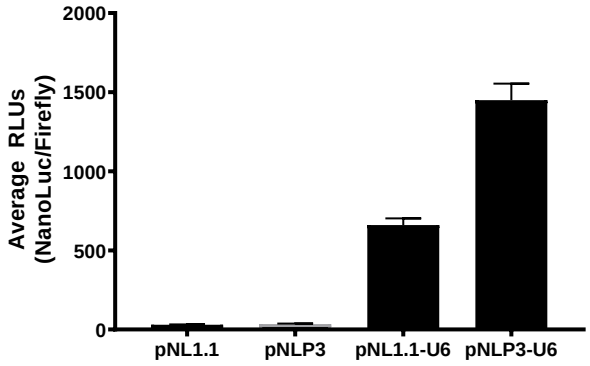
Table 1. Promoters analyzed in this study. Pol III promoters were selected from several organisms and cloned into a luciferase reporter to analyze promoter activity during infection

Origin	Promoter	Promoter Type	Promoter Insert Length (nt)	Reference
Human	5S rRNA	Type 1	42	(52)
Human	tRNA-Tyr	Type 2	53	Gene ID: 100189507
Human	Vault RNA1-1	Type 2	65	(32, 53); GenBank: AF045143.1
Human	U6 snRNA	Type 3	227	GenBank: M14486.1
Adenovirus	VA1	Type 2	58	(54)
γHV68	TMER1 full	Type 2 (3A)	91	(22)
γHV68	TMER1 minimal	Type 2	55	“ ”
γHV68	TMER4 full	Type 2 (3A)	93	“ ”
γHV68	TMER4 minimal	Type 2	55	“ ”
γHV68	TMER5 full	Type 2 (3A)	94	“ ”
γHV68	TMER5 minimal	Type 2	53	“ ”
EBV	EBER1	Type 2 (+ upstream)	157	(55-57)
EBV	EBER1 minimal	Type 2 (no upstream)	70	“ ”
EBV	EBER2	Type 2 (+ upstream)	156	“ ”
EBV	EBER2 minimal	Type 2 (no upstream)	68	“ ”

A)



B)



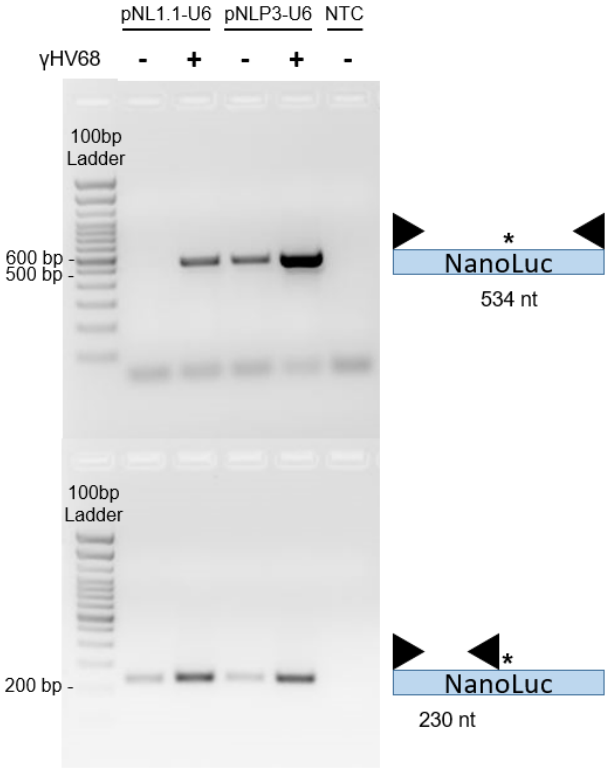


Figure 1
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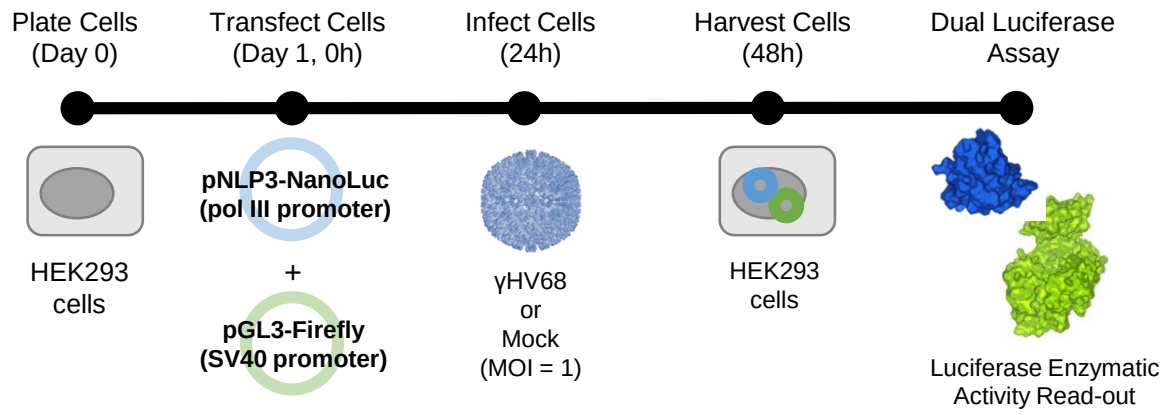
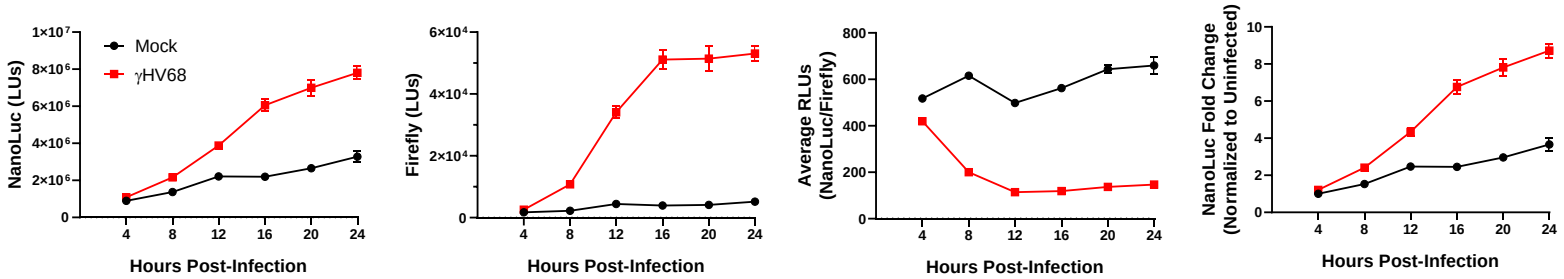
A)



B)

U6 Promoter-Driven Luciferase Activity



C)

TMER1 Promoter-Driven Luciferase Activity

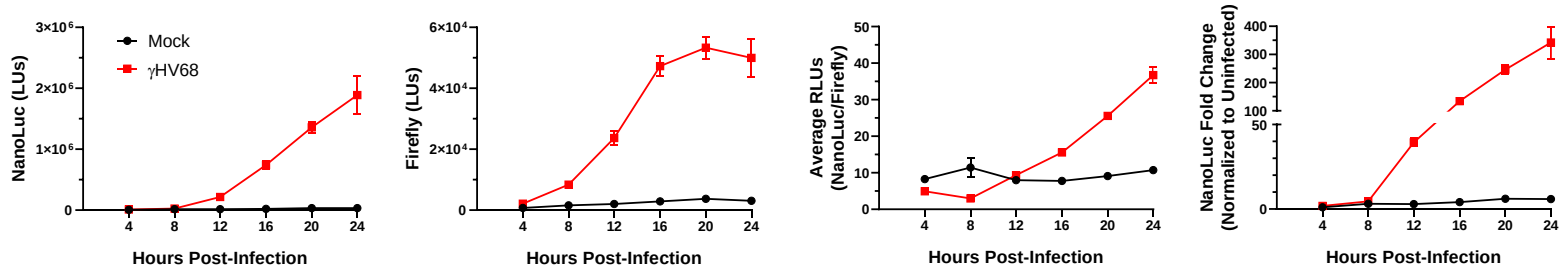


Figure 2
Knox et al

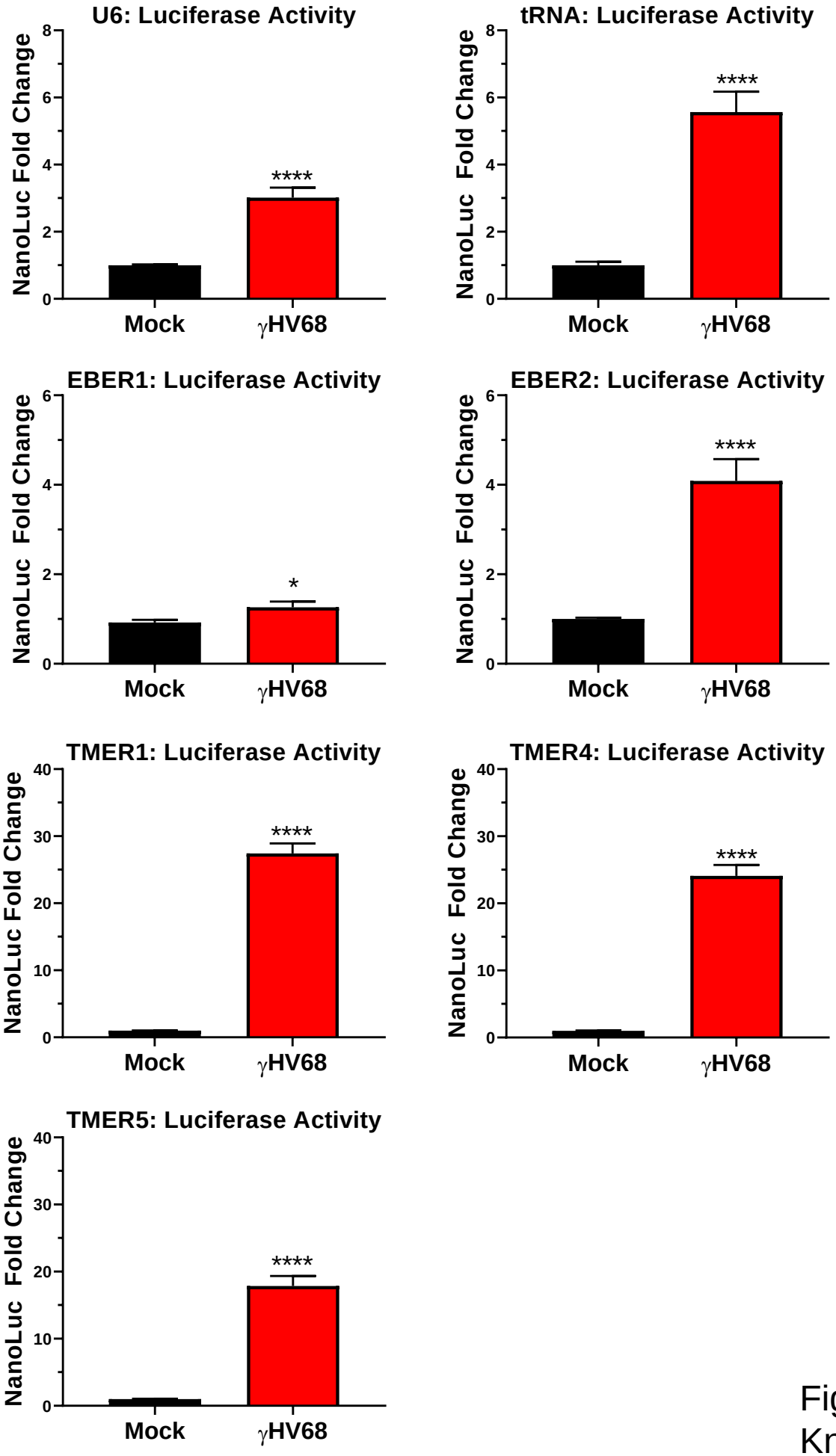


Figure 3
Knox et al

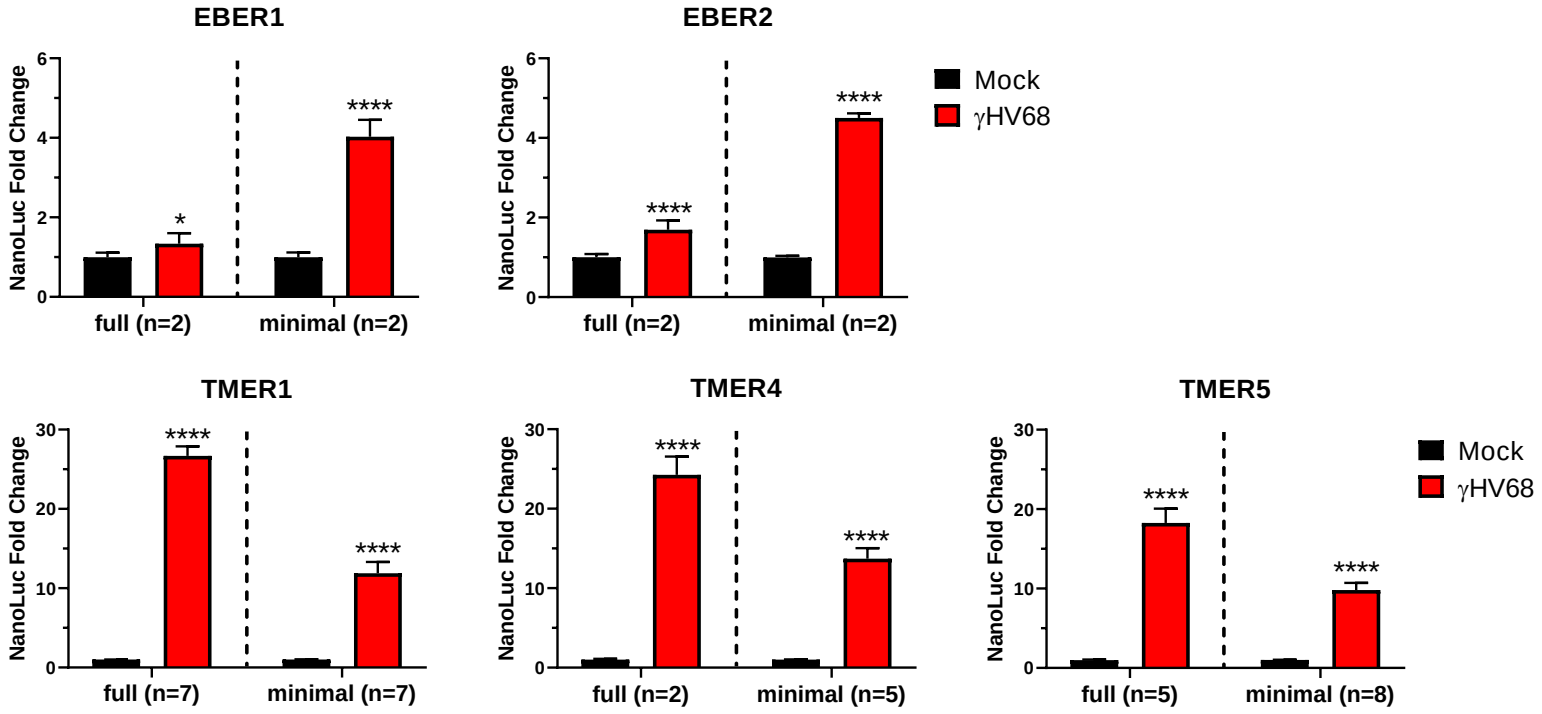
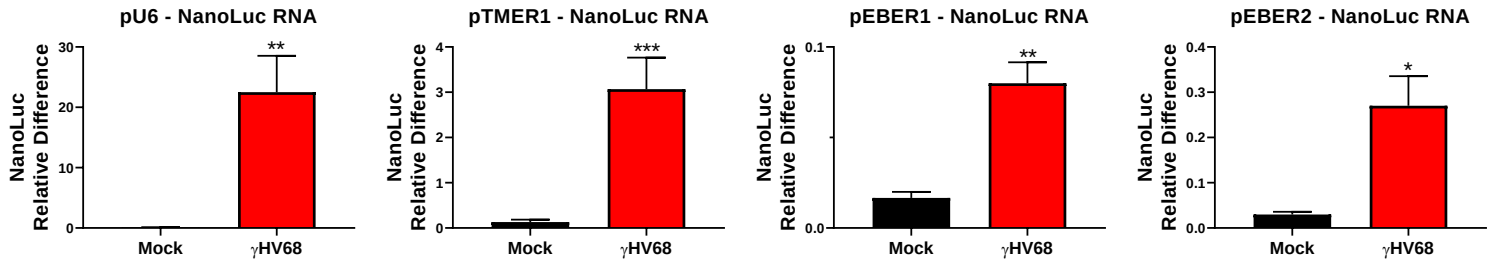


Figure 4
Knox et al

A)



B)

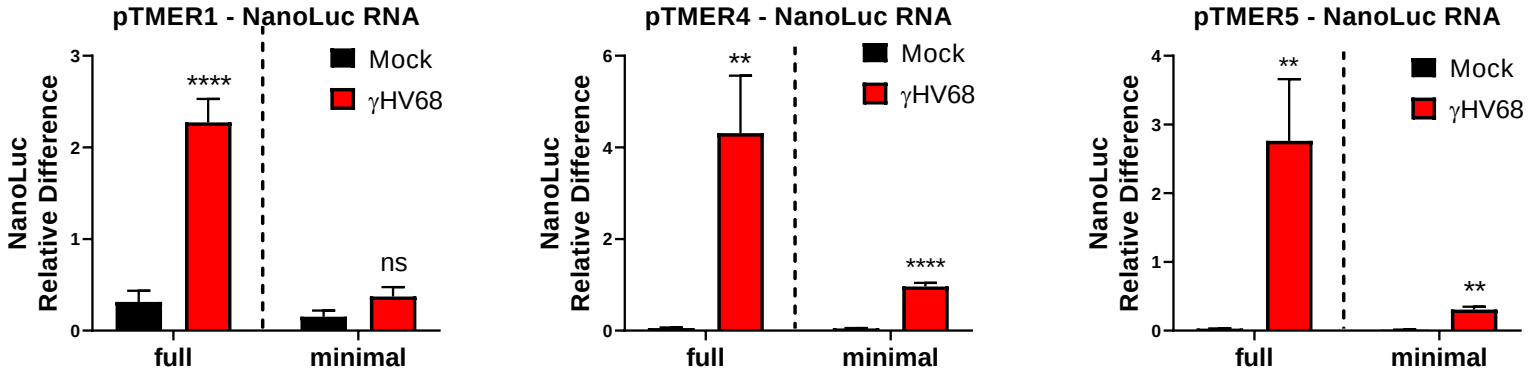


Figure 5
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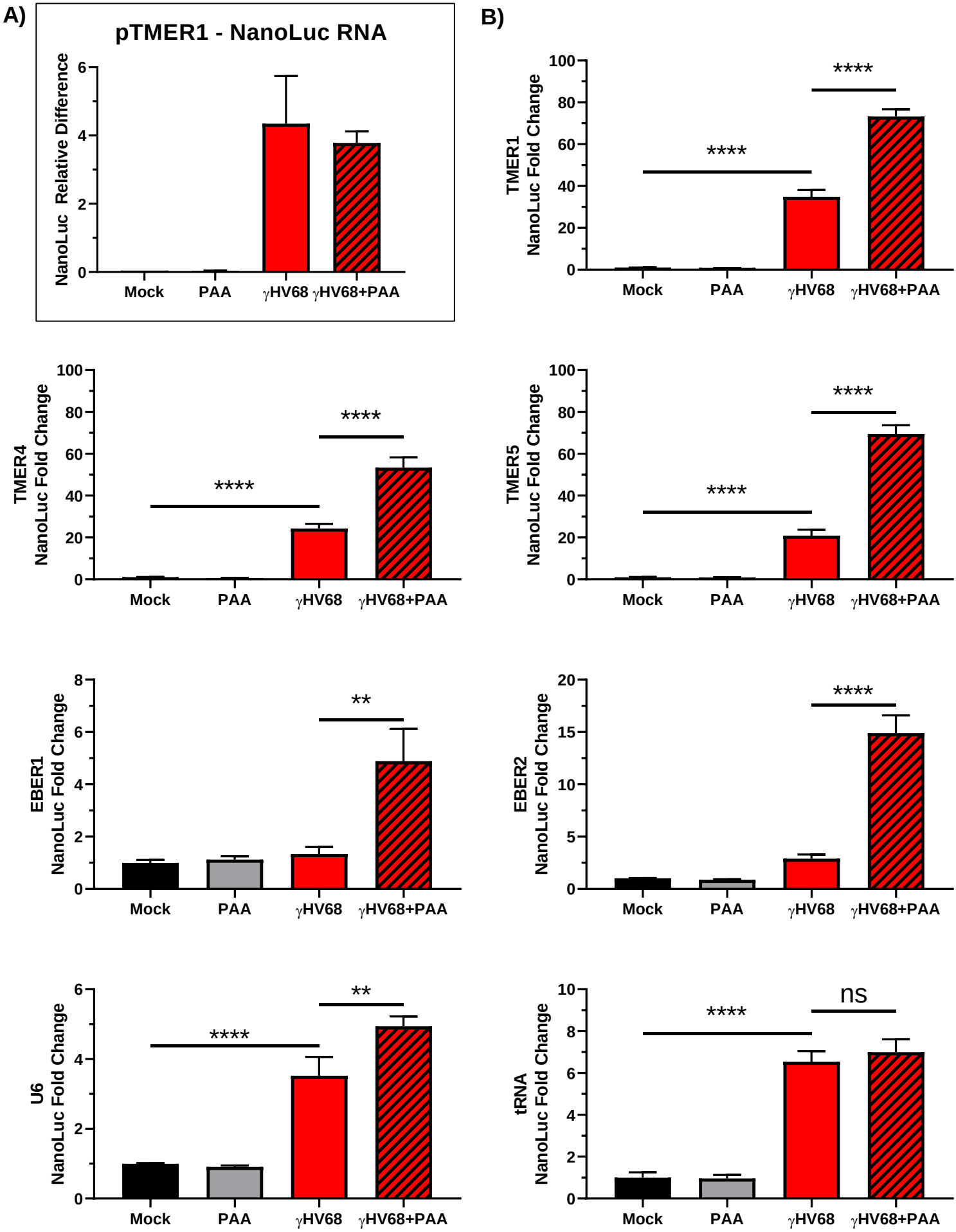

Figure 6
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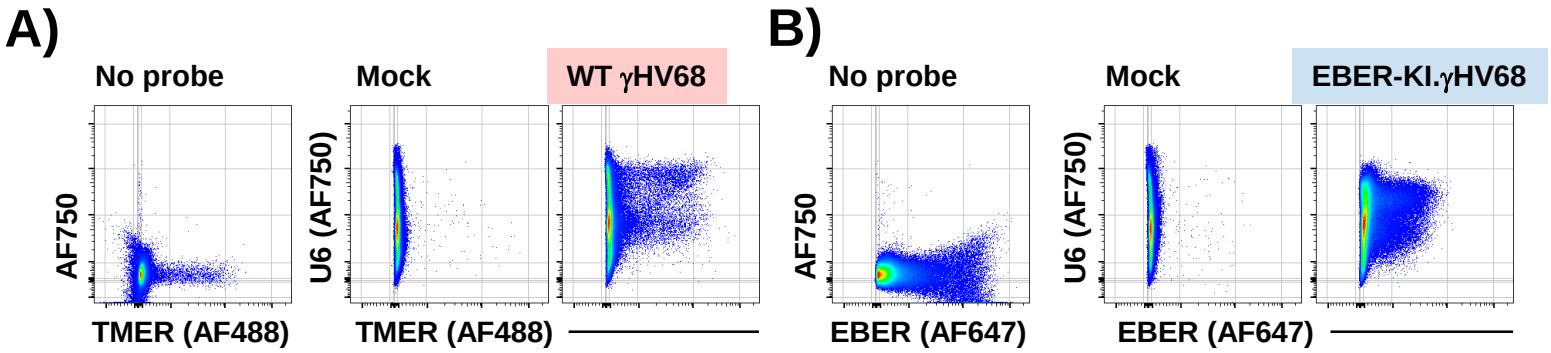
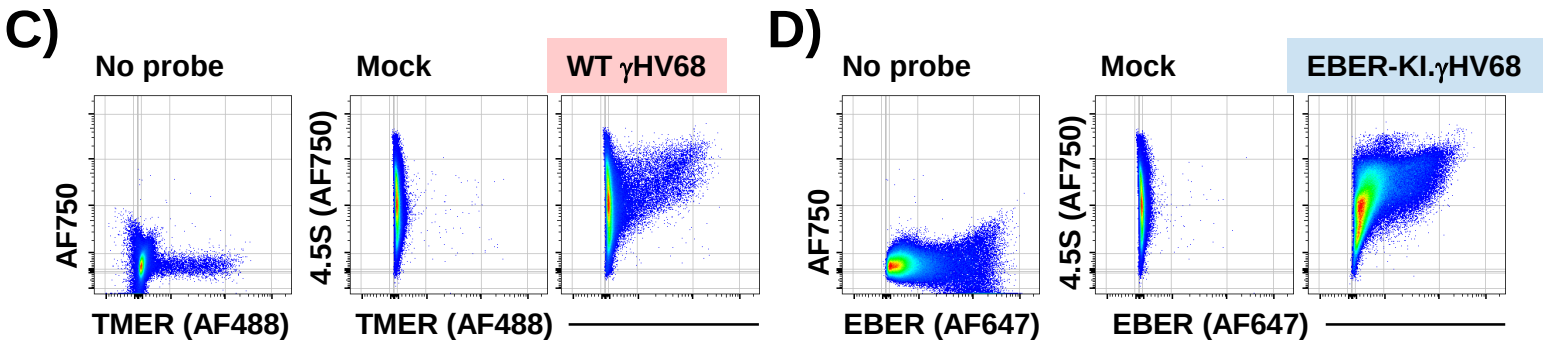
Endogenous U6 RNA expression



Endogenous 4.5S RNA expression



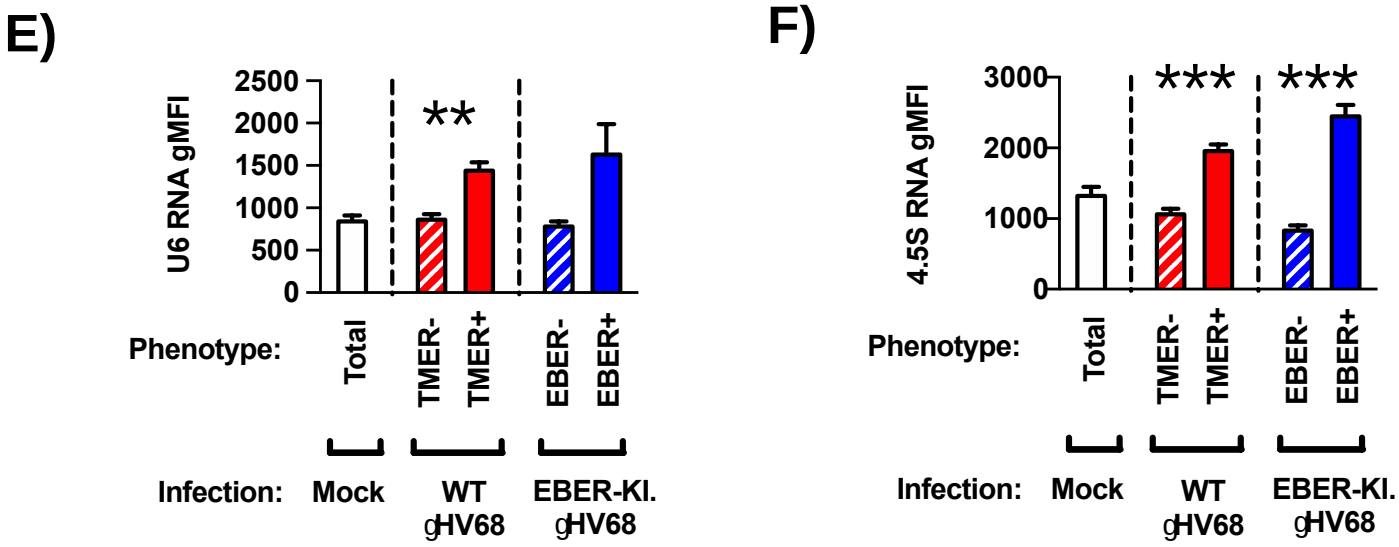


Figure 7
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