Video Article

Arbovirus Infections As Screening Tools for the Identification of Viral Immunomodulators and Host Antiviral Factors

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Abstract

RNA interference- and genome editing-based screening platforms have been widely used to identify host cell factors that restrict virus replication. However, these screens are typically conducted in cells that are naturally permissive to the viral pathogen under study. Therefore, the robust replication of viruses in control conditions may limit the dynamic range of these screens. Furthermore, these screens may be unable to easily identify cellular defense pathways that restrict virus replication if the virus is well-adapted to the host and capable of countering antiviral defenses. In this article, we describe a new paradigm for exploring virus-host interactions through the use of screens that center on naturally abortive infections by arboviruses such as vesicular stomatitis virus (VSV). Despite the ability of VSV to replicate in a wide range of dipteran insect and mammalian hosts, VSV undergoes a post-entry, abortive infection in a variety of cell lines derived from lepidopteran insects, such as the gypsy moth (*Lymantria dispar*). However, these abortive VSV infections can be "rescued" when host cell antiviral defenses are compromised. We describe how VSV strains encoding convenient reporter genes and restrictive *L. dispar* cell lines can be paired to set-up screens to identify host factors involved in arbovirus restriction. Furthermore, we also show the utility of these screening tools in the identification of virally encoded factors that rescue VSV replication during coinfection or through ectopic expression, including those encoded by mammalian viruses. The natural restriction of VSV replication in *L. dispar* cells provides a high signal-to-noise ratio when screening for the conditions that promote VSV rescue, thus enabling the use of simplistic luminescence- and fluorescence-based assays to monitor the changes in VSV replication. These methodologies are valuable for understanding the interplay between host antiviral responses and viral immune evasion factors.

Video Link

The video component of this article can be found at https://www.jove.com/video/58244/

Introduction

The ability of a virus to productively replicate in a particular host is in part governed by the availability of host cell factors that support viral entry and replication[1]. The virus-host range can also be dictated by the capacity of a virus to counter cellular antiviral defenses that would otherwise impede viral replication[2,3]. It is the outcome of these complex virus-host interactions that ultimately decide whether a virus will be able to complete its life cycle in a particular host. Given the potentially pathogenic consequences for the host if viral replication ensues, it is critical to develop experimental strategies to further our understanding of the key virus-host interactions that may tip the balance between abortive and productive infections. Elucidating the molecular features of virus-host interplay will be instrumental in the development of new and alternative antiviral therapeutic strategies.

With the advent of RNA interference (RNAi)[4,5] and genome-editing tools (*e.g.*, CRISPR-Cas9, Zinc finger nucleases, TALENs)[6,7], it has become experimentally feasible to alter the expression of cellular factors on genome-wide scales and explore the impact of these alterations on virus replication. Indeed, numerous RNAi and genome-editing-based screens have been conducted in invertebrate and vertebrate host cell types that have unveiled new facets of virus-host interactions[8,9,10,11,12]. These screens typically employ viruses encoding reporters, such as firefly luciferase (LUC) or fluorescent proteins (*e.g.*, GFP, DsRed), that provide convenient means of quantitatively assessing viral gene expression as a readout for viral replication[9,12]. This strategy allows researchers to identify host factors that either promote or antagonize viral replication as evidenced by increases or decreases, respectively, in viral reporter signals[9,12]. However, in the vast majority of cases, these screens have been conducted using viruses that are well-adapted to the host cell type in which they are being studied. While this strategy can be important for understanding coevolutionary relationships between viral pathogens and their natural hosts, it does pose fundamental concerns regarding their use in uncovering host antiviral factors. In these cases, an enhancement in virus reporter signal upon RNAi knockdown is being looked for, or the inactivation of a cellular factor that normally impedes viral replication. First, if a virus is already able to robustly replicate in the host cell being examined under control conditions, the dynamic range of the screen (*i.e.*, the ability to distinguish between background and enhanced viral reporter signals) may be limited. Second, this issue is further compounded by the situations in which the virus is well-adapted to the host cell and effective at countering host defense pathways that are being targeted in the screen.

Due to the above concerns regarding traditional virus-host interaction screening methods, we developed a new paradigm for studying virus-host interactions that exploit naturally abortive arbovirus infections in lepidopteran insect cells. This strategy derives from an observation that the well-studied human arbovirus, VSV, undergoes an abortive infection in cells derived from the gypsy moth (*L. dispar*)[13]. VSV is naturally transmitted by dipteran insects (*i.e.*, sand flies) to mammalian hosts, and has been shown experimentally to infect a wide range of invertebrate and vertebrate hosts both in cell culture and *in vivo*[14]. The 11-kb negative-sense single-stranded RNA genome of VSV encodes five subgenomic mRNAs that are each translated into the proteins that make up the enveloped virion. However, VSV reverse genetic systems have allowed for the creation of replication-competent strains encoding LUC or fluorescent proteins, in addition to the five natural VSV gene products[15,16,17]. Because these reporter proteins are not incorporated into the VSV virion, they provide a convenient readout for VSV gene expression that occurs post-entry. Using VSV strains encoding GFP or LUC, we have previously shown that VSV gene expression is severely restricted upon the entry of LD652 cells and that VSV titers do not increase by 72 hours post-infection (hpi). In contrast, the coinfection of LD652 cells with VSV and the mammalian poxvirus, vaccinia virus (VACV), leads to logarithmic increases in both VSV gene expression and titers by this time point. VACV undergoes early gene expression, DNA replication, and late gene expression in LD652 cell infections, but the VACV replication cycle is ultimately abortive due to incomplete virion morphogenesis[18]. The large ~192-kb DNA genome of VACV encodes > 200 proteins, many of which display immunomodulatory properties that promote viral replication through the suppression of host immune responses[19]. Therefore, we hypothesized that the "rescue" of VSV replication in LD652 cells by VACV coinfection was likely mediated by VACV immunomodulators that inhibited *L. dispar* responses normally restricting VSV replication. In support of this, the treatment of LD652 cells with the host RNA polymerase II inhibitor actinomycin D also rescues VSV replication in LD652 cells, indicating that the transcription-dependent host responses block VSV replication post-entry[13].

The above observations suggest that the naturally restrictive nature of LD652 cells to VSV infection may provide a relatively low background when screening for the conditions that enhance VSV-encoded reporter signals (*i.e.*, those that inhibit host antiviral defenses). Here, we provide the methods for using fluorescence or LUC-based assays to screen for conditions that relieve VSV restriction in lepidopteran cells. First, we show how these assays can be used to identify virally encoded immunomodulatory factors that break VSV restriction during either coinfection experiments or through ectopic expression of candidate viral factors. As an example, we illustrate how we used these screening techniques to identify poxvirus-encoded A51R proteins as a new family of immunomodulatory factors that rescue VSV replication in the absence of other poxvirus factors[13]. Second, we illustrate how RNAi screening in restrictive VSV-LD652 cell infections can be used to directly identify eukaryotic host factors involved in arbovirus restriction[13].

Protocol

1. General *Lymantria dispar* (LD652) Cell and Virus Culture

1. **LD652 cell culturing and plating**
   1. To culture *L. dispar*-derived LD652 cells, maintain a monolayer of cells in a growth medium (**Table of Materials**) incubated at 27 °C under normal atmosphere. Maintain the cells in 10 cm tissue-culture-treated dishes and passage the cells upon reaching 80% confluency.
   2. To plate, dislodge adherent LD652 cells from the plate by pipetting the media repeatedly onto the monolayer (these cells do not require trypsinization to dislodge), and dilute the sample in growth media to an approximate density of 1 x 10^5 cells/mL. Pipette 1 mL of diluted culture/well of a 24-well plate. Seed a minimum of three replicate wells/treatment type for each time point (a maximum of eight treatments/plate).
   3. Allow the cells to settle for a minimum of 1 h.

2. **Vaccinia virus preparation**
   1. To prepare the stocks of VACV, amplify the virus in HeLa cells[20] or African Green Monkey kidney cells (BSC-1 or BSC-40 cells)[20,21,22].
      NOTE: VACV strain Western Reserve (VACV-WR) works well in the assays described here.
   2. Titrate the VACV strain *via* plaque assay on BSC-1 or BSC-40 cells[20,22].
      NOTE: All indicated VACV multiplicity of infection (MOI) described here for LD652 cell infections are based on titers obtained from BSC-40 plaque assays.

3. **Vesicular stomatitis virus preparation**
   1. To prepare VSV stocks, amplify the virus in BHK cells[23].
   2. Titrate VSV by plaque assay on BSC-40 or BHK cell monolayers[13,23].
      NOTE: All VSV MOIs described here for LD652 cell infections are based on titers obtained from BSC-40 plaque assays.

2. Fluorescence-based VSV Rescue Assay Using Co-infection and Live-cell Imaging

1. **Seeding and infection of LD652 cells**
   1. Plate 40,000 LD652 cells/well of an 8-well chamber.
   2. For fluorescence-based live-cell imaging, use VSV strains encoding fluorescent proteins. The experiments presented here use VSV-DsRed[17], a recombinant VSV strain that expresses free DsRed protein that is not fused to any other VSV protein.
      NOTE: VSV infection of LD652 cells is not cytopathic by 96 hpi and, thus, the cell death is not a confounding factor when evaluating VSV replication within this time frame.
   3. Prepare VSV-DsRed and VACV-FL-GFP in a serum-free media (SFM; **Table of Materials**) at an MOI of 1 and 25, respectively.
      NOTE: VACV-FL-GFP is a recombinant VACV strain that expresses a fusion between LUC and GFP[24]. Using an MOI of 25 or greater for VACV strains ensures that all LD652 cells are infected[13]. If using a different coinfecting virus, the MOI will have to be determined empirically by the user. Each infection treatment should be set up in duplicate wells and include mock-infected treatments in which only SFM is added to the cells.

4. Incubate the cells in 0.2 mL of inoculum for 2 h at 27 °C.
5. Wash the cells with 0.5 mL/well of growth media.
6. Incubate the cells in 25 µM of cell viability dye (**Table of Materials**) in growth media for 45 min at 27 °C.
7. Aspirate the media and wash the wells 1x with 0.5 mL/well to remove excess dye.
8. Maintain the cells in 0.3 mL/well of the growth media.

2. **Live cell image capturing**
   1. Turn on a confocal microscope 30 min in advance and load an 8-well chamber dish.
      NOTE: LD652 cells can be imaged at room temperatures ranging from 20–25 °C, but for optimal conditions, the temperature of the room should be adjusted to 27 °C.
   2. Set up the appropriate excitation/emission settings for each fluorescent marker protein to be imaged, as well as the phase contrast image settings.
   3. Adjust the laser intensity for each channel.
      NOTE: This may require running a pilot experiment to determine the range of fluorescent signal intensities observed throughout the time course to be used in the final experiment.
   4. Using the 10X objective, capture the phase contrast and fluorescence images of each well every 1–5 h for the duration of the infection up to a desired time point (*e.g.*, 48–72 hpi).
      NOTE: It is important to capture multiple fields of view in each well to accurately estimate the infection rates throughout the culture.

3. **Image analysis**
   1. Use image analysis software for automated image analysis of appropriate fluorescent channels (*e.g.*, 405 nm, 488 nm, 568 nm).
   2. To calculate the percentage of cells that are infected, divide the total number of fluorescent cells indicating VSV infection (*e.g.*, DsRed-positive cells for VSV-DsRed infections) by the total number of viable cells labeled by cell viability dye for each field of view across all treatments.

3. General Viral Infection Protocol for Luminescence-based VSV Rescue Assays in LD652 Cells

1. **Preparation of inoculum**
   1. 30 min prior to the infection, thaw the stocks of VSV-LUC[16] (a recombinant VSV strain that expresses free firefly luciferase protein not fused to any other VSV protein). If assaying for VSV-LUC rescue during VACV coinfection, also thaw the VACV-WR virus on ice.
      NOTE: Other viruses (besides VACV-WR) may rescue VSV-LUC during the coinfection, but this will have to be determined empirically by each user.
   2. Prepare the inoculum by diluting VSV-LUC in the presence or absence of VACV-WR into SFM such that an MOI of 10 and 25, respectively, is achieved when adding a total inoculum volume of 0.2 mL/well.

2. **Infection of the cells**
   1. Aspirate mature LD652 media carefully to avoid disturbing the monolayer and inoculate with 0.2 mL of virus/well. This time is defined as 0 hpi. Add sterile SFM to additional wells to serve as “mock-infected” negative control treatments.
   2. Incubate the cells in inoculum for 2 h at 27 °C.
   3. At 2 hpi, remove inoculum by aspiration and replace with 1 mL/well LD652 growth media.
   4. Allow the infection to proceed 24–72 hpi.

4. Luciferase Assay

1. **Preparation of cell lysates**
   1. Carefully aspirate the supernatant and add 1 mL of Dulbecco's phosphate-buffered saline (DPBS)/well.
   2. Using the plunger of a 1 mL syringe, scrape the cells into DPBS.
   3. Transfer the cells to a microcentrifuge tube, and centrifuge at 400 x *g* for 20 min at 4 °C.
   4. Meanwhile, prepare 1x dilution of 5x reporter lysis buffer (RLB) in sterile H_2O.
   5. Following the centrifugation, aspirate the DPBS without disturbing the cell pellet.
   6. Resuspend each pellet in 150 µL of 1x RLB.
   7. Freeze-thaw the samples 1x using a 5-min incubation in a -80 °C freezer followed by a rapid thaw in a room temperature water bath. Store the lysates at -80 °C until ready to analyze.

2. **Luciferase assay**
   1. Thaw the lysates on ice.
   2. Pipette 20 µL of lysate into a well of a solid black or white 96-well microplate.
   3. Add 100 µL of luciferase assay reagent to each well.
   4. Immediately measure the light intensity using a luminometer (appropriate settings for specific luminometers will have to be determined empirically by the user).

3. **Luciferase assay analysis**

1. Normalize LU signals for each treatment to LU readings obtained from control treatments (**Representative Results**). After at least three independent experiments have been performed, the mean LU obtained from each treatment/control group can be analyzed by appropriate statistical tests.

5. Immunoblot

1. Use lysates extracted for the LUC assays for SDS-PAGE and subsequent immunoblotting, using appropriate reagents and instrumentation.

6. Titer of VSV from LD652 Cell Cultures

1. Plate LD652 cells according to step 1.1.
2. Viral infect the cells according to step 3.
3. At desired time points, collect the supernatants into sterile microcentrifuge tubes.
4. Pellet the cells using 1,000 x *g* for 10 min at 4 °C and transfer the supernatants to new tubes.
5. Store the supernatants at -80 °C or proceed with titration by plaque assay on BSC-40 cells.
   NOTE: If titrating VSV from LD652 cell cultures that contained VACV, it is advisable to add 100 µg/mL cytosine arabinoside (AraC) to the BSC-40 culture media within 2 hpi, to prevent residual VACV particles from forming plaques on BSC-40 monolayers[13]. AraC is a viral DNA polymerase inhibitor that blocks VACV DNA replication[25] but does not affect VSV replication.

7. Variations of Luminescence-based VSV Rescue Assays in LD652 Cells: RNAi and Plasmid Transfection Experiments

1. **Preparation of dsRNA for RNAi-mediated knockdown of viral or host transcripts**
   1. Design gene-specific primers tailed at the 5' end with the T7 promoter sequence (TAATACGACTCACTATAGGG) to target either the coinfecting virus (*e.g.*, VACV) or *L. dispar* mRNA transcripts of interest. These primers should produce a PCR product of 400–600 bp for effective RNAi-mediated knockdown. See Gammon *et al.*[13] for primer sequences used below to knockdown VACV or *L. dispar* transcripts by dsRNA-mediated RNAi in LD652 cells.
      NOTE: *L. dispar* mRNA transcripts can be identified from published *L. dispar* transcriptome databases[26,27,28].
   2. Generate a DNA template *via* RT-PCR (cDNA synthesis: 1 cycle of 50 °C for 30 min; PCR: 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 68 °C for 45 s each).
   3. Purify the PCR product using a PCR purification kit.
   4. Using the purified PCR product as a template, *in vitro* transcribe and purify dsRNA using an *in vitro* transcription and purification kit.

2. **Transfection of dsRNA or plasmid DNA into LD652 cells**
   1. Seed 1 x 10^5 cells/well of a 24-well plate and let the cells settle for at least 1 h.
   2. For each well to be transfected, dilute 4 µL of transfection reagent into 100 µL of SFM. Incubate for up to 15 min at room temperature. This can be scaled to make a master mix for all transfections to be performed.
   3. Dilute up to 1 µg of dsRNA or total plasmid DNA with 100 µL of SFM for each well to be transfected. Incubate for up to 15 min at room temperature.
      NOTE: We have previously found that a transfection of 1 µg of dsRNA/10^5 LD652 cells is sufficient for >80% knockdown of either viral or host transcripts[13], but dsRNA doses needed for modified experimental set-ups/conditions will have to be determined empirically.
   4. Combine transfection reagent and dsRNA/plasmid DNA dilutions with a 1:1 ratio (*e.g.*, 100 µL of diluted dsRNA is mixed with 100 µL of diluted transfection reagent) and incubate for 20 min at room temperature.
   5. Meanwhile, wash the wells with 1 mL/well of SFM, and then add 500 µL of SFM to each well.
   6. Slowly add the transfection reagent dsRNA (or plasmid DNA) mixture dropwise into each well.
   7. Incubate the transfection reagent with the cells for 5 h.
      1. For RNAi experiments involving the knockdown of transcripts encoded by a coinfecting virus (*e.g.*, VACV), replace the transfection reagent after the 5-h transfection incubation period with virus inoculum containing VSV-LUC (with or without VACV or another coinfecting virus) (step 3). Subsequently, replace the virus inoculum containing VSV-LUC with complete growth media 2 hpi. LUC assays (step 4) can then be performed on extracted lysates at various time points to determine if the knockdown of coinfecting virus transcripts leads to a loss of VSV-LUC rescue.
      2. For RNAi experiments involving RNAi of *L. dispar* transcripts, replace the transfection mix with complete growth media and allow knockdown to ensue for 24 h prior to infection with VSV-LUC (step 4). LUC assays (step 4) can then be performed on extracted lysates at various time points to determine if the knockdown of *L. dispar* transcripts promotes VSV-LUC rescue.
      3. For experiments involving transfections of plasmid expression vectors expressing candidate immunomodulators, replace the transfection reagent and allow for protein expression for 24 h prior to the infection with VSV-LUC (step 4). LUC assays (step 4) can then be performed on extracted lysates at various time points to determine if the expression of candidate immunomodulatory proteins promotes VSV-LUC rescue.
         NOTE: The transfection conditions described here using 1 µg/well of plasmid DNA result in transfection efficiencies of 40–60%[13].

Representative Results

As an example of live-cell imaging applications to monitor VSV rescue upon VACV coinfection, LD652 cells were plated in an 8-well chambered dish and then mock-infected or infected with VSV-DsRed (MOI = 1) in the presence or absence of VACV-FL-GFP (MOI = 25). Because VSV-DsRed expresses DsRed as a free protein and is not fused to structural VSV proteins (**Figure 1A**), it is only detected after VSV entry and gene expression initiates. All cells were then labeled with cell viability dye that freely passes through the plasma membrane of cells, where it is converted into a membrane-impermeant product, which promotes the retention of the fluorescent signal in labeled cells. Images were acquired every 5 h up to 65 hpi, using the 405 nm, 488 nm, 568 nm, and white light filters to capture cell viability dye, VACV-FL-GFP, VSV-DsRed, and phase contrast (PC) channels, respectively. Under single infection conditions, LD652 cells restrict VSV-DsRed replication; therefore, only a small number of cells exhibit a DsRed signal. However, coinfection of VSV-DsRed with VACV-FL-GFP results in most cells displaying DsRed signals by the end of the time course (**Figure 1B**). Images captured at each time point were subjected to image analysis software, where 405 nm and 568 nm channel images were used to automatically determine total and DsRed-positive cell numbers, respectively. The percentage of cells displaying a DsRed signal for each treatment is calculated by dividing objects (cells) with a positive signal in the 568-nm channel by the number of objects identified in the 405 nm channel for each time point, followed by multiplication by 100%. As shown in **Figure 1C**, ~2% of the cells from VSV-DsRed single infections were DsRed-positive by 65 hpi. In contrast, ~77% of the cells in VSV-DsRed + VACV-FL-GFP coinfections were DsRed-positive at this time point. **Movies S1 and S2** show the progression of VSV-DsRed infection over the entire 65-h time course in single infection and coinfection conditions, respectively. It is important to note that the GFP expression by VACV-FL-GFP became detectable by 10 hpi, prior to the DsRed signal, indicating that sufficient VACV gene expression is required prior to VSV-DsRed rescue (not shown). Collectively, these results clearly indicate a rescue of VSV-DsRed replication by VACV coinfection. If a coinfecting virus was unable to rescue VSV-DsRed, we would expect equal percentages of DsRed-positive cells between single infection and coinfection treatments.

VSV replication in LD652 cells can alternatively be quantified using luminescence-based assays when using VSV-LUC strains (**Figure 2A**). As an example, **Figure 2B** shows the results of a LUC assay using lysates prepared over a 72 h time course from mock-infected cells or cells infected with VSV-LUC (MOI =10) in the presence or absence of VACV-WR (MOI = 25). A positive rescue of VSV-LUC replication is indicated by the logarithmic increase in arbitrary LU detected with lysates prepared from coinfected cells, compared to lysates from single VSV-LUC infections. A negative result would be indicated in this assay by the failure of a coinfection to alter LU readings from those observed in single VSV-LUC infection treatments. If desired, prepared lysates can also be used for immunoblotting, to confirm enhanced VSV gene expression in coinfection treatments. For example, **Figure 2C** shows a typical immunoblot result for VSV-encoded LUC and matrix (M) proteins in lysates prepared from mock-, VSV-LUC, and VSV-LUC + VACV-WR treatments 72 hpi. Immunoblotting for VACV I3L protein served as a marker of VACV infection, and immunoblotting for cellular actin was used as a loading control. The clear enhancement of VSV-encoded LUC and M proteins in the coinfection lysates further confirm VSV rescue by VACV. Finally, productive VSV replication can be confirmed by collecting supernatants from these LD652 cell cultures and titrating an infectious virus on BSC-40 monolayers (**Figure 2D**). This result illustrates that only during VACV coinfection does VSV productively replicate.

Once a coinfecting virus is shown capable of rescuing VSV replication in LD652 cells, RNAi screening can be used to identify factors encoded by the coinfecting virus that contribute to VSV rescue. In this experiment, screen for an RNAi condition that leads to a "loss of rescue" phenotype during VSV-LUC coinfection with a rescuing virus. We previously identified the VACV A51R protein as a VSV rescue factor through an RNAi screening of dozens of VACV-encoded transcripts[13]. As an example, we have recapitulated a smaller scale version of this screen (**Figure 3**). RNAi is mediated by transfection of *in vitro* transcribed dsRNAs that target transcripts encoded by the coinfecting virus. In the data shown here, viral transcripts encoding VACV A50R, A51R, and A52R proteins were targeted for RNAi knockdown. As a negative control for loss of rescue, cells were also transfected with dsRNAs targeting GFP-encoding transcripts. As a positive control for a loss of rescue phenotype, cells were transfected with dsRNAs against LUC-encoding transcripts. This treatment produces a strong loss of LU signal during coinfection and helps researchers confirm that transfection/RNAi protocols are working. After 5 h of dsRNA transfection, cells were coinfected with VSV-LUC (MOI = 10) and VACV-WR (MOI = 25). Separate infections with only VSV-LUC were also performed to establish a background level of LU signal. Lysates were then harvested 72 hpi and used in a LUC assay. Comparing the level of VSV rescue across dsRNA treatments to the GFP dsRNA control treatment, the results indicate that the knockdown of VACV A51R produces a strong loss of rescue phenotype, whereas the knockdown of A50R and A52R produces a negative result (no loss of rescue compared to GFP dsRNA).

As an alternative to RNAi screening to identify viral factors that contribute to VSV rescue, overexpress candidate viral factors in LD652 cells and then assay for VSV-LUC rescue. This can be accomplished by cloning candidate genes of interest into appropriate expression vectors such as p166[29] and transfecting these plasmids into LD652 cells prior to the VSV-LUC challenge. As an example, **Figure 4A** shows a rescue experiment in which FLAG-tagged GFP (FGFP) or FLAG-tagged A51R (FA51R) p166 vectors were transfected into LD652 cells at various concentrations, followed by VSV-LUC infection (MO = 10) 24 h later. As a negative control for VSV rescue, additional cultures were mock-transfected and did not receive plasmid DNA. Lysates were collected from cultures 72 hpi and were subjected to LUC assays. FA51R treatments produced a positive VSV rescue result as demonstrated by enhanced LU signals over mock-transfected treatments at multiple doses. In contrast, FGFP treatments were negative for VSV rescue at any dose tested. Immunoblotting of the lysates from **Figure 4A** confirmed a similar expression of FGFP and FA51R proteins (**Figure 4B**).

Using an RNAi screening of candidate *L. dispar* factors, we have shown that the restriction of VSV in LD652 cells is mediated by various cellular factors belonging to antiviral RNAi pathways (*e.g.*, *AGO2 and Dicer-2), the* Nuclear Factor kappa B (NF-κB)-related IMD pathway (*e.g.*, *Relish)*, and the ubiquitin-proteasome system (*e.g.*, polyubiquitin)[13]. As an example, we have repeated a smaller scale version of these RNAi experiments with dsRNAs targeting *L. dispar* transcripts that enhanced VSV-LUC replication upon knockdown (*e.g.*, AGO2, Dicer-2, Relish, polyubiquitin) or had no effect (AGO1)[13]. After 24 h of dsRNA transfection, cells were challenged with VSV-LUC for 72 h to determine if RNAi treatments enhanced LU signals over negative control GFP dsRNA treatments. RNAi treatments that enhance LU signals indicate that the factor encoded by the targeted transcript restricts VSV replication. As a positive control for RNAi knockdown, dsRNAs targeting LUC-encoding transcripts were also transfected in parallel treatments. Due to the low background LU signals detected in GFP dsRNA control treatments, it was relatively straightforward to identify host-encoded restriction factors using RNAi screening because their knockdown produces ~10- to 1,000-fold increases in LU signals (**Figure 5**). Thus, it is possible to take advantage of the relatively low background level of VSV gene expression in LD652 cells to screen for host RNAi knockdown conditions that relieve VSV restriction.

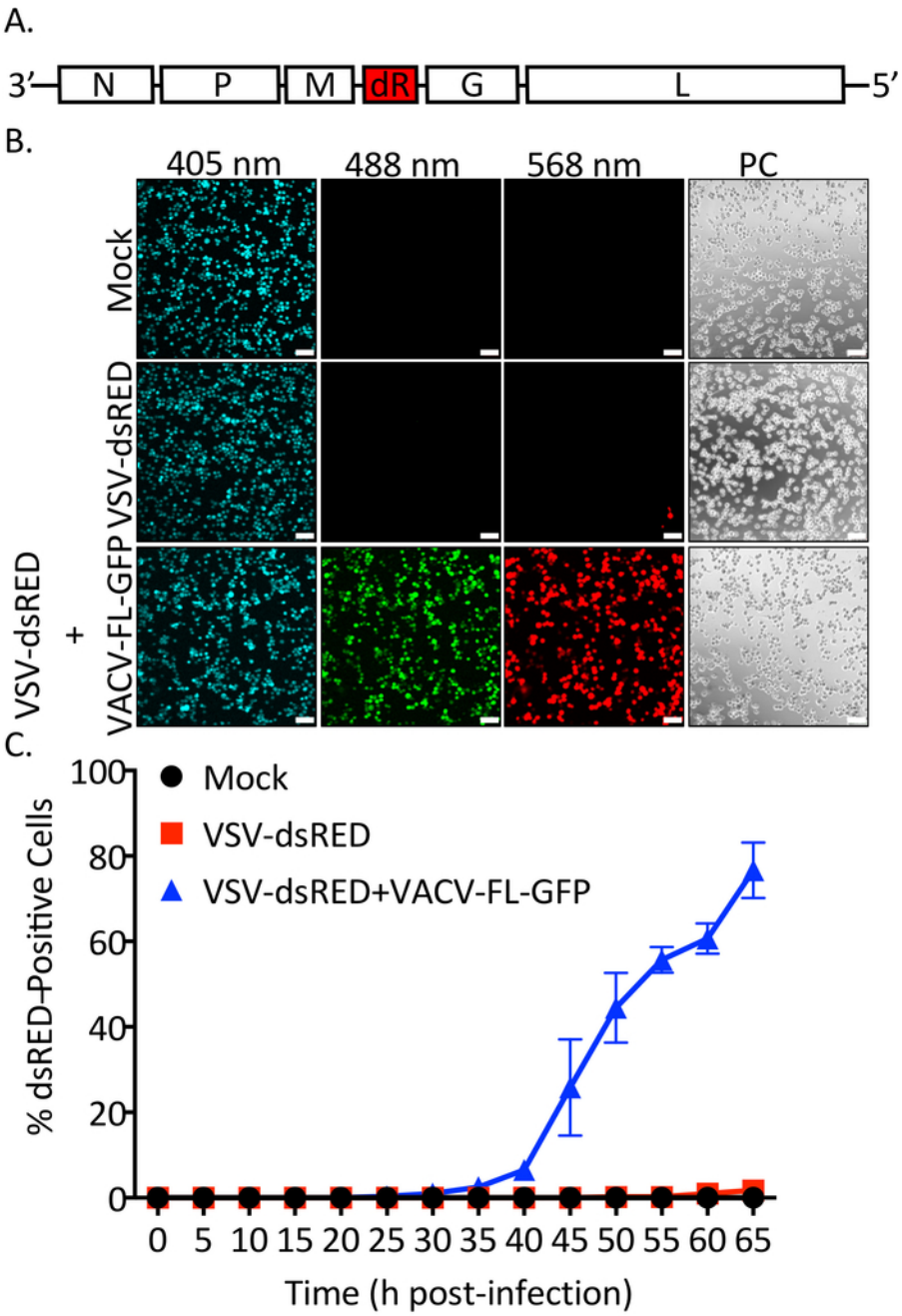


Figure 1: Identification of VSV rescue by virus coinfection of LD652 cells using fluorescence-based live-cell imaging. (**A**) This panel shows a schematic of a VSV-DsRed genome indicating the DsRed (dR) gene location. (**B**) These representative 10X confocal microscopy images are captured 60 hpi. The 405 nm channel indicates a stain for viable cells, the 488-nm channel indicates VACV-FL-GFP infection, and the 568 nm channel indicates VSV-DsRed infection. Phase contrast (PC) images are also shown. Scale bars = 100 µm. (**C**) This panel shows the percentage of DsRed-positive LD652 cells over the entire 65 h infection time course. The mean (SD) percentage of the cells showing a DsRed signal for each time point is shown. Please click here to view a larger version of this figure.

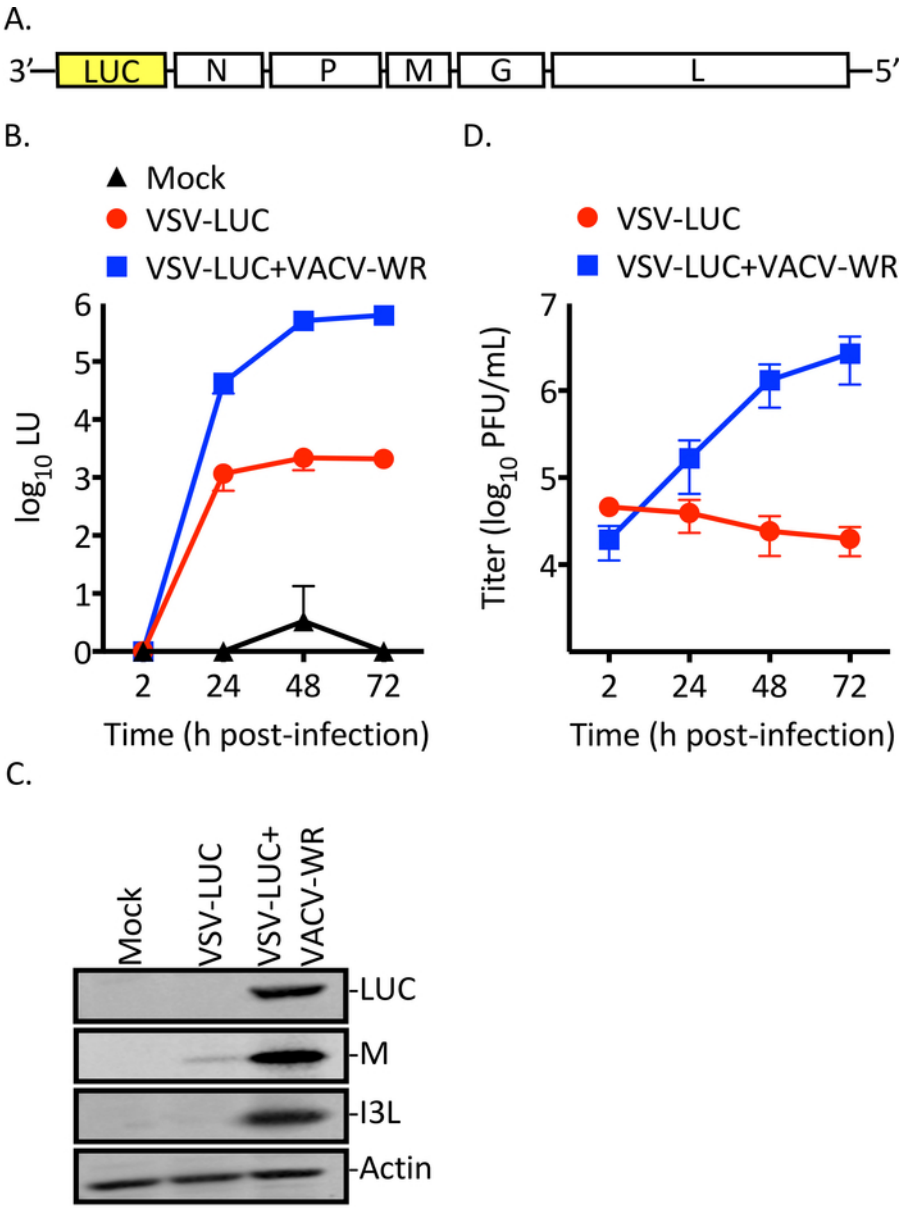


Figure 2: Assessment of VSV rescue in LD652 cells using LUC assays. (**A**) This panel shows a schematic of a VSV-LUC genome. (**B**) This panel shows arbitrary light units (LUC) assays of lysates from mock-infected cells or cells infected with VSV-LUC, in the absence or presence of VACV-WR. (**C**) This panel shows an immunoblot of LUC, VSV M, VACV I3L, and cellular actin proteins in the lysates from panel **A** collected 72 hpi. (**D**) This panel shows VSV-LUC titers in culture supernatants obtained from panel **B**. The quantitative data in panels **B** and **D** represent the means (± SD) from experiments performed in triplicate. Please click here to view a larger version of this figure.

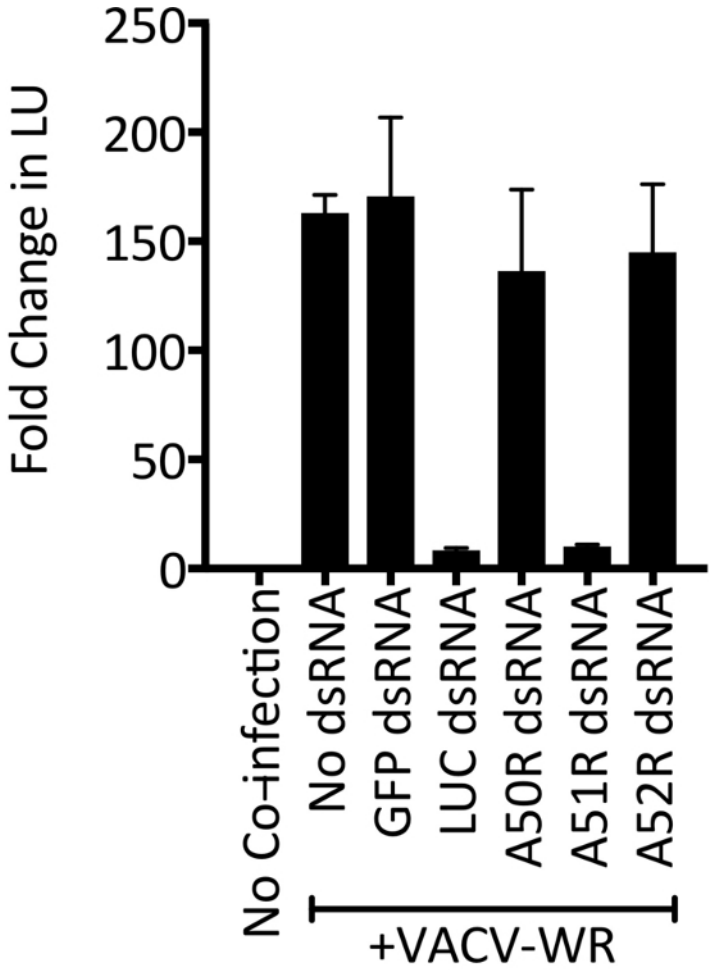
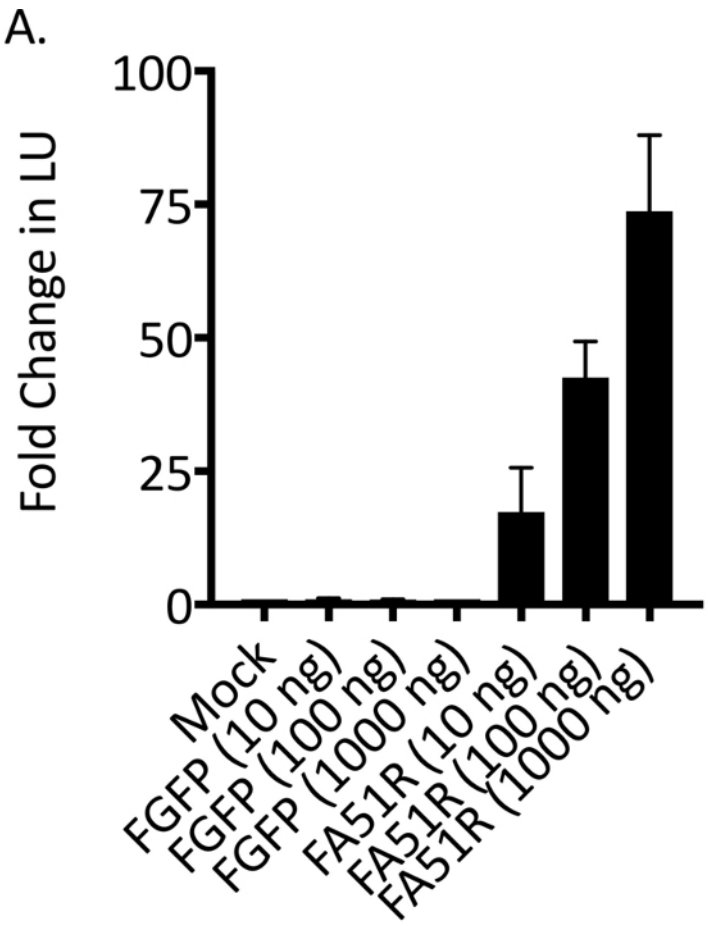


Figure 3: Identification of a virally-encoded VSV rescue factor through RNAi screening during the coinfection of LD652 cells. This panel shows fold changes in LU detected in lysates from cells 72 hpi with VSV-LUC and VACV-WR after the indicated RNAi treatment, relative to LU detected in lysates from cells singly infected with VSV-LUC. The data represent the means (± SD) from experiments performed in triplicate. Please click here to view a larger version of this figure.



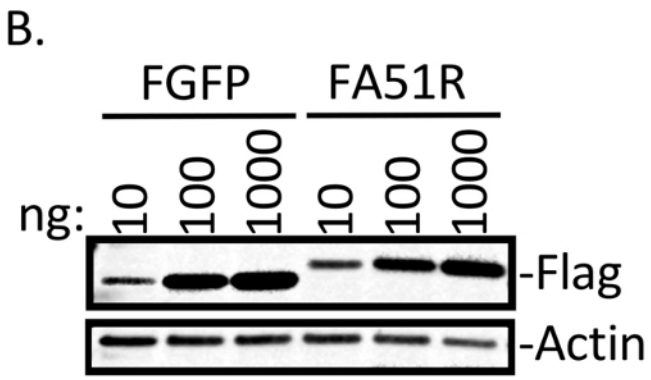


Figure 4: Rescue of VSV by the overexpression of a viral immunomodulator in LD652 cells. (**A**) This panel shows a LUC assay from VSV-LUC-infected cells 72 hpi that were mock-transfected (mock) or transfected with either FGFP or FA51R p166 expression plasmids. LU signals from each treatment were normalized to signals detected in mock-transfected control treatments. The data represent the means (± SD) from experiments performed in triplicate. (**B**) This panel shows lysates from panel **A**, immunoblotted with FLAG and actin antibodies. Please click here to view a larger version of this figure.

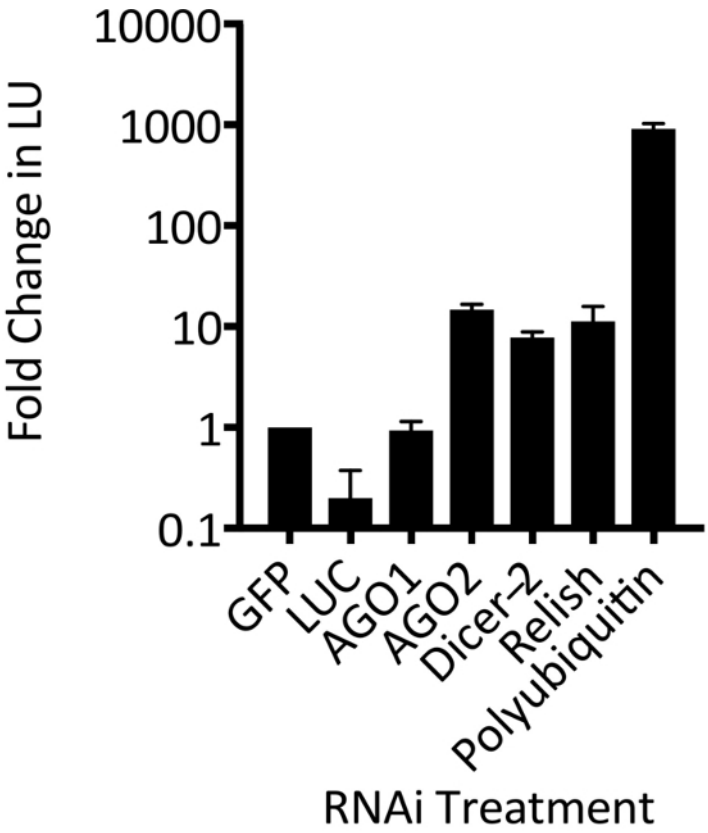


Figure 5: Identification of host factors restricting VSV replication in LD652 cells through RNAi screening. This panel shows the fold change in LU signals in indicated RNAi treatments relative to the GFP dsRNA control treatments 72 hpi with VSV-LUC. The data represent the means (± SD) from experiments performed in triplicate. Please click here to view a larger version of this figure.

Movie S1: Representative 405-nm (cell viability dye) and 568 nm (DsRed) channel images captured over a 65 h time course (each frame = 5 h interval) after VSV-DsRed infection of LD652 cells. Please click here to download this file.

Movie S2: Representative 405-nm (cell viability dye) and 568 nm (DsRed) channel images captured over a 65 h time course (each frame = 5 h interval) after coinfection of LD652 cells with VSV-DsRed and VACV-FL-GFP. Please click here to download this file.

Discussion

Here we have described simple fluorescence- and luminescence-based assays to screen for conditions that rescue VSV replication in restrictive lepidopteran cell cultures. The abortive infection of VSV in lepidopteran cells creates an excellent signal-to-noise ratio when assaying for VSV gene expression. For example, the LU signals detected in lysates from single VSV-LUC infections were ~1,000-fold higher than in mock-infected lysates, yet these signals only changed approximately twofold over a 72-h time course. In contrast, coinfection of VSV-LUC with VACV enhanced LU signals ~300-fold over single VSV-LUC infections by 72 hpi. Thus, VSV rescue assays display an excellent dynamic range, encompassing several orders of magnitude.

One of the critical steps in setting up these assays involves the decision to assay for VSV rescue using fluorescence- or luminescence-based approaches. We have shown examples of how VSV-encoded DsRed signals can be assayed quantitatively using live-cell imaging techniques and software packages that facilitate the automated quantification of DsRed-positive cells in the absence or presence of a rescuing coinfection. If researchers have access to live-cell imaging capabilities, these assays are inexpensive to set up and allow them to capture a wide range of time points that may aid in the detection of differences in VSV replication kinetics that are only observable in narrow time windows. In contrast, luminescence-based approaches are essentially end-point assays that require the preparation of cell lysates at predetermined time points, and lysate preparation can be time-consuming. On the other hand, the luminescence-based assays do not require sophisticated microscopy equipment—only a plate reader capable of reading luminescence signals. Furthermore, luminescence-based assays have a greater dynamic range than microscopy-based assays that calculate the percentage of cells infected. For example, in microscopy assays, DsRed-positive cells (indicating VSV-DsRed infection) can only range from 0–100% of analyzed cells in a field of view. In contrast, LU signals between single VSV-LUC and coinfection conditions (or other rescue conditions) can range over several orders of magnitude.

A key advantage of using the VSV-*L. dispar* cell system presented here to screen for mammalian virus-encoded immunomodulators is that it inherently selects for the identification of viral factors that suppress what are likely to be conserved and ancient antiviral responses that predate the vertebrate-specific interferon response. Indeed, preliminary observations suggest that A51R proteins inhibit conserved antiviral ubiquitin-proteasome-related cellular responses (see Gammon *et al.*[13] and unpublished data). The discovery of VACV A51R rescue of VSV was the first example of a heterologous virus rescue by a vertebrate virus in an invertebrate host[13], and it is likely that these screening systems will uncover additional vertebrate virus-encoded immunomodulators. It is important to note that the key to using VSV rescue as a readout for conditions that

inhibit host immunity is that VSV replication must be heavily restricted (or abortive) in the cell type that VSV replication is being examined in. Therefore, most mammalian cell types would likely be unsuitable for these types of assays, given that VSV replicates well in mammalian cells. This is why *L. dispar* cells were used here as a naturally restrictive host cell type for VSV.

A prior study in *Drosophila* cells showed that viral suppressors of antiviral RNAi responses could be identified by cotransfection of expression plasmids encoding candidate RNAi suppressors and a self-replicating Flock House virus genomic RNA that encodes GFP in place of B2, its natural RNAi suppressor[30]. The replication of the Flock House genomic RNA and production of GFP was dependent upon the suppression of RNAi by the cotransfected candidate factor and, thus, the rescue of GFP expression indicated RNAi suppression[30]. Our unpublished work indicates that the overexpression of virus-encoded RNAi inhibitors also rescues VSV-LUC gene expression in *L. dispar* cells, suggesting that this system can also be used to identify suppressors of RNAi responses in the context of infection by a *bona fide* viral pathogen as opposed to a replicon. Indeed, the finding that RNAi-, IMD-, and ubiquitin-proteasome-related pathways contribute to the restriction of VSV replication in *L. dispar* cells[13] suggests that viral antagonists of several antiviral pathways may be identified by the VSV rescue assays presented here.

A potential limitation of these screening methods with regard to the identification of host antiviral proteins is that the *L. dispar* genome sequence is not yet available. However, there are publicly available *L. dispar* transcriptomes that can be used to identify candidate host targets for RNAi knockdown[26,27,28]. Additionally, we have shown previously that VSV is restricted in cell lines derived from other lepidopterans[13] that now have a publicly available genome (*e.g.*, *Manduca sexta*)[31]. Therefore, cell lines derived from other lepidopterans with sequenced genomes may substitute the *L. dispar* cells described in the protocol here.

Given the growing economic and public health threat of arboviruses[32], the screening assays presented here may provide novel strategies to identify new features of arbovirus-host interactions that may have value in the design of new antiviral therapeutics. Furthermore, because of our relatively limited understanding of lepidopteran mechanisms for restricting RNA virus replication, the tools presented here provide new opportunities to probe the host defense mechanisms encoded by this economically important order of insects.

Disclosures

The authors have nothing to disclose.

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