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14. ABSTRACT
Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by "Old World" viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "New World" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize high throughput genetic screens to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection.

15. SUBJECT TERMS
Hantavirus, Andes virus, Sin Nombre virus, Puumala virus, Dobrava virus, sterol regulatory complex, haploid genetic screen, cholesterol inhibitors,

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1. **INTRODUCTION**: Hantaviruses are negative-sense RNA enveloped viruses that are transmitted to humans in aerosols of rodent excreta. Hantaviral infections are associated with two significant human diseases: hemorrhagic fever with renal syndrome (HFRS) which is caused by viruses found in Europe and Asia (Hantaan, Seoul, Puumala and Dobrava viruses) or hantavirus pulmonary syndrome (HPS) caused by the "new world" viruses of the Americas (Sin Nombre and Andes viruses). A goal of this project is to utilize a powerful haploid cell genetic screening technology (3,4) to define common cellular pathways, and broadly effective inhibitors targeting these pathways, that impact numerous hantaviruses. In the longer run, we hypothesize that the host factors identified by the proposed research will lead to new druggable targets for combating hantaviral infection. We summarize below the accomplishments on this project and products produced.
2. **KEYWORDS**: Hantavirus, Andes virus (ANDV), Sin Nombre virus (SNV), Puumala virus (PUUV), Dobrava virus (DOBV), Haploid screen, sterol regulatory complex (SRC), haploid genetic screen, cholesterol inhibitors, Major Task (MT).

3. ACCOMPLISHMENTS:

- What were the major goals of the project?
- The major goals of this project are as follows:
- 1) Analysis of the requirement for cholesterol synthesis by New and Old World hantavirus.
- 2) Utilize a genetic screening protocol to identify and then characterize host factors involved in Old World Hantavirus entry.
- 3) Identify and characterize host factors involved in New World Hantavirus entry. Each of these major goals was further divided into tasks and sub-tasks.
- What was accomplished under these goals?

Identification of additional factors needed for New World Hantavirus infection. Previously we had demonstrated that Andes virus infection was exquisitely sensitive to genetic and pharmacologic perturbations that affected cholesterol synthesis. Herein we sought to identify host factors in addition to sterol regulatory complex (SRC) needed for efficient Andes virus infection using genetically modified HAP1 cells. First, human haploid (HAP1) cells stably expressing the low-density lipoprotein receptor (LDLR) were produced and characterized by flow cytometry using an antibody against LDLR. Specifically, the LDLR cDNA was inserted into the FCIV (FM5) lentiviral vector (generous gift of Dr. Jeffrey Milbrandt lab, Washington University). This vector uses the ubiquitin promoter to express the gene of interest and also expresses the Venus fluorescent protein via an internal ribosome entry site (Figure 1).

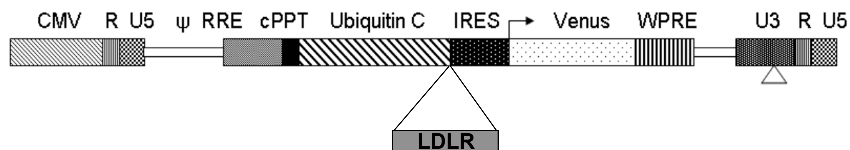


Figure 1. Vector for production of HAP1 cells constitutively expressing LDLR. *The LDLR cDNA was inserted into the FCIV (FM5) lentiviral vector (generous gift of Dr. Jeffrey Milbrandt lab, Washington University). This vector uses the ubiquitin promoter to express the gene of interest and also expresses the Venus fluorescent protein via an internal ribosome entry site. The sequence and orientation of the insert was verified by complete sequencing. Lentivirus (FCIV-LDLR) was produced by transient co-transfection of 293T cells with psPAX2 and pCMV-VSV-G then used to transduce HAP1 cells.*

Lentivirus (FCIV-LDLR) was produced by transient co-transfection of 293T cells with psPAX2 and pCMV-VSV-G then used to transduce HAP1 cells. Haploid, Venus+ cells were isolated by FACS sorting (Figure 2A). We had previously confirmed by flow cytometry of cell surface staining with an LDLR antibody that as expected LDLR is co-expressed in Venus+ cells. The smaller size of haploid cells by side scatter was employed as a gate along with Venus expression for cell sorting. The sorted cells were expanded and characterized by flow cytometry. In this analysis we found that the majority of the cells express Venus and display a Hoechst staining profile consistent with 59% 1n (haploid) and 40% 2n (diploid or S phase haploid) cells (Figure 2B). Notably there is no 4n peak as would be expected for dividing diploid cells. Roughly 75 million **HAP1-LDLR(+)** cells were then used to construct an insertionally mutagenized library using the LentiRET gene trap vector as described in the original proposal. Because these cells express LDLR they can efficiently scavenge cholesterol from the media and **are not reliant upon cholesterol synthesis or the sterol regulatory complex**. The mutagenized population was minimally expanded (2 doublings) and approximately 75 cells were selected by lethal infection with rVSV-ANDV as previously described (1). The remainder of the library was frozen for future use.

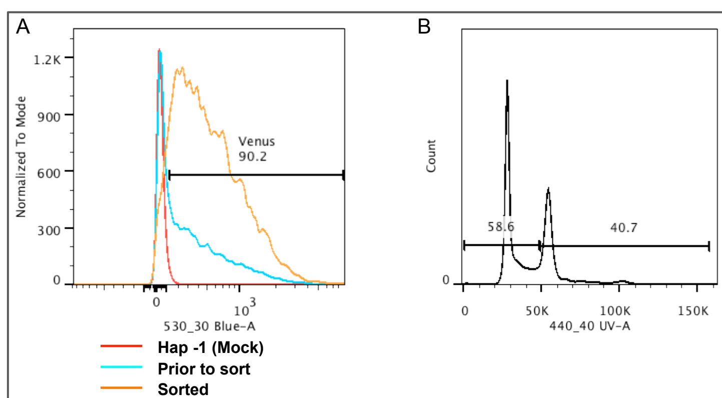


Figure 2. Generation of HAP1-LDLR(+) cells. *HAP1 cells transduced with FCIV-LDLR were expanded and then 75 million cells were sorted for Venus expression and small size by side scatter. After expansion, the cells were characterized by flow cytometry for A) Venus expression and B) DNA content upon Hoechst staining.*

HAP1-LDLR(+) cells surviving d10 post infection with rVSV-ANDV were collected and DNA was prepared for identification of the insertion sites (mutagenized genes). Using a PCR procedure that amplifies the LTR of the lentiviral mutagen and the flanking DNA, the insertion sites within the resistant cells were mapped onto the human genome and compared the insertion frequencies with those of our non-selected HAP1-LDLR(+) library cells. To identify gene integrations enriched in the rVSV-ANDV surviving cells we compared integrations sites in this selected population to >300,000 unique integrations in the unselected library. The mapped integration sites corresponding to 30,011 unique reads and 6617 unique genes including 3864 genes with more than 1 unique integration. Table 1 lists the top hits that were identified in this screen after significance values were calculated and corrected for false discovery rate. Notably, no hits were found within the genes encoding components of the sterol regulatory complex which were found when the parent HAP1 cells were screened previously. This observation supports the strategy for this screen showing that we have by-passed the need for the SRC by continuously supplying cholesterol via the constitutively expressed LDLR.

Analysis of the integration sites in HAP1-LDLR(+) cells surviving VSV-ANDV infection reveals ATP6V0A1, a gene encoding a subunit of a vacuolar ATPase that is required to acidify

endosomes, as a top hit (Table 1). Identification of ATP6V0A1 validates the screen as an acidic endosome compartment is known to be essential for hantaviral infection and because of this we did not further pursue this hit. To interrogate the other hits from this screen, we used siRNA knockdown of the candidate genes. We have initially concentrated on Suppressor with Morphological effect on Genitalia 1 (SMG1) because the screen identified several SMG1 related genes (SMG1, SMG1P2 and SMG1P5). SMG1 is a phosphatidylinositol 3-kinase-related protein kinase (PIKK). Although no role has been previously described for SMG1 in viral infection, phosphatidylinositol kinases are known to be involved in endocytic uptake and trafficking prompting us to investigate SMG1 more fully.

Table 1. Hits from the HAP1-LDLR(+) rVSV-ANDV screen.

GENE	p-value	# unique integrations
ATP6V0A1	0.0003	66
RHOA	0.0003	54
SMG1P2	0.0003	33
SMG1	0.0005	19
NOTCH2	0.0024	12
ANKRD36C	0.0025	25
LINC01128	0.0044	23
SMG1P5	0.0909	8

To investigate the potential role of SMG1 in hantaviral infection, two siRNA's specific for SMG1 were transfected into human U2OS cells, then the cells were challenged with VSV-ANDV encoding GFP. Infection was measured by flow cytometry and normalized to the non-targeting siRNA. An siRNA targeting the vacuolar ATPase, which was also identified in this screen, was employed as a positive control as it is well established that hantaviral infection is pH-dependent. A non-targeting siRNA was the negative control. Both siRNAs targeting SMG1 significantly impaired rVSV-ANDV infection yielding a 60-70% reduction while the positive control vATPase reduced infection by 80% (Figure 3). We confirmed knockdown of SMG1 at the RNA level by RT-qPCR and at the protein level by Western blot.

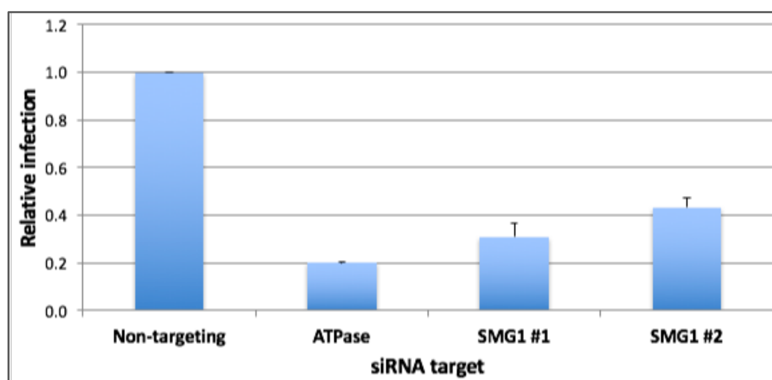


Figure 3. siRNAs targeting SMG1 inhibit Andes virus infection. *Human U2OS cells were transfected with siRNAs (Dharmacon) specific for SMG1 or the vacuolar ATPase. 48 hours after transfection the cells were infected with VSV carrying the Andes virus glycoproteins. 8 hours after infection the cells were fixed and analyzed for flow cytometry for the GFP marker encoded by VSV. Infection is normalized to control siRNA. Average of 2 independent experiments.*

SMG1 is a nonsense mediated mRNA decay associated PI3K related kinase. It is a serine/threonine protein kinase found to be involved in both mRNA surveillance and genotoxic stress response pathways. SMG1 is best characterized for its central role in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons by phosphorylating UPF1/RENT1. Caffeine and

wortmannin are relatively non-specific inhibitors of SMG1 and were used to address the role of SMG1 in hantaviral infection. Neither of these drugs showed a significant effect on rVSV-ANDV infection in the concentration ranges that are reported to inhibit SMG1 (data not shown).

To determine if the nonsense-mediated RNA decay function of SMG1 is needed for the effect on hantavirus infection, pharmacologic and genetic inhibitors of NMD were employed. These inhibitors do not target SMG1, but rather affect other components of the NMD complex. Treatment of A549 or Vero cells with a specific NMD inhibitor, Ethyl 2-(((6,7-dimethyl-3-oxo-1,2,3,4-tetrahydro-2-quinoxalinyl)acetyl)amino)-4,5-dimethyl-3-thiophenecarboxylate (NMD-14 from Calbiochem), did not significantly impair infection by rVSV-ANDV (data not shown).

To further examine if NMD activity of SMG1 was important for the observed effect on hantaviral infection, an siRNA to another component of the NMD complex, UPF1, had no significant effect on rVSV-ANDV infection. Although our data point to a significant and reproducible effect on SMG1 knockdown on infection by hantaviruses, this seems to be independent of SMG1's role in NMD.

We have since attempted to define the stage at which SMG1 might function during Andes virus entry using microscopy on U2OS cells treated with SMG1 or control siRNAs. Staining for the VSV core antigens at various time post infection of these cells did not demonstrate any gross observable alteration in trafficking of rVSV-ANDV in the knockdown cells compared to control. In the future we will continue to discern the role of SMG1 during ANDV infection and will expand these studies to determine if SMG1 is required for both New and Old World hantaviruses.

Identification of host factors needed for Old World hantaviral infection. For Specific Aim 2 of the proposal we performed a genome-wide loss-of-function genetic screen in haploid human cells utilizing a library of insertionally mutagenized human haploid cells (1). This library was created by using flow cytometry to sort a human haploid cell line (HAP1) to enrich for cells that retained the haploid phenotype, then modestly expanding the sorted cells to ~200 million before infection with a lentiviral vector that randomly inserts and disrupts gene function. After infection with the lentivirus the cell library was briefly cultured for 36 hours before being aliquoted and frozen at -80 for subsequent screening.

Viral challenge of mutagenized cells creates a genetic bottleneck, whereby cells harboring mutations in pro-viral genes undergo positive selection. To analyze the requirements for Old World hantavirus infection we cloned and then genetically rescued a recombinant vesicular stomatitis virus expressing the glycoproteins of the Old World hantavirus, Puumala (rVSV-PUUV). Approximately 75 million mutagenized HAP1 cells were infected with rVSV-PUUV, a cytopathic, replication competent recombinant virus, and cell death was allowed to proceed for 10 days. Several hundred colonies were observed 7-10 days after infection of mutagenized cells, while a parallel selection of non-mutagenized HAP1 cells yielded no survivors. We have previously observed a lack of survivors after challenging mutagenized HAP1 cells with a replication competent recombinant VSV bearing its endogenous glycoprotein (rVSV-VSV). Thus, the mutagenized HAP1 cells that survived rVSV-PUUV challenge likely carry mutations that confer specific resistance to infection mediated by the PUUV glycoprotein. Surviving cells were collected at day 10 and DNA prepared for identification of the insertion sites (mutagenized genes). Using a PCR procedure that amplifies the LTR of the lentiviral mutagen and the flanking DNA, the insertion sites within the resistant cells were

mapped onto the human genome and compared the insertion frequencies with those of our non-selected library cells. 388,743 integration sites were mapped corresponding to 1229 unique reads and 726 unique genes. To identify gene integrations enriched in the VSV-PUUV surviving cells we compared integrations sites in this selected population to >1.5 million unique integrations in the unselected library. Significance values were calculated and corrected for false discovery rate. Analysis showed that, of those genes significantly enriched in integrations in the selected cells, four of the top five genes belong to the cholesterol regulatory pathway (Figure 4). These genes, Sterol Regulatory Element Binding transcription Factor 2 (SREBF2), Sterol Regulatory Element Binding transcription Factor Cleavage Activating Protein (SCAP), Site 1 Protease (S1P), and Site 2 Protease (S2P), are involved in the sensing and homeostasis of intracellular cholesterol, and had previously been identified in HAP1 genetic screens of factors needed for infection by the Old World hantavirus Andes (1). The p-values for the enrichment of these genes ranged from 2.3x10-5 (S2P) to 2.7x10-41 (SREBF2), supporting the conclusion that their disruption in our selected cells was not due to chance.

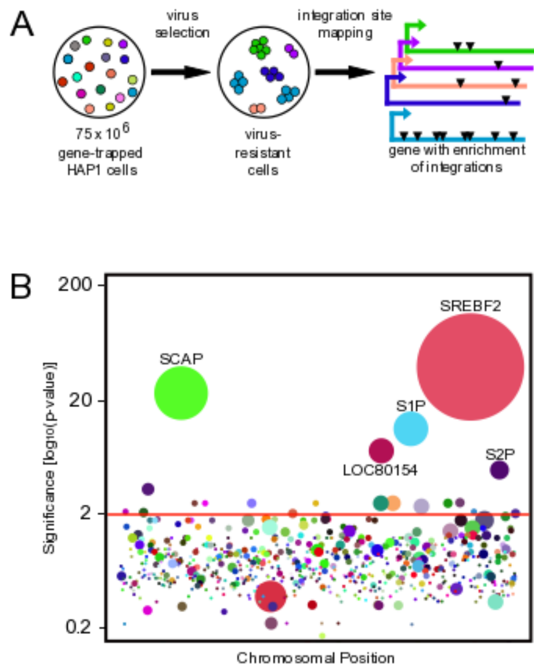


Figure 4. Haploid genetic screen identifies Puumala virus entry factors. *(A) A diagram of the HAP1 forward genetic screening process. HAP1 cells were insertionally mutated with LentiRET then selected by infection with recombinant rVSV-PUUV. Following expansion, DNA was extracted and ligation mediated PCR was performed followed by Illumina sequencing. Mapping of the insertion sites of selected cells and unselected cells and comparison of these sites in each sample allowed the identification of genes enriched in integrations in the selected sample relative to the unselected. (B) A dot plot showing the approximate chromosomal position and statistical significance of the genes identified in the HAP1 rVSV-PUUV screen. The gene names of the five most significant hits are noted. The size of the dot indicates the number of unique integrations mapped to that gene, while the orange horizontal line marks a p value of 0.01. P values have been corrected for false discovery rate.*

Having identified four members (SREBF2, SCAP, S1P, and S2P) of the cholesterol regulatory pathway in our genetic screen, we next sought to determine the importance of the genes for rVSV-PUUV infection. Instead of using CRISPR mutagenized cells as originally proposed, we instead utilized a set of well characterized Chinese Hamster Ovary (CHO) cell lines that are individually null for S1P, S2P, or SCAP. These cells are routinely utilized by the cholesterol synthesis field and thus results using these cell lines are readily comparable to that of others in this field. We challenged the CHO knockout cell lines as well as parental CHO with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV. As expected, rVSV-VSV infection was at most only moderately inhibited, showing approximately 70% less infection in SCAP null compared to parental cells (Figure 5). The effect of pathway disruption upon rVSV-PUUV infection was far more robust, with at least 95% less infection in all null cell types relative to parental. This result is similar to that observed for the New World hantavirus, Andes

(ANDV) (1). Our results using these mutant cells further support the cholesterol regulatory complex as important for rVSV-PUUV infection, showing a robust dependence of the virus upon all observed genes.

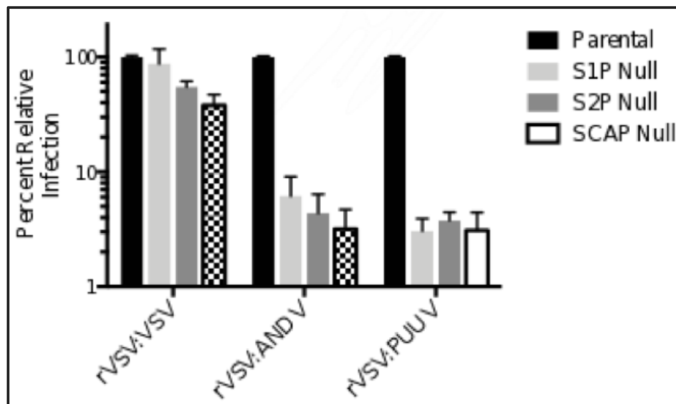


Figure 5. Genetic disruption of the sterol regulatory complex inhibits hantaviral entry. *CHO cells null for SCAP, S1P, or S2P were infected with recombinant VSV bearing the glycoproteins of PUUV, ANDV, or VSV. Infection was quantified by flow cytometry and normalized to infection of wild-type cells (CHO-K1). N=6 Bars=SEM.*

Figure 6 presents an analysis of the requirement for the sterol regulatory complex activity by prototypic New and Old World hantaviruses using the SRC inhibitor PF-429242. VSV carrying its own glycoprotein is used as a control. Infection mediated by the glycoproteins from both ANDV and PUUV was dramatically impaired in a dose-dependent manner compared to the control.

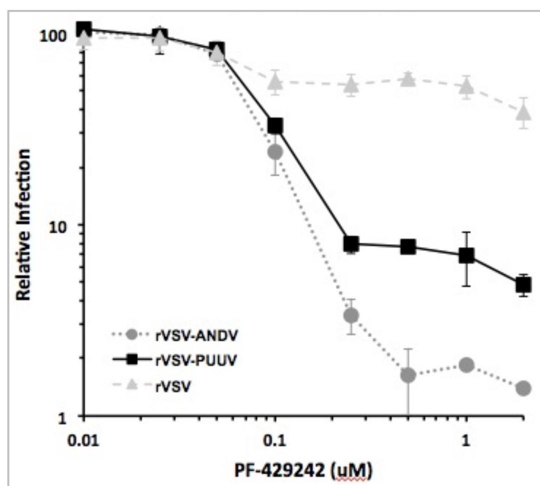
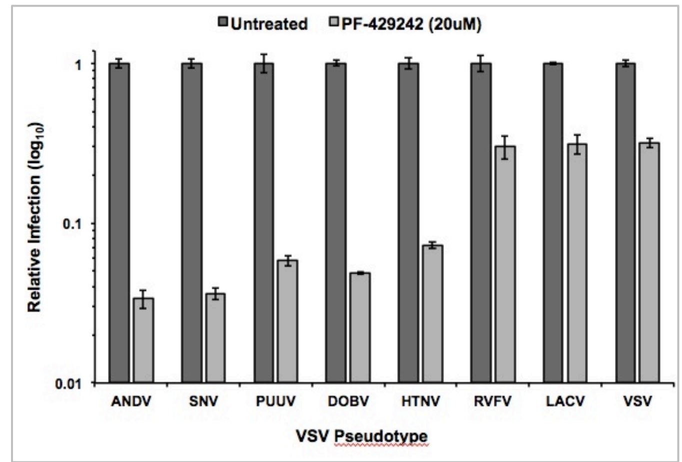


Figure 6. The Sterol Regulatory Complex inhibitor PF-429242 inhibits infection by prototypic New & Old World hantaviruses. *A range of concentrations of the compound PF-429242, which inhibits the Site 1 Protease needed to activate the SRC, was used to treat A549 cells for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with the viral glycoproteins from either the New World Andes virus (ANDV) or the Old World Puumala virus (PUUV).*

To extend this finding we analyzed the effect of SRC inhibitors on infection mediated by the glycoproteins for several additional New and Old World hantaviruses as well as more distantly related bunyaviruses, again using VSV glycoprotein as a control (MT1 subtask 3). Figure 7 clearly demonstrates that an additional New World hantaviruses, Sin Nombre (SNV) as well as the Old World hantaviruses (Dobrava; DOBV, Hantaan; HTNV) are impaired similarly to ANDV and PUUV by PF-429242. In contrast the bunyaviruses Rift Valley fever virus (RFTV) and LaCrosse virus (LACV) are minimally impacted by this inhibitor and behave similarly to the control VSV-G.

Figure 7. ***PF-429242 inhibits infection mediated by numerous New & Old World hantavirus glycoproteins.*** *Human A549 cells were treated with 20uM of the compound PF-429242 for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with glycoproteins from several hantaviruses. Glycoproteins from distantly related bunyaviruses (RVFV & LACV) Entry by VSV-G glycoprotein was analyzed as a control.*



Overall, the genes identified as critical for infection by viruses carrying the glycoproteins of an Old World hantavirus, Puumala, are nearly identical to those found using the New World hantavirus Andes virus that we previously described (1). ***These results extend our previous work by demonstrating that all hantaviruses have a profound requirement for cholesterol synthesis and suggest that perturbation of cholesterol levels may prove effective at preventing hantaviral infection in vivo***. This requirement for cholesterol synthesis by prototypic New and Old World hantaviruses is further demonstrated in Figure 8 using mevastatin, an FDA-approved pharmacologic inhibitor of cholesterol synthesis in cultured cells. VSV carrying its own glycoprotein is used as a control. Figure 8 clearly demonstrates that New World hantaviruses, Andes (ANDV) and Sin Nombre (SNV) as well as the Old World hantaviruses (Puumala; PUUV, Dobrava; DOBV, Hantaan; HTNV) are impaired similarly to ANDV and PUUV by mevastatin. In contrast, the more distantly related bunyaviruses Rift Valley fever virus (RFTV) and LaCrosse virus (LACV) are minimally impacted by this inhibitor while the controls VSV-G mediated and vaccinia virus infection show no effect.

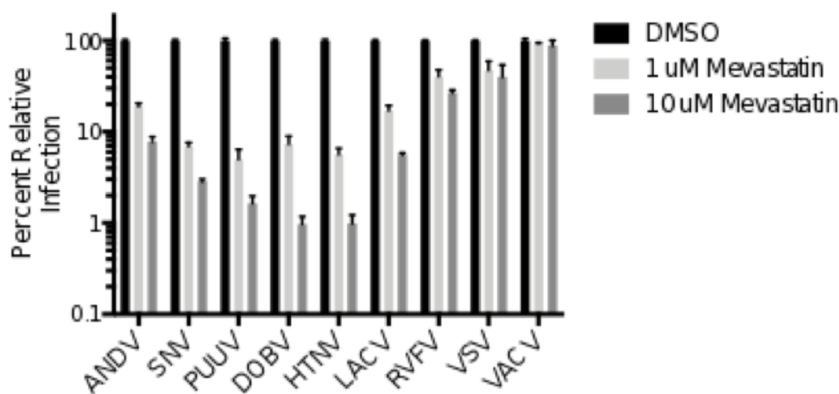


Figure 8. The FDA-approved cholesterol synthesis inhibitor Mevastatin inhibits infection by prototypic New & Old World hantaviruses. *Two concentration of mevastatin were used to treat A549 cells for 12 hours prior to infection with a GFP-expressing VSV vector pseudotyped with the viral glycoproteins from either New World or Old World hantaviruses. VSV and Vaccinia viruses were used as controls.*

Overall, our analysis has clearly demonstrated a role for cholesterol biosynthetic pathway in infection mediated New and Old World hantaviral glycoproteins. To address the mechanism of the cholesterol requirement for hantaviral infection (Major tasks 2 and 6) we have used a series of inhibitors of the cholesterol synthesis pathway to determine if there is a direct requirement for cholesterol or another sterol or by-product. To address this question two inhibitors of steps late in the cholesterol synthesis pathway (Figure 9) were examined for their effect upon VSV-PUUV infection.

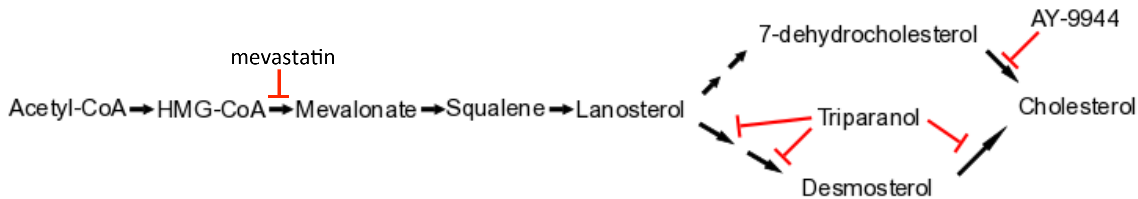


Figure 9. A diagram of the sterol biosynthetic pathway. *The enzymatic steps targeted by the drugs utilized in this study are noted.*

We find that perturbation of late steps in cholesterol synthesis dramatically reduces PUUV infection (Figure 10). Triperonol, which inhibits the synthesis pathway that utilizes desmosterol as a cholesterol precursor, demonstrates a clear dose dependent inhibition of infection by both rVSV-PUUV (Figure 10A) as well as authentic Puumala virus (Figure 10B). The compound AY-9944 blocks synthesis via 7-dehydrocholesterol and impairs infection in a dose dependent manner of both the VSV and authentic Puumala viruses (Figures 10C and 10D). In addition, we characterized entry of the New World hantavirus Andes in cell treated with these compounds. As seen with Puumala, infection by both the rVSV-ANDV and the authentic Andes hantavirus are impaired when the downstream cholesterol synthesis pathway is blocked.

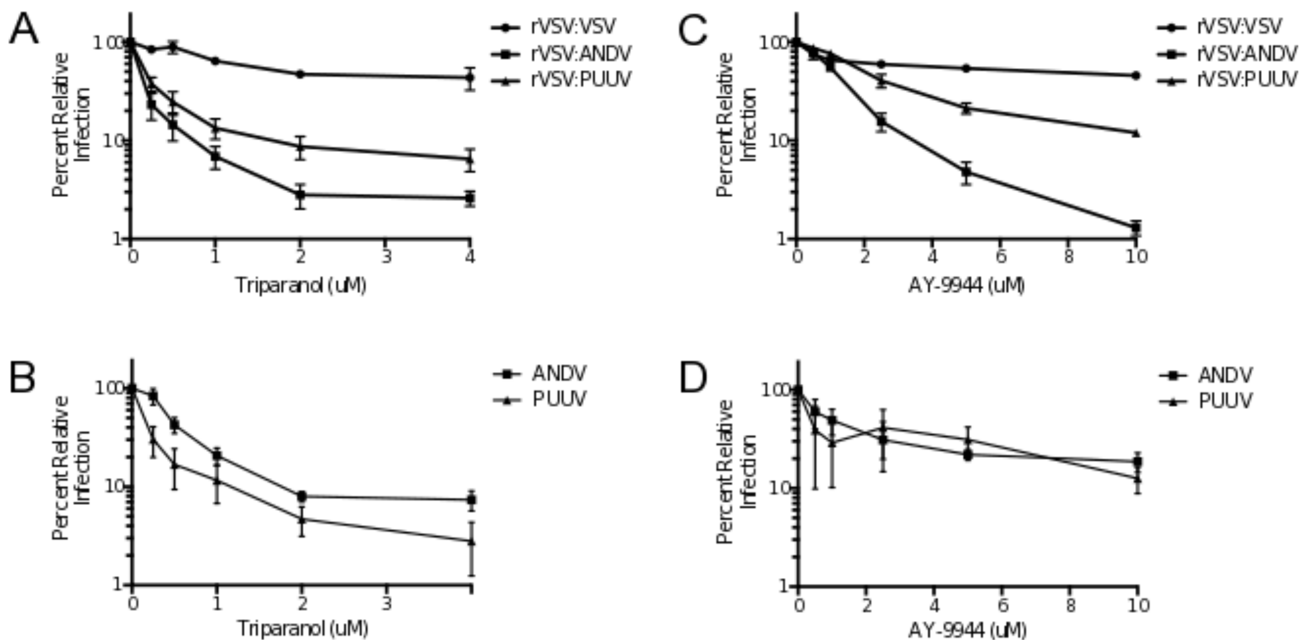


Figure 10. Disruption of late events in the cholesterol biosynthetic pathway inhibits hantavirus entry. *(A) and (B) Inhibition of reactions downstream of lanosterol decreases PUUV entry. A549 cells were cultured in delipidated media with vehicle or increasing concentrations of Triparanol, an inhibitor of various reactions in the desmosterol-containing branch of the cholesterol biosynthesis pathway. Cells were subsequently infected with (A) recombinant VSV expressing PUUV, ANDV, or VSV glycoproteins, or (B) wild type PUUV or ANDV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM. (C) and (D) Inhibiting the conversion of 7-dehydrocholesterol to cholesterol decreases PUUV entry. A549 cells were pretreated with vehicle or increasing concentrations of AY-9944, which inhibits the production of cholesterol from 7-dehydrocholesterol. Cells were then infected with (C) recombinant VSV expressing PUUV, ANDV, or VSV glycoproteins, or (D) WT PUUV or ANDV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM.*

From our analysis we surmise that a functional cholesterol synthesis pathway is a common requirement for entry by the hantavirus family. Overall, this work clearly demonstrates that this shared requirement for may present an opportunity to affect all hantaviruses by employing SRC or cholesterol synthesis inhibitors.

What is the mechanism by which cholesterol depletion impairs hantaviral entry? Based upon these results, we hypothesized that cholesterol itself might be required for efficient endosomal transport of incoming virus or fusion of the viral and host membranes. We have previously reported a defect in internalization of Andes virus when cholesterol synthesis is pharmacologically inhibited (Petersen et al 2104). To further support our findings and expand our understanding of hantaviral dependence upon cellular cholesterol for optimal infection, we utilized the sterol-binding agent methyl-β-cyclodextrin (MBCD). Depending on the relative ratios of free and MBCD-bound cholesterol, MBCD is able to extract from or add to the cholesterol content of membranes. Because of this, the direct effects of cholesterol depletion and reconstitution upon viral infection can be readily observed.

Human A549 cells were treated with MBCD or vehicle in delipidated media, followed by rinsing and a 45-minute incubation with delipidated media containing either vehicle alone or MBCD loaded with cholesterol. After treatment, cells were infected with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV in delipidated media. Cholesterol depletion had only a moderate impact on rVSV-VSV infection, decreasing it by approximately 50% (Fig. 11A). A more robust inhibition of infection was seen for both rVSV-PUUV and rVSV-ANDV, with infection of these viruses decreased by greater than 80% and 94%, respectively (Figure 11A). Importantly, infection of all three viruses was rescued by cholesterol reconstitution, supporting the specific role of cholesterol in the phenotype.

We next sought to illuminate the step of the viral lifecycle involved in the phenotype. As mentioned we have previously shown that pharmacologic or genetic cholesterol depletion impedes hantaviral endocytosis. However, considering the importance of cholesterol in membrane content, organization, and fluidity, we hypothesized that cholesterol depletion might be acting at several steps during hantaviral entry and thus chose to directly investigate the impact of cholesterol depletion upon hantaviral membrane fusion with the host membrane.

To do so, we again utilized MBCD treatment and observed the ability of recombinant viruses to fuse with the cellular membrane at low pH following cholesterol depletion or supplementation. It is well established that hantaviruses require a low pH environment to trigger virus-host membrane fusion and we mimicked this using a brief low pH treatment. Human A549 cells were incubated with delipidated media containing vehicle, MBCD, or MBCD loaded with cholesterol. Cells were subsequently rinsed and rVSV-PUUV, rVSV-ANDV, or rVSV-VSV was added in cold delipidated media and allowed to bind before cells were rinsed and 37 C media adjusted to pH 4.9 was added for 90 seconds. Following acid bypass, we incubated the cells in delipidated media containing NH_4Cl to limit infections to those initiated by low pH fusion at the cell mem10brane.

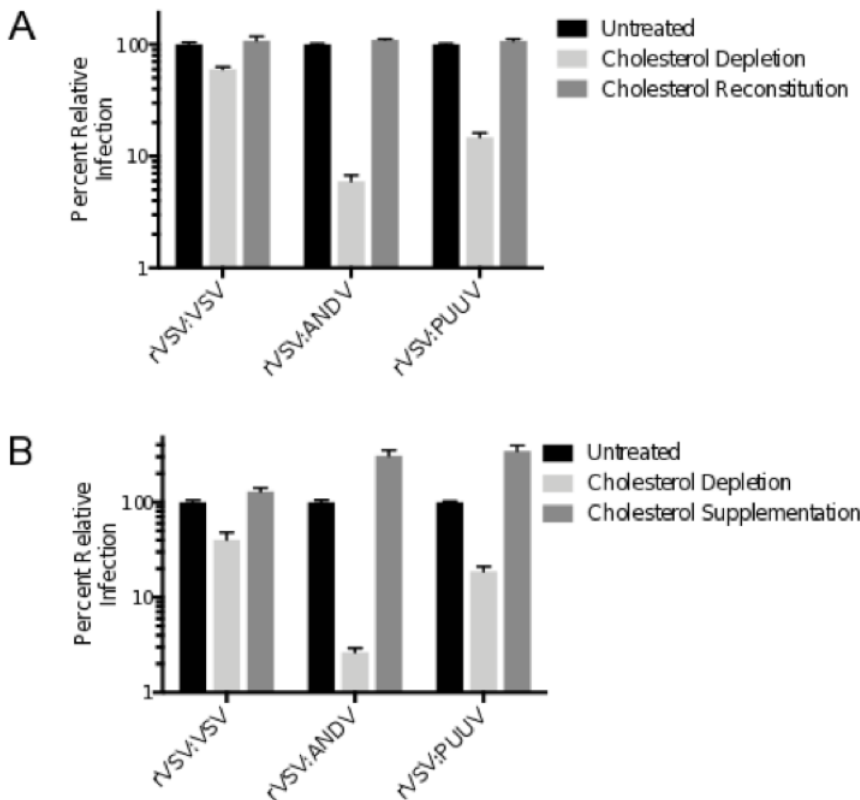


Figure 11. Hantaviral infection requires cholesterol for optimal membrane fusion. ***(A)*** *A549 cells were treated with vehicle or MBCD or deplete plasma membrane cholesterol. Depleted cells were further treated with vehicle or cholesterol-loaded MBCD to reconstitute cellular cholesterol before all cells were infected with rVSV-PUUV, rVSV-ANDV, or rVSV-VSV. Infection was quantified by flow cytometry and normalized to vehicle controls. N=6 Bars=SEM.* ***(B)*** *A549 cells were either depleted of membrane cholesterol with treatment with MBCD, had their cholesterol content supplemented by incubation with cholesterol-loaded MBCD, or treated with vehicle. Acid bypass of the VSV pseudotypes was then carried out at pH 4.9, followed by incubation in NH_4Cl-containing media and quantification of infection by flow cytometry. Infection was normalized to vehicle controls. N=6 Bars=SEM.*

The results of infection by acid-bypass following cholesterol depletion or supplementation closely parallels the previous MBCD experiment. rVSV-VSV showed a moderate decrease in fusion following cholesterol depletion, with slightly greater than 60% inhibition (Figure 11B). PUUV infection was decreased approximately 80% in fusion following cholesterol depletion, while rVSV-ANDV showed more than 97% decreased fusion. The greater cholesterol dependence of the hantaviruses compared to rVSV-VSV was also observed in their response to cholesterol supplementation, where we observed both rVSV-PUUV and rVSV-ANDV fusion increase by approximately three fold compared to untreated cells. Together, our results utilizing MBCD to alter cellular cholesterol levels support our earlier drug and genetic results, showing a significant dependence of the New and Old World hantaviruses upon cellular cholesterol for infection. The acid bypass data identifies a major site of this dependence, supporting a block to virus fusion with the host membrane to be a key point of the observed inhibition. Coupled with our prior observation showing impaired trafficking (1), this suggests that cholesterol may work at several points during hantaviral infection.

Development of s system to analyze the stage at which cholesterol depletion impairs hantaviral entry. To analyze the point at which the SRC is required for hantaviral infection we developed a system that utilized an epitope tagged protein that is co-incorporated along with the hantaviral glycoproteins into the VSV pseudotypes for microscopic analysis of viral entry. The APEX technology (2, 3) we have employed is based upon the generation of short-lived free radicals that covalently bind electron-rich amino acids in close proximity (~20nm) to the APEX enzyme and can therefore be used to label the area of the cell or cellular compartment in which the incoming virus localizes. Substrates for fluorescent microscopy (as proposed in the original grant) or dense labels for use in electron microscopic analysis are available. The ability to utilize EM or fluorescence

microscopy makes this a much more flexible system to determine the point at which the cholesterol pathway is needed in hantaviral infection.

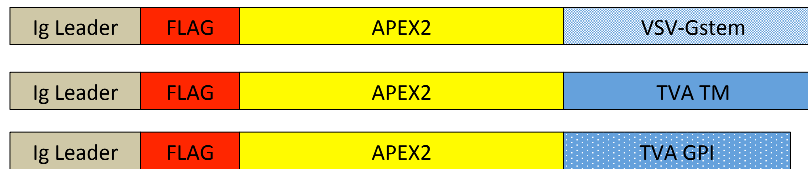


Figure 12. VaAPEX constructs to be employed in analyzing the stage at which cholesterol is needed during hantaviral entry. *These simple proteins consist of a leader sequence (brown), FLAG epitope tag (red), modified ascorbate peroxidase coding region (APEX, yellow), membrane anchor (blue) from either VSV-G, the host protein Tva, or a splice variant of Tva that encodes a GPI-linked lipid anchor*

Originally, we proposed using a simple protein consisting of a membrane anchor (the VSV-G stem region) with an appended HA epitope tag on the extracellular surface (HA-G stem). We modified this plan to include a newly described small peroxidase protein, APEX (2,3), in addition to G stem and an epitope tag. In addition, 2 other constructs with differing membrane anchoring units were developed with a goal of identifying the one that is most effectively incorporated into virions. We have called these proteins Virion-associated APEX (VaAPEX , Figure 12). The 3 constructs being evaluated are VaAPEX-G, (VSV transmembrane anchor), VaAPEX-TM (Tva membrane anchor) and VaAPEX-GPI (glycolipid membrane anchor). (MT2, subtasks 1 &2). All three of these proteins are effectively incorporated into VSV, HIV or MLV based viral particles along with hantaviral glycoproteins (MT2, subtask 2, data not shown). We find that transient expression gives much greater incorporation that stable cell lines and will therefore not generate stable Vero lines as was proposed. We tested the three variants of the APEX tagging constructs and evaluated their cell surface expression (required for incorporation into VSV) by both flow cytometry and microscopy and the tested functionality of the APEX peroxide on the cell surface. All three are well expressed at the cell surface and functional as determined by biotin-labeling of the cell surface (Figure 13). Single channel analysis of the fluorescently stained HeLa cells expressing the VaAPEX constructs demonstrates intense GFP signal indicating very efficient surface labeling. Indeed, in Figure 7 where both channels are displayed the red fluorescence for the epitope tag is not easily seen because of the intense GFP (biotin) signal.

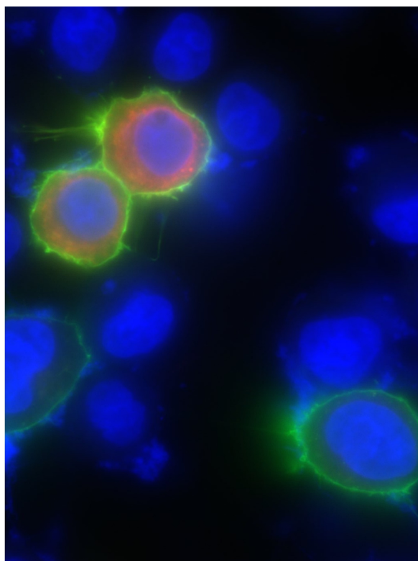


Figure 13. Function of the VaAPEX system in HeLa cells. *HeLa cells were transfected with 400 ng plasmid expressing VaAPEX-TM then labeled and fixed 26 hours post transfection. Biotin-phenol was used to label cell surface proteins in cell that express VaAPEX-TM. Cells were then permeabilized and stained for biotin (green) or the FLAG epitope tag (red) on VaAPEX.*

VaAPEX-G, VaAPEX-TM and VaAPEX-GPI were evaluated for their ability to incorporate into pseudotyped particles by western blot of released virions and also single particle analysis by microscopy (data not shown). All three are effectively incorporated into VSV, HIV or MLV based viral particles along with hantaviral glycoproteins. We find that transient expression gives much greater incorporation than stable cell lines and will therefore not generate stable Vero lines as

was proposed. Moreover, using biotin labeling of the virions themselves we have found that APEX is enzymatically active in the released viral particles (data not shown). Thus, under this funding we have developed and optimized a system to follow incoming viral particles which should be broadly useful for many different viruses. In the context of this proposal, we are poised to analyze the intracellular fate of hantaviral virions in cells where cholesterol homeostasis or SMG1 are impaired.

References

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- What opportunities for training and professional development has the project provided?
  - "Nothing to Report."
- How were the results disseminated to communities of interest?
  - Results of the initial experiments analyzing Puumala virus were presented by Ben Dyer in July at the 2015 American Society for Virology Meeting in Ontario, Canada.
  - The APEX technology for proximity labeling of viral interacting proteins was presented by Ben Dyer in July at the 2016 American Society for Virology Meeting at Virginia Tech University.
  - Dr. Bates has presented this work in several public seminars:
    - July 2014 , Keynote address @ Mount Sinai-NYU School of Medicine 25th Symposium on Virus-Host Interactions.
    - October 2014, Keynote Speaker, Rutgers Graduate Student Symposium, Newark, NJ

- May 2016, Invited seminar, University of Missouri, Columbia, MO
- April 2016, Invited seminar, Bryn Mawr College, Bryn Mawr, PA
-
- What do you plan to do during the next reporting period to accomplish the goals?
- We have requested a short no-cost extension to perform the requested experiments that will permit publication of the Old World hantavirus (rVSV-PUUV) genetic screen in mutagenized HAP1 cells. In this publication we will demonstrate the highly specific requirement for cholesterol , as opposed to any other sterols, in both New and Old World hantaviral infections. We also define the mechanism of action of cholesterol by showing it is need for membrane fusion either at the plasma membrane or in endosomal compartments. Currently, we are re-doing and analyzing a subset of the membrane fusion assays and re-writing the text of the manuscript to make it acceptable for publication.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

- What was the impact on the development of the principal discipline(s) of the project?
- These are the first forward genetic experiments designed to elucidate cellular factors needed for hantaviral entry into host cells. Thus, the knowledge gained here will not only inform the basic biology of hantaviral replication but should also describe potential inhibitors of hantaviral infection. To date we have found cholesterol synthesis inhibitors are broadly applicable against New and Old World hantaviruses. Recent results hint that protein kinase (specifically PIKK) inhibitors may also be useful for combating hantaviral infection. Additionally, we have developed replication-competent recombinant viruses that carry the hantaviral glycoproteins but are safer and can therefore be handled in lower biosafety levels. Although we developed them to facilitate genetic screening, these viruses should be of utility for developing vaccines as a similar VSV construct forms the basis for an effective Ebola vaccine that is currently in use in Africa. The Puumala and Hantaan recombinant VSV's developed in this project are currently being tested as vaccines.
- What was the impact on other disciplines?
- "Nothing to Report."
- What was the impact on technology transfer?
- "Nothing to Report."
- What was the impact on society beyond science and technology?
- "Nothing to Report."

5. **CHANGES/PROBLEMS**: A cryogenic storage system failure modestly delayed our progress early in the tenure of this research program, however we re-generated the lost libraries and screened them making up for this "lost time". In 2017 two of the personnel working on the project left the lab somewhat unexpectedly. Similarly This delayed our progress.

- Changes in approach and reasons for change
- As noted previously, a minor change is that we utilized a new technology (APEX) that allowed us to more effectively perform the studies on hantaviral entry and

the mechanism of various inhibitors. Additionally, based on the results presented, we identified SMG1 as a new potential host factor needed for VSV-ANDV infections. Experiments aimed at unraveling the mechanism by which SMG1 acts will be critical if we want to develop this as a potential target for intervention in hantaviral infections. We are still investigating SMG1.

- Actual or anticipated problems or delays and actions or plans to resolve them
    - A June 2015 a cryogenic storage unit failure caused the loss of a large HAP1 lentiviral mutagenized library and also a LDLR expressing HAP1 library. This delayed experiments described in Major Task 3 and Major Task 7. Using backup HAP1 cells from a separate cryogenic storage facility we were able to re-construct these reagents however we were delayed by several months.
    - Dr. Natalia Shalginskikh, the postdoctoral fellow working on this project left the lab at the beginning of 2016 and her departure affected the timeline for completion of the aims.
    - Mr. Ben Dyer abruptly left the graduate program in early 2017. His departure further disrupted this research program specifically affecting the timeline for completion of the Old World hantavirus experiments.
- Changes that had a significant impact on expenditures
    - Nothing to Report
- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
    - Nothing to Report
- Significant changes in use or care of human subjects
    - Nothing to Report
- Significant changes in use or care of vertebrate animals.
    - Nothing to Report
- Significant changes in use of biohazards and/or select agents
    - Nothing to Report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- Publications, conference papers, and presentations
    - Journal publications. NA
    - Books or other non-periodical, one-time publications. NA
    - Other publications, conference papers, and presentations.
        - Presentation entitled Discovery of "Host Factors Required for Hantaviral Infection" by Ben Dyer (student) at the 34th Annual Meeting of the American Society for Virology. July 2015.
        - Presentation entitled Discovery of "Developing a novel proximity-based assay for discovering host-virus interactions" by Ben Dyer (student) at the 35th Annual Meeting of the American Society for Virology. July 2016.
- Website(s) or other Internet site(s) NA
- Technologies or techniques We have generated the technology to produce lentiviral

mutagenized HAP1 libraries that can be used to identify important genes for numerous cellular processes. This technology will be shared freely with the scientific community. Similarly, the APEX constrcuts that are incorporated into budding virions will be mae available.

- Inventions, patent applications, and/or licenses NA
- Other Products We have generated recombinant VSV viruses that carry various hantaviral glycoproteins. These viruses will be of general utility to groups analyzing hantaviral entry and/or inhibitors of hantaviral infection including antibodies and vaccines. In this regard, we are pursuing Puumala and Hantaan recombinant VSVs as vaccine platforms. Additionally, we have created cell lines in which genes encoding SRC factors have been inactivated by CRISPR technology which will be of utility to others who work in this area. These reagents will be shared freely with the scientific community

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- What individuals have worked on the project? (Entire period of support)

▪ Name:	Paul Bates
▪ Project Role:	PI
▪ Researcher Identifier (e.g. ORCID ID):	PFBATES (eRA Commons ID)
▪ Nearest person month worked:	12
▪ Contribution to Project:	Directed Project, coordinated team, analyzed data
Funding Support:	NIH R01AI081913, NIH P51OD011104-53S1, NIH 1R21 AI 129531, this award
▪ Name:	MJ Drake
▪ Project Role:	Graduate Student
▪ Researcher Identifier:	MJDRAKE (eRA Commons ID)
▪ Nearest person month worked:	12
▪ Contribution to Project:	Performed experiments, analyzed data
Funding Support:	NIH R01AI081913, NIH T32AI007324, this award
Name:	Natalia Shalginskikh
Project Role:	Postdoctoral Fellow
Researcher Identifier:	
Nearest person month worked:	25
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award
Name:	Ben Dyer

Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	28
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, NSF Fellowship
Name:	Steven Bart
Project Role:	Graduate Student
Researcher Identifier:	SMBART (eRA Commons ID)
Nearest person month worked:	19
Contribution to Project:	Performed experiments, analyzed data
Funding Support:	This award, Institutional Funds, NIH T32AI007324
Name:	Justin Hoffman
Project Role:	Technician
Researcher Identifier:	
Nearest person month worked:	11
Contribution to Project:	Performed experiments
Funding Support:	this award, NIH 1R21 AI 129531

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
- Yes, PR182551 from CDRMP was awarded.
- What other organizations were involved as partners?
- Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- COLLABORATIVE AWARDS:
- Nothing to Report.