Herpes Simplex Virus 1 Amplicon Vectors

by

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ABSTRACT

Globally, about two-thirds of the population is latently infected with herpes simplex virus type 1 (HSV-1). HSV-1 is a large double stranded DNA virus with a genome size of ~150kbp. Small defective genomes, which minimally contain an HSV-1 origin of replication and packaging signal, arise naturally via recombination during viral DNA replication. These small defective genomes can be mimicked by constructing a bacterial plasmid containing the HSV-1 origin of replication and packaging signal, transfecting these recombinant plasmids into mammalian cells, and infecting with a replicating helper virus. The absence of most viral genes in the amplicon vector allows large pieces of foreign DNA (up to 150kbp) to be incorporated. The HSV-1 amplicon is replicated and packaged by the helper virus to form HSV-1 particles containing the amplicon DNA. We constructed a novel HSV-1 amplicon vector system containing lambda phage-derived attR sites to facilitate insertion of transgenes by Invitrogen Gateway recombination. To demonstrate that the amplicon vectors work as expected, we packaged the vector constructs expressing Emerald GFP using the replication-competent helper viruses OK-14 or HSV-mScartlet-I-UL25 in Vero cells and demonstrate that the vector stock can subsequently transduce and express Emerald GFP. In further work, we will insert transgenes into the amplicon vector using Invitrogen Gateway recombination to study their functionality.

DEDICATION

Dedicated to my first love, my daughter Kylie Velarde, for she is my inspiration to be the best mother and human I can be. I want to thank the love of my life and soulmate, my fiancé Christian Becerra for always believing and supporting me throughout all these years. I have been blessed with many great friends that have always believed in me and provided me with kind words of support. Therefore, I want to dedicate my work to my best friends Diana, Allyson, David, and Crystal, for our wonderful friendships. My lovely little sister Miriam Velarde whom I am so proud of and love with all my heart. At last, I want to thank my parents Jesus and Maria for their love and wisdom, and I hope to make them proud in my greatest achievement.

No importa de donde venimos, sino cuantas veces nos levantaremos para seguir adelante, y hasta que tan lejos nos permitimos llegar.

Los quiero mucho, y gracias a todos ustedes por siempre apoyarme!

(It does not matter where we come from, but how many times we get up to move forward, and how far we allow ourselves to go).

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INTRODUCTION

Viruses have co-evolved with humans, and we have been impacted by them long before the discovery of the first viruses in the 1890s. As such, scientists have tried to understand how viruses work for more than a century. While viruses are obligate intracellular parasites that can cause disease and death, they have also proven to be useful for the study of molecular and cellular biology and for various medical treatments. In particular, viral vectors are now a well-established technology, vector-based gene therapy, vaccines, and oncolytics currently in clinical use. This has led to the improvement of vector engineering methods for gene therapy to increase in recent years, with up to 70% of gene therapy research targeting cancer (Young et al., 2006). These oncolytic viruses are modified viral vectors that can selectively infect, replicate in, and kill tumor cells (Conry et al., 2018).

Viruses like Herpes Simplex Virus 1 have been successfully developed as an oncolytic virus. For example, the first genetically engineered oncolytic virus was an HSV-1 mutant used to treat glioblastoma multiforme in the early 90's (Hulou et al., 2016). In 2015, Talimogene laherparepvec (T-VEC) became the first and only HSV-1 oncolytic virus immunotherapy approved by the U.S., Europe, and Australia for the treatment of advanced melanoma (Conry et al., 2018; Rehman et al., 2016). This new class of drugs targeting tumor cells has shown to be a great achievement of the use of viral vectors for gene therapy. Optimistically, T-VEC will become the foundation to constructing a safe and highly efficient clinical treatment for other types of cancers, thus, providing viral vector research to continue and ultimately lead to further discoveries.

More broadly, viral vectors are easily manipulated to efficiently deliver and express therapeutic genes in target cells. They can be genetically engineered to

replicate in specific cell lines (Young et al., 2006). When considering diseases caused by genetic malfunction, viral vectors can be constructed to express the desired gene of interest to restore function. Some examples of genetic diseases targeted by virus vector-based gene therapy include neurodegenerative diseases, cardiovascular disease, cancer, metabolic syndromes, muscular disease, hematological disease, and a variety of other chronic genetic diseases (Lundstrom, 2018; Wu & Ataai, 2000). Furthermore, HSV-1 amplicon vectors have been designed as gene-transfer vehicles for expression in the central nervous system for neurodegenerative disorders and potentially neuropsychiatric illnesses like drug addiction and depression (Neve et al., 2005; Roizman, 1996). These applications of viral vectors have led to great interest in other potential uses for modified viruses.

More recent studies have used HSV-1 vectors to efficiently express light chains of botulinum neurotoxins upon infection of both adult and embryonic rat dorsal root ganglia neurons, ultimately cleaving SNARE proteins and causing a decrease in neurosecretion of calcitonin gene-related protein in embryonic rat sensory neurons (Joussain et al., 2019). In 2020, a study conducted by *Fernández-Frías et al*., supplemented culture medium with amino acids, polyamines, reduced glutathione and antioxidants causing an increase in HSV-1 viral production, thus HSV-1 amplicons were isolated from culture supernatants and not cell lysates (Fernández-Frías et al., 2020). These modifications resulted in purer viral preparations by avoiding cellular debris caused by breakage of host cells (Fernández-Frías et al., 2020). These examples demonstrate viral vector research is currently in progress and is important to continue improving both the structures and uses of HSV-1 amplicon vectors.

Viral Amplicon Vectors

Viral vectors are a technology that harnesses viruses as molecular tools to deliver genetic information (Bouard et al., 2009). These viral vectors can then either introduce or replace specific cellular functions, and in the case of vaccinations, they are used to inhibit disease in both humans and animals (Bouard et al., 2009). The idea of gene therapy using viruses such as the Shope papilloma virus, was first proposed by Stanfield Rogers in the mid-1960's to regulate cellular arginase enzyme activity. Moreover, a human clinical trial was established in which the virus was introduced into patients (Friedmann, 2001). While this clinical trial did not progress, the potential use of viruses as therapeutic vectors became very appealing. Soon after, scientists begin to look at various other viruses like retroviruses, SV40, bovine papilloma virus, vaccinia virus, and herpes simplex virus as potential tools for gene therapy (Finer & Glorioso, 2017). A successful first-generation retroviral vector was constructed in 1981 by *Shimotohno et. al*., capable of introducing foreign DNA sequences into cells (Bouard et al., 2009). A helper virus was used to replicate the defective vector carrying foreign DNA (Shimotohno & Temin, 1981). Another success took place 15 years later, when the first generation lentiviral vector, derived from HIV-1, was created to transduce neurons (Naldini et al., 2016). Consequently, research on viral vectors continued to expand over the years.

Regrettably, the progress of viral vector research for gene therapy has been negatively impacted by safety and ethical concerns. For instance, a clinical trial using a very high dosage of human adenovirus type 5 to treat ornithine transcarbamylase (OTC) deficiency caused a major systemic immune response, intravascular coagulation, and multiple organ system failure, resulting in the death of the patient (Raper et al., 2003). In another unfortunate event, a retroviral vector designed to treat X-linked severe combined immunodeficiency (X-SCID) caused leukemia in 3 out of 17 underage patients. Aside from this severe side-effect, the vector was otherwise

successful in treating X-SCID (Young et al., 2006). Situations like these have demonstrated that the use of viral vectors as a therapeutic approach still has challenges to overcome. However, there have been successful viral gene therapy treatments that are safe and effective, leading the way to new and improved gene therapies. As one example, in 2003, a recombinant Ad-p53 gene therapy treatment for head and neck squamous cell carcinoma was licensed in China (Pearson et al., 2004). Both successes and failures have contributed to the improvement of scientific discoveries and applications.

Currently, a wide variety of viruses, including adenoviruses, adeno-associated viruses (AAV), alphaviruses, flaviviruses, herpesviruses, measles virus, rhabdoviruses, retroviruses, Newcastle disease virus, poxviruses, and picornaviruses are used as viral vectors for potential gene therapy applications (Lundstrom, 2018). There are a variety of design considerations that influence which viruses are suitable for particular purposes: their potential cytotoxicity, their efficiency in transgene delivery to target cells, and the amount and duration of transgene expression poses additional challenges (Goins et al., 2014). Viral vectors generally must be non-pathogenic, may need to be replication-defective (depending on their application), and must have minimal to no cell toxicity to produce a stable and safe gene therapeutic (Bouard et al., 2009).

Viral vectors have distinct characteristics of viral-encoded functions known as *cis* and *trans* elements. Origin of replication and packaging signals are examples of *cis*-acting elements: these sequences/structures must be present on a vector genome for it to be replicated and packaged into virus particles. Meanwhile, *trans*-acting factors, such as viral enzymes and structural proteins, can be expressed separately, from host genome, plasmid transfection, or a helper virus (Wu & Ataai, 2000). Retroviral vectors were the first type of viral vectors used, most commonly for tumor therapy, as they have the advantage to infect actively proliferating cells

(Grandi, 2004; Wu & Ataai, 2000). Retrovirus particles contain positive-stranded RNA genomes, from which nearly all of the protein-coding sequences can be removed, and replaced with around 8 kb of foreign genetic sequences (Merten & Gaillet, 2016). Retroviral vectors created from lentiviruses (namely, HIV-1) are capable of transducing non-dividing cells in tissues including the liver, heart, muscle, and brain. Although, there have been biosafety concerns over the use of HIV-derived vectors, due to the very rare possibility of recombination generating a replication-competent virus (Wu & Ataai, 2000). Retroviral vectors are also beneficial as they can integrate into the host DNA genome, thus ensuring the expression of the genes of interest throughout the life of the host cell (Robbins & Ghivizzani, 1998). This ability to integrate unpredictably into the host genome is also a limitation because integration into the host genome is mutagenic, limiting their use in human therapeutics. Unfortunately, retrovirus vectors also produce a relatively low yield, and the relative fragility of retrovirus particles limits the ability to concentrate and purify vector stocks (Merten & Gaillet, 2016; Wu & Ataai, 2000). Another very common viral vector is based on the parvovirus, adeno-associated virus (AAV), which contains a small, relatively simple, single-stranded DNA genome of 4.7 kb in size (Hareendran et al., 2013). AAV vectors have very low pathogenicity and toxicity, broad cell tropism, and long-term transgene expression (Bouard et al., 2009). The major limitation of AAV vectors is their very limited packaging capacity of foreign DNA (Bouard et al., 2009; Finer & Glorioso, 2017). AAVs are naturally replication deficient and require co-infection with a helper virus, like adenovirus; therefore, both AAV as well as adenovirus genes must be provided in *trans* to replicate and package AAV vectors (Hareendran et al., 2013; Penrod et al., 2015).

Herpes Simplex Virus 1 Amplicon Vectors

Herpes simplex virus 1 (HSV-1) possess a double-stranded DNA genome of 152 kb. This relatively large genome is the major benefit of HSV-1 viral vectors, allowing insertion and expression of multiple transgenes up to 150 kb in size (Goins et al., 2014). If transgenes less than 150 kb in size are used, virus particles will package multiple copies of the vector genome, up to the ~150 kb limit. HSV-1 amplicon vectors consist of a plasmid containing the HSV-1 origin of replication and packaging signals (Neve et al., 2005; Robbins & Ghivizzani, 1998). HSV-1 amplicon vectors typically require a replication-defective HSV-1 mutant as a helper virus to replicate and package the vector, and this helper virus, as well as biologically-active tegument proteins packaged into virus particles, causes some amount of cytotoxicity (Neve & Lim, 2013). The helper virus provides the viral genes needed for replication and packaging of the vector (and the helper virus) genome (Roizman, 1996). Helper viruses typically contain deletions in immediate-early virus genes, such as ICP4 or ICP27, causing the helper virus to replicate only in complementing cell lines (Robbins & Ghivizzani, 1998). *Saeki et al*., were the first to achieve the production of helper virus-free amplicon vector system, nevertheless, this amplicon vector stock provided limited yield (Saeki et al., 2001). A study conducted by *Neve et. al*., showed that in HSV-1 amplicon vectors, a 1:1 vector to helper virus ratio allows for a higher yield of vector titers (Neve & Lim, 1999). Both approaches must be further studied to confirm biosafety and long-lasting gene expression.

Herpes amplicon vectors consist of an Escherichia coli (*E.* Coli) plasmid containing one copy of the HSV-1 *ori*-S origin of replication, the HSV-1 DNA cleavage/packaging signal (*pac*), and a transgene of interest (Epstein, 2005). The amplicon contains only HSV-1 *cis*-acting sequences, and no HSV-1 protein-coding genes, reducing cytotoxicity and immunogenicity (Neve & Lim, 1999). Advantageous qualities of HSV-1 amplicon vectors include: being able to infect many cell types,

relative ease of construction, and a large transgene capacity (Grandi, 2004). This unique combination of features makes HSV-1 amplicon vectors most appealing for transgene delivery.

The early progress of HSV-1 amplicon vectors consisted of using mutant HSV helper viruses to aid in replication and delivery of transgenes to targeted cells. In 1982, R. R. Spaete and N. Frenkel reported the construction of the first HSV-1 amplicon vector with success in propagating foreign DNA with the assistance of a helper virus (Spaete, 1982). Soon after, a second version of the amplicon vector packaging system was constructed by *Geller et al.,* involving the development of a temperature sensitive helper virus HSV-1 *tsK* from HSV-1 strain 17. It was used to infect rat superior cervical ganglia and dorsal root ganglia (Alfred I. Geller & Breakefield, 1988). The helper virus contained a point mutation in the ICP4 gene, which rendered it non-functional at 37-39°C, allowing the vector and helper virus to be propagated at low permissive temperatures, but prevented pathogenesis at higher body temperatures (A. I. Geller et al., 1990; Alfred I. Geller & Breakefield, 1988). The viral ICP4 gene is required for subsequent expression of other viral genes (Neve et al., 2005).

The use of a helper virus limits the use of HSV-1 amplicon vectors in a clinical setting, because, despite mutations that render the helper virus non-pathogenic, the helper virus can still cause some cytotoxicity and inflammation. To resolve this issue, helper-free amplicon vector systems have been developed (Epstein, 2005). These typically consist of a bacterial artificial chromosome (BAC) containing a helper virus genome with deletions in the cleavage and packaging signal (pac) sequence, preventing this helper genome from being packaged into virus particles. These BAC-based packaging systems may also contain mutations in viral immediate-early genes, similarly to defective helper viruses (Federoff, 1995). For example, a BAC-based packaging system with *pac* and ICP27 deletions successfully generated helper-

virus free amplicon vectors (Saeki et al., 2001). Currently, amplicons replicated and packaged by replication-defective helper viruses or helper-free systems can only be produced in specific cell lines that have been constructed to complement immediate-early gene functions missing from helper viruses and helper-free packaging systems (Roizman, 1996).

To study the molecular and cell biology of HSV-1 exocytosis, we need a method to introduce fluorescent protein-tagged transgenes into cells that will be infected with HSV-1. One potential method is use plasmid transfection to introduce a transgene, followed by HSV-1 infection. However, because it is difficult to infect previously transfected cells, there are not enough co-transfected/infected cells for direct microscopy experiments. Another potential method is to recombine the transgene into the HSV-1 genome; however, it is laborious and time-consuming to generate recombinant virus strains for each experiment. The current method used in the Hogue laboratory is to introduce transgenes using non-replicating adenovirus vectors, followed by HSV-1 infection (Hogue et al., 2014). This method, however, still has problems: There are still relatively few co-transduced/infected cells for microscopy experiments, and transducing with too much of the adenovirus vector leads to severe over-expression of the transgene of interest. To try to overcome these difficulties, we generated herpes amplicon vectors which can be amplified and packaged using a replication-competent HSV-1 helper virus. Importantly, we can use as the helper virus the very same HSV-1 strain we plan to use to perform experiments, streamlining our experimental protocol.

Herpesviruses and Herpes Simplex Virus 1

The human herpesviruses are among the most prevalent in the world, with some reaching nearly universal seroprevalence. Two-thirds of the world population are affected by herpes simplex virus 1 alone (Wertheim et al., 2014). Carriers are

often asymptomatic, and continual virus shedding causes this virus to spread unnoticed (Federoff, 1995). Herpesviruses belong to an ancient virus lineage, and most herpesviruses have co-evolved and co-speciated with their natural hosts. Recent evidence of evolutionary origin of herpesviruses in primates using phylogenetic and molecular dating analyses suggests that these viruses have been present 44.2 million years ago (mya) in ancestors of New World and Old World primates (Wertheim et al., 2014). It is believed that HSV-1 diverged from chimpanzee herpes virus (ChHV) around 6 mya, at the same time that the human lineage diverged from chimpanzees (Wertheim et al., 2014). Then, around 1.6 mya, there was a cross-species transmission event, where ChHV was transmitted to a modern human ancestor, leading to the evolution of herpes simplex virus 2 (Wertheim et al., 2014). Humans are the only primate known to harbor two separate simplex viruses.

Mammalian and avian herpesviruses are divided into three subfamilies of alpha, beta and gamma which are classified based on their host range, the distinct cell types they infect, site of latency and replication cycles (Conn, 2013; Whitley, 1996). The *Herpesviridae* family contains over 100 viruses, with likely many more viruses yet to be characterized. Only eight herpesviruses naturally infect humans: Alpha herpesvirus subfamily (HHV-1, HHV-2 and HHV-3), beta herpesvirus subfamily (HHV-5, HHV-6 and HHV-7) and gamma herpesvirus subfamily (HHV-4 and HHV-8) (Griffiths, 1997). These viruses are associated with a variety of different diseases like varicella (chicken pox), zoster (shingles, neuralgia, and various other neuropathies), herpes labialis (cold sores, pharyngitis), genital herpes, and a variety of cancers (Goins et al., 1997; Nováková et al., 2018; Whitley, 1996).

All herpesviruses contain common features and conserved genes that encode for structural proteins (capsid, tegument, and envelope proteins), regulatory proteins, DNA synthesis and nucleotide metabolism, a system that regulates a

temporal cascade of gene expression, and ability to establish latency or quiescent state in particular cells in their natural host (Griffiths, 1997). Both alpha and gamma herpesvirus subfamilies can express viral thymidine kinase (Nováková et al., 2018). Several antiviral drugs target viral DNA synthesis and the viral thymidine kinase enzyme, including acyclovir, penciclovir, ganciclovir, and foscarnet; however, these drugs only suppress active viral replication and do not eliminate latent, non-replicating virus (Griffiths, 1997; Nováková et al., 2018).

Herpes simplex virus 1, a member of the alpha herpesvirus subfamily, is typically transmitted orally between close family members early in childhood (Whitley, 1996). This causes oro-facial mucocutaneous lesions, and establishes lifelong latent infection in the peripheral nervous system (Bradley et al., 2014; Dai & Zhou, 2018; Ramchandani et al., 2016; Whitley, 1996). Approximately two-thirds of the world population is latently infected with HSV-1 (Bradley et al., 2014). When virus reactivation occurs, the virus typically returns to the oral cavity and lips causing episodic ulcerative lesions (Diefenbach & Fraefel, 2014). Although rare, it can also be present in tears and nasal mucosa (Ramchandani et al., 2016). Common external factors such as stress, immunosuppression, nerve trauma, fever, menstruation, and exposure to ultraviolet light can contribute to viral reactivation. In some patients reactivation can occur asymptomatically (Traylen et al., 2011). Immunocompromised patients are often severely affected by herpes simplex virus 1, resulting in keratoconjunctivitis causing blindness, meningoencephalitis, nerve damage, and death (Diefenbach & Fraefel, 2014). Although these serious conditions are rare, because of their very high prevalence in the population, the alpha herpesviruses are the leading cause of viral encephalitis. For this reason, it is important to better understand the molecular and cell biology of alpha herpesviruses, like HSV-1.

Herpes Simplex Virus I Structure

Herpesvirus virions are organized in distinctive layers as follows: (i) an ~150-kb linear double-stranded DNA genome, enclosed within (ii) an approximately 125 nm icosahedral capsid composed of 161 capsomeres formed from six different viral capsid proteins; (iii) a proteinaceous tegument layer containing 22 viral proteins that surround the viral capsid; and, (iv) an external lipid bilayer envelope containing 16 viral membrane proteins, most of which are glycosylated (glycoproteins) (Diefenbach & Fraefel, 2014; Karasneh & Shukla, 2011; Weed & Nicola, 2017). Over 50% of herpesvirus genes encode structural proteins that form the virion (Subak-Sharpe & Dargan, n.d., 1998).

Virus entry is mediated by viral membrane glycoproteins that function in a cascade: gC mediates cell attachment by binding to cellular heparan sulfate proteoglycans, gD engages cellular receptors including nectin proteins, and gB and the gH/gL heterodimer mediate membrane fusion (Griffiths, 1997; Karasneh & Shukla, 2011; Nicola, 2016; Rahn et al., 2011). In most cell types, entry occurs by pH-independent membrane fusion at the plasma membrane; however, HSV-1 can also utilize endocytic mechanisms and pH-dependent entry (Nicola, 2016; Rahn et al., 2011; Tebaldi et al., 2020). In Vero cells, HSV-1 uses a pH-independent pathway by fusing with the host cell plasma membrane, whereas HSV-1 is reported to use pH-dependent endocytic entry in HeLa cells (Rahn et al., 2011). Following entry, capsid-associated tegument proteins recruit the minus end-directed microtubule motor protein dynein, to traffic to the nucleus. There, the virus capsid docks at the nuclear pore complex, and releases its viral DNA into the host cell nucleus. In the nucleus, immediate-early promoters are recognized by host transcription machinery, and this begins a cascade of immediate-early, early, and late gene expression (Conn, 2013; Copeland et al., 2009). Following viral DNA replication and late gene expression, the newly synthesized viral DNA is packaged into newly-assembled capsids. Virus capsids

are too big to exit the nucleus via nuclear pore complexes, and instead exit by budding through the nuclear envelope membranes. Once in the cytoplasm, the capsid binds tegument proteins and membrane proteins, which mediate its envelopment into intracellular organelles, most likely the trans-Golgi network. Finally the enveloped virion traffics towards the periphery of the host cell, where it is released by exocytosis (Hogue, 2020).

All known members of the *Herpesviridae* family establish latency in particular cell types of their natural hosts and can periodically reactivate. This allows the virus to avoid running out of susceptible hosts, allowing these viruses to persist in the population, even with near universal prevalence. Infection with HSV-1 begins by infecting the mucocutaneous linings or the oronasal or genital tract. It then efficiently invades the peripheral nervous system, and establishes a lifelong latent infection (Möckel et al., 2019). During this process, HSV-1 virus particles travel by retrograde axonal transport from sensory or autonomic nerve endings to the neuron cell body, to establish latency in the nucleus of these peripheral nervous system neurons (Dai & Zhou, 2018). Upon reactivation, newly assembled HSV-1 particles return to the site of initial infection, traveling from the neuron cell body to nerve terminals near epithelial cells, via anterograde axonal transport (Dai & Zhou, 2018; Diefenbach & Fraefel, 2014). During latency, herpesviruses may replicate at very low levels or not at all, and infectious virus remains undetectable in peripheral ganglia (Fink & Giorioso, 1997). Most viral genes are transcriptionally silent during latency, with the exception of one major non-coding Latency-Associated Transcript (LAT) (Goins et al., 2014). This tactic allows the virus to avoid detection and elimination by the host's immune system during latency. Upon reactivation, HSV-1 starts full lytic replication cycle, with a cascade of immediate-early, early, and late gene expression, DNA genome replication, and production of progeny virus (Amen & Griffiths, 2011; Diefenbach & Fraefel, 2014; Traylen et al., 2011). Recent studies have shown that

neurons undergoing virus reactivation are ultimately eliminated by the host immune system (Doll et al., 2019); however, the subsequent reinfection of epithelial tissues re-seeds the peripheral ganglia, ensuring life-long persistence of the latent infection.

It is still unclear how HSV-1 is capable of successfully infecting and replicating various cell types, particularly in the nervous system. As an obligate intracellular parasite that has co-evolved with its natural hosts for at least tens of millions of years, this virus is surely a master of its host's molecular and cell biology. In this Master's Thesis, I present an improved HSV-1 viral vector that will provide a useful molecular tool to transduce over 150 kb of foreign DNA into cells of our choosing. The main goal of this research project was to construct and characterize new HSV-1 amplicon vectors, optimize their propagation using a replication-competent helper virus, and demonstrate how co-infection of amplicon and helper virus can be used to study the molecular and cell biology of HSV-1 infection in mammalian cells. The use of these novel amplicon vectors will provide further information as to how HSV-1 uses the host's cell machinery to effectively conduct exocytosis.

To develop a better experimental system to study the molecular and cell biology of HSV-1 egress, we pursued the following two specific aims: In Aim 1, we optimized the production of amplicon vectors using a replication-competent helper virus. Currently, HSV-1 amplicons are replication-defective and can be either helper-dependent or helper-free (Epstein, 2005). Recent studies have demonstrated effective ways to produce high helper-dependent amplicon vector stocks with low helper virus contamination (Diefenbach & Fraefel, 2014). For this project, replication-competent helper viruses were used to package and replicate the amplicon vectors without contamination concerns.

Following our optimization in aim 1, in aim 2 we investigated the use of amplicon vectors as a biological tool to study and monitor the trafficking of HSV-1 viral particles in exocytosis. Identifying what proteins aid and contribute to HSV-1

exocytosis in various cell types has been difficult to assess, as we are still unsure as to what triggers HSV-1 replication and egress. To further understand how HSV-1 uses the host cell machinery to effectively leave the infected cell, we will use these amplicon vectors to introduce proteins that can potentially aid in viral egress. Ultimately, the development of this new tool will allow us to determine not only what proteins contribute to HSV-1 exocytosis, but also being able to differentiate if the same mechanisms are used for various mammalian and neuronal cells. If we can establish what proteins aid in HSV-1 egress, we can determine which proteins can be inhibited to prevent exocytosis and eventually new and improved antivirals can be produced for the treatment of HSV-1 infection.

MATERIALS AND METHODS

HSV-1 Amplicon Vector Construct

Our novel HSV-1 amplicon vectors consist of a plasmid backbone containing an *E. coli* origin of replication, an ampicillin antibiotic resistance gene, and two non-coding, *cis*-acting viral sequences, the *OriS* origin of replication, and *pac* cleavage/packaging signals. In addition, the amplicon vectors contain a transgene expression cassette that consists of a human cytomegalovirus (CMV) promoter and enhancer, the coding sequence of emerald green fluorescent protein (EmGFP), and the HSV-1 thymidine kinase polyadenylation signal. To facilitate insertion of any desired protein coding sequence, immediately before or after the EmGFP coding sequence, there are *att*R1 and *att*R2 recombination sites for Invitrogen Gateway cloning. The Gateway recombination sites flank a chloramphenicol antibiotic resistance gene and *E. coli* negative-selection marker, the *ccdB* gene, to facilitate selection of *E. coli* clones harboring recombined amplicon plasmids containing the transgene of interest. The HSV-1 amplicon plasmid is transfected into HEK293 cells,

followed by infection with a replication-competent helper virus (Federoff, 1995; Neve & Lim, 1999).

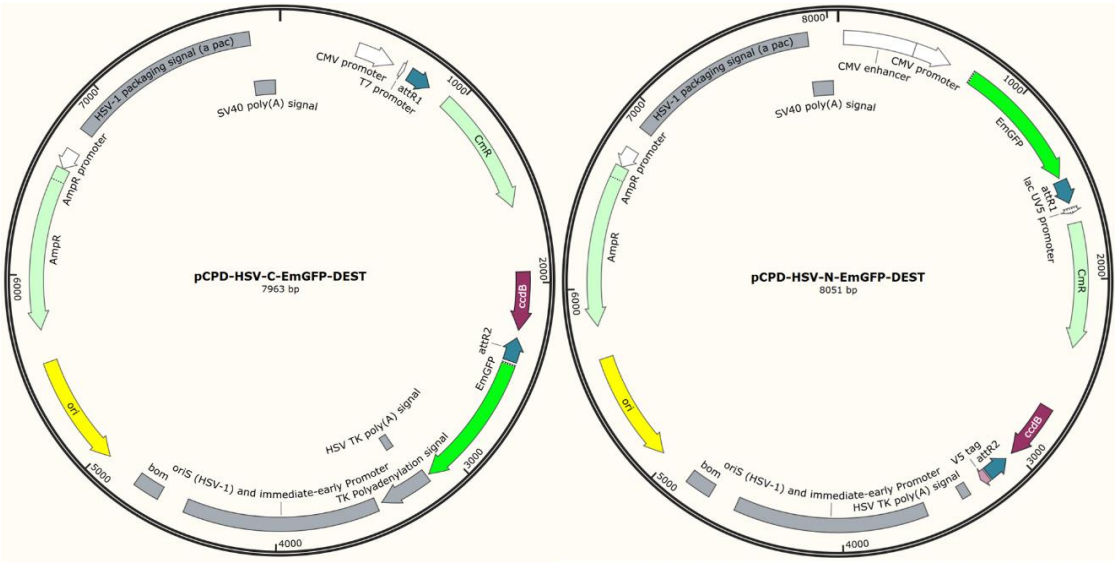

Figure 1. Diagram of pCPD-HSV-C-EmGFP-DEST (left) and pCPD-HSV-N-EmGFP-DEST (right) Amplicon Vector Constructs. The HSV-1 amplicon on the left is designed to create an in-frame fusion of EmGFP to the C-terminus of a gene of interest, and the HSV-1 amplicon on the right creates an in-frame fusion of EmGFP on the N-terminus of the gene of interest.

The two HSV-1 amplicon vector constructs pCPD-HSV-C-EmGFP-DEST (HSV-C-EmGFP amplicon) and pCPD-HSV-N-EmGFP-DEST (EmGFP-HSV-N amplicon) were synthesized at the DNASU Plasmid Repository in the Biodesign Institute (Arizona State University, Tempe AZ). To test whether these amplicon vectors are capable of being replicated and packaged by the helper virus and are capable of expressing EmGFP in transduced cells, a Gateway recombination LR reaction was performed to insert a 5-residue glycine-serine linker (Gly–Gly–Gly–Gly–Ser) peptide onto the N- or C-terminus of EmGFP in the respective amplicon plasmid.

The Invitrogen Gateway recombination system is a multi- or single-fragment recombination cloning technique that uses λ phage integration and excision enzymes to perform recombination at specific *att* sites, which are naturally found in the bacterial host genome (Fu et al., 2008; Petersen & Stowers, 2011). This system

allows DNA sequences to be quickly, easily, and accurately swapped between different plasmids (Kunec et al., 2009). Recombination occurs via two symmetrical reactions: the Gateway BP reaction (recombination between *att*B and *att*P sites, which generates *att*L and *att*R sites) and the LR reaction (recombination between *att*L and *att*R, which generates *att*B and *att*P sites) (Fu et al., 2008). In particular, the *att*B sites are small, so can be incorporated into PCR primers, and can be used to create an in-frame fusion between coding sequences, encoding a short linker peptide. During a Gateway LR reaction, the *ccdB* negative selection marker is replaced by the gene of interest. Parental amplicon plasmids that did not recombine, as well as recombination byproducts, will not propagate because the *ccdB* gene product is toxic to *E. coli*. For this project, we recombined two genes of interest into the amplicon vectors, Rab6a and VAMP2, to study their involvement in egress/exocytosis of HSV-1 virions.

Bacterial strains, enzymes, and plasmids

Amplicon plasmids were constructed by the DNASU Plasmid Repository (Biodesign Institute, Arizona State University, Tempe AZ). To propagate the plasmids, a small sample of the glycerol stock was grown overnight in LB medium (Caisson laboratories, INC, AR, USA) containing Carbenicillin (100 µg/ml) and placed in shaking incubator (200 RPM at 37°C). Amplicon plasmid DNA was purified using Qiagen QIAprep Spin Miniprep Kit, following the manufacturer's protocol. Concentration of amplicon plasmids were measured using NanoDrop ND-1000 Spectrophotometer.

Cell lines and cell culture

Human embryonic kidney cells (HEK293) were obtained from ATCC and used for plasmid transfection. African green monkey kidney cells (Vero) were obtained

from ATCC and used for HSV-1 and amplicon vector propagation. The Vero-derived 3-3 cell line, generously provided by Dr. David Davido (Kansas University, United States), expresses ICP27 necessary for replication of the ICP27-deleted helper virus, 5dl1.2. Ultimately, the 3-3 cell line could not be transfected efficiently, so it was not used for further experiments.

HEK293, Vero, and 3-3 cells were cultured in complete high-glucose Dulbecco's modified essential medium (DMEM), containing 4mM glutamine, 4500 mg/L glucose and sodium pyruvate (HyClone, GE Healthcare Life Sciences, PA, USA) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific, inc., CA, USA) and 1% of Penicillin-Streptomycin (P/S) solution (HyClone, GE Healthcare Life Sciences, PA, USA). Viral propagation medium consisted of the same complete DMEM medium, except supplemented with 2% FBS. All cell cultures were maintained at 37°C in a humidified incubator containing 5% CO_2.

Helper virus

The first helper virus chosen was a replication-defective mutant of HSV-1 called 5dl1.2. 5dl1.2 contains a deletion in the IE2 gene, which encodes the ICP27 immediate-early protein. The 5dl1.2 mutant was constructed by removing the ICP27 promoter sequences, transcriptional start site, and amino terminus sequence encoding 262 amino acids (McCarthy et al., 1989). This helper virus can only replicate in a complementing cell line expressing the ICP27 protein. Dr. David Davido (Kansas University, United States) kindly provided the defective 5dl1.2 helper virus. The viral titer for 5dl1.2 was 1.0×10^7 PFU/mL.

A second helper virus chosen was a replication-competent recombinant HSV-1 strain, OK-14, kindly provided by Dr. Lynn W. Enquist (Princeton University, United States). OK-14 was derived from the HSV-1 lab strain 17(syn+) and expresses an mRFP-VP26 capsid fusion. Viral titers of OK-14 helper virus were 3.4×10^8 PFU/mL.

A third replication-competent helper virus, HSV-UL25-mScarlet-I, was constructed in Dr. Ian Hogue's lab by homologous recombination between HSV-1 17 (syn+) nucleocapsid DNA and a plasmid containing an mScarlet-I-UL25 fusion protein. The HSV-mScartlet-I-UL25 construct consists of the following in order: the last 83 bps of UL24, the 267 bps between UL24 and UL25, the first 50 bps of UL25, the 696 bp of mScarlet-I, and the following 500 bps of UL25 (bp 51-551). This construct was co-transfected with viral nucleocapsid DNA, and resulting recombinant virus was plaque picked and purified. Viral titers of HSV-UL25-mScarlet-I helper virus were 9.4×10^7 PFU/mL. Table 1 lists the purpose of each helper virus that was used for this project.

Table 1. Helper Viruses. Left column lists the helper virus constructs used for this project. Right column describes the purpose of each helper virus. Both helper viruses contain a red fluorescent protein fusion to a viral capsid protein for easier localization of viral particles.

Helper Virus	**Purpose**
HSV-UL25-mScarlet-I	Replication-competent recombined HSV-1 strain containing a UL25-mScarlet-I fusion protein was used for the amplification and packaging of amplicon DNA into HSV-1 particles.
OK-14	Replication-competent recombined HSV-1 strain containing a mRFP-VP26 capsid fusion was used for the amplification and packaging of amplicon DNA into HSV-1 particles.

Genes of interest

Rab (Ras-related protein) GTPase proteins regulate essentially all intracellular membrane traffic. Previously, Dr. Ian Hogue showed that a related alpha herpesvirus, pseudorabies virus, uses post-Golgi secretory vesicles marked by Rab6a for egress by exocytosis (Ambrosini et al., 2019; Hogue et al., 2014). Invitrogen Gateway cloning LR reaction of the Rab6a plasmid (Clone: HsCD00296778 from DNASU Plasmid Repository) containing *att*L sites was conducted by DNASU Plasmid Repository to introduce Rab6a into a destination vector amplicon containing the *att*R

sites. The insertion of Rab6a created *att*B sites, one of which formed a protein coding linker lacking stop codons located between the N-terminus of Rab6 and EmGFP.

Synaptobrevins, vesicle associated membrane proteins (VAMPs), or v-SNARES are common names for proteins responsible for fusing exocytotic vesicles with the plasma membrane (Bhattacharya et al., 2002). VAMP2, also known as synaptobrevin-2, is a SNARE protein that facilitates the fusion of synaptic vesicles to release neurotransmitters in neurons (Salpietro et al., 2019). An Invitrogen Gateway cloning LR reaction of the VAMP2 (Clone: HsCD00719004 from DNASU Plasmid Repository) plasmid containing *att*L sites was conducted by DNASU Plasmid Repository to introduce VAMP2 into a destination vector amplicon expressing *att*R sites. The insertion of VAMP2 resulted in *att*B sites forming a protein coding linker lacking stop codons between the C-terminus of VAMP2 and EmGFP. Table 2 lists the purpose of each amplicon vector tested for this project.

Table 2. Amplicon Vectors. Left column lists the three amplicon vector constructs used for this project. Right column lists the purpose of using each amplicon vector pertaining to this project.

Amplicon	**Purpose**
HSV-C-EmGFP	To test if amplicon is capable of being replicated and packaged by the helper virus and express EmGFP in transduced cells.
EmGFP-HSV-N	To test if amplicon is capable of being replicated and packaged by the helper virus and express EmGFP in transduced cells.
EmGFP-Rab6	Constructed to study Rab6 protein interactions with HSV-1 viral particles and verify if amplicon is capable of being replicated and packaged by the helper virus in addition to expressing EmGFP in transduced cells.

Transfection

HEK293 cells were seeded at 3.5 x 10^5 cells/well in a six-well plate (Cell Treat) with 2 mL of complete DMEM (10% FBS, 1% P/S) overnight. Twenty-four

hours later, cells were transfected using Lipofectamine 2000 reagent (Invitrogen Inc.). We transfected 2, 3, 5, or 10 µg of HSV-1 amplicon DNA in duplicates. OPTI-MEM (Gibco Life Technologies) containing GlutaMAX, HEPES, and 2.4g/L sodium bicarbonate was used to dilute plasmids and Lipofectamine 2000 reagent. A mixture of 12 µL Lipofectamine 2000 and 500 µL of OPTI-MEM was prepared in a sterile 1.5-mL microcentrifuge tube, followed by a 5-minute incubation at room temperature. In a separate sterile 1.5-mL microcentrifuge tube, the amplicon DNA was mixed with 500 µL of OPTI-MEM. The DNA and Lipofectamine 2000 solutions were then combined into one sterile 1.5-mL microcentrifuge tube and incubated at room temperature for 25-30 minutes. Prior to adding the transfection mixture to cells, the cell culture medium was replaced with 2 mL of fresh room temperature complete viral DMEM (2% FBS, 1% P/S). For each well, the transfection mix was added dropwise evenly throughout the culture well. The six-well plate was maintained at 37°C in a humidified incubator containing 5% CO2 overnight.

Superinfection with helper virus

At twenty-four hours post-transfection, observable EmGFP expression in over 70-90% of cells was confirmed by inverted fluorescent microscopy (AmScope Model: XYL403). Next, a stock of helper virus HSV-UL25-mScarlet-I or OK-14 was thawed in a 37°C water bath, sonicated using a Fisher Scientific, model: FB-505 sonicator set to 80% amplitude, 50% duty cycle of 1s on and 1s off, for 10s total. At last, the sample was briefly vortexed, and centrifuged (3,000 x *g* for 3 min). For each sample well, the helper virus supernatant (1 or 10 µl) was transferred to a sterile 1.5-mL microcentrifuge tube containing 1 mL of room temperature complete viral DMEM (2% FBS, 1% P/S). Transfected HEK293 cell medium was replaced with 1 mL of fresh complete viral DMEM (2% FBS, 1% P/S). Lastly, the diluted helper virus mixture was

added to each well. The plate was stored at 37°C in a humidified incubator containing 5% CO_2.

Fluorescence microscopy

Cytopathic effect (CPE) was visualized by phase contrast light microscopy (approximately 48 h.p.i.). Fluorescence microscopy was performed using a Nikon Ti2-E Inverted Fluorescence Microscope equipped with a SpectraX LED lightsource, and appropriate filters to detect green and red fluorescent proteins. To detect EmGFP, the FITC channel was set at approximately 5-20% power and exposure time of 60-600 ms. For detection of mRFP, the TRITC channel was set at approximately 20-25% power and exposure time of 100-900 ms.

Amplification of HSV-1 amplicon vector stocks

Vector stocks were passaged several times to increase virus titer and approach a 1:1 amplicon to helper virus ratio.

P0: After imaging using Nikon Ti2-E Inverted Fluorescence Microscope, the samples were harvested. Each co-infection was conducted in duplicates, as a result, a total of approximately 3.5-4 mL was harvested from both wells using a cell lifter to scrape up infected cells. Next, the duplicated samples were combined in a 15 mL conical tube with HEPES (50X, Gibco Life Technologies) and stored at -80℃.

P1: Fresh Vero cells (70-80% confluency) in a 100-cm tissue culture plate must be ready for each sample. The complete DMEM (10% FBS, 1% P/S) media was replaced with 8 mL of room temperature fresh complete DMEM (2% FBS, 1% P/S). The cell lysates were thawed in a 37℃-water bath, sonicated, and centrifugated (3,000 x *g* for 3 min). Next, the supernatant (totaling 3.5-4 mL) was added to the 100-cm dish containing Vero cells and incubated at 37◦C in a humidified incubator containing 5% CO_2. At last, when approximately 95% of cells demonstrated CPE, a cell lifter was

used to harvest the samples into a 15 mL conical tube with HEPES (50x) and stored at -80℃.

P2: Two 100-cm tissue culture plates of fresh Vero cells (70-80% confluency) was prepared for each sample. For each plate, the supplemented DMEM (10% FBS, 1% P/S) was removed and replaced with 6mL of fresh room temperature supplemented DMEM (2% FBS, 1% P/S). The harvested stock was thawed in a 37℃-water bath, sonicated, and centrifugated (3,000 x *g* for 3 min). Finally, 5 mL of supernatant amplicon viral stock was added to each plate and incubated at 37℃ until cells demonstrated 95% CPE. Using a cell lifter, each plate of infected cells was harvested, transferred to a 15 mL conical tube with HEPES (50x) and stored at -80℃.

P3: Two 100-cm tissue culture plates of fresh Vero cells (70-80% confluency) was prepared for each sample. For each plate, the supplemented DMEM (10% FBS, 1% P/S) was removed and replaced with 6mL of fresh room temperature supplemented DMEM (2% FBS, 1% P/S). The harvested stock was thawed in a 37℃-water bath, sonicated, and centrifugated (3,000 x *g* for 3 min). Finally, 5 mL of supernatant amplicon viral stock was added to each plate and incubated at 37℃ until cells demonstrated 95% CPE. Using a cell lifter, infected cells were harvested, transferred to a 50 mL conical tube with HEPES (50x). At this time, the 50 mL conical tube was placed in -80℃ overnight.

The next day, the harvested stock was thawed in a 37℃-water bath, sonicated, and centrifugated (3,000 x *g* for 3 min). Aliquots of 1mL of supernatant were made using sterile 1.5-mL microcentrifuge tubes and stored at -80℃.

Total internal reflection fluorescence (TIRF)

Vero cells were seeded at 3.5 x 10^5 cells/mL in a 30mm x 10mm tissue culture dish with 15 mm glass bottom (Mattek or Cell Treat) overnight. The next day, the amplified HSV-1 amplicon vector stock was thawed in a 37℃-water bath,

sonicated, and centrifugated (3,000 x *g* for 3 min). At last, Vero cells were infected with 1mL of amplified HSV-1 amplicon vector stock supernatant and imaged using Nikon Ti2-E Inverted Fluorescence Microscope. This microscope is equipped with a heated stage and objective warmer for live-cell imaging, a TIRF illuminator and 60X high-NA TIRF objective for TIRF imaging, and 488nm and 561nm lasers.

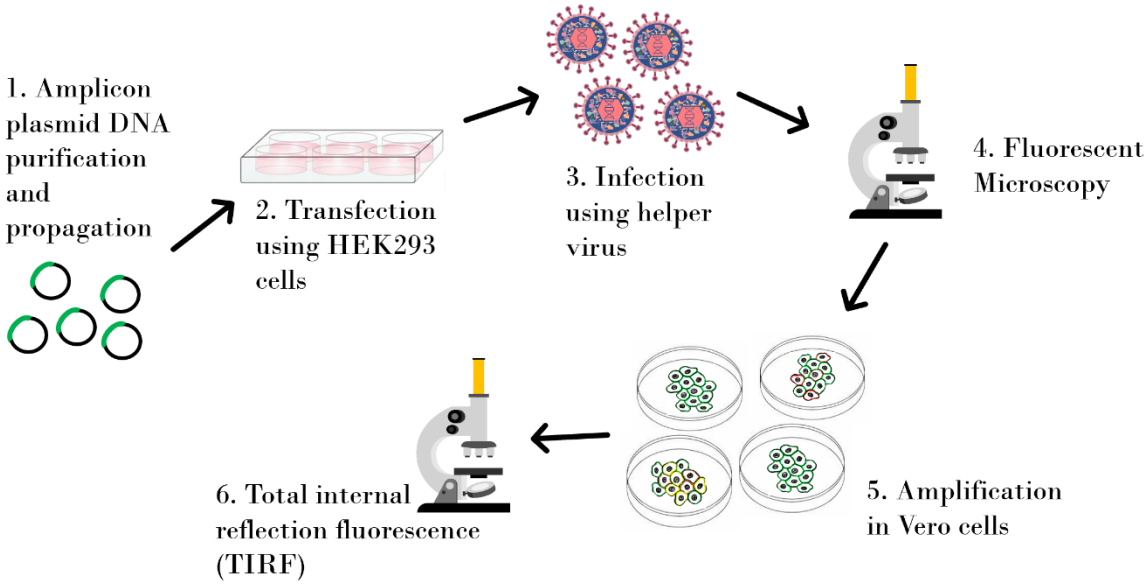

Figure 2. Diagram of Experimental Design. For each amplicon vector, steps 1-6 were conducted to acquire amplified stocks and determine if stocks can successfully co-infect Vero cells to achieve amplicon: helper virus ratio.

Multiple experiments were conducted to obtain amplified amplicon vector stocks of EmGFP-Rab6, HSV-C-EmGFP and EmGFP-HSV-N (Figure 2). 1) Purification and propagation of the amplicon plasmid DNA. 2) Transfection of the amplicon plasmid DNA into HEK293 cells using Lipofectamine 2000 transfection reagent. 3) At 24-hrs post-transfection, superinfection using helper virus OK-14 or HSV-UL25-mScarlet-I was conducted. 4) At 2 days post infection, samples were imaged using fluorescent microscopy to confirm co-infection followed by harvesting sample for storage in -80℃. 5) Three rounds of amplification using Vero cells was conducted and 1mL aliquots made. 6) The 1mL aliquots were used to infect Vero cells seeded in a 30mm x 10mm tissue culture dish with 15 mm glass bottom for TIRF microscopy.

RESULTS

In aim 1, we attempted to optimize the production of amplicon vectors using a replication-competent helper virus. Transfection efficiency of the 3-3 cell line was poor, with less than 5% of cells transfected with the amplicon plasmids. This limitation prevented us from packaging amplicon vectors using the ICP27-deleted HSV-1 5dl1.2 helper virus on complementing 3-3 cells. Ultimately, we plan to do experiments combining vector transduction with replication-competent HSV-1 infection, to study the molecular and cell biology of HSV-1. Therefore, we next sought to determine whether the amplicon vectors can be replicated, packaged, and co-propagated with replication-competent HSV-1. Because these replication-competent HSV-1 variants do not require a complementing cell line, we were able to use cell lines that can be more efficiently transfected.

HEK293 cells are widely used in molecular biology in part because of their high transfection efficiency. We transfected the amplicon plasmids into HEK293 cells using Lipofectamine 2000. Because the amplicon plasmids express EmGFP constitutively, we were able to observe GFP fluorescence to assess transfection efficiency. HEK293 cells exhibited high transfection efficiency for nearly all of the amplicon plasmids. The VAMP2-EmGFP amplicon did not produce any EmGFP expression. We concluded that a stop codon was mistakenly inserted at the end of the VAMP2 coding sequence, preventing EmGFP expression, so we discontinued the use of the VAMP2-EmGFP amplicon vectors for further experiments.

We tested numerous combinations of amplicon DNA and helper virus in transfection/infection experiments to optimize vector production. The following amounts of amplicon plasmid DNA were tested: 2 µg (~$2x10^{11}$ copies), 3 µg (~$4x10^{11}$ copies), 5 µg (~$6x10^{11}$ copies), and 10 µg (~$1x10^{12}$ copies) of plasmid. For OK-14 helper virus the amount of 10 µl was tested. Two days post-infection, we acquired images using fluorescent microscopy (FITC and TRITC channels at 4x

magnification) to assess co-transfection/infection of the HEK293 cells (Figure 3). For comparison, we also tested a different replication-competent HSV-1 helper virus, by testing 1 µl of HSV-UL25-mScarlet-I, with 2 µg of amplicon DNA. The resulting virus/vector stocks were then tested on Vero cells to assess co-transduction/infection efficiency and estimate the vector to helper virus ratio.

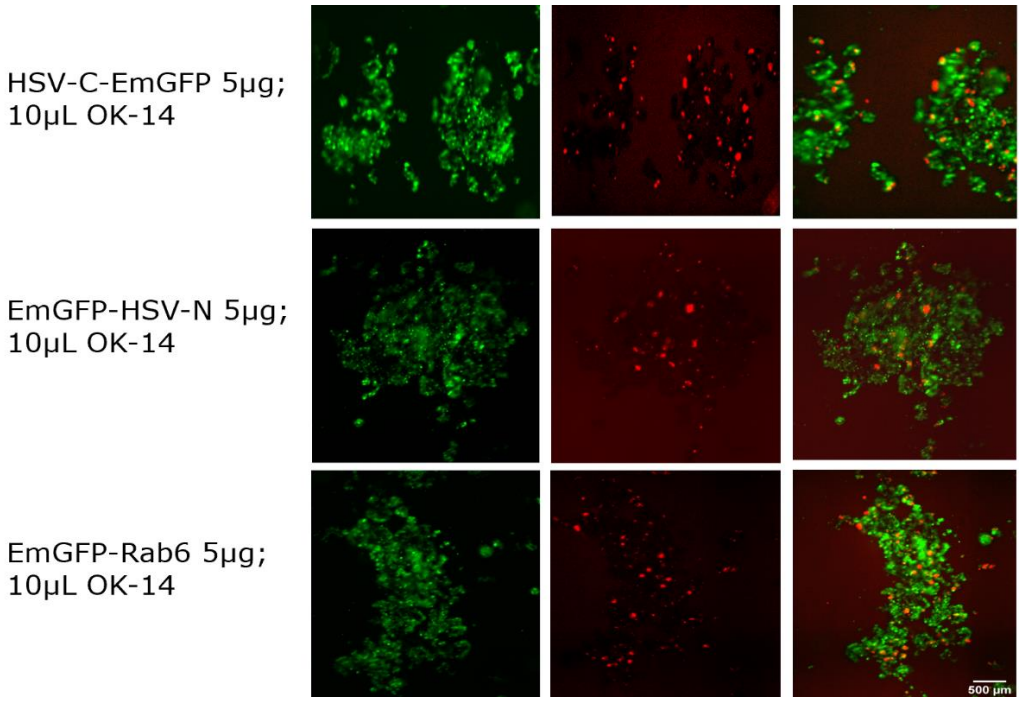

Figure 3. HEK293 Cells Transfected With 5µg of Amplicon Plasmid DNA, and Co-infected With 10µl OK-14 Helper Virus. Images were taken by fluorescent microscopy at 2 days post-infection. The left column demonstrates the amplicon vector EmGFP expression. The middle column demonstrates mRFP expression of the OK-14 helper virus. The right column shows overlay of both images.

To confirm amplification of co-infected cells, we imaged Vero cells co-transduced/infected with EmGFP-Rab6 and HSV-UL25-mScarlet-I vector stocks, produced from 2µg plasmid and 1µl helper virus in HEK293 cells (Figure 4). The viral titer used for HSV-UL25-mScarlet-I helper virus was 9.4×10^4 PFU. At 8 h.p.i., fluorescent microscopy images were taken, thus demonstrating this condition showed few co-infected cells. The UL25-mScarlet-I fusion, a red fluorescent protein fusion to a viral capsid protein, accumulated in the cell nucleus, but also localized

throughout the cytoplasm. In addition, the mScarlet-I fluorophore appeared to exhibit faster photobleaching compared to other HSV-1 capsid protein fusions. Therefore, the HSV-UL25-mScarlet-I helper virus was not used for subsequent experiments, and was replaced by OK-14, which expresses an mRFP1-VP26 capsid protein fusion instead.

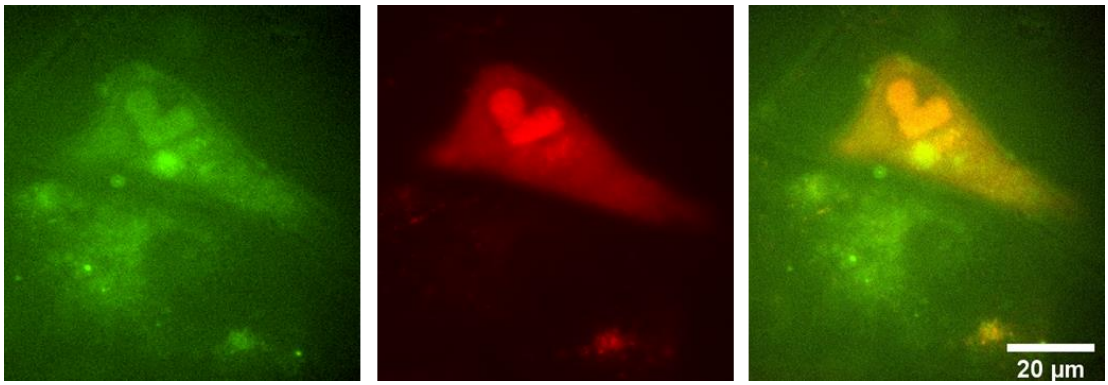

Figure 4. Fluorescence Microscopy of a Vero Cell Co-infected With EmGFP-Rab6 Amplicon Vector and HSV-UL25-mScarlet-I at 8 h.p.i. Left: EmGFP-Rab6 expression. Note that EmGFP-Rab6 localizes strongly to the Golgi and to small vesicles throughout the cytoplasm (individual vesicles cannot be discerned in this image). Middle: UL25-mScarlet-I expression. Note that UL25-mScarlet-I localizes strongly to the nucleus and also in the cytoplasm. Right: Overlay of fluorescence channels.

Vero cells were co-transduced/infected with 1mL of vector stocks produced from 5 µg EmGFP-Rab6 amplicon and 10 µl OK-14 in HEK293 cells for assessment of co-infection. The viral titer used for OK-14 helper virus was 3.4×10^6 PFU. At 7 h.p.i., fluorescent microscopy demonstrated co-infection with both amplicon and helper virus. The mRFP-VP26 fusion protein expressed by OK-14 localized strongly to the cell nucleus, EmGFP-Rab6 localized strongly to the Golgi apparatus, and EmGFP-Rab6 secretory vesicles could be observed traveling to and accumulating at the cell periphery (Figure 5).

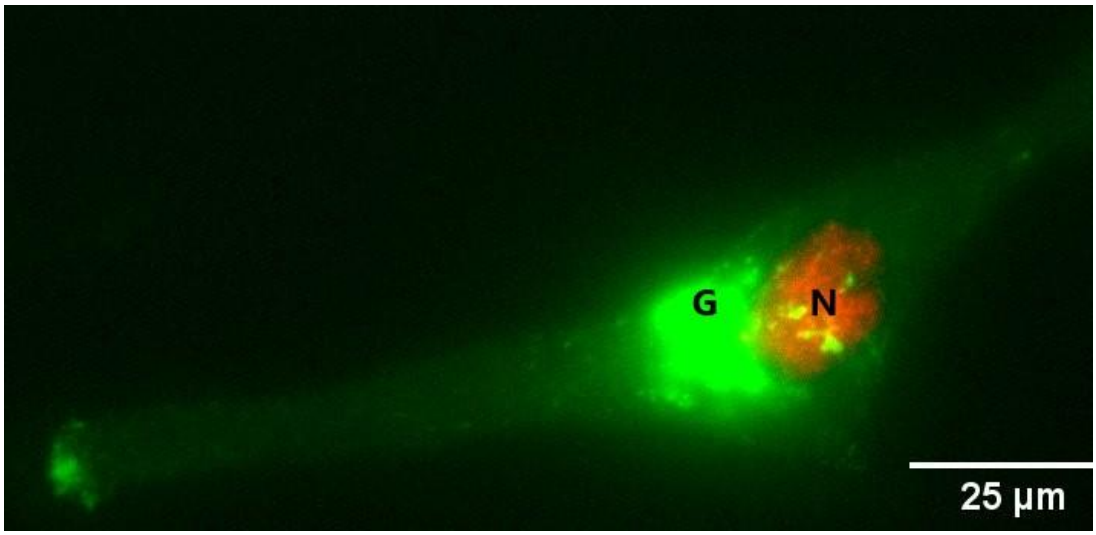

Figure 5. Vero Cell Co-infected With EmGFP-Rab6 Amplicon Vector and OK-14. The nucleus (N) of the Vero cell demonstrated infection of OK-14 by mRFP expression. The Golgi apparatus (G) is expressing EmGFP found on the Rab6 amplicon vector. Image was taken by fluorescent microscopy at 7 h.p.i.

For aim 2, we used the amplicon vectors as a biological tool to study and monitor the trafficking of HSV-1 viral particles in exocytosis. We next sought to determine whether co-transduction/infection with these amplicon stocks can be used to study intracellular trafficking and exocytosis of virus particles. The helper virus viral titers used for subsequent experiments were as follows: OK-14 helper virus was 3.4×10^6 PFU. To better observe vesicle transport and exocytosis, we used Total Internal Reflection Fluorescence (TIRF) microscopy. In TIRF, the excitation laser beam is reflected off the coverslip to excite fluorescence only where a cell is in contact with the coverslip. Because the fluorescent virus capsid protein accumulates strongly in the nucleus and Rab6 accumulates strongly in the Golgi (Figure 6, right), TIRF allows us to disregard the high background fluorescence from these organelles, to be able to see smaller, dimmer individual virus particles and secretory vesicles (Figure 6, left).

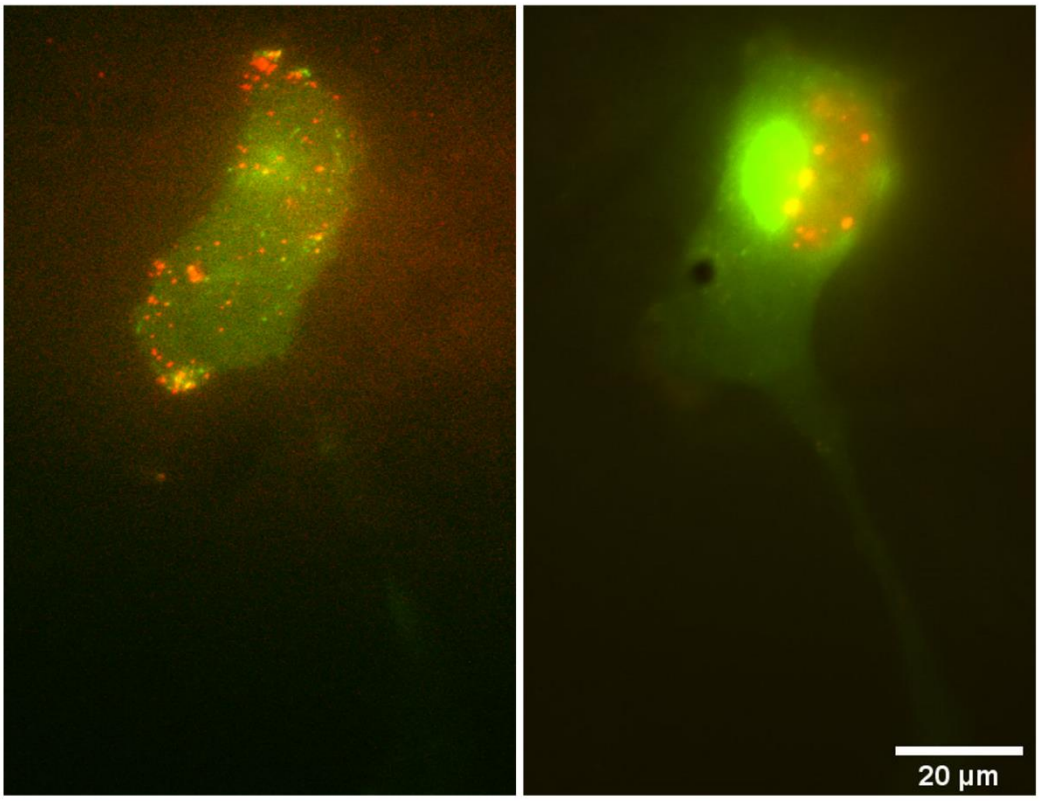

Figure 6. TIRF and Widefield Comparison of Amplicon Stock Produced From 3 µg EmGFP-Rab6 Plasmid and 10 µl OK-14. TIRF image shows only individual fluorescent particles near the coverslip that are excited by the laser beam (left). Widefield image shows fluorescent virus capsid protein accumulation in the nucleus and Rab6 accumulation in the Golgi (right). Image was taken by fluorescent microscopy at 7-8 h.p.i.

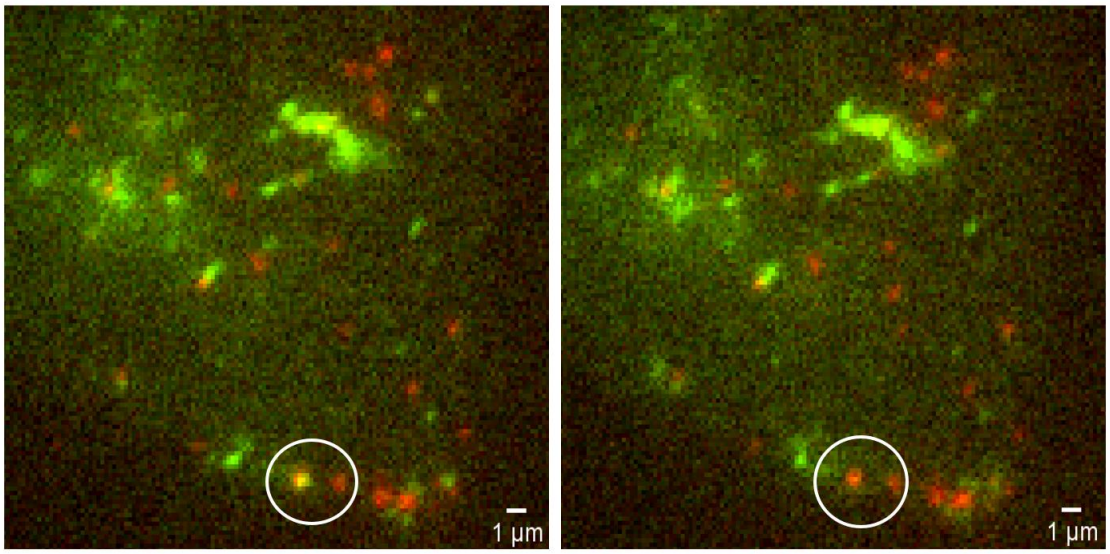

Figure 7. Exocytosis Event of Amplicon Stock Produced From 3 µg EmGFP-Rab6 Plasmid and 10 µl OK-14 Taken by TIRF Microscopy. EmGFP-Rab6 secretory vesicle

transporting a red viral particle exhibiting a yellow color, as it traffics inside the cell (left). Exocytosis of red viral particle results in diffusion of EmGFP-Rab6 secretory vesicle (right). Image was taken by fluorescent microscopy at 7-8 h.p.i.

Using the amplicon stock produced from 3 µg EmGFP-Rab6 plasmid and 10 µl OK-14 (1 mL of amplified stock), we were able to capture virus particles exiting the cell via exocytosis from secretory vesicles labeled with EmGFP-Rab6 at 7-8 h.p.i. (Figure 7). Vero cells expressing only the EmGFP-Rab6 amplicon vector also exhibited transport of EmGFP-Rab6 vesicles in the cytoplasm.

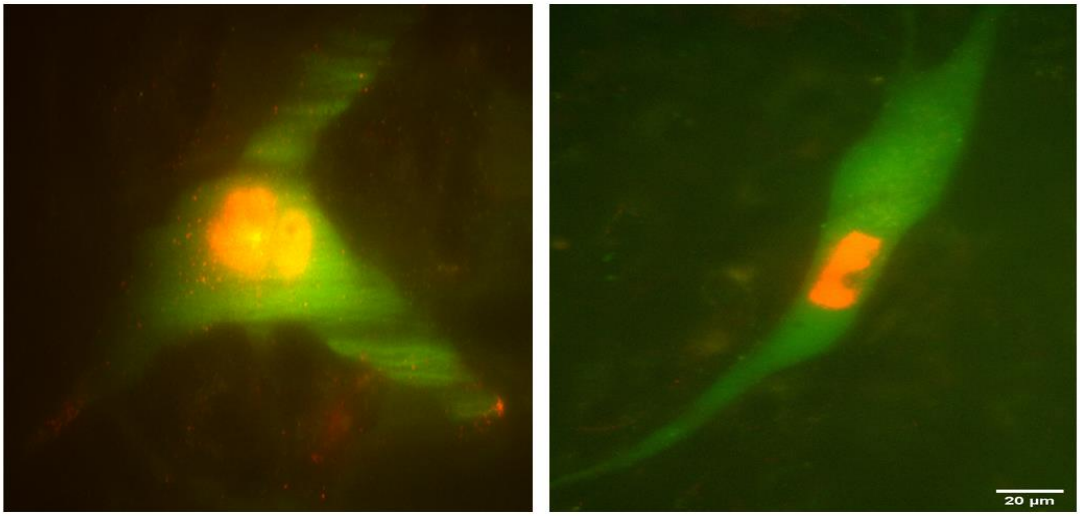

Figure 8. HSV-C-EmGFP and EmGFP-HSV-N Amplicon Vectors Superinfected With OK-14. HSV-C-EmGFP (left) and EmGFP-HSV-N (right) amplicon vectors demonstrate expression of EmGFP in the cytoplasm and mRFP in the nucleus. Image was taken by fluorescent microscopy at 7-8 h.p.i.

Both the HSV-C-EmGFP and EmGFP-HSV-N amplicon vector, replicated and packaged using OK-14 helper virus, exhibited co-infection in Vero cells. Both amplicon vectors demonstrated EmGFP expression in the cytoplasm in conjunction with mRFP expression of OK-14 viral particles in the nucleus of host cell (Figure 8).

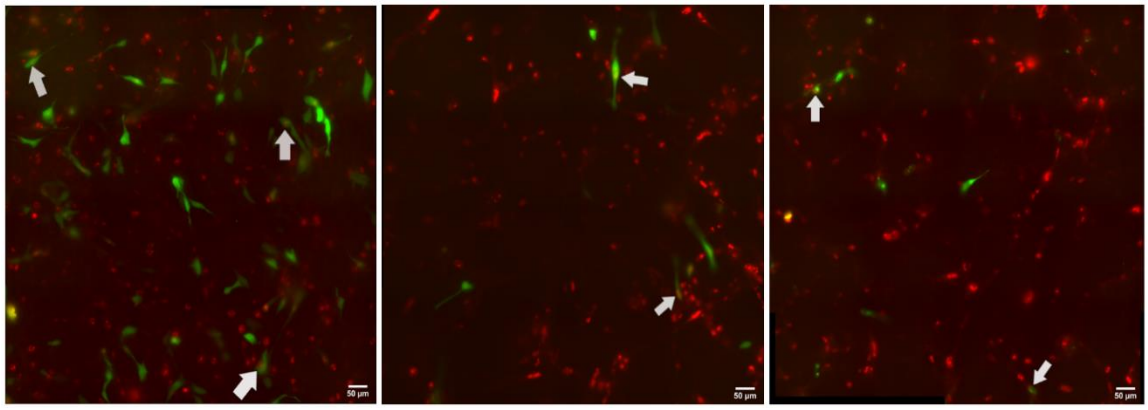

Figure 9. Vero Cells Co-infected With 10 µg of Each Amplicon Vector DNA and 10 µl of OK-14 by Fluorescent Microscopy. HSV-C-EmGFP (left) and EmGFP-HSV-N (middle) and EmGFP-Rab6 (right) amplicon vector stocks show high numbers of co-infected Vero cells. Image was taken by fluorescent microscopy at 7-8 h.p.i.

Using fluorescent microscopy, Vero cells were imaged 8 h.p.i. to verify the number of cells co-infected with both amplicon vector and helper virus. HSV-C-EmGFP amplicon vector stock resulted in 30 co-infected cells out of 50 EmGFP expressing Vero cells (Figure 9, left). EmGFP-HSV-N amplicon vector stock showed 9 co-infected cells out of 10 EmGFP expressing Vero cells (Figure 9, middle). EmGFP-Rab6 showed 5 co-infected cells out of 14 EmGFP expressing Vero cells (Figure 9, right).

Altogether, these data demonstrate that amplicon vectors can be successfully replicated and packaged by a replication-competent HSV-1 helper virus, to produce amplicon vector stocks capable of efficiently co-transducing/infecting cells. These results are semi-quantitative, additionally, there are enough double-positive co-transduced/infected cells to easily find them for subsequent microscopy analysis. We further demonstrate that these amplicon vectors can be used to study the intracellular trafficking and exocytosis of HSV-1 virus particles in infected cells by TIRF microscopy.

DISCUSSION

The advantages provided by HSV-1 amplicon vectors to carry up to 150 kb of foreign DNA into various mammalian cell lines makes them an ideal gene-delivery vehicle. Additionally, using amplicon vectors that contain Invitrogen Gateway cloning system will allow for a quick and efficient way for insertion of any genes of our choosing. The HSV-1 amplicon vector stocks constructed for this project can efficiently transduce DNA into host cells with the aid of a helper virus. Importantly, this helper virus can be the very same replication-competent virus we use for subsequent experiments in infected cells. Thus, we have constructed a molecular tool to study host proteins that mediate in HSV-1 trafficking and egress, and demonstrate their interaction with HSV-1 viral particles.

After multiple failed transfections, 3-3 Vero cells were too inefficient for successfully packaging the amplicons. It is possible that ICP-27 overexpression in 3-3 Vero cells could be affecting the cells in some way that renders them less transfectable. Consequently, we moved forward by using HEK293 cells, as this cell line demonstrated much greater transfection efficiency. The replication-defective helper virus 5dl1.2 was also replaced, as it can only competently replicate in the complementing 3-3 Vero cells. The HSV-UL25-mScarlet-I helper virus showed unexpected localization of the UL25-mScarlet-I fusion protein in the cytoplasm. The host cell nucleus expressed a higher amount of red fluorescence than the rest of the cell, but unlike OK-14, we were unable to distinguish individual viral particles against the high cytosolic background. In addition, laser illumination, as used for TIRF microscopy, caused the mScarlet-I fluorophore to photobleach quickly. Therefore, we focused on using OK-14 as the helper virus in subsequent experiments. However, Vero cells were able to be co-transduced/infected with amplicon and HSV-UL25-mScarlet-I, confirming their capacity to be used as a helper virus. The HSV-UL25-

mScarlet-I virus requires further analysis to determine if this is an appropriate helper virus for these amplicon vectors.

The HSV-1 amplicon vector expressing EmGFP-Rab6a replicated and packaged using OK-14 demonstrated the highest amount of co-infected Vero cells throughout the dish. Using TIRF microscopy, the Vero cells infected with vector stock produced from 3 µg EmGFP-Rab6 plasmid and 10 µl OK-14 (1 mL of amplified stock) illustrated Rab6 proteins aiding in the exocytosis of HSV-1 viral particles. The EmGFP-Rab6 amplicon vector showed Rab6-positive secretory vesicles traveling, most likely by kinesin motor proteins, from the Golgi apparatus to the corners of the Vero cells. Exocytosis events of HSV-1 particles with EmGFP-Rab6 could be seen by TIRF microscopy. Virus particles labeled with a red fluorescent capsid protein co-localizing with the green EmGFP-Rab6 exhibits a yellow color, as the virus particle traffics inside the cell. Once the virus particle exits the cell by exocytosis, the red virus particle remains on the cell surface, outside of the plasma membrane. These exocytosis events show the importance of Rab6 in facilitating HSV-1 egress from infected cells.

Fluorescence microscopy of Vero cells confirmed the number of cells co-transduced/infected with both amplicon vector and helper virus. HSV-C-EmGFP amplicon vector stock demonstrated the highest number of cells with 30 co-infected cells out of 50 Vero cells expressing EmGFP. EmGFP-HSV-N amplicon vector stock showed up to 9 co-infected cells out of 10, and EmGFP-Rab6 showed 5 co-infected cells out of 14. Although there is variability in the number of cells expressing EmGFP and the amount of co-infected cells, the results show the amplification of the amplicon vectors with helper virus OK-14 works best when they are produced using higher DNA concentrations.

Expression of EmGFP in both HSV-C-EmGFP and EmGFP-HSV-N amplicon vector stocks, after amplification with OK-14, verified the constructs are stable. The

parental HSV-C-EmGFP and EmGFP-HSV-N amplicon vectors are ready to be used to insert additional desired genes of interest using Invitrogen Gateway cloning. The use of these amplicon vectors to study other important proteins will provide further insight into the molecular and cell biology of HSV-1 infection for potential future use as gene therapeutics.

FUTURE DIRECTIONS

This project still has some outstanding questions and areas for improvement. The HSV-1 amplicon vectors will be used in the future to express other genes of interest using the Invitrogen Gateway cloning system. Furthermore, the HSV-1 amplicon vector with the Rab6a insert will continue to provide insight into how Rab GTPases play a role in HSV-1 egress. Although the VAMP2-EmGFP amplicon construct was incorrect, VAMP2 is an important SNARE protein that should be further studied. The VAMP2-EmGFP amplicon will be reconstructed at DNASU Plasmid Repository, and this revised construct may produce better results than the ones used in these experiments. The HSV-UL25-mScarlet-I helper virus will be further tested to determine its suitability in these experiments. More data will be collected to quantify the co-transduction/infection of mammalian cells, using these amplicon stocks.

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