Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Francine Scott Date

A Tissue Culture Model of Murine Gammaherpesvirus Replication Reveals Roles for the Viral Cyclin in Both Virus Replication and Egress.

By
Francine Scott

Doctor of Philosophy

Emory University, Microbiology and Molecular Genetics

_____ [Advisor's signature]
Dr. Sam Speck
Advisor

_____ [Member's signature]
Dr. Dan Kalman
Committee Member

_____ [Member's signature]
Dr. Dave Steinhauer
Committee Member

_____ [Member's signature]
Dr. Arash Grakoui
Committee Member

_____ [Member's signature]
Dr. Marty Moore
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

A Tissue Culture Model of Murine Gammaherpesvirus Replication Reveals Roles for the Viral Cyclin in Virus Replication and Egress.

By

Francine Scott

B.S., California State University 2005

Advisor: Samuel H. Speck, PhD

An abstract of
A dissertation submitted to the James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Microbiology and Molecular Genetics
2014

Abstract

A Tissue Culture Model of Murine Gammaherpesvirus Replication Reveals Roles for the Viral Cyclin in Virus Replication and Egress.

By: Francine Scott

The *Herpesviridae* are a family of clinically relevant viruses that are unique in that they can remain latent in their host cells and may reactivate to cause disease. Gammaherpesviruses, in particular, are often associated with oncogenic conditions that are influenced by the state of the host cell cycle. Passage through the eukaryotic cellular cycle is regulated by cyclins and cyclin dependant kinases (CDKs). Kaposi's sarcoma-associated herpesvirus (KSHV) and murine gammaherpesvirus 68 (MHV68) are two gammaherpesviruses that bring with them a virally encoded homologue to eukaryotic cyclin D (k-cyclin and m-cyclin). In MHV68, m-cyclin can interact with host CDK2 and CDK1/CDC2 this activity can influence lytic replication in the lungs. In-vitro attempts to study this defect have been limited by the lack of available tissue culture models that mimic the growth defect. It is hypothesized that the study of MHV68 replication in a polarized tissue culture cell line would provide a suitable environment to characterize the role of the v-cyclin in virus replication. We report here MHV68 replication in the rat lung cell line RL-65, a spontaneously immortalized, non-transformed polarizable epithelial cell line. These analyses mirror the in-vivo findings that m-cyclin is important in acute virus replication, as well shed new light on its influence on virus egress from infected cells. We also show that the KSHV v-cyclin (k-cyclin), which can functionally interact with different CDK partners (CDK4 and CDK6) than the MHV68 v-cyclin (CDK2 and CDC2), can partially rescue the replication defect observed with a v-cyclin null mutant. In summary, the RL-65 cell line provides an attractive in vitro model that mimics critical aspects of MHV68 replication in the lungs.

A Tissue Culture Model of Murine Gammaherpesvirus Replication Reveals Roles for the Viral Cyclin in Virus Replication and Egress.

By

Francine Scott

B.S., California State University 2005

Advisor: Samuel H. Speck, PhD

A dissertation submitted to the James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Microbiology and Molecular Genetics
2014

ACKNOWLEDGEMENTS

I would like to extend my gracious thanks and appreciation to several individuals and departments at Emory University for their support during my graduate career. Firstly, I would like to thank the Emory Laney Graduate School and the Department of Microbiology and Molecular Genetics for giving me this opportunity to pursue a graduate degree. A special thank you goes out to Dr. Sam Speck as well as the entire Speck lab. Over the past several years they have provided kind support and encouragement. Also, members of my thesis committee deserve acknowledgement: Dr. Arash Grakoui, Dr. Dan Kalman, Dr. Martin Moore, and Dr. Dave Steinhauer, for their thoughtful suggestions along the way. Finally, my family and friends deserve noted thanks as well for their support and encouragement.

Table of Contents

List of Figures

CHAPTER 1–INTRODUCTION

1.1 Herpesviridae-Overview

1.1 a) Morphology and Biological Properties

The word "herpes" is Greek in origin and means "to creep or crawl"; it was first used by Hippocrates to describe skin lesions that seemed to spread across the skin. Centuries later, Burnet and Williams noted that herpes simplex virus (HSV) remains latent and infections persist for life [1,2]. Viral latency has since become a trademark characteristic of herpesvirus infections, but it is not the only feature that herpesviruses share. The *Herpesviridae* are a collection of 120-250 kbp double stranded DNA viruses each containing characteristic architecture and biological properties. In a typical virion; DNA is toroid in form and can be suspended by fibrous material anchored to the capsid. The icosahedral symmetry of the capsid is comprised of 162 capsomers with a triangulation number of 16 [1-3]. A proteinaceous tegument encases the capsid and may be variable in its thickness, while the outermost lipoprotein envelope is fragile and studded with glycoproteins [1]. In its mature form, the virion can range in size from 120-260nm [1,3]. The biological features that all herpesviruses share are: a) virally encoded enzymes for DNA synthesis, protein synthesis, and protein processing; b) nuclear synthesized viral DNA and capsid assembly; c) cellular destruction upon infectious virus generation; and d) the ability to undergo latency followed by periodic episodes of reactivation and replication [1]. Additionally, their genomes can encode 20-200 genes and among these are 40 genes that are "herpesvirus core genes". These core genes are conserved across all subfamilies and can include gene products for virion structure and virus replication in culture [1,4,5]. Throughout the natural world, herpesviruses are highly disseminated with vertebrates constituting the predominant host type. There is, however, an instance of an invertebrate species hosting herpesviruses: the oyster [6].

Herpesviridae are divided into three subfamilies; the alpha, beta, and gammaherpesviruses which each contain their own additional unique properties and associated diseases.

1.1 b) Alphaherpesvirinae

The *Alphaherpesvirinae* include viruses such as herpes simplex virus (HSV) and varicella-zoster virus (VZV). As a group, alphaherpesviruses have a broad host range and cause an abundant degree of cellular destruction upon infection. HSV, the prototypic alphaherpesvirus, induces severe pathological changes to the host. These include: ballooning of the cell, condensation of the chromatin, fragmenting of the golgi, and rearrangement of the cytoskeleton [1]. Alphaherpesviruses also maintain the distinguishing ability to establish latency primarily in sensory nerve ganglia. To establish latency, the alphaherpesvirus must first fuse with the neural axon and retrograde transport must then carry the nucleocapsid to the nucleus where it will persist as a circular episome. Upon a reactivating stimulus, anterograde transport shuttles the virus back to the axonal termini where it can shed and infect surrounding tissue [1,7].

1.1 c) Betaherpesvirinae

The Betaherpesvirinae include viruses such as human cytomegalovirus (CMV) and roseolavirus. Betaherpesviruses have a fairly narrow host range with susceptibility being limited to cells belonging to their origin host species [1]. They characteristically remain cell associated and contribute to cytomegaly (enlarged cells) in their host. Latency occurs in many cell types includeing secretory and lymphatic tissue or kidney cells. Additionally, in myeoloid in vitro models, the differentiated state of the cell appears to be an important factor regulating reactivation events [8].

1.2 Gammaherpesvirinae

1.2 a) Taxonomy

The herpesvirus subfamily Gammaherpesvirinae are further divided into two subdivisions based on genomic sequence: the lymphocryptoviruses (γ1) or the rhadinoviruses (γ2). Included in the γ1 group are Epstein-Barr virus (EBV) and rhesus lymphocryptovirus (rhLCV). While the γ2 group consists of Kaposi's sarcoma associated herpesvirus (KSHV or formally, human herpesvirus 8), herpesvirus saimiri (HVS), and murine gammaherpesvirus 68 (MHV68). Recent work has suggested that two new species, the macaviruses and percaviruses, should be added to the γ2 genus [1,9].

1.2 b) Virus Lytic Replication

(Many replication events described in this section are not unique to gammaherpesviruses, where applicable- unique gammaherpesvirus characteristics are noted.)

A gammaherpesvirus virion must first bind to a host receptor before entry and replication can take place. For EBV, its glycoprotein 350/220 is important for binding to the CD21 ligand on B lymphocytes [10]. The abundant levels of CD21 ligand on B lymphocytes combined with the high affinity of gp350/220 towards CD21 makes this interaction an important contributor to EBV's host range restriction [11,12]. For KSHV host binding, its glycoprotein B contains an integrin binding domain that interacts with α3β1 integrins on host surfaces, though this interaction is not fully sufficient for entry [13]. It has recently been proposed that xCT (cystine/glutamte transporter) may be another possible receptor that KSHV may use [14]. After

attachment to the host, a fusion event occurs in which the virion fuses with the plasma membrane and its capsid is released into the cytoplasm. For EBV, its capsids are then transported to the nucleus via the endocytic pathway [15]. Once at the nuclear pore, the virus DNA enters the nucleus where gene transcription occurs in a specific ordered fashion. Immediate-early genes are transcribed first and include regulatory genes and those that are important for creating a suitable host environment for virus replication. BZLF1 is one of EBV's immediate early genes that can transactivate other gene promoters and may influence activities of host NF-κB or p53 activities [1]. RTA (ORF50) is a KSHV imediate-early gene and may influence host STAT3 transcription and regulation subsequent viral gene products [1,16]. Early genes are the next to be transcribed and prepare the cell for virus DNA replication. The viral DNA polymerases BALF5(EBV) or Pol8 (KSHV) function much like their host cellular counterparts but may contain additional unique attributes such as the ability to withstand high amounts of salt [1]. Following DNA replication into concatemeric form, late gene transcription ensues and often these late genes encode for products necessary for structural elements of the virion such as glycoproteins or tegument proteins. Mature nucleocapsids traverse through the golgi in order for the viral envelope to mature and then the virus can egress through exocytosis [3]. Virion egress will be examined with greater detail in subsequent sections.

1.2 c) Virus Latency

(Many latency events described in this section are not unique to gammaherpesviruses, where applicable unique gammaherpesvirus characteristics are noted.)

During latency the gammaherpesviruses maintain the ability to exist as circular molecules with only a small portion of their viral genes being expressed. This latency program is also the

most abundant transcription pathway in EBV and KSHV in vitro infections [1]. Latency genes that are expressed during this time period serve many functions and can have lasting effects on the host. EBV's EBNA-2 can transactivate other EBV latency genes such as LMP1 or LMP2A or even host cell genes such as CD21 or c-myc [1]. LMP1 acts as a tumor necrosis factor (TNF) receptor and may also function as an oncogene under certain conditions [17,18]. Additionally, B cell differentiation can be influenced by LMP2A [1]. In KSHV latency, expression of its latency associated nuclear antigen (LANA) gene is important for maintaining the viral episome, segregation of the viral episome in daughter cells, and binding to host tumor suppressor Rb [1,19]. Host inflammatory pathways, such as that for NF-κB, may also be modulated in reponse to KSHV latent gene product Kaposin B. Currently, the detailed mechanisms that govern the gammaherpesvirus switch from latency to lytic replication are still being worked out. From what has been determined so far, BZLF1 (EBV) and RTA (KSHV) induction is critical for these gammaherpesviruses to undergo reactivation from latency [20,21].

1.2 d) Associated Diseases in Humans

In individuals with a healthy immune system, infection with a gammaherpesvirus is rarely a cause for concern. Across the globe >90% of humans have been infected with EBV by the age of 40 with many of the initial infections occurring before 3 years of age [22]. Rarely, about 25% of the time, primary infections with EBV that occur in adolescence or later can result in infectious mononucleosis (IM) [23]. IM manifests as a period of fever, pharyngitis, malaise, and CD8 T cell lymphocytosis [1,24]. The abundance of proinflammatory cytokines (TNF-α, IFN-γ, IL-1) produced by those T cells is believed to be responsible for influencing symptomatic progression [25]. For persons with compromised immune systems, such as transplant recipients

or AIDS patients, the outcome of an EBV infection can be much more severe. As much as 50% of AIDS associated lymphomas are attributed to an association with EBV [26]. These lymphomas most often appear in later stages of AIDS when the immune system is particularly compromised. Tumor examination from these patients often reveals positive identification of EBV genes such as EBNA-2 or LMP1 [27]. KSHV infections are similarly found all over the world but infection patterns largely vary based on geographic location. In North America, Northern Europe, and Asia the prevalence is less than 5%; while in Africa or parts of the Amazon in can be greater than 50% [1]. As with EBV, in healthy persons an infection with KSHV rarely causes severe pathology. However, immune compromise leads to increased risk of KSHV associated diseases. Kaposi's sarcoma (KS) is the most well documented of these conditions. This disease is characterized by tumors that are largely localized to the skin and are purple-blue or red-brown in color [28]. These lesions are driven by KSHV infected endothelial cells that present with a distinct spindle shape [29]. KSHV infection alters the endothelial program of these cells and can cause up regulation of angeogenesis [30]. The viral g-protein coupled receptor (vGPCR) has been shown to upregulate host vascular endothelial growth factor (VEGF) implying this viral gene product may be critical for tumor proliferation new blood vessel formation [31,32]. Patients with AIDS have increased KS risk compared to the general population but the exact mechanism in which AIDS influences KS is still under investigation. Although diseases associated with gammaherpesvirus infections are often influenced by the compromised immune status of the host, there are some instances where other factors may contribute to disease outcome. In sub-Saharan Africa, the most common cause of childhood cancer is Burkitt's lymphoma (BL). This particular type of lymphoma presents at unusual anatomical sites, such as the jaw, and the lesions contain numerous macrophage cells which give a histological "starry sky" appearance [1]. The EBV viral genome is present in all of the associated tumor cells and the strong geographical location of sub-Saharan Africa within the malaria belt suggests that malarial

co-infection influences Burkitt's lymphoma incidence [33,34]. In southeast Asia, the incidence of Nasopharyngeal Carcinoma (NPC) is up to 100X greater than in North America or Europe [1]. Characteristically, tumors from these patients show high levels of the EBV genome; suggesting a viral association with the disease [35] . Additionally, other environmental factors such as exposure to certain carcinogens and a diet rich in salted fish may also contribute to the risk of this disease [1].

1.2 e) Animal Models

Humans are not the only primates susceptible to infection by gammaherpesviruses. Herpesvirus saimiri (HVS) was extracted from squirrel monkeys in the 1970s [36]. This virus establishes a persistent infection in most of these monkeys but does not induce disease. Additionally, the prevalence of HVS antibodies in squirrel monkeys indicates this virus is indigenous to these animals in the same fashion that has been observed for EBV antibody distribution in humans [37] . In New World monkeys, on the other hand, infection with HVS causes a fatal T cell lymphoma [37]. Analysis of tumor samples indicates that the HVS viral genome can be recovered from them, which is also true of EBV recovery from BL and NPC tumors [37] . Finally EBV, KSHV, and HVS all share many blocks of conserved genes as well as conserved gene functions. Each of these viruses contain viral gene products that can modulate either B or T cell signal transduction or function as chemokine homologues in the host [38] .

A natural pathogen of murid rodents, murine gammaherpesvirus 68 (MHV68), was first isolated from bank voles in the 1980s [39]. Since then, its genomic structure and sequence analysis of its genome have placed it the herpesvirus subfamily gammaherpesviridae [40-42]. MHV68 also shares many blocks of conserved genes with EBV, KSHV, and HVS. With KSHV & HVS being more closely related to MHV68 than EBV [42]. In addition to conserved genes,

MHV68 does contain genetic information that is not conserved in the other gammaherpesviruses. These virus specific genes are located interspersed between the conserved genetic blocks and encode information important for virus latency and modulation of host immune function [40-42]. MHV68 can readily infect in-bred strains of mice which is one important aspect that makes this animal model an attractive option for in-vivo studies. At this time it is still not known what route constitutes the natural spread of this virus, but the respiratory route has been speculated to have some involvement [43]. Acute infection via the intranasal route is characterized by productive infection in lung alveolar and mononuclear cells [44]. This infection is largely cleared by day 9-12 post infection. Continued spread to distal organs relies on the presence of B cells, though only after intranasal inoculation and not inoculation through other routes [45]. The spleen is the major anatomical site of MHV68 latency with analysis of latently infected splenocytes routinely occurring at day 16-18 post infection. In terms of specific cell type: B cells, macrophages, and dendritic cells (the professional antigen presenting cells) are the main cell types of MHV68 latency [46].

In immune competent mice, infection with MHV68 leads to a chronic infection that persists for the lifetime of the animal. Acute infection causes an interstitial pneumonia and splenomegaly upon dissemination of the virus [44] . This particular spleomegaly is characterized by an increase in $CD4^+$ T cells and $CD8^+$ T cells which is very much reminiscent of EBV induced infectious mononucleosis in humans [47] . Chronic infection with MHV68 is also associated with lymphoproliferative disease healthy mice [48]. In another aspect similar to the human gammaherpesvirus conditions, mice with various immune deficits can have a more drastic pathological outcome upon infection with MHV68. In mice immunosuppressed by the administration of cyclosporine A, MHV68 infection is associated with the increased incidence of lymphoproliferation [48]. One example of how immune function is important for controlling infection can be seen when examining the interferon pathway. In interferon gamma receptor

knock-out mice, infection with MHV68 induces severe multi-organ fibrosis [49]. Also, in mice lacking the interferon αβ receptor, the animals have a hard time controlling infection and up to 50% of them succumb to infection at low dose [50].

The ease by which MHV68 can grow to high titers in fibroblast cell lines combined with the availability of a bacterial artificial chromosome (BAC) containing its genome have strengthened the ability to utilize this virus as a model for learning more about the human gammaherpesviruses [51]. The genetic manipulation of the MHV68 BAC in the creation of virus mutants has shed light on the function of various MHV68 genes in viral pathogenesis. The study of MHV68 LANA is one example. MHV68 contains a homologue to KSHV LANA and this gene product is shorter than its KSHV homologue but shares a similar C-terminal domain [45] . The generation of MHV68 LANA null viruses and the analysis of their infection in mice compared to WT MHV68 showed that this viral product is important for establishment and reactivation from latency in splenocytes [52-54]. Additionally, recent advances in molecular cloning techniques have allowed for the insertion of the yellow fluorescent protein (YFP) into the MHV68 genome. The creation of this transgenic virus allows for the expression of the YFP reporter gene in infected cells increasing the efficiency at which virus infected cells can be visually identified and analyzed in flow cytometry [55,56].

1.3 Eukaryotic Cell Cycle & Cyclins

1.3 a) Cell cycle, Cyclin, and CDK Overview

The eukaryotic cell cycle is a tightly regulated series of events in which DNA is replicated and distributed to daughter cells. In quiescent cells, or G_0, transcription of genes, such as quiescins, prevent the timely entrance into the cell cycle [57-59]. Upon mitogenic stimulation, the Ras-Raf-MEK-ERK pathways are activated and cyclin D is upregulated [57,60]. Cyclins are the regulatory subunits of the heterodimeric Cyclin:CDK complexes that control passage through the cell cycle [57]. CDKs constitute the catalytic portion of that complex and must be associated with a cyclin for this catalysis activity [57]. Cyclin D interacts with CDKs 4 and 6 to facilitate entry and progression through G_1 phase. This phase is marked by Cyclin D: CDK 4/6 phosphorylation of Rb and transcription of cyclin E. Cyclin E interacts with CDK2 to further hyperphosphorylate Rb and E2f release which pushes transcription of cyclin A and entry into S phase [57,59] . During the S phase, or DNA synthesis, cyclin A interacts with CDK 2 to promote chromosome replication [61]. Also promoted by cyclin A:CDK2 is entry into G_2 phase, or the early events of mitosis, which include cyclin B:CDK1/CDC2 activity and eventual full passage to mitosis [57].

1.3 b) Cell cycle CKIs

Cyclin:CDK activity is crucial to the ordered cascade of the cell cycle. The eukaryotic cell has employed CDK inhibitors (CKIs) as one way to uphold regularity in the cell cycle events. The INK4 and Cip/Kip are two classes of CKIs. INK4 CKIs have ankyrin repeats that are crucial for restraining CDK4/6 complexes from cylin D [62,63]. The resulting inhibition of the mid-G1

phase cyclin:CDK complexes results in decreased phosphorylation of Rb, and decreased transcriptional activity of E2F [57]. The Cip/Kip class of CKIs contain amino terminal residues that enable them to suppress CDKs who complex with cyclins D,E, and A [63,64]. Their specific binding to CDKs 4 or 6 antagonizes the assembly of the cyclin:CDK complex, and therefore must be degraded before DNA replication ensues [63].

1.3 c) Cyclin and CDK Homologies

There are four different classes of mammalian cyclins: A, B, D, and E with the distinction between classes based on specific behavior during the cell cycle [57,65]. At the C terminal end of all cyclins exists a region of approximatly 100 amino acids termed the "cyclin box" [66]. Within this region are highly conserved residues important for binding to and activation of cyclin dependant kinases [65]. Additionally, all cyclins retain a similar tertiary structure described as the "cyclin fold" [65]. As described previously, each stage of the cell cycle is associated with the activity of a different cyclin. Because these different cyclins share many important homologies the significance of any one particular cyclin in a given cell cycle stage has been debated. Recently, the availability of knockout mice has allowed for the evaluation of compensatory roles amongst the cyclins. There are three D-type cyclins and in mice that cannot make any cyclin D, cellular division can still occur with survival occuring until midgestation [67]. This ability to continue to divide without cyclin D was determined to be based on cyclin E and its ability to compensate in cyclin D's absence [68]. On the other hand, there are two types of cyclin E, and knock-out of both types results in mice cannot survive beyond embryogenesis [69]. Thus, cyclin E can compensate for cyclin D but not vice versa. With cyclin A there are also two types, and only A2 is essential for development beyond the embryonic stage with none of the other cyclins

being able to compensate for its function [70,71]. Of the three B type cyclins, mice knock-outs for B1 lead to embryonic lethality but B1 can compensate for loss of B2 as B2 knock-out mice develop normally [72]. In terms of CDKs, there are currently 11 different mammalian CDKs [73]. All CDKs contain the following conserved properties: the PSTAIRE α1 helix that will go through a reorientation upon cyclin binding exposing residues that interact with ATP; the L12 helix that will change to a beta strand upon cyclin binding; and the T loop where the activating phosphorylation site is found [65]. This abundance of shared properties amongst the CDKs has also prompted the evaluation of compensatory abilities of many cell cycle CDKs. Mice that cannot make CDK4/6 exhibit extensive anemia and embryonic lethality, however in single knockouts CDK4 can compensate for CDK6 or vice versa [74,75]. CDK2 knock-out mice exhibit viability due to compensatory mechanisms of CDK1/CDC2 [75]. CDK1/CDC2 deletion in mice exerts an embryonic lethality that cannot be compensated by any other CDK [75,76]. Even with these recent insights many questions still remain as to the specific mechanism(s) of compensation in cyclins and CDKs.

1.4 Virus Modulation of Cell Cycle

1.4 a) Virus Modulation of the Cell Cycle-Overview

Viruses rely on their host to provide an environment fit to replicate their genomes and produce progeny virions. Many viruses have exploited the eukaryotic cell cycle in this process, and as such, elicit complex cellular consequences during infection. Human T cell leukemia virus contains a TAX protein that can bind to p16 (INK4 CKI) preventing its ability to induce inhibition on cyclin D:CDK complexes [77] . Human immunodeficiency virus (HIV) has a Vpr protein that arrests the cell at the G_2/M phase by preventing cyclinB:CDK1/CDC2 activities [78]. Human pappilloma virus (HPV) encodes a protein (E7) that can de-stablize Rb and interfers with p27's (Cip/Kip CKI) cyclin E:CDK2 inhibition leading to bypassing of G_0 arrest [79]. Herpesviruses can also modulate the host cell cycle. The human cytomegalovirus (HCMV) encodes a protein (IE86) which interacts with sequences in the cyclinE promoter. Another HCMV protein (IE72) activates an E2F dependent promoter and in early CMV infection there is an increase in cyclinE/CDK2 activity [80-82]. Additionally, many of the gammaherpesviruses modulate cyclin D as described below.

1.4 b) Gammaherpesvirus Modulation of the Cell Cycle

HVS, KSHV, and MHV68 each possess a virally encoded homologue to eukaryotic cyclin D. These virally encoded cyclins are approximately 25-31% identical to mammalian cyclin D and 26-32% identical to each other [42,83]. The area of highest homology amongst them is in the cyclin box [42] . Despite these similarities, the viral cyclins do display different CDK association properties. HVS cyclin, the first identified gammaherpes viral cyclin, associates

with CDK6 [84]. KSHVs' cyclin, k-cyclin, associates with CDK4/6 primarily, but has also been shown to interact with CDK 2, 5, and 9 [85-87]. MHV68s' viral cyclin, m-cyclin, interacts with CDK2 and CDK1/CDC2 [88]. Cellular CKIs, which would normally inhibit cyclin D activities, do not hinder these viral cyclins. K-cyclin is not held back by INK4 and Cip/Kip, and m-cyclin phosphorylates Kip [88,89] . All three viral cyclins facilitate phosphorylation of Rb, an action crucial for cell cycle progression [84,86,88]. Additionally, the viral cyclin:CDK complexes possess a broad substrate arsenal including Orc1 (K-cyclin), Bcl2 (K-cyclin & M-cyclin), p53 (K-cyclin & M-cyclin), and histone H1 (K-cyclin &HVS cyclin) [85-88,90,91]. EBV does not encode a cyclin D homologue but its' EBNA3C protein enhances the function of cellular cyclin D and promotes passage through G_1-S [92] .

1.5 MHV68-Viral cyclin

1.5 a) MHV68-cyclin Expression

The MHV68 genome is approximately 118kb of unique sequence flanked by terminal repeats [42]. The m-cyclin (ORF 72) gene is located near the right end of the viral genome within a block of gammaherpesvirus conserved genes containing ORFs 72-75 [42]. The m-cyclin gene was determined to be a leaky-late gene based on expression elimination after protein synthesis blockage and its continued expression after viral DNA synthesis ablation at specific time points [83]. The promoter region of m-cyclin is Rta (MHV68 major viral lytic transactivator) responsive due to a cis-element in its promoter region [93]. Thus, m-cyclin is downstream of Rta cascade during lytic infection. M-cyclin transcripts have been recovered from both lytically as well as latently infected cells [94,95]. These transcripts may have alternatively spliced forms and can include elements of MHV68 LANA [96]. The details about the expression pattern and importance of these alternatively spliced transcripts during MHV68 infection remain to be elucidated.

1.5 b) MHV68-cyclin Biochemical Properties

The m-cyclin protein contains 252 amino acids (Genbank U97553) and crystallization studies have revealed many unique aspects of its interaction with CDK2. The CDK2 binding site is contained within the confines of the m-cyclin cyclin-box, however, these sites confer different residue interaction than the eukaryotic cyclin A [97]. Salt bridge formation by the m-cyclin residues E133 and K104 are particularly critical for m-cyclins bond to CDK2 [97]. Other m-cyclin unique residues and interfaces underlie m-cyclins $p27^{KIP}$ resistance. Acidic residues in

eukaryotic cyclin A that are important for $p27^{KIP}$ binding are uncharged in the m-cyclin equivalent residues [97]. Unique m-cyclin structural properties, such as its H4-H5 loop, may also prevent $p27^{KIP}$ interactions [97].

1.5 c) MHV68-cyclin During Infection

M-cyclins' importance was first identified in its function as an oncogene. It is sufficient to drive cell cycle progression in T lymphocytes and induce the development of lymphoma in M-cyclin-T-G mice [83]. Reactivation from latency is another parameter of infection where m-cyclin contributes sufficient influence on virus behavior. Without m-cyclin, MHV68 is not hindered it its ablilty to establish latency but it cannot reactivate from latency to WT levels from both splenocytes and peritoneal exudates cells (PECs) [98]. Viral mutants that harbor point mutations in the cyclin box of m-cyclin have revealed that the m-cyclin CDK interaction is not required for the ability to support WT levels of reactivation from latently infected PECs and to some level from latently infected splenocytes [99]. At this time it is unclear what these CDK independent functions of the m-cyclin are. Interestingly, the m-cyclin partnership with CDK2 and CDK1/CDC2 is critical for acute replication in the lungs at low dose [99]. Additionally, recent studies have revealed that endothelial cells can support persistent MHV68 infection and MHV68 infection can drive immortalization of fetal liver progenitor B cells with both of these phenomenon requiring m-cyclin [100,101]. Other attempts to study the m-cyclin's influence on acute replication in-vitro have been unsuccessful. The in-vivo phenotype of acute replication defects at low dose without m-cyclin have failed to be recapitulated in multiple cell types analyzed [99]. One hypothesis has been that the differences in epithelial state between conventional cell lines and that of host lungs is important for revealing m-cyclins function [99].

1.6 Epithelial Tissue in the Lungs

The epithelium lining the lungs serves not only as a medium for gas exchange but as a barrier protecting the host from the outside environment. This lining is often only a single cell thick and its cells are polarized with distinct apical and basolateral domains [102]. Facing the lumen is the apical domain which functions as the interface between organism and environment. The basolateral domain is comprised of the surface of the cell that adjoins the underlying basement membrane as well as cellular neighbors [102-104]. Separating these two surfaces are a variety of junction proteins that form strong connections between neighboring cells. E-cadherin and Zona Occludens are two such proteins that are critical for stability of cellular junctions [105,106]. E-cadherin is a major component of epithelial adherans junctions and functions to strengthen contacts with neighboring cells as well as actin cytoskeleton organization [107]. Zona Occludens is a tight junction protein that also provides structure to the cytoskeletal framework and works to control paracellular passage of ions [108]. This polarization of epithelium can influence viral behavior.

1.7 Viral Egress and the Role of Epithelium

1.7 a) Virus egress

Upon assembly of mature virions, a virus must then leave its host cell. This process generally occurs via extracellular diffusion and/or through contact mediated movement across neighboring cells [109]. Though cell-free virus spread has the characteristic advantage of allowing for the passage of virus across large distances and/or hosts there are often many barriers to its efficiency such as virus stability or concentration of target cells and their receptors. Cell-cell movement of virus, on the other hand, is highly efficient [109]. Many viruses have evolved unique mechanisms of egress that contribute to the efficiency of spread to its neighbors. HIV virus can bud from infected cells at the site of pseudopod (microfilament projection from the cell membrane) formation [110,111]. This is facilitated by HIV Gag and Env protein products joining with cellular lipid rafts that settle at the pseudopods [112]. HSV virons assemble in compartments of the trans-Golgi network (TGN) that will sort to basolateral surfaces allowing the virions to egress to the intercellular space of neighboring cells [113]. Additionally, several other viruses actively remodel the host cytoskeleton during their egress. Vaccinia virus cell associated enveloped virions (CEV) utilize tyrosine kinases in the production of actin protrusions that extend to neighboring cells [114]. HCMV utilizes the cyclin/CDK pathway in disassembly of the host lamina network in virus egress [115].

1.7 b) Epithelial Role in Egress

Viruses generally egress from host epithelial tissues in a directional manner either apically or basolaterally; with typically the same preference for release shared within a virus

family [116]. Additionally, egress directionality can be an important contributor to pathogenesis. Apically released viruses may laterally infect neighboring cells without ever making the journey to the other side, whereas basolateral release exposes the virus to underlying tissues and provides the opportunity for dissemination throughout the host [116]. Interestingly, EBV has been described as egressing both apically and basolaterally. With apical shedding having implications of viral shed in saliva and basolateral shedding as a means of access to basement membrane and underlying lymphoid aggregates [117-119]. Additionally, in EBV infection of polarized tonsil epithelium, the virus can rapidly move via macropinocytosis mediated transcytosis from the apical to basolateral direction and also via caveolae from basolateral to apical direction in the absence of productive virus replication [119].

CHAPTER 2-A Tissue Culture Model of Murine Gammaherpesvirus Replication Reveals Roles for the Viral Cyclin in both Virus Replication and Egress from Infected Cells.

This chapter is a reproduction with minor modifications of a manuscript that was accepted by Public Library of Science (PLoS One) on March 11, 2014. The title of the manuscript is "A tissue culture model of murine gammaherpesvirus replication reveals roles for the viral cyclin in both virus replication and egress from infected cells." Permission will not need to be obtained from the publisher for reproduction because the journal is open access.

2.1 Abstract

Passage through the eukaryotic cell cycle is regulated by the activity of cyclins and their cyclin-dependent kinase partners. Rhadinoviruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV) and murine gammaherpesvirus 68 (MHV68), encode a viral homologue of mammalian D-type cyclins. In MHV68, the interaction of the viral cyclin with its CDK partners is important for acute replication in the lungs following low dose inoculation. Attempts to further study this requirement in vitro have been limited by the lack of available tissue culture models that mimic the growth defect observed in vivo. It is hypothesized that analysis of virus replication in a cell line that displays properties of primary airway epithelium, such as the ability to polarize, might provide a suitable environment to characterize the role of the v-cyclin in virus replication. We report here MHV68 replication in the rat lung cell line RL-65, a non-transformed polarizable epithelial cell line. These analyses reveal a role for the v-cyclin in both virus replication, as well as virus egress from infected cells. As observed for acute replication in vivo, efficient replication in RL-65 cells requires CDK binding. However, we show that the KSHV v-cyclin (K-cyclin), which utilizes different CDK partners (CDK4 and CDK6) than the MHV68 v-cyclin (CDK2 and CDC2), can partially rescue the replication defect observed with a v-cyclin null mutant – both in vitro and in vivo. Finally, we show that MHV68 is shed from both the apical and basolateral surfaces of polarized RL-65 cells. In summary, the RL-65 cell line provides an attractive in vitro model that mimics critical aspects of MHV68 replication in the lungs.

2.2 Introduction

The eukaryotic cell cycle is a tightly regulated and sequentially executed pathway by which DNA is duplicated and partitioned into daughter cells. Cyclins and their cyclin-dependant kinases (CDKs) form active complexes that facilitate this progression. Each phase of the cell cycle is associated with its own unique cyclin activity and control mechanisms. Low RNA and protein synthesis occurs in quiescent cells, and this state is referred to as G_0. It is upon mitogenic stimulation that many of these cells upregulate cyclin D which partners with CDK4 and/or 6 to drive entry into the G1 phase of the cell cycle. Cell growth, phosphorylation of retinoblastoma protein (pRb), cyclin E synthesis, and factors required for DNA synthesis and passage through the restriction point are all properties of the G1 phase [60]. Entry into the next phase of the cycle, S phase, is driven by cyclin E. Cyclin E interacts with CDK2 to drive E2F transcriptional activity which pushes the cell farther into the cycle and drives the production of yet another cyclin, cyclin A. Cyclin A also partners with CDK2 and will promote DNA replication and progression through S phase, as well as entry into the G2 phase of the cell cycle. The early events of mitosis occur during G2, which include cyclin B:CDK1 activity that facilitates full progression into mitosis. Eukaryotic cells also employ mechanisms to regulate cyclin:CDK activities. Catalytic subunit inhibition and cyclin:CDK assembly promotion by CKIs are just two examples of how the cell cycle can be kept in check [63].

Although the eukaryotic cell cycle is very tightly controlled, this has not stopped many viruses from exploiting various steps in this pathway to create a cellular environment conducive for their replication. Gammaherpesviruses, which include Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) and murine gammerherpesvirus 68 (MHV68), are just

one example of a virus subfamily that has evolved strategies for modulating the host cell cycle in their favor. For example, the rhadinovirus subgroup of the gammaherpesvirus family (e.g., KSHV and MHV68) encode a viral homologue of eukaryotic D type cyclins. These homologues all share conserved residues with D type cyclins, most prominently in the cyclin box – a domain that is critical for CDK binding [42]. The KSHV viral cyclin (K-cyclin) associates predominately with cellular CDK 6, but can also interact with CDKs 2, 4, 5, and 9 [85,120]. The MHV68 viral cyclin has been shown to preferentially interact with cellular CDK2 and CDC2/CDK1 [88]. Unlike their eukaryotic counterparts, both KSHV and MHV68 v-cyclins are resistant to the action of some CKIs and thus can evade normal host cell control [88,121]. Despite encoding these specific mechanisms to manipulate the host cell cycle, much of how this modulation of cyclinactivity is directly connected with viral pathogenesis remains to be elucidated. The species specificity of the human rhadinovirus KSHV makes in vivo studies challenging, thus making the rodent model utilizing MHV68 infection of laboratory strains of mice an attractive alternative.

MHV68 is a natural pathogen of murid rodents that has been shown to establish chronic infections in in-bred strains of laboratory mice. Acute MHV68 infection following intranasal inoculation is characterized by productive infection in the lung, spleen, and liver. MHV68 establishes latency in professional antigen presenting cells: B cells, macrophages, and dendritic cells [46,122]. Immune impairment of mice is correlated with various conditions after infection including fibrosis, vasulitis, or neurological disease [44,49,123-125]. In mice with healthy immune systems the primary pathology after MHV68 infection is interstitial pneumonia, which is largely cleared by days 9-12 post-infection [45].

The MHV68 viral cyclin homologue (v-cyclin) is an important regulator of reactivation from latency, replication in the lungs (at low dose) and, when expressed as a transgene, is a potent oncogene [83,98,99]. Generation of CDK binding mutants in the v-cyclin have shown that the

viral cyclin:CDK interaction is necessary for the virus to replicate to WT levels in the lungs after low dose. However, those same CDK binding mutants, in contrast to the v-cyclin null virus, are able to reactivate from latency to near wild type virus levels - indicating a CDK-independent function of the v-cyclin important for virus reactivation [99]. In interferon-γ deficient mice on a BALB/c background the v-cyclin critically contributes to acute lethal pneumonia and fibrosis of the lungs [126,127]. However, attempts to further study the importance of the MHV68 viral cyclin using tissue culture models have failed to reveal a role for v-cyclin [99]. One possible explanation for this disconnect between virus behavior in vivo and what is observed in vitro is postulated to be the differences in epithelial state between established cell lines and that of host lungs [99]. MHV68 infection via the intranasal route leads to viral engagement with only the surface, superficial epithelium, which is highly differentiated [128]. The process by which an epithelial cell becomes fully differentiated involves exit from the cell cycle, acquisition of epithelial specific molecular markers, and asymmetric separation of various cellular properties (polarization) [129,130]. Many conventional epithelial cell lines are derived from a transformed progenitor and do not polarize; thus they can continue to cycle and do not take on many of the polarization properties inherent of airway epithelium. Therefore, it seems likely that one or more of the properties unique to differentiated epithelium are critical for an environment in which the function of the MHV68 cyclin D homologue becomes most apparent. Here we report analyses of MHV68 replication, and the role of the viral cyclin, utilizing an epithelial cell line (RL-65) that exhibits many properties of airway epithelium - including the ability to form polarized monolayers on transwells [131,132].

2.3 Results and Discussion

MHV68 requires the viral cyclin for efficient replication in RL-65 epithelial cells

MHV68 requires the viral cyclin to replicate efficiently in the lungs of mice after low dose inoculation [99]. After screening a number of fibroblast and epithelial cell lines in which we failed to identify a significant replication defect of the v-cyclin null virus, we report here MHV68 growth in the rat lung cell line RL-65. RL-65 cells are a spontaneously immortalized, non-transformed epithelial cell line that was originally derived from neonatal rat lungs by careful manipulation of microenvironment to select for a cell type that maintained highly differentiated features in vitro [132]. They are propagated in media supplemented with growth factors rather than sera in order to more accurately mimic the surroundings the lung epithelium would be subjected to in vivo [132]. To address whether the RL-65 cell line would provide an environment in which the dependence on the v-cyclin in viral growth would be apparent, confluent monolayers of RL-65 epithelium were infected at an MOI of 0.05. The v-cyclin null virus (vCyclin.stop), which has been extensively characterized [98,99], harbors a translation stop codon near the amino-terminus of the v-cyclin open reading frame. After infection of the monolayer, cells and supernatants were harvested every 24 hours for a total of 144 hours for analysis of total virus growth (Figure 1). Notably, there was a significant defect in vCyclin.stop virus replication compared to WT MHV68 at early times post-infection (at 48 hrs post-infection there was a ca. 100-fold defect in virus replication). However, the vCyclin.stop virus was able to generate nearly equivalent virus titers as WT MHV68 by late time post-infection (Figure 1).

MHV68 is shed from both the apical and basolateral surfaces of polarized RL-65 cells

In polarized lung epithelium, the separation of the apical and basolateral surfaces by adhesive connections allows the tissue to form a distinct barrier between the outer surface and the

lumen. In tissue culture models, the strength of this boundary can be measured by trans-epithelial resistance (TER). It has previously been shown that the RL-65 cell line can form high resistance epithelial monolayers when grown on transwell filters [131]. To confirm this, $2x10^5$cells were plated onto transwells and transepithelial resistance (TER) was measured every 24 hours. As the monolayer continued to grow, TER readings steadily increased to levels typical for this cell type (Figure 2A) [131]. As a negative control, NIH 3T12 fibroblasts were plated on transwell filters and TER measurements assessed over the same timeframe (Figure 2A). As expected, little increase in resistance was observed. Junctions between polarized epithelial tissues are composed of specific adhesion protein complexes; adherans junctions and tight junctions. To visualize the integrity of these junctions in RL-65 cells, confluent monolayers were stained for E-cadherin (adherans junction marker) and zona occludens 1 (tight junction marker) (Figure 2B, 2C). E-cadherin and zona occludens were visualized to localize predominately to the region between adjacent cells forming a "cobblestone" pattern. This is a strong visual indicator of the formation of intact junctions [131,133]. Taken together, along with the high TER measurements, indicate properties of highly polarized epithelium.

Prior to characterizing MHV68 egress from polarized RL-65 cells, we assessed whether there was any binding of virus to transwell membranes as such binding might alter our interpretations of these analyses. As shown in figure 3A, when virus as added to empty transwells (i.e., in the absence of a cell monolayer) over a 4 hour timecourse the total amount of virus recovered was equivalent to the amount of input. Thus, there was no evidence of virus trapping/binding to the transwell membrane. To study the apical/basolateral egress of MHV68, RL-65 monolayer formation on the transwell membranes was monitored by TER every 24 hours as well as visual inspection. 168 hours post plating, confluent RL-65 cells were infected at an MOI of 0.05 with either MHV68 WT or vCyclin.stop virus apically. Every 24 hours, the supernatant was gathered from either the apical or basolateral chamber for quantification via

plaque assay (Figure 3B). WT MHV68 was shed from both the apical and basolateral surfaces – with larger amount of virus being shed basolaterally at late times post-infection (Figure 3B). Notably, the v-cyclin null mutant showed a profound defect in egress from polarized RL-65 cells (Figure 3B). At 24 and 48 hours post-infection the amount of virus shed either apically or basolaterally was at or below the limit of detection (dotted line, Figure 3B). By 72 and 96hrs post-infection a small amount of v-cyclin null virus was detectable and was mostly shed from basolateral surface of the polarized RL-65 cells.

TER measurements taken throughout the course of the experiment showed viral infection caused little change to the resistance of infected monolayer (Figure 2A), indicating MHV68 doesn't significantly destroy the integrity of the epithelium during infection. However, during the course of all experiments in RL-65 cells it was observed that infection with MHV68 causes apparent changes in the morphology of the epithelium, with infected cells appearing to "pile" on top of each other and were visualized to be "out of plane" with the rest of the monolayer - yet never detaching from the monolayer, even up to 120 hours post-infection (Figure 4A , 4B, and unpublished observations). It is unclear at this time whether the underlying epithelium is intact and whether this phenomenon influences the amount of virus that is detected in the apical and basolateral chambers. Future analyses of this phenomenon will benefit from the use of confocal microscopy/Z stack image acquisition for a 3-D visualization. However, we speculate that some of the apically shed virus arises from infected cells that are no longer part of the polarized epithelial monolayer and, as such, may confound the determination of amount of apically shed virus.

The defect in egress of the v-cyclin null virus from polarized RL-65 cells was much greater than the defect in virus replication observed when total virus replication (cell associated plus released virus) was analyzed (compare Figures 1 and 3B). To further explore this issue, we

repeated the analysis to compare total virus replication (cell associated plus released virus) to virus released from polarized RL-65 cells (Fig. 5). As previously observed, there was a significant defect in total virus replication which was most apparent are early times post-infection. However, when the amount of release virus was measure (apical plus basolateral), a much more profound defect was observed. Notably, the same analysis for WT MHV68 showed nearly equivalent levels of total and shed virus – indicating very efficient release of virus from polarized RL-65 cells (Figure 5). Thus, we conclude that the v-cyclin null virus has a profound defect in virus egress, as well as a defect in overall virus replication.

Finally, because the role of the v-cyclin in acute MHV68 replication in the lungs of intranasally inoculated mice has previously been shown to be CDK-dependent, we compared egress of the vCyclin.stop and a CDK binding mutant v-cyclin (mCyclin/E133V) from polarized RL-65 cells (Figure 6). The CDK binding mutant v-cyclin (mCyclin/E133V) exhibited a similar egress phenotype to the v-cyclin null virus mutant (vCyclin.stop) (Figure 6). Thus, as expected the roles of the v-cyclin in virus replication and egress are both dependent on interaction with appropriate cellular CDKs. Other viruses, such as HCMV, have been shown to utlize the cyclin/CDK pathway in efficient dissassembly of the lamina for virus egress [115]. Additionally, many viruses remodel the host cytoskelton during egress [113], and cyclin has been shown to be an important contributor to cellular remodeling[102,134]. How specifically MHV68 cyclin is important for virus egress remains to be elucidated.

2.4 Materials and Methods

Tissue culture

RL-65 cells were purchased from ATCC. Culture media consisted of a 1:1 mixture of F12/DMEM supplemented with 0.15mg/mL Bovine Pituitary extract (VWR), 0.005mg/mL Porcine Insulin (Sigma), 0.01mg/mL Human Transferrin (Sigma), 0.1mM Ethanolamine (Sigma), 0.1mM phosphoethanolamine (Sigma), 25 nM selenium (Sigma), 500nM hydrocortisone (Sigma), 0.005 mM forskolin (Sigma), 0.05μM retinoic acid (Sigma) as previously described [132]. Cells were maintained by passaging no less than 1:20 in RL-65 media, media changed every other day, with experiments consisting of cells from passages 2-10.

NIH 3T12 fibroblasts were obtained from ATCC. Culture media consisted of DMEM supplemented with 10% Fetal Bovine Serum (Lonza) or Calf Serum (Lonza), 10 IU/mL Penicillin (Cellgro), 10μg/mL Streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). Cells grown in FBS were maintained by passaging no more than 1:5 in 3T12 media, media changed every other day, with experiments consisting of cells from passages 5-10. Cells grown in CS were maintained by passaging no less than 1:10 in 3T12 media, with media changed every other day, with experiments consisting of cells from passages 3-6.

Costar 0.4μM 12 mm diameter polyester transwell permeable supports were used for all experiments utilizing transwell setups. Volumes in each chamber were kept at 0.4mL in apical chamber and 1mL in basolateral chamber. $2X10^5$ RL-65 epithelial cells or NIH 3T12 fibroblasts were plated per transwell and trans-epithelial resistance (TER) was measured every 24 hours using a Millipore Millicell-ERS. Briefly, the resistance (ohms) of blank transwells with media

but no cells was measured and its value subtracted from that of wells with cells at each time point. This number was then multiplied by the effective membrane area (cm^2) of the transwell membrane to determine TER as ohms/cm^2.

Immunofluorescence

E-Cadherin and Z0-1. RL-65 cells were plated at a concentration of $2x10^5$/well onto transwell membranes. Confluency was assessed by visual inspection coupled with measuring resistance across the transwell membrane (TER measurements). Confluent monolayers were fixed with 3.7% formaldehyde for 20 minutes at room temperature. Cells were permeabilized using 0.2% Triton X-100 treatment for 10 minutes at room temperature. All samples were incubated in blocking buffer (3% BSA/0.05% Tween) for 1 hour at room temperature before addition of specific antibodies. Specific antibodies [mouse anti - E-cadherin (BD Biosciences) or rabbit anti - Zona Occludens (Invitrogen)] were added at a concentration of 5ug/mL in blocking buffer, and incubated for 1 hour at room temperature. Finally, secondary Alexa Fluor conjugated- antibodies [Alexa Fluor 488 goat anti-mouse or Alexa Fluor 568 goat anti-rabbit] were added at a concentration of 0.4μg/mL in blocking buffer and incubated in the dark for 1 hour at room temperature. Samples were mounted with antifade reagent containing DAPI (Invitrogen).

YFP. RL-65 cells were plated at a concentration of $2X10^5$ onto 1.7cm^2 glass chamberslides (Millipore). After visual confirmation of confluency, cells were infected at a MOI of 0.05 with MHV68-H2bYFP, a virus that expresses the yellow fluorescent protein from infected cells [56]. 120 hours post infection cells were fixed with 3.7% formaldehyde for 20 minutes at room

temperature and mounted with antifade reagent containing DAPI (Invitrogen) and visualized for YFP expression.

Virus growth assays

For replication assays in standard 24 well tissue culture plates, RL-65 cells were plated at a concentration of $4x10^5$ cells/well. After visual inspection to confirm full monolayer formation, cells were infected at MOI 0.05 with either MHV68 WT or V.cyclin.stop. Every 24 hours cells plus supernatants were collected. Samples were subjected to freeze/thaw twice to release any intracellular virus. Total virus titer was calculated from plaque assays on NIH 3T12 fibroblasts.

Plaque assays were perfomed on NIH 3T12 fibroblasts. Briefly, 24 hours prior to assay $2x10^5$ NIH 3T12 cells were plated per well onto standard 6 well tissue culture plates. After fibroblasts reached ~80% confluency samples ready to titer were serially diluted and plated onto fibroblast monolayer. Plates were incubated at 37°C for one hour with gently rocking back and forth to envenly distribute inoculum. After one hour a 2.5% serum/methocelluose solution was overlayed and plates were incubated for approximately one week until plaques were visualized. Plaques were stained with 0.1% crystal violet in 20% methanol.

For growth analyses on polarized RL-65 cell plated on transwells (Costar 0.4μM 12 mm diameter), cells were plated at a concentration of $2x10^5$ cells per transwell. Monolayer growth was monitored by TER and visual inspection. Confluent monolayers were infected apically at an MOI of 0.05 with either MHV68 WT, V.cyclin.stop, or V-cyclin/E133V. Every 24 hours supernatant from apical and basolateral chamber was collected, or in the case of determining total virus replication cells were scraped off membrane using a sterile pipette tip blunt end and cells plus supernatant from apical and basolateral chamber was collected. Samples were subjected to

two rounds of freeze/thaw to release any intracellular virus. Total virus was quantified by plaque assay on NIH 3T12 fibroblasts.

To determine whether any virus becomes trapped in empty transwells, virus was added to apical chamber of transwell setups that contained media only (no cells). WT MHV68 (4×10^5 pfu) in a volume of 50 μL was added to 350μL media in apical chambers. Basolateral chambers contained 1mL of media. Plates were incubated at 37°C and samples from the apical and basolateral chambers were taken at 30min, 2hr, and 4hr after virus addition, and subsequent virus titer determined by plaque assay on NIH 3T12 fibroblasts.

Statistical analysis

Statistical data analysis was performed using GraphPad Prism software. Data shown represents one of at least triplicate experiments. Error bars represent standard error mean. Significance was determined by two-tailed, unpaired Student's t-test with a confidence level of 95%.

2.5 Conclusions

The epithelium lining the lungs serves not only as a medium for gas exchange, but also as a barrier protecting the host from the outside environment. This lining is often only a single cell thick and its cells are polarized with distinct apical and basolateral domains [102]. Facing the lumen is the apical domain which characteristically contains properties to interact with the environment. The basolateral domain interfaces the underlying basement membrane, as well as cellular neighbors [102]. Separating these two surfaces are a variety of junction proteins that form strong connections between neighboring cells. It seems very likely that these cells serve as the initial barrier through which intranasally inoculated MHV68 must traverse to establish infection. Here we have shown that the characterization of MHV68 replication in a polarized, non-transformed lung epithelial cell line (RL-65) not only recapitulates the CDK-dependent role of the v-cyclin in virus replication, but also identifies a previously unknown role for the v-cyclin in egress from polarlized epithelium.Future studies will focus on the nature of the latter defect, which are likely to provide new insights into how MHV68 traffics in infected cells.

2.6 Figures

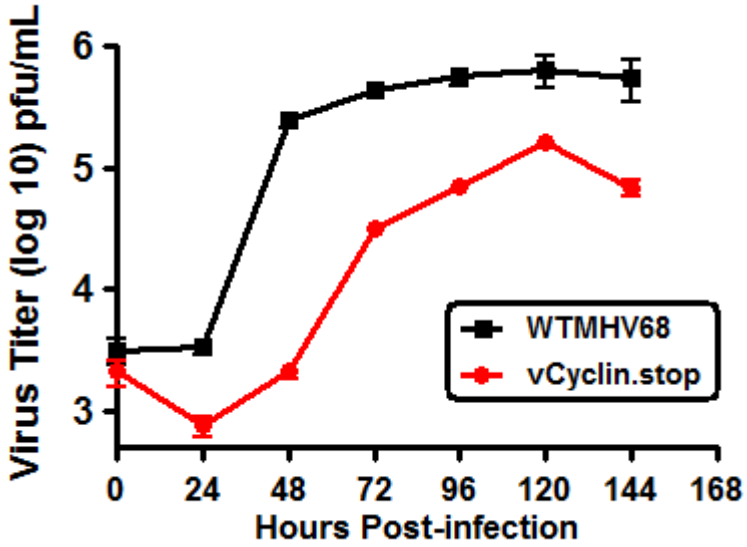

Figure 1 MHV68 requires v-cyclin for robust growth at early time points in growth arrested RL-65 cells.

RL-65 cells were plated to confluency and then infected at an MOI of 0.05 with either WT MHV68 or vCyclin.stop virus. Cells plus supernatants were collected every 24 hours and subjected to freeze/thaw twice to release any intracellular virus. Total virus at each time point was determined by plaque assays on NIH 3T12 fibroblasts. The data shown was compiled from 3 separate experiments, each done in triplicate. Standard error of the mean is shown.

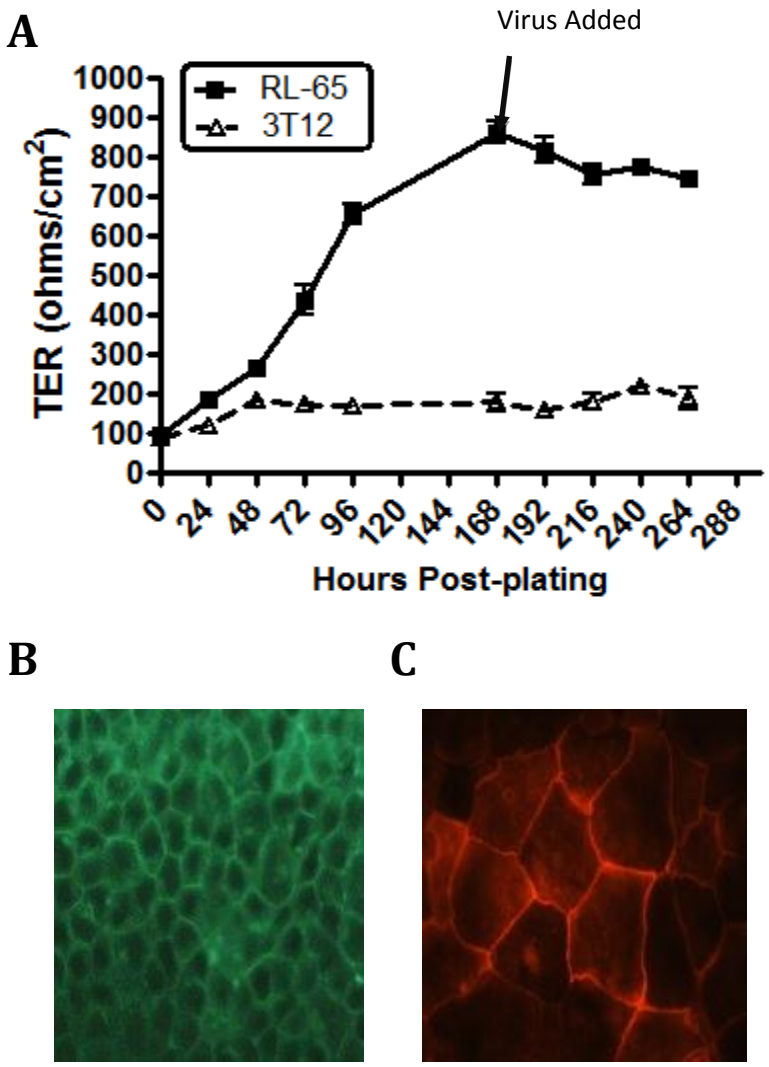

Figure 2 RL-65 cells form polarized monolayers in transwells.

(A) RL-65 cells or, as a negative control, NIH 3T12 cells were plated at a concentration of $2X10^5$ cells/well onto 0.4μM pore size transwell polyester membranes. Every 24 hours transepithelial resistance (TER) was measured. The RL-65 monolayer was infected with MHV68 (MOI=0.05) at 168 hours post-plating, as indicated. The data shown was compiled from 3 separate experiments. Standard error of the mean is shown. (B & C) Confluent monolayers of RL-65 cells were stained for adherens junction marker E-cadherin (B) or tight junction marker zona occludens (C).

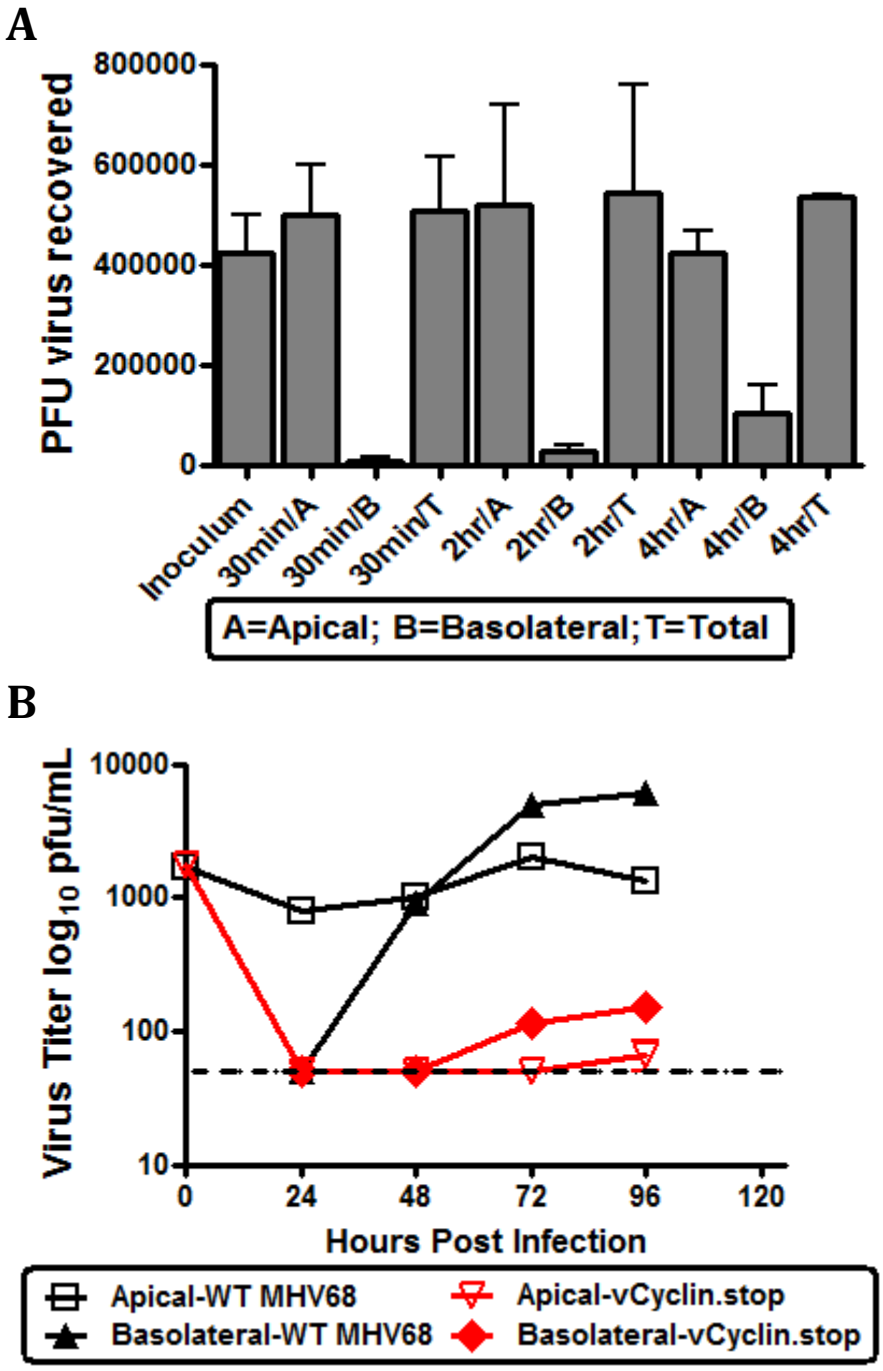

Figure 3 Basolateral shedding of MHV68

Figure 4 Basolateral shedding of MHV68 (continued)

(A) RL-65 cells were plated at a concentration of $2x10^5$ cells per transwell. To assess virus diffusion, as well as potential virus trapping in transwell membranes, a known quantity of MHV68 (inoculum) was added to the apical chamber of empty transwells (no cells) (168 hrs post-plating cells). Samples from the apical and basolateral chambers were collected at various time points to determine number amount of virus present. Total virus reflects the sum of virus titers determined for the apical and basolateral chambers. (B) RL-65 cells were plated at a concentration of $2x10^5$ cells per transwell. Confluent monolayers of RL-65 cells (168 hours post plating) were infected at an MOI of 0.05 with WT or v.Cyclin.stop virus. Every 24 hours post-infection supernatant from the apical and basolateral chambers was collected and the amount of virus presented determined by plaque assay on NIH 3T12 fibroblasts. The data shown was compiled from 3 separate experiments, each done in triplicate. Standard error of the mean is shown.

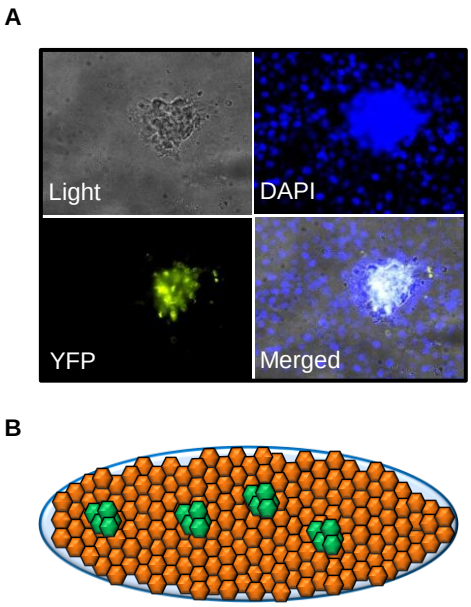

Figure 5 MHV68 infection of polarized RL-65 cells leads to piles of infected cells that appear to be extruded from cell monolayer.

(A) RL-65 cells plated to confluency onto glass chamberslides and infected at an MOI of 0.05 with MHV68-H2bYFP virus. 120 hours post-infection samples were fixed as described in Materials and Methods. Nuclei were stained with DAPI and virus infected cells detected by YFP expression. (B) Cartoon illustrating visual of MHV68 infected cells (green) forming piles out of plane with the on the polarized RL-65 cell monolayer.

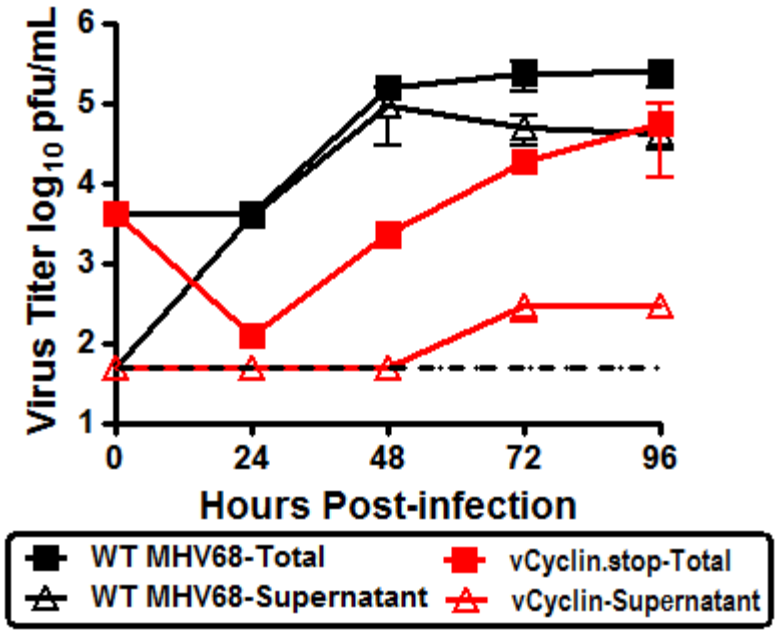

Figure 6 The vCyclin.stop mutant exhibits a severe defect in egress from growth arrested RL-65 cells.

RL-65 cells were plated at a concentration of $2x10^5$ cells per transwell. Confluent monolayers were infected with an MOI of 0.05 with either MHV68 WT or vCyclin.stop. Every 24 hours either shed virus (supernatant from both apical and basolateral chambers) or total virus (cells plus supernatant from both apical and basolateral chambers) were collected for titer on NIH 3T12 fibroblasts. The data shown was compiled from 3 separate experiments, each done in triplicate. Standard error of the mean is shown

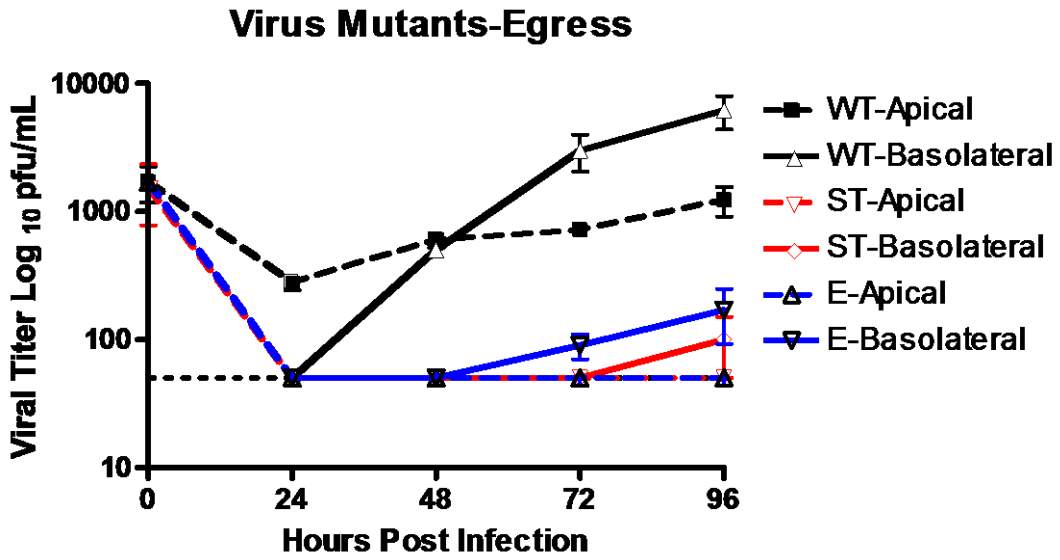

Figure 7 CDK binding is required for efficient MHV68 replication in RL-65 cells.

Confluent monolayers of RL-65 cells (168 hours post plating) were infected at an MOI of 0.05 with the indicated viruses. Every 24 hours post-infection supernatant from the apical (closed symbols) and basolateral (open symbols) chambers was collected and the amount of virus presented determined by plaque assay on NIH 3T12 fibroblasts. The data shown was compiled from a single experiment done in triplicate. Standard error of the mean is shown.

CHAPTER 3-KSHV-cyclin and MHV68-cyclin conserved functions.

For this chapter all figures are an excerpt from the manuscript that is detailed in Chapter 2. The title of this manuscript is "A tissue culture model of murine gammaherpesvirus replication reveals roles for the viral cyclin in both virus replication and egress from infected cells." This manuscript was accepted by PLoS One on March 11, 2014. The written portion of this chapter is a separate body (abstract/introduction/conclusions) that is not submitted for publication but presented here to specifically highlight the unique implications of the results from these experiments as they were a separate project. Permission will not need to be obtained from the author and publisher for reproduction because the journal is open access.

3.1 Abstract

The development of oncogenic conditions often arises from dis-ordered events in the cellular cycle. Cell cycle progression is regulated by the heterdimeric complex consisting of cyclins and cyclin dependant kinases (CDKs). The human gammaherpesvirus KSHV is associated with the development of tumorous lesions and has been shown to modulate the host cell cycle. The KSHV viral gene product, viral cyclin, is a homologue to eukaryotic cyclin D and can influence key regulators of cell cycle events *in –vitro*. The murine model of human gammaherpesvirus infection (MHV68) also contains a viral cyclin and shares many conserved *in-vitro* mechanisms of perturbing the host cell cycle. Because much remains to be answered about how k-cyclin influences infection, *in-vivo*, and whether the *in-vivo* functions of m-cyclin might be conserved in the m-cyclin; a chimeric virus was generated. The chimeric MHV68 virus encodes the k-cyclin instead of the m-cyclin and *in-vitro* and *in-vivo* studies reveal there are some conserved properties. K-cyclin was sufficient to partially rescue the growth defect and egress defect of the m-cyclin in RL-65 cells. Additionally, the k-cyclin was sufficient to partially rescue the growth defect from acute infection in the lungs of C57/BL6 mice. However, it was not sufficient to rescue reactivation from latency from spelnocytes. These conserved properties exist, despite the noted differences in CDK biding preference. These studies support the use of MHV68 model as a means to study the human k-cyclin.

3.2 Introduction

Gammaherpesviruses often contribute to the development of lymphoproliferative disorders and certain tumors. Kaposi's sarcoma associated herpesvirus (KSHV) is highly implicated as influencing the development of Kaposi's sarcoma. Though tumors can arise from any number(s) of initiating events; uncontrolled cellular regulation is usually involved in an oncogenic progression [57] . In the eukaryotic cell cycle, normal progression from a quiescent cell to mitosis is governed by a heterodimeric complex consisting of cyclins and cyclin dependent kinases (CDKs). Each stage of the cell cycle is governed by a particular type of cyclin and CDK complex. G1 is influenced by the action of cyclin D and CDK 4/6. Entry into S phase is associated with cyclin E and or CDK2. Additionally, G2 to M is governed by cyclins A and B in conjunction with CDK1/CDC1. The classification of cyclins into a particular class is based on this functional difference of action [65]. All cyclins, regardless of class, share a conserved core domain termed the “cyclin box” which consists of approximately 100 amino acids that are important for their binding to CDKs [66]. Additionally, they also share conserved areas of protein structure [65,66]. These shared similarities have led to much debate about the importance of any one particular type of cyclin in a given cell cycle stage. Interestingly, recent reports utilizing knock-out mice have revealed that many cyclins can functionally compensate for one another [72,75,76]. Often mice lacking a necessary cyclin that is not functionally compensated will not survive beyond certain stages of embryogenesis [75]. Because cyclins are such a critical regulator of cellular progression, and strictly ordered cellular progression is highly crucial for organismal survival, it follows that the eukaryotic cell should employ several mechanisms to keep cyclin actions in check. In fact, INK4 and CIP/KIP CKIs are two regulatory measures that the

cell uses to ensure cyclin:CDK activities remain ordered. INK 4 CKIs specifically interact with CDK 4/6 and this interaction prevents their binding to cyclin D [63]. CIP/KIP CKIs have a much broader range of inhibitory action in that they can influence suppression on the action of cyclins D, A, or E [63]. Another mechanism the cell uses to keeps its cycle under control is employing the tumor-suppressor gene Rb, which functions to prevent untimely G1-S phase transition and promote cellular cycle exit [135].

Due to the strong association between cell cycle control and oncogenesis, much care has been undertaken to examine the mechanisms by which KSHV interacts with its hosts' cell cycle as a means to unravel the possible mechanisms in which it contributes to KS tumors. KSHV encodes a basic-leucine zipper protein, K-bZIP, which contains CDK recognition sites and can be phosphorylated by CDKs [136,137]. Additionally, K-bZIP can interact with and down regulate tumor suppressor p53 by interacting with p53's carboxy-terminal domain [138]. This region of p53 is quite important for restricting growth in some cancerous cell lines [139]. KHSV also encodes another gene product that can perturb the host cell cycle. A virally encoded homologue with eukaryotic cyclin D (k-cyclin) [86]. Since k-cyclins discovery, many other unique attributes of this viral cyclin have been revealed. Not only can it function like a traditional cyclin D and bind to CDK 4/6, k-cyclin can interact with CDKs 2, 5, and 9 [84-86]. Interestingly, its interaction with CDK 9 can functionally phosphorylate Rb [85]. Rb phosphorylation is a characteristic that inactivates the tumor-suppressor (promotes cycle progression) [135]. Unlike eukaryotic cyclins, K-cyclin can evade restriction by INK4 and CIP/KIP [89]. Characterization of k-cyclin has largely been carried out *in-vitro*, due to the strict species specificity of KSHV. The questions of how k-cyclin influences viral pathogenesis *in-vivo* remain largely unanswered. There have been a few studies that have utilized transgenic mice in attempts to and answer these questions. Under the control of the VEGF-3 promoter, the k-cyclin transgene induces lymphatic dysfunction and mortality at a young age in the transgenic mice [140]. In k-cyclin transgenic

mice the k-cyclin is also sufficient to induce tumor formation in the absence of p53 [141] . While these k-cyclin studies are, infact, crucial to studying the in-vivo impacts of k-cyclin. They do not address the issue of how k-cyclin might impact pathogenesis in the context of a viral infection. We hypothesize that the use of the murine gammaherpesvirus 68 (MHV68) model system would be a useful tool to address such a question.

Murid rodents can be naturally infected with MHV68, and this virus can also readily infect inbred strains of mice (*Mus Musculus*) [45]. Intranasal infection with MHV68 establishes a productive infection in lung mononuclear and alveolar cells [48]. The major site of MHV68 latency is the spleen with latency routinely occurring in the major antigen presenting cells: B cells, macrophages, and dendritic cells [46]. There are large blocks of conserved genomic sequence and conserved gene function between MHV68 and the human gammaherpesviruses [42,45]. Once of these conserved genes is MHV68 viral-cyclin (m-cyclin) [42]. M-cyclin shares between 25-31% identities with eukaryotic cyclin D and between 26-32% identity with k-cyclin of KSHV [42,83]. Much of this similarity in identity is localized to the region of the cyclin-box [42]. There are other conservations of function between the two viral cyclins as noted by *in-vitro* experiments. MHV68 can also phosphorylate Rb, and evade CKIs much like k-cyclin [88]. The major noted area of dissimilarity lies in the viral cyclins preferred CDK binding partner. In-vitro experiments show that m-cyclin prefers to interact with CDK2 or CDK1/CDC2 [88]. Because many cyclins can functionally compensate for each other the importance of this CDK preference is a subject of debate [75].

Unlike k-cyclin, much more is known about how m-cyclin contributes to pathogensis *in-vivo.* As a transgene, m-cyclin can function as an oncogene and can influence T-lymphocyte cell cycle expression [83]. Additionally, in the context of a viral infection, m-cyclin is required for reactivation from latency in both spenocytes and PECs [98]. CDK binding mutants of m-cyclin

have revealed that CDK interaction is not required for this reactivation from latency [99]. Dissimilar to this, lytic infection in the lungs (at low dose) is highly influenced by m-cyclins' CDK interaction [99]. To study MHV68 viral cyclin in the lungs, a rat lung epithelial cell 65 (RL-65) tissue culture model of murine gammaherpesvirus 68 infection has recently been developed and used to study aspects of virus infection and egress (Chapter 2). Noted findings were that v-cyclin is important for virus replication and egress in these cells. It remains to be addressed how much of these *in-vivo* and *in-vitro* functions ascribed to m-cyclin are conserved in the k-cyclin. The availability of an MHV68 bacterial chromosomes [51] enables the efficient generation of genetically modified MHV68 virus and it is hypothesized her that the generation of a transgenic MHV68 virus that encodes k-cyclin instead of its own would provide a means by which to analyze k-cyclin and m-cyclin conserved functions.

3.3 Results and Discussion

The KSHV v-cyclin can partially rescue replication of MHV68 lacking a functional v-cyclin, both in vitro and in vivo

As discussed above, it is notable that the MHV68 and KSHV v-cyclins interact with different cellular CDKs, raising the question of whether this represents a divergence in their functions during virus infection. To begin to address this issue, we generated a recombinant MHV68 in which the v-cyclin open reading frame was replaced with the KSHV v-cyclin coding sequence. Upon low MOI infection of RL-65 cells the K-cyclin virus exhibited an intermediate phenotype in that it replicated faster at early times post-infection than the v-cyclin null mutant, but replicated more slowly than WT MHV68 (most apparent at 48 and 72hrs post-infection) (Figure 7B). We extended this analysis to assess replication of the MHV68/K-cyclin virus in vivo. After intranasal inoclulation of C57Bl/6 mice with 1,000 pfu of either WT MHV68, vCyclin.stop or the MHV68/K-cyclin virus, lungs were harvested at days 4 and 9 post-infection. As observed in the in vitro growth analyses in RL-65 cells, the K-cyclin virus exhibited an intermediate phenotype in vivo (Figure 8A, 8B). These results suggest that either there is a function of MHV68 v-cyclin that is not recapitulated by the K-cyclin (which could reflect utilization of different CDK partners), and/or there is a technical issue related to regulation of K-cyclin expression from the MHV68 v-cyclin locus. Additionally, characterization of K-cyclin CDK interaction has been extensively characterized in human, monkey, and insectcells [85-87]. Whether any differences in binding occur in cells from rodents is unclear, though the K cyclin %

similarity is roughly identical (~54%) when compared to mouse, human, and rat cyclin D [86]. Regardless, these results demonstrate that the K-cyclin can significantly enhance replication of MHV68 in the absence of the MHV68 v-cyclin. To address whether K-cyclin enhancement of MHV68 replication in the absence of the MHV68 v-cyclin would rescue the v-cyclin null virus reactivation defect from splenocytes [99], we assessed virus reactivation at day 18 post-infection. Notably, the K-cyclin failed to increase the frequency of splenocytes reactivating virus at day 18 when compared to the vCyclin.stop mutant (Figure 9A, 9B). This indicates that although acute replication in the lungs is enhanced with the K-cyclin chimeric virus, this does not enhance virus reactivation from splenocytes – suggesting a function of the MHV68 v-cyclin involved in virus reactivation that is not conserved in the K-cyclin. Overall, the analyses of the vCyclin.stop and K-cyclin viruses in vivo, as well as in RL-65 cells, argue that replication in RL-65 cells accurately mimics the requirements for MHV68 replication in the lungs at the acute stage of virus infection.

K-cyclin exhibits both apical and basolateral shed from RL-65 cells.

The RL-65 cell line has provided important information about the egress phenotype of the v.Cyclin mutant and WT MHV68 virus. This phenotype has been shown to be dependent on the v.Cyclin interaction with a CDK [99]. We set out to compare the K-cyclin chimeric virus egress patterns to that of WT and vCyclin.stop. Importantly, although the K-cyclin chimeric MHV68 exhibited a modest defect in virus replication (see Figure 7), it did not exhibit a defect in virus egress (Figure 10). In contrast, the v.Cyclin stop virus exhibits significantly lower amounts of egressed virus. Because this egress defect is related to the CDK binding ability of the vCyclin (see Figure). These results suggest that the CDK binding ability necessary to egress to WT levels in RL-65 cells is sufficiently conserved in the K-cyclin. Because both viral cyclins have been shown to interact with different CDK partners is remains to be addressed whether this noted

phenomenon is due to a form of CDK compensation or perhaps downstream signals in the G1-Sphase transition of the cell cycle (where both cyclins have conserved properties)that is the key mediator in the egress ability of MHV68 [88,120].

3.4 Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Emory University Institutional Animal Care and Use Committee and in accordance with established guidelines and policies at Emory University School of

Medicine (Protocol no. YER-2002245-031416GN).

Tissue culture

RL-65 cells were purchased from ATCC. Media composition was generated as specified in guidelines from both ATCC and Roberts et.al [132] . The media components were a 1:1 mixture of F12/DMEM supplemented with 0.15mg/mL Bovine Pituitary extract (VWR), 0.005mg/mL Porcine Insulin (Sigma), 0.01mg/mL Human Transferrin (Sigma), 0.1mM Ethanolamine (Sigma), 0.1mM phosphoethanolamine (Sigma), 25 nM selenium (Sigma), 500nM hydrocortisone (Sigma), 0.005 mM forskolin (Sigma), 0.05μM retinoic acid (Sigma) as previously described [132]. The epithelial cells were passaged approximately 1:20 in the appropriately formulated RL-65 media. Media changes occurred every other day with experimentally cells not exceeding passage 10.

ATCC was also the source for NIH 3T12 fibroblasts. DMEM media was the source of growth media and it was supplemented with 10% Fetal Bovine Serum (Lonza) or Calf Serum (Lonza), 10 IU/mL Penicillin (Cellgro), 10µg/mL Streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). Cells grown in FBS were maintained by a 1:5 passage in 3T12 media, media changes occurring every other day, and experiments consisting of cells from passages 5-10. CS supplemented cells were passaged in a larger split ration (1:10) in 3T12 media. Media change for these cells occurred every other day, with passages 3-6 as the source for experiments.

Transwell experiments were conducted on Costar 0.4µM 12mm diameter transwell permeable membranes. These membranes were of polyester formulation. During experiments the volumes in each chamber were maintained at the Costar recommended amounts. For this membrane size it was 400 µL in the apical chamber and 1mL in the basolateral chamber. Approximately $2X10^5$ RL-65 cells or NIH 3T12 fibroblasts were added per transwell. The trans-epithelial resistance (TER) was measured using a Millipore-Millicell-ERS every 24 hours. To calculate the TER specific calculations of ohms in a blank transwell was determined and this value was subtracted from the value of a well containing cells. The value was multiplied by the effective membrane area (cm^2) of the membrane. The final resulting value(s) was reflective of ohms/cm^2.

Replication analyses were conducted in standard tissue culture plates of 24 well size. The concentration of cells plated at beginning of experiment was $4x10^5$ cells/well. To confirm full monolayer formation, visual inspection was performed. Upon confluent monolayer conformation, cells were infected at MOI 0.05 with WT MHV68 or MHV68 v.cyclin.stop or K-cyclin mutant (described below). Cells plus supernatants (total virus) was assessed every 24

hours. Freezing and thawing occurred twice in order to release any intracellular virus. The total virus titers from each time point was calculated by plaque assay on NIH 3T12 fibroblasts.

To perform plaque assay, 24 hours prior to assay $2x10^5$ NIH 3T12 cells were plated per well onto 6 well standard tissue culture plates. Upon reaching 80% confluency, the samples ready to titer were serially diluted and then plated onto the 80% confluent cell monolayer. Incubation occurred at 37°C with gently rocking back and forth. This was necessary in order to ensure that the inoculum was evenly distributed. A 2.5% serum/methocelluose solution was overlayed after 1 hours and a 7 day incubation (or until visual confirmation of plaques) occurred. To stain the plaques a 0.1% crystal violet in 20% methanol staining solution was used.

Egressed virus (total verses shed virus) on polarized RL-65 cells occurred by first plating $2x10^5$ cells onto Costar 0.4μM 12 mm diameter membranes. TER (as described above) was used to confirm full monolayer formation. Upon complete monolayer formation, MHV68 WT, V.cyclin.stop, or K-cyclin virus was infected apically. Supernatant from either the apical or basolateral chamber was sampled every 24 hours. Total virus was quantified by plaque assay on NIH 3T12 fibroblasts.

Generation of MHV68/K-cyclin virus

PCR was used to clone the KSHV v-cyclin open reading frame (K-cyclin) from the KSHV latently infected BCBL cell line (reference for KSHV cyclin region-GenBank U79416). The K-cyclin coding sequences were cloned using the following primers: 5'-cttgtcgtccttgtagtcatagctgt ccagaat-3' and 5'-tatatggcaactgccaataacccgccc-3'. PCR was then used to amplify the left and right flanking arms of the MHV68 v-cyclin ORF with designed overlapping sequences matching that of K-cyclin ORF (reference for MHV68 cyclin region-

GenBank U97553). Left flanking arm primers: 5'-gggggatcccacacatcaagttatcacttttg-3' and 5'-gactacaaggacgacggacgacaagttaaaaaat aaatgcc-3'. Right flanking arm primers: 5'-gggcgggttattggcagttgccatata-3' and 5'-gggtctagaat tgttttcaataaaaaagtg-3'. The flanking arm primers also contained restriction sites BamHI and XbaI for subsequent subcloning. Overlapping-extension PCR was used to generate the final PCR fragment that contained the K-cyclin ORF with flanking sequences from the MHV68 v-cyclin region such that an exact replacement of MHV68 v-cyclin open reading frame with the K-cyclin open reading frame would be generated (i.e., an exact open reading frame replacement from start codon to stop codon was constructed). Chimeric DNA was inserted into pCR-Blunt using BamHI and XbaI and then cloned into the allelic exchange targeting vector pGS284MHV68 using the same restriction sites. MHV68-BAC was generated and bacteria mediated allelic exchange was performed as described previously for MHV68 mutants [99]. After confirming the appropriate recombinants, the BAC vector was removed by passage through Vero-CRE cells as previously described. High titer viral stocks were then generated from infected NIH 3T12 cells as described previously for MHV68 mutants [99].

Infections and Analysis of Acute Virus Replication in Lungs

C57/Bl6 mice (Jackson laboratories) were placed under isofluorane anesthesia before intranasal inoculation with 20μL complete media containing 1,000pfu of MHV68 WT, or MHV68 mutants V-cyclin.stop or K-cyclin. During the course of infection mice were visually monitored for signs of distress and routine care and feeding of animals was carried out by the Emory University Veterinary Staff in Accordance with the Animal Care and Use Guidelines. On days 4 and 9 mice were sacrificed and lungs harvested. Lungs were placed in complete media (1mL) and frozen at -80°C. Upon thawing, lungs were subjected to mechanical disruption and

dilutions of the resulting lysate plated on NIH 3T12 fibroblasts for plaque assay analysis as previously described [99].

Limiting dilution analyses for assessing ex-vivo MHV68 reactivation

To determine the frequency of splenocytes capable of reactivating virus from latency, a limiting dilution ex-vivo reactivation assay was performed as previously described [99]. Briefly, splenocytes from infected mice were plated in a two-fold serial dilution fashion (starting with 10^5 splenocytes per well) on to MEF monolayers in 96-well tissue culture plates. Twenty-four wells were plated per dilution and 12 dilutions were plated per sample. Wells were scored microscopically for cytopathic effect (CPE) at 14-21 days post-explant. Preformed infectious virus was detected by plating parallel samples of mechanically disrupted cells onto MEF monolayers alongside intact cells.

Statistical analysis

Statistical data analysis was performed using GraphPad Prism software. Data shown represents one of at least triplicate experiments. Error bars represent standard error mean. Significance was determined by two-tailed, unpaired Student's t-test with a confidence level of 95%.

3.5 Conclusions

Human gammaherpesviruses, such as KSHV induce tumors and modulate the host cell cycle. KSHVs homologue to eukaryotic cyclin D can interact with host CDKs and evade restriction by CKIs [89,120]. Though much is known about how k-cyclin influences the cell cycle *in-vitro*, much less is known about its importance in pathogenesis *in-vivo*. To this end we described here the generation of a chimeric MHV68 virus that encodes the k-cyclin from KSHV instead of its own m-cyclin. Analyses revealed that the chimeric virus sufficiently rescued the m-cyclin ascribed growth defect in RL-65cells and from acute infection in the lungs of mice (Figures 7 and 8). This acute growth defect has been implicated as being dependent on CDK action [99] and the ability of k-cyclin to rescue the acute growth defect implies that there are conserved CDK initiated events between the two viral cyclins. This is important because the two viral cyclins preferentially interact with different CDK binding partners [88,120]. The difference in CDK binding partners combined with the apparent conserved functions leads to the speculation that the mechanism of conserved function might lie in the area of overlap in the CDK4/6 and CDK2 pathways, such as Rb phosphorylation [65]. Of course it is formally possible that despite the CDK binding partner preference is not relevant and the two viral cyclins can simply compensate for one another as with some eukaryotic cyclins [75]. Additionally, the k-cyclin did not rescue the m-cyclin growth defect to WT levels perhaps indicating there might be more than one mechanism of action necessary for the growth phenotype of k-cyclin expression in this model influence these results. Further evaluations will be needed to address this.

The chimeric k-cyclin virus did not rescue the reactivation defect from splenocytes (Figure 9). This m-cyclin reactivation defect is known to be a CDK independent phenomenon [99]. These CDK independent events are elusive at this time and perhaps could be related to the mechanisms of eukaryotic CDK independent functions. Eukaryotic cyclin D (cyclin D1) can

activate the estrogen receptor independent of CDK activation [142]. However, at this time this would be a speculation and further studies utilizing the m-cyclin CDK binding mutants should be used to analyze this.

Finally, the k-cyclin chimeric virus did not show a preference for apical or basolateral egress in RL-65 cells. This egress defect has been implicated to include CDK action (Figure 6), and this suggests that the CDK action events necessary for viral egress in this cell line are conserved in the K-cycin. As a whole, this body of work highlights the importance of the MHV68 mouse model system for studying aspects of the human gammaherpesviruses and provides further evidence of conserved functionality between the two viral cyclins (specifically *in-vivo*).

3.6 Figures

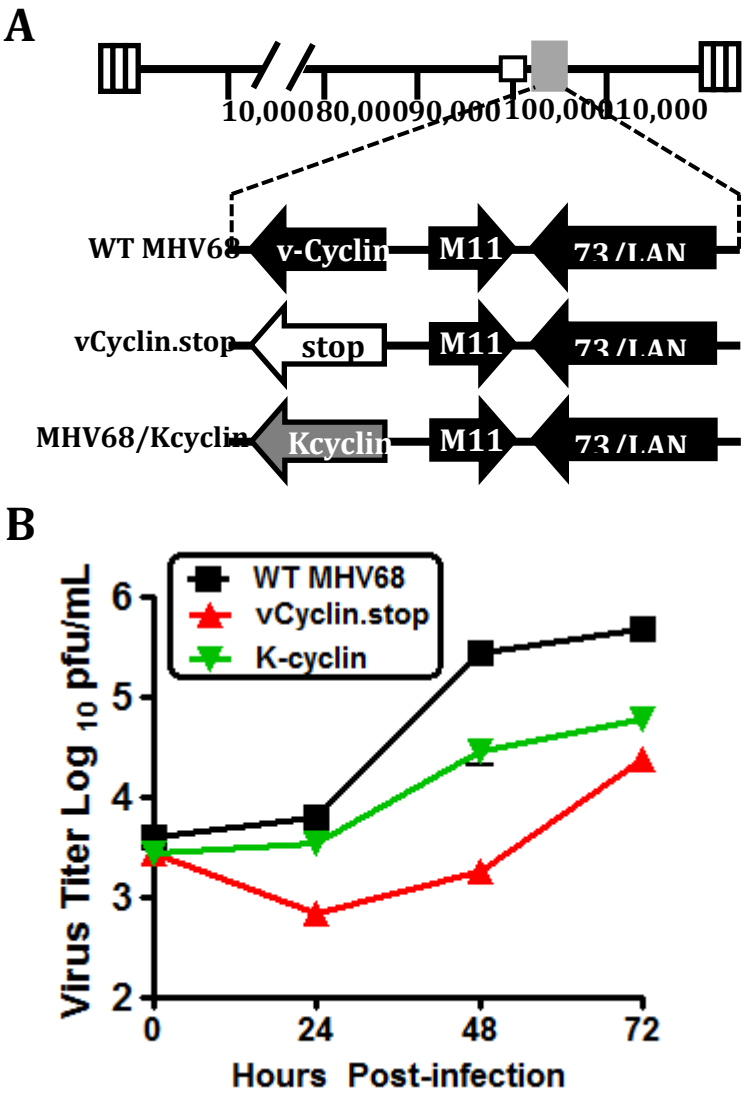

Figure 8 KSHV v-cyclin can partially rescue MHV68 v-cyclin null virus replication defect in growth arrested RL-65 cells.

(A) Schematic illustration depicting the v-cyclin locus and insertion of KSHV v-cyclin in place of the MHV68 v-cyclin open reading frame. (B) RL-65 cells were plated to confluency and then infected at an MOI of 0.05 with either WT MHV68, vCyclin.stop or a recombinant MHV68 expressing the KSHV v-cyclin from the MHV68 v-cyclin locus (K-cyclin). Cells plus supernatants were collected every 24 hours and subjected to freeze/thaw twice to release any intracellular virus. Total virus at each time point was determined by plaque assays on NIH 3T12 fibroblasts. The data shown was compiled from 3 separate experiments, each done in triplicate. Standard error of the mean is shown

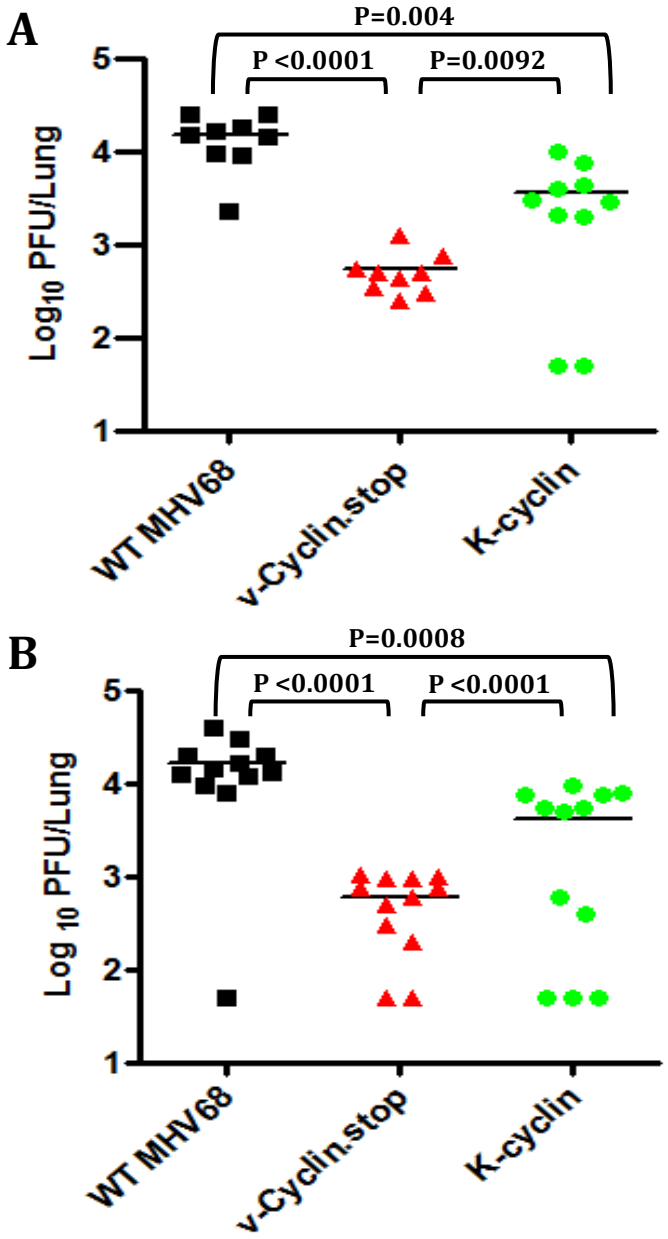

Figure 9 KSHV v-cyclin can partially rescue MHV68 v-cyclin null virus replication defect in lungs following intranasal inoculation

C57Bl/6 mice were infected via intranasal inoculation with 1,000 pfu of either WT MHV68, vCyclin.stop or a recombinant MHV68 expressing the KSHV v-cyclin from the MHV68 v-cyclin

locus (K-cyclin). Lungs were harvested at days 4 (panel A) and 9 (panel B) post-infection, and total virus at each time point was determined by plaque assays on NIH 3T12 fibroblasts as described in Materials and Methods. Each symbol represents analysis of an individual infected mouse.

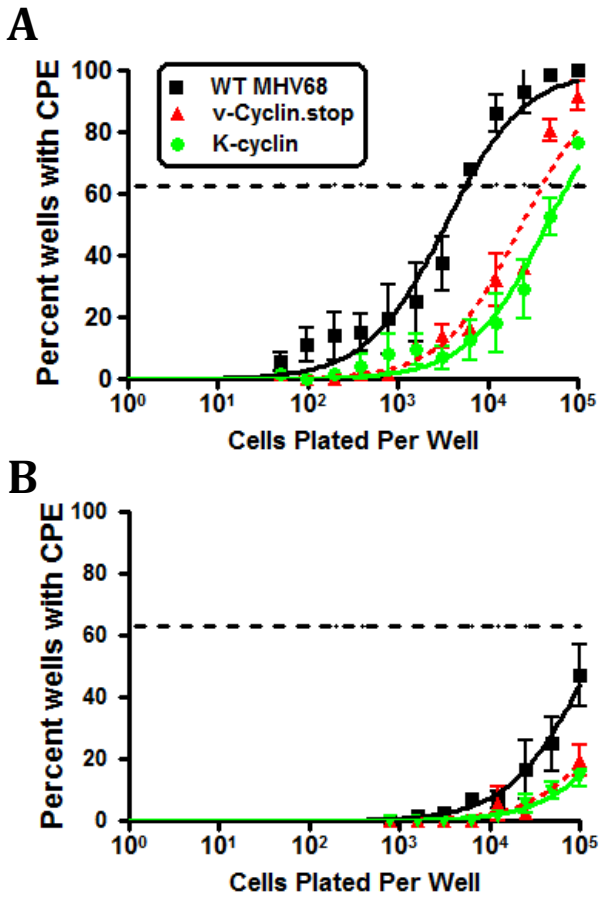

Figure 10 KSHV K-cyclin does not rescue MHV68 v-cyclin null virus reactivation from splenocytes following intranasal inoculation.

C57Bl/6 mice were infected via intranasal inoculation with 1,000 pfu of either WT MHV68, vCyclin.stop or a recombinant MHV68 expressing the KSHV v-cyclin from the MHV68 v-cyclin locus (K-cyclin). (A) Reactivation from splenocytes on day 18 post infection was determined by assessing the percent of wells positive for viral CPE after limiting dilution onto MEF indicator monolayers as described in Materials and Methods. (B) Amount of preformed infectious virus

was determined by assessing mechanically disrupted splenocytes. The analyses shown were compiled from 3 separate experiments, using 5 mice per experiment for each virus. Standard error of the mean is shown.

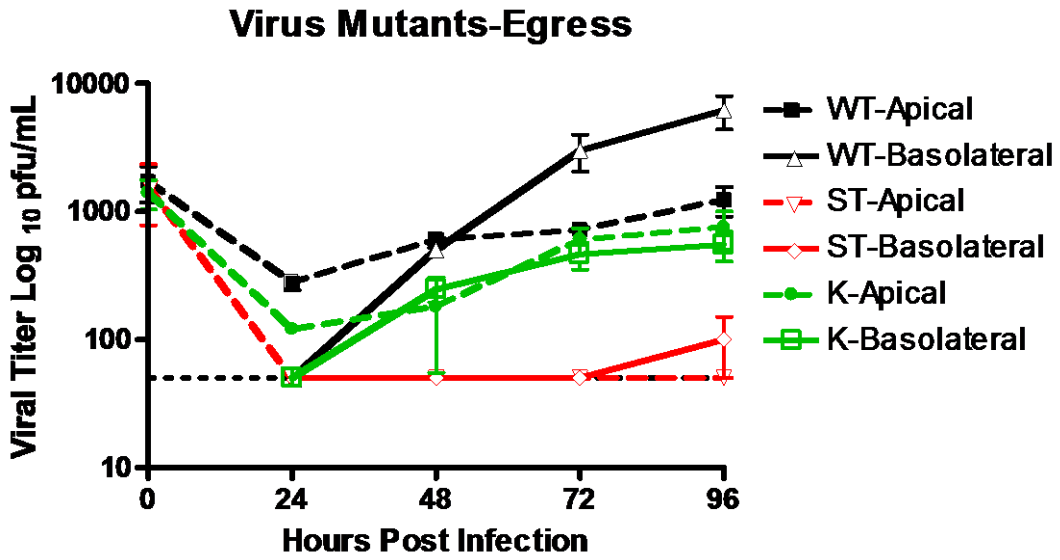

Figure 11 K-cyclin Virus does not exhibit a preferred Egress Pattern on Polarized RL-65 cells.

*WT and 72. stop controls and first part of figure description are the same as in Chapter 2 figure 6. This was performed as one experiment.

Confluent monolayers of RL-65 cells (168 hours post plating) were infected at an MOI of 0.05 with the indicated viruses. Every 24 hours post-infection supernatant from the apical (closed symbols) and basolateral (open symbols) chambers was collected and the amount of virus presented determined by plaque assay on NIH 3T12 fibroblasts. The data shown depict a pattern of egress that is equal for both basolateral and apical shed virus in the k-cyclin mutant was compiled from a single experiment done in triplicate. Standard error of the mean is shown.

CHAPTER 4-SUMMARY OF WORK AND FUTURE DIRECTIONS

This chapter is a summary of Chapters 1-4 and comments on future directions.

The Herpesviridae are a collection of clinically important viruses not only because they can illicit disease, but also due to the fact that they can persist for the lifetime of the host. As such, the gammaherpesviridae are associated with conditions that can be particularly detrimental. Burkitts lymphoma tumors can be notably aggressive in adults older than 60 years of age. Even intensive chemotherapy or rituximab antibody therapy often does not provide much benefit to this population of individuals [143]. Kaposi's sarcoma outcome can be reasonably managed by highly active antiretroviral therapy (HAART) [144,145]. However, certain individuals such as those with systemic KS or advanced AIDS are still likely to have a poor prognostic outcome [144]. The continued need to analyze gammaherpesviruses and unravel the mechanisms by which they cause pathogenesis is often hindered by their strong species specificity. Primate models for EBV and KSHV do exist; however, they often are extremely costly and may be limited in their ability to reproduce key aspects of infection. Such is the case with EBV and its tamarin and marmoset models. These models cannot be infected via EBV's natural mode; the oropharynegeal route [146].

The isolation of the MHV68 virus over 30 years ago, and the sequencing of its genome a decade later, paved the way for MHV68 infection of mice to be established as has an important animal model system. The continued study of MHV68 specific genes and how they influence pathogenesis in the host is constantly providing us with new insights into mechanisms of disease outcome [45]. Particularly important to gammaherpesvirus disease is the ORF72, or viral cyclin. This gene product not only influences virus infection, it can also alter the state of its hosts cellular cycle [84,88,98,121]. Included in this is the understanding of how cyclins may evade restriction by host CKIs. The unique properties displayed by the viral cyclins provide novel biochemical evidence of host evasion [97]. The fact that MHV68s m-cyclin shares high homology to the viral cyclin of KSHV and HVS and shares mechanisms of CDK interaction and CKI restriction warrants continued study of this cyclin and its impact on infection [83,120,121].

Virus replication in the lungs is a distinctive event that is influenced by the state of the host epithelium. In pulmonary epithelium, the polarized state of the cell is characterized by specific properties and expression of adhesion proteins such as E-cadherin and Zona occludens [102-104]. These polarization attributes may influence virus infection [116]. The fact that MHV68's viral cyclin is important for virus replication in the lungs at low dose and this characteristic could not be replicated in conventional tissue culture cell lines, led to the hypothesis that host cell polarization could be an important contributor to the need for m-cyclin [99]. It was predicted that an epithelial cell line that contained many of the properties of primary epithelium, such as the ability to be polarized, would make a more appropriate model than conventional tissue culture lines. This is because epithelial differentiation was speculated to be critical for exposing the virus' need for its cyclin [128] . To test this hypothesis, the RL-65 cell line was chosen due to its demonstrated polarity on transwell culture plates [131,132]. Additionally, their ease of availability from ATCC makes them particularly accessible.

The analysis of RL-65 cells in this study confirmed that the cells asymmetrically position in orientation and that the junctional barriers are firmly intact as indicated by high TER readings and visual inspection of E-cadherin and Z0-1 staining (Fig. 2A, 2B, 2C). As noted in Figure 1, MHV68 requires its viral cyclin to sufficiently replicate in RL-65 cells. Currently, this is the first identified tissue culture model that recapitulates the acute growth in-vivo defect attributed to the cyclin homologue. It was also observed that the need for the cyclin D homologue was most apparent at early times post-infection, specifically between 48 and 96 hours post infection (Figure 1). This would suggest that the need for cyclin is most crucial early on when the cells monolayers are not actively cycling; similar to what was noted previously with an MHV68 TK mutant [128]. The MHV68 TK is also important for replicating in quiescent cells but as of yet has not been implicated in the same pathway as v-cyclin [99]. Currently, the full breadth of cell types in the lung that are infected after intranasal inoculation with MHV68 is not known. It is

possible that the growth defect attributed to v-cyclin after low dose inoculation is related to virus infection of another cell type in addition to epithelium. Additionally, it is not known if any specific immune components of infection influence this CDK dependent phenotype. Therefore, care should be used when interpreting data from these RL-65 cells and making attributions to the specific pathways implicated in the in-vivo growth defect. Nonetheless, the fact that this cell type is unique in providing an environment fit for the v-cyclin mutant to fail to replicate sufficiently warrants the experimental examination.

As mentioned previously, KSHV and MHV68 both possess a homologue to eukaryotic cyclin D. There are several silimilarities as well as differences between the two viral cyclins. Both viral cyclins are by sequence, most similar to cyclin D, and share several conserved residues [97]. They also both have been shown to, in conjunction with their CDK partners, phosphorylate pRb [87,88]. Those CDK partners that they both prefer to interact with, however, are different. K-cyclin prefers CDK 4 or 6 while MHV68's cyclin prefers CDK2 or 1. The different eukaryotic cyclin:CDK complexes are each uniquely involved in different stages of the cell cycle but have been shown to functionally substitute for one another [75,147]. As such, there appears to be much overlap in these cyclin:CDK pathways. As such, it is not clear at this time how important the specific CDKs are in the phenotypes outlined here. It was thought that by generating a MHV68 mutant that encodes the k-cyclin instead of its own, a deeper insight into the conserved functions of these gammaherpesvirus cyclins could be gained. Figure 7B, 8A, and 8B indicate that indeed some properties unique to both viral cyclins are sufficient to rescue the acute growth defect attributed to MHV68 V-cyclin, at least partially. Reactivation from splenocytes at 18 days following intranasal inoculation was not rescued by the k-cyclin mutant virus (Figure 9A). As reactivation is attributed to an unknown CDK independent function of the viral cyclin [99], these results indicate that this property does not appear to be conserved in the k-cyclin under these parameters tested. While conducting these experiments, Lee et.al reported the generation of

a similar MHV68 mutant that was engineered to encode the K-cyclin [148]. In their analysis they analyzed the acute infection in IFN-g$^{-/-}$ mice on BALB/c background with their mutant and found it rescued the ascribed MHV68 cyclin acute growth defect [148] . They further reported that their mutant rescued the reactivation defect attributed to MHV68's cyclin from peritoneal exudates cells (PECs) in C57/Bl6 mice following IP inoculation [148]. Chapter 3 of this manuscript, along with Lee et.al's reported findings together indicate that in the context of an MHV68 acute infection the MHV68 cyclin and k-cyclin can at least partially substitute for each other. Since the virus growth in RL-65 cells appears to mirror aspects of acute replication in the lungs this host cell line should serve useful in future in-vitro studies to elucidate the conserved viral cyclin properties. For virus reactivation, the apparent differences in the ability of the k-cyclin to substitute for the MHV68 cyclin following reactivation from splenocytes or PECs could possibly be indicative of the different cell types latently infected.

Virus egress direction is a preference that is shared within virus families [116]. This directionality can play a role in virus pathogeneisis as with the case of EBV [118,119]. For MHV68, egress hadn't yet been evaluated and it was hoped that the RL-65 transwell set ups could provide useful insights into the viruses egress patterns. Similar to that of EBV, Figure 3B shows that MHV68 can egress in both directions; while having a slight preference for basolateral egress. This suggests that if the same were to occur in the host this pattern would allow access to the underlying basement membrane and means for dissemination through the host. Additionally, basolateral release, in general, can have implications in viral pathogenicity. Certain strains of influenza (H5N1) can infect epithelium apically and shed basolaterally or infect basolaterally have critical and implications on viral dissemination to underling organs and bloodstream [149]. Without the viral cyclin, it was difficult to detect much MHV68 virus egress in either direction over the limit of detection (though values for basolateral egress were marginally higher than apical). It should be noted that these results were from apical infections, as that route of entry

would be most closely correlated to an intranasal inoculation of a host mouse. It is unknown at this time how these MHV68 egress patterns would be different following basolateral infection of RL-65 cells.

Viral titers from the egress experiments in RL-65 cells indicated a dramatically lower amount of virus shed when compared to previous experiments examining total virus growth. (Figures 1 and 3) In an attempt to determine whether there was a disproportionate amount of MHV68 remaining cell associated in this model, cell association was examined in RL-65 transwell setups. Large numbers of MHV68 remains cell associated but even more striking is the apparent proportion of total virus to shed virus from V.cyclin.stop infections. These numbers indicate the v-cyclin may be playing some role in cell to cell spread. Additionally, the m-cyclin CDK binding mutant exhibited egress patterns similar to v.cyclin stop (Figure 5). This suggests the CDK binding ablility, specifically, is an important contributor to MHV68 egress. Other herpesviruses, such as HCMV utilize the cyclin pathway in egress from host cells [115]. Further research would be needed to explore how exactly the v-cyclin is involved in this.

In summary, these experiments have shown the RL-65 cell line to be useful for studying various aspects of MHV68 infection particularly related to the viral cyclin mutants including the k-cyclin virus. They are also useful in analysis of virus egress, and mirror aspects of MHV68 in-vivo acute replication seen in mice.

REFERENCES:

1. Fields BN, Knipe DM, Howley PM Fields' virology. 2 volumes p.
2. Strauss JH, Strauss EG Viruses and human disease. vii, 383 pages p.
3. Flint SJ (2004) Principles of virology : molecular biology, pathogenesis, and control of animal viruses. Washington, D.C.: ASM Press. xxvi, 918 p. p.
4. Arvin A, Abendroth A (2007) VZV: immunobiology and host response. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge.
5. McGeoch DJ, Rixon FJ, Davison AJ (2006) Topics in herpesvirus genomics and evolution. Virus Res 117: 90-104.
6. Farley CA, Banfield WG, Kasnic G, Jr., Foster WS (1972) Oyster herpes-type virus. Science 178: 759-760.
7. Dohner K, Wolfstein A, Prank U, Echeverri C, Dujardin D, et al. (2002) Function of dynein and dynactin in herpes simplex virus capsid transport. Mol Biol Cell 13: 2795-2809.
8. Sinclair J (2008) Human cytomegalovirus: Latency and reactivation in the myeloid lineage. J Clin Virol 41: 180-185.
9. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, et al. (2009) The order Herpesvirales. Arch Virol 154: 171-177.
10. Nemerow GR, Mold C, Schwend VK, Tollefson V, Cooper NR (1987) Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. J Virol 61: 1416-1420.
11. Tanner J, Whang Y, Sample J, Sears A, Kieff E (1988) Soluble gp350/220 and deletion mutant glycoproteins block Epstein-Barr virus adsorption to lymphocytes. J Virol 62: 4452-4464.
12. Nemerow GR, Wolfert R, McNaughton ME, Cooper NR (1985) Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). J Virol 55: 347-351.
13. Akula SM, Pramod NP, Wang FZ, Chandran B (2002) Integrin alpha3beta1 (CD 49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. Cell 108: 407-419.
14. Kaleeba JA, Berger EA (2006) Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT. Science 311: 1921-1924.
15. Nemerow GR, Cooper NR (1984) Early events in the infection of human B lymphocytes by Epstein-Barr virus: the internalization process. Virology 132: 186-198.
16. Gwack Y, Hwang S, Lim C, Won YS, Lee CH, et al. (2002) Kaposi's Sarcoma-associated herpesvirus open reading frame 50 stimulates the transcriptional activity of STAT3. J Biol Chem 277: 6438-6442.
17. Izumi KM, Kieff ED (1997) The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to

mediate B lymphocyte growth transformation and activate NF-kappaB. Proc Natl Acad Sci U S A 94: 12592-12597.
18. Wang D, Liebowitz D, Kieff E (1985) An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 43: 831-840.
19. Ballestas ME, Kaye KM (2001) Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. J Virol 75: 3250-3258.
20. Gradoville L, Gerlach J, Grogan E, Shedd D, Nikiforow S, et al. (2000) Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire viral lytic cycle in the HH-B2 primary effusion lymphoma cell line. J Virol 74: 6207-6212.
21. Jenkins PJ, Binne UK, Farrell PJ (2000) Histone acetylation and reactivation of Epstein-Barr virus from latency. J Virol 74: 710-720.
22. Henle G, Henle W, Clifford P, Diehl V, Kafuko GW, et al. (1969) Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J Natl Cancer Inst 43: 1147-1157.
23. Crawford DH, Macsween KF, Higgins CD, Thomas R, McAulay K, et al. (2006) A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis. Clin Infect Dis 43: 276-282.
24. Hoshino Y, Morishima T, Kimura H, Nishikawa K, Tsurumi T, et al. (1999) Antigen-driven expansion and contraction of CD8+-activated T cells in primary EBV infection. J Immunol 163: 5735-5740.
25. Wright-Browne V, Schnee AM, Jenkins MA, Thall PF, Aggarwal BB, et al. (1998) Serum cytokine levels in infectious mononucleosis at diagnosis and convalescence. Leuk Lymphoma 30: 583-589.
26. Carbone A (2003) Emerging pathways in the development of AIDS-related lymphomas. Lancet Oncol 4: 22-29.
27. Hamilton-Dutoit SJ, Rea D, Raphael M, Sandvej K, Delecluse HJ, et al. (1993) Epstein-Barr virus-latent gene expression and tumor cell phenotype in acquired immunodeficiency syndrome-related non-Hodgkin's lymphoma. Correlation of lymphoma phenotype with three distinct patterns of viral latency. Am J Pathol 143: 1072-1085.
28. Antman K, Chang Y (2000) Kaposi's sarcoma. N Engl J Med 342: 1027-1038.
29. Staskus KA, Zhong W, Gebhard K, Herndier B, Wang H, et al. (1997) Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J Virol 71: 715-719.
30. Hong YK, Foreman K, Shin JW, Hirakawa S, Curry CL, et al. (2004) Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. Nat Genet 36: 683-685.
31. Sodhi A, Montaner S, Patel V, Zohar M, Bais C, et al. (2000) The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res 60: 4873-4880.
32. Montaner S, Sodhi A, Molinolo A, Bugge TH, Sawai ET, et al. (2003) Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. Cancer Cell 3: 23-36.
33. Magrath I (1990) The pathogenesis of Burkitt's lymphoma. Adv Cancer Res 55: 133-270.
34. Moormann AM, Snider CJ, Chelimo K (2011) The company malaria keeps: how co-infection with Epstein-Barr virus leads to endemic Burkitt lymphoma. Curr Opin Infect Dis 24: 435-441.

35. Wolf H, zur Hausen H, Becker V (1973) EB viral genomes in epithelial nasopharyngeal carcinoma cells. Nat New Biol 244: 245-247.
36. Falk LA, Wolfe LG, Deinhardt F (1972) Isolation of Herpesvirus saimiri from blood of squirrel monkeys (Saimiri sciureus). J Natl Cancer Inst 48: 1499-1505.
37. Fickenscher H, Fleckenstein B (2001) Herpesvirus saimiri. Philos Trans R Soc Lond B Biol Sci 356: 545-567.
38. Damania B (2004) Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis. Nat Rev Microbiol 2: 656-668.
39. Blaskovic D, Stancekova M, Svobodova J, Mistrikova J (1980) Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol 24: 468.
40. Efstathiou S, Ho YM, Hall S, Styles CJ, Scott SD, et al. (1990) Murine herpesvirus 68 is genetically related to the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. J Gen Virol 71 (Pt 6): 1365-1372.
41. Efstathiou S, Ho YM, Minson AC (1990) Cloning and molecular characterization of the murine herpesvirus 68 genome. J Gen Virol 71 (Pt 6): 1355-1364.
42. Virgin HWt, Latreille P, Wamsley P, Hallsworth K, Weck KE, et al. (1997) Complete sequence and genomic analysis of murine gammaherpesvirus 68. J Virol 71: 5894-5904.
43. Nash AA, Dutia BM, Stewart JP, Davison AJ (2001) Natural history of murine gamma-herpesvirus infection. Philos Trans R Soc Lond B Biol Sci 356: 569-579.
44. Sunil-Chandra NP, Efstathiou S, Arno J, Nash AA (1992) Virological and pathological features of mice infected with murine gamma-herpesvirus 68. J Gen Virol 73 (Pt 9): 2347-2356.
45. Barton E, Mandal P, Speck SH (2011) Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu Rev Immunol 29: 351-397.
46. Flano E, Husain SM, Sample JT, Woodland DL, Blackman MA (2000) Latent murine gamma-herpesvirus infection is established in activated B cells, dendritic cells, and macrophages. J Immunol 165: 1074-1081.
47. Tripp RA, Hamilton-Easton AM, Cardin RD, Nguyen P, Behm FG, et al. (1997) Pathogenesis of an infectious mononucleosis-like disease induced by a murine gamma-herpesvirus: role for a viral superantigen? J Exp Med 185: 1641-1650.
48. Sunil-Chandra NP, Arno J, Fazakerley J, Nash AA (1994) Lymphoproliferative disease in mice infected with murine gammaherpesvirus 68. Am J Pathol 145: 818-826.
49. Ebrahimi B, Dutia BM, Brownstein DG, Nash AA (2001) Murine gammaherpesvirus-68 infection causes multi-organ fibrosis and alters leukocyte trafficking in interferon-gamma receptor knockout mice. Am J Pathol 158: 2117-2125.
50. Barton ES, Lutzke ML, Rochford R, Virgin HWt (2005) Alpha/beta interferons regulate murine gammaherpesvirus latent gene expression and reactivation from latency. J Virol 79: 14149-14160.
51. Adler H, Messerle M, Wagner M, Koszinowski UH (2000) Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J Virol 74: 6964-6974.
52. Fowler P, Marques S, Simas JP, Efstathiou S (2003) ORF73 of murine herpesvirus-68 is critical for the establishment and maintenance of latency. J Gen Virol 84: 3405-3416.
53. Moorman NJ, Willer DO, Speck SH (2003) The gammaherpesvirus 68 latency-associated nuclear antigen homolog is critical for the establishment of splenic latency. J Virol 77: 10295-10303.

54. Paden CR, Forrest JC, Moorman NJ, Speck SH (2010) Murine gammaherpesvirus 68 LANA is essential for virus reactivation from splenocytes but not long-term carriage of viral genome. J Virol 84: 7214-7224.
55. Collins CM, Boss JM, Speck SH (2009) Identification of infected B-cell populations by using a recombinant murine gammaherpesvirus 68 expressing a fluorescent protein. J Virol 83: 6484-6493.
56. Collins CM, Speck SH (2012) Tracking murine gammaherpesvirus 68 infection of germinal center B cells in vivo. PLoS One 7: e33230.
57. Lodish HF Molecular cell biology. 1 volume (various pagings) p.
58. Coppock DL, Kopman C, Scandalis S, Gilleran S (1993) Preferential gene expression in quiescent human lung fibroblasts. Cell Growth Differ 4: 483-493.
59. Coller HA (2007) What's taking so long? S-phase entry from quiescence versus proliferation. Nat Rev Mol Cell Biol 8: 667-670.
60. Blagosklonny MV (2006) Cell senescence: hypertrophic arrest beyond the restriction point. J Cell Physiol 209: 592-597.
61. Coverley D, Laman H, Laskey RA (2002) Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat Cell Biol 4: 523-528.
62. Roussel MF (1999) The INK4 family of cell cycle inhibitors in cancer. Oncogene 18: 5311-5317.
63. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501-1512.
64. Murray AW (2004) Recycling the cell cycle: cyclins revisited. Cell 116: 221-234.
65. Morgan DO (2007) The cell cycle : principles of control. London

Sunderland, MA: New Science Press ;

Sinauer Associates. xxvii, 297 p. p.
66. Nugent JH, Alfa CE, Young T, Hyams JS (1991) Conserved structural motifs in cyclins identified by sequence analysis. J Cell Sci 99 (Pt 3): 669-674.
67. Kozar K, Ciemerych MA, Rebel VI, Shigematsu H, Zagozdzon A, et al. (2004) Mouse development and cell proliferation in the absence of D-cyclins. Cell 118: 477-491.
68. Geng Y, Whoriskey W, Park MY, Bronson RT, Medema RH, et al. (1999) Rescue of cyclin D1 deficiency by knockin cyclin E. Cell 97: 767-777.
69. Parisi T, Beck AR, Rougier N, McNeil T, Lucian L, et al. (2003) Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. EMBO J 22: 4794-4803.
70. Murphy M, Stinnakre MG, Senamaud-Beaufort C, Winston NJ, Sweeney C, et al. (1997) Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. Nat Genet 15: 83-86.
71. Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, et al. (1998) Cyclin A1 is required for meiosis in the male mouse. Nat Genet 20: 377-380.
72. Brandeis M, Rosewell I, Carrington M, Crompton T, Jacobs MA, et al. (1998) Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. Proc Natl Acad Sci U S A 95: 4344-4349.
73. Malumbres M, Harlow E, Hunt T, Hunter T, Lahti JM, et al. (2009) Cyclin-dependent kinases: a family portrait. Nat Cell Biol 11: 1275-1276.

74. Tsutsui T, Hesabi B, Moons DS, Pandolfi PP, Hansel KS, et al. (1999) Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol Cell Biol 19: 7011-7019.
75. Satyanarayana A, Kaldis P (2009) Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene 28: 2925-2939.
76. Santamaria D, Barriere C, Cerqueira A, Hunt S, Tardy C, et al. (2007) Cdk1 is sufficient to drive the mammalian cell cycle. Nature 448: 811-815.
77. Low KG, Dorner LF, Fernando DB, Grossman J, Jeang KT, et al. (1997) Human T-cell leukemia virus type 1 Tax releases cell cycle arrest induced by p16INK4a. J Virol 71: 1956-1962.
78. Re F, Braaten D, Franke EK, Luban J (1995) Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. J Virol 69: 6859-6864.
79. Boyer SN, Wazer DE, Band V (1996) E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res 56: 4620-4624.
80. Bresnahan WA, Albrecht T, Thompson EA (1998) The cyclin E promoter is activated by human cytomegalovirus 86-kDa immediate early protein. J Biol Chem 273: 22075-22082.
81. Pajovic S, Wong EL, Black AR, Azizkhan JC (1997) Identification of a viral kinase that phosphorylates specific E2Fs and pocket proteins. Mol Cell Biol 17: 6459-6464.
82. Bresnahan WA, Thompson EA, Albrecht T (1997) Human cytomegalovirus infection results in altered Cdk2 subcellular localization. J Gen Virol 78 (Pt 8): 1993-1997.
83. van Dyk LF, Hess JL, Katz JD, Jacoby M, Speck SH, et al. (1999) The murine gammaherpesvirus 68 v-cyclin gene is an oncogene that promotes cell cycle progression in primary lymphocytes. J Virol 73: 5110-5122.
84. Jung JU, Stager M, Desrosiers RC (1994) Virus-encoded cyclin. Mol Cell Biol 14: 7235-7244.
85. Chang PC, Li M (2008) Kaposi's sarcoma-associated herpesvirus K-cyclin interacts with Cdk9 and stimulates Cdk9-mediated phosphorylation of p53 tumor suppressor. J Virol 82: 278-290.
86. Li M, Lee H, Yoon DW, Albrecht JC, Fleckenstein B, et al. (1997) Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. J Virol 71: 1984-1991.
87. Godden-Kent D, Talbot SJ, Boshoff C, Chang Y, Moore P, et al. (1997) The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J Virol 71: 4193-4198.
88. Upton JW, van Dyk LF, Speck SH (2005) Characterization of murine gammaherpesvirus 68 v-cyclin interactions with cellular cdks. Virology 341: 271-283.
89. Swanton C, Mann DJ, Fleckenstein B, Neipel F, Peters G, et al. (1997) Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature 390: 184-187.
90. Ojala PM, Yamamoto K, Castanos-Velez E, Biberfeld P, Korsmeyer SJ, et al. (2000) The apoptotic v-cyclin-CDK6 complex phosphorylates and inactivates Bcl-2. Nat Cell Biol 2: 819-825.
91. Laman H, Coverley D, Krude T, Laskey R, Jones N (2001) Viral cyclin-cyclin-dependent kinase 6 complexes initiate nuclear DNA replication. Mol Cell Biol 21: 624-635.
92. Saha A, Halder S, Upadhyay SK, Lu J, Kumar P, et al. (2011) Epstein-Barr virus nuclear antigen 3C facilitates G1-S transition by stabilizing and enhancing the function of cyclin D1. PLoS Pathog 7: e1001275.
93. Allen RD, 3rd, DeZalia MN, Speck SH (2007) Identification of an Rta responsive promoter involved in driving gammaHV68 v-cyclin expression during virus replication. Virology 365: 250-259.

94. Martinez-Guzman D, Rickabaugh T, Wu TT, Brown H, Cole S, et al. (2003) Transcription program of murine gammaherpesvirus 68. J Virol 77: 10488-10503.
95. Ebrahimi B, Dutia BM, Roberts KL, Garcia-Ramirez JJ, Dickinson P, et al. (2003) Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J Gen Virol 84: 99-109.
96. Allen RD, 3rd, Dickerson S, Speck SH (2006) Identification of spliced gammaherpesvirus 68 LANA and v-cyclin transcripts and analysis of their expression in vivo during latent infection. J Virol 80: 2055-2062.
97. Card GL, Knowles P, Laman H, Jones N, McDonald NQ (2000) Crystal structure of a gamma-herpesvirus cyclin-cdk complex. EMBO J 19: 2877-2888.
98. van Dyk LF, Virgin HWt, Speck SH (2000) The murine gammaherpesvirus 68 v-cyclin is a critical regulator of reactivation from latency. J Virol 74: 7451-7461.
99. Upton JW, Speck SH (2006) Evidence for CDK-dependent and CDK-independent functions of the murine gammaherpesvirus 68 v-cyclin. J Virol 80: 11946-11959.
100. Liang X, Paden CR, Morales FM, Powers RP, Jacob J, et al. (2011) Murine gamma-herpesvirus immortalization of fetal liver-derived B cells requires both the viral cyclin D homolog and latency-associated nuclear antigen. PLoS Pathog 7: e1002220.
101. Suarez AL, van Dyk LF (2008) Endothelial cells support persistent gammaherpesvirus 68 infection. PLoS Pathog 4: e1000152.
102. Kazmierczak BI, Mostov K, Engel JN (2001) Interaction of bacterial pathogens with polarized epithelium. Annu Rev Microbiol 55: 407-435.
103. Bryant DM, Mostov KE (2008) From cells to organs: building polarized tissue. Nat Rev Mol Cell Biol 9: 887-901.
104. Harris TJ, Tepass U (2010) Adherens junctions: from molecules to morphogenesis. Nat Rev Mol Cell Biol 11: 502-514.
105. Stevens T (2008) Epithelium: sticking it out, together. Am J Physiol Lung Cell Mol Physiol 294: L440-441.
106. Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103: 755-766.
107. Hartsock A, Nelson WJ (2008) Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 1778: 660-669.
108. Bauer H, Zweimueller-Mayer J, Steinbacher P, Lametschwandtner A, Bauer HC (2010) The dual role of zonula occludens (ZO) proteins. J Biomed Biotechnol 2010: 402593.
109. Zhong P, Agosto LM, Munro JB, Mothes W (2013) Cell-to-cell transmission of viruses. Current Opinion in Virology 3: 44-50.
110. Pearcepratt R, Malamud D, Phillips DM (1994) Role of the Cytoskeleton in Cell-to-Cell Transmission of Human-Immunodeficiency-Virus. Journal of Virology 68: 2898-2905.
111. Perotti ME, Tan X, Phillips DM (1996) Directional budding of human immunodeficiency virus from monocytes. Journal of Virology 70: 5916-5921.
112. Nguyen DH, Hildreth JEK (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. Journal of Virology 74: 3264-3272.
113. Johnson DC, Huber MT (2002) Directed egress of animal viruses promotes cell-to-cell spread. Journal of Virology 76: 1-8.
114. Reeves PM, Bommarius B, Lebeis S, McNulty S, Christensen J, et al. (2005) Disabling poxvirus pathogenesis by inhibition of Abl-family tyrosine kinases (vol 11, pg 731, 2005). Nature Medicine 11: 1361-1361.

115. Hamirally S, Kamil JP, Ndassa-Colday YM, Lin AJ, Jahng WJ, et al. (2009) Viral Mimicry of Cdc2/Cyclin-Dependent Kinase 1 Mediates Disruption of Nuclear Lamina during Human Cytomegalovirus Nuclear Egress. Plos Pathogens 5.
116. Blau DM, Compans RW (1996) Polarization of viral entry and release in epithelial cells. Seminars in Virology 7: 245-253.
117. Chodosh J, Gan YJ, Holder VP, Sixbey JW (2000) Patterned entry and egress by Epstein-Barr virus in polarized CR2-positive epithelial cells. Virology 266: 387-396.
118. Tugizov SM, Berline JW, Palefsky JM (2003) Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells (vol 9, pg 307, 2003). Nature Medicine 9: 477-477.
119. Tugizov SM, Herrera R, Palefsky JM (2013) Epstein-Barr Virus Transcytosis through Polarized Oral Epithelial Cells. Journal of Virology 87: 8179-8194.
120. Verschuren EW, Jones N, Evan GI (2004) The cell cycle and how it is steered by Kaposi's sarcoma-associated herpesvirus cyclin. J Gen Virol 85: 1347-1361.
121. Swanton C, Jones N (2001) Strategies in subversion: de-regulation of the mammalian cell cycle by viral gene products. Int J Exp Pathol 82: 3-13.
122. Rajcani J, Blaskovic D, Svobodova J, Ciampor F, Huckova D, et al. (1985) Pathogenesis of acute and persistent murine herpesvirus infection in mice. Acta Virol 29: 51-60.
123. Terry LA, Stewart JP, Nash AA, Fazakerley JK (2000) Murine gammaherpesvirus-68 infection of and persistence in the central nervous system. J Gen Virol 81: 2635-2643.
124. Tarakanova VL, Suarez F, Tibbetts SA, Jacoby MA, Weck KE, et al. (2005) Murine gammaherpesvirus 68 infection is associated with lymphoproliferative disease and lymphoma in BALB beta2 microglobulin-deficient mice. J Virol 79: 14668-14679.
125. Dal Canto AJ, Virgin HWt, Speck SH (2000) Ongoing viral replication is required for gammaherpesvirus 68-induced vascular damage. J Virol 74: 11304-11310.
126. Lee KS, Cool CD, van Dyk LF (2009) Murine gammaherpesvirus 68 infection of gamma interferon-deficient mice on a BALB/c background results in acute lethal pneumonia that is dependent on specific viral genes. J Virol 83: 11397-11401.
127. Mora AL, Torres-Gonzalez E, Rojas M, Xu J, Ritzenthaler J, et al. (2007) Control of virus reactivation arrests pulmonary herpesvirus-induced fibrosis in IFN-gamma receptor-deficient mice. Am J Respir Crit Care Med 175: 1139-1150.
128. Coleman HM, de Lima B, Morton V, Stevenson PG (2003) Murine gammaherpesvirus 68 lacking thymidine kinase shows severe attenuation of lytic cycle replication in vivo but still establishes latency. J Virol 77: 2410-2417.
129. Caldon CE, Sutherland RL, Musgrove E (2010) Cell cycle proteins in epithelial cell differentiation: implications for breast cancer. Cell Cycle 9: 1918-1928.
130. Nelson WJ (2009) Remodeling epithelial cell organization: transitions between front-rear and apical-basal polarity. Cold Spring Harb Perspect Biol 1: a000513.
131. Wang XQ, Li H, Van Putten V, Winn RA, Heasley LE, et al. (2009) Oncogenic K-Ras regulates proliferation and cell junctions in lung epithelial cells through induction of cyclooxygenase-2 and activation of metalloproteinase-9. Mol Biol Cell 20: 791-800.
132. Roberts PE, Phillips DM, Mather JP (1990) A novel epithelial cell from neonatal rat lung: isolation and differentiated phenotype. Am J Physiol 259: L415-425.
133. Ryeom SW, Paul D, Goodenough DA (2000) Truncation mutants of the tight junction protein ZO-1 disrupt corneal epithelial cell morphology. Mol Biol Cell 11: 1687-1696.
134. Xie Z, Samuels BA, Tsai LH (2006) Cyclin-dependent kinase 5 permits efficient cytoskeletal remodeling--a hypothesis on neuronal migration. Cereb Cortex 16 Suppl 1: i64-68.

135. Hatakeyama M, Weinberg RA (1995) The role of RB in cell cycle control. Prog Cell Cycle Res 1: 9-19.
136. Lin SF, Robinson DR, Miller G, Kung HJ (1999) Kaposi's sarcoma-associated herpesvirus encodes a bZIP protein with homology to BZLF1 of Epstein-Barr virus. J Virol 73: 1909-1917.
137. Polson AG, Huang L, Lukac DM, Blethrow JD, Morgan DO, et al. (2001) Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. J Virol 75: 3175-3184.
138. Park J, Seo T, Hwang S, Lee D, Gwack Y, et al. (2000) The K-bZIP protein from Kaposi's sarcoma-associated herpesvirus interacts with p53 and represses its transcriptional activity. J Virol 74: 11977-11982.
139. Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, et al. (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol 15: 3032-3040.
140. Sugaya M, Watanabe T, Yang A, Starost MF, Kobayashi H, et al. (2005) Lymphatic dysfunction in transgenic mice expressing KSHV k-cyclin under the control of the VEGFR-3 promoter. Blood 105: 2356-2363.
141. Verschuren EW, Hodgson JG, Gray JW, Kogan S, Jones N, et al. (2004) The role of p53 in suppression of KSHV cyclin-induced lymphomagenesis. Cancer Res 64: 581-589.
142. Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, et al. (1997) Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. Mol Cell Biol 17: 5338-5347.
143. Castillo JJ, Nadeem O (2014) Improving the accuracy in prognosis for Burkitt lymphoma patients. Expert Rev Anticancer Ther 14: 125-127.
144. Radu O, Pantanowitz L (2013) Kaposi sarcoma. Arch Pathol Lab Med 137: 289-294.
145. Martin-Carbonero L, Palacios R, Valencia E, Saballs P, Sirera G, et al. (2008) Long-term prognosis of HIV-infected patients with Kaposi sarcoma treated with pegylated liposomal doxorubicin. Clin Infect Dis 47: 410-417.
146. Hayashi K, Teramoto N, Akagi T (2002) Animal in vivo models of EBV-associated lymphoproliferative diseases: special references to rabbit models. Histol Histopathol 17: 1293-1310.
147. Sherr CJ, Roberts JM (2004) Living with or without cyclins and cyclin-dependent kinases. Genes Dev 18: 2699-2711.
148. Lee KS, Suarez AL, Claypool DJ, Armstrong TK, Buckingham EM, et al. (2012) Viral cyclins mediate separate phases of infection by integrating functions of distinct mammalian cyclins. PLoS Pathog 8: e1002496.
149. Chan MC, Chan RW, Yu WC, Ho CC, Chui WH, et al. (2009) Influenza H5N1 virus infection of polarized human alveolar epithelial cells and lung microvascular endothelial cells. Respir Res 10: 102.