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MOLECULAR DETERMINANTS OF
LATENCY-ASSOCIATED NUCLEAR ANTIGEN
FUNCTION IN GAMMAHERPESVIRUS PATHOGENESIS

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MOLECULAR DETERMINANTS OF LATENCY-ASSOCIATED NUCLEAR ANTIGEN FUNCTION IN GAMMAHERPESVIRUS PATHOGENESIS

By

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B.S, Vanderbilt University, 2005

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Abstract

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Gammaherpesviruses establish a lifelong latent infection in the host and are associated with a myriad of lymphoprolilferative disorders. Kaposi's sarcoma-associated herpesvirus (KSHV) is tightly associated with the AIDS-defining illness Kaposi's Sarcoma, as well as other diseases. The Latency-Associated Nuclear Antigen (LANA) is the only viral gene expressed in every KSHV-associated malignancy, so it is of great interest in the gammaherpesvirus field as a candidate viral oncogene. However, due to the tight species-specificity of the herpesviruses, little is known about its role in infection, before and during transition to disease.

Murine gammaherpesvirus 68 (MHV68) is a now well-described rodent pathogen we can use to do controlled pathogenesis experiments. In this dissertation, I describe my work on the MHV68 LANA (mLANA) during natural infection.

We found that mLANA is expressed throughout lytic infection and that it regulates the cascade of lytic gene expression required for efficient virus replication. In the absence of mLANA, there is a 90-99% reduction in output virus.

Previously, our lab determined that mLANA was necessary to establish latency after intranasal infection, supporting hypotheses based on observations from KSHV. To extend this work, we infected mice intraperitoneally with an mLANA-null virus to expose the natural latency reservoir to the virus. We found the mLANA-null virus actually establishes a chronic infection, but it is incapable of reactivating from those cells. Further, we demonstrated that the mLANA-null genome is not episomal, but the virus apparently integrates into the host chromosome. Despite that unusual arrangement, the mLANA-null virus expressed latency genes similar to wild type, causing us to rethink traditional descriptions and molecular definitions of latency

Of the many described functions of LANA, we chose to examine its role as a transcriptional modulator. We generated a library of random point mutants and designed an assay to screen them for loss of function. Many of these mutations occurred in a conserved region of LANA genes that is predicted to be a DNA-binding domain. Notably, each of these mutations, when cloned into the virus, results in mLANA-null phenotypes, leading us to hypothesize that this LANA function is absolutely essential.

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CHAPTER 1.

Introduction

A) The Herpesviruses

i. Background

a. Basic information

The *Herpesviridae* family is comprised of a large number of viruses, and members of this family are thought to infect all vertebrate species. Herpesviruses are characterized by their capacity to establish lifelong infections of their host through the establishment of a quiescent latent infection in specific cell types. The double-stranded DNA genomes of the herpesviruses range in size from around 100 to 250 kilobases, and encode an estimated 70 to over 100 genes. As with other viruses, the viral genome is packaged within a virally-encoded protein capsid. In addition, the viral capsid is enveloped in a lipid bilayer that is studded with viral glycoproteins, some of which serve to facilitate attachment and entry of virus into host cells (Pellett and Roizman 2006).

Herpesvirus genomes are comprised of one or more large regions of non-repeating sequence (which encode most of the viral genes), termed unique sequence, and one or more regions of repeated sequence. The ends of herpesviral DNA genomes contain multiple copies of a GC-rich sequence (terminal repeats), which recombine upon cell entry, forming a closed circular genome. This circular genome is subsequently the substrate for rolling-circle DNA replication, or, in cells where latency is established, the circular genome persists as an extra-chromosomal episome that replicates during mitosis along with the host genome (Kieff and Rickenson 2006; Pellett and Roizman 2006).

b. Taxonomy

The *Herpesviridae* family is divided into three subfamilies: alpha-, beta-, and gammaherpesviruses, based on both general cellular tropism (i.e., where they establish latency) and genetic similarity. The alphaherpesviruses are typically neurotropic, and they are represented by viruses such as Herpes Simplex Virus (HSV)-1 and HSV-2. The betaherpesviruses are broad in their tropism. Some, like cytomegalovirus (CMV), establish latency primarily in leukocytes (e.g., monocytes), while others, such as Human Herpesvirus (HHV)-6, can establish latency in neuronal tissue. The gammaherpesviruses, which include Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), establish latency primarily in lymphocytes (Pellett and Roizman 2006; Davison *et al.* 2009).

Based on sequence homology, the gammaherpesvirus subfamily is further divided into lymphocryptoviruses and rhadinoviruses (additionally, two more recent groups have been proposed: macaviruses and percaviruses) (Davison *et al.* 2009). The lymphocryptoviruses include EBV, as well as the primate virus Rhesus lymphocryptovirus (RhLCV). The rhadinoviruses include KSHV, the primate viruses Herpesvirus saimiri (HVS) and Rhesus rhadinovirus (RRV), and the rodent pathogen Murine gammaherpesvirus 68 (MHV68). With the exception of HVS, all of these viruses primarily establish long-term latency in B cells (HVS establishes latency in T lymphocytes) (Pellett and Roizman 2006; Davison *et al.* 2009).

b. Replication and Life Cycle

All viruses go through a productive replication, or lytic, cycle. Productive replication consists of the orderly expression of a cascade of viral proteins, replication of the viral genome, and assembly and release of infections virus particles. Herpesviruses are no different in this respect, and they have three distinct temporally-regulated sets of genes. Upon entry into the cell, the immediate-early (IE or α) genes are expressed. This set includes genes whose products prepare the cell for infection and those that activate transcription of other cellular and viral genes. They are defined by examining virus transcripts active after virus infection in the presence of a protein synthesis inhibitor such as cycloheximide or anisomycin. The set of viral genes activated by IE gene expression are termed the early (E or β) genes and typically include those gene products required for viral genome replication (e.g., the viral DNA-dependent DNA polymerase). These genes are defined as those that are expressed after infection in the presence of a viral DNA synthesis inhibitor (e.g., phosphonoacetic acid), but not in the presence of cycloheximide. The remaining genes, the late (L or γ) genes, typically encode proteins involved in virus assembly (e.g., capsid proteins, surface glycoproteins, and other gene products required for assembly, packaging, and release of virions). For the most part, late genes are expressed during or after viral DNA synthesis has initiated (Kieff and Rickenson 2006; Knipe *et al.* 2006; Pellett and Roizman 2006).

Herpesviruses, similar to polyomaviruses and, in some ways, to lysogenic bacteriophages, have the capacity to enter a quiescent phase called latency. During herpesvirus latency, the viral genome is maintained as a nuclear episome in the absence of any synthesis of infectious virions. Typically, latency is established in long-lived,

slowly-replicating cells. For example, HSV-1 and EBV establish long-term latency in neurons and memory B cells, respectively. With some herpesviruses, such as EBV, the virus genome retains the capacity replicate along with the host cell DNA during S phase. Some EBV genes, such as EBNA-1, are hypothesized to be involved in this process, but their role(s) have not been explicitly defined (Kieff and Rickenson 2006; Knipe *et al.* 2006; Pellett and Roizman 2006).

Due to host immune pressures, very few, if any, viral proteins are expressed during latency. At early times of gammaherpesvirus latency, there are sets of genes that are expressed and progressively shut off as the virus directs the infected B lymphocyte into the memory reservoir (Speck and Ganem 2010). Another feature of latency is the capacity of the viral genome to return to a state of productive replication, a process called reactivation. Cues for the virus to undergo reactivation are thought to be primarily stress-related, but they have not been comprehensively defined. Reactivation may be induced in cell culture by a variety of signals, ranging from 12-O-Tetradecanoylphorbol-13-acetate (TPA) treatment, to DNA methyltransferase inhibitors, to DNA damaging agents such as etoposide, to cross-linking the B cell receptor, all of which activate various cellular pathways (Daibata *et al.* 1990; Robertson *et al.* 1995; Yu *et al.* 1999).

ii. Human Gammaherpesviruses: Pathogenesis and Disease Associations

a. Epstein-Barr Virus

There are two known human gammaherpesviruses: Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus [KSHV; also know as human herpesvirus 8 (HHV8)]. Gammaherpesvirus infections are frequently asymptomatic, both during

primary infection and throughout the course of life-long chronic infection where the virus largely persists in latently infected cells (Crawford 2001). However, primary EBV infection, particularly in adolescents, can lead to a severe mononucleosis syndrome (Henle *et al.* 1968). Little is known about primary KSHV infection in humans.

Importantly, there are a significant number of malignancies that are associated with latent gammaherpesvirus infections. In the case of EBV, chronic infection is associated with Hodgkin's disease, nasopharyngeal carcinoma, Burkitt's lymphoma, breast and gastric carcinomas, and body cavity-based lymphomas (BCBLs) (Epstein *et al.* 1964; Raab-Traub *et al.* 1987; Weiss *et al.* 1989; Magrath 1990; Preciado *et al.* 1995; Bonnet *et al.* 1999; Yanai *et al.* 1999; Koriyama *et al.* 2001). Despite 95% of the population of the United States being latently infected with EBV, EBV-related diseases are rare in all but a few subsets of the population. Those at risk for Burkitt's lymphoma, for example, are those people who are on immunosuppressive therapy, have AIDS, or live in areas where *Plasmodium falciparum* malaria is hyperendemic. Though these diseases are relatively uncommon, prognosis, until recently has been poor. In the United States, though, the quality of treatment is steadily increasing with the development of new chemotherapy and antiviral therapeutics (Perkins and Friedberg 2008).

EBV can readily immortalize human B cells, and this process has been a focus of research on EBV for over 30 years. Myriad studies have been done to understand how EBV transforms cells, what viral genes are involved, and how the cell and virus coexist (Henle *et al.* 1968; Speck and Ganem 2010). It is now known that expression of a set of EBV latency-associated genes is activated upon *in vitro* infection of B cells, giving rise to the establishment of the "growth program," which leads to B cell immortalization.

However, while early studies on the role of EBV in Burkitt's lymphoma speculated that EBV B cell immortalization was directly linked to lymphomagenesis, careful characterization of Burkitt's lymphoma tumors revealed a very restricted pattern of EBV latency-associated gene expression (EBNA1-only program) that was quite distinct from the "growth program" (Rowe *et al.* 1987). This has led to a deeper understanding of the relationship between EBV infection and B cell differentiation. Notably, a reduced requirement for viral pro-growth factors is likely due to the hallmark chromosomal translocation present in Burkitt's lymphoma tumors, not found in EBV *in vitro*-immortalized B cells, which involves translocation of the pro-growth c-*myc* gene into the either the immunoglobulin heavy chain or light chain locus (Taub *et al.* 1982; Okubo *et al.* 2001; Takahashi *et al.* 2003).

b. Kaposi's Sarcoma-associated Herpesvirus (KSHV/HHV-8)

KSHV, a more recently discovered gammaherpesvirus, is the etiological agent of Kaposi's Sarcoma (KS), HIV-associated Castleman's Disease, and Primary Effusion Lymphoma (PEL)—a rare B cell lymphoma generally only observed in AIDS patients (Chang *et al.* 1994; Cesarman *et al.* 1995a; Boshoff and Weiss 1998; Laurent *et al.* 2008). Kaposi's Sarcoma is an unusual disorder marked by purplish discoloration of the skin, first described in elderly Mediterranean men in the 1870's (Kaposi 1872). The disease became more prominent as it began to be observed in apparently healthy, young gay men in the United States, concurrent with the appearance of the AIDS pandemic in the US (Jaffe *et al.* 1983). In the 1980's and 90's, this non-classical Kaposi's Sarcoma was recognized as an AIDS-defining condition, one of a few illnesses to which a large

percentage of AIDS patients succumbed. Studies showed that KS, alone or in concert with other opportunistic pathogens, was involved in 17-22% of AIDS-related deaths (Rothenberg *et al.* 1987; Stewart *et al.* 2012). Thus, much effort has been put into understanding KS, and the discovery of herpesvirus sequences in these malignancies was an important step forward (Chang *et al.* 1994). That discovery paved the way for greatly expanded interest in the pathogenesis of rhadinoviruses.

KS is an unusual malignancy in that it is a virally-induced angioproliferative disease of a polyclonal nature, rather than through one transformation event that metastasizes (Delabesse *et al.* 1997). Additionally, unlike typical cancers, it does not display an aggressive, progressive growth phenotype. KS originates presumably from infection of the vascular endothelium, proceeding with angiogenesis, inflammatory cell infiltration, and edema (Tappero *et al.* 1993). There is tight association of the malignancy with KSHV, and of intense research focus is to define the role that KSHV, and individual viral genes, play in: (i) the transition of the infected cell to a "spindle" cell phenotype, the distinctive proliferating cell of KS; and (ii) the development and sustenance of the lesions themselves. Notably, development of non-classical KS is clearly dependent on immune suppression, usually through HIV/AIDS or post-transplant immunosuppressive drugs, and regaining immune function can frequently cause regression of the disease (Ganem 2006).

Unlike EBV-associated Burkitt's lymphoma, where a distinctly latent gene program is active, it appears that KS requires expression of a variety of genes, both lytic and latency-associated, adding to the complexity of this disease (Parravicini *et al.* 2000; Grundhoff and Ganem 2004; Thorley-Lawson and Gross 2004; Ganem 2006). Further,

KS spindle cells maintain the KSHV genome *in vivo*, but when explanted into tissue culture, they require a rich medium for growth and subsequently lose the viral genome over several passages. In contrast, PEL is a tumor which seems to be sustained by KSHV latency-associated genes alone, and when explanted, the cells maintain the viral genome (Katano *et al.* 2000). The genes expressed in PEL cells include ORF73/LANA, ORF72/v-cyclin, K13/v-FLIP, and K12/kaposin, all of which are also expressed in KS and are each hypothesized to contribute to anti-apoptotic or pro-growth phenotypes (Rainbow *et al.* 1997; Parravicini *et al.* 2000)

iii. Non-Human Gammaherpesviruses

A growing number of gammaherpesviruses that infect non-human primates and other mammalian species have been identified. However, few have been studied in any detail. It is notable that several lymphocryptoviruses which infect non-human primate species have been identified, but no examples of lymphocryptoviruses in non-primate mammalian species have identified (Carville and Mansfield 2008; Davison *et al.* 2009). In contrast, there are numerous examples of rhadinoviruses in both non-human primates and non-primate mammalian species (Davison *et al.* 2009). I will briefly discuss here the best studied of these: Herpesvirus saimiri and Murine gammaherpesvirus 68.

a. Herpesvirus saimiri (HVS)

Subsequent to the discovery of EBV, other transforming herpesviruses were discovered, including Herpesvirus saimiri (HVS), which was isolated from squirrel monkeys (Melendez *et al.* 1968; Morgan *et al.* 1970). HVS was found to readily transform cells in culture and to induce fulminant T cell lymphomas in marmosets (Hunt

et al. 1970; Falk *et al.* 1973; Miller *et al.* 1977; Johnson and Jondal 1981). Because of the strict species specificity of EBV, and later KSHV, which severely limit studies requiring experimental infection, HVS was a major source of interest for investigating mechanisms of herpesvirus transformation and latency *in vivo*. Much has been learned from the virus, both from studying particular genes and from pathogenesis work. However, there are significant differences between HVS and the human herpesviruses, in tropism (HVS establishes latency in T, rather than B lymphocytes), in genetics (the most potent transforming proteins STP and TIP are not present in the human viruses), and in pathogenicity (Fleckenstein 1979; Trimble and Desrosiers 1991; Damania 2004). Due partially to those differences, species specificity, cost, and ethical considerations, it is very difficult, though not impossible, to perform detailed pathogenesis experiments with HVS in non-human primates.

b. Murine gammaherpesvirus 68 (MHV68)

A rodent herpesvirus Murine gammaherpesvirus 68 (MHV68, also *Murid herpesvirus 4*, MuHV-4) was isolated from a bank vole in 1976. Over the next decade, the virus was characterized and found to be genetically similar to EBV (Blaskovic *et al.* 1980; Efstathiou *et al.* 1990a; Efstathiou *et al.* 1990b). Further genetic analysis determined that MHV68 is a rhadinovirus, more closely related to KSHV and HVS than to EBV. Complete sequence analysis further revealed that many MHV68 open reading frames share high sequence and positional identity with the known rhadinoviruses (Figure 1.1) (Virgin *et al.* 1997). It is also notable that there are a number of genes present in the MHV68 genome that are not present in KSHV, and likewise, KSHV

encodes a similar number of genes that are unique. It was proposed, and has been partially borne out, that these unique genes are important for manipulating host immune response and facilitating host-specific latency and replication requirements (Virgin *et al.* 1997; Barton *et al.* 2011).

Importantly, experimental infection of mice demonstrated a similar course of infection to human gammaherpesviruses, including a mononucleosis-like expansion of T lymphocytes following acute infection, and establishment of long-term latency primarily in memory B cells (Tripp *et al.* 1997; Weck *et al.* 1999a; Nash *et al.* 2001; Willer and Speck 2003). After intranasal inoculation, the virus was found to replicate in the nose and lungs, reaching peak titers by days 7-9 post-infection. After that time, the virus is disseminated to the spleen, apparently by B cells, where a secondary lytic phase is initiated and resolved by days 16-18 post-infection (Weck *et al.* 1996; Milho *et al.* 2009). Following this period, virus genome could be detected in latently infected B cells, macrophages, and dendritic cells up to and beyond a year post-infection (Weck *et al.* 1996; Weck *et al.* 1999b). During the earlier phase of latency (ca. days 14-28), reactivation could be observed by explanting latently-infected cells and observing cytopathic effect on a fibroblast monolayer (Weck *et al.* 1999a; Willer and Speck 2003).

MHV68 was found to induce lymphoproliferative disease in varous immunocompromised mice (Sunil-Chandra *et al.* 1994; Tarakanova *et al.* 2005; Hrabovska *et al.* 2010), and further, like EBV, MHV68 can immortalize host cells in tissue culture and direct their differentiation (Liang *et al.* 2011). These observations argue that MHV68 provides a strong model to study gammaherpesvirus pathogenesis and disease in the context of a natural virus/host infection.

Prior to using MHV68, one of the major limitations in gammaherpesvirus pathogenesis research was the difficulty in characterizing natural infection of a naïve host. Importantly, though many valuable and informative EBV and KSHV cell culture studies have been done, a great majority of them use cell lines that are already infected and transformed with the viruses being studied. Thus, although a significant amount of information has been obtained regarding the cancer cells themselves and the viral genes expressed in those cells, significantly less was known about the process of *de novo* infection and the progression to disease state. MHV68, on the other hand, provides a system to study *de novo* infection in culture and in animals. The virus readily infects naïve laboratory mice and establishes a chronic infection which can be assayed for levels of latency and spontaneous reactivation. Additionally, in mice, immune responses, response to drugs or stimuli, and physical condition of the mice can be monitored throughout the course of infection.

Importantly, experimental infection of laboratory mice provides an opportunity for detailed analyses of gammaherpesvirus pathogenesis. First, the MHV68 genome has been cloned into a bacterial artificial chromosome, making the generation of MHV68 mutants very straightforward using standard molecular biology tools (Adler *et al.* 2000). In addition, MHV68, unlike EBV and KSHV, grows rapidly and to high titer in fibroblasts in tissue culture, even after transfection of cells with MHV68 BAC DNA. Together, these features allow for easy loss-of-function gene studies, progressing rapidly to infection and *in vivo* pathogenesis studies. Second, an extensive panel of knockout and transgenic mice is available, most notably in host genes involved in immune responses. These mice have facilitated studies identifying and characterizing how host genes

contribute to gammaherpesvirus infections, both with respect to controlling acute infection and the response to latent infection (Doherty *et al.* 2001; Barton *et al.* 2011).

Over the course of the past decade and a half, MHV68 has been used to identify and confirm suspected functions of the many conserved genes among gammaherpesviruses, as well as tease apart functions of the genes unique to MHV68 (Barton *et al.* 2011). For the conserved lytic transactivator protein, encoded by ORF50, the MHV68 knockout virus confirmed that the gene is absolutely required for lytic replication and aided subsequent study of the KSHV homolog (Pavlova *et al.* 2003; Xu *et al.* 2005). Other genetic knockouts gave surprising phenotypes—knocking out the v-cyclin gene, encoded by ORF72, revealed for the first time that the viral cyclin was important in lytic replication in the lung, and further, that it has important functions aside from those related to CDK binding (van Dyk *et al.* 2000; Upton and Speck 2006).

Studies of the unique genes have pointed to interesting examples of conservation of function in the absence of any significant sequence homology, demonstrating the importance of specific strategies to gammaherpesvirus pathogenesis. The MHV68 M2 protein, which shares no homology with any protein outside of the rodent gammaherpesviruses, when introduced into B cells, activates a pathway that results in cell differentiation and survival, as well as the production of interleukin-10 (IL-10) (Siegel *et al.* 2008). EBV contains no M2 homolog, but it does encode a viral IL-10 (BCRF1) as well as proteins (LMP1 and LMP2a) that provide signals that lead to cell survival and differentiation. Thus, through surprisingly different methods, these viruses have arrived at generally similar mechanisms, arguing for the importance of those mechanisms in the virus life cycle.

B) Latency-Associated Nuclear Antigen (LANA)

i. Identification and Background

The Latency-Associated Nuclear Antigen (LANA) of KSHV was discovered in 1996, in efforts to develop a diagnostic serological test for KSHV. Sera from KS patients was used to stain a KSHV+/EBV- body cavity-based lymphoma cell line (termed BCBL-1) to see whether there was an antigen common to many of these patients. Sera from over 80% of these individuals bound and detected an apparent 220-230 kD antigen in the nucleus of the BCBL-1 cells (Gao *et al.* 1996a; Kedes *et al.* 1996). Further work revealed that this antigen is encoded by ORF73 (Rainbow *et al.* 1997). These studies demonstrated that LANA is expressed in nearly every case of KS. Later studies showed that a handful of other latency-associated genes, such as the viral cyclin and IRF3 homologues, vFLIP, and vIL-6 are expressed in discrete disease states. Notably, though, only LANA is present in every KSHV-associated malignancy, including Castleman's disease and PEL (Dupin *et al.* 1999; Parravicini *et al.* 2000), establishing LANA as a major determinant of KSHV-associated disease.

Importantly, LANA is one of the latency genes that is conserved in each characterized rhadinovirus. It is found in KSHV, HVS, and MHV68, as well as the primate viruses retroperitoneal fibromatosis herpesvirus (RFHVMn) and rhesus rhadinovirus (RRV), and the rodent wood mouse herpesvirus (WMHV). Interestingly, LANA homologs vary significantly in length and domain organization. KSHV and RFHMn LANA proteins are very large, 1162 and 1072 amino acids, respectively, and they contain a long, variable-length, amino acid repeat. HVS LANA also contains a repeat region, but it is shorter and contains a different sequence of amino acids. RRV,

MHV68, and WMHV LANA proteins are considerably shorter overall (448, 314, and 327 amino acids, respectively) (Figure 1.2) (Virgin *et al.* 1997; Alexander *et al.* 2000; Burnside *et al.* 2006; Hughes *et al.* 2010b). In contrast to these differences, all of the LANA proteins share a conserved C-terminal domain. Borne out in deletion studies of KSHV and HVS LANA, it appears that this C-terminal region is responsible for binding DNA (Hall *et al.* 2000; Kelley-Clarke *et al.* 2007).

While LANA homologs are present in all known rhadinoviruses, there is no obvious sequence or positional homolog in the lymphocryptoviruses. However, the 3D-PSSM algorithm (Kelley and Sternberg 2009) predicts structural similarity of the LANA C-terminal domain to the solved structure of the EBV protein EBNA-1 (Grundhoff and Ganem 2003). Interestingly, EBNA-1 shares many of the same characteristics and functions proposed for LANA proteins (facilitating genome replication, transcriptional modulation, latency/episome maintenance), and EBNA-1, similar to LANA, is the only detectable proteins expressed in many EBV latently-infected tumor cells (Middleton *et al.* 1991; Leight and Sugden 2000).

ii. LANA in disease and pathogenesis

a. Introduction

Much work has gone into characterizing the transcription, expression, and interaction network of LANA. It has been implicated in disrupting anti-apoptotic pathways, altering transcription of many genes, and maintaining the virus episome during latency (Ballestas *et al.* 1999; Friborg *et al.* 1999; Fujimuro and Hayward 2003; Ottinger *et al.* 2006). As part of maintaining the virus episome, it has been implicated in recruiting

the origin recognition complex (ORC) to facilitate viral DNA replication during latent infection (Hu *et al.* 2002). In addition, LANA is thought to tether the viral episome to the host chromosome to facilitate segregation of the genome to daughter cells during mitosis (Ballestas *et al.* 1999). With all the disparate functions attributed to LANA, it is doubtful that a single interaction or effect is the one that is crucial to its function. Rather, it is likely the sum of cellular pathways and functions affected by LANA expression as a whole that is important to the role of LANA in the virus life cycle and pathogenesis.

To illustrate the potential important role(s) that KSHV LANA plays in viral pathogenesis, the region of KSHV containing the LANA promoter and ORF was used to generate transgenic mice (Jeong *et al.* 2001; Jeong *et al.* 2002; Fakhari *et al.* 2006). The mice, expressing the LANA transgene under the control of its own promoter (Dittmer *et al.* 1998; Sarid *et al.* 1998), develop distinct B cell hyperplasia and have increased numbers of activated B cells—this benign lymphoproliferative disorder increased the chances of a second site mutation and led to the development of frank lymphomas (Fakhari *et al.* 2006). Thus, LANA alone is able to promote the transition of B cells from their normal physiologic state to a disease state. Importantly, the role that KSHV LANA plays in disease induction/progression in the LANA transgenic mice has not been rigorously defined.

To understand what tissues express LANA, transgenic mice expressing LacZ under the control of the LANA promoter (LANAp) were bred and whole organs and tissues were assayed for β-galactosidase activity. LANAp activity was found in B cells, as well as in kidney epithelium, but not T cells or endothelial tissues (Jeong *et al.* 2002). Thus, these data are consistent with the idea that LANA, as a latency maintenance

protein, facilitates maintenance of long-term latency in B cells. However, these findings do not explain the high level of LANA expression, or the presence of latent KSHV in KS lesions, which appear to be of endothelial cell origin. Thus, either KS is not of endothelial origin (even though cell surface markers indicate it is) or there is more complexity to both expression of LANA and to the mechanisms KSHV uses to establish latency. LANA has at least one additional promoter, which is induced by the viral lytic transactivator ORF50/RTA, and data indicate that KS requires low-level induction of the lytic cycle (Jeong *et al.* 2001; Grundhoff and Ganem 2004). Together, these data argue for a much higher expression of LANA in KS tissues than during normal latency, and studies in other systems suggest LANA expression may actually be quite low during the course of asymptomatic latent infection.

b. Protein-Protein Interactions

It is advantageous for a herpesvirus to extend the life of its host cell, as well as prevent death of those cells indefinitely, or at least long enough to reinitiate lytic replication. To this end, LANA has been shown to prevent or delay cell death by binding to and altering the downstream functions of several key mediators of tumor suppression and the cell cycle. One recent study used a LANA pull-down/mass spectrometry approach to identify protein complexes that have the capacity to interact with LANA and found 109 interacting proteins (Kaul *et al.* 2007). These include cell cycle and cell division proteins, cytoskeletal proteins, transcription factors, DNA and RNA polymerases, kinases, and a whole host of other factors. While interactions alone do not

demonstrate functional importance, it makes for an intriguing dataset from which to investigate individual phenotypes.

PEL cells show an increased level of β-catenin, and it was also shown that levels of β-catenin are upregulated in KS compared to surrounding tissue (Fujimuro *et al.* 2003). β-catenin is a mediator of the highly conserved Wnt signaling pathway, and when β-catenin levels are high, the protein translocates to the nucleus and forms a complex with TCF/LEF transcription family members to initiate transcription of genes such as *MYC, JUN, and CCND1* (Barker *et al.* 2000). This pathway, and particularly β-catenin expression, are dysregulated in a number of cancers (He *et al.* 1998; Tetsu and McCormick 1999). GSK-3β is a kinase that phosphorylates β-catenin to mark it for proteasomal degradation. LANA has been shown to interact with GSK-3β, altering its localization and allowing β-catenin levels to rise (Fujimuro and Hayward 2003; Fujimuro *et al.* 2003). This process is thought to remove part of the checkpoint for cells to enter S phase.

LANA also interacts with the retinoblastoma protein (pRb) (Radkov *et al.* 2000). pRb is a cell cycle checkpoint protein that acts as a negative regulator of the G1/S transition. The hypophosphorylated form binds the transcription factor E2F, and during G1, pRb is phosphorylated by cyclin D/CDK complexes, resulting in the release of E2F to initiate transcription of cyclin E and other genes (Hatakeyama and Weinberg 1995; Keyomarsi *et al.* 1995). LANA expression leads to an upregulation of E2F-responsive promoters, including that of cyclin E, due at least in part to LANA binding pRb and releasing E2F. When expressed along with *Hras*, LANA can transform primary cells,

which, when injected into nude mice, form tumors (Radkov *et al.* 2000). This is another example of blunting a checkpoint in the transition to S phase.

Many cancers involve mutations or alterations in the tumor suppressor p53 (Hollstein *et al.* 1991). Virally induced cancers are no different, and there are several famous examples of viral proteins that interact with and/or alter the function of p53. Simian Virus 40 (SV40) Large T antigen interacts with and inactivates p53 (Jay *et al.* 1981). Human Papilloma Virus 16 (HPV-16) E6 protein enhances ubiquitination of p53, causing it to be degraded (Werness *et al.* 1990). Adenovirus E1A and E1B proteins actually work to stabilize p53 while inhibiting the downstream effectors of apoptosis (Debbas and White 1993; Lowe and Ruley 1993; Han *et al.* 1996). Similarly, KSHV LANA has been shown to interact with p53, blocking p53-dependent transcriptional activation. Furthermore, LANA is able to prevent p53-induced apoptosis, presumably through this interaction (Friborg *et al.* 1999). Part of the function of p53 is in responding to DNA damage detected during the G1 phase. When p53 is activated in response to DNA damage, the transition to S phase is delayed in order to initiate repair or apoptosis (Kastan *et al.* 1992; Lowe *et al.* 1993; Wahl and Carr 2001). Typically, this is due to damage by environmental radiation (e.g. UV radiation), but chromosomal instability can be another signal detected and propagated through p53. Indeed, it has been observed that LANA-expressing cells may have increased genomic instability—the cells are allowed to live in part by LANA blunting the p53-dependent cellular response to chromosomal breaks (Si *et al.* 2008). G1/S checkpoint proteins are thus a common target of many known LANA interactions.

c. Transcriptional regulation

LANA binds DNA in a sequence-specific manner. This interaction affects the transcription of many genes, similar to a transcription factor, with both activating and repressive phenotypes (Cotter *et al.* 2001; Renne *et al.* 2001; Garber *et al.* 2002; Lu *et al.* 2012). This is likely a mechanism used by the virus to prepare the cell for infection (e.g. blunting anti-viral responses and influencing the cell cycle), or to drive cell differentiation to a state suited for latency (i.e. from naïve to memory B cells). In addition, it is likely that direct or indirect LANA-mediated dysregulation of cellular genes is one of the factors that leads to cellular transformation. In one study, LANA expression was found to modestly upregulate expression of reporter genes downstream of a TATA box (i.e. a minimal promoter). Similarly, when CAAT or Sp1 transcription factor binding sequences are placed upstream of the TATA box, the reporter gene is activated 6-7 fold (Renne *et al.* 2001). Interestingly, LANA may also modestly upregulate the expression of human telomerase (hTERT), similarly to HPV-16 E6, contributing to cell transformation (Klingelhutz *et al.* 1996; Groves *et al.* 2001).

LANA also alters transcription of viral genes by binding promoter regions of DNA, including that of ORF50, which encodes the major lytic transactivator (Lu *et al.* 2006). Importantly, KSHV LANA activates its own promoter, which initiates a transcript shared with the viral cyclin gene (Dittmer *et al.* 1998; Renne *et al.* 2001). This indicates that there is some auto-feedback in LANA expression, similar to EBV latency genes (Speck and Ganem 2010). It is notable that LANA expression positively influences the expression of v-cyclin, another gene implicated in cell transformation. LANA expression also activates promoters of other viruses. The Human Immunodeficiency Virus (HIV)

long terminal repeat (LTR) is of particular interest, as KSHV and HIV diseases are so tightly linked. One group found that in the BJAB cell line, in cooperation with HIV *tat* protein, transcription from the HIV LTR is activated by LANA up to 25-fold. Another study, which used COS-7 cells and no *tat,* found that LANA actually represses the LTR-driven transcription 30-fold (Hyun *et al.* 2001; Renne *et al.* 2001). Thus, this relationship is complex, and it is not known whether LANA is binding directly to LTR sequences or if one or both of these are indirect effects. Investigating LANA's influence on EBV gene expression, LANA expression was shown to activate the EBV LMP1 and BamHI C promoters, both of which drive transcription of genes implicated in cell immortalization. A truncated LANA protein consisting of only the repeat and C-terminal regions retained this activity, demonstrating both the importance of the C-terminal domain of LANA in transcriptional regulation and in possible crosstalk between viruses during EBV coinfection (Groves *et al.* 2001).

LANA also serves as a transcriptional repressor in some contexts. It may perform this function in a number of ways. First, LANA has been shown to interact with and sequester cAMP-Response Element Binding (CREB) Protein (CBP), effectively interfering with the CBP/c-Fos interaction, and disrupting that complex as a transcriptional activator (Gwack *et al.* 2001). Second, LANA may facilitate epigenetic modification of a promoter by tethering DNA-modifying proteins to specific DNA sequences. It has been shown that LANA expression downregulates several promoters, including cyclin D2, H-cadherin, and lactate dehydrogenase B promoters, among others. LANA expression results in LANA and *De novo* methyltransferase 3a (Dnmt3a) occupancy at the promoters and increased methylation of the promoter sequences. LANA

also interacts with Dnmt3a itself (Shamay *et al.* 2006). Epigenetic modifications may also be through LANA directing histone deacetylases (HDACs) to specific promoters. LANA interacts with SAP30, a member of the mSin3 HDAC complex, and LANA-mediated repression of EBV Q and C promoters results in a corresponding reduction in acetylation (Krithivas *et al.* 2000). Notably, the effect on the EBV C promoter is different than the repression observed by Groves *et al.,* and likely reflects a complex set of interactions. Finally, the terminal repeat (TR) sequence of KSHV acts as an enhancer and it is likely important in enhancing transcription of genes near the left and right ends of the unique sequence. However, LANA expression inhibits this enhancer activity, and this function is dependent on the DNA-binding function of the C terminus of LANA (Garber *et al.* 2001; Han *et al.* 2010).

d. Latent replication and episome maintenance

During latency, virus DNA must be replicated and segregated into new daughter cells. The latent origin of replication has not been rigorously defined for any rhadinovirus, but the terminal repeat region has been shown to contain sequences that confer the ability to replicate DNA in a LANA-dependent manner (Ballestas *et al.* 1999; Hu *et al.* 2002; Grundhoff and Ganem 2003). Transfected TR-containing plasmids, which are otherwise devoid of mammalian origins of replication, are lost over the course of cell division in the absence of LANA expression, indicating that LANA is required for initiating replication on whatever origin sequence the TR contains. The C terminus of LANA has been shown to interact with proteins of the origin replication complex in the context of the LANA binding site sequence in PEL cells, bolstering the argument for a

LANA-dependent origin in the terminal repeat (Verma *et al.* 2006). However, there appear to be other LANA-independent origins in the KSHV genome, so LANA may not be absolutely required for viral genome replication during latent infection, and rather, may simply increase the rate of replication in certain situations (Verma *et al.* 2007a).

One of the functions of LANA that has received considerable notoriety is the proposed episomal tethering function, which is a novel mechanism for separation of DNA during mitosis, independent of centrosome/centromere attachment. KSHV genomes do not have obvious centromeres, which would provide attachment to the microtubule spindle and govern separation of eukaryotic chromosomes. The C terminus of LANA binds specific DNA sequences in the KSHV TR, and it is thought that the N terminus simultaneously binds to host chromatin via a protein-protein interaction with histone H2A-H2B. Thus, this mechanism may be what ensures that the viral genome is effectively partitioned into the daughter cells during and following anaphase. The evidence for this function is that LANA can mediate replication of a TR-containing plasmid under drug selection (which is dependent on LANA binding the TR sequence to initiate replication), but only if the sequences in the N terminus, which were demonstrated to be necessary for histone H2A-H2B and chromatin association, are intact (Ballestas *et al.* 1999; Barbera *et al.* 2006). Further, fluorescence in situ hybridization (FISH), combined with immunofluorescent detection of LANA, shows that KSHV genomes, LANA, and mitotic chromosomes all colocalize (Ballestas *et al.* 1999).

C) MHV68 LANA

i. Introduction

MHV68 ORF73 encodes a positional and partial sequence homolog of KSHV LANA, termed mLANA (Virgin *et al.* 1997). As depicted in Figure 1.2, mLANA is significantly shorter than KSHV LANA (314 amino acids vs. 1162), and the two proteins share very little overall primary sequence homology. However, both proteins do have a proline rich region in the N-terminal region, and they share a C-terminal domain with about 40% similarity (29% identity). In a MHV68-positive lymphoma (S11) isolated from a mouse, ORF73 is the most abundant viral transcript (Usherwood *et al.* 1996a; Martinez-Guzman *et al.* 2003), consistent with a critical role for LANA in rhadinovirus lymphomagenesis.

The development of MHV68 as a model to understand gammaherpesvirus pathogenesis provides an unmatched opportunity to study the role of LANA proteins during the course of natural infection. LANA is of great interest because of its presence in KSHV-associated tumors, coupled with evidence that LANA is required for their survival (Corte-Real *et al.* 2005; Godfrey *et al.* 2005). Prior to experimental infection of mice with MHV68, there was a complete absence of any information regarding: (i) what cells express LANA during infection *in vivo*; (ii) what role LANA plays in acute replication during gammaherpesvirus infection; (iii) what role LANA plays in a non-pathogenic latent infection; and (iv) what role LANA has in the transition from latently-infected cell to tumor. To understand gammaherpesvirus-associated disease pathogenesis, it is important to fully understand the biology of the virus and the natural host responses to it.

ii. Transcription and cell-type expression of mLANA

MHV68, unlike many other gammaherpesviruses, undergoes a robust lytic infection in tissue culture infection of fibroblasts, allowing easy characterization of lytic transcripts and kinetics of viral gene expression. Infection of fibroblasts in the presence of the protein synthesis inhibitor cycloheximide showed that ORF73 is transcribed with immediate early kinetics, detectable as early as five hours post-infection (Roy *et al.* 2000; Rochford *et al.* 2001; Ahn *et al.* 2002; Martinez-Guzman *et al.* 2003). Indeed, one study found ORF73 to be not only one of the most robustly expressed immediate-early genes, but the only one readily detected at early times post-infection (Ahn *et al.* 2002). This finding was somewhat surprising in light of KSHV and HVS studies that indicate that LANA inhibits lytic replication and the lytic transactivator directly (Schafer *et al.* 2003; Lan *et al.* 2004; Lu *et al.* 2006).

In vivo analyses of mLANA expression have had varying results. An early analysis used an RNase protection assay and found expression of mLANA during lytic infection of the lungs, but not in the spleen 10 days post infection (Rochford *et al.* 2001). Another study used fluorescence activated cell sorting (FACS) of cells from mice infected with MHV68 to characterize cell types that express mLANA via RT-PCR. That study found mLANA expression in germinal center and marginal zone B cells and dendritic cells, but they found no mLANA transcripts in follicular or newly formed B cells or macrophages (Marques *et al.* 2003). This result is interesting because it is consistent with the earlier observations in KSHV and HVS tumor cells that LANA is needed for replication of the viral episome in rapidly dividing cells (Kung and

Medveczky 1996; Ballestas *et al.* 1999; Cotter and Robertson 1999). However, because MHV68 also establishes latency in macrophages, and those cells show no detectable mLANA expression, it suggests there is an mLANA-independent mechanism for maintaining latency in these cells (Weck *et al.* 1999b; Marques *et al.* 2003).

Contrasting with what is known about transcription of LANA in KSHV-infected tumor lines, *in vivo* analyses of splenocytes from latently infected mice demonstrate that there are at least two transcripts that splice into ORF73 (Dittmer *et al.* 1998; Coleman *et al.* 2005b; Allen *et al.* 2006). One of these transcripts (p1-initiated) initiates within the terminal repeat region and splices to include several copies of the TR-encoded exon (E1) before splicing to an exon encoded by unique sequence adjacent to the TR (E2), which in turn splices to an exon containing ORF73 (Figure 1.3). The second transcript (p2-initiated) initiates immediately upstream of the exon E2, and it contains the splice to the ORF73 exon. The promoter elements for this transcript are in the left-hand end of the TR, and they may initiate other transcripts in distal copies of the TR. Both p1- and p2-initiated transcripts can be detected during latent and lytic infection. Interestingly, unlike KSHV LANA, a promoter proximal to MHV68 ORF73 was not detected *in vivo*, but analysis of the 5' end of mLANA transcripts of lytically-infected fibroblasts indicates that there are likely transcripts that initiate between ORF73 and ORF74. Notably, this promoter has modest activity in either orientation in a reporter assay (i.e., a bi-direction promoter), reminiscent of the KSHV LANA promoters (Dittmer *et al.* 1998; Jeong *et al.* 2001; Coleman *et al.* 2005b; Cheng *et al.* 2012). The differences between promoter usage in KSHV and MHV68 may reflect a preference for one promoter in rapidly dividing cells, or perhaps one or more of the promoters are subject to methylation. Indeed, cells

immortalized with MHV68 show low, but detectable levels of *orf73* transcript, however the frequency of those cells expressing spliced 73E1-containing transcripts is very low (Liang *et al.* 2011) (Liang and Speck, unpublished observation). Thus, this may reflect selective promoter usage, or differing, context-dependent requirements for LANA expression. The situation is not entirely clear and warrants further study.

iii. Pathogenesis studies of MHV68 mLANA-null mutants

With the development of protocols for facile mutagenesis of the MHV68 genome and quantitative assays for latency and reactivation, mLANA was a compelling choice for early loss-of-function mutagenesis. It was hypothesized that since LANA is proposed to be required for maintenance of latency and persistence of the viral episome, that an mLANA-null virus would fail to establish latency. Mutants of ORF73 containing stop codons and frameshift mutations early in the ORF were introduced in the MHV68 genome by BAC recombineering by two separate research groups. Both groups observed that when mice were infected intranasally with mLANA-null mutants, the virus failed to establish a latent infection in the spleen (Fowler *et al.* 2003; Moorman *et al.* 2003) (Figure 1.4). Additionally, consistent with data showing that mLANA is robustly expressed during the lytic cycle, there was a 1.5-log defect in acute replication observed in the lungs of infected mice four days post-infection.

MHV68 has recently been shown to immortalize and direct differentiation of murine fetal liver cells (Liang *et al.* 2011). Given the importance and prevalence of LANA in KSHV tumor cells, it was thought that mLANA would be important in the immortalization process. Consistent with this hypothesis, the mLANA-null virus failed to

immortalize murine fetal liver-derived B cells (Liang *et al.* 2011). These data provide evidence for a role for mLANA in immortalization and transformation. Importantly, the failure of the mLANA-null virus to immortalize murine fetal liver-derived B cells may not be absolute, similar to the EBNA1-null EBV, which immortalizes cells ca. 1,000 times less efficiently than wild type EBV and apparently requires integration of the viral genome into the host genome (Humme *et al.* 2003).

D) FIGURES AND LEGENDS

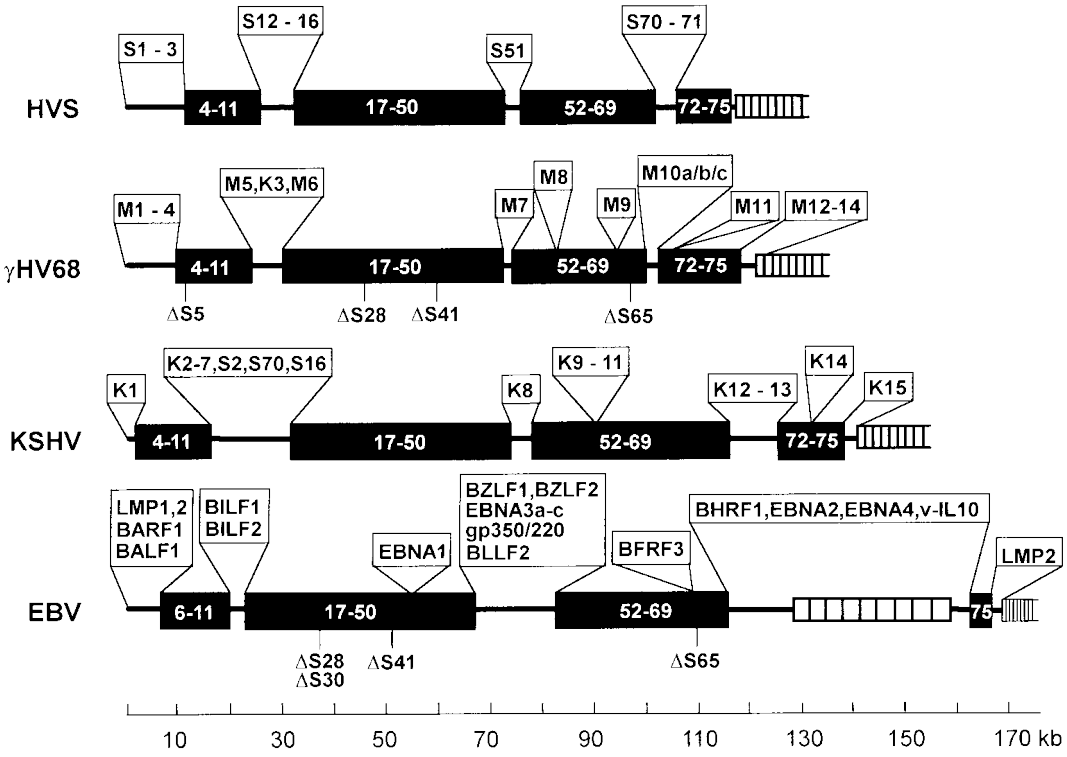

Figure 1.1. Comparison of genome organization among gammaherpesviruses. The structures of the indicated viruses were compared to the HVS genome structure. The conserved blocks of genes are indicated in the shaded rectangles, and the numbers correspond to the HVS gene numbers. Genes missing from particular blocks are indicated below each block (specific genes which are missing are indicated with a D), while genes inserted within a particular block of genes are indicated above each block. The terminal repeats located at the ends of the gammaherpesvirus genomes are shown only at the right-hand end of the respective genomes. The major internal repeat of EBV is shown near the right-hand end of the genome as a series of connected open rectangles. ORFs designated with an S are largely unique to HVS; ORFs designated with an M are largely unique to gHV68; ORFs designated with a K are largely unique to KSHV (Virgin *et al.* 1997).

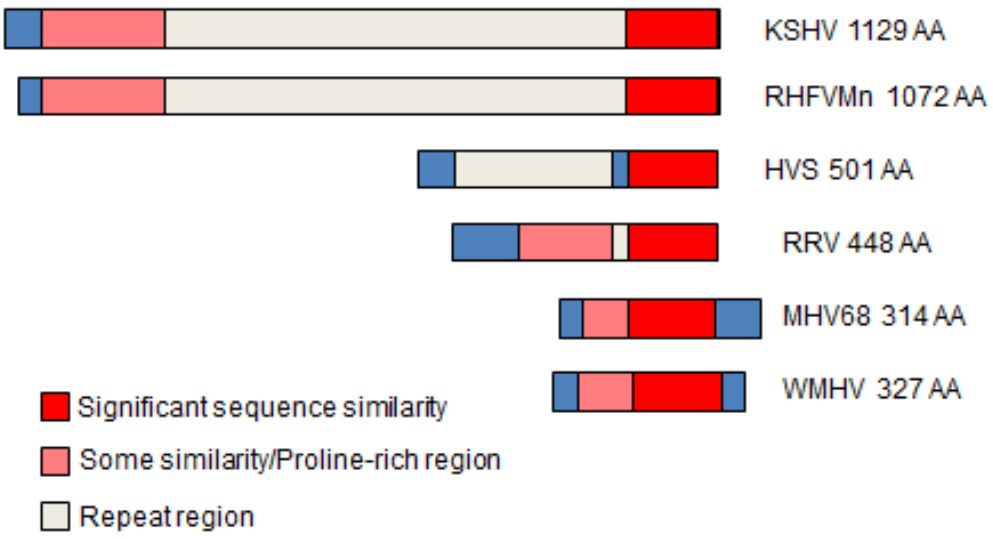

Figure 1.2. Schematic diagram of LANA proteins from different rhadinoviruses. Depicted are schematics of LANA from KSHV, the primate viruses RHFVMn, HVS, and RRV, and the rodent viruses MHV68 and WMHV showing general regions of conservation and diversity. Adapted from (Grundhoff and Ganem 2003; Hughes *et al.* 2010a).

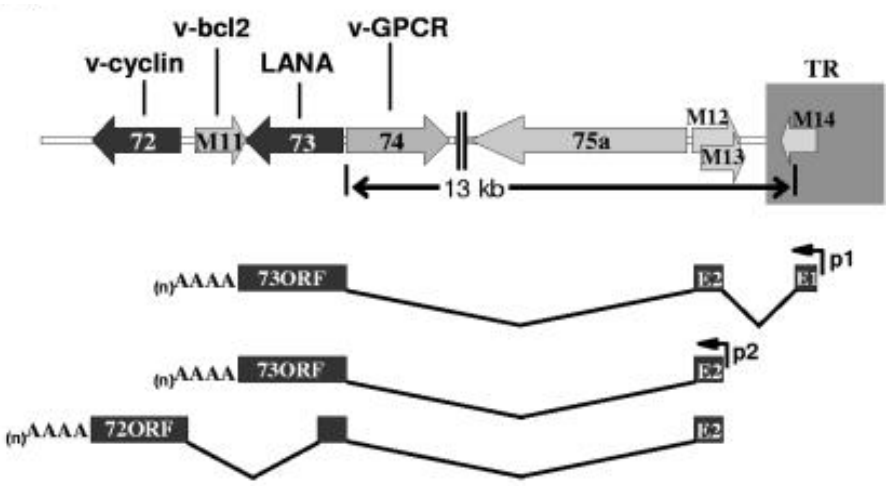

Figure 1.3. The region of the genome encoding γHV68 orf72 and orf73 is shown. Two major spliced orf73 transcripts were identified. The larger orf73 spliced transcript contained at least one copy of a 91-bp exon (E1) located within the viral terminal repeat, a 106-bp exon (E2) and the orf73 coding exon (E3). The second, smaller orf73 transcript identified contained only E2 and E3. RT-PCR performed with a primer specific to orf72 (72RTi, TCAACATCAACATCTGGTGATGGTG) and a primer specific to exon 2 of the orf73 transcript (73E2i, TCCCGACTCGTGAGTAGCGCCGACTAG) amplified a spliced product that contains the sequences encoding orf72 as well as an additional exon from within the orf73 coding region. Products were subsequently subcloned and sequenced. The positions of the 73p1 and 73p2 promoters are also indicated. (Allen *et al.* 2006)

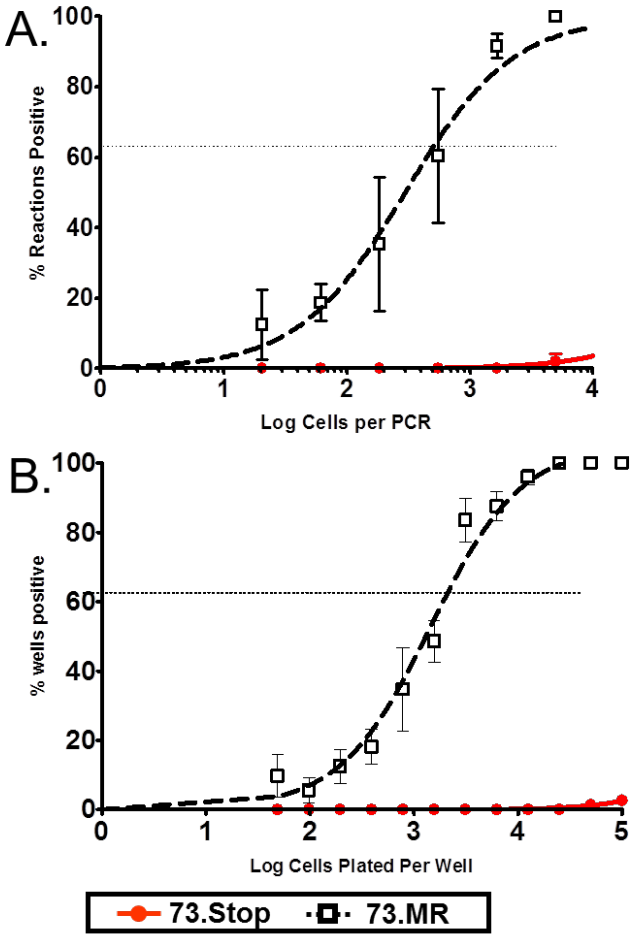

Figure 1.4. Inefficient establishment of latency after intranasal inoculation of MHV68.73.Stop. Mice were inoculated with 1000PFU of 73.Stop or the genetically repaired 7.MR virus. Splenocytes were harvested 18 days post-infection. (A) 73.Stop virus does not establish latency, as determined by limiting-dilution PCR, and (B) there is no reactivation of 73.Stop-infected cells, scored by a limiting-dilution CPE assay, due to paucity of infected splenocytes following intranasal inoculation.

CHAPTER 2.

Roles for mLANA and p53 in MHV68 replication

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This study is included in this dissertation as I was heavily involved in the planning and execution of much of the work, and the theme fits well with and sets the stage for the rest of my dissertation work. I have noted in the figure legends who generated the data in each figure. The text is written primarily by J.C. Forrest.

A) INTRODUCTION

Murine gammaherpesvirus 68 (MHV68) is a member of the gammaherpesvirinae subfamily of viruses that naturally infects rodents. Following infection of inbred mice by various routes, MHV68 undergoes acute replication at the primary site of inoculation, a process that facilitates the seeding of several cell types including macrophages, dendritic cells, epithelial cells, and B cells for latent or persistent infection (Sunil-Chandra *et al.* 1992; Stewart *et al.* 1998; Weck *et al.* 1999b; Flano *et al.* 2000; Willer and Speck 2003). In latent infections the viral genomes are maintained in a quiescent state for the lifetime of the host, but retain the capacity to reactivate the lytic replication cycle and produce progeny virions. Like all gammaherpesviruses, MHV68 is defined by its capacity to establish latent infections in host lymphocytes.

During latent gammaherpesvirus infections viral transcription is restricted, and only those genes proposed to influence maintenance and immune evasion are transcribed. For MHV68, the latent-transcription program varies with cell type (Marques *et al.* 2003), likely reflecting cell-type-specific needs for viral maintenance and demonstrating the dynamic nature of gammaherpesvirus latency. Of the viral transcripts expressed during latent infections, mLANA [homologue of the Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA)] is critically required, as mLANA-null viruses are unable to establish and maintain latent infections following intranasal inoculation of mice (Fowler *et al.* 2003; Moorman *et al.* 2003). This is consistent with a proposed role for mLANA in episome maintenance during latency. However, mLANA influences more than just the capacity of MHAV68 to persist during latency, as we and

others observed acute replication deficits in the lungs of mice infected mLANA-null mutants (Moorman *et al.* 2003; Song *et al.* 2005).

Although a replication deficit was not originally borne out in cell-culture growth assays, several lines of evidence suggest a role for mLANA in facilitating acute-phase viral replication in addition to the demonstrated role in regulating viral latency. First, in global and gene-specific MHV68 transcription analyses, *orf73* was detected at very early times after infections in culture, and *orf73* transcription occurred in the presence of cycloheximide, classifying *orf73* as an immediate-early transcript (Ahn *et al.* 2002; Martinez-Guzman *et al.* 2003; Coleman *et al.* 2005a). Further, Herpesvirus saimiri (HVS) and Rhesus rhadinovirus (RRV) LANAs suppress the progression of lytic replication in culture by inhibiting transcription of the major lytic transactivator protein, RTA (Schafer *et al.* 2003; DeWire and Damania 2005). KSHV LANA can either repress activity of the promoter for RTA (Lan *et al.* 2004) or conversely contribute to its activation via HIF-1α under hypoxic conditions (Cai *et al.* 2006; Carroll *et al.* 2006).

In addition to regulating transcription of viral lytic genes, KSHV LANA inhibits the activity of several host cell-cycle regulatory proteins. The tumor suppressor protein p53 functions as a transcriptional regulator of cell-cycle checkpoints and apoptosis in response to numerous cellular stressors, including DNA damage and viral infection (Vogelstein *et al.* 2000; Harris and Levine 2005; Lavin and Gueven 2006). Consistent with these roles, loss of p53 function is a major contributor to cellular transformation and cancer (Vogelstein *et al.* 2000). Biochemical analyses demonstrated that p53, as well as the tumor suppressor pRb, are targets of KSHV LANA and HVS LANA-mediated repression (Friborg *et al.* 1999; Radkov *et al.* 2000; Borah *et al.* 2004), which may

contribute to LANA inhibition of p16INK4a-induced cell-cycle arrest (An *et al.* 2005). Moreover, KSHV LANA contributes to H-ras-induced transformation of rat fibroblasts and tumor growth in SCID mice (Radkov *et al.* 2000), and KSHV LANA expressed from its cognate promoter induces lymphoproliferative disease in transgenic mice (Fakhari *et al.* 2006). Together with the observations that LANA is highly expressed in KS and KSHV-derived tumor cell lines (Kedes *et al.* 1997), these data demonstrate the capacity of LANA proteins to promote oncogenesis by deregulating cellular tumor suppressors, in addition to their proposed role in maintaining γ2-herpesvirus episomes during latency. However, LANA-expressing KSHV-derived cell lines are sensitive to p53 agonistic chemotherapeutic agents (Curreli *et al.* 2005; Petre *et al.* 2006), perhaps illustrating the complexity of extending the results of isolated biochemical analyses to the setting of virus infection. Moreover, a role for LANA proteins in regulating cellular stress responses, such as p53 activation or cell-cycle arrest, during lytic viral replication has not been analyzed.

In this report we examined the role of MHV68 mLANA during productive virus replication. We found that mLANA is expressed with diverse localization patterns throughout the MHV68 replication cycle and is required for efficient replication, especially at low multiplicities of infection (MOI), in murine fibroblasts. Infection with mLANA-null (73.Stop) MHV68 more readily induced cell death as compared to marker rescue virus (73.MR) at high MOI. The death occurred with p53 activation and enhanced kinetics of viral gene expression, even in the presence of inhibitors of viral DNA replication. Stable mLANA expression correlated with reductions in both p53 stabilization and cell death upon treatment with p53-inducing stimuli, and p53-deficient

MEFs were modestly protected from 73.Stop-induced death at early times after infection. The absence of p53 equilibrated 73.Stop and 73.MR replication, but led to an overall delay in the progression of viral gene expression and replication compared to p53-expressing cells. Consistent with these findings, lytic-cycle viral genes were inducible by p53 overexpression or treatment with p53-inducing drugs. These data provide the first evidence that LANA proteins regulate p53 induction during productive gammaherpesvirus replication to control the viral gene expression cascade. Furthermore, we demonstrate that p53 expression actively influences the gammaherpesvirus life-cycle.

B) MATERIALS AND METHODS

Cell culture and viruses. NIH 3T12, NIH 3T3, and 293T cells were purchased from ATCC. BOSC 23 cells were a gift from Joshy Jacob (Emory University, Atlanta, GA). Vero cells used in this report express Cre recombinase and are commonly used by our laboratory and others to propagate BAC-derived recombinant MHV68 (Moorman *et al.* 2003). Vero-cre cells were a gift from David Leib (Washington University, St. Louis, MO). C57BL/6 MEFs were prepared from d13.5 embryos as described (Weck *et al.* 1996). *Trp53*$^{-/-}$ (p53$^{-/-}$) MEFs were a gift from Vera Tarakanova and Herbert W. Virgin, IV (Washington University, St. Louis, MO.) All cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (cMEM). NIH 3T3 cells transduced with empty MSCV or mLANA-GFP MSCV or p53$^{-/-}$ MEFs transduced with DsRed MSCV or p53-DsRed MSCV were selected with 1 μg/ml puromycin (Sigma). Wild-type MHV68 was strain WUMS (ATCC VR1465). The derivation of 50.Stop, 73.Stop, and 73.MR viruses was previously described (Moorman *et al.* 2003; Pavlova *et al.* 2003). P2 stocks propagated on Vero-cre cells were used for all experiments in this paper. Recombinant mLANA-GFP MHV68 was derived by allelic exchange in the MHV68 BAC as previously described (Adler *et al.* 2000; Moorman *et al.* 2003). To derive the targeting construct, GFP from pIRES2-EGFP (Clontech) was fused to the carboxyl terminus of mLANA by overlap-extension PCR using the following oligos: 73GFPFUS P1, 5'-CGAG ATCTGGA GAGACAGACGATCCC-3'; 73GFPFUS P2, 5'-CTCGCCCTTGCTCACTGTCTGAGA CCCTTG-3'; 73GFPFUS P3, 5'-GTGAGCAAGGGCGAGGAGCTGTTCACCGGG-3'; 73GFPFUS P4, 5'- ACCCT

TACACTAAATTTACTTGTACAGCTC-3'; 73GFPFUS P5, 5'- ATTTAGTGTAAG GGTCTTATATA GGACCCG-3'; 73GFPFUS P6, 5'- CGGCGGCCGCCACGCGTCGATAAAATT-3'. The overlap-extension PCR product was cloned into pCR-blunt (Invitrogen), and the fidelity of cloning was verified by automated sequencing. The construct was subcloned into pGS284 and allelic exchange was performed as described (Adler *et al.* 2000; Moorman *et al.* 2003). Appropriate recombination was determined by colony PCR. Recombinant mLANA-GFP MHV68 virus was derived by BAC transfection into Vero-cre cells.

Vectors, transfections, and antibodies. The -310 *orf50* promoter and -800 *v-cyclin* promoter constructs were previously described (Liu *et al.* 2000; Allen *et al.* 2007). The p53 expression vector pFC-p53 was purchased from Stratagene. mLANA-GFP was cloned via PCR from recombinant mLANA-GFP MHV68 with primers 73-1_Bam, 5'- GATCGGATCCCTT GACCCACACCCTTCCTGTGC-3' and 73-3_Eco, 5'- GATCGAATTCCATGCCCTGGCGAAGGTGTTG-3' and restriction digested with *BamHI* and *EcoRI* for transfer into the *BglII* and *EcoRI* sites in pMSCV-puro (Stratagene). Fidelity of cloning was verified by automated sequencing. DSRED was cloned from pDsRed Monomer-N1 (Clontech) by PCR with primers DSRED_US, 5'- GATCGGATCCGCCACCATGGACAACACCGAG-3' and DSRED_DS, 5'- GATCGAATTCCTACTGGGAGCCGGAGTGGCGG-3'. To derive P53-DsRed, DSRED from pDsRed Monomer-N1 was fused to the carboxyl terminus of wild-type p53 from pFC-p53 by overlap-extension PCR using the following oligos: P53_US, 5'- GATCGGATCCCTGCCATGGAGGAG CCGCAG-3'; P53-DSRED_comp, 5'- GTTGTCCATGGTGGCGTCTGAGTCAGGCCCTTCTGTC-3'; P53-DSRED, 5'-

GGGCCTGACTCAGACGCCACCATGGACAACACCGAG-3' and DSRED_DS (above). Products were digested with *BamHI* and *EcoRI* for transfer into the *BglII* and *EcoRI* sites in pMSCV-puro. Retroviruses were generated by transfecting pMSCV constructs into BOSC 23 cells. Transfections were performed using Lipofectamine (Invitrogen). The antibodies used in this study include chicken anti-ORF59 (Upton *et al.* 2005), rabbit anti-v-cyclin (van Dyk *et al.* 1999), anti-PARP #9542, anti-p53 #2524, anti-phospho S15 p53 #9284S (Cell Signaling Technology), goat polyclonal and mouse monoclonal anti-GFP (Rockland Immunochemicals), and anti-β-actin A-5316 (Sigma).

Viral infections. Viral stocks were diluted in cMEM and adsorbed to monolayers of cells plated the previous day. The time of adsorption was considered t=0. For MOI=2-20 infections, cells in 6-well plates were adsorbed in 200μl inocula with rocking every 10-15 min for 1 h. Inocula were removed for single-step growth curves. After adsorption cells were incubated in a normal culture volume of cMEM for the indicated times at 37^{o}C. Cells were harvested by direct lysis for immunoblots or by freezing at -80^{o}C for titers. Progeny virions were liberated by freeze-thaw lysis, lysates were serially diluted, and titers were determined by MHV68 plaque assay as described (Upton and Speck 2006).

Drug and UV treatments. Cidofovir (Vistide, Gilead Sciences, Inc.) was diluted to 20 ng/ml in cMEM. Viral stocks were diluted in Cidofovir containing media for adsorption, and incubations were carried out at 37^{o}C for the indicated times in the presence of Cidofovir. Drug efficacy was verified by parallel 24 h growth and subsequent plaque assay. Etoposide (Sigma) was dissolved in DMSO and diluted to the appropriate concentration in cMEM. Cells were treated with DMSO as a negative control or with

etoposide for the indicated times. UV treatments were performed on cells plated to ~ 70% confluence in 6-well plates. Culture media was removed and replaced with 400µl warm phosphate buffered saline (PBS). Cells were exposed to 100 J/m^2 UV light in a Stratalinker 2400 (Stratagene). PBS was removed and replaced with cMEM, and cells were incubated at 37^{o}C for the indicated times. Mock treatments were identically manipulated plates without UV exposure.

Imaging and immunofluorescence. Fixed and crystal-violet stained cells (described below) for mock, 50.Stop, 73.Stop, and 73.MR infection were viewed by light microscopy, and mLANA-GFP transfected 293T cells were imaged by fluorescence microscopy on a Leica DMRB microscope equipped with a Retiga EXi digital camera. For infections with recombinant mLANA-GFP MHV68, cells were plated on glass coverslips the day before infections. Cells were infected at an MOI of 10 PFU/cell. Cells were fixed with 10% formalin, washed with PBS, permeabilized and blocked with 0.1% Triton X-100 (TX100) in PBS containing 5% BSA (blocking buffer). Primary antibodies [anti-GFP (to increase the sensitivity of detection for mLANA-GFP), ORF59, or polyclonal MHV68 antiserum] were diluted in blocking buffer with 1% normal donkey serum and incubated for 1 h at 37^{o}C. For mLANA-GFP detection, staining specificity was verified by colocalizing true GFP fluorescence with red (Alexa 568) anti-GFP immunofluorescence. Cells were washed 3 times with 0.1% TX100 in PBS (wash buffer) and incubated with species-appropriate Alexa fluor-conjugated secondary antibodies (488 for mLANA-GFP, 568 for ORF59, or 350 for MHV68 antiserum; Molecular Probes/Invitrogen) diluted in blocking buffer at 37^{o}C for 45 min. Cells were washed 3 times with wash buffer, once with PBS and mounted on slides using ProLong Anti-fade

Gold reagent (Molecular Probes/Invitrogen). DNA was detected with Hoechst 33342 (Molecular Probes/Invitrogen) or with DAPI in the mounting media. MSCV or mLANA-GFP-transduced cells for p53 induction were plated on glass cover-slips the day before treatments. Cells were fixed with 10% formalin for 15-30 min, washed with PBS, then permeabilized with 0.5% TX100-PBS. Samples were incubated in blocking buffer. Samples were incubated overnight at 4^{o}C with primary antibody (anti-p53, see above) diluted 1:2000 in blocking buffer containing 1 μg/ml normal goat IgG (Santa Cruz Biotech). Cells were washed 3 times in wash buffer then incubated for 1 h at room temperature with secondary antibody (Alexa-fluor 568 goat anti-mouse) diluted in blocking buffer. After washing 3 times with wash buffer and once with PBS, samples were mounted on slides using DAPI-containing ProLong Anti-fade Gold reagent. Images were captured using a Zeiss Axio A1 Imager fluorescent microscope.

Immunoblot analyses. Cells were lysed with alternative RIPA buffer (150 mM NaCl, 20 mM Tris, 2 mM EDTA, 1% NP-40, 0.25% DOC, 1 mM NaF, and 1 mM Na_3VO_4 supplemented with complete mini-EDTA free protease inhibitors (Roche)) and quantitated using the BioRad DC protein assay prior to resuspending in Laemmli sample buffer, or equivalent numbers of cells (1-2 X 10^5) were directly lysed with 100 μl Laemmli sample buffer (Laemmli 1970). Samples were heated to 100^{o}C for 10 min and resolved by SDS-PAGE. Resolved proteins were transferred to nitrocellulose and identified with the indicated antibodies. Immobilized antigen and antibody were detected with HRP-conjugated secondary antibodies and ECL reagents (Amersham/GE Healthcare) and exposed to film. Densitometric analysis was performed by scanning

developed films using a Typhoon scanner (GE Healthcare) and analyzed using Image Quant Software (Amersham/GE Healthcare).

Reporter assays. NIH 3T3 cells were transfected with the indicated plasmids and incubated for 24 h, at 37^{o}C. A GFP reporter plasmid was included at 1:10 dilution as a transfection control. Parallel assays using a pp53-TA-luc reporter verified the function of the p53 expression construct. Cells were scraped, pelleted by centrifugation, and lysed using Promega passive lysis buffer. Lysates were clarified, and luciferase activity was determined using luciferase reporter assay kits (Promega). Luciferase activity was measured using a Turner Designs 2000 luminometer.

RNA Isolation and Quantitative RT-PCR. NIH 3T3 cells were infected at an MOI of 10 PFU/cell with MHV-68 ORF50-deficient virus (50.Stop, (Pavlova *et al.* 2003). 90 min post-infection, cells were treated with either DMSO or 100 μM etoposide. All treatments were performed in duplicate. Cells were harvested in TRIzol (Invitrogen) at four and eight hours post drug treatment. Total RNA was extracted according to the TRIzol RNA extraction protocol and quantified with an ND-1000 (NanoDrop Tech.). Following extraction, 2 μg of RNA was treated for 22 minutes with amplification grade DNase I (1 U; Invitrogen) in a 10 μL reaction containing 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl. Reactions were quenched by adding 1 μL 25 mM EDTA and heating for 10 minutes at 70°C. One microgram of treated RNA was reverse transcribed with SuperScript II (Invitrogen) in a total volume of 20 μL containing 180 ng random

hexamers. No-RT controls were carried out in parallel. Resulting cDNAs were diluted 1:3 in water.

Quantitative PCR was carried out on the resulting cDNA using a Bio-Rad iCycler. Each reaction contained 25 μL iQ SYBR Green Supermix (Bio-Rad), 10 pmol each primer, 2 μL cDNA and water to a final volume of 50μL and was performed in triplicate in optical 96-well plates. Primers for the reactions were as follows: *orf73*, 5'-AAGGGTTGTCTTGGCCTACTGTG-3', 5'-AGAGATGCTGTGGGACCATGTTG-5'; *orf50*, 5'-GGCCGCAGACATTTAATGAC-3', 5'-GCCTCAACTTCTCTGGATATGCC-3'; *glyceraldehyde-3-phosphate dehydrgenase (GAPDH)* 5'-CCTGCACCACCAACTGCTTAG-3' and 5'-GTGGATGCAGGGATGATGTTC-5'; and murine *p21* (Chipuk *et al.* 2004). Cycling conditions were as follows: 95°C for 5 minutes and then 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

Data were analyzed using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001), where $\Delta\Delta C_t = [C_t(\text{gene}) - C_t(\text{GAPDH})]_{\text{treated}} - [C_t(\text{gene}) - C_t(\text{GAPDH})]_{\text{mock-treated}}$. The data are presented as the evaluation of $2^{-\Delta\Delta Ct}$ for each sample, representing the fold change in transcript level of each gene in the treated samples, normalized to GAPDH, relative to the normalized level of that gene in the untreated control.

Cell viability assays. For trypan blue exclusion, cell-culture supernatants, which included non-adherent dead cells, were collected, and the remaining cells were detached by trypsinization. Supernatants and detached cells were combined and pelleted by centrifugation. Cells were resuspended in PBS and kept on ice until quantitation. Trypan

blue solution (Sigma) was added to the cells at a final concentration of 0.1 %, and positive and negative cells were identified by light microscopy. A modification of a previously described protocol was used for imaging of infected cells (Garcia *et al.* 2002; Garcia *et al.* 2006). Media and detached cells were removed by aspiration, followed by washing two times with PBS. Adherent cells were fixed for 15-30 min in 10% formalin. Cells were washed and stained with 0.1% crystal violet solution for 1-2 h. Stained cells were washed 3 times in deionized H_20 and allowed to dry. Cells were imaged or retained crystal violet was extracted with 10% acetic acid, transferred to microtiter plates, and quantified by spectrophotometry at 630 nm using a plate reader (Biotek). For p53-deficient MEFs, cells were fixed with 10% formalin and washed with PBS. Cells were permeabilized with 0.1% TX100-PBS and nuclei were stained with 1 μg/ml ethidium bromide. Nuclei visualized by fluorescence microscopy were quantitated from 3 random fields of vision. For etoposide-treated cells, cell viability was determined using the CellTiter-Glo assay (Promega) according to the manufacturer's instructions.

C) RESULTS

MHV68 LANA is required for efficient viral replication in primary MEFs at low multiplicities of infection. We previously demonstrated growth deficiencies for 73.Stop virus in the lungs of mice infected by intranasal inoculation compared to wild-type (WT) and 73.MR viruses. However, in our original analysis of 73.Stop replication in tissue culture we did not appreciate a replication defect, despite modestly reduced titers at late time-points in multi-step viral growth curves in NIH 3T12 fibroblasts (Moorman *et al.* 2003). To more thoroughly assess the role of mLANA in MHV68 replication in culture, we compared the capacities of 73.Stop and 73.MR viruses to replicate over time in Vero cells or in primary murine embryonic fibroblasts (MEFs) at an MOI of 0.05 (Fig. 2.1A and B). While 73.Stop replicates efficiently in Vero cells (replication kinetics and yields were enhanced compared to 73.MR at the times analyzed), 73.Stop replication was 7-fold reduced relative to 73.MR in MEFs 96 hours after infection at an MOI of 0.05 PFU/cell. We further assessed the nature of the 73.Stop replication defect by infecting MEFS and NIH 3T3 fibroblasts (the cell line used by Song et al.) at very-low MOI (0.001 PFU/cell) and observed an even greater dimunition of 73.Stop replication, with 73.Stop yielding 1.8 and 1.5 log average reductions, respectively, relative to 73.MR at time-points corresponding to peak output titers (Fig. 2.1C and D). These results demonstrate a role for mLANA in virus replication, especially following low multiplicity infection of both primary and immortalized murine fibroblasts. This correlates with the observed growth defect of the 73.Stop mutant *in vivo*.

MHV68 LANA is a nuclear/cytoplasmic protein expressed during productive virus replication. LANA proteins of KSHV, HVS, and RRV localize to the nuclei of host cells, where they regulate a variety of processes from modulating host and viral transcription to facilitating episome maintenance (Collins 2007). The requirement of MHV68 LANA for efficient viral replication, paired with data demonstrating that *orf73* is an immediate-early transcript present throughout lytic replication (Ahn *et al.* 2002; Martinez-Guzman *et al.* 2003; Coleman *et al.* 2005b), suggests an important role in the initial stages of viral replication. However, the localization of mLANA protein or its expression during productive replication has not been examined. To determine the expression and localization of mLANA during infection, we generated a recombinant mLANA-GFP fusion-expressing MHV68 (MHV68-73GFP). NIH 3T3 fibroblasts were infected with MHV68-73GFP and observed over time by fluorescence microscopy. These experiments revealed mLANA-GFP expression throughout the course of infection with diverse and time-dependent patterns of localization (Fig. 2.2A). Expression was restricted to the nucleus early during infection and was largely diffuse with focal areas of intensity. By 8 hours post-infection, globular staining was detected in some cells. mLANA-GFP localization by 12 hours post-infection was predominately diffuse exhibiting nuclear and some cytoplasmic staining. By 18 and 24 hours, mLANA localization varied dramatically. An interesting feature at these times was mLANA-GFP staining patterns that resembled herpesvirus replication foci (see especially 18 hour time-point in Fig. 2.2A) (Zhong and Hayward 1997; Wu *et al.* 2001), a pattern also seen for KSHV LANA in productively-infected endothelial cells (Krug *et al.* 2004). Expression and localization of mLANA relative to the viral processivity factor ORF59 at high magnification (Fig.

2.2B) and in cells that stain with MHV68 antiserum (Fig. 2.2C) depict mLANA localization during various stages of the replication process and provide further evidence that mLANA is expressed during productive viral replication. Taken together, these data demonstrate the unexpected diversity of mLANA localization during the course of a productive MHV68 infection, and its presence very early in infection suggests a role in modulating the viral replication process.

mLANA-deficient MHV68 exhibits increased kinetics of cell death during viral replication in tissue culture. In contrast to the low MOI replication deficit observed for 73.Stop (Fig. 2.1), high MOI 73.Stop infections of MEFs did not reveal an overt replication defect (Moorman *et al.* 2003). However, 73.Stop-infected cultures display cytopathic effect (CPE) much more rapidly than WT or 73.MR-infected cells following high MOI infections. Representative images of this phenotype at an MOI of 2 PFU/cell are shown 24 hours after infection of early passage NIH 3T3 fibroblasts (Fig. 2.3A). The uniformity of CPE exhibited by NIH 3T3 fibroblasts and reduced basal levels of apoptosis made them more amenable than MEFs to these studies, although MEFs also exhibit increased CPE following high MOI 73.Stop infections (data not shown). We quantitated the viability of infected cells by trypan blue exclusion and found that >50% of 73.Stop-infected cultures were non-viable 24 hours after infection compared to approximately 10% for cultures infected with 73.MR at equivalent MOI (Fig. 2.3B). Moreover, infection by 73.MR at a higher MOI of 5.0 PFU/cell still resulted in less than 30% cell death at this time-point (data not shown).

To complement the viability experiments, we examined the integrity of poly-ADP ribosylating polymerase (PARP) over time following infection with 73.Stop or 73.MR at equivalent MOI (Fig. 2.3C). During apoptosis and necrosis, PARP is specifically cleaved to smaller, faster migrating products that are readily detected by immunoblot, and thus serves as a biochemical marker for cell death (Duriez and Shah 1997; Soldani and Scovassi 2002). These experiments demonstrated that 73.Stop infection results in very rapid PARP cleavage, beginning between 6-12 hours post-infection, with complete cleavage by 24 hours post-infection. Conversely, 73.MR-infected cells exhibited PARP cleavage predominantly between 24-36 hours post-infection at timepoints corresponding to peak output titers (Fig. 2.3C). Full-length PARP was detected throughout the time-course for mock-infected cells. Intriguingly, we did not detect evidence of activated caspase 3, the effector caspase in the apoptotic cascade, nor did treatment with the pan-caspase inhibitor z-vad-fmk ameliorate cell death or rescue replication following 73.Stop infection (data not shown). These data suggest a non-apoptotic death pathway or reflect the functional activity of the viral bcl-2 orthologue (Virgin *et al.* 1997; Wang *et al.* 1999; Loh *et al.* 2005) in MHV68-infected cells. Together these data demonstrate that 73.Stop infection leads to increased cell death relative to 73.MR.

To assess the potential contribution of virus replication to 73.Stop-induced cell death, we analyzed the replication kinetics of 73.Stop and 73.MR in NIH 3T3 fibroblasts in a comprehensive time-course analysis over a single replication cycle following infection at an MOI of 2 PFU/cell (Fig. 2.4A). This analysis revealed that 73.Stop replication proceeds with increased kinetics compared to 73.MR, with 73.Stop virus yields being 0.5 log higher than those of 73.MR by 12 hours post-infection. With time,

however, the virus yields from 73.MR-infected cells surpass those of 73.Stop, with maximal virus production from 73.Stop-infected cells occurring by ca. 18-24 hours post-infection while titers from 73.MR-infected cells continued to increase until ca. 30 hours post-infection. Consistent with these data, we also detect more abundant cell-associated lytic antigens in 73.Stop-infected cells (data not shown), although it is unclear if this reflects retention due to cell death or truly correlates with increased early titers. Importantly, with the exception of the 18 h time-point, these results are statistically significant, and were consistent in multiple experiments. It is interesting to note that we observed a reduced eclipse phase in MEFs during 73.Stop infection at MOI=5 in our original study (Moorman *et al.* 2003), which further supports the observations reported here. These data reveal the mLANA-dependent single-cycle replicative differences in murine fibroblasts and predict that differences in viral titers following low MOI infections (Fig. 2.1) are the product of multiple inefficient rounds of 73.Stop replication. These data also suggest that in the absence of mLANA either deregulation of the lytic gene-expression cascade occurs, leading to increased replication kinetics and subsequent cell death, or that mLANA is required to control an infection-related stress response that otherwise influences the course of the viral replication cycle.

73.Stop infection is characterized by deregulated viral gene expression and activation of p53. Given the increased cell death and expedited replication kinetics of 73.Stop, we asked if viral early gene expression is more rapidly detected during 73.Stop infection in culture. For these experiments, lysates of NIH 3T3 fibroblasts infected with 73.Stop or 73.MR at equivalent MOI were analyzed by immunoblot for the presence of v-

cyclin and the viral processivity factor, ORF59. Relative to 73.MR, infection by 73.Stop resulted in more rapid and more robust expression of both v-cyclin and ORF59 (Fig. 2.4B), suggesting that normal viral gene expression is dysregulated in the absence of mLANA.

KSHV LANA and HVS LANA previously were demonstrated to inhibit activation of the tumor suppressor p53 (Friborg *et al.* 1999; Borah *et al.* 2004), which led us to hypothesize that p53 is activated by MHV68 in the absence of mLANA. Activation of p53 leads to its stabilization mediated by a variety of modifications including acetylation, sumoylation, and phosphorylation on a variety of serine residues [reviewed in (Harris and Levine 2005; Lavin and Gueven 2006)]. To assess and compare the activation status of p53 during the course of infection with either 73.Stop or 73.MR viruses, we performed immunoblots for total p53 and p53 phosphorylated on serine 18 (serine 15 in human p53), a residue commonly phosphorylated in response to DNA damage stimuli and observed in other herpesvirus infections (Jault *et al.* 1995; Boutell and Everett 2004; Shirata *et al.* 2005; Gaspar and Shenk 2006; Lavin and Gueven 2006). Untreated or UV-exposed cells served as respective negative and positive controls for p53 induction. Increased stabilization and more robust phosphorylation of p53 on serine 18 was observed in 73.Stop-infected cells compared to 73.MR infections (Fig. 2.4B), suggesting that MHV68 infection is recognized by the host cell as an agonist of p53 and further that mLANA is involved in regulating p53 activation. Moreover, to our knowledge these data provide the first evidence of a potential role for LANA proteins in regulating p53 during any aspect of gammaherpesvirus replication.

Increased cell death is independent of viral replication. To help determine whether expedited virus replication and/or cell stress is the cause of 73.Stop-induced cell death, we tested the capacity of a pharmacologic inhibitor of MHV68 DNA replication to inhibit 73.Stop-induced cell death. Cidofovir is a nucleoside analog that potently inhibits viral DNA synthesis at concentrations that have no observable toxic effects on cultured cells [(Neyts and De Clercq 1998), Fig. 2.5A and data not shown]. NIH 3T3 fibroblasts were mock infected or infected with 73.Stop (MOI=2) or 73.MR (MOI=2-20) in the presence of 20 ng/ml Cidofovir, a concentration previously demonstrated and verified in these experiments (see Materials and Methods) to efficiently block MHV68 replication (Neyts and De Clercq 1998), and cell viability was assessed 24-26 hours post-infection. Cidofovir treatment did not inhibit 73.Stop-induced CPE (Fig. 2.5A), nor did treatment inhibit the infection-associated loss of cell viability (Fig. 2.5B), while mock and 73.MR-infected cells displayed no observable CPE or loss of viability. Importantly, infection with 73.MR in the presence of Cidofovir at multiplicities up to 20 PFU/cell, or infection with a replication-incompetent ORF50-deficient virus (50.Stop, titer determined by plaque assay on complementing cells), exhibit much less CPE than 73.Stop at MOI=2 (Fig. 2.5A), providing confidence that 73.Stop-related cellular death is not simply the result of a particle effect due to an increased particle/PFU ratio. In addition, these data indicate that cell death associated with 73.Stop infection is not a direct result of virus replication (i.e., generation of progeny virus), and further suggest that mLANA inhibits an infection-related cellular stress response that would otherwise promote cell death.

We also tested the capacity of Cidofovir to inhibit the enhanced expression of viral early genes and activation of p53 observed following 73.Stop infection as

demonstrated in figure 2.4. Lysates of NIH 3T3 fibroblasts infected with 73.Stop or 73.MR in the presence or absence of Cidofovir were analyzed by immunoblot. Notably, even in the presence of Cidofovir, infection with 73.Stop virus resulted in robust expression of v-cyclin and ORF59, as well as eliciting p53 phosphorylation on serine 18, by 6 hours post-infection (Fig. 2.5C). These data indicate that viral replication is not the cause of death and further suggest that the observed dysregulation of viral gene expression in the absence of mLANA may be a direct consequence of very early events in MHV68 infection that trigger a cell-stress response leading to p53 activation – a process that is modulated or inhibited by expression of mLANA.

Expression of mLANA correlates with decreased p53 stabilization and cell death following etoposide treatment. KSHV LANA was shown to prevent cell death triggered by a variety of p53-inducing agonists (Friborg *et al.* 1999). The increased activation of p53, and correlative induction of viral gene expression described above, suggested a role for mLANA in limiting p53 induction to facilitate survival of an infected cell. To analyze the capacity of mLANA to limit p53 induction, NIH 3T3 fibroblasts stably transduced with either empty vector (MSCV) or mLANA-GFP-expressing retroviruses were treated with the potent p53-inducing radio-mimetic drug, etoposide, and p53 stabilization was examined by immunofluorescence (Fig. 2.6A). In these experiments, cells with the highest expression of mLANA-GFP correlated with the lowest levels of p53 accumulation in the nucleus, which coupled mLANA expression to p53 destabilization. As a functional complement to the immunofluorescence experiments, we examined the capacity of NIH 3T3 fibroblasts expressing mLANA-GFP to resist

death relative to empty vector transduced control cells following treatment with increasing concentrations of etoposide (Fig. 2.6B). The presence of mLANA-GFP provided modest protection from death induced by the p53-inducing stimulus. Of note, the greatest protection from etoposide-induced cell death occurred with the lowest concentrations tested (≤ 25 μM). We also observed mLANA-related protection from death following UV exposure and in NIH 3T12 fibroblasts expressing untagged mLANA (data not shown). To provide a more detailed analysis of this phenomenon, we extended our experiments to a time-course analysis of cell viability following etoposide treatment at concentrations of 12.5 and 25 μM (Fig. 2.6C). In support of the dose-response experiments, mLANA-GFP provided increased protection from etoposide-induced cell death with the protection becoming more evident throughout the time-course. These data provide functional evidence that MHV68 mLANA singularly has the capacity to increase the threshold for toxicity following treatment with classic p53 agonists and suggest that this is accomplished by limiting p53 induction.

To gain additional insight into a role for mLANA in limiting p53 induction and death during MHV68 infection, we sought to determine the effects of 73.Stop infection in p53-deficient cells. In preliminary experiments, the differences in cellular morphology and growth rate made direct comparisons of cell death in WT and $p53^{-/-}$ MEFs difficult. To normalize the system, $p53^{-/-}$ MEFs were transduced with retroviruses encoding DsRed as a negative control ($p53^{-/-}$) or a p53-DsRed fusion protein ($p53^{comp}$) to reintroduce p53 expression. The function of p53 fusion proteins has been previously described (Ferbeyre *et al.* 2000; Rowland *et al.* 2002; Casavant *et al.* 2006) and is demonstrated for our construct (data not shown). Immediately following puromycin selection and expansion,

cells were infected with 73.Stop or 73.MR at a MOI of 2 PFU/cell. Infections were performed in the presence of Cidofovir to block contribution of virus replication to cell death. Viability determination 12 hours post-infection demonstrated a reduction in cell death associated with 73.Stop infection in $p53^{-/-}$ cells relative to $p53^{comp}$ cells (Fig. 2.6D). The difference, however was completely lost by 24 hours post-infection (data not shown), suggesting that the contribution of p53 to 73.Stop-induced death occurs early during infection. These data additionally suggest roles for the unchecked viral gene expression or a currently undefined cellular stress in 73.Stop-related cellular injury.

P53 plays a general role in virus replication that influences the efficiency of 73.Stop replication. The capacity of mLANA to regulate p53 induction paired with activation of p53 by 73.Stop virus suggests that p53 induction contributes to the replication deficit observed for 73.Stop following low MOI infection. To understand the role of p53 activation in 73.Stop replication, we performed multi-step growth analyses using the $p53^{-/-}$ or $p53^{comp}$ cells described above (see Fig. 2.6). Consistent with experiments in WT MEFs or NIH 3T3 cells (Fig. 2.1), viral titers in $p53^{comp}$ MEFs for 73.Stop peaked at a 1.4 log deficit relative to 73.MR (Fig. 2.7A). Conversely, although 73.Stop displayed a delay in the onset of productive replication in $p53^{-/-}$ MEFs, by 168 hours post-infection titers for both 73.Stop and 73.MR were virtually identical [73.Stop: 6.7 +/- 0.4 PFU/ml; 73.MR: 7.1 +/- 0.5 PFU/ml (Fig. 2.7B)]. Remarkably, this equilibration was not due to an overt increase in 73.Stop replication. Rather, it represented an 8-fold reduction in 73.MR output (7.9 +/- 0.1 PFU/ml in $p53^{comp}$ cells). As further evidence that p53 directly impacts MHV68 replication, while titers began to

increase between 24 and 48 hours post-infection in $p53^{comp}$ cells, the "eclipse" phase was prolonged a further 24 hours in $p53^{-/-}$ MEFs for both 73.Stop and 73.MR in these assays. These data indicate that p53 regulates the timing and efficiency of MHV68 replication.

P53 promotes viral gene expression. The data presented above, in conjunction with the correlation between p53 induction and increased viral gene expression following 73.Stop infection, suggested the capacity of p53 to promote expression of viral genes. To more precisely define a role for p53 in augmenting MHV68 replication, we compared viral antigen production over time in WT and p53-deficient MEFs infected at an MOI of 2 PFU/cell with WT MHV68, 73.Stop, or 73.MR (Fig. 2.7C). In this assay, while ORF59 and v-cyclin were readily detectable 6-8 hours after infection with WT MHV68 and 73.MR in WT MEFs, signal was not evident for these antigens in $p53^{-/-}$ MEFs. Identical blots performed 14 hours post-infection demonstrated that the $p53^{-/-}$ MEFs supported viral gene expression from the WT and MR viruses in these experiments (data not shown). Although 73.Stop still displayed the increased viral gene expression portrayed in Figures 2.4 and 2.5, expression of ORF59 and v-cyclin similarly exhibited relative delays in the absence of p53, which is in agreement with the early function of p53 in 73.Stop-induced death described in Figure 2.6D. Together, these data support a role for p53 in enhancing MHV68 replication and potentially define the basis for increased viral gene expression and advanced replication kinetics at high MOI for mLANA-deficient virus.

To more specifically determine a mechanism for p53-related induction of viral gene expression and replication, we tested the capacity of p53 and p53-inducing agonists to drive viral gene expression. Since v-cyclin and ORF59 were more rapidly and more

abundantly expressed, and p53 was activated during infection with mLANA-deficient virus, we hypothesized that p53 expression could induce activity of the proximal *v-cyclin* lytic-cycle-associated promoter [a promoter active during MHV68 replication in permissive fibroblasts (Allen *et al.* 2007)] in the absence of any viral gene products. Since this promoter is responsive to the immediate-early transactivator RTA, we further hypothesized that induction of early viral gene expression could alternatively be triggered through p53-mediated induction of *orf50*, leading to RTA expression and subsequent induction of viral early genes. To test these hypotheses, NIH 3T3 fibroblasts were transfected with *v-cyclin* and *orf50* promoter-driven reporter constructs in the presence or absence of a p53 expression vector. Transfection of the p53 expression vector slightly increased *v-cyclin* promoter activity (2-fold), while eliciting an approximate 5-fold induction of the *orf50* promoter (Fig. 2.8A). These data suggest an RTA-mediated positive feedback loop for increased v-cyclin expression in response to p53. Transfection efficiencies in these analyses were similar, as monitored by co-transfection of a GFP expression vector, and complementary experiments with a p53-responsive reporter vector verified the functionality of the p53 expression construct (data not shown). To gain additional insight into a potential role for p53 in MHV68 infection, we asked whether an exogenous p53-inducing stressor could influence viral gene expression by treating NIH 3T3 fibroblasts with etoposide following infection with the RTA-null 50.Stop MHV68 mutant (Pavlova *et al.* 2003) (Fig. 2.8C). 50.Stop virus was used to minimize potential influences of RTA-responsive genes. Analysis by quantitative RT-PCR demonstrated substantially increased *orf50* transcription that accumulated over time following etoposide treatment. Induction of the p53-responsive cyclin inhibitor *p21* served as a

positive control for drug treatment (Chipuk *et al.* 2004) (Fig. 2.8C). Interestingly, we observed a 2-fold decrease in *orf73* transcripts following etoposide treatment, perhaps suggesting a negative feedback loop that facilitates the progression of the lytic gene expression cascade. Further, etoposide treatment of cells infected with WT MHV68 promoted a reproducible 2-fold increase in v-cyclin protein expression relative to vehicle-treated samples as assessed by densitometry (Fig. 2.8D). Together, these data provide strong evidence that MHV68 is poised to respond to either p53 itself or p53-activating stimuli, and further implicate p53 as a factor that promotes viral gene expression toward enhanced lytic replication. Moreover, these findings suggest that mLANA critically regulates this process in a manner that limits host cell injury to permit efficient viral replication.

D) DISCUSSION

In this paper we have examined the role of the MHV68 LANA homologue, mLANA, in virus replication. We found that mLANA-null virus is attenuated in primary and immortalized fibroblasts at low MOI and that mLANA is a nuclear/cytoplasmic protein expressed during virus replication in permissive fibroblasts. At higher multiplicities of infection, 73.Stop resulted in increased kinetics of cell death with apparently protracted entry into the productive replication phase, but an overall deficit in viral yield. Consistent with these findings, we detect increased viral gene expression in the absence of mLANA and determined that 73.Stop-induced cell death occurred in the presence of Cidofovir, demonstrating that the induction of cell death was independent of viral replication. We further observed that infection with 73.Stop resulted in more robust activation of p53 than 73.MR, and that p53 activation also was independent of virus replication. In agreement with a role for mLANA in modulating p53 induction, expression of an mLANA-GFP fusion protein in fibroblasts provided modest protection from etoposide-induced death, characterized at the cellular level by diminished p53 stabilization. Moreover, p53-deficient cells were less susceptible than p53-competent cells to 73.Stop-induced death at early times post-infection. We found that 73-null virus replicated to the same level as 73.MR in p53-deficient cells, but more remarkably, that p53 generally promoted MHV68 replication. To this end, we found that p53 over-expression or treatment with the potent p53-inducing drug etoposide could increase promoter activity, transcription, and expression of viral genes that promote lytic replication. Together, these data further define the replication defect previously demonstrated for mLANA-null virus *in vivo*, reveal a previously unappreciated role for

p53 in the MHV68 life cycle, and illuminate a role for mLANA in managing a cellular stress response to viral infection to prevent cellular injury and coordinate the MHV68 lytic gene-expression cascade.

Based on these studies, we propose a model for the roles played by mLANA to regulate MHV68 replication (Fig. 2.9). In this model, the immediate-early mLANA limits the induction of cellular stress responses that would otherwise activate p53 and currently undefined pathways that drive viral gene expression. We envision mLANA either reducing the stressors directly or indirectly by counteracting feedback loops. We hypothesize that mLANA in this capacity orchestrates the cell and virus during infection in a manner that limits cellular injury and maximizes viral yield. We think it likely that unregulated cell stress and viral gene expression form a self-perpetuating loop that drives the host cell to death. We also illustrate the newly defined role for p53 in promoting lytic viral replication and that mLANA may alter p53 function by preventing activation, blocking downstream functions, or both. Our experiments did not delineate these possibilities, nor did we pinpoint an exact role for p53 in 73.Stop-induced cell death, although experiments with mLANA transduced cells suggest an attenuation in p53-related death pathways (Fig. 2.6). Aside from a role for mLANA in episomal tethering to facilitate viral genome maintenance during latency, our data support the hypothesis that mLANA critically influences host-cell survival and/or viral gene expression, not only to allow efficient replication, but also to promote establishment and maintenance of latent MHV68 infection.

MHV68 LANA and viral replication. In previous studies we demonstrated that ORF73-mutant viruses exhibit a replication defect in the lungs of infected mice, and Song and colleagues reported a transposon mutant in ORF73 that was attenuated in NIH 3T3 fibroblasts (Song *et al.* 2005). Moreover, the immediate-early transcription pattern described for *orf73* (Coleman *et al.* 2005b) suggests an important role in facilitating MHV68 replication. The experiments reported here extend the previous findings by demonstrating an MOI-dependent requirement for mLANA in viral replication in fibroblasts (Fig. 2.1), but do not offer an immediate mechanism for potential cell-type specific differences in 73.Stop replication. There are numerous possible explanations for 73.Stop replicating efficiently in Vero (Fig. 2.1) or BHK-21 (Fowler *et al.* 2003) cells including, but not limited to, species and tissue of origin, lack of expression of host inhibitory factors, and length of cell-cycle. The data we present, particularly complementation of 73.Stop replication at low MOI in p53-deficient MEFs, indicate the importance of p53 in limiting 73.Stop replication. It is interesting to note that the NIH 3T12 immortalization regimen for MEFs can promote mutations in p53 (Rittling and Denhardt 1992), which perhaps offers an explanation for why we did not originally appreciate a replication deficiency for 73.Stop in culture (Moorman *et al.* 2003).

Unlike herpesvirus immediate-early genes with transactivator functions that are absolutely required for lytic replication, such as HSV1 ICP4 (DeLuca *et al.* 1985), HCMV IE2 (Marchini *et al.* 2001), and MHV68 RTA [ORF50, (Pavlova *et al.* 2003)], a second “class” of immediate-early genes display their defects only at low MOI, such as ICP0 of HSV1 (Hagglund and Roizman 2004) or HCMV IE1 (Mocarski *et al.* 1996). Perhaps this reveals requirements for these genes in regulating cellular stresses very early

in the infection process in the absence of high-MOI virus particle effects or in the face of an active cellular or host immune response. Detection of mLANA in the nucleus very early in infected cells (as early as 2 h post-infection in separate experiments, J.C.F and S.H.S unpublished results) suggests the importance of mLANA in modulating viral and host gene expression or limiting activation of nuclear stress pathways, such as a DNA damage response, to provide an ideal cellular environment for viral replication.

Role of p53 in MHV68 replication. Activation of p53 occurs in response to numerous cellular stresses, ranging from contact inhibition to nutrient deprivation to viral infection, and is central to maintaining metabolic homeostasis and genetic integrity (Harris and Levine 2005; Lavin and Gueven 2006). In its role as a regulator of cell-stress responses, p53 enforces cell-cycle checkpoints to allow recovery from the sensed stress, but p53 can also direct initiation of apoptosis by promoting expression of apoptosis-initiating genes, repressing expression of anti-apoptotic genes, or direct insult to mitochondrial integrity (Baptiste and Prives 2004; Chipuk *et al.* 2004; Harris and Levine 2005). Being critically involved in recognizing and responding to cellular events that could elicit nutrient crisis or genomic instability, it stands to reason that nuclear invasion and exponential synthesis of large, foreign DNAs would elicit a p53 response. Conversely, the rationale for viral inactivation or deregulation of such a gatekeeper is also quite clear. Indeed, the characterization of DNA tumor virus replication (e.g., adenovirus, papillomavirus, and SV40) helped define the regulation of several p53-mediated-tumor-suppression related pathways through the analysis of early gene functions (E1B, E6, and large T antigen, respectively) that either inhibit p53 activation or

promote p53 degradation (Nevins 2001). Importantly, adenovirus and SV40 with mutations in E1B and large T antigen, respectively, exhibit attenuated replication and increased cell death (Stillman 1986; Sullivan and Pipas 2002).

In the case of herpesvirus replication the role of p53 is less clear. Both HCMV (Jault *et al.* 1995; Gaspar and Shenk 2006) and HSV1 (Boutell and Everett 2004; Shirata *et al.* 2005) induce p53 during infection. During HSV1 infection, p53 is sequestered into viral replication complexes (Wilcock and Lane 1991; Zhong and Hayward 1997), although p53 status does not appear to regulate HSV1 replication (Boutell and Everett 2004). The functional presence of p53 positively influences the kinetics and productivity of HCMV replication (Casavant *et al.* 2006), in a manner analogous to that for MHV68 (Fig. 2.7). This remarkable functional conservation across herpesvirus families suggests similar mechanisms of p53 regulation and utilization during productive viral replication. Similar to HSV1, HCMV recruits p53 into viral replication compartments (Fortunato and Spector 1998; Rosenke *et al.* 2006), presumably in a manner that requires the IE2 viral protein and inactivates p53 function (Tsai *et al.* 1996; Castillo and Kowalik 2002). Although the mechanism whereby mLANA inhibits p53 activation is not apparent from our studies, it is tempting to speculate that gammaherpesvirus LANA proteins directly recruit p53 to viral replication compartments to inhibit p53 transcriptional regulation of host genes. This hypothesis is consistent with data demonstrating that KSHV LANA and HVS LANA engage p53 to inhibit its function (Friborg *et al.* 1999; Borah *et al.* 2004). KSHV LANA also limits p53 expression and activation of a *p53* promoter (Si and Robertson 2006), a finding consistent with the decrease in levels of nuclear p53 we observed in mLANA-GFP-expressing cells following UV (data not shown) or etoposide

treatment (Fig. 2.6B). Alternatively, mLANA may limit p53 induction by altering one or more other host stress pathways, numerous of which have been described for KSHV LANA (Collins 2007). A more refined interpretation will involve the temporal determination of viral and host proteins and genes targeted by LANA during productive rhadinovirus replication.

Our finding that mLANA limits p53 activation during productive MHV68 replication, but that p53 promotes viral gene expression and replication, are likely reflections of the tenuous relationship between herpesviruses and the host cell. Cassavant and colleagues suggest that p53 positively influences HCMV gene expression and subsequent replication as a transcriptional activator for viral replication and structural genes (Casavant *et al.* 2006). In this case, sequestration of p53 into replication complexes would facilitate viral gene transactivation while limiting induction of p53-responsive host genes.

In light of mLANA limiting p53 activation during MHV68 replication, we propose additional hypotheses for p53 in the herpesvirus life-cycle. LANA proteins of KSHV, HVS, and RRV inhibit activation of promoters for the lytic transactivator protein, RTA (Schafer *et al.* 2003; Lan *et al.* 2004; DeWire and Damania 2005). As a corollary for HVS in particular, mLANA induction limits the progression of lytic replication in culture (Schafer *et al.* 2003). We did not detect mLANA-mediated inhibition of an *orf50* promoter (Fig 2.8B), but we did demonstrate p53-related *orf50* induction (Fig. 2.8). In addition, the absence of mLANA promoted increased p53 activation and viral gene expression (Figs. 2.4 and 2.5). Together these findings suggest that LANA proteins limit p53-dependent activation of lytic-phase viral genes and viral replication, thereby

depicting the importance of mLANA in regulating the p53 response to facilitate efficient viral replication. Moreover considering the delay in these events in cells lacking p53 (Fig. 2.7), these data predict that p53 serves as a catalyst for viral replication, perhaps even dictating the switch from latency to lytic replication.

Cell death and v-cyclin. The mechanism of cell death induction by 73.Stop infection is not clear. Our results in Figure 2.6 suggest that p53 contributes to the cell death phenotype, especially early during infection, and to this end mLANA can protect cells from death caused by p53-inducing treatments. However, the absence of p53 is not protective to 73.Stop-infected cells at later timepoints (data not shown) even when viral replication is pharmacologically inhibited, indicating the execution of p53-independent death pathways. These results are not necessarily surprising, and probably reflect the delay, but not total shutdown, in viral gene expression observed in p53-deficient MEFs (Fig. 2.7). To be sure, the DNA damage caused by UV light and etoposide induce numerous stress pathways that inhibit cell cycle progression and mediate repair or cell death, even in the absence of p53 [for example, see (Reinhardt *et al.* 2007)]. Perhaps insight into other stress pathways altered by LANA functions can be gathered from studies in p53-deficient cells. Furthermore, as p53 mutations are very common in human cancer (Vogelstein *et al.* 2000) and p53-independent cell death pathways thus form the basis for many cancer treatment regimens, understanding how gammaherpesvirus-infected cells limit death in the absence of p53 may offer new insights into treatments for gammaherpesvirus related tumors.

Given the anti-growth and cell-cycle inhibitory properties of p53, it is remarkable that v-cyclin expression is so dramatically increased early during 73.Stop infection (Figs.

2.4 and 2.5). MHV68 v-cyclin expression promotes tumor development in transgenic mice (van Dyk *et al.* 1999) and interacts with CDK1 and CDK2 to positively support pRb phosphorylation in vitro and cell-cycle progression in serum-starved infected cells (Upton *et al.* 2005). Since p53-dependent transcription of the CDK inhibitor p21 potently blocks cellular cyclin-dependent pRb phosphorylation and progression from G1 to S-phase (Vidal and Koff 2000; Vogelstein *et al.* 2000), we speculate that the robust v-cyclin expression observed in 73.Stop-infected cells is a compensation mechanism that facilitates replication when the cell-cycle is arrested by p53 activation in the absence of mLANA expression. Indeed, despite p53 induction, we detect more rapid pRb hyper-phosphorylation [which promotes E2F activity and cellular DNA synthesis (Cobrinik 2005)] in 73.Stop-infected cells relative to 73.MR (J.C.F and S.H.S unpublished), which is consistent with increased v-cyclin expression. These ideas support our data demonstrating RTA and v-cyclin induction by p53-related cellular stresses (Fig. 2.8). Since v-cyclin is a requirement for MHV68 reactivation following *ex vivo* plating in some settings (van Dyk *et al.* 2000; Upton and Speck 2006), these data further suggest that cell-cycle arrest and/or p53 activation contribute to viral reactivation from latency.

Similar to mLANA-mutant viruses, v-cyclin mutants exhibit replication deficits in the lungs of infected mice, although the effect is dose dependent for v-cyclin mutants (Upton and Speck 2006). It will be interesting to determine if mLANA similarly compensates for v-cyclin-mutant MHV68 by increasing expression. If our hypotheses regarding compensatory functional mechanisms between v-cyclin and mLANA are correct, we would further speculate that a virus lacking both genes will be severely attenuated, perhaps even replication incompetent in some experimental settings.

Moreover, as oncogene expression or deregulated expression of cell-cycle control proteins, including cyclin E, can trigger p53 activation and cell-cycle arrest (Lowe and Ruley 1993; Serrano *et al.* 1997; Lin *et al.* 1998; Bartkova *et al.* 2006; Di Micco *et al.* 2006), it is tempting to speculate that, while partially rescuing 73.Stop replication, v-cyclin "overexpression" during 73.Stop infection actually contributes to the infection-related cellular injury, thereby promoting increased cell death and an ensuing defect in virus replication. Indeed, we observed striking v-cyclin expression and death following 73.Stop infection, even in the presence of Cidofovir (Fig. 2.5). Such a precarious balance between survival and death may exemplify the tightly regulated nature of herpesvirus gene expression.

E) FIGURES AND LEGENDS

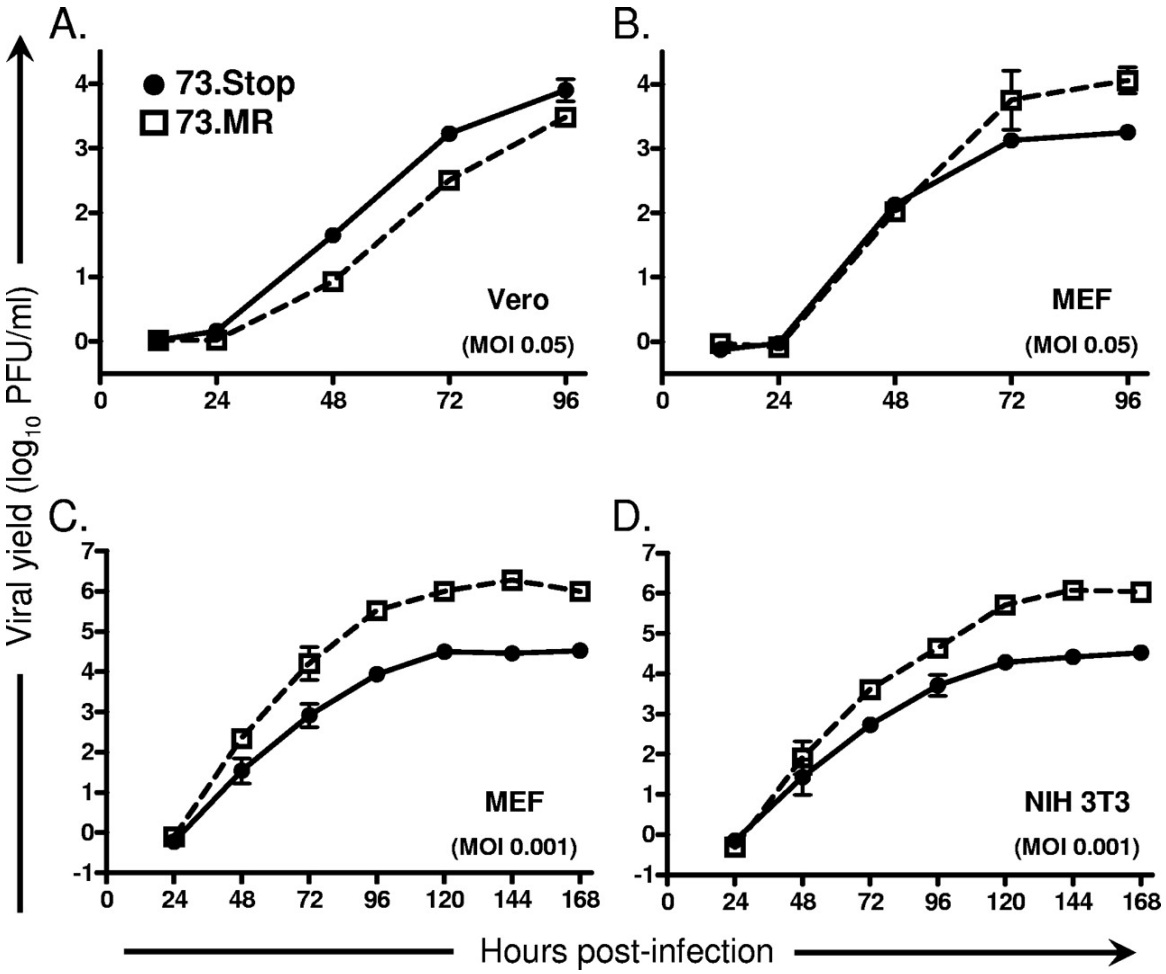

Figure 2.1. mLANA promotes efficient viral replication in primary murine fibroblasts. Vero cells (A), MEFs (B and C), or NIH 3T3 fibroblasts were infected with 73.Stop or 73.MR viruses at multiplicities of 0.05 (A and B) or 0.001 PFU/cell (C and D). Cells were harvested at the indicated times post-infection, and viral titers were determined by plaque assay. Results are the means of triplicate samples. Error bars represent standard deviations. This data was generated by C.R. Paden and J.C. Forrest.

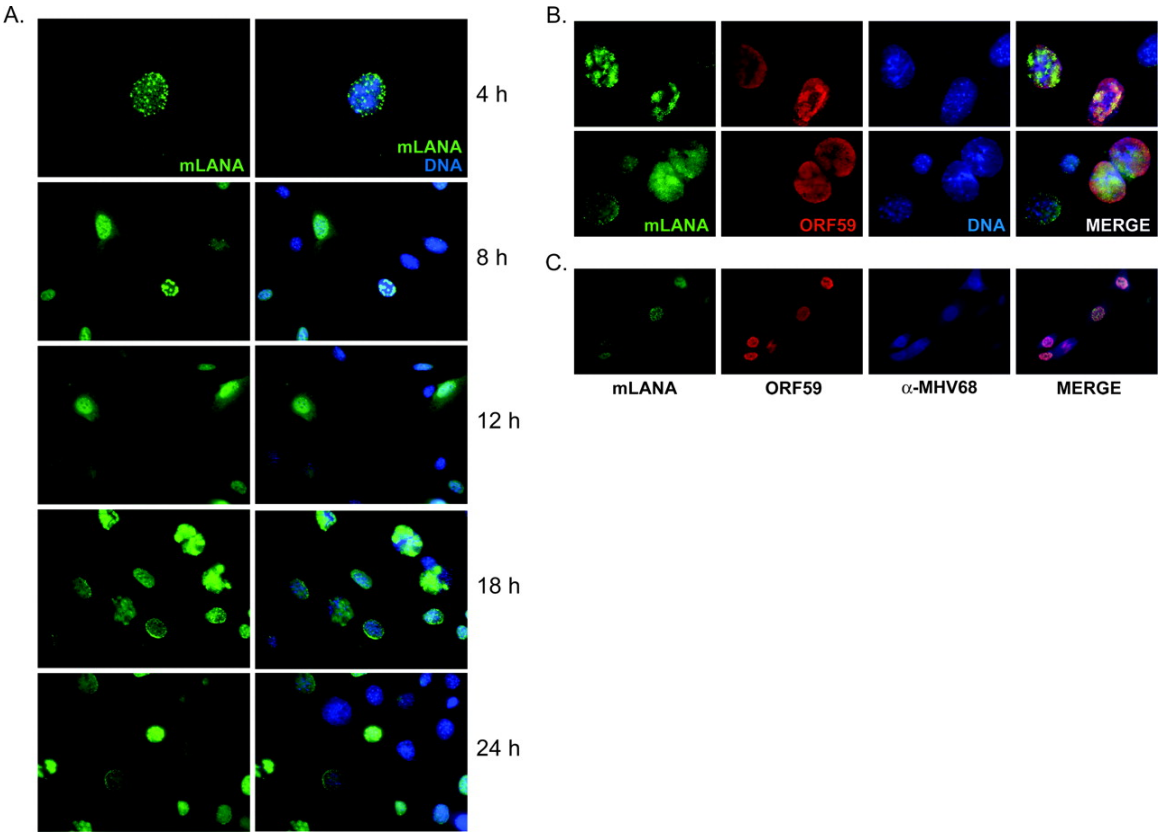

Figure 2.2. mLANA is a nuclear/cytoplasmic protein expressed during lytic replication. NIH 3T3 fibroblasts were infected with recombinant MHV68-73GFP at an MOI of 10 PFU/cell. (A) Cells were fixed at the indicated times post-infection and stained with GFP-directed antiserum to visualize mLANA-GFP subcellular localization by fluorescence microscopy. Cells were stained with antibodies to GFP or ORF59 (B) or GFP, ORF59, and MHV68 antiserum (C) 24 h post-infection. DNA was stained with DAPI (A) or Hoechst 33342 (B). The images in panel A at 4 h and in panel B were captured at 100X magnification. All others were captured at 40X magnification. This data was generated entirely by J.C. Forrest.

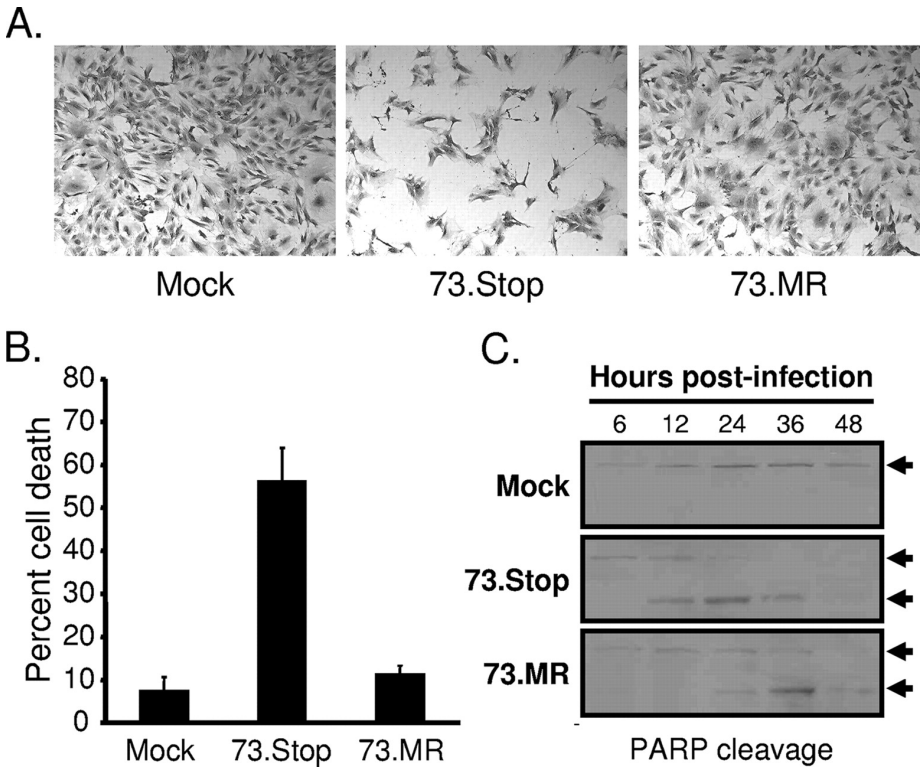

Figure 2.3. mLANA-deficient virus exhibits increased kinetics of cell death and PARP cleavage. (A and B) NIH 3T3 fibroblasts were mock infected or infected with 73.Stop or 73.MR viruses at multiplicities of 2 PFU/cell and harvested 24 h post-infection. Representative images of cells that were fixed and stained with crystal violet for imaging by light microscopy are shown (A), or cell viability of triplicate samples was determined by trypan blue exclusion (B). A minimum of 200 cells was counted per sample. Results are the means of triplicate samples. Error bars represent standard deviations. (C) NIH 3T3 fibroblasts were mock infected or infected with 73.Stop or 73.MR viruses at multiplicities of 2 PFU/cell and harvested at the indicated times post-infection. Equivalent amounts of total protein were resolved by SDS-PAGE and the integrity of PARP was determined by immunoblot. Arrows denote intact (upper) or cleaved (lower) PARP. This data was generated by J.C. Forrest.

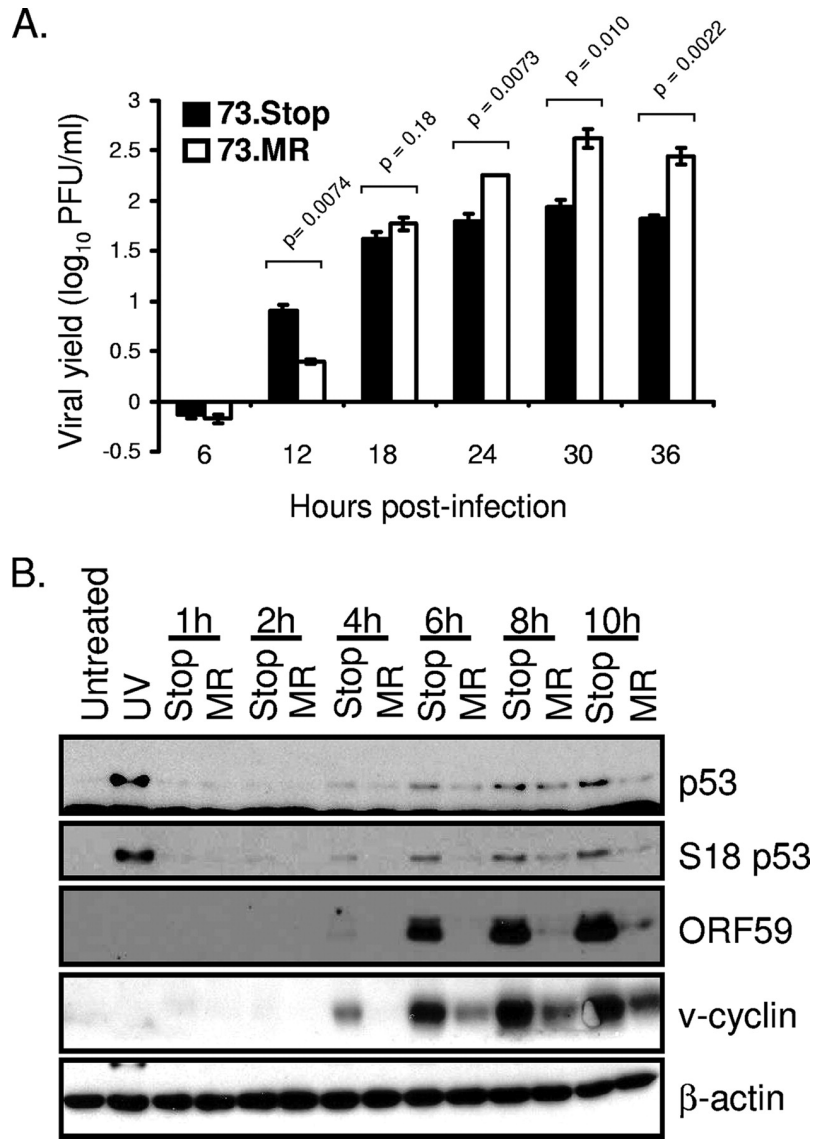

Figure 2.4. 73.Stop virus exhibits increased replication, viral antigen expression, and p53 induction.

Figure 2.4. (cont) (A) MEFs were infected with 73.Stop or 73.MR viruses at multiplicities of 2 PFU/cell, and cells were harvested at the indicated times post-infection. Viral yields were determined by plaque assay. Results are the means of triplicate samples. Error bars represent standard deviations. P-values were determined using a two-tailed, paired student's t-test. (B) NIH 3T3 fibroblasts were infected with 73.Stop or 73.MR virus at multiplicities of 2 PFU/cell. Equivalent numbers of cells were harvested directly by addition of Laemmli sample buffer at the indicated times post-infection. Total cell lysates were resolved by SDS-PAGE and analyzed by immunoblot with antibodies to the indicated proteins. Untreated and UV-exposed cells (100 J/m^2) serve as respective negative and positive controls for p53 stabilization and activation. The blot shown is representative of three independent experiments. Panel A as generated by C.R. Paden; Panel B was generated by J.C. Forrest.

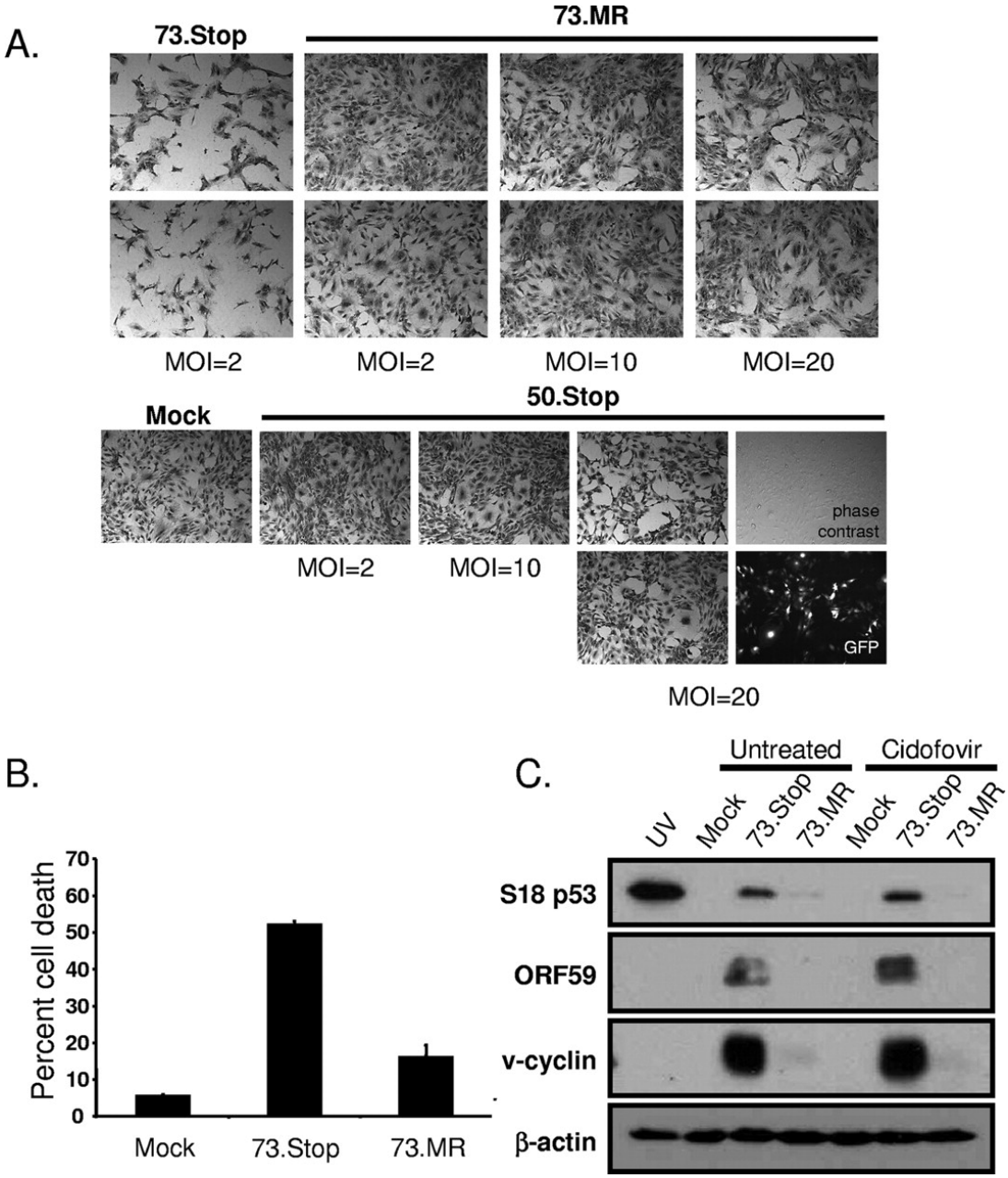

Figure 2.5. 73.Stop-induced cell death, p53 phosphorylation, and dysregulated early gene expression occur independent of viral replication.

Figure 2.5. (cont) (A-C) NIH 3T3 fibroblasts were mock infected, infected with 73.Stop or 73.MR virus at multiplicities of 2-20 PFU/cell in the presence of 20 ng/ml Cidofovir (A and B) or with 50.Stop virus at multiplicities of 2-20 PFU/cell (A). Representative images of cells that were fixed and stained with crystal violet 24 h after infection for imaging by light microscopy are shown (A), or cell viability was determined by trypan blue exclusion 26 h after infection (B). A minimum of 200 cells per sample was counted. Results are the means of triplicate samples. Error bars represent standard deviations. (C) NIH 3T3 fibroblasts were mock infected or infected with 73.Stop or 73.MR viruses in the presence or absence of 20 ng/ml Cidofovir at multiplicities of 2 PFU/cell. Equivalent numbers of cells were harvested directly by addition of Laemmli sample buffer 6 h post-infection. Total cell lysates were resolved by SDS-PAGE and analyzed by immunoblot with antibodies to the indicated proteins. UV-exposed cells (100 J/m^2) serve as a positive control for p53 activation. The blot shown is representative of three independent experiments. The data in this figure was generated by J.C. Forrest.

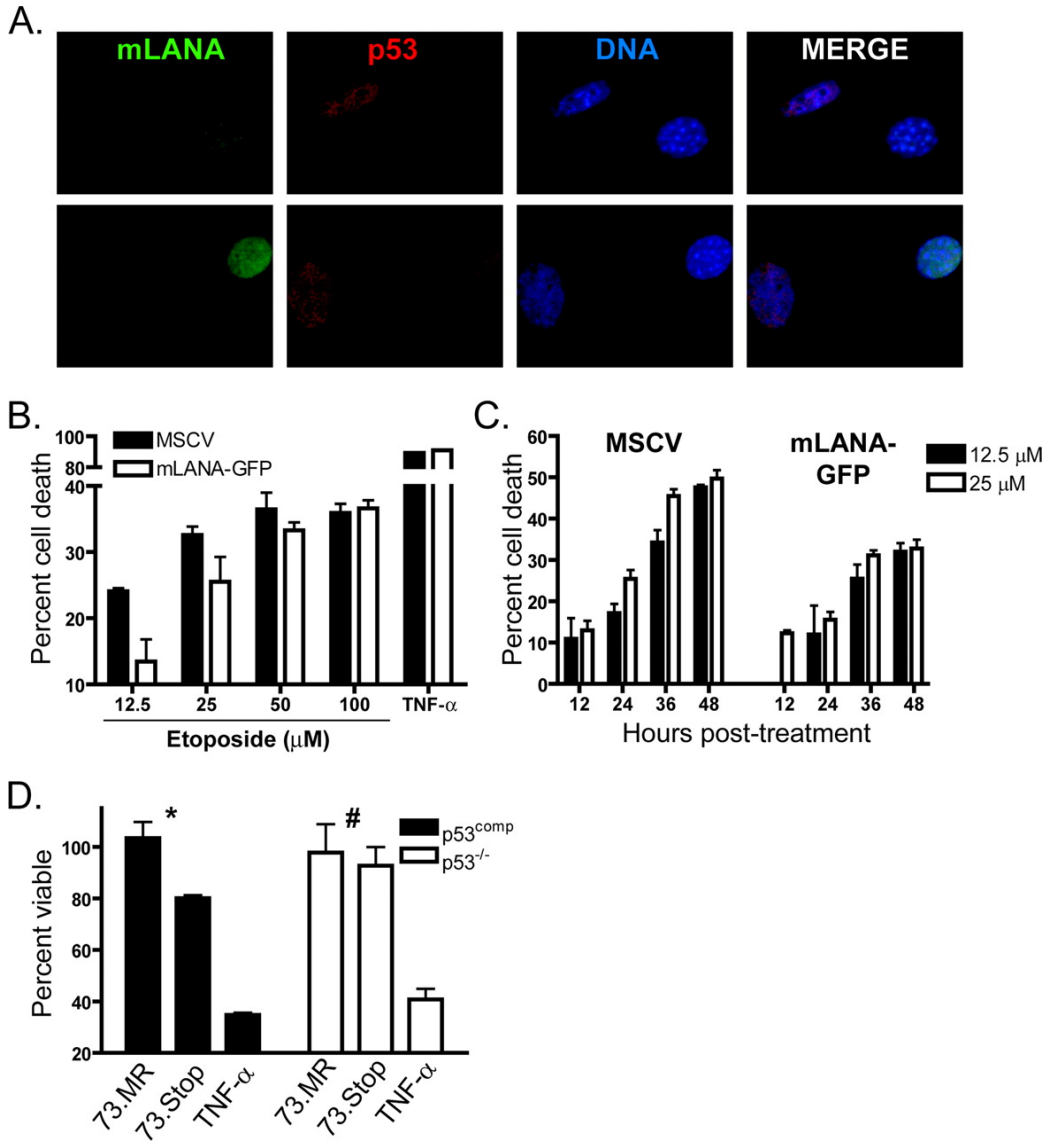

Figure 2.6. mLANA inhibits p53 induction and reduces etoposide-induced cell death.

Figure 2.6 (cont) (A) mLANA-GFP-transduced NIH 3T3 fibroblasts were treated with etoposide (100 μM). Cells were fixed and stained for p53 4 h post-treatment. Two representative images are shown at 100X magnification. (B and C) Empty vector (MSCV) or mLANA-GFP-transduced NIH 3T3 fibroblasts were treated with etoposide at the indicated concentrations. Cell viability was analyzed 28 h post-treatment (B) or over a time-course (C). Data are presented as the percentage of cell death with treatment relative to mock-treated controls. Results are the means of triplicate samples. Error bars represent standard deviations. (D) $P53^{comp}$ or $p53^{-/-}$ MEFs were infected with 73.Stop or 73.MR viruses at multiplicities of 2 PFU/cell in the presence of 20 ng/ml Cidofovir. Cell viability was determined 12 h post-infection. Data represent the percentage of cell death relative to mock-infected samples. Results are the means of triplicate samples. Error bars represent standard error of the mean. Statistical analyses of differences for 73.MR and 73.Stop-infected cells were performed using a two-tailed, paired student's t-test (*, p=0.05; #, p=0.66). TNF-α samples were treated with 25 ng/ml TNF-α and 10 μg/ml cycloheximide as a positive control for cell death induction in B and D. The entirety of this figure was generated by J.C. Forrest.

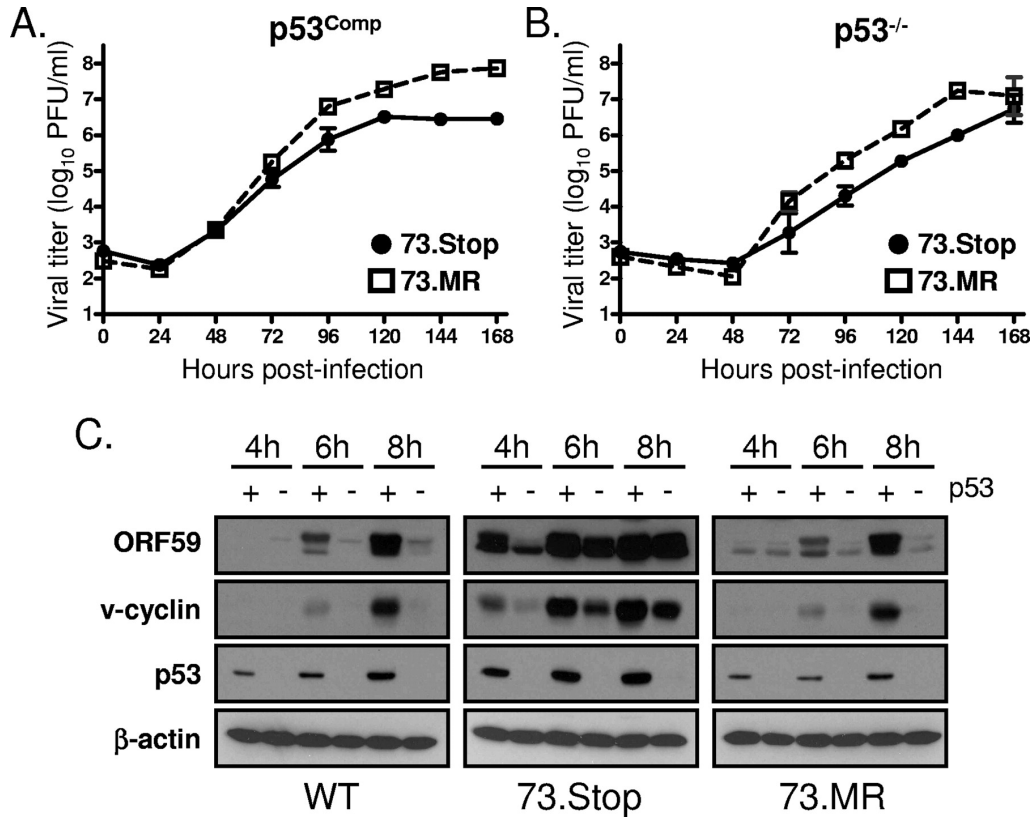

Figure 2.7. p53 regulates MHV68 replication. $p53^{comp}$ MEFs (panel A) or $p53^{-/-}$ MEFs (panel B) were infected with 73.Stop or 73.MR viruses at multiplicities of 0.001 PFU/cell. Cells were harvested at the indicated times post-infection, and viral titers were determined by plaque assay. Results are the means of triplicate samples. Error bars represent standard deviations. (C) Wild-type or $p53^{-/-}$ MEFs were infected with WT, 73.Stop, or 73.MR MHV68 at multiplicities of 2 PFU/cell. Equivalent numbers of cells were harvested directly by addition of Laemmli sample buffer at the indicated times post-infection. Total cell lysates were resolved by SDS-PAGE and analyzed by immunoblot with antibodies to the indicated proteins. Panel A was generated by C.R. Paden and J.C. Forrest; Panel B was generated by J.C. Forrest.

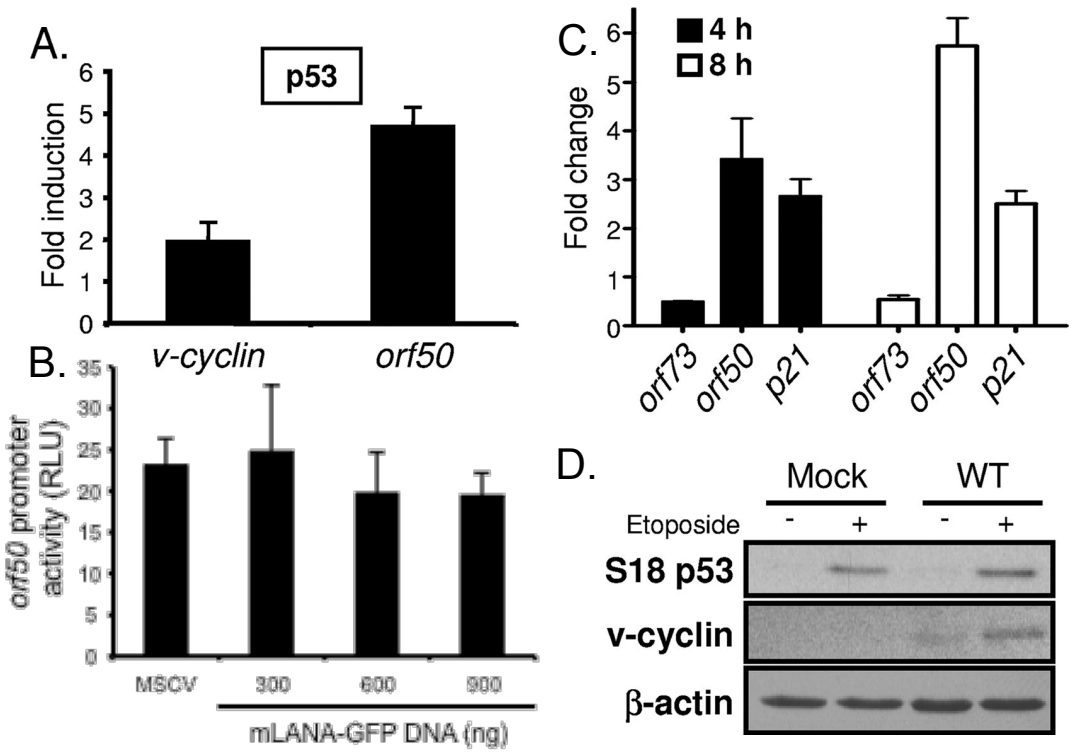

Figure 2.8. Overexpression of p53 and etoposide treatment induce viral gene expression. (A) NIH 3T3 fibroblasts were transfected with luciferase-reporter promoter constructs for *v-cyclin* or *orf50* in conjunction with either empty vector or p53 expression vector. Cells were harvested 24 h after transfection, and luciferase activity was determined. The data represent the fold difference between vector-transfected and p53-transfected cells. Results are the means of triplicate samples. Error bars represent standard deviations.

Figure 2.8. (cont) (B) 293T cells were transiently transfected with a firefly luciferase-reporter promoter construct for orf50 and increasing amounts of an mLANA-GFP expression vector. Cells were harvested 48 h after transfection, and luciferase activity was determined. Data were normalized by co-transfection of an SV40-renilla luciferase reporter. Results are the means of triplicate samples. Error bars represent standard deviations. (C) NIH 3T3 fibroblasts were infected with ORF50-deficient (50.Stop) MHV68 at a multiplicity of 10 PFU/cell. 90 min post-adsorption, cells were treated with DMSO or with etoposide (100 μM). Cells were harvested at 4 and 8 h post-treatment. Quantitative RT-PCR was performed for the indicated genes. Data represent the fold change in transcript levels normalized to *GAPDH* as determined using the $\Delta\Delta C_t$ method for etoposide treated samples relative to DMSO treated samples. Data are the average of duplicate samples. Error bars represent the range of data. (D) NIH 3T3 fibroblasts were mock infected or infected with WT MHV68 at an MOI of 2 PFU/cell. One hour post-adsorption, cells were treated with either DMSO or etoposide (100 μM). Cells were harvested 12 h post-infection, and equivalent amounts of protein were resolved by SDS-PAGE. Samples were examined by immunoblot analysis with antibodies to the indicated proteins. The blot shown is representative of four independent experiments harvested between 8-12 h. Panel A was generated by J.C. Forrest and C.R. Paden; Panel B by C.R. Paden; Panels C and D by J.C. Forrest.

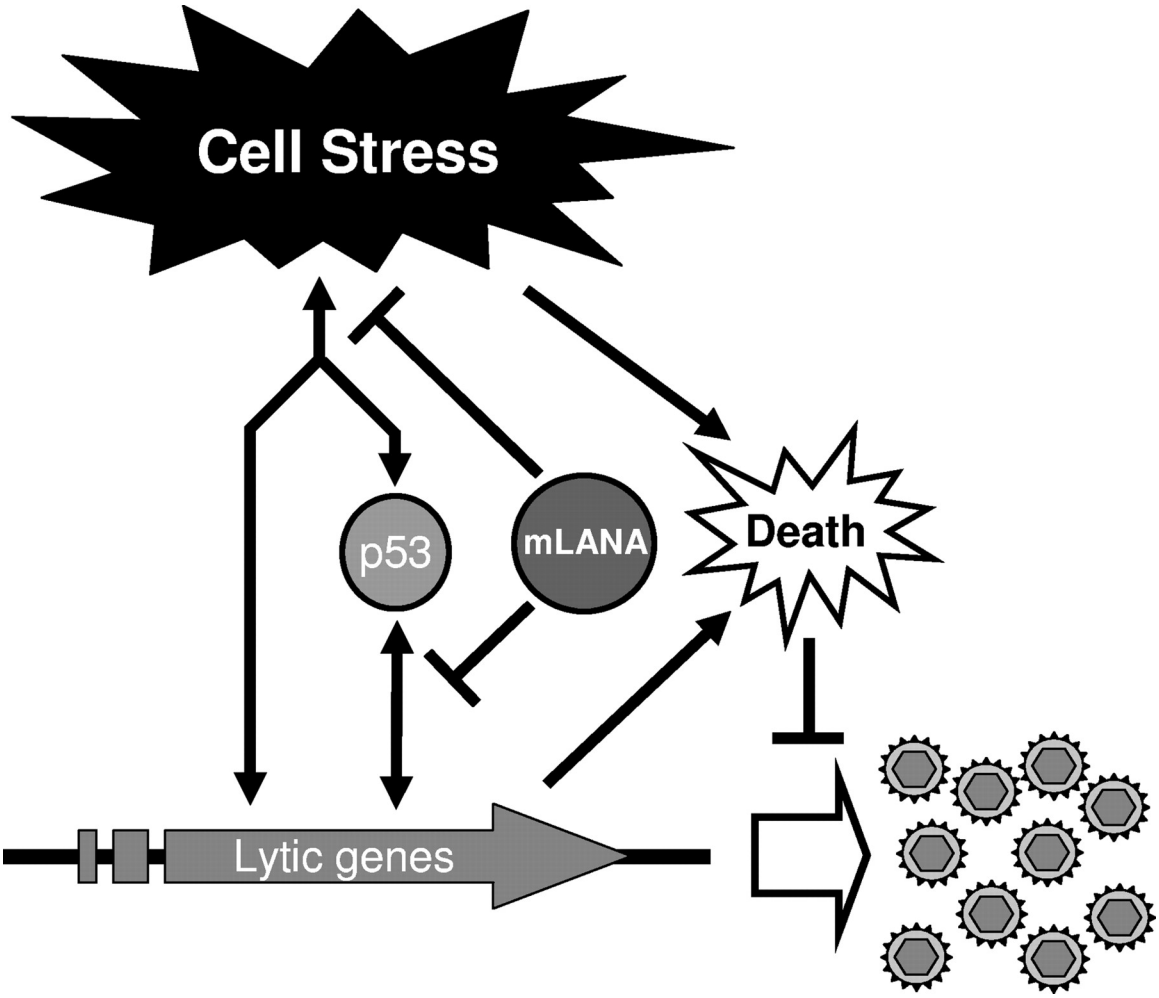

Figure 9. Model of predicted mLANA functions to regulate host-cell stress and promote efficient viral replication. MHV68 infection elicits the activation of cellular stress pathways that may promote p53 activation. Cell stress and/or p53 activity enhances expression of viral lytic genes, and likely cellular targets, in a manner that is deleterious to both the virus and host cell. We hypothesize that mLANA functions to limit propagation of the p53-inducing stressor or directly alters the activity of p53 to regulate viral gene expression and limit host-cell injury allowing for efficient viral replication. This diagram was generated by J.C. Forrest.

CHAPTER 3.

MHV68 LANA is essential for virus reactivation from splenocytes, but not long-term carriage of viral genome

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A) INTRODUCTION

Murine gammaherpesvirus 68 (MHV68) is a natural rodent pathogen in the rhadinovirus subfamily of gammaherpesviruses. Other members of this subfamily include the well-studied primate pathogen herpesvirus saimiri (HVS) and the important human virus Kaposi's sarcoma- associated herpesvirus (KSHV or HHV-8). These viruses are characterized by a biphasic life cycle – an acute phase of virus replication, amplification at the site of initial infection and spread to distal sites, followed by the establishment of quiescence infection (latency) that is sustained throughout the life of the host (Fickenscher and Fleckenstein 2001; Nash *et al.* 2001). At various times, perhaps spontaneously or in response to certain stimuli, herpesviruses are capable of exiting latency and re-entering the virus replication cycle - a process termed reactivation (Simas and Efstathiou 1998; Speck and Virgin 1999; Gargano *et al.* 2009). There are a small handful of genes expressed during latency, and they are not always present, depending on the time point, cell type, and host - implying that different genes are needed at different stages of latency (Virgin *et al.* 1999; Marques *et al.* 2003; Martinez-Guzman *et al.* 2003). Some of these latency genes, most notably the latency-associated nuclear antigen (LANA) encoded by ORF73 of rhadinoviruses (Rainbow *et al.* 1997), are robustly expressed in malignancies associated with the virus (Cesarman *et al.* 1995b; Soulier *et al.* 1995; Gao *et al.* 1996a; Rettig *et al.* 1997; Verma *et al.* 2007b).

LANA is transcribed as an immediate-early gene during lytic replication, and is detectable in replicating infected cells—both in culture with KSHV and *in vivo* in mice infected with MHV68 (Sun *et al.* 1999; Martinez-Guzman *et al.* 2003). In addition, LANA is detectable in every KSHV-associated malignancy (Parravicini *et al.* 2000).

Thus, it seems that LANA proteins have key functions in every aspect of the rhadinovirus life cycle. Indeed, MHV68 has borne out several findings not afforded in tumor studies with the other gammaherpesviruses, including that MHV68 LANA (mLANA) is required for establishment of latency after intranasal infection, mLANA-null virus can vaccinate against wildtype infection and that mLANA is necessary for efficient lytic replication in both the lungs of mice and in tissue culture (Moorman *et al.* 2003; Fowler and Efstathiou 2004; Forrest *et al.* 2007). MHV68 has also been used to map the mLANA transcript *in vivo*, which is quite distinct from the kLANA promoter identified from tumor cell lines (Dittmer *et al.* 1998; Jeong *et al.* 2001; Coleman *et al.* 2005b; Allen *et al.* 2006).

Perhaps the most well-known proposed function of LANA proteins is that of episomal maintenance. The data show that LANA is necessary to maintain an HVS minigenome as a circular, extrachromosomal plasmid when introduced into replicating cells under selection. Absence of LANA led to loss of detectable episomal minigenome (Collins *et al.* 2002). Similar data has been generated for KSHV as well (Ballestas *et al.* 1999; Ballestas and Kaye 2001). Further, it has been proposed that the mechanism for this maintenance is through physically tethering the viral episome to host histones so that episomes are distributed evenly to daughter cells (Cotter and Robertson 1999; Barbera *et al.* 2006). Given the variety of contexts in which LANA is transcribed during the virus life cycle, it is clear that the LANA homologs also have other important functions during the rhadinovirus life cycle. These other functions include manipulating the DNA-damage response and other tumor-suppressor pathways (Forrest *et al.* 2007; Kaul *et al.* 2007; Verma *et al.* 2007b), transcriptional regulation (Garber *et al.* 2001; Renne *et al.* 2001), and loading origins (Hu *et al.* 2002; Grundhoff and Ganem 2003; Verma *et al.* 2006).

Previously we (Moorman *et al.* 2003) and others (Fowler *et al.* 2003) reported the inability of mLANA null MHV68 mutants to establish latency following intranasal inoculation of wildtype mice. Here we report studies demonstrating that either altering the route of inoculation in immunocompetent C57Bl/6 mice or intranasal inoculation of type I interferon receptor knockout (IFNα/βR-/-) mice with a mLANA-null MVH68 mutant (73.Stop) results in animals becoming persistently infected and harboring viral genome positive splenocyte for at least 6 months post-infection. These studies also revealed an essential role for mLANA in virus reactivation from splenocytes, and a critical role peritoneal exudate cells (PECs).

B) MATERIALS AND METHODS

Cell lines and viruses. MHV68 BAC-derived strains 73.Stop and 73.MR are described previously (Moorman *et al.* 2003). Briefly, 73.Stop contains two stop codons and a frameshift early in the open reading frame to disrupt mLANA expression; 73.MR rescues 73.Stop to wildtype sequence. Viral stocks were grown to P2 in Vero-Cre cells, and titers were determined by plaque assay on NIH 3T12 monolayers.

NIH 3T12 and MEF (mouse embryonic fibroblast) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100 mg of streptomycin per ml, and 2 mM L-glutamine (complete DMEM). Cells were maintained in a 5% CO2 tissue culture incubator at 37℃. MEFs were obtained from C57BL/6 mouse embryos as described previously (Weck *et al.* 1996). Vero-Cre cells were a gift from David Leib. Cells were passaged in DMEM supplemented with 10% FCS and 300 μg of hygromycin B/ml.

Mice and infections. Female C57Bl/6 mice 6 to 8 weeks of age were purchased from the Jackson Laboratory and 129S2/SvPas.IFNαβR$^{-/-}$ were bred and maintained at Emory University. Mice were sterile housed and treated according to the guidelines at Emory University School of Medicine (Atlanta, GA). Mice were infected intraperitoneally with 1000PFU of virus diluted into 0.5mL of complete DMEM or intranasally with 100PFU of virus diluted into 20μL complete DMEM.

Limiting dilution analyses. Limiting dilution assays for frequency of latent were performed as previously described (Weck *et al.* 1996; Weck *et al.* 1999a). To determine the frequency of cells harboring latent viral genomes, single-copy-sensitive nested PCR was performed. Splenocytes or PECs were plated in three-fold serial dilutions in a

background of 10^4 NIH 3T12 cells in 96 well plates. Cells were lysed by protease K digestion for six hours at 56℃. Two rounds of nested PCR were performed per sample with twelve samples per dilution, and the products were resolved on 2% agarose gels. To measure the frequency of reactivating cells, splenocytes or PECs were resuspended in complete DMEM and plated in serial two-fold dilutions on mouse embryonic fibroblast (MEF) monolayers in 96-well tissue culture plates. Parallel samples of mechanically disrupted cells were plated to detect preformed infectious virus. Wells were scored for cytopathic effect 14 to 21 days post-explant.

Isolation of splenocytes and peritoneal cells, and purification of B cells. Mice were sacrificed by asphyxiation at the specified days. Peritoneal cells were recovered by injecting 10mL DMEM (without serum) into the peritoneal cavity, agitating the mouse, and recovering the DMEM using a 16g needle. Spleens were Dounce homogenized into a single-cell suspension and erythrocytes were lysed with ammonium chloride. B cells were enriched from total splenocytes using magnetic beads (Miltenyi B cell isolation antibody cocktail) and an AutoMACS, per manufacturer's instructions (Miltenyi).

RNA isolation and RT-PCR. RNA was harvested from splenocytes by lysing in TRIzol (Invitrogen) according to manufacturer's instructions. Three micrograms of RNA was treated with DNaseI (Invitrogen), and 1.5 μg of DNase-treated RNA was reverse transcribed using of random-hexamer primed RNA and Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. Minus RT controls were performed in parallel. The resulting cDNA was serially diluted to 1/5 and 1/25. For PCR amplification of transcripts, 1 μL of each cDNA dilution was subjected to a nested 25 μL PCR reaction using primer sets previously described (Virgin *et al.* 1999; Moorman

et al. 2003; Allen *et al.* 2006; DeZalia and Speck 2008). The PCR program was as follows: 25 cycles of 94˚ 1 min, 62˚ 1 min, 72˚ 1min; 2μL of round 1 product was put into a 50 μL round 2 reaction with the same conditions for 45 cycles.

BAC Transfections. 3T12 cells were plated and transfected the next day with 0.5μg BAC DNA with Superfect (Qiagen), per manufacturer's instructions. Transfection efficiency was monitored by expression of the HCMV IE promoter-driven GFP cassette engineered into the BAC. At 6 and 9 days post transfection, supernatants were harvested and titered by plaque assay.

Immunoblot analyses. Cells were lysed with alternative radioimmunoprecipitation assay buffer (150mM NaCl, 20mM Tris, 2mM EDTA, 1%NP-40,0.25% deoxycholate, 1mM NaF, and 1mM Na3VO4 supplemented with complete mini-EDTA-freeproteaseinhibitors[Roche]) and quantitated using the Bio-Rad DC protein assay prior to resuspension of 45ug protein in Laemmli sample buffer. Samples were heated to 100℃ for 10 minutes and were resolved by SDS-PAGE.Resolved proteins were transferred to nitrocellulose and were identified with the indicated antibodies. Immobilized antigen and antibody were detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham/GEHealthcare) and were exposed to film.

Plaque assay. Plaque assays were performed as previously described (Weck *et al.* 1996). NIH 3T12 cells were plated in six-well pates 1 day prior to infection at 2 x 10^5 cells per well. Organs were subjected to four rounds of mechanical disruption of 1 min each using 1.0-mm zirconia/silica beads (Biospec Products, Bartsville, OK) in a Mini-Beadbeater-8 (Biospec Products). Serial 10- fold dilutions of organ homogenate were

plated on NIH 3T12 monolayers in a 200-µl volume. Infections were performed for 1 h at 37℃ with rocking every 15 min. Immediately after infection, plates were overlaid with 1.5% methylcellulose in complete DMEM. After 6-7 days, cells were stained with 0.12% (final) Neutral Red. The next day, methyl-cellulose was aspirated and plaques were counted. The sensitivity of the assay is 50PFU/organ.

Digestion-Circularization-PCR. Genomic DNA was prepared from splenocytes using standard overnight proteinase K digestion followed by phenol:chloroform extraction. 6µg of gDNA was digested overnight with EcoRI or BamHI in a 100µL reaction. Enzymes were inactivated and DNA was purified using GeneCleanII (BIO 101). 10%, 1%, or 0.1% of the digested DNA was placed into a 100µL ligation reaction overnight at 16˚C, with or without 2µL T4 DNA ligase (New England Biolabs). Following ligation, a nested PCR reaction was performed using the following primer sets. Round 1: DCTR-Bam-Lout 5'-CTCTCAACTAAC ACTAACAGAGGATTT-3', DCTR-Bam-Rout 5'-ATGTCTACACCTACATGCCCGCATC-3', DC64-Bam-Lout 5'-CAACCACAGAATATAACA CCCATCTACTG-3', DC64-Bam-Rout 5'-TGATTTTTGCTGGAATTGCACCTG-3'; Round 2: DCTR-Bam-Lin 5'-GGCTTTGTGGTCGTT CACACCTC-3', DCTR-Bam-Rin 5'-TAGCGCCACCATGGTGGTAAA CAA-3', DC64-Bam-Lin 5'-AACCAGTCCCCAACTGAAAGAACG-3', DC64-Bam-Rin 5'-ATTGCTGTAAGCATGTAATT AATA-3'. Probes spanning the junctions were made from oligonucleotides that span the junctions created by the digestion/ligation protocol: DCTR-Bam-Probe 5'-TGCCTGGCTTTTATCGTGTTCGAACCACCGTTAACTGTG-

AAATTGTAGAC-3' and DC64-Bam-Probe 5'-TGGAAATCACAGTTGCAAGAAC-TTGAGGAGGCTGTCAAAACTACTACACA-3'. The probes were 5' labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs). Gels with DC-PCR products were transferred to nylon membranes and hybridized overnight in hybridization buffer containing 50% formamide, 5x SSC, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 15 mM sodium phosphate(pH 6.5), 10% dextran sulfate, 0.01% denatured salmon sperm DNA, and labeled oligonucleotide. Membbranes were washed in 2X SSC/0.2%SDS and visualized on a Typhoon phosphorimager (GE Healthcare).

Statistical analyses. All data were analyzed using GraphPad Prism (GraphPad Software). Titer data were statistically analyzed using the unpaired *t* test. The frequencies of reactivation and genome-positive cells were statistically analyzed using the paired *t* test. To accurately obtain the frequency for each limiting dilution, data were subjected to nonlinear regression (using a sigmoidal dose curve with nonvariable slope to fit the data). Frequencies of reactivation and genome-positive cells were obtained by calculating the cell density at which 63.2% of the wells scored positive for reactivating virus based on a Poisson distribution.

C) RESULTS

Innate immunity prevents dissemination of mLANA null virus from the lung. Previous studies with mLANA-deficient viruses demonstrated that mLANA is required for establishment of latency following intranasal inoculation (Fowler *et al.* 2003; Moorman *et al.* 2003). Notably, a 1- 2 log defect in acute virus replication in the lung was also observed following intranasal inoculation (Moorman *et al.* 2003). More recently a replication defect was also observed *in vitro* at low multiplicities of infection of mouse embryo fibroblasts with an mLANA null virus (Forrest *et al.* 2007). Because we do not have a clear understanding of the relationship between the levels of acute virus replication in the lungs and establishment of latency at distal sites, we initially set out to assess whether innate immunity, specifically the type I interferon response, might be involved in limiting the spread of mLANA- null MHV68 from the lung to the spleen. Type I interferon receptor knockout (IFNαβR-/-) and Stat1 knockout mice were previously shown to be hyper-susceptible to MHV68 infection (Weck *et al.* 1997; Barton *et al.* 2005). Thus, we inoculated IFNαβR-/- mice with 100 PFU of a mLANA-null virus (73.Stop) or a genetically repaired marker rescue virus (73.MR) intranasally and harvested lungs at day 9 post-infection to assess acute virus replication, and spleens at day 28 post-infection to assess the establishment of latency (Fig. 3.1). Notably, the lack of type I interferon-mediated control of replication did not ameliorate the difference in acute replication of 73.Stop and 73.MR, although significantly more virus production was observed in the lungs of interferon-nonresponsive animals (Fig. 3.1A). In addition, about half of the IFNαβR-/- mice succumbed to 73.Stop or 73.MR infection between days 10 and 20 post-infection, with the greatest drop at day 14 (Fig. 3.1B). Importantly, no

differences in the kinetics of virus-induced death or the percentage of mice that survived was observed between mice infected with 73.Stop vs. 73.MR (Fig. 3.1B).

To assess the establishment of latency in the spleen, we waited for lytic replication to clear, which takes 7-10 days longer in IFNαβR-/- mice (Barton *et al.* 2005). Of the mice that survived, spleens were harvested at day 28 post-infection and subjected to limiting-dilution PCR (Fig. 3.1C) and ex vivo reactivation analyses (Fig.1D). These analyses revealed that 73.Stop can establish infection splenocytes at a 7.7-fold reduced frequency compared to 73.MR. However, no virus reactivation upon explant of splenocytes from 73.Stop infected IFNαβR-/- mice could be detected (Fig. 3.1D). This raised the question of whether we were detecting some form of detective/non-productive 73.Stop virus infection in the spleens of IFNαβR-/- mice. Thus, to further address 73.Stop virus infection, we focused on infection of immunocompetent C56Bl/6 mice.

Intraperitoneal inoculation of immunocompetent mice overcomes the requirement for mLANA to establish a chronic infection, but reveals a role for mLANA in virus reactivation. To extend the analyses in IFNαβR-/- mice, we investigated alternative routes of inoculation with a mLANA null virus (73.stop) in immunocompetent mice in an effort to overcome any restriction virus replication in the lungs may have had on the establishment of latency. We inoculated C57Bl/6 mice intraperitoneally with 1,000 PFU of either 73.Stop or 73.MR. Eighteen days post-infection, the mice were sacrificed and peritoneal exudate cells (PECs) and spleens were harvested. LD-PCR revealed that the 73.Stop mutant established infection in both PECs and splenocytes, exhibiting 1.6-fold and 4.6-fold reduced frequencies, respectively, of

viral genome positive cells compared to the marker rescue virus (Fig. 3.2A & 3.2B). In parallel, we assessed the capacity of splenocytes and PECs harvested from 73.Stop infected mice to reactivate virus by plating live cells onto MEF monolayers in a limiting dilution analysis. The 73.Stop-infected PECs demonstrated a significant, almost 2-log, decrease in the frequency of cells reactivating virus compared to PECs recovered from 73.MR infected mice (Fig. 3.2C). Based on the frequency of infected cells and the frequency of reactivating cells, this means that nearly 100% of 73.MR-infected PECs reactivate virus, while only a small fraction of 73.Stop- infected PECs (1 in 51) spontaneously reactivate upon explant. Even more profound was the reactivation defect observed with splenocytes (Fig. 3.2D). While the frequency of splenocytes from 73.MR infected mice that reactivated virus upon explant (1 in 5,902) was similar to that previously reported for wt MHV68, little or no virus reactivation was observed with splenocytes recovered from 73.Stop infected mice (Fig. 3.2D). Attempts to stimulate 73.Stop reactivation using LPS did not increase the frequency of reactivating splenocytes (Paden and Speck, unpublished observation), further highlighting this defect.

B cells are the major cell type harboring mLANA-null virus in the spleen, where it establishes an infection that persists for more than 6 months. Because there are many aspects of early establishment of latency that we do not yet understand, we asked whether there were any gross differences in the cell types that have the capacity to be latently infected in the absence of mLANA. To do this, we enriched for B cells from the spleens of mice infected with 73.Stop and 73.MR at day 18. We determined the frequency of infected B cells, using purified B cells (average 90% purity), by LD-PCR

and in parallel with analyses of the bulk unsorted populations. As expected, these analyses revealed that 73.Stop does indeed establish a chronic infection in B cells (Fig. 3.3) at a frequency essentially equivalent (4.5-fold decrease vs. 5.4-fold decrease) to that determined for the respective unsorted splenocytes. Thus, these results demonstrate that the majority of virus infection for both 73.Stop and 73.MR viruses is in B cells, arguing against the persistence of the mLANA-null virus in a cell population that is physiologically irrelevant for maintenance of chronic wt MHV68 infection.

One of the many functions proposed for KSHV LANA is that of the episomal maintenance protein, responsible for faithful partitioning of KSHV latent episomes to daughter cells during cell division. Fitting with this idea, we hypothesized that in the absence of mLANA, long-term MHV68 latency would not be maintained. To test this, we again infected C57Bl/6 mice intraperitoneally with 73.MR or 73.Stop and sacrificed groups of mice at 6 weeks and 6 months post-infection. Unexpectedly, we observed that the frequency of 73.Stop and 73.MR rescue viruses were equivalent at both time points (Fig. 3.4), demonstrating that the absence of mLANA did not lead to a loss of virus-infected splenoctyes. Instead, the frequency of 73.MR infected cells decreased to an often observed setpoint (Tibbetts *et al.* 2003), around 1/5,000 at 6 weeks (Fig. 3.4A) and 1/19,000 at 6 months (Fig. 3.4B), where frequencies of 73.Stop-infected splenocytes also settled. This was very surprising given our previous observation that the majority of MHV68 infected B cells in the spleen are actively proliferating at 6 weeks post-infection (Moser *et al.* 2005), and suggests a mLANA-independent mechanism must be involved in maintaining the viral genome.

Detection of viral transcripts in splenocytes infected with mLANA null virus. Characteristics of latent gammaherpesvirus infection include limited expression of viral antigens, absence of infectious virus, the capacity to reactivate, and distinct expression of a very limited repertoire of viral transcripts (Simas *et al.* 1999; Virgin *et al.* 1999; Marques *et al.* 2003). We assessed whether the 73.Stop virus infected splenocytes express other known latency-associated viral genes *in vivo*. Three genes transcribed during early latency, at least in some splenocyte populations, are the unique M2 and M9 genes and ORF73 (encoding mLANA) (Rochford *et al.* 2001; Allen *et al.* 2006; DeZalia and Speck 2008; Forrest and Speck 2008). To demonstrate an active form of latency in the absence of mLANA expression, we isolated RNA from spleens of individual mice between 25 and 28 days post-infection with 73.Stop or 73.MR and reverse-transcribed it to make cDNA. To understand how levels of transcripts vary between 73.Stop and 73.MR, we assayed five-fold serial dilutions of cDNA. PCR analysis of cDNA detected both spliced M2 and *orf73* transcripts, as well as M9, M1 and viral DNA polymerase transcripts in 73.MR and 73.Stop-infected splenocytes (Fig. 3.5A). GAPDH was used as a control for integrity for RNA, while RNA prepared from a naïve mouse was used as a control for specificity of RT-PCR amplification. These results demonstrate that the mLANA-null virus, though crippled for reactivation, is transcriptionally active, expressing viral genes that are implicated in both latency as well as transcripts that are upregulated during virus reactivation/repllication. However, although *orf73* transcripts are present, mLANA protein is not made in the 73.Stop-infected cells.

Finally, we assessed whether the initial virus-induced B cell response was similar between 73.Stop and 73.MR infection, since this might impact the frequency of latently

infected splenocytes. MHV68 elicits a potent germinal center response, which is dependent on M2- expression (Siegel and Speck, unpublished observation). We obtained splenocytes from infected mice at day 18 post-infection and determined the frequency of CD19+ B cells that stained positive for the germinal center markers GL7 and CD95. As expected, we observed that 73.Stop and 73.MR generated similar germinal center responses: ca. 4.5% of splenic B cells harvested from either 73.Stop or 73.MR infected mice exhibited a germinal center phenotype compared with ca. 0.25% of B cells harvested from naïve mice (Fig. 3.6). This data argues against the defect in establishment of 73.Stop latency in the spleen being linked to a diminished germinal center response.

Failure of mLANA null virus to reactivate from splenocytes correlates with the absence of viral episomes. We considered two possibilities that might account for the failure of mLANA-null virus infected-splenocytes to reactivate upon explant. First, we addressed whether the absence of mLANA might impact initiation of the viral replication cycle in the absence of virion entry and release of tegument proteins, which would be anticipated to mimic aspects of virus reactivation. Thus, to assess the capacity of mLANA-null virus to initiate *de novo* virion production from naked viral DNA, we transfected either 73.Stop-BAC or 73.MR-BAC DNA into permissive Vero cells and harvested supernatants at 3, 7, and 9 days post-transfection. To control for transfection efficiency, several replicate experiments were performed and GFP expression in the transfected Vero cells, arising from CMV IE promoter- driven GFP expression cassette present in the BAC, was monitored and shown to be equivalent (data not shown). Tissue culture supernatants harvested from each transfection were titered by plaque assay on

NIH 3T12 fibroblasts. Notably, the 73.Stop-BAC-transfected cells displayed a severe, three-log replication defect at day three post-transfection (Fig. 3.7A). Importantly, a replication defect of this magnitude is not observed in fibroblasts infected with mLANA null virus (Fowler *et al.* 2003; Moorman *et al.* 2003). The observed replication defect was slightly ameliorated by day nine, most likely due to subsequent rounds of infection with progeny virus produced from the transfected cells. To show that this effect happens very early in the transition from DNA to virus production, and is not due to a failure to efficiently plaque 73.Stop virus, we transfected Vero cells with 73.Stop-BAC or 73.MR-BAC in duplicate and harvested lysates at 24, 72, and 96 hours post-transfection (Fig. 3.7B). We observed that the LANA-null BAC displayed a significant lag in the expression of lytic antigens, consistent with the observed decrease in viral titers. This is in contrast to the over-expression of lytic antigens seen under some experimental conditions upon infection of permissive fibroblasts with the 73.Stop virus (Forrest *et al.* 2007).

Second, we considered whether in the absence of mLANA expression the viral genome might integrate into the host chromosome, as it has been shown that HVS and KSHV LANA are important for facilitating episomal maintenance of the viral genome in rapidly dividing cells (Collins *et al.* 2002; Hu *et al.* 2002; Grundhoff and Ganem 2003). Furthermore, it has been suggested that KSHV terminal repeat-containing plasmids integrate into the cellular genome under antibiotic selection when LANA is not present (Grundhoff and Ganem 2003). Notably, many characterized gammaherpesvirus integration events have shown that the viral genomes preferentially integrate into chromosomes through their terminal repeats (Henderson *et al.* 1983; Gulley *et al.* 1992).

In contrast, episomal virus genomes have fused terminal repeats, and the left and right ends of the viral unique sequences are thus physically connected through fusion of the terminal repeats. To assess the presence of viral episomes, we employed a digestion-and-circularization-mediated PCR (DC-PCR) strategy (Chu *et al.* 1992) to detect the presence of fused terminal repeats. Splenocytes from mice infected intraperitoneally with 73.Stop or 73.MR virus were harvested at day 18 post- infection and total genomic DNA was prepared. The recovered genomic DNA was digested with either EcoRI or BamHI, neither of which cuts within the terminal repeats, followed by dilution of the digested DNA and ligation under conditions which favors intramolecular ligation (i.e., circle formation) (see Fig. 3.8A). Nested sets of PCR primers were designed a short distance upstream of the respective enzyme digestion sites, oriented towards the cut site, such that a PCR product could only be generated following ligation of each end to form a circle (Fig. 3.8A). One set of primers was designed to detect fused terminal repeats, and another was designed to detect the digested and circularized EcoRI-H or BamHI-A2 fragments, containing ORF64, in the middle of the unique region, as a control for detecting viral DNA following digestion and circularization. We observed in four independent sets of infections with both 73.Stop and 73.MR that we could readily detect the ORF64-containing circles (Fig. 3.8B & 3.8C). However, while we also consistently observed the fused terminal repeat product in splenocytes recovered from 73.MR infected mice, we never observed the presence of the fused terminal repeat product in 73.Stop infected splenocytes (Fig. 3.8B and 3.8C). This implies that mLANA is needed *in vivo* at early times of latency to facilitate maintenance of MHV68 as a circular genome in latently infected splenocytes. This does not, however, necessarily demonstrate integration of the

viral genome. Another possibility is that the virus may be present in a linear, extrachromosomal form.

D) DISCUSSION

In this report we identified a role for the MHV68 LANA homolog in virus reactivation from splenocytes and PECs, as well as provide initial evidence that mLANA plays a critical role in establishing and/or maintaining the viral genome as an episome *in vivo*. A striking finding here was the ability of the mLANA null virus to establish latency in the spleen following intraperitoneal inoculation of C57Bl/6 mice, in contrast to earlier studies which demonstrated a complete failure to establish latency following intranasal inoculation (Fowler *et al.* 2003; Moorman *et al.* 2003). As discussed above, we had previously observed a significant acute replication defect with the mLANA null virus in the lungs following intranasal inoculation (Moorman *et al.* 2003). However, substantially increasing the inoculating dose of virus, which greatly increased the peak titers of virus in the lungs, did not overcome the defect in establishment of latency (Moorman *et al.* 2003). This suggested a more complex relationship between route of administration and establishment of latency in the spleen in immunocompetent mice. This complexity is underscored by the previous observation that establishment of latency in the spleen following intranasal inoculation is severely impaired in B cell-deficient mice (MuMT), but splenic latency is robustly established in these mice following intraperitoneal inoculation (Usherwood *et al.* 1996b; Weck *et al.* 1996). Similarly, M2-null mutants also exhibit a more severe establishment of latency phenotype in the spleen following intranasal inoculation compared to intraperitoneal inoculation (Jacoby *et al.* 2002; Herskowitz *et al.* 2005), and M2-null mutants also exhibit a profound defect in reactivation from B cells (Herskowitz *et al.* 2005; Liang *et al.* 2009). Taken together, these data suggest that latently infected B cells that traffic to the spleen and reactivate play a

pivotal role in the initial establishment of latency following intranasal inoculation, but not following intraperitoneal inoculation (Siegel *et al.* 2010). Thus, the inability of mLANA null virus to reactivate from splenocytes may be directly linked to the failure of this mutant virus to establish latency following intranasal inoculation of immunocompetent mice.

LANA was originally identified in a KSHV latently infected tumor cell line using serum from patients with Kaposi's Sarcoma (Gao *et al.* 1996a). Subsequent studies demonstrated that LANA-expression could be detected in every KS-associated tumor and proliferative disease (Cesarman *et al.* 1995b; Soulier *et al.* 1995; Gao *et al.* 1996a; Rettig *et al.* 1997; Parravicini *et al.* 2000; Verma *et al.* 2007b). These studies and others established a correlation between LANA and KSHV disease, a notion further bolstered by studies that identified numerous functions of LANA, many of which appeared to be consistent with its putative role as a viral oncogene. Functions attributed to LANA proteins include regulating transcription of cell-cycle genes (Garber *et al.* 2001), blunting cellular responses to virus infection and DNA-damage (Forrest *et al.* 2007), interaction with p53 and other tumor suppressors (Kaul *et al.* 2007; Verma *et al.* 2007b), and preventing its own presentation by MHC on infected cells (Kwun *et al.* 2007). LANA expression is associated with these tumor-like phenotypes, and thus much of what is known about LANA in the context of KSHV-infection has been worked out in cancer cells. However, there is no basis for assuming that constitutive expression of LANA is normal during chronic rhadinovirus infections. Like other herpesvirus latency-associated genes including EBNA-1, another well-studied gammaherpesvirus protein suggested to

promote episome maintenance (Yates *et al.* 1985; Schaefer *et al.* 1991), its expression is likely tightly controlled.

As discussed above, episomal maintenance is perhaps the most well-known function attributed to LANA homologs. Similar to what has been shown for EBNA-1 of EBV (Yates *et al.* 1984; Yates *et al.* 1985), it has been suggested by studies with both HVS LANA and KSHV LANA that LANA: (i) maintains viral genomes or minigenomes as extrachromosomal episomes; and (ii) physically associates, or tethers, the viral genome to host DNA to ensure faithful partitioning of the viral genome into daughter cells (Cotter and Robertson 1999; Ballestas and Kaye 2001; Collins *et al.* 2002; Verma and Robertson 2003). We speculated that in the absence of LANA the virus would integrate into host cells and/or eventually be lost with host cell division. Consistent with episome maintenance assays performed for HVS and KSHV LANA, we were unable to detect episomes with fused terminal repeats *in vivo* at day 18 in the absence of mLANA. At this time point, a large percentage of MHV68-positive cells exhibit a germinal center phenotype and are actively proliferating (Moser *et al.* 2005; Collins *et al.* 2009). Thus, this may be the point at which *in vitro* maintenance assay data may most strongly correlate with virus infection of B cells *in vivo*, and in the absence of mLANA, the integrated viruses are the ones that survive the rapid cell division in the germinal center. Notably, it is the germinal center subset of B cells that have the most robust expression of *orf73* transcripts (Marques *et al.* 2003). Furthermore, germinal center B cells that survive become either memory cells or plasma cells; memory B cells are the long lived latency reservoir (Flano *et al.* 2002; Willer and Speck 2003) and plasma cells are a major cell type reactivating virus (Liang *et al.* 2009). It is possible that mLANA thus plays a central role

in retaining the virus in a state (i.e. viral episome) that can rapidly switch from latency to reactivation.

Our data demonstrate that gammaherpesvirus infection can persist *in vivo* in the absence of an episomal form, either maintained as a linear piece of DNA or integrated into the host chromosome. There is precedent both for MHV68 being carried as linear DNA upon infection of primary lymphocytes (Dutia *et al.* 1999) and for gammaherpesvirus integration (Hurley *et al.* 1991; Delecluse *et al.* 1992; Delecluse *et al.* 1993a). Both possibilities may lead to a block in virus reactivation from splenocytes (which largely reflect infection of B cells), likely due to the absence of a mechanism for circularization or for excision of the viral DNA from the host chromosome. Notably, 73.Stop virus can reactivate, albeit at a greatly reduced efficiency, from infected peritoneal cells and perhaps from an undetectable population of splenocytes. This minor reactivation may reflect reactivation from a population of infected macrophages that do not require mLANA to maintain the capacity of the virus to reactivate, due either to the lack of active proliferation of those cells or another cell-type specific factor. Similarly, when permissive cells are transfected with MHV68 BAC DNA, 73.STOP-BAC lags behind the 73.MR-BAC in both lytic antigen production and virion production, indicating a secondary role for mLANA early in the transition from latency to reactivation.

These data may confound the working definition of latency commonly used in the herpesvirus field. It is generally accepted that a latent virus is one that "may be induced to multiply and that does not exist in an infectious form" (Roizman and Sears 1987). Further, episomal herpesvirus genomes are associated with latently infected cells (Lindahl *et al.* 1976; Roizman and Sears 1987). However, a definitive molecular definition of

latency is still lacking in the gammaherpesvirus field, and though we know there is little viral gene expression during latency, there are distinct programs of a limited number of tightly regulated genes observable in EBV-transformed cells and in disease (Rogers *et al.* 1992; Speck 2002). It stands to reason that transcription of viral genes must be an important aspect of the establishment and maintenance of latency, giving some advantage to virus-infected cells to survive and become a long-term reservoir for the virus. With the LANA-null virus, we observe the presence of the viral genome in the absence of infectious virus, but the viral genome does not appear to be episomal. We do, however, observe similar patterns of viral gene expression in mutant and wildtype virus infected cells, as well as long-term carriage of the viral genome *in vivo*. Thus, the LANA-null virus is indeed present within the host indefinitely, in the preferred cell-type and at levels equivalent to wild type virus. In addition, because the LANA-null infected cells are capable of expressing viral genes, the mutant virus presumably retains the capacity to alter the cell, even though it is incapable of efficiently re-entering the lytic cycle to produce progeny virions. Thus, the ability of the LANA null virus to ultimately access the same cellular reservoirs that are latently infected with wildtype MHV68 argue in support of a broader definition of latency.

This idea of a more complex definition of latency has been building for several years in both KSHV and EBV tissue culture models. KSHV-infected primary effusion lymphoma cells (PEL) cells have been treated with lentiviruses that knockdown LANA, and while the genome copy number is reduced, latency persists (Godfrey *et al.* 2005). Furthermore, in both EBV (Norio and Schildkraut 2004) and KSHV (Verma *et al.* 2007a), it has been shown that viral replication origins exist and function outside of the

EBNA-1-dependent OriP and proposed LANA-dependent terminal repeat (TR) origin, respectively. In the same regard, it is also now appreciated that LANA itself is not sufficient to maintain a TR-containing plasmid, suggesting that specific cellular mechanisms are required for this function (Grundhoff and Ganem 2004). Finally, a study using an EBV mutant lacking EBNA1 showed that this virus not only can latently infected B cells, but can also drive B cell immortalization - albeit with a significantly reduced efficiency (likely due to the need for the virus to integrate into the host genome) (Humme *et al.* 2003). The data presented in the present study argue that what we observe *in vivo* with the 73.Stop virus is a form of latency in the absence of both viral episomes and the latency-maintenance protein mLANA, because: (i) we observe a similar pattern of latency gene expression; (ii) the frequency of viral genome-positive cells persists and is equivalent long-term with wildtype virus; and (iii) it is B cells that are predominantly infected with 73.Stop, similar to wildtype.

With respect to persistence of herpesviruses in the absence of episome formation, it has been shown that human herpesvirus-6 (HHV-6), a betaherpesvirus, integrates into host chromosomes as a normal part of its life cycle (Daibata *et al.* 1999). Furthermore, HHV-6 is apparently able to reactivate from this integrated state as virus is frequently detected in the saliva of healthy people and in the blood of bone marrow transplant patients (Levy *et al.* 1990; Carrigan and Knox 1994). Integrated HHV-6 has also been observed in vertical transmission from parent to child, arguing for integration as an efficient mechanism of survival (Arbuckle *et al.* 2010). Additionally, the alphaherpesvirus Marek's Disease Virus (MDV), which is highly pathogenic in chickens, is exclusively found integrated into host chromosomes, but also retains the capacity to be

excised and replicate (Delecluse *et al.* 1993a; Delecluse and Hammerschmidt 1993). Notably, with respect to gammaherpesvirus biology, integration of the viral genome at the terminal repeats would prevent expression of viral genes encoded across the fused terminal repeats (e.g., EBV LMP2a and KSHV K15) (Bernasconi *et al.* 2006). The consequences of the loss of such gene products on either establishment or maintenance of viral latency *in vivo* are currently unknown. In the case of LMP2a, it has been hypothesized that it plays a critical role in survival of EBV infected germinal center B cells (Caldwell *et al.* 1998) and thus likely plays a critical role in the generation of those virus infected memory B cells that arise from virus driven differentiation of infected naïve B cells (Caldwell *et al.* 1998; Babcock and Thorley-Lawson 2000). At present, no such viral gene product encoded across the fused terminal repeats of MHV68 has been identified.

In summary, the results presented here reveal a crucial role for mLANA in reactivation and provide *in vivo* support for the notion that an episome is a prerequisite for virus reactivation, but not long-term carriage of MHV68. The data also show that mLANA is important early in infection, both in mounting an aggressive lytic infection at the site of inoculation and in establishing episomal latency. Our data further demonstrate the resilience of herpesvirus latency - the virus can continue to persist in the host even in the absence of the proposed latency maintenance protein.

E) FIGURES AND LEGENDS

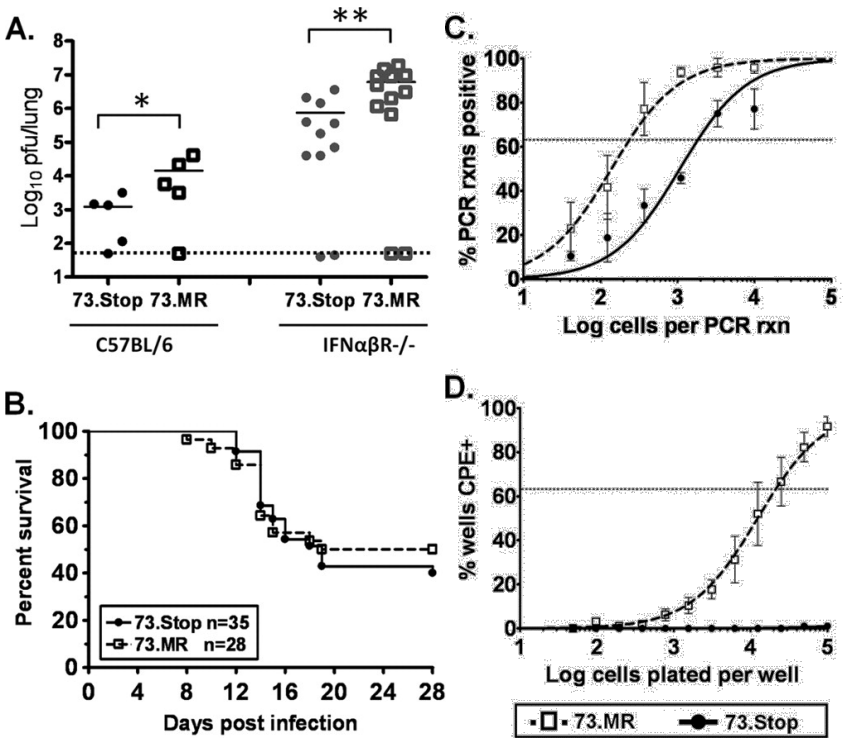


Figure 3.1. Intranasal infection of IFNαβR-/- mice with 73.Stop allows for greater lytic replication and mLANA-independent seeding of latency in the spleen, but not reactivation of virus. (A) Either C57Bl/6 (black symbols) mice or IFNαβR-/- mice (grey symbols) were infected intranasally with 100 PFU of 73.Stop or 73.MR virus. Lungs were harvested nine days later and infectious virus was titerd by plaque assay. **(B)** Kaplan-Meier curve of IFNαβR-/- mice infected intranasally with 73.Stop or 73.MR viruses. Chi-squared analysis revealed no significant difference in survival between these experimental groups.

Figure 3.1. (cont.) (C and D) Surviving IFNαβR-/- mice infected with either 73.Stop or 73.MR virus were sacrificed at day 28 post-infection and spleen harvested for analysis. Splenocytes were subjected to limiting dilution analyses to determine the frequency of cells harboring viral genome **(C)** or spontaneously reactivating virus upon explant onto monolayers of mouse embryo fibroblasts **(D)**.

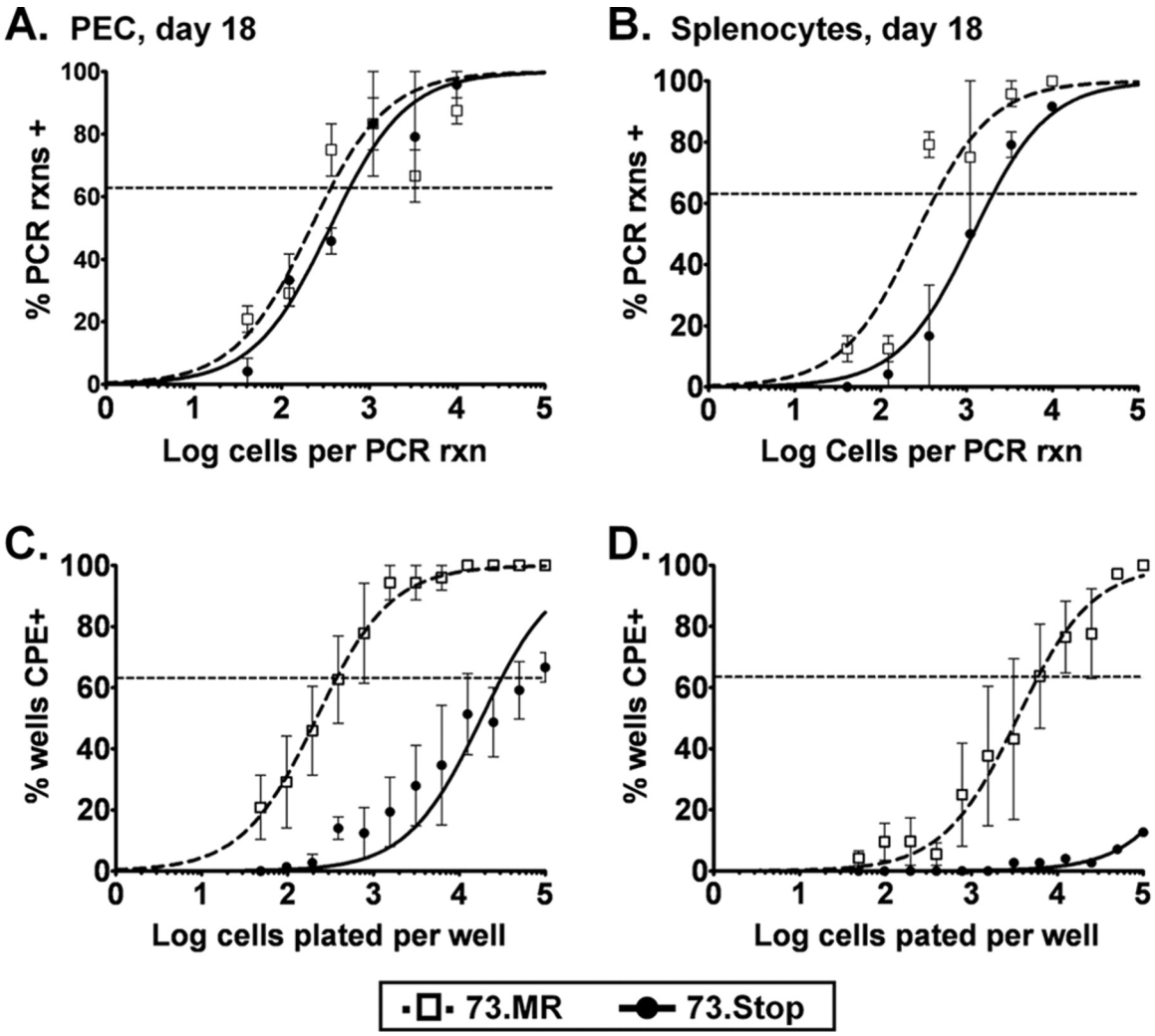


Figure 3.2. mLANA is required for efficient reactivation, but not establishment of latency, in PECs and splenocytes following intraperitoneal inoculation of immunocompetent C57Bl/6 mice. Groups of 3-5 female C57Bl/6 mice were inoculated intraperitoneally with 1000 PFU of 73.Stop or 73.MR virus. At day 18 post-infection, PECs **(A and C)** and splenocytes **(B and D)** were harvested and assayed for latency and reactivation by limiting dilution analyses. Results are the mean of four independent experimental groups, and error bars represent standard deviation between separate groups.

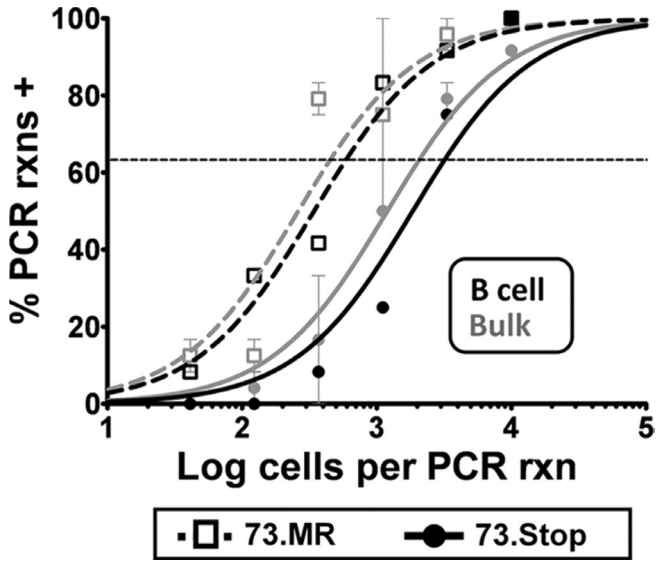


Figure 3.3. B cells account for the majority of mLANA null virus infected splenocytes. Splenocytes were harvested from mice at day 18 post-infection and sorted into B cell and non-B cell populations using a commercially available magnetic-bead based B cell purification kit (Miltenyi). The bulk and B cell-positive splenocyte populations were then analyzed by limiting dilution PCR analysis to determine the frequency of viral genome-positive cells. Purified B cells are shown in black symbols and lines, and the B cell depleted splenocyte population is shown in grey symbols and lines.

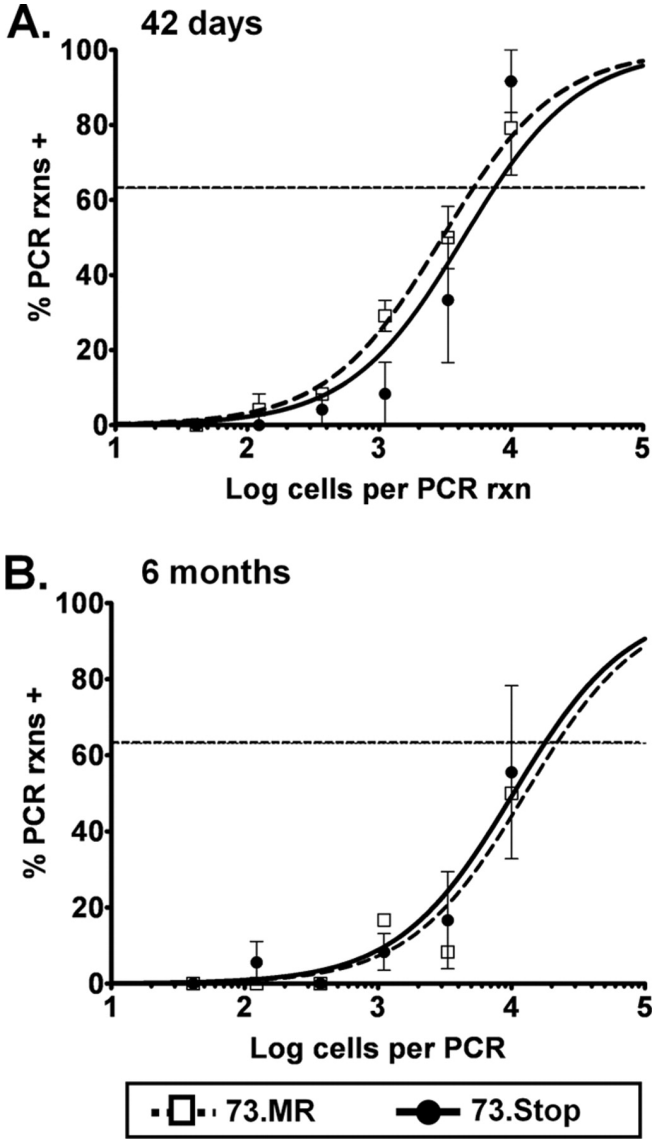
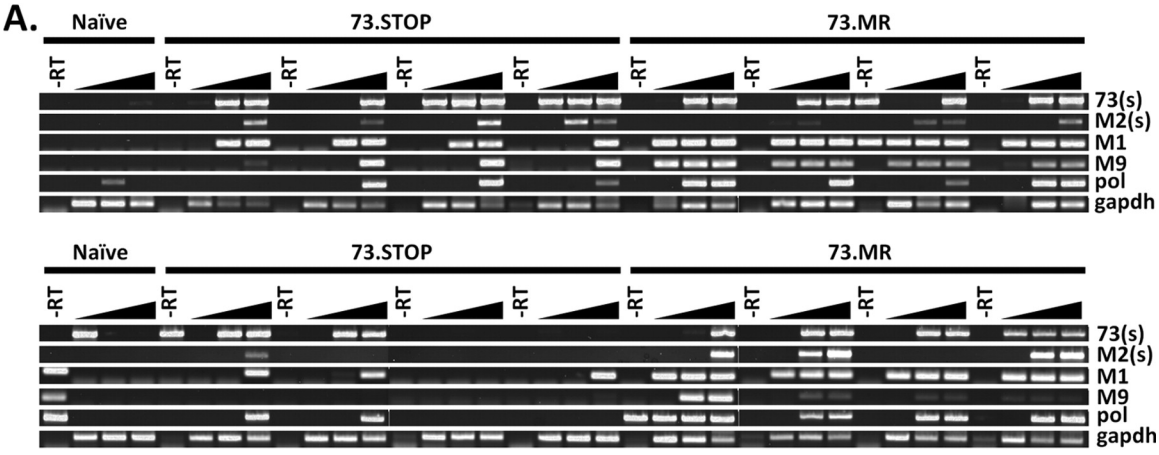


Figure 3.4. Viral genomes are maintained long-term in mice in the absence of mLANA. C57Bl/6 mice were inoculated intraperitoneally with 1000PFU of the indicated virus. At 42 days **(A)** and 6 months **(B)** post-infection, mice were sacrificed, and splenocytes were assayed by limiting dilution PCR for the frequency of cells harboring viral genome.



B.

		73(s)	M2(s)	M1	M9	pol	gapdh
73.MR	-RT	1/8	0/8	1/8	0/8	1/8	1/8
	+RT	15/24	10/24	24/24	21/24	15/24	23/24
73.STOP	-RT	1/8	0/8	0/8	0/8	0/8	1/8
	+RT	13/24	6/24	10/24	4/24	5/24	24/24

Figure 3.5. Latency-associated MHV68 transcripts can be detected in mLANA-null virus infected splenocytes. (A) Semi-quantitative RT-PCR analysis of MHV68 latency-associated gene expression in 73.Stop and 73.MR infected splenocytes. RNA from individual spleens was reverse transcribed, and dilutions (undiluted, 1:5 or 1:25 dilutions) of the cDNA from each spleen was analyzed by PCR for the indicated genes. Shown are amplification of products arising from spliced M2 or *orf73* transcripts (these RT-PCR products cross the known splice junctions in these viral transcripts), M9, viral DNA polymerase (pol), M1 or the cellular GADPH transcript using RNA prepared at from infected mice harvested at days 25-28 post-infection. **(B)** Compiled data from the RT-PCR analyses shown in panel A, indicating the number of PCR positive reactions and the total number of reactions analyzed.

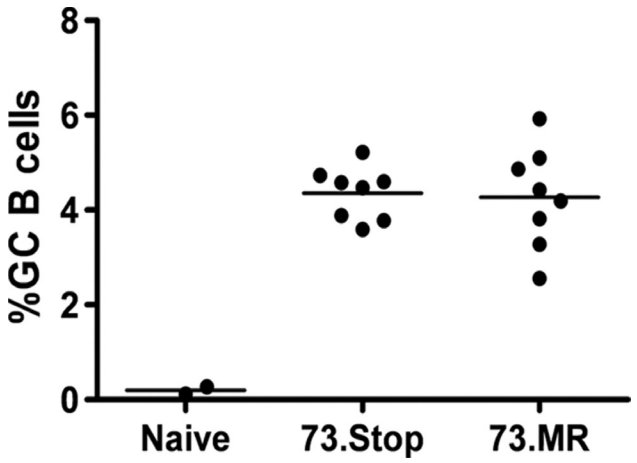


Figure 3.6. mLANA-deficient virus is not impaired in the induction of a strong germinal center response. Compiled data from flow cytometry analyses of the germinal center response (expressed as a percentage of total B cells that exhibit a germinal center phenotype) in the spleen 18 days post-infection. Splenocytes from individual mice were stained with anti-CD19, GL7, and anti-CD95.

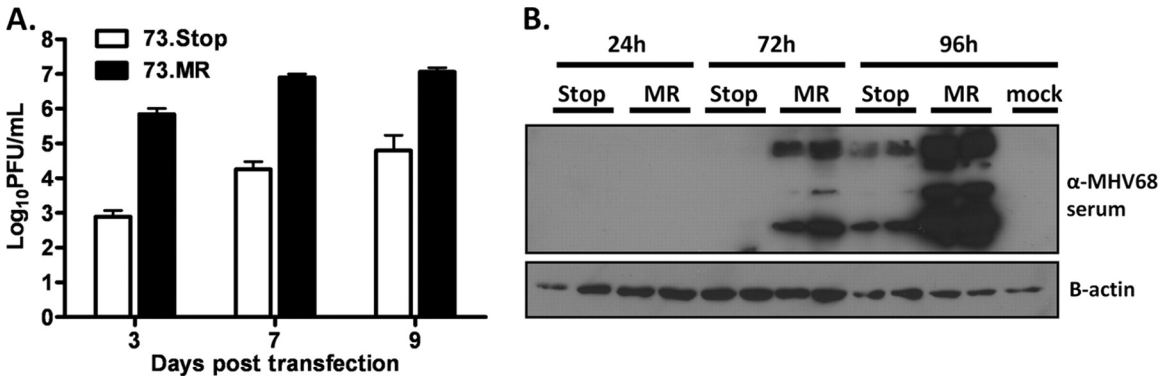


Figure 3.7. A functional mLANA gene is required for efficient replication of MHV68 following transfection of viral DNA in permissive fibroblasts. (A) NIH 3T12 fibroblasts were transfected with 0.5µg of either 73.Stop-BAC or 73.MR-BAC DNA. At 3, 7, and 9 days post-transfection, supernatants were harvested and titered for levels of infectious virus produced. The data is representative of at least three replicate experiments. **(B)** Vero cells were transfected in duplicate as above and lysates were harvested at 24, 72, and 96-hours. Western blots for lytic MHV68 antigen and β-actin are shown.

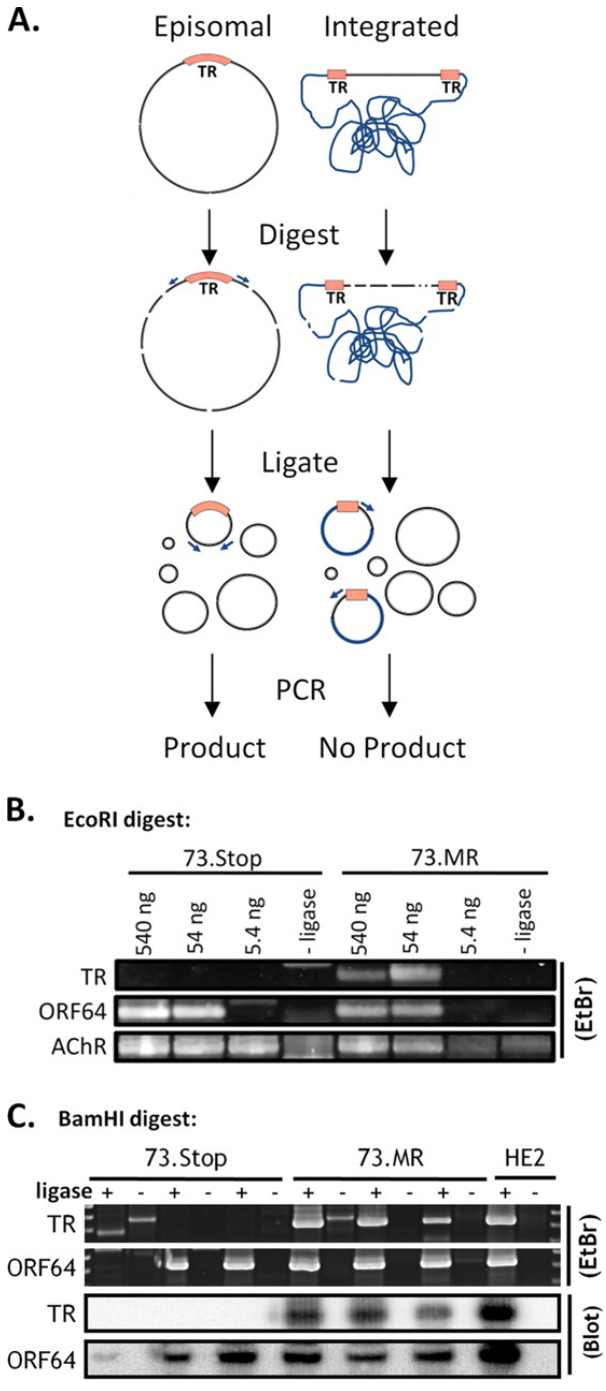


Figure 3.8. Digestion-Circularization PCR reveals mLANA-dependent episome formation during early latency in the spleen.

Figure 3.8 (cont.) (A) Diagram of DC-PCR method. Primers are designed facing away from each other on opposite sides of the TR. After digestion and intramolecular circular formation and ligation, the primers will produce a product during PCR. If the virus is integrated, i.e. does not have fused terminal repeats, only one primer binding site will be contained in the resulting circles, and no product is formed. **(B)** EcoRI digestion and circularization. Numbers above the gel represent the amount of digested DNA that went into each ligation. One set of mice is analyzed for 73.MR and 73.Stop infections. Murine acetyl choline receptor (AChR) is a control for digestion and intramolecular circularization. **(C)** BamHI digestion and circularization. 60ng of DNA was placed into each ligation reaction. Each pair of lanes (ligase +/-) represents a separate group of mice. Ligase negative reactions are used as a negative control. DNA from the latently infected HE2 line (Forrest and Speck 2008) is used as a positive control. Southern blots of the PCR products using probes that span the ligated junction further confirm the specificity of the PCR reaction.

CHAPTER 4.

Unbiased mutagenesis of MHV68 LANA reveals a DNA-binding domain required for LANA function *in vitro* and *in vivo*

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A) INTRODUCTION

Rhadinovirus infections are associated with a number of lymphoproliferative diseases. In the case of the human virus, Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8), there is tight association between KSHV and Kaposi's sarcoma (sporadic, endemic and HIV-associated forms of Kaposi's sarcoma) (Chang *et al.* 1994), as well as multicentric Castleman's disease and primary effusion lymphoma (Moore 2000; Wen and Damania 2010). Herpesvirus saimiri (HVS) has been shown to induce T cell lymphomas (Kiyotaki *et al.* 1986) and Murine gammaherpesvirus 68 (MHV68) has been shown to induce B cell lymphomas (Sunil-Chandra *et al.* 1994). In addition, both HVS and MHV68 can immortalize specific populations of lymphocytes in tissue culture (Melendez *et al.* 1971; Liang *et al.* 2011). A common feature of the KSHV-associated malignancies, in addition to harboring the latent viral genome, is the consistent detection of latency-associated nuclear antigen (LANA) expression (Gao *et al.* 1996a; Parravicini *et al.* 2000).

LANA, encoded by ORF 73 in the viral genome, is thought to be involved in many aspects of gammaherpesvirus infection. It was discovered as an antigen that speckles the chromosomes of KSHV-infected tumor cells when the tumor cells were stained with KS patient serum (Gao *et al.* 1996b; Kedes *et al.* 1996). KSHV LANA interacts with a number of cellular proteins that influence cellular signaling events, including interaction with the tumor suppressor p53 (Gao *et al.* 1996a; Friborg *et al.* 1999; Sarid *et al.* 1999; Verma *et al.* 2007b). KSHV LANA has also been shown to bind DNA, which is hypothesized to have importance in loading of replication origins

(Ballestas *et al.* 1999; Ballestas and Kaye 2001; Cotter *et al.* 2001; Garber *et al.* 2001), maintaining the virus genome as an episome (Ballestas *et al.* 1999; Collins and Medveczky 2002; Hu *et al.* 2002; Fejér *et al.* 2003; Verma *et al.* 2007b; Paden *et al.* 2010) and regulating gene transcription (Garber *et al.* 2001; Renne *et al.* 2001; Han *et al.* 2010). Domains of the KSHV LANA required for regulating gene transcription and DNA binding have been identified (Komatsu *et al.* 2004; Srinivasan *et al.* 2004; Han *et al.* 2010).

We have previously shown that the MHV68 LANA (mLANA) is required for replication fitness *in vivo* and *in vitro*, and for virus reactivation from latency (Moorman *et al.* 2003; Forrest *et al.* 2007; Paden *et al.* 2010). To begin to investigate how mLANA influences virus replication, we have assessed whether mLANA can modulate gene transcription and have identified a promoter in the terminal repeat of MHV68 that is potently repressed by mLANA. We have used this as a tool to identify residues in mLANA that are required for the transcriptional repressor function. Notably, many of the identified mutations that eliminate mLANA-mediated transcriptional repression are within the conserved (among rhadinovirus LANA proteins) C-terminal region of mLANA, corresponding to a putative DNA-binding domain. Investigation of these mutations reveals that the DNA-binding function is indeed disrupted. We further engineered several of these mutations into the MHV68 genome and determined that mLANA mutants deficient in transcriptional repressor function have a profound effect on both virus replication and reactivation from latency.

B) MATERIALS AND METHODS

Cell lines, transfections and reporter assays. NIH 3T12, RAW24.7, HEK 293T, and MEF (mouse embryonic fibroblast) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100mg of streptomycin per ml, and 2mM L-glutamine (complete DMEM). Cells were maintained in a 5% CO2 tissue culture incubator at 37°C. MEFs were obtained from C57BL/6 mouse embryos as described previously (Weck *et al.* 1996). Vero-Cre cells were a gift from David Leib. Cells were passaged in DMEM supplemented with 10% FCS and 300 μg of hygromycin B/ml.

Transfections were performed, except where noted otherwise, using the lipid-based reagent TransIT LT-1 (Mirus) according to manufacturer's instructions. For those analyses carried out in 12-well plates (format used for most transfections), 100ng of reporter and 50 to 500ng of the relevant expression vector were transfected per well. For some experiments 10ng of a SV40-driven *Renilla* luciferase, phRL-SV40, was co-transfected with the firefly luciferase reporter vector and served as an internal control. All transfections were harvested 48h post-transfection. For those transfections carried out in 96-well plates, 50ng expression vector, 15ng pGL-TR reporter vector, and 0.2ng phRL-SV40 were introduced to each well.

For co-immunoprecipitation experiments, 7.5μg of each expression construct was used. The cells were harvested 48h post transfection

Reporter assays. All dual reporter assays were performed using the Dual Luciferase kit (Promega), according to manufacturer's instructions. For single luciferase assays, cells were lysed in an appropriate volume of lysis buffer (25mM Tris-phosphate, pH 7.8, 2mM DTT, 2mM DCTA, 10% glycerol, 1% Triton X-100), and then 10µL lysate was mixed with 50µL luciferase assay reagent (1.5mM HEPES, pH8, 80µM MgSO4, 0.4mM DTT, 2µM EDTA, 10.6µM ATP, 5.4µM Coenzyme A, and 9.4µM beetle Luciferin) and light units were read on either TD-20/20 luminometer (Turner BioSystems) or a plate reader (BioTek), for large format assays.

Cloning and plasmids. The mLANA-GFP fusion protein expression plasmid pMSCV-73GFP was described previously (Forrest *et al.* 2007). The control plasmid, expressing only EGFP pMSCV-EGFP was made by amplifying the EGFP sequence from pIRES-EGFP using the oligos EGFP_5'_BglII (5'-ggaagatctATGGTGAGCAAGGGCGAGG-3') and EGFP_3'_EcoRI (5'-tagaattcTTACTTGTAC AGCTCGTCC-3') and cloning it into the BamHI/EcoRI sites of pMSCVpuro. The mLANA-3XFLAG epitope tagged plasmid was made by PCR amplifying ORF73 sequence from pL3700 (van Dyk *et al.* 2000) using 73-1_BAM (5'-GATCGGATCCCTTGACCCACACCCTTCCTGTGC-3') and 73-3_nostop_BAM (5'-gatcggatccTGTCTGAGACCC-3'). The PCR product was cloned into the BamHI site in p3XFLAG-CMV-14 (Sigma). The KSHV LANA expression construct was created by excising the LANA sequence from pDD105 (Jeong *et al.* 2004) and inserting it into the NotI site of pcDNA3.1(+) (Invitrogen).

The reporter plasmid pGL-TR(NotI) (pGL-TR) was created by digesting MHV68 BAC with NotI to obtain the 1240bp TR fragment. This fragment was cloned into the NotI site of pcDNA3.1(+) (Invitrogen) to create pcDNA-TRnot. The resulting vector was then digested to determine orientation, and then digested with XhoI and EcoRV to liberate a fragment that was then cloned directionally into pGL4.10 (Promega) using the corresponding restriction sites. The TR is oriented towards the luciferase open reading frame in reverse with respect to the annotated sequence.

Serial deletions of the TR were made by amplifying portions of the TR using PCR and then cloning those fragments into the KpnI/BglII sites. The primers used were, for 5' deletions: TRn-3'BglII (5'-atatagatctGCGCCTGGGGCGCCATGC-3') in combination with 173F-Kpn (5'-ttaggtacc cccggggccccccacaagcctc-3'), 311F-Kpn (5'-ttaggtacc ccgggggccccgctacgagc-3'), 353F-Kpn (5'-ttaggtacc aagccccgggcccgcccc-3'), 395F-Kpn (5'-ttaggtacc cgtgcccctcccccctgcag-3'), 456F-Kpn (5'-ttaggtacc cctccgggacccgcccacag-3'), 590F-Kpn (5'-ttaggtaccgagggccagagtctgaa ctg-3'), 702F-Kpn (5'-ttaggtacc gagggccagagtctgaactg-3'), or 995F-Kpn (5'-ttaggtaccgtggccgc gctggcctagc-3'); and for 3' deletions, TRn-5'KpnI (5'-ttaggtaccGGCCGCTAC CGCCCGGGCC-3') in combination with 311R-Bgl (5'-atatagatctGCTCGTAGCGGGGCCCCCGG-3'), 353R-Bgl (5'-atatagatctCGGGGGCGGGCCCGGGGCTTAC-3'), 526R-Bgl (5'-atatagatctCCCCCTCGGC GGCTCCCCTAC-3'), 737R-Bgl (5'-atatagatctAGGGGGCTGGAGGGGGGAAG-3'), 875R-Bgl (5'-atatagatctCCCCCACCCCCCAAGAAGAG-3'), 1014R-Bg (5'-atatagatctAGCTAGGCCAG CGCGGCCAC-3'), 1161R-Bgl (5'-

atatagatctCCCTACCGGGCTGCCGCTAC-3'), or 1205R-Bgl (5'-atatagatctCACGCGGCGCGCCCTGGAG-3').

The bacterial expression vector pET22b-73dN was generated by amplifying ORF73 from pL3700 using the following primers: 73_5'_ATG2_Bam_2 (5'-gatcg gatccgATGCCTCCTAAAA GACGCC-3') and 73_3'_nostop_Xho (5'-gatcctcgagTGTCTGAGACCCTTGTCCCTG-3'). The PCR product was cloned into the BamHI/XhoI sites of pET22b (Novagen).

All PCR amplifications for cloning were performed using the high-fidelity Phusion polymerase (New England Biolabs).

Protein expression and purification. The vectors pET22b-73dN (and also ORF73 mutants) and pRARE (Stratagene) were both transformed into the BL21(DE3) strain of E. coli (New England Biolabs). After bringing 1 L cultures to log-phase growth, expression was induced by the addition of 1mM IPTG for 5 hours at 37°C. Bacteria were harvested by centrifugation, and lysates were prepared for Ni-NTA purification under native conditions according to the manufacturer's instructions (QIAGEN). Briefly, the pellet was resuspended in lysis buffer, and cells were lysed in the presence of 1mg/mL lysozyme and by sonication. Following sonication, lysates were treated with RNase A (10µg/mL) and DNase I (5µg/mL) prior to clearing. Lysate was incubated with 10mL Ni-NTA agarose slurry in batch for 1 hour and subjected to column-based washing and elution. 500µL fractions were collected and analyzed by SDS-PAGE. Those fractions containing the majority of mLANA protein, determined by Coomassie Blue staining,

were pooled and dialyzed overnight into buffer BFD (20mM HEPES, 20% glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM DTT, and 0.5mM PMSF) and stored at -80°C.

Radiolabeling and DNase I footprinting. DNase I footprinting was performed essentially as previously described, with minor modifications (Lee *et al.* 1987; Flemington and Speck 1990). Probes were made by digesting pcDNA-TRnot with the blunt-cutter PmeI and either XhoI or BamHI to create probes with overhangs on one end or the other. These fragments were then labeled with [α-32]P using the Klenow fragment (3'-5'exo-) of DNA Polymerase I (New England Biolabs) in an overhang fill in reaction. Free nucleotides were removed by gel filtration spin columns (GE Healthcare) followed by precipitation of the probes.

The binding reaction was set up in 50µL containing a total 100µg protein (0-100µg mLANA-6XHis, with the remainder made up with bovine serum albumin [BSA]), 1µg poly(dI:dC), and binding buffer (2% polyvinylethanol, 2.5% glycerol, 10mM Tris pH 8, 0.5mM EDTA, and 0.5mM DTT). After a 10 minute incubation on ice, the reactions were brought to room temperature, and 50,000 cpm labeled probe was added to each reaction, and then they were incubated at room temperature for 20 minutes. DNase I (DPRF) (Worthington) was diluted into 10mM MgCl2 and then, each reaction was exposed to 100µL of the appropriate concentration of diluted DNase I for 30 seconds before the addition of DNase Stop buffer (8M Urea, 0.5% SDS, 5mM EDTA) followed by two phenol extractions, three phenol-chloroform extractions, a chloroform extraction, and then ethanol precipitation. These digests were separated on an 8% denaturing acrylamide sequencing gel.

The G+A ladder was made using a modified Maxam-Gilbert protocol. 50,000 cpm probe was mixed with 2µg salmon sperm DNA in 10µL reaction, adding 1µL 4% formic acid, and incubating 25 minutes at 37°C. Next, 150µL of a 1:10 dilution of pre-chilled piperidine was added and the reaction was incubated 30 minutes at 90°C. Then, the reaction was placed on ice, and 1mL 1-butanol was added and vortexed. The precipitated DNA was centrifuged. The pellet was washed in 150µL 1%SDS, then 1mL 1-butanol was added, and the reaction which was centrifuged again. After a final wash in 0.5mL 1-butanol, centrifugation, and removal, the pellet was dried under a vacuum and resuspended in formamide loading buffer.

Mutagenesis of ORF73 plasmid. The plasmid pMSCV-73GFP was used to make mutants of ORF73 by subjecting it to error-prone PCR using the GeneMorph II kit (Stratagene). Conditions were set, in accordance with the manufacturer's recommendation, to create 1-2 mutations on each PCR product. The primers 73_5'ATG2 (5'-ATGCCTCCTAAAAGACGCCG-3') and 73_3'noSTOP (5'-TGTCTGAGACCCTTGTCCCTGTT-3') were used to amplify ORF73 from the pMSCV-73GFP (6448 ng) using the following PCR conditions: 2' 95°, [30" 95°, 30" 55°, 60" 72°] x 30, 10' 72°. After precipitation of the PCR product, it was used as a "megaprimer" in a site-directed mutagenesis reaction (Stratagene) on pMSCV-73GFP to create the library of mutants in the expression plasmid. Following DpnI digestion of the newly-created mutant expression, the plasmids were transformed into *E. coli*, colonies were picked, and DNA was prepared by alkaline lysis followed by silica membrane

purification in 96-welll format (Zymo Research). These DNA preps were used directly for transfection and for automated DNA sequencing.

Mutagenesis of MHV68 BAC and production of virus. The MHV68 BAC was mutated using the galK-mediated Red recombineering, as described previously for other BACs (Warming *et al.* 2005). Briefly, we introduced MHV68 BAC into the galk strain of bacteria SW102. In order to create and ORF73/GalK replacement BAC, we amplified the galK gene from the plasmid pGalK using the primers galK-US-73 (5'-catgccctggcgaaggtgttgccca ggatatattctgggaatgtgatttaCCTGTTGACAATTAATCATCGGCA-3') and galK-DS-73(5'-acccttcctgtgctaaaagttgtgactgtgtactttatctctttcagataTCAGCACTGTCCTGCT CCTT-3'); the resulting PCR product includes 50bp of the sequence upstream and downstream of ORF73 for use in homologous recombination. The PCR product was introduced via electroporation to SW102/MHV68 cells and those recombinants were selected by using minimal media with galactose as the sole carbon source. Once ORF73/GalK replacement BAC mutants (MHV68.Δ73galK) were confirmed by restriction digest, ORF73 point mutant PCR products were made to swap out GalK. The PCR products were made by amplifying each mutant pMSCV-73GFP plasmid with the following primers, which contain the same 50bp of homology as the primers above: 73-res-ds (5'-ACCCTTCCTGTGCTAAAAGTTGTGACTGTGTACTT TATCTCTTTCAGATAATGCCCACATCCCCAC-3') and 73-res-us (5'-catgccctggcgaaggtgt tgcccaggatatattctgggaatgtgatttatgtctgagacccttg-3'). The mutant ORF73 PCR product was electroporated into SW102/MHV68.Δ73galK and after

recombination, were selected on minimal media plates containing glycerol and 2-deoxy-D-galactose, which is toxic to bacteria that metabolize galactose. The resulting colonies were screened by colony PCR (primers 5'-ACAC AACCTCAGGCAAAAC-3' and 5'-CCTTCAACATCAACATCTGG-3') and then verified by restriction digest. Mutations were confirmed by PCR of ORF73 and automated DNA sequencing of the PCR product.

Resultant BACs were transfected into Vero-Cre cells. When cells reached 70% CPE, cells and supernatants were harvested by freeze/thaw lysis, and fresh Vero-Cre cells were infected with this stock to ensure excision of the BAC. One further passage in Vero-Cre cells was performed in order to expand the virus stocks.

Virus infections and plaque assay. Virus stocks were diluted in complete media and adsorbed on cells plated the previous day. For MOI 3 infections, virus was adsorbed for one hour, the inoculum was removed, and fresh, complete media was replaced. For low MOI (0.001) infections, virus was diluted, adsorbed, and left on the cells. Virus was harvested from cells and supernatant by 3X freeze/thaw lysis. Plaque assay was performed as previously described (Upton and Speck 2006).

Immunoblot and immunoprecipitation analyses and antibodies. Immunoblots were performed as described (Forrest *et al.* 2007). Cells were lysed in alternative radioimmunoprecipitation assay (RIPA) buffer (150mM NaCl, 20mM Tris, 2mM EDTA, 1% NP-40, 0.25% deoxycholate, 1mM NaF, and 1mM Na3VO4 supplemented with complete mini-EDTA-free protease inhibitors [Roche]). Antibodies used were mouse MHV68 antiserum, rabbit mLANA antiserum, mouse anti-β-actin (Sigma), as well as

HRP-conjugated anti-rabbit and anti-mouse Ig antibodies(Jackson ImmunoResearch). mLANA-6XHis was detected using mouse anti-penta-His antibody (QIAGEN).

For co-immunoprecipitation experiments, lysates were prepared from 10 cm dishes of 293T cells co-transfected with p73-3XFLAG and pMSCV-73GFP (and mutants) by lysing in 1.1mL of alt. RIPA buffer and shearing with a 20G needle. After clearing by centrifugation at 4°C, 0.1mL was saved for whole cell lysate analysis, and the remaining 1mL lysate was incubated overnight at 4°C with anti-FLAG (M2) agarose beads (Sigma) to pull down mLANA-3XFLAG complexes. Beads were washed according to manufacturer's instructions, and then separated on 10% SDS-PAGE gel and transferred to nitrocellulose. Blots were probed with either HRP-conjugated mouse anti-FLAG M2 (Sigma) or goat anti-GFP (Rockland Immunochemicals) and HRP-conjugated donkey anti-goat Ig (Jackson ImmunoResearch).

To generate rabbit antiserum to mLANA, the sequence of mLANA encoding amino acids 173-314 of the C-terminus was cloned into pGEX-6P-1 (GE LifeSciences), and the resulting GST fusion protein was produced in ArcticExpress BL21 cells (Agilent Technologies) then purified on Glutathione Sepharose 4B columns (GE LifeSciences).

Mice and infections. Female C57Bl/6 mice 6 to 8 weeks of age were purchased from the Jackson Laboratory and and maintained at Emory University. Mice were sterile housed and treated according to the guidelines at Emory University School of Medicine (Atlanta, GA). Mice were infected intraperitoneally with 1000PFU of virus diluted into 0.5mL of complete DMEM or intranasally with 1000PFU of virus diluted into 20μL

complete DMEM. For intranasal infection, mice were sedated, and after infection allowed to recover before returning to their cages.

Limiting dilution assays. Limiting dilution assays for frequency of latently infected cells or cells reactivating virus were performed as previously described (Weck *et al.* 1996; Weck *et al.* 1999b). Briefly, to determine the frequency of cells harboring latent viral genomes, single-copy-sensitive nested PCR was performed on wells of serially-diluted, erythrocyte-free splenocytes. Cells were lysed by protease K digestion, and two rounds of PCR were performed per sample with twelve samples per dilution, and the products were resolved on 2% agarose gels. To measure the frequency of cells reactivating virus, splenocytes were resuspended in complete DMEM and plated in serial two-fold dilutions on mouse embryonic fibroblast (MEF) monolayers in 96-well tissue culture plates. Parallel samples of mechanically disrupted cells were plated to detect preformed infectious virus. Wells were scored for cytopathic effect 14 to 21 days post-explant. Frequencies are determined using a Poisson distribution.

C) RESULTS

mLANA-mediated repression of transcription from the MHV68 terminal repeat In previous work, our lab showed that the mLANA-null virus (73.Stop) displays dysregulated gene expression, characterized by hyper-expression of all temporal classes of viral genes at early time points post-infection of murine fibroblasts compared to the genetically-repaired marker rescue virus (73.MR), as measured by western blot (Forrest *et al.* 2007) and RT-PCR (Paden and Speck unpublished observation). Included in these hyper-expressed genes is the *gene 73* transcript, which encodes mLANA. It is known that at least one of the promoters for mLANA expression is contained within the terminal repeat, as identified *in vivo* from infected cells (Allen *et al.* 2006) (see schematic diagram of mLANA transcripts shown in Fig. 4..1A). It is also known that the KSHV LANA can bind DNA in the terminal repeat of the KSHV genome (Ballestas and Kaye 2001; Cotter *et al.* 2001; Garber *et al.* 2001; Garber *et al.* 2002), and the KSHV LANA can modulate expression of a variety of genes (Renne *et al.* 2001). Taken together, we reasoned that mLANA is likely directly regulating transcription of viral genes, including the mLANA promoter within the terminal repeat of MHV68. To test this hypothesis, we cloned a complete copy of the MHV68 terminal repeat (using NotI, which cuts only once within the terminal repeat, liberating a 1213bp restriction endonuclease fragment) into the reporter vector pGL4.10 upstream of the firefly luciferase gene (pGL-TR). The pGL-TR plasmid was co-transfected into cell lines with either the control parental expression vector or a mLANA expression vector, and luciferase activity assessed at 48 hours post-transfection (Fig. 4.1B).The pGL-TR reporter vector exhibited significant activity in the absence of mLANA expression. Furthermore, luciferase expression from the pGL-TR

plasmid was potently inhibited in the presence of mLANA-GFP (pMSCV-73GFP) (Fig. 4..1B). Notably, the observed repression of activity mediated by mLANA appeared specific since co-transfecting the mLANA-GFP expression plasmid had no effect on reporter activity driven by the HSV-TK promoter (Fig. 4..1B).

Because there are significant differences in the N-terminus of KSHV LANA and mLANA, we tested whether there is enough conservation of sequence and structure between the KSHV and MHV68 LANA homologs to extrapolate findings on mLANA to the human virus. As such, we assessed whether KSHV LANA can functionally substitute in the MHV68 TR repression assay. We co-transfected HEK 293T cells with the MHV68 TR reporter construct (pGL-TR) and either empty expression vector, the MHV68 LANA expression vector, or the KSHV LANA expression vector (Fig. 4..1C). Importantly, we observed (using an equivalent amount of input of KSHV LANA or mLANA expression vector) that both the KSHV LANA and mLANA strongly repressed transcription from the MHV68 TR. This indicates that though there is significant amino acid sequence divergence between these homologs, along with the fact that the known KSHV LANA binding sites are not precisely conserved in the MHV68 TR, there is enough functional similarity between the two homologs to reveal a conserved transcriptional repression function. Thus, this provides additional confidence that studies on the MHV68 LANA will likely provide insights into KSHV LANA function—particularly with respect to functions that map to the conserved C-terminal domain.

To address whether LANA repression of transcription from the MHV68 TR is detected in the context of MHV68 infection, we introduced the pGL-TR reporter construct into NIH 3T12 fibroblasts by electroporation, followed by MHV68 infection at

a multiplicity of 3 PFU/cell (viral infection was initiated once the cells had adhered to the culture plates approximately 12 hours post-electroporation). Cells were harvested 18 to 24 hours post-infection and assayed for luciferase activity (Fig. 4..1D). Comparing luciferase induction to cells that were mock infected, we observed that WT virus (73.MR) infected cells exhibited ca. a 5-fold increase in TR-driven luciferase activity, indicating that overall transcription arising from the TR is stimulated by viral infection. However, cells infected with the mLANA-null mutant virus (73.Stop) exhibited significantly higher levels of TR-driven luciferase activity (30- to 35-fold induction) compared to mock infected cells. The latter provides evidence that mLANA plays an important role in regulating transcription initiating from the TR and likely plays a critical role in preventing over-expression of mLANA during infection of permissive fibroblasts (Fig. 4..1D).

We extended these initial studies to assess mLANA repression of the MHV TR promoter(s) in several different cell lines. Notably, we observed that the promoter contained in the TR had significant activity in a variety of cell types, including NIH 3T12 mouse fibroblasts (Fig. 4..1E), the murine macrophage cell line RAW264.7 (Fig. 4..1F), and the human embryonic kidney cell line HEK 293T (Fig. 4..1G). Furthermore, when the pGL-TR reporter plasmid was co-transfected along with increasing amounts of the mLANA-GFP expression vector, in all cell lines examined the expression of luciferase decreased in a dose-dependent manner (Fig. 4..1, panels E-G). Taken together these data demonstrate that mLANA represses transcription initiating from the TR—an observation that is consistent with the observed hyper-transcription of mLANA transcripts (as well as

other viral genes) in the absence of mLANA expression in cells infected with the mLANA-null mutant virus (73.Stop) (Forrest *et al.* 2007).

Identification of DNA sequences required for mLANA repression of MHV68 TR-driven luciferase expression. To determine what sequences within the MHV68 TR are involved in mediating mLANA repression we took two independent approaches: (i) assaying mLANA repression of a panel of TR deletion constructs (Fig. 4..2); and (ii) DNase I footprinting of the TR using recombinant mLANA protein (Fig. 4..3). First, nested deletions from either end of the TR NotI restriction endonuclease fragment were generated and cloned into the pGL4.10 luciferase reporter vector (Fig. 4..2B). Notably, the TR is cloned into the reporter vector with respect to the luciferase open reading frame in reverse orientation from the annotated sequence (Virgin *et al.* 1997) (GenBank U97553) (i.e., the orientation of the TR fragment is consistent with promoter activity observed in the context of the viral genome that would lead to transcripts extending into the righthand end of the unique region of the MHV68 genome, such as previously characterized for *gene 73* and *gene 72* transcripts (Coleman *et al.* 2005b; Allen *et al.* 2006))(Fig. 4..2A). As noted above, the NotI fragment in the reporter vector is a 1213 bp fragment corresponding to bp 119,105 to bp 118,238, and continuing from bp 119,450 to bp 119,106 (the junction between copies of the TR is at bp 118,238/119,450 as defined from the complete sequences of the WUMS MHV68 isolate (Virgin *et al.* 1997). To simplify the discussion of mapping TR sequences involved in mediating mLANA repression, we have renumbered the NotI TR fragment as nucleotides 1-1213 as shown in Figure 4.2A.

TR deletion constructs were co-transfected into HEK 293T cells with either an mLANA-GFP expression vector or the parental empty expression vector, and assayed for luciferase activity (Fig. 4..2B). Data were normalized as fold over the pGL4.10 empty reporter vector controls. Deleting sequences from the upstream end of the TR (bp 1-585) had little impact on either basal promoter activity or the capacity of mLANA to repress activity. Notably, deleting the sequences from bp 390 to 451, which had no discernible impact on luciferase activity, removed the region encoding one of the previously characterized LANA promoters [transcription initiation site (i) in Fig. 4..2A] indicating the presence of other transcriptional start sites within the MHV68 TR. Indeed, deletion of the sequences from bp 585 to 963 resulted in a nearly complete loss of reporter gene activity (Fig. 4..2B, compare activities of Δ1-585 and Δ1-963 reporter constructs) , implicating sequences within this region as critical for the observed activity in the absence of upstream TR sequences [e.g., transcription initiation site (ii) shown in Fig. 4..2A]. That other sites within the TR can be involved in driving luciferase expression was further underscored by analysis of the nested downstream deletions where the Δ369-1213 deletion exhibited basal activity as high as the full length reporter construct (Fig. 4..2B). This deletion removes both of the previously mapped transcription initiation sites within the TR [sites (i) and (ii) shown in Fig. 4..2A]. However, as noted by Coleman et al, there is a distinct AT-rich sequence 5' of the 73E1 start site. When the AT-rich sequences (bp 328-350, or MHV68 genomic coordinates118,778-118,756) are removed in the Δ327-1213 TR reporter construct, basal activity for this promoter is lost. Thus, it appears that there are several potential transcription initiation sites within the TR whose

contribution to the observed luciferase activity are likely context dependent (i.e., the presence or absence of other TR sequences).

Deletion of TR sequences from bp 1174 to 1213 resulted in a significant increase (ca. 8-fold) in basal luciferase activity (Fig. 4..2B), revealing the presence of a potent negative cis-element in this region of the TR. Notably, the Δ1174-1213 reporter construct was repressed by mLANA to the same extent as the full length TR reporter construct (Fig. 4..2B). However, a further deletion which removes the sequences from bp 1130-1174 resulted in the complete loss of mLANA repression. The lack of mLANA repression was also observed with all the other downstream nested deletion mutants that lacked the sequences from bp 1130-1174 (Fig. 4..2B). Thus, within the 1213bp TR fragment, a single region was mapped that mediates mLANA repression of TR-driven luciferase activity.

To assess whether mLANA, like KSHV LANA, mediates repression through directly binding a target site(s) within the TR, as opposed to being dependent on associations with other DNA-binding proteins, we performed DNase I footprinting on the MHV68 TR NotI fragment using recombinant mLANA-6XHis tagged protein purified from E. coli lysates. Using recombinant mLANA affinity purified from bacteria ensured that any mLANA-dependent DNA binding observed would not reflect binding of any mLANA-associated eukaryotic protein(s), but rather DNA binding activity of mLANA. We radiolabeled labeled each end of the TR separately and performed footprinting assays using each of these labeled fragments and varying amounts of recombinant mLANA-6XHis in the presence of BSA, and with BSA alone as a negative control. We found a single region of the TR, composed of a cluster of ca. three distinct sites (flanked by

DNAse I hypersensitive sites), that was protected from DNAse I digestion (Fig. 4..3A). Notably, the region of mLANA binding overlapped substantially with the region identified as critical for mLANA repression of the pGL-TR luciferase reporter constructs (see Fig. 4..3D). Thus, these data together provide strong evidence that mLANA-mediated repression of the pGL-TR luciferase reporter construct involves direct interaction of mLANA with the identified sequences within the MHV68 TR.

Mutagenesis of mLANA reveals residues required for repression of transcription. Having established the TR reporter assay as a robust readout of mLANA-mediated transcriptional modulation, we used this assay to screen for mLANA mutants impaired in repressing transcription. To identify the region or regions of mLANA that are important in regulating transcription, we took an approach that afforded an unbiased analysis of the entire mLANA coding sequence. Low-fidelity/error-prone PCR was used to amplify the mLANA ORF, using the polymerase Mutazyme II (Stratagene) and PCR conditions that favor one to two errors per amplified template. The resulting PCR products were then cloned into an expression vector to generate C-terminal GFP fusion proteins, and single colonies were grown up for DNA isolation. These mutated mLANA-GFP plasmids were subsequently transfected into HEK 293T cells in 96 well plates, along with the pGL-TR reporter vector and the internal control phRL-SV40 which expresses *Renilla* luciferase. Forty-eight hours post-transfection each well was examined for GFP expression, to identify and exclude those wells in which either a nonsense mutation was incorporated into the mLANA coding sequence or that the mutation(s) introduced led to the formation of an unstable fusion protein. Thus, those wells with

robust GFP expression were subsequently analyzed for luciferase activity to identify clones that were incapable of inhibiting transcription from the MHV68 TR. As such, those clones that were GFP-positive and luciferase-high were regrown in *E. coli* and retested for loss of transcriptional repression. Those clones in which the phenotype was confirmed were then sent for DNA sequence analysis to determine the mutations introduced into the mLANA coding sequence.

Approximately 1500 clones isolated from the mutagenesis protocol were screened, and 14 clones (~0.93%) displayed expression and a robust enough change in phenotype from wild-type mLANA to warrant further analysis (Table 4.1). Though the random mutagenesis strategy was not designed to be an exhaustive search, we found that the majority of the mutations fell within the domain of mLANA (highlighted in grey in Fig. 4..4A) that is relatively well conserved among the sequenced LANA homologs. Notably, 3 of the clones recovered contained 2 missense mutations in the mLANA coding sequence, while a fourth clone had incorporated 3 missense mutations (Table 4.1). The remainder of the mLANA mutants analyzed harbored a single missense mutation (Table 4.1). For the complex mutants, several individual point mutations were introduced into mLANA to pinpoint the residue involved in loss of transcriptional repression.

A number of these mutations were changes in residues conserved between KSHV and MHV68 LANA proteins, and others were in residues that share similar charge or hydrophobicity. Notably, many of these randomly-generated mutations overlap with regions of KSHV LANA targeted by Han *et al.* to disrupt LANA DNA binding (Fig. 4..4B). Shown in red in Figure 4.4B are mutations that attenuate mLANA-mediated

transcriptional repression in our hands (MHV68_LANA) and mutations in KSHV LANA that result in ≤50% of WT KSHV transcriptional repression (Han *et al.* 2010).

To assess how the mutations that disrupt mLANA repression are distributed on a three-dimensional model of mLANA (to date there is no available crystal structure of any LANA homolog), we used the Phyre (Protein Homology/analogy Recognition Engine) 0.2 algorithm and online search tool to indentify similar structures. We queried the Phyre tool (http://www.sbg.bio.ic.ac.uk/~phyre/) using the full-length amino acid sequence of mLANA and searching against profiles of proteins with known structures(Kelley and Sternberg 2009). This algorithm determined that the top hit was for the shaded region of the mLANA sequence shown in Figure 4.4A, which matched most closely to the DNA-binding domain (DBD) of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) protein—the presumed functional homolog (encoded by the lymphocryptovovirus subclass of gammaherpesviruses) of the rhadinovirus LANA proteins (Bochkarev *et al.* 1995; Dittmer *et al.* 1998). Notably, this region of EBNA1 shares almost no homology and little similarity to the primary sequence of mLANA. This is similar to what was found using KSHV LANA as a query sequence in an older 3D-PSSM algorithm (Grundhoff and Ganem 2003). Using this model, we mapped the loss of repression mutations in this region on the EBNA-1 DBD/mLANA hybrid model (Fig. 4.4C). The sequence shaded in grey in Figure 4.4A is shown in the model, with the mutations colored in green. For reference, an additional mutant we generated that does not disrupt activity (A171V) is colored in red (Fig. 4..4C). From this analysis we hypothesized that the mLANA loss of function mutations identified in this region disrupt DNA binding, either by causing the β-

barrel structure of the core DNA-binding domain (Cruickshank *et al.* 2000) to misfold slightly, misaligning contact residues, or by directly mutating a contact residue.

Mutations in the predicted mLANA DNA-binding domain disrupt mLANA-DNA interaction. Based on the EBNA-1 structure prediction, we chose six mutants to further characterize. The mutants chosen were: (i) P179T, which is predicted to be in helix 2, considered part of the core DNA-binding region; (ii) A171V, which would be in the turn between the flanking and core domains and has no measured effect; (iii) R128G, which would be in or near the minor groove contact loop of the flanking domain; (iv) V194E, which would be in the turn between the two helices of the core domain; (v) A293G, which is predicted to be downstream of the core DNA-binding domain; and (vi) L150P, which would be part of the flanking domain, near the major groove contact residues. Their effects on repression of the TR-driven luciferase activity are all significantly different than wild-type mLANA, save for the A171V mutant (Fig. 4..5A). Notably, the relatively small differences in the activities of the mLANA mutants that have lost repression compared to the empty vector (EV) control were not statistically significant (Fig. 4..5A).

To assess whether or not the mutations we identified that exhibit diminished transcriptional repression activity actually lose the capacity to bind DNA, we generated recombinant protein for three of the mutants, P179T, A171V, and L150P (Fig. 4..3C). We performed DNase I footprinting on these mutant mLANA proteins, along with WT mLANA, and observed that while there may be subtle differences between the P179T and L150P mutants—both of which fail to repress transcription—neither produced robust

protection from DNase I digestion demonstrating decreased DNA-binding (Fig. 4..3B). In contrast, both the wild-type mLANA and the A171V mutant, which exhibits wild-type phenotype in the pGL-TR luciferase repression assay (Fig. 4..5A), show robust sequence-specific DNA binding (Fig. 4..3B). It should be noted that the absence of DNA binding observed with the P197T and L150P mutants is unlikely to reflect inadequate levels of recombinant protein used in the DNAse I footprinting assay, based on SDS PAGE analyses of the purified mLANA proteins (Fig. 4..3C).

Since it has been shown that the DNA-binding domain and the dimerization domain in EBNA-1 are near each other (Ambinder *et al.* 1991; Bochkarev *et al.* 1995; Cruickshank *et al.* 2000), we wanted to know: (i) if mLANA also forms dimers, and (ii) whether any of the identified mLANA mutants are defective in dimerization, which would likely render them non-functional as it has been shown that KSHV LANA binds DNA as a dimer (Garber *et al.* 2002). To assess dimerization, we generated a construct that expresses mLANA with a C-terminal 3XFLAG epitope tag (p73-3XFLAG) and co-expressed that construct along with five mLANA mutants (expressed as GFP-fusion proteins), WT mLANA-GFP, or GFP alone. After immunoprecipitating with an anti-FLAG antibody, we resolved the complexes by SDS-PAGE and probed immunoblots with an anti-GFP antibody (Fig. 4..5B). Notably, as expected we observed that WT mLANA-3XFLAG was capable of interacting with and pulling down WT mLANA-GFP, demonstrating formation of mLANA dimers (Fig. 4..5B). In addition, WT mLANA-3XFLAG was also capable of pulling down each of the mLANA mutants, but not GFP alone (Fig. 4..5B). Additionally, as a control we co-expressed the empty 3XFLAG vector and WT mLANA-GFP and demonstrated that the anti-FLAG antibody did not

immunoprecipitate mLANA-GFP (Fig. 4..5B). These analyses demonstrate: (i) dimerization of mLANA; and (ii) all of the identified mLANA mutants are able to dimerize with wild-type mLANA. Notably, while the L150P mutant (and to a lesser extent the A171V mutant) were expressed at lower levels, similar levels of mutant protein appeared to be recovered by co-immunoprecipitation of wt mLANA. It is likely that this reflects that the co-immunoprecipitation assay is not quantitative and that antibody is the limiting, leading to similar levels of mLANA dimers being recovered following immunoprecipitation.

MHV68 LANA DNA-binding and/or transcriptional repression null mutant viruses exhibit a mLANA-null phenotype in vivo. The advantage of using MHV68 to study LANA biology is that: (i) it is straightforward to create mutant viruses, as MHV68 replicates robustly in tissue culture and the viral genome has been cloned into a bacterial artificial chromosome (BAC); and (ii) the virus readily infects naïve laboratory mice, allowing detailed viral pathogenesis studies. For these reasons, we chose six mLANA mutations to introduce into the viral genome to assess the impact that the transcriptional repression and/or DNA-binding functions of mLANA have on the overall pathogenesis of MHV68. To construct the mutant BACs, we employed λRed-mediated homologous recombination using *galK* to facilitate selection in the *E. coli* strain SW102 (Warming *et al.* 2005). The MHV68-YFP BAC (Collins *et al.* 2009) was transformed into the *E. coli* strain SW102. Next, the entirety of ORF73 was replaced with the *galK* cassette, flanked by 50 bp arms homologous to the region upstream and downstream of ORF73, and selected on galactose-containing minimal media to create pMHV68.73galK. Clones were

verified by restriction digest. PCR products of ORF73 containing each mutation, as well as the 73.Stop mutation (Moorman *et al.* 2003), and WT ORF73 were introduced into SW102 harboring MHV68.73galK BAC for recombination onto the galK BAC. Resulting colonies were verified by colony PCR and restriction digest. Since there are no diagnostic restriction sites in these point mutants, the ORF73 region of each BAC was further PCR amplified and sequenced (data not shown). The resulting mLANA-transcription-repression-null mutant viruses (73TRN) are designated 73.P179T, 73.R128G, 73.V194E, 73.A293G, and 73.L150P, along with 73.A171V, 73.Stop-2, which contains a stop codon near the beginning of the ORF identical to 73.Stop (Moorman *et al.* 2003), and the genetically-repaired 73.galKMR.

To ensure that our mLANA mutants would express in the context of infection, we infected NIH3T3 cells at an MOI of 3 PFU/cell and collected lysates at seven hours post-infection. We readily observed mLANA expression by immunoblot, using a rabbit polyclonal anti-mLANA antibody, in all mutants except for A293G (the basis for failure to detect the latter mutant is unclear) (Fig. 4..6A). Further, a phenotype of 73.Stop virus we previously observed was dysregulation of lytic gene expression, which is manifested as hyperexpression of all lytic genes. Each mutant also displayed expression of antigens detectable by lytic antiserum, except for the 73.galK marker rescue (73.MR) and 73.A171V viruses, which behave as WT (Fig. 4..6A).

We have previously shown that mLANA is required for efficient replication in tissue culture following low MOI infection—the mLANA-null virus 73.Stop showed a 1- to 2-log defect in output virus from permissive fibroblasts upon low MOI infection (between 0.05 and 0.001 PFU/cell) compared to the genetically repaired 73.MR virus

(Forrest *et al.* 2007). To determine whether mLANA DNA binding is required for efficient virus replication, we infected NIH3T3 fibroblasts with each 73TRN virus at an MOI of 0.001 PFU/cell. Cells and supernatants were collected by freeze/thaw lysis seven days post-infection and titered on NIH 3T12 fibroblasts. We determined that the 73TRN viruses, like 73.Stop, exhibited a significant decrease in output titers over the course of several rounds of virus infection, whereas the 73.A171V mutant was equivalent to 73.galK marker rescue (73.MR) virus (Fig. 4..6B).

In vivo analyses of 73TRN viruses demonstrate a requirement for mLANA DNA-binding domain. As with the in vitro replication defect known for the mLANA-null 73.Stop virus, we have previously shown that MHV68 requires mLANA expression for efficient reactivation from latently-infected cells (Paden *et al.* 2010). To examine whether the transcription repressor function of mLANA is required for reactivation from latency, we used the 73TRN mutant viruses to infect mice and assay for establishment of and reactivation from latency. Each 73TRN virus was used to infect groups of C57Bl/6 mice intraperitoneally with 1000 PFU of virus per mouse. The intraperitoneal route was used since the 73.Stop virus is capable of establishing latency in immunocompetent mice via this route and not by the intranasal route (Fowler *et al.* 2003; Moorman *et al.* 2003; Paden *et al.* 2010). Eighteen days post-infection, mice were sacrificed and spleens and peritoneal exudate cells were harvested and subjected to limiting dilution PCR (Fig. 4..7B) and an ex vivo limiting dilution reactivation assay (Fig. 4..7C). Somewhat surprisingly, we found that every mLANA mutant that was null in the TR transcription repression assay when introduced into MHV68, displayed limited splenomegaly, similar

to the mLANA-null mutant (73.Stop) (Fig. 4..7A), and a reactivation phenotype comparable to the mLANA-null mutant (Fig. 4..7C) (i.e., these virus-infected cells displayed no detectable reactivation in the limiting dilution assay). Under these experimental conditions, the 73.Stop mutant also displays a slight decrease in the frequency of latently infected cells—ca. 3 to 5-fold lower than the MR virus—which is borne out here in each of the 73TRN mutant viruses (Fig. 4..7B). These data point to the conclusion that mLANA DNA-binding and/or transcriptional repressor functions play a critical role in viral pathogenesis.

Intranasal infection of mice with these mutant viruses also led to an ablation of reactivating virus from the spleen (Fig. 4..7D), not surprising based on intraperitoneal infection results, as well as previous data regarding 73.Stop intranasal infection (Fowler *et al.* 2003; Moorman *et al.* 2003).

D) DISCUSSION

Here we report that mLANA can function to repress transcription driven from the MHV68 TR via binding to a specific site within the TR. In addition, we report the development of a rapid method for generating and screening loss-of-function mLANA mutants, and have used this approach to identify and characterize mLANA mutants that have lost the capacity to repress transcription from the MHV68 TR. Finally, we have used this information to generate mLANA mutants in the context of the viral genome and shown that the role of mLANA in: (i) virus replication in permissive fibroblasts in vitro; (ii) regulation of viral gene expression during entry into the lytic cycle; and (iii) virus reactivation from latency, are all dependent on mLANA DNA binding. Indeed, all the mLANA mutants characterized which do not repress TR transcription exhibit a phenotype indistinguishable from the mLANA-null mutant virus. Thus, these studies on mLANA repression of transcription highlight the role of the mLANA DNA-binding domain and its function during viral infection.

The promoters and regulatory regions within the terminal repeat identified here are consistent with various elements previously mapped by others (Coleman *et al.* 2005b; Allen *et al.* 2006). There exist at least two promoters within the terminal repeats, 73p1 (Allen *et al.* 2006), which initiates transcription of exon 1 of gene 73, and an ORF75a promoter (which shares cis-elements with the 73p2 promoter and may also drive transcription of ORF73 in vivo (Husain *et al.* 1999; Coleman *et al.* 2005b)), identified during reactivation from infected splenocytes, as well as during de novo infection of fibroblasts. Results from our deletion analysis are consistent with data from these studies identifying a promoter driving a transcript initiating between bp 118683-118640.

Interestingly, deleting these coordinates did not ablate transcription from this area, but when we deleted the A/T-rich region between bp 118780-118738 (TR coordinates 327-369), luciferase activity was reduced to background levels, indicating that this region (-73 relative to the most 5' TSS) is critical for transcribing exon 1 of gene 73. The deletion analysis also confirms the existence of a second promoter which initiates a transcript between bp 119434-119429. Note that the latter sequence is homologous to bp 118221-118216 in the viral genome, which is within the short 27bp partial TR sequence at the righthand end of the MHV68 unique sequence immediately adjacent to the first complete copy of the TR, as defined by the sequence of the MHV68 WUMS sequence (Virgin *et al.* 1997) [Fig. 4..2; shown as transcription initiation site (ii)].

Importantly, all of the regions that appear to be involved in TR-driven reporter gene activity lie upstream of the mLANA-binding site(s), which were mapped at genomic coordinates 119169-119122. In this context, mLANA potently repressed reporter gene activity. KSHV LANA has been shown to bind to sequences in the KSHV TR and to have a similar negative effect on transcription from promoters placed near the TR in a reporter vector (Garber *et al.* 2001). Unlike the MHV68 TR, where one copy of the TR is sufficient to observe both promoter activity and its efficient repression by mLANA, the KSHV TR has not been shown to contain promoter activity on its own, and multiple copies of the TR are required to observe LANA repression of a heterologous promoter (Garber *et al.* 2001).

Consistent with our observation that mLANA can repress transcription arising from the TR, it is important to note that the presence of mLANA causes a downregulation of ORF73 transcript levels during virus infection in permissive fibroblasts, as detected by

RT-PCR of the ORF73 coding exon. Similarly, in a study of Rhesus rhadinovirus (RRV) LANA, many observed viral transcript levels, including ORF73, were elevated during infection with a LANA null RRV mutant (Wen *et al.* 2009). These studies are seemingly contrary to what has been observed with respect to the impact of KSHV LANA in transient transfection analyses, in which LANA has been shown to upregulate its promoter (Krithivas *et al.* 2000; Garber *et al.* 2001; Renne *et al.* 2001). However, in line with observations with MHV68 and RRV, a KSHV LANA null mutant virus does display hyperexpression of viral replication-associated genes, although the authors did not assess levels of LANA transcripts (Li *et al.* 2008b). Thus, the differences between the behavior of the LANA encoded by RRV and MHV68 and KSHV LANA may simply reflect a paucity of data regarding transcription of LANA during infection. Notably, with respect to this issue, there have not been any studies published demonstrating KSHV LANA transcripts initiating from the terminal repeats. Similarly, there is very little data on the MHV68 LANA promoter located in the unique region of the viral genome (Coleman *et al.* 2005b; Cheng *et al.* 2012). As such, this underscores a need for further careful analyses of the role of LANA homologs in regulating transcription of ORF73, both during the establishment of latency and acute virus replication.

DNA-binding domains have been identified in KSHV LANA (Garber *et al.* 2001; Garber *et al.* 2002; Srinivasan *et al.* 2004; Han *et al.* 2010) and EBV EBNA-1 (Rawlins *et al.* 1985; Ambinder *et al.* 1991; Inoue *et al.* 1991)—the latter appearing to be the functional homolog of the rhadinovirus LANA proteins. We have shown here that mLANA, like KSHV LANA and EBNA-1, physically interacts with DNA in a sequence-specific manner. It is of note that we identified this sequence using two distinct assays,

with both approaches identifying the same region of the MHV68 TR as being required for both mLANA DNA binding and repression of TR-driven transcription. Briefly, the TR deletion assay identified the DNA sequence between bp 1130 and 1174 of the TR NotI fragment as required for mLANA-dependent repression. DNase I footprinting analyses of both ends of this TR fragment identified a 48bp protected region that overlaps with the 44bp region identified from the analyses of TR-Luciferase deletions constructs. KSHV LANA footprinting of KSHV TR sequences shows a similarly-sized protected region (Garber *et al.* 2002). Further analysis showed that the large footprint was actually two distinct binding sequences, being occupied simultaneously by a LANA multimer (Garber *et al.* 2002). We suspect mLANA binds DNA in a similar fashion, however, further work will be needed to confirm this experimentally.

Notably, with respect to the mLANA DNA binding sites, there is some homology between the MHV68 mLANA TR binding sequence and one of the known KSHV LANA binding sites (LBS) (Cotter *et al.* 2001; Garber *et al.* 2002). KSHV LBS1 and the TR binding site share 14 of 16 nucleotides, although there is no significant homology between the MHV68 TR mLANA binding site and KSHV LBS2. However, despite the fact that the exact KSHV LBS1 or LBS2 sites are not present in the MHV68 TR, we observed that KSHV LANA was fully capable of repressing transcription initiated from the MHV68 TR.

The C-terminal portion of mLANA, which is the region with most sequence conservation between rhadinovirus LANA proteins, is predicted to have a tertiary structure similar to that of the EBV EBNA-1 DNA-binding domain. The homologous region of the KSHV LANA is also predicted to share this same similarity with the

EBNA-1 DNA binding domain (Garber *et al.* 2002; Grundhoff and Ganem 2003). This argues that studies on mLANA structure and function will likely be informative with respect to KSHV LANA function. Importantly, the majority of loss of repression mutants that were identified from the unbiased mutagenesis screen mapped to this region of mLANA. There has been discussion with respect to the EBNA-1 DNA-binding domain the contribution that the second and third helices (considered the "core" domain), and the first helix (considered a "flanking" domain) in sequence-specific DNA binding (Bochkarev *et al.* 1995; Bochkarev *et al.* 1996; Cruickshank *et al.* 2000). Here, we have identified from a random pool of mutants, loss-of-function mutations that fall within each of these three helices, indicating that all three helices are critical for the formation of a functional DNA-binding domain. Furthermore, our analysis of DNA binding using the mLANA mutants P179T (mutation in helix 2—thought to be in the core region) and L150P (mutation in helix 1—thought to be the flanking region) demonstrated that both mutant proteins failed to protect the mLANA binding site from cleavage by DNase I, further supporting the data obtained from TR-driven luciferase repression assay.

Knowing that the mLANA-null virus exhibits several phenotypes, including: (i) in vitro and in vivo replication defects; (ii) dysregulated viral gene expression; and (iii) failure to reactivate from latently-infected splenocytes (Moorman *et al.* 2003; Forrest *et al.* 2007; Paden *et al.* 2010), we tested whether these phenotypes were dependent on the transcription modulating/DNA binding function of mLANA. Importantly, all of the point-mutant virus analyses revealed that this domain of mLANA is critical for each of these phenotypes. One complicating factor may be that disrupting the DNA-binding capacity of mLANA could have an impact on other functions proposed for LANA homologues aside

from transcriptional regulation. For example, mLANA may be involved in viral DNA replication, as it, like EBNA-1, is proposed to be an origin binding protein with the capacity to recruit cellular DNA replication machinery (Goldsmith *et al.* 1993; Hu *et al.* 2002). However, it is unclear from studies of KSHV LANA whether this is required for virus replication and whether it functions in lytic and/or latent replication (Verma *et al.* 2006; Verma *et al.* 2007a). Alternatively, it may be that mLANA, which binds very strongly in the terminal repeat region and dimerizes, may facilitate circularization of the genome prior to rolling circle replication or episome formation. Indeed, we have previously reported that in vivo the 73.Stop virus established a life-long infection in mice, but that the viral genome fails to form episomes (Paden *et al.* 2010).

mLANA may be utilizing a variety of mechanisms to downregulate transcription, either directly or indirectly. It may directly interfere with transcription, either during the initiation phase or physically during the elongation of a nascent transcript. Another possibility is that mLANA interacts with and recruits proteins that shut off transcription through chromatin modification, similar to KSHV LANA which has been shown to interact with and recruit de novo methyltransferases (Shamay *et al.* 2006) and members of the mSin3 co-repressor complex(Krithivas *et al.* 2000). Give the high G+C content of the TR, the cellular transcriptional activator Sp1 may be required for efficient activation of transcription initiation from the TR. It has been shown in EBV that EBNA-1 displaces Sp1 from the TR (Sun *et al.* 1997), and it could be that mLANA performs a similar function—mLANA may displace Sp1, or some other cellular transcription-enhancing protein, from key sites, thereby downregulating transcription.

It is proposed that multiple TR copies may function as added attachment sites for strongly tethering the virus genome to the host chromosome via an interaction with host histone protein (Grundhoff and Ganem 2003; Barbera *et al.* 2004; You *et al.* 2006; Kelley-Clarke *et al.* 2007). In addition, we propose that multiple copies of the TR (and mLANA binding sites) allow mLANA to control transcription of the latency locus (genes 72, 73, and possibly others) during lytic and latent replication—perhaps before this region becomes methylated during latency (K.S. Gray and S.H. Speck, unpublished observation), which also shuts down transcription. This would be reminiscent to the regulation of EBV EBNA transcripts from the C and W promoters during the early phase of latency—viral promoters that are subsequently silenced by DNA methylation as EBV latency transitions to germinal center and then memory B cells (Jansson *et al.* 1992; Jin and Speck 1992; Tierney *et al.* 2000). It is likely very important to have tight, semi-redundant control over transcription of the latency locus—robust unchecked expression of this locus from KSHV leads to tumor formation in mice (Jeong *et al.* 2002; Fakhari *et al.* 2006) and is apparently required for growth transformation/immortalization of B cells by MHV68 (Liang *et al.* 2011). Ultimately, we do not yet have a sufficiently detailed understanding of transcription in this region of the viral genome to appropriately model how mLANA regulation of latency and lytic cycle-associated gene expression impact virus infection.

Finally, the random mutagenesis method we employed in this study may prove useful for developing treatments of gammaherpesvirus-associated diseases. Effective dominant negative forms of EBNA-1 have been identified (Kirchmaier and Sugden 1997), and small modifications to the mLANA mutagenesis protocol could result in

selectively targeting mutations to regions of mLANA other than the DNA binding domain. The latter may lead to the generation mLANA mutants that can occupy LANA binding sites without recruiting additional factors involved in modulating viral gene expression and subsequent cellular transformation. Small molecule inhibitors of EBNA-1 have recently been identified (Li *et al.* 2010; Thompson *et al.* 2010) and proposed as therapeutic for treatment of EBV-induced disease. Similarly, the screening approach described here has the capacity to rapidly determine the impact of a large number of individual compounds on mLANA function. We also have the advantage in studying MHV68 mLANA that we can test these strategies in vivo to directly assess effects on pathogenesis and disease. Given the apparent conservation of functions between mLANA and KSHV LANA, such studies could ultimately prove beneficial in the treatment of KSHV-associated diseases.

E) FIGURES AND LEGENDS

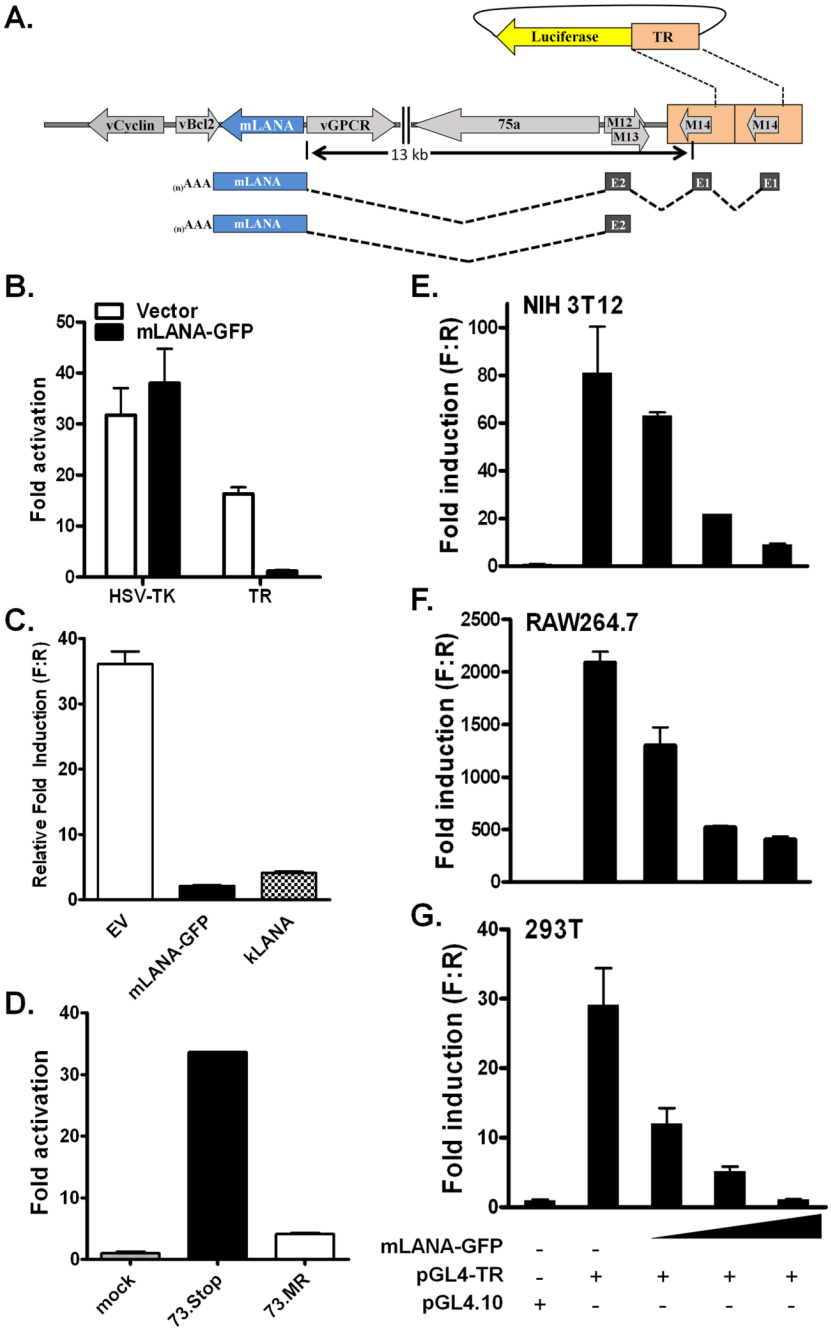

Figure 4.1. mLANA represses a promoter that initiates transcription within the MHV68 terminal repeat (TR).

Figure 4.1 (cont.) (A) Schematic illustration of mLANA gene transcription arising from 2 distinct promoters, one in the unique region adjacent the MHV68 TR, and the other within the TR. A firefly luciferase reporter construct was generated in which a single copy of the MHV68 TR (Not I fragment) was cloned upstream of the reporter gene. This reporter plasmid was used in the assays described in panels B to G. (B) A reporter vector containing one copy of the NotI fragment of the TR (pGL-TR) exhibits significant activation over background in HEK 293T cells. Co-transfection of an mLANA expression vector (pMSCV-73GFP) significantly represses the TR-associated promoter activity, but does not repress the HSV-TK promoter. (C) Co-transfecting an equivalent amount of KSHV LANA expression plasmid represses TR transcription as well as mLANA. (D) Infection with MHV68 further activates TR-driven luciferase activity 5-fold, and in infection with an mLANA-null virus (73.Stop), the TR-driven luciferase reporter construct is activated further still (33-fold), indicating a role for mLANA in controlling transcription from the TR during lytic infection. (E-G) The MHV68 TR promoter(s) is/are active in various cell lines and mLANA represses it in a dose-dependent manner. (E) NIH3T12 murine fibroblasts, (F) RAW264.7 murine macrophage, and (G) HEK 293T human epithelial cells were co-transfected with 100ng pGL-TR or the empty reporter vector (pGL4.10), along with 0, 5, 50, or 500ng of an pMSCV-73GFP, each balanced to a total of 500ng with empty expression vector (pMSCV-EFGP). Cell lysates were assayed for luciferase expression at 48h. The TR promoter(s) is/are active in each cell type, and activity is diminished, in a dose-dependent fashion, with the addition of mLANA expression. Each set is performed in triplicate, and data are presented a ratio

of normalized RLU (ratio of firefly to *Renilla* luciferase) over the empty expression vector (pGL4.10) normalized RLU.

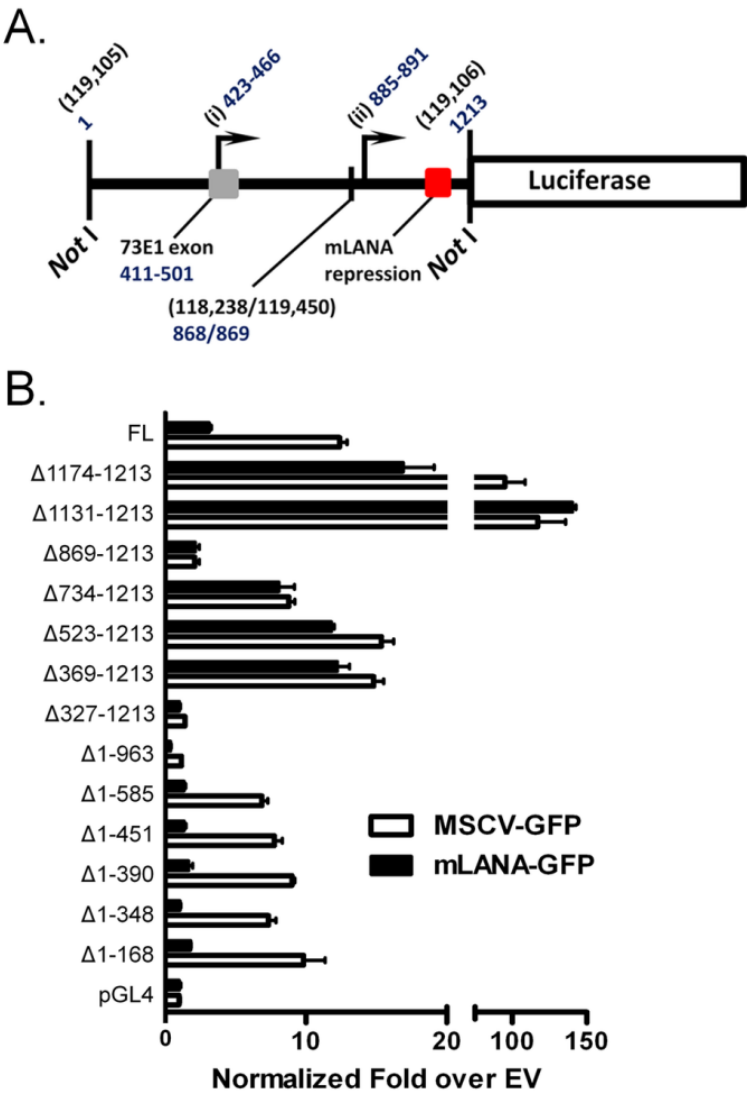

Figure 4.2. Deletion analysis of TR reveals DNA sequence required for mLANA-mediated repression.

Figure 4.2 (cont.) (A) Schematic of pGL-TR, noting the numbering scheme with regard to the NotI site. Coordinate 1 is corresponds to MHV68 WUMS (Virgin *et al.* 1997) bp 119,105, proceeding in order towards the right end of the unique sequence. Coordinate 894 is the end of one TR unit fused to the beginning of the next. Indicated on the diagram are two known sites of transcription initiation: (i) (73p1) starts transcription between coordinates 423-466 (118,683-118,640) depending on cell type (Coleman *et al.* 2005b; Allen *et al.* 2006) and the partial exon splices to the full 73E1 exon (118,695-118,605) in the next repeat unit; (ii) initiates transcription of one species of ORF75a beginning between coordinates 885-891 (118221-118216) (Coleman *et al.* 2005b) (promoter elements are likely shared between ORF75a and ORF73 transcripts that initiate at 73E2(Allen *et al.* 2006), and in distal copies may splice into 73E1). (B) Serial deletions of the TR were made in the pGL-TR vector by PCR and are named accordingly. Each TR deletion, including the full-length TR (FL) was co-transfected into 293T cells, along with either an mLANA-GFP or empty GFP expression vector, as in Figure 4.1, in triplicate. Here, data are normalized by taking the ratio of each deletion construct over its respective pGL4.10 empty reporter control, setting pGL4.10 in each case to 1. Deleting the 3' end gives a large uptick in activity, which is still repressed by mLANA, until the second deletion removes mLANA sensitivity. 5' deletions had no effect on mLANA sensitivity.

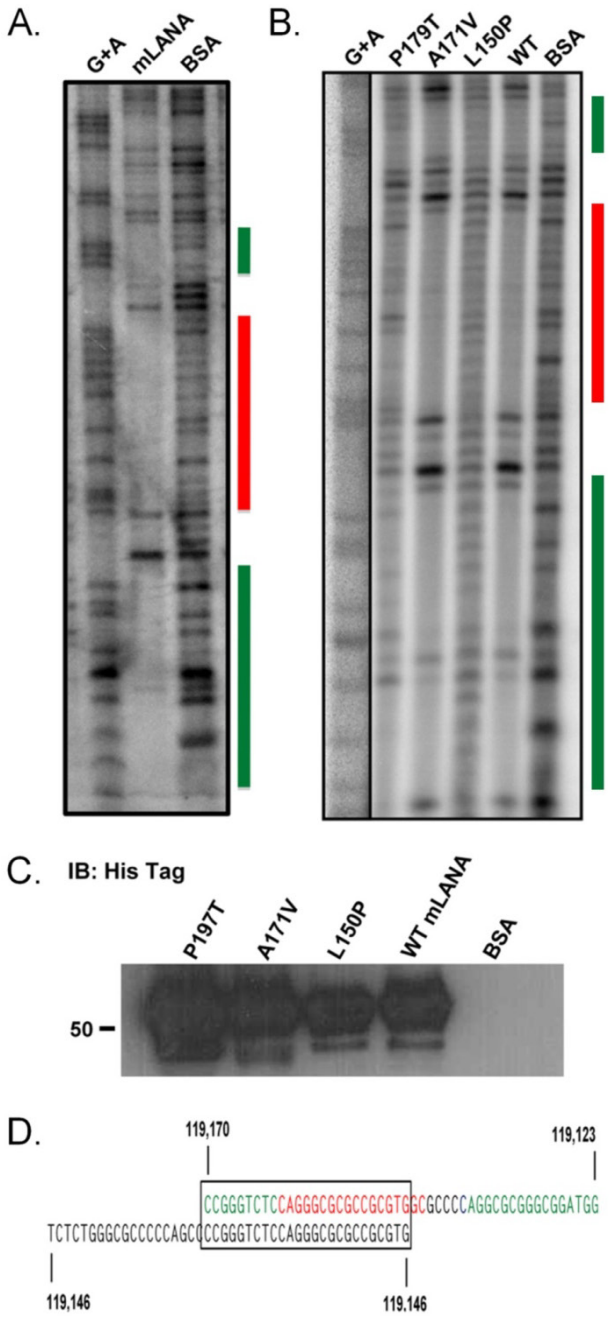

Figure 4.3. DNaseI footprinting of the terminal repeat with recombinant mLANA shows sequence-specific DNA binding.

Figure 4.3 (cont.) (A) After a binding reaction with the terminal repeat NotI fragment from pGL-TR, which was radiolabeled at the 3' end, and either 50µg purified mLANA-6XHis + 50µg BSA, or 100µg BSA alone, as a negative control, the protein/DNA mix was subjected to DNaseI digestion, and after DNA recovery, loaded onto an 8% denaturing polyacrylamide gel. The cluster of three protected regions is denoted by the colored bars to the side. This image is representative of several repeats, as well as the result of optimizing mLANA, BSA, and TR concentrations. (B) DNase I footprint analysis of the mLANA mutants P179T, A117V, and L150P, along with WT mLANA and BSA, performed as in (A). The mutants which do not repress transcription do not bind DNA. (C) Immonoblot of recombinant mLANA-6XHis affinity-purified from *E. coli.* Protein was run on an SDS-PAGE gel, transferred to nitrocellulose and probed with an anto-His tag HRP-conjugated antibody. (D) The sequence protected in the footprint analysis is shown here, deciphered using the G+A tracks and color coded, reflecting the colored bars in (A). Below the protected sequence in black is the sequence identified in Figure 4.2 as being required for mLANA-sensitivity. The box shows the regions that overlap, and the coordinates are genomic coordinates from GenBank U97553.

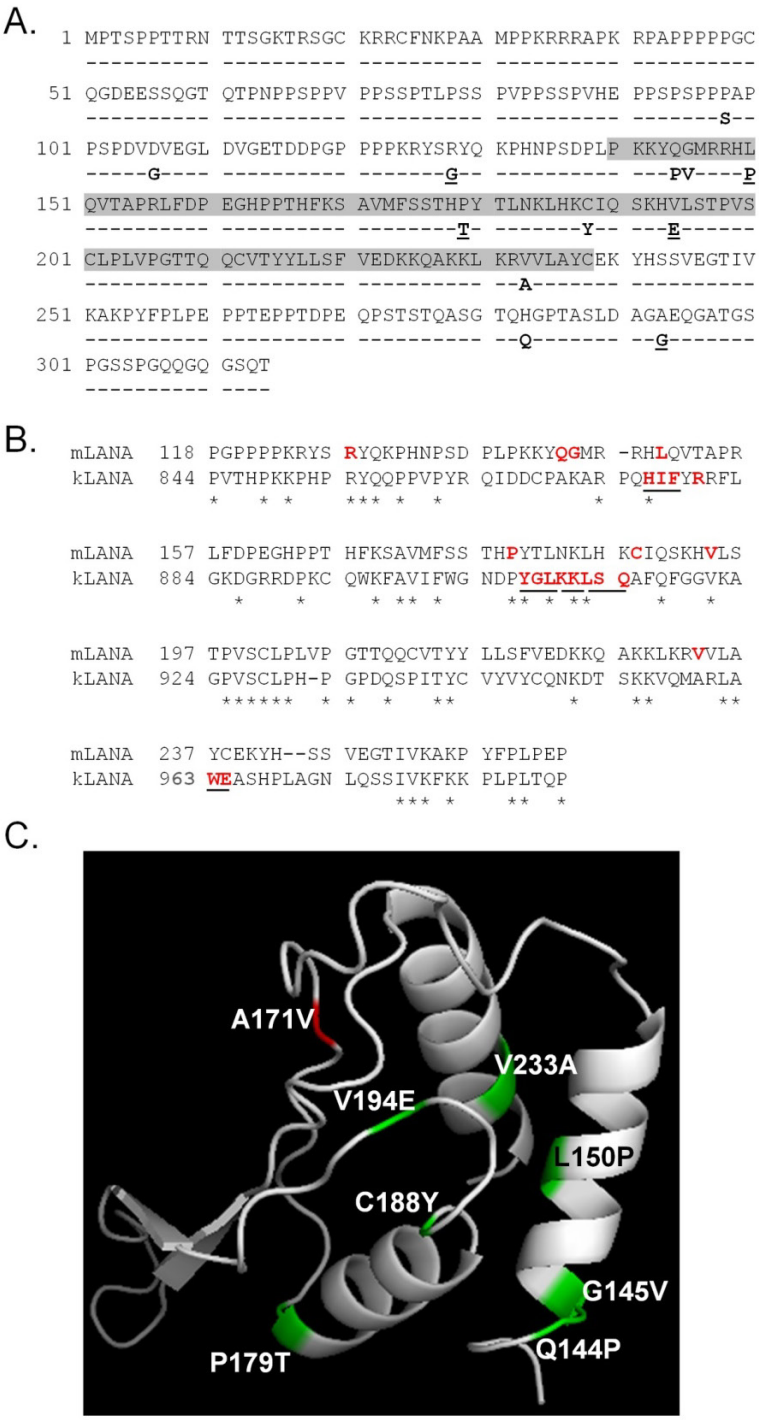

Figure 4.4. Random mutagenesis of mLANA reveals residues important for transcriptional repression.

Figure 4.4 (cont.) Loss of function mutants generated at random by error-prone PCR and defined by their failure to repress TR-luciferase reporter activity are shown here. (A) The mLANA primary amino acid sequence is shown, and underneath, each amino acid difference shown represents an amino acid changed in a mutant that had lost its capacity to repress transcription. Highlighted in grey is the region that has homology to other LANA proteins and is the domain that is predicted to fold like the EBV EBNA1 DNA binding domain, as shown in panel C. Underlined are mutations that were introduced into the MHV68-YFP BAC in subsequent experiments. (B) Sequence alignment of the conserved C-terminal domains of mLANA and KSHV LANA (kLANA). Shown in red are mutations in mLANA and kLANA that diminish LANA-mediated repression of transcription (those mutations introduced into kLANA that altered more than 1 residue are underlined). (C) The PHYRE protein structure prediction algorithm modeling of the conserved C-terminal domain (shaded in grey in panel A) of mLANA based on the solved structure of the EBNA-1 DNA-binding domain (Bochkarev *et al.* 1995; Kelley and Sternberg 2009). This depiction of the predicted three-dimensional structure of the mLANA conserved C-terminal domain has highlighted in green mutations that ablate the transcription repression function of mLANA. Also shown in red is a mutation, A171V, that does not impact mLANA transcriptional repression (has wild type phenotype) and was used in subsequent experiments as a negative control mLANA mutant.

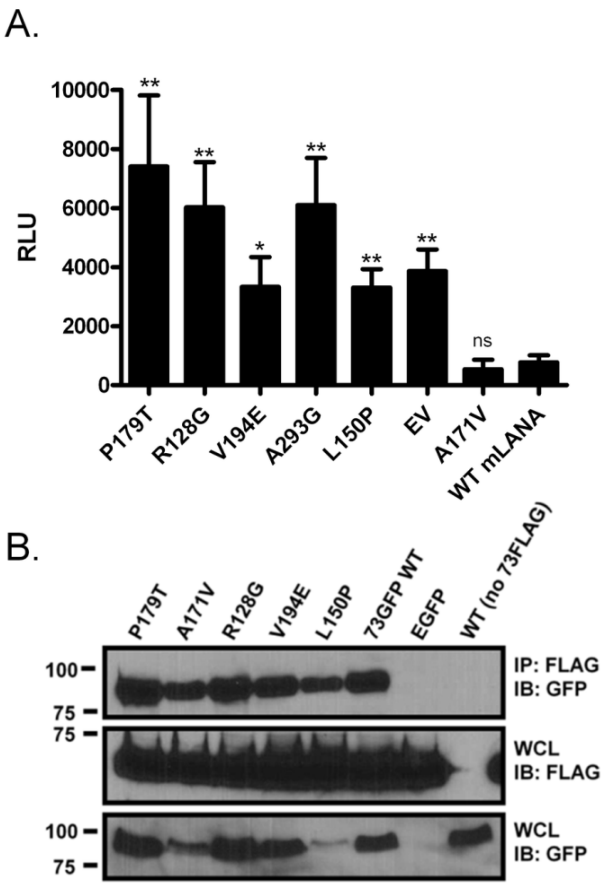

Figure 4.5. mLANA mutants lose transcription repression function, but not the capacity to dimerize. (A) Analysis of mLANA mutants that exhibited diminished repression of the MHV68 TR-driven luciferase activity. The mutants chosen, with the exception of the A171V mutant, have dimished capacity to repress transcription from the TR promoter(s). Data are expressed as relative light units. Comparisons to WT mLANA, with respect to the effectiveness of repressing transcription, were assessed using a student's t test: *, $P<0.05$; **, $P<0.01$; ns, not significant. Differences between each non-repressive mutant and empty vector (EV) were not significant ($P >0.05$).

Figure 4.5 (cont.) (B) Expression vectors for WT mLANA-3XFLAG, along with WT mLANA-GFP or the indicated mLANA-GFP mutants were cotransfected into 293T cells. Lysates were collected and co-immunoprecipitated using FLAG-agarose beads and the recovered complexes resolved by SDS-PAGE. Control pulldowns included EGFP with mLANA-3XFLAG and WT mLANA-GFP and the empty FLAG tag expression-vector, which fail to co-immunoprecipitate. Shown are anti-GFP blots for the pulldown and whole cell lysates, as well as an anti-FLAG blot for the whole cell lysates.

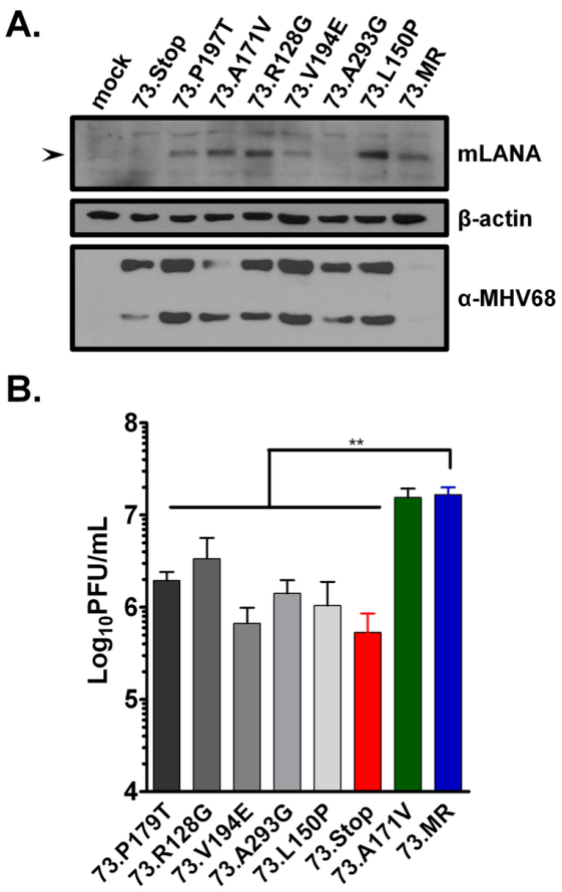

Figure 4.6. mLANA mutants that have lost the capacity to repress transcription from the MHV68 TR-driven luciferase reporter construct (MHV68-73TRN mutants) display similar growth kinetics and virus output as the mLANA null mutant virus (73.Stop virus).

Figure 4.6 (cont.) Six mLANA mutants were introduced into the MHV68 genome using BAC recombineering. (A) After infection of NIH3T3 fibroblasts with each MHV68-73TRN virus at an MOI of 3 PFU/mL, cell lysates were harvested at one and seven hours post-infection. The lysates were immunoblotted with rabbit mLANA antiserum, mouse MHV68 antiserum, and anti-β-actin antibodies. (B) NIH3T3 fibroblasts were infected at an MOI of 0.001 PFU/cell, and cells and supernatants were harvested at day 7 post-infection and titered on NIH3T12 cells. The resulting data shows that the MHV68-73TRN viruses grow to titers similar to that of 73.Stop, about 10-fold lower than the genetically-repaired 73galkMR virus. **, $P<0.01$. Panel A was generated by J.C. Forrest.

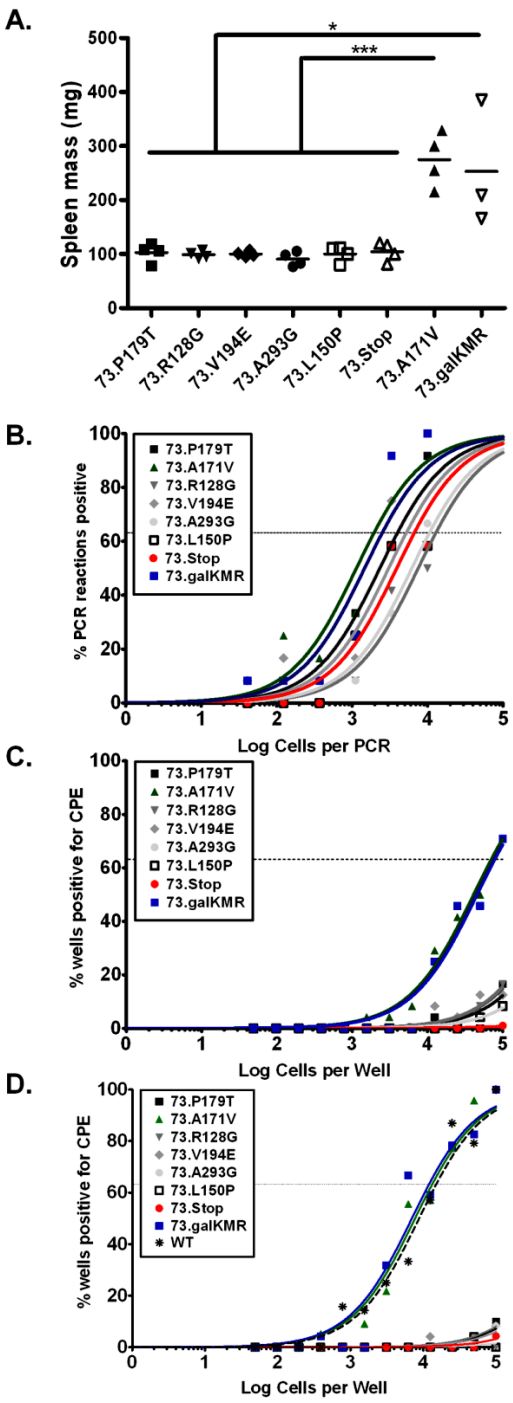

Figure 4.7. Intraperitoneal infection of C57Bl/6 mice with MHV68-73TRN mutants reveals mLANA DNA-binding/transcriptional repression function is essential for reactivation from splenocytes.

Figure 4.7 (cont.) Groups of 3-4 mice were infected intraperitoneally with 1000 PFU of each MHV68-73TRN virus. Eighteen days post-infection mice were sacrificed, and (A) spleens were weighed, (B) the frequency of infected splenocytes was determined by limiting-dilution PCR, and (C) the frequency of cells that reactivate virus was determined by the limiting-dilution CPE assay. In all cases the MHV68-73TRN viruses behaved similar to the mLANA null mutant 73.Stop. (D) Similarly, mice infected intranasally with 1000PFU each virus were assayed for cells reactivating virus 18 days post infection. *, $P<0.05$, ***, $P<0.001$.

F) TABLE

Clone	Mutation	Activity*	Dimerize
3C4	S86L/Q144P/S300P	-	nd
13C2	P98S	+	nd
2E4	D106G	-	nd
14A9	D106V	+	nd
3H8	R128G	-	nd
15G3	G145V	-	yes
16A10	L150P	-	yes
3D7	A171V/Q308P	-	yes
1C7	P179T/E260D	-	yes
1G5	C188Y/S273F	-	yes
4A6	V194E	+	yes
2C11	V233A	-	nd
16G4	H283Q	-	nd
16A3	A293G	-	nd
3C7b	Q144P	-	nd
1C7a	P179T	-	yes
1G5a	C188Y	-	nd
4H12	P72H	+++	nd
16F9	V70M	+++	yes
3H7	D111E	+++	nd
3D7a	A171V	+++	yes

Table 4.1. Repression activity of mLANA mutants in the MHV68 TR-driven luciferase reporter assay. * -, 0-20% repression; +, 20-50% repression; ++, 50-70% repression; +++, 80-100% repression

CHAPTER 5.

General Discussion and Future Possibilities

Understanding basic virology of gammaherpesvirus infection, and the interplay between virus and host, is of utmost importance in understanding the biology and etiology of gammaherpesvirus-associated disease. With decades of research on the human gammaherpesviruses KSHV and EBV in tissue culture, there are a wealth of theories regarding the functions of numerous viral proteins and their effects on the outcome of infection. However, due to the absence of appropriate animal models, most of these hypotheses remain largely untested. Neither research in EBV nor KSHV fields have made compelling strides in infecting non-human primates to study *in vivo* latency or the role of the immune system in regulating latency. Issues of species specificity, combined with the chronic, complex relationship a herpesvirus shares with its host—*tempus fugit; herpes manet*—make these questions compelling, but challenging to test using human herpesviruses.

MHV68 has been so far a powerful tool in deciphering the importance of gammaherpesvirus strategies and of particular viral genes in the life cycle and pathogenesis of gammaherpesviruses (Forrest *et al.* 2009; Barton *et al.* 2011). There are indeed significant differences between KSHV and MHV68, but as has been demonstrated repeatedly, basic mechanisms of persistence, seem to be largely the same. MHV68 is quite useful because it: (i) infects laboratory mice of myriad genotypes; (ii) is itself genetically malleable; (iii) replicates prodigiously in tissue culture; and (iv) has robust assays for quantifying latency and viral reactivation. However, like any study of pathogenesis, the outcome of such experiments can be quite complex and difficult to understand in terms of the molecular mechanisms involved. The body of work done thus

far in KSHV, EBV, and HVS tumor lines has been invaluable in helping to interpret the sometimes esoteric results of *in vivo* pathogenesis studies. Using the strengths of both cell culture-based human gammaherpesvirus studies and *in vivo* infection studies with the mouse virus helps to construct a more holistic and compelling understanding of how gammaherpesvirus pathogenesis works, both at a molecular and an organismal level.

When using mice and a mouse virus to understand the pathogenesis of a human virus in humans, it is critically important to allow human virus studies to inform the mouse virus studies and *vice versa.* Largely, with MHV68 and KSHV/EBV this has been the case. Some examples of this include studies of the conserved gammaherpesvirus gene ORF50, and the nonconserved MHV68 gene M2. ORF50 encodes a gene that has been known in KSHV to be absolutely essential for KSHV replication—it is the master transactivator for the next set of genes expressed in lytic replication, and is the same in MHV68 (Pavlova *et al.* 2003; Staudt and Dittmer 2007). MHV68 ORF50 was found to have multiple, context-sensitive promoters, subject to cell type-specific epigenetic regulation during normal infection (Gray *et al.* 2009; Gray *et al.* 2010). Using this knowledge, both KSHV ORF50 and EBV BRLF1 (an ORF50 homolog) were found to have very similar exon layouts (Gray *et al.* 2009). These studies were made possible in part because of the robust replication exhibited by MHV68 in tissue culture. Further, a virus expressing ORF50 constitutively as a transgene, which cannot establish latency, shows potential as a strategy for vaccination against secondary infection (Rickabaugh *et al.* 2004). Thus, with respect to the regulation of virus replication and reactivation, there appear to be well-conserved mechanisms that are shared between the rodent and human

gammaherpesviruses and, as such, the possibilities for applying the results from the mouse model to human disease and vaccination are very good.

The story for the MHV68 M2 gene, however, is somewhat more complex. M2, a unique MHV68 gene, was knocked out of the virus, and upon infection, it was observed that cells from M2-null-infected mice were severely hindered in reactivation. In addition, infected cells displayed a delay in transitioning from germinal center or naïve phenotype to memory phenotype (where both MHV68 and EBV establish latency) (Willer and Speck 2003; Herskowitz *et al.* 2005). It was also determined that primary murine B cells expressing M2 displayed higher rates of survival and proliferation (Siegel *et al.* 2008). EBV is known to encode a viral IL-10 (vIL-10), which has been shown to perform several of the functions ascribed to M2 in MHV68. Analysis from the M2-expressing cells showed increased levels of IL-10, which, in combination with intracellular M2, was required for the cells' proliferation, survival, and differentiation in culture (Siegel *et al.* 2008; Liang *et al.* 2009). Thus, despite the absence of a direct homolog of the vIL-10 gene, MHV68 employs a similar strategy, which results in signaling through the IL10R to facilitate cell survival and differentiation into cell types that support long term latency.

Analyses of LANA function have led to both expected and unexpected results. In Chapter 2, we describe an important role of mLANA in lytic replication, which was somewhat surprising, and which was unappreciated from studies on KSHV LANA, due to a lack of appropriate system for accurately measuring lytic replication. We observed a hyperlytic phenotype, but unlike in KSHV, we did not observe any effect of mLANA on the MHV68 ORF50 promoter. We expanded on the work of Friborg *et al*., who first identified the interaction of KSHV LANA with the tumor suppressor p53. Somewhat

surprisingly, we discovered that p53 is required for efficient replication, and in the absence of p53, the difference in replication between 73.Stop and 73.MR virus is abrogated. Stabilization of p53 actually activates the ORF50 promoter, likely leading to normal levels of virus replication.

This work led to interest in testing the importance of LANA in lytic replication of KSHV and RRV by other groups. It was found that for both these viruses that deletion of LANA causes a hyper-lytic phenotype, similar to that of MHV68, although some of that is attributed to ORF50 overexpression in the absence of LANA-dependent repression of ORF50 in those systems (Li *et al.* 2008b; Wen *et al.* 2009). Based on data from knockout viruses and cells from knockout mice, the study described in Chapter 2 argues that the situation is more complex. There is a DNA-damage response induced during infection that stabilizes p53. We hypothesize that mLANA blunts the p53-mediated cell death response while allowing for p53-dependent activation of replication, due, in part, to p53-dependent upregulation of ORF50.

Chapter 3 addresses issues regarding the involvement of mLANA in the maintenance of MHV68 latency. Though both Moorman *et al.* and Fowler *et al.* describe similar infection studies with mLANA-null viruses, there were some unresolved complicating issues, including the significant lytic replication deficit of the 73.Stop virus in the lungs. KSHV LANA had been thought to be necessary for replication of the viral episome and for separation of the episomes during cell division. Thus, it was assumed that in the absence of mLANA, latency would not be established, or that it would quickly recede. Both studies found no latency in the spleen after intranasal inoculation, which seemed at first to confirm the hypothesis. It is possible that a virus that replicates

inefficiently during the acute phase (or that does not reactivate to seed latency reservoirs) would have significant problems accessing the latency reservoir, complicating analysis of the role of mLANA in long term latency (Fowler *et al.* 2003; Moorman *et al.* 2003; Moser *et al.* 2005; Moser *et al.* 2006). Further, there is evidence for LANA-independent latent origins of replication (Verma *et al.* 2007a; Verma *et al.* 2011). Thus, we used intraperitoneal inoculation of virus to allow more direct access to the latency reservoirs and allow us to assess whether the mLANA-null virus can maintain latency. What we observed in this instance was complicated—not only did the virus establish latency, as determined by the presence of cells harboring the viral genome (as assessed by PCR), but the mLANA-null mutant virus (73.Stop) persisted in the infected host in the same frequency of cells as wild type MHV68. Further, the mLANA-null virus persisted in the spleen primarily in B cells, similar to wild type virus. However, we discovered that this virus was incapable of reactivating from splenocytes, and it was severely crippled for reactivation from peritoneal cells (likely macrophages). Interestingly, there is data to show that the frequency of latently infected cells that express mLANA is the same frequency of cells that reactivate from latency (Allen *et al.* 2006; Nealy *et al.* 2010). This led us to the idea that mLANA was either performing some function to prepare the cell for reactivation, or it is necessary in some way to set up latency in the first place.

To test whether the 73.Stop-infected cells harbored MHV68 episomes, we developed a new, sensitive method to test whether virus episomes are present. In the 73.Stop-infected mice, we failed to detect fused terminal repeats, while they were readily detected in 73.MR-infected animals. This was the first demonstration *in vivo* that LANA expression is correlated with episome persistence. The 73.Stop virus genome was,

however, still present, and at relatively the same frequency as the 73.MR genome. In addition, the same set of viral genes was being expressed by 73.Stop non-episomal virus genomes and 73.MR virus.

The fact that both viruses persist at the same frequency of infected cells long term was unexpected since it appears that are maintained by very different mechanisms (autonomously replicating episome vs. integrated/linear viral genome). This finding raises a few very interesting questions. First, what determines the "set point" for gammaherpesvirus infection? That is, is there some sort of sensing/counting mechanism? Is it immunologically based? Second, does this infection, though by a seemingly-stable, integrated virus, represent a subset of natural, chronic gammaherpesvirus infections? The 73.Stop virus is transctiptionally active and does appear to express the same viral transcripts as wild type MHV68. Is the physical status of the viral genome important to pathogenesis of disease? Notably, the alphaherpesvirus Marek's Disease Virus actually integrated into the host genome as a matter of course, and through a currently undefined mechanism is excised during virus reactivation from latency (Delecluse *et al.* 1993b; Morgan *et al.* 2001). The betaherpesvirus Human Herpesvirus-6 may also use integration and excision as a major mechanism of maintaining latency (Arbuckle *et al.* 2010; Kaufer *et al.* 2011). Finally, it is known that EBV frequently integrates into the host cell genome upon infection of cells in culture (Hurley *et al.* 1991; Delecluse *et al.* 1993a; Lestou *et al.* 1993). While it is unclear whether EBV integration is incidental or a natural part of latency, it is interesting to consider whether integration and excision is an evolutionarily relevant mechanism for maintaining herpesvirus genomes during infection. Epigenetic regulation of gene expression and latency is a well-known process in the

gammaherpesvirus field (Paulson and Speck 1999; Elliott *et al.* 2004; Miller *et al.* 2007; Pantry and Medveczky 2009). It is possible that maintaining the genome as an episome, through a viral protein dependent or independent manner, evolved as a mechanism to take advantage of epigenetic gene regulation in the virus, where it would not be influenced by surrounding host chromatin marks. Certainly also, an episome may also provide the virus a rapid platform for reentering the lytic cycle upon reactivation, omitting a time-consuming excision/repair step.

It is also of interest to consider mechanistic explanations of what mLANA is doing *in vivo* to "maintain the episome." Studied in HVS have shown that deletion the terminal repeat region (H-DNA) from the HVS genome results in a viral genome that neither replicate lytically nor undergo LANA-dependent latent replication (Collins *et al.* 2002; White *et al.* 2007). LANA binds the KSHV TR with high affinity and homodimerizes. Herpesvirus virion DNA is linear (Poffenberger and Roizman 1985), and upon entering the nucleus, initiates and blunts a DNA-damage response that seems to be necessary for replication (Kudoh *et al.* 2005; Tarakanova *et al.* 2007). We and others have shown mLANA is expressed with immediate-early kinetics. One hypothesis, taking this data together, is that LANA proteins assist in the circularization of the viral genome directly after *de novo* infection of a cell. That is, LANA, through homodimerization and DNA-protein interaction with the terminal repeats on either side of the virus, brings each opposite end of the genome in proximity during a DNA repair process. This may encourage the intramolecular interactions during DNA repair to facilitate circularization prior to either rolling circle lytic replication or episomal quiescence.

The data reported in Chapter 3 also raise the question of whether there are any viral genes required to establish and maintain latency. It is clear from our in vivo analyses that the mLANA-null virus is able to establish a long-term infection. Notably, every other MHV68 mutant that has been tested *in vivo* which establishes latency also ultimately persists at the same frequency of latently-infected cells long term (>42 days post infection) as WT MHV68. More broadly, does the mLANA-null phenotype of chronic infection in the apparent absence of virus reactivation reflect a biologically active form of gammaherpesvirus latency? Both ORF50.Stop and ORF6.Stop (ssDNAbp-null, which is an essential gene for lytic replication) viruses establish chronic infections in the absence of reactivation, with the latter shown to express viral latency transcripts; neither have the capacity to reactivate (Moser *et al.* 2006; Li *et al.* 2008a). While these viruses do not fit the currently accepted operational definition of latency ["a latent virus is one that must be induced to multiply and that does not exist in infectious form" (Roizman and Sears 1987)], they do have many of the characteristics of latency including latent gene expression, genome persistence, and reaching a setpoint in the frequency of infected cells.

In Chapter 4, we addressed the mechanism of mLANA repression of transcription. Using random mutagenesis coupled with a simple screen, we identified several single amino acid changes that could ablate mLANA-dependent transcriptional repression. The majority of these mutations fell within the relatively well-conserved C-terminal portion of mLANA. As discussed in Chapter 1, many of the proposed LANA protein-protein interactions that are thought to result in altered transcription map to the proline-rich N terminus of LANA. The conserved C-terminal domain of both KSHV

LANA and mLANA has predicted structural homology to the DNA-binding domain of EBV EBNA-1. As borne out in Chapter 4, mutations in the C-terminal domain of mLANA disrupt mLANA DNA binding, without affecting stability and homdimerization. Importantly, loss of mLANA DNA binding resulted in mLANA-null phenotypes in each assay examined, including hyperexpression of lytic genes, *in vitro* replication defect, failure to establish latency after intranasal inoculation of mice, and failure to reactivate from latency after intraperitoneal infection. Thus, we concluded that DNA-binding is essential for mLANA function in MHV68 pathogenesis.

An EBNA-1 deletion analysis beautifully delineated regions of the protein required for DNA replication, mitotic segregation, and transcriptional activation functions (Wu *et al.* 2002). We had originally hoped that by targeting a single function to knock out in mLANA, we would be able to observe separation of phenotypes to see which ones are linked to one another (i.e., whether the transcriptional repression phenotype is linked the mLANA role in acute virus replication, virus reactivation from latency, and/or establishment of latency). That turned out not to be the case—the DNA-binding function is required for every phenotype observed with the mLANA null mutant, save for the homodimerization function [notably, the latter is linked to DNA binding in EBNA-1 (Bochkarev *et al.* 1995)].

While somewhat disappointing at first, finding that mLANA DNA binding (as read out in our transcriptional repression assay) is linked to all the phenotypes we interrogated may prove to be useful information on learning how to treat KSHV-associated diseases. The screen for mLANA transcriptional repression is a very simple, rapid assay that can easily be made into a higher-throughput screen. The presence or

absence of the transcriptional repression function (attributed presumably to the ability to stably bind DNA), in our hands, seems to be an excellent predictor of mLANA functionality in pathogenesis. If this is indeed the case, it is very easy to imagine employing a similar assay to screen large banks of small molecule drugs to identify compounds that can inhibit KSHV LANA and/or mLANA function. Further work is needed to test whether LANA is necessary to maintain KSHV tumors after transformation and, if it is, anti-LANA drugs may be a powerful tool in combating these diseases.

MHV68 has the capacity to immortalize fetal liver cells (FLCs) and is thought to direct differentiation into mature, isotype-switched B cells. In efforts to define genes required for B cell immortalization, we attempted to immortalize FLCs with the 73.Stop virus with no success (Liang *et al.* 2011). This result was not particularly surprising, given the potent pro-proliferative effects KSHV LANA alone, as assessed in the LANA transgenic mouse B cells (Fakhari *et al.* 2006). However, the MHV68 *in vitro* immortalization process is thought to be quite inefficient, and to date we do not have a way of measuring efficiency. Thus, it is formally possible that 73.Stop can immortalize FLCs at a significantly lower efficiency that wild type MHV68. The latter has been observed with an EBNA1 null mutant (Humme *et al.* 2003). In the future, it will be interesting to test whether any of the mLANA DNA-binding mutant viruses have the capacity to immortalize FLCs. This sort of study will be particularly interesting if we can first separate the functions of genome maintenance and transcriptional repression with further mutagenesis studies.

Another important question relates to whether mLANA is required to maintain MHV68 immortalized B cell lines. To address this we will need to construct a virus where, once cells are immortalized, ORF73 can be excised (e.g., with Flp-recombinase) and observe whether or not they remain immortalized and retain the capacity to form tumors in mice. Thus far, experiments in KSHV tumor lines attempting to test whether or not LANA is required to maintain an immortalized or transformed state have been at best inconclusive (Corte-Real *et al.* 2005; Godfrey *et al.* 2005). Since we know that mLANA is not required for establishing a long term, stable infection, these experiments will help understand in a tumor or KS whether LANA is continually required and if LANA may be an appropriate target for therapy.

The work presented here has helped open up questions into the *in vivo* importance of LANA proteins in rhadinovirus infection. However, there remain significant differences to be resolved between MHV68 and KSHV LANA. First, what role does the acidic repeat of KSHV LANA play in human pathogenesis that is not necessary in the mouse virus? The repeat region has been shown to be necessary to prevent, in *cis*, peptide presentation on MHC Class I, presumably to prevent immune surveillance from deleting latently infected cells (Kwun *et al.* 2011). A tenuous study in MHV68 showed a similar function, but mLANA contains no repeat region (Bennett *et al.* 2005). Thus, this function may be executed by some other part of the protein, perhaps utilizing an entirely different mechanism than KSHV LANA. In addition, are there other functions carried out by the nonconserved regions of KSHV LANA, and if identified, are those functions also carried out by mLANA? Second, does mLANA facilitate replication in the same way that KSHV LANA does? To date, TR replication/episome maintenance assays have not been

developed for mLANA/MHV68 TR, as they have for KSHV and HVS. As such, it is currently not known whether mLANA utilizes the same mechanism(s). Efforts in our lab toward developing an mLANA-dependent episome replication assay have been unsuccessful to date. Third, what is the significance of the differences in transcription between MHV68 and KSHV LANA? MHV68 LANA is most prominently expressed via a promoter in the terminal repeat (Allen *et al.* 2006), and there is evidence of a proximal ORF73 promoter in reactivating MHV68-infected cells, similar to the inducible KSHV LANA promoter (Jeong *et al.* 2001; Cheng *et al.* 2012). However, to date no spliced LANA transcripts arising from the KSHV TR have been reported.

More broadly, questions arise from this work on the nature of latency and the role of LANA in infection and disease. In Chapter 3 we show that 73.Stop virus does not reactivate from splenocytes. However, though attenuated, we did observe reactivation of the LANA null mutant (73.Stop) virus from peritoneal macrophages. A major site of latency in PECs is in peritoneal macrophages (Weck *et al.* 1999b), and notably, *orf73* transcripts are not always detected in macrophages (Marques *et al.* 2003). Thus, it is worth considering how latency in macrophages may be different from latency in B cells, and whether there are different molecular requirements for gammaherpesvirus latency in non-dividing cells.

The work presented in this thesis has paired *in vitro* characterization of LANA gene function with *in vivo* pathogenesis studies in mice. The major conclusions of this work are: (i) mLANA is critical in acute replication and reactivation; (ii) mLANA can function as a transcriptional repressor; (iii) mLANA is a DNA binding protein; (iv) the MHV68 TR contains specific DNA sequences that constitute an mLANA binding site;

(v) the conserved C-terminal domain of mLANA contains many single residues that are critical for DNA binding; and (vi) mLANA mutants that have lost the ability to repress transcription/bind DNA exhibit the same phenotype *in vivo* as an mLANA-null mutant MHV68, suggesting that DNA binding is critical for the known functions of mLANA *in vivo*.

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