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Lyndsey Moore

Bcl-xL is required to protect endothelial cells latently infected with KSHV from virus induced intrinsic apoptosis

Lyndsey Moore

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Reading Committee:

Michael Lagunoff, Chair

Susan Fink

Adam Geballe

Program Authorized to Offer Degree:

Department of Microbiology

University of Washington

Abstract

Bcl-xL is required to protect endothelial cells latently infected with KSHV from virus induced intrinsic apoptosis

Lyndsey Moore

Chair of the Supervisory Committee:
Michael Lagunoff
Department of Microbiology

Kaposi's Sarcoma Herpesvirus (KSHV) is an oncogenic gammaherpesvirus that, like other herpesviruses, can establish both lytic and latent replication programs. It is the etiologic agent of Kaposi's Sarcoma (KS), Multicentric Castleman's Disease (MCD), and Primary Effusion Lymphoma (PEL). Within Kaposi's Sarcoma tumors, cells are predominantly latently infected. This poses a significant problem, as all current antivirals for herpesviruses target cells that are lytically infected, KSHV included. It is necessary to develop therapeutics that can target latently infected cells to effectively eradicate herpesvirus infections. Unfortunately, latent viruses are difficult to target since they do not produce infectious virions and they dramatically repress expression of viral genes. Targeting host cell requirement for latency, though, is a potentially viable course of action. Previously our lab performed a CRISPR/Cas9 essentiality screen in endothelial cells latently infected with KSHV. In this thesis work, I follow up on the most promising hit from that screen, Bcl-xL. Bcl-xL is an anti-apoptotic protein in the Bcl-2 protein family. The Bcl-2 family proteins are the master regulators of intrinsic apoptosis. I found that Bcl-xL is required for the survival of KSHV latently infected endothelial cells. Bcl-xL is needed

during KSHV infection to inhibit virus induced apoptosis by sequestering the pro-apoptotic pore former Bax. Bcl-xL, but not other anti-apoptotic Bcl-2 family proteins, is uniquely required because endothelial cells do not express additional anti-apoptotic proteins, such as Mcl-1 and Bcl-2, like other cell types. No other cell types were found to require Bcl-xL for survival during KSHV latent infection, likely due to the fact that they express several Bcl-2 family anti-apoptotic proteins. It is unlikely that the virus has evolved to induce apoptosis itself, rather it is more likely that the virus is inducing some host cell change that results in the activation of apoptosis. I found that the KSHV latent locus alone is sufficient to induce cell death in the absence of Bcl-xL. This led me to investigate if there was a specific KSHV latent gene that was inducing apoptosis during KSHV infection. I found that the kaposins, but no other KSHV latent genes or microRNAs (miRNAs), are necessary to render Bcl-xL necessary for survival. In addition to Bcl-xL, I sought to validate other top hits from the previous CRISPR/Cas9 screen. I examined the requirement of several genes for the survival and proliferation of KSHV latently infected cells, and determined that CYP27A1, a sterol hydroxylase, is needed for the proliferation of KSHV infected endothelial cells. Following the introduction and background (chapter 1) and materials and methods (chapter 2), chapter 3 details the validation of the top hit from our CRISPR/Cas9 screen, Bcl-xL. Chapter 4 describes how KSHV induces apoptosis during latent infection. In chapter 5, I validate the necessity of three other genes, *Cyp27a1*, *Ylpm1*, and *Cmklr1*, and determine that CYP27A1 is required for the proliferation of KSHV latently infected cells. Together this work identifies promising potential therapeutics for the specific treatment of Kaposi's Sarcoma, improves our understanding of how KSHV changes the host cell during latent infection, and provides insight into the accuracy of global CRISPR/Cas9 screens.

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DEDICATION

I dedicate this thesis to me, Diet Coke, and Wellbutrin.

Chapter 1

INTRODUCTION

1.1 Herpes Viruses

The *Herpesviridae* family has historically been defined by virion architecture. The typical herpesvirus virion has four components: (1) a core containing the large, linear double-stranded DNA genome, with size ranging from 124-295 kb, (2) an icosahedral capsid approximately 125 nm in diameter, (3) an amorphous proteinaceous region between the nucleocapsid and envelope known as the tegument, and (4) an envelope with viral glycoprotein spikes on the surface (1,2). Herpesviruses are highly disseminated in nature with most animal species having at least one herpesvirus. However, most herpesviruses only infect one species, thus they have a very limited host range. Members of the *Herpesviridae* share four significant biological properties. First, they have a large number of enzymes involved in nuclei acid metabolism, DNA synthesis, and processing of proteins. Second, synthesis of viral DNAs and capsid assembly occurs in the nucleus and final processing of the virion takes place in the cytoplasm. Third, the production of infectious progeny is always accompanied by destruction of the infected host cell. And fourth, the herpesviruses observed to date can remain latent in their natural hosts. Herpesviruses have been classified into three subfamilies based on biological properties. The *alphaherpesvirinae* are

classified by their variable host range, short reproductive cycles, rapid spread in culture, efficient destruction of the infected cell, and their capacity to establish latent infections primarily in sensory ganglia. *Betaherpesvirinae* have a more restricted host range. Their reproductive cycles are longer, and progression of infection is slow. The infected cells frequently become enlarged and carrier cultures, or passage of cultures with recoverable virus, are easily established. These viruses maintain latent infections in secretory glands, lymphoreticular cells, and kidneys. The *gammaherpesvirinae* have very restricted host range as they only infect their natural hosts. They are usually specific to B or T lymphocytes and latency is established in lymphoid tissues (1). The largest difference between gammaherpesviruses and the other subfamilies is their oncogenic capacity (3).

All members of the family have both latent and lytic replication schemes. Replication of all herpesviruses is a multi-step process. Following the onset of infection, DNA is uncoated and transported to the nucleus of the cell, which initiates viral gene transcription. There are four major groups of genes that are temporally regulated and follow a typical regulatory cascade. Lytic infection begins with expression of the immediate early (IE) genes, which require no new viral or cellular proteins to be synthesized for expression and these encode regulatory proteins. The transcription and translation of IE genes induce the expression of the early genes, whose transcription is independent of viral DNA synthesis. These genes encode enzymes for replicating viral DNA. Lastly are the leaky late genes whose expression is augmented by the onset of viral DNA synthesis and the true late genes whose expression is totally dependent upon viral DNA synthesis and encode the structural proteins (1). Assembly of the viral core and capsid takes place within the nucleus, which is then enveloped at the nuclear membrane. It is transported out of the nucleus to the endoplasmic reticulum and then to the golgi, where glycosylation of the

membrane occurs. Mature virions are then transported to the outer membrane of the host cell via vesicles, which is followed by virion release and cell death (2).

The herpesvirus biological cycle at the cellular level begins with the initiation of infection after which a decision will be made to either establish latency or undergo lytic replication. Lytic replication will lead to both host cell death and aid in cell-to-cell transmission of the virus. Latent infection allows for persistence within the host cell. The viral genomes are maintained as episomes, or circular genetic elements, that get replicated by host cell machinery. During latency only a small number of viral genes are expressed. Those viruses that go latent still retain the capacity to replicate and cause disease upon reactivation, but the mechanisms that lead to this reactivation are not fully understood.

To date, eight human herpesviruses have been discovered and have been given the nomenclature Human Herpesvirus (HHV) 1-8. The alphaherpesviruses include Herpes Simplex Virus 1 (HSV-1 or HHV-1) and Herpes Simplex Virus 2 (HSV-2 or HHV-2), both of which cause oral cold sores and genital sores. Varicella-Zoster Virus (VZV or HHV-3) is also an alphaherpesvirus and is responsible for chicken pox and shingles infections. The betaherpesviruses are Human Cytomegalovirus (HCMV or HHV-5), HHV-6, and HHV-7. HCMV infection can be problematic for children if acquired before or shortly after birth. It can be transmitted vertically and occurs in an estimated 0.6-6% of pregnancies depending on which population is sampled. When transmitted vertically HCMV can lead to a number of birth defects, such as neurocognitive impairment and microcephaly (4). HCMV is also the leading cause of sensorineural hearing loss in children (4). HCMV can also manifest as mononucleosis or cause serious disease in immunocompromised individuals. HHV-6 and HHV-7 are associated with roseola. Finally, the gammaherpesviruses are Epstein-Barr Virus (EBV or HHV-4) and Kaposi's

Sarcoma Herpesvirus (KSHV or HHV-8). EBV causes mononucleosis and Burkitt's Lymphoma (5), while KSHV is the etiologic agent of Kaposi's Sarcoma (KS) and two other B-cell malignancies, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

1.2 Kaposi's Sarcoma

Kaposi's Sarcoma (KS) was first described by Moritz Kaposi in 1872. He detailed several cases of a multifocal pigmented sarcoma of the skin in elderly European men, all of whom died shortly thereafter. Today, four main epidemiological forms of KS are recognized. Classical KS, the disease first observed by Moritz Kaposi, occurs in middle-aged and elderly individuals, with men from the Middle East, eastern Europe, and the Mediterranean being at higher risk. It is usually indolent and rarely disseminates or becomes aggressive. Classical KS is normally confined to the lower extremities. AIDS-related/epidemic KS is one of the most prevalent AIDS defining illnesses. Multiple cutaneous lesions occur on the face, trunk, and limbs and mucosal lesions are also common. Iatrogenic KS occurs in individuals with iatrogenic immunodeficiency, such as those who have undergone organ transplant. And lastly, endemic KS is found in central and southern Africa in those who are seronegative for HIV. Endemic KS is often very aggressive in children with widespread lymphadenopathy and visceral dissemination. In adults, disease progression is often indolent or more locally invasive (6).

The clinical manifestations of the different forms of KS have significant overlap. All forms of KS result in the formation of cutaneous lesions, which usually present as multiple, pigmented, raised or flat, painless lesions. These tumors are unlike classical tumors which arise from a single cell type. KS lesions are complex and composed of many different cell types. The main

proliferating agent is the *spindle cell*, named for its elongated shape. KS tumors also contain other cell types like T lymphocytes, macrophages, erythrocytes, monocytes, and dendritic cells. The spindle cells express markers of the endothelium such as CD31 and CD34 and much work has been done to pinpoint the spindle cell origin. Endothelial cells are divided into blood vascular and lymphatic endothelial cells, which are related but distinct cell types. Spindle cells have gene expression profiles that more closely match that of lymphatic endothelial cells, but they also express blood vascular endothelial and mesenchymal cell markers(7–9) . More recent work has identified endothelial colony forming cells (ECFCs) as potential progenitors of the spindle cell (10).

The cause of KS was not discovered until 1994. After the first description of KS in 1872, KS went largely undescribed until the 1950s when a large number of cases started being reported in Africa, which would later be described as endemic KS. In the 1960s, KS emerged in immunosuppressed individuals following organ transplant. KS was most prominently talked about in the 1980s when there was a large increase in KS cases that were concomitant with the AIDS epidemic. In the United States, KS was 20,000 times more frequent in patients with AIDS than the general population (6). An epidemiological examination in 1990 found HIV was not the direct cause of KS. The KS agent also had low general prevalence in Europe and North America, but was common in some geographic regions, like sub-Saharan Africa. In the US, the KS agent was apparently sexually transmitted among gay and bisexual men, but unlike HIV there was low risk for parental transmission. The geographic variability and high occurrence of AIDS-KS in homosexual men suggested the evidence of a unique “KS infectious agent” (11). In 1994 Chang *et al* amplified two novel DNA sequences from an AIDS-associated KS lesion. Observation of DNA sequences absent from uninfected tissue displayed homology to herpesvirus capsid and

tegument genes. They were ultimately able to achieve complete sequencing of the viral genome, thus KSHV was identified using molecular techniques (12). This combined with the epidemiological evidence that KSHV DNA was always found in KS tumors, KSHV infection tracked strongly with KSHV risk, and infection with KSHV preceded the onset of KS led to the conclusion that KSHV is the etiologic agent of KS.

1.3 Primary Effusion Lymphoma and Multicentric Castleman's Disease

KSHV is also associated with two other proliferative disorders. Multicentric Castleman's Disease (MCD) is an aggressive and rare B-cell polyclonal lymphoproliferative disorder that tends to affect multiple organ systems. It commonly arises in HIV-infected individuals or those who are immunocompromised. MCD presents with intermittent inflammatory symptoms (fever, night sweats, weight loss, fatigue, GI symptoms), anemia, splenomegaly, and swollen lymph nodes. It weakens the body's immune system and increases the risk of developing lymphoma. MCD can also progress to failure of multiple organs and is lethal if untreated. KSHV-MCD can occur alone, but it can also occur concurrent with KS or PEL or both (13). There are no MCD specific treatments; treatment regiments consist of antiretroviral therapies and immune-chemotherapy (14,15). In patients with MCD, about 50% are KSHV+ while the cause is unknown in the other half (16).

Primary effusion lymphoma (PEL) is a rare and aggressive monoclonal B-cell lymphoma. It also commonly affects those who are immunocompromised, particularly those with HIV, and predominantly occurs within body cavities leading to the development of malignant effusions. PEL can affect the pleural, peritoneal, or pericardial spaces and specific symptoms will depend on which body cavity is affected. PEL was initially described during the AIDS epidemic, and it

was later discovered that KSHV was the etiologic agent of PEL. Additionally, about 80% of individuals with PEL are also coinfected with EBV, another oncogenic herpesvirus. Prognosis after diagnosis with PEL is poor as there are no definitive guidelines for treatment, but options include chemotherapy and other drug cocktails used for aggressive lymphomas (17,18).

1.4 KSHV

KSHV is a double-stranded linear DNA virus with a genome size of 165 kb (19,20). The genome has a large unique central region containing all the viral open reading frames (ORFs) flanked by highly GC-rich direct terminal repeats. Most isolates have 20-25 kb of total terminal repeats per genome, but the number of repeats at each terminus can vary. It has an enveloped icosahedral capsid surrounded by tegument which is typical of a herpesvirus. The envelope is studded with glycoproteins that are responsible for initial host-virus interactions. The glycoproteins K8.1A, glycoprotein-B (gB), and the heterodimer of glycoproteins-H and -L (gHgL) are thought to be most important for viral entry (21).

KSHV infection begins with the binding of the viral envelope glycoproteins to cellular receptors, specifically heparan sulfate (HS). Additional receptors required for tighter binding and membrane fusion include multiple integrins and Eph receptors, cystine/glutamate antiporter (xCT), and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). It is this diverse range of potential binding partners and entry sites that gives KSHV its broad cell tropism (22). A membrane fusion event occurs next which delivers the capsid and tegument into the cytosol of the host cell. The capsid is then transported to the nuclear envelope where the viral genome is delivered to the nucleus. Once inside the nucleus, the viral genome is quickly circularized, forming an episome. After this, the virus will enter either the latent or lytic

transcriptional program. For KSHV, the default program in cell culture is latency with greater than 90% of infected cells establishing latency. Most of these observations have been made using *in vitro* studies, but 24-48 hours post infection, most cells display gene expression profiles and characteristics of latently infected cells. However, before latency is established, cells will transiently express some lytic genes, but their expression will subside by 24 hours post infection. While the latency program dominates cells infected in culture, there will be a small percentage of cells lytically active, about 1-3% (20). In KS spindle cells, cells are also predominantly latently infected with 1-5% of cells supporting lytic infection. The same is true for PELs (23).

During latency, the full viral genome will be retained within the host cell, but viral gene expression is dramatically reduced. Latency must be both persistent and reversible. There is no general mechanism for how viruses establish and maintain latency, but in general, viral gene products that promote virus reproduction will not be made, cells carrying latent genomes will be poorly recognized by the host immune system, and the viral genome will persist so productive infection can be reinitiated at a later time.

During KSHV latency, there is one major latent locus that is expressed. The latent locus consists of the latency associated nuclear antigen (LANA), a viral cyclin (vCyc), a viral FLICE inhibitory protein (vFLIP), the kaposin locus expressing a family of proteins using different start sites, and 12 miRNA loci expressing 25 mature miRNAs. Two major latency promoters exist and through alternative splicing lead to the expression of LANA, v-cyc, and v-FLIP. The kaposins are expressed through a separate promoter and the miRNAs are expressed by the processing of other latent transcripts (20,24,25).

The major difference in gene expression during latency is the expression of the LANA2/vIRF3 gene in PEL and MCD cells, whereas this protein is not expressed in KS tumor

cells or cultured endothelial cells. The expression of LANA2 appears to be lymphoid specific (23). vIRF3 associates with other interferon-regulatory factor (IRF) proteins and is able to modulate the innate antiviral response by interfering with their functions. The vIRF3 protein has also been shown to interact with IKK where it impaired translocation of NF-κB to the nucleus and reduced NF-κB-mediated transcription (26).

LANA is a large multifunctional protein that is found in the nucleus of infected cells as punctate foci. Its primary function is to establish and maintain the nuclear latent genome, but it is also involved in dysregulation of cell growth and survival, inhibition of apoptosis, and repression of viral lytic genes. LANA binds directly to the conserved terminal repeat sequences through its C-terminal domain and tethers the viral genome to the host chromosome. This facilitates the latent viral episomes being divided into each daughter cell when the host cell divides. LANA also binds other regions of the KSHV genome. It directly binds to several epigenetic regulatory proteins, like topoisomerases. The LANA protein also binds to several lytic promoters to inhibit viral lytic gene transcription, thus helping maintain latency (27). LANA has also been found to interact with several cellular proteins, like the tumor suppressor p53 and cell cycle protein pRb and many other proteins (28).

V-cyclin is a homologue of cellular cyclin D. It binds and activates CDK6 to regulate cell cycle progression to sustain cell proliferation. The v-cyclin also mediates the phosphorylation of pRb and p27. Work has also been done that shows v-cyclin is required for KSHV-induced bypass of senescence in LECs, suggesting it is also a critical oncogene for KS tumor development (29).

V-FLIP upregulates the anti-apoptotic NF-κB pathway, a key cellular survival pathway, to promote cell survival and proliferation. It activates the pathway by binding to the inhibitor of kB-

kinase γ (IKK γ). The induction of the NF-κB pathway is important for both viral latency and oncogenesis. Activation of the NF-κB pathway has been linked to KSHV lytic replication because a KSHV Δv-FLIP mutant inhibited expression of RTA. Enhanced activation of the pathway could also be important for the transformation and oncogenic potential of v-FLIP as demonstrated in Rat-1 fibroblasts. V-FLIP expression has also been shown to induce anoikis, which is detachment induced apoptosis.

The kaposins are less well understood, but the kaposin locus encodes three proteins, Kaposin A, B, and C, which are also highly upregulated during lytic infection. Not much is known about the individual kaposins, but Kaposin A has been shown to be capable of transforming Rat-3 fibroblasts, thus demonstrating oncogenic potential and Kaposin B has been shown to activate the p38/MAPK signaling pathway and enhance the stability of Prox1, a regulator of endothelial differentiation. Overall, the kaposins are thought to contribute to the pro-inflammatory KS tumor microenvironment (28,30).

KSHV encodes 12 pre-miRNAs. miRNAs are a group of non-coding small single-stranded RNAs, about 19-22 nucleotides long. The primary function of miRNAs is inhibition of target gene expression by binding to complementary regions on the target gene. They typically bind the 3' UTR and modulate expression post-transcriptionally. miRNAs are promiscuous and alter expression of hundreds to thousands of genes. Additionally, the same gene can be targeted by multiple miRNAs. KSHV miRNAs play significant roles during infection and the development of KSHV-associated diseases. All 12 miRNAs are expressed during latent infection and pre-miR-K10 and pre-miR-K12 are known to be induced during lytic infection. The levels and activities of the miRNAs vary, and even more so between different cell types. Expression of the miRNAs will vary with different phases of the viral life cycle and their expression is highly

regulated. Much work has been done to identify targets of the KSHV miRNAs, but much is still unknown (25).

1.5 KSHV Culture Systems

To date, there are no robust or reliable animal models of KSHV infection that mimic symptoms and pathology observed in humans. For this reason, KSHV is primarily studied using *in vitro* cell culture systems. There are several cell lines and systems that have been developed to study KSHV. The first successful cultivation of KSHV was in PEL cell lines taken from patients with advanced AIDS. These cells readily grow in cell culture, unlike many other lymphomas. In these PEL cell lines, the majority of cells are latently infected, but 1-5% of the cells are lytically active (20). The B cell lines derived from these PEL patients include BCBL-1s, BC-1s, JSC-1s, and BC-3s among others. These cell lines stably maintain the KSHV genome and serve as a model for KSHV lytic reactivation (31). BJABs are also sometimes used as they are KSHV negative control lymphoma cell line. However, there are two major drawbacks to using these cells for the study of KS. First, KS is an endothelial cell-based tumor, so these B cells are not the most representative cell type to study. Second, since these cells are already infected with KSHV, *de novo* infection cannot be studied.

Our lab has developed a model for studying KSHV using immortalized endothelial cells. TIME (tert-immortalized microvascular endothelial) cells were first made by Venetsanakos et al. by transducing dermal microvascular endothelial cells with a retrovirus expressing hTERT. These TIME cells grow continuously in culture and maintain many properties of primary endothelial cells (32). When *de novo* infected with KSHV, TIME cells can reach infection levels that reflect what is observed in KS tumors (33). TIME cells are still an immortalized cell line

though, so while they are a useful model, their relevance can be limited. Because of this, primary cell lines can also be used. Blood endothelial (BECs) and lymphatic endothelial (LECs) cells are often used to study KSHV and KS.

An additional cell line that should be recognized is the iSLK line, which was originally reported to be isolated from KS tumor samples (34). However, short tandem repeat profiling revealed iSLK cells to be indistinguishable from renal-cell carcinoma cells. Since these cells are not of endothelial origin, their use as a model for KSHV has dropped off (35). However, these iSLK cells are still a valuable tool to produce recombinant viruses and study lytic replication (36).

Cell culture models have been used to show KSHV alterations of host cell biology and virus host interactions. For example, cell culture systems have extensively been used to study angiogenesis and various signaling pathways. They have also been used to identify cellular pathways essential for KSHV latency, like glycolysis and fatty acid synthesis (37). A key tool in studying these host cell changes and molecular interactions is gene editing using CRISPR/Cas9 systems.

1.6 CRISPR/Cas9 Screening

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated endonuclease 9 (Cas9) protein was first reported in 2012. The targeted DNA cleaving mechanism was made by taking CRISPR RNAs (crRNAs) and integrating them with synthetic trans-activating crRNA (tracrRNA) to form single-guide RNAs (sgRNAs). The CRISPR/Cas9 system has two major components: 1) the sgRNA, which is a specific RNA sequence that recognizes the target DNA region of interest and directs the Cas protein to that site and 2) the

Cas9 protein, which is an endonuclease that binds and cuts DNA. The Cas9-sgRNA complex cuts DNA at the region of interest and inserts or deletes DNA sequences, known as indels. These indels lead to the target gene being shut off (38). This CRISPR/Cas9 system is a convenient, rapid, and versatile genome editing technology that can be used for library screening to identify essential genes under various forms of positive or negative selection.

CRISPR/Cas9 has been used extensively to knockout genes in mammalian culture systems. An *in vivo* genome wide CRISPR screen in SUM159s, a triple-negative breast cancer cell line, was able to identify breast cancer vulnerabilities (39). Another genome-wide screen in human mesenchymal precursor cells (hMPCs) was able to identify a specific gene driving cellular senescence (40). And a CRISPR/Cas9 screen in human colon organoids identified tumor suppressors related to colorectal cancer (41).

To date, there are no KSHV specific treatments that are effective against KS tumors. All current drug treatments only target lytically active cells, making them ineffective for diseases characterized by latent infection. A direct treatment for KSHV is difficult due to the limited gene expression and lack of viral targets, however, targeting specific pathologic changes to the host cell during latent infection presents viable treatment option. A global CRISPR/Cas9 essentiality screen is a viable method for identifying host genes necessary for KSHV infection.

A global CRISPR/Cas9 screen was performed in PEL cells to determine genes essential for the survival of those lymphoma cells. Manzano *et al.* used eight different PEL cell lines and identified over 200 genes as PEL-specific oncogenic dependencies. The genetic requirements for PELs were also heavily dependent upon co-infection with EBV. They demonstrated the requirements for IRF4 and MDM2. Further, they found PEL cells were addicted to high levels of MCL1 expression (42). However, this screen could not differentiate genes necessary for

lymphoma survival in general to those necessary for the survival of KSHV latency as there is no uninfected control for PEL cell lines.

Our lab performed a global CRISPR/Cas9 essentiality screen in KSHV latently infected endothelial cells. We used half of the Human Activity-Optimized CRISPR-Cas9 lentivirus library to generate a mutant pool of cells containing sgRNAs and Cas9-expressing lentiviruses targeting 18,166 human genes with 5 sgRNAs each. TIME cells were transduced with this library and the mock or KSHV infected. Dead cells were collected each time cells were passaged and live cells were harvested 8 days post-infection. The genomic DNA was harvested from the live and dead cell populations and sequenced. MAGeCK analysis was used to identify changes in representation of guides targeting different genes between the infected and uninfected samples. This global screen identified 146 genes that were significantly depleted from the live cell population using a false discovery rate of 0.25. It also identified 1,600 genes that were enriched by more than 2.75-fold in the dead cell screen (43). A screen of this magnitude would be extremely laborious to repeat, so a sub-pool screen was performed to validate the original global screen. The sub-pool screen was performed in duplicate, and this time 13 sgRNAs were used to target the top 350 hits from the original live cell screen, the top 350 hits from the dead cell screen, 100 genes that were unchanged in the live and dead cell screens, and 500 non-targeting control sgRNAs. Many hits from the original screen were similarly depleted in this more extensive sub-pool screen (24). There was not complete correlation between the two sub-pool replicates, but there were a number of genes that were depleted in both replicates (Fig. 1.1). The top hit was Bcl-xL, an anti-apoptotic protein.

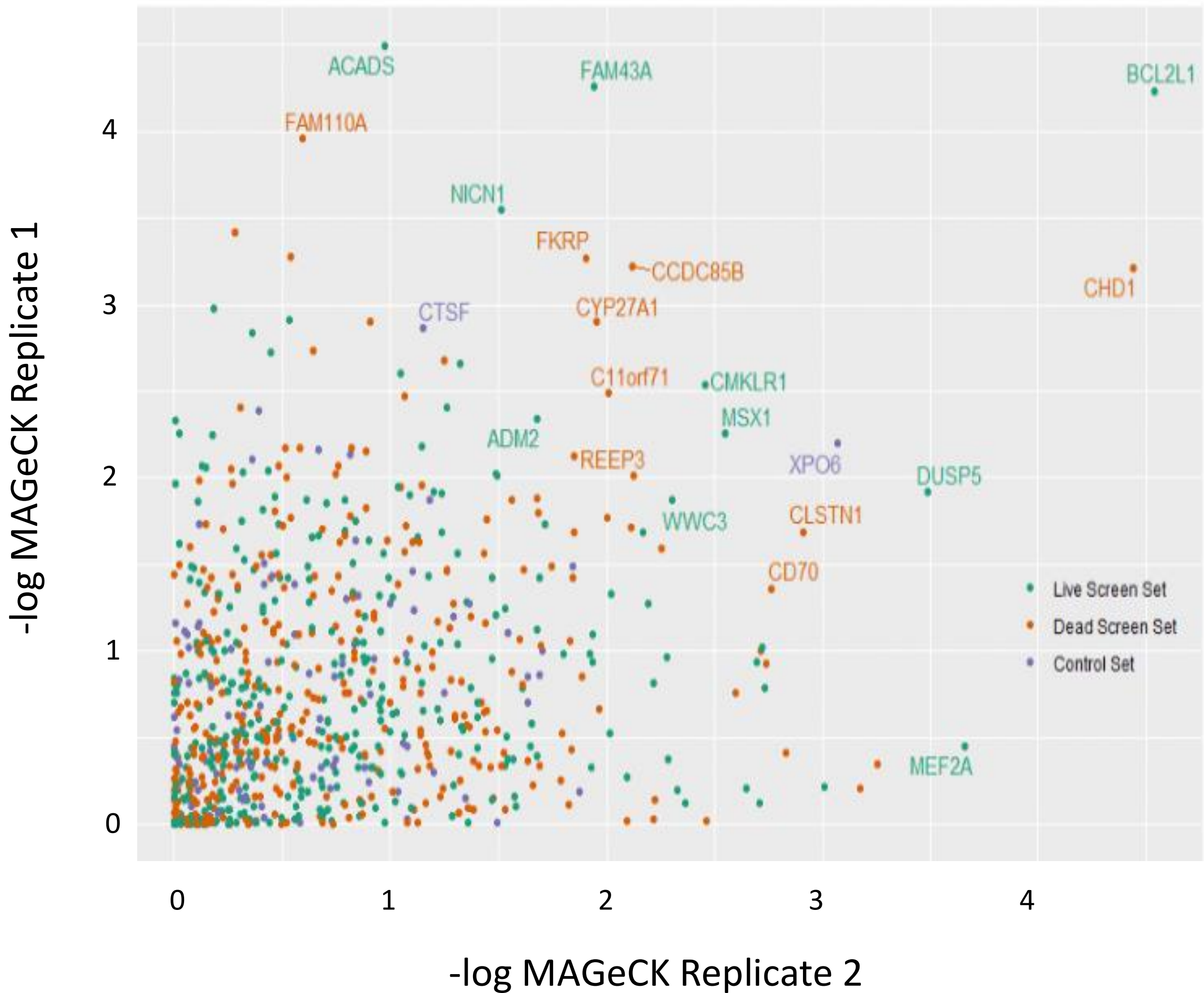


Figure 1.1: CRISPR/Cas9 Screening for Host Genes Required for KSHV latency: Each axis represents -log MAGeCK scores from an independent experiment comparing the mock population to the KSHV infected population for each screen. Each point represents an individual gene score. Points are colored based on the source of the target gene, with green being from the whole genome live cell screen, orange from the whole genome dead cell screen, and blue from the unchanged control gene set.

1.7 Cell Death

Cell death is a critical and active process needed to maintain homeostasis and eliminate potential threats in organisms. Historically, cell death was divided into three major morphologically distinct types of cell death: 1) apoptosis, 2) autophagic cell death, and 3) necrosis. However, in more recent years, more cell death modalities have been discovered and the types of cell death have expanded. Overall, cell death processes can be described as programmed or non-programmed. Programmed cell death (PCD) is driven by tightly regulated intracellular signaling pathways. Alternatively, accidental cell death, or non-PCD, occurs as a result of cell injury. PCD can be further subdivided into apoptotic versus non-apoptotic cell death. Apoptosis keeps the cell membrane intact and is dependent on caspases. Non-apoptotic cell death will be characterized by membrane rupture and caspase-independency (44).

1.7.1 Non-Programmed Cell Death

Necrosis is characterized by cell swelling, plasma membrane rupture, and a loss of organellar structure. It is mediated by interference with cell energy supply and damage to the cell membrane. This irreparable cell damage can be caused by things like freeze-thawing or mechanical damage. This form of necrosis is energy independent and leaves the cell a passive victim. It tends to be uncontrolled and will affect large fields of cells at one time. The rupture of the membrane during necrosis releases chemotactic signals that will recruit inflammatory cells.

Necrosis can be detected by looking for lactate dehydrogenase (LDH) activity, an enzyme that will be released from the cell upon necrosis. Membrane integrity loss can be observed using cell-impermeable DNA dyes.

1.7.2 Programmed Apoptotic Cell Death

Apoptosis is a vital and normal component of development, aging, and maintaining homeostasis in cell populations, but it can also be used as a defense mechanism. Apoptosis is characterized by cell shrinkage, membrane blebbing, and pyknosis (condensation of the chromatin). As the cell undergoes apoptosis it will form apoptotic bodies in a process known as budding. It is these apoptotic bodies that are phagocytosed by other cells. Importantly, there is essentially no inflammatory reaction associated with apoptosis because apoptotic cells do not release their constituents into surrounding tissue, and they are quicky engulfed by phagocytic cells that do not produce any inflammatory cytokines themselves (45). Worth mentioning, anoikis is a specific type of apoptosis and it shares the same pathways. The major difference between apoptosis and anoikis is that anoikis is triggered by dysregulated cell-matrix interactions.

Many of the genes involved in apoptosis have been identified and the apoptotic mechanisms are highly conserved. Apoptosis is generally considered irreversible with the activation of caspases committing a cell to death. But there are arguments for it being reversible (45). Tang et al. describe apoptotic cancer cells that were able to regain their normal morphology and proliferate when apoptotic inducers were removed (46). And there is evidence that early p53-induced apoptotic cells can be rescued from death through DNA repair (47).

Apoptotic cells have several distinct biochemical features. Caspases, which are widely expressed in their inactive proenzyme form, are endoproteases activated during apoptosis. Once activated, caspases can activate other caspases and they can also autoactivate, thus initiating a proteolytic caspase cascade where one caspase activates other caspases and amplifies apoptotic signaling leading to rapid cell death. There are 10 major caspases that have been identified to date and they are subdivided into initiators (-2, -8, -9, -10), executioners (-3, -6, -7), and

inflammatory caspases (-1, -4, -5). Once caspases have been activated, the path towards cell death appears irreversible. Apoptotic cells also have extensive protein cross-linking and DNA breakdown. There will also be the expression of cell surface markers that target apoptotic cells for phagocytic uptake. The normally inward-facing phosphatidylserine on the cell's lipid bilayer will be moved to the outer layers of the cell membrane. The now outward-facing phosphatidylserine is a popular recognition ligand for the phagocytic cells. Annexin V is a protein that interacts strongly and specifically with phosphatidylserine residues. It is commonly used as a marker for apoptosis (45).

Two interconnected pathways are responsible for regulating apoptosis. The extrinsic pathway involves transmembrane receptor-mediated interactions. When extrinsic apoptosis is initiated, a cell surface receptor will bind pro-apoptotic ligands. These cell surface receptors are members of the tumor necrosis factor (TNF) superfamily. TNF receptors have two common features: an extracellular domain that is rich in cysteines and a cytoplasmic domain of about 80 amino acids known as a death domain (48). The death domain is responsible for transmitting the death signal at the cell surface to the intracellular signaling pathways. Some of the more well studied ligand/death receptor pairs are FasL/FasR and TNF-α/TNFR1. Briefly, the sequence of events during extrinsic apoptosis is as follows: 1) there will be a clustering of receptors binding the death signal ligand at the cellular membrane, 2) cytoplasmic adaptor proteins with death domains are recruited that bind the death domain of the receptor, 3) these protein complexes associate with pro-caspase-8 via death domains, 4) pro-caspase-8 is activated to caspase-8, and 5) caspase-8 initiates the execution phase (45).

The intrinsic apoptosis pathway is initiated by many different non-receptor-mediated stimuli. These stimuli produce intracellular signals that directly act on targets inside the cell and are

initiated at the mitochondria. Such signals may be either positive, such as radiation, hypoxia, viral infections, and toxins, or negative. Negative signals are more of a withdrawal of factors leading to loss of apoptotic suppression and subsequent activation of apoptosis. Such factors include absence of specific growth factors or cytokines. Regardless of their origin, all stimuli cause changes to the inner mitochondrial membrane resulting in the formation of mitochondrial permeability transition pores (MTPs). These pores cause a loss of mitochondrial transmembrane potential and release two main groups of pro-apoptotic proteins that are normally sequestered from that intermembrane region. The first group consists of cytochrome *c*, Smac/DIABLO, and HtrA2/Omi, a serine protease. Cytochrome *c* binds and activates both Apaf-1 and procaspase-9 to form an apoptosome. The proximity of all the procaspase-9 proteins leads to caspase-9 activation. The Smac/DIABLO proteins are thought to promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAPs). The second group of proteins do not lead to apoptosis but are released during apoptosis. The proteins AIF, endonuclease G, and CAD are released from the mitochondria after the cell has committed to death. AIF moves to the nucleus and results in DNA fragmentation and condensation of chromatin. Endonuclease G cleaves chromatin to produce DNA fragments in the nucleus as well. Both AIF and endonuclease G are caspase-independent, but CAD is cleaved by caspase-3 and then causes more DNA fragmentation and advanced chromatin condensation (45).

These two pathways are not discrete and can intersect throughout the stress response, but they will converge at the same point. Whether it is done by caspase-8 from the extrinsic pathway or caspase-9 from the intrinsic pathway, both pathways converge on the activation of caspase-3, an executioner caspase. Activation of caspase-3 leads to the activation of cytoplasmic

endonucleases and proteases which work to degrade DNA, condense chromatin, and rearrange the cytoskeleton all of which ultimately lead to the formation of apoptotic bodies.

Apoptosis can be detected with a variety of methods. The terminal deoxynucleotidyl transferase dUPT nick-end labeling (TUNEL) assay and comet assays are able to detect the presence of fragmented DNA. Annexin V, mentioned earlier, in combination with a cell-impermeable DNA staining dye is used to detect the outwards exposed phosphatidylserine on the cell membrane and cellular integrity. One can also look for intermediates in the apoptosis pathway, such as caspase activation and PARP cleavage (44).

1.7.3 Programmed Non-Apoptotic Cell Death: Vacuole Presenting

Autophagy is characterized by the appearance of large intracellular vesicles, plasma membrane blebbing, enlarged organelles, and engagement of autophagy machinery. Autophagic cell death is a catabolic process that will be engaged during metabolic stress, and it is more so a survival process. Autophagy usually represents a failed attempt to overcome lethal stress, and disruption of this promotes cell death rather than inhibiting it. So, it would be most accurate to say autophagy accompanies cell death rather than causing it. It is also important for the removal of damaged organelles, protein aggregates, and infecting organisms (49). Autophagy can be detected by looking for autophagy-specific proteins via Western blot or LDH sequestration.

Entosis is cellular cannibalism and is hence characterized by cell-in-cell formation. After being internalized, an entoic cell can remain viable for a short time whereafter it is frequently degraded by the lysosome. Entosis is thought to be caused by integrin-extracellular matrix (ECM) detachment. No entosis specific assays exist, but it can be detected using microscopy to observe the presence of the cell-in-cell structures.

Methuosis is characterized by excessive accumulation of large fluid-filled vacuoles. It resembles necrosis in morphology with cell swelling and plasma membrane integrity loss. The massive vacuoles formed cannot be degraded by the lysosomes and will eventually lead to cell death. Electron microscopy is typically needed to observe methuosis.

Paraptosis is characterized by extensive cytoplasmic vacuoles that have been derived from the endoplasmic reticulum or mitochondria. Paraptosis has been reported to be induced by the activation of insulin-like growth factor 1 receptor (IGF1R) and its downstream signaling activity that incorporates MAPK and JNK pathways. It has also been associated with ROS and dysregulation of calcium in the mitochondria. There are no specific assays to detect paraptosis, but it can be observed using microscopy.

1.7.4 Programmed Non-Apoptotic Cell Death: Mitochondria Dependent

Mitoptosis, also known as mitochondrial suicide, is a separate process from mitophagy. The process involves the programmed fission and fusion of the mitochondria accompanied by disruption of ATP supply. These degraded mitochondria will either become autophagosomes or mitoptotic bodies to be extruded by the cell. So, while mitoptosis is more of a mitochondrial death pathway, the extensive mitochondrial fission will eventually lead to cell death. These fragmented mitochondria can be visualized using mitochondria-specific dyes, such as MitoTracker.

Parthanatos is characterized by the hyperactivation of PARP and is linked to the mitochondria but is caspase-independent. PARP mediates the production of poly(ADP-ribose) (PAR), which binds to mitochondrial proteins and releases apoptosis-inducing factor (AIF). This AIF moves from the cytoplasm to the nucleus and induces chromatin condensation and DNA

breakage. So, the hyperactivation of PARP leads to large scale DNA fragmentation. PAR accumulation, PARP-1 activation, and nuclear AIF are common biomarkers of parthanatos.

Ferroptosis is associated with cells that have normal morphology, but they have unusually small mitochondria that have reduced cristae and ruptured membranes. This results in free iron that can interact with lipid hydroperoxides to form lipid ROS, which can lead to cell death. Ferroptosis specific inhibitors can be used for detection.

1.7.5 Programmed Non-Apoptotic Cell Death: Immune Reactive

Pyroptosis is an inflammatory form of cell death induced by the recognition of intracellular pathogens in immune cells. Inflammasomes are innate immune signaling platforms that will respond to microbial or damage-associated stimuli and activate caspase-1. The pattern recognition receptors in the NOD-like receptor (NLR) and AIM2-like receptor (ALR) families will oligomerize when these stimuli are detected. This will then recruit adapter proteins and pro-caspase-1. Caspase-1 becomes auto-activated due to its proximity to itself (50). Caspase-1, in addition to producing mature inflammatory cytokines, will initiate formation of membrane pores by cleaving gasdermin D. Gasdermin D facilitates membrane rupture. The cell membrane rupture will also be accompanied by DNA condensation and fragmentation. Pyroptosis can be detected with a variety of methods including LDH release, caspase activation, and gasdermin D cleavage by western blot (44,51).

1.7.6 Necroptosis

Necroptosis is programmed necrosis. It involves the activation of RIPKs after their recruitment by death receptors, TLKRs, and T-cell receptor. It will elicit an inflammatory response and typically happens in response to pathogen interaction. It is characterized by cell swelling, membrane rupture, loss of organelles, and mitochondrial swelling. Necroptosis can be

detected by using cell-impermeable DNA binding dyes to assess membrane integrity, LDH release, and observing changes to morphology with electron microscopy (44).

1.8 Regulation and Induction of Apoptosis

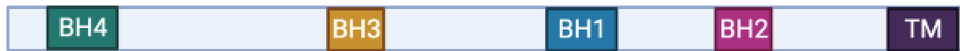
1.8.1 The Bcl-2 Protein Family

Intrinsic apoptosis is regulated by the Bcl-2 protein family. This family of proteins controls apoptotic cell death through their direct binding interactions that regulate mitochondrial outer membrane permeabilization (MOMP), which leads to release of those intermembrane space proteins, and irreversible caspase activation and subsequent death. The interactions are influenced by both the affinities and relative abundance of Bcl-2 family proteins. The family is divided into three sub-groups based on function: 1) anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, BFL-1/A1), 2) pro-apoptotic pore-formers (Bax, Bok, Bak), and 3) pro-apoptotic BH3-only proteins (Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa, Puma). The BH3-only proteins are further subdivided into activators and sensitizers. The interactions between these proteins are regulated by BH domains (Figure 1.2A-B). There are four different BH domains and the entire family shares a BH3 domain. The anti-apoptotic contain all four BH domains and the pro-apoptotic contain domain BH1-3, but some evidence shows them containing the BH4 domain as well. The domains form a highly conserved tertiary structure with a groove that serves as a receptor for other BH3 domains (52–54).

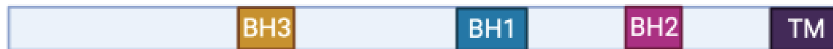
There are several competing models to describe how the interactions between these proteins regulate MOMP. The “embedded together” model emphasizes the mitochondrial membrane as the site of action for most of the Bcl-2 family members. The BH3 domain of activator BH3-only proteins binds the groove in Bax/Bak proteins leading to their activation. Their activation elicits

A

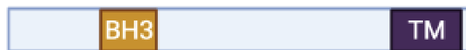
Anti-Apoptotic proteins: Bcl-2, Mcl-1, Bcl-xL, Bcl-w, A1



Pro-Apoptotic proteins: Bak, Bax, Bok



BH3-Only proteins: Bid, Bim, Bad, Bik, Bmf, Bad, PUMA, HRK, etc.



B

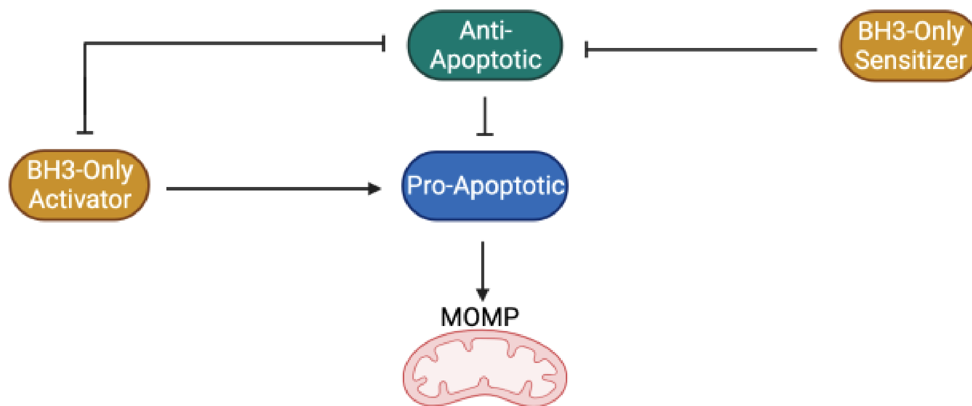


Figure 1.2: Bcl-2 Protein Family and Domain Structures: *(A)* Representative model of Bcl-2 family proteins and their BH domains. *(B)* Simplified summary of the interactions between the Bcl-2 family proteins.

conformational changes that result in Bax/Bak homo-oligomerization and formation of the pore in the outer mitochondrial membrane. The BH3 domain of anti-apoptotic proteins binds either pro-apoptotic pore-formers or activators and inhibits their function by sequestering them. The BH3 domain of sensitizer BH3-only proteins can bind the groove of the anti-apoptotic proteins and inactivate them. The binding of BH3-only sensitizers and BH3-only activators and pore-formers is competitive, so many complex and transient interactions are occurring. Most of these interactions will physically occur either on or within the mitochondrial outer membrane and the membrane even affects structural changes of the Bcl-2 proteins that can alter their affinities and thus interactions.

The original model describing Bcl-2 family interactions is the rheostat model and it is much simpler. It reasons that the decision for a cell to undergo apoptosis is dependent upon the ratio of pro-apoptotic to pro-survival proteins within the cell. Despite being proposed when only Bax and Bcl-2 had been discovered, the main principles underlying the model hold relevant for the embedded together model: if all the interactions result in Bax/Bak oligomers and MOMP then the cell will die.

Determining specific affinities amongst the family members is rather complex. It has been shown that certain members will preferentially bind one member over another. However, these interactions are also influenced by other Bcl-2 family members present, different affinities of these proteins for the different membranes, changing conformations of the proteins, and the binding of the Bcl-2 family proteins to non-family proteins in different subcellular locations. Further, the Bcl-2 family proteins can be transcriptionally and post-translationally modified which also affects their abundance and affinities (55). A lot remains to be understood regarding the protein-protein interactions of these Bcl-2 family members.

The Bcl-2 family proteins can be found all over the cell, though most seem to have a predominant location under non-apoptotic or apoptotic conditions. The localization of the proteins will determine available binding partners and responses to different death stimuli. The subcellular localization of the proteins could be related to the monitoring of different processes or could be related to the potential alternate functions of these proteins in non-apoptotic cells (55). More recent work has identified several non-canonical functions of the Bcl-2 family members that occur both dependently and independently of apoptosis. They have been shown to regulate mitochondrial dynamics, regulate metabolism, calcium homeostasis, and the DNA damage response (56–59).

1.8.2 Viruses and Apoptosis

Viruses induce apoptosis in a variety of ways. Their alteration of the cellular membrane during fusion or endocytosis, disruption of signal transduction pathways, or degradation and damage of DNA are just a few ways in which viral infection can activate apoptotic pathways. Consequently, many viruses have evolved mechanisms to inhibit apoptosis. Many viruses express Bcl-2 family homologues or proteins that interact with the Bcl-2 family. Some of the earliest evidence of this was observed in adenoviruses. Adenoviruses lacking a functional EIB 19K protein could not prevent apoptosis, which resulted in premature cell death and reduced production of viral progeny. Interestingly, loss of EIB 19K could be complemented by overexpressing Bcl-2. When the amino acid sequences were compared it was found that these proteins shared sequence homology associated with a BH domain (60). Other virus families also encode homologues to members of the Bcl-2 protein family. Importantly, herpesviruses, in particular the gammaherpesviruses, express Bcl-2 protein homologues. This is especially interesting considering their association with cancer in their natural hosts. EBV encodes LMP-1,

a multifunctional protein, that upregulates cellular anti-apoptotic proteins Bcl-2, A20, and bfl-1. EBV also encodes a Bcl-2 homolog BHRF1, but it is interesting that cellular Bcl-2 and the viral homologue are expressed at different times. The upregulation of cellular Bcl-2 by LMP-1 happens during latent infection, while BHRF1 is seen at high levels during productive infection, so it probably plays a greater role in promoting release of progeny as opposed to playing a role in transformation. The KSHV homolog of Bcl-2 is also expressed mainly during lytic infection.

Viruses also encode proteins to modulate the activity of p53. p53 is a tumor suppressor that will induce cell cycle arrest or induce apoptosis in response to DNA damage, aberrant growth signals, or other cytotoxic signals. Adenoviruses express a E1B-55K protein, which will bind directly to p53 and prevent any p53-mediated transcription. p53 is known to induce the transcription of genes like Bax and Bak1. Human papillomavirus simply encodes the E6 protein which degrades p53 by marking it for ubiquitination.

The TNF receptor superfamily are also major inducers of apoptosis and so viruses will also target these receptors. Many TNFR orthologs have been identified in poxviruses and human CMV. Viruses can also alter expression of death receptors and ligands to inhibit apoptosis. Viruses will hijack the extrinsic apoptosis pathway using their viral FLICE inhibitory proteins, or v-FLIPs. These proteins contain homologues to the death domains of FADD and procaspase-8 and thus interfere with death receptor signaling pathways. They were discovered independently by three different groups in the gammaherpesvirus HVS, equineherpesvirus 2, bovine herpesvirus 4, and a poxvirus. These v-FLIPs have been shown to bind directly to FADD or procaspase-8 (61). The v-FLIPs also play a role in regulating NF-κB expression through interaction with transcription factors critical to inhibiting apoptosis.

Caspases play a critical role in regulating apoptosis, and viruses can take advantage of them as well. The enzymatic activity of caspases can be inhibited by a family of proteins known as inhibitor of apoptosis proteins (IAPs). They were originally discovered in baculovirus and at least eight cellular IAPs have been identified since. Mammalian viruses contain orthologs to these cellular IAPs and this is used, although less frequently, to inhibit apoptosis. For example, CrmA, which is present in most poxviruses, can inhibit caspases (62).

1.8.3 Cancer and Apoptosis

All cancer cells display the "hallmarks of cancer" regardless of the cause or type of cancer. These markers include uncontrolled growth, angiogenesis, and apoptosis evasion among others. Cells rely on apoptosis to prevent the development of cancer, so evasion of apoptosis is vital to carcinogenesis. Cells can evade apoptosis through several mechanisms.

Cells can disrupt the balance of pro- and anti-apoptotic proteins. The Bcl-2 protein family are crucial when it comes to regulation of apoptosis. Cancerous cells have a tendency to upregulate the anti-apoptotic proteins and promote the loss of Bax and Bak. Raffo *et al* showed overexpression of Bcl-2 protected prostate cancer cells from apoptosis (63). The overexpression of Bcl-xL has been shown to provide multi-drug resistance in tumor cells and prevent apoptosis (64). Another study showed that apoptosis was reduced in colorectal cancer cells due to mutations in the *bax* gene (65). The p53 protein, which is one of the most well-known tumor suppressor genes, is another target of cancerous cells. Defects in the gene have been linked to a large number of cancers, as well as the dysregulated expression of targets of p53. Dysregulation of IAPs is also common in many cancers. Lopes *et al* showed abnormal expression of IAPs in pancreatic cancer cells, which were also linked to resistance to chemotherapy (66).

Cancer cells also avoid apoptosis through the downregulation of caspases. Downregulation of caspase-9 is frequently seen in patients in the advanced stages of cancer and can correlate with poor treatment outcome. Caspase-3 mRNA levels in breast, ovarian, and cervical tumors were found to be dramatically decreased or undetectable. Overall, research suggests lower levels of caspases or impaired function of caspases may lead to decreased apoptosis and increased carcinogenesis.

In addition to dysregulating intrinsic apoptosis, cancer cells will often target the extrinsic pathway as well. Downregulation of the receptors or impairment of the receptor function can lead to reduced levels of death signals and subsequent reduction in apoptosis. Several cancers have been shown to employ reduced membrane expression of death receptors or expression of decoy receptors to evade activation of the death signaling pathways (67).Importantly, targeting apoptosis and apoptotic pathways could serve as a promising therapeutic for several cancers. Indeed, many drugs exist that target both the intrinsic and extrinsic pathways (67–69).

1.9 Hypotheses

A global CRISPR/Cas9 essentiality screen performed in endothelial cells identified Bcl-xL as potentially required for cells latently infected with KSHV. Bcl-xL is an anti-apoptotic protein and cells need to evade apoptosis for tumorigenesis. While latent infection is thought to be fairly quiescent a number of viral genes are expressed that could alter the host cell leading to the induction of apoptosis. I hypothesize that Bcl-xL is required for the survival of KSHV latently infected cells, and this hypothesis will form the basis of the work described in chapter 3. In chapter 4, I explore how KSHV induces apoptosis in latently infected cells. I hypothesize a single latent gene is inducing a cellular pathway that leads to the induction of apoptosis. Chapter

5 follows up on the CRISPR/Cas9 sub-pool screen by validating additional top hits from the screen. I hypothesize the sub-pool screen was able to identify several essential genes and additional genes from the screen will be required for the survival and/or proliferation of KSHV latently infected cells, but not uninfected cells.

Chapter 2

MATERIALS AND METHODS

Cell Lines

TIME (Tert-Immortalized Microvascular Endothelial) and primary endothelial cells were maintained in endothelial cell basal medium-2 (EBM-2) media (Lonza), which was supplemented with an EGM-2 MV SingleQuot Microvascular Endothelial Cell Growth Medium Bullet Kit (Lonza) containing 5% fetal bovine serum (FBS), hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, and hEGF, as well as gentamycin and amphoteric-B. All BCBL-1, JSC-1, and BC1 cells were grown in Roswell Park Memorial Institute media (RPMI) 1640 (+L-glutamine, +penicillin-streptomycin, +2-mercaptoethanol, +10% FBS). 293T and HFF cells were grown in Dulbecco's modified Eagle media (DMEM) (+L-glutamine, +penicillin-streptomycin, +4.5g/L glucose, +sodium pyruvate, + 10% FBS, Fisher). iSLK cells for BAC16 virus production were grown in DMEM (+L-glutamine, +penicillin-streptomycin, +4.5g/L glucose, +sodium pyruvate, + 10% FBS, +250μg/mL G418, +1200μg/mL hygromycin B, +1μg/mL puromycin).

Viruses and infection

Extracellular KSHV particles were obtained from BCBL-1 cells (5 x 10^5 cells/mL) induced with 20ng of TPA (12-*O*-tetradecanoylphorbol-13-acetate; Sigma)/mL as described previously. After 5 days, cells were pelleted, and the supernatant was run through a 0.45µm-pore-size filter (Whatman). Virions were pelleted at 30,000xg for 2 h in a JA-14 rotor, Avanti-J-25 centrifuge (Beckman Coulter). The viral pellet was resuspended in EBM-2 without supplements. The ad-GFP and KLAR viruses were made as described previously (70). The recombinant KSHV BAC16 originally made in the Jung lab was obtained from the Renne lab (71). The KSHV BAC16 mutant viruses were obtained from the Renne lab and are described elsewhere (72,73). BAC16 mutant viruses were passaged in iSLK cells as described (72). Lytic replication was induced by adding 1 µg/mL of doxycycline and 1 mM of sodium butyrate. Virus was harvested from the supernatant 4 days post induction as described above.

KSHV infections of all cell types were performed in serum-free EBM-2 supplemented with 8µg/mL polybrene for 4 h, after which the medium was replaced with complete EGM-2. Mock infections were performed identically except that concentrated virus was omitted from the inoculum. Infections with ad-GFP and KLAR were performed the same as above except serum-free EBM-2 was supplemented with 1µg/mL poly-L-lysine instead of polybrene.

Infections of TIME cells using spin-oculation were performed in 6-well plates. Cells were seeded in serum-free EBM-2 supplemented with 8µg/mL polybrene and virus. The plates were placed in a Sorvall Legend XTR Centrifuge (Thermo Scientific) and centrifuged for 45 minutes at 800g. Plates were removed and infection continued for a total of 3 hours.

Immunofluorescence

Mock- or KSHV-infected cells were seeded on LabTek Permanox four-well chamber slides (Fisher Scientific) and fixed with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline. Immunofluorescence was performed as described previously (74). Briefly, cells were incubated in Tris-Buffered Saline (20mM Tris, 150mM NaCl, pH 7.6; TBS) containing 1% normal goat serum followed by incubation with primary antisera at a dilution of 1:1000 (rabbit anti-LANA, a kind gift from the Ganem lab) diluted in TBS containing 1% BSA for 1 h. Cells were then incubated with fluor-conjugated secondary antibodies (Molecular Probes/Invitrogen) for 1 h. Cells were mounted in medium containing DAPI (4',6'-diamidino-2-phenylindole) before being viewed under a Keyence BZ-X710.

CRIPSR/Cas9 gene targeting of Bcl-xL and other genes

We obtained a pRRL plasmid expressing a Cas9-T2A cassette from Daniel Stetson (University of Washington), described in Gray et al., 2016 (75). Guide RNAs specific to each gene were inserted into pRRL using the In-Fusion cloning system (Takara Bio). Briefly sense and anti-sense sgRNAs were annealed to form a gRNA. These gRNAs were cloned into the pRRL plasmid using the In-Fusion enzyme (Takara Bio). Cloned plasmids were then transformed into Stellar Competent *E. Coli* cells and antibiotic selected. Single colonies were grown up and plasmids were isolated using the Qiagen Mini-Prep Kit. Lentivirus targeting Bcl-xL or the non-targeting control was generated by co-transfection of 293T cells.

Transfection

293T cells were seeded at 5.5 x 10^6 cells per 10 cm dish the night before transfection. Transit 293T (Mirus Bio) was used to transfect plasmids as indicated by the manufacturer. For lentivirus production, the masses of each plasmid added was 8μg of psPAX2, 4μg of PMD2.G, and 8μg of the pRRL vector. After 24 h, the media on the cells was replaced with fresh serum containing media. Culture supernatants were collected at 48 and 72 h post transfection and filtered through 0.45-μm filters before aliquoting and freezing.

Transduction

Lentiviral infections were done by incubating 1 mL of virus preparation with TIME, HUVEC, 293T, and HFF cells for 6 h with 8μg/mL polybrene. Transduced cells were selected for 2-5 days with 1 μg/mL puromycin. For the transduction of BCBL-1 and other non-adherent cell types, 1 x 10^5 cells/mL were seeded the night before transduction. Cells were resuspended in 1 mL of lentivirus preparation for 6 h. Cells were selected with 1 μg/mL puromycin for 2 days and dead cells were removed with a slow spin in centrifuge (500 rpm, 3 min) where supernatant was removed, and remaining cells were resuspended in growth media.

Overexpression of Bcl-2

We obtained a pCDH plasmid expressing Bcl-2 (Addgene #46971) and an empty pCDH plasmid (Addgene #64874) both with puromycin resistance. These plasmids were transduced into TIME cells using the transduction protocols detailed above. Transduced cells were selected

for using puromycin selection for 2 days and Bcl-2 expression was determined by western blot analysis.

Western Blot Analysis

Cells were harvested (using trypsin to remove adherent cells) and then pelleted and washed once with PBS. Cell pellets were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Trist-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 40 mM β-glycerophosphate, Complete Mini protease inhibitor tablet; Roche). Cell lysate was quantified using the Peirce BCA assay (ThermoFisher Scientific), and equal masses of protein were loaded to a 4-20% polyacrylamide gel (BioRad). The protein was transferred to a polyvinylidene difluoride membrane and blotted using the appropriate primary antibody. Blots were treated with LI-COR IRdye secondary antibodies prior to imaging on Odyssey Fc system.

Trypan Blue Assay

To measure cell viability, TIME cells were trypsinized and collected. Prior to trypsinization, the supernatant was collected along with the PBS from one wash. The trypsinized cells, supernatant, and PBS wash were combined and cells were pelleted. The cell pellet was resuspended in 50-100 μL of growth media. 10 μL was removed and combined with equal volume trypan blue. Cells were then counted using a TC-20 Cell Counter (Bio-rad).

Cell Death Assay with Cell-Cyte

To determine percent cell death, cells were plated and treated in 6-well dishes. Cells infected with BCBL-1 derived virus were incubated with YoYo-1 (200 nM) and Syto59 (50 nM) dyes (Life Technologies). The plates were placed into a Cell-Cyte (Cytena) and phase contrast and fluorescent photographs were taken. Fluorescence intensity was normalized among images using Cell-Cyte software, and the number of YoYo-1 and Syto59 positive cells was measured to calculate percent dead cells.

Inhibitor Studies

For infections combined with drug inhibitors, the inhibitor was added to growth media and added to cells 4 h post-infection or 48 h post-infection as indicated. A-1331852 (Selleck) was dissolved in DMSO and used at a final concentration of 1 or 10 nM. QVD-OPH (SM Biochemicals) was dissolved in DMSO and used at a final concentration of 20 nM.

Caspase-3/7 Activation

To observe caspase-3/7 activation the NucView caspase-3 enzyme substrate (Biotium) consisting of a fluorogenic DNA dye coupled to the caspase-3/7 DEVD recognition sequence was added to media at concentration of 5 μM. The substrate is initially non-fluorescent and able to penetrate the plasma membrane. In apoptotic cells, caspase-3/7 will cleave the substrate releasing the DNA dye which will then migrate to the cell nucleus and fluorescently stain DNA. This fluorescence was detected using Cell-Cyte (Cytena).

Crystal Violet Assays

Cells were seeded at 40,000 cells/well into 12-well dishes. Cells were allowed to proliferate for 8 days. During those 8 days, when one well reached confluence, all wells were split 1:2. At the end of 8 days, cells were first put onto ice. Cells were then washed 2X with 0.5 mL cold DPBS. Cells were fixed for 10 minutes with 0.5 mL ice cold 100% MeOH. Cells were moved to room temperature and stained with 0.5 mL 0.5% crystal violet solution for 10 minutes. Crystal violet was washed off by washing with 0.5 mL diH2O 4X. Plates were dried overnight. Plates were scanned on Sapphire Biomolecular Image (Azure Biosystems) using the following settings: Pixel Size (50 uM), Sample Type (Plate +3.00), Dye (AzureSpectra 650), Intensity (8). Images were then analyzed using ImageJ.

ICE Analysis

Transduced cells were harvested and genomic DNA was extracted using DNeasy Blood & Tissue kit (Qiagen). DNA was PCR amplified using primers targeting the region directly upstream and downstream of the sgRNA cut site. DNA was collected from the PCR using QIAquick PCR Purification Kit (Qiagen). DNA was sequenced using Sanger Sequencing and analyzed using Synthego's ICE Analysis software.

Chapter 3

BCL-XL IS REQUIRED TO PROTECT ENDOTHELIAL CELLS LATENTLY INFECTED WITH KSHV FROM VIRUS-INDUCED INTRINSIC APOPTOSIS

This chapter is adapted from a published manuscript.

Lyndsey N. Moore, Daniel L. Holmes, Anjali Sharma, Joselyn Landazuri Vinueza, Michael Lagunoff

Department of Microbiology, University of Washington, Seattle, WA, USA.

3.1 Abstract

Kaposi's Sarcoma herpesvirus (KSHV) is the etiologic agent of Kaposi's Sarcoma (KS), a highly vascularized tumor common in AIDS patients and many countries in Africa. KSHV is predominantly in the latent state in the main KS tumor cell, the spindle cell, a cell expressing endothelial cell markers. To identify host genes important for KSHV latent infection of endothelial cells we previously used a global CRISPR/Cas9 and subsequent rescreen to identify genes necessary for the survival or proliferation of latently infected cells. We found that the highest scoring gene necessary for infected cell survival is the anti-apoptotic Bcl-2 family member Bcl-xL. Knockout of Bcl-xL or treatment with a Bcl-xL inhibitor leads to high levels of

cell death in latently infected endothelial cells but not their mock counterparts. Cell death occurs through apoptosis as shown by increased PARP cleavage and activation of caspase-3/7. Knockout of the pro-apoptotic protein, Bax, eliminates the requirement for Bcl-xL. Interestingly, neither Bcl-2 nor Mcl-1, related and often redundant anti-apoptotic proteins of the Bcl-2 protein family, are necessary for the survival of latently infected endothelial cells, likely due to their lack of expression in all the endothelial cell types we have examined. Bcl-xL is not required for the survival of latently infected primary effusion lymphoma (PEL) cells or other cell types tested. The critical requirement of Bcl-xL during KSHV latency makes it an intriguing therapeutic target for KS tumors.

3.2 Introduction

Kaposi's Sarcoma (KS) is an angioproliferative tumor and is one of the most common tumors among individuals infected with HIV-AIDS. It is also endemic to many countries in sub-Saharan Africa, where it afflicts both HIV-positive and HIV-negative individuals (76). The KS tumor is complex and highly vascularized. The main proliferating agent of the KS tumor is the spindle cell, a cell expressing markers of the endothelium (6,77,78). Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of KS and is also the cause of other cancers, including primary effusion lymphoma (PEL), a B cell lymphoma that occurs in the pleural cavity (18). Spindle cells in the KS tumor are predominantly latently infected with KSHV with only a small percentage of cells expressing markers of lytic infection (77,78).

Infection of primary endothelial cells in culture, as well as a tert-immortalized microvascular endothelial cell line (TIME cells), leads to high levels of latent infection with a very low level of cells undergoing lytic replication, recapitulating what is found in spindle cells

in the KS tumor (79). In endothelial cells there is one major latent locus expressed during latency in endothelial cells, though other genes can be detected at extremely low levels or under specific conditions (80).The major latent locus consists of the latency associated nuclear antigen (LANA), a viral cyclin (vCyc), a viral flice inhibitory protein (vFLIP), the Kaposin locus expressing a family of proteins using different start sites, and 12 miRNA loci expressing a larger number of mature miRNAs. In primary effusion lymphoma cell lines, a viral interferon regulatory protein (vIRF3 or LANA2) is also expressed but this is not routinely detected in latently infected endothelial cells or in KS spindle cells (81). During lytic infection most of the over 80 viral genes are expressed (76,82).

Apoptosis, one form of programmed cell death, is often induced by viral infection and many viruses have mechanisms to inhibit apoptosis (45,83). KSHV expresses several genes capable of inhibiting apoptosis. For example, during lytic infection the virus encodes a viral Bcl2 homolog (vBcl2) that can inhibit intrinsic apoptosis induced by pro-apoptotic cellular proteins at the mitochondrial membrane (84,85). There are additional lytic genes that can block apoptosis including vIRF1, which inhibits p53 induced apoptosis, and K1, which inhibits apoptosis through the release of growth factors like VEGF and many others (86,87). During latent infection, vFLIP is known to block extrinsic apoptosis, programmed cell death mediated by death receptors on the cell membrane. The vFLIP induces NF-kB expression to inhibit apoptosis and also contains a domain capable of blocking apoptosis induced by extracellular death inducing factors (88–90). It was recently shown that Mcl1, an inhibitor of intrinsic apoptosis is required for the survival of PEL cell lines (42). However, it is not known if inhibition of intrinsic apoptosis is needed during latent infection of endothelial cells or if latent infection activates intrinsic apoptotic pathways or

if the dependency found in PEL cell lines was part of lymphoma formation and not a direct result of KSHV infection.

There are no KSHV specific treatments that are effective for KS tumors. All current drug treatments for herpesviruses only target lytic replication, making them ineffective for diseases characterized by latent infection. KS tumors are most often treated with general anti-cancer treatments (78). A direct treatment for latent KSHV is difficult due to the limited gene expression. However, the possibility to specifically target pathologic changes to the host cell during latent infection is intriguing. Previously, we performed a global CRISPR/Cas9 screen to investigate the requirement of all human genes during KSHV latency in KSHV infected TIME cells. This screen identified 146 genes that were required for survival and/or proliferation of KSHV latently infected TIME cells, however, there were several other genes of interest just outside our cutoff of significance (43). For this study, the top hits, as well as additional genes with a slightly higher false discovery rate, were tested for their essential nature in a directed sub-pool CRISPR/Cas9 screen performed in duplicate with 13 guide RNAs for each gene. *Bcl2l1* was the top hit in both replicates. *Bcl2l1* encodes the anti-apoptotic protein Bcl-xL (91).

Bcl-xL is a member of the Bcl-2 protein family. The Bcl-2 protein family regulates intrinsic or mitochondrial-mediated apoptosis. The family is broken down into three groups: anti-apoptotic proteins, pro-apoptotic proteins, and BH3 only proteins. Anti-apoptotic proteins, including Bcl-2, Mcl-1, and Bcl-xL, bind and sequester the pro-apoptotic pore-forming proteins, Bax and Bak, thus preventing them from gathering at the mitochondrial membrane where they induce pore formation and the subsequent activation of caspase cascades that lead to apoptosis (55,92–94).

We found that specific knockout of Bcl-xL, but not the other anti-apoptotic Bcl-2 family members, Bcl-2 or Mcl-1, leads to rapid cell death of latently infected endothelial cells via a caspase dependent mechanism. Other cell types including KSHV infected primary effusion lymphoma cells were not sensitive to Bcl-xL knockout nor Bcl-xL inhibitors. Interestingly, all the endothelial cells we tested do not express detectable levels of Bcl-2 or Mcl-1. These studies demonstrate that Bcl-xL is an attractive therapeutic target for KS tumors where the main tumor cells express endothelial cell markers.

3.3 Results

3.3.1 Bcl-xL is required for the survival of KSHV latently infected endothelial cells.

To determine if Bcl-xL is required for survival during latent KSHV infection, TIME cells were transduced with a non-targeting control (NTC) sgRNA or one of two sgRNAs targeting Bcl-xL (Table 3.1). Bcl-xL was efficiently knocked out in the TIME cells by both targeting sgRNAs as determined by western blot analysis, though the knockout was slightly more efficient with guide RNA 1 (Figure 3.1A). Control and knockout cells were infected with KSHV. 48 hours post-infection cells were harvested, and cell viability was measured using a trypan blue assay. The knockout of Bcl-xL had little effect on the survival of TIME cells but the population of cells in which Bcl-xL was knocked out showed significantly reduced survival when latently infected with KSHV as compared to infected cells that had been transduced with the NTC sgRNA (Figure 3.1B). We next utilized a specific Bcl-xL inhibitor to ensure the results were not due to off-target effects from using the CRISPR system for knocking out Bcl-xL. We treated latently infected TIME cells with A-1331852 (A-133), a Bcl-xL specific inhibitor. According to the manufacturer, this inhibitor has a 600-fold higher Ki for Bcl-xL over Bcl-2 and an over

Table 3.1 Oligonucleotides

Name	Sequence
gRNA_Bcl-xL_1	GAGTAAAGCAAGCGCTGAGGG
gRNA_Bcl-xL_2	GCAGCAGTAAAGCAAGCGCTG
gRNA_Bcl-2_1	GCGGCGGGAGAAGTCGTCGC
gRNA_Bcl-2_2	GTGGAGGAGCTCTTCAGGGA
gRNA_Mcl-1_1	GGAGCTGGACGGGTACGAGC
gRNA_Mcl-1_2	GCCGCCAGCAGAGGAGGAGG
gRNA_Bax_1	GATCGAGCAGGGCGAATGGG
gRNA_Bax_2	GGCTGGATCCAAGACCAGGG
gRNA_Cyp27a1_1	GCCAGCGGTTGCTGAAGCCAG
gRNA_Cyp27a1_2	GCCTGCAGCGATCCATCCCCG
gRNA_Ylpm1_1	GACTGGGGCTGCTGTTAACTG
gRNA_Ylpm1_2	GATGGACCAGCTGATTGCAA
gRNA_Cmklr1_1	AAGCCAGGCGAACGCTGCGG
gRNA_Cmklr1_2	GGTGACCAGGATCTTCCTGG

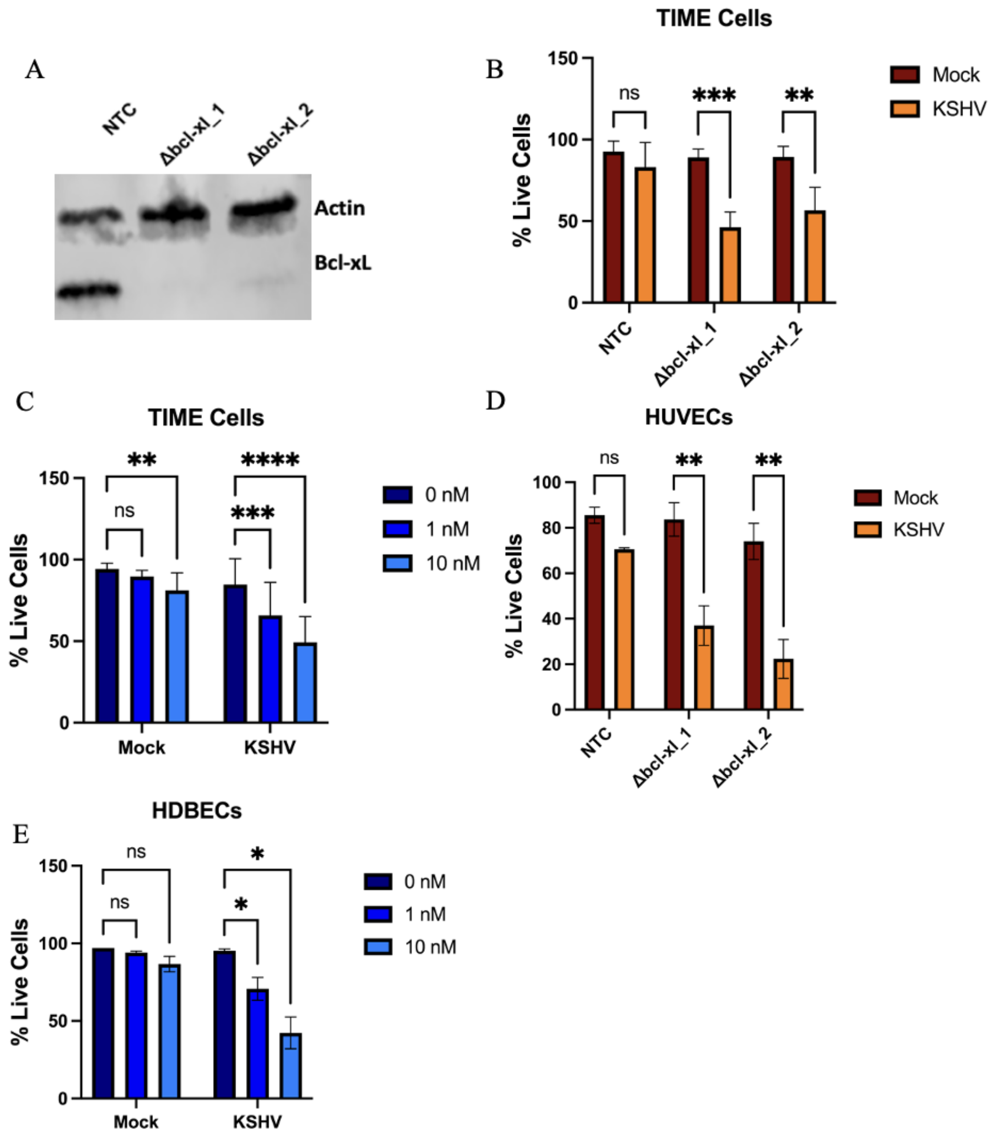


Figure 3.1: Bcl-xL is required for the survival of KSHV latently infected endothelial cells: (*A*) Western blot analysis with an antibody to Bcl-xL in TIME cells treated with sgRNAs targeting Bcl-xL or a non-targeting control (NTC) sgRNA. (*B*) TIME cells transduced with the indicated sgRNAs were mock or KSHV infected for 48 hours and cell viability was measured using trypan blue assay 48 hpi. (*C*) Mock or KSHV infected TIME cells were supplemented with Bcl-xL inhibitor A-1331852 (A-133) at 1 nM or 10 nM or vehicle control and 48 hpi cell viability was measured with a trypan blue assay. (*D*) Primary HUVEC cells were transduced with the indicated sgRNAs, subsequently mock or KSHV infected for 48h and cell viability was measured using trypan blue assay. (*E*) HDBEC cells were infected with KSHV then supplemented with the Bcl-xL inhibitor A-133 at 1 nM or 10 nM or vehicle control. Cell viability was measured using the Cell-Cyte 48 hpi by counting live (Syto59) and dead (YoYo-1) cells using fluorescent dyes. Data are presented as mean ± s.d. (2-way ANOVA, $n \geq 3$) *$P<0.05$.

10,000-fold Ki over Mcl-1 (95,96). TIME cells were either mock or KSHV-infected and 4 hours post-infection, growth media containing A-133 was added to cells. 48 hours post-infection, cells were harvested, and cell viability was measured. Latently infected TIME cells treated with increasing amounts of A-133 showed increased cell death compared to mock infected cells treated with A-133, showing that the inhibition of Bcl-xL during KSHV latent infection also results in decreased cell survival (Figure 3.1C). To ensure the requirement of Bcl-xL in latently infected TIME cells was not an artifact of the immortalization process, we next performed the same experiments in primary human umbilical vein endothelial cells (HUVECs). As with the TIME cells, Bcl-xL was found to be required for the survival of latently infected cells but not the non-targeting control treated cells, (Figure 3.1D). To test another primary endothelial cell type, human dermal blood endothelial cells (HDBECs) were either mock or KSHV-infected and 4 hours post-infection, growth media containing A-133 was added to the cells. Cell viability was measured 48 hours post-infection, and similar to the TIME cells, inhibition with A-133 led to increased cell death in the cells latently infected with KSHV (Figure 3.1E).

3.3.2 Bcl-xL is required for the maintenance of KSHV latent infection.

The knockout experiments and early drug treatment experiments do not differentiate between whether Bcl-xL is required during the establishment of latency or if it is required for the maintenance of latent infection. To determine if the main driver rendering Bcl-xL necessary was establishment of latency or if Bcl-xL is required post-establishment of latency, TIME cells were either mock or KSHV infected and the A-133 inhibitor was not added until 48 hours post-infection when latency has already been established. Cell death was then measured only 6 hours after the addition of the drug. There was rapid death of KSHV latently infected cells but not

mock after 6 hours of Bcl-xL inhibition suggesting that Bcl-xL is required for maintenance of latent infection not just during the establishment phase of latency (Figure 3.2A). A time course experiment performed on TIME cells infected with KSHV and treated with A-133 4 hpi was used to determine at which point during infection TIME cells become dependent on Bcl-xL (Figure 3.2B). Infected cells experienced a dramatic increase in cell death around 12 hpi that continues to increase as latency is established. This finding mirrors what is seen in knockout cells as well (Figure 3.2C). While cells start to become reliant on Bcl-xL before latency has been fully established, the fact that cells treated with A-133 at 48 hpi die rapidly and in large amounts shows cells are still dependent on Bcl-xL after the establishment phase of latency.

3.3.3 Bcl-xL inhibits cell death in latently infected endothelial cells via a caspase-mediated pathway.

Bcl-xL is known to inhibit apoptosis via the intrinsic (mitochondrial-mediated) apoptosis pathway and prevent activation of two convergent caspase cascades (45,55). However, recent studies have shown that Bcl-xL has additional functions in the cell not dependent on activation of the caspase cascade (56,91,97–99). To determine the mechanism for the requirement of Bcl-xL we tested if Bcl-xL was inhibiting cell death by preventing caspase-mediated activation of death. As before, CRISPR/Cas9 was used to knockout Bcl-xL in TIME cells and the knockout cells were infected with KSHV. 4 hours post-infection, growth media containing QVD, a pan-caspase inhibitor, was added to cells and infection continued for a total of 48 hours. Cells were then harvested, and cell viability was measured using trypan blue assay. As seen previously, when Bcl-xL is knocked out in infected TIME cells, there is a marked increase in cell death. But, when infected Bcl-xL knockout cells are treated with QVD, cells are rescued from death (Figure

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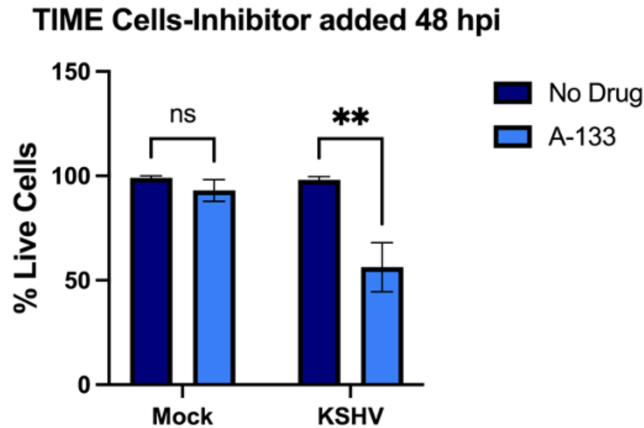

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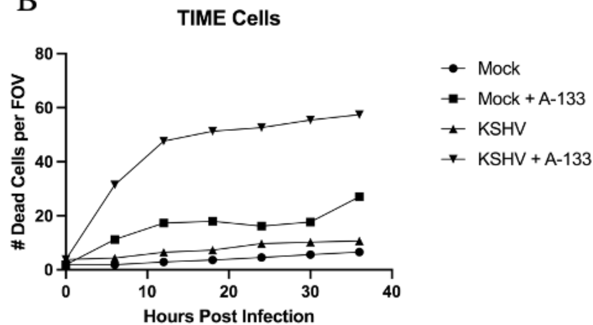

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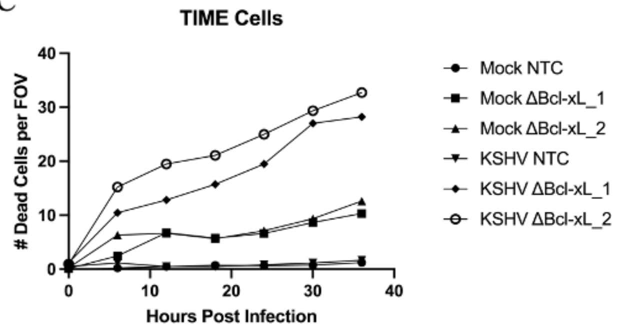

Figure 3.2: Bcl-xL is required for the maintenance of KSHV latent infection: *(A)* TIME cells were mock or KSHV infected and 48 hpi media was supplemented with or without A-133 at 10 nM. Cell viability was measured 6 hr after drug addition by counting live (Syto59) and dead (YoYo-1) cells using the Cell-Cyte. Data are presented as mean ± s.d. (2-way ANOVA, $n \geq 3$) *$P<0.05$. *(B)* TIME cells were mock or KSHV infected and A-133 was added 4 hpi. Number of dead cells (YoYo-1) was counted using the Cell-Cyte. *(C)* TIME cells transduced with the indicated sgRNAs were mock or KSHV infected. Dead cells (YoYo-1) were counted using Cell-Cyte. Data in *(B)* and *(C)* are representative graphs from 2 separate experiments.

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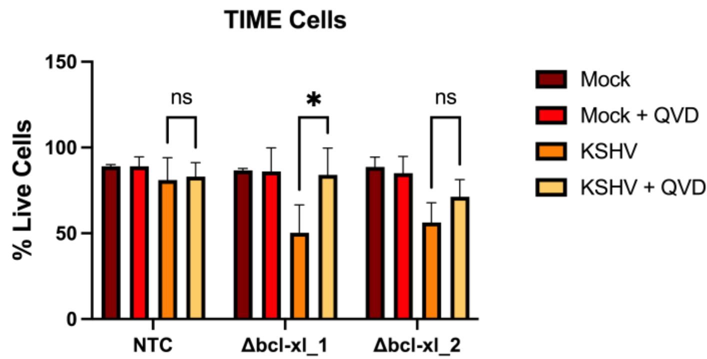

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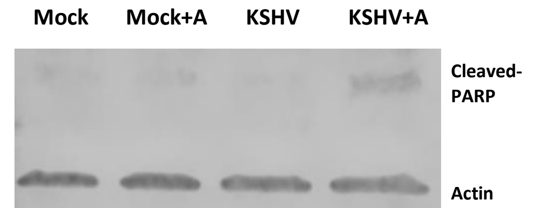

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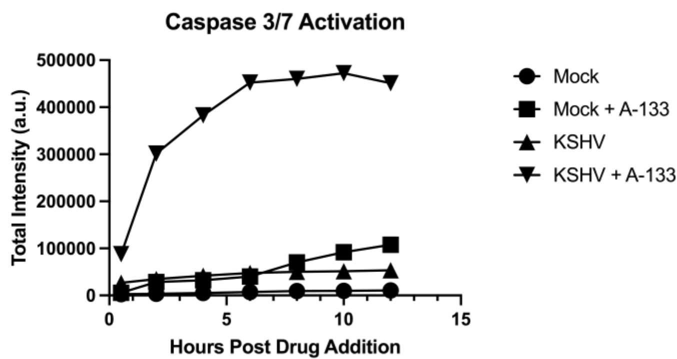

Figure 3.3: Bcl-xL inhibits cell death in latently infected endothelial cells by inhibiting apoptosis: (*A*) TIME cells transduced with sgRNAs were selected for using puromycin resistance for 2 days. Transduced cells were infected with KSHV and 4 hpi media was supplemented with or without QVD at 20 nM. Cell viability was measured with a trypan blue assay 48 hpi. Data are presented as mean ± s.d. (2-way ANOVA, n=3) *P<0.05. (*B*) Western blot analysis with antibody to PARP in TIME cells mock or KSHV-infected and treated with A-133 (10 nM). (*C*) TIME cells were either mock or KSHV infected. 48 hpi cells were treated with A-133 or vehicle control. All cells were treated with caspase-3 enzyme substrate (5 µM). Cells were placed in Cell-Cyte and fluorescence was measured over time. Graph is representative of two replicates.

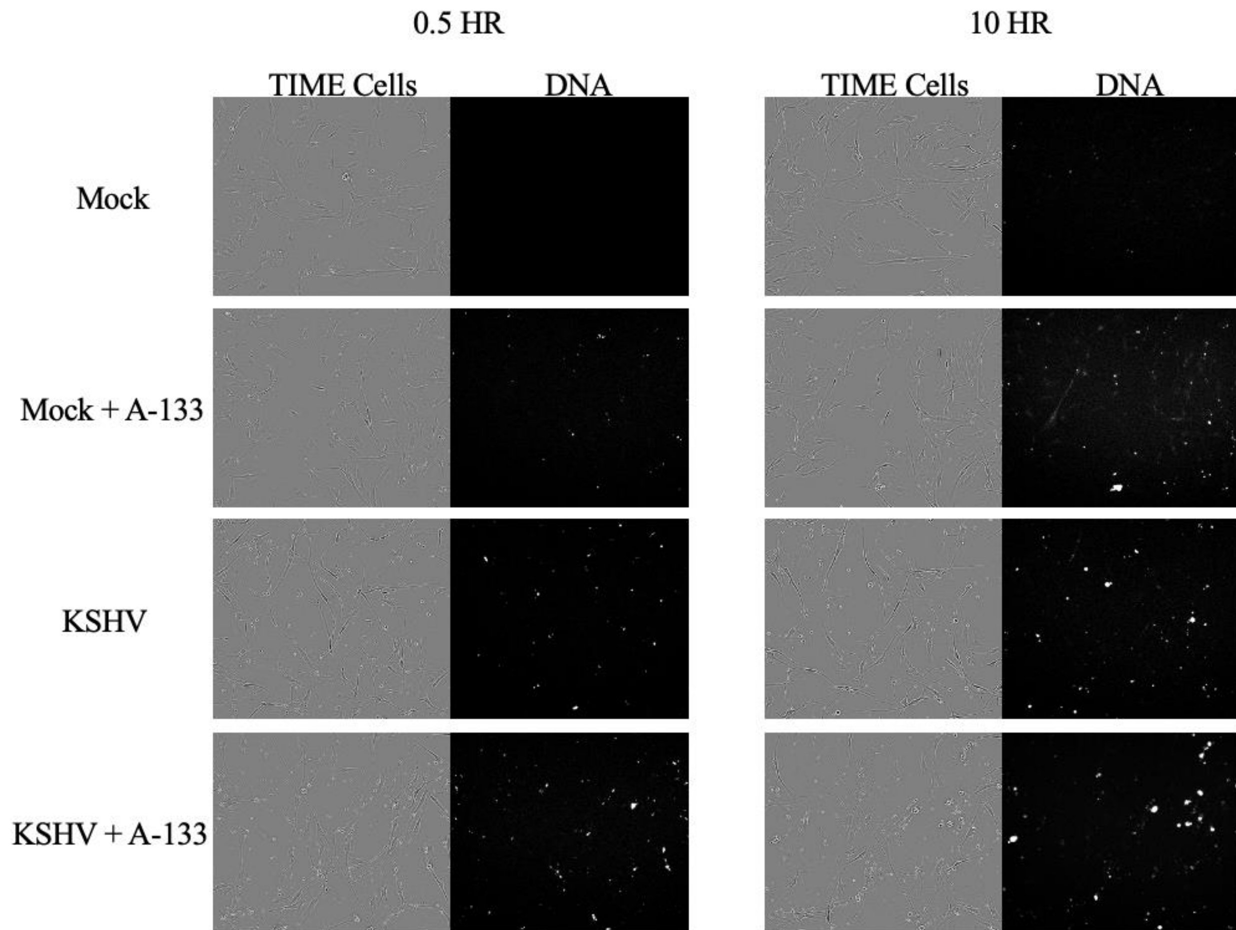

Figure 3.4: Caspase-3/7 activity observed during KSHV infection when Bcl-xL is inhibited: Representative images of mock or KSHV infected TIME cells that have been treated with vehicle control or A-133 at 48 hpi. A caspase-3/7 substrate was added to all cells at the same time as vehicle control or A-133. The substrate, when cleaved by caspase-3/7 releases a high affinity fluorogenic DNA dye. Left column contains enhanced contour images of cells and right column shows fluorescent DNA.

3.3A). The comparison between KSHV and KSHV + QVD is significant for the first CRISPR guide RNA (ΔBcl-xL_1), however, data from the weaker of the two CRISPR guide RNAs (ΔBcl-xL_2) does not meet significance but the trend still indicates rescue from cell death upon treatment with QVD. The smaller difference can likely be attributed to the fact that the second gRNA for Bcl-xL was less effective than the first. This shows that Bcl-xL is preventing caspase-mediated death during latent infection.

Caspases also mediate other methods of programmed cell death, such as necroptosis and pyroptosis, in addition to apoptosis. Different caspases regulate different pathways. For example, caspase-3/7, caspase-1, and caspase-8 regulate apoptosis, pyroptosis, and necroptosis respectively (100). Since Bcl-xL is known to inhibit apoptosis, we also used the presence of PARP cleavage to further corroborate Bcl-xL is inhibiting apoptosis in KSHV infected cells. Cleavage of PARP by caspases is considered a hallmark of apoptosis (101). KSHV-infected cells, but not mock infected, treated with the A-133 inhibitor show evidence of significantly increased PARP cleavage (Figure 3.3B).

To orthogonally corroborate that Bcl-xL was preventing cell death by inhibiting apoptosis, activation of caspase-3/7 was assessed. TIME cells were infected for 48 hours and then the A-133 inhibitor was added. A caspase-3 enzyme substrate was added to the media. The substrate releases a high-affinity fluorescent DNA dye when cleaved by caspase-3/7, so caspase-3/7 activity can be observed by quantifying fluorescence over time (Figure 3.4). In infected cells treated with the A-133 inhibitor, there was an immediate and dramatic increase in caspase-3/7 activity consistent with the onset of apoptosis, while untreated infected cells saw no change in caspase-3/7 activity. Mock infected cells treated with the A-133 inhibitor experienced a small

increase in caspase-3/7 activity around 8 hours after the drug and substrate were added, which was not unexpected considering the minor toxicity of the A-133 inhibitor (Figure 3.3C).

3.3.4 Bcl-xL inhibits the pore-former Bax to inhibit apoptosis in KSHV infected endothelial cells.

Bcl-xL is known to inhibit apoptosis by binding and sequestering Bax, one of the pro-apoptotic pore-formers. To further investigate the mechanism by which Bcl-xL is inhibiting apoptosis in our system we tested to see if knocking out Bax would alleviate the need for Bcl-xL during latent infection. TIME cells were transduced with a non-targeting control (NTC) sgRNA or one of two sgRNAs targeting Bax (Table 3.1) (Figure 3.5A). Transduced cells were infected with KSHV. 4 hours post-infection, growth media supplemented with the A-133 inhibitor was added to cells and infection continued for a total of 24 hours and cell viability was measured. Knocking out Bax while inhibiting Bcl-xL rescued cells from death compared to cells with Bax still expressed (Figure 3.5B). This indicates that Bcl-xL is inhibiting cell death by inhibiting Bax's ability to permeabilize the mitochondrial membrane. Together this data shows that by inhibiting Bax, Bcl-xL is able to prevent apoptotic cell death that is induced during KSHV latent infection.

3.3.5 Bcl-xL is specifically required for survival in endothelial cells due to limited expression of other anti-apoptotic proteins.

There is significant functional redundancy in the anti-apoptotic Bcl-2 protein family members (55). However, other Bcl-2 family anti-apoptotic proteins were not identified in our CRISPR screen. To determine if other Bcl-2 family members are required for survival of latently

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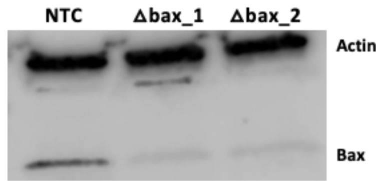

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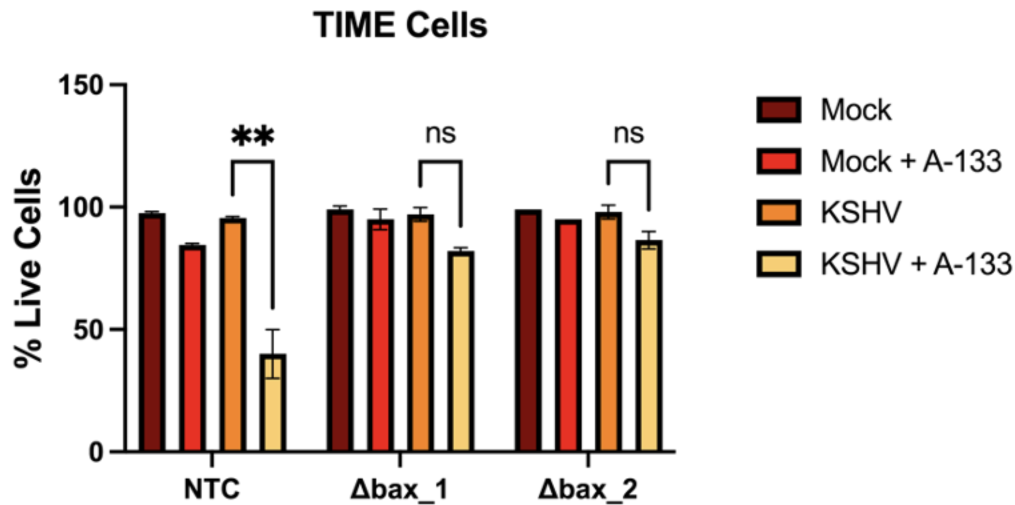


Figure 3.5: Bcl-xl inhibits the pore-former bax to inhibit apoptosis in KSHV infected endothelial cells: (*A*) Western blot analysis with an antibody to Bax in TIME cells treated with sgRNAs targeting Bax or a non-targeting control (NTC) sgRNA. (*B*) TIME cells transduced with sgRNAs were selected using puromycin resistance. Transduced cells were either mock or KSHV infected. 48 hpi A-133 (10 nM) or vehicle control was added to cells and cell viability was measured 12 hr after A-133 addition using Cell-Cyte. Data are presented as mean ± s.d. (2-way ANOVA, n=2) *P<0.05.

infected endothelial cells, we used CRISPR/Cas9 to knockout Bcl-2 or Mcl-1 using two guides for each gene (Table 3.1). Knockout of Bcl-2 and Mcl-1 had no significant effect on the survival of latently infected cells (Figure 3.6A). However, we were unable to determine the level of knockout of Bcl-2 or Mcl-1 by western blot due to lack of protein in TIME cells (Figure 3.6B). We examined our previous RNA sequencing data for TIME cells and found mRNA expression of Bcl-2 was near the limits of detection. While read counts for Mcl-1 were higher than Bcl2l1, the gene that encodes Bcl-xL, we do not see expression of Mcl-1 at the protein level, suggesting Mcl-1 is active transcriptionally, but not translationally. We also examined RNA sequencing data for both primary blood and lymphatic endothelial cells and found the same limited read depth for Bcl-2 (Table 3.2). Bcl-2 and Mcl-1 were also undetectable on western blots for primary endothelial cells (Figure 3.6C). Masri *et al* investigated differences in Bcl-2 and Mcl-1 protein levels in idiopathic pulmonary arterial hypertension (IPAH) pulmonary artery endothelial cells (PAEC) vs control PAEC and there is little to no expression of these proteins in control PAECs (102). Nör *et al* investigated the impact of VEGF on expression of Bcl-2 proteins in HDMECs and control cells show expression of Bcl-xL but near undetectable levels of Bcl-2 (103). RNAseq data of KS lesions from Tso *et al* shows expression of Bcl-xL and Mcl-1, but lower read counts for Bcl-2 in whole tumors. It should be noted that the tumors consist of multiple cell types, not just endothelial (104). Using the CELLxGENE Database, a comparison of hundreds of public datasets of single-cell sequencing of blood, lung, vasculature, and skin tissues also shows minimal expression of Bcl-2 in endothelial cells (105). Overall, these findings support the finding that Bcl-xL is the predominant, and often only, intrinsic anti-apoptotic protein expressed in endothelial cells.

Table 2: RNAseq Data of Endothelial Cells

		Raw Read Count Mock			Raw Read Count KSHV		
	Gene Symbol	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
TIME	ACTB	272254	250615	211350	155623	98234	133551
	BCL2L1	13304	12474	10840	9965	5111	6159
	BCL2	54	80	85	31	18	33
	MCL1	26847	28148	25504	24685	15526	20522
BEC	ACTB	223131	278812	296647	135683	147443	205780
	BCL2L1	9073	10394	11984	7740	6628	10578
	BCL2	12	23	23	11	19	11
	MCL1	14789	18857	24599	16311	16163	22260
LEC	ACTB	275183	248862	291521	176084	124272	167083
	BCL2L1	9745	10664	11015	6444	6045	6850
	BCL2	48	32	52	13	23	44
	MCL1	18634	18909	21704	16427	14351	17615

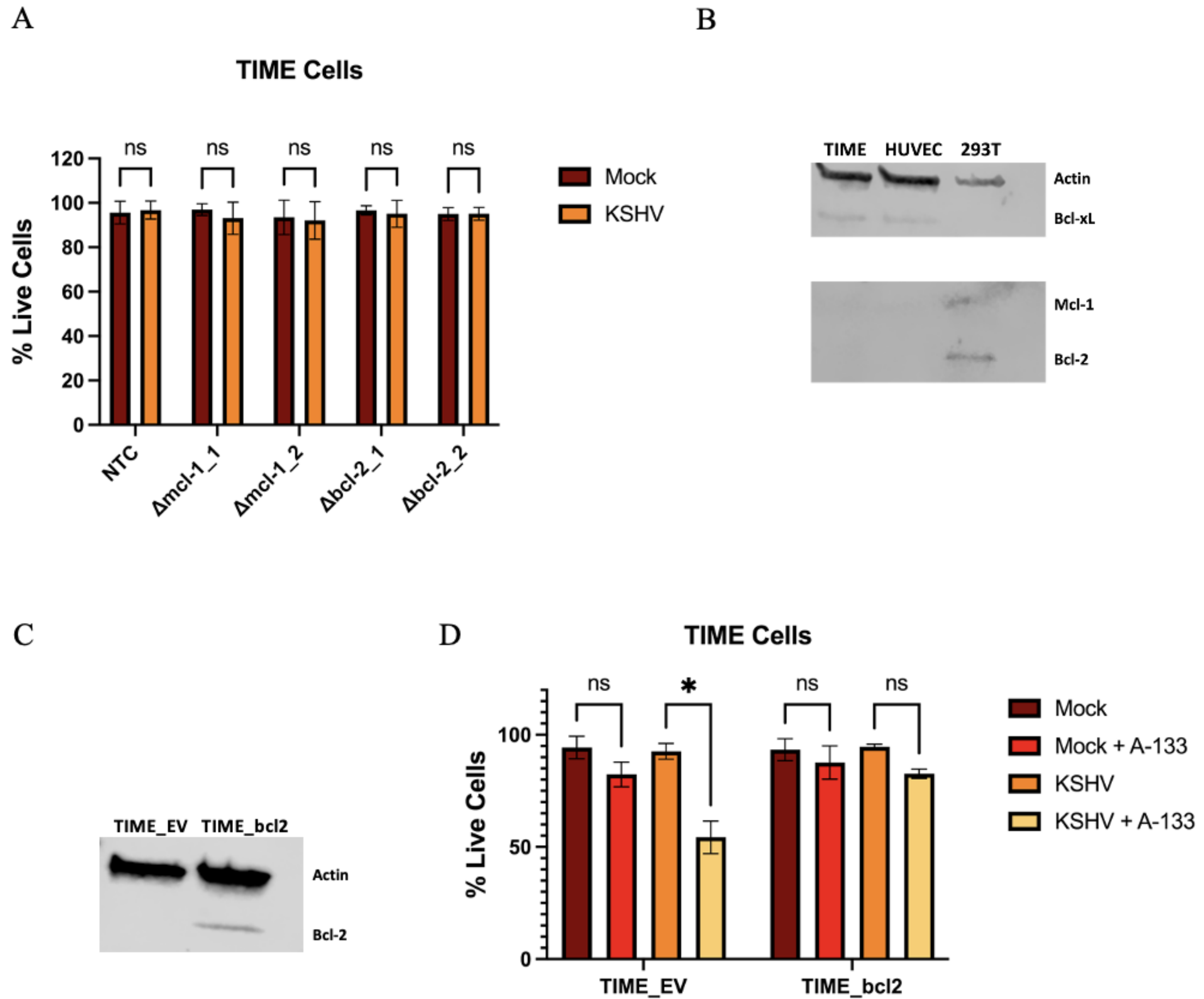


Figure 3.6: Bcl-xl is specifically required for survival in endothelial cells due to limited expression of other Bcl-2 anti-apoptotic proteins: (*A*) TIME cells were transduced with the indicated sgRNAs and selected as before and were subsequently mock or KSHV infected. Cell viability was measured with a trypan blue assay 48 hpi. (*B*) Western blot analysis of TIME, HUVEC (primary endothelial), or 293T cells with antibodies to Bcl-xL, Mcl-1, and Bcl-2. (*C*) Western blot analysis with an antibody to Bcl-2 in TIME cells transduced with empty vector (EV) or plasmid overexpressing Bcl-2. (*D*) TIME cells were transduced with empty vector or plasmid overexpressing Bcl-2 and selected for using puromycin. Transduced cells were mock or KSHV infected. 48 hpi A-133 (10 nM) or vehicle control was added to cells and cell viability was measured 12 hr after A-133 addition using Cell-Cyte. Data are presented as mean ± s.d. (2-way ANOVA, n = 3) *P<0.05.

Since other anti-apoptotic proteins are not expressed in endothelial cells, we wanted to determine if exogenous expression of Bcl-2 could rescue infected cells with Bcl-xL inhibited from death. TIME cells were transduced with an empty vector or plasmid overexpressing Bcl-2 (Figure 3.6C). Cells were then infected with KSHV for 48 hours and then A-133 was added to cells. Infected cells expressing Bcl-2 were protected from death when Bcl-xL was inhibited (Figure 3.6D). Since another anti-apoptotic protein from the same family rescues Bcl-xL deficient cells from death, these findings further show that Bcl-xL is needed for the survival of infected cells because of its anti-apoptotic activity, not a specific function of the Bcl-xL protein.

3.3.6 Bcl-xL is not required for survival during KSHV latent infection in B-cells.

Since KSHV is also known to cause lymphomas in B cells, we next wanted to determine if Bcl-xL was required for the survival of B cells. We knocked out Bcl-xL in BCBL-1 cells, a KSHV-infected cell line derived from a patient diagnosed with PEL. BCBL-1 cells transduced with either the non-targeting control or Bcl-xL sgRNAs were seeded at 1 x 10^5 cells/mL and allowed to grow for 4 days. After 4 days, cells were harvested, and cell viability was measured. When Bcl-xL was knocked out in BCBL-1 cells, we saw no marked differences in cell survival when compared to the nontargeting control (Figure 3.7A). Cell survival appears lower for both the control and Bcl-xL knockout cells likely due to an artifact of the antibiotic selection the BCBL-1 cells used to select for retroviral transduction of the guide RNAs. We also treated BCBL-1 cells with the A-133 inhibitor. BCBL-1 cells were seeded at 1 x 10^5 cells/mL and 24 hours later the cells were resuspended in growth media supplemented with A-133. 4 days post-treatment cells were harvested, and viability was measured. There was no difference in survival rates between BCBL-1 cells treated with growth media alone or growth media supplemented

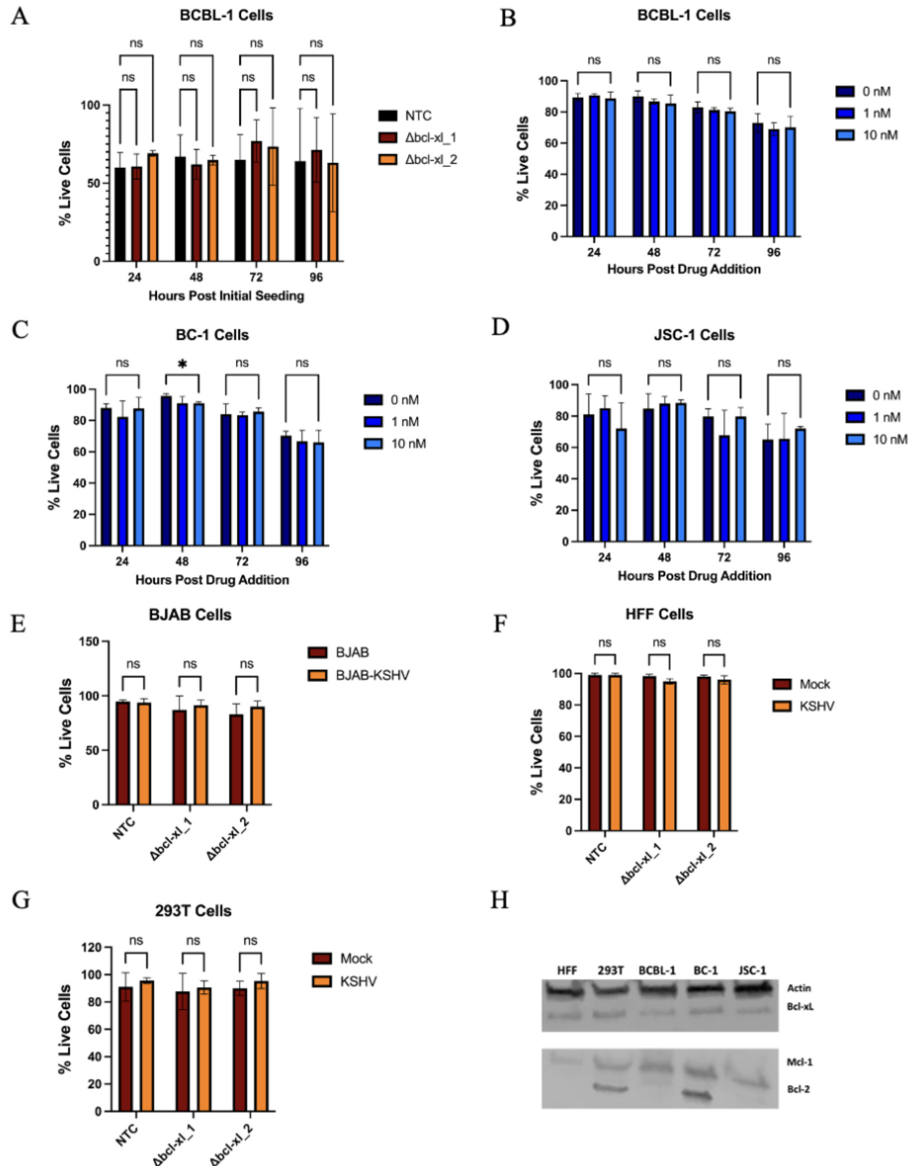


Figure 3.7: Bcl-xL is not required for survival during KSHV latent infection in B cells: (*A*) BCBL-1, cells were transduced with sgRNAs selected for using puromycin resistance for 4 days, then seeded at 1 x 10^5 cells/mL. Cell viability was measured using a trypan blue assay at 24, 48, 72, and 96 h post seeding. (*B*) BCBL-1 cells were seeded at an initial concentration of 1 x 10^5 cells/mL and growth media was supplemented with or without A-133 at 1 nM or 10 nM. Cell viability was measured using a trypan blue assay at 24, 48, 72, and 96 hpi. (*C*) BC-1 or (*D*) JSC-1 were treated with A-133 as in (B). (*E*) BJABs were transduced with sgRNAs and treated as in (*A*). (*F*) 293T cells or (G) HFF cells were transduced and selected for sgRNAs to Bcl-xL. Transduced cells were mock or KSHV infected and cell viability was measured using a trypan blue assay 48 hpi (F) or measured by counting live (Syto59) and dead (YoYo-1) cells using Cell-Cyte (G). (*H*) Western blot analysis with antibodies to Bcl-xL, Mcl-1, and Bcl-2 in cell lines tested above. Data are presented as mean ± s.d. (2-way ANOVA, n = 3) *P<0.05.

with A-133 (Figure 3.7B). To determine if this was unique to BCBL-1 cells, we looked at a range of different B cells. Two additional KSHV positive PEL lines, BC-1 and JSC-1s, as well as a KSHV and EBV negative B-cell line, BJAB, were also not found to require Bcl-xL for survival during infection (Figure 3.7C-E). We also knocked out Bcl-xL in additional cell types that were latently infected with KSHV. Bcl-xL was also not required for the survival of KSHV latently infected primary human foreskin fibroblasts (HFF cells), nor for latently infected HEK-293T cells, an immortalized cell type (Figure 3.7F-G). This suggests the requirement of Bcl-xL for survival during latent infection is specific to endothelial cells. We investigated the expression of Bcl-xL, Bcl-2, and Mcl-1 in these cell types. All cell types express Bcl-xL and they all also express Mcl-1. A more limited number of these lines also expressed Bcl-2 (Figure 3.7H). The expression of multiple anti-apoptotic proteins could explain why these cell types do not require Bcl-xL during infection, as they have another Bcl-2 family anti-apoptotic protein acting in a redundant fashion to Bcl-xL to protect latently infected cells from death.

3.4 Discussion

The anti-apoptotic Bcl-2 family member, Bcl-xL, is specifically required for the survival of endothelial cells latently infected with KSHV. Bcl-xL is required to inhibit apoptosis during KSHV infection as caspase-3/7 was activated by inhibition of Bcl-xL, PARP cleavage was induced, and caspase inhibitors blocked the induced cell death in the absence of Bcl-xL. Knockout of Bcl-xL demonstrated that it was required during the establishment of latency. Importantly, inhibition of Bcl-xL after the establishment of latency led to death of over half of the cells within six hours indicating that Bcl-xL is required for the maintenance of latency. Thus, KSHV latent infection must induce and maintain the induction of intrinsic apoptosis in the

infected endothelial cell and require the cellular anti-apoptotic protein, Bcl-xL, to prevent cell death throughout the course of latency.

Other anti-apoptotic Bcl-2 family members have many redundant functions in common with Bcl-xL; in particular, binding to pro-apoptotic Bcl-2 family members to prevent release of cytochrome C which initiates the caspase cascade. Other anti-apoptotic Bcl-2 family members including Bcl-2 and Mcl-1 are not required for the survival of KSHV infected endothelial cells. Interestingly, neither Bcl-2 protein nor Mcl-1 protein could be detected in multiple types of cultured endothelial cells. There was negligible detection of the mRNA for Bcl-2 in RNAseq data from multiple endothelial cell types and much lower Bcl-2 reads in RNAseq data from KS tumors (Table 3.2). Despite significant redundant functions with Bcl-xL, latently infected endothelial cells are only reliant on Bcl-xL and not Mcl-1 nor Bcl-2. While KSHV is known to cause both KS and PEL, Bcl-xL was not required for survival in PEL cells, nor any other cell type examined likely due to higher expression of Mcl-1 and/or Bcl-2 in addition to Bcl-xL in these cell types. In support of this, it was recently shown that Mcl-1 was required for the survival of multiple primary effusion lymphoma lines while Bcl-2 and Bcl-xL were not found in that study (19). However, there are no KSHV negative PEL cell lines so, in contrast to the current studies, the studies in PEL lines cannot differentiate if KSHV infection directly leads to the requirement of anti-apoptotic proteins or if this requirement arose during the formation of the lymphoma after infection (42). Using uninfected and KSHV infected primary and immortalized endothelial cells, we unequivocally demonstrated that KSHV latent gene expression drives the requirement for Bcl-xL by inducing intrinsic apoptosis during latency. Overall, it appears that inhibition of intrinsic apoptosis is required for survival of cells latently infected with KSHV, but

different cell types support expression of different anti-apoptotic Bcl-2 family members to achieve this.

These findings present Bcl-xL as a potential therapeutic target for KSHV infections of endothelial cells. KS tumors are composed predominantly of latently infected spindle cells, cells expressing endothelial cell markers. Since Bcl-xL is not required for survival in uninfected cells or other cell types infected with KSHV, treatment with a Bcl-xL inhibitor would be more selective for killing of endothelial cells latently infected with KSHV, thus providing a more KSHV specific treatment. The anti-apoptotic Bcl-2 family members have been proposed in many cancer studies as strong therapeutic targets (57,91). Venetoclax, a Bcl-2 inhibitor, has shown robust anti-tumor effects against several malignancies, and is already FDA approved (106,107). Dual Bcl-2/Bcl-xL inhibitors, like ABT-737 and APG-1252, have also been used to treat various malignancies (108,109). Early Bcl-xL studies have had issues with cytotoxic effects (110,111). However, AstraZeneca has recently developed AZD0466, a dual Bcl-2/Bcl-xL inhibitor conjugated to a PEGylated poly-lysine dendrimer, that has reduced the cardiovascular issues and thrombocytopenia seen with other Bcl-2/Bcl-xL inhibitors (112–114). Pre-clinical trials using AZD0466 have shown AZD0466 can inhibit tumor growth in mouse xenograft studies, providing the potential of using a drug to inhibit Bcl-xL to treat KS in the future.

Chapter 4

THE KSHV KAPOSINS INDUCE APOPTOSIS DURING LATENT INFECTION MAKING BCL-XL REQUIRED FOR THE SURVIVAL OF LATENTLY INFECTED CELLS

Lyndsey N. Moore, Michael Lagunoff

Department of Microbiology, University of Washington, Seattle, WA, USA

4.1 Abstract

Bcl-xL is required for the survival of KSHV latently infected endothelial cells. In this study we explored mechanisms behind why Bcl-xL is required for survival. Expression of the KSHV major latent locus alone in the absence of KSHV infection led to sensitivity to the absence of Bcl-xL. This indicates that viral gene expression from the latent locus is sufficient to induce intrinsic apoptosis leading to the requirement for Bcl-xL in endothelial cells. Further, knockout of single KSHV latent genes during infection revealed that the kaposins, but no other latent gene or miRNA, were necessary for the requirement of Bcl-xL. The finding that a single KSHV latent gene is sufficient to induce apoptosis during latent infection is interesting, as it

provides evidence that latent infection is far from quiescent. Rather, this study provides further evidence that KSHV latent infection induces dramatic changes to the host cell during infection.

4.2 Introduction

The observation that Bcl-xL is required for survival in endothelial cells during latent infection with KSHV is interesting and merits further study as a potential therapeutic target. However, the previous work does not address why Bcl-xL is required, only that it is. Viruses are known to alter the host cell, but most findings are in the context of lytic infection. Since Bcl-xL, a host gene, is required only during latent KSHV infection, this suggests the virus induces cellular changes that render Bcl-xL necessary. More specifically, I hypothesize KSHV latent infection induces cellular changes that trigger the activation of the intrinsic apoptotic pathway.

When describing viral infections, latency and dormancy are often used interchangeably. However, more recent work has furthered our understanding of latency-associated changes to the host cell. It has been a wide-held belief that during latency, the viral infection is quiescent and has little effect on the host cell it has infected, most likely as a strategy of immune avoidance. Indeed, a totally quiescent viral genome would be the most logical route to avoid triggering the host immune response. Instead, HCMV has been shown to express select number of viral transcripts that alters the host cell in a way that would support cell survival and preservation of the latent genome (115,116). KSHV's miRNAs have been shown to inhibit cholesterol synthesis, possibly suppressing cellular innate immune functions (117). EBV's LMP1 protein enhances glycolysis, which has been associated with infected cells' transformation characteristics and increased cell viability and proliferation (118). So, it is apparent that latent viruses are changing the host cell environment for their own benefit.

I found that KSHV's latent locus is sufficient to induce the requirement of Bcl-xL for survival. This suggests a KSHV viral gene(s) is changing the host cellular environment in such a way that it is activating intrinsic apoptosis. It is unlikely the virus has evolved to induce apoptosis during latent infection as that would be antagonistic to its survival. So, it reasons that KSHV is inducing a cellular change that triggers the apoptotic pathway but does not lead to cell death since Bcl-xL is present.

4.3 Results

4.3.1 The KSHV major latent locus alone induces the requirement for Bcl-xL.

To determine if KSHV latent gene expression causes the host cellular changes rendering Bcl-xL necessary for survival, we determined if expression of the KSHV latent locus was sufficient to render Bcl-xL necessary for survival. We previously generated a gutted adenovirus that expresses the KSHV-latency associated region (KLAR) but no adenovirus genes (70). This virus expresses the entire major latent locus including LANA, vCyclin, vFLIP, the Kaposin locus, and the 12 KSHV miRNA loci all under their native promoters. The same gutted adenovirus containing only the GFP marker (ad-GFP) was used as a negative control during infection. TIME cells were transduced with sgRNAs to Bcl-xL or a nontargeting control. Transduced cells were infected with either the ad-GFP or ad-KLAR virus for 48 hours. At 48 hours post-infection, cells were harvested, and viability was measured. Successful high-level infection was verified by using IFA to look for GFP-positive or LANA positive cells (data not shown). Bcl-xL knockout cells that were infected with ad-GFP showed no increase in cell death while those infected with ad-KLAR experienced a significant increase in cell death, showing the

expression of the KSHV latent locus alone is sufficient to induce host cell changes that render Bcl-xL necessary (Figure 4.1).

4.3.2 The KSHV Kaposins are required to render Bcl-xL necessary.

To determine if a specific KSHV latent gene is required for the requirement of Bcl-xL we used a series of mutant viruses lacking single KSHV latent genes. These mutant viruses were produced using bacterial artificial chromosome (BAC) technology. We used a wild-type BAC16 virus (WT), viruses lacking either the v-cyclin (Δv-cyc) or vFLIP (ΔvFLIP) genes, and a mutant virus lacking the 12 KSHV miRNAs (Δ12). TIME cells were infected with mutant viruses. 48 hpi, cells were treated with the A-133 inhibitor at 10 nM and cell death was monitored using the Cell-cyte as described previously. The survival rates for the mutant viruses were similar except for the A-133 treated cells infected with the Δ12 virus (Figure 4.2A). However, it is worth noting that infection rates for the Δ12 virus were ~10% lower than the other viruses, so the decreased amount of cell death could be attributed to the lower infection rates.

To further evaluate if the KSHV miRNAs are required to render Bcl-xL necessary we determined whether any single miRNA was sufficient to induce cell death when Bcl-xL was inhibited. TIME cells were transduced with plasmids overexpressing single miRNAs that contained a GFP marker. Successful transduction was determined by looking at GFP expression. Transduced TIME cells were then treated with a vehicle control or the A-133 inhibitor. Cell death was measured 24 hours after the addition of A-133 using a trypan blue assay. The expression of no single miRNA was sufficient to induce cell death when Bcl-xL was inhibited (Figure 4.2B). This suggests that either the reduced cell death observed in cells infected with Δ12

virus is in fact due to reduced infection levels, or that multiple miRNAs are required to induce death in cells lacking functional Bcl-xL.

To determine if the KSHV kaposins were essential for the requirement of Bcl-xL TIME cells were infected with WT BAC16 KSHV or a mutant lacking kaposins A, B, and C (ΔKap) using spin-oculation. The A-133 inhibitor was added 4 hpi and cell death was measured using a trypan blue assay 48 hpi. There was a significant increase in cell death observed in cells infected with the WT virus that had been treated with the A-133 inhibitor. The increased cell death observed in cells treated with the vehicle control is an artifact of the spin-oculation process. There was no significant difference in survival in cells infected with ΔKap when Bcl-xL was inhibited (Figure 4.2C). This suggests the kaposins are required to render Bcl-xL necessary.

4.3.3 Evidence the kaposins could be inducing mitochondrial stress during KSHV latent infection through its interaction with mitochondrial protein CHCHD2.

To determine the mechanism by which the kaposins are inducing apoptosis, we looked for potential protein interactions between the kaposins and host cell proteins. Work from Davis *et al* mapped herpesvirus-host protein complexes for KSHV. The kaposins B and C, also denoted as K12B and K12C were found to interact with a small subset of proteins, most of which were heat shock proteins. However, one protein, CHCHD2, was of particular interest (119). CHCHD2 is a mitochondrial protein that plays a role in regulating electron flow in the electron transport chain and acts as transcription factor (120).

To determine if KSHV infection had any impact on CHCHD2, cells were infected with the KSHV WT BAC16 virus. Cells were harvested 24- and 48-hours post-infection and stained for CHCHD2. In mock infected cells, CHCHD2 stains in a very distinct pattern that is

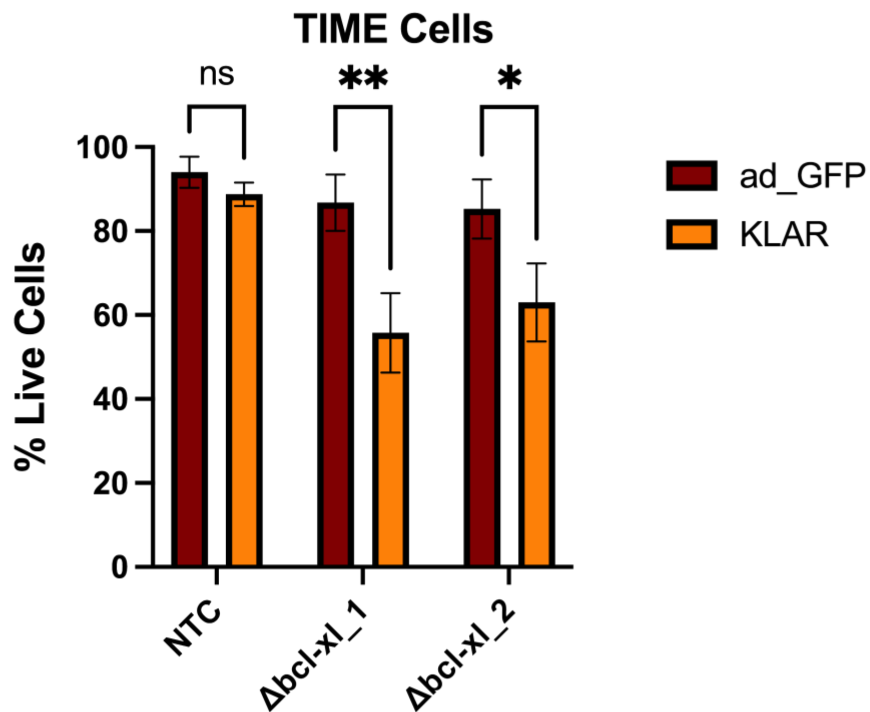


Figure 4.1: Expression of KSHV latent locus is sufficient to render Bcl-xL necessary for survival: TIME cells were transduced and selected for sgRNAs to Bcl-xL or non-targeting control and infected with either a control gutted adenovirus expressing only GFP (ad-GFP) or a gutted adenovirus virus expressing only the KSHV latent locus (KLAR). 48 hpi cell viability was measured using a trypan blue assay. Data are presented as mean ± s.d. (2-way ANOVA, n = 4) *P<0.05.

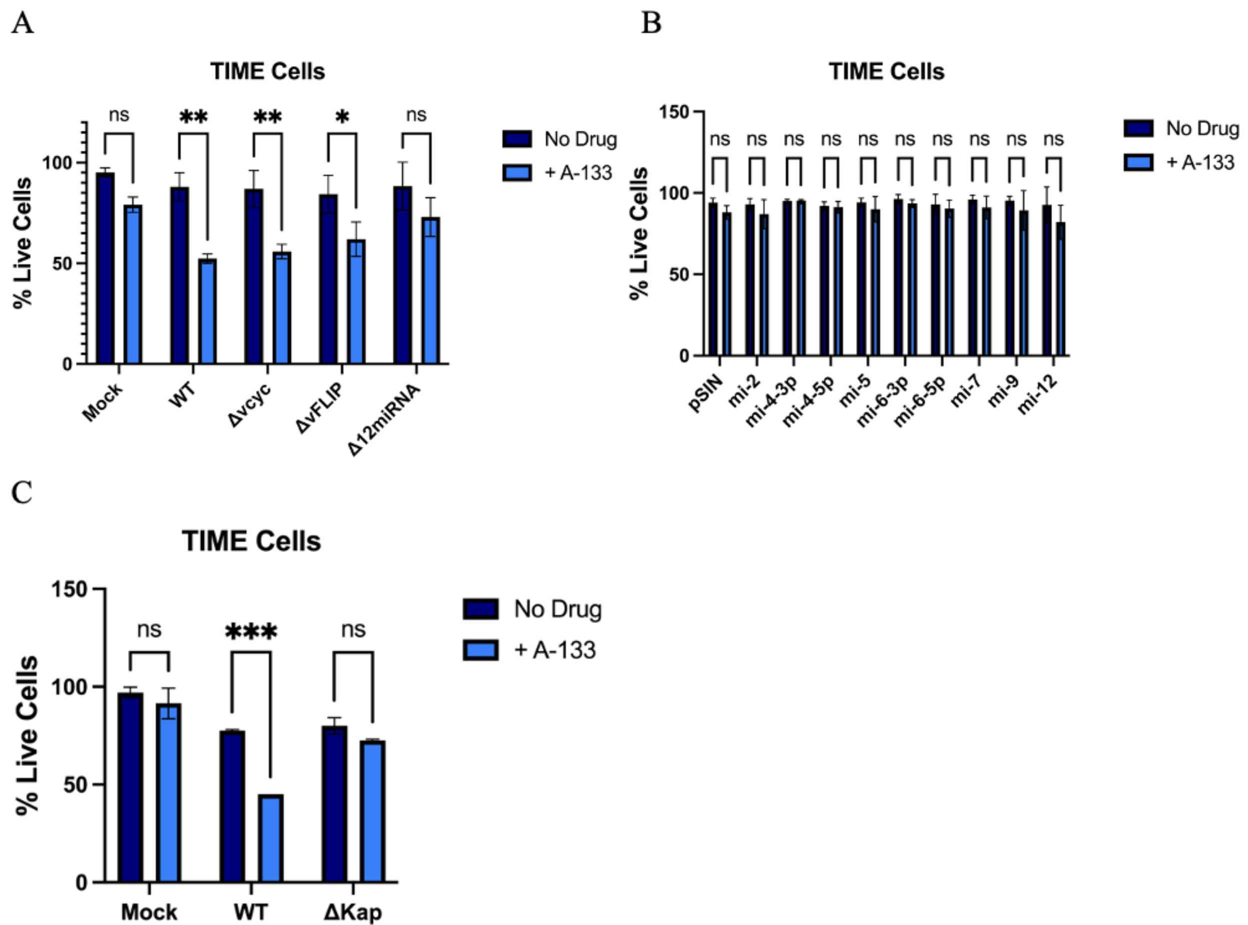


Figure 4.2: The KSHV Kaposins are required for the induction of apoptosis in the absence of Bcl-xL: *(A)* TIME cells were infected with one of the BAC16 mutant viruses or mock infected. A-133 (1 0nM) was added 48 hpi and cell death was measured 24 hours after drug addtion using Cell-Cyte. *(B)* TIME cells were transduced with lentivirus overexpressing the indicated miRNA or control empty vector (pSIN). 48 hours after transduction A-133 (10 nM) was added and cell death was measured 24 hours after drug addition using trypan blue assay. *(C)* TIME cells were infected with WT or ΔKap mutant virus or mock infected using spin-oculation. A-133 (10 nM) was added 4 hpi and cell death was measured 48 hpi using trypan blue assay. Data are presented as mean ± s.d. (2-way ANOVA, n ≥2) *P<0.05.

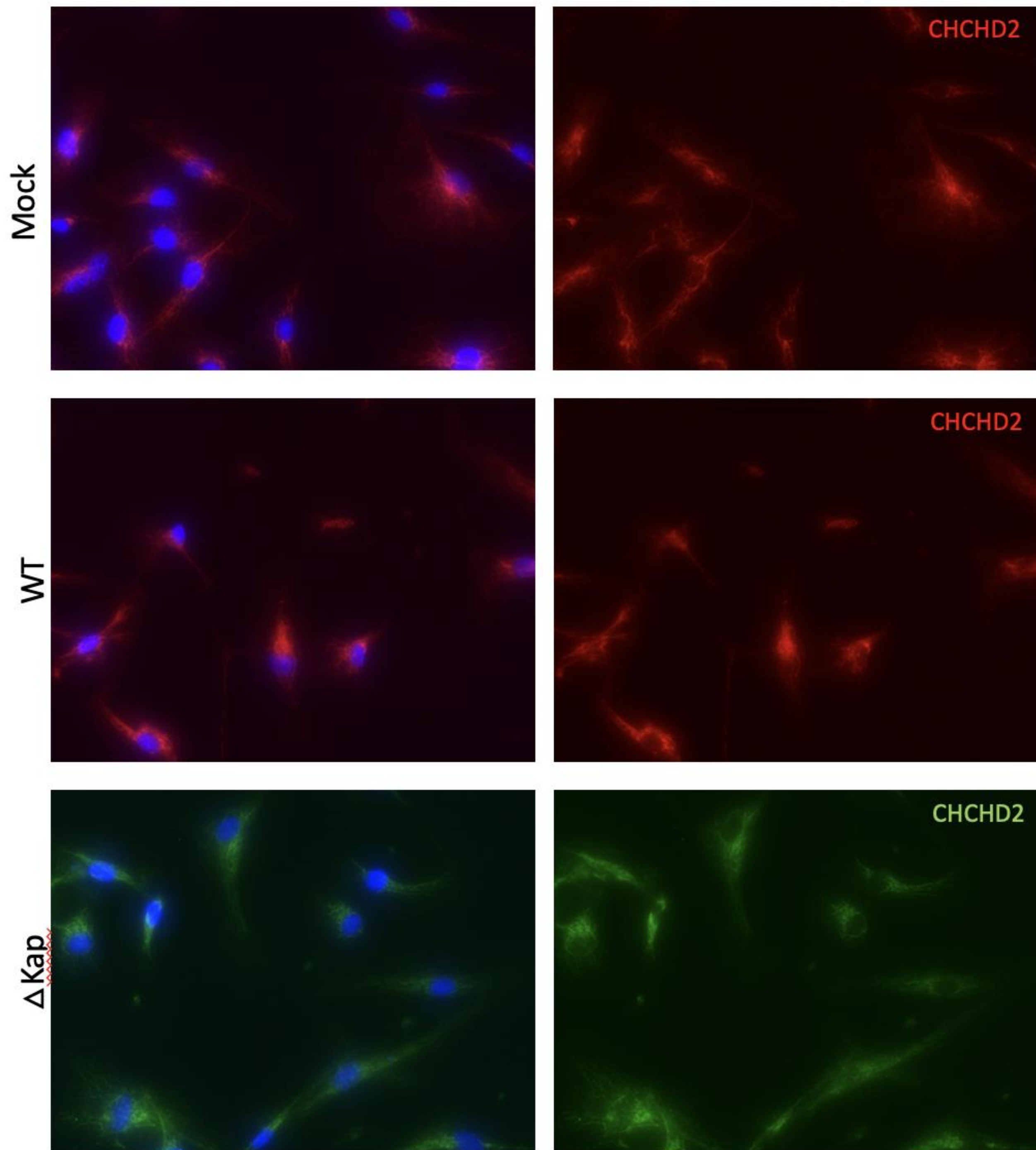


Figure 4.3: CHCHD2 expression in KSHV infected TIME cells 24 hours-post-infection. Representative images of mock, WT, and ΔKap infected TIME cells at 24 hpi stained with antibody to CHCHD2 and DAPI to identify nuclei. Merged images on left.

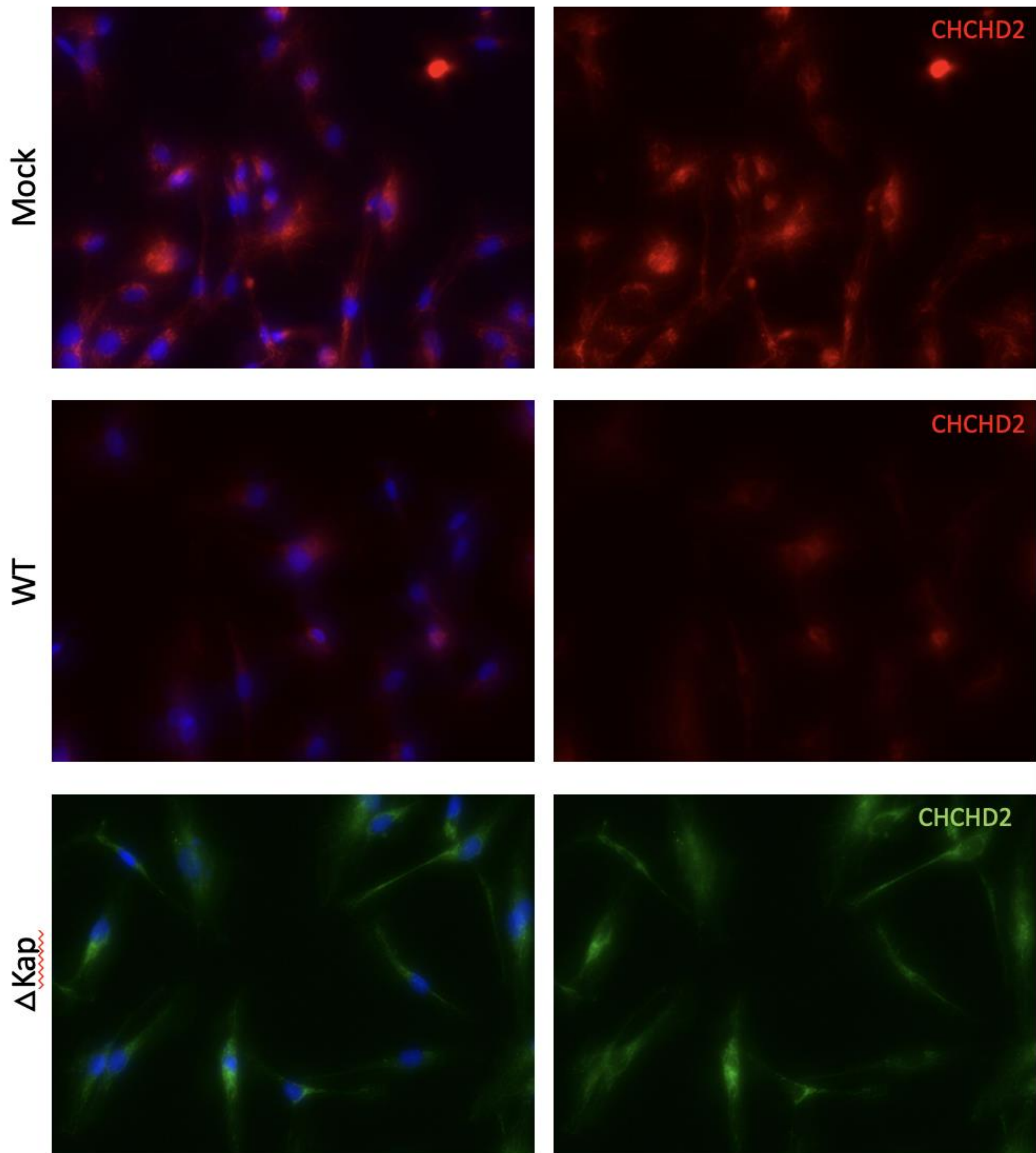


Figure 4.4: CHCHD2 expression in KSHV infected TIME cells 48 hours-post-infection. Representative images of mock, WT, and ΔKap infected TIME cells at 48 hpi stained with antibody to CHCHD2 and DAPI to identify nuclei. Merged images on left.

indicative of it being localized to mitochondrial structures (Figure 4.3). Interestingly, at 48 hours post infection, the distinct staining pattern for CHCHD2 is lost in WT infected cells, but not mock infected. The staining appears far more diffuse and suggests CHCHD2 has relocated to the cytosol (Figure 4.4). Intriguingly, in the cells infected with the ΔKap mutant, CHCHD2 appears to still be staining distinct structures, with far less cells displaying diffused CHCHD2 staining. This suggests the kaposins could be involved in regulating CHCHD2 during latent infection.

4.4 Discussion

Expression of the KSHV latent locus in the absence of KSHV infection was sufficient to render Bcl-xL necessary for survival. This suggests a KSHV latent gene induces intrinsic apoptosis and must rely on cellular anti-apoptotic functions to prevent cell death. Of the four gene loci and 12 miRNA loci expressed from the major latent locus, only the kaposins were found to be required to make Bcl-xL necessary for survival during KSHV latent infection. It is unlikely that the virus has evolved to induce intrinsic apoptosis, therefore, the kaposins likely activate a pathway sensed by the host cell that leads to an apoptotic response.

Several changes occur in the host cell during latent viral infection that could lead to induction of intrinsic apoptosis. We have previously identified major alterations in cellular metabolism during latent infection as well as the activation of oncogenic signaling pathways and changes in endothelial cell differentiation during latent infection of endothelial cells (37,70). It is possible that activation of one or more of these cellular pathways leads the cell to respond to the dramatic changes in cellular signaling by inducing mitochondrial apoptosis. Interestingly, we recently found that KSHV latent infection changes the size and number of mitochondria, indicating that the mitochondria themselves are altered by KSHV infection (43). The

mitochondria are intimately associated with intrinsic apoptosis and therefore, changes to the mitochondria could lead to activation of pro-apoptotic genes located in the mitochondrial membrane. Activation of these proteins must be blocked by the anti-apoptotic protein Bcl-xL for the cell to survive.

David *et al* identified over 500 virus-host interactions using mass spectrometry to identify herpesvirus-host protein interactions. The KSHV K12B and K12C proteins, also known as the kaposins, were found to interact with six host proteins: CHCHD2, BAG2, DNAJC7, FBL, HSPA7, and HSPA9 (119). The majority of these are heat shock proteins, except for CHCHD2. CHCHD2 belongs to the mitochondrial coiled-coil-helix-coiled-coil-helix (CHCH) domain protein family. CHCHD2 and CHCHD10, another CHCH protein, localize primarily to the mitochondrial cristae (121). These proteins likely play a role in mitochondrial respiration, and there is evidence that CHCHD2-CHCHD10 complexes are necessary for efficient mitochondrial respiration. Interestingly, loss of CHCHD10 has been shown to result in a hyperfused mitochondrial network. Mitochondrial fusion is a common response to nutrient starvation and increased oxidative stress (122). The hyperfusion that occurs in stressed cells is hypothesized to be protective since it can temporarily increase ATP production, block mitophagy, and delay cell death (123). We have observed evidence that there is a decreased number of mitochondria that are larger in size during KSHV infection. These findings suggest KSHV induces increased mitochondrial fusion. The increased mitochondrial fusion could be in response to the increased ROS present during KSHV infection (124). Regardless of what initiates the mitochondrial fusion, I would argue that increased mitochondrial fusion is evidence of mitochondrial stress.

It could be possible that the kaposins interact with CHCHD2 and this induces mitochondrial stress. The mitochondrial stress response would be enough to induce apoptosis in

cells. However, with Bcl-xL present, KSHV infected cells do not undergo apoptosis induced by mitochondrial stress because they have this anti-apoptotic protein to inhibit it.

The finding that KSHV kaposins are required to make Bcl-xL necessary for survival during infection is interesting on several fronts. First, it supports the idea that latent KSHV infection is not quiescent, but instead, is a process that actively changes the host environment. Second, the association of kaposin B and C with the CHCHD2 protein provides rationale that some mitochondrial-mediated event is the intrinsic death stimuli that activates the intrinsic apoptosis pathway. Indeed, it appears that KSHV latent infection changes the localization of CHCHD2 from mitochondrial to cytosolic. Additionally, infection with the ΔKap mutant maintains CHCHD2 localization similar to mock infected, suggesting the kaposins are either directly or indirectly regulating CHCHD2. CHCHD2 and its paralogue CHCHD10 play a role in mitochondrial respiration and form complexes with each other (121). A depletion of CHCHD10 has been linked to mitochondrial hyperfusion and our lab has shown preliminary evidence of increased mitochondrial fusion during KSHV infection (24,122). There is evidence that prolonged mitochondrial fusion can lead to caspase-dependent death (125). It is possible that KSHV-induced mitochondrial stress is the apoptotic trigger during latent infection that Bcl-xL is inhibiting.

Chapter 5

VALIDATION OF TOP HITS FOR ESSENTIALITY FROM A CRISPR/CAS9 SUB-POOL SCREEN OF KSHV LATENTLY INFECTED ENDOTHELIAL CELLS

Lyndsey N. Moore, Daniel L. Holmes, Jessica Lee, Michael Lagunoff

Department of Microbiology, University of Washington, Seattle, WA, USA.

5.1 Abstract

Our lab previously performed a global CRISPR/Cas9 screen and subsequent sub-pool screen to identify genes necessary for survival or proliferation of latently infected cells. While Bcl-xL has been characterized, additional hits were identified which could provide therapeutic targets. Validation will provide additional therapeutic targets for treatment of KSHV latent infection. It will also provide insight to the accuracy of the sub-pool screen. This work investigates three additional genes from the top 25 scored hits from the sub-pool screen. CYP27A1 is identified as necessary for the proliferation of KSHV latently infected cells, while two other hits, YLPM1 and CMKLR1, are found to be non-essential for KSHV latent infection.

The validation of CYP27A1 presents another potential therapeutic for KSHV treatment and will eventually provide more insight into how KSHV changes the host cell.

5.2 Introduction

The sub-pool screen identified many genes that could potentially be required during KSHV latent infection. The Bcl-xL project was proof that the screen could accurately identify a gene required for survival during KSHV latent infection. However, it is unknown if any of the other genes identified would also be essential during infection. *Cyp27a1*, *Ylpm1*, and *Cmklr1* were among the top 10 hits from the sub-pool screen.

CYP27A1, also known as sterol 27-hydroxylase, is a cytochrome P450 enzyme responsible for catalyzing hydroxylations in bile acid synthesis and bioactivation of vitamin D_3. It has also been discovered that CYP27A1 plays a role in cholesterol homeostasis through the formation of oxysterols and the elimination of cholesterol from cells (126). Little is known about the role of CYP27A1 in KSHV latent infection, but previous work has shown that cholesterol biogenesis is downregulated during by KSHV latent genes. Another cholesterol derivative, 25-hydroxycholesterol (25HC) has been shown to block KSHV *de novo* infection of primary endothelial cells, as well as downregulate LANA expression (127).

YLPM1, or YLP motif-containing protein 1, has not been very well studied. It is a cytosolic protein known to enable RNA binding activity. It has also been predicted to be involved in the regulation of telomere maintenance (128).

CMKLR1, or chemerin chemokine-like receptor 1, protein is a G-protein-coupled receptor for chemerin, which plays a role in the recruitment and activation of macrophages, natural killer cells, and dendritic cells in inflammatory diseases. It has been shown to contribute

to inflammation in the lungs (129). However, chemerin does not appear to be strictly pro or anti-inflammatory, rather it depends on the biological system. Studies have shown that chemerin acts directly on its receptor CMKLR1 to affect adipogenesis, angiogenesis, and inflammation in adipose tissue (130).

5.3 Results

5.3.1 Cyp27a1 is required for the proliferation of KSHV latently infected cells

The CRISPR/Cas9 live cell screen cannot differentiate between whether a gene is necessary for survival or proliferation. To determine if CYP27A1 was required for survival during latent KSHV infection, TIME cells were transduced with a non-targeting control (NTC) sgRNA or one of two sgRNAs targeting CYP27A1 (Table 5.1). We determined that there was sufficient knockout of CYP27A1 by using Inference of CRISPR Edits (ICE) Analysis (Table 5.2). Briefly, ICE analysis uses Sanger sequencing data to predict the frequency of indel outcomes and provides a score that reflects knockout efficiency of the sgRNA used. Control and knockout cells were infected with KSHV. 24 hours post-infection cells were reseeded. Upon reseeding, the media was supplemented with Syto59 and Yoyo-1, live and dead cell dyes respectively. Cells were placed into Cell-Cyte and images were taken every 12 hours for 4 days. Total live and dead cells were enumerated, and viability was assessed. Knockout of CYP27A1 had no effect on the survival of KSHV latently infected cells compared to uninfected (Figure 5.1A). There is a slight reduction in the survival of knockout cells around 72-84 hours, but the difference was not significant. To assess effects on cell proliferation, NTC or knockout cells were mock or KSHV infected. 24 hpi, cells were seeded and allowed to proliferate over the course of 8 days after which the cells were fixed and stained with crystal violet.

Table 5.1 ICE Analysis Scores

Gene	Guide	ICE Score
Cyp27a1	1	81
	2	80
Ylpm1	1	81
	2	47
Cmklr1	1	72
	2	86

The area stained was used as measure of cell confluence. Knockout of CYP27A1 caused a significant proliferation defect in the Δcyp27a1_1 cells, and a similar but not statistically significant defect in Δcyp27a1_2 cells when mock infected, demonstrating a general growth defect (Figure 5.1B). The decreased proliferation observed in knockout cells that were latently infected with KSHV was far more pronounced (Figure 5.1C). This suggests KSHV infection contributed to further decreasing growth rates and CYP27A1 is required for the proliferation of KSHV latently infected cells.

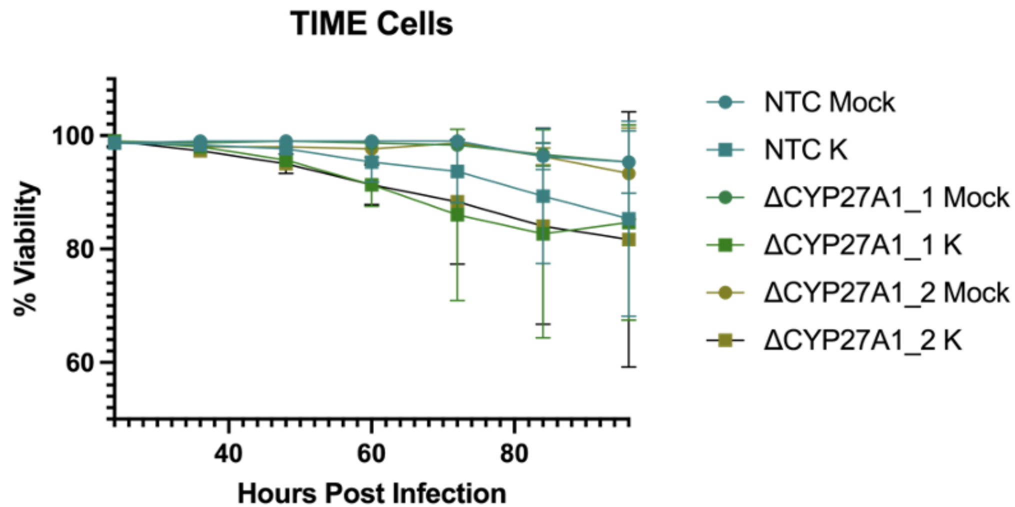
5.3.2 Ylpm1 is not essential for KSHV latently infected cells.

To determine if YLPM1 is essential during latent KSHV infection, TIME cells were transduced with a non-targeting control (NTC) sgRNA or one of two sgRNAs targeting YLPM1 (Table 5.1). It was determined that there was sufficient knockout of YLPM1 using ICE analysis (Table 5.2). Control and knockout cells were infected with KSHV. 24 hours post-infection cells were reseeded for survival and proliferation assays as performed previously. There was no observable difference in survival rates when YLPM1 was knocked out (Figure 5.2A). Knocking out YLPM1 also had no effect on the proliferation rates of mock or KSHV infected cells (Figure 5.2B-C). One caveat to these findings is the low ICE analysis score for YLMP1 sgRNA 2.

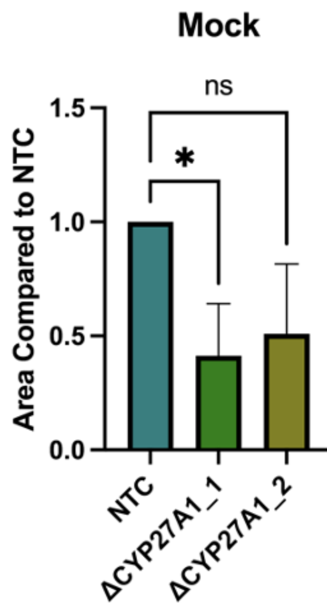
5.3.3 Cmklr1 is not essential for KSHV latently infected cells.

To determine if CMKLR1 was essential during latent KSHV infection, TIME cells were transduced with a non-targeting control (NTC) sgRNA or one of two sgRNAs targeting CMKLR1 (Table 5.1). It was determined that there was sufficient knockout of CMKLR1 by ICE

A



B



C

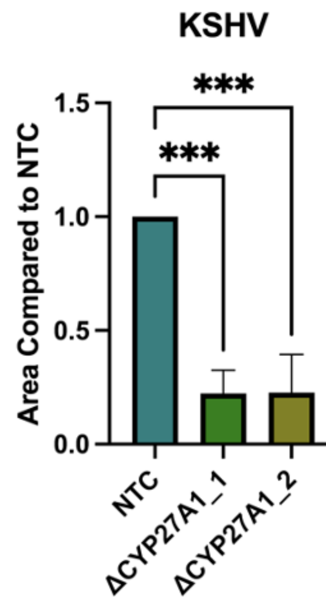
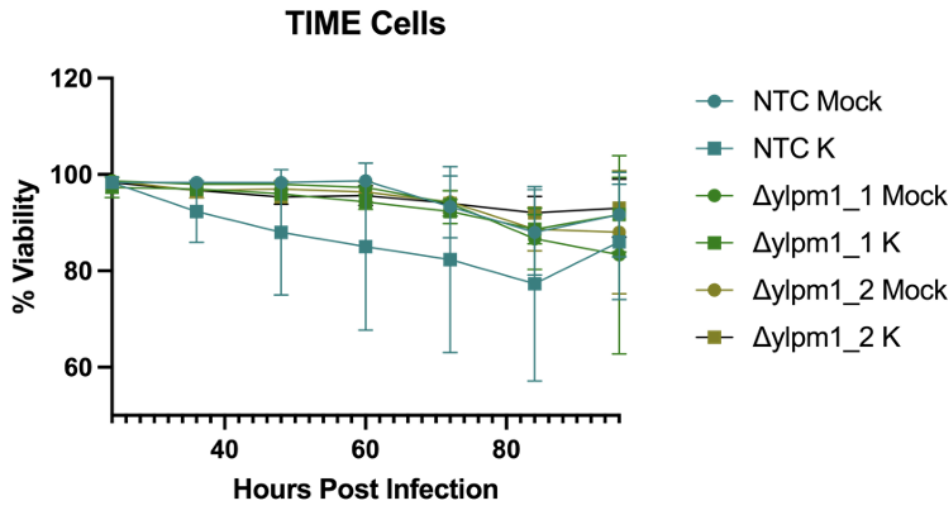
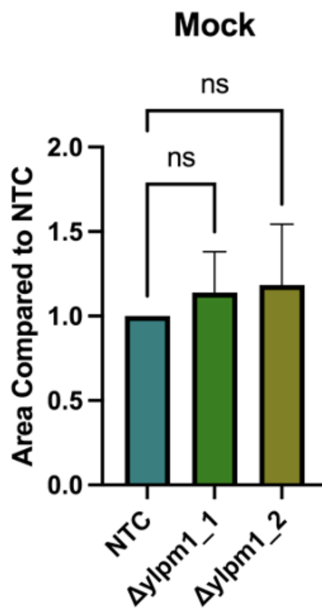


Figure 5.1: CYP27A1 is required for the proliferation of KSHV latently infected cells: *(A)* TIME cells were transduced with the indicated sgRNAs and mock or KSHV infected. Cells were stained with Syto59 (live cell) and YoYo-1 (dead cell) dyes. Total live and dead cells were calculated using Cell-Cyte to determine cell viability over course of 96 hours. TIME cells were transduced with indicated sgRNAs and mock or KSHV infected. Cells were allowed to proliferate for 8 days and were fixed and stained with crystal violet. Area of crystal violet was measured. The area of knockout cells was compared to NTC during mock *(B)* or KSHV *(C)* infection.

A



B



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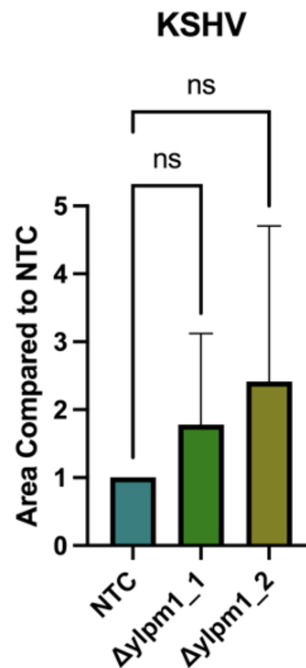


Figure 5.2: YLPM1 is not essential for KSHV latently infected cells: *(A)* TIME cells were transduced with the indicated sgRNAs and mock or KSHV infected. Cells were stained with Syto59 (live cell) and YoYo-1 (dead cell) dyes. Total live and dead cells were calculated using Cell-Cyte to determine cell viability over course of 96 hours. TIME cells were transduced with indicated sgRNAs and mock or KSHV infected. Cells were allowed to proliferate for 8 days and were fixed and stained with crystal violet. Area of crystal violet was measured. The area of knockout cells was compared to NTC during mock *(B)* or KSHV *(C)* infection.

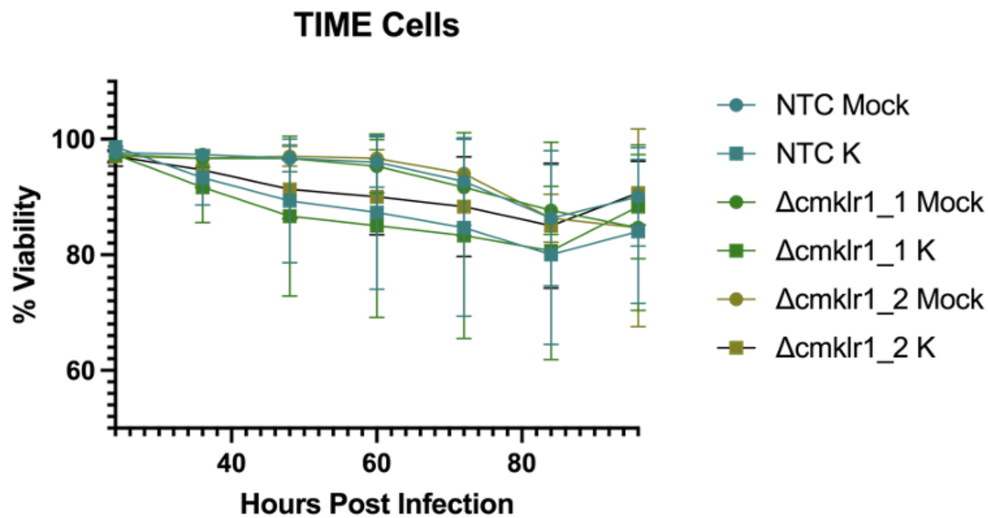
analysis (Table 5.2). Control and knockout cells were infected with KSHV. 24 hours post-infection cells were reseeded for survival and proliferation assays as before. There were no observable differences in survival when CMKLR1 was knocked out during infection (Figure 5.3A). Knocking out CMKLR1 also had no effect on the proliferation rates of mock or KSHV infected cells (Figure 5.3B-C). Interestingly, if anything, the knockout of CMKLR1 during infection trended towards increasing proliferation rates.

5.4 Discussion

The sub-pool screen identified a large number of genes that could potentially be essential during KSHV latent infection. This study sought to validate some of those top hits for their potential as therapeutic targets. Knockout of CYP27A1 was found to inhibit proliferation of KSHV latently infected TIME cells significantly more than uninfected, and while not conclusive, these experiments warrant further study of CYP27A1. Other studies have shown that KSHV miRNAs repress several genes in the mevalonate pathway. The mevalonate pathway is a sequence of reactions leading to the production of farnesyl pyrophosphate, a common substrate for the synthesis of cholesterol. Not only are genes in this pathway repressed, but there is evidence that *de novo* KSHV infection, or the KSHV miRNAs alone, will reduce total and free cholesterol levels in cells (117). This suggests that some product of the mevalonate pathway is antiviral and KSHV is attempting to decrease levels of this antiviral by repressing the mevalonate pathway. 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), and 24-hydroxycholesterol (24HC) are all cholesterol derivatives formed as cholesterol is used to synthesize bile acids. There is evidence that 25HC has antiviral properties, as it could be

decreasing KSHV viral gene expression. It has also been shown to decrease proliferation and induce apoptosis in cells infected with EBV (127). CYP27A1 is the enzyme responsible for

A



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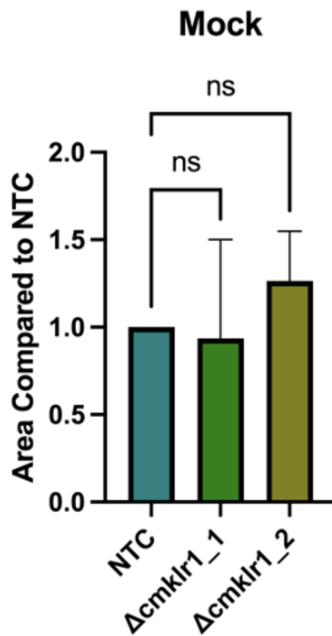

C

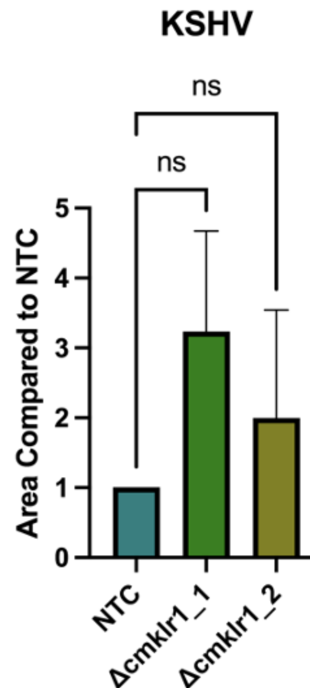

Figure 5.3: CMKLR1 is not essential for KSHV latently infected cells: *(A)* TIME cells were transduced with the indicated sgRNAs and mock or KSHV infected. Cells were stained with Syto59 (live cell) and YoYo-1 (dead cell) dyes. Total live and dead cells were calculated using Cell-Cyte to determine cell viability over course of 96 hours. TIME cells were transduced with indicated sgRNAs and mock or KSHV infected. Cells were allowed to proliferate for 8 days and were fixed and stained with crystal violet. Area of crystal violet was measured. The area of knockout cells was compared to NTC during mock *(B*) or KSHV *(C)* infection.

converting cholesterol into 27HC. It is interesting that one cholesterol derivative, 25HC, would be antiviral against KSHV, but 27HC would seemingly be required in cells latently infected with KSHV. It could be possible that deleting the enzyme that converts cholesterol to 27HC shunts more cholesterol in the 25HC direction, thereby increasing the amount of antiviral 25HC in the cells. Or perhaps 27HC is proviral as opposed to its antiviral counterpart, since its role in KSHV has not been studied.

The work in this chapter also highlights important details regarding CRISPR/Cas9 screens. As CRISPR screens gain popularity for their unbiased and efficient approach to identifying genes/pathways involved in certain phenotypes and biological processes, additional validation will be important to eliminate false positives. It is not unusual for top-ranked hits to contain false positives, which can arise from off-target effects, random fluctuations, or any biases present in the screen. Further, the precise ranking of hits can be noisy even in successful screens, especially if observed differences are small (131). Two groups have shown that gene-independent targeting of genomic amplifications in human cancer cell lines reduces proliferation and/or survival. They suggest the lethality occurs from an excessive DNA damage response due to CRISPR cutting rather than gene inactivation (132). Overall, this chapter highlights the importance of validating top hits in CRISPR/Cas9 screens.

Chapter 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

KSHV is the etiologic agent of KS. KS gained notoriety during the AIDS epidemic of the 1980s. During the 1980s, individuals with HIV-AIDS were 20,000 times more likely to have KS than someone in the general population. While the incidence of KS has fallen since the late 90s in the United States, KS still remains a major public health issue in Africa. KS is endemic in regions of central and southern Africa where it is among the most common tumors and a leading cause of death for both men and women. KSHV also causes two other B cell lymphomas, PEL and MCD. Unfortunately, there are no great treatments for KSHV-associated diseases.

The main proliferating agent in KS is the spindle cell. While its exact origins are unknown, the spindle cell is thought to be of endothelial origin. KSHV, like other herpesviruses, can establish both lytic and latent infections. In KS, however, spindle cells infected with KSHV are predominantly latently infected rather than lytically infected. More than 90% of infected cells are latently infected. During latency, the full viral genome is maintained within the host cell, but gene expression is dramatically restricted, and no viral particles are produced. This is particularly problematic when it comes to developing treatment options. Current antivirals do not work for

KSHV because they target lytically infected cells, and therapeutics targeting latent infection are difficult to develop because of the reduced gene expression and lack of virions produced.

As opposed to looking at viral factors, our lab has focused on targeting host cellular factors needed by KSHV latently infected cells. Previously, a lot of focus has been on metabolic requirements for KSHV latent infection. The lab has shown KSHV both induces and requires several metabolic pathways, including glycolysis, glutaminolysis, and fatty acid synthesis, for the survival of KSHV latently infected cells (37,133). To continue identifying host factors and processes necessary for KSHV latently infected cells, the lab took a broad approach and performed a genome wide CRISPR/Cas9 screen that targeted over 18,000 human genes to identify genes essential only during latent infection. The top hits from this screen were used to perform a subsequent sub-pool screen. Those screens have provided a long list of potential essential genes for KSHV latent infection. Further, we have identified mitochondrial translation as an essential process for KSHV infection in endothelial cells (43).

This work follows up on the top hit identified in the duplicate sub-pool screens, Bcl-xL. Bcl-xl is an anti-apoptotic protein from the Bcl-2 protein family whose main function is to inhibit intrinsic, or mitochondrial-mediated, apoptosis. The intrinsic apoptosis pathway is signaled by a variety of signals, not limited to absence of particular growth factors, cytokines, radiation, hypoxia, and viral infections. Once stimulated, pro-apoptotic pore-formers from the Bcl-2 protein family will cause permeabilization of the mitochondrial membrane leading to release of cytochrome c, which binds Apaf-1 and pro-caspase-9 to form an apoptosome complex. This activates caspase-9 which goes on to activate caspase-3, an executioner caspase, and results in apoptotic cell death (45,92).

I found that Bcl-xL is required for the survival of KSHV latently infected endothelial cells. Inhibiting Bcl-xL with the Bcl-xL specific inhibitor A-1331852 (A-133) has the same effect as knocking it out using CRISPR/Cas9. When A-133 is added to TIME cells 48 hours post-infection, there is rapid death of infected, but not uninfected cells. This combined with time course experiments following total cell death in knockout and A-133 treated cells beginning 4 hours post-infection suggests that Bcl-xL is required for the maintenance of KSHV latent infection.

Bcl-xL was found to be inhibiting a form of caspase-mediated cell death during KSHV latent infection. Since there are several caspase-mediated death modalities, additional experiments were performed to demonstrate evidence of PARP cleavage and caspase-3/7 activation indicating Bcl-xL is inhibiting apoptosis. While these experiments do not differentiate between extrinsic and intrinsic apoptosis, the extensive literature that characterizes Bcl-xL's role in mitochondrial-mediated apoptosis allows us to surmise that Bcl-xL is specifically inhibiting intrinsic apoptosis during KSHV latent infection.

Surprisingly, neither Mcl-1 nor Bcl-2 were found to be required for the survival of KSHV infected cells, which was interesting considering their redundant functions. However, there was no expression of Bcl-2 nor Mcl-1 at the protein level in any endothelial cells tested. Bcl-2 is also not expressed at the transcriptional level, which is further supported by single cell sequencing data from various projects studying endothelial cells. Several other groups have looked at the protein expression of Bcl-2 and Mcl-1 in endothelial cells and those studies also provide evidence that those proteins are not expressed in endothelial cells (102,103). All of this supports the novel discovery that Bcl-xL is the only anti-apoptotic Bcl-2 family protein expressed in endothelial cells.

KSHV, in addition to KS, causes PELs, which are a B cell lymphoma. This led to the question of whether or not Bcl-xL was required during KSHV latent infection in any other cell types. Bcl-xL was not found to be required in any of the other cell types tested, which included various B cells, 293Ts, and HFFs. However, all of these cell types expressed Mcl-1 and some expressed Mcl-1 and Bcl-2, in addition to Bcl-xL. These cells most likely do not require Bcl-xL because they express additional anti-apoptotic proteins that can compensate for loss of Bcl-xL during latent infection.

Chapter 3 of this thesis determined that Bcl-xL is required for the survival of KSHV latently infected endothelial cells. In Chapter 4, the reason behind why Bcl-xL is required is explored further. Experiments using the KLAR virus, a gutted adenovirus that only expresses the KSHV latent locus, determined that the latent locus alone is sufficient to induce cell death in cells lacking Bcl-xL. Thus, it can be surmised that one or more KSHV latent genes are responsible for inducing apoptosis.

Using a series of BAC16 mutant viruses lacking individual KSHV latent genes, the kaposins were the only latent gene found to be required to induce cell death in cells where Bcl-xL had been inhibited. While one could argue that the miRNAs are involved in inducing apoptosis, the lower infection rates combined with the experiments where single miRNAs were expressed to test if any miRNA was sufficient to induce apoptosis provide further credence to the conclusion that they are not involved in the phenotype being studied here.

It is unlikely the virus would evolve to induce apoptosis. Rather, the virus is likely altering the host cell in a way that is beneficial for the virus, but triggers apoptosis in the host cell. It does not matter to the virus that apoptosis has been induced since it has Bcl-xL present to inhibit any apoptosis that is triggered. For this reason, the role of the kaposins in inducing

apoptosis was explored. Previous research identified CHCHD2 as a host protein that interacts with KSHV's Kaposin B and Kaposin C. CHCHD2 is a mitochondrial protein involved in regulating the electron transport chain amongst other functions. Immunofluorescence experiments provided some evidence that during KSHV latent infection CHCHD2 translocated from the mitochondria to the cytosol.

The sub-pool CRISPR/Cas9 screen was able to successfully identify a gene that is essential for KSHV latently infected cells but not uninfected. Chapter 5 attempts to validate several other top hits from that same screen. *Ylpm1* and *Cmklr1* were amongst the top ten hits in the sub-pool screen. Neither were found to be essential for the survival or proliferation of KSHV latently infected TIME cells. However, *Cyp27a1* was found to be required for the proliferation of KSHV latently infected TIME cells.

6.2 Future Directions

6.2.1 Bcl-xL

This body of work demonstrates that Bcl-xL is required for the survival of KSHV latently infected endothelial cells. While it was apparent endothelial cells did not require Bcl-2 nor Mcl-1, the fact that knocking out Bcl-xL only resulted in approximately 50% of the infected cells dying is interesting. While much of this could be explained by the fact that not all cells are transduced with the Bcl-xL gRNA and not 100% of cells are infected, it could also mean that an additional anti-apoptotic protein is inhibiting cell death during latent infection. Bcl-w, Bfl-1, and Bcl-B are the other Bcl-2 family anti-apoptotic proteins. These are less studied than Bcl-xL, Mcl-1, and Bcl-2, but one of those three could still be inhibiting apoptosis during KSHV infection. It would be simple to answer that question by using sgRNAs to knockout those proteins and observe cell death during infection. If deletion of Bcl-w, Bfl-1, or Bcl-B also results in cell death

during infection then a double knockout should be made to determine if there is a synergistic effect. These experiments would be important to conclude that no other anti-apoptotic Bcl-2 proteins are needed for survival during infection.

Qualitative observations during Bcl-xL knockout survival assays identified some debris in the supernatant concurrent with cell death. Apoptotic cells do not have any rupture of their membrane, so it would be unexpected to see cellular content debris result from death occurring solely from apoptosis. These debris could also be debris from the viral infection. A simple LDH assay could be used to determine if any membrane rupture is occurring, which would help identify whether secondary necrosis is occurring as well. It is possible that other secondary forms of cell death are occurring after the initial apoptosis, such as pyroptosis. This could be assessed by using caspase-specific inhibitors to determine which specific caspase is needed for cell death to occur in the absence of Bcl-xL during KSHV latent infection.

6.2.2 Kaposins

It is extremely intriguing that the kaposins are required for survival during KSHV latent infection. It would be critical to perform additional experiments using the Δ12 miRNA virus where 90% infection rates are achieved to conclusively say they are not involved. The overexpression experiments indicate no single miRNA is sufficient to induce cell death in the absence of Bcl-xL, however, the nature of miRNAs makes it extremely unlikely that a single miRNA would be involved. Rather a combination of miRNAs would likely be necessary and/or sufficient.

There are three individual kaposins in the kaposin locus: Kaposin A, B, and C. More experiments can be done to ascertain if one specific kaposin is involved. The lab has several BAC16 mutant viruses that can answer this question. There is the ΔKap mutant in which

kaposins A, B, and C are all deleted. Then there are mutants lacking kaposins B and C, just kaposin B, and just kaposin C. I would hypothesize that kaposins B and C will both be necessary as they are both known to interact with the CHCHD2 protein. Results from these experiments could be further corroborated using kaposin overexpression experiments. Regardless of which specific kaposin(s) are necessary, the results will shed further light onto the way in which the KSHV kaposins are inducing apoptosis. I can additionally test to see if one or multiple kaposins are sufficient to induce apoptosis by overexpressing them in the absence of Bcl-xL.

Most importantly, the mechanism behind how KSHV is inducing apoptosis should be followed up on. There is significant evidence that it is the kaposins, and the experiments with the ΔKap mutant and localization of CHCHD2 suggest the mitochondria could be involved in the intrinsic death stimulus. First, the immunofluorescence assays should be repeated using confocal microscopy since greater resolution is needed to accurately depict mitochondrial structures. Additionally, the CHCHD2 stain should be used alongside a mitochondria specific stain, such as mitotracker, to confirm lack of colocalization of CHCHD2 with the mitochondria in KSHV infected cells. This can be approached orthogonally by using differential centrifugation to assess a loss of CHCHD2 from the mitochondrial fraction and an increase in CHCHD2 in the whole cell fraction in infected cells.

Since CHCHD2 is found in complex with its paralog CHCHD10, I would also investigate the localization and abundance of this protein as well. Especially since loss of these proteins has been shown to be associated with mitochondrial hyperfusion, which the lab has shown evidence of during infection. All of these experiments will undoubtedly shed more light onto how KSHV is inducing apoptosis during infection and whether or not mitochondrial stress is responsible for triggering induction of apoptosis.

6.2.3 CRISPR/Cas9 Sub-Pool Screen

While some work has been completed to validate the results of the sub-pool CRISPR/Cas9 screen on an individual gene basis, there is much left to be done. The top 25 hits from the sub-pool screen should be validated. Those genes should be knocked out using a minimum of two sgRNAs and their effects on the survival and proliferation of KSHV infected cells should be assessed. The screen has already successfully identified two targets, Bcl-xL and CYP27A1, and it is extremely likely other essential genes will be found amongst the top 25 hits.

6.3 Conclusion

This body of work has conclusively shown that Bcl-xL is required for the survival of KSHV latently infected endothelial cells. Importantly, it is not required in any other cell types. KS tumors are comprised primarily of latently infected spindle cells, which are thought to be of endothelial origin. The experiments in this thesis support the conclusion that Bcl-xL would be an attractive therapeutic target that would be specific to KS tumors. Excitingly, Bcl-2 family inhibitors have already been shown to be effective in treating disease. Previously, ABT-737 was discovered as the first dual inhibitor of Bcl-2 and Bcl-xL, but it had unfavorable pharmacokinetic properties. This led to the development of ABT-263, which made it to clinical trials in 2006. However, ABT-263 induced rapid and dose-dependent thrombocytopenia. The failures of ABT-737 and ABT-263 are what led to the development of ABT-199. ABT-199 is a Bcl-2 specific inhibitor, also known as Venetoclax, and is now FDA-approved. It has shown antitumor efficacy in adults and preliminary studies show it also has antitumor effects in pediatric malignancies as well (134). Specifically, it has successfully been used for the treatment of chronic lymphocytic leukemia and is actively being investigated for other hematological malignancies and breast cancer. More recently a new dual Bcl-xL/Bcl-2 inhibitor has been develop by AstraZeneca,

AZD0466, that has not demonstrated any long-term thrombocytopenia or cardiovascular issues like other similar drugs (135). This provides great promise for using a Bcl-xL inhibitor to combat KS.

Determining that the KSHV latent locus is sufficient to induce apoptosis is intriguing because it furthers our knowledge about how latent viruses change the host cells they infect. Latent viruses are anything but inert. KSHV has been shown to alter several aspects of the host cell, like host cell metabolism. And now, there is further evidence that KSHV is changing the host cell, and it is likely that one such change is what induces the apoptotic pathway. The association between the kaposins and the mitochondrial protein CHCHD2 once again highlights the mitochondria as playing a critical role in the survival of KSHV latently infected cells. More work needs to be done to tease apart how KSHV is changing the mitochondria either directly or indirectly. However, I hypothesize that the KSHV kaposins are interacting with the mitochondrial protein CHCHD2 and changing its localization from the mitochondria to the cytosol. This could be causing mitochondrial stress that is resulting in hyperfusion of the mitochondria. This mitochondrial stress could be the intrinsic death stimulus that Bcl-xL is protecting against during KSHV latent infection.

Finally, this work has identified a new potential therapeutic target for KSHV, CYP27A1. While much more work needs to be done to characterize the essentiality of this gene, the validation of another hit from the CRISPR/Cas9 screen is proof that the screen was able to identify several essential genes for KSHV latently infected cells.

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