Latent and Lytic KSHV Infection Require Altered Host Cell Metabolism

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Abstract

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Viruses are obligate intracellular parasites that depend on host cell machinery for their production and spread. Host metabolism is dramatically altered by several viruses to provide metabolic intermediates for biosynthetic as well as bioenergetic precursors. Understanding how a virus establishes and depends on a specific metabolic signature is critical to revealing possible therapeutic targets of infected cells. In this thesis, I further examined metabolic alterations during infection with the oncogenic virus, Kaposi's Sarcoma Associated Herpesvirus (KSHV). Like all herpesviruses, KSHV has both a latent and lytic viral life stage. Both latent and lytic cells are found in the KS tumor, therefore, I identified the metabolic alterations in both latent and lytic cells. Previous research indicated that glycolysis and fatty acid synthesis (FAS) were required during latent KSHV infection for the survival of latently infected endothelial cells. In Chapter 1, I confirmed the requirement for FAS during latent infection. After presenting the background (Chapter 1) and Methods (Chapter 2), in Chapter 3, I defined the critical role glutamine metabolism plays in endothelial cells infected with KSHV. I also identify the cellular mechanism by which glutamine

addiction is established during latency. Finally, in Chapter 4, I demonstrated that glycolysis, glutamine metabolism, and FAS are all required for maximal infectious KSHV virion production in lytically replicating cells. We show that each of these metabolic pathways contribute to virus production at a different step in lytic replication. Therefore, this body of work provides evidence that both latent and lytic KSHV infection depend on central carbon metabolism. These data may support the development or improvement of clinical therapies that target these carbon utilization pathways, thereby treating all infected cells of the KS tumor.

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DEDICATION

I dedicate this thesis to my parents, Laura and Robert Sanchez, who have supported and loved me unconditionally not only throughout graduate school, but my whole life. To you Mom and Dad, the hardest working people I know.
I love you!

Chapter 1. INTRODUCTION

1.1 HERPESVIRUSES

Herpesviridae is a large family of DNA viruses in animals and humans. The host-specific infectivity of herpesviruses signifies they have evolved with their hosts over long periods of time and are exceptionally well adapted to each host(1). Herpesvirus virions range from approximately 120-260 nm in diameter and contain large double stranded DNA genomes which range from 124-230 kb in length, encoding for between 70 and possibly 750 protein-coding genes(2). There are four distinguishing morphological features of herpesvirus virions: an inner core containing the viral genomic DNA, an icosahedral protein capsid, the presence of an amorphous tegument region surrounding the nucleocapsid, and finally, a lipid envelope in which virally encoded glycoproteins are embedded.

All herpesviruses demonstrate both a latent and lytic viral program. Latency is a defining feature of herpesviruses, characterized by the expression of very few, if any, viral genes(2). During this life stage, the viral genome is only replicated during cell division. All latently infected cells have the potential to reactivate to productive, lytic infection. However, triggers of lytic replication are poorly understood. During productive lytic infection, herpesvirus gene expression represents a classic regulatory cascade, with genes being transcribed temporally. Lytic gene expression begins with the transcription of immediate-early genes that require no new protein synthesis for their expression. Herpesviruses typically encode a few key immediate-early genes, which encode proteins required for activation of early gene transcription, regulation of the cell cycle, RNA transport and splicing, and modulation of host innate and adaptive immune responses. This is followed by expression of early genes whose transcription is independent of viral DNA synthesis, but requires immediate early protein synthesis. Most early proteins are involved in viral genome replication, helping to provide adequate pools of nucleotides for viral DNA synthesis, as well as

proteins and enzymes required for viral DNA synthesis. Finally, late gene expression is dependent on the replication of viral DNA. Late genes include structural proteins and glycoproteins for assembly of the virion. Viral gene expression during the lytic life cycle provides the proteins required for assembly of new infectious virions.

There are eight known human-specific *Herpesviridae* family members, Human Herpesvirus (HHV) 1-8. Furthermore, all human herpesviruses fall into one of three subfamilies based on their tropism and growth in culture: alphaherpesviruses, betaherpesviruses and gammaherpesviruses. Alphaherpesviruses include Herpes Simplex Virus 1 (HSV-1 or HHV-1), which typically causes oral cold sores, Herpes Simplex Virus 2 (HSV-2 or HHV-2), which characteristically causes genital sores, as well as Varicella-Zoster Virus (VZV or HHV-3), which causes chicken pox and when reactivated to lytic replication later in life can cause shingles. Betaherpesviruses include Human Cytomegalovirus (HCMV or HHV-5), HHV-6 and HHV-7. HCMV can cause birth defects, childhood fever, and in immunocompromised patients can cause pneumonia, gastroenteritis, retinitis and other diseases. HHV-6 causes roseola and childhood rash and HHV-7 is not known to cause disease. Finally, gammaherpesviruses include Epstein-Barr Virus (EBV or HHV-4), which results in mononucleosis and in some cases Burkitt's lymphoma, and Kaposi's Sarcoma Herpesvirus (KSHV or HHV-8) which causes Kaposi's Sarcoma (KS), an endothelial-cell based tumor, as well as two lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and multicentric Castleman's disease (MCD).

1.2 KSHV AND KS

While KS was first described in 1872 by Hungarian dermatologist Moritz Kaposi, it was not until 1994 that KSHV was identified as the etiologic agent of KS(3). In 1994, KSHV genomic DNA was identified in KS lesions, but not in normal tissue analyzed by representational difference analysis. After cloning and sequencing the entire KSHV genome, the virus was classified as a member of the lymphotropic herpesviruses. There are two groups within the lymphotropic

herpesviruses, the *lymphocrytoviruses* and the *rhadinoviruses*(2). The *Lymphocryptovirus* group includes EBV and its simian relatives, and the *rhadinovirus* group includes KSHV and the prototype primate virus, *Herpesvirus saimiri*, among other simian viruses. The KSHV genome is roughly 165-170 kb. Approximately 145 kb of the KSHV genome is comprised of a unique region where the viral genes are encoded. This gene rich region is surrounded by GC-rich terminal repeats on each side ranging from 20-25 kb each.

Most genes encoded by KSHV are conserved with other herpesviruses in six large gene blocks(2). These include viral genes necessary for DNA replication, viral gene expression and virion structural proteins. However, there are more than 15 genes within the KSHV genome that include the prefix "K" to identify that these loci are unique to KSHV. Many of these genes encode homologs of cellular genes that affect host cell growth, alter cell signaling and promote immune evasion(4). For example, K3 and K5, which encode modulators of immune recognition 1 and 2, specifically downregulate the MHC class I receptor, but not the MHC class II receptor, to inhibit the cytolytic arm of the immune system.

Like all herpesviruses, KSHV has both a latent and lytic phase of its life cycle. Latency has been confirmed as the default mode of infection of most cells in culture based on immunofluorescence (IFA) to detect viral proteins(5). The latent genome is maintained as a circular episome in the host cell nucleus and replicated during cell division. However, latent infection has the potential to reactivate to lytic replication and actively produce virus. Importantly, in endothelial tissue culture, as well as in KS tumors, KSHV establishes latency in >90% of infected cells, while only 1-5% of cells support lytic replication of the virus(6). Studies have shown that both latent and lytic viral cycles likely contribute significantly, however differently, to KS development(5).

During latency, only a few viral genes are expressed, including the latency-associated nuclear antigen (LANA-1), viral cyclin (vCyc), viral FLICE (FADD-like interleukin-1-β–converting enzyme) inhibitory protein (vFLIP), the Kaposin family members A, B and C, as well as a number of

viral microRNAs (miRNAs) expressed from 12 loci(7–10). While these transcripts provide signals for maintenance of the host cell, evasion of apoptosis, proangiogenic and inflammatory signals, as well as limit replicative potential, together, they have not been shown to fully transform endothelial cells or any other cell types *in vitro*(5). LANA is proposed to have several cellular functions as part of KSHV pathogenesis. For example, LANA tethers the viral episome to cellular chromatin to ensure proper segregation of viral genomes during cell division, and also promotes the replication of the latent viral genome through binding to the terminal repeats. Additionally, LANA binds and inhibits the function of the tumor suppressors p53 and pRb, and has been reported to cooperate with Myc to assist in Myc regulation during latent infection(11–13). Specifically, LANA was found to stabilize c-Myc by preventing the phosphorylation of c-Myc at a key tyrosine residue, which when phosphorylated leads to Myc-induced apoptosis. Furthermore, LANA was found to independently stimulate phosphorylation of c-Myc at a key serine residue, which transcriptionally activates c-Myc. Together, these activities most likely allow for the continued expression of Myc-induced cellular genes and for the continuation of host cell cycle progression(11).

vCyc is expressed from the LANA promoter, and is the product of a specific splice variant of the mRNA transcript. vCyc activates cyclin-dependent kinase 6, but has also been shown to play other roles. vCyc expression can lead to cell division defects and polyploidy which can activate p53 and induce apoptosis. However, in the absence of functional p53, vCyc expressing cells will survive(14). In culture, vCyc overexpression can lead to senescence of mammalian cells(15).

vFLIP binds to the inhibitor of κB kinase-gamma (IKKγ), thereby activating the nuclear factor-kB (NF-κB)(2). This role has been linked to anti-apoptotic activity during oncogenesis. vFLIP expression also reduces autophagy and interestingly, is responsible for the spindle cell morphology of KSHV-infected endothelial cells. vFLIP was also shown by one group to cooperate with c-Myc to aid in the formation of primary effusion lymphoma (PEL) in mice(16). In this study, c-Myc overexpressing mice were crossed with vFLIP expressing mice. Because progeny developed distinct

tumors in a shorter time period, therefore the study proposed that there is a connection between vFLIP regulation of NF-κB and Myc in tumorigenesis.

The primary roles proposed for Kaposin A and Kaposin B, both transcribed from ORF K12, are their contribution to the inflammatory microenvironment established in the KS tumor(17). Finally, the latent KSHV miRNAs are thought to be involved in suppressing lytic reactivation of the virus and to influence endothelial cell differentiation and angiogenesis(5). Interestingly, the miRNA cluster has recently been credited with modulating glycolysis during latent infection as described more extensively later in the introduction(18).

During KSHV lytic infection, the entire viral genome is expressed in a temporally regulated transcriptional cascade(19). Comparable to other herpesviruses, KSHV immediate-early genes are expressed first. Immediate-early genes encode for transcription factors and regulatory proteins. The Replication and Transcription Activator (RTA/Orf50) protein, an immediate-early gene, is referred to as the lytic switch protein because it is sufficient for initiation of the lytic program by transactivating the lytic gene promoters. Early genes are expressed next in the temporal cascade, encoding proteins involved in viral DNA replication, as well as proteins that regulate host cell transcription and immune evasion. Once early genes are expressed, viral genome replication begins to produce many copies of the viral genetic material. Only once the viral genome has been replicated will late gene synthesis begin. Late genes encode viral structural proteins which are often needed in large amounts during virion assembly. Therefore, sufficient viral genome replication needs to take place before these proteins are synthesized, as these viral genomes are used for late gene transcription(2).

As stated above, the KS tumor is comprised of predominantly latently infected cells, though there is a low percentage of lytically replicating endothelial cells as well, and it is thought that both cell populations contribute to the development and maintenance of disease(5). KS is categorized into four classes of the disease: Classic, endemic, iatrogenic, and epidemic KS(4). Classic KS is a rare

neoplasm often characterized as a slow growing disease, occurring most often in elderly Mediterranean men, manifesting as skin lesions on the lower extremities. This form of KS is rarely life-threatening. Endemic (or African) KS is often more aggressive. This disease is seen in young children in endemic zones in sub-Saharan Africa. Epidemic or AIDS-related KS is frequently described as the most aggressive form of KS, occurring in immunodeficient patients infected with HIV. AIDS-related KS often affects more than the skin, commonly involving sites such as the lungs and gastrointestinal tract(5). Iatrogenic (or transplant KS) results in similar symptoms to that of epidemic KS, and is often more aggressive than classic KS due to the reduced immune system activity of patients on immunosuppressive therapy.

Although KS has different epidemiological forms, all forms display the same histopathology(20). The main proliferating cell in KS is called the spindle cell, named after its spindle-like shape. Spindle cells are of endothelial origin, expressing several markers of endothelium. Clinically, KS has been described to have three main stages of development: patch, plaque, and nodule. However, patients can present with lesions in several different stages of development. KS lesions contain aberrant vascularization with extravasated red blood cells and infiltrating inflammatory cells, resulting in the characteristic red and purple bruise-like appearance(5).

1.3 KSHV Latent and Lytic Cell Culture Systems

Many of the host cell modulations induced by KSHV are similarly activated in many cancer cells. Therefore, it is important that *in vitro* studies are conducted in primary or minimally immortalized cells. While information can still be gleaned from transformed cells, some cellular changes can be camouflaged by switches that are initiated by oncogenesis(21).

To this day, there are no reliable animal models of KSHV infection that lead to pathology similar to what is seen in human infection. To study KSHV *in vitro*, a few culture systems have been

established. Primary Effusion Lymphoma (PEL) cell-lines, also called body cavity based lymphomas can be isolated from patients and grown in culture. These lines stably maintain the KSHV viral episome in culture and can be passaged for *in vitro* experiments(22). One such line, BCBL-1 cells, has been used extensively. These cells can also be chemically induced to produce large amounts of WT KSHV for laboratory use to infect other cell types.

An efficient endothelial cell culture system has been established to study how KSHV infection causes KS at the cellular level. Tert-immortalized microvascular endothelial (TIME) cells recapitulate infection rates observed *in vivo* in KS tumors(6). Additionally, primary human dermal microvascular endothelial cells (1° hDMVEC) are also used. KSHV infection of both TIME cells and 1° hDMVECs support greater than 90% latent infection and 1-5% lytic replication. TIME cells are advantageous for lab use as they can be grown to over 100 passages in culture, while primary cells will senesce after 10-15 passages. Similarly to what is observed in cultured spindle cells, passage of KSHV latently infected endothelial cells results in gradual loss of virus as cells divide(23).

To examine KSHV lytic replication in the laboratory, TIME cells can be infected with WT KSHV and then superinfected with an adenovirus expressing the lytic switch protein RTA to induce the lytic life cycle. Recently, a new tissue culture system for virus production using inducible SLK cells (iSLK), a renal cell carcinoma that stably maintains the KSHV episome, has been established(24). With this inducible system, large amounts of virus can be produced for use in studying latent infection, but the iSLK cells can also be harvested to query changes in host cell molecular biology during virus replication, assembly and egress. However, this cell line is fully transformed and is not optimal for studies of oncogenic changes induced by KSHV.

1.4 ALTERED METABOLISM AND VIRAL INDUCED ONCOGENESIS

1.4.1 Cancer Cell Metabolism

Metabolic changes of human cells were first reported through examining metabolic modification of tumor cells. Several key metabolic pathways are dramatically altered in cancer cells(25–27). These alterations are thought to provide transformed cells with the necessary energy and biosynthetic substrates for rapid cell division and survival. In the 1920's, Otto Warburg determined that increased glycolysis and decreased oxidative phosphorylation were both characteristic metabolic alterations of solid tumors(28). Since these initial observations, several cancer cell models have shown similar changes in glycolysis and oxidative phosphorylation, which is now referred to as the Warburg effect.

Two additional carbon utilization pathways, Fatty Acid Synthesis (FAS) and glutamine metabolism or glutaminolysis, are also induced by carcinogenesis(29–31). Fatty acid synthesis is critical for the creation of lipid material for cellular membrane production (32). The core of fatty acid synthesis is the production of palmitate from acetyl-CoA and malonyl-CoA in a reaction that requires NADPH and is catalyzed by fatty acid synthase (FASN). In mammalian cells, the carbon substrates for fatty acid synthesis are generally derived from citrate, an intermediate of the Tri-carboxylic Acid (TCA) cycle(32). Once synthesized, palmitate can be further metabolized into a number of long chain fatty acids that can then be used in lipid production for membrane biosynthesis and lipid droplet formation. Lipid droplets are storage organelles for lipids, triacylglycerides and sterol esters and are also beneficial as energy storage for cells(33,34). Lipid droplet formation can be indicative of increased fatty acid synthesis. Increased lipid droplet formation prepares the cell for rapid membrane generation and provides an energy cache. Glutamine is a non-essential amino acid, however extracellular glutamine is often imported for multiple cellular metabolic pathways in cancer cells. Glutamine can be utilized for glutathione production, ammonia production, and purine synthesis through nitrogen donation among other

uses(35,36). Importantly, glutamine can be utilized in glutaminolysis, the catabolism of glutamine. In glutaminolysis, glutamine is converted to glutamate and then to alpha-ketoglutarate. Alpha-ketoglutarate can enter the mitochondria where it can be utilized as an intermediate of the TCA cycle. While glutamine is a non-essential amino acid, cancer cells often become glutamine addicted. Therefore, glutamine is considered "conditionally essential" in cancer cells which significantly increase the requirement for glutamine utilization compared to normal cells(29). Glutamine addiction is thought to occur due to the need for TCA cycle intermediates in cells where glycolysis predominates over oxidative phosphorylation. In many cancer cells, glucose carbon is shunted away from the TCA cycle both into lactic acid production as well as fatty acid synthesis. Glutamine is then required as an anaplerotic substrate to replenish the TCA cycle(35). Overall, alterations in glycolysis, FAS and glutaminolysis have been well established as hallmarks of cancer cell metabolism(37).

1.4.2 Viruses and Host Cell Metabolism

Viruses are non-living entities, and therefore do not inherently have their own metabolism. However, within the last decade, it has become clear that viruses dramatically modify cellular metabolism upon entry into a cell. Virus-induced metabolism can provide substrates needed for both latent maintenance of certain viruses as well as replication of the viral genome. Maximal viral genome replication, as well as, sufficient production of virion proteins and viral envelope lipids may all require altered metabolism.

While there were clear indications from studies in the 1950s and 60s that virus infection required altered metabolism for replication(38,39), large-scale identification of virus-induced metabolic pathways has been facilitated by recent advances in techniques such as mass spectrometry to measure global metabolite levels and measure carbon flux(40–43). The term metabolomics was coined to describe experiments where global cellular metabolite levels are

measured using a systems-level approach via mass spectrometry(44). These studies can yield a broader comparison of mock-infected and virus-infected cell metabolite levels and can identify changes in metabolic flux by following an isotope-labeled carbon substrate.

In 2006, the first eukaryotic virus-infected cell metabolomics study led the resurgence of the virus-induced metabolism field(40). The study analyzed over 60 metabolites during Human Cytomegalovirus infection (HCMV). Since this first studies, a number of viruses have been shown to alter multiple major metabolic pathways as shown by metabolomics studies and have expanded the number of metabolites measured(45,43,46–49,42). Core cellular metabolic pathways, including glycolysis, fatty acid synthesis and glutaminolysis, are significantly altered by multiple virus families, some of which have been well studied over the past few decades (Table 1.1). There appear to be both shared and unique metabolic signatures for each virus tested showing the importance of examining changes in metabolism for each virus species (Figure 1.1).

Global metabolite analyses have been bolstered by observations that drug inhibition of these major carbon pathways can specifically target infected cells to either significantly reduce viral titer or even induce cell death of infected cells(21). Inhibition of carbon metabolism via glycolysis, glutaminolysis and fatty acid synthesis have shown consistent effects on reducing production of infectious virions as well as inducing apoptosis of infected cells, but not uninfected cells.

1.4.3 KSHV and Host Cell Metabolism

In 2010, our lab was the first to report that endothelial cells latently infected with KSHV induce and require glycolysis and the establishment of the Warburg effect for their survival(50). In this paper, it was shown that infected endothelial cells increase the expression of glucose transporter protein 3 (GLUT3) and increase the amount of glucose uptake. Latently infected cells also increase the expression of the rate-limiting glycolytic enzyme hexokinase 2 (HK2) as well as the production and export of lactic acid, suggesting that glucose carbon is being metabolized via

glycolysis rather than oxidative phosphorylation for ATP production. These data were confirmed utilizing the Seahorse XF extracellular flux analyzer to sensitively and quantitatively measure and compare the acidification rate and oxygen consumption rate of mock-infected and KSHV latently infected cells. These data showed an increase in extracellular acidification during KSHV infection and a decrease in oxygen consumption, reinforcing the conclusion that the Warburg effect is established during latent KSHV infection of endothelial cells.

This paper also explored the requirement of aerobic glycolysis for the survival of endothelial cells during latent KSHV infection(50). Infected cells were sensitive to the glycolytic inhibitors 2-deoxy-glucose (2DG) and oxamate. 2DG inhibits the processing of glucose whereas oxamate inhibits the enzyme lactate dehydrogenase (LDH)(51–54). Both inhibitors induced significant levels of cell death of KSHV-infected cells, compared to mock-infected counterparts. Finally, it was found that inhibition of glycolysis induced apoptosis of KSHV-infected endothelial cells by first identifying an increase in the cleavage of the classic apoptosis markers Caspase-3 and PARP while also showing that cells treated with the pan-caspase inhibitor ZVAD were rescued from cell death upon treatment with oxamate. Together, these data confirmed that KSHV-infected endothelial cells induce and require the Warburg effect for their survival. This was the first evidence showing that altered metabolism is significant during latency. It has since been shown by another research group that the KSHV miRNAs are sufficient to induce the Warburg effect during latent KSHV infection(18), though it is not clear if they are necessary for this effect during latent infection.

Finding that the establishment of the Warburg effect was imperative to the survival of KSHV-infected endothelial cells, our lab was inspired to conduct a global metabolomics analysis to compare other metabolic changes during latent infection. These studies compared mock- and KSHV-infected samples via mass-spectrometry to measure the levels of nearly 200 metabolites, nearly one-third of which were altered by 96 hours post infection(43). Metabolites of several major

metabolic pathways commonly dysregulated by tumor cells were modified by infection, including glycolysis, amino acid metabolism, nucleotide metabolism and lipid metabolism. One of the most significant findings from this study was that over half of the long chain fatty acids measured were elevated by latent KSHV infection.

I confirmed that KSHV latent infection significantly increases the formation of lipid droplets in endothelial cells (Figure 1.2). Lipid droplets store cellular triacylglycerols, phospholipids and sterols, often providing a readily-available source of accessible energy as well as cellular membrane material. Furthermore, I found that UV-irradiated KSHV virus, which can bind and enter cells, but does not express any latent viral transcripts, did not induce the increase in lipid droplets. Therefore, we concluded that latent viral gene expression is required for establishing and maintaining an increase in lipid storage in lipid droplets. In this study, we also found that KSHV-infected endothelial cells are sensitive to drug inhibition of FAS. C75, a drug that inhibits the activity of fatty acid synthase (FASN) and TOFA, a drug that specifically inhibits the rate-limiting enzyme acetyl-coA carboxylase (ACC1) both induce cell death in KSHV-infected cells, but do not significantly kill mock-infected cells. Furthermore, I found that if KSHV-infected endothelial cells treated with TOFA were supplemented with the downstream metabolic intermediate of fatty acid synthesis, palmitic acid, a significant percentage of the cell population was protected from cell death (Figure 1.3). Together, these data indicate that the induction of lipid synthesis is required for the survival of endothelial cells latently infected with KSHV. Work completed in PEL cells confirmed that fatty acid synthesis is also required for the survival of several B-cell lymphomas(55). Current work in our lab is focused on identifying the viral gene or gene-set responsible for establishing an endothelial cell dependence on long chain fatty acids.

1.5 HYPOTHESIS

Cancer cells require alterations in metabolism for their survival. Induction of metabolism is now accepted as a hallmark of cancer(56). KSHV is an oncogenic virus that results in a tumor where over 90% of the endothelial cells are latently infected and approximately 1-5% of the cells are undergoing lytic replication. Our lab has previously shown that latent KSHV infected cells require both glycolysis and fatty acid synthesis for their survival(43,50). Additionally, other groups have found that HCMV, a betaherpesvirus that lytically replicates in culture, requires glycolysis, fatty acid synthesis and glutamine metabolism for maximal virus production(40,42,41,57–63). I hypothesize that the reprogramming of host cellular metabolism is required for both the maintenance of latent KSHV infection as well as productive KSHV lytic replication. Identifying alterations in metabolism shared by both latently infected cells and lytically replicating cells could reveal therapeutic targets to treat the entire KS tumor.

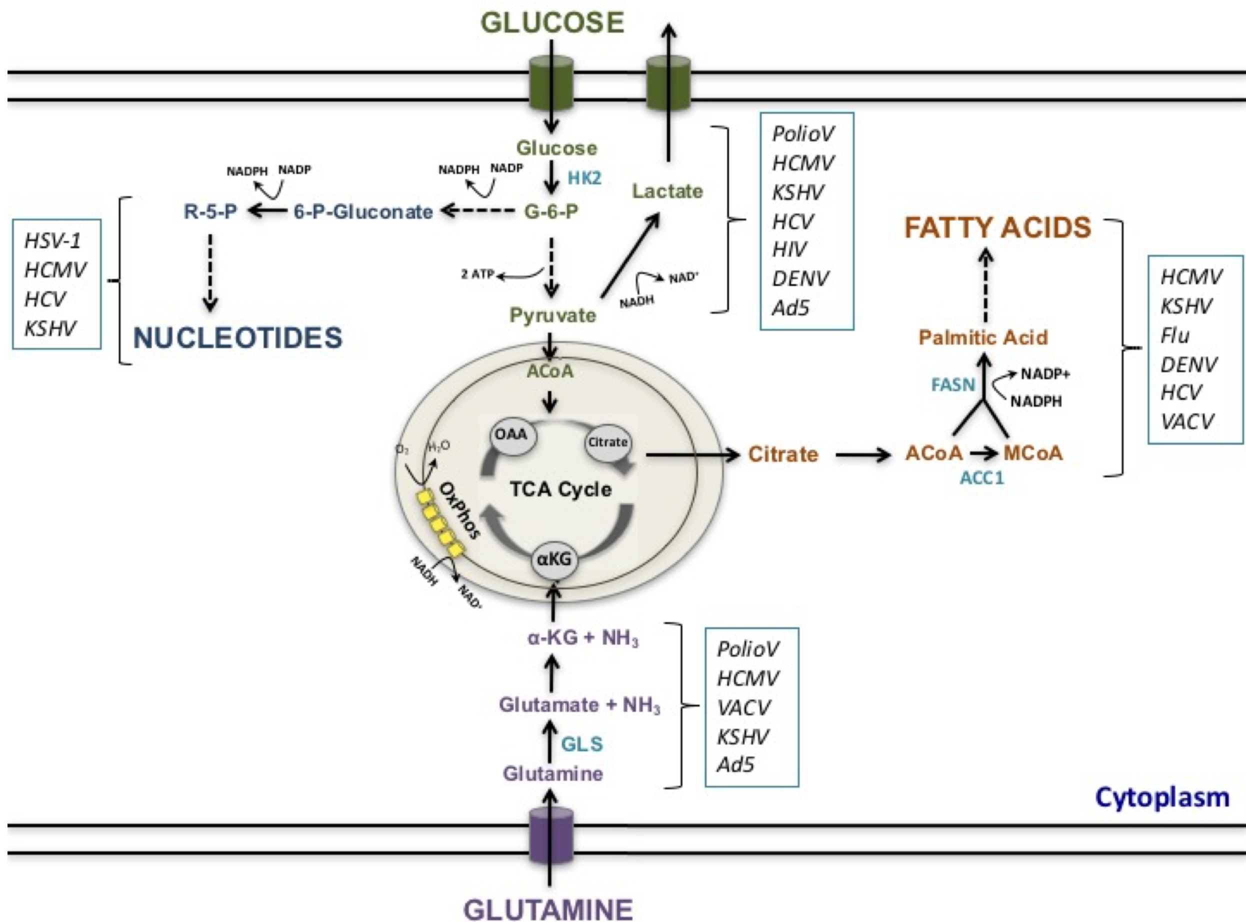

Figure 1.1: Virus infection alters host cell metabolism
Major metabolic pathways altered by virus infection are highlighted, glycolysis (green), fatty acid synthesis, FAS (orange), glutaminolysis (purple), pentose phosphate pathway, PPP (dark blue). Glucose enters the cell and is metabolized to glucose-6-phosphate (G-6-P) which can be shunted to the PPP to support nucleotide synthesis, or to pyruvate. Pyruvate can be converted to lactate via glycolysis, which is secreted from the cell, or acetyl-CoA (ACoA), which enters the TCA cycle. Citrate can be shunted out of the mitochondria to enter FAS. Glutamine enters the cell and is de-aminated twice to form αKG and ammonia (NH_3). αKG can then enter the TCA cycle. Major enzymes of carbon metabolism are in turquoise and discussed throughout thesis(21).

Virus	Essential Carbon Source/ Important metabolic pathway	Major publications
Poliovirus	Glucose Glutamine Fatty Acid Synthesis	Eagle H. and Habel K, (1956) Darnell JE and Eagle H, (1958) Nchoutmboube JA, (2013)
HCMV	Fatty Acid Synthesis Glucose and Glycolysis Glutamine and Glutaminolysis	Munger J, et al. (2006) Munger J, et al. (2008) Chambers J, et al. (2009) Vastag L, et al. (2011)
DENV	Fatty Acid synthesis Glycolysis	Heaton NS, et al. (2010) Perera R, et al. (2012) Fontaine KA, et al. (2014)
HCV	Fatty Acid Synthesis Glycolysis	Kapadia SB and Chisari FV, (2005) Ripoli S, et al. (2010) Diamond DL, et al. (2010) Ramière C, et al. (2014)
VACV	Glutaminolysis Fatty Acid Synthesis	Fontaine KA, et al. (2014) Greseth M. and Traktman P. (2014)
HSV-1	Glucose and Glycolysis?	Lewis VJ. and Scott LV, (1962) Courtney RJ, et al. (1973) McArdle J, et al. (2011) Vastag L, et. al. (2011)
Adenovirus	Glycolysis Glutaminolysis	Thai M, et. al. (2014) Thai M, et al. (2015)
KSHV (Latency)	Glycolysis Fatty Acid Synthesis Glutamine and Glutaminolysis	Delgado T, et al. (2010) Bhatt AP, et al. (2012) Delgado T, et al. (2012) Yogev O, et al. (2014) Sanchez EL, et al. (2015)

Table 1.1: Virus Induced Metabolism
Major publications examining virus-induced metabolic signatures for Poliovirus, Human Cytomegalovirus (HCMV), Dengue Virus (DENV), Hepatitis C Virus (HCV), Vaccinia Virus (VACV), Herpes Simplex Virus 1 (HSV-1), Adenovirus, and latent Kaposi's Sarcoma Associated Herpesvirus (KSHV)(21).

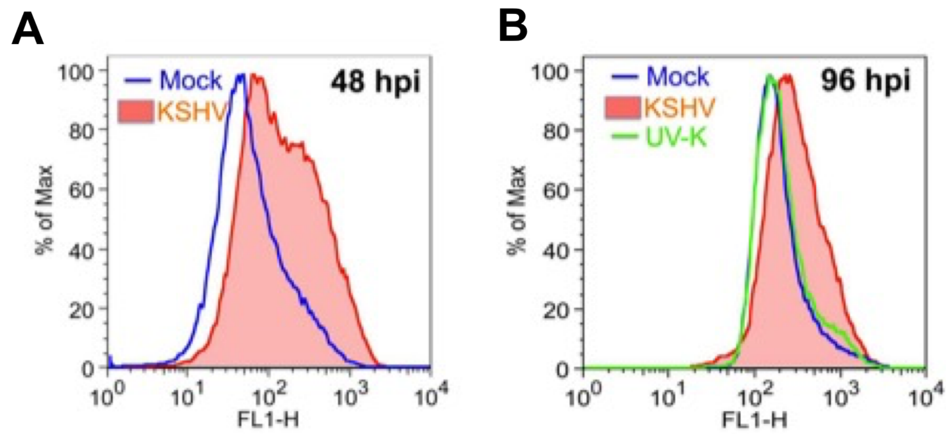

Figure 1.2: KSHV-infected cells induce the formation of lipid droplet organelles
(A) Mock- and KSHV- infected TIME cells were harvested at 48 hpi, fixed and stained with Nile Red, a specific fluorescent stain for lipid droplets. Staining was analyzed by flow cytometry. **(B)** Mock-, KSHV- and UV-irradiated-KSHV- infected TIME cells were harvested at 96 hpi and stained for lipid droplets(43).

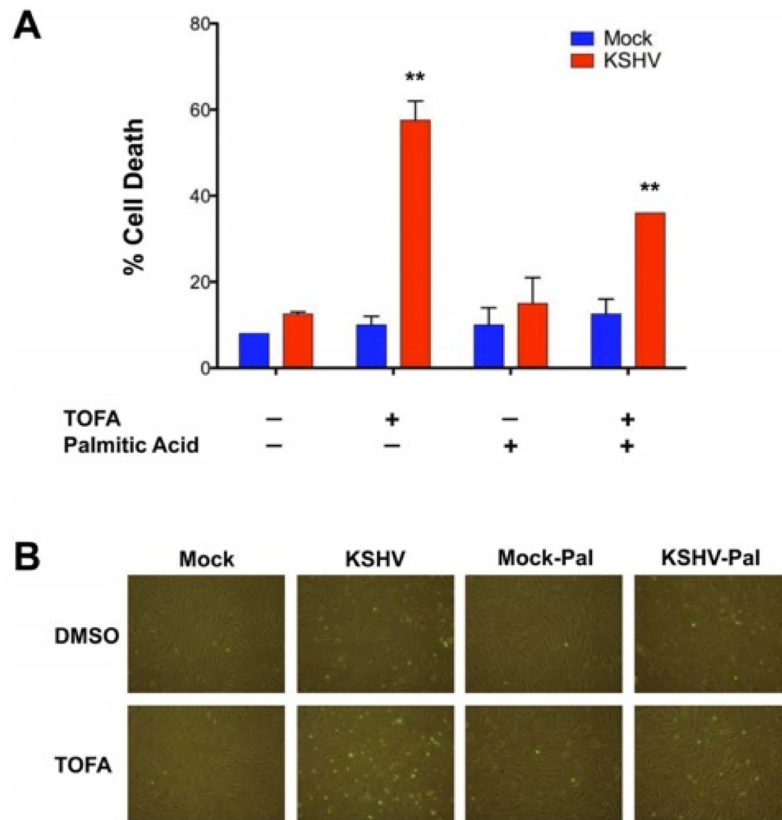

Figure 1.3: Palmitate rescues TOFA-induced cell death in KSHV latently infected endothelial cells

(A) At 48 hpi, mock- and KSHV-infected TIME cells were treated with DMSO (control) or 20 mg/mL of TOFA, in the presence or absence of 24 mM palmitic acid, and incubated for an additional 48 hours. Cell death rates were then determined by a trypan blue exclusion assay. Cell death rate (%) is # of dead cells/# of total cells. **(B)** At 48 hpi, TIME cells were treated as in panel A. Cells were then treated with Image-It Dead Green viability stain and visualized on an inverted fluorescent microscope(43).

Chapter 2. MATERIALS AND METHODS

2.1 CELL CULTURE SYSTEMS

Tert-Immortalized Microvascular Endothelial (TIME) cells(64) and primary human dermal microvascular endothelial cells (1° hDMVECs) (Lonza, MD) were maintained as monolayer cultures in EBM-2 media (Lonza or Cellgro) or EndoGrow media (Millipore) supplemented with a bullet kit containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor 3, epidermal growth, hydrocortisone, and 5% FBS. Millipore EndoGrow media, supplemented with dialyzed FBS (depleted of small molecules including glucose and glutamine) was used for all experiments that compare replete (4 mM L-glutamine) and glutamine-free media. iSLK cells (a kind gift from Jae Jung), stably maintaining a selectable GFP-expressing WT KSHV genome (KSHV.219), were maintained in Dulbecco's Modified Eagle Medium (DMEM) media containing 5% FBS, 1% Pen-strep, and 1% L-glutamine selected with puromycin (10 mg/mL), G418 (95 mg/mL) and Hygromycin B (50 mg/mL), as previously described(24,65). All iSLK lytic experiments were performed using treatment DMEM media without glucose, L-glutamine and sodium pyruvate (Corning) supplemented with 10% dialyzed FBS (depleted of small molecules including glucose and glutamine), 1 g/L D-glucose (SIGMA) with or without 4mM L-glutamine.

2.2 VIRUSES

KSHV inoculum from induced BCBL-1 cells was titered and used to infect TIME cells or 1° hDMVECs as previously described(66). Adenoviruses expressing the lytic replication and transcription activator (RTA) were used in lytic replication assays. Adenovirus RTA (AdRTA) was produced previously by Lagunoff lab. Helper-dependent RTA (HD_RTA), a gutted-adenovirus containing no adenovirus viral coding sequences, was produced subsequently following a modified version of a previously described published protocols(67,68). Briefly, the coding region of KSHV RTA driven by a CMV promoter was cloned into the pBluescript II SK-plasmid (Stratagene) to create

a shuttle vector (pBShuttle) flanked by adenovirus sequences. The RTA/adenovirus expression cassette was excised from this plasmid and electroporated into BJ5183 cells (Stratagene) along with pC4HSu helper adenovirus vector (Microbix Biosystems) to allow for homologous recombination. The resulting plasmid (HD_RTA) was transfected into 293Cre4 cells, which stably express a Cre recombinase enzyme, selected with puromycin. Cells were passaged in the presence of Helper Adenovirus (HD14; Microbix), which contains all adenovirus-coding regions and allows for the production of HD_RTA adenovirus. The Helper Adenovirus contains a modified packaging sequence flanked by loxP sites, therefore the helper adenovirus is not replicated along with the HD_RTA. Upon final passage and expansion of adenovirus producing cells, cells were collected, pelleted, and freeze-thawed three times using liquid nitrogen and 37°C water bath. Cell debris was then spun out at 2000 rpm and the cell free supernatant was taken for next steps. The cleared lysate was layered onto a continuous 15% to 40% CsCl gradient and centrifuged for 2 to 3 h at 25,000g using an SW41Ti rotor (Beckman Coulter, Inc., Fullerton, CA). The mature virus band was collected and purified in a second CsCl density gradient. The mature virus band was collected, dialyzed against three changes of A195 buffer, flash frozen in liquid nitrogen, and stored at 80°C.

2.3 LATENT KSHV INFECTIONS

Latent infections were performed in serum-free EBM-2 media and subsequently overlaid with complete EBM-2 media. Infection rates were assessed for each experiment by immunofluorescence and only experiments where greater than 85% of the cells expressed LANA, a latent marker, and less than 1% of the cells expressed ORF59, a lytic marker, were used. In a subset of the siRNA transfection experiments, where larger quantities of siRNA were used, there was a slight increase in the cells expressing ORF59, but this always occurred in both the control and gene specific siRNA transfections and did not alter the results of the experiments.

2.4 IMMUNOFLUORESCENCE

Prior to harvesting cells for immunoblot, RT-PCR, functional assays, or when determining titer for TIME cell lytic assays, an aliquot of 1º hDMVECs, TIME or iSLK cells were seeded on four-well chamber slides and fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline. Infection rates were monitored using antibodies against the latent KSHV protein LANA (a kind gift from A. Polson and D. Ganem) and the lytic protein ORF59 (Advanced Biotechnologies Incorporated) as described previously(50). Early and late protein expression was monitored using antibodies for ORF59, ORF45 or K8.1. Cells were incubated with fluor-conjugated secondary antibodies goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 Molecular Probes/Invitrogen). Cells were mounted in medium containing DAPI (4',6'-diamidino-2-phenylindole) before being viewed under a Nikon Eclipse E400 fluorescence microscope (Nikon, Inc.).

2.5 REAGENTS

YOYO-1 and SytoGreen24 were diluted in DMSO and used at a final concentration of 100 nM and 50 nM respectively (Life Technologies). Dimethyl-α-ketoglutarate (alpha-ketoglutarate or αKG) and pyruvate were purchased from Sigma and used at 3.5 mM and 8 mM respectively. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide, or BPTES (Sigma) was solubilized in DMSO, used at a final concentration of 2.5 μM. QVD-OPH (SMBiochemicals) and was dissolved in DMSO and used at a final concentration of 20 μM. L-γ-glutamyl-*p*-nitroanilide (GPNA) (Sigma), was prepared in DMSO in a 1 M stock solution and used at a final concentration of 5mM. Oxamate (Sigma) was prepared in EBM-2 or DMEM media at a final concentration of 25 or 50 mM. 5- (tetradecyloxy)-2-furancarboxylic acid, or TOFA (Sigma or Santa Cruz) was solubilized in DMSO and used at a final concentration of 2 or 10 ug/mL.

2.6 GLUTAMINE UPTAKE ASSAY

Twenty-four hours post mock- or KSHV-infection; TIME cells were re-seeded into 12-well plates at equal numbers in triplicate. At 96 hpi, cells were overlaid with serum-free media for 2 hours. Cells were then washed three times with PBS before the addition of 1mL of serum-free media containing 0.5μCi (10 pmol) of [^{3}H]-L-glutamine (Perkin Elmer #NET551). Cells were incubated for 10 minutes at 37°C. Following incubation, the medium was removed and each well was washed twice with 1mL of ice-cold DPBS and 200μL of lysis buffer (1% SDS in PBS) was added to each well and incubated at room temperature with occasional agitation for 5 minutes. Lysates were transferred to microcentrifuge tubes and mixed by vortexing. 150μL of each lysate was transferred to a vial containing 4mL of Biofluor Plus scintillation fluid (Perkin Elmer). Each vial was mixed by vortexing and counted in a Beckman LS6500 liquid scintillation counter. The remaining lysate was quantified by BCA Protein Assay Reagent Kit (Pierce) for normalization.

2.7 GLUTAMINE STARVATION, BPTES, AND GPNA TREATMENT STUDIES

At 20 hpi, mock- and KSHV-infected TIME cells were re-seeded into 24-well plates. At 24 hpi, cells were treated with Replete (4 mM glutamine), glutamine-free media or replete media with 2.5 μM BPTES in triplicate. Of note, no changes in latent or lytic infection rates were observed after glutamine starvation. YOYO-1, to identify dead cells, or SytoGreen24, to mark all cell nuclei, were added at this step. For rescue studies, supplementation with 3.5 mM αKG, 8 mM pyruvate or 20 μM QVD were added at this step. Plates were then placed on the IncuCyte (Essen Biosciences), a live-cell phase-contrast and fluorescent imaging system and recorded for cell death and total cell number for 48 hours (24 hpi through 72 hpi). GPNA experiments were prepared according to the same protocol, but scanned on the Typhoon 9400 variable mode imager (GE Healthcare) and analyzed with ImageJ software for relative fluorescence at 48 hours post treatment. Apoptosis experiments conducted with the apoptosis marker, Caspase-3/7 substrate, were prepared

according to the same protocol, but the Caspase-3/7 Cell Event reagent was added, plates were scanned at 48 hours post treatment on the Typhoon 9400 and ImageJ software and normalized to relative florescence for Styogreen24. Primary hDMVEC experiments were re-seeded into 24-well plates and at 48 or 72 hpi were treated with Replete (4 mM glutamine) or glutamine-free media and 48 hours post treatment (96 or 120 hpi) were scanned on the Typhoon 9400 variable mode imager (GE Healthcare) and analyzed with ImageJ software for relative fluorescence.

2.8 WESTERN BLOT ANALYSIS

All cells were lysed in RIPA buffer and protein was quantified using BCA Assay (Pierce). 10-50ug were subjected to SDS-PAGE on a 4-12% Gel (Life Technologies or Bio-Rad) then transferred to 0.2um nitrocellulose membrane (Bio-Rad) or Immobilon F polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% Non-Fat Dry Milk in Tris-buffered saline with 0.1% Tween (TBST) or Odyssey Blocking Buffer (LI-COR) for at least an hour then probed with the indicated primary antibodies diluted in 5% milk in TBST or LI-COR for 2 hours at RT, or overnight at 4C (anti-c-Myc (Abcam), anti-Max (Santa Cruz Biotechnology), anti-MondoA (Proteintech), anti-Mlx (Santa Cruz Biotechnology), anti-SCL1A5 (Cell Signaling) and anti-TXNIP (MBL, JY1). For viral protein analysis, we used the lytic antibodies anti-ORF45 (kind gift from Yan Yuan, Feng Zhou) or anti-K8.1 (Santa Cruz Biotechnology). Blots were washed 3 times in TBST, then probed with HRP-conjugated secondary antibody (Cell Signaling) diluted in 5% milk in TBST or IRDye secondary antibody (LI-COR) diluted in LICOR Buffer for 1 hour at RT. Blots were washed 3 times in TBST, then subjected to chemiluminescence, exposed to blue autoradiography film (GeneMate), and processed in an autoprocessor or scanned and quantified with the Odyssey CLx Infrared Imaging System (LI-COR) for fluorescent blots.

2.9 QUANTITATIVE RT-PCR

For latent experiments in Chapter 3, total RNA was isolated from TIME cells 72 hours post siRNA transfection using the Nucleospin RNA II Kit (Macherey-Nagel). Two-step quantitative real-time reverse transcription PCR (BioRad) was used to measure expression levels of SLC1A5 and the housekeeping gene GAPDH. iScript Reverse Transcription Supermix and SsoAdvanced SYBR Green Supermix (BioRad) were used according to manufacturer's protocols. The primers used were: SLC1A5-F '5-TTATCCGCTTCTTCAACTCCTT-3', SLC1A5-R '5-ACATCCTCCATCTCCACGAT-3', or GAPDH-F: '5-GGACTCATGACCACAGTCCA-3', GAPDH-R '5-CCAGTAGAGGCAGGGATGAT-3'. Relative levels of SLC1A5 mRNA were normalized by the delta threshold cycle method to the abundance of GAPDH mRNA. For analysis of lytic transcripts in Chapter 4, primers for early and late viral gene expression were used. Early gene primers used were: ORF45-F '5-CAACTCTCCGGACGTGAACA-3', ORF45-R '5-GGAGATTGGGTTGGGAGGTG-3', ORF59-F '5-GACAGCGTCTCGCTGACAGA-3', ORF59-R '5-CACACGCGTGAGCTATTCGG-3', ORF26-F '5-AGCCGAAAGGATTCCACCATT-3', ORF26-R '5-CCGTGTTGTCTACGTCCAGA-3', K8.1-F '5-AAAGCGTCCAGGCCACCACAG-3', K8.1-R '3-GGCAGAAAATGGCACACGGTT-5'. Relative levels of each transcript were normalized by the delta threshold cycle method to the abundance of GAPDH mRNA.

2.10 SIRNA TRANSFECTION AND CELL SURVIVAL

A set of four siRNAs specific to the glutamine transporter SLC1A5 (siSLC1A5) and MondoA (siMondoA) were purchased (Qiagen, Flexitube GeneSolution #GS6510 and #GS22877 respectively). A negative-control siRNA (siControl) was designed and synthesized by Ambion. TIME cells were transfected with siRNA at a final concentration of 200 nM, using the Amaxa Nucleofector Kit (Lonza) according to the manufacturer's protocol. At 24 hours post transfection, cells were mock- or KSHV-infected. Upon completion of the infection, cells were washed and treated with

Replete media containing YOYO-1 or SytoGreen24. Relative fluorescence was measured 48 hours post treatment using a Typhoon 9400 variable mode imager (GE Healthcare) and ImageJ software.

2.11 Lytic KSHV Assay

For TIME cell lytic assays, immediately following 4 hours KSHV infection, infection with an Adenovirus expressing Rta was performed in serum free EBM-2 containing 0.2 ug poly-L-lysine/mL for 1.5 hours in a 37°C incubator. After infection, cells were washed three times with PBS. Treatment EBM media containing metabolic inhibitors or vehicle controls were added to cells. Forty-eight hours post AdRTA or HD_RTA infection, cell supernatant was collected and spun down at 2000 rpm to remove cellular debris. Infectious supernatant was titered on fresh TIME cells by incubating cells with cell-free supernatant supplemented with polybrene (1:1000) in 6- or 12-well plates for 4 hours. Forty-eight hours post titer infection, cells were harvested and seeded for IFA analysis of LANA and ORF59 expression. For iSLK lytic assays, cells were induced with Doxycycline (Dox) and Sodium Butyrate (NaB) in the presence or absence of metabolic inhibitors. After 24 hours of lytic induction, the supernatant from all samples was collected spun down at 2000 rpm to remove cellular debris. Supernatant was titered on fresh TIME cells by incubating cells with cell-free supernatant supplemented with polybrene (1:1000) in 6- or 12-well plates for 4 hours. At 48 hours post titer infection, plates were scanned on the Typhoon for relative GFP fluorescence of each sample and analyzed using Image-J software.

2.12 Statistical analysis

Standard errors of the means are shown, and statistical differences between groups were analyzed with Student's t test (two-tailed) or one-way ANOVA. A p-value of <0.05 was considered significant and is indicated by an asterisk in the figures. A p-value of <0.01 is indicated by a double asterisk, and a p-value of <0.0001 is indicated by a triple asterisk in the figures.

Chapter 3. LATENT KSHV INFECTED ENDOTHELIAL CELLS ARE GLUTAMINE ADDICTED AND REQUIRE GLUTAMINOLYSIS FOR SURVIVAL

Adapted from an article originally published in the scientific journal PloS Pathogens

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3.1 ABSTRACT

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is the etiologic agent of Kaposi's Sarcoma (KS). KSHV establishes a predominantly latent infection in the main KS tumor cell type, the spindle cell, which is of endothelial cell origin. KSHV requires the induction of multiple metabolic pathways, including glycolysis and fatty acid synthesis, for the survival of latently infected endothelial cells. Here we demonstrate that latent KSHV infection leads to increased levels of intracellular glutamine and enhanced glutamine uptake. Depletion of glutamine from the culture media leads to a significant increase in apoptotic cell death in latently infected endothelial cells, but not in their mock-infected counterparts. In cancer cells, glutamine is often required for glutaminolysis to provide intermediates for the tri-carboxylic acid (TCA) cycle and support for the production of biosynthetic and bioenergetic precursors. In the absence of glutamine, the TCA cycle intermediates alpha-ketoglutarate (αKG) and pyruvate prevent the death of latently infected cells. Targeted drug inhibition of glutaminolysis also induces increased cell death in latently infected cells. KSHV infection of endothelial cells induces protein expression of the glutamine transporter, SLC1A5. Chemical inhibition of SLC1A5, or knockdown by siRNA, leads to similar cell death rates as glutamine deprivation and, similarly, can be rescued by αKG. KSHV also induces expression of the heterodimeric transcription factors c-Myc-Max and related heterodimer MondoA-Mlx. Knockdown of MondoA inhibits expression of both Mlx and SLC1A5 and induces a significant increase in cell

death of only cells latently infected with KSHV, again, fully rescued by the supplementation of αKG. Therefore, during latent infection of endothelial cells, KSHV activates and requires the Myc/MondoA-network to upregulate the glutamine transporter, SLC1A5, leading to increased glutamine uptake for glutaminolysis. These findings expand our understanding of the required metabolic pathways that are activated during latent KSHV infection of endothelial cells, and demonstrate a novel role for the extended Myc-regulatory network, specifically MondoA, during latent KSHV infection.

3.2 AUTHORS SUMMARY

KSHV is the etiologic agent of KS, the most common tumor of AIDS patients worldwide. Currently, there are no therapeutics available to directly treat latent KSHV infection. This study reveals that latent KSHV infection induces endothelial cells to become glutamine addicted, similarly to cancer cells. Extracellular glutamine is required to feed the TCA cycle through glutaminolysis, a process called anaplerosis. KSHV induces protein expression of the glutamine transporter SLC1A5 and SLC1A5 expression is required for the survival of latently infected cells. KSHV also induces the expression of the proto-oncogene Myc and its binding partner Max, as well as, the nutrient-sensing transcription factor, MondoA and its binding partner Mlx. MondoA regulates SLC1A5 and glutaminolysis during latent KSHV infection, and its expression is required for the survival of latently infected endothelial cells. These studies show that glutaminolysis and a single glutamine transporter, under the regulation of MondoA, are required for the survival of latently infected cells, providing novel druggable targets for latently infected endothelial cells. This work supports that a cancer-like metabolic signature is established by latent KSHV infection, opening the door to further therapeutic targeting specifically of KSHV latently infected cells.

3.3 INTRODUCTION

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is a human γ-herpesvirus and the etiologic agent of several malignancies, including two B-cell lymphomas, primary effusion lymphoma (PEL) and Multicentric Castleman's Disease (MCD), as well as Kaposi's Sarcoma (KS), an angioproliferative tumor(4,20). KS is the most common tumor of AIDS patients worldwide and also commonly occurs in non-AIDS patients in central Africa and the Mediterranean(20,69,70). KS is a highly vascularized tumor comprised predominantly of spindle cells of endothelial origin. In both KS spindle cells and endothelial cells in culture, KSHV establishes a primarily latent infection, with only a small percentage of the tumor cells undergoing lytic replication(6,71).

How KSHV alters endothelial cells to lead to cancer is still an open question. Previous work from our lab and others has demonstrated that KSHV, similarly to cancer cells, induces several major metabolic pathways. These alterations in cellular metabolism are imperative to the survival of cells latently infected with KSHV(43,50,55). During latent KSHV infection, glucose uptake is induced and lactate production is significantly increased(50). This switch to aerobic glycolysis is characteristic of the Warburg effect, a hallmark of cancer cell metabolism(72). Interestingly, KSHV-infected endothelial cells require the Warburg effect for their survival, as latently infected endothelial cells are extremely sensitive to drug inhibition of glycolysis(50). Recent evidence supports that the viral miRNAs expressed during latency are sufficient for the induction of the Warburg effect in KSHV-infected cells(18).

Our lab has also shown that KSHV induces the production of lipids via fatty acid synthesis (FAS) during latent infection(43). Over half of the long-chain fatty acids detected in our metabolomics screen were elevated following latent KSHV infection. Lipid droplet organelles were also increased by latent KSHV infection of endothelial cells, evidence of increased fatty acid synthesis. Inhibition of FAS leads to apoptosis of KSHV-infected cells, which was rescued with supplementation of palmitate, a downstream metabolic intermediate of FAS. These data indicated

that downstream intermediates of FAS are required for endothelial cell survival during latent infection. Induction of both glycolysis and FAS are also required in primary effusion lymphoma cells where KSHV is present(55).

Both the Warburg effect and increased FAS are metabolic signatures found in most cancer cells(37). In these cells, glucose is primarily being utilized to produce lactic acid and fatty acids and is therefore diverted away from the tri-carboxylic acid (TCA) cycle. The TCA cycle metabolizes carbon to produce both bioenergetic and biosynthetic precursors. Importantly, glutamine carbon can be utilized to replenish the TCA cycle through a process termed anaplerosis(29). Glutamine is the most abundant amino acid available to mammalian cells. Cancer cells induce glutamine uptake to support a glutamine requirement that exceeds the amount that cells can synthesize. Cancer studies have shown that transformed cells become glutamine addicted, or dependent on this exogenous glutamine and its catabolism via glutaminolysis for their survival(73,74). Recent evidence demonstrates that glutamine addiction in some cancers is enabled by the extended Myc network. Together, Myc-Max, with MondoA, a nutrient-sensing transcription factor, and its heterodimerization partner, the Max-like protein X (Mlx), facilitate the reprogramming of cellular metabolism in Myc-overexpressing cells(75–77).

A number of lytically replicating viruses also require glutamine for maximal viral replication(57,78,38). Previous studies have shown that poliomyelitis virus and human cytomegalovirus depend on both glucose and glutamine for efficient virus replication. Interestingly, during vaccinia virus infection, glucose is completely dispensable for viral replication, but viral infection is reliant on glutamine for maximal virion production(78). However, no studies have examined glutamine dependence during *de novo* KSHV infection.

We show that latent KSHV infection of endothelial cells induces glutamine uptake and that infected cells are dependent on the catabolism of glutamine for their survival. In the absence of exogenous glutamine, a significant percentage of KSHV-infected endothelial cells undergo apoptosis

unless supplemented with TCA cycle intermediates such as alpha-ketoglutarate (αKG) or pyruvate. Targeted drug inhibition of glutamine uptake or glutaminolysis during latent infection recapitulates the findings from the glutamine-deprived conditions.

Additionally, we show that KSHV infection induces protein expression of c-Myc, its dimerization partner Max, MondoA, and its dimerization partner, Mlx. KSHV infection also induces protein expression of the glutamine-transporter protein SLC1A5. c-Myc coordinately with MondoA/Mlx is essential for regulation of glutaminolysis in cancer cells(75,76) and is also necessary for the induction of SLC1A5 in KSHV-infected endothelial cells. Inhibition of MondoA or SLC1A5 induces cell death in KSHV-infected cells, but not mock-infected cells, and can be rescued with supplementation of αKG. Therefore, latent KSHV infection induces and requires glutamine uptake and subsequent glutaminolysis, regulated by MondoA and the glutamine transporter SLC1A5, for the survival of latently infected endothelial cells.

3.4 Results

3.4.1 Latent KSHV Infection of Endothelial Cells Induces Increased Glutamine Uptake

A global metabolomics screen identified that glutamine levels are significantly elevated at both 48 and 96 hours post latent KSHV infection(43). Intracellular glutamine abundance is elevated 2.2 fold at 48 hours post infection (hpi) and 2.7 fold at 96 hpi, as compared to mock-infected cells (Figure 3.1A). To determine if the increased levels of glutamine in infected cells was due to increased uptake during latent infection, a radiolabeled glutamine molecule, [^{3}H]-Glutamine, was added to the media of mock- and KSHV-infected Tert-Immortalized Microvascular Endothelial (TIME) cells at 96 hpi. Intracellular radiolabeled glutamine levels were then determined 10 min post treatment by scintillation. Latent KSHV infection induces glutamine uptake by approximately 35% compared to mock-infected cells (Figure 3.1B). These data indicate that elevated levels of

glutamine during latent KSHV infection are in part a result of an increase in exogenous glutamine uptake.

3.4.2 Exogenous Glutamine is Required for the Survival of Endothelial Cells Latently Infected with KSHV

To determine if exogenous glutamine is a required carbon source for the survival of endothelial cells latently infected with KSHV, we quantified cell death over time in the presence or absence of exogenous glutamine. TIME cells were mock- or KSHV-infected and allowed to establish latency for 24 hours. Cells were re-seeded into 24-well plates, and overlaid with replete media, which contains 4mM glutamine, or glutamine-free media. Both treatment medias were prepared with dialyzed FBS, depleted of small molecules, including glutamine, and experiments were performed in triplicate. Average cell death over time was measured using the live-cell Essen Bioscience IncuCyte imaging system, which records both phase-contrast as well as fluorescent images over time. Dead cells were identified using the fluorescent nuclear dye YOYO-1, a cell impermeable dye that only enters cells with compromised membranes. Total cell number was determined by using SytoGreen24, a cell permeable dye that enters all cell nuclei. Percent cell death was calculated by dividing the total number of dead cells (YOYO-1 positive) by the total number of cells (SytoGreen24 positive). Cell death was monitored for 48 hours (24 hpi through 72 hpi). Figure 3.2A shows the average percent cell death recorded every 2 hours over 48 hours of monitoring for three biological replicate infections. The bar graph shows the average cell death at 0, 24, and 48 hours post treatment for each condition. In mock-infected cells, with replete or glutamine-deprived media, there is less than 5 percent cell death over the time monitored (Figure 3.2A). KSHV-infected cells in replete media have a slight increase in cell death over the time course. However, glutamine starvation of KSHV-infected cells induces a significant increase in cell death, approximately 25-30% after 48 hours of treatment (72 hpi). Microscopy images were analyzed for positive cell nuclei

based on size and fluorescence intensity for both YOYO-1 and SytoGreen24. Representative images of YOYO-1 positive cells at 48 hours post treatment are shown in Figure 3.2B.

To ensure that glutamine addiction is not simply due to virus binding and entry, we repeated the experiments with UV-irradiated KSHV. UV-irradiated virus is able to bind and enter cells, but does not support viral gene expression. KSHV-infected cells show a 10-fold increase in cell death upon glutamine starvation, whereas UV-irradiated KSHV-infected cells show similar levels of cell death to mock-infected cells (Figure 3.2C). Therefore, KSHV viral gene expression is required to induce the dependence on glutamine and establish a state of glutamine addiction in endothelial cells.

To show that KSHV glutamine addiction was not limited to TIME cells, we conducted similar cell death experiments upon depletion of glutamine in mock- and KSHV-infected primary human dermal microvascular endothelial cells (1° hDMVECs). Mock- and KSHV-infected 1° hDMVECs were overlaid with glutamine depleted media at 48 or 72 hpi. Fourty-eight hours post glutamine depletion, YOYO-1 and SytoGreen24 counts were measured on a Typhoon scanner to determine relative fluorescence for cell death. These experiments reveal a significant increase in cell death only in KSHV-infected 1° hDMVECs, substantiating that KSHV infection of 1° hDMVECs also induces glutamine addiction (Figure 3.2D).

3.4.3 Glutamine Starvation Leads to Apoptosis of KSHV Infected Endothelial Cells

We have previously shown that inhibition of glycolysis and fatty acid synthesis leads to cell death via apoptosis of endothelial cells latently infected with KSHV(43,50). It has also been shown that glutamine deprivation leads to apoptosis of cancer cells(77). To determine if glutamine starvation induces cell death via activation of an apoptotic pathway, we performed the previously described cell death assay using YOYO-1 and SytoGreen24 counts in the presence or absence of the pan-caspase inhibitor QVD. Upon supplementation with QVD, KSHV-infected cells deprived of

glutamine are rescued from cell death (Figure 3.3A), indicating that cell death is due to caspase-dependent apoptosis.

To confirm that cells are dying via apoptosis, we utilized a fluorogenic Caspase-3/7 substrate, which contains the caspase cleavage site, a short four amino acid peptide (DEVD), conjugated to a nucleic acid binding dye. This cleavage site is specifically targeted by activated executioner caspases 3 and 7. When caspase 3 and/or 7 are activated during apoptosis, the DEVD site is cleaved, resulting in the release of the DNA dye, translocation to the nucleus and fluorescence. For these experiments, the Caspase-3/7 substrate was added to mock- and KSHV-infected cells in the presence or absence of glutamine at 24 hpi. After 48 hours of treatment, plates were scanned for relative fluorescence using a Typhoon 9400 variable mode imager. Caspase-3/7-mediated relative fluorescence was normalized to SytoGreen24 relative fluorescence from the same experiment. Only KSHV-infected cells starved of glutamine showed significant detection of fluorescence from the Caspase-3/7 substrate, indicating that latent infection induces Caspase 3 and/or 7 activation, which in turn results in an elevated level of DEVD cleavage and nuclear fluorescence (Figure 3.3B). We also included samples supplemented with QVD. These samples showed no increased fluorescence even in the absence of glutamine (Figure 3.3B). Representative microscopy images at 48 hours post treatment were captured with the Cellomics ArrayScan Vti (Figure 3.3C). Overall, these data indicate that when deprived of glutamine, KSHV-infected endothelial cells activate apoptosis in a Caspase-3/7 dependent manner.

3.4.4 Glutaminolysis is Required for KSHV Infected Endothelial Cell Survival

Upon entering the cell, glutamine is catabolized via glutaminolysis. Glutaminolysis consists of two consecutive deamination steps. First, glutamine is converted to glutamate by glutaminase (GLS). Second, glutamate is converted to αKG by one of three enzymes: glutamate dehydrogenase (GDH), glutamate pyruvate transaminase (GPT) or glutamate oxaloacetate transaminase (GOT)(73,79). At

this stage, αKG can enter and replenish the TCA cycle. To determine if glutamine is required to maintain the TCA cycle in KSHV-infected cells, we added either membrane-soluble αKG or pyruvate, both of which can enter the TCA cycle, to the treatment medium of glutamine-deprived cells during latent KSHV infection. After mock- or KSHV-infection of TIME cells for 24 hours to allow the establishment latency, cells were re-seeded as before and overlaid with replete media, glutamine-free media, or glutamine-free media supplemented with either 3.5 mM αKG or 8 mM pyruvate. Supplementation with αKG completely rescues the glutamine-deprived KSHV-infected cells from cell death and supplementation with pyruvate significantly rescues cell death in the glutamine-deprived infected cells (Figure 3.4A). These metabolite rescue data support the model that the exogenous glutamine taken up by KSHV-infected endothelial cells is necessary to support glutaminolytic metabolism for replenishment of the TCA cycle.

BPTES is a specific inhibitor of GLS, the first enzyme of glutaminolysis. When treated with BPTES in the presence of 4mM glutamine (replete media), KSHV-infected endothelial cells died at similar levels to those deprived of glutamine, while having little effect on mock-infected cells (Figure 3.4B). These data recapitulate our findings with glutamine-deprived media. Taken together, these data validate that glutamine is essential for glutaminolysis in KSHV-infected cells.

3.4.5 KSHV Induces Expression of the Myc/MondoA Network and Their Targets, Including the Glutamine Transporter SLC1A5

Glutamine metabolism is regulated by oncogenic c-Myc in many cancer cells(75,77,80). Additionally, there is evidence that c-Myc is regulated by latent KSHV infection(81,82). Recently, it was shown that c-Myc(83), and N-Myc(76), manipulate metabolic gene expression coordinately with the Myc-bHLHZ superfamily members MondoA, a nutrient-sensing protein, and its dimerization partner, Mlx. MondoA/Mlx or the paralogue ChREBP/Mlx constitute the "nutrient-sensing" arm of the extended Myc network(84). Protein expression of c-Myc, Max, MondoA and Mlx are increased during latent KSHV infection of TIME cells, as determined by immunoblot analysis of

whole cell lysates harvested at 48 hpi (Figure 3.5). Additionally, a known target of activated MondoA/Mlx, TXNIP, is upregulated at the protein level during latent KSHV infection (Figure 3.5).

It has been shown that Myc/MondoA controls glutamine metabolism by inducing the expression of the major glutamine transporter, SLC1A5(76). SLC1A5 is a neutral amino acid transporter which localizes to the cellular membrane, and is known to primarily import glutamine(85). SLC1A5 is upregulated in many cancer cells(75,76,85). There is a small, but reproducible, increase in SLC1A5 protein in TIME cells latently infected with KSHV when whole cell lysates are compared by immunoblot analysis at 48 hpi (Figure 3.5). Together, these data suggest that latent KSHV infection induces changes to the Max/Mlx-regulation network consistent with coordinate regulation of metabolism, including glutamine uptake through SLC1A5.

3.4.6 The Glutamine Transporter SLC1A5 is Required for Survival of Endothelial Cells Latently Infected with KSHV

To determine the role of the glutamine transporter SLC1A5 during latent infection, the SLC1A5 specific inhibitor L-γ-Glutamyl-*p*-nitroanilide (GPNA) was used(86). Mock- and KSHV-infected TIME cells were re-seeded at 24 hpi and overlaid with replete media or replete media treated with 5mM GPNA. YOYO-1 or SytoGreen24 were added to compare the relative florescence of dead cells and the relative fluorescence of total cells, respectively, at 48 hours post treatment. These experiments were conducted using the Typhoon 9400 variable mode imager to measure relative fluorescence of all samples. GPNA treatment leads to increased cell death only in KSHV-infected cells but not their mock counterparts (Figure 3.6A). Importantly, when supplemented with 3.5 mM αKG, cell death induced by GPNA treatment of KSHV-infected endothelial cells was rescued to KSHV replete control treatment levels, indicating that the drug-induced cell death was due to the requirement of glutamine metabolism via glutaminolysis and not off-target effects.

To further confirm the drug studies, a validated siRNA set directed to SLC1A5 was used to knockdown SLC1A5 expression(76). SLC1A5 expression was reduced by approximately 70% in

TIME cells transfected with a mix of four siRNAs specific for SLC1A5 (siSLC1A5), as compared to cells transfected with a scrambled non-target control (siControl) (Figure 3.6B). Twenty-four hours post transfection with the SLC1A5 or control siRNA, cells were either mock- or KSHV-infected and subsequently provided replete media containing YOYO-1 for cell death or SytoGreen24 to identify all cells. Plates were scanned at 48 hours post treatment (72 hpi) for relative fluorescence on the Typhoon 9400 variable mode imager. Minimal cell death was observed in both mock- and KSHV-infected cells treated with siControl. KSHV-infected cells, but not mock-infected cells, transfected with the siSLC1A5 show an increase in cell death. The fold change in relative fluorescence for cell death of KSHV-infected cells over mock-infected cells is increased in cells transfected with siSLC1A5 compared to cells transfected with siControl (Figure 3.6B). Together, these data support that KSHV-infected endothelial cells rely on the expression of the glutamine transporter SLC1A5 for survival.

3.4.7 MondoA Regulation of Glutaminolysis is Required for Survival of Endothelial Cells Latently Infected with KSHV

SLC1A5 is directly regulated by the nutrient-sensing Myc extended network member MondoA in many human cancer cells(76). To determine if MondoA controls SLC1A5 expression during latent KSHV infection of endothelial cells, we examined the expression of SLC1A5 upon siRNA knockdown of MondoA in mock- and KSHV-infected endothelial cells. MondoA protein expression was significantly reduced in both mock and KSHV-infected TIME cells transfected with a mix of four siRNAs specific for MondoA (siMondoA), as compared to cells transfected with a scrambled non-target control (siControl) (Figure 3.7A). While SLC1A5 protein levels are elevated by KSHV infection, loss of MondoA results in a reduction in detected SLC1A5 in all samples. Additionally, protein levels of Mlx, a co-stabilized MondoA binding partner(76), and TXNIP, a known downstream target of MondoA/Mlx regulation, are also reduced upon loss of MondoA.

These data support the hypothesis that MondoA is directly regulating SLC1A5, the major glutamine transporter, during latent KSHV infection of endothelial cells.

To determine if MondoA is required for endothelial cell survival during latent KSHV infection, we examined cell death in the presence of control siRNA or siRNA directed against MondoA. As shown in Figure 3.7B, only KSHV-infected endothelial cells in the absence of MondoA show a significant increase in cell death at 48 hpi, indicating that MondoA is indeed required for the survival of latently infected cells. Importantly, this significant increase in cell death is fully rescued upon supplementation with αKG, indicating that the cell death that occurs in KSHV-infected cells where MondoA is knocked down is due to a loss of TCA cycle intermediates and not an unrelated function of MondoA.

3.5 DISCUSSION

Transformed cells were first described as 'glutamine addicted' in the 1950's(74). It is now well established that glutamine, the most abundant amino acid in plasma, is 'conditionally essential' for cancer cell growth and survival(29). More recent evidence shows that lytically replicating viruses orchestrate specific cellular metabolic modifications to support the unique requirements for their viral replication(40,42,41,57,78,87). We demonstrate that latent infection with KSHV, an oncogenic virus, induces glutaminolysis in endothelial cells. In addition to showing that latent KSHV infection enhances glutamine uptake during infection, we have shown that a significant percentage of latently infected endothelial cells become glutamine addicted, and that glutaminolysis is required for the survival of these cells. Deprivation of glutamine in both TIME cells and 1°hDMVECs leads to significant increases in apoptosis unless they are supplemented with TCA cycle intermediates.

Glutaminolysis is an important anaplerotic reaction that produces αKG, which can enter the TCA cycle (Figure 3.8). TCA cycle intermediates support the production of both bioenergetic and biosynthetic precursors; therefore, glutamine is potentially required for a variety of downstream

cellular processes including ATP and NADPH production and fatty acid synthesis(88). There is substantial evidence in cancer biology that glutamine metabolism is required to replenish the TCA cycle when glucose is being metabolized to lactic acid as part of the Warburg effect(29). Previous research from our lab has shown that induction of the Warburg effect is required for the survival of endothelial cells during latent KSHV infection. Therefore, we were interested in the role glutamine metabolism may play in KSHV-infected endothelial cells. Human cytomegalovirus and vaccinia virus require glutamine to support the TCA cycle for maximal virus replication and media supplemented with TCA cycle intermediates, such as αKG or pyruvate, rescued replication in the absence of glutamine(57,78). Our data supports that glutamine is a vital carbon source during latent infection with KSHV.

A recent study reported an increase in glutamate secretion during latent KSHV infection(82). Glutamate is produced intracellularly through the deamination of glutamine (Figure 3.8). When glutamate secretion was inhibited, cell proliferation was reduced; however, apoptosis was not reported upon treatment with glutamate secretion inhibitors. Therefore, the increase in glutamine uptake that we observe during latent KSHV infection could be supporting the pleiotropic role of glutamine during infection to support multiple cellular processes, including anaplerosis to support the TCA cycle as well as signaling to the extracellular environment.

We demonstrate that the glutamine transporter SLC1A5 is upregulated during latent KSHV infection of endothelial cells, and that specifically the latently infected cells are dependent upon SLC1A5 for survival. This was of specific interest because previous studies have shown that oncogenic c-Myc, or N-Myc, induces increased expression of the glutamine transporter SLC1A5, and dependency upon it for survival in Myc-activated cells(83). Multiple studies have reported that c-Myc is upregulated during KSHV infection(11,82). We observed an upregulation in c-Myc during infection of endothelial cells, but also identified a significant upregulation in the related proteins MondoA and Mlx. These proteins are a part of the expanded Myc network, known as the Max/Mlx

network. MondoA and Mlx form an important glucose-responsive heterodimer that participates in regulating cellular metabolism, specifically glucose, lipid and glutamine metabolism in collaboration with c-Myc or N-Myc. It was recently described that both Myc overexpression and MondoA expression are required to induce the expression of glutamine transporters, including SLC1A5, as well as induce glutaminolysis(76). We find that MondoA regulation is required for the survival of latently infected endothelial cells and that supplementation with αKG, the immediate downstream intermediate of glutaminolysis and TCA cycle metabolite, promotes cell survival, similarly to our findings upon glutamine deprivation. This is the first evidence of the requirement for MondoA metabolic regulation during human viral infection.

While we have delineated the cellular mechanism of KSHV-induced glutamine addiction, the latent viral gene or set of genes sufficient to induce the MondoA-mediated metabolic switch to glutamine addiction has not yet been determined. Previous research has identified that the latent KSHV protein LANA collaborates with Myc to stabilize and activate the transcriptional regulator during infection(11). However, this story may be more complicated. It was recently shown that expression of the latent KSHV miRNA cluster is sufficient to induce glucose uptake and glycolysis(18). If alterations in glucose and glutamine metabolism are interconnected, such as a requirement for glucose to activate MondoA/Mlx, it is likely that multiple viral genes are involved and more work is needed to identify which latent factors are necessary to activate the overall metabolic signature that is required during latent KSHV infection of endothelial cells.

Several major metabolic switches are required during latent KSHV infection; however, the question remains whether induction of cancer cell metabolism is pre-adapting cells for a cancer microenvironment or if these alterations are helping drive oncogenesis when cells are placed in the correct microenvironment. Our findings are in agreement with metabolic signatures described by many cancer studies, which would be predicted if latent KSHV infection is indeed predisposing cells for oncogenesis. However, these models are not necessarily mutually exclusive. Comparing induced

metabolic phenotypes, such as the Warburg effect and glutamine addiction in a viral system, where we can include mock controls, provides a unique model to identify the initial drivers of oncogenesis as well as characterize the suitable microenvironment established. Glutamine addiction may be induced early in oncogenesis, yet also be a characteristic of long-term tumor maintenance. Drug inhibitors specifically targeting glutamine-addicted cells could also provide novel therapeutic treatments to specifically target endothelial cells latently infected with KSHV.

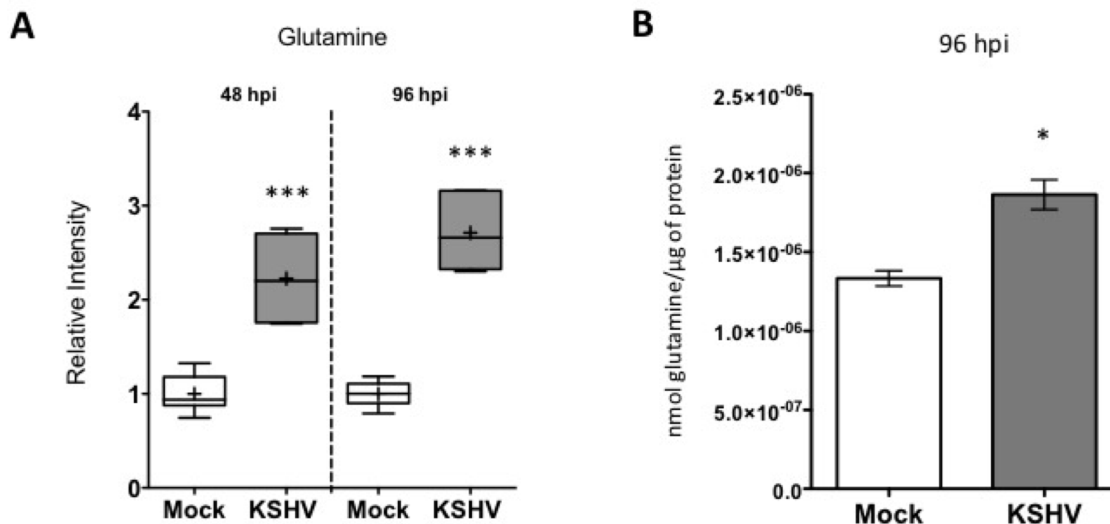

Figure 3.1: Glutamine uptake is increased by latent KSHV infection
(A) Intracellular glutamine levels are elevated following KSHV infection. Box and whisker plot showing relative level of intracellular glutamine determined in a metabolomics screen of Mock- (white) and KSHV-infected (grey) TIME cells at 48 and 96 hpi(43). The average intensity of intracellular glutamine from six biological replicates is denoted by (+) sign. *P*-value < 0.0001 at both time points. **(B)** Glutamine uptake is increased during latent KSHV-infection. Ninety-six hpi, Mock- and KSHV-infected TIME cells were incubated with [^{3}H]-Glutamine for 10 min, followed by intracellular quantification of radioactivity, normalized to total protein. Error bars represent the SEM of three independent experiments, *p*-value < 0.05.

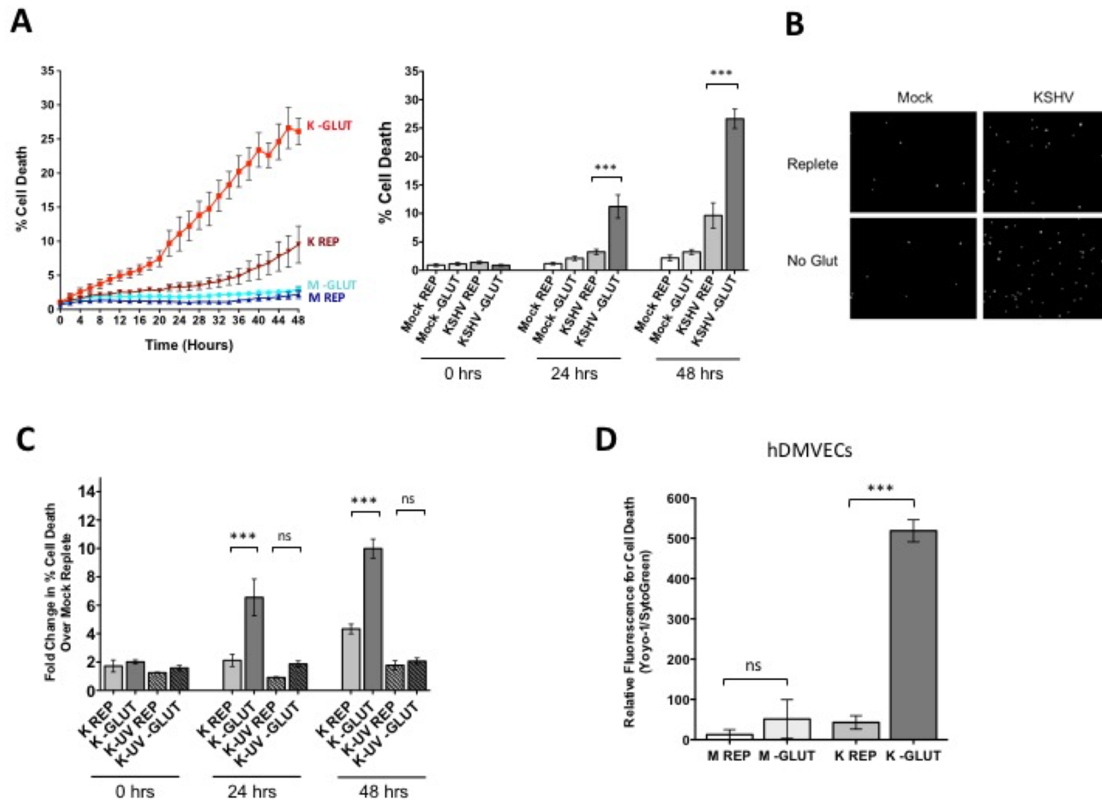

Figure 3.2: Glutamine metabolism is required for survival of latently infected endothelial cells

(A) Mock- or KSHV-infected TIME cells were seeded into 24-well plates in triplicate and treated with replete (REP) or glutamine-free (-GLUT) media containing the fluorescent dye YOYO-1 to identify dead cells or SytoGreen24 to identify total cell number. Cells were imaged every 2 hours for 48 hours on the Essen BioScience IncuCyte. Line graph displays percent cell death (Average YOYO-1 positive cells/Average SytoGreen positive cells) for every time point over 48 hours of treatment, while the bar graph shows the percent cell death at t=0, 24, and 48 hours post treatment. **(B)** IncuCyte microscopy images identifying dead cell nuclei (YOYO-1) for Mock- and KSHV-infected cells in REP or –GLUT media at 48 hours post treatment (72 hpi). Essen software was used to identify cell nuclei by size and fluorescence intensity, with background subtracted. **(C)** TIME cells were Mock-, KSHV-, or KSHV-UV (UV-irradiated) infected and prepared as described in panel A. Cells were imaged every 2 hours for 48 hours on IncuCyte. Fold increase in cell death over Mock replete at t=0 is shown. Error bars represent SEM, *p*-value < 0.0001 and "ns" is shown where the averages are not significantly different. **(D)** 1° hDMVECs were re-seeded into 24-well plates at 48 or 72 hpi and were treated with REP or –GLUT media and 48 hours post treatment were scanned on the Typhoon 9400 variable mode imager and analyzed with ImageJ software for relative fluorescence. Data shown represent the average of three independent experiments. Error bars are SEM. A *p*-value < 0.0001 is represented by three asterisks and "ns" is shown where the averages are not significantly different.

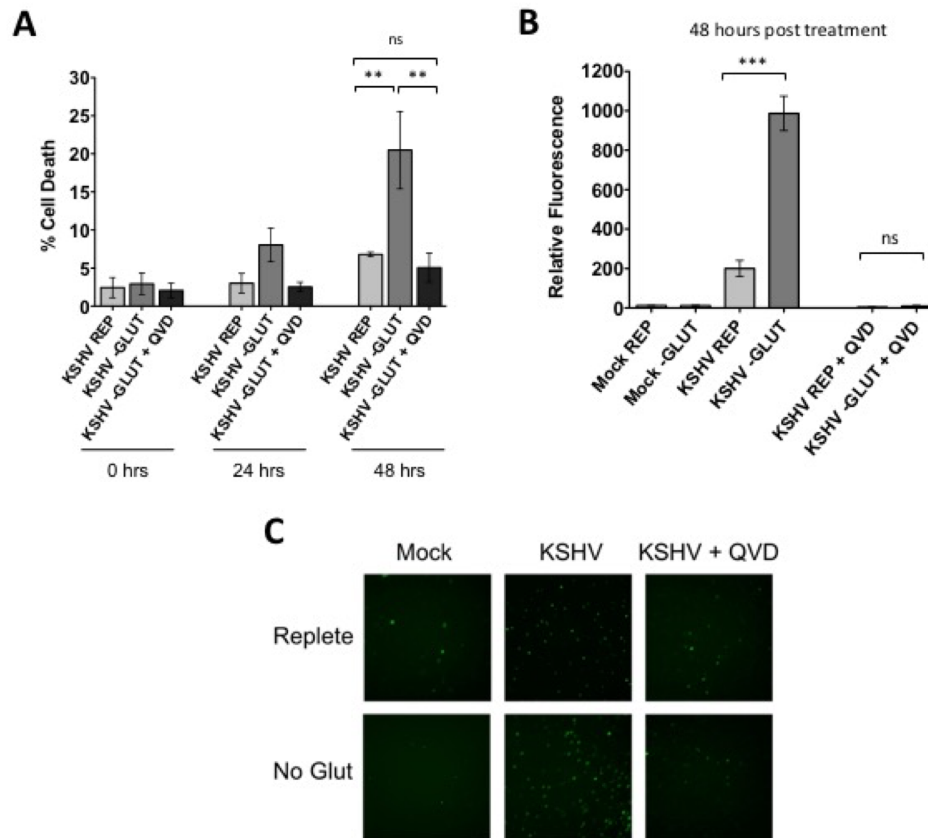

Figure 3.3: Glutamine starvation leads to apoptosis of KSHV-infected endothelial cells
At 20 hpi, Mock- or KSHV-infected TIME cells were re-seeded into 24-well plates in triplicate. Cells were treated with replete (REP) or glutamine-free (-GLUT) media in the presence or absence of 20 μM QVD (pancaspase-inhibitor) and **(A)** YOYO-1 or SytoGreen24 were added to wells to quantify dead cells and total cell numbers, respectively. Each condition was examined in triplicate, using the Essen BioSciences IncuCyte. Percent cell death was calculated (YOYO-1 positive cells/SytoGreen24 positive cells). Data represents the average of three independent experiments. Error bars represent SEM. A p-value < 0.01 is denoted by two asterisks and "ns" is shown for averages that are not significantly different. **(B)** The Cell Event Caspase-3/7 substrate was added to all wells . Each condition was examined in triplicate at 48 hours post treatment, and scanned for relative fluorescence on the Typhoon. Relative fluorescence shown is normalized to SytoGreen24 levels (total cell count). Data represents the average of three independent experiments. Error bars represent SEM. A p-value < 0.0001 is denoted by three asterisks and "ns" is shown where two averages are not significantly different. **(C)** Representative microscopy images of Caspase-3/7 samples at 48 hours post treatment (72 hpi). Live cell imaging was captured on the Cellomics ArrayScan Vti.

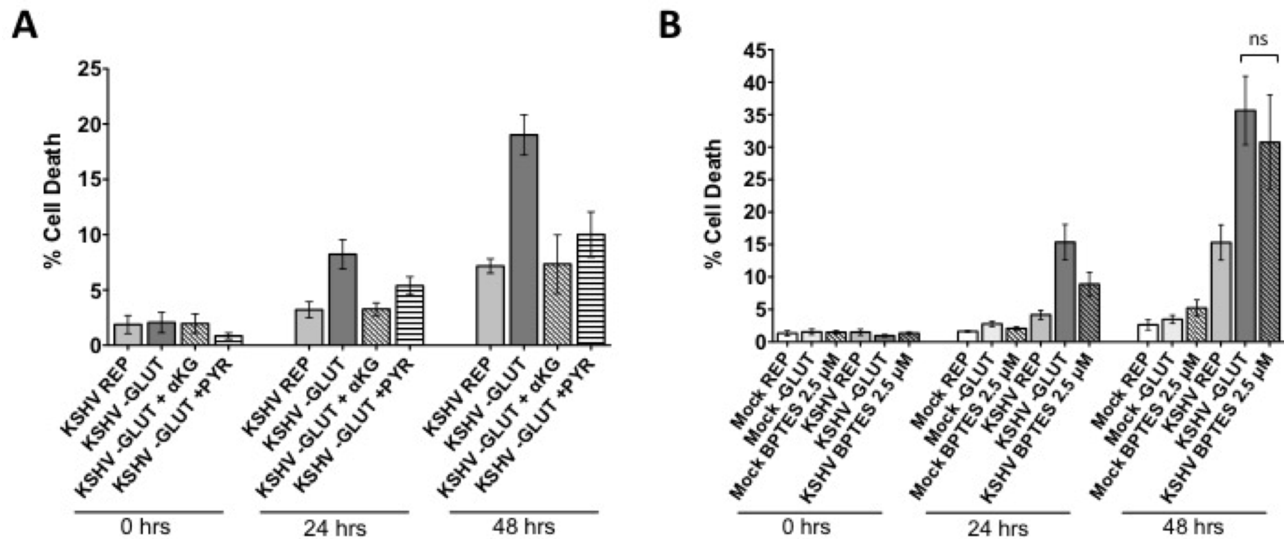

Figure 3.4: Glutamine is required for glutaminolysis in KSHV-infected endothelial cells
TCA cycle intermediates rescue KSHV-infected cells when starved of glutamine. At 20 hpi, Mock- or KSHV-infected TIME cells were re-seeded into 24-well plates in triplicate. **(A)** Cells were treated with replete (REP) or glutamine-free (-GLUT) media, supplemented with either 3.5 mM αKG or 8 mM Pyruvate and imaged on the Essen BioSciences IncuCyte for 48 hours. Percent cell death (YOYO-1 positive/SytoGree24 positive cells) is shown for t=0, 24, and 48 hours post treatment from at least two independent experiments. Error bars represent the SEM. Mock samples showed no increase in cell death. **(B)** Treatment with 2.5 μM BPTES, a specific glutaminase inhibitor, induces cell death to the same level as glutamine-deprivation in KSHV-infected cells. Data represents the average of three independent experiments and error bars represent the SEM. The average cell death quantified in the –GLUT samples and the BPTES-treated samples are not significantly different, denoted by “ns” in the graph.

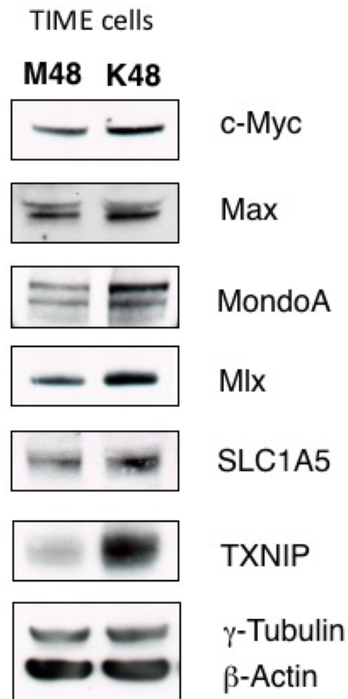

Figure 3.5: KSHV infection of endothelial cells increases protein expression of the Myc/MondoA network and downstream targets, including the glutamine transporter SLC1A5
TIME cells were Mock- or KSHV-infected and whole-cell lysates were harvested at 48 hpi. Lysates were subjected to immunoblot analysis using the indicated antibodies. ϒ-tubulin and β-actin standards were included as loading controls.

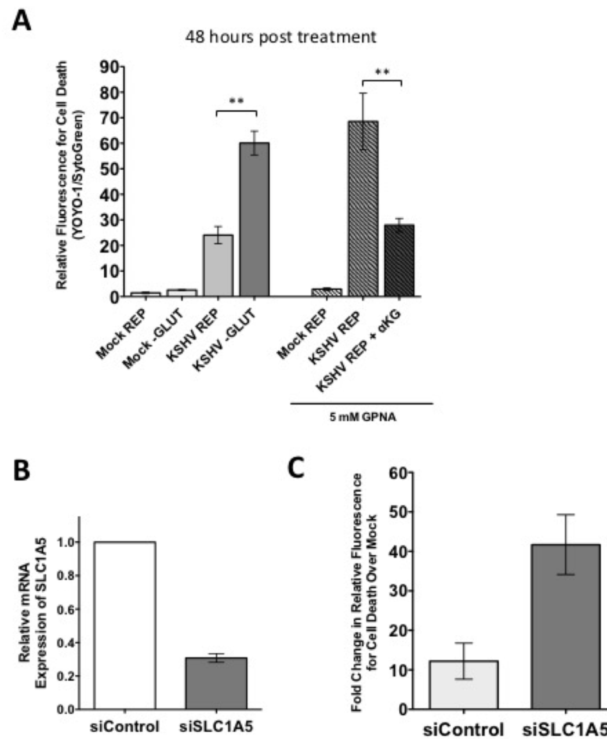

Figure 3.6: Endothelial cells latently infected with KSHV require the glutamine transporter SLC1A5 for survival

(A) 20 hpi, Mock- or KSHV-infected TIME cells were re-seeded into 24-well plates in triplicate. Cells were treated with replete (REP) media in the presence or absence of 5mM GPNA, a specific inhibitor of SLC1A5, in the presence or absence of 3.5 mM αKG and scanned on the Typhoon at 48 hours post treatment (72 hpi). Glutamine-deprived (-GLUT) controls were included for comparison. Data shown is the average relative fluorescence (YOYO-1 positive cells/SytoGreen24 positive cells) from three independent experiments. Error bars represent the SEM. A *p*-value < 0.01 is denoted by two asterisks. **(B)** TIME cells were transfected with siControl or siSLC1A5. siSLC1A5 treatment leads to an approximately 70% reduction in SLC1A5 expression, determined by qRT-PCR for SLC1A5. Expression was normalized to the housekeeping gene GAPDH. **(C)** Twenty-four hours post transfection of TIME cells with siControl or siSLC1A5, cells were Mock- or KSHV-infected. Upon completion of the infection, cells were treated with replete media containing YOYO-1 to identify dead cells or SytoGreen24 to identify total cell number. 48 hpi (72 hours post transfection), cells were scanned on the Typhoon. Data shown is the average fold change in relative fluorescence of KSHV over mock cells (YOYO-1 positive cells/SytoGreen24 positive cells) from two independent experiments. Error bars represent the SEM.

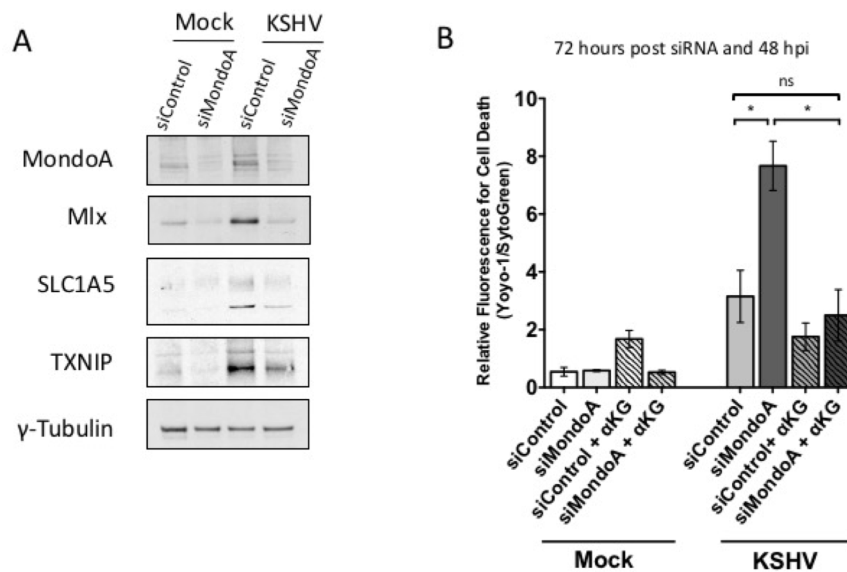

Figure 3.7: MondoA regulation of glutaminolysis is required for the survival of endothelial cells latently infected with KSHV

(A) TIME cells were transfected with siControl or siMondoA and then Mock- or KSHV-infected 24 hours post transfection. Whole-cell lysates were harvested at 48 hpi. Lysates were subjected to immunoblot analysis for MondoA, Mlx, SLC1A5 and TXNIP. KSHV infection elevates levels of all four proteins and loss of MondoA reduces the observed increase in protein expression. The standard γ-tubulin was included as a loading control. **(B)** Twenty-four hours post transfection of TIME cells with siControl or siMondoA, cells were Mock- or KSHV-infected and overlaid with replete media in the presence or absence of αKG. YOYO-1 or SytoGreen was added to the media to monitor relative fluorescence for cell death or total cells, respectively. Forty-eight hpi (72 hours post transfection), cells were scanned on the Typhoon. Data shown is the average fold change in relative fluorescence of KSHV over mock cells (YOYO-1 positive cells/SytoGreen24 positive cells) from two independent experiments. Error bars represent the SEM. A $p < 0.05$ is denoted by one asterisk and "ns" is shown where two averages are not significantly different.

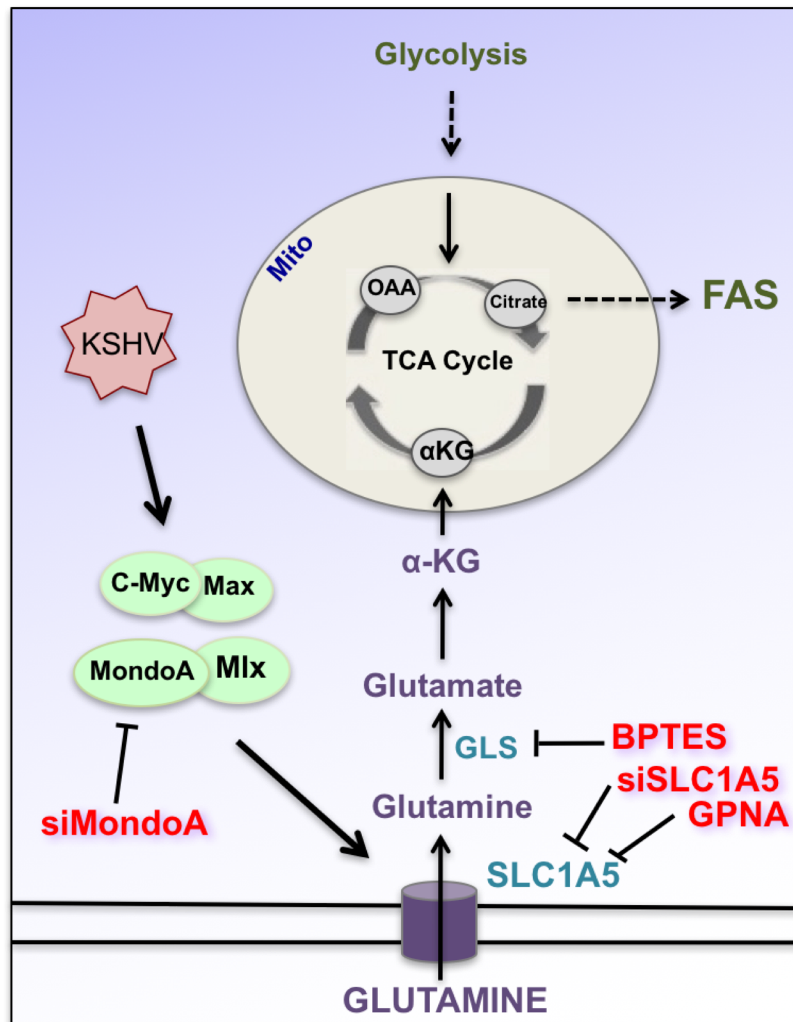

Figure 3.8: Schematic of glutamine metabolism via glutaminolysis during KSHV infection of endothelial cells

Latent KSHV infection induces and requires the Myc/Max and MondoA/Mlx heterodimers leading to the induction of the glutamine transporter SLC1A5 during latent KSHV infection. Upon entering the cell, glutamine is deaminated twice to form αKG. αKG can enter the TCA cycle where it can be utilized to support bioenergetics and the metabolism of biosynthetic precursors. GPNA or siSLC1A5 treatment was used to specifically inhibit glutamine transport via SLC1A5. BPTES is a specific inhibitor of glutaminase (GLS), the first enzyme of glutaminolysis. siMondoA treatment was used to specifically inhibit MondoA-mediated activation of glutaminolysis.

Chapter 4. HOST METABOLISM IS REQUIRED FOR MAXIMAL KSHV VIRION PRODUCTION

Adapted from manuscript in preparation

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4.1 ABSTRACT

Kaposi's Sarcoma-associated Herpesvirus (KSHV) is the infectious cause of Kaposi's Sarcoma (KS). KSHV establishes a predominantly latent infection, characterized by minimal KSHV gene expression, and no production of new infectious virus. However, 1-5% of the KS tumor cells show lytic replication, characterized by a cascade of KSHV gene expression, viral genome replication, virion assembly and ultimately mature virus egress. Latent KSHV infection requires the induction of multiple metabolic pathways, including glycolysis, glutaminolysis and fatty acid synthesis (FAS) for the survival of latently infected endothelial cells. We were interested in whether productive lytic KSHV infection required altered metabolism for maximal virion production. Here we show that glycolysis, glutaminolysis and FAS are all required for KSHV virus production and that these pathways appear to participate in virus production at different stages of the viral life cycle. Our data suggest that glycolysis and glutaminolysis are required for early steps of virus production, specifically for the synthesis of early lytic gene products. Interestingly, our data suggest that FAS is not required for early or late gene expression, therefore we hypothesize that it may be playing a role in virion assembly or egress. These data, together with our previous findings that these same pathways are vital for the survival of latently infected cells, provide the first evidence that metabolic inhibition can be used to treat both latent and lytic KSHV infection. Drugs targeting metabolism could thus potentially treat all infected cells of the KS tumor.

4.2 INTRODUCTION

Kaposi's Sarcoma (KS) is caused by Kaposi's Sarcoma-associated Herpesvirus (KSHV), a human γ-herpesvirus. KSHV is also the etiologic agent of two B-cell lymphomas, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD). KS is currently the most common tumor of AIDS patients worldwide and frequently occurs in HIV-negative patients in sub-Saharan Africa and the Mediterranean(20,69,70). KS is a highly vascularized tumor comprised predominantly of spindle cells of endothelial cell origin.

KS spindle cells and infected endothelial cells in culture establish a predominantly latent infection, with a small percentage of cells undergoing lytic replication(6,71). Approximately 90% of endothelial cells in the KS tumor are latently infected, and 1-5% of the tumor cells are lytically replicating. Since its discovery as the etiologic agent of KS, numerous studies have suggested that both latent and lytic viral replicative cycles of KSHV likely contribute to KS tumor development(5). While the majority of our work has focused on understanding the virus-host interaction during latency, the default form of infection, the most effective therapeutics would target both latent and lytic KSHV-infected cells.

During latent infection, only 4-5 KSHV genes, and a few miRNAs are expressed from the latency associated genomic region. Upon lytic reactivation, temporal gene expression, viral genome replication and virion assembly are induced. The KSHV immediate-early gene, the Replication and Transcription Activator (RTA) induces the transcription of early genes which facilitate viral genome replication, followed by late gene expression and finally virus assembly. RTA is referred to as the lytic switch protein because its sufficient for initiation of the lytic program by transactivating the lytic gene promoters(19). Early genes are expressed next in the temporal cascade, encoding proteins involved in viral DNA replication, as well as proteins that regulate host cell transcription and immune evasion(2). Once early genes are expressed, viral genome replication begins to produce many copies of the viral genetic material. Only once the viral genome has been replicated

will late-gene synthesis begin. Late genes encode viral structural proteins which are often needed in large amounts during virion assembly. Therefore, sufficient viral genome replication needs to take place before these proteins are synthesized, as these viral genomes are used for late gene transcription.

Previous work from our lab has explored alterations in host cell metabolism during latent KSHV infection of endothelial cells. Altered central carbon metabolism is a hallmark of cancer(37,72,89–91). Three of the major pathways of central carbon metabolism are glycolysis, fatty acid synthesis, and glutaminolysis. Our work, and the work of others, has demonstrated that latent KSHV infection, similarly to cancer cells, induces central carbon metabolism. These alterations in cellular metabolism are critical for the survival of cells latently infected with KSHV(43,50,55). Our lab has shown in a global metabolomics analysis that endothelial host cell metabolism is significantly altered during latent KSHV infection(43). We have also shown that increased glycolysis, fatty acid synthesis and glutaminolysis are all required for the survival of latently infected endothelial cells (38,44). Furthermore, PEL cells appear to also require both glycolysis and FAS for their survival. It was recently shown that the KSHV latent miRNA cluster is sufficient to induce glycolysis and reduce oxidative phosphorylation, supporting previous data showing that latent KSHV infection induces the Warburg effect(18,55).

Host metabolic requirements for maximal virus production have been examined in other virus models, including the DNA viruses HCMV, HSV1, Adenovirus and Vaccinia virus(42,41,78,93,94). Interestingly, while similarities exist, these studies surprisingly find virus-specific metabolic signatures are established during infection. While central carbon metabolic pathways (glycolysis, fatty acid synthesis and glutaminolysis) are altered by several of these viruses, significant differences in carbon utilization have been identified. For example, HCMV has been found by several groups to alter and require glycolysis, glutaminolysis and FAS for maximal virus production(42,41,57–59,95). Surprisingly, HCMV, but not HSV-1, has been shown to induce

glycolytic flux. HCMV increased TCA metabolites fueling FAS, while HSV-1 increased flux to the TCA cycle to feed pyrimidine synthesis(42). Recent work has shown that a model Adenovirus (Ad5) depends on both glycolysis and glutamine metabolism for its replication, and the viral protein E4ORF1, which interacts with cellular Myc, has been implicated in modulating these cellular metabolic pathways during infection(93,94). Fascinatingly, Vaccinia virus replication depends only on glutamine metabolism, while glucose metabolism is dispensable(78).

We set out to determine the KSHV lytic replication metabolic requirements during lytic replication. We found that KSHV lytic replication also depends on the major central carbon metabolic pathways. Inhibition of glycolysis, glutaminolysis and FAS all reduce KSHV virus production in both an endothelial system, as well as the iSLK inducible KSHV system. Furthermore, we show that inhibition of glycolysis, glutamine metabolism and FAS block lytic replication at different stages of virus production. Whereas glycolysis and glutamine metabolism contribute to virus production at or before viral genome replication, inhibition of FAS has little to no effect on early or late gene expression, suggesting that lipogenesis is required for virion assembly, egress or both. Identification of how viruses alter cellular metabolism and where in the virus life cycle these metabolic changes are necessary will provide a deeper understanding of virus replication needs and potentially provide cellular targets for inhibition of viruses. This is the first evidence that both the latent and lytic life cycle of an oncogenic herpesvirus share druggable therapeutic targets.

4.3 RESULTS

4.3.1 Glycolysis is Required for Maximal KSHV Virion Production

We first asked if glycolysis was a required metabolic pathway for maximal KSHV lytic replication. To test this, we conducted a KSHV lytic replication assay in the presence or absence of two concentrations of the glycolysis inhibitor, oxamate. Oxamate inhibits the enzyme Lactate Dehydrogenase (LDH), thereby reducing the available NAD+ required for early steps of glycolysis

and blocking the production of ATP and downstream intermediates of glycolysis(54). For experiments with TIME cells, we treated cells with 25 and 50 mM oxamate, two concentrations that do not affect cell viability. For the TIME cell lytic assay, we infected cells with KSHV, then immediately superinfected the cells with an adenovirus expressing RTA. This approach induces lytic replication without allowing cells to establish latent infection first. We allowed cells to produce virus for 48 hours in the presence or absence of increasing concentrations of oxamate, then harvested the supernatant from each sample. After pelleting any cellular debris in the supernatant, we tittered virus in the cell-free supernatant on fresh TIME cells. Forty-eight hours post titer infection, we harvested cells for IFA and counted LANA positive cells for infection rate. Our results show a dose dependent response to oxamate treatment, with increasing concentrations of the glycolytic inhibitor reducing the percent LANA positive cells to approximately 50% of the control sample (Figure 4.1A).

We also performed a lytic replication assay using iSLK cells, an inducible cell line that stably maintains the BAC16-KSHV viral genome, as well as an integrated, inducible RTA expressing locus(96,65). These cells can be induced upon treatment with doxycycline (Dox) and sodium butyrate (NaB). After 24 hours of chemical induction of iSLK cells in the presence of 50 mM oxamate, we harvested the supernatant and determined viral titers on TIME cells. When induced, iSLK cells produce GFP-expressing BAC16-KSHV. Therefore, after 48 hours of infection, we scanned titer plates on the Typhoon and compared relative fluorescence. Oxamate treatment significantly reduced relative fluorescence by more than 90% compared to control samples, supporting the results from the TIME cell lytic assay (Figure 4.1B). Together, these data indicate that glycolysis is a required metabolic pathway for maximal KSHV virion production.

4.3.2 Glutaminolysis is Required for Maximal KSHV Virion Production

We next examined whether glutamine metabolism is a required metabolic pathway for maximal KSHV virus production. We first performed the KSHV lytic assay in TIME cells, in the presence or absence of exogenous glutamine. When titered, glutamine deprived supernatant results in a significantly reduced percent of LANA positive cells, approximately 25% of replete samples (Figure 4.2A). We also performed this assay in the absence of glutamine, but with supplementation of alpha-ketoglutarate. Alpha-ketoglutarate is a downstream intermediate of glutamine metabolism and can enter the Tri-Carboxylic Acid (TCA) cycle(29). Supplementation with αKG is sufficient to rescue a significant percentage of virion production in the absence of glutamine, with the percent of LANA positive cells observed at approximately 75% of replete samples (Figure 4.3A). This was the first evidence that glutaminolysis, the catabolism of glutamine to fill the TCA cycle through an anaplerotic pathway, is required for maximal replication of infectious KSHV virus. We next removed glutamine from the iSLK lytic replication system and found that in the absence of glutamine, iSLK cells produced significantly less virus as well. Relative fluorescence of cells titered with glutamine-deprived supernatant was reduced by over 90% compared to control sample fluorescence (Figure 4.2B).

To confirm that glutaminolysis is important for KSHV virion production, we performed the TIME cell lytic assay in the presence of BPTES, a specific inhibitor of glutaminase (GLS), the first enzyme of glutaminolysis. After titer infection of sample supernatants, we find that LANA positive cells are significantly reduced in the presence of BPTES to about 50% of replete samples (Figure 4.2C). Additionally, we observed a partial rescue upon supplementation with the TCA cycle intermediate αKG (Figure 4.2C), or pyruvate (Figure 4.2D), a metabolite that can also support the TCA cycle. Together, the above data indicate that glutamine metabolism via glutaminolysis is a required metabolic pathway for maximal KSHV lytic replication.

4.3.3 Fatty Acid Synthesis is Required for Maximal KSHV Virion Production

We finally asked whether fatty acid synthesis (FAS) is a required metabolic pathway during lytic replication of KSHV. We again performed the KSHV lytic replication assay in TIME cells, in the presence or absence of the FAS inhibitor TOFA. TOFA inhibits acetyl-CoA carboxylase (ACC1), the rate limiting enzyme of FAS(97). TOFA treatment significantly reduced virion production, as the TIME cell titer shows an average 80% reduction in LANA-positive cells compared to control samples (Figure 4.3A). Importantly, when we supplemented TOFA-treated samples with the long chain fatty acid, palmitic acid, a downstream intermediate of FAS, we observed a significant rescue of virus titer to about 60% of control samples (Figure 4.3A).

We confirmed these findings, using the inducible iSLK system. When iSLKs were chemically induced, with Dox and NaB, in the presence of TOFA, infectious virus production was reduced. Compared to control samples, TOFA treated supernatant resulted in about a 90% reduction in relative fluorescence. This reduced virus production was also substantially rescued when supplemented with 32 uM palmitic acid, increasing relative fluorescence to about 50% of control samples (Figure 4.3B). The results of both the TIME cell lytic assay and the iSLK virus production assay support the conclusion that lipogenesis via FAS is a required metabolic pathway for maximal KSHV virion production.

4.3.4 Glycolysis is Required for Early and Late Gene Expression

Using the iSLK KSHV virus production cell line, we asked where in the lytic life cycle cellular metabolism was required. First, we examined early and late KSHV gene expression upon inhibition of glycolysis via oxamate treatment. After 24 hours of lytic replication in the presence or absence of 50 mM oxamate, supernatant was collected for titering and cells were harvested to extract RNA for qRT-PCR analysis to determine relative levels of viral transcripts. qRT-PCR for the immediate early gene ORF45, a tegument protein(98,99), and the early gene ORF59, a key viral processivity

factor(100–102), revealed a significant reduction in early transcript levels, when normalized to the host housekeeping gene GAPDH (Figure 4.4A). Furthermore, expression of the late viral genes, ORF26, a capsid protein and K8.1, a KSHV virion glycoprotein(103), were almost completely lost compared to the induced control samples (Figure 4.4B).

4.3.5 Glutamine Metabolism is Required for Late KSHV Gene Expression

Next, we examined viral transcript levels in the presence and absence of glutamine during iSLK induction. For these experiments, we induced iSLK cells with replete media, containing 4mM glutamine, or glutamine-deprived media. Twenty-four hours post induction, cell free supernatant was harvested for titering, and iSLK cells were harvested for RNA. Interestingly, qRT-PCR for the early genes ORF45 and ORF59 show no change in gene expression (Figure 4.5A). In contrast, relative transcript levels of both late genes, ORF26 and K8.1, were substantially reduced compared to expression levels of induced control cells (Figure 4.5B). These data suggest that virus production in the absence of glutamine metabolism is blocked after early viral gene transcription and at or before late viral gene expression.

4.3.6 Fatty Acid Synthesis is Not Required for Early or Late KSHV Gene Expression

Finally, we examined whether FAS is required for early and late KSHV gene expression by conducting iSLK induction in the presence or absence of the ACC1 inhibitor, TOFA. Supernatant was collected at 24 hours post induction and cells were harvested for qRT-PCR analysis of viral transcript levels. Titering confirmed that virus production was significantly reduced in each TOFA treated sample (data not shown). Transcript levels for both early viral genes, ORF45 and ORF59, were not reduced (Figure 4.6A). Surprisingly, transcript levels of both late viral genes, ORF26 and K8.1, were not reduced either (Figure 4.6B). These results suggest that KSHV viral gene transcription is unaltered by the loss of cellular lipid production.

4.3.7 Glycolysis and Glutamine Metabolism, but Not Fatty Acid Synthesis, are Required for Early and Late Protein Synthesis

We next analyzed the protein levels of the immediate-early protein, ORF45 and the late gene, K8.1 in the presence or absence of oxamate, TOFA or glutamine. Protein levels of the immediate early protein, ORF45, were significantly reduced in oxamate treated samples compared to induced controls (Figure 4.7A and B). Additionally, protein levels of the late virion glycoprotein, K8.1, were almost completely eliminated by oxamate treatment, compared to induced control samples (Figure 4.7A and B). With these results, we hypothesize that viral genome replication is severely reduced or not occurring at all, as late gene expression is dependent on viral DNA synthesis. These data indicate that glycolysis is required for early KSHV viral gene expression.

In the absence of glutamine, we probed for the immediate early protein ORF45 and the late protein K8.1. Similarly to what was observed during oxamate treatment, glutamine-deprived samples show a marked loss of both early and late protein levels compared to control samples (Figure 4.7A and B). These data are interesting, as they suggest that while early gene transcription is occurring as shown in Figure 4.5A, early protein translation may be blocked. This block would be expected to prevent viral DNA synthesis and thereby inhibit all late gene production.

Finally, we examined protein levels of the early protein, ORF45 and the late protein, K8.1 in the presence or absence of a FAS inhibitor. TOFA-treated samples show no significant change in protein production of either the early or late proteins (Figure 4.7A and B). Together, our qRT-PCR and protein data indicate that fatty acid synthesis is not required for early or late gene transcription or translation. Inhibition of FAS may block virion production at a later stage, possibly during assembly or egress.

4.4 DISCUSSION

Here we show that central carbon metabolism is required for maximal KSHV virion production. Inhibition of glycolysis, glutaminolysis or fatty acid synthesis leads to significant loss of infectious KSHV virus production. We examined virion production in TIME cells and confirmed these results in iSLK cells, an inducible BAC16-KSHV virus producing cell line(24,65). Our data indicate that glycolysis and glutaminolysis are important for early viral gene transcription and protein synthesis, respectively (Figure 4.8). However, FAS appears dispensable for early and late protein synthesis, suggesting lipids are required for a later stage of virion production (Figure 4.8).

We are currently investigating virion assembly and egress by transmission electron microscopy of iSLK cells induced in the presence or absence of metabolic inhibition. Preliminary results surprisingly show little to no significant change in virion assembly in TOFA treated samples. However, our early results clearly show that loss of KSHV titer in the presence of TOFA is rescued by supplementation with palmitic acid, therefore, supporting the conclusion that lipids are important for infectious virus production. Current work is designed to determine if either cellular or virus envelope lipid composition is altered upon inhibition FAS. It is possible that lipid membrane components are altered in the absence of natural levels of metabolism and that these modifications inhibit virus egress. We are currently expanding our analysis of early and late viral genes expression. We are quantifying expression to determine if inhibition of metabolic pathways has universal effects on each viral gene class or if there are some genes that are not inhibited. This will help to identify how each inhibitor is specifically blocking gene expression. Additionally, we are quantifying the levels of viral DNA to determine if the inhibition of glycolysis and glutaminolysis prevent viral replication. Further studies will determine where inhibition of each specific metabolic pathway blocks viral production.

We will also determine if other metabolic changes occur during KSHV lytic replication. Current work to examine global metabolic alterations during lytic replication by a mass-

spectrometry approach is underway. We have identified that three major metabolic pathways are required for maximal KSHV virion production, but anticipate that others, including nucleotide synthesis, and possibly other amino acid metabolic pathways could also be altered during lytic replication. These data could expand our list of current plausible therapeutic targets of metabolism and yield a deeper overall picture of why different metabolic pathways are required.

We hypothesize that other pathways are altered during KSHV lytic replication based on data from other herpesviruses DNA virus studies where metabolism has been examined, including HSV1, HCMV(40,42). There are specific metabolic signatures for each virus. For example, when compared at a global metabolite level and by metabolic flux analysis, it was found that HSV1 is feeding carbon into nucleotide synthesis via the pentose phosphate pathway, while HCMV is taking up increasing amounts of carbon to feed fatty acid synthesis. These herpesviruses show distinct differences in their influence on cellular metabolism. We propose to use metabolomics to determine important additional metabolic pathways altered by KSHV lytic replication as these may be different from those identified by our global analysis of cells in the latent state. As KS tumor spindle cells maintain both latent and lytic infection, identification of targets that exist for both latent and lytic KSHV infection would reveal the strongest targets for drug design to treat the KS tumor.

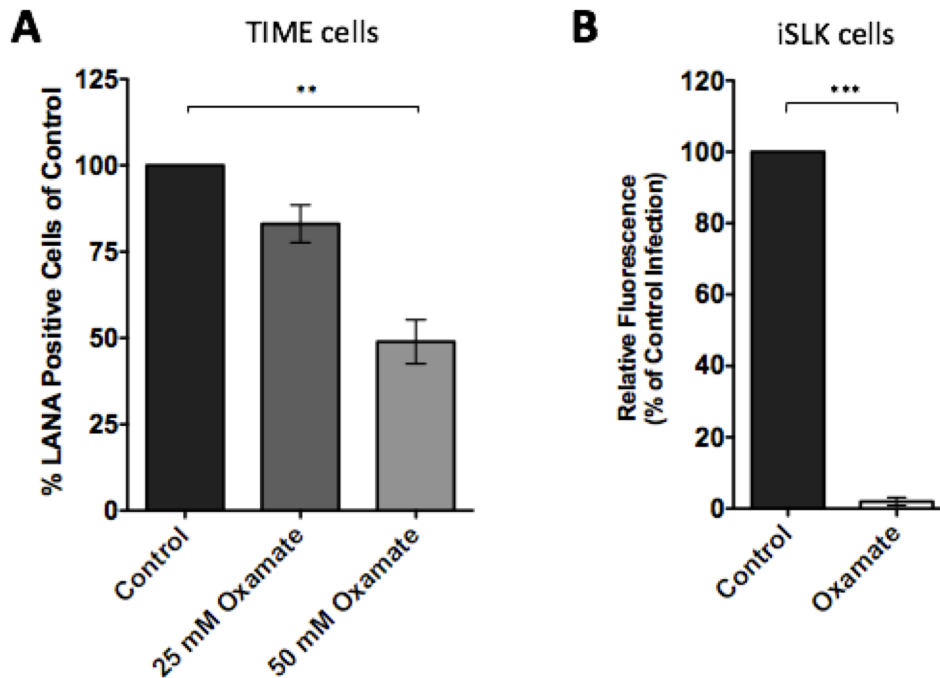

Figure 4.1: Glycolysis is required for maximal KSHV virion production
(A) TIME cells were infected with KSHV and then superinfected with an RTA-expressing adenovirus. Upon completion of infection, cells were overlaid with treatment media with or without the glycolytic inhibitor, oxamate at 25 mM and 50 mM. After 48 hours, supernatants were collected and titered on fresh TIME cells. Titer was measured by IFA for LANA expressing cells. Data shown is the average percent LANA expressing titer cells of at least 3 biological replicate experiments. 25 mM oxamate slightly reduces infectious virion production, and 50 mM reduces infectious virion production by 50%. **(B)** iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of oxamate (50mM). After 24 hours, supernatant was harvested and titered on TIME cells. WT KSHV BAC16 expresses GFP, therefore, titer was assessed at 48 hpi by scanning plates on the Typhoon. Data shown in the average of 3 biological replicate experiments with control set to 100%. Oxamate treatment significantly reduces KSHV virion production.

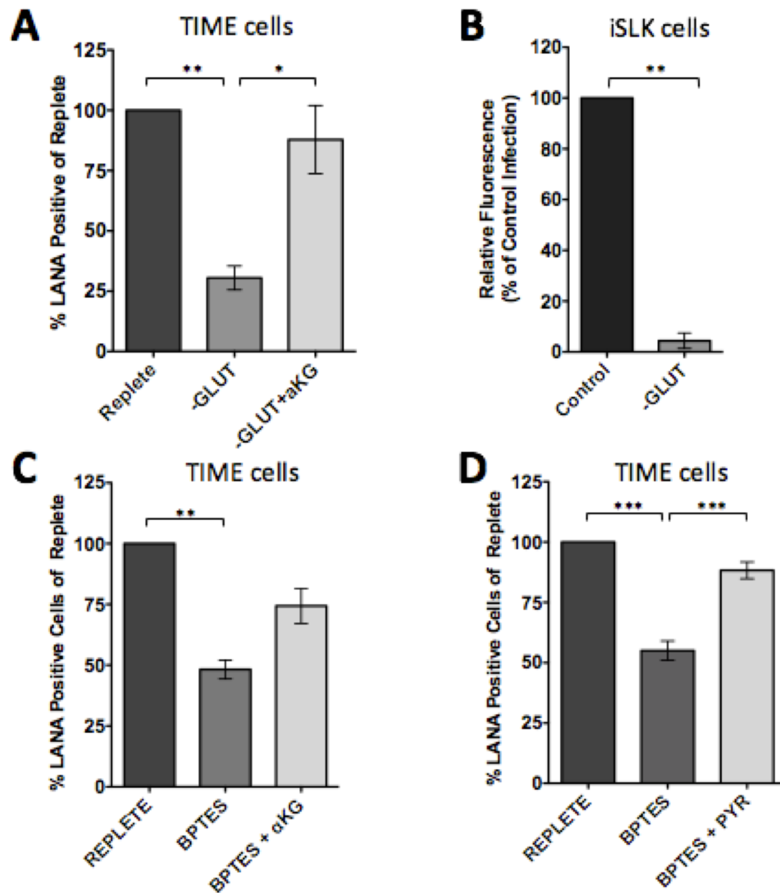

Figure 4.2: Glutaminolysis is required for maximal KSHV virion production
(A) TIME cells were infected with KSHV and superinfected with an RTA-expressing adenovirus. Cells were overlaid with treatment media with or without glutamine. Replete media contains 4 mM glutamine. One glutamine free (-GLUT) sample was supplemented with the TCA cycle intermediate alpha-ketoglutarate (αKG). At 48 hours, supernatants were titered on TIME cells. Data shown is the average percent LANA expressing titer cells. Glutamine-deprivation significantly reduces infectious virion production. αKG rescues virion production in the absence of glutamine. **(B)** iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of glutamine (4mM). At 24 hours, supernatants were titered on TIME cells. Relative fluorescence of WT BAC16-KSHV-GFP was assessed at 48 hpi by scanning plates on the Typhoon. Data shown is the average of 3 biological replicate experiments. Glutamine-deprivation significantly reduces KSHV virion production. **(C-D)** TIME cells were infected with KSHV and superinfected with an RTA-expressing adenovirus. Cells were overlaid with replete media in the presence or absence of the glutaminase inhibitor, BPTES, supplemented with the TCA cycle intermediates 3.5 mM αKG **(C)** or 8 mM pyruvate (PYR) **(D)**. At 48 hours, supernatants were titered on fresh TIME cells. Data shown is the average percent LANA expressing titer cells of 3 biological replicates. BPTES treatment reduces infectious virion production. αKG and pyruvate rescue virion production when glutaminolysis is inhibited.

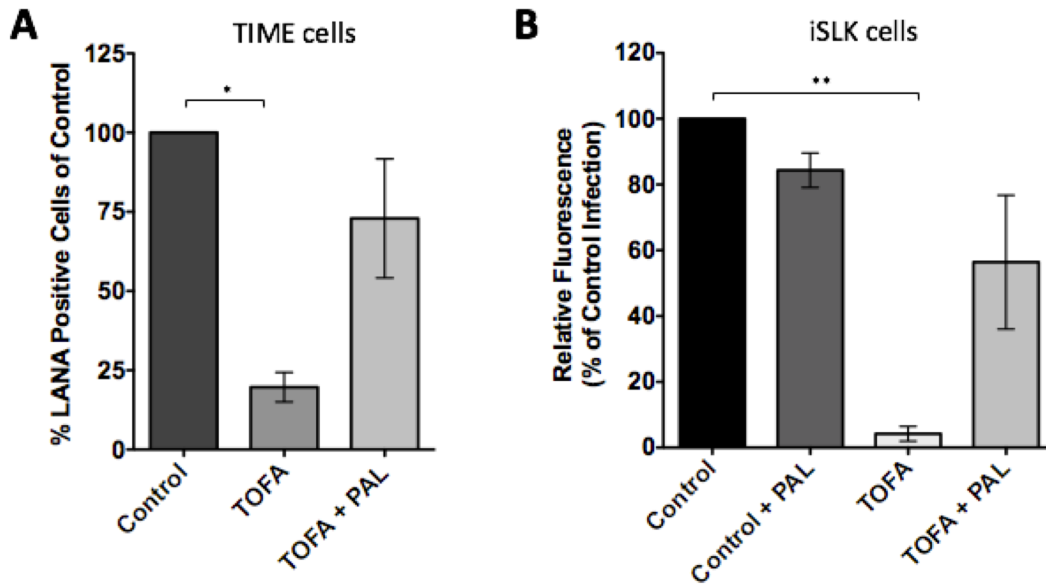

Figure 4.3: Fatty Acid Synthesis is required for maximal KSHV virion production
(A) TIME cells were infected with KSHV and then superinfected with an RTA-expressing adenovirus. Upon completion of infection, cells were overlaid with treatment media with or without the FAS inhibitor, TOFA at 2 ug/mL. One TOFA treated sample was supplemented with the long chain fatty acid palmitic acid (PAL). After 48 hours, supernatants were collected and titered on fresh TIME cells. Data shown is the average percent LANA expressing titer cells of 3 biological replicate experiments. TOFA treatment significantly reduces infectious virion production. Palmitic acid partially rescues virion production when FAS is inhibited by TOFA treatment. **(B)** iSLK cells were chemically induced with doxycycline and sodium butyrate in the presence or absence of TOFA (10 ug/mL) and supplemented with palmitic acid. After 24 hours, supernatant was harvested and titered on TIME cells. WT KSHV BAC16 expresses GFP, therefore, titer was assessed at 48 hpi by scanning plates on the Typhoon. Data shown in the average of 3 biological replicate experiments with control set to 100%. TOFA treatment significantly reduces KSHV virion production. Palmitic acid partially rescues virion production to approximately 60% of control infection when FAS is inhibited by TOFA treatment.

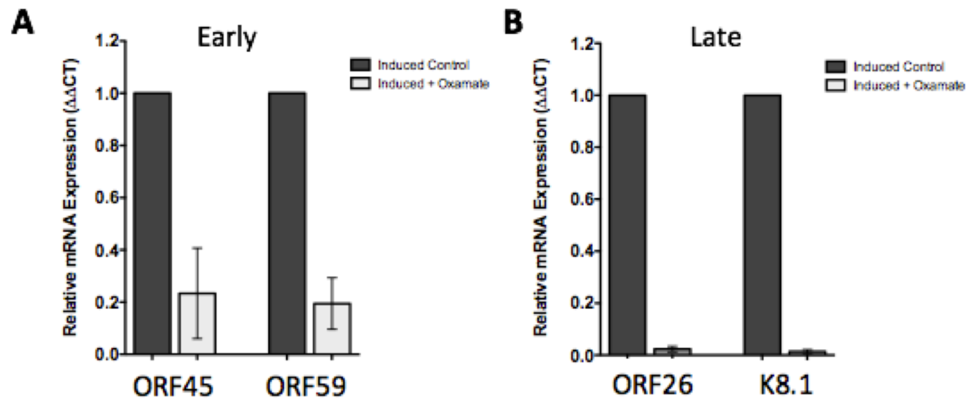

Figure 4.4: Glycolysis is required for early and late KSHV gene expression
iSLK cells induced in the presence or absence of oxamate (50mM) were harvested at 24 hours post induction. RNA was extracted and qRT-PCR was performed on cDNA for **(A)** early KSHV transcripts (ORF45 and ORF59) and **(B)** late KSHV transcripts (ORF26 and K8.1). Relative mRNA expression for each viral transcript, normalized to the housekeeping gene GAPDH is shown. Error bars are representative of standard error from at least 2 biological replicate experiments.

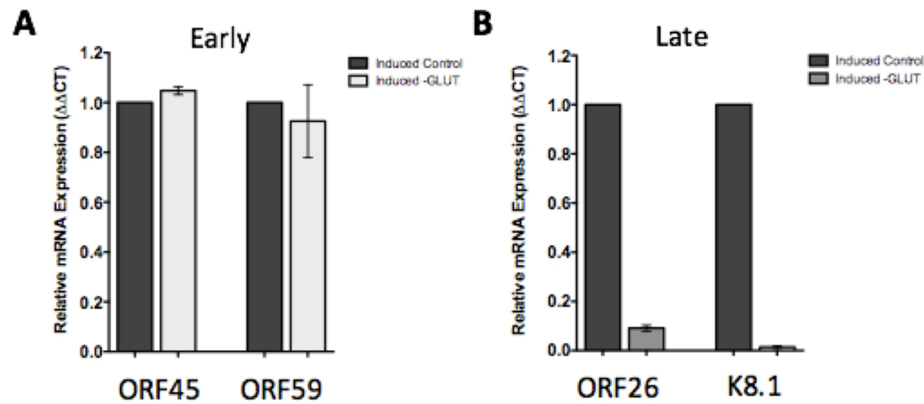

Figure 4.5: Glutamine metabolism is required for late KSHV gene expression
iSLK cells induced in the presence or absence of glutamine (4mM) were harvested at 24 hours post induction. RNA was extracted and qRT-PCR was performed on cDNA for **(A)** early KSHV transcripts (ORF45 and ORF59) and **(B)** late KSHV transcripts (ORF26 and K8.1). Relative mRNA expression for each viral transcript, normalized to the housekeeping gene GAPDH is shown. Error bars are representative of standard error from at least 2 biological replicate experiments.

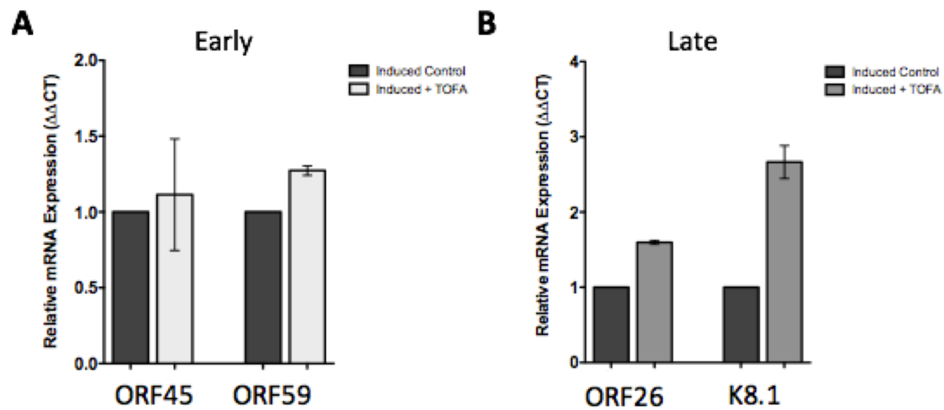

Figure 4.6: Fatty Acid Synthesis is not required for early or late KSHV gene expression
iSLK cells induced in the presence or absence of TOFA (10 ug/ml) were harvested at 24 hours post induction. RNA was extracted and qRT-PCR was performed on cDNA for **(A)** early KSHV transcripts (ORF45 and ORF59) and **(B)** late KSHV transcripts (ORF26 and K8.1). Relative mRNA expression for each viral transcript, normalized to the housekeeping gene GAPDH is shown. Error bars are representative of standard error from at least 2 biological replicate experiments.

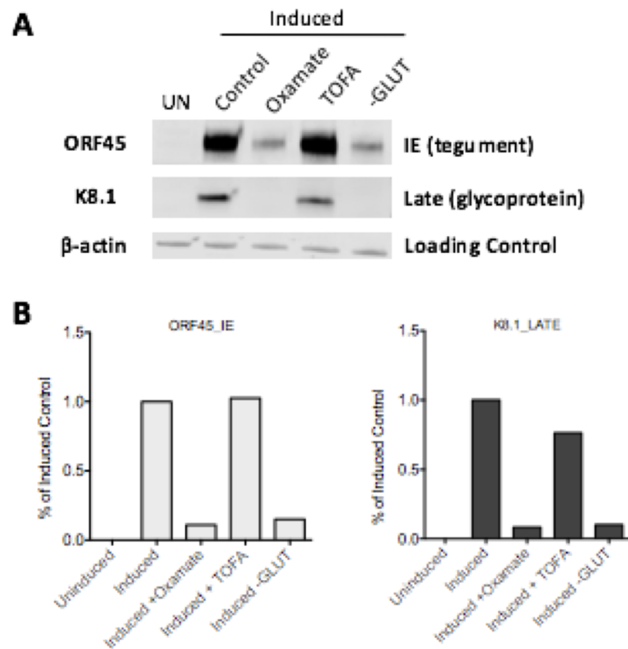

Figure 4.7: Glycolysis and Glutamine metabolism are required for KSHV protein synthesis
(A) iSLK cells induced in the presence or absence of oxamate (50 mM), TOFA (10 ug/mL) or glutamine (4mM) were harvested at 24 hours post induction. An uninduced (UN) control was also included. Total protein was extracted and examined for the immediate early (IE) KSHV protein ORF45 and late KSHV protein K8.1 by immunoblot analysis. Both early and late protein synthesis is markedly reduced in the presence of oxamate and in the absence of glutamine. Inhibition of FAS via TOFA treatment did not noticeably reduce early or late protein levels. β-actin was included as a loading control. Data shown is a representative experiment of three biological replicate experiments. **(B)** Quantitation of ORF45 and K8.1 protein levels from immunoblot in panel A.

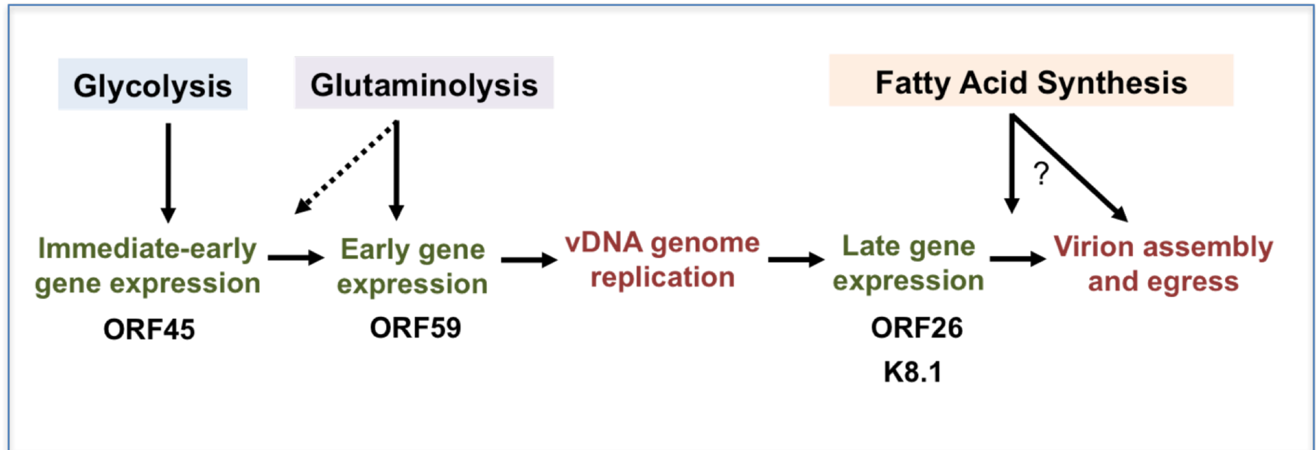

Figure 4.8: Carbon metabolism and KSHV virus production
Glycolysis, glutaminolysis and Fatty Acid Synthesis (FAS) appear to participate at different stages of KSHV virus production. Glycolysis is required for early KSHV gene expression, whereas Glutaminolysis is required for early protein synthesis. Interestingly, FAS is not required for early or late KSHV gene expression or protein synthesis. FAS may be required for later steps in virion production, possibly virion assembly or egress.

Chapter 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY

It has been known for several decades that cancer cells exhibit specific metabolic signatures compared to healthy cells. Common alterations in carbon metabolism have since been recognized as hallmarks of cancer. Three of the most studied altered cellular pathways are glycolysis, glutaminolysis and FAS. These three carbon metabolic pathways are increased in most tumors and in transformed cell lines(56). Moreover, cancer cells depend on altered and induced metabolism for their survival(25–27). Several drugs currently in therapeutic use or currently undergoing clinical trial have been designed to target cellular metabolism(25).

Work in our lab has shown that carbon utilization is altered and required during latent KSHV infection of endothelial cells. We first showed that increased glycolysis and the establishment of the Warburg effect were required for the survival of KSHV latently infected endothelial cells(50). When glycolysis was blocked with cellular inhibitors, only latently infected cells succumbed to apoptosis, while mock control cells survived. The lab then examined global changes in metabolite levels using a mass-spectrometry approach. This work showed that over half of the long-chain fatty acids detected were elevated during infection(43). We went on to show that fatty acid synthesis is also required for the survival of latently infected endothelial cells. As part of this study, I showed that latent KSHV infected cells treated with the FAS inhibitor TOFA were rescued from cell death when supplemented with the downstream metabolic intermediate, palmitic acid. I have since found that glutamine metabolism is also required for the survival of endothelial cells during latent KSHV infection. Exogenous glutamine is required to support the TCA cycle, a process regulated by MondoA, a Myc-related factor. MondoA regulates the protein synthesis of SLC1A5, the glutamine transporter required for cell survival during latent infection.

I have also found that all three major carbon metabolic pathways, glycolysis, glutaminolysis and FAS are required for maximal KSHV virion production during lytic infection. Our findings suggest that these metabolic pathways most likely contribute to virion production at different steps in viral gene transcription, translation, viral genome replication or assembly and egress. This body of work demonstrates that cellular metabolism is required for both the survival of cells latently infected with KSHV, and for KSHV lytic replication. We believe that metabolic targets represent some of the strongest druggable targets of both latent and lytic KSHV infected cells. Treating with drugs designed to target metabolism would allow for treatment of all cells of the KS tumor, and eventually the clearance all infected cells in KSHV-infected patients prior to tumor development (Figure 5.1).

5.2 LATENT KSHV INFECTION REQUIRES GLUTAMINE METABOLISM VIA GLUTAMINOLYSIS

Our global metabolomics analysis showed that the levels of the amino acid glutamine were significantly elevated by latent KSHV infection. My thesis work has shown that endothelial cells latently infected with KSHV are glutamine addicted(92). Infected cells increase the uptake of exogenous glutamine, and require glutamine metabolism for their survival. Without glutamine, endothelial cells latently infected with KSHV will die via apoptosis, unless supplemented with a downstream metabolic intermediate such as aKG or pyruvate. Both aKG and pyruvate can enter the TCA cycle and support the production of essential biosynthetic and bioenergetic metabolic intermediates.

My work has also revealed that glutaminolysis is regulated during latent infection by the nutrient-sensing protein MondoA. MondoA is a member of the Myc-extended network, and when bound to Mlx, a Max-like heterodimerization partner, has been shown to regulate glutaminolysis in cancer cells(76,84). We found that when MondoA expression is inhibited, KSHV-infected endothelial cells die unless supplemented with alpha-ketoglutarate. This evidence suggests that

KSHV is modulating glutaminolysis through MondoA. We also found that the major glutamine transporter, SLC1A5, is both regulated by MondoA during latent KSHV infection and also required for survival of infected cells. Together, these results highlight that glutamine addiction and induced glutaminolysis, regulated by MondoA through SLC1A5, is an oncogenic phenotype established early during latent KSHV infection of endothelial cells. This is the first evidence indicating a virus-dependence on MondoA regulation. These findings are important for general biology, as we know now that we can use infection with latent KSHV as a tool to unveil specific connections of metabolic regulation. Furthermore, we have revealed that as normal cells can utilize multiple transporters, KSHV infected cells have become reliant on SLC1A5. This vulnerability highlights a potential therapeutic target.

5.3 CARBON METABOLISM AND LYTIC KSHV INFECTION

We have recently explored the metabolic requirements of cells undergoing KSHV lytic replication. I found that all three major metabolic pathways we have previously examined during latent KSHV infection (glycolysis, glutaminolysis and FAS), are indeed also required for maximal virion production. I have shown that inhibition of glycolysis with Oxamate, a specific inhibitor of the enzyme Lactate Dehydrogenase, significantly reduces viral titer in both our TIME cell lytic assay as well as in iSLK cells, the BAC16-KSHV inducible cell system. Furthermore, I found that both viral gene transcription and protein synthesis are extremely reduced in the absence of glycolysis. These data indicate that glycolysis is required for virus production at or before viral genome replication. Upon glutamine-deprivation, early viral gene transcription was maintained, however late transcripts were significantly decreased. Interestingly, both early and late protein synthesis were reduced in the absence of glutamine, suggesting that glutamine-deprivation is inhibiting early protein synthesis. Therefore virus production requires glutamine metabolism at or before viral genome replication as well. Finally, we were surprised to find that inhibition of FAS with TOFA

treatment had no significant effects on early or late viral gene transcription or protein synthesis. These results are very interesting as they suggest that FAS is playing a role after late viral protein synthesis, possibly as part of virion assembly or egress. Overall, we have found that these three major carbon metabolic pathways are participating in KSHV virion production, however, that each pathway may be playing an independent role at a different stage of virus production.

5.4 FUTURE DIRECTIONS

5.4.1 Latent KSHV and Glutaminolysis

I have shown that glutaminolysis is required for endothelial cell survival during latent KSHV infection, however, we have not yet identified the viral mechanism by which KSHV modulates glutaminolysis. Previous work has shown that LANA cooperates with Myc during latent infection, therefore, I would hypothesize that it might be LANA that is regulating glutamine metabolism(11,12). It is possible that LANA could be interacting either directly or indirectly with the MondoA regulation system. Alternatively, KSHV encodes vFLIP, which activates NF-κB which could potentially lead to the activation of Myc and MondoA to promote cell survival during infection. To test these hypotheses, we can utilize a few approaches. First, we can examine the singular expression of each latent gene, using a lentivirus gene expression approach, and investigate if LANA, vFLIP, or any other latent gene product are sufficient to induce glutamine addiction through MondoA and SLC1A5. Second, we can utilize a new construct in the lab, an adenovirus that expresses all the genes and microRNAs of the KSHV Latency Associate Region (Ad_KLAR) and ask if glutamine dependence is established. If cells infected with Ad_KLAR are glutamine addicted, we would then mutate different loci in Ad_KLAR to determine the genes that are necessary for glutamine metabolism during latent infection.

We are also interested in determining how glutamine is specifically being utilized during latent KSHV infection. To explore glutamine utilization, we would conduct a systems-level

metabolic flux analysis, by supplementing cells with ^{13}C-labeled glutamine and determining its incorporation in downstream intermediates by mass-spectrometry. Metabolic flux is defined as the rate at which material passes through a given metabolic pathway(104). Our global metabolomic data reveal that concentrations of many metabolites are elevated during infection. However, these data do not allow us to distinguish whether the elevated concentrations are due to increased production or decreased consumption of these intermediates. Also, cells can use both glucose and glutamine as extracellular carbon sources, but our current metabolomics data cannot identify if downstream intermediates are derived from one substrate or the other. To investigate how glutamine consumed by KSHV infected cells is ultimately being utilized, it is necessary to measure metabolic flux. While our data indicate that glutamine is supporting the TCA cycle, we would like to identify where glutamine carbon is ultimately being utilized, whether for energy metabolites, biosynthetic intermediates, or both. Using ^{13}C-labeled glutamine, we would compare mock-and KSHV-infected cells over the course of a latent infection. Similar studies have been successfully conducted for both HCMV and HSV-1 infected cells and have shown that these herpesviruses induce distinct metabolic flux profiles during infection(42). My initial studies showed that glutamine utilization for TCA cycle versus oxidative carboxylation did not change following infection, however, further studies are warranted. Finally, we are very interested in exploring additional amino acid transporters during latent KSHV infection. Preliminary data has shown that two other transporters, SLC3A2 and SLC7A5 are highly upregulated during latent KSHV infection (Figure 5.2). SLC3A2 and SLC7A5 dimerize to form the bidirectional transporter CD98-xCT(105). It has been shown that CD98-xCT, effluxes small amounts of glutamine in exchange for essential amino acids such as L-leucine. It would be interesting to see if an increase in glutamine uptake is partially due to its role in increasing essential amino acid uptake during latent KSHV infection. Interestingly, CD98-xCT has also been proposed as possible receptor for the KSHV virion(106). This study suggests that CD98-xCT participates in the formation of a multi-molecular complex made of several integrins in

addition to SLC3A2 and SLC7A5. Future experiments will have to be conducted to identify the specific role of this bidirectional transporter, however, it would be very interesting if a major role for glutamine uptake was to support the uptake of essential amino acids from the extracellular environment, as these metabolites cannot be synthesized by cells. Other groups have shown that certain cancer cells induce this bidirectional transport to bring in essential amino acid which can control the activation of mTORC1 and trigger autophagy. Future work could provide more insight into whether CD98-xCT is upregulated during latent KSHV infection in order to regulate autophagy.

5.4.2 Lytic KSHV and Cellular Metabolism

My work has found that inhibition of glycolysis, glutaminolysis and fatty acid synthesis significantly reduces KSHV virion production. We also found that glycolysis and glutaminolysis seem to participating early in virus replication, most likely prior to viral genome replication. However, FAS is seemingly unnecessary for viral gene expression and protein synthesis, but most likely is required for later steps in virion assembly or egress. Current work is comparing viral DNA genomes by qPCR for episomal targets during lytic replication in the presence or absence of metabolic inhibition. We hypothesize that these data will show a decrease in viral DNA genomes in the presence of Oxamate or absence of glutamine, but show no change in the presence of TOFA, compared to induced control samples. I am also utilizing transmission electron microscopy to examine virion assembly during metabolic inhibition. I would hypothesize that these images will show a clear reduction in assembled virions in the presence of Oxamate or absence of glutamine. My preliminary data examining TOFA treated samples show little to no change in virion presence compared to induced control cells (Figure 5.3). This data would indicate that the block is after virion production and possibly due to a block in egress or maturation. It may be that only non-infectious particles are released. Current work is being done to tease out these different possibilities. We are now interested in examining intracellular virus in the presence and absence of

TOFA. For this experiment, we will lyse lytically replicating iSLK cells and harvest intracellular virus. We will titer intracellular virus on TIME cells to determine if indeed TOFA treatment is reducing the amount of infectious virus produced within cells. If inhibition of fatty acid synthesis is inhibiting the production of viable virus particles, we should observe reduced titer in the presence of TOFA, while the number of KSHV viral genomes is maintained. We will measure KSHV viral genomes by qPCR for a viral locus. It is possible that lipids are participating in a very specific stage of egress or that altering the lipid composition of the virion is reducing infectivity. Other groups have examined both cellular and virion lipid composition via lipidomics analysis via mass spectrometry for lipid species during viral replication. Interestingly, when the lipidome of HCMV infected cells was compared to control cells, very few changes were observed(107). However, the lipidome of HCMV virions was very distinct compared to the overall lipidome of infected cells, suggesting that virions bud from a membrane with a specific lipid composition. Identifying the lipid composition required by KSHV virions may provide further insight into the specific role of lipids in cells actively replicating KSHV.

Finally, we are also interested in conducting a global metabolomics analysis of lytically replicating cells to identify any other altered pathways during the KSHV lytic life stage. This analysis would reveal any other metabolic pathways altered and possibly required for KSHV lytic replication. These data should reinforce our current findings, highlighting alterations in major carbon metabolism, but could also unveil new druggable cellular targets to treat lytically replicating cells. One pathway that could be revealed as important to KSHV lytic replication could be nucleotide synthesis. Pyrimidine biosynthesis was recently found to support glycosylation of viral proteins during HCMV infection(108). It would be interesting if KSHV also depended on this pathway, as we have shown that the glycoprotein K8.1 is downregulated when glycolysis and glutamine metabolism are inhibited, but have not specifically examined protein glycosylation. We could preform molecular biology experiments to examine this question by supplementing cells with

pyrimidine precursors to see if in the absence of glucose or glutamine metabolism if these metabolites rescued KSHV viral titer. Overall, a metabolomics analysis would expand our current understanding of the cellular alterations that occur during KSHV lytic replication.

5.5 CONCLUSIONS

In conclusion, both latent and lytic KSHV infection require central carbon metabolism. I have found that inhibition of fatty acid synthesis and glutaminolysis induces cell death of latently infected endothelial cells unless supplemented with downstream metabolic intermediates of each pathway. These results indicate that the products of these metabolic pathways are critical for successful latent infection, and most likely are contributing to KS development. I have also shown that lytic replication and infectious KSHV virion production depends on glycolysis, glutaminolysis and FAS. Most interestingly, our work suggests that these pathways are contributing to virion production at different stages of lytic replication. It is clear that central carbon metabolism is playing a significant role or even multiple roles throughout KSHV infection. These alterations in metabolism are possibly being established early during latent infection to facilitate growth of latently infected cells for the expansion of latency, or possibly as a response to cell stress of latent infection. These metabolic signatures may also be established in preparation for their required participation in active lytic replication. Either way, their role during KSHV lytic replication needs to be further examined. Importantly, we may be able to identify a single therapeutic target for both latent and lytic KSHV infection. Alternatively, we could identify two metabolic targets, one required for survival of KSHV infected cells and the other required for maximal virus production. These basic research findings provide several attractive targets for therapeutic intervention of both latency and lytic replication. Drugs designed to target cellular metabolism could, for the first time, treat the entire KS tumor and all KSHV-infected cells in the body (Figure 5.1).

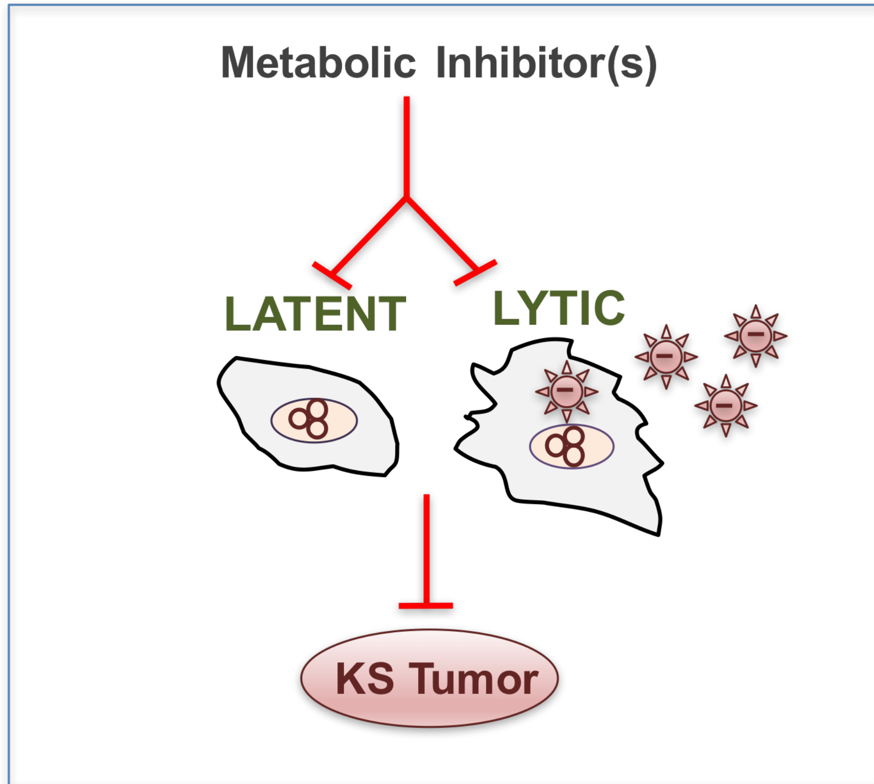

Figure 5.1: Metabolic targets of both latent and lytic KSHV infection exist
Previous data from the Lagunoff lab, as well as data from Chapter 3 and Chapter 4, provide strong evidence that there are metabolic targets of both latent and lytic KSHV infection. These basic research findings have the potential to influence drug design to target required metabolic pathways such as glycolysis, glutaminolysis and fatty acid synthesis to inhibit both latent and lytic KSHV infection. By killing latently infected cells and inhibiting lytically replicating cells from producing virus, we will be capable of treating all infected cells of the KS tumor.

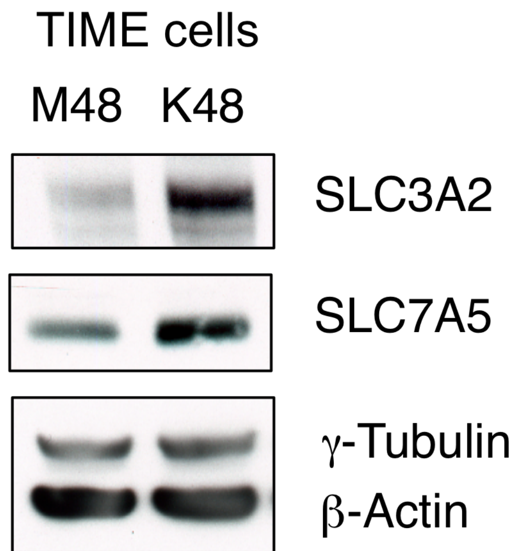

Figure 5.2: Solute carrier transport proteins, SLC3A2 and SLC7A5, are upregulated by KSHV infection

TIME cells were Mock- or KSHV-infected and whole-cell lysates were harvested at 48 hpi. Lysates were subjected to immunoblot analysis using the indicated antibodies. ϒ-tubulin and β-actin standards were included as loading controls.

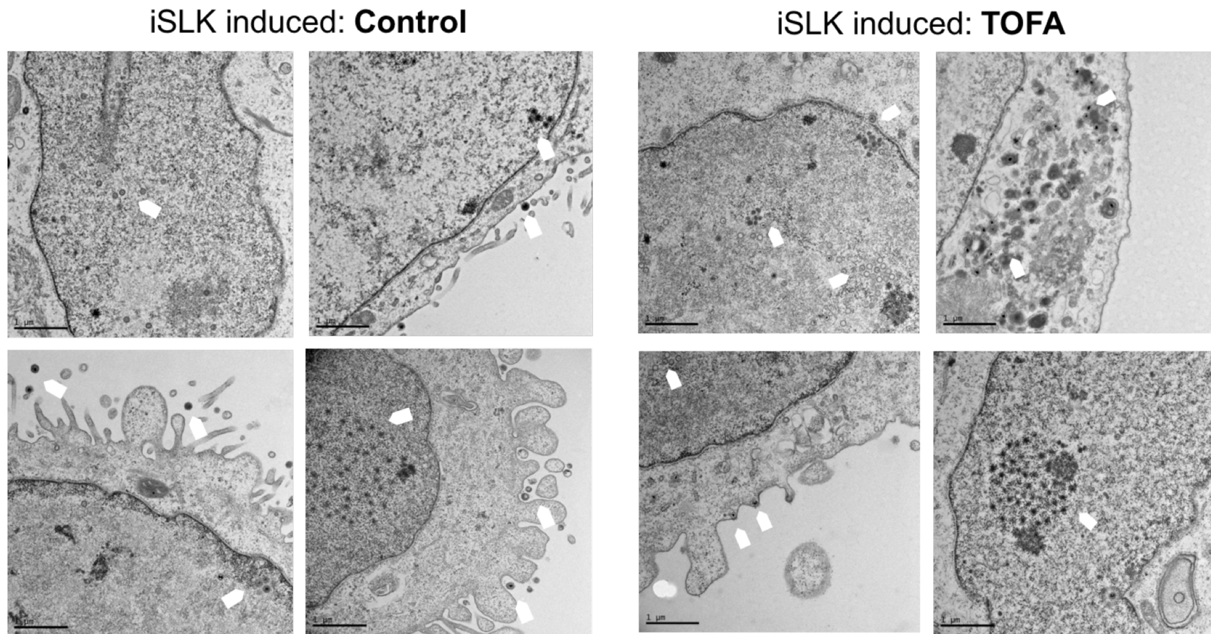

Figure 5.3: Virion assembly appears unchanged by FAS inhibition
Transmission electron microscopy of iSLK cells induced in the presence or absence of TOFA for 48 hours. Cells were fixed and harvested in ½ Karnovsky's fixative, and processed by the Fred Hutch EM core facility. White arrows indicate assembling and maturing virus particles in nucleus and cytoplasm. Budding virus particles are also indicated by white arrows.

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Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M (2015) "Latent KSHV Infected Endothelial Cells Are Glutamine Addicted and Require Glutaminolysis for Survival" PLoS Pathog 11 (7):e1005052. doi:10.1371/journal.ppat.1005052, Open access journal.

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2009-2010 **Postbaccalaureate Research**
NIH Academy-Intramural Research Training Award (IRTA)
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2007-2009 **Undergraduate Researcher: UC Davis Genome Center**
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Research Fellowships and Funding

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Awards

2016	University of Washington Graduate School Medal
2015	SACNAS National Conf Graduate Oral Presentation Award
2015	18th Annual International KSHV Workshop Travel Award
2015	Keystone Conference Travel Award (Viruses and Cancer)
2015	Keystone Conference Travel Award (Tumors and Metabolism)
2014	SACNAS National Lifetime Membership Award
2014	SACNAS National Conf Graduate Poster Presentation Award
2008	ABRCMS Undergraduate Poster Presentation Award

Publications

Sanchez EL, Thalhofer AB, Lagunoff M, "Lytic Replication of KSHV Requires Altered Host Cell Metabolism for Maximal Virion Production" (*manuscript in preparation*).

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M (2015) "Latent KSHV Infected Endothelial Cells Are Glutamine Addicted and Require Glutaminolysis for Survival" PLoS Pathog 11 (7):e1005052. doi:10.1371/journal.ppat.1005052

Sanchez EL, Lagunoff M, "Viral activation of cellular metabolism" Virology, Volumes 479–480, May 2015,Pages 609-618, ISSN 0042-6822

Fontaine KA, **Sanchez EL**, Camarda R, Lagunoff M. "Dengue Virus Induces and Requires Glycolysis for Optimal Replication." Journal of Virology. 2015;89 (4):2358-2366. doi:10.1128/JVI.02309-14.

Delgado T, **Sanchez EL**, Camarda R, Lagunoff M (2012) Global Metabolic Profiling of Infection by an Oncogenic Virus: KSHV Induces and Requires Lipogenesis for Survival of Latent Infection. PLoS Pathogens 8 (8): e1002866. doi: 10.1371/journal.ppat.1002866

Lee MS, Bonner JR, Bernard DJ, **Sanchez EL**, et al. "Disruption of the Folate Pathway in Zebrafish Causes Specific Developmental Defects" BMC Developmental Biology, 12:12 2012.

Blahnik KR, Dou L, Echipare L, Iyengar S, O'Geen H, **Sanchez E**, Zhao Y, Marro MA, Hirst M, Costello JF, Korf I, Farnham PJ. (2011) Characterization of the Contradictory Chromatin Signatures at the 3′ Exons of Zinc Finger Genes. PLoS ONE 6 (2): e17121. doi: 10.1371/journal.pone.0017121

Oral Presentations

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M, **"**Glutaminolysis is Required for Survival of Endothelial Cells Infected with an Oncogenic Virus," SACNAS, October 2015. Washington, DC.

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M, **"**Glutaminolysis is Required for Latent KSHV Infection of Endothelial Cells" 18th Annual International KSHV Workshop, July 2015. Miami, FL.

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M, **"**Glutamine Metabolism Is Required for KSHV Infected Endothelial Cell Survival" Keystone: Viruses and Cancer, Big Sky, MT. March, 2015.

Poster Presentations

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M, **"**Glutaminolysis is Required for Endothelial Cells Infected with an Oncogenic Virus," ABRCMS, Seattle WA. Nov 2015. Abstract accepted.

Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M, **"**Glutaminolysis is Required for Endothelial Cells Infected with an Oncogenic Virus" Keystone: Tumors & Metabolism, January, 2015. Vancouver, BC**.**

Sanchez EL, Thalhofer AB, Lagunoff M, "Glutaminolysis is Required for both Latent and Lytic KSHV Infection," ABRCMS Conference, November 2014. San Antonio, TX.

Sanchez EL, Thalhofer AB, Lagunoff M,"Glutaminolysis is Required for both Latent and Lytic KSHV Infection," SACNAS National Conference, Oct 2014. Los Angeles, CA.

Sanchez EL, Lagunoff M, "Glutaminolysis is Required for Latent KSHV Infected Cell Survival," ABRCMS, Nov 2013. Nashville, TN.

Sanchez, EL, Laguoff M., "Glutaminolysis is Required for Latent KSHV Infected Cell Survival," SACNAS National Conference, October 2013. San Antonio, TX.

Sanchez, EL, Lagunoff M., "Glutaminolysis is Required for Latent KSHV Infected Cell Survival" 16th Annual International KSHV Workshop. Puerta Vallarta, Mexico. June, 2013.

Sanchez, EL, Delgado T, Lagunoff M, "Global Metabolic Profiling of Infection by an Oncogenic Virus: KSHV Induces and Requires Lipogenesis for Survival of Latent Infection," American Society for Virology Conference, Madison WI. July, 2012.

Mentoring and Outreach

Mentoring Experience (2012-2016)

Winter 2016-present: rotation student, Mollie McDonnell (MCB program)
Summer 2015-present: Postbaccalaureate Fellow, Mona M. Ahmed
Winter 2013-2015: Undergraduate researcher, Hanna S. Hong
Winter 2013-Summer 2014: Postbaccalaureate Fellow, Angel B.Thalhofer
Winter 2014: rotation student, Daniel Holmes (Microbiology program, joined lab)
Fall 2013: rotation student, Katie Slavens (MCB program)
Winter 2012: rotation student, Zoi Sychev (MCB program, joined lab)

Formal Teaching Experience

Winter 2012: Biology 302: Molecular Techniques Laboratory

Society for the Advancement of Chicanos & Native Americans in Science (2010-2016)

SACNAS is a STEM student-led chapter that connect students with opportunities to mentor, network with students and faculty in their field, and present their independent research. I have helped organize and lead several professional/academic workshops and I have held several leadership roles in our UW Chapter.

UW SACNAS Leadership Positions
Chapter President: 2013-2014
Vice President: 2012-2013
Secretary: 2011-2012

UW SACNAS Chapter of the Year Awards: SACNAS National Conference
2015: Chapter Pillar Award
2014: Graduate School Chapter of the Year
2013: Distinguished Chapter Award
2012: Graduate School Chapter of the Year

Graduate Opportunities for Minority Achievement Program (2011-2016)

GO-MAP is a UW resource and community for underrepresented graduate students of color. GO-MAP aims to foster a culturally & ethnically diverse academic, professional & social environment for all UW students.

2013-2016 Graduate Student Ambassador (GSA): As a GSA, I have participated in several graduate recruitment events and have helped welcome new graduate students to campus in the fall.

BIBLIOGRAPHY

1. Davison AJ. Evolution of the herpesviruses. Vet Microbiol [Internet]. 2002 Apr [cited 2016 May 5];86(1-2):69–88. Available from: http://www.sciencedirect.com/science/article/pii/S0378113501004928

2. Fields BN, Knipe DM, Howley PM. Fields Virology [Internet]. Knipe DM, Howley PM, editors. Fields Virology. Lippincott Williams & Wilkins; 2007. 3177 p. Available from: http://www.loc.gov/catdir/toc/ecip072/2006032230.html

3. Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, et al. Identification of Herpesvirus-Like DNA Sequences in AIDS-Associated Kaposi's Sarcoma. Science (80-). 1994;266(5192):1865–9.

4. Dourmishev LA, Dourmishev AL, Schwartz RA, Lukac DM, Palmeri D. Molecular Genetics of Kaposi ' s Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Epidemiology and Pathogenesis. Microbiol Mol Biol Rev. 2003;67(2):175–212.

5. Ganem D. KSHV Infection and the Pathogenesis of Kaposi's Sarcoma. Annu Rev Pathol [Internet]. 2006 Jan [cited 2012 Dec 10];1:273–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18039116

6. Lagunoff M, Bechtel J, Venetsanakos E, Roy AM, Abbey N, Herndier B, et al. De Novo Infection and Serial Transmission of Kaposi ' s Sarcoma-Associated Herpesvirus in Cultured Endothelial Cells. J Virol. 2002;76(5):2440–8.

7. Dittmer D, Lagunoff M, Renne R, Staskus K, Haase A, Ganem D. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol [Internet]. 1998;72(10):8309–15. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9733875

8. Kedes DH, Lagunoff M, Renne R, Ganem D. Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma-associated herpesvirus. J Clin Invest. 1997;100(10):2606–10.

9. Cai X, Lu S, Zhang Z, Gonzalez CM, Damania B, Cullen BR. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proc Natl Acad Sci U S A [Internet]. 2005;102(15):5570–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15800047

10. Sun Q, Zachariah S, Chaudhary PM. The human herpes virus 8-encoded viral FLICE-inhibitory protein induces cellular transformation via NF-kappaB activation. J Biol Chem [Internet]. 2003;278(52):52437–45. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14563855

11. Liu J, Martin HJ, Liao G, Hayward SD. The Kaposi's sarcoma-associated herpesvirus LANA protein stabilizes and activates c-Myc. J Virol [Internet]. 2007;81(19):10451–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17634226

12. Bubman D, Guasparri I, Cesarman E. Deregulation of c-Myc in primary effusion lymphoma by Kaposi's sarcoma herpesvirus latency-associated nuclear antigen. Oncogene [Internet]. 2007;26(34):4979–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17310999

13. Friborg Jr. J, Kong W, Hottiger MO, Nabel GJ. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature [Internet]. 1999;402(6764):889–94. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10622254

14. Ojala PM, Tiainen M, Salven P, Veikkola T, Castanos-Velez E, Sarid R, et al. Kaposi's sarcoma-associated herpesvirus-encoded v-cyclin triggers apoptosis in cells with high levels of cyclin-dependent kinase 6. Cancer Res [Internet]. 1999;59(19):4984–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10519412

15. Leidal AM, Cyr DP, Hill RJ, Lee PW, McCormick C. Subversion of autophagy by Kaposi's sarcoma-associated herpesvirus impairs oncogene-induced senescence. Cell Host Microbe [Internet]. 2012;11(2):167–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22341465

16. Ahmad A, Groshong JS, Matta H, Schamus S, Punj V, Robinson LJ, et al. Kaposi sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 cooperates with Myc to promote lymphoma in mice. Cancer Biol Ther [Internet]. 2010;10(10):1033–40. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20818173

17. Muralidhar S, Veytsmann G, Chandran B, Ablashi D, Doniger J, Rosenthal LJ. Characterization of the human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) oncogene, Kaposin (ORF K12). J Clin Virol. 2000;16(3):203–13.

18. Yogev O, Lagos D, Enver T, Boshoff C. Kaposi's sarcoma herpesvirus microRNAs induce metabolic transformation of infected cells. PLoS Pathog [Internet]. 2014;10(9):e1004400. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25255370

19. Greene W, Kuhne K, Ye F, Chen J, Zhou F, Lei X, et al. Molecular biology of KSHV in relation to AIDS-associated oncogenesis. Cancer Treat Res [Internet]. 2007;133:69–127. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17672038

20. Mesri E a, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpesvirus. Nat Rev Cancer [Internet]. Nature Publishing Group; 2010 Oct [cited 2012 Dec 3];10(10):707–19. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20865011

21. Sanchez EL, Lagunoff M. Viral activation of cellular metabolism. Virology [Internet]. 2015 May [cited 2016 Feb 25];479–480:609–18. Available from: http://www.sciencedirect.com/science/article/pii/S0042682215000847

22. Renne R, Zhong W, Herndier B, McGrath M, Abbey N, Kedes D, et al. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat Med [Internet]. 1996;2(3):342–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8612236

23. Grundhoff A, Ganem D. Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. J Clin Invest [Internet]. 2004;113(1):124–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14702116

24. Myoung J, Ganem D. Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: maintenance of tight latency with efficient reactivation upon induction. J Virol Methods [Internet]. 2011 Jun [cited 2016 May 1];174(1-2):12–21. Available from: http://www.sciencedirect.com/science/article/pii/S0166093411001029

25. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov [Internet]. 2011;10(9):671–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21878982

26. Cairns R a, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer [Internet]. Nature Publishing Group; 2011 Feb [cited 2012 Nov 2];11(2):85–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21258394

27. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab [Internet]. 2008/01/08 ed. 2008;7(1):11–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18177721

28. Warburg O. On the origin of cancer cells. Science (80-) [Internet]. 1956;123(3191):309–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13298683

29. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene [Internet]. Nature Publishing Group; 2010 Jan 21 [cited 2012 Nov 1];29(3):313–24. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2809806&tool=pmcentrez&rendertype=abstract

30. Currie E, Schulze A, Zechner R, Walther TC, Farese Jr. R V. Cellular fatty acid metabolism and cancer. Cell Metab [Internet]. 2013;18(2):153–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23791484

31. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, et al. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci [Internet]. 2007 Dec 4;104(49):19345–50. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2148292&tool=pmcentrez&rendertype=abstract

32. Menendez J a, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer [Internet]. 2007 Oct [cited 2012 Nov 4];7(10):763–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17882277

33. Farese R V, Walther TC. Lipid droplets finally get a little R-E-S-P-E-C-T. Cell [Internet]. 2009 Nov 25 [cited 2012 Nov 1];139(5):855–60. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3097139&tool=pmcentrez&rendertype=abstract

34. Guo Y, Cordes KR, Farese R V, Walther TC. Lipid droplets at a glance. J Cell Sci [Internet]. 2009 Mar 15 [cited 2012 Nov 28];122(Pt 6):749–52. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714424&tool=pmcentrez&rendertype=abstract

35. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci [Internet]. Elsevier Ltd; 2010 Aug 1 [cited 2012 Oct 26];35(8):427–33. Available from: http://www.cell.com/article/S0968000410000915/fulltext

36. Dang C V. Glutaminolysis: Supplying carbon or nitrogen, or both for cancer cells? Cell Cycle

[Internet]. 2010 Oct 1 [cited 2012 Nov 18];9(19):3884–6. Available from: http://www.landesbioscience.com/journals/cc/article/13302/

37. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science [Internet]. 2009 May 22 [cited 2012 Oct 29];324(5930):1029–33. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2849637&tool=pmcentrez&rendertype=abstract

38. Eagle BYH, Habel AK. The Importance of Glucose and Glutamine for the Elaboration of Poliomyditis Virus by HeLa Cell. J Exp Med. 1956;104(2):271–87.

39. Lewis Jr. VJ, Scott L V. Nutritional requirements for the production of herpes simplex virus. I. Influence of glucose and glutamine of herpes simplex virus production by HeLa cells. J Bacteriol. 1962/03/01 ed. 1962;83:475–82.

40. Munger J, Bajad SU, Coller HA, Shenk T, Rabinowitz JD. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog [Internet]. 2006 Dec [cited 2012 Nov 20];2(12):e132. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1698944&tool=pmcentrez&rendertype=abstract

41. Munger J, Bennett BD, Parikh A, Feng X-JJ, McArdle J, Rabitz H a, et al. Systems-level metabolic flux profiling identifies fatty acid synthesis as a target for antiviral therapy. Nat Biotechnol [Internet]. 2008 Oct [cited 2012 Nov 1];26(10):1179–86. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2825756&tool=pmcentrez&rendertype=abstract

42. Vastag L, Koyuncu E, Grady SL, Shenk TE, Rabinowitz JD. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. PLoS Pathog [Internet]. 2011 Jul [cited 2012 Dec 1];7(7):e1002124. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3136460&tool=pmcentrez&rendertype=abstract

43. Delgado T, Sanchez EL, Camarda R, Lagunoff M. Global metabolic profiling of infection by an oncogenic virus: KSHV induces and requires lipogenesis for survival of latent infection. PLoS Pathog [Internet]. 2012 Aug [cited 2012 Dec 1];8(8):e1002866. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22916018

44. Weljie AM, Jirik FR. Hypoxia-induced metabolic shifts in cancer cells: moving beyond the Warburg effect. Int J Biochem Cell Biol [Internet]. Elsevier Ltd; 2011 Jul [cited 2012 Nov 18];43(7):981–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20797448

45. Birungi G, Chen SM, Loy BP, Ng ML, Fong S, Li Y, et al. Metabolomics approach for investigation of effects of dengue virus infection using the EA.hy926 cell line. J Proteome Res. 2010/10/20 ed. 2010;9(12):6523–34.

46. Hollenbaugh J a, Munger J, Kim B. Metabolite profiles of human immunodeficiency virus infected CD4+ T cells and macrophages using LC-MS/MS analysis. Virology [Internet]. 2011/05/14 ed. Elsevier Inc.; 2011 Jul 5 [cited 2012 Dec 1];415(2):153–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3107887&tool=pmcentrez&rendertype

=abstract

47. Lin S, Liu N, Yang Z, Song W, Wang P, Chen H, et al. GC/MS-based metabolomics reveals fatty acid biosynthesis and cholesterol metabolism in cell lines infected with influenza A virus. Talanta [Internet]. Elsevier B.V.; 2010 Nov 15 [cited 2012 Nov 13];83(1):262–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21035673

48. Ritter JB, Wahl AS, Freund S, Genzel Y, Reichl U. Metabolic effects of influenza virus infection in cultured animal cells: Intra- and extracellular metabolite profiling. BMC Syst Biol. 2010/05/15 ed. 2010;4:61.

49. Roe B, Kensicki E, Mohney R, Hall WW. Metabolomic profile of hepatitis C virus-infected hepatocytes. PLoS One [Internet]. 2011/08/20 ed. 2011 Jan [cited 2012 Dec 1];6(8):e23641. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3154941&tool=pmcentrez&rendertype=abstract

50. Delgado T, Carroll PA, Punjabi AS, Margineantu D, Hockenbery DM, Lagunoff M. Induction of the Warburg effect by Kaposi ' s sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. Proc Natl Acad Sci [Internet]. 2010;107(23):10696–701. Available from: http://0-www.pnas.org.innopac.up.ac.za/content/107/23/10696.full

51. Coleman MC, Asbury CR, Daniels D, Du J, Aykin-Burns N, Smith BJ, et al. 2-deoxy-D-glucose causes cytotoxicity, oxidative stress, and radiosensitization in pancreatic cancer. Free Radic Biol Med [Internet]. 2008 Feb 1 [cited 2012 Dec 1];44(3):322–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18215740

52. Elwood JC. Effect of Oxamate on Glycolysis and Respiration in Sarcoma 37 Ascites Cells. Cancer Res. 1968;28:2056–60.

53. Liu H, Hu YP, Savaraj N, Priebe W, Lampidis TJ. Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry [Internet]. 2001 May 8;40(18):5542–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11331019

54. Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, et al. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol Cancer [Internet]. BioMed Central; 2010 Jan 9 [cited 2016 May 1];9(1):33. Available from: http://molecular-cancer.biomedcentral.com/articles/10.1186/1476-4598-9-33

55. Bhatt AP, Jacobs SR, Freemerman AJ, Makowski L, Rathmell JC, Dittmer DP, et al. Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma. Proc Natl Acad Sci U S A [Internet]. 2012 Jul 17 [cited 2012 Dec 1];109(29):11818–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22752304

56. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell [Internet]. 2011;144(5):646–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21376230

57. Chambers JW, Maguire TG, Alwine JC. Glutamine metabolism is essential for human cytomegalovirus infection. J Virol [Internet]. 2009/11/27 ed. 2010 Feb [cited 2012 Dec 1];84(4):1867–73. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2812398&tool=pmcentrez&rendertype

=abstract

58. Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus activates glucose transporter 4 expression to increase glucose uptake during infection. J Virol [Internet]. 2010/12/15 ed. 2011 Feb [cited 2012 Dec 1];85(4):1573–80. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3028904&tool=pmcentrez&rendertype=abstract

59. Spencer CM, Schafer XL, Moorman NJ, Munger J. Human cytomegalovirus induces the activity and expression of acetyl-coenzyme A carboxylase, a fatty acid biosynthetic enzyme whose inhibition attenuates viral replication. J Virol [Internet]. 2011 Jun [cited 2012 Dec 1];85(12):5814–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21471234

60. Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces adipocyte-like lipogenesis through activation of sterol regulatory element binding protein 1. J Virol [Internet]. 2012/01/20 ed. 2012 Mar [cited 2012 Dec 1];86(6):2942–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3302344&tool=pmcentrez&rendertype=abstract

61. Koyuncu E, Purdy JG, Rabinowitz JD, Shenk T. Saturated very long chain fatty acids are required for the production of infectious human cytomegalovirus progeny. PLoS Pathog. 2013/05/23 ed. 2013;9(5):e1003333.

62. Yu Y, Pierciey Jr. FJ, Maguire TG, Alwine JC. PKR-like endoplasmic reticulum kinase is necessary for lipogenic activation during HCMV infection. PLoS Pathog. 2013/04/18 ed. 2013;9(4):e1003266.

63. Yu Y, Maguire TG, Alwine JC. ChREBP, a glucose-responsive transcriptional factor, enhances glucose metabolism to support biosynthesis in human cytomegalovirus-infected cells. Proc Natl Acad Sci U S A. 2014/01/23 ed. 2014;111(5):1951–6.

64. Venetsanakos E, Mirza A, Fanton C, Romanov SR, Tlsty T, McMahon M. Induction of tubulogenesis in telomerase-immortalized human microvascular endothelial cells by glioblastoma cells. Exp Cell Res [Internet]. 2002 Feb 1 [cited 2016 May 5];273(1):21–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11795943

65. Jain V, Plaisance-Bonstaff K, Sangani R, Lanier C, Dolce A, Hu J, et al. A Toolbox for Herpesvirus miRNA Research: Construction of a Complete Set of KSHV miRNA Deletion Mutants. Viruses [Internet]. 2016 Jan [cited 2016 May 5];8(2). Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4776209&tool=pmcentrez&rendertype=abstract

66. Punjabi AS, Carroll PA, Chen L, Lagunoff M. Persistent activation of STAT3 by latent Kaposi's sarcoma-associated herpesvirus infection of endothelial cells. J Virol [Internet]. 2007;81(5):2449–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17151100

67. Dronadula N, Du L, Flynn R, Buckler J, Kho J, Jiang Z, et al. Construction of a novel expression cassette for increasing transgene expression in vivo in endothelial cells of large blood vessels. Gene Ther [Internet]. Macmillan Publishers Limited; 2011 May [cited 2016 May 2];18(5):501–8. Available from: http://dx.doi.org/10.1038/gt.2010.173

68. Sandig V, Youil R, Bett AJ, Franlin LL, Oshima M, Maione D, et al. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc Natl Acad Sci [Internet]. 2000 Feb 1 [cited 2016 May 2];97(3):1002–7. Available from: http://www.pnas.org/content/97/3/1002.long

69. Wabinga HR, Parkin DM, Wabwire-Mangen F, Mugerwa JW. Cancer in Kampala, Uganda, in 1989-91: changes in incidence in the era of AIDS. Int J Cancer [Internet]. 1993;54(1):26–36. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8478145

70. Chokunonga E, Levy LM, Bassett MT, Mauchaza BG, Thomas DB, Parkin DM. Cancer incidence in the African population of Harare, Zimbabwe: second results from the cancer registry 1993-1995. Int J cancer [Internet]. 2000 Jan 1 [cited 2016 May 5];85(1):54–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10585583

71. Zhong W, Wang H, Herndier B, Ganem D. Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc Natl Acad Sci U S A [Internet]. 1996;93(13):6641–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8692871

72. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell [Internet]. 2008 Sep 5 [cited 2012 Nov 2];134(5):703–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18775299

73. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. J Clin Invest [Internet]. 2013 Sep [cited 2016 Apr 15];123(9):3678–84. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3754270&tool=pmcentrez&rendertype=abstract

74. Eagle BYH. Nutrition Needs of Mammalian Cells in Tissue Culture. Science (80-). 1955;122(3168):501–4.

75. Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang X-Y, Pfeiffer HK, et al. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci [Internet]. 2008 Dec 2;105(48):18782–7. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2596212&tool=pmcentrez&rendertype=abstract

76. Carroll PA, Diolaiti D, McFerrin L, Gu H, Djukovic D, Du J, et al. Deregulated Myc requires MondoA/Mlx for metabolic reprogramming and tumorigenesis. Cancer Cell [Internet]. Elsevier; 2015 Feb 9 [cited 2016 May 5];27(2):271–85. Available from: http://www.cell.com/article/S1535610814004759/fulltext

77. Yuneva M, Zamboni N, Oefner P, Sachidanandam R, Lazebnik Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. J Cell Biol [Internet]. 2007 Jul 2 [cited 2016 Jan 14];178(1):93–105. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2064426&tool=pmcentrez&rendertype=abstract

78. Fontaine KA, Camarda R, Lagunoff M. Vaccinia virus requires glutamine but not glucose for efficient replication. J Virol [Internet]. 2014;88(8):4366–74. Available from: http://jvi.asm.org/content/88/8/4366

79. Daye D, Wellen KE. Metabolic reprogramming in cancer: Unraveling the role of glutamine in tumorigenesis. Seminars in Cell and Developmental Biology. 2012. p. 362–9.

80. Dang C V. MYC, metabolism, cell growth, and tumorigenesis. Cold Spring Harb Perspect Med [Internet]. 2013 Aug [cited 2016 Apr 5];3(8). Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3721271&tool=pmcentrez&rendertype=abstract

81. Li X, Chen S, Feng J, Deng H, Sun R. Myc is required for the maintenance of Kaposi's sarcoma-associated herpesvirus latency. J Virol [Internet]. 2010;84(17):8945–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20573831

82. Valiya Veettil M, Dutta D, Bottero V, Bandyopadhyay C, Gjyshi O, Sharma-Walia N, et al. Glutamate secretion and metabotropic glutamate receptor 1 expression during Kaposi's sarcoma-associated herpesvirus infection promotes cell proliferation. PLoS Pathog [Internet]. 2014;10(10):e1004389. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25299066

83. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity [Internet]. 2011 Dec 23 [cited 2016 Mar 17];35(6):871–82. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3248798&tool=pmcentrez&rendertype=abstract

84. Diolaiti D, McFerrin L, Carroll PA, Eisenman RN. Functional interactions among members of the MAX and MLX transcriptional network during oncogenesis. Biochim Biophys Acta [Internet]. 2015 May [cited 2016 Mar 24];1849(5):484–500. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4241192&tool=pmcentrez&rendertype=abstract

85. Pochini L, Scalise M, Galluccio M, Indiveri C. Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. Front Chem [Internet]. 2014 Jan [cited 2016 May 5];2:61. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4127817&tool=pmcentrez&rendertype=abstract

86. Ren P, Yue M, Xiao D, Xiu R, Gan L, Liu H, et al. ATF4 and N-Myc coordinate glutamine metabolism in MYCN-amplified neuroblastoma cells through ASCT2 activation. J Pathol [Internet]. 2015 Jan [cited 2016 May 5];235(1):90–100. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25142020

87. Fontaine KA, Sanchez EL, Camarda R, Lagunoff M. Dengue virus induces and requires glycolysis for optimal replication. J Virol [Internet]. 2015;89(4):2358–66. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25505078

88. Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. Curr Opin Genet Dev [Internet]. 2008 Feb [cited 2016 Mar 25];18(1):54–61. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2476215&tool=pmcentrez&rendertype=abstract

89. Anastasiou D, Cantley LC. Breathless cancer cells get fat on glutamine. Cell Res. 2012;22(3):443–

6.

90. Hirschey MD, DeBerardinis RJ, Diehl AM, Drew JE, Frezza C, Green MF, et al. Dysregulated metabolism contributes to oncogenesis. Semin Cancer Biol [Internet]. 2015;35 Suppl:S129–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26454069

91. Cantor JR, Sabatini DM. Cancer cell metabolism: one hallmark, many faces. Cancer Discov [Internet]. 2012;2(10):881–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23009760

92. Sanchez EL, Carroll PA, Thalhofer AB, Lagunoff M. Latent KSHV Infected Endothelial Cells Are Glutamine Addicted and Require Glutaminolysis for Survival. PLoS Pathog [Internet]. 2015;11(7):e1005052. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4510438&tool=pmcentrez&rendertype=abstract

93. Thai M, Graham NA, Braas D, Nehil M, Komisopoulou E, Kurdistani SK, et al. Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication. Cell Metab. 2014;19(4):694–701.

94. Thai M, Thaker SK, Feng J, Du Y, Hu H, Ting Wu T, et al. MYC-induced reprogramming of glutamine catabolism supports optimal virus replication. Nat Commun [Internet]. 2015;6:8873. Available from: http://www.nature.com/doifinder/10.1038/ncomms9873

95. Yu Y, Clippinger AJ, Alwine JC. Viral effects on metabolism: changes in glucose and glutamine utilization during human cytomegalovirus infection. Trends Microbiol [Internet]. 2011/05/17 ed. Elsevier Ltd; 2011 Jul [cited 2012 Nov 16];19(7):360–7. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3130066&tool=pmcentrez&rendertype=abstract

96. Kuang E, Fu B, Liang Q, Myoung J, Zhu F. Phosphorylation of eukaryotic translation initiation factor 4B (EIF4B) by open reading frame 45/p90 ribosomal S6 kinase (ORF45/RSK) signaling axis facilitates protein translation during Kaposi sarcoma-associated herpesvirus (KSHV) lytic replication. J Biol Chem [Internet]. 2011;286(48):41171–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21994950

97. Wang C, Xu C, Sun M, Luo D, Liao D-F, Cao D. Acetyl-CoA carboxylase-alpha inhibitor TOFA induces human cancer cell apoptosis. Biochem Biophys Res Commun [Internet]. Elsevier Inc.; 2009 Jul 31 [cited 2012 Nov 17];385(3):302–6. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2724073&tool=pmcentrez&rendertype=abstract

98. Zhu FX, Yuan Y. The ORF45 protein of Kaposi's sarcoma-associated herpesvirus is associated with purified virions. J Virol [Internet]. 2003;77(7):4221–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12634379

99. Zhu FX, Cusano T, Yuan Y. Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. J Virol [Internet]. 1999;73(7):5556–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10364304

100. Bruce AG, Bakke AM, Gravett CA, DeMaster LK, Bielefeldt-Ohmann H, Burnside KL, et al. The ORF59 DNA polymerase processivity factor homologs of Old World primate RV2 rhadinoviruses

are highly conserved nuclear antigens expressed in differentiated epithelium in infected macaques. Virol J [Internet]. 2009;6:205. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19922662

101. Chen Y, Ciustea M, Ricciardi RP. Processivity factor of KSHV contains a nuclear localization signal and binding domains for transporting viral DNA polymerase into the nucleus. Virology [Internet]. 2005;340(2):183–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16043206

102. Chan SR, Chandran B. Characterization of human herpesvirus 8 ORF59 protein (PF-8) and mapping of the processivity and viral DNA polymerase-interacting domains. J Virol [Internet]. 2000;74(23):10920–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11069986

103. Arias C, Weisburd B, Stern-Ginossar N, Mercier A, Madrid AS, Bellare P, et al. KSHV 2.0: a comprehensive annotation of the Kaposi's sarcoma-associated herpesvirus genome using next-generation sequencing reveals novel genomic and functional features. PLoS Pathog [Internet]. 2014;10(1):e1003847. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24453964

104. Griffin JL, Shockcor JP. Metabolic profiles of cancer cells. Nat Rev Cancer [Internet]. 2004 Jul [cited 2012 Nov 3];4(7):551–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15229480

105. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, et al. Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy. Cell [Internet]. 2009 Feb 6 [cited 2015 Oct 19];136(3):521–34. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3733119&tool=pmcentrez&rendertype=abstract

106. Veettil M V, Sadagopan S, Sharma-Walia N, Wang FZ, Raghu H, Varga L, et al. Kaposi's sarcoma-associated herpesvirus forms a multimolecular complex of integrins (alphaVbeta5, alphaVbeta3, and alpha3beta1) and CD98-xCT during infection of human dermal microvascular endothelial cells, and CD98-xCT is essential for the postentry stag. J Virol [Internet]. 2008;82(24):12126–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18829766

107. Liu STH, Sharon-Friling R, Ivanova P, Milne SB, Myers DS, Rabinowitz JD, et al. Synaptic vesicle-like lipidome of human cytomegalovirus virions reveals a role for SNARE machinery in virion egress. Proc Natl Acad Sci U S A [Internet]. 2011 Aug 2 [cited 2012 Nov 6];108(31):12869–74. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3150939&tool=pmcentrez&rendertype=abstract

108. DeVito SR, Ortiz-Riano E, Martinez-Sobrido L, Munger J. Cytomegalovirus-mediated activation of pyrimidine biosynthesis drives UDP-sugar synthesis to support viral protein glycosylation. Proc Natl Acad Sci U S A. 2014/12/05 ed. 2014;111(50):18019–24.