HIPPO PATHWAY CONTROL OF KSHV LATENCY

by

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is the infectious cause of Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease. It is not known how KSHV causes cancer, but studies have shown that many viral gene products, including proteins and miRNAs, inactivate human tumour suppressor pathways. One putative KSHV oncoprotein, vGPCR, has been shown to inactivate the Hippo tumour suppressor pathway, but little is known about how this pathway might be controlled in the context of infection and expression of the full complement of viral gene products. Using an inducible model of KSHV lytic replication, I demonstrated that reactivation from latency and progression through the lytic cycle is associated with Hippo pathway activation. Silencing of the Hippo pathway transcription factor YAP1 increased reactivation from latency, lytic protein accumulation, and production of infectious virions. Accordingly, ectopic expression of the YAP1-deactivating kinase LATS1 also increased reactivation from latency. By contrast, LATS1 silencing reduced viral protein accumulation during late stages of lytic replication. Although the precise mechanism of Hippo-mediated control of the KSHV latent/lytic switch remains unclear, increased numbers of nuclear foci containing the viral latency-associated nuclear antigen (LANA) were observed in YAP1-silenced cells. LANA maintains latent viral episomes in the nucleus, suggesting that YAP1 deficiency may be linked to increased load of latency viral genomes, which may in turn influence the frequency of reactivation from latency. Finally, using a genetic screen to identify viral modulates of transcription of a luciferase reporter driven by the YAP1-binding protein TEAD, I demonstrated that several viral proteins can stimulate TEAD transcription, as well as two viral proteins, ORF21 and ORF71, that could inhibit it. Together, these findings suggest that KSHV latency is sensitive to perturbations in Hippo signal transduction, and that efficient lytic replication is aided by Hippo pathway activation. This presence of multiple Hippo pathway modulators in the KSHV genome suggests that the virus has evolved complex mechanisms to regulate this pathway.

LIST OF ABBREVIATIONS USED

AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
BCBL	Body Cavity Based Lymphoma
bp	Base Pairs
BSA	Bovine Serum Albumin
BSD	Blasticidin
CMDI	Cellular Microscopy Digital Imaging
CMV	Cytomegalovirus
CO2	Carbon Dioxide
CORES	Centralized Operation of Research Equipment and Support
DMEM	Dulbeccos Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EV	Empty Vector
FACS	Fluorescent associated cell sorting
FBS	Fetal Bovine Serum
g	Gravity
GFP	Green Fluorescent Protein
GPCR	G-protein coupled receptor
h	Hours
HAART	Highly Active Anti-Retroviral Therapy
HCMV	Human Cytomegalovirus
HDAC	Histone deacetylase
HI	Heat Inactivated
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRE	Hypoxia Responsive Element
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
IL	Interleukin
IRF	Interferon Regulatory Factor
ITAM	Immunoreceptor Tyrosine-based Activation Motif
KS	Kaposi's Sarcoma
KSHV	Kaposi' sarcoma-associated herpesvirus
LANA	Latent Nuclear Antigen
LAR	Luciferase Assay Reagent
LATS	Large tumour suppressor
luc	Luciferase
MCD	Multicentric Castlemans Disease

MEM	Minimal Essential Media
mg	milligram
MHV-68	Murine herpesvirus 68
miRNA	micro RNA
mL	milliliter
mM	millimolar
mRNA	Messenger RNA
MST	Mammalian STE20 -like kinase
MT	Middle T Antigen
NaB	Sodium Butyrate
NF2	Neurofibromatosis 2
NHDF	Newborn human dermal fibroblasts
NK	Natural Killer
OE	Overexpression
ORF	Open Reading Frame
PAN	Polyadenylated RNA
PBS	Phosphate Buffered Saline
PEI	Polyethlenimine
PEL	Primary Effusion Lymphoma
pen-strep	Penicillen and Streptomycin
PLB	Passive Lysis Buffer
PML	Promyelocytic leukemia
Puro	Puromycin
PVDF	Polyvinylidene fluoride
qPCR	Quantitative Polymerase Chain Reaction
RBP-Jk	Recombinant Signal Binding Protein
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTA	Enhancer of Regulation and Transcription
SCR	Scramble
shRNA	Short Hairpin Ribonucleic Acid
SIM	SUMO-interacting motif
ST	Small T Antigen
STUbL	SUMO-targeting Ubiquitin Ligase
SUMO	Small Ubiquitin-like Modifier
T	Tween-20
TAZ	Transcriptional Co-activator with PDZ Domain
TBS	Tris-buffered Saline
TEAD	TEA-domain transcription factor
TLR	Toll-like Receptor

TPA	12-O-tetradecanoylphorbol-13-acetate
μg	microgram
μL	microliter
UTR	Untranslated Region
V	Volts
VEGF	Vascular Endothelial Growth Factor
WT	Wild-type
YAP	Yes-associated protein

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Chapter 1 Introduction

1.1 Herpesviruses

Herpesviruses are a ubiquitous family of viruses that infect a wide range of eukaryotic organisms (Roizman and Furlong, 1974). All herpesviruses share a general structure including a genome core, a capsid, a protein layer of tegument, and a host-derived lipid bilayer containing both host and viral glycoproteins (Whitley, 1996). Of the greater than 100 herpesviruses characterized to date, only 8 of them can infect humans. All herpesviruses have a biphasic replication cycle, are able to establish a life-long latency in their infected host, and reactivate under stress to lytic replication (Stevens, 1989). Latency is characterized by limited viral gene expression to prevent immune detection, and not viral progeny are produced. Viral DNA is tethered to the host genome by viral proteins, promoting segregation of viral genomes into daughter cells following cell division. Through this tethering, the viral episome is passively replicated alongside host DNA using canonical host DNA replication machinery.

The family *Herpesviridae* is further split into three separate classifications, α, β and γ-herpesviruses. Each class of herpesvirus is separated based on morphological and tissue tropism differences. Alpha-herpesviruses, which includes Herpes simplex virus (HSV), establish latency in neurons and reactivates to infect epithelial cells on the skin (Whitley, 1996). Beta-herpesviruses, which include the highly prevalent cytomegalovirus (CMV), establish latency in leukocytes and causes severe immune system disruption by infecting several other immune cell lineages (Adams and Carstens, 2012). The final subclass of herpesviruses, the γ-herpesviruses, includes Kaposi's sarcoma-associated

herpesvirus (KSHV), which preferentially establishes latency in the B-cell compartment, and has been implicated in several endothelial and B-cell malignancies (Mesri et al., 2010).

1.2 Kaposi's Sarcoma-associated Herpesvirus

1.2.1 Introduction

KSHV is a γ-herpesvirus with a complex latent and lytic replication cycle. KSHV was first discovered during the HIV epidemic of the 1980/1990s. Using tissues extracted from Kaposi's sarcoma (KS) lesions from HIV+ patients, Dr. Patrick Moore and Dr. Yuan Chang discovered that KS tissue contained DNA from an uncharacterized herpesvirus utilizing a technique called representational difference analysis (Chang et al., 1994). Kaposi's sarcoma had been described for decades prior to isolating its causative agent. It is thought that HIV-associated immunosuppression increases viral load in KSHV infection. KS incidence is higher in HIV-susceptible populations, likely due to enhanced angioproliferative effects in the context of immune suppression. Infection by KSHV has also been implicated in the development of two rare lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

The KSHV genome is approximately 140 kilobases, which encodes at least 90 proteins and 18 miRNAs. In its mature form, an infectious KSHV virion contains a tightly packed core of viral DNA, with an icosahedral capsid containing five different capsid proteins (Trus et al., 2001). Between the capsid and host-derived membrane is a section of tegument protein: using both predictive and functional experiments,

approximately 9 KSHV ORFs encode for tegument proteins with various functions (Sathish et al., 2012). Tegument protein function includes antagonization of innate immune responses during primary infection, structural roles in virion production, and roles in viral egress. The activity of several of these tegument proteins is required for establishment of secondary infection, and many are required for efficient virion production (Gallo et al., 2017). The viral envelope is a host-derived lipid bilayer membrane, studded with viral glycoproteins.

1.2.2 KSHV Infectious Cycle

Establishment of viral latency in the B-cell compartment is not well understood. KSHV likely infects epithelial cells in the mouth, which allows for the passage of virus through to the bloodstream, where it can then infect B-cells; this route of infection is similar to what is seen with Epstein-Barr virus (EBV) infection (Odumade et al., 2011). It is thought that primary infection of the tonsils allows KSHV to establish latency in tonsillar B-cells, and B-cell movement between lymphatic compartments might transmit these latently infected cells to other B-cell compartments in the body (Hassman et al., 2011).

Several studies have shown that KSHV can be detected in healthy individuals, but is asymptomatic (Andreoni et al., 2002; Lagos and Boshoff, 2007). In these cases, it is likely that KSHV establishes latency initially, but virus spread would be contained by a competent immune system. Several immune subsets are highly active against KSHV infected cells – including natural killer (NK) cells and cytotoxic T-cells – both classically

known anti-viral immune cells. NK cells derived from healthy individuals are able to effectively lyse Primary Effusion Lymphoma (PEL) cells from KSHV infected individuals, and several subsets of cytotoxic T-cells have been isolated that are adapted to target cells expressing KSHV epitopes; it is clear that under healthy immune function, infection by KSHV will be cleared (Lagos and Boshoff, 2007). Under conditions of immune suppression, KSHV infection leads to severe disease. When the underlying cause of immunosuppression is treated – either with highly active anti-retroviral therapy (HAART) in the case of HIV co-infection, or by discontinuing immunosuppressive agents following organ transplant – KSHV-driven diseases go into remission. In the case of HIV co-infection, viral gene products have been shown to directly influence the ability of KSHV infection to persist – by treating patients with HAART, KSHV will also resolve (Thakker and Verma, 2016).

Upon initial infection of B-cells by KSHV, the default replication cycle is latency, but a small proportion of cells undergo lytic reactivation. Primary latent gene products are transcribed from the latency locus of the viral episome, including several genes and miRNAs (Figure 1). The latency- associated nuclear antigen (LANA) is one of the most important latency gene products. Functionally, LANA works to physically tether the viral episome to the host by binding histone proteins, and it has several roles in controlling host cell mechanisms in order to support latency (Liu et al., 2007). LANA targets several lytic program proteins that potentiate reactivation, effectively acting as a pro-latency protein by dampening reactivation (Lan et al., 2004). A more in-depth analysis of the latent/lytic switch will be described in Section 1.2.6. In addition to LANA, there are

several other latency-associated genes that have key roles in regulating cellular events to promote latency. The viral encoded cyclin, (v-cyclin, cellular D-cyclin homolog) allows for continued proliferation of latent cells (Van Dross et al., 2005). The viral FLICE - inhibitory protein (vFLIP) upregulates the expression of several anti-apoptotic proteins (Cavallin et al., 2014), and regulates the effects of another latent protein, v-cyclin (Leidal et al., 2012) promoting cell survival.

The lytic replication cycle is a stepwise process through immediate early, early, and late lytic reactivation. Gene expression at each of these time points differs, and viral gene accumulation is required for successful reactivation. Immediate early gene expression includes proteins required to control the latent/lytic switch, including ORF50. ORF50 will be discussed in detail below. Early lytic gene expression encodes for the viral polymerase, ORF9, which is required for high levels of viral genome replication, and other viral products required for genome replication. Gene expression during the late lytic phase requires an abundance of episomal DNA, which triggers the expression of structural and glycoprotein genes required for the nucleation and formation of the viral capsid (Lukac and Yuan, 2007). The exact process of viral egress in a productive KSHV infection is not well known, but several viral proteins are required for this process, including glycoprotein B and tegument protein ORF45 (Krishnan et al., 2005).

1.2.3 Lymphoproliferative Disorders and KSHV

Infection of B-cells by KSHV can lead to two lymphoproliferative disorders: Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD).

Though these disorders are markedly different in terms of disease progression and symptoms, both diseases share two main characteristics: infection by KSHV, and host immunosuppression.

PEL is a non-Hodgkins form of lymphoma, often referred to as a body cavity based lymphoma, or BCBL. Naïve and undifferentiated B-cells are the primary cell infected, and clonal populations proliferate to form PEL (Kati et al., 2013). Establishment of PEL is very rare, but highly fatal, and there are very few treatments available if immune reconstitution fails (Wen and Damania, 2010).

MCD has several disease variants classified by severity and dissemination. A plasmablastic variant is highly aggressive and characterized by disordered B-cell proliferation in multiple body compartments. This disordered growth is not often seen clonally, and many cellular lineages are likely involved (Wen and Damania, 2010). MCD requires high levels of both human and viral IL-6 to drive constitutive activation of B-cells (Parravinci et al., 1997).

As described, both PEL and MCD share several characteristics, and further understanding of KSHV dynamics within the B-cell lineages primarily infected in these disorders is ongoing. Interestingly, viral gene expression in each of these disorders is quite different, and regulation of latency and lytic reactivation varies greatly (Parravicini et al., 2000). The lymphoproliferative disorders described here are not as well studied as

the third disease caused by KSHV infection: the endothelial lesion known as Kaposi's sarcoma.

1.2.4 Kaposi's Sarcoma

1.2.4.1 Introduction

Kaposi's sarcoma was first identified in 1872 by Moritz Kaposi, who described the disease as an "idiopathic multiple pigmented sarcoma of the skin" (Sternbach and Varon, 1995). Until the 1980s, incidence of KS was rare, and only regions of endemic disease showed high rates of this cancer. It wasn't until the HIV/AIDS epidemic of the 1980s that Kaposi's sarcoma came into the public eye, as incidence of this cancer increased in populations susceptible to HIV infection. Increased rates of Kaposi's sarcoma are also seen among populations with suppressed immune systems, as in those following organ transplant.

1.2.4.2 Epidemiology

There are several different manifestations of KS, and though all require infection by KSHV, the populations' in which the disease develops differentiates each group, as well as the aggressiveness and severity of tumours. AIDs-associated KS arises in individuals that are also infected by HIV; it is most prevalent among males who have sex with men, and is also the most aggressive form of KS. The classic form of KS, as described by Moritz Kaposi, is most commonly seen in middle-aged Mediterranean or eastern European men. An endemic form of KS exists in Africa, largely with HIV co-infection. And lastly, an iatrogenic form of KS has been seen among patients undergoing

immunosuppressive therapy; though, near full recovery is seen when immune activity is restored (Curtiss et al., 2016).

1.2.4.3 Complexity of KS Lesions

The endothelial tumour of Kaposi's sarcoma is a complex lesion that consists of several different cell types, and the majority of infected cells within a KS lesion are latently infected (Zhong et al., 1996). Paradoxically, certain characteristics of KS, namely high levels of angiogenesis and inflammation, require lytic gene expression. There are two main theories that attempt to explain the paradox in the latent versus lytic gene expression in a KS lesion. The first theory describes a paracrine effect, where neighbouring cells in the lesion are undergoing a productive lytic infection; not only does this increase the spread of virus throughout the lesion, it also allows for the expression of several pro-inflammatory viral genes in neighbouring cells that drive tumour progression (Chang and Ganem, 2013). For example, vGPCR, a constitutively active G-protein coupled receptor, increases the secretion of VEGF into the microenvironment of a KS lesion. VEGF expression increases the vascularity of KS tumours, and is a hallmark of KS tumour progression. Expression analysis of vGPCR indicates it is active only during the lytic program, therefore, some subset of cells within the KS lesion must be actively expressing lytic program genes, undergoing a productive lytic reactivation (Cavallin et al., 2014). Because a small fraction of cells in each KS lesion express lytic gene products like vGPCR, it is hypothesized that lytic gene products contribute to the tumour microenvironment. The second theory is that of abortive lytic infection; a subset of cells in the lesion undergoes the initial stages of the lytic gene expression, but reactivation is

aborted, and no viral progeny are produced. This allows for the expression of early lytic genes like vGPCR, but not structural proteins required for viral release, preventing immune detection or protein stress in cells (Bais et al., 2003). These theories are supported by evidence showing that the paracrine effects of vGPCR alone are able to elicit tumour formation (Jensen et al., 2005), as well as paracrine lytic gene products influencing gene expression in latently infected cells. Also, difference in viral gene expression has been shown dependent on the cell type infected by KSHV, adding to the complexity in understanding tumour progression (Chang and Ganem, 2013). As shown by Gutkind's group, targeting of vGPCR-expression lesion cells alone allows for regression of KS tumours (Montaner et al., 2006).

The complexity of Kaposi's sarcoma lesions makes studying KS *in vitro* especially challenging; most of what is known about KSHV infected cells has been learned outside of the context of a KS lesion, and as such, may not be easily translated to the *in vivo* setting. The greatest barrier to understanding the *in vivo* activity of KSHV is the lack of an easily accessible model; KSHV does not efficiently infect mice, and though there are some murine gammaherpesviruses (murine gammaherpesvirus-68, MHV-68) that resemble KSHV activity, hypotheses derived from MHV-68 work cannot be directly related to KSHV activity due to differences in key viral proteins. Some groups have worked to established non-human primate models with some success, but accessibility to these models is a challenge for many groups (Chang et al., 2009). Developing a murine model of KSHV remains essential to advance *in vivo* studies.

1.2.5 Molecular Mechanisms of KS Tumour Formation

1.2.5.1 Introduction and Current Model Systems

Establishing a model to understand the oncogenic mechanisms of infection by KSHV is challenging; there are several models in use, but each with limitations. Several versions of recombinant KSHV have been developed, each with its own set of unique challenges. There are several cell-culture models of primary B-cell infection, but culturing endothelial cells derived from KS lesions has been unsuccessful to date (Chang et al., 2009), and as such several groups have developed *in vitro* models that act to recapitulate the KS spindle cell. Using the recombinant KSHV virus, rKSHV.219 (Vieira and O'Hearn, 2004), an epithelial cell line SLK was infected, and a very tight control of latency was established through the use of a mammalian antibiotic selection marker - puromycin. The iSLK.219 cell line is often used in studies of the latent/lytic switch of KSHV reactivation, and will be used extensively in this thesis (Myoung and Ganem, 2011). More about this cell model will be described below.

KSHV also naturally infects B-cells, and lifelong latency is maintained in the B-cell population. There are several cell lines that are most often used to study B-cell infections of KSHV, and both have been derived from patients infected by KSHV and adapted for growth in culture systems. The BCBL-1 model is often used to understand the infection in a B-cell, but levels of spontaneous reactivation are much higher than the iSLK.219 model of epithelial infection.

Work to establish KSHV genes as hallmark oncogenes has been ongoing, and though several candidate oncogenes have been studied, it is unlikely that one specific viral gene alone drives development of KS lesions or B-cell lymphomas. Both latent and lytic gene products have been studied for their ability to transform cultured cells, or form tumours in immunocompromised mice; including LANA, vFLIP, Kaposin, vGPCR, K1, vIL-6 and vIRF-1. This group of viral genes has wide-ranging functions, and expression profiles that vary throughout the viral replication cycles. It is likely that the activity of latent genes help prime cells for stimulation by paracrine lytic effectors, allowing for the establishment of a KS lesion (Wen and Damania, 2010).

1.2.5.1.1 iSLK.219 Cell Model

The cell line primarily used within this thesis is the iSLK.219 cell line, developed in the Ganem laboratory (Myoung and Ganem, 2011). This cell model utilizes the rKSHV.219 recombinant virus that constitutively expresses GFP under the control of an EIF1A promoter. Upon lytic reactivation, an RFP construct under the control of the ORF50-inducible PAN promoter is expressed, and the cells fluoresce both GFP and RFP – allowing for an easy analysis of replication cycle status. To control lytic reactivation, the iSLK.219 model contains a retrovirally inserted doxycycline-inducible ORF50 construct. Through the addition of doxycycline, high levels of ORF50 are expressed, which induces potent lytic reactivation. iSLK.219 cells were selected for tight latency; spontaneous lytic reactivation is not seen in this model.

1.2.5.2 LANA

As described earlier, LANA is a multifunctional latent protein, with oncogenic properties. LANA can bind and inhibit p53, preventing p53-mediated apoptosis - a key step for preventing oncogenic cellular changes (Friborg et al., 1999). Though LANA and p53 have been shown by several groups to form complexes, active p53 is still found in KSHV infected cells, suggesting that cellular pools of p53 are not completely inactivated by LANA binding (Chen et al., 2010). LANA has also been shown to bind and inhibit the retinoblastoma (Rb), a tumour suppressor protein thereby promoting uncontrolled entry into the cell cycle (Radkov et al., 2000). Though able to actively target two main proteins often antagonized during the early stages of cancer development, LANA alone is not sufficient to cause cellular transformation, but can work in conjunction with other well-characterized oncogenes, the human papillomavirus (HPV) E6 and E7 proteins, as well as the small GTPase Hras (Wen and Damania, 2010) to transform cultured cells.

1.2.5.3 vGPCR

vGPCR is encoded by KSHV ORF74, and is a viral homolog of the cellular IL-8 receptor. vGPCR is constitutively active; it does not require the binding of a ligand to trigger a signaling cascade. vGPCR signals through multiple cellular growth pathways, including PI3K and Akt (Wen and Damania, 2010), and the upregulation of VEGF induced by vGPCR can transform several cell lines, as well as primary endothelial cells (Bais et al., 2003). *In vivo*, vGPCR drives the formation of epithelial tumours in mice that are phenotypically similar to KS lesions, with high levels of both angiogenesis and inflammation (Montaner et al., 2003). The Montaner *et al* 2003 study also highlighted the

role for vGPCR as a paracrine modulator of latently infected cells. In a study of vGPCR expression in a KS lesion, a small percentage of cells had detectable levels of protein, providing evidence that vGPCR is likely stimulating proliferation in KS lesions in a paracrine manner (Lukac and Yuan, 2007; Montaner et al., 2003). More recently, the Guan lab has showed that vGPCR-driven tumourigenesis requires activity of two recently identified cellular oncogenes, YAP and TAZ, to promote tumour formation in mice (Liu et al., 2015).

1.2.5.4 K1

The KSHV K1 encodes for a single-pass transmembrane protein that contains an immunoreceptor tyrosine-based activation motif (ITAM) with functional homology to a domain in the B-cell receptor signaling complex (Lee et al., 1998a). Like vGPCR, K1 is constitutively active, and does not rely on external signaling. In several models, K1 expression alone can immortalize rat cells, and transgenic mice expressing K1 show a tendency to form tumours phenotypically similar to KS lesions (Lee et al., 1998b; Prakash et al., 2002). Through the upregulation of several secreted growth factors, it is likely that the oncogenic activity of K1 is acting in a paracrine manner (Wang et al., 2004). K1 is an early lytic protein, but there is evidence showing K1 mRNA is present at low levels during latent infection, suggesting that there could be an opportunity for K1 to contribute to the latent phenotype (Bowser et al., 2002).

1.2.6 Latent/Lytic Switch of KSHV

KSHV has two distinct replication cycles: a persistent and life-long latency, and lytic replication. Following primary infection, KSHV establishes reversible latency in most infected cells, and is the default mechanism following primary infection. In the context of a KS lesion, a small pool of lytically infected cells is required to produce both viral and host proteins to support latently infected cells; in cell culture systems without tight selection of latency, viral episomes are slowly lost over time. Lytic reactivation is thought to occur in three stages: reactivation from latency, lytic DNA replication, and virion production (Purushothaman et al., 2015). A complex cascade of viral gene expression follows reactivation, and there are three distinct subsets of gene expression: immediately early, early, and late lytic, with the majority of viral genes expressed during lytic reactivation (Toth et al., 2010). Latency allows for long-term persistence of viral infection, and the small repertoire of viral gene expression prevents immune detection of infected cells.

Maintaining tight control over lytic reactivation is important in persistence of latency, and several mechanisms are employed by KSHV to maintain regulation of the latent lytic switch.

1.2.6.1 ORF50 - Enhancer of Regulation and Transcription (RTA)

Immediate early protein ORF50 is both necessary and sufficient to reactivate latently infected cells and trigger the cascade of lytic reactivation events (Wang et al., 2003). ORF50 is a transcription factor that can act on viral and host genes. Activation of

ORF50 is complex, and latent viral genes work to keep ORF50 activity low to prevent reactivation of the lytic program.

The transcription factor activity of ORF50 has been widely studied, and its activating ability can be divided into Recombinant Signal Binding protein (RBP-Jk) -dependent and -independent interactions. RBP-Jk is a DNA binding protein within the Notch signaling pathway that identifies Notch responsive promoters (Guito and Lukac, 2012), and is a required host component of lytic reactivation (Liang and Ganem, 2003). RBP-Jk-independent ORF50 activity upregulates the KSHV PAN transcript, a long polyadenylated RNA that is the most abundant transcript following lytic reactivation, and another essential KSHV gene for lytic reactivation (Borah et al., 2011). In a RBP-Jk-dependent manner, ORF50 can drive the expression of a number of immediate early lytic proteins including ORF57, or mRNA transcript accumulation (Mta), which is required for export of unspliced viral mRNAs from the nucleus during viral replication (Majerciak and Zheng, 2015). Acting as a transcription factor, ORF50 triggers a cascade of viral gene expression, leading to a productive reactivation and release of infectious virions. Not only does ORF50 regulate the activity and expression of viral proteins, but it can act on a number of cellular proteins via its SUMO-targeting ubiquitin ligase (STUbL) activity, which is required for efficient lytic reactivation (Izumiya et al., 2013). This STUbL activity reduces the pool of SUMOylated protein in the cell, degrading many host proteins, thereby contributing to host shut-off. Regulation of this potent viral gene activator is complex, but two major latent gene products have been shown to tightly control the activity of ORF50, LANA and miR-K9.

1.2.6.1.1 LANA and ORF50

Interactions between LANA and ORF50 are context-dependent; during *de novo* infection, ORF50 upregulates LANA expression, allowing for establishment of latency as LANA functionally links the viral episome to the host genome (Lan et al., 2005). During latency, LANA binds and inhibits ORF50 (Lan et al., 2004). The Roberston group has shown that, in a dose-responsive manner, LANA can block activity at the endogenous ORF50 promoter, preventing transcription and activation of this viral lytic switch gene, effectively promoting maintenance of latency. In a similar dose-dependent fashion, increasing levels of LANA co-expressed with ORF50 showed that LANA can physically block ORF50 transactivation, and reduce the lytic reactivation in TPA-induced B-cells (Lan et al., 2004). This example of a viral gene working to regulate another viral gene shows the complexity in control of KSHV lytic reactivation. See Figure 1 for a summary of stresses that control both latency and lytic reactivation.

1.2.6.1.2 miRNAs and ORF50

More recently, a KSHV-encoded miRNA expressed during both latent and lytic replication cycles has been demonstrated to regulate the protein level of ORF50. miR-K9, one of the 18 known miRNAs encoded by KSHV, affects the translation efficiency of ORF50, reducing overall levels of protein in cells induced for lytic reactivation by binding to the 3' untranslated region (UTR) of ORF50 mRNA (Bellare and Ganem, 2009). By reducing levels of miR-K9 in latently infected cells, spontaneous lytic reactivation was significantly increased, suggesting that miR-K9 acts to reduce levels of

ORF50 during latency, preventing spontaneous lytic reactivation. Though not a major player in the control of the latent/lytic switch, miR-K9 likely functions to reduce basal levels of ORF50 transcription to tightly repress lytic reactivation in the absence of stress.

1.2.6.2 Triggers that Cause Lytic Reactivation

Understanding the role of stress in lytic reactivation of KSHV is a major area of research – it has implications in understanding what triggers latently infected cells to switch to a productive infection, causing further infection and dissemination of disease.

Viral co-infection is a well-studied phenomenon that has the potential to trigger reactivation of the KSHV lytic program. Co-infection by several herpesviruses including herpes simplex virus (HSV-1, HSV-2), EBV, and HCMV can potentiate lytic reactivation, as well as co-infection by retrovirus HIV (Purushothaman et al., 2015). Interestingly, HIV protein Tat and Nef are able to reactivate KSHV from latency alone – adding complexity to the interplay of HIV and KSHV co-infection (Zhu et al., 2014). Not only are viral proteins able to reactivate KSHV from latency, but the activation of anti-viral signaling networks in the cell, namely Toll-like receptor (TLR) 7 and 8, can trigger reactivation (Gregory et al., 2009), though the signaling mechanism behind this remains unclear.

Cellular stresses such as hypoxia and reactive oxygen species (ROS) have also been implicated in the regulation of lytic reactivation. ORF50, the major latent/lytic switch protein, contains a hypoxia responsive element (HRE) within its promoter;

reduced oxygen conditions within cells or tissues will upregulate the expression of ORF50, triggering lytic reactivation (Purushothaman et al., 2015). High levels of ROS in KSHV infected cells is a well-described phenomenon, and activation of several cellular signaling cascades by excess hydrogen peroxide has been shown to trigger lytic reactivation (Ye et al., 2011). Whether ROS production in KSHV infected cells is a consequence of viral infection, or being triggered by viral gene expression, has yet to be fully understood.

KSHV infected cells are also remarkably sensitive to chromatin modulation; treatment with histone deacetylase (HDAc) inhibitors can activate the lytic program to a higher degree than seen with other treatments; exogenous expression of ORF50 is not required. This sensitivity is based on the chromatin surrounding the promoter for ORF50 – gene expression is selectively controlled by several HDAcs (Shin et al., 2014). In the presence of HDAc inhibitors – such as sodium butyrate (NaB) – histone acetylation is increased, allowing for greater access of transcription machinery to the ORF50 promoter, driving gene expression (Lu et al., 2003).

Though extensively studied *in vitro*, the greater challenge to this work is to understand the role of these cellular stresses in reactivation *in vivo*. Understanding the process behind reactivation of life-long latency in humans is ongoing, and likely consists of a complex interplay between several of the reactivation triggers discussed above, as well as immune evasion strategies.

1.3 The Hippo Signaling Pathway

1.3.1 Introduction

The Hippo signaling pathway consists of a myriad of both upstream and downstream signals and effectors; but at its core, contains two kinases and a set of nuclear effectors. The Hippo pathway acts as a phosphorylation cascade that controls the activity of nuclear effectors – Yes-associated protein (YAP) and a paralog transcriptional co-activator with a PDZ domain (TAZ). Mammalian STE20 like kinase (MST1/2) is a core Hippo kinase that phosphorylates large tumour suppressor kinase (LATS1/2) through its scaffolding protein SAV1. MST1/2 interacts with several other cellular proteins, integrating several signaling networks. When activated by MST1/2, LATS1/2 phosphorylates YAP/TAZ via its scaffolding protein MOB1. LATS1/2 has several roles outside of Hippo signaling, and is the canonical kinase that controls YAP/TAZ phosphorylation. When phosphorylated, YAP/TAZ are sequestered in the cytoplasm either by binding cellular protein 14-3-3 for cytoplasmic sequestration or being targeted for degradation via the ubiquitin-lysosomal degradation pathway. In general, the Hippo pathway is a tumour suppressor pathway that regulates the activity of two oncogenes, YAP and TAZ. When Hippo signaling is ‘on’, YAP/TAZ are inactive and retained in the cytoplasm; when ‘off’, YAP/TAZ can transit to the nucleus to induce expression of a subset of genes activated by the TEA-domain (TEAD) set of transcription factors, upregulating expression of many genes required for cell growth and proliferation. Figure 2 depicts important aspects of the Hippo signaling pathway.

1.3.2 Discovery of Hippo Pathway Proteins

Before understanding the cascade of Hippo signaling, several components were discovered using genetic screens in *Drosophila melanogaster*, hunting for as-yet undiscovered tumour suppressor genes. Further characterization of pathway dynamics occurred in both *Drosophila* and mammalian cells, but most initial findings are supported by genetic studies in the fruit fly – most Hippo pathway proteins identified in *Drosophila* have homologs in murine and human cell lineages – this pathway is remarkably well conserved.

In 1995, two groups independently identified wts (mammalian homolog LATS) as a key protein regulating cell growth; when disrupted, large tissue outgrowth was observed in *Drosophila* (Justice et al., 1995; Xu et al., 1995) .The genetic approach used by these groups – genetic mosaics, common to the field of *Drosophila* research, was used to understand the roles of several other Hippo pathway proteins in the following years. This strategy allows for inducing genetic changes in certain tissue subsets to study gene knockouts that could be lethal for development (Blair, 2003) In 2003, Drosophila gene Hpo, and its mammalian homolog MST, were identified as core Hippo components (Harvey et al., 2003; Wu et al., 2003). Several groups' linked MST activity to reduction in cell proliferation and promotion of apoptosis, activities that indicate the Hippo pathway may function similarly to known tumour suppressor pathways. Throughout discovery of these proteins, researchers linked activity back to mammalian homologs in nearly every instance, but it was not until 2007-2008 that the Hippo pathway as a whole was understood in mammals, when LATS1/2 was shown to negatively regulate the

activity of YAP/TAZ (Hao et al., 2008; Zhao et al., 2007). Following these major discoveries, research surrounding the understanding of signals that both activate and impede the tumour suppressor activity of the pathway have been ongoing, and understanding the implication of these signaling proteins in a wide range of cancers has continued.

1.3.3 Canonical Regulation of Hippo Pathway Signaling

There are many physical and chemical cues that can modulate signaling through the Hippo pathway – in both healthy and disease models. Regulation of Hippo activity is important to maintain healthy tissues and organs, and to protect the cell from stress.

The main physical signal that modulates Hippo pathway signaling is cell-cell contact. In the context of a tissue or organ, cell-cell contact acts to maintain tissue size, and the architecture of the tissue alone is enough to restrict further proliferation. High cell density is able to potently activate LATS1/2, which then phosphorylates YAP, leading to its cytoplasmic sequestration (Zhao et al., 2007). Though the exact mechanism behind cell-cell contact and increased activity of LATS1/2 is not fully understood, it seems that an upregulation of tight junctions in these cells contributes to LATS1/2 activation (Silvis et al., 2011). Both extracellular matrix (ECM) rigidity and cell shape are potent regulators of YAP/TAZ localization, but it is unclear whether these regulatory signals act in a LATS1/2-dependent or -independent manner (Codelia et al., 2014; Dupont et al., 2011).

G-protein coupled receptors (GPCRs), ubiquitous cellular proteins that act as signal transducers for paracrine signaling molecules, are potent regulators of the Hippo pathway. Specifically, the $G\alpha_{12/13}$ and $G\alpha_{q/11}$ GPCR effector proteins have been shown to regulate activity through LATS1/2, via several different intermediary proteins – including RhoA, which is activated by actin dynamics (Yu et al., 2014). KSHV vGPCR regulates the activity of LATS1/2, reducing its ability to phosphorylate YAP/TAZ, potentially contributing to the oncogenic nature of the virus (Liu et al., 2015).

Several cellular stresses have been studied in their ability to regulate signaling through the Hippo pathway, including hypoxia, oxidative stress, and nutrient stress. Under hypoxic conditions, LATS1/2 is targeted for degradation via the upregulation of an E3 ubiquitin ligase that destabilizes the protein; as such, YAP has higher levels of nuclear activity (Ma et al., 2015). Several groups identified that YAP can act as a ROS scavenger, and that MST1/2 is activated during cellular responses to oxidative stress – though the role for Hippo during oxidative stress is not fully understood – as previous studies have hypothesized different roles (Meng et al., 2016). Nutrient stress is a major regulator of Hippo signaling which is expected, as the pathway controls the expression of growth and cell division genes. Activation of LATS1/2 during glucose deprivation works to phosphorylate and deactivate YAP, but other kinases activated via nutrient stress signals can regulate YAP localization independent of LATS1/2 phosphorylation (DeRan et al., 2014; Mo et al., 2015). The implication of YAP activity in this broad range of cellular stress signals suggests it is tightly regulated within the cell, and that dysregulation at any point has the potential to cause severe disease. Recently, Hippo pathway signaling has

been implicated in maintaining innate immune function, and will be described further in Section 1.3.5.1.

1.3.4 Hippo Signaling and Cancer

Many Hippo pathway proteins were discovered via screens for tumour suppressors in *Drosophila*; from discovery, the activity of these proteins was implicated in the regulation of cell growth and division. Research focusing on the role of Hippo pathway proteins in the development of tumours in various models has exploded over the last decade, and it is becoming clear that Hippo pathway regulation is key in maintaining cellular integrity, as YAP has the potential to be a highly aggressive oncogene.

1.3.4.1 Initial Observations

In the early 1990s, a mutation within gene NF2 was identified as the causative agent behind neurofibromatosis 2, a genetic disease that increases the susceptibility of carriers to the development of neural tumours (Rouleau et al., 1993). Following its discovery, NF2 was described to be a classical tumour suppressor, and mutation hampered its ability to regulate cellular pathways to prevent uncontrolled growth; though the mechanism of this was mostly unclear. In 2006 – more than 10 years after discovery, the NF2 gene product Merlin was shown to regulate activity of the Hippo signaling pathway (Hamaratoglu et al., 2006). By antagonizing an ubiquitin ligase that destabilizes LATS1/2, Merlin functions to support the integrity and function of LATS1/2, thereby preventing nuclear translocation of YAP/TAZ. Merlin is now characterized as a Hippo pathway protein, upstream of MST1/2, and was one of the first to be fully characterized

as a tumour suppressor protein within this pathway. Many of the other canonical Hippo proteins have been identified as having many functional roles in regulated cellular processes key to maintaining cellular integrity – through both YAP dependent and independent mechanisms.

1.3.4.2 Dysregulation of Hippo Proteins Drive Tumourigenesis

Merlin, as described above, is one of the few Hippo pathway genes impacted by genetic mutation. Though dysregulated in a wide range of cancers, as will be described in Section 1.3.4.3, very few Hippo genes have somatic mutations behind the dysregulation. Only the largest Hippo genes show a potential increase in mutations, and this is likely an artifact of gene size alone, not indicative of a mutation hotspot. Harvey and colleagues, in their 2013 review demonstrate that, though mutations in these genes have been seen in select analyses, they are not high-risk for mutational damage (Harvey et al., 2013). Several groups have characterized hypermethylation at both LATS1/2 and MST1/2 promoters, and a reduction in gene expression due to methylation is seen in several cancer indications, but the cellular signals driving this chromatin remodeling remain unknown (Seidel et al., 2007; Takahashi et al., 2005). Knowing that dysregulation is seen in a wide number of cancer indications – but, that somatic mutations are likely not behind this dysfunction begs the question: what is driving the activation of YAP? It is likely that other cellular proteins that are upregulated or mutated in cancer are impacting pathway components to manipulate the tumour suppressor function of core Hippo kinases, driving uncontrolled cell growth and proliferation. Considering the wide range of cellular inputs that can both activate and deactivate the Hippo pathway, it is interesting to note that

many are implicated in cellular processes that are important in tumour development. Sensitivity to nutrient stress, loss of contact inhibition, and loss of cell cycle control are hallmark features of cancerous cells, and each of these features can be linked to Hippo pathway signaling.

1.3.4.3 Hippo Dysfunction in Numerous Cancer Types

Most studies of the role of Hippo components in cancer have done so using mutated Hippo pathway genes, and analyzing the resulting tumours that develop, usually in mice models. There are some major limitations to these studies, primarily that mutations in these genes rarely occur in tumours, as well as the fact that the translation of understanding between a mouse and human context is often very challenging. Nevertheless, these studies have led to breakthroughs in understanding the roles of Hippo proteins in tumourigenesis, and have led into studies looking at primary tissues for a greater depth of knowledge. The implication of dysregulated Hippo signaling in different cancer types is vast, and as researchers continue screening both cancer cell lines and patient derived samples for Hippo signaling status, is it likely that this number will grow. Aberrant Hippo signaling has been very well-described in breast and prostate cancer, as well as hepatocellular carcinoma (Chan et al., 2008; Li et al., 2011; Xu et al., 2009). More recently, highly active YAP has been identified in a cancer of viral origin, HPV+ oropharyngeal squamous cell carcinoma. (Alzahrani et al., 2017).

1.3.5 Hippo Signaling and Viral Infection

Understanding the role of Hippo signaling in viral infection is an under-studied field; few groups have done any type of analysis of Hippo pathway effectors in the context of virus infection. The strong data linking Hippo pathway activity to tumour suppression proves and ample justification for prioritizing studies of the known human tumour viruses. Interestingly, within the last few months, there have been several breakthrough studies examining the role of YAP in antiviral immunity, adding yet another functional role for this highly active oncogene. These breakthroughs are key in understanding how viral proteins may antagonize Hippo pathway components to establish infection, evade immune defenses, or promote oncogenic growth.

The few papers published looking at Hippo pathway dynamics during viral infection focus on a handful of viruses that target different tissues and cell types. Interestingly, much of what is known about Hippo dynamics *in vivo* come from studies of induced hepatocellular carcinoma, and as such many groups are looking at the interplay between hepatitis virus proteins and Hippo proteins as drivers for this tumour development (Hu et al., 2017; Wu et al., 2016; Zhang et al., 2012). The other class of viruses that has been studied in the context of Hippo signaling is oncogenic viruses, including polyoma virus, human papilloma virus (HPV) and KSHV, which will be described in more detail below.

1.3.5.1 Hippo Signaling and Innate Immunity

Very recently, several papers have been published analyzing the effect of Hippo pathway manipulation on innate immune signaling in cells. The general consensus, from two papers, is that YAP works as a negative regulator of innate immune signaling. The first publication tied YAP activity to nucleic acid sensing in cells, a potent activator of innate immune signaling and one that is especially important in the context of viral infection. In March of 2017, Zhang and colleagues showed that YAP/TAZ physically associated with TBK1, a protein activated following cytoplasmic sensing of nucleic acids and required for passage of this innate signal (Zhang et al., 2017). YAP/TAZ bind TBK1 at a site that prevents a dimerization event required for activation, thereby blocking transduction of innate signals. Using shRNA and CRISPR-based genetic manipulations, this group demonstrated that YAP/TAZ depletion decreases replication levels of both RNA and DNA viruses. A few months later, a paper by Wang and colleagues was published describing a role for well-known innate immune kinase IKKε in phosphorylating YAP at S403, targeting it for lysosomal degradation. This second group saw similar effects of low-level YAP activity, including decreased viral replication. Several independent groups have demonstrated that YAP seems to have a pro-viral role in suppressing antiviral immunity, which is even more cemented by the fact that an innate immune signaling kinase can phosphorylate YAP, leading to degradation.

1.3.5.2 The Hippo Pathway and Oncogenic Viruses

Only a handful of studies have been published that look at the activity of Hippo pathway proteins in the context of viral infection, and the pool gets even smaller when focusing on oncogenic viruses.

Murine polyomavirus, a non-enveloped dsDNA virus, has been intensively studied to understand the oncogenic role of its two main proteins; small T (ST) antigen and middle T (MT) antigen. A recently published paper showed that YAP has a key role in MT-induced transformation of primary cells. MT works to both upregulate signaling pathways that increase the activation of YAP as well as physically bind and stabilize the protein. YAP1 silencing decreased the ability of MT to effectively transform cells, but it remains unclear whether MT binding to YAP elicits a yet-unknown function of YAP, as it is trafficked to the membrane in the cell (Rouleau et al., 2016).

HPV is the causative agent of both cervical and head and neck cancers, and encodes two highly oncogenic proteins, E6 and E7. Previous work has shown that these proteins can target Scribble – an upstream Hippo pathway protein that recruits MST1/2, LATS1/2 and TAZ proteins to the plasma membrane, allowing phosphorylation of each component, and therefore nuclear exclusion of TAZ (Cordenonsi et al., 2011). When Scribble is lost – through genetic manipulation, or via degradation by HPV E6, levels of phosphorylated YAP/TAZ are reduced, allowing these effectors to transit into the nucleus and upregulate TEAD responsive genes (He et al., 2015; Nakagawa and Huibregtse, 2000). More recently, upregulation and nuclear localization of YAP has been shown in

primary samples derived from HPV+ oropharyngeal squamous cell carcinoma tumours, giving more support for the idea that YAP is likely tightly implicated in cancer progression of HPV-infected cells (Alzahrani et al., 2017).

The final oncogenic virus that has been studied in the context of Hippo pathway signaling, and is the focus of this thesis, is KSHV. As described in depth in Section 1.2 above, KSHV is an oncogenic herpesvirus that can cause both B-cell and epithelial tumours. Several KSHV genes have been studied in great detail to understand their ability to drive tumour formation, with a particular focus on vGPCR. vGPCR is a G-protein coupled receptor that is constitutively active, and as such does not require ligand binding for activation. A few years ago, a prominent Hippo research lab – the Guan lab – published a paper looking at the ability of vGPCR to signal through the Hippo pathway. In this paper they showed that vGPCR signaling reduced levels of YAP/TAZ phosphorylation, and when KS-like tumours were induced in mice using vGPCR expressing cells alone, YAP/TAZ silencing was sufficient to reduce tumour size (Liu et al., 2015). As described in Section 1.2.5.1.2, it is unlikely that vGPCR is solely responsible for KS tumour formation because it is expressed as an early lytic gene product in cells that will succumb to viral infection.

It is quite clear from these examples that Hippo pathway components are tightly linked to the activity of oncogenic viruses. The majority of work has been done using single oncogenes or proteins of interest, while Hippo pathway activation in the context of whole virus infection has not been studied. Understanding the status of Hippo pathway

signaling in the complex environment of viral and host protein interaction may reveal both new roles for this pathway, and yield a better understanding of KSHV infection.

1.4 Rationale and Objectives

The study of oncogenic viruses has expanded our understanding of what types of cellular disruption has the potential to drive tumour formation. Many of these viruses have been well characterized, with potent oncogenes discovered and function dissected; but this is not true for all oncogenic viruses. Since its discovery in the early 1990s, the mechanism behind development of tumours following infection by KSHV has been heavily studied, but is still not well understood. Recent developments have linked activity of this virus to dysregulated signaling of the Hippo pathway. The only current published study that looks at Hippo pathway dynamics in the context of KSHV does so by isolating one potential oncogene – vGPCR – and studying its effects on the pathway. It is unclear what the role for Hippo pathway effectors are in the context of KSHV infection, and whether or not targeting this pathway may be suitable for treating patients with KSHV induced malignancies. We hypothesize that modulating Hippo pathway effectors – namely YAP1 and LATS1, will induce changes in KSHV replication dynamics, but it is unclear whether this will be pro- or anti-viral. In this thesis, a thorough analysis of Hippo pathway effectors in KSHV infected cells will be described, and data presented showing that YAP1 may be a cellular factor working to maintain KSHV latency. A screen of all known KSHV encoded proteins will be shown in an effort to understand if several proteins, similar to vGPCR, have activity in regulating the dynamics of this core signaling pathway.

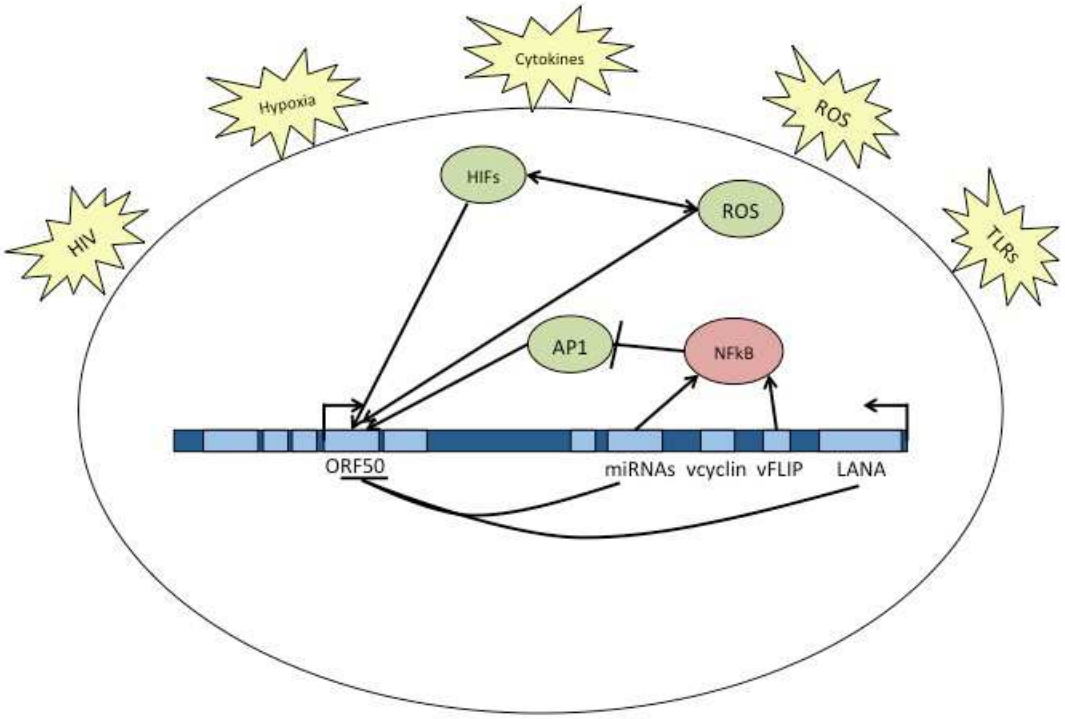

Figure 1. Host and viral proteins converge to regulate the activity of the ORF50 promoter. Latent gene products – LANA, vFLIP, and several miRNAs downregulate expression of ORF50 through various mechanisms, including the induction of NFkB signaling. Cellular stresses such as HIV co-infection, hypoxia, cytokines, reactive oxygen species, and engaging Toll-like receptor signaling can induce activation of the ORF50 promoter and drive lytic reactivation. Adapted from Ye et al., 2011b.

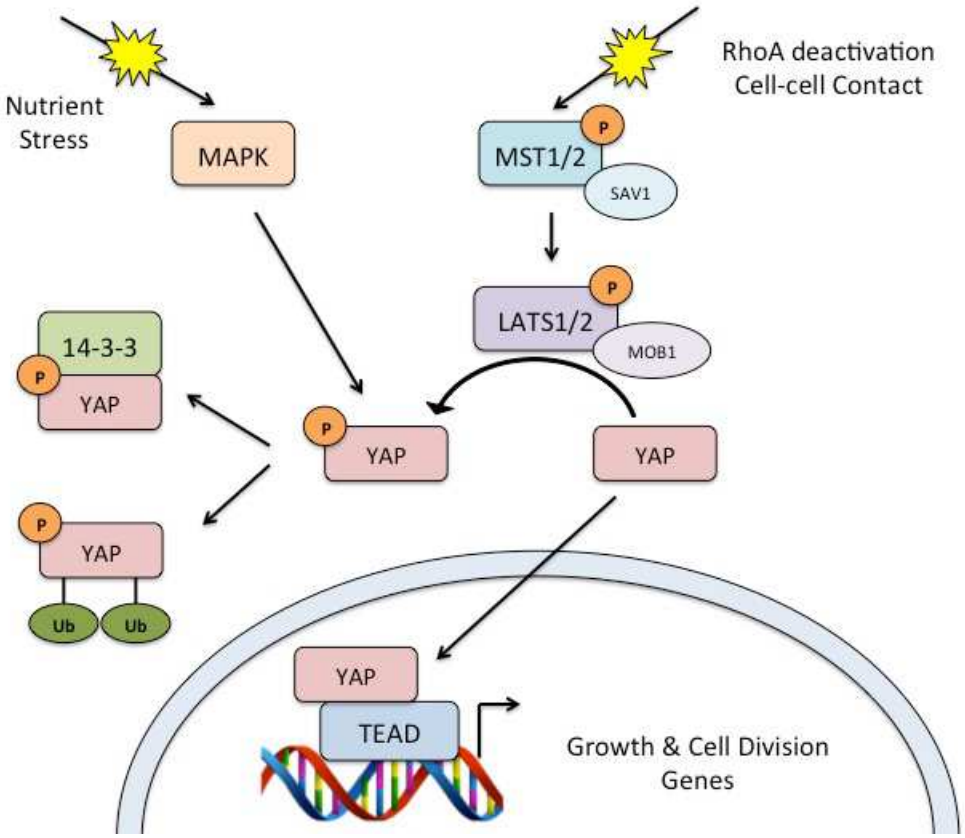

Figure 2. There are several mechanisms that control the phosphorylation and therefore cellular localization of Hippo pathway effector, YAP. Canonically, RhoA deactivation via cytoskeletal rearrangement, or cell-cell contact, activates the MST1/2 kinases. MST1/2, scaffolded by SAV1, can then phosphorylate LATS1/2 – MOB1 is the LATS1/2 scaffolding protein. LATS1/2 can then phosphorylate YAP, leading to its cytoplasmic retention via binding 14-3-3, or degradation through the ubiquitin-lysosomal degradation pathway. Non-canonically, MAPK can also phosphorylate YAP in response to nutrient stress. Unphosphorylated YAP can enter the nucleus to act on TEAD transcription factors, driving the expression of cellular growth and cell division genes.

Chapter 2 Materials and Methods

2.1 Cell Culture

All cell lines were subcultured at 1/10 every 2-3 days, and incubated at 37 °C, 5% CO_2. iSLK.puro cells, a gift from Dr. Jae Jungs laboratory, were cultured in DMEM (Fisher) + 10% heat-inactivated FBS (Invitrogen) 1% penicillin-streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) with 1 µg/µl Puromycin (Invitrogen). iSLK.219 cells (Myoung and Ganem, 2011), provided by Dr. Don Ganem, were cultured as above, but with 10 µg/ml puromycin. Both HEK293A (ATCC) and HEK293T (ATCC) cell lines were cultured in standard media described above. BCBL-1 cells (Picchio et al., 1997), obtained from Dr. Rolf Renne were cultured as described above, in RPMI-1640 media (Gibco) with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 2 mM L-glutamine and 1% beta-mercaptoethanol (Sigma).

2.2 Lentiviral Production and Treatment

Two lentiviral plasmids were used, a modified version of pLJM1 (Sancak et al., 2008) with both puromycin and blasticidin resistance cassettes, and the pLKO.1 TRC cloning vector (Addgene). Following sequence verification of cloned constructs, HEK 293T cells were used to produce lentiviral particles. HEK293T cells were subcloned at 80% confluency, 1/10 in complete media, as described in section 2.1. The following day, media was aspirated, and cell monolayers washed twice with phosphate buffered saline (PBS; Wisent). Monolayers were overlaid with 5 mL DMEM with no supplements. Transfection mixtures using polyethylenimine (PEI, Sigma) were set-up as follows: 18 µL PEI in 500 µL serum-free Opti-MEM (Invitrogen) per transfection, 3.3 µg of gene of

interest, 2 μg pSPAX (Addgene) and 1 μg pMD.2G (Addgene). Cells were transfected for 4-6 hours, and media was replaced with complete media lacking antibiotics. 24 h post-transfection, supernatant was harvested, passed through a 0.45 μM filter, and stored in 1 mL aliquots at -80 °C.

Prior to transduction, iSLK.219 or HEK293A cells were seeded from 80% confluency into 6-well dishes; a 1/15 dilution of cells into 2 mL of media. The following day, fresh complete media plus the addition of 4 μg/ml polybrene (Sigma) was overlaid on cells. Lentiviral aliquots were thawed in a 37 °C water bath, and a series of virus dilutions added to cells, ranging from 1/25 to 1/200. One well remained untransduced as an antibiotic selection control. 24 h post-transduction, either 1 μg/μL of puromycin (Invitrogen) (HEK293A) or 10 μg/mL of blasticidin (Invitrogen) (iSLK.219) was added to cells, in complete media. Puromycin-treated cells selected for 2-3 d, while blasticidin selection maintained for 4-5 d. The transduced well with viral dilutions resulting in approximately 20-30% surviving cells was carried forward.

2.3 Antibodies

Antibody	Supplier	Dilution
YAP	Santa Cruz Biotech	1/1000
pYAP S127	Cell Signaling	1/1000
LATS1/2	Cell Signaling	1/1000
pLATS 1/2	Cell Signaling	1/1000
ORF65	A gift from Dr. SJ Gao	1/500
ORF45	Thermo Fisher	Immnoblot: 1/1000 FACS: 1/250
ORF50	A gift from Dr. David Lukac	1/2000
β-actin-HRP	Cell Signaling	1/8000
LANA	A gift from Dr. Don Ganem	1/500
Anti-rabbit HRP	Cell Signaling	1/4000
Anti-mouse HRP	Cell Signaling	1/4000
Anti-mouse A555	Thermo Fisher	1/1000
Anti-rabbit A647	Thermo Fisher	1/1000

Table 1. Antibody dilutions.

2.4 Primers

Primer	Sequence (5'→3')	Type	Reference
shYAP#1 sense	CCGGCTGGTCAGAGATACTTCTTAACTCGAG TTAAGAAGTATCTCTGACCAGTTTTTC	Hairpin	(Zhao et al., 2008)
shYAP#1 antisense	AATTGAAAAACTGGTCAGAGATACTTCTTAA CTCGAGTTAAGAAGTATCTCTGACCAG	Hairpin	(Zhao et al., 2008)
shYAP#2 sense	CCGGAAGCTTTGAGTTCTGACATCCCTCGAG GGATGTCAGAACTCAAAGCTTTTTTTC	Hairpin	(Zhao et al., 2008)
shYAP#2 antisense	AATTGAAAAAAAGCTTTGAGTTCTGACATCC CTCGAGGGATGTCAGAACTCAAAGCTT	Hairpin	(Zhao et al., 2008)
shLATS#1 forward	CCGGGTCTGCTTCATACATTCCTAACTCGAG TTAGGAATGTATGAAGCAGACTTTTT	Hairpin	(Zhang et al., 2008)
shLATS#1 reverse	AATTCAAAAAGTCTGCTTCATACATTCCTAA CTCGAGTTAGGAATGTATGAAGCAGAC	Hairpin	(Zhang et al., 2008)
shLATS#2 forward	CCGGGAGAAATTAAGCCATCGTGTTCTCGAG AACACGATGGCTTAATTTCTCTTTTT	Hairpin	(Zhang et al., 2008)
shLATS#2 reverse	AATTCAAAAAGAGAAATTAAGCCATCGTGTT CTCGAGAACACGATGGCTTAATTTCTC	Hairpin	(Zhang et al., 2008)
LATS1 forward	GTCAACCGAAGATCCTCGACAAGTCAGAAAT CCACCC	PCR, cloning	
LATS2 reverse	GTCAGTCGACTAGTCAGACAAAATGATGCAA CTTAATTTT	PCR, cloning	
ORF26 forward	CAGTTGAGCGTCCCAGATGA	qPCR	
ORF26 reverse	GGAATACCAACAGGAGGCCG	qPCR	
Beta-actin forward	CTTCCAGCAGATGTGGATCA	qPCR	(Leidal et al., 2012)
Beta-actin reverse	AAAGCCATGCCAATCTCATC	qPCR	(Leidal et al., 2012)

Table 2. Primers used in this thesis.

2.5 Immunoblotting

Cell lysates were harvested using 2X Laemmli (Cold Spring Harbour) buffer, in one of two methods. iSLK.219 cells were harvested by removing and retaining supernatant, washing 1X in ice-cold PBS (retain PBS) and scraping cell monolayers in 500 µL 1X PBS. Cells were pelleted at 800 g in a 4°C centrifuge. Cell pellets were washed once with ice-cold PBS, and lysed in 250 µL of 2X Laemmli buffer. Lysates were stored at -80°C prior to further processing. HEK293A cells were washed 2x with ice-cold PBS, 250 µL Laemmli buffer was added to each well of a 6-well dish, and cells were directly scraped and collected. Lysates were stored at -80°C until processing. Before protein quantification, lysates were homogenized by passing the sample 4-6 times through a 21-gauge needle. Protein content was quantified using the standard 96-well plate protocol of the BioRad DC Protein Assay (BioRad). Following quantification, 5 µL of a 3 M DTT solution, and 5 µL of a saturated solution of Bromophenol blue (Sigma) was added to samples. Lysates were boiled at 96°C for 5 minutes. 10 µg of protein was loaded in hand-cast polyacrylamide gels. Gels were run at 100 V until the dye front ran off the end of the gel. Protein was transferred to PVDF membranes using the BioRad Turbo Blot transfer system. Blots were blocked on a rocker at room temperature in blocking buffer (5% bovine-serum albumin [BioShop] in Tris-buffered saline with 0.05% Tween-20 [Fisher]) for one hour. Primary antibody was incubated overnight, rocking, at 4°C in blocking buffer – see antibody dilution table for details. Blots were washed 4X for 5 minutes each in TBS-T, and secondary antibody incubated for 1 hour in TBS-T + 5% skim milk powder – see antibody dilution table for details. Blots were washed again as

described above, and developed using Clarity-ECL (BioRad). Images were acquired using BioRad Chemi-doc Touch.

2.6 Viral Titering

iSLK.219 cell supernatants were harvested at 0-24 h post-induction with doxycycline. Supernatants were frozen at -80 °C immediately following harvest, and stored until use. HEK293A cells were seeded at 150,000 cells/well in a 12-well dish in complete medium with the addition of 40 mM HEPES (Invitrogen). The following day, cells were treated with dilutions of harvested supernatant at the following concentrations: 1/10, and 1/50. Plates were sealed, and centrifuged at 800 g, 37 °C, for 2 h in a Beckman-Coulter Avanti JE rotor. Following spinoculation, media was refreshed, and plates incubated at standard growth conditions. The following day, media was aspirated and cells were washed with 1X PBS. 200 µL of 0.25% trypsin + 1% EDTA (Invitrogen) was added to each well. Cells were collected by adding 1 mL of complete media, and placed in 5 mL FACS tubes. Tubes were centrifuged at 500 g for 5 minutes; supernatant was removed, and cells washed in 2mL PBS. Tubes were centrifuged again at 500 g for 5 minutes, supernatant was removed, and cells were resuspended in 500 µL 1% paraformaldehyde in PBS (Electron Microscopy Sciences). Tubes were stored at 4 °C until acquisition. Cells were counted using a BD FACScalibur cytometer. GFP+ cells were counted in FL1, and data analyzed using FCS Express 6.

2.7 Viral Genome Quantification

iSLK.219 cells were seeded at 100,000 cells/ml into 12-well dishes. 24 h following seeding, cells were induced by the addition of 1 μg/μl doxycycline (Sigma). Cells were harvested every 24 h to 96 h via the following method. Media was collected, cells washed with 1X PBS and collected. 500 μL PBS was added to the cell layer, and cells were scraped and collected, all samples were handled on ice. Cells, PBS, and media were centrifuged at 1000 g for 5 minutes at 4 °C. Supernatant was aspirated, and cell pellet washed 1X in PBS. Supernatant was aspirated, and cell pellet immediately frozen at -20 °C until processing. Episomal DNA was extracted using the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen). DNA was eluted using 100 μL nuclease-free water, and used in a standard qPCR reaction. Reactions were set up as follows: 2 μL extracted DNA, 1 μL primer (ORF26 or β-actin – see primer table for details), and 5 μL 2X Sybr green qPCR master mix (Promega). qPCR reaction performed using a BioRad CFX Connect under standard cycling conditions. All data was normalized to β-actin, and scramble hairpin control. Statistical analysis performed using GraphPad Prism; Students t-test performed of each time-point with Sidak-Bonferonni adjustment.

2.8 Fluorescent Microscopy

Live cells were imaged in their culture containers using the EVOS Cell imager (Invitrogen). iSLK.219 cells were seeded at 100,000 cells/ml in complete DMEM, and following induction images were obtained every 24 h.

2.8.1 Immunofluorescence

iSLK.219 cells were seeded at 50,000 cells/ml onto gelatin coated (0.1% gelatin [Sigma] in PBS) 18 mm coverslips (Zeiss). The following day, cells were washed 1X in PBS, and fixed in 4% paraformaldehyde in PBS for 15 minutes at 37 °C. Following fixation, coverslips were washed, and stored in PBS at 4 °C until further processing. Coverslips were permeablized in 0.1% Triton X-100 (Fisher) in PBS for 15 minutes at RT. Following incubation, coverslips were washed 2X in PBS, and blocked for 1hour in 5% BSA in PBS at RT. Cells were washed 2X, and incubated in primary antibody (see Table 1 for dilutions) overnight at 4 °C in a humidified chamber. The following day, coverslips were washed 2X, and incubated with secondary antibody in PBS + 5% BSA for 1 hour in a humidified chamber, in the dark. Coverslips were washed 2X, and incubated with a 1/1000 dilution of DRAQ5 (Fisher) in PBS for 15 minutes. Coverslips washed 1X and mounted to slides using a droplet of Prolong Gold Anti-fade Reagent (Fisher). Slides incubated at RT overnight in the dark to allow mounting solution to dry.

2.8.2 LANA Dot Imaging & Enumeration

iSLK.219 cells, stained for LANA by immunofluorescence, were imaged and LANA dot counts per cell was determined. Cells were stained following protocol previously described. Slides were imaged using a Zeiss 510 META Confocal Microscope (Zeiss) imaged at 63X objective. 10 images were captured of each treatment, and 10 cells per field were counted using Cell Profiler. The nuclear speckles pipeline in Cell Profiler was selected, and cells selected based on nuclear staining using Hoescht. Within the nuclear staining border, LANA dots were counted on a per-cell basis, with a threshold set

to discard background staining. Statistical analysis was performed using GraphPad Prism, a repeated measures ANOVA was run, followed by a Students' two-tailed t-test.

2.9 Luciferase-based Screen for Viral Hippo Pathway Modulating Proteins

HEK293A cells were subcultured from 80% confluency, and seeded into 96-well plates, at 2500 cells/well in antibiotic-free DMEM. Triplicates of each ORF or control being tested were seeded. The following day, transfection mixtures of each test article were set up as follows: 250 ng ORF/control DNA, 200 ng 8X-GTIIC luciferase (Addgene) 50 ng CMV-Renilla (modified from Addgene), 1.5 µL FuGENE HD transfection reagent (Promega) made up to 150 µL in Opti-MEM. 36 hours following transfection, cells were starved by incubating with DMEM + 2 mM L-glutamine only, for 12 h. Following serum starvation, media was aspirated, and cells were washed with 1X PBS. Cells were lysed using 25 µL Passive Lysis Buffer (PLB – Promega) per well, incubated at room temperature, shaking, for 20 minutes. White-walled 96-well plates were pre-aliquoted with 25 µL of Luciferase assay reagent (LAR - Promega). 10 µL of cell lysate was transferred to white-walled plate containing the pre-aliquoted LAR. Luciferase activity was measured using a Tecan Infinite 200Pro (Thermo Fisher) plate reader. 25 µL of Stop and Glo solution (Promega) was added to each well, and luminescence was read again. This second read corresponds to the expression of the CMV-Renilla construct, while the first read is a measure of the activity of the 8X-GTIIC luciferase reporter. Data was normalized to Renilla expression, and values of the empty vector control. A non-phosphorylatable YAP construct (YAP5SA, Addgene) was used as a positive control.

2.10 GFP/RFP Quantification

iSLK.219 cells were seeded at 100,000 cells/ml in 12-well plates, in complete media. The following day, media was aspirated, and replaced with complete DMEM containing 1 μg/μl doxycycline. Cells were harvested every 24 h following induction using the method described above. Fixed cells were stored in 1% paraformaldehyde at 4 °C until sample acquisition. Samples were acquired using a BD Fortessa, run by Derek Rowter in the Dalhousie Medical Flow Core Facility. Samples were gated on GFP+ (FL-1) and RFP + (FL-2) and 1% compensation was used to exclude GFP emission in the RFP channel.

2.11 BCBL-1 Reactivation

BCBL-1 cells were cultured as described above. Cells were seeded at 100,000 cells/ml in a 6-well dish. Polybrene was added to a final concentration of 4 μg/ml, and a 1/10 dilution of lentivirus containing shRNA targeting sequences was added. Cells were incubated with virus for 24 h. Following transduction, cells were centrifuged at 500 g for 5 minutes, and cell pellets were resuspended in complete medium + 1 μg/ml puromycin for 48 h. Following selection, cells were spun at 500 g for 5 minutes, and pellets resuspended in ice-cold PBS. Cells were washed, as described, and resuspended in 1% paraformaldehyde and incubated at 4 °C for 1 hour. Cells washed 2x following fixation, in cold PBS. Cells were permeabilized in PBS containing 0.1% Saponin (Sigma), and 1% heat-inactivation FBS (Invitrogen) for 1 h at room temperature. This wash buffer was maintained through the remaining washing and staining procedures.

Chapter 3 Results

3.1 Hippo Pathway Signal Transduction is Altered During the KSHV Lytic Cycle

To understand how KSHV latency and lytic replication influence Hippo pathway signal transduction, iSLK.219 cells were left untreated (latent, time = 0) or treated with doxycycline for 72 hours to reactivate KSHV from latency. Lysates were harvested at 24 h intervals and immunoblotted with a panel of antibodies directed against Hippo pathway proteins or their respective phosphorylated forms. Basal Hippo pathway activation was evident during KSHV latency, whereas reactivation was characterized by increased levels of both phosphorylated LATS and YAP over time, with a corresponding decrease in the total levels of YAP detected most obvious at 48 hours following reactivation (Figure 3). LATS1 decreases at 48 and 72 h is likely an artifact due to the reduced ability of this antibody to bind the phosphorylated form of LATS1, and increase in the pLATS1 suggests an active form of this protein is present. A reduction in both MST1 and MST2 proteins was also observed; it is unclear whether this reduction is due to host shut-off induced by viral reactivation, or an inability of the antibody used to detect the phosphorylated form of MST1/2. Progress through lytic reactivation was measured via protein accumulation of ORF45, an early lytic protein. In sum, these data suggest that reactivation from latency and progression through the lytic cycle coincides with Hippo pathway activation, but that levels of Hippo pathway proteins decreases as the lytic cycle progresses.

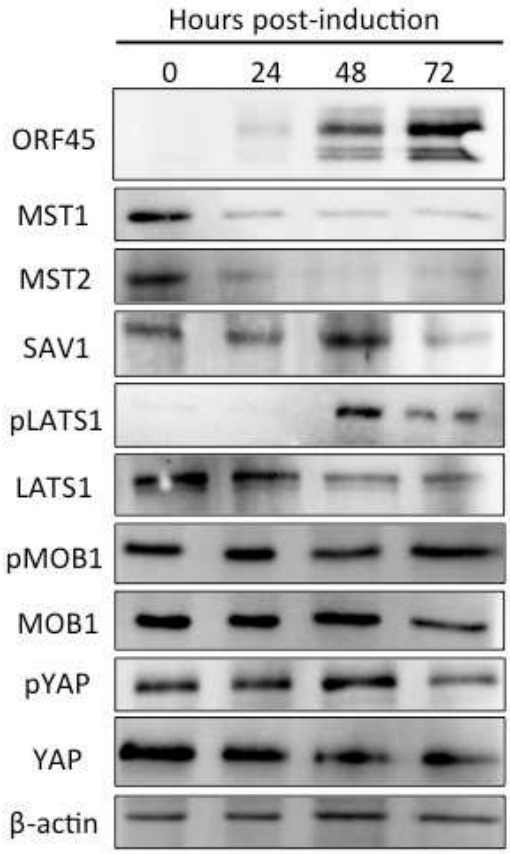

Figure 3. Phosphorylation of both YAP and LATS1 increases through lytic reactivation of iSLK.219 cells, causing a decrease in the total amount of YAP in cells at 72 h post-addition of doxycycline. iSLK.219 cells were treated with doxycycline and harvested every 24 h post-induction. 10 μg of protein was loaded per lane, and probed with a panel of antibodies (See Section 2.3 for details) n=1.

3.2 YAP1 Knockdown Cells Show Accelerated Progression Through Lytic Replication

The suggested increased levels of phosphorylated YAP during KSHV lytic replication would be expected to limit nuclear accumulation of YAP and YAP-dependent transactivation of genes that regulate cell growth. We hypothesized that activation of Hippo signal transduction and suppression of YAP nuclear accumulation could support KSHV lytic replication. We took a genetic approach to further investigate the role of YAP1 in KSHV lytic replication. Two lentiviral vectors encoding short hairpin RNAs (shRNAs) targeting YAP1, named shYAP#1 and shYAP#2, were designed and constructed. Latently infected iSLK.219 cells were transduced with shYAP#1 and shYAP#2 lentiviruses and a non-targeting hairpin control (shSCR) for 24 hours in the presence of polybrene. Stable transductants were selected for 4 days in blasticidin. Cell lysates were harvested for immunoblotting with an anti-YAP1 antibody. Selected cells were treated with doxycycline to activate ORF50 expression and trigger lytic reactivation. RFP expression was determined using flow cytometry at several time-points following doxycycline addition (Figure 4). When normalized to the shSCR control at each time-point, both shYAP1 hairpin constructs demonstrated an increased level of RFP+ cells, with shYAP#2 displaying highest RFP levels, consistent with our previous findings. Taken together, these observations suggest that YAP1 silencing may increase the proportion of KSHV reactivation from latency in this model. A student's t-test comparing shSCR and the shYAP1 knockdowns was performed, but statistical significance was not determined due to high levels of inter-experiment variability.

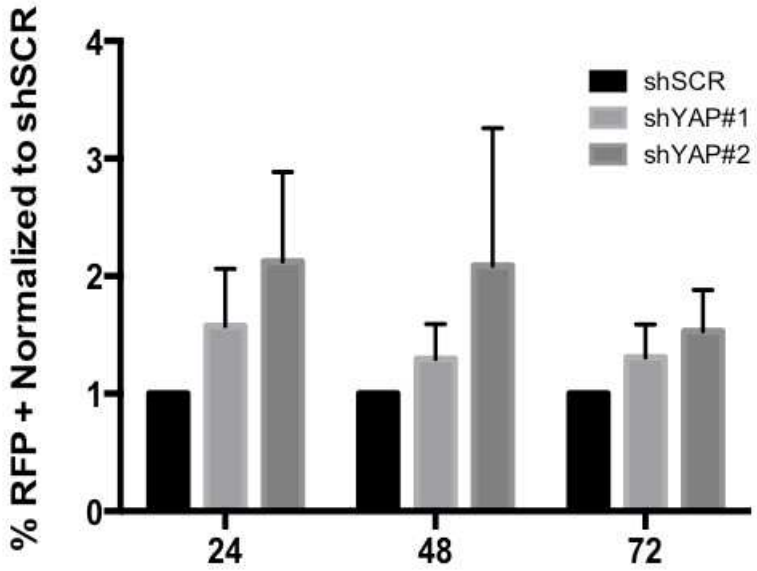

Figure 4. YAP1-silenced iSLK.219 cells show increased reactivation from latency compared to untreated control as measured by RFP+ cells. Quantification of RFP+ cells via flow cytometry over time post-induction. Counts normalized to shSCR control at each timepoint, n=3. Samples run by Derek Rowter at Dalhousie FACS Core Facility. Data analyzed using FCS Express 6.

To confirm our observations of increased reactivation from latency in the iSLK.219 model upon YAP1 silencing, shSCR, shYAP#1 and shYAP#2 cells were treated with doxycycline over a time-course as above, and cell lysates were harvested and immunoblotted with antibodies directed against immediate-early (ORF50), early (ORF45), and late (ORF65) viral antigens. At each time-point, the knockdown efficiency of YAP1 was also assessed. YAP1 silencing was maintained throughout the time-course, again with slightly increased YAP1 silencing by the shYAP#2 construct (Figure 5), consistent with earlier observations (Figure 4A). Moreover, total YAP1 levels in the shSCR control lane decreased as the lytic cycle progressed (Figure 5), consistent with observations in parental iSLK.219 cells (Figure 3). YAP1 silencing increased ORF50 and ORF45 protein accumulation at each time point compared to the shSCR control (Figure 5). A slight increase in ORF65 accumulation at 48 h is suggested by the data, but more replicates with quantification are required for further analysis. Together, this data suggests that YAP1 silencing causes accelerated accumulation of viral gene products.

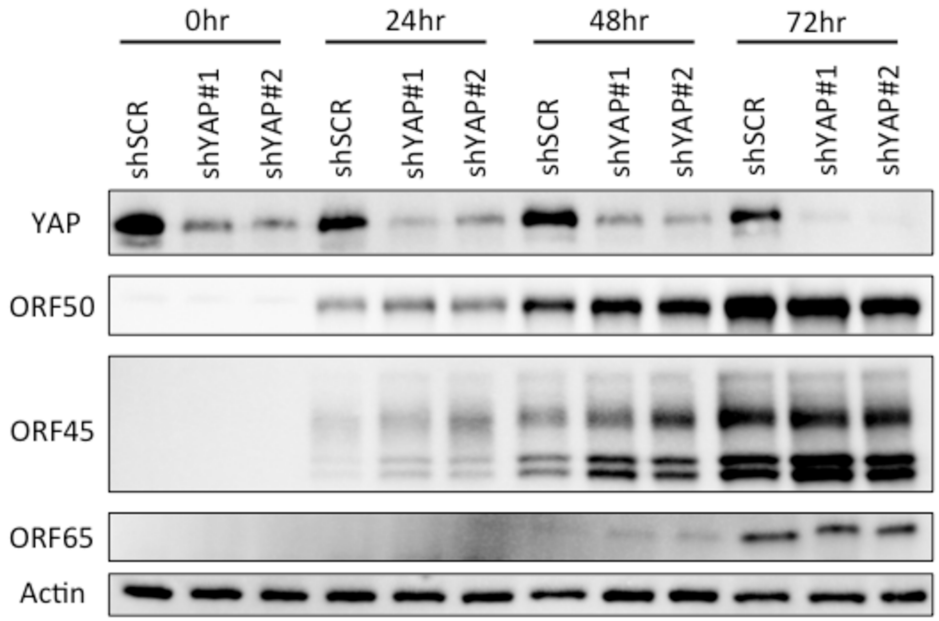

Figure 5. YAP1-silenced iSLK.219 cells show an increase in lytic protein accumulation at 48 and 72 h following initiation of lytic reactivation. Immunoblot of iSLK.219 transduced with lentiviruses expressing two separate knockdown constructs targeting YAP1. Cells treated with doxycycline and harvested every 24 h. 10 µg protein loaded per well. See antibody table in Section 2.3 for details. Representative blot of n=3.

To determine whether increased production of lytic viral proteins corresponds to more efficient viral replication in YAP1-deficient cells, viral genome replication and production of infectious viral progeny was measured using standard assays. Reactivation from latency was triggered by doxycycline treatment in YAP1-deficient and control iSLK.219 cells as described previously. Viral genomes were isolated from cells 96 h post-induction, and amplified by quantitative PCR, using oligonucleotide primers that hybridize to the ORF26 gene. Viral genome copy number was normalized to β-actin, and control cells were treated with phosphonoacetic acid (PAA), a selective inhibitor of the KSHV DNA polymerase, to block genome replication (Figure 6). Following addition of doxycycline, viral genome copy number did not increase over the first 24 h, as expected, and were consistent with levels observed in the PAA control. These background levels represent the pre-existing latent viral episomes. From 24 h to 72 h post-reactivation, viral genome copy numbers were comparable between YAP1-silenced and control cells, but by 96 h, statistically-significant increases in viral genome copies in the YAP1-silenced cells were observed, compared to controls. Thus, YAP1 silencing causes modest increases in viral genome replication that is only apparent at very late stages of infection.

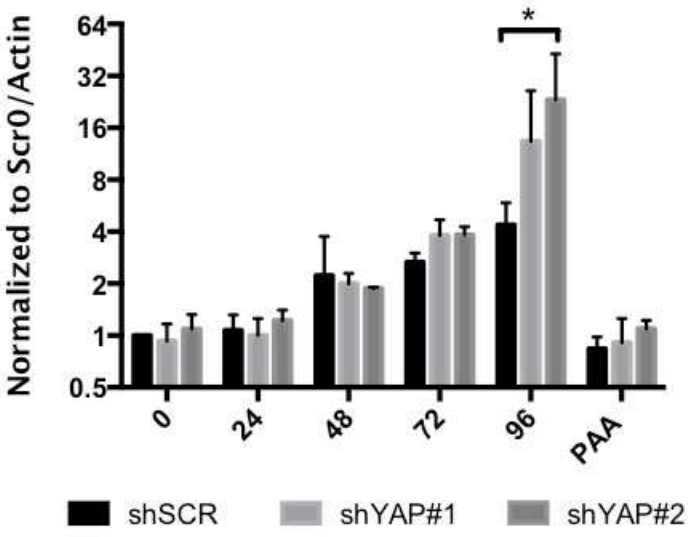

Figure 6. YAP1-silenced iSLK.219 cells showed significantly higher genome copy numbers at 96 h following initiation of lytic reactivation. Cells collected at 24 h intervals following induction with doxycycline, genomic DNA isolated, qPCR detection of genome copy normalized to β-actin and the shSCR latent control. Statistical significance determined using Students t-test, n=3.

To determine whether YAP1 silencing causes increased release of infectious viral progeny, cell supernatants from doxycycline-treated, YAP1-deficient iSLK.219 cells, or controls, were harvested over time, clarified by centrifugation, and used to inoculate naïve HEK293A cell monolayers. The recombinant KSHV virions produced by iSLK.219 cells contain a GFP transgene, and infection of HEK293A cells causes GFP expression, which can be rapidly assessed by microscopy and flow cytometry. At 24 h post-infection, HEK293A cells were harvested and GFP+ cells were enumerated via flow cytometry. Counts were normalized to the shSCR control at each time-point. As shown in Figure 7, YAP1 silencing showed a trend to increased virion release. However, these findings must be interpreted with caution because the dada did not attain statistical significance due to variability. Taken together, these findings suggest that YAP1 silencing has a positive impact on reactivation from latency, and does not impede later events in the lytic replication cycle.

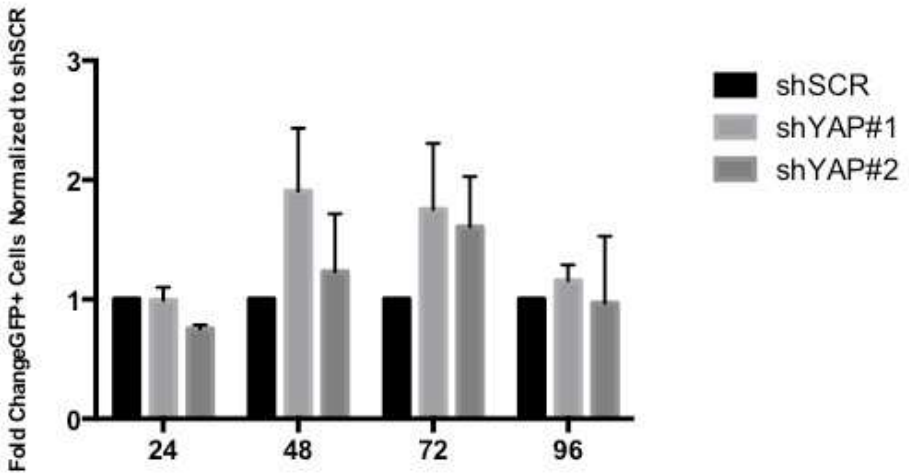

Figure 7. YAP1-silenced iSLK.219 cells trend towards an increase in infectious viral release at multiple time-points following initiation of lytic reactivation. Supernatants collected from doxycycline-induced cells at 24 h intervals. Secondary monolayer of HEK293A cells treated with supernatant, harvested for flow cytometry. GFP+ cells counted on BD FACSCalibur. GFP counts normalized to shSCR at each time-point, n=3.

With stable latency, and drug-inducible lytic reactivation, the iSLK.219 model is an excellent tool for studying the latent/lytic switch and understanding the dynamics of KSHV infection in epithelial cells. However, the primary latent reservoir for KSHV in humans is B-lymphocytes, and there are several excellent B-lymphocyte models of infection. Body cavity-based lymphoma-1 (BCBL-1) cells are a KSHV-infected B-lymphocyte cell line isolated from a patient with the rare KSHV malignancy, Primary Effusion Lymphoma. This cell line was one of the first laboratory models of KSHV infection, primarily displaying latent infection, with low-level spontaneous lytic reactivation in the culture as well. Experimental reactivation from latency can be achieved by treatment of cells with phorbol esters (e.g. TPA) and/or histone deacetylase inhibitors (e.g. sodium butyrate).

To determine whether YAP1 silencing could affect latent/lytic switch in B-cells, BCBL-1 cells were transduced with lentiviruses expressing the previously described shYAP#2 construct, or the shSCR control, puromycin resistant constructs were used for the BCBL-1 cells due to their sensitivity and short treatment required for effective selection. Following lentiviral transduction, cells were selected in puromycin for 2 days, and treated with TPA to promote reactivation from latency. Cells were harvested for flow cytometry, and stained using an anti-ORF45 antibody to identify cells in which the virus had reactivated from latency. Consistent with observations in the iSLK.219 model, YAP1 silencing in BCBL-1 cells resulted in an increased number of cells expressing ORF45 (Figure 8) when lytic reactivation was induced with TPA. Remarkably, YAP1 silencing also caused increases in ORF45-positive cells in the absence of TPA likely reflecting

increased spontaneous lytic reactivation in this model. While more work needs to be done to investigate the role of YAP1 in KSHV latent/lytic switch control in B cells, these findings are generally consistent with our findings in the iSLK.219 model.

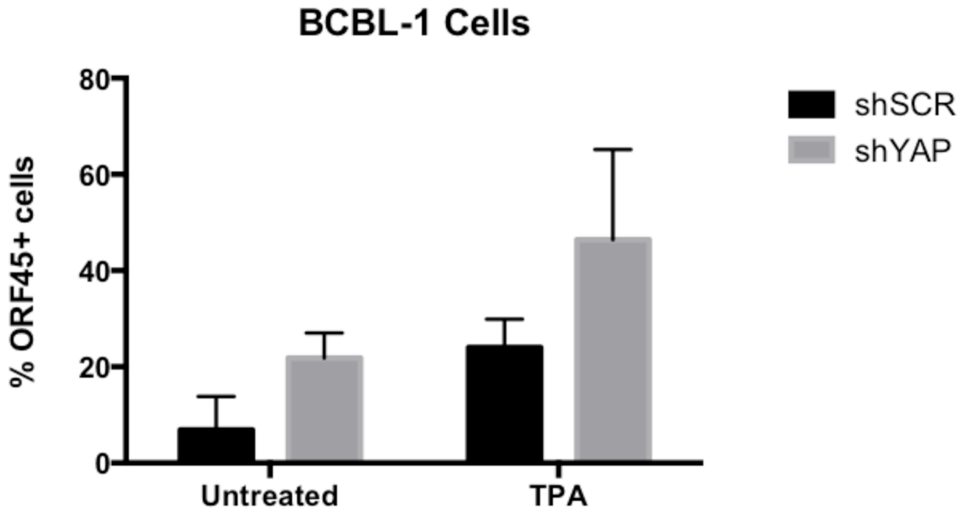

Figure 8. YAP1 silencing of BCBL-1 cells increase both spontaneous lytic reactivation and TPA-induced lytic reactivation. BCBL-1 cells transduced with lentivirus containing YAP1 knockdown construct (shYAP#2) and shSCR control. 2 days following selection, cells treated +/- TPA for 24 h. Cells harvested and fixed for flow cytometry, primary stained using anti-ORF45 antibody at 1/250 dilution (Thermo Fisher), secondary anti-mouse AlexaFluor488 at 1/500 dilution (Thermo Fisher). ORF45 positive cells counted on BD FACSCalibur, data analyzed using FCS Express 6, n=2.

From the data presented thus far, it suggests that removing YAP1 from the iSLK.219 model of latency and lytic reactivation changes the dynamics of reactivation in a positive way, by increasing the amount of infectious virus being released by these cells. The replication of this enhanced lytic reactivation phenotype in BCBL-1 cells (Figure 8) shows that YAP1 likely has some function at suppressing lytic reactivation – both induced and spontaneous. To understand if this observed function of YAP1 is happening in a Hippo pathway-dependent or independent manner, the core regulatory kinase of YAP1, LATS1, was studied via RNA silencing and overexpression to determine if modulating this kinase could recapitulate the effects of YAP1 silencing on KSHV infection.

3.3 Ectopic expression of LATS1 promotes reactivation from latency

To understand if the effects seen with YAP1 silencing could be controlled via its regulatory kinase, a lentiviral construct containing the LATS1 gene was used to transduce iSLK.219 cells as described. Following selection, cells were seeded for immunoblotting and flow cytometry assays. As the core kinase that phosphorylates YAP1 and promotes its nuclear exclusion, increased levels of LATS1 should recapitulate conditions of YAP1 silencing. Levels of LATS1 are slightly higher in the overexpression lanes, and an increase in levels of phosphorylated YAP1 at residue S127 increase through lytic replication, and are markedly higher in the LATS1 overexpression cells, a similar trend to what is seen in parental iSLK.219 cells (Figure 3). Accumulation of lytic proteins is also seen in the LATS1 overexpression – similar to the YAP1 silencing, with both ORF45 and ORF50 protein levels accumulating earlier and to a higher degree in the overexpression

treatments. To determine whether this increased level of protein accumulation corresponds to increased progression through the lytic cycle, the number of RFP+ cells in each treatment was enumerated via flow cytometry. At 72 h post-doxycycline addition, there is an increase in the number RFP+ cells in the LATS1 overexpression treatment compared to the empty vector control, though further replicates are needed to determine significance. Further characterization of the cells overexpressing LATS1 is needed to understand whether this potentially increased rate of lytic reactivation also corresponds with greater release of infectious virus.

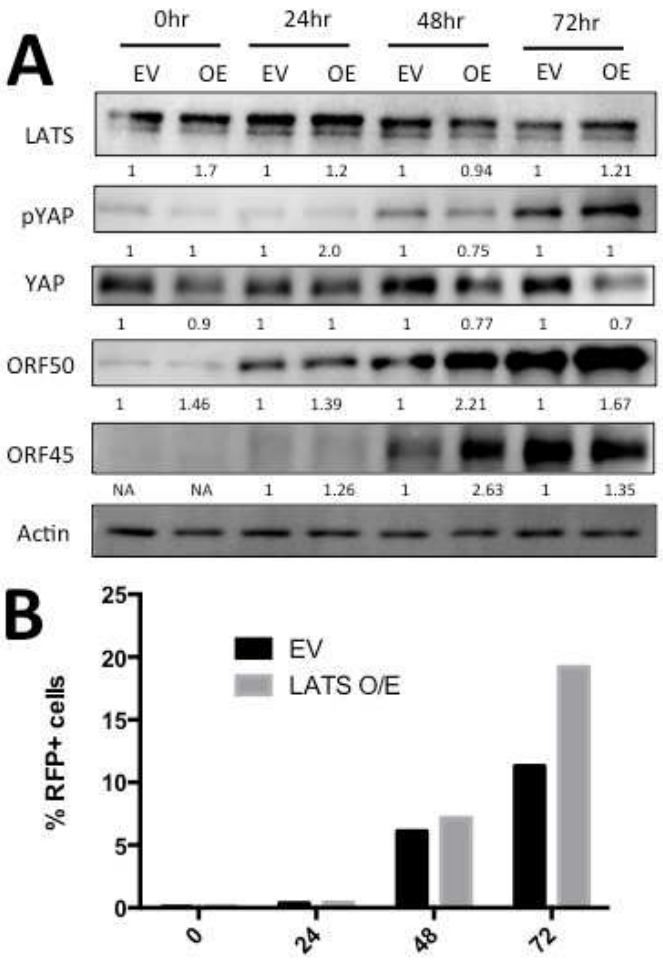

Figure 9. LATS1-overexpressing cells have an increase in lytic protein accumulation and percent of RFP+ cells. iSLK.219 cells transduced with lentivirus carrying LATS1 overexpression construct, and empty vector (EV) control. Lytic reactivation induced via doxycycline addition. Cells harvested at 24 h intervals for immunoblotting and flow cytometry analysis. (A) Immunoblot of harvested cell lysates, 10 µg of protein loaded per lane, see antibody dilution table in Section 2.3 for details. Blots imaged using ChemiDoc Touch (BioRad), n=1. Quantification of signal intensity using BioRad ImageLab, normalized to β-actin and EV at each timepoint. (B) Quantification of RFP+ cells via flow cytometry. Samples run by Derek Rowter at Dalhousie FACS Core Facility. Data analyzed using FCS Express 6, n=1.

3.4 LATS1/2 Knockdown Reverses Accelerated Lytic Production

So far, the data suggests that reduction in YAP1 via both genetic and regulatory approaches can increase the rate of early lytic protein accumulation. To understand if relieving the LATS1/2 regulation of YAP1 would show an opposite phenotype – a reduction in lytic reactivation - two short hairpin RNAs were designed against LATS1. As LATS1 is the core kinase for regulating YAP1, it is expected that a reduction in LATS1 protein levels would increase the amount of active, or non-phosphorylated YAP1. iSLK.219 cells were transduced with lentiviruses containing two different shRNAs targeting LATS1: viral protein accumulation and viral titer was assessed following selection. The efficiency of knockdown for each shRNA was quite different, with shLATS1#1 being the most efficient of the pair (Figure 10A). Treatment with shLATS1#1 showed a striking decrease in the amount of the late viral ORF65 protein at 72 h post-doxycycline addition (Figure 10A) – the reverse of what is observed with YAP1 silencing (Figure 5). When assessing viral titer, shLATS1#1 also severely reduced the amount of virus being released (Figure 10B). These experiments are consistent with the idea that increasing the amount of active YAP1 in cells by reducing protein levels of regulatory kinase LATS1 is sufficient to block effective lytic reactivation. Combined with our previous observations, these findings strongly suggest that Hippo pathway signal transduction can regulate KSHV latent/lytic switch in this model.

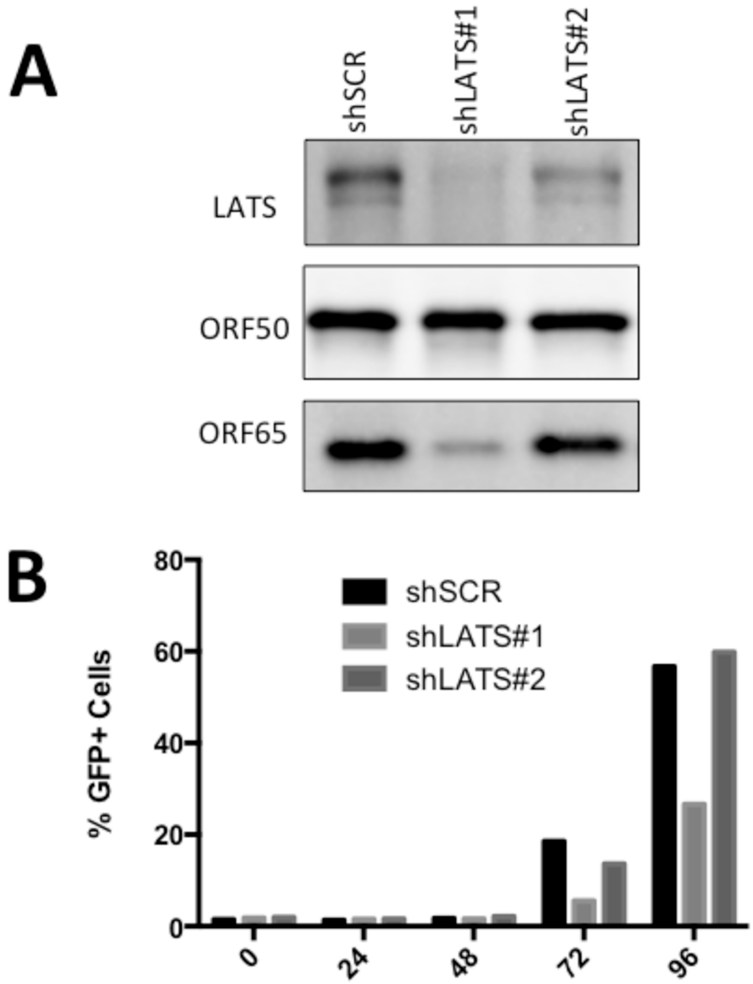

Figure 10. LATS1 silencing reduces accumulation of ORF65 and release of infectious virions. iSLK.219 cells transduced with lentivirus containing two separate shRNA constructs targeting LATS1. Cells selected and lytic reactivation initiation via doxycycline addition; cells harvested at 24 h intervals (A) Immunoblot of cell lysates harvested 72 h, 10 μg of protein loaded per lane, see antibody dilution table in Section 2.3 for details. (B) Viral titer assay. Supernatants collected from doxycycline-induced cells, clarified, and used to infect a secondary monolayer of HEK293A cells. After 24 h cells were dissociated and examined for GFP fluorescence by flow cytometryharvested for flow cytometry. n=1.

3.5 YAP1 Reduction Increases LANA Dots in Latent iSLK.219 Cells

Up to this point, several experiments have assessed changes in iSLK.219 cells upon lytic reactivation in various contexts of Hippo pathway modulation. We hypothesized that the increase in lytic replication seen in YAP1-silenced cells could be due to an increase in the amount of viral genome present in latent cells.

To test whether YAP1-silenced cells had an altered ability to maintain viral genomes, or replicate, both levels of LANA protein and LANA dot counts per cell were assessed. Counting LANA dots per cell gives a rough estimate of episome number. iSLK.219 cells were transduced with lentivirus expressing shSCR, shYAP#1 and shYAP#2 constructs as described. Following selection, cells were seeded onto gelatin-coated glass coverslips and stained for LANA dots. Cells were imaged, and counted using Cell Profiler. Five duplicates were used, with between 400-430 cells counted per treatment in total. Statistical significance was assessed using a repeated measured ANOVA, followed by a Students t-test. Strikingly, the number of LANA dots is modestly increased between the shSCR group and both the shYAP#1 and shYAP#2 treatments, with high confidence ($p<0.0001$) (Figure 11A). The average LANA dot count per cell in each treatment is 31.96, 35.81 and 36.25 in the shSCR, shYAP#1, and shYAP#2 groups respectively. A significant increase in LANA dot counts per cell was measured by immunofluorescence, but there was no change in the amount of LANA protein detected in YAP1-silenced cells (Figure 11B) and the differences did not coincide with major changes in LANA dot morphology or size (Figure 11C). The functional consequence of this is unclear, but the increase in LANA dot counts may explain the increase in lytic

protein accumulation previously seen (Figure 5), as it suggests there are more viral genomes in YAP1-silenced cells that could potentially be reactivated from the latent state.

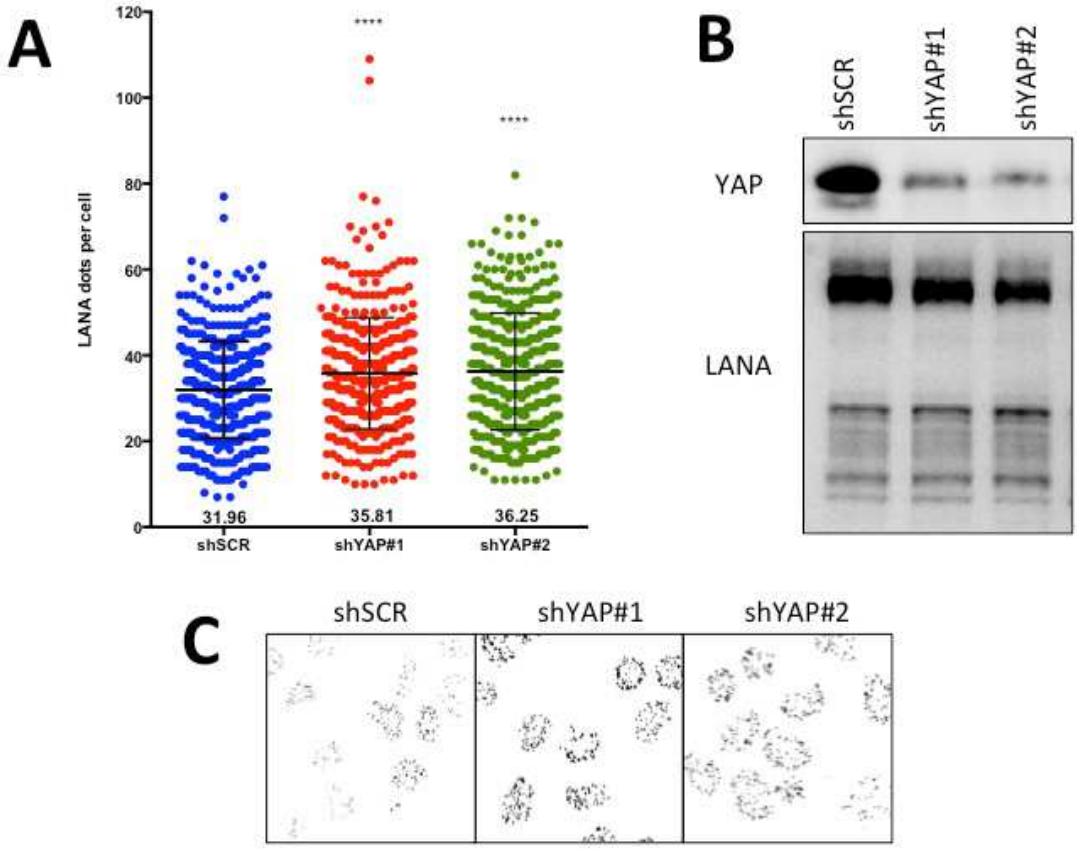

Figure 11. YAP1-silenced latent iSLK.219 cells have a striking increase in LANA dot per cell counts, with no change in LANA protein levels or LANA dot morphology. Cells transduced with lentiviruses expressing two separate knockdown constructs targeting YAP1. (A). Cells seeded onto gelatin-coated coverslips, stained with anti-LANA antibody, secondary anti-Rabbit Alexafluor647, and Hoescht as a nuclear stain. 10 fields of view imaged per treatment using Zeiss 510 Confocal Microscope (CMDI, Dalhousie CORES Facility). LANA dots numerated using Cell Profiler, graph depicts 5 replicate experiments with 400-430 cells counted per treatment. Statistical significance determined using repeated measures ANOVA followed by a Students t-test. (B). Immunoblot of cell lysates harvested 72 h post-induction, 10 µg of protein loaded per lane, see antibody dilution table in Section 2.3 for details. (C) Representative immunofluorescent images from each treatment showing LANA dots in black.

3.6 Several KSHV ORFs Modulate Transcription of TEAD-Response Element-Driven Reporter

It is clear that the activity of YAP1 is important for controlling lytic reactivation events in both an epithelial and B-lymphocyte model of KSHV infection. What remains to be understood is if certain viral proteins may be interacting with the Hippo signaling pathway in order to drive the phenotype observed in YAP1 silencing and LATS1 modulation studies shown previously. In order to address this discrepancy in understanding, the McCormick lab KSHV ORF library was screened using a luciferase-based TEAD reporter. When YAP1 is nuclear, and therefore active, expression of the luciferase protein is increased – as the promoter contains 8 binding sites for TEAD, the transcription factor activated by YAP1. Figure 12 depicts a schematic of the screen set-up, and a more in-depth description of methods is described in Section 2.9. Using a constitutively active CMV-Renilla construct as a transfection control, ORF DNA and TEAD-luc reporter plasmid were transfected into HEK293A cells using FuGENE HD Transfection Reagent. 36 hours following transfection, cells were starved of serum for 12 hours, and harvested for luciferase readout using the Promega Dual-Glo luciferase assay kit. Luminescence of both luciferase and renilla were measured on a Tecan Infinite 200Pro plate reader (Thermo Fisher). All values were normalized to expression of CMV-Renilla, and the empty vector control. Three biological replicates of the screen were performed and plotted. Using a non-phosphorylatable form of YAP1 (YAP5SA) as a positive control, several KSHV ORFs increased expression of the TEAD-driven luciferase reporter under serum starvation conditions. Remarkably, K10 drove expression of the TEAD reporter to levels similar to the positive control YAP5SA, and several other ORFs showed very high induction: ORF75, K10.5, and K8. A handful of ORFs also

decreased the activity of the TEAD reporter to less than basal levels of YAP1 activity following serum starvation – including ORF21 and ORF71.

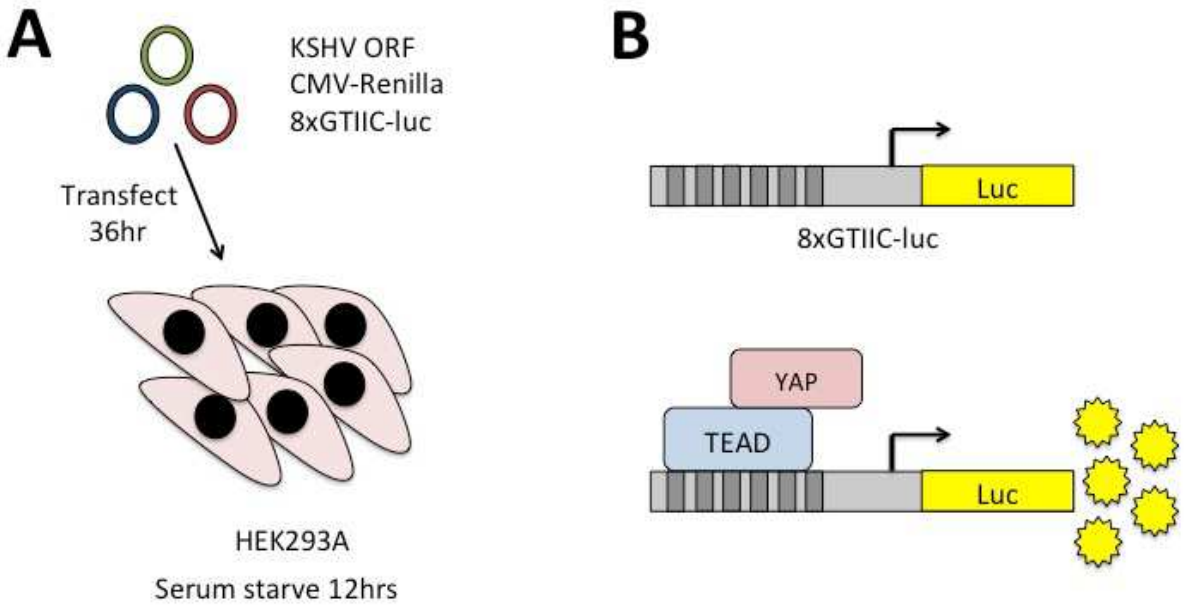

Figure 12. Schematic representation of ORF library screen. (A) HEK293A cells transfected with three separate plasmids, starved of serum for 12 h before harvest. (B) Expression of luciferase requires TEAD activation.

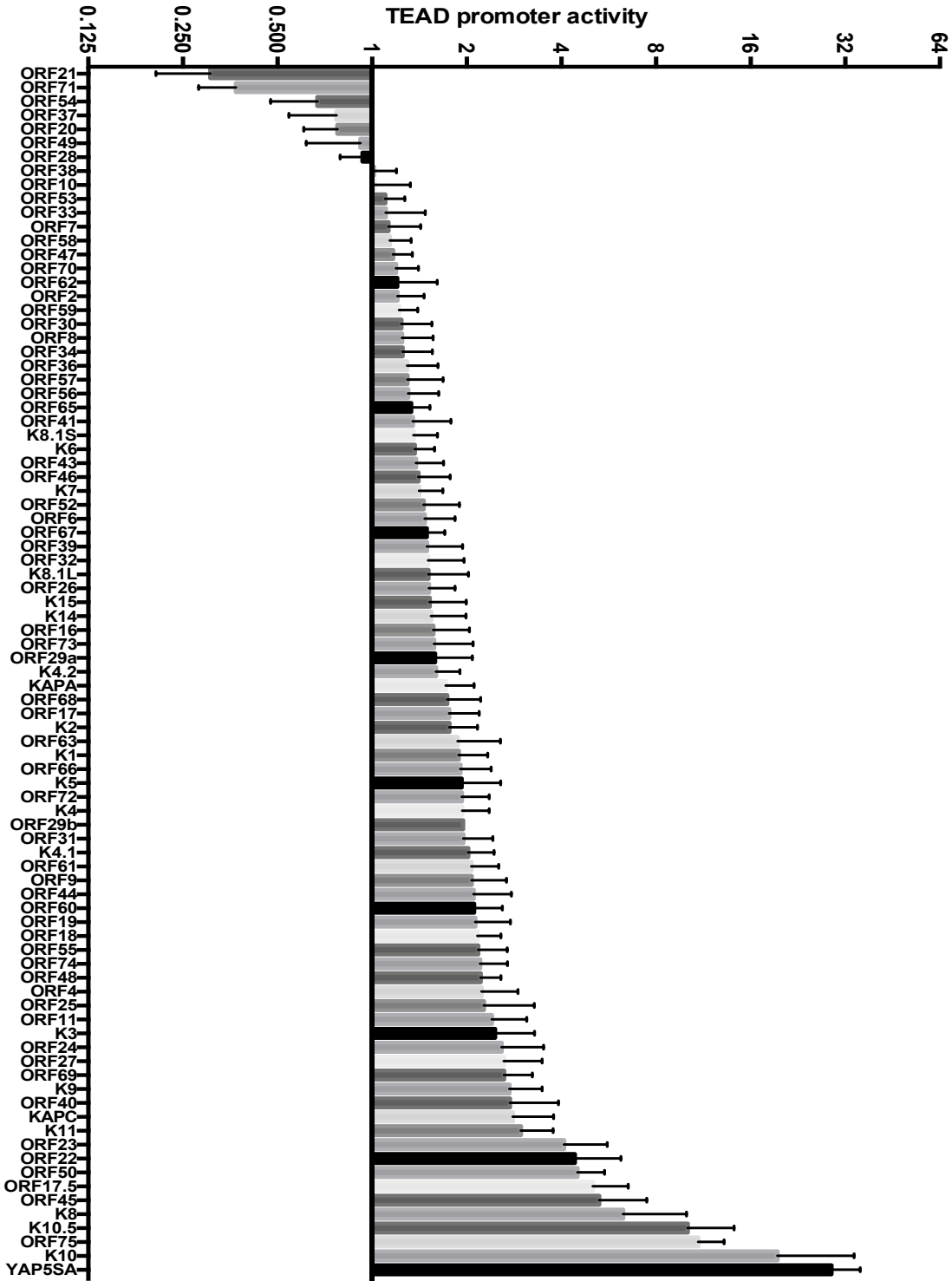

Figure 13. Several KSHV ORFs modulate activity through TEAD-luciferase reporter. McCormick lab KSHV ORF library screened in HEK293A cells for ability to modulate activity of TEAD-luciferase reporter. Data normalized to CMV-Renilla, and empty vector transfected cells, n=3.

3.6.1 Several KSHV ORFs modulate TEAD activity in a YAP1-independent fashion

The nature of the TEAD-luciferase reporter screen does not directly address whether nuclear YAP1, the canonical activator of TEAD promoter activation, is driving expression of the reporter construct – but it is assumed that nuclear YAP1 is driving upregulation. To determine if any of the viral ORFs that showed high levels of TEAD reporter activation were acting in a YAP1-independent manner, a secondary screen in HEK293A cells stably expressing the shYAP#1 and shYAP#2 silencing constructs was conducted. As expected, the activity of the luciferase reporter in YAP1-silenced cells was markedly reduced – basal activity can be seen in the empty vector treatment, and the majority of ORFs tested showed highly reduced levels of activity (Figure 14A). Non-normalized values for TEAD reporter activation in wild-type HEK293A cells are shown in Figure 14B. Highly potent ORF75 and ORF74 show very largely decreased activity in the knockdown cells, suggesting their activation of the TEAD reporter is through YAP1 activation. Interestingly, several ORFs seem unaffected or only slightly affected by the YAP1 silencing including ORF17.5, a scaffolding protein required for assembly of virions prior to budding. It is possible that these ORFs may be upregulating TEAD activity via a YAP1-independent mechanism: a phenomenon that has not been described in the literature.

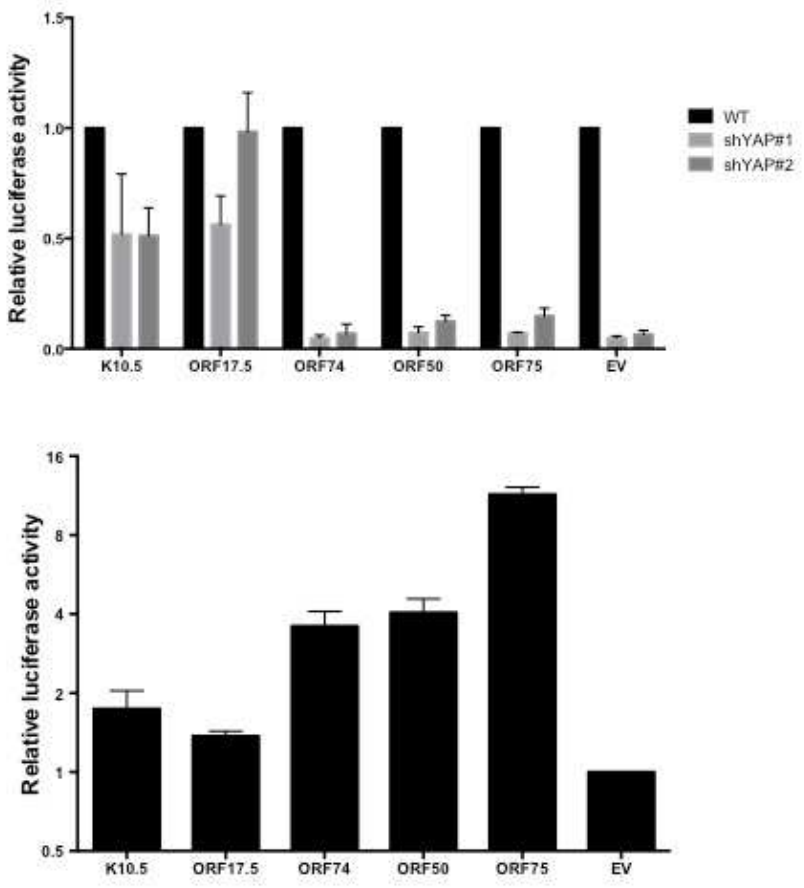

Figure 14. Majority of KSHV ORFs that increase activity of TEAD reporter are sensitive to YAP1 silencing. TEAD-luciferase reporter screen in HEK293A cells stably YAP1 silencing constructs, and scramble control performed as described in Section 2.9. Following 36 h transfection, cells starved in DMEM+2 mM L-glutamine for 12 h prior to harvest. Luminescence measured using Promega Dual-Glo assay kit, read on Tecan Infinite 200Pro plate reader. (A) Luciferase activity values normalized to CMV-Renilla, empty vector, and shSCR cell. (B) Luciferase activity values normalized to CMV-Renilla and empty vector only.

Chapter 4 Discussion

4.1 YAP1 may maintain latency and prevent lytic reactivation

The role of Hippo pathway signaling in tumour virus infection is very poorly understood, and though many cancer indications have seen Hippo pathway implication, viral cancers have not been thoroughly studied. In this thesis, I demonstrated that Hippo pathway signaling via its main effector YAP1 regulates KSHV reactivation from latency. Specifically, reduction in YAP1 levels via RNA silencing and increasing regulatory kinase LATS1 activity can each trigger an increase in lytic reactivation. This is the first work to critically analyze the effects of Hippo pathway modulation on KSHV replication dynamics, and adds to the current understanding how cellular transduction pathways can regulate the latent/lytic switch.

4.1.1 Reactivation from latency stimulates the Hippo pathway

Others have shown that the KSHV early lytic protein vGPCR can modulate Hippo pathway activity and thereby promote nuclear translocation of YAP1 (Liu et al., 2015). Using a primary KSHV infection of HEK293A cells and robust reactivation of the lytic program using sodium butyrate, this group observed an increase in YAP1 nuclear localization and YAP1/TEAD-driven gene expression through lytic reactivation, suggesting that vGPCR, and potentially other lytic proteins, may be inhibiting the Hippo pathway. Interestingly, our findings suggest that YAP1 is active during latency, and through lytic reactivation there is an increase in phosphorylated LATS1/2 – the chief regulatory kinase that phosphorylates and inactivates YAP1 (Figure 3). Accordingly, YAP1 serine 127 phosphorylation of YAP1 increased as well during lytic reactivation,

which corresponded to a decrease in the total YAP1 levels as viral replication progressed, (Figure 3). Thus, our findings are at odds with the established literature. There are stark differences in the models used for each of these studies; Liu and colleagues used a *de novo* infection model, with an aggressive treatment to induce lytic replication. Specifically, they triggered KSHV reactivation from latency by addition of the HDAc inhibitor sodium butyrate. This differs considerably from the tight control of latency in iSLK.219 cells used in this thesis, and a gentle induction of lytic reactivation using doxycycline alone. The effect of sodium butyrate on Hippo pathway components has not been studied, and may be contributing to some of the effects seen by Liu and colleagues. Sodium butyrate treatment of KSHV-infected cells causes a robust lytic reactivation, and the percentage of cells undergoing sodium butyrate-induced reactivation is much higher compared to the doxycycline-inducible iSLK.219 system.

It is possible that the reduction in YAP1 protein levels, shown in Figure 3, is caused by general host-shutoff induced during KSHV lytic replication. Host shut-off is a mechanism used by many viruses to prioritize production of viral proteins while blocking translation of host proteins, freeing up translation machinery for viral mRNAs. Alternatively, a lytic protein could be directly affecting Hippo pathway activation, and YAP1 phosphorylation. It is also possible that as cells continued to divide, cell-cell contact inhibition drove Hippo pathway activation at later time points. In order to be absolutely certain that the results of this experiment were not due to changing cell densities over time – a potent regulator of Hippo pathway activation – several cell densities should be assayed in parallel in follow-up experiments. From our data, latency

seems to be the replication cycle in which YAP1 is the most active. The lack of published research in this area prevents an informed hypothesis regarding Hippo dynamics during both latent and lytic replication cycles, and a major goal of this thesis is to address this gap in understanding.

4.1.2 YAP1 silencing potentiates lytic reactivation in iSLK.219 cells

From the data presented in this thesis, it is clear that YAP1 plays some role in controlling the latent/lytic switch. It is worthwhile to note that this role does not impede efficient reactivation in this cell line; the iSLK.219 model of KSHV infection has been studied widely, and is well known for potent reactivation. When YAP1 was silenced in the iSLK.219 cell epithelial model of infection, an increase in the amount of cells undergoing lytic reactivation was observed (Figure 4). This was characterized by lytic protein accumulation (Figure 5), an increase in the amount of virions being released from cells (Figure 7), and significantly higher genome copy numbers (Figure 6). The iSLK.219 cells have a doxycycline-inducible ORF50 transcript that triggers lytic reactivation when ORF50 accumulates in these cells; it is worthwhile to note that the accumulation of ORF50 shown in Figure 5 is both the doxycycline-inducible transcript as well as the episomal transcript of ORF50.

An exciting finding, the increase in lytic reactivation shown with YAP1 silencing was not expected. YAP1 reduction has severe consequences on cell growth; it was not anticipated that removal of a pro-growth factor would increase viral propagation. The lack of major growth inhibition in these YAP1-silenced iSLK.219 cells may be due to

expression of latent viral genes bypassing signals for growth inhibition. It is worthwhile to note the iSLK.219 model reactivates from latency in an asynchronous manner – in any one population of cells, individual cells can be at different stages of reactivation (Adang et al., 2006). It is unclear whether YAP1 silencing is changing the percentage of cells able to reactivate – as if it was priming more cells for efficient reactivation, or instead increasing the potency of reactivation in the same percentage of cells, leading to more viral gene production and viral release. Episome copy number and chromatin dynamics in these cells can influence the ability to reactivate, as well as changes in cell cycle status – these potential implications of YAP1 silencing will be discussed.

4.1.3 LATS1 overexpression and silencing support YAP1 silencing model

To validate our initial findings, LATS1, the regulatory kinase of YAP1, which functions to phosphorylate YAP1 and promote cytoplasmic retention, was studied by both silencing and overexpression experiments. LATS1 overexpression in iSLK.219 cells phenocopied that of YAP1 silencing, an increase in lytic protein accumulation was seen, with more cells entering the lytic reactivation program (Figure 9). Strikingly, LATS1 silencing blocked accumulation of late lytic capsid protein ORF65, and reduced the release of infectious virions (Figure 10), the phenotypic reverse of what was seen with overexpression. This phenotype was only observed with more efficient shRNA – shLATS1#1, suggesting that even moderate levels of LATS1 is sufficient for effective lytic replication. Technical challenges accompanied both LATS1 overexpression and silencing studies; further replicates and experimentation is needed to fully understand if this phenotype can be tracked back to LATS1 regulation of YAP1.

Whether LATS1 silencing caused a block in late lytic protein accumulation due less phosphorylation and degradation of YAP1 is unclear, as LATS1 has a range of functions outside of core Hippo pathway signaling. The activity of LATS1 in this model follows the phenotype seen with YAP1 silencing; less active YAP1 through an increase in the amount of its negative regulatory kinase LATS1 shows an increase in lytic replication potency. LATS1 reduction shows a block in lytic replication, suggesting that constitutively active YAP1 may block some lytic reactivation events.

4.1.4 YAP1-silenced BCBL-1 cells show increased spontaneous and induced reactivation

YAP1 silencing also induced changes in the dynamics of lytic reactivation in BCBL-1 cells, a wild-type model of KSHV infection derived from a patient with PEL. Not only did YAP1-silenced cells show an increase in TPA-induced lytic reactivation, levels of spontaneous reactivation increased as well (Figure 8). The majority of research surrounding Hippo pathway dynamics in cell growth and division utilizes solid tumour models - very little work has been done to understand the role of this pathway in lymphocytes. In this regard, it was uncertain if YAP1 silencing in B-lymphocytes would have any effect at all. It is clear from these experiments that whatever role YAP1 may be playing during latency, the BCBL-1 model is also susceptible to YAP1 antagonization. More in-depth experiments using the BCBL-1 model, including immunoblotting to analyze progression through lytic reactivation is a very necessary next step in order to have full confidence in the phenotype shown thus far.

It is interesting to note that the YAP1-silenced, but untreated cells, and control cells treated with TPA (Figure 8) show very similar levels of lytic reactivation. Recently, a report using a liver cancer model showed that TPA treatment of cells blocked the interaction between YAP1 and TEAD, reducing proliferation (Zhu et al., 2017). It is possible that the effects of TPA at reactivating BCBL-1 cells from latency may be through its antagonization of YAP. TPA has a multitude of pleotropic effects on cells, and though suggested by the data shown here, more experimentation is needed to fully understand the effects of TPA on YAP1 in a B-cell context. Immunoprecipitation of nuclear YAP with and without TPA treatment, and analysis of TEAD content by western blot could be used to determine if this complex is being disrupted in the BCBL-1 model of KSHV reactivation.

4.1.5 YAP1 silencing impacts LANA dot counts in latent iSLK.219 cells

Following initial results suggesting that cells with reduced levels of YAP1 showed a more robust reactivation from latency when treated with doxycycline, latent iSLK.219 cells were assessed. We hypothesized that, considering the increase in reactivation was trending upward but not quite a log-fold increase, small changes in genome stability during latency could be contributing to this phenotype. The amount of LANA dots per cell in the YAP1-silenced latent iSLK.219s was significantly increased compared to control cells (Figure 11A). Interestingly, there was no detectable change in the amount of LANA protein in these cell treatment groups, and no obvious change in LANA dot morphology was observed (Figure 11B,C). The functional consequence of this remains unclear, but an increase in LANA dots in these YAP1-silenced cells may be

contributing to the increases in lytic reactivation seen. Further experimentation is required to understand how YAP1 may be modulating levels of LANA, or chromatin remodeling of the KSHV episome; this will be discussed further in Section 4.3.1.

4.2 Unanticipated consequences of YAP1 knockdown

Beyond its central role in regulating cellular growth, recent work has shown that YAP1 can regulate antiviral responses and chromatin remodeling. These additional YAP1 phenotypes need to be formally investigated in the context of ongoing KSHV studies. Reducing YAP1 levels through RNA silencing could have severe effects in these non-canonical roles of YAP1, contributing to the phenotype observed.

4.2.1 DNA Sensing

A wide range of cellular stresses can reactivate KSHV from latency, as described in depth in Introduction Section 1.2.6.2. One of these triggers is Toll-Like Receptor (TLR) 7/8 activation, induced by secondary viral infection of latent cells (Gregory et al., 2009). This activation triggers an upregulation of lytic switch protein ORF50, inducing lytic reactivation.

As described in depth in Introduction Section 1.3.5.1, YAP1 and Hippo pathway components have been recently identified as potent regulators of interferon responses in virally infected cells through several mechanisms (Wang et al., 2017; Zhang et al., 2017). Interestingly, under YAP1 silencing conditions there was an increase in innate immune signaling of anti-viral networks, suggesting that YAP1 functions as a negative regulator

or suppressor of innate immune activation. YAP1 silencing in susceptible cells reduced the ability of two very different viruses – RNA virus vesicular stomatitis virus (VSV), and herpesvirus HSV, to infect and replicate in these cells. Considering this role for YAP1 in innate immune signaling, in the context of YAP1 silencing, TLR7/8 may be more sensitive to activation, which could stimulate reactivation of the lytic replication program. Though this may be a player in the increase in lytic reactivation seen in the model used in this thesis, it is worthwhile to note that spontaneous reactivation – or reactivation without the addition of doxycycline – was not induced to any measurable extent in the YAP1-silenced iSLK.219 cells. These cells in effect were more primed for lytic reactivation, but YAP1 modulation alone was not sufficient to initiate reactivation.

At first glance, the viral replication data presented by Zhang and Wang seems in conflict with the data presented in this thesis, but it is worthwhile to note one major difference in the cellular models utilized in each study. Both Zhang and colleagues, and Wang and colleagues used a primary infection model of susceptible cells; in this thesis, a previously constructed model of latent viral infection was used. An enhanced innate immune system is more potent at restricting primary viral infection, and this is likely a major factor in the reduction in viral replication seen in both studies. A thorough analysis of innate immune signaling with particular attention paid to TLR7/8 activation in the YAP1-silenced iSLK.219 cells is a necessary next step to understand if this signaling axis may have a role in the YAP1 silencing phenotype observed.

There is a particular challenge to recapitulating the primary infection models used in these publications to understand if YAP1 silencing also affects KSHV replication; latency is the default replication cycle following primary infection with this virus. There are several tools that have been developed in an attempt to study one-step replication kinetics of KSHV, including a LANA knockout viral genome, or primary infection in cells expressing ORF50. Viruses with LANA removed from the genome are unable to form episomes, and as such chromatinization of the genome is not possible, leading to lytic gene expression upon primary infection (Li et al., 2008). With ORF50 overexpression, primary infection will lead to immediate lytic gene expression - similar to the LANA knockout virus.

4.2.2 Chromatin remodeling

It is well known that KSHV latency is very sensitive to treatment with HDAC inhibitors, and a wide range of these drugs can drive reactivation from latency. Latent episomes are bound by facultative chromatin, and reactivation from latency requires histone acetylation and chromatin de-repression.

Typically thought of as a transcriptional activation complex, YAP/TAZ-TEAD has also been shown to have genomic co-repressor function in conjunction with the nucleosome remodeling and histone deacetylase (NuRD) complex (Kim et al., 2015). Kim and colleagues showed that YAP/TAZ-TEAD acts to functionally repress the expression of nearly 100 genes, including several tumour suppressors. The binding of this multi-protein complex changed the acetylation status at promoters through recruitment of

the NuRD complex, and in YAP-silenced cells, the amount of acetylated histone H3 was globally increased.

Interestingly, the YAP/TAZ-TEAD complex described by Kim and colleagues was sensitive to trichostatin A (TSA) treatment – in the presence of TSA; this repressor complex was no longer active. Treatment of KSHV infected cells with TSA can stimulate a proportion of cells to undergo productive lytic infection (Shin et al., 2014) characterized by a loss of repressive LANA at the ORF50 promoter (Lu et al., 2006) a potentially intriguing link. TSA treatment recapitulates the phenotype seen in YAP/TAZ depletion when assaying for repression of gene expression; antagonizing this repressive function of the YAP/TAZ-TEAD complex may be playing a role in the more robust lytic reactivation shown in this thesis in YAP1-silenced latently-infected cells. YAP1 depletion may counteract repression of the ORF50 promoter in latently infected cells via this mechanism, but further experimentation is required to understand what is at play. Chromatin immunoprecipitation assays of the ORF50 promoter during latency under YAP1 silencing conditions would be the most clear-cut path to understand if chromatin remodeling induced by YAP1 silencing is driving this increase in lytic reactivation. Infecting YAP1-silenced cells and measuring the percentage of cells that establish latency versus reactivate to the lytic program would also assess whether chromatinzation of the episome by the YAP/TAZ-TEAD repressor complex is required for latency.

4.3 YAP1 in episome maintenance

4.3.1 Episome dynamics during lytic reactivation

Maintenance of the KSHV episome during latency is a highly regulated and important event, necessary for life-long latent infection of susceptible hosts. Latency is complex: herpesviruses do not integrate their genome, but instead tether it to the host genome via viral proteins; LANA maintains this role in KSHV infected cells. As described in Introduction Section 1.2.2, LANA is the master latent protein, and has a variety of functions from episome maintenance to antagonization of tumour suppressor proteins. LANA physically tethers the KSHV episome to host chromatin, and maintains this association through DNA replication and cell division to propagate viral episomes to daughter cells. Replication of the viral episome during latency uses host DNA replication machinery – LANA also plays a key role in this process. LANA recruits host replication machinery to the Ori-P site on the KSHV episome, and through a complex process reviewed nicely in Purushothaman et al, 2016; the viral episome is fully replicated. Following DNA replication, LANA works to bind KSHV episomes to centromeres during mitosis, effectively segregating episomes into daughter cells (Purushothaman et al., 2016).

It is very clear from the literature that LANA plays several required roles in both episome maintenance and persistence during cell division. It is unclear why, under YAP1 silencing conditions, the amount of LANA dots in these cells would change (Figure 11A). Comparing this data to that of the genome replication experiments (Figure 6), it is unlikely that this increase in LANA dots is corresponding to higher genome copy

numbers during latency due to the sensitivity of the genome replication assay, but further experimentation is needed. Could YAP1 silencing induce the loss of LANA at ORF50 promoters, therefore increase the amount of LANA dots in each cell via chromatin remodeling? This would likely not be detectable via immunofluorescence. As there seems to be no change in LANA protein level (Figure 11B), could the increase in LANA dots mean there is less LANA at each episome or tethering site, therefore reducing its ability to adequately bind and repress lytic reactivation to some level? To fully understand this phenotype, a more in-depth analysis of episome dynamics and LANA changes through lytic reactivation is needed.

4.4 Growth and cell cycle effects of Hippo pathway modulation

4.4.1 YAP1

As a major player in normal growth and differentiation of cells, YAP1 silencing has definitive effects on cellular growth, especially in cell culture systems. YAP1-null mice only survive between 8-10 days as embryos; a clear indication that YAP1 is required for normal growth and differentiation (Zhao et al., 2010). Cell growth following YAP1 silencing has major defects – most apparent is the slow down in cell division. It is possible that the cellular stress used in this thesis - removing a potent growth pathway from cultured cells - could be disrupting KSHV latency, leading to a more potent reactivation. Similar to nutrient stress and hypoxia, changes in growth conditions in cells can trigger reactivation of the lytic program so the virus can ‘escape’ the damaged host cell. Interestingly, compared to HEK293A cells, the iSLK.219 cell line was much less susceptible to a slowed growth phenotype under YAP1 silencing conditions.

4.4.2. LATS1

Several technical challenges accompanied studying LATS1-overexpressing iSLK.219 cells, namely that growth was very heavily inhibited. As a tumour suppressor protein, LATS1 functions to regulate cell cycle dynamics, and promotes arrest at the G_2/M stage in the cell cycle. LATS1 induces growth arrest through inhibition of the CDC2 kinase, and a direct downregulation of both Cyclin A and B – required for the transition of cells into mitosis (Xia et al., 2002). Considering long-term culture of iSLK.219 cells, even under antibiotic selection for episome maintenance, causes episome loss, we hypothesized that the YAP1 silencing was slowing growth of these cells in a manner that prevented normal rates of episome loss. If YAP1-silenced cells underwent fewer cell divisions than the scrambled control, episome loss would be slowed. Inducing cell-cycle arrest effectively reduces the number of replicative cycles these cells have undergone, which may lead to higher genome copy numbers compared to the scrambled control where cell growth is not affected. I have shown that this may not be the case; changes in LANA dots per cell was measured through three replication cycles in both YAP1-silenced and control latent cells – no measurable difference in episome loss was observed between treatments and controls.

4.5 Several KSHV ORFs interact with the Hippo pathway

KSHV is an oncogenic virus with a very large repertoire of genetic coding potential, and gene expression varies dependent on the replication cycle of the virus. One lytic gene, vGPCR, has been shown to have potent effects on regulation of the Hippo signal transduction pathway, driving growth and development through preventing

phosphorylation of YAP1. Considering the immense coding potential of the ~140 kilobase KSHV genome, we hypothesized that several other KSHV ORFs would likely show activity at promoting or inhibiting the nuclear localization of YAP1. Following a luciferase based screen of close to 90 KSHV ORFs, it is clear that several ORFs are able to influence very high activation of the TEAD reporter; suggesting that vGPCR is not the only KSHV protein that has functional activity at regulating Hippo pathway signaling dynamics (Figure 12). This list includes K10, ORF75, and K8. Deactivation, or reduction past basal levels of TEAD activity under serum starvation conditions was also observed; both ORF21 and ORF71 strikingly decreased the activity of this reporter. A more thorough discussion of potential hits will be discussed below.

4.5.1 TEAD-luc activators and PML

Following the first replicate of this screen, a literature review was completed to understand if any of the top hits had known shared functions. Remarkably, ORF75, ORF45, K10, K10.5, ORF50 and K8 – all ORFs that scored highly following three replicates of the screening assay – have published (or observed in our lab) interactions with promyelocytic leukemia (PML) bodies. PML bodies are innate immune signaling hubs found within the nucleus of most cells and tissues; they are a known restriction factor for KSHV infection (Full et al., 2014). PML bodies act as a an organizing centre for a wide range of cellular – and viral – proteins, and what these proteins share in common is either a SUMOylation consensus site, or SUMO-interacting motif. SUMO, a small ubiquitin-like modifier, is a very common post-translational modification of many proteins – but unlike ubiquitin, it often modifies proteins to increase their half-life, or

target them to certain subcellular compartments (Wilkinson and Henley, 2010). PML bodies are a site for protein SUMOylation. Proteins that interact with SUMO, or have SUMO-modifications localize to PML bodies, and SUMOylation of PML proteins themselves are required for establishment of PML bodies (Gao et al., 2008).

An ongoing collaboration between the McCormick lab and the Dellaire lab has shown that ORF45 localizes to PML bodies throughout KSHV lytic reactivation, and Kanno and colleagues have observed K10 also localizes to PML bodies (Kanno et al., 2006); the function of these interactions remain unclear, but are under investigation. ORF75 has been shown to antagonize major components of PML bodies, SP100 and ATRX (Full et al., 2014), whereas K10.5 increases the SUMO2 modification of PML body components, targeting them for proteasomal degradation (Marcos-Villar et al., 2009). ORF50, the master transcriptional regulator for lytic reactivation, also has SUMO-targeted ubiquitin ligase (STUbL) activity; it functions to degrade proteins that have been SUMO-modified (Izumiya et al., 2013). Finally, K8 has been identified as an E3-ligase for SUMO2/3 modification of proteins (Chang et al., 2010) and has been shown by several groups to target to PML bodies (Wu et al., 2001). Wu and colleagues also demonstrated that KSHV can replicate at PML bodies, and suggests they may be a site for virion assembly; similar to what is seen with related γ-herpesvirus, EBV (Wang et al., 2015).

It is not surprising that several KSHV proteins have published interactions with different components of PML bodies; the study of PML bodies and DNA viruses is a

broad field, and many groups have demonstrated that DNA viruses often utilize PML bodies as sites of genome replication or transcription of viral genes (Everett, 2001). It is also worthwhile to note that when viral ORFs were assessed for SUMOylation consensus sites or SUMO-interacting motifs (SIMS) using GPS-SUMO bioinformatics software (Zhao et al., 2014), all of these proteins contain a SIM or SUMO-conjugation site. What is more surprising is that the proteins that show published interactions with PML bodies and components, are also potently activating TEAD promoter transcription. Why this may be happening is discussed below.

4.5.1.1 Hippo and PML

Understanding how PML bodies and Hippo signaling may interact is not a new field, and several clear interactions between PML and YAP1 have been identified, primarily in understanding the p73 axis of the DNA-damage response and cellular apoptosis. p73 is a protein in the p53 family; it is a tumour-suppressor transcription factor that drives expression of pro-apoptotic genes, canonically triggered by irreparable DNA damage. YAP1 binds to p73 in a manner that stabilizes both proteins, and the YAP1-p73 complex upregulates pro-apoptotic genes, inducing apoptosis in damaged cells. Similarly, PML binds p73 at PML bodies, also stabilizing its transcriptional activity. Where this interaction gets more complex, is that PML is a direct transcriptional target of the YAP1-p73 complex; PML is upregulated when YAP1 and p73 are in association in the nucleus (Lapi et al., 2008). YAP1 and PML have also been observed as interacting proteins, and SUMOylation of YAP1 by PML prevents ubiquitylation of YAP1 thereby increasing its stability. It is unclear what this complex web of interaction means functionally, and has

not been studied in depth in the context of viral infection. It may be that the PML-degrading activity of some of these viral ORFs increases the transcription of more PML protein, which is activated by nuclear YAP1– this nuclear YAP1 will then also subsequently increase activity at the TEAD reporter. Manipulating components of the SUMOylation machinery in the context of these viral proteins will allows us to understand the link between this post-translational modification and activity.

Considering the recent discoveries that YAP1 has several functional roles at regulating innate immune signaling, and that PML is often thought of as a hub for innate signaling proteins, it is likely that YAP1 and PML cross signaling-paths in ways that have yet to be discovered. Elucidating the function of these viral ORFs in terms of their ability to drive TEAD activation is an important step to understand these interactions. Some preliminary work has shown that KSHV ORFs that show both TEAD reporter activation and antagonization of PML components (ORF75, K10.5) do not require PML to drive TEAD activation; TEAD activity remained high in PML knockout cell lines, suggesting that PML antagonization is not required for TEAD activity. Careful dissection of both Hippo pathway and PML body components in the context of viral gene expression is an important next step.

4.5.2 ORF21 and ORF71

Both ORF21 and ORF71 showed consisten reduction in TEAD luciferase reporter activity under serum starvation conditions. ORF21 is a viral homolog of a thymidine kinase, and is likely a tegument protein associated with mature virions(Sathish et al.,

2012). More recently, activity of ORF21 has been linked to alterations in cell morphology. In a manner dependent on RhoA activation of myosin II, ORF21 initiates actin stress fiber formation and cell contraction (Gill et al., 2015). Considering actin stress fiber formation is a potent activator of Hippo pathway signaling, it is not obvious how ORF21 may be decreasing activity of this reporter past serum-starvation levels. It is likely that following cell contraction and rounding caused by ORF21 expression, cells that have lost solid-state support now have a highly active Hippo signaling pathway, further reducing the amount of nuclear YAP1 even in the context of serum starvation. How this function of ORF21 may play into lytic reactivation is not clear, but as a lytic protein, it may be contributing to the increase in phosphorylated YAP1 observed during late lytic reactivation (Figure 3).

On the other hand, ORF71 – also known as vFLIP – is a latently expressed protein, briefly described in introduction Section 1.2.2. vFLIP has several cellular functions, including induction of anti-apoptotic signaling, and potent activation of the NFkB pathway. It is unclear how vFLIP may be reducing TEAD-reporter activity, and further testing of a potential overlap in NFkB and Hippo pathway activation is needed to further understand this phenotype.

4.5.3 YAP1-independent activation of TEAD promoter

A limitation of the TEAD-based luciferase reporter used for the ORF screen is that it is unclear whether the activation of the TEAD reporter is happening in a YAP1-dependent manner. It is a fair assumption that use of this reporter signifies increased levels of nuclear YAP1, as YAP1 (and its paralog TAZ) are the primary activators of

TEAD transcription factors. Considering viral genes have not been studied in the context of Hippo signaling, we decided to test if the activation seen was YAP1-dependent. As expected, the majority of ORFs tested had heavily reduced activity in the YAP1-silenced cells tests (Figure 13) but three maintained at least 50% activity in reduced YAP1 conditions; K10, K10.5, and ORF17.5. Both K10 and K10.5 are viral homologs of interferon regulatory factors (vIRFs), termed vIRF4 and vIRF3 respectively. As transcription factors, it is not completely surprising that these proteins have the potential to drive expression of this reporter. Direct activation of promoters containing TEAD activation domains by vIRFs has not been reported, and further experimentation is required to fully understand the phenomenon driving this YAP1-independent TEAD activation in this way. Understanding if expression of these vIRFs increase downstream TEAD-driven genes via quantitative PCR would allow for further understanding if this phenotype is an artifact of screening conditions. More surprising, is the YAP1-independent activity of ORF17.5, a structural protein required for assembly of virions. ORF17.5 has not been linked to the activity of any signaling pathway, and viral structural proteins often do not have functional roles in modulating host effectors. More in-depth validation of K10, K10.5 and ORF17.5 for their ability to drive activation of the TEAD reporter in both wild-type and YAP1-silenced contexts is required to fully understand the nature of activation, and potentially uncover a non-canonical mechanism of TEAD activation.

4.5.4 Limitations of screening methodology

The small number of very highly active ORFs suggests a good specificity for this assay, but there are several technical challenges and considerations to be made. The set-up of this screen is biased in that TEAD-activators will be more easily detected; by starving cells for 12 hours prior to harvest for luciferase readout, we are testing the ability of ORFs to bypass this strong signal for Hippo pathway activation, and reduction in nuclear YAP1. In cultured cells, the Hippo pathway is not often active; YAP1 is nuclear, driving continued growth and cell proliferation. Because of this, activity of any ORF at promoting nuclear YAP1 would not be seen due to high levels of basal activity. To surpass this, cells were starved of serum before readout, reducing basal levels quite extensively, allowing for the identification of ORFs able to bypass pathway activation induced by starvation. In this context, the ability of both ORF21 and ORF71 to highly reduce activity at the TEAD reporter past basal levels is striking, and suggests that mechanical cues are a more potent regulator of the Hippo pathway than nutrient deprivation.

The use of CMV-Renilla as a transfection control came with a major assumption; that KSHV ORFs would have no effect on translation of this reporter construct. For the purpose of this screen, this assumption allows us to select a certain number of interesting ORFs to more carefully dissect their function at regulating Hippo pathway dynamics. For the most part, most viral ORFs did not markedly change levels of CMV-Renilla expression, but some variability was observed.

4.5.5 Further experimentation

Secondary validation of these potential screen hits is required to be fully confident in their function at modulating Hippo pathway signaling. Utilizing immunofluorescence to detect the nuclear or cytoplasmic localization of YAP1 in the presence of these viral genes, as well as immunoblotting on cells expressing these genes to understand the levels and phosphorylation of several key Hippo pathway components is a necessary next step. To test for negative regulators of YAP1 activation, screening conditions in full serum would potentially indicate if a viral ORF is able to reduce activity of the highly active YAP1; further testing of ORF21 and ORF71 in this way is pertinent for understanding how potent these viral genes are. Similarly, utilizing a non-starved control well to compare levels of highly active YAP1 to ORFs able to drive activation of the TEAD reporter would give great insight into the functional limits of the reporter assay, and understand the magnitude of activation driven by these viral genes.

4.6 Conclusions

It is abundantly clear, through data presented in this thesis and the current understanding in the published literature, that the Hippo pathway plays a key role in KSHV infection dynamics. Reduction of core Hippo effector YAP1 in two very different latent models of KSHV infection drives a more robust reactivation of the lytic program, and inducing high nuclear activity of YAP1 through regulation of its core kinase abolishes late gene production. Not only is modulation of this pathway effective at changing KSHV reactivation dynamics, but several latent and lytic proteins show potent control over activation of TEAD-driven genes; in both a YAP1-dependent and

independent manner. The functional significance of many of these interactions have yet to be elucidated, but the work thus far suggests that this ubiquitous signaling pathway may prove to be an important piece in understanding the complexity of KSHV replication mechanisms.

REFERENCES

Adams, M.J., and Carstens, E.B. (2012). Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2012). Arch. Virol. *157*, 1411–1422.

Adang, L.A., Parsons, C.H., and Kedes, D.H. (2006). Asynchronous Progression through the Lytic Cascade and Variations in Intracellular Viral Loads Revealed by High-Throughput Single-Cell Analysis of Kaposi's Sarcoma-Associated Herpesvirus Infection. J. Virol. *80*, 10073–10082.

Alzahrani, F., Clattenburg, L., Muruganandan, S., Bullock, M., MacIsaac, K., Wigerius, M., Williams, B.A., Graham, M.E.R., Rigby, M.H., Trites, J.R.B., et al. (2017). The Hippo component YAP localizes in the nucleus of human papilloma virus positive oropharyngeal squamous cell carcinoma. J. Otolaryngol. - Head Neck Surg. *46*.

Andreoni, M., Sarmati, L., Nicastri, E., El Sawaf, G., El Zalabani, M., Uccella, I., Bugarini, R., Parisi, S.G., and Rezza, G. (2002). Primary human herpesvirus 8 infection in immunocompetent children. JAMA *287*, 1295–1300.

Bais, C., Van Geelen, A., Eroles, P., Mutlu, A., Chiozzini, C., Dias, S., Silverstein, R.L., Rafii, S., and Mesri, E.A. (2003). Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/ KDR. Cancer Cell *3*, 131–143.

Bellare, P., and Ganem, D. (2009). Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. Cell Host Microbe *6*, 570.

Blair, S.S. (2003). Genetic mosaic techniques for studying Drosophila development. Development *130*, 5065–5072.

Borah, S., Darricarrère, N., Darnell, A., Myoung, J., and Steitz, J.A. (2011). A Viral Nuclear Noncoding RNA Binds Re-localized Poly(A) Binding Protein and Is Required for Late KSHV Gene Expression. PLoS Pathog. *7*.

Bowser, B.S., DeWire, S.M., and Damania, B. (2002). Transcriptional Regulation of the K1 Gene Product of Kaposi's Sarcoma-Associated Herpesvirus. J. Virol. *76*, 12574–12583.

Cavallin, L.E., Goldschmidt-Clermont, P., and Mesri, E.A. (2014). Molecular and Cellular Mechanisms of KSHV Oncogenesis of Kaposi's Sarcoma Associated with HIV/AIDS. PLoS Pathog. *10*.

Chan, S.W., Lim, C.J., Guo, K., Ng, C.P., Lee, I., Hunziker, W., Zeng, Q., and Hong, W. (2008). A Role for TAZ in Migration, Invasion, and Tumorigenesis of Breast Cancer Cells. Cancer Res. *68*, 2592–2598.

Chang, H.H., and Ganem, D. (2013). A unique herpesviral transcriptional program in KSHV-infected lymphatic endothelial cells leads to mTORC1 activation and rapamycin sensitivity. Cell Host Microbe *13*, 429–440.

Chang, H., Wachtman, L.M., Pearson, C.B., Lee, J.-S., Lee, H.-R., Lee, S.H., Vieira, J., Mansfield, K.G., and Jung, J.U. (2009). Non-Human Primate Model of Kaposi's Sarcoma-Associated Herpesvirus Infection. PLOS Pathog. *5*, e1000606.

Chang, P.-C., Izumiya, Y., Wu, C.-Y., Fitzgerald, L.D., Campbell, M., Ellison, T.J., Lam, K.S., Luciw, P.A., and Kung, H.-J. (2010). Kaposi's Sarcoma-associated Herpesvirus (KSHV) Encodes a SUMO E3 ligase That Is SIM-dependent and SUMO-2/3-specific. J. Biol. Chem. *285*, 5266–5273.

Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M., and Moore, P.S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science *266*, 1865–1869.

Chen, W., Hilton, I.B., Staudt, M.R., Burd, C.E., and Dittmer, D.P. (2010). Distinct p53, p53:LANA, and LANA Complexes in Kaposi's Sarcoma-Associated Herpesvirus Lymphomas. J. Virol. *84*, 3898–3908.

Codelia, V.A., Sun, G., and Irvine, K.D. (2014). Regulation of YAP by Mechanical Strain through Jnk and Hippo Signaling. Curr. Biol. *24*, 2012–2017.

Cordenonsi, M., Zanconato, F., Azzolin, L., Forcato, M., Rosato, A., Frasson, C., Inui, M., Montagner, M., Parenti, A.R., Poletti, A., et al. (2011). The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. Cell *147*, 759–772.

Curtiss, P., Strazzulla, L.C., and Friedman-Kien, A.E. (2016). An Update on Kaposi's Sarcoma: Epidemiology, Pathogenesis and Treatment. Dermatol. Ther. *6*, 465–470.

DeRan, M., Yang, J., Shen, C.-H., Peters, E.C., Fitamant, J., Chan, P., Hsieh, M., Zhu, S., Asara, J.M., Zheng, B., et al. (2014). Energy Stress Regulates Hippo-YAP Signaling Involving AMPK-Mediated Regulation of Angiomotin-like 1 Protein. Cell Rep. *9*, 495–503.

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., Zanconato, F., Le Digabel, J., Forcato, M., Bicciato, S., et al. (2011). Role of YAP/TAZ in mechanotransduction. Nature *474*, 179–183.

Everett, R.D. (2001). DNA viruses and viral proteins that interact with PML nuclear bodies. Oncogene *20*, 7266–7273.

Friborg, J., Kong, W., Hottiger, M.O., and Nabel, G.J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. Nature *402*, 889–894.

Full, F., Jungnickl, D., Reuter, N., Bogner, E., Brulois, K., Scholz, B., Stürzl, M., Myoung, J., Jung, J.U., Stamminger, T., et al. (2014). Kaposi's Sarcoma Associated Herpesvirus Tegument Protein ORF75 Is Essential for Viral Lytic Replication and Plays a Critical Role in the Antagonization of ND10-Instituted Intrinsic Immunity. PLOS Pathog. *10*, e1003863.

Gallo, A., Lampe, M., Günther, T., and Brune, W. (2017). The Viral Bcl-2 Homologs of Kaposi's Sarcoma-Associated Herpesvirus and Rhesus Rhadinovirus Share an Essential Role for Viral Replication. J. Virol. *91*.

Gao, C., Ho, C.-C., Reineke, E., Lam, M., Cheng, X., Stanya, K.J., Liu, Y., Chakraborty, S., Shih, H.-M., and Kao, H.-Y. (2008). Histone Deacetylase 7 Promotes PML Sumoylation and Is Essential for PML Nuclear Body Formation. Mol. Cell. Biol. *28*, 5658–5667.

Gill, M.B., Turner, R., Stevenson, P.G., and Way, M. (2015). KSHV-TK is a tyrosine kinase that disrupts focal adhesions and induces Rho-mediated cell contraction. EMBO J. *34*, 448–465.

Gregory, S.M., West, J.A., Dillon, P.J., Hilscher, C., Dittmer, D.P., and Damania, B. (2009). Toll-like receptor signaling controls reactivation of KSHV from latency. Proc. Natl. Acad. Sci. U. S. A. *106*, 11725–11730.

Guito, J., and Lukac, D.M. (2012). KSHV Rta Promoter Specification and Viral Reactivation. Front. Microbiol. *3*.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. (2006). The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat. Cell Biol. *8*, 27–36.

Hao, Y., Chun, A., Cheung, K., Rashidi, B., and Yang, X. (2008). Tumor suppressor LATS1 is a negative regulator of oncogene YAP. J. Biol. Chem. *283*, 5496–5509.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457–467.

Harvey, K.F., Zhang, X., and Thomas, D.M. (2013). The Hippo pathway and human cancer. Nat. Rev. Cancer *13*, 246–257.

Hassman, L.M., Ellison, T.J., and Kedes, D.H. (2011). KSHV infects a subset of human tonsillar B cells, driving proliferation and plasmablast differentiation. J. Clin. Invest. *121*, 752–768.

He, C., Mao, D., Hua, G., Lv, X., Chen, X., Angeletti, P.C., Dong, J., Remmenga, S.W., Rodabaugh, K.J., Zhou, J., et al. (2015). The Hippo/YAP pathway interacts with EGFR signaling and HPV oncoproteins to regulate cervical cancer progression. EMBO Mol. Med. *7*, 1426–1449.

Hu, B., Xie, S., Hu, Y., Chen, W., Chen, X., Zheng, Y., and Wu, X. (2017). Hepatitis C virus NS4B protein induces epithelial-mesenchymal transition by upregulation of Snail. Virol. J. *14*, 83.

Izumiya, Y., Kobayashi, K., Kim, K.Y., Pochampalli, M., Izumiya, C., Shevchenko, B., Wang, D.-H., Huerta, S.B., Martinez, A., Campbell, M., et al. (2013). Kaposi's Sarcoma-Associated Herpesvirus K-Rta Exhibits SUMO-Targeting Ubiquitin Ligase (STUbL) Like Activity and Is Essential for Viral Reactivation. PLOS Pathog. *9*, e1003506.

Jensen, K.K., Manfra, D.J., Grisotto, M.G., Martin, A.P., Vassileva, G., Kelley, K., Schwartz, T.W., and Lira, S.A. (2005). The human herpes virus 8-encoded chemokine receptor is required for angioproliferation in a murine model of Kaposi's sarcoma. J. Immunol. Baltim. Md 1950 *174*, 3686–3694.

Jiao, S., Li, C., Hao, Q., Miao, H., Zhang, L., Li, L., and Zhou, Z. (2017). VGLL4 targets a TCF4–TEAD4 complex to coregulate Wnt and Hippo signalling in colorectal cancer. Nat. Commun. *8*.

Justice, R.W., Zilian, O., Woods, D.F., Noll, M., and Bryant, P.J. (1995). The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. *9*, 534–546.

Kanno, T., Sato, Y., Sata, T., and Katano, H. (2006). Expression of Kaposi's sarcoma-associated herpesvirus-encoded K10/10.1 protein in tissues and its interaction with poly(A)-binding protein. Virology *352*, 100–109.

Kati, S., Tsao, E.H., Günther, T., Weidner-Glunde, M., Rothämel, T., Grundhoff, A., Kellam, P., and Schulz, T.F. (2013). Activation of the B Cell Antigen Receptor Triggers Reactivation of Latent Kaposi's Sarcoma-Associated Herpesvirus in B Cells. J. Virol. *87*, 8004–8016.

Kim, M., Kim, T., Johnson, R.L., and Lim, D.-S. (2015). Transcriptional Co-repressor Function of the Hippo Pathway Transducers YAP and TAZ. Cell Rep. *11*, 270–282.

Krishnan, H.H., Sharma-Walia, N., Zeng, L., Gao, S.-J., and Chandran, B. (2005). Envelope Glycoprotein gB of Kaposi's Sarcoma-Associated Herpesvirus Is Essential for Egress from Infected Cells. J. Virol. *79*, 10952–10967.

Lagos, D., and Boshoff, C. (2007). Immunobiology and host response to KSHV infection. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, A. Arvin, G. Campadelli-Fiume, E. Mocarski, P.S. Moore, B. Roizman, R. Whitley, and K. Yamanishi, eds. (Cambridge: Cambridge University Press), p.

Lan, K., Kuppers, D.A., Verma, S.C., and Robertson, E.S. (2004). Kaposi's Sarcoma-Associated Herpesvirus-Encoded Latency-Associated Nuclear Antigen Inhibits Lytic Replication by Targeting Rta: a Potential Mechanism for Virus-Mediated Control of Latency. J. Virol. *78*, 6585–6594.

Lan, K., Kuppers, D.A., Verma, S.C., Sharma, N., Murakami, M., and Robertson, E.S. (2005). Induction of Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen by the Lytic Transactivator RTA: a Novel Mechanism for Establishment of Latency. J. Virol. *79*, 7453–7465.

Lapi, E., Di Agostino, S., Donzelli, S., Gal, H., Domany, E., Rechavi, G., Pandolfi, P.P., Givol, D., Strano, S., Lu, X., et al. (2008). PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop. Mol. Cell *32*, 803–814.

Lee, H., Guo, J., Li, M., Choi, J.-K., DeMaria, M., Rosenzweig, M., and Jung, J.U. (1998a). Identification of an Immunoreceptor Tyrosine-Based Activation Motif of K1 Transforming Protein of Kaposi's Sarcoma-Associated Herpesvirus. Mol. Cell. Biol. *18*, 5219–5228.

Lee, H., Veazey, R., Williams, K., Li, M., Guo, J., Neipel, F., Fleckenstein, B., Lackner, A., Desrosiers, R.C., and Jung, J.U. (1998b). Deregulation of cell growth by the K1 gene of Kaposi's sarcoma-associated herpesvirus. Nat. Med. *4*, 435–440.

Leidal, A.M., Cyr, D.P., Hill, R.J., Lee, P.W.K., and McCormick, C. (2012). Subversion of autophagy by Kaposi's sarcoma-associated herpesvirus impairs oncogene-induced senescence. Cell Host Microbe *11*, 167–180.

Li, Q., Zhou, F., Ye, F., and Gao, S.-J. (2008). Genetic disruption of KSHV major latent nuclear antigen LANA enhances viral lytic transcriptional program. Virology *379*, 234–244.

Li, W., Wang, L., Katoh, H., Liu, R., Zheng, P., and Liu, Y. (2011). Identification of a Tumor Suppressor Relay between the FOXP3 and the Hippo Pathways in Breast and Prostate Cancers. Cancer Res. *71*, 2162–2171.

Liang, Y., and Ganem, D. (2003). Lytic but not latent infection by Kaposi's sarcoma-associated herpesvirus requires host CSL protein, the mediator of Notch signaling. Proc. Natl. Acad. Sci. U. S. A. *100*, 8490–8495.

Liu, G., Yu, F.-X., Kim, Y.C., Meng, Z., Naipauer, J., Looney, D.J., Liu, X., Gutkind, J.S., Mesri, E.A., and Guan, K.-L. (2015). Kaposi sarcoma-associated herpesvirus promotes tumorigenesis by modulating the Hippo pathway. Oncogene *34*, 3536–3546.

Liu, J., Martin, H.J., Liao, G., and Hayward, S.D. (2007). The Kaposi's Sarcoma-Associated Herpesvirus LANA Protein Stabilizes and Activates c-Myc. J. Virol. *81*, 10451–10459.

Lu, F., Zhou, J., Wiedmer, A., Madden, K., Yuan, Y., and Lieberman, P.M. (2003). Chromatin Remodeling of the Kaposi's Sarcoma-Associated Herpesvirus ORF50 Promoter Correlates with Reactivation from Latency. J. Virol. *77*, 11425–11435.

Lu, F., Day, L., Gao, S.-J., and Lieberman, P.M. (2006). Acetylation of the latency-associated nuclear antigen regulates repression of Kaposi's sarcoma-associated herpesvirus lytic transcription. J. Virol. *80*, 5273–5282.

Lukac, D.M., and Yuan, Y. (2007). Reactivation and lytic replication of KSHV. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, A. Arvin, G. Campadelli-Fiume, E. Mocarski, P.S. Moore, B. Roizman, R. Whitley, and K. Yamanishi, eds. (Cambridge: Cambridge University Press), p.

Ma, B., Chen, Y., Chen, L., Cheng, H., Mu, C., Li, J., Gao, R., Zhou, C., Cao, L., Liu, J., et al. (2015). Hypoxia regulates Hippo signalling through the SIAH2 ubiquitin E3 ligase. Nat. Cell Biol. *17*, 95–103.

Majerciak, V., and Zheng, Z.-M. (2015). KSHV ORF57, a protein of many faces. Viruses *7*, 604–633.

Marcos-Villar, L., Lopitz-Otsoa, F., Gallego, P., Muñoz-Fontela, C., González-Santamaría, J., Campagna, M., Shou-Jiang, G., Rodriguez, M.S., and Rivas, C. (2009). Kaposi's sarcoma-associated herpesvirus protein LANA2 disrupts PML oncogenic domains and inhibits PML-mediated transcriptional repression of the survivin gene. J. Virol. *83*, 8849–8858.

Meng, Z., Moroishi, T., and Guan, K.-L. (2016). Mechanisms of Hippo pathway regulation. Genes Dev. *30*, 1–17.

Mesri, E.A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. Nat. Rev. Cancer *10*, 707–719.

Mo, J.-S., Meng, Z., Kim, Y.C., Park, H.W., Hansen, C.G., Kim, S., Lim, D.-S., and Guan, K.-L. (2015). Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. Nat. Cell Biol. *17*, 500–510.

Montaner, S., Sodhi, A., Molinolo, A., Bugge, T.H., Sawai, E.T., He, Y., Li, Y., Ray, P.E., and Gutkind, J.S. (2003). Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. Cancer Cell *3*, 23–36.

Montaner, S., Sodhi, A., Ramsdell, A.K., Martin, D., Hu, J., Sawai, E.T., and Gutkind, J.S. (2006). The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor as a therapeutic target for the treatment of Kaposi's sarcoma. Cancer Res. *66*, 168–174.

Myoung, J., and Ganem, D. (2011). Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: maintenance of tight latency with efficient reactivation upon induction. J. Virol. Methods *174*, 12–21.

Nakagawa, S., and Huibregtse, J.M. (2000). Human Scribble (Vartul) Is Targeted for Ubiquitin-Mediated Degradation by the High-Risk Papillomavirus E6 Proteins and the E6AP Ubiquitin-Protein Ligase. Mol. Cell. Biol. *20*, 8244–8253.

Odumade, O.A., Hogquist, K.A., and Balfour, H.H. (2011). Progress and Problems in Understanding and Managing Primary Epstein-Barr Virus Infections. Clin. Microbiol. Rev. *24*, 193–209.

Parravicini, C., Chandran, B., Corbellino, M., Berti, E., Paulli, M., Moore, P.S., and Chang, Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Am. J. Pathol. *156*, 743–749.

Parravinci, C., Corbellino, M., Paulli, M., Magrini, U., Lazzarino, M., Moore, P.S., and Chang, Y. (1997). Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. Am. J. Pathol. *151*, 1517–1522.

Picchio, G.R., Sabbe, R.E., Gulizia, R.J., McGrath, M., Herndier, B.G., and Mosier, D.E. (1997). The KSHV/HHV8-infected BCBL-1 lymphoma line causes tumors in SCID mice but fails to transmit virus to a human peripheral blood mononuclear cell graft. Virology *238*, 22–29.

Prakash, O., Tang, Z.-Y., Peng, X., Coleman, R., Gill, J., Farr, G., and Samaniego, F. (2002). Tumorigenesis and aberrant signaling in transgenic mice expressing the human herpesvirus-8 K1 gene. J. Natl. Cancer Inst. *94*, 926–935.

Purushothaman, P., Uppal, T., and Verma, S.C. (2015). Molecular Biology of KSHV Lytic Reactivation. Viruses *7*, 116–153.

Purushothaman, P., Dabral, P., Gupta, N., Sarkar, R., and Verma, S.C. (2016). KSHV Genome Replication and Maintenance. Front. Microbiol. *7*.

Radkov, S.A., Kellam, P., and Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma–E2F pathway and with the oncogene Hras transforms primary rat cells. Nat. Med. *6*, 1121–1127.

Roizman, B., and Furlong, D. (1974). The Replication of Herpesviruses. In Reproduction, H. Fraenkel-Conrat, and R.R. Wagner, eds. (Springer US), pp. 229–403.

Rouleau, C., Pores Fernando, A.T., Hwang, J.H., Faure, N., Jiang, T., White, E.A., Roberts, T.M., and Schaffhausen, B.S. (2016). Transformation by Polyomavirus Middle T Antigen Involves a Unique Bimodal Interaction with the Hippo Effector YAP. J. Virol. *90*, 7032–7045.

Rouleau, G.A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., et al. (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. Nature *363*, 515–521.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496–1501.

Sathish, N., Wang, X., and Yuan, Y. (2012). Tegument Proteins of Kaposi's Sarcoma-Associated Herpesvirus and Related Gamma-Herpesviruses. Front. Microbiol. *3*.

Seidel, C., Schagdarsurengin, U., Blümke, K., Würl, P., Pfeifer, G.P., Hauptmann, S., Taubert, H., and Dammann, R. (2007). Frequent hypermethylation of MST1 and MST2 in soft tissue sarcoma. Mol. Carcinog. *46*, 865–871.

Shin, H.J., DeCotiis, J., Giron, M., Palmeri, D., and Lukac, D.M. (2014). Histone Deacetylase Classes I and II Regulate Kaposi's Sarcoma-Associated Herpesvirus Reactivation. J. Virol. *88*, 1281–1292.

Silvis, M.R., Kreger, B.T., Lien, W.-H., Klezovitch, O., Rudakova, G.M., Camargo, F.D., Lantz, D.M., Seykora, J.T., and Vasioukhin, V. (2011). α-Catenin Is a Tumor Suppressor That Controls Cell Accumulation by Regulating the Localization and Activity of the Transcriptional Coactivator Yap1. Sci Signal *4*, ra33-ra33.

Sternbach, G., and Varon, J. (1995). Moritz Kaposi: idiopathic pigmented sarcoma of the skin. J. Emerg. Med. *13*, 671–674.

Stevens, J.G. (1989). Human herpesviruses: a consideration of the latent state. Microbiol. Rev. *53*, 318–332.

Takahashi, Y., Miyoshi, Y., Takahata, C., Irahara, N., Taguchi, T., Tamaki, Y., and Noguchi, S. (2005). Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *11*, 1380–1385.

Thakker, S., and Verma, S.C. (2016). Co-infections and Pathogenesis of KSHV-Associated Malignancies. Front. Microbiol. *7*.

Toth, Z., Maglinte, D.T., Lee, S.H., Lee, H.-R., Wong, L.-Y., Brulois, K.F., Lee, S., Buckley, J.D., Laird, P.W., Marquez, V.E., et al. (2010). Epigenetic Analysis of KSHV Latent and Lytic Genomes. PLoS Pathog. *6*.

Trus, B.L., Heymann, J.B., Nealon, K., Cheng, N., Newcomb, W.W., Brown, J.C., Kedes, D.H., and Steven, A.C. (2001). Capsid Structure of Kaposi's Sarcoma-Associated Herpesvirus, a Gammaherpesvirus, Compared to Those of an Alphaherpesvirus, Herpes Simplex Virus Type 1, and a Betaherpesvirus, Cytomegalovirus. J. Virol. *75*, 2879–2890.

Van Dross, R., Yao, S., Asad, S., Westlake, G., Mays, D.J., Barquero, L., Duell, S., Pietenpol, J.A., and Browning, P.J. (2005). Constitutively active K-cyclin/cdk6 kinase in Kaposi sarcoma-associated herpesvirus-infected cells. J. Natl. Cancer Inst. *97*, 656–666.

Vieira, J., and O'Hearn, P.M. (2004). Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. Virology *325*, 225–240.

Wang, L., Wakisaka, N., Tomlinson, C.C., DeWire, S.M., Krall, S., Pagano, J.S., and Damania, B. (2004). The Kaposi's Sarcoma-Associated Herpesvirus (KSHV/HHV-8) K1 Protein Induces Expression of Angiogenic and Invasion Factors. Cancer Res. *64*, 2774–2781.

Wang, S., Xie, F., Chu, F., Zhang, Z., Yang, B., Dai, T., Gao, L., Wang, L., Ling, L., Jia, J., et al. (2017). YAP antagonizes innate antiviral immunity and is targeted for lysosomal degradation through IKKε-mediated phosphorylation. Nat. Immunol.

Wang, S.E., Wu, F.Y., Yu, Y., and Hayward, G.S. (2003). CCAAT/enhancer-binding protein-alpha is induced during the early stages of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic cycle reactivation and together with the KSHV replication and transcription activator (RTA) cooperatively stimulates the viral RTA, MTA, and PAN promoters. J. Virol. *77*, 9590–9612.

Wang, W.-H., Kuo, C.-W., Chang, L.-K., Hung, C.-C., Chang, T.-H., and Liu, S.-T. (2015). Assembly of Epstein-Barr Virus Capsid in Promyelocytic Leukemia Nuclear Bodies. J. Virol. *89*, 8922–8931.

Wen, K.W., and Damania, B. (2010). Kaposi Sarcoma-associated Herpesvirus (KSHV): Molecular Biology and Oncogenesis. Cancer Lett. *289*, 140–150.

Whitley, R.J. (1996). Herpesviruses. In Medical Microbiology, S. Baron, ed. (Galveston (TX): University of Texas Medical Branch at Galveston), p.

Wilkinson, K.A., and Henley, J.M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. Biochem. J. *428*, 133–145.

Wu, F.Y., Ahn, J.-H., Alcendor, D.J., Jang, W.-J., Xiao, J., Hayward, S.D., and Hayward, G.S. (2001). Origin-Independent Assembly of Kaposi's Sarcoma-Associated Herpesvirus DNA Replication Compartments in Transient Cotransfection Assays and Association with the ORF-K8 Protein and Cellular PML. J. Virol. *75*, 1487–1506.

Wu, S., Huang, J., Dong, J., and Pan, D. (2003). hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell *114*, 445–456.

Wu, Y., Zhang, J., Zhang, H., and Zhai, Y. (2016). Hepatitis B virus X protein mediates yes-associated protein 1 upregulation in hepatocellular carcinoma. Oncol. Lett. *12*, 1971–1974.

Xia, H., Qi, H., Li, Y., Pei, J., Barton, J., Blackstad, M., Xu, T., and Tao, W. (2002). LATS1 tumor suppressor regulates G2/M transition and apoptosis. Oncogene *21*, 1233–1241.

Xu, M.Z., Yao, T.-J., Lee, N.P.Y., Ng, I.O.L., Chan, Y.-T., Zender, L., Lowe, S.W., Poon, R.T.P., and Luk, J.M. (2009). Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. Cancer *115*, 4576–4585.

Xu, T., Wang, W., Zhang, S., Stewart, R.A., and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Dev. Camb. Engl. *121*, 1053–1063.

Ye, F., Zhou, F., Bedolla, R.G., Jones, T., Lei, X., Kang, T., Guadalupe, M., and Gao, S.-J. (2011). Reactive oxygen species hydrogen peroxide mediates Kaposi's sarcoma-associated herpesvirus reactivation from latency. PLoS Pathog. *7*, e1002054.

Yu, F.-X., Luo, J., Mo, J.-S., Liu, G., Kim, Y.C., Meng, Z., Zhao, L., Peyman, G., Ouyang, H., Jiang, W., et al. (2014). Mutant Gq/11 Promote Uveal Melanoma Tumorigenesis by Activating YAP. Cancer Cell *25*, 822–830.

Zhang, J., Smolen, G.A., and Haber, D.A. (2008). Negative regulation of YAP by LATS1 underscores evolutionary conservation of the Drosophila Hippo pathway. Cancer Res. *68*, 2789–2794.

Zhang, Q., Meng, F., Chen, S., Plouffe, S.W., Wu, S., Liu, S., Li, X., Zhou, R., Wang, J., Zhao, B., et al. (2017). Hippo signalling governs cytosolic nucleic acid sensing through YAP/TAZ-mediated TBK1 blockade. Nat. Cell Biol. *19*, 362–374.

Zhang, T., Zhang, J., You, X., Liu, Q., Du, Y., Gao, Y., Shan, C., Kong, G., Wang, Y., Yang, X., et al. (2012). Hepatitis B virus X protein modulates oncogene Yes-associated protein by CREB to promote growth of hepatoma cells. Hepatol. Baltim. Md *56*, 2051–2059.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. *21*, 2747–2761.

Zhao, B., Ye, X., Yu, J., Li, L., Li, W., Li, S., Yu, J., Lin, J.D., Wang, C.-Y., Chinnaiyan, A.M., et al. (2008). TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. *22*, 1962–1971.

Zhao, B., Li, L., Lei, Q., and Guan, K.-L. (2010). The Hippo–YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev. *24*, 862–874.

Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y., and Ren, J. (2014). GPS-SUMO: a tool for the prediction of sumoylation sites and SUMO-interaction motifs. Nucleic Acids Res. *42*, W325–W330.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc. Natl. Acad. Sci. *93*, 6641–6646.

Zhu, G., Chen, Y., Zhang, X., Wu, Q., Zhao, Y., Chen, Y., Sun, F., Qiao, Y., and Wang, J. (2017). 12-O-Tetradecanoylphorbol-13-acetate (TPA) is anti-tumorigenic in liver cancer cells via inhibiting YAP through AMOT. Sci. Rep. *7*.

Zhu, X., Guo, Y., Yao, S., Yan, Q., Xue, M., Hao, T., Zhou, F., Zhu, J., Qin, D., and Lu, C. (2014). Synergy between Kaposi's sarcoma-associated herpesvirus (KSHV) vIL-6 and HIV-1 Nef protein in promotion of angiogenesis and oncogenesis: role of the AKT signaling pathway. Oncogene *33*, 1986–1996.