UC Berkeley
UC Berkeley Electronic Theses and Dissertations

**Title**
Characterization of receptor use and entry mechanisms in two KSHV infection systems

**Permalink**
https://escholarship.org/uc/item/96g8q5mk

**Author**
TerBush, Allison Alwan

**Publication Date**
2018

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

# Characterization of receptor use and entry mechanisms in two KSHV infection systems

by

Allison Alwan TerBush

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Laurent Coscoy, Chair
Professor Britt Glaunsinger
Professor Eva Harris
Professor Ellen Robey

Spring 2018

Abstract

Characterization of receptor use and entry mechanisms in two KSHV infection systems

by

Allison Alwan TerBush

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Dr. Laurent Coscoy, Chair

Viruses initiate infection at the cell surface, where they use viral proteins to contact and manipulate naturally occurring host receptors in the plasma membrane. Through this interaction, viruses negotiate internalization and begin their infection cycle. These virus-receptor interactions can be surprisingly complex, sometimes coordinating many receptors using several viral proteins simultaneously. Cytoskeletal rearrangements, a multitude of intracellular signaling cascades, and even transcriptional changes can be triggered through the host receptors by this initial interaction and influence the outcome of the attempted infection. Thus, viral entry is a nuanced process evolved to ensure that viruses can infect the right cells at the right time, while successfully evading host defenses.

Kaposi's Sarcoma-Associated Herpesvirus (KSHV) is an important human pathogen. It is the causative agent of several cancers and inflammatory disease which together, in the context of the global HIV epidemic, are a major public health burden. KSHV is the most recent of the human herpesviruses to be discovered, but research on KSHV entry mechanisms has almost a twenty-year history. Eight receptors for KSHV have been described, and it has become apparent that the step-by-step details of KSHV entry mechanisms are likely to be unique in every cell line. By interacting with the same set of receptors on human foreskin fibroblasts or primary microvascular endothelial cells, for example, the virion is internalized by clathrin-mediated endocytosis or clathrin-independent macropinocytosis, respectively.

Here we investigated KSHV receptor usage in cell types that are relatively understudied in the field: epithelial cells and lymphocytes. We uncovered novel variability in receptor use across many susceptible cell lines, particularly that infection of epithelial cells and lymphocytes was independent of known KSHV integrin receptors and likely all known integrins. Additionally, we found that infection of Caki-1 and HeLa cells did not require EphA2 signaling, and infection of primary oral keratinocytes did not depend on Eph receptor interactions whatsoever. We hypothesize that there is at least one more KSHV receptor required for infection in the epithelial cells we studied.

Furthermore, we showed that coculture-mediated infection of BJAB cells required heparan sulfate and Eph receptor interactions, despite the fact that BJAB cells do not express heparan sulfate and manipulation of Eph receptor expression did not affect infection. These results are evocative of a "transfer infection" mechanism akin to Epstein-Barr Virus, which requires receptor interactions on adjacent cells to promote infection of an otherwise non-susceptible cell type. We identified KSHV orf28 as a potential player in determining lymphocyte tropism.

Our work reveals another layer of complexity beyond receptor availability on cells. It is now clear that even when KSHV receptors are expressed by a cell, additional contextual factors determine whether they play a role during infection. Going forward, this will be very important to understand, especially since virus-receptor interactions are often targeted by small molecules or biologics in the hopes of slowing viral dissemination.

Dedication

Dedicated to my parents, Joe and Mary Ann Alwan. You laid a strong foundation which has withstood the test of a PhD.

To my dear sister, Grace Alwan. You are truly an inspirational paragon of hard work and kindness.

And to my loving husband, Ryan TerBush. I can't thank you enough for your unconditional love and encouragement. I couldn't have done this without you.

Acknowldegements

First, I would like to thank my committee Britt Glaunsinger, Ellen Robey, Eva Harris, and especially my advisor Laurent Coscoy. You presided over the most transformative years of my life and helped guide my transition from a fledgling new college graduate to a confident scientist. The way I look at the world and approach problems has been shaped by my graduate training for the better.

Thank you to my lab mates, past and present: Elena Berkerman, Andrew Birnberg, Trever Greene, Kristi Geiger, Emilie Gios, Florianne Hafkamp, Hee Jun Lee, and Valerie Vargas-Zapata. You each brought a unique brand of enthusiasm and passion to lab and to life, and your friendships mean so much to me.

I must additionally thank Hector Nolla and Alma Valeros from the Flow Cytometry Core. My work wouldn't have been possible without you both. Thank you to the IGI for helping us get additional CRISPR methods off the ground in our lab. And finally, thank you to the members of the Glaunsinger Lab for countless hours of entertaining and illuminating lab meeting discussion.

## Table of Contents

**Chapter 1 - KSHV Receptors and Entry: Background and History ..... 1**
**1.1 KSHV Basics and History ..... 2**
**1.2 KSHV Infection and Life Cycle ..... 4**
**1.3 KSHV Glycoproteins and Receptors ..... 6**
**1.4 Mechanisms of Entry ..... 11**
**Chapter 2 - Discovery and Characterization of a Novel KSHV Entry Mechanism in Epithelial Cells ..... 17**
**2.1 Introduction ..... 18**
**2.2 Results ..... 20**
Caki-1 and HeLa cells express most known KSHV receptors ..... 20
Heparan sulfate interactions are required for KSHV infection of Caki-1 and HeLa cells ..... 21
Single-cell clones of EXT1 KO Caki-1 cells lose KSHV infection phenotype ..... 22
KSHV infection of Caki-1 and HeLa cells is independent of canonical KSHV integrin receptors ..... 23
FAK inhibitors do not affect KSHV infection of Caki-1 or HeLa cells ..... 28
EphA2 is necessary for KSHV infection of Caki-1 and HeLa cells ..... 30
EphA4 and EphB2 are dispensable for KSHV infection in Caki-1 cells. ..... 32
Multiple Eph receptors rescue KSHV infection of EphA2 KO cells. ..... 34
Ectodomains of EphA2 and EphA4 are sufficient to rescue KSHV infection in EphA2 KO cells. ..... 37
Infection of primary gingival keratinocytes requires HS interactions. ..... 39
Dynamin is required during KSHV infection of Caki-1 and HeLa cells ..... 41
Two mutant cell lines lack surface EphA2 expression and are highly susceptible to KSHV infection. ..... 42
**2.3 Discussion ..... 45**
**2.4 Materials and Methods ..... 49**
Cell lines and culture ..... 49
KSHV production and infection ..... 50
CRISPR-Cas9 genome editing ..... 50
Antibodies ..... 50
Blocking reagents ..... 51
Constructs and cloning ..... 51
Transfection and transduction ..... 51
Flow cytometry and sorting ..... 51
Western blotting ..... 51
Statistical analysis ..... 52
**Chapter 3 - Receptor Use During Infection of B Cells in Coculture ..... 53**
**3.1 Introduction ..... 54**
**3.2 Results ..... 57**
B cell lines are resistant to high-titer cell-free KSHV, but some can be latently infected by coculture ..... 57

Lymphocyte cell lines lack cell surface expression of most known KSHV receptors . 60
Coculture infection of BJAB cells is independent of integrin expression. 60
Coculture infection of BJAB cells is blocked by an ephrin ligand and heparin. 64
Manipulation of Eph receptor expression in BJAB cells does not affect KSHV infection rate in coculture 64
Extracellular virions infect B cells in coculture with reactivated iSLK.KSHV cells 67
**3.3 Discussion 69**
**3.4 Materials and Methods 72**
Cells and Culture 72
Antibodies 73
CRISPR-Cas9 genome editing 73
Flow cytometry and sorting 73
Cell-free KSHV infection 74
Coculture KSHV infection 74
Constructs and cloning 74
Transfection and transduction 74
**Chapter 4 - The Search for a KSHV Tropism Switch Glycoprotein 75**
**4.1 Introduction 76**
**4.2 Results 79**
Creation of stop cassette glycoprotein mutant KSHV BACs 79
Infection assays reveal K8.1, orf27, and orf28 to be nonessential KSHV proteins 80
**4.3 Discussion 84**
**4.4 Materials and Methods 86**
KSHV BACs, bacterial strains, and cell lines 86
Red-based scarless BAC recombineering 87
Transfection 88
Coculture method for creation of latently infected iSLK.KSHV lines 88
Cell-free KSHV infection 88
Coculture KSHV infection 88
**Chapter 5 – Concluding Remarks 89**
**References 94**

## List of Figures

2.1 Surface expression of known KSHV receptors on Caki-1 and HeLa cells....20
2.2 Detailed receptor expression histograms for WT Caki-1 and HeLa cells. ....21
2.3 Heparan sulfate interactions are required for infection of Caki-1 and HeLa cells ....22
2.4 Cloned *EXT1* KO Caki-1 cells lose KSHV infection phenotype....23
2.5 Integrins are not required for infection of Caki-1 and HeLa cells....25
2.6 Perturbations in KSHV receptor expression do not affect other known receptors ...27
2.7 Integrin-blocking reagents have mild effects on the infection of Caki-1 and HeLa cells ....28
2.8 FAK inhibitors are not sufficient to block KSHV infection in multiple cell lines....29
2.9 EphA2 is required for efficient infection of Caki-1 and HeLa cells ....31
2.10 Caki-1 *EPHA2* KO Clone A1 lacks EphA2 protein expression....31
2.11 EphA4 and EphB2 are dispensable for infection of Caki-1 cells ....33
2.12 EphA2, EphA4, and EphA5 rescue KSHV infection in *EPHA2* KO Caki-1 cells....35
2.13 EphB2 rescues KSHV infection in *EPHA2* KO Caki-1 cells....36
2.14 EphA2 ectodomain is sufficient to rescue infection rate in EPHA2 KO cells....38
2.15 EphA4 ΔCT enhances infection in EPHA2 KO cells....39
2.16 KSHV infection of PGKs depends on HS interactions but is not inhibited by integrin- or Eph-blocking agents .... 40
2.17 KSHV infection of Caki-1 and HeLa cells requires dynamin....42
2.18 *ITGB3*/*ITGB1* double KO Caki-1 cells have altered morphology and expression of known KSHV receptors but are still susceptible to KSHV infection....43
2.19 An *EPHA4*/*EPHA2* DKO clone is superinfectable with KSHV and may carry a mutation in EphA6....44
3.1 Nonspecific transfer of fluorescent reporter proteins during KSHV coculture infection....58
3.2 Infection rates of B cell lines in coculture....59
3.3 Expression of known KSHV receptors on a panel of B cell lines and primary tonsillar CD19+ cells .... 61
3.4 Chemical stimulation of BJAB cells fails to induce DC-SIGN expression ....62
3.5 Coculture infection does not depend on αV- or β1-family integrins ....63
3.6 Coculture infection does not require β2-family integrins....63
3.7 Infection of BJAB cells in coculture depends on HS and ephrin interactions....65
3.8 Alterations in Eph receptor expression does not affect coculture infection rate ..... 66
3.9 Extracellular virions can infect target B cells in coculture .... 68
3.10 iSLK.BAC16 cells can be superinfected with KSHV .... 69
4.1 Engineering premature stop codon mutants into orfs of interest .... 80
4.2 Infection rates of nonlethal KSHV glycoprotein mutants on Caki-1 and BJAB cells .83
4.3 Additional orf28 infection experiments .... 84

## List of Tables

2.1 CRISPR-Cas9 guide RNA sequences used to target the indicated genes ..... 50
3.1 CRISPR-Cas9 guide RNA sequences used to target integrin genes in BJAB cells ..... 73
4.1 Summary of infection phenotypes of glycoprotein mutant KSHV strains ..... 82
4.2 Oligos used to amplify recombination cassettes from pEP-KanS ..... 87

# I

# KSHV Receptors and Entry: Background and History

## 1.1 KSHV Basics and History

Kaposi's Sarcoma-Associated Herpesvirus (KSHV, or HHV-8) is a human oncovirus which became notorious for its association with the HIV/AIDS epidemic. The discovery of KSHV was published in 1994 after an intensive hunt for the infectious cause of AIDS-associated Kaposi's Sarcoma (KS), but the virus has a natural history that far predates HIV. KS was formally reported in 1872 by Moritz Kaposi, who described it as an "idiopathic multiple pigmented sarcoma of the skin" (Sternbach *et al.*, 1995). At the time, it was largely restricted to elderly men of Mediterranean or Ashkenazi Jewish descent and was slow-growing and rarely fatal. This non-HIV-associated form of the disease is referred to as "classic KS". In the 1950's, a more aggressive form of KS was found to be quite common in sub-Saharan Africa, where it afflicted both male and female children and adults (Cook-Mozaffari *et al.*, 1998). More recently, high rates of both KSHV infection and classic KS have been measured in Amerindians of South America and certain geographic and ethnic groups in Asia (Minhas *et al.*, 2014). Today, the seroprevalence of the virus in these endemic regions can range from 20% to 80% (Minhas *et al.*, 2014).

The seroprevalence of KSHV is very low in the United States (<10%) and classic KS is exceedingly rare, which made it even more striking when very aggressive, fatal KS and non-Hodgkin lymphomas became defining illnesses of HIV/AIDS in the early days of the HIV epidemic (Minhas *et al.*, 2014). Most other AIDS-defining illnesses are opportunistic infections, and it was proposed that KS may be caused by a sexually transmitted infectious agent based on epidemiological analysis of KS before and during the HIV/AIDS outbreak (Beral *et al.*, 1990). In the United States at the time of this analysis (1989), an AIDS patient was 20,000 times more likely to develop KS when compared to the general population, whereas most known carcinogens only increase cancer risk by about 100-fold (Beral *et al.*, 1990). In the fall of 1993, Chang and Moore at Columbia University used representational difference analysis to pinpoint the novel KSHV sequence in clinical samples of KS in New York City (Chang *et al.*, 1994, Schulz *et al.*, 1995, Chang *et al.*, 2014). Early validation experiments occasionally revealed KSHV DNA within non-KS "control tissues" from AIDS patients, leading to the timely discovery of the two other KSHV-driven malignancies: primary effusion lymphoma (PEL, previously called body cavity B cell lymphoma or BCBL) and multicentric Castleman's disease (MCD) (Cesarman *et al.*, 1995, Soulier *et al.*, 1995).

KSHV has since been characterized as a rhadnovirus with a ~200kb double-stranded DNA genome which encodes over 80 canonical open reading frames (ORFs) (Russo *et al.*, 1996). The genome consists of a core of about 60 ORFs that are largely homologous and syntenic with other rhadnoviruses such as herpesvirus saimiri and Epstein-Barr virus (EBV) and about 20 KSHV-specific ORFs (K genes) (Russo *et al.*, 1996, Arias *et al.*, 2014). Like all herpesviruses, the genome is also flanked by GC-rich terminal repeats that aid in genome circularization (Russo *et al.*, 1996). Within this basic genomic

layout, numerous miRNAs, ncRNAs, sORFs, uORFS, and spliced genes have been identified, which allude to complex mechanisms of transcriptional regulation and host manipulation (Chandriani *et al.*, 2010, Xu *et al.*, 2010, Gottwein *et al.*, 2012, Jaber *et al.,* 2013, Arias *et al.*, 2014).

KSHV is only one of two human herpesviruses that cause cancer, and the mechanisms behind the virally-driven transformations that lead to KS, PEL, and MCD have been the subject of intense study. KSHV encodes several genes and miRNAs that may contribute to cell transformation in KSHV-driven malignancies (reviewed most recently in Dittmer *et al.*, 2016 and Wong *et al.*, 2017). Some of these are unique to the virus, while others appear to have been co-opted from the host genome. As an example, KSHV latency associated nuclear antigen (LANA) has been shown to regulate both tumor suppressors and proto-oncogenes through transcriptional and direct interaction mechanisms (reviewed most recently in Wei *et al.*, 2016). The virus also notoriously encodes v-cyclin, a homolog of cyclin D which likely interferes with cell cycle regulation, and v-FLIP, a homolog of FLICE-inhibitory proteins which induces an antiapoptotic cellular state through NF-κB activation and induces cytoskeletal rearrangements that are characteristic of KS spindle cells (reviewed in Ganem, 2010). KSHV has even mimicked several transforming miRNAs from its host (Gottwein *et al.*, 2007, Stalsky *et al.*, 2007, Forte *et al.*, 2015). The virus also uses several tools to promote angiogenesis and inflammation which are crucial to the development of KS lesions (reviewed in Ganem, 2010).

While KS is etiologically linked to KSHV, infection is not sufficient to drive KSHV-associated malignancies. Cofactors and triggers of KSHV-related disease remain mysterious, with one clear exception in the case of HIV/AIDS. The immunosuppressed state of AIDS patients appears to contribute to KS progression, as there have been cases of KS regression upon HIV/AIDS treatment with antiretroviral therapy and cases of KS following artificial immunosuppression in transplant patients (Penn *et al.*, 1979, Gill *et al.*, 2002). It is well-documented that KSHV induces a myriad of innate and adaptive immune responses and encodes many tools to counter this response, and it is thought that the robust immune response may be required for the establishment and maintenance of KSHV latency (most recently reviewed in Dittmer *et al.*, 2016). However, there is increasing evidence that HIV-induced cytokines and HIV infection itself can drive reactivation of KSHV from its latent reservoirs, but few mechanistic details of this process have been reported (Harrington *et al.*, 1997, Mercader *et al.*, 2000, Merat *et al.*, 2002, Zhou, 2013).

Recently, KSHV has been implicated in a systemic inflammation in HIV patients termed KSHV-inflammatory cytokine syndrome (KICS) characterized by an overabundance of IL-6, IL-10 and the viral cytokine vIL-6 and a high mortality rate (Uldrick *et al.*, 2010, Polizzotto *et al.*, 2016). Rapid progression of KS has also been

observed as an outcome of immune reconstitution inflammatory syndrome (KS-IRIS), a condition in HIV patients who have received antiretroviral therapy (Bower *et al.,* 2005, Volkow *et al.*, 2017). While the common knowledge is that the immune system exerts control over KSHV and actively infected cells, in KS-IRIS it appears that the reconstituted immune response to existing pathogens paradoxically leads to reactivation of latent KSHV.

## 1.2 KSHV Infection and Life Cycle

A biphasic life cycle is characteristic of all herpesviruses, including KSHV. During the productive phase, referred to as the lytic phase, all viral genes are expressed, the genome is actively replicated, and new infectious virions are assembled and emitted from the cell. KSHV virions are thought to egress by either budding at the plasma membrane or into vesicles that are exocytosed so the "lytic" phase is somewhat of a misnomer, but cells infected with actively replicating KSHV do eventually die (Wang *et al.*, 2015). To achieve lifelong infection, herpesviruses can enter an alternative infection state called latency during which a minimal set of genes are expressed, but most of the genome is chromatinized and silenced. During latency, this minimal viral program ensures that the viral genome is replicated and maintained as host cells divide (Ballestas *et al.*, 1999). Latency seems to be the default program upon KSHV infection of diverse cells in tissue culture, and is regulated by the essential, multifunctional protein LANA (most recently reviewed in Weidner-Glunde *et al.*, 2017 and Aneja *et al.*, 2017).

Through experiments with various chemicals, several cellular processes have been linked to KSHV reactivation from latency in tissue culture. Sodium butyrate induces the lytic cycle through histone deacetylation. 12-O-tetradecanoyl-phorbol-13-acetate (TPA or PMA) stimulates reactivation through a kinase cascade and the activation of the AP-1 transcription factor complex. Additionally, calcium flux, the neurotransmitters epinephrine and norepinephrine, and host cell apoptosis, and hypoxia can promote reactivation from latency (reviewed in Aneja *et al.*, 2017). Together, such studies show that changes in episome chromatin and the activities of several signaling pathways and transcription factors are important mechanistic factors for KSHV reactivation *in vitro* (reviewed in Dittmer *et al.*, 2016, and Aneja *et al.*, 2017).

In the context of an infected host, however, precise drivers of reactivation have been difficult to characterize. It has been noted that co-infection with several viruses, including HIV, appears to drive KSHV reactivation and likely involves inflammatory cytokines and immune signaling (Harrington *et al.*, 1997, Mercader *et al.*, 2000, Viera *et al.*, 2001, Merat *et al.*, 2002, Wells *et al.*, 2009, Gregory *et al.*, 2009, Tang *et al.*, 2012, Zhou *et al.*, 2013). Additionally, immune system control seems to promote latency and thus immune system impairment likely also factors into KSHV reactivation. This has been

shown both in a laboratory setting and by clinical and epidemiological analysis (Penn *et al.*, 1979, Gill *et al.*, 2002, Myoung *et al.*, 2011, and reviewed further in Aneja *et al.*, 2017).

In addition, KS-related disease states are not explicitly tied to either life cycle stage. The spindle cells that make up the bulk of KS lesions are mostly latently infected but are not able to stably retain the KSHV genome in tissue culture (Grundhoff *et al.*, 2004). A small population of lytic-phase infected spindle cells can also be detected in KS lesions, and these cells are thought to serve as a reservoir of continuous infection within this microenvironment (reviewed in Aneja *et al.*, 2017). KS lesions are usually polyclonal, emphasizing the continuous infection and partial transformation of cells within the tumor. In contrast, KSHV-infected B cells in PEL are tightly restricted to latency and do not lose the viral genome in tissue culture. This does not mean, though, that B cell infection is always latent. Fewer B cells in KSHV-related MCD are infected, but they express several viral transcripts consistent with the lytic phase (reviewed in Giffin *et al.*, 2015). As a result, MCD is often associated with high viral loads and historically poor prognosis (reviewed in Polizzotto *et al.*, 2012).

Upon colonization of a new host, KSHV likely first encounters epithelial cells. Experimentally, the virus is able to infect epithelial cell lines such as HEK293, HeLa, Caki-1/SLK, HepG2 (Betchel *et al.*, 2003, Hahn *et al.*, 2012, Stürzl *et al.*, 2013, and personal observations), Caco-2, Calu-3 (personal observations), and primary epithelial cells and keratinocytes (Diamond *et al.*, 1998, Cerimele *et al.*, 2001, Duus *et al.*, 2004, Johnson *et al.*, 2005, Tiwari *et al.,* 2009, Seifi *et al.*, 2011, Gong *et al.*, 2014). One group also observed latent transcripts in naturally infected tonsillar epithelium, although these samples proved to be rare (Chagas *et al.*, 2006). This group also showed compelling evidence that the tonsillar epithelium is a gateway to B cell infection, as KSHV-infected lymphocytes were observed directly below the epithelial cells and disseminated with patient age (Chagas *et al.*, 2006).

B cells are the primary target of KSHV for lifelong latency, so characterizing the route of B cell infection is of great interest (reviewed in Knowlton *et al.*, 2012). KSHV genomes can be detected in circulating PBMC's of healthy individuals, in addition to the malignant B cells of KSHV-related PEL and MCD (Ambroziak *et al.*, 1995, Soulier *et al.*, 1995, Cesarman *et al.*, 1995, Blackbourn *et al.*, 1997). Studies investigating the immunological phenotypes of PEL and MCD have revealed that KSHV infects germinal center B cells (PEL) and naïve B cells (MCD) and, mysteriously, infected cells almost exclusively express the lambda light chain of the BCR (Du *et al.*, 2001, Chadburn *et al.*, 2008). However, B cell infection has been notoriously difficult to study in the laboratory setting. B cell lines are almost entirely resistant to KSHV in solution (Renne *et al.*, 1998, Friborg *et al.*, 1998, Blackbourn *et al.*, 2000, Betchel *et al.*, 2003, Rappocciolo *et al.*, 2008), and are only slightly infectible in a coculture model (Myoung *et al.*, 2011c, Hahn *et al.*, 2013). Primary B cells are slightly more susceptible to infection, especially when

stimulated or activated with cytokines (Mesri *et al.*, 1996, Blackbourn *et al.*, 1997, Renne *et al.*, 1998, Kliche *et al.*, 1998, Blackbourn *et al.*, 2000, Rappocciolo *et al.*, 2008, Hassman *et al.*, 2011, Myoung *et al.*, 2011a, Myoung *et al.*, 2011d, Knowlton *et al.*, 2014, Nicol *et al.*, 2016) and the infection rate is elevated in coculture (Myoung *et al.*, 2011c). Still, the field lacks a model to study the infection of naïve, unstimulated B cells. Notably, human B cells also become infection after several routes of KSHV inoculation in humanized-BLT mice, although very few studies have been done using this animal model (Wang *et al.*, 2014).

Early on in KS research, there was a strong incentive to characterize the origin of the spindle cells that are pervasive in KS tumors (Dupin *et al.*, 1999). Spindle cells were found to express endothelial markers, and it was shown that KSHV infection *in vitro* drove a morphological change in endothelial cells that matched spindle cell morphology (Flore *et al.*, 1998, Ciufo *et al.*, 2001, and reviewed in Ganem, 2010). A debate continues about whether these spindle cells arise from lymphatic or vascular endothelium because infected vascular endothelial cells upregulate markers of lymphatic endothelium and vice versa (reviewed in Ganem, 2010). KSHV efficiently infects primary endothelial cells and cell lines such as BB19, BMEC, DMVEC, HUVEC, TIME, and mesenchymal stem cells (Boshoff *et al.*, 1995, Flore *et al.*, 1998, Panyutich *et al.*, 1998, Blackbourn *et al.*, 2000, Ciufo *et al.*, 2001, Lagunoff *et al.*, 2002, Lee *et al.*, 2016).

There are additional cell types that can be infected in vitro and in vivo and likely play important roles in the KSHV life cycle and KS pathogenesis. Fibroblasts are found infiltrating KS lesions, and the virus can infect both primary oral fibroblasts and fibroblast cell lines from humans and other species (Bechtel *et al.*, 2003, Dai *et al.*, 2012). Immune cells such as monocytes, macrophages, and dendritic cells (DCs) are also found within KS tumors and can be infected *in vitro* (reviewed in Knowlton *et al.*, 2012). KSHV alters the function of these cells, interfering with the normal immune response and promoting a pro-inflammatory tumor microenvironment. One curiosity of KS spindle cells is their dependence on cytokines for growth *in vitro*, and these immune cell subtypes are a likely source of such factors *in vivo* (Rappocciolo *et al.*, 2017, Host *et al.*, 2017, and further reviewed in Knowlton *et al.*, 2012).

## 1.3 KSHV Glycoproteins and Receptors

The very first stage in a *de novo* viral infection is entry. Enveloped viruses must breach the plasma membrane in order to deliver their virion contents and genetic material to the cell. In general, viruses utilize viral glycoproteins embedded in the virion envelope to engage host proteins on the surface of an uninfected cell. These intricate interactions result in either direct membrane fusion at the cell surface, or endocytic uptake of the virion and subsequent membrane fusion with the endosome wall. The

fusion of the viral and host membranes allows the contents of the virion to access host cell cytoplasm, and viral takeover begins.

Herpesviruses express five conserved glycoproteins: gB, gH, gL, gM, and gN (Russo *et al.*, 1996, Neipel *et al.*, 1997, Zhu *et al.*, 2005). gB is a trimeric fusion protein and is thought to be the main executor of membrane fusion (Pereira *et al.*, 1994, Pertel *et al.*, 2002). gB has several conformations in which the fusion peptides are embedded within the protein, extended toward the target membrane, or folded back toward the primary membrane. It is through these sequential conformational changes that the primary and target membranes are brought into close proximity such that fusion becomes energetically favorable (most recently reviewed in Cooper *et al.*, 2015). The single-pass transmembrane protein gH forms a heterodimer with the untethered gL which is generally involved in receptor binding and gB activation (most recently reviewed in Cooper *et al.*, 2015). gB, gH, and gL are often referred to as the core fusion glycoproteins. gM and gN also form a complex that is found in the virion envelope and may contribute to entry and cell-cell fusion, though the functions of this complex are more divergent between individual herpesviruses (Zhu *et al.,* 2005, and summarized in Koyano *et al.*, 2003). In the lone published study on KSHV gM/gN, the heterodimer was found to inhibit membrane fusion between cells (Koyano *et al.*, 2003).

Herpesviruses also encode accessory glycoproteins, which are sometimes unique to the virus and carry out diverse functions and often contribute to receptor binding activity and modulate the essential functions of gH/gL and gB. Several accessory glycoproteins have been found to be incorporated into the KSHV envelope, including K8.1, orf4, orf27, and orf28 (Neipel *et al.*, 1997, Zhu *et al.*, 1999, Jenner *et al.*, 2001, Spiller *et al.*, 2003, Zhu *et al.,* 2005). KSHV orf27 and orf28 have never been studied and have no ascribed function as of this writing. K8.1 and orf4 both bind a cellular proteoglycan, heparan sulfate, but the significance of these proteins in the virion envelope is not well understood, and K8.1 is even dispensable for KSHV replication and infection (Akula *et al.*, 2001b, Wang *et al.*, 2001, Birkmann *et al.*, 2001, Luna *et al.*, 2004, Mark *et al.*, 2006, Spiller *et al.*, 2006).

Receptors for KSHV have been quite well-studied in several model infection systems. The first receptor to be identified in 2001 was heparan sulfate (HS), a highly negatively charged proteoglycan modification that can be found on many proteins (Akula *et al.*, 2001a, Akula *et al.*, 2001b, Wang *et al.*, 2001, Birkmann *et al.*, 2001). HS is widely used by viruses such as HIV, HPV, RSV, Dengue, and herpesviruses to attach to the target cell membrane and promote subsequent receptor engagement (Patel *et al.*, 1993, Chen *et al.*, 1997, Feldman *et al.*, 2000, Shukla *et al.*, 2001, Cruz *et al.*, 2013). For KSHV, HS is necessary for infection of endothelial cells, fibroblasts, and HT1080 epithelial cells (Akula *et al.,* 2001a, Akula *et al.,* 2001b, Wang *et al.*, 2001, Birkmann *et al.*, 2001, Akula *et al.*, 2003, Garrigues *et al.*, 2014a).

Several KSHV glycoproteins have HS-binding activity, including gB, K8.1, and orf4 (Akula *et al.*, 2001b, Wang *et al.*, 2001, Birkmann *et al.*, 2001, Mark *et al.*, 2006, Spiller *et al.*, 2006, Hahn *et al.,* 2009). Modern imaging techniques revealed that HS and KSHV do not exclusively colocalize during infection of HT1080 epithelial cells, suggesting that multiple factors may contribute to KSHV attachment (Garrigues *et al.*, 2014b). It is also possible that HS plays a more nuanced role in infection than a simple attachment factor, as it has been shown to modulate gB/gH/gL membrane fusion with HEK293T, CHO, and HCjE cells (Tiwari *et al.*, 2009). Potential involvement of the core proteins to which HS is attached has never been explored in the context of KSHV infection except a single study which found a potential role for syndecans during infection of HEK293T cells (Hahn *et al.*, 2009).

B cell lines are notably deficient in HS expression because a critical enzyme in the biosynthetic pathway is not expressed (Jarousse *et al.*, 2008). When HS biosynthesis is artificially restored, B cells remain resistant to KSHV despite enhanced cell surface attachment (Jarousse *et al.*, 2008). This emphasizes that while HS is necessary for infection of adherent cells, it is neither necessary nor sufficient for lymphocyte infection.

Shortly afterward, integrin α3β1 was found to be a post-attachment entry receptor required for infection of HFF and HMVEC-d cells (Akula *et al.*, 2002). α3β1 is one of twenty-four known integrins which are broadly expressed and regulate cell migration and adhesion to extracellular matrix proteins (see Hynes *et al.*, 2002, and Barczyk *et al.*, 2010 for excellent reviews). Although some studies disputed the requirement for α3β1 in several other cell types, it was clear that integrin-associated signaling was important for KSHV entry processes, discussed in further detail below. Two other integrins, αVβ3 and αVβ5, were later found to be involved in KSHV entry of CHO, HT1080, HSG(HeLa), HFF, and HMVEC-d cells (Garrigues *et al.*, 2008, Veettil *et al.*, 2008). Integrins αVβ3 and αVβ5 are RGD-binding integrins, meaning they share a common motif of the amino acids R-G-D in their ligands such as fibronectin. Integrin α3β1 is a laminin-binding integrin that may also have RGD-binding function. KSHV gB contains an RGD motif that binds integrins by mimicking natural ligands, and this gB-integrin interaction can be blocked by RGD peptides (Wang *et al.*, 2003, Garrigues *et al.*, 2008). In many studies, gB was sufficient to activate integrin-associated signaling molecules such as focal adhesion kinase (FAK) (Akula *et al.*, 2002, Wang *et al.*, 2003, Sharma-Walia *et al.*, 2004, Zhang *et al.*, 2005). Cell migration and adhesion can also be induced by gB (Wang *et al.*, 2003, Garrigues *et al.*, 2008). It is likely that one or more of these integrins are required in complex to facilitate KSHV infection, although the potential for functional redundancy has not been explored in depth using depletion studies in any infection model.

KSHV gB also contains a second integrin interaction motif called a disintegrin-like domain (DLD) (Walker *et al.*, 2014). DLDs are an important part of ADAM (a disintegrin and metalloprotease) proteins and named for their similarity to disintegrins, potent

inhibitory proteins found in snake venom that disrupt integrin function (recently reviewed in Giebeler *et al.*, 2016). The DLD of KSHV gB was shown to specifically bind a non-RGD integrin heterodimer, α9β1 (Walker *et al.*, 2014). Disrupting the gB-α9β1 interaction modestly reduced infection of HFF and HMVEC-d cells (Walker *et al.*, 2014). Additionally, the DLD and RGD domains may have antagonistic functions (Hussein *et al.*, 2016). While KSHV gB is unique among herpesviruses for its RGD domain, the DLD domain is conserved throughout beta- and gammaherpesviruses (Walker *et al.*, 2014). Accordingly, herpesviruses in all families have been found to use integrins as entry receptors. The interactions with integrins are not only mediated by gB, as the gH/gL complexes of some herpesviruses have also been shown to bind certain integrins. However, a link between KSHV gH/gL and integrins has never been described. It is clear that interactions with integrins are a conserved theme in herpesvirus entry mechanisms, and that herpesviruses have evolved multiple ways of achieving these interactions (most recently reviewed in Campadelli-Flume *et al.*, 2016).

Erythropoietin-producing hepatocellular (Eph) Receptor A2 (EphA2) was reported to be a KSHV receptor by two independent groups in 2012 (Hahn *et al.*, 2012, Chakraborty *et al.*, 2012). EphA2 is one of fourteen members of the Eph family of receptor tyrosine kinases which coordinate with ephrin ligands to regulate cell adhesion and repulsion and maintain borders between tissues (recently reviewed in Lisabeth *et al.*, 2013). Eph receptors are divided into two types, A or B, based on their ability to bind A- or B-type ligands (Lisabeth *et al.*, 2013). KSHV gH/gL binds EphA2 in the ligand-binding domain, again mimicking a natural ligand to hijack cellular receptors (Hahn *et al.*, 2014 Großkopf *et al.*, 2018). This interaction occurs alongside virus-integrin binding and results in the amplification of virally induced integrin signaling and the recruitment of endocytosis effectors that lead to virion internalization (Chakraborty *et al.*, 2012, Dutta *et al.*, 2013, Bandyopadhyay *et al.*, 2014a, Bandyopadhyay *et al.*, 2014b). Additionally, the activity of EphA2 as a KSHV receptor may be modulated by other proteins which may provide links to known epidemiological risk factors or infection patterns. This is exemplified by a recent study showing that androgen receptor (AR) binds EphA2, is activated during infection, and was essential for EphA2-mediated entry in endothelial and SLK/Caki-1 cells (Stürzl *et al.*, 2013, Wang *et al.*, 2017).

It has been shown that gH/gL may bind other A-type Eph receptors, but the use of another Eph besides EphA2 as an entry receptor has not been demonstrated (Hahn *et al.*, 2013). Recently, a domain within KSHV gH that binds to EphA2 was identified, and interestingly a recombinant virus with mutations in this domain remained infectious in several cell types, albeit at drastically reduced levels compared to WT virus (Großkopf *et al.*, 2018). Thus, while EphA2 is clearly a very important KSHV receptor, it is possible that another mechanism alone is sufficient for viral entry. Whether this EphA2-independent infection is dependent on integrins has not been explored.

xCT/SLC7A11 is a subunit of a cysteine and glutamate transporter and was identified in a screen as a fusion receptor for KSHV in 2006 (Kaleeba *et al.*, 2006b). In this study, xCT antisera blocked fusion of some cell lines with a KSHV-infected PEL cell line, BCBL-1. However, no xCT depletion or KO studies have ever been published. Follow-up studies from an independent group reported that xCT can be found in the KSHV entry complex during infection in pulldown experiments, but viral entry was not blocked by xCT antibodies (Veettil *et al.*, 2008, Chakraborty *et al.*, 2011). Transcriptional defects were reported when xCT antibodies were present during infection, and the group concluded that xCT may have a role in a post-entry stage of infection (Veettil *et al.*, 2008). Another group reported that xCT was upregulated in primary B cells upon activation, but anti-xCT antisera did not block the infection of activated B cells (Rappocciolo *et al.*, 2008). No KHSV glycoproteins have been reported to bind xCT.

Finally, dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) has been implicated as a receptor for KSHV in cells of the immune system such as dendritic cells, macrophages, and activated B cells (Rappocciolo *et al.*, 2006, Rappocciolo *et al.*, 2008, Kerur *et al.*, 2010). DC-SIGN is a C-type lectin that binds mannose-containing glycoproteins and is a common target of viral glycoproteins (Lozach *et al.*, 2007). KSHV gB has been found to be mannosylated, and likely binds DC-SIGN through these sugar modifications (Hensler *et al.*, 2014). DC-SIGN is expressed by activated primary B cells, primary monocyte-derived DCs and macrophages, and the THP-1 cell line, and KSHV infection of these cells can be blocked by mannan or anti-DC-SIGN antibodies (Rappocciolo *et al.*, 2006, Rappocciolo *et al.*, 2008, Kerur *et al.*, 2010). In THP-1 cells, integrins and HS are also necessary for KSHV infection, and the signaling pathways activated in these cells appear similar to previously characterized KSHV-triggered integrin signaling (Kerur *et al.*, 2010). Thus, it is still unknown whether DC-SIGN functions simply as an attachment receptor (especially in the absence of HS on B cells), or if it is used to trigger intracellular events.

Kinetic differences in pulldown experiments have revealed a putative order of receptor engagement, discussed in more detail below. How this receptor engagement impacts the activation of KSHV glycoproteins, and gB in particular, is still unknown due to a lack of structural studies of the KSHV glycoproteins. On the other hand, the glycoproteins of the related related human gammaherpesvirus Epstein-Barr Virus (EBV, or HHV-4) have been studied more extensively and interact with some of the same receptors for adherent cell entry. Thus, there is likely much to learn about KSHV glycoprotein triggering by studying the same processes in EBV.

EBV utilizes two sets of drastically different receptors to enter B cells and epithelial cells. For epithelial cell entry, several αV-family integrins have been identified as receptors, although two recent papers describe an integrin-independent, EphA2 ectodomain-dependent EBV entry mechanism (Chen *et al.*, 2018, Zhang *et al.*, 2018, and

reviewed in Connolly *et al.*, 2011 and Chesnokova *et al.*, 2014). CD21 and HLA class II are receptors for EBV on B cells. EBV encodes a unique tropism switch protein, gp42, which binds to gH/gL and induces conformational changes that likely activate gB into the extended conformation upon gp42 binding to HLA class II (Sathiyamoorthy *et al.*, 2014, and reviewed in Connolly *et al.*, 2011, Chesnokova *et al.*, 2014). EBV gH/gL contains a putative integrin-binding KGD motif and since gp42 potently inhibits epithelial cell infection, it was thought that gp42 may sterically interfere with this KGD motif. However, structural studies revealed that the C terminus of gp42 binds the KGD motif, while experimentally the N terminus of the protein provides much more potent inhibition of epithelial cell infection (Sathiyamoorthy *et al.*, 2016). Moreover, the KGD motif is important for infection of both cell types (Chen *et al.*, 2012). Finally, the newest studies which identified EphA2 as an EBV receptor call into question the necessity of integrin receptors in the first place (Chen *et al.*, 2018, Zhang *et al.*, 2018). Thus, the roles of integrins and the KGD motif of gH/gL during EBV infection remain unclear.

It should be emphasized that in KSHV, the RGD motif is present in gB instead of gH/gL and whether the interactions with integrins, HS, or DC-SIGN directly contribute to gB activation independent of gH/gL is not known. It is possible that there are multiple functionally redundant routes of gB activation that are used in different cellular contexts, which seems to be the general case for herpesviruses. Notably, a tropism switch accessory glycoprotein has not been identified for KSHV, nor has a second distinct receptor set been described for its distinct B cell tropism.

## 1.4 Mechanisms of Entry

The ultimate goal of interactions between viral glycoproteins and cellular receptors is to achieve membrane fusion and the introduction of virus contents into the host cell cytoplasm. However, not all viruses may productively fuse directly with the cell membrane, often due to the fact that gB can only fully execute fusion at the low pH found in endosomes and lysosomes (reviewed in Cooper *et al.*, 2015). Thus, a second critical function of virus-receptor interactions is the mobilization of endocytosis effectors that eventually direct the virion to an endosomal compartment where it can complete the process of membrane fusion. All known KSHV entry mechanisms require virion internalization.

In HFF cells, KSHV induces the colocalization of EphA2 and integrins $\alpha3\beta1$, $\alpha V\beta3$, and $\alpha V\beta5$ in non-lipid raft membrane domains (Dutta *et al.*, 2013). The interaction between gB and these integrins induces the sequential activation of focal adhesion kinase (FAK), Src, and phosphoinositide 3-kinase (PI-3K) (Naranatt *et al.*, 2003 Wang *et al.*, 2003 Sharma-Walia *et al.*, 2004). EphA2 also becomes phosphorylated in this complex and binds these signaling proteins to the receptor complex (Dutta *et al.*, 2013). EphA2 also

associates with myosin IIa and the E3 ubiquitin ligase c-Cbl which polyubiquitinates EphA2 in the receptor complex (Dutta *et al.*, 2013). This modification likely serves as an internalization signal. Finally, EphA2 recruits Eps15 and AP-2, which subsequently assemble clathrin and clathrin-mediated endocytosis (CME) effectors that ultimately internalize EphA2 and the virion (Akula *et al.*, 2003 Dutta *et al.*, 2013).

A small series of studies from an independent group have also examined receptor usage on a different fibrosarcoma cell line, HT1080. KSHV binding to the surface of these cells is blocked by treatment with heparin (Garrigues *et al.*, 2014a). RGD peptides (including a cyclic RGD peptide with specific affinity for integrin αVβ3) and a function-blocking αVβ3 antibody block KSHV infection of these cells. Additionally, KSHV initially colocalizes well with microdomains containing all three integrins α3β1, αVβ3, and αVβ5, but HS and CD98 (the heavy chain of the xCT-CD98 complex) were only present in these microdomains some of the time (Garrigues *et al.*, 2014b). It is unclear why KSHV did not colocalize with HS in this imaging assay, while heparin effectively abolishes virion binding to HT1080 cells. This group also reported KSHV binding to HT1080 cells independent of apparently highly variable HS expression in the population. This curious observation was not confirmed (by flow cytometry, for example) or explored further by combining the imaging assay with either heparin blocking or heparinase treatment of the cells. The colocalization of KSHV with EphA2 in these initial attachment microdomains was also not examined in this study.

In microvascular endothelial cells, the initial events upon virus binding are quite similar to what has been characterized for HFF cells. Interaction with integrins in the non-lipid raft membrane region triggers the FAK-Scr-PI-3K signaling cascade which is recruited into the receptor complex with integrins α3β1, αVβ3, and αVβ5, and EphA2. EphA2 recruits myosin IIa and c-Cbl which either mono- or polyubiquitinates the integrin β subunits (Valiya-Veetil *et al.*, 2010, Chakraborty *et al.*, 2011 Greene *et al.*, 2012, Chakraborty *et al.*, 2012). This seems to be a signal for sorting and internalization as polyubiquitinated αVβ5 is retained in non-lipid raft membrane regions, whereas monoubiquitinated α3β1, αVβ3, and the associated virion, EphA2, xCT, and signaling molecules are translocated into lipid rafts (Chakraborty *et al.*, 2011). When parts of this complex are impaired, including knock down of EphA2 or c-Cbl, the complex and bound virus remains in the non-lipid raft membrane portion and are internalized by CME into lysosomes from which the virus apparently cannot escape (Chakraborty *et al.*, 2011 Chakraborty *et al.*, 2012).

Once translocated to lipid rafts, the scaffold and signaling proteins CIB1, Crk, and p130Cas are recruited to the complex (Bandyopadhyay *et al.*, 2014a, Bandyopadhyay *et al.*, 2014b). Membrane blebs begin to form around the KSHV receptor and signaling complex, and the ESCRT protein Hrs and downstream effectors are recruited to facilitate macropinocytosis (Raghu *et al.*, 2009, Valiya-Veettil *et al.*, 2010, Veettil *et al.*, 2016, Kumar

*et al.*, 2016b). However, at least one study has disputed the use of macropinocytosis in endothelial cells, favoring a CME-dependent model (Greene *et al.*, 2009). The authors of this study discuss that the internalization mechanism could be influenced by the multiplicity of infection (MOI). It is possible that KSHV-induced events that occur in and out of the context of lipid rafts are uncoupled (Raghu *et al.*, 2007).

In addition to the primary FAK-Src-PI-3K signaling cascade that is induced by KSHV, several other signaling pathways are activated. Downstream of PI-3K, protein kinase C zeta (PKCζ), MEK1/2, and ERK1/2 are activated in both infection systems (Naranatt *et al.*, 2003). The receptor-induced ERK1/2 and several associated transcription factors are important for the expression of both host and latent viral genes (Naranatt *et al.*, 2004 Sharma-Walia *et al.*, 2005). Although many KSHV proteins have been found to modulate sustained NF-κB activity during the viral life cycle (well-summarized in Grossmann *et al.*, 2008), NF-κB is phosphorylated just minutes after cells are exposed to KSHV, suggesting that this initial activation is mediated by receptor binding (Sadagopan *et al.*, 2007). Recently, KSHV has been found to induce reactive oxygen species (ROS) early on during infection, and in endothelial cells ROS is important for the proper membrane trafficking of the KSHV-receptor complex (Ma *et al.*, 2013, Bottero *et al.*, 2013). Furthermore, ROS induction and the Src-PI-3K signaling axis in these cells leads to the activation of the transcription factor Nrf2 which induces host gene expression, binds KSHV LANA, and promotes viral latency (Gjyshi *et al.*, 2014 Gjyshi *et al.*, 2015).

Finally, KSHV also triggers cytoskeletal responses downstream of the virus-induced FAK-Src-PI-3K cascade through Rho GTPases in both infection systems. In fibroblasts, extracellular gB is sufficient to draw RhoA and Cdc42 to the plasma membrane where they activate the actin cytoskeleton tethering protein ezrin (Sharma-Walia *et al.*, 2004). During infection in both model cell types, Rho GTPases mediate the acetylation and stabilization of microtubules through downstream effectors such as diaphanous-2 which are required to transport the KSHV capsid to the nucleus (Naranatt *et al.*, 2005, Veettil *et al.*, 2006). Rho GTPases additionally direct actin dynamics and the formation of structures such as filopodia, lamellipodia, and stress fibers that are essential for viral entry through CME or macropinocytosis (Naranatt *et al.*, 2003, Raghu *et al.*, 2009, Greene *et al.*, 2009).

The KSHV entry mechanisms for infection of epithelial cells and DC-SIGN-expressing immune cells are much less well-characterized, hindered by the relatively small number of studies that have been done, the experimental approaches of these studies, and the variety of cell types used (instead of focusing on specific cell lines or primary cells). Given these limitations, it is not currently possible to thoroughly describe the entry mechanisms that govern KSHV infection in any of these cell types.

Receptor assays have occasionally been performed on HEK293 epithelial cells. It was noted early on that heparin blocks KSHV infection of HEK293 cells, as is the case

with most other adherent cells (Akula *et al.*, 2001a, also reported in Inoue *et al.*, 2003 and Veettil *et al.*, 2008). Heparinase treatment of the cell surface, or the addition of soluble heparin also reduced fusion with glycoprotein-expressing effector cells (Tiwari *et al.*, 2009). There is conflicting evidence on the use of integrins for HEK293 entry. One group reported that treatment with soluble integrin heterodimers α3β1, αVβ3, and αVβ5 partially inhibited KSHV entry, while a different group using a constructed reporter cell line derived from HEK293 reported that fibronectin, several RGD peptides, and soluble α3β1 were all unable to block KSHV infection (Inoue *et al.*, 2003, Veettil *et al.*, 2008). A third group additionally reported that function-blocking antibodies targeting integrins β1 and αV did not block infection of HEK293 cells (Walker *et al.*, 2014). The first research group has also reported the activation of several signaling molecules downstream of HEK293 infection, including FAK, ERK 1/2, and RhoA (Naranatt *et al.*, 2003, Veettil *et al.*, 2006). Another group also reported phosphorylation of EphA2 in response to KSHV or gH/gL, and that soluble EphA2 or ephrin-A4 block HEK293 infection (Hahn *et al.*, 2012, Hahn *et al.*, 2013). Several groups have also enhanced the KSHV infection rate in HEK293 cells by overexpressing certain proteins, including VEGFR, EphA2, CIB1, and syndecans 1, 2, and 4 (Zhang *et al.*, 2005, Hahn *et al.*, 2009, Hahn *et al.*, 2012, Bandyopadhyay *et al.*, 2014a). While many of these studies suggest the use of a similar integrin- and EphA2-dependent entry pathway, the evidence is far from definitive and no receptor depletion studies have ever been performed.

SLK/Caki-1 cells have also been used in a handful of receptor studies. Several studies reported that soluble EphA2 and soluble ephrin-A4 block infection of SLK/Caki-1 cells by interfering with the KSHV gH/gL-EphA2 interaction (Hahn *et al.*, 2012, Hahn *et al.*, 2013, Hahn *et al.*, 2014, Großkopf *et al.*, 2018). Additionally, siRNA mediated knock down of either EphA2 or androgen receptor (AR) reduced infection rate of SLK/Caki-1 cells (Hahn *et al.*, 2012, Wang *et al.*, 2017). It has also been shown that EphA2 becomes phosphorylated upon KSHV infection in SLK cells, and this phosphorylation is dependent on AR-recruited Src (Wang *et al.*, 2017). One study has reported that soluble integrins α3β1 and αVβ3 have no effect on infection of SLK/Caki-1 cells (Hahn *et al.*, 2012).

Very select experiments have been performed on even more epithelial cell lines. Heparinase treatment or soluble heparin blocked fusion of human conjunctival epithelial cells with KSHV glycoprotein-expressing effector cells (Tiwari *et al.*, 2009). Overexpression of EphA2 on the human lung epithelial cell line H1299 enhanced KSHV infection (Hahn *et al.*, 2012). Soluble EphA2 blocks the infection of other miscellaneous epithelial cells: HeLa and HepG2 (Hahn *et al.*, 2012), and KSHV gH/gL is sufficient to trigger endocytosis in EphA2-transduced HeLa cells (Hahn *et al.*, 2012). Interestingly, a study in mouse keratinocytes suggested that the presence of α3β1 inhibited KSHV infection (Garrigues *et al.*, 2008). Finally, a HeLa derivative cell line mislabeled as human salivary gland epithelial cells (HSG), HeLa(HSG), was found to express all known receptors except for integrin β3 and was resistant to KSHV infection (Garrigues *et al.*,

2014b, Garrigues *et al.*, 2018). Infection of these HeLa(HSG) cells was increased significantly by expressing integrin β3 (Garrigues *et al.*, 2014b). It should be noted that the behavior of this HeLa derivative cell line does not match our experience, although our HeLa cells also lack integrin β3 expression at the cell surface (personal observations).

Finally, a handful of contradictory experiments have been performed with animal epithelial cells. One group reported in two publications that overexpression of integrin α3 in CHO cells increased KSHV infection (Akula *et al.*, 2002, Sharma-Walia *et al.*, 2004). Later, a second group reported that overexpression of integrin α3 in CHO cells reduced the surface expression of integrin αVβ3 and reduced the ability of the cells to bind RGD-containing ligands (Garrigues *et al.*, 2008). They also reported that expression of integrin α3 in *ITGA3* KO mouse keratinocytes reduced infection rate (Garrigues *et al.*, 2008).

Infection of certain DC-SIGN-expressing cells may be independent of α3β1, as B cells transfected with DC-SIGN were infectible but did not express α3β1, and macrophages expressing α3β1 but not DC-SIGN were resistant to KSHV (Rappocciolo *et al.*, 2006a). It is also unclear whether xCT is required, as there is a similar lack of correlation between xCT expression and KSHV permissiveness in primary B cells and B cell lines (Rappocciolo *et al.*, 2008). It should be noted that DC-SIGN is important for surface adhesion, likely in the absence of HS, but the role of several receptors including integrins αVβ3, αVβ5, and EphA2 has not been studied by this group in the context of DC-SIGN-mediated infection (Rappocciolo *et al.*, 2006a, Rappocciolo *et al.*, 2008). An independent group examined receptor use during KSHV infection of the DC-SIGN expressing monocyte cell line THP-1 (Kerur *et al.*, 2010). This study reported that both HS and DC-SIGN were required for infection (Kerur *et al.*, 2010). Soluble integrins blocked infection, and the virus both colocalized with integrins and initiated integrin-related signaling in THP-1 cells (Kerur *et al.*, 2010). Importantly, EphA2 had not yet been formally described as a KSHV receptor until 2010, but it was observed that receptor tyrosine kinase (RTK) inhibitors reduced the infection rate in these cells (Kerur *et al.*, 2012). In summary, the infection mechanism used in THP-1 cells may be similar to that in HFF and primary endothelial cells, but receptor use beyond DC-SIGN in B cells and other antigen-presenting cells is still unclear.

Undoubtedly, our collective knowledge about KSHV entry pathways is incomplete and in-depth characterization has been limited to a select few model infection systems. New research groups with different approaches and techniques have much to offer in the field of KSHV entry. For example, an independent group recently published a kinome screen to identify cellular kinases that become phosphorylated within fifteen minutes of endothelial cell infection (Cheng *et al.*, 2015). In this study, over twenty new kinases were identified to be activated in response to infection. Clearly, the cellular response to *de novo* infection is extensive, complex, and cell-type dependent. Thus, it is critically important to

specifically define receptors and entry mechanisms in each infection system to best allow us to untangle the web of host responses to this important human pathogen.

# II

# Discovery and Characterization of a Novel KSHV Entry Mechanism in Epithelial Cells

A version of this chapter appeared in TerBush AA, Hafkamp F, Hartenian E, Lee HJ, Glaunsinger BA, Coscoy, L. 2018. An Integrin-Independent Kaposi's Sarcoma-Associated Herpesvirus Infection Mechanism. bioRXIV https://doi.org/10.1101/270108

## 2.1 Introduction

In the decades since its discovery, it has been observed that KSHV has broad tropism and can efficiently infect many types of human primary cells and cell lines (Renne *et al.*, 1998, Blackbourn *et al.*, 2000, Bechtel *et al.*, 2003). KSHV entry mechanisms have been most thoroughly studied in endothelial cells and fibroblasts, which were of particular interest to understand the origin of the KSHV-infected spindle cells that make up the distinct, highly vascularized KS tumors (reviewed in Kumar *et al.*, 2016a). Infection of monocytes and dendritic cells has also been observed within KS tumors and in tissue culture models (Blasig *et al.*, 1997, Rappocciolo *et al.*, 2006a, Rappocciolo *et al.*, 2017). B cells are thought to be the latently infected reservoir of KSHV (Mesri *et al.*, 1996, Blackbourn *et al.*, 1997), but modeling their infection in a laboratory setting has proven to be technically challenging.

However, it is reasonable to assume that the first cells infected in a new host upon transmission are epithelial cells. While KSHV was first considered to be a sexually transmitted infection because of its co-infection pattern with HIV, it is now widely recognized that KSHV can be transmitted through saliva and close contact between individuals (reviewed in Minhas *et al.*, 2014). Multiple studies have shown that KSHV infects primary human epithelial cells and cell lines including oral keratinocytes (Renne *et al.*, 1998, Bechtel *et al.*, 2003, Cerimele *et al.*, 2001, Duus *et al.*, 2004, Johnson *et al.*, 2005, Seifi, 2011, Hahn *et al.*, 2012, Gong *et al.*, 2014) and another clinical report provides compelling clinical evidence that infection of the tonsillar epithelium could provide a gateway through which the virus might access the underlying lymphocytes to establish the reservoir of latently infected B cells (Chagas *et al.*, 2006).

KSHV interacts with a variety of receptors on the surface of host cells. Heparan sulfate (HS) is thought to be a major cell attachment factor and several KSHV glycoproteins have HS-binding activities (Akula *et al.*, 2001a, Akula *et al.*, 2001b, Wang *et al.*, 2001, Birkmann *et al.*, 2001, Mark *et al.*, 2006, Spiller *et al.*, 2006, Hahn *et al.*, 2009). KSHV also coordinates a complex of integrins α3β1, αVβ3, αVβ5, erythropoietin-producing hepatocellular (Eph) receptor A2 (EphA2), and SLC7A11/xct to trigger clathrin-mediated endocytosis or macropinocytosis of the virion in HFF cells and primary endothelial cells, respectively (most recently reviewed in Kumar *et al.*, 2016, Kumar *et al.*, 2018). Some questions have been raised over precisely which integrins are required for the infection of individual cell lines (Inoue *et al.*, 2003, Kaleeba *et al.*, 2006, Garrigues *et al.*, 2008, Garrigues *et al.*, 2014b). However, in these two well-characterized infection models, the interaction between KSHV gB and the canonical integrin receptors initiates a signaling cascade of FAK, Src, and PI-3K (Kumar *et al.*, 2016, Akula *et al.*, 2002, Naranatt *et al.*, 2003, Sharma-Walia *et al.*, 2004). KSHV gH/gL binds EphA2 which amplifies this cascade and coordinates endocytosis effectors together with c-Cbl and myosin IIA (Hahn *et al.*, 2012, Chakraborty *et al.*, 2012, Dutta *et al.*, 2013, Bandyopadhyay *et al.*, 2014a,

Bandyopadhyay *et al.*, 2014b Hahn *et al.*, 2014). Still, there are important differences in the entry mechanisms used during infection of HFF and primary endothelial cells, such as the form of endocytosis used to ultimately internalize the virion, hinting that KSHV initiates different entry processes in different types of cells while using the same receptors.

A smaller number of receptor studies have been performed on a variety of epithelial cell lines, but such a unified model of KSHV receptor usage and entry mechanism has not yet been assembled for any individual cell line. Soluble heparin or enzymatic removal of HS from the cell surface inhibits KSHV infection of human embryonic kidney (HEK) 293 cells and human conjunctival epithelial cells, suggesting that HS is necessary for epithelial cell infection (Akula *et al.*, 2001a, Inoue *et al.*, 2003, Veettil *et al.*, 2008, Tiwari *et al.*, 2009). EphA2 is also clearly important for KSHV infection of several cell lines. Soluble EphA2 or Eph-blocking ligands inhibit infection of HEK293 and SLK cells, and EphA2 becomes phosphorylated upon infection in these two cell lines (Hahn *et al.*, 2012, Hahn *et al.*, 2013, Wang *et al.*, 2017, Großkopf *et al.*, 2018). Furthermore, siRNA knock down of EphA2 significantly reduces infection of SLK cells (Hahn *et al.*, 2012, Wang *et al.*, 2017). Soluble EphA2 inhibits infection of two additional epithelial cell lines (HeLa and HepG2), and overexpression of EphA2 enhances infection of HEK293 cells and the human lung epithelial cell line H1299 (Hahn *et al.*, 2012).

The evidence for integrin involvement during infection of epithelial cell lines is mixed. Two groups have reported that integrin ligands, RGD peptides, soluble α3β1, or function-blocking integrin αV and β1 antibodies did not block KSHV infection of a HEK293-derived reporter cell line or HEK293 cells (Inoue *et al.*, 2003, Walker *et al.*, 2014). A third group reported that soluble integrins α3β1 and αVβ3 and a function-blocking αVβ3 antibody did not block KSHV infection of SLK cells (Hahn *et al.*, 2012). However, a fourth group reported that soluble integrins α3β1, αVβ3, and αVβ5 reduced the infection rate of HEK293 cells and that the signaling proteins FAK, ERK1/2, and RhoA were activated upon KSHV infection (Naranatt *et al.*, 2003, Veettil *et al.*, 2006, Veettil *et al.*, 2008). Finally, a fifth study of a HeLa-derivative cell line misidentified as human salivary gland epithelial cells HSG(HeLa) reported that the cells were resistant to KSHV despite expressing all known receptors except integrin β3, and expression of integrin β3 (and restoration of integrin αVβ3) greatly increased the susceptibility of the cells to KHSV infection (Garrigues *et al.*, 2014b, Garrigues *et al.*, 2018).

Here we used CRISPR-Cas9 to comprehensively examine the use of this KSHV receptor complex in two highly infectible epithelial cell lines: Caki-1 kidney epithelial cells, and HeLa cervical epithelial cells. Caki-1 cells are significant as they have contaminated all known stocks of the SLK cell line used in KSHV research (Stürzl *et al.*, 2013). We found that HS and EphA2 were required for infection of both Caki-1 and HeLa cells, while αV- and β1-family integrins were dispensable. Interestingly, we also found that FAK and the intracellular domain of EphA2 were not required for infection of these cells,

despite a reliance on dynamin-mediated endocytosis. Moreover, the ectopic expression of EphA5 and overexpression of EphA4 and EphB2 promoted infection in *EPHA2* knock out (KO) cells but knock out of endogenous EphA4 lead to an elevated infection rate in both WT and EPHA2 KO contexts. Finally, we also showed that infection of primary gingival keratinocytes (PGKs) was unaffected by integrin- or Eph-blocking reagents, which together with experiments reported by us and others strongly suggests the existence of yet another unknown KSHV receptor which could trigger intracellular signaling and virion uptake in all three of the cell types we investigated. Overall, our studies revealed a novel KSHV infection mode that is independent of integrins α3β1, αVβ3, and αVβ5 and suggest that Eph receptors may play more diverse and complex roles during infection than was previously known.

## 2.2 Results

### Caki-1 and HeLa cells express most known KSHV receptors.

It has been shown that KSHV uses a multimolecular complex of attachment molecules and receptors, including HS, EphA2, xct, DC-SIGN (in some immune cells), and the integrin heterodimers α3β1, αVβ3, and αVβ5, to enter cells in several different infection models (reviewed in Kumar *et al.*, 2016a). The expression of these known KSHV receptors on the surface of Caki-1 and HeLa cells was examined by flow cytometry. Most of the KSHV receptors were expressed on the surface of both cell lines: EphA2, HS, and integrin subunits α3, αV, β1, and β5 (summarized in Fig. 2.1 and in detail in Fig. 2.2). Integrin β3 was additionally detected on the surface of Caki-1 cells but not HeLa cells (Figs. 2.1, 2.2). However, neither the myeloid cell marker DC-SIGN nor xct were detected on the surface of either cell line (Figs. 2.1, 2.2).

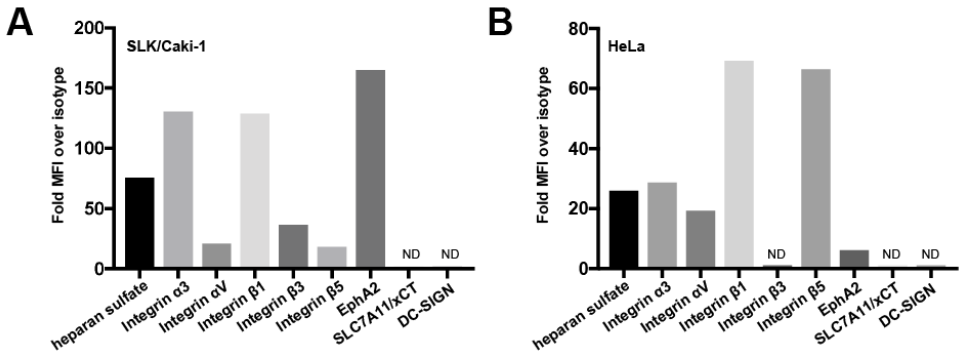



Figure 2.1. Surface expression of known KSHV receptors on Caki-1 and HeLa cells. Live Caki-1 (A) and HeLa (B) cells were tested for surface expression of known KSHV receptors by immunostaining and flow cytometry. The mean fluorescence intensity (MFI) of each receptor stain was normalized to that of the appropriate primary antibody isotype control. ND, not detected.

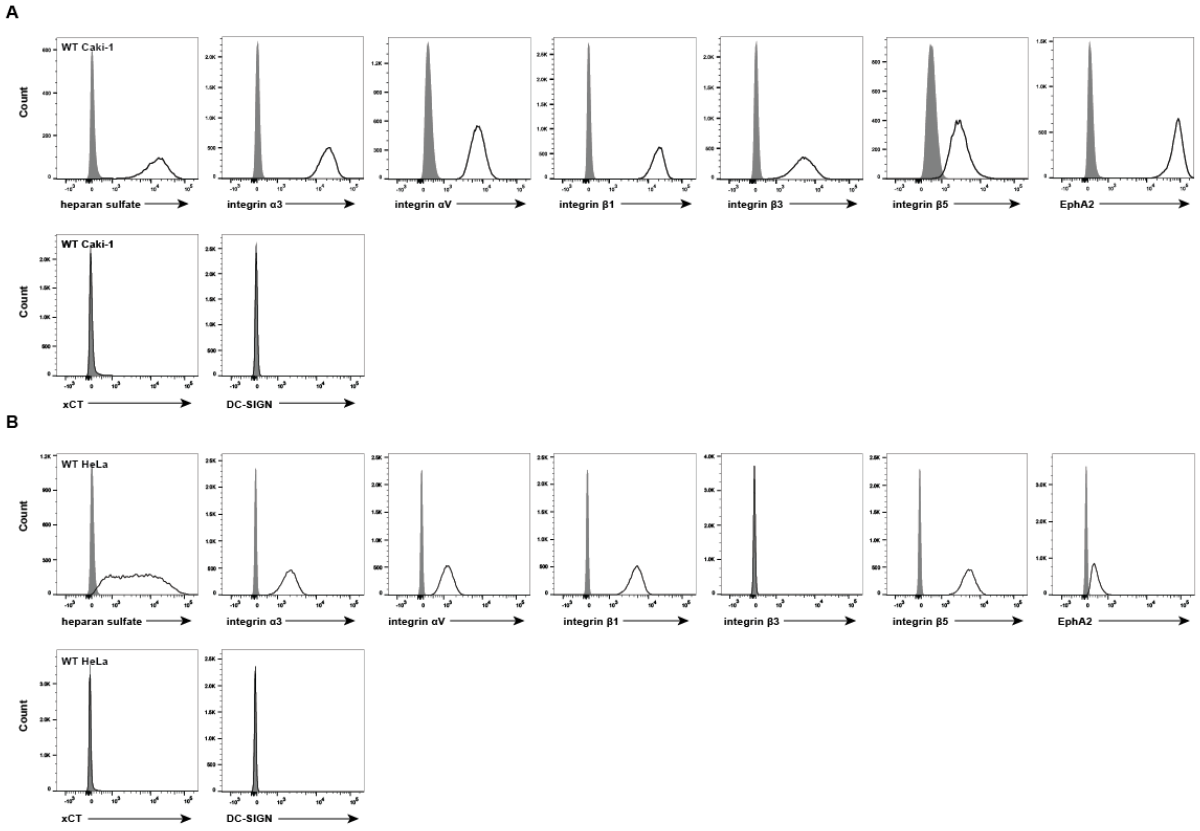



Figure 2.2. Detailed receptor expression histograms for WT Caki-1 and HeLa cells. Cells were stained for the indicated receptors and analyzed by flow cytometry. Grey histograms represent isotype controls.

## Heparan sulfate interactions are required for KSHV infection of Caki-1 and HeLa cells.

The role of HS in adhering virions to the cell surface and promoting viral entry is well documented across many virus families. Caki-1 and HeLa cells express HS on the cell surface and we expected this proteoglycan to play a major role during KSHV infection. We have previously shown that a deficiency in the enzyme Ext1 rendered cells unable to synthesize HS (Jarousse *et al.*, 2008), so we could use *EXT1* KO cells to confirm the requirement for HS during KSHV entry. An *EXT1*-specific guide sequence was cloned into px330, a Cas9 and sgRNA delivery plasmid, which was then transfected into Caki-1 cells (Table 2.1). After four days, a subpopulation of HS-low mutant cells was discernable by flow cytometry. The mutant population was purified by fluorescence-activated cell sorting (FACS), then passaged until the immunostaining of HS in the pool decayed to isotype levels (Fig. 2.3A).

The purified *EXT1* KO Caki-1 pool and WT Caki-1 cells were infected with KSHV.BAC16 which encodes a constitutive GFP reporter (Brulois *et al.*, 2012), and the

infection percentage was quantified by measuring GFP+ cells by flow cytometry after two days. As expected, the infection rate of HS-deficient cells was drastically reduced compared to WT cells (Fig. 2.3B).

As an orthogonal approach, we used soluble heparin to competitively block KSHV infection, as this has been used extensively to investigate HS usage in a variety of cell types (Akula *et al.*, 2001a, Birkmann *et al.*, 2001, Akula *et al.*, 2003, Kerur *et al.*, 2010). Purified virus was pre-incubated with increasing concentrations of heparin and then used to infect Caki-1 cells. In agreement with our results using *EXT1* KO cells, soluble heparin inhibited infection in a dose-dependent manner but approached a non-zero asymptote (Fig. 2.3C). We additionally used the 500 ug/mL concentration to pre-block KSHV before infecting HeLa cells, which completely blocked infection (Fig. 2.3D) Collectively, these results show that HS is required for efficient infection of both Caki-1 and HeLa cells and underscore the value of CRISPR-Cas9 to study viral receptors.

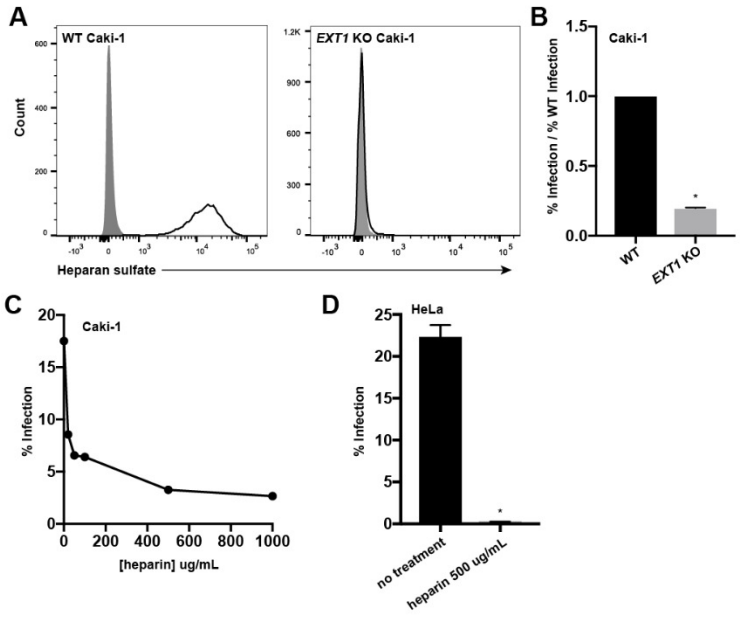



Figure 2.3. Heparan sulfate interactions are required for infection of Caki-1 and HeLa cells. (A) WT and *EXT1* KO Caki-1 cells were immunostained for surface heparan sulfate (HS) expression. Grey histograms represent isotype controls. (B) WT and *EXT1* KO Caki-1 cells were infected with KSHV in duplicate and infection rates were measured by flow cytometry. The infection rate of the KO was normalized to the average WT infection rate and data was pooled from multiple experiments. (C) Filtered KSHV was pre-incubated with the indicated concentrations of soluble heparin which was maintained during infection. Infection rates were measured by flow cytometry. (D) Filtered KSHV was pre-blocked with the indicated concentration of heparin, then used to infect WT HeLa cells in triplicate for two hours. Infection percentage was measured by flow cytometry two days post infection. *, $p < 0.05$.

### Single-cell clones of *EXT1* KO Caki-1 cells lose KSHV infection phenotype.

*EXT1* KO Caki-1 cells from the pool enriched in Fig. 2.3A were single-cell cloned in order to isolate a clonal population with which to perform additional experiments. The

vast majority of isolated clones lacked surface HS expression, two of which are shown (Fig. 2.4A). To confirm that these clones maintained the phenotype of the parent population, WT Caki-1 cells and two clonal *EXT1* KO Caki-1 cell lines were infected with KSHV. Surprisingly, we found that these two *EXT1* KO clones and others (data not shown) were infected at rates similar to WT Caki-1 cells, despite the total loss of HS at the cell surface (Fig. 2.4B). This was an extremely puzzling result, but was the same in every clone we tested, so we concluded that the process of single-cell cloning may have universally triggered a homeostatic change in the context of *EXT1* KO. We hypothesized that a different glycosaminoglycan may have been upregulated which is able to compensate for the loss of HS and which can promote KSHV entry in the absence of HS, but were not able to investigate this phenomenon further.

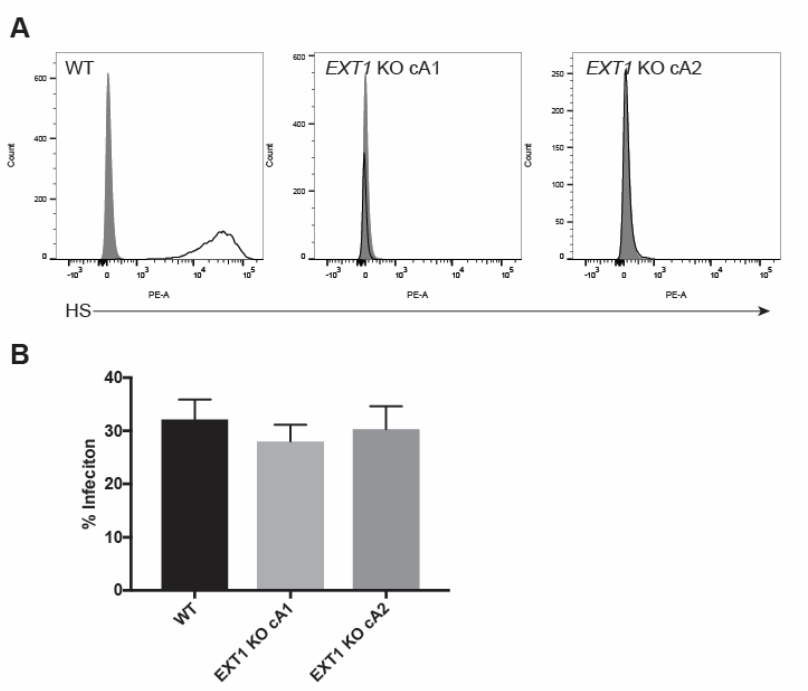



Figure 2.4. Cloned *EXT1* KO Caki-1 cells lose KSHV infection phenotype. (A) WT, *EXT1* KO clone A1, and *EXT1* KO clone A1 Caki-1 cells were immunostained for surface HS expression and analyzed by flow cytometry. Grey histogram represents matched isotype control. (B) WT, *EXT1* KO clone A1, and *EXT1* KO clone A1 Caki-1 cells were infected with KSHV and infection percentage was analyzed after two days by flow cytometry.

### KSHV infection of Caki-1 and HeLa cells is independent of canonical KSHV integrin receptors.

KSHV coordinates several integrin heterodimers to initiate signaling events that are required for infection of fibroblasts and endothelial cells (reviewed in Kumar *et al.*, 2016a). Because we observed the expression of all proposed integrin receptors for KSHV at the surface of Caki-1 cells and all, except integrin β3, on the surface of HeLa cells (Figs. 2.1,

2.2), we investigated whether these integrins were required for KSHV to infect these cell lines. Both the α and β subunits contribute to the unique ligand-binding surface of a given integrin heterodimer. Therefore, we reasoned that infecting cells with reciprocal subunits of KSHV-associated integrins knocked out would reveal precisely which heterodimers were required for infection.

Single KO pools of integrins α3, αV, β1, β3, and β5 were created by transfection of Caki-1 cells with px330 plasmids containing guide sequences that targeted the genes encoding each integrin subunit (Table 2.1). The mutant populations were enriched as described for *EXT1* to generate integrin KO Caki-1 pools (Fig. 2.5A). Lacking integrin αV protein, *ITGAV* KO cells lost the ability to adhere to tissue-culture treated polystyrene dishes, but normal morphology and growth returned when they were plated on fibronectin-coated plates. The *ITGAV* KO cells were grown on fibronectin for passaging and infection experiments. The infection rates of both WT Caki-1 and HeLa cells were unchanged in the presence of a fibronectin coat (data not shown). WT HeLa cells were additionally transfected with px330 plasmids targeting *ITGAV or ITGB1*, but left unpurified. The cells were passaged until the receptor staining of the mutant population decayed to near isotype levels, generating the mixed integrin KO pool that we used for our experiments. (Fig. 2.5G). The mixed *ITGAV* KO HeLa pool was also grown on fibronectin.

WT Caki-1, the single integrin KO Caki-1 pools, and the mixed integrin KO HeLa cell pools were then infected with KSHV. The mixed KO HeLa pools were additionally stained for the appropriate integrin at the cell surface to allow for gating on WT and KO subpopulations. Overall, the infection rates of the integrin KO pools or subpopulations were not significantly reduced compared to that of WT cells for both cell lines (Fig. 2.5B, 2.5H). The slight decline in infection rate of the *ITGAV* KO HeLa subpopulation compared to the WT subpopulation reached statistical significance, but the magnitude of difference was similar to the other integrin KO Caki-1 pools. Since the KO pools were enriched by FACS, it is likely that there are still a small number of cells that express WT levels of each integrin receptor. Nevertheless, these data suggest that KSHV infection of Caki-1 and HeLa cells does not require integrin α3β1, αVβ3, or αVβ5 alone, or any other single integrin in the αV and β1 families.

Although targeting single integrins has yielded clear infection defects in past studies (Akula *et al.*, 2002, Garrigues *et al.*, 2008, Veettil *et al.*, 2008, Garrigues *et al.*, 2014b), we considered that our strategy of knocking out individual integrin subunits would not reveal fully redundant involvement of α3β1, αVβ3, and αVβ5 during infection of Caki-1 cells. To address this, an *ITGA3*/*ITGAV* double KO Caki-1 pool was generated to effectively remove integrin α3β1 and the entire integrin αV family, including αVβ3 and αVβ5, from the cell surface (Fig. 2.5C). This *ITGA3*/*ITGAV* DKO was enriched but not purified, since a very small population of cells expressing WT levels of integrin α3 was still

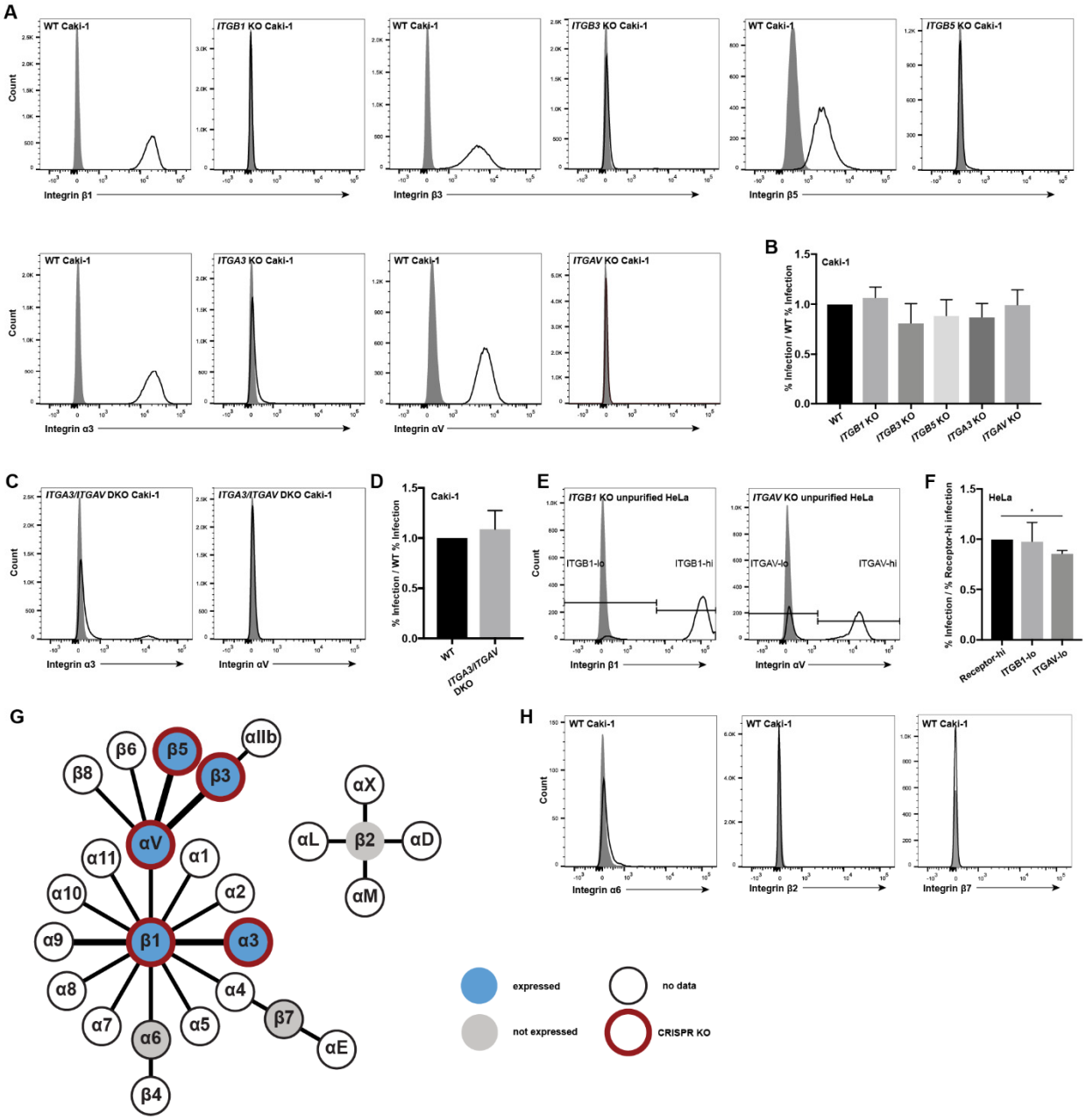



Figure 2.5. Integrins are not required for infection of Caki-1 and HeLa cells. (A, H) WT and indicated integrin subunit KO Caki-1 cells were immunostained for surface expression of the indicated integrins. Grey histograms represent isotype controls. (B) WT and integrin KO Caki-1 pools were infected with KSHV in duplicate and infection rate was quantified by flow cytometry. The infection rates of the KO pools were normalized to the average WT infection rate and data was pooled from multiple experiments. (C) *ITGA3*/*ITGAV* double KO Caki-1 cells were immunostained for surface integrin α3 and αV expression. Grey histograms represent the isotype controls. (D) WT and *ITGA3*/*ITGAV* double KO Caki-1 cells were infected with KSHV in triplicate and infection rate was quantified by flow cytometry. The infection rate of the DKO pool was normalized to the average WT infection rate and data was pooled from multiple experiments. (E) Mixed *ITGAV* and *ITGB1* KO HeLa cells were immunostained for surface integrin αV and β1 expression. Grey histograms represent the isotype controls. (F) Mixed integrin KO HeLa pools were infected with KSHV

in triplicate and infection rates were quantified by flow cytometry. The pools were also immunostained for the corresponding integrins and gated on integrin-high or -low populations as indicated in (E). The infection rates of integrin-low cells were normalized to integrin-high cells in each well and data was pooled from multiple experiments. (G) Schematic integrin pairing diagram (adapted from Hynes, 2002) showing expression data measured by surface immunostaining and flow cytometry. Bold connections denote heterodimers previously implicated in KSHV infection. *, $p < 0.05$.

visible by flow cytometry (Fig. 2.5C). WT and *ITGA3*/*ITGAV* double KO Caki-1 cells were then infected with KSHV. Still, the infection rate of *ITGA3*/*ITGAV* double KO Caki-1 cells was not reduced compared to WT cells (Fig. 2.5D). These results further indicate that integrins α3β1, αVβ3, and αVβ5 are not required for KSHV infection of Caki-1 cells.

We also considered whether our genetic disruptions in the integrin network were altering the expression level of other KSHV receptors, potentially obscuring an infection defect in integrin subunit KO cells. To address this, we examined the expression of all known KSHV receptors on *ITGB1* KO and *ITGAV*/*ITGA3* DKO Caki-1 cells (Fig. 2.6). We observed that *ITGB1* KO cells lost surface expression of integrin α3, which is not unexpected since integrin α3 does not bind to any other known integrin β subunits (Fig. 2.6). Likewise, we found that *ITGAV*/*ITGA3* DKO Caki-1 cells lost surface expression of integrins β3 and β5 (Fig. 2.6). Otherwise, we did not observe any large changes in the surface expression of unrelated integrin subunits, HS, or EphA2 (Fig. 2.6).

Past studies have utilized integrin-blocking reagents to show that certain classes of integrins are required for KSHV entry in a variety of cell types (Akula *et al.*, 2002, Wang *et al.*, 2003, Veettil *et al.*, 2008, Garrigues *et al.*, 2008). However, at least three publications have reported that several integrin-blocking reagents failed to inhibit KSHV infection in HEK239 and SLK cells (Inoue *et al.*, 2003, Hahn *et al.*, 2012, Walker *et al.*, 2014). To confirm that our results were not unique to the CRISPR-Cas9 KO approach, we repeated key integrin-blocking methods from these publications. WT Caki-1 and HeLa cells were pre-incubated with the RGD-containing integrin ligand fibronectin, the non-RGD-containing integrin ligand laminin, GRGDSP and GRGESP peptides, or a 50% DMSO control for the peptide resuspension solution for one hour, then infected with KSHV for two hours. Infection rate was quantified by flow cytometry two days post infection. Fibronectin, which contains an RGD sequence and binds αV-family integrins, did not significantly alter infection rate of either cell line (Fig. 2.7). Laminin, which binds to a subset of integrins including α3β1, slightly inhibited infection of HeLa cells but not Caki-1 cells (Fig. 2.7). Neither the RGD-containing peptide GRGDSP nor the control peptide GRGESP significantly affected KSHV infection of HeLa cells (Fig. 2.7). GRGDSP very slightly inhibited infection of Caki-1 cells, but the effect was not significantly different compared to GRGESP, suggesting that the inhibitory effect of the peptide was nonspecific, which has been previously suggested (Inoue *et al.*, 2003) (Fig. 2.7). Overall,

we found that these blocking reagents had little or no effect on KSHV infection in Caki-1 and HeLa cells which is consistent with the results of our KO studies.

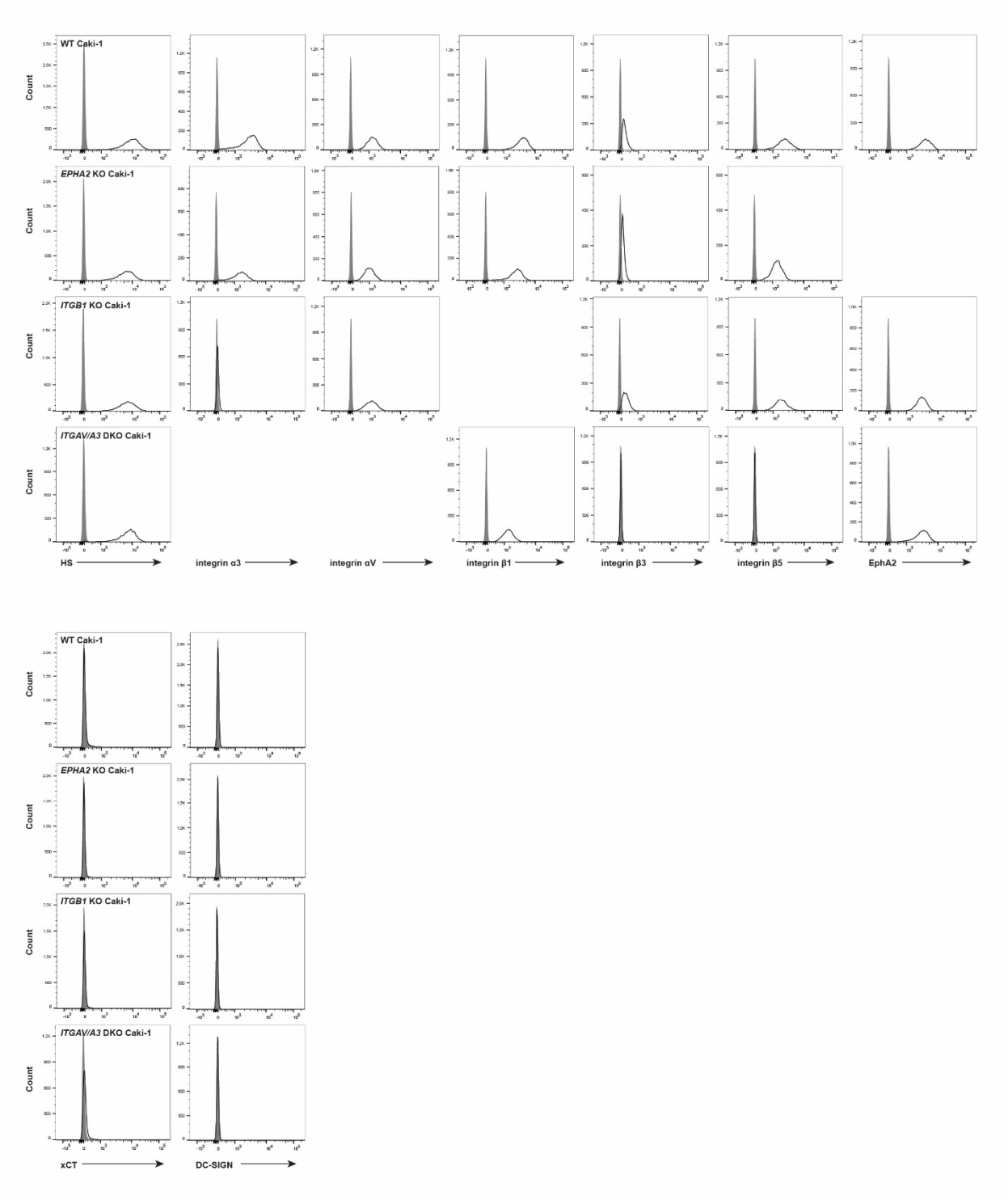



Figure 2.6. Perturbations in KSHV receptor expression do not affect other known receptors. WT, *EPHA2* KO, *ITGB1* KO, and *ITGAv*/*ITGA3* DKO cells were concurrently immunostained for all known KSHV receptors and analyzed by flow cytometry. Grey histogram represents the matched isotype control.

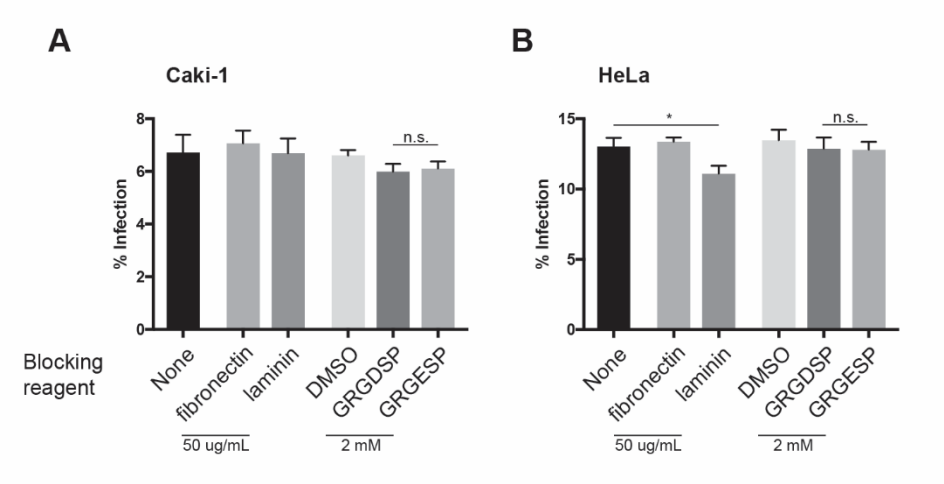



Figure 2.7. Integrin-blocking reagents have mild effects on the infection of Caki-1 and HeLa cells. WT Caki-1 (A) or HeLa (B) cells were pre-incubated with media alone, 50 μg/mL of fibronectin or laminin in media, or 2 mM of the peptides GRGDSP, GRGESP, or a volume control of 50% DMSO for one hour at 4°C. Cells were subsequently washed and infected with KSHV for two hours. Percent infection was measured after two days by flow cytometry.

A non-RGD-binding integrin, α9β1, has been shown to bind a disintegrin-like domain (DLD) in KSHV gB and is important for infection of HFF and primary microvascular endothelial cells, but not HEK293 cells (Walker *et al.*, 2014). Our data demonstrate that KSHV infection of Caki-1 and HeLa cells is independent of the twelve β1-containing integrins and the five αV-containing integrins, however we considered that other integrins could still be required for KSHV infection of Caki-1 cells. There are eight integrins that do not contain the αV or β1 subunits: αIIbβ3, α6β4, α4β7, αEβ7, and four β2-containing integrins (Fig. 2.5F). Neither integrin α6, integrin β7, nor integrin β2 were detected on the surface of WT Caki-1 cells by flow cytometry (Fig. 2.5E). Additionally, integrin β3 was lost from the cell surface of *ITGAV*/*ITGA3* DKO Caki-1 cells implying that integrin αIIbβ3 is not expressed in Caki-1 cells (Fig. 2.6). Altogether these data indicate that none of the eight non-αV, non-β1 integrin heterodimers are expressed in Caki-1 cells, so these integrins are unlikely to play a role in this KSHV infection mechanism in the absence of αV- or β1-family integrins.

### FAK inhibitors do not affect KSHV infection of Caki-1 or HeLa cells.

In HFF and microvascular endothelial cells, FAK is a key effector activated downstream of the interaction between KSHV gB and integrin receptors (reviewed in Kumar *et al.*, 2016a). Since we found that infection of Caki-1 and HeLa cells did not depend on canonical KSHV integrin receptors, we asked whether FAK activation was necessary for infection through the use of inhibitors.

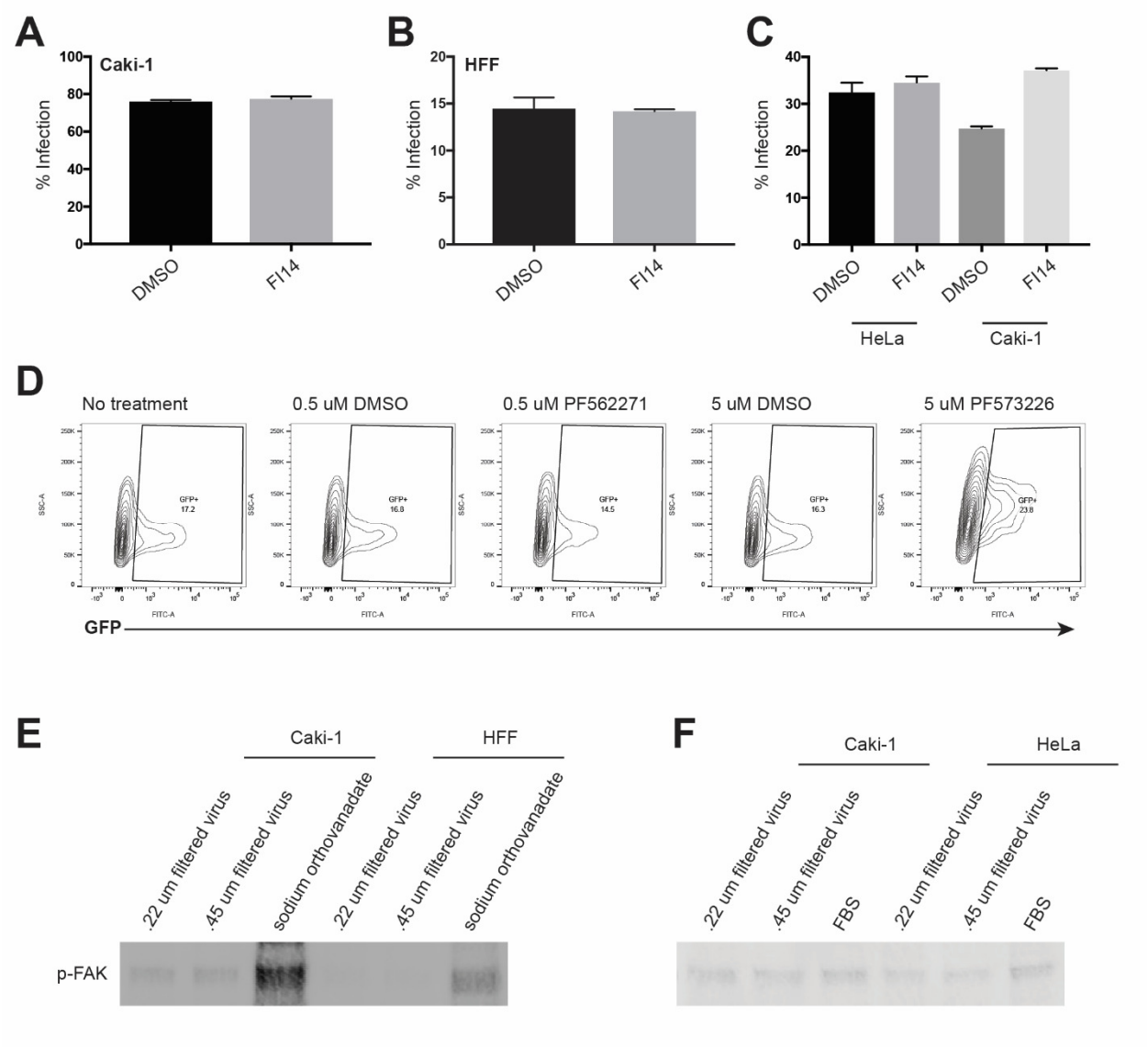



Figure 2.8 FAK inhibitors are not sufficient to block KSHV infection in multiple cell lines. Caki-1 (A, C), HFF (B), and HeLa (C) cells were pre-treated with 5 μM FI14 or a volume control of DMSO for one hour at 37°C. Cells were then infected with KSHV for four hours in the presence of the same concentration of FI14 or DMSO. Infection percentage was quantified by flow cytometry two days post infection. (D) Caki-1 cells were pre-treated with the indicated concentrations of PF562271, PF573226, or the appropriate DMSO volume controls for one hour at 37°C. Cells were then infected with KSHV for four hours in the presence of the same concentrations of the indicated drugs or DMSO. Infection percentage was quantified by flow cytometry two days post infection. (E) HFF or Caki-1 cells were serum starved for 24 hours, then treated with sterilized viral supernatant, viral supernatant, or sodium orthovanadate for 10 minutes. Whole cell lysates were run on an SDS-PAGE gel and blotted for p-FAK at Y-397. (F) HeLa or Caki-1 cells were serum starved for 24 hours, then treated with sterilized viral supernatant, viral supernatant, or 100% FBS for 10 minutes. Whole cell lysates were run on an SDS-PAGE gel and blotted for p-FAK at Y-397.

Caki-1, HeLa, and HFF cells were pre-treated with FAK Inhibitor 14 (FI14) at 5 μM or a volume control of DMSO for one hour. The cells were then infected with KSHV while maintaining the drug concentration for an additional four hours. Cells were then washed and cultured in normal culture medium until infection percentage was analyzed two days later. We found that FI14 had no effect on infection of Caki-1, HeLa, and surprisingly HFF cells (Figs. 2.8A, 2.8B, 2.8C). This was concerning, as the role of FAK in HFF infection has been well-defined by decades of research (reviewed in Kumar *et al.,* 2016a). We additionally tested the effects of two other FAK inhibitor compounds (PF562271 and PF573226) on Caki-1 infection, but we were unable to detect any significant impairment of KSHV infection (Fig. 2.8D).

Since our experiments with HFF cells were not in agreement with previous studies, we attempted to replicate additional experiments from the literature in which FAK phosphorylation was detected by western blot. Caki-1 and HFF cells were serum-starved for 24 hours, then treated with sterilized viral supernatant, viral supernatant, or the phosphatase inhibitor sodium orthovanadate as a positive control for 10 minutes. After 10 minutes, cells were put on ice, washed three times, and lysed for protein immediately. A small amount of phosphorylated FAK was detected by western blot which did not increase upon treatment with viral supernatant but was enhanced by treatment with sodium orthovanadate (Fig. 2.8E). This indicated that while the antibody was specific for phosphorylated FAK, KSHV virions were not inducing FAK phosphorylation above the baseline level. Fetal bovine serum (FBS) alone activates FAK through the extracellular matrix proteins it contains and has been used as a positive control for FAK activation. Thus, we again serum-starved Caki-1 and HeLa cells for 24 hours, then treated the cells with sterilized viral supe, raw vrial supe, and FBS. However, we found that, like treatment with KSHV supe, FBS failed to induce FAK phosphorylation above baseline (Fig. 2.8F). Since we were unsuccessful in replicating previously reported results using HFF cells, we cannot confidently conclude that infection of Caki-1 and HeLa cells does not require FAK. This question should be further investigated, as FAK is a major hub for cytoskeletal organization and has been reported to be involved in cellular responses downstream of Eph receptors (Miao *et al.,* 2000, Carter *et al.,* 2002 Shi *et al.,* 2009, and reviewed in Zhao *et al*., 2011)

**EphA2 is necessary for KSHV infection of Caki-1 and HeLa cells.**

EphA2 has been well-characterized as a receptor for KSHV and binds to the envelope glycoprotein complex gH/gL (Hahn *et al.*, 2012, Chakraborty *et al.*, 2012, Hahn *et al.*, 2013 Hahn *et al.*, 2014, Großkopf *et al.*, 2018). Together with integrins, EphA2 helps propagate virus-induced signaling and mobilize endocytosis effectors which leads to viral entry in multiple cell types (Hahn *et al.*, 2012, Chakraborty *et al.*, 2012, Dutta *et al.*, 2013, Bandyopadhyay *et al.*, 2014a, Bandyopadhyay *et al.*, 2014b, Wang *et al.*, 2017). However, we found that KSHV infection of Caki-1 and HeLa cells does not require canonical KSHV

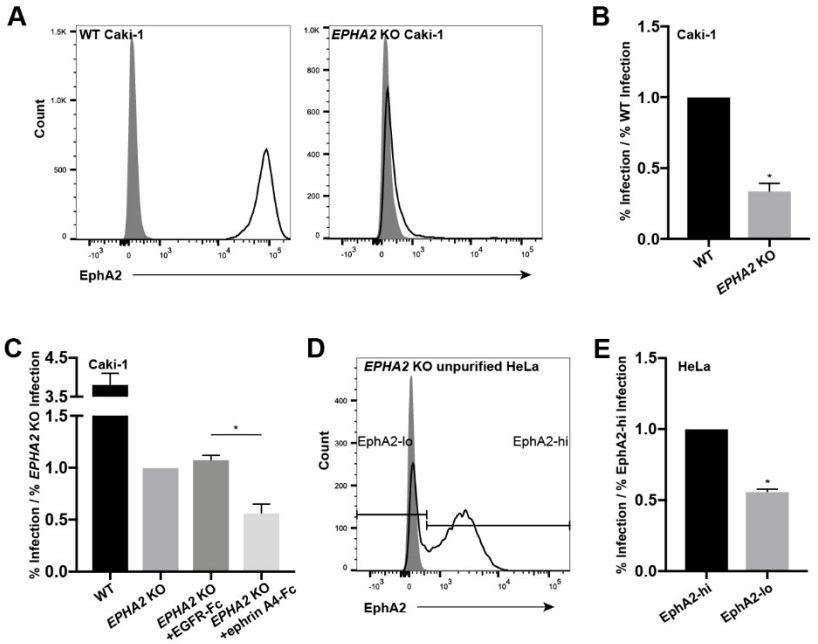



Figure 2.9. EphA2 is required for efficient infection of Caki-1 and HeLa cells. (A) WT and *EPHA2* KO Caki-1 cells were immunostained for surface EphA2 expression. Grey histogram represents isotype control. (B) WT and *EPHA2* KO Caki-1 cells were infected with KSHV in duplicate and infection rates were quantified by flow cytometry. The infection rate of the *EPHA2* KO pool was normalized to the average WT infection rate and data was pooled from multiple experiments. (C) *EPHA2* KO Caki-1 cells were pre-blocked with EGFR-Fc, ephrin-A4-Fc, or an equal volume of PBS in media at 10 μg/mL and then infected in triplicate in the presence of EGFR-Fc or ephrin-A4-Fc at 5 μg/mL. Infection rate was measured by flow cytometry and infection rates were normalized to the average *EPHA2* KO infection rate. (D) Mixed *EPHA2* KO HeLa cells were immunostained for surface EphA2 expression. Grey histograms represent the isotype controls. (E) The mixed *EPHA2* KO HeLa cells were infected with KSHV in triplicate and GFP+ cells were quantified by flow cytometry. The cells were also immunostained for EphA2 and gated on EphA2-high or -low as indicated in (D). The infection rates of EphA2-low cells were normalized to EphA2-high cells in each well and data was pooled from multiple experiments. *, $p < 0.05$.

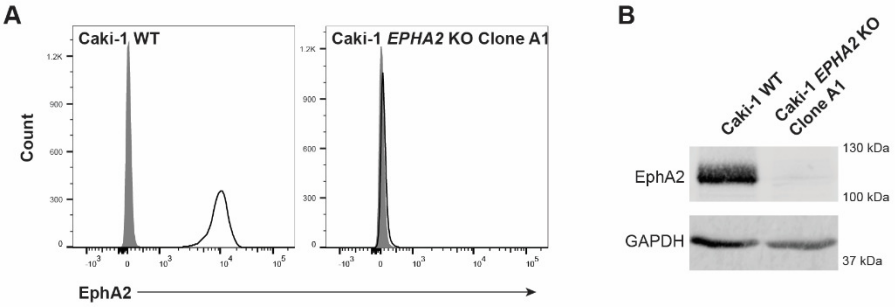



Figure 2.10. Caki-1 *EPHA2* KO Clone A1 lacks EphA2 protein expression. (A) WT Caki-1 and *EPHA2* KO Clone A1 were immunostained for surface EphA2 with a second antibody and analyzed by flow cytometry. Filled grey histogram represents isotype control. (B) Whole lysate from WT Caki-1 and *EPHA2* KO Clone A1 were run on an SDS-PAGE gel and blotted for EphA2 and GAPDH.

integrin receptors, so we investigated whether EphA2 was required for infection in these cell lines.

Caki-1 cells were transfected with a px330 plasmid containing a guide sequence targeting *EPHA2* and an *EPHA2* KO pool was enriched as described for EXT1 (Table 2.1, Fig. 2.9A). In addition, a mixed WT/*EPHA2* KO pool was generated in HeLa cells as described for *ITGAV* and *ITGB1* (Fig. 2.9D). WT and *EPHA2* KO Caki-1 cells and the mixed WT/KO HeLa pool were then infected with KSHV. The mixed WT/KO HeLa pool was additionally stained for surface EphA2 expression to distinguish the KO and WT subpopulations. *EPHA2* KO cells were significantly less susceptible to infection compared to WT cells in both cell lines, though they were not completely resistant to the virus (Figs. 2.9B, 2.9E). These results indicate that EphA2 is necessary for efficient infection of both Caki-1 and HeLa cells.

To ensure that the KO of *EPHA2* did not alter the expression of any other known KSHV receptors, WT and *EPHA2* KO Caki-1 cells were examined for surface receptor expression by flow cytometry. We did not observe any unexpected changes in the surface expression of any other known receptors (Fig. 2.6).

A prior study demonstrated that KSHV gH/gL has the ability to bind other Eph receptors besides EphA2 in multiple assays (Hahn *et al.*, 2013). We hypothesized that the residual KSHV infection of *EPHA2* KO cells could depend on other Eph receptors that may be expressed by Caki-1 cells. A clonal *EPHA2* KO Caki-1 cell line was isolated from single-cell clones of the *EPHA2* KO pool and was used for this experiment. The clone lacked surface expression of EphA2 and the infection defect compared to WT cells was similar to the parent population (Figs. 2.9C, 2.12E). To ensure this *EPHA2* KO clone did not produce EphA2 protein, surface and total EphA2 were examined by flow cytometry and western blot, respectively, using a second EphA2-specific antibody (Fig. 2.10).

To test whether additional Eph receptors were required for infection in these cells, we attempted to block KSHV infection using ephrin-A4 or the unrelated protein EGFR, as reported previously (Hahn et al., 2012 and Hahn et al., 2013). Clonal *EPHA2* KO cells were pre-incubated with soluble forms of either the A-type Eph ligand ephrin-A4 or EGFR as a control, then infected with KSHV in the presence of these blocking agents. The infection rate of *EPHA2* KO cells was further reduced in the presence of ephrin-A4-Fc compared to the unrelated EGFR-Fc (Fig. 2.9C). Since ephrin ligands, including ephrin-A4, can broadly bind to and block interactions with Eph receptors of the same type, these data suggest that another A-type Eph receptor may be required for infection of Caki-1 cells in the absence of EphA2.

### EphA4 and EphB2 are dispensable for KSHV infection in Caki-1 cells.

Since we found that residual infection in *EPHA2* KO Caki-1 cells could be further blocked by a soluble ephrin ligand, we investigated whether additional Eph receptors

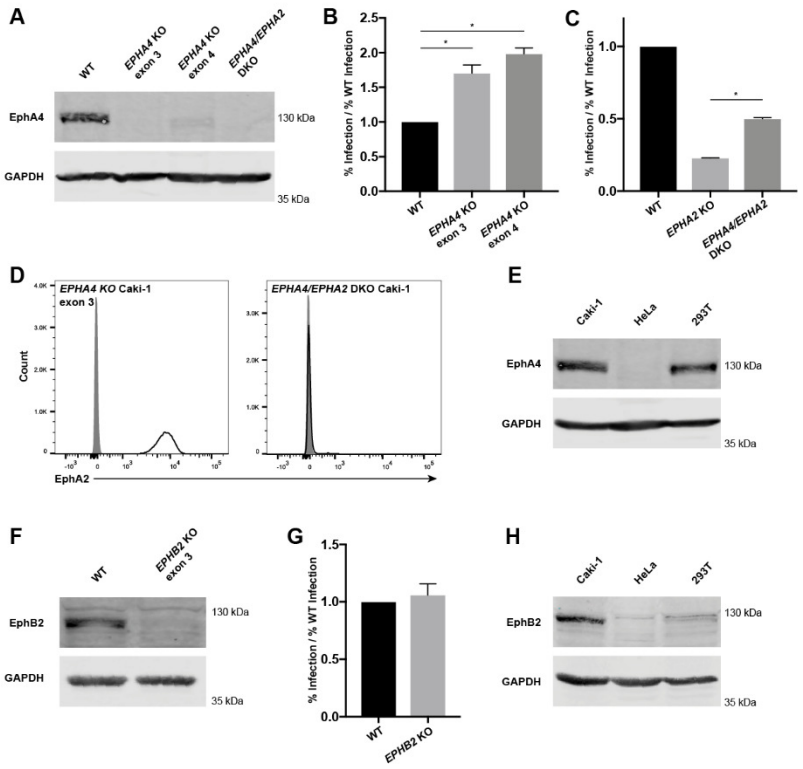



Figure 2.11. EphA4 and EphB2 are dispensable for infection of Caki-1 cells. (A, E, H) 120 μg or (F) 50 ug of the indicated cell lysates were run on a 10% SDS-PAGE gel and blotted for EphA4 (A, E) or EphB2 (F, H) and GAPDH as a loading control. WT and *EPHA4* KO Caki-1 cells (B), or WT, *EPHA2* KO, and *EPHA4*/*EPHA2* DKO Caki-1 cells (C) were infected with KSHV in triplicate and infection rate was quantified by flow cytometry. The infection rates of KO cell lines were normalized to the average infection rate of WT cells and a representative experiment is shown. (D) *EPHA4* KO and *EPHA4*/*EPHA2* DKO Caki-1 cells were immunostained for surface EphA2 expression. Grey histograms represent isotype controls. (G) WT and *EPHB2* KO Caki-1 cells were infected with KSHV in triplicate and infection rates were quantified by flow cytometry. The infection rates of the KO line were normalized to the average WT infection rate and a representative experiment is shown. *, $p < 0.05$.

were expressed by Caki-1 cells and if they were required for KHSV infection of Caki-1 cells. EphA4 and EphB2 transcripts were found in an RNA sequencing dataset from iSLK.219 cells (C. Arias, personal communication) and we confirmed the expression of these two proteins by western blot (Figs. 2.11E, 2.11H).

WT Caki-1 cells were transfected with px330 plasmids containing guide sequences targeting *EPHA4* and *EPHB2* (Table 2.1). We were unable to find an antibody that reliably detected EphA4 or EphB2 by surface immunostaining of live cells, so single-cell clones were derived from the transfected populations and screened for loss of EphA4 or EphB2 by western blot. Two *EPHA4* KO Caki-1 cell lines and one *EPHB2* KO Caki-1 cell line were isolated (Figs. 2.11A, 2.11F). WT, *EPHA4* KO, and *EPHB2* KO Caki-1 cell lines were then infected with KSHV. Surprisingly, the infection rate of *EPHA4* KO cells was elevated

compared to WT cells, while the infection rate of *EPHB2* KO cells was not significantly different (Figs. 2.11B, 2.11G). The same results were observed in *EPHA4* and *EPHB2* KO Caki-1 cell pools created with a lentiviral CRISPR-Cas9 system (data not shown).

To further understand the infection phenotype of *EPHA4* KO cells, one of the *EPHA4* KO Caki-1 cell lines was transfected with the *EPHA2*-targeted px330 plasmid (Table 2.1) and a pool of *EPHA2*/*EPHA4* DKO cells was isolated by FACS (Figs. 2.11A, 2.11D). When these cells were infected with KSHV, the infection rate was reduced compared to WT cells, but significantly elevated compared to *EPHA2* single KO cells (Fig. 2.11C). These data indicate that either EphA4 is a negative regulator of KSHV infection, or that Caki-1 cells compensate for the loss of EphA4 in a way that enhances KSHV infection.

EphA4 and EphB2 were detected in Caki-1 lysate and EphA4 was additionally found in 293T lysate. Importantly, both of these proteins were lacking in HeLa cell lysate, even though EphA2-independent infection was observed in both Caki-1 and HeLa cells (Figs. 2.11E, 2.11H). Furthermore, these results show that EphA4 and EphB2 are dispensable for KSHV infection and are unlikely to be the functional targets of ephrin-A4-Fc blocking during infection of *EPHA2* KO Caki-1 cells.

**Multiple Eph receptors rescue KSHV infection of EphA2 KO cells.**

Although we found that two endogenous Eph receptors besides EphA2 were not required for KSHV infection in WT Caki-1 cells, the significant infection defect of *EPHA2* KO Caki-1 cells provided an ideal platform to test the effects of transduced Eph receptors on KSHV infection. The clonal *EPHA2* KO Caki-1 cell line, described above, was used for these experiments.

To ensure that expression levels of different Eph receptors could be compared, mature forms of *EPHA2*, *EPHA4*, *EPHA5*, and *EPHB2* lacking endogenous signal peptides were cloned into p3xFlag-CMV-9 following the preprotrypsin leader sequence and a 3xFlag tag (Fig. 2.12A). This cloning scheme ensured that the proteins would be properly oriented in the membrane during translation and ultimately be N-terminally tagged with 3xFlag. The 3xFlag-tagged Eph receptor constructs were cloned into a retroviral vector and transduced into *EPHA2* KO Caki-1 cells.

The 3xFlag tag was detected on the surface of each cell line by flow cytometry, although the magnitude of expression varied with each receptor (Figs. 2.12B, 2.13C). However, when the cell lines were stained for intracellular 3xFlag, the overall expression levels of the receptors appeared to be similar to each other (Fig. 2.12D). Additionally, when the expression of the receptors was examined by western blot, the intensities of the 3x-Flag-tagged bands were similar across the three transduced cell lines (Figs. 2.12C, 2.13A). These data show that all three constructs were expressed to a similar degree but that cell surface trafficking of the three receptors was different.

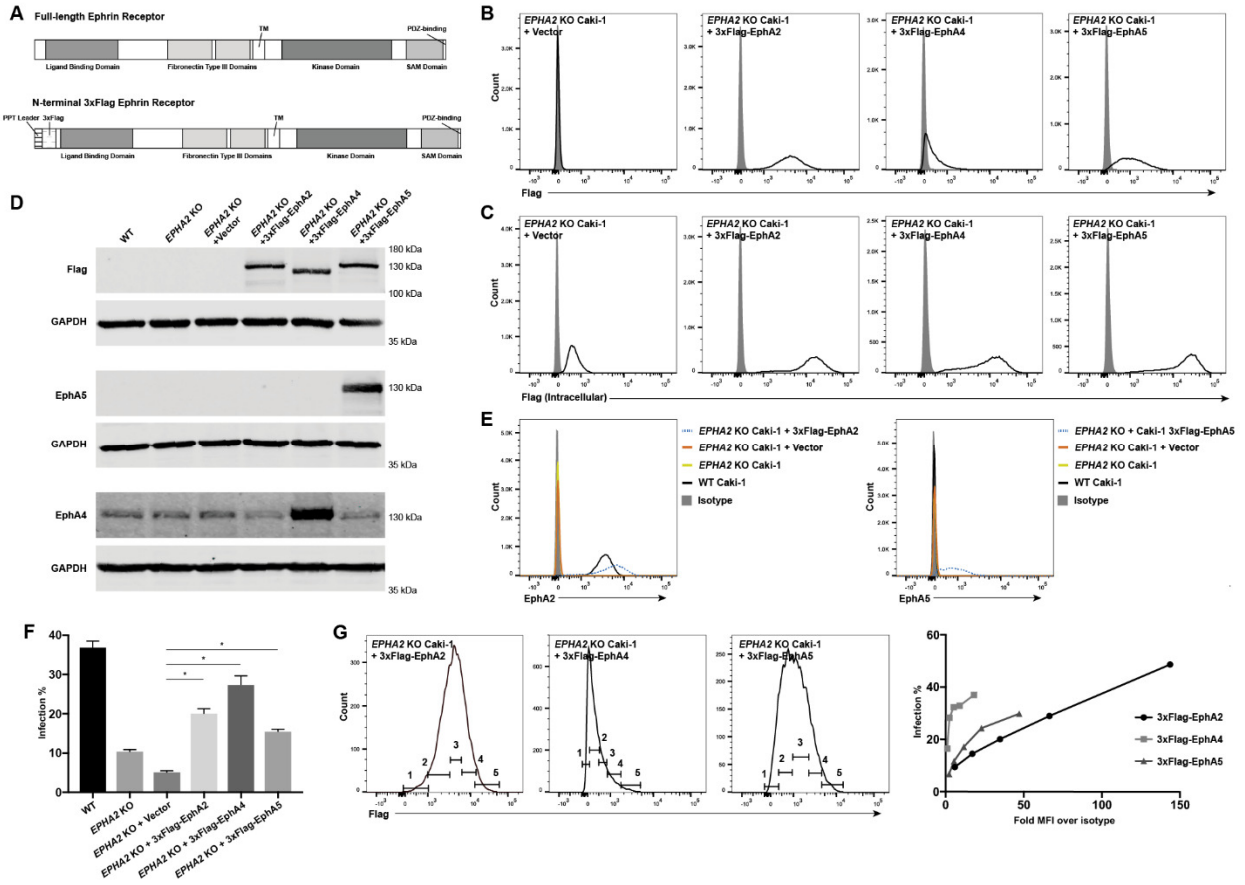



Figure 2.12. EphA2, EphA4, and EphA5 rescue KSHV infection in *EPHA2* KO Caki-1 cells. (A) Diagram of generalized full-length and PPT-3xFlag-mature ephrin receptor constructs. Live (B) or fixed and permeabilized (C) 3xFlag-tagged ephrin receptor transduced *EPHA2* KO cells and a vector control were immunostained for surface (B) or intracellular (C) 3xFlag expression and analyzed by flow cytometry. Grey histograms represent isotype controls. (D) The indicated cell lysates were run on 10% SDS-PAGE gels and blotted for 3xFlag, EphA4, and EphA5 with matched GAPDH as a loading control. For the Flag and EphA5 blots, 15 µg of lysate was loaded. For the EphA4 blot, 120 µg of lysate was loaded. (E) The indicated cell lines were immunostained for surface EphA2 or EphA5 expression and analyzed by flow cytometry. Grey histograms represent isotype controls. (F) The indicated cell lines were infected with KSHV in triplicate and infection rate was quantified by flow cytometry. A representative experiment is shown. (G) The 3xFlag expression histograms of infected 3xFlag-tagged ephrin receptor transduced cell lines were divided into five successive gates as shown. The infection rate within each gate was plotted against the fold MFI over isotype of each gate. *, $p < 0.05$.

The expression of the 3x-Flag-tagged Eph receptors was also compared to the corresponding endogenous protein. The peak of surface expression of transduced 3xFlag-EphA2 was slightly higher than endogenous EphA2 as measured by flow cytometry, but the range of EphA2 expression in the population of 3xFlag-EphA2-transduced cells was much greater compared to WT cells (Fig. 2.12E). EphA5 was not naturally expressed by Caki-1 cells, but EphA5 was readily detected in 3xFlag-EphA5-transduced cells as a wide

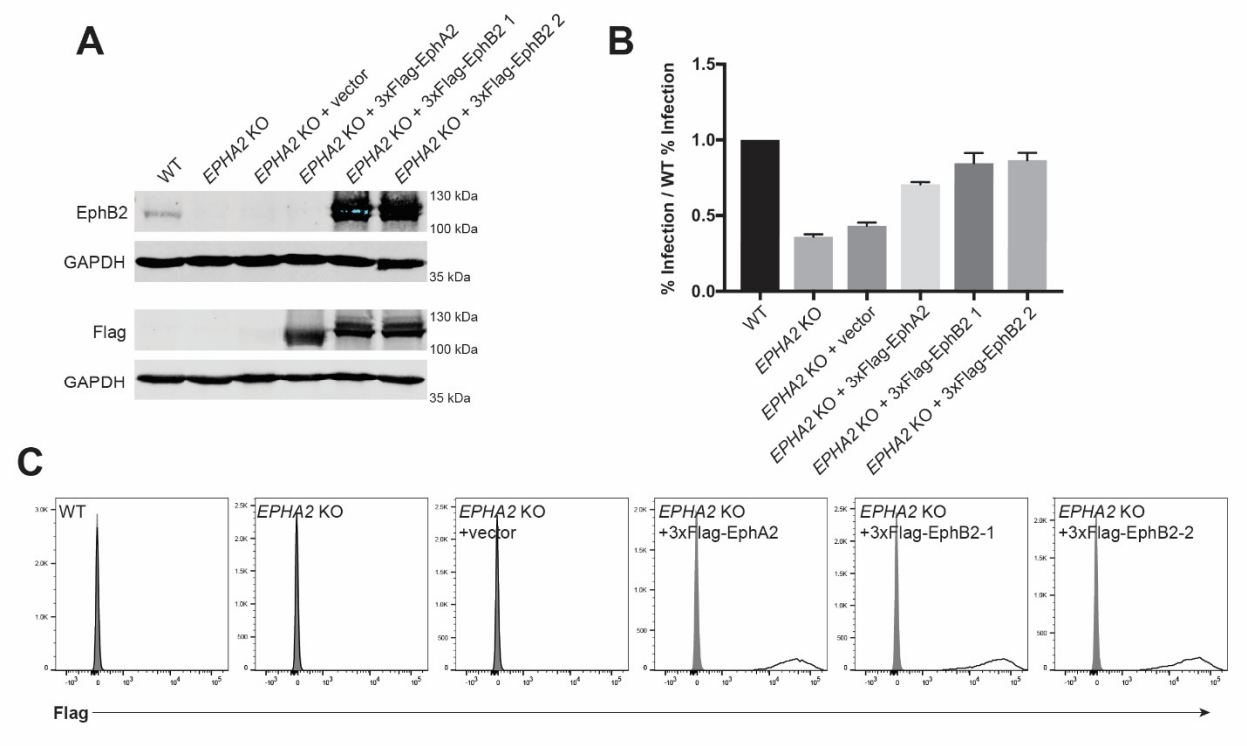



Figure 2.13 EphB2 rescues KSHV infection in *EPHA2* KO Caki-1 cells. (A) Whole cell lysates of WT, *EPHA2* KO, *EPHA2* KO + 3xFlag-EphA2, *EPHA2* KO + 3xFlag-EphB2, and a vector control were run on an SDS-PAGE gel and then blotted for EphB2, Flag, and GAPDH. (B) WT, *EPHA2* KO, *EPHA2* KO + 3xFlag-EphA2, *EPHA2* KO + 3xFlag-EphB2, and vector control Caki-1 cells were infected with KSHV. Infection was measured two days post infection by flow cytometry. (C) WT, *EPHA2* KO, *EPHA2* KO + 3xFlag-EphA2, *EPHA2* KO + 3xFlag-EphB2, and vector control Caki-1 cells were immunostained for surface expression of 3xFlag and analyzed by flow cytometry. Grey histogram represents an isotype control.

peak by flow cytometry and also as a strong band by western blot (Figs. 2.12E, 2.12D). In contrast, EphA4 was found to be expressed endogenously by Caki-1 cells by western blot and the EphA4 band became more pronounced in the 3xFlag-EphA4-transduced cell lysate (Fig. 2.12D). Since we did not find an antibody that reliably detected EphB2 on the cell surface by flow cytometry, we examined the total protein expression level of WT and *EPHA2* KO + 3xFlag-EphB2 by western blot. The transduced EphB2 produced vastly more protein compared to endogenous EphB2 (Fig. 2.13A).

WT Caki-1, *EPHA2* KO Caki-1, and the 3xFlag-tagged Eph receptor transduced *EPHA2* KO cell lines and a vector control were infected with KSHV. For EphA2, EphA4, and EphA5, the surface 3xFlag expression was measured concurrently with infection rate by flow cytometry. 3xFlag-EphA2, 3xFlag-EphA4, and 3xFlag-EphA5 all significantly rescued the infection rate to varying degrees compared to the vector control (Fig. 2.12F, 2.13B). Because of the broad range of flag-tagged receptor expression within the populations of the transduced cell lines, we also examined how the infection rate changed

with surface protein level. The histograms of 3xFlag expression from one replicate well of the experiment were divided into five successive gates (Fig. 2.12G). The percent GFP+ cells in each bin was plotted against the fold geometric mean of the fluorescence intensity (MFI) over isotype MFI of each bin (Fig. 2.12G). Surprisingly, we found that both EphA4 and EphA5 mediated higher KSHV infection rates at lower amounts of surface protein compared to EphA2. However, at very high surface expression levels that were only attained by EphA2, the infection rate surpassed that of WT cells from the same experiment (Fig. 2.12F).

Altogether these data show that EphA2, EphA4, EphA5, and EphB2 can rescue the infection rate phenotype of *EPHA2* KO cells which suggests that the function of EphA2 in this infection mechanism may not be specific to EphA2. Moreover, the overexpression of EphA4 in this context strongly enhanced KSHV infection, which is not what we expected based on our EPHA4 KO experiments. The precise role of endogenous EphA4 cannot be discerned from these studies alone. Finally, while KO of *EPHB2* did not affect KSHV infection percentage (Fig. 2.11), overexpression of EphB2 in *EPHA2* KO cells promoted infection. We concluded that either the endogenous expression of EphB2 is too low to have a significant impact on infection, or like EphA4, the transduced EphB2 construct has a different effect from endogenous EphB2.

**Ectodomains of EphA2 and EphA4 are sufficient to rescue KSHV infection in EphA2 KO cells.**

In endothelial and fibroblast KSHV infection models, several studies have reported that downstream effector proteins co-immunoprecipitate and colocalize with EphA2 during infection, implying involvement of the cytoplasmic tail of EphA2 which contains a kinase and several protein-protein interaction domains (Chakraborty *et al.*, 2012, Dutta *et al.*, 2013, Bandyopadhyay *et al.*, 2014a, Bandyopadhyay *et al.*, 2014b, Wang *et al.*, 2017). EphA2 is also phosphorylated upon KSHV infection in HEK293 and SLK cells (Hahn *et al.*, 2012, Wang *et al.*, 2017) Here we have shown that KSHV infection of Caki-1 and HeLa cells is independent of canonical KSHV integrin receptors, but still dependent on EphA2. Eph receptors can naturally trigger endocytosis in response to ephrin ligand binding by several mechanisms (reviewed in Pitulescu *et al.*, 2010). Since KSHV gH/gL binds to the ephrin-binding domain of EphA2 (Hahn *et al.*, 2014, Großkopf *et al.*, 2018), we hypothesized that the signaling domains in the cytoplasmic tail of EphA2 would be necessary for infection and would provide clues about how the virus might use EphA2 to enter cells without canonical integrin receptors.

To this end, truncation mutants of EphA2 and EphA4 were generated which lacked all cytoplasmic signaling domains. These cytoplasmic truncation (ΔCT) contained the entire peptide signal and ectodomain (amino acids 1-537) and the transmembrane (TM) domain (aa 538-558) (Fig. 2.14A). Full length *EPHA2* (FL) and *EPHA2* ΔCT were cloned into retroviral vectors and stably transduced into *EPHA2* KO Caki-1 and HeLa cells. Both

EphA2 FL and EphA2 ΔCT were expressed on the cell surface to a slightly higher degree than endogenous EphA2 on Caki-1 (Fig. 2.14D) and HeLa cells (data not shown). When infected with KSHV, both EphA2 FL and EphA2 ΔCT significantly rescued infection to nearly identical levels compared to the vector control (Figs. 2.14B, 2.14C).

Eph receptor clustering is essential for natural signaling events, and a homodimerization region has been found within the transmembrane domain of EphA1 by nuclear magnetic resonance spectroscopy (Bocharov *et al.*, 2008). To test whether the transmembrane domain was required for EphA2 ectodomain function during KSHV infection, we performed additional domain swaps with the EphA2 ΔCT construct and replaced the EphA2 TM domain with that of integrin β1 or HLA-B7, two unrelated single-pass transmembrane proteins (Fig. 2.14A). These domain-swapped constructs were also cloned into retroviral vectors and transduced into *EPHA2* KO Caki-1 cells. The EphA2/HLA-B7 chimeric CT and EphA2/integrin β1 chimeric ΔCT constructs were expressed at the cell surface to the same degree as the FL and CT constructs, and they also significantly rescued KSHV infection of *EPHA2* KO cells (Figs. 2.14D, 2.14B). Together, these data show that only the ectodomain of EphA2 is required to rescue KSHV infection in *EPHA2* KO Caki-1 cells.

To further investigate the role of EphA2 during infection of *EPHA2* KO cells, 3x-Flag-tagged *EPHA4* ΔCT was also cloned into a retroviral vector and stably transduced into WT and *EPHA2* KO Caki-1 cells. Unlike full-length 3xFlag-tagged EphA4, 3xFlag-tagged EphA4 ΔCT was expressed to high levels at the cell surface (Fig. 2.15A). However, when infected with KSHV, EphA4 ΔCT only promoted infection in the *EPHA2* KO context and did not have an effect on WT cells (Fig. 2.15B).

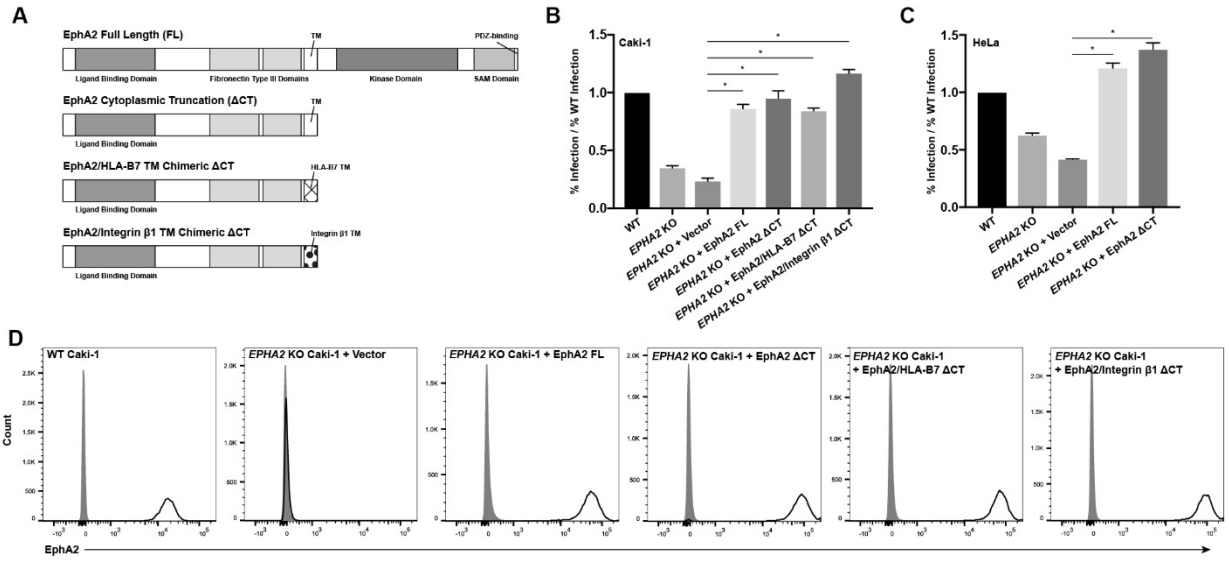



Figure 2.14. EphA2 ectodomain is sufficient to rescue infection rate in *EPHA2* KO cells. (A) Diagram of EphA2 truncation and domain swap constructs. (B, C) WT, *EPHA2* KO, and *EPHA2* KO cells transduced

with the EphA2 constructs indicated in (A) were infected with KSHV in triplicate and infection rate was quantified by flow cytometry. The infection rates were normalized to the average infection rate of WT cells and a representative experiment is shown. (D) WT, *EPHA2* KO, and the indicated transduced *EPHA2* KO Caki-1 cells were immunostained for surface EphA2 expression and analyzed by flow cytometry. Grey histograms represent the isotype controls. *, $p < 0.05$.

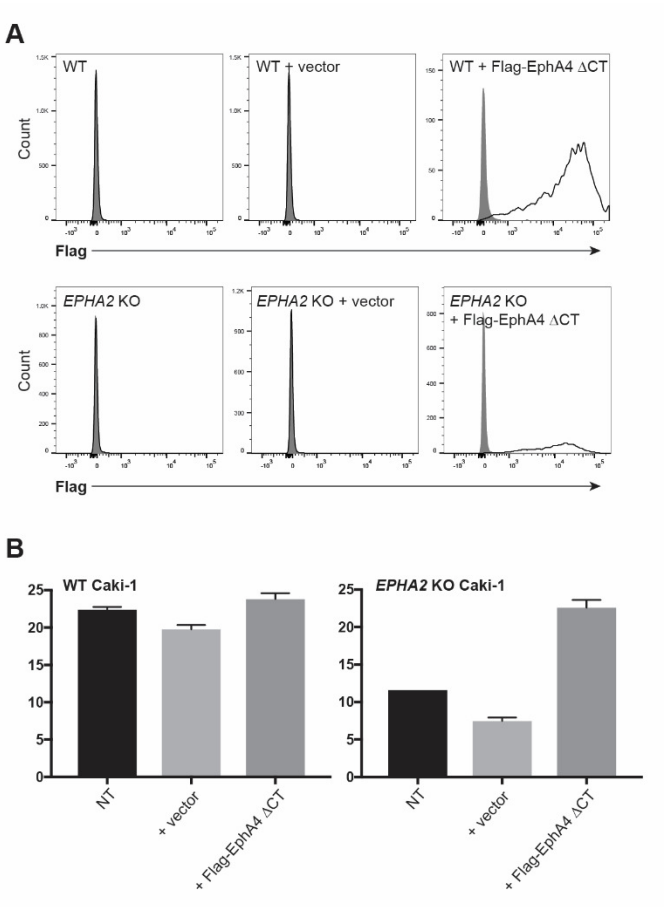



Figure 2.15 EphA4 ΔCT enhances infection in *EPHA2* KO cells. (A) WT, WT + 3xFlag-EphA4, *EPHA2* KO, and *EPHA2* KO + 3xFlag-EphA4 Caki-1 cells and appropriate vector controls were immunostained for surface Flag expression. Grey histogram represents isotype control. (B) WT, WT + 3xFlag-EphA4, *EPHA2* KO, and *EPHA2* KO + 3xFlag-EphA4 Caki-1 cells and appropriate vector controls were infected with KSHV and infection percentage was quantified by flow cytometry two days post infection.

## Infection of primary gingival keratinocytes requires HS interactions.

Since transmission through saliva is now thought to be the major route of KSHV transmission, we examined the expression and use of known KSHV receptors in primary gingival keratinocytes (PGKs). First, PGKs were analyzed for surface expression of KSHV receptors by flow cytometry. We found that HS, EphA2, and the integrin subunits α3, αV, β1, and β5 were readily detected at the cell surface (Fig. 2.16). Like HeLa cells, PGKs did not express integrin β3 at the cell surface, nor did we detect xCT or DC-SIGN (Fig. 2.16).

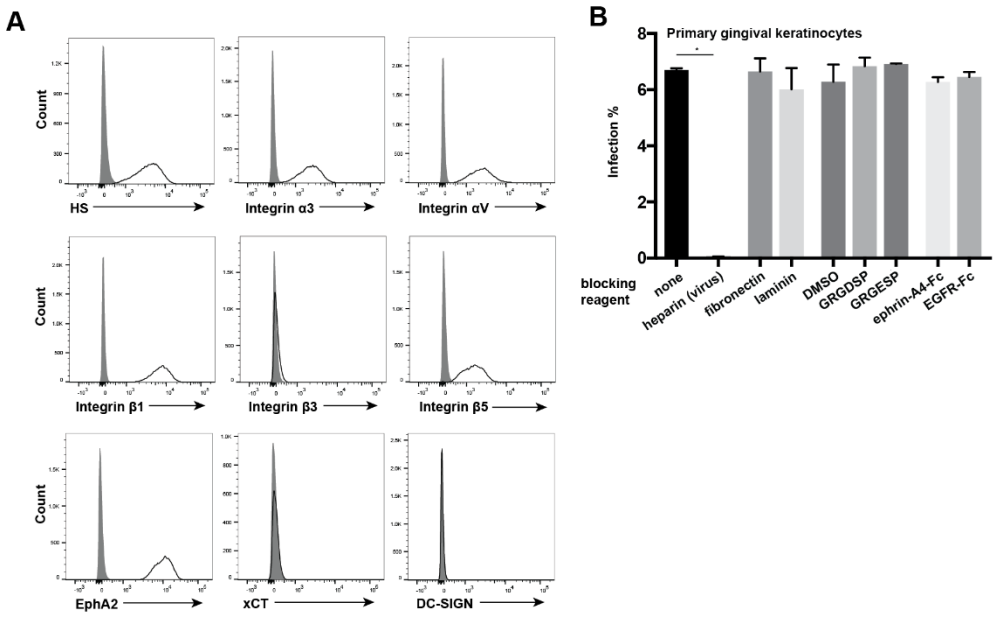



Figure 2.16. KSHV infection of PGKs depends on HS interactions but is not inhibited by integrin- or Eph-blocking agents. (A) PGKs were immunostained for surface expression of known KSHV receptors and analyzed by flow cytometry. Grey histogram represents the appropriate isotype control. (B) PGK cells were pre-incubated with fibronectin or laminin at 50 μg/L, GRGDSP or GRGESP peptides at 2 mM or an appropriate volume control of DMSO, and ephrin-A4-Fc or EGFR-Fc as a control at 5 μg/mL for one hour at 4°C. For the no treatment and heparin condition, cells were pre-incubated in normal media at 4°C. For the heparin block condition, virus was blocked with heparin at 500 μg/mL for one hour at 37°C. Cells were then washed and infected in triplicate with KSHV, or heparin-blocked KSHV for two hours at 37°C. Ephrin-A4-Fc and EGFR-Fc concentrations were maintained during the infection. Infection percentage was quantified by flow cytometry two days post infection.

Next, we utilized the blocking experiments that we had replicated from existing KSHV receptor literature to probe which of these receptors were utilized during infection of PGKs. To test whether HS interactions were required for infection, KSHV was pre-blocked with soluble heparin before infection. To investigate whether any canonical integrin receptors were required for infection, we pre-blocked cells with fibronectin, laminin, or the RGD-containing peptide GRGDSP. The peptide GRGESP and a DMSO condition were included as additional controls. Finally, to investigate whether Eph receptor interactions were required for infection, we pre-blocked cells with ephrin-A4-Fc or EGFR-Fc as a control.

We found that soluble heparin completely abrogated the infection of PGKs (Fig. 2.16). Similar to our results with Caki-1 and HeLa cells, the integrin ligands and RGD peptide had no significant effects on infection rate (Fig. 2.16). Surprisingly, the ephrin-A4 ligand also had no effect on infection rate (Fig. 2.16). These data suggest that KSHV

infection of PGKs does not require interactions with the laminin-binding integrin α3β1, the RGD-binding integrin αVβ5, nor EphA2 which is competitively blocked by ephrin-A4 (Hahn *et al.,* 2012, Hahn *et al.,* 2013). However, PGK infection clearly requires heparan sulfate interactions.

**Dynamin is required during KSHV infection of Caki-1 and HeLa cells.**

It is well-known that clathrin-mediated endocytosis and lipid raft-dependent micropinocytosis are the routes of virion internalization triggered by KSHV-receptor interactions in fibroblasts and endothelial cells, respectively (reviewed in Kumar *et al.*, 2016). In this study we have discovered an infection mechanism that does not seem to use the same KSHV-induced integrin/EphA2 signaling axis that has been characterized in these types. However, we hypothesized that endocytosis would still be a necessary step of this novel infection mechanism regardless of how it was induced.

Chlorpromazine, dynasore, and nystatin were each tested for their ability to inhibit KSHV infection of both WT and *EPHA2* KO Caki-1 cells and HeLa cells. Chlorpromazine disrupts the assembly of clathrin coated vesicles at the cell surface and dynasore inhibits the activity of dynamin which is required for newly formed endocytic vesicles to pinch off from the plasma membrane. Nystatin sequesters cholesterol and disrupts lipid raft structures. Cells were pre-treated with the indicated concentrations of these three drugs, then infected with KSHV while drug concentrations were maintained. In all three cell lines, we found that chlorpromazine and dynasore significantly reduced KSHV infection (Fig. 2.17). These data suggest that KHSV infection in all three cell lines requires clathrin-mediated endocytosis. Notably, while nystatin did not affect the infection rate in WT Caki-1 and HeLa cells, it seemed to enhance infection of *EPHA2* KO Caki-1 cells (Fig. 2.17B).

To further confirm the role of dynamin during infection of Caki-1 cells, mCherry-tagged WT and DN dynamin constructs were transfected into WT Caki-1 cells. One day post transfection, the cells were infected with KSHV. One day post infection, infection percentage was analyzed in mCherry+ and mCherry- populations within each well. Overexpression of the WT dynamin construct may have slightly enhanced infection, but overexpression of DN dynamin clearly inhibited infection (Fig. 2.17D). Together, these results indicated that KSHV requires a dynamin-dependent form of endyocytosis to be internalized by Caki-1 and HeLa cells, likely clathrin-mediated endocytosis (CME).

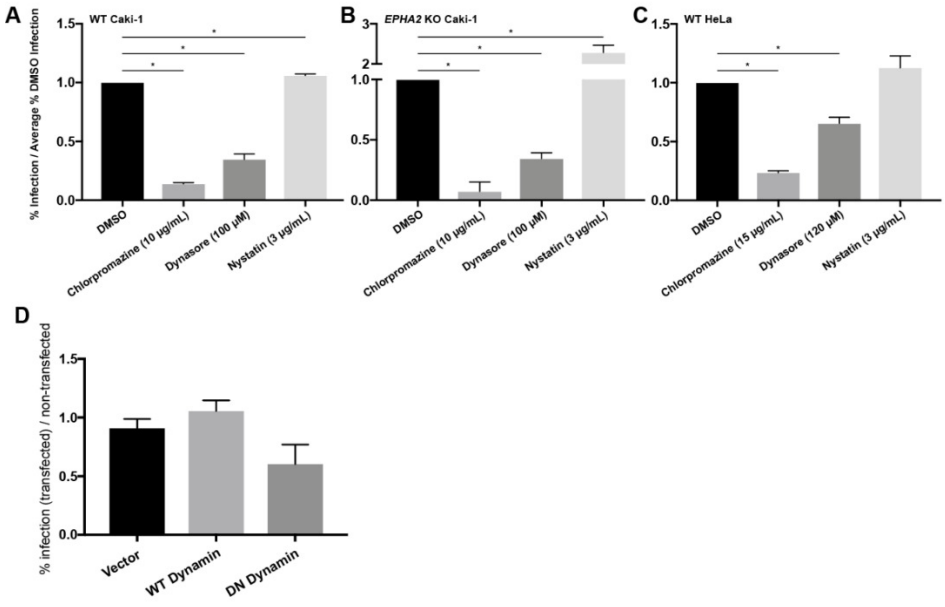



Figure 2.17. KSHV infection of Caki-1 and HeLa cells requires dynamin. WT Caki-1 (A), *EPHA2* KO Caki-1 (B), and WT HeLa (C) cells were pretreated with the indicated concentrations of chlorpromazine, dynasore, nystatin, or an appropriate volume control of DMSO, then infected with KSHV in triplicate while maintaining the indicated drug concentrations. Infection rate was quantified by flow cytometry. The infection rates of the drug-treated cells were normalized to the average infection rate of the corresponding DMSO-treated cells and a representative experiment is shown for each cell line. (D) WT Caki-1 transfected WT dynamin, DN dynamin, or a vector control were infected with KSHV in triplicate. One day post infection, infection rate was analyzed by flow cytometry. Infection percentage in the transfected mCherry+ population within each well was normalized to the infection percentage in the non-transfected mCherry- population. *, $p < 0.05$.

## Two mutant cell lines lack surface EphA2 expression and are highly susceptible to KSHV infection.

During the course of our studies, we inadvertently came across two distinct mutant cell lines which had unusually high infectivity relative to the apparent lack of EphA2 expression. At the time we regarded them as potentially interesting but idiosyncratic scraps of research. However, in the light of our studies in PGKs which revealed yet another infection mechanism—this time independent of both EphA2 and canonical integrin receptors, we felt it was important to describe these unusual mutant cell lines.

Before we had worked out that *ITGAV*/*ITGA3* DKO Caki-1 cells, which lack the canonical KSHV integrin receptors α3β1, αVβ3, and αVβ5, did not have a defect in KSHV infection percentage, we had been creating several combinations of DKOs of integrins β1, β3, and β5 in Caki-1 cells. While most of these DKO cell lines were unremarkable, one stood out with an exceptional morphological and surface expression phenotype. *ITGB3*

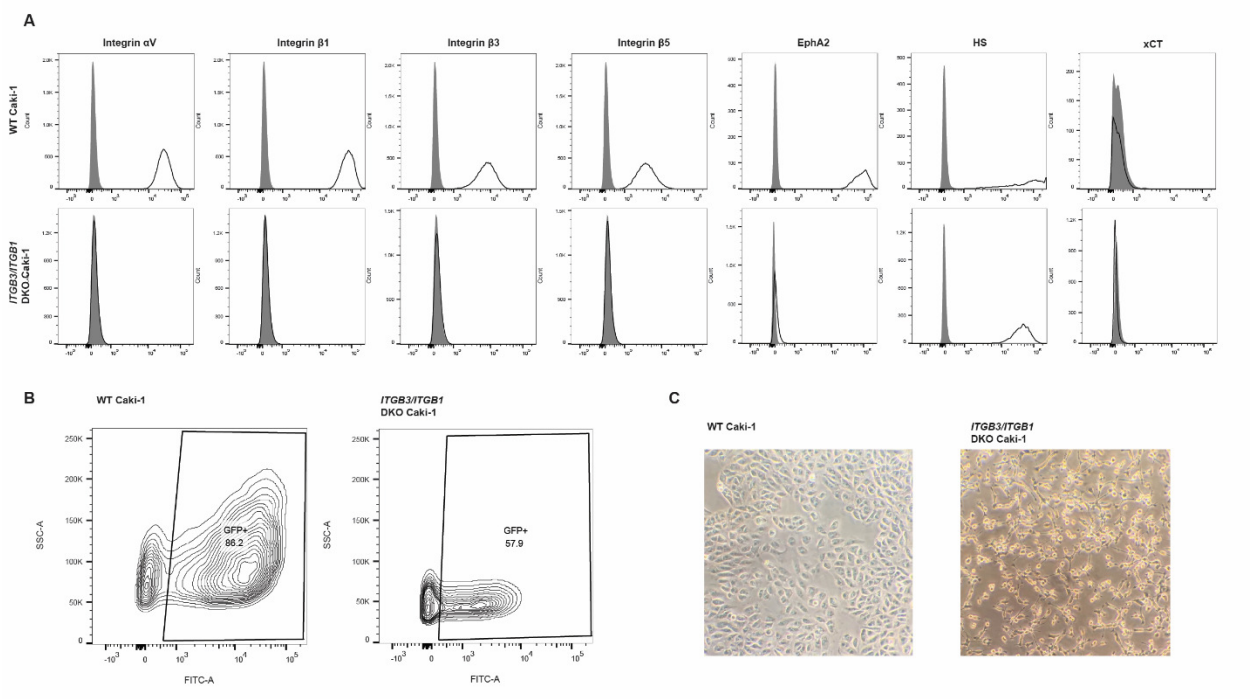



Figure 2.18. *ITGB3*/*ITGB1* double KO Caki-1 cells have altered morphology and expression of known KSHV receptors but are still susceptible to KSHV infection. (A) WT and *ITGB3*/*ITGB1* double KO Caki-1 cells were immunostained for the indicated receptors at the cell surface and analyzed by flow cytometry. Grey histogram represents isotype control. (B) WT and *ITGB3*/*ITGB1* double KO Caki-1 cells were infected with KSHV and infection percentage was measured by flow cytometry two days post infection. (C) Bright-field images of WT and *ITGB3*/*ITGB1* double KO Caki-1 cell morphology at low magnification.

KO cells were transfected with *ITGB1*-targeted px330, and the *ITGB3*/*ITGB1* DKO population was enriched by flow cytometry. The first unusual thing we noticed was that the cells had lost the cobblestone-like morphology of WT Caki-1 cells (and which was shared by every other receptor KO Caki-1 cell line we generated) and instead adopted a more rounded, spiked shape reminiscent of HEK293T cells (Fig. 2.18C). The cells were derived as described a second time to ensure that they were not contaminated with HEK293T cells, but we observed the same morphological change (not shown). The *ITGB3*/*ITGB1* DKO Caki-1 cells were then stained for surface expression of the known KSHV receptors. Surprisingly, although only integrins β1 and β3 were targeted with CRISPR-Cas9, we observed an unexplained loss of EphA2 and the additional integrin subunits αV and β5 from the cell surface (Fig. 2.18A).

Given this pattern of receptor expression, we were then curious whether *ITGB3*/*ITGB1* DKO Caki-1 cells were still susceptible to KSHV infection. We found that the infection percentage of these cells was only reduced by about 1/3 compared to WT Caki-1 cells, which is a much milder defect than we documented for EphA2 KO Caki-1 cells (Figs. 2.18B, 2.9B). Together, these data show that the simultaneous loss of integrins β1 and β3

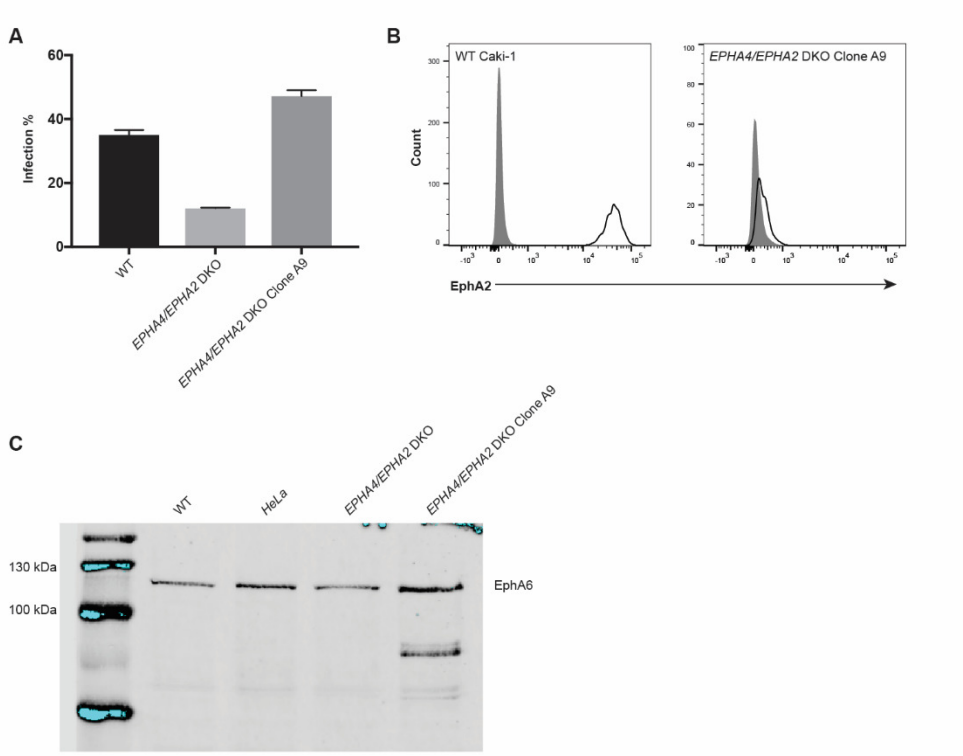



Figure. 2.19. An *EPHA4*/*EPHA2* DKO clone is superinfectable with KSHV and may carry a mutation in EphA6. (A) WT, parental *EPHA4*/*EPHA2* DKO Caki-1 cells, and *EPHA4*/*EPHA2* DKO clone A9 cells were infected with KSHV and infection percentage was measured by flow cytometry two days post infection. (B) WT and *EPHA4*/*EPHA2* DKO clone A9 cells were immunostained for surface EphA2 expression and analyzed by flow cytometry. Grey histogram represents isotype control. (C) WT HeLa, and WT, *EPHA4*/*EPHA2* DKO, and *EPHA4*/*EPHA2* DKO clone A9 Caki-1 whole cell lysates were run on an SDS-PAGE gel and blotted for EphA6.

in Caki-1 cells triggered profound changes in protein expression and cellular morphology. Nevertheless, KSHV efficiently infects these cells, suggesting that either HS alone is sufficient for infection in this context, or that another internalization receptor is used by KSHV during infection of these cells.

The second unusual cell line we uncovered was a single clone of *EPHA4*/*EPHA2* DKO Caki-1 cells: clone A9. Clones of *EPHA4*/*EPHA2* DKO cells were being screened for KSHV infectivity related to another protein, and the infection percentage of clone A9 was approximately five times higher than the parent population and in fact slightly elevated compared to WT cells (Fig. 2.19A). To ensure that this clone didn't express EphA2 as a side effect of the *EPHA4*/*EPHA2* parent population being enriched by flow cytometry but not purified, WT and *EPHA4*/*EPHA2* DKO clone A9 cells were examined for surface EphA2 expression. *EPHA4*/*EPHA2* DKO clone A9 cells still lacked expression of EphA2 (Fig. 2.19B). We hypothesized that *EPHA4*/*EPHA2* DKO clone A9 cells may have a

mutation in another Eph receptor such as EphA4 that may repress *de novo* KSHV infections, so it was included on a western blot for EphA6. Thus, we coincidentally discovered that *EPHA4*/*EPHA2* DKO clone A9 contained strong extra bands in the EphA6 western blot, suggesting that this clone may carry a mutation in EphA6 that could be related to the infection phenotype (Fig. 2.19C). It is possible that endogenous EphA6 functions similarly to EphA4 and inhibits infection. Also like *ITGB3*/*ITGB1* DKO cells, either HS is sufficient for infection in Clone A9, or another receptor could be required for the process of virion internalization.

## 2.3 Discussion

In this report, we describe a novel KSHV infection mechanism in Caki-1 and HeLa cells which requires HS and the ectodomain of EphA2 but is independent of the canonical KSHV integrin receptors. We also present evidence that infection of PGK cells is dependent on HS but not EphA2 or canonical KSHV integrin receptors. Finally, we found that that several other Eph receptors may regulate KSHV infection in various contexts. CRISPR-Cas9 proved to be a valuable tool to dissect the roles of individual receptors during KSHV infection.

We found that infection of Caki-1 cells was significantly reduced in the *EXT1* KO context or when KSHV was pre-blocked with heparin. Additionally, the infection of HeLa and PGK cells was abolished when KSHV was pre-blocked with heparin. It is thought that HS broadly acts as an attachment factor for many viruses including KSHV, but some publications indicate that HS may have additional functions during KSHV infection of several cell types. One study reported that HS was required on target HEK293, CHO, and human conjunctival epithelial cells in a virus-free fusion assay with effector cells that expressed KSHV gB, gH, and gL, suggesting that HS is involved in the interactions between KSHV glycoproteins and entry receptors (Tiwari *et al.*, 2009). A second study used advanced imaging to reveal that upon initial binding to HT1080 fibrosarcoma cells KSHV only colocalized with HS about 50% of the time, while colocalization with integrin receptors was much more robust (Garrigues *et al.*, 2014b). However, soluble heparin still abolishes virion binding to these cells (Garrigues *et al.*, 2014a). Our experiments demonstrated that HS is required for KSHV to infect Caki-1, HeLa, and PGK cells, but the precise role of this molecule during infection remains an open question. Interestingly, the blocking effect of soluble heparin appeared to be more severe in the two cell lines that lacked integrin β3 expression: HeLa and PGK. A possible explanation for this result is that integrin αVβ3 can provide some functionally redundant attachment activity during infection of Caki-1 cells, especially in the *EXT1* KO context. This phenomenon should be further explored.

Surprisingly, we found that KSHV infection was completely unaffected by perturbations in the integrin network in Caki-1 and HeLa cells despite the well-characterized roles that integrins α3β1, αVβ3, and αVβ5 play during infection of HFF and microvascular endothelial cells (reviewed in Kumar *et al.*, 2016). However, these results are in agreement with several studies in which integrin-blocking reagents failed to inhibit KSHV infection of HEK293 and SLK cells (Inoue *et al.*, 2003, Hahn *et al.*, 2012, Walker *et al.*, 2014). We found that Caki-1 and HeLa cells lacking either integrin αV or β1—abolishing the expression of five and twelve integrin heterodimers, respectively—were infected at rates similar to WT cells. The same result was also found for Caki-1 cells knocked out for both integrin subunits α3 and αV, effectively lacking all three canonical KSHV integrin receptors: α3β1, αVβ3, and αVβ5. Furthermore, a panel of integrin ligands and RGD peptides had little or no effect on the percent of Caki-1, HeLa, or PGK cells infected by KSHV.

Our CRISPR-Cas9 KO studies covered sixteen of the twenty-four known integrin heterodimers, and we further determined that the remaining eight heterodimers were not expressed in Caki-1 cells. It is still conceivable that an αV-family and one or more β1-family integrins besides α3β1 are fully redundant receptors of KSHV in this system, although such a situation would not be consistent with several past studies where a KSHV infection phenotype was recorded after targeting only a single integrin heterodimer with a blocking antibody (Akula *et al.*, 2001, Garrigues *et al.*, 2008, Veettil *et al.*, 2008).

It should be noted that the results of our experiments with HeLa cells may not be in agreement with a recent KSHV receptor study on a HeLa-derivative cell line that was misidentified as human salivary gland epithelial cells (HSG[HeLa]) (Garrigues *et al.*, 2014b, Garrigues *et al.*, 2018). This study reported that HSG(HeLa) cells were mostly resistant to infection, despite expressing HS, EphA2, xCT, and integrins α3β1 and αVβ5 (Garrigues *et al.*, 2014b, Garrigues *et al.*, 2018). Like our HeLa CCL-2 cells, HSG(HeLa) cells did not express integrin αVβ3. The infection rate of HSG(HeLa) cells was greatly increased upon expression of integrin β3 leading the authors to conclude that integrin αVβ3 was a crucial receptor for KSHV in these cells (Garrigues *et al.*, 2014b, Garrigues *et al.*, 2018). The differing conclusions from this study and ours may be attributed to the experimental approaches used, since our work focused on depleting receptors from WT cells instead of overexpressing them. It is also possible that HSG(HeLa) cells and our HeLa CCL-2 cells are too divergent to be comparable, as it is unclear how far removed HSG(HeLa) cells are from parental HeLa strains.

The KSHV glycoprotein gB binds integrins through an RGD domain that mimics natural integrin ligands, as well as a DLD domain (Akula *et al.*, 2001, Garrigues *et al.*, 2008, Walker *et al.*, 2014, Wang *et al.*, 2003). In HFF and primary microvascular endothelial cells, this gB-integrin interaction is required to initiate the KSHV-induced signaling cascade through the activation of focal adhesion kinase and other downstream

effectors that eventually lead to virion endocytosis (Akula *et al.*, 2002, Naranatt *et al.*, 2003, Sharma-Walia *et al.*, 2004). This leads to the outstanding question of how KSHV initiates endocytosis of the virion in Caki-1, HeLa, and PGK cells without canonical integrin receptors. We hypothesized that KSHV might directly induce endocytosis through EphA2, mimicking natural ephrin ligand-receptor binding events. Several studies report phosphorylation of EphA2 during KHSV infection and suggest that the cytoplasmic domain of EphA2 is essential to propagate KSHV-induced signaling events and recruit effectors of macropinocytosis and clathrin-mediated endocytosis, but this idea has never been directly tested in the context of *EPHA2* KO cells (Hahn *et al.*, 2012, Chakraborty *et al.*, 2012, Dutta *et al.*, 2013, Bandyopadhyay *et al.*, 2014a, Bandyopadhyay *et al.*, 2014b, Wang *et al.*, 2017). While we found that infection of Caki-1 and HeLa cells required EphA2, remarkably an EphA2 construct truncated after the TM domain rescued infection in *EPHA2* KO cells as efficiently as the full-length EphA2 construct. Furthermore, infection of PGK cells was unaffected by the Eph receptor blocking agent ephrin-A4 which has been previously shown to efficiently inhibit infection in multiple cell types (Hahn *et al.*, 2012, Hahn *et al.*, 2013, Wang *et al.*, 2017, Großkopf *et al.*, 2018).

Together, our results suggest that in Caki-1, HeLa, and PGK cells, KSHV does not trigger the same integrin-EphA2 signaling axis that is so crucial for viral entry into HFF and primary microvascular endothelial cells. However, this conclusion must be rectified with the significant infection defect we observed in EPHA2 KO Caki-1 and HeLa cells. The KSHV membrane glycoprotein gH/gL binds strongly to EphA2 (Hahn *et al.*, 2012, Hahn *et al.*, 2013, Hahn *et al.*, 2014, Großkopf *et al.*, 2018), so one interpretation of our data is simply that the ectodomain of EphA2 acts as an adhesion receptor in the cellular context of Caki-1 and Hela cells.

Taken together with our experiments investigating potential roles for different Eph receptors during KSHV infection, more speculative hypotheses can also be made. The result that we were able to further inhibit infection of *EPHA2* KO Caki-1 cells with soluble ephrin-A4 suggests that another factor which is blocked by ephrin-A4, most likely an Eph receptor, promotes KSHV infection. We ruled out that endogenous EphA4 and EphB2 were necessary for infection of Caki-1 infection, but also found that these Eph receptors were not expressed by HeLa cells. This is important because like Caki-1 cells, HeLa cells exhibit a significant amount of EphA2-independent KSHV infection. It is possible that additional Eph receptors are expressed by both cell lines and affect KSHV infection in *EPHA2* KO and WT contexts.

In support of this idea, we demonstrated that transduced EphA4 and EphA5 constructs rescued infection rates in *EPHA2* KO cells to levels comparable with transduced EphA2. In fact, at low amounts of surface expression, EphA4 and EphA5 constructs outperformed EphA2 in this assay. It is unclear why EphA4 is dispensable for infection in the endogenous setting, while it promoted KSHV infection in this context.

Spliced or modified forms of EphA4 produced from the endogenous gene could account for this discrepancy. Alternatively, EphA4 may be part of a homeostatic network that ultimately impacts KSHV infection efficiency and cellular adaptation to the loss of EphA4 could be responsible for the KO phenotype. Whether EphA4, EphA5, and other Ephs act as true cellular receptors for KSHV should be further investigated.

A striking property of Eph receptors is that they form heterotetramers with their ligands as well as large oligomerized arrays through Eph-Eph interactions in their ectodomains which are critical to trigger forward signaling in response to ligands (Seiradake *et al.*, 2010, Himanen *et al.*, 2010, Seiradake *et al.*, 2013, Xu *et al.*, 2013). These signaling arrays can contain multiple types of Eph receptors, Eph receptors that are not bound to ligands, and Eph receptor ectodomains (Seiradake *et al.*, 2010, Himanen *et al.*, 2010, Xu *et al.*, 2013, Wimmer-Kleikamp *et al.*, 2004, Janes *et al.*, 2011). Moreover, Eph cluster size, composition, and the presence of alternatively spliced Eph receptor forms may all influence the cellular outcomes of Eph signaling (Holmberg *et al.*, 2000, Greene *et al.*, 2014, Schaupp *et al.*, 2014). Importantly, one study showed that the ectodomain of EphA2 was sufficient to localize the protein to cell-cell contacts (Seiradake *et al.*, 2010), and another study of chimeric EphA2 and EphA4 constructs suggested that the ectodomain may be a stronger determinant of cellular responses than the attached cytoplasmic domain (Seiradake *et al.*, 2013). Thus, it is conceivable that in the presence of other signaling-competent Eph receptors, the ectodomain of EphA2 could promote clustering and signaling during KSHV infection just as well as the full-length receptor as we observed in our experiments.

However, it is also possible that an unknown receptor—not an Eph receptor—is required for initiating virion endocytosis and EphA2-independent infection in Caki-1 and HeLa cells. In support of this, a new study has identified a motif in gH of KSHV and rhesus rhadinovirus (RRV) that is required for Eph receptor binding (Großkopf *et al.*, 2018). When this motif was mutated, *de novo* KSHV infection of HFF and endothelial cells was severely attenuated at the post-attachment stage, but not completely blocked (Großkopf *et al.*, 2018). Not only is this study consistent with our *EPHA2* KO data in Caki-1 and HeLa cells, the existence of another KHSV receptor may explain why infection of PGK cells was not inhibited by soluble ephrin-A4. This unknown receptor hypothesis is not exclusive to the potential involvement of other Eph receptors. It is possible that some Eph receptors such as EphA4 and EphA6 negatively regulate the unknown receptor, while others such as EphA2 or other transduced Eph receptors inhibit this regulation though interactions between Eph extracellular domains.

It is still unclear why targeting EphA2 with either CRISPR-Cas9 or ephrin-A4 had such differential effects on Caki-1 and HeLa cells versus PGK cells. Eph signaling is known to be quite cell type-dependent, and therefore the availability of EphA2 in the cell membrane or its intracellular signaling outcomes may naturally differ in PGK cells.

However, EphA2 has also been found to be upregulated in many types of solid tumors and its intracellular signaling functions may also be dysregulated, confounding the interpretation of our results in the Caki-1 and HeLa cell lines (reviewed in Beauchamp and Debinski, 2011, and Wykoski and Debinski, 2008).

Interestingly, two independent groups recently discovered that EphA2 is a receptor for the gammaherpesvirus Epstein-Barr Virus (EBV) on epithelial cells (Chen *et al.*, 2018, Zhang *et al.*, 2018). While integrins αVβ5, αVβ6, and αVβ8 had previously been identified as epithelial cell receptors for EBV (Chesnokova *et al.*, 2009, Chesnoknova *et al.*, 2011), one of these groups demonstrated with CRISPR-Cas9 KO cells that αV-family integrins were not required for EBV glycoprotein-mediated fusion with HEK293 cells (Chen *et al.*, 2018). Furthermore, these studies demonstrated that the kinase activity of EphA2 and indeed the entire intracellular domain were dispensable for EBV glycoprotein fusion and infection, respectively, which is quite similar to results we report here for Caki-1 and HeLa cells (Chen *et al.*, 2018, Zhang *et al.*, 2018). Both model infection systems will need to be further characterized before more comparisons can be drawn.

Given the importance of epithelial cell infection for host colonization, it will be valuable to further characterize this infection mechanism and its impact on the viral life cycle. Our data support the notion that KSHV receptor usage and entry mechanisms vary between cell types. We propose that KSHV infection is not restricted by integrin and EphA2 expression and that the virus may utilize several members of both the integrin and Eph receptor families in various combinations for entry into a broad variety of cell types throughout the body. Modern gene editing technologies such as CRISPR-Cas9 will facilitate detailed studies of KSHV receptors in the future and have the potential to rapidly expand the field of virus-host interactions.

## 2.4 Materials and Methods

### Cell lines and culture

SLK/Caki-1 (ATCC HTB-46) cells were a gift from D. Ganem. HeLa cells (ATCC CCL-2) were obtained from the UC Berkeley BDS Cell Culture Facility. HEK293T cells (ATCC CRL-1573), Phoenix cells (ATCC CRL-3213), and primary gingival keratinocytes (ATCC PCS-200-014) were purchased from the ATCC. Primary gingival keratinocytes were grown in Dermal Cell Basal Medium (ATCC PCS-200-030) supplemented with Keratinocyte Growth Kit (ATCC PCS-200-040) at 37°C with 5% $CO_2$. All other cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, SeraDigm) at 37°C with 5% $CO_2$.

## KSHV production and infection

iSLK.Bac16 (gift from J. Jung, USC) cells harboring latent KSHV.BAC16 infection were cultured under selection with 1.2 mg/mL of hygromycin B (Invitrogen). The cells were induced to produce virus with 1 mM sodium butyrate (Alfa Aesar) and 1 ug/mL doxycycline (Sigma-Aldrich). Three days after reactivation, supernatant was collected and filtered through a 0.45 μm syringe filter. The unconcentrated viral supernatant was stored at 4°C and diluted with standard culture medium for use in infection experiments. The dilution was calculated for each batch to produce an approximate infection rate of 30% on WT Caki-1 cells, measured in GFP+ events by flow cytometry. Cells were incubated with virus for 12-24 hours, then viral supernatant was removed and replaced with fresh medium until analysis two days post infection.

## CRISPR-Cas9 genome editing

Guide sequences were designed using the online tool crispr.mit.edu and are provided in Table 2.1. A 5' G was added to sequences that didn't already contain one and then the appropriate adaptors were appended to both forward and reverse oligos to facilitate cloning into px330 (Addgene #42230) according to the protocol provided at genome-engineering.org. Assembled px330 plasmids were transfected into cells of interest and mutant cells were sorted by FACS or subcloned to obtain genetic KO cell pools or cell lines, respectively.

| Gene | Exon | Guide sequence 5'-3' |
|---|---|---|
| *EXT1* | 1 | GTGGACGAACGACTACTCCA |
| *ITGA3* | 4 | GTCAGAAGACCAGCGGCGCA |
| *ITGAV* | 2 | GTGACTGGTCTTCTACCCGC |
| *ITGB1* | 3 | AATGTAACCAACCGTAGCAA |
| *ITGB3* | 3 | GAGCCGGAGTGCAATCCTCT |
| *ITGB5* | 1 | GCAGGCGTACAGCGGCGCCG |
| *EPHA2* | 4 | GTGTGCAAGGCATCGACGCT |
| *EPHA4* | 3 | TCCACTCACAGTCCGCAATC |
| | 4 | TCTGAAAAAGCCTCGGTCAC |
| *EPHB2* | 3 | GAACACGATCCGCACGTACC |

Table 2.1 CRISPR-Cas9 guide RNA sequences used to target the indicated genes.

## Antibodies

Heparan sulfate antibody (F58-10E4) was purchased from Amsbio, integrin α3 antibody (P1B5 from Calbiochem, integrin αV, integrin β7, and EphA5 antibodies (MAB12191, MAB4669, and MAB541, respectively) from R&D Systems, integrin β1 and integrin β3 antibodies (T2S/16 and PM6/13, respectively) from Novus Biologicals, integrin β5 and EphA2 antibodies (AST-3T and SHM16, respectively) from BioLegend, xct and GAPDH antibodies (ab37185 and ab181602, respectively) from Abcam, DC-SIGN antibody (DCN47.5) from Miltenyi Biotec, EphA4 antibody (4C8H5) from ThermoFisher, EphB2

and EphA6 antibody (2D12C6 and 1426CT591.205.91.119, respectively) from Santa Cruz Biotech, and Flag antibody (M2) from Sigma-Aldrich. Purified isotype control antibodies (MAB002, MAB003, MAB004, AB-105-C, MAB006) were purchased from R&D Systems except mouse IgM, κ (MM-30) was from BioLegend.

### Blocking reagents

Recombinant ephrin-A4-Fc or EGFR-Fc were purchased from R&D Systems. GRGDSP and GRGESP peptides were purchased from Anaspec. Heparin sodium salt was purchased from Sigma-Aldrich.

### Constructs and cloning

Eph receptors were amplified from BJAB (EphA4, EphA5) or Caki-1 (EphA2) cDNA and directly cloned into pQCXIN (Clontech) or cloned into p3xFlag-CMV-9 (Sigma-Aldrich) and subsequently cloned into pQCXIN to add an N-terminal 3xFlag tag preceded by the preprotrypsin leader sequence. Truncation mutants were amplified with a reverse primer in the indicated position containing an artificial stop codon. Chimeric TM domain EphA2 constructs were made using SOEing PCR.

### Transfection and transduction

Caki-1 and HeLa cells were transfected with px330 and phoenix cells were transfected with pQCXIN-based constructs using Fugene transfection reagent (Promega) and Optimem (Gibco) according to the manufacturer's instructions. After 2-3 days, retrovirus was collected from the phoenix cell supernatant and filtered through a 0.45 μm filter. Filtered retroviral supernatant was applied to target cells with 6 μg/mL polybrene (Santa Cruz) and spinfected at 500x g for 2 hours at room temperature. Transduced cells were selected with neomycin (Fisher Scientific) at 1.2 mg/mL.

### Flow cytometry and sorting

Cells were harvested with trypsin (Gibco) or PBS (Gibco) + 2 μM EDTA (Fisher) when staining for trypsin-sensitive epitopes. Cells were blocked, stained, and washed in 1% BSA (Fisher) in PBS. When applicable, cells were fixed in 4% PFA (ThermoFisher Pierce) in PBS and permeabilized with 0.25% Triton X-100 (EM Science) in PBS. Live cells were stained with DAPI (BioLegend) and fixed cells with Ghost Dye Violet 510 (Tonbo Biosciences) for viability according to the manufacturer's instructions. Cells were analyzed using an LSR Fortessa or LSR Fortessa X-20 cell analyzer (BD Biosciences) and sorted using a BD Influx or BD FACSARIA Fusion cell sorter (BD Biosciences). Data was processed and visualized with FlowJo 10 (BD Biosciences).

### Western blotting

Cells were harvested by scraping in PBS and lysed in RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50

mM Tris, and a protease inhibitor cocktail (Roche)). Protein concentration in lysate was quantified by BCA assay (ThermoFisher Pierce). Lysates were run on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. A buffer containing 3% BSA and 10% FBS in TBST (20 mM Tris, 150 mM sodium chloride, 0.1% Tween 20) was used for blocking and primary antibody incubation. Plain TBST was used for washing and secondary antibody incubation. Blots were visualized with IRDye 800CW and 680RD secondary antibodies from LI-COR Biosciences using a LI-COR Odyssey infrared scanner and analyzed in ImageStudio Lite 5.2 (LI-COR Biosciences).

### Statistical analysis

The indicated data sets were compared using the student's t-test in Prism 7 (GraphPad). A p value $< 0.05$ was denoted with a *.

# III

# Receptor Use During Infection of B Cells in Coculture

## 3.1 Introduction

Like all gammaherpesviruses, KSHV is lymphotropic. B cells are a valuable target for KSHV during *de novo* host colonization because it is thought that B cells harbor latent virus for the entire life of the host (reviewed in Knowlton, 2012). Not only can PEL and MCD arise from latently KSHV-infected B cells (Soulier *et al.*, 1995, Cesarman *et al.*, 1995), such infected cells can also be detected circulating in otherwise healthy patients (Blackbourn *et al.*, 1997). PEL-derived cell lines have been immensely useful in the field to study KSHV reactivation and the lytic phase within B cells, but B cell lines are incredibly refractory to infection with purified virus (Renne *et al.*, 1998, Friborg *et al.*, 1998, Blackbourn *et al.*, 2000, Betchel *et al.*, 2003, Rappocciolo *et al.*, 2008), and thus, studying the initial entry mechanism and the establishment of latency in this important cell type has been difficult.

KSHV-infected B cells from patients display curious phenotypes that as of yet, are largely unexplained. In KSHV-associated MCD, infected B cells resemble plasmablasts and are located in the mantle zone (MZ) of B cell follicles. While there is some variability in the expression of plasmablast and other immunological markers, these cells always express cytoplasmic IgMλ. These cells express markers of memory B cells, but do not have evidence of somatic hypermutation, suggesting that naïve pre-germinal center B cells are initially infected by KSHV. Furthermore, KSHV-infected B cells in MCD are polyclonal, indicating recurrent infection events into this population of B cells (reviewed in Du *et al.*, 2007). In PEL, the KSHV-infected cells express CD45, but lack most B cell makers and do not express the BCR in any form. The cells do not have uniform morphology, but express similar immunological markers. Unlike KSHV-infected MCD cells, PEL cells are monoclonal and have undergone extensive somatic hypermutation, suggesting that initial infection occurs after antigen selection in the germinal center (reviewed in Du *et al.*, 2007).

It was noted early on that primary B cells from peripheral blood or tonsils could be infected with purified KSHV at a very low level (~1-4%), but infection rate can increase when the cells were stimulated with cytokines such as CD40L and IL-4 (Mesri *et al.*, 1996, Blackbourn *et al.*, 1997, Renne *et al.*, 1998, Kliche *et al.*, 1998, Blackbourn *et al.*, 2000, Rappocciolo *et al.*, 2008, Hassman *et al.*, 2011, Myoung *et al.*, 2011a, Myoung *et al.*, 2011d, Knowlton *et al.*, 2014, Nicol *et al.*, 2016). This remains a common and reliable way to study *de novo* B cell infection in a laboratory setting. Based on *ex vivo* infection studies of activated and resting tonsillar B cells, it has been found that KSHV is capable of infecting cells representing a range of developmental stages, from naïve B cells to memory B and plasma cells (Hassman *et al.*, 2011, Knowlton *et al.*, 2014). One study reported KSHV infection of resting B cells led to latent infection and a proliferative plasmablast phenotype and markers that correspond to those seen *in vivo*, including the curious bias toward λ light chain expression (Hassman *et al.*, 2011). A recent study of infected activated

tonsillar B cells reported that infection drives unusual polyfunctionality and the secretion of IL-6, TNFα, MIP1α/β, and IL-8 (Knowlton *et al.*, 2014). In particular it was hypothesized that the secretion of these factors may contribute to the progression of KS, which has been noted to be unusually dependent on a cytokine-rich environment (Knowlton *et al.*, 2014). Recently, a transcriptome analysis was published of PBMC's infected with KSHV, but the infection was performed in the presence of polybrene which is known to facilitate viral entry independent of receptors and thus the relevance of this data to a natural infection must be carefully considered (Purushothaman *et al.*, 2015). Recently, one group reported that several B cell lines were infected by purified KSHV at an extremely high MOI, but these findings have yet to be replicated by any other groups (Dollery *et al.*, 2014).

An interesting series of papers have also been published on T cell infection and the impact of T cells on the functional outcome of B cell infection. KSHV can be detected in tonsillar CD4+ and CD8+ T cells after infection with purified virus, and the percent of cells infected increases upon T cell activation with PHA (Myoung *et al.*, 2011a, Myoung *et al.*, 2011d). However, these infected T cells do not support KSHV replication (Myoung *et al.*, 2011d). Interestingly, this group also found that infected primary tonsillar B cells alone frequently spontaneously reactivate into the lytic cycle, while the presence of activated CD4+ T cells promoted latency in the infected B cells (Myoung *et al.*, 2011a). Such studies raise important questions about the environmental and tissue context of *de novo* host colonization by KSHV, especially if the initial infection of B cells occurs in the tonsil as is suggested by Chagas *et al.*, 2006.

For the first time in 2011, it was published that coculture with reactivated KSHV-infected iSLK cells (SLK/Caki-1 cells transduced with a doxycycline-inducible RTA construct) rendered resting primary B cells and otherwise resistant lymphocyte cell lines more susceptible to KSHV infection (Myoung *et al.*, 2011b, Myoung *et al.*, 2011c and reviewed in Kang, 2017). Interestingly, this infection mechanism is exquisitely dependent on physical contact between the virus-producing cells and target lymphocytes (Myoung *et al.*, 2011c). A year later, a second group confirmed infection of a B cell line by coculture with lytically infected HEK293 cells (Cho *et al.*, 2012). B cells infected by coculture establish latency and can be selected and propagated long-term (Myoung *et al.*, 2011c). Recently, another group demonstrated that resting primary tonsillar B cells could be infected by coculture, then cultured for an extended period of time post-infection by providing IL-4 and CD40L (Nicol *et al.*, 2016). Interestingly, the bias for IgMλ expression in infected B cells was recapitulated during coculture infection *in vitro* (Nicol *et al.*, 2016). Coculture infection of primary B cells has allowed for new research addressing the cellular changes driven by *de novo* infection and the immune response to these newly infected cells (Bekerman *et al.*, 2013, Nicol *et al.*, 2016, and reviewed in Kang *et al.*, 2017).

The cellular receptors employed in B cell infection, via either the cell-free or coculture route, have been poorly studied due to the technical challenges of infecting B cells *in vitro*. A single group has reported that DC-SIGN serves as an entry receptor for KSHV in activated primary B cells, a fraction of which upregulate surface expression of this molecule upon activation stimuli (Rappocciolo *et al.*, 2006b, Rappocciolo *et al.*, 2008, Na-Ek *et al.*, 2017). Transduction of DC-SIGN into two lymphocyte cell lines also rendered them susceptible to infection with purified KSHV, while xCT expression and function was irrelevant to infection status (Rappocciolo *et al.*, 2008). KSHV gB binds to DC-SIGN, and evidence from two groups suggests that DC-SIGN can mediate cell surface binding (Rappociolo *et al.*, 2008, Kerur *et al.*, 2010, Hensler *et al.*, 2014). While two groups have studied the use of DC-SIGN as a cellular receptor for KSHV on THP-1 cells and primary monocytes, macrophages, and dendritic cells, whether DC-SIGN is used as a receptor during coculture infection of resting B cell lines or primary B cells has not been investigated (Rappocciolo *et al.*, 2006a, Kerur *et al.*, 2010, and reviewed in Knowlton *et al.*, 2013).

Given the complex entry mechanisms utilized by KSHV for entry into other cell types, there may be other entry receptors besides DC-SIGN that are necessary for B cell infection and T cell entry. In THP-1 cells, for example, KSHV infection is dependent on DC-SIGN but also heparan sulfate and the canonical KSHV integrin receptors, and integrin-associated signaling molecules were found to be activated in response to infection (Kerur *et al.*, 2010). Furthermore, there is evidence that activation and thus, DC-SIGN expression, may not be required for KSHV infection in tonsillar cells and particularly in the coculture model (Myoung *et al.*, 2011, Nicol *et al.*, 2016, and discussed in Kang *et al.*, 2017). One group reported that blocking Eph-ephrin interactions during coculture severely inhibited KSHV infection of a B cell line in coculture, but precisely which protein is necessary in which cell in this system has not been explored further (Hahn *et al.*, 2013). Whether additional receptors are involved in these multiple *in vitro* B cell infection contexts is an important open question.

In this study, we set out to characterize the coculture-dependent B cell entry mechanism. We demonstrated that extracellular virions infect B cells in coculture, supporting the idea that cellular receptors are likely required for viral entry into these cells. Next, we examined the expression of known KSHV receptors on coculture-infectible B cell lines and used CRISPR-Cas9 to demonstrate that coculture infection is independent of these receptors. Finally, we investigated the role of several Eph receptors during coculture infection. Interestingly, unlike the behavior of canonical KSHV receptors in other systems, expression of EphA2 did not render cells susceptible to infection with purified virus or in coculture. Overexpression of two other endogenously expressed Eph receptors slightly elevated the infection rate in coculture, but we found no effect on infection rate when one of these Eph receptors, EphA5, was knocked out. Thus, we were unable to pinpoint the functional target of ephrin ligand blocking in the coculture KSHV

infection system. We hypothesize that EphA2 is required on the iSLK cells for coculture infection, while an unknown receptor is required on B cells for infection.

## 3.2 Results

### B cell lines are resistant to high-titer cell-free KSHV, but some can be latently infected by coculture.

Historically, it has been observed that B cell lines are quite resistant to purified KSHV, with only a single recent publication claiming to have achieved moderate levels of infection in MC116 cells, and a small but expandable population of infected BJAB and Reh cells at a very high MOI (Renne *et al.*, 1998, Friborg *et al.*, 1998, Blackbourn *et al.*, 2000, Betchel *et al.*, 2003, Rappocciolo *et al.*, 2008, Dollery *et al.*, 2014). Recently, it was reported that a number of different lymphocyte cell lines (BCBL-1, JSC-1, BJAB, Ramos, Jurkat, and SUP-T1) became positive for a GFP reporter-marked KSHV strain after being cocultured in direct contact with KSHV-infected iSLK cells, both in resting and reactivated conditions (Myoung *et al.*, 2011). A second group has also published successful infection of BJAB cells in coculture (Hahn *et al.*, 2013).

In these studies, the GFP reporter contained within KSHV.BAC16 or KSHV.219 was used to measure infection rate of target lymphocyte cell lines by flow cytometry. However, it has not yet been published whether this reporter exclusively marks KSHV-infected B cells at early timepoints. This is a major concern, as the target cells are cocultured for an extended period of time with dying, highly GFP-expressing cells. To test whether fluorescent material was transferred to the target cells independent of KSHV replication, we infected BJAB cells and primary tonsillar B cells in coculture with reactivated iSLK.BAC16 cells in the presence of the viral replication inhibitor phosphonoacetic acid (PAA). PAA treatment completely abolished the production of infectious virions, measured by viral supernatant transfer onto uninfected Caki-1 cells (Fig. 3.1A). However, a small percentage of BJAB cells and almost 20% of tonsillar B cells became GFP+ in this condition, measured by flow cytometry (Fig 3.1B, C). This is significant because the reported rates of B cell infection in coculture are generally quite low (<10%) and so, even a small amount of nonspecific fluorescence is a major confounding factor when measuring infection rate. To ensure the transfer of fluorescence was nonspecific, we transduced iSLK.BAC16 cells with a lentiviral vector expressing mCherry. After BJAB cells were cocultured with reactivated iSLK.BAC16-mCherry cells for three days, a small percentage of the B cells became positive for mCherry in both the presence and absence of PAA, comparable to the number of cells which become positive for KSHV-derived GFP (Fig 3.1D). Thus, we have shown that during coculture, both GFP from the KSHV.BAC16 reporter and a different fluorescent protein from an unrelated lentiviral construct were both transferred nonspecifically to target lymphocytes during

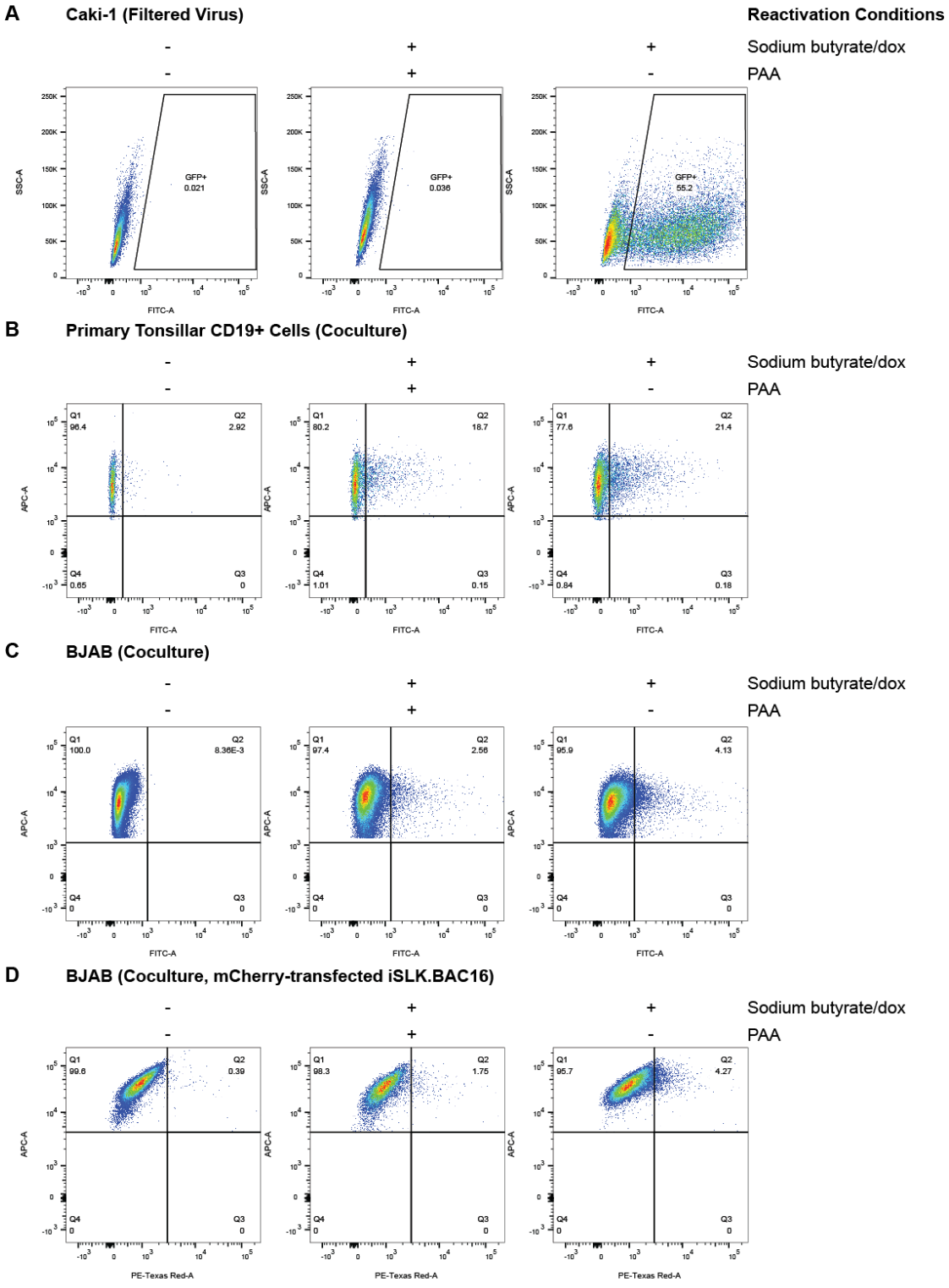



Figure 3.1. Nonspecific transfer of fluorescent reporter proteins during KSHV coculture infection. (A) Phosphonoacetic acid (PAA) is used as a control to inhibit replication of the viral genome during

reactivation induced by doxycycline and sodium butyrate. Infectious particles are not produced in the presence of PAA, demonstrated by supernatant transfer onto highly infectible monolayers of Caki-1 cells after thee days of reactivation with the indicated conditions. Primary tonsillar lymphocytes (B) and BJAB cells (C, D) were cocultured with iSLK.BAC16 (B, C) or iSLK.BAC16-mCherry(D) cells reactivated with the indicated conditions. After three days in coculture, the B cells were harvested, stained with an APC-anti-Cd19 antibody, and analyzed by flow cytometry.

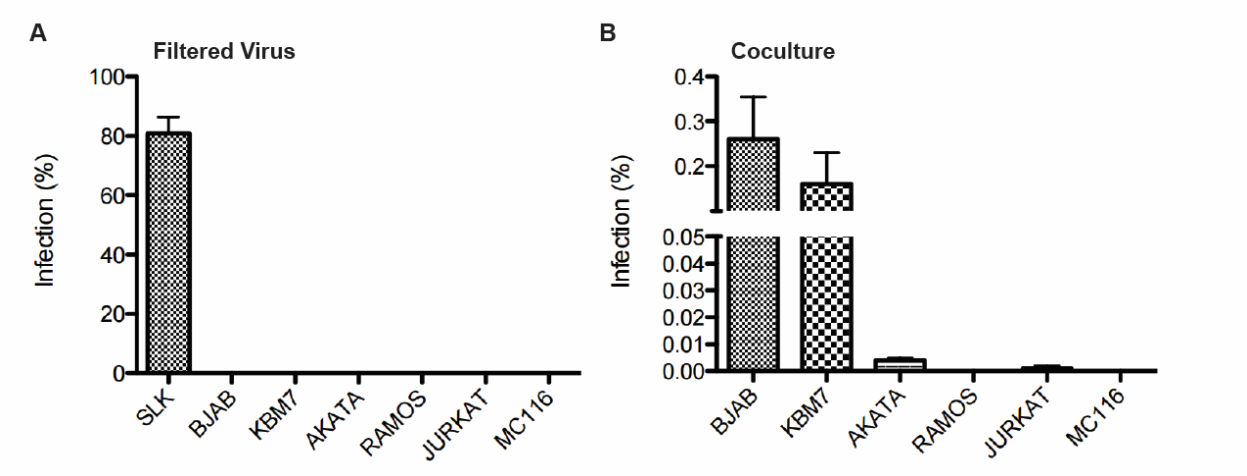



Figure 3.2. Infection rates of B cell lines in coculture. An equal number of cells for each indicated cell line was infected with filtered KSHV (A) or in coculture with reactivated iSLK.BAC16 cells (B) for three days. Infection rate was measured by flow cytometry (A) or limiting dilution with hygromycin selection (B).

coculture. Thus, the measurement of B cell infection rate by flow cytometry should not be considered a reliable assay for true latent or lytic KSHV infection.

To more accurately measure coculture infection rate, we relied on the second reporter encoded in KSHV.BAC16: hygromycin resistance. We attempted to infect a panel of lymphocyte cell lines: BJAB, Ramos, Akata, Jurkat, KBM7, and MC116. The target cells were infected in coculture with reactivated iSLK.BAC16 cells for three days, then harvested by gentle pipetting and counted. The cells were then plated to a limiting dilution with hygromycin in 96-well plates at 1000 cells/well, 100 cells/well, and 10 cells/well. Two weeks later, lymphocyte colonies were counted at each dilution and infection rate was calculated by dividing the number of colonies at each dilution by the total number of cells plated at each dilution. If more than one dilution was informative, the infection rates calculated at each dilution were averaged. As a control, the same number of lymphocytes were also infected with filtered, cell-free virus for two days and infection rate was measured by flow cytometry. By this method, we found that several of the cell lines reported to be infectible in coculture by Myoung and Ganem, 2011 were not infected in our hands (Fig. 3.2B). Only BJAB and KBM7 cells became robustly infected, and we also observed occasional infected colonies of Jurkat and EBV-negative AKATA

cells (Fig. 3.2B). All lymphocyte cell lines were refractory to infection will cell-free virus as reported previously (Fig 3.2A). Furthermore, we were unable to infect MC116 cells as reported in Dollery *et al*, 2014, although in our experiments the cells were infected at a lower MOI (Fig. 3.2A).

**Lymphocyte cell lines lack cell surface expression of most known KSHV receptors.**

Since we were able to infect some lymphocyte cell lines with KSHV in coculture as previously reported, we set out to examine whether any known KSHV receptors were involved in this infection. The entire panel of lymphocyte cell lines and unstimulated primary CD19+ tonsillar B cells were immunostained for known KSHV receptors and quantified by flow cytometry. None of the cell lines or primary cells tested expressed xCT, EphA2, or DC-SIGN (Fig. 3.3A). All of the cell lines and primary cells expressed integrins α3, and β1 (Fig 3.3A). The expression of integrin αV was mixed among the cell lines, and integrins β3 and β5 were largely not detected in the panel (Fig. 3.3A). Since BJAB cells were most robustly infected in coculture in our hands, we used them as a model coculture target for the rest of the experiments. The detailed receptor expression profile for BJAB cells is shown in Fig 3.3B.

In the resting state, the panel of cells did not express most known KSHV receptors. However, we considered that it was possible that receptor expression could change during the three days of coculture. Both primary tonsillar B cells and BJAB cells were infected in coculture, then harvested after three days. The cells were stained for CD19 to differentiate BJAB cells from iSLK cells or debris, and then also stained for known KSHV receptors. No gross changes were observed in receptor expression after coculture, including DC-SIGN (data not shown). The same result was observed in the GFP+ subset, marking cells that either were truly infected or had internalized GFP+ debris (data not shown). Thus, receptor expression did not appear to transiently change during coculture. We additionally attempted to induce DC-SIGN expression in BJAB cells by treatment with 12-O-Tetradecanoylphorbol-13-acetate (PMA) and ionomycin but failed to detect any upregulation of surface DC-SIGN after 6 or 24 hours (Fig. 3.4).

**Coculture infection of BJAB cells is independent of integrin expression.**

Interestingly, integrins were the only known KSHV receptors that we observed to be expressed by many B and T cell lines and primary tonsillar lymphocytes. Although it was clear that expression of these canonical KSHV integrin receptors did not correlate specifically with susceptibility to coculture infection (Figs. 3.2, 3.3A), we hypothesized that integrins could still play an important role during infection, perhaps in combination with another unknown receptor.

To test whether integrins α3β1, αVβ, or αVβ5 were required for KSHV infection in coculture, we generated BJAB cells lacking the essential subunits integrin αV or β1. To generate *ITGB1* KO BJAB cells, WT cells were electroporated with two different px330

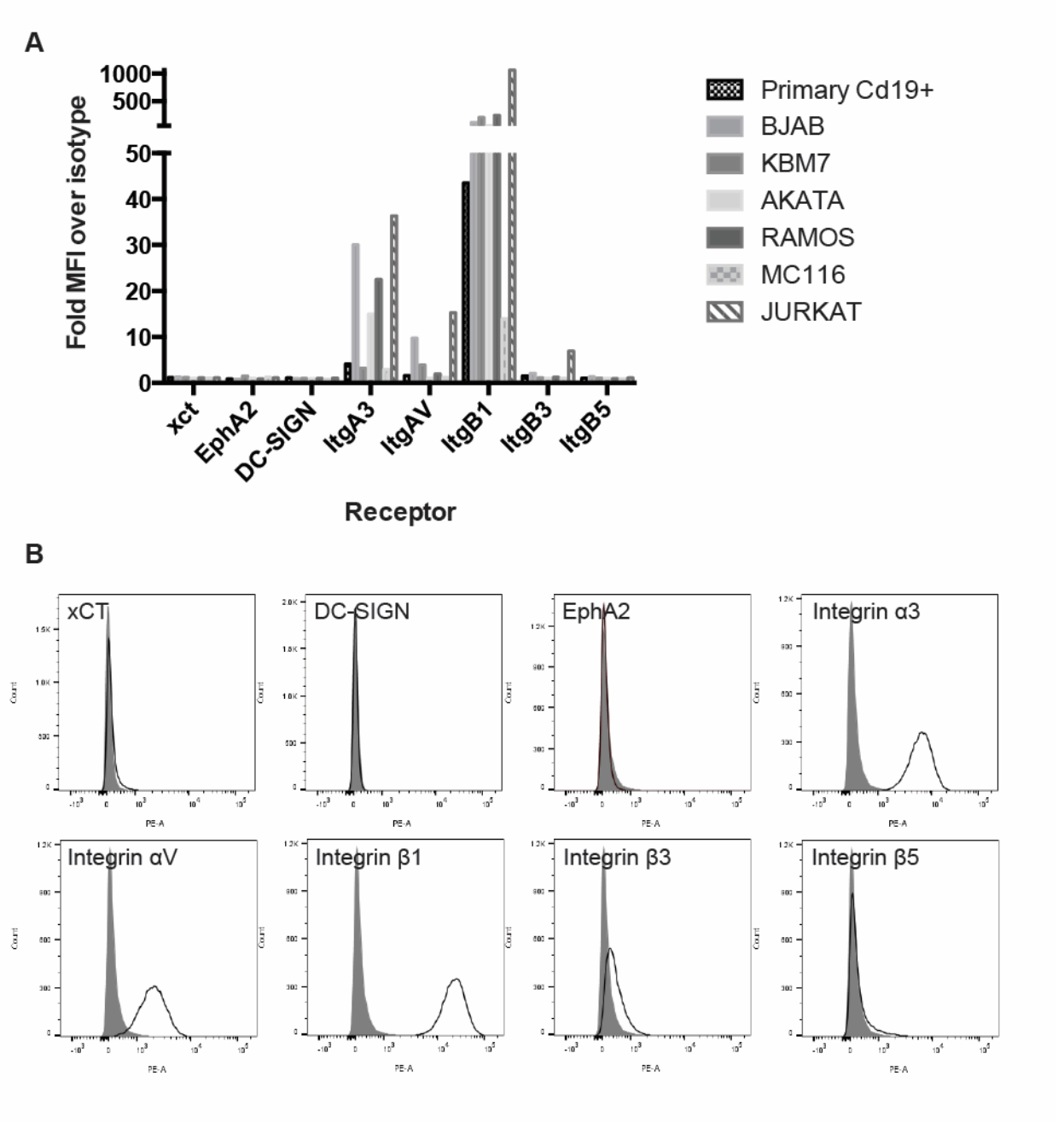



Figure 3.3. Expression of known KSHV receptors on a panel of B cell lines and primary tonsillar CD19+ cells. (A) Each cell line was immunostained for the indicated receptors and compared with matched isotype controls. The mean fluorescence intensity (MFI) of the receptor stain was divided by the MFI of the matched isotype and graphed. (B) Detailed receptor staining histograms for BJAB cells, out model cell line. Black line represents receptor stain and filled grey area represents the corresponding isotype control.

plasmids targeting *ITGB1* (Table 3.1). After four days, a mutant population was visible by flow cytometry and was purified by FACS (Fig. 3.5C). The expression of integrin αV on

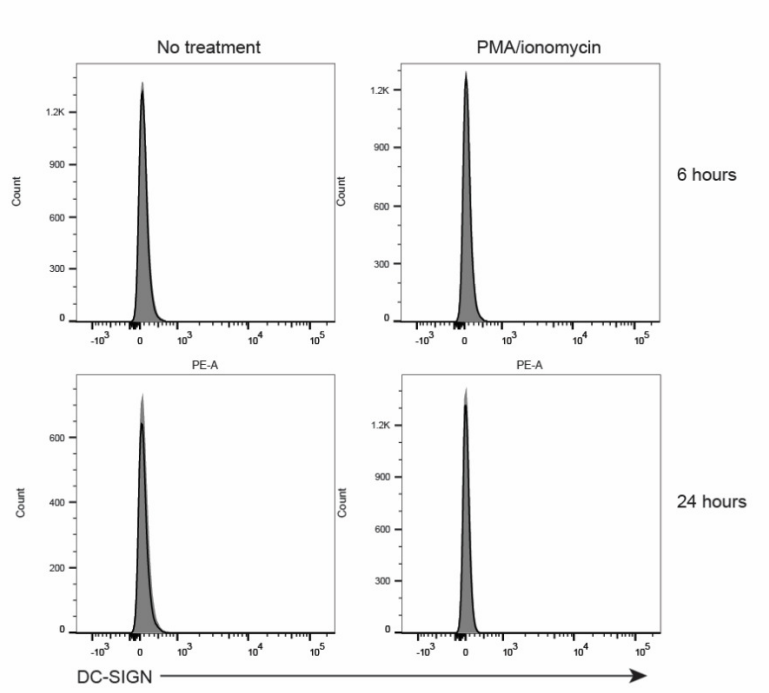



Figure 3.4. Chemical stimulation of BJAB cells fails to induce DC-SIGN expression. BJAB cells were stimulated with PMA/ionomycin for 6 or 24 hours. At these two timepoints, cells were harvested and immunostained for DC-SIGN.

BJAB cells was too low to purify a KO population by FACS, so an alternative approach was used to generate *ITGAV* KO BJAB cells. First two *ITGAV*-specific gRNAs were *in vitro* transcribed (IVT) according to the UC Berkeley IGI protocols referenced in the methods section. CRISPR-Cas9 ribonucleoproteins (RNPs) were assembled by mixing recombinant purified Cas9 (IGI) and one or two of the IVT guides (Table 3.1). WT BJAB cells were then nucleofected with these RNPs. This approach resulted in a very high amount of cell death, but the population of cells that eventually outgrew from the nucleofected population were found to be close to 100% edited and lacked surface integrin αV (Fig. 3.5A). These populations of cells were referred to as *ITGAV* KO.

Two independently generated pools each of *ITGB1* KO and *ITGAV* KO BJAB and WT BJAB were infected with KSHV in coculture. Infection rate was measured by limiting dilution with hygromycin selection. Although the average infection rate of individual KO pools sometimes varied compared to WT cells, there was no unifying trend in either direction across the both KO pools for either *ITGB1* or *ITGAV* KO (Fig. 3.5B, D). Thus, we concluded that infection of BJAB cells in coculture did not require β1- or αV-family integrins. We additionally tested whether coculture infection required expression of the four lymphocyte integrins which contain the integrin β2 subunit, but we found that *ITGB2* KO BJAB cells were infected to the same degree as WT (Fig. 3.6).

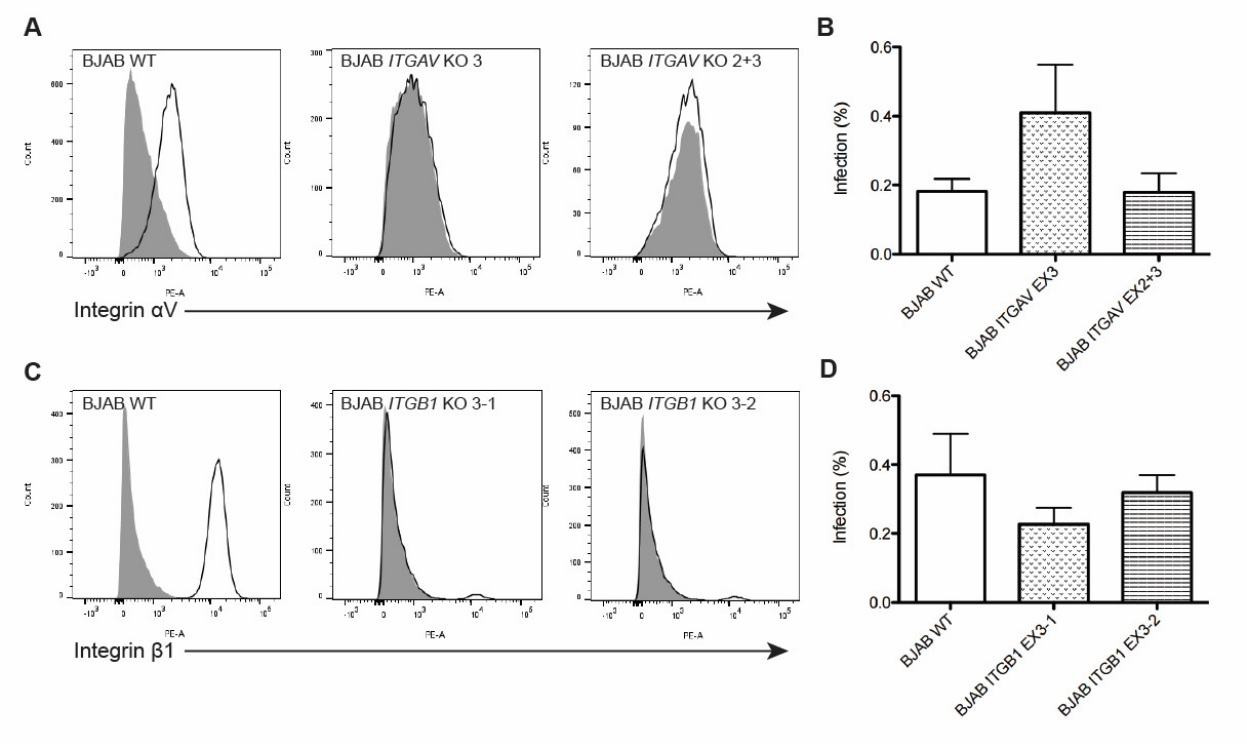



Fig 3.5. Coculture infection does not depend on αV- or β1-family integrins. Two independent cell pools for each integrin target gene were generated using either px330 transfection followed by sorting (C) or Cas9-RNP nucleofection (A). (A, C) WT and integrin KO pools were immunostained and analyzed by flow cytometry. The black trace represents receptor staining and the filled grey histogram represents isotype control staining. (B, D) WT and indicated integrin KO populations were infected in coculture with reactivated iSLK.BAC16 cells and infection rate was measured by limiting dilution with hygromycin selection. The mean and standard deviation of three replicates is shown.

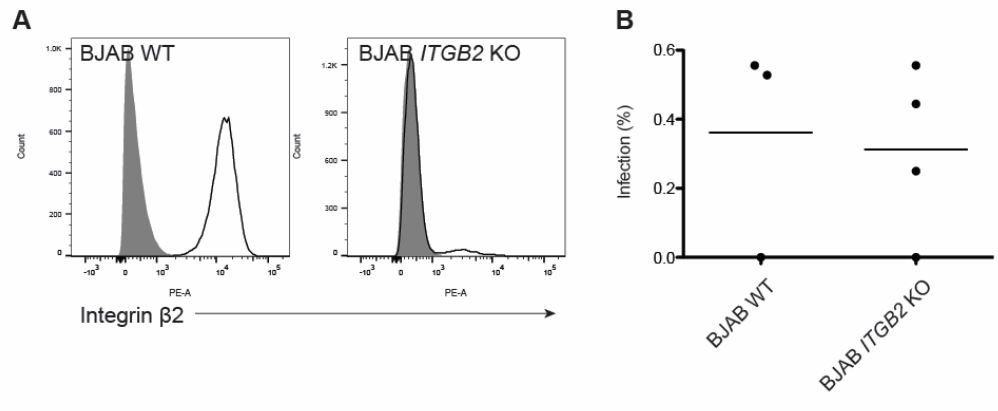



Fig. 3.6 Coculture infection does not require β2-family integrins. (A) WT and *ITGB2* KO BJAB cells were immunostained for integrin β2. Black trace represents integrin staining and the filled grey histogram represents the isotype control. (B) WT and *ITGB2* KO BJAB cells were infected in coculture with iSLK.BAC16 cells and infection rate was measured by limiting dilution with hygromycin selection.

**Coculture infection of BJAB cells is blocked by an ephrin ligand and heparin.**

A single important clue about the possible receptor mechanism behind KSHV infection in coculture was published by Hahn, *et al.,* 2013. In this publication, the authors reported that recombinant soluble ephrin ligands or EphA2 very efficiently blocked infection of BJAB cells in coculture with reactivated iSLK.219 cells. Infection percentage was measured by flow cytometry, which we have shown is prone to contamination with non-specific fluorescence.

To test whether these reagents truly blocked coculture infection, or just the nonspecific uptake of fluorescent debris, we repeated this experiment using limiting dilution in hygromycin as a readout of infection rate. WT BJAB cells were infected in coculture with reactivated iSLK.BAC16 cells for two days with no treatment, soluble ephrin-A4-Fc, or soluble heparin. Infection rate was measured by limiting dilution with hygromycin selection (Fig. 3.7A, B). Surprisingly, we found a similarly robust inhibition of coculture infection with ephrin-A4-Fc using hygromycin resistance as an infection readout compared to what was reported in Hahn *et al.,* 2013. Perhaps even more surprising was the fact that soluble heparin also almost completely blocked BJAB infection in coculture, despite prior work in our lab demonstrating that B cells do not synthesize HS (Fig. 3.7B, Jarousse *et al.*, 2008).

These results suggest that coculture infection requires both HS interactions and an interaction that can be blocked by ephrin-A4—likely between and Eph receptor and a ligand. This interaction is most likely the well-characterized one between KSHV gH/gL and an Eph receptor (Hahn *et* al., 2012, Hahn *et* al., 2013, Hahn *et al.,* 2014, Großkopf *et al*., 2018). However, more experiments are required to determine which Eph receptors are required on which cell type in this two-cell system.

**Manipulation of Eph receptor expression in BJAB cells does not affect KSHV infection rate in coculture.**

We have shown that B cell infection in coculture does not require the β1- or αV-family of integrins, however, in Chapter 2 we described a KSHV infection mechanism in epithelial cell lines that is also independent of integrins but dependent on EphA2 and possibly additional Eph receptors. *In vivo*, the expression of Eph receptors by B cells is somewhat variable and dynamic, but in general this aspect of B cell biology has not been well studied (Alonso-C, *et al*., 2009). Additionally, it has been documented that Eph receptor genes can be epigenetically silenced in lymphomas—a possible explanation for why transformed lymphocyte cell lines are so refractory to KSHV infection *in vitro* (Kuang *et al.*, 2010). Thus, we investigated the use of Eph receptors during B cell infection.

BJAB cells, primary unstimulated tonsillar B cells, and indeed all lymphocyte cell lines tested in our panel did not express EphA2 (Fig 3.3A). Several studies have reported rendering KSHV-resistant cells permissive by reconstituting missing receptors. In

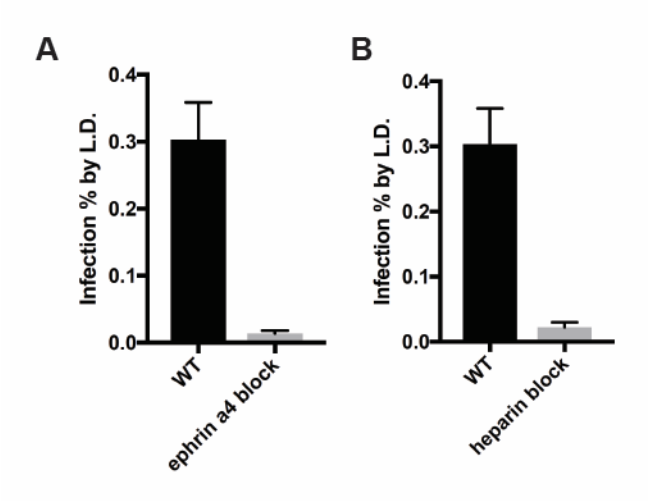



Fig 3.7. Infection of BJAB cells in coculture depends on HS and ephrin interactions. WT BJAB cells were infected in coculture with reactivated iSLK.BAC16 cells in the presence of no treatment, 5 ug/mL ephrin-A4-Fc (A), or 1000 ug/mL of heparin (B). Infection rate was measured by limiting dilution with hygromycin selection.

HSG(HeLa) cells, expression of the missing integrin subunit β3 greatly enhanced infection rate (Garrigues *et al.*, 2014). In Raji and K562 lymphocyte cell lines, expression of DC-SIGN rendered them permissible to purified KSHV (Rappocciolo *et al.*, 2008). Thus, we tested whether the expression of EphA2 would render BJAB cells more susceptible to KSHV infection.

EphA2 was cloned into a retroviral vector containing a neomycin resistance selection marker and transduced into WT BJAB cells, along with an empty control vector. Transduced cells were selected with neomycin and the transduced population was stained for surface EphA2 expression (Fig. 3.8A). WT BJAB, BJAB-vector, and BJAB-EphA2 cells were infected with cell-free KSHV and in coculture with iSLK.BAC16 cells. The transduced BJAB cells remained resistant to cell-free KSHV, measured by flow cytometry (data not shown). The infection rate of the transduced BJAB cells in coculture was also not significantly changed compared to WT (Fig. 3.8B).

Primary B cells and BJAB cells express other Eph receptors (Aasheim *et al.*, 1997, Aasheim *et al.*, 2000, Alonso-C *et al.*, 2009, Fig. 3.8D). We did not complete a full expression panel, but initial screening by RT-PCR suggested that EphA4, EphA5, and EphA7 were naturally expressed by these cells (data not shown). Full length cDNAs of these three Eph receptors could be cloned from BJAB RNA. Finally, EphA5 was robustly detected on the cell surface by flow cytometry (Fig. 3.8D).

EphA4 and EphA5 were cloned into retroviral vectors containing a neomycin resistance marker. EphA4, EphA5, and an empty vector control were transduced into WT

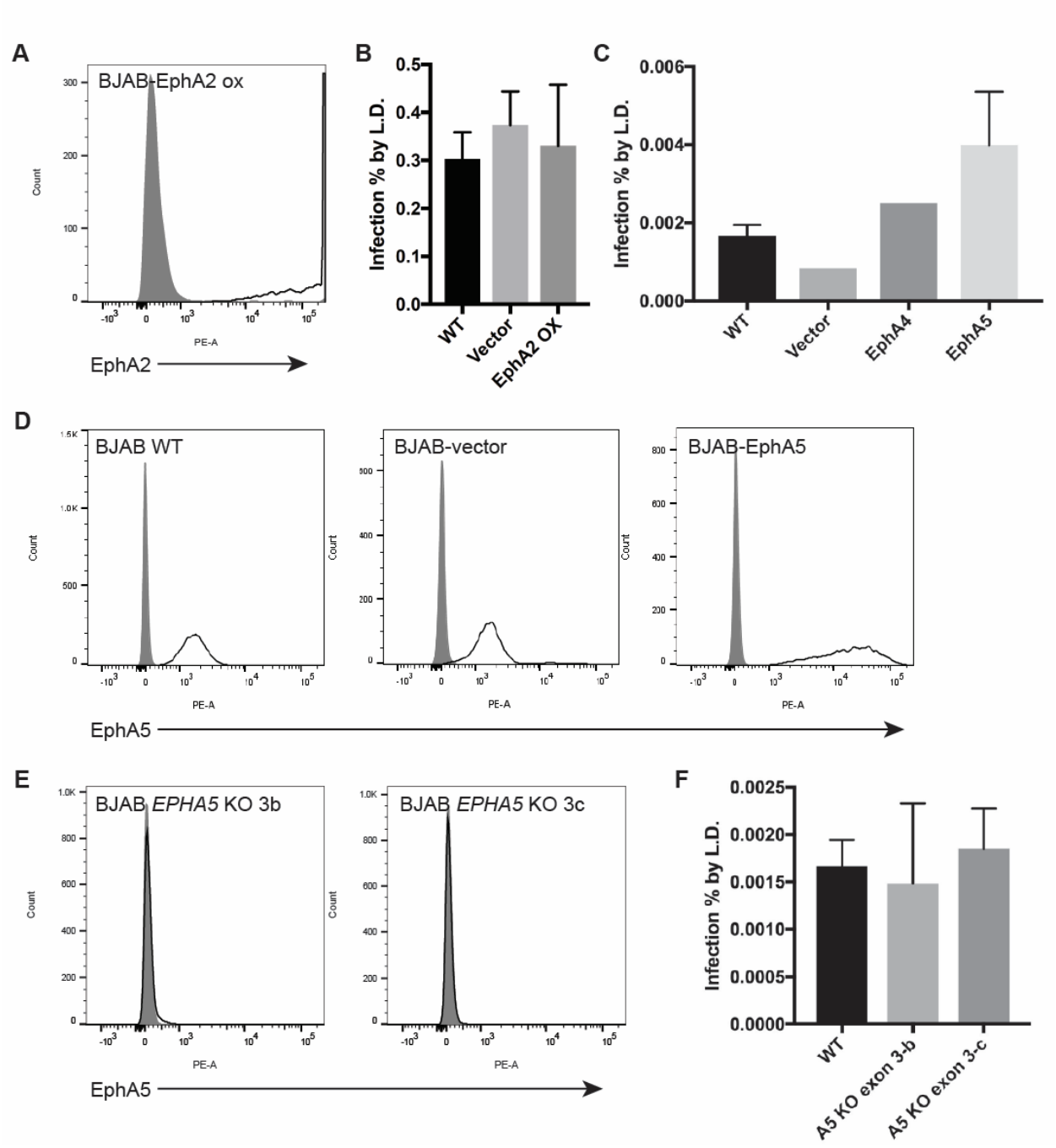



Figure 3.8. Alterations in Eph receptor expression does not affect coculture infection rate. (A) BJAB cells transduced with EphA2 were immunostained for surface EphA2. The black trace represents receptor staining and the filled histogram is an isotype control. (B) WT, BJAB-EphA2, and a vector control and (C) WT, BJAB-EphA4, BJAB-EphA5, and a vector control were infected in coculture with iSLK.BAC16 cells and infection rate was measured by limiting dilution with hygromycin selection. (D) WT, EphA5-transduced, and vector-transduced BJAB cells were immunostained for surface EphA5. The black trace represents receptor staining and the filled histogram is an isotype control. (E) Two independently generated *EPHA5* KO populations were immunostained for surface EphA5. The black trace represents receptor staining and

the filled histogram is an isotype control. (F) WT and two independently generated *EPHA5* KO populations were infected in coculture with iSLK.BAC16 cells and infection rate was measured by limiting dilution.

BJAB cells and the transduced populations were selected with neomycin (Fig. 3.8D). WT BJAB, BJAB-vector, BJAB-EphA4, and BJAB-EphA5 were infected with cell-free KSHV and in coculture with reactivated iSLK.BAC16 cells. Overexpression of EphA4 or EphA5 did not render cells susceptible to infection with cell-free virus, but slightly enhanced coculture infection rate (Fig. 3.8C). To test whether endogenous EphA5 was required for KSHV infection in coculture, a px330 construct targeting *EPHA5* was electroporated into WT BJAB cells. After four days, the mutant population became visible by flow cytometry and was purified by FACS (Fig. 3.8E). WT and *EPHA5* KO BJAB were then infected in coculture with iSLK.BAC16 cells. Surprisingly, the loss of EphA5 had no effect on coculture infection rate (Fig. 3.8F). KO studies of EphA4 and EphA7 were not completed. Thus, while coculture infection is efficiently blocked by ephrin-A4 (Hahn *et al.*, 2013, and Fig 3.7), we found that perturbations in Eph receptor expression in BJAB cells generally did not affect infection rates in coculture.

**Extracellular virions infect B cells in coculture with reactivated iSLK.KSHV cells.**

In their discovery of KSHV coculture infection, Myoung and Ganem demonstrated that cell-cell contact between reactivated iSLK.219 and B cells was required for this infection event to take place (Myoung *et al.*, 2011c). That is, infection was abolished when the two cell types were separated by a .45 μm transwell insert but otherwise shared the same media, secreted factors, and extracellular virions (Myoung *et al.*, 2011c). The mechanism behind these results has yet to be investigated.

It has been well-documented that many viruses have the ability to spread directly between cells, eliminating the need for virus release and travel through extracellular space to reach target cells (see Sattentau *et al.*, 2008 and Mothes *et al.*, 2010 for excellent reviews on this subject). The mechanisms of direct transmission between cells are diverse, however, they can be separated into two major categories. First, fully formed and released virions can be trapped or confined within natural or induced cellular synapses, especially in the case of spread between neurons and cells of the immune system. Second, viral genomes can be spread without complete virion formation via cytoplasmic bridges or induced fusion with uninfected neighboring cells. To begin to characterize the physical requirement for cell-cell contact during coculture infection of BJAB cells, we asked whether virions that ultimately infected BJAB cells necessarily originate from the infected monolayer of reactivated iSLK.BAC16 cells.

To this end, we made use of a recombinant strain of KSHV.BAC16 that constitutively expresses mCherry instead of GFP from the reporter locus (BAC16-mCherry). Cell-free mCherry-expressing KSHV was isolated from reactivated iSLK.BAC16-

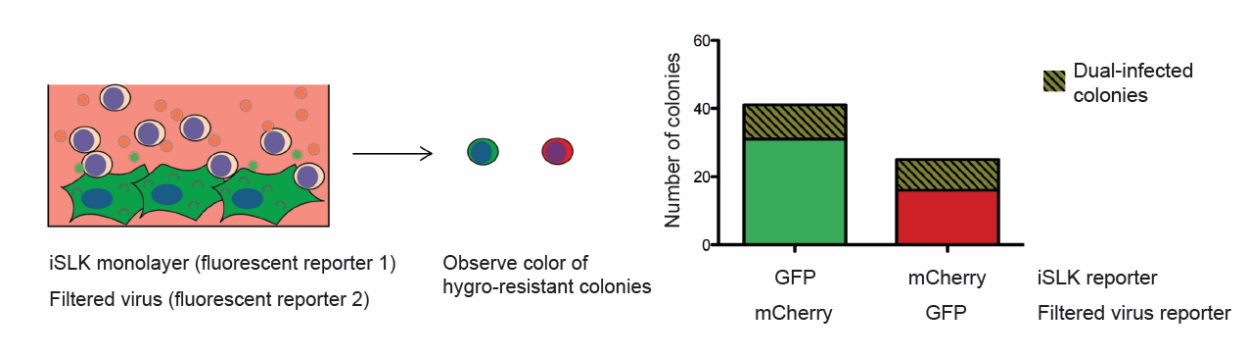



Figure 3.9. Extracellular virions can infect target B cells in coculture. BJAB cells were infected in coculture with reactivated iSLK.BAC16 (GFP) cells and filtered KSHV.BAC16-mCherry virions, or vice versa. Infected BJAB cells were plated at limiting dilutions and selected with hygromycin. Colonies and colony color (or the presence of both colors) were tallied.

mCherry cells and filtered through a 0.45 μm syringe filter to remove cellular debris. WT BJAB cells were then infected in coculture with reactivated iSLK.BAC16 cells in the presence of filtered mCherry-expressing KSHV. When infected colonies were counted after limiting dilution and hygromycin selection, the color of the infected B cell colonies was also recorded. While the infected colonies were mostly GFP+, double GFP+/mCherry+ colonies were also detected (Fig. 3.9). The same, but inverse pattern of colony color was recorded when GFP-expressing virus was purified and the coculture was conducted in the presence of reactivated iSLK.BAC16-mCherry cells (Fig 3.9).

These data suggest that coculture promotes B cell infection with fully formed extracellular virions, rather than harnessing a direct cytoplasmic connection between the two cell types. However, there still seemed to be a bias toward the virions produced from the cellular monolayer during coculture. Interestingly, we also observed that in dually-infected B cell colonies, the KSHV episomes did not appear to be uniformly distributed and propagated within the colony. While some cells in these colonies expressed both GFP and mCherry, often the cells at the periphery of the colonies only expressed either GFP or mCherry, but not both (data not shown).

However, we considered that in our experimental design it was possible that the reactivated monolayer of iSLK cells in the coculture could be superinfected with KSHV expressing the opposite reporter. The second KSHV strain could then possibly be replicated and directly transmitted from the cell monolayer. To test whether reactivated iSLK cells can become superinfected with KSHV, reactivated iSLK.BAC16 cells were incubated with filtered KSHV.BAC16-mCherry for two days. The iSLK.BAC16 cells were then analyzed for mCherry expression by flow cytometry. We found that reactivated iSLK.BAC16 cells are robustly superinfected with KSHV.BAC16-mCherry, and vice versa

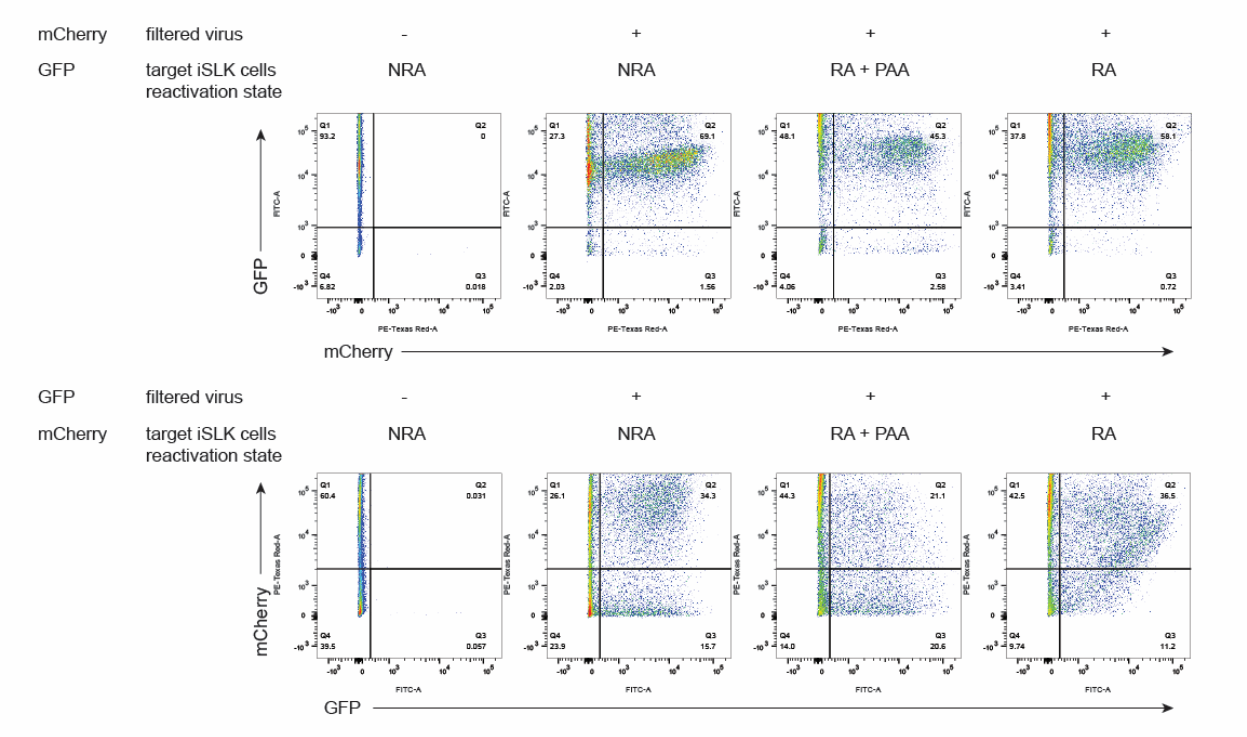



Figure 3.10 iSLK.BAC16 cells can be superinfected with KSHV. iSLK.BAC16 cells carrying GFP- or mCherry-reporter KSHV were left unreactivated, reactivated, or reactivated in the presence of PAA, then infected with filtered KSHV of the opposite reporter strain. Infection was analyzed by flow cytometry.

(Fig 3.10). Thus, we cannot rule out that KSHV infection of BJAB cells occurs through direct cell-cell spread involving some form of a cytoplasmic bridge.

## 3.3 Discussion

Here we have begun to explore the mechanistic basis for contact-dependent KSHV infection of B cells, a pressing and important question in the KSHV field. B cells are thought to be the lifelong reservoir of latent KSHV and thus understanding how these cells are targeted and infected is crucial to designing effective interventions. We studied the infection of BJAB cells as a model system. This Burkitt's Lymphoma cell line is completely resistant to cell-free KSHV, but susceptible to infection in coculture.

Curiously, we found that coculture infection of BJAB cells was independent of all known KSHV receptors. As we found to be the case for several different epithelial cells in Chapter 2, although canonical KSHV integrin receptors were expressed, they were dispensable for infection. Furthermore, we demonstrated that the ectopic expression of the known receptor EphA2 did not alter the infection rate of BJAB cells. Additionally, our group has previously published a study concerning HS expression in B cells and its implication for KSHV and MHV68 infection (Jarousse *et al.*, 2008). In this publication, it

was reported that B cells do not express an essential HS biosynthetic enzyme (Ext1) and are therefore unable to synthesize HS. Interestingly, expression of Ext1 rendered mouse B cell lines permissive to MHV68, while in the human BJAB cell line it promoted virus adhesion to the cell surface, but the cells remained resistant to infection with cell-free virus (Jarousse *et al.*, 2008). Together, these data indicate that unlike the infection mechanism we characterized in epithelial cells, the contact-dependent infection mechanism of B cells is fundamentally unique.

Interestingly, we also found that BJAB infection in coculture was independent of DC-SIGN. DC-SIGN has been the only KSHV receptor reported and studied in any B cell infection model and it is also important for the infection of myeloid-lineage cells such as dendritic cells, macrophages, and monocytes (Rappocciolo *et al.*, 2006a, Rappocciolo *et al.*, 2008, Kerur *et al.*, 2010). DC-SIGN as a receptor has only been studied in peripheral blood B cells and is largely upregulated upon stimulation of these cells with cytokines (Rappocciolo *et al.*, 2006b), however subsequent work demonstrated that in the absence of stimulation, tonsillar B cells can be infected with KSHV in both cell-free and coculture contexts (Myoung *et al.*, 2011a, Myoung *et al.*, 2011c). Here we confirmed that unstimulated primary tonsillar B cells and our model BJAB cell line do not express DC-SIGN. Furthermore, we were unable to induce DC-SIGN expression in BJAB cells by PMA/ionomycin treatment or during coculture with lytically infected iSLK.KSHV cells.

The necessity of DC-SIGN for infection could represent a physiological difference between circulating blood B cells and tonsillar B cells, or DC-SIGN expression may enhance a core virus-receptor interaction common to B cells. We did not test whether ectopic expression of DC-SIGN in BJAB cells increased infection rate, which has been reported for certain other lymphocyte cell lines (Rappocciolo *et al.,* 2008). In THP-1 cells, blocking antibodies to DC-SIGN reduced viral entry but did not affect binding to the cell surface, suggesting that the role of DC-SIGN is more than just as an adhesion molecule for the virus (Kerur *et al.*, 2010). However, THP-1 cells also expressed HS and several KSHV integrin receptors which were also found to be essential for infection of these cells (Kerur *et al.*, 2010). It is possible that the role of DC-SIGN varies depending on the other receptors being used for entry in a given cell type.

Given that coculture infection of BJAB cells can be almost completely blocked in the presence of a soluble ephrin ligand (Hahn *et al.*, 20103, and replicated here), we also assessed the potential role of Eph receptors besides EphA2 that are naturally expressed by BJAB cells as viral receptors in coculture. While this topic is severely understudied, it has been found that primary B cells dynamically express Eph receptors, including EphA4 and EphA7 *in vivo* (Aasheim *et al.*, 1997, Aasheim *et al.*, 2000, Alonso-C *et al.*, 2009). Hahn *et al.*, 2013 and our own studies in Chapter 2 present strong evidence that KSHV gH/gL interacts broadly with EphA receptors, not just EphA2, and that these other Eph receptors may play a role in KSHV infection. Thus, we hypothesized that these other Eph receptors

could be required for KSHV infection of B cells, a mechanism which is enhanced by cell-cell contact. However, while overexpression of EphA4 and EphA5 may have slightly enhanced the coculture infection rate of BJAB cells, infection was unaffected by EphA5 KO. The effects of EphA4 and EphA7 KO were not tested. It is possible that these Eph receptors are functionally redundant, and a phenotype may only be observed in a multiple KO context.

Another explanation for these results is that a virus-Eph receptor interaction is required on the reactivated iSLK cells in order for B cell infection to take place. Indeed, we observed that filtered virions originating from the extracellular space were ultimately able to infect BJAB cells in coculture, perhaps suggesting that this infection mechanism involves trapping virions in a temporary synapse or cell-to-cell contact. However, additional experiments are required to rule out the possibility of pass-through infection in our original assay. In the ideal assay, virions that passed through the reactivated iSLK cells would be marked to distinguish if virus ultimately infecting BJABs truly originated from the extracellular space. GFP-targeted CRISPR-Cas9 could be introduced into iSLK.BAC16 cells in order to render all cellular-based KSHV genomes colorless, while KSHV.BAC16 from the extracellular space would have an intact GFP reporter gene. Additionally, mCherry-targeted CRISPR-Cas9 could be introduced into iSLK.BAC16 cells, mutating the mCherry reporter of any KSHV.BAC16-mCherry that infected the iSLK.BAC16 monolayer.

Interestingly, we would hypothesize that in the coculture system, virions would adhere much better to iSLK cells than the target BJAB cells. The iSLK cell surface, abundant in HS and EphA2, could provide a platform on which to artificially concentrate virions proximal to the B cell membrane—a function that the surface of B cells cannot perform in isolation due to the lack of high-affinity receptors HS and EphA2.

However, we have shown here and in Jarousse *et al.*,2008 that neither ectopic expression of EphA2 or Ext1 (leading to reconstituted surface HS) is sufficient to render BJAB cells susceptible to cell-free KSHV which suggests that the lack of an adhesion molecule on the B cell surface is not the only block to infection with purified virus. Furthermore, the result that soluble ephrin ligands and EphA2 so efficiently block infection suggests that the function of this interaction is not redundant with HS and thus is unlikely to be simply attachment.

Together, our results and other published observations about KSHV coculture infection are highly evocative of the contact-dependent "transfer infection" that mediates the entry of EBV into epithelial cells. Twenty years ago, it was noted by several research groups that while epithelial cells were mostly resistant to infection with purified EBV, infection was greatly enhanced by direct contact with EBV-producing B cell lines (Imai *et al.*, 1998). This finding was subsequently confirmed by several independent groups (Chang *et al.*, 1999, Speck *et al.*, 2000, Tugizov *et al.*, 2003, Shannon-Lowe *et al.*, 2006,

Shannon-Lowe *et al.*, 2011, Nanbo *et al.*, 2016). Furthermore, these infection events were independent of the known receptors for EBV on B cells (Imai *et al.*, 1998, Chang *et al.*, 1999, Speck *et al.*, 2000). A later study found that EBV could be held at the surface of B cells without being internalized, promoting conjugate formation between the two cell types and facilitating subsequent contact-mediated epithelial cell infection through the action of a subset of accessory glycoproteins (Shannon-Lowe *et al.*, 2006). Although the precise mechanisms of epithelial cell infection and even the receptors that are required continues to be debated, it is currently thought that EBV accessory glycoproteins modulate the binding capacity of the core fusion glycoproteins as a tropism switch. The B cell surface provides a sink for certain types of modulated glycoprotein complexes which promote B cell binding but inhibit interaction with epithelial cells, thereby greatly enhancing infection of epithelial cells (Shannon-Lowe *et al.*, 2014).

Given the abundant similarities in receptor use and contact-dependent tropism between EBV and KSHV, it is a reasonable hypothesis that KSHV employs a similar blueprint of glycoprotein-receptor interactions to mediate infection of distinct sets of cells with common sets of viral receptors. Like EBV, the "non-permissive" cell type for KSHV is not truly non-permissive since cell-free infection can be detected in primary B cells, but infection is greatly enhanced in the presence of reactivated infected iSLK cells. Additionally, EphA2 and integrins have been implicated in epithelial cell infection by both viruses. In our studies, we ruled out a role for any of the known KSHV receptors in B cell infection, which strongly suggests that a completely different set of receptors is utilized for the infection of lymphocytes which is also the case for EBV. As of this writing, tropism switching glycoprotein activity has never been explored or reported for KSHV. However, it is a reasonable hypothesis that KSHV may employ modulated core glycoprotein complexes, and binding of certain complexes or accessory glycoproteins to EphA2 and/or HS in the iSLK membrane during coculture may subsequently promote an otherwise inefficient interaction with the hypothetical lymphocyte receptors. Better understanding of the KSHV B cell receptors and the creation of additional tools for studying KSHV infection in coculture will help to address these outstanding questions.

## 3.4 Materials and Methods

### Cells and culture

iSLK.BAC16 cells were a gift from J. Jung. SLK/Caki-1 (ATCC HTB-46) and BJAB (DSMZ ACC757) cells were gifts from D. Ganem. KBM-7 (CVCL_A426) cells were a gift from J. Carrette. HEK293T (ATCC CRL-1573) and MC116 (ATCC CRL-1649) cells were purchased from the ATCC. Ramos (ATCC CRL-1596), EBV-negative Akata (CVCL_0148), and Jurkat (ATCC TIB-152) cells were purchased from the University of California, Berkeley Cell Culture Facility. Adherent cells were grown in high glucose Dulbecco's

Modified Eagle's Medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, SeraDigm). BJAB, Ramos, Jurkat, and MC116 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 Media (Gibco) supplemented with 5% FBS (SeraDigm). KMB-7 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 5% FBS (SeraDigm). Primary tonsillar lymphocytes were isolated and grown as described in Bekerman *et al*., 2013.

### Antibodies

Integrin α3 antibody (P1B5) was purchased from Calbiochem, integrin αV and EphA5 antibodies (MAB12191 and MAB541, respectively) from R&D Systems, integrin β1 and integrin β3 antibodies (T2S/16 and PM6/13, respectively) from Novus Biologicals, integrin β5 and EphA2 antibodies (AST-3T and SHM16, respectively) from BioLegend, xct and GAPDH antibodies (ab37185 and ab181602, respectively) from Abcam, DC-SIGN antibody (DCN47.5) from Miltenyi Biotec. Purified isotype control antibodies (MAB002, MAB003, MAB004, AB-105-C, MAB006) were purchased from R&D Systems.

### CRISPR-Cas9 genome editing

Two gRNAs each targeting *ITGB1 and ITGAV* were designed using crispr.mit.edu (Table 3.1). A 5' G was added to the *ITGB1* guides, then adaptors were added to form oligos which were annealed and cloned into px330 according to the protocol at genome-engineering.org. The *ITGAV* guides were incorporated into primers and used to *in vitro* transcribe (IVT) gRNAs according to the following protocol from the UC Berkeley IGI: dx.doi.org/10.17504/protocols.io.dwr7d5. The IVT gRNA was then mixed with recombinant Cas9 protein produced by the UC Berkeley IGI and nucleofected into BJAB cells according to the following protocol using a Lonza 4D Nucleofector: dx.doi.org/10.17504/protocols.io.dm448v.

| Target/Exon | gRNA sequence |
|---|---|
| *ITGB1*/exon 3-1 | AATGTAACCAACCGTAGCAA |
| *ITGB1*/exon 3-2 | TGCTGTTCCTTTGCTACGGT |
| *ITGAV*/exon 2 | GTGACTGGTCTTCTACCCGC |
| *ITGAV*/exon 3 | AGCATCTGTGAGGTCGAAAC |

Table 3.1. CRISPR-Cas9 guide RNA sequences used to target integrin genes in BJAB cells.

### Flow cytometry and sorting

Cells were harvested by centrifugation. Cells were blocked in 1% BSA (Fisher) in PBS (Gibco) plus Fc block (BD Biosciences). Cells were washed and stained in 1% BSA in PBS solution. When applicable, cells were fixed in 4% PFA (ThermoFisher Pierce) in PBS and permeabilized with 0.25% Triton X-100 (EM Science) in PBS. Live cells were stained with DAPI (BioLegend) and fixed cells with Ghost Dye Violet 510 (Tonbo Biosciences) for viability according to the manufacturer's instructions. Cells were analyzed using an LSR Fortessa or LSR Fortessa X-20 cell analyzer (BD Biosciences) and sorted using a BD Influx

or BD FACSARIA Fusion cell sorter (BD Biosciences). Data was processed and visualized with FlowJo 10 (BD Biosciences).

### Cell-free KSHV infection

iSLK.KSHV cells were plated in 6-well plates and reactivated with 1 μM sodium butyrate and 1 μg/mL doxycycline when the cells were ~80% confluent. After three days, the supernatant was collected and filtered through a 0.45 μm syringe filter directly onto 50% confluent target Caki-1 or BJAB cells. Media was changed on the target cells after 24 hours of infection, and infection rate was measured by flow cytometry two days post infection.

### Coculture KSHV infection

iSLK.KSHV cells were plated in 6-well plates and reactivated with 1 μM sodium butyrate and 1 μg/mL doxycycline when the cells were ~80% confluent. After two days, the reactivation media was removed and 110,000 BJAB cells were added to the well in a 50:50 mixture of complete DMEM and RPMI 1640 containing 1 μg/mL doxycycline. After three days of coculture media, BJAB cells were carefully pipetted off the iSLK monolayer, counted, and plated to limiting dilution in complete RPMI 1640 containing 1.2 mg/mL hyromycin.

### Constructs and cloning

Eph receptors were amplified from BJAB (EphA4, EphA5) or Caki-1 (EphA2) cDNA and cloned into pQCXIN (Clontech) using restriction enzyme sites.

### Transfection and transduction

Phoenix cells were transfected with pQCXIN-based constructs using Fugene transfection reagent (Promega) and Optimem (Gibco) according to the manufacturer's instructions. After 2-3 days, retrovirus was collected from the phoenix cell supernatant and filtered through a 0.45 μm filter. Filtered retroviral supernatant was applied to target cells with 6 μg/mL polybrene (Santa Cruz) and spinfected at 500x g for 2 hours at room temperature. Transduced cells were selected with neomycin (Fisher Scientific) at 1.2 mg/mL.

# IV

# The Search for a KSHV Tropism Switch Glycoprotein

## 4.1 Introduction

An important reason why herpesviruses are such successful pathogens is their ability to infect many different cell types. To disseminate upon a *de novo* infection, they must navigate through the body to cells that are targeted for lifelong latent infection. In general, herpesviruses use distinct sets of receptors to enter different cell types. The alphaherpesvirus HSV-1 serves as a prototypical example, encoding the bifunctional accessory glycoprotein gD which binds two different tissue-specific receptors (reviewed in Eisenberg *et al.*, 2012). Several herpesviruses have an extra layer of complexity in their tropism determination mechanisms and encode accessory glycoproteins that act as tropism switches—conditionally promoting engagement with one set of receptors over another. For example, the betaherpesvirus HCMV uses two different modulated gH/gL complexes—a trimer and a pentamer—which confer fibroblast or epithelial and endothelial tropism, respectively, and the balance of these two complexes in the viral envelope is controlled by an HCMV tropism determinant (reviewed in Li and Kamil, 2016).

Perhaps the best known gammaherpesvirus tropism switch mechanism has been described in EBV. EBV coordinates several sets of receptors with a number of different accessory glycoproteins in order to promote fusion with either B cells or epithelial cells (expertly reviewed in Shannon-Lowe *et al.*, 2014). EBV gp42 in complex with gH/gL binds HLA-II and is indispensable for B cell infection. EBV gH/gL alone binds to certain αV-family integrins to promote epithelial cell entry, but gp42 inhibits this interaction. Thus, the gH/gL complex alone promotes epithelial cell entry but is insufficient for B cell entry, while the gp42/gH/gL complex promotes B cell entry and inhibits interaction with epithelial cells. A similar effect has also been documented for the gp350 protein which does not bind gH/gL but interacts with coreceptors during B cell infection (Shannon-Lowe *et al.*, 2006, Turk *et al.*, 2006). Additionally, the amount of gp42 incorporated into virions is differentially modulated during replication in B cells and epithelial cells such that virions produced from B cells are more permissive for epithelial cell infection, and vice versa. Having a proximal sink for inhibitory gp42/gH/gL complexes and gp350 proteins is also a likely mechanism by which direct cell-to-cell contact between infected B cells and target epithelial cells enhances the infection rate of epithelial cells (reviewed in Shannon-Lowe *et al.*, 2014).

Interestingly, a functionally similar process was recently described in bovine herpesvirus 4 (BoHV-4). The BoHV-4 positional homolog of EBV gp350 is gp180 and is encoded by the Bo10 gene. Bo10 is alternatively spliced in a cell-type specific manner, and this splicing event dictates whether the virus infects cells lacking glycosaminoglycans, particularly CD14+ circulating monocytes *in vivo* (Machiels *et al.*, 2011, Machiels *et al.*, 2013). The murid herpesvirus 4 (MuHV-4 or MHV68) also encodes a positional homolog of EBV gp350 and BoHV-4 gp180 called gp150 which also binds glycosaminoglycans (de

Lima *et al.*, 2004). It has been shown that gp150 KO MHV68 is less efficiently released from cell surfaces, but infects cells lacking glycosaminoglycans better than WT virus. Surprisingly, the gp150 KO MHV68 has a very mild *in vivo* infection defect and still establishes latency (Stewart *et al.*, 2004, de Lima *et al.*, 2004, Gillet *et al.*, 2009, and reviewed in Gillet *et al.*, 2015).

There has been little research published on tropism determinants in KSHV. Like EBV, KSHV has at least two drastically distinct modes of infection: adherent cell infection dependent on a combination of integrins, HS, and EphA2, and B cell infection which requires CD21 and HLA-II for EBV and unknown receptors (perhaps including DC-SIGN) for KSHV. Given the abundance of tropism switch mechanisms described in the gammaherpesvirus family, we hypothesized that KSHV encodes a tropism switch. A new KSHV bacterial artificial chromosome (BAC) has recently been created which enables targeted scarless mutation of viral genes by a red recombination system (Tischer *et al.*, 2006, Tischer *et al.*, 2010, Brulois *et al.*, 2012). We used recombineering of KSHV.BAC16 to manually screen candidates for a tropism determinant between cell-free infection of Caki-1 cells and coculture infection of BJAB cells.

A clear candidate for this study was KSHV K8.1, the positional homolog of the EBV gp350, BoHV-4 gp180, and MHV68 gp150 proteins discussed above. K8.1 is alternatively spliced into K8.1A and K8.1B, which are both single-pass glycosylated transmembrane proteins and are distinctly recognized as a doublet by patient sera by western blot (Raab *et al.*, 1998, Chandran *et al.*, 1998, Li *et al.*, 1999, Zhu *et al.*, 1999, Wu *et al.*, 2000, Tang *et al.*, 2002). K8.1 localizes to the surface of cells during lytic replication and is incorporated into the viral envelope (Li *et al.*, 1999, Zhu *et al.*, 1999, Wu *et al.*, 2000, Zhu *et al.*, 2005). It is a robust target of both antibody and T cell responses during natural infection, and most KSHV diagnostic tools still rely on detection of anti-K8.1 antibodies (Raab *et al.*, 1998, Chandran *et al.*, 1998, Li *et al.*, 1999, Osman *et al.*, 1999, Lang *et al.*, 1999, Zhu *et al.*, 1999b, Juhász *et al.*, 2001, Corchero *et al.*, 2001, Lam *et al.*, 2002, Robey *et al.*, 2009, Mbisa *et al.*, 2010, Robey *et al.*, 2011, Labo *et al.*, 2014).

The major known function of K8.1 is that it binds the glycosaminoglycan heparan sulfate (Birkmann *et al.*, 2001, Wang *et al.*, 2001). Additionally, extracellular recombinant K8.1 induces IRF-3, interferon beta, and expression of interferon-stimulated genes in fibroblasts through an unknown mechanism (Perry *et al.*, 2006). Another group has shown that while K8.1 is dispensable for viral entry into HEK293T cells, it is important for efficient virion egress from reactivated BCBL-1 cells, reminiscent of some of the published studies on MHV68 gp150 (Luna *et al.*, 2004, Subramanian *et al.*, 2010). Interestingly, K8.1 can also stimulate VEGF and vIL-6 expression in infected cells, a hallmark signature of the highly vascularized and cytokine-dependent KS tumors (Subramanian *et al.*, 2010). Given that K8.1 is so highly immunogenic, its function must be absolutely essential to the KSHV life cycle. Although it binds to HS, it seems to be mostly dispensable for both

replication and infection of HS-expressing HEK293T cells, so we hypothesized that K8.1 could be involved in the regulation of B cell entry (Luna *et al.*, 2004).

As other candidates, we included the core herpesvirus fusion glycoproteins gB, gH, and gL. We also tested gM and gN, another conserved heterodimer of herpesvirus glycoproteins with somewhat divergent functions. In alphaherpesviruses, gM and gN are involved in viral entry and virally-induced cellular fusion but are generally dispensable for replication, especially in tissue culture (Osterrieder *et al.*, 1996, Dijkstra *et al.*, 1997, Fuchs *et al.*, 1999, Brack *et al.*, 1999, Klupp *et al.*, 2000, König *et al.*, 2002, Tischer *et al.*, 2002, Fuchs *et al.*, 2005, Ziegler *et al.*, 2005, Yamagishi *et al.*, 2008, Leege *et al.*, 2008, Ren *et al.*, 2012, Kim *et al.*, 2013, El Kasmi and Lippé, 2015). In betaherpesviruses, gM and gN form a complex and are essential for viral growth in tissue culture (Mach *et al.*, 2000, Mach *et al.*, 2005, Krzyzaniak *et al.*, 2007, Kawabata *et al.*, 2012). Interestingly, a potential role for gM or gN in betaherpesvirus syncitia formation has not been studied, despite these proteins having a similar function in alphaherpesvirus cell-to-cell spread and membrane fusion. In the gammaherpesvirus MHV68, gM is required for replication (May *et al.*, 2005). gN KO EBV is severely impaired in both replication and infection (Lake and Hutt-Fletcher, 2000). Only a single paper has been published on the function KSHV gM/gN, which reports that the glycoprotein complex inhibits natural fusion between HEK293 cells (Koyano *et al.*, 2003). KSHV gM and gN are also incorporated into the virion envelope (Zhu *et al.*, 2005).

Finally, we screened two other small predicted single-pass transmembrane glycoproteins of unknown function: orf27 and orf28. Like K8.1, orf28 is another KSHV protein that generates robust CD8+ T cell responses during infection, implying a critical function for the virus (Robey *et al.*, 2009, Robey *et al.*, 2011, Labo *et al.*, 2014). KSHV orf28 is a virion envelope protein and is the positional homolog of EBV gp150/BDLF3, but it has no amino acid sequence similarity to this protein (Zhu *et al.*, 2005). EBV gp150/BDLF3 is a nonessential virion glycoprotein and binds heparan sulfate (Borza and Hutt-Fletcher, 1998, Chesnokova *et al.*, 2016). Interestingly, KO of gp150/BDLF3 had no effect on B cell infection rate but enhanced the infection rate of an epithelial cell line (Borza and Hutt-Fletcher, 1998). MHV68 orf28 is also incorporated into the virion but is nonessential for replication in tissue culture (Bortz *et al.*, 2003, May *et al.*, 2005). As of this writing, no studies have been published concerning the function of KSHV orf27. The orf27 gene product in EBV is BDLF2 which can be found in the virion and induces morphological changes in infected cells (Johannsen, 2004, Loesing *et al.*, 2009). In MHV68, orf27 produces a small virion-associated protein called gp48. MHV68 gp48 also promotes actin cytoskeletal rearrangements in infected cells and is implicated in direct cell-to-cell spread, although it is dispensable for viral replication (May *et al.*, 2005, Gill *et al.*, 2008). Both homologous orf27 gene products from MHV68 and EBV require complex formation with a second viral protein for complete maturation and surface trafficking (May *et al.*, 2005, Gill *et al.*, 2008, Gore *et al.*, 2009, Loesing *et al.*, 2009).

Recombinant KSHV strains with stop cassette mutations of gB, gH, gL, gM, gN, K8.1, orf27, and orf28 were tested for their ability to produce infectious virions, measured by the infection rate of Caki-1 cells using filtered cell-free virus and BJAB cells in coculture. Stop cassettes inserted into the orfs encoding gB, gH, gL, gM, and gN appeared to be lethal, as no infectious virions were detected from reactivated iSLK cells transfected with the mutant KSHV BACs. Although our studies were plagued by technical issues in reactivation efficiency, we confirmed the findings reported in Luna *et al.*, 2004 that K8.1 is dispensable for replication and infection. We also found that orf27 and orf28 are likely not required for replication and infection, while gB, gH, gL, gM, and gN mutants produced no infectious virions in any assay. Interestingly, we found in multiple experiments that orf28-stop KSHV infected Caki-1 cells at a slightly lower rate than WT, but infected BJAB cells in coculture more efficiently. However, this effect was largely nullified at high MOI which we hypothesize is a limitation of the BJAB coculture infection system. Still, our results suggest that KSHV orf28 is a candidate tropism determinant and perhaps even a tropism switch and the role of this protein should be further characterized in additional infection systems.

## 4.2 Results

### Creation of stop cassette glycoprotein mutant KSHV BACs.

To investigate the role of the selected KSHV proteins in tropism determination between epithelial and B cell infection, we first created scarless mutant strains carrying small insertions of a "stop cassette" in the orfs of interest. The stop cassette consisted of dual premature termination codons (PTC) and an EcoRI restriction enzyme site which facilitated genetic screening of clones. The stop cassettes were targeted in-frame within the first ~20 nucleotides of each orf, which should interrupt translation and cause degradation of the small RNA product by nonsense mediated decay (Fig. 1).

To insert the stop cassettes, we used the red recombination-based scarless mutation system described in Tischer *et al.*, 2006 and Tischer *et al.*, 2010. Linear double-stranded DNA recombination cassettes containing the I-SceI nuclease site and a kanamycin resistance positive selection marker (PSM) flanked by homology arms containing the stop cassette were prepared by PCR using the gene-specific primers listed in Table 4.2 and the plasmid pEP-KanS as a template. The cassettes were gel purified, then electroporated into competent GS1783 E. coli expressing red recombinase and containing the WT KSHV.BAC16. These first recombinants were screened by selection on kanamycin plates. The presence of the ~1200 bp recombination cassette within the gene of interest was confirmed by PCR and sequencing of at least one homology arm.

The GS1783 E. coli containing first recombinants were then induced to express red recombinase for a second time, as well as the I-SceI meganuclease. This results in a

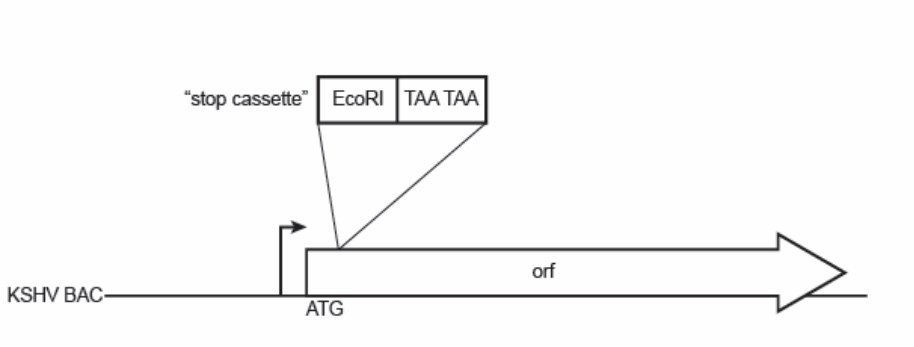



Figure 4.1. Engineering premature stop codon mutants into orfs of interest.

double-strand cut proximal to the PSM and between the two homologous regions containing the stop cassette, which then recombine to scarlessly excise the I-SceI site and the PSM. These second recombinants were then replica plated on chloramphenicol plates with or without kanamycin to screen for loss of the PSM. Second recombinants were screened for loss of the recombination cassette by PCR and subsequently sequenced to confirm the presence of the stop cassette.

Finally, structural integrity of the KSHV.BAC16 mutants was confirmed by restriction enzyme digestion. Screened second recombinants and WT KSHV.BAC16 were digested with RsrII, then run on an 8% agarose gel at 20V overnight. The digested band pattern was compared to the parent KSHV.BAC16 to ensure that no other large rearrangements had occurred elsewhere. For orf28, a revertant was constructed in the manner described above to restore the WT sequence to the target gene to control for unintended changes elsewhere in the BAC that were not detected by the RsrII digest.

**Infection assays reveal K8.1, orf27, and orf28 to be nonessential KSHV proteins.**

The KSHV glycoprotein mutant BACs were transfected in parallel with WT KSHV.BAC16 into uninfected iSLK cells. After two days, newly latently infected iSLK cells were selected with hygromycin. As soon as cells were selected and expanded to sufficient numbers, the infected iSLK cells were plated in 6-well plates for concurrent cell-free virus production or coculture infection of BJAB cells. For cell-free infection, virus-containing supernatant was collected and filtered after three days of reactivation with doxycycline and sodium butyrate. The filtered virus was then transferred onto 50% confluent monolayers of Caki-1 cells. Infection rate was measured after two days with GFP expression by flow cytometry. For coculture infection, infected iSLK cells were reactivated with doxycycline and sodium butyrate. After two days, the reactivation media was removed and replaced with a 50:50 mixture of DMEM and RPMI with 5% FBS, doxycycline. 110,000 WT BJAB cells were added to each well and cocultured for three days. After three days, BJABs were gently pipetted off the iSLK monolayer, counted, and plated at limiting dilutions with hygromycin selection, as described in Chapter 3.

Infection rate was calculated from the number of B cell colonies at each dilution approximately 2 weeks post infection.

Since Caki-1 cells are highly permissive to cell-free infection, this assay served as an initial readout of whether any virions were made and released by cells containing the stop mutant KSHV strains. We were unable to detect any infectious virus when stop cassettes were inserted into orf8 (gB), orf22 (gH), orf47 (gL), orf39 (gM), or orf53 (gN), suggesting that these genes are essential for viral replication and/or virion assembly (Table 4.1). Furthermore, no infected BJAB colonies were detected after coculture with gB-stop, gH-stop, gL-stop, gM-stop, or gN-stop KSHV. Thus, our data suggest that these five stop mutants have significant defects in either viral egress or entry regardless of infection system. We did not further characterize the nature of these defects.

During our studies, we noticed that the basic transfection method of making latently infected iSLK cells renders them prone to becoming permanently unable to reactivate, even in cells containing the WT BAC. This effect also seemed to be exacerbated in cells transfected with all recombineered BACs regardless of sequence—both mutants and revertants with WT sequence inactivated faster than the WT parent BAC. The reason for this effect is unknown, but it confounded the following analysis of the non-lethal glycoprotein mutants. Thus, we refer to the relative ratio of WT to coculture infection in addition to the raw infection rate.

In line with prior reports that K8.1 was dispensable for viral replication and entry, we found that the K8.1-stop virus infected both Caki-1 cells and BJAB cells in coculture (Fig. 4.2). While the raw infection rates of the KSHV.BAC16-K8.1-stop virus were reduced compared to WT KSHV.BAC16, the infection rate was reduced comparably in each context, suggesting that K8.1 does not play a differential role in these two types of infections (Fig. 4.2). This was quite surprising, given that the K8.1 positional homologs in other gammaherpesviruses play prominent roles in tropism determination related to heparan sulfate interactions (Stewart *et al.*, 2004, de Lima *et al.*, 2004, Shannon-Lowe *et al.*, 2006, Turk *et al.*, 2006, Gillet *et al.*, 2009, Machiels *et al.*, 2011, Machiels *et al.*, 2013, Gillet *et al.*, 2015). We believe the reduced infection rates to be attributed to the inactivation of the iSLK cells rather than a true phenotype, as K8.1 was previously reported to be completely dispensable for replication and entry into adherent cells.

We found that like K8.1-stop virus, both orf27-stop and orf28-stop virus-containing cells produced less infectious virus in the cell-free Caki-1 infection assay compared to cells transfected with WT KSHV.BAC16 (Fig. 4.2). Again, we believe this to be due to inactivation of the iSLK cells as opposed to a real phenotype but were not able to confirm this. Interestingly, while orf27-stop virus showed a proportional decrease in coculture infection rate as well, or28-stop virus infected BJAB cells in coculture at a rate exceeding that of WT virus despite the significantly reduced Caki-1infection rate. This result suggests that orf28 is involved in KSHV tropism switching and may inhibit B cell

| BAC | Caki-1 Infection | B cell infection | Phenotype |
|---|---|---|---|
| orf8-stop (gB) | - | - | presumed lethal |
| orf22-stop (gH) | - | - | lethal or severe entry defect |
| orf47-stop (gL) | - | - | lethal or severe entry defect |
| orf39-stop (gM) | - | - | presumed lethal |
| orf53-stop (gN) | - | - | presumed lethal |
| K8.1-stop | + | + | possible reduced infectivity (both) |
| orf27-stop | + | + | possible reduced infectivity (both) |
| orf28-stop | + | + | enhanced B cell infectivity |
| orf28-revert | - | - | dead? |

Table 4.1 Summary of infection phenotypes of glycoprotein mutant KSHV strains.

infection. It is unclear if the reduced rates of cell-free infection from orf27- and orf28-stop viruses are due to the natural inactivation phenomenon that we repeatedly observed, or due to real phenotypes. Regardless, orf27 seems to be nonessential but may have a role in replication or egress. The interpretation of the orf28-stop phenotype is more interesting. Either there is no effect on cell-free infection and coculture infection is drastically elevated, or coculture infection is unaffected while cell-free infection is impaired.

Since the original populations of transfected cells quickly lost the ability to reactivate, to attempt to validate the phenotype, we made single cell clones of the original transfected populations of WT and orf28-stop cells and selected highly reactivating clones for further experiments. Unfortunately, these clones also quickly lost the ability to reactivate, but we were able to perform one set of infection experiments with the orf28-stop iSLK clone. In this experiment, the orf28-stop virus infected a lower percentage of Caki-1 cells (Fig. 4.3A). Interestingly, again the orf28-stop virus infected BJAB cells at an elevated rate (Fig. 4.3B).

We attempted to further validate the phenotype of KSHV.BAC16-orf28-stop virus by modifying the way that new latently infected iSLK cells were generated in a manner that is much less prone to inactivation (M. Gardner, personal communication). First, HEK293T cells were transfected with KSHV BACs. The next day, the transfected HEK293T cells were mixed with uninfected target iSLK cells. Virion production was stimulated from the HEK293T cells by treatment with PMA and sodium butyrate. After four days of coculture infection, the coculture is selected such that all HEK293T cells and uninfected

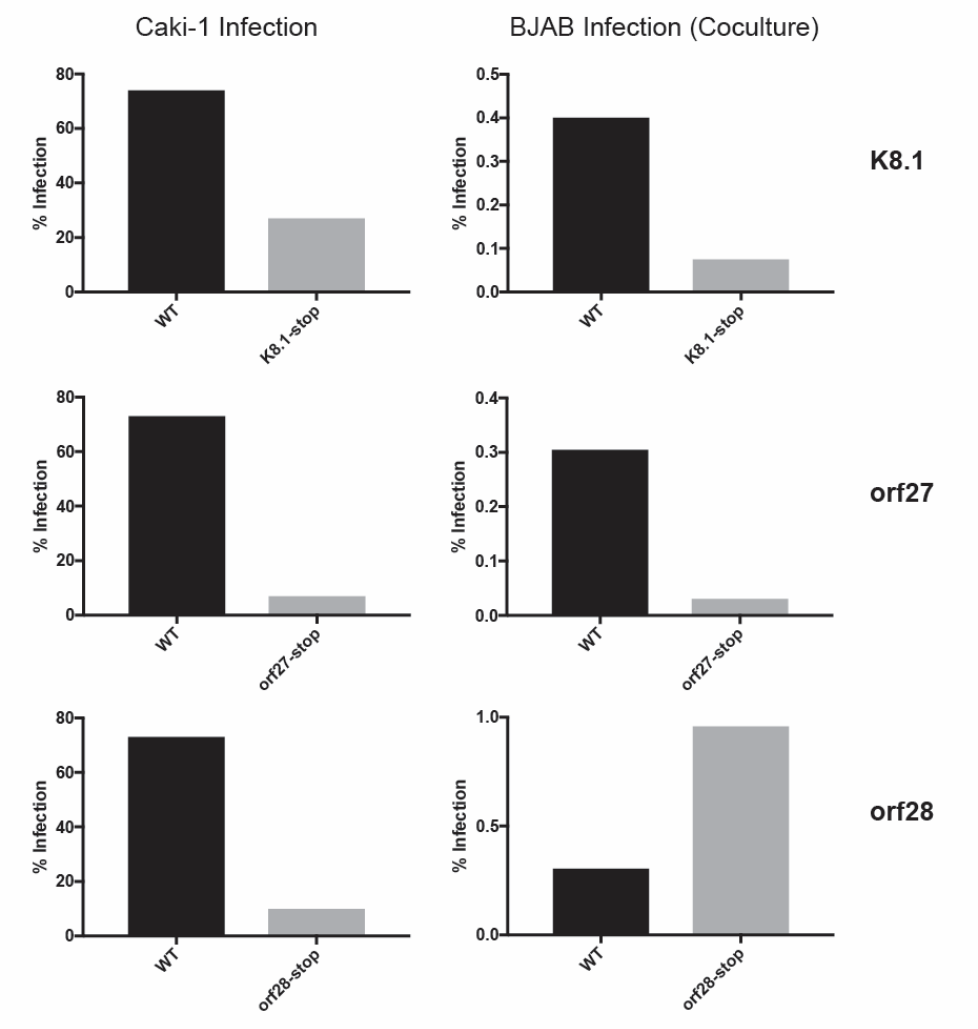



Fig. 4.2. Infection rates of nonlethal KSHV glycoprotein mutants on Caki-1 and BJAB cells. iSLK cells containing each mutant KSHV.BAC16 strain were reactivated. Virus was either collected after three days and used to infect Caki-1 cells, or BJAB cells were cocultured for three days after two days of initial reactivation. Infection rate of Caki-1 cells was quantified by flow cytometry. Infection rate of BJAB cells was measured by limiting dilution with hygromycin selection.

iSLK cells die off. The concentration of hygromycin is then slowly increased after two weeks in culture with the initial selection media. iSLK cells infected with WT and orf28-stop BAC16 generated in this manner reactivated well and produced very high-titer virus compared to previous experiments, measured by infection of Caki-1 cells (Fig. 4.3C). Surprisingly, the infection percentage of BJAB cells in coculture was only elevated about two-fold compared to previous infections (Fig. 4.3D). Furthermore, while orf28-stop virus still appeared to infect BJABs to a slightly higher percentage than WT cells, the effect was less dramatic than in previous experiments and was not statistically significant (Fig. 4.3D). Further work is required to understand the phenotype of orf28-stop KSHV.

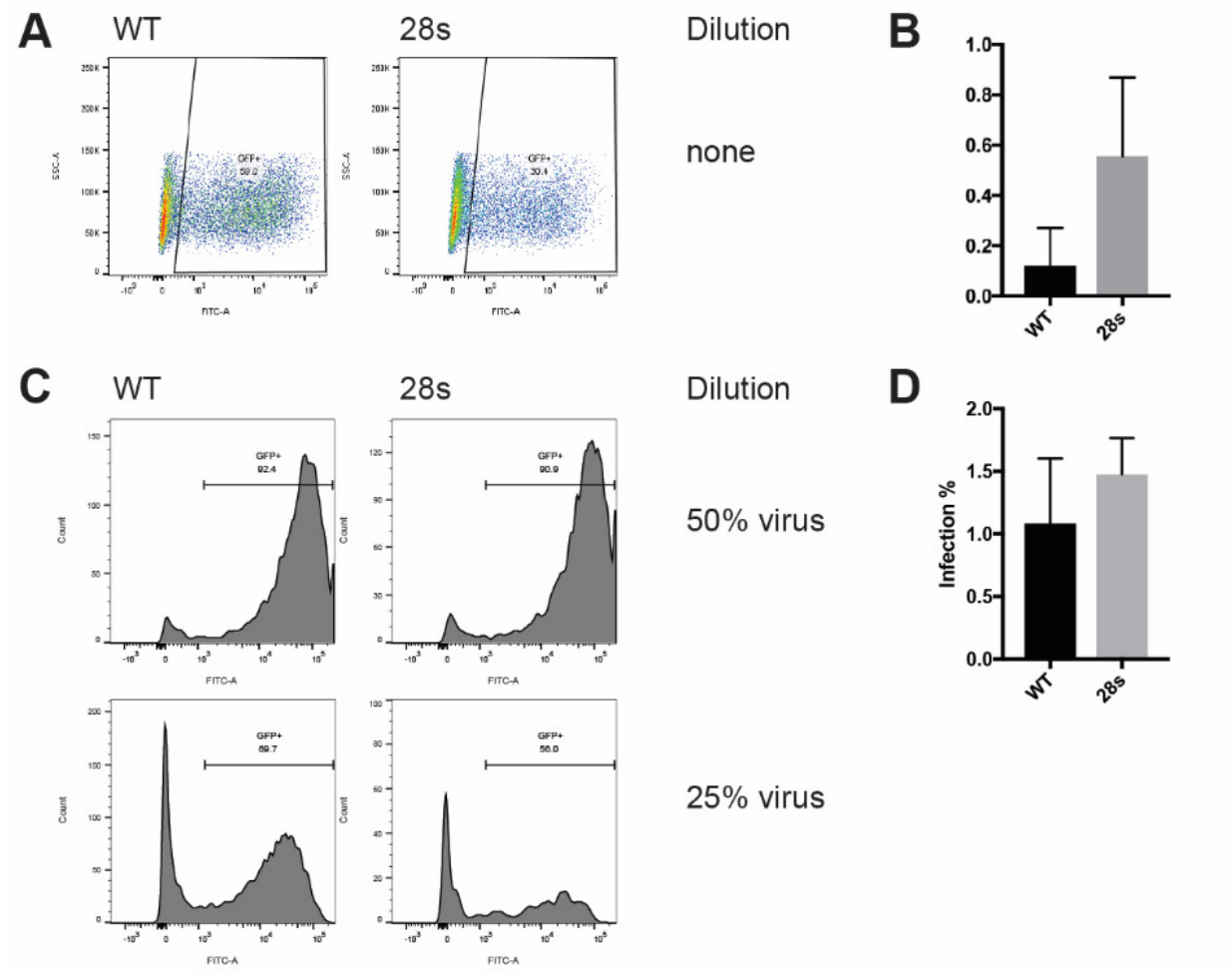



Figure 4.3. Additional orf28 infection experiments. The original orf28-stop-transfected iSLK cells were single-cell clones and highly reactivating clones were isolated. The clone was expanded and then simultaneously reactivated from 6-well plates (A) to produce cell-free virus used to infect Caki-1 cells or (B) to infect BJAB cells in coculture. (A) Cell-free infection percentage was measured by flow cytometry two days post infection. (B) Coculture infection percentage was measured by limiting dilution with hygromycin selection. WT and orf28-stop iSLK cells generated by coculture were simultaneously reactivated from 6-well plates (A) to produce cell-free virus used to infect Caki-1 cells or (B) to infect BJAB cells in coculture. (A) Cell-free infection percentage with diluted, filtered viral supernatant was measured by flow cytometry two days post infection. (B) Coculture infection percentage was measured by limiting dilution with hygromycin selection.

## 4.3 Discussion

Here we screened selected candidate KSHV genes for tropism determination by comparing two infection models: highly permissive cell-free infection of Caki-1 cells and the relatively inefficient infection of BJAB cells in coculture. As a family, gammaherpesviruses seem to employ functionally redundant and somewhat homologous

proteins that function as tropism determinants or switches, and the expression of these proteins can be modulated to influence efficiency of subsequent infections. Such mechanisms are likely highly advantageous for dissemination in a new host, since herpesviruses have to navigate through many different cell types and tissues during initial infection and colonization. Thus, we hypothesized that KSHV encodes tropism determinants that are functionally similar to those that have been characterized in EBV and MHV68.

In screening the core herpesvirus glycoproteins, we observed that mutant KSHV BACs with interrupted gB, gH, gL, gM, or gN genes were unable to make new infectious virions in either infection assay. These results indicate that these glycoproteins are essential for either viral egress or subsequent infection. These results are not unprecedented. Limited studies of gM and gN in gammaherpesviruses have documented severe-lethal phenotypes when the expression of either protein is disrupted (Lake and Hutt-Fletcher, 2000, May *et al.*, 2005). gB is required for KSHV egress from infected HEK293T cells (Krishnan *et al.*, 2005). The roles of gH and gL in herpesvirus egress are not well-studied, but data from the alphaherpesvirus HSV-1 suggests that gH, along with gB, is involved in trafficking viral capsids across the nuclear membrane (Farnsworth *et al.*, 2007). However, KO of gH in EBV has no effect on egress, just subsequent *de novo* infection (Molesworth *et al.*, 2000).

During these studies we discovered that latently infected iSLK cells made by transfection of KSHV BACs are prone to rapidly losing the ability to reactivate, especially when the BACs had been modified by recombineering. We suspect that this is related to the selection step following transfected during which the cells are treated with a high concentration of hygromycin. Thus, it is important to consider that this phenomenon could have factored in to the apparent lack of infectious virus produced by these five mutant KSHV strains.

Surprisingly, we found that K8.1-stop virus infected cells in both cell-free and coculture infection models at reduced levels but a similar ratio as WT KSHV. K8.1 was a prime candidate for a KSHV tropism determinant given the functions of its positional homologs in other gammaherpesviruses and the robust immune response against this protein. However, our assays only compared two KSHV infection models. To infect adherent cells, we now know that there are at least two versions of the HS- and EphA2-dependent infection mechanisms used by KSHV to infect adherent cells which vary in their requirement for integrin receptors and EphA2 signaling. Furthermore, the infection of BJAB cells in coculture does not entirely recapitulate the characteristics of primary B cell infection, specifically: that cell-free infection of B cells is possible at a lower rate than coculture infection, that the presence of T cells may influence cell-free infection of B cells, and that activated B cells may be infected by cell-free KSHV in a DC-SIGN-dependent manner. Thus, the effects of the K8.1-stop mutant strain should be more thoroughly

examined in other infection models, especially in cell-free infection of activated and resting primary B cells.

Additionally, we found compelling evidence that orf28, but not orf27, may have a novel tropism determining or switching activity during KSHV infection. Although we were limited by technical challenges of the viral BAC system, we documented in multiple experiments that orf28-stop KSHV seems to have a slight defect in Caki-1 cell infection, while infection of BJAB cells in coculture is elevated. Unfortunately, once we had solved some technical issues with the iSLK cells infected with the recombineered BACs, we encountered a new limitation of the coculture system which is that there seems to be a maximum infection rate for unknown reasons. When BJABs were infected in coculture with new iSLK.BAC16 and iSLK.BAC16-orf28-stop cells producing a much higher titer of KSHV, the enhanced infection phenotype was blunted and the infection percentage of the BJAB cells did not increase proportionately to the number of free virions being produced by the iSLK cells. It is possible that the BJAB infection system was saturated in this experiment, or that the tropism regulation function of orf28 is inversely related to virus concentration.

However, using these new iSLK cells it should be relatively simple validate the orf28 phenotype either by reducing the amount of virions produced into the coculture either by titrating back the reactivation agents doxycycline and sodium butyrate, or by diluting the monolayer seeded for coculture with uninfected iSLK cells. Furthermore, it would be very informative in future experiments to normalize these experiments to genome copy number and then test the WT and orf28-stop KSHV for their relative infectivity on a variety of different cell types which we now know differ significantly in receptor use, namely: HFF, HUVEC, PGK, and resting and activated tonsillar and peripheral blood CD19+ B cells.

## 4.4 Materials and Methods

### KSHV BACs, bacterial strains, and cell lines

GS1783 E. coli (Tischer *et al.*, 2010) carrying the KSHV.Bac16 (Brulois *et al.*, 2012) were grown in LB media containing 20 μg/mL chloramphenicol. iSLK and SLK/Caki-1 (ATCC HTB-46) cells were a gift from D. Ganem. HEK293T cells (ATCC CRL-1573) were purchased from the ATCC. All adherent cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, SeraDigm) at 37°C with 5% $CO_2$. BJAB cells (DSMZ ACC757) were a gift of D. Ganem and grown in Roswell Park Memorial Institute (RPMI) 1640 Media (Gibco) supplemented with 5% FBS (SeraDigm) at 37°C with 5% $CO_2$.

### Red-based scarless BAC recombineering

For each orf, in-frame stop cassettes were designed to be inserted within the first 20 nucleotides from the start codon annotated in the NCBI HHV-8 reference genome NC_009333.1. Oligos were designed containing the stop cassette insertion, the appropriate amount of flanking genomic sequence, and the PSM F and R amplification sequences listed in the Table 4.2. Recombination cassettes were created by using these oligos to amplify the PSM from pEP-KanS by PCR. These dsDNA cassettes were electroporated into GS1783 cells containing KSHV.BAC16 and the stepwise recombination induction was carried out as described in Tischer *et al*., 2010.

| Name | Sequence |
|---|---|
| PSM F | AGGATGACGACGATAAGTAGGG |
| PSM R | AACCAATTAACCAATTCTGATTAG |
| orf8 recombination cassette F | ctgcaattgagcaaccacaatgactcccaggtctagattggaattctaataa gccaccctggggactgtcatAGGATGACGACGATAAGTAGGG |
| orf8 recombination cassette R | cgcaaaagcagaccaacaggatgacagtccccagggtggcttattagaattc caatctagacctgggagtcaAACCAATTAACCAATTCTGATTAG |
| K8.1 recombination cassette F | tcctctgggattaataaccatgagttccacacagattcgcgaattctaataa acagaaatccctgtggcgctAGGATGACGACGATAAGTAGGG |
| K8.1 recombination cassette R | gacaaaggcataggattaggagcgccacagggatttctgtttattagaattc gcgaatctgtgtggaactcaAACCAATTAACCAATTCTGATTAG |
| orf22 recombination cassette F | TAGAGGAGACATGCAGGGTCtagccttcttggcggcccttgaattctaataa gcatgctggcgatgcatatcAGGATGACGACGATAAGTAGGG |
| orf22 recombination cassette R | cagtggctccacatgtcaacgatatgcatcgccagcatgcttattagaattc aagggccgccaagaaggctaAACCAATTAACCAATTCTGATTAG |
| orf27 recombination cassette F | ctaacgatttgaagcgggggggggtatggcgtcatctgatgaattctaataa attctgtcggttgcaaggacAGGATGACGACGATAAGTAGGG |
| orf27 recombination cassette R | cacagacggagccgtcatccgtccttgcaaccgacagaatttattagaattc atcagatgacgccataccccAACCAATTAACCAATTCTGATTAG |
| orf28 recombination cassette F | tcagagaatacagtgctaatcagggtagatgagcatgactGAATTCTAATAA tccccgtctccagtcaccggAGGATGACGACGATAAGTAGGG |
| orf28 recombination cassette R | cggagccgtccaccattcctccggtgactggagacggggaTTATTAGAATTC agtcatgctcatctaccctgAACCAATTAACCAATTCTGATTAG |
| orf28 revertant cassette F | tcagagaatacagtgctaatcagggtagatgagcatgacttccccgtctcca gtcaccggAGGATGACGACGATAAGTAGGG |
| orf28 revertant cassette R | cggagccgtccaccattcctccggtgactggagacggggaagtcatgctcat ctaccctgAACCAATTAACCAATTCTGATTAG |
| orf39 recombination cassette F | cagagcaaatgtacataattacagccacaaacaacagcttttattagaattc ggaggacatgagaaaacggtAGGATGACGACGATAAGTAGGG |
| orf39 recombination cassette R | catgcgcgcttcaaagagcgaccgttttctcatgtcctccgaattctaataa aagctgttgtttgtggctgtAACCAATTAACCAATTCTGATTAG |
| orf47 recombination cassette F | atgtgaccaatagggtggtccacaggacggcaaatagcgcttattagaattc aaagatccccatggggcaaaAGGATGACGACGATAAGTAGGG |
| orf47 recombination cassette R | acacaagggtgaaacccggatttgccccatggggatctttgaattctaataa gcgctatttgccgtcctgtgAACCAATTAACCAATTCTGATTAG |
| orf53 recombination cassette F | gacctagtatcgaggccacaaataaagccagggccaccgtttattagaattc ggacgctgtcattatgaacaAGGATGACGACGATAAGTAGGG |
| orf53 recombination cassette R | cggcttggagcctcggcggttgttcataatgacagcgtccgaattctaataa acggtggccctggctttattAACCAATTAACCAATTCTGATTAG |
| orf58 recombination cassette F | tcccgctcagataactgaagagcgacagagcgcgctcactttattagaattc gtccaggcggcacatggtgtAGGATGACGACGATAAGTAGGG |
| orf58 recombination cassette R | acatttaacccccctgatttgacaccatgtgccgcctggacgaattctaataa agtgagcgcgctctgtcgctAACCAATTAACCAATTCTGATTAG |

Table 4.2. Oligos used to amplify recombination cassettes from pEP-KanS.

### Transfection

Uninfected iSLK cells were transfected with 2 μg of BAC DNA in 6-well plates using the Fugene transfection reagent (Promega). iSLK cells were transfected at a 3.5:1 ratio of Fugene to DNA.

### Coculture method for creation of latently infected iSLK.KSHV lines

HEK293T cells at 70% confluence in 100 mm dishes were transfected with 5 μg fresh BAC DNA prepared with a Macherey-Nagel Nucelobond BAC 100 kit using a 3:1 ratio of Fugene to DNA. After 1 day, 1.5 million transfected HEK293T cells were co-plated with an equal number of uninfected iSLK cells into a fresh 100 mm plate. After 12-24 hours, virus production was induced using 25 nM PMA and 1 mM sodium butyrate in 10 mL of culture medium. After 2 days, an additional 5 mL of induction media was added to the coculture. After 2 additional days, selection was begun by replacing the induction medium with fresh medium containing 300 μg/mL hygromycin, 1 μg/mL puromycin, and 250 μg/mL G418/neomycin. Selection media was changed every two days until all HEK293T cells were dead and selected infected iSLK cells filled the 100 mm dish. After two weeks, the hygromycin concentration was raised in increments of 200 μg/mL until reaching the final concentration of 1 mg/mL.

### Cell-free KSHV infection

iSLK.KSHV cells were plated in 6-well plates and reactivated with 1 μM sodium butyrate and 1 μg/mL doxycycline when the cells were ~80% confluent. After three days, the supernatant was collected and filtered through a 0.45 μm syringe filter directly onto target Caki-1 cells. Media was changed on the target cells after 24 hours of infection, and infection rate was measured by flow cytometry two days post infection.

### Coculture KSHV Infection

iSLK.KSHV cells were plated in 6-well plates and reactivated with 1 μM sodium butyrate and 1 μg/mL doxycycline when the cells were ~80% confluent. After two days, the reactivation media was removed and 110,000 BJAB cells were added to the well in a 50:50 mixture of complete DMEM and RPMI 1640 containing 1 μg/mL doxycycline. After three days of coculture media, BJAB cells were carefully pipetted off the iSLK monolayer, counted, and plated to limiting dilution in complete RPMI 1640 containing 1.2 mg/mL hyromycin.

# V

# Concluding Remarks

Here we provide an in-depth report into the nuance of KSHV receptor use across multiple infection systems. A comprehensive and accurate model of receptor function during KSHV entry is critical to understand downstream biological activities and functional consequences that result from receptor engagement at the cell surface. Our work, presented here, underscores that the set of available receptors on any given cell type alone does not necessarily directly indicate permissiveness for infection, nor the ultimate infection mechanism used to penetrate the cell surface. It is clear that additional environmental and cellular factors are taken into account by the KSHV glycoproteins which negotiate virion uptake and entry with host receptors at the cell surface.

A level of simplification is required when summarizing and reviewing twenty years' worth of studies regarding KSHV receptor use, but we would argue that going forward, the cell types used in individual experiments should be discussed separately. The KSHV receptor field has been fortunate to have a handful research groups thoroughly characterize KSHV receptor use and entry mechanisms in several cell lines. However, the cell lines investigated by each research group rarely overlap. This fact means that very little independent replication and validation of experimental results has been reported. It also has made it difficult to synthesize the results of these studies into common models of receptor use and infection. Perhaps most problematic is that cell-type-specific variation in the details of entry mechanisms are often presented as contradictory to the existing KSHV receptor dogma. An additional confounding factor is the lengths of time separating the discovery of different KSHV receptors. For example, EphA2 was not described as a KSHV receptor until 2012, and thus, studies published prior to 2012 make conclusions based on incomplete contextual knowledge of the expression and functional status of what we now know is an extremely important receptor.

The most sensible approach will be to model receptor use and entry mechanisms with fine details confined exclusively to individual cell lines or primary cell types. Then, unifying characteristics can be used to classify these entry mechanisms into model groups. Infection of HFF cells, HUVEC, and HMVEC-d cells depends on HS, EphA2, and some combination of integrins α3β1, αVβ3, and αVβ5. Within this group, details of integrin use and internalization mechanisms diverge. Infection of HT1080 fibroblasts, primary mouse keratinocytes, and HSG(HeLa) may fall into this category as well, although this series of studies were mostly focused on gain-of-function studies of integrin αVβ3 and questioned the requirement for HS. However, the larger receptor context was not explored through loss-of-function studies.

Another major group of infection mechanisms is identified and characterized in more detail in this report. The unifying feature of Caki-1, HeLa, and PGK infection is that integrin receptors are not required for infection. We found that infection of SLK/Caki-1 and HeLa cells requires HS and only the extracellular domain of EphA2, but not integrins. There were several experiments published over the years which had suggested that

infection of SLK/Caki-1 and HEK293 cells did not involve integrins, so we were pleased to replicate these findings and further characterize this type of infection event. Using blocking reagents, we identified yet another mode of KSHV infection with unique receptor involvement in PGK cells. Despite the fact that PGK cells expressed a pattern of known KHSV receptors identical to that of HeLa cells, we found that infection appeared to be independent of both canonical KSHV integrin receptors and Eph receptors but was critically dependent on heparan sulfate interactions.

Based on our studies, we strongly believe there are other additional receptors involved in the infection of Caki-1, HeLa, and especially PGK cells, and that virion internalization is completely independent of the integrin-EphA2 signaling axis. Given the relatively high susceptibility of Caki-1 and HeLa cells to infection with raw, unconcentrated KSHV produced from infected iSLK cells, it would be relatively simple and fruitful to perform a screen for host factors required for *de novo* infection including novel receptors. Given that the novel infection mechanism seems to be common to both cell lines, comparing the results obtained from each cell line would help drastically narrow down candidate hits for follow-up manual confirmation. In this report, we demonstrate that CRISPR-Cas9 is an easy and efficient tool to evaluate the requirement for proposed receptors during KSHV infection, and thus is a great platform for validation of new receptor candidates.

DC-SIGN was discovered as a KSHV receptor used during the infection of primary CD14+ monocyte-derived DCs and macrophages. A follow up study examined the use of DC-SIGN in the context of the other known KSHV receptors in the monocyte cell line THP-1 and found that HS and KSHV integrin receptors were required in addition to DC-SIGN for infection in these cells. More work is required to understand precisely which other receptors are required for infection of monocyte-derived DCs and macrophages and determine whether usage of DC-SIGN, HS, and integrins delineates a third category of infection mechanisms. It is also worth noting that all of this work was performed before the discovery of EphA2 as a receptor for KSHV.

Finally, B cell infection has been the subject of difficult but important studies. The severe limitations of KSHV B cell infection *in vitro* have hindered studies of receptor use. Our studies show that KSHV infection of a B cell line in coculture is completely independent of the previously identified KSHV receptors through comprehensive examination of receptor expression on both primary tonsillar B cell lines with follow up CRISPR-Cas9 KO of putative receptors on the model cell line BJAB. Given our results, we hypothesize that KSHV must interact with a completely new receptor or set of receptors in order to infect lymphocytes. In addition, we propose that coculture enhances B cell infection in a manner similar to EBV transfer infection, requiring an interaction between the virus and HS and/or Eph receptors on the surface lytically infected iSLK cells. Based on precedents in the gammaherpesvirus family, this contact-dependent infection likely

involves specific coordination or modulation of a tropism-determining KSHV glycoprotein.

Requirement for DC-SIGN expression for lymphocyte infection may represent a dividing line between two different groups of infection mechanisms. It has been demonstrated that activated B cells are susceptible to cell-free KSHV infection *in vitro* via the upregulation of DC-SIGN, but these results have not been independently verified by another research group. It has also not been investigated whether this mode of infection requires the integrins that we showed are expressed on B cells, as is reported for DC-SIGN-expressing THP-1 cells. We believe it is more likely that DC-SIGN synergizes with or enhances infection through additional unknown receptors that are expressed in common between activated B cells, resting B cells, and perhaps T cells.

Discovery of KSHV lymphocyte receptors will be challenging but important task for the field going forward. We have shown that the true infection rate of B cells in coculture is likely far lower than what has been reported due to the previously unrecognized fact that fluorescent debris seems to transfer to B cells nonspecifically during coculture infection. The infection rate of any known model B cell line is too low to perform a canonical loss-of-function screen for B cell entry factors. However, it is possible that some lymmphocyte cell lines may be much more susceptible to infection with orf28-stop KSHV. In addition, further study of the mechanism of tropism determination by KSHV orf28 may lead to potential candidates for lymphocyte receptors. It may also be worth considering a gain-of-function screen for host factors that restrict B cell infection.

We also believe it is quite likely that the mechanism of coculture-enhanced B cell infection is similar to EBV transfer infection. Our laboratory has previously shown that B cells do not make HS, and here in Chapter 3 we present evidence that Eph receptors do not function as entry receptors for KSHV during B cell infection. Despite this, we show that the coculture infection rate is highly impacted by the presence of soluble heparin or ephrin-A4. Based on these results, we hypothesize that HS and EphA2 or other Eph receptors on the surface of actively replicating iSLK.KSHV cells are required for coculture infection. This is a potentially fruitful path of investigation which would begin with utilizing our robust CRISPR-Cas9 KO platform to specifically target HS and EphA2 in infected iSLK cells and assess the effects of these knock outs on subsequent Caki-1 and BJAB infection.

Finally, it is also interesting to note that in both model infection systems we studied, the effects of inhibiting virus interactions with HS and EphA2 on infection rate was always quite similar. Several KSHV glycoproteins are known to bind HS, and gH/gL has been shown to bind quite well to EphA2. While HS is commonly referred to in the literature as a simple attachment receptor, Tiwari *et al*., 2009 reported that soluble heparin interfered with a virus-free cell-cell fusion assay between effector cells transduced with KSHV core fusion glycoproteins gB, gH, and gL and target cells. It was

also reported in this publication that heparinase treatment of the target cells reduced fusion with effector cells as well. Especially when taken together with our data regarding this topic, these results suggest that HS plays a role in the coordination and execution of membrane fusion by gB, gH, and gL. In the context of our coculture inhibition data, it may also indicate that a hypothetical B cell infection-inhibitory glycoprotein complex that is proximally soaked by the iSLK cell membrane during coculture may interact with both HS and EphA2.

The ability to generate surface receptor KO cell lines with such ease using CRISPR-Cas9 technology is unprecedented in the field of virus-receptor interactions and will facilitate the study of many of these outstanding questions and hypotheses surrounding KSHV receptors.

# References

Aasheim HC, Terstappen LW, Logtenberg T. 1997. Regulated expression of the Eph-related receptor tyrosine kinase Hek11 in early human B lymphopoiesis. Blood 90:3613-22.

Aasheim HC, Munthe E, Funderud S, Smeland EB, Beiske K, Logtenberg T. 2000. A splice variant of human ephrin-A4 encodes a soluble molecule that is secreted by activated human B lymphocytes. Blood 95:221-30.

Akula SM, Pramod NP, Wang FZ, Chandran B. 2001a. Human herpesvirus 8 envelope-associated glycoprotein B interacts with heparan sulfate-like moieties. Virology 284:235-249.

Akula SM, Wang FZ, Vieira J, Chandran B. 2001b. Human herpesvirus 8 interaction with target cells involves heparan sulfate. Virology 282:245-255.

Akula SM, Pramod NP, Wang FZ, Chandran B. 2002. Integrin alpha3beta1 (CD 49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. Cell 108:407-419.

Akula SM, Naranatt PP, Walia NS, Wang FZ, Fegley B, Chandran B. 2003. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) infection of human fibroblast cells occurs through endocytosis. J Virol 77:7978-7990.

Alonso-C LM, Trinidad EM, de Garcillan B, Ballesteros M, Castellanos M, Cotillo I, Muñoz JJ, Zapata AG. 2009. Expression profile of Eph receptors and ephrin ligands in healthy human B lymphocytes and chronic lymphocytic leukemia B-cells. Leuk Res 33:395-406.

Ambroziak JA, Blackbourn DJ, Herndier BG, Glogau RG, Gullett JH, McDonald AR, Lennette ET, Levy JA. 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science 268:582-3.

Aneja KK, Yuan Y. 2017. Reactivation and Lytic Replication of Kaposi's Sarcoma-Associated Herpesvirus: An Update. Front Microbiol 8:613.

Arias C, Weisburd B, Stern-Ginossar N, Mercier A, Madrid AS, Bellare P, Holdorf M, Weissman JS, Ganem D. 2014. KSHV 2.0: a comprehensive annotation of the Kaposi's sarcoma-associated herpesvirus genome using next-generation sequencing reveals novel genomic and functional features. PLoS Pathog 10:e1003847.

Ballestas ME, Chatis PA, Kaye KM. 1999. Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science 284:641-4.

Bandyopadhyay C, Valiya-Veettil M, Dutta D, Chakraborty S, Chandran B. 2014a. CIB1 synergizes with EphrinA2 to regulate Kaposi's sarcoma-associated herpesvirus

macropinocytic entry in human microvascular dermal endothelial cells. PLoS Pathog 10:e1003941.

Bandyopadhyay C, Veettil MV, Dutta S, Chandran B. 2014b. p130Cas scaffolds the signalosome to direct adaptor-effector cross talk during Kaposi's sarcoma-associated herpesvirus trafficking in human microvascular dermal endothelial cells. J Virol 88:13858-13878.

Barczyk M, Carracedo S, Gullberg D. 2010. Integrins. Cell Tissue Res 339:269-80.

Beauchamp A, Debinski W. 2012. Ephs and ephrins in cancer: ephrin-A1 signalling. Semin Cell Dev Biol 23:109-15.

Bechtel JT, Liang Y, Hvidding J, Ganem D. 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. J Virol 77:6474-81.

Bekerman E, Jeon D, Ardolino M, Coscoy L. 2013. A role for host activation-induced cytidine deaminase in innate immune defense against KSHV. PLoS Pathog 9:e1003748.

Beral V, Peterman TA, Berkelman RL, Jaffe HW. 1990. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet 335:123-8.

Birkmann A, Mahr K, Ensser A, Yağuboğlu S, Titgemeyer F, Fleckenstein B, Neipel F. 2001. Cell surface heparan sulfate is a receptor for human herpesvirus 8 and interacts with envelope glycoprotein K8.1. J Virol 75:11583-11593.

Blackbourn DJ, Ambroziak J, Lennette E, Adams M, Ramachandran B, Levy JA. 1997. Infectious human herpesvirus 8 in a healthy North American blood donor. Lancet 349:609–611.

Blackbourn DJ, Lennette E, Klencke B, Moses A, Chandran B, Weinstein M, Glogau RG, Witte MH, Way DL, Kutzkey T, Herndier B, Levy JA. 2000. The restricted cellular host range of human herpesvirus 8. AIDS 14:1123-33.

Blasig C, Zietz C, Haar B, Neipel F, Esser S, Brockmeyer NH, Tschachler E, Colombini S, Ensoli B, Stürzl M. 1997. Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J Virol 71:7963-7968.

Bocharov EV, Mayzel ML, Volynsky PE, Goncharuk MV, Ermolyuk YS, Schulga AA, Artemenko EO, Efremov RG, Arseniev AS. 2008. Spatial structure and pH-dependent conformational diversity of dimeric transmembrane domain of the receptor tyrosine kinase EphA1. J Biol Chem 283:29385-29395.

Bortz E, Whitelegge JP, Jia Q, Zhou ZH, Stewart JP, Wu TT, Sun R. 2003. Identification of proteins associated with murine gammaherpesvirus 68 virions. J Virol 77:13425-32.

Borza CM, Hutt-Fletcher LM. 1998. Epstein-Barr virus recombinant lacking expression of glycoprotein gp150 infects B cells normally but is enhanced for infection of epithelial cells. J Virol 72:7577-82.

Boshoff C, Schulz TF, Kennedy MM, Graham AK, Fisher C, Thomas A, McGee JO, Weiss RA, O'Leary JJ. 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat Med 1:1274-8.

Bottero V, Chakraborty S, Chandran B. 2013. Reactive oxygen species are induced by Kaposi's sarcoma-associated herpesvirus early during primary infection of endothelial cells to promote virus entry. J Virol 87:1733-49.

Bower M, Nelson M, Young AM, Thirlwell C, Newsom-Davis T, Mandalia S, Dhillon T, Holmes P, Gazzard BG, Stebbing J. 2005. Immune reconstitution inflammatory syndrome associated with Kaposi's sarcoma. J Clin Oncol 23:5224-5228.

Brack AR, Dijkstra JM, Granzow H, Klupp BG, Mettenleiter TC. 1999. Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. J Virol 73:5364-72.

Brulois KF, Chang H, Lee AS, Ensser A, Wong LY, Toth Z, Lee SH, Lee HR, Myoung J, Ganem D, Oh TK, Kim JF, Gao SJ, Jung JU. 2012. Construction and manipulation of a new Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. J Virol 86:9708-9720.

Campadelli-Fiume G, Collins-McMillen D, Gianni T, Yurochko AD. 2016. Integrins as Herpesvirus Receptors and Mediators of the Host Signalosome. Annu Rev Virol 3:215-236.

Campbell DM, Rappocciolo G, Jenkins FJ, Rinaldo CR. 2014. Dendritic cells: key players in human herpesvirus 8 infection and pathogenesis. Front Microbiol 5:452.

Carter N, Nakamoto T, Hirai H, Hunter T. 2002. EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). Nat Cell Biol 4:565-73.

Cerimele F, Curreli F, Ely S, Friedman-Kien AE, Cesarman E, Flore O. 2001. Kaposi's Sarcoma-Associated Herpesvirus Can Productively Infect Primary Human Keratinocytes and Alter Their Growth Properties. J Virol 75:2435-2443.

Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med 332:1186-1191.

Chadburn A, Hyjek EM, Tam W, Liu Y, Rengifo T, Cesarman E, Knowles DM. 2008. Immunophenotypic analysis of the Kaposi sarcoma herpesvirus (KSHV; HHV-8)-

infected B cells in HIV+ multicentric Castleman disease (MCD). Histopathology 53:513-24.

Chagas CA, Endo LH, Sakano E, Pinto GA, Brousset P, Vassallo J. 2006. Detection of herpesvirus type 8 (HHV8) in children's tonsils and adenoids by immunohistochemistry and in situ hybridization. Int J Pediatr Otorhinolaryngol 70:65-72.

Chakraborty S, ValiyaVeettil M, Sadagopan S, Paudel N, Chandran B. 2011. c-Cbl-mediated selective virus-receptor translocations into lipid rafts regulate productive Kaposi's sarcoma-associated herpesvirus infection in endothelial cells. J Virol 85:12410-30.

Chakraborty S, Veettil MV, Bottero V, Chandran B. 2012. Kaposi's sarcoma-associated herpesvirus interacts with EphrinA2 receptor to amplify signaling essential for productive infection. Proc Natl Acad Sci U S A 109:E1163-1172.

Chandran B, Bloomer C, Chan SR, Zhu L, Goldstein E, Horvat R. 1998. Human herpesvirus-8 ORF K8.1 gene encodes immunogenic glycoproteins generated by spliced transcripts. Virology 249:140-9.

Chandriani S, Xu Y, Ganem D. 2010. The lytic transcriptome of Kaposi's sarcoma-associated herpesvirus reveals extensive transcription of noncoding regions, including regions antisense to important genes. J Virol 84:7934-42.

Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science 266:1865-1869.

Chang Y, Moore P. 2014. Twenty years of KSHV. Viruses 6:4258-64.

Chang Y, Tung CH, Huang YT, Lu J, Chen JY, Tsai CH. 1999. Requirement for cell-to-cell contact in Epstein-Barr virus infection of nasopharyngeal carcinoma cells and keratinocytes. J Virol 73:8857-66.

Chen J, Rowe CL, Jardetzky TS, Longnecker R. 2012. The KGD motif of Epstein-Barr virus gH/gL is bifunctional, orchestrating infection of B cells and epithelial cells. MBio 3.

Chen J, Sathiyamoorthy K, Zhang X, Schaller S, Perez White BE, Jardetzky TS, Longnecker R. 2018. Ephrin receptor A2 is a functional entry receptor for Epstein-Barr virus. Nat Microbiol 3:172-180.

Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3:866-71.

Chesnokova LS, Nishimura SL, Hutt-Fletcher LM. 2009. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins alphavbeta6 or alphavbeta8. Proc Natl Acad Sci U S A 106:20464-9.

Chesnokova LS, Hutt-Fletcher LM. 2011. Fusion of Epstein-Barr virus with epithelial cells can be triggered by αvβ5 in addition to αvβ6 and αvβ8, and integrin binding triggers a conformational change in glycoproteins gHgL. J Virol 85:13214-23.

Chesnokova LS, Hutt-Fletcher LM. 2014. Epstein-Barr virus infection mechanisms. Chin J Cancer 33:545-8.

Chesnokova LS, Valencia SM, Hutt-Fletcher LM. 2016. The BDLF3 gene product of Epstein-Barr virus, gp150, mediates non-productive binding to heparan sulfate on epithelial cells and only the binding domain of CD21 is required for infection. Virology 494:23-8.

Cho H, Kang H. 2012. KSHV infection of B-cell lymphoma using a modified KSHV BAC36 and coculturing system. J Microbiol 50:285-92.

Ciufo DM, Cannon JS, Poole LJ, Wu FY, Murray P, Ambinder RF, Hayward GS. 2001. Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. J Virol 75:5614-26.

Connolly SA, Jackson JO, Jardetzky TS, Longnecker R. 2011. Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol 9:369-81.

Cook-Mozaffari P, Newton R, Beral V, Burkitt DP. 1998. The geographical distribution of Kaposi's sarcoma and of lymphomas in Africa before the AIDS epidemic. Br J Cancer 78:1521-8.

Cooper RS, Heldwein EE. 2015. Herpesvirus gB: A Finely Tuned Fusion Machine. Viruses 7:6552-69.

Corchero JL, Mar EC, Spira TJ, Pellett PE, Inoue N. 2001. Comparison of serologic assays for detection of antibodies against human herpesvirus 8. Clin Diagn Lab Immunol 8:913-21.

Dai L, Qin Z, Defee M, Toole BP, Kirkwood KL, Parsons C. 2012. Kaposi sarcoma-associated herpesvirus (KSHV) induces a functional tumor-associated phenotype for oral fibroblasts. Cancer Lett 318:214-20.

de Lima BD, May JS, Stevenson PG. 2004. Murine gammaherpesvirus 68 lacking gp150 shows defective virion release but establishes normal latency in vivo. J Virol 78:5103-12.

Diamond C, Brodie SJ, Krieger JN, Huang ML, Koelle DM, Diem K, Muthui D, Corey L. 1998. Human herpesvirus 8 in the prostate glands of men with Kaposi's sarcoma. J Virol 72:6223-7.

Dijkstra JM, Gerdts V, Klupp BG, Mettenleiter TC. 1997. Deletion of glycoprotein gM of pseudorabies virus results in attenuation for the natural host. J Gen Virol 78 ( Pt 9):2147-51.

Dittmer DP, Damania B. 2016. Kaposi sarcoma-associated herpesvirus: immunobiology, oncogenesis, and therapy. J Clin Invest 126:3165-75.

Dollery SJ, Santiago-Crespo RJ, Kardava L, Moir S, Berger EA. 2014. Efficient infection of a human B cell line with cell-free Kaposi's sarcoma-associated herpesvirus. J Virol 88:1748-57.

Du MQ, Liu H, Diss TC, Ye H, Hamoudi RA, Dupin N, Meignin V, Oksenhendler E, Boshoff C, Isaacson PG. 2001. Kaposi sarcoma-associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. Blood 97:2130-6.

Du MQ, Bacon CM, Isaacson PG. 2007. Kaposi sarcoma-associated herpesvirus/human herpesvirus 8 and lymphoproliferative disorders. J Clin Pathol 60:1350-7.

Dupin N, Fisher C, Kellam P, Ariad S, Tulliez M, Franck N, van Marck E, Salmon D, Gorin I, Escande JP, Weiss RA, Alitalo K, Boshoff C. 1999. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc Natl Acad Sci U S A 96:4546-51.

Dutta D, Chakraborty S, Bandyopadhyay C, Valiya Veettil M, Ansari MA, Singh VV, Chandran B. 2013. EphrinA2 regulates clathrin mediated KSHV endocytosis in fibroblast cells by coordinating integrin-associated signaling and c-Cbl directed polyubiquitination. PLoS Pathog 9:e1003510.

Duus KM, Lentchitsky V, Wagenaar T, Grose C, Webster-Cyriaque J. 2004. Wild-type Kaposi's sarcoma-associated herpesvirus isolated from the oropharynx of immune-competent individuals has tropism for cultured oral epithelial cells. J Virol 78:4074-84.

Eisenberg RJ, Atanasiu D, Cairns TM, Gallagher JR, Krummenacher C, Cohen GH. 2012. Herpes virus fusion and entry: a story with many characters. Viruses 4:800-32.

El Kasmi I, Lippé R. 2015. Herpes simplex virus 1 gN partners with gM to modulate the viral fusion machinery. J Virol 89:2313-23.

Farnsworth A, Wisner TW, Webb M, Roller R, Cohen G, Eisenberg R, Johnson DC. 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the

virion envelope and the outer nuclear membrane. Proc Natl Acad Sci U S A 104:10187-92.

Feldman SA, Audet S, Beeler JA. 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. J Virol 74:6442-7.

Flore O, Rafii S, Ely S, O'Leary JJ, Hyjek EM, Cesarman E. 1998. Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. Nature 394:588-92.

Forte E, Raja AN, Shamulailatpam P, Manzano M, Schipma MJ, Casey JL, Gottwein E. 2015. MicroRNA-mediated transformation by the Kaposi's sarcoma-associated herpesvirus Kaposin locus. J Virol 89:2333-41.

Friborg J, Kong WP, Flowers CC, Flowers SL, Sun Y, Foreman KE, Nickoloff BJ, Nabel GJ. 1998. Distinct biology of Kaposi's sarcoma-associated herpesvirus from primary lesions and body cavity lymphomas. J Virol 72:10073-82.

Fuchs W, Mettenleiter TC. 1999. DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL10 gene product as a nonglycosylated and nonessential virion protein. J Gen Virol 80 ( Pt 8):2173-82.

Fuchs W, Mettenleiter TC. 2005. The nonessential UL49.5 gene of infectious laryngotracheitis virus encodes an O-glycosylated protein which forms a complex with the non-glycosylated UL10 gene product. Virus Res 112:108-14.

Ganem D. 2010. KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. J Clin Invest 120:939-49.

Garrigues HJ, Rubinchikova YE, Dipersio CM, Rose TM. 2008. Integrin alphaVbeta3 Binds to the RGD motif of glycoprotein B of Kaposi's sarcoma-associated herpesvirus and functions as an RGD-dependent entry receptor. J Virol 82:1570-1580.

Garrigues HJ, Rubinchikova YE, Rose TM. 2014a. KSHV cell attachment sites revealed by ultra sensitive tyramide signal amplification (TSA) localize to membrane microdomains that are up-regulated on mitotic cells. Virology 452-453:75-85.

Garrigues HJ, DeMaster LK, Rubinchikova YE, Rose TM. 2014b. KSHV attachment and entry are dependent on αVβ3 integrin localized to specific cell surface microdomains and do not correlate with the presence of heparan sulfate. Virology 464-465:118-133.

Garrigues HJ, DeMaster LK, Rubinchikova YE, Rose TM. 2018. Corrigendum to: "KSHV attachment and entry are dependent on αVβ3 integrin localized to specific cell

surface microdomains and do not correlate with the presence of heparan sulfate" [Virology 464-465 (2014) 118-133]. Virology 515:264-265.

Giebeler N, Zigrino P. 2016. A Disintegrin and Metalloprotease (ADAM): Historical Overview of Their Functions. Toxins (Basel) 8:122.

Giffin L, Damania B. 2014. KSHV: pathways to tumorigenesis and persistent infection. Adv Virus Res 88:111-59.

Gill J, Bourboulia D, Wilkinson J, Hayes P, Cope A, Marcelin AG, Calvez V, Gotch F, Boshoff C, Gazzard B. 2002. Prospective study of the effects of antiretroviral therapy on Kaposi sarcoma--associated herpesvirus infection in patients with and without Kaposi sarcoma. J Acquir Immune Defic Syndr 31:384-90.

Gill MB, Edgar R, May JS, Stevenson PG. 2008. A gamma-herpesvirus glycoprotein complex manipulates actin to promote viral spread. PLoS One 3:e1808.

Gillet L, May JS, Stevenson PG. 2009. In vivo importance of heparan sulfate-binding glycoproteins for murid herpesvirus-4 infection. J Gen Virol 90:602-13.

Gillet L, Frederico B, Stevenson PG. 2015. Host entry by gamma-herpesviruses--lessons from animal viruses? Curr Opin Virol 15:34-40.

Gjyshi O, Bottero V, Veettil MV, Dutta S, Singh VV, Chikoti L, Chandran B. 2014. Kaposi's sarcoma-associated herpesvirus induces Nrf2 during de novo infection of endothelial cells to create a microenvironment conducive to infection. PLoS Pathog 10:e1004460.

Gjyshi O, Roy A, Dutta S, Veettil MV, Dutta D, Chandran B. 2015. Activated Nrf2 Interacts with Kaposi's Sarcoma-Associated Herpesvirus Latency Protein LANA-1 and Host Protein KAP1 To Mediate Global Lytic Gene Repression. J Virol 89:7874-92.

Gong D, Wu NC, Xie Y, Feng J, Tong L, Brulois KF, Luan H, Du Y, Jung JU, Wang CY, Kang MK, Park NH, Sun R, Wu TT. 2014. Kaposi's sarcoma-associated herpesvirus ORF18 and ORF30 are essential for late gene expression during lytic replication. J Virol 88:11369-11382.

Gore M, Hutt-Fletcher LM. 2009. The BDLF2 protein of Epstein-Barr virus is a type II glycosylated envelope protein whose processing is dependent on coexpression with the BMRF2 protein. Virology 383:162-7.

Gottwein E, Mukherjee N, Sachse C, Frenzel C, Majoros WH, Chi JT, Braich R, Manoharan M, Soutschek J, Ohler U, Cullen BR. 2007. A viral microRNA functions as an orthologue of cellular miR-155. Nature 450:1096-9.

Gottwein E. 2012. Kaposi's Sarcoma-Associated Herpesvirus microRNAs. Front Microbiol 3:165.

Greene AC, Lord SJ, Tian A, Rhodes C, Kai H, Groves JT. 2014. Spatial organization of EphA2 at the cell-cell interface modulates trans-endocytosis of ephrinA1. Biophys J 106:2196-2205.

Greene W, Gao SJ. 2009. Actin dynamics regulate multiple endosomal steps during Kaposi's sarcoma-associated herpesvirus entry and trafficking in endothelial cells. PLoS Pathog 5:e1000512.

Greene W, Zhang W, He M, Witt C, Ye F, Gao SJ. 2012. The ubiquitin/proteasome system mediates entry and endosomal trafficking of Kaposi's sarcoma-associated herpesvirus in endothelial cells. PLoS Pathog 8:e1002703.

Gregory SM, West JA, Dillon PJ, Hilscher C, Dittmer DP, Damania B. 2009. Toll-like receptor signaling controls reactivation of KSHV from latency. Proc Natl Acad Sci U S A 106:11725-30.

Großkopf AK, Ensser A, Neipel F, Jungnickl D, Schlagowski S, Desrosiers RC, Hahn AS. 2018. A conserved Eph family receptor-binding motif on the gH/gL complex of Kaposi's sarcoma-associated herpesvirus and rhesus monkey rhadinovirus. PLoS Pathog 14:e1006912.

Grossmann C, Ganem D. 2008. Effects of NFkappaB activation on KSHV latency and lytic reactivation are complex and context-dependent. Virology 375:94-102.

Grundhoff A, Ganem D. 2004. Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. J Clin Invest 113:124-36.

Hahn A, Birkmann A, Wies E, Dorer D, Mahr K, Stürzl M, Titgemeyer F, Neipel F. 2009. Kaposi's sarcoma-associated herpesvirus gH/gL: glycoprotein export and interaction with cellular receptors. J Virol 83:396-407.

Hahn AS, Kaufmann JK, Wies E, Naschberger E, Panteleev-Ivlev J, Schmidt K, Holzer A, Schmidt M, Chen J, König S, Ensser A, Myoung J, Brockmeyer NH, Stürzl M, Fleckenstein B, Neipel F. 2012. The Ephrin Receptor Tyrosine Kinase A2 is a Cellular Receptor for Kaposi's Sarcoma-Associated Herpesvirus. Nat Med 18:961-966.

Hahn AS, Desrosiers RC. 2013. Rhesus Monkey Rhadinovirus Uses Eph Family Receptors for Entry into B Cells and Endothelial Cells but Not Fibroblasts. PLoS Pathog 9:e1003360.

Hahn AS, Desrosiers RC. 2014. Binding of the Kaposi's sarcoma-associated herpesvirus to the ephrin binding surface of the EphA2 receptor and its inhibition by a small molecule. J Virol 88:8724-8734.

Harrington W, Sieczkowski L, Sosa C, Chan-a-Sue S, Cai JP, Cabral L, Wood C. 1997. Activation of HHV-8 by HIV-1 tat. Lancet 349:774-5.

Hassman LM, Ellison TJ, Kedes DH. 2011. KSHV infects a subset of human tonsillar B cells, driving proliferation and plasmablast differentiation. J Clin Invest 121:752-68.

Hensler HR, Tomaszewski MJ, Rappocciolo G, Rinaldo CR, Jenkins FJ. 2014. Human herpesvirus 8 glycoprotein B binds the entry receptor DC-SIGN. Virus Res 190:97-103.

Himanen JP, Yermekbayeva L, Janes PW, Walker JR, Xu K, Atapattu L, Rajashankar KR, Mensinga A, Lackmann M, Nikolov DB, Dhe-Paganon S. 2010. Architecture of Eph receptor clusters. Proc Natl Acad Sci U S A 107:10860-10865.

Holmberg J, Clarke DL, Frisén J. 2000. Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. Nature 408:203-206.

Host KM, Jacobs SR, West JA, Zhang Z, Costantini LM, Stopford CM, Dittmer DP, Damania B. 2017. Kaposi's Sarcoma-Associated Herpesvirus Increases PD-L1 and Proinflammatory Cytokine Expression in Human Monocytes. MBio 8.

Hynes RO. 2002. Integrins: bidirectional, allosteric signaling machines. Cell 110:673-687.

Imai S, Nishikawa J, Takada K. 1998. Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells. J Virol 72:4371-8.

Inoue N, Winter J, Lal RB, Offermann MK, Koyano S. 2003. Characterization of entry mechanisms of human herpesvirus 8 by using an Rta-dependent reporter cell line. J Virol 77:8147-52.

Jaber T, Yuan Y. 2013. A virally encoded small peptide regulates RTA stability and facilitates Kaposi's sarcoma-associated herpesvirus lytic replication. J Virol 87:3461-70.

Janes PW, Griesshaber B, Atapattu L, Nievergall E, Hii LL, Mensinga A, Chheang C, Day BW, Boyd AW, Bastiaens PI, Jørgensen C, Pawson T, Lackmann M. 2011. Eph receptor function is modulated by heterooligomerization of A and B type Eph receptors. J Cell Biol 195:1033-1145.

Jarousse N, Chandran B, Coscoy L. 2008. Lack of heparan sulfate expression in B-cell lines: implications for Kaposi's sarcoma-associated herpesvirus and murine gammaherpesvirus 68 infections. J Virol 82:12591-12597.

Jenner RG, Albà MM, Boshoff C, Kellam P. 2001. Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J Virol 75:891-902.

Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, Illanes D, Sarracino D, Kieff E. 2004. Proteins of purified Epstein-Barr virus. Proc Natl Acad Sci U S A 101:16286-91.

Johnson AS, Maronian N, Vieira J. 2005. Activation of Kaposi's sarcoma-associated herpesvirus lytic gene expression during epithelial differentiation. J Virol 79:13769-77.

Juhász A, Kónya J, Beck Z, Remenyik E, Veress G, Bégány A, Medgyessy I, Hunyadi J, Gergely L. 2001. HHV-8 ELISA based on a one-step affinity capture of biotinylated K8.1 antigen. J Virol Methods 94:163-72.

Kaleeba JA, Berger EA. 2006a. Broad target cell selectivity of Kaposi's sarcoma-associated herpesvirus glycoprotein-mediated cell fusion and virion entry. Virology 354:7-14.

Kaleeba JA, Berger EA. 2006b. Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT. Science 311:1921-4.

Kang S, Myoung J. 2017. Primary lymphocyte infection models for KSHV and its putative tumorigenesis mechanisms in B cell lymphomas. J Microbiol 55:319-329.

Kawabata A, Jasirwan C, Yamanishi K, Mori Y. 2012. Human herpesvirus 6 glycoprotein M is essential for virus growth and requires glycoprotein N for its maturation. Virology 429:21-8.

Kerur N, Veettil MV, Sharma-Walia N, Sadagopan S, Bottero V, Paul AG, Chandran B. 2010. Characterization of entry and infection of monocytic THP-1 cells by Kaposi's sarcoma associated herpesvirus (KSHV): role of heparan sulfate, DC-SIGN, integrins and signaling. Virology 406:103-16.

Kim IJ, Chouljenko VN, Walker JD, Kousoulas KG. 2013. Herpes simplex virus 1 glycoprotein M and the membrane-associated protein UL11 are required for virus-induced cell fusion and efficient virus entry. J Virol 87:8029-37.

Kliche S, Kremmer E, Hammerschmidt W, Koszinowski U, Haas J. 1998. Persistent infection of Epstein-Barr virus-positive B lymphocytes by human herpesvirus 8. J Virol 72:8143-9.

Klupp BG, Nixdorf R, Mettenleiter TC. 2000. Pseudorabies virus glycoprotein M inhibits membrane fusion. J Virol 74:6760-8.

Knowlton ER, Lepone LM, Li J, Rappocciolo G, Jenkins FJ, Rinaldo CR. 2012. Professional antigen presenting cells in human herpesvirus 8 infection. Front Immunol 3:427.

Knowlton ER, Rappocciolo G, Piazza P, Lepone LM, Nadgir SV, Bullotta A, Berendam SJ, Li J, Reinhart TA, Jenkins FJ, Rinaldo CR. 2014. Human herpesvirus 8 induces polyfunctional B lymphocytes that drive Kaposi's sarcoma. MBio 5:e01277-14.

König P, Giesow K, Keil GM. 2002. Glycoprotein M of bovine herpesvirus 1 (BHV-1) is nonessential for replication in cell culture and is involved in inhibition of bovine respiratory syncytial virus F protein induced syncytium formation in recombinant BHV-1 infected cells. Vet Microbiol 86:37-49.

Koyano S, Mar EC, Stamey FR, Inoue N. 2003. Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion. J Gen Virol 84:1485-91.

Krishnan HH, Sharma-Walia N, Zeng L, Gao SJ, Chandran B. 2005. Envelope glycoprotein gB of Kaposi's sarcoma-associated herpesvirus is essential for egress from infected cells. J Virol 79:10952-67.

Krzyzaniak M, Mach M, Britt WJ. 2007. The cytoplasmic tail of glycoprotein M (gpUL100) expresses trafficking signals required for human cytomegalovirus assembly and replication. J Virol 81:10316-28.

Kuang SQ, Bai H, Fang ZH, Lopez G, Yang H, Tong W, Wang ZZ, Garcia-Manero G. 2010. Aberrant DNA methylation and epigenetic inactivation of Eph receptor tyrosine kinases and ephrin ligands in acute lymphoblastic leukemia. Blood 115:2412-9.

Kumar B, Chandran B. 2016a. KSHV Entry and Trafficking in Target Cells-Hijacking of Cell Signal Pathways, Actin and Membrane Dynamics. Viruses 8:305.

Kumar B, Dutta D, Iqbal J, Ansari MA, Roy A, Chikoti L, Pisano G, Veettil MV, Chandran B. 2016b. ESCRT-I Protein Tsg101 Plays a Role in the Post-macropinocytic Trafficking and Infection of Endothelial Cells by Kaposi's Sarcoma-Associated Herpesvirus. PLoS Pathog 12:e1005960.

Kumar B, Roy A, Veettil MV, Chandran B. 2018. Insight into the Roles of E3 Ubiquitin Ligase c-Cbl, ESCRT Machinery, and Host Cell Signaling in Kaposi's Sarcoma-Associated Herpesvirus Entry and Trafficking. J Virol 92.

Labo N, Miley W, Marshall V, Gillette W, Esposito D, Bess M, Turano A, Uldrick T, Polizzotto MN, Wyvill KM, Bagni R, Yarchoan R, Whitby D. 2014. Heterogeneity and breadth of host antibody response to KSHV infection demonstrated by systematic analysis of the KSHV proteome. PLoS Pathog 10:e1004046.

Lagunoff M, Bechtel J, Venetsanakos E, Roy AM, Abbey N, Herndier B, McMahon M, Ganem D. 2002. De novo infection and serial transmission of Kaposi's sarcoma-associated herpesvirus in cultured endothelial cells. J Virol 76:2440-8.

Lake CM, Hutt-Fletcher LM. 2000. Epstein-Barr virus that lacks glycoprotein gN is impaired in assembly and infection. J Virol 74:11162-72.

Lam LL, Pau CP, Dollard SC, Pellett PE, Spira TJ. 2002. Highly sensitive assay for human herpesvirus 8 antibodies that uses a multiple antigenic peptide derived from open reading frame K8.1. J Clin Microbiol 40:325-9.

Lang D, Hinderer W, Rothe M, Sonneborn HH, Neipel F, Raab M, Rabenau H, Masquelier B, Fleury H. 1999. Comparison of the immunoglobulin-G-specific seroreactivity of different recombinant antigens of the human herpesvirus 8. Virology 260:47-54.

Leege T, Fuchs W, Granzow H, Kopp M, Klupp BG, Mettenleiter TC. 2009. Effects of simultaneous deletion of pUL11 and glycoprotein M on virion maturation of herpes simplex virus type 1. J Virol 83:896-907.

Li G, Kamil JP. 2016. Viral Regulation of Cell Tropism in Human Cytomegalovirus. J Virol 90:626-9.

Li M, MacKey J, Czajak SC, Desrosiers RC, Lackner AA, Jung JU. 1999. Identification and characterization of Kaposi's sarcoma-associated herpesvirus K8.1 virion glycoprotein. J Virol 73:1341-9.

Lisabeth EM, Falivelli G, Pasquale EB. 2013. Eph receptor signaling and ephrins. Cold Spring Harb Perspect Biol 5.

Loesing JB, Di Fiore S, Ritter K, Fischer R, Kleines M. 2009. Epstein-Barr virus BDLF2-BMRF2 complex affects cellular morphology. J Gen Virol 90:1440-9.

Lozach PY, Burleigh L, Staropoli I, Amara A. 2007. The C Type Lectins DC-SIGN and L-SIGN. Methods Mol Biol 379:51-68.

Luna RE, Zhou F, Baghian A, Chouljenko V, Forghani B, Gao SJ, Kousoulas KG. 2004. Kaposi's sarcoma-associated herpesvirus glycoprotein K8.1 is dispensable for virus entry. J Virol 78:6389-98.

Ma Q, Cavallin LE, Leung HJ, Chiozzini C, Goldschmidt-Clermont PJ, Mesri EA. 2013. A role for virally induced reactive oxygen species in Kaposi's sarcoma herpesvirus tumorigenesis. Antioxid Redox Signal 18:80-90.

Mach M, Kropff B, Dal Monte P, Britt W. 2000. Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). J Virol 74:11881-92.

Mach M, Kropff B, Kryzaniak M, Britt W. 2005. Complex formation by glycoproteins M and N of human cytomegalovirus: structural and functional aspects. J Virol 79:2160-70.

Machiels B, Lété C, de Fays K, Mast J, Dewals B, Stevenson PG, Vanderplasschen A, Gillet L. 2011. The bovine herpesvirus 4 Bo10 gene encodes a nonessential viral envelope protein that regulates viral tropism through both positive and negative effects. J Virol 85:1011-24.

Machiels B, Stevenson PG, Vanderplasschen A, Gillet L. 2013. A gammaherpesvirus uses alternative splicing to regulate its tropism and its sensitivity to neutralization. PLoS Pathog 9:e1003753.

Mark L, Lee WH, Spiller OB, Villoutreix BO, Blom AM. 2006. The Kaposi's sarcoma-associated herpesvirus complement control protein (KCP) binds to heparin and cell surfaces via positively charged amino acids in CCP1-2. Mol Immunol 43:1665-1675.

May JS, Colaco S, Stevenson PG. 2005a. Glycoprotein M is an essential lytic replication protein of the murine gammaherpesvirus 68. J Virol 79:3459-67.

May JS, Coleman HM, Boname JM, Stevenson PG. 2005b. Murine gammaherpesvirus-68 ORF28 encodes a non-essential virion glycoprotein. J Gen Virol 86:919-28.

May JS, de Lima BD, Colaco S, Stevenson PG. 2005c. Intercellular gamma-herpesvirus dissemination involves co-ordinated intracellular membrane protein transport. Traffic 6:780-93.

Mbisa GL, Miley W, Gamache CJ, Gillette WK, Esposito D, Hopkins R, Busch MP, Schreiber GB, Little RF, Yarchoan R, Ortiz-Conde BA, Labò N, Whitby D. 2010. Detection of antibodies to Kaposi's sarcoma-associated herpesvirus: a new approach using K8.1 ELISA and a newly developed recombinant LANA ELISA. J Immunol Methods 356:39-46.

Merat R, Amara A, Lebbe C, de The H, Morel P, Saib A. 2002. HIV-1 infection of primary effusion lymphoma cell line triggers Kaposi's sarcoma-associated herpesvirus (KSHV) reactivation. Int J Cancer 97:791-5.

Mercader M, Taddeo B, Panella JR, Chandran B, Nickoloff BJ, Foreman KE. 2000. Induction of HHV-8 lytic cycle replication by inflammatory cytokines produced by HIV-1-infected T cells. Am J Pathol 156:1961-71.

Mesri EA, Cesarman E, Arvanitakis L, Rafii S, Moore MA, Posnett DN, Knowles DM, Asch AS. 1996. Human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus is a new transmissible virus that infects B cells. J Exp Med 183:2385-90.

Miao H, Burnett E, Kinch M, Simon E, Wang B. 2000. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat Cell Biol 2:62-9.

Minhas V, Wood C. 2014. Epidemiology and Transmission of Kaposi's Sarcoma-Associated Herpesvirus. Viruses 6:4178-4194.

Molesworth SJ, Lake CM, Borza CM, Turk SM, Hutt-Fletcher LM. 2000. Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. J Virol 74:6324-32.

Mothes W, Sherer NM, Jin J, Zhong P. 2010. Virus cell-to-cell transmission. J Virol 84:8360-8.

Myoung J, Ganem D. 2011a. Active lytic infection of human primary tonsillar B cells by KSHV and its noncytolytic control by activated CD4+ T cells. J Clin Invest 121:1130-40.

Myoung J, Ganem D. 2011b. 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.

Myoung J, Ganem D. 2011c. Infection of lymphoblastoid cell lines by Kaposi's sarcoma-associated herpesvirus: critical role of cell-associated virus. J Virol 85:9767-77.

Myoung J, Ganem D. 2011d. Infection of primary human tonsillar lymphoid cells by KSHV reveals frequent but abortive infection of T cells. Virology 413:1-11.

Na-Ek P, Thewsoongnoen J, Thanunchai M, Wiboon-Ut S, Sa-Ard-Iam N, Mahanonda R, Thitithanyanont A. 2017. The activation of B cells enhances DC-SIGN expression and promotes susceptibility of B cells to HPAI H5N1 infection. Biochem Biophys Res Commun 490:1301-1306.

Naranatt PP, Akula SM, Zien CA, Krishnan HH, Chandran B. 2003. Kaposi's sarcoma-associated herpesvirus induces the phosphatidylinositol 3-kinase-PKC-zeta-MEK-ERK signaling pathway in target cells early during infection: implications for infectivity. J Virol 77:1524-1539.

Naranatt PP, Krishnan HH, Svojanovsky SR, Bloomer C, Mathur S, Chandran B. 2004. Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. Cancer Res 64:72-84.

Naranatt PP, Krishnan HH, Smith MS, Chandran B. 2005. Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. J Virol 79:1191-206.

Neipel F, Albrecht JC, Fleckenstein B. 1997. Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J Virol 71:4187-92.

Nicol SM, Sabbah S, Brulois KF, Jung JU, Bell AI, Hislop AD. 2016. Primary B Lymphocytes Infected with Kaposi's Sarcoma-Associated Herpesvirus Can Be Expanded In Vitro and Are Recognized by LANA-Specific CD4(+) T Cells. J Virol 90:3849-59.

Osman M, Kubo T, Gill J, Neipel F, Becker M, Smith G, Weiss R, Gazzard B, Boshoff C, Gotch F. 1999. Identification of human herpesvirus 8-specific cytotoxic T-cell responses. J Virol 73:6136-40.

Osterrieder N, Neubauer A, Brandmuller C, Braun B, Kaaden OR, Baines JD. 1996. The equine herpesvirus 1 glycoprotein gp21/22a, the herpes simplex virus type 1 gM homolog, is involved in virus penetration and cell-to-cell spread of virions. J Virol 70:4110-5.

Panyutich EA, Said JW, Miles SA. 1998. Infection of primary dermal microvascular endothelial cells by Kaposi's sarcoma-associated herpesvirus. AIDS 12:467-72.

Patel M, Yanagishita M, Roderiquez G, Bou-Habib DC, Oravecz T, Hascall VC, Norcross MA. 1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of T-cell lines. AIDS Res Hum Retroviruses 9:167-74.

Penn I. 1979. Kaposi's sarcoma in organ transplant recipients: report of 20 cases. Transplantation 27:8-11.

Pereira L. 1994. Function of glycoprotein B homologues of the family herpesviridae. Infect Agents Dis 3:9-28.

Perry ST, Compton T. 2006. Kaposi's sarcoma-associated herpesvirus virions inhibit interferon responses induced by envelope glycoprotein gpK8.1. J Virol 80:11105-14.

Pertel PE. 2002. Human herpesvirus 8 glycoprotein B (gB), gH, and gL can mediate cell fusion. J Virol 76:4390-400.

Pitulescu ME, Adams RH. 2010. Eph/ephrin molecules—a hub for signaling and endocytosis. Genes Dev 24:2480-2492.

Polizzotto MN, Uldrick TS, Hu D, Yarchoan R. 2012. Clinical Manifestations of Kaposi Sarcoma Herpesvirus Lytic Activation: Multicentric Castleman Disease (KSHV-MCD) and the KSHV Inflammatory Cytokine Syndrome. Front Microbiol 3:73.

Polizzotto MN, Uldrick TS, Wyvill KM, Aleman K, Marshall V, Wang V, Whitby D, Pittaluga S, Jaffe ES, Millo C, Tosato G, Little RF, Steinberg SM, Sereti I, Yarchoan R. 2016. Clinical Features and Outcomes of Patients With Symptomatic Kaposi

Sarcoma Herpesvirus (KSHV)-associated Inflammation: Prospective Characterization of KSHV Inflammatory Cytokine Syndrome (KICS). Clin Infect Dis 62:730-738.

Purushothaman P, Thakker S, Verma SC. 2015. Transcriptome analysis of Kaposi's sarcoma-associated herpesvirus during de novo primary infection of human B and endothelial cells. J Virol 89:3093-111.

Raab MS, Albrecht JC, Birkmann A, Yağuboğlu S, Lang D, Fleckenstein B, Neipel F. 1998. The immunogenic glycoprotein gp35-37 of human herpesvirus 8 is encoded by open reading frame K8.1. J Virol 72:6725-31.

Raghu H, Sharma-Walia N, Veettil MV, Sadagopan S, Caballero A, Sivakumar R, Varga L, Bottero V, Chandran B. 2007. Lipid rafts of primary endothelial cells are essential for Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8-induced phosphatidylinositol 3-kinase and RhoA-GTPases critical for microtubule dynamics and nuclear delivery of viral DNA but dispensable for binding and entry. J Virol 81:7941-59.

Raghu H, Sharma-Walia N, Veettil MV, Sadagopan S, Chandran B. 2009. Kaposi's sarcoma-associated herpesvirus utilizes an actin polymerization-dependent macropinocytic pathway to enter human dermal microvascular endothelial and human umbilical vein endothelial cells. J Virol 83:4895-911.

Rappocciolo G, Jenkins FJ, Hensler HR, Piazza P, Jais M, Borowski L, Watkins SC, Rinaldo CR. 2006a. DC-SIGN is a receptor for human herpesvirus 8 on dendritic cells and macrophages. J Immunol 176:1741-1749.

Rappocciolo G, Piazza P, Fuller CL, Reinhart TA, Watkins SC, Rowe DT, Jais M, Gupta P, Rinaldo CR. 2006b. DC-SIGN on B lymphocytes is required for transmission of HIV-1 to T lymphocytes. PLoS Pathog 2:e70.

Rappocciolo G, Hensler HR, Jais M, Reinhart TA, Pegu A, Jenkins FJ, Rinaldo CR. 2008. Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN. J Virol 82:4793-806.

Rappocciolo G, Jais M, Piazza PA, DeLucia DC, Jenkins FJ, Rinaldo CR. 2017. Human Herpesvirus 8 Infects and Replicates in Langerhans Cells and Interstitial Dermal Dendritic Cells and Impairs Their Function. J Virol 91.

Ren Y, Bell S, Zenner HL, Lau SY, Crump CM. 2012. Glycoprotein M is important for the efficient incorporation of glycoprotein H-L into herpes simplex virus type 1 particles. J Gen Virol 93:319-29.

Renne R, Blackbourn D, Whitby D, Levy J, Ganem D. 1998. Limited transmission of Kaposi's sarcoma-associated herpesvirus in cultured cells. J Virol 72:5182-8.

Robey RC, Lagos D, Gratrix F, Henderson S, Matthews NC, Vart RJ, Bower M, Boshoff C, Gotch FM. 2009. The CD8 and CD4 T-cell response against Kaposi's sarcoma-associated herpesvirus is skewed towards early and late lytic antigens. PLoS One 4:e5890.

Robey RC, Mletzko S, Bower M, Meys R, Boffito M, Nelson M, Bunker CB, Gotch FM. 2011. Ex-vivo recognition of late-lytic CD8 epitopes specific for Kaposi's sarcoma-associated herpesvirus (KSHV) by HIV/KSHV-coinfected individuals. Viral Immunol 24:211-20.

Russo J J, Bohenzky R A, Chien MC, Chen J, Yan M, Maddalena D, Parry J P, Peruzzi D, Edelman I S, Chang Y, Moore P S. 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A 93:14862-7.

Sadagopan S, Sharma-Walia N, Veettil MV, Raghu H, Sivakumar R, Bottero V, Chandran B. 2007. Kaposi's sarcoma-associated herpesvirus induces sustained NF-kappaB activation during de novo infection of primary human dermal microvascular endothelial cells that is essential for viral gene expression. J Virol 81:3949-68.

Sathiyamoorthy K, Hu YX, Möhl BS, Chen J, Longnecker R, Jardetzky TS. 2016. Structural basis for Epstein-Barr virus host cell tropism mediated by gp42 and gHgL entry glycoproteins. Nat Commun 7:13557.

Sathiyamoorthy K, Jiang J, Hu YX, Rowe CL, Möhl BS, Chen J, Jiang W, Mellins ED, Longnecker R, Zhou ZH, Jardetzky TS. 2014. Assembly and architecture of the EBV B cell entry triggering complex. PLoS Pathog 10:e1004309.

Sattentau Q. 2008. Avoiding the void: cell-to-cell spread of human viruses. Nat Rev Microbiol 6:815-26.

Schaupp A, Sabet O, Dudanova I, Ponserre M, Bastiaens P, Klein R. 2014. The composition of EphB2 clusters determines the strength in the cellular repulsion response. J Cell Biol 204:409-422.

Schulz TF, Weiss RA. 1995. Kaposi's sarcoma. A finger on the culprit. Nature 373:17-8.

Seifi A, Weaver EM, Whipple ME, Ikoma M, Farrenberg J, Huang ML, Vieira J. 2011. The lytic activation of KSHV during keratinocyte differentiation is dependent upon a suprabasal position, the loss of integrin engagement, and calcium, but not the interaction of cadherins. Virology 410:17-29.

Seiradake E, Harlos K, Sutton G, Aricescu AR, Jones EY. 2010. An extracellular steric seeding mechanism for Eph-ephrin signalling platform assembly. Nat Struct Mol Biol 17:398-402.

Seiradake E, Schaupp A, del Toro Ruiz D, Kaufmann R, Mitakidis N, Harlos K, Aricescu AR, Klein R, Jones EY. 2013. Structurally encoded intraclass differences in EphA clusters drive distinct cell responses. Nat Struct Mol Biol 20:958-964.

Shannon-Lowe CD, Neuhierl B, Baldwin G, Rickinson AB, Delecluse HJ. 2006. Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells. Proc Natl Acad Sci U S A 103:7065-70.

Shannon-Lowe C, Rowe M. 2011. Epstein-Barr virus infection of polarized epithelial cells via the basolateral surface by memory B cell-mediated transfer infection. PLoS Pathog 7:e1001338.

Shannon-Lowe C, Rowe M. 2014. Epstein Barr virus entry; kissing and conjugation. Curr Opin Virol 4:78-84.

Sharma-Walia N, Naranatt PP, Krishnan HH, Zeng L, Chandran B. 2004. Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 envelope glycoprotein gB induces the integrin-dependent focal adhesion kinase-Src-phosphatidylinositol 3-kinase-rho GTPase signal pathways and cytoskeletal rearrangements. J Virol 78:4207-4223.

Sharma-Walia N, Krishnan HH, Naranatt PP, Zeng L, Smith MS, Chandran B. 2005. ERK1/2 and MEK1/2 induced by Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) early during infection of target cells are essential for expression of viral genes and for establishment of infection. J Virol 79:10308-29.

Shi Y, Pontrello CG, DeFea KA, Reichardt LF, Ethell IM. 2009. Focal adhesion kinase acts downstream of EphB receptors to maintain mature dendritic spines by regulating cofilin activity. J Neurosci 29:8129-42.

Shukla D, Spear PG. 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. J Clin Invest 108:503-10.

Skalsky RL, Samols MA, Plaisance KB, Boss IW, Riva A, Lopez MC, Baker HV, Renne R. 2007. Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. J Virol 81:12836-45.

Soulier J, Grollet L, Oksenhendler E, Cacoub P, Cazals-Hatem D, Babinet P, d'Agay MF, Clauvel JP, Raphael M, Degos L. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood 86:1276-1280.

Speck P, Longnecker R. 2000. Infection of breast epithelial cells with Epstein-Barr virus via cell-to-cell contact. J Natl Cancer Inst 92:1849-51.

Spiller OB, Robinson M, O'Donnell E, Milligan S, Morgan BP, Davison AJ, Blackbourn DJ. 2003. Complement regulation by Kaposi's sarcoma-associated herpesvirus ORF4 protein. J Virol 77:592-9.

Spiller OB, Mark L, Blue CE, Proctor DG, Aitken JA, Blom AM, Blackbourn DJ. 2006. Dissecting the regions of virion-associated Kaposi's sarcoma-associated herpesvirus complement control protein required for complement regulation and cell binding. J Virol 80:4068-4078.

Sternbach G, Varon J. 1995. Moritz Kaposi: idiopathic pigmented sarcoma of the skin. J Emerg Med 13:671-4.

Stewart JP, Silvia OJ, Atkin IM, Hughes DJ, Ebrahimi B, Adler H. 2004. In vivo function of a gammaherpesvirus virion glycoprotein: influence on B-cell infection and mononucleosis. J Virol 78:10449-59.

Stürzl M, Gaus D, Dirks WG, Ganem D, Jochmann R, G S. 2013. Kaposi's sarcoma-derived cell line SLK is not of endothelial origin, but is a contaminant from a known renal carcinoma cell line. Int J Cancer 132:1954–1958.

Subramanian R, Sehgal I, D'Auvergne O, Kousoulas KG. 2010. Kaposi's sarcoma-associated herpesvirus glycoproteins B and K8.1 regulate virion egress and synthesis of vascular endothelial growth factor and viral interleukin-6 in BCBL-1 cells. J Virol 84:1704-14.

Tang Q, Qin D, Lv Z, Zhu X, Ma X, Yan Q, Zeng Y, Guo Y, Feng N, Lu C. 2012. Herpes simplex virus type 2 triggers reactivation of Kaposi's sarcoma-associated herpesvirus from latency and collaborates with HIV-1 Tat. PLoS One 7:e31652.

Tang S, Zheng ZM. 2002. Kaposi's sarcoma-associated herpesvirus K8 exon 3 contains three 5'-splice sites and harbors a K8.1 transcription start site. J Biol Chem 277:14547-56.

Tischer BK, Schumacher D, Messerle M, Wagner M, Osterrieder N. 2002. The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J Gen Virol 83:997-1003.

Tischer BK, von Einem J, Kaufer B, Osterrieder N. 2006. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40:191-7.

Tischer BK, Smith GA, Osterrieder N. 2010. En passant mutagenesis: a two step markerless red recombination system. Methods Mol Biol 634:421-30.

Tiwari V, Darmani NA, Thrush GR, Shukla D. 2009. An unusual dependence of human herpesvirus-8 glycoproteins-induced cell-to-cell fusion on heparan sulfate. Biochem Biophys Res Commun 390:382-7.

Tugizov SM, Berline JW, Palefsky JM. 2003. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. Nat Med 9:307-14.

Turk SM, Jiang R, Chesnokova LS, Hutt-Fletcher LM. 2006. Antibodies to gp350/220 enhance the ability of Epstein-Barr virus to infect epithelial cells. J Virol 80:9628-33.

Uldrick TS, Wang V, O'Mahony D, Aleman K, Wyvill KM, Marshall V, Steinberg SM, Pittaluga S, Maric I, Whitby D, Tosato G, Little RF, Yarchoan R. 2010. An interleukin-6-related systemic inflammatory syndrome in patients co-infected with Kaposi sarcoma-associated herpesvirus and HIV but without Multicentric Castleman disease. Clin Infect Dis 51:350-8.

Valiya Veettil M, Sadagopan S, Kerur N, Chakraborty S, Chandran B. 2010. Interaction of c-Cbl with myosin IIA regulates Bleb associated macropinocytosis of Kaposi's sarcoma-associated herpesvirus. PLoS Pathog 6:e1001238.

Veettil MV, Sharma-Walia N, Sadagopan S, Raghu H, Sivakumar R, Naranatt PP, Chandran B. 2006. RhoA-GTPase facilitates entry of Kaposi's sarcoma-associated herpesvirus into adherent target cells in a Src-dependent manner. J Virol 80:11432-46.

Veettil MV, Sadagopan S, Sharma-Walia N, Wang FZ, Raghu H, Varga L, Chandran B. 2008. Kaposi's sarcoma-associated herpesvirus forms a multimolecular complex of integrins (alphaVbeta5, alphaVbeta3, and alpha3beta1) and CD98-xCT during infection of human dermal microvascular endothelial cells, and CD98-xCT is essential for the postentry stage of infection. J Virol 82:12126-12144.

Veettil MV, Kumar B, Ansari MA, Dutta D, Iqbal J, Gjyshi O, Bottero V, Chandran B. 2016. ESCRT-0 Component Hrs Promotes Macropinocytosis of Kaposi's Sarcoma-Associated Herpesvirus in Human Dermal Microvascular Endothelial Cells. J Virol 90:3860-72.

Vieira J, O'Hearn P, Kimball L, Chandran B, Corey L. 2001. Activation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) lytic replication by human cytomegalovirus. J Virol 75:1378-86.

Volkow P, Cesarman-Maus G, Garciadiego-Fossas P, Rojas-Marin E, Cornejo-Juárez P. 2017. Clinical characteristics, predictors of immune reconstitution inflammatory syndrome and long-term prognosis in patients with Kaposi sarcoma. AIDS Res Ther 14:30.

Walker LR, Hussein HA, Akula SM. 2014. Disintegrin-like domain of glycoprotein B regulates Kaposi's sarcoma-associated herpesvirus infection of cells. J Gen Virol 95:1770-1782.

Wang FZ, Akula SM, Pramod NP, Zeng L, Chandran B. 2001. Human herpesvirus 8 envelope glycoprotein K8.1A interaction with the target cells involves heparan sulfate. J Virol 75:7517-7527.

Wang FZ, Akula SM, Sharma-Walia N, Zeng L, Chandran B. 2003. Human herpesvirus 8 envelope glycoprotein B mediates cell adhesion via its RGD sequence. J Virol 77:3131-3147.

Wang LX, Kang G, Kumar P, Lu W, Li Y, Zhou Y, Li Q, Wood C. 2014. Humanized-BLT mouse model of Kaposi's sarcoma-associated herpesvirus infection. Proc Natl Acad Sci U S A 111:3146-51.

Wang X, Zhu N, Li W, Zhu F, Wang Y, Yuan Y. 2015. Mono-ubiquitylated ORF45 Mediates Association of KSHV Particles with Internal Lipid Rafts for Viral Assembly and Egress. PLoS Pathog 11:e1005332.

Wang X, Zou Z, Deng Z, Liang D, Zhou X, Sun R, Lan K. 2017. Male hormones activate EphA2 to facilitate Kaposi's sarcoma-associated herpesvirus infection: Implications for gender disparity in Kaposi's sarcoma. PLoS Pathog 13:e1006580.

Wei F, Gan J, Wang C, Zhu C, Cai Q. 2016. Cell Cycle Regulatory Functions of the KSHV Oncoprotein LANA. Front Microbiol 7:334.

Weidner-Glunde M, Mariggiò G, Schulz TF. 2017. Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen: Replicating and Shielding Viral DNA during Viral Persistence. J Virol 91.

Wells R, Stensland L, Vieira J. 2009. The human cytomegalovirus UL112-113 locus can activate the full Kaposi's sarcoma-associated herpesvirus lytic replication cycle. J Virol 83:4695-9.

Wimmer-Kleikamp SH, Janes PW, Squire A, Bastiaens PIH, Lackmann M. 2004. Recruitment of Eph receptors into signaling clusters does not require ephrin contact. J Cell Biol 164:661-666.

Wong JP, Damania B. 2017. Modulation of oncogenic signaling networks by Kaposi's sarcoma-associated herpesvirus. Biol Chem 398:911-918.

Wu L, Renne R, Ganem D, Forghani B. 2000. Human herpesvirus 8 glycoprotein K8.1: expression, post-translational modification and localization analyzed by monoclonal antibody. J Clin Virol 17:127-36.

Wykosky J, Debinski W. 2008. The EphA2 receptor and ephrinA1 ligand in solid tumors: function and therapeutic targeting. Mol Cancer Res 6:1795-806.

Xu K, Tzvetkova-Robev D, Xu Y, Goldgur Y, Chan YP, Himanen JP, Nikolov DB. 2013. Insights into Eph receptor tyrosine kinase activation from crystal structures of the EphA4 ectodomain and its complex with ephrin-A5. Proc Natl Acad Sci U S A 110:14634-14639.

Xu Y, Ganem D. 2010. Making sense of antisense: seemingly noncoding RNAs antisense to the master regulator of Kaposi's sarcoma-associated herpesvirus lytic replication do not regulate that transcript but serve as mRNAs encoding small peptides. J Virol 84:5465-75.

Yamagishi Y, Sadaoka T, Yoshii H, Somboonthum P, Imazawa T, Nagaike K, Ozono K, Yamanishi K, Mori Y. 2008. Varicella-zoster virus glycoprotein M homolog is glycosylated, is expressed on the viral envelope, and functions in virus cell-to-cell spread. J Virol 82:795-804.

Zhang H, Li Y, Wang HB, Zhang A, Chen ML, Fang ZX, Dong XD, Li SB, Du Y, Xiong D, He JY, Li MZ, Liu YM, Zhou AJ, Zhong Q, Zeng YX, Kieff E, Zhang Z, Gewurz BE, Zhao B, Zeng MS. 2018. Ephrin receptor A2 is an epithelial cell receptor for Epstein-Barr virus entry. Nat Microbiol 3:164-171.

Zhang X, Wang JF, Chandran B, Persaud K, Pytowski B, Fingeroth J, Groopman JE. 2005. Kaposi's sarcoma-associated herpesvirus activation of vascular endothelial growth factor receptor 3 alters endothelial function and enhances infection. J Biol Chem 280:26216-24.

Zhao X, Guan JL. 2011. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. Adv Drug Deliv Rev 63:610-5.

Zhou F, Xue M, Qin D, Zhu X, Wang C, Zhu J, Hao T, Cheng L, Chen X, Bai Z, Feng N, Gao SJ, Lu C. 2013. HIV-1 Tat promotes Kaposi's sarcoma-associated herpesvirus (KSHV) vIL-6-induced angiogenesis and tumorigenesis by regulating PI3K/PTEN/AKT/GSK-3β signaling pathway. PLoS One 8:e53145.

Zhu FX, Chong JM, Wu L, Yuan Y. 2005. Virion proteins of Kaposi's sarcoma-associated herpesvirus. J Virol 79:800-11.

Zhu L, Puri V, Chandran B. 1999. Characterization of human herpesvirus-8 K8.1A/B glycoproteins by monoclonal antibodies. Virology 262:237-49.

Zhu L, Wang R, Sweat A, Goldstein E, Horvat R, Chandran B. 1999. Comparison of human sera reactivities in immunoblots with recombinant human herpesvirus (HHV)-8 proteins associated with the latent (ORF73) and lytic (ORFs 65, K8.1A, and K8.1B)

replicative cycles and in immunofluorescence assays with HHV-8-infected BCBL-1 cells. Virology 256:381-92.

Ziegler C, Just FT, Lischewski A, Elbers K, Neubauer A. 2005. A glycoprotein M-deleted equid herpesvirus 4 is severely impaired in virus egress and cell-to-cell spread. J Gen Virol 86:11-21.