Role of MEK/ERK and PI3K/Akt Signalling Cascades and Kaposi Sarcoma Herpesvirus K1 Protein During the Progression of the Viral Lytic Replication Cycle.

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades einer
DOKTORIN DER NATURWISSENSCHAFTEN
Dr. rer. nat.
genehmigte Dissertation

von
Diplombiologin Silke Hartmann
geboren am 6. Oktober 1977, in Siegen

2010

Referent: Prof. Dr. med. Thomas F. Schulz
Koreferentin: Prof. Dr. rer. nat. Christine Josenhans

Tag der Promotion: 01. Juni 2010

Zusammenfassung

Das Kaposi-Sarkom assoziierte Herpesvirus (KSHV oder auch humanes Herpesvirus 8, HHV-8) ist kausal an der Entstehung des Kaposi-Sarkoms (KS) sowie zwei menschlicher B-Zell-Lymphome beteiligt. Es gibt zahlreiche Hinweise darauf, dass neben der latenten Virusinfektion auch der lytische, produktive Replikationszyklus von KSHV bei der Onkogenese eine bedeutende Rolle spielt. Eine Schlüsselfunktion beim Übergang vom latenten zum lytischen Replikationszyklus besitzt das sehr frühe (*immediate-early*) virale Protein RTA, welches die Expression weiterer Virusproteine aktiviert und in der Lage ist den vollständigen produktiven Lebenszyklus von KSHV auszulösen. In der vorliegenden Arbeit wurde untersucht, welche intrazellulären Signaltransduktionswege unterhalb von RTA entscheidend zum Fortschreiten des lytischen Zyklus beitragen. Es konnte eine verstärkte Aktivierung von ERK2 und Akt während der frühen und späten Phase der lytischen Replikation festgestellt werden. Eine Hemmung der MEK/ERK Signalkaskade durch chemische Inhibitoren führte zu einer stark reduzierten Expression später Virusproteine und erheblich verminderter Virusproduktion in verschiedenen Zelllinien.

Um herauszufinden welche Virusproteine an der Aktivierung von ERK und Akt und/oder dem stufenweisen Fortschreiten des produktiven Vermehrungszyklus beteiligt sind, wurde die Synthese mehrerer aussichtsreicher viraler Proteine mittels inhibitorischer RNA unterdrückt. Es zeigte sich, dass ein Mangel an KSHV-eigenem K1-Protein die Bildung infektiöser Viruspartikel erheblich verringerte. Untersuchungen mit einer K1-Deletionsmutante eines rekombinanten KSHV (BAC36 ΔK1) bestätigten die verstärkende Wirkung von K1 auf den produktiven Lebenszyklus von KSHV. In HEK293 Zellen, welche anstelle mit BAC36 Wildtyp mit BAC36 ΔK1 transfiziert worden waren, wurden die viralen Gene K8/b-ZIP, vIL-6 und vCCL-1 nicht oder nur in verringertem Ausmaß exprimiert. Die Virusproduktion war stark herabgesetzt und auch die Menge an RTA-Protein war deutlich reduziert. Dies ging einher mit einer fehlenden oder stark verringerten Aktivierung von ERK und Akt. Zusammenfassend weisen diese Ergebnisse auf eine wichtige Rolle des K1-Proteins während des lytischen Replikationszyklus von KSHV hin.

Schlagwörter: *KSHV, Signaltransduktionswege, lytischer Zyklus*

Summary

Lytic (productive) replication of the Kaposi sarcoma herpesvirus (KSHV) promotes progression of Kaposi sarcoma (KS), a common malignancy in sub-Saharan Africa and patients with AIDS worldwide. Activation of the KSHV lytic pathway is initiated by the viral immediate-early protein RTA, which activates the expression of several viral early genes. I analysed which signalling pathways and viral proteins are important for the progression of the KSHV lytic cycle downstream of RTA. I observed that ERK2 and Akt activation increased during the viral lytic cycle following RTA expression and that chemical inhibition of the ERK pathway reduced late viral protein expression and virus production in various cell lines. These observations suggest an important role for MEK/ERK signalling cascade in the progression of the KSHV lytic life cycle.

In order to identify viral lytic cycle proteins required for the activation of ERK2 and Akt, and/or the progression of the lytic cycle, I used RNA interference to down-regulate the expression of individual candidate proteins. Knockdown of the KSHV K1 protein significantly reduced the production of infectious virus particles in the supernatant of reactivated Vero rKSHV.219 and EAhy rKSHV.219 cells. To confirm these results, I deleted the K1 open reading frame from a recombinant KSHV genome (BAC36). In HEK293 cells transfected with BAC36 ΔK1, expression of the early viral genes K8/b-ZIP, vIL-6, vCCL-1 was lacking or strongly reduced, protein levels of RTA were diminished and significantly less viral progeny was produced. This was accompanied by a lacking activation of ERK and Akt in KSHVΔK1 containing cells. These results suggest an essential role of the K1 protein during the early stage of the KSHV lytic (productive) replication cycle.

Keywords: *KSHV, signalling pathways, lytic cycle progression*

Contents

Zusammenfassung I
Summary II
Contents III
Abbreviations used: VI

1 Introduction 1
1.1 The family *Herpesviridae* 1
1.2.1 Diseases associated with KSHV 3
1.2.2 Epidemiology 5
1.2.4 Transmission 5
1.2.5 Tropism of KSHV and virus entry 6
1.2.6 Organization and expression of the KSHV genome 7
1.3 KSHV and signalling pathways 12
1.3.1 MAPK and PI3K/Akt signalling cascades and Syk protein kinase 12
1.3.2 Viruses and MEK-ERK1/2 and PI3K-Akt signalling cascades 17
1.3.3 Signalling pathways involved in KSHV infection and activation of the lytic cycle 17
1.3.4 KSHV lytic proteins involved in signalling 18
1.4 Protein kinase inhibitors 32
1.4.1 Protein kinase inhibitors in the treatment of KSHV-associated diseases 33
1.5 Objectives 34

2 Materials and Methods 35
2.1 Reagents and Chemicals 35
2.1.1 Antibodies 35
2.2 Vectors, primers, inhibitor peptide and small inhibitory RNAs (siRNAs) 38
2.2.1 Expression vectors 38
2.2.2 Primers and probes 39
2.2.3 Inhibitor peptide 40
2.2.4 siRNAs 41
2.3 Eukaryotic cell culture methods 43

2.3.1 General components for cell culture 43
2.3.2 Eukaryotic cell lines 44
2.3.3 Cell culture conditions 46
2.3.4 Cryoconservation 46
2.3.5 Amplification of baculovirus Orf50/RTA in SF9 cells 47
2.3.6 Lytic cycle induction 47
2.3.7 Infectivity assay 49
2.3.8 Transient transfection 50
2.4 Prokaryotic culture methods 51
2.4.1 Culture media and growth conditions 51
2.4.2 Bacterial strains 51
2.4.6 Cryokonservation of bacteria 52
2.4.7 Preparation of competent cells and transformation 52
2.5 Molecular biology methods 53
2.5.1 Isolation of plasmid and BACmid DNA 53
2.5.2 Enzymatic modification of DNA 53
2.5.3 PCR amplification of DNA 53
2.5.4 Preparation of RNA and quantification by qPCR 54
2.5.5 Electrophoresis of DNA and extraction from agarose gels 55
2.5.6 DNA sequencing 55
2.5.7 Generation of BAC36 ΔK1 56
2.5.8 One-colour microarray-based gene expression analysis 56
2.6 Biochemical and cell biology methods 57
2.6.1 Preparation of cell lysates 57
2.6.2 SDS polyacrylamide gelelectophoresis (SDS-PAGE) 58
2.6.3 Western blot analysis 59
2.6.4 Immunofluorescence assay 60
2.6.5 Flow cytometry-based cell cycle analysis 60

3 Results 62
3.1 Results I: The MEK/ERK Signalling Cascade Contributes to Successful KSHV Lytic Replication Downstream of RTA Expression. 62
3.2 Results II: Essential Role of Kaposi Sarcoma Herpesvirus K1 Protein During the Progression of the Lytic Replication Cycle. 81

4 Discussion 98
4.1 Role of MEK/ERK and PI3K/Akt signalling pathways during the KSHV lytic replication cycle 98
4.2 Role of K1 protein in the viral lytic replication cycle 102

References 108

Erklärung zur Dissertation i
Danksagung ii
Curriculum vitae iv
Scientific publications and presentations v

Abbreviations used:

Ab, antibody;

AP-1, activator protein 1;

cAMP, cyclic adenosine monophosphate;

BAC, bacterial artificial chromosome;

CCR, CC-motif chemokine receptor;

Cox-2, cyclooxygenase-2;

CREB, cAMP-response element binding protein;

CBP, CREB binding protein;

Dscr-1, Down syndrome critical region gene 1;

EBV, Epstein Barr virus;

ERK, extracellular signal-regulated kinase;

FGF-2, basic fibroblast growth factor;

FKHR, member of the FoxO subfamily of forkhead transcription factors (also known as FoxO1a);

GM-CSF, granulocyte-macrophage colony-stimulating factor;

Gro-α, growth regulated oncogene alpha;

GSK3, glycogen synthase kinase 3;

HHV, human herpesvirus;

HIF1α, hypoxia induced factor 1 alpha;

hIL-6, human interleukine 6;

HSV, herpesvirus saimiri;

IKK, IkappaB kinase;

IL, interleukine;

ITAM, immunoreceptor tyrosine-based activation motif;

JAK, janus kinase;

JNK, c-Jun N-terminal kinase;

KSHV, Kaposi sarcoma herpesvirus;

MAPK, mitogen activated protein kinase;

Mdm2, murine double minute 2;

MKK, MAPK kinase (also called MEK);

MEF-2, myocyte enhancer factor 2;

MIP-1, macrophage inflammatory protein 1;

MMP, matrix metalloproteinase;

MSK1, mitogen- and stress-activated protein kinase 1;

mTOR, mammalian target of rapamycin;

MSK1/2, mitogen- and stress-activated protein kinase 1/2;

NFAT, nuclear factor of activated T cells;

NF-κB, nuclear factor kappa B;

Oct-2, octamer binding protein 2;

p70RSK/p90RSK, approximately 70/90-kDa ribosomal protein S6 kinase;

PDK1/2, PI3K-dependent kinase 1/2;

PI3K, phosphatidylinositol 3-kinase;

PKA, protein kinase A (also known as cAMP-dependent kinase);

PKB, protein kinase B (also known as Akt);

PKC, protein kinase C;

PLCγ, phospholipase C-gamma;

PTEN, phosphatase and tensin homologue;

RANTES, regulated upon activation, normal T cell expressed and secreted (also known as CCL5);

RasGAP, Ras GTPase activating protein;

SH2, src homology 2;

SP-1, specificity protein 1 (transcription factor);

STAT, signal transducers and activators of transcription;

Syk, spleen tyrosine kinase;

TAK1, transforming growth factor-β-activated protein kinase 1;

VEGF, vascular endothelial growth factor.

1 Introduction

1.1 The family *Herpesviridae*

Herpesviruses are widely distributed in nature and are prevalent in most species throughout the animal kingdom. They are highly adapted to their hosts and are thought to have coevolved with their hosts for millions of years. Up to date, more than 130 herpesviruses have been described, eight of which have been isolated from humans (Wang et al. 2007a).

All herpesviruses have a characteristic virion morphology, which consists of the envelope, tegument, capsid and core (Mettenleiter 2002). The size of the virion varies from 120 to nearly 300 nm. The viral genomes consist of linear, double-stranded DNA molecules that range in size from about 125 to 240 kbp and in the nucleotide composition from 32 to 75% G+C, depending on the virus species (Honess 1984), and the most extensively characterized contain from about 70 to 165 genes. Replication of the viral DNA and capsid assembly occurs in the nucleus. All herpesviruses encode a variety of enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing. Herpesviruses are able to establish a latent infection in their natural hosts. In the latent stage, viral gene expression is heavily restricted to a small number of genes (latency associated genes); the viral DNA is retained as circular episomes in the nucleus and no progeny virus is produced. Reactivation from latency induces expression of a large number of viral (lytic) genes, viral DNA replication and production of new infectious progeny, in the course of which the host cell dies.

On the basis of their biological characteristics and genome sequences, human herpesviruses (HHV-1 to -8) are classified into three subfamilies: *Alpha*-, *Beta*- and *Gammaherpesvirinae* (McGeoch et al. 1995).

- *α-herpesvirina*e, which include *Simplexvirus* herpes simplex virus 1 and 2 (HSV-1 and -2 or HHV-1 and -2) and *Varicellovirus* Varizella zoster virus (VZV or HHV-3), have a wide host cell range, multiply efficiently and rapidly spread in culture. Primarily, but not exclusively, they establish latency in sensory ganglia.
- *β-herpesvirinae* show a more restricted host range and grow slowly in cell culture. They establish latent infection in lymphocytes, secretory glands and other tissue. Representatives of this subfamily are the genera *Cytomegalovirus* (HCMV or HHV-5) and *Roseolovirus* (HHV-6 and -7).

- *γ-herpesvirinae* are lymphotropic but some are also capable of infecting epithelial, eindothelial and/or fibroblast cells. The subfamily contains two genera: *Lymphocryptovirus* (γ-1) (Epstein-Barr virus, EBV or HHV-4) and *Rhadinovirus* (γ-2) (Kaposi sarcoma herpesvirus, KSHV or HHV-8). A striking characteristic of members of the γ-herpesvirinae, including EBV and KSHV, is their strong association with neoplastic disease. Both EBV and KSHV are associated with different types of cancer and also related with lymphoproliferative diseases of B and/or T cells (Damania 2004).

1.2 Kaposi sarcoma herpesvirus (KSHV)

Kaposi sarcoma herpesvirus was discovered in Kaposi sarcoma (KS) lesions in 1994 (Chang et al. 1994), and soon epidemiologic evidence accumulated showing that KSHV (or human herpesvirus 8, HHV-8) is the infectious cause of KS. Before the early 1980s, KS was a rare disease restricted to East and Central Africa, the Mediterranean and Eastern European regions. However, in the course of the then-emerging HIV-pandemic, KS has evolved into the most common malignancy in some African countries, and in patients with AIDS in other parts of the world (Dedicoat and Newton 2003). Today, KS is the most common cancer in sub-Saharan Africa, and poses an important health problem in HIV-infected and uninfected individuals worldwide (Orem et al. 2004; Parkin 2006; Greene et al. 2007). In addition to KS, KSHV is associated with primary effusion lymphoma (PEL) (Cesarman et al. 1995), a rare form of non-Hodgkin's lymphoma mostly seen in AIDS patients, and the plasma cell variant of multicentric Castleman's disease (MCD) (Soulier et al. 1995).

Like other herpesviruses, KSHV is able to establish a lifelong infection in its host. The viral life cycle is divided into two phases: latent and lytic (Dourmishev et al. 2003). During latency, the KSHV genome persists as a circular DNA episome, and protein expression is tightly restricted. The lytic (productive) phase is characterized by wide-spread gene activation, classically in an ordered cascade of sequential induction, in the course of which new infectious progeny virus is produced. In KSHV associated tumours, the vast majority of cells are latently infected by KSHV. However, a small percentage of cells (usually in the order of 1-5%) spontaneously switch into the lytic replication cycle (Zhong et al. 1996; Staskus et al. 1997; Katano et al. 2000; Parravicini et al. 2000).

1.2.1 Diseases associated with KSHV

Kaposi sarcoma

Kaposi sarcoma (KS) is an unusual neoplasm which was first described in Eastern Europe by the dermatologist Moritz Kaposi in 1872 (Kaposi 1872) but later recognized to be endemic in Mediterranian countries and sub-Saharan Africa. The classic form of KS is a rare, benign disease seen in elderly males and usually only affecting the skin. Endemic KS can be more widely disseminated and occurs in East and Central Africa. An aggressive form of endemic KS (also known as lymphadenopathic form) is found in young children in endemic regions with high fatality rates (Ziegler and Katongole-Mbidde 1996). AIDS-KS is the most common and aggressive variant of KS with the most lymph node/visceral spreading amongst all KS subtypes (Mitsuyasu 1987; Friedman-Kien and Saltzman 1990). Another form of KS, known as iatrogenic/post-transplant KS, is seen in patients under immunosuppressive therapy after solid organ transplantation (Penn 1988).

Unlike most classical cancers, which arise from the clonal outgrowth of a single cell type, KS lesions are histologically complex (Herndier and Ganem 2001). The principal proliferating cell in advanced lesions is the so-called spindle cell, named for its dramatically elongated morphology. The cellular origin and nature of KS spindle cells have long been debated. Evidence indicates a lymphatic endothelial cell origin for KS spindle cells. However, recently it has been shown that KSHV can reprogram endothelial cells to express lymphatic endothelial markers, which complicates attempts to discern the lineage of these KSHV infected cells (Hong et al. 2004; Wang et al. 2004a). All KS lesions also contain significant numbers of inflammatory cells (B- and T-cells, plasma cells, and monocytes) and a profusion of aberrant, slit-like neovascular spaces. Hence, KS appears to be composed of three parallel processes: a proliferative component (involving chiefly spindle cells), an inflammatory component, and an angiogenic component. It is likely that these processes are interdependent and perhaps mutually reinforcing (Ganem 2006).

KSHV DNA is present in all KS tumours, irrespectively of clinical type. Viral infection selectively targets spindle cells (Boshoff et al. 1995; Staskus et al. 1997; Dupin et al. 1999), with little or no infection of other cell types. *In situ* hybridization studies have shown that most KS spindle cells are latently infected; only a small subpopulation of spindle cells (1% - 3%) display lytic replicative markers (Staskus et al. 1997; Sturzl et al. 1997; Dupin et al. 1999; Katano et al. 2000; Dittmer 2003). Although latent KSHV infection certainly plays a

role in the development of KS and KSHV-associated B-cell lymphomas (Dupin et al. 1999; Parravicini et al. 2000), there is also a role for lytic replication. This may involve the propagation of infectious virus to new target cells, as well as autocrine and paracrine effects induced directly by KSHV-encoded cytokines and regulatory molecules, or indirectly through the induction of cellular cytokines (Martin et al. 1999; Goudsmit et al. 2000; Grundhoff and Ganem 2004; Schulz 2006).

Primary effusion lymphoma

Primary effusion lymphoma (PEL), sometimes referred to as body cavity-based lymphoma (BCBL), is a unique form of non-Hodgkin's lymphoma found more commonly in immunocompromised AIDS patients. PEL is derived from clonally expanded malignant B-cells and presents as a lymphomatous effusion tumour contained in various body cavities such as the pericardium, pleura, and peritoneum. PEL is aggressive and rapidly progressing, and rapidly fatal. Unlike KS tumours, which harbour generally less than two viral genome copies per cell (this is due to the mixed cellularity of KS lesions – see above – with only endothelial and spindle cells being infected by KSHV), KSHV genomes are found in PEL cells at a high copy number (50-150 viral genomes per infected cell) (Cesarman et al. 1995; Renne et al. 1996a; Staudt et al. 2004). In PEL cells, KSHV gene expression is restricted largely to the latency locus that encodes LANA-1, v-cyclin and vFLIP, and the kaposin locus. Additionally, LANA-2/vIRF-3 expression has been observed in some PEL cell lines (Rivas et al. 2001). Lytic gene expression has been detected in a small subset of cells: the viral interleukin 6 homologue (vIL-6) was detected in two to five percent of tumour cells, whereas other lytic transcripts such as K8/bZIP or the late lytic glycoprotein K8.1 were detected in less than one percent of PEL cells (Katano et al. 2000; Parravicini et al. 2000).

Multicentric Castleman's disease

MCD is a lymphoproliferative disorder, characterized by the proliferation of polyclonal B-cells. Its plasmablastic variant is highly associated with KSHV; however, the hyaline variant of MCD is not. In MCD lesions, the ratio of lytically infected to latently infected cells is much greater than in PEL or KS, suggesting that the pathogenic role of KSHV in these different diseases might be attributable to different gene expression programs of the virus (Dourmishev et al. 2003). Dysregulated IL-6 levels, probably induced by KSHV proteins such

as the virally encoded IL-6 (vIL-6) (Parravicini et al. 1997), play a role in the clinico-pathophysiology of MCD.

1.2.2 Epidemiology

In regions in the world where KS is not endemic, including Northern, Western and Central Europe, USA, Canada, as well as Asian countries, the seroprevalence of KSHV is low with estimates of < 5% (Bagni and Whitby 2009). The prevalence of KSHV is elevated in Mediterranean countries but varies geographically and thereby mirrors the incidence of classic KS in different geographical regions. The highest prevalence of KSHV in the Mediterranean area is observed in Southern Italy, particularly the Islands of Sicily and Sardinia (24-35%) (Bagni and Whitby 2009). In other Mediterranean regions, seropositivity for the virus ranges from 8% to 25%. Elevated KSHV prevalence may also be present in parts of Asia (Dilnur et al. 2001; Chen et al. 2004). However, by far the highest prevalence of KSHV is found in most parts of sub-Saharan Africa where 40-50% of the general population carries KSHV antibodies. Higher prevalence estimates are reported for Congo (82%), Botswana (76%), Malawi (67%), and the West African country Gambia (75%) (Ariyoshi et al. 1998; Engels et al. 2000; DeSantis et al. 2002; Whitby et al. 2004). Lower estimates are reported for South Africa (30%) and some West African countries (≤ 25%) (Sitas et al. 1999; Dedicoat et al. 2004; Volpi et al. 2004; Collenberg et al. 2006; Malope et al. 2007).

1.2.4 Transmission

In KS endemic regions, infection with KSHV occurs in childhood, and prevalence increases with age (Mayama et al. 1998; Andreoni et al. 1999; Gessain et al. 1999; Plancoulaine et al. 2000; Whitby et al. 2000). Evidence from numerous studies suggests that KSHV transmission occurs from mother to child or from sibling to sibling, most likely via infected saliva, and it has been shown that mothers with high levels of detectable KSHV DNA in saliva are more likely to have KSHV-infected children (Lyall et al. 1999; Plancoulaine et al. 2000; Mantina et al. 2001; Andreoni et al. 2002; Mbulaiteye et al. 2003; Dedicoat et al. 2004; Plancoulaine et al. 2004). Vertical transmission from mother to child might also, albeit very rarely, occur in endemic countries (Gutierrez-Ortega et al. 1989; Mantina et al. 2001; Brayfield et al. 2003).

Horizontal transmission by saliva is believed to be the most common route of infection also among high-risk groups in Western countries since viral loads found in vaginal, seminal and prostatic secretions are much lower than in saliva (Pauk et al. 2000; Martin 2003; Widmer et al. 2006). However, sexual, blood and transplant-related transmission remain a significant concern worldwide (Bagni and Whitby 2009).

1.2.5 Tropism of KSHV and virus entry

In vivo, KSHV DNA and transcripts have been detected in human B-cells, macrophages, keratinocytes, endothelial cells and epithelial cells. A number of PEL cells have been isolated and grown in culture, and have been the main subject to study the KSHV life cycle. In PEL cells, the switch from latency to lytic replication can be induced by various chemicals such as phorbol esters and sodium butyrate (see below). Virus from induced PEL cell lines has been used to infect many cell types in culture, including epithelial and endothelial cells, fibroblasts, keratinocytes, B- and CD34+ stem cell precursors of dendritic cells. KSHV also infects numerous animal cells such as monkey (Vero, CV-1), hamster (BHK-21, CHO) and mouse (Du17, 3T3) cells (Renne et al. 1998; Moses et al. 1999; Blackbourn et al. 2000; Akula et al. 2001b; Ciufo et al. 2001; Vieira et al. 2001; Dezube et al. 2002; Bechtel et al. 2003; Naranatt et al. 2003; Gasperini et al. 2005; Wu et al. 2006; Rappocciolo et al. 2008). The broad *in vitro* cellular tropism of KSHV may be due in part to its interaction with the ubiquitous cell surface heparin sulphate (HS-)-like molecule via the viral envelope glycoproteins gpK8.1A and glycoprotein B (gB) (Akula et al. 2001a; Akula et al. 2001b; Birkmann et al. 2001; Wang et al. 2001a). Subsequently, gB binds via its Arginine-Glycine-Aspartic Acid (RGD) motif to the $\alpha_3\beta_1$ integrin, which serves as one of the cellular receptors for KSHV entry into target cells. Interaction of gB with $\alpha_3\beta_1$ integrin leads to the autophosphorylation of focal adhesion kinase (FAK) and subsequent activation of members of Src family kinases, phosphatidylinositol-3-kinase (PI3K) and RhoGTPases (Akula et al. 2002; Naranatt et al. 2003; Sharma-Walia et al. 2004; Veettil et al. 2006). The induction of intracellular signalling pathways may facilitate virus entry through induction of cytoskeletal rearrangements and endocytosis and, in the following, may support a suitable environment for successful infection of the host cell (Naranatt et al. 2003; Naranatt et al. 2004; Sharma-Walia et al. 2004). It has been shown that KSHV infects fibroblasts, epithelial and endothelial cells via endocytosis (Akula et al. 2003; Inoue et al. 2003; Naranatt et al. 2005; Greene and Gao 2009). In fibroblast cells as well as endothelial cells endocytosis was shown to be clathrin-

dependent. However, in fibroblast cells endocytosis was suggested to predominantly depend on microtubule rearrangements (Naranatt et al. 2005; Raghu et al. 2007), while in endothelial cells the process may be primarily regulated through actin dynamics (Greene and Gao 2009).

A recent study reported that DC-SIGN, a C-type lectin first identified on dendritic cells (DC), may serve as an entry receptor for KSHV on DC and macrophages (Rappocciolo et al. 2006). B lymphocytes from peripheral blood and tonsils were found to express DC-SIGN and this expression significantly increased after B-cell activation mediated by CD40 ligand (CD40L) and interleukin 4 (IL-4) (Rappocciolo et al. 2006). It was demonstrated that activated blood and tonsillar B-cells expressing DC-SIGN can be productively infected with KSHV, as determined by an increase in the level of viral DNA, the expression of lytic and latency-associated viral proteins, and the production of infectious virus. Furthermore, infection could be blocked by the pretreatment of the B-cells with anti-DC-SIGN monoclonal antibody (MAb) (Rappocciolo et al. 2008).

In most infected cell lines, KSHV quickly establishes a latent infection as defined by the expression of the latency associated genes and the absence of productive lytic replication. Detection of LANA (Orf73) expression two days after infection, has led to the notion that the establishment of latency is the default pathway of infection (Schulz et al. 2002; Bechtel et al. 2003).

1.2.6 Organization and expression of the KSHV genome

Genome structure

KSHV has a double-stranded DNA genome and its size ranges from 165 to 170 kb (Renne et al. 1996a; Neipel et al. 1998). The long unique region (LUR) is about 138 to 140.5 kb in length, contains all of the KSHV open reading frames (orfs) and is flanked by terminal repeat (TR) sequences at both ends of the linear viral genome (Figure 2). Each TR is 801 bp in length and highly GC-rich (Russo et al. 1996). The number of TRs varies among KSHV isolates, ranging from 16 to 75 (Duprez et al. 2007), which accounts for the variations in the genome size of KSHV isolates. The KSHV genome shows high similarity to retroperitoneal fibromatosis-asociated herpesvirus (RFHV) and rhesus monkey rhadinovirus (RRV) among the rhadinovirus genus (O'Connor and Kedes 2007) and shares a somewhat co-linear genetic organization with herpesvirus saimiri (HVS). KSHV encodes for over 86 orfs which have been given serial numbers from left to right. Although many of the KSHV genes are

conserved among the family of herpesviridae, the virus contains a significant number of unique orf's not found in other herpesviruses. These KSHV-specific orfs are named K1 to K15, based on their relative locations - from left to right – in the viral genome (Russo et al. 1996) (see also Figure 1). Moreover, KSHV contains a large number of accessory genes including many homologues of cellular proteins. These cellular homologues are believed to be captured by the virus from the host cellular genome and are mainly implicated in signal transduction, cell cycle control and apoptosis (Neipel et al. 1997; Nicholas et al. 1997).

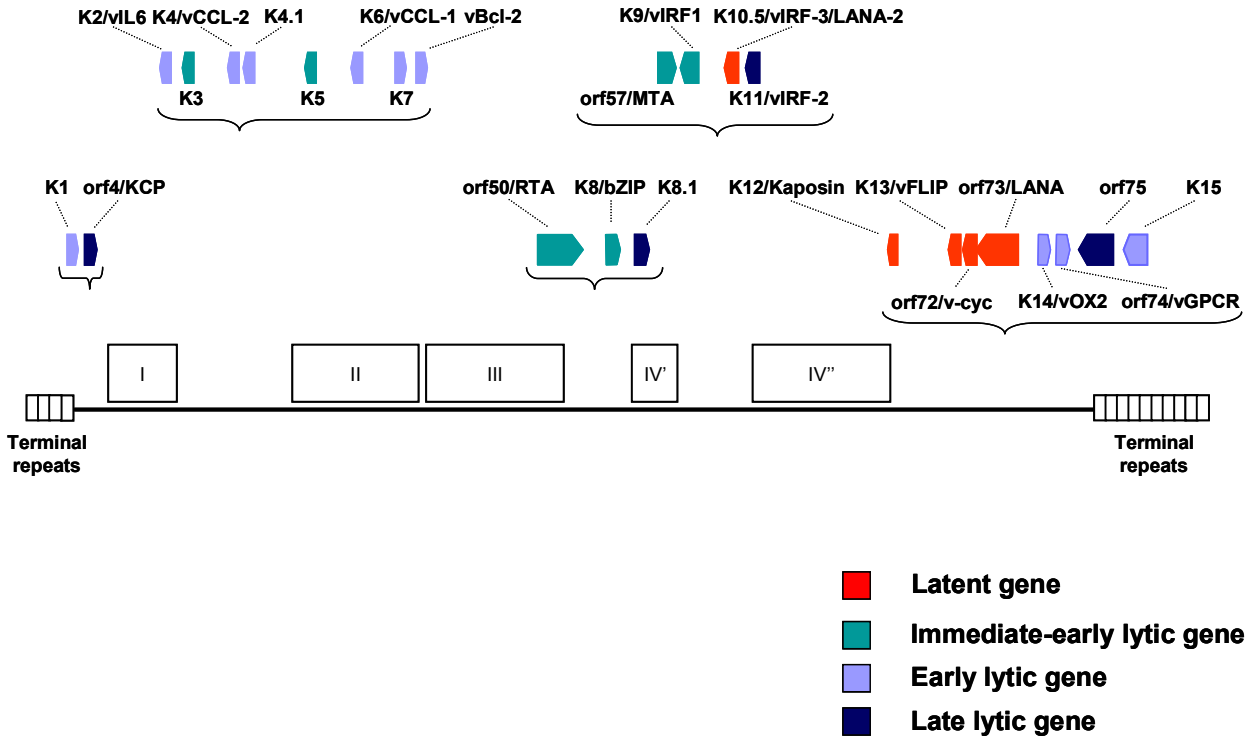


Figure 1: Schematic representation of the KSHV genome. The long unique region comprises nearly 90 open reading frames (orfs) and is flanked by terminal repeat sequences. Conserved gene blocks within the herpesvirus family are labelled by roman numbers (I-IV'') from left to right. Genes specific to KSHV carry the prefix K. Kinetic classes of gene expression are indicated by colours as depicted at the bottom of the figure.

Viral gene expression

KSHV genes are broadly classified as latent or lytic genes, reflecting latent and lytic replication phases of the virus, respectively. Based on the temporal onset of expression and their response to chemical inducers and inhibitors, lytic genes are further divided into immediate-early, early and late genes.

KSHV gene expression has been extensively studied by Northern blot analysis and later by cDNA arrays and real-time quantitative PCR covering the entire viral genome (Sarid et al. 1998; Sun et al. 1999; Jenner et al. 2001; Paulose-Murphy et al. 2001; Fakhari and Dittmer 2002; Dittmer 2003; Nakamura et al. 2003; Lu et al. 2004; Yoo et al. 2005).

Latent gene expression and the major latent protein LANA-1

Viral proteins expressed during latent infection include the latency-associated nuclear antigen (LANA-1, encoded by orf73), a viral homologue of a D-type cyclin (vCyc, encoded by orf72), a viral homologue of a FLICE-inhibitory protein (vFLIP or K13), kaposin (K12) and one of the viral interferon-regulatory factors (vIRFs) LANA-2 (K10.1 or IRF-3) (Sadler et al. 1999; Katano et al. 2000; Parravicini et al. 2000; Fakhari and Dittmer 2002; Dittmer 2003). Except for LANA-2, which is B-cell specific, all other latent genes have been detected in all KSHV-associated tumors and experimental models of KSHV latency (Rivas et al. 2001; Dittmer 2003). Interestingly, another vIRF, vIRF-1/K9, was found to be expressed during latency in primary KS lesions and its transcription profile clustered with LANA-1, vFLIP and vCyc (Dittmer 2003). These findings suggests that different vIRFs are important during latency in different target cells during latency and/or other stages of viral infection and point to an essential role for vIRFs in KSHV pathogenesis. In addition to protein-encoding genes, 12 KSHV-encoded microRNAs (miRNAs) have been identified in the viral latency region, and were shown to be expressed during latent infection in B-cells (Cai et al. 2005; Pfeffer et al. 2005; Samols et al. 2005; Grundhoff et al. 2006).

LANA-1 can be detected in all latently infected cells, and LANA-specific antibodies have been found in most infected individuals (Dupin et al. 1999; Zhu et al. 1999). Therefore, it is the most widely used marker for KSHV infection (Bagni and Whitby 2009). LANA-1 is a large (222-234 kDa), nuclear protein, which has an important function in the establishment and maintenance of the viral episome in the nucleus and is required for its replication (Verma et al. 2007). The protein tethers the viral episomal DNA to the host chromosomes by directly binding to two adjacent 16-bp motifs within the TR region of the KSHV genome through its C terminus and to cellular nucleosomes via its N terminus. Tethering to the host chromosomes facilitates efficient segregation of the viral episomes during host cell devision (Kedes et al. 1997; Ballestas et al. 1999; Cotter and Robertson 1999; Hu et al. 2002). Disruptions of LANA-1 expression lead to loss of KSHV episomes from latently infected cells, supporting its role in persistence of the viral DNA (Ye et al. 2004; Kelley-Clarke et al. 2009). However,

viral latent episomes are rapidly lost from infected culture cells in the absence of genetic selection (Grundhoff and Ganem 2004). This also applies to short-term cultures of cells explanted from KS lesions (Aluigi et al. 1996; Lebbe et al. 1997). Thus, LANA-1 is necessary but may not always be sufficient for viral episomal persistence.

In addition to its role in episome maintenance, LANA-1 acts as a transcriptional repressor/activator of both viral and cellular gene expression (Renne et al. 2001; An et al. 2005; Verma et al. 2006). For example, LANA-1 upregulates expression from its own promoter while blocking the expression of RTA protein, which is critical for the switch from latency to lytic replication, and thus helps in maintaining viral latency. Furthermore, LANA-1 can perturb several cellular pathways to contribute to viral oncogenesis. In transfected cells, the protein binds to and inhibits p53 function, conferring increased resistance to p53-dependent apoptosis (Friborg et al. 1999). LANA-1 can also inactivate the tumor suppressor retinoblastoma (Rb) gene and release E2F transactivator which induces cells to transit through the G1/S cell cycle checkpoint (Radkov et al. 2000). Another potential role for LANA-1 in tumorigenesis derives from its interaction with glycogen synthase kinase (GSK)-3β, which prevents GSK-3β from complexing with, and degrading, β-catenin in the cytoplasm, and ultimately leads to the activation of genes implicated in cell cycle regulation and oncogenesis (e.g. cyclin D, c-myc) (Fujimuro and Hayward 2003; Fujimuro et al. 2003). Finally, LANA-1 was demonstrated to upregulate human telomerase reverse transcriptase (hTERT) gene expression, to increase the life-span of primary human umbrilical vein endothelial cells (HUVEC), and transgenic mice expressing LANA-1 were shown to develop splenic follicular hyperplasia and lymphomas (Watanabe et al. 2003; Verma et al. 2004; Fakhari et al. 2006).

The lytic replication cycle

Reactivation of KSHV from latency and induction of the full lytic cycle leads to an ordered sequence of gene expression and viral DNA replication and finally to the production and release of new infectious virions. The biological signals that promote lytic cycle induction are not fully understood, though proinflammatory cytokines (e.g. IFN-γ) (Monini et al. 1999; Chang et al. 2000; Mercader et al. 2000), hypoxia (Davis et al. 2001), and co-infection with other viruses (Harrington et al. 1997; Vieira et al. 2001; Lu et al. 2005; Gregory et al. 2009) have been implicated. *In vitro*, KSHV lytic replication can be induced by various chemicals such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), or sodium butyrate (Na-butyrate, NaB) (Renne et al. 1996b; Miller et al. 1997).

The central protein in KSHV lytic replication is the *r*eactivation and *t*ranscription *a*ctivater (RTA) encoded by orf50. KSHV RTA alone is sufficient to induce the complete lytic cascade (Lukac et al. 1998; Sun et al. 1998; Lukac et al. 1999; Gradoville et al. 2000; Bechtel et al. 2003; Zhu et al. 2004; Xu et al. 2005).

RTA is a potent transcriptional activator. In addition to its own promoter, RTA activates a number of genes that are involved in lytic replication, including replication proteins (Orf6, K8/bZIP, and Orf21), transcriptional modulators (K8/bZIP, Orf57, and vIRF1), signal inducing membrane proteins (vOX2, vGPCR, and kaposins), viral cytokines (vIL-6, vCCL-1), immunomodulatory proteins (K5, K3), viral structural proteins (glycoprotein B (gB)), the lytic origin of DNA replication (oriLyt), polyadenylated nuclear (PAN) RNA as well as latency-associated genes (LANA, vCyclin, vFLIP) (Zhang et al. 1998; Lukac et al. 1999; Chen et al. 2000; Deng et al. 2000; Jeong et al. 2001; Lukac et al. 2001; Song et al. 2001; Wang et al. 2001b; Chang et al. 2002; Deng et al. 2002; AuCoin et al. 2004; Damania et al. 2004; Matsumura et al. 2005; Wang and Yuan 2007). The molecular mechanisms of transactivation by RTA include direct binding of RTA to specific DNA sequences as well as interaction of RTA with cellular proteins (e.g. Oct-1, C/EBPα, SP-1, AP-1, RBP-Jk (also known as CSL or CBF-1)) bound to promoter DNA (Zhang et al. 1998; Sakakibara et al. 2001; Byun et al. 2002; Ueda et al. 2002; Wang et al. 2003a; Wang et al. 2003b; Liang and Ganem 2004; Chang et al. 2005b). RTA has also been implicated in induction of some cellular genes (Chang et al. 2005a; Zhang et al. 2005), initiation of viral DNA replication (AuCoin et al. 2004; Wang et al. 2004c), and was shown to possess ubiquitin E3 ligase activity (Yu et al. 2005).

K8/bZIP (or replication associated protein, RAP) and MTA (Orf57) are two additional viral proteins that have been implicated in transcriptional and posttranscriptional regulation, respectively. K8/bZIP, a basic region-leucine zipper (bZIP) protein and a positional homologue of BZLF1 (also known as ZTA or ZEBRA) in EBV (Gruffat et al. 1999; Lin et al. 1999), negatively regulates the RTA-mediated activation of the K8, Orf57/MTA and K15 promoter (Izumiya et al. 2003; Liao et al. 2003; Ellison et al. 2009). Sumoylation and phosphorylation of K8/bZIP are critical in regulating this transcriptional repression activity. Phosphorylation of K8/bZIP by vPK/Orf36 reduces its repression function (Izumiya et al. 2005; Izumiya et al. 2007), while sumoylation of K8/bZIP through its cellular interaction partner Ubc 9 (E2 SUMO conjugation enzyme) positively influences its repression activity (Izumiya et al. 2005). Recent evidence suggests that K8/bZIP may also synergize with RTA in the activation of lytic promoters, as the majority of RTA-responsive promoters showed

enhanced activity when RTA and K8/bZIP were co-expressed in a reporter assay-based system (Ellison et al. 2009). Moreover, Lefort and Flamand (2009) showed that in the absence of K8/bZIP expression, expression of orf50, orf57, and orf26 transcripts was highly decreased as were the protein levels for RTA and the late lytic glycoprotein K8.1. These findings suggest that K8/bZIP may function as a positive regulator of viral lytic gene expression.

K8/bZIP has also been found to be important for origin-dependent lytic DNA replication (Izumiya et al. 2003; Wang et al. 2003a; Wang et al. 2003b; AuCoin et al. 2004; Rossetto et al. 2007; Lefort and Flamand 2009; Rossetto et al. 2009) and was shown to be involved in cell cycle regulation, inhibition of apoptosis and host antiviral response (Park et al. 2000; Izumiya et al. 2007; Lefort et al. 2007).

The KSHV Orf57 protein, also known as mRNA transport and accumulation protein (MTA) and homologue of the EBV SM (MTA, EB2, BMLF1) protein, has been reported to enhance several posttranscriptional processes including viral mRNA export, RNA stability and pre-mRNA splicing (Conrad 2009; Majerciak and Zheng 2009). Additionally, KSHV Orf57 functions as a transcriptional activator or co-activator of several viral genes (Kirshner et al. 2000; Malik et al. 2004; Palmeri et al. 2007) and is essential for the production of infectious virus (Han and Swaminathan 2006; Majerciak et al. 2007).

1.3 KSHV and signalling pathways

1.3.1 MAPK and PI3K/Akt signalling cascades and Syk protein kinase

Mitogen-activated protein kinase (MAPK) pathways

Mitogen-activated protein kinase (MAPK) cascades are important signalling pathways that convert extracellular signals into cellular responses. They regulate proliferation, differentiation, cell activation and immune responses. Up to now, four different members organized in separate cascades have been identified: (i) ERK (extracellular-signal-regulated kinase), (ii) JNK (c-Jun N-terminal kinase), (iii) p38 and (iv) ERK5 (Figure 2). For each MAPK different isoforms are known. MAP kinases are activated upon tyrosine and threonine phosphorylation within a conserved Thr –Xxx-Tyr motif in the activation loop of the kinase domain. This phosphorylation is catalysed by dual-specificity kinases, MAPK kinases (MAPKK, MKK or MEK), which again are regulated by serine/threonine phosphorylation within a conserved motif in the activation loop, catalysed by MAPKK kinases (MAPKKK or

MEKK) (Figure 2). This three layer signalling cascade is activated by various upstream activators, including kinases and GTP-binding proteins.

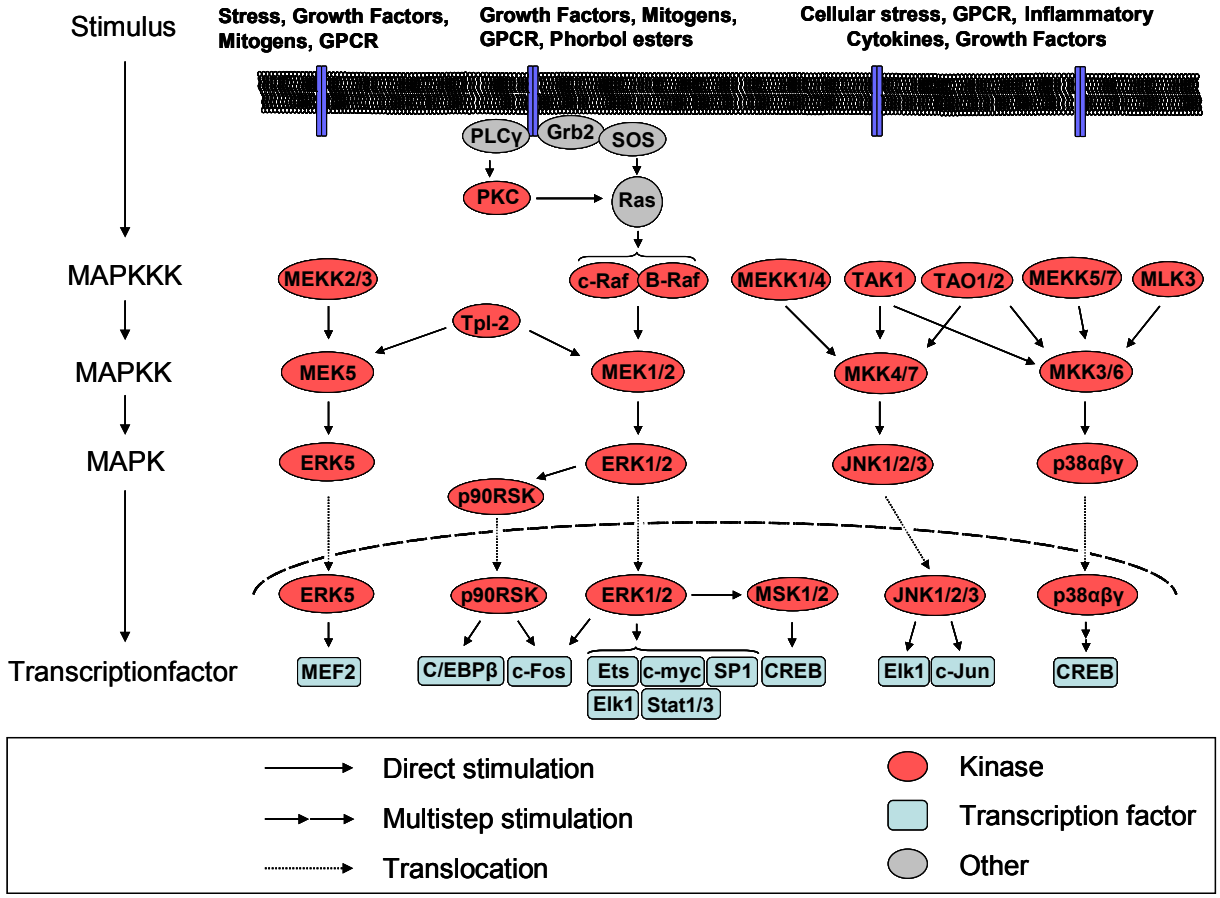


Figure 2. Schematic depiction of mitogen-activated protein kinase (MAPK) cascades. MAPK cascades are induced in response to various extracellular stimuli including among others growth factors and cytokines, phorbol esters, stress and G protein-coupled receptors (GPCR). Various intracellular upstream activators, including kinases and small GTP-binding proteins then activate a characteristic phosphorylation cascade in which a series of three protein kinases phosphorylate and activate one another. MAPKs serve as phosphorylation substrate for MAPK kinases (MAPKKs, MKKs or MEKs), which in turn are phosphorylated by MAPKK kinases (MAPKKK or MEKKs). Activated MAPKs (ERK, JNK, p38, ERK5) translocate to the nucleus, where they phosphorylate and activate various transcription factors. ERK1 and ERK2 further phosphorylate multiple cytoplasmic and cytoskeletal targets, including p90RSK, that also activates several transcription factors in the nucleus.

The Ras/Raf/MEK/ERK pathway has been particularly well studied, because mutations in the *ras* gene occur in many cancers. A wide variety of extracellular stimuli induce activation of this signalling cascade including growth factors, phorbol esters and cytokines. The canonical ERK MAPK cascade is stimulated by binding of a growth factor to its cell surface receptor which leads to the self-phosphorylation of the receptor. The src homology 2 (SH2) domain of an adaptor protein, Grb2, then binds to the phosphorylated tyrosine residue

of the receptor; Grb2 thereby links the activated receptor to the guanine exchange factor SOS that regulates the small GTPase Ras. This is followed by the sequential recruitment and activation of the kinases Raf, MEK, and ERK. ERK phosphorylates both cytoplasmic and nuclear substrates, including many enzymes, cytoskeletal proteins and transcription factors.

A number of Ras/ERK signalling-related proteins have been identified, such as scaffold proteins and inhibitor proteins of this pathway. These proteins provide variations in ERK signalling by modulating the duration, magnitude and subcellular compartmentalization of ERK activity (Marshall 1995; Ebisuya et al. 2005). Accumulating evidence suggests that such differences in ERK activity generate variations in signalling outputs that regulate cell fate dicisions.

ERK1 (also called $p44^{ERK1}$) and 2 (also called $p42^{ERK2}$) are highly conserved serine/threonine kinases that are 84% identical and share many functions (Lloyd 2006). They are activated by MEK1/2 via phosphorylation of both threonine and tyrosine residues within the Thr-Glu-Tyr (TEY) sequence in the activation loop. Substrates of ERK1/2 are members of the family of ~90-kDa ribosomal S6 kinases (p90RSK), mitogen- and stress-activated protein kinase 1/2 (MSK1/2), and transcription factors including Elk-1, c-Fos, c-Myc and Stat1/3 among others (Figure 2).

Regulation and cellular functions of the PI3K/Akt pathway

The phosphatidylinositol-3-kinase (PI3K)-Akt pathway is activated by several hormones, growth factors, cellular stress, signals derived from receptors for extracellular matrix molecules (e.g. integrins), and by activation of Ras. The induced signalling cascade regulates various cellular processes, such as metabolic regulation, cell growth, proliferation and survival (Cantley 2002; Franke et al. 2003; Brazil et al. 2004).

PI3K consists of regulatory (p85) and enzymatic (p110) subunits and exhibits both a protein kinase and a lipid kinase activity (Dhand et al. 1994; Hawkins et al. 2006). The serine-threonine protein kinase Akt (also known as protein kinase B, PKB) mediates main downstream effects of PI3K. Akt exists in three closely related enzymatic isoforms, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). They are similar both in structure and size and are thought to be activated by a common mechanism (Okano et al. 2000; Gonzalez and McGraw 2009). The three Akt proteins (in the following refered to as Akt) contain an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory region. Activation of Akt is a multi-step process involving both membrane binding and

phosphorylation. Briefly, activated PI3K catalyses the conversion of phosphatidylinositol-3,4-P_2 (PIP_2) to phosphatidylinositol-3,4,5-P_3 (PIP_3). Cytoplasmic Akt is recruited to the plasma membrane where it binds to PIP_3 through its PH domain (Franke et al. 1997), and is then phosphorylated by PI3K-dependent kinase-1 (PDK1) at the threonine residue 308, and by another PI3K-dependent kinase (PDK2) at the serine residue 473; phosphorylation at both sites is required for full induction of Akt activity (Figure 3). The tumour suppressor protein PTEN (*ph*osphatase and *ten*sin homolog deleted on chromosome 10) antagonizes the PI3K/Akt pathway by dephosphorylating PIP_3, resulting in decreased translocation of Akt to cellular membranes and subsequent downregulation of the activity of Akt. Active Akt phosphorylates a number of substrates both in the cytoplasm and in the nucleus which are involved in the regulation of key cellular functions such as cell growth and survival, glucose metabolism and protein translation. These targets include GSK3, BAD, caspase 9, forkhead and NF-κB transcription factors, mTOR, and p21 (Figure 3).

The PI3K/Akt pathway is commonly dysregulated in cancer (Liu et al. 2009). In this respect, Akt has been recognized as an essential link between the PI3K pathway and the mammalian target of rapamycin (mTOR) through the inactivation of the tuberous sclerosis complex (TSC) (Manning and Cantley 2003). The TSC complex, formed by hamartin (TSC1) and tuberin (TSC2), functions as a GTPase-activating protein (GAP) for Rheb, a Ras-related small GTP-binding protein that promotes the activation of mTOR (Garami et al. 2003; Inoki et al. 2003; Tee et al. 2003). Phosphorylation of TSC2 by Akt results in its inactivation, thereby promoting the accumulation of (active) Rheb-GTP and the induction of mTOR activity. mTOR then triggers the phosphorylation of key regulators of the cellular translation machinery, including ribosomal p70 S6 kinase (p70S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1) (Gingras et al. 1998; Aoki et al. 2001). Emerging evidence suggest that the TSC/mTOR pathway may play a critical role in the development of tumours dependent on Akt activation, highlighting its impact on the control of cell growth and proliferation and emphasizing its importance in human tumourigenesis (Luo et al. 2003; Majumder et al. 2004; Wislez et al. 2005).

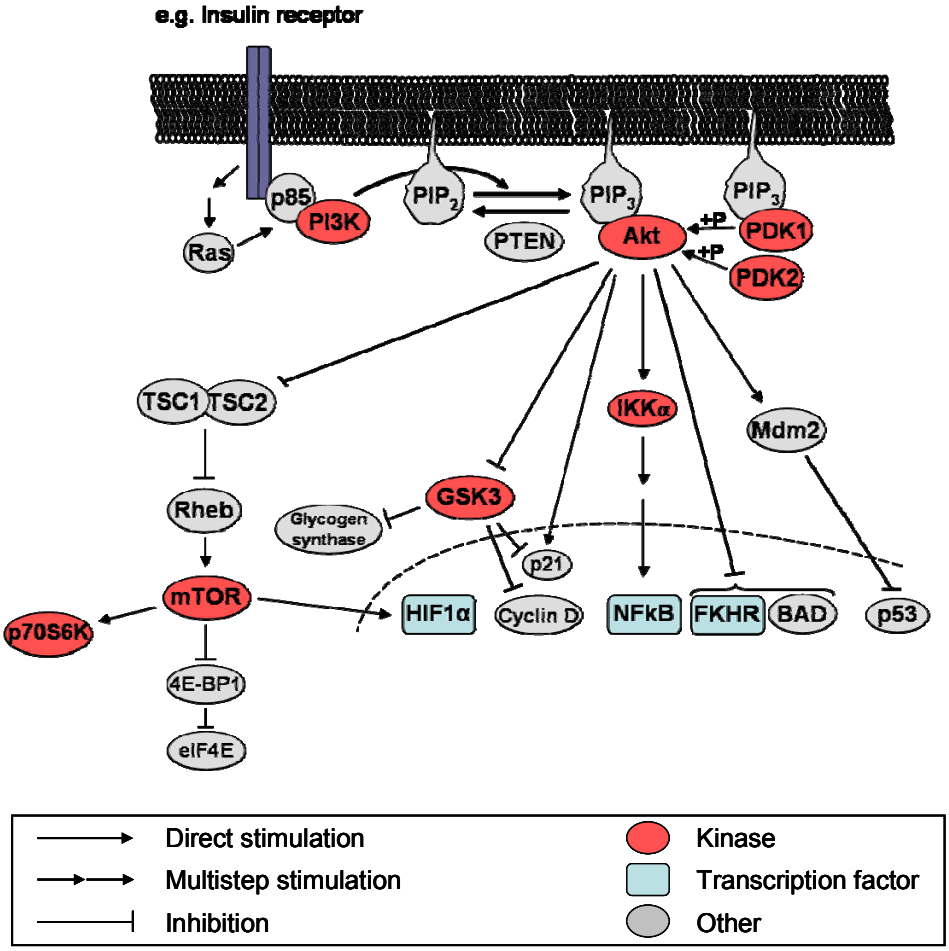


Figure 3. Schematic depiction of Akt signalling. Akt is the major downstream target of phosphatidylinositol-3-kinase (PI3K). PI3K activation is achieved by direct or indirect binding of its regulatory subunit p85 to an activated receptor. Alternatively, PI3K can be activated through active Ras. Activated PI3K then catalyses the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2), giving rise to the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the cell membrane. This process is antagonized by the tumour suppressor protein PTEN, which dephosphorylates PIP_3. PIP_3 serves as a docking platform for Akt, which is then activated by dual phosphorylation through PI3K-dependent kinases 1 and 2 (PDK-1 and -2). Active Akt directly phosphorylates and regulates – both positively and negatively – the activity of a wide variety of proteins and transcription factors, some of which are represented in this illustration.

The protein kinase Syk

Syk (spleen tyrosine kinase) is a small cytoplasmatic non-receptor protein tyrosine kinase (PTK) that is widely expressed in haematopoietic cells and has been detected in fibroblasts, epithelial cells, breast tissue, hepatocytes, neuronal cells, and vascular endothelial cells (Yanagi et al. 2001). In hematopoietic cells, including B- and T-cells, Syk is activated by binding of its tandemly arranged SH2 domains to immunoreceptor tyrosine-based activation motifs (ITAMs) in immune response receptors (Turner et al. 2000; Sada et al. 2001). In non-

hematopoietic cells, Syk has been demonstrated to be activated by integrins and G protein-coupled receptors (Yanagi et al. 1994; Wan et al. 1996; Gao et al. 1997; Woodside et al. 2001; Mocsai et al. 2002). Signalling pathways activated by Syk include MAPK (Ras-ERK, JNK) and PI3K-Akt cascades, as well as PKC and PLCγ1/2 (Jabril-Cuenod et al. 1996; Law et al. 1996; Jiang et al. 1998; Craxton et al. 1999; Kawakami et al. 2003; Ruschel and Ullrich 2004; Moon et al. 2005) (see also Figure 2). The kinase has been implicated in immunoreceptor signalling events that mediate diverse cellular responses including proliferation, differentiation, and phagocytosis. In non-hematopoietic tissue, Syk signalling has been implicated in several cellular mechanisms such as cell growth, proliferation, differentiation, and migration and is assumed to act as a tumour suppressor (Coopman and Mueller 2006).

1.3.2 Viruses and MEK-ERK1/2 and PI3K-Akt signalling cascades

Many viruses activate signalling pathways as a result of cellular response to the infection and virus modulation of its environment. For several viruses, including RNA and DNA viruses, it has been shown that they largely depend on intact MEK1/2-ERK1/2 signalling for efficient replication and protein expression (Rodems and Spector 1998; Andrade et al. 2004; Lim et al. 2005; Pleschka 2008), while activation of PI3K signalling is a strategy of several viruses to facilitate virus entry at early stages of infection and to suppress premature apoptosis at later stages of infection (Ehrhardt et al. 2006; Saeed et al. 2008; Soares et al. 2009).

1.3.3 Signalling pathways involved in KSHV infection and activation of the lytic cycle

Multiple upstream inducers of KSHV lytic cycle share the Raf/MEK/ERK/Ets-1 pathway to activate the expression of the immediate-early RTA protein and the same pathway also mediates spontaneous reactivation in BC-3 cells (Yu et al. 2007). TPA induction of KSHV lytic cycle is mediated by various pathways, including calcium-dependent calcineurin signalling, protein kinase C (PKC) δ pathway, and MAPK (ERK, JNK, and p38) pathways (Zoeteweij et al. 2001; Deutsch et al. 2004; Cohen et al. 2006; Ford et al. 2006; Xie et al. 2008). MEK/ERK, JNK and p38 mediate lytic cycle induction through their downstream target AP-1, which binds to the RTA promoter (Wang et al. 2004c; Xie et al. 2008). All three MAPK pathways are also required for productive lytic replication during primary infection

(Sharma-Walia et al. 2005; Xie et al. 2005; Pan et al. 2006). Epinephrine and norepinephrine efficiently reactivate KSHV from latency via *β*-adrenergic activation of the adenylyl cyclase/cAMP/protein kinase A (PKA) signalling pathway (Chang et al. 2005c). The role of NF-kB pathway in lytic replication remains unclear, but there is evidence suggesting that NF-kB is both a positive and negative regulator of lytic cycle: High level NF-kB inhibits immediate-early protein expression and, thus, helps to maintain the latent state of infection (Brown et al. 2003; Ye et al. 2008; Izumiya et al. 2009), while the expression of lytic genes required for efficient *de novo* infection seems to depend on NF-kB activity later on during the lytic cycle (Sgarbanti et al. 2004).

1.3.4 KSHV lytic proteins involved in signalling

Several lytic proteins of KSHV are capable of inducing signalling pathways. These proteins include homologues of cellular cytokines (vIL-6 (K2), vCCL-1 (K6), vCCL-2 (K4), vCCL-3 (K4.1)), a homologue of G protein-coupled IL-8 receptor (vGPCR, encoded by orf74), two integral membrane signalling proteins (K1 and K15), a serine/threonine protein kinase (vPK, encoded by orf36) and a homologue of the cellular glycoprotein CD200/OX2 (vOX2). Some of them have been shown to contribute to angiogenesis and/or the production of cellular mitogenic and survival factors, and thus, as a by-product, to KSHV pathogenesis (Schulz et al. 2002; Schulz 2006; Hartmann et al. 2009; Wen and Damania 2009). Some of these proteins may also directly modulate the lytic replication cycle. The virus-encoded kinase vPK/Orf36 was shown to activate the JNK MAPK pathway, and the cellular transcription factor c-Jun. Chemical inhibition of JNK activity resulted in the inhibition of late viral gene expression (Hamza et al. 2004). vPK/Orf36 localizes to KSHV replication/transcription complexes and interacts with, and thereby modulates, the transcriptional regulator K8/b-ZIP in productively infected cells (Izumiya et al. 2007). The KSHV-encoded chemokines vCCL-1 and vCCL-2 (see also below) promote KSHV lytic replication in endothelial cells and it was suggested that this activity involves pro-survival signalling via paracrine as well as autocrine mechanisms (Choi and Nicholas 2008). Additionally, two recently published studies have shown that vGPCR signalling via $G\alpha_q$-subunits of heterotrimeric G-protein positively regulates the lytic replication cycle (Bottero et al. 2009; Sandford et al. 2009), which may be due to the induction of increased promoter activity of Orf50/RTA and other early lytic genes (Bottero et al. 2009) (see also below).

Viral cytokines

vCCLs

The viral CC-chemokines of KSHV, vCCL-1, vCCL-2 and vCCL-3, are encoded by orfs K6, K4 and K4.1, respectively (Russo et al. 1996; Neipel et al. 1997; Nicholas et al. 1997b). While vCCL-1 and vCCL-2 are structurally most closely related to CCL3 and CCL4, vCCL3 has no clear cellular counterpart but is related to CCL2. Functionally, these viral chemokines are distinct from their cellular homologues. vCCL-1 and vCCL-2 are agonists for CCR8, the receptor for CCL1 (I-309), and vCCL-2 has been reported also to interact productively with CCR3 (Boshoff et al. 1997; Dairaghi et al. 1999; Endres et al. 1999). vCCL-3 signals through CCR4 and XCR1 (Stine et al. 2000; Luttichau et al. 2007). Additionally, vCCL-2 binds as a neutral (non-signalling) ligand to a variety of CC- and CXC receptors and to XCR1, thereby acting as an antagonist for cognate ligand signalling (Kledal et al. 1997; Shan et al. 2000; Luttichau et al. 2001).

vCCL-1 as well as vCCL-2 were shown to activate ERK and Akt signalling pathways in endothelial cells (Choi and Nicholas 2008). A key property shared by both viral chemokines is their anti-apoptotic activity, demonstrated in dexamethasone-treated PEL cells and serum-deprived primary and telomerase-immortalized human microvascular endothelial cells (Liu et al. 2001). CCR8-mediated anti-apoptotic signalling has been noted in murine thymic lymphoma cells, BW5147, and Ras/MAPK signalling has been implicated in this function (Louahed et al. 2003; Spinetti et al. 2003). CCR8 also mediates vCCL-1 and vCCL-2 induced anti-apoptotic signalling in endothelial cells, and it was suggested that this process involves the suppression of the proapoptotic Bcl-2 family protein Bim (Choi and Nicholas 2008). In addition to Bim, it was shown that Bcl-2 is targeted by v-chemokine signalling, proposing that vCCL-1 and vCCL-2 may contribute to prevention of stress-induced apoptosis (Choi and Nicholas 2008). The v-chemokines are able to mediate pro-survival signalling in a paracrine manner. Thus, their expression in a minority of lytically infected cells within a predominantly latent cell population would allow for their contribution to viral pathogenesis via promoting the survival of latently infected cells and thereby cooperating with latency functions.

Recent evidence suggests that vCCL-1 and vCCL-2 also directly promote viral lytic replication via both autocrine and paracrine-mediated signalling (Choi and Nicholas 2008). Other likely biological functions of the v-chemokines may include immune evasion and virus dissemination via Th2 polarization and lymphocyte/monocyte recruitment mediated by their

various agonist and neutral agonist activities. Several studies suggest a role in angiogenesis, and it is known that vCCL-1 can induce VEGF production in latently infected PEL cell lines (Boshoff et al. 1997; Stine et al. 2000).

vIL-6

Cellular (or human) IL-6 (hIL-6), a multifunctional cytokine that acts on a wide variety of cell types, had been implicated as a contributing factor in KS and MCD prior to the discovery of KSHV. KS lesions showed elevated IL-6 levels and KS cells in culture were reported to show increased proliferation rates in response to IL-6; levels of serum IL-6 were found to correspond with disease severity in MCD patients and to decrease following excision of diseased lymph nodes (Yoshizaki et al. 1989; Miles et al. 1990; Burger et al. 1994; Ishiyama et al. 1994). hIL-6 is a known mitogen and survival factor for B-cells, and STAT3, the main downstream effector of IL-6 signalling, is found at elevated levels in many cancers.

The IL-6 homologue encoded by orf K2 shares only 25% amino acid identity with its human counterpart but is structurally highly conserved and activates the same downstream signalling pathways as hIL-6 (Molden et al. 1997). Both human and viral (v) IL-6 activate signalling through the JAK/STAT pathway, specifically activating STAT3 transcriptional activity, and induce Ras/MAP kinase pathways (Molden et al. 1997; Osborne et al. 1999; Chatterjee et al. 2002). To activate signalling in a cell, human IL-6 requires gp130, a surface receptor common to many cytokines, and gp80, an IL-6 specific component. In contrast to its cellular homologue, vIL-6 can signal independently of gp80 (Burger et al. 1998). This makes the viral IL-6 a more promiscuous activator of signalling since gp130 is present on many cell types while gp80 is limited to specific cells (Heinrich et al. 2003). However, gp80 can be incorporated into vIL-6-induced signalling complexes, and may contribute to complex stability and influence vIL-6 signal transduction (Wan et al. 1999; Boulanger et al. 2004; Chen and Nicholas 2006; Hu and Nicholas 2006).

Another major difference between vIL-6 and hIL-6 is that the viral cytokine is very inefficiently secreted, retained largely within the endoplasmic reticulum (ER), where it is able to transduce signalling intracellularly (Meads and Medveczky 2004; Kovaleva et al. 2006). hIL-6 cannot signal in the ER, even when targeted to this compartment (Chen et al. 2009).

vIL-6 was shown to be expressed with early kinetics during lytic replication in PEL cells and is directly induced by the lytic switch protein RTA (Sun et al. 1999; Jenner et al. 2001; Paulose-Murphy et al. 2001b; Song et al. 2003). However, vIL-6 also can be expressed

independently of other lytic genes (Nicholas et al. 1997c; Chatterjee et al. 2002; Chiou et al. 2002; Chang et al. 2005a). The cytokine has been found to be expressed in a higher percentage of cells than other lytic proteins in KS and PEL tissues and together with latency-associated nuclear antigen (LANA) in individual cells of KS and MCD (Moore et al. 1996; Cannon et al. 1999; Brousset et al. 2001; Chiou et al. 2002), and Chang et al. (2005) identified vIL-6 as one of several lytic genes that are specifically induced via Notch signalling, independently of RTA (Chang et al. 2005a). vIL-6 may therefore function as an autocrine proliferation and survival factor even in the absence of the full lytic replication cycle. In fact, it was shown that PEL cells grow with reduced kinetics and display higher rates of aopotosis upon shRNA-mediated depletion of vIL-6 when compared to non-transduced cells (Chen et al. 2009). Thus, intracellular signalling by vIL-6 may be functional in the autocrine promotion of proliferation and survival of latently infected PEL cells.

vIL-6 could potentially have a role in neoplasia also via paracrine signalling. Effects could be exerted directly, for example via mitogenic and survival signalling in surrounding latently infected and uninfected cells, and also indirectly via the induction of cellular cytokines or other proteins, such as receptors. Examples might include hIL-6, VEGFs and other angiogenic factors, which have been demonstrated to be induced by vIL-6 in some cell types (Aoki et al. 1999; Mori et al. 2000; Liu et al. 2001), as well as growth factor and cytokine receptors. Inflammatory and angiogenic cytokines are known to play a role in KS, and VEGF and IL-6 appear to be important for PEL and MCD (Yoshizaki et al. 1989; Burger et al. 1994; Ishiyama et al. 1994; Aoki and Tosato 1999). *In vitro*, both vIL-6 and hIL-6 support PEL cell growth (Foussat et al. 1999; Jones et al. 1999).

Membrane proteins

The viral G protein-coupled receptor (vGPCR)

The vGCPR of KSHV is a constitutively active (ligand-independent) G protein-coupled receptor that is most related structurally to the CXCL8 (IL-8) receptor CXCR2 (Russo et al. 1996). The receptor is promiscuous in its Gα coupling, activating i, q and 12/13 classes of Gα proteins that lead to a broad range of signalling pathway activation (Couty et al. 2001; Shepard et al. 2001; Cannon et al. 2003; Liu et al. 2004). Kinases and transcription factors activated by vGPCR include MAPK (Erk, p38, Jnk), Akt, NFAT, CREB, NF-κB, AP-1 and HIF-1α (Figure 4), and these are relevant to promotion of cell proliferation and survival and to

induction of angiogenic responses via cytokine gene activation (Arvanitakis et al. 1997; Bais et al. 1998; Sodhi et al. 2000; Montaner et al. 2001; Pati et al. 2001; Schwarz and Murphy 2001; Shepard et al. 2001) (see below). Although constitutively active, vGPCR activity can be modulated both positively and negatively by cellular chemokines such as agonists GROα and IL-8 and inverse agonists IP-10 and SDF-1α (Geras-Raaka et al. 1998a; Geras-Raaka et al. 1998b; Gershengorn et al. 1998). In addition, the KSHV-encoded chemokine vCCL-2 acts as an inverse agonist (Geras-Raaka et al. 1998a). Several 'neutral' ligands bind to vGPCR without affecting signal transduction; these include CC-ligands CCL1 (I-309), CCL5 (RANTES) and CCL3 (MIP-1a), and CXC-ligands CXCL7 (NAP-2), CXCL5 (ENA-78) and CXCL4 (PF-4) (Arvanitakis et al. 1997; Rosenkilde et al. 1999). The biological relevance of the vGPCR-chemokine functional and neutral interactions is unresolved. It is known, however, that agonist stimulation of vGPCR leads to a switch to predominantly $G\alpha_q$-initiated signalling, and this is likely to be relevant to KS pathogenesis (see below).

Of all the KSHV-encoded proteins vGPCR is probably the one for which there is most evidence for a role in virus neoplasia, specifically KS. Ectopic expression of vGPCR in NIH3T3 cells causes oncogenic transformation and leads to the upregulation of VEGF (Bais et al. 1998). It has been suggested that vGPCR might participate in KS pathogenesis through autocrine and paracrine mechanisms by inducing the secretion of VEGF and by maintaining an angiogenic phenotype by signalling via a VEGFR2/VEGF loop (Bais et al. 2003).

Investigations on vGPCR transgenic mice identified the viral receptor as an inducer of KS-like lesions (Yang et al. 2000; Holst et al. 2001; Montaner et al. 2003), and it was shown that chemokine stimulation of vGPCR is necessary for efficient induction of KS tumours in the murine model (Holst et al. 2001). Additionally, Akt-mediated signalling has been identified as important for murine sarcomagenesis in transgenic animals and for tumorigenicity of vGPCR-expressing endothelial cells in recipient athymic mice (Sodhi et al. 2004). In this respect, activation of mTOR via Akt-mediated inactivation of the negative regulator TSC2 appears to be centrally important to vGPCR-induced endothelial cell growth in culture and to tumorigenesis in the allograft model (Sodhi et al. 2006).

Recently, two independent studies provided evidence for a functional relevance of vGPCR in KSHV biology: Bottero *et al.* (2009) showed that vGPCR signalling activates the Orf50/RTA promoter in a Sp1 and Sp3 transcription factor-dependent manner and decreases histone deacetylase (HDAC) activity. This activity was demonstrated to involve the $G\alpha_q$ and $G\alpha_{12}$ subunits of heterotrimeric G-Protein (Bottero et al. 2009). In agreement with these findings, Sandford *et al.* (2009) suggest that vGPCR promotes KSHV lytic replication

through $G\alpha_q$-mediated activation of MAPK signalling cascades. Thus, vGPCR signalling may facilitate sustained expression of Orf50/RTA during the lytic replication cycle, which may lead to continued expression of lytic cycle genes and ultimately to the release of new infectious virions.

vOX2

The KSHV vOX2 protein shows 36% identity with a cellular protein of the group of leukocyte glycoproteins, called CD200 or OX2. The protein is expressed early during lytic replication from a bicistronic mRNA containing the orfs 74 (vGPCR) and K14 (vOX2) (Kirshner et al. 1999; Talbot et al. 1999; Jenner et al. 2001; Paulose-Murphy et al. 2001).

CD200-like sequences have been identified in genomes of several evolutionary diverse viruses, which include gamma- and betaherpesviruses, yaba- and leporipoxviruses, and small viruses such as duck adeno virus (Foster-Cuevas et al. 2004). This apparently independent capture of the host CD200 gene indicates a strong selective advantage conveyed by members of this family. CD200 is expressed on the surface of a wide variety of cell types including endothelial cells, B lymphocytes, T cells, and neuronal cells (Wright et al. 2001) and provides immunomodulatory functions. Signal delivery of CD200 occurs through binding to a CD200 receptor (CD200R). This interaction results in inhibition of CD200R downstream signalling (including ERK, JNK and p38 MAPK activity) in monocytes and thus limiting their activation (Hoek et al. 2000; Zhang and Phillips 2006). As CD200R expression is restricted to cells of the myeloid lineage, it is suggested that CD200-CD200R interaction acts locally to modulate inflammatory-cell activity at sites of infection.

However, studies on vOX2 function have so far pointed in different directions. While Chung et al. (Chung et al. 2002) demonstrated that vOX2 stimulates inflammatory cytokine production including IL-1β, monocyte chemoattractant protein 1 (MCP-1), and TNF-α from primary monocytes, macrophages and dendritic cells, more recent studies by two groups show the opposite, suggesting a similar function for vOX2 as for its cellular homologue CD200 (Foster-Cuevas et al. 2004; Rezaee et al. 2005). Foster-Cuevas et al. (2004) observed that vOX2 interacts with almost identical kinetics with human CD200R as shown for CD200, and cells expressing vOX2 or CD200 on their surface were able to inhibit secretion of TNF-α from activated macrophages. In KSHV biology and tumorigenesis the local inhibition of macrophages, which are abundant within KSHV-infected tissue, might be important to prevent a host response against lytically-infected cells. Several lytic proteins, of which,

notably, the vOX2 co-expressed vGPCR is of major importance, have been shown to induce paracrine effects on latently and uninfected cells that may have a role in viral maintenance and spread.

Rezaee et al. (2005) confirmed the immunosuppressive function of vOX2 by demonstrating that a fusion protein of vOX2 and the Fc domain of human immunoglobulin G1 (vOX2:Fc) downregulated the activity of neutrophils directly and indirectly via inhibiting the production of the potent neutrophil chemotactic proteins IL-8 and MCP-1 from monocytes and macrophages *in vitro*. *In vivo* studies in a carrageen-mouse model of acute inflammation demonstrated, that vOX2:Fc reduced cell infiltration (Rezaee et al. 2005), suggesting that vOX2 may modulate chemotaxis.

K1

The K1 open reading frame is located at the left end of the KSHV genome. This position is equivalent to that of genes in other γ-herpesviruses, which are known to encode proteins involved in signalling and transformation. These include the herpesvirus saimiri (HVS) transforming protein (STP) (Murthy et al. 1989), the latent membrane protein-1 (LMP-1) of Epstein Barr virus (Kaye et al. 1993), and the R1 protein of rhesus monkey rhadinovirus (RRV) (Damania et al. 1999). Like these proteins, K1 activates intracellular signalling cascades and induces proliferative, anti-apoptotic and inflammatory responses.

Structurally, K1 is a type-I transmembrane protein of 46 kDa. Its extracellular domain (228 aa) features regional homology with the immunoglobulin (Ig) superfamily, and consists of two conserved regions (C1 and C2) as well as two highly variable regions (V1 and V2). The cytoplasmic tail (38 aa) contains an ITAM (Lee et al. 1998a), analogous to motifs within the Ig*α* and Ig*β* chains of the B-cell receptor (BCR). Although the K1 cytoplasmic tail is highly variable among different KSHV isolates around the world, the Src homology 2 (SH2) binding motifs comprising the ITAM are always conserved (Zong et al. 1999).

Unlike the BCR, K1 signalling activity is constitutive in the absence of exogenous ligands, presumably through multimerization of its cysteine-rich extracellular domain, which then results in phosphorylation of the tyrosine residues in the ITAM. The ITAM is necessary for phosphorylation and activation of SH2-containing signalling proteins, such as Syk, Lyn, Cbl, Vav, and the p85-subunit of phosphatidylinositol-3'-OH kinase (PI3K) as well as for K1-mediated upregulation of PI3K pathway signalling, and NF-κB and NFAT promoter-dependent activity (Lee et al. 1998a; Lagunoff et al. 1999; Prakash et al. 2002; Tomlinson and

Damania 2004; Prakash et al. 2005). Activation of PLCγ2, RasGAP, SH2 domain-containing protein tyrosine phosphatase 1/2 (SH2-PTP1/2), and Grb2 has been observed upon stimulation with a monoclonal antibody directed against the extracellular domain of K1 (Lee et al. 2005).

K1 is expressed as an early gene during the KSHV lytic cycle (Sarid et al. 1998; Jenner et al. 2001; Paulose-Murphy et al. 2001; Bowser et al. 2002; Fakhari and Dittmer 2002; Nakamura et al. 2003). Although low level expression has been reported in latently infected PEL cell lines (Samaniego et al. 2001), this could have been due to a small proportion of cells in these cultures that undergo productive (lytic) replication. K1 expression has been observed in tumour tissues of KS and MCD (Samaniego et al. 2001; Lee and Merchant 2003) and in KS cells expressing K1, the induction of secretion of inflammatory cytokines implicated in KS lesion formation, such as IL-6, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF), was observed (Samaniego et al. 2001; Prakash et al. 2002).

First evidence for the ability of K1 to transform cells emerged from studies in rodent cells: K1 expression transformed rodent fibroblasts *in vitro*, and recombinant herpesvirus saimiri strains, in which the STP had been replaced with the K1 gene, induced lymphomas *in vivo* (Lee et al. 1998b). Further experiments indicated that a minority of K1 transgenic mice develop tumours with features of a spindle-cell sarcomatoid tumour and malignant plasmaplastic lymphomas (Prakash et al. 2002). In these mice, IL-12 levels were severely decreased and basic fibroblast growth factor (FGF2) expression was upregulated in lymphocytes and tumours (Prakash et al. 2002). Interestingly, FGF2 has been described as an autocrine growth factor for endothelial cells that promotes growth and angiogenesis of AIDS-KS cells (Samaniego et al. 1995).

Lymphocytes isolated from transgenic mice expressing K1 showed constitutive activation of transcription factors NF-κB and Oct-2 as well as enhanced Lyn kinase activity (Prakash et al. 2002). Biochemical and *in vitro* studies confirmed that NF-κB-dependent-promoter activity is mediated by K1, and for B-cells, Prakash *et al*. (2002) demonstrated that this activation further depends on the interaction of the ITAM with Lyn kinase. Additionally, the transcription factor NFAT was identified as a downstream target of K1-induced PI3K, Syk and PLCγ signalling (Lee et al. 1998b; Lagunoff et al. 1999; Samaniego et al. 2001; Lee et al. 2005; Prakash et al. 2005), and Wang *et al*. (Wang et al. 2004b) provided evidence that the transcription factor AP-1 is activated by K1-mediated activation of PI3K-MAPK signalling. NF-κB and NFAT are known to increase the expression of cytokines associated with inflammation and proliferation, and Lee *at al*. (2005) identified numerous inflammatory

cytokines (MDC, IL-8, IL-10, VEGF, IL-1α/β, and RANTES), which all contain NF-κB and/or NFAT and/or AP-1 binding sites in their promoter sequences and were induced by K1-mediated signal transduction. K1 may therefore activate uninfected or latently infected cells in a paracrine manner through activation of NF-κB-, NFAT- and AP-1-dependent promoters and secretion of inflammatory cytokines. Co-expression of the HIV-1-transactivation gene tat with K1 results in an additive effect on NF-κB-dependent transcription, suggesting cooperative signal activation of K1 and Tat. It has therefore been postulated that these two proteins might converge to reach an enhanced level of inflammation that may underlie progressive KS in AIDS patients (Prakash et al. 2000).

K1 utilizes several mechanisms to prevent cells from undergoing apoptosis and to support cell proliferation. These processes are mediated by increased anti-apoptotic and survival signalling via direct inhibition of proteins involved in apoptosis, or by expression and secretion of growth factors such as VEGF. A role for K1 in the survival of KSHV-infected cells was first described by Lee et al. (Lee et al. 2000), who found out that the N-terminal extracellular domain of K1 interacts with the μ (heavy) chains of BCR complexes to retain them in the endoplasmatic reticulum (ER), resulting in downregulation of their surface expression. Later, Tomlinson & Damania (2004) demonstrated that K1 mediated-upregulation of the PI3K signalling cascade in B-cells results in the phosphorylation of Akt and PTEN, which further leads to increased phosphorylation, and thereby inactivation, of forkhead (FKHR) transcription factors. This would enhance cell survival signals and protect cells from apoptosis. Wang *et al.* (Wang et al. 2006) showed, that K1 can immortalize primary human umbrilical vein endothelial cells (HUVEC) in culture, and suggested that this is a consequence of the activation of the PI3K pathway. In addition to the PI3K downstream signals in B-cells, this group observed K1-induced activation of mTOR and inactivation of GSK-3 and Bad, which are all events indicative of cell survival. Furthermore, Wang *et al*. (2007) showed that Fas is an interaction partner of K1 *in vitro* and *in vivo* (Wang et al. 2007b), suggesting that K1 may directly inhibit Fas-mediated apoptosis to promote cell survival.

Inhibition of pro-apoptotic factors within the cell represents a direct effect of K1 on the infected cell, which may ensure host cell survival during the lytic cycle of KSHV for efficient virus production. In addition, paracrine mechanisms apparently mediated by K1 may also contribute to KSHV maintenance and pathogenesis. It has been demonstrated, that K1 induces the expression of VEGF, matrix metalloproteinase-9 (MMP-9) and basic fibroblast growth factor (FGF2) (Prakash et al. 2002; Wang et al. 2004b), and VEGF is secreted in the

surrounding matrix (Wang et al. 2004b; Prakash et al. 2005). Angiogenesis is an essential feature of KS, and VEGF and FGF2 have been described to be involved in this process (Samaniego et al. 1998; Sgadari et al. 2002). Thus, the signalling activity of K1 may play an important role in VEGF-mediated angiogenesis in KS tumours.

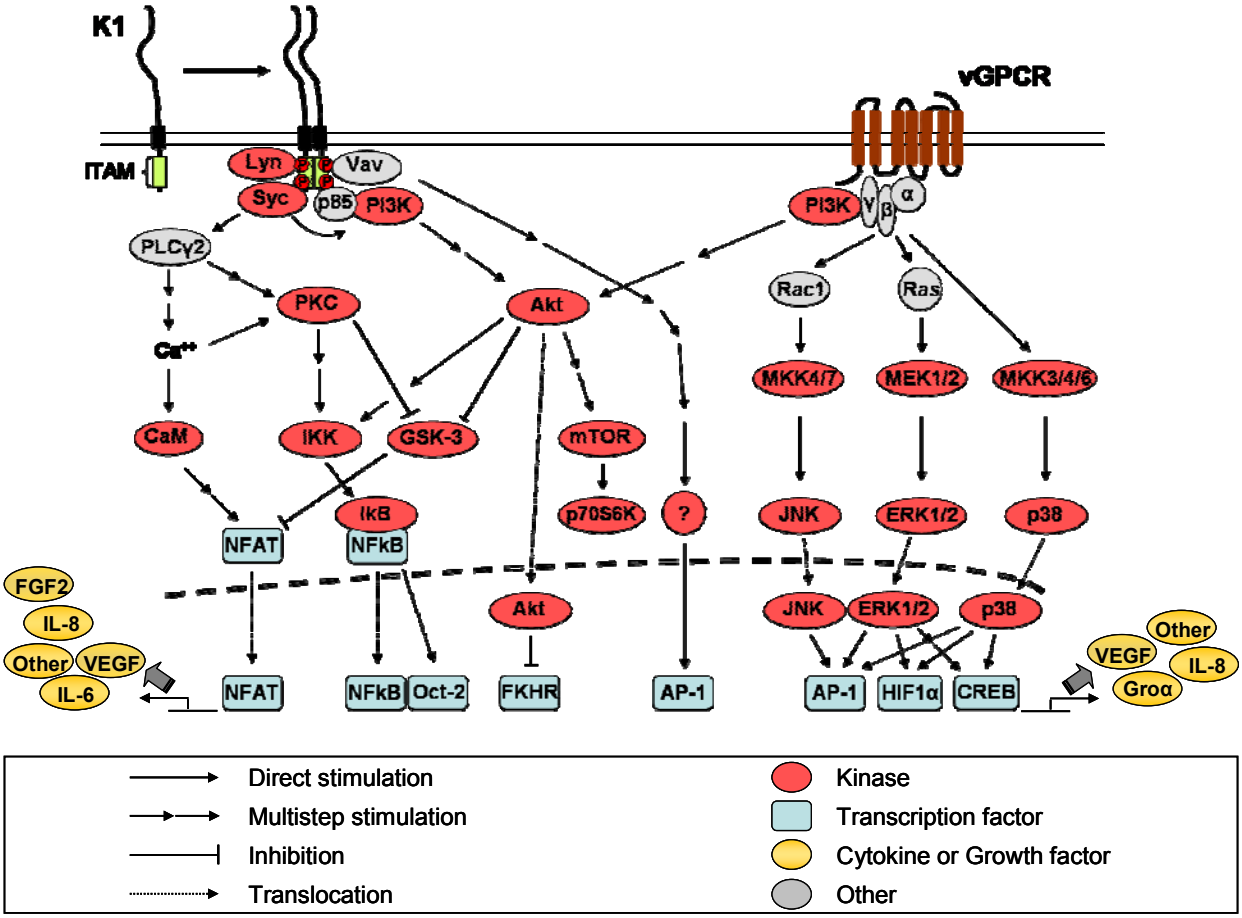


Figure 4. Schematic representation of the intracellular signalling pathways engaged by the KSHV transmembrane proteins K1 and vGPCR. Both K1 and vGPCR activate the PI3K/Akt cascade, AP-1 and NF-kB transcription factors. In addition, K1 activates members of the Src kinase family and Syc kinase in an ITAM-dependent manner, while vGPCR activates MAPK cascades. Their downstream effects include the activation of a broad range of inflammatory and angiogenic cytokines, particularly VEGF and IL-8 (both K1 and vGPCR), IL-6 (K1), and Gro*α* (vGPCR).

Whether K1 plays a role in lytic replication cycle is controversial: Overexpression of a CD8/K1 chimera suppressed TPA-induced lytic replication in B-cells (Lee et al. 2002). In contrast, a dominant-negative K1 mutant lacking the ITAM motif reduced RTA-induced lytic replication in B-cells up to 80 percent (Lagunoff et al. 2001). Furthermore, it was shown that K1 inhibits NF-κB activation induced by latently expressed viral FLICE inhibitory protein (vFLIP)/K13 and Orf75 when transiently co-expressed in HEK293-T cells (Konrad et al.

2009). In consideration of the finding by others that NF-kB suppresses lytic replication (Brown et al. 2003) the authors suggest that K1 may promote the productive replication cycle.

K15

Open reading frame K15 is located at the right end of the KSHV genome, adjacent to the terminal repeat region. Multiple and alternative splicing of its eight exons gives rise to a family of proteins that share a common C-terminal cytoplasmic domain but vary in the number of membrane anchor domains. So far, only the largest K15 protein with predicted 12 transmembrane domains linked to the cytoplamic region has been shown to activate signalling pathways within host cells. Signalling motifs found in the cytoplasmic tail include two potential SH2- and one SH3-binding site as well as a TRAF-binding region (Glenn et al. 1999; Choi et al. 2000; Brinkmann et al. 2003). These motifs are conserved among the two highly divergent P (predominant) and M (minor) genotypes of K15 (Glenn et al. 1999; Poole et al. 1999), suggesting the conservation of associated functional properties.

Biochemical studies with a CD8-K15 (C-Tail) chimera expressed in KSHV-negative BJAB-cells performed by Choi *et al*. (2000) showed, that the tyrosine residue (Y_{481}) within the putative SH2-B domain Y_{481}EEV is the major site of phosphorylation by cellular tyrosine kinases and that this tyrosine phosphorylation is independent of antibody stimulation.

Structurally and functionally, K15 shows similarities to LMP1 and LMP2A of Epstein-Barr virus (Nicholas 2003; Brinkmann and Schulz 2006). Like LMP1, K15 interact with TRAF-1, TRAF-2, and TRAF-3, activates the MAP kinases ERK2 and JNK as well as the transcription factors NF-κB and AP-1 (Glenn et al. 1999; Brinkmann et al. 2003). Reminiscent of LMP2A, K15 interacts with members of the Src family of protein tyrosine kinases, which phosphorylate the tyrosine residue Y_{481} in a YEEV SH2-binding domain (Brinkmann et al. 2003). In a further parallel to LMP2A, K15 is able to inhibit B-cell receptor signalling, as shown by the suppression of tyrosine phosphorylation and intracellular calcium mobilization in the B-cell line BJAB (Choi et al. 2000). Inhibition of BCR signalling involves the SH2-B (Y_{481}EEV) and the SH3-B domains, as mutants with point mutations in these motifs do not suppress mobilization of intracellular free calcium upon anti-IgM stimulation (Choi et al. 2000). The second putative SH2-B motif (Y_{431}SI) is not significantly phosphorylated and may therefore not serve as a SH2-B domain but provide other functions.

K15 is expressed during the lytic cycle of KSHV with low expression levels observed in unstimulated PEL cells (Glenn et al. 1999; Poole et al. 1999; Choi et al. 2000; Brinkmann et

al. 2007). This is reminiscent of the K1 expression pattern (see above), and clustering of gene expression array data grouped K15 together with K1, indicating a similar expression pattern for these two proteins (Jenner et al. 2001; Paulose-Murphy et al. 2001; Nakamura et al. 2003). It was demonstrated that TPA-induced lytic infection and the RTA protein activate K15 promoter elements (Brinkmann et al. 2003; Wong and Damania 2006). Furthermore, a 45 kDa K15-derived protein has been shown to be expressed after the activation of the lytic replication cycle in HEK293 cells carrying a recombinant viral genome (Brinkmann et al. 2007). These findings indicate a predominant role for K15 during the KSHV lytic cycle.

K15 is capable of inducing the expression of multiple cytokines, including in particular IL-8, IL-6, CXCL3 (Gro-γ), which have been shown to play a role in KSHV-associated pathogenesis (Brinkmann et al. 2007). While strongly inducing the expression and secretion of the angiogenic chemokine IL8, K15 does not, unlike K1, show a marked effect on VEGF expression, but activates a number of VEGF target genes directly, e.g. dscr1 and cox-2 (Brinkmann et al. 2007). Expression of the majority of K15-induced genes depends on an intact YEEV SH2 binding site, underlining the importance of this motif, and presumably the recruitment of members of the src kinase family, for K15-mediated signalling (Brinkmann et al. 2003; Brinkmann et al. 2007).

Therefore, as suggested for K1, K15 signalling may contribute to the hypothesized paracrine effects of lytically infected cells in KS lesions on latently infected or uninfected neighbouring cells. In addition, K15 may also have an antiapoptotic role. Sharp *et al*. (2002) observed that K15 interacts with HAX-1 (HS1 associated protein X-1), a protein with anti-apoptotic functions. A K15 protein and HAX-1 were shown to co-localize in the ER and, consistent with apoptotic regulatory activity, in mitochondria (Sharp et al. 2002). However, to date there is no functional evidence that K15 association with HAX-1 affects cell survival. In addition, K15 has recently been shown to upregulate the expression of anti-apoptotic genes such as *tnfaip3/A20*, *bf*, *birc3*, *birc2*, and *bcl2a1*, of which, notably, the last shows sequence homologies with HAX-1 (Brinkmann et al. 2007). Another interesting factor shown to be upregulated through K15-induced signalling pathways is Cox-2 (Brinkmann et al. 2007). Cox-2 expression after KSHV infection had already been reported (Naranatt et al. 2004) and its association with other herpesviruses, including EBV, CMV and HHV-6, has been observed (Speir et al. 1998; Murono et al. 2001; Janelle et al. 2002; Zhu et al. 2002). Cox-2 may contribute to viral replication by increasing prostaglandin E_2 synthesis, which has been shown to increase multiple gene products of the KSHV related γ-herpesvirus MHV68 (Symensma et al. 2003).

Table 1. Characteristics and functional properties of some important KSHV lytic signalling proteins.

Characteristics	Transmembrane proteins			Serine-threonine protein kinase	Viral homologues of cellular cytokines		
	K1	K15	vGPCR/Orf74	vPK/Orf36	vIL-6/K2	vCCL-1/K6	vCCL-2/K4
Expression kinetics	early lytic	early lytic	early lytic	early lytic (3.6 kb transcript) early-late lytic (4.2 kb transcript)	early lytic	early lytic	early lytic
Activated by Orf50/RTA (molecular mechanism of transactivation)	yes (Oct-1)	yes	yes (RBP-Jk)	not known; induced by hypoxia	yes (direct mechanism); also RTA-independent regulation via Notch signalling pathway	yes (RBP-Jk)	not known
Induction of the activation of	PI3K-Akt, Syk, Lyn, Cbl, Vav, RasGAP, PLCγ2, NFAT, NF-kB, AP-1, Oct-2	MAPK (ERK, JNK), NF-kB, AP-1	MAPK (ERK, p38, JNK), Akt, JAK2, NFAT, CREB, NF-kB, AP-1, HIF-1α, SP-1/3	JNK, MKK4, MKK7	JAK1, STAT1/3, Ras-MAPK	Agonist of CCR8; Akt, ERK	Agonist of CCR8 and CCR3; Akt, ERK

Characteristics	Transmembrane proteins			Serine-threonine protein kinase	Viral homologues of cellular cytokines		
	K1	K15	vGPCR/Orf74	Orf36/vPK	vIL-6 (K2)	vCCL-1 (K6)	vCCL-2 (K4)
Induction of the expression of	VEGF, FGF-2, GM-CSF, MMP-9, IL-6, IL-8, IL-10, IL-12, IL-1α/β; Rantes	IL-8, Dscr-1, Cox-2	VEGF, IL-6, Gro-α, MIP-1α/β	not known	hIL-6, VEGF	VEGF	not known
Role in lytic replication cycle	yes, but both positive and negative regulation of KSHV lytic replication have been described	not known	yes; positive regulator	yes; involved in lytic DNA replication	no	yes; promotes survival signalling	yes; promotes survival signalling

AP-1, activator protein 1; CCR, CC-motif chemokine receptor; Cox-2, cyclooxygenase-2; CREB, cAMP-response element binding protein; Dscr-1, Down syndrome critical region gene 1; ERK, extracellular signal-regulated kinase; FGF-2, basic fibroblast growth fctor; GM-CSF, granulocyte-macrophage colony-stimulating factor; Gro-α, growth regulated oncogene alpha, HIF1α, hypoxia induced factor 1 alpha; hIL-6, human interleukine 6; IL, interleukine; JAK, janus kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MKK, MAPK kinase (also called MEK); MEF-2, myocyte enhancer factor 2; MIP-1, macrophage inflammatory protein 1; MMP, matrix metalloproteinase; MSK1, mitogen- and stress-activated protein kinase 1; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; Oct-2, octamer binding protein 2, PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase C-gamma; Rantes, regulated upon activation, normal T cell expressed and secreted (also known as CCL5), RasGAP, Ras GTPase activating protein; SP-1, specificity protein 1; STAT, signal transducers and activators of transcription; Syk, spleen tyrosine kinase; VEGF, vascular endothelial growth factor.

1.4 Protein kinase inhibitors

Using protein kinase inhibitors to study cellular signalling pathways

Protein kinase inhibitors represent a powerful tool to study the physiological substrates and cellular functions of their target kinases and related signalling pathways. Since their discovery in the early 1980s (Cohen 2002), a broad spectrum of small cell permeable inhibitors has been developed that exhibit a relatively high degree of specificity for a particular protein kinase. The advantage of using these chemical compounds for the study of signalling pathways is that (i) they can be used simply and rapidly to assess the physiological roles of protein kinases in normal cells and tissue, and (ii) they inhibit the endogenous protein kinases, avoiding the need for overexpression of dominant-negative and constitutively active protein kinases, which can cause the specificity of signalling to break down.

Two widely used inhibitors of the MEK-ERK1/2 signalling cascade are PD98059 and U0126 (see also Table 3 in Results I). Both inhibitors bind to (inactive) MEK1 and thereby prevent its activation by upstream kinases such as c-Raf (Alessi et al. 1995; Dudley et al. 1995; Favata et al. 1998; Davies et al. 2000). A commonly used inhibitor of PI3K is LY294002. Unlike PD98059 and U0126, LY294002 is ATP-competitive and acts by binding to the ATP-binding site of its target kinase (Vlahos et al. 1994; Vlahos et al. 1995; Baumann and West 1998). Piceatannol (trans-3,4,3',5'-tetrahydroxystilbene) is a naturally occurring stilbene present in sugar cane, berries, peanuts, red wine and the skin of grapes (Cantos et al. 2003; Rimando et al. 2004), that has been shown to act as a potent inhibitor of the tyrosine kinase Syk (Thakkar et al. 1993; Burke 1994) (see also Table 3).

Protein kinases as major drug targets

Dysregulated protein kinases are involved in the development of a variety of human neoplastic diseases. Hence, much of the effort to develop drugs that target specific protein kinases has been concentrated in the field of cancer. A landmark event occurred in May 2001 when Gleevec – the first important drug to be developed by targeting a protein kinase specifically (the Abelson tyrosine kinase, ABL) – was approved for clinical use (Druker and Lydon 2000). In nearly all cases of chronic myelogenous leukaemia (CML), a chromosomal rearrangement leads to enhanced activity of ABL, which might cause CML by eliciting uncontrolled activation of the MAPK cascade.

Today, protein kinases are the second largest group of drug targets after G protein-coupled receptors, and they account for 20 to 30 percent of the drug discovery programs of many companies (Cohen 2002). In cancer therapy, protein kinase inhibitors are proving to be well tolerated compared with conventional chemotherapeutic treatments.

1.4.1 Protein kinase inhibitors in the treatment of KSHV-associated diseases

A number of reports have been published describing the effectiveness of rapamycin (sirolimus) in the treatment of transplant recipients with (iatrogenic) KS (Gutierrez-Dalmau et al. 2005; McCaffrey 2005; Mohsin et al. 2005; Stallone et al. 2005; Zmonarski et al. 2005; Kolhe et al. 2006). Rapamycin is widely used as an immunosuppressive agent. It inhibits mTOR, which is a target of the PI3K-Akt signalling cascade (see also Figure 3) and has a crucial role in cell-cycle progression from G1- to S-phase, as well as in IL-2 stimulated T-cell proliferation. It has been demonstrated that in patients undergoing a kidney-transplant, rapamycin (sirolimus) inhibited the progression of dermal KS while providing effective immunosuppression. *In vivo* experiments using a cell based (allograft) animal model for KS showed that activation of the TSC2/mTOR pathway was sufficient to promote the tumorigenic potential of endothelial cells. Furthermore, Sin *et al.* (Sin et al. 2007) showed that mTOR, Akt and p70S6K are phosphorylated in PEL cells and that rapamycin was efficacious against PEL in culture and in a murine xenograft model.

Others found that expression of the proto-oncogene c-Kit, a member of the PDGF family of receptor protein tyrosine kinases and receptor for stem cell factor, is upregulated in KS tumours and KSHV latently infected endothelial cells (Moses et al. 2002; Pantanowitz and Dezube 2004; Pantanowitz et al. 2005). Limited experience with the *c-kit* inhibitor imantinib showed a partial response to the drug in patients with epidemic KS (Koon et al. 2005). Clinical trials are currently ongoing to examine the role of other *c-kit* inhibitors, such as sorafenib, in the treatment of KS.

1.5 Objectives

Accumulating evidence suggests that ongoing viral lytic replication in a small number of cells in KSHV associated tumours significantly contributes to KSHV pathogenesis and is necessary to maintain stable viral infection. Identification of the molecular mechanisms that regulate the viral lytic life cycle may therefore provide new targets for antiviral and tumour therapy. Additionally, understanding virus manipulation of cellular signalling pathways and their functional relevance to KSHV biology may contribute to our knowledge and appreciation of viral strategies developed during the long co-evolution with their host.

Previous reports had concentrated on the role of signalling pathways during primary infection and reactivation of KSHV from latency and had identified Raf/MEK/ERK and PI3K signalling cascades as key regulators of KSHV infection and activation of the RTA promoter. I now investigated the activation of these signalling pathways during progression of viral lytic replication downstream of RTA expression.

Since several viral lytic proteins are capable of inducing signalling pathways within their host cell, I asked whether these proteins contribute to the activation of signalling pathways during KSHV productive phase and analysed their contribution to successful viral protein expression and virus production in siRNA-mediated depletion experiments and by genetic knockout.

2 Materials and Methods

2.1 Reagents and Chemicals

Chemicals were purchased from the following companies: Amersham, AppliChem, Biochrom, Biomol, BioRad, Calbiochem, Difco, Fluka, Invitrogene, Life Technologies, Merck, New England Biolabs, Pierce, Promega, Riedel-deHaën, Roche, Roth, Santa Cruz, Sigma and Stratagene.
Consumables were obtained from Amersham, Beckman, Biozym, Costar, Eppendorf, Falcon, Gilson, Greiner, Hartmann, Kodak, Machery-Nagel, Millipore, Nerbe Plus, Qiagen, Sarstedt and Whatman.
Restriction enzymes came from Amersham, New England Biolabs and Life Technologies. Oligonucleotides were purchased from Ambion, Dharmacon, MWG Biotech and Sigma.

2.1.1 Antibodies

Primary antibodies:

α-*β*-actin
Chemicon, mouse monoclonal, $IgG_{1\kappa}$, used in a 1:2000 dilution, specific for the N-terminal two-thirds of the actin molecule near aa residues 50-70 in primary sequence.

α-Akt1
Cellsignaling, rabbit polyclonal, raised against a synthetic peptide (KLH-coupled) derived from the carboxy-terminal sequence of mouse Akt. Antibodies are purified by protein A and peptide affinity chromatography, used in a 1:1000 dilution. MW of Akt = 60 kDa.

α-BrdU
BD Biosciences, mouse monoclonal, $IgG_{1,k}$, purified, used in a 1:5 dilution.

α-ERK2 D-2
Santa Cruz, mouse monoclonal, IgG_{2b}, raised against a peptide at the C-terminus of human ERK2-encoded MAP kinase, used in a 1:2000 dilution. MW of ERK2 = 42 kDa.

α-K1 (2H5)

(Lee et al. 2003), mouse monoclonal, purified from mouse ascites by affinity chromatography on recombinant protein G columns, recognizes an epitope of K1 in the C2 region overlapping with the Ig region, used in a 1:500 dilution for Western blotting and a 1:200 dilution for immunofluorescence assay; provided by J.Jung. MW of K1 = 46 kDa.

α-K8

(Wu et al. 2001), rabbit polyclonal, the antibody was generated by immunization with the N-terminal peptide (16-DNSEKDEAVIEED-28) bounded by N-Y and C-CS residues, used in a 1:200 dilution; provided by G. S. Hayward. K8 is a 237-aa nuclear protein.

α-K8.1 A/B

Advanced Biotechnologies (ABI), mouse monoclonal, IgG_1, originated from serum-free hybridoma cell culture medium, purified by protein G agarose affinity chromatography. K8.1 is highly glycosylated, used in a 1:1000 dilution. A 37 kDa- and a 25 kDa-K8.1 protein are detected by Western blotting in most KSHV lytic infected cell lines.

α-c-myc 9E10

Biomol, mouse monoclonal, IgG1; raised against a synthetic peptide corresponding to residue 408-439 of the human p62c-myc protein and recognizes an epitope located in the aa residue 410 to 419; used in a 1:500 dilution.

α-Orf50/RTA

(Lukac et al. 1998), rabbit polyclonal, raised against a recombinant Orf50/RTA fragment (aa 484 to 691), rabbit serum, used in a 1:1000 dilution, provided by D. M. Lukac. MW of RTA: predicted: 73.7 kDa. However, the expressed protein when anlysed by Western blot appears to be about 110 kDa, suggesting post-translational modification (Lukac et al. 1999).

α-Orf59

Advanced Biotechnologies (ABI), mouse monoclonal, IgG, originated from ascites fluid and is purified by protein A agarose affinity chromatography, used in a 1:2000 dilution. MW of Orf59 = 50 kDa.

α-phospho Akt (Ser473) (193H12)

Cellsignaling, rabbit monoclonal, IgG, detects endogenous levels of Akt only when phosphorylated at serine 473, used in a 1:1000 dilution. MW of Akt = 60 kDa.

α-phospho p42/44 MAPK (Thr202/Tyr204) (E10)

Cellsignaling, mouse monoclonal, detects endogenous levels of p42 (ERK2) and p44 (ERK1) MAP kinase dually phosphorylated at threonine 202 and tyrosine 204, used in a 1:2000 dilution. MW of ERK1 = 44 kDa and of ERK2 = 42 kDa.

α-Vimentin

Chemicon, mouse monoclonal, IgG_1, used in a 1:1000 dilution, recognizes the 57-60 kDa vimentin protein.

Secondary antibodies:

α-goat, HRP-conjugated

DakoCytomation, rabbit monoclonal, IgG, used in a 1:2000 dilution.

α-mouse, Cy5-conjugated

Jackson ImmunoResearch, goat polyclonal, IgG, used in a 1:200 dilution for immunofluorescence and 1:50 for FACS analysis.

α-mouse, HRP-conjugated

DakoCytomation, rabbit polyclonal, IgG, used in a 1:1000 dilution.

α-mouse, Rhodamine X-conjugated

Jackson ImmunoResearch, goat polyclonal IgG, used in a 1:200 dilution.

α-rabbit, HRP-conjugated

Cellsignaling, mouse polyclonal IgG, used in a 1:2000 dilution.

α-rabbit, Rhodamine X-conjugated

Jackson ImmunoResearch, goat polyclonal, IgG, used in a 1:200 dilution.

2.2 Vectors, primers, inhibitor peptide and small inhibitory RNAs (siRNAs)

2.2.1 Expression vectors

Eukaryotic expression vectors

pcDNA3

T7 and SP6 promoter for in vitro transcription, CMV promoter for expression in mammalian cells; bovine growth hormone polyadenylated signal; Ampicillin and Neomycin resistance gene. Invitrogen.

pcDNA3.1(-)/Myc-His

Derived from pcDNA3.1. With a C-terminal peptide containing the c-myc epitope and a polyhistidine metal binding tag. Invitrogen.

pcDNA4/myc-His B

CMV promoter for expression in mammalian cells; three reading frames with a C-terminal peptide encoding c-myc epitope and a polyhistidine (6xHis) metal-binding tag; Zeocin resistance gene for selection. Invitrogen.

pK1

K1 expressing plasmid, the cDNA sequence of K1 was cloned between the *BamH*I-*Hind*III restriction sites into pcDNA3.1(-)/Myc-His. Provided by M. Stürzl.

pOrf74

vGPCR (Orf74) expressing plasmid; Orf74 was cloned by *Eco*RI and *Sac*II digestion of pcDNA4/myc-His B. Provided by M. Stürzl.

pTpl-2 wt.MT

Tpl2 expressing plasmid. Provided by A. Kieser.

Prokaryotic expression vector

pKD46

(Datsenko and Wanner 2000); Red recombinase expression plasmid, includes 2.154 nt (31088-33241) of phage λ (GenBank accession no. J02459).

2.2.2 Primers and probes

Primer pair for K1-knockout construction

K1 ko-forward

Target position: nt 44-104 of KSHV genome sequence (Russo et al. 1996) and nt 1-24 (underlined) of the rpsL-neo cassette (GeneBridges).

Sequence: 5'-TTC TGG CGG CCC TTG TGT AAA CCT GTC TTT CAG ACC TTG TTG GAC ATC CTG TAC ATC AAG GGC CTG GTG ATG ATG GCG GGA TCG-3'.

K1 ko-reverse

Target position: nt 971-1034 of KSHV genome sequence (Russo et al. 1996) and nt 1298-1319 of the rpsL-neo cassette sequence (GeneBridges).

Sequence: 5'-TAA ACA TAA AAC ACA TTA CAA TTA TGT TAT AGA GAA TAT TTA GAT TAT CTT ACC TGA ATG TCA GAA GAA CTC GTC AAG AAG GCG-3'.

Primer pairs used for verification of BAC36 ΔK1:

neo1 (forward)

Targets position nt 541-560 in the rpsL-neo cassette (GeneBridges).

Sequence: 5'-GAT TGC ACG CAG GTT CTC CG-3'.

neo2 (reverse)

Targets position nt 1298-1319 in the rpsL-neo cassette (GeneBridges).

Sequence: 5'-TCA GAA GAA CTC GTC AAG AAG G-3'.

K1 ko transitional region right forward

Target position: nt 981-1000 of the rpsL-neo cassette (GeneBridges).

Sequence: 5'-GTC GAT CAG GAT GAT CTG GA-3'.

K1 ko transitional region right reverse

Target position: nt 1084-1104 in the KSHV genome sequence (Russo et al. 1996).

Sequence: 5'-CTG TAA CAT ATT GGC ACG ATG-3'.

Table 2: Primers used to quantify transcripts of KSHV early lytic proteins by reverse transcription real-time quantitative PCR (qPCR).

primer pair	target position	product size (bp)	forward primer sequence (5'-3')	reverse primer sequence (5'-3')
K1	152-256[a]	104	TATTAAGCCTTTATGTGCTATCGTCTC	CCAGGATATTGGCAAGGATGAATC
K2/vIL-6	17709-17841[b]	132	CCCATAGCATCCAATTGAGTCTC	ATGCGCTGGTTCAAGTTGTG
K6/vCCL-1	27358-27445[b]	87	ACGCCCTCGCTTGGTCAG	CCGCCCGTCCAAATTCTAAAAG
K8/bZIP	75142-75278[b]	137	AATTCCACATCCCCGATCCTTC	CTTAGTGCATAAGCGTTCCTCTTC
GAPDH[c]			GAAGGTGAAGGTCGGATGC	GAAGATGGTGATGGGATTTC

Probe	length (nt)	Sequence (5'-3')[d]
K1	25	[6FAM]TGTGCCCTGGAGTGATTTCAACGCC[TAM]
K2/vIL-6	25	[6FAM]CTATCTTGCTGGTCGGTTCACTGCT[TAM]
K6/vCCL-1	23	[6FAM]CGTCCCCAGCGTGCCCAAAACCC[TAM]
K8/bZIP	25	[6FAM]CACACACCACCAAGAGGACCACACA[TAM]
GAPDH	20	[6FAM]CAAGCTTCCCGTTCTCAGCC[TAM]

[a] Based on the coding sequence of K1 derived from BC-1 cells (GenBank accession no. KSU86667).

[b] Based on the genomic sequence of a KSHV P-type (GenBank accession no. NC_009333).

[c] Glyceraldehydes-3-phosphate dehydrogenase primer pair was used as an internal control.

[d] All taqman probes were labelled with 6-FAM at their 5' end and with TAMRA at their 3' end.

2.2.3 Inhibitor peptide

ERK Activation Inhibitor peptide I

Purchased from Calbiochem; alternate name: Ste-MEK1$_{13}$ or Ste-MPKKKPTPIQLNP-NH$_3$; a cell-permeable, stereated, 13-aa peptide corresponding to the N-terminus of MEK; acts as a specific inhibitor of ERK activation and blocks transcriptional activity of Elk1; selectively binds to ERK2 and prevents it interaction with MEK (IC_{50}=2.5 µM according to the

manufacturer). MW: 1757.3. Reconstituted in ddH_2O (2.5 mM stock solution). Aliquotted and stored at -20°C.

2.2.4 siRNAs

siRNAs targeting early-lytic transcripts of KSHV

<u>siRNAs K1</u>

siRNA1 was purchased from Ambion, siRNA2 and siRNA3 were purchased from Dharmacon. siRNA1 and 3 were designed based on a K1 sequence obtained from a patient with classic KS (GenBank accession no. AF148805); siRNA2 was designed with the use of a K1 sequence derived from JSC-1 cells (PEL cells) (GenBank accession no. DQ984721).

siRNA1: Targets position nt 319-339 in the K1 transcript;
sequence: 5'-GUCACUUGUGGUCAGCAUGTT-3'

siRNA2: Targets position nt 111-131 in the K1 transcript;
sequence: 5'-CCAUUGUGCUCGAAUCCAAUU-3'.

siRNA3: Targets position nt 403-423 in the K1 transcript;
sequence: 5'-GAAACCGUGUCACAAACUAUU-3'.

<u>siRNAs K2 (vIL-6)</u>

Purchased from Ambion; siRNA design was based on the coding sequence for K2/vIL-6 derived from a PEL cell line, published by Nicholas et al. (Nicholas et al. 1997) (GenBank accession no. U67774).

siRNA1: Targets position nt 234-254 in the K2 transcript;
sequence: 5'-CGAUACUGAUCACUGCGGGUU-3' (K2_1),

siRNA2: Targets position nt 426-444 in the K2 transcript;
sequence: 5'-GCUGACUAAGACGCACUACUU-3' (K2_2).

<u>K4 (vCCL-2)</u>

Purchased from Ambion; designed based on the K4 sequence from a KSHV P-type complete genomic sequence (GenBank accession no. NC_009333).

siRNA1: Targets position nt 147-167 in the K4 transcript;

sequence 5'-GCACCUGUGGUAAUGGUCUU-3'.

siRNA2: Targets position nt 19-39 in the K4 transcript;
sequence: 5'-UAAUUGCUGCAUCAGCUUCUU-3'.

K6 (vCCL-1)

Purchased from Ambion; designed based on the coding sequence of K6 published by Sun et al. (1996) (GenBank accession no. U50138).

siRNA1: Targets position nt 131-149 in the K6 coding sequence;
sequence: 5'-CACCGCCCGUCCAAAUUCUUU-3'.

siRNA2: Targets position nt 153-171 in the K6 coding sequence;
sequence: 5'-AGAGUGGUAUCCCACGUCCUU-3'.

K14 (vOX2)

Purchased from Ambion; designed based on the K14 mRNA sequence published by Nador et al. (Nador et al. 2001) (GenBank accession no. AF367766).

siRNA1: Targets position nt 225-543 in the K14 transcript;
sequence: 5'-CCUUGCAGUGGAUGAUGAGUU-3'.

siRNA2: Targets position nt 104-122 in the K14 transcript;
sequence: 5'-CUGAAGAAGGCGUGUCUGCUU-3'.

K15 siRNA

Purchased from Dharmacon; targets exon 8 of the K15 transcript.
Sequence: 5'-CAACCACCUUGGCAAUAAU-3'.

Orf36 (vPK)

Purchased from Ambion; designed based on the Orf36 coding sequence from a KSHV P-type complete genomic sequence (GenBank accession no. NC_009333).

siRNA1: Targets position nt 362-380 in the Orf36 transcript;
sequence: 5'-UUGCCAACGACCUGAUGCAUU-3'.

siRNA2: Targets position nt 109-129 in the Orf36 transcript;
sequence: 5'-CUCCCUAAGGCUACCCGGAUU-3'.

Orf74 (vGPCR)

Purchased from Ambion (siRNA1) and Dharmacon (siRNA2 and siRNA3); designed based on the Orf74 sequence published by Nador et al. (Nador et al. 2001) (GenBank accession no. AF367766).

siRNA1: Targets position nt 638-656 in the Orf74 transcript;
sequence: 5'-CCGUGUCAGUUACUGCAGGUU-3'.

siRNA2: Targets position nt 191-209 in the Orf74 transcript;
sequence: 5'-GAAAUGGAUUGGUCACCUAUU-3'.

siRNA3: Targets position nt 276-294 in the Orf74 transcript;
sequence: 5'-CUCGCUGUGUCUUAGCAUAUU-3'.

Control siRNA

siCONTROL Non-Targeting siRNA#1

Dharmacon; negative control siRNA with at least four mismatches to any human gene.
Sequence: 5'-UAGCGACUAAACACAUCAA-3'.

2.3 Eukaryotic cell culture methods

2.3.1 General components for cell culture

DMEM (Gibco)	+ 4.5 g/l glucose, L-glutamine, pyruvate
MEM (Cytogen)	+ Eagle's BSS, NEAA, L-glutamine, 2.2 g/l $NaHCO_3$
RPMI 1640 (Gibco)	+ L-glutamine
Grace's Insect medium (Gibco)	+ L-amino acids
L-Glutamine (Cytogene)	200 mM
NEAA (Gibco)	100x
β-Mercaptoethanol (Gibco)	50 mM
PBS,10x (Gibco)	without $CaCl_2$ and $MgCl_2$
Trypsin/EDTA (Cytogen)	0.05/0.02% in PBS
Trypsin/EDTA (Biochrom)	0,25/0.02% in PBS
FCS (Gibco)	heat-inactivated for 30 min. at 56°C
FCS (PAA)	heat-inactivated for 30 min. at 56°C

Hygomycin B (Signal)	50 mg/ml in PBS
Puromycin (Sigma)	5 mg/ml
DMSO (AppliChem)	cell culture grade

Protein kinase inhibitors

MEK inhibitors

U0126 Purchased from Calbiochem, MW: 380.5, reconstituted in DMSO just prior to use (25 mM stock solution).

PD98059 Purchased from Calbiochem, MW: 267.3, reconstituted in DMSO (25 mM stock solution), stored at -20°C.

U0124, the non-active analogue of U0126, was purchased from Biomol; MW: 226.3, reconstituted in DMSO (25 mM stock solution), stored at -20°C.

PI3K inhibitor

LY294002 Purchased from Biomol, MW: 307.4, reconstituted in DMSO (25 mM stock solution), stored at -20°C.

Syk inhibitor

Piceatannol Purchased from Biomol and Calbiochem; MW: 244.2, reconstituted in DMSO (25 mM stock solution), stored at -20°C.

After reconstitution, all kinase inhibitors were aliquotted and stored for up to six months. All inhibitors were handled under low-light conditions.

2.3.2 Eukaryotic cell lines

Adherent cell lines

HEK293

human embryonic kidney cell line; adherent fibroblastoid cells which were established from a human primary embryonal kidney transformed by adenovirus type 5 (Ad 5). ACC 305 (German Collection of Microorganisms and Cell Cultures (DSMZ)).

EAhy926

(Edgell et al. 1983); derived by fusing human umbrilical vein endothelial cells (HUVEC) with the permanent human lung carcinoma cell line A549. Provided by P. Ojala.

Vero

African green monkey kidney cell line; adherent-elongated, fibroblast-like cells, which were established from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*). ACC 33 (DSMZ).

Insect cells SF9

Cells isolated from *Spodoptera frugiperda* (ATCC CRL-1711).

Adherent cell lines carrying a recombinant KSHV genome

Vero rKSHV.219

Vero cells stably infected with a recombinant KSHV (rKSHV.219) containing the red-fluorescent protein (RFP) gene under the control of the viral lytic PAN promoter, the green-fluorescent protein (GFP) gene expressed by the human elongation factor 1-α (EF-1α) promoter, and the *pac* gene for resistance to puromycin expressed by the RSV promoter. The RFP/GFP/PURO cassette is inserted between ORF K9 and ORF 57 at a site that sequence data indicated does not contain a gene (Vieira and O'Hearn 2004). Provided by J. Vieira.

EAhy rKSHV

EAhy926 cells were stably infected with rKSHV.219 produced from Vero rKSHV.219 cells (see 2.3.6).

HEK293 BAC36

HEK293 stably transfected with recombinant bacterial artificial chromosome (BAC) KSHV (BAC36) containing hygromycin resistance and GFP markers. The BAC vector/Hyg/GFP

cassette is integrated into the *Pme*I site between orf18 and orf19 in the long unique region of the viral genome (Zhou et al. 2002).

Suspension cells

TREx BCBL-1-pcDNA5/FRT/TO-RTA/myc/his (TREx BCBL-1-RTA)
PEL cell line (BCBL-1) stably transfected with a vector containing myc/his-tagged RTA cDNA downstream of the CMV promoter and tetracycline operator sequences. The vector further contains the hygromycin resistance gene as selectable marker (Nakamura et al. 2003). Provided by J. Jung.

2.3.3 Cell culture conditions

Vero:	MEM with 10% FCS
HEK293, EAhy926:	DMEM with 10% FCS
SF9:	Grace's insect medium with 10% FCS
Vero rKSHV.219:	MEM with 10% FCS, puromycin 5 µg/ml
EAhy rKSHV:	DMEM with 10% FCS, puromycin 1 µg/ml
HEK293 BAC36:	DMEM with 10% FCS, hygromycin B 150 µg/ml
TREx BCBL-1-RTA	RPMI 1640 with 20% FCS, hygromycin B 100 µg/ml

Cells were kept in humidified air with 5% CO_2 at 37°C. Adherent cell lines were split at 80-90% confluency at a ratio of 1:6 to 1:10. BCBL-1 cells were usually split every three days at a ratio of 1:5. Insect cells were cultured at 27°C and split at 100% confluency at a ratio of 1:10.

2.3.4 Cryoconservation

For conservation, cells were resuspended in 1 ml of the appropriate cell culture medium with 20% FCS and 10% DMSO, transferred to a cryo tube and frozen in a polystyrene box or an isopropanol-jacketed freezing box at -80°C. After two days, the cells were transferred to

liquid nitrogen. For recovery, cells were thawn in handwarm water, washed once in 10 ml pre-warmed medium and transferred to a tissue culture flask.

2.3.5 Amplification of baculovirus Orf50/RTA in SF9 cells

Baculovirus (BV) Orf50/RTA was provided by J. Vieira. For production of new BV Orf50/RTA, SF9 insect cells were grown in 125 ml suspension culture flasks up to a cell density of $1{,}6x10^6$ cells/ml. The cells were then infected with SF9 cell supernatant containing high titer BV Orf50/RTA. The added amount corresponded to 5% of the final volume. When a cytopathic effect was observed – usually two to three days after infection - the culture was aliquoted to 50 ml-falcon tubes and centrifuged at 1500 rpm for 10 minutes at 4°C to remove cells and cell debris. Supernatant was filtered through a 0.45 µm- filter, transferred to fresh tubes, and stored at 4°C for later use.

2.3.6 Lytic cycle induction

Lytic cycle induction in Vero rKSHV.219 cells

For reactivation of KSHV from latency, Vero rKSHV.219 cells were subcultured to $1.5x10^5$ cells per well in 6-well plates (Greiner) in medium without puromycin over night. The next day, lytic cycle was induced using baculovirus (BV) Orf50/RTA in combination with 1 mM sodium (Na-) butyrate (NaB, Sigma-Aldrich). For inhibition of signalling pathways during lytic replication, Vero rKSHV cells were incubated with protein kinase inhibitors at a final concentration of 12.5 and 25 µM one hour prior to reactivation (if not indicated otherwise). Subsequently, BV Orf50/RTA and NaB were added without medium change. After 48 h, the supernatant was collected, and the number of newly produced infectious virions (infectious units (IU)/ml) was determined by infectivity assay. Cells were washed once in PBS, lysed, subjected to Western blot analysis or stored at -20°C for later use.

To investigate the activation pattern of the ERK and Akt kinases during the KSHV lytic cycle, Vero rKSHV.219 cells were lysed 4, 8, 12, 16, 20, 24, 28, and 40 h after induction of the lytic replication cycle (h.p.i.). Supernatants were collected 20, 24, 28, and 40 h.p.i..

To inhibit viral DNA replication during the KSHV lytic cycle, phosphonoacetic acid (PAA, 98%, Sigma-Aldrich) was applied at a final concentration of 1 mM at the time of

inducing the lytic cycle. To inhibit viral lytic protein synthesis, cycloheximide (CHX, Sigma-Aldrich) was applied at 50 µg/ml 4, 8, 10, 12, 13, 14, or 16 h.p.i..

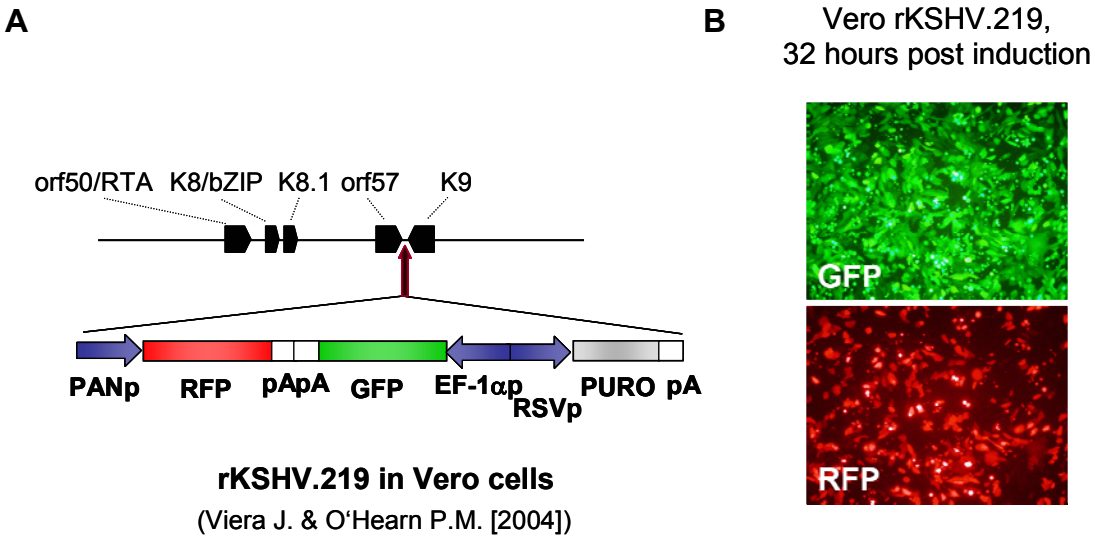


Figure 5: (A) Schematic diagram of the structure and insertion site of the RFP/GFP/PURO construct in the recombinant KSHV.219 genome. rKSHV.219 was constructed by insertion of a gene cassette between orf K9 and orf 57, that comprises the RFP genetic marker under the control of the lytic PAN promoter, along with the GFP gene expressed by the human elongation factor 1-α promoter (EF-1α p), and a gene for resistance to puromycin (PURO) expressed by the RSV promoter. pA, poly A. (B) GFP expression (upper picture) and RFP expression (lower picture) in Vero rKSHV.219 cells 32 h after induction of the lytic replication cycle using baculovirus Orf50/RTA in combination with sodium butyrate [1 mM].

Generation and reactivation of EAhy rKSHV

To establish EAhy rKSHV cell lines, EAhy926 cells (see 2.3.2) were plated at $1.5X10^5$ cells per well on 6-well plates (Greiner) and grown over night. The next day, the medium was removed, cells were infected with supernatant of reactivated Vero rKSHV.219 cells (1ml/ well), incubated for 2 h while rocking at room temperature (RT), the volume of the medium in each well was adjusted to 2 ml with fresh cell culture medium, and cells were transferred to 37°C. The medium was exchanged the next day. The infection efficiency was monitored by expression of GFP. 48 hours after infection (h.p.i.), cells were split 1:3 in medium containing 1 µM puromycin for selection. In the following, medium was exchanged every two days, and when foci developed, individual clones were picked and transferred to 96-well (Greiner) under selection of puromycin. Cell lines were established from 12 individual clones which

were resistant to puromycin, expressed GFP to nearly 100 %, and produced infectious virions after induction of lytic replication using BV Orf50/RTA and Na-butyrate.

To induce the KSHV lytic cycle, EAhy rKSHV cells were subcultured at $1*10^5$ cells/ml in 6-well plates in medium without puromycin. After 24 h, the medium was removed, and cells were incubated with BV Orf50/RTA (1ml/well) for 2 h while rocking at RT. Then, the supernatant baculovirus was replaced with 2 ml of fresh medium containing 1 mM Na-butyrate. 48 h after reactivation from latency, culture supernatants were collected in 2 ml-reaction tubes (Eppendorf), centrifuged for 10 minutes at 2.000 rpm, and transferred to new tubes. Virus titer (IU/ml) was determined by infectivity assay on HEK293 cells. Cells were washed once in PBS, lysed, analysed by Western blotting or stored at -20°C for later use.

2.3.7 Infectivity assay

For determination of virus titer (IU/ml) in supernatant of cells undergoing KSHV lytic infection (see 2.3.6), HEK293 cells were seeded to 70% confluency in a 96-well plate. The next day, cells were infected in triplicates with 25 µl/well of culture supernatant diluted 1:2; 1:5, and 1:10. Total volume per well was adjusted to 100 µl with fresh medium. After incubation for 2 h at RT, plates were centrifuged at 450x g for 25 min at 30°C, the culture volume per well was adjusted to 200 µl with fresh medium, and plates were incubated at 37°C. After 48 h, the plates were examined on a Nikon Eclipse TS100 fluorescence microscope with a 10x objective for GFP-expressing cells. For counting, we chose the dilution that gave between 100 and 400 GFP-positive cells per well. These numbers were then converted to GFP-positive cells per ml to reflect the virus titer (IU/ml).

To relatively quantify the number of infectious virions in supernatant of inhibitor-treated and reactivated cells, HEK293 cells were plated at 2x105 cells per ml on black-bottom 96-well plates (Nunc), and infected 24 h later with 50-100 µl supernatant as described above. 48 hours after infection, the overall fluorescence intensity of GFP in each well was measured at a BioTek plate reader. Data was processed using Gen5 software and analysed in Excel.

Entry control assay for kinase inhibitors

To ensure that inhibitors applied during reactivation of KSHV in Vero rKSHV.219 or EAhy rKSHV cells no longer interfered - under the experimental conditions used - with

KSHV entry into HEK293 cell in infectivity assay, inhibitors were incubated for 48 h at 37°C in normal culture medium supplemented with 10% FCS, BV Orf50/RTA, and 1 mM NaB. These pre-incubated inhibitors were then mixed with virus inoculum, which was subsequently used to infect HEK293 cells as described above. After 48 h, infected (GFP-positive) cells were counted and virus titers were calculated. The titers obtained for pre-treated virus inoculum were compared to those determined for non-treated and DMSO-treated virus inoculum.

2.3.8 Transient transfection

Transient expression of K1 in HEK293 cells

pcDNA 3.1(-)-K1/myc/His, and pcDNA4-Orf74/myc/His were kindly provided by M. Stuerzl. Tpl-2 wt.MT (myc-tagged) (Patriotis et al. 2001) was a kind gift of A. Kieser. HEK293 cells were plated at $2x10^5$ cells per well of 6-well plates, and grown over night. The cells were transiently transfected with 1 µg of DNA per well using FuGENE 6 transfection reagent (Roche) at a ratio of 1:3 (DNA to transfection reagent) according to the manufacturer's protocol. The cells were incubated in complete medium. Protein samples were taken 24 h and 48 h posttransfection to be assayed by immunoblotting for activation of ERK signalling. Expression of K1, Orf74/vGPCR, and Tpl-2 was analysed using myc-specific antibodies.

Transfection of siRNAs to downregulate lytic gene expression

Expression of KSHV early-lytic proteins was downregulated using RNA interference. Therefore, Vero rKSHV.219 or EAhy rKSHV cells were transfected with individual siRNAs targeting viral early-lytic transcripts using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Briefly, Vero rKSHV.219 or EAhy rKSHV cells were seeded to 40-50 % confluency on 6-well plates one day prior to transfection. Each siRNA (160 pmol) was diluted with 5 µl Lipofectamine 2000 in 500 µl reduced serum medium (OptiMEM I, Gibco), incubated for 20 min at RT, and then added to the cells. Medium was replaced after 6-8 h, and lytic cycle was induced simultaneously using BV Orf50/RTA in combination with Na-

butyrate as described in 2.3.6. 48 h after reactivation, culture supernatant was collected for infectivity assay, and protein extracts were taken for Western blot analysis.

2.4 Prokaryotic culture methods

2.4.1 Culture media and growth conditions

Luria Bertani (LB) -medium:
1% (w/v) Trypton (Becton Dickinson)
0.5% (w/v) Yeast extract (Difco)
1% (w/v) NaCl
pH 7.0 with NaOH

Agar plates:
LB medium with 1.5% (w/v) Bacto-Agar (Becton Dickinson)

Overnight cultures were usually grown in LB medium at 37°C and 220 rpm. The medium was inoculated with bacteria kept on agar plates at 4°C or from cryoconserved cultures. The medium/agar plates were supplemented with antibiotics according to the properties of the plasmid being introduced. Antibiotics were used in the following final concentration:

Ampicillin:	100 µg/ml
Kanamycin:	15 µg/ml
Chloramphenicol:	15 µg/ml

2.4.2 Bacterial strains

E. coli TG-2
supE hsdΔ5 thiΔ(lac-proAG)Δ(srl-recA)306::Tn10(tet^r)F´[traD36 proAB+ laclq lacZΔM15].

E.coli DH10B
F$^-$ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ$^-$ (Durfee et al. 2008).

2.4.6 Cryokonservation of bacteria

500 µl of a bacterial overnight culture were mixed with 500 µl glycerol in a cryo tube and stored at -80°C.

2.4.7 Preparation of competent cells and transformation

Competent cells were prepared using the rubidium chloride method. An overnight culture was diluted 1:100 in LB medium and grown at 37°C and 200 rpm to an OD_{600} between 0.6 and 0.8. In the following, all steps were performed at 4°C and buffers were ice cold. Cells were incubated on ice for 15 min, centrifuged at 1000 x g for 10 min, cell pellets were resuspended gently and thoroughly in 20 ml RF1 buffer per 50 ml of starting culture, cells were again incubated on ice for 15 min, centrifuged as before, resuspended in 2 ml RF2 per 50 ml of starting culture and aliquotted to 200 µl. After 15 min incubation on ice, the now competent cells were used for transformation or stored at -80°C.

For each transformation, 200 µl of competent bacteria were mixed with DNA (e.g. 100 ng plasmid DNA) in a 1.5 ml tube, incubated on ice for 1 h, heat-shocked for 45 sec at 42°C and kept on ice for 5 min. LB medium (800 µl, pre-warmed to 42°C) was then added and cells were incubated at 37°C and 200 rpm for 45 min. 100 µl of the cell suspension was subsequently plated onto LB agar plates containing the appropriate antibiotic for selection and plates were incubated at 37°C over night.

RF1:	RF2:
100 mM $RbCl_2$	10 mM MOPS
30 mM K-acetate	75 mM $CaCl_2$
10 mM CaCl2	10 mM $RbCl_2$
50 mM MnCl2	15% (v/v) glycerol
15% (v/v) glycerol	pH 6.5 with KOH
pH 5.8 with acetic acid	

2.5 Molecular biology methods

2.5.1 Isolation of plasmid and BACmid DNA

Plasmid and small amounts of BACmid DNA were isolated from 5-10 ml overnight cultures with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Large DNA preparations of BAC36 wild types or mutants were obtained from 500 ml bacterial cultures and isolated with the Nucleobond BAC 100 Kit (Machary-Nagel, Germany) following the manufacturer's protocol.

2.5.2 Enzymatic modification of DNA

Digestion of DNA with specific restriction endonucleases and ligations were performed with enzymes and suitable provided buffers from Roche, New England Biolabs of Life Sciences according to the manufacturer's instructions.

2.5.3 PCR amplification of DNA

Generation of the rpsL-neo PCR product flanked by homology arms

To generate the rpsL-neo counter selection cassette PCR product flanked by 60 or 63 nucleotides homologous to the region immediately upstream of the KSHV K1 start codon or downstream of the K1 stop codon, respectively, PCR reactions were carried out in a final volume of 100 µl using the following components: 20 ng of rpsL-neo template DNA, 1x *Pfu* polymerase buffer, 200 µM dNTPs, 0.2 µM of each K1 ko forward and reverse primer, 9 µl DMSO, 10 units *Pfu*-polymerase and H_20 (PCR grade). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR system 2400 thermocycler with the following cycling parameters: 95°C for 5 min (initial denaturation), followed by 35 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (annealing) and 72°C for 4 min (elongation), and was completed with 72°C for 10 min.

Colony-PCR

PCR reactions were carried out in a final volume of 50 µl. The reaction mixture contained 1x PCR buffer (10x stock), 2.5 mM $MgCl_2$, 500 µM dNTPs, 0.3 µM of each forward and reverse primer, 3 units/50 µl Taq DNA polymerase, 1 µl/50 µl DMSO and H_20 (PCR grade). A small amount of bacteria of individual bacterial clones grown on an agar plate substituted for DNA template. PCR reactions were performed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler or in a ABI Gene Amp PCR System 9700. To verify the insertion of the rpsL-Kan/neo cassette into the genome of BAC36, we used primers - neo1 (forward) and neo2 (reverse) - located in the rpsL-neo cassette and the following cycling parameters: 95°C for 7 min (initial denaturation), followed by 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (primer annealing) and 72°C for 1.5 min (elongation).

2.5.4 Preparation of RNA and quantification by qPCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Residual DNA was digested using RNase-free DNase I (rDNase I, Ambion). RNA concentration was measured using a NanoDrop 1000 spectrophotometer. 1 µg of the DNA-free RNA was reverse transcribed for 1 h at 43°C in 20 µl reaction buffer containing 50 units Expand Reverse Transcriptase (Roche), 1x Expand Reverse Transcriptase buffer, 12.5 µM oligo-dT's, 10 mM DTT, 1mM deoxynucleoside triphosphates, and 20 U RNasin (Promega). A control experiment without reverse transcriptase was conducted in parallel. qPCR was performed with the cDNA. Primers and probes used for the quantification of K1, K8, K2/vIL-6, K6/vCCL-1, and GAPDH are listed in Table 1 (see 2.2.2 Primers and probes). Taqman probes were labeled with 6-FAM at their 5' end and with TAMRA at their 3' end.

qPCR was carried out in a total volume of 25 µl containing 1x JumpStart Taq ReadyMix (Sigma), 5 mM $MgCl_2$, 1x reference dye (Sigma), 500 nM of each primer, 250 nM probe, and 4 µl cDNA at 500 ng/µl. Thermal amplification was performed in a Stratagene MX3000P and using the following linked profile: 94°C for 2 min for initial denaturation followed by 40 amplification cycles, each with denaturation (94°C for 15 sec), and annealing (60°C for 1 min) periods. Fluorescence data were viewed and analysed using MxPro QPCR software. The cycle threshold (C_T) value shows the number of cycles required for the

fluorescent signal to cross the threshold (i.e. exceed background level), and was set automatically by the program. Each sample was assayed in duplicates. cDNA extracted from transiently K1-expressing HEK293 cells (see 2.3.8 Transient expression of K1 in HEK293 cells) was amplified as positive control for K1 expression.

2.5.5 Electrophoresis of DNA and extraction from agarose gels

DNA loading buffer (5x; 100 mM Tris-HCl, pH 7.4, 100 mM EDTA pH 8.0, 45 % (v/v) glycerol, orange G) was added to DNA fragments and the DNA was separated electrophoretically with 1 % agarose gels in TAE running buffer (1x; 40 mM Tris-acetate, 1 mM EDTA). Restriction-enzyme digested BAC36 DNA was separated electrophoretically with large 0.4 % agarose gels in TBE running buffer (0.5x; 45 mM Tris-borate, 1 mM EDTA, pH 8.5) for 14-20 h at 2.5 V/cm.
DNA fragments were purified with the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The 1 kb plus DNA ladder (Invitrogen) was used for molecular weight marker.

2.5.6 DNA sequencing

DNA fragments were sequenced with the BI Prism dRhodamine terminatof cycle sequencing ready reaction kit (Applied Biosystems). Per sequencing reation, 500 ng of DNA were added to 4 µl terminator ready reaction mix and 1.6 pmole specific primer and the total reaction volume was adjusted to 10 µl with H_2O. Thermal cycling was performed with the following parameters for 25 cycles with the annealing temperature depending on the specific sequencing primer: 96°C for 10 sec (denaturation), 56°C (variable) for 10 sec (annealing) and 60°C for 4 min (elongation). Excess of dye terminators was then removed by ethanol precipitation as follows: 10 µl of the PCR product was added to 37 µl precipitation mix (70 % EtOH, 0.5 mM $MgCl_2$) in a 1.5 ml high yield nucleic acid recovery tube (Robbins), vortexed and incubated for 15 min at RT. After centrifugation (14.000 x g, 15 min) the supernatant was removed and the pellet was dried at 95°C for 1 min. The sample pellet was resuspended in 6 µl of template suppression reagent, heated for 2 min at 95°C, cooled on ice, centrifuged and

transferred to a 0.5 ml sequencing sample tube (Applied Biosystems). Sequencing was carried out with the ABI Prism 310 Genetic Analyser.

2.5.7 Generation of BAC36 ΔK1

The BAC36 ΔK1 mutant was generated by ET-cloning (Gene Bridges) following the manufacturer's protocol. Briefly, BAC36 (Zhou et al. 2002) was transformed into *E. coli* strain DH10B, and selected with chloramphenicol to generate DH10B-BAC36. A Red/ET expression plasmid (pkD46; Datsenko and Wanner 2000), was transformed into DH10B-BAC36. The rpsL-neo cassette carrying 60 nucleotides upstream of the K1-start codon, and 63 nucleotides downstream of the K1-stop codon was obtained by PCR using the primers K1 ko-Forward and K1 ko-Reverse (see 2.2.2). To insert the rpsL-neo cassette into BAC36, a liquid overnight culture of DH10B BAC36 containing the pKD46 plasmid was induced with 0.2% L-arabinose for 1 h at 37°C to allow expression of the recombination enzymes. Bacteria were washed three times with ice-chilled water to render them electrocompetent. After electroporation with the homology-arms carrying rpsL-neo cassette (2.5 KV and 25 μF in a 2 mm-cuvette), DH10B BAC36 were incubated for 70 min at 37°C to allow recombination. The resulted mutant was selected by kanamycin-resistance on LB-agar plates. Colony PCR was performed to verify the presence of the rpsL-neo cassette in each bacterial clone (see above). BAC plasmid DNA was isolated from 10-ml overnight cultures by the alkaline lysis method and analysed by restriction digestion. Large DNA preparations of BAC36 wild types or mutants were used for direct sequencing (see 2.5.1). To reconstitute infectious virus, purified BAC36ΔK1 DNA was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogene) according to the manufacturer's instructions. Individual cell colonies were selected by hygromycin-resistance and GFP-expression, and cells stably harbouring the BAC36ΔK1 DNA were propagated in culture medium containing 150 μg/ml hygromycin B.

2.5.8 One-colour microarray-based gene expression analysis

The Agilent Whole Human Genome Oligo Microarray is provided in a 4x44 k format and covers more than 41.000 validated unique human genes and transcripts. The length of each oligonucleotide probe is 60-mer.

Cellular gene expression in HEK293 BAC36 wt and ΔK1 cell lines was measured using the one-colour protocol. Therefore, HEK293 BAC36 wt and ΔK1 cell lines as well as non-infected parental HEK293 cells were seeded to 6-well plates at 1.5 to $2x10^5$ cells/well and grown for 24 h. Susequently, cells were treated with baculovirus Orf50/RTA and 1 mM Na-butyrate in order to induce KSHV lytic replication and total RNA was purified 48 h after reactivation from lytic cycle-induced as well as latently infected cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions followed by on-column DNaseI-digestion (Qiagen). cRNA was synthesized following the Agilent Quick Amp one-colour protocol version 5.7. Briefly, a total of 0.5 µg RNA used to generates fluorescent cRNA by oligo(dT)-T7-primed cDNA synthesis followed by in vitro transcription with T7 RNA polymerase. The T7 RNA polymerase simultaneously amplifies target material and incorporated Cy3-labeled CTP. cRNA yields were measured photometrically using a NanoDrop ND 1000. 1 µg of Cy3-labeled cRNA of every experimental and control sample was fragmented, repurified and hybridized to individual microarrays for 17 h at 65°C. Subsequently, arrays were washed according to the manufacturer's protocol using provided Gene Expression (GE) Wash buffers 1 and 2.

Hybridized arrays were scanned on a Microarray Scanner 62505 B at XDR 100 and 5 (photomultiplier set to 100 or 5 % intensity). Data were processed using Agilent's Feature Extraction software version 9.5.3.1 and normalized according to an "inter-array" normalization approach (global linear scaling to reference array "non-infected, non-induction mixture-treated parental HEK293 cells" according to 75th percentiles of intensity distribution). Additionally, a lower intensity threshold of 39 was used as surrogate value for all measurements that fall below the intensity threshold. Data were analysed Genedata Expressionist and Excel software. Ratios of gene expression were calculated using Excel macros and. Additional information on the microarrays and methodology used can be obtained at http://www.agilent.com.

2.6 Biochemical and cell biology methods

2.6.1 Preparation of cell lysates

Cells were washed once with PBS and lysed on ice in 1 x SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% bromphenol blue) for 5

min. Afterwards, lysates were either directly analysed by Western blot or stored at -20°C for later use.

To analyse the expression of KSHV membrane proteins, cells were lysed in RIPA 100 buffer (20 mM Tris, pH 7.5, 1 mM EDTA, pH 7.5, 10 mM NaCl, 1% Triton X-100, 0.5 % sodium deoxycholate (DOC), 0.1 % SDS) for 10 min on ice. RIPA 100 also contained proteinase inhibitors that were added just prior to use giving the following final concentrations: 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 µM leupeptin, 100 U/ml aprotinin, 200 µM benzamidine and 1 µM pepstatin A (in DMSO). Lysates were cleared from cell debris by centrifugation at 14.000 rpm for 10 min at 4°C. Protein concentration in each sample was determined by Bradford analyses using the Bradford reagent (Biorad) according to the manufacturer's instructions. This analysis is based on binding of Coomassie brilliant blue G-250 to proteins, which alters the maximum of absorbance of the dye from 465 nm without protein to 595 nm with protein (Bradford 1976). Protein concentrations of different cell lysates that should be analysed by Western blot and compared with each other were adjusted to the lowest protein concentration of all samples with H_2O.

2.6.2 SDS polyacrylamide gelelectophoresis (SDS-PAGE)

Whole cell lysates were sonicated for 10 sec on ice, boiled at 100°C for 5 min, cooled on ice, and shortly centrifuged. Protein samples in RIPA 100 buffer were not boiled, when expression of KSHV membrane proteins should be determined. Cell lysates obtained using 1x SDS sample buffer were loaded directly on SDS polyacrylamide gels at 20 µl per lane, while lysates obtained using RIPA 100 buffer were diluted with 5x protein loading buffer prior to analysis by SDS-PAGE. Accoding to the size of the protein of interest, the separation gel contained 10-15% and the stacking gel 3-4% polyacrylamide. Gels were run at 25 mM in a Hoefer mighty small electrophoresis chamber (Amersham). To determine the size of separated proteins we included a prestained 10-200 kDa-molecular weight standard (Biorad) on every SDS-polyacrylamide gel during electrophoresis.

Protein loading buffer (1x)	Electrophoresis buffer
50 mM Tris-HCl, pH 6.8	25 mM Tris-Base
10 % (v/v) glycerol	250 mM glycine
2 % (w/v) SDS	0.1 % (w/v) SDS
100 mM DTT	pH 8.3
0.05 % (w/v) bromphenolblue	

2.6.3 Western blot analysis

Proteins separated by SDS- PAGE were electrophoretically transferred onto nitrocellulose membranes in cold blotting buffer (25 mM Tris-Base, 230 mM glycine, 20 % v/v methanol, pH 8.3) for 1.5 h at 350 mA using a Mini Trans Blot Cell (Biorad).

To analyse the activation status of cellular protein kinases Western blotting was performed with phosphospecific primary antibodies, i.e. phospho-p44/42 (Thr202/Tyr204) mAb and phospho-Akt (S473) mAb (both purchased from Cell Signaling Technology) following the manufacturer's instructions. Briefly, membranes were incubated in blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dried milk) for 1 h at RT, incubated first over night at 4°C with primary antibody specific for the phosphorylated forms of ERK1/2 or Akt, followed by HRP-conjugated secondary antibody for 1 h at RT. Immunoreactive bands were visualized using a standard enhanced chemiluminescence (ECL) reaction kit (Perkin Elmer). After detection, membranes were stripped and reprobed with antibodies specific to total ERK2 (p42 MAP kinase), or total Akt1.

For detection of K8.1 A/B, c-myc, or β-actin, membranes were blocked in PBS with 0.1% v/v Tween 20 (PBS-T) and 5% nonfat dried milk for 1 h at RT, incubated for 1 h at RT with primary antibody diluted in blocking buffer, washed three times for 10 min each in PBS-T, incubated with a HRP-conjugated secondary antibody diluted in blocking buffer for 30 min at RT, washed twice in PBS-T and once in PBS for 10 min each, and immunoreactive bands were visualized by chemiluminescence. For detection of Orf50/RTA membranes were blocked and antibodies diluted in PBS-T supplemented with 3% BSA and 1% glycine.

10X TBS (1 liter)	Stripping buffer
50 mM Tris	100 mM β-mercaptoethanol
150 mM NaCl	2% w/v SDS
adjust to pH 7.6 with HCl	62.5 mM Tris, pH 6.8

2.6.4 Immunofluorescence assay

HEK293 BAC36 wt and HEK293 BAC36 ΔK1 cells were seeded at 70% confluency on poly-L-lysine -coated glass coverslips in 6-well plates one day prior to reactivation. The lytic cycle was induced using baculovirus Orf50/RTA in combination with Na-butyrate (1 mM). 72 h after induction, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT, washed three times with PBS for 5 min each, quenched with 50 mM NH_4Cl for 10 min at RT, permeabilized with 0.2% Triton X-100 in PBS for 10 min at RT, washed once with PBS, and blocked with 10% goat serum in PBS for 10 min at RT. Then, cells were first incubated with rabbit anti-K8 pAb, mouse anti-Orf59 mAb, or rabbit anti-K8 pAb in combination with mouse anti-K1 mAb for 1 h in a damp and wet chamber at 37°C. Afterwards, cells were washed three times with 0.5% goat serum in PBS for 5 min each, and incubated with Rhodamine X-conjugated goat-anti-mouse IgG, or Rhodamine X-conjugated goat-anti-rabbit IgG and Cy5-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch) for 45 min in a damp and wet chamber at 37°C. Cells were washed twice with 0.5% goat serum in PBS, and twice with PBS for 5 min each. DNA was stained using DAPI (1:1000 in PBS) for 30 min at RT. As controls for cross-reactions and background staining, cells were stained with secondary antibody only, or cells, which were stained with primary antibodies specific for K8, were incubated with secondary antibody for K1, and vice versa. Immunofluorescence was examined with a Zeiss AxioObserver Microscope.

2.6.5 Flow cytometry-based cell cycle analysis

Vero rKSHV.219 cells were seeded at $5x10^5$ cells per well of a 6-well plate. The next day, the KSHV lytic replication cycle was induced as described in 2.3.5 in the presence or absence of the MEK inhibitor U0126 (20 μM). 32 to 36 hours after induction, 5-bromo-2'-deoxyuridine (BrdU) was added to the medium of the growing population of cells to a final

concentration of 10 µM and incubated for 1 h at 37°C. Cells were then trypsinized, washed in PBS, and washed twice with PBS containing 1% w/v bovine serum albumin (BSA). Cells were fixed with 4% paraformaldehyde, pH 7.4, for 15 min, permeabilized with 0.1% Triton X-100 in PBS 1% BSA at RT for 10 min, washed once in PBS 1% BSA, resuspended in 1 ml PBS containing 20 µg/ml DNase-free RNase A and incubated at 37°C for 30 min. Cells were then subjected to acidic DNA denaturation with 1 ml 2 N HCl containing 0.5 % Triton X-100 for 30 min at RT, followed by a neutralization step with 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5. Subsequently, cells were labeled with a α-BrdU antibody (final concentration: 500 ng/ml) in 950 µl of PBS containing 0.5% Tween 20 (v/v) and 1% BSA (w/v) for 30 min at RT, followed by incubation with a secondary Cy5-conjugated antibody (final concentration: 15 µg/ml) in 100 µl of PBS containing 0.5% Tween 20 and 1% BSA for 30 min at RT. After two washing steps with PBS 1% BSA, cells were resuspended in PBS containing 100 µg/ml propidium iodide to stain DNA. Flow cytometric cell cycle analysis with a total number of 10^5 events per sample was performed on a BD FACSCalibur. Raw data were recorded and analyzed using the software Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI2.8) available at http://facs.scripps.edu/software.html. For cell cycle analysis, the BrdU content of the whole cell population was quantified in relation to its DNA content. Thereby, the percentage of cells in each phase of the cell cycle can be determined.

3 Results

3.1 Results I: The MEK/ERK Signalling Cascade Contributes to Successful KSHV Lytic Replication Downstream of RTA Expression.

ERK and Akt signalling are activated, respectively, during the early and late stages of the KSHV lytic cycle.

In earlier work, it was shown that PI3K and the MAP kinases ERK, JNK and p38 are activated early during KSHV primary infection, and have important functions in virus entry and/or subsequent viral gene expression (Naranatt et al. 2003; Sharma-Walia et al. 2005; Xie et al. 2005; Pan et al. 2006). Furthermore, it was shown that signalling cascades involving MEK/ERK, JNK and p38 mediate TPA-induced reactivation of KSHV from latency and activation of the immediate-early RTA promoter (Cohen et al. 2006; Pan et al. 2006; Yu et al. 2007; Xie et al. 2008). Two studies further suggest an impact of all three MAP kinases on the production of new infectious KSHV virions, as inhibition of MEK, JNK and p38 reduces the yield of progeny virus during lytic replication (Pan et al. 2006; Xie et al. 2008). The aim of the current study was to investigate the role of cellular signalling pathways during the progression of the lytic replication cycle and to identify additional viral proteins required for the completion of viral productive replication.

To investigate which signalling pathways are activated during the KSHV productive cycle, I induced lytic replication using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB) in Vero rKSHV.219 cells, a cell line stably infected with recombinant KSHV expressing green-fluorescent protein (GFP) from the EF-1α promoter and red-fluorescent protein (RFP) from the lytic PAN promoter (Vieira et al. 2004, Figure 5). Cells were lysed 4, 8, 12, 16, 20, 24, 28, and 40 hours post induction (h.p.i.), and whole protein extracts were immunoblotted with anti-phospho p42/44 (ERK2/1), and anti-phospho Akt-S473 antibody. ERK activation strongly increased around 8 h.p.i., while activation of Akt was first seen at 4 h.p.i. and strongly increased around 20-24 h.p.i. (Fig. 6 A). To exclude the possibility that the activation of the ERK pathway could have been the result of the baculoviral infection, I infected Vero rKSHV.219 cells with baculovirus expressing the Orf73 protein of murine herpesvirus 68 (baculovirus (BV) MHV68 Orf73). As shown in Fig. 6 B, ERK activation was not induced when the cells were infected by this irrelevant baculovirus.

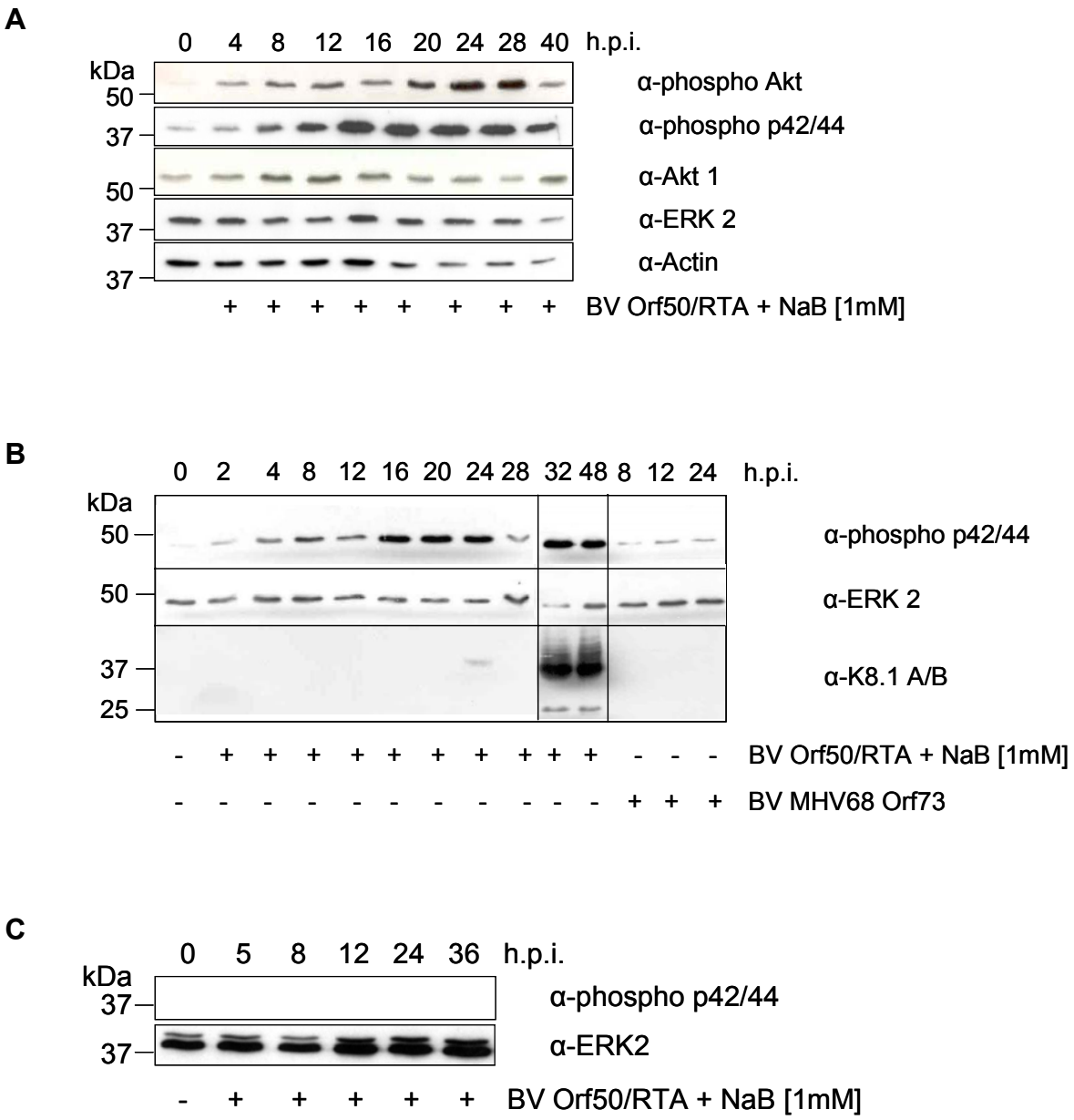


Figure 6. ERK and Akt signalling pathways are activated during the KSHV lytic cycle. (A) Lytic replication was induced in Vero rKSHV.219 cells using baculovirus Orf50/RTA (BV Orf50/RTA) in combination with Na-butyrate (NaB) [1mM]. Cells were lysed 4, 8, 12, 16, 20, 24, 28, and 40 hours post induction (h.p.i.), subjected to Western blot analysis. Equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose, probed with phosphospecific antibody to Akt (S473), or p42/44 (ERK 2/1), respectively, stripped and reprobed with antibody that recognized total Akt levels, or ERK 2 levels, respectively. The membranes were stripped a second time and reprobed with antibody to *β*-actin. (B) Expression of the viral late lytic protein K8.1 was detected 24 h.p.i. and was strongly increased 32 and 48 h.p.i., indicating successful viral DNA replication and the progression of the lytic replication cycle. A baculovirus expressing the Orf73 protein of murine herpesvirus 68 (BV MHV68 Orf73) only very weakly induced ERK activation in Vero rKSHV.219 cells. (C) BV Orf50/RTA and NaB did not activate ERK signalling in uninfected (KSHV negative) Vero cells.

Furthermore, I exposed uninfected (i.e. KSHV-negative) Vero cells to BV Orf50/RTA and Na-butyrate as above, and no activation of the ERK pathway was seen in this experiment.

(Fig. 6 C). Therefore, I concluded that new viral protein synthesis is necessary for lytic cycle-induced ERK activation and that this activation may be mediated by KSHV early genes.

To investigate whether activation of the ERK signalling pathway during lytic replication of KSHV occurs independently of the infected cell type and the induction system used in our experiments, I reactivated KSHV in a PEL cell line (BCBL-1) that harbours a tetracycline-inducible RTA expression system (TREx BCBL-1-RTA (Nakamura et al. 2003)). To induce the lytic replication cycle, TREx BCBL1-RTA were stimulated with 1 µg of tetracycline/ml for 2, 4, 8, 10, 16, 20, 24, 27, 32, or 48 h. Whole cell lysates were analysed by immunoblotting with phospho-specific antibodies for ERK1/2. As shown in Fig. 7, activation of ERK was detected after 4 h of tetracycline treatment, and strongly increased after 20 h of tetracycline treatment. These findings suggested that activation of ERK during the lytic replication cycle is independent of both cell type and the method used to reactivate KSHV from latency.

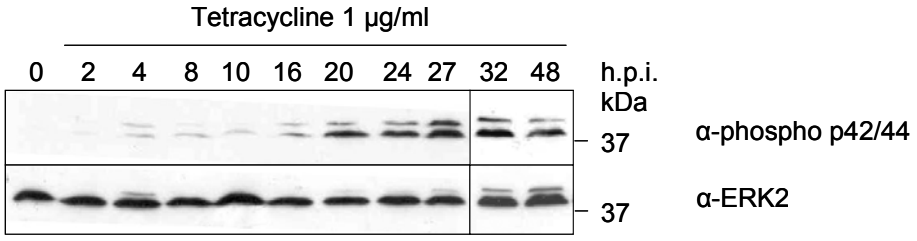


Figure 7. The ERK signalling pathway is activated during lytic replication in the tetracycline-inducible PEL cell line TREx BCBL1-RTA. KSHV lytic replication was induced in TREx BCBL1-RTA by stimulation with 1 µg/ml tetracycline. Cells were lysed at different time points after induction of the lytic cycle (h.p.i.), and protein extracts were analysed by Western blotting with phosphospecific antibodies to p42/44 (ERK2/1). Membranes were stripped and reprobed with antibody to total ERK2.

Activation of ERK is independent of KSHV genome replication while activation of Akt may depend on successful replication of viral DNA.

The temporal pattern of ERK and Akt activation suggests activation of ERK and Akt pathway during the early or late stages of the lytic replication cycle. To investigate, if activation of ERK and Akt signalling depends on viral genome replication, I reactivated KSHV from latency in the presence of phosphonoacetic acid (PAA), an inhibitor of herpesviral DNA polymerase (Chang and Ganem 2000). Vero rKSHV.219 cells were pre-incubated with PAA one hour prior to induction of lytic replication, lytic cycle was induced using baculovirus Orf50/RTA in combination with Na-butyrate, and cells were lysed at 4, 8, 10, 12, 16, 20, 24, and 48 h.p.i..

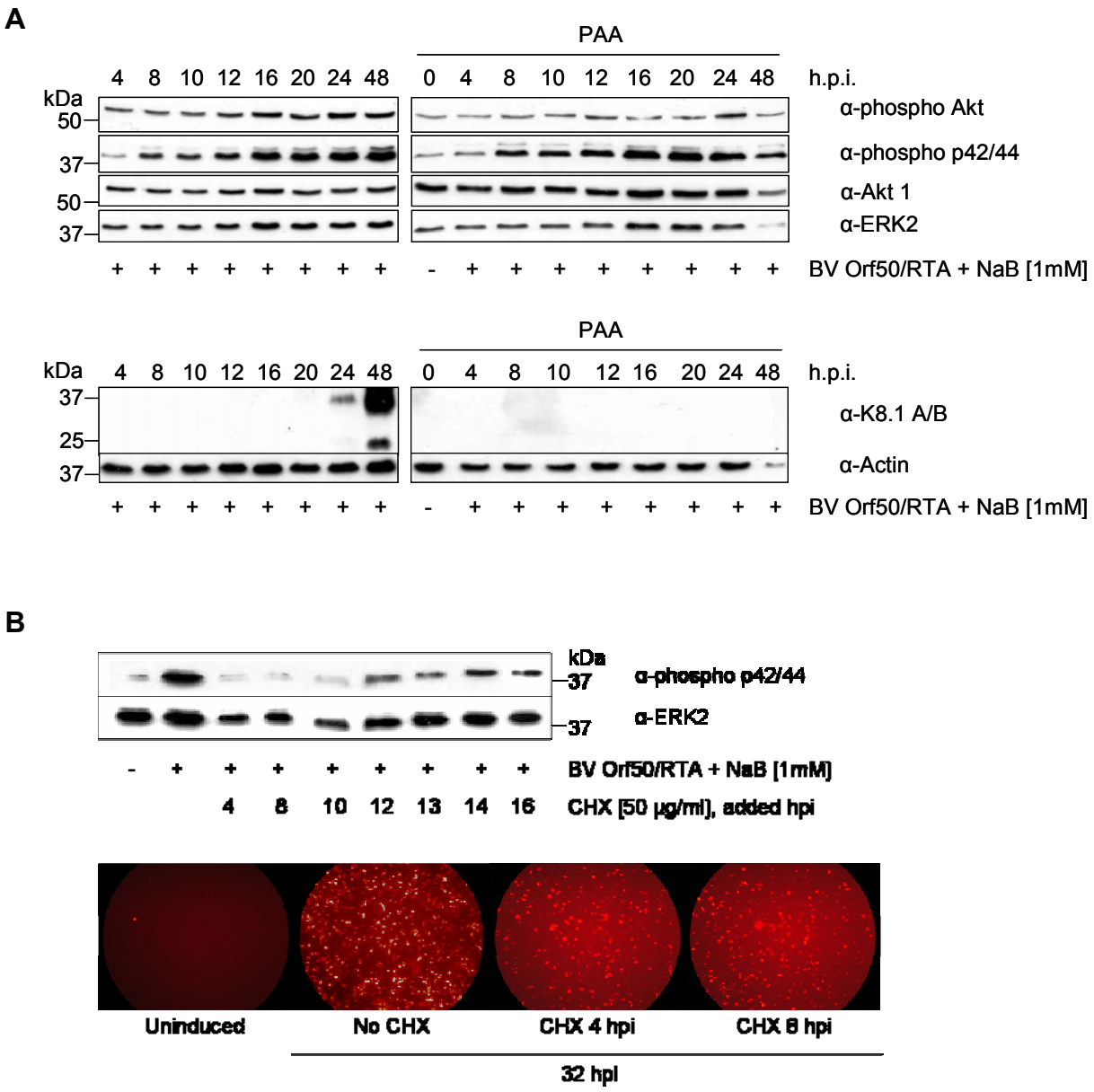


Figure 8. Effect of chemical manipulation of the KSHV lytic replication cascade on ERK and Akt activation. (A) Akt activation may depend on viral DNA replication, while ERK activation does not. Lytic replication was induced in Vero rKSHV.219 cells in the presence (right panel) or absence (left panel) of phosphonoacetic acid (PAA, 1 mM). Cells were lysed 4, 8, 12, 16, 20, 24, and 48 hours after induction (h.p.i.). Lysates were analysed by Western blot analysis with phosphospecific antibody to Akt, or ERK2/1 (p42/44), or antibody to K8.1 A/B. Membranes were stripped and reprobed with antibody to total Akt 1, or total ERK2, or antibody to *β*-actin. (B) Activation of ERK requires *de novo* protein expression in the first 10 h post induction of lytic cycle. Vero rKSHV.219 cells were reactivated from latency using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB, 1 mM). Cycloheximide (CHX) [50 µg/ml] was added to individual wells 4, 8, 10, 12, 13, 14, or 16 h.p.i.. 32 h.p.i., expression of red-fluorescent protein (RFP) from the lytic PAN promoter was visualized by fluorescence microscopy, cells were lysed and analysed by Western blot with phosphospecific antibodies to ERK2/1 (p42/44). The membrane was stripped and reprobed with antibodies to total ERK2 levels.

Lysates were immunoblotted with anti-phospho Akt and anti-phospho p42/44. The inhibition of viral DNA synthesis by PAA was checked indirectly by immunoblotting with antibody for

the late lytic protein K8.1, which should not be expressed in the absence of KSHV DNA replication (Chang and Ganem 2000). As shown in Fig. 8 A, K8.1 was not expressed in the presence of PAA, indicating that PAA efficiently blocked KSHV genome replication. Whereas activation of ERK was not affected by PAA, Akt activation – although relatively low also in the non-PAA-treated control experiment (Fig. 8 A, left panel) - was reduced in the presence of the viral polymerase inhibitor (Fig. 8 A, right panel); suggesting that activation of ERK is independent of viral DNA replication, while activation of Akt signalling at least partially depends on successful KSHV genome replication.

To narrow down to the time point of initial ERK activation, I reactivated rKSHV in Vero cells in the presence of cycloheximide (CHX), which is traditionally used to distinguish between the immediate-early (independent of *de novo* protein synthesis) and early (requiring synthesis of viral proteins) stages of the herpesviral replication cycle. I induced lytic replication in Vero rKSHV.219 cells and added CHX 4, 8, 10, 12, 13, 14, or 16 h.p.i.. I found that ERK activation during lytic replication was completely inhibited, if CHX was added in the first 10 h after induction, and was still reduced, if CHX was added between 12 and 16 h after reactivation from latency (Fig. 8 B). The present but strongly reduced expression of RFP from the lytic PAN promoter in Vero rKSHV.219 cells in the presence of CHX shows that the KSHV lytic cycle was induced in all samples, but widely inhibited at the early phase by CHX (Fig. 8 B). From this experiment I concluded that new viral protein synthesis is necessary for lytic cycle-induced ERK activation and suggest that activation of ERK may be mediated by KSHV early genes.

Chemical inhibition of MEK reduces late lytic protein expression, and the production of infectious progeny virus.

To determine whether efficient KSHV lytic replication and virus production depend on the activation of ERK and Akt signalling cascades, I induced the KSHV lytic cycle in Vero rKSHV.219 cells in the presence of chemical inhibitors of these pathways (Table 3). Cells were pretreated with the inhibitors for one hour prior to reactivation. KSHV lytic cycle was induced as described above. Cells were lysed 48 h.p.i., and protein extracts were immunoblotted with antibodies for Orf50/RTA, and for the late lytic protein K8.1. Supernatants were collected, filtered, and used to infect HEK293 cells. After two days, the number of GFP-positive (i.e. infected) HEK293 cells was counted to determine the number of infectious virions released from Vero rKSHV.219 cells during the productive cycle.

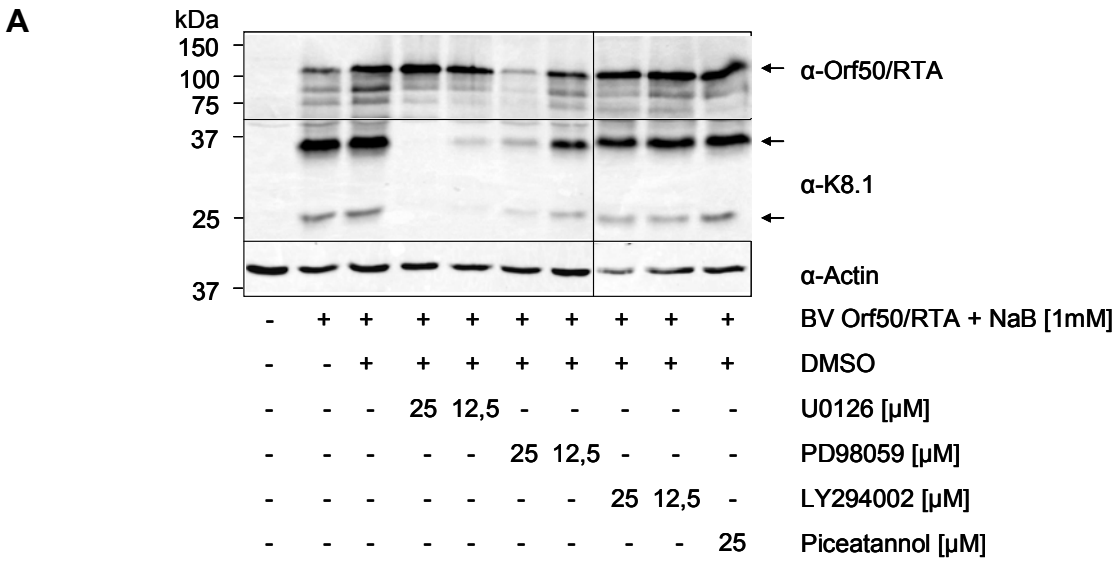
Table 3. Protein kinase inhibitors used to study the role of signalling pathways during KSHV lytic cycle.

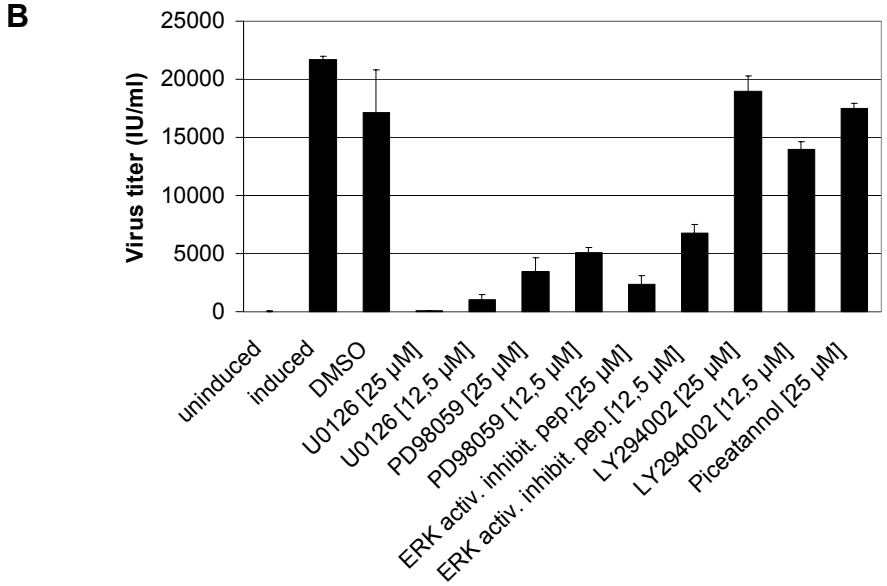
Inhibitor name	Target kinase	Mode of action/description	Target signalling pathway	Signalling pathway involved in
U0126[1] PD98059[2]	MEK1/2	Non-ATP-competitive; prevent the activation of MEK1/2	Ras-Raf-MEK1/2-ERK1/2	Cell growth, differentiation
ERK Activation Inhibitor Peptide I[3]	MEK/ERK1/2	prevents the interaction of ERK1/2 and MEK1/2	Ras-Raf-MEK1/2-ERK1/2	Cell growth, differentiation
LY294002[4]	PI3K	ATP-competitive	PI3K-Akt	Anti-apoptotic processes, cell survival
Piceatannol[5]	Syk	trans-3 ,4 ,3 ',5'-tetrahydroxystilbene (plant metabolite)	MAPK pathways; NFAT activation	Proliferation, differentiation

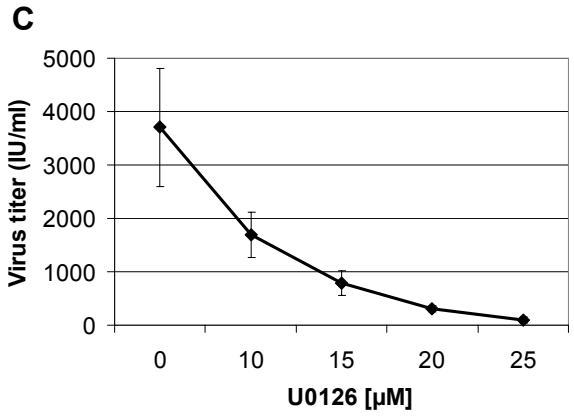
[1] DeSilva et al. 1998; Duncia et al. 1998; Favata et al. 1998; Davies et al. 2000
[2] Kültz et al. 1998; Dudley et al. 1995; Langlois et al. 1995; Davies et al. 2000
[3] Kelemen et al. 2002
[4] Baumann and West, 1998; Vlahos et al. 1995; 1994; Davies et al. 2000
[5] Oliver et al. 1994; Geahlen et al. 1989

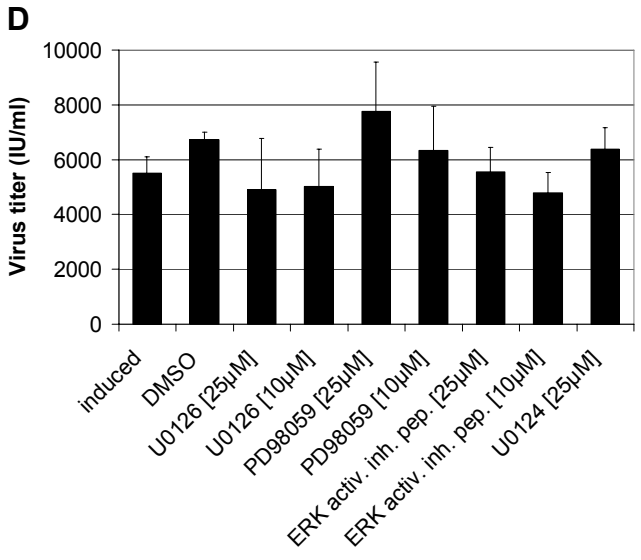
After induction of the KSHV lytic cycle, RTA was strongly expressed in Vero rKSHV.219 cells, except in the presence of PD98059 at a concentration of 25 μM (Fig. 9 A). In contrast, expression of the late lytic protein K8.1 was strongly reduced by both MEK1/2 inhibitors, U0126 and PD98059, in a dose dependent manner (Fig. 9 A). For PD98059 at a concentration of 25 μM it is assumed that at least part of the reduced amounts of K8.1 protein was due to less efficient RTA expression. The inhibiton of the PI3K-Akt pathway by LY294002 and inhibiton of Syk kinase by Piceatannol affected neither RTA nor K8.1 expression (Fig. 9 A).

To confirm that the inhibitors used in these experiments exerted the expected effects on their kinase targets, Vero rKSHV.219 cells were pre-incubated with U0126, PD98059, LY294002 and Piceatannol for one hour prior to reactivation, induced lytic replication as above, lysed the cells 30 h.p.i., and immunoblotted lysates with phosphospecific antibodies (Fig. 9 E). ERK phosphorylation was more strongly inhibited by U0126 than by PD98059, while Akt phosphorylation was not affected by either MEK inhibitor (Fig. 9 E). LY294002 inhibited lytic cycle-induced Akt phosphorylation as expected (Fig. 9 E). Inhibition of Syk by Piceatannol also reduced Akt phosphorylation, while ERK phosphorylation was not negatively affected by Piceatannol (Fig. 9 E)


A
kDa
150
100
75
37
25
37
α-Orf50/RTA
α-K8.1
α-Actin
- + + + + + + + + + BV Orf50/RTA + NaB [1mM]
- - + + + + + + + + DMSO
- - - 25 12,5 - - - - - U0126 [µM]
- - - - - 25 12,5 - - - PD98059 [µM]
- - - - - - - 25 12,5 - LY294002 [µM]
- - - - - - - - - 25 Piceatannol [µM]


B
Virus titer (IU/ml)
25000
20000
15000
10000
5000
0
uninduced
induced
DMSO
U0126 [25 µM]
U0126 [12,5 µM]
PD98059 [25 µM]
PD98059 [12,5 µM]
ERK activ. inhibit. pep.[25 µM]
ERK activ. inhibit. pep.[12,5 µM]
LY294002 [25 µM]
LY294002 [12,5 µM]
Piceatannol [25 µM]


C
Virus titer (IU/ml)
5000
4000
3000
2000
1000
0
0
10
15
20
25
U0126 [µM]


D
Virus titer (IU/ml)
10000
8000
6000
4000
2000
0
induced
DMSO
U0126 [25µM]
U0126 [10µM]
PD98059 [25µM]
PD98059 [10µM]
ERK activ. inh. pep. [25µM]
ERK activ. inh. pep. [10µM]
U0124 [25µM]

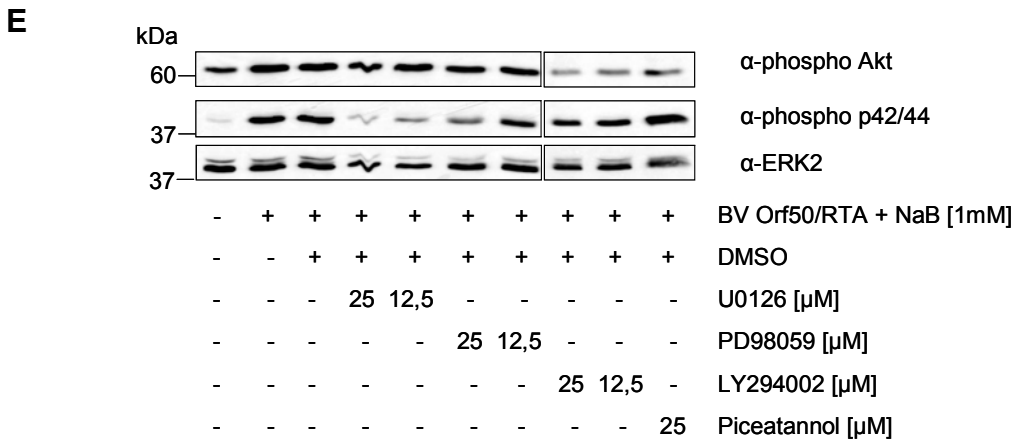
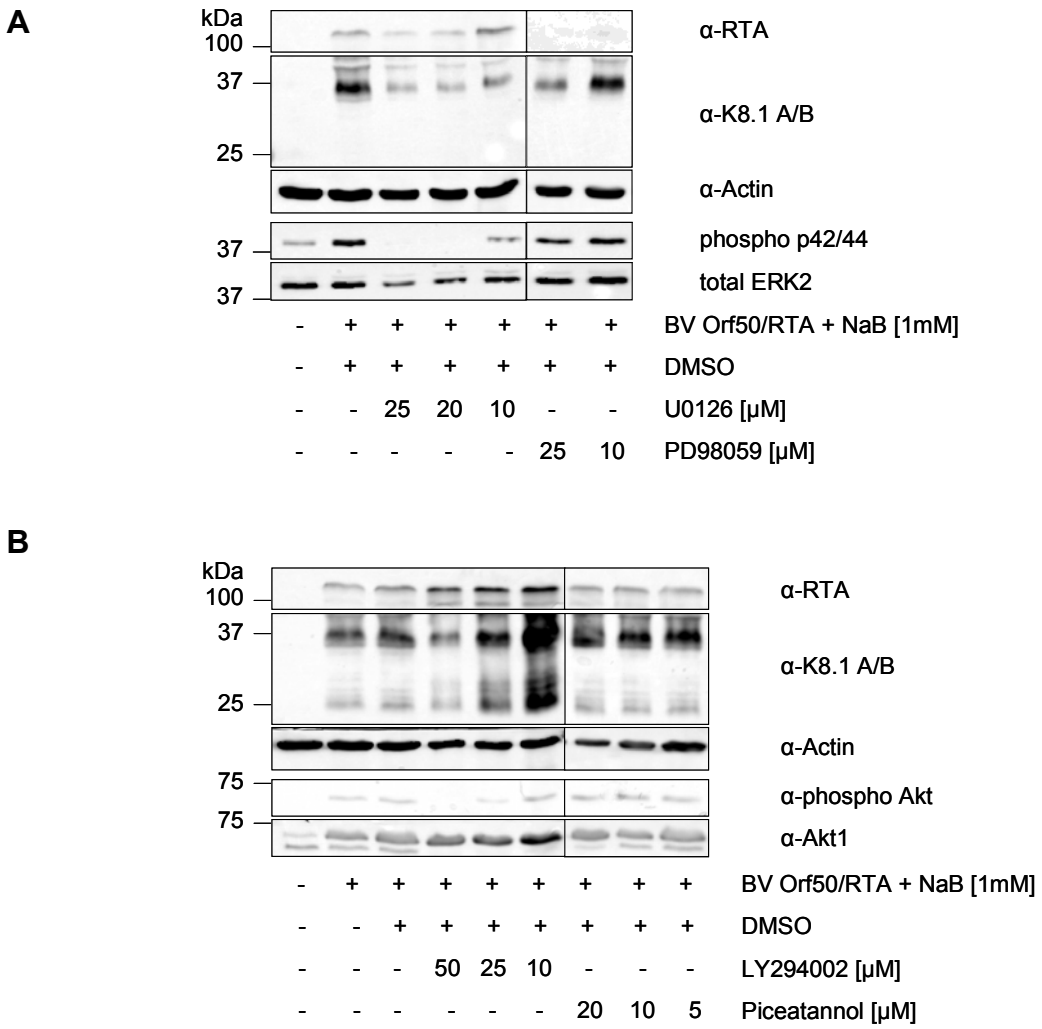


Figure 9. Inhibition of MEK/ERK signalling during KSHV lytic cycle reduces expression the viral late lytic K8.1 protein and virus production. (A) Expression of the late lytic protein K8.1 and is reduced by inhibition of MEK1/2 signlling. Vero rKSHV.219 cells were incubated with chemical kinase inhibitors at two concentrations 1h prior to induction of lytic replication. DMSO (i.e. solvent of all inhibitors) was applied for negative control. Lytic cycle was induced using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB, 1mM). 48h after induction (h.p.i.), cells were lysed and analysed by Western blotting with antibodies to Orf50/RTA, K8.1 A/B, and *β*-actin. (B) Inhibition of MEK1/2-ERK signalling reduces the yield of infectious progeny virus from Vero rKSHV.219 cells. Vero rKSHV.219 cells were incubated with chemical inhibitors or ERK activation inhibitor peptide I (ERK activ. inhibit. pep.) and the lytic replication cycle was induced as above. 48 h.p.i., culture supernatants were collected and used to infect HEK293 cells. Two days after infection, the number of infected (i.e. green-fluorescent) HEK293 cells was counted, and virus titers (infectious units (IU)/ml) were calculated. Results represent the averages and standard deviations from one experiment carried out in triplicates. (C) U0126 inhibits virus production in a dose-dependent manner. Shown are the results of an infectivity assay on HEK293 cells with supernatant of Vero rKSHV.219 cells. (D) MEK inhibitors have no significant effect on virus entry in infectivity assay. U0126, its inactive analogue U0124, PD98059, and the ERK activation inhibitor peptide I were incubated with BV Orf50/RTA and NaB for 48h at 37°C. These pre-incubated inhibitors were then applied at their indicated final concentrations to HEK293 cells, which were then infected with rKSHV. The number of infected target cells was determined after two days, and virus titers were calculated. Bars represent average virus titers and standard deviations of one experiment carried out in triplicates. (E) Chemical kinase inhibitors reduce lytic cycle-induced activation of ERK and Akt in Vero rKSHV.219 cells. Vero rKSHV.219 cells were incubated with chemical kinase inhibitors and the lytic cycle was induced as described above. 36 h.p.i., cells were lysed and analysed by Western blot with phosphospecific antibodies to Akt (S473) and p42/44 (ERK2/1). The membrane was then stripped, and reprobed with antibody that recognized total ERK2 levels.

Inhibition of the MEK-ERK signalling cascade by U0126 and PD98059 also reduced late-lytic K8.1 expression in the endothelial cell line EAhy rKSHV (see Materials and Methods) (Fig. 10 A). However, the effect of both inhibitors on K8.1 expression was weaker than observed in Vero rKSHV.219 cells, although U0126 completely abolished lytic cycle-induced ERK activation in EAhy rKSHV cells, when applied at a final concentration of 25 or 20 μM (Fig 10 A).

The infectivity assay showed similar results. Inhibition of MEK1/2 by U0126 or PD98059 drastically reduced the titer of infectious virus released from treated Vero rKSHV.219 cells in a dose-dependent manner (Fig. 9 B, C). In accordance with the reduction in KSHV K8.1 protein expression (Fig. 9 A), U0126 showed a more pronounced inhibition than PD98059. In addition, peptide-based inhibition of ERK activation also reduced virus production (Figure 9 B). Since inhibition of ERK by this peptide is thought to be specific because of its ability to compete with the MEK-ERK2 interaction (Kelemen et al. 2002), these findings provide strong evidence for a role of MEK/ERK pathway during the progression of the lytic replication cycle. No effect on virus production from Vero rKSHV.219 cells was seen after inhibition of PI3K using LY294002, nor after inhibition of Syk kinase using Piceatannol (Fig. 9 B).



C

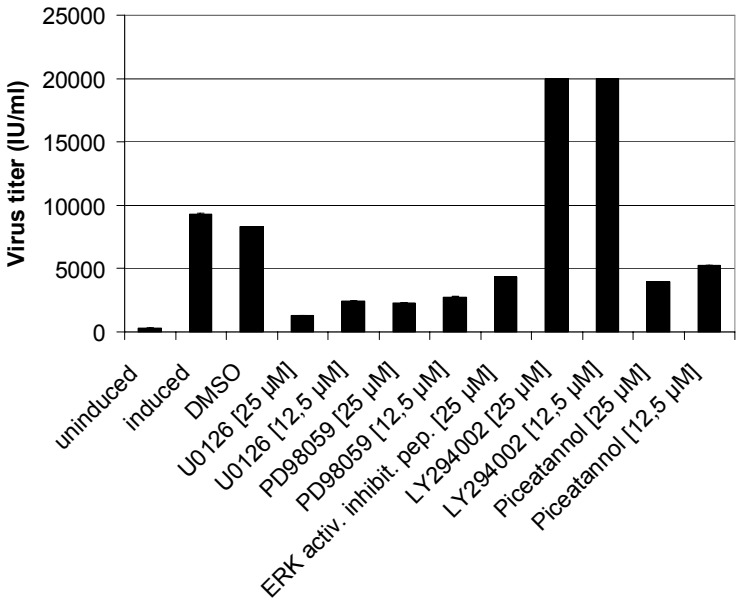


Figure 10. Inhibition of MEK/ERK signalling during KSHV lytic cycle reduces late lytic K8.1 protein expression in EAhy rKSHV cells. (A) Expression of the late lytic protein K8.1 and is reduced by inhibition of MEK1/2 signal transduction. The MEK inhibitors U0126 and PD98059 were applied individually at different concentrations to EAhy rKSHV cells. DMSO (i.e. solvent of all inhibitors) was applied for negative control. After 1h, the lytic replication cycle was induced using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB, 1mM). 48 hours after induction (h.p.i.), cell lysates were analysed by Western blotting with antibodies to Orf50/RTA, K8.1 A/B, and a phosphospecific antibody to p42/44. Membranes were stripped and reprobed with antibodies to *β*-actin and total ERK2. (B) Inhibition of Akt and Syk signalling does not reduce K8.1 expression in EAhy rKSHV cells. EAhy rKSHV cells were incubated with LY294004, which targets PI3 kinase, or with Piceatannol, which targets Syk kinase, at different final concentrations 1h prior to reactivation. Lytic cycle was induced as in A, cells were lysed 48 h.p.i., and analysed by Western blotting with antibodies to Orf50/RTA, K8.1 A/B, and a phosphospecific antibody to Akt. Membranes were stripped and reprobed with antibodies to *β*-actin and total Akt1. (C) Inhibition of the MEK/ERK pathway reduces virus production in EAhy rKSHV cells, while inhibition of PI3K by LY294002 enhances the yield of infectious progeny virus. EAhy rKSHV cells were treated with individual kinase inhibitors and the lytic replication cycle was induced as described above. Culture supernatants were harvested 48 h.p.i., centrifuged, and used to infect HEK293 cells. The number of infected (i.e. green-fluorescent) 293 cells was determined after 2 days, and virus titers were calculated. Bars represent averages and standard deviations of one experiment carried out in triplicates.

In contrast, when EAhy rKSHV cells were used in a similar experiment, inhibition of PI3K by LY294002 consistently produced an enhancement of virus production (Fig. 10 C,), while inhibition of Syk kinase by Piceatannol reduced the yield of infectious progeny virus from EAhy rKSHV cells by 30-50 % in a dose-dependent fashion. Western blot analysis showed that also expression of the RTA and the K8.1 protein was enhanced in the presence of LY294002 during the lytic replication cycle in EAhy rKSHV cells (Fig. 10 B), whereas Piceatannol had no effect on RTA and K8.1 protein levels (Fig. 10 B).

To exclude the possibility that the reduced numbers of GFP-positive target HEK293 cells in our infectivity assay in the presence of ERK inhibitors were simply due to inhibition of virus entry (Pan et al. 2006), mediated by carry-over of MEK inhibitors, I incubated U0126, its inactive analogue U0124, PD98059, and the ERK activation inhibitor peptide I at a concentration of 25 μM and 10 μM together with baculovirus Orf50/RTA and Na-butyrate in regular growth medium for two days at 37°C (i.e. the duration of the incubation phase used in Figs. 9 and 10). The inhibitors were then transferred to HEK293 cell cultures, which were subsequently infected with newly produced rKSHV. In this experiment I did not observe a significant reduction of infectivity by U0126, PD98059 and the ERK activation inhibitor peptide that had been pre-incubated at 37°C for two days (Fig. 9 D).

Since ERK activity during the lytic cycle did not considerably increase before eight hours after reactivation from latency (Fig. 6 A), the effect of U0126 on late lytic protein synthesis and virus production was investigated when the inhibitor was applied at different time points after induction of the lytic replication cycle. Lytic replication in Vero rKSHV.219 cells was induced using baculovirus Orf50/RTA, and U0126 was applied at a final concentration of 25 μM at 0 (t_0), 1 (t_1), 2 (t_2), 4 (t_3), 8 (t_4), 16 (t_5), or 24 (t_6) hours after induction. 48 hours after induction, cells were lysed and protein extracts were subjected to Western blot analysis. Culture supernatants were used to infect HEK293 cells. Although the level of K8.1 expression and virus production varied slightly in a non-specific order among the different samples (Fig. 11 A, B), I found that U0126 severely inhibited K8.1 protein expression and virus production when added until eight hours after reactivation from latency (Fig. 11 A, B). When the inhibitor was applied later than eight hours after induction of the lytic replication cycle, the reducing effect on K8.1 protein expression and virus production was less prominent but still significant even when U0126 was applied at 16 or 24 hours after reactivation (Fig. 11 A, B). Since U0126 is unstable in solution, I analysed, if the inhibitor was still fully active at t_5 and t_6 (i.e. 16 and 24 hours after its reconstitution). Therefore, U0126 was applied at t_5 and t_6 to Vero rKSHV.219 cells, in which KSHV lytic replication was just then induced. Cells were lysed and culture supernatant was harvested 48 hours after reactivation. As shown in Fig. 11 A and B, U0126 completely blocked late-lytic K8.1 expression and virus production 16 or 24 hours after its reconstitution when applied to freshly reactivated cells, indicating that the activity of the inhibitor had not deteriorated at t_5 and t_6. These findings suggest that inhibition of ERK activity after eight and even after 16 hours postreactivation sufficiently blocks KSHV lytic replication.

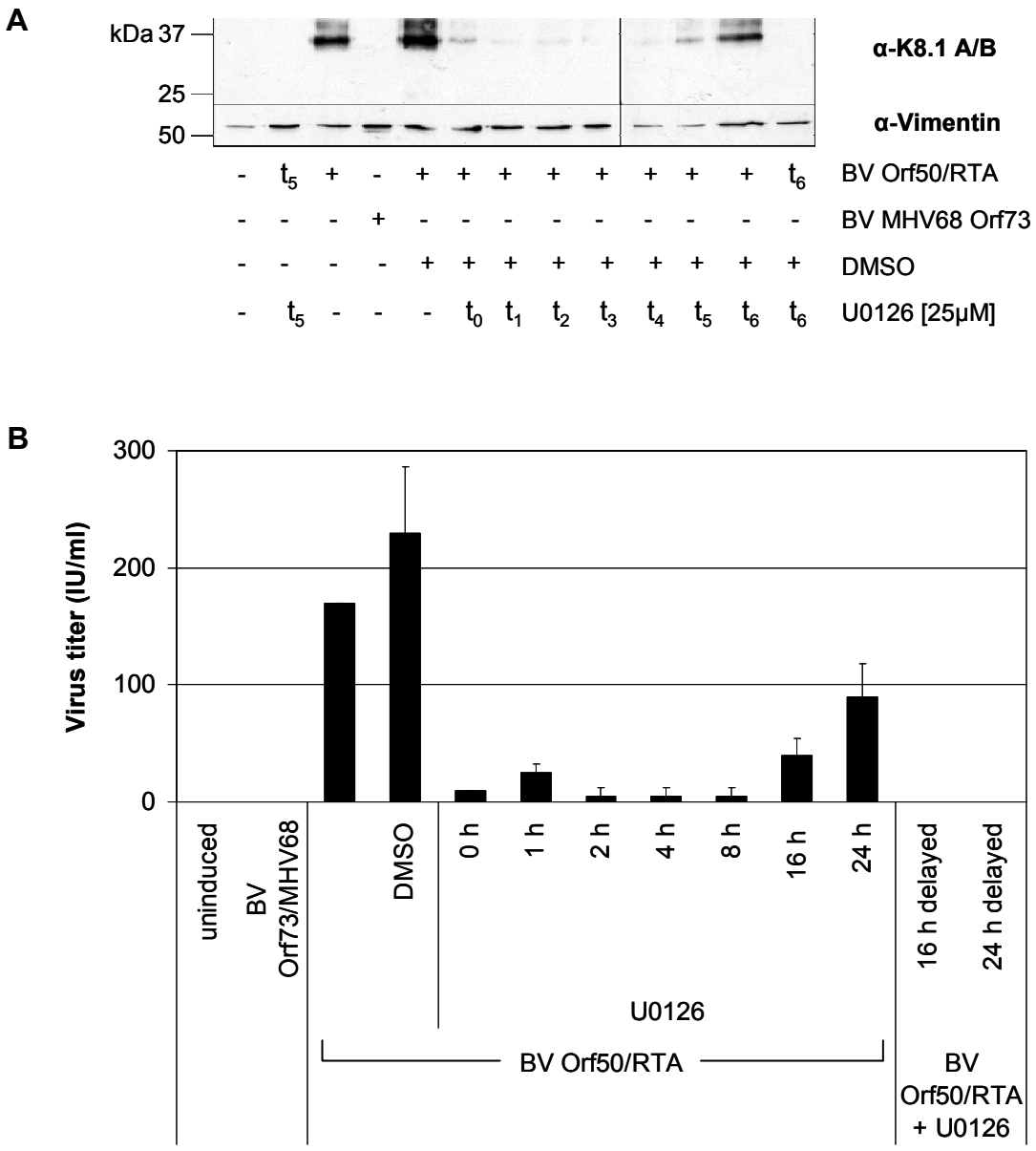


Figure 11: U0126 still reduces expression of the K8.1 protein and virus production when applied at the early or late phase of the lytic replication cycle. Vero rKSHV.219 cells were reactivated with baculovirus (BV) Orf50/RTA and treated with U0126 (25 µM) at 0 (t_0), 1 (t_1), 2 (t_2), 4 (t_3), 8 (t_4), 16 (t_5), or 24 (t_6) hours after induction (h.p.i.) of the lytic replication cycle. 16 h.p.i. (t_5) and 24 h.p.i. (t_6), U0126 was applied in parallel to Vero rKSHV.219 cells, which were just then reactivated from latency. 48 h after reactivation, the cells were lysed and supernatants were harvested. (A) Protein extracts were analysed by Western blotting with an antibody to late lytic K8.1 glycoprotein. Membranes were stripped and reprobed with an antibody to vimentin. (B) Culture supernatants were centrifuged and used to infect HEK293 cells. Infected cells were counted after two days, and virus titers were calculated. By analysing the effect of the inhibitor on K8.1 expression and virus production at postponed t_0, we determined the remaining activity of U0126 16 or 24 hours, respectively, after its reconstitution. A baculovirus expressing the Orf73 protein of murine herpesvirus 68 (BV MHV68 Orf73) did not induce lytic replication in Vero rKSHV.219 cells. DMSO (i.e. solvent of U0126) was used for negative control. Bars represent average titers and standard deviations of one experiment carried out in triplicates.

The effect of the two MEK inhibitors U0126 and PD98059 on late lytic protein expression was also determined in the tetracycline-inducible PEL cell line TREx BCBL1-RTA. To induce the lytic replication cycle, TREx BCBL1-RTA cells were stimulated with 0.5 µl tetracycline/ml. U0126 and PD98059 were applied at a final concentration of 20 µM or 25 µM, respectively, one hour prior to reactivation (t_{-1}) or together with tetracycline (t_0). 48 hours after reactivation, the cells were lysed, and expression of the K8.1 protein was determined by immunoblotting. Both MEK inhibitors efficiently blocked K8.1 expression during tetracycline-induced lytic replication in TREx BCBL1-RTA cells (Fig. 12). However, when I additionally stimulated the cells with baculovirus Orf50/RTA, the block in late lytic K8.1 expression mediated by inhibition of MEK was overcome (Fig. 12). Accordingly, when the cells were stimulated with high concentrations of tetracycline, i.e. ≥ 1 µg/ml, U0126 and PD98059 were not sufficient to significantly reduce K8.1 expression (data not shown). Therefore, I suggest that (i) U0126 and PD98059 are less efficient MEK inhibitors in BCBL-1 cells, or (ii) strong stimulation of KSHV reactivation induces other factors, e.g. other signalling pathways, which can substitute for the role of MEK signalling for efficient lytic replication in this cell line.

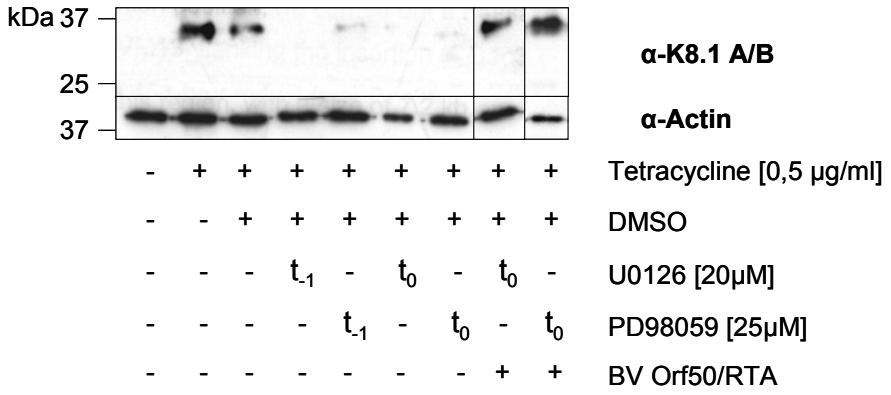


Figure 12. Inhibitors of MEK/ERK signalling reduce expression of K8.1 protein in the tetracycline-inducible PEL cell line TREx BCBL1-RTA. U0126 (20 µM) and PD98059 (25 µM) were applied individually to TREx BCBL1-RTA cells one hour prior (t_{-1}) or simultaneously (t_0) to stimulation with 0.5 µg/ml tetracycline or to combined treatment with 0.5 µg/ml tetracycline and baculovirus (BV) Orf50/RTA. Cells were lysed 48 h after reactivation, and protein extracts were analysed by immunoblotting with antibody to K8.1. Membranes were stripped and reprobed with antibody to *β*-actin.

Together, these findings suggest that activation of MEK1/2-ERK1/2 signalling is important for KSHV lytic replication downstream of RTA expression, and that inhibition of MEK1/2 strongly reduces KSHV lytic protein expression and virus production, even when

RTA is abundant in the cell. In contrast, activation of the Akt pathway appears dispensable for efficient lytic replication of KSHV and may even suppress KSHV lytic replication in certain cell types (e.g. endothelial cells).

U0126 does not inhibit cell cycle progression from G_0/G_1 to S phase.

The Ras/ERK signalling pathway is known to regulate various cellular responses. In particular, its role in cell cycle progression in G_1 phase and cell proliferation is well established (Massague 2004; Torii et al. 2006; Yamamoto et al. 2006). It has been shown that PD98059 inhibits stimulation of cell growth in fibroblast (Dudley et al. 1995), and that PD184352 (CI-1040), another inhibitor of the MEK-ERK signalling cascade, inhibits serum-stimulated cyclin D1 expression and DNA synthesis in an ERK 1/2-dependent manner (Squires et al. 2002). Therefore, I tested the possible effect of U0126 on cell cycle progression.

Since it was shown that BCBL-1 cells are most susceptible to induction of lytic replication during S-phase (McAllister et al. 2005), a block of S-phase entry mediated by U0126 might create unfavourable conditions for efficient KSHV lytic replication. To determine the proportion of cells in each phase of the cell cycle, I used a bromodeoxyuridine (BrdU) incorporation assay, and analysed U0126-treated and non-treated Vero rKSHV.219 cells in a fluorescence-activated cell sorter (FACS)-based cell cycle assay. BrdU is an analogue of thymidine and commonly used in the detection of proliferating cells in living tissues. It works by substituting for thymidine during DNA replication and incorporating itself into the newly synthesized DNA (during S-phase). Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. Propidium iodide was used to stain the total amount of DNA in the cells. Vero rKSHV.219 cells were seeded at $1x10^5$ cells/well on six-well plates. The lytic replication cycle was induced after 24 h using baculovirus Orf50/RTA in the presence or absence of U0126 at a final concentration of 25 μM. 48 hours after reactivation, cells were identified at a proportion of ~ 50% in G_1/G_0 phase, ~ 18% in S phase, and ~ 7 % in G_2/M phase (Fig. 13 A, cells located in region (R) 1, 2, and 3, respectively; Fig. 13 B). The remaining percentages laid outside the regions that were set for each cell cycle phase for analysis (Fig. 13 A, upper panel). U0126-treated cells showed cell cycle distributions similar to those of non-inhibitor treated cells (Fig. 13 A, B), implying that the inhibitor does not affect the cell cycle distribution of Vero rKSHV.219 cells.

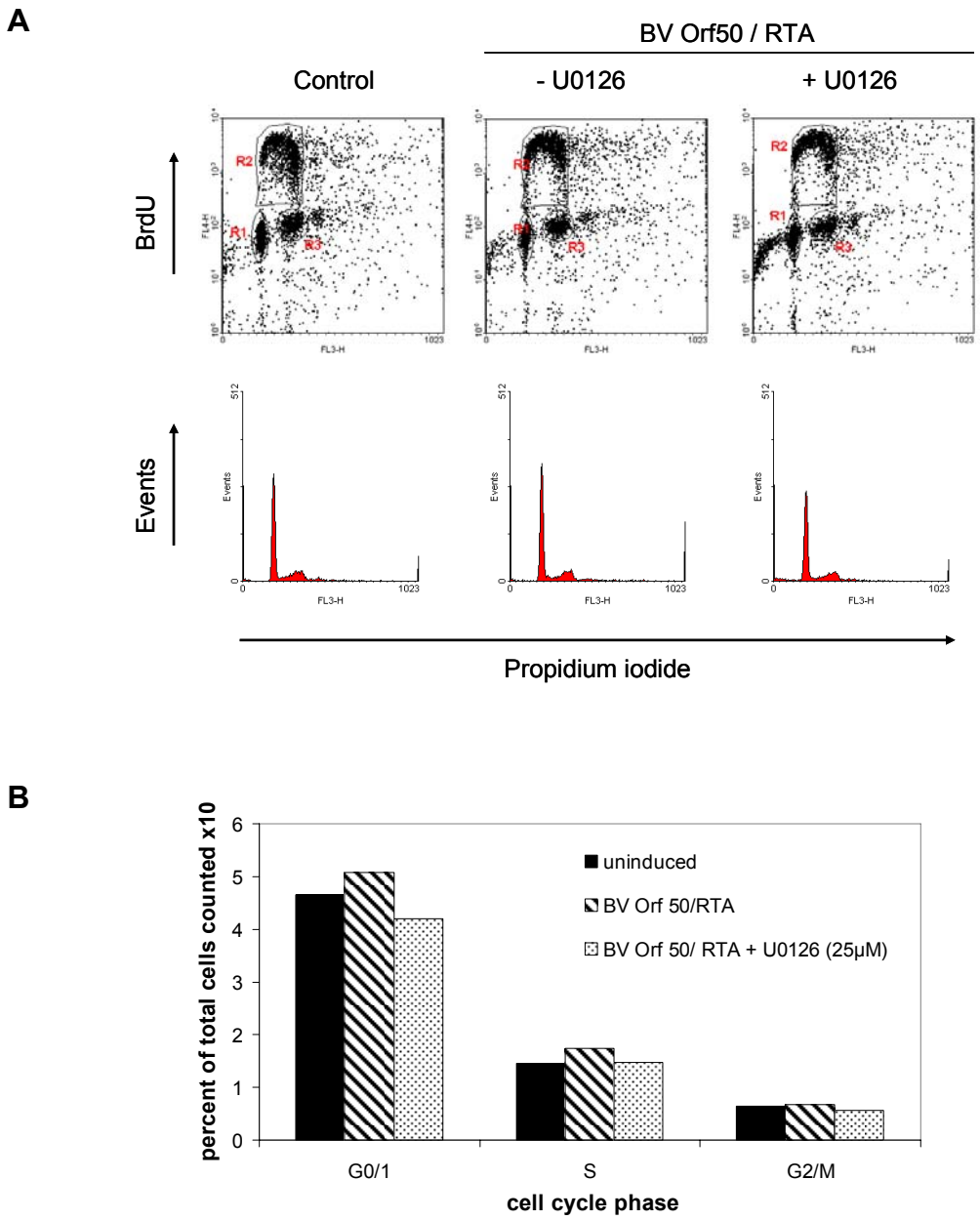


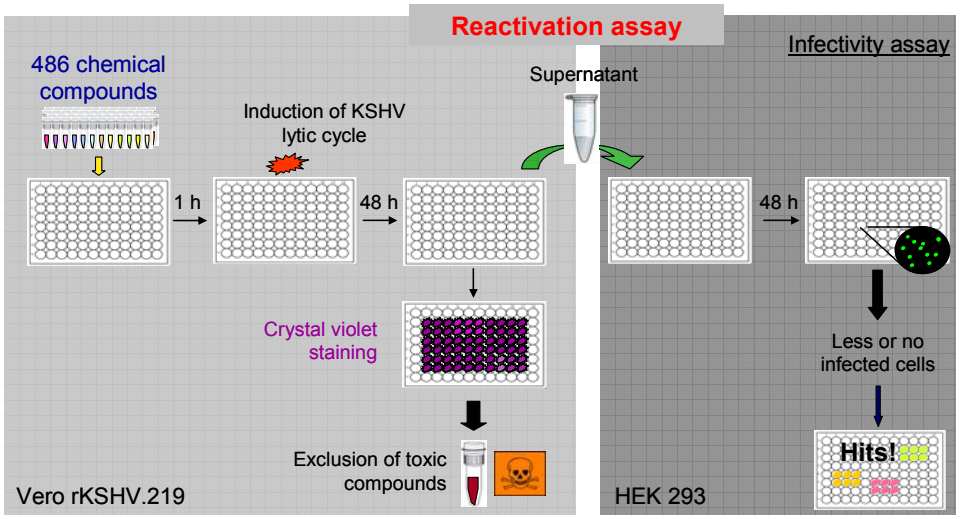
Figure 13. U0126 does not induce G0/1 cell cycle arrest in Vero rKSHV.219 cells. (A, B) Cell cycle analysis of U0126-treated and non-treated Vero rKSHV.219 cells. Briefly, Vero rKSHV.219 cells were incubated with U0126 at a finl concentration of 25µM one hour prior to reactivation using baculovirus (BV) Orf50/RTA. 48 hours after induction, cells were BrdU pulsed for 45 min by adding BrdU to a final concentration of 10 µM. Subsequently, cells were paraformaldehyde fixed, permeabilized, and labeled with a primary BrdU-specific antibody and a secondary Cy5-conjugated antibody. Finally, cells were resuspended in PBS containing propidium iodide (PI) and flow cytometric analysis was performed. A total of 10^5 cells were measured in each sample by using a BD FACSCalibur. Data were analyzed using the software Windows Multiple Document Interface for Flow Cytometry 2.8 (WinMDI2.8). (A, upper panel) Cells were analyzed for BrdU positivity (*y* axis, log scale) in relation to their DNA content (*x* axis, linear scale) depicted as dot plot. Cells with a DNA content of 2n (diploid) and negative for BrdU are shown in region R1 (i.e. cells in G0/1 phase); cells with a DNA content of ≥2n and ≤4n (tetraploid) and which were positive for BrdU are shown in region R2 (i.e. cells in S-phase); cells with a DNA content of 4n and which were negative for BrdU are found in region R3 (G2/M phase). The lower panel shows the distribution of all cells counted (y-axis, linear scale) to individual cell cycle phases (DNA content, x-axis, linear scale). Control = uninduced, no inhibitor. (B) Percentage of cells in each individual cell cycle state.

A systematic screen of chemical compounds to identify new inhibitors of KSHV lytic replication.

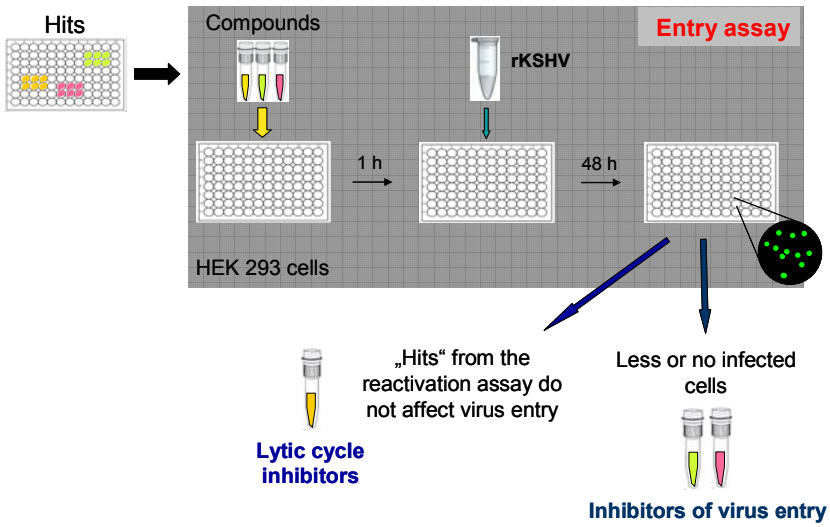
In order to identify novel kinase inhibitors that could interfere with the production of infectious virions we screened 486 chemical compounds from the core validation library of the biotech research company VICHEM CHEMIE (Budapest, Hungary) in a virus reactivation assay. The screen was conducted in Vero rKSHV.219 cells in a 96-well format. Figure 14 shows a schematic illustration of the screening system and representative results from the initial screening phase. Briefly, 486 kinase inhibitors were applied individually at final concentrations of 10 µM and 5 µM to Vero rKSHV.219 cells. After one hour of incubation, the lytic replication cycle was induced using baculovirus Orf50/RTA in combination with Na-butyrate. Culture supernatants were harvested 48 hours after induction, centrifuged, and used to infect HEK293 cells. The relative number of infected (i.e. green fluorescent) target HEK293 cells was determined after two days by measuring the overall fluorescence intensity in the wells using a Biotek plate reader. Diminished fluorescence intensity in comparison to controls (i.e. infection with supernatant of non-inhibitor-treated cells) reflected fewer rKSHV-infected cells, suggesting that virus production had been inhibited during lytic replication in Vero rKSHV.219 cells in the presence of the respective compound. To exclude toxic compounds, reactivated and inhibitor-treated Vero rKSHV.219 cells were fixed with paraformaldehyde (PFA), stained with crystal (Gentiana) violet, and total absorbance was measured using a Biotek plate reader. Reduced absorbance in comparison to non-inhibitor treated controls reflected reduced cell numbers, indicating toxicity of the compound that had been applied to the respective well. Toxic compounds were excluded from the list of potential "hits" identified in the first round of the screen, but were further titrated in a second round of reactivation assay to identify non-toxic concentrations and analyse their effect on lytic replication.

To differentiate between true inhibitors of the lytic replication cycle and inhibitors of virus entry, we tested compounds with inhibitory activity in a virus-entry assay. Therefore, target HEK293 cells were incubated with fresh inhibitors one hour prior to infection with an rKSHV virus preparation and the number of infected cells was determined after two days as described above. Figure 14 C shows representative results of the first screening round. Compounds 3617 and 4550 were identified as "hits" in reactivation assay and were subjected to further analysis and titrations (Fig. 14 D, not shown for 3617). Compound 5802 was toxic to the cells when applied at a final concentration of 10 or 5 µM, and was therefore further titrated in a second screening round (data not shown).

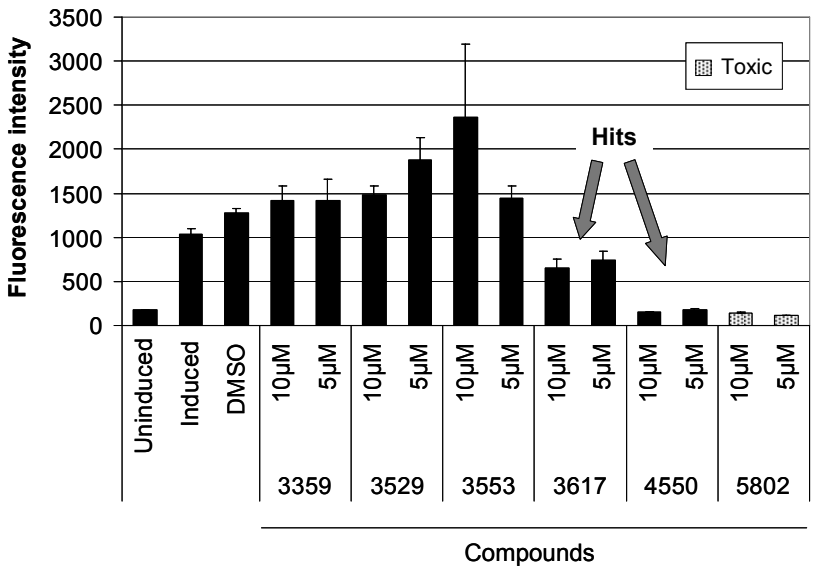
A


Reactivation assay
Infectivity assay
486 chemical compounds
Induction of KSHV lytic cycle
Supernatant
1 h
48 h
48 h
Crystal violet staining
Less or no infected cells
Exclusion of toxic compounds
Hits!
Vero rKSHV.219
HEK 293

B


Hits
Compounds
Entry assay
rKSHV
1 h
48 h
HEK 293 cells
„Hits“ from the reactivation assay do not affect virus entry
Less or no infected cells
Lytic cycle inhibitors
Inhibitors of virus entry

C


Fluorescence intensity
3500
3000
2500
2000
1500
1000
500
0
Toxic
Hits
Uninduced
Induced
DMSO
10µM
5µM
3359
3529
3553
3617
4550
5802
Compounds

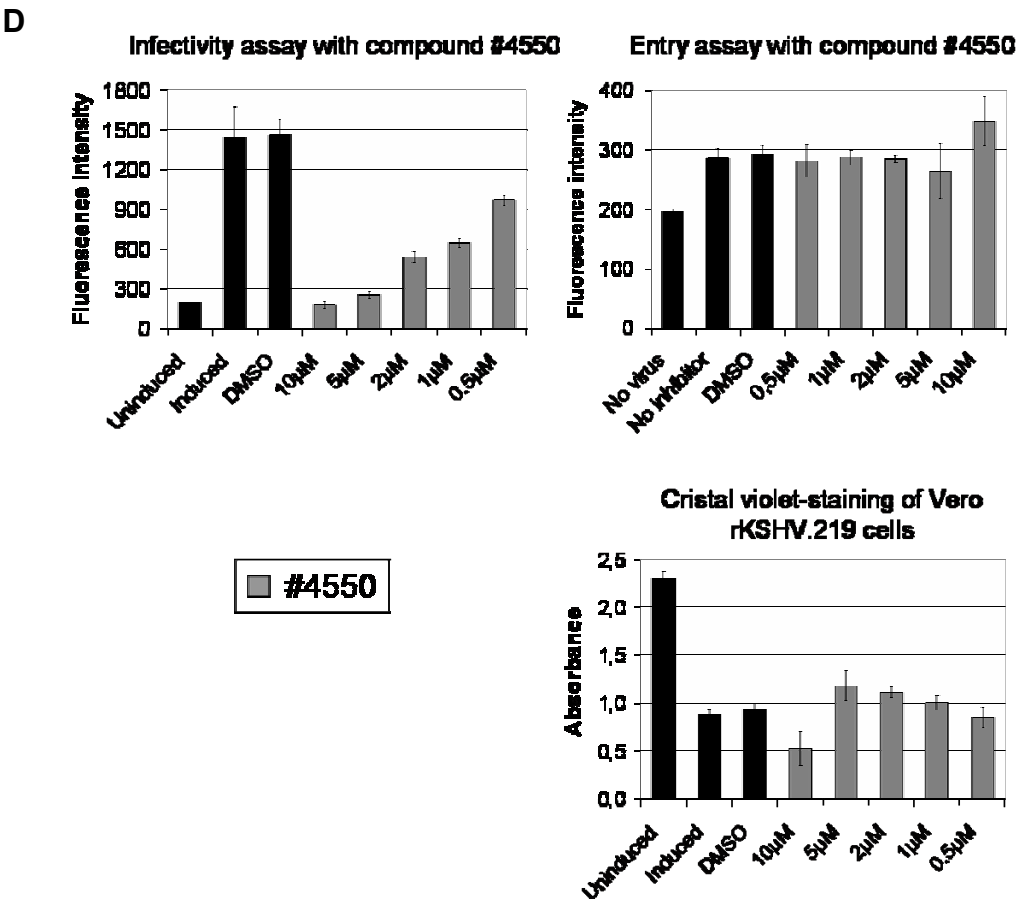


Figure 14. Schematic illustration of the screen system and primary results.
(A, B) Overview of the reactivation assay and the virus-entry assay. (A) Vero rKSHV.219 cells were incubated with individual kinase inhibitors at two different concentrations one hour prior to reactivation using baculovirus Orf50/RTA in combination with Na-butyrate (1mM). Culture supernatants were harvested after 48 hours, centrifuged, and used to infect 293 cells. Two days after infection, the overall fluorescence intensity was measured in each well using a Biotek plate reader. Inhibitor-treated and reactivated Vero rKSHV.219 cells were fixed, stained with crystal violet, and absorbance at 590 nm was measured using a Biotek plate reader. Inhibitors of lytic viral replication reduce the number of infectious virus in culture supernatant, which is reflected by reduced numbers of infected target 293 cells i.e. reduced fluorescence intensity in the respective wells. Toxic compounds induce increased cell death of Vero rKSHV.219 cells, which is reflected by reduced absorbance in the respective well. All experiments were carried out in triplicates. (B) Potential inhibitors of KSHV lytic replication identified in the reactivation assay ("hits") were analysed in a virus-entry assay for their effect on virus infectivity. Therefore, 293 cells were incubated with fresh inhibitors one hour prior to infection with an rKSHV virus preparation. The overall fluorescence intensity in each well was measured 48 hours postinfection using a Biotek plate reader. Reduced fluorescence intensity indicated that the respective compound reduced virus infectivity and was therefore classified as virus entry inhibitor. All experiments were carried out in triplicates. (C) Histogram of representative results from the reactivation assay. The reference value for all fluorescence intensities is represented by the fluorescence intensity of the DMSO control (i.e. solvent of all compounds). The arrows indicate the signals and respective inhibitors that showed significantly reduced fluorescence intensity in comparison to DMSO control. Compound 5802 had toxic effects on Vero rKSHV.219 cells during reactivation assay and could therefore not be classified as a "hit". (D) Further analysis of compound 4550. Entry assay shows that the inhibitor had no effect on KSHV infectivity (upper right panel). Therefore, we classified compound 4550 as a lytic cycle inhibitor. Further titration of 4550 in the reactivation assay demonstrated that the inhibitor reduced virus production during lytic replication in a dose-dependent manner with an $IC_{50} \leq 1\mu M$ (left panel), while the inhibitor only showed some toxicity to the cells when applied at a concentration of 10 µM (lower right panel). Bars represent average fluorescence (or absorbance, respectively) and standard deviations of one experiment carried out in triplicates. Similar results were obtained for compound 3617 (data not shown).

After setting up the system and protocol for the screen with 20 initial compounds, screening of all further compounds as well as all entry assays and further titrations were carried out by a technician. In summary, we identified 13 compounds that appeared to specifically target the production of new infectious virus. These are currently being investigated for their cellular and viral targets.

Table 4. Total number of inhibitors of the KSHV lytic replication cycle and virus entry identified by systematic screening.

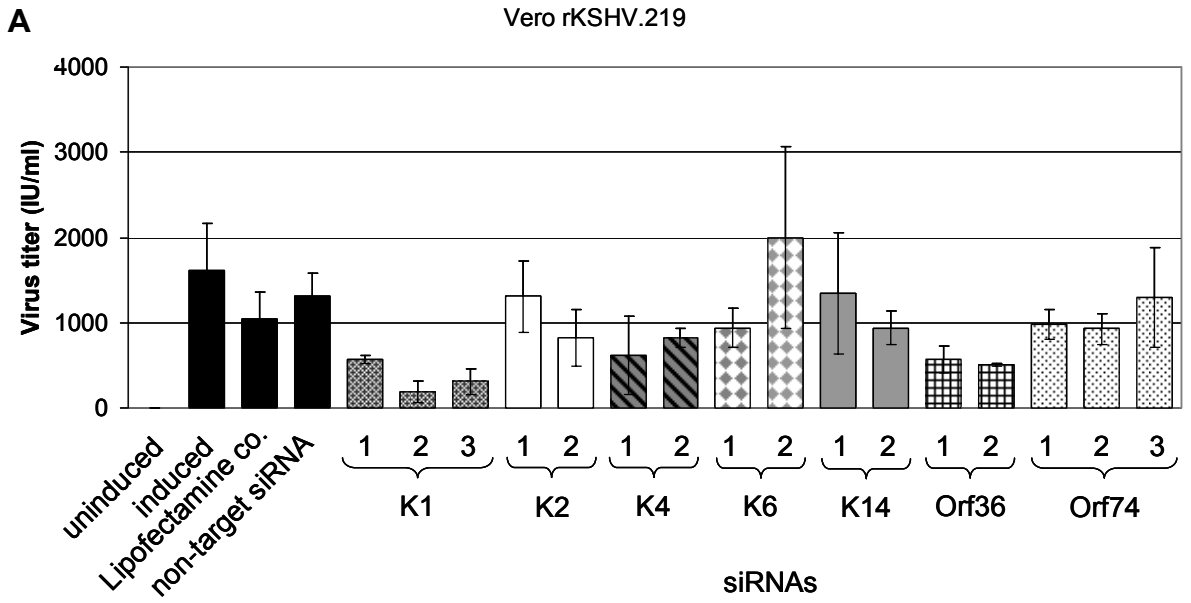
Total number of compounds	Inhibitors of KSHV lytic replication	Inhibitors of virus entry
486	13	33

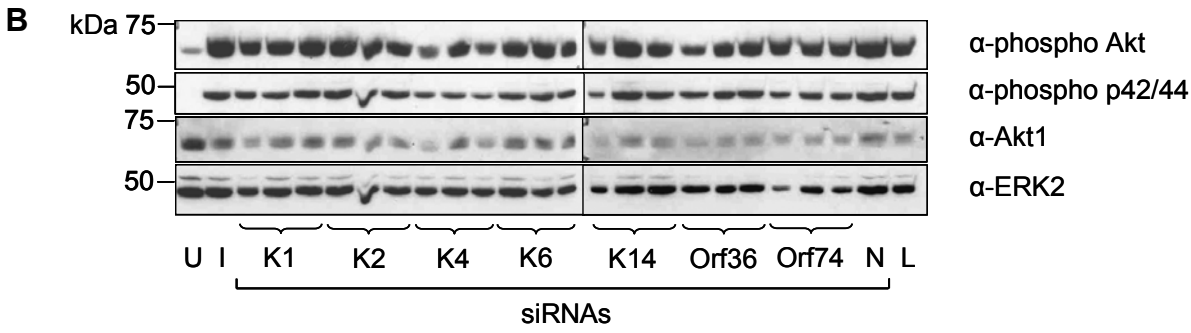
3.2 Results II: Essential Role of Kaposi Sarcoma Herpesvirus K1 Protein During the Progression of the Lytic Replication Cycle.

SiRNA-mediated depletion of K1 reduces the yield of new infectious virions.

Transient transfection of several early lytic proteins of KSHV has been reported to induce the activation of signalling pathways. Among them are transmembrane proteins such as the viral G-protein coupled receptor (vGPCR, encoded by orf74), the K15 and the K1 protein. Kinases and transcription factors activated by vGPCR include MAPK (ERK, JNK, and p38), Akt, NF-kB, NFAT, CREB, AP-1, and HIF-1α (Sodhi et al. 2000; Couty et al. 2001; Shepard et al. 2001; Cannon et al. 2003; Liu et al. 2004) (see also Fig. 4). K15 induces ERK and JNK signalling as well as activation of the transcription factors NF-kB and AP-1 in HEK293 cells (Brinkmann et al. 2003; Brinkmann et al. 2007). The K1 protein interacts among others (see also Fig. 4) with PLC-γ2, and the p85 subunit of PI3K, and induces activation of Akt signalling and the transcription factors NFAT, Ap-1 and NF-kB in B-cells and endothelial cells (Lagunoff et al. 1999; Prakash et al. 2002; Tomlinson and Damania 2004; Wang et al. 2004b; Lee et al. 2005). Activation of signalling pathways has further been shown for viral homologues of cellular cytokines including the viral interleukin 6 (vIL-6, encoded by orf K2), which activates STAT3, JAK1 and the Ras-MAPK pathway (Molden et al. 1997; Osborne et al. 1999; Hideshima et al. 2000), and the viral CC-chemokines 1 and 2 (vCCL-1/K6 and vCCL-2/K4), that are capable to induce Akt and MAPK signalling in endothelial cells (Choi and Nicholas 2008) (see also Table 1).

To test whether any of these candidate proteins contribute to ERK and Akt activation during the early phase of KSHV lytic cycle, I designed at least two distinct siRNAs for each of these genes. I also included two siRNAs each for the viral serine/threonine protein kinase (vPK) encoded by Orf36 (Park et al. 2000) and for viral OX2 (vOX2). Orf36 activates JNK MAPK signalling, and was shown to have an important function in KSHV DNA replication and progression of lytic cycle (Hamza et al. 2004; Izumiya et al. 2007). vOX2 is expressed from the same transcript as vGPCR and has been shown to possess immunomodulatory functions (Foster-Cuevas et al. 2004; Rezaee et al. 2005). These siRNAs were then transfected into Vero rKSHV.219 cells, induction of lytic replication was initiated eight hours after transfection, cells were lysed 48 h after induction of lytic cycle, and supernatants were collected.


A
Vero rKSHV.219
Virus titer (IU/ml)
4000
3000
2000
1000
0
uninduced
induced
Lipofectamine co.
non-target siRNA
1 2 3
K1
1 2
K2
1 2
K4
1 2
K6
1 2
K14
1 2
Orf36
1 2 3
Orf74
siRNAs


B
kDa 75
50
75
50
α-phospho Akt
α-phospho p42/44
α-Akt1
α-ERK2
U I
K1
K2
K4
K6
K14
Orf36
Orf74
N L
siRNAs

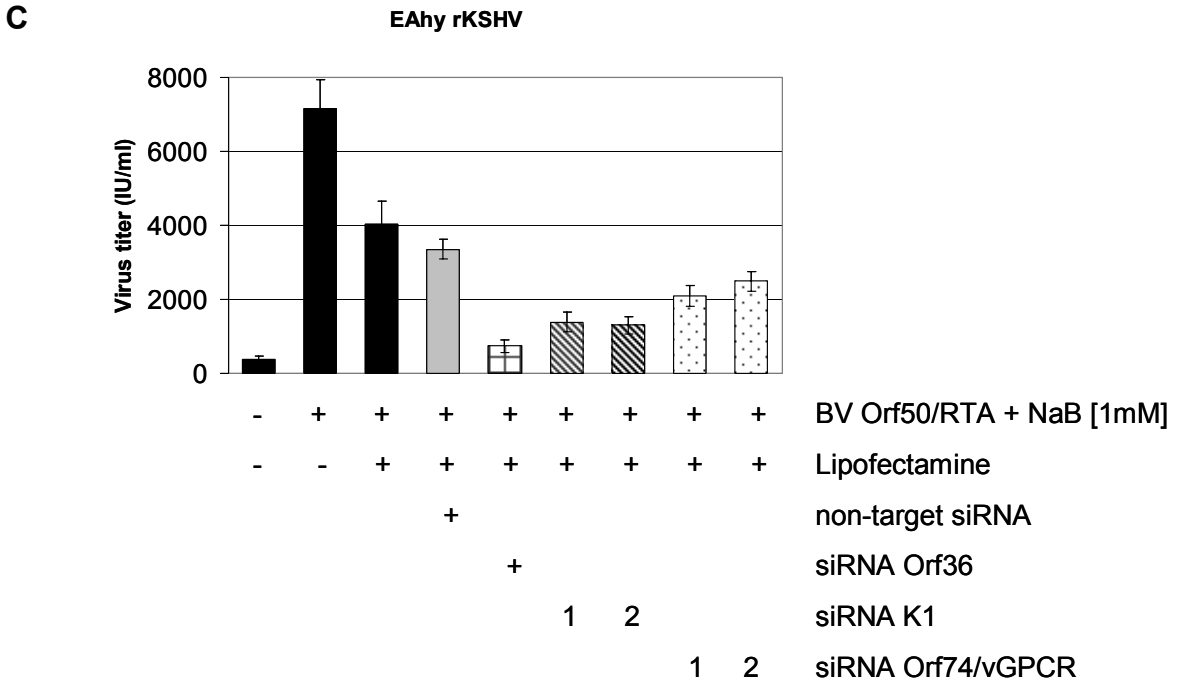

C
EAhy rKSHV
Virus titer (IU/ml)
8000
6000
4000
2000
0
- + + + + + + + + BV Orf50/RTA + NaB [1mM]
- - + + + + + + + Lipofectamine
+ non-target siRNA
+ siRNA Orf36
1 2 siRNA K1
1 2 siRNA Orf74/vGPCR

Figure 15. SiRNA-mediated downregulation of early-lytic protein expression reduces production of new infectious virions. Vero rKSHV.219 cells or EAhy rKSHV cells were transfected with individual siRNAs targeting the KSHV early-lytic proteins K1, K2 (vIL-6), K4 (vCCL-2), K6 (vCCL-1), K14 (vOX2), Orf36 (vPK) and Orf74 (vGPCR) or K1, Orf36 and Orf74, respectively. Non-target siRNA (N) was used for negative control. Six to eight hours after transfection, the lytic cycle was induced using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB). Culture supernatants were collected 48 h after reactivation from latency, centrifuged, and used to infect HEK293 cells. After two days, the number of infected (i.e. green fluorescent) target HEK293 cells was counted using fluorescence microscopy, and virus titers (infectious units (IU)/ml) were calculated. (A) Infectivity assay with culture supernatant of siRNA-transfected and reactivated Vero rKSHV.219 or (C) Eahy rKSHV cells. Experiments were carried out independently six times in Vero rKSHV.219 cells, and four times in EAhy rKSHV cells, each in triplicate. Bars represent the average and standard deviation from one experiment. (B) siRNA-transfected and reactivated Vero rKSHV.219 cells were lysed 48 h after induction of the lytic replication cycle and analysed by Western blotting with phosphospecific antibodies to Akt and p42/44 (ERK2/1). Afterwards, the membranes were stripped and reprobed with antibodies detecting total levels of Akt1 and ERK2. For each KSHV protein targeted, siRNAs were transfected in the following order (from left to right): siRNA1, siRNA2, combination of siRNA1 and 2.
U, uninduced; I, induced; L, lipofectamine.

Immunoblots of cellular lysates were performed with antibodies to phospho-p42/44 and phospho-Akt, and supernatants were transfered to HEK293 cells to measure infectivity. None of the siRNAs tested showed a reproducible and significant inhibition of phospho ERK or phospho Akt levels. Results from one experiment are shown in Figure 15 B.

Only siRNA-mediated silencing of K1 resulted in a more than 50 % reduction of infected HEK293 cells in all experiments. Representative results of one experiment are shown in Figure 15 A (K15 siRNA was not included in this experiment). Downregulation of Orf36 also consistently and strongly reduced the number of GFP-positive HEK293 cells, while transfection of non-target siRNA did not lower the yield of infectious virions during lytic cycle in Vero rKSHV.219 cells (Fig. 15 A). In most experiments I also observed reduced numbers of rKSHV-infected HEK293 cells in infectivity assay after RNA interference with other early lytic transcripts of KSHV, e.g. the viral CC-chemokines and vGPCR. This was in line with the findings by others, who showed that vCCL-1, vCCL-2 and vGPCR contribute to the progression of the lytic replication cycle (Choi and Nicholas 2008; Bottero et al. 2009; Sandford et al. 2009). However, downregulation of K1 expression had the most consistent effect on KSHV reactivation. I repeated the siRNA silencing experiments in EAhy.926 endothelial cells that had been stably infected with rKSHV.219 (see Materials and Methods) and obtained similar results (Figure 15 C). Again, the highest and most consistent reduction in virus yield was seen, if expression of Orf36 (i.e. positive control) or of K1 was downregulated by siRNA interference (Fig. 15 C). These observations indicated that the early-lytic protein K1 may contribute significantly to successful KSHV lytic replication in endothelial cells.

Generation of a KSHV mutant with a deletion of orf K1.

In order to corroborate the role of K1 during the lytic replication cycle of KSHV and to the activation of the ERK and Akt signalling pathway, I constructed a K1 deletion mutant in a recombinant KSHV genome cloned into a bacterial artificial chromosome (BAC36, Zhou et al. 2002). To delete the complete orf K1 (i.e. nt 105 – 974, Russo et al. 1996) from the BAC36 genome, I induced homologous recombination with a bacterial double selection cassette, rpsL-kan/neo, flanked by sequences homologous to 60 nt upstream of the K1 start codon and 63 nt downstream of the stop codon of orf K1 at two ends, in *E. coli* DH10B-cells carrying BAC36 wt (Figure 16; see Materials and Methods). Transformants were selected by kanamycin resistance. DNA was isolated from kanamycin-resistant colonies, checked by colony PCR for integration of the rpsL-kan/neo cassette, digested with restriction enzymes, and analysed on 0.4% agarose gels. *Eco*RI, *Kpn*I, *Not*I, and *Sal*I digestion of episomal DNA from mutant clones showed that they had all the restriction bands observed in wildtype BAC36 (Fig. 16 B and data not shown). To confirm the correct insertion of the rpsL-kan/neo cassette, I sequenced the 3'-transitional region by placing one primer (forward) in the rpsL-kan/neo cassette and a second primer (reverse) in the flanking KSHV genomic region on the right. Nucleotide alignment of the sequences of BAC36 ΔK1 clones to the predicted sequence in the transitional region showed that the rpsL-kan/neo counter selection cassette was inserted at its correct target position (Fig. 16 A). To verify the integrity of the rest of the KSHV genome, one BAC36 ΔK1 clone (bacterial clone 6) was completely sequenced using 454 technology (not shown).

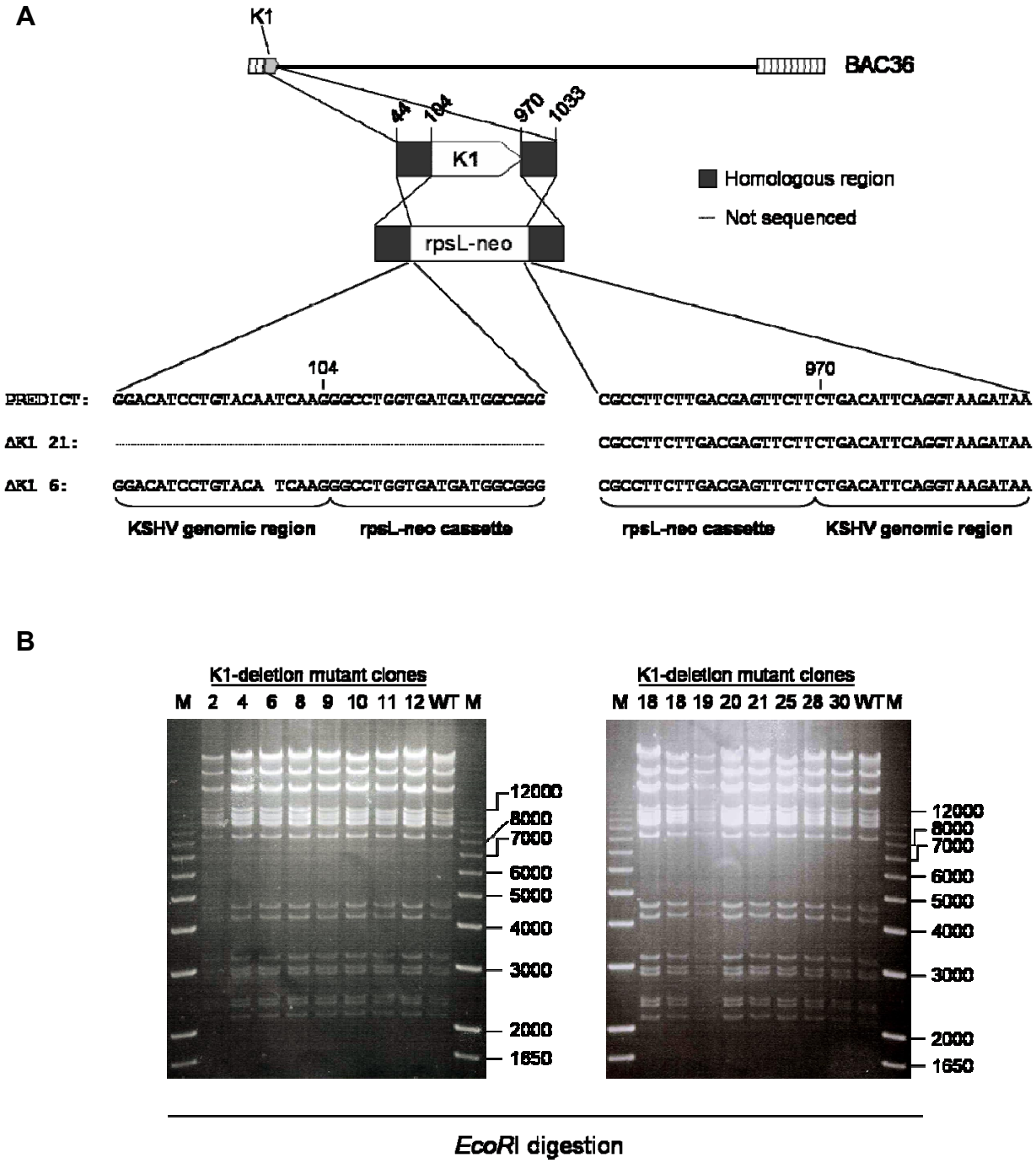


Figure 16. Schematic illustration of the generation of a KSHV mutant with a deletion of the K1 gene. (A) BAC36ΔK1 was generated by ET-cloning. The K1 gene was replaced with a Kan/Neo cassette (rpsL-neo) PCR product flanking sequences from outside the area to be knocked out by homologous recombination in *E. coli* strain DH10B, which had been previously transformed with wildtype BAC36. The correct insertion of the rpsL-neo cassette was verified by sequencing of a PCR fragment spanning the 3' end of the rpsL-neo cassette and the flanking KSHV sequence, as well as by complete sequencing of one BAC clone (ΔK1 clone 6) using 454 technology. Gaps indicate missing nucleotides. (B) *EcoR*I-digested BAC DNA from bacterial colonies was resolved on a 0.4% agarose gel. All restriction bands present in the wild type BAC36 were also present in the K1-deletion mutant clones.

K1 promotes KSHV lytic replication in BAC36-infected HEK293 cells and contributes to lytic cycle-induced activation of ERK and Akt signalling.

BAC36 ΔK1 DNA from two independent clones (bacterial clone 6 and 21), verified by restriction profiling and PCR, was transfected into HEK293 cells, which were then subjected to hygromycin selection. Of each KSHV ΔK1 DNA, 15 to 25 individual GFP-expressing cell clones were isolated, and amplified under hygromycin selection.

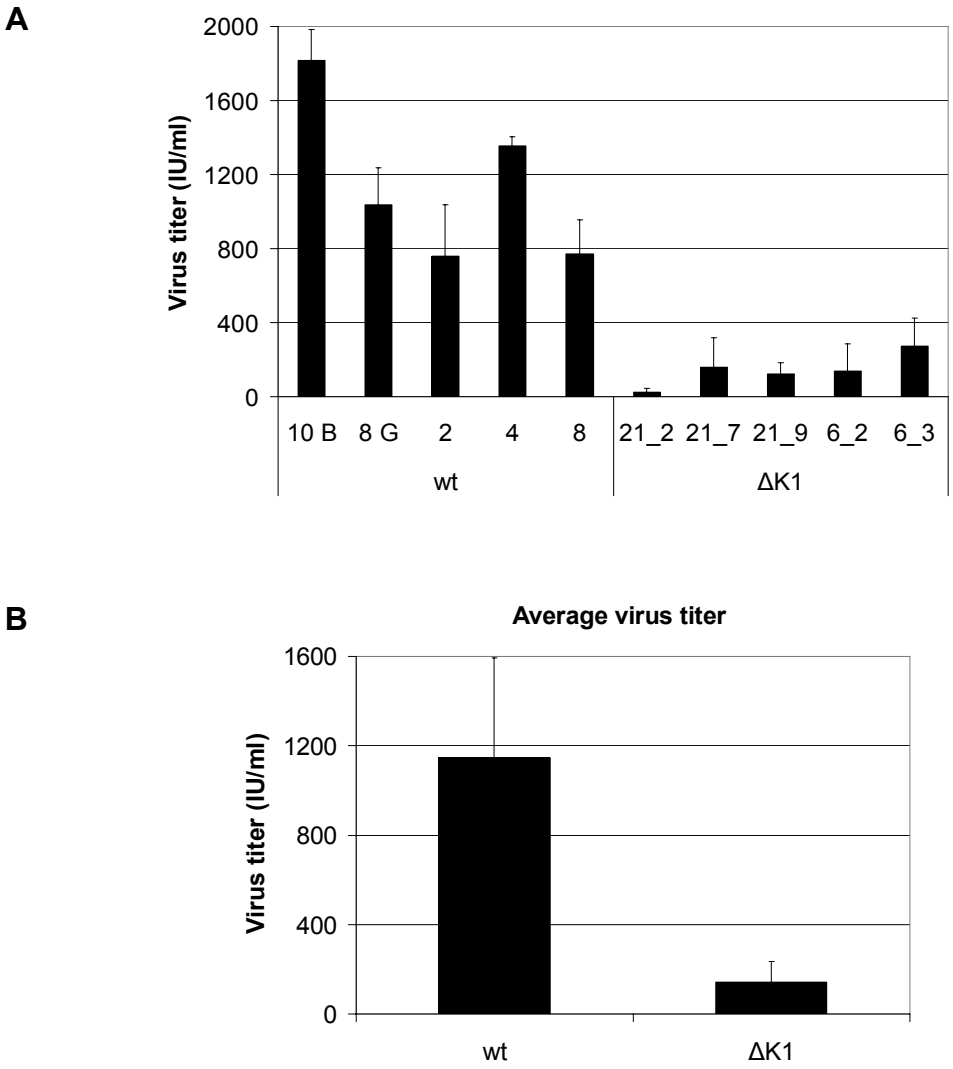


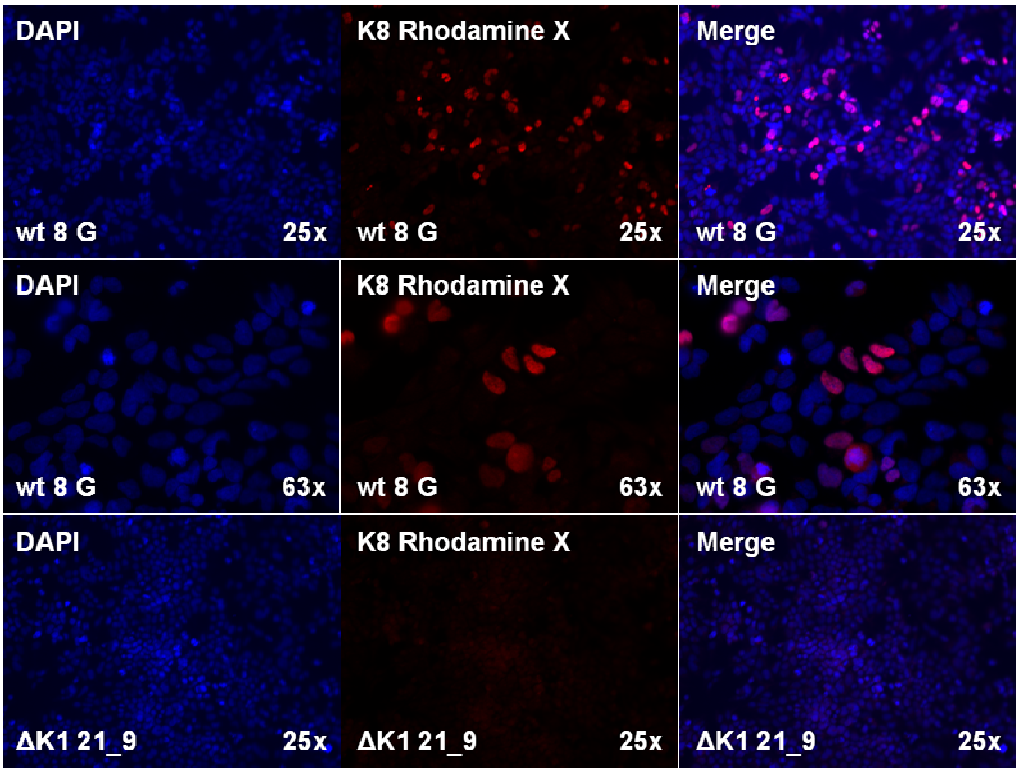
Figure 17. Production of infectious virions is reduced in HEK293 BAC36 ΔK1 cells. (A) Lytic replication was induced in BAC36 wt-infected and BAC36 ΔK1-infected HEK293 cells in parallel using baculovirus Orf50/RTA in combination with Na-butyrate [1mM]. Three days after reactivation, culture supernatants were harvested, centrifuged, and used to infect HEK293 cells. After two days, the number of infected target HEK293 cells was counted using fluorescence microscopy, and virus titers were calculated. B: Average virus titers of BAC36 wt and BAC36 ΔK1 clones depicted in Fig. 17 A were calculated. Two individual experiments were carried out, each in triplicate. Results represent averages and standard deviations from one experiment.

I examined ten cell lines derived from BAC36 ΔK1 clone 21 and two cell lines derived from BAC36 ΔK1 clone 6 for production of infectious virus and activation of ERK and Akt signalling after induction of lytic replication using baculovirus Orf50/RTA in combination with Na-butyrate. In parallel, I generated and analysed ten individual HEK293 cell lines harbouring the BAC36 wt genome. In all cell lines, KSHV lytic replication was induced at low cell passage number (between p4 and p10), and using the same batches of baculovirus Orf50/RTA and Na-butyrate. Cells were lysed at 5, 12, 24, 48, and 72 h.p.i., and protein extracts were immunoblotted with anti-phospho p42/44 and anti-phospho Akt antibody. Supernatants were collected, and used to infect HEK293 cells to determine the yield of infectious progeny virus. Production of infectious virus was clearly reduced in all BAC36 ΔK1 clones (Fig. 17). Virus titers in BAC36 ΔK1 culture supernatant ranged in all cell lines from 23 infectious units (IU)/ml (clone 21_2) to 273 IU/ml (clone 6_3) with an average number of 144 IU/ml, compared to titers of 760 IU/ml (clone 2) to 1816 IU/ml (clone 10 B) for BAC36 wt culture supernatant (average 1148 IU/ml). Therefore, in spite of marked variations in the yield of infectious progeny virus among individual BAC36 wt or BAC36 ΔK1 lines, the average number of infectious virions produced from BAC36 ΔK1 lines was eight-times lower than the average number produced from BAC36 wt lines (Fig. 17 B). These results confirm the importance of the K1 protein for successful KSHV lytic replication.

KSHV lytic gene expression is reduced in K1-deficient HEK293 BAC36 cells.

I next investigated the expression of KSHV lytic genes in KSHV wt and ΔK1 cell lines. I induced KSHV reactivation in three individual clones of each BAC36 wt and BAC36 ΔK1, and measured the accumulation of the viral proteins K8/-bZIP and Orf59. The early-lytic protein K8/-bZIP is the proposed homologue of Epstein-Barr virus (EBV) Zta, and it has been suggested that K8/K-bZIP modulates RTA function (Izumiya et al. 2003; Kato-Noah et al. 2007). Orf59 expresses the KSHV homologue of other herpesvirus polymerase accessory proteins, and is expressed with delayed early kinetics (Chan et al. 1998). Both K8/K-bZIP and Orf59 are nuclear proteins. Additionally, I measured the expression of K1, and analysed the correlation of K1 expression to the expression of K8 and Orf59. 72 h after induction of the lytic cycle, cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100, and stained with either anti-Orf59 antibody or anti-K8 antibody alone, or double-stained with anti-K8 and anti-K1 antibody. Cells were then analysed by fluorescent microscopy.

A



B

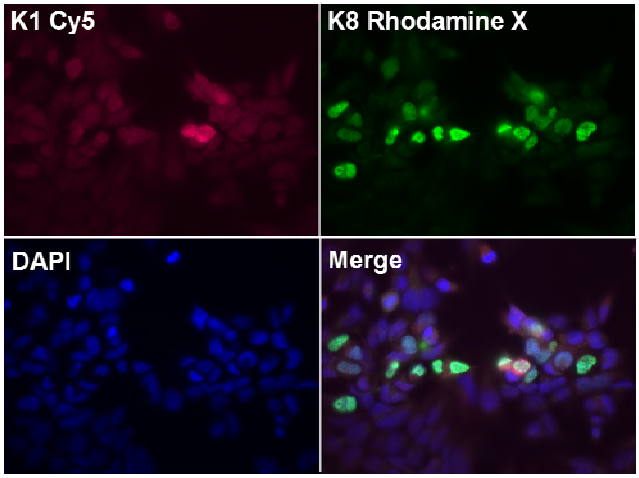


Figure 18. K8 expression was not detected by immunofluorescence assay in BAC36 ΔK1-infected HEK293 cell lines. (A) Three individual lines of each BAC36 wt- (two top rows) and BAC36 ΔK1-infected (bottom row) HEK293 cells were stained for K8 protein expression 72 h after reactivation from latency. DNA was stained by DAPI. Among BAC36 wt clones, ten to fifteen percent of the cells expressed K8 three days after induction of lytic cycle, while no K8 expression was detected in cells that were infected with K1-deletion mutant. Pictures show representative results from one wildtype-infected line and one K1-deletion mutant-infected line. (B) Seven to ten percent of K8-expressing BAC36 wt-infected HEK293 cells also show K1 expression. BAC36 wt-infected HEK293 cells were double stained for K8 and K1 protein expression at 72 h after reactivation from latency. DNA was stained by DAPI. Pictures show representative results from BAC36 wt clone 8 G-infected HEK293 cells.

For BAC36 wt, 15 ± 3 % of the cells of each clone (10 B, 8 G, and 8) expressed K8 after activation of the lytic cycle (Fig. 18 A, B). Similar percentages were obtained for Orf59 expressing cells (data not shown). Five to ten percent of the K8-expressing cells also expressed the K1 protein (Fig. 18 B). As expected, K1 was not expressed in HEK293 BAC36 ΔK1 cells, nor did I detect K8 or Orf59 expression in any of the three K1-deletion mutant clones analysed (21_7, 21_9, 6_2) (Fig. 18 A and data not shown).

In addition I measured the level of K8/bZIP transcripts by quantitative real-time PCR in four independent BAC36 wt lines and five independent K1-deletion mutant lines after reactivation from latency. I further determined the transcript levels of the KSHV chemokines vIL-6 and vCCL-1 in the same BAC36 wt and BAC36 ΔK1 lines. GAPDH was amplified in parallel as an internal control and the expression of RTA in the same BAC36 wt and ΔK1 clones was monitored by immunoblotting. For HEK293 BAC36 wt lines, the average cycle threshold (C_T) value for K1 was in the range of 23 to 24 (Fig. 7 A), indicating that 72 hours post reactivation of KSHV from latency K1 is highly expressed in all BAC36 wt clones. In contrast, the fluorescent signal for K1 detection in samples of induced HEK293 BAC36 ΔK1 lines did not exceed background levels within 40 qPCR cycles, confirming the absence of K1 expression in all HEK293 BAC36 ΔK1 clones (Fig. 19 A). For K8/bZIP, the C_T value was in the range of 20 to 22 for BAC36 wt lines with an average C_T value of 21, and varied from 27 (clone 21_9) to greater than 40 (clone 6_3) for BAC36 ΔK1 (Fig. 19 B). To calculate the average C_T value for K8 for BAC36 ΔK1, I set the C_T value for clone 6_3 to 40, and thereby obtained an average C_T value of 31. Thus, 72 hours after induction of the KSHV lytic cycle, expression of K8/bZIP in BAC 36 ΔK1 lines is approx. 500 to 1000 fold lower than in BAC36 wt lines. These expression levels are probably too low to be detected by immunofluorescent staining.

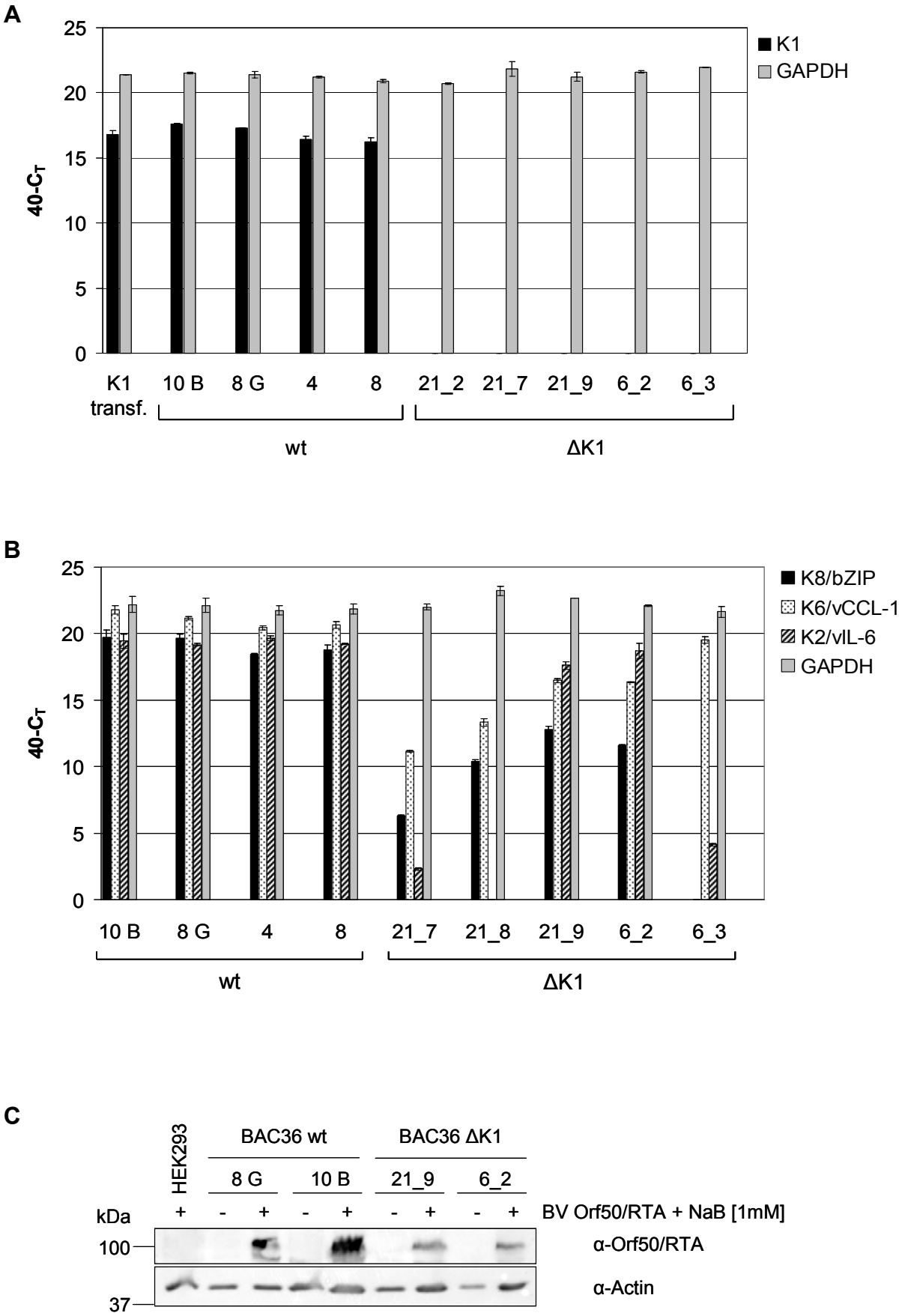

A
25
20
15
10
5
0
40-C_T
K1
GAPDH
K1
10 B
8 G
4
8
21_2
21_7
21_9
6_2
6_3
transf.
wt
ΔK1
B
25
20
15
10
5
0
40-C_T
K8/bZIP
K6/vCCL-1
K2/vIL-6
GAPDH
10 B
8 G
4
8
21_7
21_8
21_9
6_2
6_3
wt
ΔK1
C
HEK293
BAC36 wt
BAC36 ΔK1
8 G
10 B
21_9
6_2
kDa
+ - + - + - + - +
BV Orf50/RTA + NaB [1mM]
100
37
α-Orf50/RTA
α-Actin

Figure 19. Expression of KSHV early lytic genes is reduced in HEK293 BAC36 ΔK1 cells. Lytic replication was induced in BAC36 wt- and BAC36 ΔK1-infected HEK293 cells. 72 h after reactivation, total RNA was isolated, treated with DNase I, reverse transcriped, and subjected to real-time quantitative PCR with specific primers and Taqman probes to early-lytic proteins. GAPDH was used as internal control, and was strongly detected in all samples. Each reaction was done in duplicate, and each bar represents the average and standard deviation of one experiment. C_T = cycle threshold. (A) K1 is highly expressed in BAC36 wt-infected cells. cDNA from K1-transfected HEK293 cells was used as a positive control. As expected, K1 expression was not detected in BAC36 ΔK1-infected HEK293 cells at 72 h after induction of lytic cycle up to cycle 40 of the real-time quantitative PCR run. (B) K8, vIL-6, and vCCL-1 protein expression is reduced in HEK293 BAC36 ΔK1 cell lines. Ct values of K8, vIL-6, and vCCL-1 were compared among individual BAC36 wt lines and BAC36 ΔK1 lines. (C) RTA expression is reduced in HEK293 BAC36 ΔK1 cell lines. Two independent lines of each BAC36 wt-infected and BAC36 ΔK1-infected HEK293 cells were reactivated using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB). 48 h after induction, cells were lysed. Proteins were separated by SDS-PAGE, blotted to nitrocellulose, and probed with antibodies to Orf50/RTA and antibodies to *β*-actin. Expression of RTA was not detected in uninfected HEK293 cells after treatment with BV Orf50/RTA in combination with NaB in parallel, indicating that the RTA detected by Western blotting reflects RTA expressed from the KSHV genome and not from the baculovirus RTA vector.

Expression of vIL-6 and vCCL-1 after induction of the lytic cycle was constitutively high in all KSHV wt lines (C_T values of 17 to 22) but varies markedly in the four KSHV ΔK1 lines (Fig. 19 B). The average C_T value for vIL-6 was 32 for BAC36 ΔK1 and 21 for BAC36 wt lines equivalent to an approx. 500 to 1000 fold lower expression of vIL-6 in ΔK1 cell lines, while the average C_T value for vCCL-1 was 25 for the K1-deletion mutant and 18 for the wild type-virus (approx. 100 fold difference in expression).

Figure 19 C shows the RTA expression for two HEK293 BAC36 wt lines and two HEK293 BAC36 ΔK1 lines 72 h after induction of the KSHV lytic cycle. Expression of RTA is reduced in both HEK293 BAC36 ΔK1 clones compared to the two KSHV wt lines (Fig 19 C). Similar results were obtained, when cells were lysed two days after induction (data not shown). As RTA was not expressed in uninfected HEK293 cells, which were treated with baculovirus Orf50/RTA and Na-butyrate in parallel (Fig. 19 C), the levels of RTA seen in this experiment reflect RTA expression from the KSHV genome and not the baculovirus RTA vector. Therefore, expression of the early-lytic proteins K8/bZIP, vIL-6, and vCCL-1, as well as of the immediate-early protein RTA is reduced in HEK293 BAC36 ΔK1 compared to BAC36 wt cells. These findings suggest the expression of early lytic genes is reduced in the absence of K1 expression and that K1 promotes KSHV early lytic gene expression.

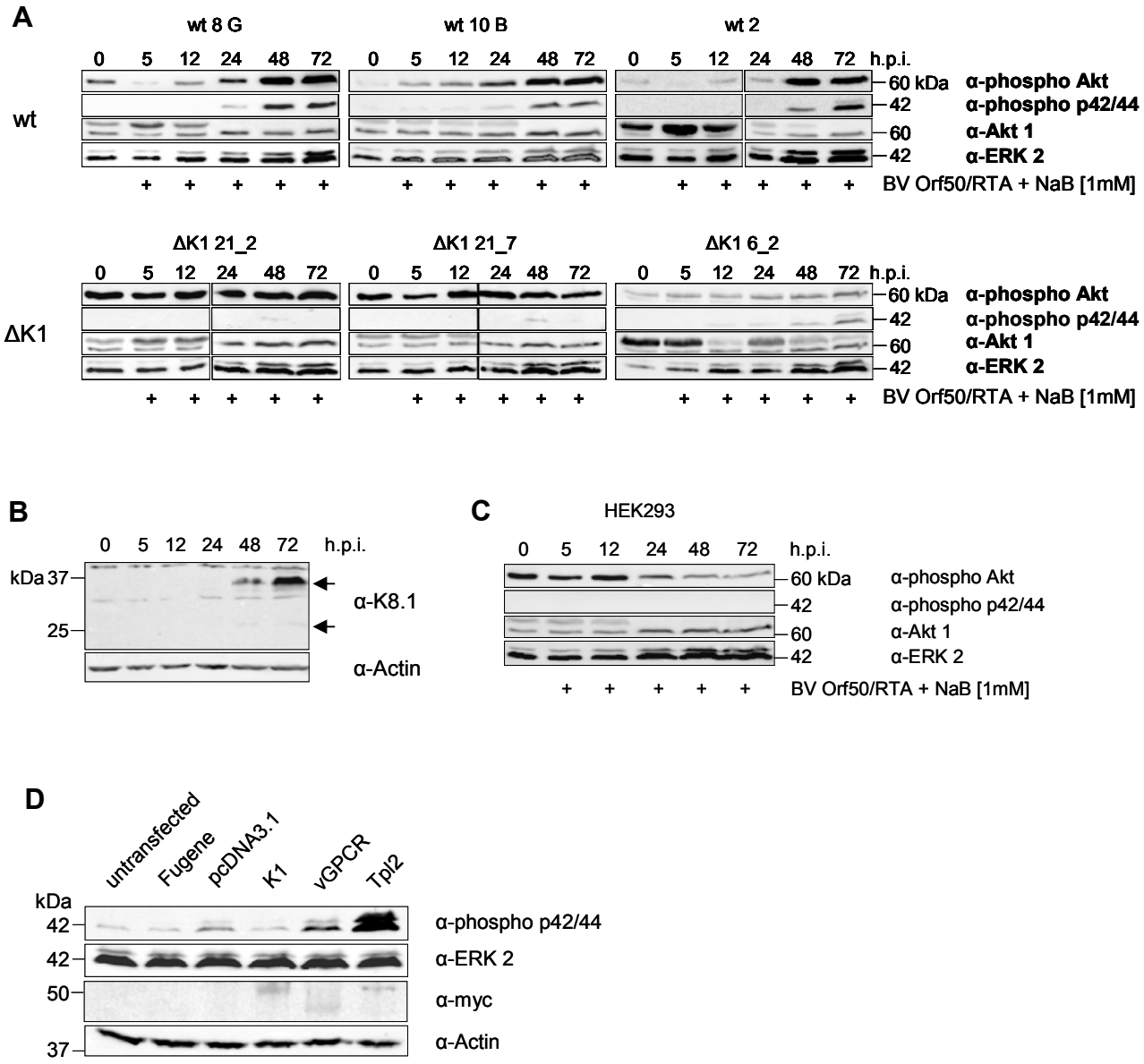


Figure 20. Reduced activation of signalling pathways in K1-deletion mutants. (A) Lytic cycle-induced activation of ERK is reduced in BAC36 ΔK1-infected HEK293 cell lines. Akt activation, although constitutively high in many K1-deletion mutant lines, does not increase during lytic replication. BAC36 wt-infected and BAC36 ΔK1-infected HEK293 cells were reactivated using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB). 5, 12, 24, 48, and 72 h after induction (h.p.i.), cells were lysed. Proteins were separated by SDS-PAGE, blotted to nitrocellulose, probed with phosphospecific antibodies to Akt or ERK2/1 (p42/44), stripped and reprobed with antibodies to total Akt1 or total ERK2 levels. (B) Expression of the late lytic glycoprotein K8.1 in BAC36 wt 8 G-infected HEK293 cells after induction of lytic replication using BV Orf50/RTA in combination with NaB. (C) BV Orf50/RTA and Na-butyrate do not induce ERK or Akt signalling in uninfected (KSHV-negative) parental HEK293 cells. Uninfected HEK293 cells were treated with BV Orf50/RTA in combination with NaB, cells were lysed in parallel to BAC36-infected HEK293 cells, and analysed by Western blot analysis for activation of ERK and Akt. (C) K1 does not directly induce ERK activity. HEK293 cells were transfected with pcDNA3.1-K1/myc/his, pcDNA4-vGPCR/myc/his, pCMV5-Tpl2/myc, or pcDNA3.1. 48 h after transfection, cells were lysed and assayed for ERK activation by Western blotting with phosphospecific antibodies to ERK2/1 (p42/44). Protein expression of K1, vGPCR, and Tpl2 was analysed using anti-myc antibody.

Having shown that the absence of K1 leads to an inefficient activation of the lytic replication cycle, I investigated the activation of the ERK and Akt pathways in the absence of K1. I observed activation of both ERK2 and Akt kinase in all BAC36 wt-infected HEK293 cell lines similar to Vero rKSHV.219 cells (Fig. 20 A and data not shown), although ERK2 activation occurred somewhat later in HEK293 BAC36 wt cells as activation was not detected before 24 h.p.i., and both ERK and Akt activation were prolonged up to 72 after reactivation, the last time point measured in our experiments (Fig. 20 A). I also did not detect expression of the late lytic protein K8.1 in BAC36 wt cell lines nor infectious virions in culture supernatant before 48 h after reactivation from latency (Fig. 20 B and data not shown), while first viral progeny appeared around 24 h.p.i. in culture supernatant of Vero rKSHV cells (data not shown). Baculovirus Orf50/RTA and Na-butyrate alone did not induce ERK2 or Akt signalling in the cells, as phospho p42/44 ERK was not found, and pAkt did not increase in non-infected HEK293 cells incubated with the induction cocktail (Fig. 20 C). Interestingly, ERK2 activation was strongly reduced in all K1 deletion mutants, and activation of Akt, although constitutively high in the majority of HEK293 BAC36 ΔK1 clones, did not increase after induction of KSHV lytic cycle (Fig. 20 A and data not shown).

While this result suggests that the presence of K1 is required for the activation of ERK and Akt in KSHV-infected cells, K1 does not appear to activate ERK directly. Transfection of a K1 expression vector did not result in an increased ERK phosphorylation in HEK293 cells, whereas transfection of vGPCR did (Fig. 20 D). From these results I conclude that K1 is required at an early point in the lytic replication cycle which precedes the activation of the ERK and Akt pathways.

Differential regulation of cellular gene expression in HEK293 BAC36 wt and HEK293 BAC36 ΔK1 cell lines.

In order to explore the cellular targets of K1 signalling, I performed high-density (HD) DNA microarrays (Agilent Whole Human Genome Oligo Microarrays) on HEK293 BAC36 wt and HEK293 BAC36 ΔK1 lines with latently as well as with productively (lytically) infected cells. The Agilent microarray design is based on sources such as RefSeq, Goldenpath, Ensembl, Unigene and the Human Genome Build 33, and covers more than 41.000 unique genes and transcripts which have been verified and optimized by alignment to the human genome assembly and by Agilent's Empirical Validation process. The lytic replication cycle was induced in each three individual lines of BAC36 wt- and BAC36 ΔK1-

containing HEK293 cells using baculovirus Orf50/RTA in combination with Na-butyrate [1mM].

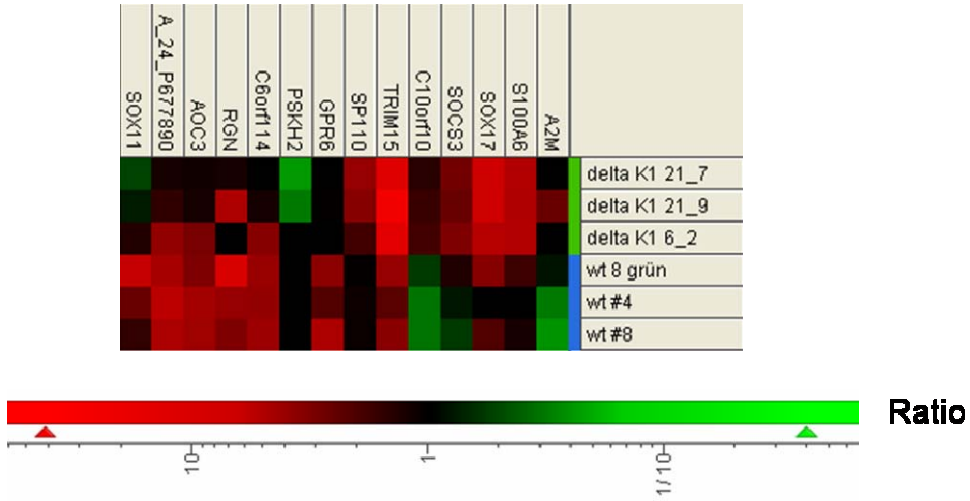


Figure 21: Differentially regulated genes in BAC36 wildtype (wt) and BAC36 delta K1 lines 48 h after induction of the lytic replication cycle. Each three HEK293 BAC36 wt and HEK293 BAC36 ΔK1 cell lines were reactivated from latency using baculovirus (BV) Orf50/RTA in combination with Na-butyrate (NaB, 1mM). 48 h after reactivation, RNA was harvested. High density (HD) microarray experiments were performed as "one-colour hybridizations". For each cRNA population generated from reactivated cells, we hybridized cRNA of non-induced cells in parallel. Additionally, we hybridized cRNA that was generated from BV Orf50/RTA and NaB-treated and non-treated parental (KSHV-negative) HEK293 cells. Thus, 14 HD microarrays were hybridized altogether. After hybridization and data extraction, the whole data set was clamped to a minimal intensity threshold of 39, each analysis pair (induced/non-induced) was normalized by pointwise division, i.e. the "induced" expression values were divided by the "non-induced" values. The resulting ratios were grouped according to the experimental background (wt and ΔK1 lines), and these groups were analyzed for genes that were differentially regulated at least threefold in the BAC36 ΔK1 group compared to the BAC36 wt group. Depicted are manually selected probes from a total number of 17 probes with a fold change larger three that were separated in up- and down-regulated profiles by k-means clustering. The profile with a label starting with 'A_' is not annotated with a Gene Symbol. Therefore, the Agilent ID is displayed instead. wt 8 grün = BAC36 wt 8 G.

Non-infected, parental HEK293 cells were exposed to the induction cocktail in parallel to analyse the impact of the baculovirus infection and Na-butyrate on the cellular transcription profile. 48 hours after induction of the lytic cycle, reactivated and non-induced cells were lysed for RNA extraction, and cDNA was prepared as described in Materials and Methods. Overall, 14 Agilent arrays were hybridized using the one-colour protocol and analysed in order to find genes that are differentially regulated between BAC36 wt and BAC36 ΔK1 lines. Since K1 is a lytic gene, which is strongly induced after reactivation from latency (Paulose-Murphy et al. 2001; Fakhari and Dittmer 2002; Nakamura et al. 2003), I analysed the differential induction of cellular genes after reactivation from latency. Therefore, the data were clamped to a minimal intensity of 39, and each analysis pair (induced/non-induced) was

then normalized by point-wise division, i.e. the induced expression values were divided by the non-induced values. By this the cell clone effect and a possible hybridization date effect on the cellular transcription profile should be removed. The resulting ratios were then subdivided into a wildtype and a K1-deletion group. For each group the median was calculated, and the ΔK1 value was then divided by the wt value calculating the ratio and analyzed by fold change analysis and t-test.

Filtering for a fold change of larger two resulted in 171 probes, among which 17 probes showed a fold change of more than three. However, t-test analysis with a p-value cutoff of 4E-4 showed no overlap with the fold change analysis, indicating a rather low significance for these results. Setting the p-value cutoff to 0.05 resulted in 41 genes, which are depicted in Table 5. Among the genes that were upregulated in the BAC36 wt group compared to the BAC36 ΔK1 group was a G protein-coupled receptor (GPR6, 3.6-fold upregulated), while genes with a lower expression in BAC36 wt lines compared to BAC36 ΔK1 lines included two members of the family of tripartite motif-containing (TRIM) proteins, namely TRIM15 and TRIM10 (4-fold and 2.8-fold downregulated, respectively) and a suppressor of cytokine signalling (SOCS3, 3.6-fold downregulated) among others (Table 5).

Absolute data was also analysed (i.e. the gene expression values of induced cells were compared without setting an intensity threshold of 39 and without normalization to the expression values of non-induced cells) by fold-change analysis and t-test, and thereby 273 genes were identified with a fold-change of larger two, among which 55 genes showed a fold-change of larger three and nine genes of larger five (data not shown). The genes identified in this analysis partially overlapped with those from the ratio data above, including GPR6, which was found to be upregulated by 4.6-fold in wt cell lines compared to K1-deletion cell lines (not shown).

I further compared the non-induced experiments of ΔK1 and wt cell lines to get an impression on the impact of K1 deletion on latently infected cells. Filtering for a fold-change of ≥ three resulted in 73 genes (not shown). A p-value cutoff of 4E-4 resulted in 53 items, but showed no overlap with the fold change analysis (not shown). Nevertheless, a significant difference between the BAC36 wt and BAC36 ΔK1 group during latency seemed probable. In summary, I found a rather high number of genes differentially regulated between ΔK1 and wt cell lines. However, t-test analysis did not allow to statistically verify the fold change results because of high variance in the profile and the low number of experiments.

Table 5. Identification of differentially regulated genes between HEK293 BAC36 wt and ΔK1 lines after induction of the lytic replication cycle.[a]

Identifier	Gene symbol	Gene name	Gene expression (Ratio of Medians)[b]	T-Test: P-Value	T-Test: BH q-Value
NM_005284	GPR6	G protein-coupled receptor 6	3.6	0.0069	0.77
BP871540	NA	NA	3.0	0.0114	0.8
NM_000444	PHEX	phosphate regulating endopeptidase homolog, X-linked (hypophosphatemia, vitamin D resistant rickets)	2.77	0.0084	0.78
AK002200	SMC4	structural maintenance of chromosomes 4	2.75	0.00198	0.69
ENST00000369158	NA	NA	2.61	0.0134	0.81
NR_003003	SCARNA17	small Cajal body-specific RNA 17	2.56	0.0216	0.83
A_32_P169383	NA	NA	2.4	0.0389	0.89
AB067523	SMYD4	SET and MYND domain containing 4	2.27	0.0293	0.86
NM_004302	ACVR1B	activin A receptor, type IB	2.25	0.0469	0.89
BC065547	GUSBL2	glucuronidase, beta-like 2	2.22	0.0268	0.86
AL137653	CTBP1	C-terminal binding protein 1	2.2	0.0101	0.79
NM_013305	ST8SIA5	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 5	2.08	0.0131	0.80
THC2498220	NA	NA	2.08	0.0066	0.77
NM_197941	ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	2.07	0.0082	0.78
THC2685727	NA	NA	2.01	0.0399	0.89
A_24_P565503	NA	NA	2.01	0.0478	0.89
NM_007021	C10orf10[c]	chromosome 10 open reading frame 10	0.20	0.0019	0.69
NM_022454	SOX17	SRY (sex determining region Y)-box 17	0.23	0.0296	0.86
NM_014624	S100A6	S100 calcium binding protein A6	0.24	0.0010	0.69
NM_033229	TRIM15	tripartite motif-containing 15	0.25	0.0040	0.72
NM_003955	SOCS3	suppressor of cytokine signaling 3	0.28	0.0088	0.78
NM_004510	SP110	SP110 nuclear body protein	0.32	0.0147	0.82
NM_007021	C10orf10[c]	chromosome 10 open reading frame 10	0.33	0.0016	0.69

Identifier	Gene symbol	Gene name	Gene expression (Ratio of Medians)[b]	T-Test: P-Value	BH q-Value
NM_079422	MYL1	myosin, light chain 1, alkali; skeletal, fast	0.34	0.0495	0.90
NM_052828	TRIM10	tripartite motif-containing 10	0.35	0.0085	0.78
NM_152403	EGFLAM	EGF-like, fibronectin type III and laminin G domains	0.40	0.0234	0.84
NM_177403	RAB7B	RAB7B, member RAS oncogene family	0.43	0.0260	0.86
L33930	CD24	CD24 molecule	0.43	0.0085	0.78
NM_000204	CFI	complement factor I	0.44	0.0273	0.86
NM_002928	RGS16	regulator of G-protein signalling 16	0.45	0.0028	0.72
NM_174936	PCSK9	proprotein convertase subtilisin/kexin type 9	0.46	0.0434	0.89
NM_015085	GARNL4	GTPase activating Rap/RanGAP domain-like 4	0.47	0.0009	0.69
NM_001037582	SCD5	stearoyl-CoA desaturase 5	0.48	0.0406	0.89
NM_001012984	LOC388284	hypothetical gene supported by BC032064; BC041612	0.48	0.0470	0.89
NM_002318	LOXL2	lysyl oxidase-like 2	0.48	0.0375	0.88
NM_005756	GPR64	G protein-coupled receptor 64	0.48	0.0420	0.89
NM_000204	CFI	complement factor I	0.49	0.0156	0.82
THC2586092	NA	NA	0.49	0.0329	0.87
NM_022124	CDH23	cadherin-like 23	0.49	0.0084	0.78
AB075837	KIAA1957	KIAA1957	0.5	0.0250	0.85
NM_013369	DNMT3L	DNA (cytosine-5-)-methyltransferase 3-like	0.5	0.0356	0.88

[a] cDNA of latently infected and reactivated cells of each three individual HEK293 BAC36 wt and ΔK1 cells lines was analysed by Agilent Whole Human Genome Oligo Microarrays, which were performed as “one-colour hybridizations”. Gene expression values of reactivated cells were divided by the respective values of non-induced cells. The resulting ratios were then grouped into “wt-lines” and “K1-deletion lines”, and for each group the median expression values for all individual probes were calculated. The median expression values of the wt group were divided by the median expression values of the ΔK1 group.
[b] The ratio of medians describes the fold expression of the respective gene in BAC36 wt cell lines in relation to BAC36 ΔK1 cell lines. Marked in red are the values which indicate a higher expression of the respective gene in BAC36 wt cell lines (ratio of medians >1), while values marked in green show a lower gene expression in the wt group compared to the K1-deletion group (ratio of medians <1).
[c] The gene C10orf10 was identified by hybridization to two different oligonucleotide sequences on the microarrays.
NA=not available.

4 Discussion

4.1 Role of MEK/ERK and PI3K/Akt signalling pathways during the KSHV lytic replication cycle.

As obligate intracellular parasites, viruses exploit diverse cellular signalling machineries during infection of their host. Many viruses, including herpesviruses, have evolved to depend on specific modulations of signalling pathways for efficient primary infection and viral DNA replication (Rodems and Spector 1998; McLean and Bachenheimer 1999; Johnson et al. 2001a; Johnson et al. 2001b; Pleschka et al. 2001; Jiang et al. 2004; Rahaus et al. 2006; Schumann and Dobbelstein 2006; Rahaus et al. 2007). Previous studies showed that PI3K signalling and MAPK (ERK, JNK, p38) pathways play an important role during KSHV primary infection. All three MAPK pathways modulate the expression of RTA, the key transactivator of KSHV lytic replication, during both primary infection and TPA-induced reactivation from latency (Cohen et al. 2006; Pan et al. 2006; Yu et al. 2007; Xie et al. 2008).

In the present study, I have shown that KSHV induces the ERK pathway at a point downstream of RTA expression during the early stage of lytic replication (Fig. 6, 7). As shown in Figs. 6 C and 19 C, activation of the ERK pathway requires the presence of the KSHV genome in Vero or HEK293 cells, and is therefore not the result of infection with the recombinant baculovirus used here to transduce Orf50/RTA. Furthermore, the ERK pathway was also activated after tetracycline-mediated induction of the lytic replication cycle in PEL-cells (Fig. 7), suggesting that activation of ERK during lytic replication occurs independently of the infected cell type.

Inhibition of the ERK pathway by the specific MEK inhibitor U0126, and by a second MEK inhibitor, PD98059, reduced late lytic protein expression (Fig. 9 A) and virus production in a dose-dependent manner (Fig. 9 B, C). U0126 did not affect RTA expression, suggesting that its inhibitory effect on late viral glycoprotein expression and virus production occurred downstream of RTA expression. As shown in Fig. 19 C, the RTA expression in the experiments shown here occurs from the KSHV genome in all cell lines used in this study, not from the baculovirus RTA vector, which was used at a concentration below that needed to express Western blot-detectable RTA. Activation of the ERK pathway was not susceptible to an inhibitor of the viral DNA polymerase, but to cycloheximide treatment and therefore occurred during the early phase of the lytic replication cycle. However, since expression of

K8.1 and virus production were also reduced when ERK activity was inhibited not until the delayed-early or late phase of the lytic replication cycle (Fig. 11), intact ERK signalling may be crucial also for progression and successful completion of the lytic cycle at subsequent phases.

Activation of ERK pathway has been implicated in viral DNA replication and gene expression during productive primary infection of several herpesviruses (Johnson et al. 1999; Johnson et al. 2001a; Jiang et al. 2004; Xie et al. 2005; Xie et al. 2008). For example, chemical inhibition of ERK activity reduced viral DNA synthesis and immediate-early protein synthesis during permissive primary infection of the γ-herpesvirus bovine herpesvirus 4 (BHV-4) (Jiang et al. 2004). Further analysis showed that ERK regulated the promoter activity of immediate-early IE-2 protein expression of BHV-4, and thereby influenced the expression of early-lytic proteins involved in viral DNA replication (Jiang et al. 2004). For human cytomegalovirus (HCMV) it was shown that U0126 inhibited increased expression of the early genes UL44 and UL84, which are required for viral DNA replication (Johnson et al. 2001a). These findings suggest that regulation of viral gene expression might be a common feature of ERK signalling pathway during the herpesviral productive phase. ERK activity may promote viral DNA replication and virus production during the lytic cycle downstream of RTA expression by modulating the expression of lytic proteins which are involved in transcriptional activation and/or viral DNA replication.

I also observed an activation of the Akt pathway during the late phase of the KSHV productive replication cycle (Fig. 6 A, 20 A). A specific PI3K inhibitor, LY294002, strongly reduced lytic cycle-induced Akt activation (Fig. 9 E, 10 B), but did not reduce lytic protein expression or KSHV virus production (Fig. 9 A and B, 10 B and C). On the contrary, inhibition of PI3K by LY294002 resulted in increased expression of RTA and K8.1 protein (in an inverse dose-dependent manner, Fig. 10 B) and virus production (Fig. 10 C) in the endothelial cell line EAhy rKSHV. Notably, a very recent study conducted by Peng *et al*. has shown that inhibition of Akt facilitates reactivation of KSHV from latency in the PEL cell line BC-3 and that RTA activity as well as subsequent lytic gene expression are increased by reducing Akt 1 activity (Peng et al. 2009). Together, these findings suggest that the PI3K-Akt signalling cascade may suppress KSHV productive replication at least in some cell lines (e.g. endothelial cells, B-cells) very early during the lytic cycle through Akt-mediated repression of RTA activity. However, I observed that Akt activation strongly increased during the delayed-early or late phase of the lytic replication cycle in Vero rKSHV.219 (Fig. 6 A) and HEK293 BAC36 wt cell lines (Fig. 20 A) and appeared to be partially dependent on viral DNA

replication (8 A). Since activating PI3K-Akt signalling is a strategy employed by several viruses to slow down apoptosis and prolong viral replication (Groskreutz et al. 2007; Ehrhardt and Ludwig 2009), it is conceivable that activation of the PI3K-Akt signalling cascade at the late stage of the viral lytic cycle may also support cell survival and/or viral egress during KSHV lytic infection.

In summary, my results suggest that MEK/ERK pathway plays an important role during KSHV lytic cycle downstream of RTA expression, while activation of Akt may not be essential for successful virus production.

The identification of novel kinase inhibitors that interfere with KSHV lytic replication may reveal additional kinases, substrates and/or signalling pathways targeted during the viral productive cycle and may help to characterize the function of these signalling pathways in the KSHV life cycle. Screening of large panels of protein kinase inhibitors and their derivatives will aid the development of even more specific protein-kinase inhibitors.

Independent evidence from clinical studies suggests that lytic replication plays a pivotal role in KS development. The presence of replicating KSHV in peripheral blood has been shown to be one of the strongest predictors for the development of KS (Smith et al. 1997; Ziegler et al. 2003). In patients with advanced AIDS, treatment with ganciclovir, a nucleoside analogue that blocks lytic but not latent KSHV infection, reduces the appearance of new KS lesions (Martin et al. 1999). Since most of these patients have carried HIV and KSHV for many years, the impact of ganciclovir is not attributable simply to the reduction of early dissemination of KSHV to target cells. Furthermore, *in vitro* studies demonstrated that latent KSHV episomes are rapidly lost from a wide variety of proliferating cells in culture, including spindle cells explanted directly from KS biopsies, suggesting that ongoing lytic replication in a small amount of cells may be required for viral persistence and, thus, for initiation and maintenance of KS tumours (Aluigi et al. 1996; Lebbe et al. 1997; Grundhoff and Ganem 2004). Several recent studies have highlighted the crucial role of PI3K and MAPK pathways in KSHV infection and pathogenesis. The present study further underlines the essential function of the MEK/ERK cascade in KSHV lytic replication. Targeting this pathway, e.g. by MEK inhibitors, may inhibit viral replication and prevent subsequent pathogenic processes such as the production of angiogenic cytokines and growth factors. Thus, MEK inhibitors may have several advantages in KSHV antiviral therapy. Besides strong and broad antiviral activity, they may further inhibit production of contributing pathogenic factors such as angiogenic and inflammatory cytokines, and surprisingly showed very little toxicity in clinical trials for use as an anticancer agent (Cohen 2002; Wong 2009).

Moreover, a combined targeting of viral and cellular factors that are critical for viral replication may alleviate the emergence of drug-resistant virus variants.

4.2 Role of K1 protein in the viral lytic replication cycle

Several KSHV-encoded proteins, which are expressed during the early phase of viral lytic replication, are capable of inducing MAPK signalling pathways and/or transcription factors downstream of MAPK cascades. Using siRNAs to knock down expression of these proteins during the lytic cycle, I obtained the most consistent results with siRNAs to KSHV K1 in Vero rKSHV.219 and EAhy rKSHV cells. Genetic knockout of the K1 gene in a bacmid containing the KSHV genome confirmed the importance of K1 protein during the productive cycle as virus production was severely diminished in BAC36 ΔK1 cell lines induced with baculovirus Orf50/RTA and Na-butyrate (Fig. 17). An involvement of K1 during KSHV lytic reactivation had previously been suggested by Lagunoff et al. (2001) who showed that introduction of dominant-negative K1 mutants into BCBL-1 cells, a cultured PEL cell line, reduces RTA-induced lytic reactivation by 80 percent at a relatively early stage, prior to onset of viral DNA synthesis. In this study, stimulation with TPA could overcome the block imposed by K1 dominant negative mutants, suggesting that K1 and TPA might activate similar signalling cascades, or that other pathways activated by TPA can substitute for K1-mediated stimulation. In contrast, another study conducted by Lee et al. (2002) shows that TPA-induced lytic gene expression is strongly diminished when a CD8/K1-C chimera is expressed in BCBL-1 cells, suggesting an inhibitory role of K1 in the lytic replication cycle. The authors provide evidence that K1-signalling downregulates TPA-induced activation of AP-1, NF-κB, and Oct-1 activities, and suggest that this may confer the observed inhibitory effect of K1 on viral lytic protein expression.

My results show that K1 is required for an efficient activation of the lytic cycle in epithelial and endothelial cell lines. In agreement with Lagunoff *et al*., the present findings suggest that K1 expression is not indispensable for lytic cycle progression as the majority of K1-deletion mutants produced low numbers of infectious virus after reactivation from latency (Fig. 17). Rather, K1 may act as a major amplifier of lytic replication. Real-time quantitative PCR and Western blot analysis demonstrate that expression of immediate-early and early viral genes, including RTA, is reduced in ΔK1 mutants (Fig. 18, 19). These findings indicate that K1 is important at the early or even immediate-early phase of lytic cascade. Since RTA is required for expression of early viral genes and initiation of viral DNA replication, I assume that at least part of the block in virus production and reduced expression of early viral proteins is due to decreased RTA expression. However, studies conducted by Bu *et al.* (2008) showed that RTA activated only eight KSHV genes in the absence of *de novo* protein synthesis. Direct

transcriptional targets included the genes PAN, orf57/MTA, orf56/Primase, K2/vIL-6, orf37/SOX, K14/vOX2, K9/vIRF1 and orf52 (Bu et al. 2008). These findings suggest that the induction of most lytic cycle genes, including K8/bZIP and the viral CC-chemokines, requires additional protein expression after the expression of RTA and may involve further transactivators. Both cellular and viral proteins could satisfy this proposed role as RTA-cooperating factors.

Activation of ERK was strongly reduced in K1-deletion mutants, and Akt activation, although constitutively high in many clones, did not further increase during progression of the lytic cycle (Fig. 20 A). Activation of Akt signalling by K1 has repeatedly been shown (Tomlinson and Damania 2004; Wang et al. 2006). However, since my results (Fig. 8 A) suggest that activation of the Akt pathway occurs during the late phase of the lytic replication cycle, while the lack of K1 affects the expression of early proteins (Fig. 19, 20), I conclude that the absence of an increased Akt activity is due to reduced viral replication rather than a lacking direct activation of Akt by K1. In contrast to Akt, K1 does not activate ERK signalling (Fig. 20 D). Therefore, I suggest that the lytic cycle block observed in the absence of K1 occurs before the ERK pathway is activated, for example by other viral proteins (e.g. viral cytokines) or by cellular genes induced by K1. In this context, it is interesting to note that I observed a reduction of vCCL-1 and vIL-6 expression in BAC36 ΔK1 lines (Fig. 19 B), which have been implicated in activation of ERK/MAPK pathway.

Investigations of RTA expression profile during reactivation from latency and permissive *de novo* infection show that RTA expression is low during the immediate-early phase of lytic cycle but that it strongly increases during early and late phase (Paulose-Murphy et al. 2001; Nakamura et al. 2003; Yoo et al. 2005). Since K1 is expressed with early kinetics during the productive cycle, it is unlikely that K1 affects immediate-early RTA expression. I rather suggest that deletion of K1 inhibits increased RTA expression at subsequent stages of the lytic cycle. This is in accordance with the observation that RTA is not completely blocked in BAC36 ΔK1 mutants. Hence I conclude that K1 signalling might, directly or indirectly, augment RTA expression during early phase of lytic replication. It was shown that MAPK pathways activate the RTA promoter activity through AP-1 during primary infection and TPA-induced reactivation from latency (Sharma-Walia et al. 2005; Pan et al. 2006) and that ectopic expression of c-Jun and c-Fos, which together form the active AP-1 complex, activated the RTA promoter in transient transfections and promoted expression of RTA in PEL cells (Wang et al. 2004c). Activation of AP-1 activity may therefore provide a possible mechanism how K1 augments RTA expression during lytic cycle. Interestingly, AP-1 activity

has also been implicated in early activation of the K8 promoter (Wang et al. 2004c). I found the K8 gene was to be significantly reduced in K1-deletion mutants (Fig. 18 A, 19 B). Hence, K1-induced activation of AP-1 might be involved in activation of the K8 promoter.

Anti-apoptotic/pro-survival mechanisms are important during KSHV lytic cycle to prevent premature host cell dying and thereby to ensure efficient virus replication. It was shown that K1 inhibits apoptosis via Akt-mediated cell survival signalling (Tomlinson and Damania 2004; Wang et al. 2006). However, inhibition of PI3K-Akt signalling by LY294002 did not significantly affect viral progeny production in Vero rKSHV.219 cells (Fig. 9), and I did not observe increased cell death in BAC36 ΔK1 during lytic replication (not shown).

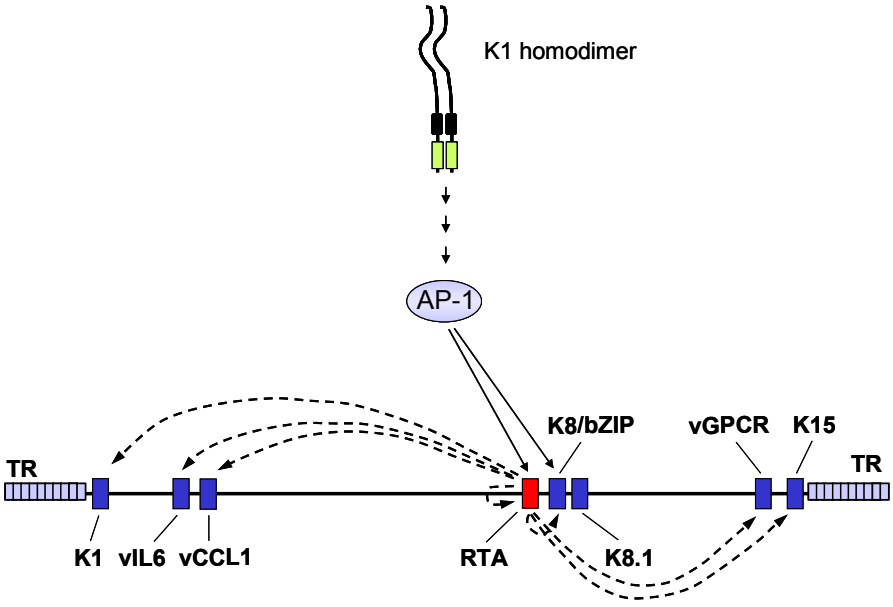


Figure 22. Schematic model of K1's function in lytic cycle progression. Intracellular signalling of K1 activates several cellular proteins including AP-1 transcription factor. Active AP-1 has been shown to induce expression of viral RTA and K8/bZIP proteins by targeting specific binding sites in their promoter region. These proteins in turn transactivate expression of other viral proteins such as vGPCR and the viral cytokines which have been shown to positively regulate KSHV lytic replication. Thus, K1 signalling may promote progression through the lytic cycle by upregulating expression of viral proteins that facilitate KSHV productive replication.

Immunofluorescence in BAC36 wt cell lines showed that only about ten percent of cells undergoing KSHV lytic replication (indicated by expression of K8/bZIP) also express K1 protein (Fig. 19 B). Therefore, I suggest that K1-mediated promotion of lytic replication may include paracrine mechanisms. Several studies show that K1-mediated signal transduction

induces numerous cellular cytokines and growth factors, including macrophage derived cytokine (MCD), IL-8, IL-10, VEGF, basic fibroblast growth factor (FGF-2), IL-αβ, and RANTES (Samaniego et al. 2001; Wang et al. 2004b; Lee et al. 2005). It has been suggested that K1 activates the promoter of VEGF and probably also promoters of other cytokines in an AP-1-dependent manner (Lagunoff et al. 2001; Wang et al. 2004b; Lee et al. 2005). Activation of ERK signalling cascade by K1-induced cellular cytokines might therefore significantly contribute to lytic gene expression and viral DNA replication. The details of the signalling cascade underlying the stimulatory effects of K1 in KSHV lytic cycle remain to be elucidated.

I further analysed the cellular gene expression profile of BAC36 wt– and BAC36 ΔK1-containing HEK293 cells before and after induction of the viral lytic cycle in order to identify genes that are differentially regulated by BAC36 wildtype and K1-deletion mutant virus. One of the transcripts that were most significantly and consistently upregulated in BAC36 wt-containing cell lines compared to BAC36 K1-deletion mutant lines encoded for G protein-coupled receptor 6 (GPR6). GPR6 belongs to a family of G protein-coupled receptors that also includes GPR3 and GPR12. Receptors of this family have been shown to be constitutively active, stimulate cyclic adenosine monophosphate (cAMP) production via $G\alpha_s$-protein activation and activate intracellular Ca^{2+} mobilization (Eggerickx et al. 1995; Uhlenbrock et al. 2002; Ignatov et al. 2003). Although constitutive activity of these receptors has been known for more than a decade, their physiological roles are just beginning to be identified. All three GPR6, GPR3 and GPR12 have been implicated in cAMP-mediated neurite outgrowth (Tanaka et al. 2007). Expression of GPR3 and GPR12 in ovary is responsible for maintaining high cAMP concentrations required for meiotic arrest in mammalian and rodent oocytes (Mehlmann et al. 2004; Freudzon et al. 2005; Hinckley et al. 2005; Mehlmann 2005). Thus, promotion of cAMP-mediated signalling may be an important function of these receptors. cAMP stimulates serine-threonine protein kinase A (PKA), also referred to as cAMP-dependent protein kinase, which in turn activates the transcription factor CREB (cAMP response element binding) in the nucleus. Activated CREB then binds to cAMP response elements (CRE) in the DNA. Studies performed by Chang *et al.* have shown that activation of cAMP signalling with a cell permeable cAMP analogue or a cAMP inducer activated KSHV lytic gene expression in PEL cells. Furthermore, overexpression of the PKA catalytic subunit upregulated RTA gene expression, enhanced activity of the RTA promoter and posttranslationlly promoted the trans-activating capacity of RTA for its own promoter and heterologous lytic promoters (e.g., the viral PAN gene) (Chang et al. 2005). Additionally, it

was shown that RTA binds to CREB-binding protein (CBP) (Gwack et al. 2001). CBP acts as a co-activator of CREB, and evidence suggests that binding of RTA to CBP activates RTA-mediated viral transcription (Gwack et al. 2001). Increased cAMP levels via K1-mediated upregulation of GPR6 expression may therefore facilitate KSHV lytic replication.

Among the cellular genes that showed a reduced expression in HEK293 BAC36 wt cell lines compared to BAC36 ΔK1 cell lines were TRIM15, TRIM10 and SOCS3 (4-, 2.8- and 3.5-fold downregulated, respectively).

Tripartite motif (TRIM) proteins comprise a family of E3 ligases with over 70 members implicated in a variety of cellular functions, including differentiation, apoptosis and immunity (Reymond et al. 2001). An increasing number of TRIM proteins have been found to display antiviral activities, targeting retroviruses in particular (Nisole et al. 2005; Ozato et al. 2008; Uchil et al. 2008). The antiviral activities affect both early and late stages of the viral life cycle. For example, TRIM11 and TRIM31 were demonstrated to inhibit HIV entry, while TRIM11 and TRIM15 reduced virus release in HEK293 cells (Uchil et al. 2008). TRIM5α was shown to be responsible for a species-specific post-entry restriction of N-tropic murine leukaemia virus (N-MLV) and HIV-1 in primate cells (Hatziioannou et al. 2004; Perron et al. 2004; Stremlau et al. 2004; Yap et al. 2004), whereas TRIM22, also known as Staf50, has been shown to inhibit HIV-1 replication (Bouazzaoui et al. 2006; Barr et al. 2008). TRIM28 restricts MLV LTR-driven transcription in murine embryonic cells (Wolf and Goff 2007; Wolf et al. 2008). Broad antiviral activities have further been reported for TRIM19, the defining component of PML bodies in the nucleus. Viruses inhibited by TRIM19 include - among others - influenza A virus, HIV, human cytomegalovirus and herpes simplex type 1 (Nisole et al. 2005; Everett and Chelbi-Alix 2007). The finding that two TRIM proteins, including TRIM15, which has already been implicated in antiviral function (Uchil et al. 2008), are differentially regulated between BAC36 wildtype and K1-deletion mutant lines, may indicate that members of this protein family also play a role in the KSHV life cycle. Downregulation of TRIM proteins by K1 signalling possibly facilitates certain steps in viral lytic replication.

The family of suppressors of cytokine signalling (SOCS) consists of eight members (SOCS-1 to SOCS-7 and the cytokine-inducible SH2-containing protein (CIS)) all sharing a central SH2 domain and a C-terminal SOCS box (Kile and Alexander 2001). SOCS proteins play key roles in the negative regulation of cytokine signal transduction. Transcripts encoding CIS, SOCS1, SOCS2, and SOCS3 are upregulated in response to cytokine stimulation, and the corresponding SOCS proteins inhibit cytokine-induced signalling pathways. SOCS

proteins therefore form part of a classical negative feedback circuit. SOCS family members modulate signalling by several mechanisms, which include inactivation of JAKs, blocking access of the STAT proteins to receptor binding sites, and ubiquitination of signalling proteins and their subsequent targeting to the proteasome (Kile and Alexander 2001; Krebs and Hilton 2001). In addition to their role as inhibitors of cytokine signalling, SOCS proteins may also play a role in the desensitization of cytokine signalling (Fischer et al. 2004). Mice conditionally deficient for *Socs3* revealed SOCS3 to be a potent physiological suppressor of IL-6 signalling (Croker et al. 2003; Lang et al. 2003; Yasukawa et al. 2003), and it was demonstrated that SOCS3 desensitized murine primary bone marrow-derived macrophages to IL-6 signalling (Wormald et al. 2006). The present microarray data may therefore provide evidence for a role of K1 in continuous cytokine signalling and sensitivity of KSHV-infected cells to viral and cellular IL-6. Previously, it was shown that RTA and the major latent protein LANA interact with STAT3 and stimulate transcription of STAT-driven reporter genes (Gwack et al. 2002; Muromoto et al. 2006), indicating that KSHV may modify STATs activities to increase viral persistence and/or replication. Since it is assumed that IL-6 and STAT3 signalling significantly contribute to malignant progression of PEL (Yoshizaki et al. 1989; Miles et al. 1990; Burger et al. 1994; Ishiyama et al. 1994; Aoki et al. 2003), my findings may further reveal a novel mechanism of K1 signalling in KSHV pathogenesis. However, since microarray experiments were performed in the context of the whole KSHV genome, gene expression profiles obtained do not allow differentiation between effects caused by K1 directly and subsequent K1-dependent mechanisms. Therefore, it is possible that other lytic proteins, which depend on K1 signalling for their expression, are responsible for the effects on cellular gene expression described above.

Although analysis of the microarray data provided some promising genes for cellular targets of K1 signalling, further investigations are necessary to verify the differential regulation of genes between BAC36 wildtype and K1-deletion cell lines (e.g. using real-time quantitative PCR). Because of the high variations in gene expression profiles among different HEK293 BAC36 wt and ΔK1 lines, future experiments should include larger numbers of individual cell lines to improve statistical verification.

In summary, my results suggest that the K1 protein of KSHV may stimulate viral protein expression and viral DNA replication during the lytic replication cycle by direct or indirect activation of ERK signalling cascade via autocrine and/or paracrine mechanisms. Furthermore, K1 signalling may stimulate the expression of cellular signalling proteins and interfere with antiviral processes and thereby support KSHV replication and persistence.

References

Aluigi, M. G., A. Albini, S. Carlone, L. Repetto, R. De Marchi, A. Icardi, M. Moro, D. Noonan and R. Benelli (1996). "KSHV sequences in biopsies and cultured spindle cells of epidemic, iatrogenic and Mediterranean forms of Kaposi's sarcoma." Res Virol 147(5): 267-75.

An, F. Q., N. Compitello, E. Horwitz, M. Sramkoski, E. S. Knudsen and R. Renne (2005). "The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest." J Biol Chem 280(5): 3862-74.

Aoki, Y., G. M. Feldman and G. Tosato (2003). "Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma." Blood 101(4): 1535-42.

Aoki, Y., E. S. Jaffe, Y. Chang, K. Jones, J. Teruya-Feldstein, P. S. Moore and G. Tosato (1999). "Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6." Blood 93(12): 4034-43.

Bagni, R. and D. Whitby (2009). "Kaposi's sarcoma-associated herpesvirus transmission and primary infection." Curr Opin HIV AIDS 4(1): 22-6.

Ballestas, M. E., P. A. Chatis and K. M. Kaye (1999). "Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen." Science 284(5414): 641-4.

Barr, S. D., J. R. Smiley and F. D. Bushman (2008). "The interferon response inhibits HIV particle production by induction of TRIM22." PLoS Pathog 4(2): e1000007.

Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss and J. J. O'Leary (1995). "Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells." Nat Med 1(12): 1274-8.

Bouazzaoui, A., M. Kreutz, V. Eisert, N. Dinauer, A. Heinzelmann, S. Hallenberger, J. Strayle, R. Walker, H. Rubsamen-Waigmann, R. Andreesen and H. von Briesen (2006). "Stimulated trans-acting factor of 50 kDa (Staf50) inhibits HIV-1 replication in human monocyte-derived macrophages." Virology 356(1-2): 79-94.

Brinkmann, M. M., M. Glenn, L. Rainbow, A. Kieser, C. Henke-Gendo and T. F. Schulz (2003). "Activation of mitogen-activated protein kinase and NF-kappaB pathways by a Kaposi's sarcoma-associated herpesvirus K15 membrane protein." J Virol 77(17): 9346-58.

Brinkmann, M. M., M. Pietrek, O. Dittrich-Breiholz, M. Kracht and T. F. Schulz (2007). "Modulation of host gene expression by the K15 protein of Kaposi's sarcoma-associated herpesvirus." J Virol 81(1): 42-58.

Brown, H. J., M. J. Song, H. Deng, T. T. Wu, G. Cheng and R. Sun (2003). "NF-kappaB inhibits gammaherpesvirus lytic replication." J Virol 77(15): 8532-40.

Bu, W., D. Palmeri, R. Krishnan, R. Marin, V. M. Aris, P. Soteropoulos and D. M. Lukac (2008). "Identification of direct transcriptional targets of the Kaposi's sarcoma-associated herpesvirus Rta lytic switch protein by conditional nuclear localization." J Virol 82(21): 10709-23.

Burger, R., J. Wendler, K. Antoni, G. Helm, J. R. Kalden and M. Gramatzki (1994). "Interleukin-6 production in B-cell neoplasias and Castleman's disease: evidence for an additional paracrine loop." Ann Hematol 69(1): 25-31.

Cesarman, E., Y. Chang, P. S. Moore, J. W. Said and D. M. Knowles (1995). "Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas." N Engl J Med 332(18): 1186-91.

Chang, J. and D. Ganem (2000). "On the control of late gene expression in Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8)." J Gen Virol 81(Pt 8): 2039-47.

Chang, M., H. J. Brown, A. Collado-Hidalgo, J. M. Arevalo, Z. Galic, T. L. Symensma, L. Tanaka, H. Deng, J. A. Zack, R. Sun and S. W. Cole (2005). "beta-Adrenoreceptors reactivate Kaposi's sarcoma-associated herpesvirus lytic replication via PKA-dependent control of viral RTA." J Virol 79(21): 13538-47.

Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles and P. S. Moore (1994). "Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma." Science 266(5192): 1865-9.

Chen, D., Y. B. Choi, G. Sandford and J. Nicholas (2009). "Determinants of secretion and intracellular localization of human herpesvirus 8 interleukin-6." J Virol 83(13): 6874-82.

Choi, J. K., B. S. Lee, S. N. Shim, M. Li and J. U. Jung (2000). "Identification of the novel K15 gene at the rightmost end of the Kaposi's sarcoma-associated herpesvirus genome." J Virol 74(1): 436-46.

Cohen, P. (2002). "Protein kinases--the major drug targets of the twenty-first century?" Nat Rev Drug Discov 1(4): 309-15.

Coopman, P. J. and S. C. Mueller (2006). "The Syk tyrosine kinase: a new negative regulator in tumor growth and progression." Cancer Lett 241(2): 159-73.

Cotter, M. A., 2nd and E. S. Robertson (1999). "The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells." Virology 264(2): 254-64.

Croker, B. A., D. L. Krebs, J. G. Zhang, S. Wormald, T. A. Willson, E. G. Stanley, L. Robb, C. J. Greenhalgh, I. Forster, B. E. Clausen, N. A. Nicola, D. Metcalf, D. J. Hilton, A. W. Roberts and W. S. Alexander (2003). "SOCS3 negatively regulates IL-6 signaling in vivo." Nat Immunol 4(6): 540-5.

Damania, B. (2004). "Oncogenic gamma-herpesviruses: comparison of viral proteins involved in tumorigenesis." Nat Rev Microbiol 2(8): 656-68.

Damania, B., M. Li, J. K. Choi, L. Alexander, J. U. Jung and R. C. Desrosiers (1999). "Identification of the R1 oncogene and its protein product from the rhadinovirus of rhesus monkeys." J Virol 73(6): 5123-31.

Datsenko, K. A. and B. L. Wanner (2000). "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products." Proc Natl Acad Sci U S A 97(12): 6640-5.

Dedicoat, M. and R. Newton (2003). "Review of the distribution of Kaposi's sarcoma-associated herpesvirus (KSHV) in Africa in relation to the incidence of Kaposi's sarcoma." Br J Cancer 88(1): 1-3.

Dittmer, D. P. (2003). "Transcription profile of Kaposi's sarcoma-associated herpesvirus in primary Kaposi's sarcoma lesions as determined by real-time PCR arrays." Cancer Res 63(9): 2010-5.

Dourmishev, L. A., A. L. Dourmishev, D. Palmeri, R. A. Schwartz and D. M. Lukac (2003). "Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis." Microbiol Mol Biol Rev 67(2): 175-212, table of contents.

Druker, B. J. and N. B. Lydon (2000). "Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia." J Clin Invest 105(1): 3-7.

Dupin, N., C. Fisher, P. Kellam, S. Ariad, M. Tulliez, N. Franck, E. van Marck, D. Salmon, I. Gorin, J. P. Escande, R. A. Weiss, K. Alitalo and C. Boshoff (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(8): 4546-51.

Durfee, T., R. Nelson, S. Baldwin, G. Plunkett, 3rd, V. Burland, B. Mau, J. F. Petrosino, X. Qin, D. M. Muzny, M. Ayele, R. A. Gibbs, B. Csorgo, G. Posfai, G. M. Weinstock and F. R. Blattner (2008). "The complete genome sequence of Escherichia coli DH10B: insights into the biology of a laboratory workhorse." J Bacteriol 190(7): 2597-606.

Edgell, C. J., C. C. McDonald and J. B. Graham (1983). "Permanent cell line expressing human factor VIII-related antigen established by hybridization." Proc Natl Acad Sci U S A 80(12): 3734-7.

Eggerickx, D., J. F. Denef, O. Labbe, Y. Hayashi, S. Refetoff, G. Vassart, M. Parmentier and F. Libert (1995). "Molecular cloning of an orphan G-protein-coupled receptor that constitutively activates adenylate cyclase." Biochem J 309 (Pt 3): 837-43.

Everett, R. D. and M. K. Chelbi-Alix (2007). "PML and PML nuclear bodies: implications in antiviral defence." Biochimie 89(6-7): 819-30.

Fakhari, F. D. and D. P. Dittmer (2002). "Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR." J Virol 76(12): 6213-23.

Fakhari, F. D., J. H. Jeong, Y. Kanan and D. P. Dittmer (2006). "The latency-associated nuclear antigen of Kaposi sarcoma-associated herpesvirus induces B cell hyperplasia and lymphoma." J Clin Invest 116(3): 735-42.

Fischer, P., U. Lehmann, R. M. Sobota, J. Schmitz, C. Niemand, S. Linnemann, S. Haan, I. Behrmann, A. Yoshimura, J. A. Johnston, G. Muller-Newen, P. C. Heinrich and F. Schaper (2004). "The role of the inhibitors of interleukin-6 signal transduction SHP2 and SOCS3 for desensitization of interleukin-6 signalling." Biochem J 378(Pt 2): 449-60.

Freudzon, L., R. P. Norris, A. R. Hand, S. Tanaka, Y. Saeki, T. L. Jones, M. M. Rasenick, C. H. Berlot, L. M. Mehlmann and L. A. Jaffe (2005). "Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein." J Cell Biol 171(2): 255-65.

Friborg, J., Jr., W. Kong, M. O. Hottiger and G. J. Nabel (1999). "p53 inhibition by the LANA protein of KSHV protects against cell death." Nature 402(6764): 889-94.

Friedman-Kien, A. E. and B. R. Saltzman (1990). "Clinical manifestations of classical, endemic African, and epidemic AIDS-associated Kaposi's sarcoma." J Am Acad Dermatol 22(6 Pt 2): 1237-50.

Fujimuro, M. and S. D. Hayward (2003). "The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3beta." J Virol 77(14): 8019-30.

Fujimuro, M., F. Y. Wu, C. ApRhys, H. Kajumbula, D. B. Young, G. S. Hayward and S. D. Hayward (2003). "A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency." Nat Med 9(3): 300-6.

Ganem, D. (2006). "KSHV infection and the pathogenesis of Kaposi's sarcoma." Annu Rev Pathol 1: 273-96.

Glenn, M., L. Rainbow, F. Aurade, A. Davison and T. F. Schulz (1999). "Identification of a spliced gene from Kaposi's sarcoma-associated herpesvirus encoding a protein with similarities to latent membrane proteins 1 and 2A of Epstein-Barr virus." J Virol 73(8): 6953-63.

Greene, W., K. Kuhne, F. Ye, J. Chen, F. Zhou, X. Lei and S. J. Gao (2007). "Molecular biology of KSHV in relation to AIDS-associated oncogenesis." Cancer Treat Res 133: 69-127.

Grundhoff, A. and D. Ganem (2004). "Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis." J Clin Invest 113(1): 124-36.

Gwack, Y., H. Byun, S. Hwang, C. Lim and J. Choe (2001). "CREB-binding protein and histone deacetylase regulate the transcriptional activity of Kaposi's sarcoma-associated herpesvirus open reading frame 50." J Virol 75(4): 1909-17.

Gwack, Y., S. Hwang, C. Lim, Y. S. Won, C. H. Lee and J. Choe (2002). "Kaposi's Sarcoma-associated herpesvirus open reading frame 50 stimulates the transcriptional activity of STAT3." J Biol Chem 277(8): 6438-42.

Hartmann, S., T. F. Schulz and J. Nicholas (2009). HHV-8/KSHV Proteins Involved in Signaling and Transformation. DNA Tumor Viruses. B. Damania and J. M. Pipas, Springer Science+Business Media.

Hatziioannou, T., D. Perez-Caballero, A. Yang, S. Cowan and P. D. Bieniasz (2004). "Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha." Proc Natl Acad Sci U S A 101(29): 10774-9.

Heinrich, P. C., I. Behrmann, S. Haan, H. M. Hermanns, G. Muller-Newen and F. Schaper (2003). "Principles of interleukin (IL)-6-type cytokine signalling and its regulation." Biochem J 374(Pt 1): 1-20.

Herndier, B. and D. Ganem (2001). "The biology of Kaposi's sarcoma." Cancer Treat Res 104: 89-126.

Hinckley, M., S. Vaccari, K. Horner, R. Chen and M. Conti (2005). "The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes." Dev Biol 287(2): 249-61.

Honess, R. W. (1984). "Herpes simplex and 'the herpes complex': diverse observations and a unifying hypothesis. The eighth Fleming lecture." J Gen Virol 65 (Pt 12): 2077-107.

Hong, Y. K., K. Foreman, J. W. Shin, S. Hirakawa, C. L. Curry, D. R. Sage, T. Libermann, B. J. Dezube, J. D. Fingeroth and M. Detmar (2004). "Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus." Nat Genet 36(7): 683-5.

Hu, J., A. C. Garber and R. Renne (2002). "The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells." J Virol 76(22): 11677-87.

Ignatov, A., J. Lintzel, H. J. Kreienkamp and H. C. Schaller (2003). "Sphingosine-1-phosphate is a high-affinity ligand for the G protein-coupled receptor GPR6 from mouse and induces intracellular Ca2+ release by activating the sphingosine-kinase pathway." Biochem Biophys Res Commun 311(2): 329-36.

Ishiyama, T., S. Nakamura, Y. Akimoto, M. Koike, S. Tomoyasu, N. Tsuruoka, Y. Murata, T. Sato, Y. Wakabayashi and S. Chiba (1994). "Immunodeficiency and IL-6 production by peripheral blood monocytes in multicentric Castleman's disease." Br J Haematol 86(3): 483-9.

Izumiya, Y., C. Izumiya, A. Van Geelen, D. H. Wang, K. S. Lam, P. A. Luciw and H. J. Kung (2007). "Kaposi's sarcoma-associated herpesvirus-encoded protein kinase and its interaction with K-bZIP." J Virol 81(3): 1072-82.

Izumiya, Y., S. F. Lin, T. Ellison, L. Y. Chen, C. Izumiya, P. Luciw and H. J. Kung (2003). "Kaposi's sarcoma-associated herpesvirus K-bZIP is a coregulator of K-Rta: physical association and promoter-dependent transcriptional repression." J Virol 77(2): 1441-51.

Kaposi, M. (1872). "Idiopathisches multiples Pigmentsarcom der Haut. (English translation: Idiopathic multiple pigmented sarcoma of the skin.)." Arch Dermatol und Syphillis 4: 265-273.

Katano, H., Y. Sato, T. Kurata, S. Mori and T. Sata (2000). "Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease." Virology 269(2): 335-44.

Kato-Noah, T., Y. Xu, C. C. Rossetto, K. Colletti, I. Papouskova and G. S. Pari (2007). "Overexpression of the kaposi's sarcoma-associated herpesvirus transactivator K-Rta can complement a K-bZIP deletion BACmid and yields an enhanced growth phenotype." J Virol 81(24): 13519-32.

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

Kelley-Clarke, B., E. De Leon-Vazquez, K. Slain, A. J. Barbera and K. M. Kaye (2009). "Role of Kaposi's sarcoma-associated herpesvirus C-terminal LANA chromosome binding in episome persistence." J Virol 83(9): 4326-37.

Kile, B. T. and W. S. Alexander (2001). "The suppressors of cytokine signalling (SOCS)." Cell Mol Life Sci 58(11): 1627-35.

Krebs, D. L. and D. J. Hilton (2001). "SOCS proteins: negative regulators of cytokine signaling." Stem Cells 19(5): 378-87.

Lagunoff, M., D. M. Lukac and D. Ganem (2001). "Immunoreceptor tyrosine-based activation motif-dependent signaling by Kaposi's sarcoma-associated herpesvirus K1 protein: effects on lytic viral replication." J Virol 75(13): 5891-8.

Lang, R., A. L. Pauleau, E. Parganas, Y. Takahashi, J. Mages, J. N. Ihle, R. Rutschman and P. J. Murray (2003). "SOCS3 regulates the plasticity of gp130 signaling." Nat Immunol 4(6): 546-50.

Lebbe, C., P. de Cremoux, G. Millot, M. P. Podgorniak, O. Verola, R. Berger, P. Morel and F. Calvo (1997). "Characterization of in vitro culture of HIV-negative Kaposi's sarcoma-derived cells. In vitro responses to alfa interferon." Arch Dermatol Res 289(7): 421-8.

Lee, B. S., S. H. Lee, P. Feng, H. Chang, N. H. Cho and J. U. Jung (2005). "Characterization of the Kaposi's sarcoma-associated herpesvirus K1 signalosome." J Virol 79(19): 12173-84.

Liu, C., Y. Okruzhnov, H. Li and J. Nicholas (2001). "Human herpesvirus 8 (HHV-8)-encoded cytokines induce expression of and autocrine signaling by vascular endothelial growth factor (VEGF) in HHV-8-infected primary-effusion lymphoma cell lines and mediate VEGF-independent antiapoptotic effects." J Virol 75(22): 10933-40.

Liu, P., H. Cheng, T. M. Roberts and J. J. Zhao (2009). "Targeting the phosphoinositide 3-kinase pathway in cancer." Nat Rev Drug Discov 8(8): 627-44.

Lloyd, A. C. (2006). "Distinct functions for ERKs?" J Biol 5(5): 13.

Lukac, D. M., J. R. Kirshner and D. Ganem (1999). "Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells." J Virol 73(11): 9348-61.

Martin, D. F., B. D. Kuppermann, R. A. Wolitz, A. G. Palestine, H. Li and C. A. Robinson (1999). "Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group." N Engl J Med 340(14): 1063-70.

McAllister, S. C., S. G. Hansen, I. Messaoudi, J. Nikolich-Zugich and A. V. Moses (2005). "Increased efficiency of phorbol ester-induced lytic reactivation of Kaposi's sarcoma-associated herpesvirus during S phase." J Virol 79(4): 2626-30.

McGeoch, D. J., S. Cook, A. Dolan, F. E. Jamieson and E. A. Telford (1995). "Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses." J Mol Biol 247(3): 443-58.

Mehlmann, L. M. (2005). "Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes." Dev Biol 288(2): 397-404.

Mehlmann, L. M., Y. Saeki, S. Tanaka, T. J. Brennan, A. V. Evsikov, F. L. Pendola, B. B. Knowles, J. J. Eppig and L. A. Jaffe (2004). "The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes." Science 306(5703): 1947-50.

Mettenleiter, T. C. (2002). "Herpesvirus assembly and egress." J Virol 76(4): 1537-47.

Miles, S. A., A. R. Rezai, J. F. Salazar-Gonzalez, M. Vander Meyden, R. H. Stevens, D. M. Logan, R. T. Mitsuyasu, T. Taga, T. Hirano, T. Kishimoto and et al. (1990). "AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6." Proc Natl Acad Sci U S A 87(11): 4068-72.

Mitsuyasu, R. T. (1987). "Clinical variants and staging of Kaposi's sarcoma." Semin Oncol 14(2 Suppl 3): 13-8.

Mori, Y., N. Nishimoto, M. Ohno, R. Inagi, P. Dhepakson, K. Amou, K. Yoshizaki and K. Yamanishi (2000). "Human herpesvirus 8-encoded interleukin-6 homologue (viral IL-6) induces endogenous human IL-6 secretion." J Med Virol 61(3): 332-5.

Muromoto, R., K. Okabe, M. Fujimuro, K. Sugiyama, H. Yokosawa, T. Seya and T. Matsuda (2006). "Physical and functional interactions between STAT3 and Kaposi's sarcoma-associated herpesvirus-encoded LANA." FEBS Lett 580(1): 93-8.

Nakamura, H., M. Lu, Y. Gwack, J. Souvlis, S. L. Zeichner and J. U. Jung (2003). "Global changes in Kaposi's sarcoma-associated virus gene expression patterns following expression of a tetracycline-inducible Rta transactivator." J Virol 77(7): 4205-20.

Nisole, S., J. P. Stoye and A. Saib (2005). "TRIM family proteins: retroviral restriction and antiviral defence." Nat Rev Microbiol 3(10): 799-808.

O'Connor, C. M. and D. H. Kedes (2007). "Rhesus monkey rhadinovirus: a model for the study of KSHV." Curr Top Microbiol Immunol 312: 43-69.

Orem, J., M. W. Otieno and S. C. Remick (2004). "AIDS-associated cancer in developing nations." Curr Opin Oncol 16(5): 468-76.

Ozato, K., D. M. Shin, T. H. Chang and H. C. Morse, 3rd (2008). "TRIM family proteins and their emerging roles in innate immunity." Nat Rev Immunol 8(11): 849-60.

Pan, H., J. Xie, F. Ye and S. J. Gao (2006). "Modulation of Kaposi's sarcoma-associated herpesvirus infection and replication by MEK/ERK, JNK, and p38 multiple mitogen-activated protein kinase pathways during primary infection." J Virol 80(11): 5371-82.

Park, J., D. Lee, T. Seo, J. Chung and J. Choe (2000). "Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) open reading frame 36 protein is a serine protein kinase." J Gen Virol 81(Pt 4): 1067-71.

Parkin, D. M. (2006). "The global health burden of infection-associated cancers in the year 2002." Int J Cancer 118(12): 3030-44.

Parravicini, C., B. Chandran, M. Corbellino, E. Berti, M. Paulli, P. S. Moore and Y. Chang (2000). "Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease." Am J Pathol 156(3): 743-9.

Paulose-Murphy, M., N. K. Ha, C. Xiang, Y. Chen, L. Gillim, R. Yarchoan, P. Meltzer, M. Bittner, J. Trent and S. Zeichner (2001). "Transcription program of human herpesvirus 8 (kaposi's sarcoma-associated herpesvirus)." J Virol 75(10): 4843-53.

Peng, L., T. T. Wu, J. H. Tchieu, J. Feng, H. J. Brown, J. Feng, X. Li, J. Qi, H. Deng, I. Vivanco, I. K. Mellinghoff, C. Jamieson and R. Sun (2009). "The Inhibition of PI3K-Akt Pathway Enhances Gamma-2 Herpesvirus Lytic Replication and Facilitates Reactivation from Latency." J Gen Virol.

Penn, I. (1988). "Secondary neoplasms as a consequence of transplantation and cancer therapy." Cancer Detect Prev 12(1-6): 39-57.

Perron, M. J., M. Stremlau, B. Song, W. Ulm, R. C. Mulligan and J. Sodroski (2004). "TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells." Proc Natl Acad Sci U S A 101(32): 11827-32.

Poole, L. J., J. C. Zong, D. M. Ciufo, D. J. Alcendor, J. S. Cannon, R. Ambinder, J. M. Orenstein, M. S. Reitz and G. S. Hayward (1999). "Comparison of genetic variability at multiple loci across the genomes of the major subtypes of Kaposi's sarcoma-associated herpesvirus reveals evidence for recombination and for two distinct types of open reading frame K15 alleles at the right-hand end." J Virol 73(8): 6646-60.

Radkov, S. A., P. Kellam and C. Boshoff (2000). "The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells." Nat Med 6(10): 1121-7.

Renne, R., C. Barry, D. Dittmer, N. Compitello, P. O. Brown and D. Ganem (2001). "Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus." J Virol 75(1): 458-68.

Reymond, A., G. Meroni, A. Fantozzi, G. Merla, S. Cairo, L. Luzi, D. Riganelli, E. Zanaria, S. Messali, S. Cainarca, A. Guffanti, S. Minucci, P. G. Pelicci and A. Ballabio (2001). "The tripartite motif family identifies cell compartments." Embo J 20(9): 2140-51.

Rezaee, S. A., J. A. Gracie, I. B. McInnes and D. J. Blackbourn (2005). "Inhibition of neutrophil function by the Kaposi's sarcoma-associated herpesvirus vOX2 protein." Aids 19(16): 1907-10.

Rivas, C., A. E. Thlick, C. Parravicini, P. S. Moore and Y. Chang (2001). "Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53." J Virol 75(1): 429-38.

Sadler, R., L. Wu, B. Forghani, R. Renne, W. Zhong, B. Herndier and D. Ganem (1999). "A complex translational program generates multiple novel proteins from the latently expressed kaposin (K12) locus of Kaposi's sarcoma-associated herpesvirus." J Virol 73(7): 5722-30.

Samaniego, F., P. D. Markham, R. C. Gallo and B. Ensoli (1995). "Inflammatory cytokines induce AIDS-Kaposi's sarcoma-derived spindle cells to produce and release basic fibroblast growth factor and enhance Kaposi's sarcoma-like lesion formation in nude mice." J Immunol 154(7): 3582-92.

Samaniego, F., S. Pati, J. E. Karp, O. Prakash and D. Bose (2001). "Human herpesvirus 8 K1-associated nuclear factor-kappa B-dependent promoter activity: role in Kaposi's sarcoma inflammation?" J Natl Cancer Inst Monogr(28): 15-23.

Schulz, T. F. (2006). "The pleiotropic effects of Kaposi's sarcoma herpesvirus." J Pathol 208(2): 187-98.

Schulz, T. F., J. Sheldon and J. Greensill (2002). "Kaposi's sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8)." Virus Res 82(1-2): 115-26.

Sgarbanti, M., M. Arguello, B. R. tenOever, A. Battistini, R. Lin and J. Hiscott (2004). "A requirement for NF-kappaB induction in the production of replication-competent HHV-8 virions." Oncogene 23(34): 5770-80.

Sharma-Walia, N., H. H. Krishnan, P. P. Naranatt, L. Zeng, M. S. Smith and B. Chandran (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(16): 10308-29.

Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos and et al. (1995). "Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease." Blood 86(4): 1276-80.

Staskus, K. A., W. Zhong, K. Gebhard, B. Herndier, H. Wang, R. Renne, J. Beneke, J. Pudney, D. J. Anderson, D. Ganem and A. T. Haase (1997). "Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells." J Virol 71(1): 715-9.

Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier and J. Sodroski (2004). "The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys." Nature 427(6977): 848-53.

Sturzl, M., C. Blasig, A. Schreier, F. Neipel, C. Hohenadl, E. Cornali, G. Ascherl, S. Esser, N. H. Brockmeyer, M. Ekman, E. E. Kaaya, E. Tschachler and P. Biberfeld (1997). "Expression of HHV-8 latency-associated T0.7 RNA in spindle cells and endothelial cells of AIDS-associated, classical and African Kaposi's sarcoma." Int J Cancer 72(1): 68-71.

Symensma, T. L., D. Martinez-Guzman, Q. Jia, E. Bortz, T. T. Wu, N. Rudra-Ganguly, S. Cole, H. Herschman and R. Sun (2003). "COX-2 induction during murine gammaherpesvirus 68 infection leads to enhancement of viral gene expression." J Virol 77(23): 12753-63.

Tanaka, S., K. Ishii, K. Kasai, S. O. Yoon and Y. Saeki (2007). "Neural expression of G protein-coupled receptors GPR3, GPR6, and GPR12 up-regulates cyclic AMP levels and promotes neurite outgrowth." J Biol Chem 282(14): 10506-15.

Tomlinson, C. C. and B. Damania (2004). "The K1 protein of Kaposi's sarcoma-associated herpesvirus activates the Akt signaling pathway." J Virol 78(4): 1918-27.

Uchil, P. D., B. D. Quinlan, W. T. Chan, J. M. Luna and W. Mothes (2008). "TRIM E3 ligases interfere with early and late stages of the retroviral life cycle." PLoS Pathog 4(2): e16.

Uhlenbrock, K., H. Gassenhuber and E. Kostenis (2002). "Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors." Cell Signal 14(11): 941-53.

Verma, S. C., S. Borah and E. S. Robertson (2004). "Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus up-regulates transcription of human telomerase reverse transcriptase promoter through interaction with transcription factor Sp1." J Virol 78(19): 10348-59.

Verma, S. C., K. Lan, T. Choudhuri and E. S. Robertson (2006). "Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen modulates K1 expression through its cis-acting elements within the terminal repeats." J Virol 80(7): 3445-58.

Verma, S. C., K. Lan and E. Robertson (2007). "Structure and function of latency-associated nuclear antigen." Curr Top Microbiol Immunol 312: 101-36.

Vieira, J. and P. M. O'Hearn (2004). "Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression." Virology 325(2): 225-40.

Wang, H. W., M. W. Trotter, D. Lagos, D. Bourboulia, S. Henderson, T. Makinen, S. Elliman, A. M. Flanagan, K. Alitalo and C. Boshoff (2004a). "Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma." Nat Genet 36(7): 687-93.

Wang, L., D. P. Dittmer, C. C. Tomlinson, F. D. Fakhari and B. Damania (2006). "Immortalization of primary endothelial cells by the K1 protein of Kaposi's sarcoma-associated herpesvirus." Cancer Res 66(7): 3658-66.

Wang, L., N. Wakisaka, C. C. Tomlinson, S. M. DeWire, S. Krall, J. S. Pagano and B. Damania (2004b). "The Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) K1 protein induces expression of angiogenic and invasion factors." Cancer Res 64(8): 2774-81.

Wang, N., P. F. Baldi and B. S. Gaut (2007a). "Phylogenetic analysis, genome evolution and the rate of gene gain in the Herpesviridae." Mol Phylogenet Evol 43(3): 1066-75.

Wang, S., S. Wang, H. Maeng, D. P. Young, O. Prakash, L. E. Fayad, A. Younes and F. Samaniego (2007b). "K1 protein of human herpesvirus 8 suppresses lymphoma cell Fas-mediated apoptosis." Blood 109(5): 2174-82.

Wang, S. E., F. Y. Wu, H. Chen, M. Shamay, Q. Zheng and G. S. Hayward (2004c). "Early activation of the Kaposi's sarcoma-associated herpesvirus RTA, RAP, and MTA promoters by the tetradecanoyl phorbol acetate-induced AP1 pathway." J Virol 78(8): 4248-67.

Watanabe, T., M. Sugaya, A. M. Atkins, E. A. Aquilino, A. Yang, D. L. Borris, J. Brady and A. Blauvelt (2003). "Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen prolongs the life span of primary human umbilical vein endothelial cells." J Virol 77(11): 6188-96.

Wen, K. W. and B. Damania (2009). "Kaposi sarcoma-associated herpesvirus (KSHV): Molecular biology and oncogenesis." Cancer Lett.

Wolf, D. and S. P. Goff (2007). "TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells." Cell 131(1): 46-57.

Wolf, D., K. Hug and S. P. Goff (2008). "TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells." Proc Natl Acad Sci U S A 105(34): 12521-6.

Wong, E. L. and B. Damania (2006). "Transcriptional regulation of the Kaposi's sarcoma-associated herpesvirus K15 gene." J Virol 80(3): 1385-92.

Wormald, S., J. G. Zhang, D. L. Krebs, L. A. Mielke, J. Silver, W. S. Alexander, T. P. Speed, N. A. Nicola and D. J. Hilton (2006). "The comparative roles of suppressor of cytokine signaling-1 and -3 in the inhibition and desensitization of cytokine signaling." J Biol Chem 281(16): 11135-43.

Wright, G. J., M. Jones, M. J. Puklavec, M. H. Brown and A. N. Barclay (2001). "The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans." Immunology 102(2): 173-9.

Yanagi, S., R. Inatome, T. Takano and H. Yamamura (2001). "Syk expression and novel function in a wide variety of tissues." Biochem Biophys Res Commun 288(3): 495-8.

Yap, M. W., S. Nisole, C. Lynch and J. P. Stoye (2004). "Trim5alpha protein restricts both HIV-1 and murine leukemia virus." Proc Natl Acad Sci U S A 101(29): 10786-91.

Yasukawa, H., M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, T. Hirano, K. R. Chien and A. Yoshimura (2003). "IL-6

induces an anti-inflammatory response in the absence of SOCS3 in macrophages." Nat Immunol 4(6): 551-6.

Ye, F. C., F. C. Zhou, S. M. Yoo, J. P. Xie, P. J. Browning and S. J. Gao (2004). "Disruption of Kaposi's sarcoma-associated herpesvirus latent nuclear antigen leads to abortive episome persistence." J Virol 78(20): 11121-9.

Yoo, S. M., F. C. Zhou, F. C. Ye, H. Y. Pan and S. J. Gao (2005). "Early and sustained expression of latent and host modulating genes in coordinated transcriptional program of KSHV productive primary infection of human primary endothelial cells." Virology 343(1): 47-64.

Yoshizaki, K., T. Matsuda, N. Nishimoto, T. Kuritani, L. Taeho, K. Aozasa, T. Nakahata, H. Kawai, H. Tagoh, T. Komori and et al. (1989). "Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease." Blood 74(4): 1360-7.

Zhong, W., H. Wang, B. Herndier and D. Ganem (1996). "Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma." Proc Natl Acad Sci U S A 93(13): 6641-6.

Zhu, L., R. Wang, A. Sweat, E. Goldstein, R. Horvat and B. Chandran (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(2): 381-92.

Ziegler, J. L. and E. Katongole-Mbidde (1996). "Kaposi's sarcoma in childhood: an analysis of 100 cases from Uganda and relationship to HIV infection." Int J Cancer 65(2): 200-3.

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel
Role of MEK/ERK and PI3K/Akt signalling Cascades and Kaposi Sarcoma Herpesvirus K1 Protein During the Progression of the Viral Lytic Replication Cycle.

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, _____

Silke Hartmann

Danksagung

Viele Menschen haben mich während meiner Doktorandenzeit begleitet und dabei auf unterschiedliche Weise zum Entstehen dieser Dissertation beigetragen.

Allen voran möchte ich meinem Doktorvater und Betreuer Prof. Thomas F. Schulz dafür danken, dass er meine Absage für die Stelle in seinem Labor nicht einfach akzeptiert, sondern meinen Zweifeln Gehör geschenkt hat und mich in einem langen Telefongespräch letztendlich doch noch davon überzeugen konnte nach Hannover zu kommen. Ich hatte eine sehr interessante und lehrreiche Doktorandenzeit in seinem Labor. Der unermüdliche wissenschaftliche Geist und das rege Denken von Herrn Schulz haben meine Begeisterung für die Wissenschaft vertieft und waren stets anregend für weiterführende Experimente und Gedanken. Seine hohen Ansprüche an die eigene und die Arbeit anderer werden für mich auch weiterhin Maßstab und Herausforderung sein. Ich möchte Herrn Schulz auch für die vielen Möglichkeiten danken an wissenschaftlichen Tagungen und Workshops teilzunehmen, meine eigenen Daten zu präsentieren und wunderbare Wissenschaftler kennen zu lernen, deren Denken, Erfahrung und Verhalten prägend und bereichernd waren.

Ich danke Frau Prof. Christine Josenhans und Herrn Prof. Helmut Holtmann für die Begutachtung dieser Dissertation sowie – zusammen mit Herrn Prof. Thomas F. Schulz und Prof. Edgar Maiß – für die faire und kollegiale Gestaltung der Disputation.

Weiterhin danke ich Frau Prof. Beate Sodeik für hilfreiche Diskussionen, ihre moralische Unterstützung und die Hilfe bei organisatorischen Problemen im Labor.

Mein großer Dank gilt all den hervorragenden Kollegen und Menschen, die mich täglich im Labor begleitet haben und von denen ich sehr profitiert habe. Besonders danke ich Melanie und Matthias, die mich ins Labor eingeführt haben und auch später wichtige Ansprech- und Diskussionspartner bei allgemeinen und methodischen Fragen waren. Unverzichtbar war auch Khaled mit seiner umfassenden Erfahrung, der ständigen Bereitschaft zu wissenschaftlichen Hilfeleistungen, seiner persönlichen Unterstützung und seinem Humor. كل اركش oder „Mamnuun“, Khaled!

Conny, Fabienne, Oliver, Jutta, Heidi, Sabine und Gabi danke ich für ihre guten Ratschläge und tatkräftige Unterstützung bei der Planung und Durchführung von Experimenten zum Nachweis von DNA und RNA sowie bei Sequenzierungen. Bei Jessika bedanke ich mich vor allem für ihre hervorragende Hilfe bei der Charakterisierung der BAC36 K1-Deletionsmutante und der Durchführung des Inhibitor-Screens. Ich danke Magda für die Einführung am Fluoreszenzmikroskop und die hilfreichen Ratschläge zur

Fluoreszenzfärbung. Irina danke ich für all die praktische und moralische Unterstützung während der letzten fünf Jahre, für die Unmenge an Gelen, die sie während meiner Schwanger- und Stillzeit für mich gegossen hat und besonders für ihre Freundschaft. Während meiner Schwangerschaft und in der Zeit danach haben mich auch viele andere Institutsmitglieder enorm unterstützt, insbesondere Herr Schulz, Marcel, Anika, Raffaella, Khaled, Jessica und Susann. Ich danke allen von Herzen für ihre Hilfe und diese gute Erfahrung.

Mein inniger Dank gilt Eva, deren Unterstützung so vielfältig war, dass ich sie im Einzelnen hier nicht aufführen kann. Danke, Eva, Du bist ein wunderbarer Mensch! Ohne Dich und Deine Freundschaft wäre vieles in den letzten Jahren sehr, sehr viel schwerer zu meistern gewesen.

Ich möchte mich auch bei den Sekretärinnen Frau Kloth, Frau Meyer und Frau Böttcher bedanken, die sich weit über ihren Job hinaus für ein gutes, persönliches Arbeitsverhältnis eingesetzt und sich um all die dazugehörigen Formalitäten gekümmert haben. Herrn Scharr, Achim Steinkopf, Frau Kraut und Herrn Verhagen danke ich dafür, dass sie sich schnell und kompetent um Nachschub an Arbeits- und Verbrauchsmaterial und die Einsatzfähigkeit von Geräten gekümmert und somit für einen reibungslosen Ablauf des Institutsalltags gesorgt haben.

Großer Dank gilt meiner Familie, insbesondere meinen Eltern, die mir immer die Freiheit gegeben und mich bestärkt haben meinen Weg zu gehen und mich moralisch und finanziell sehr unterstützt haben. Ich habe wirklich tolle Eltern! Auch meine Oma hat mir in ihren wöchentlichen Anrufen viel Beistand geleistet. Meinem Sohn Til danke ich, dass er sich während der Schwangerschaft so kooperativ verhalten hat und mir jeden Tag soviel Lachen und Liebe entgegen bringt. Sven danke ich für seine ruhige und zuversichtliche Art während der angespannten Phasen der Doktorarbeit und für seinen Humor, der mir selbst in Momenten größter Niedergeschlagenheit ein Lachen entlockt hat. Außerdem danke ich allen Freunden, die sich auch nach der zehnten „Keine Zeit“-Absage noch gemeldet und sich als so treue Gefährten erwiesen haben.

Zum Schluss möchte ich all jenen danken, die mir während der letzten fünf Jahre ein Lächeln, ein aufbauendes Wort oder eine helfende Hand geschenkt haben. Es sind oft die kleinen Dinge, die einen Tag, sein Gelingen und letztendlich das eines ganzen Werkes bestimmen.

Curriculum vitae

Personal information

Silke Hartmann
born October 6, 1977 in Siegen, Germany
German citizenship
single, one child (born in 2007)

Education

Oct. 2004- PhD student in the group of Prof. Thomas F. Schulz, Institute of Virology, Hanover Medical School
Oct. 2004 - Sept. 2007: Scholarship holder, GRK 745 "Mukosale Wirts-Pathogen-Interaktionen" from the Deutsche Forschungsgemeinschaft (DFG).

June 2004 Diploma in Biology, Friedrich Schiller University, Jena

Concentrations:	Microbiology, Medical Microbiology
Minor:	Ecology
Diploma thesis:	Etablierung durchflusszytometrischer Methoden zum Nachweis von tumorspezifischen T-Zellen. (Establishment of flow cytometry-based methods to analyse tumour-specific T-cells.)

Sept. 2001 Intermediate diploma, University of Rostock

June 1997 University-entrance diploma, Grammar School Kreuztal

Courses and work experience

Sept. 6-8, 2006 RNAi training course „The nitty-gritty of RNAi", Max Planck Institute of Infection Biology, Department of Molecular Biology.

Feb. – Aug. 1998 Work experience at DRK Paediatric Clinic Siegen, ward for infectious disease.

Scientific publications and presentations

Publications

Hartmann S., Schulz T. F., Nicholas J. HHV-8/KSHV Proteins Involved in Signaling and Transformation. In: DNA Tumor Viruses. B. Damania and J. M. Pipas, Springer Science+Business Media.

Hartmann, S., Alkarsah, K. R., Yakushko, Y., Henke-Gendo, C. and Schulz, T. F.. Essential Role of Kaposi Sarcoma Herpesvirus K1 Protein During the Progression of the Lytic Replication Cycle. Manuscipt to be submitted.

Oral presentations

Hartmann S., Schulz T.F.. Signaling Pathways Involved In KSHV Reactivation And The Maturation Of New Infectious Virions. "GRK 745 Klausurtagung", March 23-24, 2006, Bergkirchen.

Hartmann S., Pliquet D., Schulz T.F.. Modulation of Erk and Akt signalling by lytic proteins of KSHV contributes to successful viral replication. INCA Annual Meeting, March 12-14, 2007, Sesimbra, Portugal.

Hartmann S., Pliquet D., Schulz T.F.. Modulation of ERK and Akt signalling by lytic proteins of KSHV contributes to successful viral replication. "GRK 745 Klausurtagung", April 20-21, 2007, Ruthe.

Hartmann S., Pliquet D., Schulz T.F.. Modulation of Erk and Akt signalling by lytic proteins of KSHV contributes to successful viral replication. Virological Retreat, June 5, 2007, Hannover.

Hartmann S., Pliquet D., Schulz T.F.. Modulation of Erk and Akt signalling by lytic proteins of KSHV contributes to successful viral replication. 10th International Workshop on KSHV and Related Agents, August 1-5, 2007, Portland, Oregon, USA.

Hartmann S., Rückert J., Fischer I.A., Kéri G., Varga Z., Schulz T.F.. Modulation of ERK and Akt signaling by the KSHV lytic protein K1 contributes to successful viral replication. INCA Annual Meeting, March 13-16, 2009, Cascais, Portugal.

Hartmann S., Alkarsah K.R., Schulz T.F.. Essential Role of the K1 Protein During the Progression of the Lytic Replication Cycle. 12th International Workshop on KSHV and Related Agents, September 13-16, 2009, Charleston, South Carolina, USA.

Poster presentations

Hartmann S., Fischer I.A., Schulz T.F.. The role of Ras-Raf-Mek1/2-Erk signalling in lytic replication of KSHV, Jahrestagung der Gesellschaft für Virologie, March 2006, Munich.

Hartmann S., Schulz T.F.. Activation of Erk and Akt during lytic replication of KSHV, 9th International Workshop on KSHV and Related Agents, July 12-15, 2006, Hyannis, Massachusetts, USA.

Hartmann S., Rückert J., Fischer I.A., Kéri G., Varga Z., Schulz T.F.. Modulation of ERK and Akt signaling by the KSHV lytic protein K1 contributes to successful viral replication. INCA Annual Meeting, March 13-16, 2009, Cascais, Portugal.