ORFome-based arrays in eukaryotic expression vectors – a new approach to screen for the function of viral proteins (LANA-1 meets the mediator)

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To my parents

1. Summary

Since its first description in 1994 by Yuan Chang and Patrick Moore, Kaposi's sarcoma-associated herpesvirus (KSHV) or Human Herpesvirus 8 (HHV-8) has emerged as a pathogen of international public health importance. KSHV belongs to the *γ-herpesvirinae* subfamily and shows similarity to EBV (Epstein - Barr Virus) and HVS (Herpesvirus saimiri). It has been detected in biopsies of all forms of Kaposi's sarcoma (KS), irrespective of geographic origin, age, or gender of the patient[13]. Moreover, KSHV has been shown to be associated with two other diseases, multicentric Castleman's disease (MCD) and primary effusion lymphoma (PEL).

In comparison to α and β-herpesvirinae, the understanding of KSHV-related pathogenesis has been hampered by inefficient virus replication *in vitro*, poor cell culture systems and the lack of an animal model. Thus, many basic questions concerning the biology of KSHV infection remain open. For example, the primary target cell of KSHV and the function of more than 50% of the viral proteins are still unknown. Since the investigation of viral gene functions by virus mutants did not prove to be very efficient for KSHV, a system for a genome-wide screening of viral gene functions by cloning the complete KSHV ORFeome (all open reading frames) and by generating KSHV arrays in a variety of different expression vectors was established in this project.

The intimate relationship between viruses and their host cells dictates that viruses exploit key cellular regulatory systems and usurp important cellular processes to carry out a successful infection. Very often they regulate cellular signalling pathways, which favour viral infection and replication in the host cells. In cells infected with KSHV, the activation of the *serum response element* (SRE) is crucial for virus infection. The SRE is a transcription factor binding site present in promoters of many genes involved in cell growth and transformation. In this study, a genome-wide screen for KSHV genes inducing the SRE element and AP-1 was performed. A strong induction of SRE by the latency-associated nuclear antigen 1 (LANA-1) was observed. LANA-1 is a multifunctional protein which interacts with the p53 and RB tumor suppressor proteins. LANA-1 is mandatory for the maintenance of viral episomes. This study reveals several novel functions of LANA-1. It was found that LANA-1 also stimulated the *interferon-*

stimulated response element (ISRE) present in promoters activated by type I interferons. LANA-1 led to an activation of the ERK-1/2 MAP kinase, but also bound to the Mediator, a multi-subunit transcriptional coactivator complex for RNA polymerase II, via the ARC92/ACID1 subunit. Since LANA-1 interacted with SRF, one of the two transcription factors binding to the bipartite SRE element, a model for LANA-1 as an adaptor between specific transcription factors and the basal transcriptional machinery was hypothesized.

2. Introduction

2.1. Kaposi's Sarcoma-Associated Herpesvirus (KSHV)

Classical Kaposi's sarcoma (KS) was originally described in 1872 by the Hungarian dermatologist Moritz Kaposi as an idiopathic, multi-pigmented sarcoma of the skin[16]. For more than a century, KS remained an interesting neoplasm for only a few clinicians due to its rare occurrence and very restricted outbreaks primarily in Mediterranean white men. In addition, this complex proliferative lesion slowly progresses and presents with only mild clinical manifestation. The explosion of the AIDS epidemic around the mid 1980s brought increased attention to KS as it is the major malignancy among AIDS patients, occurring in approximately 20% of male HIV patients[136]. The strong linkage between KS and HIV initially led researchers to suspect HIV as the single etiological cause for KS. The hunt for the causative agent came to an end and shifted to a new stage when Drs Chang and Moore discovered fragments of DNA specific to KS lesions that were similar to other known herpesviruses including herpesvirus saimiri (HVS) and Epstein-Barr virus (EBV)[32,33]. This new herpesvirus named Kaposi's sarcoma-associated herpesvirus (KSHV) or Human Herpesvirus 8 (HHV-8) was soon found in all epidemiological forms of KS, and defined as an etiological agent of KS[13,37,112]. This discovery opened new avenues in the research on, therapy for, and prevention of KS.

2.1.1. KSHV – associated diseases

KSHV is etiologically linked to Kaposi's sarcoma, which is of endothelial origin, and two lymphoproliferative disorders, "body cavity-based" or "primary effusion lymphoma" (BCBL/PEL) and multicentric Castleman's disease (MCD) (Fig. 1).

2.1.1.1. Kaposi's sarcoma (KS) - clinical and morphological features, epidemiology

Usually classical KS first manifests as bluish-red, well demarcated, painless maculae on distal portions of the lower limbs. These KS early patches and plaque lesions often resemble granulated tissues. In the majority of instances the lesions progress slowly and merge into large plaques. However, solitary lesions occasionally progress to form nodular and fungi-form, brownish-red tumors[35]. During long-lasting courses of KS, the

lesions may erode, bleed, and even ulcerate. In addition to the skin, KS manifestation can involve mucosal surfaces, especially in the oral cavity and gastrointestinal tract. Involvement of internal organs is infrequent in classical KS and occurs in only approximately 10% of cases[153,154]. Paracrine effects play an important role in the pathogenesis of this tumor, as tumor tissue contains high concentrations of inflammatory cytokines (e.g. IL-6, IL-10) and growth factors (e.g. VEGF, bFGF)[28]. The KS lesions contain spindle cells that share features with endothelial cells and smooth muscle cells[118]. The vast majority of spindle cells is latently infected and expresses a small subgroup of viral proteins including Lana-1, v-IRF3 (Lana-2), v-cyclin, v-FLIP and kaposin A[45,78]. Most other viral proteins are expressed by lytically infected cells constituting less than 1% of the tumor.

KS can be classified into four distinct clinical varieties: classical, endemic (African), iatrogenic (immunosuppression-associated), and AIDS-associated (epidemic). Classical KS typically arises in elderly men of Mediterranean and eastern European Jewish decent and presents as indolent disease with paranodular skin lesions with little or no visceral involvement[113]. Endemic KS is widespread in southern equatorial Africa among young black adult males and prepubescent children, systemic involvement can occur and disease progression is often rapid[97]. Iatrogenic immunosuppression-associated KS includes corticosteroid-induced KS and post-transplant KS and gives rise to systemic involvement[47,58]. AIDS-associated KS is the most aggressive form, lesions occurring both on the skin and the viscera, with variable disease progression[158]. Epidemiological evidence has suggested that all forms of KS have an infectious etiology[158].

Geographic location, ethnicity, age, and gender heavily influence the incidence rate of classical KS. Low rates were reported in England and Wales (0.14 per million in both males and females between 1971-1980)[62], intermediate rates were reported in Sweden (4.0 per million in men and 1.4 per million in women between 1952-1982)[42], whereas higher rates were reported in Italy (10.5 per million in men and 2.7 per million in women between 1976-1984)[53], and Greece (5.8 per million in men and 3.7 per million in women between 1979-1983)[155]. The incidence rate of classic KS in Israeli Arabs (7 per million and 2 per million in men and women, respectively) was 3-fold lower than Israeli Jews[73]. KS was reported to occur more frequently in individuals during their fifth and sixth decades of life, with a lower incidence at other ages and only sporadic cases occurring

before the age of thirty[70]. The higher incidence of KS among men compared to women suggests that gender is a risk factor for KS.

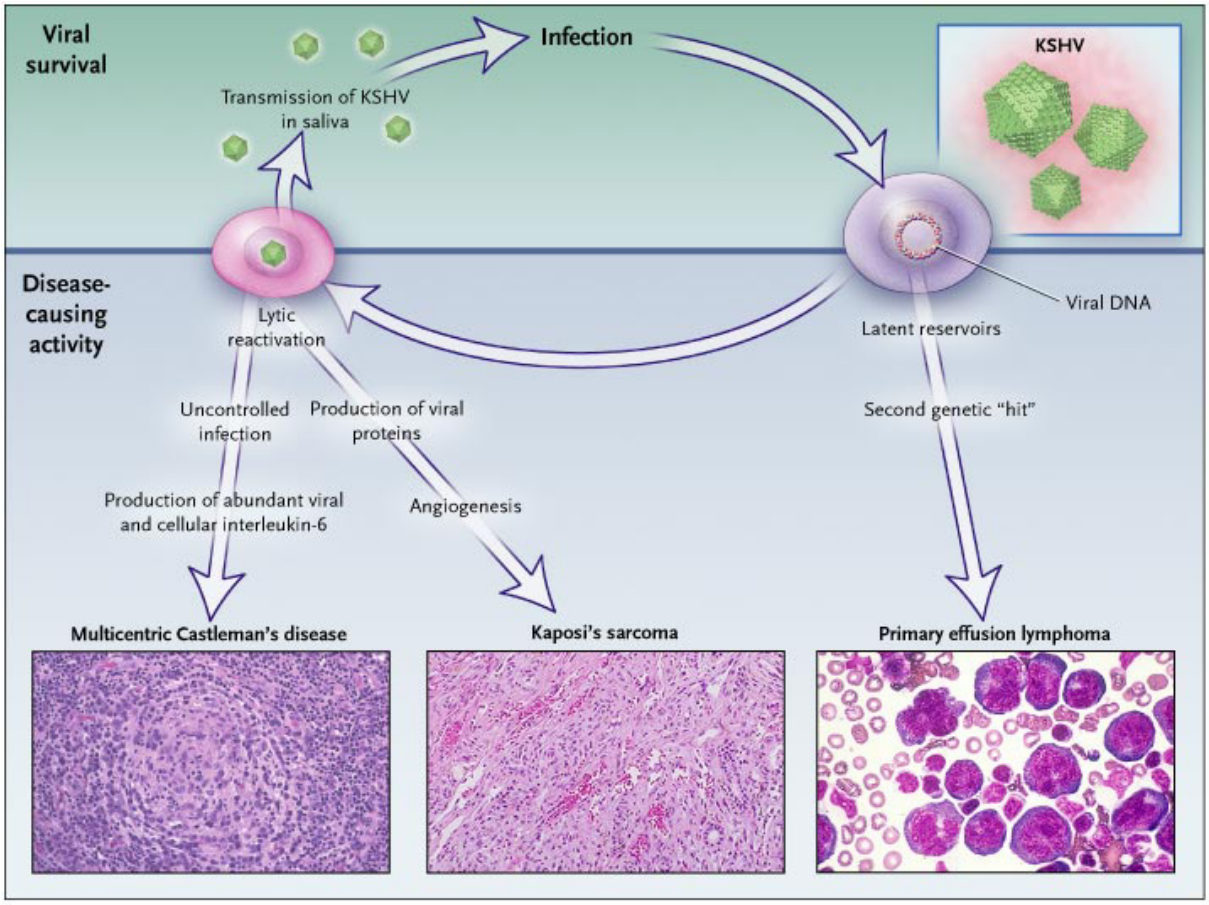


Fig. 1: KSHV-associated diseases as a side effect of viral persistence.
Transmission of Kaposi's sarcoma–associated herpesvirus (KSHV) leads to a lifelong infection, in which the virus remains latent in cellular reservoirs. Reactivation of the virus leads to the infection of new cells within the host and secretion in saliva, and thus, possibly, transmission of the virus. In a minority of infected persons, and often in the setting of immunodeficiency, KSHV-associated diseases may arise. These result from the production of viral proteins, which in turn may induce cellular growth factors and angiogenic factors. Infected cells may also contain the Epstein–Barr virus and cellular genetic alterations (second genetic "hits"). Histologic images of the three diseases for which a causal association with KSHV has been firmly established are shown: multicentric Castleman's disease (lymph-node specimen; hematoxylin and eosin, x200), Kaposi's sarcoma (skin specimen; hematoxylin and eosin, x200), and primary effusion lymphoma (pleural fluid; hematoxylin and eosin, x1000) (adapted from Cesarman, 2003)[27].

The frequency of KS in HIV disease is strikingly higher in homosexual or bisexual males than in patients with haemophilia, transfusion recipients or injecting drug users[11,66]. Classical KS is more common in non-HIV-infected homosexual men than the general

population, suggesting transmission of an infectious etiological agent via sexual routes or close fecal contact[10].

2.1.1.2. Primary effusion lymphoma (PEL)

PEL is a rare lymphoma which occurs in the peritoneal, pleural or pericardial space, often in the absence of an obvious tumor mass[51]. The lymphoma cells have usually monoclonal and B cell origin with pleomorphic and anaplastic features, and express only few of the usual markers of B cell differentiation[25,132]. In most PEL cases, the lymphoma cells are co-infected with Epstein-Barr virus (EBV)[24,29]. EBV is present in PEL cells in a "monoclonal" form, i.e. EBV episomes in different lymphoma cells all have the identical terminal repeat pattern, indicating that infection by a single EBV particle preceded the emergence of the malignant clonal lymphoma[137]. Immunohistochemistry and *in situ* hybridisation studies on several PEL cell lines indicate that five KSHV genes, *LANA-1, v-cyclin, v-FLIP, kaposin, LANA-2*, are expressed in a substantial proportion of lymphoma cells[45,105,122,134,162].

2.1.1.3. Multicentric Castleman's disease (MCD)

MCD is a lymphoproliferative disease found in the mediastinum, mesenterial or peripheral lymph nodes and is characterized histologically by expanded germinal centres with B cell and vascular proliferation[137]. MCD in AIDS patients is often associated with Kaposi's sarcoma. KSHV can nearly always be detected by PCR in the lymph nodes or spleen affected by MCD in AIDS patients, but is much less common in Castleman's disease without HIV infection[44,145]. KSHV-infected B cells express, as assessed by immunohistochemistry, LANA-1, a hallmark of all KSHV-infected cells[44,45,78]. MCD is associated with interleukin-6 (IL-6) overexpression[1].

2.1.2. KSHV virion structure

KSHV shows the typical morphology of herpesviruses. The virus particle has a diameter of approximately 125 nm (1250 A°)[125,161]. The core of the mature virion contains the viral DNA in the form of a torus. The capsid has an icosahedral shape and consists of 162 hexagonal capsomers. Three types of capsids A, B and C are released from PEL cells after TPA or sodium butyrate treatment. C-capsids are fully mature capsids and contain the polypeptide ORF25/MCP (major capsid protein), ORF65/SCIP (small capsomer interacting protein), ORF26/TRI-2 (triplex-2), ORF62/TRI-1 and the 160- to 170-kb viral genome. They have a total mass of approximately 300 megadaltons[110]. A and B capsids contain the four proteins listed above but lack viral genomic DNA.

The envelope has a typical trilaminar appearance and contains glycoproteins and lipids. The tegument consisting of various viral and cellular proteins is located between the capsid and the envelope (Fig. 2).

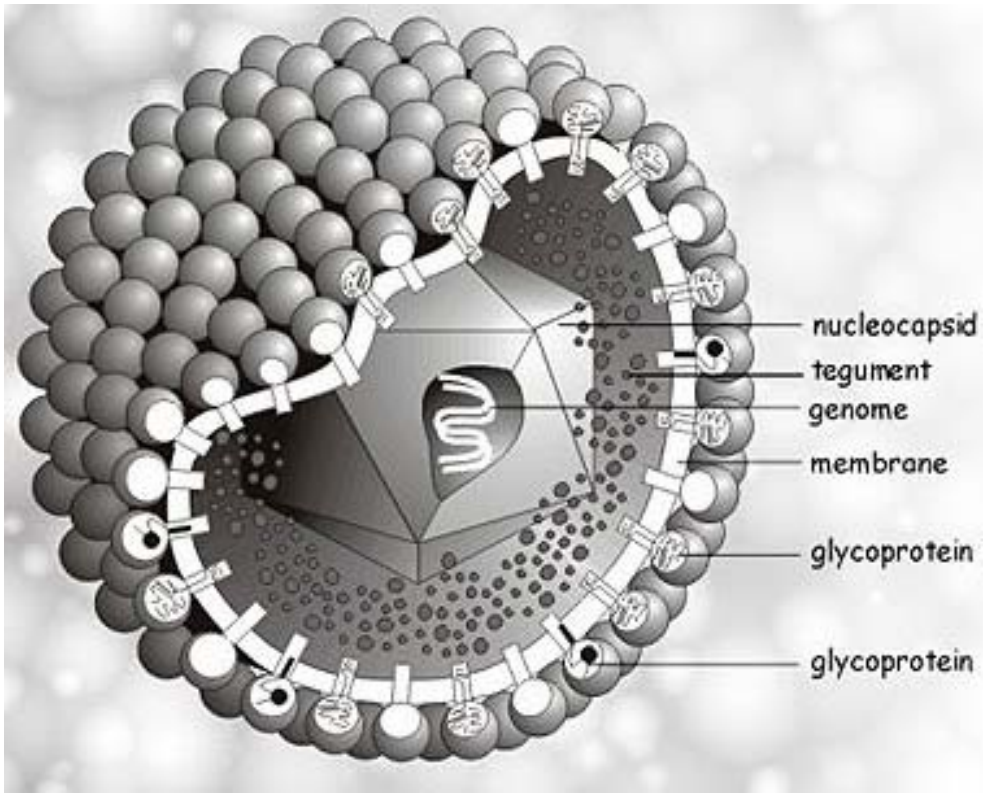


Fig. 2: The herpesvirus particle.
Schematic model of a herpesvirus particle (adapted from Reschke, 1994)[126]. Major virion components are indicated.

2.1.3. KSHV genome organization

Members of γ herpesviruses can be further divided into γ 1 and γ 2 subgroups, based on sequence homology. Whereas EBV belongs to the γ 1 subgroup, KSHV, HVS, rhesus monkey rhadinovirus (RRV), retinofibromatosis-associated herpesvirus (RFHV), and mouse herpesvirus 68 (MHV-68) fall into the γ 2 subgroup.

KSHV was initially identified by Moore and Chang[32]. The genome of KSHV was mapped with cosmid and phage genomic libraries from the BC-1 cell line[111,130]. BC-1 is a PEL cell line containing a high KSHV genome copy number and is co-infected with EBV. The KSHV genome has the standard features of rhadinovirus genomes. The latent KSHV genome appears to be in a circular conformation, while the encapsidated products of lytic replication are linear[125]. The BC-1 KSHV genome consists of a 140.5-kb-long unique coding region flanked by multiple G+C rich 801-bp terminal repeat sequences which are the presumed sites for genome circularisation[86] (Fig. 3). The number of repeats at each end varies. Some molecules contain as few as no or 1 repeat at one terminus and as many as 35-45 at the other terminus; most, however, contain multiple repeats at each end[86]. During the lytic replication, the viral progeny DNA is synthesized by a rolling-circle mechanism[125].

The genome of KSHV contains at least 89 open reading frames (ORFs)[111,130]. The viral genome can be divided into highly conserved blocks, which are interrupted by interspersed regions of unique genes. The conserved genes have been marked by the prefix “ORF”, while the unique genes have been designated K1 to K15[130]. The genes with high conservation degree are predicted to have metabolitic and catalytic functions in the replication of the viral DNA and are taken together in a set of “ancient” genes conserved in all mammalian herpesviruses[142].

KSHV harbors numerous ORFs with homology to cellular genes which are involved in modulating cellular signal transduction pathways, cytokine-regulated cell growth, apoptosis, and cell cycle control. To achieve persistent infection, KSHV has evolved elaborate mechanisms that target and modulate different aspects of the host’s immune system.

The KSHV genome also contains two lytic origins of DNA replication that are inverted duplications of each other: the left is located between K4.2 and K.5, and the right between K12 and ORF71[7,92].

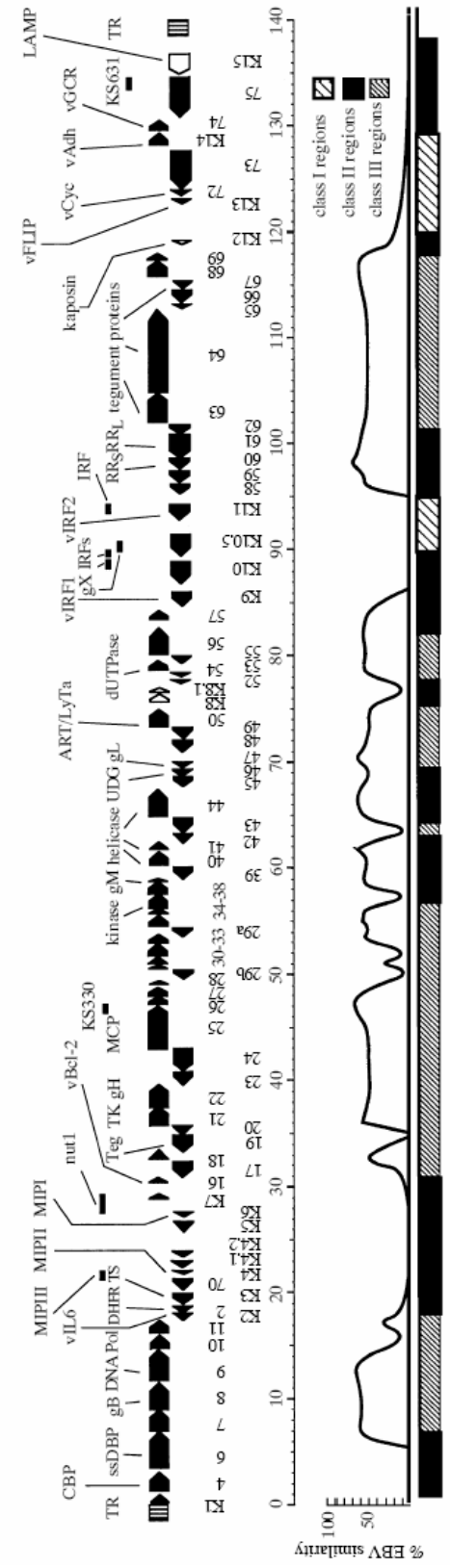


Fig. 3: A representation of the KSHV genome showing the major ORFs in the long unique region (LUR). Below the genomic representation is a chart figure showing the percentage amino - acid identity represented for each KSHV ORF and the corresponding ORFs in EBV. This graph shows the highly conserved genes, mainly encoding structural and virion replication proteins, tend to cluster together while intervening areas are rich in latency genes that have little or no similarity between the two viruses. Clusters of KSHV genes also tend to be regulated similarly with distinct clusters of genes having expression patterns in PEL cells falling into either class I (constitutive), class II (constitutive-TPA inducible) or class III (only expressed after TPA induction) patterns (adapted from Moore and Chang, 2001).

2.1.4. Gene expression program of KSHV

Subsequent to primary infection, herpesviruses persist life-long in the host. The gene expression program of KSHV can be divided into two phases: non-productive (latent) phase and productive (lytic) phase. The viral gene expression program can be switched from latent to lytic by phorbol ester and sodium butyrate. KSHV gene expression in PEL cells was analysed during the latent and lytic phase and the viral genes were grouped into 3 classes[134]:

- Class I genes are expressed during the latent phase and are not induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). The following genes belong to this group: ORF73 [latency-associated nuclear antigen-1 (LANA-1)], ORF72 (viral cyclin D) and ORF K13 (v-FLIP).
- Class II genes are primarily clustered in nonconserved regions of the genome and include most of the virus-encoded cytokines. These genes are transcribed without TPA treatment, but are further induced to higher transcription levels by chemical treatment. The following genes belong to this group: three CC family chemokines (ORF K4, ORF K4.1, ORF K6), a BCL-2 analog (ORF16), etc.
- Class III genes represent ORFs that cannot be detected without TPA and are induced by this chemical. This class includes lytic genes which are transcribed during active infection and are necessary for efficient viral replication and virion particle production [e. g. ORF21 (thymidine kinase), ORF25 (major capsid protein), ORF26 (capsid protein), ORF50 (the replication and transcriptional activator Rta)][134].

Rta (ORF50) of KSHV is able to reactivate the virus from latency in PEL cell lines[59,94,152]. Most of the KSHV - infected cells in PEL and KS clinical samples are latently infected with the virus and only few cells show spontaneous lytic replication[31,46].

2.2. Viral modulation of host signalling pathways activating the SRE element

A major emphasis of current research is to determine which genes are responsible for KSHV-driven tumor cell growth. Several studies on single KSHV genes have shown that KSHV possesses a complex molecular capacity to regulate cell proliferation by interfering with different cell signalling pathways. Many of these signal transduction pathways converge at transcription factors that bind to specific enhancer elements [e.g. SRE (serum response element), AP-1 (activating protein-1), etc.] found in the promoters of various genes and modulate the transcription of these genes. Since this study focuses on a viral protein which modulates a signalling pathway via SRE (see results), more information about this enhancer element will be given in this part of the thesis.

2.2.1. Serum response element (SRE)

The SRE binding site is a key regulatory element in the promoters of many cellular early response genes involved in cell growth (c-*fos*, FosB, JunB, Egr-1, Egr-2, IL2Rα, etc.), differentiation (Cyr61, Pip92), as well as muscle development and function (cardiac α-actin, skeletal α – actin, smooth muscle γ – actin)[30]. The SRE element contains two distinct binding sites for serum response factor (SRF) and ternary complex factor (TCF) (Fig. 4). Upon phosphorylation a dimer of SRF and one molecule of TCF form a ternary complex, which binds to the SRE element[139].

2.2.1.1. Serum Response Factor (SRF)

SRF has a molecular weight of 67 kD and contains a DNA binding domain, a transactivation domain and several phosphorylation sites[30]. SRF can be activated by a variety of agents, including serum, lysophosphatidic acid (LPA), lypopolysaccharide (LPS), 12-O-tetradecanoylphorbol-13-acetate (TPA), cytokines, tumor necrosis factor-α (TNF-α), T-cell virus 1 activator protein, hepatitis B virus activator proteins pX, activated proto-oncogenes and oncogenes as well as antioxidants and UV light[8,30]. SRF is regulated by interactions with other transcription factors e.g. Sp1, ATF6 and myogenic regulatory factors[100,146].

2.2.1.2. Ternary Complex Factor (TCF)

TCFs are a subgroup of the ETS protein family, which was first described in the context of c-*fos* gene regulation[138]. The transcription factors ELK-1, Sap1a and Net/Erp/Sap2, which share a characteristic "winged helix – turn - helix" DNA – binding domain belong to this group[17]. TCFs are targets for mitogen-activated protein kinases (MAPK)[138]. Several kinases, including extracellular–signal regulated kinase (ERK), Jun N–terminal kinase [known as stress-activated protein kinase (SAPKs)] and other subfamilies of MAPK take part in the phosphorylation of TCFs[30]. Transcriptional activation by ELK-1 was shown to depend on several consensus sites in the carboxy-terminal domain of ELK-1 that are rapidly phosphorylated following mitogen stimulation of cells and by MAPKs *in vitro*[56,82,96]. Although several sites are phosphorylated *in vivo*, Ser383 appears to be critical both for trans-activation and for ternary complex formation[56,56,74,96,150].

There are two general classes of signalling mechanisms involved in SRE activation, a TCF-dependent and a TCF-independent pathway[30] (Fig. 4).

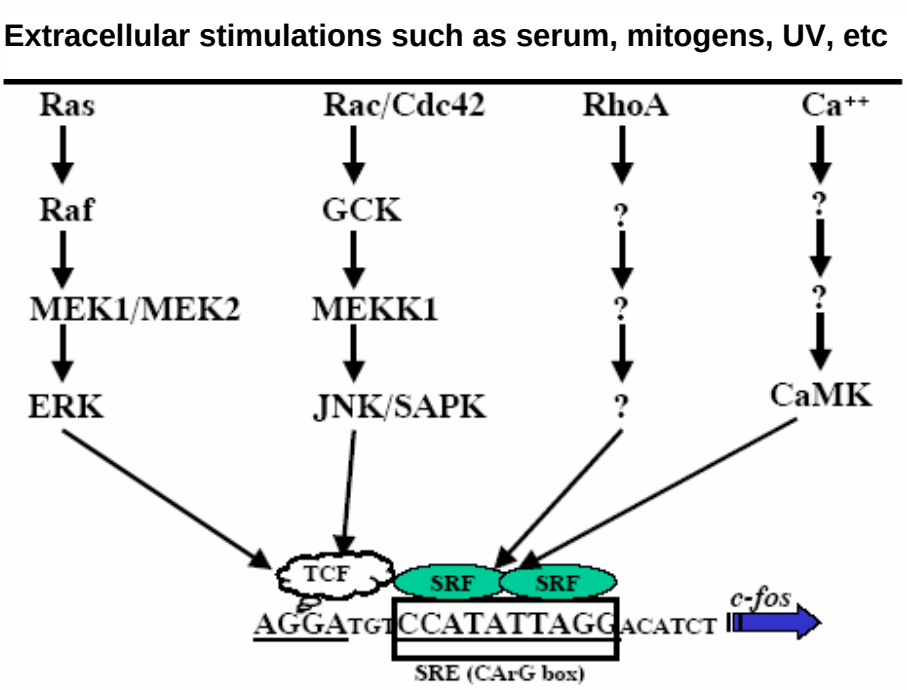


Fig. 4: Diagrammatical presentation of the pathways involved in c-*fos* gene activation.
Four signalling pathways leading to the activation of the *c-fos* promoter via the SRE element are indicated. The activation of SRE by the RAS-RAF-MAPK-ERK and Rac/Cdc42/GCK/MEKK1/JNK/SAPK pathways requires the phosphorylation of both TCF and SRF. The RhoA and Ca^{2+} calmodulin-dependent kinase (CaMK) induced activation of SRE depends only on the phosphorylation of SRF. Question marks indicate unknown pathways (adapted from Chai and Tarnawski, 2002)[30].

The TCF-dependent pathway involves the RAS-RAF-MEK-ERK and Rac/Cdc42/GCK/MEKK1/JNK/SAPK cascades[75]. Both the phosphorylation of TCFs and the phosphorylation of SRF are required for the activation of SRE by this pathway (Fig. 4).

The TCF–independent pathway activated by the Rho family of GTPases or Ca^{2+} calmodulin-dependent kinase (CaMK) does not lead to the phosphorylation of TCFs (Fig. 4). The activation of SRE by the TCF-independent pathway requires only the phosphorylation of SRF.

Recently, it was observed that during infection of HUVEC (human umbilical vein endothelial cells) cells KSHV activates multiple MAPK pathways including MEK1/MEK2, JNK and p38 (Gao et al., *unpublished*). Moreover, the infectivity of KSHV and the viral lytic replication and production of infectious virions can be reduced up to 80% in the presence of specific inhibitors of MAPKs (Gao et al., *unpublished*). This demonstrates that the activation of multiple MAPK pathways is essential for successful KSHV infection and replication.

2.2.2. Viral proteins modulating host signalling pathways related to SRE

Several viral proteins have been identified as modulators of host signalling pathways activating the SRE binding site. In each case the mechanism of modulation is different: (i) some viral proteins activate the phosphorylation of specific transcription factors binding to SRE, (ii) others bind directly to SRF or ELK-1, and (iii) there are viral proteins with a complex mechanism of activation of SRE. Several examples of viral proteins activating SRE are listed in the text below.

In 1997, it was reported that the Tat protein of HIV (Human Immunodeficiency virus) activates the c-*fos* promoter via the SRE binding site[55]. Three years later the Tax protein of T-cell leukemia virus type 1 (HTLV-1) was shown to have similar properties[141]. Tax activates the expression of cellular immediate early genes controlled by the SRE element via direct binding to TCFs. In addition, Tax interacts with CREB binding protein (CBP), which is essential for Tax activation of SRF-mediated transcription[141].

The hepatitis C virus (HCV) core protein has oncogenic potential. The MAPK-ERK pathway and its downstream target, SRE, are activated in BALB/3T3 cells producing the HCV core protein[50]. In the presence of a mitogenic signal, the HCV core protein

enhances ELK-1 activation by acting downstream of MEK, however without affecting ERK activity and ELK-1 phosphorylation. This suggests that HCV core protein activates ELK-1 through a pathway alternative to the typical phosphorylation cascade[50,54].

Hepatitis delta virus (HDV) is a viroid whose replication is dependent on hepatitis B virus (HBV). There are only a few studies on the effects of HBV and HDV proteins [HDV antigens (HDAgs)] on intracellular signalling pathways. One of them reveals that HBV X protein (HBx), alone or with the large isoform of HDAg (LHDAg), synergistically activates the SRE-dependent pathway. HBx enhances the transcriptional ability of ELK-1, whereas LHDAg activates the transcriptional ability of SRF[57].

Finally, the VP3 protein of hepatitis A virus (HAV) has recently been added to the group of viral proteins activating cellular signalling pathways which induce SRE[77].

2.3. The Mediator complex

The regulation of cellular gene expression requires the carefully choreographed binding of general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH to the RNA polymerase II (Pol II)[65]. The response to activators of transcription (e. g. Gal4, VP16 of HSV, the adenoviral E1A protein, etc.) requires additional factors referred to as coactivators. A group of these factors is involved in the regulated alteration of chromatin structure. One such coactivator, the Mediator, is an approximately 2 megadaltons multi-subunit complex capable of responding to different activators[95,107,119]. The Mediator plays a key role in bridging DNA-bound activators to the general transcriptional machinery, especially RNA polymerase II, and the core promoter[88]. The subunits of the Mediator are necessary for a variety of positive and negative regulatory processes and serve as direct targets for the activators. The general requirement for individual Mediator subunits in global gene regulation differs. Some components are needed for the regulated expression of nearly all genes, whereas others are only needed for a certain subset of genes[63,67]. The exact molecular mechanism of Mediator-dependent transcriptional activation still remains unclear. Analyses by electron microscopy and image processing demonstrated striking similarities between the Mediator complexes isolated from yeast, mouse and human cells[6]. The regulation and magnitude of transcription is a function of the promoter architecture and the activator's influence on the Mediator conformation[88]. In two-dimensional projections, the Mediator alone appears

compact but in the presence of RNA polymerase II, the Mediator is altered to an extended structure consisting of three domains: a tail followed by middle and head domains. The head and middle regions, which contain a subset of conserved Mediator proteins, make contact to RNA polymerase II[6] (Fig. 5). The tail domain represented by non-conserved subunits is needed for the function of a wide variety of activators[63].

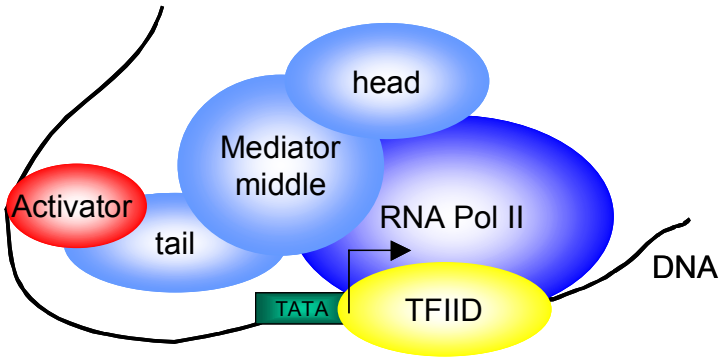


Fig. 5: The Mediator model of activator-dependent transcription.
Shown is a hypothetical promoter containing a canonical TATA box and interacting with the TFIID complex. The arrow within the TFIID complex represents the start site of transcription. The activator is shown binding to its DNA sites and recruiting the Mediator to the promoter via a physical interaction with a Mediator module (indicated by the overlap between the activator and the respective Mediator module). The head, middle and tail domains of the Mediator are indicated (adapted from Lewis and Reinberg, 2003)[88].

2.3.1. Viral proteins directly interacting with subunits of the Mediator complex

2.3.1.1. Human activator-recruited cofactor (ARC92/ACID1) and VP16

ARC92/ACID1 is a subunit of the Mediator. The structure of ARC92/ACID1 gene is of ancient origin and is conserved in mammals and higher eukaryotes. Using an affinity purification procedure ARC92/ACID1 was identified as a novel specific target of the herpes simplex transactivator VP16[102]. This interaction serves as an important transducer of transcription activating signals from the VP16 activation domain to the RNA polymerase II transcriptional machinery. Depletion of the ARC92/ACID1 - Mediator (A-Med) complex from HeLa nuclear extracts abolishes activation by VP16[102]. The Mediator-binding module of ARC92/ACID1 is a von Willebrand factor type A (VWA) domain which possesses the typical features of a protein-protein interaction module. It

consists of a rigid scaffold composed of helices grouped around a core that is formed from β-sheets[102]. The VP16 binding region is the ACID (activator interaction domain) domain which is still poorly characterized (see Fig. 6). The overexpressed N-terminal 290 amino acids of ARC92/ACID-1 (including the VWA domain), called NTD have a strong dominant-negative effect on the VP16 activation in mammalian cells[102].

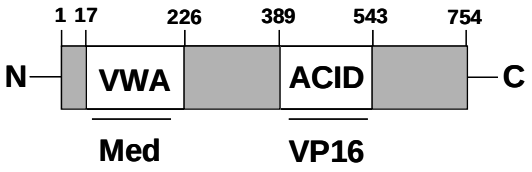


Fig. 6: Schematic representation of ARC92/ACID1.
The von Willebrand factor type A (VWA) domain and the VP16 binding domain are indicated[102].

2.3.1.2. Suppressor of RAS protein (Sur-2) and E1A

The Mediator subunit Sur-2 was originally discovered in the vulval signalling pathway of the nematode *Caenorhaditis elegans*[143]. Sur-2 acts downstream of the RAF/MAP kinase pathway. A null Sur-2 mutation gives rise to pleiotropic phenotypes, including partial larval lethality and sterile adults[143]. The human homologue of Sur-2 is a binding target of the adenoviral E1A transcriptional activator and interacts with the MAPK-modified form of the transcription factor ELK-1[15]. A knockout of murine Sur-2 in embryonic stem cells specifically prevents gene activation by ELK-1, but not by multiple other factors[149]. The activation of immediate early genes (c-*fos, egr-1,* etc.) in response to the RTK-RAS-RAF-MEK-ERK pathway is defective in *sur2*$^{-/-}$ cells because ELK-1 must interact with the Sur-2 Mediator subunit to activate transcription[149].

2.3.1.3. CDK8/cyclin C and ORF A

The ORF A protein of Walley dermal sarcoma virus (WDSV) also belongs to the list of viral proteins interacting with subunits of the Mediator complex. It was reported that the ORF A protein co-immunoprecipitates with CDK8 (cyclin-dependent kinase 8) and cyclin C, which form part of the Mediator complex[129].

2.4. Different strategies to generate herpesvirus mutants

Traditionally, biological functions of herpesviruses have been examined by using viral mutants. Recombinant viruses can be generated by different methods. In the past chemical mutagenesis was one classical genetic approach used for the generation of temperature-sensitive (ts) virus mutants[135]. Using mutagenic chemicals mutants with random point mutations were generated and screened for interesting phenotypes. The disadvantage of this technique is the difficulty to precisely identify the mutation responsible for an observed phenotype and to exclude second-site mutations. Another method that was developed in the early 1980s for herpes simplex virus is based on site-directed mutagenesis by homologous recombination[103]. Using this method the desired mutation is introduced into the viral genome together with a selectable marker using the recombination and repair machinery of the cell. This technique also has the disadvantage that enriching and purifying mutants which have restricted growth capacity in comparison to the wild-type is difficult and can only be achieved with complementing cell lines that provide the essential gene product[40,41]. Another approach is to clone the entire genome of the virus as sets of five or more overlapping cosmid clones, which allows the manipulation of a cloned segment with the tools of molecular biology[128]. Similar to the above-mentioned techniques, the cosmid technology has several disadvantages: recombinant viruses are genetically unstable, it is difficult to identify suitable restriction site in large cosmids, etc. In the last few years new vehicles have been developed as tools for mammalian genetics such as yeast artificial chromosomes (YACs), PACs (P1-derived artificial chromosome) based on the origin of replication (ori) of bacteriophage P1, and BACs (bacterial artificial chromosome) based on the bacterial F plasmids[72,140,148]. These cloning vectors can accept foreign DNA sequences of up to 300 kbp while remaining remarkably stable[140]. The first herpesvirus genome cloned as an infectious BAC was mouse cytomegalovirus (CMV) followed by other viruses such as EBV, HSV, pseudorabies virus, human CMV and KSHV[112,38,39,69,131,144,147]. The mechanisms of recombination in *Escherichia coli* are well studied and easily controlled and their application is less time consuming than recombination procedures in mammalian cells[18]. However, the presence of repetitive sequences in herpesvirus genomes have raised doubts about the stability of herpesvirus BACs[18].

In comparison to members of β–herpesvirinae (hCMV, mCMV), the genetic analysis of KSHV, which belongs to the γ-herpesvirinae, has been hampered by severe difficulties in producing infectious viral particles and modifying the viral genome. The lack of a cellular model for efficient KSHV infection has impeded the understanding of KSHV-related pathogenesis. Many basic questions concerning KSHV remain open. The primary target cell of KSHV infection *in vivo* as well as the function of more than 50% of the viral proteins is still unknown. The role of proteins controlling the activation and completion of the viral lytic cycle is also only partly understood[39]. KSHV was cultured by using cell lines established directly from primary effusion lymphoma or peripheral blood mononuclear cells of PEL patients[5,21,23,29,43,52]. KSHV can infect different cell types, including a variety of human cell types (B, T, endothelial, epithelial, fibroblast, keratinocyte cells) and non-human cell types (owl monkey kidney and baby hamster kidney fibroblast cells)[26,34,48,81,101,106,114] but the primary KSHV infection rates in these systems remain very low (< 10%)[124]. Cloning KSHV as a BAC was first performed by the group of Hammerschmidt[39]. The primary rate of infection with this BAC clone was below 1% and the reconstitution of viral mutants was inefficient[39]. Creating KSHV mutants for genome-wide screening is of little value using the above mentioned KSHV BAC clone. So far, the BAC technology has not been very powerful for the manipulation of KSHV. Therefore new alternatives must be developed for better and efficient wide screening of the KSHV genome.

2.5. Objectives

The function of a majority of KSHV proteins is currently unknown, or is only predicted due to sequence homology. Although many viral genes show homology to cellular genes and share structural similarity, their functional properties may differ from those of their cellular counterparts. The objective of this project was the establishment of an efficient screening system to identify and characterize new functions of KSHV proteins. In order to reach this goal, the following aims were defined:

- *the generation of a KSHV array containing all herpesviral ORFs in an eukaryotic expression vector by recombinatorial cloning,*

- *the screening for viral proteins modulating the SRE and AP-1 related signalling pathways, and*
- *the analysis of the mechanism driving the observed modulation.*

3. Results

The result section is divided into two parts. The first part describes the generation of an expression array containing all KSHV open reading frames (ORFs) by recombinatorial cloning and the screening of the array for viral proteins modulating signal pathways dependent on SRE and AP-1 binding sites. The second part focuses on the mechanism driving the observed modulation.

3.1. Identification of LANA-1 as an activator of SRE

3.1.1. Generation of a KSHV expression array by recombinatorial cloning

The generation of KSHV arrays in a variety of different expression vectors provides the possibility to perform genome-wide screens for viral-host and viral-viral protein interactions, localization, induction of signalling pathways, etc. Viruses, but especially herpesviruses, offer an interesting genome size for such projects, because their limited number of genes allows to study several aspects of the herpesvirus biology simultaneously by different methods.

In order to generate the KSHV array in different expression vectors, the advantages of recombinatorial cloning (GATEWAY cloning technology), which facilitates the cloning of large numbers of DNA fragments by restriction endonucleases and ligase, were used. Recombinatorial cloning is based on site-specific recombination reactions of the λ bacteriophage in *E. coli* during lysogenization. Proteins encoded by λ phage and *E. coli* mediate the integrative and excisive recombination reactions of λ phage. These recombination reactions, performed *in vitro*, are the basis of the GATEWAY cloning technology. The BP reaction is a recombination reaction between a PCR product containing terminal *att*B sites and a donor vector mediated by a BP clonase mix of recombination proteins (Fig. 7a). The BP reaction results in the generation of an entry clone. The LR reaction is a recombination reaction between an entry clone and a destination vector, mediated by LR clonase. The *attB* (*attL*) sites are specifically recognized by the recombination proteins that constitute the clonase enzyme mix cocktails. These proteins cut to the left and to the right of the gene in the entry clone and ligate it into the destination vector, creating a new expression clone (Fig. 7b). There is a

counterselection against destination vectors without the gene of interest since they carry the *ccd*B *gene,* which is lethal to most *E. coli* strains. Thus, selection for ampicillin resistance chooses for *E. coli* cells that carry the desired product, which usually comprise 70-90% of the colonies. Once a DNA segment is cloned in the entry vector, it can easily be transferred into a destination vector (via the LR reaction) to generate an expression clone.

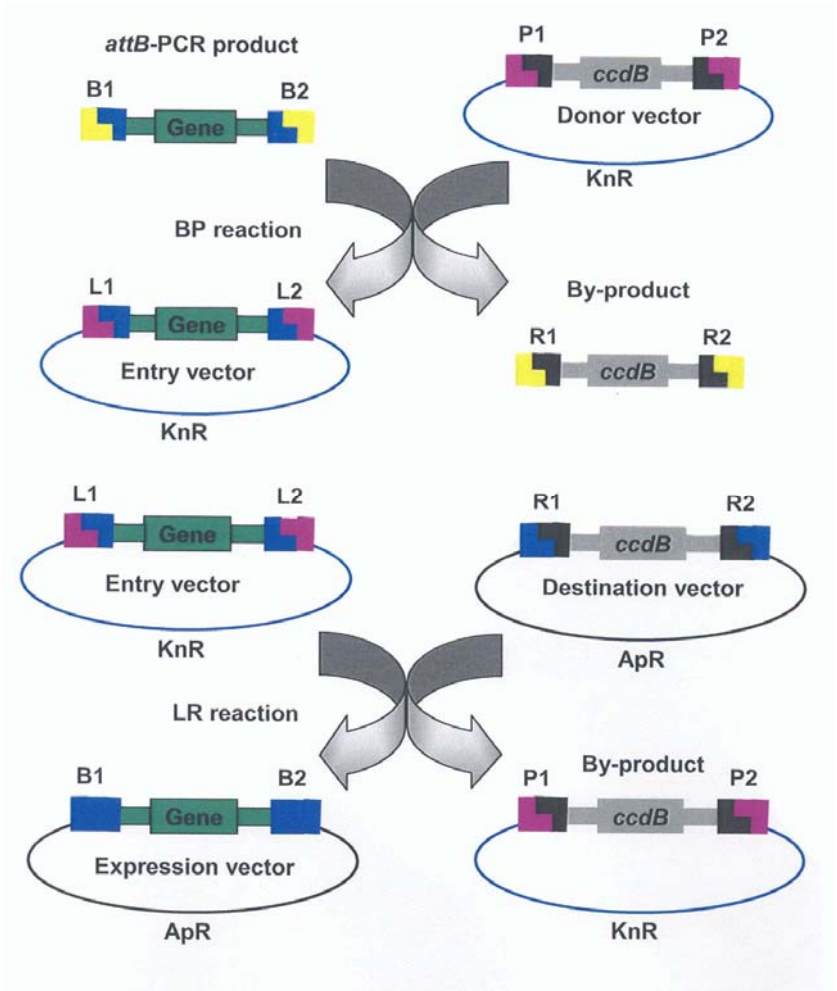


Fig. 7: GATEWAY cloning reactions.
(a) The BP reaction. The BP reaction can transfer a PCR gene flanked by the attB sites into a donor vector to yield an entry vector. Only plasmids without the *ccd*B gene that are also kanamycin resistant (KnR) will yield colonies. (b) The LR reaction. An entry vector, containing a gene flanked by recombination sites, recombines with a destination vector to yield an expression vector and a by-product plasmid. The result is that a gene sequence in the entry vector is transferred into an expression vector, donated by the destination vector. Only plasmids without the *ccd*B gene that are also ampicillin (ApR) resistant will yield colonies.

PCR products flanked by the attB sites for recombination were generated of all KSHV ORFs and cloned into the entry vector pDONR207 by BP clonase (Uetz et al. *unpublished*). 114 DNA fragments representing full-length or partial viral genes from the KSHV genome were cloned into the entry vector. Subsequently, all ORFs of KSHV were subcloned into three different destination vectors: the *yeast two-hybrid* (Y2H) plasmids pGADT7 (ampicillin resistance) and pGBKT7 (kanamycin resistance), which were used to screen for viral protein-protein interactions in the yeast-two hybrid system and the eukaryotic expression vector pDEST-script. The rationale was to obtain a viral array, which can be expressed in mammalian cells and can be screened for the modulation of signalling pathways. The pDEST-script plasmid was generated by insertion of a cassette containing the two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance (CmR) gene and a *ccd*B gene, into the unique EcoRV site of the pCMV-script vector (Fig. 8).

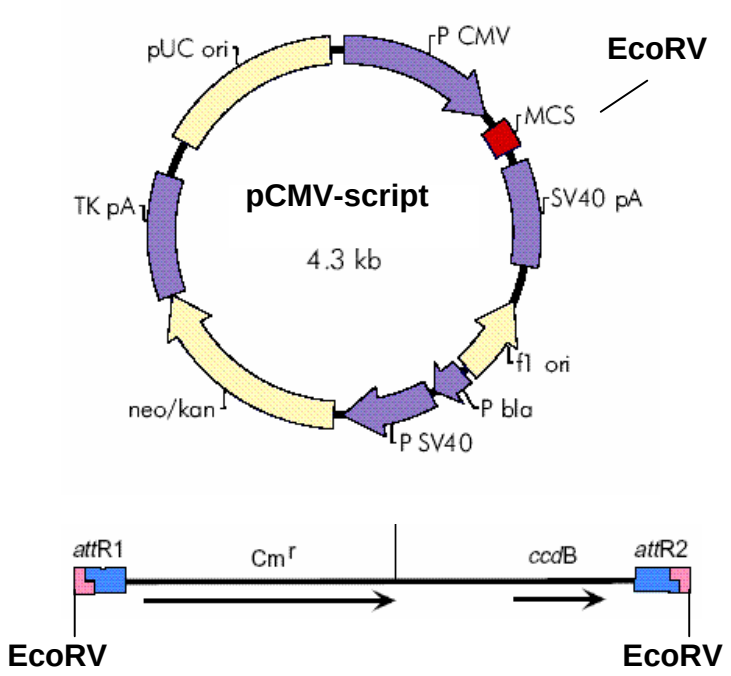


Fig. 8: Scheme of the pDEST-script vector and the recombinatorial cassette.
Blunt-end B cassette was inserted into the EcoRV unique site in the MCS (multiple cloning site) of pCMV-script converting it into a destination vector. The B-cassette contains an *att*R1 site at the 5'-end followed by the chloramphenicol resistance gene (Cmr) and *ccd*B gene. The *att*R2 site is at the 3'- end of the cassette.

For each of the generated expression constructs three *E. coli* colonies were picked up from the selection agar plates, the plasmid DNA was isolated and analysed by several restriction digests using different endonucleases. The efficiency of the recombinatorial cloning of the KSHV ORFs into different destination vectors is illustrated in Fig. 9. Approximately 85-95% of the analysed bacterial colonies carried the desired gene product.

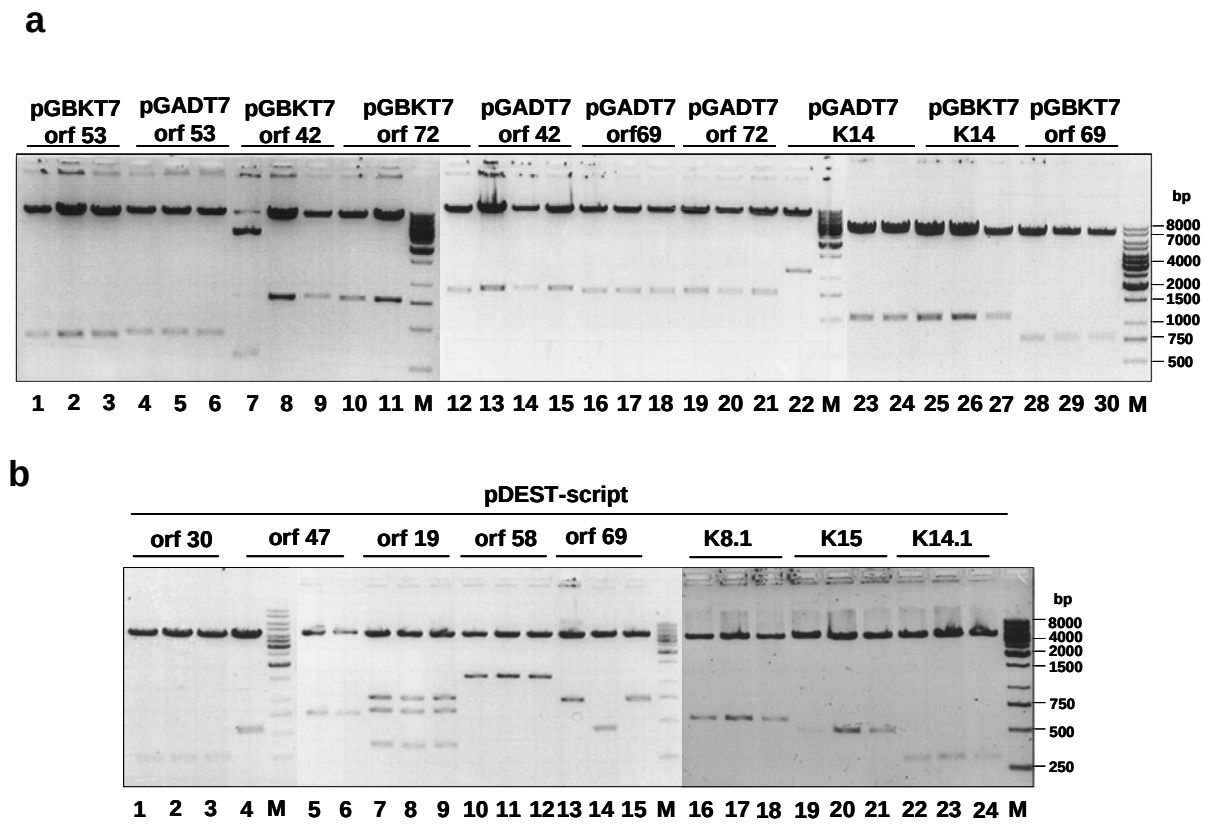


Fig. 9: Restriction digest of different destination vectors by endonucleases.
Three different bacterial colonies from each selection agar plate were analysed for the presence of the cloned gene of interest. DNA preparations were carried out and a restriction digest by several endonucleases was performed. The yeast expression vectors pGADT7 and pGBKT7 were digested by EcoRI and BamHI restriction enzymes (panel a). The eukaryotic expression plasmid pDEST-script was cut by EcoRI and HindIII restriction enzymes (panel b). Most of the *E. coli* clones carried the desired viral product (52/54=96%). Only the plasmid DNA samples with number 7 from panel a and number 14 from panel b showed a wrong pattern of DNA fragments after the restriction digest. M indicates the 1 kb DNA marker loaded on the agarose gel.

The correct DNA sequence of the generated KSHV ORFs cloned into the entry vector was evaluated by sequence analyses. To test that the viral genes are properly expressed by pDEST-script immunofluorescence (data not shown) and Western blot analyses (Fig. 10) were carried out. The correct reading frame of the cloned KSHV ORFs was also evaluated by cloning of the gene for green fluorescence protein (GFP) into pDEST-script.

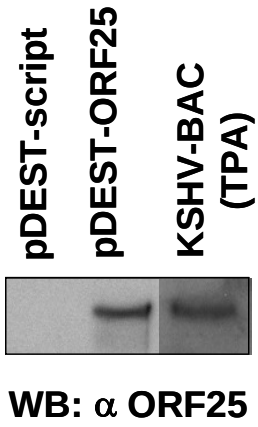


Fig. 10: Western blot analysis for the expression of KSHV ORF25 from pDEST-script.
HEK 293 cells were transfected by pDEST-ORF25 and the lysate was stained by an antibody specific for ORF25 (KS 330A). As a negative control, a lysate from HEK 293 cells transfected with the empty pDEST-script was included. A lysate of HEK 293 cells stably transfected with the KSHV-BAC and stimulated with TPA was used as a positive control.

3.1.2. Screening of the KSHV expression array for viral proteins activating SRE and AP-1

SRE and AP-1 binding sites are enhancer elements present in many promoters of genes related to cell growth, differentiation, development and transformation. SRE is present in the promoters of proto-oncogenes such as c-*fos,* FosB and JunB[87,115,156]. The dimeric AP-1 transcription factor is composed of members of the Jun, Fos, and activating transcription factor (ATF) families, and the constituents of this complex may govern cell proliferation and related responses[83].

The activation of a signal transduction pathway by a single viral protein can be monitored by the expression level of the luciferase reporter gene controlled by a

promoter containing these enhancer elements. Therefore, the KSHV expression array was screened for viral genes acting on signalling pathways which lead to an activation of SRE or AP-1 by luciferase reporter assays.

Individual viral ORFs were cotransfected into HEK 293 cells together with a luciferase reporter plasmid containing five copies of the SRE element derived from the promoter of the c-*fos* proto-oncogene or five copies of an AP-1 binding site (Fig. 11).

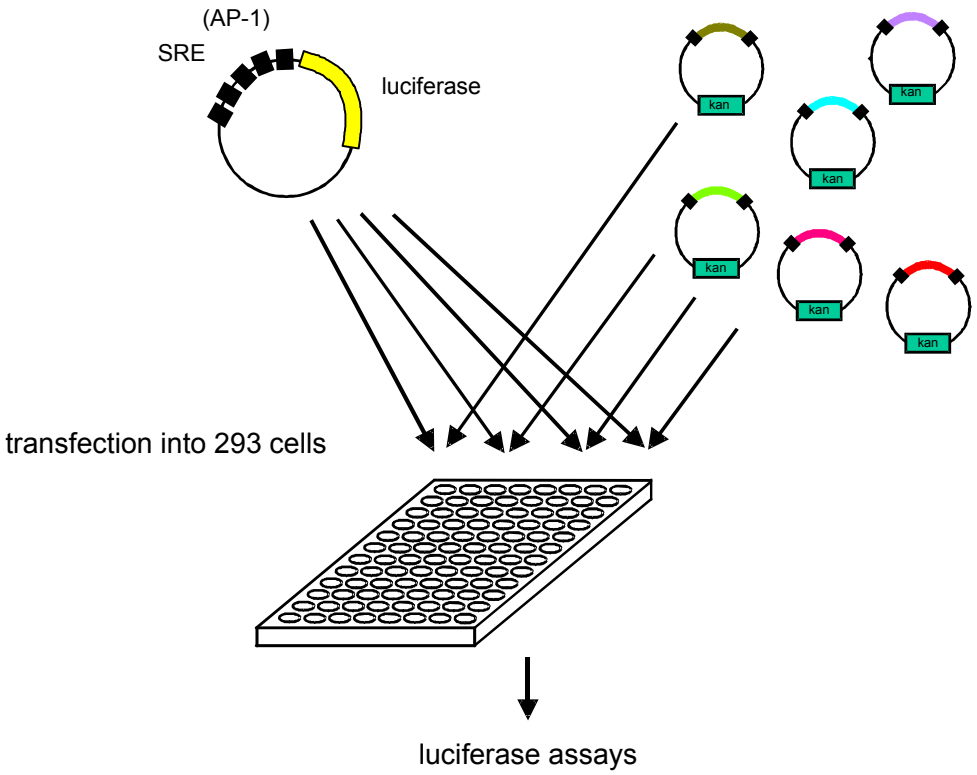


Fig. 11: Schematic representation of the screening in HEK 293 cells.
Test of the KSHV expression array for the induction of signalling pathways activating SRE and AP-1. Individual viral ORFs were cotransfected into HEK 293 cells together with a luciferase reporter plasmid containing five copies of the SRE element derived from the promoter of the c-*fos* proto-oncogene or five copies of an AP-1 binding site. 48h after transfection the cells were lysed and the luciferase activity was measured.

The empty pDEST-script vector was used as a negative control. The dominant positive RAS V12, which is a constitutively active mutant of RAS, was included as a positive control. Two days after transfection the cells were lysed and the luciferase activity was

measured (Fig. 12). The transactivation of the luciferase reporter constructs by each viral protein is indicated as fold activation compared to the negative control.

One viral protein, LANA-1 (ORF73), was identified as a strong activator of SRE. LANA-1 activated SRE approximately 16- and 20-fold above background (Fig. 12a). Several viral proteins induced a low level activation (2- to 4-fold) of SRE, which was considered to be either unspecific or of minor importance (Fig. 12a). In the screening for signal transduction pathways inducing AP-1, no viral gene activating the luciferase reporter construct more than 2- to 3-fold above background was detected (Fig. 12b).

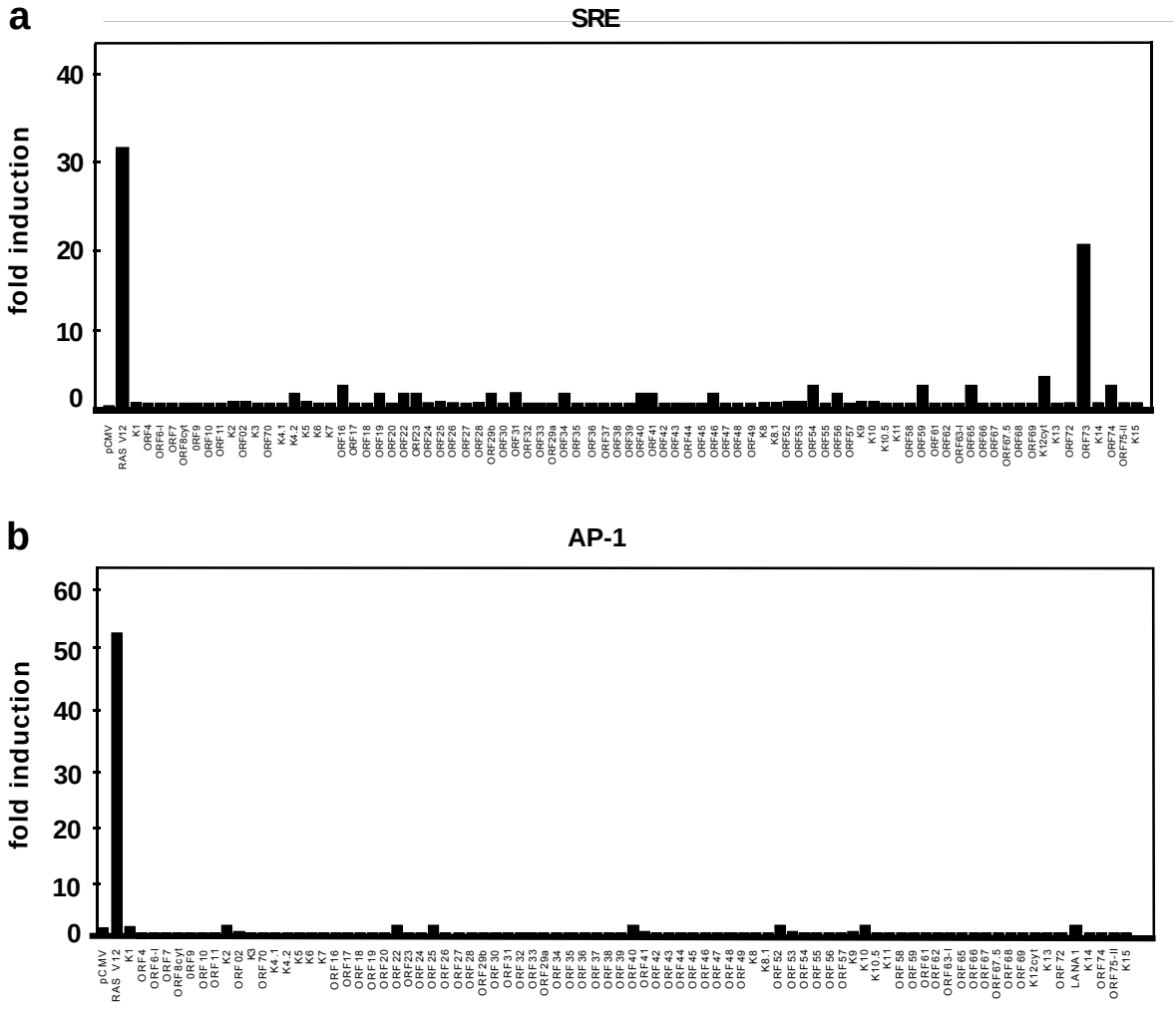


Fig. 12: LANA-1 is an activator of the serum response element (SRE).
LANA-1 was identified as an activator of the SRE element. The KSHV expression array consisting of all viral ORFs cloned into an eukaryotic expression vector was screened in HEK 293 cells using a luciferase plasmid with 5 SRE binding sites (a) and luciferase plasmid containing 5 AP-1 binding sites (b).

This observation differs from the data presented by An et al. on LANA-1 - mediated IL-6 promoter activation via AP-1[3]. The different results are probably caused by the fact that An and colleagues used a luciferase plasmid containing 6 different AP-1 binding sites (TGAGTCAG TGAGTCAC TGACTCAC TGACTCA TGAGTCAGC TGACTC) in the promoter, in comparison to the AP-1 binding site (7x TGACTAA) present in the plasmid used in this study or, alternatively, by differences in the vector backbones used.

3.2. Mechanism of LANA-1-induced SRE activation

In the current study, the mechanism of LANA-1-mediated activation of the SRE binding site was explored considering the following possible mechanisms:

- LANA-1 functions as a transcription factor that directly binds to the SRE binding site and activates transcription.
- LANA-1 induces a signal transduction pathway that leads to activation of transcription factors binding to the SRE element.
- LANA-1 acts as a transcriptional coactivator of a SRE family member by directly binding to SRF or TCFs.
- LANA-1 directly modulates the basal transcription machinery by interfering with some of its subunits.

3.2.1. Specificity of the LANA-1 effect on SRE

To examine the specificity of SRE activation by LANA-1 increasing amounts of the LANA-1 plasmid (50 to 250 ng per well) were cotransfected with 50 ng of the luciferase reporter vector. Three independent experiments showed a dose-dependent effect of LANA-1 on SRE (Fig. 13a). With 50 ng of the LANA-1 plasmid the SRE activity was increased 5-fold. With increasing concentrations of the LANA-1 plasmid the effect was stronger and with 250 ng of LANA-1 plasmid DNA an induction between 20- and 25-fold was detected. To correct for variations in transfection efficiency, the *Renilla* luciferase encoding plasmid pRL-TK was used as a normalization control.

To ensure that the effect seen was not due to sequences present in the backbone of the plasmid used, LANA-1 vectors with different backbone were tested for SRE activation. A similar effect was observed with every construct indicating that LANA-1 and not the vector induced SRE (data not shown).

Similar results were obtained in HeLa cells, but neither in DG75 B-cells nor Jurkat T lymphocytes, suggesting that a cell-type specific cofactor present in epithelial/endothelial but missing in lymphocytes is mandatory for stimulation (data not shown).

Previously, it was reported that LANA-1 is a transcriptional modulator of a variety of viral and cellular promoters including EBV Cp, LANA-1, HIV-1 LTR (in some cell lines), telomerase reverse transcriptase and promoters containing SP1 and CAAT binding sites[123]. Thus, the effect of LANA-1 on a variety of promoters was compared. Several vectors expressing the firefly luciferase gene controlled by synthetic promoters containing a basic TATA element and various binding sites for AP-1, NF-kB, SRE and ISRE were evaluated in cotransfection experiments with an expression vector for LANA-1 (Fig. 13b). A luciferase reporter vector with the HIV-1 LTR was also included. It was found that LANA-1 strongly activated another promoter besides SRE which contained 5 *interferon-stimulated response elements* (ISRE). Several other promoters containing AP-1 or NF-κB binding sites or the HIV-1 LTR, on the other hand, were not activated but repressed, indicating that LANA-1 is a promiscous, but still selective transcriptional modulator.

The activation of ISRE by LANA-1 which was as strong as the induction by 500 units/ml of IFN α indicated a possible role of LANA-1 in IFN type I-dependent signalling pathways. The functional relevance of stimulation of type I interferon signalling by LANA-1 is currently unknown and is an object of investigation in another study in our laboratory.

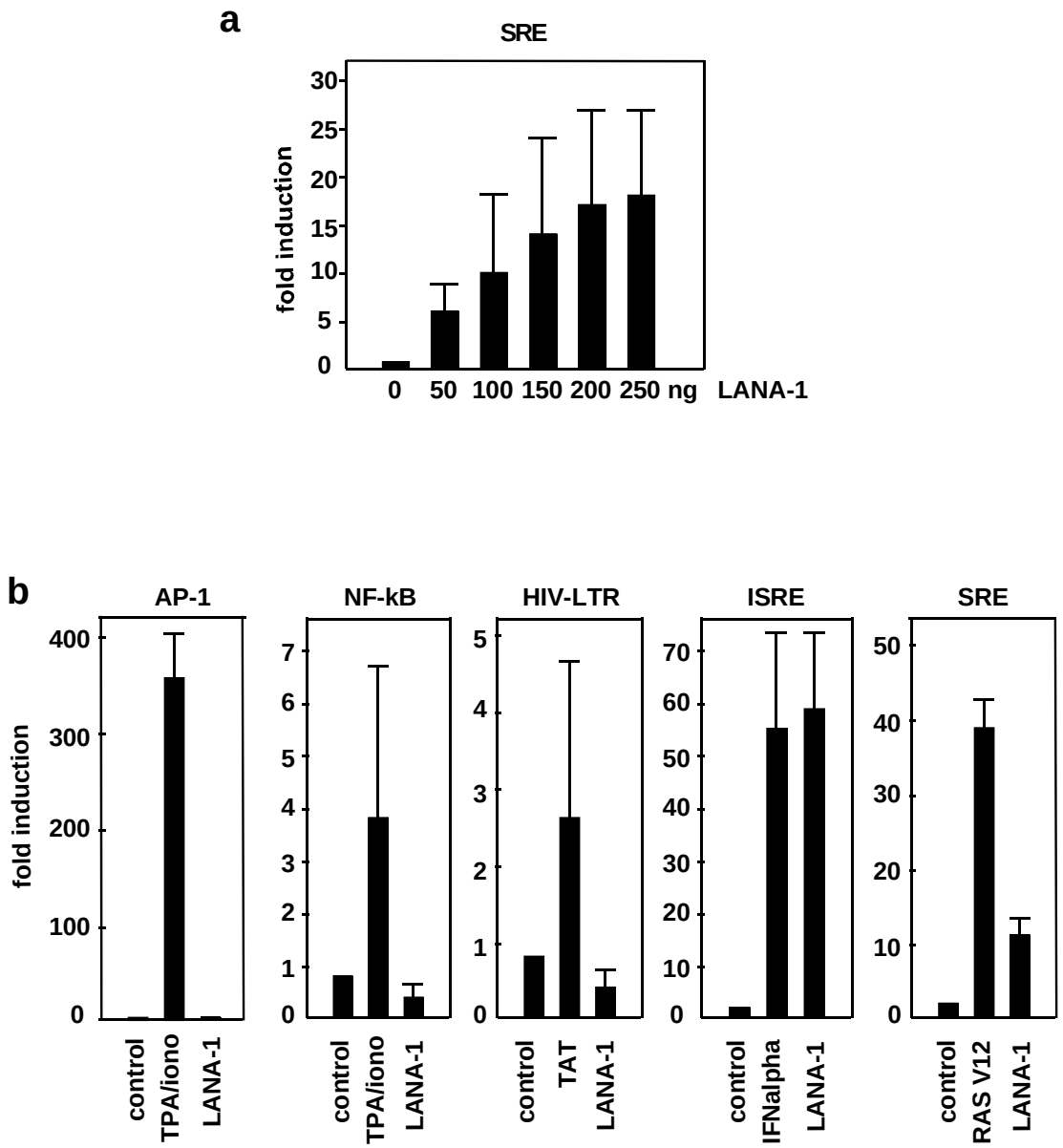


Fig. 13: Specificity of the LANA-1 - related activation of SRE.
(a) Dose-dependent effect of LANA-1 on SRE. Increasing amounts of LANA-1 plasmid DNA were cotransfected with the SRE luciferase plasmid. (b) LANA-1 transactivation of cellular promoters is specific but promiscous. An expression vector for LANA-1 was cotransfected into HEK 293 cells with a variety of luciferase reporter plasmids containing AP-1, NF-kB, SRE, ISRE binding sites or the HIV-1 LTR. As negative controls, cells cotransfected with the empty pCR3 vector were included. The positive controls were cells cotransfected with plasmids expressing either RAS V12 or HIV-1 Tat, or stimulated with either IFN α or with phorbol ester (TPA) and ionomycin (iono). Data are representative of three independent experiments, and error bars represent standard error values. To correct for variations in transfection efficiency, the *Renilla* luciferase encoding plasmid pRL-TK was used as a normalization control.

3.2.2. LANA-1 is not a general processivity factor

Subsequently, it was evaluated whether LANA-1 would stimulate activated promoters independently of the transcription factor binding site. A similar set of luciferase reporter vectors as in Fig. 13 was used and stimulated with the respective positive controls in the absence or the presence of LANA-1 (Fig. 14). As a negative control HEK 293 cells were cotransfected with the empty pDEST-script plasmid. 48 h post transfection the cells were harvested and the luciferase activity was measured. To correct for variations in transfection efficiency, the *Renilla* luciferase encoding plasmid pRL-TK was used as a normalization control. LANA-1 was suppressive for the promoter containing AP-1 binding sites and for the IL-6 promoter, but had no or slight suppressive effect on the promoter containing NF-κB binding sites (see discussion). Moreover, LANA-1 further increased the activity of stimulated SRE and ISRE promoters (Fig. 2b), suggesting (i) that it only acts on specific promoters and (ii) that LANA-1 probably has a different mode of action as the transcription factors themselves (Fig. 14).

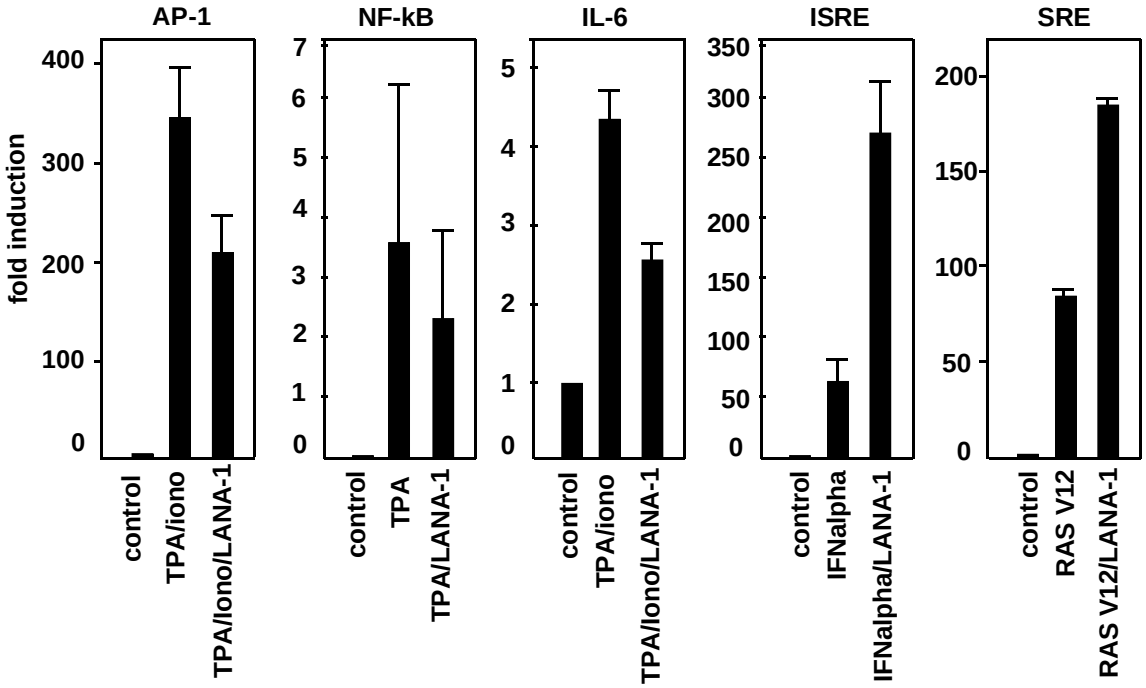


Fig. 14: LANA-1 is not a general processivity factor.
HEK 293 cells transfected with AP-1, NF-kB, SRE, ISRE and IL-6 promoter luciferase reporter plasmids were either cotransfected with the positive control plasmid RAS V12 or stimulated with either IFN α or phorbol ester (TPA) and ionomycin (iono). Additionally, LANA-1 was transfected to evaluate whether it is a general processivity factor or specific for SRE and ISRE.

In order to assess if LANA-1 directly binds to DNA, an electromobility shift assay was carried out. The experiment did not demonstrate a direct binding of LANA-1 to SRE (data not shown). Other possible mechanisms of action of LANA-1 were therefore investigated.

3.2.3. The role of the MEK/ERK-1/2 MAP kinase pathway in LANA-1-mediated activation of SRE

Several reports demonstrated that KSHV also affects cell cycle regulation through mitogenic pathways[9,22,104,108,117]. The activation of promoters containing SRE binding sites occurs via different pathways (see introduction). RAS-induced activation of SRE is mediated by a sequential phosphorylation of RAF, MEK-1/2, ERK-1/2 and the ternary complex factor ELK-1 (Fig. 15a). In order to investigate whether the MEK-ERK pathway plays a role in the activation of SRE by LANA-1 the following experiments were carried out. The effect of LANA-1 on SRE was evaluated in the presence and absence of MEK1/2 inhibitors. Two different chemicals were used, PD98059 and U0126. PD98059 inhibits the phosphorylation activation of MEK1 by upstream kinases. U0126 is a specific inhibitor of the phosphorylation activation of MEK1 and MEK2 by upstream kinases. The SRE luciferase reporter vector was cotransfected with empty pCR3, LANA-1 and RAS V12, respectively, and the specific MEK1/2 inhibitors were added 24 h later (Fig. 15b). The luciferase activity was determined 40 h post transfection. The two MEK-1/2 inhibitors U0126 and PD98059 inhibited the activation of SRE by LANA-1 to a similar extent as they reduced the induction of SRE by RAS V12 (Fig. 15b). This observation indicated that the phosphorylation of MEK1/2 kinases might be necessary for the activation of SRE by LANA-1.

In addition to the luciferase assays, a Western blot experiment with anti-phospho-MEK1/2 antibody was performed to assess the effect of MEK1/2 inhibitors. An increased phosphorylation of MEK-1/2 by LANA-1 (Fig. 15c, line 3) and a very strong band for RAS V12 induced phosphorylation of MEK1/2 was detected, as was expected. The specific inhibitor PD98059 reduced both the RAS V12-, as well as the LANA-1-induced MEK1/2 phosphorylation (Fig. 15, lines 2, 4 and 6).

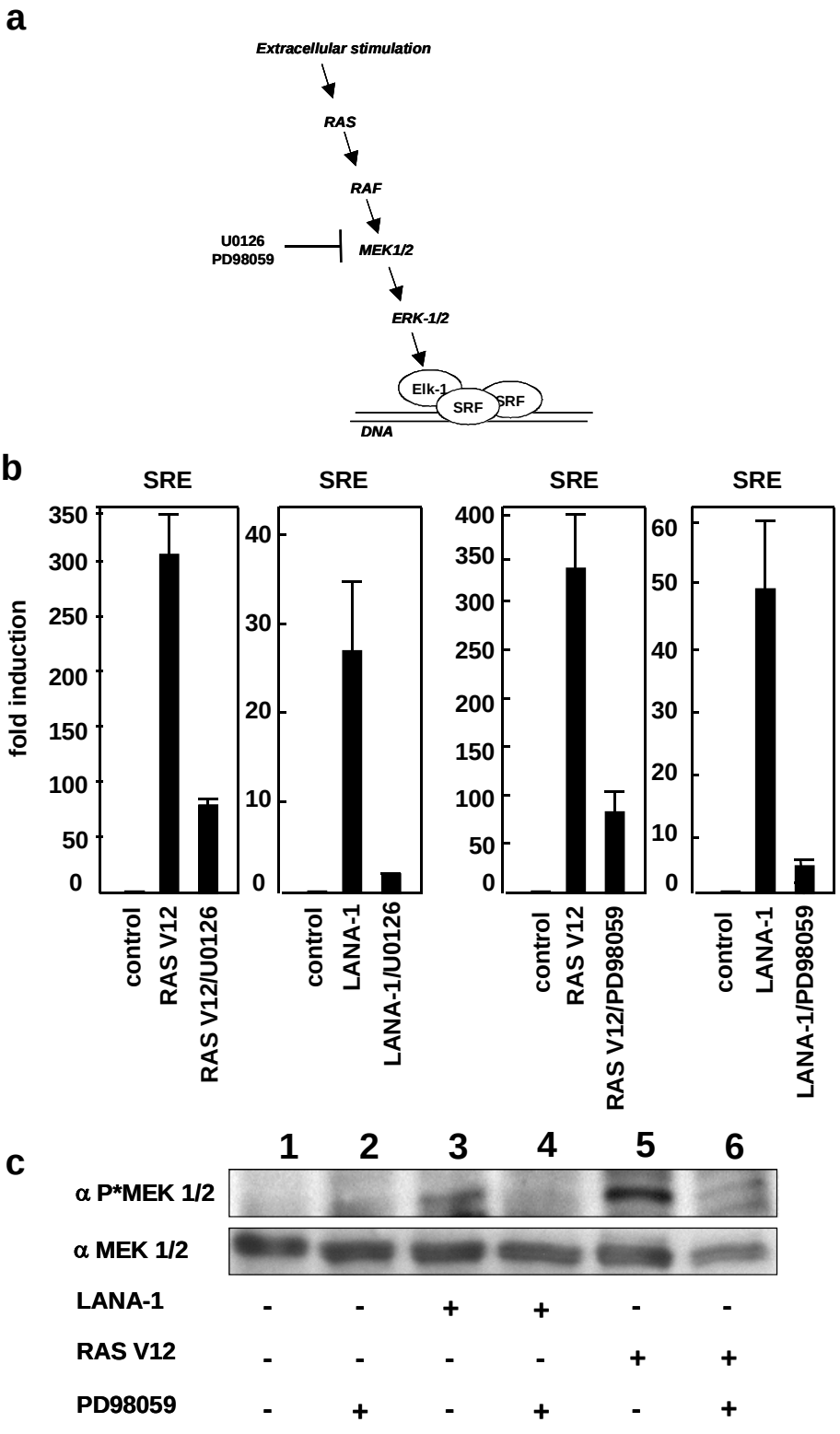


Fig. 15: LANA-1 induces the MEK/ERK-1/2 MAP kinase pathway.
(a) Scheme of the RAS induced activation of SRE binding site. (b) Inhibition of LANA-1-induced SRE induction by inhibitors of MEK-1/2 phosphorylation. HEK 293 cells were transfected with pSRE-luc and RAS V12 in the presence or absence of U0126 or PD98059 MEK-1/2 inhibitors. (c) LANA-1 induces the phosphorylation of MEK-1/2 as shown by Western blot analysis using a mAB specific for phosphorylated MEK-1/2 (α P* MEK1/2) and an α MEK1/2 antibody recognizing all forms of MEK1/2.

These experiments revealed that the phosphorylation of MEK1/2 might play an important role in the LANA-1-mediated activation of SRE.

3.2.4. LANA-1 induces the phosphorylation of the ternary complex factor ELK-1

In the RAS-induced MAPK cascade the phosphorylated MEK1/2 kinases subsequently activate ERK1/2 (Fig. 15a). ERK1/2 mediate the phosphorylation of the ternary complex factor ELK-1 on Ser383 (see introduction). Thus, it was investigated if the phosphorylation of ELK-1 on Ser383 is enhanced in the presence of LANA-1. A p42/44 *in vitro* MAP kinase assay was performed using lysates of HEK 293 cells transfected either with LANA-1, RAS V12 or the empty pCR3 vector. Active phosphorylated ERK-1/2 was selectively precipitated using an immobilized antibody, the precipitates were incubated with ELK-1 fusion protein and ATP and subsequently the ELK-1 phosphorylation at Ser383 was analysed by Western blot using an anti-phospho ELK-1 specific antibody. An approximately 3-fold enhanced ELK-1 phosphorylation on Ser383 in the lysates of cells transfected with LANA-1 was detected (Fig. 16a, lane 2), which was even more enhanced than in cells transfected with the two positive controls ERK-2 and RAS V12 (Fig. 16b, lanes 3 and 4).

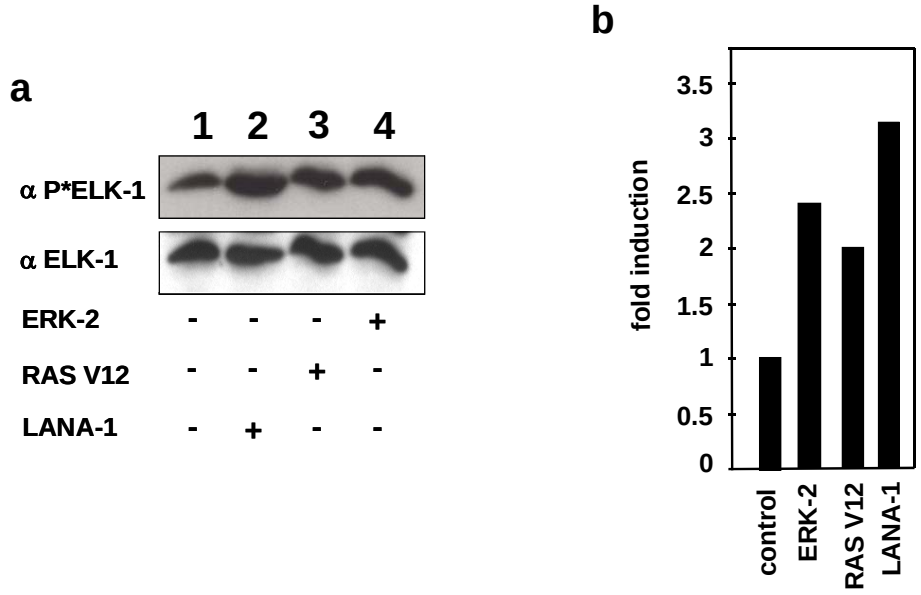


Fig. 16: LANA-1 induces a phosphorylation of the ternary complex factor ELK-1.
(a) LANA-1-induced increased ERK-1/2 activity indicated by an *in vitro* kinase assay. Cells transfected with pCR3, ERK-2, RAS V12 or LANA-1 were lysed and analysed for ERK-1/2 activity in an *in vitro* kinase assay using a mAB specific for phosphorylated ELK-1 (α P* ELK-1). (b) Quantitative analysis of the LANA-1-induced ELK-1 phosphorylation. The x-ray film shown in (a) was scanned and analysed by the quantification software Quantity One.

3.2.5. LANA-1 acts through both SRF and TCF binding sites

In order to determine whether the SRF or TCF binding sites are required for the induction of the SRE element in response to LANA-1, HEK 293 cells were transiently transfected with the wild-type or mutated SRE-luciferase plasmids (Fig. 17a). Each reporter plasmid contained a single copy of the wild-type or mutated SRE element. As a positive control dominant positive RAS V12 and as a negative control the empty pCR3 vector were used. The RAS V12-induced SRE activation can be mediated by TCF-dependent or independent pathways, both of which require SRF (see introduction). As expected, dominant positive RAS V12 was still able to activate the promoter containing the mutated TCF binding site, but not the one with the mutated SRF binding site (Fig. 17b). LANA-1, however, showed a completely different picture. Mutation of either the SRF or TCF binding site did not block the ability of LANA-1 to activate the promoter and LANA-1 was still able to induce both mutant promoters to approximately 50% of the level of the wild-type SRE promoter activated by LANA-1 (Fig. 17b), suggesting that LANA-1 either modulates both TCF and SRF pathways or acts downstream of these two transcription factors.

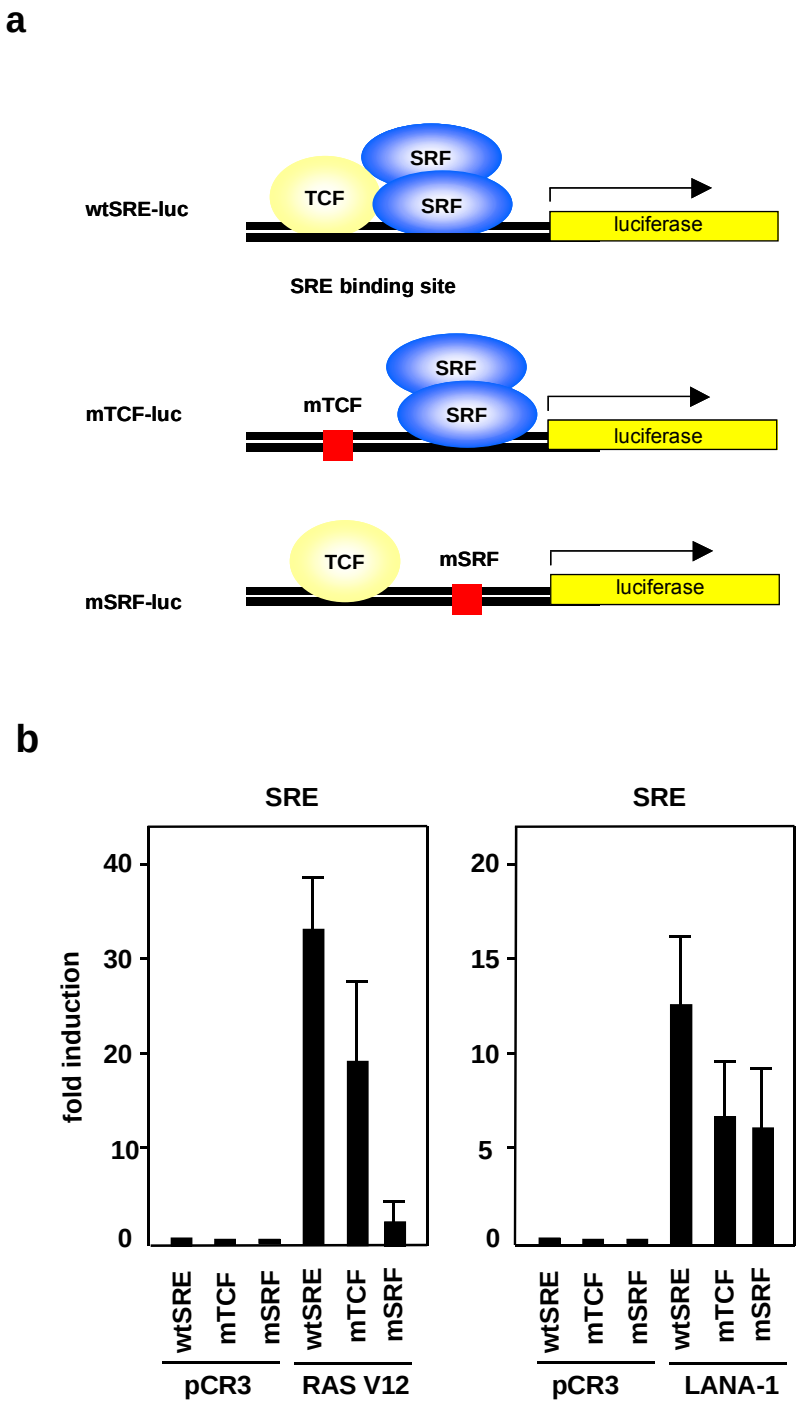


Fig. 17: LANA-1 acts through both SRF and TCF binding sites.
(a) Schematic representation of the luciferase reporter constructs used in the transfection assay: wtSRE-luc with wild-type SRE binding site from the c-*fos* promoter, mSRF-luc with mutated SRF binding site and mTCF-luc with mutated TCF binding site. (b) LANA-1 acts via both SRF and TCF binding sites in the SRE element. HEK 293 cells were transiently transfected with luciferase reporter constructs containing either the wild-type SRE binding site from the *c-fos* promoter or SRE sites deficient for SRF and TCF binding.

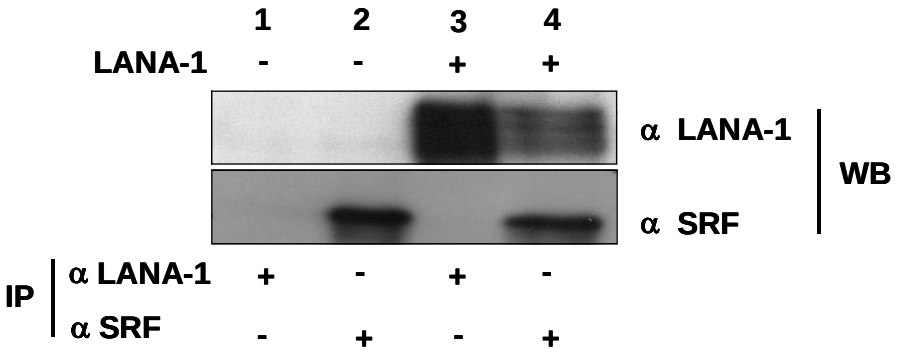
3.2.6. LANA-1 interacts with the specific transcription factor SRF

Next, the cellular counterparts of LANA-1 in the activation of the SRE inducing signalling pathway were investigated. First, it was tested whether LANA-1 interacts directly with some of the specific transcription factors. To assess whether SRF and LANA-1 interact, a co-immunoprecipitation was carried out using a lysate from HEK 293 cells that were transiently transfected with LANA-1. As a negative control, cells transfected with the empty pCR3 vector were used. In these cells LANA-1 was coprecipitated with endogenous SRF (Fig. 18a). Subsequently it was tested whether the transcriotion factors ELK-1, STAT-1 or SpiB would interact with LANA-1.

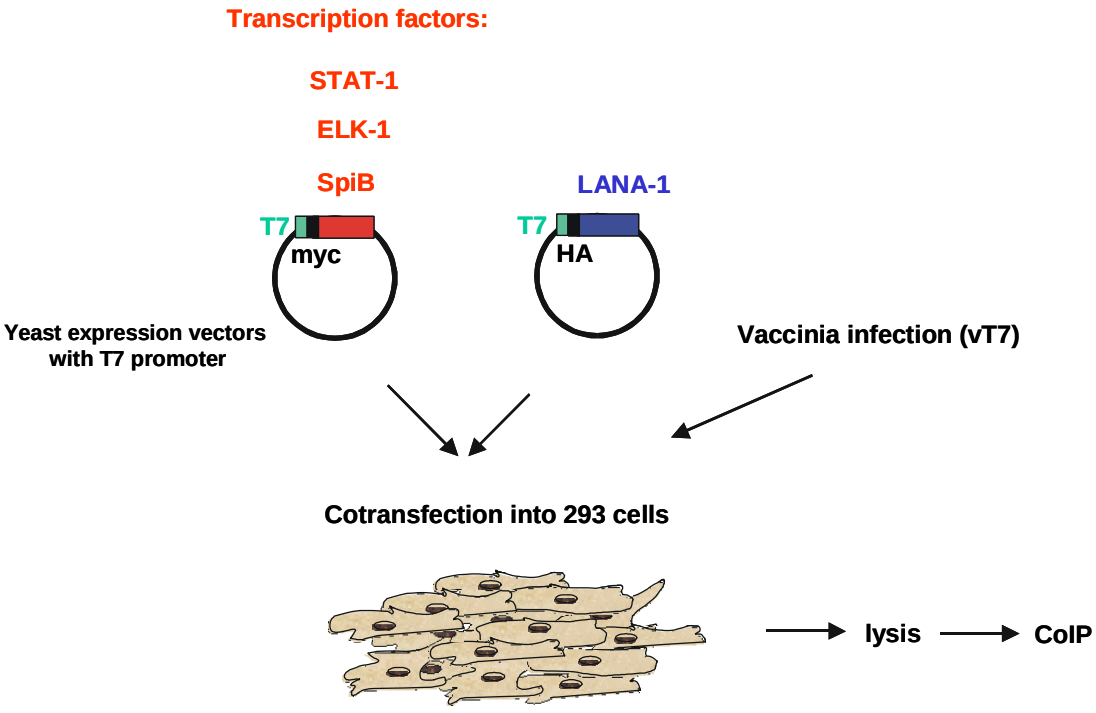
ELK-1, STAT-1 and SpiB were cloned by recombinatorial cloning into the pDONR207 vector and subsequently into the expression vector pGBKT7. LANA-1 was similarly subcloned into the pGADT7 vector. Proteins expressed from these plasmids are tagged either with c-Myc (pGBKT7) or hemagglutinin (HA) (pGADT7 vector) epitopes. The expression of the cloned genes in these two plasmids is driven by a T7 promoter, which can be activated by a simultaneous infection with a recombinant vaccinia virus expressing the T7 RNA polymerase (Fig. 18b).

To assess the interaction between LANA-1 and ELK-1, STAT-1, and SpiB, HEK 293 cells were first infected by recombinant vaccinia virus expressing T7 RNA polymerase and subsequently cotransfected with LANA-1 and the vector expressing the respective transcription factor. 48 h post transfection the cells were lysed and the overexpressed Myc-tagged transcription factors and HA-tagged LANA-1 were immunoprecipitated either with anti-HA (lanes 1-4) or anti-Myc (lanes 5-8) antibodies as indicated in Fig. 18c. After SDS-PAGE electrophoresis, a Western blot was carried out with anti-c-Myc and anti-HA antibodies (Fig. 18c, on the right). As a control for each experiment lysates from HEK 293 cells transfected only with single expression vectors were included (Fig. 18c, lanes 2, 3, 6 and 7). As an additional control a lysate of HEK 293 cells infected with vaccinia virus only was used (Fig. 18c, lanes 1 and 5). In summary, these co-immunoprecipitation assays indicated that LANA-1 binds directly to SRF transcription factor (Fig. 18a), but not to ELK-1, STAT-1 and SpiB (Fig. 18c).

a


1
2
3
4
LANA-1
-
-
+
+
α LANA-1
α SRF
WB
IP
α LANA-1
α SRF
+
-
+
-
-
+
-
+

b


Transcription factors:
STAT-1
ELK-1
SpiB
LANA-1
T7
myc
T7
HA
Yeast expression vectors
with T7 promoter
Vaccinia infection (vT7)
Cotransfection into 293 cells
lysis
CoIP

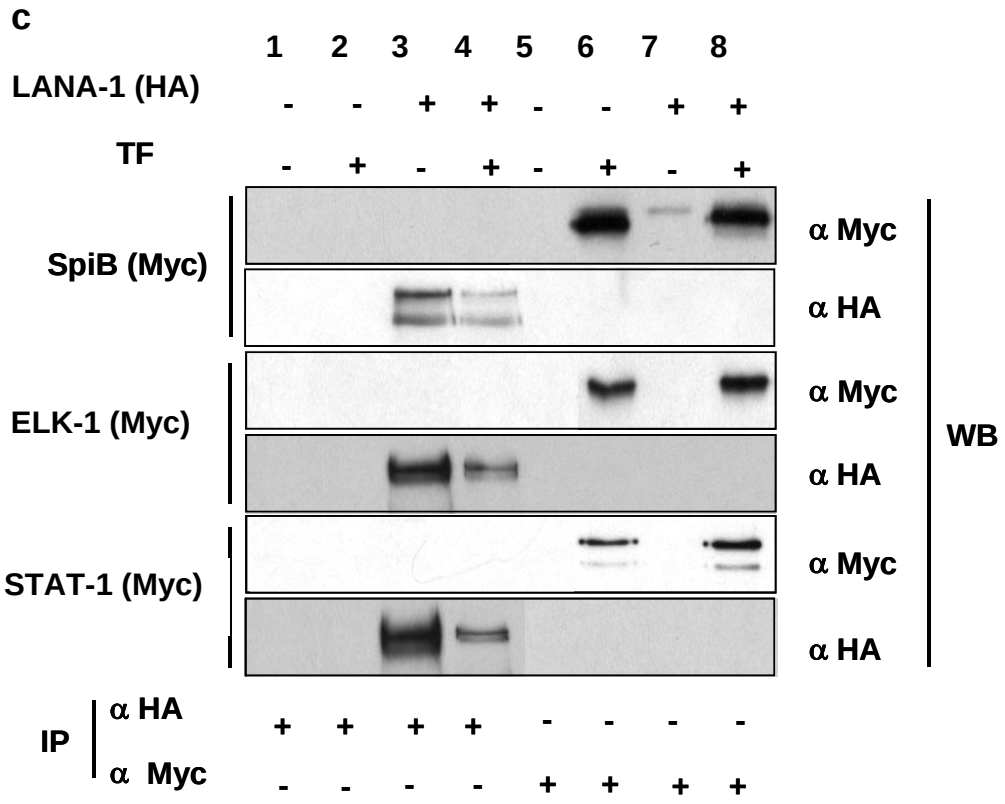


Fig. 18: LANA-1 interacts with SRF.
(a) LANA-1 interacts with the transcription factor SRF as shown by CoIP. Endogenous SRF was precipitated from lysates of HEK 293 cells transiently transfected with LANA-1. (b) Schematic representation how the experiment shown in (c) was performed. (c) LANA-1 does not interact with SpiB, ELK-1 or STAT-1 in CoIP experiments. HEK 293 cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase and subsequently cotransfected with LANA-1 and the vector expressing the respective transcription factor, as indicated. The proteins were precipitated and detected in Western blot with anti-HA and anti-myc antibodies. TF indicates whether the transcription factors SpiB, ELK-1 or STAT-1 were transfected or not.

3.2.7. LANA-1 acts through the Mediator complex

Since Lana-1 has a pleitropic effect on several promoters and since LANA-1-induced SRE activation was only partially blocked by either of the two mutations in the SRF and TCF binding sites (Fig. 17b), we hypothesized that LANA-1 acts downstream of the SRE binding site and has a more direct effect on the transcriptional activation. Transcriptional activation by the herpesviral VP16 protein requires the Mediator subunit ARC92/ACID-1 (MED 25)[14,102] which also participates in the activation of type I-interferon inducible promoters (Meisterernst et al. *unpublished*). We thus speculated that the Mediator is involved in LANA-1-induced transcriptional activation as well. The Mediator complex serves as a "bridge" for transferring positive as well as negative signals from different activators or inhibitors (specific transcription factors) to the polymerase.

To investigate if the effect of LANA-1 on SRE is the result of a direct interaction with Mediator proteins a variety of co-immunoprecipitation assays were carried out. In fact, it was found that LANA-1 interacts with the Mediator complex. In co-immunoprecipitation (CoIP) experiments the overexpressed LANA-1 protein was precipitated with several different Mediator subunits including PCQAP (MED 15), ARC92/ACID-1 and Sur-2 (Fig. 19a-c). Both LANA-1 and GFP-tagged LANA-1 coprecipitated with the endogenously expressed Mediator subunit PCQAP (Fig. 19a). As a negative control, a lysate from HEK 293 cells transfected with the empty pCMV-script vector was included (Fig. 19a, lines 1 and 4). LANA-1 was also coprecipitated with overexpressed (Fig. 19b, lanes 5-8) as well as endogenous ARC92/ACID1 (lanes 1-4).

VP-16 can control transcriptional activation in mammalian cells by direct binding to the ARC92/ACID-1 protein of the Mediator complex[102]. The NTD domain of ARC92/ACID1 has a dominant negative effect on the activation by VP16[102] (see introduction). When overexpressed in mammalian cells NTD replaces the endogenous ARC92/ACID-1 and blocks the transcriptional activation by VP-16. To evaluate if the mechanism of binding of LANA-1 to the ARC92/ACID-1 protein is similar as for the HSV-1 transactivator VP16, luciferase assays were carried out, in which the SRE or ISRE reporter constructs were cotransfected together with LANA-1 in the absence or the presence of the overexpressed dominant negative NTD domain of ARC92/ACID-1. As a positive control for the NTD domain, the pGLMRG5 plasmid containing Gal4 binding sites and Gal4 fusion proteins of full length VP16 and the VP16 H1 domain were used (Fig. 20a and b).

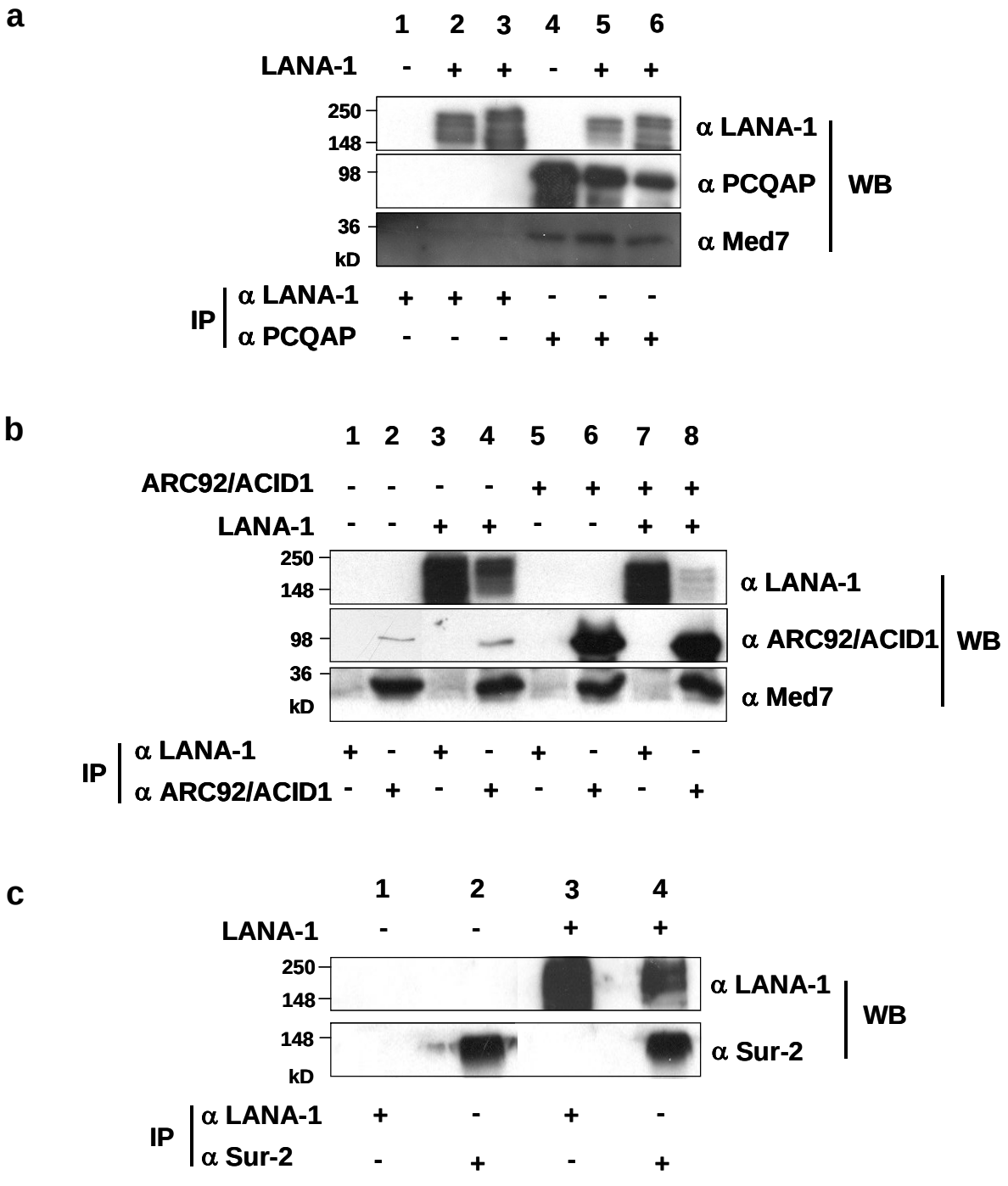


Fig. 19: LANA-1 interacts with the Mediator.
(a) LANA-1 interacts with the Mediator protein PCQAP as shown by CoIP. Lysates of HEK 293 cells transiently transfected with LANA-1 were either precipitated with the anti-LANA-1 mAB or an anti-PCQAP mAB. The Western blot analyses were carried out with either of the two antibodies or an antibody against the Med7 Mediator protein. Lines 2 and 5 refer to the lysates from cells expressing wild-type LANA-1 used in the CoIP. Lines 3 and 6 refer to the lysates from cells expressing GFP-tagged LANA-1. (b) LANA-1 interacts with the Mediator protein ARC92/ACID1 as demonstrated by CoIP. (c) LANA-1 interacts with the Mediator protein Sur-2 as shown by CoIP.

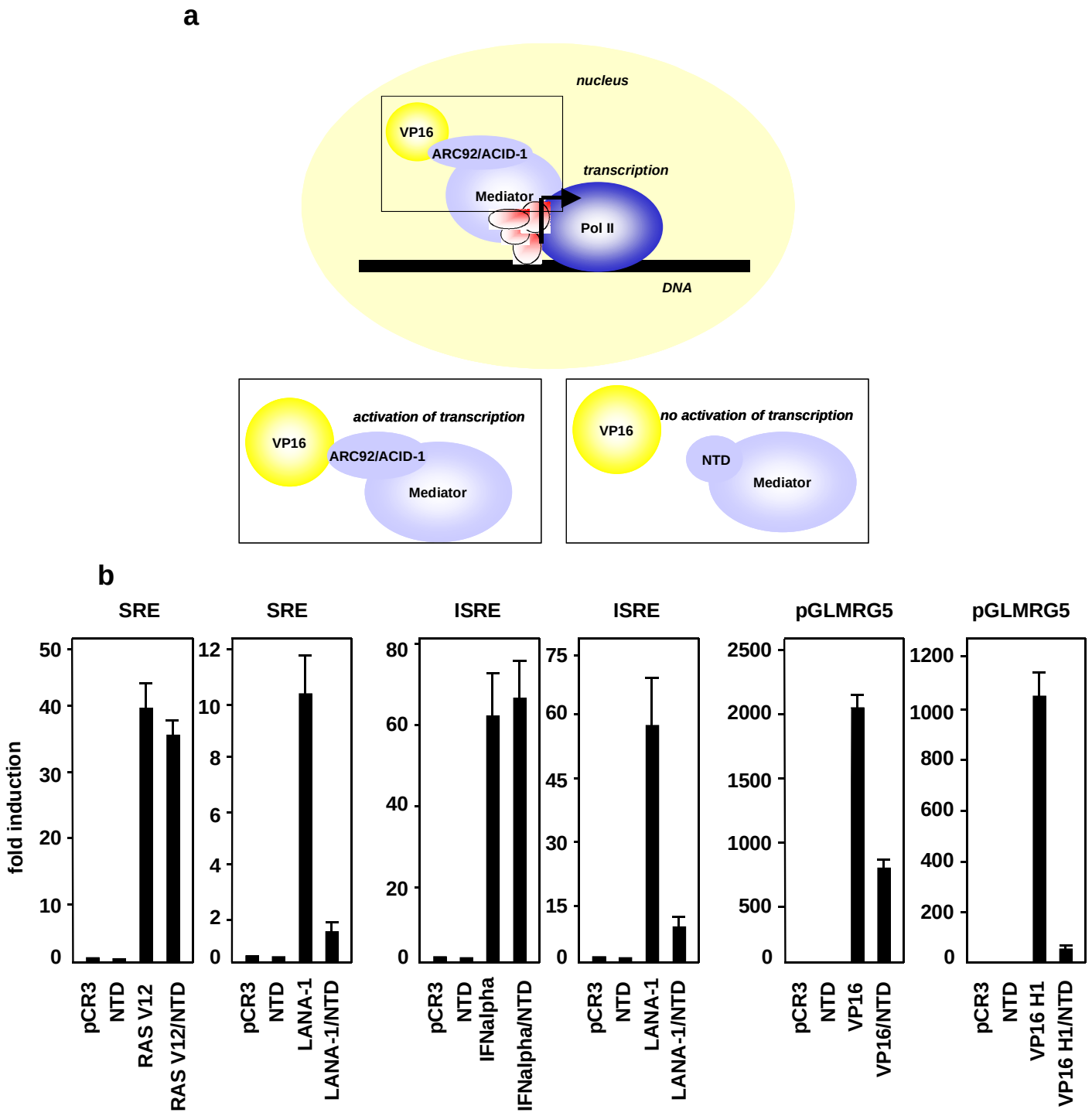


Fig. 20: Dominant negative effect of NTD on LANA-1 activation.
(a) Model for NTD–related inhibition of transcriptional activation by VP16. (b) The LANA-1-mediated activation of SRE and ISRE is abrogated by the N-terminal domain (NTD) of ARC92/ACID1. HEK 293 cells were transfected with pSRE-luc, pISRE-luc or pGLMRG5 luciferase plasmids and LANA-1, dominant positive RAS V12, full-length VP16 or the H1 domain of VP16.

The LANA-1-induced activation of both SRE and ISRE was inhibited by NTD similar to the VP16 control. NTD lead to a 84% inhibition of LANA-1-induced SRE activation and to a 85% inhibition of LANA-1-induced ISRE activation. In a parallel experiment, different amounts of the NTD expression vector were evaluated. A dose-dependent effect of NTD was observed, in that increasing concentrations of NTD led to stronger inhibition of the LANA-1-induced SRE and ISRE activation (data not shown).

To prove that the inhibition by NTD is specific for the viral transactivators VP16 and LANA-1, additional experiments were performed. RAS V12 was cotransfected with SRE reporter vector in presence or absence of NTD. The same was done for ISRE reporter vector activated by IFN α. Intriguingly, NTD had no effect on SRE activation by RAS V12 and ISRE activation by exogenous IFN α (Fig. 20b), suggesting that here ARC92/ACID-1 is not involved as a Mediator subunit (see discussion). As expected, NTD also had no effect on v-FLIP-induced activation of a NF-kB luciferase construct[98], indicating that the observed effect is specific to LANA-1 but not to other KSHV proteins (data not shown).

3.2.8. The N-terminal domain of LANA-1 binds to the Mediator subunit ARC92/ACID1

To identify the domains within LANA-1 which bind to the Mediator a variety of His- and GST-tagged mutants of LANA-1 were used in CoIPs and GST pull-down assays. The C-terminal deletion mutants of LANA-1 used in the CoIP experiments are illustrated in Fig. 21a. The CoIPs were carried out using lysates of HEK 293 cells transfected with N-terminally His-tagged wild-type LANA-1, L25, or L26. As a negative control, HEK 293 cells were transfected with the empty pCR3 vector. The lysates were immunoprecipitated with the respective antibodies as is indicated in Fig. 21b (indicated on the left). Coprecipitated proteins were detected by Western blot using anti – ARC92/ACID-1 (9C2) and anti-LANA-1 antibodies (Fig. 21b, indicated on the right). The two C-terminal deletion mutants L25 and L26 were still able to precipitate the endogenous ARC92/ACID-1 protein (Fig. 21b), which demonstrated that the region between 1006 and 1162 AA at the C-terminus of LANA-1 is not mandatory for the interaction with the Mediator.

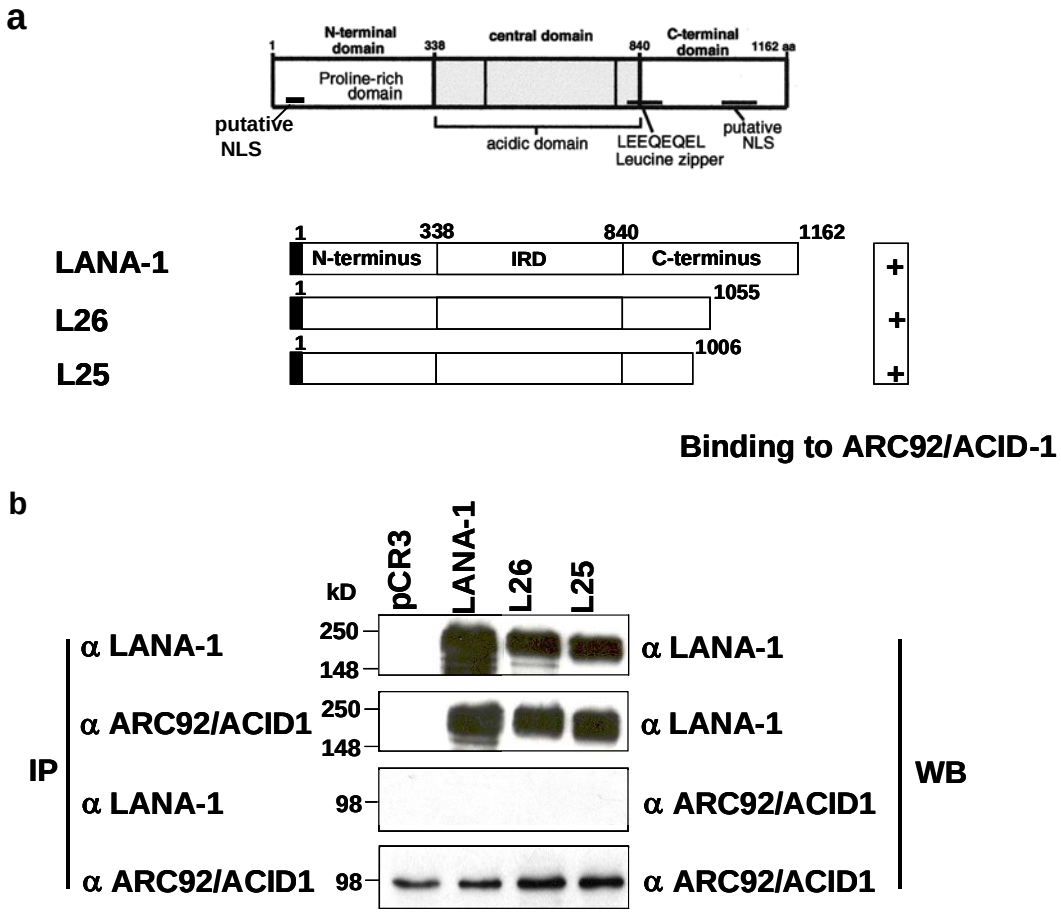


Fig. 21: The C-terminal LANA-1 domain is not mandatory for the interaction with the Mediator protein ARC92/ACID1.
(a) Schematic diagram of the LANA-1 C-terminal deletion constructs used in the CoIP experiment shown. The ability to bind to ARC92/ACID-1 is summarized on the right. (b) The C-terminal domain of LANA-1 is not mandatory for the interaction with ARC92/ACID1 in CoIP. Plasmids encoding the LANA-1 mutants were transfected into HEK 293 cells, immunoprecipitated as indicated, separated by SDS-PAGE, blotted, and examined by immunostaining (top two panels, anti-LANA-1; lower two panels, anti-ARC92/ACID1).

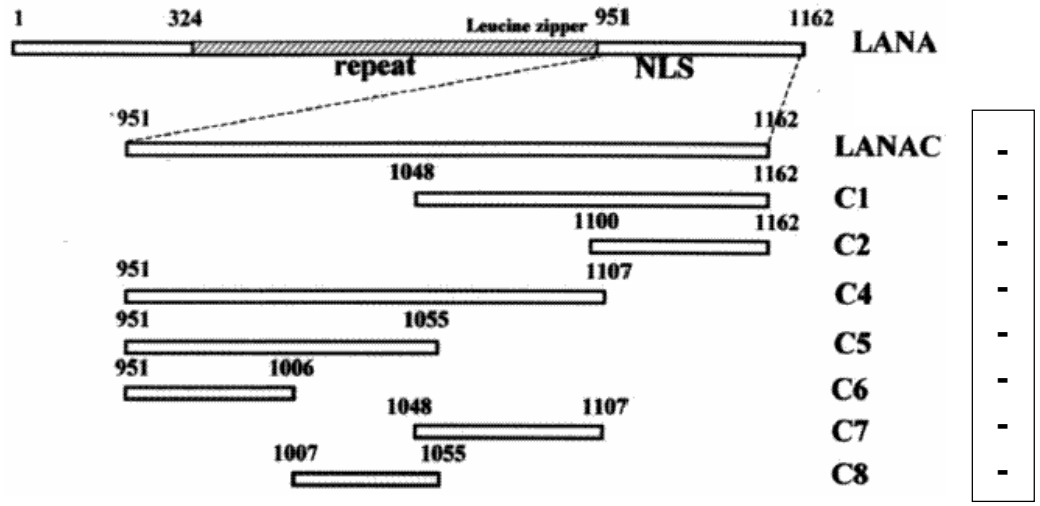
In order to assess which domain of LANA-1 interacts with ARC92/ACID-1, a set of different LANA-1 GST-fusion mutants was used. The carboxy-terminally tagged deletion mutants of LANA-1 used in the GST pull-down assay are illustrated in Fig. 22a. A schematic representation of the GST-tagged LANA-1 mutants pGL9, pGL12 and pGL13 is shown in Fig. 22b. The pGL9 construct expresses the N- plus C-terminus of LANA-1 protein without the middle domain. pGL12 expresses the C-terminal domain of LANA-1 and pGL13 the N-terminal domain of LANA-1.

The GST alone and GST-tagged proteins were expressed in BL21 bacteria, as described in Materials and Methods, and equivalent amounts were used in GST pull-down experiments. The GST-tagged proteins were immobilized with glutathione beads and incubated overnight with cell lysates of HEK 293 cells transfected with a plasmid expressing ARC92/ACID-1. After SDS-PAGE, bound proteins were assessed by Western blot analysis using an anti-ARC92/ACID-1 antibody (Fig. 22c, top panel).

The GST alone didn't precipitate ARC92/ACID-1. None of the C-terminal fragments of LANA-1 precipitated ARC92/ACID-1 (Fig. 22c, top panel). This GST pull-down experiment thus confirmed that the C-terminus of LANA-1 does not bind to the Mediator complex.

The Western blot analysis demonstrated that the LANA-1 mutants pGL9 and pGL13 still bound to ARC92/ACID-1, whereas the mutant pGL12 lacking the N-terminal domain did not, indicating that the N-terminal domain is responsible for the binding to the ARC92/ACID-1 subunit of the Mediator complex (Fig. 22c).

a



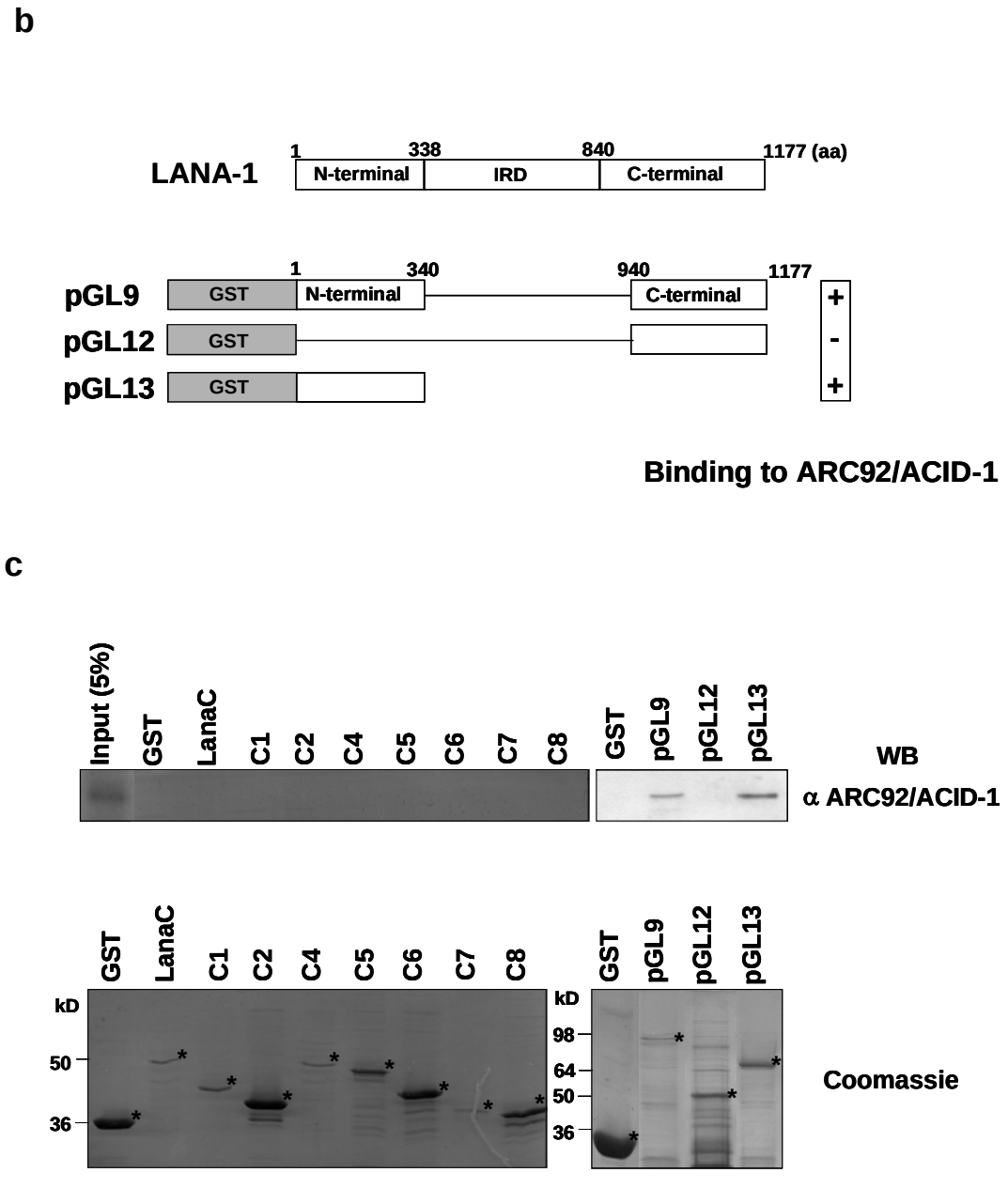


Fig. 22: The N-terminal domain of LANA-1 binds to the Mediator protein ARC92/ACID1. (a) Schematic diagram of the C-terminal LANA-1 fragments expressed as fusion proteins. (b) Schematic representation of the LANA-1 deletion constructs pGL9, pGL12 and pGL13. (c) The N-terminal LANA-1 domain interacts with ARC92/ACID1 in GST pull-down assays. None of the C-terminal fragments of LANA-1 precipitated ARC92/ACID-1 in GST pull-down assays. The GST-tagged proteins expressed in BL21 bacteria and bound to glutathione beads were incubated overnight with the cell lysates of HEK 293 cells transfected with ARC92/ACID1. The binding reaction was analysed by Western blot using an anti-ARC92/ACID1 antibody (top panel). The GST-LANA-1 fusion proteins used in the binding are shown on a Coomassie blue-stained SDS-polyacrylamide gel.

4. Discussion

4.1. ORFeome-based arrays in eukaryotic expression vectors - a new approach to screen for the function of viral genes

KSHV is the eighth and the most recently identified Human Herpesvirus. Currently 89 genes have been identified in the long unique region of the viral genome. So far, approximately 50% of the KSHV genes have no function assigned. An emerging challenge is to rapidly uncover the functions of previously uncharacterised viral genes and to discover viral proteins that alter cellular physiology. As an alternative strategy to DNA microarrays for transcriptional profiling and to yeast two-hybrid arrays for the determination of protein-protein interactions, a system for the functional screening of a large number of viral gene products in mammalian cells in parallel has been developed in this thesis.

In the present study, the generation of a KSHV array consisting of all ORFs of KSHV cloned into an eukaryotic expression vector by recombinatorial cloning was presented. The expression of KSHV proteins in mammalian cells allows their analysis in the natural host cell. The effect of viral proteins on the cellular physiology can be investigated by a variety of functional assays. The KSHV array cloned in an eukaryotic expression vector can be screened by simple detection methods for diverse cellular phenotypes to assign functions to previous uncharacterised viral proteins. For example, the KSHV ORFeome cloned into the eukaryotic expression vector can be used for systematic analyses of virus-host interaction, protein localization, protein phosphorylation or apoptosis induction. To identify viral gene products that induce cytoskeletal changes, the array could be examined for cells with abnormal morphology by immunofluorescence. In this study, the KSHV array was screened for the activation of signalling pathways by cotransfection of the individual viral ORFs with luciferase reporter vectors. It was shown by the screening for viral proteins activating signalling pathways that this strategy is a powerful approach and an efficient alternative.

4.2. LANA-1 was identified as an activator of SRE

There are several reports on KSHV proteins which modulate cell signalling pathways[9,22,104,108,117]. However, our study is the first in which all genes of a complete organism were systematically screened for a modulation of cell signalling pathways. Since SRE and AP-1 binding sites are important regulatory element of many cellular genes involved in cell growth, differentiation and development, the KSHV array was screened for KSHV proteins activating promoters via SRE and AP-1. Transient transfection experiments in HEK 293 cells identified LANA-1 as a strong activator of promoters containing SRE (Fig. 12a). The effect of LANA-1 on SRE was dose-dependent, supporting its specificity (Fig. 13a). LANA-1, the protein product of the open reading frame ORF73, is a latent viral protein expressed in all KSHV-infected cells investigated so far[79,80,121]. Previous studies indicated that LANA-1 shares functions with both EBV nuclear antigens EBNA-1 and EBNA-2[61]. LANA-1 is essential for the maintenance of viral episomes by binding to the two 17-bp nucleotide motifs LBS 1 and 2 present in the terminal repeat of the KSHV genome. It associates with histone H1 in BCBL-1 cells and tethers the viral episome to the host chromatin[36]. Moreover, LANA-1 is a multifunctional protein which interacts with the p53 and RB tumor suppressor proteins, blocks apoptosis and stimulates cellular transformation[49,120].

In the present study, it was shown that LANA-1 is able to activate a promoter containing a single wild-type SRE binding site derived from c-*fos* (Fig. 17). The expression of c-*fos* may affect the subsequent expression of other genes involved in the regulation of cell growth, differentiation, or transformation[4,60,83]. Recently, it was found that KSHV infection leads to an upregulation of the c-*fos* gene (Gao et al., *personal communication*). Moreover, mutational analysis of the c-*fos* promoter identified SRE as the dominant cis-acting element responding to KSHV. A number of experiments have suggested that an aberrant production of the c-*fos* protein can lead to oncogenesis. Thus, LANA-1 may be a carcinogenic cofactor by causing aberrant c-*fos* production. In KSHV infection, LANA-1-related SRE activation could contribute to the activation of cell division and to the stimulation of viral transcription.

In HEK 293 cells stably transfected with LANA-1, 33% of the upregulated genes have a SRE binding site in their promoters[2]. The following genes belong to this group:

- Transducer 1 of ERB-B2
- osteoclast stimulating factor 1
- TGFB inducible early growth response factor
- DEAD (Asp-Glu-Ala-Asp) box polypeptide 17

In BJAB B-cell line stably transfected with LANA-1 the picture is similar but not exactly the same: about 23% of the upregulated genes have a SRE binding motif in their promoters[2]. The following genes belong to this group:

- POU domain, class 2
- transcription factor 2
- nuclear factor (erythroid-derived 2)-like 3
- FK506 binding protein 5
- cell division cycle 25A
- DnaJ (Hsp40) homolog, subfamily A, member 2
- major histocompatibility complex, class II, DR beta 1
- B-cell lymphoma 6 protein
- ubiquitin-conjugating enzyme E2M (UBC12 homolog, yeast)
- mitogen-activated protein kinase-activated protein kinase 2
- neuroblastoma RAS viral (v-ras) oncogene homolog
- GM2 ganglioside activator protein
- cytochrome b-245, beta polypeptide
- ubiquitin C
- phosphoribosyl pyrophosphate synthetase 1
- elastase 2A
- solute carrier family 25

In cells infected with KSHV, similar observations can be obtained. KSHV induces a transcriptional reprogramming of blood vascular (BEC) to lymphatic endothelial cells (LEC), which is similar to the expression profile of KS tumors[68,160]. Eleven of 33 upregulated genes detected in these cells contain a SRE binding site in their promoter:

- GIPC2 (PDZ domain protein)

- UBD (ubiquitin D)
- FST (follistatin)
- TNFSF10 (tumor necrosis factor superfamily 10)
- SULF1 (sulfatase 1)
- GHR (growth hormone receptor)
- SEPP1 (selenoprotein P, plasma 1)
- IL6ST (interleukin 6 signal transducer)
- IL7
- CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1-biliary glycoprotein)
- CH25H (cholesterol 25-hydroxylase)

These data suggest that LANA-1-related SRE activation might be important in the regulation of KSHV infection and tumorigenesis.

4.3. LANA-1 is a promiscous but selective transcriptional modulator acting in a cell-type specific manner

In DG75 and Jurkat T cells the activation of SRE by LANA-1 was not enhanced (data not shown). Several possible mechanisms might explain this observation. It could be that LANA-1 targets promoters with SRE via cellular proteins, which are specific for the type of cell line used. It could also be that LANA-1 binds to an inhibitory factor in B- and T-cell lines which is not present in HEK 293 cells and HeLa cells. In fact, under our experimental conditions, a significant activation of SRE in B- and T-cell lines was not detected (data not shown).

Several studies showed that LANA-1 can both activate and repress transcription of cellular, as well as viral promoters. Moreover, in Cos-7 cells LANA-1 can activate transcription directed by a wide array of simple, synthetic promoters containing binding sites for the cellular proteins ATF, AP-1, CAAT, or Sp1, linked to a TATA box[123]. LANA-1 activates the HIV-1 LTR promoter in BJAB cells[71] but represses the same promoter in Cos-7 cells[123]. In both cases the modulation of transcription by LANA-1 is mediated via the NF-κB binding site in the HIV-1 LTR promoter. These reports suggest that the

interaction of LANA-1 with the cellular transcription apparatus is very likely to be cell-type specific.

In the present study, a set of promoters was compared for transcriptional modulation by LANA-1. LANA-1 was suppressive for the promoters containing AP-1 and NF-kB binding sites, and for the IL-6 promoter (Fig. 14). It has been reported that LANA-1 activates the cellular IL-6 promoter via the AP-1 binding site in HEK 293 cells[3]. In the present study, however a very strong activation of AP-1 by LANA-1 was not obtained (Fig. 13). The different outcome is probably caused by the fact that An and colleagues used a luciferase plasmid containing different AP-1 binding sites than in the reporter plasmid used in our study. The transcriptional repression of the promoter with NF-κB binding sites and the HIV-1 LTR promoter by LANA-1 observed in our experimental system corresponds to reported data on repression of these promoters by LANA-1 in Cos-7 cells[123] (Fig. 13).

In the present study, LANA-1 also activated a reporter plasmid containing the ISRE element, which is responsive to type 1 interferons[133]. This effect was seen in HEK 293 cells but not in DG75 or Jurkat T cells, which suggests a cell type specific behaviour of LANA-1. In a microarray screen of cellular cDNAs after KSHV infection, a robust increase in the expression of IFN-induced genes is observed in human adult dermal microvascular endothelial cells (HMVEC) and human foreskin fibroblast (HFF) cells, whereas there is a total lack of induction of these genes in BJAB B-cells [109]. This confirms that KSHV-induced host gene modulation is different in B-cells compared to endothelial and fibroblast cells[109].

Interferons (IFNs) are a unique class of cytokines that stimulate an antiviral and antitumor response by inducing the expression of a variety of cellular genes. A number of genes induced by IFNs participate in cell growth regulation and apoptosis[76]. The ISRE element is activated by a trimer termed ISGF-3 constituted of phosphorylated STAT-1, phosphorylated STAT-2, and p48/IRF-9[133]. Currently, we do not know the biological impact of the finding that LANA-1 activates promoters with ISRE, as it contrasts to the fact that KSHV encodes several viral interferon regulatory factors (vIRF) that block interferon signalling[19,20,93,127]. These data are preliminary and the relevance of the role of LANA-1 in the response to type 1 interferons is investigated in another study in our laboratory.

In conclusion, our data suggest a cell-type specific manner of transcriptional modulation by LANA-1, similar to the published literature.

4.4. The molecular mechanism of the LANA-1-induced SRE activation

The present study was focused on the evaluation of the molecular mechanism of activation of SRE by LANA-1. They are several reports in the literature on viral proteins modulating signalling pathways, which lead to an activation of promoters via the SRE element (see introduction). However, the mechanism of modulation differs in all of the cases described. Therefore, several strategies for investigation of the effect of LANA-1 on SRE were pursued.

4.4.1. The role of the MEK/ERK-1/2 pathway in the activation of SRE by LANA-1

It has been reported that the HCV core protein activates the MAPK/ERK-1/2 pathway and its downstream target, SRE, in BALB/3T3 cells [50]. Since MAPKs take part in one of the main cascades leading to an activation of promoters with SRE binding sites, the question was addressed whether LANA-1–induced activation of SRE is dependent on MAPK activity. To answer the question the phosphorylation of MEK1/2 kinases in HEK 293 transiently expressing LANA-1 was evaluated in the presence or absence of specific MEK1/2 inhibitors. Two different chemicals were used, PD98059 and U0126. PD98059 is known to block the phosphorylation of MEK 1 by upstream kinases and U016 inhibits both MEK1 and MEK2 phosphorylation. Since both of the inhibitors considerably decreased the activation of SRE by LANA-1 (Fig. 15b), an important role of MEK1/2 kinases in LANA-1-related SRE activation was suggested. By Western blot analyses it was demonstrated that LANA-1 enhanced the phosphorylation of MEK1/2 in HEK 293 cells (Fig. 15c, line 3). ERK1/2 are phosphorylated by activated MEK1/2 and ELK-1, which is one of the transcription factors binding to SRE, is a substrate of ERK1/2 kinases. In a p44/42 MAP kinase assay an increased phosphorylation of ELK-1 at Ser383 in LANA-1 transfected cells *in vitro* was observed, which was even stronger than the positive control ERK-2 (Fig. 16). These observations indicate that the MEK/ERK cascade might play an important role in the LANA-1-induced activation of SRE.

4.4.2. Does the activation of SRE by LANA-1 require the binding to specific transcription factors?

A luciferase assay with SRE reporter plasmids with mutated binding sites for SRF and TCF respectively revealed that LANA-1 could activate the SRE element via both SRF and TCF (Fig. 17). LANA-1 is located in the nuclei of KSHV-infected cells. Therefore, it is possible that LANA-1 activates the promoter containing SRE via direct binding to DNA or one of the two transcription factors SRF or TCF. An EMSA experiment demonstrated that LANA-1 does not bind directly to SRE (data not shown). Subsequently, it was evaluated whether LANA-1 interacts with specific transcription factors. Co-immunoprecipitation experiments with several transcription factors including ELK-1, SRF, STAT-1 and SpiB showed that LANA-1 interacts with SRF (Fig. 18). LANA-1 was immunoprecipitated with an antibody specific for SRF (Fig. 18a, line 4, upper panel). However, the reciprocal experiment did not work. This could be due to the stochiochemistry of the complex formed between the two interacting proteins (SRF and LANA-1), which prevents the binding of the antibody to the specific epitope. LANA-1 interacts with a variety of transcriptional regulators including the nucleic mitogen-activated kinase Ring3[99,116], ATF/CREB2[91] and CBP[90], and acts as a transcriptional activator or suppressor of cellular and viral genes.

An interaction between LANA-1 and STAT-1 similar to SRF was not detected, indicating either technical restraints or that LANA-1-mediated ISRE activation is caused by another mechanism, possibly as a secondary phenomenon. The interaction of LANA-1 with SRF, however, does not seem to be mandatory for the LANA-1 related SRE activation, since the c-*fos* promoter with the mutated SRF binding site could still be activated by LANA-1 (Fig. 17). On the other hand, in luciferase reporter gene experiments LANA-1 acted as an enhancer of RAS V12-induced transcriptional activation of the SRE promoter (Fig. 14). When LANA-1 was cotransfected together with RAS V12 the activation of the promoter was 2-fold stronger than the induction with RAS V12 alone. A similar effect was seen with the promoter ISRE promoter. A prestimulated ISRE promoter was further activated when LANA-1 was cotransfected into the cells. The presence of LANA-1 led to 4-fold stronger induction of the reporter vector compared to the stimulation with IFN α alone (Fig. 14). This observation and the studies published so far led us to the

hypothesis that LANA-1 could have a more global effect on cellular gene expression through a direct interaction with the basal transcription machinery.

4.4.3. LANA-1 meets the Mediator

The Mediator is a multi-subunit complex of approximately 2 megadaltons capable of responding to different activators[95,107,119]. It plays a key role in bridging DNA-bound activators of chromatin remodeling with the general transcriptional machinery, particularly RNA polymerase II[88]. Several other viral proteins are known to interact with the Mediator and thereby modulate transcription: adenovirus large E1A protein interacts with the Sur-2 subunit from the Mediator complex via its E1A-CR3 zinc finger[159], ORFA protein of Walley dermal sarcoma virus co-immunoprecipitates with Cdk8 and cyclin C which are part of the Mediator[129] and herpes simplex virus type 1 VP16 interacts with ARC92/ACID1 via its activation domain[102]. In this study, it was shown by luciferase assays and CoIP that LANA-1 interacts with the Mediator (Fig. 19 and 20). The interaction between LANA-1 and the Mediator complex was detected by CoIP using antibodies against several different Mediator subunits including ARC92/ACID1, PCQAP and Sur-2 (suppressor of ras) (Fig. 19).

Transcriptional activation by the herpesviral VP16 protein requires the Mediator subunit ARC92/ACID-1 (MED 25)[14,102]. Similar to LANA-1, VP-16 also participates in the activation of type I-interferon inducible promoters (Meisterernst et al. *unpublished*). Overexpression of the ARC92/ACID1 NTD domain strongly reduces the activation of pGLMRG5 (synthetic promoter containing five GAL4-binding sites upstream of a core promoter) by GAL4-VP-16[102]. This effect is a result of the direct binding of NTD to the Mediator and the loss of the endogenous ARC92/ACID-1 from the Mediator complex[102] (Fig. 20a). In this study, the overexpressed ARC92/ACID1 NTD domain inhibited the LANA-1-induced activation of promoters carrying SRE and ISRE, but not the activation of these promoters by their respective positive controls (Fig. 20b). This outcome indicates that it is very likely the viral transactivator LANA-1 to use the same Mediator subunit (ARC92/ACID-1) as the HSV-1 VP16 protein[102] (Fig. 20). The fact that NTD did not block the activation of SRE by RAS V12 confirmed the specificity of the transcriptional modulation by the overexpressed negative dominant domain of

ARC92/ACID-1 (Fig. 20b). The activation of endogenous immediate early genes in response to the RAS-RAF-MEK-ERK pathway occurs via another subunit of the Mediator complex, Sur-2[149].

LANA-1 has a complex domain structure (Fig. 23). The N-terminal domain is proline-rich and a binding site for SH3-domain proteins. The N-terminal domain of LANA-1 contains a nuclear localization sequence (NLS) and binds the methyl CpG-binding protein MeCP2[84,85]. The acidic internal repeat domain (IRD) is mandatory for the transcriptional activation of cellular and viral promoters[123]. The C-terminal domain contains a leucine zipper oligomerization sequence and a second NLS[61]. In order to map the domain of the LANA-1 protein, which interacts with the ARC92/ACID-1 subunit, GST pull-down experiments were carried out (Fig. 22). These experiments revealed that the N-terminal domain of LANA-1 interacts with the Mediator subunit ARC92/ACID1 (Fig. 22). Co-immunoprecipitations using C-terminal deletion mutants of LANA-1 confirmed that the C-terminus of LANA-1 is not mandatory for this interaction (Fig. 21).

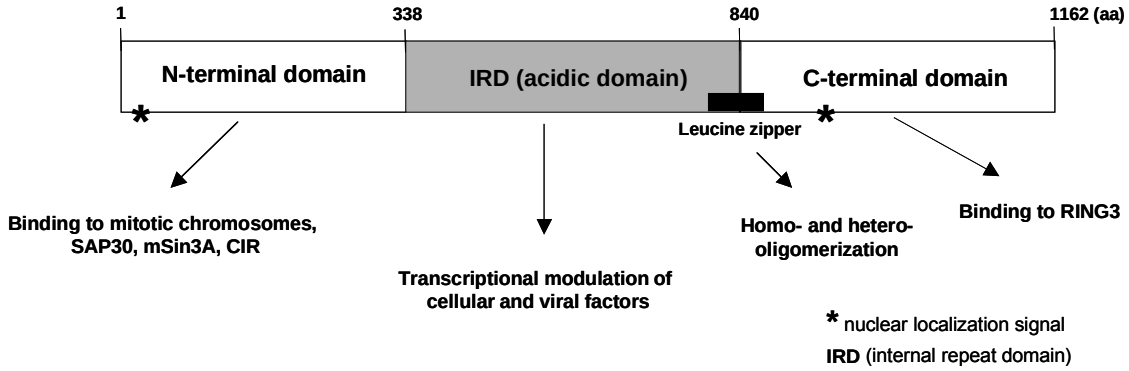


Fig. 23: The domain structure of LANA-1. The three main domains of the LANA-1 protein, namely N-terminal, central and C-terminal, are indicated.

In previous studies the corepressor protein SAP30 was identified as a LANA-1 binding protein in an Y2H screen[85]. SAP30 is a constituent of a large multicomponent complex that recruits histone deacetylases to the promoter. LANA-1 binds to two other members

of the corepressor complex, mSin3A and CIR, via its N-terminal domain and represses the expression from the latent EBV Qp and Cp promoters[85]. This indicates that LANA-1 can mediate a transcriptional repression through the recruitment of the mSin3 corepressor complex [85]. In our study, it was shown that LANA-1 activates transcription of promoters with SRE and ISRE binding sites by binding to the Mediator complex via its N-terminal domain. Based on these two observations, one could speculate that the transcriptional modulator LANA-1 uses the same N-terminal domain to bind either a corepressor complex or the Mediator. Our investigations together with published data suggest that LANA-1 can switch between repression and activation of a variety of promoters. Like many viral regulators LANA-1 interacts with cellular proteins to recruit the machinery needed to support viral propagation. Complexes with LANA-1 may have extended substrate specificity or changes in activity toward specific transcription factors, thereby increasing or decreasing transcription from a subset of promoters. *In vivo*, binding affinities are probably modulated differentially at promoters with SRE and ISRE binding sites by posttranscriptional modifications and by interactions with different cofactors and accessory proteins. Such mechanisms may have oncogenic potential through the enhancement or inhibition of proto-oncogene or tumor suppressor expression. It is anticipated that information derived from the function of LANA-1 will also help to delineate the role of the Mediator complex in the control of transcription.

4.4.4. Proposed model for action of the transcriptional modulator LANA-1

A schematic model for the molecular mechanism of LANA-1-mediated activation of promoters containing the SRE element is presented in Fig. 24. LANA-1 interacts with the Mediator most likely via a direct binding to the ARC92/ACID-1 protein. At promoters with an SRE element, LANA-1 additionally binds to SRF.

There are still open questions, for example which domain of the Mediator subunit ARC92/ACID-1 binds to the N-terminal domain of LANA-1 and which part of LANA-1 interacts with SRF. The answers of these questions will shed light on how this complex of viral and cellular proteins assemble on SRE promoters and drive the transcription.

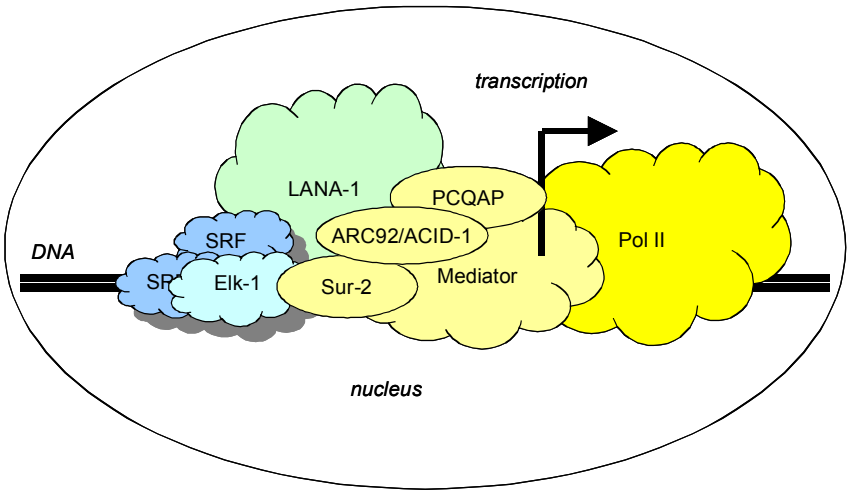


Fig. 24: Proposed model for action of LANA-1.
The N-terminal domain of LANA-1 interacts with ARC-92/ACID-1 of the Mediator complex. The Mediator transfers the activation signal to the polymerase II. At promoters with an SRE element, LANA-1 additionally binds to the specific transcription factor SRF.

The activation of the MAPK/ERK pathway and the interaction of LANA-1 with the Mediator complex presented in this study can be independent phenomena. Alternatively, both effects could be cooperatively linked. The RAF/MEK1/2 pathway leads to an interaction between ELK-1 and the Mediator subunit Sur-2 that transfers the activation signal to the basal transcription machinery[149]. The results presented in this study indicate that LANA-1 leads to a phophorylation of ELK-1 (Fig. 16), binds to SRF (Fig. 18a), and coprecipitates with the Sur-2 protein of the Mediator complex (Fig. 19c). This suggests that the two phenomena are likely to be connected to each other.

It is possible that other proteins of cellular or viral origin contribute to the high complexity of this effect and modify it in a specific way in different cell types. Our study demonstrated that LANA-1 associates and cooperates with the Mediator and thus influences the basal transcription machinery on promoters with SRE and ISRE binding sites. In cells infected with KSHV other viral proteins might modulate the effect of LANA-

1. The RTA protein of KSHV, which acts as a molecular switch for lytic reactivation, activates the expression of viral promoters by recruiting the Mediator complex[64]. It may also be that RTA and LANA-1 synergistically interact to regulate the virus infection.

Currently, neither the stochiochemistry of the interaction of LANA-1 with the Mediator complex nor if additional cellular factors are involved is known. Further studies are necessary to define the specific order of transcription factor assembly on the promoters with SRE and ISRE binding sites and to clarify the role and function of LANA-1 in this process. These analyses, however, will not only provide insight into a crucial step of KS oncogenesis, but also shed light on the way how transcriptional processes work in non-transformed cells. An increased understanding of signalling pathways induced by KSHV proteins may eventually lead to the development of novel therapies to control KS lesions.

5. Materials and Methods

5.1. Materials

5.1.1. Equipment

Bacterial Shaker	Kühner, Bürsfelden, Switzerland
Balances	Sartorius, Göttingen, Germany
Centrifuge GP	Beckman, Palo Alto, USA
Centrifuge J2-21	Beckman, Palo Alto, USA
Centrifuge Varifuge 3.0R	Heraeus, Hanau, Germany
Centrifuge Minifuge RF	Heraeus, Hanau, Germany
Centrifuge Labofuge T	Heraeus, Hanau, Germany
Centrifuge, refrigerated and non-refrigerated	Heraeus, Hanau, Germany
Confocal laser scanning microscope	Leica, Bensheim, Germany
Confocal laser scanner	Leica, Bensheim, Germany
Eagle eye	Stratagene, The Netherlands
Elisa Reader	Tecan Labinstruments, Germany
Film developing machine	Optimax Typ TR MS Laborgeräte, Germany
Fluorescence/light microscope Axiovert 35	Zeiss, Oberkochen, Germany
Fluorescence/light microscope Axiovert 200M	Zeiss, Oberkochen, Germany
Fridge (4°C)	Liebherr, Ochsenhausen, Germany
Freezer (-20°C)	Liebherr, Ochsenhausen, Germany
Freezer (-80°C)	Forma Scientific, Inc., Marietta, USA
Cryo 1°C Freezing Container	Nalgene Nunc, Wiesbaden, Germany
Gel dryer	Bio-Rad, Munich, Germany
GelAir drying system	Bio-Rad, Munich, Germany

Incubators for cell culture (37°C)	Forma Scientific, Inc., Marietta, USA
Inverted microscope TMS	Nikon, Düsseldorf, Germany
Laminar Flow Hood Steril Gard II A/B3	The Baker Company, USA
Magnetic stirrer with heating block	Janke & Kunkel, Staufen, Germany
Microwave	AEG, Berlin, Germany
Overhead mixer	Heidolph, Schwabach, Germany
PCR Thermal Cycler GeneAmp 2400	Perkin Elmer, Weiterstadt, Germany
pH-Meter	WTW, Weilheim, Germany
Photometer Gene Quant II	Pharmacia/LKB, Freiburg, Germany
Pipettes	Gilson, Villies Le Bel, France
	Eppendorf, Hamburg, Germany
Pipetting aid	Technomara, Zürich, Switzerland
Electrophoresis Power supply EPS200	Amersham-Pharmacia, Germany
Sonifier 450	Branson Ultrasonics Corp., Danbury, USA
Thermomixer	Eppendorf, Hamburg, Germany
UV-transilluminator (366 nm)	Vetter, Wiesloch, Germany
(254 nm)	Konrad Benda, Wiesloch, Germany
Vortex mixer	IKA Works, Inc, Wirmington, USA
Water bath	Julabo, Seelbach, Germany
	GFL, Burgwedel, Germany

5.1.2. Reagents

5.1.2.1. Chemicals

Acetic Acid	Roth, Karlsruhe, Germany
Acrylamide/Bisacrylamide 37,5/1 (Rotiphorese Gel 30)	Roth, Karlsruhe, Germany
Agar for plates	BD Biosciences Clontech, Heidelberg, Germany
Agarose electrophoresis grade	Invitrogen, Karlsruhe, Germany
Ammonium persulfate (APS)	Sigma, Munich, Germany

Ampicillin	Roche Diagnostics, Germany
Bacto peptone	BD Biosciences Clontech, Germany
Bacto tryptone	BD Biosciences Clontech, Germany
Bacto yeast extract	BD Biosciences Clontech, Germany
Bromophenol blue	Serva, Heidelberg, Germany
Bovine serum albumin (BSA)	Sigma, Munich, Germany
Calcium chloride	Merck, Darmstadt, Germany
Chloramphenicole	Sigma, Munich, Germany
Coomassie brilliant blue R-250	Bio-Rad, Munich, Germany
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
Disodiumhydrogenphosphate	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
dNTPs	Roche Diagnostics,Germany
Dulbecco's modified Eagle's medium (DMEM)	Gibco BRL, Karlsruhe, Germany
Ethanol (EtOH)	Riedel-de Haën, Seelze, Germany
Ethidium bromide	Sigma, Munich, Germany
Ethylenediamintetraacetate disodium salt (EDTA)	Roth, Karlsruhe, Germany
Ethylene glycol	Sigma, Munich, Germany
Fetal calf serum (FCS)	Gibco BRL, Karlsruhe, Germany
Glucose	Merck, Darmstadt, Germany
Glutathione-Sepharose 4B	Amersham-Pharmacia, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Serva, Heidelberg, Germany
Histogel	Linaris, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Interferon (IFN) α	PBL Biomedical Laboratories, USA
Ionomycin	Sigma, Munich, Germany
Isopropanol	Riedel-de Haën, Seelze, Germany
Isopropylthio-b-D-galactosid (IPTG)	Roth, Karlsruhe, Germany
Kanamycin	Serva, Heidelberg, Germany

L-glutamine	Gibco BRL, Karlsruhe, Germany
L-Glutathione (reduced)	Sigma, Munich, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Magnesium sulfate	Merck, Darmstadt, Germany
2-mercaptoethanol	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
N-butyrate	Sigma, Munich, Germany
Nonidet P40 (NP-40)	Fluka, Seelze, Germany
Pefabloc	Roche Diagnostics,Germany
Polyethylene glycol (PEG 1000)	Sigma, Munich, Germany
Penicillin-Streptomycin	Gibco BRL, Karlsruhe, Germany
Phenylmethylsulfonfluoride (PMSF)	Roche Diagnostics, Germany
Phosphate buffered saline (PBS) Dulbecco's	Gibco BRL,Germany
Ponceau S	Sigma, Munich, Germany
Potassium acetate	Riedel-de Haën, Seelze, Germany
Potassium chloride	Merck, Darmstadt, Germany
Protein G-Sepharose Fast Flow	Amersham-Pharmacia, Germany
Rosswell Park Memorial Institute (RPMI)1640	Gibco BRL, Karlsruhe, Germany
SD Base medium	BD Biosciences Clontech, Germany
Skim milk powder	Merck, Darmstadt, Germany
Sodium acetate	Riedel-de Haën, Seelze, Germany
Sodium azide	Serva, Heidelberg, Germany
Sodium chloride	Riedel-de Haën, Seelze, Germany
Sodium dodecylsulfate (SDS)	Merck, Darmstadt, Germany
Sodium hydroxid	J.T.Baker B.V., Deventer, Holland
Sorbitol	Sigma, Munich, Germany
Tetramethylethylenediamin (TEMED)	Amersham-Pharmacia, Germany
12-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma, Munich, Germany
Tris(hydroxymethyl)aminomethan	Roth, Karlsruhe, Germany

(Tris)	
Triton X-100	Serva, Heidelberg, Germany
Trypsin	Gibco BRL, Karlsruhe, Germany
Tween 20	Merck, Darmstadt, Germany
Western Blue® Stabilized Substrate for alkaline phosphatase	Promega, Mannheim, Germany

5.1.3. Additional materials

Autoradiography films BIOMAX-MR	Eastman-Kodak, USA
Cell culture plastic ware	Greiner, Nürtingen, Germany
	Nunc, Wiesbaden, Germany
	Falcon/Becton Dickinson, Germany
Filter paper (3 mm)	Whatman Ltd., Maidstone, England
Glass slides for IF	Marienfeld, Germany
Protran nitrocellulose transfer membranes	Schleicher & Schuell, Germany
Sterile filter units	Millipore

5.1.4. Enzymes

AmpliTag Gold® DNA polymerase	Applied Biosystems, USA
Calf intestinal alkaline phosphatase (CIP)	New England Biolabs
Expand High Fidelity PCR system	Roche
Klenow fragment	Roche
Pfu DNA polymerase	Stratagene
Restriction enzymes	New England Biolabs, Roche
RNase A	Roche
T4 DNA ligase	New England Biolabs
T4 DNA polymerase	New England Biolabs
T4 polynucleotide	kinase New England Biolabs
Taq polymerase	Roche
Vent Polymerase	New England Biolabs

5.1.5. General buffers

HeBS (HEPES-buffered saline) solution, 2x: 16.4 g NaCl, 11.9 g HEPES, 0.21 g Na_2HPO_4 in 1 L dH_2O. Titrate to pH 7.05 with 5 N NaOH. Filter sterilize through a 0.45-µm nitrocellulose filter (Millipore). Store in aliquots at –20°C.

TBE (Tris/borate/EDTA) electrophoresis buffer (10x): 890 mM Tris base, 890 mM boric acid, 20 mM EDTA.

SDS electrophoresis (Tris/glycine) buffer, 5x: 15.1 g Tris base, 72.0 g glycine, 5.0 g SDS in 1 L dH_2O.

SOC: 2.0 g Bacto-Tryptone, 0.5 g Bacto-Yeast extract, 1 ml 1 M NaCl, 0.25 ml 1.M KCl, 1 ml 2 M Mg stock (1 M $MgCl_2$ – 6 H_2O, 1 M $MgSO_4$ – 7 H_2O), 1 ml 2 M Glucose, up to 100 ml ddH_2O. Added Bacto-Tryptone, Bacto-Yeast extract, NaCl and KCl to 97 ml dH_2O. Stirred to dissolve. Autoclaved and cooled to RT. Added 2 M Mg stock and 2 M Glucose, each to a final concentration 20 mM. Filtered the complete medium through a 0.2 µm filter. The pH should be 7.0.

LB medium was prepared according to Sambrook and Russel: 10 g Bacto tryptone, 5 g Bacto yeast extract and 5 g NaCl for 1 L medium. For agar plates 15 g agar was added.

Adjusted the pH to 7.0 with NaOH (~200 ul 5 M NaOH). Autoclaved and allowed to cool to 50°C before adding antibiotics. Autoclaved media was stored at room temperature if top is tightened and then had been used by microwaving at 70 % for ~15 min. For liquid media leave out the agar.

5.1.6. Viruses

Recombinant vaccinia virus T7 (vT7) expressing T7 polymerase was provided by the NIH reagent program (Fuerst et al., 1986).

5.1.7. Bacterial strains

BL21 RIL	kindly provided by Dr. K.-P. Hopfner, München, Germany
DH10B	obtained from Invitrogen, Germany
DH5α	obtained from Gibco BRL, Karlsruhe, Germany

5.1.8. Cell lines

BCBL-1	KSHV positive body-cavity-based lymphoma cell line
BJAB	EBV negative human B lymphoblastoid cell line
DG75	EBV positive human Burkitt lymphoma cell line
HEK 293	human embryonic kidney cells
HeLa	human epithelial cell line originating from a cervical carcinoma
Jurkat	human T lymphoblastoid cell line

5.1.9. Oligonucleotides

5.1.9.1. Oligonucleotides used for generation of the KSHV PCR products cloned into pDONR207 by BP reaction (see 5.2.2.7 and 5.2.2.8).

attB1 internal for	AAAAAGCAGGCTCCGCC**ATG**XXXXXXXXXXXXXXXXXX
attB1 internal rev	AGAAAGCTGGGT**CTA**XXXXXXXXXXXXXXXXXXX
attB1 external for	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2 external rev	GGGGACCACTTTGTACAAGAAAGCTGGGT

yellow box: attB1 sequence
red letters: Kozak sequence
bold letters: ATG (for) and stop (rev) codon
XXXXX: homologous for and rev sequence

Internal primers:

Orf4 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GCCTTTTTAAGACAAAC
Orf4 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAACGA AAGAACAGATAG
Orf6 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GCGCTAAAGGGACCAC
Orf6 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACAAAT CCAGGTCAGAG
Orf7 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG

	GCAAAGGAACTGGCG
Orf7 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGACC TGGGAGTCATTG
Orf8 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCCGC CGCACCAATACCATAGC
Orf8 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCACTCC CCCGTTTCCGG
Orf9 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GATTTTTTCAATCCATTTATC
Orf9 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGGGC GTGGGAAAAGTC
Orf10 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG CAGACAGAGGCAACG
Orf10 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCACGATT GCATGGGTTC
Orf11 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GCGCAGGAGTCAGAG
Orf11 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAACTG CGTCCGGTGGC
K2 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG TGCTGGTTCAAGTTG
K2 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACTTAT CGTGGACGTC
Orf02 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GATCCTACACTTTAC
Orf02 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACGAA GTCTCACTGAAG
K3 for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCATG GAAGATGAGGATGTTC
K3 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAATGAA ACATAAGGGC
orf6 for	AAAAAGCAGGCTCCATGGCGCTAAAGGGACCAC
orf6 rev	AGAAAGCTGGGTCTACAAATCCAGGTCAGAG
orf7 for	AAAAAGCAGGCTCCGCCATGGCAAAGGAACTGGCGGC G
orf7 rev	AGAAAGCTGGGTCTAGACCTGGGAGTCATTGTGG
Orf70 for	AAAAAGCAGGCTCCGCCATGTTTCCGTTTGTACCTTTA AG
Orf70 rev	AGAAAGCTGGGTCTATACTGCCATTTCCATACG
K4 for	AAAAAGCAGGCTCCGCCATGGACACCAAGGGCATC
K4 rev	AGAAAGCTGGGTTCAGCGAGCAGTGACTGG
K4.1 for	AAAAAGCAGGCTCCGCCATGTGGAGCATGTGCTGGG
K4.1 rev	AGAAAGCTGGGTCTAGGGGCATAACCCTTTAC
k4.2 for	AAAAAGCAGGCTCCGCCATGCAAATTAGCTTTGCCGAA G
K4.2 rev	AGAAAGCTGGGTTTATTGAAGCCCAGGCGACC
K5 for	AAAAAGCAGGCTCCGCCATGGCGTCTAAGGACGTAG
K5 rev	AGAAAGCTGGGTTCAACCGTTGTTTTTTGGATG

K6 for	AAAAAGCAGGCTCCGCCATGGCCCCCGTCCACGTT
K6 rev	AGAAAGCTGGGTCTAAGCTATGGCAGGCAGC
K7 for	AAAAAGCAGGCTCCGCCATGGGAACACTGGAGATAAA AG
K7 rev	AGAAAGCTGGGTCTACAACTGGCCTGGAGATTG
Orf 16 for	AAAAAGCAGGCTCCGCCATGGACGAGGACGTTTTGC
Orf 16 rev	AGAAAGCTGGGTTTATCTCCTGCTCATCGCG
Orf 17 for	AAAAAGCAGGCTCCGCCATGAGCCTCCTAAGCCCC
Orf 17 rev	AGAAAGCTGGGTCTACTGCTTGTTCAGGAGC
Orf 18 for	AAAAAGCAGGCTCCGCCATGCTCGGAAAATACGTGTG
Orf 18 rev	AGAAAGCTGGGTTTAAACCGCGTTGTTGTTAAAC
Orf 67 for	AAAAAGCAGGCTCCGCCATGAGTGTCGTTGGTAAGCG
Orf 67 rev	AGAAAGCTGGGTTTATGACCTTCTCACCAGCGC
Orf 19 for	AAAAAGCAGGCTCCGCCATGCTGACATCAGAAAGGTC
Orf 19 rev	AGAAAGCTGGGTTTAAACGACCGCGAGGACC
Orf 20 for	AAAAAGCAGGCTCCGCCATGTACGAGGTTTTTACAGAC
Orf 20 rev	AGAAAGCTGGGTTCATGGACCTGAACAAGCC
Orf 21 for	AAAAAGCAGGCTCCGCCATGGCAGAAGGCGGTTTTG
Orf 21 rev	AGAAAGCTGGGTCTAGACCCTGCATGTCTC
Orf 22 rev cyt	AGAAAGCTGGGTCTAATAAAGGATGGAAAACAG
Orf 23 for	AAAAAGCAGGCTCCGCCATGTTACGAGTTCCGGACG
Orf 23 rev	AGAAAGCTGGGTTTAGACGGTCAATAAAGCG
Orf 24 for	AAAAAGCAGGCTCCGCCATGGCAGCGCTCGAGGG
Orf 24 rev	AGAAAGCTGGGTTTAGACCAGCGGACGGAC
Orf 25 for	AAAAAGCAGGCTCCGCCATGGAGGCGACCTTGGAG
Orf 25 rev	AGAAAGCTGGGTCTAATACACCACCTTGTTTC
Orf 26 for	AAAAAGCAGGCTCCGCCATGGCACTCGACAAGAGTATA G
Orf 26 rev	AGAAAGCTGGGTTTAGCGTGGGGAATACCAAC
Orf 27 for	AAAAAGCAGGCTCCGCCATGGCGTCATCTGATATTC
Orf 27 rev	AGAAAGCTGGGTTTATTTAAAATTTAGAATCAAGGGAG G
Orf 28 for cyt	AAAAAGCAGGCTCCGCCATTCGCGTGTTCCTGGCGGC
Orf 28 rev cyt	AGAAAGCTGGGTCTAATCTGGCATGTATATTG
Orf 29b for	AAAAAGCAGGCTCCGCCATGCTTCAGAAAGACGCCAA G
Orf 29b rev	AGAAAGCTGGGTTTATTGTGGGGATATGGGC
Orf 30 for	AAAAAGCAGGCTCCGCCATGGGTGAGCCAGTGGATC
Orf 30 rev	AGAAAGCTGGGTTCATTTCGCACCGGTGTC
Orf 31 for	AAAAAGCAGGCTCCGCCATGTCACAAAACAGAAAGACT C
Orf 31 rev	AGAAAGCTGGGTCTACGTATCTTTCGTTGATAG
Orf 32 for	AAAAAGCAGGCTCCGCCATGGATGCGCATGCTATCAAC
Orf 32 rev	AGAAAGCTGGGTCTAGCCATAGCGGCCTCG
Orf 33 for	AAAAAGCAGGCTCCGCCATGGCTAGCCGGAGGCGC
Orf 33 rev	AGAAAGCTGGGTTCAGACATTCGTAAGAGGACC
Orf 29a for	AAAAAGCAGGCTCCGCCATGCTGCTCAGCCGTCAC
Orf 29a rev	AGAAAGCTGGGTTTAAGGCCCTGGGCTTACG

Orf 34 for	AAAAAGCAGGCTCCGCCATGTTTGCTTTGAGCTCGTG
Orf 34 rev	AGAAAGCTGGGTTTAGAGTTGGTTGAGTCCATTC
Orf 35 for	AAAAAGCAGGCTCCGCCATGGACTCAACCAACTCTAAA AG
Orf 35 rev	AGAAAGCTGGGTTTAGGGAGTTTCAGGGCAC
Orf 36 for	AAAAAGCAGGCTCCGCCATGCGCTGGAAGAGAATGG
Orf 36 rev	AGAAAGCTGGGTTCAGAAAACAAGTCCGCGG
Orf 37 for	AAAAAGCAGGCTCCGCCATGGAGGCCACCCCCAC
Orf 37 rev	AGAAAGCTGGGTCTACGGGCTGTGAGGGACG
Orf 38 for	AAAAAGCAGGCTCCGCCATGGGATTTCTCCTATCTATC
Orf 38 rev	AGAAAGCTGGGTTTAATAAATTGCTTCTTTATTTTTTTTC
Orf 39 for cyt	AAAAAGCAGGCTCCGCCCGCGTTATAAGGAGCGACTG
Orf 39 rev cyt	AGAAAGCTGGGTTCTAAATGAATATCATTTGCGTTTC
Orf 40 for	AAAAAGCAGGCTCCGCCATGGCAACGAGCGAAGAAAC
Orf 40 rev	AGAAAGCTGGGTCAAGCAGGGACAGTAGGTC
Orf 41 for	AAAAAGCAGGCTCCGCCATGGCCGGGTTTACTCTG
Orf 41 rev	AGAAAGCTGGGTTCAAAATAAAGATAAAAGCCTGG
Orf 42 for	AAAAAGCAGGCTCCGCCATGTCCCTGGAAAGGGCC
Orf 42 rev	AGAAAGCTGGGTTTATTTTGAAAAAAGGGAAACAATG
Orf 43 for	AAAAAGCAGGCTCCGCCATGTTGAGGATGAACCCGG
Orf 43 rev	AGAAAGCTGGGTCTATGCACTTCCAGGACAAG
Orf 44 for	AAAAAGCAGGCTCCGCCATGGACAGCTCGGAAGGG
Orf 44 rev	AGAAAGCTGGGTTCAGTAGATCAGAGTAGTC
Orf 45 for	AAAAAGCAGGCTCCGCCATGGCGATGTTTGTGAGGAC
Orf 45 rev	AGAAAGCTGGGTTCAGTCCAGCCACGGCCAG
Orf 46 for	AAAAAGCAGGCTCCGCCATGGACGCATGGTTGCAAC
Orf 46 rev	AGAAAGCTGGGTTTACTGCTCCAACAGGCCC
Orf 47 for gL	AAAAAGCAGGCTCCGCCCATGGGGATCTTTGCGCTATT TG
Orf 47 rev gL	AGAAAGCTGGGTTTATTTTCCCTTTTGACCTGCG
Orf 48 for cyt	AAAAAGCAGGCTCCGCCATGGAGGTGTGTATCCCAATT C
Orf 48 rev cyt	AGAAAGCTGGGTCCGCCGGAACTCCACATC
Orf 48 for ext	AAAAAGCAGGCTCCGCCATTACCTCGGACGTGAGAC
Orf 48 rev ext	AGAAAGCTGGGTTCAATCATACTCATCGTCGG
Orf 49 for	AAAAAGCAGGCTCCGCCATGACATCGAGAAGGCCCC
Orf 49 rev	AGAAAGCTGGGTTTATTGTATACTGAACAATGCG
Orf 50 for	AAAAAGCAGGCTCCGCCATGAAAGAATGTTCCAAGCTT G
Orf 50 rev	AGAAAGCTGGGTTCAGTCTCGGAAGTAATTACG
K8 for	AAAAAGCAGGCTCCGCCATGCCCAGAATGAAGGAC
K8 rev	AGAAAGCTGGGTCTATACCTGCTGCAGCTG
Orf 52 for	AAAAAGCAGGCTCCGCCATGGCCGCGCCCAGGGG
Orf 52 rev	AGAAAGCTGGGTTCAGTCATCAACCCCCGC
Orf 53 for	AAAAAGCAGGCTCCGCCATGACAGCGTCCACGGTGG
Orf 53 rev	AGAAAGCTGGGTCTATGCATGGACCACCTCG
Orf 54 for	AAAAAGCAGGCTCCGCCATGAACAACCGCCGAGGC
Orf 54 rev	AGAAAGCTGGGTCTAAAACCCAGACGACCCC

Orf 55 for	AAAAAGCAGGCTCCGCCATGTCGTCTCCATGGTACAC
Orf 55 rev	AGAAAGCTGGGTCTATGTCGAACCTATCGCG
Orf 56 for	AAAAAGCAGGCTCCGCCATGGAGACGACATACCGCC
Orf 56 rev	AGAAAGCTGGGTTTAACTGGCCAGTCCCACTG
Orf 57 for	AAAAAGCAGGCTCCGCCATGATAATTGACGGTGAGAG CC
Orf 57 rev	AGAAAGCTGGGTTTAAGAAAGTGGATAAAAGAATAAAC
K9 for	AAAAAGCAGGCTCCGCCATGGACCCAGGCCAAAGAC
K9 rev	AGAAAGCTGGGTTTATTGCATGGCATCCCATAAC
K10 for	AAAAAGCAGGCTCCGCCATGGGGTCCTCTGGGACG
K10 rev	AGAAAGCTGGGTTCAATGTAGACTATCCCAAATG
K10.5 for	AAAAAGCAGGCTCCGCCATGTACCACGTGGGACAGG
K10.5 rev	AAAAAGCAGGCTTTAGTCATCACATGTAACTGAAC
K11 for	AAAAAGCAGGCTCCGCCATGCACAGTTTGTTTTTTGAA G
K11 rev	AGAAAGCTGGGTTTAGTCTCTGTGGTAAAATGG
Orf 58 for	AAAAAGCAGGCTCCGCCATGTGCCGCCTGGACAGTG
Orf 58 rev	AGAAAGCTGGGTTTAGCCAACAACTTTATTTATTAC
Orf 59 for	AAAAAGCAGGCTCCGCCATGCCTGTGGATTTTCACTAT G
Orf 59 rev	AGAAAGCTGGGTTCAAATCAGGGGGTTAAATG
Orf 60 for	AAAAAGCAGGCTCCGCCATGGATTCAGTTGATCGATTT C
Orf 60 rev	AGAAAGCTGGGTTCACAAATCGTCAGTCACAC
Orf 61 for	AAAAAGCAGGCTCCGCCATGTCTGTCCGGACATTTTG
Orf 61 rev	AGAAAGCTGGGTCTACTGACAGACCAGGCAC
Orf 62 for	AAAAAGCAGGCTCCGCCATGAAGGTGCAGGCTGAAAA TG
Orf 62 rev	AGAAAGCTGGGTTTACAGAAACACAGTCCAGG
Orf 63 for	AAAAAGCAGGCTCCGCCATGGACGGCACAGACGCTC
Orf 63 rev	AGAAAGCTGGGTCTATTCGACAAACAGTTTCCG
Orf 64 for	AAAAAGCAGGCTCCGCCATGGCAGCCCAGCCTCTG
Orf 64 rev	AGAAAGCTGGGTTCACAAGTACCACTTCTTTATTC
Orf 65 for	AAAAAGCAGGCTCCGCCATGTCCAACTTTAAGGTGAGA G
Orf 65 rev	AGAAAGCTGGGTCTATTTCTTTTTGCCAGAGGG
Orf 66 for	AAAAAGCAGGCTCCGCCATGGCCCTGGATCAGCGC
Orf 66 rev	AGAAAGCTGGGTTCAGGAGGAACACTTCCCGC
ORF 67.5 for	AAAAAGCAGGCTCCGCCATGGAGTACGCGTCTGACCA G
ORF 67.5 rev	AGAAAGCTGGGTTCAGGGCCGTGCCCGCGC
Orf 68 for ext	AAAAAGCAGGCTCCGCCATGTCACGAGGCAGAAGC
Orf 68 rev ext	AGAAAGCTGGGTTTAGGCATAGTCCTCGAAGAAAC
Orf 68 for cyt	AAAAAGCAGGCTCCGCCGCAGACCTGTGCGAGATC
Orf 68 rev cyt	AGAAAGCTGGGTTCAAGCGTACAAGTGTGACGTC
Orf 69 for	AAAAAGCAGGCTCCGCCATGGAGACCCCGATATGCAC
Orf 69 rev	AGAAAGCTGGGTTTATAGGGCGTTGACAAGTGC
K13/Orf 71 for	AAAAAGCAGGCTCCGCCATGGCCACTTACGAGGTTC

K13/Orf 71 rev	AGAAAGCTGGGTCTATGGTGTATGGCGATAG
K12 for cyt	AAAAAGCAGGCTCCGCCAGTGGCCAGCGTGGCCCC
K12 rev cyt	AGAAAGCTGGGTTCAGTGCGCGCCCGTTGC
K12 for ext	AAAAAGCAGGCTCCGCCATGGATAGAGGCTTAACGGTG
K12 rev ext	AGAAAGCTGGGTCCTCCAGCGCCACCCATTTAG
Orf 72 for	AAAAAGCAGGCTCCGCCATGGCAACTGCCAATAACCC
Orf 72 rev	AGAAAGCTGGGTTTAATAGCTGTCCAGAATGCG
Orf 73 for	AAAAAGCAGGCTCCGCCATGGCGCCCCCGGGAATG
Orf 73 rev	AGAAAGCTGGGTTTATGTCATTTCCTGTGGAGAG
K14 for	AAAAAGCAGGCTCCGCCATGATACACACATTTTTTGATTG
K14 rev	AGAAAGCTGGGTTCACTGGGTGGATAGGGG
K14.1 for	AAAAAGCAGGCTCCGCCATGCCTAGAGGGCGCACC
K14.1 rev	AGAAAGCTGGGTCTAGGACCACAGCATTTTTC
Orf 74 for	AAAAAGCAGGCTCCGCCTCCCTCTTTAGGCAGAGGATG
Orf 74 rev	AGAAAGCTGGGTCTACGTGGTGGCGCCGGAC
Orf 75 for	AAAAAGCAGGCTCCGCCATGGCCTACGACGTCACTG
Orf 75 rev	AGAAAGCTGGGTTTAGTGGTGGTCGTTGATC
Orf 9 for	AAAAAGCAGGCTCCGCCATGGATTTTTTCAATCCATTTATC
Orf 9 rev	AGAAAGCTGGGTCTAGGGCGTGGGAAAAGTC
K15 for cyt	AAAAAGCAGGCTCCGCCAAGGAAAAAAAAAGTGGTGGC
K15 rev cyt	AGAAAGCTGGGTCTAGTTCCTGGGAAATAAAACC
Orf 22 rev ext	AGAAAGCTGGGTTTAACTGGCTGCGCGTCTTC
Orf 22 for cyt	AAAAAGCAGGCTCCGCCTACAGACTGTTTTCCATC
Orf 28 for ext	AAAAAGCAGGCTCCGCCATGAGCATGACTTCCCCGTCTCC
Orf 28 rev ext	AGAAAGCTGGGTTTAGGGAGGCTTGGTGGCCATTC
Orf8 for ext	AAAAAGCAGGCTCCGCCATGACTCCCAGGTCTAGATTG
Orf8 rev ext	AGAAAGCTGGGTTTAGCCACCTAGGGGGTGTTT
Orf 22 for ext	AAAAAGCAGGCTCCGCCATGCAGGGTCTAGCCTTC
K 8.1 for	AAAAAGCAGGCTCCGCCCAGGACCACGCGAATTCG
K 8.1 rev	AGAAAGCTGGGTCTATTTCTGCCGTTTTCTG
Orf 64 I rev	CAGGGCGTCCAGGGCATC
Orf 64 II for	ATTAGGATCGCCTCGCGTGAG
Orf 64 II rev	CGCGTAATGCAGGGCTGG
Orf 64 III for	GTCGCGGATTATGTGGAG
Orf 64 III rev	CTTCCGTGGGTCAATGAC
Orf 64 IV for	TACCCCTACGGCCTTGCC
Orf 64 ½ for (Kpn1)	AAAAAGCAGGCTCCGCCGGTACCCCCCTGGAGCTCCCTGCCACAG
Orf 64 ½ rev (Kpn1)	AGAAAGCTGGGTTAGGTACCCACCGTGGCCTGCTGCAATCG
K3 cyt1 rev	AGAAAGCTGGGTTATTCCAGACCCTCCTGGTAAG
K3 cyt2 for	AAAAAGCAGGCTCCGCCCACATGATGCGCCACGTGGGG

K5 cyt1 rev	AGAAAGCTGGGTTATTCAAAAATTTCTTGGCGCTCC
K5 cyt2 for	AAAAAGCAGGCTCCGCCGGCGGCATATGCCGCGTAAG TGG
K1 for	AAAAAGCAGGCTCCGCCATGTTCCTGTATGTTGTCTGC
K1 rev	AGAAAGCTGGGTTCAGTACCAATCCACTGGTTGC
Orf 47 for	AAAAAGCAGGCTCCGCCCACTCTATCCACTTCGCC
Orf 39 for	AAAAAGCAGGCTCCGCCCATGCGCGCTTCAAAGAGCG ACC
K8.1 cyt for	AAAAAGCAGGCTCCTACCTGTGCGTTCCACGATGC
K8.1 rev	AGAAAGCTGGGTTACACTATGTAGGGTTTCTTACG
vIRF3 for	AAAAAGCAGGCTCCGCCATGGCGGGACGCAGGCTTAC C
CD19 for	CAGTCCTATGAGGATATGAGAGGAA
Orf 74 for (FL)	AAAAAGCAGGCTCCGCCATGGCGGCCGAGGATTTCC
Orf 22 cyt (Adapter)for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCTACA GACTGTTTTCCATCCTTTATTAGACCCAGCTTTCTTGTA CAAAGTGGTCCCC
Orf 22 cyt (Adapter) rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATAAAG GATGGAAAACAGTCTGTAGGCGGAGCCTGCTTTTTTGT ACAAACTTGTCCCC
Orf 53 cyt (Adapter)for	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCGCCAAG TTTGTGGACGAGGTGGTCCATGCATAGACCCAGCTTTC TTGTACAAAGTGGTCCCC
Orf 53 cyt (Adapter) rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATGCATG GACCACCTCGTCCACAAACTTGGCGGAGCCTGCTTTTT TGTACAAACTTGTCCCC
k1 ext rev	AGAAAGCTGGGTTATACAAGAAAATGCACTTCAAT
k1 cyt for	AAAAAGCAGGCTCCGCCCATTGTCAAAAACAACGTGAC
K15(Lamp,P-Allel)for	AAAAAGCAGGCTCCGCCATGAAGACACTCATATTC
Orf 57 spliced for	AAAAAGCAGGCTCCGCCATGGTACAAGCAATGATAG
Orf 75I rev(StuI)	AGAAAGCTGGGTTAGTAGGCCTGCTCCCCCATGGCC
Orf 75II for(StuI)	AAAAAGCAGGCTCCGCCATGGCCAACCTCCTACACAA G
Orf 64 II Spe1 for	AAAAAGCAGGCTCCGCCATGTGCGAGATCGTCCAACA G
Orf 64 I Spe1 rev	AGAAAGCTGGGTTACAGGGACTTGAAGTCCTGACG
Orf 64 IV Not1 for	AAAAAGCAGGCTCCGCCATGCGGCCGCTTACTGCGGA TGAATCCG
Orf 64 III Not1 rev	AGAAAGCTGGGTTAAAGCGGCCGCAAGGTGGACCACA GCTGCC
Zeo Mlu1 for	CGCGGGACGCGTCGTTTACAATTTCGCCTG
Zeo Xba1 rev	CGCGGGTCTAGACTGCTGATCTTCAGATCC
Orf 6 I Sac1 rev	AGAAAGCTGGGTTAATGGACGGATTTGATGTTCTCTCT G
Orf 6 II Sac1 for	AAAAAGCAGGCTCCGCCATGGCAGAGCTCCTAAAGTTT ATTAAC
Orf 63 I Mlu1 rev	AGAAAGCTGGGTTACAAGTGACTCTTGGTAAACGC
Orf 63 II Mlu1 for	AAAAAGCAGGCTCCGCCATGCGGGCCATCTTCGAGAT

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5.1.9.2. Other oligonucleotides used in the study

ELK-1 forward	AAA AAG CAG GCT CCG CCA TGG ACC CAT CTG TGA CGC
ELK-1 reverse	AGA AAG CTG GGT TCA TGG CTT CTG GGG CCC TG
SpiB forward	AAA AAG CAG GCT CCG CCA TGC TCG CCC TGG AGG CTG C
SpiB reverse	AGA AAG CTG GGT TCA GTA GCC CCA GCA GGA ACT G
STAT-1 forward	AAA AAG CAG GCT CCG CCA TGT CTC AGT GGT ACG AAC
STAT-1 reverse	AGA AAG CTG GGT TTA CAC TTC AGA CAC AGA AAT C

The oligonucleotides were obtained from Invitrogen (Germany) and Metabion (Martinsried, Germany).

5.1.10. Plasmids

5.1.10.1. Constructs used for reporter gene analysis

The luciferase reporter constructs pAP-1-luc, pSRE-luc and pISRE-luc were purchased from PathDetect® *in Vivo* Signal Transduction Pathway cis-Reporting Systems (Stratagene):

pAP-1-luc	7x AP-1 (TGACTAA) binding sites
pISRE-luc	5x ISRE (TAGTTTCACTTTCCC) binding sites
pSRE-luc	5x SRE (AGGATGTCCATATTAGGACATCT) binding sites

5.1.10.2. Other plasmids used in the study

pGLMR5 GAL4-reporter	kindly provided by Michael Meisterernst, GSF, München[102]
IMAG p958D03310Q2	obtained from the Rzpd, Heidelberg
IRAK p961L1793Q2	obtained from the Rzpd, Heidelberg
L25	kindly provided by Thomas Schulz, Medizinische Hochschule Hannover[157]
L26	kindly provided by Thomas Schulz, Medizinische Hochschule Hannover[157]
mSRF-luc	kindly provided by Jessica Schwartz, University of Michigan Medical School, Michigan[89]
mTCF-luc	kindly provided by Jessica Schwartz, University of Michigan Medical School, Michigan[89]
pARC92/ACID-1	kindly provided by Michael Meisterernst, GSF, München[102]
pARC92/ACID-1-NTD	kindly provided by Michael Meisterernst, GSF, München[102]
pcDNA3.1 zeo	obtained from Invitrogen, Karlsruhe, Germany
pcDNA3.1-LANA-1	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover
pCR3	obtained from Invitrogen, Karlsruhe, Germany
pDEST-script	kindly provided by Ulrich Hentschel
pGADT7	obtained from BD Biosciences Clontech, Heidelberg, Germany
pGAL4-VP16	kindly provided by Michael Meisterernst, GSF, München[102]
pGAL4-VP16 H1	kindly provided by Michael Meisterernst, GSF, München[102]
pGBKT7	obtained from BD Biosciences Clontech, Heidelberg, Germany
pGEX-1 C1	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]

pGEX-1 C2	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 C4	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 C5	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 C6	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 C7	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 C8	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 GST	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGEX-1 LANAC	kindly provided by Thomas Schulz, Medizinische Hochschule, Hannover [116]
pGL12	kindly provided by Diane Hayward, Johns Hopkins School of Medicine, Baltimore [85]
pGL13	kindly provided by Diane Hayward, Johns Hopkins School of Medicine, Baltimore [85]
pGL9	kindly provided by Diane Hayward, Johns Hopkins School of Medicine, Baltimore [85]
pHIVluc	kindly provided by Michael Meisterernst, GSF, München
p-IL6 -Luc	kindly provided by Gergana Iotzova, Max von Pettenkover Institut, München
pLANA-1-gfp	kindly provided by Matthew Rettig, VA Greater Los Angeles Healthcare System, Los Angeles[3]
pNFκB-Luc	obtained from Stratagene, Amsterdam, The Netherlands
pRK5c-mycRasV12	kindly provided by Dr.Alan Hall, MRC, London, UK
pSpiB	kindly provided by Celeste Simon, Department of

	Medicine, University of Chicago[151]
pSV2tat72	NIH AIDS reagent program
wtSRE-luc	kindly provided by Jessica Schwartz, University of Michigan Medical School, Michigan[89]

5.1.11. Molecular weight markers

5.1.11.1. Markers for measurements of protein size

See blue 2 prestained protein standard low range obtained from Invitrogen, Karlsruhe, Germany.

5.1.11.2. Markers for measurements of DNA size

Gene Ruler 100 bp DNA ladder obtained from MBI Fermentas, Germany

Gene Ruler DNA 1 kb ladder obtained from MBI Fermentas, Germany

5.1.12. Kits

Nucleofector™ Transfection Kit	Amaxa, Biosystems, Germany
ECL Western blotting detection system	Amersham-Pharmacia, Germany
Pharmacia GFX PCR DNA Gel Purification Kit	Amersham-Pharmacia, Germany
Nucleobond AX® Plasmid DNA Purification Kit	Macherey-Nagel, Germany
Nucleobond AX® BAC DNA Purification Kit	Macherey-Nagel, Germany
BCA Protein	Assay Pierce, Rockford, USA
Luciferase Assay System	Promega, Mannheim, Germany
Dual-Luciferase®Reporter Assay System	Promega, Mannheim, Germany
Effectene Transfection Reagent	Qiagen, Hilden,Germany
Qiafilter Plasmid Maxi Kit	Qiagen, Hilden, Germany

5.1.13. Antibodies

Primary antibody	Clone/Catalogue number	Company/Provider
Goat polyclonal α-SpiB	sc-5947 x	Santa Cruz
Mouse monoclonal α-GST monoclonal (B-14)	sc-138	Santa Cruz
Mouse monoclonal α-myc (9E10)	sc-40	Santa Cruz
Mouse monoclonal α-human Sur-2	550429	BD Pharmingen
Rabbit α- caspase 3	9662	Cell Signaling
Rabbit α-actin	A-2066	Sigma
Rabbit polyclonal α-ELK-1*	9181s	Cell Signaling
Rabbit polyclonal α-ELK1-1	9182	Cell Signaling
Rabbit polyclonal α-GFP	sc-8334	Santa Cruz
Rabbit polyclonal α-human SRF against C-terminal domain	sc-335	Santa Cruz
Rabbit polyclonal α-MEK1/2	9122	Cell Signaling
Rabbit polyclonal α-MEK1/2*	9121	Cell Signaling
Rabbit polyclonal α-STAT-1	9172	Cell Signaling
Rabbit polyclonal α-STAT-1*	9171s	Cell signaling
Rat α-KS 330A	4F8-1	E. Kremmer
Rat α-KSHV ORF25	4G2-1-5-1	E. Kremmer
KSB 330B IgG2a (2003)		
Rat monoclonal α-ARC92/ACID-1	(9C2)	M. Meisterernst E. Kremmer

Rat monoclonal α-HA tag	1867423	Roche
Rat monoclonal α-human Mediator7	Med7	M. Meisterernst E. Kremmer
Rat monoclonal α-KSHV ORF73 against latent nuclear antigen LANA-1	13-210-100	ABI
Rat monoclonal α-PCQAP	(6C9)	M. Meisterernst E. Kremmer
α-goat-POX	305035003	Dianova
α-human-POX	109035088	Dianova
α-mouse-POX	115035146	Dianova
α-rabbit-POX	111035144	Dianova
α-rat-POX	112035062	Dianova

* recognizes the phosphorylated forms of the protein
POX - peroxidase-conjugated antibodies

5.2. Methods

5. 2. . Bacteria related methods

5.2.1.1. Preparation of competent *E. coli*

50 ml LB medium were inoculated with 0.5 ml overnight culture of *E. coli* DH5α or DH10B and grown to A_{600} of 0.5. The bacteria were then centrifuged in a 50 ml conical tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at 3000 rpm, 5 min, 4°C, resuspended in 25 ml cold sterile 0.1 M $CaCl_2$ and stored on ice for 30 min. After a second centrifugation as above, the bacteria were resuspended in 1 ml cold 0.1 M $CaCl_2$ and frozen in 15 % sterile glycerol at –80°C, in aliquots.

5.2.1.2. Transformation and growth of transformed bacteria

For the transformation of *E. coli* either chemically competent bacteria or electrocompetent bacteria were used depending on the DNA (ligation mix, plasmid, BAC DNA, etc.).

100 µl of the chemical competent bacteria were mixed with 0.1–100 ng of plasmid DNA and incubated on ice for 30 min. The cells were heat shocked by placing in a 42°C water bath for 90 s. 1 ml LB medium was added to the transformation mix and the tubes were placed in a 37°C shaker for 1 h. Afterwards the bacteria were spread onto LB agar plates containing the respective antibiotic: 100 µg/ml ampicillin, 50 µg/ml kanamycin, or 34 µg/ml chloramphenicol and grown overnight at 37°C. Single colonies were used to inoculate 2 ml LB cultures. These cultures were expanded to 400 ml and grown overnight at 37°C, shaking at 200 rpm.

100 µl of the electrocompetent bacteria were mixed with the ligation mixture or plasmid DNA and incubated on ice for 10 min. Then the bacteria were placed in a 2 mm Gene Pulser® cuvette (Bio-Rad Laboratories**,** Hercules, CA) and a pulse of 2.5 kV and 25 µF was applied. The next steps were as for the chemical competent bacteria described above.

5.2.1.3. Preparation of glycerol stocks

Bacterial overnight cultures were mixed 1:1 with 50 % glycerol to an end concentration of 20 % (v/v) and stored in 1 ml aliquots at –80°C.

5.2.2. Methods for DNA isolation, purification and analysis

5.2.2.1. "Mini" and "maxi" plasmid preparation

Maxi preps were done using kits from Qiagen (Chatsworth, CA) according to the manufacturer's instructions. Mini preps were done according to Sambrook et al. (Molecular Cloning, A Laboratory Manual, 1989).

5.2.2.2. Restriction digests

Restriction digests were typically incubated for 2 h at 37°C with 5-10 units restriction endonuclease per µg DNA in the appropriate buffer as recommended by the manufacturer (New England Biolabs, Beverly, MA).

5.2.2.3. Ligation

To avoid religation of incompletely digested vector, 5' ends were dephosphorylated by calf intestinal alkaline phosphatase (CIP). 50 U CIP were used for dephosphorylation of about 1 µg vector DNA. The reaction was performed for 40 min at 37°C and afterwards blocked. Then the CIP-ed vector was mixed with the purified fragment. Approximately 40-60 ng vector DNA was used. The molar ratio of vector/insert was between 1:3-1:8 (depending on the size of the fragment). The reaction was performed in a total volume of 30 µl 1x reaction buffer (MBI Fermentas) with 5 to 10 U T4 DNA ligase (MBI Fermentas). First vector and insert were mixed in reaction buffer subsequently the ligase was added. After incubation overnight in a water bath at 16°C the ligation was either directly transformed into competent bacteria or stored at –20°C until further usage.

5.2.2.4. Polymerase chain reaction (PCR)

The standard PCR reaction is assembled by mixing the following components:

Reagent	**Amount**
DNA	2-200 ng
10× PCR buffer	5 µl
25 mM $MgCl_2$	5 µl
dNTPs (10 mM)	2 µl
Primers (forward and reverse)	20 pmol (each)
Polymerase	0,5 µl
ddH_2O	up to final volume 50µl

In most cases the following program was used: 94°C for 5 min (or 2 min depending on the enzyme used); 94°C for 1 min, 54°C for 30 sec, 72°C for 1 min 30 sec (or longer); 72°C for 7 min.

5.2.2.5. Agarose gel electrophoresis

Agarose (electrophoresis grade, GibcoBRL, Grand Island, NY) was dissolved in 0.5x TBE buffer to the desired concentration (1 to 2.5 % depending on the size of DNA fragments) by boiling in a microwave oven. After cooling down, ethidium bromide was added to a final concentration of 0.5 µg/ml and the agarose solution poured in a gel chamber (Peqlab Biotechnologie GmbH, Erlangen). The DNA sample was mixed 1:10 with 10x DNA sample buffer (30 % glycerol, 0.25 % Bromphenol Blue, 0.25 % Xylene Cyanole in TBE buffer). The gels were run in 0.5xTBE buffer and visualized on a UV transilluminator.

5.2.2.6. Purification of DNA from agarose gel

DNA was purified from agarose gel according to the protocol from the manual of GFX-TM PCR DNA and Gel Band Purification Kit (Pharmacia).

5.2.2.7. Recombinatorial cloning - generation of a KSHV array in the eukaryotic expression vector pDEST-script

The KSHV array was generated into the eukaryotic expression vector pDEST-script (B-cassette) using the GATEWAY cloning technology. The recombination reactions of the GATEWAY cloning technology are based on the site specific recombination reactions by endonucleases and ligase of bacteriophage λ in *E. coli*. The LR reaction is a recombination reaction between an entry clone (pDONR207) and a destination vector (pDEST-script-B-cassette), mediated by the LR clonase mix of recombination proteins. Previously, all the KSHV ORFs have been cloned into the entry vector pDONR207 (Invitrogen) (Uetz et al., *unpublished*). In this project, all the ORFs of the KSHV genome were subsequently subcloned into the destination vector pDEST-script (B-cassette). The B-cassette containing the two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance (CmR) gene and a *ccd*B gene were previously included into pCMV-script vector in EcoRV unique site converting it into a Gateway destination vector. The subcloning was done according to the manufacture's protocol (Gibco BRL, Invitrogen, Life Technologies) with small modifications. 200 ng from each donor (entry) vector containing ORF of KSHV were mixed with 300 ng from the destination vector pDEST-script. 1 µl LR buffer, 1 µl LR clonase mix and 1 µl DNA topoisomerase I (10 U/µl-MBI Fermentas) were added, mixed well and left at RT for 1 h or overnight (depending on the size of the fragments for subcloning). The total volume of the reaction mix was between 5 and 8 µl. After 1 h (or overnight incubation) the LR reaction was stopped by adding 2 µl proteinase K (Gibco BRL, Invitrogen, Life Technologies) and incubating for 10 min at 37°C. Next step was to transform bacteria (electrocompetent DH10B or DH5α) with 1 µl from the LR mix. After 1h incubation in 1 ml SOC medium or LB at 37°C in a shaker 150 µl of the bacterial culture was plated on kanamycin plates and left overnight at 37°C for selection of positive clones. The next day 3 to 5 bacteria colonies were picked up, minipreps were performed and the DNA was checked by

restriction digest. One of the positive clones was then used to perform a maxiprep of DNA, which was kept at –20°C.

5.2.2.8. Generation of constructs expressing transcription factors using recombinatorial cloning

The transcription factors STAT-1, ELK-1 and SpiB were generated as PCR products using primers specific for each transcription factor carrying the *attB* sites. The templates used were IMAG p958D03310Q2 (rzpd) for STAT-1, IRAK p961L1793Q2 (rzpd) for ELK-1 and pSpiB expression vector for SpiB. The following primers were used: STAT-1 forward**,** STAT-1 reverse, ELK-1 forward, ELK-1 reverse, SpiB forward and SpiB reverse the sequences of which are indicated in Materials and Methods.

The PCR was done in two steps. The first PCR were performed using primers specific for each transcription factor with the following program: 1x 2.5 min 94°C; 10-15 cycles: 15 sec 94°C; 60 sec 55°C; 15-180 sec (60 sec per 1000 bp) 72°C (hot start: add polymerase added after 2.5 min 94°C denaturation). The second PCR step was done using the same pair of external primers for each transcription factor namely attB1 external forward and attB2 external reverse. The sequences are indicated in Materials and Methods.

The PCR program was carried out using the following scheme: 5 cycles 15 sec 94°C; 30 sec 45°C; 15-180 sec (60 sec per 1000 bp) 68°C 10-15 cycles 15 sec 94°C; 30 sec 55°C; 15-180 sec (60 sec per 1000 bp) 68°C. The PCR products were than run on a 1 % agarose gel and purified by GFX-TM PCR DNA and Gel Band Purification Kit (Amersham-Bioscience). The purified PCR products were cloned into pDONR207 vector by BP clonase reaction and subsequently subcloned into destination vector pGBKT7 by LR clonase reaction.

5.2.2.9. Generation of the pGADT7-LANA-1 expression construct

LANA-1 was first cloned into pDONR207 and subsequently into pGADT7 by recombinatorial cloning.

5.2.3. Tissues culture and related techniques

5.2.3.1. Culture conditions

The cell lines used in this study were grown in the following media:

HeLa and HEK 293 cells: Dulbecco's modified Eagle medium (DMEM), containing 1 g/l glucose and supplemented with 10 % FCS, 4 mM L-glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate (Invitrogen Life Technologies).

Jurkat, BJAB, LCLs, DG75 and BCBL-1: RPMI 1640 medium, supplemented with 20 % heat-inactivated fetal calf serum (FCS) and 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 4 mM L-glutamine.

The adherent cell lines used in this study were usually grown in 10-cm or 15-cm tissue culture dishes (Falcon, Becton Dickinson Labware) in a tissue culture incubator with 5 % CO_2 atmosphere at 37°C. Subculturing of the adherent cells was done by trypsinization with 0.25 % trypsin, 0.2 % EDTA (Invitrogen Life Technologies).

Suspension cell lines were grown in 25, 75 or 175 cm^2 tissue culture flasks. Subculturing of suspension cells was done by diluting with fresh medium.

5.2.3.2. Cryoconservation

Cells were frozen in 90 % FCS and 10 % (v/v) dimethyl-sulfoxide (DMSO) in a freezing box at -80°C. The frozen aliquots were first stored at –80°C and subsequently in a liquid nitrogen tank.

Thawing of the cells was done by submerging the frozen vial in a 37°C water bath. The cells were then washed with medium by centrifugation at 1200 rpm for 3 min and resuspended in pre-warmed medium before transferring them to culture plates or flasks.

5.2.3.3. Lipofection

HEK 293 cells or HeLa cells were split into 6, 24 or 48 - well plates the day before transfection. The plasmid DNAs were transiently transfected using Effectene (Qiagen) according to the protocol in the manual. One day post transfection the DMEM was replaced with fresh medium containing 1 % fetal calf serum (FCS) and supplements, and

cells were starved for another 24 h. After 48 h cells were harvested, lysed in 1x passive lysis buffer and luciferase activity was measured by using a commercial luciferase assay system (Promega) and a tube luminometer (Berthold). The transfection efficiency was normalized by co-transfection of the RL-TK vector (Promega) containing *Renilla* luciferase.

5.2.3.4. Calcium phosphate transfection

One day before transfection HEK 293 were seeded in 10 cm tissue culture dishes at 30 % confluency. 10 µg total DNA was used, 500 µl 2.5 M $CaCl_2$ was added, the tubes were vortexed, and left at room temperature for 5 min. 500 µl of 2x HEBS were added dropwise while gently vortexing, and the solution was incubated another 20 min at room temperature. Calcium phosphate-DNA solution was added dropwise onto the cell culture plate while swirling. The plates were incubated overnight in a 5 % CO_2-humidified incubator at 37°C. On the next day the medium was exchanged with fresh MDEM medium containing 1 % FCS and the cells were starved for another 24 h. 48 h after transfection the cells were harvested and lysed in the appropriate lysis buffer.

5.2.3.5. Transfection by electroporation

For electroporation of cells, 5×10^6 cells per transfection were spun down and resuspended in 400 µl pre-warmed FCS. 40 µg total plasmid DNA (the total DNA concentration in each transfection experiment was kept constant by adding vector plasmid DNA) was added to the cell suspension and mixed carefully. The sample was transferred to a 4 mm Gene Pulser® cuvette (Bio-Rad Laboratories**,** Hercules, CA) and a pulse of 250 mV and 1200 µF was applied. The cell suspension was then transferred to a 10 cm tissue culture dish, containing 10 ml pre-warmed RPMI medium and the cells were incubated for the desired period of time (at least 36 h).

This protocol was applied successfully for the transfection of Jurkat, BJAB and DG75 cells.

5.2.3.6. Luciferase reporter assays

Cells were collected, washed once with 1x PBS and resuspended in 100 µl 1x Passive Lysis Buffer (Promega, Madison, WI). The cell suspension was incubated 15 min at room temperature. The cell debris were spun down at 13000 rpm for 5 min 20 µl of the protein extract was used for measurement of luciferase activity using the Dual Luciferase assay system (Promega, Madison, WI), following the manufacturer's instructions. The measurement was performed with the luminometer LUMAT LB9507 (EG&G® BERTHOLD, Wellesley, MA).

5.2.3.7. Stimulation/inhibition of cell signal transduction pathways

For the transfection experiments with stimulation of main signal transduction pathways the following chemicals were used:

IFN α or **IFN** β activate the type I interferon-mediated pathway. They were used at a concentration of 500 U/ml.

TPA activates protein kinase C *in vivo* and *in vitro*. Active concentration 20 ng/ml.

Ionomycin is a Ca2+ ionophore, a mobile ion carrier for Ca2+, which induces apoptotic degeneration. It was used at a concentration of 20 ng/ml.

For transfection experiments, inhibitors were added, 10 h before harvesting the cells, at the concentration mentioned below. The following inhibitors were used:

PD98059 (MEK1 inhibitor) inhibits phosphorylation activation of MEK1 by upstream kinases. Active concentration 10-20 µM.

U0126 (MEK1/2 inhibitor) inhibits phosphorylation activation of MEK1 and MEK2 by upstream kinases. Active concentration 10-20 µM.

5.2.4. Protein purification and analysis

5.2.4.1. Cell extracts

Plates of adherent cells were washed twice with Dulbecco's PBS, scraped off with a rubber policeman and transferred to an Eppendorf tube. Suspension cells were collected by centrifugation, washed twice in Dulbecco's PBS without Ca^{2+} and Mg^{2+}, and transferred to Eppendorf tubes. All subsequent steps were performed on ice. The cells were resuspended in an appropriate volume (depending on the cell pellet) NP-40 lysis buffer: 1 % NP-40, 140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris pH 7.6 and 1 mM phenylmethylsulfonyl fluoride (PMSF) (freshly added). The samples were incubated on ice for 20 - 30 min and then centrifuged for 15 min at 13000 rpm, 4 °C. The supernatant, containing the total protein extract, was transferred to a fresh Eppendorf tube and the total protein extract was stored at –20°C or –80°C.

5.2.4.2. Nucleic extracts preparation

All steps were done at 4°C or on ice! First, the cells were washed in cold 1x PBS. Then they were resuspended in 200 µl buffer A (1 mM PMSF, 1 mM DTT, 0.4 mM NaVan, 1 mM NaF) and incubated for 15 min on ice. Afterwards 12 µl TRITON-100 (10 %) was added and the tube was vortexed. After 1 min the cells were centrifuged for 5 min at 2000 rpm. The nuclei were washed 2 times with 500 µl buffer A (plus inhibitors) and centrifuged for another 2 min at 4°C at 1750 rpm. Next the nuclei were resuspended in 200 µl buffer C (plus inhibitors) and rolled at 4°C for 15 min. Finally, a centrifugation for 10 min at 4°C at 14 000 rpm was done, the supernatants were collected in new tubes and stored at –80°C.

5.2.4.3. Measurement of protein concentration

The protein concentration was determined using the Bradford assay (Pierce).

5.2.4.4. Western blotting (immunoblotting)

Proteins separated by SDS-PAGE were transferred on a nitrocellulose filter (membrane) by either wet or semi-dry blot transfer.

For semi-dry transfer the ROTH „SEMI-DRY-BLOT“ apparatus was used applying constant current of 0.8 mA per cm^2 for 1 h. For protein transfer, the gel was sandwiched between gel-sized Whatmann 3 MM papers soaked in transfer buffer (20 % methanol in 1× Tris/glycine buffer).

The wet blot was carefully set up under transfer buffer to avoid air pockets. The transfer was run in a cold room at 100 V for 1 h and 30 min or at 40 V overnight.

After transfer, nitrocellulose filters were incubated for 1 h in a Blocking solution (TBS containing 5 % milk powder and 0.1 % Tween-20) in order to reduce the unspecific background. The membrane was sealed in a plastic bag and incubated for 1 h with an appropriate dilution of the primary antibodies, directed against the protein of interest.

The membrane was washed 3 times for 10 min in TBST (0.1 % Tween-20 in TBS) and incubated for an additional 1 h with the appropriate secondary antibody conjugated to horseradish peroxidase. After 3 washes in TBST, antigen-antibody complexes were detected using the enhanced chemiluminescence detection system (NEN, Boston, MA or Amersham Biosciences), according to the manufacturer’s instructions and exposed on Biomax-MR film (Eastman Kodak, Rochester, NY).

5.2.4.5. Co-immunoprecipitation

Co-immunoprecipitation (CoIP) was performed using lysates from HEK 293 cells transfected with the expression plasmids pcDNA-LANA-1 and pLANA-1-gfp for LANA-1 and expression plasmid pARC92/ACID-1 for the Mediator protein ARC92/ACID-1. HEK 293 cells were cultured in 10 cm dishes and transfected with 10 µg of each of the two expression plasmids by the calcium phosphate transfection method. Expression was controlled using a GFP plasmid under the control of a T7. After 24 h, the medium was changed with fresh medium containing 1 % FCS and the cells were starved for another 24 h. After 48 h they were harvested and lysed by incubation for 30 min in 1 ml of NP-40 lysis-buffer (1 % NP-40, 140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris pH 7,6, 1 mM PMSF) on ice. Lysates were centrifuged for 15 min at 20,000 g and 4°C to remove unsolubilized material and pre-cleared with 25 µl of protein G-sepharose (Pharmacia) for 1h at 4°C. Beads were washed three times with ice-cold 1x PBS. Proteins were precipitated respectively from the monoclonal rat anti-ORF73 antibody, anti-ARC92/ACID-1 and anti-PCQAP for 4 h. Subsequently, new precleared 30 µl of protein G-sepharose beads (30

µl per sample) were added and incubated overnight at 4°C. Co-immunoprecipitations and transfected HEK 293 cells were resuspended in 2x SDS protein sample buffer. Samples were boiled for 5 min, electrophoresed in 7.5 to 10 % SDS-PAGE gels and transferred onto nitrocellulose (Schleicher and Schuell). Protein bands were visualized with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pharmacia).

5.2.4.6. Co-immunoprecipitation using recombinant vaccinia virus (vT7)

Co-immunoprecipitations between LANA-1 (in pGADT7) and the transcription factors ELK-1, STAT-1 and SpiB (all in pGBKT7) were performed using total cell lysates of HEK 293 cells transfected using recombinant vaccinia virus vT7 expressing the T7 RNA polymerase. HEK 293 cells were cultured on 10 cm dishes and infected with vT7 at a MOI of 10 in serum-free medium. The cells were incubated at 37°C for 1 h and 30 min. Immediately after infection cells were transfected with 10 µg of each of the two expression plasmids by calcium phosphate transfection (as described above) and were harvested 24 h post-transfection.

5.2.4.7. Measuring of p44/42 MAP Kinase Activity

For measuring MAP kinase activity the non-radioactive method from Cell Signaling Technology has been used. The assay was performed according to the protocol in the manual (p44/42 MAP Kinase Assay Kit, Cell Signaling Technology). Briefly, the cells were lysed and active (phosphorylated) MAPK was precipitated using immobilized antibody from the kit. The IP pellets were incubated in kinase buffer containing ELK-1 fusion protein and cold ATP and then the ELK-1 phosphorylation at Ser383 was analysed by WB and chemiluminescent detection using phospho-antibodies.

5.2.4.8. Coomassie blue staining

The protein lysates to be analysed were loaded on SDS-PAGE gels and incubated for 30 min with Coomassie blue staining solution: 0,25 % Coomassie brilliant blue R-250, 45 % methanol, 10 % acetic acid. Afterwards they gel was destained by several washes with 10 % methanol, 10 % acetic acid solution until the desired protein staining was visible.

5.2.4.9. Purification of GST-tagged fusion proteins, binding reaction

1ml of overnight culture of BL21 transformed with constructs expressing GST-fusion proteins was inoculated into 9 ml of fresh LB containing ampicillin (100 µg/ml) and incubated for 1 h at 37°C. IPTG was added to a final concentration of 1 mM and incubated for an additional 3 - 4 h at 30°C. The bacteria were harvested at 4°C by centrifugation for 15 min at 5000 rpm. The pellet was dissolved in 500 µl PBS contaning 1 mM PMSF and 4 mM Pefablock and sonicated 3 times for 5 sec (output control level 7, 100 %) with a Branson Sonifier 450. Triton X-100 was added to a final concentration of 1 %, solutions were mixed and centrifuged at 4°C for 10 min at 14000 rpm. The supernatants were retained and mixed with 50 % slurry of glutathione beads washed previously 3x with PBS. The protein lysates were incubated with the glutathione beads for 30 min at RT. The beads were then centrifuged and washed 3 times with washing buffer (50 mM Tris pH 7.8, 150 mM NaCl, 0.5 mM EDTA, 0.1 % NP40). For the analysis of protein expression by Coomassie blue 2x protein lysis buffer was added and the samples were boiled at 95°C for 10 min. For the pull-down assays the beads were mixed with the cell lysates of HEK 293 cells obtained from one 10cm culture dish and incubated for 1 h at RT. Subsequently, the beads were centrifuged, washed 3x with washing buffer and boiled for 10 min at 95°C in 2x protein lysis buffer. The binding reaction was analysed by Western blot analysis.

5.2.5. Sequence analysis

Protein and DNA sequences were retrieved from the NCBI GenBank Entrez server (http://www.ncbi.nlm.nih.gov/Entrez/) and analysed for open reading frames (ORF) and restriction sites using the DNA Strider software (Douglas, 1995). Sequence homology searches and alignments were done on the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1997). The analysis of promoters was performed with the Gene2Promoter algorithm (Genomatix, Münich).

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7. Abbreviations

°C	degree Celsius
µ	micro (10-6)
Å	Angström
AIDS	aquired immune deficiency syndrome
AP-1	activator protein 1
APS	ammonium persulfate
ARC92/ACID-1	Human activator-recruited cofactor
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BCBL-1	body cavity-based lymphoma cell line 1
bp	base pair
C	cytosine
CmR	chloramphenicol resistance
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
E. coli	Escherichia coli
e.g.	exempli gratia (Lat. = for instance)
E1A	adenoviral transcriptional activator
EBV	Epstein Barr Virus
EDTA	ethylenediamine tetraacetic acid
eGFP	enhanced green fluorescent protein
et al.	et alii (Lat. = and others)
EtOH	ethanol
f.l.	full length
FCS	fetal calf serum
Fig.	figure

g	gram
G	guanine
GTFs	general transcription factors
h	hour(s)
HAV	hepatitis A virus
HBV	hepatitis B virus
HBx	HBV X protein
hCMV	human cytomegalovirus
HDAgs	HDV antigens
HDV	hepatitis D virus
HeBS	HEPES-buffered saline solution
HEPES	2-[4-(2-Hydoxyethyl)-1-piperazinyl]-ethane sulfonic acid
HHV-8	human herpesvirus-8
His	Histidine
HIV	human immunodeficiency virus
HSV	herpes simplex virus
HTLV-1	human T-cell leukemia virus type 1
HUVEC	human umbilical vein endothelial cells
IF	immunofluorescence
IFN	interferon
IgG	immunglobulin G
IL	interleukin
IP	immunoprecipitation
IPTG	isopropylthiogalactoside
IRD	internal repeat domain
ISRE	IFN-stimulated response element
kb	kilo bases
kbp	kilo base pairs
kd	kilodaltons
KS	Kaposi’s Sarcoma
KSHV	Kaposi’s Sarcoma associated herpesvirus

L	liter
LANA-1	Latency-Associated Nuclear Antigen 1
LANA-2	Latency-Associated Nuclear Antigen 2
LB	Luria-Bertani
LBS	LANA-1 binding site
LPS	lypopolysaccharide
LTR	long terminal repeats
m	milli (10-3)
m	meter
M	mol/liter, molar
MAPK	mitogen-activated protein kinase
MCD	multicentric Castleman's disease
MCMV	murine cytomegalovirus
MCP	major capsid protein
MeCP2	methyl CpG binding protein
mHV-68	mouse herpesvirus 68
min	minute(s)
n	nano (10-9)
NFkB	nuclear factor κB
NLS	nuclear localization signal
NTD	N-terminal domain of ARC92/ACID-1
o/n	overnight
OD	optical density
ORF	open reading frame
p	pico (10-12)
PACs	bacteriophage P1-derived artificial chromosome
PAGE	polyacrylamide gel electrophoresis
PBS	phoshate buffered saline
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
PMSF	phenylmethylsulfonfluoride
R	receptor

RB	retinoblastoma
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rosswell Park Memorial Institute
RT	room temperature
SAPK	stress-activated protein kinase
SCIP	small capsomer interacting protein
SDS	sodium dodecylsulfate
sec	second(s)
Ser	serine
SH3 domain	Src homology 3 domain
SRE	serum response element
SRF	serum response factor
Sur-2	Suppressor of ras protein
SV-40	Simian virus 40
TAE	Tris-Acetate-EDTA
TBE	Tris/borate/EDTA
TBST	Tris buffered saline with Tween 20
TCF	ternary complex factor
TEMED	N, N, N', N'-tetramethylenediamine
TF	transcription factor
TNF-α	tumor necrosis factor- α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRI-1	triplex-1
Tris	Tris(hydroxymethyl)aminomethan
ts	temperature-sensitive
U	unit(s), enzyme activity
UV	ultraviolet
V	Volt
v/v	volumen/volumen
v-FLIP	viral FAS-ligand IL-1 *β*-converting enzyme inhibitory protein

VP16	virion protein of herpes simplex virus
vT7	recombinant vaccinia virus T7
VWA	Willebrand factor type A
VZV	varicella zoster virus
w/v	weight/volumen
WB	Western blot
WDSV	Walley dermal sarcoma virus
wt	wild type
YACs	yeast artificial chromosomes

8. Curriculum Vitae

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Date of birth: 10 July 1975, Nationality: Bulgarian

EDUCATION:

Aug. 2001 – present *Ludwig – Maximilians University, Munich, Germany*

Pre-Doctoral Fellow/Ph.D. Research in Virology

Dissertation: ORFeome-based arrays in eukaryotic expression vectors – a new approach to screen for the function of viral proteins (LANA-1 meets the Mediator)

Supervisor: PD Dr. Dr. Jürgen Haas

April 2000 – Aug. 2001 *Eberhard Karls University, Tübingen, Germany*

Faculty of Medicine, Stipend from *"Tübingen Fortüne Programme"*

Characterization of the polymorphic nature of the IL-10 (interleukin 10) promoter in relation to infectious diseases as *Plasmodium falciparum* malaria and Epstein-Barr virus-associated tumors

Supervisor: Prof. P. Kremsner

Oct. 1994 – July 1999 *St. Kliment Ohridski Sofia University, Sofia, Bulgaria*

Master of Science

Majored in: Molecular Biology, Grade 5.67 (highest 6)

Field of specialization: Virology

Diploma thesis: Anti – Herpes simplex virus – type I activity of complexes of Platinum (II) and Palladium (II) with Thiosemicarbazones, Grade 6 (highest 6)

Supervisor: Prof. T. Varadinova

Attended conferences and seminars

Fifth International Workshop on KSHV/HHV8 and Related Rhadinoviruses, Germany, 2002

Poster presentation: Generation of recombinant HHV-8 using BAC technology

Gene Center Retreat 2002, Wildbadkreuth, Germany

Poster presentation: Generation of recombinant HHV-8 using BAC technology

Gene Center Retreat 2003, Wildbadkreuth, Germany

Poster presentation: New approaches for a systematic analysis of γ-herpesvirus gene function: Generation and screening of a KSHV expression library

Presentation: Genome-wide analysis of protein-protein interactions in Kaposi`s sarcoma associated herpesvirus (KSHV/HHV-8)

Annual Meeting of the «Gesellschaft für Virologie», 2004, Tübingen, Germany

Poster presentation: Systematic analysis of KSHV signalling pathways by viral expression array