Role of BET-Proteins in the Function of Rhadinoviral Orf73-Proteins

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Zusammenfassung

Das Kaposi Sarkom assoziierte Herpesvirus (KSHV) gehört zusammen mit dem murinen γ-Herpesvirus 68 (MHV68) zur Gattung der *Rhadinoviren* in der Ordnung *Herpesvirales*. Beide Viren persistieren im lymphatischen Gewebe und spielen eine Rolle bei der Entstehung lymphoproliferativer Erkrankungen. Eines der wichtigsten Latenzproteine ist im offenen Leserahmen (orf) 73 kodiert, bei KSHV wird das Protein auch als Latenz assoziiertes nukleäres Antigen 1 (LANA-1) bezeichnet. Beide orf73 Proteine werden in der Latenzphase exprimiert und erfüllen verschiedene wichtige Funktionen für das Virus. Eine dieser Funktionen ist die Interaktion mit zellulären BET Proteinen. In dieser Arbeit wurde die molekulare Natur dieser Interaktion und deren Auswirkungen untersucht.

Es gelang uns, zwei zuvor in einem Peptid Interaktionsversuch identifizierte Bindungsstellen für BET Proteine innerhalb des MHV68 orf73 Proteins zu bestätigen. Die Bindungsstellen wurden auf verschiedene Weise mutiert, um den Einfluss der Bindungsstellen auf Funktionen des MHV68 orf73 Proteins zu untersuchen. Das Aminosäuremotiv KKLK in der C-terminalen BET Bindungsstelle ist notwendig für die Interaktion des MHV68 orf73 Proteins mit den BET Proteinen Brd2 und Brd4. Mutationen in diesem Motiv verursachen keine Veränderung bei der Bindung des MHV68 orf73 Proteins an mitotische Chromosomen, das schliesst eine Beteiligung von BET Proteinen bei der Anheftung der viralen Genome an Chromosomen während der Mitose aus. Aber die Mutation des Bindungsmotivs verringert die Fähigkeit des MHV68 orf73 Proteins, die Transkription von Cyclin Promotoren zu aktivieren. Die Interaktion mit den BET Proteinen Brd2 und Brd4 spielt also eine Rolle bei der Aktivierung von transkriptionellen Prozessen durch das MHV68 orf73 Protein, ist aber nicht an der Persistenz des viralen Genoms in der Zelle beteiligt.

Das LANA-1 Protein von KSHV wurde in einem Yeast two Hybrid Interaktionsversuch mit einer Bibliothek von Peptidaptameren verwendet. Es konnten drei Peptidaptamere identifiziert werden, die mit dem C-terminalen Teil des LANA-1 Proteins von Aminosäure 1090 bis 1162 interagieren, der auch die Region mit der höchsten Sequenzähnlichkeit zum MHV68 orf73 Protein und die Bindungsstelle für die BET Proteine im MHV68 orf73 Protein beinhaltet. Die interagierenden Peptidaptamere inhibierten sowohl die von LANA-1 verursachte Aktivierung des Cyclin E Promotors als auch eines Promotors, der auf Serum ansprechende Sequenzen enthält. Eine generelle Inhibition der Transkription wurde ausgeschlossen, da die interagierenden Peptidaptamere zwei virale Promotoren nicht beeinflussten. Die Peptidaptamere veränderten die Aktivierung des LTR Promotor von HIV durch das TAT Protein, welche durch Brd4 vermittelt wird, nicht.

Die Erkenntnisse dieser Arbeit betonen die wichtige Rolle der Interaktion von BET Proteinen mit orf73 Proteinen für deren Funktion während des viralen Lebenszyklus.

Summary

The Kaposi sarcoma-associated herpesvirus (KSHV) belongs, together with murine γ-herpesvirus 68 (MHV68), to the genus of *Rhadinoviruses* within the order of *Herpesvirales*. Both viruses persist in lymphoid tissues and are involved in lymphoproliferative disease. A main factor for the latent infection is encoded in open reading frame (orf) 73, the protein is termed latency-associated nuclear antigen 1 (LANA-1) for KSHV. Both orf73 proteins are expressed during latency and fulfil several important functions in the viral life cycle. One of the conserved functions is the interaction with cellular BET proteins. In this thesis we investigated the molecular nature of the interaction of the MHV68 orf73 protein with the BET proteins Brd2 and Brd4 and the consequences of this interaction.

We were able to verify two potential BET binding sites within the MHV68 orf73 protein, which were identified by an *in vitro* peptide interaction assay. These BET binding sites were mutated in several variants to adress the impact of the interaction with BET proteins on MHV68 orf73 functions. The motif of the amino acids (aa) KKLK in the C-terminal BET binding site mediated interaction with the two BET proteins Brd2 and Brd4. Mutation of this motif did not alter tethering of the MHV68 orf73 protein to mitotic chromosomes and therefore BET proteins are not involved in tethering of the MHV68 viral genome to chromosomes during mitosis. However, the mutation of BET binding sites impaired the induction of transcription from cyclin promotors by the MHV68 orf73. Therefore the interaction with the BET proteins Brd2 and/or Brd4 is involved in transcriptional activation processes of the MHV68 orf73, but not in viral genome maintenance.

The KSHV LANA-1 protein was subject of a yeast two hybrid screen with a library of peptide aptamers. We identified three peptide aptamers, which bind to aa 1090-1162 of the LANA-1 protein, which encompasses the region with the highest sequence similarity to the MHV68 orf73 protein and the binding site for BET proteins. The interacting peptide aptamers were able to impair LANA-1 mediated activation of transcription from the cyclin E promotor and a serum reponse element containing promotor, in contrast to a not interacting peptide aptamer or the empty expression vector. This downregulation is not caused by a general inhibition of transcription, as the transcription from the long terminal repeat promotor (LTR) of HIV and the immediate early promotor of human cytomegalovirus (CMV) are not affected by the expression of any peptide aptamer. Also a general inhibition of the positive effect of Brd4 in the P-TEFb complex can be excluded, since the induction of the LTR promotor by the HIV transactivator of transcription (TAT) was not altered by any peptide aptamer.

These findings indicate the importance of the interaction between BET proteins and the orf73 proteins for their regular function during the viral life cycle.

Schlagwörter:

Herpesviren, BET-Proteine, Zellzyklus

Keywords:

Herpesvirus, BET-Proteins, Cellcycle

Table of content:

1. Introduction

1.1. The family of *Herpesviridae*

Very recently the family of *Herpesviridae* was split into three new families within the new order of *Herpesvirales* (Davison, Eberle et al. 2009). The new families *Alloherpesviridae* and *Malacoherpesviridae* were introduced to integrate far related herpesviruses like Oyster Herpesvirus or Koi Herpesvirus into the taxonomy. The family of *Herpesviridae* with its division into three subfamilies was maintained, but several new genera were introduced. The order of *Herpesvirales* is widespread across animal species, nearly every species features at least one herpesvirus. All members of *Herpesvirales* share some typical structural characteristics. Common structural features of a herpesvirus include a linear double stranded DNA genome, which is enclosed inside an icosahedral capsid. The capsid consists of 162 capsomers and has a diameter of about 100 to 110 nm. Between the capsid and the host cell derived envelope a protein layer called tegument is located. Viral glycoproteins are embedded within the double lipid membrane envelope. In addition all members of *Herpesviridae* exhibit some common biological properties:

1. A wide set of viral proteins enable the herpesviruses to interfere with nucleic acid metabolism, DNA synthesis and protein processing of the host cell.
2. The assembly of new virions and the previous replication of viral DNA occurs in the nucleus of the host cell.
3. Upon replication of the herpesvirus the host cell is destroyed to release the newly produced virions (lytic viral lifecycle)
4. In their natural host herpesviruses may establish a latent infection (latent viral lifecycle). The viral genome is maintained within the nucleus of the host cell. The array of expressed proteins is greatly reduced to the required minimum. Following reactivation of latent genome a herpesvirus can switch back into the lytic cycle.

The *Herpesviridae* are further classified into three subfamilies, according to their nucleotide sequence, host range, replication properties and pathogenicity:

α-*Herpesviridae* are able to infect a wide variety of species in experimental animal systems. They spread fast and because of their efficient multiplication they cause the destruction of the host cells. They establish latent infections mainly but not solely in neurons of sensory ganglia.

β-Herpesviridae are more restricted in their range of host cells and their growth in cell culture is slower compared to α-*Herpesviridae*. A prototypic human *β-herpesvirus*, cytomegalovirus, induces the formation of enlarged cells and nuclear inclusion bodies, hence the name cytomegalovirus. Most of latently infected cells are of bone marrow origin, but the viruses are also able to infect tissues like the salivary glands and kidneys.

γ-Herpesviridae infect only a very narrow range of host cells, they grow in lymphoid B- and T-cells *in vitro* as *in vivo*. The subfamily is further devided into four genera: the newly introduced *Percavirus* and *Macavirus* plus the *Lymphocrytovirus* and the *Rhadinovirus*. The genera of *Percavirus* and *Macavirus* do not contain any human herpesviruses, the Epstein-Barr virus (EBV or Human Herpesvirus 4) is a member of the *Lymphocrytovirus* genus, while Kaposi's sarcoma-associated herpesvirus (KSHV or Human Herpesvirus 8) and the Murine gammaherpesvirus 68 (MHV68 or Murid Herpesvirus 4) belong to the *Rhadinovirus* genus.

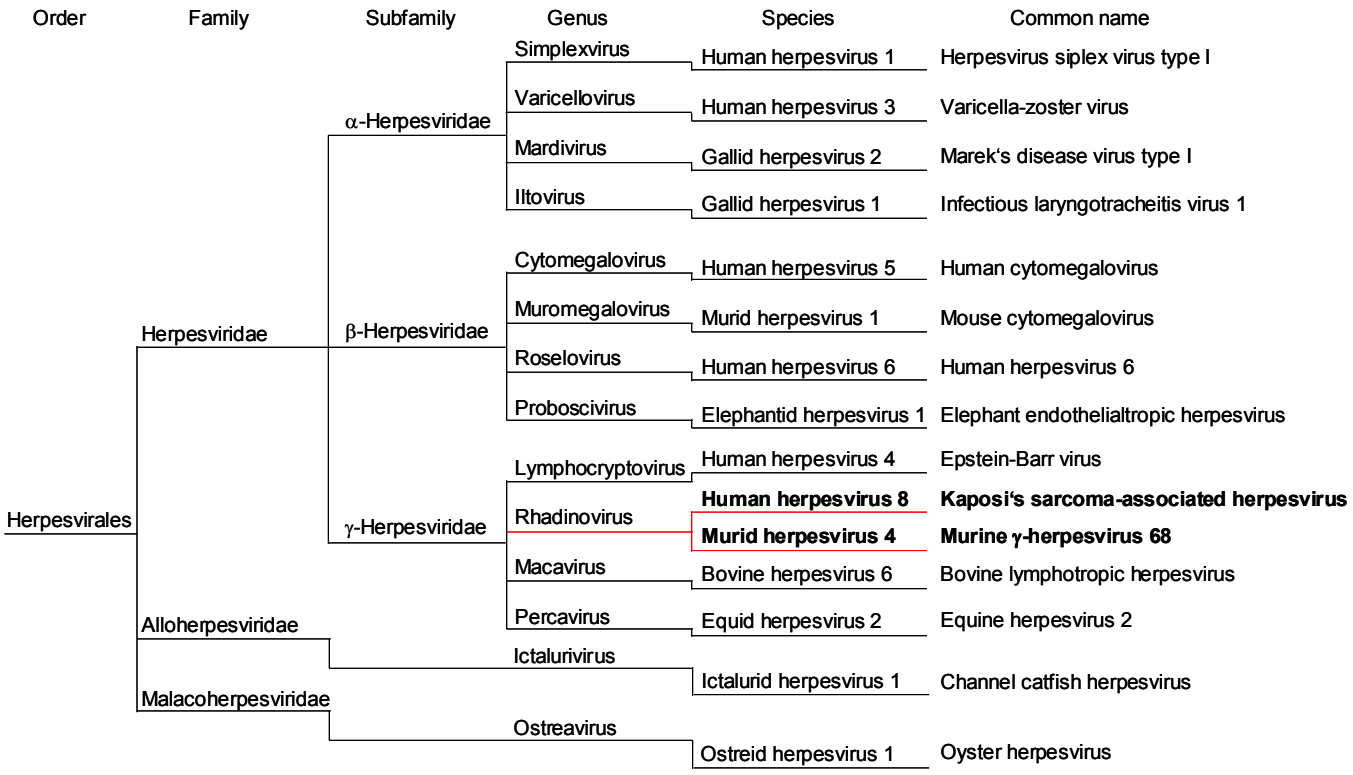


Figure 1. Phylogenetic tree of the order of *Herpesvirales.*

The order of *Herpesvirales* is divided into three families, the family *Herpesviridae* and the more distantly related families *Alloherpesviridae* and *Malacoherpesviridae*, which contain herpesviruses infecting fishes and invertebrates. The family *Herpesviridae* further splits up into three subfamilies, named α-, β- and γ-*Herpesviridae*. Each subfamily encompasses four genera. One species of each genera is presented in the figure, except for the genus of *Rhadinovirus* in the γ-*Herpesviridae*, which is in the focus of this thesis. Two species are highlighted in this figure: the Human herpesvirus 8 (HHV8), also designated as Kaposi's sarcoma-associated herpesvirus (KSHV) and a murine relative, the Murid herpesvirus 4 (MHV4) or Murine γ-herpesvirus 68 (MHV68). The distances in the tree do not correlate with the degree of relationship.

1.2. The subfamily of *γ-Herpesviridae*

Members of the subfamily of *γ-Herpesviridae* are characterized by their narrow range of host cells. They establish a latent infection in B or T lymphocytes. Depending on the virus lytic infection may involve epithelial and fibroblastic cells as well. In the following sections an introduction to the two *Rhadinovirus* species KSHV and MHV68 will be given.

1.2.1. Kaposi's sarcoma-associated herpesvirus (KSHV)

History of KSHV

In 1872 Moritz Kaposi described for the first time six patients, who all showed purplish colored idiopathic sarcoma of the skin. He stated that the disease was incurable and often resulted in death within two years after appearance of the sarcomas. 20 years after this publication the disease was named after its discoverer. Nearly a century later in the 1960s is was realized, that Kaposi's sarcoma (KS) is endemic in some regions of Africa. In 1972 first hints suggesting a link between KS and an infectious agent were found, when herpesviruslike particles were detected in KS biopsies by electron microscopic analysis (Giraldo 1972). These particles were assumed to be Cytomegalovirus (CMV), which is highly prevalent in the human population. With the outbreak of the Human Immunodeficiency Virus (HIV) epidemic in the early 1980s the incidence of KS increased, which led to the hypothesis that HIV infection is the critical step to develop acquired immunodeficiency disease syndrome (AIDS) related KS. However, different KS case rates among different HIV transmission groups (homosexual men, recipients of HIV-contaminated factor VIII, intravenous drug users, women) pointed to a transmissible agent other than HIV being responsible for KS development (Beral 1990). For example, while homo- and bisexual AIDS patients had KS case rates of more than 20%, age- and sex-matched patients with haemophilia had KS case rates of 1%, suggesting a sexual transmissible agent as cause for KS.
Direct proof of this prediction was obtained in 1994, when Y. Chang and coworkers detected herpesvirus-like DNA fragments in KS biopsies of an AIDS patient (Chang, Cesarman et al. 1994). The new herpesvirus was named Kaposi's sarcoma-associated herpesvirus, with the formal, taxonomic designation of Human Herpesvirus 8 (HHV8).

Soon the new herpesvirus was linked to two other rare proliferating diseases of lymphatic origin found in AIDS patients: primary effusion lymphoma (PEL) and the plasma cell variant of the multicentric Castleman's disease (MCD) (Cesarman, Chang et al. 1995; Soulier, Grollet et al. 1995). Upon sequence analysis KSHV was classified as a member of the *Rhadinovirus* genus (Moore, Gao et al. 1996; Russo, Bohenzky et al. 1996).

Epidemiology of KSHV

The first challenge after the discovery of KSHV was the lack of sensitive tests to detect KSHV and hence generate epidemiological data. Several groups used PCR-based methods to detect KSHV DNA within PEL and MCD cases and to show the presence of KSHV in all forms of KS (Cesarman, Chang et al. 1995; Soulier, Grollet et al. 1995; Schulz 1999). In addition detection of KSHV DNA in the peripheral blood of homosexual men predicted the subsequent progression to KS and thus highlighted the causative role of KSHV in a prospective setting (Whitby, Howard et al. 1995).

In 1996 the first serologial tests were established, which used detection of antibodies to the latency-associated nuclear antigen 1 (LANA-1) by either immunoblot or immuno-fluorescence (Gao, Kingsley et al. 1996; Kedes, Operskalski et al. 1996). Another assay used the structural protein encoded in the open reading frame 65, which was expressed as a recombinant protein and served as target antigen in an ELISA (Simpson, Schulz et al. 1996). In 1998 antibodies to the lytic glycoprotein K8.1 were used for setting up an immunoblot based test, which is very stable and specific (Raab, Albrecht et al. 1998). Further improvements were made by combining different tests and using recombinant proteins expressed in bacteria or mammalian cells (Laney, Peters et al. 2006). The early assays showed the uneven distribution of KS infection among different HIV infected populations. In regions nonendemic for KS, KSHV seroprevalence was clearly increased in the group of homosexual men (seroprevalence rates between 20% and 40%) in comparison to HIV positive intravenous drug users, women and person suffering from haemophilia, who had seroprevalence rates of usually below 10% (Kedes, Operskalski et al. 1996; Martin, Ganem et al. 1998). Later cohort studies confirmed these differences in KSHV seroprevalence and additionally showed an increased risk for developing KS, if the KSHV infection occurred after the infection with HIV (Renwick, Halaby et al. 1998; Jacobson, Jenkins et al. 2000).

The immunspression caused by HIV may lead to faster progression to KS development, this hypothesis was drawn from several case reports, that described rapid progression to KS or

other malignancies in already immunocompromised patients, who got infected with KSHV and is further supported by cohort studies that showed increased progression rates to KS in HIV patients as well (Parravicini, Olsen et al. 1997; Oksenhendler, Cazals-Hatem et al. 1998; Renwick, Halaby et al. 1998; Luppi, Barozzi et al. 2000).

KSHV is endemic at an intermediate level in the mediterranian area with seroprevalence rates between 3% and 25% (Angeloni, Heston et al. 1998; Rezza, Lennette et al. 1998; Whitby, Luppi et al. 1998; Cattani, Cerimele et al. 2003). KSHV seroprevalence correlates with incidence of "classic" KS in elderly men in these regions. However, even in Sicily the incidence of classic KS is in the order of 3/100,000, in spite of KSHV seroprevalence rates of about 30% (Calabro, Sheldon et al. 1998; Whitby, Luppi et al. 1998). Several studies showed high KSHV seroprevalence rates ranging from 30% to 70% in the populations of many African countries (Mayama, Cuevas et al. 1998; Olsen, Chang et al. 1998; Andreoni, El-Sawaf et al. 1999; Gessain, Mauclere et al. 1999).

It is believed that KSHV is an ancient virus, as different genotypes correlate with the geographic and ethnical background of the infected patients. The variety of the virus therefore resembles the divergence in the human population through the migration patterns during the past 35,000 to 60,000 years (Hayward 1999). This hypothesis is supported by existence of endemic spots with high KSHV prevalence in distinct and isolated populations (Biggar, Whitby et al. 2000; Rezza, Danaya et al. 2001; Whitby, Marshall et al. 2004).

Transmission of KSHV

The transmission of KSHV is complex and includes several possible routes. The significance of the different routes varies between non endemic and endemic areas.

The increased prevalence in homosexual men in non endemic countries points to sexual transmission routes (Kedes, Operskalski et al. 1996; Martin, Ganem et al. 1998). The fact that the number of sexual partners and contacs with men positive correlated with KSHV prevalence further supported this hypothesis (Dukers, Renwick et al. 2000; Diamond, Thiede et al. 2001; Casper, Wald et al. 2002). The number of KSHV particles varies between the different body fluids. The highest viral load was determined in saliva, while it was low in semen (Diamond, Huang et al. 1997; Koelle, Huang et al. 1997; Pauk, Huang et al. 2000). Therefore the transfer of saliva, may play a major role in transmission.

Evidence for heterosexual transmission also exists, but is less strong. Two studies restricted to women and not including other risk groups like intravenous drug users, obtained opposing

results (Cannon, Dollard et al. 2001; Goedert, Charurat et al. 2003). The transmission through intravenous drug abuse is still under discussion, but several studies indicate a role for intravenous drug abuse in transmission, despite the lower KSHV prevalence in intravenous drug users in comparison to homosexual men (Diamond, Thiede et al. 2001; Atkinson, Edlin et al. 2003; Goedert, Charurat et al. 2003). In contrast, other studies have not found any evidence in support of intravenous drug abuse (Renwick, Dukers et al. 2002; Bernstein, Jacobson et al. 2003).

Concerns about KSHV transmission via blood transfusion were raised (Blackbourn, Ambroziak et al. 1997). Small studies failed to demonstrate a relation between blood transfusion and infection with KSHV (Operskalski, Busch et al. 1997; Engels, Eastman et al. 1999), nevertheless transmission via blood could not be excluded. Several studies showed a KSHV transmission from infected donor organs to the recipient (Luppi, Barozzi et al. 2000; Barozzi, Luppi et al. 2003; Marcelin, Roque-Afonso et al. 2004).

In endemic areas there is clear evidence for a transmission to children, as studies showed an increasing rate of KSHV prevalence in children (Mayama, Cuevas et al. 1998; Andreoni, El-Sawaf et al. 1999; Gessain, Mauclere et al. 1999). Transmission via saliva in conjunction with host factors favoring shedding of KSHV into saliva seems to account for one possible route of infection (Dedicoat, Newton et al. 2004; Alkharsah, Dedicoat et al. 2007).

A very recent publication demonstrates the presence of KSHV in the placenta, but epidemiological studies failed to show evidence for a vertical transmission (Di Stefano, Calabro et al. 2008; Wojcicki, Mwanahamuntu et al. 2008). The high prevalence of KSHV in endemic regions increases the likelihood of transmisson via blood transfusion as indicated by two studies (Mbulaiteye, Biggar et al. 2003; Hladik, Dollard et al. 2006). However, in KSHV endemic areas the blood transfusions are more likely to be directly used and not stored before use, but storage of the blood units seem to reduce the risk of transmission (Hladik, Dollard et al. 2006).

KSHV and neoplastic disorders

Kaposi's sarcoma

Four variants of Kaposi's sarcoma (KS) are distinguished on the basis of their epidemiological context. "Classic" KS was originally described by Moritz Kaposi and is mainly found in elderly men of Mediterranean origin. It is characterized by purple spots at the

feets and lower libs, an involvement of internal organs is uncommon but possible (10%). The cancer is rare in immunocompetent KHSV infected individuals.
"Endemic" KS is mostly seen in sub Saharan Africa were KSHV prevalence is high. The clinical symtoms are similar to "classic" KS, but a visceral involvement is more likely.
"Post-transplant" KS was described first after solid organ transplantation in 1969 (Siegel 1969), with a disseminated phenotype including cutanous as well as visceral involvement. After the discovery of KSHV as the causative agent for KS it was demonstrated that cells within the KS lesion can be of donor origin (Barozzi, Luppi et al. 2003).
The last, today most common variant is "AIDS-associated" KS. Originally described in young homosexual men during the beginning of HIV epidemic (Hymes, Cheung et al. 1981), it spread with the HIV pandemic and is also termed "epidemic" KS. It quickly became the most common HIV-associated malignancy. First presentations are often disseminated cutaneous KS with bulky lesions. Later a visceral involvement especially of the respiratory and gastrointestinal tract is very frequently observed (Friedman, Wright et al. 1985; Mitchell, Fleming et al. 1992). Prior to the introduction of highly active antiretroviral therapy (HAART) the prognosis was poor with a median survial time of 15 months in case of visceral involvement (Krown, Testa et al. 1997). It is today still the most prevalent cancer in Africa and represents a significant clinical problem in regions, where no effective HAART is available.
The KS neoplastic component are spindle cells of endothelial origin, which habour the mainly latent KSHV (Boshoff, Schulz et al. 1995; Rainbow, Platt et al. 1997). A small fraction of KSHV infected cells in the tumours undergoes reactivation of KSHV (Staskus, Zhong et al. 1997). It is hypothesised, that this reactivation is necessary to sustain KSHV infection, since latently infected primary endothelial cells do not persist in cell culture (Gao, Deng et al. 2003; Grundhoff and Ganem 2004; Grossmann, Podgrabinska et al. 2006).

Multicentric Castleman's disease

Castleman's disease (CD) is a rare polyclonal lymphoproliferative disorder. Two histological forms of CD are known, the "plasma cell" variant, which is identified by plasma cell proliferation, while preserving a nodal architecture, and the "hyaline vascular" form characterized by abnormal germinal centers and neoangionesis. The localized form of CD is not correlated with KSHV, but the more aggressive multicentric form is. Most studies showed a connection of KSHV to the "plasma cell" variant of MCD, in contrast only one study

presented a case of “hyaline vascular” MCD (Staskus, Sun et al. 1999; Larroche, Agbalika et al. 2003). In MCD lesions the fraction of cells, undergoing lytic KSHV reactivation is higher than in KS or PEL (Staskus, Sun et al. 1999; Katano, Sato et al. 2000). It is thought that expression of the viral Interleukin-6 (vIL-6) gene as part of the lytic cycle may contribute to oncogenesis in a paracrine manner, which would explain the polyclonality of MCD lesions.

Primary effusion lymphoma

Primary effusion lymphoma (PEL) is a rare lymphoid malignancy, which is mainly found in HIV infected patients. Most PELs are characterized by the absence of a solid tumour mass, instead enlarged cells of B cell lineage with abundant cytoplasm and inconsistently shaped nuclei are observed as effusions in the pleura or peritoneum (Cesarman, Chang et al. 1995; Nador, Cesarman et al. 1996). PEL cells are frequently coinfected with EBV, but the relevance of this coinfection in the pathogenesis of PEL is still debated (Mack and Sugden 2008). Like in KS the percentage of cells undergoing lytic KSHV reactivation is quite low, vIL-6 may be expressed in about 5% of PEL cells without detectable levels of other lytic proteins (Katano, Sato et al. 2000; Parravicini, Chandran et al. 2000).
An involvement of vIL-6 in PEL pathogenesis is very likely, since vIL6 proliferation of PEL cell lines could be inhibited with antibodies against vIL-6 but not IL-6 antibodies (Jones, Aoki et al. 1999; Chatterjee, Osborne et al. 2002).

KSHV lifecycle

Cell entry

KSHV is capable of infecting a broad range of different human cell types *in vitro* like human primary endothelial cells and B cells, as well as several animal cell lines from various species (Flore, Rafii et al. 1998; Renne, Blackbourn et al. 1998; Akula, Wang et al. 2001; Bechtel, Liang et al. 2003). The first cellular factor identified as necessary for KSHV entry was heparan sulfate (HS) (Akula, Pramod et al. 2001; Akula, Wang et al. 2001). Later it was demonstrated, that three different types of integrins $\alpha 3\beta 1$, $\alpha V\beta 3$ and $\alpha V\beta 5$ are involved in KSHV entry (Akula, Pramod et al. 2002; Inoue, Winter et al. 2003; Garrigues, Rubinchikova et al. 2008). Two other cellular membrane proteins are also involved in KSHV entry: the 12-

transmembrane transporter protein xCT, which is part of the CD98 complex and contributes to amino acid transport (Kaleeba and Berger 2006) and DC-SIGN, an ICAM-3 binding non-integrin receptor specific for dendritic cells (DC) (Rappocciolo, Jenkins et al. 2006).

The great variety of involved cellular receptors led to the hypothesis that KSHV attaches to cells via HS and then interacts with either one or more of the identified integrins via the RGD motif of the capsid protein gB (Akula, Pramod et al. 2002). These integrins are often associated with the CD98 complex by their β1-subunit, upon recruitment of CD98 other events of entry and early signal transduction may be induced (Veettil, Sadagopan et al. 2008). This model was suggested for endothelial cells, in B cell the entry seem to be mediated by DC-SIGN alone, as KSHV entry in B cells was inhibited by antibodies to DC-SIGN but not with antibodies to xCT (Rappocciolo, Hensler et al. 2008).

KSHV virions are endocytosed by different modes in B cells, fibroblast and endothelial cells. In B cells and fibroblasts the virions are taken up by clathrin mediated endocytosis, as inhibitors of this pathway like chlorpromazine impair KSHV entry in B cells and fibroblasts (Akula, Naranatt et al. 2003; Rappocciolo, Hensler et al. 2008). For endothelial cells chlorpromazine showed no inhibitory effects on KSHV entry, in contrast to inhibitors of macropinocytosis or inhibitors of the required actin polymerisation. In addition KSHV virions colocalized with markers of macropinocytosis, but not with markers of clathrin mediated endocytosis (Raghu, Sharma-Walia et al. 2009). However both entry ways are dependent on acidification of the vesicles to iniate fusion of virion and vesicle membranes (Akula, Naranatt et al. 2003; Raghu, Sharma-Walia et al. 2009).

Viral capsids released from intracellular vesicles utilize the micotubule (MT) associated motor protein dynein to travel to the nucleus of infected cells, where they release the KSHV genome into the nucleus (Naranatt, Krishnan et al. 2005). The binding of KSHV virions to the different α-β-integrins also activates the focal adhesion kinase (FAK), a non-receptor protein-tyrosine kinase, one of the first activated proteins in integrin signaling (Giancotti 2000). The fast activation of FAK is indispensible for a sucessful infection (Akula, Pramod et al. 2002; Naranatt, Akula et al. 2003). Several pathways are induced by FAK, leading to activation of Rho-GTPases like Rho, Rac and Cdc42, which cause morphological changes by actin remodelling (Naranatt, Akula et al. 2003; Naranatt, Krishnan et al. 2005; Sharma-Walia, Krishnan et al. 2005; Krishnan, Sharma-Walia et al. 2006). The reduction of KSHV infection by abrogation of lipid rafts suggests a critical role of the phosphotadylinotisol 3 kinase (PI3) PKC-ζ, which is associated with lipid rafts (Naranatt, Akula et al. 2003; Raghu, Sharma-Walia et al. 2007).

Replication

The replication of KSHV can be devided into two separate processes: latent and lytic (productive) replication. A detailed descripition of these two forms of replication is presented in section 1.3, here only a brief introduction is given. Following viral entry the KSHV genome is delivered to the nucleus. The genome of KSHV consists of a long unique region (LUR) encoding the proteins of the virus, which is flanked by a varying number of terminal repeats (TR). The TRs enable the KSHV genome to circurlarize to an episome, which is the genome form during latency. During latency only a minimal set of proteins is expressed and the episome is maintained at a low number in the host cell. During lytic infection a well coordinated sequence of gene and protein expression is started, which results in replication of the linear genome by the rolling circle mechanism.

Assembly and egress

The available data about the assembly and egress of KSHV are poor, due to the low rates of lytic replication of KSHV in cell culture systems. At the end of the lytic replication cycle three different kinds of capsid are found, all composed of the major capsid protein (MCP)/orf25, the small capsomer interacting protein (SCIP)/orf65 and a triplex of one unit of triplex protein 1 (TRI-1)/orf62 and two units triplex protein 2 (TRI-2)/orf26 (Nealon, Newcomb et al. 2001). So termed A-capsids are empty, while the B-capsids contain varying amounts of the scaffold protein (SCAF)/orf17.5, and the C-capsids contain the KSHV genome and are fully functional virions capable of infecting new cells (Nealon, Newcomb et al. 2001).

Deng and colleagues proposed a model for capsid assembly after their investigations on KHSV capsids with cryo electron tomography. The MCP associates with SCAF and binds to one portal vertex complex, which later serves as package portal for the KSHV DNA. Other MCP/SCAF complexes are bound and additionally heterotrimers of TRI-1 and TRI-2 attach. A procapsid is formed, which resembles the form of the subsequent three capsid variants, but has a more spherical and less angular shell. At this point the presence of KSHV DNA determines the further fate of the procapsid. In the presence of KSHV DNA SCAF is digested, while the DNA is inserted into the maturing capsid, resulting in a C-capsid. When no DNA is present SCAF is also degraded, and B-capsids remain as intermediate products, containing

differnent amounts of SCAF. If SCAF is completely degraded, the maturing capsid yields an empty A-capsid (Deng, O'Connor et al. 2008).
In addition to the whole DNA genome several RNAs, representing full length gene transcripts, were detected in KSHV virions (Bechtel, Grundhoff et al. 2005). They correlate with several lytic transcripts found during early infection and may contribute to a successful establishment of infection in the host cell (Krishnan, Naranatt et al. 2004).
The capsids leave the nucleus and receive their tegument protein layer in the cytoplasm. The kinesin-2 motor protein KIF3A interacts with the tegument protein Orf45 and mediates thereby transport of the capsids to the cellular periphery (Sathish, Zhu et al. 2009). The virions may bud through the cellular membrane and obtain their envelope or be released upon death of the host cell (Whitman, Dyson et al. 2007).

Immune evasion by KSHV

The natural host cells of KSHV are part of of human immune system and processes of immune defense are believed to contribute to KSHV pathogenesis. Therefore it is not surprising, that KSHV inferes with the host immune system in various ways. The immune system can be roughly divided into two parts, the innate immunity, forming a general first line of cellular defense and the adaptive immunity, which delivers a highly specific response to a certain pathogen. KSHV is by several well adapted viral proteins capable of manipulating both branches of the immune response (for a schematic overview see figure 2).
The complement system, which is part of innate immunity, involves a cascade of protein protein interactions that result either in phagocytosis of the infected cell or the death of the infected cell by membrane disruption. KSHV expresses a viral homologue of the cellular complement control protein CD46 in orf4, also termed KSHV complement control protein (KCP) (Neipel, Albrecht et al. 1997; Neipel, Albrecht et al. 1997). KCP promotes the decay of classical convertase C3 and aids as cofactor for Factor I the degradation of convertases C3b and C4b (Spiller, Blackbourn et al. 2003; Spiller, Robinson et al. 2003). This results in bybassing the complement system and due to the incorparation into KSHV virions and binding to heperan sulfate KCP might protect the virus from attacks of the complement system even before entering a host cell (Mark, Lee et al. 2006; Mark, Proctor et al. 2008).

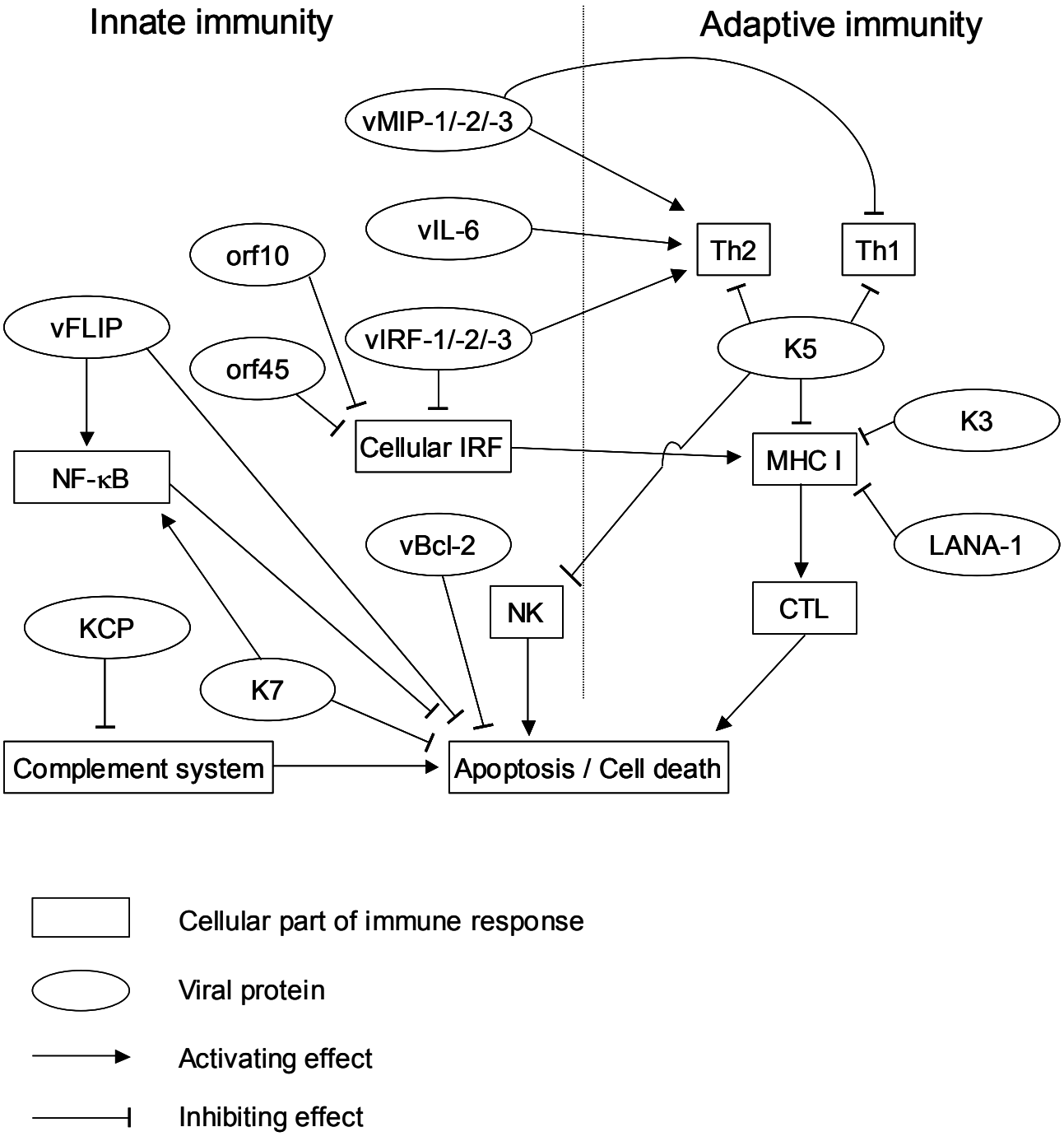


Figure 2. Immuneevasion by KSHV proteins.

Boxes display cellular proteins, pathways or processes or immune cells, while the elipses indicate viral proteins. The following abrevations are used: CTL=Cytotoxic T lymphocyte, IL=Interleukin, IRF=Interferon reponse factor, K=KSHV specific protein, KCP=KSHV complement control protein, LANA=Latency-associated nuclear antigen, MHC=Major histocompatibility complex, MIP=Macrophage inflammatory protein, NK=Natural killer cell, Th=T helper cell, v=Viral

KSHV features a broad range of viral proteins, that interact with various parts of the immune response of the host. Thereby KSHV ensures the survival or at least an extended lifetime of the infected cell, which allows generation of progeny virus.

The secretion of cytokines by cells to influence themselves (autocrine effects) and surrounding cells (paracrine effects) is another important mechanism of innate immunity. KSHV captured and modified several cellular genes for cytokines and use them to alter the host cell and its environment. The viral homologues of macrophage inflammatory proteins (MIP) vMIP-I, -II and –III are encoded in the orfs K6, K4 and K4.1 and were shown to have angiogenic effects and promote proliferation of cells (Boshoff, Endo et al. 1997; Liu, Okruzhnov et al. 2001). They drive the adaptive immune response into the direction of T helper (Th) 2 cells, by increasing chemotaxis for Th 2 cells via the chemokine receptors CCR3, CCR4 and CCR8, while inbiting chemotaxis of Th 1 cells by RANTES (Stine, Wood et al. 2000; Weber, Grone et al. 2001).

Another important inducer of the Th 2 response is the viral homologue for interleukin-6 vIL-6, encoded in gene K2. vIL-6 enhances proliferation of IL-6 dependent B cells (Moore, Boshoff et al. 1996) and activates several signal transduction pathways like Janus protein kinases (JAK) / signal transducers and activators of transcription (STAT) and mitogen-activated protein kinase (MAPK) through binding to the gp130 receptor subunit of the cellular IL-6 receptor (Molden, Chang et al. 1997; Osborne, Moore et al. 1999; Hideshima, Chauhan et al. 2000). The other subunit of that heterodimer, gp80 seem to modulate the signaling of vIL-6 upon binding to the vIL-6/gp130 complex, which nevertheless is capable of signaling on its own (Hu and Nicholas 2006). The induction of vascular endothelial growth factor (VEGF) B by vIL-6 drives proliferation of PEL cell lines in combination with cellular IL-6 and IL-10 (Aoki, Jaffe et al. 1999; Foussat, Wijdenes et al. 1999; Jones, Aoki et al. 1999). vIL-6 is linked to KSHV pathogenesis, as all three KSHV associated neoplasias are characterized by vIL-6 expression (Parravicini, Chandran et al. 2000). Although classified as a lytic gene vIL-6 expression was detected after exogenous stimuli like Notch or interferon signaling without reactivation of the lytic cycle, suggesting an additional role in evading host responses to viral infection (Chatterjee, Osborne et al. 2002; Chang 2006).

The interferon signaling provides another layer of cellular defense against incomming pathogens. After induction of various cellular receptors by either interferon (IFN) α/β, which are produced by the virus infected cells itself, or IFN γ, which is secreted by innate immunity effector cells, JAK/STAT pathways activate interferon response factors (IRF). IRFs then induce several cellular promotors with IFN stimulating response elements, transferring the cell into an antiviral state with elevated level of major histocompatibility complex (MHC) I and II, protein kinases and tumor necrosis factor (TNF). KSHV encodes three different viral homologues of IRFs and two other proteins interfering with IFN signaling. The homologues

vIRF-1/K9, vIRF-2/K11.1 and vIRF-3/K10.5/LANA-2 interact with many of the cellular IRFs and transcription factors like cyclic AMP response element binding protein (CREB) and thereby inhibit the correct interferon signaling (Gao, Boshoff et al. 1997; Burysek, Yeow et al. 1999; Li, Damania et al. 2000; Seo, Lee et al. 2000; Kirchhoff, Sebens et al. 2002). The other two proteins capable of inhibiting the function of IRFs are encoded in orfs 10 and 45. The orf10 protein inhibits signaling of IFN α/β via binding to their receptors, while the orf45 protein interacts with cellular IRF-7 and thereby antagonizes its function (Zhu, King et al. 2002; Bisson, Page et al. 2009).

Programmed cell death (apoptosis) is another mechanism to limit the spread of viral infection and may be caused by either intrisic or extrinsic signals. A central switch for this process is the Bcl-2 protein, which is present as a viral homologue in the KSHV genome as orf16/vBcl-2. The impact of the vBcl-2 on cellular targets is discussed controversially (Cheng, Nicholas et al. 1997; Sarid, Sato et al. 1997), but it saves cells from viral cyclin (vCyclin) induced apoptosis, which is caused by degradation of cellular Bcl-2 (Ojala, Tiainen et al. 1999; Ojala, Yamamoto et al. 2000). The viral homologue of the FLICE inhibitory protein (vFLIP)/K13 is encoded in a latent cluster with LANA-1 and vCyclin and blocks FAS receptor mediated proapoptotic signaling, which results in decreased levels of apoptosis inducers caspase-3, -8 and –9 (Thome, Schneider et al. 1997; Sarid, Flore et al. 1998; Djerbi, Screpanti et al. 1999; Belanger, Gravel et al. 2001). By direct binding to IKKγ vFLIP activates the NFκB pathway, which is necessary for survival of PEL cells and avoidance of apoptosis in primary endothelial cells (Guasparri, Keller et al. 2004; Efklidou, Bailey et al. 2008). It was reported, that vFLIP also inhibits superoxide induced cell death by upregulation manganese superoxide dismutase (Thurau, Marquardt et al. 2009).

By interaction with the p53 related transcription factor p300 vIRF-1 leads to less acetylation and phosphorylation of the p53 protein, thus decreasing its effects on the transcription of apoptosis mediators (Seo, Park et al. 2001). The product of gene K7 is a mitochondrial membrane protein, capable of increasing cytosolic calcium concentration and thereby prevents apoptosis by depolarisation of the mitochondrial membrane (Feng, Park et al. 2002). By interaction with cellular Bcl-2 and caspase 3 K7 enables Bcl-2 to inhibit caspase 3 mediated apoptosis (Wang, Sharp et al. 2002). The interaction of K7 with the cellular PLIC-1 protein, which stabilizes ubiqutinated proteins, leads to a rapid degradation of IκB and therefore an induction of the NFκB pathway as with vFLIP (Feng, Scott et al. 2004).

Natural killer (NK) cells play a critical role in innate immunity, as they release perforin and granzyme into virally infected cells, leading to their death. KSHV posses one specific protein,

the K5 protein, capable of inhibiting the NK cell mediated cell death. NK cells decide by an excessive network of cell cell contacts, if a cell should be target of programmed cell death. The K5 protein influences this network by modifying the presence of several cell surface markers. K5 was shown to act as an E3 ubiquitin ligase and thereby mediate proteosomal degradation or retargeting of the human leukocyte antigens (HLA)-A and –B, which are part of the MHC I complex. K5 additionally downregulates the NK cell receptors B7.2, ICAM-1, NKG2D and NKp80 (Coscoy and Ganem 2000; Ishido, Choi et al. 2000; Coscoy and Ganem 2001; Means, Lang et al. 2007; Thomas, Boname et al. 2008). The downregulation of surface receptors for NK cells decreases the time NK cells bind to the virally infected cell, which then is left unharmed by the NK.

The other part of the immune system, adaptive immunity, features a more pathogen specific and, if memory cells are involved, a very fast response to intruders. The biggest threat for a virally infected cell is the recognition by a cytotoxic T lymphocyte (CTL), which induces either apoptosis or direct lysis of the infected cell. The CTLs recognize their targets via the presentation of nonhost peptide sequences by MHC I complexes on the target cell surface. Hence the downregulation of MHC I is the most feasible way to hide an infected cell from the adaptive immune response. The decrease of surface MHC I mediated by K5 protein, is further aided by the K3 protein, which is not restricted to two HLA subtypes, but enhences endocytosis of a broader range of HLAs (Ishido, Wang et al. 2000; Means, Ishido et al. 2002; Sanchez, Coscoy et al. 2002). It was speculated, that the central acidic repeat region of LANA-1 interferes with proteasomal degradation necessary for MHC I antigen presentation and prevents recognition of latently infected cells by CTLs (Zaldumbide, Ossevoort et al. 2007).

The other branch of adaptive immunity, which is affected by KSHV proteins, is the activation of Th cells. Activated Th 1 cells attract macrophages and induce B cells to produce certain subclasses of antibodies, while the activated Th 2 cells enable B cells to synthesize a wide variety of immunoglobulins. The protein K5 limits the activation of Th cells, by downregulation of the ICAM and B7.2 (Coscoy and Ganem 2001).

1.2.2. Murine gammaherpesvirus 68 (MHV68)

MHV68 was discovered by Blaskovic and colleagues during field studies in Slovakia with the goal to identify vectors for flaviviruses. Instead five herpesviruses were obtained from two wild living murid rodents, which were initially classified as α-*Herpesvirinae* (Blaskovic, Stancekova et al. 1980; Svobodova, Blaskovic et al. 1982). The strains 60, 68 (later refered MHV68) and 72 of murid herpesvirus 4 were isolated from the bank vole (*Clethrionomys glareolus*), while the strains 76 and 78 were obtained from the wood mouse (*Apodemus flavicollis*) (Blaskovic, Stancekova et al. 1980). Ten years later the MHV68 was shown to align better with sequences of the γ-*Herpesviruses* EBV and Herpesvirus saimiri (HVS) than with α/β-H*erpesviruses* Herpes simplex virus (HSV) and CMV (Efstathiou, Ho et al. 1990). The cloning and mapping of MHV68 DNA sequences revealed a very related genome organisation to HVS, suggesting, that MHV68 is a *Rhadinovirus* (Efstathiou, Ho et al. 1990). This opinion was further supported, when the whole genome of MHV68 was sequenced and aligned with, when it became available later, KSHV genome sequences (see figure 3 for an alignment of KSHV and MHV68 genome structures (Virgin, Latreille et al. 1997; Nash, Dutia et al. 2001)).

The MHV68 genome contains a long unique region (LUR) of 118kbp length encoding for at least 73 genes, which is flanked by variable number of TR elements each 1.2kbp in size . The overall G/C content in LUR is 46%, the TR elements posses a G/C content of 78% (Virgin, Latreille et al. 1997).

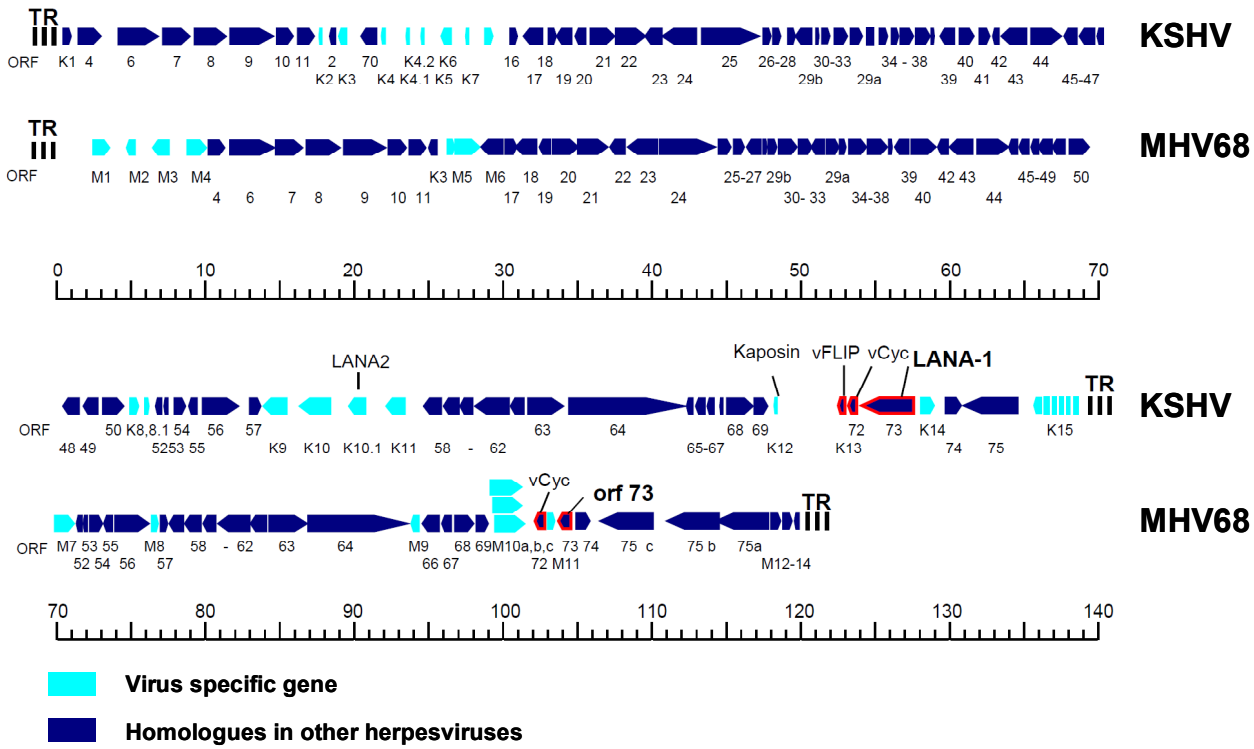


Figure 3. Alignment of the genomic structures of KSHV and MHV68.

The Boxes display identified open reading frames (orfs) with the arrowheads indicating the direction of transcription. Numbers represent the orfs, while K denotes genes first identified in KSHV and M designates unique genes in MHV68. Turquoise color represents unique genes, while the dark blue indicates that homologous genes exist in other herpesviruses. The red bordered boxes highlight latent genes, for KSHV: LANA-1, vCyclin and vFLIP; and for MHV68: the orf73 and vCyclin. The comparison of the two genomes emphasizes the similarity in genome organisation between this two Rhadinoviruses (regarding sequences for KSHV and MHV68 (Russo, Bohenzky et al. 1996; Virgin, Latreille et al. 1997); figure modified from (Greensill and Schulz 2000)).

Primary infection of mice via the intranasal route resulted in infection of lung epithelium and monocytes (Sunil-Chandra, Efstathiou et al. 1992). The primary infection of lungs is cleared in a cytotoxic T lymphocyte dependent manner about ten days after inoculation, but MHV68 DNA could be detected up to 30 days later (Sunil-Chandra, Efstathiou et al. 1992; Ehtisham, Sunil-Chandra et al. 1993). The virus spreads from lungs to many other tissues like heart, kidneys adrernal glands and the spleen. The spleen seem to be the major site of latency, where it can infect dendric cells (DC), macrophages and B cells (Sunil-Chandra, Efstathiou et al. 1992; Usherwood, Stewart et al. 1996; Flano, Husain et al. 2000). In the absence of B cells no splenic latency is established in mice, but the virus could be still maintained in the lungs after clearence of the primary infection (Usherwood, Stewart et al. 1996). Stewart and coworkers revealed the presence of MHV68 genomes in epithelial lung tissue, which provide further

evidence for latent infection of alternative cell types in the absence of B cells (Stewart, Usherwood et al. 1998). The general pattern of MHV68 pathogenesis was supported, when mice infected with a recombinant MHV68 virus expressing the firefly luciferase and thereby allowed the tracking of the virus within the living animal (Hwang, Wu et al. 2008). The salivary glands were identified as novel sites of virus proliferation, proposing a potential route of transmission to other animals (Hwang, Wu et al. 2008).

The cloning of the MHV68 genome into a bacterial artifical chromosomes (BAC) enabled efficient manipulation of the MHV68 genome and simplified the generation of mutant viruses (Adler, Messerle et al. 2000). Nearly every gene within the MHV68 genome exhibits homology to either rhadinoviral or cellular genes (Pepper, Stewart et al. 1996; Stewart, Janjua et al. 1996; Virgin, Latreille et al. 1997). In combination with the relative easy availability of mice for research purposes and a broad range of existing transgenic mice, MHV68 allows a detailed disection of the interplay between a γ-*herpesvirus* and its host. Hence MHV68 provides a suitable small animal model for *Rhadinoviruses*.

Like KSHV the MHV68 genome contains a viral homologue of the cellular apoptosis regulator Bcl-2, which is capable of inhibiting FAS or TNF-α induced apoptosis, but probably uses a different mechanism than the KSHV vBcl-2 (Wang, Garvey et al. 1999; Roy, Ebrahimi et al. 2000). The knockout of the gene demonstrated normal replication *in vitro*, lytic infection of mice was comparable to the wt virus as well, however the splenic latent peak was decreased *in vivo*, while longterm latency was unaffected (de Lima, May et al. 2005). This suggests a role for vBcl-2 in evading apoptosis in B cells, prolonging the window of virion production in these cells.

MHV68 also features a cyclin homologue, which is required for efficent reactivation from latency *in vivo* (Hoge, Hendrickson et al. 2000; van Dyk, Virgin et al. 2000; van Dyk, Virgin et al. 2003). It was shown to be a potent oncogene in mice and may influence the host cell cycle in a similar manner like the vCyclin of KSHV (see 1.4.2.).

Another viral homologue present in MHV68 as well as in KSHV is the viral G-protein coupled receptor (vGPCR). Both are encoded as orf74 in the right end of the genomes and display the ability to transform cells (Siegel 1969; Arvanitakis, Geras-Raaka et al. 1997; Wakeling, Roy et al. 2001). Other functions seem to be more diverse: the KSHV vGPCR is one of the many components leading to an enhanced NFκB activity during KSHV infection to aid KSHV infection (Couty, Geras-Raaka et al. 2001; Schwarz and Murphy 2001; Cannon, Philpott et al. 2003). In contrast high NFκB levels inhibit the lytic MHV68 infection (Brown, Song et al. 2003). Like with vCyclin, it was demonstrated, that vGPCR is necessary for an

efficient reactivation of the lytic cycle *in vivo* (Lee, Koszinowski et al. 2003; Moorman, Virgin et al. 2003). The murine vGPCR is still dependent on binding of chemokines to induce signal cascades, while the KSHV vGPCR was shown to be constitutively active without stimulation by chemokines (Holst, Rosenkilde et al. 2001; Verzijl, Fitzsimons et al. 2004).
The product of the orf50 is like its KSHV counterpart termed RTA and the main regulator of lytic reactivation (Sun, Lin et al. 1998; Lukac, Kirshner et al. 1999; Wu, Usherwood et al. 2000). The deletion of RTA in MHV68 leads to severe impairment during lytic infection and a decreased ability to establish latency in the spleen, which resulted in a greatly reduced host colonization (Boname, Coleman et al. 2004; May, Coleman et al. 2004; Rickabaugh, Brown et al. 2004). The infection of virus with deregulated RTA protected to a certain degree against infection with wt virus, suggesting the use of deregulated orf50 herpesvirus as a vaccine (Boname, Coleman et al. 2004; Rickabaugh, Brown et al. 2004).
MHV68 encodes like KSHV a multifunctional latent protein in orf73, which shares several functions with KSHV LANA-1. The deletion of the orf73 in the MHV68 virus results in a severe deficiency of splenic latency *in vivo*, which supports the importance of orf73 proteins for virus genome maintainance during latent phase of infection (Fowler, Marques et al. 2003; Moorman, Willer et al. 2003). The orf73 knockout virus was able to prevent a challenging wt virus infection and therefore could be used as a vaccine (Fowler and Efstathiou 2004). A more detailed description of orf73 proteins can be found in section 1.5.

1.3. Replication of *Rhadinoviruses*

The members of the subfamily of *Rhadinovirus* have, like all *Herpesviridae,* two modes of replication. Lytic replication results in the production of progeny virions and spread of the virus infection to new host cells, whereas latent replication is characterized by the expression of only few essential genes and the maintainance of a low number of viral episomes.
Both variants of replication nevertheless follow the general course of every replication of DNA. The starting point of replication are special sites of the genome calles origins, here the unwinding of the two DNA strands by accessory proteins takes place. A small template is generated on the leading strand to facilitate binding of the DNA dependent DNA polymerase, which catalyzes the incorporation of desoxynucleoside triphosphate (dNTP) units into the new DNA strand by separation of pyrophosphate . The new desoxynucleosid monophosphate is incorporated at 3'-OH group of the growing DNA strand. The DNA is dublicated in a

semiconservative fashion, as each strand of the old DNA serves as a template for one newly synthesized strand. DNA synthesis stops at specific sites, termini. Besides the DNA-dependent DNA polymerase several other proteins are required to recruit the polymerase to the origin, promote processity during elongation of the new DNA strand, stabilize DNA single strands and perfom various other tasks.

In a latently infected cell the viral genome is present in a circularized form termed episome. Episomes could be detected in KS lesions as well as in PEL cell lines (Renne, Lagunoff et al. 1996). The number of maintained viral episomes was constant over time, but varied between 25 to several 100s in different PEL cell lines (Cesarman, Moore et al. 1995; Renne, Zhong et al. 1996; Cannon, Ciufo et al. 2000). The episome is maintained as an extrachromosomal entity in the host cell nucleus.

The viral gene expression pattern during latency is very restricted, with very few exceptions only six genes of KSHV are expressed: the latency associated nuclear antigen 1 (LANA-1), the viral cyclin homologue (vCyclin), the viral apoptosis inhibitor (vFLIP), a group of membrane associated proteins called Kaposin-A, -B and –C, the viral interferon regulator 3 (vIRF-3) also termed LANA-2, and a cluster of KSHV micro RNAs (miRNA) (Jenner, Alba et al. 2001; Rivas, Thlick et al. 2001). Only the LANA-1 protein could be detected in samples of all KSHV infected cells, providing a reliable marker for cells latently infected with KSHV (Rainbow, Platt et al. 1997; Katano, Sato et al. 2000). LANA-2 was only detected in latently infected B cells, suggesting a tissue specific expression pattern for this protein (Rivas, Thlick et al. 2001).

The latent expression profile for MHV68 is slightly different. The LANA-1 homologue encoded in orf73 is present as well, the other detected transcripts are: the MHV68 specific gene M2, the viral apoptosis inhibitor vBcl-2, the viral interleukin homologue vIL-8 and three transcripts of orf75 (Martinez-Guzman, Rickabaugh et al. 2003).

The first evidence for a latent origin within the KSHV genome was obtained, when a cosmid encompasing the left end of the genome including TRs could be maintained for a long time period in the presence of LANA-1 (Ballestas, Chatis et al. 1999). Further mapping revealed, that a single TR is suffient to mediate longterm persistence of extrachromosomal DNA plasmids (Ballestas and Kaye 2001; Garber, Shu et al. 2001; Hu, Garber et al. 2002). The C-terminal amino acids (aa) 1026 to 1129 of LANA-1 were determined as minimal interaction site with the TR (Komatsu, Ballestas et al. 2004; Kelley-Clarke, Ballestas et al. 2007). The TR was in the focus of additional studies, which identified two binding sites for LANA-1 within the TR. The first LANA binding site (LBS) is an 17 bp long imperfect palindrome,

separated by a five bp spacer from the second LBS, which is except for three nucleotide changes identical to LBS1 (Garber, Hu et al. 2002). Previously Schwam and colleagues demonstrated, that the C-terminal DNA binding domain of LANA-1 dimerizes in solution (Schwam, Luciano et al. 2000). These findings imply, that LANA-1 is recruited to the latent origin in similar manner like the EBV nuclear antigen 1 (EBNA-1) to the EBV latent origin (Yates, Camiolo et al. 2000). A homodimer of the LANA-1 proteins binds with high affinity to LBS1 and permits binding of another LANA-1 homodimer to the LBS2 (Garber, Hu et al. 2002). Deletion analyses of the TR showed, that a plasmid containing an 105bp subfragment of the TR replicates in the presence of LANA-1 as well as plasmids with one or two compelete TR units (Hu, Garber et al. 2002). Despite the two LBS, this fragment featured a 29 to 32bp long GC rich element upstream of the LBSs, termed as replication element due to its necessity for replication (Hu and Renne 2005). A LANA-1 mutant containing the C-terminus is capable to mediate replication at a basal level, however the presence of the full length protein greatly enhances the replication ability (Hu, Garber et al. 2002). LANA-1 was shown to recruit different proteins, which play a role in DNA replication like origin recognition complexes (Orc), histones and DNA modifying enzymes (Lim, Sohn et al. 2002; Stedman, Deng et al. 2004; Verma, Bajaj et al. 2006; Hu, Liu et al. 2009). One study identified an additional DNA sequence within the KSHV genome capable of mediating autonomous replication independent of LANA-1 (Verma, Lan et al. 2007). This additional latent origin explains the failure to inhibit growth in PEL cells by LANA-1 knockdown (Godfrey, Anderson et al. 2005).

For MHV68 to date no studies exist, that identify a potential latent origin within the MHV68 genome. However by the use of an orf73 deficient virus, the requirement of the murine homologue of LANA-1 for establishment of a longterm latent infection was demonstrated, which suggest MHV68 latent replication might be similar to that of KSHV (Fowler, Marques et al. 2003; Moorman, Willer et al. 2003).

Lytic replication is completely different compared to latent replication. It induces a broad cascade of viral genes to be expressed, which result in production of progeny virions and finally death of the host cell. The preferential state of KSHV seem to be the latentcy, as the majority of KHSV infected cells are in the latent state in the absence of a exogenous stimilus. But a certain fraction of cells undergo lytic reactivation, that is characterized by expression of the main lytic switch protein regulator of transcriptional activation (RTA), which is encoded in orf50 (Lukac, Renne et al. 1998; Sun, Lin et al. 1998). The expression of RTA alone is sufficient to induce the lytic reactivation and results in production of new virions . The

reactivation can be blocked by ectopic expression of a dominant negative RTA mutant (Lukac, Kirshner et al. 1999). RTA activates several viral and cellular genes by binding to RTA recognition sites within their promotors, including its own promotor, thereby featuring an autonomous feedback loop for RTA (Deng, Young et al. 2000; Gwack, Hwang et al. 2001; Byun, Gwack et al. 2002; Chang, Shedd et al. 2002; Deng, Chu et al. 2002; Gwack, Hwang et al. 2002; Liang, Chang et al. 2002; Wang, Wu et al. 2003). RTA expression promotes proliferation of infected cells by inhibiting p53 mediated apoptosis, this effect is mediated by interaction of RTA with STAT3 and the following downregulation of p53 transcription (Gwack, Hwang et al. 2001; Gwack, Hwang et al. 2002). RTA recruits various cellular transcription factors to activate different promotors. For its own promotor it cooperates with the cellular protein Octamer-1 (Sakakibara, Ueda et al. 2001), but RTA also interacts with the cellular proteins C/EBPα, CREB binding protein (CBP) and RBP-Jk (Gwack, Byun et al. 2001; Liang, Chang et al. 2002; Wang, Wu et al. 2003). The binding of RTA to promotors allows the recruitment of SWI/SNF chromatin remodeling and TRAP/mediator complexes to the DNA, which is required for a sucessful reactivation (Gwack, Baek et al. 2003).

Another level of modulation is mediated by the interaction with the viral K8/Kb-ZIP protein, which is another viral modulator of lytic transcription but unable to induce reactivation alone (Polson, Huang et al. 2001). K8/KbZIP supresses RTA mediated activation of K8 and orf57 promotors (Izumiya, Lin et al. 2003; Liao, Tang et al. 2003), otherwise it was shown to cooperate with RTA to activate K8 and RTA promotors (Wang, Wu et al. 2003).

The process of reactivation can be triggered by several mechanisms. Treatment with tetra phorbol acetate (TPA) induces the AP-1 pathway and the C/EBPα protein leading to expression of RTA, which releases the lytic cycle .(Renne, Zhong et al. 1996; Miller, Heston et al. 1997; Wang, Wu et al. 2003; Wang, Wu et al. 2003; Wang, Wu et al. 2004). Another class of chemicals, used as activators of lytic cycle, are histone deacetylase (HDAC) inhibitors e.g. sodium butyrate (Miller, Heston et al. 1997; Lu, Zhou et al. 2003). The study of Lu and coworkers revealed, that the RTA promotor is associated with cellular HDACs. Treatment with HDAC inhibitors releases the nucleosome from the RTA promotor and enables start of RTA transcription equivalent with lytic reactivation (Lu, Zhou et al. 2003). Hypoxia seems to be a physiological condition for reactivation, as the accumulation of hypoxia inducible factor 1 (HIF-1) induces the transcription of RTA and orf34 by HIF responding elements (HRE) in their promotors (Davis, Rinderknecht et al. 2001; Haque, Davis et al. 2003).

Like expected from the massive inference of KSHV with the immune system, several cytokines and soluble factors could led to lytic reactivation including IFN γ, oncostatin M and HIV TAT (Varthakavi, Browning et al. 1999; Blackbourn, Fujimura et al. 2000; Mercader, Taddeo et al. 2000).

The DNA replication during lytic infection is started from two lytic orgins in the left and right half of the KSHV LUR. Both origins consist of a nearly identical core sequence of 1.1kbp each flanked by a 600bp long G/C-rich repeat (AuCoin, Colletti et al. 2002; Lin, Li et al. 2003). The viral proteins RTA and KbZIP bind via specific response elements to the DNA and mediate loading of the core replication machinery complex onto the DNA (Wang, Tang et al. 2006). This core machinery is neccesary to replicate KSHV DNA and contains six viral proteins: a DNA single stand binding protein, a DNA helicase-primase complex composed of helicase, primase and primase-associated factor, the DNA dependent DNA polymerase and a polymerase processivity factor (AuCoin, Colletti et al. 2004). The DNA is then replicated, most probably in the "rolling circle" mechanism. The DNA is packaged into virions and the virions egress the nucelus (see 1.2.1. KSHV life cycle, assembly, and egress).

The lytic replication of MHV68 is driven by the protein encoded in orf50 as with KSHV. Also called RTA, it is responsible for viral DNA replication as well as inducing the lytic gene cascade (Wu, Usherwood et al. 2000; Wu, Tong et al. 2001; Pavlova, Virgin et al. 2003). The blocking of RTA by ectopic expression of a dominant negative RTA or use of a RTA deficient MHV68 virus to infect mice both demonstrated the absolute necessity of RTA for lytic reactivation (Wu, Tong et al. 2001; Pavlova, Virgin et al. 2003). The KSHV RTA can activate murine RTA dependent promotors and induce reactivation in cells latently infected with MHV68, displaying the close similarity between human and murine RTA (Rickabaugh, Brown et al. 2005). This close relation in control of lytic reactivation between the two viruses is further supported by the finding, that HDAC inhibitors induce lytic reactivation of cells latently infected with MHV68 *in vitro* as well as *in vivo* (Yang, Tang et al. 2009). The organisation of the lytic origins in the MHV68 genome is analogous to KSHV, with one origin at each end of the LUR of the MHV68 genome, the functionality of both origins has been proven by knockout viruses (Deng, Chu et al. 2004; Flach, Steer et al. 2009; Gong, Qi et al. 2009).

1.4. Cell cycle and its alteration by *Rhadinoviruses*

Proliferation is one of the fundamental characteristics of life, in a multicellular organism the control of proliferation respectively over cell division is an essential task to ensure the integrity of the organism. Therefore eukaryotic and particular human cells evolved a well regulated sequence, which is completed by dividing cells with every cycle of cell division. This cell cycle is divided into four general phases. For most of their lifetime cells stay in the G1 (gap 1) phase, in this phase the cells execute its tissue specific tasks e.g. provide metabolic catalysis. Some cells stay in G1 phase throughout their lifetime and do not divide, in this setting this phase is also termed G0 to indicate the stop of the cell cycle. In late G1 phase the cells start to prepare the replication of its genomes. This is performed in the S (synthesis) phase, where the replication machinery generates a copy of the cellular genome. The G2 phase is a control phase, in which the two copies of the genome are checked for their integrity. A noticed DNA damage results in the activation of DNA repair pathways and if they fail to repair the damage, induction of apoptosis. When the integrity of DNA was ensured, the cell cycle proceeds to the M (mitosis) phase, during which the cell division is executed. With the successful completion of the M phase the cell cycle closes and can start again from the beginning (see figure 4).

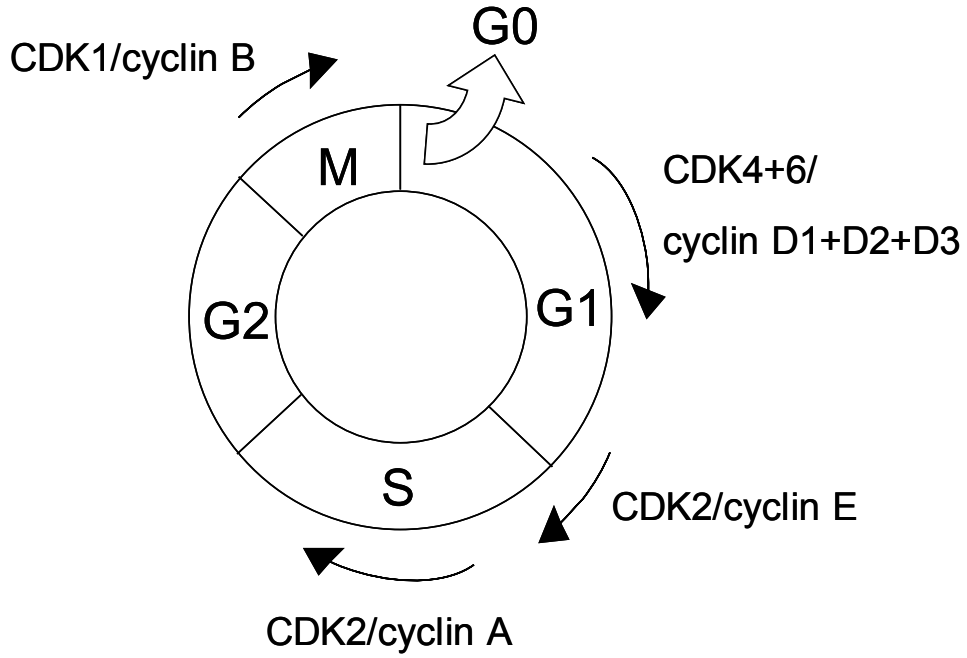


Figure 4. Scheme of the cell cycle.

In the figure the circle illustrates the four phases of the cell cycle together with complexes of cyclins and cyclin dependent kinases (CDKs) required for cell cycle progression. In addition the resting G0 phase is presented as an exit arrow out of the cell cycle.

1.4.1. Cell cycle regulation

Cyclins and cyclin dependent kinases (CDK)

The progression of the cell cycle is well regulated, as an uncontrolled cell division would ultimately result in the development of cancer. The main backbone of cell cycle regulation is formed by the cyclin dependent kinases (CDK) and their interaction partners, the cyclins. The CDKs are only functionally active, if a cyclin is interacting with them. The protein levels of CDKs are generally constant, while the protein levels of the different cyclins alter during the progression of the cell cycle. The CDKs differ in their affinity for the existing cyclins and thereby allow a regulated sequence of progression steps. CDKs 4 and 6 display a specifity for D-type cyclins, CDK2 interacts with cyclin A and cyclin E and CDK1 binds to cyclin B. The binding of CDK2 to the different cyclins alters its substrate specificity (see figure 4 (Sarcevic, Lilischkis et al. 1997; Zarkowska and Mittnacht 1997)).

CDK activity can be modified by phosphorylation, the phosphorylation can be activating as well as repressing. To enable the regulation via phosphorylation various CDK activating kinases and CDK activating phosphatases exist (Nigg 1995; Russo, Jeffrey et al. 1996; Endicott, Noble et al. 1999).

Another important regulator of CDK activity are the CDK inhibitors (CKI), which repress the activity of CDK/cyclin complexes by interaction with them. One of the major CKI families consists of four members with molecular masses of 16, 15, 18 and 19kDa, which are capable of inhibiting CDK4 and therefore named $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The INK4 CKI interact specifically with CDK4 and CDK6, but not CDK1 and CDK2. The binding of INK4 CKIs prevents binding of D-type cyclins to CDK4 and CDK6 (Russo, Tong et al. 1998; Jeffrey, Tong et al. 2000). The second major CKI family is named cyclin inhibitory protein or CDK inhibitory protein or short Cip/Kip. Three different proteins are described and termed $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ according to their molecular masses. By binding of cyclin D, E and A the CDK activity is greatly reduced by $p21^{Cip1}$and $p27^{Kip1}$, while cyclin B dependent kinase activity is only weakly affected (el-Deiry, Tokino et al. 1993; Gu, Turck et al. 1993; Harper, Adami et al. 1993; Xiong, Hannon et al. 1993; Toyoshima and Hunter 1994). The Cip/Kip inhibitors function by binding to CDK cyclin complexes with high affinity, but also interact with separate CDKs and cyclins with lower affinity (Harper, Elledge et al. 1995; Chen, Saha et al. 1996; Lin, Reichner et al. 1996).

The localization of cyclins is another alternative to regulate CDK activity. To execute their function CDK/cyclin complexes are dependent on their nuclear localization. The cyclins A and E, expressed during G1 and S phase, are predominantly nuclear, in keeping with their functions in S phase progression and DNA synthesis. The cyclin B responsible for progression to mitosis is detected in the cytoplasm during G1/S phase and is relocated to the nucleus upon start of mitosis (Pines and Hunter 1991; Ohtsubo, Theodoras et al. 1995; Takizawa and Morgan 2000)

The transition from one phase of cell cycle to the next phase is correlated with a fast reduction of protein levels of certain proteins, which is how cyclins were first observed (Evans, Rosenthal et al. 1983). This degradation is mediated by ubiquitin dependent proteolysis. The cyclins are targets of specific E3 ubiquitin ligases, which connect ubiqutin chains to lysines in the target protein. The ubiquitin chain is the signal for transport to proteasomes and the following degradation by the proteasomes. The activity of the E3 ubiquitin ligases is tightly regulated by phosphorylation and inhibitory proteins and is only activated, if all prerequisites for the cell cycle progression were accomplished (Harper, Burton et al. 2002).

The retinoblastoma protein (Rb) and progression to S phase

The checkpoint at the transition from G1 to S phase is very important to ensure integrity of the cell cycle, as many cancers are characterized by a deregulation of this checkpoint. A major player in the progression to S phase is the retinoblastoma protein (Rb). The Rb protein has a molecular weight of 105 kDa, two other variants of Rb are known and termed p107 and p130 according to their molecular masses. These three proteins form the “pocket” protein family, named after a pocket like structure, which is formed by the two subunits of the proteins connected via a linker region (Giordano, Rossi et al. 2007). This pocket represents a site for protein protein interactions and is the supposed binding site for proteins, which interfere with chromatin structure like HDACs or the SWI/SNF chromatin remodeling complex (Dunaief, Strober et al. 1994; Singh, Coe et al. 1995; Kouzarides 1999; Zhang, Gavin et al. 2000). Another well characterized interaction partner are transcription factors of the E2F family. The E2F transcription factors can be roughly divided into activating factors as E2F-1, -2 and -3 or inhibitory factors like E2F-4 and -5. At the beginning of the G1 phase p130 interacts with E2F-4 and -5 and blocks E2F dependent promotors, while the Rb protein binds to E2F-1, -2 and -3 in solution, thereby inhibiting E2F dependent promotors are cooperatively. The direct interaction between Rb and E2F factors was demonstrated to block E2F promoted

transcription (Helin, Harlow et al. 1993). In the middle of G1 phase the p130 levels start to decrease, which partially releases E2F promotors. With progression of the cell cycle by mitogenic signals the CDK4/6 cyclin D complexes start to phosphorylate Rb. The Rb protein features 16 different phosphorylation sites (Knudsen and Wang 1997). At the G1/S transition the CDK2/cyclin E complex phosphorylates all three pocket proteins and finishes the successive phosphorylation of Rb. This interrupts the binding of the activating E2F factors to Rb and transcription of E2F dependent genes is enabled. The inhibitory E2F factors lack a nuclear localization signal, in contrast to their activating counterparts. They are only localized in the nucleus in combination with p107 and p130. So the abrogation of the interaction between the inhibitory E2F factors and p105/p130, combined with the proteosomal degradation of p105/p130, further supports the transcription of E2F dependent genes and marks the transition from G1 into S phase (Sun, Bagella et al. 2007).

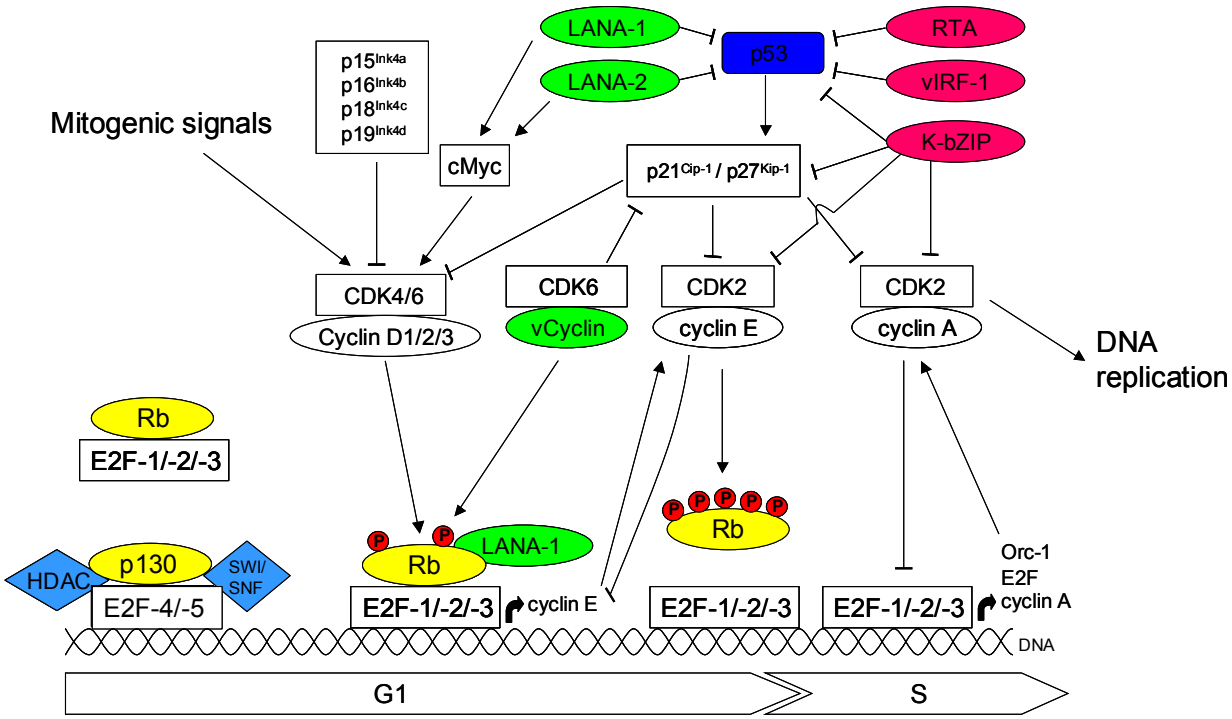


Figure 5. Representation of main events during G1 to S transition of cell cycle.

Proteins labeled green mark viral latent proteins, while a purple colour indicates lytic proteins of KSHV. Members of the pocket protein family are coloured in yellow and DNA modifing enzymes are drawn in light blue. The repressing p130/E2F-47-5 complexes block E2F dependent promotors in the beginning of G1. Through partial phosphorylation of Rb protein, which is mediated by CDK4 and CDK6 in complexes with D-type cyclins upon activating mitogenic signals, the complex of Rb with activating E2F-1/-2/-3 is translocated to E2F dependent promotors. Expression of cyclin E and subsequent formation of CDK2/cyclin E complexes results in hyperphosphorylation of the Rb protein and its dissociation from E2F-1/-2/-3. This allows transcription of E2F dependent genes like cyclin A, origin recognition complex protein 1 (ORC-1) and E2F itself, leading to progression into S phase with active DNA replication.

1.4.2. Cell cycle modification by *Rhadinoviruses*

The members of the genus *Rhadinovirus* have evolved several strategies to manipulate the cell cycle of their host cells to favour either latent or lytic infection.

Viral Cyclin

The viral Cyclin (vCyclin) is the most obvious protein, capable of interfering with the host cell cycle. Both KSHV and MHV68 express a viral cyclin from their latent gene cluster at the right end of the genome (Cesarman, Nador et al. 1996; Virgin, Latreille et al. 1997). The KSHV vCyclin interacts with human CDK2, 4, 6 and 9, but forms only an active complex with the CDKs 6 and 9 . The vCyclin act like a D-type cyclin, phosphorylates Rb and promotes progression to S phase (Swanton, Mann et al. 1997). However the broader substrate specificity of the CDK6/vCyclin complexes also enables phosphorylation of $p21^{Cip1}$, $p27^{Kip1}$, which abrogates their function as CKI (see figure 4 (Ellis, Chew et al. 1999; Mann, Child et al. 1999; Sarek, Jarviluoma et al. 2006)). The expression of vCyclin was verified in KS lesions, suggesting an oncogenic potential for vCyclin (Ojala, Tiainen et al. 1999). This is in line with the observation, that the expression of vCyclin in primary cells induces DNA synthesis, but the cells are not immortalized and actually exhibit p53 mediated growth arrest and defects in cytokinesis (Verschuren, Klefstrom et al. 2002). The inhibitory effects could be explained by a recent study, that demonstrated an interaction between vCyclin and CDK9, which is a regulator of p53. CDK9/vCyclin complexes enhances phosphorylation of p53 leading to a growth arrest (Chang and Li 2008). The vCyclin of MHV68 has a different binding capacity than the KSHV vCyclin, preferrentially interacting with CDK2. Nevertheless the CDK2/vCyclin complexes mediates phosphorylation of Rb and the CKI $p27^{Kip1}$ *in vivo* and of $p21^{Cip1}$, Bcl-2, and p53 in *in vitro* kinase assays (Upton, van Dyk et al. 2005). Whether the murine vCyclin inhibits $p21^{Cip1}$ and is sufficent for S phase progression is still debated (Yarmishyn, Child et al. 2008). The murine vCyclin was demonstrated to be necessary for lytic reactivation in the spleen upon intranasal infection of mice, but not when the mice were infected intraperintoneally (van Dyk, Virgin et al. 2000; Upton and Speck 2006). The murine vCyclin is required to induce a lymphoproliferate disease in immunocompromised mice (Tarakanova, Kreisel et al. 2008).

Latency-associated nuclear antigen 1 (LANA-1)

As a key component of the latent infection LANA-1 interfers with cell cycle control in various ways. A direct involvement of LANA-1 in cell cycle manipulation was proven, when Radkov and collegues discovered a direct interaction between LANA-1 and the active hypophosphorylated Rb (Radkov, Kellam et al. 2000). LANA-1 interacts with the Rb pocket and thereby abrogates the ability of the Rb protein to block E2F dependent promotors. A dysregulation of the G1/S checkpoint was demonstrated by transformation of rodent cells upon coexpression of LANA-1 with activated oncogene H-Ras (Radkov, Kellam et al. 2000).
Another advance into S phase is delivered by the LANA-1 mediated activation of Wnt pathway. LANA-1 is capable of binding to glycogen synthase kinase 3β (GSK3β), which is responsible for phosphorylation of β-catenin, resulting in the degradation of the protooncogene β-catenin (Fujimuro and Hayward 2003). One of the several target genes of β-catenin is cMyc, which itself activates the human telomerase promotor, contributing to a LANA-1 mediated induction of telomerase activity in KSHV infected cells (Wu, Grandori et al. 1999; Verma, Borah et al. 2004). In addition cMyc induction may cause cytoplasmic accumulation of D-type cyclins, due to their phosphorylation, further promoting progression into the S phase (see figure 4 (Verschuren, Jones et al. 2004)).
The induction of a cell cycle arrest and apoptosis by the cellular p53 protein is circumvented by LANA-1. The direct interaction of LANA-1 with p53 lead to an inhibition of p53 dependent transcription and p53 mediated apoptosis (Friborg, Kong et al. 1999). This mechanism could potentially prevent the induction of cell cycle arrest or apoptosis caused by vCyclin.
The MHV68 homologue of LANA-1 encoded in orf73 is much less investigated. An interaction of MHV68 orf73 with p53 was observed in *in vitro* pulldown experiments (Ottinger, Pliquet et al. 2009). A study of Forrest and coworkers showed an impact of MHV68 orf73 on p53, as a p53 knockout decreases the cytotoxicity observed upon infection of mice with a orf73 deficient MHV68 virus (Forrest, Paden et al. 2007).

LANA-2/vIRF-3

The viral interferon response factor 3 (vIRF-3) shares some functional similarities with LANA-1. The interaction of vIRF-3 with p53 results in a downregulation of p53 dependent transcription as well as p53 mediated apoptosis (Rivas, Thlick et al. 2001). In addition vIRF-3

was found to upregulate cMyc dependent transcription by interaction with the cMyc inhibitor MM-1α. The upregulation leads to elevated CDK4 levels and therefore promote the deregulation of the cell cycle (see figure 4 (Lubyova, Kellum et al. 2007)). vIRF-3 expression is restricted to B cells and reflects a cell type specific latency expression pattern, which is further supported by the necessity of vIRF-3 for survival of PEL cell lines (Wies, Mori et al. 2008).

Lytic proteins

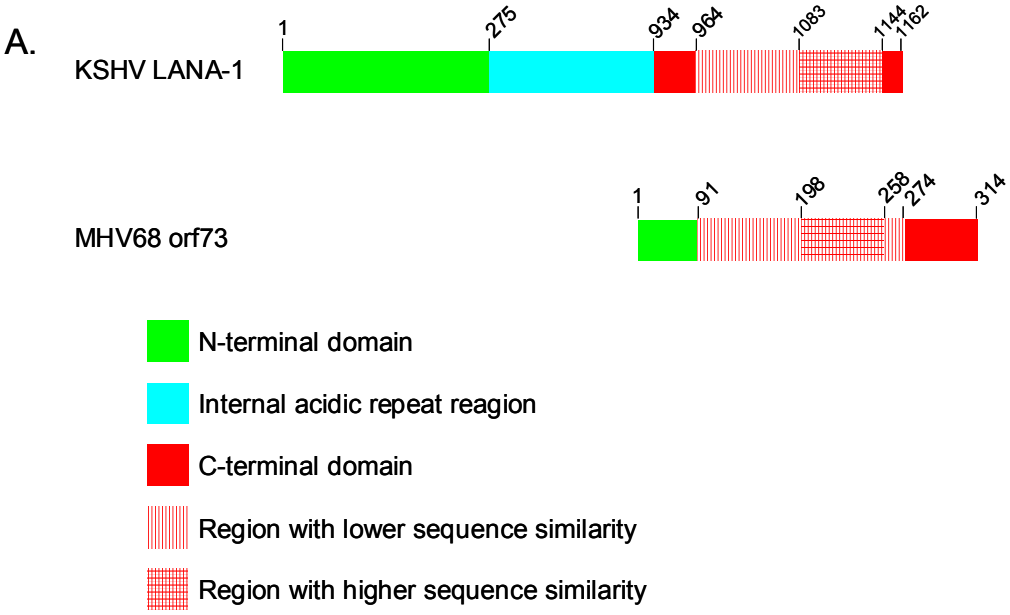
Three lytic proteins of KSHV have been demonstrated to interfere with the cell cycle of the host cell, the Regulator of transcriptional activation (RTA)/orf50, K-bZIP/orfK8 and the vIRF-1/orfK9. All of them interact with members of p300/CBP family of histone acetyltransferases (HAT), that serve as coadaptors to enable p53 dependent transcription (Park, Seo et al. 2000; Gwack, Hwang et al. 2001; Nakamura, Li et al. 2001). The HAT activity is required to acetylate p53 and histones at p53 dependent promotors to prepare transcription. Direct interactions of vIRF-1 and K-bZIP with p53 may occur and further decreases p53 mediated apoptosis. K-bZIP relocates p53 to promyelocytic leukemia protein (PML) bodies, abolishing regular p53 function in apoptosis (Katano, Ogawa-Goto et al. 2001). The vIRF-1 additionally directs p53 for degradation, possibly by enhancing the E3 ubiquitin ligase activiy of p300/CBP (Shin, Nakamura et al. 2006). This is also connected to a prevention of induction of the DNA damage pathway ATM, which would activate p53 mediated apoptosis (Shin, Nakamura et al. 2006). The K-bZIP further alters the cell cycle by binding to $p21^{Cip1}$ and therefore block cells in late G1 phase (Wu, Tang et al. 2002; Wu, Wang et al. 2003). This block is further supported by binding of K-bZIP to CDK2, which inactivates the CDK responsible for transition into S phase (see figure 4 (Izumiya, Lin et al. 2003)). Together all actions of this lytic proteins result in a prolonged G1 phase during lytic phase of infection, ensuring a supporting environment for a productive virus replication.

1.5. Orf73 encoded proteins of *Rhadinoviruses*

Both *Rhadinoviruses* investigated in this thesis encode a latent protein in the open reading frame 73 (orf73). The orf73 protein expressed by KSHV, also termed latency-associated nuclear antigen 1 (LANA-1), is 1162 aa long, in contrast to the much shorter orf73 protein of MHV68, which consists only of 314 aa. The MHV68 orf73 protein lacks the internal acidic repeat region of LANA-1 and the N-terminal part of the MHV68 orf73 protein exhibits only minimal sequence homology to LANA-1. In contrast, the C-terminal half of the MHV68 orf73 protein features a sequence similarity of 36% to LANA-1, if amino acids 964-1162 of LANA-1 are compared with amino acids 91-274 of the MHV68 orf73 protein. When the compared region is reduced to amino acids 1083-1144 of LANA-1 and amino acids 198-258 of the MHV68 orf73 protein the degree of sequence similarity even raises to 48% (see figure 6). This implies a functional similarity between the LANA-1 protein of KSHV and the orf73 protein of MHV68, which are introduced in details in the following paragraph.

1.5.1. Latency-associated nuclear antigen 1 of KSHV

KSHV encodes only a few proteins, which are expressed during the latent phase of infection, one of them is the latency-associated nuclear antigen 1 (LANA-1) encoded in the orf73 at the right end of the long unique region (LUR) of the KSHV genome (Russo, Bohenzky et al. 1996). LANA-1 is transcribed together with two other latent genes, vCyclin/orf72 and vFLIP/K13, at the right end of LUR in a polycistronic messenger RNA (Dittmer, Lagunoff et al. 1998). Protein expression of LANA-1 is detected in the overwhelming majority cells of KSHV associated neoplesias: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) or multicentric Castleman's disease (MCD) (Gao, Kingsley et al. 1996; Kedes, Operskalski et al. 1996; Rainbow, Platt et al. 1997; Dupin, Fisher et al. 1999). Due to its ubiquitous nature LANA-1 protein expression is today correlated with KSHV infection and antibodies to LANA-1 form the backbone of most current serological assays for KSHV diagnosis (Olsen, Sarid et al. 2000).



B.

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KSHV    931  TVEEPIILHG SSSEDEMEVD YPVVSTGEQI ASSPPGDNTP DDDPQPGPSR   980
MHV68    58  QGTQTPNPPS PPVPPSSPTL PSSPVPPSSP VHEPPSPSPP PAPPSPDVDV   107

KSHV    981  EYRYVLRTSP PHRPGVRMRR VPVTHPKKPH PRYQQPPVPY RQIDDCPAKA  1030
MHV68   108  EGLDVGETDD PGPPP----- ----------P KRYSRYQKPH NPSDPLPKKY   143

KSHV   1031  RPQHIFYRRF LG--KDGRRD PKCQWKFAVI FWGNDPYGLK KLSQAFQFGG  1078
MHV68   144  QGMRRHLQVT APRLFDPEGH PPTHFKSAVM FSSTHPYTLN KLHKCIQSKH   193

KSHV   1079  VKAGPVSCLP H-PGPDQSPI TYCVYVYCQN KDTSKKVQMA RLAWEASHPL  1127
MHV68   194  VLSTPVSCLP LVPGTTQQCV TYYLLSFVED KKQAKKLKRV VLAYCEKYHS   243

KSHV   1128  AGNLQSSIVK FKKPLPLTQP GENQGPGDSP QEMT                   1162
MHV68   244  S--VEGTIVK AKPYFPLPEP PTEP-PTDPE QPSTSTQASG              280

Identical: 55 aa

Similar:   13 aa
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Figure 6. Comparison of KSHV LANA-1 with MHV68 orf73 protein.

(A) Schematic diagram of LANA-1 consisting of a N-terminal domain, a central acidic repeat region and a C-terminal region, in total 1162 aa. The MHV orf73 protein is much smaller with 314 aa and lacks the internal repeat region. Red stripes indicate areas with lower degree of protein sequence similarity, while a red cross pattern marks regions with higher sequence similarity.

(B) The alignment of the protein sequences of KSHV LANA-1 and MHV68 orf73 reveals regions with different degrees of similarity, in total 68 aa are either identical or at least similar in the region of aa 964-1162 in KSHV LANA-1 compared with aa 91-264 in MHV68 orf73, corresponding to 36% similarity. The degree of similarity raises to 48%, regarding only the region of aa 1083-1144 in KSHV LANA-1 and respectively aa 198-258 in MHV68 orf73.

The structure of the LANA-1 protein can be roughly separated into three parts: a N-terminal part, an internal repeat region with a variable number of repeats containing acidic amino acids and a C-terminal part composed of a proline rich region and a region with increased number of acidic and hydrophobic amino acids (Russo, Bohenzky et al. 1996). The different number of acidic repeats in the internal repeat region results in LANA-1 proteins with various lengths, ranging from 982 to 1162 aa. The LANA-1 protein investigated in this thesis was derived from the PEL cell line BC-1 and consists of 1162 aa (Russo, Bohenzky et al. 1996).

Like the name of LANA-1 indicates, it can be detected in the nuclei of KSHV infected cells and exhibits a specific speckled pattern within the nucleus (Gao, Kingsley et al. 1996; Kedes, Operskalski et al. 1996; Rainbow, Platt et al. 1997). The LANA-1 protein is capable of attaching to interphase chromatin as well as mitotic chromosomes, suggesting a constant function in nuclear environment during the whole cell cycle (Piolot, Tramier et al. 2001; Viejo-Borbolla, Kati et al. 2003). For the association with mitotic chromosomes the N-terminal amino acids 5-20 are essential, as the crystallization of a histone nucleosome with a peptide encompassing the first 23 aa of LANA-1 revealed a tight interaction of this LANA-1 peptide with an acidic patch formed by the histones H2A and H2B (Barbera, Chodaparambil et al. 2006). This interaction explains the requirement for aa 5 to 15 of LANA-1 for chromosomal attachment, which had been reported previously by other groups (Piolot, Tramier et al. 2001; Krithivas, Fujimuro et al. 2002). The demonstrated interactions with histone H1 and Methyl CpG binding protein 2 (MeCP2) seem therefore not to mediate attachment of LANA-1 to cellular chromosomes (Cotter and Robertson 1999; Krithivas, Fujimuro et al. 2002).

The C-terminal part of LANA-1 posseses an additional binding site for chromosomes, which is dispensable in the presence of the N-terminal binding site, but mediates LANA-1 chromosomal attachment and episomal persistence when the N-terminal binding site is mutated (Kelley-Clarke, De Leon-Vazquez et al. 2009). While full length LANA-1 is distributed over the whole chromosomes like its interaction partners H2A and H2B, the LANA C-terminus is located at the pericentromeric and peritelomeric regions of the mitotic chromosomes, suggesting a different interaction partner for the C-terminal chromosomal binding site (Kelley-Clarke, Ballestas et al. 2007).

Our group identified the BET protein Brd2/RING3 (see 1.6. for details) as a binding partner of LANA-1, interacting with two C-terminal regions of LANA-1 aa 1007-1055 and aa 1048-1162 (Platt, Simpson et al. 1999). Another BET protein, the Brd4 protein, was demonstrated to interact with the LANA-1 C-terminus as well, both variants $Brd4^{S}$/Hunk and $Brd4^{L}$/MCAP

showed that ability (Ottinger, Christalla et al. 2006; You, Srinivasan et al. 2006). As both BET proteins feature binding to acetylated histones via their bromodomains, they are possible candidates to mediate binding to mitotic chromosomes. The study by You and coworkers showed a colocolization of LANA-1 and Brd4 but not Brd2 on mitotic chromosomes and therefore the interaction with Brd4 may support episomal persistence of KSHV genomes (You, Srinivasan et al. 2006).

The N-terminus as well as the C-terminus contain nuclear localization signals (NLS), which correlate with nuclear localization of isolated N- and C-terminal LANA-1 constructs (Schwam, Luciano et al. 2000; Piolot, Tramier et al. 2001). Only the C-terminal constructs showed the speckled staining pattern like LANA-1 wt, the N-terminal constructs showed a diffuse staining pattern (Schwam, Luciano et al. 2000). This phenotype is connected with the ability of LANA-1 to form dimers respectively oligomers, only C-terminal but not N-terminal constructs were able to oligomerize (Schwam, Luciano et al. 2000). This hypothesis is further supported by the observation of our group, that truncations from the C-terminal end of the LANA-1 protein lead to abrogation of a speckled nuclear distribution, in parallel resulted in a loss of oligomerization (Viejo-Borbolla, Kati et al. 2003; Viejo-Borbolla, Ottinger et al. 2005).

The LANA-1 protein is capable of interacting with a broad range of cellular proteins, thereby interfering with various functions of the cell and providing an environment favourable for viral latency. The major keystone of the regulation of apoptosis, the p53 protein, is bound by LANA-1 and p53 dependent apoptosis decreases (Friborg, Kong et al. 1999). The interaction was confirmed in KSHV associated malignancies, were apoptosis was found to be rare, while no mutations of p53 were detectable (Katano, Sato et al. 2001). LANA-1 was shown to form a complex with p53 and its regulator MDM2, upon treatment with an MDM2 inhibitor p53 was released, which lead to apoptosis of KSHV infected cells (Sarek, Kurki et al. 2007). In addition Cai and collegues demonstrated, that LANA-1 is a component in a EC_5S ubiquitin complex, this complex mediates ubiquitination of p53 and another cellular tumour suppressor protein, von Hippel Lindau (VHL), and targets them for protesomal degradation (Cai, Knight et al. 2006). The LANA-1 protein promotes formation of the EC_5S ubiquitin complex via its SOCS motif, which consists of a BC-box in the N-terminus, interacting with Elongin and a Cullin-box in the C-terminus, that mediates binding to Cullin (Cai, Knight et al. 2006). Therefore LANA-1 protects KSHV infected cells from induced cell death.

Another important cellular protein, the retinoblastoma protein (Rb, described in 1.4.1.) is interacting with the LANA-1 protein (Radkov, Kellam et al. 2000). The interaction was shown by *in vitro* assays as well as in PEL cells and the interaction domain was mapped to aa 770 to 930 of LANA-1, which bind to the 'pocket' domain of Rb formed by aa 379-792 (Radkov, Kellam et al. 2000). The observed upregulation of E2F dependent promotors was later confirmed by a microarray, that revealed LANA-1 mediated induction of several Rb/E2F dependent genes (Radkov, Kellam et al. 2000; An, Compitello et al. 2005). The overexpresion of LANA-1 promotes, in cooperation with the cellular oncogene Harvery rat sarcoma viral oncogene homologue (H-Ras), the transformation of primary rat embryonic fibroblasts and prevents a cell cycle arrest in cells overexpressing the CDK inhibitor $p16^{INK4a}$ (Radkov, Kellam et al. 2000; An, Compitello et al. 2005).

LANA-1 also targets the cell cycle of the host cell via by binding to glycogen synthase kinase 3β (GSK3β), which is part of the Wnt signaling pathway and responsible for degradation of β-catenin by phosphorylation (Fujimuro, Wu et al. 2003). The binding of LANA-1 to GSK3β requires the N-terminal aa 241-275 and a C-terminal region encompassing aa 1133-1147 of LANA-1, through this interaction GSK3β is relocated to the nucleus, resulting in elevated β-catenin levels by its increased stability (Fujimuro and Hayward 2003; Fujimuro, Wu et al. 2003). The C-terminal LANA-1 binding domain mediates binding in a similar way like the cellular GSK3β interaction partner axin, while the N-terminal LANA-1 binding site is phosphorylated by GSK3β, which is necessary for the interaction, but abolishes the ability of GSK3β to phosphorylate other targets (Fujimuro, Liu et al. 2005). Despite the elevated levels of β-catenin, the abrogation of GSK3β activity by LANA-1 results in activated cMyc. On one hand the missing phosphorylation of cMyc by GSK3β prevents its degradation, on the other hand LANA-1 directly interacts with cMyc and promotes cMyc phosphorylation by ERK1, leading to transcriptional active cMyc (Bubman, Guasparri et al. 2007; Liu, Martin et al. 2007). Both effects contribute to the increase in S phase progression observed in LANA-1 expressing primary endothelial cells (Fujimuro, Wu et al. 2003).

LANA-1 enhences its own expression by a direct interaction of LANA-1 with its promotor, leading to a constant LANA-1 expression during viral latency (Krithivas, Young et al. 2000; Jeong, Papin et al. 2001; Renne, Barry et al. 2001; Jeong, Orvis et al. 2004). LANA-1 also targets several other cellular and viral promotors and either activates or reduces their transcriptional activity. Depending on the conditions used, LANA-1 was shown either to activate the latent EBV C promotor or on the other hand repress the transcription from it (Krithivas, Young et al. 2000; Groves, Cotter et al. 2001; Viejo-Borbolla, Kati et al. 2003). A

strong repression was observed, when LANA-1 was tethered to constitutively active promotors by heterologous DNA binding domain, this effect was linked to domains in LANA-1 N- and C-terminus (Schwam, Luciano et al. 2000). By binding to p53 LANA-1 prevents transcription of p53 dependent promotors (Friborg, Kong et al. 1999). The transcription factor activating transcription factor 4 (ATF4)/ cAMP response element binding protein 2 (CREB2) is bound by LANA-1, thereby abrogating the induction mediated by ATF4/CREB2 (Lim, Sohn et al. 2000). Renne and coworkers demonstrated the inhibition of NFκB dependent genes by LANA-1 and in addition observed a repression of long terminal repeat (LTR) promotor of HIV (Renne, Barry et al. 2001). Furthermore LANA-1 was shown to abolish the transcription activity of a single KSHV terminal repeat (TR) element by interaction with the 801bp long TR element (Garber, Shu et al. 2001).

On the other hand LANA-1 acts as an activator of several promotors. Cellular interleukin 6 (cIL-6) is an important growth factor in KSHV associated diseases (Screpanti, Musiani et al. 1996; Asou, Said et al. 1998). The transcription of cIL-6 is upregulated in the presence of LANA-1, by an interaction of LANA-1 with the transcription factor cJun, a member of the activator protein 1 (AP-1) family (An, Lichtenstein et al. 2002; An, Sun et al. 2004). This upregulation is further supported by the LANA-1 interaction with the transcription factor Signal transducers and activators of transcription 3 (STAT3), which results in an increased transcriptonal activity of STAT3 also targeting the cIL-6 promotor (Muromoto, Okabe et al. 2006). Another target of STAT3 is the survivin gene, which was shown to be upregulated and crucial for inhibition of apoptosis in KSHV associated malignancies (Aoki, Feldman et al. 2003; Lu, Verma et al. 2009). Several artifical promotors containing only the minimal TATA box were induced by the LANA-1 protein, indicating, that LANA-1 is able to manipulate the basal transcription machinery (Renne, Barry et al. 2001). As mentioned above LANA-1 upregulates the latent EBV LMP-1 and C promotors (Groves, Cotter et al. 2001; Viejo-Borbolla, Kati et al. 2003). The transcription of E2F and Rb dependent genes is upregulated upon ectopic expression of LANA-1, like mentioned before (Radkov, Kellam et al. 2000; An, Compitello et al. 2005). A possible role in the reactivation plays the interaction of LANA-1 with the hypoxia-inducible factor 1α (HIF-1α), which is mediated by the N-terminus of LANA-1 and results in increased transcription, protein stability and nuclear relocalization of the HIF-1α protein (Cai, Lan et al. 2006; Cai, Murakami et al. 2007). The elevated levels of HIF-1α lead to induction of hypoxia responsive elements (HRE) containing promotors, as the promotor of the lytic switch protein RTA features several of this HRE, it is induced upon hypoxia causing lytic reactivation (Davis, Rinderknecht et al. 2001; Cai, Lan et al. 2006). This

induction is further supported by ectopic LANA-1 expression in a dose dependent manner (Cai, Lan et al. 2006). Therefore LANA-1 is a bifunctional regulator of transcription, capable of induction and repression of promotors. This allows LANA-1 to modulate a broad network of cellular and viral targets and thus to support the latent infection by KSHV.

As described in the section 1.3. LANA-1 is essential for the replication of KSHV in multiple ways. It binds to histones via its N-terminal domain, while its C-terminal domain mediates interaction with TR elements of KSHV episomes (Ballestas, Chatis et al. 1999; Garber, Hu et al. 2002; Barbera, Chodaparambil et al. 2006). Therefore LANA-1 achieves a successful distribution of viral episomes into daughter cells during cell division, and is thus, in combination with TR containing episomes, a minimal prerequisite for latent persistence (Ballestas, Chatis et al. 1999; Ballestas and Kaye 2001; Garber, Hu et al. 2002; Hu, Garber et al. 2002; Viejo-Borbolla, Kati et al. 2003).

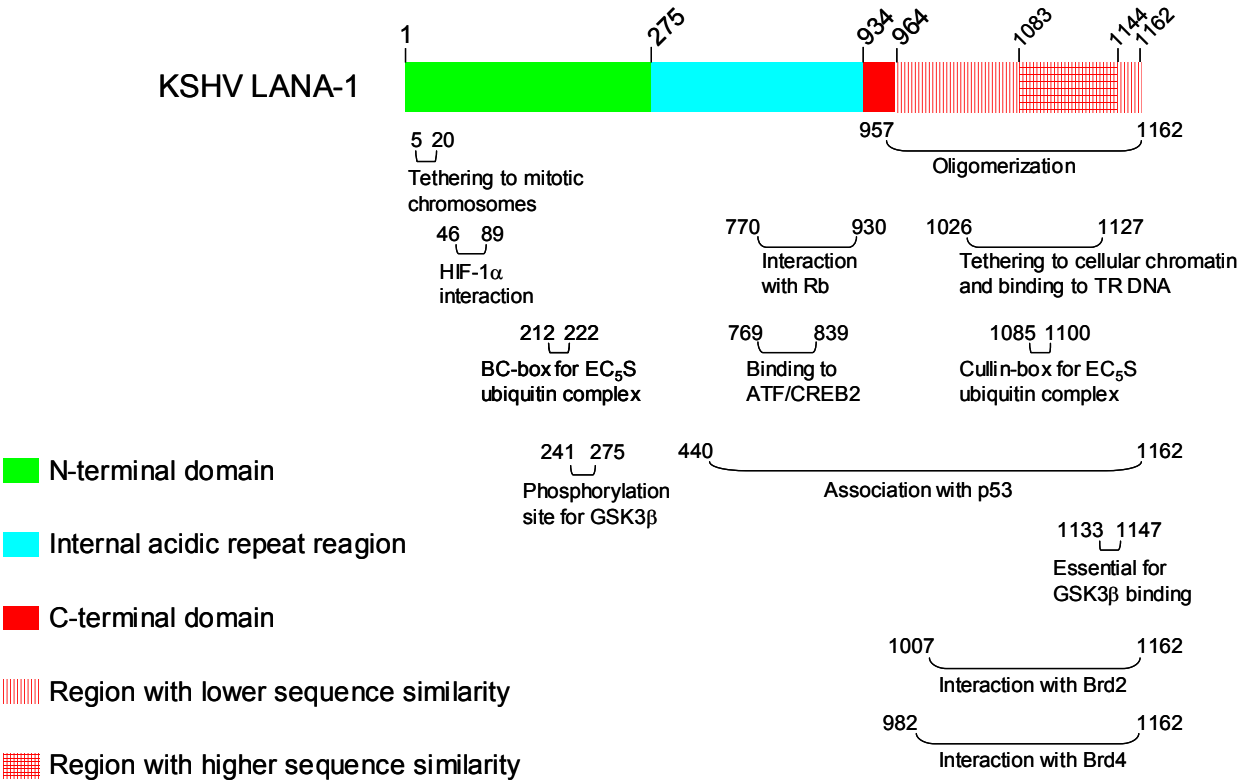


Figure 7. Overview of LANA-1 mediated functions.

Representation of LANA-1 protein with selected features of the LANA-1 protein

1.5.2. The orf73 protein of MHV68

The orf73 protein of MHV68 consists of only 314 aa and is the shortest of *Rhadinoviral* orf73 proteins known today. It lacks the internal acidic repeat region, present in the centre of the LANA-1 protein. When protein sequences of LANA-1 and MHV68 orf73 are aligned, aa 198-258 of MHV68 orf73 resemble the LANA-1 sequence to a quite high degree of 48%, implying functional similarities between the two proteins. It also features an additional C-terminal tail, which has so far not been linked to a specific function. In general the MHV68 orf73 protein is far less characterized than the LANA-1 protein.

The MHV68 orf73 gene was classified by two studies as an immediate early gene (Rochford, Lutzke et al. 2001; Ebrahimi, Dutia et al. 2003), but a study on the S11 cell line, which is latently infected with MHV68, showed transcription of MHV68 orf73 during latentcy (Martinez-Guzman, Rickabaugh et al. 2003). The implied classification of MHV68 orf73 as a latent gene was further supported, as a later study was able to prove the presence of MHV68 orf73 transcripts in cells extracted from the spleen of latently MHV68 infected mice (Allen, Dickerson et al. 2006).

The regulation of MHV68 orf73 transcription is complicated, with three different identified promotors, the first lies within the terminal repeats (TR) of MHV68 over 10kbp away from the initiation codon of the orf73 gene. The second promotor was located at the very right end of the LUR of MHV68 just in front of the TRs, both promotors were identified by random amplification of cDNA ends (RACE) in murine 3T3 cells infected with MHV68. A third, less potent, promotor directly upstream of the initiaton codon of orf73 gene and overlapping with the orf75 gene was identified by a RNAse protection assay (Coleman, Efstathiou et al. 2005). The transcripts of the first and second promotor were also detected in splenocytes of latently infected mice and were further spliced, which results in two different mRNAs from the two distant promotors (Allen, Dickerson et al. 2006). The third proximal promotor has till now not be further investigated.

The elimination of the orf73 gene in recombinant MHV68 viruses showed no effects in *in vitro* infection, however the establishment of splenic latency during *in vivo* infection was severly impaired (Fowler, Marques et al. 2003; Moorman, Willer et al. 2003). A MHV68 orf73 knock out virus exhibits a vaccine potential, as a later challenge with MHV68 wt virus could be prevented (Fowler and Efstathiou 2004). The involvement in immune evasion processes was later proven by a study, that demonstrated the failure of T helper cells specific for MHV68 orf73 to control the MHV68 infection *in vivo* (Smith, Rosa et al. 2006). The very

low MHV68 orf73 protein level and its remarkably stability might contribute to this evasion function (Smith, Rosa et al. 2006).

A later study with a MHV68 orf73 knock out virus showed an impaired viral replication at very low multiplicity of infection (MOI) (Forrest, Paden et al. 2007). In contrast, there was again no diffence compared to MHV68 wt virus in infection at high MOI, but the cells infected with the orf73 deficient virus presented a much higher percentage of dead cells caused by the infection (Forrest, Paden et al. 2007). The increased cell death aligned with induction of the apoptosis by p53 and a deregulated viral gene expression (Forrest, Paden et al. 2007).

Recently it was revealed, that the MHV68 orf73 protein is capable of inhibiting tumour necrosis factor (TNF) mediated induction of NFκB (Rodrigues, Filipe et al. 2009). The MHV68 orf73 features a Cullin-box in its C-terminus similar to the one identified in KSHV LANA-1, but lacks the BC-box necessary for interaction with Elongin. Nevertheless MHV68 orf73 was able to ubiquitinate the p65 member of the NFκB family in a Cullin and Elongin dependent manner (Rodrigues, Filipe et al. 2009). The mutation of the Cullin-box in MHV68 orf73 results in abrogation of p65 ubiquitination and restoration of TNF induced NFκB activation *in vitro* (Rodrigues, Filipe et al. 2009). When this mutation was introduced in a recombinant MHV68 virus, a strong reduction in splenic latency similar to the MHV68 orf73 knockout is observed upon infection of mice, displaying the significance of NFκB inhibition for the *in vivo* infection (Rodrigues, Filipe et al. 2009).

1.6. Bromodomain and extraterminal domain containing (BET) proteins

The family of BET proteins is a group of transcriptional regulators, present in animal, fungi and plants. Like the name indicates, all members of the BET protein family are characterized by the presence of one (in plants) or two (in animal and yeast) bromodomains (BD1 and BD2) in the N-terminal part and a single extraterminal (ET) domain in the C-terminal part of the protein (Florence and Faller 2001). The first member identified in the BET protein family was the female sterile homeotic gene (fsh) in *drosophila melanogaster* followed by the mammalian really interesting gene 3 (RING-3) and the yeast protein Bdf-1 (Haynes, Mozer et al. 1989; Beck, Hanson et al. 1992; Lygerou, Conesa et al. 1994). Other members were later identified and will be described later in this section.

The first three identified BET proteins are potent modulators of the transcription of several promotors, suggesting a sequence unspecific involvement of BET proteins in transcriptional control (Lygerou, Conesa et al. 1994; Denis, Vaziri et al. 2000; Sinha, Faller et al. 2005).

Bromodomain

The bromodomain is a conserved sequence motif, which can be found in various protein families and which mediates tethering to cellular chromatin. Proteins, that exhibit bromodomains, are transcriptional regulators and histone acetyl transferases (HAT) (Gansheroff, Dollard et al. 1995; Mizzen, Yang et al. 1996).
The bromodomain itself is a 110 aa long motif, interacting with acetylated lysines residues of other proteins. Acetylated lysines can be found in transcription factors like the viral TAT protein from HIV or within the cellular p53 protein, but especially common at the tails of histones forming nucleosomes (Jacobson, Ladurner et al. 2000; Owen, Ornaghi et al. 2000; Dorr, Kiermer et al. 2002; Mujtaba, He et al. 2002; Matangkasombut and Buratowski 2003; Mujtaba, He et al. 2004). In the last two years the structures of both bromodomains in several members of the BET protein family have been solved, they will be discussed in details later in this section.

Extraterminal (ET) domain

The ET domain of BET proteins is a site of protein protein interactions and is highly conserved in the case of the mammalian BET proteins. It consists of 64 aa, which share a sequence identity of over 90%. The ET domains of the mammalian BET proteins Brd2, Brd3 and Brd4 are suffiencent for an interaction with the KSHV LANA-1 protein and its orf73 homologue in MHV68 (Platt, Simpson et al. 1999; Ottinger, Christalla et al. 2006; You, Srinivasan et al. 2006; Ottinger, Pliquet et al. 2009). In addition the ET domain is the assumed binding site for E2F transcription factors (Denis, Vaziri et al. 2000). The C-terminus including the ET domain of the yeast BET protein Bdf-1 was demonstrated to interact with the TBP-associated factor (TAF) 67, which forms together with TATA-binding protein (TBP) the basal transcription factor $TF_{II}D$ (Matangkasombut, Buratowski et al. 2000). Recently the structure of the murine Brd4 ET domain has been solved, which showed no obvious similarity to known structures. The ET-domain itself was not capable to oligomerize and and consists of

three α-helices and a loop structure, which present an unknown but well defined structure (Lin, Umehara et al. 2008).

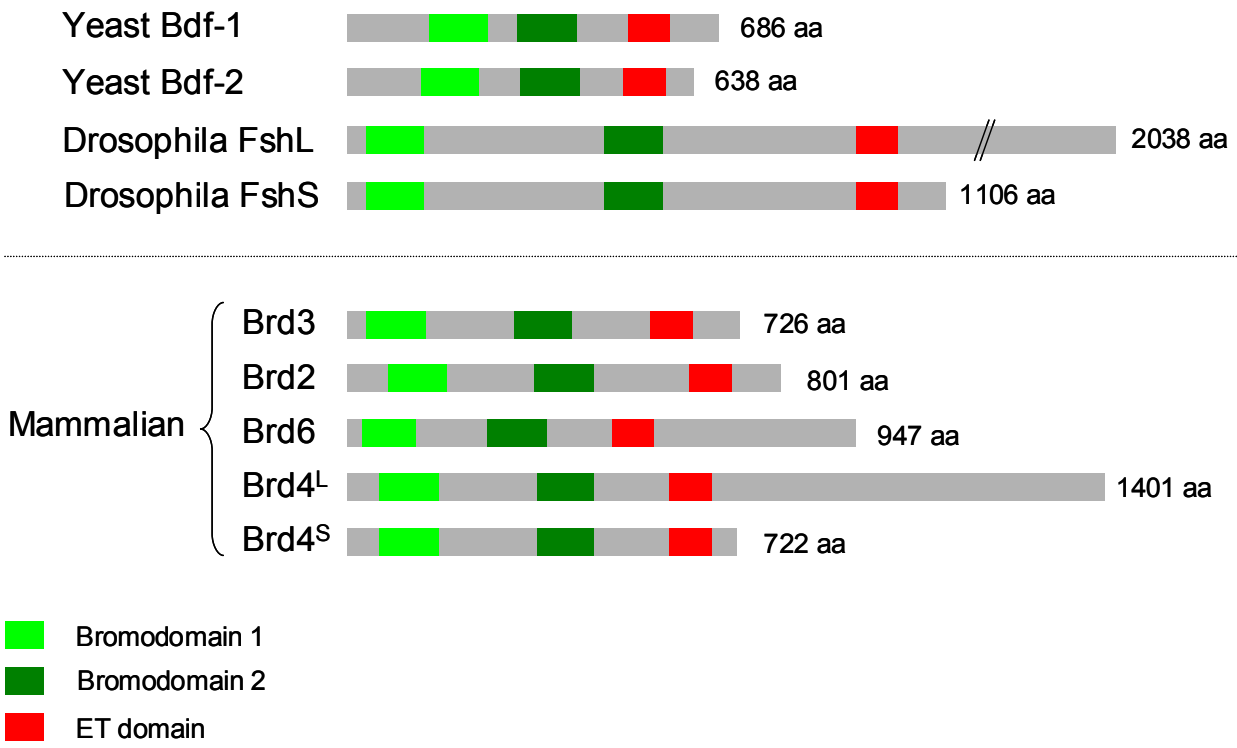


Figure 8. Comparison of mammalian BET proteins with other eukaryotic homologues.
All BET protein feature two bromodomains, coloured in light respectively dark green and an extraterminal (ET) domain, highlighted in red

1.6.1. BET proteins in yeast and drosophila

Yeast Bdf-1 and Bdf-2 proteins

Yeast expresses two BET proteins termed Bdf-1 and Bdf-2. Yeast cells deficient in Bdf-2 exhibit no obvious phenotype, in contrast to Bdf-1 knock out cells, which show defects in snRNA transcription, sugar metabolism and meiosis as well as a temperature sensitive lethality (Chua and Roeder 1995). The abrogation of both Bdf genes has a lethal outcome, pointing at a certain degree of redundancy of the two Bdf genes in some functions (Chua and Roeder 1995). The Bdf-1 protein features, apart from its association with TAF67, an ATP binding site and extracts containing Bdf-1 were demonstrated to have kinase activity, similar to the mammalian Brd2 protein (Matangkasombut, Buratowski et al. 2000). Through the interaction with TAF67, which is part of the basic transcription factor $TF_{II}D$, Bdf-1 and Bdf-2 are involved in transcriptional control (Matangkasombut, Buratowski et al. 2000). Later it was

proven, that the binding of Bdf-1 to chromatin was mediated by interaction of the bromodomains with acetylated histone H4 and the chromatin tethering is required for transcriptional control functions of Bdf-1 (Matangkasombut and Buratowski 2003).

Drosophila female sterile homoetic (fsh) protein

More than 30 years ago the first member of the BET protein family was identified, when genetic studies to detect genes linked to female sterility in *drosophila melanogater* were performed (Gans, Audit et al. 1975; Gans, Forquignon et al. 1980). The characterization of the gene termed female sterile homeotic (fsh) revealed the existence of several fsh isoforms (Digan, Haynes et al. 1986; Haynes, Mozer et al. 1989). A study of Haynes and coworkers characterized two of them. The short fsh isoform fsh^S consists of 1106 aa, while the long isoform fsh^L, which is 2038 aa long, comprises nearly the fsh^S, but use an alternative splicing site in the last exon of fsh^S (Haynes, Mozer et al. 1989). The splicing pattern of the fsh gene resembles the splicing pattern of the two variants of the mammalian Brd4 gene, described later in this section. Both isoforms of fsh possess a predicted ATP binding site and therefore might function as kinases, like the yeast Bdf proteins or the mammalian Brd2 protein (Florence and Faller 2001). Drosophila mutants that completely lack the fsh gene exhibit embryonic lethality, while different point mutations of the fsh gene correlated with defects in meiosis, oogenisis and development in the progeny drosophila (Gans, Forquignon et al. 1980; Digan, Haynes et al. 1986; Florence and McGinnis 1998).

1.6.2. Mammalian BET-proteins

Four different BET genes were detected in mammals so far and result in the expression of five BET proteins as the Brd4 encodes two isoforms of the Brd4 protein. The four genes found in humans and mouse are termed Brd2/RING3, Brd3/OrfX, Brd4/HUNK respectively MCAP and Brd6/BrdT and all contain two Bromodomains in their N-terminus and an ET-domain in the C-terminal part of the proteins. The Brd2 encodes for a protein of 801aa length, the Brd3 protein is 726aa long and the Brd6 protein 947aa, while Brd4 encodes for a short isoform designated as $Brd4^S$ or HUNK with 722aa and a long isoform called $Brd4^L$ or MCAP (Mitotic chromosome-associated protein) with 1401aa.

Brd3/OrfX

The Brd3 gene of humans is located on chromosome 9 (9q34) and its expression was observed ubiquitously in foetal tissues as well as in adult tissues (Thorpe, Gorman et al. 1997). However in murine foetal tissue Brd3 expression was linked with sex differentiation as Brd3 overexpression was observed in the developing testis but not ovary (Boyer, Lussier et al. 2004). In adult mice the Brd3 expression is widespread, but seem to be well orchestrated with the other BET proteins in spermatogenesis (Shang, Salazar et al. 2004). Like the other BET proteins, Brd3 was shown to interact with chromatin via acetylated histones and thereby facilitate RNA polymerase II mediated transcription (LeRoy, Rickards et al. 2008). A genetic screen on murine embryonic stem cells by Ishii and colleagues revealed a downregulation of Brd3 during differentiation of endothelial cells. In view of the altered Brd3 expression due to genomic rearrangement of its genomic locus in 4 out 12 patients with bladder cancer they propose a role for Brd3 expression in angiogenisis during oncogenesis (Ishii, Mimori et al. 2005). Another carcinoma with participitation of Brd3 is the NUT midline carcinoma (NMC), in which the nuclear protein in testis (NUT) is the target of a genomic rearrangement to either Brd4 or Brd3 (French 2008; French, Ramirez et al. 2008). Upon this relocation a NUT-Brd3 fusion protein is expressed, resulting in enhanced nuclear localization compared to NUT wt protein (French 2008; French, Ramirez et al. 2008). A downregulation of this NUT fusion proteins in NMC cell lines causes cell cycle arrest, indicating the importance of this fusion proteins for this carcinoma (French, Ramirez et al. 2008).

Brd6/BrdT

A Brd6 cDNA was identified in a testis specific cDNA library and therefore is also designated as BrdT (T for testis specific) (Jones, Numata et al. 1997). The gene is located on the human chromosome 1p and two different transcripts can be detected (Jones, Numata et al. 1997). The longer mRNA of 4kb contains a PEST motif, which is believed to cause protein instability, the PEST motif is spliced out in the more abundant 3.5kb mRNA, leading to a more stable Brd6 protein with a length of 947aa (Pivot-Pajot, Caron et al. 2003). The Brd6 protein interacts with acetylated histone H4 and induction of histone acetylation by trichostatin A mediates chromatin condensation in cells with ectopic Brd6 expression (Pivot-Pajot, Caron et al. 2003). Later studies correlated this effect with the developmental process of spermatogenesis, as Brd6 is expressed in mid to late spermatocytes corresponding with

acetylation of chromatin prior to chromatin condensation (Shang, Salazar et al. 2004; Govin, Lestrat et al. 2006). The functional necessary part of the BRD6 protein was mapped to the Bromodomain (BD) 1, as male mice carrying a Brd6 mutant lacking the BD1 but not BD2 produce abnormal sperms and are infertile (Shang, Nickerson et al. 2007). Very recently the molecular structure of the interaction of the Brd6 BD1 with histone H4 was elucidated, the BD1 preferentially interacts with a diacetylated histone H4 peptide (Moriniere, Rousseaux et al. 2009). The structure solution revealed a specific recognition of histone H4 acetylated at the lysines 5 and 8 by a distinct binding pocket (Moriniere, Rousseaux et al. 2009). Two studies identified uncommon Brd6 expression in cancer tissue of non-small cell lung cancer, suggesting a supportive role for Brd6 in oncogenesis, since Brd6 expression was not seen in all cases and frequently in combination with other genes regulating developing processes (Scanlan, Altorki et al. 2000; Grunwald, Koslowski et al. 2006).

Brd2/RING3

The Brd2 gene was discovered during a screen of a T cell cDNA library as homologue of the fsh gene in drosophila in 1992 and its location was determined to the short arm of chromosome 6 (6p21.3) (Beck, Hanson et al. 1992). High expression of Brd2 was detected in reproductive tissues of both genders and in established lymphoma cell lines as well as in lymphocytes isolated from leukemia patients (Denis and Green 1996; Rhee, Brunori et al. 1998; Shang, Salazar et al. 2004; Trousdale and Wolgemuth 2004). The lymphocyte specific overexpression of Brd2 from the Eμ promotor in transgenic mice led to the formation of B cell lymphoma and leukemia (Greenwald, Tumang et al. 2004). The active Brd2 protein is located in the nucleus and associated with euchromatin in proliferating tissue, but was shown to be cytoplasmic and inactive in resting neural cells, implying that Brd2 acts as a transcriptional regulator during cell cycle progression and developmental processes (Guo, Faller et al. 2000; Crowley, Kaine et al. 2002; Crowley, Brunori et al. 2004). In line with this hypothesis Brd2 was demonstrated to activate E2F dependent promotors of cell cycle regulation, resulting in elevated Cyclin A levels and accelarated cell cycle progression (Denis, Vaziri et al. 2000; Sinha, Faller et al. 2005; Denis, McComb et al. 2006). The binding to chromatin is mediated by attachment of the Brd2 to acetylated histones, as Kanno and coworkers were able to visualize an interaction between Brd2 and histone H4 acetylated at lysine 12 in living cells (Kanno, Kanno et al. 2004). Meanwhile the molecular structures of both bromodomains of the Brd2 protein have been solved: both resemble the general

conformation of other known bromodomains with four α-helices arranged in a tight left-handed bundle (Huang, Zhang et al. 2007; Nakamura, Umehara et al. 2007). Due to lower degree of similarity between the BD1 and BD2 of Brd2 in contrast to BD1 and BD2 of other BET proteins, differences in tertiary structure could be proposed and indeed the BD2 was observed to be monomeric, while evidence for a dimerization of the BD1 was presented (Huang, Zhang et al. 2007; Nakamura, Umehara et al. 2007). The suggested role in sensing the histone code, which is described as trancriptional regulation of genes through secondary modification of their associated histones like acetylation or phosphorylation, was further supported by the observed binding of Brd2 to the chromatin region of the Cyclin D1 gene (LeRoy, Rickards et al. 2008). This interaction was indispensable for Cyclin D1 transcription *in vivo* and Brd2 association facilitated RNA polymerase II transcription through hyperacetylated nucleosomes (LeRoy, Rickards et al. 2008). Studies investigating potential interaction partners of Brd2 identified several proteins related to transcriptional regulation like TATA binding protein (TBP), E2F-1 and its cofactor DP-1 and RNA polymerase II and chromatin remodeling proteins like members of Swi/Snf complex and HDAC 11 (Denis, McComb et al. 2006; Peng, Dong et al. 2007).

The impact of transcriptional regulation through Brd2 on development was studied mainly in mice, but recently two Brd2 related genes have been discovered in zebrafish (Dibenedetto, Guinto et al. 2008). However, in mice Brd2 was shown to be involved in spermatogenesis and development of neural tissue (Crowley, Kaine et al. 2002; Crowley, Brunori et al. 2004; Shang, Salazar et al. 2004). The significance for neural development was confirmed by two recent studies that demonstrated a knockout of Brd2 gene to be lethal during embryogenesis and be essential for correct closure of the neural tube (Gyuris, Donovan et al. 2009; Shang, Wang et al. 2009). This might explain a recently postulated association between two forms of epilepsy and Brd2 gene polymorphisms. However, this association could not be reproduced in all studies, implying that more factors are linked to these diseases (Pal, Evgrafov et al. 2003; Lorenz, Taylor et al. 2006; Cavalleri, Walley et al. 2007; de Kovel, Pinto et al. 2007).

The Brd2 protein was the first BET protein shown to interact with a viral protein, when our group was able to identify Brd2 as a binding partner of the KSHV LANA-1 protein in a yeast-two-hybrid assay (Platt, Simpson et al. 1999). The interaction led to phosphorylation of LANA-1 and due to the unique presence of an ATP binding site in the Brd2 protein, one could assume a kinase activity of Brd2 (Platt, Simpson et al. 1999). The originally observed kinase activity of Brd2 by Denis and colleagues could not be reproduced by later studies, suggesting a Brd2 associated kinase is responsible for the phosphorylation (Denis and Green

1996; Rhee, Brunori et al. 1998). The interaction of Brd2 with LANA-1 is mediated via the ET-domain of the Brd2 protein and a part of C-terminal domain in the LANA-1 protein (Platt, Simpson et al. 1999; Viejo-Borbolla, Ottinger et al. 2005). The overexpression of Brd2 led to a G1 arrest of cell cycle, which is partially reversed by coxpression of LANA-1 (Ottinger, Christalla et al. 2006).

Brd4/HUNK/MCAP

Two different transcripts are produced by the human Brd4 gene on chromosome 19 (19p13.1). The shorther one encompasses 11 exons and encodes for protein with 722 aa, which is termed Brd^S or HUNK, the longer transcript uses an alternative splicing site and consists of 19 exons in total. Exon 11 of HUNK is replaced by an alternative exon 11, resulting in the longer mRNA, which is translated into a 1401aa long protein, designated as Brd^L or MCAP. Most of the studies on Brd4 performed so far have been done with the murine $Brd4^L$ protein.

During embryogenesis of mice $Brd4^L$ is essential, as an abrogation of the Brd4 gene in transgenic mice leads to death shortly after implantation and is linked to deficiency in maintaining an inner cell mass *in vitro* (Houzelstein, Bullock et al. 2002). The transcripts of Brd4 have been detected in several murine tissues, indicating, together with other results, that Brd4 is involved in ensuring normal cell proliferation (Shang, Salazar et al. 2004).

$Brd4^L$ is a nuclear protein associated with euchromatin throughout the whole cell cycle including mitosis, this association is mediated by interaction of the bromodomains of Brd4 with acetylated histones (Dey, Ellenberg et al. 2000; Dey, Chitsaz et al. 2003). Binding experiments with histone peptides identified di- or tetraacetylated histone H4 and diacetylated histone H3 as interaction partners of the Brd4 protein (Dey, Chitsaz et al. 2003). Very recently the crystal structure of both bromodomains of Brd4 was determined and together with the previously published molecular structure of the second bromodomain confirms the interaction of Brd4 with the acetylated histones (Liu, Wang et al. 2008; Vollmuth, Blankenfeldt et al. 2009). In contrast to the Brd2 protein both studies demonstrated a monomeric status of both bromodomains, however a dimerization upon binding to acetylated histones is postulated for BD1 (Liu, Wang et al. 2008; Vollmuth, Blankenfeldt et al. 2009).

The ectopic overexpression of $Brd4^L$ in cell lines caused a cell cycle arrest in G1 phase, while depletion of $Brd4^L$ by injection of a $Brd4^L$ antibody led to inhibition of progression from G2 to M phase (Dey, Ellenberg et al. 2000; Maruyama, Farina et al. 2002; Dey, Chitsaz et al. 2003). The abrogation of Brd4 expression by small RNA interference also resulted in G1

arrest (Mochizuki, Nishiyama et al. 2008). Evidence for the assumed role as transcriptional regulator was reported in two studies that showed an interaction of $Brd4^{L}$ with the positive transcription elongation factor b (P-TEFb), which is a heterodimer of CDK9 and CyclinT (Jang, Mochizuki et al. 2005; Yang, Yik et al. 2005). Upon recruitment of the P-TEFb to $Brd4^{L}$ the inhibitory subunit of P-TEFb is replaced by $Brd4^{L}$ and RNA polymerase II dependent transcription is enhanced (Jang, Mochizuki et al. 2005; Yang, Yik et al. 2005). One of the two bromodomains is sufficient for binding of CyclinT to Brd4, in line with the recent observation that BD2 interacts with equal binding properties with acetylated CyclinT or histone H4 (Jang, Mochizuki et al. 2005; Vollmuth, Blankenfeldt et al. 2009). The equilibrium of inactive and active Brd4 associated P-TEFb is tightly regulated and linked with cell cycle progression and cell growth, respectively (He, Pezda et al. 2006).

The chromosomal rearrangement of the Brd4 gene in combination with the NUT gene (also see Brd3 section) led to expression of a Brd4 NUT fusion protein with abnormal stable nuclear localization, correlating with highly aggressive NUT midline carcinomas (French, Miyoshi et al. 2001; French, Miyoshi et al. 2003; French 2008). In addition a study showed, that the cellular Brd4 levels in breast cancer patients could predict their survival, implying a possible involvement of Brd4 in breast cancer development (Crawford, Alsarraj et al. 2008).

The Brd4 protein is a target of several viral proteins that employ Brd4 functions for their own purposes. The first viral proteins demonstrated to interact with the $Brd4^{L}$ protein were the E2 proteins of bovine papillomavirus (BPV) 1 and human papillomavirus (HPV) 16 (You, Croyle et al. 2004). Due to the fact, that Brd4 by that time was only known to associate with mitotic chromosomes, it was suggested, that Brd4 mediates tethering to mitotic chromosomes during cell segregation via its binding to the E2 protein, which was already identified as a necessary step in viral genome maintainance. This assumption was further validated by several studies for the BPV-1, but could not be observed with various other HPV types, suggesting a different cellular factor to be involved in the maintainance of at least some HPV genomes (Brannon, Maresca et al. 2005; McPhillips, Ozato et al. 2005; You, Schweiger et al. 2005; Abbate, Voitenleitner et al. 2006; Dao, Duffy et al. 2006; McPhillips, Oliveira et al. 2006). In fact, Parish and colleagues found an E2 mutant, that still interacts with Brd4 and transactivate E2 specific promotors, but was unable to stably maintain viral genomes. This mutant was defective in binding to the cellular protein ChIR1, which was suggested to be the cellular component for viral episome persistence (Parish, Bean et al. 2006). While a function for Brd4 in HPV genome maintainance seems unlikely, the necessity of the interaction of the viral E2 protein with the $Brd4^{L}$ protein for transcriptional activation was confirmed by several studies

(McPhillips, Oliveira et al. 2006; Schweiger, You et al. 2006; Senechal, Poirier et al. 2007; Jang, Kwon et al. 2009). The interaction with the $Brd4^{L}$ protein increases protein stability of the E2 protein, by inhibition of its ubiquitinylation, resulting in enhanced tethering of the E2 protein to chromatin and therefore elevated transcription of E2 dependent genes (Gagnon, Joubert et al. 2009; Lee and Chiang 2009; Zheng, Schweiger et al. 2009). The transcriptional repression function of the E2 protein is independent of Brd4, as E2 repression functions were shown to be unaffected by Brd4 depletion (Schweiger, Ottinger et al. 2007).

The second viral protein shown to interact with Brd4, was the KSHV LANA-1 protein, which was found to bind to the $Brd4^{L}$ as well as the $Brd4^{S}$ protein via the ET domain of the Brd4 protein (Ottinger, Christalla et al. 2006; You, Srinivasan et al. 2006). The LANA-1 and Brd4 proteins colocalize on mitotic chromosomes in the presence of artifical KSHV episomes, but Brd4 whethers participates in the maintainance of the KSHV episome is still unclear and most likely other factor are involved as well (You, Srinivasan et al. 2006). However ectopic LANA-1 expression partially rescued epithelial and B cells from G1 cell cycle arrest induced by overexpression of the $Brd4^{S}$ protein (Ottinger, Christalla et al. 2006). The atomic structure of the Brd4 ET domain necessary for the binding of LANA-1 has been published, revealing that the ET domain is composed of a bundle of three α-helices arranged in a unique fashion with no known homologue. The group failed in demonstrating binding of their ET domain construct to an immobilized LANA-1 peptide, however this could be easily explained by the requirement of LANA-1 to oligomerize to ensure correct function (Viejo-Borbolla, Ottinger et al. 2005; Stedman, Kang et al. 2008). Using the structure of the ET domain our group could recently identify individual residues involved in the interaction with LANA (Weidner-Glunde et al.; unpublished results).

Another two viral proteins associated with Brd4 are the TAT protein of HIV and the Tax protein of the Human T-lymphotropic Virus (HTLV). In both cases the viral proteins are responsible for transcriptional activation of the long terminal repeat (LTR) promotor in their genomes and this function is linked to activation of the P-TEFb complex by Brd4. Overexpression of the $Brd4^{L}$ protein led to reduced activation of the HTLV LTR promotor by Tax, while overexpression of only the carboxy terminal region of the $Brd4^{L}$ protein, which interacts with the P-TEFb complex, was sufficient to inhibit HIV replication (Bisgrove, Mahmoudi et al. 2007; Cho, Zhou et al. 2007; Urano, Kariya et al. 2008).

Recently the latent antigen protein of EBV EBNA-1 has been demonstrated to interact with $Brd4^{L}$ protein as well, not affecting the tethering of EBNA-1 to mitotic chromosomes (Lin, Wang et al. 2008). In contrast, dysregulation of the equilibrium between active and inactive P-

TEFb complexes by either Brd4 depletion or overexpression, resulted in decreased EBNA-1 dependent transcription, indicating again a role for Brd4 in transcriptional regulation (Lin, Wang et al. 2008).

1.7. Objectives

The best characterized orf73 protein in the subfamily of *Rhadinoviruses* is the LANA-1 protein of KSHV. However, most of the knowledge gained so far is based on *in vitro* experiments, due to the lack of an animal model for KSHV infection. Therefore the MHV68 virus and its orf73 protein may serve as a potential model system for KSHV infection and dissection of orf73 proteins *in vivo*.

Relative little is known about the MHV68 orf73 protein and its function. Previous studies performed in our group already identified some of the fundamental parameters of the MHV68 orf73 protein and demonstrated that it is able to assume several important tasks for viral latency, similar to its homologue LANA-1 in KSHV. Our group had shown that the MHV68 orf73 protein interacts with cellular BET proteins, in particular the Brd2 and Brd4^{S} proteins, via their ET domains. This observation was the starting point to address the first main topic of this thesis:

What is the molecular basis for the interaction between the MHV68 orf73 protein and cellular BET proteins?

Directly from the results of the first question arises the second question, which could be answered with specific MHV68 orf73 mutants not interacting with BET proteins:

What cellular processes are altered by the MHV68 orf73 protein via its binding to BET proteins?

Another important task was to further investigate the role of the interaction between BET proteins and the KSHV LANA-1 protein. A new method ot examine LANA-1 functions would be the interuption of LANA-1 functions with specific peptides, which interact with LANA-1.

We wondered if such peptides first exist and if they do, would they be able to abrogate known LANA-1 functions that are assummed to depend on the interaction between LANA-1and BET proteins?

2. Materials and Methods

2.1. Reagents

Chemicals were purchased from the following companies: Amersham, Applichem, Biomol, BioRad, Boehringer Mannheim, Fluka, Gibco, ICN, Invitrogen, Merck, New England Biolabs, Pierce, Promega, Roche, Roth, Santa Cruz, Seromed, Serva, Sigma, and Stratagene
Consumables from the following companies were used: Amersham, Beckman, Biozym, Eppendorf, Falcon, Gilson, Greiner, Kodak, Nalgene, Sarstedt, and Whatman
Restriction enzymes were bought from New England Biolabs.

2.1.1. Antibodies

αcMyc 9E10:
Biomol, mouse monoclonal, protein A purified IgG, used at a 1:700 dilution. Immunogen: human pp62 c-myc (408-432) peptide. Targets the cMyc epitope EQKLISEEDL.

αGFP antibody, clone JL-8:
BD Biosciences, mouse monoclonal antibody, IgG raised against *Aequorea Victoria* green fluorescent protein (GFP), recognizes native and denatured forms of wildtype or recombinant N- or C-terminal fusion proteins. Used at a 1:5000 dilution.

αHA antibody, clone 3F10:
Roche, rat monoclonal IgG, used at a 1:2000 dilution for immunoblots . Detects the peptide sequence YPYDVPDYA derived from the human influenza hemagglutinin protein.

αHA antibody, clone 12CA5:
Roche, mouse monoclonal IgG, used for immunoprecipitation and at a 1:200 dilution for IFA. Detects the peptide sequence YPYDVPDYA derived from the human influenza hemagglutinin protein.

αHHV8-orf73 antibody:

ABI, rat monoclonal IgG, used in immunoblots at a dilution of 1:2000, detects KSHV LANA protein.

αHHV8-orf73 antibody:

Novo Castra Laboratories, mouse monoclonal antibody, used at a 1:50 dilution for IFA, detects KSHV LANA protein.

αRb antibody, clone 4H1:

Cell Signaling, mouse monoclonal antibody, detects retinoblastoma protein at a dilution 1:2000 in immunoblots.

αThioredoxin antibody:

Sigma, rabbit polyclonal antibody, used at 1:1000 dilution for immunoblotting and 1:100 for IFA and recognizes the thioredoxin protein.

αHHV8-orf73 antisera:

Human antisera from KSHV patients have been used at different dilutions (1:500 to 1:5000) to detect KSHV LANA-1 by immunoblotting.

αRING3 antiserum:

rabbits were immunized with recombinant GST RING3 fusion protein; the resulting polyclonal rabbit antiserum (R29, 1999) was used at a 1:5000 dilution to verify Brd2/RING3 and/or GST expression. This antibody may crossreact with Brd4/HUNK.

αMouse-FITC antibody:

Dako, rabbit polyclonal antibody, secondary antibody for IFA, that detects mouse antibodies, coupled to FITC and used at a 1:40 dilution.

αMouse-Rhodamin X antibody:

Jackson Immuno Research, goat polyclonal antibody, secondary antibody for IFA, that detects mouse antibodies, coupled to Rhodamin X fluorophore and used at a 1:200 dilution.

αRabbit-FITC antibody:

Jackson Immuno Research, donkey polyclonal antibody, secondary antibody for IFA, that detects rabbit antibodies, coupled to FITC fluorophore and used at a 1:200 dilution.

2.2. Vectors and Primers

2.2.1. Eukaryotic expression vectors

pcDNA3:

With T7 and SP6 promotor for *in vitro* transcription, a CMV promotor for expression in mammalian cells, provides a bovine growth polyadenylation signal, ampicillin and neomycin resistance; Invitrogen.

pcDNA3-9E10:

Derived from pcDNA3, features the 9E10 myc epitope (EQLISEEDL), ampicillin and neomycin resistance; Vector was kindly provided by S. Mittnacht (London).

pcDNA3.1(+):

Derived from pcDNA3, lacks the SP6 promoter and is altered in the MCS, ampicillin and neomycin resistance; Invitrogen.

pcDNA3.1(-):

same vector as pcDNA3.1(+) but the MCS is in opposite orientation, ampicillin and neomycin resistance; Invitrogen.

pEGFP-C1:

Vector suitable for generation of GFP fusion proteins for expression and/or localization studies or expression of GFP alone in mammalian cells. The gene of interest is inserted in the MCS and expressed as a C-terminal fusion to C-terminal enhanced GFP, expression is driven by a CMV promotor, kanamycin- and neomycin resistance; Clonetech.

pVR1255-HA:

Empty expression vector for cloning of MHV68 orf73 C-terminal deletion constructs coding for a double HA-tag at the C-terminus of the cloned deletion contructs. By *Bam*HI/*Not*I digest of pVR1255-HA-MHV-orf73 wt generated empty pVR1255 plasmid was ligated to a ca. 100bp PCR fragment representing two HA-tags. The fragment was produced with the primers 73HA NotI F and 73HA BHI R and pVR1255-HA-MHV-orf73 wt as template; kanamycin resistance.

pEG202:

Expression vector for yeast-2-hybrid screens. The gene of interest is cloned in the MCS and then expressed as a LexA fusion protein; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

pCI-His10tag:

Expression vector for eukaryotic cells based on the pCI vector from Promega with a CMV promotor in front of a MCS and followed by a SV40 polyadenylation sequence, the sequence for the protein thioredoxin was inserted via the *Xba* I site in the MCS; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

<u>MHV68 orf73 vectors</u>

pVR1255-HA-MHV68-orf73 wt (MHV orf73 wt):

The orf73 gene of MHV68 was amplified by PCR (primers ORF73START and ORF73STOP) from cDNA of murid herpesvirus 4 strain 68, digested by *Not* I and *Bam* H I and ligated into the pVR1255 vector. Under use of the reverse primer ORF73STOP the sequence for a double HA-tag was added at the 3'end of the gene; kanamycin resistance; This plasmid was kindly provided by J.P. Stewart (Liverpool).

pVR1255-HA-MHV68-orf73 Δ262-314 (MHV orf73 Δ262-314):

With MHV orf73 wt as template and the primers ORF73 START NotI and R261 NotI a shorter orf73 sequence only coding for amino acids 1-261 of MHV68 was generated. The PCR product and pVR1255-HA were *Not*I digested and ligated; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 Δ253-314 (MHV orf73 Δ253-314):

With MHV orf73 wt as template and the primers ORF73 START NotI and R252 NotI a shorter orf73 sequence only coding for amino acids 1-252 of MHV68 was generated. The PCR product and pVR1255-HA were *Not*I digested and ligated; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 Δ237-314 (MHV orf73 Δ237-314):

With MHV orf73 wt as template and the primers ORF73 START NotI and R236 NotI a shorter orf73 sequence only coding for amino acids 1-236 of MHV68 was generated. The PCR product and pVR1255-HA were *Not*I digested and ligated; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 Δ225-314 (MHV orf73 Δ225-314):

With MHV orf73 wt as template and the primers ORF73 START NotI and R224 NotI a shorter orf73 sequence only coding for amino acids 1-224 of MHV68 was generated. The PCR product and pVR1255-HA were *Not*I digested and ligated; kanamycin resistance; generated by M. Ottinger.

MHV68 orf73 full length constructs with changes in the aminoacid sequence

All mutants have been generated with site directed mutagenesis kit by Stratagene with use of the indicated primers and templates

pVR1255-HA-MHV68-orf73 200-A (MHV orf73 S200A):

The sequence for amino acid 200 serine had been changed to an alanine residue by site directed mutagenesis with the primers MHV S 200 A F/R and MHV orf73 wt as template; kanamycin resistance.

pVR1255-HA-MHV68-orf73 228-A (MHV orf73 K228A):

The sequence for amino acid 228 lysine had been changed to an alanine residue by site directed mutagenesis with the primers MHV K 228 A F/R and MHV orf73 wt as template; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 228-4A (MHV orf73 228-4A):

The sequence for amino acids 228-231 (KKLK) had been changed to four alanine residues by site directed mutagenesis with the primers MHV 228 4A 231 F/R and MHV orf73 K228A as template; kanamycin resistance.

pVR1255-HA-MHV68-orf73 226-4A (MHV orf73 226-4A):

The sequence for amino acids 226-229 (QAKK) had been changed to four alanine residues by site directed mutagenesis with the primers MHV 226 4A 229 F/R and MHV orf73 K228A as template; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 225-7A (MHV orf73 225-7A):

The sequence for amino acids 225-231 (KQAKKLK) had been changed to seven alanine residues by site directed mutagenesis with the primers MHV 225 7A 231 F/R and MHV orf73 228-4A as template; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 226-DAADLE (MHV orf73 DAADLE):

The sequence for amino acids 226-231 (QAKKLK) had been changed to the sequence DAADLE by site directed mutagenesis with the primers MHV 226 DAADLE 231 F/R and MHV orf73 K228A as template, as result the polarity of the charged residues was inverted; kanamycin resistance; generated by M. Ottinger.

pVR1255-HA-MHV68-orf73 20-4A (MHV orf73 20-4A):

The sequence for amino acids 20-23 (CKRR) had been changed to four alanine residues by two succesive site directed mutagenesis reactions with the primer pair MHV68ORF73-22RR-AA Fw/Rv and MHV orf73 wt as template for the first reaction and the end product of the first PCR as template and the primer pair MHV68ORF73-20CKAA-4A F/R for the second PCR; kanamycin resistance.

pVR1255-HA-MHV68-orf73 20-4A + 225-7A (MHV orf73 20-4A+225-7A):

The sequence for amino acids 20-23 (CKRR) had been changed to four alanine residues by two succesive site directed mutagenesis reactions with the primer pair MHV68ORF73-22RR-AA Fw/Rv and MHV orf73 225-7A as template for the first reaction and the end product of the first PCR as template and the primer pair MHV68ORF73-20CKAA-4A F/R for the second PCR; kanamycin resistance.

Other constructs

L122:

The sequence of the LANA wt was obtained by PCR from BCP-1 (a PEL cell line stably infected with KSHV) and via subcloning into Bluescript cloned into pcDNA3.1(+); ampicillin resistance.

pEG202-AC-LANA:

For Y2H screen aa 2-275 and 934-1162 of LANA have been cloned via *Eco*R I in the MCS of the pEG202 vector; ampicillin resistance.

pEG202-LANA-Cterm:

For Y2H screen aa 934-1162 of LANA have been cloned via *Eco*R I in the MCS of the pEG202 vector; ampicillin resistance.

pEG202-LANA-1050:

For Y2H screen aa 1050-1162 of LANA have been cloned via *Eco*R I in the MCS of the pEG202 vector; ampicillin resistance.

pEG202-LANA-1090:

For Y2H screen aa 1090-1162 of LANA have been cloned via *Eco*R I in the MCS of the pEG202 vector; ampicillin resistance.

pcDNA3-9E10-Rb:

The wt sequence of the retinoblastoma protein was cloned in front of the 9E10 myc epitope (EQLISEEDL); ampicillin and neomycin resistance; Vector was kindly provided by S. Mittnacht (London).

pCGCG-HIV-TAT:

The transactivator of transcription (TAT) protein of HIV was cloned into the pCGCG vector. The expression is driven by CMV promotor and the TAT insert is followed by a GFP gene with IRES site for separate translation of GFP protein; ampicillin resistence; vector was kindly provided by J. Münch (Ulm)

pCI Aptamer 3:

Expression vector for the peptide aptamer 3 fused to the thioredoxin protein as a scaffold, the peptide sequence was digested with Ava II and ligated into the Rsr II site within the thioredoxin sequence; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

pCI Aptamer 24:

Expression vector for the peptide aptamer 24 fused to the thioredoxin protein as a scaffold, the peptide sequence was digested with Ava II and ligated into the Rsr II site within the thioredoxin sequence; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

pCI Aptamer 70:

Expression vector for the peptide aptamer 70 fused to the thioredoxin protein as a scaffold, the peptide sequence was digested with Ava II and ligated into the Rsr II site within the thioredoxin sequence; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

pCI Aptamer 26:

Expression vector for the peptide aptamer 26 fused to the thioredoxin protein as a scaffold, the peptide sequence was digested with Ava II and ligated into the Rsr II site within the thioredoxin sequence; ampicillin resistance; Vector was kindly provided by W. Zwerschke (Innsbruck).

2.2.2. Prokaryotic expression vectors

pGEX-1:

Expression vector from Amersham, that allows generation of Glutathion S transferase (GST) fusion proteins by cloning the gene of interest in the MCS downstream of the GST gene. It features a tac promotor, which is inducible by addition of IPTG, and a thrombin cleaveage site to allow separation of GST from the expressed fusion protein; ampicillin resistance.

pGEX-2T:

Improved version of pGEX-1, which features a more versatile MCS, but retains all other details of pGEX-1; ampicillin resistance.

GST-MHV68 orf73 fusion proteins

The following MHV68 orf73 vectors are all based on pGEX-2T, into which MHV orf73 sequences of different length were inserted. These inserts were generated by PCR with MHV-orf73 wt as template and the primer pairs listed below, digested with *Bam*HI/*Eco*RI and ligated into the similarly digested pGEX-2T, resulting in GST MHV orf73 fusion proteins of different length with the GST protein at the N-terminus.

GST-MHV orf73 wt (aa166-314):

Construct containing the C-terminal half of the MHV68 orf73 protein in the pGEX-2T vector; ampicillin resistance; generated by K.Nathan.

GST-MHV orf73 Δ262-314 (aa166-261):

Vector expressing a GST fusion protein with aa 166-261 of the MHV68 orf73 protein; primers: MHV F166 and MHV R261; ampicillin resistance; generated by M. Ottinger.

GST-MHV orf73 Δ257-314 (aa166-256):

Vector expressing a GST fusion protein with aa 166-256 of the MHV68 orf73 protein; primers: MHV F166 and MHV R256; ampicillin resistance; generated by M. Ottinger.

GST-MHV orf73 Δ253-314 (aa166-252):

Vector expressing a GST fusion protein with aa 166-252 of the MHV68 orf73 protein; primers: MHV F166 and MHV R252; ampicillin resistance; generated by M. Ottinger.

GST-MHV orf73 Δ248-314 (aa166-247):

Vector expressing a GST fusion protein with aa 166-247 of the MHV68 orf73 protein; primers: MHV F166 and MHV R247; ampicillin resistance; generated by M. Ottinger.

GST-MHV orf73 Δ242-314 (aa166-241):

Vector expressing a GST fusion protein with aa 166-241 of the MHV68 orf73 protein; primers: MHV F166 and MHV R241; ampicillin resistance; generated by M. Ottinger.

GST-MHV orf73 Δ212-314 (aa166-211):

Vector expressing a GST fusion protein with aa 166-211 of the MHV68 orf73 protein; primers: MHV F166 and MHV R211; ampicillin resistance; generated by M. Ottinger.

2.2.3. Reporter vectors

pGL2basic:

Reporter vector from Promega with a luciferase gene without eukaryotic promotor and enhancer sequences, therefore expression of luciferase depends on insertation and orientation of a functional promotor sequence in front of the luciferase gene; ampicillin resistance.

pGL3basic:

Reporter vector from Promega with a luciferase gene without eukaryotic promotor and enhancer sequences, therefore expression of luciferase depends on insertation and orientation of a functional promotor sequence in front of the luciferase gene. Improved luciferase expression in comparison to pGL2basic, due to deletion of a SV40 small T-intron downstream of the luciferase sequence; ampicillin resistance.

pGL2-basic-cyclin D1:

A 7.9 kbp encompassing region of the murine cyclin D1 promotor was inserted upstream of the luciferase gene in pGL2-basic; ampicillin resistance; kindly provided by M. Eilers (Muller, H. et al., 1994).

pGL2-basic-cyclin D2:

A 2.3 kbp encompassing region of the murine cyclin D2 promotor was inserted upstream of the luciferase gene in pGL2-basic; ampicillin resistance; kindly provided by M. Eilers (Bouchard, C. et al., 1999).

pGL2-basic-cyclin E:

The human cyclin E promotor region was inserted upstream of the luciferase gene in pGL2-basic; ampicillin resistance; kindly provided by R. Weinberg (Geng, Y. et al., 1996).

pGL2-basic-EBV Cp2.0:

A 2.3 kbp encompassing region of the Epstein Barr Virus (EBV) C promotor was inserted upstream of the luciferase gene in pGL2-basic; ampicillin resistance; construct generated by K. Nathan (Viejo-Borbolla, A. et al., 2003).

pFR-SRE:

Five serum response element (SRE) enhancer were cloned into the pFR luciferase reporter vector (from Stratagene); ampicillin resistence; kindly provided by J. Haas (München).

HIV NL4-3 LTR:

The proviral HIV reporter construct carries a frameshift mutation in the envelope gene to inactivate the virus. In addition the nef gene is replaced by a luciferase gene, which allows to detect transcription from the long terminal reporter (LTR) promotor; ampicillin resistence; kindly provided by J. Münch (Ulm).

pCMV-β-Gal:

This reporter vector contains a β-galactosidase gene under control of the immediate early promotor of the human cytomegalovirus (CMV); ampicillin resistence.

2.2.4. Primers

Name	5'-3' sequence
ORF73 START NotI	ATGCGGCCGCCGCCGCCACCATG
R261 NotI	ATGCGGCCGCGGGGCTCTGGTAAGGGA
R252 NotI	ATGCGGCCGCGTGCCTTGACTATAGTACCTTC
R236 NotI	ATGCGGCCGCGGGCCAAGACAACCCTTTTTAG
R224 NotI	ATGCGGCCGCGCTTGTCTTCAACAAATGAAAG
MHV S 200 A F	GTACTCTCAACACCAGTTGCCTGTTTACCCTTGGTACC
MHV S 200 A R	GGTACCAAGGGTAAACAGGCAACTGGTGTTGAGAGTAC
MHV K 228 A F	GACAAGAAACAGGCCGCAAAACTAAAAAGGGTTGTCTTGGCC
MHV K 228 A R	GGCCAAGACAACCCTTTTTAGTTTTGCGGCCTGTTTCTTGTC
MHV 228 4A 231 F	GAAGACAAGAAACAGGCCGCAGCAGCAGCAAGGGTTGTCTTGGCC
MHV 228 4A 231 R	GGCCAAGACAACCCTTGCTGCTGCTGCGGCCTGTTTCTTGTCTTC
MHV 226 4A 229 F	GTTGAAGACAAGAAAGCGGCCGCAGCACTAAAAAGGGTTGTCTTG
MHV 226 4A 229 R	CAAGACAACCCTTTTTAGTGCTGCGGCCGCTTTCTTGTCTTCAAC
MHV 225 7A 231 F	GTTGAAGACAAGGCAGCGGCAGCAGCAGCAGCAAGGGTTGTCTTG
MHV 225 7A 231 R	CAAGACAACCCTTGCTGCTGCTGCTGCCGCTGCCTTGTCTTCAAC
MHV 226 DAADLE 231 F	GTTGAAGACAAGAAAGACGCCGCAGACCTAGAAAGGGTTGTCTTG
MHV 226 DAADLE 231 R	CAAGACAACCCTTTCTAGGTCTGCGGCGTCTTTCTTGTCTTCAAC
MHV68ORF73-22RR-AA-Fw	CCAGATCAGGGTGCAAAGCTGCGTGCTTCAACAAACCAGCAG
MHV68ORF73-22RR-AA-Rv	CTGCTGGTTTGTTGAAGCACGCAGCTTTGCACCCTGATCTGG
MHV68ORF73-20CKAA-4AF	CCAGATCAGGGGCCGCAGCTGCGTGCTTCAACAAACCAG
MHV68ORF73-20CKAA-4A-R	CTGGTTTGTTGAAGCACGCAGCTGCGGCCCCTGATCTGG
MHV F166	GAGGGATCCACACATTTTAAGTCAGCTG
MHV R261	CTCGAATTCGGGCTCTGGTAAGGGAA
MHV R256	TGGGAATTCTTAAAAATAAGGCTTTGCCTTGAC
MHV R252	ATAGAATTCTTATGCCTTGACTATAGTACC
MHV R247	GACGAATTCTTAACCTTCTACGCTGCTGTG
MHV R241	GCTGAATTCGTATTTTTCACAGTAGGCCAAG
MHV R211	TGTGAATTCCTGTTGTGTTGTGCCTG
MHV 73 BAC F	AGAGGATCCACCATGGACTACAAGGACGACGACGACAGGCCCACATCCCCACCG
MHV 73 BAC R	AGACTCGAGGATGTCTGAGACCCTTGTC
LANA_aa1050_EcoRI_fw	AGAGAATTCGATCCAAAGTGT
LANA_aa1090_EcoRI_fw	AGAGAATTCCCCCACCTT
LANA end EcoRI	ATAGAATTCTTATGTCATTTCCTGTGGAGAG

2.2.5. Recombinant baculovirus

MHV68 orf73 baculovirus:

The recombinant baculovirus was generated by an *in vitro* recombination of BaculoDirect C-Term Linear DNA (from Invitrogen) with pENTR1A-MHV68-orf73 wt. pENTR1A-MHV68-orf73 wt is the product of a PCR with the primerpair MHV 73 BAC F/R and the template pVR1255-HA-MHV68-orf73 wt. The expression after infection of SF9 cells results in a recombinant MHV68 orf73 protein encompassing aa 2-314 with a N-terminal flag tag and a C-terminal 6xhis tag. The expression is driven by the strong baculoviral polyhedrin promotor (generated by Matthias Ottinger).

2.3. Cell culture methods for eukaryotic cells

2.3.1. Supplies used for propagation

DMEM (Gibco)	high glucose, with sodium pyruvate and pyrodoxine
Grace's Insect medium (Gibco, PAN)	with L-aminoacids
Penicillin/Streptomycin (Cytogen)	10,000 U/ml / 10 mg/ml
Trypsin/EDTA (Cytogen)	0,05/0,02% in PBS
PBS (Gibco; 10x)	1.37 M NaCl
	27 mM KCl
	66 mM $Na_2HPO_4x2H_2O$
	15 mM KH_2PO_4
Fetal calf serum (Biozym, PAN)	Heat inactivated at 56°C for 30 min

2.3.2. Eukaryotic cell lines

HEK 293:

Human cells derived from embryonal kidney tissue and transformed with the IE region of Adenovirus Type 5 (Ad 5) (ACC305 in the German collection of microorganisms and cell cultures)

HEK 293T:

HEK 293 cells in which a temperature sensitve version of the Large T-Antigen of SV40 was inserted, thereby the expression from vectors containing a SV40 replication origin is increased (CRL-11268 American type culture collection)

HeLa:

HeLa cells are human cells of cervical adenocarcinoma origin and are infected with human papillomavirus type 18. They were used for immunofluorescence and luciferase reporter assays (CCL-2 American type culture collection)

NIH 3T3:

NIH 3T3 cells were derived from a murine embryo and are fibroblasts. They were used to perform luciferase reporter assays in a murine background (CRL-1658 American type culture collection)

Insect cells SF9:

A cell line gained from the Fall Armyworm *Spodoptera frugiperda*, which is used for protein overexpression with baculoviruses (CRL-1711 American type culture collection)

2.3.3. Cell culture conditions

All media contained 10% (v/v) FCS, 50 U penicillin and 50 µg streptomycin. With the exception of the insect cells all cell lines were propagated in DMEM at 37°C and 5% CO_2 in humified air. The insect cells were kept at 27°C without CO_2 and split at 100% confluency. All other cell lines were split at subconfluency (80-90%).

2.3.4. Cryopreservation of eukaryotic cells

Cells were pelleted and resuspended in 1 ml of the appropriate medium with an increased amount of FCS (20%) and additional 10% (v/v) DMSO. The cell suspension was transferred into a cryotube and frozen in a styrofoam box at –20°C for 4 h. Then the box with the tube was transferred to –80°C for a period of 24 h and finally the cells were stored in liquid

nitrogen. For recovery of the cells, the tube was removed from the liquid nitrogen and thawn for 10 min at 37°C. The cells were washed with their normal medium to remove DMSO and transferred into a tissue culture flask with the suitable amount of medium.

2.3.5. Baculovirus expression system

An inserted protein is overexpressed by infection of insect cells, such as the SF9 cells, described above (see 2.3.2.), with a recombinant Baculovirus (see 2.2.4.). To obtain small amounts of protein an adherent culture was used. $1x10^7$ SF9 cells were plated in a 75 cm^2 cell flask with 9 ml medium and left for 1 h at 27°C to allow attachment of the cells. Then 1 ml of cell culture supernatant containing the baculovirus was added to the cells. The cells were incubated for 3 days at 27°C and the cells were pelleted for protein purification (see 2.6.7.), while the supernatant was stored at 4°C for later infection. For larger amounts of protein the SF9 cells were grown in spinner flasks as suspension culture. In a spinner flask the cell suspension was diluted to a concentration of $1.6x10^6$ cells/ml including 10% (v/v) baculovirus supernatant. After diluting of the cells the baculovirus supernatant was added. The spinner flask was incubated for 3 days at 27°C on a magnetic stirrer to avoid attachment and clumping of the cells. The cells were pelleted and used for protein purification (see 2.6.7.), while the supernatant was stored at 4°C for later infections.

2.3.6. Transient transfection of plasmid DNA

In order to express an exogenous protein in eukaryotic cells, the cells were transfected with a plasmid encompassing the sequence for the protein. On the first day an appropriate number of cells was plated in a 6 well plate (e.g. $4x10^5$ cells/well for 293 and 293T). 24 h later the cells should have reached about 50% confluency to be transfected. The transfection reagent Fugene6 from Roche was used according to the manufacturer's manual. The DNA:Fugene ratio was constantly kept at 1:3, which resulted in good transfection rates. In most cases cells were kept in the incubator for 48 h post transfection and then used for further experiments.

2.4. Cell culture methods for prokaryotic cells

2.4.1. Medium used for propagation

LB medium: 1% (w/v) Trypton (Becton Dickson)
0,5% (w/v) Yeast extract (Difco)
1% (w/v) NaCl
adjust to pH 7.0 with NaOH

Agar plates: LB-medium with 1,5% (w/v) Bacto-Agar (Becton Dickson)

Antibiotics:		
	Ampicillin	100 μg/ml
	Kanamycin	25 μg/ml
	Chloramphenicol	20 μg/ml

Other additives:		
	IPTG	0.5 mM
	X-Gal	100 mM

For overnight cultures LB medium with the appropriate antibiotic agent were inoculated either from a cryoconserved stock culture or a colony from an agar plate. The inoculated cultures were shaked at 200 rpm at 37°C.

2.4.2. Bacterial strains

E. Coli TG-2:

TG-2 cells were transformed with all plasmids except members of the pGEX family; supE hsdΔ5 thi Δ(lac-proAB) Δ(srl-recA)306: Tn10(tet^r)F'[traD36 proAB$^+$ lacl^{q}lacZΔM15].

E. Coli M15:

M15 cells were transformed with expression vectors of the pGEX family and used for expression of recombinant GST fusion proteins. To regulate the expression of the proteins the cells contain pRep4, a plasmid coding for the lac repressor in trans. pRep4 features a kanamycin resistance

E. Coli Rosetta:

Rosetta cells were also used for the pGEX family of expression vectors. They produce generally more GST fusion protein than the M15 cells. They possess a plasmid containing six additional tRNAs for translation (chloramphenicol resistance) and are depleted of the proteases *lon* and *ompT*.

2.4.3. Cryopreservation of prokaryotic cells

To store transformed cells for longer time, 500 µl of an overnight culture were mixed with 500 µl sterile glycerol (87%) in a cryotube and frozen at –80°C.

2.4.4. Preparation of competent cells and transformation

Competent bacteria were prepared by usage of the Rubidium chloride method. A 5 ml overnight culture was diluted 1:100 with fresh LB medium and shaken for another 2 to 3 h at 37°C and 200 rpm. When the culture reached an OD_{600} of 0.6 to 0.8 the cells were pelleted at 1000 g and 4°C for 15 min, from this point on, all steps were performed at 4°C with ice cold buffers. The cell pellet was gently and thoroughly resuspended in 200 ml RF1 buffer and incubated on ice for 15 min. After centrifuging again as described above, the pellet was resuspended in 20 ml RF2 buffer and incubated for another 15 min on ice. Then the cells were competent and aliquoted à 200 µl. The aliquots could be either used directly for transformation or stored at –80°C for later usage.

For each transformation one 200 µl aliquot was used. If a frozen aliquot was used, it was first thawed on ice. The DNA to be transformed was added to the thawed bacteria (1 to 5 µl corresponding to 20 to 100 ng). The solution was mixed by shortly flipping the tube and then incubated for 30 min on ice. Then a heat shock for 45 sec at 42°C was performed. After the cells were put back on ice for 2 min, 500 µl of LB medium were added and the tube was shaken at 37°C and 200 rpm for 1 h. Subsequently 20 µl of the transformation were plated on LB agar plate with the appropiate antibiotic for selection. The remaining bacterial suspension was centrifuged for 3 min at 5000 g, the pellet resuspended in 200 µl LB medium and then plated on another agar plate. Plates were incubated overnight at 37°C to obtain colonies of transformed bacteria.

RF1 buffer:	RF2 buffer:
100 mM $RbCl_2$	10 mM MOPS
30 mM K acetate	10 mM $RbCl_2$
10 mM $CaCl_2$	75 mM $CaCl_2$
50 mM $MnCl_2$	15% (v/v) glycerol
15% (v/v) glycerol	
adjust to pH 5.8 with acetic acid	adjust to pH 6.5 with KOH

2.5. Molecular biology methods

2.5.1. Isolation of plasmid DNA

Plasmid DNA was obtained from overnight cultures with the QIAprep Spin Mini kit from Qiagen. The kit was used according to the manual of the manufacturer. For larger amounts of DNA the QIAprep Maxi kit from Qiagen was used as described in the company's manual.

2.5.2. Enzymatic modification of DNA

For the digestion by endonucleases, dephosphorylation with alkaline phosphatase and ligation by T4 DNA ligase the enzymes needed and the suitable buffers from the following companies were used: Boehringer Mannheim, Fermentas, New England Biolabs, Promega and Roche. All reactions were performed according to company's manuals.

2.5.3. PCR amplification of DNA

The following components and concentrations were used in a standard PCR with 50 µl volume: 100 ng of the template DNA, 250 µM of each dNTP, 10 pmol of each of the two primers, 5 mM $MgCl_2$, 1x polymerase buffer, 3 U Taq polymerase and filled up to 50 µl with ddH_2O. The mixture was prepared in a PCR tube, this tube transferred into one of the following PCR cyclers from Applied Biosystems: GeneAmp PCR System 2400 thermocycler, GeneAmp PCR System 9700 thermocycler and Veriti 96well Thermal Cycler. The

amplification program depended on different factors: first the sequence length and the GC content determined the annealing temperature of the primer and the duration of this step, second the charateristics of the template DNA (genomic or plasmid, GC content) defined the number of amplification cycles and the denaturation temperature, finally the length of the desired PCR product dictated the duration of the elongation step at 72°C.

2.5.4. Electrophoresis and extraction of DNA in agarose gels

To separate DNA fragments or molecules of different sizes an 1% agarose gel was normally used. Therefore the required amount of agarose (0.4 g for a small and 1 g for a large gel) was heated together with the proper amount of water (40 ml / 100 ml) and 50x TAE buffer (0,4 ml /2 ml) in a microwave oven till the agarose dissolved. 8 µl / 20 µl of a ethidium bromide solution (1 mg/ml) was added while stirring and the gel was then cast into a fitting tray with the appropiate comb. After polymerisation the gel was transferred into the electrophoresis chamber and the comb was removed. The DNA samples were prepared by adding an appropiate amount of the 5x DNA loading buffer and then pipetting them into the wells. 1 kb plus DNA ladder from Invitrogen served as size standard. The electrophoresis was performed with a constant voltage of 100 V for small and 130 V for large gels. The run was stopped depending on the desired degree of separation. The gel was then either photographed in the gel documentation (MultiImage Light Cabinet, Alpha Innotech) or was subject to gel extraction.

For extraction of DNA from agarose gels the QIAquick gel extraction kit from Qiagen was used according to the manufacturer's instructions and the DNA was eluted in 50 µl of ddH_2O.

50x TAE buffer:
2 M Tris acetate
50 mM EDTA

5x DNA loading buffer
100 mM Tris HCl; pH 7.4
10 mM EDTA
45% (v/v) glycerol
Orange G

2.5.5. DNA sequencing

All plasmids generated in this study were sequenced to ensure that there were no mutations in the inserted DNA sequences. The ABI Prism dRhodamine terminator cycle sequencing ready reaction kit from Applied Biosystem was used for sequencing. Per sequencing reaction: 500 ng of plasmid DNA were mixed with 4 µl of the terminator cycle sequencing reaction kit, 1.6 pmol of the sequencing primer and filled up with H_2O to a final volume of 10 µl. The cycling reaction was performed with the following parameters: 96°C for 10 sec; annealing temperature of the primer (variable) for 10 sec, 60°C for 4 min, for 25 cycles. To remove the excess of unprocessed nucleotides an ethanol precipitation was performed.
The PCR product was added to 37 µl precipitation mix (70% ethanol, 0.5 mM $MgCl_2$) in a 1.5 ml high yield nucleic acid recovery tube (Robbins), vortexed and left for 15 min at RT. The pellet obtained after centrifugation with 14,000 g for 15 min was dried at 94°C for 1 min to remove traces of ethanol. The dry pellet was resuspended in 6 µl template suppression reagent, heated for 2 min to 95°C, cooled on ice, centrifuged and transferred into a 0.5 ml sequencing sample tube (Applied Biosystems). The sequencing was conducted with an ABI Prism 310 Genetic Analyzer and later with a 3130 Genetic Analyzer from Applied Biosystems.

2.6. Biochemical and cell biology methods

2.6.1. Preparation of cell lysates

For most experiments, transfected cells needed to be lysed. For this purpose the 6 well plates, in which the cells were usually grown, were put on ice and washed once with cold PBS. Then one of the lysis buffers described below was added (300 µl per well for a 6 well plate) to the cells, incubated for 10 min on ice and the raw lysates were transferred into 1.5 ml tubes. After a centrifugation step (14,000 g, 10 min, 4°C) to remove cellular debris cleared lysates were transferred into new 1.5 ml tubes and either used directly for assays or stored at –20°C. To minimize potential protein degration all lysis buffers were mixed prior to use with five protease inhibitor listed below.

TBS-T:	20 mM	Tris HCl, pH 7.4
	150 mM	NaCl
	1 mM	EDTA
	1% (v/v)	TritonX-100
NP40 lysis buffer:	50 mM	Tris pH, pH 8.0
(for SF9 insect cells)	1% (v/v)	NP40
Protease inhibitors:	1 mM	PMSF
	50 µM	Leupeptin
	100 U/ml	Aprotinin
	200 µM	Benzamidine
	1 µM	Pepstatin A (in DMSO)

2.6.2. SDS polyacrylamide gel electrophoresis (PAGE) and Coomassie staining

SDS PAGE was performed to separate proteins of different size and to detect them either by Coomassie staining or immunoblotting (see 2.6.3.). Gels were casted with a 8 or 10% content of acrylamide in the separation part (depending on the size of the protein to be detected) and 4% acrylamide in the stacking part of the gel. Cleared cell lysates containing proteins were mixed with an appropiate volume of 5x protein loading buffer and boiled for 3 min at 95°C. After short centrifugation 10 or 20 µl (depending on the size of the wells) of the protein solutions were loaded into the wells. In order to be able later determine the size of the detected protein, 5 µl of Precision Plus All Blue prestained Protein Marker (BioRad) were loaded onto the gels as size standard. The proteins were separated using a Hoefer mighty small electrophoresis chamber (Amersham) with the running buffer described below at constant current of 25 mA per gel.

Gels for immunoblotting were further processed as described in 2.6.3.

If the gels were to be stained with Coomassie solution, they were first washed three times for 5 min with H_2O, shaken for 1 h at RT with Coomassie solution (BioRad) and destained by washing five times for 10 min with H_2O. Stained gels were stored after drying for 1 h at 80°C on filter paper (Whatman).

5x protein loading buffer:		SDS PAGE running buffer:	
300 mM	Tris HCL, pH 6.8	25 mM	Tris base
50% (v/v)	Glycerol	250 mM	Glycine
10% (w/v)	SDS	0.1% (w/v)	SDS
0.1%(w/v)	Bromphenolblue	pH 8.3	
300 mM	β-Mercaptoethanol		

2.6.3. Immunoblotting

After electrophoresis gels were assembled into a plastic frame for the transfer to a nitrocellulose membrane in the following manner: the side of the frame facing the cathode was put first into a plastic container filled with blotting buffer, then a sponge and two filter papers were placed, the gel was positioned onto that stack, a nitrocellulose membrane (HyBond ECL from Amersham) was exactly fitted to the gel and another stack of two filter papers and a sponge was placed upon the membrane, at last the other plastic frame was put on top and the whole casette was transferred into the blotting chamber. The chamber was filled with blotting buffer and blotting was carried out either at 250 mA for 1.5 h for proteins larger than 150 kDa or at 350 mA for 1 h for smaller proteins.

After blotting the cassette was disassembled and the membrane was transferred into a 50 ml tube containing 10 ml PBS-M. The tube with the membrane was incubated on a roller for either 1 h at RT or overnight at 4°C to block unspecific binding sites on the membrane. When the blocking step was finished, the membrane was transferred into another 50 ml tube containing 5 ml PBS-M and the first antibody to detect the desired protein at an appropiate dilution (see 2.1.1. for details). The membrane was again incubated on a roller either 1 h at RT or overnight at 4°C. Then the membrane was put into a small plastic container and washed three times with PBS-T, each washing step lasting 10 min. The washed membrane was placed into a new 50 ml tube with the appropiate secondary antibody (HRP coupled polyclonal antibodies for different species all from Dako, except αrat-HRP from Harlan) diluted in 5 ml PBS-M. When the 1 h incubation at RT on a roller with the secondary antibody was finished, the membrane was again washed three times 10 min each step. For the first two steps again PBS-T was used, while the last washing step was performed with PBS.

The membrane was at this point placed onto a plastic tablet and incubated with 3 ml freshly mixed ECL (1:1 mixture of ECL solutions 1 and 2) per membrane for 1 min and then the chemiluminescence was detected either by putting films on the membrane and developing of the exposed films or in a chemiluminesce detector (LAS 3000 system with intelligent dark box from Fujifilm).

Sometimes it was necessary to detect multiple proteins sequentially, for this purpose the membrane was stripped, which removed all previous bound antibodies. The membrane was washed with PBS for about 10 min after the chemiluminesce detection and put into a 50 ml tube filled with stripping buffer. The tube was incubated for 10 min at 50°C in a water bath. Then the membrane was washed in a plastic container with a constant flow of deionized water till the smell of β-mercaptoethanol disappeared. Then the membrane was ready for another round of immunoblotting performed as described above, starting again with the blocking step.

Blotting buffer:	25 mM	Tris base
	250 mM	glycine
	20% (v/v)	methanol
PBS-T:	PBS + 5% (v/v) Tween 20	
PBS-M	PBS-T + 5% (w/v) non-fat dried milk	
Stripping buffer:	PBS with	
	4% (w/v)	SDS
	180 mM	β-mercaptoethanol
ECL solution 1:	100 mM	Tris HCl, pH 8.5
	2.5 mM	Luminol
	0.4 mM	p-cumaric acid
ECL solution 2:	100 mM	Tris HCl, pH 8.5
	0.02% (v/v)	H_2O_2

2.6.4. Luciferase reporter assay

To examine the effect of proteins on specific promotors luciferase reporter assays were performed. Different promotors were cloned in front of a luciferase gene. The effect of the protein of interest could be determined by cotransfection of an expression vector for this protein with the vector containing luciferase under control of the promotor of interest and measuring of the luciferase activity in the lysates of the cotransfected cells.

The cell line used in the experiment was plated in 6 well plates and transfected at 50% confluency with 50 or 200 ng of the reporter vector (noted for each experiment) and different amounts of expression vector for the protein or empty vector. All transfections were filled up to the same amount of DNA with the empty vector of the protein expression vector as a control and were performed as duplicates. The cells were harvested 48 h post transfection, at that time the plates were placed on ice, washed once with cold PBS and then lysed with 300 µl of 1x reporter lysis buffer (Promega) per well for 10 min on ice. The lysates were transferred into 1.5 ml tubes, cleared by centrifugation at 14,000 g for 1 min and then either directly used for measurement or frozen at –20°C.

For measurement of the luciferase activity 20 µl of the lysate supernatant were mixed with 100 µl luciferase buffer and the light emmision was measured for 10 sec in a luminometer (DCR-1 from Digene). To calculate the relative activation or repressive activity of the protein on the promotor, the value of sample of the empty expression vector was set to 1 and all other sample values were divided by the value of the empty expression vector.

Luciferase buffer:	40 mM	Tricine HCl, pH 7.8
	10 mM	$MgSO_4$
	10 mM	DTT
	0.5 mM	EDTA
	0.5 mM	ATP
	0.5 mM	Coenzyme A
	0.5 mM	D-Luciferin

2.6.5. β-galactosidase assay

Similar to the luciferase reporter assay the β-galactosidase assay was used to examine the effect of proteins on the immediate early promotor of human cytomegalovirus (CMV). The effect of the protein of interest could be determined by cotransfection of an expression vector for this protein with the pCMV-β-Gal vector containing a β-galactosidase gene under control the CMV promotor and measuring of the β-galactosidase activity in the lysates of the cotransfected cells.

HEK 293 cells were plated in 6 well plates and transfected at 50% confluency with 50 ng of pCMV-β-Gal vector and different amounts of expression vector for the protein or empty vector. All transfections were filled up to the same amount of DNA with the empty vector of the protein expression vector as a control and were performed as duplicates. The cells were harvested 48 h post transfection, at that time the plates were placed on ice, washed once with cold PBS and then lysed with 300 µl of 1x reporter lysis buffer (Promega) per well for 10 min on ice. The lysates were transferred into 1.5 ml tubes, cleared by centrifugation at 14,000 g for 1 min and then either directly used for measurement or frozen at –20°C.

For measurement of the β-galactosidase activity 5 µl of the lysate supernatant were diluted with 45 µl 1x reporter lysis buffer in a 96 well plate. Each diluted sample was mixed with 50 µl of 2x assay buffer, the plate was covered and incubated for 30 min at 37°C. The reaction was stopped by addition of 150 µl 1 M sodium carbonate solution. The absorbance at 420 nm was determined with an Anthos htIII plate reader. To calculate the relative activation or repressive activity of the protein on the CMV promotor, the value of sample of the empty expression vector was set to 1 and all other sample values were divided by the value of the empty expression vector.

2x assay buffer:	200 mM	Na_2HPO_4, pH 7.3
	2 mM	$MgCl_2$
	100 mM	β-mercaptoethanol
	1.33 mg/ml	ortho-nitrophenyl-β-galactoside (ONPG)

2.6.6. Immunofluorescence microscopy

Two different types of immunofluorescence microscopy were performed, one in which HeLa cells were grown on coverslips, transfected, fixed and stained on the coverslips (standard) and one where the cells were grown in the 6 well plate, transfected, trypsinized, counted and spun down onto the slide, where they were fixed and stained (chromosomal spreads).

For the standard immunofluorescence HeLa cells were plated on 20x20 mm coverslips in a 6 well plate and transfected with an expression vector for the protein of interest (usually 1 µg) at 50% confluency. 48 h post transfection the cells were washed with PBS and then fixed by putting 500 µl of a 4% PFA solution onto each coverslip for 10 min. The PFA was removed and the cells were washed three times with 1 ml PBS per coverslip. To inactivate any traces of PFA the coverslips were incubated with 1 ml of a 50 mM NH_4Cl solution per well for 10 min. After another three washing steps with PBS the coverslips were either stored at 4°C in PBS or were directly stained.
To permeablize the cell membranes the cells were incubated for 10 min with 500 µl of a 0.2% (v/v) TritonX-100 in PBS solution per well and washed three times with PBS. To block unspecific binding sites for antibodies, the cells were blocked with 1% (v/v) FCS in PBS (PBS-F) solution and 1 ml per well for at least 15 min. Then the staining with the primary antibodies, either one or sometimes two, was performed. The antibodies were diluted (see 2.1.1. for details) in PBS-F and pipetted (100 µl for each coverslip) onto a piece of Parafilm in a plastic container. The coverslips were placed upside down (cells facing the liquid) onto the antibody solution drops using a forceps. The plastic container was transferred into a humid chamber and incubated at 37°C for 1 h. The coverslips were put back into the 6 well plate and washed four times for 5 min with 1 ml PBS-F per well.
Then the secondary antibody staining was performed, antibodies binding to the primary antibodies were diluted in PBS-F and again for each coverslip 100 µl of the solution was placed on a new sheet of Parafilm. The coverslips were positioned onto the droplets, like before, and the container incubated in the dark and at 37°C in a humid chamber for 45 min. Meanwhile an aliquot of Mowiol was thawn, DABCO was added to a concentration of 25 mg/ml and incubated on a roller, till the DABCO dissolved. After finishing the staining with secondary antibodies the coverslips were put back into the 6 well plate and washed four times for 5 min each step and the two initial washes were done with PBS-F and the other two with PBS. The cellular DNA was stained by incubating the cells with a 1:1000 dilution of Topro-3

in PBS, 1 ml per well for 10 min in the dark at RT. Meanwhile the slides were cleaned with 70% (v/v) ethanol and labeled. The coverslips were submerged ten times into a glass with ddH_2O and then mounted onto the slides with 20 µl of Mowiol. The slides were dried overnight in a dark drawer and then directly investigated or stored at 4°C.

In the chromosomal spread variant of immunofluorescence staining, HeLa cells were directly plated in a 6 well plate and transfected at 50% confluency. The cells were blocked in mitosis by exchanging the normal medium to one that contained colchicine (0.1 mg/ml) 42 h post transfection and incubating them another 6 h at 37°C. The cells were then washed with PBS, detached from the plastic surface by incubating 10 to 20 min with 300 µl trypsin/EDTA solution per well (see 2.3.1.), washed again with PBS and resuspended in 1 ml PBS. The cell concentration was determined with a Neubauer counting chamber and the cells were diluted to a concentration of $5x10^4$ cells/ml with hypotonic solution (75 mM KCl), which was added dropwise to avoid formation of clumps. During the incubation time of 5 min the required number of slides were cleaned with 70% (v/v) ethanol and labeled.
The slides were fixed to a Cytospin funnel by a clamp and positioned into a cytospin centrifuge, 200 µl of each cell suspension were pipetted into each of the funnels. By centrifuging for 10 min at 1500 rpm the cells were spun down onto slides in form of a spot of about 5 mm diameter. Cell spots were dried and surrounded with a PAP pen to keep liquid solutions on the spots. The cells were fixed to the slides by adding 40 µl 4% PFA solution to each spot and incubating for 10 min at RT. All of the washing steps that follow were performed by using a tray for the slides, which was placed into a plastic container filled with the required buffer. The slides were washed twice with H_2O, then twice with KCM 1 min for each step and stored overnight at 4°C in KCM.
On the next day the slides were dried with paper, except the cell spot, which was again surrounded by a circle drawn with a PAP pen. A dilution of the primary antibody in KCM was prepared and pipetted onto the cell spots (30 µl per spot). The slides were incubated for 1 h at 37°C in a dark humid chamber. After the incubation the cells were washed three times with KCM for 10 min each step without shaking. The slides were dried again and the PAP pen marking was renewed, before 30 µl of the secondary antibody diluted in KCM was added to each spot. The slides were incubated at 37°C in a dark humid chamber for another hour. Meanwhile an aliquot of Moviol was thawn, DABCO was added to a concentration of 25 mg/ml and incubated on a roller till the DABCO dissolved. The cells were again washed three times with KCM for 10 min each step without shaking and then twice 1 min with PBS. To

stain the cellular DNA, Topro3 was diluted 1:2000 in PBS and 30 µl were added per spot. The cells were incubated for 10 min in a dark drawer at RT. The slides were rinsed twice in H_2O and then small round coverslips were mounted onto the cell spots with 10 µl Mowiol per spot. The slides were dried overnight in a dark drawer and then directly investigated or stored at 4°C.

PFA:	4% (w/v) paraformaldehyde dissolved in H_2O	
PBS-F:	PBS + 1% (v/v) FCS	
KCM:	120 mM	KCl
	20 mM	NaCl
	10 mM	Tris HCl, pH 7.7
	0,1% (v/v)	TritonX-100

2.6.7. Purification of His tagged proteins

MHV68 orf73 protein was overexpressed *via* a baculovirus in SF9 insect cells. The recombinant protein contained a His tag, which is able to form a complex binding to an unsaturated nickel ion. Nickel ions were immobilized on a sepharose matrix to allow seperation of nickel protein complexes from the crude protein mixture. All steps were carried out at 4°C or on ice to avoid protein degradation, in addition all buffers were mixed with protease inhibitors (see 2.6.1. for details).

The starting material were the insect cell pellets from infections with baculovirus encoding the MHV68 orf73 protein (see 2.3.5. for infection procedure). The pellets were thawn on ice and resuspended with 10 ml lysis buffer per 100 ml volume of the original insect cell suspension culture. The lysates were sonicated five times for 20 s with 20 s breaks in between with about 30% output power. The lysis of the cells was verified by examination of 5 µl of the sample in a light microscope. Solubilization of DNA bound proteins such as the MHV68 orf73 protein was enhanced by adding LiCl to a concentration of 1 M and stirring the sample for 30 min.

To separate the proteins in solution from cell debris, the lysate was centrifuged for 30 min at 15,000 rpm in a Ti70 rotor in an ultracentrifuge (both from Beckman). The supernatant of the

centrifugation was transferred into a 500 ml polycorbonate tube and mixed with a 50% (v/v) Ni bead solution. The beads had previously been washed three times with lysis buffer. 3 ml of beads solution per 100 ml lysate supernatant were added. The mixture of beads and lysates was incubated on a roller for at least 3 h. Subsequently the solution was centrifuged for 10 min at 1000 g in a JA-10 rotor, the supernatant was replaced by 100 ml washing buffer 1 per 100 ml supernatant and the beads were again incubated for 1 h on a roller. This washing step was repeated once with washing buffer 1 and twice with washing buffer 2. After the last washing the beads were resuspended with 20 ml Washing buffer 2 and transferred into a 25 ml plastic column (from BioRad). The beads were left to settle down onto the filter at the bottom of the column and then the washing buffer was run through till the beads went nearly dry. The protein was eluted with 10 ml elution buffer, which was added to the column, incubated with the beads for 10 min and run through. The elution step was performed five times, which resulted in five different protein fractions. Through the whole process all material was kept, stored at 4°C and samples for SDS PAGE with Coomassie staining were taken. In this way, it was possible to follow the whole course of protein purification and not only to detect the end product.

Lysis buffer:	50 mM	Tris pH, pH 8.0
	1% (v/v)	NP40
Washing buffer 1:	50 mM	Tris pH, pH 8.0
	500 mM	NaCl
Washing buffer 2:	50 mM	Tris pH, pH 8.0
	500 mM	NaCl
	5 mM	Imidazole
Elution buffer:	50 mM	Tris pH, pH 8.0
	100 mM	NaCl
	400 mM	Imidazole

2.6.8. Interaction assays with GST fusion proteins (GST pulldowns)

In a GST pulldown the interaction between two different proteins was investigated. One was expressed from a pGEX vector in M15 or Rosetta bacteria as a GST fusion protein and was bound to sepharose beads coated with glutathione. The other protein was either overexpressed in transfected HEK 293T cells (see 2.3.6.) or was a purified His tagged protein (see 2.6.7.), which was incubated with the GST fusion protein bound to glutathione beads. After washing of the beads, they were subjected to SDS PAGE, which was followed by immunoblot with an antibody for the second protein. In case of an interaction, the second protein is detectable.

To express a GST fusion protein, a 5 ml overnight culture of bacteria containing the pGEX vector with the protein of interest was inoculated and shaken overnight at 37°C. On the next day the OD_{600} for the overnight culture was determined, a new 10 ml culture was set up with a starting OD_{600} of 0.1 and was shaken at 37°C. When the culture reached OD_{600} of 0.4 to 0.5, it was induced by adding 50 µl of IPTG (200 mM stock), from this point the culture was incubated at 30°C. After growing for 5 to 6 h the bacteria were centrifuged at 1000 g and 4°C for 30 min and the pellet was frozen at –80°C or directly used for lysis.
If required the pellet was thawn on ice and resuspended with 500 µl PBS with protease inhibitors per pellet. The suspension was sonicated three times for 30 s with 30 s breaks in between on ice and with 30% output power of the sonicator. The lysate was mixed with 50 µl of a 10% (v/v) TritonX-100 solution and incubated for 10 min on ice. During the subsequent 10 min centrifugation at 14,000 g and 4°C to remove debris, glutathione beads (from Amersham) were washed three times with PBS and resuspended with an equal amount of PBS to obtain a 50% solution of glutathione beads. A cut tip was used to pipet beads. The 500 µl of lysate supernatant were mixed in a 1.5 ml tube with 20 µl washed glutathione beads and filled up to 750 µl with PBS. This tube was incubated at RT for 1 h on a roller. The beads were washed three times with 1 ml PBS per washing step and resuspended to a volume of 250 µl with PBS. An aliquot of 5 µl was separated, mixed with 5x protein loading buffer and a SDS PAGE followed by Coomassie staining was performed to ensure that the GST fusion protein had bound to the glutathione beads.
After this verification the incubation with the second protein could be set up. Either a lysate of transfected HEK 293T cells (see 2.6.1. for lysis) or a purified protein was used to test its interaction with the GST fusion protein. The protein concentration levels of the second protein, which should be tested for binding to the GST fusion proteins, varies over a wide

range, so the required volume of the second protein was determined by dilution series. Volumes between 20 and 500 µl of second lysate/protein solution were used depending on determined protein concentrations. The required solution of the second protein was mixed with 100 µl beads with bound GST fusion protein and filled up to an end volume of 750 µl with PBS. The second incubation was also performed for 1 h at RT. The beads were washed five times with 500 µl PBS, resuspended with 20 µl 5x protein loading buffer and either directly used for a SDS PAGE followed by immunoblot or stored at –20°C.

2.6.9. Coimmunoprecipitation

Another possibility to test the interaction between two proteins is coimmunoprecipitation. In this assay two different expression vectors for the two proteins of interest are cotransfected into cells, the lysates of these cells are incubated with beads bound to antibodies specific to one of the proteins (either to the native protein itself or to a recombinant tag). After washing the beads an interaction with the second protein can be detected by Western blotting.

HEK 293T cells were transfected at 50% confluency with the two expression vectors for the proteins or their empty vectors as a control. 48 h later the cells were lysed in TBS-T as described (see 2.6.1.). 40 µl of the cleared lysate was mixed with 10 µl 5x protein loading buffer and frozen at –20°C, to have an input control. The remaining 240 µl were mixed in a 1.5 ml tube with 10 µl beads bound to the required antibody and were filled up to 500 µl with TBS-T containing proteasome inhibitors. The tube was incubated on a roller overnight at 4°C. The beads were centrifuged at 5000 g and 4°C for 2 min, the supernatant was removed and the beads were resuspended with 500 µl with TBS-T containing proteasome inhibitors. This washing was performed five times, then the beads were mixed with 20 µl 5x protein loading buffer and either directly used for SDS PAGE followed by immunoblot or stored at –20°C.

2.6.10. Nuclear fractionation assay

In a nuclear fractionation assay the relative strength of binding to cellular chromatin for an overexpressed nuclear protein can be determined. Transfected cells were lysed and the pellet containing the cellular chromatin was extracted several times with buffers with increasing concentrations of KCl. Samples of the extracts were analysed by SDS PAGE followed by immunoblot and the relative concentrations of the recovered protein could be observed.

HEK 293T were plated in a 6 well plate and transfected with different MHV68 orf73 mutants, the wt of MHV68 orf73 and the empty vector at 50% confluency. Two days after transfection the plate was placed on ice, the cells were washed once with cold PBS and lysed with 300 µl lysis puffer per well for 30 min. All steps from now on were performed on ice and all buffers used contained protease inhibitors to avoid protein degradation. The lysates were transferred into 1.5 ml tubes and centrifuged for 10 min at 14,000 g and 4°C. The supernatants were pipetted into new 1.5 ml tubes, from which aliquots of 40 µl mixed with 10 µl 5x protein loading buffer were prepared. Each of the pellets was mixed with 300 µl of buffer 1, vortexed and incubated another 30 min. The lysates were again centrifuged for 10 min at 14,000 g and 4°C and the separation of supernatants and pellets was repeated. This extraction procedure was repeated with the buffers 2 to 7. After the last centrifugation the pellets were resuspended with 50 µl 5x protein loading buffer and all samples were frozen at –20°C. At a later time the aliquots and the pellet samples were used in a SDS PAGE followed by immunoblot.

Lysis buffer: 10 mM Tris HCl, pH 8.0
0.2 mM EDTA
1% (v/v) NP40
5% (v/v) Glycerol

Buffer 1: as the lysis buffer, but contains additionally
50 mM KCl

Buffer 2: as the lysis buffer, but contains additionally
100 mM KCl

Buffer 3: as the lysis buffer, but contains additionally

150 mM KCl

Buffer 4: as the lysis buffer, but contains additionally

200 mM KCl

Buffer 5: as the lysis buffer, but contains additionally

300 mM KCl

Buffer 6: as the lysis buffer, but contains additionally

400 mM KCl

Buffer 7: as the lysis buffer, but contains additionally

500 mM KCl

3. Results

3.1. Functional consequences of the interaction between cellular BET proteins and the MHV68 orf73 protein

3.1.1. Identification of binding sites for BET proteins within the MHV68 orf73 protein and charcterization of their interaction patterns

Identification of two potential binding sites for BET proteins by a peptide array allowed us to generate several mutants, which are altered in these regions to a different degree (Ottinger, Pliquet et al. 2009). An overview of all mutants is shown in figure 9. The mutants include: single amino acid (aa) changes, four aa substitutions, a mutation of the whole binding site and a conversion to opposite polarity of all charged residues all for the first identified C-terminal binding site. The potential binding site for BET proteins in the N-terminus later identified, was mutated alone and in combination with the complete C-terminal binding site. For control purposes the amino acid 200 (serine) was changed to an alanine**.**

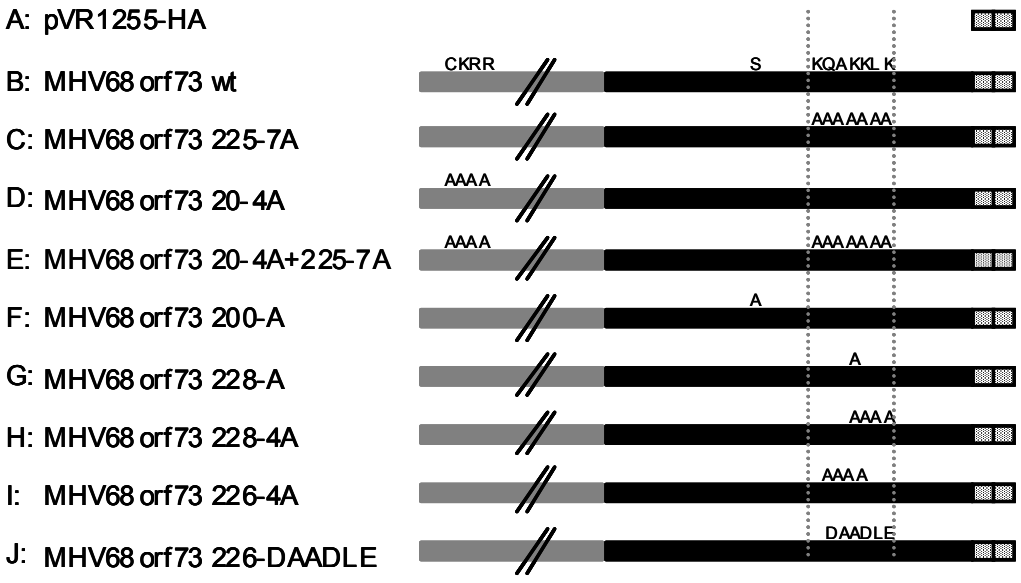


Figure 9. Overview of the used MHV68 orf73 constructs.

In this figure all MHV68 orf73 constructs are labeled with a single letter and also a schematic drawing of the expressed protein is presented. In the scheme for the MHV68 orf73 wt protein at the position of mutations in other constructs the wt aa are written in one letter code. In the case of the mutants the new aa are depicted. Grey boxes represent the N-terminal and black boxes the C-terminal part of the MHV68 orf73 protein. The small double boxes illustrate the double HA-tag. The vertical light grey dotted lines mark the potential BET protein binding site between aa 225 and 231.

The aa 228-231 of MHV68 Orf73 interact with Brd2

Different mutants of the MHV68 orf73 protein were tested in a coimmunoprecipitation experiment with the BET protein Brd2, which was overexpressed as GFP fusion protein. The lysates of 293T cells transfected with one of the MHV68 orf73 constructs and the Brd2 were immunoprecipitated with αHA coated beads and subjected to a SDS PAGE followed by an immunoblot against GFP. The results are shown in figure 10.

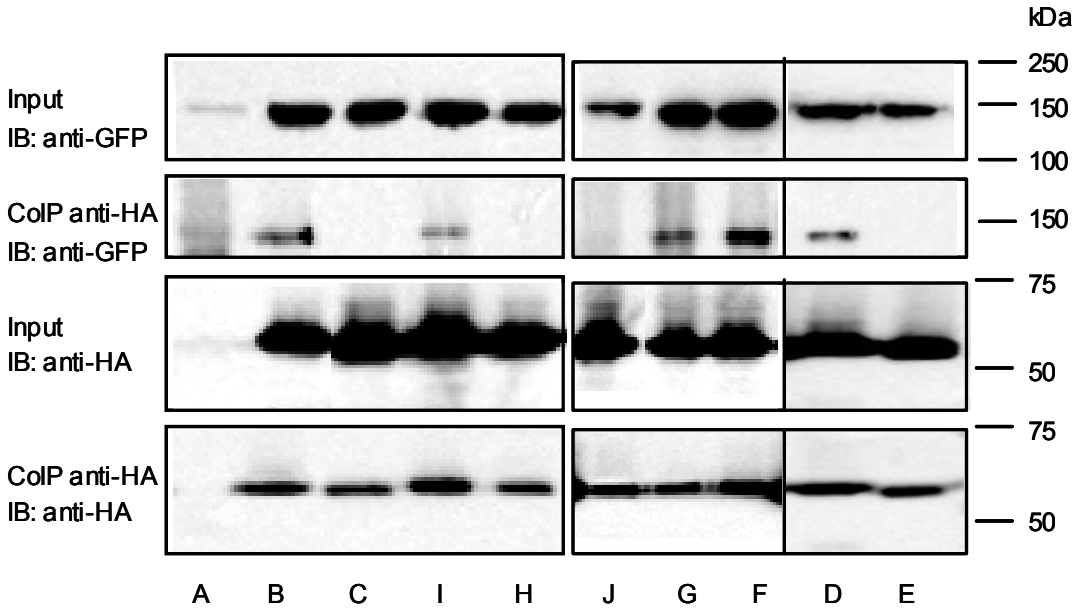


Figure 10. MHV68 orf73 interacts with BRD2 via its aa 225-231.

HEK 293T cells were transfected with 1 µg of Brd2-GFP plasmid DNA and 1 µg plasmid DNA of a MHV68 orf73 construct or the empty vector pVR1255HA. 48 h after transfection the cells were harvested and coimmunoprecipitation was performed with αHA beads (described in 2.6.9.). In the top panel an immunoblot of input lysates with αGFP antibody is shown. In the second panel an immunoblot with αGFP antibody of the samples after precipitation with αHA beads is shown. To evaluate expression of MHV68 orf73 proteins the membranes of the first and second immunoblot were stripped and immunoblotted again with an αHA antibody. The results of these immunoblots are presented in two bottom rows of blots. The lanes are labeled according to figure 9.

In the presence of MHV68 orf73 wt the band for Brd2-GFP at about 140 kDa is visible in the input lysate as well as after immunoprecipation (figure 10, lane B). This is also true for the single point mutations MHV68 orf73 200-A and 228-A (figure 10, lanes F and G) and the mutant of the N-terminal BET binding site MHV68 orf73 20-4A (figure 10, lane D). The mutant MHV68 orf73 226-4A showed an impaired binding to Brd2 as the intensity of band after immunoprecipitation is lower (figure 10, lane I). All MHV68 orf73 proteins mutated in the aa 228-231 displayed no binding to Brd2 (figure 10, lanes C, E, H, and J). The expression

of all MHV68 orf73 proteins was equal (figure 10, third row), the immunoprecipitation worked for all of them to the same extent (figure 10, fourth row). Therefore we conclude, that the aa 228-231 of the MHV68 orf73 protein are necessary for the interaction with the BET protein Brd2.

The interaction between the MHV68 orf73 protein and the Brd4^S protein depends on aa 20-23 and aa 228-231 of MHV68 orf73

To elucidate binding characteristics of another BET protein with the MHV68 orf73 protein, we repeated the coimmunoprecipitation experiment of the preceding paragraph, but substituted the Brd2-GFP expression plasmid by a Brd4^S-GFP plasmid. The other experimental parameters were not changed. The outcome of that experiment is presented in figure 11.

The MHV68 orf73 wt and the control mutant MHV68 orf73 200-A clearly interacted with the Brd4^S protein, as evident by the presence of strong bands of Brd4^S at 140 kDa in the immunoblot after immunoprecipitation (figure 11, lanes B and F). The point mutant MHV68 orf73 228-A and N-terminal binding site mutant MHV68 orf73 20-4A showed a decreased ability to interact with Brd4^S, as proven by the lower intensity of the Brd4^S band after immunoprecipitation with these samples (figure 11, lanes G and D). More extensive mutations of the C-terminal binding site, as the ones present in the remaining mutants, led to abrogation of Brd4^S binding (figure 11, lanes C, I, H, and J). This led us to the conclusion that the Brd4^S protein could interact via both identified binding sites, but the C-terminal one seemed to be more important as a mutation of the N-terminal site only decreased the amount of bound Brd4^S, while even the partial mutation of the C-terminal binding site resulted in a total abrogation of interaction.

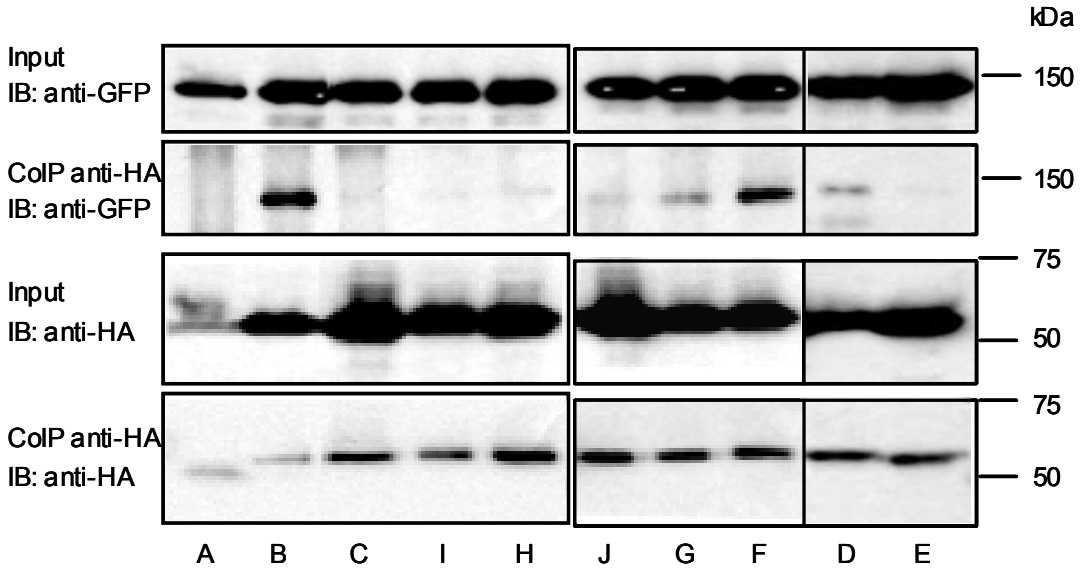


Figure 11. Binding of Brd4^{S} to MHV68 orf73 is mediated by both BET binding sites.

HEK 293T cells were transfected with 1 µg of Brd4^{S}-GFP plasmid DNA and 1 µg plasmid DNA of a MHV68 orf73 construct or the empty vector pVR1255HA. 48 h after transfection the cells were harvested and coimmunoprecipitation was performed with αHA beads (described in 2.6.9.). In the top panel an immunoblot of input lysates with αGFP antibody is shown. In the second panel an immunoblot with αGFP antibody of the samples after precipitation with αHA beads is shown.. To evaluate expression of MHV68 orf73 proteins the membranes of the first and second immunoblot were stripped and immunoblotted again with an αHA antibody. The results of these immunoblots are presented in two bottom rows of blots. The lanes are labeled according to figure 9.

Endogenous Brd4 binding to the MHV68 orf73 protein is mediated by aa 228-231 of MHV68 orf73

In the experiments presented above the BET proteins and the MHV68 orf73 protein were overexpressed in HEK 293T cells. To provide more natural conditions, we performed an coimmunoprecipitation in the B cell line BJAB. Either MHV68 orf73 wt, the mutant MHV68 orf73 228-4A or the empty vector pVR1255HA were introduced into BJAB cells via electroporation, cells were harvested 72 h after electroporation and the lysates incubated with either αHA, αBrd4 or αrabbit antibody beads. The proteins were separated by PAGE and detected by immunoblotting with the indicated antibodies. The results of the experiments are presented in figure 12. The input lysates of the first experiment show equal protein amounts for both variants of Brd4, the MHV68 orf73 wt and the 228-4A mutant, and of cellular actin, which served as loading control (figure 12, part A, input lanes). In contrast to the input lysates the samples of the immunoprecipitation exhibit different Brd4 levels. The MHV68 orf73 wt

protein was able to coprecipitate a clearly detectable amount of Brd4, in contrast to the empty vector and the 228-4A mutant, where no Brd4 was detectable (figure 12, part A, IP lanes). The levels of precipitated MHV68 orf73 wt and 228-4A protein were identical, therefore the lower rate of precipitation with the mutant is not a result of weaker binding of the mutant protein to the αHA beads.

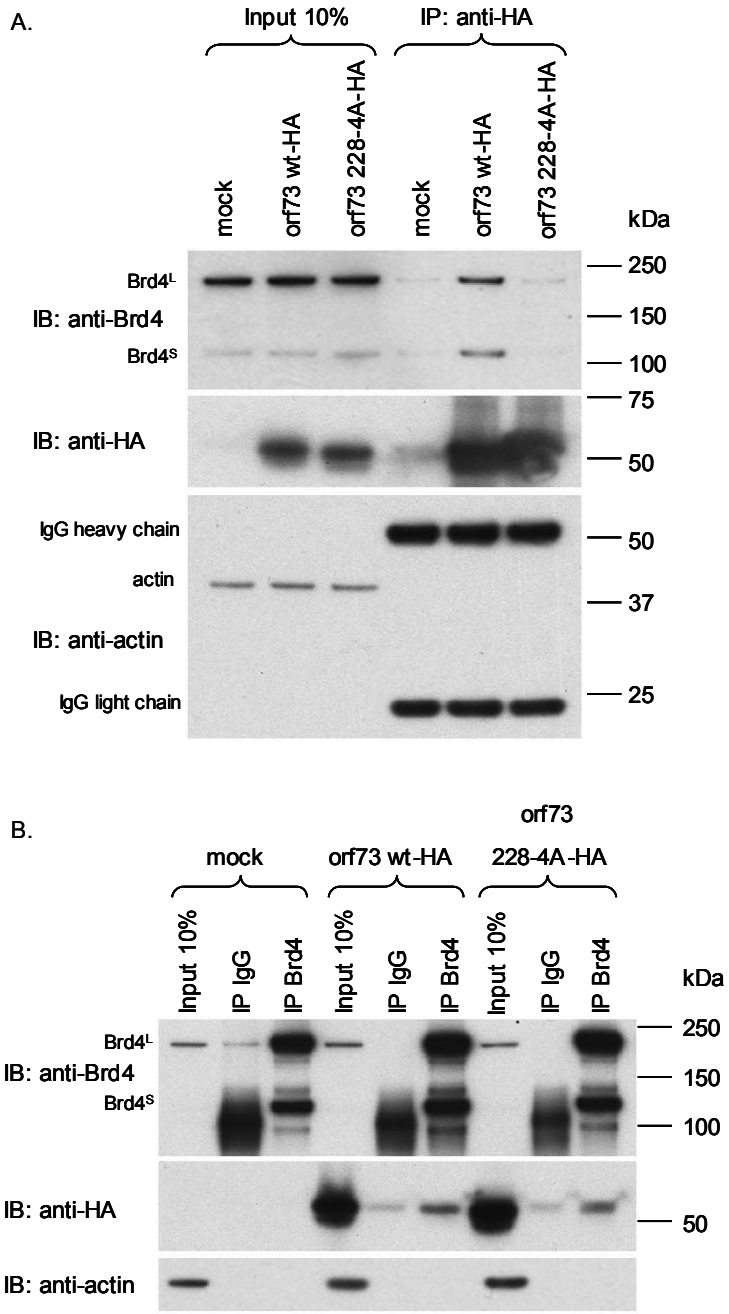


Figure 12. Interaction of endogenous Brd4 with MHV68 orf73.

BJAB cells were electroporated with either pVR1255HA DNA (mock) or MHV68 orf73 wt or 228-4A DNA (orf73 wt-HA and orf73 228-4A-HA), lysates were immunoprecipitated with αHA beads (part A) or αBrd4 and αrabbit (IgG) beads (part B). The proteins were separated by PAGE and detected by immunoblotting with the indicated antibodies (for detailed procedure see (Ottinger, Pliquet et al. 2009); experiment performed by M. Ottinger)

To complete the picture αrabbit (as negative control) and αBrd4 antibody beads were used for immunoprecipitation. None of the MHV68 orf73 proteins were coprecipitated with the αrabbit antibody (figure 12, part B, IP IgG lanes). After immunoprecipitation with the αBrd4 beads a substantial amount of MHV68 orf73 wt protein was detectable (figure 12, part B lane orf73 wt-HA IP Brd4). In contrast, the level of coprecipitated MHV68 orf73 228-4A protein was lower (figure 12, part B lane orf73 228-4A-HA IP Brd4).

Mutation of the C-terminal BET protein binding site does not reduce the oligomerization capability of the MHV68 orf73 protein

The MHV68 orf73 protein has the ability to form homooligomers. During my diploma thesis we were able to prove, that the region between aa 211-236 is necessary for this function and that the oligomerization seems to be a prerequisite for all other functions of the MHV68 orf73 protein. Therefore we decided to investigate the oligomerization capability of chosen mutants in a GST interaction assay. The MHV68 orf73 wt protein and the three mutants were overexpressed in 293T cells and lysates from these cells were tested for interaction with either GST protein alone or two C-terminal parts of the MHV68 orf73 protein (aa 166-314 and 166-211) as GST fusion proteins. The results of the GST Pulldown are shown in figure 13. The interactions between the MHV68 orf73 wt protein and the GST proteins were as estimated from former data. There is no interaction with GST alone and the MHV68 orf73 aa 166-211 and a clear interaction with the MHV68 orf73 aa 166-314 (figure 13, part A, wt). No bands were visible for the empty expression vector pVR1255HA (figure 13, part A, mock). The three mutants MHV68 orf73 225-7A, 228-4A and 228-A showed an identical interaction pattern as the MHV68 orf73 wt protein (figure 13, part A, 225-7A, 228-4A and K228A), leading to the conclusion, that the C-terminal BET protein binding site is not required for the oligomerization process of the MHV68 orf73 protein. This result is the first prove, that the introduced mutations did not led to a misfolded protein, as the prerequisite for all other functions, namely the oligomerization, is still present.

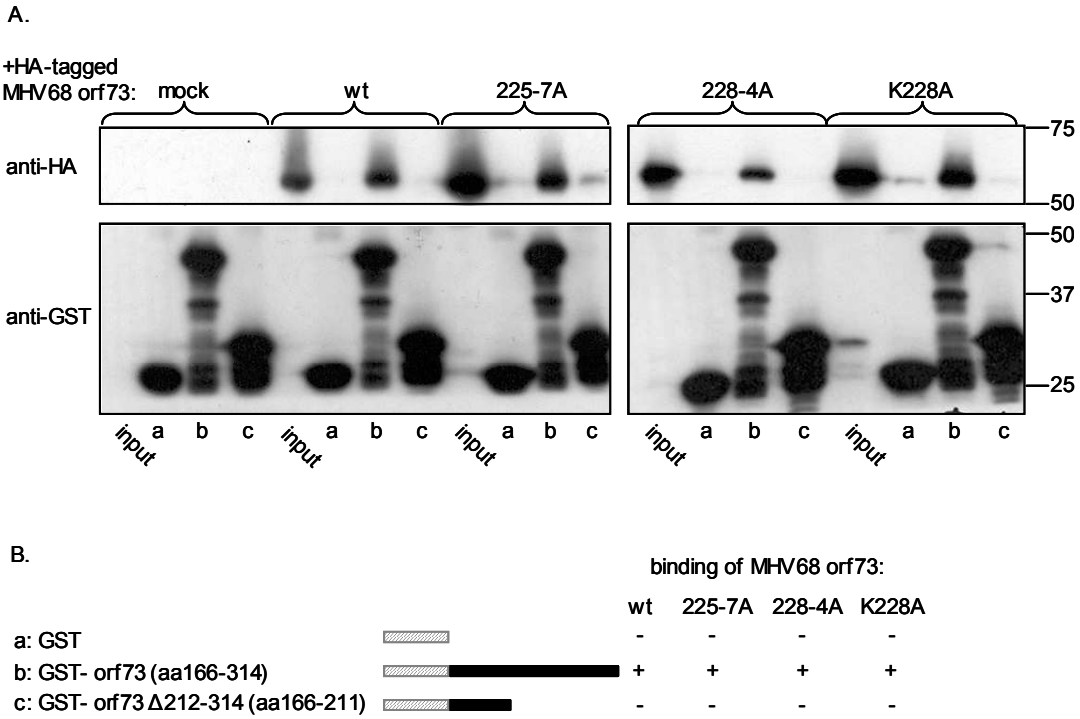


Figure 13. MHV68 orf73 mutants are capable of oligomerization similar to the wt protein.

HEK 293T cells were transfected with 1 µg of either one of MHV68 orf73 plasmids or the pVR1255HA and lysates were prepared 48 h post transfection (see 2.6.1.). These lysates were incubated with two different MHV68 orf73 GST fusion proteins and GST coupled to glutathione beads (detailed description in 2.6.8.). Part A represents two sequential immunoblots performed after the GST pulldown. First the full length MHV68 orf73 proteins were detected with an αHA antibody. Afterwards the membranes were stripped and a second immunoblotting with an αRing3 antiserum was performed to detect the GST proteins. Part B depicts a summanry of the GST proteins used in this experiment, the key to their labeling in part A and a summary of their interaction properties with the full length MHV68 orf73 proteins.

Interaction of the MHV68 orf73 protein with Rb is not affected by mutation of the BET protein binding sites

The retinoblastoma (Rb) protein is a known interaction partner of KSHV LANA-1 (Radkov, Kellam et al. 2000). Our laboratory was able to show, that the MHV68 orf73 protein is capable of interacting with Rb as well (Ottinger, Pliquet et al. 2009). To determine if mutation of identified BET protein binding sites has an impact on this interaction, we performed coimmunoprecipitation with the different MHV68 orf73 mutants. We overexpressed the mutants in combination with Rb protein bearing a myc tag. The recombinant Rb protein was precipitated with αcmyc antibody beads and the amounts of bound MHV68 orf73 protein were evaluated by an αHA immunoblot.

The first panel in figure 14 shows, that the different MHV68 orf73 proteins were present in the lysates in equal amounts. The levels of MHV68 orf73 protein after immunoprecipitation were approximately equal with the exception of the two mutants MHV68 orf73 20-4A and 20-4A+225-7A. The amount of coprecipitated protein was slightly reduced with these two mutants (figure 14, second panel, lanes D and E). The binding of the Rb protein to the αcmyc antibody beads was approximately equal throughout the samples, as seen on the blot for myc tag after immunoprecipitation (figure 14, bottom panel). Therefore we could exclude any influence of the C-terminal BET binding site on the interaction between the Rb protein and the MHV68 orf73 protein.

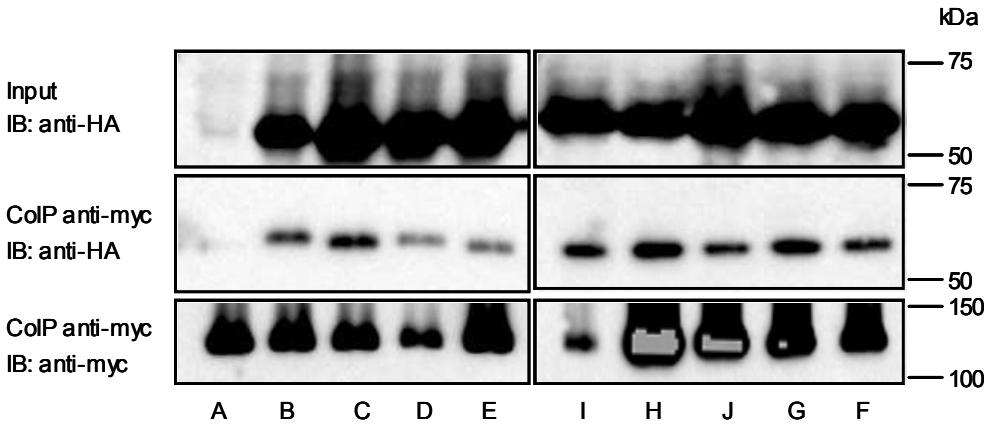


Figure 14. All MHV68 orf73 mutants coprecipitate with Rb.

HEK 293T cells were transfected with 1 µg of pcDNA-9E10-Rb plasmid DNA and 1 µg plasmid DNA of a MHV68 orf73 construct or the empty vector pVR1255HA. 48 h after transfection the cells were harvested and coimmunoprecipitation was performed with αcmyc antibody beads (described in 2.6.9.). In the first panel an immunoblot of the input lysates with αHA antibody is shown. The second panel displays an immunoblot with αHA antibody after the immunoprecipitation with αcmyc antibody beads. To evaluate expression of Rb protein the membranes of the immunoprecipitation were stripped and immunoblotted again with αcmyc antibody. The results of these immunoblots are presented in the third panel. Numbering of the constructs in invidual lanes as in figure 9, 10, and 11.

3.1.2. Investigation of the nuclear localization properties of MHV68 orf73

Mutation of BET binding sites does not alter the intracellular distribution of the MHV68 orf73 protein

The orf73 proteins of all so far investigated *Rhadinoviruses* are localized in the nucleus. To check the nuclear distribution of the MHV68 orf73 protein may be dependent on the

interaction with BET proteins, we performed immunofluorescence microscopy with cells overexpressing either the MHV68 orf73 wt protein or different mutants.

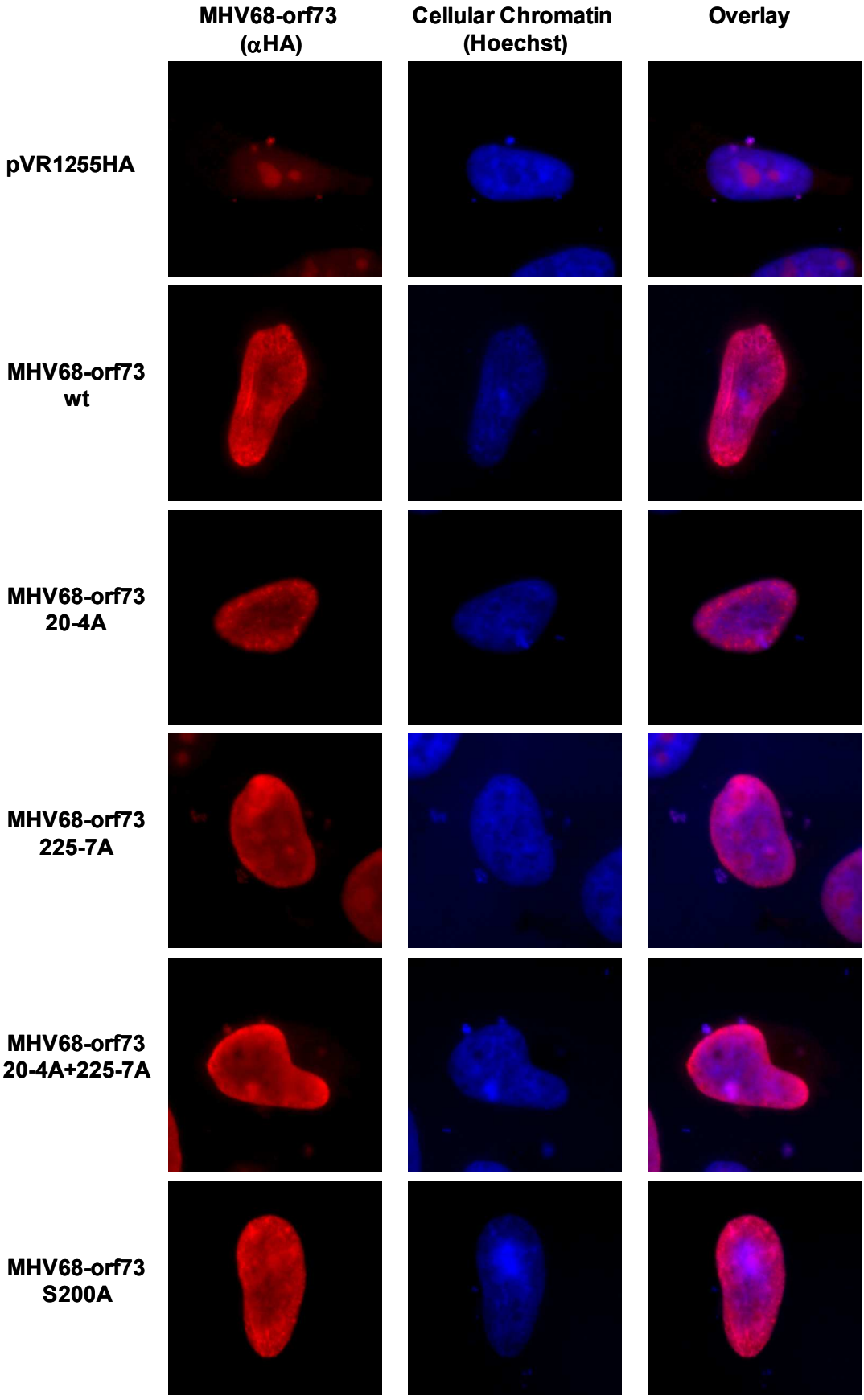


Figure 15. Nuclear localization changes upon mutation of BET binding sites within the MHV68 orf73.
Hela cells were plated on coverslips and transfected with 1 µg of MHV68 orf73 plasmid DNA, 48 h later cells were fixed with PFA and stained with an αHA antibody, the DNA was stained with Hoechst dye.

Figure 15 shows that the wt protein and the single aa exchange mutant S200A display an equal nuclear localization. Also the mutants 20-4A, 225-7A and the double mutant 20-4A+225-7A display a nuclear staining pattern, which looks comparable to that of the wt MHV68 orf73 protein. Therefore the BET proteins do not play a role in the nuclear localization of the MHV68 orf73 protein.

BET proteins do not mediate attachment of the MHV68 orf73 protein to mitotic chomomosomes during cell division

BET proteins like Brd4 are postulated to mediate the attachment of BPV genomes to cellular chromosomes during cell division. For LANA-1 a similar correlation was published (Kelley-Clarke, Ballestas et al. 2007) and so we decided to investigate, if mutation of the BET binding sites has an influence of the attachment of the MHV68 orf73 protein to mitotic chromosomes. Therefore we performed an immunofluorescene staining on cells, which were fixed in mitosis two days after transfection with MHV68 orf73 expression plasmids and chromosomes spread by cytospin (for details see 2.6.5.). The results are presented in figure 16. In the channel for Topro-3 the single chromosomes spread onto the slides are visible. In case of the empty vector pVR1255HA no staining in the αHA channel is recognizeable (figure 16, αHA channel for pVR1255HA). But with the MHV68 orf73 wt protein all chromosomes are surrounded with a red staining for the MHV68 orf73 wt protein. This results in a pink color for all chromosomes in the overlay of both channels (figure 16, αHA channel and overlay for MHV68 orf73 wt). The same pictures were obtained with all investigated MHV68 orf73 mutants (figure 16). The more prominent staining of some telomers or centromers (figure 16, αHA channel and overlay for MHV68 orf73 20-4A) were also visible with all other MHV68 orf73 mutants and the wt and thereby does not seem to be linked to a specific interaction. We conclude that in contrast to KSHV LANA-1 the MHV68 orf73 protein interacts with mitotic chromosomes in a manner independent of BET proteins.

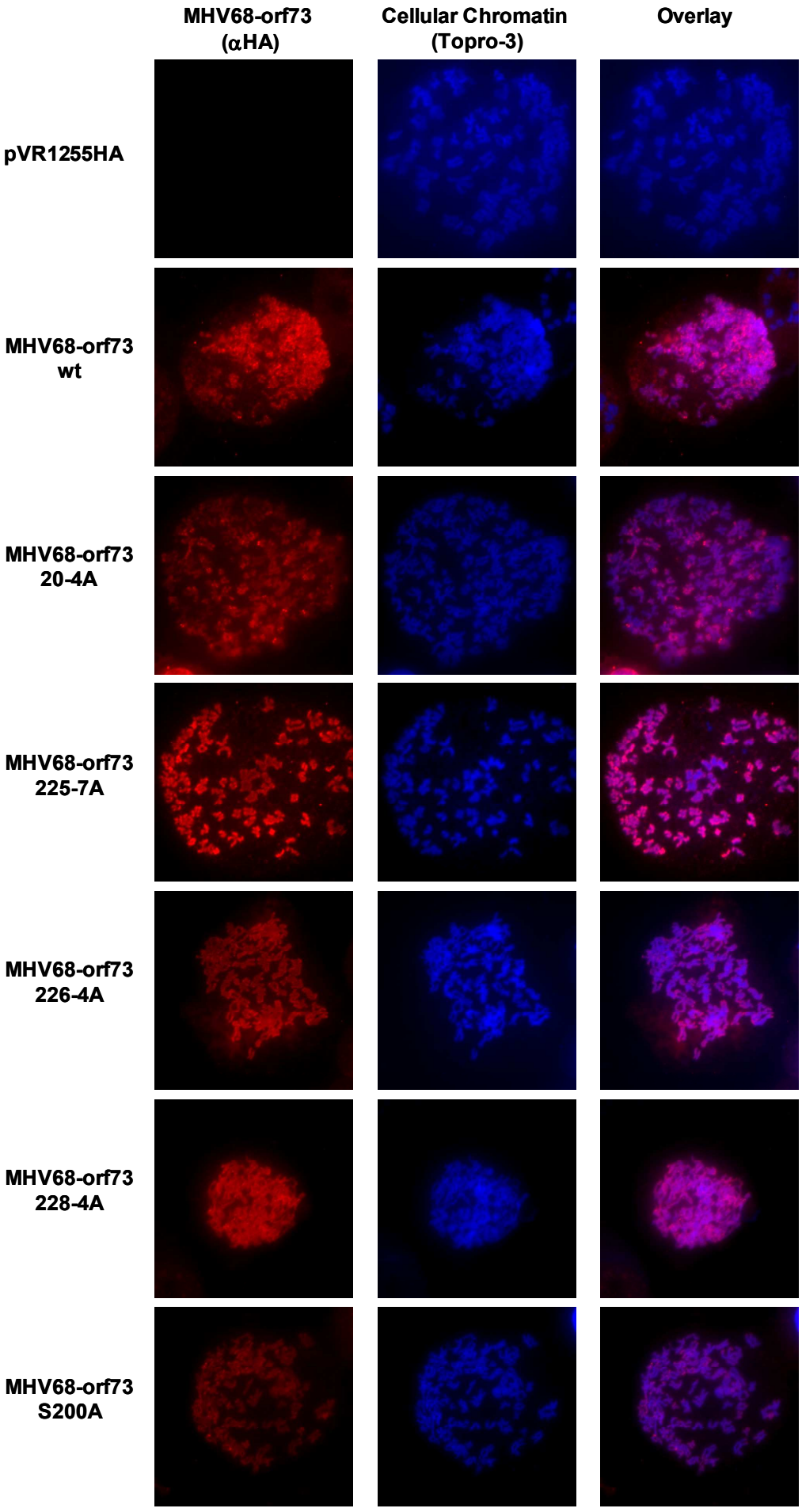

MHV68-orf73
(αHA)
Cellular Chromatin
(Topro-3)
Overlay
pVR1255HA
MHV68-orf73
wt
MHV68-orf73
20-4A
MHV68-orf73
225-7A
MHV68-orf73
226-4A
MHV68-orf73
228-4A
MHV68-orf73
S200A

Figure 16. Binding of MHV68 orf73 to mitotic chromosomes is not linked to interaction with BET protein. Hela cells were transfected with 1 μg of MHV68 orf73 plasmid DNA, 42 h later cells were blocked in M phase by a 6 h colchicine treatment. Cells were spun onto slides, fixed with PFA and stained with an αHA antibody, the DNA was stained with Topro-3 dye.

Binding of MHV68 orf73 protein to cellular chromatin is associated with its interaction with BET proteins

Although we did not find a connection between binding of the MHV68 orf73 protein to mitotic chromosomes and its interaction with BET proteins, we were interested, if there is a link between the MHV68 orf73 protein interaction with BET proteins and the attachment of MHV68 orf73 to interphase chromatin. To evaluate this, we performed a salt extraction assay, in which proteins were extracted from the nuclear fraction of cell lysates by buffers with increasing concentration of KCl. The level of MHV68 orf73 protein is equal for the wt and all mutant protein in the lysis buffer, which contains no KCl. With increasing concentration of KCl differences between the wt and the mutant protein levels extracted start to appear. The two KCl concentrations displaying the most clear difference (200 mM and 300 mM) are shown in the second and the third panel of figure 17. The mutant of the C-terminal BET binding site (MHV68 orf73 225-7A) showed an elevated level of extracted protein in comparison to MHV68 orf73 wt (figure 17, second and third panel, compare lanes C and B). Due to the obvious difference between these two samples, they were used as reference points for the other MHV68 orf73 mutants. The control mutant MHV68 orf73 200-A presented the same protein levels as the wt throughout the whole course of extraction (figure 17, lane F). As with the control mutant the single amino acid change of the MHV68 orf73 228-A mutant did not showed significantly increased levels of extracted protein (figure 17, lane G).
The partial mutation of the C-terminal BET binding site in the constructs MHV68 orf73 228-4A resulted in an intermediate phenotype, which was characterized by a clear increase of extracted protein, but not as strong as in the case of the MHV68 orf73 225-7A (figure 17, lanes H). The opposite polarity mutant of the C-terminal and double mutant of the N- and the C-terminal BET binding site showed an increased amount of extracted protein to the same extent as MHV68 orf73 225-7A (figure 17, lanes J and E). While the MHV68 orf73 protein does not rely on BET proteins to bind chromosomes during mitosis, we could therefore demonstrate a linkage between the attachment of the MHV68 orf73 protein to cellular chromatin in interphase via interaction with BET proteins.

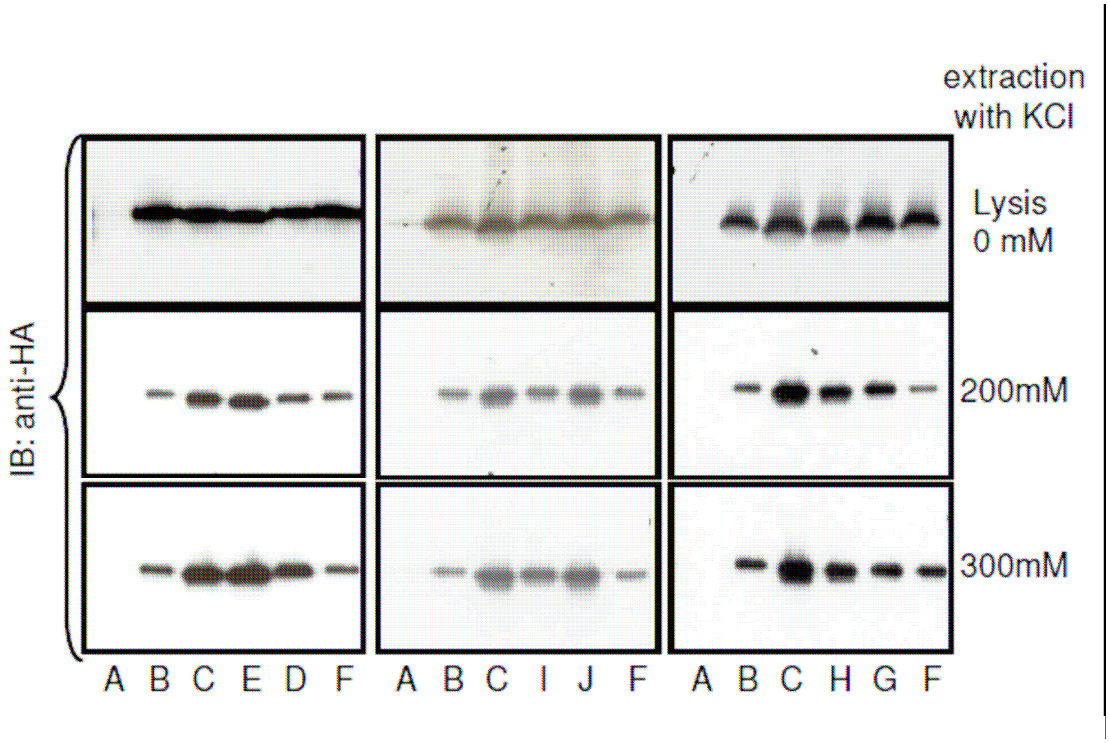


Figure 17. The attachment of MHV68 orf73 to cellular chromatin is affected by mutation of its BET binding sites.

HEK 293T cells have been transfected with 1 µg of MHV68 orf73 or empty vector plasmid DNA. The cells were lysted 48 h after transfection in a buffer without KCl. The nuclear pellet was extracted with buffers with increasing concentration of KCl (detailed instructions see 2.6.10.). Proteins in the extracts were separated by a SDS PAGE and detected with an αHA immunoblot. The lanes are labelled as explained in figures 9, 10, and 11. The figure shows three individual experiments (left, middle, right column), performed with the same and different mutants.

3.1.3. Characterization of effects of mutations of BET binding sites within MHV68 orf73 on its transcriptional function

The BET protein binding sites identified in the MHV68 orf73 protein have, so far, been connected to an altered pattern of nuclear localization during interphase. One of the proposal roles of BET proteins, the attachment to mitotic chromosomes, does not seem to be significant in the case of the MHV68 orf73 protein. Therefore we wondered, if another known function of the BET proteins could be important for the correct function of the MHV68 orf73 protein. Brd2 as well as both forms of Brd4 serve as effectors in transcriptional control (LeRoy, Rickards et al. 2008; Mochizuki, Nishiyama et al. 2008). To test this hypothesis, we performed luciferase reporter assays with different cyclin promotors.

The cyclin D1 promotor activation by the MHV68 orf73 protein is connected to the KKLK motif in the C-terminal BET binding site

The MHV68 orf73 wt protein increases the transcription from the cyclin D1 promotor by three fold in relation to the empty vector pVR1255HA (figure 18, wt and pVR1255). The mutation of the N-terminal BET binding site or a single amino acid exchange (control mutant S200A or the C-terminal K228A mutant) does not lead to a significant alteration of the activation level of the cyclin D1 promotor (figure 18, 20-4A, K228A and S200A). The inversion of polarity of the charged aa in the C-terminal BET binding site or the mutation of the first part of C-terminal BET binding site to alanines lower the activation to a level slightly above the background activity of the empty vector (figure 18, 226-DAADLE and 226-4A). All mutants, which contain changes at the amino acids KKLK of the C-terminal BET binding site, not only do not activate at all, but even lead to repression of the cyclin D1 promotor in comparison to pVR1255HA (figure 18, 225-7A, 20-4A+225-7A and 228-4A)

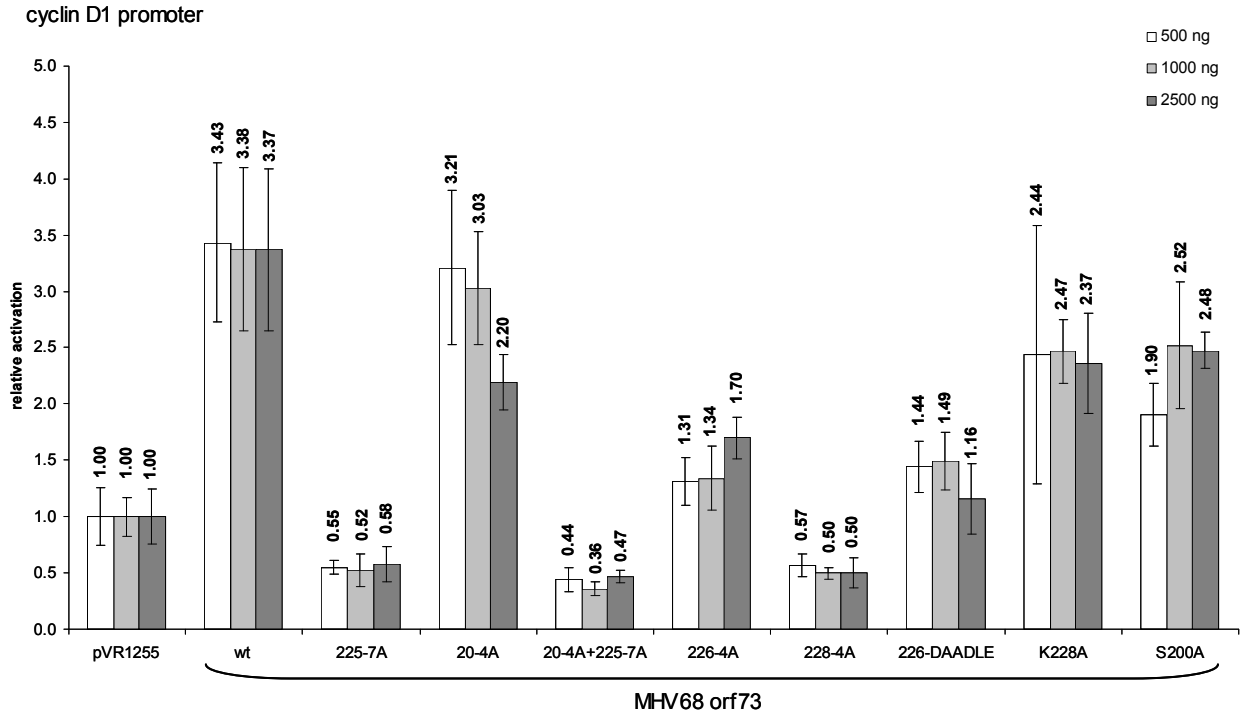


Figure 18. Activation of cyclin D1 promotor by the MHV68 orf73 protein is linked to the C-terminal BET binding site.

NIH 3T3 cells were transfected with 200 ng of pGL2basic-cyclin D1, the indicated amounts of MHV68 orf73 constructs (500 to 2500 ng). The total amount of plasmid DNA transfected per sample was adjusted to 2500 ng by addition of pVR1255 DNA. 48 h later cells were harvested, lysed and the luciferase activity was determined with a luminometer (for a detailed description see 2.6.4.)

Activation of the cyclin D2 promotor via the MHV68 orf73 protein is mediated by the KKLK motif in the C-terminal BET binding site

The experiment presented in figure 18 was repeated with another reporter plasmid, namely the cyclin D2 promotor region. The wt protein of MHV68 orf73 activates the transcription from the cyclin D2 promotor similar to the cyclin D1, about three fold as compared to the empty vector pVR1255HA (figure 19, wt and pVR1255). Single point mutants like MHV68 orf73 200-A and 228-A show an activation comparable to wt (figure 19, S200A, K228A). The abrogation of the N-terminal BET binding site in the mutant MHV68 orf73 20-4A surprisingly boosted the activation by to approximately five fold, i.e. lies above the wt level. The partial mutation or inversion of the polarity of the C-terminal BET binding site in the mutants MHV68 orf73 226-4A and 226-DAADLE led to slight reduction of transcription in comparison to the activation by the wt protein (figure 19, 226-4A and 226-DAADLE). All of the mutants, in which the KKLK motif is converted to four alanines, lost the ability to activate or even repressed the transcription as compared to the empty vector (figure 19, 228-4A, 225-7A and 20-4A+225-7A). The observed activation pattern is very similar to the one obtained with the cyclin D1 promotor. The activation of both promotors seems to be mediated by the KKLK motif in the MHV68 orf73 C-terminus, while the N-terminal BET binding site is dispensable for this function.

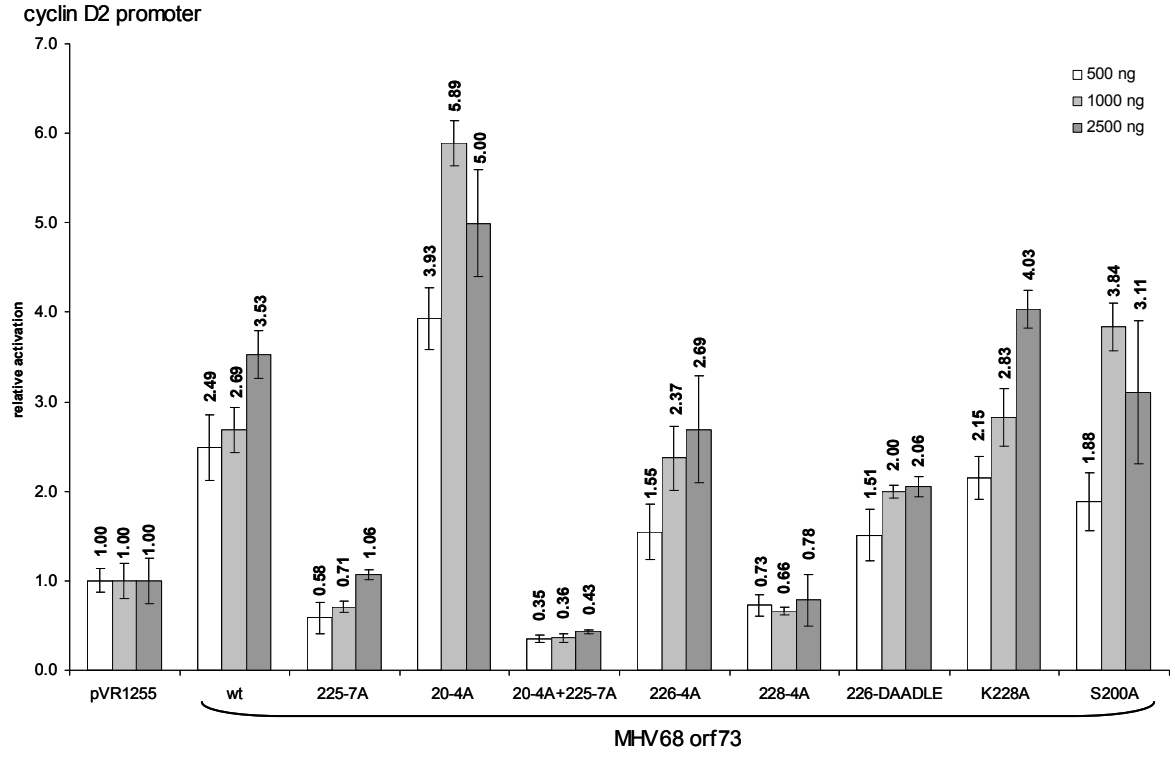


Figure 19. Cyclin D2 promotor activation by MHV68 orf73 is mediated by the C-terminal KKLK motif.
NIH 3T3 cells were transfected with 200 ng of pGL2basic-cyclin D2, the indicated amounts of MHV68 orf73 constructs (500 to 2500 ng). The total amount of plasmid DNA transfected per sample was adjusted to 2500 ng by addition of pVR1255 DNA. 48 h later cells were harvested, lysed and the luciferase activity was determined with a luminometer (for a detailed description see 2.6.4.)

The BET binding sites of the MHV68 orf73 protein are required for activation of the cyclin E promotor

Cyclin E is a key factor regulating the transition from G1 phase into S phase during the progression of the cell cycle.Since we could show that MHV68 orf73 was able to increase transcription from cyclin D1 and D2 promotor, we wanted to elucidate effects of MHV68 orf73 on the cyclin E promotor as well. The results of a luciferase reporter assay with a cyclin E luciferase reporter plasmid are depicted in figure 20.

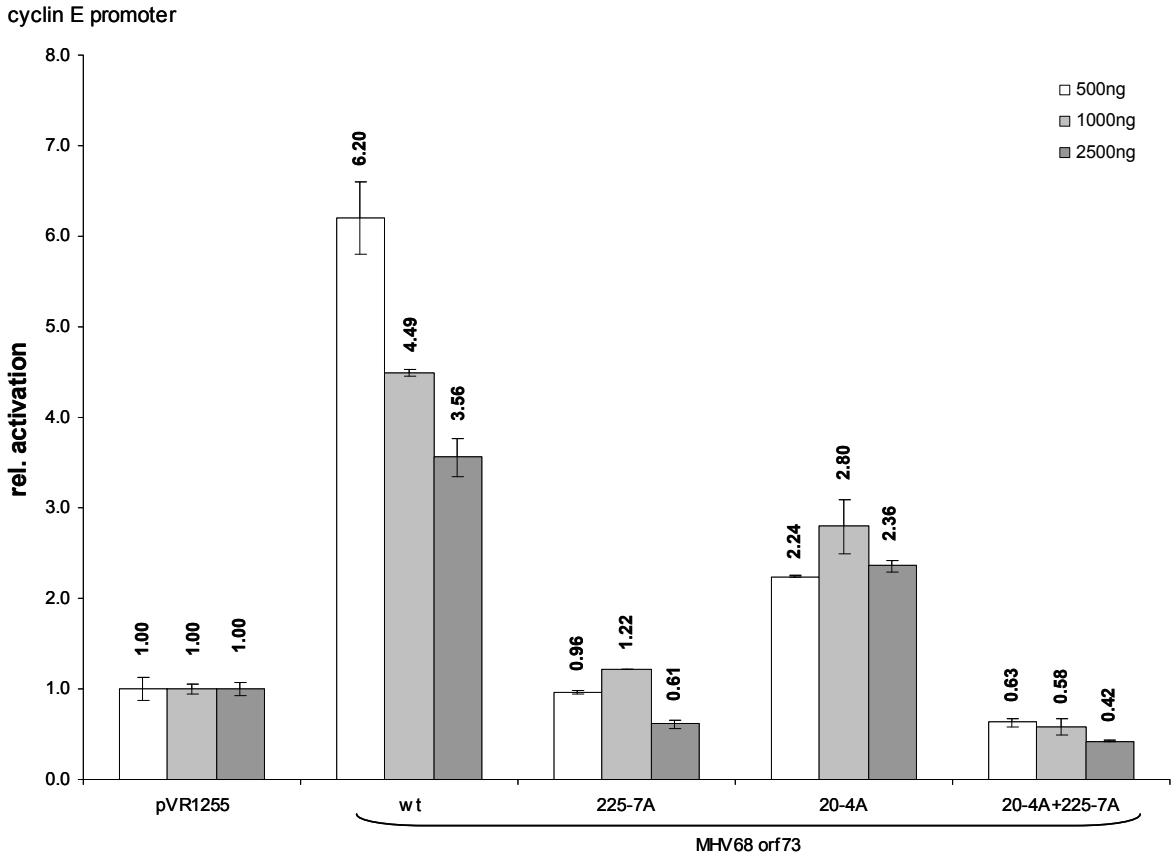


Figure 20. Both BET binding sites of the MHV68 orf73 are required for activation of transcription from the cyclin E promotor.
NIH 3T3 cells were transfected with 200 ng of pGL2basic-cyclin E, the indicated amounts of MHV68 orf73 constructs (500 to 2500 ng). The total amount of plasmid DNA transfected per sample was adjusted to 2500 ng by addition of pVR1255 DNA. 48 h later cells were harvested, lysed and the luciferase activity was determined with a luminometer (for a detailed description see 2.6.4.)

The presence of the wt MHV68 orf73 protein increased the transcription level from the cyclin E promotor up to six fold (figure 20, wt). The mutation of the N-terminal BET binding site led to a decreased activation of about 2.5 fold (figure 20, 20-4A). The mutation of the C-terminal BET binding site led to the same level of activation of the cyclin E promotor as the empty vector pVR1255 (figure 20, 225-7A). The mutation of both binding sites slightly repressed the promotor (figure 20, 20-4A+225-7A). In the case of the cyclin E promotor activation, both BET binding sites seem to play a role, but the C-terminal site is clearly more important, as the effect of a conversion into alanines is more dramatic than with the N-terminal BET binding site.

Differences in transcriptional activation are not due to unequal expression level of the MHV68 orf73 protein in different samples

To exclude the possibility, that the observed effects on different promotors were the result of unequal protein expression, lysates of luciferase reporter assays were used for SDS PAGE followed by an immunoblot. An example is shown in figure 21, where the different MHV68 orf73 proteins were detected with an αHA antibody. All proteins were present in equal amounts (figure 21).

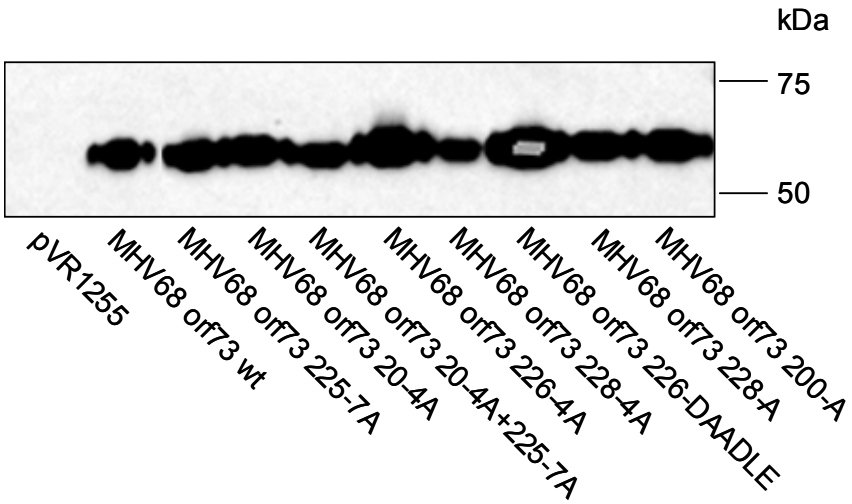


Figure 21. All MHV68 orf73 mutants display equal protein expression.

40 µl of cell lysate supernatant containing the cyclin D2 reporter were mixed with 10 µl 5x protein loading buffer and 10 µl of each lysate mixture was loaded on a 10% SDS gel. The gel was blotted onto a nitrocellulose membrane, which was then subjected to an immunoblot with an αHA antibody.

3.2. Discovery of peptide aptamers binding to the KSHV LANA-1 protein and investigation of their impact on LANA-1 functions

3.2.1. Yeast two hybrid (Y2H) screen with LANA-1 as bait and a peptide aptamer library as prey

KSHV LANA-1 is one of the few proteins expressed during the latent phase of KSHV life cycle, but it is also present during lytic phase (Sun, Lin et al. 1999). LANA-1 mediates attachment of the viral episome during cell division and therefore ensures distribution of viral genomes into both daugther cells (Ottinger, Christalla et al. 2006; Kelley-Clarke, Ballestas et al. 2007). LANA-1 represses lytic reactivation mediated by RTA (Lan, Kuppers et al. 2004), but on the other hand LANA-1 is able to activate several cellular promotors, e.g. cyclin E (Viejo-Borbolla, Ottinger et al. 2005). LANA-1 interacts with a variety of cellular proteins, one important example is p53, which looses its ability to induce apoptosis upon binding by LANA-1 (Friborg, Kong et al. 1999). Hence LANA-1 is an excellent target to interfere with the life cycle of KSHV. LANA-1 is indispensable for maintainance of KSHV genome, as shown by use of a KSHV bacterial artifical chromosome (BAC) deleted for LANA-1 (Ye, Zhou et al. 2004). The collaborating group of W. Zwerschke in Innsbruck posseses a library of peptide aptamers, which can be used in a Y2H screen.

Design and cloning of LANA-1 vectors for the Y2H screen

Before the Y2H screen we prepared four different vectors with different parts of LANA-1. We decided to focus mainly on the C-terminus of LANA-1, as most of the described functions for LANA-1 are linked to the C-terminus. All constructs based on the pEG202 vector, which allows expression of a gene of interest as a LexA fusion protein by in frame insertation of that gene in the MCS of the vector. The longest construct is a fusion of the LANA N-terminal domain with aa 2 to 275 and the C-terminal domain from aa 934–1162 lacking the internal acidic repeat region, which so far is not linked to a specific function. The expressionvector was named pEG-AC-LANA. The next construct covers the whole C-terminus with aa 934 till 1162 of the LANA-1 protein and was named pEG-LANA-Cterm. The other two encompass shorter fragments of the C-terminus (aa 1050-1162 and aa 1090-1162) and were labeled with pEG-LANA-1050 and pEG-LANA-1090. The correctness of the sequences was verified by sequencing of the inserts.

Three peptide aptamers interact with the amino acids 1090 to 1162 of the LANA-1 protein in a Y2H screen

The Y2H screen was carried out in Innsbruck by the group of W. Zwerschke. First the four cloned LANA-1 LexA fusion protein constructs were tested for protein expression to ensure a sufficent protein expression for the screen. Figure 22 represents the results of this test. The three C-terminal constructs showed an easily detectable level in immunoblots with an αLexA antibody and could be used in the Y2H screen. In case of the pEG-AC-LANA construct no LexA fusion protein was detectable, which excludes this construct from the screen, as potential interaction partners may not be detected due to the low LexA expression.
For the pEG-LANA-Cterm bait no interacting peptide aptamers were identified within the peptide prey library. But with the pEG-LANA-1090 construct they obtained three transformants, which were positive for an interaction with the bait. In figure 23 an overview of the results of the Y2H screen with pEG-LANA-1090 is displayed.

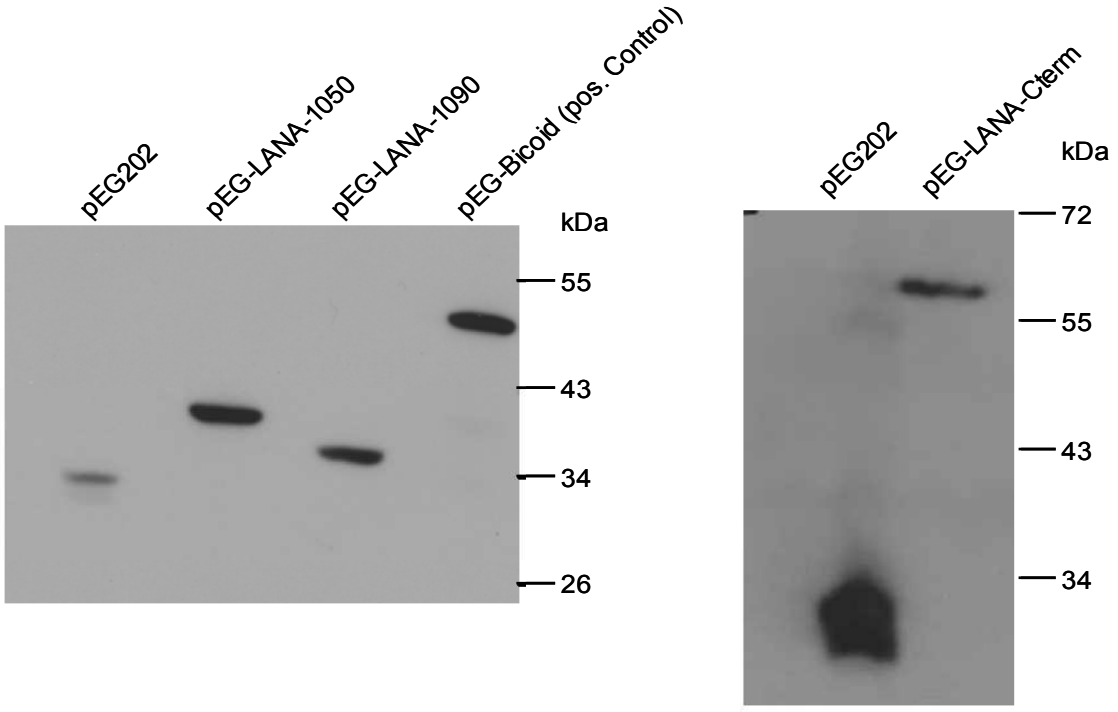


Figure 22. Fusion proteins of LexA and the LANA-1 C-terminal domain are expressed at sufficent levels. Yeast were transformed with either the empty vector pEG202, the positive control pEG-Bicoid or one of the pEG-LANA constructs. Cells were lysed and proteins separated by SDS PAGE and immunoblotted with an αLexA antibody at a dilution of 1:500 (experiment was performed in the group of W. Zwerschke)

The first column is a general growth control. The next column represents the negative control, as yeast transformants should not be able to grow on a plate with glucose and without leucine. When glucose is replaced by galactose, the interaction of bait and prey allows the growth despite the lack of leucine. The next two columns display the second selection parameter for an interaction between bait and prey. In case of an interaction the dye X-Gal will colour the grown colony blue in the presecence of galactose and leucine but not glucose and leucine. The first row shows a general positive control, in which the bait LexA-M2PK is transformed in combination with peptide aptamer M2PK 15 known to bind to M2PK. There is no growth in the column with negative control, but all other show a colony. With galactose, leucine and X-Gal the colony turned blue (figure 23, first row).

The next panel displays the result of three different baits pEG-LANA-1090, pEG-Bicoid and pEG-c-myc-Cterm, the last two baits are used for control purpose, with the peptide aptamer 3. The pattern of the positive control is seen with pEG-LANA-1090 and the Aptamer 3 as well. The transformant is able to grow with galactose in the absence of leucine and stains the colony blue in presence of galactose, leucine and X-Gal (figure 23, first panel, first row). The unrelated control bait were neither able to grow without leucine nor stain their colonies (figure 23, first panel, second and third row). This supports the specificity of the detected interaction between the LANA-1 aa 1090-1162 and the peptide aptamer 3.

The second panel features the same three baits as the first but they are combined with the peptide aptamer 24 as prey. The results are same as with the aptamer 3, an interacting pattern with pEG-LANA-1090 and no interaction with the two control baits (figure 23, second panel). The last panel was received from transformants with the peptide aptamer 70 and the three baits used before. The pEG-LANA-1090 binds to peptide aptamer 70 and therefore overcomes the growth repression in the absence of leucine and is able to stain the colony blue in the presence of X-Gal. The two unrelated baits neither grew on leucine deficient galactose plates nor colour their colonies blue in the presence of X-Gal.

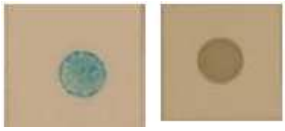
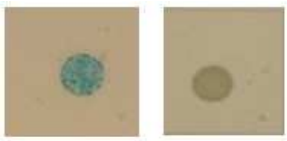
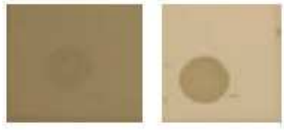
Bait	Prey	Control	Glucose -Leu	Galactose -Leu	Galactose +Leu +X-Gal	Glucose +Leu +X-Gal
pEG-M2PK	Aptamer M2PK 15					
pEG-LANA-1090	Aptamer 3					
pEG-Bicoid	Aptamer 3					
pEG-c-myc-Cterm	Aptamer 3					
pEG-LANA-1090	Aptamer 24					
pEG-Bicoid	Aptamer 24					
pEG-c-myc-Cterm	Aptamer 24					
pEG-LANA-1090	Aptamer 70					
pEG-Bicoid	Aptamer 70					
pEG-c-myc-Cterm	Aptamer 70					

Figure 23. Three peptide aptamers interact with LANA-1 aa 1090-1162.
Yeast cells were transformed with above indicated combinations of bait and prey expressions vectors and then plated on the different growth plates (experiment was performed in the group of W. Zwerschke).

Not all peptide aptamers bind to pEG-LANA-1090 as shown in figure 24, where the results of the combination of two other peptide aptamers with pEG-LANA-1090 are presented. The two aptamers are not able to overcome the growth restriction by the lack of leucine in contrast to the aptamer 70, which functions as a positive control (figure 24).

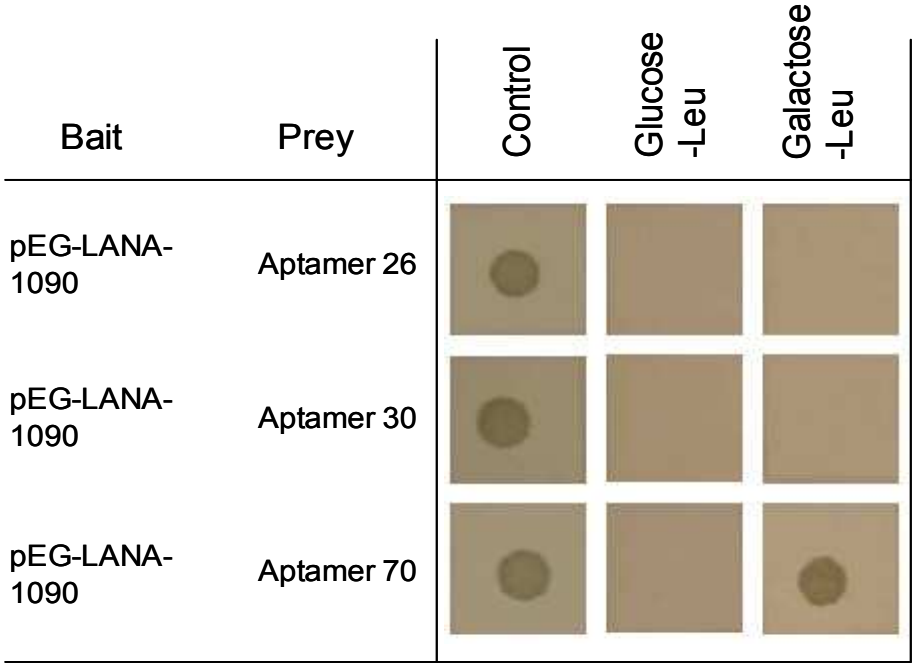


Figure 24. The interaction between peptide aptamers and LANA-1 aa 1090-1162 is specific.
Yeast cells were transformed with the indicated combinations of bait and prey expressions vectors and then plated on the different growth plates (experiment was performed in the group of W. Zwerschke).

3.2.2. Investigations on the functional properties of the peptide aptamers capable of interacting with the KSHV LANA-1 protein

The three identified peptide aptamers, which interact with LANA-1, and the aptamer 26 as a negative control were cloned into two different expression vector systems. First for overexpression in bacteria the pET-19b vector was used, for overexpression in eukaryotic cells the vector pCI with an additional His tag was used. In both cases the insert bearing the

sequence for the peptide fused to the thioredoxin protein as a scaffold was gained via a digest with the restrictionenzyme *Ava* II. The inserts were ligated into the vectors, which were digested with *Ava* II as well. The expression vectors were prepared in the group of W. Zerschke and then sent to our group for further characterization.

The peptide aptamers are expressed in eukaryotic cells upon transfection

As a prerequisite for all other experiments the expression of the peptide aptamers in eukaryotic cells was tested. Therefore HEK 293T cells were transfected with vectors and cell lysates from 48 h post transfection were immunoblotted with an αThioredoxin antibody to detect the fusion protein of thioredoxin and the peptide aptamers. The thioredoxin alone in case of the empty vector pCI is expressed at a very high level (figure 25, first lane). The aptamers 24, 70 and 26 are expressed at about the same level (figure 25, last three lanes). The expression level of the aptamer 3 is significantly lower (figure 25, second lane). For judging further results, we have to keep in mind, that a minor effect of aptamer 3 might result from the low expression level compared to the other peptide aptamers.

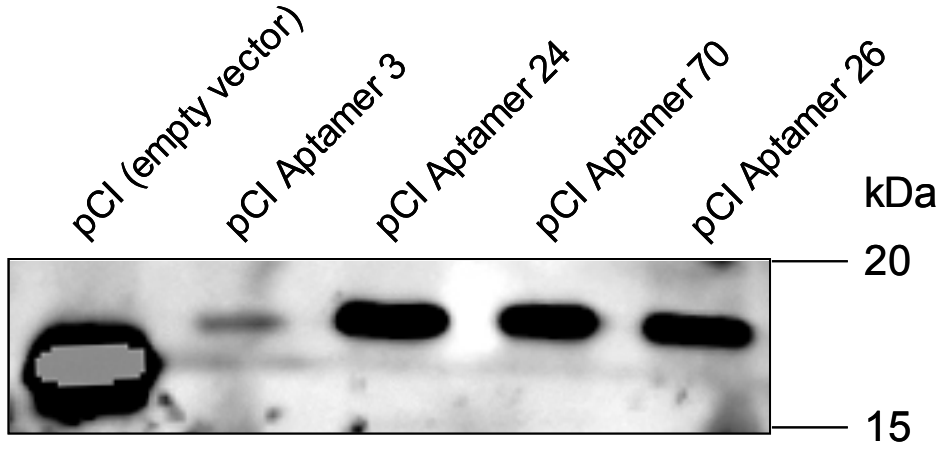


Figure 25. All peptide aptamers are expressed in HEK 293T cells.

1 µg of each vector DNA was used for transfection of HEK 293T cells in a 6 well plate. 48 h after transfection cells were lysed with TBS-T, proteins separated by SDS PAGE and thioredoxin peptide fusion proteins were detected by an immunoblot with an αThioredoxin antibody at a dilution of 1:1000.

Activation of the serum response element (SRE) by LANA-1 is reduced by the interacting peptide aptamers

LANA-1 is capable of activating a promotor containing five copies of SRE enhancer elements in front of a luciferase gene (Roupelieva, Griffiths et al.). The SRE is present in several cellular promotors linked to genes of cellular growth and motility. We transfected HEK 293 cells with pFR-SRE, which is a luciferase reporter vector, either full length KSHV LANA-1 or the empty vector pcDNA3 and the different peptide aptamers or their empty vector pCI. At a concentration of 50 ng of the aptamers we observed no significant effects of any aptamer, but LANA-1 was able to activate the transcription from the SRE by about five fold relative to the empty vector pcDNA3.1 (figure 26, first two column blocks).

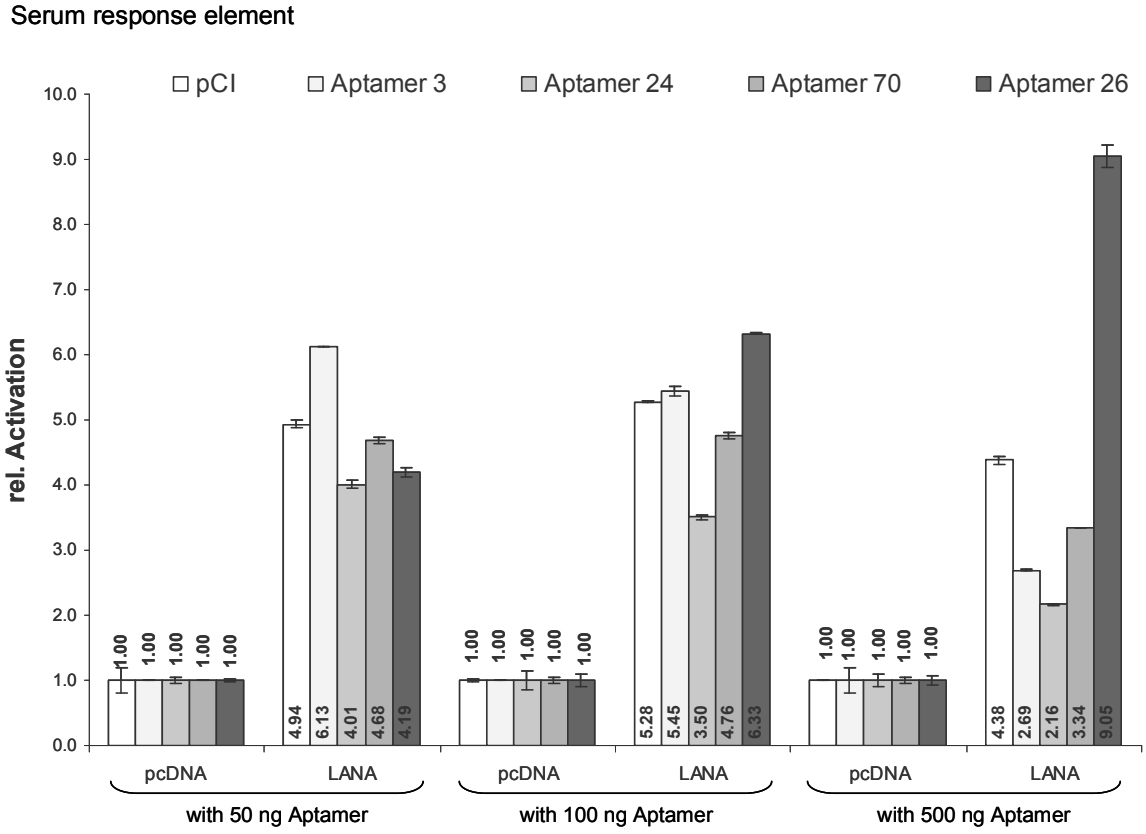


Figure 26. Activation of SRE by LANA-1 could be inhibited with interacting peptide aptamers.
HEK 293 cells were transfected with 50 ng the reporter plasmid pFR-SRE, 500 ng of LANA plasmid DNA or the empty vector pcDNA3 and the above indicated amounts of the peptide aptamer expression vectors. The cells were lysed 48 h after transfection in reporter lysis buffer and the luciferase activity was determined. The experiments were performed in duplicates.

Upon increase the aptamer concentration to 100 ng the activation with the empty vector pCI and the aptamers 3 and 70 stayed at circa five fold, while it decreased to 3.5 fold with the interacting aptamer 24. The control aptamer 26 showed a slight increase of the activation to 6.3 fold (figure 26, two middle column blocks). The hightest aptamer concentration led to a impairment of the activation of LANA-1 with all three interacting aptamers, the strongest effect was observed with aptamer 24. The control aptamer 26 was boosting the activation compared to the empty vector pCI (figure 26, last two column stacks).

Peptide aptamers interacting with LANA-1 impair the cyclin E activation by LANA-1

The increased transcription from the cyclin E promotor in the presence of LANA-1 was described before (Viejo-Borbolla, Ottinger et al. 2005). The connection of this LANA-1 function to the C-terminal part of LANA-1 led to the assumption, that the LANA-1 binding peptide aptamers could interfere with this function as well. To test this hypothesis we performed a luciferase reporter assay with a cyclin E reporter. HeLa cells were transfected with the cyclin E reporter, LANA-1 or the empty vector pcDNA3 and increasing amounts of the peptide aptamers. The results in figure 27 show, that LANA-1 mediated activation of the cyclin E promotor is not significantly reduced in the presence of 100 ng of the peptide aptamers, although a slight decrease is obtained with the interacting peptide aptamer 70 (figure 27, first two column stacks). When 500 ng of the peptide aptamers are present, the cyclin E promotor activation by LANA-1 is still slightly inhibited by the peptide aptamer 70, the aptamer 24 displays a moderate inhibition of the cyclin E induction by LANA-1 (figure 27, the two column stacks in the middle). At the highest concentration of peptide aptamer the aptamer 24 reduces the cyclin E transcription in the presence nearly to the background level with the empty vector, the other two interacting peptide aptamers lead to a moderate decrease of the cyclin E activation by LANA-1, the control aptamer 26 shows no difference in cyclin E activation by LANA-1 compared to the empty vector pCI (figure 27, last two column stacks).
To eliminate the possibility that the peptide aptamers directly influence the protein level of LANA-1 upon their coexpression, aliquots of the samples of the cyclin E luciferase reporter assay were subject of a SDS PAGE followed by immunoblot with antibodies against LANA-1, the peptide aptamers and actin. Figure 28 shows the picture of the samples with the highest peptide aptamer concentration of 1000 ng. LANA-1 expression is equal with the aptamers 24, 70 and 26 (figure 28, top panel, lanes 3-5), the level of LANA-1 protein with the empty aptamer vector pCI is slightly reduced, in the presence of the interacting aptamer 3 the

LANA-1 protein level is increased (figure 28, top panel, lanes 1 and 2). Due to the equal protein levels of actin uneqaul loading or harvesting of cells could be excluded (figure 28, middle panel). The reason for the uneven LANA-1 expression is propably the different efficiency of protein expression of the peptide aptamers. The aptamers 24, 70 and 26 are expressed to roughly equal protein levels, the aptamer 3 is nearly not detectable and the empty vector displays a very strong expression of thioredoxin protein (figure 28, bottom panel).

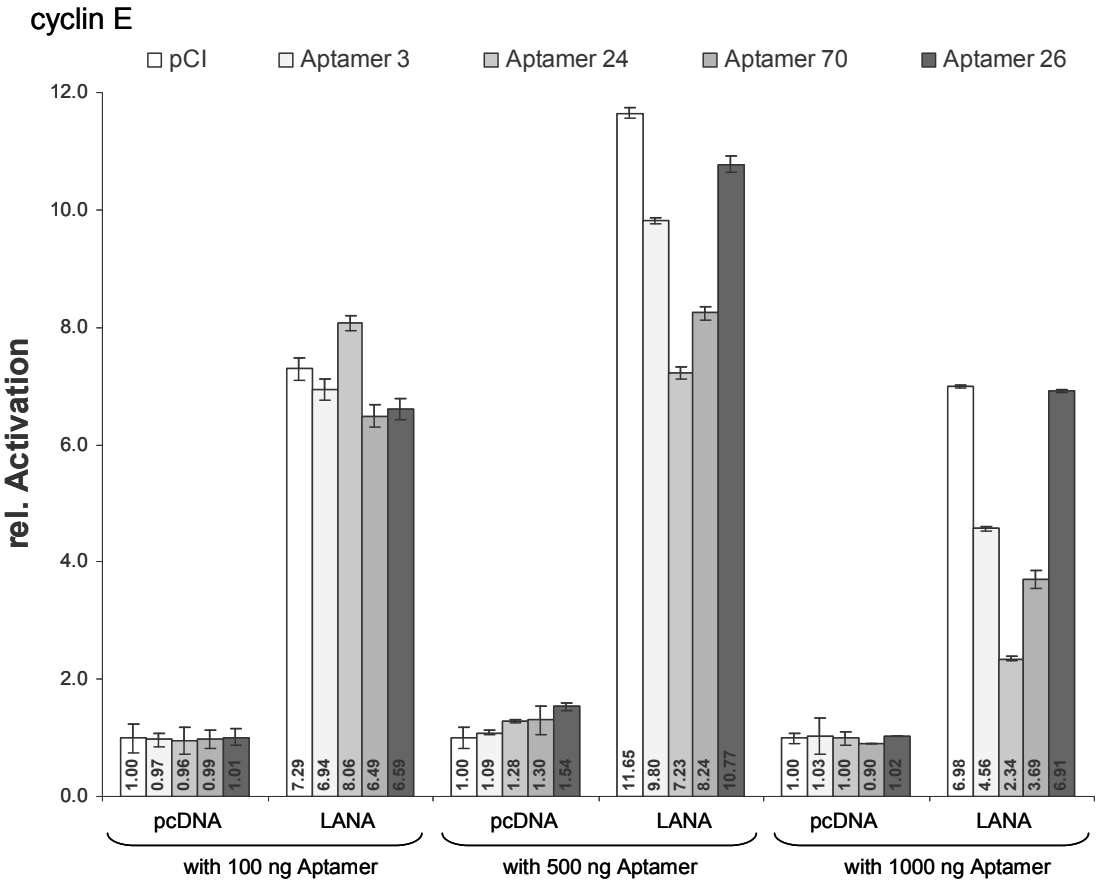


Figure 27. Cyclin E activation by LANA-1 is decreased by interacting peptide aptamers.

HeLa cells were transfected with 100 ng the reporter plasmid pGL2b-cyclin E, 500 ng of LANA plasmid DNA or the empty vector pcDNA3 and the above indicated amounts of the peptide aptamer expression vectors. The cells were lysed 48 h after transfection in reporter lysis buffer and the luciferase activity was determined. The experiments were performed in duplicates (experiment was performed by S. Kati).

The strong expression of the thioredoxin correlates with a lower LANA-1 protein level, while the very low expression of aptamer 3 results in an elevated LANA-1 level. The variations in the LANA-1 protein levels do not invalidate the inhibition of LANA-1 mediated cyclin E activation by interacting peptide aptamers, as the inhibition is obtained even in the presence of higher levels of LANA-1. The only point to consider is that aptamer 3 might have a bigger inhibitory potential, which can not be measured, due to its very low expression.

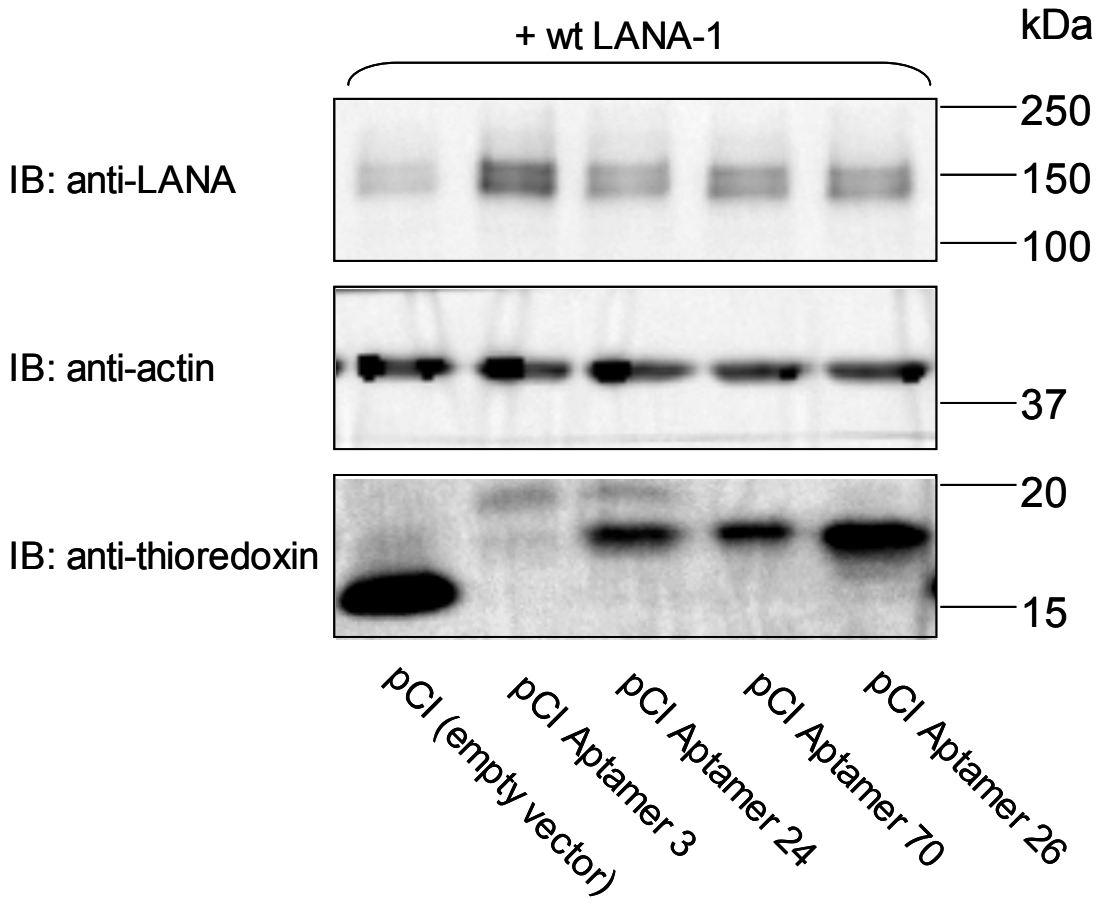


Figure 28. LANA-1 protein levels inversely correlate with protein expression of the peptide aptamers.
40 µl of lysate supernatants of luciferase reporter assay with cyclin E reporter were mixed with 10 µl 5x protein loading buffer and 10 µl of each lysate mixture was loaded on a 10% SDS gel. The gel was blotted onto a nitrocellulose membrane, cutted into three pieces, which were immunoblotted with αLANA, αactin and αThioredoxin antibodies (experiment was performed by S. Kati).

The blocking of enhanced transcription by LANA-1 with the interacting peptide aptamers is not based on a general transcriptional repression by the peptide aptamers

To rule out the possibility, that the peptide aptamers downregulate transcription in a LANA-1 independent function, we performed two additional experiments. First we tested the peptide aptamers in another luciferase reporter assay with a HIV LTR reporter plasmid and cotransfected LANA-1, pcDNA3 or a expression plasmid for the transactivator of transcription (TAT) of HIV. The transcription from the HIV LTR reporter was not altered considerably with any of the peptide aptamer neither in the presence of the LTR activator TAT or in its absence (figure 29).

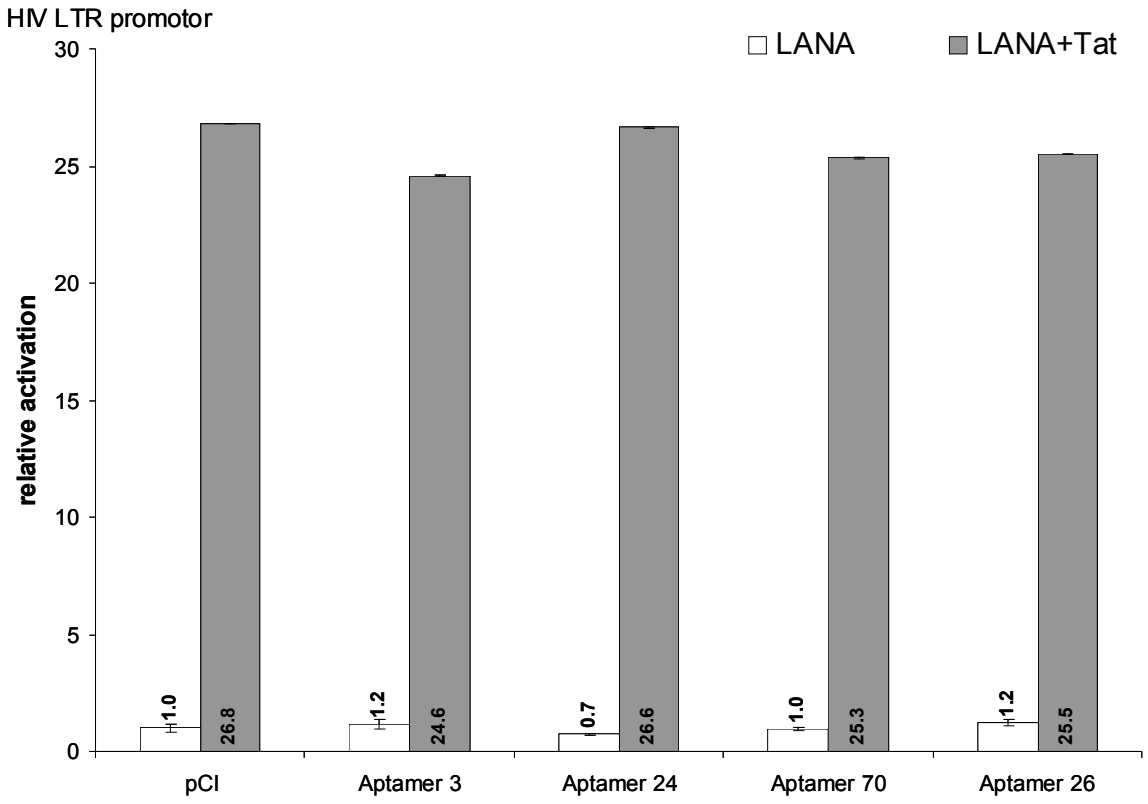


Figure 29. The peptide aptamers do not alter LTR promotor activation.

HEK 293 cells were transfected with 50 ng of the reporter plasmid HIV NL4-3 LTR, 500 ng of LANA plasmid DNA, 50 ng pcDNA3.1 or the TAT expression plasmid and 500 ng of the peptide aptamer expression vectors. The cells were lysed 48 h after transfection in reporter lysis buffer and the luciferase activity was determined. The experiments were performed in duplicates.

To provide another control a β-galactosidase assay was performed. HEK 293 cells were cotransfected with 50 ng of pCMV-β-Gal reporter plasmid and the indicated amount of peptide aptamer expression vectors. The cells were harvested 48 h after transfection, lysed and the amount of expressed β-galactosidase was measured (details see 2.6.5.).

The results, presented in figure 30, show no significant alteration of the β-galactosidase activity. Therefore the peptide aptamers do not influence transcription of the consitutively active CMV promotor of the pCMV-β-Gal reporter vector.

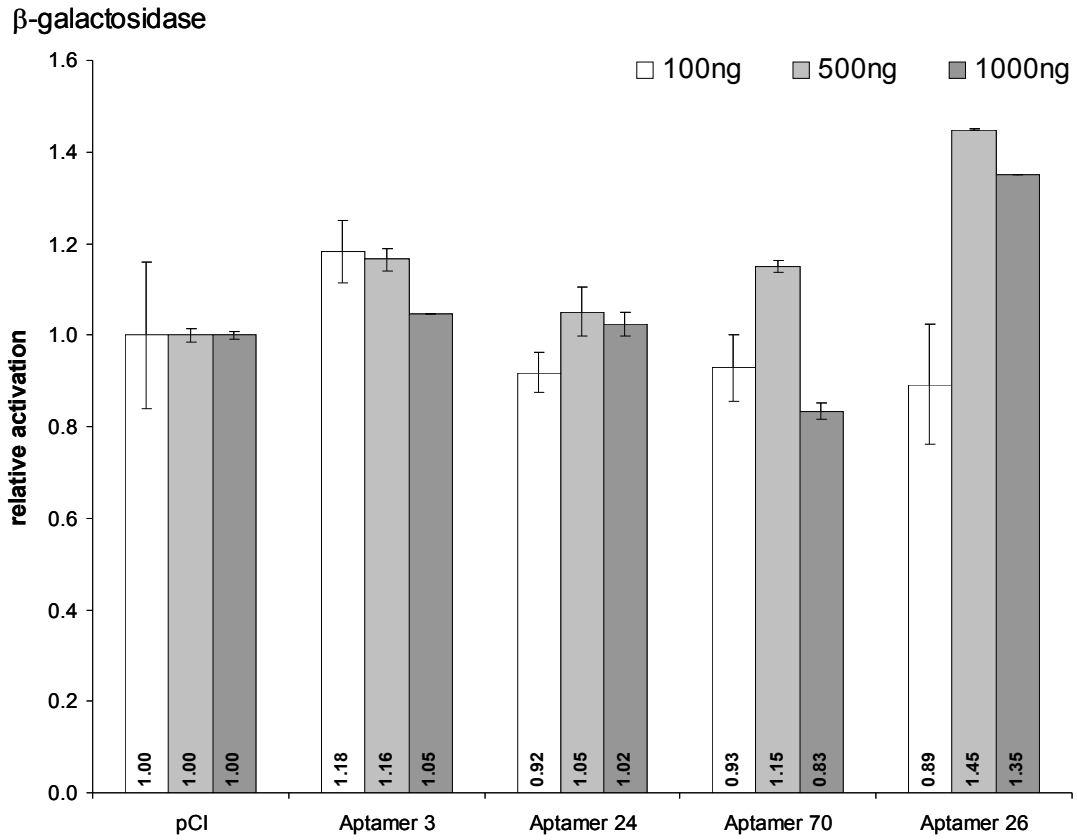


Figure 30. The peptide aptamers do not influence β-galactosidase activity.

HEK 293 cells were transfected with 50 ng of the reporter plasmid pCMV-β-Gal and indicated amounts of the peptide aptamer expression vectors. The cells were lysed 48 h after transfection in reporter lysis buffer and the β-galactosidase activity was determined. The experiments were performed in duplicates.

Nuclear loclization pattern of LANA-1 is not altered by the interacting peptide aptamers

We wanted to investigate if, by binding to LANA-1, the peptide aptamers modify its nuclear localization. We performed an immunofluorescence staining on HeLa cells cotransfected with LANA-1 and either the peptide aptamers or the empty vector pCI. The results are displayed in figure 31. All peptide aptamers present a similar staining pattern and are found throughout the whole cell. However, the nuclear staining is more intense than the cytoplasmic staining and additional brighter spots are visible within the nucleus (figure 31, αThioredoxin channel). The LANA-1 localization in the absence of any peptide aptamer or the empty pCI vector showed the expected nuclear staining pattern (figure 31, first panel). The coexpression of none of the peptide aptamers nor pCI altered the observed localization of LANA-1 (figure 31, αLANA channel). The observed nuclear spots of LANA-1 and the peptide aptamers do not colocalize (figure 31, LANA+pCI and aptamers). The impairment of the transcriptional activation

mediated by LANA-1 upon coexpression of the interacting peptide aptamers does not correlate with a modified nuclear localization of LANA-1.

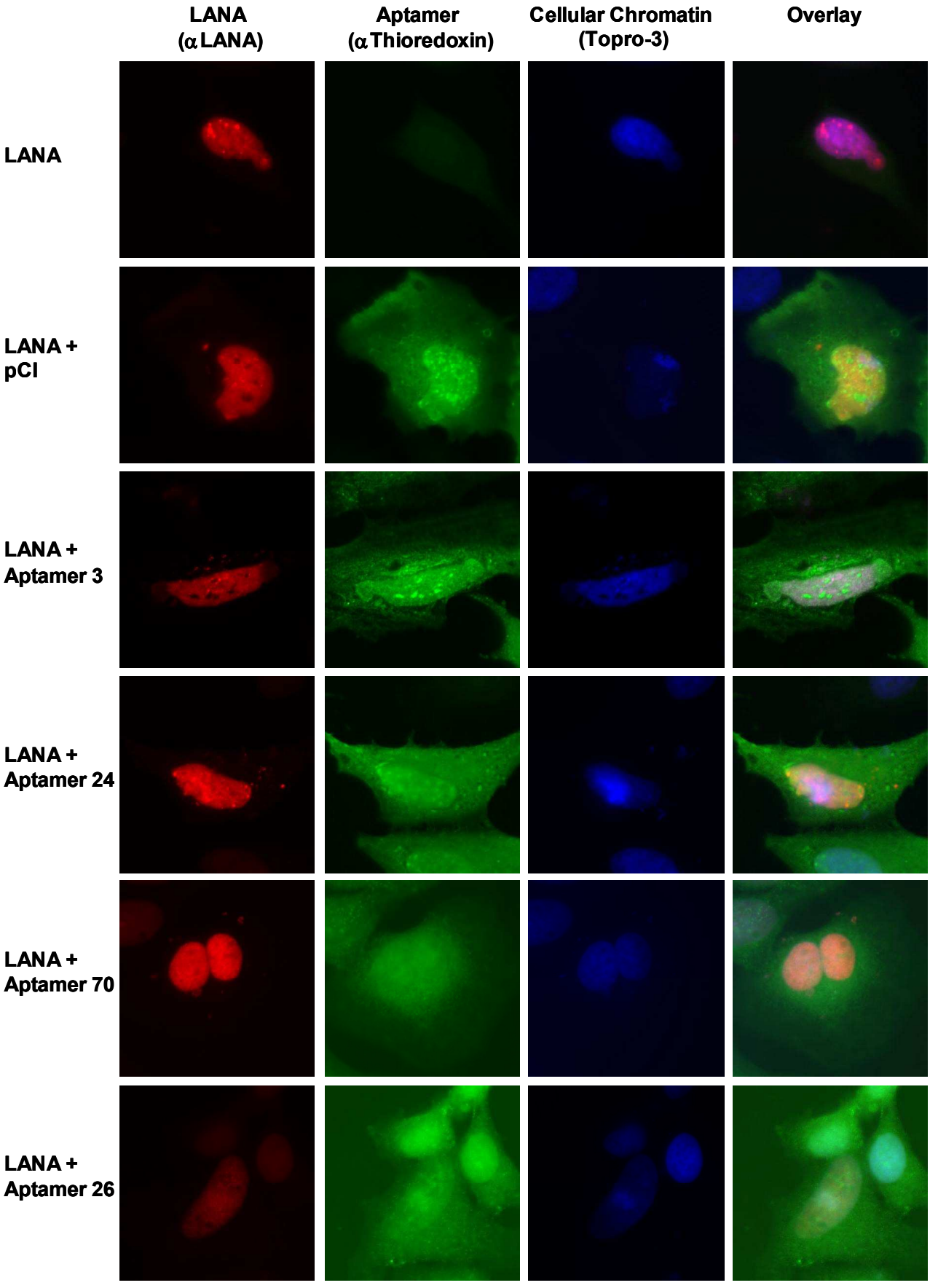


Figure 31. Peptide aptamers binding to LANA-1 do not change the localization of LANA-1.

HeLa cells grown on coverslips were cotransfected with 1 μg of LANA DNA and either 1 μg of the empty vector pCI or the peptide aptamer vectors. The cells were fixed with 4% PFA 48 h after transfection and stained with αLANA and αThioredoxin antibodies.

4. Discussion

Kaposi's sarcoma (KS) is the most frequently seen tumour in AIDS patients in Africa and now the commonest tumour in African man. Its causative agent Kaposi's sarcoma-associated herpesvirus (KSHV) therefore is a human pathogen with severe impact. Although it was detected already fifteen years ago by Chang and colleagues, the vast majority of data is generated *in vitro* in cell culture systems. Due to the narrow host range of *Rhadinoviruses*, infection of regular used small animals like rats and mice with KSHV is impossible and can not be employed to investigate KSHV infection *in vivo*. However, the infection of mice with murine γ-herpesvirus 68 (MHV68) may close that gap and provide a widely available animal model to study rhadinoviral pathogenesis, in contrast to other primate based animal models, which are much less available for research. To evaluate *in vivo* data obtained with MHV68 in regard to KSHV, the similarities and differences between the two *Rhadinoviruses* need to be addressed. As both viruses persists in a latent state with a restricted protein expression pattern, these proteins play a major role in rhadinoviral pathogenesis. Among the latent proteins the proteins encoded in open reading frame (orf) 73 fulfil several essential tasks for viral persistence. KSHV LANA-1 interacts with chromatin associated histones via its N-terminus and binds with the C-terminus to TR elements in the viral episome to support maintainance of the viral genome in the host cell. LANA-1 alters transcription of several cellular and viral promotors, promotes cell cycle progression and interacts with a broad variety of cellular proteins. Fewer studies have investigated the MHV68 orf73 protein, but together with previous experiments in our group, they demonstrated, that the MHV68 orf73 protein can carry out many functions of LANA-1 like transcriptional regulation, viral episome maintainance, cell cycle promotion and interaction with several cellular proteins (unpublished results of M. Ottinger).

One family of proteins KSHV LANA-1 as well as MHV68 orf73 protein have been shown to interact with, are the cellular BET proteins. All mammalian BET proteins are involved in development, transcriptional control and oncogenesis, therefore these proteins seem to be interesting targets for viral proteins. And indeed several viruses benefit from an interaction of a viral protein with cellular BET proteins. Following the report from our group that Brd2 interacts with LANA-1 (Platt, Simpson et al. 1999), the interaction of the E2 protein of papillomavirus with the $Brd4^{L}$ protein was described (You, Croyle et al. 2004). In addition two members of the *Retroviridae* family HIV and HTLV feature viral proteins capable of

binding to Brd4^{L} (Bisgrove, Mahmoudi et al. 2007; Cho, Zhou et al. 2007; Urano, Kariya et al. 2008). This implies a general involvement of the Brd4^{L} protein in many viral life cycles, as very different orders of viruses target the BET proteins to capitalize on their functions. In the view of the association of Brd4 with cellular chromatin throughout the whole cell cycle it was hypothesized, that Brd4 may be responsible for tethering viral genomes to cellular chromatin and thereby ensure proper distribution of the viral genomes during cell division (You, Croyle et al. 2004; You, Srinivasan et al. 2006). However, after the discovery, that Brd4 is part of positive transcription elongation factor b (P-TEFb), many studies question this hypothesis and postulated the involvement in transcriptional control to be the more important parameter (McPhillips, Oliveira et al. 2006; Parish, Bean et al. 2006). Still it is possible that Brd4 can play different roles in the lifecycle of different viruses.

Our results obtained with the MHV68 orf73 protein support an involvement in transcriptional control regulation. A previously performed peptide array identified two potential binding sites within the MHV68 orf73 protein, the first is a four amino acids long peptide in the N-terminus, starting at amino acid (aa) 20 with sequence cystein-lysine-argenine-argenine (CKRR), interacting with purified recombinant Brd4^{S} protein. The second interaction site was detected with Brd2 as well as Brd4^{S} protein, indicating a general BET interaction domain and starts at aa 226 with the sequence glutamine-alanine-lysine-lysine-leucine-lysine (QAKKLK). The second BET binding site lies within the C-terminal domain of the MHV68 orf73 protein and in the region with the highest sequence similarity to the KSHV LANA-1 protein (see figure 5 in the introduction). A closer look at the amino acid composition of the second BET binding site uncovers a high percentage of positively charged amino acid residues (four aa out of six aa). The recently published solution structure of the ET domain of the Brd4 protein, which was the mapped binding part of the Brd4 protein, exhibits an acidic stretch on its surface, this could enable an electrostatic interaction between the two proteins (Lin, Umehara et al. 2008).

In this publication a binding assay with the immobilized ET domain of Brd4 and a KSHV LANA-1 peptide encompassing the aa 1139-1162 failed to show an interaction (Lin, Umehara et al. 2008). This apparent contradiction can be easily explained by two facts: First, regarding our alignment of the MHV68 orf73 protein with the KSHV LANA-1 protein, the second BET binding site would correspond to aa 1110-1115, which are not included in the LANA-1 peptide used. Second the used amino acids 1139-1162 have been demonstrated to be required for oligomerization of the LANA-1 protein, which is a prerequisite for correct function of LANA-1 like interacting with BET proteins (Schwam, Luciano et al. 2000; Viejo-Borbolla,

Ottinger et al. 2005; You, Srinivasan et al. 2006). Therefore the assay failed, due to the lack of a potential BET binding site and the monomeric state of the LANA-1 peptide.

The identified BET binding sites were mutated in a vector for ectopic expression of the MHV68 orf73 protein, which was the tool to dissect the involvement of Brd2 and Brd4 in MHV68 orf73 functions. The obtained results are summerized in figure 32, which presents an overview of all investigated functions of the MHV68 orf73 protein.

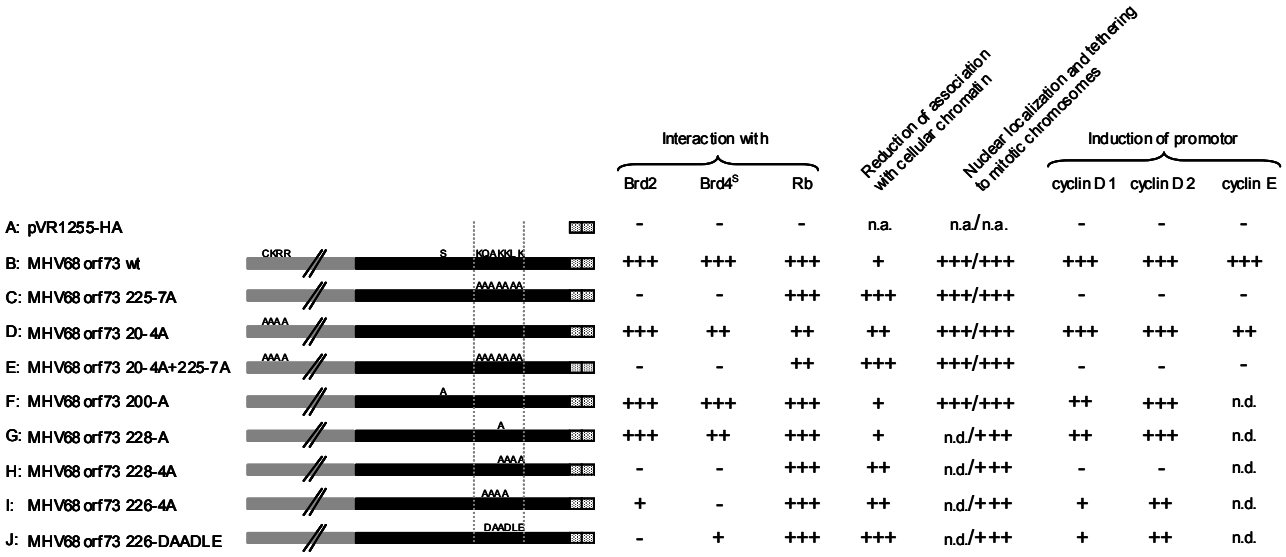


Figure 32. Summary of observed parameters of MHV68 orf73 mutants.

The figure summarizes the results of different experiments from the results section 3.1. n.a., not applicable; n.d., not determined; -, no interaction/induction; +, weak interaction/induction/reduction; ++, intermediate interaction/induction/reduction; +++, strong interaction/induction/reduction

My first task was to verify that the interaction of theMHV68 orf73 protein with the two BET proteins was mediated by the sites identified with the peptide array. To adress this issue, we performed coimmunoprecipitation experiments. These clearly showed the necessity of the C-terminal BET binding site for interaction with both Brd2 and Brd4 proteins, as mutation of the aa KKLK to alanines resulted in complete abrogation of the interaction in both cases. The mutation of the N-terminal BET binding site only affected the Brd4 to a lesser degree and the still available C-terminal BET binding site continues to interact with most of the Brd4^S protein. This implies, that the C-terminal BET binding site is more important, as the N-terminal BET binding site could not compensate the loss of the C-terminal BET binding site. The mutation of functional domains may not only result in abrogation of that specific function, but may also prevent the proper folding of the protein and therefore may result in a complete loss of functions of the mutant protein. To exclude this possibility, we investigated

the binding of the MHV68 orf73 protein to the retinoblastoma (Rb) protein and in addition checked the nuclear localization of the mutant proteins. Neither mutation caused impairment in binding to Rb protein nor led to an abrogation of nuclear localization of the proteins. This makes the possibility of a totally misfolded protein as a result of the mutations unlikely.

The BET protein Brd4 stays associated with cellular chromatin throughout the whole cycle, implying, that it might be resposible for tethering viral genomes to mitotic chromosomes. Although Brd4 colocalizes with the E2 protein of papillomavirus and facilitates maintaince of viral genomes, it is not sufficient for viral genome maintainance in all papillomaviruses and is probably an accessory factor in the tethering of viral genomes to chromosomes during mitosis. The colocalization of $Brd4^{L}$ protein with KSHV LANA-1 protein on mitotic chromosomes was previously published and we were interested to investigate, if the abrogation of BET protein binding in the MHV68 orf73 protein results in any phenotype during mitosis. We therefore investigated the state of the MHV68 orf73 protein in mitosis by preparing chromosomal spreads of cells stalled in mitosis and stained the chromosomal spreads for MHV68 orf73 protein. We observed no alteration in the association of any MHV68 orf73 protein, wt nor mutant, with mitotic chromosomes, providing evidence, that the tethering of MHV68 orf73 to mitotic chromosomes is independent of binding to the BET proteins Brd2 and Brd4.

Nevertheless we obtained a clear reduction of chromatin association, when we extracted nuclear preparations of cells overexpressing MHV68 orf73 proteins with buffers, with an increased KCl concentration. Mutants, whose interaction with BET proteins was disabled, showed elevated levels of MHV68 orf73 protein in the extracts compared to the wt protein. The increased protein levels in the extracts reflects a less tight association with cellular chromatin. As we did not notice any differences in the tethering of MHV68 orf73 to chromosomes during mitosis, the observed decreased binding to cellular chromatin therefore needs to occur during the interphase of the cell cycle. The interaction of MHV68 orf73 with BET proteins and thereby cellular interphase chromatin may allow the virus to alter transcription of cellular genes, by reeling in the P-TEFb complex via binding to Brd4 and thereby start transcription independent of cellular signals to initiate transcription for that particular gene. We investigated three different cell cycle based cyclin promotors, if their transcription is altered by the presence of the MHV68 orf73 protein and if a possible alteration is depending on BET protein interaction. We examined cyclin D2 and cyclin E, two E2F dependent genes, and cyclin D1, which is not responding to E2F stimulation. With all three promotors we found an increased transcription, if the MHV68 orf73 wt protein was

ectopically expressed. While the mutation of the N-terminal Brd4 binding site only slightly decreased the ability to activate cyclin promotors, the abrogation of the KKLK motif in the C-terminal BET binding site resulted in a complete loss of transcription from the investigaed cyclin promotors. Therefore the KKLK motif clearly mediates the interaction of the MHV68 orf73 protein with Brd2 and Brd4 and through this interaction the promotors of cyclin D1, D2 and E are positively regulated (see figure 33).

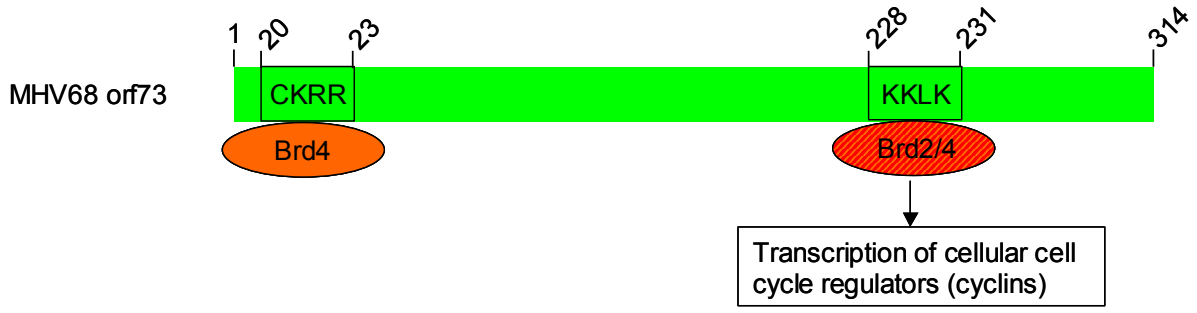


Figure 33. Model of transcriptional activation by the MHV68 orf73 protein.
Via the KKLK motif in the C-terminal BET binding site the MHV68 orf73 recruits the two BET proteins Brd2 and Brd4, after establishment of interaction with the BET proteins transcription from cyclin promotors is activated. The N-terminal BET binding site interacts with Brd4, but is not required for the transcriptional activation process nor does it interact with Brd2.

The observation that the interaction with the Rb protein is not affected by any of our mutations of the MHV68 orf73 protein argues against a possible involvement of the Rb protein in the MHV68 orf73 mediated transcriptional regulation process of these promotors. In addition, the E2F independent cyclin D1 promotor is induced in an equal manner like the E2F responding promotor of cyclin D2, making a participation of Rb in transcriptional regulation by the MHV68 orf73 very improbable.

The discrimination between Brd2- and Brd4-mediated effects is not feasible with the employed methods. Brd2 has been shown to be required for the cyclin D1 transcription *in vivo* as well as recruiting the TATA box binding protein to E2F dependent promotors and thereby enhance their transcription (Peng, Dong et al. 2007; LeRoy, Rickards et al. 2008). The Brd4 protein also influences the cell cycle progression, as modification of the equilibrium between active and inactive P-TEFb alters general cell growth (Mochizuki, Nishiyama et al. 2008; Yang, He et al. 2008). The KLKK motif was necessary for interaction with each of the two BET proteins, due to the ability of the MHV68 orf73 protein to oligomerize, the functional active complex may consists of a mixture of MHV68 orf73 protein with Brd2 and Brd4 in unknown ratios. Therefore a certain degree of redundancy can not be eleminated and a future

attempt to determine the single contribution of each BET protein by abrogation of the other BET protein via small RNA interference may not result in a clear phenotype.
A more valuable prospective task is the *in vivo* investigation of a MHV68 virus with a disabled BET binding site. To accomplish this goal I constructed a bacterial artifical chromosome containing the MHV68 virus genome with a mutated in the orf73 gene. I used a mutant, in which the KKLK binding motif was replaced by four alanines. After finishing the revertant mutant, to exclude accidental introduction of additional mutations, first characterizations like growth kinetics in cell culture would be performed. But the longterm goal would be comprehensive infection experiments in living mice, to adress the involvement of BET proteins in rhadinoviral pathogenesis *in vivo*.

In the second part of this thesis a possible inhibition of KSHV LANA-1 functions with peptide aptamers, which interact with the LANA-1 protein, was investigated. In cooperation with the group of W. Zwerschke a Yeast two hybrid assay was performed to identify peptide aptamers, capable of binding to the LANA-1 protein. While several LANA-1 constructs failed to uncover LANA-1 interacting peptide aptamers, finally a construct encompassing aa 1090-1162 of the LANA-1 protein revealed three interacting peptide aptamers. Although this construct is relatively short, it contains the region with hightest sequence similarity between KSHV LANA-1 and MHV68 orf73 (see figure 6), which is responsible for several LANA-1 mediated functions. To investigate the impact of the identified peptide aptamers on LANA-1 functions, the sequences of the peptide aptamers were cloned into an eukaryotic expression vector.
After verifying the expression of the peptide aptamers upon transfection of eukaryotic cells, the first examined LANA-1 function was the activation of transcription of cellular promotors. A promotor, which contains five copies of the serum response element (SRE), was induced to higher levels in the presence of LANA-1 protein together with the empty aptamer vector pCI and the not interacting peptide aptamer 26, than upon coexpression of LANA-1 with the interacting peptide aptamers 3, 24 and 70. This inhibition of LANA-1 mediated transcriptional activation was tested again with a reporter vector containing the cyclin E promotor. In line with the results obtained with the SRE promotor the LANA-1 mediated induction of the cyclin E promotor was decreased in the presence of the interacting peptide aptamers. As the LANA-1 binding peptide aptamers have inhibited LANA-1 mediated transcriptional induction twice, one could argue, that the interacting peptide aptamers might have a potential to generally inhibit transcriptional processes. To exclude this possibility, we performed another two reporter assays, one with a β-galactosidase reporter under control of a

CMV promotor and with a luciferase reporter vector featuring the long terminal repeat (LTR) promotor of HIV. None of the peptide aptamers changed transcription from the constitutively active CMV promotor, showing, that the interacting peptide aptamers are not capable to suppress transcription in general. The luciferase activity under control of the LTR promotor was not altered by any peptide aptamer either, neither upon coexpresision with the LANA-1 protein nor the transactivator of transcription (TAT) of HIV, which greatly induces transcription from the LTR promotor in comparison to LANA-1. In addition to the obvious conclusion that the interacting peptide aptamers were not generally inhibiting transcription, a second conclusion can be drawn from that experiment. As we demonstrated in the first part of this thesis, the activation of the cyclin E promotor by the MHV68 orf73 protein is mediated via the interaction with the BET proteins Brd2 and/or Brd4. Due to the high degree of similarity in the identified BET binding site it is very likely, that the KSHV LANA-1 protein utilizes BET proteins for the induction of transcription as well. The induction of transcription from the LTR promotor by TAT is dependent on the recruitment of Brd4 by the TAT protein and could be inhibited by overexpression of a C-terminal Brd4 protein fragment, which is interacting with the TAT protein (Zhou and Yik 2006; Bisgrove, Mahmoudi et al. 2007). As the TAT mediated induction of the LTR promotor activity is not reduced by the LANA-1 binding peptide aptamers, the peptide aptamers are not able to abrogate the Brd4 mediated activation of transcription in general. Nevertheless they might still prevent the interaction of the LANA-1 protein with the BET proteins by blocking their binding site at the LANA-1 protein.

Another possible mode of interference with LANA-1 functions may be the downregulation of the LANA-1 protein levels either by reducing protein translation or acceleration of protein degradation for the LANA-1 protein. The LANA-1 protein levels were not equal, when samples of the cyclin E luciferase reporter assay were immunoblotted with an αLANA antibody. The LANA-1 protein level was lower upon coexpression of the empty aptamer vector pCI and higher if the interacting peptide aptamer 3 was coexpressed, compared to the equal LANA-1 protein levels with coexpressed interacting peptide aptamers 24 and 70 and the non binding peptide aptamer 26. In both cases, where the LANA-1 protein level is altered, the peptide aptamer expression is altered as well. The transfection of the empty aptamer vector resulted in a very strong expression of the thioredoxin protein, which serves as a scaffold for the peptide aptamers, the expression of the peptide aptamer 3 is very low, close to loss of detection. Therefore the altered LANA-1 protein expression seem to correlate inversely with

the peptide aptamer expression. As the interacting peptide aptamers 24 and 70 are expressed to equal levels like the non interacting peptide aptamer 26 and the LANA-1 protein levels are comparable as well, the observed inhibition of LANA-1 function is not caused by altered LANA-1 protein levels. The only point one needs to consider, is a possible underestimation of the inhibitory potential of the peptide aptamer 3, due to its very low expression.

The nuclear localization of the LANA-1 protein is another requirement for the correct function of the LANA-1 protein. An inhibition of the nuclear localization of LANA-1 by the interacting peptide aptamers would result in an abrogation of LANA-1 function like transcriptional regulation, which occurs in the nucleus. An immunoflourescence revealed no changes in the localization of the LANA-1 protein neither in the prescence of the empty aptamer vector and the non interacting peptide aptamer 26, nor with the binding peptide aptamers 3, 24 and 70. Therefore we eliminated a modified LANA-1 protein localization as a possible reason for the repression of LANA-1 functions by the interacting peptide aptamers.

To further address the value of the LANA-1 binding peptide aptamers to interfere with the KSHV life cycle, a possible experiment to perform would be the generation of KSHV habouring cell lines, that stablely express the peptide aptamers. If the interacting peptide aptamers would be able to interfere with LANA-1 functions, this would provide further evidence for the inhibition of LANA-1 in living cells.

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6. Appendix

6.1. Abreviations

aa	Amino acids
AIDS	Acquired immunodeficiency disease syndrome
AP-1	Activator protein 1
ATCC	American type culture collection
ATF	Activating transcription factor
ATP	Adenosine-triphosphate
BAC	Bacterial artifical chromosome
BCBL	Body cavity based lymphoma
BET	Bromodomain, extra terminal domain
bp	Basepair
BPV	Bovine papillomavirus
CBP	CREB binding protein
CD	Castleman's disease
cDNA	Complementary DNA
CMV	Cytomegalovirus
CREB	Cyclic AMP response element binding protein
CTL	Cytotoxic T lymphocyte
DABCO	1,4-Diazabicyclo[2.2.2]octane
DC	Dendritic cells
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
EBV	Epstein Barr virus
EBNA	EBV nuclear antigen
E. Coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme linked immunosorbent assay
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluorescien isothiocyanate
Fsh	Female sterile homeotic
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GSK3β	Glycogen synthase kinase 3β
GST	Gluathione-S-transferase
HA	Hemagglutinin
HDAC	Histone deacetylase
HAT	Histone acetyltransferase
HHV	Human herpesvirus
HIF	Hypoxia-inducible factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRas	Harvey rat sarcoma viral oncogene homologue

HRE	Hypoxia responsive elements
HRP	Horseraddish peroxidase
HS	Heparan sulfate
HSV	Herpes simplex virus
HTLV	Human T-lymphotropic Virus
HVS	Herpesvirus Saimiri
IFA	Immuno fluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropylthio-β-D-galactoside
IRF	Interferon response factors
JAK	Janus protein kinases
kb	Kilo bases
kbp	Kilo basepairs
KCP	KSHV complement control protein
kDa	Kilo dalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	Latency-associated nuclear antigen
LBS	LANA binding site
LTR	Long terminal repeat
LUR	Long unique region
MAPK	Mitogen-activated protein kinase
MCAP	Mitotic chromosome-associated protein
MCD	Multimeric Castleman's disease
MCP	Major capsid protein
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MHV68	Murine gammaherpesvirus 68
MIP	Macrophage inflammatory protein
miRNA	micro RNA
MOI	Multiplicity of infection
mRNA	Messenger RNA
MT	Microtubule
myc	Myc gene or protein
NLS	Nuclear localisation signal
NMC	NUT midline carcinoma
NUT	Nuclear protein in testis
OD	Optical density
ONPG	ortho-nitrophenyl-β-galactoside
ORC	Origin recognition complex
orf	open reading frame
PAGE	Polyacrylamide gelelectophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PFA	Paraformaldehyde
PML	Promyelocytic protein
PMSF	Phenylmethylsufonyl fluoride
P-TEFb	Positive transcription elongation factor b

RB	Retinoblastoma protein
RNA	Ribonucleic acid
rpm	Rounds per minute
RRV	Rhesus rhadinovirus
RT	Room temperature
RTA	Regulator of transcriptional activation
SCAF	Scaffold protein
SCIP	Small capsomer interacting protein
SDS	Sodium dodecyl sulfate
SRE	Serum response element
STAT	Signal transducers and activators of transcription
SV40	Simian Virus 40
TAE	Tris acetic acid EDTA buffer
TAF	TBP-associated factor
TAT	Transactivator of transcription
TBP	TATA-binding protein
Th	T helper cell
TNF	Tumor necrosis factor
TR	Terminal repeat
TRI	Triplex protein
Tris	Tri(hydroxymethyl)aminomethan
U	Unit
v	Viral
vEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau
wt	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
Y2H	Yeast two hybrid

6.2. Publications

Matthias Ottinger*, Daniel Pliquet*, Thomas Christalla, Ronald Frank, James P. Stewart, and Thomas F. Schulz. (2009). "The interaction of the gammaherpesvirus 68 orf73 protein with cellular BET proteins affects the activation of cell cycle promoters." J Virol **83**(9): 4423-34.

(* these authors contributed equally)

6.3. Curriculum vitae

Personal data

Name:	Dipl. Biochem. Daniel Pliquet
Address:	Kranckestr. 5, 30161 Hannover
Telephone:	0049-(0)511-1248144
Date of birth and place:	14.12.1977 in Pinneberg
Marrial status:	married, 1 child

Scientific carrier:

04/2005 – today	doing a PhD in the group of Prof. Dr. T.F. Schulz with the title: „*Role of Bet-Proteins in the Function of Rhandinoviral Orf73-Proteins*“; organisation and supervision of practical courses with up to 30 participants
03/2005	Diploma (grade: 1.8)
07/2004 – 01/2005	Diploma thesis at the Medical School Hannover, Institute of Virology in group of Prof. Dr. T.F. Schulz
10/2000	Intermediate diploma (grade: 1.9)
10/1998	**Start of studying biochemistry at the Leibniz University Hannover**

Publications:

02/2009 Articel in *Journal of Virology*

“The interaction of the gammaherpesvirus 68 orf73 protein with cellular BET proteins affects the activation of cell cycle promoters.”
Matthias Ottinger §, Daniel Pliquet §, Thomas Christalla, Ronald Frank, James P. Stewart und Thomas F. Schulz §: equal contributors

03/2009 Poster and presentation at the annual meeting of the Infection and Cancer (INCA) program of the EU in Cascais (Portugal)

03/2009 Poster at the annual meeting of the German Society for Virology in Leipzig

School education:

06/1997	Abitur (grade: 1.7)
1988 - 1997	**Comprehensive school Elmshorn; advanced courses in chemistry und mathematics, forgein languages: English, Latin**
1984 - 1988	Elementary school Seester

Additional skills:

Computing skills:	Windows and MS-Office, Adobe Photoshop
Languages:	German native speaker, English fluently spoken and written, basics in Swedish und Japanese, Latin
Professional trainings:	Security in genetic engineering (according to German laws) Practical course on working with radioactive materials (requisite qualification according to German laws)

6.4. Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover für die Promotion zum Dr. rer. nat.
Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Role of BET-Proteins in the Function of Rhadinoviral Orf73-Proteins

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.
Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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(Unterschrift)

Name:Daniel Pliquet

6.5. Acknowlegdements

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