Aus dem Institut für Virologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Cellular factor ND10 complex is involved in repression of lytic replication of human herpesvirus 6A

Inaugural-Dissertation

zur Erlangung des akademischen Grades
Doctor of Philosophy (Ph.D.) in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Anirban Sanyal
aus Siliguri, Indien

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Zweiter Gutachter: Univ.- Prof. Dr. Klaus Osterrieder

Dritter Gutachter: - PD Dr. Sandra Blome

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To the memories of my grandparents,

Prakriti & Sailendranath Sanyal and Purnima & Binoybhushan Lahiri

यद्यदाचरति श्रेष्ठस्तत्तदेवेतरो जनः।
स यत्प्रमाणं कुरुते लोकस्तदनुवर्तते॥

-भगवद्गीता ३.२१

Yad yad ācarati śreṣṭhaḥ tad tad eva itaraḥ janaḥ
Sa yad pramāṇam kurute lokaḥ tad anuvartate.

-Bhagavad Gītā 3.21

“However a great man conducts himself, common men will follow. Accordingly, whatever standards he sets by his actions, others will follow in his footsteps.”.

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2. Abbreviations

AAV-2	Adeno-associated virus 2
BAC	Bacterial artificial chromosome
bp	Base pairs
BSA	Bovine serum albumin
CAM	Chloramphenicol
CBMC	Cord blood mononuclear cell
d	Day
ddH_2O	Double distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
DR	Direct repeat
dsDNA	Double-stranded deoxyribonucleic acid
E	Early genes
E. col	Escherichia coli
EDTA	Ethylendiamine tetraacetic acid
EBV	Epstein-Barr virus
FBS	Fetal bovine serum
FCS	Fetal calf serum
FISH	Fluorescence *in situ* hybridization
for	Forward
gH/gL/gQ1/gQ2	Glycoprotein H, L, Q1 and Q2 complex
GFP	Green fluorescent protein
h	Hour
HCMV	Human Cytomegalovirus
HDAC	Histone deacetylase
hDaxx	Human death domain-associated protein 6
HHV	Human herpesvirus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HSV-1	Herpes simplex virus 1
HR	Homologous recombination
IE	Immediate early
IL-2	Interleukin 2

impTMR	Imperfect telomeric repeats
Kana	Kanamycin
Kb	Kilo base pairs
Kbp	Kilobase pairs
L	Late
LB	Luria-Bertrani medium or lysogeny broth
LATs	Latency-associated transcripts
min	Minutes
miRNA	Micro ribonucleic acid
MDV	Marek's disease virus
MEM	Minimum essential Medium Eagle
ND10	Nuclear domain 10
o/n	Overnight
OD_{600}	Optical density, 600 nm wavelength
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
p.i.	Post infection
PML	Promyelocytic leukemia
pTMR	Perfect telomeric repeats
P/S	Penicillin/streptomycin
qPCR	Quantitative real Time PCR
rev	Reverse
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Seconds
SEM	Standard error of the mean
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
Sp100	Speckled protein of 100 kDa
ssDNA	Single-stranded deoxyribonucleic acid
TAE	Tris-acetate-EDTA buffer

TGN	Trans-Golgi network
TMR	Telomeric repeat
TPA	Tetradecanoylphorbol acetate
TSA	Trichostatin A
U	Unique
wt	Wild type

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5. Introduction

5.1. Herpesvirales

The order Herpesvirales comprises of some of the largest and complex viruses known to mankind. The list of herpesviruses is ever growing and more than 100 different species are well documented and classified by the International committee on Taxonomy of Viruses (ICTV). It is also interesting to know that many herpesvirus species have also become extinct over the course of evolution alongside the extinction of their respective hosts. Moreover, a considerable number of these viruses still remain unidentified as a vast majority of living organisms are yet to be investigated for the presence of these viruses (1).

Owing to their large size, the herpesvirus virions have a diameter range between 200-250 nm. Within the core of the virion is a linear, double stranded DNA (dsDNA) genome. The size of the genome ranges between 125-245 kbp, depending on the species of the virus. The genome is packed within an icosahedral capsid, which in turn is surrounded by virus encoded proteins called the tegument. The outermost layer of the virion consists of the lipid envelope which again harbors numerous viral glycoproteins on its surface (Figure 1.) (1, 2).

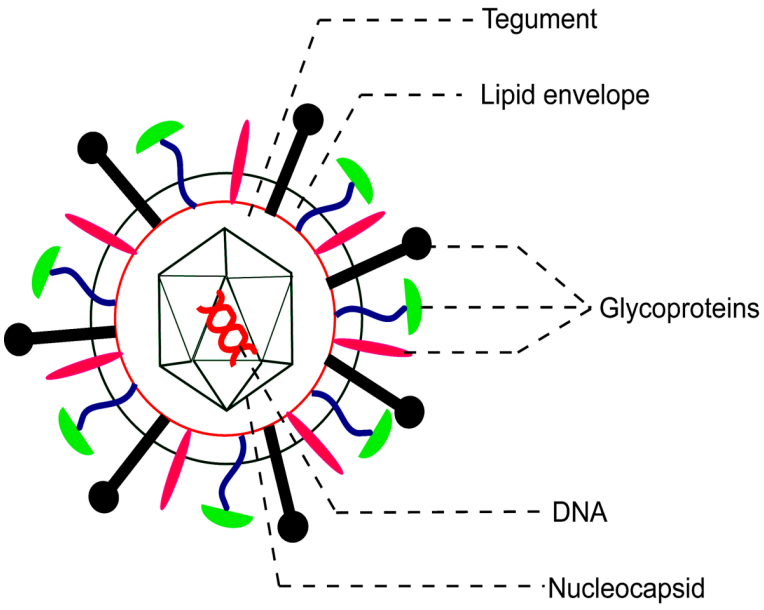

Figure 1: Schematic representation of a herpesvirus virion.
The dsDNA genome of the virus and the viral capsid together form the nucleocapsid. The nucleocapsid is embedded surrounded by the the viral tegument. The tegument in turn is surrounded by the lipid envelope, which is spiked with virally encoded glycoproteins.

According to the Baltimore scheme of classification of all known viruses, herpesviruses are categorized as class I owing to their double-stranded DNA genome (3). The ICTV further

updated the classification of herpesviruses by classifying them under the new order of *Herpesvirales*. *Herpesvirales* further comprises of three families namely, *Malacoherpesviridae*, consisting of invertebrate hosts like molluscs (4, 5), the *Alloherpesviridae* which includes hosts fish and frogs and the *Herpesviridae*, that encompasses viruses infecting mammals, birds and reptiles (6, 7). *Herpesviridae* is further subdivided into three sub-families based on the biological properties of the member viruses. These three sub-families are the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae*. Furthermore, another subfamily called the *Deltaherpesvirinae* has recently (2014) been proposed that will consist of six elephant endotheliotropic herpesviruses (EEHVs) (8).

Although herpesviruses have an enormous and extremely diverse host range as well as variable complexity in their genome, yet the most defining feature of all herpesviruses is their ability to establish lifelong persistence or latency within their respective hosts. Also, reactivation can periodically occur from the latent infection state leading to virus replication and spread into new hosts. The subfamily *Alphaherpesvirinae* (α) exhibits a broader host range yet relatively short reproductive cycle. The sensory ganglia of the infected hosts serve as the primary latency reservoir for these viruses. While, the subfamily *Betaherpesvirinae* (β) has a restricted range of hosts yet have longer reproductive cycles. They primarily establish latency in differentiating cells such as hematopoietic stem cells. On the other hand the subfamily of *Gammaherpesvirinae* (γ) infects T- or B-lymphocytes of the host and establishes latency in lymphoid tissue (2). Nine members of the herpesviridae from the *α*, *β* and *γ* subfamilies have been known until now to infect humans, they are also termed as human herpesviruses (HHV) and are listed in the following table:

Table 1: Classification of human herpesvirus

HHV-1 / HSV-1	Herpes Simplex Virus 1	α
HHV-2 / HSV-2	Herpes Simplex Virus 2	α
HHV-3 / VZV	Varicella-Zoster Virus	α
HHV-4 / EBV	Epstein-Barr Virus	γ
HHV-5 / CMV	Cytomegalovirus	β
HHV-6A	Human Herpesvirus 6A	β
HHV-6B	Human Herpesvirus 6B	β
HHV-7	Human Herpesvirus 7	β
HHV-8 / KSHV	Kaposi’s Sarcoma-associated Herpesvirus	γ

Over the course of evolution for millions of years the herpesviruses have learnt to adapt accurately with respect to their hosts. This high adaptability has led to limited pathogenicity and higher potential of establishing latency thereby striking the perfect equilibrium between the host and the virus. Hence, primary infection by herpesviruses is usually asymptomatic in immunocompetent hosts. In order to achieve long term persistence within the host the maintenance of this equilibrium is of prime importance for herpesviruses (1).

5.2. Human herpesvirus 6

5.2.1. Background and general features

Salahuddin *et al*. discovered human B-lymphotrophic virus within patients with lymphoproliferative disorders and AIDS. This was classified as the first member of the genus *Roseolovirus* within the *Betaherpesvirinae* subfamily. The nomenclature was renamed as human herpesvirus 6 (HHV-6) in the future (9). Initially HHV-6 was further divided in between two distinct variants, HHV-6A and HHV-6B owing to major differences in their genetic, biological, immunological and epidemiological features (10, 11), but later, because of major discrepancies, in 2012 the ICTV classified HHV-6A and 6B as two separate species (12) (Figure 2.).

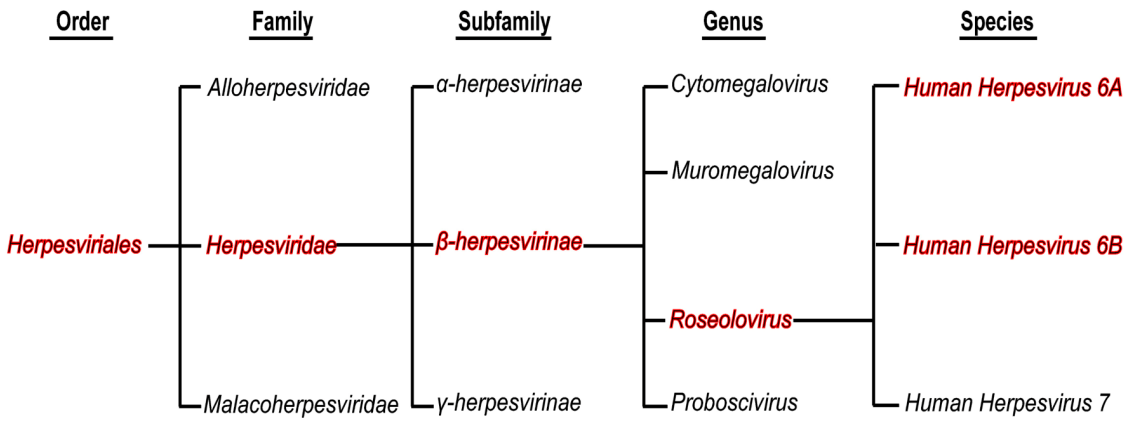

Figure 2: Human herpesvirus 6 classification hierarchy

The global seroprevelance of HHV-6 in the human population is more than 90% thus making it a potential ubiquitous pathogen. Following the loss of maternal antibodies, primary infection by HHV-6 occurs in newborns and children under the age of 3 years (13, 14). Moreover, ~1 % of children in the world are born with congenital HHV-6 infection (15, 16). Among the two different species of HHV-6, 6B is considered to be the major causative agent for illness in children. Disease symptoms are usually characterized by febrile illness associated with skin rash over the trunk, neck and face. The condition is clinically termed as *roseola infantum*, or *exanthema subitum* and more commonly as the sixth disease (17). Severe neurological

complications like seizures and encephalitis can also manifest during the infection but such cases are rare in occurrence (16, 18). In few cases, primary infection in adults has also been reported earlier. In comparison to 6B, HHV-6A infection is not so commonly detected, although, majority of infants in an HIV-endemic region in sub-Saharan Africa were diagnosed positively for HHV-6A infection (19). In all aspects, HHV-6 poses as an opportunistic pathogen causing asymptomatic infection followed by lifelong persistence/establishing latency in immunocompetent individuals. There is no known HHV-6 specific antiviral drug available with due approval until now. HHV-6 infections are thus, currently treated with anti-CMV antivirals like valganciclovir, cidofovir or foscarnet (20).

In case of individuals who are immunocompromised, reactivation of HHV-6 leads to severe disease manifestation. This is the reason behind HHV-6 being associated with a plethora of medical conditions like encephalitis, graft rejection after transplantation (solid organ, bone marrow or stem cell therapy), multiple sclerosis and also associated with rapid AIDS progression (21-23). Similar to HIV, HHV-6 can also infect CD4+ T-lymphocytes which has been demonstrated *in vitro*. HHV-6 can also upregulate CD4 expression on T-lymphocytes and also trans-activate the long terminal repeat promoter of HIV, thereby enhancing HIV infection (24, 25). The biological significance of co-infection of HHV-6 in HIV positive individuals is a complex area of research and hence further investigations are currently ongoing on these issues.

5.2.2. Animal models

A suitable animal model/natural host-virus model is lacking in the field of HHV-6 research. This poses as a big challenge to get in depth knowledge of viral pathogenesis. Moreover, a stable animal model would propel the development of advanced diagnostic tools as well as antivirals or vaccines. A few mouse as well as ape models have been developed over the recent past (26, 27) but none are considered to be the ideal gold standard for research until now. CD46 transgenic/humanized mice (28) as well as a marmoset model (29) have been developed to study the neuropathology of the virus. A pig-tailed macaque model was also developed to study immunomodulation and immunodeficiency as well as HHV-6 facilitated HIV progression (30). Neutralizing antibodies against HHV-6 was discovered in different primates (31) followed by the discovery of HHV-6 homologs in drill monkeys as well chimpanzees (32, 33). Very recently, homologs of both HHV-6 and HHV-7 were discovered in pig-tailed monkeys (34). These findings are quite intriguing and have led to the proposal for development of a chimpanzee model. The establishment of such a model would greatly advance our insight into host-pathogen interactions in the future.

5.2.3. HHV-6 genome structure

HHV-6 has a class A genome which is approximately 160 kilo base pairs in length. The viral genome was completely sequenced in 1995 (35). The viral genome consists of a unique segment (U), approximately 144 kilo base pairs long, which is flanked on either side by approximately 8 -10 kilo base pairs of direct repeats (DR) (Figure 3).

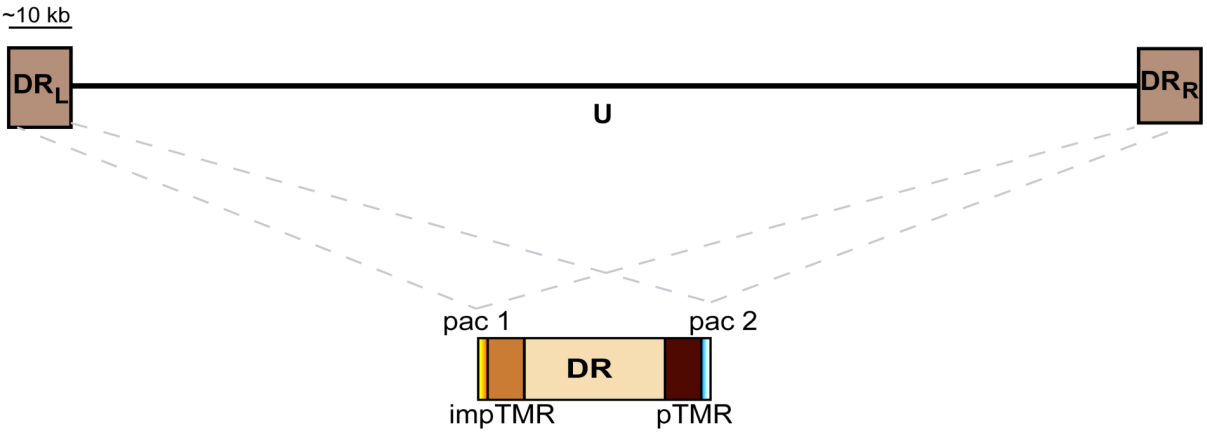

Figure 3: Schematic representation of the HHV-6 genome explaining the direct repeat region.

The HHV-6A genome contains a unique region (U), flanked on either side by terminal direct repeats (DR_L and DR_R). DR regions are shown as grey boxes. The DR region is zoomed into to depict the telomeric repeats (imperfect TMRs (impTMRs) and perfect TMRs (pTMRs)) and the packaging sites pac1 and pac2 (image is not drawn to scale).

Genomes HHV-6A and 6B have a very low percentage (<1%) of variability between them. Nucleotide sequence identity between both the species is as high as 90% (21). The maximum diversity between the two species lies in the IE genes, the region between U86 and DR_R and also in the glycoprotein encoding gp82/105 gene. These variabilities could be relevant for the biological differences between the two species of viruses, as has been hypothesized earlier (36, 37).

The entire genome can be segregated into 119 ORFs (open reading frames) as predicted earlier (21). Out of these, 43 forms the core genes which forms the seven major blocks as can be seen in all herpesvirus genomes. It is possible that these core genes in all herpesviruses must have come from a common ancestor. These core genes code for proteins that are highly essential for virus replication, cleavage as well as packaging. The rest of the non-core genes are possibly virus specific and probably plays important role in delineating an evolutionary niche for the particular pathogen. They are responsible for the following functions: a) cellular tropism, b) manipulation of cellular processes, c) evasion of host immune system and d) latency (38). The non-core genes include betaherpesvirus specific genes, especially those

which are present in the members of the *Roseoloviruses*, some of which are unique to HHV-6A or 6B.

Genes are coded from both strands of the DNA. Some of the viral genes are also spliced post-transcriptionally resulting in differential regulation of gene expression (21). The genome also codes for latency associated transcripts (LATs) and micro RNAs (miRNAs) which are types of non-coding RNAs. Also if one considers factors like alternative splicing, translational frame shifting, internal translational initiation sites, or antisense open reading frames then the number of genes expressed could have a higher tally (2). For example, ribosomal profiling of HCMV revealed numerous unidentified ORFs, a few years back (39, 40).

5.2.4. Immunobiology

Herpesvirus genomes have evolved tremendously over the course of evolution mostly in order to infect their respective hosts. A lot of this adaptation has been dedicated to evolving genes in the virus that are responsible in the cross-talk between the immune system of the host and the viral genome. The virus-host interaction process is complex and key to the establishment of a successful infection by the virus. Firstly, incoming viruses have to face the host's innate defense mechanism which includes antiviral cytokines or chemokines that in turn signals natural killer cells and other granulocytes to destroy virus infected cells. Secondly, the infected cells release type II interferons which activates the cytotoxic T-cell response of the adaptive immune system of the host. Finally, in some cases the virus infected cells can induce programmed cell death or apoptosis thereby clearing the virus.

Substantial genetic resources of the herpesviruses could be dedicated in order to escape such multipronged defense mechanism of the host. A barrage of escape strategies has evolved in herpesviruses to counter host immune system (41, 42). Some of them like, molecular mimicry, expression of cellular interleukin homologs or chemokine receptors are seen in a wide variety of herpesviruses. Some herpesviruses can also induce the downregulation of the expression of major histocompatibility complex (MHC) in infected host cells thereby subduing the presentation of viral antigens to the adaptive immune system of the host (43).

Striking the perfect balance between stimulation and evasion of the host immune system is key to perpetuity of the viral genome in the infected host, and it is a key characteristic of the members of the *Roseolovirus* subfamily (44). In case of HHV-6 only a few genes have been known to exhibit these functions. There are quite a few uncharacterized genes as well that maybe involved in the immune evasion process and are yet to be characterized (41). Relevant data available denotes the immunomodulatory functions of proteins encoded by HHV-6 genes, which are involved in suppressing both the innate and adaptive immunity in the host,

manipulation of host cell death/apoptosis and T-cell signaling (41). HHV-6 IE1 protein is of key importance in this context. It is one of the first gene products following the entry of the virus into the host cell nucleus. It has been found to abrogate antiviral responses in the host by impairing IFN-β gene induction and IFN-stimulated gene activation. This proves to be a key step in the protection of the viral genome in the early infection phase thereby facilitating efficient virus replication. The viral U54 tegument protein has been reported to suppress cellular interleukin-2 (IL-2) synthesis thereby impairing host cell proliferation. The downregulation of MHC-I in host cells is caused by the viral U21 gene product (20, 41). This prevents HHV-6 antigen presentation thereby dampening cell mediated immune response. Simultaneously, the viral U24 gene expression causes the internalization of T-cell receptor/CD3 complex in T-cells which abrogates T-cell activation by antigen-presenting cells (45, 46). HHV-6 also exhibits molecular mimicry in order to enhance its immune evasion capabilities. Viral U83 protein binds and blocks host chemokine receptors while the viral U12 and U51 genes encode for host chemokine receptors, thereby abrogating chemokine signaling in hosts (41).

5.2.5. Cell tropism

As discussed earlier, HHV-6 has a preferential tropism towards lymphocytes, essentially activated CD4+ T-cells in the host (47, 48). Apart from lymphocytes, it can also infect other cell types like CD8+ T-cells, Macrophages/monocytes, natural killer cells, endothelial cells, astrocytes, dendritic cells, fibroblasts, bone-marrow progenitor cells and epithelial cells. It is important to note that there is limited productive replication in the cell types other than CD4+ T-lymphocytes (25, 49). HHV-6 infection in cells is characterized by strong cytopathic effect and formation of large syncytia (21).

5.2.6. Virus replication

Replication of genome during the life cycle of herpesviruses takes place inside the host cell nucleus. During the lytic phase of infection viral genome is amplified and new progeny viruses are released, whereas, during the latent/quiescent phase of infection the viral genome is not amplified and rather maintained within the nucleus of the infected host cell. Both the phases are discussed in detail below.

5.2.6.1. Genome replication in lytic phase

Almost all nucleated cells in a human host expresses the type I transmembrane glycoprotein CD46. This receptor is the key to the attachment of HHV-6 to the target host cells. This

attachment of the virus with the host cell receptor is the first step in the replication cycle of the virus (50, 51). CD46 also acts a receptor for measles virus, Neisseria gonorrhoeae and streptococcus A (52-54). Also, HHV-6B specifically binds to CD134 (TNF receptor superfamily) receptor on host cells (55). The viral glycoprotein complex gH/gL/gQ1/gQ2 is involved in binding with the host cell receptor. This process of binding is followed by the internalization of the virion inside the cell via endocytosis. Subsequently, the the viral envelope and the cellular membranes fuse together thereby releasing the nucleocapsid into the cytoplasm of the host cell. Following which, the cellular tubulin microtubules then transports the nucleocapsid to the outer nuclear membrane when the viral DNA enters the nucleus through the nuclear pore complex and with the help of the importin-β activity. The viral DNA then circularizes inside the nucleus post entry (51, 56). DNA replication, viral transcription and nucleocapsid assembly events happen inside the host cell nucleus from here on.

Viral genes are transcribed into messenger RNAs (mRNAs) utilizing the cellular RNA polymerase II enzyme, which carries out the 5'- capping and 3'- polyadenylation of the transcripts (56). The regulatory cascade of herpesvirus gene expression during lytic replication is initiated by a trans-activator brought along with the viral particle during infection. The immediate-early (IE) genes of the virus is expressed irrespective any other viral protein synthesis, and it helps to activate the expression of early (E) genes of the virus. The E genes are responsible for processes like viral replication, metabolism and also in abrogating antigen processing. The E genes are expressed independent of viral DNA synthesis, whereas, the expression of late (L) genes is very much dependent on viral DNA synthesis. They are therefore expressed at the end and the late gene transcripts usually code for structural proteins of the virus like capsids, tegument or the viral envelope (2, 43). Some of the viral proteins are also responsible for degrading cellular mRNAs in order to facilitate viral gene expression (56).

The DNA replication process begins within specific sites in the viral genome called the origin of lytic replication (oriLyt) which lies between the U41 and U42 region of the viral genome (2, 56, 57). As discussed earlier, a set of core genes (7 in number), which are conserved among herpesviruses, then facilitate the entire replication process. HHV-6 U38 gene encodes the viral DNA polymerase. It is the most important of all the core genes. It has a 3'-5'proofreading exonuclease as well as RNaseH activity. It functions in tandem with the viral DNA polymerase processivity factor which is encoded by the HHV-6 U27 gene. Furthermore, HHV-6 U43, U74 and U77 encode for the heterotrimeric helicase-primase complex and is essential for the replication process. Additionally, HHV-6 U41 encodes for a single-strand (ss) binding protein which protects the ssDNA template during replication. Finally the HHV-6 U73 gene which encodes for a protein that recognizes the origin of DNA synthesis is also highly essential for the viral genome replication process (38, 56). 'Rolling circle replication' method is observed in

HHV-6 genome replication. This type of replication gives rise to multiple unit-length viral genomes which a called concatemers, linked in a head-to-tail manner. The cleavage of the concatemers takes place at the *cis*-acting regions pac1 and pac2 that are present in the terminal *a* sequence of all herpesviruses. The unit-length viral genomes are then encapsidated into already assembled capsids, thus making the viral nucleocapsid (38, 56, 58). Subsequently, the viral nucleopcapsids exit the nucleus following a sequence of envelopment and de-envelopment steps. Budding into the perinuclear cisternae the nucleocapsids are incorporated with a temporary envelope (envelopment) devoid of glycoproteins. The capsids are then released from the perinuclear cisternae into the cytoplasm by the removal of the temporary envelope (de-envelopment). In the cytoplasm, the nucleocapsid is equipped with the tegument protein. This is then followed by the re-envelopment of the viruses at the trans-Golgi network or at the exit of trans-Golgi network membranes. The envelope thus obtained is now embedded with glycoproteins. The mature virions then finally exit the cell either by exocytosis or through cell lysis (38, 51). It takes approximately 72 hours to complete one cycle of replication of HHV-6 from the point of infection until the release of progeny virions (22).

5.2.6.2. Genome replication in latent phase

As mentioned in the beginning, a characteristic feature of herpesviruses is its capability to establish a lifelong/persistent infection in the host. This is commonly termed as latency. Latent phase of infection is characterized by the maintenance of the viral genome in the host cell nucleus without the production of progeny virions/amplification of the genome. The maintenance of this stage is augmented by the activity of both viral and some cellular factors which ensures the suppression of viral gene expression (56). Also the latent phase might involve a period of chronic replication leading to continuous or periodic release of infectious virions (21). It is important to note that betaherpesviruses are capable of establishing latency in a variety of leukocyte subsets (43).

During the latent stage of infection, most herpesvirus genomes exist in a typical circularized form called episomes which are associated with histones (43). The histone packaging of DNA has regulatory as well as repressive influence as has been revealed from studies on CMV. It was also found that distinct methylation/acetylation marks might be involved in the switch between lytic and latent infection (59). During latency, the production of viral proteins is highly restricted in order to avoid detection by host immune system. For example, the IE genes or the other important genes required for productive infection are required to be trancriptionally silent in latently infected cells. Histones associated with the latent genome shows distinct heterochromatic modifications like de-methylation of H3K4 and de-acetylation of H3K9 or mono- and di-methylation of H3K9 which is caused by co-repressor complexes like histone

deacetylases (HDACs). Viral genes that are expressed during the latent infection stage are associated with histones with euchromatic modifications (59, 60). Cellular protein complex (subnuclear) called nuclear domain 10 (ND10) is a transcriptional regulator complex which has a multivalent role in the cell cycle processes. It can mediate a transcriptional repression role as well and hence maybe also involved in the maintenance of latency of herpesvirus genome (59). The possible involvement of ND10 in lytic/latent infection of HHV-6 will be discussed in the upcoming paragraphs.

In addition to the above factors, micro RNAs (miRNAs) also plays a major role in the establishment or maintenance of latency in case of herpesviruses. About 100 different miRNAs have been discovered until now in human herpesviruses. miRNAs are small non-coding RNAs that can target mRNAs thereby leading to translational repression and reduction in protein levels. miRNAs encoded by herpesviruses are expressed in both the lytic as well as latently infected cells. Few of these miRNAs can target viral mRNAs thus regulating the switch between lytic phase and latent phase of infection. Most of the viral miRNAs are involved in targeting cellular mRNAs by which they can dampen the host's antiviral immune responses (61, 62). In case of EBV and KSHV expression of several miRNAs are observed during latency. It has also been found that the LAT locus of HSV-1 codes for several miRNAs (62). Herpesvirus miRNAs mostly targets IE genes thus shutting down lytic replication and assisting in entrance or maintenance of latency (63). The direct repeat (DR) region of HHV-6B encodes for four different miRNAs, which were recently discovered (64). In case of HHV-6A only one miRNA has been identified yet that is encoded from the complementary strand of the IE gene U86. It has been observed to have a regulatory role in viral replication (65). miRNAs that are expressed during the latency in case of HHV-6 are yet to be identified.

HHV-6 has been found to establish latency in cells of macrophage and monocytes lineage (66). Moreover they are also capable of establishing latency in bone marrow progenitor cells (67). There are also a few continuous cell lines which are latently infected by HHV-6 thus making them necessary tools for *in vitro* experiments (68-70). HHV-6 harbors 4 sets of latency associated transcripts that are located between the IE1/IE2 gene locus. These also bear structural homology with HCMV latency transcripts (71, 72). Although they are situated within the immediate early genes yet the expression of these genes are exclusively latency associated (73).

Reactivation of HHV-6 from latency can be triggered in immunocompromised individuals such as in case of AIDS patients, during solid organ transplantation, elderly individuals and in individuals on immunosuppressive drugs. Reactivation can also happen in individuals going through a phase of physical or psychological stress. The suppression of the host's immune

system can act as a trigger for HHV-6 to shift from latency into a fully fledged lytic replication thus giving rise to progeny viruses which can then infect new cells (43). *In vitro*, reactivation of HHV-6 as well as other herpesviruses from latency can be achieved by treating cells with HDAC inhibitors like trichostaton A (TSA), tetradecanoylphorbolacetate (TPA), and sodium butyrate or protein kinase C activator (43, 59, 66).

5.3. HHV-6 integration

While most herpesviruses, during latency, exist as circular episomes in the nucleus of the infected host cell, HHV-6 has the ability to integrate its entire genome into the telomeres of latently infected cells (74-78). It can also readily reactivate from the integrated status into active lytic infection giving rise to infectious progeny. The integration step assists the virus in a stable maintenance of its genome in the infected host cell and also ensuring automatic replication alongside the replication of host cellular DNA. Moreover, the silencing of the integrated viral genome reduces the risk of detection by host immune system thereby prolonging the infection in the host. Among other herpesviruses, EBV has been found to integrate its whole genome in transformed host cells, but in this case reactivation of EBV does not occur (79, 80). The avian oncogenic alphaherpesvirus MDV also integrates it genome into the telomeres of host chromosomes (81). Similar to HHV-6 integration, in this case efficient reactivation from latency is also observed (82). Our group has shown earlier that the perfect TMR sequences which flank the DR regions of HHV-6 genome, is crucial for integration of the virus in the telomeres of the host cellular chromosomes (83). Other viral factors that possibly facilitate in the integration process are yet to be discovered. Additionally, there is possibility of involvement of numerous yet undetermined cellular factors which facilitate the integration of HHV-6 genome into the host cell.

5.3.1. Germline integration of HHV-6 or iciHHV-6

When HHV-6 integrates its genome into the telomeres into the germline of infected host then virus gets vertically transmitted on to the future progeny. This condition is termed as inherited chromosomally infected HHV-6 (iciHHV-6) (78, 84-88). This phenomenon is unique to HHV-6 (6A and 6B) and has been substantiated by numerous research groups earlier. Approximately 1% of the global human population is estimated to have iciHHV-6 (88-91). In this condition, the infected individual carries the HHV-6 genome in every nucleated cell of their body (92, 93). The ramifications of such a condition are quite poorly understood. The possibility of severe clinical implications in these individuals during the reactivation of the virus is currently under investigation by a number of research groups. The association of various clinical conditions such as severe neurological complications (94), development of angina pectoris (95) and

myocarditis (89) has already been established. Moreover, ciHHV-6 puts individuals in high risk for organ transplantation procedures (96).

5.4. Viral and cellular factors facilitating HHV-6 latency

5.4.1. Viral factors:

5.4.1.1. HHV-6 telomeric repeat sequence (TMRs)

The integration of HHV-6 genome in the host chromosome leads to the silencing of viral genome thereby establishing latency. As discussed earlier, this process is beneficial for the virus in order to constantly maintain its genetic material over long courses of time as well as protecting it from getting detected by the host's immune system. The viral genome is believed to harbor certain factors that would facilitate its integration process into the host's chromosome. One such factor is the viral TMR sequence present on either ends of the DR region of the viral genome (Figure 3) (58, 97). TMRs are repeats of the hexanucleotide sequence TTAGGG which resembles the human telomeric sequence. Our group had recently established that the TMR region (especially the perfect TMR sequences) is essential for the integration of HHV-6A genome into the telomeres of host chromosomes. Wallascheck *et al* created TMR mutant HHV-6A in which the TMR sequences were deleted as well as revertant viruses in which the TMR sequence was restored. It was clearly observed that the integration efficiency of HHV-6A was severely reduced in the absence of the TMR sequences as compared to the wild type and the revertant virus (83).

5.4.1.2. Viral integrase protein U94

A second viral factor, the putative viral integrase protein U94 was speculated for a while to be critical for HHV-6 integration. The sequence homology of almost 98% between HHV-6A and -6B denoted that it is an important gene for both the viruses (36). It also had high levels of homology (amino acid homology ~ 24%) with the Rep78/68 protein of human parvovirus adeno-associated virus 2 (AAV-2) (98). Since Rep78/68 protein is highly essential for site specific integration of AAV-2 into its host's genome, it was believed that U94 might play similar role in case of HHV-6. But our group had recently demonstrated that U94 is dispensable for HHV-6A integration (99). Wallascheck *et al* showed that U94 deleted mutant HHV-6A virus had integration efficiency similar to that of the wild type and the revertant virus (U94 sequence restored).

5.4.1.3. HHV-6 recombinase system U41 and U70

The TMRs being essential for viral integration process suggested that the integration event may occur through homologous recombination between viral TMRs and host telomeres. The

viral recombinase system consisting of the single-strand DNA binding protein U41 and a 5'-3' exonuclease U70 (in case of both HHV-6a and -6B) was thus speculated to play a role in HHV-6 integration. The putative orthologues of these proteins in case of HSV -1 (UL29 and UL12 respectively) has been shown to mediate recombination of HSV-1 genome (100-103). Our group eventually demonstrated though that both U41 and U70 are dispensable for HHV-6 integration into host telomeres (104). This was indeed very surprising and did indicate the possible involvement of other viral or cellular factors.

There are quite a few uncharacterized genes present within HHV-6A and -6B genomes whose potential role in virus integration needs to be elucidated. Investigation into these uncharacterized viral proteins could lead to a better understanding of HHV-6 latent replication process.

5.4.2. Cellular factors:

5.4.2.1. Cellular recombinase protein Rad51

While investigating the role of viral integrase U94 as well as viral recombinase system the role of cellular recombinase Rad51 was also studied. Our group again demonstrated repeatedly that the telomere integration of HHV-6A was not affected by additionally blocking cellular Rad51 activity with inhibitors alongside abrogation of the above mentioned viral factors (104).

5.4.2.2. Nuclear domain 10 (ND10) protein complex

ND10 protein complex is a set of constitutively expressed cellular proteins. It is a subnuclear structure and also commonly called as PML nuclear bodies (PML-NBs). ND10 complex has been known to mediate different cellular functions especially in mediating intrinsic immunity against viral infections (105). ND10 bodies are macromolecular structures composed of multiple nuclear proteins that assemble as distinct foci within inter-chromosomal space. The frequency of ND10 bodies varies between 2-30 per cell, depending on cell type and status (105). Among various proteins associated with ND10 complex, there are some which permanently localize in ND10 and are called as major component of the complex. The major components includes promyelocytic leukemia protein (PML), human death domain-associated protein 6 (hDaxx, transcriptional co-repressor) and speckled protein of 100 kDa (Sp100, transcriptional repressor). These proteins accumulate together within interchromosomal spaces of the nucleus forming distinct foci.

Substantial research links the role of ND10 with various cellular events like regulation of gene expression, DNA damage repair or stress responses, although the complete range of functions of ND10 are not well understood. It is now widely believed that ND10 complex can indirectly

control transcription level events epigenetically by getting involved in chromatin remodeling (106-108). Studies further reveal the association of transcriptional repressors like HDACs or DNA methyltransferases with ND10 components like PML and hDaxx (109-112). Taken together, it consolidates the possible function of ND10 as a site of transcriptional repression.

A major function of the ND10 complex is its role as an antiviral defense mediator. The antiviral activity is mostly interferon driven, as both type I and type II interferons can stimulate the expression of PML and Sp100 through interferon-stimulated response element (ISRE) and interferon-gamma activation site (GAS) that are present in the promoter of their genes (113-116). Additionally, PML transcription is enhanced by the IFN-regulatory factor 3 (IRF3) that binds to the PML promoter directly (117). Interestingly, a large number of viruses, especially herpesviruses have also evolved strategies to evade antiviral activities of the ND10 complex, in order to enhance their survivability within the host.

HSV-1 infection leads to the complete disassociation of the ND10 complex (118, 119). This observation initiated the interest in understanding the interaction between ND10 and viruses. It was also shown that HSV-1 parental genomes and replication complexes closely associated with ND10 (120, 121). This observation was further extended and finally included members of all subfamilies of the herpesviridae (122, 123). This intimate association of the herpesviruses and ND10 aroused significant interest amongst researchers to understand the functional consequences of such a tight interaction. Over the years, progress has been made to elucidate the function of ND10 complex with respect to numerous herpesviruses. The role of ND10 complex in relation to HHV-6 still remains poorly understood.

5.5. Major components of the ND10 protein complex

5.5.1. Promyelocyitic Leukemia protein (PML)

PML is the most essential component of the ND10 complex as it helps to localize other ND10 associated proteins (105, 122, 124-126). PML or TRIM19 belongs to the tri-partite motif family (TRIM) of proteins. It has one zinc-finger RING domain, between 1-2 B-boxes and a predicted α-helical coiled coil domain (127). This structural configuration of PML allows it to interact with other proteins as well as homo-oligomerize, which is essential for ND10 assembly (128). PML also has 7 different isoforms which arises due to differential splicing of PML gene transcript. The specific function of each isoform of PML is still unclear. Since it is critical for the ND10 complex assembly, numerous herpesviruses have evolved strategies to counter PML in order to establish either lytic replication or latency within the host (Figure 4).

5.5.2. Human death domain-associated protein 6 (hDaxx)

hDaxx is a highly conserved nuclear protein. It consists of a serine/proline/threonine-rich domain, an acidic domain, a coiled coil region and two paired amphipathic helices (129-131). hDaxx is widely attributed with functions like regulation of apoptosis and gene expression (132). It functions as a transcriptional co-repressor in gene regulation. It has been identified to suppress activity of numerous transcriptional factors, for example, NF-κB (133), Smad4 (134) and p53 (135) family members. It can associate itself with SUMOylated DNA-binding transcriptional factors (136) and recruits transcriptional repressors like histone deacetylases HDAC1 (137), HDAC2 (138) or DNA methyltransferase 1 (DNMT1) (139). In due course of evolution, several herpesviruses have acquired strategies to target hDaxx (Figure 4) in order to prolong their survival in the host.

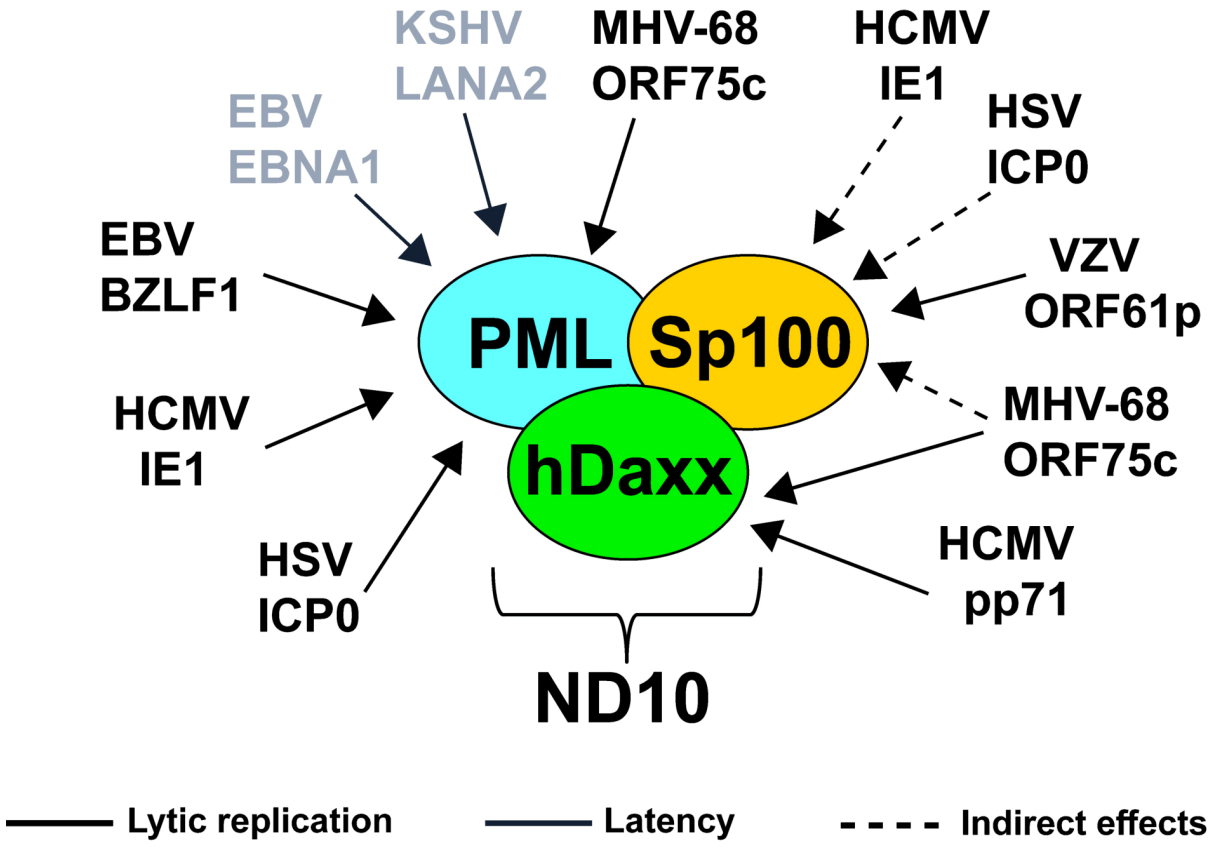

Figure 4: Major ND10 proteins targeted by herpesviral regulatory factors.
A concise overview of different herpesviral proteins which are known to target individual ND10 components. Modified from Tavalai *et al*

5.5.3. Speckled protein of 100 kDa (Sp100)

Sp100 has four isoforms arising from the alternative splicing of a single gene transcript (124). All Sp100 isoforms consist of a N-terminus harboring a homogeneously staining region (HSR) motif. This motif helps in the homo-oligomerization of Sp100 and also facilitates in targeting it to the ND10 complex (124). Sp100 is involved in transcriptional regulation, Sp100B isoform has been identified as a transcriptional repressor for both cellular and viral promoter as document in transient expression experiments (140). A range of herpesviruses are capable of blocking Sp100 (Figure 4) in order to establish successful infection within the host.

5.6. Project outline

As discussed in earlier sections, the incoming viral genome into a target cell is encountered by the ND10 complex of the host cell. ND10 possesses antiviral activity against a wide range of viruses yet many herpesviruses have evolved strategies to evade suppression by the host ND10 complex. For example, ICP0 of HSV-1 induces degradation ND10 major components like PML and Sp100. Similarly, in case of HCMV, the viral IE1 protein interacts with PML and causes dissociation of ND10 complex. In addition, HCMV pp71 causes degradation of hDaxx which is a crucial step for productive viral gene expression. Interestingly, the effect of ND10 complex on HHV-6 infection is not well studied and remains poorly understood.

In this project I have addressed the role of ND10 complex as a factor involved in mediating the switch over of HHV-6A lytic replication to latency. I infected human T-cell line (JJHan) with the infectious HHV-6A culture, following which I stained the cells to investigate the status of the ND10 bodies in comparison to uninfected parental cells. To better address the exact role of ND10 complex I knocked down the ND10 major components PML, hDaxx and Sp100 in JJHan (JJHan-KD) cells using shRNAs. Lytic replication of the virus was evaluated in these knocked down cells as compared to parental cells. Spread of infection was assessed by FACS and efficiency of virus replication with respect to virus copy number was assessed by qPCR. The efficiency of lytic infection of HHV-6A in the absence of ND10 complex was estimated by fluorescence *in situ* hybridization (FISH). In order to determine if the ND10 complex induces HHV-6A genome silencing, I assessed viral protein expression in 293T cells which only allows limited viral replication. For this purpose I knocked down the previously mentioned major ND10 components in 293T cells (293T-KD) using shRNAs. I also used a late gene reporter virus (HHV-6A infectious BAC) where the U57 gene (major capsid protein) of the virus is tagged with GFP via a P2a ribosome skipping motif. Clonal cell lines were generated as per requirement for in-depth experiments. My project addresses and gives first evidence of the role

of cellular factor ND10 in the switch between lytic and latent infection by HHV-6A, and will enable us to understand the silencing of herpesviral genome by cellular factors.

6. Materials and methods

6.1. Materials

All following chemicals listed were used according to the manufacturer´s instructions. Buffers and media for cell and bacterial culture are indicated separately.

6.1.1. List of chemicals, consumables and equipment

6.1.1.1. Chemicals:

Name	Catalogue. No.	Manufacturer
Acetic acid	Cat.No. 20103.295	VWR, Dresden
Agar	Cat.No. 2266.2	Carl-Roth, Karlsruhe
Agarose- Standard Roti® grade	Cat.No. 3810.4	Carl-Roth, Karlsruhe
Ampicillin Sodium-salt	Cat.No. K029.2	Carl-Roth, Karlsruhe
Arabinose L (+)	Cat.No. A11921	Alfa Aesar, Karlsruhe
BSA (albumin bovine fraction V)	Cat.No. A6588.0100	Applichem, Darmstadt
CH_3COOH (acetic acid)	Cat.No. A3686, 2500	Applichem, Darmstadt
Chloramphenicol	Cat.No. 3886.1	Carl-Roth, Karlsruhe
Dextran Sulphate Sodium salt	Cat.No. 17-0340-01	Pharmacia Biotech, Uppsala
Dimethyl sulfoxide (DMSO)	Cat.No. 1.02952.2500	Merck, Darmstadt
dNTP Mix (10mM total)	Cat.No. BIO-39053	Bioline, Luckenwalde
EDTA(ethylendiamine tetraacetic acid)	Cat.No. A2937, 1000	Applichem, Darmstadt
Ethidium bromide 1%	Cat.No. 2218.2	Carl-Roth, Karlsruhe
EtOH Absolute	Cat.No. A1613	Applichem, Darmstadt
Formamide deionized Molecular biology grade	Cat No. A2156	Applichem, Darmstadt
Glycerol	Cat No. A2926,2500	Applichem, Darmstadt
HCl 37% (hydrochloric acid)	Cat.No. 4625.2	Carl-Roth, Karlsruhe
Isopropyl alcohol (2-propanol)	Cat.No. A0892	Applichem, Darmstadt
Kanamycin sulphate	Cat.No. T832.2	Carl-Roth, Karlsruhe
KCH_3CO_2 (potassium acetate)	Cat.No. A4279,0100	Applichem, Darmstadt
β-mercaptoethanol	Cat.No. 28625	Serva, Heidelberg
Methanol	Cat.No. 20847.320	VWR, Dresden
$MgCl_2$ (magnesium chloride hexahydrate)	Cat.No. 5833.025	Merck, Darmstadt

Mounting Medium Vectashield with DAPI	Cat.No. H-1200	Vector Laboratories Inc, Burlingame
NaCl (sodium chloride)	Cat.No. A3597,5000	Applichem, Darmstadt
NaOH (sodium hydroxide)	Cat.No. 1.06462	Merck, Darmstadt
Nonfat dried milk powder	Cat.No. A0830	Applichem, Darmstadt
Nonfat dried milk powder	Cat.No. A0830	Applichem, Darmstadt
OptiMEM	Cat.No. 31985062	Life Tech., Carlsbad
Paraformaldehyde	Cat.No. P6148	Sigma-Aldrich, St. Louis
Pepsine from porcine gastric mucosa	Cat.No. P7012	Sigma-Aldrich, St. Louis
Peptone/Tryptone	Cat.No. A2210,0250	Applichem, Darmstadt
Roti® Mount FluorCare DAPI	Cat.No. HP20.1	Roth, Karlsruhe
Roti™-Phenol	Cat.No. 0038.3	Sigma-Aldrich, St. Louis
Sodium butyrate	Cat.No. 286367-68-8	Sigma-Aldrich, St. Louis
SDS (sodium dodecyl sulfate)	Cat.No. 75746	Sigma-Aldrich, St Louis
Sodium Phosphate, monobasic, monohydrate	Cat.No. S9638	Sigma-Aldrich, St Louis
di-Sodium Hydrogenphsohate dodecahydrate	Cat.No. A3906	Applichem, Darmstadt
tri-Sodium Citrate dehydrate	Cat.No. A1357	Applichem, Darmstadt
Temed	Cat.No. 2367.3	Roth, Karlsruhe
Tetradecanoylphorbol acetate	Cat.No. P8139	Sigma-Aldrich, St. Louis
Tris	Cat.No. A1086,5000	Applichem, Darmstadt
Triton X-100 detergent	Cat.No. 8603	Merck, Darmstadt
Tween-20	Cat.No. 9127.2	Roth, Karlsruhe
Water Molecular biology grade	Cat.No. A7398	Applichem, Darmstadt

6.1.1.2. Consumables:

Name	**Features/Cat.No.**	**Manufacturer**
Cell culture dishes	6-well, 24-well, 96-well	Sarstedt, Nümbrecht
Cell culture flasks	25 ml, 75 ml	Sarstedt, Nümbrecht
Conical test tubes 17x120	15 ml	Sarstedt, Nümbrecht
Conical test tubes 30x115	50 ml, with and without feet	Sarstedt, Nümbrecht
Cryotubes	1.8 ml	Nunc, Roskilde

Eppendorf tubes 1.5 and 2 ml	1.5 and 2 ml	Sarstedt, Nümbrecht
Expendable cuvettes	1mm	Biodeal, Markkleeberg
Latex gloves	Size S	Unigloves, Troisdorf
Kimtech Science, Precision Wipes	Cat.No. 05511	Kimberly-Clark, Roswell
Microscope cover glasses	Cat.No. ECN631-1569	VWR, Sacramento
Nitrile gloves	Size S	Hansa-Medical 24, Hamburg
Nytran®SPC	Cat.No. 10416296	Whatman, Maidstone
Parafilm® M		Bems, Neenah
Pipettes for Pipetboy	5, 10, 25 ml	Sarstedt, Nümbrecht
Pipette tips	P1000, 200, 100 and 10	VWR International, West Chester
Pierce™ Concentrators, 150 MWCO, 20ml	Cat.No. 89921	ThermoFisher, Waltham
Petri dishes for cell culture	60 mm, 100 mm, 150 mm	Sarstedt, Nümbrecht
Petri dishes for bacteria		Sarstedt, Nümbrecht
Polystyrene round-bottom tube 5ml	Cat.No. 352063	VWR, Dresden
PVDF membrane	Cat.No. T830	Roth, Karlsruhe
Sterile syringe filters PVDF	0,45 µm	VWR International, West Chester
SuperFrost® Plus	Cat.No. J1800AMNZ	Menzel Glaser, Braunschweig
Transfection polypropylene tubes		TPP, Trasadingen
Whatman blotting paper	3MM	GE Healthcare, Freiburg

6.1.1.3. Equipment:

Name	**Features/Cat. No.**	**Manufacturer**
General equipment		
Fast Real-time PCR system	ABI Prism 7500	Invitrogen Life Technologies, Grand Island
Bacterial incubator	07-26860	Binder, Turtlingen
Bacterial incubator shaker	Innova 44	New Brunswick Scientific, New Jersey
Bunsen burner	Type 1020	Usbeck, Radevormwald

Cell incubators	Excella ECO-1	New Brunswick Scientific, New Jersey
Centrifuge 5424	Rotor FA-45-24-11	Eppendorf, Hamburg
Centrifuge 5804R	Rotors A-4-44 and F45-30-11	Eppendorf, Hamburg
Centrifuge Sorvall RC 6+		Thermo Scientific, Dreieich
Cytospin3	Shandon	Thermo Scientific, Dreieich
Imaging system	Chemismart 5100	Peqlab, Erlangen
Electroporator	Genepulser Xcell	Bio-Rad, München
Electrophoresis power supply		VWR International, West Chester
Flow cytometer	Cytoflex	Beckmann Coulter, Krefeld
FACSsorter	FACS AriaIII	BD Bioscience, San Jose
Freezer	-20°C	Liebherr, Bulle
Freezer	-80°C	GFL, Burgwedel
Mini centrifuge	Galaxy	VWR International, West Chester
Gel electrophoresis chamber		VWR International, West Chester
Gel electrophoresis chamber	SUB-Cell GT	Bio-Rad, München
Hybridization Oven	HB-1000	UVP Laboratory Product, Cambridge
Ice machine AF100	AF100	Scotsman, Vernon Hills
Pipetboy	INTEGRA	IBS Integrated Biosciences, Fernwald
Magnetic stirrer	RH basic KT/C	IKA, Staufen
Gel chambers	Mini Protean 2D	Bio-Rad, München
Protean Tetra Cell chambers		Bio-Rad, München
Nanodrop 1000		Peqlab, Erlangen
Newbauer counting chamber		Assistant, Sondheim/Rhön
Nitrogen tank	ARPEGE70	Air liquide, Düsseldorf
Nucleofector™ II		Lonza, Basel
Orbital shaker	0S-10	Peqlab, Erlangen
Pipets	P1000, P100, P10	Eppendorf, Hamburg
Horizontal Maxi-Gel System	Perfect Blue™	Peqlab, Erlangen
pH-meter	RHBKT/C WTW pH level 1	Inolab, Weilheim
Sterile laminar flow	ScanLaf, Mars Safety Classe 2	LMS, Brigachtal
Sterile laminar flow		Bleymehl, Inden

Thermocycler	T-Gradient	Biometra, Göttingen
UV Transiluminator	Bio-Vision-3026	Peqlab, Erlangen
Transiluminator printer P93D	P93D	Mitsubishi, Rüsselsheim
Transiluminator	VL-4C, 1x4W-254 nm	Vilber-Lourmat, Eberhardzell
Vortex Genie 2™		Bender&Hobein AG, Zurich
Water baths	TW2 and TW12	Julabo, Seelbach
Water bath shaker	C76	New Brunswick Scientific, New Jersey
Microscopes and associated equipment		
Fluorescence microscope	Axiovert S 100	Carl Zeiss MicroImaging GmbH, Jena
Fluorescence microscope	Axio Imager M1	Carl Zeiss MicroImaging GmbH, Jena
Microscope	AE31	Motic, Wetzlar
Software		
Axiovision 4.8 software		Carl Zeiss MicroImaging GmbH, Jena
Chemi-Capt		Vilber-Lourmat, Eberhardzell
Cytexpert		Beckman Coulter, Krefeld
FlowJo	Version 7	FlowJo LLC. USA
Graphpad Prism	Version 5	Graphpad Software Inc, La Jolla
Vector NTI	Version 9	Invitrogen Life Technologies, Grand Island
Vision-Capt		Vilber-Lourmat, Eberhardzell

6.1.1.4. Enzymes and markers:

Enzyme	**Catalog Number**	**Manufacturer**
Antarctic phosphatase	Cat. No. M0289L	New England Biolabs, Ipswich
*Bam*HI	Cat.No. R0136	New England Biolabs, Ipswich
Benzonase	Cat.No. D00111784	Novagen, San Diego
*Dpn*I	Cat.No. ER1701	New England Biolabs, Ipswich
*Hind*III	Cat.No. R0104	New England Biolabs, Ipswich
*Xho*I	Cat.No.R0146	New England Biolabs, Ipswich
Phusion Hot Start High-Fidelity DNA Polymerase	Cat.No. M0530S	Finnzymes, Thermo Scientific, Rochester

Proteinase K	Cat.No. 7528.2	Carl-Roth, Karlsruhe
RNase A	Cat.No. 2326466	Applichem, Darmstadt
T4 DNA Ligase	Cat.No. 01-1020	Peqlab, Erlangen
Taq DNA-Polymerase	Cat.No. 01-1020	Peqlab, Erlangen
Marker		
Generuler™ 1kb Plus DNA	Cat.No. SM0311	Fermentas, Mannheim
Protein Prestained plus marker	Cat.No. 26619	Thermo Scientific, Darmstadt

6.1.1.5. Anitibodies:

Name	Catalog Number	Company
Anti-β-Actin	Cat.No. 49705	CellSignaling, Cambridge
Anti-hDaxx	Cat.No. 631301	Biolegend, San Diego
Anti-DIG-FITC	Cat.No. 11207741910	Roche, Mannheim
Anti-DIG-ALP	Cat.No. 11093274910	Roche, Mannheim
Anti-PML	Cat.No. A301-167A	Bethyl, Montgomery
Goat-anti-mouse HRP	Cat.No. Sc-2031	Santa Cruz, Santa Cruz
Goat-anti-rabbit AF488	Cat.No. A11008	Santa Cruz, Santa Cruz
Goat-anti-rabbit HRP	Cat.No. 7074S	CellSignalling, Cambridge
Streptavidin-Cy3	Cat.No. PA43001	GE Healthcare, Berlin

6.1.1.6. Kits:

Name	Catalog Number	Company
Amaxa Nucleofector kit V	Cat.No. VCA-1003	Lonza, Basel
CellVue Claret far red dye		Sigma Aldrich
ECL Plus Detection kit	Cat.No. RPN2232	GE Healthcare, Berlin
GF-1 AmbiClean PCR/Gel purification kit	Cat.No. GF-GC-200	Vivantis, USA
Hi Yield Gel/PCR DNA Fragments Extraction Kit	Cat.No. HYDF100-1	SLG, Gauting
Nucleobond BAC100 Midi kit	Cat.No. 740579	Macherey-Nagel, Düren
RTP® DNA/RNA Virus Mini Kit	Cat.No. 1040100300	STRATEC Molecular GmbH, Berlin

6.1.1.7. Antibiotics:

Name	Working concentration	Manufacturer
Ampicillin (Amp) [Cat. No. K0292]	100 µg/ml in ddH_2O	Roth, Karlsruhe
Kanamycin sulphate (Kana) [Cat. No. T832.3]	50 µg/ml in ddH_2O	Roth, Karlsruhe
Chloramphenicol (Cam) [Cat. No. 3886.3]	30 µg/ml in 96 % EtOH	Roth, Karlsruhe
Penicillin (P) [Cat. No. A1837]	100 U/ml in MEM	Applichem, Darmstadt
Streptomycin (S) [Cat. No. A1852]	100 U/ml in MEM	Applichem, Darmstadt
Puromycin (Puro) [Cat. No. A11138-03]	8 µg/ml in RPMI	Invitrogen, Carlsbad

6.1.1.8. Bacteria, cells, viruses and plasmids:

Name	Features	Reference
Bacteria		
Top10	F^- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1	Invitrogen
GS1783	DH10B λ cl857 Δ(cro–bioA)<>araC-P_{BAD}I-SceI	(141)
Stbl3	F^-mcrB mrrhsdS20(r_B^-, m_B^-) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(Str^R) xyl-5 λ^-leumtl-1	ThermoScientific,Waltham
Cells		
HEK293T	Human epithelial kidney cell line, SV40 T-antigen	ATCC CRL-11268
JJHan	Acute T-cell leukemia-derived cell line and a subclone of the JURKAT line	HHV-6 foundation
Viruses		
HHV-6A BAC	Bacterial artificial chromosome (BAC) of HHV-6A strain U1102	(142)

Plasmids		
pcDNA3	Mammalian expression vector; T7prom, f1 ori, pBR322 ori, AmpR, pCMV, pSV40, NeoR	Invitrogen
pEP Kan-S	Mammalian expression vector, T7prom, f1 ori, SV40 prom, KanR, I-Sce-I restriction site, AmpR, ColE1 ori, NeoR	(143)
pLKO-shDPS	Mammalian expression vector for shRNAs, 7SK, mU6, U6, shPML, shDaxx, shSp100, CPPT, hPGK, PuroR, sin 3'LTR, f1 ori, AmpR, pUC ori, 5'LTR, RRE	(144)
pCMV-dR8.91	Mammalian expression vector for envelope, AmpR, CMV promoter, VSV-G, SV40 polyA	(144)
pCMV-VSV-G	Mammalian expression vector for packaging, CMV promotor, Gag-Pol, CPPT, RRE, SP6, AmpR, pBR322 ori	(144)

6.1.1.9. Buffers and media:

General buffers

1x Phosphate buffer saline (1xPBS)	**1x Tris-acetate-EDTA buffer (TAE)**
2 mM KH_2PO_4	40 mM Tris
10 mM Na_2HPO_4	1 mM $Na_2EDTAx2H_2O$
137 mM NaCl	20 mM Acetic acid 99 %, pH 8.0
2.7 mM KCl, pH 7.3	

Media and supplements to cultivate bacteria (E.coli)

LB medium (1l)	**SOB medium (1l)**	**SOC medium**
10 g Bacto™ Tryptone	20 g Bacto™ Tryptone	SOB medium
5 g Bacto™ Yeast Extract	5 g Bacto™ Yeast Extract	20 mM Glucose
10 g NaCl	0.584 g NaCl	
15 g Bacto™ Agar	0.186 g KCl	
	pH to 7.0	

Plasmid preparation solution

Buffer (P1)

50 mM Tris HCL pH 8.0

10 mM EDTA

100 µg/ml RNAse

Neutralization Buffer (P3)

3 M K-Acetate pH 5.5

Lysis Buffer (P2)

200 mM NaOH

1 % SDS

Buffer TE

10 mM Tris HCl pH 7.4

1 mM Na_2EDTA

Media and supplements to cultivate mammalian cells

Name	Catalogue number	Manufacturer
Biocoll Separating solution, density 1.077 g/ml	Cat.No. L6115	Biochrom AG, Berlin Sigma-Aldrich, St Louis
Fetal bovine serum (FBS)	Cat.No. S 0415	Biochrom AG, Berlin
Fetal calf serum (FCS)	Cat. No. P30-1506	Pan Biotech, Aidenbach
Minimum essential Medium Eagle (MEM)	Cat.No. F 0315	Biochrom AG, Berlin
RPMI 1640 (w/o Glutamine)	Cat.No. F 1215	Biochrom AG, Berlin
Trypsin	Cat.No. L 2103-20G	Biochrom AG, Berlin

Trypsin

137 mM NaCl

2.7 mM KCl

8 mM $Na_2HPO_4 * 2H_2O$

1.8 mM KH_2PO_4

Trypsin 1:250

Fluorescence *in situ* hybridization (FISH) solutions

Hypotonic solution

0.075 M KCl

autoclaved

20 x SSC

87.6 g NaCl

44.1 g TriNaCitrate dehydrate

to 500 ml in ddH_2O, pH to 7.4

Fixative

Methanol:Acetic acid 3:1

Stringency wash solution

50 % 2x SSC

50 % Deionized formamide

Detergent wash solution

4 x SSC

0.5 % Tween-20

Pepsine solution

0.01 % Pepsin in 10 mM HCl

Hybridization buffer

50 % Deoinized formamide (v/v)

10 % Dextran sufate (v/v)

2 x SSC

1 x Phosphate buffer, pH 7.0

Probe

Salmon sperm

Western blot solutions

10 x SDS PAGE running buffer

250 mM Tris

1.9 M Glycine

1 %SDS

2 x stripping buffer

50 mM Glycine

2 % SDS

pH 2

Ripa buffer

20 mM Tris

150 mM NaCl

1% (v/v) Nonidet P-40

0.5% (w/v) Sodium Deoxycholate

0.1% (w/v) SDS

Complete® Mini protease/phosphatase

Inhibitor cocktail

6.1.1.10. Primers

Table 2: List of primers

Mutagenesis primer		
HHV-6-U57-P2a-GFP	for	GTTTGTGATCGAAAGTGCAGTAGACGGTTTCCATTTTACTT GTACAGCTCGTCCATGCCG
HHV-6-U57-P2a-GFP	rev	GAGAAACCATACCTTTCCAACTCATTATCGAATCATCCATA GGATCTGGAGCGACCAATT
Sequencing primers		
HHV-6 U57 sequencing	for	CTTTGTTGGAGGAGACGATGG
HHV-6 U57 sequencing	rev	GCCTCTTCACTGTTCATCCAA
qPCR primers		
β2M	for	CCAGCAGAGAATGGAAAGTCAA
	rev	TCTCCATTCTTCAGTAAGTCAACTTCA
β2M probe		FAM-ATGTGTCTGGGTTTCATCCATCCGACA-TAMRA
U86	for	TGTACATGGGCTGTAGGAGTTGA
	rev	ACATCCTCTGCTTCCAATCTACAATC
U86 probe		FAM-TTCCGAAGCAAAGCGCACCTGG-TAMRA

6.2. Methods

6.2.1. Molecular biology methods

6.2.1.1. DNA mini and midi preparation

Isolation of bacterial DNA was performed by standard alkaline lysis protocol. Bacterial cultures (5ml) were grown overnight (o/n) at 32°C in lysogeny broth (LB) in presence of appropriate antibiotic in a shaker incubator at 220 rpm. The bacterial cells were then pelleted at 5000 rpm in a table top centrifuge on the following day, the supernatant was discarded and the pellets were resuspended in 300 µl of P1 buffer. This was followed by the addition of 300 µl of P2 buffer (lysis buffer) and the mixture was allowed to incubate at room temperature for 5 minutes (min) for alkaline lysis. The reaction was neutralized with the addition of 300 µl P3 buffer and the mxture was incubated on ice for 10 min. After incubation proteins and cellular debris were removed by centrifugation for 10 min at 10,000 rpm. The supernatant was then transferred into a fresh new tube, to which 900 µl of Tris-buffered phenol:chloroform solution was added to ensure the elimination of any proteins. The mixture was then briefly vortexed and the centrifuged for 10 min at 10,000 rpm, the aqueous phase was then transferred into a fresh tube. To this 450 µl of isopropanol was added and incubated for 10 min at -20°C. The DNA was then precipitated by centrifugation of the samples for 15 min at 10,000 rpm at 4°C. this was followed by two wash steps using 70% ethanol (ice-cold), residual ethanol was then completely removed by warming the samples for 5 min at 37°C. Finally the DNA was dissolved in TE-buffer containing RNase A (final concentration 100 µg/ml) at 37°C for 30 min. The extracted DNA was stored at -20°C until further use.

Small scale plasmid DNA preparation was carried out with the help of peqGOLD plasmid miniprep kit, VWR Peqlab. The preparation of midi DNA was performed using the BAC100 Kit (Macherey-Nagel, Düren) following the manufacturer's instructions for BAC DNA. The DNA quality was assessed using a nanodrop spectrophotometer.

6.2.1.2. Electrocompetent bacteria

GS1783 strain of *E. coli* harboring the HHV-6A strain U1102 BAC clone was grown o/n at 32°C in 1 ml LB medium supplemented with chloramphenicol (Cam). On the very next day, 100 ml of LB medium (with CAM) was inoculated with the o/n culture. The culture was incubated at 32°C in a shaker incubator, shakint at 220 rpm. After 3-4 hours, when the culture reached a logarithmic growth phase (OD_{600} 0.5 - 0.7), the culture was provided with a heat shock for 15 min at 42°C, still shaking at 220 rpm. This activates the Red-recombination system. Following the heat shock, the culture was cooled on ice for 20 min with continuous shaking. This was followed by pelleting the culture by centrifugation for 5 min at 4°C at 5,000 rpm. The pellet was

washed three times with ice-cold 10% glycerol solution. Finally the bacteria was resuspended in 1 ml of 10% glycerol, 50µl aliquots were prepared and finally snap-frozen for storage at -80°C.

6.2.1.3. Generation of reporter gene virus

Working with Bacterial artificial chromosomes (BACs) assists in efficient and rapid manipulation of cloned DNA using already established techniques under perfectly monitored conditions (145). We utilized the "*en passant*" mutagenesis technique that has been developed in our laboratory by Tischer *et al*. (143) to generate the reporter gene virus. *En passant* mutagenesis is conceptualized on the Red mediated recombination of dsDNA. Red recombination system is a derivative of the λ-phage, and is commonly used to manipulate BACs. It can be used to introduce modifications like insertions, deletions, point mutations or tagging of genes in the BACs. The recombination system consists of three proteins – Exo, Bet and Gam whose expression is controlled by a temperature inducible promoter (146-148). Gam inhibits the degradation of free dsDNA induced by the Rec B/C/D system of *E. coli* (149). The 5' – 3' exonuclease Exo is responsible for generating free single stranded 3' –overhangs (150). These are further protected by Bet. Furthermore, Bet promotes the annealing of complementary single strand (151, 152). Red recombination needs on 20-50 base pairs (bp) long homologous sequences because of which PCR amplified products can be used as targeting cassettes, which are introduced in the bacteria by electroporation (153). An *E.coli* strain, GS1783 was used in our project. The GS1783 strain harbors both temperature inducible λ-phage Rec system and *S. cerevisiae* derived homing endonuclease I-SceI which in turn is controlled by an arabinose inducible promoter. I-SceI has an 18 bp long recognition/cleavage site (154) which ensures site-specific cleavage of mutated region. This in turn allows the efficient removal of the kanamycin cassette which underlines the second recombination event. Hence this two-step method is advantageous in obtaining scarless recombination.

Thus, by utilizing the two-step Red-mediated mutagenesis, we constructed a late gene reporter virus, where GFP was fused with a P2a ribosome skipping motif to the major capsid protein U57 in pHHV-6A (vU57-P2a-GFP) (Figure 5) (155), an infectious BAC clone of HHV-6A (strain U1102). The recombinant virus clones were confirmed by restriction fragment length polymorphism (RFLP) and Sanger sequencing. The mutagenesis and sequencing primers used are listed in Table 2. The mutagenesis PCR protocol for the reporter virus is listed in Table 3 and the sequencing PCR protocol is listed in Table 4.

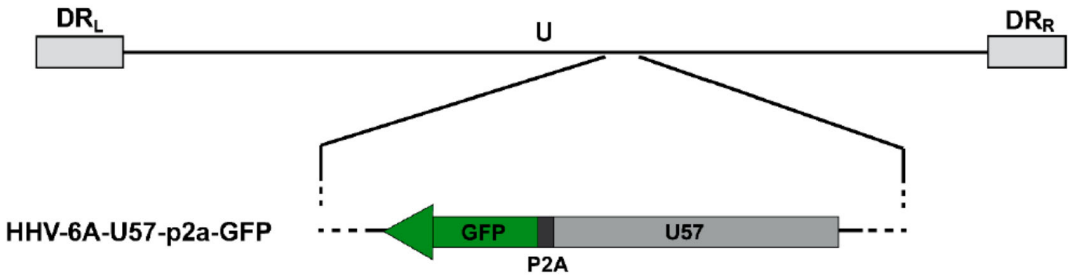

Figure 5: Schematic representation of the HHV-6A-U57-P2a-GFP BAC generated by *en passant* mutagenesis

Table 3: Two-step PCR protocol for the construction of reporter gene virus

Temperature (°C)	Time	PCR step	Cycles
94 °C	5 min	Initial denaturation	
94 °C	30 s	Denaturation	
52 °C	30 s	Annealing	10 cycles
72 °C	2 min	Elongation	
94 °C	30 s	Denaturation	
68 °C	30 s	Annealing	30 cycles
72 °C	2 min	Elongation	
72 °C	8 min	Extension	

Table 4: Sequencing PCR protocol

Temperature (°C)	Time	PCR step	Cycles
95 °C	5 min	Initial denaturation	
95 °C	3 s	Denaturation	
52 °C	30 s	Annealing	30 cycles
72 °C	1 min	Elongation	
72 °C	8 min	Extension	

6.2.1.4. qPCR

qPCR assays were performed for HHV-6A genome quantification. DNA from respective samples was isolated using the RTP® DNA/RNA Virus Mini Kit according to manufacturer's instructions. For the qPCR reaction, a master mix was prepared containing 10 µl Master Mix, 0.12 µl of each primer (100 µM), 0.5 µl probe (10 µM) and 10 µl of DNA sample. The qPCR assays were done on a 7500 Fast Real-Time PCR System (Invitrogen, Grand Island) using primers and a TaqMan probe specific for the HHV-6A U86 gene. Viral U86 gene copies were

normalized against cellular genome copies of the β2M gene. Primers and probe sequences are listed in Table 2 and cycling conditions are given in Table 3. To generate standard curves for U86 and β2M, HHV-6A BAC DNA and a plasmid containing cellular β2M were used, respectively, as serial 10-fold dilutions. The coefficient of regression was always >0.99 for standard curves.

Table 5: qPCR cycling conditions

Temperature (°C)	Time	PCR step	Cycles
95 °C	20 s	Initial denaturation	
95 °C	30 s	Denaturation	
60 °C	30 s	Annealing	40 cycles

6.2.2.Cell culture methods

6.2.2.1. Culture of mammalian cell lines

The mammalian cell lines used in this project were cultured and maintained at 37 °C and under 5 % CO_2 atmosphere. JJHan cell line which is of T-cell lineage was maintained in suspension. These were grown in RPMI media supplemented with 10% FBS and 1% Penicillin/Streptomycin. The cells were maintained at a constant density of 1x 10^6 cells/ml and were routinely passaged by a 1:4 split in every three days. The adherent 293T cells were cultured in DMEM media supplemented with 10% FBS and 1% Penicillin/Streptomycin. The cells were allowed to grow upto confluency in the culture flasks. Routine passaging involved 1x PBS wash of the confluent monolayer followed by detachment using 0.25% trypsin-EDTA. Inactivation of trypsin was done by adding 10% FBS in growth media to detached cells. Cells were regularly split in 1:5 for maintenance of passage. All mammalian cell lines were also frozen in 1.8 ml cryotubes in growth media containing 10% DMSO for long term storage in liquid nitrogen.

6.2.2.2. Propagation of infectious HHV-6A stock

HHV-6A is highly cell associated and propagates lytically in the JJHan cell line. The stocks were maintained at a high infection level (~ 90-95% infected cells in suspension). For our experiments, frozen aliquots of the highly infected stock were thawed and resuspended in serum supplemented RPMI media. The virus culture was washed with fresh RPMI media in every second day in order to get rid of dead cell debris and split by 1:2 to maintain a constant density of 1-2 x 10^6 cells/ml.

6.2.2.3. Lentiviral transduction

In order to knockdown key ND10 components PML, hDaxx and Sp100 shRNAs against these targets were delivered by a lentivirus transduction system into both JJHan as well as the 293T cell lines. The lentiviruses were produced by co-transfection of 293T cells with a pLKO-shDPS vector harboring specific shRNAs against the key ND10 components PML, hDaxx and Sp100, pCMV-VSV-G (expressing the vesicular stomatitis virus (VSV) envelope protein), and pCMV-dR8.91 (expressing lentivirus helper functions), as described previously (144). Virus supernatants were harvested 24 h post transfection and filtered before use. This was followed by transduction of JJHan as well as 293T cells with these lentiviruses expressing shRNAs against the major ND10 protein components. Post lentiviral transduction, the cell lines were kept under puromycin selection (8 µg/ml for JJHan cells and 1 µg/ml for 293T cells) to obtain stable cell lines. The polyclonal cells obtained were further selected by single cell suspension in order to obtain monoclonal cell lines stably expressing the shRNAs. The extent of PML and hDaxx knock down was estimated by Western blotting. Furthermore, PML knockdown was also confirmed by confocal microscopy.

6.2.2.4. Western blotting

ND10 knocked down JJHan or 293T cell lines as well as their respective parental cell lines were harvested and lysed using radioimmunoprecipitation assay buffer (RIPA). The lysates were then separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using a 10 % gel for 20 min at 80 V and 120 min at 130 V. This was followed by transfer of the proteins from the gel onto a nitrocellulose membrane (Roth, Karlsruhe) using the BioRad wet blot system for 1 h at 100 V. Post transfer the membranes were blocked for 1 hour at RT with 5% nonfat dried milk powder in PBS-T (blocking buffer). Subsequently, the membranes were incubated o/n at 4°C with either rabbit anti-PML antibody or mouse anti-hDaxx antibody. Both the primary antibodies were diluted 1:1000 in blocking buffer. After 3x washing with PBS-T, the membranes were incubated for 1 hour at RT using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody. The secondary antibodies were diluted to 1:10,000 using blocking buffer. Subsequently, the membranes were washed 3x in PBS-T and stained with enhanced chemiluminescence (ECL) Plus western blot detection reagent and the signal was recorded using the Chemi-Smart 5100 detection system (Peqlab, Erlangen). Post image acquisition, membranes were incubated twice for 10 min with stripping buffer at RT to remove bound antibodies, washed 2x with PBS-T, blocked with blocking buffer and re-probed with the rabbit anti-Actin (1:1,000) antibody.

6.2.2.5. Immunofluorescence

To assess the effect of HHV-6A infection on the ND10 complex, JJHan cells were infected with HHV-6A-GFP. Cells were fixed with 4% paraformaldehyde (PFA) at 24 hours post infection (hpi), permeabilized with 0.1 % Triton-X 100 and blocked with 10 % BSA. Cells were then stained with rabbit anti-PML (Bethyl Laboratories) and mouse anti-gp82 antibodies (clone 2-D6; HHV-6 foundation repository) at 1:1000 dilutions and further stained using goat anti-rabbit Alexa 568 and goat anti-mouse Alexa 647 antibodies, respectively. Images were acquired on an Andor iXon888 EMCCD using a Nikon-based spinning-disk confocal microscope at 100X magnification (Visitron Systems GmbH). Images were processed by ImageJ, Adobe Photoshop and Illustrator software. One hundred cells were imaged and the number of PML foci per nucleus counted in a blinded manner. The HHV-6A infected cells were further grouped with respect to the stage of infection; (i) immediate early infected cells that only express GFP and (ii) GFP/gp82 double positive indicative of late replication. In addition, immunofluorescence was also used to confirm the knockdown of PML in JJHan cells after lentiviral transduction as described above.

6.2.2.6. CellVue infection assay

An infection assay using the CellVue Claret far-red fluorescent membrane dye (Sigma-Aldrich) was developed in order to determine the replication properties of HHV-6A in the absence of ND10 complex in the ND10KD JJHan cells. The CellVue Claret far-red fluorescent membrane dye's chemical properties dictate it to stably incorporate a far-red fluorophore harbouring long aliphatic tails into the lipid bi-layer of cell membranes thereby facilitating in-vitro cell labeling. $2.5x10^5$ HHV-6A-GFP infected JJHan cells in a falcon were 2x washed with serum free RPMI media follwed by 1x wash with PBS. The cells were then resuspended in 250 µl of Diluent C provided with the staining kit. Simultaneously, 2 µl of the CellVue dye was also diluted in 250 µl of Diluent C. Following the dilution of the dye it was then mixed with the resuspended virus infected cells and the mix was incubated at RT for 10 minutes with intermittent flicking of the cells in the falcon for homogeneous staining. After incubation the staining process was halted by adding 1 ml of FBS into the falcon, then incubated at RT for 1 minute. The cells were then washed 3X with serum supplemented RPMI and finally resuspended in 250 µl of serum supplemented media, thereby concluding the CellVue far-red dye labeling of the virus infected cells. Simultaneous staining of uninfected JJHan cells was also done which were later used as negative control. These CellVue labeled virus infected cells/uninfected JJHan cells were then co-cultured with $1x10^6$ unstained JJHan or JJHan-KD target cells. The cells were cultured for 5 days which allowed sufficient time for the infection to spread from the stained infected cells into the unstained target cells.

5 days post infection the spread of HHV-6A into the uninfected cells was assessed by flow cytometry, as described later. Subsequently, CellVue negative cells were sorted from the infection assay and HHV-6A genome copies were quantified by qPCR, as described later. Simultaneously, GFP positive CellVue negative cells (GFP+CellVue/APC-) from the infection assay were isolated by FACS to obtain a pure infected target population. These cells were then analyzed by fluorescence in situ hybridization (FISH) assay to determine the number of lytically infected cells. The FISH assay is described below in details.

6.2.2.7. Flow cytometry analysis

The spread of HHV-6A into uninfected cells was assessed by flow cytometry 5 days post infection. Live cell population was gated from the forward scatter (FSC) vs side scatter plot (SSC). The target live population was further plotted between FSC-height (FSC-H) vs FSC-area (FSC-A) in order to delineate only the singlet population. The singlet population was the further plotted between APC (indicates CellVue positive population) vs FITC (indicates virus infected/GFP positive population). Finally, the APC negative – FITC positive population indicated the percentage of freshly infected cells. After initial estimation this APC negative – FITC positive population was sorted into respective falcons and later analysed by fluorescence *in situ* hybridization assay (FISH). Furthermore APC negative cells from replicate CellVue assays were sorted in respective falcons and later analysed by qPCR to determine the virus copy number.

6.2.2.8. Fluorescence *in situ* hybridization (FISH) assay

Cells were then pelleted for 8 min at 400 g, the supernatant was removed and the pellet was washed twice with 5 ml ice-cold fixative. Finally, the pellet was resuspended in an adequate amount of ice-cold fixative and chromosomes were stored at -20 °C until further use.

On the day of preparing the slides, the water bath was set at 98 °C. The pellets were washed twice in ice cold fixative. The respective microscope slides were quickly passed through the water vapour from the water bath and then 10µl of the cell suspension was dropped at the centre of the glass slides from a reasonable height in order to spread the cells better. The slides were again passed through the water vapor quickly and then placed on the side of a metallic plate over the water bath to dry. The slides were then left at RT o/n for aeging. The next day, samples were dehydrated for 5 min at RT in 100 % ethanol. They were then incubated in pre-warmed pepsin solution at 37 °C for 7 min in order to remove protein debris from the slides. In the following step, the RNA was degraded by incubation for 2 min in 2x SSC with RNAse A. Subsequently, the slides were washed twice for 1 min with 2x SSC, rinsed in Millipore water and finally dehydrated by subsequent ethanol washes, twice for 2 min in 70 %,

twice for 2 min in 90 % and once for 4 min in 100 % ethanol. These were then incubated for 1 h at 65 °C for further ageing. To generate the HHV-6A probe, 1.5 µg HHV-6A BAC DNA were first digested for 3 h at 37 °C with *Hae*III and *Dpn*I, then purified with HI-Yield Gel purification kit (SLG, Gauting) and eluted in water. The purified DNA was then labeled with Digoxigenin (DIG) using DIG-High Prime (Roche, Mannheim) according to the manufacturer´s instructions. 300 ng of digested DNA was dissolved in a final volume of 16 µl and denatured for 10 min at 98 °C. The sample was cooled on ice, 4 µl of DIG-High prime was added to the DNA solution. The reaction was continued o/n at 37 °C and stopped by heat inactivation (10 min at 65 °C). Finally the probe was purified once more with High-Yield PCR Purification kit (SLG, Gauting).

In the hybridization step, 1.2 µl of the HHV-6A probe was mixed with 30 µl of hybridization buffer. 0.5 µl of salmon sperm DNA per slide was used as competitor DNA to reduce background. The probe mixture was initially denatured for 10 min at 75 °C, then cooled down on ice and dropped on the aged slides. A coverslip was placed on the probe of each slide and sealed with rubber cement to prevent evaporation. To facilitate hybridization efficiency, slides were placed into a 80 °C incubator for 90 seconds, then gradually cooled to 42 °C and left o/n. the following day, coverslips were removed and the slides were washed sequentially at 44 °C twice for 5 min in 2x SSC, twice for 5 min in stringency wash, twice for 5 min in 2x SSC and finally twice for 5 min in detergent wash. This was followed by incubation of the slides for 30 min at 37 °C with 75 µl of anti-DIG-FITC antibody (1:500) diluted in detergent wash. Finally the slides were washed three times of 4 min incubations in detergent wash followed by a brief rinse in water. Mounting media containing DAPI was added and samples were sealed with a coverslip prior to detection with Axio Imager M1 (Zeiss, Jena).

6.2.3. Statistical analysis

Statistical analysis for all experimental data was performed using the GraphPad Prism Software. Immunofluorescence estimation, qPCR data on HHV-6 genome copies, FISH interphase integration counts as well as FACS results were analyzed using Mann-Whitney U test.

7. Results

7.1. Status analysis of ND10 complex in HHV-6 infected cells

Most human herpesviruses known to man is capable of the degradation of PML which leads to the complete disassembly of the ND10 complex in the host cells. This allows the viruses to overcome the anti-viral activity of the ND10 complex. Since the interaction of HHV-6 with the host ND10 complex is not well studied we wanted to determine if HHV-6A has similar capabilities to disrupt the ND10 complex in the host cells. Subsequently, we analyzed cells infected with HHV-6A-GFP, expressing GFP under the control of HCMV major immediate early promoter. A kinetics study of PML in host cells upon infection with HHV-6A-GFP was

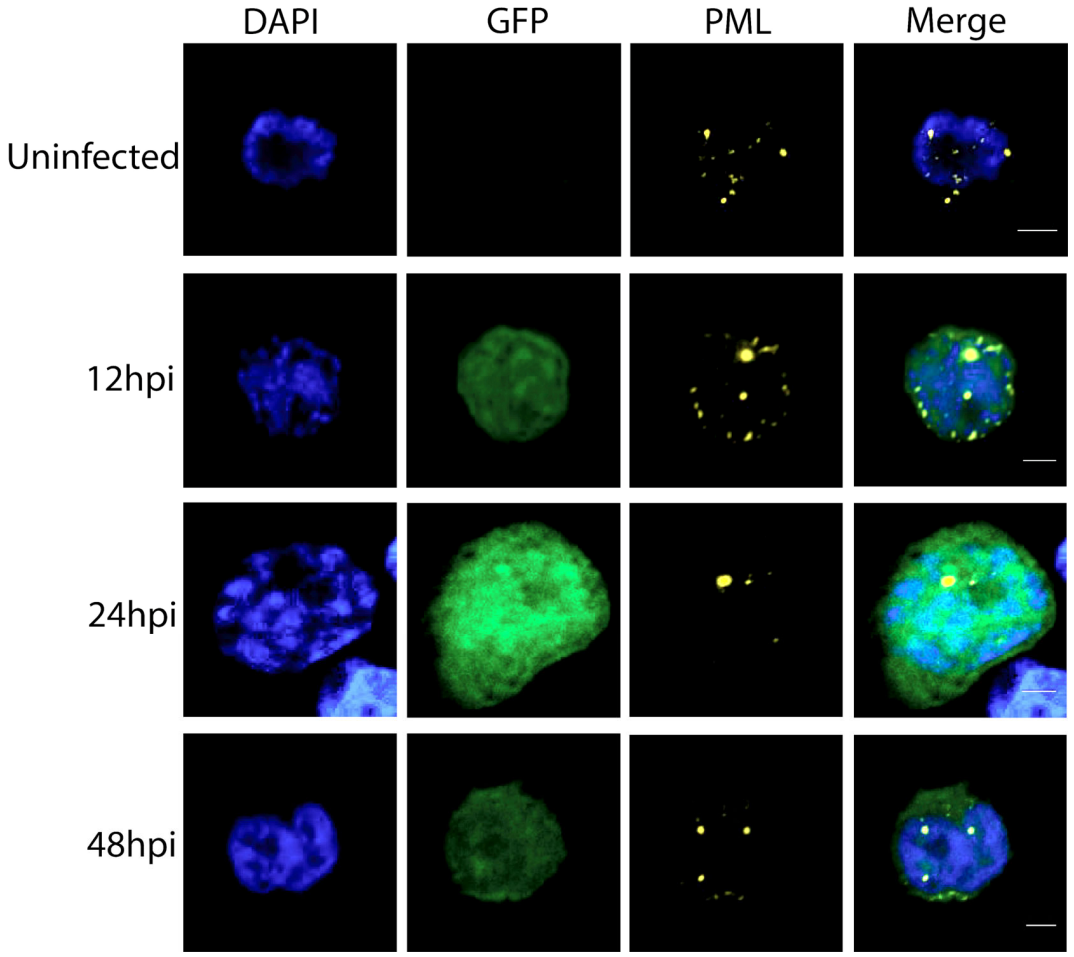

Figure 6: HHV-6A is incapable of complete suppression of PML in infected JJHan cells
HHV-6A-GFP and mock infected JJHan cells were immunostained for PML (yellow) and analyzed by confocal microscopy. Virus infected cells appears green (GFP) and nuclei were stained with DAPI (blue). Representative images are shown for HHV-6A-GFP and mock infected cells at 12, 24 and 48 hours post infection. The scale bars correspond to 3µm.

performed (Figure 6). The experiment revealed that the number of PML foci in the virus infected cells gradually decrease over a period of time but never completely lost as compared to the mock infected cells.

In a follow up experiment, it was also observed that the decrease in PML staining was more drastic in the virus infected cells expressing the viral late gene product gp82, indicative of late lytic replication, compared to cells expressing only the immediate early promoter driven GFP (Figure 7A). The decrease of PML foci in the virus infected cells was also found to be statistically significant upon further quantification (Figure 7B). Our data showed that unlike other human herpesviruses, that abrogates ND10 complex to promote virus replication, HHV-6A is not capable of completely disrupting the anti-viral ND10 complex.

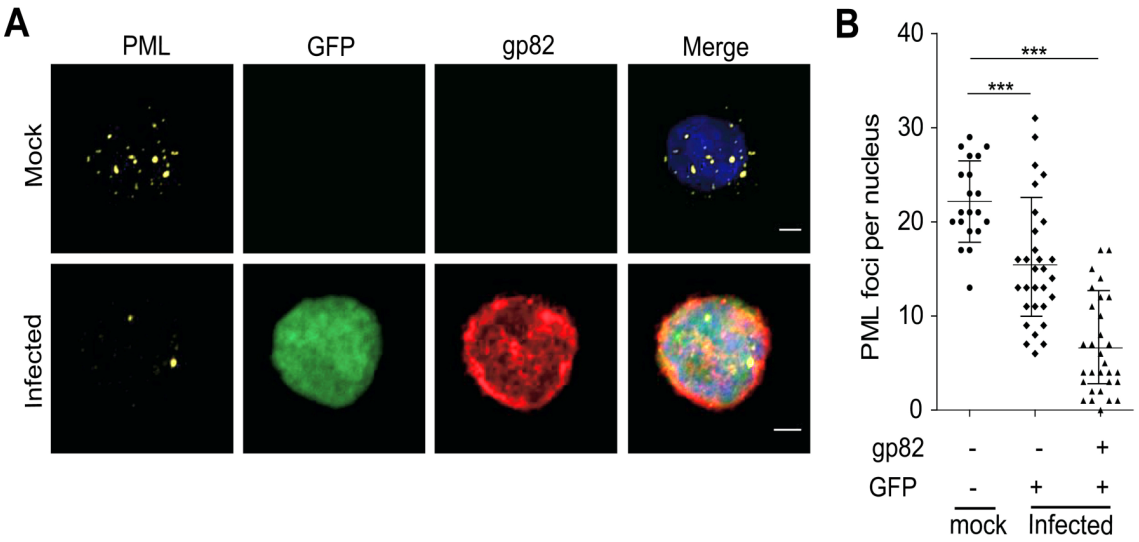

Figure 7: HHV-6A infection reduces the number of PML bodies.

(A) HHV-6A-GFP and mock infected JJHan cells were immunostained for PML (yellow) and gp82 (red; late viral gene) and analyzed by confocal microscopy. Virus infected cells could be identified by GFP (green) and nuclei were stained with DAPI (blue). Representative images are shown for HHV-6A-GFP and mock infected cells. The scale bars correspond to 3µm. **(B)** Quantification of PML foci in the nucleus of HHV-6A-GFP and mock infected JJHan cells (n=100). Infected cells were grouped by the stage of infection; GFP is expressed during the early stage of infection, while both GFP and gp82 are expressed during late lytic replication. Results are shown as the mean of three independent experiments with standard errors (***, P<0.001).

7.2. Generation and analysis of ND10 knockdown cells

The role of ND10 complex as an intrinsic anti-viral response mediator has been well documented in the past. From our initial experiments it was evident that HHV-6A is not capable of complete degradation of ND10 complex in the host cells. Hence to determine whether the

ND10 complex inhibits HHV-6A replication we knocked down the major components of the ND10 complex, namely PML, Daxx and Sp100 in JJHan cells (JJHan-KD) using a single shRNA lentivirus vector. Lentiviruses harboring the shRNAs against PML, hDaxx and Sp100 were produced in HEK293T cells. JJHan cells were transduced with these lentiviruses and cultured under puromycin selection. Previous reports clearly verify stable knockdown of PML, Daxx and Sp100 in cells transduced with this lentivirus (106). The knockdown of PML was confirmed by western blotting (Figure 8A) and immunofluorescence (Figure 8B), this was indicative of the loss of all three ND10 component proteins (106).

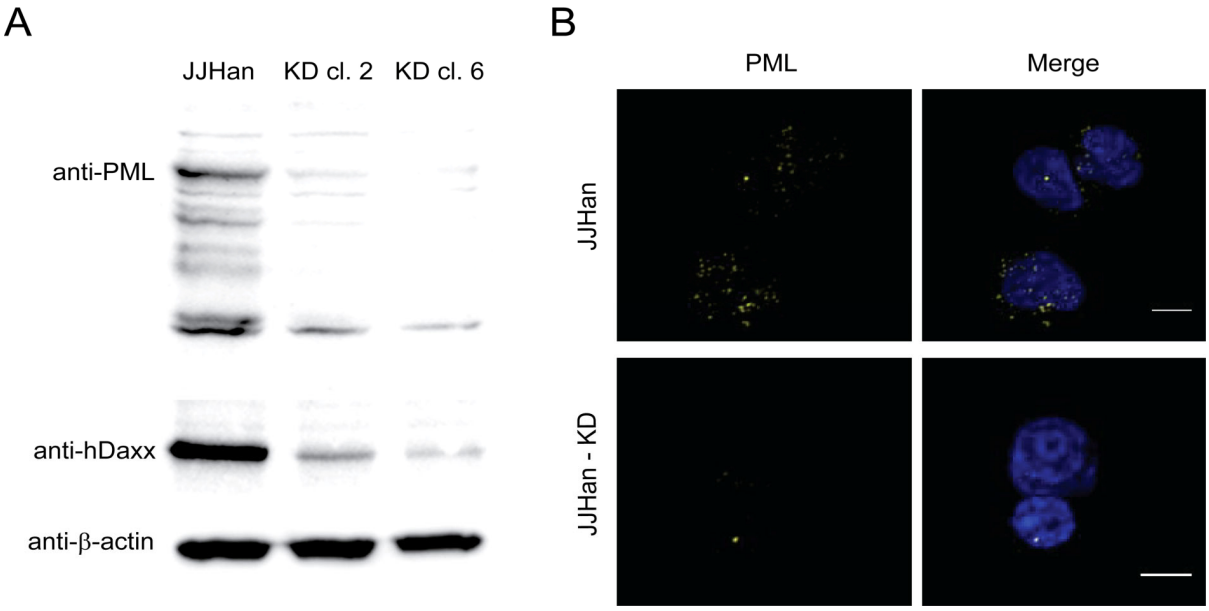

Figure 8: Knockdown of ND10 complex in JJHan cells.

(A) PML and hDaxx knockdown were assessed in two independent knockdown JJHan clones by western blotting. **(B)** PML knockdown was confirmed in JJHan clone 6 by indirect immunofluorescence against PML protein (yellow). Representative images are shown where the nuclei were stained with DAPI (blue) (scale bars correspond to 3µm).

We used JJHan-KD clone 6 for our further experiments as the knockdown efficiency was maximum for this clone. In order to determine the effect of ND10 knockdown on HHV-6A replication, we infected JJHan-KD clone 6 and control cells with HHV-6A-GFP virus. Five days post infection the spread of infection, indicated by GFP was analyzed by flow cytometry. Analysis of data obtained revealed that the percentage of infected cells was significantly increased in the absence of the ND10 protein complex (Figure 9A). We then performed qPCR to further quantify virus replication and found that HHV-6A genome copies were significantly higher in the JJHan-KD cells as compared to the control cells (Figure 9B). Hence it was established that the removal of the ND10 complex facilitated towards more efficient HHV-6A lytic replication.

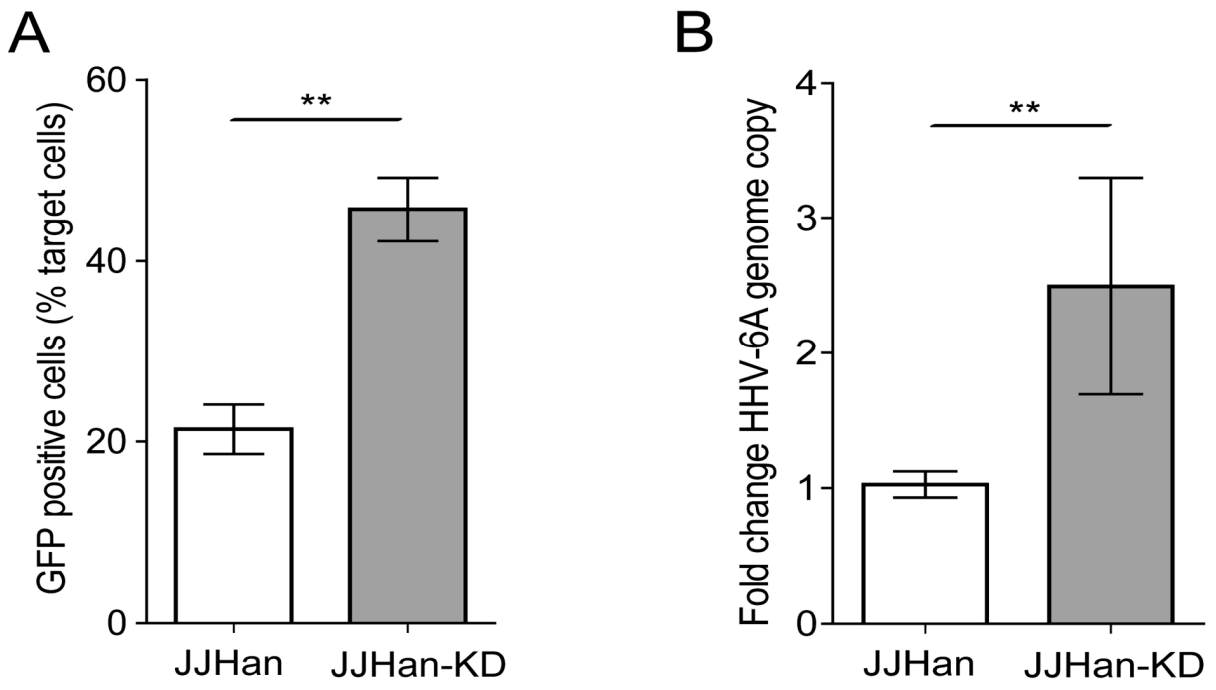

Figure 9: Effect of ND10 complex knockdown in JJHan cells on HHV-6A replication.
(A) Flow cytometry analysis to quantify the number of GFP expressing cells upon infection of JJHan or JJHan-KD clone 6. Results are shown as the mean of three independent experiments with standard errors (**, P<0.01). **(B)** qPCR analysis to determine the HHV-6A genome copies in infected JJHan and JJHan-KD clone 6 cells. Results are shown as the mean of five independent experiments with standard errors (**, P<0.01).

7.3. Influence of ND10 complex on HHV-6A lytic replication

JJHan cells support lytic replication of HHV-6A virus. In spite of this HHV-6A can also establish latent infection in these cells (83). We were curious to know if the ND10 complex influences this decision. Hence, to determine if the ND10 complex was involved in suppressing lytic replication of HHV-6A we stained HHV-6A-GFP infected JJHans with a membrane dye (CellVue) and co-cultured them with uninfected and unstained JJHans-KD or parental JJHan cells. Subsequently, we sorted a pure population of newly infected target cells (GFP+ Cellvue –) (Figure 10A). From the sorted target cell population we determined the percentage of lytically infected cells by fluorescence in situ hybridization (FISH) (Figure 10B). Our data revealed that the percentage of lytically infected cells was significantly higher in the absence of the ND10 complex (Figure 10C).

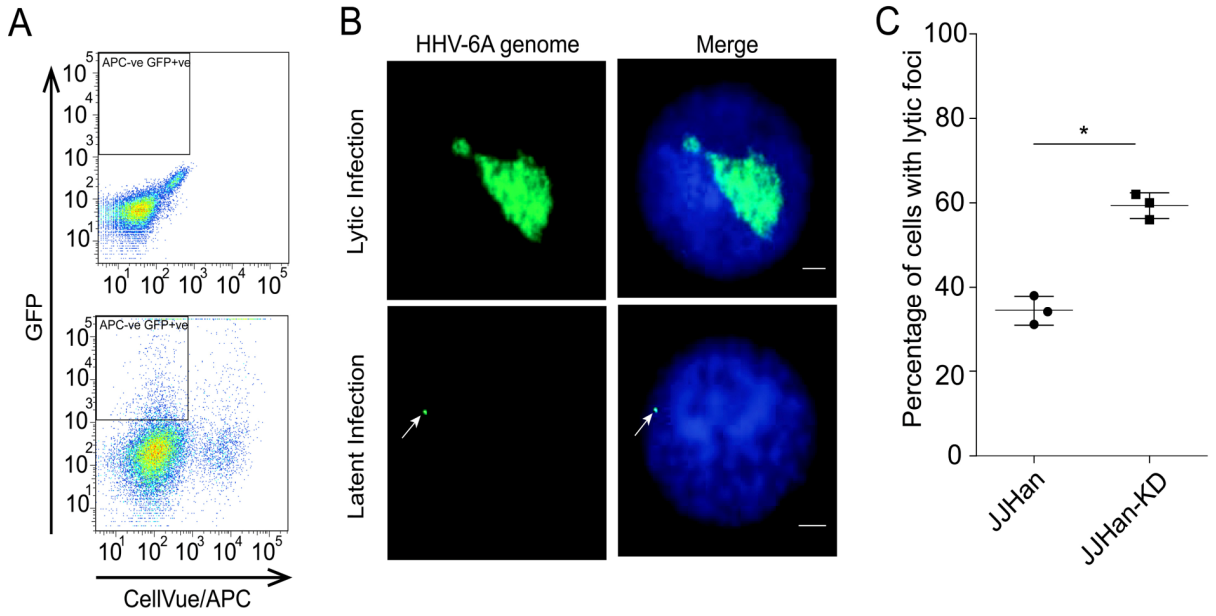

Figure 10: Quantification of lytic replication in JJHan-KD cells.

(A) Gating strategy to isolate pure HHV-6A infected cell population. Infected GFP positive target cells were sorted and subsequently analyzed by FISH. **(B)** Representative FISH images showing the HHV-6A genome (green) in interphase nuclei (DAPI, blue) in lytically and latently infected cells. **(C)** The percentage of lytically infected cells was quantified in JJHan and JJHan-KD cells (n=100) in a blinded manner. Results are shown as the mean of three independent experiments with standard errors (*, P<0.05).

7.4. ND10-mediated silencing of viral protein expression in latently infected cells

The results from our fluorescence *in situ* hybridization clearly indicated that the lytic replication of HHV-6A was enhanced in the absence of the ND10 complex, which led us to wonder whether the ND10 complex also contributes to the sliencing of viral lytic gene expression. To answer this question we assessed the viral protein expression in 293T cells. 293T cells are capable of allowing limited virus replication and are used to assess HHV-6 integration. We knocked down the three ND10 components in the 293T cells (293T – KD) as well, similarly as in JJHan cells. We confirmed the knock down of PML by western blotting as well as by immunofluorescence (Figure 11A). 293T-KD clone 2 showed maximum knockdown efficiency and was used for further experiments. We transfected 293T- KD alongside control cells with the HHV-6A – GFP genome and three days post transfection expression of GFP was analyzed by flow cytometry. The results showed that GFP expression was significantly higher in the ND10 knockdown cells as compared to the parental cells (Figure 11B).We wanted to ensure whether or not HHV-6A genes are also silenced because of the ND10 complex. In order to do so, we used a late gene reporter virus genome expressing GFP. The GFP motif was fused to

the late major capsid protein U57 via a P2a ribosome skipping motif (HHV-6A-U57-p2a-GFP). 293T – KD and control cells were transfected with the HHV-6A-U57-p2a-GFP genome. Subsequently, the percentage of cells expressing U57-GFP was analyzed by FACS five days post transfection. We could observe that the expression of U57-GFP was significantly high in the absence of ND10 components (Figure 11C). Our cumulative data clearly shows that the ND10 complex contributes to the silencing of HHV-6A genome.

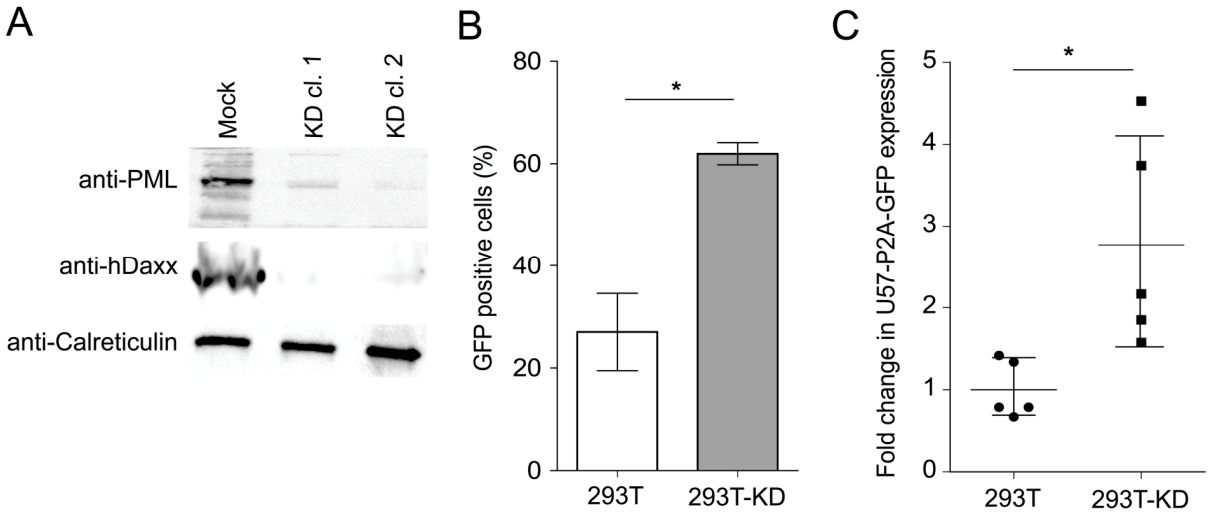

Figure 11: Depletion of ND10 components in 293T cells and its effects on HHV-6A gene expression.

(A) PML and hDaxx knockdown was assessed in two independent 293T knockdown cell clones by western blotting. **(B)** Quantification of the GFP expression in 293T and 293T-KD clone 2 cells at three days post-transfection with the HHV-6A-GFP BAC by FACS. Results are shown as the mean of three independent experiments with standard errors (*, P<0.05). **(C)** Quantification of the expression of the major capsid protein U57 (late gene) by FACS in 293T and 293T-KD clone 2 cells five days post HHV-6A-U57-p2a-GFP BAC transfection by FACS. Results are shown as the mean of three independent experiments with standard errors (*. P<0.05).

8. Discussion

Among the DNA viruses herpesviruses have one of the widest ranges of hosts. The successful infection and persistence of viral genome within the host for prolonged periods is owed to the ability of herpesviruses to establish latency within the host. Host cells have numerous antiviral immune mechanisms to identify and destroy incoming viral genomes. But the herpesviruses have also evolved to counter many of these host defense mechanisms in order to prolong the survival of the viral genome within the host. The ND10 protein complex in the host cells is one such intrinsic antiviral immune entity which has been known to interact and abrogate incoming viral genomes (156). As stated in the introduction chapter, ND10 protein complex comprises of three key components, PML, hDaxx and Sp100. The various components of the ND10 complex have been documented to be involved in silencing incoming viral genomes (124). PML is the most crucial contributing factor to the maintenance of the ND10 structure. SUMO modification of PML is a key step for it to be associated within the ND10 complex (157). This step further assists in the recruitment of other necessary protein components to the complex. PML also consists of a SUMO binding motif which is independent of the SUMO modification sites. This motif allows it to recruit other SUMO-modified proteins into the complex (158). Hence both these covalent and non-covalent modifications of PML by SUMO are indispensable for ND10 complex formation (136). It is also important to note that PML expression is enhanced by type 1 and type 2 interferons (IFNs) through the interferon stimulated response element (ISRE) and IFN-gamma activation sites (GAS) that are located within the PML promoter region. hDaxx is a highly conserved nuclear protein entity and has been found to be involved in the regulation of gene expression and apoptosis (129). It mostly functions as a transcriptional co-repressor and negatively modulates gene expression through the suppression of numerous transcription factors (133-135). It is recruited to the ND10 complex by PML through non-covalent interactions (136). The SUMO-interacting motif (SIM) of hDaxx helps to associate it with SUMOylated DNA-binding interaction factors thus allowing the recruitment of other proteins involved in transcriptional repression like histone deacetylases and DNA methyltranferases (136-138). The recruitment of hDaxx in the ND10 complex attenuates its transrepressive effects (137). When not associated with the ND10 complex, hDaxx can localize within the nucleolus (159), centromeres or heterochromatin . Hence, with respect to its sub-nuclear localization hDaxx can fulfill different functions. Sp100 is another permanent member of the ND10 complex. Similar to PML, Sp100 expression is also enhanced by type 1 interferon as it also harbors GAS and ISRE sites within its promoters (114). Similar to PML, Sp100 also has numerous splice variants such as Sp100A (160), Sp100B (161), Sp100C (162) and Sp100-HMG (163). All the different isoforms of Sp100 have a common N-terminus region that allows them to target ND10 as well as acts as a binding site

for non-histone chromosomal DNA-binding protein HP1 (heterochromatin protein 1) (164). Sp100 also undergoes post-translational modification by SUMO which enhances its interaction with HP1 (165). This indicates the possible regulation of the interplay between ND10 and heterochromatin by Sp100. It has also been reported to be a transcriptional regulator as well (140). Splice variant Sp100B has been shown to associate with hypomethylated DNA like incoming foreign DNA or viral DNA (140).

Genomes of nuclear replicating viruses like herpesviruses (for example HCMV, HSV-1) interact with the ND10 complex the moment they enter the host cell nucleus. This sometimes leads to epigenetic silencing of the viral genomes thus affecting lifecycle of herpesviruses. Evolutionarily most herpesviruses have developed mechanisms to antagonize the repressive effect of the ND10 complex. The best studied examples are ICP0 protein encoded by HSV-1 and IE1 protein encoded by HCMV that can abrogate the ND10 complex by causing disassembly and degradation of ND10 components. ICP0 of HSV-1 colocalizes with the ND10 complex at early time point of infection thereby leading to the degradation of PML and Sp100 which results in the complete dis-aggregation of the ND10 complex (166-168). In case of HCMV, the IE1 protein of the virus is known to prevent SUMOylation of PML thereby preventing ND10 complex assembly (169-171). We on the other hand observed that HHV-6A upon infection of JJHan cells was incapable of completely dissociating the ND10 complex unlike other herpesviruses during lytic replication. Our results revealed that the number of ND10 foci within the nucleus of infected cells was significantly reduced and appeared slightly larger when compared to uninfected cells. Our observations are thus concurrent with similar observations made for HHV-6B, which suggests that both these closely related betaherpesviruses does not counteract ND10 unlike HCMV and other herpesviruses.

It has been evidenced earlier that PML, hDaxx and Sp100 play an important role in heterochromatinization as well as in chromatin condensation process (172). This in turn suggests that these proteins could play a potential role in direct silencing of incoming herpesviral genomes. It has been previously documented that both PML and hDaxx induces a transcriptional repression of IE genes of HCMV in non-permissive cell lines (172, 173). Furthermore, Sp100 is known to interact with the HP1 (163, 164). HP1 is an important protein involved in establishing heterochromatin network (174, 175); hence the association of Sp100 with HP1 denotes its role as a transcriptional regulator. The S1p00B isoform of Sp100 has been found to cause transcriptional repression of both cellular and viral promoters in transient expression experiments. Moreover, it has been reported earlier that the Sp100B isoform has an affinity to associate with unmethylated CpG DNA (176). As incoming viral DNA genomes are mostly hypomethylated it makes them an easy target for association with Sp100. Sp100B has been shown to play a role of transcriptional regulator for both cellular as well as viral

promoters (140). Therefore, in all likelihood PML, hDaxx and Sp100 could potentially be involved in silencing of HHV-6A genome, thus resulting in a quiescent infection instead of a fully-fledged lytic replication.

The role of ND10 complex in suppressing HHV-6A genome replication was not evidenced earlier. In order to determine whether ND10 complex was involved in blocking HHV-6A replication, we knocked down the expression of PML, hDaxx and Sp100 using a triple shRNA vector which was earlier developed and successfully implemented by Glass and colleagues (106). We utilized this triple shRNA vector tool to successfully knockdown expression of ND10 complex in JJHan (JJHan-KD) and 293T cells (293T-KD). Upon infection of the JJHan-KD cells with HHV-6A we observed that the viral genome replicated more efficiently compared to the parental cell line as determined by both FACS and qPCR data. Additionally, we isolated infected cells by FACS and could demonstrate that there was a significant increase in lytically infected cells upon the knockdown of the ND10 components, which suggests that less number of cells are driven towards latent infection in the absence of ND10 complex.

The silencing of the expression of viral lytic proteins would be a key indicator of suppression of viral genome. Hence, to determine the contribution of the ND10 complex in silencing the expression HHV-6A, we used 293T cells which permit latent infection and integration of virus with limited lytic replication. Infection of the 293T-KD cells with our HHV-6A virus revealed significantly higher expression of GFP which was encoded by the virus genome. Additionally, the late gene reporter assay showed that the major capsid protein U57 was poorly silenced in the absence of the ND10 protein complex. Altogether, our project successfully addresses and provides the first insight on the repressive effect of the ND10 complex on HHV-6A infection. We were able to demonstrate how the ND10 complex suppresses lytic HHV-6A replication while simultaneously inducing silencing of the viral genome. Our findings could in part provide an explanation why HHV-6A predominantly establishes a quiescent infect as compared to other herpesviruses which can efficiently dissociate the ND10 complex.

The switch from lytic infection to establishment of chronic latent infection by HHV-6A is very poorly understood. The sheer number of iciHHV-6 patients (1% of global population) is quite unsettling and the clinical ramifications of such a condition are still eluding the scientific community. The association of HHV-6A with numerous other clinical conditions like multiple sclerosis (177, 178), epilepsy (179), cognitive dysfunction (180), Alzheimer’s disease (181), chronic fatigue syndrome (182) as well as myocarditis (183, 184) and cardiomyopathy (185) is quite alarming. Although the contribution of HHV-6 towards these diseases has not been delineated yet it remains an area of serious concern from the clinical point of view. This necessitates the need to understand the mechanism of HHV-6 latency as well as to underline

the various factors that might be involved in this process. The epigenetic modifications that the ND10 complex is capable of inducing on invading viral genomes such as HSV-1 and KSHV, has been documented before (60, 186, 187). This being said, the extent of epigenetic modifications induced by the ND10 complex on the HHV-6A genome remains very much elusive. Hence it provides an exciting area for further research. Studies by Merkl *et al* on HSV-1 recently suggested the involvement of cellular interferon-inducible protein 16 (IFI16), either by itself or in combination with ND10 complex in restricting virus replication (188). Such an involvement of IFI16 in terms of HHV-6 infection has not been reported yet and hence should be definitely investigated upon. There are also possibilities for the involvement of numerous other cellular as well as uncharacterized viral factors in inducing latency of HHV-6A genome which needs to be investigated alongside. Such projects would provide an in-depth understanding of HHV-6 latency.

9. Summary

Cellular factor ND10 complex is involved in repression of lytic replication of human herpesvirus 6A

Human herpesvirus-6 (HHV-6) is a betaherpesvirus that has been classified as two distinct virus species, HHV-6A and HHV-6B, based on the differences in their biological and genetic characteristics. Primary infection with HHV-6B causes a febrile illness in children called *roseola infantum* (sixth disease), which is occasionally associated with neurological issues like seizures and encephalitis. The clinical aspects and epidemiology associated with HHV-6A infections are inadequately understood. Following primary infection the virus establishes a lifelong persistence in infected individuals, termed as latency. Both HHV-6A and -6B are known to integrate their genome into host telomeres of latently infected cells. When integration of the virus occurs in germ cells it results in individuals that harbor the integrated virus in each and every nucleated cell in their body. This condition is commonly termed as inherited chromosomally integrated HHV-6 (iciHHV-6) and is present in about 1 % of the global human population. Both HHV-6A/B can reactivate from latently infected cells, as well as in iciHHV-6 patients, and is associated with several clinical conditions including encephalitis and graft rejection following transplantation.

Human cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) provide efficient replication of HHV-6A and -6B. Various T-cell lines including JJHan and SupT1 cells are permissive to HHV-6A/B lytic replication as well. It is also interesting to note that the viruses can establish latency in these cell lines; however, it remains unknown how the decision between lytic replication and latency is made. In the target cells, after infection, the virus genome is confronted with the nuclear domain 10 (ND10) complex that possesses antiviral activity against a plethora of viruses. The three major constituents of the ND10 complex are namely, promyelocytic leukemia antigen (PML), speckled protein of 100kDa (Sp100) and human death domain-associated protein 6 (hDaxx). Numerous viruses belonging to the herpesviridae family encode proteins that have been shown to manipulate these components and disrupt the ND10 complex during the establishment of infection. For example, ICP0 of herpes simplex virus 1 (HSV-1) induces degradation of PML and Sp100. Similarly, the viral immediate early protein-1 (IE1) of human cytomegalovirus (HCMV) interacts with PML and induces dissociation of the ND10 complex. Additionally, HCMV pp71 induces degradation of hDaxx, which is an important step for productive HCMV gene expression. Majority of known human herpesviruses have been found to efficiently subvert the ND10 complex to establish a successful lytic infection in the host; however, the role of the ND10 complex in HHV-6 infection remains poorly understood.

In this project I investigated the role played by the ND10 protein complex on the event of HHV-6A infection. Firstly, to determine the status of ND10 complex in HHV-6A infected cells I stained and checked for the status of PML (indicative of ND10 complex) in HHV-6A infected JJHan cells. Immunofluorescence studies revealed that ND10 bodies are not dissociated, but their number is reduced in lytically infected cells. To address the role of the ND10 complex, I knocked down the key constituents PML, Sp100 and hDaxx in HHV-6A permissive cells using shRNAs. Clonal cell lines were generated from the ploy clonal knock down stock. In the follow up step I used the clonal KD10 knock down cells in an infection assay to determine the effect of the absence of ND10 constituents on viral replication. My data revealed that lytic replication of HHV-6A was significantly enhanced upon knockdown of the ND10 complex. Finally, I also investigated the effect of ND10 complex on suppression of viral gene expression. For this purpose I HHV-6A virus with a GFP tag on the late lytic gene U57 and infected ND10 knockdown cells. The data obtained clearly showed that viral gene expression was more efficient in cells upon knockdown of ND10 complex as compared to the parental cells.

Taken altogether, my data provides the unique evidence that unlike other human herpesviruses HHV-6A replication within host cells are suppressed by the ND10 complex. Furthermore HHV-6A gene expression is also silenced because of the presence of the ND10 complex. Also, my study garners evidence of stronger lytic replication of HHV-6A in the absence of the ND10 complex, which provides a strong case for the ND10 complex as a key contributor towards HHV-6A latency.

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10. Zussamenfassung

Der zelluläre Faktor ND10-Komplex ist an der Unterdrückung der lytischen Replikation des humanen Herpesvirus 6A beteiligt

Das Humane Herpesvirus 6 (HHV-6) ist ein Betaherpesvirus und wird in zwei Virusspezies, HHV-6A und HHV-6B, klassifiziert – basierend auf Unterschieden in biologischen und genetischen Eigenschaften. Eine Primärinfektion mit HHV-6 führt zu einem febrilen Infekt, der Kinderkrankheit *Roseola Infantum* (Sechste Krankheit), die gelegentlich mit Begleiterscheinungen wie neurologischen Störungen, Krämpfen und Enzephalitiden auftritt. Klinische Aspekte und die mit HHV-6 Infektionen einhergehende Epidemiologie sind bisher nur unzureichend bekannt. Nach einer Primärinfektion kann HHV-6 in infizierten Individuen in einem Ruhezustand verbleiben, welcher Latenz genannt wird. Für beide Viren (sowohl HHV-6A als auch HHV-6B) ist hinreichend bekannt, dass sie ihr Genom in die Telomere der latent infizierten Wirtszelle integrieren können. Findet diese Integration in der Keimbahn statt führt es dazu, dass das integrierte Virus in betroffenen Individuen in jeder zellkernhaltigen Körperzelle zu finden ist. Dieser Zustand wird als chromosomal integriertes HHV-6 (*inherited chromosomally integrated HHV-6*, iciHHV-6) bezeichnet und hat eine Prävalenz von circa 1% in der gesamten Weltbevölkerung. HHV-6A und HHV-6B können in latent infizierten Zellen und in iciHHV-6 Patienten reaktiviert werden und diese Reaktivierung ist mit einer Reihe von klinischen Ausprägungen inklusive Enzephalitis und Abstoßungsreaktionen bei Organtransplantationen assoziiert.

Mononukleäre Zellen aus Nabelschnurblut (*human cord blood mononuclear cells*, CBMC) und mononukleäre Zellen des peripheren Blutes (*peripheral blood mononuclear cells*, PBMC) erlauben eine effiziente Replikation von HHV-6A und HHV-6B. Genau wie verschiedene T-Zelllinien wie JJHan und SupT1. Interessanterweise kann das Virus auch in diesen Zellen eine Latenz etablieren, wobei ungeklärt ist, wie die Entscheidung zwischen lytischer Replikation und latenter Infektion getroffen wird. Nach Infektion der Zielzelle ist das Virus mit dem sogenannten ND10 (*nuclear domain 10*)-Komplex konfrontiert, ein intrinsischer antiviraler Abwehrmechanismus mit Aktivität gegen eine Vielzahl von Viren. Der ND10-Komplex besteht aus 3 Hauptkomponenten: promyelocytic leukemia protein (PML), human death associated protein (hDaxx) und speckled protein of 100 kDa (sp100). Viele Herpesviren codieren Proteine von denen gezeigt wurde, dass diese die ND10-Komponenten beeinflussen und so den ND10-Komplex während der Etablierung einer Infektion dissoziieren. Zum Beispiel induziert das Herpes Simplex Virus 1 (HSV-1) Protein ICP0 eine Degradation von PML und Sp100. Ähnlich interagiert auch das virale *immediate early 1* Protein (IE1) des Humanen Cytomegalievirus (HCMV) mit PML und induziert so eine Dissoziation des ND10-Komplexes. Zusätzlich induziert

das HCMV-Protein pp71 eine Degradierung von hDaxx – ein wichtiger Schritt für eine produktive HCMV Genexpression. Für die Mehrzahl der bekannten humanen Herpesviren wurde beschrieben, dass diese den ND10-Komplex effektiv zersetzen können um eine lytische Replikation im Wirt zu ermöglichen. Dagegen ist die Rolle des ND10-Komplexes in der HHV-6 Infektion bisher unzureichend bekannt.

In diesem Projekt habe ich untersucht, welche Rolle der ND10-Komplex in der HHV-6 Infektion spielt. Zuerst habe ich mittels Färbung von PML (stellvertretend für den ND10-Komplex) in HHV-6 infizierten JJHan Zellen den Status von ND10 in HHV-6 infizierten Zellen bestimmet. Immunofluoreszenzstudien konnten zeigen, dass ND10-Komplexe nicht dissoziieren, deren Anzahl in lytisch infizierten Zellen jedoch reduziert ist. Um die Rolle des ND10-Komplexes weiter zu untersuchen habe ich die Kernkomponenten PML, SPp100 und hDaxx in HHV-6 permissiven Zellen per shRNA ausgeschaltet (*shRNA knockdown*). Aus polyklonalen Zellen habe ich monoklonale Knockdown-Zelllinien generiert. Mit diesen klonalen ND10 Knockdown-Zellen habe ich in einem Infektionsassay den Effekt der ND10-Abwesenheit auf die Virusreplikation untersucht. Meine Daten zeigen, dass die lytische HHV-6 Replikation in den ND10 Knockdown-Zellen signifikant zunimmt. Zusätzlich habe ich den Effekt des ND10-Komplexes auf die Unterdrückung der viralen Genexpression untersucht. Hierfür habe ich ein HHV-6A Virus mit einer GFP-Markierung des späten lytischen Gens U57 benutzt um die ND10 Knockdown-Zellen zu infizieren. Die daraus resultierenden Daten zeigen eindeutig, dass die virale Genexpression in den ND10 Knockdown-Zellen effizienter ist als in den parentalen Zellen.

Zusammenfassend geben meine Daten eindeutige Hinweise darauf, dass im Gegensatz zu anderen humanen Herpesviren die Replikation von HHV-6 in den Wirtszellen vom ND10-Komplex unterdrückt wird. Zusätzlich wird die HHV-6 Genexpression durch die Präsenz des ND10-Komplexes ausgeschaltet. Außerdem zeige ich mit dieser Studie eine stärkere lytische HHV-6 Replikation in Abwesenheit des ND10-Komplexes, ein deutlicher Hinweis dafür, dass der ND10-Komplex entscheidend zur HHV-6 Latenz beiträgt.

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12. List of publications

12.1. Research articles

Anirban Sanyal, Nina Wallaschek, Mandy Glass, Louis Flammand, Darren J. Wight and Benedikt B. Kaufer, Viruses. 2018 Jul 29; “The ND10 complex represses lytic human herpesvirus 6A replication and promotes silencing of the viral genome”: doi: 10.3390/v10080401

Darren J. Wight, Nina Wallaschek, **Anirban Sanyal**, Sandra K.Weller, Louis Flamand and Benedikt B. Kaufer, Viruses 2018 Nov 21; “Viral Proteins U41 and U70 of Human Herpesvirus 6A Are Dispensable for Telomere Integration”: doi: 10.3390/v10110656

Nina Wallaschek, **Anirban Sanyal**, Fabian Pirzer, Annie Gravel, Yasuko Mori, Louis Flammand and Benedikt B. Kaufer, PLos Pathogens 2016 May 3; “The Telomeric Repeats of Human Herpesvirus 6A (HHV-6A) Are Required for Efficient Virus Integration”: doi: 10.1371/journal.ppat.1005666

12.2. Conference proceedings

Anirban Sanyal, Darren J. Wight, Mandy Glass, Nina Wallaschek and Benedikt B. Kaufer. Role of nuclear protein complex ND10 in the decision between lytic replication and latency of Human Herpesvirus-6 (HHV-6). 42nd International herpesvirus workshop, July 29-2nd August 2017, Ghent, Belgium.

Anirban Sanyal, Darren J. Wight, Mandy Glass, Nina Wallaschek and Benedikt B. Kaufer. Role of the ND10 complex in the decision between lytic replication and latency of human herpesvirus-6. The 10th International conference on HHV-6 and 7, July 23-26 2017, Berlin Germany.

Anirban Sanyal, Darren J. Wight, Nina Wallaschek and Benedikt B. Kaufer. The nuclear protein complex ND10 plays an important role in the decision between lytic replication and latency of Human Herpesvirus 6. 27th Annual Meeting of the Society for Virology. 22-25 March 2017, Marburg, Germany.

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14. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 30. Juli 2019

Anirban Sanyal