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of Michael Melling

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The influence of SUMOylation on the adenoviral early region 4 protein Orf6/7

DISSERTATION

With the aim of achieving a doctoral degree
at the Faculty of Mathematics, Informatics and Natural Sciences

Department of Biology of the University of Hamburg

Submitted
by
Michael Melling
June 2018 in Hamburg

Statutory Declaration

I declare that this thesis is a result of my personal work and that no other than the indicated aids have been used for its completion. Furthermore, I assure that all quotations and statements that have been inferred literally or in a general manner from published or unpublished writings are marked as such. Beyond this, I assure that the work has not been used, neither completely nor in parts, to achieve an academic grading or is being published elsewhere.

Hamburg, 07.06.18 _____

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Hamburg, May 25th, 2018

The English language in Michael Melling's PhD thesis entitled „**The influence of SUMOylation on the adenoviral early region 4 protein Orf6/7"** reads fluently and the text is well written. I give my support that the English language is correctly articulated in the PhD Thesis of Michael Melling.

Sincerely,

25/05/18

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II. Abbreviations

aa	Amino acid
HAdV	Human Adenovirus
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
DAPI	4', 6-Diamidine-2'phenylindole dihydrochloride
dd	Doube distilled
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid
E	Early region
E. coli	*Escherichia coli*
EDTA	Ethylendiaminetetraacetic acid
EtOH	Ethanol
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
ffu	*focus* forming units
fw	Forward
g	Earth gravity
h	Hours
h p.i.	Hours post infection
h p.t.	Hours post transfection
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IP	Immunoprecipitation
kb	Kilobase
kBp	Kilobasepairs
kDa	Kilodalton

Abbreviations

L	Late region
min	Minutes
mRNA	Messenger RNA
MOI	Multiplicity of infection
NEM	N-ethylmaleimide
NES	Nuclear export signal
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
qPCR	Quantitative real-time PCR
RB	Retinoblastoma protein
rev	Reverse
RNA	Ribonucleic acid
RT	Room temperature
SAE	SUMO activating enzyme
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	Second
SENP	Sentrin specific protease
SIM	SUMO interaction motif
SUMO	Small ubiquitin related modifier
Tris	Tris-(hydroxmethy)-aminomethane
U	Unit
vol	Volume
v/v	Volume per volume
w/v	Weight per volume
WT	Wild type

1 Zusammenfassung

Adenoviren sind dafür bekannt posttranslationale Modifikationen zu nutzen, um eine effiziente Virusreplikation zu ermöglichen. Eine besondere Rolle spielen hierbei so genannte SUMOlierungen, welche unter anderem die Aktivität des frühen adenoviralen Proteins E1B-55K regulieren. Weiterhin wurde gezeigt, dass E1B-55K die SUMOlierung des pro-apoptotischen Proteins p53 induzieren kann. Daher wurden Zellen mit einem Wild-Typ Adenovirus oder einer E1B-55K deletierten Mutante infiziert und anschließend das SUMO-Proteom analysiert, um weitere E1B abhängig SUMOlierte Proteine zu detektieren. Hierfür wurden zunächst Proteine durch Isotopen markiert. Im Anschluss wurden SUMOlierte Proteine über Affinitätschromatographie aufgereinigt und mit einem Massenspektrometer analysiert. Insgesamt wurden in der Wild-Typ Infektion 272 SUMOlierte zelluläre Proteine detektiert, wovon 78 Proteine eine mindestens zweifach höhere SUMOlierung ausschließlich in der Wild-Typ Infektion aufwiesen. Ebenso wurden 20 SUMOlierte virale Proteine gefunden, von denen drei nur in der Wild-Typ Infektion detektiert werden konnten. Eines davon ist das, in der E4 Region kodierte, Protein Orf6/7 (E4orf6/7). Dieses 19.8 kDa große Protein ist zusammen mit dem viralen Protein E1A maßgeblich für die Aktivierung von E2F Promotoren verantwortlich, um post-mitotisch ruhende Zellen in die S-Phase zu überführen. Im Gegensatz zu E1A verfügt E4orf6/7 über die Fähigkeit zwei E2F Transkriptionsfaktoren zu binden und dadurch eine kooperative Bindung zu Promotoren mit zwei invertierten E2F Bindestellen zu ermöglichen. Vergangene Studien legen nahe, dass besonders die Regulierung des viralen E2A als auch des E2F-1 Promoters und die Lokalisation von E2F-4 in den Zellkern Hauptaufgaben von E4orf6/7 sind. Um die Auswirkungen der SUMOlierung von E4orf6/7 auf die bekannten Funktion(en) zu untersuchen, wurden in dieser Arbeit Plasmid und Virus Mutanten generiert, bei denen Lysin-Reste durch Arginin-Reste substituiert wurde. Mittels dieser E4orf6/7 Varianten war es uns möglich zu zeigen, dass E4orf6/7 am Lysin-Rest 68 SUMOliert wird. In weiteren Experimenten wurde gezeigt, dass die Substitution in der E4orf6/7 K68R Mutante keine Auswirkung auf die Transaktivierung des E2A oder des E2F-1 Promotors hat. Ebenso konnte keine Veränderung in der Lokalisierung von E4orf6/7 und dessen Interaktionspartnern detektiert werden. Es wurde jedoch wiederholt festgestellt, dass die Stabilität der K68R Mutante substanziell verringert ist. Weiterhin war es möglich, einen E2F-1 Promoter vor dem Gen FAM111B zu identifizieren und zu zeigen, dass die FAM111B mRNA Menge im Verlauf der HAdV-5 K68R Infektion stark erhöht ist. Diese Ergebnisse lassen vermuten, dass die SUMOlierung nicht nur die Stabilität von E4orf6/7 beeinflusst, sondern auch, dass der SUMO Status von E4orf6/7 für die Repression des FAM111B Promotors und vermutlich anderer zellulärer Promotoren, oder die spezifische Stabilisierung von mRNA notwendig ist.

1 Abstract

The human adenovirus type 5 (HAdV-5) early region 1B 55-kDa product (E1B-55K) is a multifunctional 496 amino acid polypeptide that exploits the host SUMO conjugation system to promote efficient viral replication. A comprehensive proteomic analysis in wild type and E1B mutant virus-infected cells was performed to reveal the effect of E1B-55K on the SUMO proteome of the host cell. Stable isotope labeling by amino acids in cell culture (SILAC) followed by Ni-NTA pulldown experiments and mass spectrometry detected 272 SUMOylated cellular proteins that were exclusively during wild type infection. Of these proteins, the abundance of 78 was increased by a factor of two or more. Besides cellular proteins, also 20 viral proteins were found to be SUMOylated whereat three of them occurred exclusively during wild type infection. One of them was the 19.8 kDa protein encoded in the early region 4 open reading frame 6/7 (E4orf6/7). This E4 protein is required for the initiation of viral DNA replication and cooperates with the early region protein 1A (E1A), to drive post-mitotic resting cells into S-phase by activating E2F responsive promoters. In contrast to E1A, E4orf6/7 dimerizes E2F transcription factors thereby increasing the activity of E2F target promoters. Previous studies have shown that dimerization of E2F transcription factors by E4orf6/7 is, in particular, important for the transactivation of the E2F-1 promoter, and the re-localization of E2F-4 from the cytoplasm into the nucleus. In order to elucidate the functional consequences of E4orf6/7 SUMOylation on known E4orf6/7 function(s), we generated several virus- and plasmid-encoded E4orf6/7 mutants leading to the substitution of lysine by an arginine, within the protein. Using these E4orf6/7 variants we confirmed SUMOylation of E4orf6/7 in plasmid-transfected cells and identified, for the first time, lysine 68 as the site of SUMO conjugation. Functional studies using transient reporter assays showed that the arginine substitution at lysine 68 (K68R) has no significant effect on the viral E2A or the cellular E2F-1 promoter. Furthermore, the intracellular localization and abundance of E4orf6/7 as well as the interaction with E4orf6/7 targets was not impaired. Intriguingly, the stability of the E4orf6/7 K68R mutant was substantially reduced, accompanied by a significant increase in the cellular FAM111B mRNA abundance, a gene which is also controlled by E2F binding sites. These results not only indicate that SUMOylation regulates the stability of the E4 protein during the course of a productive infection. But also they give rise to the assumption that E4orf6/7 either inhibits the FAM111B and may be other cellular promoters or specifically stabilizes mRNAs in a SUMOylation dependent manner.

2 Introduction

2.1 Adenoviruses

2.1.1 Classification and pathogenesis

In 1953 Adenoviruses were isolated for the first time and named according to the adenoid tissue they were obtained from (Rowe et al., 1953; Enders et al., 1956). The family of *Adenoviridae* however, was not defined before 1976 by *The International Committee on Taxonomy of Viruses.* At that time the *Adenoviridae* initially comprised the genera *Mastadenovirus* and *Aviadenovirus*. Over the last decades, more and more adenoviruses have been described and additional genera were introduced. Today, the family of *Adenoviridae* contains more than 130 species, which are subdivided into 6 genera, depending on their host specificity: *Mastadenoviruses* isolated from mammals, *Aviadenoviruses* isolated from birds, *Siadenovirus* isolated from amphibians and birds; *Atadenoviruses* isolated from ruminant reptiles, avian and marsupial hosts and *Ichtadenovirus* isolated from fish as well as *Testadenoviruses* isolated from tortoises (Doszpoly et al., 2013; Harrach et al., 2012; Davison et al., 2003; Benkö & Harrach, 1998). Since their discovery, the research on adenoviruses has enormously contributed to the understanding of virus structure, eukaryotic gene expression and organization, RNA splicing and apoptosis. Also, they are frequently used as experimental vectors for gene therapy, cancer therapy and recombinant vaccines. Nevertheless, they received most of their interest shortly after some of them were discovered to cause tumors, when inoculated into newborn rodents. Therefore, they have been used in many experimental oncogenesis studies to gain a better insight into the processes of transformation, although they have never been proven to cause tumors in their respective natural host (reviewed in Maclachlan & Dubovi, 2011).

Human adenovirus (HAdV) types of the genus *Mastadenovirus* are further subdivided into the species A to G (illustrated in Figure 1) and it is estimated, that about 90 % of the human population is seropositive for one or more serotypes (Huang & Xu, 2013; D'ambrosio et al., 1982; Wadell, 1984). In the beginning of classification, HAdV serotypes 1-51 were grouped according their agglutination properties (Wadell, 1984; Bailey & Mautner, 1994). Those from type number 52 onwards have been reported and characterized with additional classification methods, such as DNA sequence similarities, oncogenicity in rodents, relatedness of their tumor antigens or electrophoretic mobility of virion proteins (Jones et al., 2007; Robinson et al., 2011; Seto et al., 2011).

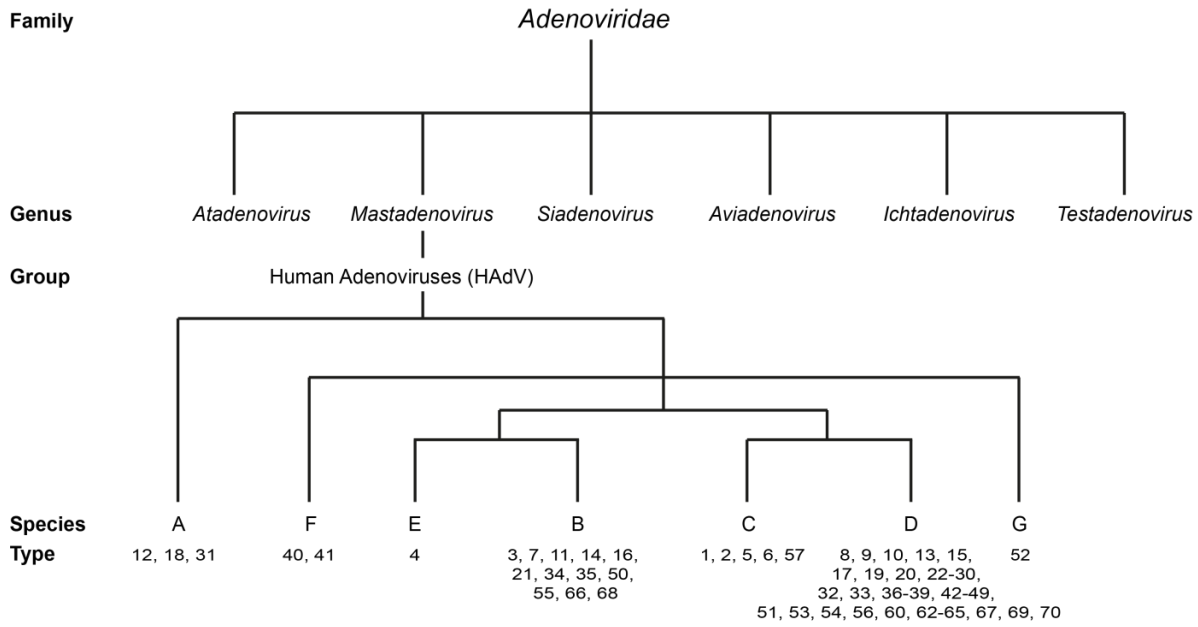


Figure 1: Classification of the family *Adenoviridae*. Simplified illustration of the *Adenoviridae* taxonomy including HAdV types 1-70. HAdV types 1-52 are classified according to Davison *et al.* and the International Committee of the Taxonomy of Viruses (ICTV) (Hage et al., 2015; Davison et al., 2003; Doszpoly et al., 2013; Harrach et al., 2012).

Depending on the type, HAdVs display a certain degree of tissue specificity and cause an array of clinical diseases, including highly contagious conjunctivitis, gastroenteritis, myocarditis and pneumonia (Flomenberg, 2014). Although most adenoviral infections are self-limiting, fatal invasive diseases might occur in immunocompromised patients. In particular those individuals receiving organ transplants, HIV patients developing AIDS, or those treated with radiation and chemotherapy against tumors, are prone to die of opportunistic HAdV infections (Flomenberg, 2014; Echavarría, 2008; Abe et al., 2003). Furthermore, HAdVs were responsible for several severe outbreaks in day care institutions and in military camps (Gaydos & Gaydos, 1995; Gray et al., 2000). Especially members of species B have been associated with epidemic outbreaks causing live threatening systemic infections leading to pneumonia, gastroenteritis or central nervous system symptomatology (Louie et al., 2008; Zhu et al., 2009).

2.1.2 Structure of human Adenoviruses

Adenoviral particles have a non-enveloped, icosahedral appearance with fibers projecting from the vertices of the icosahedron (Figure 2). They have a size of about 90-110 nm in diameter and a virion mass of 150×10^6 Da (Rux & Burnett, 2004). The whole viral particle comprises 11 known viral proteins (three major-, four minor- and four core proteins) as well as the viral genome (reviewed in Berk, 2007). The characteristic icosahedral appearance of the capsid is primarily given by 720 hexon

monomers (major protein II) that form 240 hexon trimers, which in turn form 20 capsid facets, each consisting of 12 hexon homotrimers (Stewart et al., 1991; van Oostrum & Burnett, 1985). Pentons (major protein III) form a pentameric complex at each of the 12 vertices, where the fiber proteins (major protein IV) extend as a trimer (Rux & Burnett, 2004; Stewart et al., 1993, 1991). The tip of each fiber is connected to the so called fiber knob, which is necessary for the primary contact with the cellular receptor protein *Coxsackie-Adenovirus-Receptor* (CAR). Most HAdVs encode a single type of fiber, with some exceptions as for example, HAdV 40, 41 and 52, which encode for two different fiber proteins, with one or the other bound to each penton base (de Jong et al., 2008; Jones et al., 2007; Kidd et al., 1993; Pieniazek et al., 1990). Since the fiber makes the primary contact with cells, it is assumed that the incorporation of two fiber proteins might extend the host range. After primary attachment, the penton base protein facilitates efficient virus uptake via secondary interaction with integrins on the host cell surface (Mathias et al., 1994; Wickham et al., 1994, 1993).

The minor capsid- and core proteins have quite diverse functions, but they all work together to stabilize the capsid and to package and anchor the viral genome in the capsid (reviewed in Berk, 2007). The minor capsid proteins (IIIa, VI, VIII and IX) are part of the virion and it is assumed, that they mostly stabilize the capsid. Nevertheless, apart from their structural features, more recent studies showed that they also exert crucial functions after entering the host cell and before the onset of viral gene transcription. The minor capsid protein VI, for instance, supports the disruption of the endosomal membrane after endocytosis of the viral particle, but also helps to antagonize the initial antiviral response in the cell nucleus (Schreiner et al., 2012; Vellinga et al., 2005). On the other hand, the *core proteins* (terminal protein (TP) and proteins μ, IVa2, V, VII), are associated with the viral genome inside the virion. The polypeptides μ, V and VII condense the viral DNA to form a tight nucleoprotein complex and protein IVa2 assists during the packing into the capsid. In addition to its DNA binding properties, protein V has also been shown to bind to the minor capsid protein VI, suggesting that it acts as a linker between the nucleoprotein complex and the capsid (Zhang et al., 2001; Russell & Precious, 1982; Everitt et al., 1975; Russell et al., 1971). The remaining core component, terminal *protein,* assists during viral genome replication, where it serves as a primer to initiate viral DNA synthesis (Davison et al., 2003).

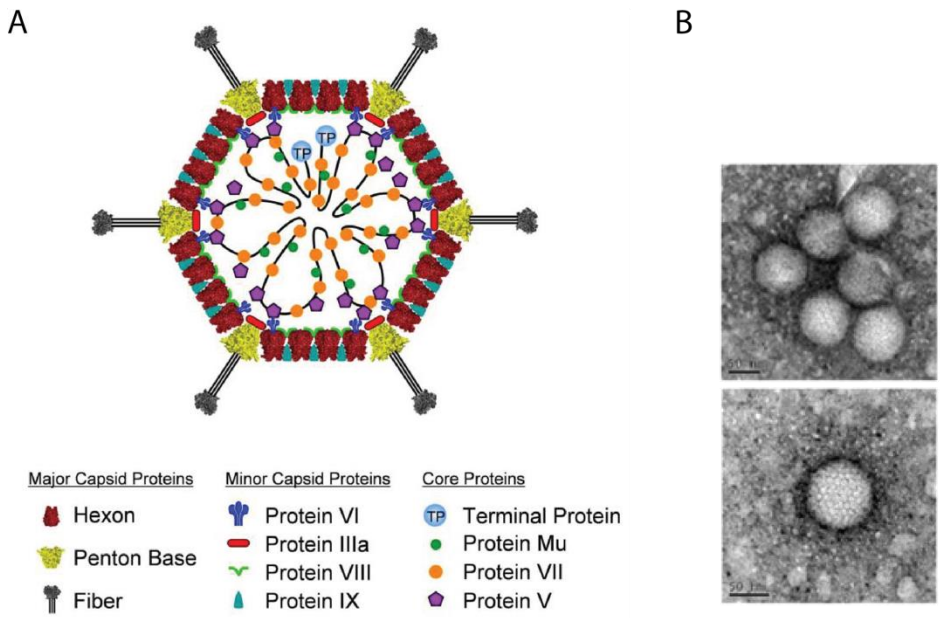


Figure 2: Structure of adenovirus particles. (A) Schematic representation of HAdV-5 according to Nemerow *et. al.*(Nemerow et al., 2009; Russell, 2009). (B) Electron microscope images illustrating the icosahedral structure of HAdV5 particles (Department of Electron Microscopy, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg).

2.1.3 Genome organization of Adenoviruses

The linear double-stranded DNA genome of HAdV-5 encodes for more than 40 regulatory and structural proteins as well as two non-coding RNAs [(virus-associated RNA)(VA-RNA)]. It is organized in nine transcription units: five early (E1A, E1B, E2, E3, E4), three delayed (IX, Iva2, E2L) and one major late transcription unit (MLTU). Early proteins are involved in transcriptional/ translational regulation, mRNA export, viral DNA replication, cell cycle control and inhibition of host antiviral response. In contrast, the delayed and late proteins serve as structural components and support maturation, but also have functions very early during infection, when capsids enter the cell (reviewed in Flint, 2001). Apart from the VA-RNAs, all HAdV-5 transcription units are transcribed by the cellular RNA polymerase II (Weinmann et al., 1974).

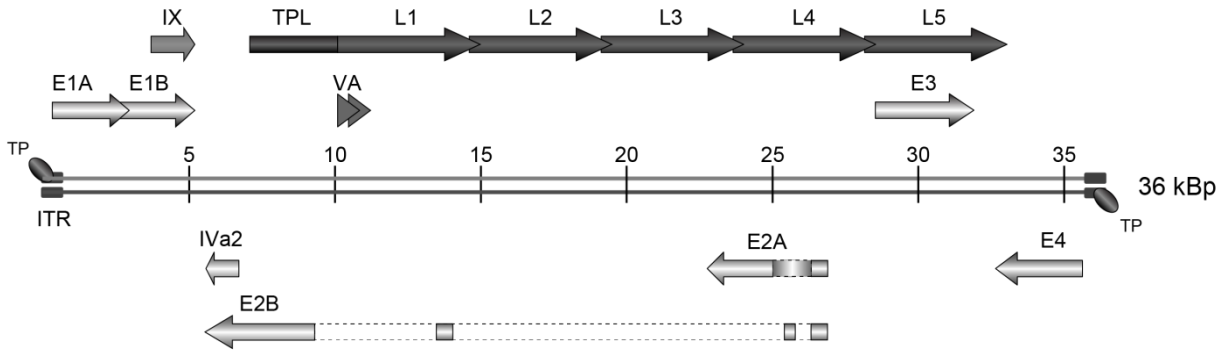


Figure 3: Genome organization of HAdV-5. Organization of early (E1A, E1B, E2A, E2B, E3, E4), delayed (IX, Iva2) and late (L1-L5) transcription units. Organization is indicated by arrows. E: *early*, L: *late; ITR: inverted terminal repeat*; VA-RNA: *Virus-associated RNA*; MLTU: *Major late transcription unit* (adopted from Täuber & Dobner, 2001b).

2.1.4 Productive infection by human Adenoviruses

2.1.4.1 Adsorption and entry

HAdVs preferentially infect post-mitotic resting, differentiated epithelial cells of the respiratory and gastrointestinal tract, *in vivo*. For the investigation of the HAdV replication cycle most studies have employed human tumor cells in culture, in which HAdV can infect several different tumor and primary cell lines. One factor that influences virus tropism is the availability, concentration and localization of certain receptors. The primary attachment of HAdV occurs through the binding of the C-terminal fiber knob to the *Coxsackie-adenovirus receptor* [(CAR)(Bergelson, 1997; Tomko et al., 1997)]. The initial binding facilitates the secondary binding of penton bases to αv-integrins, an interaction which provokes internalization by clathrin-mediated endocytosis (Varga et al., 1991; Greber et al., 1993). Once, the clathrin-coated vesicles have matured to endosomes, the virus particles escape into the cytosol by pH shift and pVI induced permeabilization of the endosomal membrane (Wiethoff et al., 2005; Greber et al., 1993). As soon as viral particles enter the cytosol, they associate with dynein motor proteins for the transport along the microtubules to the *microtubule organizing center* (MTOC) in proximity to the nucleus (Bremner et al., 2009; Dales & Chardonnet, 1973; Greber & Way, 2006; Schreiner et al., 2012). The interaction of virion proteins, especially hexon, with components of the *nuclear pore complex* (NPC) likely facilitates the final disassembly of viral particles and the translocation of the viral genome into the nucleus of the infected host cell (Dales & Chardonnet, 1973; Greber & Way, 2006; Suomalainen et al., 1999; Wodrich et al., 2010). Upon arrival in the nucleus, the transcription of viral genes follows a strict temporal sequence. By convention, early genes are those expressed before viral replication whereas the late transcription is initiated after genome replication has started, although it has been shown

that the major late promoter unit is also transcribed at low levels during the early phase of infection (Berk, 1986).

2.1.4.1 The early region 1A

There are three main goals for HAdVs to accomplish productive viral replication: 1. To forward cell cycle into S-phase, providing an optimal environment for efficient replication 2. To emplace viral systems that protect infected cells from various host antiviral defenses. 3. To synthesize viral proteins needed for viral DNA replication, production of capsids as well as viral maturation. All three goals depend on transcriptional activation of the viral genome as well as the induction of S-phase. Although all viral early promoters show a basal activity in mammalian cells, their transcription is greatly enhanced by the products of the early region 1A (E1A) gene of HAdVs (reviewed in Berk, 2013). Once the viral genome has entered the nucleus, E1A is the first gene to be transcribed, due to strong enhancer activity upstream of the E1A promoter. Two major mRNAs E1A-13S and E1A-12S are transcribed from the E1A transcription unit owing to alternative splicing (Nevins, 1981). In addition, three further splice variants (11S, 10S, 9S) accumulate at later time points, during which the levels of 13S and 12S decline (Stephens & Harlow, 1987). All E1A gene products activate viral transcription and induce reprogramming of infected cells, to provide an optimal environment for viral replication (reviewed in Gallimore & Turnell, 2001). The two major mRNA species 12S and 13S contain the same 5' and 3' ends but differ in their internal part (illustrated in Figure 4). The encoded proteins are nearly identical, except for an additional 46 amino acid segment (CR3), resembling an activation domain being present in the larger polypeptide (Moran & Mathews, 1987). *In-situ* E1A sequence alignments of different HAdV types revealed four conserved regions (CR1, CR2, CR3, CR4), separated by less conserved domains (Kimelman et al., 1985; van Ormondt et al., 1980). These regions enable protein-protein interactions that mediate the regulation of transcription, chromatin remodeling, cell proliferation and transformation. However, since both major E1A-12S and -13S proteins can promote cell cycle progression, it is believed that CR3 is not absolutely required for these activities (Haley et al., 1984).

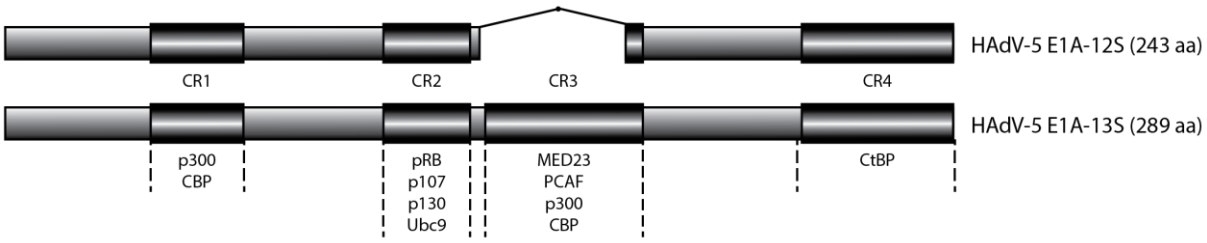


Figure 4: E1A organization and cellular proteins interacting with the conserved regions. Linear representation of E1A-12S and E1A-13S domain structure with conserved regions (Pelka et al., 2009). Proteins interacting with the CRs are listed below. CR: *conserved region* (adopted from Pelka et al., 2008).

The CR3 domain in E1A-13S is considered to be the main transactivator of the early genes and increases the E1A promoter activity up to 5-fold, the E1B promoter 10-fold and the E2, E3 as well as E4 promoters approximately 100-fold (reviewed in Berk, 2013). Activation requires stable, highly specific CR3 mediated interaction with the *mediator complex subunit 23* (MED23) and the *histone acetyl transferases* (HAT) p300 and *CREB binding protein* [(CBP)(Ablack et al., 2010)]. Interaction with MED23 subunit both increase the assembly of the preinitiation complex on promoters and stimulate transcription elongation (Vijayalingam & Chinnadurai, 2013; Berk, 2013). Once bound by the CR3 region of E1A-13S and recruited to target promoters, p300 and CBP transfer acetyl residues to histone tails, resulting in increased accessibility of the chromatin to the transcription machinery (Pelka et al., 2009; Ogryzko et al., 1996; Bannister & Kouzarides, 1996).

In contrast, the inhibition or activation of cellular genes is mostly attributed to E1A-12S, even though both major E1As contain the CR1, CR2 and CR4 region. Interestingly, CR1 of E1A-12S can also bind p300 and CBP like the CR3 of E1A-13S. However, the binding to E1A-12S CR1 results in the sequestration of p300 and DBP from cellular transcription factors and inactivation of HAT activity, leading to transcriptional repression of cellular genes (reviewed in Frisch & Mymryk, 2002). CR2 in turn binds to tumor suppressor proteins of the pRB-family, which control the entry into S-phase by the inhibition of the transcription factor E2F. Therefore, the inactivation of pRB-family members and the displacement from E2Fs by E1A-12S initiates the transcription of many important S-phase genes such as CDK2 and cyclins E and A (Dyson & Harlow, 1992; Cobrinik, 2005). The CR4 contains a nuclear localization signal in all E1A variants and acts additionally as another transcriptional regulatory region in E1A-12S (Lyons et al., 1987). CR4 binds the *E1A C-terminal binding protein* (CtBP), interacting with multiple repressors and influences the process of transformation (Boyd et al., 1993).

In summary, E1A manipulates regulatory constraints at the G1/S-phase checkpoint by acting on at least three levels: 1. Inactivation and displacement of pRB-family proteins to activate E2F transcription factors. 2. Modulation of chromatin remodeling factors such as p300, CBP and CtBP and 3. Targeting of additional cellular proteins, including downstream targets of CDK2 and transcription

factors involved in regulation of genes that participate in cell cycle control (reviewed in Ben-Israel & Kleinberger 2002; and Berk, 2005).

2.1.4.2 The early region 1B

The early region 1B (E1B) encodes two major proteins E1B-55K and E1B-19K (Perricaudet et al., 1979; Bos et al., 1981). In addition, three further minor splice variants and two N-terminally truncated E1B gene products have been characterized, which seem to partly share functions with the large E1B-55K polypeptide (Kindsmüller et al., 2009; Sieber & Dobner, 2007). Both major proteins are able to antagonize apoptosis by interacting with p53-dependent and p53-independent pathways and contribute to complete cell transformation of primary rodent cells (Debbas & White, 1993). E1B-55K inhibits the apoptotic key regulator p53, through direct and indirect interactions with the protein and/or PML-NB associated factors. E1B-19K in turn resembles a homolog of the anti-apoptotic Bcl2 protein and negatively modulates pro-apoptotic proteins such as Bax and Bak (White, 1993, 2001).
In particular, the multifunctional roles of E1B-55K have been intensively studied over the past decades and have been connected to several key steps during the early and the late phase of adenoviral infection (reviewed in Berk, 2005 and 2007). Initially, E1B-55K counteracts host cell induced anti-proliferative processes, including the activation of p53-dependent and -independent apoptosis, and the induction of cell cycle arrest. Additionally, it also stimulates antiviral mechanisms like the cellular DNA damage response, an intrinsic immune response mediated by the MRN complex (reviewed in Weitzman & Ornelles, 2005 and White, 2001). During the late phase of infection, the adenoviral protein stimulates efficient cytoplasmic accumulation and translation of viral late mRNAs and at the same time it prevents the export of cellular mRNAs (reviewed in Dobner & Kzhyshkowska, 2001 and Flint & Gonzalez, 2003). In order to accomplish these tasks, E1B-55K continuously shuttles between nucleus and cytoplasm and interacts with numerous cellular as well as viral proteins. Specifically, the association with the early viral proteins E4orf3 and E4orf6 seems to be of great importance, since deletions in these gene regions considerably affect virus replication (reviewed in Täuber & Dobner, 2001a,b).

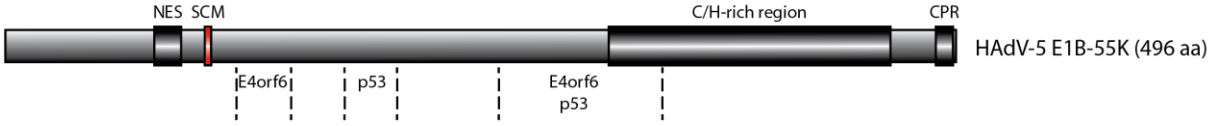


Figure 5: Schematic domain structure of HAdV-5 E1B-55K. Interaction regions with E4orf6 and p53 are indicated below. C/H-rich region: *cysteine/histidine-rich regions*; NES: *nuclear export signal*; SCM: SUMO conjugation motif; CPR: C-terminal phosphorylation region (Wimmer et al., 2013)

Sequence analysis of the E1B-55K region, revealed a characteristic arrangement of zinc ion-binding cysteine and histidine residues in the C-terminal part of the polypeptide (Härtl et al., 2008). This defined arrangement is called a *really interesting new gene finger domain* (RING finger domain) and has also been shown for some other proteins to play a key role in the ubiquitin pathway (Borden & Freemont, 1996; Deshaies & Joazeiro, 2009). In line with this observation, several groups were able to show that E1B-55K, together with E4orf6, forms an E3 ubiquitin ligase complex, comprising the cellular proteins elongin B and C, cullin 5 and RBX1 (Harada et al., 2002; Querido et al., 2001). This complex associates with various cellular proteins to target them for ubiquitin conjugation and proteasomal degradation. Multiple substrate proteins of this viral E3 ubiquitin ligase complex have been identified, such as p53, to prevent apoptosis; *Meiotic recombination 11 homolog 1* (MRE11), a component of the MRN complex, to prevent intrinsic immune response and SPOC1, to modulate chromatin remodeling and intrinsic immune response (Schreiner et al., 2013; Stracker et al., 2002; Querido et al., 2001).

Intriguingly, several functions of E1B-55K have been shown to be regulated, at least in part, by the post-translational modification with so called *small ubiquitin related modifier* [(SUMO)(Endter et al., 2001, 2005; Kindsmüller et al., 2007; Krätzer et al., 2000)]. E1B-55K is one of two published SUMO substrates among adenoviral proteins but the physiological effects and the change in biological properties upon SUMOylation have been intensively studied. It has been demonstrated that SUMO conjugation regulates the nucleo-cytoplasmic shuttling and influences the transforming potential of E1B-55K considerably (Freudenberger et al., 2018; Kindsmüller et al., 2007; Lethbridge et al., 2003; Endter et al., 2001). In addition, several studies found convincing evidence that E1B-55K is not only an E3 ubiquitin ligase but also an E3 SUMO ligase inducing the SUMOylation of p53. This modification exerts an additional repressing effect on p53 activity and stimulates its nuclear export through interactions with *promyelocytic leukemia nuclear bodies* [(PML-NB)(Pennella et al., 2010; Muller & Dobner, 2008)]. Nevertheless, p53 is for now the only published SUMO target of E1B-55K and it remains to be determined, if this process might be another general mechanism, by which HAdVs facilitate efficient virus replication.

2.1.4.3 Early region 4

The E4 gene produces at least six distinct polypeptides, named according to the order and disposition of their corresponding open reading frames as E4orf1 to E4orf6/7. Initial studies in the early 1980s have focused on the regulation of E4 gene expression and E4 gene functions during the lytic infection cycle of HAdV-2 and 5. More recent studies have elucidated the functions of individual E4 gene products and identified specific cellular interaction partners. These studies revealed interactions with

proteins involved in transcription regulation, cell cycle progression and apoptosis, DNA repair as well as cell signaling (Leppard, 1997, 1998; Ghebremedhin, 2014; Imperiale et al., 1995; Dobner & Kzhyshkowska, 2001).

Analysis of different HAdV types revealed homologous E4 regions with similar sequence organization. In particular the E4 transcription units of HAdV-2 and 5 have been most intensively studied in terms of their transcriptional and posttranscriptional regulation as well as their gene products. The E4 region is located at the 3' end between the map units 91.3 to 99.1 and is transcribed in leftward direction (Figure 7). Expression of the primary transcript is controlled by the E4 promoter and is tightly regulated at the transcriptional and post-transcriptional level (Gilardi & Perricaudet, 1986; Watanabe et al., 1988; Hérissé et al., 1981; Nevins et al., 1979). Similar to the other early transcription units, E4 gene expression is activated by E1A-13S, which contains the unique domain CR3 and the auxiliary region 1 [(AR1)(Bondesson et al., 1996; Ström et al., 1998)]. The resulting primary transcript with a length of approximately 2800 bp, is subjected to a complex pattern of differential splicing, producing at least 18 distinct mRNAs that share common 5'- as well as 3' terminal sequences (Hérissé et al., 1981; Virtanen et al., 1984; Freyer et al., 1984). However, only the following polypeptides have been reported to be expressed in infected cells: Orf1, Orf2, Orf3, Orf4, Orf6 and Orf6/7 (Downey et al., 1983; Sarnow et al., 1984; Cutt et al., 1987; Kleinberger & Shenk, 1993; Javier, 1994; Dix & Leppard, 1995; Thomas et al., 2001).

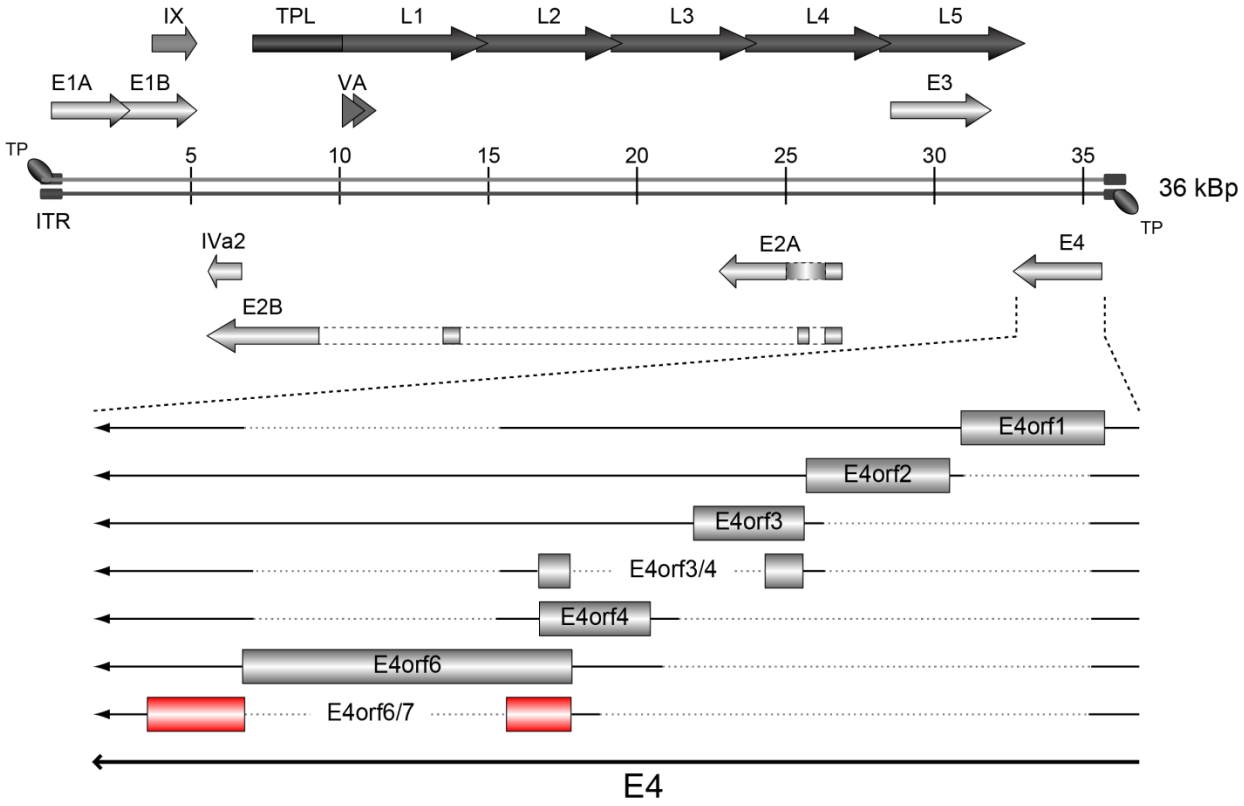


Figure 6: The genomic organization of HAdV-5 and the E4 transcription unit. Lengths are marked in kbp. Early and late transcription units are shown relative to their position and orientation in the HAdV-5 genome. The E4 unit is controlled by the E4 promoter and generates a primary transcript, which is subjected to a complex pattern of differential splicing, producing at least 18 mRNAs that share common 5'- and 3' terminal sequences. The seven different polypeptides are shown as open boxes. E4orf6/7 is indicated in red (adopted from Täuber & Dobner, 2001b).

Individual E4 mRNAs have been detected as soon as 2 h after infection and peak in a maximum approximately 4 h post infection. Although transcription declines with the onset of the late phase, E4 transcription still continues at later time points (Nevins et al., 1979). To elucidate the role of E4 gene products, a series of HAdV-2 and HAdV-5 virus insertion/deletion mutants were generated. Intriguingly, only those mutants lacking either the whole E4-region, or those that failed to express E4orf3 and/or E4orf6 proteins showed pronounced restricted progeny production (Halbert et al., 1985). These mutants exhibited a series of defects: They failed to accumulate normal nuclear and cytoplasmic levels of late messenger RNAs and were defective for late protein synthesis as well as host cell shut-off at late times of infection (Halbert et al., 1985; Weinberg & Ketner, 1986; Yoder & Berget, 1986; Falgout & Ketner, 1987; Bridge & Ketner, 1989; Huang & Hearing, 1989a; Sandler & Ketner, 1989). Additionally, these mutants showed also substantially impaired DNA replication and produce heterogeneous populations of large concatemeric viral DNAs (Weiden & Ginsberg, 1994). Further studies with virus mutants lacking individual E4 gene products or combinations of E4 proteins, revealed that also some other virus mutants show modest replication defects, indicating

that other E4 products provide additional, although minor functions required for virus progeny production (Bridge & Ketner, 1989; Huang & Hearing, 1989a).

The last of the six E4-encoded gene products is a fusion protein encoded in a spliced mRNA of the open reading frames 6 and 7. In HAdV-5 the resulting protein comprises the 58 amino-terminal residues of E4orf6 and the 92 amino acid residues encoded in E4orf7 (Figure 7). Experiments with successive E4orf6/7 deletion mutants revealed a N-terminal nuclear retention signal allowing the accumulation of E4orf6/7 in the nucleus and interaction with E1B-55K (Schaley et al., 2005; Rubenwolf et al., 1997). The C-terminus on the other hand, seems to be important for the transactivational activity of E4orf6/7 during adenoviral infection. The C-terminal 70 amino acids are essential for the binding to at least five of the known E2F transcription factors (O'Connor & Hearing, 1994; Neill & Nevins, 1991; O'Connor & Hearing, 1991). In principle, the C-terminus of E4orf6/7 can be divided into two regions required for the interaction with two E2F molecules (amino acid region 81-104 and 127-150), which flank the aa position 125, necessary for the stable double-site complex formation [(dimerization induction site) (O'Connor & Hearing, 1994)]. E2F transcription factors are heterodimers and important regulators of cell proliferation by activating the expression of cellular genes involved in DNA synthesis and cell cycle progression (reviewed in Cress & Nevins, 1996). Therefore, it is assumed that, although E1A is sufficient to initiate viral E2 early (E2A) transcription, E4orf6/7 supports and complements the function of E1A to promote the expression of the E2A transcription unit and other E2F regulated cellular genes. This assumption is additionally supported by more recent work that showed E4orf6/7 alone being sufficient to displace pRB and p107 from E2F heterodimers to activate expression of the viral E2A promoter and cellular E2F promoters. Even though this activity is much lower, these experiments demonstrate that E4orf6/7 can functionally compensate, at least partially, for the lack of E1A expression in virus infected cells (O'Connor & Hearing, 2000; Schaley et al., 2000). In this context, it is interesting to mention that, while different HAdV types conserve the two E2F binding sites for the dimerization of E2Fs, not all of these viruses carry inverted E2F-binding sites in their E2A promoter regions (Schaley et al., 2000; Obert et al., 1994). Nevertheless, each of the tested E4orf6/7 proteins is capable of inducing E2F DNA binding and transactivation of promoter regions carrying two inverted binding sites. In contrast, the E4orf6/7 dimerized E2Fs of HAdV types with a single binding site in their E2A promoter, show only weak binding affinity to their own promoter. These intriguing and contradictory observations, made Schaley and co-workers believe that the physiological relevant promoter for E4orf6/7 might be a cellular promoter with an inverted configuration of E2F binding sites that cannot be activated by E1A alone, but requires additionally the dimerizing activity of E4orf6/7. Strikingly, they found that the human E2F-1 promoter has inverted E2F binding sites and demonstrated that promoter activation requires the dimerization of E2F by E4orf6/7 (Schaley et al., 2000). The E2F-1 protein was the first

E2F family member to be cloned and plays a major role in the regulation of the cell cycle (Müller et al., 2001; Ren et al., 2002; Weinmann & Roeder, 1974). Further analysis of E4orf6/7 revealed that the N-terminus of E4orf6/7 induces nuclear localization of E2F-4, which has mostly repressive activities on cellular genes, but acts as an activator on the viral E2A promoter (Conboy et al., 2007; Litovchick et al., 2007; Kel et al., 2001; Takahashi et al., 2000; Schaley et al., 2000; Huang & Hearing, 1989b).

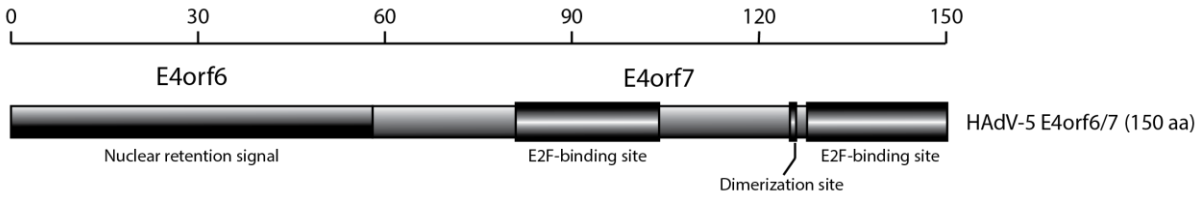


Figure 7: Schematic structure and functional domains of the HAdV-5 E4orf6/7 protein. Length is given in numbers of amino acids. The E4orf6/7 is a fusion protein of the N-terminus of E4orf6, which contains the nuclear retention signal, and the C-terminus of E4orf7, which includes the elements to bind and dimerize E2F transcription factors (Obert et al., 1994).

In summary, these observations suggest, that HAdVs have evolved at least two mechanisms to induce expression of E2F-responsive genes: On the one hand, the E1A proteins displacing pRB family proteins, thereby activating E2F transcription factors. On the other hand, E4orf6/7 re-localizes at least one E2F molecule and complements the function of E1A by stably dimerizing and recruiting active E2F to transactivate the expression of promoters with an inverted configuration of E2F binding sites (Schaley et al., 2000).

2.2 Adenoviruses modulate the cell cycle

Adenoviruses preferentially infect quiescent cells. However, early after infection they induce cell cycle transition from G_0 or G_1 into S-phase, because resting cells have only rate limiting levels of deoxynucleotides and low levels in proteins involved in DNA synthesis, both important DNA replication (Thelander & Reichard, 1979). Transition through the mammalian cell cycle requires a stringently controlled interplay of different transcription factors. The mammalian transcription factor E2F was originally found as a nuclear activity, that bound to the E2A promoter of adenoviruses (Kovesdi et al., 1986b,a). A few years later, a remarkable body of evidence supported the idea that E2F proteins play a critical role in regulating the cell cycle transition into S-phase, mediating gene expression for cell proliferation and differentiation (La Thangue, 1994; Lam & La Thangue, 1994; Nevins, 1992). The transcription factor is commonly called “E2F”, although it is actually a heterodimeric complex, containing one of eight factors (E2F-1 to E2F-8) that pair with a second subunit called *dimerization partner* [(DP-1 to DP-3)(Dyson, 1998; Ormondroyd et al., 1995)]. While some E2F species seem to have specific roles, others act globally and it has additionally become

evident that there are no simple divisions of labor among the E2F family (DeGregori & Johnson, 2006). Nevertheless, it is generally accepted that E2F-1 to E2F-3 play major roles in transcriptional activation, whereas E2F-4 to E2F-8 participate in the repression of genes (Takahashi et al., 2000).

The transcriptional regulation by E2F is further influenced by members of the retinoblastoma (pRB) family. This family of tumor suppressors comprises the protein pRB and its relatives p107 and p130, each of them binding to a certain set of E2Fs, depending on the cell cycle stage (reviewed in Dyson, 1998). E2F-1, -2 and -3 preferentially bind RB, E2F-4 preferentially binds p107 and p130, E2F-5 binds p130, while E2F-6 lacks sequences required for RB binding (Helin et al., 1993). The occurrence and interaction of E2Fs with pRB-family members happens at specific stages during the cell cycle. In G_0, the predominant E2F complexes are E2F-4 and E2F-5 with p130. Following growth factor stimulation, the E2F-5/p130 complex diminishes and E2F-1/pRB and E2F-4/p107 are evident. The activation of G_1 cyclin-dependent kinases results in hyper-phosphorylation of pRB-family members and their release from E2Fs (Stevens & La Thangue, 2003; La Thangue, 2003; Müller & Helin, 2000). Although the initial model suggested that the activating or inhibitory activity of E2F is counterbalanced by pRB-family member binding, it is nowadays clear, that the regulation by E2F is far more complex. Rather, there are three generic types of E2F complexes: 1. *Activated E2F complexes* (“free” E2F), in which pRBs family members have been displaced and E2Fs promote transcription; 2. *Inhibited E2F complexes*, in which the activation domain is masked by pRB-family proteins; 3. *Repressing E2F/pRB complexes*, in which pRB-family proteins bind to E2Fs and assemble a repressor activity and (Sahin & Sladek, 2010; Dyson, 1998).

Specifically, E2F-1 seems to play a major and dual role in the regulation of the cell cycle. It was shown to alter transcription of more than thousand genes and depending on the gene it acts as an activator or repressor (Müller et al., 2001; Ren et al., 2002; Weinmann & Roeder, 1974). For example, alteration of E2F-1 binding sites of c-myc, cdc2 and human DHFR promoters resulted in 50-90 % loss of transcription activity (Batsche et al., 1994; Jensen et al., 1997; Jun et al., 1998), whereas similar mutations in b-myb, TK and mouse DHFV caused increased activity in proliferating cells (Van Ginkel et al., 1997; Lam et al., 1995; Sahin & Sladek, 2010). Furthermore, E2F-1 has both properties, either acting as an oncogene or as a tumor suppressor, depending on the context in which E2F-1 function is analyzed. Expression of only E2F-1 is sufficient to drive quiescent cells to enter S-phase. On the other hand, it is also the only E2F family member being able to induce p53-dependent apoptosis (La Thangue, 2003; Johnson et al., 1994; Qin et al., 1994; Shan & Lee, 1994).

E2F-4 is another E2F molecule that has gained a lot of attention in the last decades (Lee et al., 2011; Crosby & Almasan, 2004; Gaubatz et al., 2001; Olgiate et al., 1999; Moberg et al., 1996). In contrast to other E2Fs, it does not possess a NLS but relies on p107 and p130 as well as the DP proteins for its re-localization into the nucleus (Gaubatz et al., 2001; Moberg et al., 1996). Further studies showed

that the re-localization is also an important regulation mechanism for E2F-4. Whereas the expression of E2F-1 to E2F-3 is highly regulated during cell cycle, E2F-4 as well as E2F-5 are constitutively expressed. Instead, regulation of E2F-4 and E2F-5 is achieved by changing the subcellular localization but also by the interaction with pRB-family proteins and by influencing translation and post-translational modifications (Yochum et al., 2007; Lindeman et al., 1997). E2F-4 has been mostly described as a repressor that binds to and regulates a specific set of proliferation and cell cycle related target genes, to maintain quiescent cells in G_0 phase (Conboy et al., 2007; Takahashi et al., 2000; Ren et al., 2002; Litovchick et al., 2007). For example, transcriptional repression of the activating E2F-1 transcription factor is achieved by p107 mediated dimerization of E2F-4 and cooperative binding to the E2F-1 promoter (O'Connor et al., 2001). On the other hand, other studies revealed also activating properties (Lee et al., 2011; Pierce et al., 1998a; Lukas et al., 1996), suggesting versatile roles of E2F-4 in the regulation of the cell cycle . Intriguingly, several studies with pathogens like HAdVs, *Human immunodeficiency virus* (HIV), and *Bovine Herpesvirus-1* were shown to utilize E2F-4 for the activation of their own viral promoters (Geiser & Jones, 2003; Ambrosino et al., 2002; O'Connor et al., 2001)

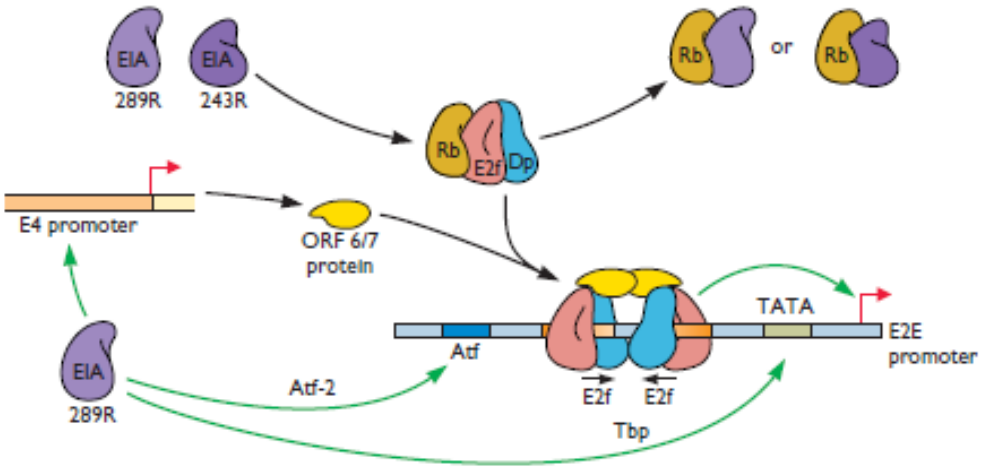


Figure 8: E1A and E4orf6/7 induced activation of the viral E2 early (E2A) promoter by dimerized E2F trasnscription factors. The viral E2A promoter contains two inverted E2F binding sites, allowing the transcriptional activation by the cellular transcription factor family E2F. During the course of infection, E1A is the first viral protein, to be expressed. E1A activates other viral early genes, by releasing E2F from its inhibitor Rb. E4orf6/7 complements the function of E1A by dimerizing active E2F molecules and increasing the affinity to the inverted E2F binding sites within the E2A promoter (adopted from Flint et al., 2009).

E1A is the first viral gene to be transcribed and is the major viral factor for the deregulation of the cell cycle by releasing E2F transcription factors and chromatin remodeling (reviewed in Ben-Israel & Kleinberger, 2002). This activity is complemented, by E4orf6/7, which primarily dimerizes free E2F proteins and increases the affinity to E2F responsive promoters (Schaley et al., 2000; Shapiro et al., 2006; O'Connor & Hearing, 1994). Consequently, E1A and E4orf6/7 orchestrate the switch from the

resting stage to S-phase to induce viral genome replication and to achieve efficient viral progeny production. At the same time, these activities also stimulate programmed cell death, because deregulation of the cell cycle activates the tumor suppressor p53, which triggers a cellular defense mechanism to implement growth inhibition and apoptosis. To avoid programmed cell death, adenoviruses employ the early proteins E1B-55K and E4orf6 to inactivate the p53 pathway, as well as E1B-19K to antagonize apoptotic downstream processes initiated by p53-dependent and p53-independent pathways (Sarnow et al., 1982a; Kao et al., 1990; Yew & Berk, 1992; Renee Yew et al., 1994; Boyd et al., 1994). Even though E1B-55K inactivates the p53 pathway by different means, the E1B-55K induced modification of p53 with *small ubiquitin related modifiers* (SUMO) is in particular of interest. During HAdV-5 infection, cellular and viral proteins are subject to dynamic post-translational modifications (PTM). Since SUMOylation plays a major role in maintaining cell homeostasis, it is not surprising that pathogens utilize the SUMO system to create a milieu that favors virus replication.

2.3 Adenovirus and the host cell SUMOylation

2.3.1 The SUMO system

Ubiquitin and its relatives, the *ubiquitin-like proteins* (Ubl), are conjugated to target proteins for post-translational modification (PTM). The PTM considerably alters the properties of proteins, increasing the complexity of the proteome in eukaryotic cells. Over the years, about a dozen Ubls have been described, among them the closely related *neural precursor cell expressed developmentally down-regulated protein 8* (NEDD8) and the previously mentioned *small ubiquitin related modifier* (SUMO), as illustrated in Figure 9. Especially SUMO has been intensively studied in the last decades, due to its functional flexibility and far reaching functional downstream consequences. In most mammals five isoforms, designated SUMO1 to SUMO5, have been described. The process of SUMOylation is essential in nearly all eukaryotes and has been implicated in the regulation of cellular functions, ranging from transcriptional regulation and chromatin remodeling to DNA repair as well as control of cell cycle progression (reviewed in Flotho & Melchior, 2013; Ulrich & Walden, 2010; Jackson & Durocher, 2013; Eifler & Vertegaal, 2015b,a). The more surprising is the observation that, although SUMO modification extensively regulates cellular pathways, only a low percentage of effector proteins are found to be actually modified (Hay, 2005). Once modified, the consequence of SUMOylation for an individual target protein is difficult to predict. In general, the underlying principle of SUMOylation is to alter inter- and/or intramolecular interactions of the substrate, influencing its stability, localization or activity (Kerscher, 2007; Song et al., 2004).

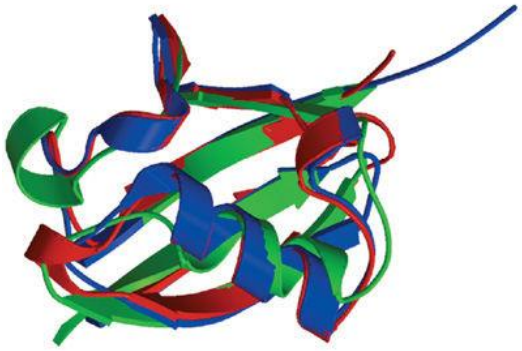


Figure 9: Ubiquitin and ubiquitin-like proteins. Ubiquitin and its relatives are related primarily by the ubiquitin superfold, which is a β-grasp fold. Blue: Ubiquitin; Green: SUMO1; Red: NEDD8. (adopted from Welchman et al., 2005).

In humans, SUMO1, SUMO2 and SUMO3 are the most abundant isoforms. SUMO2 and SUMO3 are virtually identical, except for three N-terminal residues. Therefore, they are often referred as SUMO2/3 (Wang & Dasso, 2009). SUMO2/3 occurs as a free pool in cells that is utilized during stress and shares about 50 % sequence homology with SUMO1, which is, on the other hand, mostly found engaged in conjugates (Saitoh & Hinchey, 2000). The role of the fourth SUMO family member is highly debated and is thought to be a non-expressed pseudogene, although it has been reported to be expressed in kidney cells (Bohren et al., 2004). SUMO5 is the latest identified SUMO molecule and has been revealed to have quite controversial functions on PML-NB. On the one hand, SUMO5 attachment at lysine 160 of PML has been associated with the recruitment of further components and the growth of PML-NBs. On the other hand, it also increases SUMO2/3 polySUMOylation of PML resulting in the disruption of PML-NBs (Liang et al., 2016).

Generally, SUMO and other Ubl molecules are conjugated to target proteins by an enzymatic cascade, illustrated in Figure 10, involving three enzymes: The activation enzyme E1; A conjugation enzyme E2; and typically a SUMO ligase E3. The SUMO system, however, has remained somewhat of an enigma because the pathway relies on a single E2 enzyme, the *ubiquitin-like conjugating enzyme 9* (Ubc9), which is sufficient for the transfer of SUMO to a target protein (Kerscher et al., 2006). E3 SUMO ligases seem to play an optional role, although they have been shown to support and accelerate the conjugation process (Pichler et al., 2002). This is in contrast to the ubiquitin pathway, which uses tens of E2 enzymes in unique combinations with hundreds of E3 enzymes to regulate substrate selection (Hoeller et al., 2007).

SUMO modification is initiated by a family of proteases that catalyze SUMO processing and deconjugation. These *sentrin-specific proteases* (SENP) cleave immature SUMO precursors at their C-terminus and expose a free diglycine residue that is required for efficient adenylation with adenosine monophosphate (AMP) by *SUMO activating* E1 *enzymes* (SAE/SAE2). Adenylated SUMO is the activated form and allows the formation of a thioester bond with the sulphydryl group of the

cysteine residue 173 in SAEs. In the second step, SUMO is transesterified to the SUMO conjugating enzyme Ubc9, which directly recognizes substrate proteins (Desterro et al., 1997; Johnson & Blobel, 1997). Finally, Ubc9 catalyzes the formation of an isopeptide bond between the exposed diglycine residue of SUMO and the ε-amino group of a lysine in the target protein. Global SUMO proteome approaches revealed, that modified lysines are typically found in the context of a SUMO modification consensus motif Ψ-K-x-E (where Ψ denotes a large hydrophobic and x any aa residue), although extended and slightly different conjugation motifs have also been found (Rodriguez et al., 2001; Hendriks & Vertegaal, 2016). Nonetheless, it should be mentioned that there are many SUMO modified lysine residues, where the surrounding sequence does not conform to this consensus, and the reversed case, where proteins contain a consensus motif, but are not modified (Tatham et al., 2001). In addition to the classical SUMO consensus motifs, further elements have been identified in some SUMO substrates. These include *phosphorylation-dependent SUMO motifs* (PDSM) as well as *negatively charged amino acid-dependent SUMO motifs* [(NDSM)(Stehmeier & Muller, 2009; Hietakangas et al., 2003; Yang & Grégoire, 2006)].

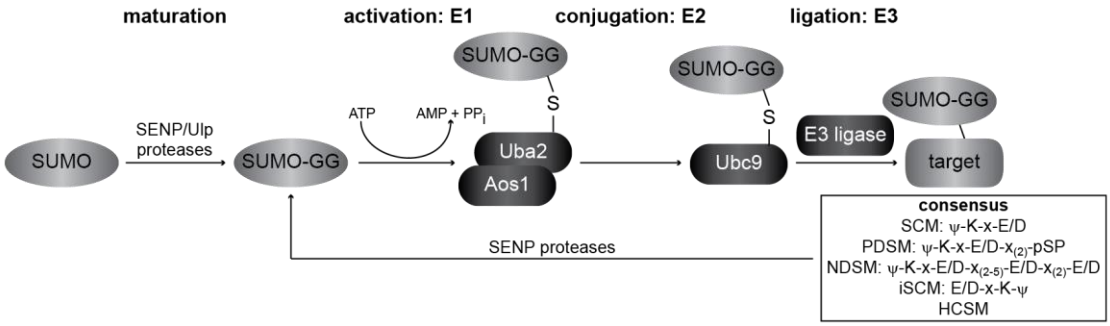


Figure 10: Scheme of the SUMO pathway. All different SUMO isoforms are expressed as immature precursors with a variable C-terminal stretch (2 to 11 aa) after an essential GG motif. After maturation via the sentrin-specific proteases (SENPs), the SUMO protein is activated in an ATP-dependent step by conjugation to the E1 heterodimer (Aos1/Uba2). SUMO is subsequently transferred to the unique E2 enzyme Ubc9, which covalently attaches the modifier to the ε-amino group of a target lysine residue in the presence of an E3 SUMO ligase. So far, four different extensions of the classic consensus SUMO conjugation motif (SCM; ψ-K-x-E/D) have been identified: the phosphorylation-dependent SUMOylation motif (PDSM; ψ-K-x-E/D-xx-pSP), the negatively charged amino-acid-dependent SUMOylation motif [NDSM; ψ-K-x-E-x-E/D-x -E/D] an inverted SUMO conjugation motif (iSCM; E/D-x-K-ψ) , and the hydrophobic cluster SUMOylation motif (HCSM) (adopted from Wimmer et al., 2012).

Even though Ubc9 can directly recognize SUMO motifs, the conjugation rate is very often not as efficient as in the presence of SUMO E3 ligases. SUMO E3 ligases contain *SUMO interaction motifs* (SIM) or *SP-RING domains* that facilitate and accelerate the SUMOylation process in a substrate specific manner (Pichler et al., 2002; Rytinki et al., 2009). However, these domains facilitate

SUMOylation by different mechanisms. For example, the SIM containing E3 ligase Ran-binding protein 2 (RanBP2) does not directly interact with the substrate, but rather, these tandem SIM elements bind the E2-SUMO thioester, stimulating E2 to discharge SUMO to the substrate (Reverter & Lima, 2005). In contrast, SP-RING domain containing E3 SUMO ligases, are thought to function in an analogous manner as ubiquitin RING E3 enzymes that bring the E2-SUMO thioester in close proximity to their substrates to promote SUMO transfer (Rytinki et al., 2009). The family of *protein inhibitor of activated STAT* (PIAS1, PIASxα, PIASxβ, PIAS3 and PIASγ) was initially found as an inhibitor of the family *signal transducer and activator of transcription* (STAT). Nevertheless, work by several research groups has demonstrated that PIAS does not only inhibit STAT, but also contains a SP-RING domain and serves as a SUMO E3 ligase (reviewed in Schmidt & Müller, 2003). The SP-RING domain in PIAS is similar to those in ubiquitin E3 ligases, which are unique Zn ligating modules that appear to participate in the recruitment of E2 proteins into the E3 complex. Thereby, these Zn modules act as adapters to bring substrate and ubiquitin thioester-loaded E2 into close proximity to accelerate SUMO conjugation (reviewed in Melchior et al., 2003).

2.3.2 Adenoviruses exploit the SUMO system

The SUMO system participates in the tight modulation of many key regulatory pathways. Therefore, it is not surprising that various intracellular pathogens have evolved strategies to take advantage of conserved host cell SUMOylation. By utilizing the SUMO system, pathogens modulate cellular pathways to evade host immune response in order to create an optimal environment that favors viral replication. So far, most research has focused on how the SUMO modification system is involved during infections of different DNA viruses, like *Adenoviridae*, *Herpesviridae, Papillomaviridae* and *Poxviridae*. Moreover, RNA viruses as well as extra- and intracellular bacteria have been convincingly associated to the SUMO system (reviewed in Wimmer et al., 2012). In particular the modification and re-organization of PML-NBs, seems to be an important step during viral replication, since these structures are targeted by several viruses to prevent intrinsic defense mechanism (Figure 11). Since many publications showed that the structural integrity and regulation of PML-NB accumulations depend on SUMO modification and especially because SUMO modification and DNA viruses are inevitably linked to PML-NBs, this framework is considered to represent the nuclear SUMOylation hot spot (van Damme & van Ostade, 2011; van Damme et al., 2010; Everett & Chelbi-Alix, 2007; Tavalai & Stamminger, 2008; Everett et al., 1998). In addition, PML contains many SIMs, contributing to the accumulation and aggregation of a complex three-dimensional structure (van Damme et al., 2010). It has been proposed that over 165 known cellular proteins can be dynamically recruited to PML-NBs,

in part depending on their SUMO modification status and/or whether they can interact with SUMO (Lang et al., 2010; Weidtkamp-Peters et al., 2008; Shen et al., 2006).

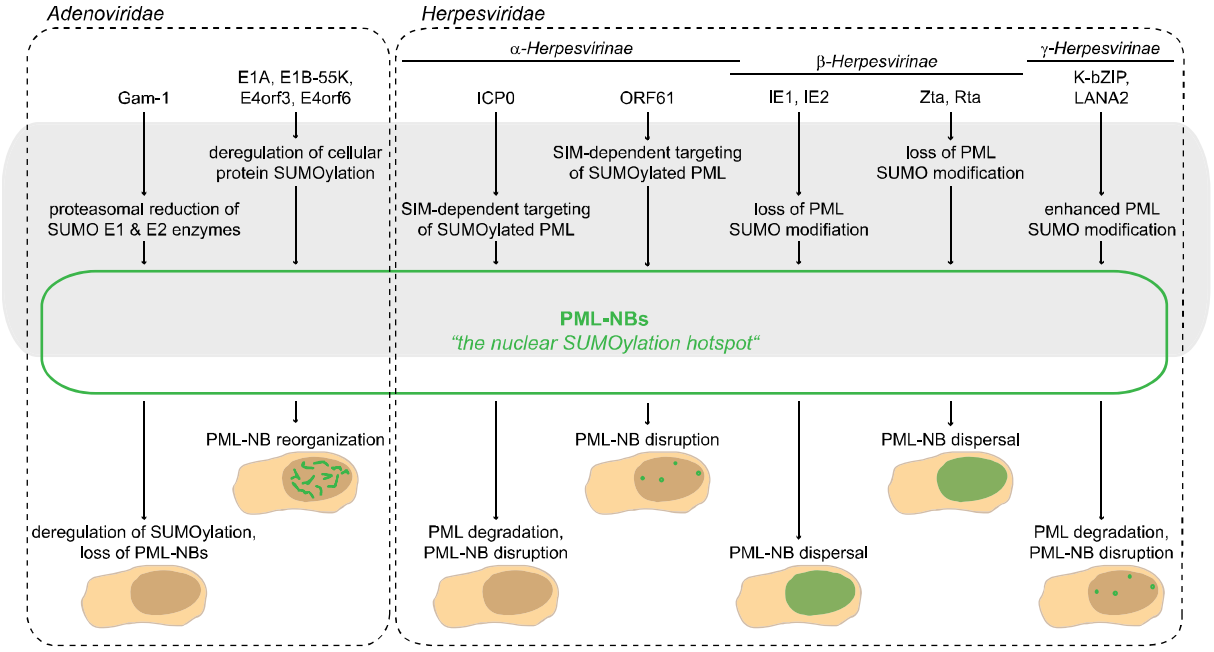


Figure 11: Modulation of the SUMO pathway and PML-NBs by DNA viruses (*Adenoviridae, Herpesviridae*). The different Adeno- and Herpesvirus proteins known to be associated with PML-NBs are shown at the top, functional alteration of the cellular SUMOylation pathway are highlighted (grey) and the resulting alterations in PML-NB morphology are illustrated at the bottom (adopted from Wimmer et al., 2012).

Many studies with HAdV-5 identified a set of early proteins, that interact with enzymes or resemble substrates of the SUMOylation pathway (reviewed in Sohn & Hearing, 2016). Initial experiments showed an interaction of E1A with murine Ubc9, which was subsequently confirmed for the human orthologue (Yousef et al., 2010; Hateboer et al., 1996). Furthermore, a specific amino acid sequence within the CR2 of E1A was identified, which is necessary and sufficient to interact with the N-terminal region of Ubc9. However, this interaction does not alter the global SUMO proteome, nor is it essential for oncogenic transformation of p53-negative mouse embryonic fibroblasts (Yousef et al., 2010). To date, pRB is the only known protein whose SUMOylation status is modulated by HAdV-5 E1A, although the exact function of pRB modification remains to be elusive, since SUMOylation deficient mutants revealed only a slightly enhanced repressive effect on E2F responsive promoters (Ledl et al., 2005). Therefore it remains unclear, whether and to what extent E1A manipulates the host cell SUMO system to mediate transcriptional regulation (Frisch & Mymryk, 2002).

In contrast, E1B-55K is a known SUMO substrate, and contains a classical SCM around lysine 104 that can be conjugated with SUMO1, SUMO2 and SUMO3 (Endter et al., 2005, 2001). Intriguingly, SUMOylation of E1B-55K is known to be necessary for several aspects of viral protein functions, such as functional inactivation of p53 and the proteasomal degradation of the chromatin remodeling

factor DAXX, two major events involved in the transformation process of primary cells (Schreiner et al., 2011, 2010, Endter et al., 2005, 2001). In contrast to the degradation of p53, which is targeted directly be the cullin-dependent E1B-55K/E4orf6 ubiquitin ligase complex, DAXX is targeted by so called *SUMO-targeted ubiquitin ligases* (STUbLs). So far, two human STUbLs, RNF4 and RNF111, have been identified, which are utilized by HAdV-5 to degrade cellular proteins. STUbLs contain several SIMs that enable the adherence to proteins with polySUMO chains to target them for ubiquitin-dependent proteasomal degradation. Another consequence of E1B-55K SUMOylation, is the differential subnuclear localization and the interaction with certain PML isoforms during HAdV-5 infection (Wimmer et al., 2010; Kindsmüller et al., 2007). The most striking observation however, which inspired the project for this doctoral thesis, was that HAdV-5 E1B-55K itself acts as a SUMO ligase, inducing the SUMOylation of p53. Thereby, E1B-55K promotes the inactivation of the tumor suppressor protein via spatial restriction to PML-NBs (Pennella et al., 2010).

In summary, these findings indicate that HAdVs are intimately connected to the SUMOylation system, although the functional consequences for individual regulatory proteins as well as the viruses themselves are diverse and far from being understood. In order to broaden our knowledge on how HAdV-5 manipulates the SUMO proteome, a *stable isotope labelling with amino acid in cell culture* (SILAC) experiment was performed by a former co-worker. This simple and straightforward MS-based approach relies on the incorporation of “heavy” and “light” amino acids into newly synthesized proteins and allows the monitoring of protein abundances under different conditions. In different experimental set ups, we aimed to investigate the SUMO proteome of infected cells to address two questions: 1. Which proteins are differently SUMOylated upon infection and 2. Are there more cellular or viral proteins which are E1B-55K dependently SUMOylated? The SILAC experiment identified 78 of SUMOylated cellular proteins whose abundance was increased by a factor of two or more during wild type infection. In addition three viral proteins were found to be exclusively SUMOylated during wild type infection and one of these was the viral early protein orf6/7 (E4orf6/7). Based on the results of to the SILAC experiment, E4orf6/7 might represent an adenoviral target of the E1B-55K SUMO ligase, since there was no SUMO modification observed in cells infected with the HAdV-5 ΔE1B virus mutant. Therefore, this work aimed to confirm and investigate the effect of SUMOylation by E1B-55K on E4orf6/7.

3 Materials

3.1 Bacteria, cells and viruses

3.1.1 Bacterial strains

Strain	Genotype
DH5α	*sup*E44, Δ*lac*U169, (80d *lac*ZΔ *M15*), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1 (Hanahan & Meselson, 1983)

3.1.2 Mammalian cell lines

Strain	Genotype
A549	Human lung carcinoma cell line expressing wild type p53 (Giard et al., 1973)
H1299	Human lung carcinoma cell line, p53 negative (Mitsudomi et al., 1992)
HEK 293	Established HAdV-5-transformed human embryonic kidney cell line stably expressing adenoviral E1A and E1B gene products (Graham et al., 1977)
HeLa	Human cervix carcinoma cell line (Gey et al., 1952)
HeLa SU2	HeLa cell line stably expressing His-tagged SUMO2 (Tatham et al., 2009)

3.1.3 Viruses

#	Adenovirus	Characteristics
100	H5*pg*4100 (HAdV-5 WT)	Wild type human Mastadenovirus type 5 (HAdV-5) carrying an 1863 bp deletion (nt 28062- 30465) in the E3 reading frame (Kindsmüller et al., 2007)
149	H4*pm*4149 (HAdV-5 E1B$^{\Delta}$)	HAdV-5 E1B-55K null mutant carrying four stop codons within the E1B-55K gene reading frame (Kindsmüller et al., 2009)
150	H5*pm*4150 (HAdV-5 E4orf3$^{\Delta}$)	HAdV-5 E4orf3 mutant with an additional thymidine at position nt 34592, causing a frame shift mutation after codon 36 (Forrester et al., 2012)
154	H5*pm*4154 (HAdV-5 E4orf6$^{\Delta}$)	HAdV-5 E4orf6 null mutant carrying a point mutation within the E4orf6 gene that introduces a stop codon and leads to the stop of translation after P66 (Blanchette et al., 2008)

268	Ad5 p15A-cm E4orf6_7 K68R (HAdV-5 K68R)	HAdV-5 mutant bearing a mutation in the gene of E4orf6/7, causing an aa exchange (K68R) within the SCM of E4orf6/7

3.2 Nucleic acids

3.2.1 Oligonucleotides

#	Name	5' to 3' Sequence	Purpose
366	cmv	CCCACTGCTTACTGGC	Sequencing
636	pcDNA3-rev	GGCACCTTCCAGGGTCAAG	Sequencing
640	E1B55K-PCR-fw	GGAGCGAAGAAACCCATCTGAGCGGGGGGTACC	qPCR
641	E1B55K-PCR-rev	GCCAAGCACCCCCGGCCACATATTTATCATGC	qPCR
1359	pSG5 Seq. Fw	CCTACAGCTCCTGGGCAACG	Sequencing
1421	GAPDH-RT-fwd	ACCACAGTCCATGCCATCAC	qPCR
1422	GAPDH-RT-rev	TCCACCACCCTGTTGCTGTA	qPCR
1688	DBP-RT-fwd	GGTCTG GGCGTTAGGATACA	pPCR
1689	DBP-RT-rev	CAATCAGTTTTCCGGCAAGT	qPCR
3013	Orf6-7 fwd BamHI	ATAGGATCCATGACTACGTCCGGCGTTCC	Cloning
3014	Orf6-7 rev EcoRI	ATATAGAATTCTCACAGAACCCTAGTATTCAACC	Cloning
3063	Orf6-7 K68R fwd	CCTTCGCCGCCCGTTAGGCAACCGCAAGTTGGACAGC	Mutagenesis
3064	Orf6-7 K68R fwd	GCTGTCCAACTTGCGGTTGCCTAACGGGCGGCGAAGG	Mutagenesis
3065	Orf6-7 K114R fwd	GGAATATAACACCTAGGAATATGTCTGTTACCC	Mutagenesis
3066	Orf6-7 K114R rev	GGGTAACAGACATATTCCTAGGTGTTATATTCC	Mutagenesis
3129	Orf6-7 K126R fwd	GATGCTTTTTAGGGCCAGCCGGGGAG	Mutagenesis
3130	Orf6-7 K126R rev	CTCCCCGGCTGGCCCTAAAAAGCATC	Mutagenesis
3411	RT FAM111B fwd	GCCCTTGAAATGCAGAATCCA	qPCR
3412	RT FAM111B rev	GCTGTAAACACACTACGGTCTAA	qPCR
3413	RT E2F1 fwd	CATCCCAGGAGGTCACTTCTG	qPCR

3414	RT E2F1 rev	GACAACAGCGGTTCTTGCTC	qPCR
3415	RT E2F4 fwd	ATCGGGCTAATCGAGAAAAAGTC	qPCR
3416	RT E2F4 rev	TGCTGGTCTAGTTCTTGCTCC	qPCR
3417	RT p73 fwd	TCGGCAGACTCGCTCTTCA	qPCR
3418	RT p73 rev	GAGCCCTCGTCTATTTTGCAG	qPCR

3.2.2 Vectors

#	Name	Purpose	Reference
32	pCMV	Expression vector for mammalian cells with a CMV promoter	Invitrogen
77	pGL2	Firefly luciferase reporter plasmid	Promega
138	pGL3	Firefly luciferase reporter plasmid	Promega
180	pRL-TK	Renilla luciferase reporter plasmid for the normalization of promoter activity	Promega
261	pLight switch	Renilla luciferase reporter plasmid	Switchgear Genomics

3.2.3 Recombinant plasmids

#	Name	Properties	Reference
1319	pcDNA3-E1B-55K	Encodes HAdV-5 E1B-55K	Group database
1667	72K DBP	HAdV5 E2A	Group database
2420	pGL3-Basic Prom E1A	HAdV-5 E1A promoter reporter gene construct	Group database
2421	pGL3-Basic Prom E1B	HAdV-5 E1B promoter reporter gene construct	Group database
2422	pGL3-Basic Prom pIX	HAdV-5 pIX promoter reporter gene construct	Group database
2423	pGL3-Basic	HAdV-5 E2A early promoter reporter gene construct	Group

	Prom E2E		database
2424	pGL3-Basic Prom MLP	HAdV-5 MLP promoter reporter gene construct	Group database
2425	pGL3-Basic Prom E3	HAdV-5 E3 promoter reporter gene construct	Group database
2428	pGL3-Basic Prom E2L	HAdV-5 E2A late promoter reporter gene construct	Group database
2476	pcDNA3-HA-E1A genomic	pcDNA expression plasmid encoding the whole E1A region	Group database
2889	pGL3-Basic Prom E4	HAdV-5 E4 promoter reporter gene construct	Group database
2946	E4orf6/7 Ad5	Encodes HAdV-5 E4orf6/7	Group database
2959	FAM111B promoter	Plight switch renilla luciferase reporter gene construct containing the cellular FAM111B	Addgene (ID: S707175)
2977	pFirefly-TK	pGL3-Basic firefly luciferase reporter plasmid for the normalization of promoter activities.	Group database
3019	Ubc9	pcDNA3 plasmid encoding human E2 conjugating enzyme Ubc9 connected to an SV5 tag	Kindly provided by R. Hay
3125	Ad5 orf6/7 pCMV K126R	Encodes HAdV-5 E4orf6/7 in which lysine 126 has been substituted by an arginine to delete SCM	This work
3142	Ad5 orf6/7 pCMV K68/126R	Encodes HAdV-5 E4orf6/7 in which lysines 68 and 126 have been substituted by an arginine to delete SCM	This work
3143	Ad5 orf6/7 pCMV K114/126R	Encodes HAdV-5 E4orf6/7 in which lysines 114 and 126 have been substituted by an arginine to delete SCM	This work
3144	Ad5 orf6/7 pCMV K68/114/126R	Encodes HAdV-5 E4orf6/7 in which lysines 68, 114 and 126 have been substituted by an arginine to delete SCM	This work
3167	Ad5 orf6/7 pCMV K68R	Encodes HAdV-5 E4orf6/7 in which lysine 68 has been substituted by an arginine to delete SCM	This work
3168	Ad5 orf6/7 pCMV K114R	Encodes HAdV-5 E4orf6/7 in which lysine 114 has been substituted by an arginine to delete SCM	This work
3169	Ad5 orf6/7 pCMV	Encodes HAdV-5 E4orf6/7 in which lysine 68 and114	This work

	K68/114R	have been substituted by an arginine to delete SCM	
3337	pcDNA3 E2F-1	pcDNA expression plasmid encoding E2F-1	
3416	pGL2-AN E2F-1 promoter	pGL2 luciferase gene construct containing the cellular E2F-1 promoter controlling a firefly luciferase gene	Addgene (ID: 20950)
3418	E2F-4-eGFP plasmid	pcDNA overexpressing plasmid encoding for cellular E2F-4 coupled to eGFP.	(Schaley et al., 2005)

3.3 Antibodies

3.3.1 Primary antibodies

#	Product	Properties	Source
1	2A6	Monoclonal mouse AB; detects N-terminus of HAdV-5 E1B-55K Ref (Sarnow et al., 1982b)	Group database
10	6A11	Monoclonal rat AB; detects HAdV-5 E4orf3 (Nevels et al., 1999)	Group database
57	Anti-Ubc9 mAb	Monoclonal mouse IgG2a AB; detects the SUMO ligase Ubc9 (cat. no. 610748)	BD Biosciences
88	β-actin (AC-15)	Monoclonal mouse AB, detects β-actin	Sigma Aldrich
94	RSA3	Monoclonal mouse AB; detects N-terminus of HAdV-5 E4orf6 and E4orf6/7 (Marton et al., 1990)	Group database
113	B6-8	Monoclonal mouse AB; detects HAdV-5 E2A (Reich et al., 1983)	Group database
275	6B10	Monoclonal rat AB; detects HAdV-5 L4-100K (Kzhyshkowska et al., 2004)	Group database
480	Anti-FAM111B (Human)	Purified polyclonal rabbit igG AB(cat. no. HPA038637)	Atlas Antibodies
551	6His	Monoclonal mouse AB; detects 6xHis-tag	Clontech
568	610	Polyclonal rabbit AB; detects HAdV-5 E1A	R. Grand
N/A	E2F-1 (KH95)	Monoclonal IgG_{2a} kappa light AB; detects transcription factor E2F-1	Santa Cruz (sc-251)
N/A	E2F-4 (D-7)	Monoclonal IgG_1 kappa light chain AB; detects the transcription factor E2F-4	Santa Cruz (sc-398543)

3.3.2 Secondary antibodies

3.3.2.1 Conjugated antibodies for western blotting

Product	Properties	Company
HRP-Anti-Mouse IgG	HRP (horseradish peroxidase)-coupled; raised in sheep	Jackson
HRP-Anti-Rabbit IgG	HRP (horseradish peroxidase)-coupled; raised in sheep	Jackson
HRP-Anti-Rat IgG	HRP (horseradish peroxidase)-coupled; raised in sheep	Jackson
HRP-Anti-Mouse IgG	HRP (horseradish peroxidase)-coupled; raised in sheep, light chain specific)	Jackson

3.3.2.2 Conjugated antibodies for immunofluorescence antibodies

Product	Properties	Company
Cy3-Anti Mouse IgG	Affinity purified, Cy3 coupled; raised in donkey (H + L)	Dianova
Cy3-AntiRabbit IgG	Affinity purified, Cy-3 coupled; raised in donkey (H + L)	Dianova
Cy3-Anti-Rat IgG	Affinity purified, Cy3 coupled; raised in donkey (H + L)	Dianova
Alexa 488 Anti-Mouse IgG	Alexa 488 antibody raised in goat (H + L) $F(ab')_2$ Fragment	Invitrogen
Alexa 488 Anti-Rabbit IgG	Alexa 488 Antibody raised in Goat (H + L) $F(ab')_2$ Fragment	Invitrogen

3.4 Standard markers

Product	company
1 kb/ 100 bp DNA ladder	New England Biolabs
PageRuler Plus Prestained Protein Ladder	Thermo Scientific

3.5 Commercial systems

Product	Company
Dual-Luciferase Reporter Assay System	Promega
Plasmid Purification Mini, Midi and Maxi Kit	Qiagen
Protein Assay	BioRad
QuikChange Site-Directed Mutagenesis Kit	Stratagene
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific

3.6 Chemicals, enzymes, reagents, equipment

Chemicals, enzymes and reagents used in this study were obtained from Agilent, Applichem, Biomol, Merck, New England Biolabs, Roche and Sigma Aldrich. Cell culture materials, general plastic material as well as equipment were supplied by BioRad, Biozym, Brand, Engelbrecht, Eppendorf GmbH, Falcon, Gibco BRL, Greiner, Hartenstein, Hellma, Nunc, PAN-Biotech, Sarstedt, Protean, Schleicher & Schuell, VWR and Whatman.

3.7 Software and databases

Software	Purpose	Source
Acrobat 9 Pro	PDF data processing	Adobe
CLC Main Workbench 5.0	Sequence data processing	CLC bio
Filemaker Pro 11	Database management	Thomson
Illustrator CS6	Layout processing	Adobe
Mendeley Desktop	Reference management	Mendeley
Photoshop CS6	Image processing	Adobe
Pubmed	Literature database, open sequence analysis software	Open software provided by NCBI
Office 2016	Text processing	Microsoft
GraphPad Prism	Data Processing and biostatistics	Statcon

4 Methods

4.1 Bacteria

4.1.1 Culture and storage

E. coli was propagated in sterile LB medium containing 100 µg/ml ampicillin and/ or 25 µg/ml kanamycin. The medium was inoculated with a single bacteria colony and incubated over night at 30 °C/37 °C and 210 rpm in an *Inova 4000 Incubator* (New Brunswick). If necessary, the concentration of bacteria was determined, measuring the optical density (OD) at 600 nm with a *SmartSpec™ Plus* (BioRad) photo spectrometer.

For the preparation of glycerol stocks, liquid cultures were pelleted for 5 min at RT and 800 *g*. Subsequently the supernatant was aspirated and the remaining pellet resuspended in 1 ml 50 % (v/v) Glycerol/LB medium. Finally, the solution was transferred into *CryoTubes* (Sarstedt) and stocked at -80 °C for long term storage.

For obtaining single colonies, bacterial solutions were plated on 100 mm agar plates containing 15 g/l agar LB medium supplemented with 100 µg/ml ampicillin and/or 25 µg/ml kanamycin. After incubation over night at 30 °C/37 °C, single colonies were picked and further propagated, as described above. The solid plate cultures were stored up to 2 weeks at 4 °C and sealed with Parafilm (*Pechiney Plastic Packaging*).

LB Medium	10 g/l	Tryptone
	5 g/l	Yeast extract
	5 g/l	NaCl
		(autoclaved)

Antibiotic solution	100 mg/ml	Ampicillin (Biochemica)
	25 mg/ml	Kanamycin (Applichem)

4.1.2 Chemical transformation of *E. coli*

For the transformation of *E. coli*, 100 µl chemical competent DH5α and 10-200 ng plasmid DNA were mixed in a round bottom tube (Falcon) and incubated on ice for 30 min. In the following step, bacteria were heat shocked for 45 s at 42 °C in a water bath and afterwards chilled on ice for further 2 min. After the addition of 1 ml LB medium, the bacteria were propagated for 1 h at 37 °C and 210 rpm in an *Inova 4000 Incubator* (New Brunswick). In order to obtain single colonies, the bacteria

were pelleted for 3 min at RT and 4000 *g*, resuspended in 100 µl LB medium and plated on LB agar plates as previously described (4.1.1).

4.2 Mammalian cells

4.2.1 Maintenance and passaging of cell cultures

Cells were grown on polystyrene cell culture dishes (12-well/6-well/100 mm/150 mm tissue culture dishes; Sarstedt/Falcon) in *Dulbecco's Modified Eagle Medium* (DMEM; Gibco). In addition, culture media contained 10 % FCS (PAN-Biotech) and 1 % penicillin/streptomycin solution [1000 U/ml penicillin & 10 mg/ml streptomycin in 0.9 % NaCl; (PAN-Biotech)]. Cells were incubated at 37 °C in Hera safe 6220 (Heraeus) incubators with 5 % CO_2 atmosphere and monolayers were grown until a confluency of 90 % was reached. To split confluent cells, the medium was aspirated, the cells were washed with sterile PBS and detached for about 5 min with trypsin/EDTA (PAN-Biotech) at 37 °C. Trypsin activity was stopped by the addition of DMEM containing FCS and antibiotics ($DMEM^{+FCS+P/S}$). Subsequently, the cell suspension was pelleted (3 min; RT; 800 *g*) in a 50 ml centrifuge tube (Sarstedt). Depending on the experimental conditions, cells were resuspended in $DMEM^{+FCS+P/S}$, and either split in an appropriate ratio (1:2-1:32) or counted with a Neubauer improved hemocytometer (4.2.3) and seeded on cell culture plates at defined cell numbers.

PBS

140 mM	NaCl
3 mM	KCl
4 mM	Na_2HPO_4
1.5 mM	KH_2PO_4
	(pH 7.0-7.7; autoclaved)

4.2.2 Storage and re-cultivation

For long term storage, cells were detached, washed and pelleted as previously described (4.2.1). Subsequently, the cells of two cell culture dishes were resuspended in 1 ml FBS containing 10 % DMSO (Sigma). Afterwards the cell suspension was aliquoted into *CryoTubes* (Sarstedt) and slowly frozen to -80 °C. For long term storage cells were stored at -80 °C or in liquid nitrogen for long term conservation. In case of re-cultivation, the frozen cell suspension was rapidly thawed at 37 °C and transferred into 10 ml fresh $DMEM^{+FCS+P/S}$ to minimize DMSO toxicity. After pelleting (3 min; RT; 800 *g*) and resuspension in 5 ml $DMEM^{+FCS+P/S}$, cells were seeded on appropriate culture dishes and incubated at standard conditions.

4.2.3 Determination of cell number

The concentration of a cell suspension was determined using a *Neubauer improved hemocytometer* (Marienfeld). Cells were detached as previously described (4.2.1). To distinguish dead and viable cells, the suspension was diluted 1:2 with *Trypan blue* solution. Afterwards 10 µl were pipetted onto the hemocytometer and the counted number of viable cells in four big squares was inserted into the formula below:

$$Cell\ concentration\ \left[\frac{cells}{ml}\right] = \frac{number\ of\ viable\ cells\ x\ dilution\ factor\ x\ 10^5}{4}$$

Trypan blue solution	0.15 %	Trypan Blue
	0.85 %	NaCl

4.2.4 Transfection of mammalian cells

4.2.4.1 Polyethylenimine transfection

The introduction of plasmid DNA into mammalian cells was performed via chemical transfection with *polyethylenimine* (PEI; Polysciences). This 25 kDa polymer was dissolved in ddH_2O at a concentration of 1 mg/ml and neutralized with 0.1 M HCl to a pH of 7.2. Afterwards the solution was sterile filtered (0.2 µm pore size), aliquoted and stored at -80 °C.

Depending on the experimental set up, appropriate amounts of cells was seeded on cell culture dishes 24 h before transfection. For the initial binding of DNA to PEI, a transfection solution was prepared, containing pre-warmed DMEM without FCS and antibiotics ($DMEM^0$), PEI and DNA in the ratio of 1:10:100. The transfection mixture was incubated for 20 min at RT. Before the transfection mix was added to the cells, the old medium was substituted by $DMEM^0$. After 4 h of incubation at 37 °C, the transfection mixture was replaced by fresh $DMEM^{+FCS+P/S}$ and further incubated for 24-48 h, before cell harvest.

4.2.4.2 Calcium-phosphate transfection

Calcium phosphate transfection was performed using the *ProFection Mammalian transfection System* (Promega). 500 µl 2x HBS was provided in a 15 ml tube (Sarstedt) and the transfection mixture was prepared in a 1.5 ml reaction tube (Eppendorf) by mixing the respective plasmids with ddH_2O in a total volume of 437.5 µl. After the addition of 26.5 µl $CaCl_2$ to the diluted plasmids, the

transfection mixture was added dropwise into the 15 ml tube, while blowing air into the solution using a Pasteur pipette and afterwards incubated for 30 min at RT. Meanwhile pre-warmed $DMEM^{0}$ was added to the cells which were about to be transfected. The transfection mixture was gently mixed and added dropwise to the cells. Transfection took place for 6-8 h, before the fresh and pre-warmed $DMEM^{+FCS+S/P}$ was added.

4.2.5 Harvesting of mammalian cells

For harvesting, cells were scraped off the cell culture dishes and transferred into 15 ml centrifuge tubes (Sarstedt). In the next step, the cells were pelleted (3 min; RT; 800 *g*), the supernatant discarded and washed in 1 ml PBS. In a final step, the cell suspension was transferred into a 1.5 ml reaction tube (Eppendorf) to pellet (3 min; RT; 800 *g*) the cells. After discarding the supernatant, cell pellets were stored at -20 °C.

4.2.6 Transformation assay of primary baby rat kidney cells

To determine the transforming potential of individual proteins, fresh pBRK cells were obtained and $3x10^{6}$ were seeded on 100 mm cell culture dishes, 24 h before transfection. Cells were grown in $DMEM^{+FCS+S/P}$ under standard conditions (4.2.1) and transfected via calcium phosphate transfection (4.2.4.2). As a negative control the carrier plasmid DNA was transfected and as a positive control the viral oncogenes E1A as well as E1A together with E1B (E1-box). Two days after transfection, cells were washed with PBS, detached with trypsin/EDTA (PAN-Biotech) and equally distributed onto three 100 m cell culture dishes. Afterwards cells were grown for 4-8 weeks, while the medium was renewed twice a week. When colony formation of cells growing in multilayers was visible, the cell culture medium was removed and the cells were fixed and stained with crystal violet solution containing methanol.

Crystal violet solution	1 %	Crystal violet (w/v)
	25 %	Methanol

4.3 Adenovirus

4.3.1 Infection with adenovirus

24 h prior to infection $4x10^{6}$ or $8x10^{6}$ cells were seeded onto 100 mm or 150 mm cell culture dishes (4.2.1 and 4.2.3). Before infection, the old $DMEM^{+FCS+P/S}$ medium was aspirated, cells were washed

with PBS and pre-warmed DMEM^{0} medium was added. Infection took place for 2 h at 37 °C and a multiplicity of infection (MOI) of 20 or 50 FFU/cell. After 2 h, the infectious medium was aspirated and pre-warmed DMEM$^{+FCS+P/S}$ was added and cells have been further incubated at 37 °C. Cells were harvested at different time points depending on the experimental set up.

$$Volume\ of\ virus\ stock\ solution\ (\mu l) = \frac{Multiplicity\ of\ infection\ x\ Total\ cell\ number}{Virus\ titer\ (focus\ forming\ units\ per\ \mu l)}$$

4.3.2 Propagation and storage of high-titer virus stocks

To establish high titer virus stocks, four to six 150 mm cell culture dishes with 8x10^{6} cells were infected with an MOI of 50 FFU/cell as described (4.3.1). After 3-5 days incubation, when all cells were dead and detached, they were harvested and transferred into a 50 ml centrifuge tube (Sarstedt). After pelleting (3 min; RT; 800 *g*) and washing with PBS, cells were again pelleted (3 min; RT; 800 *g*) and resuspended in 1000 µl DMEM^{0} per infected dish. To release the progeny viruses, the cell membrane was disrupted by three cycles of freezing & thawing. The cell debris was removed by centrifugation (20 min; RT; 3500 *g*) and the supernatant was transferred into a fresh 50 ml reaction tube (Eppendorf). The newly generated virus stock was aliquoted into *CryoTubes* (Sarstedt) and stored at 4 °C for direct use or frozen at -80 °C for long term storage.

4.4 DNA Techniques

4.4.1 Preparation of plasmid DNA from *E. coli*

To obtain high amounts of purified plasmid DNA, the plasmids were amplified in *E. coli* and isolated with the *QIAGEN plasmid Maxi Kit* (QIAGEN) according to the manufacturer's manual. A 5 ml pre-culture was inoculated with the respective plasmid transformed *E. coli* clone and propagated for 6 h. Approximately 100 µl of the pre-culture was transferred to 300-500 ml of sterile LB medium supplemented with 100 µg/ml ampicillin and/or 25 µg/ml kanamycin for selection. After overnight cultivation (16-20 h), the bacteria were pelleted (10 min; 4°C; 6300 *g*) and plasmids were isolated and purified following the principle of alkaline lysis: In three consecutive steps, the cells were resuspended in resuspension buffer, lysed in NaOH-solution, neutralized in sodium acetate solution and the released plasmid DNA was purified using an anion exchange column 500. After elution, the DNA was precipitated with 100 % isopropanol (v/v) and washed in 75% ethanol (v/v). The DNA precipitate was finally dissolved in 300-500 ml 10 mM Tris/HCl pH 8.0 buffer.

4.4.2 Quantitative determination of nucleic acid concentrations

The concentration of DNA/RNA was measured with a *NanoDrop* ND1000 (PEQLAB) spectrophotometer at a wavelength of 260 nm. The quality of DNA/RNA was assessed by the ratio of OD_{260}/OD_{280}, which was 1.8 (DNA) and 2.0 (RNA) for highly purified nucleic acids.

4.4.3 Agarose gel electrophoresis

Agarose gels were prepared either as analytical or preparative gels. Analytical gels (Gemaxxon Bioscience) served for qualitative analysis such as degradation or banding pattern of restricted nucleic acids. Preparative gels (SeaKem) in turn, were used for DNA purification of restriction enzyme digested DNA for cloning. Preparative gels additionally contained 1 mM guanosine to minimize UV radiation damage. Both were dissolved in 1x TBE buffer to a final concentration of 0.6-1.2 % (w/v) and melted in a microwave (Moulinex). For the detection of DNA, ethidium bromide (C. Roth) was added to a final concentration of 0.5 µg/ml. After pouring the gel in a suitable gel chamber, samples were mixed with 6x *loading dye* (NEB) and transferred into pre-formed wells. The DNA fragments were separated by continuous current using a voltage of 5-10 V/cm. The results were documented with a G-BOX *transilluminator system* (SynGene) using UV light at 312 nm. DNA from preparative gels was isolated with the QIAquick Gel Extraction Kit [(Qiagen)(4.4.4)].

5x TBE	450 mM	Tris
	450 mM	Boric Acid
	10 mM	EDTA
		(pH 7.8)

6x loading buffer	10 mM	EDTA
	50 % (v/v)	Glycerol
	0.25 % (w/v)	Bromphenol blue
	0.25 % (w/v)	Xylen Cyanol

4.4.4 QIAquick Gel Extraction Kit

After separation of DNA fragments on a preparative agarose gel (4.4.3), the pattern was evaluated on a UV transilluminator (Syngene). The respective fragments were excised with a clean scalpel and transferred into a 1.5 ml reaction tube (Eppendorf). Afterwards the nucleic acids were isolated with the *QIAquick Gel Extraction Kit* (Qiagen), according the manufacturer's instructions. After the gel was

dissolved and the DNA was bound to the column, samples were eluted in 20-50 µl ddH_2O for further processing.

4.4.5 Polymerase chain reaction (PCR)

A standard PCR was performed in a 0.2 ml PCR reaction tube (Sarstedt) and a total volume of 50 µl. The reaction mixture contained 25 ng DNA template, 125 ng of forward and reverse primer, 1 µl dNTP mixture [(dATP, dTTP, dCTP, dGTP; each 1 mM)(NEB)], 5 µl 10x PCR reaction buffer (Thermo Scientific) and 5 U DreamTaq DNA Polymerase (Thermo Scientific). The standard PCR program is shown in the table below. The success of a PCR was checked by loading 5 µl the sample on an analytical gel (4.4.3).

DNA denaturation	1 min	95 °C
Primer annealing	45 sec	55-70 °C
Extension	1 min/kb	72 °C (25-30 cycles)
Final extension	10 min	72 °C
Storage	∞	4 °C

4.4.6 Site-directed mutagenesis

Nucleotide exchanges were introduced using the *In-vitro QuikChange Site-Directed Mutagenesis* Kit (Agilent) according to the manufacturer's instructions. The respective mutagenesis primers were ordered at Metabion (Munich). Depending on the introduced changes and the lengths of the fragment, the standard mutagenesis PCR program in the table below, was adjusted. Original DNA strands, which did not contain the desired mutation, were removed with the restriction enzyme *DpnI* (NEB). This enzyme specifically targets methylated DNA and degrades therefore exclusively the template which was post replicationally modified by *E. coli* during propagation. Therefore, samples were incubated with 1 µl of the *DpnI* for 1 h at 37 °C. To check the success of a site-directed mutagenesis, 10 µl of the digested mixture was analyzed on an agarose gel (4.4.3). Successfully modified plasmids were transformed into chemical competent DH5α (4.1.2) and single clones were picked and propagated (4.1.1). As a final quality check, the newly isolated DNA (4.4.1) was checked on an analytical agarose gel (4.4.3) and sequenced at SeqLab (4.5.3).

DNA denaturation	1 min	95 °C
Primer annealing	45 sec	55- 70 °C
Extension	45 min/kb	68 °C (12- 16 cycles)
Final extension	10 min	72 °C
Storage	∞	4 °C

4.4.7 Isolation of DNA for the determination of viral genome replication

For the determination of viral genome replication, DNA was isolated from whole cell lysates (4.7.1). 50-100 µg protein lysate was added to a total volume of 20 µl ddH_2O. Afterwards a mix was prepared for each sample containing 50 µl 1 % Tween, 10 µl proteinase K (1 mg/ml) and 20 µl ddH_2O. After the addition to the whole cell lysates, the mixture was incubated for 60 min at 55 °C and 10 min at 100 °C. Finally, the samples were precipitated to remove residual proteins and to concentrate the DNA. Samples were mixed with 0.1 vol 3 M sodium acetate (pH = 5.2) and 1 vol 100 % isopropanol (v/v) and the DNA precipitate was pelleted (15 min; RT; 20000 *g*). Subsequently, they were washed with 400 µl 75 % ethanol (v/v), before they were pelleted (15 min; RT; 20000 *g*) and resuspended in 20 µl 10 mM Tris.

4.5 Cloning of DNA fragments

4.5.1 Enzymatic DNA restriction

Analytical restriction digestions were carried out in a 20 µl reaction mixture, containing 1 µg DNA, 0.1 vol 10x restriction buffer and 3-10 U restriction enzyme. After 2 h of incubation at 37 °C, the enzyme was heat inactivated for 20 min at 65 °C. 5 µl of the digested sample was analyzed on an agarose gel (4.4.3). In contrast, preparative restriction digests were performed for at least 3 h at 37 °C in a total volume of 50 µl, containing 10 µg DNA, 0.1 vol 10x restriction buffer and 10 U restriction enzyme. In case of a double digest with two restriction enzymes, the total amount of enzyme never exceeded 0.1 vol of the total reaction volume to avoid glycerol induced star activity. In order to isolate the fragment of interest, the mixture was separated on a preparative agarose gel (4.4.3) and the DNA was purified using the *QIAquik Gel Extraction Kit* [(QIAGEN)(4.4.4)].

4.5.2 Ligation

Before ligation, the respective DNA fragments or PCR products were enzymatically restricted and purified (4.5.1). For ligation, the *Rapid DNA Ligation Kit* (Roche) was used according the

manufacturer's instructions. The fragment was mixed in a ratio of 1:5 with 20-100 ng vector DNA in a total volume of 20 µl containing 2x ligation buffer and 1 U T4 DNA ligase. After incubation for 5 min at RT °C the newly ligated fragments were ready for transformation (4.1.2) and propagation in *E. coli* (4.1.1). Finally, the newly generated and amplified plasmids were confirmed via sequencing (4.5.3)

4.5.3 DNA sequencing

DNA samples were sent for commercial sequencing (Seqlab, Göttingen). The sequencing mixture contained 0.5-1.5 µg DNA and 30 pmol primer in ddH_2O in a total volume of 12 µl.

4.6 RNA techniques

4.6.1 Preparation of total RNA from mammalian cells

RNA was isolated using the *TRI Reagent* Kit (Sigma-Aldrich) according manufacturer's instructions. Briefly, cells were harvested (4.2.5), pelleted (3 min; RT; 800 *g*) and lysed in 1000 µl *TRI Reagent* as well as 0.1 vol chloroform (Sigma-Aldrich). Next, cell debris were pelleted (15 min; 4 °C; 1200 *g*) and the supernatant was transferred to a fresh 1.5 ml reaction tube (Eppendorf) for precipitation with 600 µl ice cold 100 % isopropanol (v/v). After another centrifugation step (15 min; 4 °C; 12000 *g*) the RNA precipitate was washed with 1 ml 75 % ethanol (v/v). Finally, the RNA was pelleted (5 min; 4 °C; 12000 *g*), air dried and re-hydrated in 20 µl DEPC H_2O. RNA concentration was determined with a spectrophotometer (4.4.2) and either stored at -80 °C or subjected to reverse transcription PCR (4.6.2) for quantitative RT-PCR (4.6.3).

4.6.2 Reverse transcription

The reverse transcription of RNA was performed with the *High-capacity cDNA Reverse Transcription Kit* (Thermo Scientific) according the manufacturer's instructions. The RNA amounts were adjusted and 0.5-1 µg of RNA was reverse transcribed. cDNA was stored at -20°C.

4.6.3 Semi-quantitative real-time PCR

Nucleic acids were quantified via real-time PCR, which was carried out in 0.1 ml STRIP tubes (LTF-Labortechnik) and measured in a *Rotor-Gene 6000* (Corbett Life Sciences). The total reaction mixture of 10 µl contained 25 ng cDNA/DNA, 5 pmol of each oligonucleotide primer (the length of amplified fragment was between 75-150 bp) and 5 µl *SensiMix SYBR* (Bioline). GAPDH was used as a

housekeeping gene for normalization with an amount of 2.5 ng cDNA/DNA per reaction. The PCR conditions are stated in the table below.

Denaturation	10 min	95 °C	
Denaturation	30 sec	95 °C	40 cycles
Primer annealing	30 sec	62 °C	
Extension	30 sec	72 °C	
	10 min	72 °C	
Storage	∞	4 °C	

Each sample was measured in triplicates and the average threshold cycle (CT) value served to calculate ΔCT for normalization. Subsequently, ΔΔCT was calculated to determine the fold induction of the RNA/DNA of interest.

$$Log_2(\Delta Ct) = mean(Ct_{ref}) - mean(Ct_{sample})$$
$$Log_2(\Delta\Delta Ct) = Log_2(\Delta Ct_{Inf}) - Log_2(\Delta Ct_{mock})$$
$$Fold\ expression = 2^{Log_2(\Delta\Delta Ct)}$$

4.7 Protein methods

4.7.1 Preparation of whole cell lysates

If not otherwise indicated, whole cell lysates were prepared with highly stringent RIPA lysis buffer containing protease inhibitors (see table below). Cell lysates were thawed on ice and lysed in 100-500 µl RIPA buffer, depending on the pellet size. To ensure efficient cell lysis, samples were incubated on ice for 30 min and vortexed every 10 min before sonification with the *Sonifier 450* [(Branson)(40 pulses; output 0.80; 0.8 impulses/s)] to shear genomic DNA. Afterwards, the cell debris as well as the insoluble fraction was pelleted (5 min; 4 °C; 20000 *g*) and the supernatant was transferred to a fresh 1.5 ml reaction tube (Eppendorf). Protein concentrations were determined via Bradford assay (4.7.2) and adjusted to a total protein concentration of 4 mg/ml. For the reduction of disulfide bonds, 5x SDS sample buffer, containing 200 mM β-mercaptoethanol, was added before heat denaturation for 4 min at 95 °C.

RIPA buffer	50 mM	Tris-HCl (pH 8.0)
	150 mM	NaCl
	5 mM	EDTA
	1 % (v/v)	Nonidet P-40
	0.1 % (w/v)	SDS
	0.5 % (w/v)	Sodium Deoxycholate
		(Protease inhibitors were added right before use)
	0.2 mM	Phenylmethylsulfonylfluorid
	1 mg/ml	Pepstatin A
	5 mg/ml	Aprotinin
	20 mg/ml	Leupeptin
5x SDS sample buffer	100 mM	Tris-HCl (pH 6.8)
	10 % (w/v)	SDS
	0.2 % (w/v)	Bromphenol blue
	50 %	Glycerol
	200 mM	β-Mercaptoethanol (added right before use)

4.7.2 Determination of protein concentration via Bradford assay

Protein concentrations of whole cell lysates were determined using the Bradford-based *BioRad Protein-Assay* (BioRad). For each sample, 1 µl protein lysate was mixed with 800 µl ddH_2O and 200 µl *Bradford Reagent* (BioRad). Samples were incubated for 5 min at RT and subsequently measured in a *SmartSpec™ Plus* photospectrometer (BioRad) at 595 nm. The unknown sample protein concentrations were determined by interpolation from a standard curve (concentration of 1-16 µg/µl).

4.7.3 Immunoprecipitation

Immunoprecipitation was performed with whole cell lysates (4.7.1), which were adjusted to equal amounts of 0.5-2 mg protein. Preclearing with *Pansorbin* [(50 µl/sample)(Calbiochem)] was performed under constant rotation (rotator 3025, GFL) for 1 h at 4 °C. For antibody coupling, sepharose/IP [(3 mg/sample)(Sigma-Aldrich)] was expanded in RIPA buffer for 30 min at 4 °C and afterwards coupled to antibodies (100 µl self-made AB; 2 µg commercial AB) for 1 h at 4 °C under

constant rotation. When antibody-coupling was finished, residual unbound antibody was removed in three consecutive pelleting (3 min; 4 °C; 4000 *g*) and washing steps with RIPA buffer and resuspended in a suitable amount of RIPA buffer. *Pansorbin* was removed by pelleting (3 min; 4 °C; 800 *g*) and the pre-cleared samples were transferred to a fresh 1.5 ml reaction tube (Eppendorf). Subsequently, coupled antibody-beads were added for immunoprecipitation of target proteins for 4 h at 4 °C. When binding was completed, unbound proteins were removed in three washing and centrifugation (3 min; 4 °C; 800 *g*) steps. Target proteins were eluted in appropriate amounts of 2x SDS sample buffer and released for 4 min at 95 °C. Storage took place at -20 °C.

2x SDS sample buffer	100 mM	Tris-HCl (pH 6.8)
	4 % (w/v)	SDS
	0.2 % (w/v)	Bromphenol blue
	20 %	Glycerol
	200 mM	β-Mercaptoethanol (added right before use)

4.7.4 Purification of 6His-SUMO2 modified proteins

HeLa cells stably expressing 6His-SUMO2 molecules were infected (4.3.1) /transfected (4.2.4) and further processed according the respective experimental set up. For harvesting, cells were scraped off the dishes, transferred to a centrifuge tube with an appropriate volume (15 or 50 ml; Sarstedt) and pelleted (3 min; RT; 900 *g*). After resuspension in 5 ml PBS, 10 % of the volume was transferred to a 1.5 ml reaction tube (Eppendorf) for the preparation of whole cell lysates (4.7.1). The remaining suspension was again pelleted (3 min; RT; 800 *g*) and the resulting cell pellet was lysed in 10 ml guanidine hydrochloride lysis (GuHCl) buffer. The samples were either directly further processed or stored at -80 °C. 6His-SUMO2 modified proteins were purified via gravity-flow chromatography, using *Ni-NTA agarose beads* (Qiagen). For purification, 60 µl/sample Ni-NTA beads were pelleted (3 min; 4 °C; 900 *g*) and washed for three times in GuHCl, and subsequently resuspended in an appropriate volume. After the addition of the beads, the samples were rotated for at least 6 h up to 24 h at 4 °C. When binding was completed, Ni-NTA beads were pelleted (5 min; 4 °C; 1700 *g*) and the supernatant discarded. The remaining pellet was resuspended in 1 ml GuHCl and transferred to a new 1.5 ml tube. Next, the beads were washed and pelleted (3 min; 4 °C; 900 *g*) in two consecutive steps with a low stringency (pH 8.0) and a high stringency (pH 6.3) washing buffer. Purified proteins were dissolved in 40 µl *elution buffer* and denatured for 4 min at 95 °C. Target proteins were separated by SDS-PAGE (4.7.5) and visualized via immunoblotting (4.7.6).

Guanodine hydrochloride buffer	6 M	Guanidine hydrochloride
	0.1 M	Na_2HPO_4
	0.1 M	NaH_2PO_4
	10 mM	Tris/HCl pH 8.0
	20 mM	Imidazole
	5 mM	β-Mercaptoethanol
Low stringency washing buffer pH 8.0	8 M0.	Urea
	1 M	Na_2HPO_4
	0.1 M	NaH_2PO_4
	10 mM	Tris/HCl pH 8.0
	20 mM	Imidazole
	5 mM	β-Mercaptoethanol
High stringency washing buffer pH 6.3	8 M0.	Urea
	1 M	Na_2HPO_4
	0.1 M	NaH_2PO_4
	10 mM	Tris/HCl pH 6.3
	20 mM	Imidazole
	5 mM	β-Mercaptoethanol
Elution buffer	200 mM	Imidazole
	0.1 % (w/v)	SDS
	150 mM	Tris-HCl pH 6.8
	30 %(v/v)	Glycerol
	720 mM	β-Mercaptoethanol
	0.001 % (w/v)	Bromphenol blue

4.7.5 SDS – Polyacrylamide gel electrophoresis

Protein samples were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels according their molecular weight. Depending on the size of the target protein, gels with different volumes of *30 % acrylamide/bisacrylamide* solution [(37.5:1)(C. Roth)] were prepared and ran at 20 mA/gel in TGS-buffer. *PageRuler Prestained Protein Ladder Plus* (Thermo Scientific) served as molecular weight marker, to estimate the size of the proteins of interest.

5 % stacking gel	17 % (v/v)	Acrylamide solution (30 %)
	120 mM	Tris-HCl (pH 6.8)
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.1 % (v/v)	TEMED
8 % separation gel	27 % (v/v)	Acrylamide solution (30 %)
	250 mM	Tris-HCl (pH 8.8)
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.04 % (v/v)	TEMED
10 % separation gel	34 % (v/v)	Acrylamide solution (30 %)
	250 mM	Tris-HCl (pH 8.8)
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.04 % (v/v)	TEMED
12 % separation gel	40 % (v/v)	Acrylamide solution (30 %)
	250 mM	Tris-HCl (pH 8.8)
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.04 % (v/v)	TEMED
15 % separation gel	50 % (v/v)	Acrylamide solution (30 %)
	250 mM	Tris-HCl (pH 8.8)
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.04 % (v/v)	TEMED
TGS running buffer	23 mM	Tris
	200 mM	Glycine
	0.1 % (w/v)	SDS

4.7.6 Western blotting

After separation via SDS-PAGE (4.7.5), proteins were blotted onto *Nitrocellulose membranes* (GE Healthcare) using the *Trans Blot Electrophoretic Transfer Cell* System (BioRad). Therefore, a so called "Gel Sandwich" was assembled on a *gel holder cassette* (BioRad). This sandwich contained several layers, composed of the SDS acrylamide gel, placed on a *nitrocellulose membrane* and two layers of Towbin buffer soaked blotting paper (Whatman) on each side. Electrophoretic transfer was performed in Towbin buffer for 90 min at continuous current of 400 mA perblotting chamber. Unspecific antibody binding sites were blocked with PBS containing 5 % non-fat dry milk powder (Frema) for at least 2 h at RT (or overnight at 4 °C). To ensure that all membranes are equally immersed in the different buffers, all incubation steps were carried out on an *orbital shaker* (GFL). After blocking, the solution was discarded and the membranes were washed three times for 5 min in PBS-Tween. For the detection of proteins, specific primary antibodies were incubated for at least 2 h (or overnight) at 4 °C. To reduce unspecific binding, some antibodies were supplemented with 1 % (w/v) non-fat dry milk powder (Frema). The primary antibody was removed, by washing three times for 10 min with PBS-Tween at RT. Afterwards the secondary HRP conjugated antibody (Amersham) was diluted 1:10.000 in 3 % non-fat dry milk powder PBS solution (Frema) and incubated for 2 h at RT. Finally, the secondary antibody was also removed in three 10 min washing steps and protein bands were visualized by *enhanced* chemiluminescence using *SuperSignal West Pico Chemiluminescent Substrate* (Thermo Scientific) according the manufacturer's instructions. The protein signals were detected using *New Medical X-Ray Films* (CEA) and developed in a *GBX Developer* (Kodak). The X-ray films were scanned for documentation and further processed using *Photoshop CS6* (Adobe) and *Illustrator CS6* (Adobe).

Towbin buffer	25 mM	Tris-HCl (pH 8.3)
	200 mM	Glycine
	0.05 % (w/v)	SDS
	20 %(v/v)	Methanol
PBS-Tween	0.1 % (v/v)	Tween 20 in 1x PBS

4.7.7 Detection of proteins via immunofluorescence staining

For indirect immunofluorescence, cells were grown on glass coverslips and transfected (4.2.4) or infected (4.3.1) depending on the experimental set up. Cells were fixed for 20 min at 4 °C with 4 % PFA and permeabilized for 30 min with PBS-Triton X-100 [5 % (v/v)] at RT. After blocking for 1 h

with TBS-BG, the primary antibodies were diluted according manufacturer's instructions (self-made antibodies were diluted 1:10) in PBS and incubated for 1 h at RT. Primary antibodies were removed in three washing steps for 10 min with TBS-BG, followed by 1.5 h of incubation with the corresponding fluorescent labelled secondary antibody together with DAPI (Leica DMI6000B Microscope= 1:1000; Spinning disc confocal microscope= 1:8000). Coverslips were again washed three times with TBS-BG and mounted onto glass slides (Menzel). Digital images were acquired with either a Leica DMI 6000B or a spinning disc microscope. The spinning disc confocal microscope was equipped with a 100x, NA 1.49 CFI Apo TIRF Nikon and a dual-camera Yokogawa W2 Spinning disc confocal scan head and Andor iXON 888 cameras. An Andor Borealis System ensured Illumination flatness of 405, 488, 561, 647 nm lasers. Pictures were further processed with NIS-*Elements AR* (Nikon), ImageJ (ImageJ), *Photoshop CS6* (Adobe) and *Illustrator* (Adobe).

PBS-Triton X-100	0.5 mM	Triton X-100 in PBS
TBS-BG	100mM	Tris-HCl pH7.6 (1 M)
	685 mM	NaCl (5 M)
	15 mM	KCl (1 M)
	7.7 mM	MgCl2 (1 M)
	0.25 %	Tween 20
	0.25 %	Sodium azide
	25 gm/ml	BSA Fraction IV
	25 mg/ml	Glycine

4.8 *In-vitro* assays

4.8.1 Reporter gene assay

Promoter activities were assessed with the *Dual-Luciferase Reporter Assay System* (Promega), according manufacturer's instructions. Target promoter regions were cloned in either pGL-3 Basic, pGL-2 Basid (Promega) or pLightswitch (Switchgear Genomics) luciferase reporter vectors, which contained a firefly (*Photinus pyralis*) or a renilla luciferase (*Renilla reniformis*) reporter gene. Additionally, a second luciferase reporter vector was transfected for normalization to avoid deviations by different cell numbers or different transfection efficiencies. This luciferase reporter vector contained the constitutively active herpes simplex virus thymidine kinase promoter (pRL-Luc). Reporter gene assays were started by the transfection with PEI of $2x10^5$ H1299 cells in 12-wells,

(4.2.4). Cells were transfected for 4 h and further incubated in DMEM containing 5 % or 0.1 % FBS, depending on the experimental set up (4.2.4). After 24 h cells were lysed with 100 µl/well *1x passive lysis buffer* (Promega) at RT under vigorous shaking. The promoter activity was determined by measuring the relative light units (RLU) of both luciferases. 10 µl of each cell lysate was subjected to sequential measuring of firefly- (10 s) and renilla luciferase activity (10 s) on a *Lumat LB 9507 luminometer* (Berthold Technologies). For normalization, each individual firefly luciferase activity was multiplied by an individual normalization factor calculated from the renilla luciferase activity. The highest activity of all renilla activities (Maximal renilla activity) was divided by each individual renilla activity. This individual normalization factor was multiplied with each individual firefly activity to achieve the normalized firefly activity (see equation below). In case of the pLightswitch luciferase reporter system, the renilla activity of the target promoter was normalized to the firefly activity.

$$Normalized \text{ firefly activity} = Individual\ firefly\ activity * \frac{Maximal\ renilla\ activity}{Individual\ renilla\ activity}$$

In order to compare the results of different experiments and to combine biological triplicates, the Relative light unit (RLU) was determined. In this last step, the normalized firefly activity of each sample was divided by the control. This was, depending on the experimental set up, either during transfection the empty vector control, or during infection or the non-infected control.

$$Relative\ light\ units\ (RLU) = \frac{Normalized\ firefly\ activity\ of\ sample}{Normalized\ firefly\ activity\ of\ control}$$

5 Results

5.1 SUMOylation of E4orf6/7 during transfection

5.1.1 E4orf6/7 is SUMOylated E1B-55K independently at lysine 68

E1B-55K is one of the major regulator proteins of adenoviruses whose functions are, at least in part, dependent on the modification with *small ubiquitin like modifiers* (SUMO). In addition, further studies showed that E1B-55K acts as a E3 SUMO ligase inducing SUMOylation of the cellular protein p53 (Pennella et al., 2010; Muller & Dobner, 2008). In an attempt to identify further E1B-55K-dependent SUMO target proteins, a *stable isotope labelling with amino acids in cell culture* (SILAC) experiment during HAdV-5 infection was performed, by a co-worker. This global approach allows the relative quantification of differential changes in complex protein samples. The method relies on the incorporation of heavy isotope labelled amino acids into newly synthesized polypeptides. Since there is almost no difference in chemical properties between the labeled and the native amino acid, the cell metabolism is not influenced and the cells behave like the control cell population. This experimental approach did not only provide the possibility to monitor changes in the SUMO proteome upon infection but also allowed the identification of SUMO substrates whose SUMOylation status is modulated by the presence of E1B-55K. In total, 272 differentially SUMOylated cellular proteins were detected during wild type infection. Of these proteins we found 78 whose abundance was increased by a factor of two or more. In addition 20 SUMOylated viral proteins were found, whereat three of them exclusively occurred during wild type infection. One of them was the early region 4 protein Orf6/7 (see red arrow in Figure 12). The observation that E4orf6/7 was highly SUMOylated in HAdV-5 WT (H5*pg*4100) infected cells in, contrast to cells infected with an E1B-55K deficient HAdV-5 (H5*pm*4149), indicated that E4orf6/7 is another E1B-55K SUMO substrate.

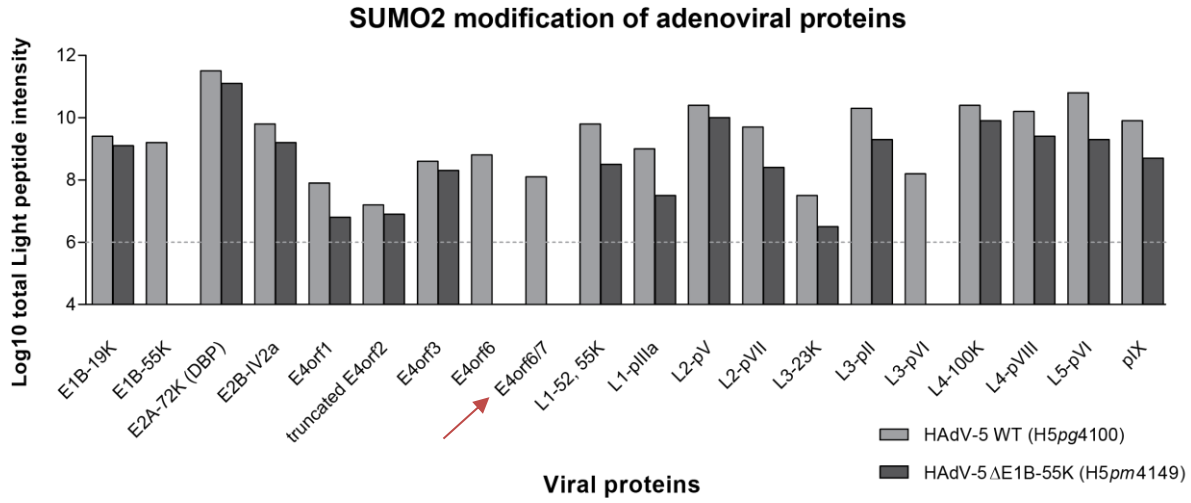


Figure 12: SUMO modified viral proteins identified in the SILAC experiment. Cells were infected with HAdV-5 WT (H5*pg*4100) and HAdV-5 ΔE1B (H5*pm*4149) viruses to determine abundance of SUMOylated viral proteins during adenoviral infection in a SILAC experiment. Light grey bars represent the abundance of SUMOylated viral proteins in HAdV-5 WT infected cells and dark grey bars represent the abundance in HAdV-5 ΔE1B-infected cells. E4orf6/7 is indicated by the arrow.

Because the SILAC experiment strongly suggested that E4orf6/7 is SUMO modified upon HAdV-5 WT infection, the next experiments aimed to confirm and identify the site of SUMOylation. *In-silico* analysis revealed three lysine residues (K68, K114, K126) within the E4orf6/7 protein, whereas K68 was predicted to be in the context of a partial SUMO conjugation motif [(SCM)(Figure 13)].

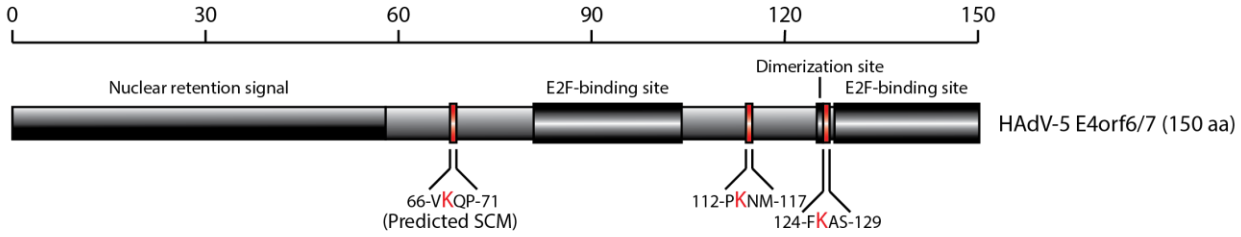


Figure 13: Functional domains of E4orf6/7 and the predicted SUMO conjugation site. *In silico* analysis was performed with: http://sumosp.biocuckoo.org/

In order to demonstrate E1B-55K-dependent SUMOylation of E4orf6/7 in cell culture, parental HeLa cells (HeLa Par) as well as HeLa cells stably expressing 6His-SUMO2 (HeLa SU2) were employed. These cells were either transfected with plasmids encoding E4orf6/7 alone or together with E1B-55K encoding plasmids. Afterwards SUMOylated proteins were purified and analyzed by immunoblotting. Since E1B-55K was shown in previous publications to be SUMO modified, it was used as a positive control (Endter et al., 2001).

The staining of E1B-55K steady state samples showed strong E1B-55K overexpression (Figure 14 A; lanes 3+6). Furthermore, the slower migrating bands in lane 6 of E1B-55K in the pulldown confirmed the successful purification of 6His-SUMO modified proteins. The steady state protein levels of E4orf6/7 (Figure 14 A; lanes 2, 3, 5, 6) showed strong overexpression in HeLa Par and HeLa SU2 cells. Intriguingly, the SUMO pulldown with HeLa SU2 cells revealed slower migrating bands of SUMOylated E4orf6/7 (Figure 14 A; lanes 5+6). However, these E4orf6/7 SUMO bands occurred in both samples, in the presence and in the absence of E1B-55K, indicating an E1B-55K-independent SUMOylation of E4orf6/7. Nevertheless, these observations support partially the results of the SILAC experiment and show that E4orf6/7 SUMOylation can also be detected via immunoblotting.

Additionally, E1B-55K-independent SUMOylation of E4orf6/7 was confirmed with HepaRG cells. These cells were derived from a hepatocellular carcinoma and have many similarities with primary hepatocytes. They have only few major chromosomal rearrangements and are pseudodiploid (Gripon et al., 2002). For the purification of SUMO modified proteins, parental HepaRG cells (HepaRG Par) and HepaRG cells stably overexpressing 6His-SUMO2 (HepaRG SU2) were used (Sloan et al., 2015).

Strikingly, although E1B-55K was not present, E4orf6/7 in showed slower migrating banding pattern, indicating SUMOylated species of E4orf6/7 (Figure 14 B; lane 4). In conclusion, E1B-55K seems not to be required for E4orf6/7 SUMO modification during the overexpression of transiently transfected cells.

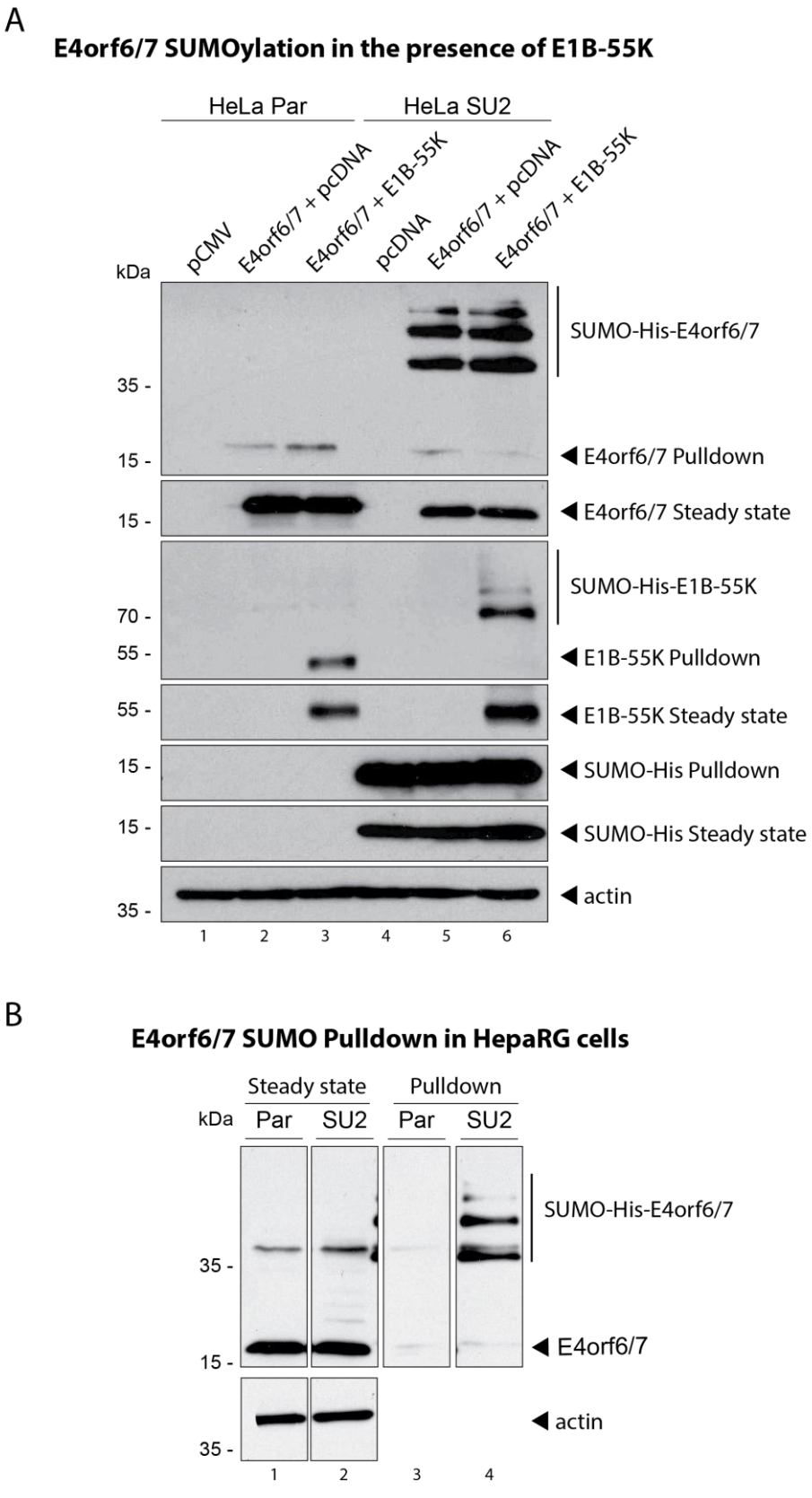


Figure 14: E4orf6/7 SUMOylation is E1B-55K-independent during transfection. Subconfluent parental HeLa **(A)** or parental HepaRG **(B)** as well as HeLa and HepaRG cells stably expressing 6His-SUMO2 (HeLa SU2/ HepaRG SU2) were transfected with 10 µg E4orf6/7 and 5 µg E1B-55K encoding plasmids and harvested after 48 h. SUMOylated proteins were extracted by Ni-NTA purification. Steady state samples and purifed proteins were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for E4orf6/7 (#94), E1B-55K (#1), SUMO-His (#551) and actin (#88). Molecular weights in kDa are indicated on the left, while the corresponding proteins are labeled on the right.

To determine the site of SUMO modification in the E4orf6/7 protein, mutant plasmids were generated. By means of site-directed mutagenesis, one, two or three mutations were introduced into the corresponding codons, leading to the substitution of the lysine by an arginine. Subsequently, these plasmids were transfected into HeLa Par and HeLa SU2 cells, SUMOylated proteins were purified and analyzed via immunoblotting.

Figure 15 illustrates the SUMO pulldown results of all lysine substituted E4orf6/7 variants in HeLa Par (Figure 15 A) and HeLa SU2 (Figure 15 B) cells. All E4orf6/7 variants were efficiently and comparably expressed as shown in the steady state protein levels (Figure 15 A+B; lanes 2-9). As in the previous experiments, E4orf6/7 WT was SUMOylated, indicated by its slower migrating bands (Figure 15 A; lane 2). In addition, only those E4orf6/7 variants showed strong SUMOylation that retained the lysine at position 68 (Figure 15 A; lanes 4, 5, 8). In contrast, the other variants showed either weak SUMOylation when lysine 68 was substituted but lysine 114 was present (Figure 15 A, lanes 3, 7) or there was no SUMOylation at all, when both lysine residues at position 68 and 114 were missing (Figure 15 A; lanes 6, 9). Consequently, as predicted in the *in-silico* analysis, these results show that lysine 68 is the major SUMOylation site of E4orf6/7 because the mutants that lacked L68 showed the most pronounced reduction in SUMOylation. Additionally, there was weak SUMOylation observed in the presence of lysine 114, which could be an indication for a minor SUMOylation site.

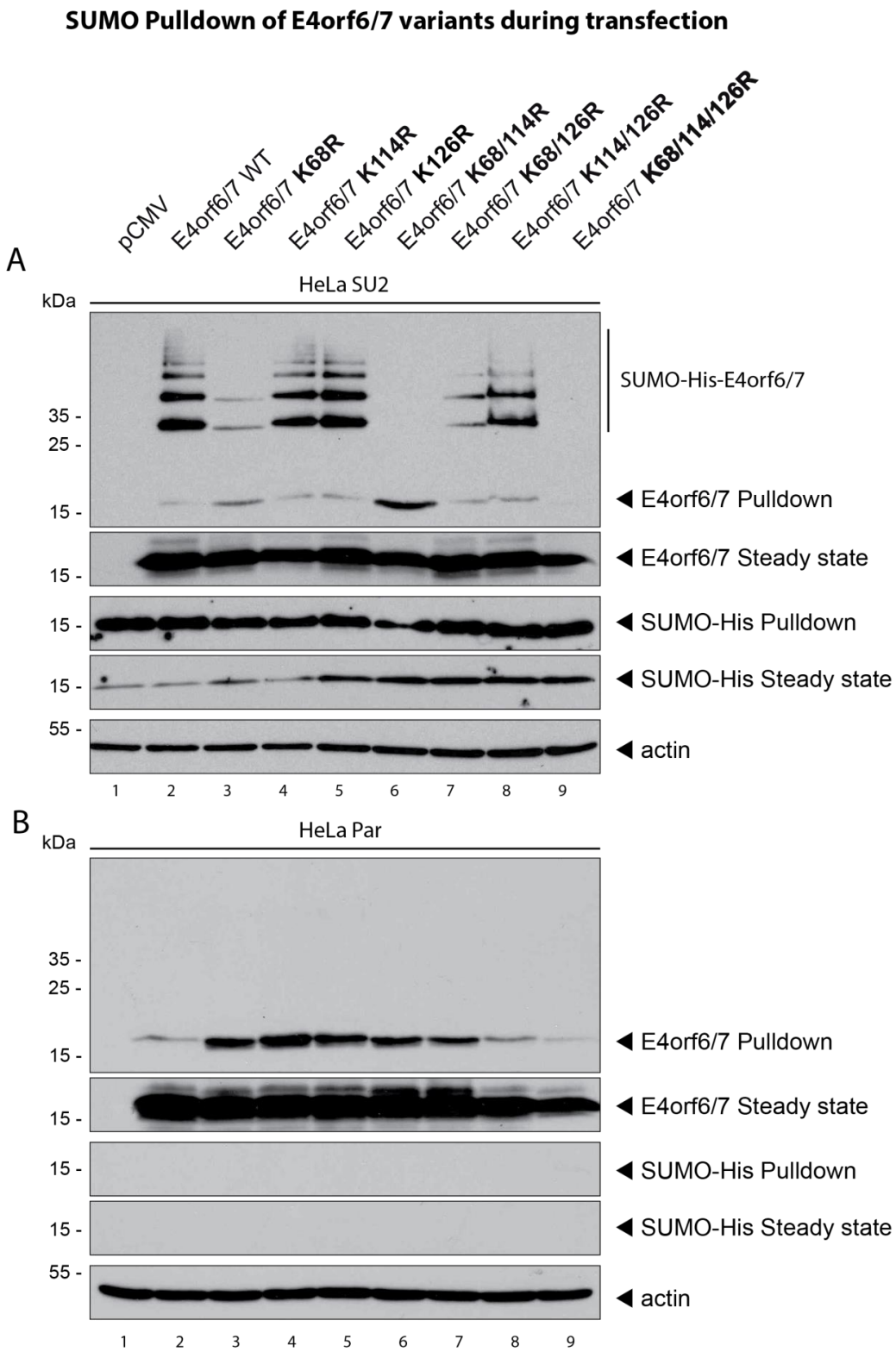


Figure 15: Lysine 68 in E4orf6/7 is the major SUMOylation site. Subconfluent **(B)** Parental HeLa (HeLa Par) and **(A)** HeLa cells stably expressing 6His-SUMO2 (HeLa SU2) were transfected with 5 µg of plasmids encoding the different E4orf6/7 variants. Cells were harvested 48 h p.t. and SUMOylated proteins were extracted by Ni-NTA purification. Steady state samples and purifed proteins were resolved via SDS-PAGE and visualized by immunoblotting using specific ABs for E4orf6/7 (#94), E1B-55K (#1), SUMO-His (#551) and actin (#88). Molecular weights in kDa are indicated on the left, while the corresponding proteins are labeled on the right.

5.1.2 Loss of SUMOylation site does not affect transactivation of viral and cellular E2F-1 promoter

E1A is the first viral gene to be transcribed, modulating the transcription of cellular and viral genes, amongst others, by displacing pRB-family members and thereby activating or inhibiting E2F transcription factors. These transcription factors are key players in the regulation of proliferation, apoptosis and differentiation in mammalian cells (Gallimore & Turnell, 2001). The function of E1A is complemented by E4orf6/7, which dimerizes two displaced E2F molecules to enhance the binding activity to inverted E2F binding sites. Since the HAdV-5 early 2A promoter (E2A promoter) contains two inverted E2F binding sites, it was aimed to address the question, whether SUMOylation regulates or influences the transactivation activity of E4orf6/7 (Schaley et al., 2000).

To shed light on this idea, the transactivation activity of E4orf6/7 WT and E4orf6/7 K68R alone as well as in cooperation with E1A on the E2A promoter was examined in a luciferase assay. H1299 cells were transfected with a luciferase reporter plasmid containing the E2A promoter together with plasmids encoding E4orf6/7 WT and E4orf6/7 K68R as well as E1A. The luciferase assay revealed that E1A and E4orf6/7 can activate the E2A promoter individually but they have a strong synergistic effect when transfected together (Figure 16 A). These observations are in line with the results of a previous publication (O'Connor & Hearing, 1991; Huang & Hearing, 1989b). However, the promoter activity between the cells co-transfected with E4orf6/7 WT or E4orf6/7 K68R were comparable, indicating that the activation of the viral E2A promoter is not influenced by E4orf6/7 SUMOylation.

In order to make sure, that the minor SUMOylation at lysine K114 has no effect on the transactivation activity of E4orf6/7, also a luciferase assay with E4orf6/7 variants was performed. H1299 cells were transfected with E4orf6/7 WT and the E4orf6/7 variants K68R, K114R and K68/114R. The results showed that none of the E4orf6/7 variants showed a significantly different transactivation activity on the E2A promoter (Figure 16 B). However, all SUMO mutants showed a slightly lower activity compared to E4orf6/7 WT.

Previous studies have shown that E1A alone can activate most of the viral promoters whereas studies on E4orf6/7 focused on the transactivation of the E2A promoter (Schaley et al., 2000; Obert et al., 1994; O'Connor & Hearing, 1991; Huang & Hearing, 1989b; Berk, 1986). However, it was also shown that E4orf6/7 can partially compensate the loss of E1A, indicating that E4orf6/7 must be able to transactivate also other viral promoters (O'Connor & Hearing, 2000). Therefore, in the following experiment the question was addressed whether E4orf6/7 can activate further viral promoters in a SUMO-dependent manner. H1299 cells were co-transfected with luciferase reporter plasmids, which

contained the different viral promoters (E1B, E2A, pIX, E2L, E3, E4 and MLP) together with plasmids encoding for E4orf6/7 WT and E4orf6/7 K68R.

The promoter activities show that E4orf6/7 was able to transactivate several viral promoters (Figure 16 C). The E1A, E2A, pIX, E2L and ML promoters were induced at least 2-fold, whereas the E1B and E4 promoter showed no significant changes. In particular, the E2L promoter is strongly activated by E4orf6/7. Nevertheless, E4orf6/7 WT and E4orf6/7 K68R showed equal transactivation activities on all viral promoters.

In summary, the previous experiments underline the importance of E4orf6/7 in cooperation with E1A in E2A promoter transactivation. Additionally, they show that E4orf6/7 can transactivate further viral promoters and not only the E2A promoter. However, all these transactivation activities were comparable in the presence of E4orf6/7 WT and E4orf6/7 K68R.

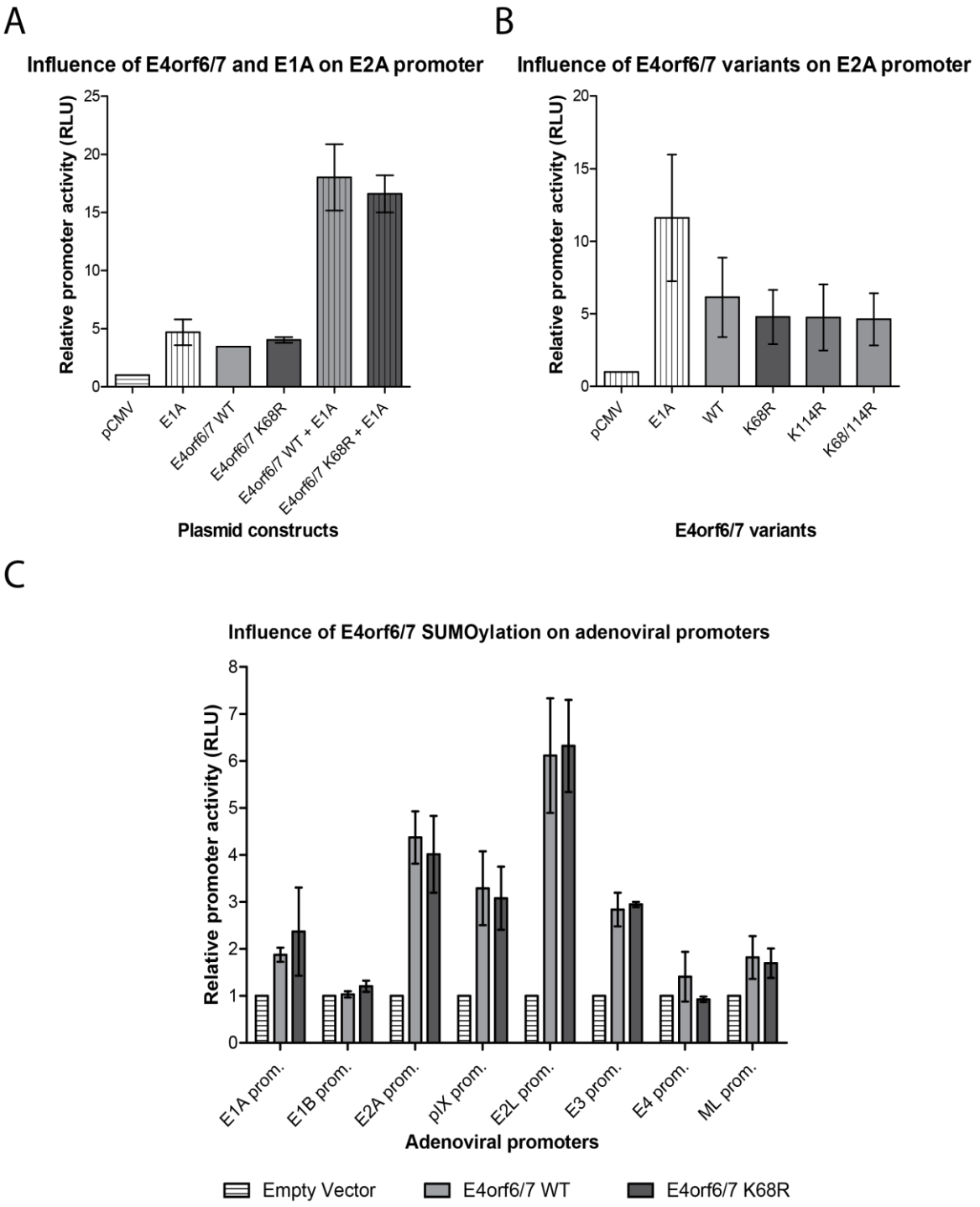


Figure 16: E4orf6/7 WT and E4orf6/7 K68R have a similar transactivation activity on the E2A and other viral promoters. H1299 cells were transfected with 0.5 µg of the different pGL3-Basic (Firefly-Luc) luciferase reporter plasmids, containing the different viral promoters, together with 0.5 µg of pRL-TK (Renilla-Luc) luciferase reporter plasmid for normalization. To determine transactivation activity, the relative light units (RLU) of both luciferases were measured 24 h p.t. in a dual luciferase assay and normalized to the overall renilla activity of all samples. The relative promoter activity describes the target promoter activity compared to the negative control. To test the transactivation activity of E4orf6/7 WT and E4orf6/7 K68R the following experiments were performed: **(A):** Transactivation activity on the E2A promoter in the presence/absence of E1A; 0.25 µg E1A as well as 0.5 µg of E4orf6/7 WT and E4orf6/7 K68R; experiment was performed in biological triplicates **(B):** Transactivation activity of different E4orf6/7 variants on E2A promoter activity; 0.5 µg E1A as well as 5 µg of each plasmid encoding E4orf6/7 WT, K68R, K114R and K68/114R. The experiment was performed in biological duplicates **(C)** Transactivation activity of different HAdV-5 promoters; 0.5 µg E4orf6/7 WT and K68R as well as the pGL3-basic luciferase plasmids, containing the viral promoter of E1A, E1B, E2A, pIX, E2L, E3, E4 and ML; Experiment was performed in technical triplicates.

Schaley and co-workers performed detailed investigations on the transactivation activity of E4orf6/7. Interestingly, they suggested that the viral E2A promoter is not the physiological relevant promoter of E4orf6/7, because it does not require the dimerization activity and E1A alone is sufficient for its transactivation. According to their hypothesis, the physiological relevant promoter must be of cellular origin having an inverted configuration of E2F binding sites that requires the dimerization of E2F transcription factors. They demonstrated that the E2F-1 promoter meets these requirements and that the protein p107 is the cellular analog of E4orf6/7, that can also dimerizes E2F molecules to modulate the E2F-1 promoter (O'Connor et al., 2001; Schaley et al., 2000).

In silico analysis performed in our group identified, another E2F promoter with E2F-1 binding sites within the promoter region of the gene FAM111B. Therefore, it was tested if the SUMOylation of E4orf6/7 influences the transactivation of these two cellular E2F promoters. H1299 cells were transfected with Luciferase reporter plasmids containing the E2F-1 or the FAM111B promoter. Additionally plasmids encoding E4orf6/7 WT and E4orf6/7 K68R were transfected alone and together with an E1A expression plasmid.

The results showed that E4orf6/7 was able to activate both promoters about 3 to 4-fold in the presence and absence of E1A (Figure 17 A+C). However, without E1A, E4orf6/7 K68R showed a slightly higher promoter activity, compared to E4orf6/7 WT. Nevertheless, this difference was only moderate and not significant.

One reason why there was only a small and non-significant difference in promoter transactivation, could be explained by the fact that the FCS, supplemented in the culture medium, stimulates cell growth and activates E2F-1 promoters (Schaley et al., 2000). To ensure that the promoters were not fully activated by the presence of FCS, the experiments were repeated in serum starved cells. Cells were synchronized in $DMEM^0$ for 24 h, transfected with the different plasmids and subsequently propagated in DMEM supplemented with 0.1 %FCS. Since serum starvation would also lead to depleted E2F-1 protein levels, E2F-1 expression plasmids were additionally co-transfected in some of the experiments.

The measured luciferase activities confirmed the results of the previous experiment (Figure 17 B+D). Although the different set ups revealed different promoter activities, depending on the presence or absence of E1A and/or E2F-1, both E4orf6/7 variants showed always comparable transactivation of the E2F-1 and FAM111B promoter. In consequence, even though both E4orf6/7 WT and E4orf6/7 K68R were able to activate the E2F-1 as well as the FAM111B promoter, the loss of the SUMOylation site does not affect the transactivation activity of E4orf6/7.

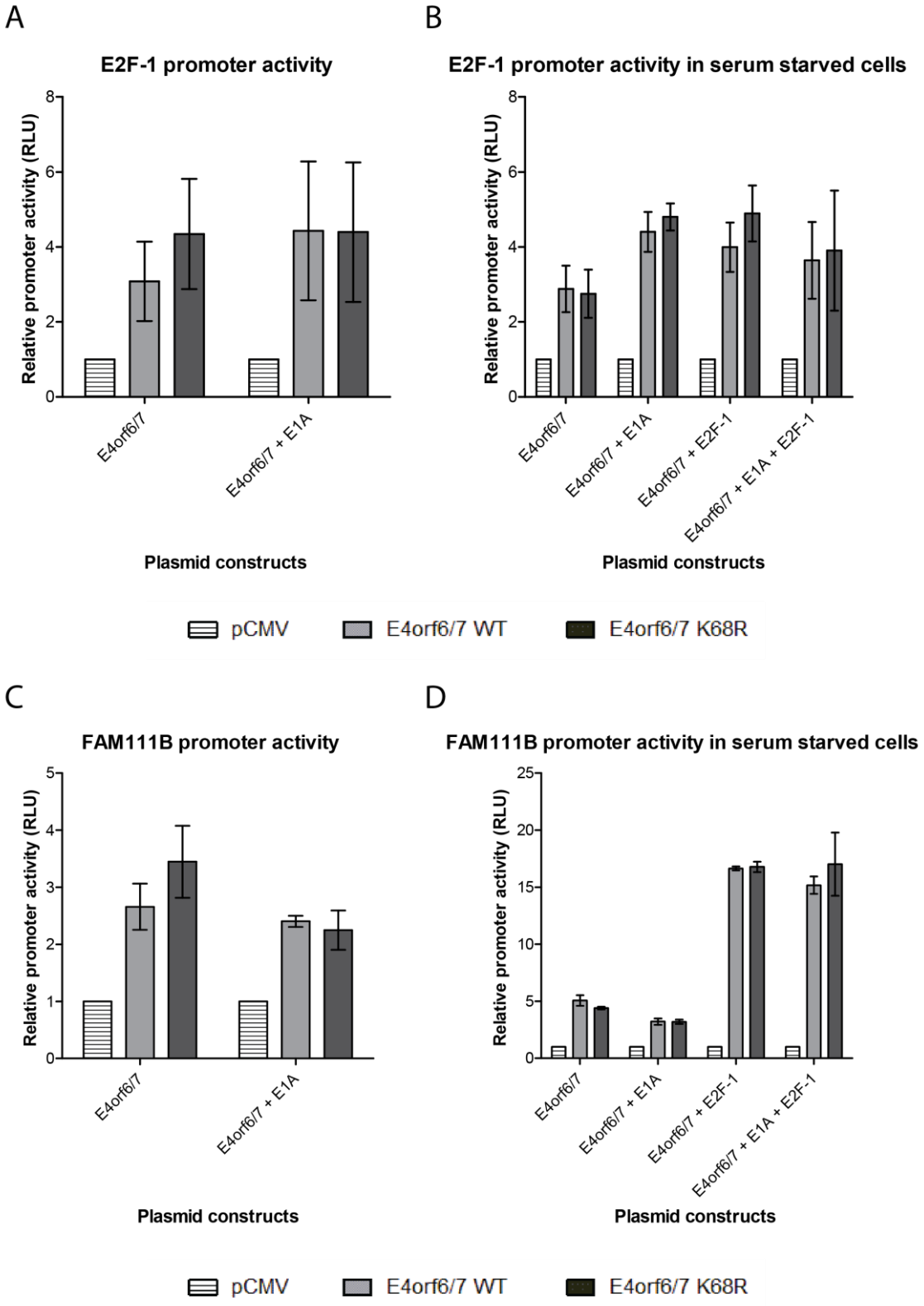


Figure 17: E4orf6/7 WT and E4orf6/7 K68 have comparable transactivation activity on E2F-1 and FAM111B promoter. H1299 cells were transfected with 0.5 µg of plasmids encoding E4orf6/7 WT or E4orf6/7 K68R or in combination with 0.25 µg of E1A encoding plasmids. Additionally, 0.5 µg of the luciferase reporter plasmids containing the E2F-1 or the FAM111B promoter were transfected together with 0.5 µg luciferase reporter plasmid containing the TK promoter for normalization. The E2F-1 promoter in the pBL3-Basic luciferase reporter plasmid was coupled to a firefly luciferase gene whereas the TK promoter was coupled to a renilla luciferase. In contrast, the FAM111B promoter in the pLightswitch luciferase reporter plasmid was coupled to a renilla luciferase, whereas the TK promoter was coupled to a firefly luciferase. Under low serum conditions some samples were additionally transfected with 0.1 µg of an E2F-1 encoding plasmid. 24 h p.t.

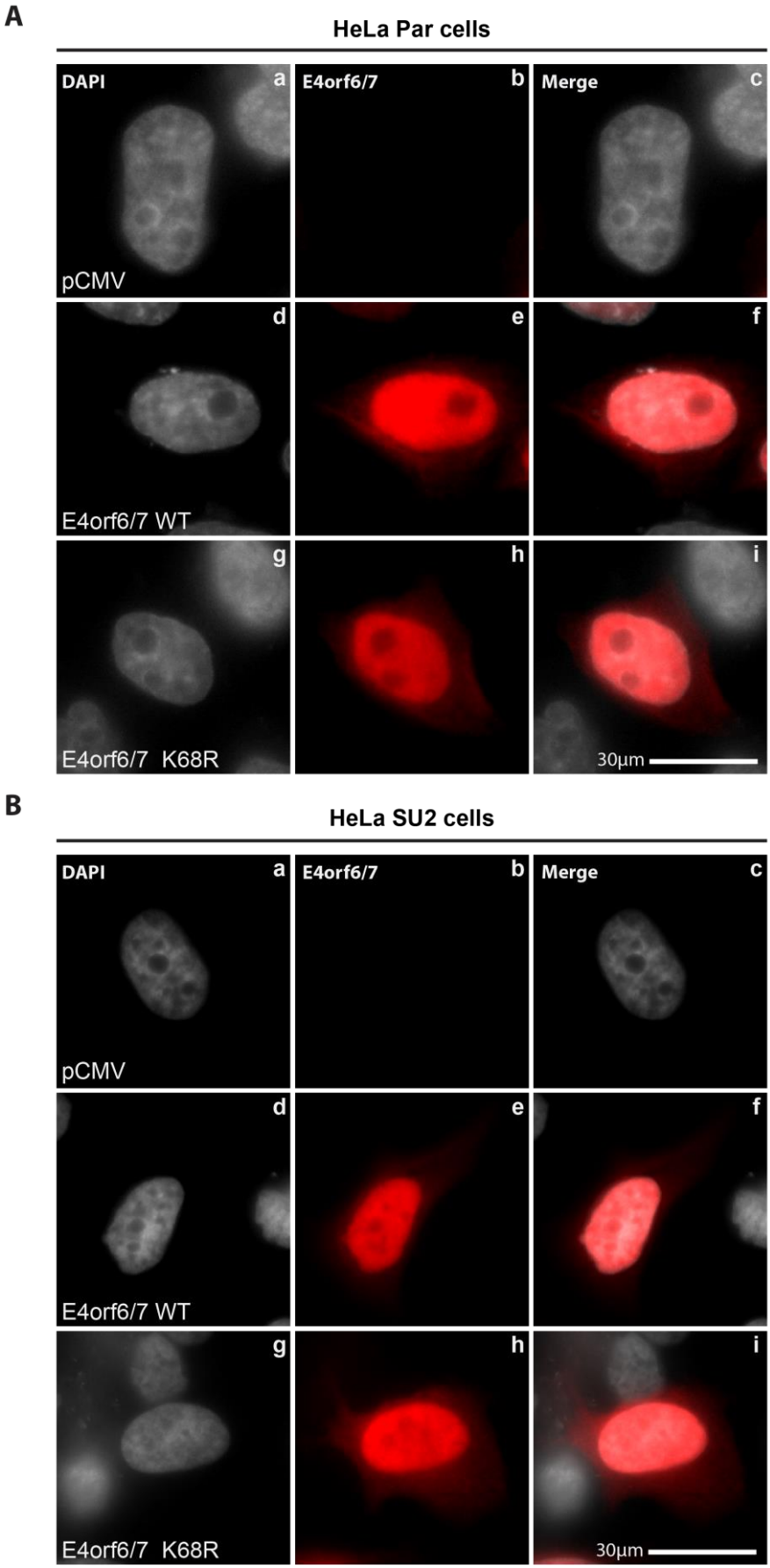
the relative light units (RLU) of both luciferases were measured in a dual luciferase assay and normalized to the overall firefly/renilla activity of all samples. Relative promoter activity describes the activity of the target promoter compared to the negative control. **(A):** Analysis of E2F-1 promoter activity in serum supplemented cells. Experiment was performed in biological triplicates **(B):** Analysis of E2F-1 promoter activity in synchronized and serum starved cells to decrease basal activity of E2F-1 promoter. Experiment was performed in technical triplicates **(C):** Analysis of FAM111B promoter activity in serum supplemented cells. Experiment was performed in biological duplicates **(D):** Analysis of FAM111B promoter activity in synchronized and serum starved cells to decrease basal activity of the FAM111B promoter. Experiment was performed in technical triplicates

5.1.3 E4orf6/7 WT and E4orf6/7 K68R localize inside the nucleus

SUMO modification can influence the subcellular localization of the target protein (van Damme et al., 2010; Shen et al., 2006; Zhong et al., 2000). It has been shown for HAdVs that the viral protein E1B-55K is re-localized into the nucleus upon SUMOylation (Pennella et al., 2010). Since the SUMOylation site in E4orf6/7 is in close proximity to the N-terminal nuclear retention signal (aa 1-58), the following experiment aimed to test, if the localization is affected by the SUMOylation (Schaley et al., 2005). To analyze differential localization upon SUMOylation, three different cell lines were used. The respective cells were transfected with plasmids encoding E4orf6/7 WT, E4orf6/7 K68R and analyzed with a microscope.

E4orf6/7 WT appeared in both HeLa cell lines diffusely localized in the nucleus, as it has been described in previous studies [(Figure 18 A+B)(Schaley et al., 2005)]. E4orf6/7 K68R showed a similar accumulation in comparable amounts to E4orf6/7 WT (Figure 18 A+B). In H1299 cells the same phenotype was observed with both E4orf6/7 variants (Figure 18 C). In summary these results suggest, that the loss of the SUMOylation site does not alter the localization and accumulation of E4orf6/7 in HeLa Par, HeLa SU2 and H1299 cells.

Localization of E4orf6/7 WT and E4orf6/7 K68R in different cell lines



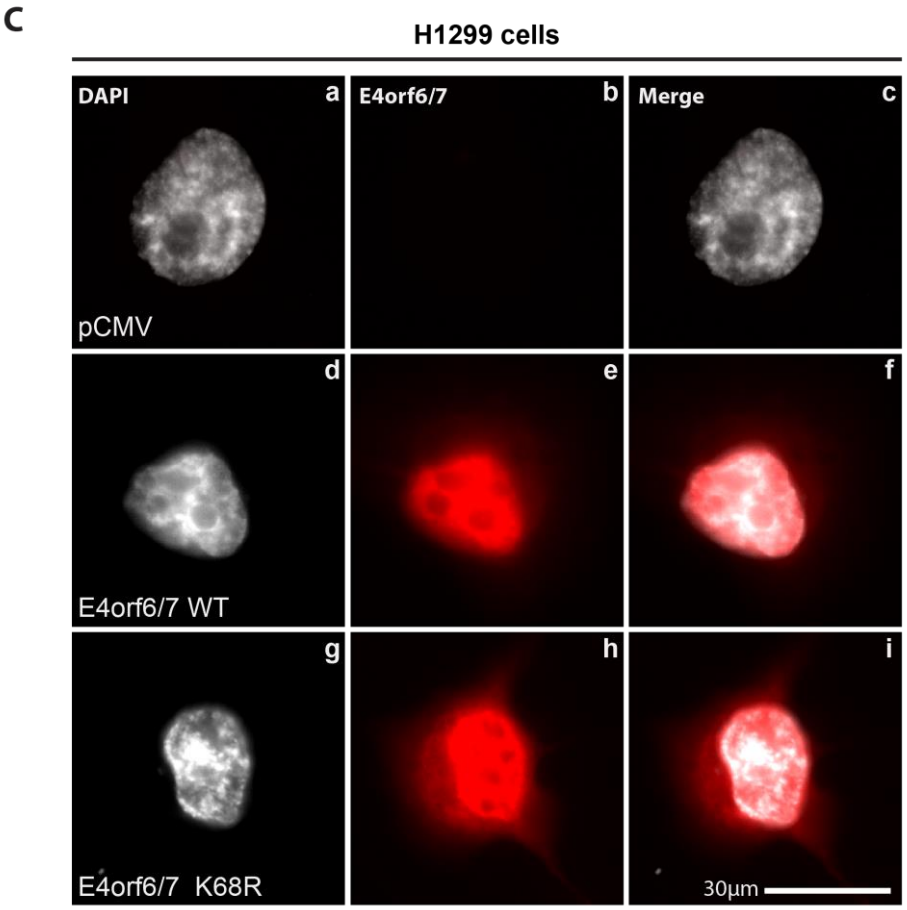


Figure 18: E4orf6/7 WT and E4orf6/7 K68R localize predominantly in the nucleus in HeLa Par (A), HeLa SU2 (B) and H1299 (C) cells. All cell lines were transfected with 5 µg of plasmids encoding E4orf6/7 WT, E4orf6/7 K68R or the vector control pCMV. Cells were fixed with 4 % PFA 48 h p.t.. Target proteins were stained with specific ABs for E4orf6/7 (#94) displayed in red, while chromatin was stained with DAPI (grey). Merge indicates the overlay of the single images in a row. Images were captured with a Leica DMI 600B microscope.

5.1.4 E4orf6/7 WT and E4orf6/7 K68R show the same interaction with E1B-55K

It is known from other experiments that the conjugation of SUMO to a target protein can influence the interaction with other proteins. With respect to adenoviral proteins, a SUMO-dependent interaction with certain PML isoforms has been observed for E1B-55K (Wimmer et al., 2010). Furthermore, E4orf6 and E4orf6/7 were shown to bind to E1B-55K as well as other E1B splicing variants via its N-terminal part (Sieber & Dobner, 2007; Rubenwolf et al., 1997). Therefore, the question was addressed if the binding to E1B-55K is influenced by the loss of the SUMOylation site in E4orf6/7. H1299 cells were transfected with plasmids encoding E1B-55K and E4orf6 as a positive control as well as together with E4orf6/7 WT and E4orf6/7 K68R.

The staining of actin shows, that equal amounts of proteins were loaded onto the gel. Additionally, the steady state of the viral protein levels indicate that cells were successfully transfected and the proteins were efficiently expressed (Figure 19; lanes 1-7). The positive control, in which E4orf6 and

E1B-55K were co-transfected, demonstrated that E1B-55K immunoprecipitation allowed the co-precipitation of E4orf6 (Figure 19; lane 12) and the immunoprecipitation of E4orf6 allowed the co-precipitation of E1B-55K (Figure 19; lane 19). Nevertheless, after immunoprecipitation of E1B-55K, no levels of E4orf6/7 WT and E4orf6/7 K68R were detected (Figure 19; lanes 13+14). In contrast, the immunoprecipitation of E4orf6/7 WT and E4orf6/7 K68R revealed co-precipitation of E1B-55K. Since equal amounts of E1B-55K were detected for both E4orf6/7 variants, these results indicate a comparable interaction E4orf6/7 WT and E4orf6/7 K68R (Figure 19; lanes 20+21). In consequence, the results of previous publications were confirmed and it was demonstrated that the binding to E1B-55K is not influenced by the SUMOylation.

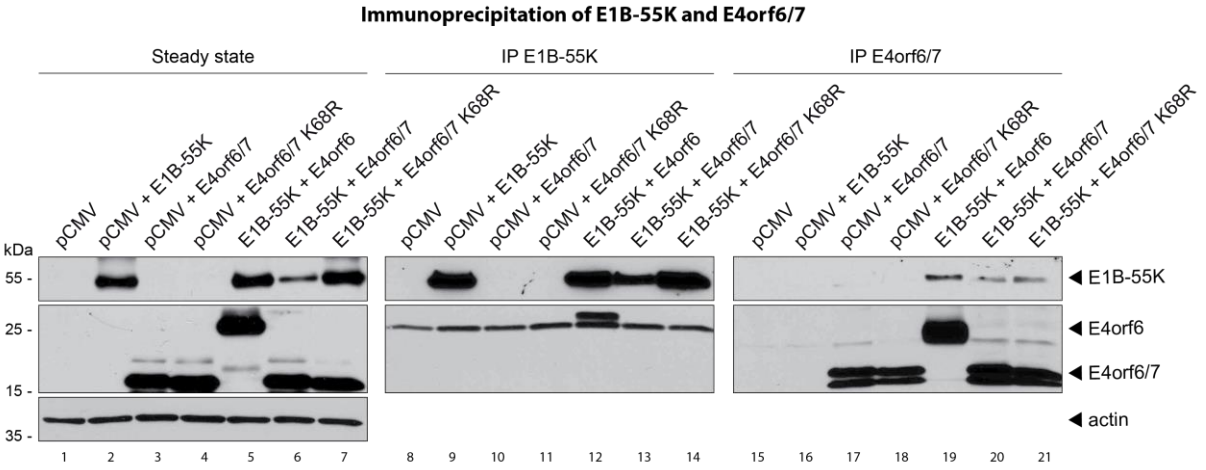


Figure 19: E1B-55K co-precipitates in equal amounts with E4orf6/7 WT and E4orf6/7 K68R. H1299 cells were transfected with 5 µg of plasmids encoding E1B-55K together with 10 µg of plasmids encoding E4orf6 or the E4orf6/7 variants WT and K68R. The cells were harvested 48 h p.t. and immunoprecipitation (IP) was performed by usage of specific ABs for E1B-55K (#1), E4orf6 and E4orf6/7 (#94). Steady state samples as well as the IP samples were resolved via SDS-PAGE and visualized by immunoblotting. Molecular weights in kDa are indicated on the left while the corresponding proteins are labeled on the right.

5.1.5 E4orf6/7 WT and E4orf6/7 K68R variants decrease transforming potential of E1A and E1B

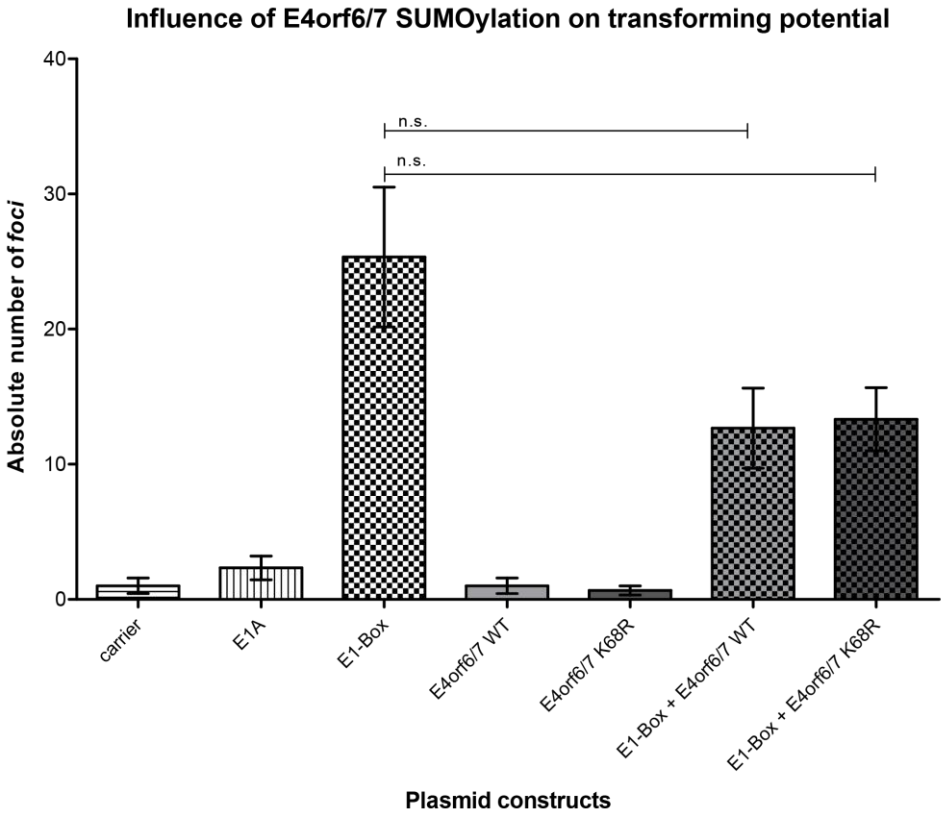
Human adenoviruses were one of the first viruses to be found to cause tumors in newborn rodents and accordingly they are categorized as highly, weakly and non-oncogenic (Trentin et al., 1962; Shenk, 2001). Until today, there is no existing proof of their tumorigenicity in humans, although HAdV genomes could be found in infiltrating lymphocytes of human sarcomas. Interestingly, these HAdVs predominantly belong to the species C, a group of adenoviruses not being classified as tumorigenic (Kosulin et al., 2013; Shenk, 2001). However, there is also overwhelming evidence that the presence of viral DNA fragments encoding the E1-region from various adenovirus species (among them HAdVs of species C) are sufficient to transform primary rodent and human cells *in-vitro*

(Speiseder et al., 2017; Endter & Dobner, 2004). Consequently, a contribution of HAdVs to the emergence of tumors in humans cannot be excluded.

Considering the redundant functions of E4orf6/7 and E1A in modulating the activity of cellular E2F responsive promoters to drive resting cells into S-phase and the fact that the SUMOylation of E1B-55K is prerequisite for its transforming ability, it was tempting to speculate that the SUMOylation of E4orf6/7 plays a relevant role during the process of transformation. In fact, it was demonstrated that E4orf6/7 induces p53-dependent apoptosis and reduces the transformation frequency of primary baby rat kidney cells (pBRK), when overexpressed with E1A and E1B-55K. In particular, the aa 39-58 in the N-term and aa 81-150 in the C-term seemed to be of major importance (Nevels et al., 1997; Yamano et al., 1999). Therefore, it was tested if the SUMOylation site, which lies exactly between these regions at position 68, has an influence on the oncogenic potential of E4orf6/7. For the transformation assay, pBRK cells were transiently transfected alone or in combination with plasmids expressing HAdV-5 E1A, the complete HAdV-5 E1 box (containing all reading frames of E1A and E1B) or one of the HAdV-5 E4orf6/7 variants E4orf6/7 WT and E4orf6/7 K68R. After several weeks of propagation, cells were fixed and stained with crystal violet to analyze the number of formed *foci* (Figure 20 A+B).

In contrast to cells transfected with the HAdV-5 E1-box, neither the E4orf6/7 WT nor the E4orf6/7 K68R transfected cells, resulted in the formation of fast growing *foci* (Figure 20 A). However, when co-transfected with the E1-box, both variants showed a reduced number of *foci* compared to the E1-box alone. Hence, the obtained data supports the finding of previous studies, which reported a reduced number of *foci* in the presence E4orf6/7 WT. Furthermore, the transformation assay revealed a comparable number of *foci* when co-transfected with the SUMO mutant E4orf6/7 K68R indicating that the SUMOylation has no effect on the transforming potential of E4orf6/7.

A



B

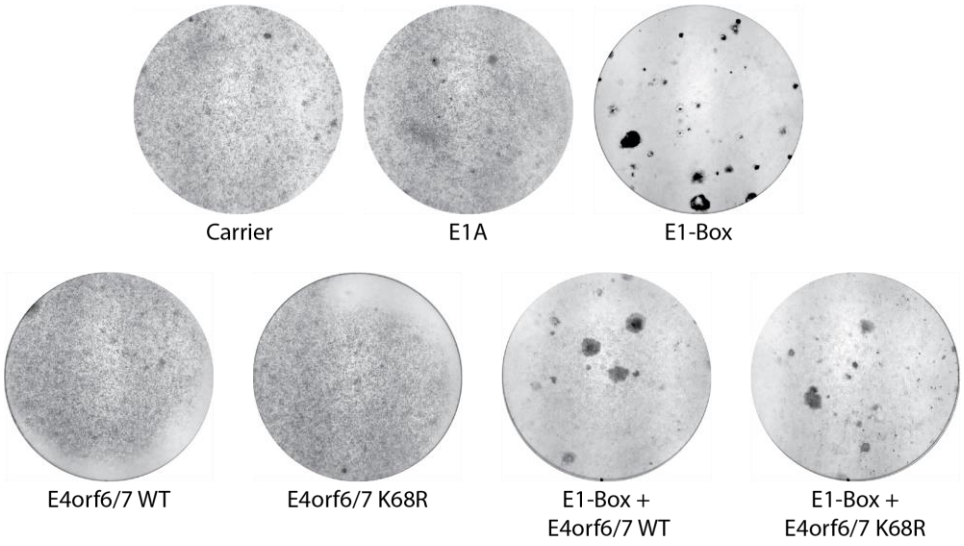


Figure 20: E4orf6/7 WT and E4orf6/7 K68R reduce the number of *foci* during E1A and E1B mediated transformation. Primary baby rat kidney cells (pBRK) were transfected with expression plasmids encoding HAdV-5 E1A, the full HAdV-5 E1-box or one of the E4orf6/7 variants. Cells were propagated for 8 weeks. Dishes were fixed with a solution containing 25 % MeOH and 1 % crystal violet in H_2O. Thereby, grown *foci* of pBRK cells were visualized and subsequently counted. The experiment was performed in technical triplicates. **(A):** *Foci* quantification in absolute numbers. **(B):** Representative images of the triplicate experiment for each

5.1.6 Loss of SUMOylation site decreases the stability of E4orf6/7 K68R

Studies with NF-κB and HIF-1α demonstrated that SUMO modification can alter the stability of proteins (Bae et al., 2004; Desterro et al., 1998). On the one hand, SUMO modification increases stability by shielding lysine residues from ubiquitination and subsequent proteasomal degradation (Desterro et al., 1998). On the other hand, SUMOylation can also decrease stability by so called *SUMO targeted ubiquitin ligases* (STUbLs). STUbLs bind to polySUMOylated proteins to target them for proteasomal degradation by ubiquitination (Sriramachandran & Dohmen, 2014). In an attempt to test the influence of the SUMO modification on the stability of E4orf6/7, H1299 cells were transfected with plasmids encoding E4orf6/7 WT and E4orf6/7 K68R. 24 h p.t. cells were treated with the translation inhibitor cycloheximide (CHX) or DMSO and cells were harvested in 1 h periods.

When treated with cycloheximide, both E4orf6/7 variants showed over time decreasing protein levels (Figure 21). However, the SUMO site deficient variant E4orf6/7 K68R showed considerable lower protein levels than E4orf6/7 WT after 4 h of treatment. Consequently, these data suggest that the SUMO modification of E4orf6/7 increased its stability.

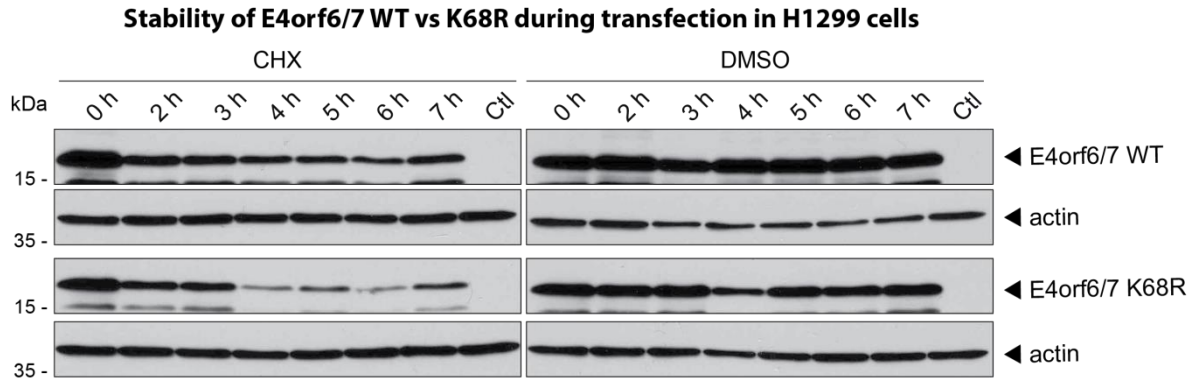


Figure 21: Loss of the SUMOylation site leads to decreased stability of E4orf6/7 K68R. H1299 cells were transfected with 5 µg of expression plasmids encoding E4orf6/7 WT or K68R. 24 h p.t. cells were treated with the translation inhibitor cycloheximide [(CHX)(100 µg/ml) or DMSO and 2 h after treatment samples were harvested in 1 h periods. Protein lysates were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for E4orf6/7 (#94) and actin (#88). Molecular weights in kDa are indicated on the left, while the corresponding proteins are labeled on the right. The experiment was performed in biological triplicates. Ctl: pCMV transfected cells

5.2 Consequences of E4orf6/7 SUMOylation site loss during HAdV-5 infection

5.2.1 No SUMOylation of E4orf6/7 detectable during HAdV-5 infection

In order to test if the SUMOylation of E4orf6/7 also plays a role during adenoviral infection, a SUMO pulldown with infected cells was performed and analyzed by immunoblotting. Therefore, an

experiment analogous to the SILAC experiment was performed, in which HeLa Par and HeLa SU2 were infected with HAdV-5 WT and a mutant virus defective for E1B-55K (HAdV-5 ΔE1B) to address two questions: 1. Does E4orf6/7 SUMOylation dependent on the presence of E1B-55K, as suggested in the SILAC experiment and 2. At which time point does the SUMOylation occur?

The slower migrating bands in the E1B-55K pulldown indicate that the 6His-SUMO2 purification was successful (Figure 22 A; lanes 4+5). However, unexpectedly there was no ladder-like pattern of slower migrating E4orf6/7 bands observed, but only a single band migrating around 25 kDa in the E4orf6/7 pulldown (Figure 22 A; lanes 1-10). Since the RSA3 AB (#94) binds to the N-terminus which is shared by E4orf6 and E4orf6/7, this band could either represent monoSUMOylated E4orf6/7 or nonspecifically purified E4orf6.

E4orf6/7 time course SUMO Pulldown in HAdV-5 WT and HAdV-5 ΔE1B-55K infected cells

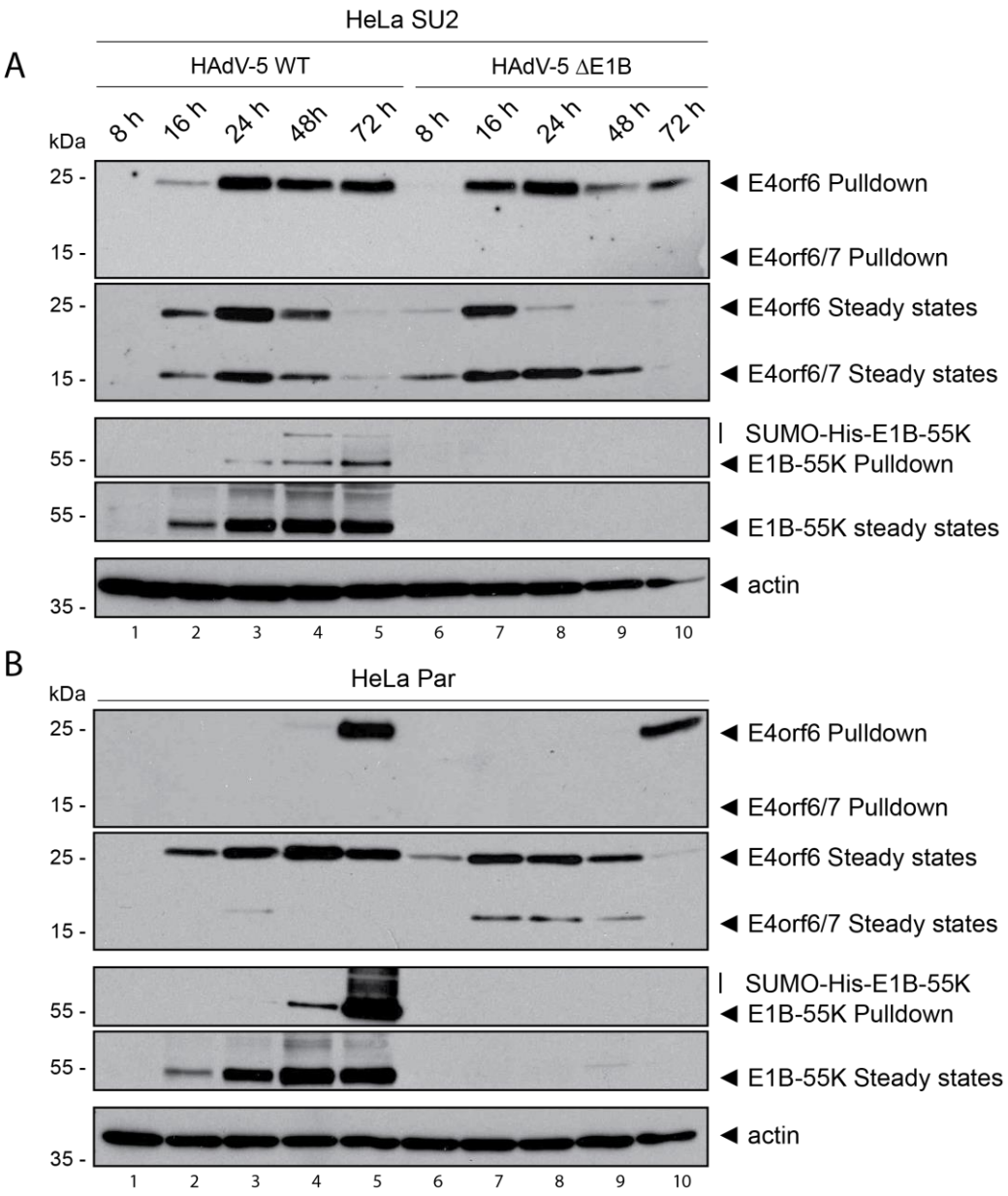


Figure 22: E4orf6/7 SUMOylation is not detectable during infection. HeLa parental **(A)** cells and HeLa cells stably expressing 6His-SUMO2 **(B)** were infected with HAdV-5 WT and an E1B-55K deficient virus mutant (HAdV-5 ΔE1B). 8, 16, 24, 48, 72 h p.i. samples were harvested and SUMOylated proteins were extracted by Ni-NTA purification. Steady state samples and purified proteins were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for E4orf6/7 (#94), E1B-55K (#) and actin (#88). Molecular weights in kDa are indicated on the left while the corresponding proteins are labeled on the right.

Because the previous E4orf6/7 pulldown revealed a slower migrating band with a molecular weight that overlaps with the molecular weight of E4orf6, the next experiment aimed to test, if this band represents monoSUMOylated E4orf6/7 or nonspecifically purified E4orf6. Therefore, another SUMO pulldown was performed, in which HeLa Par and HeLa SU2 cells were infected with HAdV-5 WT and an E4orf6 deficient virus mutant (HAdV-5 ΔE4orf6). As in the experiment before, a slower migrating

band was detected, slightly above 25 kDa, in cells infected with HAdV-5 WT (Figure 23; lanes 8+11). In contrast, this band was not detected in the SUMO pulldown of HAdV-5 ΔE4orf6 infected cells (Figure 23; lanes 9+12). Consequently, the band at approximately 25 kDa is not monoSUMOylated E4orf6/7 but rather nonspecifically Ni-NTA bound E4orf6.

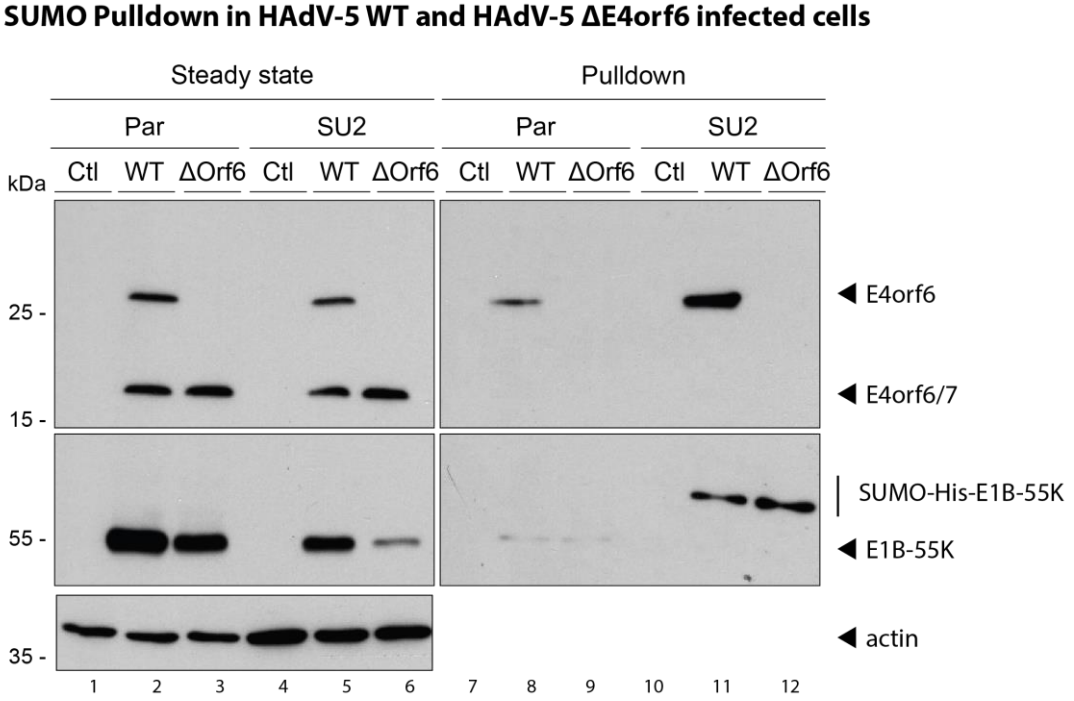


Figure 23: E4orf6 binds unspecifically to Ni-NTA beads during the purification of 6His-SUMO modified proteins. HeLa parental cells and HeLa cells stably expressing 6His-SUMO2 were infected with HAdV-5 WT and an E4orf6 deficient virus mutant (HAdV-5 ΔE4orf6). 24 h p.i. samples were harvested and SUMOylated proteins were extracted by Ni-NTA purification. Steady state samples and purified proteins were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for E4orf6/7 (#94), E1B-55K (#1) and actin (#88). Molecular weights in kDa are indicated on the left while the corresponding proteins are labeled on the right.

To enhance the SUMOylation of proteins, a SUMO Pulldown was performed with cells that overexpress the E2 SUMO conjugating enzyme Ubc9. HeLa Par and HeLa SU2 cells were transfected with plasmids encoding Ubc9 and were subsequently infected with HAdV-5 WT or HAdV-5 ΔE1B.

Although both HeLa lines show Ubc9 overexpression in their steady state levels, the expression is more efficient in HeLa Par cells (Figure 24; lanes 2, 4, 6) compared to HeLa SU2 cells (Figure 24; lanes 8, 10, 12). Also sufficient amounts of E4orf6/7 and E1B-55K were detected in the steady states (Figure 24; lanes 1-12). Intriguingly, there was more E1B-55K SUMOylation detected in the Ubc9 overexpressing cells compared to those cells transfected with pcDNA (Figure 23, lane 9+10), not only showing that the Pulldown was successful but also that overexpression of Ubc9 enhances SUMOylation. In contrast, there was no SUMOylation of E4orf6/7 detectable, but only the nonspecifically purified E4orf6, as it was observed in the previous experiments (Figure

24; lanes 8-12). In conclusion, also the overexpression of the SUMOylation enhancer Ubc9 did not enable the detection of E4orf6/7 SUMOylation by immunoblotting, although the SILAC experiment and the transfection experiments showed a strong SUMO modification. Therefore, it remains unclear if the levels of SUMOylated E4orf6/7 during infection are too low to be detected via immunoblotting, or if E4orf6/7 is not SUMOylated during the course of HAdV-5 infection.

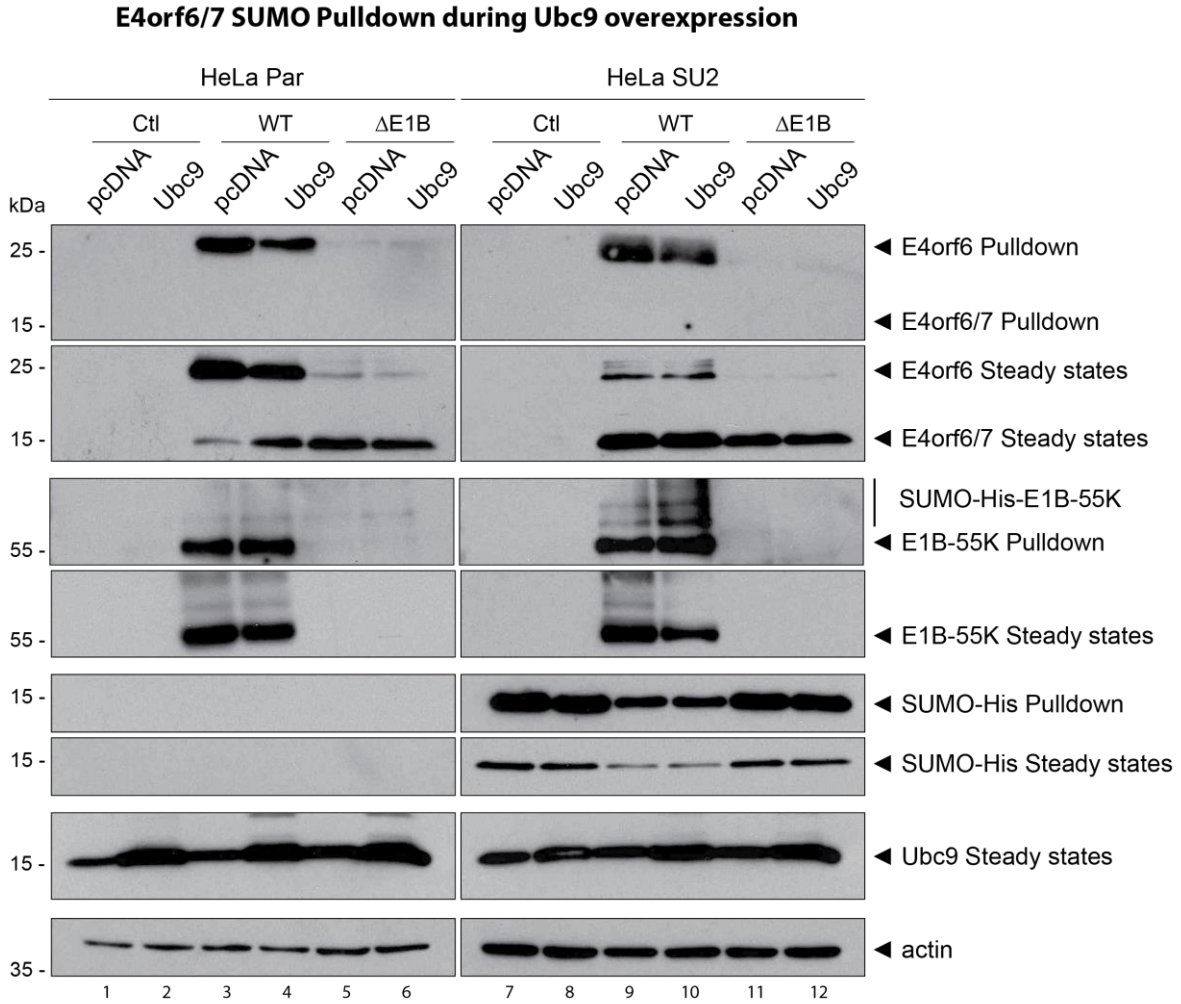


Figure 24: Overexpression of Ubc9 does not enhance SUMOylation of E4orf6/7. HeLa parental cells and HeLa cells stably expressing 6His-SUMO2 were transfected with 10 µg of an Ubc9 overexpression plasmid and 24 h p.t. infected with HAdV-5 WT and HAdV-5 ΔE1B. Samples were harvested 24 h p.i. and SUMOylated proteins were extracted by Ni-NTA purification. Steady state samples and purifed proteins were resolved via SDS-PAGE and visualized by immunoblotting using specific ABs for E4orf6 and E4orf6/7 (#94), E1B-55K (#1), SUMO-His (#551), Ubc9 (#57). Molecular weights in kDa are indicated on the left, while the corresponding proteins are labeled on the right.

5.2.2 Decreased stability of E4orf6/7 K68R during infection

Although several approaches were not successful to detect SUMOylated E4orf6/7 during infection in the previous experiments, the stability of the E4orf6/7 K68R protein during infection was tested, to

confirm the transfection stability experiments. Highly permissive A549 cells were infected with HAdV-5 WT and the HAdV-5 K68R virus mutant, which contains, in line with the transfection experiments, a point mutation in E4orf6/7 changing the codon for the lysine to an arginine.

Intriguingly, the protein dynamic of E4orf6/7 was similar to the dynamics observed during transfection (Figure 21). In all samples the abundance of E4orf6/7 decreased over time. But when treated with CHX, the protein abundance of E4orf6/7 K68R decreased considerably faster than E4orf6/7 WT (Figure 25). Hence, these results support the hypothesis that E4orf6/7 is also SUMOylated during infection leading to the stabilization of E4orf6/7 WT.

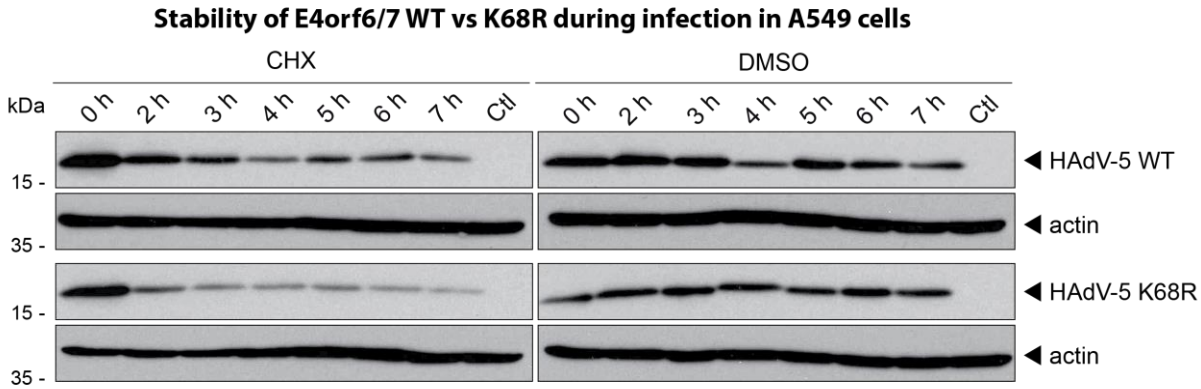


Figure 25: E4orf6/7 WT shows increased stability during infection. A549 cells were infected at an MOI of 20 with HAdV-5 WT or the HAdV-5 K68R mutant, which lacks the SUMOylation site at lysine 68 in E4orf6/7. After 24 h of infection, cells were treated with CHX and after 2 h of incubation samples were harvested in 1 h periods. Steady state samples were resolved via SDS-PAGE and visualized during immunoblotting with specific ABs for E4orf6/7 (#94) and actin (#88). Molecular weights in kDa are indicated on the left, while the corresponding proteins are labeled on the right. Ctl: non-infected cells.

5.2.3 HAdV-5 WT and HAdV-5 K68R viruses replicate comparably and produce equal amounts of infectious viral particles

The previous experiments clearly showed that the lack of the SUMOylation site at position 68 leads to a decreased stability of E4orf6/7 during transfection as well as infection. Furthermore, the switch from G_0 to S-phase mediated by E1A and E4orf6/7 is a crucial step during the course of adenoviral infection, because it provides the enzymes for genome replication (Gallimore & Turnell, 2001; Schaley et al., 2000). These facts raised the question, if the decreased stability of E4orf6/7 K68R impairs the course of HAdV-5 infection. Therefore, A549 cells were infected with HAdV-5 WT or HAdV-5 K68R and samples were collected 8, 16, 24, 48 and 72 h p.i.. Afterwards the relative amounts of genome copies as well as the number of produced infectious particles per µl were determined.

The experiments revealed slightly decreased amounts of genome copies in HAdV-5 K68R infected cells at early time points (Figure 26, graph A and B). 16 h p.i., the relative amount of genome copies in HAdV-5 K68R infected cells was about 2^3-fold (8-fold) lower than in HAdV-5 WT infected cells.

However, the reversed phenotype was observed 24 h p.i. where HAdV-5 K68R showed about 2^2-fold (4-fold) higher amount of genome copies. 48 and 72 h p.i. both viruses showed comparable amounts of genomes.

In contrast, the difference in genome replication early during infection was not reflected in the production of virus progenies (Figure 26 C). Early as well as late during infection HAdV-5 WT and HAdV-5 K68R produced comparable amounts of virus progenies. Consequently, the loss of the SUMOylation site seems to have a slight effect on genome replication, but not on the production of virus progenies.

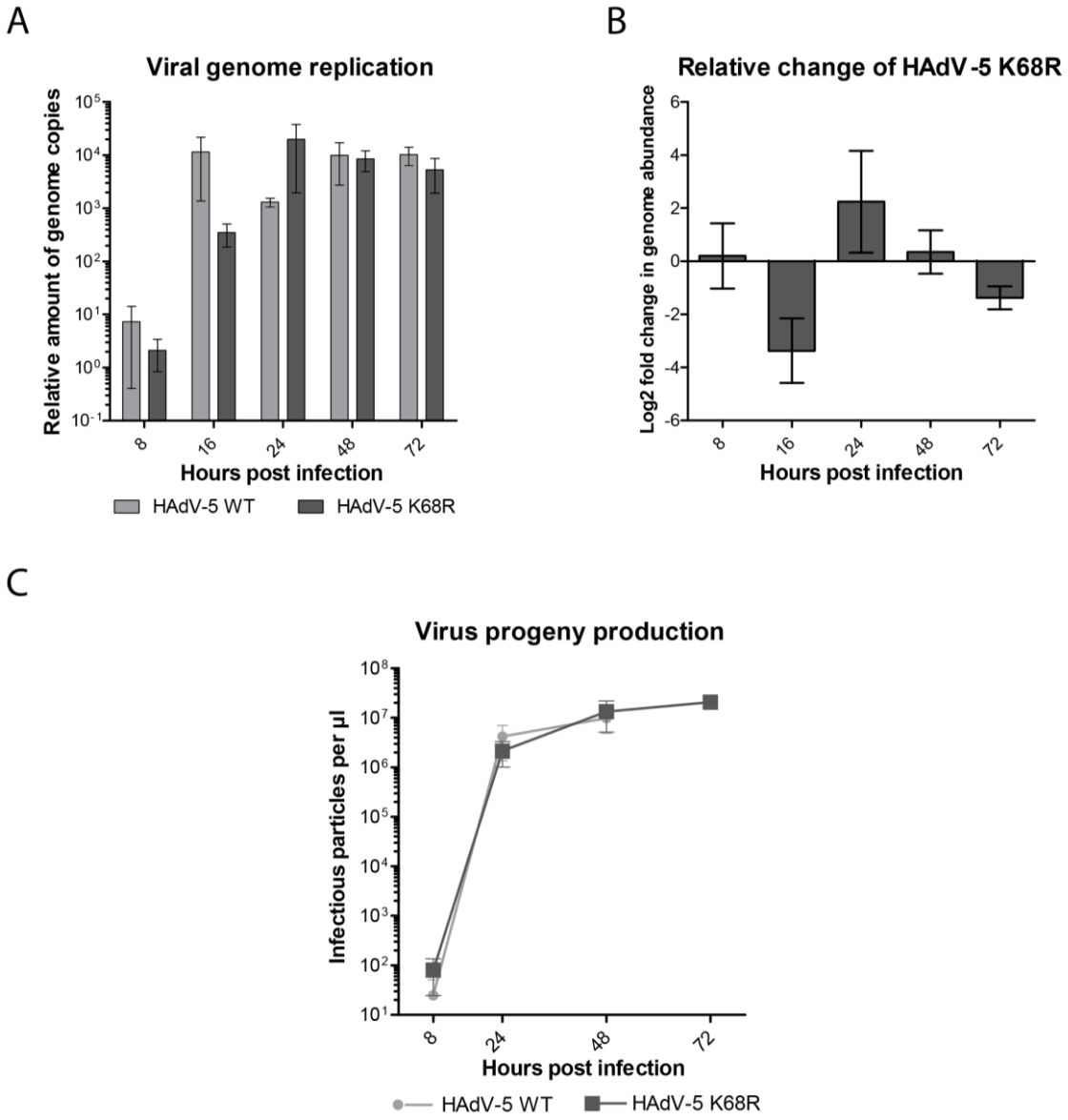


Figure 26: Virus genome replication and virus progeny production is not impaired by loss of the SUMOylation site in E4orf6/7. A549 cells were infected at an MOI of 20 with HAdV-5 WT or the HAdV-5 K68R mutant, which lacks the SUMOylation site at lysine 68 in E4orf6/7, and were harvested 8, 16, 24, 48 and 72 h p.i. After DNA isolation semi-quantitative real-time PCR allowed the relative quantification of genome copies using specific primers for E1B-55K (#640; #641) .To correlate the amount of genome copies of both viruses, the ΔΔCt value was determined given as fold change in genome amount of HAdV-5 K68R infected cells. For the determination of virus progenies, the retained viruses

were titrated on A549 cells and infected cells were detected by visualizing the viral protein E2A to calculate the infectious viral particles per µl. The experiment was performed in biological triplicates. **(A):** Relative amount of HAdV-5 genome copies given as ΔCt. **(B):** Relative abundance of genome replciation HAdV-5 WT vs. HAdV-5 K68R given as ΔΔCt. **(C):** Progression on newly produced infectious virus particles over time.

5.2.4 Consequences of E4orf6/7 SUMO modification on target promoters and protein abundance during infection

Since there was no effect observed during the general course of replication, the next infection experiments focused on published E4orf6/7 specific targets to elucidate the role of E4orf6/7 SUMOylation. In order to monitor the activation of E2F responsive promoters during the course of infection, the mRNA levels of different E2F promoter controlled genes were measured. By this approach, the endogenous promoter with all its *cis* and *trans* acting elements and in the presence of different viral proteins was monitored. In the experiments, A549 cells were infected with either HAdV-5 WT or the E4orf6/7 SUMOylation site deficient virus mutant HAdV-5 K68R and harvested together with non-infected (Mock) cells 8, 16, 24, 48 and 72 h p.i.. Afterwards mRNA was extracted to measure the mRNA level of different E2F responsive genes and protein lysates were prepared to monitor the corresponding protein abundance.

5.2.4.1 Influence of HAdV-5 WT and HAdV-5 K68R on the transactivation of the E2A promoter and the resulting protein abundance

5.2.4.1.1 Loss of SUMOylation site does not affect E2A mRNA levels

To achieve efficient transcription of E2A, E1A proteins displace and activate E2F transcription factors, which are dimerized by E4orf6/7 to increase the affinity to the E2F binding sites within the E2A promoter. Therefore, it was tested, if the loss of the SUMO site influences the transcription of E2A mRNAs and the production E2A proteins in the course of HAdV-5 infection.

As illustrated in Figure 27 A, the E2A mRNA level in HAdV-5 WT and K68R infected cells increased gradually over time until 48 h p.i.. Only 72 h p.i., the mRNA level in HAdV-5 K68R infected cells were rather low, whereas the mRNA level of HAdV-5 WT infected cells was high. However, the standard deviation of that value was extremely high, indicating a measuring error. When the mRNA levels of both infected cells were compared directly, there was no significant difference in E2A mRNA level 8, 16, 24 and 48 h p.i.. Again, only the 72 h p.i. time point showed considerable higher E2A mRNA levels in HAdV-5 WT infected cells (Figure 27 B). The protein levels of E2A reached their maximum 24 h p.i. and stayed at a constant level through the course of infection and showed comparable protein amounts in both infected cells (Figure 27 C). In summary, both experiments showed comparable

amounts in E2A mRNA level and protein abundance when comparing HAdV-5 WT or HAdV-5 K68R infected cells.

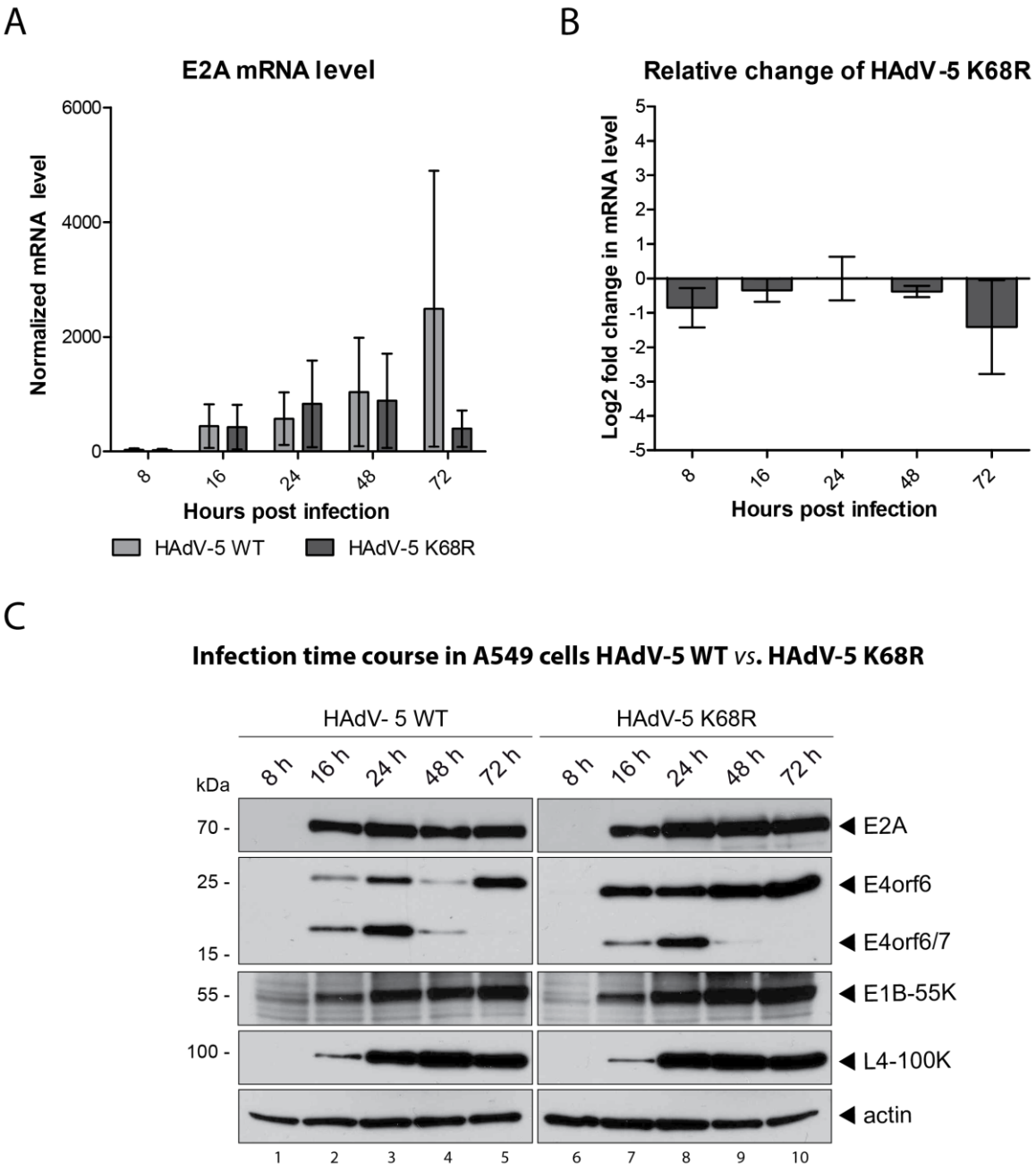


Figure 27: HAdV-5 WT and HAdV-5 K68R infection show similar mRNA and protein level of E2A. A549 cells were infected at an MOI of 20 with HAdV-5 WT or the HAdV-5 K68R mutant, which lacks the SUMOylation site at lysine 68 in E4orf6/7 and harvested after 8, 16, 24, 48 and 72 h p.i.. Afterwards RNA was isolated and after reverse transcription, specific primers (#1688; #1689) allowed the quantification of FAM111B mRNA level by semi-quantitative real-time PCR. Whole protein lysates were resolved via SDS-PAGE detected by immunoblotting with specific ABs for E2A (#113), E4orf6 and E4orf6/7 (#94), E1B-55K (#1), L4-100K (#275), and actin (88). The experiment was performed in biological duplicates **(A):** Absolute amount of E2A mRNA level of infected cells, normalized to GAPDH (ΔCt). **(B):** Relative mRNA level of HAdV-5 WT and HAdV-5 K68R infected cells given as Log2 fold change compared to HAdV-5 K68R infected cells (ΔΔCt). **(C):** Protein abundance of E2A during the course of infection.

5.2.4.1.2 *E4orf6/7 WT or E4orf6/7 K68R and E1A are sufficient for full activation of E2A promoter*

Since the E2A promoter activity was tested during transfection, it was aimed to confirm these results during infection, in the context of other viral proteins to make sure that no other viral proteins are required for SUMO-dependent transactivation of the E2A promoter. H1299 cells were transfected with luciferase reporter plasmid, containing the E2A promoter and subsequently infected with either HAdV-5 WT or HAdV-5 K68R and E2A promoter activity was measured 16 h p.i..

The infection with HAdV-5 WT and HAdV-5 K68R, showed a comparable promoter activity of about 2.5 to 3-fold (Figure 28). Therefore, these findings support the results of the transfection experiments, where also a comparable promoter activity of both E4orf6/7 variants was measured (Figure 16). In consequence, the experiments suggest that the loss of the SUMOylation site does not influence transactivation activity of E4orf6/7 during transfection or during infection.

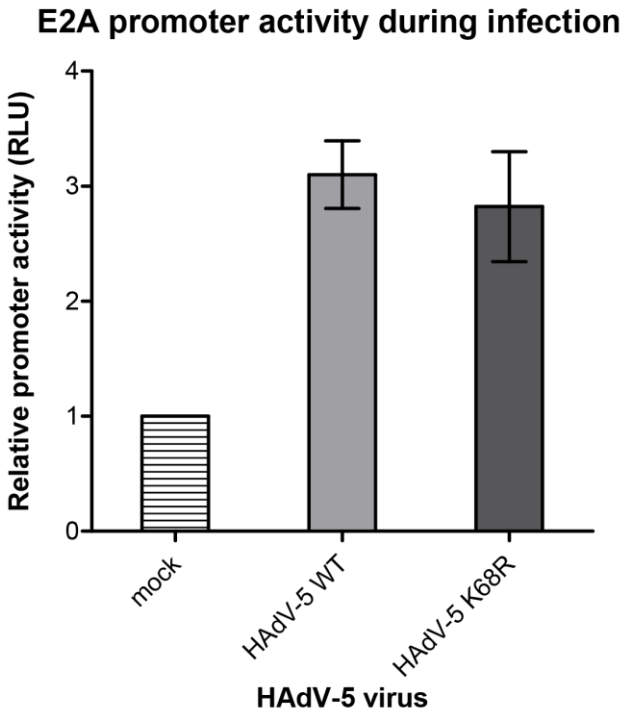


Figure 28: HAdV-5 WT and HAdV-5 K68R infected cells show comparable viral E2A promoter activity. H1299 cells were transfected with 0.5 µg pGL3-basic (firefly-Luc) luciferase reporter plasmids, containing the viral E2A and 0.5 µg of pRL-TK (renilla-Luc) containing the constitutively active TK promoter, for normalization. 6 h p.t. cells were infected at an MOI of 20 with HAdV-5 WT or the HAdV-5 K68R mutant, which lacks the SUMOylation site at lysine 68 in E4orf6/7. 16 h p.i. the relative light units (RLU) of both luciferases were measured in a dual luciferase assay and normalized to the overall renilla activity of all samples. The relative promoter activity describes the activity of the target promoter compared to the non-infected (Mock) cells. The experiment was performed in biological triplicates.

5.2.4.2 Influence of HAdV-5 WT and HAdV-5 K68R on the transactivation of the E2F-1 promoter and interaction with the E2F-1 protein

5.2.4.2.1 Comparable E2F-1 mRNA and protein levels during HAdV-5 WT and HAdV-5 K68R infection

In order to make sure that during the transfection experiments neither a component of the E2F-1 promoter in the luciferase reporter plasmid, nor a viral interaction partner of E4orf6/7 was missing for SUMO-dependent E2F-1 promoter activation, the mRNA levels of E2F-1 were measured during infection and the corresponding protein abundance was monitored by immunoblotting.

The absolute mRNA levels of the non-infected (Mock) cells show comparable levels throughout the 72 h time course experiment (Figure 29 A). In contrast, the HAdV-5 WT and HAdV-5 K68R infected cells showed increased levels of E2F-1 mRNA compared to the non-infected cells until 24 hp.i.. Afterwards E2F-1 mRNA levels of infected cells declined at time points 48 and 72 h p.i. and fell below the level of non-infected cells. However, there was no significant difference in mRNA levels when HAdV-5 WT and HAdV-5 K68R infected cells were compared directly (Figure 29 B). The log2 fold change in mRNA levels was identical at all time points. These results are supported by the protein abundance of E2F-1. Non-infected cells showed a constant protein abundance, whereas the HAdV-5 WT and HAdV-5 K68R infected cells revealed slightly increased protein abundances until 24 h p.i., which then declined at later time points (Figure 29 B).

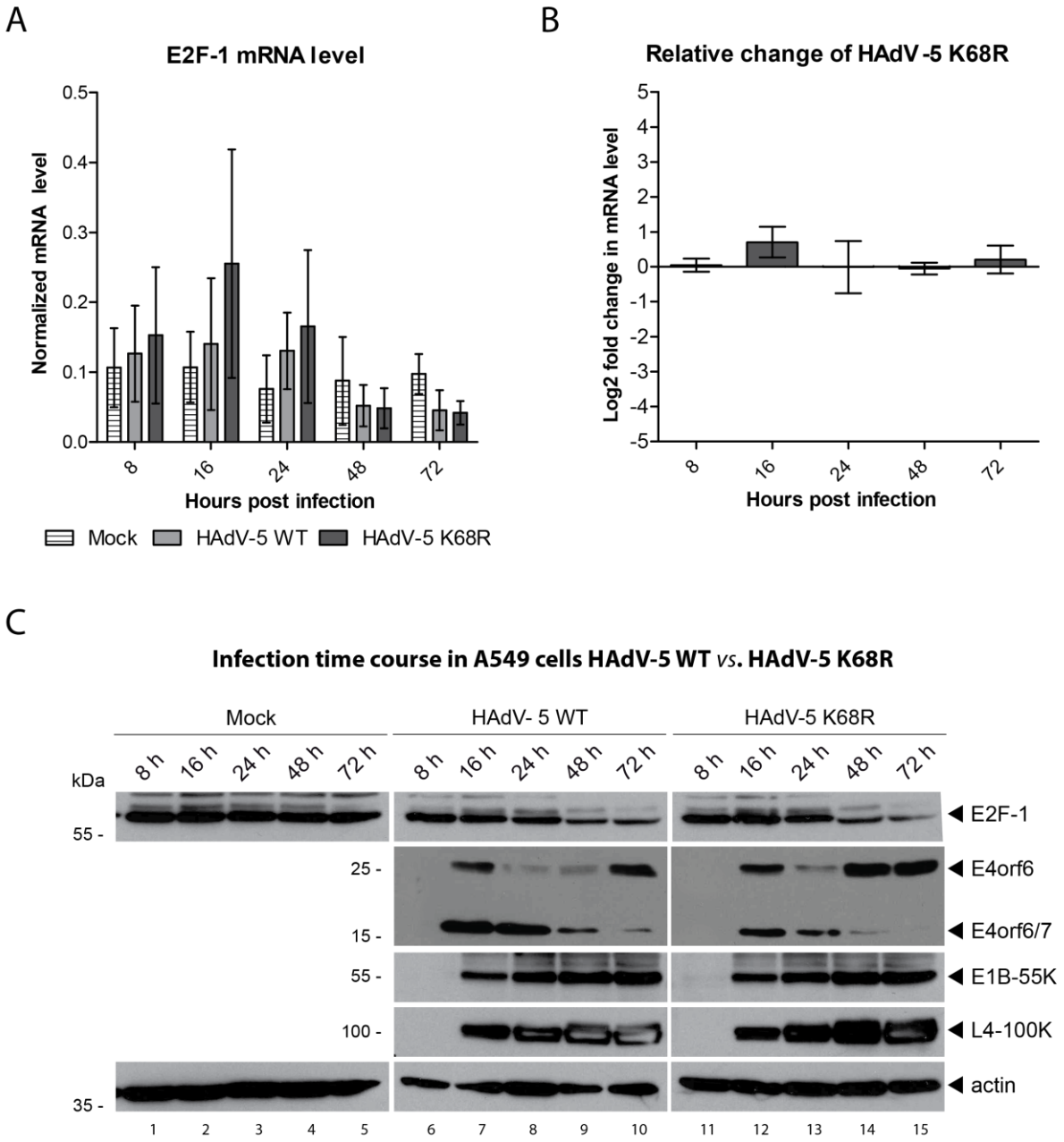


Figure 29: Comparable E2F-1 mRNA and protein levels during infection with HAdV-5 WT and HAdV-5 K68R. A549 cells were infected at an MOI of 20 with HAdV-5 WT or the HAdV-5 K68R mutant, which lacks the SUMOylation site at lysine 68 in E4orf6/7, and harvested 8, 16, 24, 48 and 72 h p.i.. Afterwards RNA was isolated and after reverse transcription, specific primers (#3413; #3414) allowed the quantification of E2F-1 mRNA levels by semi-quantitative real-time PCR. Whole cell lysates were resolved via SDS-PAGE and E2F-1 proteins were detected by immunoblotting using specific ABs for E2F-1 (sc-251), E4orf6 and E4orf6/7 (#94), E1B-55K (#1), L4-100K (#275) and actin (#88). The experiment was performed in biological triplicates. **(A):** Absolute E2F-1 mRNA levels of non-infected (Mock) and infected cells, normalized to GAPDH (ΔCt). **(B):** Relative amount of mRNA level of HAdV-5 WT and HAdV-5 K68R infected cells given as Log2 fold change compared to mRNA level of HAdV-5 K68R infected cells (ΔΔCt). **(C):** Protein abundance of E2F-1 in non-infected (Mock) as well as HAdV-5 WT and HAdV-5 K68R infected cells.

5.2.4.2.2 HAdV-5 WT and HAdV-5 K68R infection decrease E2F-1 promoter activity

In order to confirm the observations made in the luciferase assay during transfection, the E2F-1 promoter activity was additionally measured in a luciferase assay during infection. By that approach it was assured that no additional viral protein is required for E4orf6/7 SUMO-dependent promoter transactivation. H1299 cells were transfected with a luciferase reporter plasmid containing the E2F-1 promoter and subsequently infected with HAdV-5 WT or HAdV-5 K68R. E2F-1 promoter activity was measured 16 h p.i..

In contrast to the transfection experiments, where E4orf6/7 was overexpressed, the promoter activity in HAdV-5 WT and HAdV-5 K68R infected cells was slightly lower compared to the non-infected (Mock) cells at both MOIs (Figure 30). However, as in the transfection experiments, there was no significant difference in E2F-1 promoter activity between, HAdV-5 WT and HAdV-5 K68R infected cells.

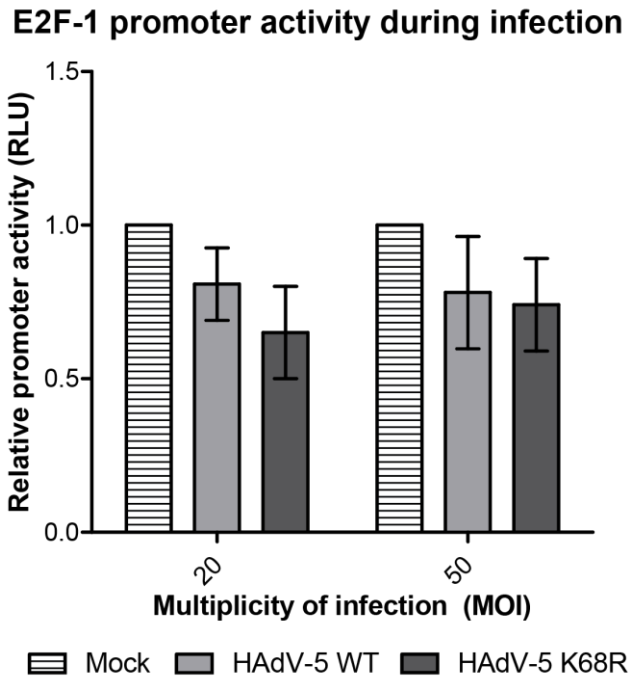


Figure 30: Comparable E2F-1 promoter activity during infection with HAdV-5 WT and HAdV-5 K68R. H1299 cells were synchronized for 24 h and then transfected with 0.5 µg pGL-3-Basic (firefly-luc) luciferase reporter plasmids containing the E2F-1 promoter and 0.5 µg of the pRL-TK (renilla-luc) luciferase reporter plasmid containing the constitutively active TK promoter, for normalization. 6 h p.t., cells were infected at an MOI of 20 or 50 with HAdV-5 WT or HAdV-5 K68R and further propagated in 5 % FCS DMEM. Luciferase activities were measured 16 h p.i. in technical triplicates and normalized to the activity of the TK promoter. The relative promoter activity describes the activity of the target promoter compared to the non-infected (Mock) cells. The experiment was performed in technical triplicates.

5.2.4.2.3 HAdV-5 infection leads to nuclear E2F-1 accumulation

Former publications showed that E4orf6/7 can bind to several E2Fs and additionally re-localizes E2F-4 from the cytoplasm into the nucleus (Schaley et al., 2005). Since it has been suggested that E2F-1 responsive promoters are the physiological relevant promoter of E4orf6/7, it was tested if the viral protein can also re-localize E2F-1 into the nucleus (Schaley et al., 2000; O'Connor & Hearing, 1994). H1299 cells were synchronized for 24 h by serum starvation and infected with HAdV-5 WT or HAdV-5 K68R. Afterwards cells were further incubated with 5 % FCS to reduce basal E2F-1 expression.

In the non-infected (Mock) cells, E2F-1 was localized in the cytoplasm and in slightly higher amounts in the nucleus (Figure 30; a, d). In the infected samples, the majority of cells expressed high amounts of viral proteins, indicating a high rate of infection and efficient virus replication (Figure 31; g, k). These cells showed no defined E2F-1 aggregation, but an increased abundance of E2F-1 in the cells and an increased accumulation in the nucleus (Figure 31; b, f, j). Nevertheless, there was no co-localization with viral proteins observed in the merged images (Figure 31; d, h, l). In summary, upon infection with HAdV-5 WT and HAdV-5 K68R, no defined aggregation of E2F-1 but a general increase in E2F-1 protein amounts and an accumulation in the nucleus was observed.

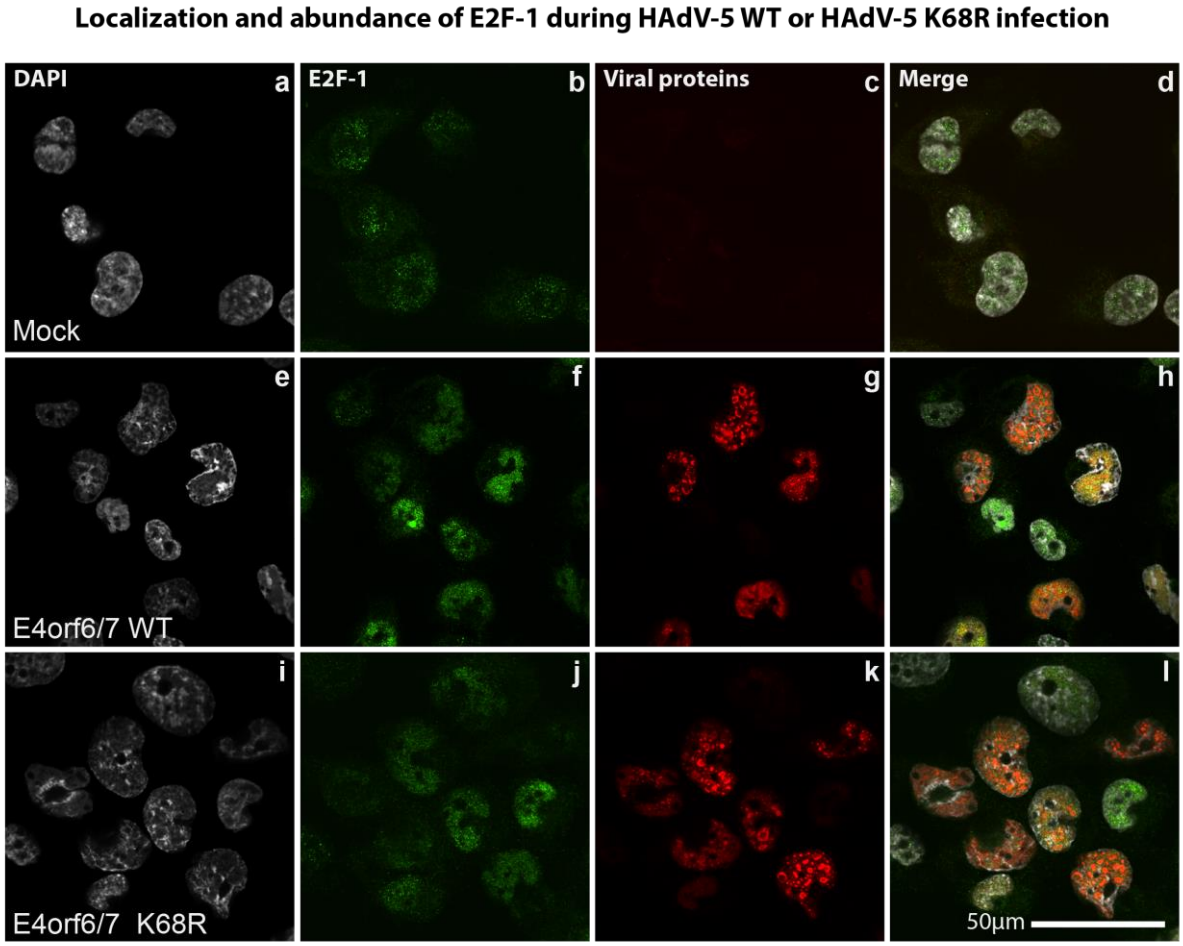


Figure 31: Comparable E2F-1 abundance and localization into the nucleus upon infection with HAdV-5 WT or HAdV-5 K68R. H1299 cells were synchronized by serum starvation for 24 h and infected with HAdV-5 WT or HAdV-5 K68R at an MOI of 20. Afterwards cells were further propagated in 5 %FCS DMEM and 24 h p.i. fixed with 4 % PFA and stained with specific ABs. Images were recorded with a spinning disc microscope. **(a, e, i):** Chromatin staining with DAPI; **(b, f, j):** E2F-1 staining (sc-251**)**; **(c, g, k):** Staining for E4orf6/7 and E4orf6 (#94); **(d, h, l):** Merged picture of all three stainings.

In order to test, if the increased nuclear accumulation of E2F-1 was mediated by E4orf6/7, a comparative transfection experiment with E4orf6/7 WT and K68R was performed. By this approach, E2F-1 localization could be observed in the presence of E4orf6/7 WT and K68R alone. H1299 cells were synchronized and transfected with plasmids encoding E4orf6/7 WT or E4orf6/7 K68R and afterwards further propagated in DMEM with 5 % FCS to reduce basal E2F-1 expression.

The immunofluorescence images showed high overexpression of both E4orf6/7 variants (Figure 32; g, k), However, in contrast to the infection experiment, the presence of E4orf6/7 WT or K68R did not lead to an accumulation of E2F-1 in the nucleus (Figure 32; f, j).

Taken both experiments together, there was a comparable E2F-1 localization between HAdV-5 WT and HAdV-5 K68R infected or the E4orf6/7 WT and E4orf6/7 K68R transfected cells. Furthermore, although the infection with both viruses led to the nuclear accumulation of E2F-1, this effect cannot

be attributed to E4orf6/7 since no nuclear accumulation was observed during transfection in the presence of E4orf6/7 WT and K68R alone.

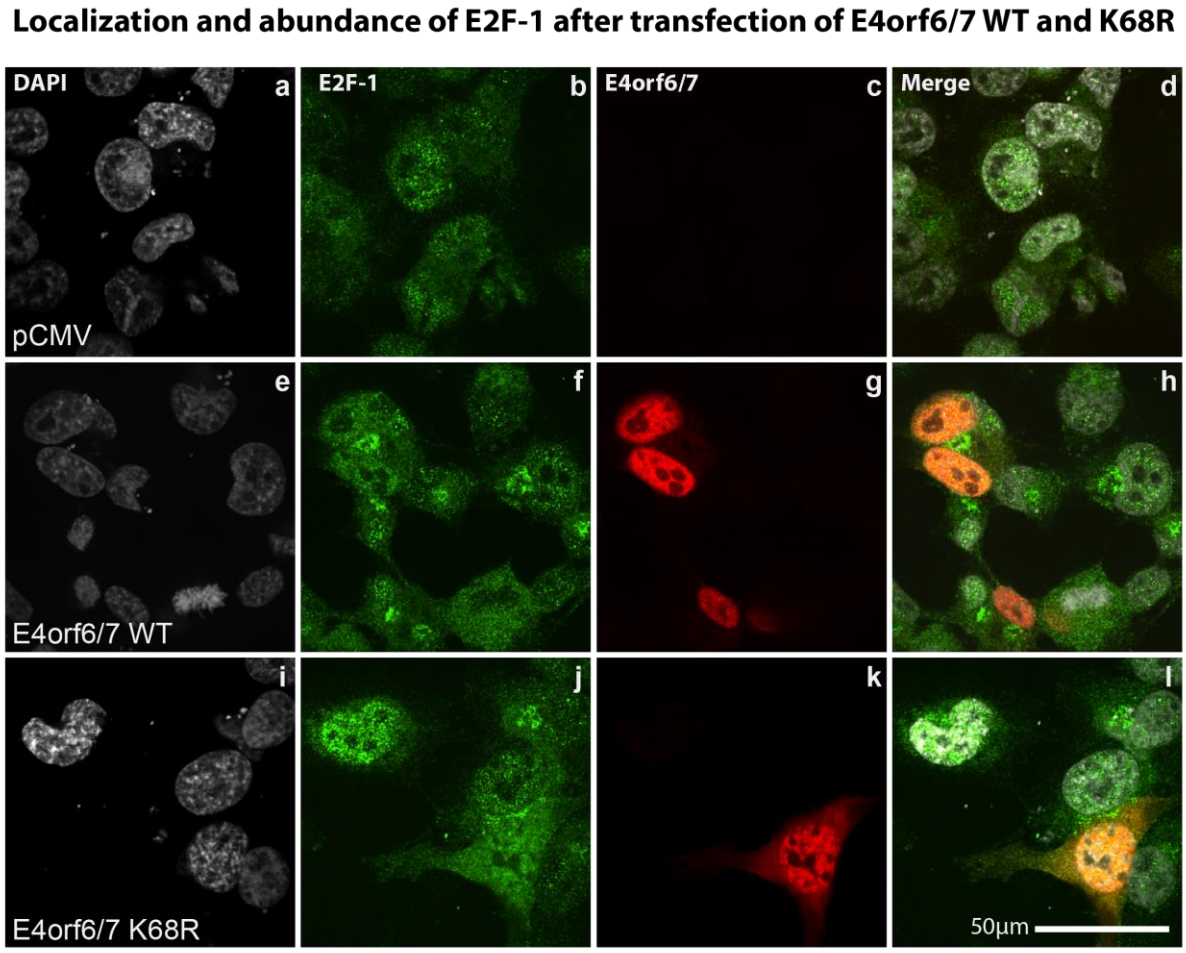


Figure 32: E4orf6/7 WT and E4orf6/7 K68R do not re-relocalize E2F-1 into the nucleus. H1299 cells were synchronized by serum starvation for 24 h and transfected with plasmids encoding for E4orf6/7 WT or K68R. 48 h p.t. cells were fixed and stained with ABs. Images were recorded with a Leica DMI 600B microscope **(a, e, i):** Chromatin staining with DAPI; **(b, f, j):** E2F-1 staining (sc-251); **(c, g, k):** Staing for E4orf6/7 and E4orf6 (#94); **(d, h, l):** Merged picture of all three stainings.

5.2.4.1 Influence of HAdV-5 WT and HAdV-5 K68R on the transactivation of the E2F-4 promoter and interaction with the E2F-4 protein

5.2.4.1.1 SUMO modification does not influence E2F-4 mRNA level and protein abundance

E2F-4 has been strongly associated with E4orf6/7 in several studies. It was already shown, that they interact and activate the viral E2A promoter, but also that E4orf6/7 is able to re-localize E2F-4 into the nucleus (Schaley et al., 2005; O'Connor et al., 2001). Since they are so closely connected, it was tested if E4orf6/7 activates the promoter of E2F-4. However, due to the lack of a suitable luciferase

reporter plasmid containing the E2F-4 promoter, E2F-4 mRNA levels were measured by qPCR and the protein abundance was monitored by immunoblotting.

The mRNA levels of the non-infected (Mock) cells gradually decreased until 24 h p.i.. Afterwards the mRNA level increased again and reached at 72 h p.i. comparable amounts as 8 h p.i. (Figure 33 A). In contrast, the mRNA levels of HAdV-5 WT and HAdV-5 K68R infected cells gradually decreased and approached the detection limit after 48 h p.i. However, there was now significant difference between HAdV-5 WT and HAdV-5 K68R infected cells (Figure 33 B). A similar dynamic was observed in the abundance of E2F-4 proteins (Figure 33 C). The non-infected cells showed over time slightly decreasing protein levels of E2F-4 and a slight increase after 72 h p.i.. The protein abundance in both infected cells decreased over time and reached the detection limit after 48 h. Taken together, HAdV-5 infection decreases the transcription of E2F-4, leading to decreased protein levels in the course of infection. Nevertheless, the modulation is not influenced by the loss of the SUMOylation site, since HAdV-5 WT and HAdV-5 K68R infected cells, show a comparable phenotype.

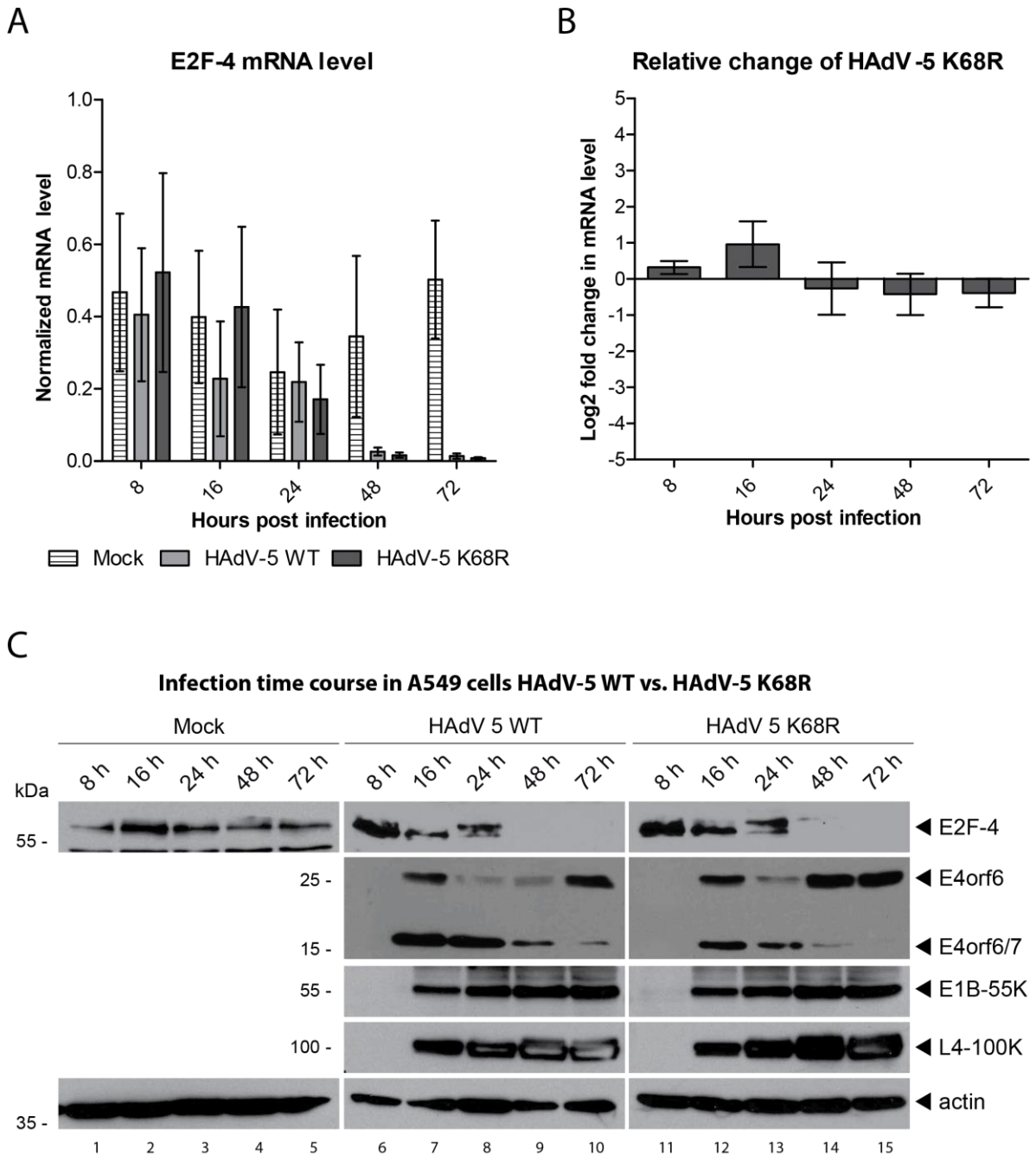


Figure 33: Comparable E2F-4 mRNA and protein levels during infection with HAdV-5 WT and HAdV-5 K68R. A549 cells were infected with HAdV-5 WT or HAdV-5 K68R, which lacks the SUMOylation site at lysine 68 in E4orf6/7. Cells were harvested 8, 16, 24, 48 and 72 h p.i.. Afterwards RNA was isolated and after reverse transcription specific primers (#3415; #3416) allowed the determination of E2F-4 mRNA level by semi-quantitative real-time PCR. Whole cell lysates were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for E2F-4 (sc-398543), E4orf6 and E4orf6/7 (#94), E1B-55K (#1), L4-100K (#275) and actin (#88). Experiments were performed in biological triplicates. **(A):** Absolute E2F-4 mRNA levels of non-infected (Mock) and infected cells, normalized to GAPDH (ΔCt). **(B)** Relative amount of mRNA level of HAdV-5 WT and HAdV-5 K68R infected cells given as Log2 fold change compared to mRNA level of HAdV-5 K68R infected cells (ΔΔCt). **(C):** Protein abundance of E2F-4 in non-infected (Mock) as well as HAdV-5 WT and HAdV-5 K68R infected cells.

5.2.4.1.2 SUMOylation does not influence the re-localization of E2F-4 by E4orf6/7

In contrast to other E2F family members, E2F-4 does not contain a nuclear localization signal. Therefore, E2F-4 relies on the pRB-family proteins p107 and 130 and the DP proteins for the re-localization into the nucleus (Allen et al., 1997; Magae et al., 1996). Schaley and his colleagues identified E4orf6/7 to be the viral analog to p107, which does not only re-localize E2F-4, but also dimerizes E2F-4 for the binding to E2F-1 responsive promoters (Schaley et al., 2005; O'Connor et al., 2001; Ginsberg et al., 1994). According to their studies, the E4orf7 C-terminus is essential for E2F-4 re-localization into the nucleus in HeLa cells (Schaley et al., 2005). As the SUMOylation site of E4orf6/7 is located in the C-terminus, it was tested if the SUMO modification impairs the re-localization of E2F-4. HeLa parental cells were transfected with an expression plasmid encoding for E2F-4 coupled to eGFP and 24 h p.t. cells were infected with either HAdV-5 WT or HAdV-5 K68R and images were recorded with a spinning disc microscope.

The intense green signal in the transfected cells confirmed the overexpression of E2F-4 coupled eGFP (Figure 33; g, l, q) and the high abundance and number of infected cells (Figure 34; m, r) indicate efficient infection and replication. In non-infected (Mock) cells, E2F-4-eGFP was exclusively present in the cytoplasm (Figure 34; g). However, upon infection with HAdV-5 WT and HAdV-5 K68R, E2F-4-eGFP was partially re-localized into the nucleus in comparable amounts (Figure 34; n, s). In conclusion, the results of Schaley *et. al.* were confirmed but the re-localization seems not to be influenced by the loss of the SUMOylation site.

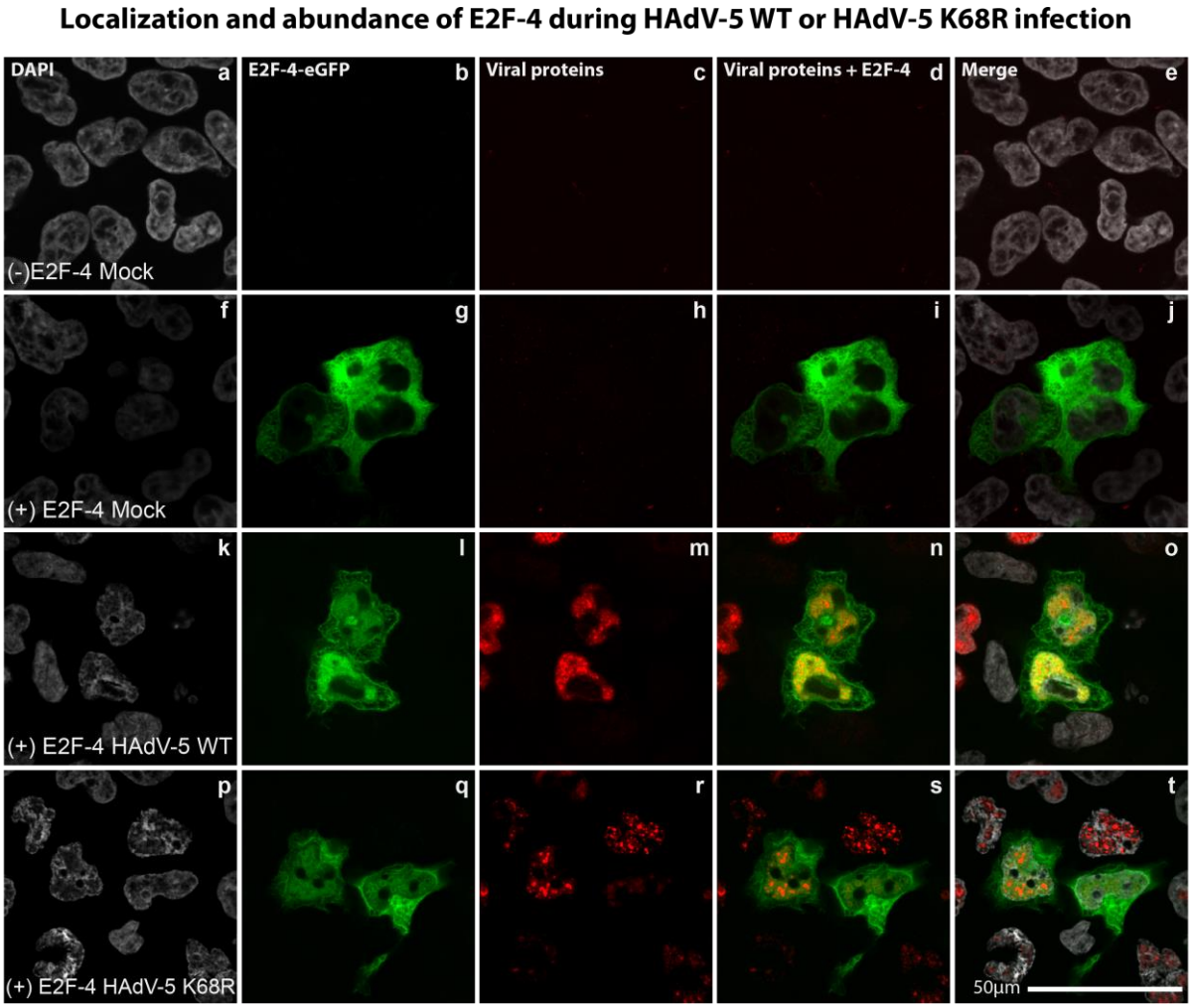


Figure 34: HAdV-5 WT and HAdV-5 K68R infection induce nuclear accumulation of E2F-4. HeLa parental cells were transfected with a plasmid encoding for E2F-4-eGFP and 24 h p.t. infected with HAdV-5 WT and HAdV-5 K68R. 24 h p.i. cells were fixed with 4 % PFA and stained with specific ABs for E4orf6 and E4orf6/7 (#94). Images were recorded with a spinning disc microscope. **(a, f, k, p):** Chromatin staining with DAPI; **(b, g, l, q):** eGFP signal; **(c, h, m, r):** Staining for E4orf6/7 and E4orf6 (#94); **(e, j, o, t):** Merged image of all three stainings.

5.2.4.1 Influence of HAdV-5 WT and HAdV-5 K68R on the transactivation of the FAM111B promoter and interaction with the FAM111B protein

5.2.4.1.1 Infection with HAdV-5 K68R site leads to a significant increase of FAM111B mRNA levels

During the transfection experiments, both E4orf6/7 variants revealed an increased but comparable transactivation activity on the FAM111B promoter. Therefore, the FAM111B mRNA levels during the course of infection were measured by qPCR and the protein abundance was monitored by immunoblotting.

In the time course of 72 h, the non-infected (Mock) cells, showed steady mRNA levels. In contrast, the infection with HAdV-5 WT and HAdV-5 K68R led to an increase in FAM111B mRNA levels early

during infection at 8 and 16 h p.i.. At later time points FAM111B mRNA levels decreased again and reached the level of non-infected cells 48 h p.i. (Figure 35 A). Intriguingly, the initial increase in FAM111B mRNA was significantly higher in HAdV-5 K68R infected cells compared to the infection with HAdV-5 WT. When compared directly, FAM111B mRNA levels were about 6 to 8-fold higher at time points 8, 16 and 24 h p.i. (Figure 35 B). However, this increase is not reflected in protein abundance. Whereas the FAM111B protein abundance in non-infected cells was at a constant level, HAdV-5 WT and HAdV-5 K68R infection caused a significant decrease in protein abundance and reached the detection limit 48 h p.i. (Figure 35 C). Consequently, the loss of the SUMOylation site seems to increase the level of FAM111B mRNA during the infection with HAdV-5 K68R, whereas the protein levels decreased comparably to HAdV-5 WT infection.

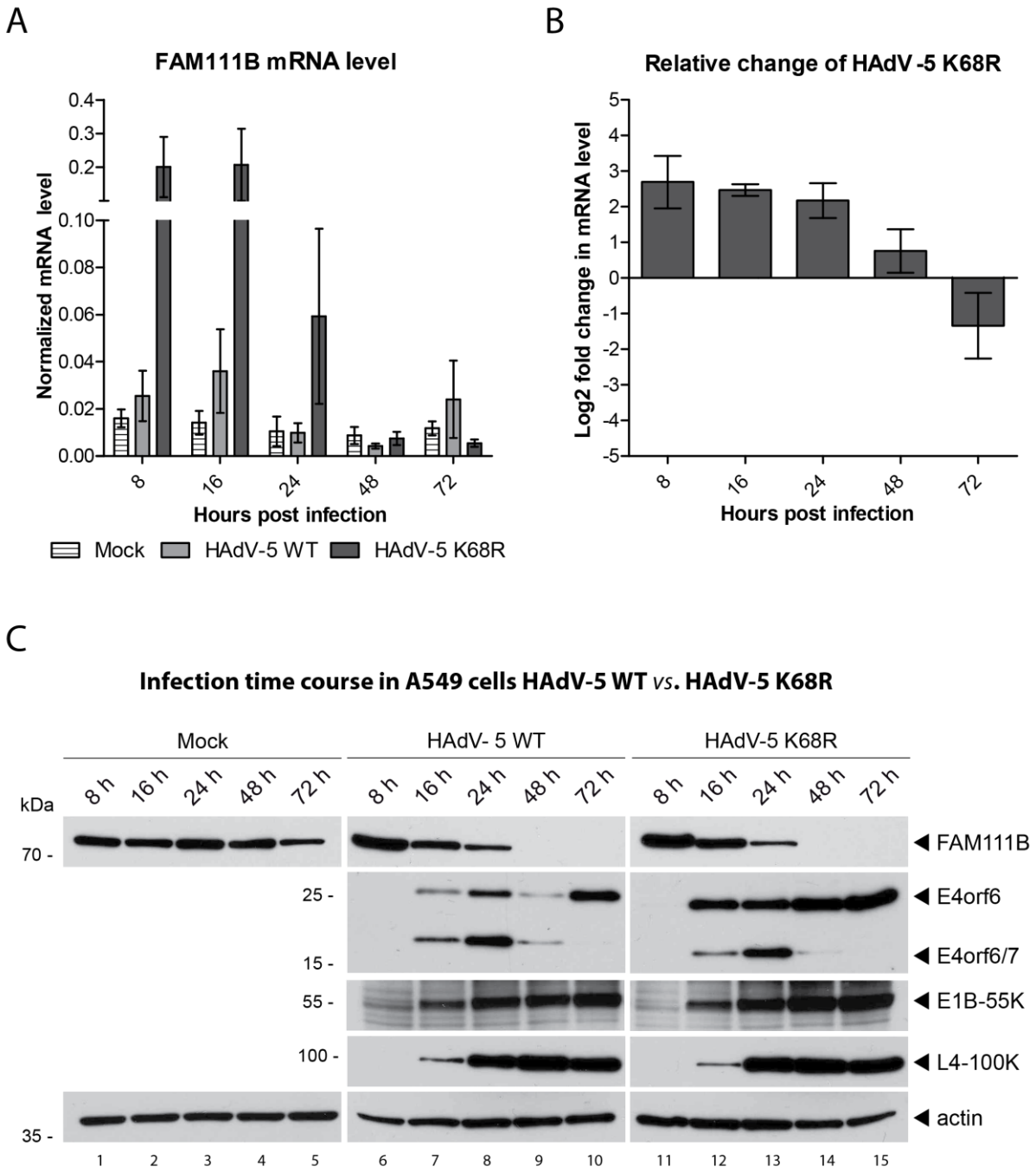


Figure 35: Significantly increased FAM111B mRNA level but comparable protein abundance in HAdV-5 WT and HAdV-5 K68R infected cells. A549 cells were infected with HAdV-5 WT or HAdV-5 K68R, which lacks the SUMOylation site at lysine 68 in E4orf6/7. Cells were harvested 8, 16, 24, 48 and 72 h p.i.. RNA was isolated and after reverse transcription specific primers (#3411;#3412) allowed the determination of FAM111B mRNA level by semi-quantitative real-time PCR. Whole cell lysates were resolved via SDS-PAGE and visualized by immunoblotting with specific ABs for FAM111B (#480), E4orf6 and E4orf6 (#94), E1B-55K (#1), L4-100K (#275) and actin (#88). The experiment was performed in biologcial triplicates. **(A):** Absolute FAM111B mRNA levels of non-infected (Mock) and infected cells, normalized to GAPDH (ΔCt). **(B)** Relative amount of mRNA level of HAdV-5 WT and HAdV-5 K68R infected cells given as Log2 fold change compared to mRNA level of HAdV-5 K68R infected cells (ΔΔCt). **(C):** Protein abundance of FAM111B in non-infected (Mock) as well as HAdV-5 WT and HAdV-5 K68R infected cells.

5.2.4.1.2 HAdV-5 K68R does not significantly increase FAM111B promoter activity in luciferase assays

Because the mRNA levels of HAdV-5 K68R infected cells were considerably increased, although there was no difference in promoter activity during transfection, FAM111B promoter activity was tested during infection in a luciferase assay. H1299 cells were transfected with a luciferase reporter plasmid containing the FAM111B promoter and 6 h p.t. infected with HAdV-5 WT or HAdV-5 K68R. Infected cells were harvested 16 h p.i..

The promoter activities of both, HAdV-5 WT and HAdV-5 K68R infected cells, were increased at both MOIs (Figure 36). However, the FAM111B mRNA levels of HAdV-5 K68R were comparable to HAdV-5 WT cells. Only at an MOI of 50 the HAdV-5 K68R infected cells showed a slightly increased promoter activity These observations are in contrast to the previous results observed in the infection time course, where HAdV-5 K68R infection caused an 8-fold increase of FAM111B mRNA levels compared to HAdV-5 WT infection (Figure 35 B).

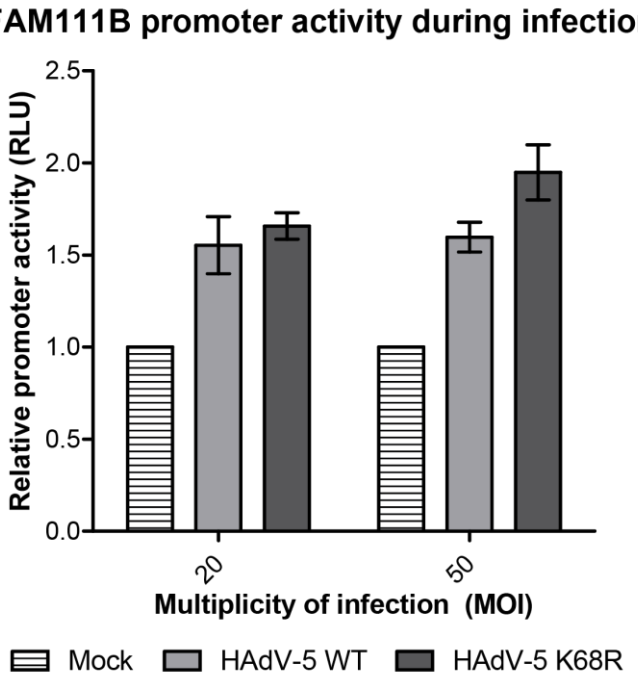


Figure 36: Comparable FAM111B promoter activity after HAdV 5 WT and HAdV 5 K68R infection. H1299 cells were synchronized for 24 h and then transfected with 0.5 µg pGL-3 Basic (firefly-luc) luciferase reporter plasmids containing the FAM111B promoter and 0.5 µg of the pRL-TK (renilla-luc) luciferase reporter plasmid, containing the constitutively active TK promoter, for normalization. 6 h p.t. cells were infected at an MOI of 20 or 50 with HAdV-5 WT or HAdV-5 K68R and further propagated in 5 % FCS DMEM. The relative light units (RLU) of both luciferases were measured 16 h p.i. and normalized to the overall firefly activity of all samples. Relative promoter activity describes the activity of the target promoter compared to non-infected (Mock) cells. The experiment was performed in technical triplicates.

5.2.4.1.3 *Infection with HAdV-5 WT and HAdV-5 K68R decreases FAM111B protein level*

So far FAM111B is a poorly investigated protein and its function is not exactly known (Goussot et al., 2017; Shaboodien et al., 2013; Mercier et al., 2013). But since there was a massive increase in FAM111B mRNA levels upon infection with HAdV-5 K68R, the localization and the abundance of FAM111B was visualized in immunofluorescence experiments. H1299 cells were synchronized for 24 h by serum starvation and subsequently infected with HAdV-5 WT or HAdV-5 K68R. Afterwards cells were further propagated in 5 % FCS DMEM to reduce basal transcription of FAM111B.

The high abundance of adenoviral proteins indicated a high rate of infection and efficient replication (Figure 37; c, g, k). In the non-infected (Mock) cells, FAM111B localized predominantly diffuse in the nucleus but also small amounts were detected diffuse in the cytoplasm (Figure 37; b). Upon infection, FAM111B amounts decreased considerably (Figure 37; f, j), which is in accordance with previous protein level analysis in the time course experiment. However, there was no domain formation or co-localization with E4orf6 or E4orf6/7 observed, nor is there any difference between HAdV-5 WT and HAdV-5 K68R infected cells (Figure 37; d, h, l). In summary, the loss of the SUMOylation site seems not to influence the interaction or the localization of FAM111B. There was neither a re-localization of FAM111B upon infection, nor is there a difference between HAdV-5 WT and HAdV-5 K68R infected cells observed, except for a comparable decrease in FAM111B level.

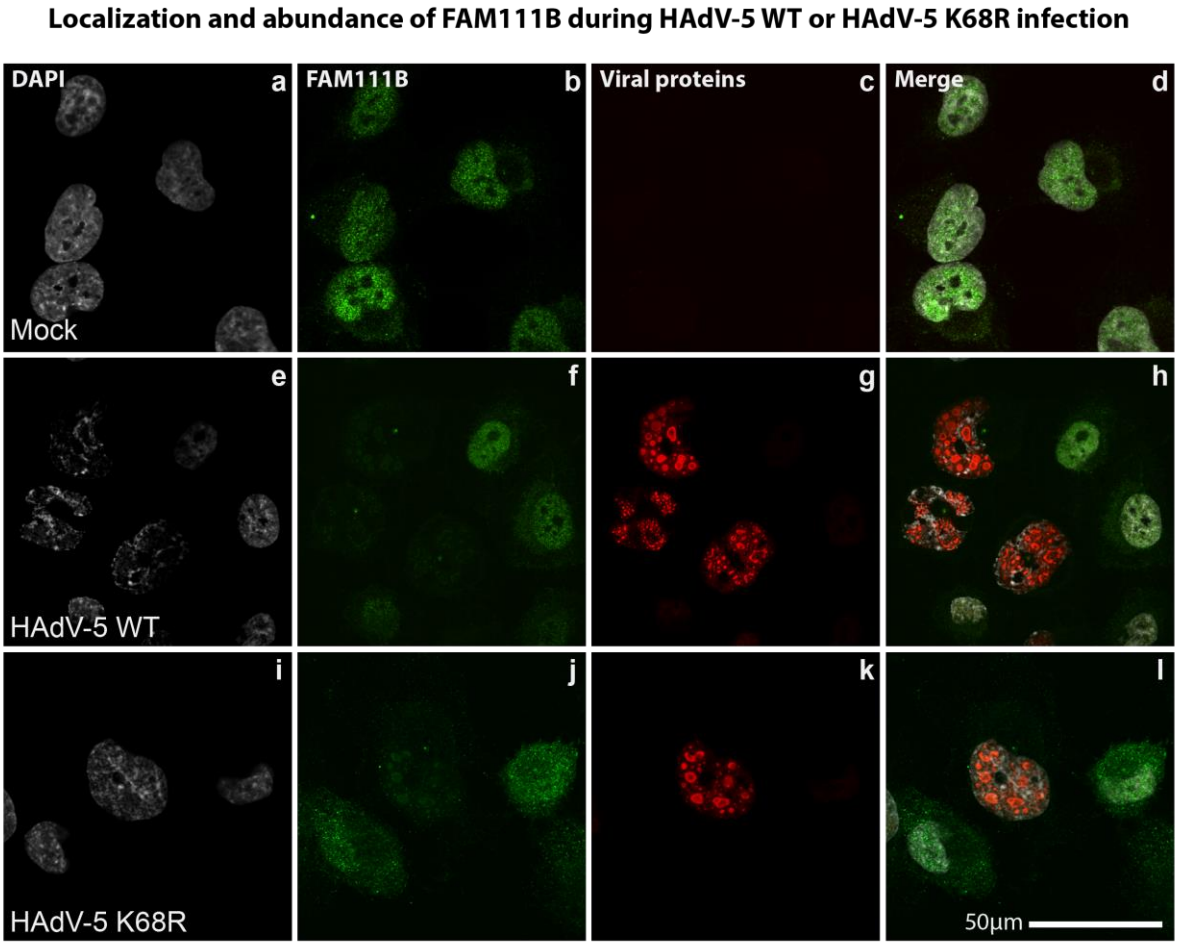


Figure 37: Decreased abundance of FAM111B upon infection with HAdV-5 WT and HAdV-5 K68R. H1299 cells were synchronized for 24 h by serum starvation and infected with HAdV-5 WT and HAdV-5 K68R at an MOI of 20 and further propagated in 5 % FCS DMEM. Cells were fixed with 4 % PFA and stained 24 h post infection with specific ABs. Images were recorded with a spinning disc microscope **a, e, i:** Chromatin staining with DAPI; **b, f, j:** E2F-1 staining (#480); **c, g, k:** Staining for E4orf6/7 and E4orf6 (#94); **d, h, l:** Merged picture of all three stainings.

6 Discussion

Ubiquitin and its family members, like the *small ubiquitin like modifiers* (SUMO), are post-translational modifications that are conjugated to proteins. The modification alters the properties of conjugated proteins and hugely increases the complexity of the proteome in eukaryotic cells. SUMO conjugation affects a lot of important biological processes and is required for cell viability in lower and higher eukaryotes as well as plants (reviewed in Hay, 2005). Besides a number of studies that have highlighted a critical role for SUMO in the regulation of the cell cycle, there is also increasing evidence that various human pathogens utilize the SUMO pathway to hijack the cell cycle control and prevent cellular antiviral defenses (reviewed in Wimmer et al., 2012; Ben-Israel & Kleinberger, 2002). Although SUMOylation has been studied intensively in the last decades, there are many open questions and enigmas. Despite many published SUMO induced functions and consequences resulting in alterations of protein activity, localization, half-life or interactome of target proteins, the molecular consequences for SUMO modified proteins are difficult to predict. More generally, the underlying principle of SUMOylation is to alter a modified substrate's inter- and/or intramolecular interaction (reviewed in Mattoscio, 2013; Geiss-Friedlander & Melchior, 2007; Hay, 2005). HAdV-5 encodes several regulatory proteins that are either targeted by the SUMO machinery or interact and influence the SUMOylation of other proteins (Freudenberger et al., 2018; Yousef et al., 2010; Muller & Dobner, 2008; Berscheminski et al., 2014). Above all, the involvement of E1B-55K with the SUMO system has been intensively studied. Besides the fact, that many functions of E1B-55K depend partially on the modification with SUMO, several publications described E1B-55K as a E3 SUMO ligase, that induces the SUMOylation of target proteins, as for example p53 (Pennella et al., 2010; Muller & Dobner, 2008). These observations tempted us to elucidate the E3 ligase activity of E1B-55K in a global *stable isotope labelling with amino acids* (SILAC) approach. Using this method, we were able to specifically analyze global virus infection with respect to E1B-55K induced changes in the SUMO proteome. Consequently, these facts underline the importance and the need to investigate the ability of human pathogens to modulate cell cycle and post-translational modifications. In an attempt to confirm the results of the SILAC experiments, which suggested that E4orf6/7 is targeted by the SUMO machinery and to identify the consequences of E4orf6/7 SUMOylation, we performed comparative experiments with E4orf6/7 WT *vs*. E4orf6/7 K68R during transfection and the course of HAdV-5 infection.

6.1 E4orf6/7 is targeted by the SUMOylation machinery

In order to detect and identify, yet unknown E1B-55K-dependent SUMO targets, cells were either infected with HAdV-5 WT or an E1B-55K deficient HAdV-5 (HAdV-5 ΔE1B-55K) virus mutant. Thereby,

SUMO targets were identified, which were significantly higher SUMOylated in the presence of E1B-55K during the course of HAdV-5 WT infection. Besides many cellular SUMO targets, we were able to detect also three viral E1B-55K-dependent SUMOylated targets. We decided to focus on the small viral fusion protein encoded in the *early region 4 orf6/7* (E4orf6/7). In particular, this protein was of interest, because it supports the switch from G_0 to S-phase, a critical step during the course of viral infection. Strikingly, we were able to confirm SUMOylation of E4orf6/7 in different set ups of SUMO pulldown experiments (Figure 12 + Figure 14). As already predicted by the *in-silico* analysis, the attachment of SUMO2 molecules at lysine residue 68 leads to a strong polySUMOylation of E4orf6/7 (Figure 13 + Figure 15). In addition, we detected small amounts of SUMOylation, in samples that lacked lysine 68 but retained lysine residue 114. We assume that the SUMO modification of this residue was artificially induced by the overexpression of 6His-SUMO2, because we detected only residual amounts of SUMOylated E4orf6/7 and lysine 114 is not in a context of a SUMO conjugation motif. Interestingly, in contrast to the SILAC experiment, E4orf6/7 seems to be SUMOylated in the presence as well as in the absence of E1B-55K. Therefore, it was unexpected, that several approaches with different viruses failed to detect SUMOylation of E4orf6/7 during the course of infection (Figure 22 - Figure 24). One putative explanation might be the so called “SUMO enigma”, which represents one of the most puzzling observations made with SUMO: Although only a low percentage of effector proteins are modified, the conjugation with SUMO has an enormous effect on the overall function of this specific protein (Muller & Dobner, 2008; Hay, 2005). Another explanation is provided by the observation that for example HAdV-5 E4orf6 has been described to be a negative regulator of E1B-55K SUMOylation (Lethbridge et al., 2003). Although an E4orf6/7 SUMO pulldown experiment with the E4orf6 deficient HAdV-5 (HAdV-5 ΔE4Orf6) virus mutant excluded E4orf6 as a negative regulator of E4orf6/7, it seems possible, that another viral factor might be involved (Figure 23). E1A, which cooperates with E4orf6/7, might be a promising candidate, because it was demonstrated to repress SUMOylation of the cellular protein pRB (Ledl et al., 2005; Huang & Hearing, 1989b). Hence, E1A could also work as a negative regulator for E4orf6/7 SUMOylation, by decreasing the abundance of SUMO modified E4orf6/7. In consequence, western blotting might be not sensitive enough, compared to mass spectrometry (MS), to detect small amounts of SUMOylated E4orf6/7. MS approaches use multiple signals that are integrated to a composite score, which is additionally verified by a number of performance characteristics, which is far more sensitive than western blotting. Therefore, Aebersold *et al.* posited that western blotting does not suffice the requirements for *state-of-the-art* research and validation of highly sensitive MS data by western blotting is no longer justified (Aebersold et al., 2013).

6.2 Consequences of E4orf6/7 SUMOylation and the importance for virus replication

6.2.1 The consequences of SUMO site inactivation on E4orf6/7 activities

6.2.1.1 Loss of SUMO site does not affect transactivation activity of E4orf6/7

Over the years, E4orf6/7 and E1A have been published to interact intensively with transcription factors of the E2F family, which are involved in the activation of E2F responsive genes (reviewed in Täuber & Dobner, 2001b; Ben-Israel & Kleinberger, 2002). Mutational analysis with E4orf6/7 identified two E2F binding sites as well as a dimerization induction site within the orf7 part, which allows the dimerization of two E2F transcription factors. Thereby, the affinity towards inverted E2F binding sites is enhanced. Interestingly, such an inverted binding site is encoded in the viral E2A promoter region (Huang & Hearing, 1989b; Obert et al., 1994). Therefore, it was initially tested, if the loss of the E4orf6/7 SUMOylation site affects the E2A promoter activity, since the SUMOylation site at position 68, is in close proximity to one of the E2F binding domains (Figure 13). Given the fact, that E4orf6/7 has a low molecular weight of 19.8 kDa, we hypothesized that: 1. The SUMOylation may blocks the dimerization of E2F molecules, thereby attenuating the transactivation activity or 2. The SUMOylation induces or increases the transactivation of other viral promoters like the E2L promoter.

The first hypothesis was tested by using functional approaches to measure the E2A promoter activity in a dual-luciferase reporter gene assay and by quantifying E2A mRNA levels by qPCR as well as by monitoring the protein dynamics during the course of infection. In the luciferase assay, the overexpression of E4orf6/7 WT and E4orf6/7 K68R increased E2A promoter activity comparably, and both variants showed a synergistic effect, when being co-transfected with E1A (Figure 16 A+B). These results were confirmed in a following luciferase assay during infection, with either HAdV-5 WT or HAdV-5 K68R, in which also comparable E2A promoter activities were measured (Figure 28). These findings are in line with the time course experiments with HAdV-5 WT or HAdV-5 K68R infected cells, where comparable mRNA and protein levels were detected (Figure 27). In consequence, these results confirm the transactivating functions of E4orf6/7 on the E2A promoter and suggest that the transactivation activity is a conserved and a robust function, which is not affected by the loss of the SUMOylation site (Obert et al., 1994; O'Connor & Hearing, 1991; Huang & Hearing, 1989b).

The second hypothesis was tested in luciferase assays using different adenoviral promoters. Studies showed that E4orf6/7 can compensate for the loss of E1A (O'Connor & Hearing, 2000). Consequently, E4orf6/7 has to be able to activate other adenoviral promoters besides the E2A promoter, although none of these contain known E2F binding sites. Intriguingly, we could show that E4orf6/7 WT and E4orf6/7 K68R indeed transactivate most of the tested adenoviral promoters (Figure 16 C). Still, an open question is how E4orf6/7 was able to activate these promoters. Most of the viral promoters

contain several binding motifs like TATA boxes, CAAT boxes as well as binding sites for general transcription factors like the *Specificity Protein 1* (SP1), *Activator Protein 1* (AP1), *Nuclear Factor 1* (NF1) and *Activating Transcription Factor* (ATF) that initiate and promote transcription. The E3 promoter is only one example, which is composed of multiple sequence elements like AP1, NF1 as well as a TATA-box (reviewed in Berk, 1986, 2013). Interestingly, these elements seem to act independently of each other, since individual deletions, as for example the TATA-box, attenuates transcription only by 20 % (Leff et al., 1985). Furthermore, many of these protein binding partners are higher expressed during cell proliferation. Since E4orf6/7 activates genes required for S-phase, one explanation for the activation of viral non-E2F promoters would be the upregulation of general transcription factors, which then activate the different adenoviral promoters, without E4orf6/7 being directly involved. A similar mechanism was described for latently Epstein-Barr Virus (EBV) infected cells. In response to EBV infection, ATF-2 is hyperphosphorylated activating not only the expression of the EBV gene EBER, but also expression of cellular transcription factors like TFIIIC and BDP1, which further enhance the transcription of EBER (Felton-Edkins et al., 2006).

In summary, the obtained results suggest that the loss of the SUMOylation site of E4orf6/7 does not affect the binding of E2F transcription factors, considering that the dimerization of E2F molecules via E4orf6/7 is a prerequisite for both: The synergistic effect of E1A and E4orf6/7 on the E2A promoter and the activation of the E2F-1 promoter. Strikingly, we were able to show that the transactivation activity of E4orf6/7 extends to other viral promoters, without known E2F binding sites but with binding sites for general transcription factors. This activation most probably occurs by S-phase induction and the increased expression of general transcription factors.

6.2.1.2 Loss of SUMO site does not impair N-terminal activities of E4orf6/7

E4orf6/7 is a fusion protein of the E4 open reading frames 6 and 7, generated by using several splice donor and acceptor combinations. The protein shares the 58 N-terminal amino acid (aa) residues of E4orf6 and the 92 aa residues encoded in orf7. Deletion experiments with E4orf6, attributed several functions to the N-terminus, like the nuclear localization and the interaction with cellular and viral proteins like E1B-55K (reviewed in Täuber & Dobner, 2001a,b). In E4orf6/7, the N-terminus is also responsible for the nuclear localization and the interaction with E1B-55K (Schaley et al., 2005; Rubenwolf et al., 1997). Since the SUMOylation site at lysine 68 is in close proximity to the N-terminus, we tested if the N-terminal activities are affected by the loss of the SUMOylation site.

For the dimerization of unbound E2F molecules and the induction of cooperative and stable binding to inverted E2F binding sites of viral and cellular promoters, E4orf6/7 has to enter the nucleus (Huang & Hearing, 1989b; Schaley et al., 2000; O'Connor & Hearing, 1991). This is accomplished by

the arginine-rich region in the N-terminus (Schaley et al., 2005). A growing list of cellular and viral proteins have been found to interact with importin β proteins via arginine-rich motifs, including human ribosomal proteins and the HIV proteins Tat and Rev. Importin β mediates the translocation of cargos to the nucleus via the nuclear core complex (Palmeri & Malim, 1999; Jäkel & Görlich, 1998; Truant & Cullen, 1999). Although this has never been shown for E4orf6/7 directly, it is believed that the re-localization of E4orf6/7 is achieved in an analogous way. The re-localization of E4orf6/7 into the nucleus was confirmed in three different cell lines and in all of them E4orf6/7 WT or E4orf6/7 K68R accumulated to comparable amounts in the nucleus (Figure 18). Even the experiments with the HeLa SU2 cell line, in which high amounts of SUMOylated E4orf6/7 were detected, support the observation that both E4orf6/7 variants accumulate to similar amounts. In conclusion, SUMOylation seems not to affect the re-localization of E4orf6/7. However, it remains an open question, if E4orf6/7 is re-localized into the nucleus by importin β, which should be further elucidated by more detailed interaction studies.

E1B-55K is a multifunctional protein mediating several critical steps during the early and late phase of infection (reviewed in Berk, 2007). Early during infection, it counteracts anti-proliferative processes, like p53-dependent apoptosis, induction of cell cycle arrest and the cellular DNA damage response (Weitzman & Ornelles, 2005; White, 2001). In the late phase of infection, E1B-55K stimulates efficient cytoplasmic accumulation and translation of late mRNAs (reviewed in Flint & Gonzalez, 2003; Dobner & Kzhyshkowska, 2001). In our studies, we could confirm the binding of E4orf6 as well as E4orf6/7 WT and E4orf6/7 K68R to E1B-55K. Therefore, we conclude, that the interaction with E1B-55K is not influenced by E4orf6/7 SUMOylation. However, compared to E4orf6, the binding affinity of E4orf6/7 WT and E4orf6/7 K68R to E1B-55K was rather weak. We assume that the stronger binding of E4orf6 was increased due to the BC-box motif, which binds, in addition to the N-terminus, to E1B-55K (Blanchette et al., 2004; Rubenwolf et al., 1997).

In summary, the loss of the SUMOylation site did not impair the N-terminal activities of E4orf6/7, as E4orf6/7 WT and E4orf6/7 K68R equally localized to the nucleus and showed a similar binding affinity to E1B-55K. Nevertheless, future experiments should address the following three questions: 1. Does E4orf6/7 interact with importin β or does it use an alternative pathway for the re-localization into the nucleus? 2. What is the importance of the E1B-55K interaction? 3. Is the binding of E1B-55K and E4orf6/7 dependent on the SUMOylation of E1B-55K?

6.2.2 The influence of SUMOylation on the viral replication cycle and the transforming potential of E4orf6/7

Efficient virus replication is achieved by the redirection of cellular pathways, involved in transcription and translation of mRNA as well as the replication of DNA. During the course of infection, viral proteins are made in a strict temporal sequence. By convention, early genes are defined as those expressed before the onset of viral DNA synthesis. These gene products are devoted to the synthesis of viral gene products, repression of host-cell immune responses and induction of S-phase, to establish an cellular environment that allows efficient viral replication (reviewed in Flint et al., 2009; Berk, 2007). In particular, the switch from G_0 to S-phase is a crucial step in the viral replication cycle. Cell proliferation is controlled by an orderly sequence of events during the mitotic cycle, where each stage is characterized by the expression of a certain set of genes. For example, during the onset of S-phase, proteins required for nucleotide biosynthesis are highly increased (Farnham et al., 1993). Many of these genes are controlled by promoters with binding sites for E2F-family transcription factors (Means et al., 1992; Pearson et al., 1991). E2F factors are encoded by at least eight genes, E2F-1 to E2F-8 and HAdV-5 E4orf6/7 was shown to bind at least five of them via its C-terminus (Schaley et al., 2000; Johnson & DeGregori, 2006). By using these transcription factors, E4orf6/7 induces S-phase to enable genome replication and increases as well as favors the transcription rate of the viral E2A promoter (reviewed in Täuber & Dobner, 2001b). Since the induction of cell proliferation is also a key step during E1A and E1B mediated transformation, we tested both, if the loss of the SUMOylation site in E4orf6/7 influences HAdV-5 replication or the process of E1A/E1B mediated cell transformation.

6.2.2.1 Comparable replication during HAdV-5 WT and HAdV-5 K68R infection

Adenoviruses show a remarkable redundancy in the functionality of their proteins. It has become apparent, that for example E4orf3 and E4orf6, independently augment viral DNA replication, late viral protein synthesis, shut-off of host protein synthesis, production of viral progenies as well as preventing concatemerization of viral genomes (Bridge & Ketner, 1989; Huang & Hearing, 1989a; Weiden & Ginsberg, 1994; Halbert et al., 1985). This is why individual deletions of E4orf3 and E4orf6 result in a moderately impaired replication, whereas a HAdV-5 double mutant, which is negative for both E4orf3 and E4orf6, has a severe replication defective phenotype (Halbert et al., 1985). A similar redundancy has been observed between E1A and E4orf6/7. However, E1A is the dominant factor that allows efficient viral replication. E1A is the first viral gene to be transcribed after the adenoviral genome entered the nucleus. The presence of the major E1As 12S and 13S does not only significantly increase the transcription of all other early genes, including the E4 region, but also activate many

genes required for driving cells into S-phase. Therefore, HAdV-5 E1A deletion mutants show severe growth defects (reviewed in Ben-Israel & Kleinberger, 2002; Berk, 1986). In contrast, individual ORF deletions or knockouts in the E4 region, like E4orf6/7, result only in moderate to weak replication defects, indicating that E4 products provide additional but rather minor functions (Bridge & Ketner, 1989; Huang & Hearing, 1989a; Halbert et al., 1985). Nevertheless, E4orf6/7 was shown to partially rescue the replication defect of E1A negative virus mutants (O'Connor & Hearing, 2000). In our experiments with HAdV-5 WT and HAdV-5 K68R, we detected comparable replication (Figure 26). Since both viruses contain E1A, we assume that the loss of the SUMOylation site did not affect the adenoviral genome replication or the viral progeny production for two reasons: 1. There was only a moderate impaired protein stability, which resulted in slightly decreased amounts of E4orf6/7 during HAdV-5 K68R infection (Figure 27 and Figure 35) and 2. E1A is very efficient in forwarding cells from G_0 to S-phase. Therefore, the dominant nature of E1A most probably compensated the reduced abundance of E4orf6/7 leading to similar HAdV-5 WT and HAdV-5 K68R genome replication and virus progeny production. In consequence, future experiments should be performed with a virus that is deficient for E1A. For example, O'Connor & Hearing employed in one of their studies a HAdV-5 that is deficient for E1A and additionally overexpresses E4orf6/7 by the introduction of a CMV promoter (O'Connor & Hearing, 2000).

6.2.2.2 E4orf6/7 WT and E4orf6/7 K68R have a similar oncogenic potential

Until now, there is no reported case in which adenoviruses are described as being the cause of tumor formation in humans. And still, adenoviruses of the group A and C were the first pathogens found to induce malignant tumors following injection into newborn hamsters (Huebner et al., 1962; Trentin et al., 1962). This seminal discovery inspired a period of intense research in the field of virus-mediated transformation. In particular, the ability of the HAdV-2 and 5 E1 gene products, which are able to immortalize and transform primary mammalian cells in cell culture, have been an immense value for elucidating key events in cellular and viral growth control. Today, it is well established that E1-mediated transformation is a multistep process that requires a complex interplay between viral and cellular proteins. The E1 region encodes two separate transcription units E1A and E1B, which have been shown to be necessary and sufficient for oncogenic transformation of primary rodent cells (reviewed Endter & Dobner, 2004). As understood at present, the major E1A species 12S and 13S mediate the most critical step in cell transformation, by driving cells into S-phase by releasing and activating E2F transcription factors and at the same time repressing other genes by sequestering limiting factors for transactivation, like histone-directed acetyltransferases and other proteins involved in chromatin remodeling (Haruta et al., 2015; Pelka et al., 2009; Cress & Nevins, 1996;

Kovesdi et al., 1986b,a). On the other hand, these activities also stimulate apoptosis and growth arrest through the stabilization of the tumor suppressor protein p53 (Debbas & White, 1993; Lowe et al., 1993). To accomplish complete transformation, these pro-apoptotic and growth arresting activities are efficiently counteracted by E1B gene products. Although, the major E1B proteins E1B-55K and E1B-19K do not have transforming properties on their own, they markedly contribute to the transformation frequency of E1A through the inhibition of p53-dependent and p53-independent apoptosis (Debbas & White, 1993). In previous studies, E4orf6/7 was identified as a factor that decreases E1 mediated transformation (Yamano et al., 1999; Nevels et al., 1997). Since SUMOylation of E1B-55K plays an important role during the transformation process, it was important to test if the loss of the SUMOylation site also affects its transforming potential of E4orf6/7 (Endter et al., 2001). Strikingly, the results obtained from *focus* formation assays support the observations described by other researchers (Yamano et al., 1999; Nevels et al., 1997). Primary baby rat kidney (pBRK) cells transfected with E1A, E4orf6/7 WT or E4orf6/7 K68R expression vectors died, whereas those transfected with plasmids encoding the whole E1 region (full length E1A and E1B), induced a high number of *focus* formation. However, upon co-transfection of E4orf6/7 WT or E4orf6/7 K68R with the E1 region, the number of *foci* decreased, compared to the *focus* forming activity of the E1-region alone (Figure 20). Former studies already showed, that the reduction of *foci* formation was mainly caused by the induction of p53-dependent apoptosis (Yamano et al., 1999). Our data support this hypothesis and we assume that p53-dependent apoptosis might be induced by E4orf6/7 mediated transactivation of E2F-1, which is by now the only E2F-family member that has been shown to induce p53-dependent apoptosis (La Thangue, 2003; Johnson et al., 1994; Qin et al., 1994; Shan & Lee, 1994; Debbas & White, 1993). Further support for this notion comes from the observation that E4orf6/7 is able to additionally induce the expression of the E2F controlled gene p73, which contributes together with p53 and p63 to the induction of apoptosis (Shapiro et al., 2006; Flores et al., 2002). Therefore, it is likely that, the expression of E2F-1 and p73 induced by E4orf6/7, in addition to the stabilization of p53 by E1A, led to enhanced pro-apoptotic signals, which drove cells into apoptosis and impaired *foci* formation. In this context it is not surprising, that both E4orf6/7 WT and E4orf6/7 K68R revealed a similar transforming potential, since they have a comparable E2F-1 transactivation activity, inducing similar E2F-1 transcript levels and E2F-1 protein abundance, which will be discussed later (6.3.1).

6.2.3 SUMO site inactivation leads to decreased stability of E4orf6/7

Protein half-lives within a cell can vary widely, from minutes to several days. Differential rates of protein turnover are an important aspect of cell cycle regulation. Rapid changes in protein stability, for example by post-translational modifications, allow the cell to quickly respond to external stimuli

(reviewed in Cooper, 2000). On the other hand, stabilization or degradation of proteins is a commonly observed phenomenon among virus infections in order to support efficient virus replication. One prominent candidate is p53, which is targeted by several human pathogenic viruses to either induce or prevent apoptosis (Wang et al., 2012; Sato et al., 2009; Querido et al., 1997). By now, it is well known, that SUMO modification is able to induce both, stabilization and destabilization of target proteins (Collot-Teixeira et al., 2004). On the one hand, SUMO molecules can shield lysine residues to prevent ubiquitination, leading to the stabilization of these proteins by inhibiting its proteasomal degradation (Bae et al., 2004; Desterro et al., 1998). On the other hand, so called *SUMO targeted ubiquitin ligases* (STUbLs) adhere to SUMOylated proteins resulting in the ubiquitination of SUMOylated proteins and their proteasomal degradation (Sriramachandran & Dohmen, 2014). E4orf6/7 WT showed a higher stability than the E4orf6/7 K68R mutant during transfection (Figure 21), which could also be confirmed in infection studies (Figure 25). These results are supported by time course experiments, in which E4orf6/7 K68R protein abundance was decreased (Figure 27 + Figure 29). The modest effect during the CHX protein half-live experiments might be due to unspecific SUMOylation of K114 that shields the two remaining lysine residues K114 and K126, thereby stabilizing the protein. In order to test, which lysine residues of E4orf6/7 are ubiquitinated, the following experiments should be performed: 1. A stability assay, in which cells where treated with CHX and the proteasome inhibitor MG132 in order to test, if E4orf6/7 is proteasomally degraded. 2. An ubiquitination assay using different E4orf6/7 SUMO variants to test, which lysine residues are ubiquitinated. 3. An ubiquitination assay using an E4orf6/7 variant, in which the lysine 68 is retained, but the SUMOylation motif is modified, in order to test if lysine 68 is not only the major SUMOylation but also the major ubiquitination site.

In summary, our results strongly suggest a stabilizing role of SUMOylation on E4orf6/7 since the CHX half-live experiments revealed a decreased stability of E4orf6/7 K68R during transfection as well as infection. Additionally these results are supported by the decreased E4orf6/7 K68R protein abundance in the time course experiments.

6.2.4 Summary on consequences of E4orf6/7 SUMOylation on viral targets

The results from our experiments underline the importance of E4orf6/7 and highlight its diverse roles during HAdV-5 infection. Nevertheless, most properties of E4orf6/7 were not affected by the substitution of lysine 68 by an arginine, leading to the inactivation of the SUMOylation site. The experiments demonstrated that E4orf6/7 was still able to localize into the nucleus, where it was capable of dimerizing E2F molecules to efficiently activate the viral E2A as well as non-E2F promoters. Most strikingly, our data demonstrate that the loss of the SUMO site impaired the

stability of E4orf6/7 (Figure 21 and Figure 25). Therefore, we assume that the SUMOylation of E4orf6/7 shields the remaining lysines almost completely and prevents ubiquitination, leading to an increased stability of the protein (Figure 38 A). In contrast, when lysine 68 is substituted by an arginine and SUMOylation is prevented, E3 ubiquitin ligases target E4orf6/7 for degradation (Figure 38 B). However, we assume that the decreased E4orf6/7 K68R abundance during the course of infection did not affect HAdV-5 replication because of the presence of E1A. Since E1A is the major factor for S-phase progression and was present in all infection experiments, the dominant nature of E1A most probably compensated the decreased abundance of E4orf6/7 K68R.

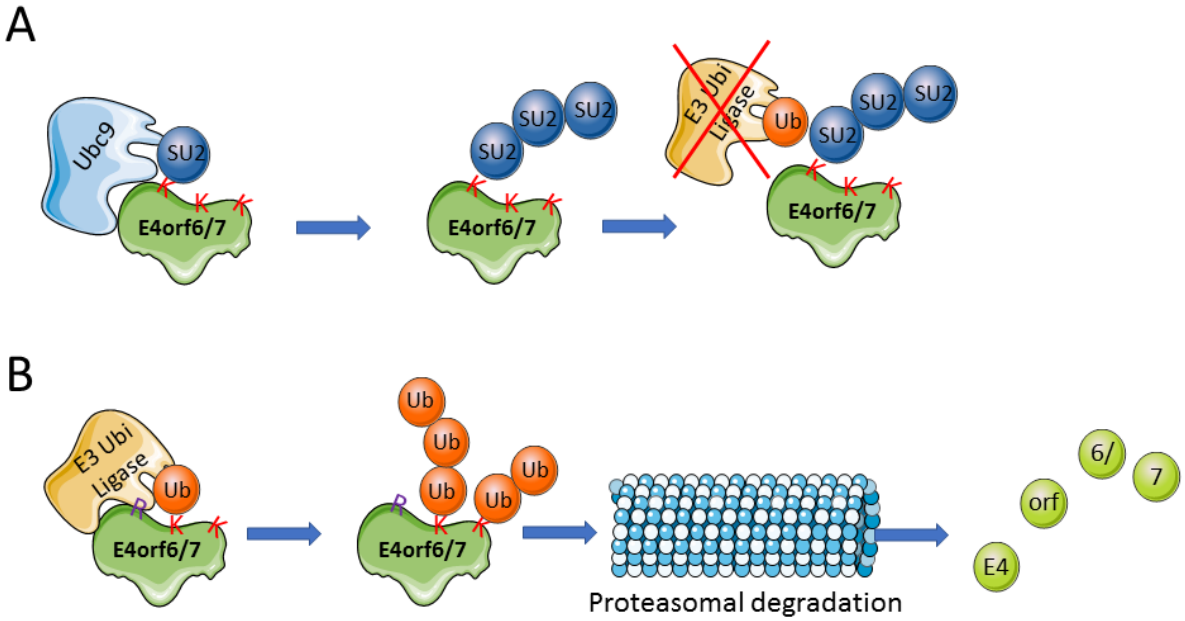


Figure 38: SUMOylation of E4orf6/7 increased its stability. (A) After SUMOylation by Ubc9, the lysine residues are shielded from E3 ubiquitin ligases, preventing targeting for proteasomal degradation and increasing the stability of E4orf6/7 WT. **(B)** When the lysine at position 68 is substituted by an arginine, E3 ubiquitin ligases target E4orf6/7 by ubiquitination for proteasomal degradation, leading to a decreased stability of the protein.

6.3 Consequences of E4orf6/7 SUMOylation and the importance on cellular targets

6.3.1 Influence of the SUMOylation site on E2F target promoters and proteins

Schaley *et. al.* suggested that the viral E2A promoter is not the physiological promoter because it does not require the dimerization activity of E4orf6/7 for its transactivation. Strikingly, they showed that the transactivation of the cellular E2F-promoter is dependent on the dimerization of E2F transcription factors, by E4orf6/7. Interestingly this promoter has a similar configuration as the E2A promoter, with inverted E2F binding sites (Schaley et al., 2000). Therefore, we tested if the loss of

the SUMOylation site in E4orf6/7 has an effect on cellular E2F-1 promoters. In addition, our laboratory identified the FAM111B gene as a putative E2F-1 regulated gene.

6.3.1.1 SUMO site inactivation does not impair transactivation activity of E4orf6/7 on E2F-1 and E2F-4 promoters

By now, eight different E2F species have been identified but specifically E2F-1 has gained a lot of attention, since it has unique roles during apoptosis and DNA repair (Johnson & DeGregori, 2006). Depending on the cell cycle, it acts as an activator or as an repressor and regulates more than 1000 genes linked to cell cycle progression (Müller et al., 2001; Ren et al., 2002; Weinmann et al., 2002). The E2F-1 promoter contains two E2F binding sites in an inverted configuration to which E4orf6/7 induces cooperative and stable binding, resulting in increased activation (Schaley et al., 2000). Therefore, we tested, analogous to the experiments on the E2A promoter, if the loss of the SUMOylation site influences the E4orf6/7 transactivating properties on the E2F-1 promoter by: 1. Measuring the transactivation activity of E4orf6/7 alone during transfection as well as in the context of HAdV-5 infection. 2. Determining the endogenous E2F promoter activity by measuring mRNA level in the course off infection to determine the peak transactivation activity and 3. Monitoring protein abundance throughout the replication cycle.

Interestingly, the E2F-1 promoter showed increased activity during transfection of E4orf6/7 WT and E4orf6/7 K68R (Figure 17), whereas infection with HAdV-5 WT and HAdV-5 K68R resulted in lower promoter activity (Figure 29). Since E2F-1 induces not only cell cycle progression but also apoptosis it is tempting to speculate that E4orf6/7 initially induces transcription of E2F-1 to drive the cells into S-phase, whereas other viral proteins inhibit the E2F-1 promoter at later time points to prevent apoptosis. This assumption is additionally supported by the mRNA analysis, which shows increased E2F-1 mRNA levels early during infection, which decrease at later time points (Figure 29). Interestingly, all transient expression experiments with E4orf6/7 WT or E4orf6/7 K68R as well as infection experiments with HAdV-5 WT and HAdV-5 K68R showed a robust transactivation leading to comparable changes in E2F-1 promoter activity, mRNA and protein levels. In conclusion, the binding and dimerization of E2F molecules seem to be a highly conserved function of E4orf6/7, which is not dependent on the SUMOylation status.

E2F-4 is another E2F family member regulating hundreds of different promoters (Conboy et al., 2007). In contrast to E2F-1, E2F-4 is thought to have crucial functions in mediating cell cycle arrest, although more recent ChIP approaches showed, that E2F-4 also acts as an activator (Lee et al., 2011; Crosby & Almasan, 2004; Meloni et al., 1999). However, whereas the expression of E2F-1 to E2F-3 is

highly regulated during the cell cycle, E2F-4 is constitutively expressed (Crosby & Almasan, 2004). Instead, regulation of E2F-4 is achieved by the interaction with pRB-family proteins, by changes at the translational level as well as post-translational modification and by changing its subcellular localization (Yochum et al., 2007; Lindeman et al., 1997). The latter is of great interest, because studies with other human pathogenic viruses showed tight implications of E2F-4 with HAdVs, Herpes Simplex Virus (HSV), Human Papillomavirus (HPV) and Human Immunodeficiency Virus [(HIV)(Decaprio, 2014; Schaley et al., 2005, 2000; Ambrosino et al., 2002; Olgiate et al., 1999; Garcia et al., 1988)]. HAdVs and HSV both re-localize E2F-4 into the nucleus upon infection and all three viruses recruit E2F-4 to specific promoters to exert multiple biological activities (Schaley et al., 2005, 2000; Ambrosino et al., 2002; Olgiate et al., 1999; Garcia et al., 1988). Consequently, it seems that E2F-4 mediates important steps in the replication cycle of different viruses. Nevertheless, its role during infection is not well defined and far from being understood. For example, HAdV-5 re-localizes E2F-4 to the nucleus by E4orf6/7 and utilizes the transcription factor for activation of the E2A promoter (Schaley et al., 2005; O'Connor et al., 2001). On the other hand, the HPV protein E7 targets repressive E2F-4 complexes for proteasomal degradation to induce cell proliferation (Rashid et al., 2015; Decaprio, 2014).

Interestingly, although dimerization of E2F-4 by p107 has been associated with repression, E4orf6/7 utilizes the same mechanism to use E2F-4 as an activator for the viral E2A promoter (O'Connor et al., 2001). The observation, that E2F-4 is transported into the nucleus by an early protein in combination with the down regulation of E2F-4 at the transcriptional and protein level later during infection (Figure 33), suggests that E2F-4 has pro- as well as anti-viral properties. Considering the activating and repressing properties and the versatile roles of E2F-4 being involved in the regulation of cell cycle, DNA repair and apoptosis, we assume that E2F-4 activates not only the E2A promoter, but also other genes involved in cell cycle regulation or DNA repair. To avoid cell cycle arrest or DNA repair, E2F-4 transcription is down regulated later during infection, for example by E1A, when the expression level of E4orf6/7 decrease. In addition, we want to raise the possibility that E4orf6/7 is not only involved in promoter activation but also in repression. E2F-4 contains binding domains for the recruitment of *Histone Deacetylases* (HDAC) for the repression of other co-repressors, resulting in the downregulation of target gene expression (Crosby & Almasan, 2004; Meloni et al., 1999). Since E4orf6/7 and E2F-4 are strongly connected, it cannot be excluded that E4orf6/7 utilizes E2F-4 for the repression of anti-viral response genes.

In summary these results suggest, that both E2F proteins are actively down regulated by the virus at the transcriptional and protein level during the course of infection with HAdV-5 WT and HAdV-5 K68R. However, it is challenging to estimate the exact influence on the viral infectious cycle

since both have activating and repressing functions. This apparent contradictory ability again reflects the diverse functions for the negative and positive regulation of thousands of genes (Sahin & Sladek, 2010; Müller et al., 2001; Weinmann et al., 2002; Ren et al., 2002; Lee et al., 2011). Given the fact, that E2F-1 is upregulated already early during infection and E2F-4 is re-localized into the nucleus, whereas both are down regulated during late infection, it is tempting to speculate, that both have dual roles during the adenoviral replication cycle. At the early phase of infection, both proteins exert pro-viral functions whereas their anti-viral activities are inhibited later during infection by repressing transcription. Furthermore, although E4orf6/7 has never been associated with the repression of genes, we want to raise the possibility, that E4orf6/7 is also involved in the down regulation of genes, by the recruitment of E2F-4.

6.3.1.2 SUMO site inactivation does not alter the localization of E2F molecules

E4orf6/7 has an arginine-rich region in the N-terminus, which is shared with E4orf6. This region was described as a nuclear targeting signal (Schaley et al., 2005). E2F-1, -2 and -3 contain nuclear localization sequences (NLS) that direct their nuclear targeting. On the other hand, E2F-4 and E2F-5 lack NLSs and rely on binding partners such as the pRB-family proteins p107 and p130 or the DP proteins, which contain a NLS themselves (Allen et al., 1997; Verona et al., 1997; Müller et al., 1997; Magae et al., 1996). Moreover, E2F-4 contains two nuclear export sequences that direct CRM1-dependent cytoplasmic transport (Gaubatz et al., 2001). In contrast to E2F-1, -2 and -3, E2F-4 is considered to be a repressing E2F-family member, which is primarily accumulated in the nucleus during S-phase, when repression of E2F target genes is initiated (Lee et al., 2011; Crosby & Almasan, 2004). Therefore, it would be anticipated that E1A displaces E2F-4/p107 complexes, inhibiting nuclear localization of E2F-4. However, it was shown that E2F-4 is utilized to transactivate the viral E2A promoter, when dimerized by E4orf6/7 (Schaley et al., 2005; Verona et al., 1997). Consistent with this idea, there is not only support that E2F-4 acts as a transcriptional activator for several cellular genes (Lang et al., 2001; Pierce et al., 1998b; Choubey & Gutterman, 1997), but also E2F-4 was found to stimulate viral gene expression of other pathogenic viruses like the Bovine Herpesviurs-1 as well as HIV (Geiser & Jones, 2003; Garcia et al., 1988). During the replication cycle of HIV, the Tat protein re-localizes E2F-4 into the nucleus and utilizes the transcription factor to stimulate the activity of E2F-dependent promoters, like the own HIV long terminal repeats (Ambrosino et al., 2002; Garcia et al., 1988). The re-localization of Tat and Rev is mediated by the interaction of the arginine-rich region with importin β and it is assumed that the same mechanism applies for E4orf6/7 (Ambrosino et al., 2002; Truant & Cullen, 1999). Since the localization of E1B-55K is influenced by its SUMOylation status and E4orf6/7 can re-localize factors that support efficient

viral infection, we aimed to investigate the importance of the SUMOylation site in E4orf6/7 on the re-localization of E2F binding partners (Pennella et al., 2010; Schaley et al., 2005). In our experiments we could show that E2F-4 was efficiently re-localized during infection (Figure 34), whereas E2F-1 showed only minor nuclear accumulation (Figure 31). However, the accumulation of E2F-1 cannot be attributed to E4orf6/7 alone, since there was no increased accumulation in cells transfected with E4orf6/7 WT and K68R (Figure 32). This observation was somewhat unexpected, because E2Fs are bound by the same region in E4orf6/7 (Schaley et al., 2005; Neill & Nevins, 1991; Huang & Hearing, 1989b). We assume that the nuclear accumulation of E2F-1 might be a rather general effect, caused by the infection and the upregulation of S-phase factors.

In conclusion, although E2Fs species are bound by the same binding region, E4orf6/7 seems to re-localize E2F-4, but not E2F-1, into the nucleus. One reason for this phenomenon could be attributed to the fact that E2F-1 has its own NLS, whereas E2F-4 relies on binding partners like pRB-family proteins or the heterodimer partner DP-1 to DP-3. Since the loss of the SUMOylation site did not affect the binding and re-localization, we assume that the interaction with E2F-4 is a robust function, which is not influenced by SUMO modification. Furthermore, the interaction with E2F-4 is an excellent example of how different ORFs within a protein work together and underline the importance of alternative splicing for viruses to guarantee efficient viral replication.

6.3.2 Influence of E4orf6/7 SUMOylation on FAM111B promoter and the FAM111B protein

So far the function and the importance of FAM111B is poorly understood (Goussot et al., 2017; Mercier et al., 2013; Shaboodien et al., 2013). Most of the mutations within the FAM111B gene have been associated to hereditary fibrosing poikiloderma with tendon contracture, myopathy and pulmonary fibrosis (Mercier et al., 2013). On the other hand, the family member FAM111A was suggested to be a restriction factor for simian virus 40 (SV40) and it was attributed to have helper functions for HAdVs, as well (Fine et al., 2012). In addition, our group observed highly increased FAM111B mRNA levels in HAdV-5 E1A/E1B transformed primary *human mesenchymal stroma cells* [(hMSC)(*Thomas Speiseder, personal communication*) and this work provides evidence that FAM111B protein levels are decreased during the course of infection. Even more interesting, *in-silico* analysis of the FAM111B gene revealed several E2F binding sites within the promoter region (*unpublished data*). Therefore, we aimed to elucidate the role of FAM111B on the adenoviral infection and the role of E4orf6/7 in FAM111B expression.

Strikingly, using luciferase reporter gene assays we were able to show that the FAM111B promoter activity is increased in both, during transfection of E4orf6/7 WT and E4orf6/7 K68R expression

plasmids (Figure 17) and during infection with HAdV-5 WT and HAdV-5 K68R [(16 h.p.i.)(Figure 36)]. This is in line with the infection time course experiments with HAdV-5 WT and HAdV-5 K68R, which revealed an initial increase in FAM111B mRNA (Figure 35 A+B) and protein levels (Figure 35 C). However, later during infection, both drop significantly below the detection level. The decrease of FAM111B protein levels in HAdV-5 WT and HAdV-5 K68R infected cells was additionally observed during immunofluorescence analysis (Figure 37). Therefore, it was somehow surprising that FAM111B mRNA levels in HAdV-5 K68R infected cells were significantly increased compared to HAdV-5 WT infection (Figure 35 A+B). Since there was no significant difference in FAM111B promoter activity observed in luciferase assays during infection, that would explain this phenomenon, we hypothesize two possible mechanisms: 1. E4orf6/7 WT might be able to selectively repress the FAM111B promoter but the luciferase construct lacks an important part of the promoter or 2. The loss of the SUMOylation site, allows E4orf6/7 to interact and stabilize FAM111B mRNA. The first hypothesis is supported by the fact, that E4orf6/7 dimerizes E2F-4, which has mainly repressing functions (Lee et al., 2011; Schaley et al., 2005; O'Connor et al., 2001). This transcription factor recruits HDACs for the repression of target genes and was observed to bind not only in promoter areas but also at distinct domains in non-promoter regions (Lee et al., 2011). Given the fact that the luciferase reporter plasmid used in our experiments contained only a minimal FAM111B promoter region, this might explain the lacking effect in the luciferase assays. The second hypothesis is supported by the observation, that the arginine-rich region of the HIV protein Tat allows the binding to mRNAs (Berkhout et al., 1989) and that binding with arginine-rich regions influences maturation and stability of mRNAs (Godin & Varani, 2007). Although we would expect that E4orf6/7 K68R induced FAM111B mRNA stabilization should be accompanied by increased FAM111B protein levels, we can argue by four additional observations why this might not be necessarily the case: 1. The virus seems to actively down regulate the FAM111B protein levels 2. Several publications have described that changes in mRNA levels do not necessarily change protein abundance to the same extend (Vogel & Marcotte, 2012) 3. Studies showed that arginine-rich domains coordinate mRNA maturation events (Godin & Varani, 2007), which could lead to the accumulation of immature FAM111B mRNA and 4. The mRNA levels comprise all four exons, whereas the FAM111B antibody used in our study binds to the first N-terminal 84 aa. However, it is also known that for example FAM111B isoform 2 lacks the first 30 aa. Therefore, it might be that the mRNA of isoform 2 is increased, the protein however was not detected properly due to inefficient binding of the antibody. Another argument against E4orf6/7 K68R induced stabilization of FAM111B mRNA is provided by the observation, that no SUMO modified E4orf6/7 was detected in the course of infection during western blot analysis (Figure 22 - Figure 24). Assuming, the fraction of SUMOylated E4orf6/7 WT was too low for the detection via western blotting, the majority exists in a non-SUMOylated form, as E4orf6/7 K68R.

Therefore, one would also expect to observe mRNA stabilization in HAdV-5 WT infected cells. Nevertheless, in this context it should be considered, that it has been frequently observed with other proteins, that the proportion of a SUMOylated protein is small in relation to the total protein pool, but the effect is high (Hay, 2005).

In summary, our infection experiments with HAdV-5 WT and HAdV-5 K68R revealed an increase in FAM111B promoter activity and increased FAM111B mRNA levels early during the viral replication cycle. However, the FAM111B mRNA levels in HAdV-5 K68R infected cells are considerably higher. Nevertheless, it remains to be further elucidated, if the increased FAM111B mRNA levels were caused by E4orf6/7 K68R mRNA stabilization or due to the inability of E4orf6/7 K68R to efficiently repress the FAM111B promoter. In order to elucidate the role of E4orf6/7 on FAM111B mRNA the following experiments should be performed: 1. RNA immunoprecipitation chip (RIP-chip) with E4orf6/7 WT and E4orf6/7 K68R, to determine if E4orf6/7 K68R binds mRNA and to elucidate if it is an exclusive interaction with FAM111B mRNA (Dölken et al., 2010) 2. Transcriptome analysis upon HAdV-5 WT and HAdV-5 K68R infection, to test if E4orf6/7 SUMO dependent repression is a general mechanism by which HAdV-5 inhibits cellular genes.

6.3.3 Summary of consequences of E4orf6/7 SUMOylation on cellular targets

E4orf6/7 and the HIV protein Tat have many common properties. Not only do both have an arginine-rich region and interact with E2F-4 to mediate the re-localization of the protein into the nucleus, but also do both utilize E2F-4 for the transactivation of their own promoters. The experiments with E2F-1, E2F-4 and FAM111B underline that E4orf6/7 has further important functions except for the dimerization of E2F transcription factors to enable cooperative and stable binding to the viral E2A promoter. Our results show that E4orf6/7 properties are quite distinct and need further investigation to get a deeper insight into its diverse functions. Previous studies have mainly focused on the activating properties of E4orf6/7 and therefore it remains uncertain, if E4orf6/7 also acts as a transcriptional repressor. The results presented in this work are specifically striking, since they provide new insights for a novel and SUMOylation dependent function of E4orf6/7. For the effect on the FAM111B mRNA level, we therefore propose two possible models: 1. Since the binding of E4orf6/7 to E2F transcription factors was not affected by the loss of the SUMOylation site, we assume that the binding of HDACs to E2F-4 is strongly enhanced by the SUMOylation of E4orf6/7. This assumption is supported by publications that showed the binding of HDACs via SUMO interaction motifs (SIM) to SUMO2/3, allowing gene specific repression (Ouyang et al., 2009). Taking this into account, we propose in our first model that complexes of SUMOylated E4orf6/7 and E2F-4 bind to a distinct, yet unknown binding domain outside the FAM111B promoter. SUMO modified

E4orf6/7 WT strongly enhances the association of HDACs to E2F-4, compared to E4orf6/7 K68R, inducing pronounced deacetylation of histones, leading to the repression of the FAM111B gene expression (Figure 39 A). 2. Publications showed that arginine-rich regions in viral proteins can bind mRNA and that these binding events affect mRNA maturation and stability (Ambrosino et al., 2002; Godin & Varani, 2007). Therefore, we propose in a second model that polySUMOylated E4orf6/7 is too bulky to interact with mRNA. The loss of the SUMOylation site allows the arginine-rich region to bind mRNA, either stabilizing or impairing the maturation of FAM111B mRNA leading to increased FAM111B mRNA levels (Figure 39 B). Finally, we want to emphasize the possibility, that these models are not FAM111B specific, but may also apply to other genes or mRNAs. Above all, the E2F-4 induced repression of genes could represent a new mechanism by which HAdVs inhibit transcription of cellular and putative anti-viral genes that do not contain E2F binding sites in their promoter.

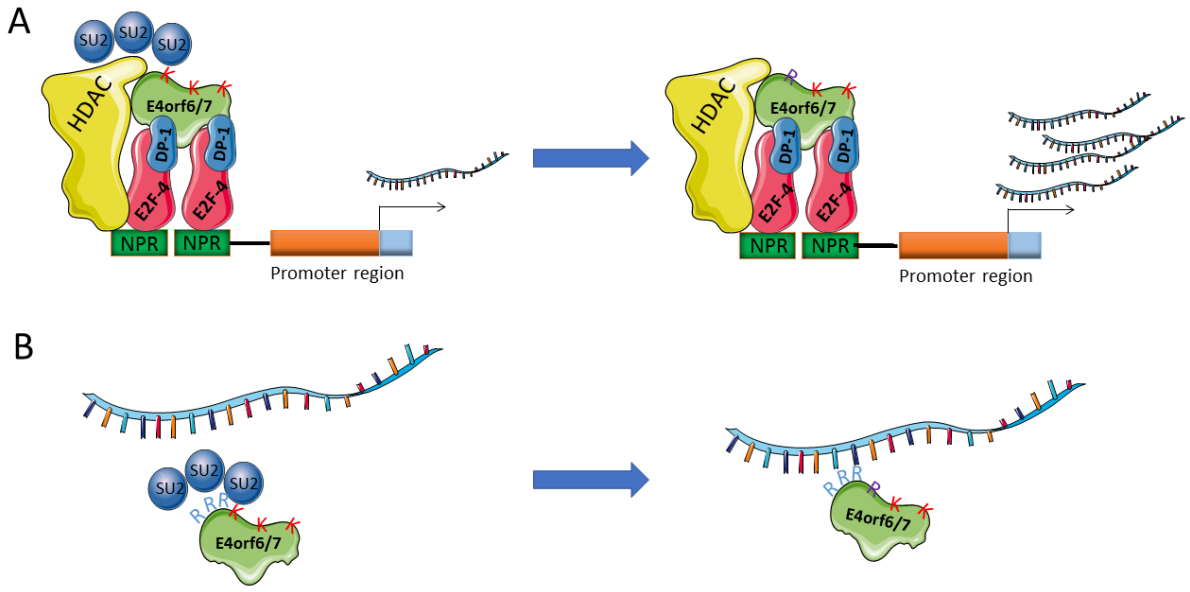


Figure 39: Infection with HAdV-5 K68R increases FAM111B mRNA level during the course of infection. The figures provides two mechanisms that would explain the increased FAM111B mRNA level during the infection with HAdV-5 K68R. **(A)** After infection with HAdV-5 WT, SUMOylated E4orf6/7 and E2F-4 bind to non-promoter regions (NPR). SUMO2 molecules strongly enhance the non-covalent association of histone deacetylases (HDACs) via SUMO-interaction motifs, leading to deacetylation of histones and the repression of target genes. In contrast, E4orf6/7 K68R and E2F-4 complex can also bind to NPR but the recruitment of HDACS is less efficient. Therefore, the repression is less efficient, leading to increased FAM111B mRNA level **(B)** SUMOylated E4orf6/7 WT cannot interact with mRNA whereas the non-SUMOylated E4orf6/7 K68R can associates with mRNA, thereby increasing the stability or influencing the maturation of FAM111B mRNA, leading to increased mRNA levels.

7 Literature

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