Functional characterization of the human adenovirus pVII protein and non-coding VA RNAI

RAVITEJA INTURI
Abstract


Human adenovirus (HAdV) is a common pathogen causing a broad spectrum of diseases. HAdV encodes the pVII protein, which is involved in nuclear delivery, protection and expression of viral DNA. To suppress the cellular interferon (IFN) and RNA interference (RNAi) systems, HAdVs encode non-coding virus-associated (VA) RNAs. In this thesis we have investigated the functional significance of the pVII protein and VA RNAI in HAdV-5 infected cells.

We report that the propeptide module is the destabilizing element targeting the precursor pVII protein for proteasomal degradation. We also found that the Cul3-based E3 ubiquitin ligase complex alter the precursor pVII protein stability via binding to the propeptide sequence. In addition, we show that inhibition of the Cul3 protein reduces HAdV-5 E1A gene expression. Collectively, our results suggest a novel function for the pVII propeptide module and involvement of Cul3 in viral E1A gene expression.

Our studies show that the cellular E3 ubiquitin ligase MKRN1 is a novel pVII interacting protein in HAdV-5 infected cells. MKRN1 expression reduced the pVII protein accumulation in virus-infected cells and affected infectious virus formation. Surprisingly, the endogenous MKRN1 protein underwent proteasomal degradation during the prolonged HAdV-5 infection. Furthermore, the precursor pVII protein enhanced MKRN1 self-ubiquitination, suggesting the direct involvement of pVII in the initiation of MKRN1 degradation. Hence, we propose that the MKRN1 is a novel antiviral protein and that HAdV-5 infection counteracts its antiviral activity.

In papers III and IV, we tested the ability of various plant and animal virus encoded RNAi/miRNA and IFN suppressor proteins to functionally substitute for the HAdV-5 VA RNAI. Our results revealed that the Vaccinia virus E3L protein was able to partially substitute for the HAdV-5 VA RNAI functions in virus-infected cells. Interestingly, the E3L protein rescued the translational defect but did not stimulate viral capsid mRNA accumulation observed with VA RNA. Additionally, we show that the HAdV-4 and HAdV-37 VA RNAI are more effective in virus replication compared to HAdV-5 and HAdV-12 VA RNAI. In paper IV, we employed a novel triplex-specific probing assay, based on the intercalating and cleaving agent benzoquinoinaxline 1,10-phenanthroline (BQQ-OP), to unravel triplex structure formation in VA RNAI. The BQQ-OP cleavage of HAdV-4 VA RNAI indicates that a potential triplex is formed involving the highly conserved stem 4 of the central domain and side stem 7. Further, the integrity of HAdV-4 VA RNAI stem 7 contributes to the virus growth in vivo.

**Keywords:** Adenovirus, VA RNA, protein VII, Ubiquitination, proteasome, anti-viral

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This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Other paper not included in this thesis

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### Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenovirus Death Protein</td>
</tr>
<tr>
<td>Avp</td>
<td>Adenoviral protease</td>
</tr>
<tr>
<td>BQQ-OP</td>
<td>Benzoquinaxline 1,10-phenanthroline</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>CRL</td>
<td>Cullin-RING E3 ubiquitin Ligases</td>
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<tr>
<td>Cul</td>
<td>Cullin</td>
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<tr>
<td>DBP</td>
<td>DNA-Binding Protein</td>
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<td>DRBD</td>
<td>Double-stranded RNA Binding Domain</td>
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<td>dsDNA</td>
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<td>dsRNA</td>
<td>Double-stranded RNA</td>
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<td>DUB</td>
<td>Deubiquitinating enzymes</td>
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<tr>
<td>eIF2</td>
<td>Eukaryotic initiation factor 2</td>
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<tr>
<td>HAdV</td>
<td>Human Adenovirus</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP carboxy terminus</td>
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<tr>
<td>HMGB</td>
<td>High Mobility Group Box protein</td>
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<tr>
<td>hpi</td>
<td>hours post-infection</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<tr>
<td>MKRN</td>
<td>Makorin</td>
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<tr>
<td>MLTU</td>
<td>Major Late Transcription Unit</td>
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<tr>
<td>Mre11</td>
<td>Meiotic recombination 11</td>
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<tr>
<td>PABP</td>
<td>Poly(A)-Binding Protein</td>
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<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
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<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
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<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
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<tr>
<td>RISC</td>
<td>RNA-induced Silencing Complex</td>
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<td>RNA interference</td>
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<td>RNA silencing suppressor</td>
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<td>Survival-time associated PHD protein in Ovarian Cancer1</td>
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<td>Template Activating Factor 1</td>
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<td>UPS</td>
<td>Ubiquitin Proteasome System</td>
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<tr>
<td>VA RNA</td>
<td>Viral Associated RNA</td>
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Introduction

I start this thesis with a very common question I often got from my friends during PhD studies: “Why study viruses?”. Although there are many ways to reply to this rhetorical question, I like to answer with a quote from the Chinese general Sun Tzu: “Know your enemy and know yourself and you can fight a hundred battles without disaster”.

Viruses are best known as disease causing agents, so not surprisingly we consider them our enemies. However, viruses can only survive within a living cell. This means that they have to establish multiple molecular connections within our cells. This extensive virus interference with the essential cellular signaling pathways is the reason why many viral infections turn into highly pathogenetic disasters. Thus, to win battles against viral infections we have to understand how viruses take advantage of the host cells and how the host cells react to virus infections.

In the present thesis I have studied the pathogenetic human adenoviruses (HAdV). More specifically, I have aimed to understand how two essential HAdV entities: the pVII protein and the non-coding VA RNAI, battle with the cellular signaling pathways. Therefore, the first part of this thesis describes the functional characteristics of the pVII protein and its interplay with the host cell factors. The second part of the thesis is focused on studying the functional significance and uniqueness of VA RNAI in HAdV lytic infections.

Human adenoviruses (HAdV)

Human adenoviruses (HAdVs) were first isolated from adenoids in young children in 1953 (1). HAdVs belong to Adenoviridae family, Mastadenoviridae genus and they were originally classified into seven subgroups based on their serological features and oncogenicity in rodents. More recent HAdV classification is based on virus genome sequence analysis, phylogenetic distance and the ability to recombine. Therefore, instead of serotypes and subgroups, individual HAdVs are designated as types belonging to specific species (2). At present, more than 70 HAdV types have been reported, which are ordered into seven species from A to G (3, 4). Of these viruses, the type 2 (HAdV-2) and 5 (HAdV-5) are best characterized. It is important to mention that as the HAdV-2 and HAdV-5 genomes are almost identical, their
encoded proteins and non-coding RNAs function in a similar fashion. Hence, the majority of examples described in the present thesis are based on studies with the HAdV-2 and/or HAdV-5.

HAdVs are pathogenic and their infections can cause a variety of acute (HAdV-4) and chronic respiratory diseases (HAdV-2 and HAdV-5), ocular diseases (HAdV-37), gastrointestinal tract diseases (HAdV-12) and urinary tract diseases (4-6). In general, HAdV infections are not lethal in immunocompetent patients. However, in immunocompromised individuals (e.g. organ transplant patients) HAdV infections can often lead to life-threatening conditions (6, 7). Curiously, the HAdVs have emerged as promising therapeutic agents. For example, HAdVs have been used as oncolytic viruses for cancer treatment and as gene delivery vehicles for gene therapy applications (8). Therefore, a detailed understanding of individual HAdV protein and non-coding RNA functions in the host cell is essential for better development of therapeutic HAdVs.

HAdV capsid and entry

HAdVs are non-enveloped, icosahedral viruses containing linear double stranded DNA (dsDNA) genomes (Figure 1). Depending on the type, the viral genome size can vary between 30-46 kbp. The HAdV-2 genome encodes more than 40 proteins and 13 of these proteins are found in the icosahedral capsid. The icosahedral capsid is composed of hexon, penton and fiber proteins, which are considered the major capsid proteins (9, 10).

Figure 1. Schematic illustration of HAdV virion, showing the structural proteins at their approximate locations. Modified from (11).
In addition, the virus particle contains four minor capsid proteins: protein IIIa, protein VI, protein VIII, and protein IX. These proteins comprise the so-called cement proteins that are mainly associated with formation of the capsid shell. The remaining six capsid proteins: protein V, protein VII (pVII), protein Mu, protein IVa2, adenoviral protease (Avp) and the terminal protein (pTP), form the viral core structure (12). While the core proteins secure condensation and stability of the viral genome, cement proteins and the major capsid proteins form an extra protective shell for the virus and facilitate virus attachment to the host cell receptors.

The majority of the HAdV types utilize the coxsackievirus and adenovirus receptor for the primary attachment to the cell surface mediated by the fibre protein (13). The internalization of the virus is facilitated through clathrin-coated vesicles after attachment of penton base to the cellular integrins (14, 15). After receptor-mediated endocytosis, the virus capsid is partially disassembled by acidic environment in the endosomes. Upon protein VI aided endosomolysis, the partially uncoated virion enters the nuclear pore via dynein-mediated transport on microtubules (16). At the nuclear pore, the viral DNA in complex with the mature VII protein is actively transported into the nucleus for subsequent transcription of viral genes (17, 18).

HAdV life cycle
The HAdV lytic life cycle can be separated into early and late phase based on the temporal expression of viral genes with DNA replication occurring in-between the two phases. The HAdV genes expressed during the early phase are: E1A, E1B, E2, E3, and E4. The delayed early genes consist of IX, IVa2 genes. During the late phase of infection, the genes encoded in the major late transcription unit (MLTU) are expressed (Figure 2). A typical HAdV lytic life cycle in HEK293 or HeLa cells takes about 24 to 36 h. Within the first hour post-infection (hpi), the virus is internalized and the early E1A gene expression is initiated. Viral DNA replication peaks around 12 hpi and capsid protein synthesis can be visualized at 16-18 hpi. After 36 hpi most of the cells start to lyse and release about $10^4$-$10^5$ virus progeny per cell (19).

Early phase
HAdV early region 1A (E1A) gene is the first viral gene to be expressed in the infected cells (20). The encoded E1A pre-mRNA undergoes extensive 5'-end alternative splicing to give rise to at least five E1A protein isoforms. The E1A proteins interact with a variety of cellular factors such as general and sequence-specific transcription factors, transcription co-factors and chromatin modifying enzymes (21, 22). The main function of the E1A protein is to push the host cell into S phase of cell cycle and thereby to provide a favorable environment for viral DNA replication. This is accomplished by the E1A protein binding directly to the retinoblastoma protein (pRb) (23).
By binding to the pRb protein, E1A releases the E2F family of transcription factors, which activate transcription of the cellular genes needed to stimulate cell cycle progression into the S phase. In addition, the E2F proteins can enhance the p14/ARF protein accumulation (24), which in turn leads to activation of the tumor suppressor protein p53 pathway (25). As a consequence, the active p53 protein can induce apoptosis of the infected cell, which would be detrimental for virus growth. To counteract p53-induced cell apoptosis, HAdV-2/5 E1B region encodes the early E1B-55K and E1B-19K proteins. The E1B-55K in association with the E4-ORF6 protein targets the p53 protein for proteasomal degradation (26). In addition to the p53 protein degradation, the E1B-55K protein can also block p53-activated transcription by direct binding to the p53 protein (27, 28). In contrast, the viral E1B-19K protein mimics a cellular anti-apoptotic Bcl-2 protein by binding to cellular pro-apoptotic proteins Bax, Bak and thereby inhibiting the apoptosis (29).

E1A also regulates transcription of other viral genes required for viral DNA replication. For example, E1A activates the E2 transcription unit, which results in expression of viral DNA polymerase, pTP and DNA-binding protein (DBP) (30). The viral DNA replication is initiated and catalyzed by the DNA polymerase and the pTP protein acts as a primer for initiation of replication. The DBP enhances the initiation step and is required for DNA replication elongation (31).

The E3 proteins encoded from the E3 region protect the virus from host immune system by blocking TNFα induced cytolysis and Fas induced cell death through internalization and by preventing virus antigen presentation on cell surface by MHC class I molecules (32). The E3-11.6K, known as adenovirus death protein (ADP), is required for cell lysis and release of new virus particles from infected cells after virus maturation (33).

Proteins encoded from the E4 region, specifically E4-ORF3 and E4-ORF6, are essential to facilitate efficient virus production by inactivating Mre11-Rad50-Nbs1 (MRN complex proteins) DNA repair complex and the p53 protein (26, 34). E4-ORF6 along with E1B-55K acts as the adaptor proteins together with Cul-5 E3 ligase complex to degrade several cellular factors through ubiquitination and proteasomal degradation (see “Ubiquitination“ section). Further, the E4-ORF6 and E1B-55K protein complex preferentially exports viral mRNAs to the cytoplasm and is suggested to block the cellular mRNA export (35, 36). The E4-ORF3 protein induces the relocalization of cellular proteins into so-called E4-ORF3 nuclear tracks (37). For example, E4-ORF3 relocates proteins of the Mre11-Rad50-Nbs1 (MRN) complex, thereby inhibiting the activation of the DNA damage response in the infected cells (34, 38). In addition, the E4-ORF3 protein can inhibit p53-activated gene expression by inducing heterochromatin formation at p53 target promoters (39). Another E4 protein, E4-ORF4, associates with cellular phosphatase PP2A and via this interaction the E4-ORF4 protein regulates HAdV-2 alternative splicing during the late phase of infection (40).
Late phase

The late phase of HAdV infection correlates with the expression of all viral late genes from a single transcription unit, the MLTU (Figure 2). The transcription of HAdV-2 MLTU generates ~28000 nucleotides long, single major late transcript (MLT). By usage of alternative polyadenylation sites, five MLT mRNA families, named as L1 to L5, are generated. Further alternative splicing of L1 to L5 mRNA transcripts results in at least 20 different mature mRNA species encoding mostly for viral structural proteins. All spliced mRNAs carry a conserved 5’ non-coding 201 nucleotide leader sequence called as the tripartite leader (41, 42). The tripartite leader consists of the first three exons present at the 5’end of the MLT pre-mRNA. The tripartite leader is required for efficient translation of late mRNAs (43).

The L1 region generates two predominant cytoplasmic mRNAs: 52,55K and IIIa mRNAs. The 52,55K protein stabilizes the association of viral DNA with empty capsids (44) and the IIIa protein acts as a minor cement protein joining the hexons in the viral capsid (9, 45).

The L2 region encodes the viral capsid protein III (penton protein), core proteins VII, mu, and V (46). The viral core proteins VII, mu and V bind the viral DNA and form nucleosome-like (also called as adenosomes) structures (47). The protein V can induce relocalization of the cellular protein nucleolin and B23, and was also reported to interact with cellular protein p32, a mitochondrial nuclear protein (48, 49). These studies suggest that protein V might have a functional role in nuclear events.
The L3 unit encodes the major HAdV structural protein hexon, the protease Avp and the cement protein pVI (50). The protease Avp is enzymatically inactive and requires viral DNA and C-terminal 11-amino acid peptide of the precursor pVI (pVIc) as the cofactors for its activation (reviewed in (51)).

The L4 unit encodes for the regulatory proteins L4-22K and L4-33K, which are involved in the regulation of HAdV-5 MLTU transcription and MLT pre-mRNA splicing (52, 53). In addition, the L4 unit encodes for the L4-100K protein, which is needed for preferential translation of the viral late mRNAs that contain tripartite leader (54).

The L5 unit expresses the fiber protein, which is needed for virus particle attachment to the cell receptors (50).

The virus capsid assembly occurs in the nucleus, hence the newly synthesized viral capsid proteins and core proteins are imported to the nucleus to form new virions. The structural protein IVa2 along with 52K, 55K protein and the pVII protein pack the viral DNA (55, 56). Several of the core and capsid proteins are synthesized as precursor proteins. The site-specific proteolytic cleavage of these precursor proteins by the Avp confers maturity, stability and infectivity upon the virion (57).

Viral Associated RNAs (VA RNAs)

To counteract the host antiviral defense and to enhance virus multiplication, HAdVs encode for a class of highly structured non-coding Viral Associated RNAs (VA RNAs). Most HAdVs (ca. 80%) encode for two VA RNAs, VA RNAI and VA RNAII, whereas the rest (20%) of HAdVs have a single VA RNA gene (58). Both VA RNA genes are transcribed by the RNA polymerase III, at relatively low levels during the early phase of infection. In contrast, the VA RNA accumulation is boosted to extremely high levels, \( \sim 10^8 \) copies/cell, at late times of lytic HAdV-2 infection (59). Deletion of the VA RNAI gene leads to defects in late protein synthesis, aberrant splicing of late mRNAs and other defects in late mRNA production, altogether severely affecting infectious virus particle formation (60-63). In contrast to the VA RNAI gene, the deletion of the VA RNAII gene was shown not to affect the lytic virus growth suggesting the pre-dominant role of VA RNAI in lytic HAdV-2 growth (64). It is important to mention that in the present thesis, the experimental work was done using the VA RNAI (Paper III and IV).

**VA RNA inhibits PKR activation**

The HAdV genome is transcribed from both DNA stands in 5´ to 3´direction. This results in accumulation of complementary RNA transcripts, which can assemble into double stranded RNA (dsRNA) species. Curiously, these viral dsRNAs can activate dsRNA-dependent protein kinase (PKR), which plays an essential antiviral role in the cells (65, 66). In addition to PKR enzymatic activation by dsRNA binding, the PKR protein accumulation is also en-
hanced in virus-infected cells. The PKR protein in cells is upregulated by a signaling cascade initiated by binding of type I interferons (IFN) to their cognate receptors (65). This ligand-receptor binding activates the JAK/STAT signaling pathway, which in turn induces transcription of several IFN-stimulated genes (ISGs) including PKR (67, 68). Hence, the PKR is regarded as the interferon-induced, dsRNA-activated protein kinase.

PKR is one of the four serine-threonine kinases shown to phosphorylate the essential α subunit (eIF2α) of the trimeric eukaryotic translation initiation factor 2 (eIF2) (69). Upon activation by dsRNA, PKR monomers are phosphorylated and dimerized to form an active enzyme (70). Activated PKR phosphorylates eIF2α on serine 51 (Ser51) residue. This site-specific phosphorylation prevents guanosine nucleotide exchange factor B (eIF2B) from exchange of GDP to GTP on eIF2. The reduced level of eIF2-GTP hampers the 43S preinitiation complex and as a consequence terminates protein synthesis (Figure 3) (69). Although this development is necessary for the host cell to achieve effective antiviral response, it is detrimental for the virus growth. Therefore, to ensure efficient production of viral proteins and to prevent translational shut down by phosphorylated eIF2α, VA RNAI binds to the dsRNA-activated PKR and thereby prevents PKR dimerization and activation by the aforementioned viral dsRNA species (71-73). The apical stem and central domain of VA RNAI are primarily known to bind and inhibit PKR, thereby allowing efficient translation of viral mRNAs during the late phase of infection (Figure 3) (74).

Since the PKR activation has a negative impact on virus growth, many mammalian viruses encode the suppressor proteins, which can interfere with the PKR protein. For example, Vaccinia virus (VACV) encodes the E3L protein, which can bind up the activating dsRNAs and thereby block PKR activation (75-77). Collectively, viruses have evolved different means, either in the form of non-coding RNA (e.g. HAdV VA RNAI) or protein (e.g. VACV E3L) to inhibit the IFN-induced dsRNA-activated PKR.
Figure 3. Mechanism of VA RNAI action: inhibition of the interferon pathway and interference with the RNAi/miRNA pathway. Binding of the viral dsRNAs activates the PKR protein. The PKR monomers are autophosphorylated and dimerized to become active. The active PKR inhibits translation by phosphorylation of eIF2α on Ser51 residue. VA RNAI also interferes with the RNAi/miRNA pathway by inhibiting the export of cellular pre-miRNAs, diminishing the processing of pre-miRNA by the Dicer enzyme and by saturating the cellular RISC complex with VA RNAI-derived small RNAs known as mivaRNAs.

VA RNA interferes with RNAi/miRNA pathway

RNA interference (RNAi) is evolutionarily conserved gene regulatory mechanism that targets and degrades specific endogenous or exogenous RNA molecules. Since its initial discovery, it has been demonstrated to function as an important antiviral defense response across eukaryotes. The central players in RNAi are the small interfering RNAs (siRNAs), which are double-stranded, 21-25 nucleotides (nt) long RNA sequences with a 2 nt 3’-overhang (78). The RNAi mechanism involves an initial recognition and cleavage of viral dsRNAs by the cytoplasmic RNase III-type ribonuclease Dicer into siRNA duplexes (79, 80). Thereafter the siRNAs are assembled into the RNA-induced silencing complex (RISC) via binding to the Argonaute (Ago) effector proteins (81). The guide strand of the siRNA duplex subsequently serves as sequence determinant by inducing specific recognition and cleavage of complementary viral RNA transcripts or genomes (78, 80, 82).

To subvert the harmful antiviral RNAi response, various plant and invertebrate viruses encode the counter-defensive RNA silencing suppressor (RSS) proteins. The RSS proteins target different stages and components of
the antiviral RNAi pathway (83). For example, the known Tomato Bushy Stunt virus p19 protein interacts and seizes siRNAs from being incorporated into RISC (84).

It is important to mention that although the RNAi system can inhibit growth of multiple plant and invertebrate viruses (85, 86), mammalian cells rely primarily on the antiviral interferon system to obstruct the virus infections.

In mammalian cells the RNAi pathway depends on small non-coding RNAs known as microRNAs (miRNAs), which undergo a similar biosynthesis cascade as the siRNAs. However, in contrast to siRNAs, the miRNAs are encoded by specific miRNA genes in the host genome and the Drosha enzyme in the cell nucleus conducts their initial processing. Therefore, the RNAi pathway in mammalian cells is also called the miRNA pathway.

Both VA RNAI and VA RNAII have been shown to act as competitive substrates suppressing the activity of Dicer cleavage of exogenous dsRNA into siRNA and loading of siRNA and cellular miRNAs into the RISC complex (87). The highly abundant VA RNAs act also as decoy substrates for the exportin-5-mediated nuclear export of pre-miRNAs and short hairpin RNAs (shRNAs) (Figure 3) (88). Further, the Dicer processes VA RNAs into small viral RNAs called mivaRNAs (89-91). The mivaRNAs are generated from different HAdV types and they are incorporated into the active RISC complex (90). However, the function of these highly abundant small RNAs in HAdV life cycle has remained enigmatic. For example, the HAdV-5 viruses where the mivaRNA sequences are mutated, replicate as efficiently as their wild-type counterpart in the established epithelial cell lines (92). This observation suggests that the functional mivaRNAs are not essential for HAdV lytic growth. Although the VA RNA cleavage by Dicer generates mivaRNAs, the same cleavage can also reduce the amount of full-length VA RNA. Hence, it has been suggested that the Dicer protein cleavage of VA RNAs may function as a cellular antiviral mechanism inhibiting HAdV replication (93).

Similar to HAdV VA RNAs, some mammalian viruses also encode suppressor of the RNAi/miRNA pathways. For example, the VACV E3L protein and the Ebola virus (EBOV) VP35, which are also known to inhibit the PKR pathway, can suppress the RNAi/miRNA pathway in mammalian cells by interfering with the individual components of this pathway (83, 94).

**HAdV core proteins VII and pVII**

Before explaining the protein VII and pVII functions, it is important to clarify the naming of these two proteins. Throughout the thesis we talk about the HAdV-2/5 precursor pVII protein (also known as the pVII(wt) protein) and the mature VII protein (also known as the pVII(Δ24) protein). The latter
lacks the N-terminal 24 amino acid module, also known as the propeptide sequence (Figure 4).

![Figure 4](Image)

**Figure 4.** Schematic view of the HAdV-5 precursor pVII protein, mature VII protein and the 1-24 propeptide.

The HAdV-2 mature VII protein is the major core protein with approximately 800 protein copies per mature virion (95). Since, the VII protein contains high proportion of basic amino acids, it resembles arginine- and lysine-rich nucleosomal histones H3 and H4 (46, 96). Hence, the VII is considered the HAdV encoded histone-like protein. Due to its basic characteristics the VII protein binds very strongly to dsDNA in a sequence-independent manner (97). In addition, the VII protein contains distinct nuclear targeting signals suggested as being important for the nuclear entry of the protein (98, 99). During the entry, the VII protein in complex with the virus DNA is imported into the cell nucleus (100). Importantly, tight binding of the VII protein to viral DNA is believed to protect the virus genome from host cell DNA damage response, which happens during the early stages of infection (101).

The majority of the studies on protein VII were focused on studying the duration, abundance and modulation of the protein VII association with viral DNA. The data from UV crosslinking of core proteins to intracellular HAdV DNA indicated that protein VII remained associated throughout the early phase of virus infection (102). Similar results were further confirmed by immunofluorescence microscopy and by chromatin immunoprecipitation (ChIP) studies (101, 103-105). While other studies indicate that the mature VII protein association with viral DNA is reduced to favor the viral gene expression as the infection proceeds (106, 107). ChIP experiments have shown that the VII protein binding to viral DNA diminishes as the infection proceeds whereas the nucleosomal histone H3 and H4 binding increases in the same viral DNA regions (108). Interestingly, the VII protein displacement with the cellular histones on HAdV-5 genome varies depending on the region of the virus genome examined (109, 110). Hence, it is plausible that
some portion of the protein VII has to be removed or remodeled to make the viral genome accessible for transcription machinery.

It has been suggested that transcription activation causes the loss of protein VII on viral DNA in the nucleus. Blocking viral gene transcription prevents the loss of protein VII suggesting that active viral gene transcription dissociates protein VII from HAdV genome (101, 103). Further, it has been suggested that the early viral protein E1A, which stimulates viral gene transcription, interacts with protein VII and causes the release of protein VII on viral DNA (103, 111).

The protein VII-DNA complex was shown to hinder efficient transcription relative to naked DNA in vitro cell-free system (112). This suggests that host cell-remodeling factors might be involved in remodeling the pHII-condensed DNA structure. Three host-derived factors; Template Activating Factor (TAF)I/SET (113), TAF-II (114) and TAF-III/nucleophosmin/B23 (115) were identified as being involved in remodeling the protein VII-DNA complex in vitro (Table 1). The TAF-I protein was further characterized and shown to bind the protein VII, decondense the HAdV DNA, remodel the chromatin structure, and stimulates transcription of HAdV-2 DNA (106, 112, 116).

HAdV-5 DNA associates with the cellular histones as early as 2 hpi (108). This observation suggests that the cellular histones may influence the mature VII protein association with viral DNA already during the early phase of infection. Deposition of cellular histones on viral DNA is further supported by the report showing that the DNA-synthesis-independent histone chaperone (HIRA) and chromo domain-helicase DNA binding protein 1 (CDH1) can recruit histone variant H3.3 to viral DNA (117). Further, recent report showed that the mature VII protein also associates with the host cell chromatin. By binding to cellular nucleosomes, protein VII alters the cellular HMGB1 and HMGB2 protein functions on host cell chromatin and thereby suppresses cellular inflammatory signaling (118).

The human SPOC1 (survival-time associated PHD protein in ovarian cancer) protein is involved in DNA damage response and chromatin modulation and was recently identified to associate with protein VII. This association was suggested to promote DNA condensation and protect the viral DNA from DNA damage response. Subsequently, transcriptional initiation could release protein VII bound SPOC1 where the SPOC1 is degraded by E1B-55K/E4-ORF6 E3 ligase complex (119).
As the infection proceeds, viral DNA is replicated, and viral late genes are expressed from the MLTU. The HAdV-2/5 protein VII is encoded from the MLTU L2 region (Figure 2) and is translated as the precursor protein (pVII) (46). During the late stage of infection, the precursor pVII protein has to replace the histones on virus DNA to allow efficient viral genome packaging. However, the replacement mechanism is not well elucidated. It has been hypothesized that reduced accumulation of the cellular histones and massive accumulation of the pVII protein allows virus DNA association with the pVII protein during the late phase of infection (109).

The pVII protein is also reported to be involved in efficient packing of virus DNA by interacting with the HAdV-5 IVa2 and 52,55K proteins (55). Further, the cellular protein nucleophosmin/B23 has been shown to interact with the pVII protein (Table 1) in the late phase of infection, however the functional consequence of this interaction is not clear (120).

The HAdV-5 pVII protein undergoes proteolytic cleavage by the Avp protease during the virus maturation step (Figure 4) (see also section “Late phase”). This site-specific cleavage generates a 24 amino acid N-terminal propeptide and the mature VII protein (amino acids 25-198 in HAdV-5) (57, 122, 123). However, it is not entirely clear how the pVII protein is cleaved during virus particle maturation step. It has been hypothesized that the precursor proteins are assembled into the immature virion and that their subsequent Avp cleavage results in mature virion formation (122).

The pVII protein was shown to localize distinctly to nucleolus similar to protamine and protein V, whereas the mature VII localized to nucleus, but not to nucleolus (118, 121). These differences in subcellular localization might indicate the distinct functional roles of the pVII and VII proteins during virus infection. Studies on kinetics of pVII processing in HAdV infections indicated that this post-translational processing is a slow process and

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SET (TAF-1)</td>
<td>Chromatin remodeling</td>
<td>(116)</td>
</tr>
<tr>
<td>pp32</td>
<td>Inhibitor of histone acetyl transferases</td>
<td>(105)</td>
</tr>
<tr>
<td>B23/nucleophosmin (TAF-III)</td>
<td>Histone chaperone</td>
<td>(120)</td>
</tr>
<tr>
<td>SPOC1</td>
<td>Chromatin-associated factor and epigenetic reader</td>
<td>(119)</td>
</tr>
<tr>
<td>HMGB1</td>
<td>Activate immune response and act as alarmin</td>
<td>(118)</td>
</tr>
<tr>
<td>HMGB2</td>
<td>Chromatin remodeling, can bend and enhance DNA flexibility</td>
<td>(118)</td>
</tr>
<tr>
<td>Cullin-3</td>
<td>E3 ubiquitin ligase</td>
<td>(121)</td>
</tr>
<tr>
<td>MKRN1</td>
<td>E3 ubiquitin ligase</td>
<td>Paper II</td>
</tr>
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Table 1. *Known cellular proteins interacting with the protein VII*
appears to be non-linear. Only a small portion of the total pVII protein was found assembled into viral particles and further processed (124, 125).

Collectively, it is obvious that temporal assembly of the mature VII and precursor pVII proteins on virus DNA assures that the viral genome is protected and expressed properly during the HAdV infectious cycle.

**Ubiquitination**

Protein ubiquitination is a complex post-translational modification where covalent attachment of a small ubiquitin molecule can alter the stability, localization and activity of the targeted substrate proteins (126). The attachment of ubiquitin moiety to the target proteins occurs sequentially in three steps and involves three key enzymes. In the first step, the ubiquitin-activating enzyme, E1, activates the C-terminal glycine residue of ubiquitin through ATP-dependent process. In the second step, the activated ubiquitin is subsequently transferred to cysteine residue of ubiquitin conjugating enzyme E2. Finally, the ubiquitin ligase E3 facilitates the transfer of ubiquitin from E2 to a lysine residue of the substrate protein (Figure 5) (127, 128).

The E3 ligases are the most prevalent and important class of enzymes that confer selectivity for protein ubiquitination by mediating interaction between the E2 and substrate protein (129). Broadly speaking, the E3 ligases are grouped into two families based on the presence of either a HECT (homologous to the E6-AP carboxy terminus) domain or a RING (really interesting new gene) finger motif. The HECT E3 ligases serve as catalytic intermediates to which ubiquitin from E2 binds before transferred to the target protein. The RING E3 ligases function as molecular scaffolds and facilitate direct transfer of ubiquitin from the E2 to the substrate protein. The fate of the protein depends on the type of ubiquitin modification. For example, attachment of a single ubiquitin (monoubiquitination) can modify protein functions and does not necessarily lead to protein degradation. In contrast, polyubiquitinated substrate protein is recognized by the 26S proteasome, which targets the protein for degradation (130). Therefore, protein elimination by the ubiquitin-mediated proteasomal degradation is also known as the ubiquitin-proteasome pathway (UPS).
Figure 5. Schematic overview of ubiquitin proteasome system (UPS) involving the RING finger E3 ligases. Ubiquitin activation is catalyzed by E1, which is ATP-dependent step. The charged ubiquitin is transferred to E2 and then ligated to the substrate protein by the E3 ligases. The ubiquitin tags (mono- or polyubiquitin) on the substrate protein lead to protein degradation or its function regulation.

Cullin-RING E3 ubiquitin ligases (CRLs)

Cullin-RING E3 ubiquitin ligases (CRLs) are the most prevalent class of E3 ligases. As the name says the key component in the CRL are the cullin (Cul) proteins. Mammalian genome encodes for seven cullins (Cul1, 2, 3, 4A, 4B, 5, and 7). The Culs contain a RING finger domain in the C-terminal region to facilitate binding of the E2 enzyme. The N-terminal region of Culs binds to specific adaptor proteins, which bind to substrate protein. Hence, Culs function as molecular scaffolds to bring substrate proteins to close proximity with the E2 protein and thereby help to attach ubiquitin moiety to the substrate protein. The CRL family of proteins play an important role in regulation of diverse eukaryotic cell processes like transcription, signal transduction, cell cycle progression and DNA replication (129, 131).

Among the different Culs, the Cul3 protein is extensively covered in the present thesis. Cul3 is a highly conserved member of the Cul family and one of the major regulators of developmental processes and stress responses in metazoans and plants (131). The defined Cul3-containing CRL complex contains the RING finger protein Rbx1, which binds the E2 enzyme and the BTB (Bric-a-brac, Tramtrack and Broad complex) domain proteins, which act as substrate specific adaptor proteins (131). The BTB domain proteins
are capable of forming dimers and can be divided into subfamilies based on their domains including BTB-zinc finger (BTB-ZF), speckle type BTB/POZ protein (SPOP), BTB-kelch, voltage-gated potassium channel T1 (T1-Kv), Meprin and TRAF homology (MATH-BTB) and others (132). The substrate proteins targeted by the cul3-CRL complex include the promyelocytic leukemia (PML), phosphatase and tensin homologue (PTEN) and Daxx (133). Not surprisingly, the presence of functional mutations or alterations in Cul3 family of E3 ligases are linked to various metabolic disorders, dystrophies and cancer (134).

Makorin ring finger protein 1 (MKRN1)

Another E3 ubiquitin ligase covered in the practical part of the thesis (Paper II) is the Makorin ring finger protein 1 (MKRN1). The MKRN1 gene was initially identified as the ancestral gene of MKRN zinc finger family of proteins. Further, the MKRN1 gene has been found in wide spectrum of species including mammals, fruitfly, roundworm (Caenorhabditis elegans), fungi and plants (135). In addition to MKRN1, also the MKRN2 and MKRN3 proteins constitute the human MKRN protein family.

The MKRN1 protein contains a distinct collection of three C₃H zinc finger motifs towards the amino terminal, followed by a C₃HC₄-type RING finger domain and a fourth C₃H zinc finger motif towards the C-terminus of the protein (Figure 6) (135).

Figure 6. Illustration of the MKRN1 protein with labeled zinc finger motifs (ZnF) and RING finger (RING) domain.

The C₃H zinc finger motifs are well known as DNA-binding motifs and are also implicated to mediate RNA-binding (136, 137). The C₃HC₄-type RING finger domain was recognized as the most significant domain of RING finger E3 ligases and appears to be involved in protein-protein interactions (138).

Accordingly, the MKRN1 protein can function as an E3 ubiquitin ligase as it contains a functional RING finger domain, which is an essential structural feature of the E3 ubiquitin ligases (139). Several cellular proteins, such as human telomerase reverse transcriptase (hTERT), p53, p21, p14ARF, peroxisome-proliferator-activated receptor (PPARγ), Fas-associated protein with death domain (FADD) and PTEN are the known substrates for
MKRN1-mediated ubiquitination (139-144). Hence, it has been suggested that MKRN1 is involved in the control of cell cycle arrest and apoptosis as it induces degradation of the p53 and p21 proteins. The MKRN1 protein was also reported to target viral proteins for proteasomal degradation. The capsid proteins from West Nile virus (WNV) and porcine circovirus type 2 (PCV2) interact with MKRN1, however only the WNV capsid protein has been shown to be ubiquitinated and proteasomally degraded in a MKRN1-dependent manner (145, 146).

Apart from MKRN1 E3 ubiquitin ligase activity, the MKRN1 protein was reported to function as a transcriptional repressor as well as transcriptional activator when bound to DNA. Interestingly, for these activities the MKRN1 does not need its E3 ligase activity (147). The MKRN1 protein short version (lacking the fourth C3H zinc finger and last six amino acids of RING finger domain) was described to positively regulate the translation of dendritic mRNAs at synapses by interacting with poly(A)-binding protein (PABP) (148). A recent report further confirmed that the MKRN1 protein assembles into a ribonucleoprotein complex in mouse embryonic stem cells. Here, the MKRN1 protein was found to interact directly with RNA and was indicated to regulate the transport of RNA transcripts involved in stress response (149).

Overall, the MKRN1 protein functions as an RNA-binding protein and as the E3 ubiquitin ligase with established roles in nucleic acid accumulation regulation and substrate protein ubiquitination.

HAdV and protein ubiquitination

The ubiquitin-proteasome system (UPS) plays an important role in various biological processes like DNA repair, cell-cycle regulation, signal transduction, immune and inflammatory responses (150). Given their diverse role in the cellular processes, it is not surprising that viruses target UPS for efficient virus production (151). For example, HAdV infections cause significant alterations in the cellular UPS. Here, the key players are the early viral proteins E1B-55K and E4-ORF6 and cellular CRL complexes (152).

In the groundbreaking report by Querido et al., the E1B-55K and E4-ORF6 proteins were demonstrated to assemble into a multiprotein complex containing cellular Cul5, Elongins B and C, and the RING-H2 finger protein Rbx1. The same study showed that the E1B-55K/E4-ORF6 together with the Cul5 complex was capable to ubiquitinate the p53 protein (153). In the follow-up studies it became clear that the E4-ORF6 protein interacts with the aforementioned cellular factors, whereas the E1B-55K serves as the substrate-recognition protein (154, 155). To date a wide range of cellular proteins are known as the substrates for the E1B-55K/E4-ORF6 CRL complexes. This list includes the p53, Mre11, DNA ligase IV, Bloom helicase, integrin α3, Tip60, ATRX and SPOC1 proteins (26, 34, 119, 156-159).
It is also evident that HAdV-5 E1B-55K alone, independently of the E4-ORF6, can form Cul5-based E3 ubiquitin complex and target a pro-apoptotic protein Daxx for proteasomal degradation (160). Similarly, the HAdV-12 E4-ORF6 protein, without the need for the E1B-55K protein, can induce degradation of the topoisomerase IIβ-binding protein 1 (TopBP1) (161). In addition to Cul5, Cul2-based CRL complexes can also interact with the E1B-55K/E4-ORF6 proteins. Here different HAdV type-specific E1B-55K/E4-ORF6 proteins display heterogeneity in selecting the Cul5 or Cul2 proteins to form functional E3 ligase complexes (162, 163). Collectively, all these findings suggest that various HAdV types employ different strategies to engage diverse CRL complexes to counteract host cell antiviral responses and to enhance virus replication.

Besides ubiquitin, there are other ubiquitin-like moieties that can be covalently attached to the substrate proteins. This includes, for example, the small ubiquitin-like modifier (SUMO) protein. Unlike ubiquitination, protein sumoylation doesn’t usually target substrate protein for direct degradation, but rather modulates protein-protein interactions. Similarly to ubiquitination, the HAdV infection interferes with the cellular sumoylation pathways. For example, the HAdV-5 E1B-55K protein can undergo sumoylation (164). Further, the E1B-55K acts as SUMO E3 ligase for p53, and sumoylates p53, which results in altered localization and inactivation of the p53 protein (165). Also, the HAdV-5 E4-ORF3 protein can induce sumoylation of the substrate proteins such as the Mre11, Nbs1 and TFII-I proteins. Interestingly, sumoylation of the TFII-I causes subsequent proteasomal degradation of the protein (166, 167). Recent reports demonstrate that attachment of a poly-SUMO2/3 chain can act as the signal for SUMO-targeted ubiquitin ligases (STUbLs), which, by binding to the sumoylated proteins, induce their ubiquitination and proteasomal degradation (168).

Ubiquitin conjugation is a reversible process. Hence, the ubiquitin moiety can be removed by a class of isopeptidases called deubiquitinating enzymes (DUBs) (128). Viruses take advantage of deubiquitination to promote efficient virus replication and some viruses encode their own viral DUB to intervene normal ubiquitination. HAdV-5 encoded Avp was found to exhibit deubiquitinating activity and its overexpression can decrease the accumulation of the ubiquitinated proteins in cells (169).
Present investigation

Paper I: Adenovirus precursor pVII protein stability is regulated by its propeptide sequence

The HAdV-5 precursor pVII protein is proteolytically cleaved by the Avp protease to generate the mature VII protein during the late phase of infection (57, 123). This site-specific cleavage releases a short 24 amino acid propeptide from the N-terminus of the precursor pVII protein (Figure 4). The mature VII protein is functionally best characterized and studied, whereas the precursor pVII protein-related studies are very limited. Therefore, in this paper we investigated the functional importance of the propeptide module in the precursor pVII protein.

The propeptide sequence regulates the precursor pVII protein stability

To test if the propeptide module per se has any functional activity, we fused the propeptide sequence to Gal4 DNA-binding domain and analyzed its ability to regulate basal transcription on the Gal4-controlled thymidine kinase promoter. Surprisingly, the observed negative effect of the 1-24Gal4 fusion protein on transcription was due to the proteasomal degradation of the 1-24Gal4 protein (Paper I, Figure 1C). Hence, we hypothesized that presence of the propeptide module in the precursor pVII (hereafter as pVII(wt)) may affect the protein stability when compared to the mature VII (hereafter as pVII(Δ24)) protein. Initial difficulties to detect the transfected precursor pVII protein expression were solved by codon optimization of the pVII DNA sequence. Upon codon optimization, the protein expression was significantly enhanced indicating that our initial protein detection failure could be due to non-optimal codon bias of the pVII mRNA sequence. Interestingly, a western blot analysis comparing the pVII(wt) and pVII(Δ24) levels indicated that the pVII(Δ24) protein lacking the propeptide sequence accumulated to much higher levels than the pVII(wt) protein (Paper I, Figure 2A).

Using HEK293 stable cell lines we performed a protein decay assay to detect the pVII(wt) and pVII(Δ24) protein accumulation in the presence of cycloheximide treatment (CHX), which blocks translation elongation. Notably, the propeptide-containing pVII(wt) protein decayed faster than the
pVII(Δ24) protein (Paper I, Figure 2B). To further elucidate if the enhanced pVII(wt) protein decay was due to proteasomal degradation, the CHX treated cells were simultaneously treated with the proteasomal inhibitor MG132. Inhibition of the ongoing pVII(wt) protein synthesis by CHX resulted in a fast decay of the pVII(wt) protein, whereas the concurrent proteasome inhibition stopped the decay (Paper I, Figure 2C). All together, the data support that the pVII(wt) protein was targeted for proteasomal degradation and the propeptide functions as the protein destabilizing element.

Lysines K26 and K27 are associated with precursor pVII protein stability

The ubiquitin-mediated protein degradation occurs through covalent linkage of the ubiquitin moieties to the lysine (K) residues of the substrate proteins (126). To identify the lysine residues involved in the pVII(wt) protein stability, we mutated the lysines at positions 20, 26, 27, 48, 97, 99 to arginines (R). By comparing the steady state protein expression of these pVII mutant proteins we found that residues K26 and K27 control the pVII(wt) protein stability. The other lysine mutants K20, K48, K97 and K99 did not remarkably elevate the protein levels (Paper I, Figure 3B). The lysines K26 and K27 are also present in the pVII(Δ24) protein, however K26R and K27R mutations in the pVII(Δ24) protein did not increase the protein stability (Paper I, Figure 3C). From these experiments, we concluded that K26 and K27 residues are important for the pVII(wt) protein stability.

Cul3-based CRL reduces the precursor pVII protein levels

To study the mechanisms involved in the pVII(wt) protein stability we decided to evaluate the involvement of CRLs as they are the most common E3 ubiquitin ligases in the mammalian cells (170). To eliminate the function of endogenous CRLs, the dominant-negative Culs (DN-Cul) were overexpressed in HEK293-pVII(wt) stable cell lines. Upon analyzing the pVII(wt) protein levels we found that inactivation of the endogenous Cul3 protein by DN-Cul3 enhanced the pVII(wt) protein stability. We further analyzed the pVII(K26R/K27R), pVII(Δ24) and pVII(wt) protein levels in the presence of DN-Cul3 and observed that only the pVII(wt) protein levels were enhanced by DN-Cul3 overexpression (Paper I, Figures 4A and 4B). Additionally, we confirmed the involvement of Cul3 in pVII(wt) protein stability in DN-Cul3 expressing and CHX treated cells (Paper I, Figure 4C). All together, these results suggest that the Cul3-based CRL complex is/are involved in the pVII(wt) protein stability control.
The propeptide enables Cul3 association and nucleolar localization of the pVII(wt) protein

Having determined the Cul3 involvement in the pVII(wt) protein stability, we then evaluated the potential protein-protein interactions between the Cul3 and pVII proteins. The GST-pVII(wt) and GST-pVII(K26R/K27R) proteins displayed enhanced association with the Flag-Cul3(wt) protein when compared to the GST-pVII(Δ24) protein (Paper I, Figure 5A). Further, we investigated the localization of pVII(wt), pVII(Δ24) and pVII(K26R/K27R) proteins in transiently transfected HeLa cells by immunofluorescence assay. Interestingly, the pVII(wt) protein specifically localized into the nucleolus whereas, the pVII(Δ24) and pVII(K26R/K27R) proteins showed notable localization to the proximity of nucleoli, hence contrasting the pVII(wt) protein localization (Paper I, Figure 5C). These differences in subcellular localization of the precursor pVII and mature VII have been recently reproduced by the Matthew D. Weitzman’s laboratory (118).

Cul3 regulates early E1A gene expression in HAdV-5-infected cells

To study the role of the Cul3 protein in HAdV-5 infection, we generated inducible HeLa stable cell line expressing the DN-Cul3-Flag protein and monitored HAdV-5 infection progress in the infected cells. Our studies revealed that the expression of DN-Cul3 reduced viral E1A protein levels as well as the E1A mRNA accumulation during the early phase of infection (Paper I, Figures 6A and 6B). Hence, the functional Cul3 protein may act as a positive factor enhancing the HAdV-5 early gene expression.

Paper II: Human adenovirus infection counteracts antiviral activity of the cellular MKRN1 E3 ubiquitin ligase

To identify the cellular interacting partners and to gain a better insight into the molecular functions of the HAdV-5 precursor pVII protein, we performed a yeast two-hybrid screen (Paper II, Table1). Further, we confirmed the identified pVII interactors using proximity ligation assay (Paper II, Figure 1). Due to our interest in protein stability regulation (Paper I), we decided to study the functional role of the identified E3 ubiquitin ligase MKRN1 and its interplay with the pVII protein in various HAdV-5 infections.
The pVII(wt) protein binds MKRN1 during HAdV-5 infection

To further confirm the pVII-MKRN1 interactions, co-immunoprecipitation in the pVII(wt)-Flag and HA-MKRN1(wt) expressing H1299 cells were performed. The experiments concluded that pVII(wt) protein can specifically interact with the MKRN1 protein and this interaction is enhanced in the presence of the proteasome inhibitor MG132 (Paper II, Figure 2A). Furthermore, pVII interaction with the endogenous MKRN1 protein was confirmed in the HAdV-5 infected cells (Paper II, Figure 2B). To determine the pVII interaction region(s) on the MKRN1, we performed GST-pVII(wt) pull-down experiments using the HA-MKRN1 expressing H1299 cell lysates. The results showed that both the N-terminal (amino acids 1-267) and C-terminal (amino acids 112-482) regions of the MKRN1 protein were able to bind to the pVII(wt) protein (Paper II, Figure 2D). Interestingly, we also found that compared to pVII(Δ24), the pVII(wt) protein binds better to the MKRN1 protein in co-immunoprecipitation and GST pull-down experiments (Paper II, Figure 2E, Supplementary figure 1A).

MKRN1 overexpression reduces the pVII protein levels and affects formation of virus progeny

Considering that MKRN1 causes substrate protein ubiquitination and degradation (139, 140), we tested if the pVII protein steady state levels were affected in the presence of the MKRN1(wt), MKRN1(H307E) and MKRN1(1-267) proteins in the HAdV-5-infected cells. We observed that the MKRN1(wt) and MKRN1(H307E) overexpression reduced the pVII protein levels at 24 hpi, but not at 48 hpi, and that this reduction was due to the proteasomal degradation (Paper II, Figures 3A and 3B). The decrease of pVII protein levels by the MKRN1 protein overexpression was also confirmed in transient transfection experiments, showing that negative effect of the MKRN1 protein on pVII does not depend on other HAdV-5 proteins (Paper II, Supplementary figure 1B). Further, we generated U2OS cell lines constitutively expressing the MKRN1(wt), MKRN1(H307E) and MKRN1(1-267) proteins and analyzed infectious virus particle production in these cell lines. Similar to the experiments in H1299 cells, MKRN1(wt) and MKRN1(H307E) expression in U2OS cells reduced the pVII protein levels as well as the HAdV-5 progeny production (Paper II, Figures 3C and 3D).

Collectively, this data suggest that MKRN1 is a potential anti-viral protein targeting HAdV-5 pVII protein for proteasomal degradation in the infected cells and overexpression of the MKRN1 protein can hinder formation of infectious HAdV-5 progeny.
The MKRN1 protein is proteolytically degraded in HAdV-5 infected cells

To investigate if the potential antiviral activity of the MKRN1 protein is counteracted by HAdV-5, we monitored the endogenous MKRN1 levels in HAdV-5 infected H1299 cells at different time points. This approach was undertaken because HAdV infections cause proteasomal degradation of several known host cell antiviral proteins (see section “HAdV and protein ubiquitination”). Surprisingly, the MKRN1 levels were gradually decreased from 16 hpi and onwards in the infected cells (Paper II, Figure 4A). The decrease in MKRN1 level was however restored in the MG132-treated cells, pointing out that HAdV-5 targets MKRN1 for proteasomal degradation (Paper II, Figure 4B, lanes 3,4). The MKRN1 degradation was not limited to H1299 cells alone, as similar phenomenon was observed in HAdV-5 infected A549, U2OS, HEK293 cells (Paper II, Supplementary Figure 2). These findings confirm that the MKRN1 protein proteasomal degradation by HAdV-5 infection is not cell line specific and strengthens the outlook that HAdV-5 has evolved counteracting mechanism against MKRN1 activity.

HAdVs express the E1B-55K and E4-ORF6 proteins, which recruit Cul-based E3 ligases to target different cellular substrates for degradation via UPS (see section “HAdV and protein ubiquitination”). However, based on our results, the MKRN1 protein degradation occurs independently of the E1B-55K and/or E4-ORF6 proteins in transient transfection experiments as well as in the HAdV-5-infected cells (Paper II, Figure 5).

The MKRN1 protein self-ubiquitination is enhanced by the pVII(wt) protein

The MKRN1 protein interacts with the substrate proteins through its C-terminal RING finger domain and induces their ubiquitination and proteasomal degradation (see section “Makorin ring finger protein 1 (MKRN1)”). Curiously, the precursor pVII(wt) protein was found to interact with both the N-terminal (amino acids 1-267) and C-terminal RING finger domain containing regions (amino acids 112-482) of the MKRN1 protein (Paper II, Figure 2D). This observation urged us to study the impact of the pVII(wt) protein on the MKRN1 ubiquitination. More specifically, we were interested to understand if the pVII protein can be the substrate for MKRN1 or if the pVII can affect MKRN1 ubiquitin E3 ligase activity. Surprisingly, we found that the precursor pVII(wt) protein, but not the pVII(A24) protein, enhanced the MKRN1(wt) protein self-ubiquitination, whereas this effect was completely absent in MKRN1(1-267) and negligible in MKRN1(H307E) expressing cells (Paper II, Figures 6A and 6B). Taken together, these results indicate that HAdV-5 counteracts the potential MKRN1 antiviral effect by causing proteasomal degradation of the MKRN1
protein and that this process might be initiated by the pVII-mediated MKRN1 self-ubiquitination. Most interestingly, the MKRN1 protein deprivation was not limited to HAdV-5 alone, as we found that also measles virus and vesicular stomatitis virus infections affect MKRN1 protein accumulation (Paper II, Figure 7).

Paper III: Complementation of the human adenovirus type 5 VA RNAI defect by the Vaccinia virus E3L protein and serotype-specific VA RNAIs

The HAdV encoded VA RNAI targets innate immune system by antagonizing the PKR protein functions as well as it can interfere with RNAi/miRNA pathway in the infected cells (Figure 3). In the paper III, we aimed to understand if the VA RNAI multifunctionality is due to its non-coding RNA origin. Therefore we decided to test various plant and animal virus encoded RNAi/miRNA and IFN suppressor proteins to evaluate if they can functionally rescue the growth of VA RNAI deficient HAdV-5 (known as dl705) virus.

Complementation of VA RNAI-deficient dl705 virus growth with various suppressor proteins

In search for proteins that can functionally complement VA RNAI-deficient HAdV dl705, we tested two plant virus proteins: p19 (Tombusvirus, TBSV), NS3 (Tenuivirus, RHBV) and two animal virus proteins: E3L (Vaccinia virus, VACV), VP35 (Ebola virus, EBOV). These proteins have been shown to bind dsRNAs, function as the RSS proteins and counteract antiviral response (79, 171-175). The VA RNAI mutant adenovirus dl705 shows reduced growth in the infected cells (64), which correlates with diminished viral capsid protein accumulation (e.g. Paper III, Figure 1, lane 13). Thereby detection of the viral capsid proteins, such as hexon and fiber, can be considered as a good quantitative measurement of virus replication in the infected cells. For this purpose HEK293 cells were transfected with increasing amounts of plasmids expressing the Flag-tagged p19, NS3, E3L, VP35 proteins and the dl705 virus growth was monitored by detection of viral capsid protein accumulation and synthesis rate. The plasmid pHindB, which expresses HAdV-2 VA RNAI and VA RNAII, was used as a positive control throughout the study.

Transient overexpression of the p19, NS3, VP35 proteins did not enhance dl705 virus capsid protein accumulation by western blotting and capsid protein synthesis by 35S-methionine pulse labeling (Paper III, Figure 1, lanes 2-5, 8-9). In contrast, overexpression of the VACV E3L protein elevated the
expression of viral late proteins, pointing to the positive effect of this protein on the growth of dl705 virus in our cell system (Paper III, Figure 1, lanes 6-7). Similarly, cells transfected with the pHindB plasmid enhanced the viral capsid protein accumulation (Paper III, Figure 1, lanes 10-11). Interestingly, complementation by the E3L protein was not as efficient as it was achieved by overexpression of the VA RNA encoding plasmid pHindB. Taken together, our data indicates that the VACV E3L protein can specifically enhance dl705 virus growth.

The VACV E3L C-terminal region is essential to enhance dl705 virus amplification

The VACV E3L is a bifunctional protein, which binds to DNA via the N-terminal Z-DNA-binding domain and to dsRNA via the C-terminal dsRNA-binding domain (DRBD) (76, 77). Both these domains are reported to inhibit PKR activity in different experimental systems (171).

Since the E3L protein enhanced the capsid protein production in dl705-infected cells, we were interested to delineate the E3L domain(s) required for this effect. To study this, HEK293 cells were transfected with plasmids encoding the wild-type E3L(wt), or mutant E3L(1-100), E3L(KRAA) proteins (176). The aforementioned mutant E3Ls lack the C-terminal DRBD (E3L(1-100)) or contain mutations at lysine 167 and arginine 168 (E3L(KRAA)), which eliminate dsRNA-binding activity of the protein. As expected, expression of the E3L(wt) protein stimulated the capsid protein synthesis, whereas the expression of the E3L(1-100) and E3L(KRAA) proteins failed to enhance dl705 capsid protein synthesis (Paper III, Figure 2B).

To further investigate the role of the E3L protein in dl705 virus amplification, we analyzed eIF2α phosphorylation at Ser51. Phosphorylation of this particular amino acid residue is a diagnostic mark of the cellular antiviral activity as it blocks translation initiation, and therefore affects virus capsid protein synthesis (see section “VA RNA inhibits PKR activation”). Notably, the E3L(wt) protein reduced eIF2α Ser51 phosphorylation in dl705-infected cells (Paper III, Figure 2C). Taken together, our results suggest that the E3L C-terminal region containing the DRBD may enhance capsid protein synthesis in dl705-infected cells by reducing the eIF2α Ser51 phosphorylation.

The E3L protein substitutes for VA RNAI and enhances formation of infectious virus progeny

Replication of dl705 can be rescued by the E3L protein, which stimulates virus capsid protein synthesis (Paper III, Figure 1). However, it is not definite that this translational rescue results in generation of infectious virus progeny. To answer this, we transiently transfected HEK293 cells with the
E3L protein encoding plasmids and infected cells with the dl705 virus for new virus formation. The total cell lysates were collected, re-infected and titrated in 911 cells to quantitate the formation of infectious virus particles. Our results indicated that the E3L(wt) protein contributes to the formation of infectious dl705 virus progeny and it was more effective when compared to the expressed E3L(1-100) and E3L(KRAA) proteins (Paper III, Figure 3C).

The E3L protein suppress PKR phosphorylation of eIF2α to enhance dl705 virus capsid protein synthesis

As described above, E3L(wt) expression affects the eIF2α Ser51 phosphorylation during dl705 virus infection and partially rescues the dl705 virus capsid protein synthesis. As the best-characterized kinase phosphorylating the eIF2α Ser51 is PKR, we investigated the E3L protein impact on PKR-mediated eIF2α phosphorylation. Here, we took advantage of the HeLa PKR knockdown (PKR<sup>kd</sup>) and control (PKR<sup>kd-con</sup>) cell lines (177). Plasmids encoding the E3L(wt) protein and HAdV-2 VA RNAs (pHindB) were transiently transfected into PKR<sup>kd</sup> and PKR<sup>kd-con</sup> cell lines followed by dl705 infection. As expected, the dl705 virus replicated well in the PKR<sup>kd</sup> cells due to the lack of antiviral activity of the PKR protein (Paper III, Figure 5A). In the PKR<sup>kd-con</sup> cells, which have active functional PKR, the dl705 virus grew poorly, but the virus growth was enhanced by expression of the E3L protein or VA RNAs (pHindB) (Paper III, Figure 5A, lanes 6 to 8). The protein lysates from this experiment were subjected to western blotting and the membrane was probed to detect eIF2α Ser51 phosphorylation signal. The quantification indicated decrease in the eIF2α phosphorylation in PKR<sup>kd-con</sup> cells transfected with the E3L(wt) protein and HAdV-2 VA RNA encoding plasmids (Paper III, Figure 5B, lanes 7 and 8). Taken together, these results suggest that the E3L(wt) protein inhibits PKR-mediated phosphorylation of eIF2α on Ser51 to enhance the dl705 viral protein synthesis.

The E3L protein does not alter the viral late mRNA levels during dl705 virus infection

From the virus titration experiments, it was evident that dl705 virus rescue by VA RNAs was at least 1 log better than in the case of the E3L protein (Paper III, Figure 3). Previous reports showed that VA RNAs could increase cytoplasmic mRNA abundance along with mRNA translation (178, 179). To study the potential effect of the E3L protein on mRNA abundance in our cell system, we analyzed HAdV-5 hexon mRNA levels in the E3L protein and VA RNA expressing, dl705 infected cells. Our results suggest that the E3L protein does not influence the hexon mRNA levels, whereas the VA RNAs can increase hexon mRNA accumulation up to 4-fold in concentration-
dependent manner (Paper III, Figure 6). Therefore, the combined effect including PKR inhibition and enhanced late viral mRNA expression, may explain why VA RNAs are more potent molecules than the E3L protein in rescuing dl705 virus replication.

HAdV-4 and HAdV-37 VA RNAI complement dl705 virus more efficiently than other HAdV type-specific VA RNAIs

The VA RNAs originating from various HAdV types show considerable variations in sequences, length and probably also in functions in the infected cells (58, 180). This outlook suggests that HAdV type-specific VA RNAIs may rescue dl705 virus growth differently. To study if the ability of VA RNAI to rescue dl705 virus replication depends on virus type origin, we generated plasmids expressing only VA RNAI from HAdV-4, HAdV-5, HAdV-12 and HAdV-37 types. These plasmids were then used in the aforementioned dl705 virus complementation assay. Our results clearly indicate that HAdV-4 and HAdV-37 VA RNAI were most prominent to enhance virus capsid protein accumulation (Paper III, Figure 7A, lanes 2 and 6) and formation of infectious dl705 virus particles (Paper III, Figure 7D). The difference in the HAdV type-specific complementation of dl705 growth was likely due to variations in the eIF2α Ser51 phosphorylation. Notably, the VA RNAI from HAdV-4 and HAdV-37 lowered the eIF2α Ser51 phosphorylation better than the other tested VA RNAIs (Paper III, Figure 7C).

Paper IV: RNA triplex formation in human adenovirus type 4 VA RNAI and its implication on virus growth

As a logical continuation of the paper III, we investigated the structural differences between VA RNAIs originating from various HAdV types. We hypothesized that HAdV type-specific VA RNAIs adopt different tertiary structures, such as RNA triplexes, which may explain the diverse activity of VA RNAIs (Paper III, Figure 7). To study the potential triplex structure formation within the VA RNAI molecule, we took advantage of a novel nucleic acid triplex probing compound, benzoquinolinaxline 1,10-phenanthroline (BQQ-OP). Hence, we demonstrated that BQQ-OP preferably cleaves the HAdV-4 VA RNAI, whereas the HAdV-12 VA RNAI was resistant to the cleavage. Importantly, this in vitro cleavage data (Paper IV, Figure 1) is in line with the functional data presented in the paper III (Figure 7). Furthermore, our data suggest that the conserved tetranucleotide motif 5’-GGGU-3’ in stem 4 and protruding stem 7 may form a potential triplex RNA structure in HAdV-4 VA RNAI. This is based on observation that mutations
within these sequence elements abolish or alter BQQ-OP \textit{in vitro} cleavage (Paper IV, Figure 2).

HAdV-4 VA-RNAI (S7M) is partially deficient in complementing dl705 virus replication

By utilizing the VA RNAI-deficient virus (dl705) based assay we tested whether the HAdV-4 VA RNAI stem 4 (hereafter as VA RNAI(S4M)) and stem 7 mutants (hereafter as VA RNAI(S7M)) are functional \textit{in vivo} in virus-infected cells. Surprisingly, our results indicated that both wild-type and stem 4 mutant VA RNAIs were able to enhance the dl705 virus replication and infectious virus progeny formation by diminishing the eIF2α Ser51 phosphorylation (Paper IV, Figure 3C). In contrast, the VA RNAI(S7M) partially failed to complement dl705 virus growth (Paper IV, Figure 3A and 3D) and it was unable to block eIF2α Ser51 phosphorylation (Paper IV, Figure 3C). This result suggest that the protruding stem 7 is important for the functional activity of the HAdV-4 VA RNAI in the infected cells.
Discussion and future perspectives

The HAdV-5 precursor pVII stability is regulated by its propeptide sequence

One of the aims of the present thesis was to study the biochemical characteristics of the HAdV-5 precursor pVII protein. Therefore, the first study (Paper I) was focused mainly on the importance of the propeptide cleavage and its role in the precursor pVII protein stability regulation.

In this study, we showed for the first time that the 24 amino acid propeptide at the N-terminus of the precursor pVII protein acts as the protein destabilizing element. Truly, the presence of propeptide sequence caused proteasomal degradation of either an artificial fusion protein (1-24Gal4) or the precursor pVII protein. The lysine residues K26 and K27 were found to be involved in the pVII stability regulation. Further, the Cul3 protein was pinpointed as the factor influencing the pVII protein stability as interference with the endogenous Cul3 protein increased the pVII protein stability. From GST-pull down experiments it was evident that the Cul3 protein preferably binds to the pVII propeptide sequence.

Collectively, we put forward a model whereby the propeptide acts a platform for Cul3-based CRL, which in turn targets the precursor pVII protein K26/K27 lysine residues for ubiquitin modification and ultimately for target protein degradation (Figure 7).

![Figure 7. Schematic model showing Cul3-mediated regulation of the HAdV-5 pVII stability through lysines K26/K27 while binding to the pVII propeptide element.](image-url)
Further, based on our findings we have proposed a hypothetical model to explain the role of Cul3 protein in E1A gene expression and the need to cleave the propeptide module from the precursor pVII protein (Figure 8).

What would be the role of Cul3 in the HAdV-5 infection? Inhibition of the endogenous Cul3 protein functions by DN-Cul3 expression resulted in reduced viral early E1A gene expression. This observation indicates that the Cul3 protein might be essential during early stages of HAdV-5 infection. Notably, the Cul3 protein was suggested to play crucial role in endosome maturation and was reported to be responsible for ubiquitination and proteasomal degradation of proteins involved in the efficient endosomal transport of the influenza A and VACV particles in the infected cells (181, 182). As the HAdV intracellular trafficking occurs via endosomes, we speculate that Cul3 might be essential factor for HAdV-5 endosomal transport and import of viral DNA into the nucleus. Further, the Cul3 protein CRL complex was shown to ubiquitinate and degrade the Daxx protein, a transcriptional repressor that inhibits E1A gene transcription (183). Similarly, Cul3 was suggested to be involved in degradation of Tip60, a lysine acetyltransferase, which suppresses E1A gene expression (184). Therefore, presence of active Cul3 seems to be important for HAdV-5 early gene, such as E1A, expression (Figure 8). Hence, future studies should be addressed to understand if the Cul3 is also needed for the expression of other early genes (e.g. E1B-55K, E1B-19K) and to delineate the exact role of Cul3 (endoso-
ormal maturation versus removal of the Daxx and/or Tip60) during early phase of infection.

From our findings it is evident that the Cul3 protein affects the precursor pVII protein stability via the propeptide module. However, the incoming virus particle contains the mature VII, and not the precursor pVII protein wrapped around the virus genome. Thus, if there is no propeptide sequence available, how can we connect Cul3 activity and the propeptide cleavage during the early phase of HAdV-5 infection? To dissect this puzzle one has to imagine a theoretical condition where the precursor pVII protein is bound to viral genome instead of the mature VII protein in the incoming virus particle (Figure 8). This may generate a situation whereby the precursor pVII is recognized by the Cul3-based CRLs and targeted for the proteasomal degradation. As a consequence, the delivery of the HAdV-5 DNA into the nucleus and virus genome stability can be affected. Thus, we believe that one reason why the HAdV-5 encoded Avp protease cleaves off the propeptide on the DNA-bound pVII during the maturation step, is to generate Cul3-resistant mature VII protein.

In summary, our report provides a potential explanation why the propeptide module has to be cleaved from the precursor pVII protein during the final stages of the virus particle maturation and underlines the propeptide module the as destabilizing element within the precursor pVII protein.

HAdV-5 pVII protein interactome and interplay with the MKRN1 protein

The pVII is well established as the DNA-interacting protein, but surprisingly little is known about its mutual interactions with viral and cellular proteins. Therefore, our study (Paper II) along with recent report from the Weizmann laboratory (118), are the latest progressive attempts to reveal the pVII protein interactome. Interestingly, the precursor pVII protein interacts with the CHD3 and Baz1a proteins, which are involved in chromatin remodeling and heterochromatin formation (185, 186). Considering the reported activities of the VII protein on gene expression (103, 111-113), it is possible that there is interplay between pVII with the CHD3 and Baz1a proteins to achieve optimal pVII-dependent chromatin remodeling in the HAdV-infected cells. In addition, we identified two other chromatin structure regulating proteins, HMGB2 and HMGB3, as the novel pVII interacting partners. This finding is in line with a recent report whereby the mature VII interacts with the HMGB1 and HMGB2 proteins (118). Although, the mature VII impacts on antiviral responses by altering the HMGB1 and HMGB2 protein functions on the host cell chromatin, it remains to be tested what is the functional impact of the pVII protein on HMGB3.
We further characterized the E3 ligase MKRN1 protein as one of the novel pVII protein interacting partners. As a logical follow-up of our Paper I we tested if MKRN1 is the E3 ligases involved in the pVII protein ubiquitination/degradation. Notably, transient or stable overexpression of the MKRN1 protein caused the pVII protein degradation at 24 hpi. However, this effect was not detectable at 48 hpi, suggesting that the potential antiviral effect of the MKRN1 protein has vanished during the prolonged infection. An interesting observation from these experiments was that the mutant protein MKRN1(H307E), shown to be deficient in self-ubiquitination and in substrate ubiquitination (139, 140), behaved as the MKRN1(wt) protein. Although, MKRN1 expression affected the pVII protein accumulation, we were unable to show that MKRN1(wt) caused pVII ubiquitination. Collectively, our results suggested the following: 1) MKRN1 exhibits potential antiviral activity by causing pVII proteasomal degradation during early stages of infection, 2) the MKRN1 RING finger domain is essential for pVII protein degradation, 3) the MKRN1 protein may not function as the E3 ligase degrading the pVII protein, but instead MKRN1 may act as a scaffold protein recruiting other, yet unknown, cellular factors resulting in the pVII protein proteasomal degradation (Figure 9).

Interestingly, analysis of the endogenous MKRN1 protein in HAdV-5 infected cells clearly indicated a gradual decrease and proteasomal degradation of the protein. Surprisingly, this degradation occurred independently of the E1B-55K/E4-ORF6 proteins suggesting a novel, uncharacterized way to degrade proteins in HAdV-5 infected cells. This observation, along with the findings that the pVII protein has dual interaction surfaces on the MKRN1 protein, intuitively suggested that pVII might influence the MKRN1 E3 ligase activity. Truly, our in vivo ubiquitination studies revealed that the precursor pVII protein, but not the mature VII, specifically enhanced the MKRN1(wt) protein ubiquitination. Puzzlingly, the MKRN1(H307E) protein displayed very strong basal ubiquitination, challenging the general view that this particular mutation eliminates the MKRN1 E3 ligase activity. Based on our in vitro and in vivo experiments we believe that the MKRN1(H307E) protein is deprived of its self-ubiquitination activity, but can still be a substrate for ubiquitination by other, yet unknown, cellular E3 ubiquitin ligases. Interestingly, our data suggest that the pVII protein expression does not enhance MKRN1(H307E) ubiquitination to the same extent as was observed for the MKRN1(wt) protein. Now, considering that MKRN1(H307E) is deficient in self-ubiquitination, we favour a view whereby the pVII protein can specifically enhance MKRN1(wt) self-ubiquitination.

Taken together, further studies are warranted to reveal the exact nature of the ubiquitin modifications (mono-, poly-, self-ubiquitination), the detailed impact of these modifications on the MKRN1 protein function, and most importantly, how the pVII(wt) protein enhances MKRN1 ubiquitination.
Based on our findings in Paper II we have proposed a working model regarding the pVII and MKRN1 proteins interplay. The model is split into two parts based on the time frame of the HAdV-5 infection. First, during the period, when the de novo synthesized pVII starts to accumulate in the infected cells, it will be bound up by the MKRN1 protein. At this stage the MKRN1 acts as a scaffold protein and by recruiting of other, yet unknown, cellular E3 ligase(s), the pVII protein degradation will be achieved. Hence, at this stage the MKRN1 exhibits its potential antiviral function. Second, as the infection proceeds and the pVII protein accumulate to high levels, the pVII protein interacts and saturates both the amino- and carboxy-terminal regions of the MKRN1 protein. This unusual binding mode of the pVII protein might result in enhanced self-ubiquitination of the MKRN1 protein and its subsequent proteasomal degradation in HAdV-5-infected cells. By this way, the virus neutralizes the antiviral activity of the MKR1 protein (Figure 9).

Figure 9. Schematic model displaying the role of the MKRN1 protein during different stages of the HAdV-5 infection. The shift from scaffold function to substrate might be pVII concentration dependent as the pVII accumulation increases during the prolonged HAdV-5 infection.

Functional complementation of HAdV-5 VA RNAI

In our third paper, we analyzed the possibility of known viral IFN and RNAi/miRNA suppressor proteins to rescue replication of the HAdV-5 VA RNAI lacking virus, dl705. We selected two plant virus proteins, TBSV p19 and RHBV NS3, which block RNAi pathway, and two animal virus proteins,
VACV E3L and EBOV VP35, which are known to block IFN and RNAi/miRNA pathways. Out of these tested suppressor proteins, we showed that only the VACV encoded E3L protein could partially rescue the dl705 virus capsid protein synthesis. Further, the E3L protein reduced PKR-mediated eIF2α Ser51 phosphorylation and the integrity of E3L C-terminal region, containing the DRBD, was important to enhance the dl705 virus amplification. Hence, we propose that the E3L DRBD binds and sequesters the dsRNA species, generated during the dl705 infection. As these dsRNA molecules are needed for the PKR protein activation, and thereby for its antiviral activity, their sequestering by E3L will block PKR antiviral activity and leads to enhanced dl705 replication. Notably, the positive effect of the VACV E3L protein on dl705 is specific as the EBOV VP35 protein, which also binds dsRNA and can block PKR activity (173, 187), was unable to rescue dl705 in our experimental system.

Interestingly, the transiently provided VA RNAs (pHindB plasmid) complemented the dl705 virus replication and infectious particle formation significantly better than the E3L protein. One potential explanation here is that the VA RNA expression, in addition to blocking the PKR function and consequently the eIF2α Ser51 phosphorylation, can also increase viral hexon mRNA accumulation. This dual effect was not observed in the case of the E3L protein expression suggesting that VA RNAs possesses additional functions compared to the E3L protein (Figure 10). Although, the positive effect of VA RNA on cytoplasmic mRNA abundance has been reported previously (178, 179), the exact molecular details have remained enigmatic. Therefore, in the follow-up studies it would be interesting to know if the VA RNAs can directly target the hexon mRNAs by RNA-RNA hybrid formation or the effect is mediated by any of the VA RNA interacting proteins.

We also took an advantage of our complementation experimental system and analyzed the ability of different HAdV VA RNAIs to rescue dl705 replication. Although all known HAdV VA RNAs adopt a characteristic stem-loop structure (see Paper IV, Figure 1), they exhibit an obvious nucleotide sequence and length variations, which may have an impact on their functions. Truly, our experiments confirmed that HAdV-4 and HAdV-37 are significantly better in rescuing the dl705 virus growth relative to other tested HAdV VA RNAIs (Figure 10).
Figure 10. Schematic overview how the VACV E3L protein and HAdV type-specific VA RNAIs complement dl705 virus growth.

Functional characterization of HAdV-4 VA RNAI

The functional characterization of the HAdV type-specific VA RNAIs revealed that HAdV-4 VA RNAI efficiently targets elf2α Ser51 phosphorylation and thereby enhances dl705 replication better compared to VA RNAIs originating from the HAdV-5 and HAdV-12. One reason for this functional difference between VA RNAIs might be their different higher order organization, such as formation of the triplex structure. Therefore, we analyzed the potential triplex structure formation of these VA RNAIs using a chemical probing reagent BQQ-OP that was previously shown to be suitable to probe potential triplex DNA structures (188). Notably, our in vitro BQQ-OP cleavage experiments revealed that HAdV-4 VA RNAI adopts structures, which are not detectable in HAdV-12 VA RNAI (Paper IV, Figure 1). Thus, the enhanced dl705 replication in the presence of the HAdV-4 VA RNAI (Paper III) might be due to specific triplex structure adopted within this particular VA RNAI molecule (Paper III).

Further, HAdV-4 VA RNAI clearly displayed a BQQ-OP cleavage pattern, which was eliminated or altered when mutations were introduced into
stem 4 (VA RNAI(S4M)) and stem 7 (VA RNAI(S7M)), respectively. This suggested that the tetranucleotide motif 5' - GGGU - 3' in the stem 4 region and the three consecutive nucleotides in the stem 7 region are essential to form triplex RNA structure in vitro.

However, to our great surprise, these VA RNAI mutants behaved differently in in vivo experimental system. Notably, the VA RNAI(S4M) rescued the dl705 virus replication similar to wild type VA RNAI whereas RNAI(S7M) partially failed to do it in the same experiment. The differences in the in vitro and in vivo data can be explained as following: 1) it is probable that the VA RNAI(S4M) can still form triplex structure in vivo despite of the mutation, whereas in vitro this single point mutation is enough to block BQQ-OP activity on the triplex structure, 2) in the case of VA RNAI(S7M), the substitution mutations can affect the stability of VA RNAI structure, resulting in partial dl705 replication rescue in vivo and altered triplex structure in vitro.

Altogether, we propose that the HAdV-4 VA RNAI may form higher order structures, such as triplex, involving the highly conserved stem 4 of the central domain and side stem 7. Further, this study also revealed that the integrity of the protruding side stem 7 is needed for HAdV-4 VA RNAI to diminish the eIF2α Ser51 phosphorylation and consequently to enhance HAdV-5 replication in the infected cells.
Conclusions

Paper I

- The propeptide is the destabilizing element targeting the precursor pVII protein for proteasomal degradation
- The lysines K26 and K27 in the pVII protein and Cul3-based CRL complex regulate the pVII protein stability
- The Cul3 protein activity may control HAdV-5 E1A early gene expression

Paper II

- The MKRN1 E3 ubiquitin ligase is a novel HAdV-5 pVII protein interacting protein
- The MKRN1 acts as a potential antiviral protein by reducing pVII protein levels during HAdV-5 infection
- The MKRN1 is proteasomally degraded during prolonged HAdV-5 infection
- The pVII may initiate MKRN1 degradation by enhancing the MKRN1 protein self-ubiquitination

Paper III

- The VACV E3L protein can partially rescue VA RNAI-deficient virus, dl705, replication
- The VACV E3L dsRNA-binding region is essential to enhance dl705 virus replication
- VA RNAs exhibit dual functions to enhance capsid protein accumulation
- The HAdV-4 and HAdV-37 VA RNAI are most effective in rescuing the dl705 virus replication

Paper IV

- The HAdV-4 VA RNAI may adopt higher order structure, such as triplex
- The integrity of HAdV-4 VA RNA stem 7 is needed for its proper in vivo function
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)