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Thema:

Influence of the exogenous virus HIV-1 on the expression of human endogenous retroviral elements (HERVs)

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Meiner Familie

und Kamyar

1. Introduction 009

1.1. Retroviruses 009

1.2. HIV as a complex retrovirus 010

1.2.1. The HIV-1 genome organization and viral proteins 010

1.2.2. HIV-1 replication cycle 013

1.2.3. HIV-1 Rev 018

1.3. Human endogenous retroviruses (HERVs) 020

1.4. HERV-K(HML-2) 023

1.5. HERVs and disease 024

1.6. Aim of the work 027

2. Material and Methods 028

2.1. Materials 028

2.1.1. Buffers 028

2.1.2. Chemicals 029

2.1.3. Antibodies 031

2.1.4. Kits 031

2.1.5. Consumable materials 032

2.1.6. Equipment 033

2.1.7. Oligonucleotides 034

2.1.8. Media 039

2.1.9. Software 040

2.1.10. Human cell lines 040

2.1.11. Plasmids 041

2.1.12. Bacteria 042

2.1.13. Enzymes 042

2.2. Methods 043

2.2.1. Cell culture and transfection 043

2.2.1.1. Cell cultivation and storage 043
2.2.1.2. Thawing and freezing of cells 043
2.2.1.3. Transfection of eukaryotic cells with FuGENE 042
2.2.1.4. Rev activity assay 043
2.2.1.5. Stimulation of Jurkat T-cells with PMA/ionomycin and CD3/CD28 044
2.2.1.6. RNA interference 045
2.2.1.7. Influence of Risp on HIV-1 production 045
2.2.1.8. Infection of LC5-CD4 cells 045
2.2.2. Molecular biology methods 046
2.2.2.1. Minipreparation of plasmid DNA 046
2.2.2.2. Maxipreparation of plasmid DNA 046
2.2.2.3. DNA restriction digestion 046
2.2.2.4. Agarose gel electophoresis 046
2.2.2.5. Isolation of DNA from agarose gels 046
2.2.2.6. pGEM®-T Easy Vector System cloning 047
2.2.2.7. RNA isolation 047
2.2.2.8. DNase digest 047
2.2.2.9. Reverse transcription PCR (RT-PCR) 049
2.2.2.10. Retrovirus-specific microarray 049
2.2.2.10.1. Amplification of hybridization probes by multiplex-PCR 050
2.2.2.11. Quantification of RNA by real-time PCR 051
2.2.2.12. Amplification, cloning and sequence analysis of HML-2 transcripts 053
2.2.3. Biochemical methods 053
2.2.3.1. Western-Blot 053
2.2.3.2. p24-antigen analysis 054
2.2.3.3. Analysis of brain tissue expression patterns 054

3. Results 055

3.1. Several HERV families are activated in persistently HIV-1 infected cells 055

3.1.1. HIV-1 production levels of the three persistently infected cell lines 055

3.1.2. HERV transcription profiles of three persistently HIV infected cell lines 057

3.1.3. Validation of HERV chip data by real-time RT-PCR 059

3.2. HIV-1 specific siRNAs reverse HERV upregulation 062

3.2.1. Experimental proof of the selected siRNAs 062

3.2.2. HERV transcription profiles after HIV-1 knockdown 064

3.2.3. Quantification of HERV transcripts after HIV-1 knockdown 066

3.3. Identification of cellular Rev-interacting HIV suppressors (Risp) 068

3.3.1. Discovery of native Risp proteins in human brain tissue samples and astrocytes 068

3.3.2. Biochemical and functional analysis of interactions of Risp proteins with Rev in human astrocytes (U138MG) 072

3.3.3. Cytoplasmic sequestration of Rev proteins by Risp in astrocytes 074

3.3.4. Influence of Risp on HIV production and expression of Rev dependent HIV transcripts in persistently HIV-infected astrocytes 076

3.3.5. Risp overexpression decreases HERV expression 078

3.4. Influence of HIV-encoded proteins on HERV expression 079

3.5. HERV-K(HML-2) expression is enhanced in cells acutely infected with a primary HIV-1 isolate 082

3.5.1. HERV expression profiles after acute HIV-1 infection 082

3.5.2. Determination of active HERV-K(HML-2) loci 084

3.6. HERV expression is activated by cellular transcription factors 083

4. Discussion 089

4.1. Upregulated expression of selected HERV-groups in persistently HIV-1 infected cells 089

4.2. Risp is a negative regulator of HIV-1 replication leading to decreased HERV expression 089

4.3. HIV-1 Nef increases expression of several HERV groups 092

4.4. Acute HIV-1 infection induces HERV-K(HML-2) expression 094

4.5. HIV-1 induced transcription factor activation (e.g. NF-κB) may be involved in increased HERV expression 097

4.6. Disease relevance of HIV-1 induced HERV expression 101

4.6.1. Expression of various cellular genes by HERV-E elements and possible association with angiogenesis 101

4.6.2. HERV-K(C4) expression in HIV-1 infected cells 102

4.6.3. Immunsuppressive activity of HERV Env proteins 102

4.6.4. HERVs and their therapeutical potential 103

5. Summary 104

6. Zusammenfassung 106

7. Conclusions and Perspectives 108

8. Abbreviations 109

9. References 111

10. Supplement 123

11. Acknowledgments 134

12. Curriculum vitae 135

13. Publications 136

14. Ehrenwörtliche Versicherung / Erklärung 138

1. Introduction

1.1. Retroviruses

Retroviruses are a large and diverse family of RNA viruses. Virions (i.e. virus particles) of retroviruses are enveloped particles about 80-100 nm in diameter. They contain two copies of a single-stranded, linear, nonsegmented RNA genome of positive polarity 7-12 kilobases (kb) in length. The characteristic of this family is its replication mechanism, which includes the reverse transcription of the single-stranded virion RNA into double-stranded DNA and the integration of this DNA into the host genome as a provirus.

All retroviral genomes contain three major coding domains, *gag*, *pol* and *env*, that carry the information for the virion proteins (Figure 1.1) [1].



Figure 1.1.: Genomic organization of a retrovirus RNA-genome.
The retroviral genes *gag, pol* and *env* are surrounded by the untranslated sequences.

Gag directs the synthesis of internal virion proteins that form the matrix, the capsid and the nucleoprotein structures. *Pol* contains the information for the reverse transcriptase and the integrase enzymes and the surface and transmembrane components of of the viral envelope protein are derived from *env*.

An additional coding domain in all retroviruses is *pro*, which encodes the virion protease. Simple retroviruses bear only this basic information, whereas complex retroviruses contain additional sequences encoding proteins with regulatory or auxiliary functions.

The family of *Retroviridae* is divided into two subfamilies, the *Orthoretrovirinae* and the *Spumaretrovirinae*. The *Orthoretroviriane* subfamily consists of 6 genera, the *Alpharetrovirus*, *Betaretrovirus*, *Gammaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus* and the *Lentivirus*. The *Spumaretroviriane* subfamily consists of a single genus, the *Spumaretrovirus*.

Genus	Example	Virion morphology [a]	Genome
Alpharetrovirus	Rous sarcoma virus (RSV)	central, spherical core "C particles"	simple
Betaretrovirus	mouse mammary tumor virus (MMTV)	eccentric, spherical core "B particles"	simple
Gammaretrovirus	Moloney murine leukemia virus (MLV)	central, spherical core "C particles"	simple
Deltaretrovirus	human T-cell leukemia virus (HTLV)	central, spherical core	complex
Epsilonretrovirus	Walleye Dermal Sarcoma Virus (WDSV)	central, spherical core "C particles"	complex
Lentivirus	human immunodeficiency virus (HIV)	cone-shaped core	complex
Spumaretrovirus	human foamy virus (HFV)	central, spherical core "C particles"	complex

Table 1.1.: Classification of Retroviruses.
[a] Distinctive features seen in transmission electron micrographs

Various members of α, β-, χ and δ-*Retroviruses* can cause cancers in humans and other mammals and in birds. Members of the *Lentivirinae* cause immunodeficiency diseases in various animals. *Spumavirinae* are not known to cause diseases.

1.2. HIV as a complex retrovirus

1.2.1. The HIV-1 genome organization and viral proteins

The best known and probably most studied retrovirus is the Human Immunodeficiency Virus (HIV), which is a Lentivirus. Figure 1.2 shows a schematic of the 9,75 kb HIV-1 proviral genome. It contains several open reading frames (i.e. genes) that code for a total of 15 proteins [2,3]. The protein-coding region is flanked by long terminal repeats (LTR). The LTRs are produced during reverse transcription and are composed of the U3 (unique, 3'-end), the R (repeated) and the U5 (unique, 5'-end) regions. The LTRs are essential for viral transcription, which initiates in the 5' LTR and terminates in the 3' LTR.

In addition to the *gag*, *pol* and *env* genes found in all functional retroviral genomes, the HIV proviral genome contains genes that encode regulatory proteins (*tat, rev*), as well as genes for "accessory" proteins (*vif, vpr, vpu, and nef*).

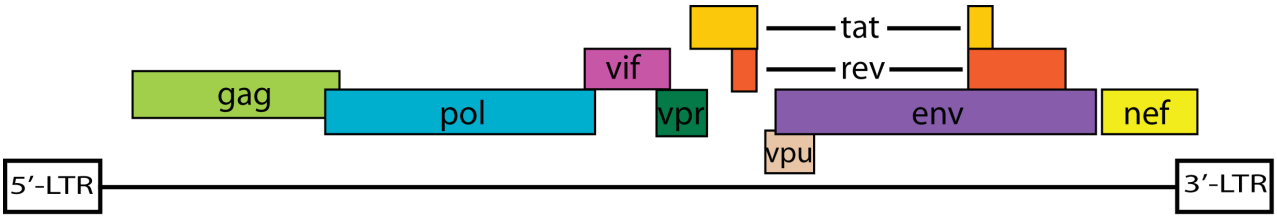


Figure 1.2.: Schematic composition of the HIV-1 genome.
Illustrated is the structure of the viral genome of HIV-1. The viral RNA contains three typical retroviral genes *gag*, *pol* and *env*, as well as lentivirus-specific regulatory and accessory genes *rev*, *tat*, *nef*, *vpr*, *vpu* and *vif*. At the 5'- and the 3'-site of the RNA LTR regions are positioned. The colored boxes symbolize the different protein-coding sequences with different reading frames.

Figure 1.3 shows a schematic of a mature HIV virion. Like all retroviruses, the HIV particle contains a lipid bilayer envelope associated with viral glycoproteins. The envelope surrounds a capsid, which contains the viral RNA genomes and enzymes. The *gag* gene codes for the Gag polyprotein precursor $Pr55^{GAG}$. Cleavage of $Pr55^{GAG}$ by the viral protease during virus maturation produces the structural proteins Matrix (MA), Capsid (CA) Nucleocapsid (NC), and the p6 protein.

Inside the capsid, displayed in Figure 1.3, two copies of the single-stranded RNA genome are stored. These RNA molecules are stabilized and packaged with the aid of the nucleocapsid proteins [3].

The information for all enzymatic proteins of the virus, the reverse transcriptase (RT, p66/p51), the protease (PR, p11) and the integrase (IN, p32) are contained in the *pol* gene. These proteins are synthesized from the Gag-Pol- polyprotein precursor protein ($Pr160^{GagPol}$), from which they are cleaved by the viral protease. During maturation of virus particles (see section 1.2.2), the protease first cleaves itself from the polypeptide chain and then cleaves the other proteins from the Gag and Gag-Pol polyproteins. The reverse transcriptase (RT) converts the single-stranded viral RNA into double-stranded DNA. The major catalytic activities of the RT are DNA polymerization from RNA and DNA templates and degradation of RNA (RNase H). The *env* gene contains the information for the viral envelope proteins that consist of the surface subunit (SU/gp120) exposed on the virion surface and the transmembrane subunit (TM/gp41) embedded in the virion envelope. The viral envelope proteins are produced from a precursor glycoprotein, gp160, which is glycosylated and cleaved into the gp120 and gp41 subunits by a host protease

during trafficking through the Endoplasmic reticulum and Golgi apparatus. The Env proteins form “spikes” on the virion consisting of three SU and three TM domains.

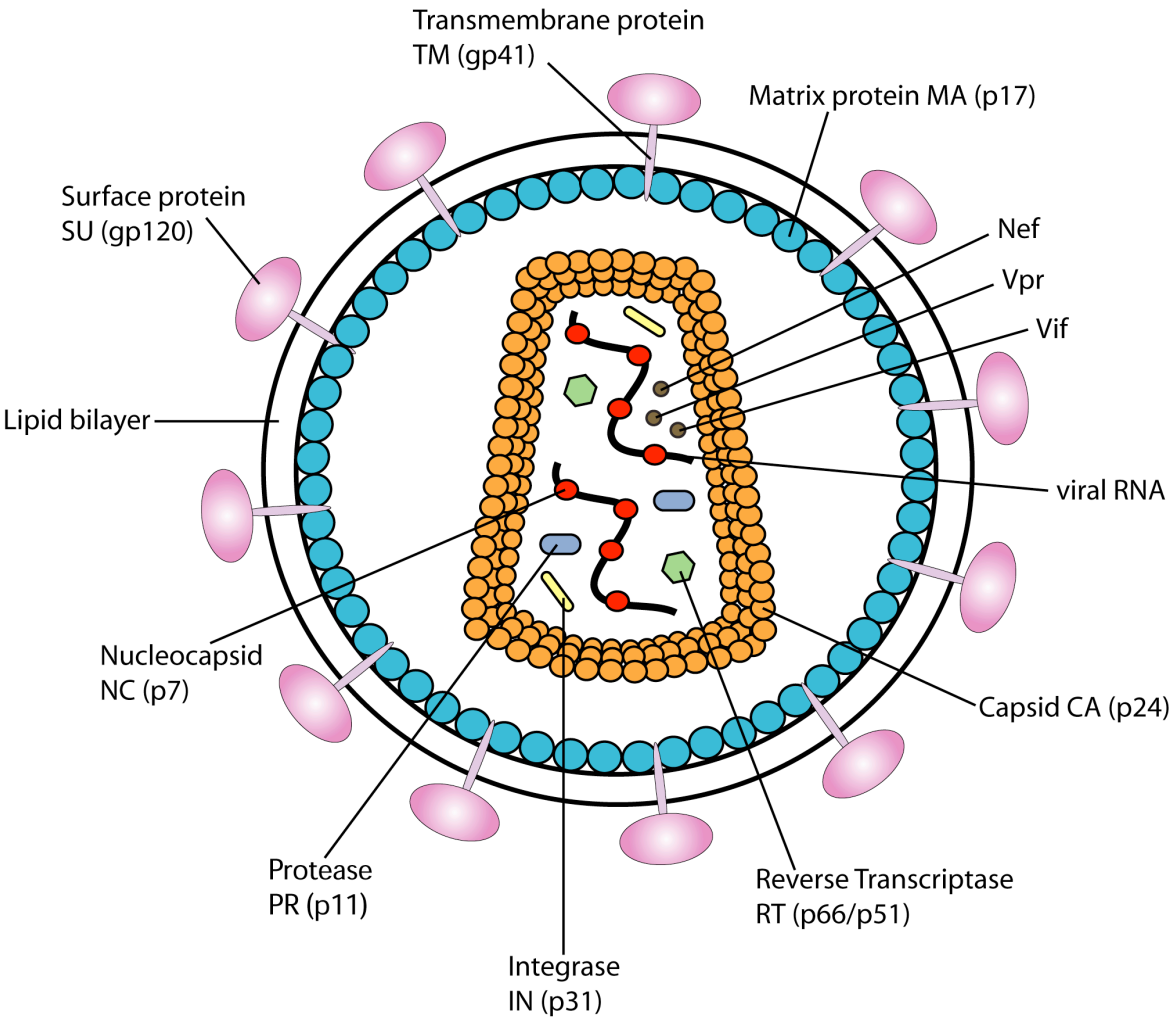


Figure 1.3.: Schematic of an retrovirus particle.
The schematic overview displays the organization of a retrovirus and its proteins. The Figure is not true to scale.

The viral regulatory proteins Tat and Rev are are essential for the replication of HIV-1 and are both stimulators of viral gene expression. Tat stimulates viral RNA production from the 5’ LTR. It binds to the TAR (transactivation response) element on the RNA after transcription has started. Subsequently it recruits Cyclin T and CDK9 resulting in phosphorylation of the RNA Polymerase II at its C-terminal domain. This increases transcription by approximately 100-fold [4,5,6]. Furthermore, Tat is capable

of loosening the chromatin structure at the 5'-LTR by recruiting HATs (Histone deacetylases) and thereby facilitating RNA transcription [7].
Rev stimulates the expression of viral structural proteins and enzymes. These proteins can only be translated if unspliced or singly spliced RNAs are exported to the cytoplasm. Rev mediates the transport of these RNAs to the cytoplasm [8]. For a more detailed description of Rev, see below (see section 1.2.3).

The HIV accessory protein Nef is another important protein for HIV-1 virus replication. Nef is a 27-34 kDa multifunctional protein. It enhances virion infectivity [9,10] and increases viral replication in primary lymphocytes and macrophages [11]. Nef can mediate down regulation of CD4 cell surface expression, an event that appears to be important for the release of HIV-1 from the cell [12,13]. Furthermore Nef can protect infected cells from being killed by cytotoxic T cells [14] by downregulating the cell surface expression of major histocompatibility complex I (MHC-I) molecules [15]. Several *in vivo* studies have demonstrated the importance of Nef for the efficiency of viral replication and for the maintenance of high viral loads [16,17]. Nef induces the release of inflammatory factors [18] and activates NF-κB [18,19]. The viral accessory proteins Vif, Vpr and Vpu are crucial for virus propagation and disease induction *in vivo*. They have multiple functions, including the defense against antiviral activities of the host [20].

1.2.2. HIV-1 replication cycle

In many of these cells, the virus can replicate, albeit with different efficiencies. The stages of HIV replication are virus entry, reverse transcription, integration, proviral gene expression, assembly of virus particles, budding and maturation of virus particles. Each stage involves many and complex virus-host interactions. Here I will just briefly outline each stage and refer the reader to various reviews for further details [2,3,21]. HIV-1 entry into the cell (Figure 1.4) begins with the interaction of the trimeric envelope complex (Env spike) and both CD4 and a chemokine receptor (generally either CCR5 or CXCR4, but others are known to interact) on the cell surface [22,23]. The Env spike contains binding domains for both CD4 and chemokine receptors [22,23].
The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp120 to CD4. Once gp120 is bound with the CD4 protein, the envelope

complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor [22,23]. This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane.

After fusion of the viral envelope with the cellular plasma membrane, the virion core is released into the cytoplasm [24]. The retroviral RNA genome undergoes reverse transcription leading to production of a double-stranded DNA molecule [25]. This complex process is catalyzed by the viral reverse transcriptase (RT) (for an overview see [2]). Initially, the RT synthesizes a short, (-) strand cDNA, using a host transfer RNA (tRNA) bound to the primer binding site (pbs) downstream of the U5-region in the viral RNA as primer. The short (-) strand cDNA anneals to complementary sequences located at the opposite end of the RNA template (first strand transfer). The RT then extends short (-) strand DNA, using the viral RNA as template. Subsequently, the RT uses the newly synthesized (-) DNA as template for generation of the (+) strand DNA. Again, the RT initially synthesizes a short DNA segment. This time, a viral RNA fragment bound to the (–) strand DNA upstream of the 3' LTR serves as primer. The short (+) strand DNA anneals to the *pbs* sequences in the newly generated (–) strand DNA (second strand transfer) and is extended. Both strand transfer and synthesis of the (+) strand DNA require degradation of RNA in RNA-DNA hybrids, which is catalyzed by the RNase H activity of the RT [26]. Errors during reverse transcription are not corrected and are a major factor for the high mutation rates of retroviruses.

The double-stranded viral DNA remains associated with cellular and viral proteins, which together form the pre-integration complex (PIC). In the PIC the integrase is bound to specific sequences (*att* sites) located at the ends of the viral double-stranded DNA, where it processes the 3' ends of the viral DNA for integration [27,28]. The PIC is subsequently transported to the nucleus, where the integrase can mediate integration of the double-stranded viral DNA into the host genome [24]. Provirus formation is essential for virus replication.

The promoter/enhancer sequences in the 5' LTR of the provirus direct transcription, the first step of proviral gene expression. The LTR contains numerous binding sites for cellular transcription factors [29]. These include multiple binding sites for SP1 (3) and NF-kB (2), which are key elements for HIV transcription. Initial viral transcription is mediated solely by the interaction of cellular transcription factors with the LTR and

is generally low. The primary transcript produced is a full-length transcript. Alternative splicing of this transcript results in the production of many different HIV transcript species (compiled in Figure 3.5; see also [30]). The HIV transcript species fall into three major classes: 1) the ~9 kb unspliced transcript which encodes the Gag- and the Gag-Pol precursor polyproteins and serves as viral RNA genome; 2) the ~5 kb partially spliced transcripts, which encode the Env, Vif, Vpr and Vpu proteins, and 3) the ~2 kb fully spliced transcripts which encode Tat, Rev and Nef.

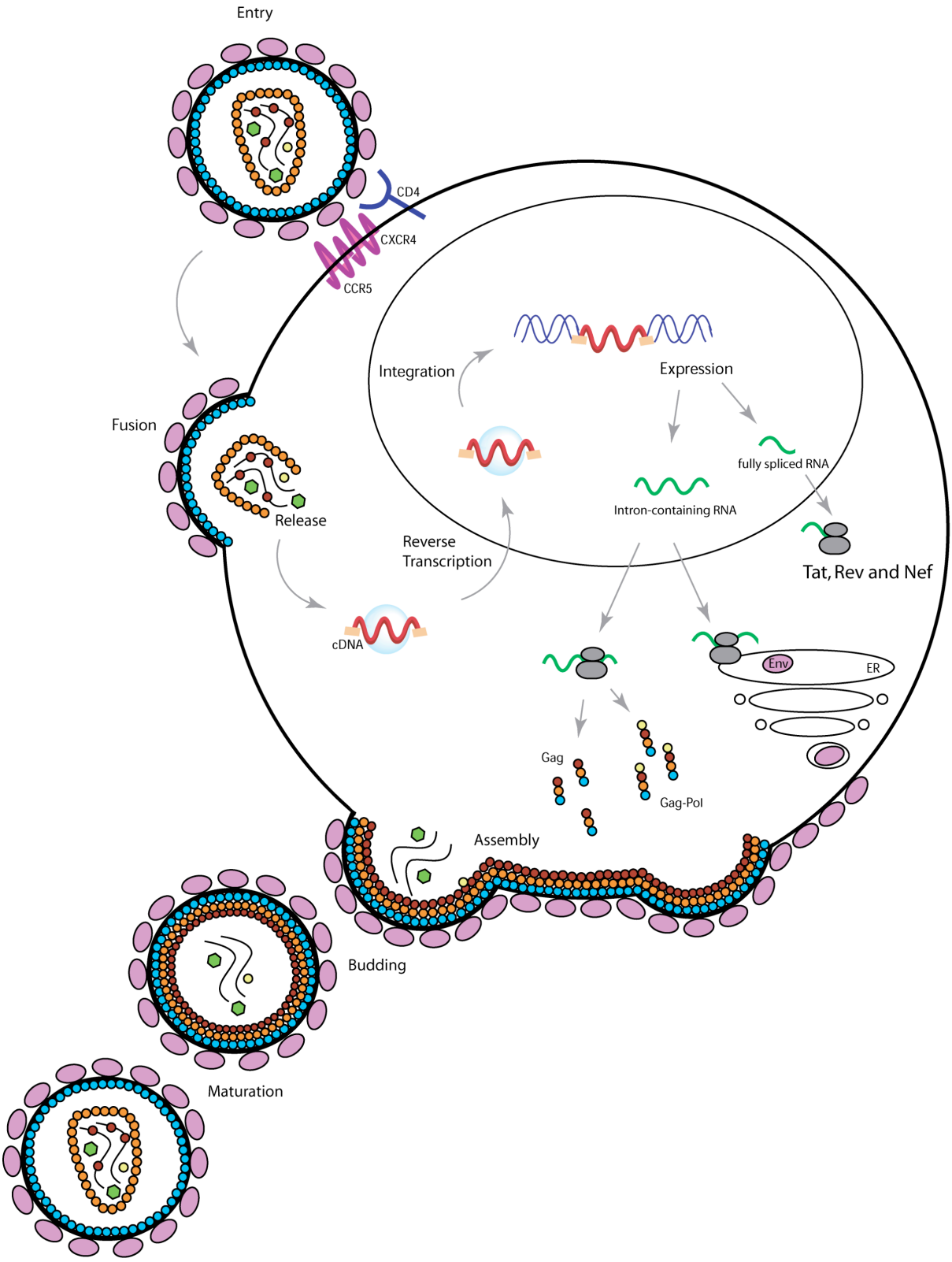
During initial transcription, the fully spliced transcript class predominates, resulting in early production of the Tat and Rev. Binding of Tat to the TAR greatly enhances, the transcription rate [24] (see 1.2.1 for a brief description of Tat activities). The Rev protein binds to the RRE (Rev responsive element) on partially and unspliced viral mRNAs and enables these mRNAs to exit the nucleus, where they are otherwise retained until spliced [31,32](see section 1.2.3 and Figure 1.5 for a description of Rev activites). Translation of the full-length mRNA at polysomes leads to production of the Gag and Gag-Pol polyprotein precursors. Production of the latter protein requires ribosomal frameshifting during translation. The Env precursor polyprotein is produced by ribosomes associated with the endoplasmic reticulum.

The production of the full set of viral proteins allows virus assembly to begin. This process takes place at the plasma membrane of the host cell and is directed by the Gag precursor protein. The Gag-protein precursor protein is inserted into the inner leaf of the lipid bilayer via a myristic acid moiety covalently attached to its N-terminus. A patch of highly basic amino acid residues in the MA domain promotes membrane attachment further. Virus assembly requires Gag-Gag interactions, which are mediated by the other domains in the Gag precursor protein, especially CA. The viral RNA genome is packaged into nascent virions by interaction of the NC-domain, which contains two zinc-finger motifs, with the packaging signal in the virus RNA. The Env polyprotein (gp160) migrates through the endoplasmic reticulum where it is glycosylated and is transported to the Golgi complex. In the Golgi, the gp160 precursor protein is cleaved by a cellular furin protease to the two HIV envelope glycoproteins gp41 and gp120. The Env proteins are transported to the plasma membrane of the host cell where gp41 anchors gp120 to the membrane of the infected cell. Gag recruits Env proteins to sites of assembly by interactions between its MA domain and the cytoplasmic tail of Env gp41.

The virion uses the cellular endosomal sorting machinery to bud from the host cell. This involves interaction of the p6 domain of the Gag precursor protein with a component of the ESCRT (endosomal sorting complex required for transport)-I protein complex.

Maturation of the virus particle to an infectious virion either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes [2].

Figure 1.4. (next page): The replication cycle of HIV-1.
After entry of the virus into the host cell through binding of gp120 to CD4 and the co-receptors the capsid is released into the nucleus of the host cell. The viral RNA genome is converted into double-stranded DNA copies by reverse transcription. These double strand copies of viral DNA are integrated into the host-cell genome, forming the provirus. Transcirption of the provirus leads to a primary full-length transcript that can serve as viral RNA genome or as substrate for alternative splicing. In the initial phase of expression, the primary transcript is fully spliced for production of key stimulators of viral gene expression, Tat, Rev and Nef. Tat activates transcription. Rev mediates the export of Intron-containing mRNAs to the cytoplasm for translation, resulting in the production of viral structural proteins and enzymes. New viral particles are assembled, bud from the plasma membrane, and are processed after release. For further details see text.


Entry
CD4
CXCR4
CCR5
Fusion
Release
Integration
Expression
fully spliced RNA
Intron-containing RNA
Reverse
Transcription
cDNA
Tat, Rev and Nef
Env
ER
Gag
Gag-Pol
Assembly
Budding
Maturation

1.2.3. HIV-1 Rev

Rev is among the first viral proteins produced in the virus replication cycle [33]. It is required for the production of HIV structural proteins and RNA progeny genomes from intron-containing viral mRNAs during HIV replication [30]. Rev is a protein of 116-amino acids that has been shown to continuously shuttle between the nucleus and the cytoplasm [34]. In HIV permissive cell lines, Rev localizes predominantly to the nucleus. Rev contains a nuclear export signal (NES) and an RNA binding domain (nuclear localization signal (NLS)) specific for the viral Rev response element (RRE). The NLS domain also serves as a nuclear import signal [8].

Rev promotes the nuclear export of partially spliced and unspliced HIV-1 mRNA species, which are specified by the presence of the Rev response element (RRE). Rev binds to the RRE, on unspliced and single spliced RNAs, stabilizes these RNAs in the nucleus and mediates their export to the cytoplasm by recruiting the nuclear export receptors CRM1/Exportin1 and RanGTP (Figure 1.5) [8]. This ternary complex (Rev-CRM1-RanGTP) binds to proteins of the nuclear pore complex, known as nucleoporins. Subsequently, the RNA is translocated to the cytoplasm, where RanGTP is hydrolyzed to RanGDP leading to dissociation of CRM1 and RanGDP from the RNA-protein-complex. The mRNA is then translated to produce viral structural proteins and enzymes, or in the case of the unspliced transcript, can serve as genome for virion formation. Rev is re-transported to the nucleus through the interaction of its nuclear localization signal (NLS) with Importin-β. In the nucleus Importin-β interacts with RanGTP resulting in the release of Rev [32].

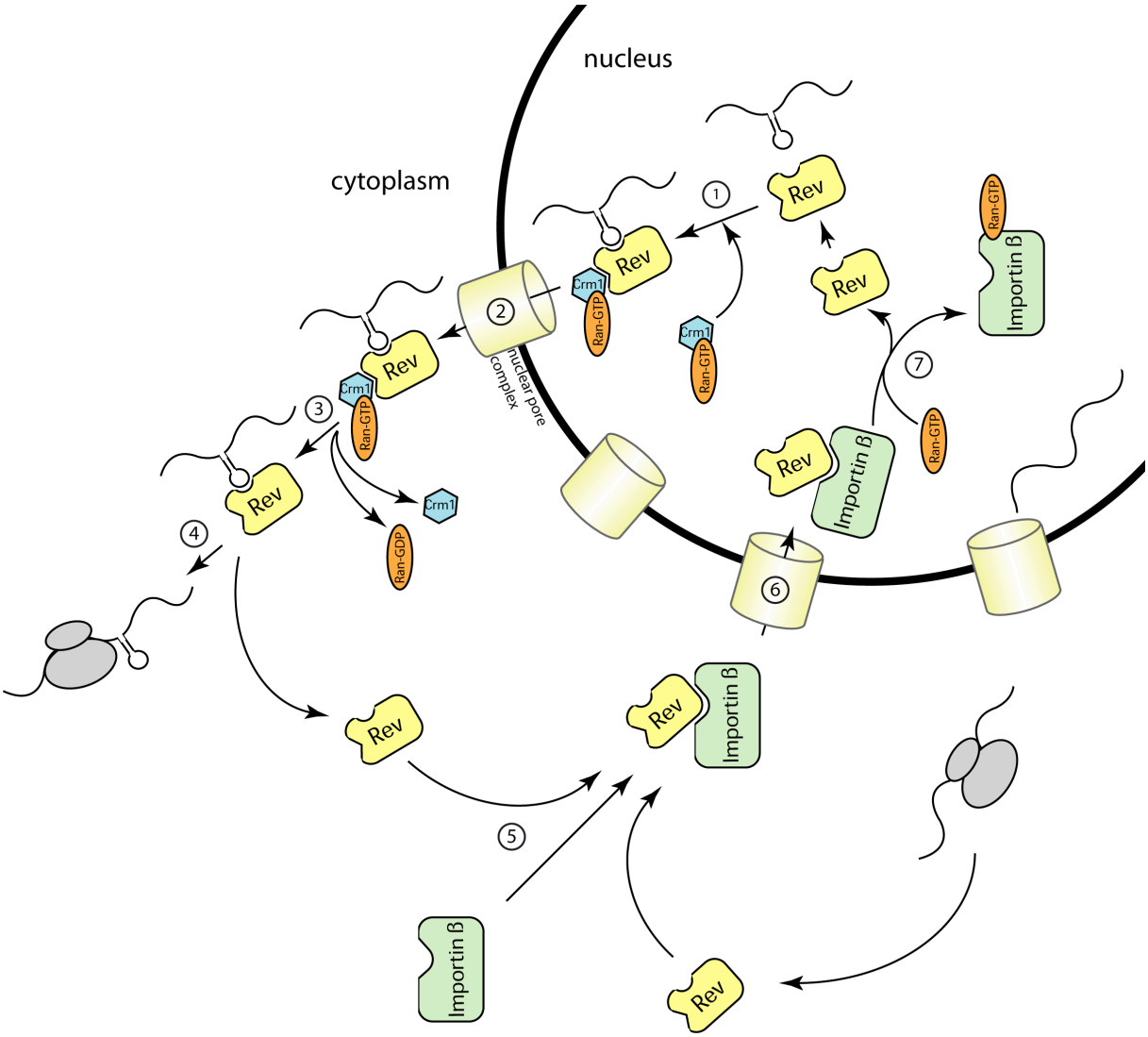


Figure 1.5.: Rev-Shuttling.
Rev binds to the RRE on unspliced and partially spliced HIV mRNAs. Rev then recruits the nuclear export protein CRM1 bound to Ran-GTP (1) for export of the entire complex through the nuclear pore (2). In the cytoplasm RanGTP is hydrolyzed to RanGDP, leading to dissociation of CRM1 and RanGDP from the RNA (3). The mRNA can than be translated at ribosomes (4). Rev binds to Importin-β (5) and shuttles back to the nucleus (6). The release of Rev in the nucleus occurs through the binding of RanGDP to Importin-β (7), freeing Rev to bind anew to viral mRNAs (1).

Rev also contributes to other processes required for the efficient utilization of HIV RNAs, including splicing, translation and packaging of the viral RNA genomes [35,36]. In astrocytes, the Rev protein exhibits reduced activity, shuttles with altered dynamics between the nucleus and cytoplasm and accumulates to abnormally high

levels in the cytoplasm [37,38]. Furthermore, Rev-dependent transcripts are selectively depleted in astrocytes persistently infected with a functional HIV provirus [39]. These results provide evidence for the existence of cellular mechanisms for regulation of Rev activity and suggest that these mechanisms can impact HIV production levels. Studies of HIV expression in astrocytes implicate the post-transcriptional HIV regulatory protein Rev as a potential target for cellular HIV suppression [37,38,39].

1.3. Human endogenous retroviruses (HERVs)

Approximately 8-9% of the human genome is composed of endogenous retroviral elements (HERVs). Endogenous retroviruses are found in all phyla and homologues of many HERVs are present among primates, representing 70 million years of evolutionary time. Complete HERV sequences possess a similar genomic organization to many exogenous retroviruses, containing three genes, *gag, pol* and *env* flanked by two long terminal repeats (LTRs). *Gag, pol and env* are often truncated through mutations and deletions and most HERVs are not able to produce proteins or replicate. It is hypothesized that during the course of primate evolution, exogenous progenitors of HERVs inserted themselves into germ-line cells, where they expanded via retrotransposition and reinfection [40,41]. HERVs are distributed among all chromosomes [42].

HERVs are classified according to sequence similarities in the polymerase (*pol*)-gene [43]. Three major classes of HERVs have been characterized, illustrated in Figure 1.6. Class I HERVs show sequence similarities to γ-retroviruses, Class II HERVs to β-retroviruses and the Class III HERVs to human spumaviruses [44]. HERV classes are subdivided into several families according to the sequence similarity of their potential primer binding site (pbs) in the 5' untranslated region with the corresponding complementary sequence of cellular tRNAs (e.g. HERV-W has a pbs matching the 3' end of the tRNA for W, tryptophane). Nevertheless this classification system fails for many HERV groups because often two HERVs primed by the same tRNA do not belong to the same phylogenetic family according to the sequence similarity of their *pol* genes.

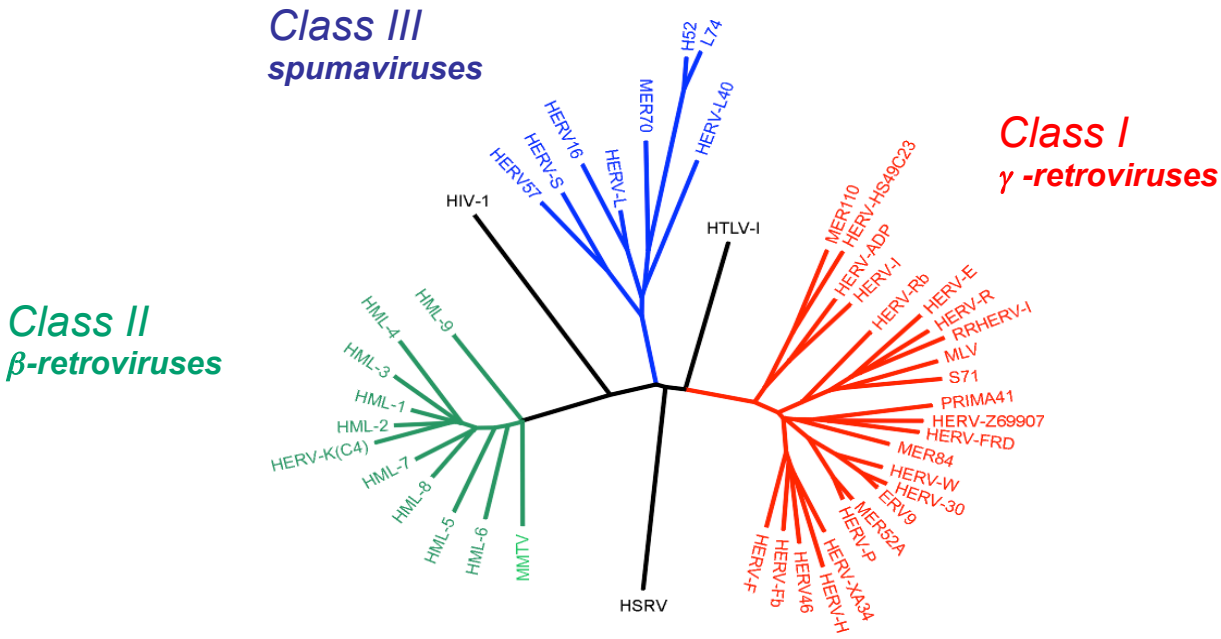


Figure 1.6.: Classification of HERVs.
The phylogenetic tree was derived from DNA sequences of the reverse transcriptase domain [44]. Displayed are class I, class II and class III HERVs as well as the exogenous retroviruses HIV (human immunodeficiency virus), HRSV (human spumavirus) and HTLV (human T-cell leukemia virus).

Figure 1.6 reveals the relationships among HERV groups. There seems to be only minor similarity between HERVs and some human exogenous retroviruses like HIV-1, HTLV-1 and HRSV. Interestingly, HERVs are more closely related to animal retroviruses such as MLV and MMTV suggesting a past interspecies transmission of progenitor exogenous retroviruses [45]. During evolution HERVs and their exogenous relative have been separated because of mutations and deletions. The analysis of the human genome has revealed only 3 HERVs with intact open reading frames for *gag*, *pol* and *env* [46], all of them belonging to the HERV-K(HML-2) family, although these HERVs possess point mutations in critical parts of the reverse transcriptase. Beside the sequence differences in the HERV-classes there exist also differences in the copy numbers of each HERV in the genome. While approximately 1000 HERV-H copies can be found in the human genome, there are only 40-115 copies of HERV-W and about 60 copies of HERV-K(HML-2) [44,47].

Most analyzed elements have been found in higher primates including Old World monkeys, but not in New World monkeys [48]. This suggests that a major invasion and expansion of *pol* containing endogenous retroviruses (ERVs) occurred after New

World Monkeys lineage separated from Old World Monkeys and apes (Figure 1.7). This interval of time dates back approximately 33 to 57 million years [49,50].

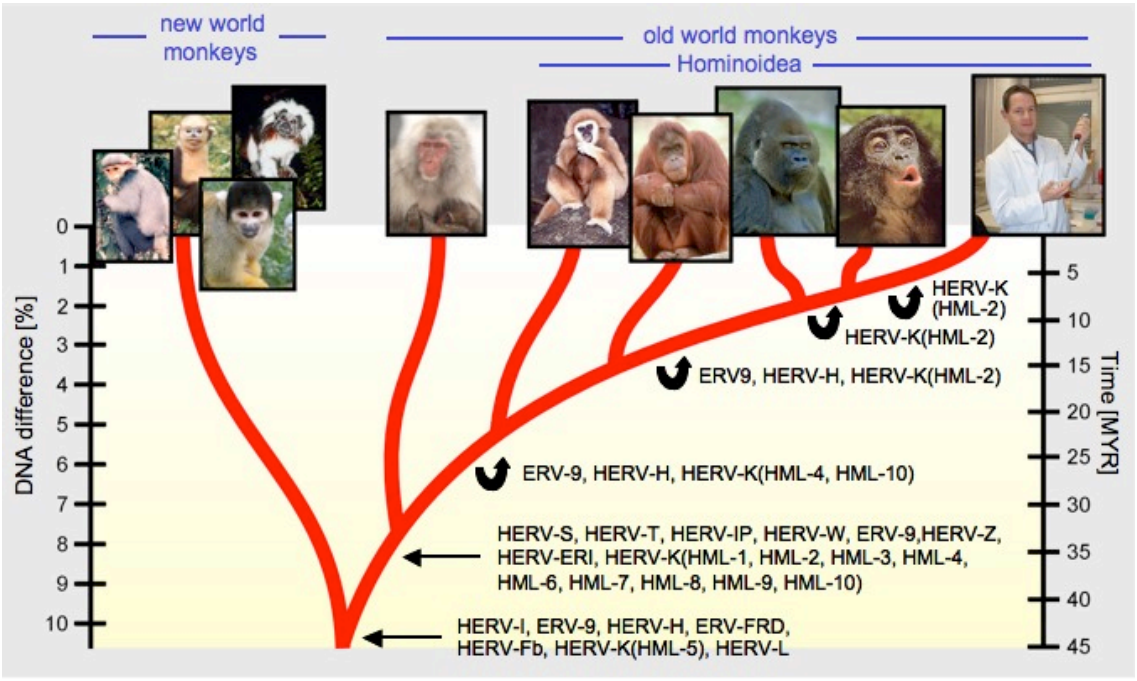


Figure 1.7.: Integration and expansion of human endogenous retroviruses in primate evolution. Displayed are the time points of integration as well as amplification in the genome. The axis on the right side shows the time in million years, the axis on the left side illustrates the DNA difference in percent between the species.

After integration and endogenization of retroviruses in the primate genome, HERV-subtypes show strong amplification and further distribution in the genome. Tristem and colleagues [41] defined 31 HERV families, which represent unique clades, each derived from a single infection of the human germ line. In recent evolutionary history HERV-K(HML-2) elements were particularly active. The most recent amplification of HML-2 started about 5 million years ago after the evolutionary split of humans from chimpanzee [51,52,53]. In humans, HERV-K(HML-2) insertion polymorphisms are known, suggesting that reinfection events must have occurred recently [41,54,55] and may still be occurring among humans. Because of this activity, HERV-K(HML-2) is one of the best-studied HERV-subtypes.

1.4. HERV-K(HML-2)

The best characterized human endogenous retroviral (HERV) family is HERV-K(HML-2). HERV-K(HML-2) proviruses are present in the genomes of humans, apes and Old World monkeys having originated about 40 million years ago. Approximately 60 full-length HERV-K(HML-2) proviruses are estimated to be present in the human germ-line [44]. Two types of HERV-K(HML-2) genomes exist. Type 1 genomes display fused *pol* and *env* genes and express exclusively unspliced mRNA [56]. HERV-K(HML-2) type 2 full-length transcripts are spliced to subgenomic *env* and two smaller mRNAs [56,57]. All human HERV-K(HML-2) proviruses described to date have mutations that inhibit viral replication, but some full-length open reading frames (ORFs) can encode selected viral proteins. These induce Gag-Pol, Env, and cORF/K-Rev suggesting that HERV-K(HML-2) might be capable of replicating by complementation and also raising the possibility of recombination among co-packaged HERV-K(HML-2) genomes [58]. In the past 2 years, one group has created an infectious clone of HERV-K(HML-2) [58], and another has shown that virus can be generated using several transcomplementary plasmids [59]. Thus, at least in its reanimated form with mutations corrected to reintroduce open reading frames, HERV-K(HML-2) can replicate. However there is a debate as to whether or not HERV-K(HML-2) is still capable of replication in humans. Some HERV-K(HML-2) proviruses have been duplicated with their cellular flanking sequences and are now present on multiple chromosomes.

HERV-K(HML-2) expression seems to be associated with testicular germ-cell tumors [60]. One of the HERV-K(HML-2) encoded proteins called Rec (synonymic cORF, K-Rev), has a Rev-like function, as it stabilizes unspliced and incompletely spliced viral transcripts and enhances their nuclear export [61,62]. The *rec* transcript is produced from the *env* gene by alternative splicing [56]. It has been shown that the expression of the HERV-K(HML-2) Rec protein induces tumor formation in nude mice [63]. Another HERV-K encoded protein is Np9. Due to a 292-bp deletion in HERV-K(HML-2) type 1 and the generation of a specific splice donor site, the *env* open reading frame in this provirus type produces Np9 instead of Rec [64]. Therefore, the *np9* transcript is transcribed exclusively from the HERV-K type 1 provirus. NP9 and Rec share the N-terminal 14 aminoacids. The 9-kDa protein localizes predominantly to the nucleus of the cell and is expressed in various tumor tissues and transformed cell lines [64,65]. Several studies indicate that Np9 constitutes an oncoprotein [65,66].

Furthermore, HERV-K(HML-2) transcripts and proteins were detected in several diseases [67,68] like rheumatoid arthritis [69], schizophrenia and bipolar disorders [70,71], and in HIV infected patients [72,73,74,75].

1.5. HERVs and disease

Exogenouse retroviruses have been shown to cause well characterized diseases, for example HIV-1 can cause AIDS. There is no current evidence that infectious HERVs are produced, suggesting they replicate exclusively as endogenous elements of the cell. This is in sharp contrast to many mammals, particularly rodents, in which the lines between endogenous and exogenous retroviruses can become very blurred [27]. However, given their interactive nature within the genome, HERVs can potentially cause diseases by a variety of mechanisms, displayed in Figure 1.8.

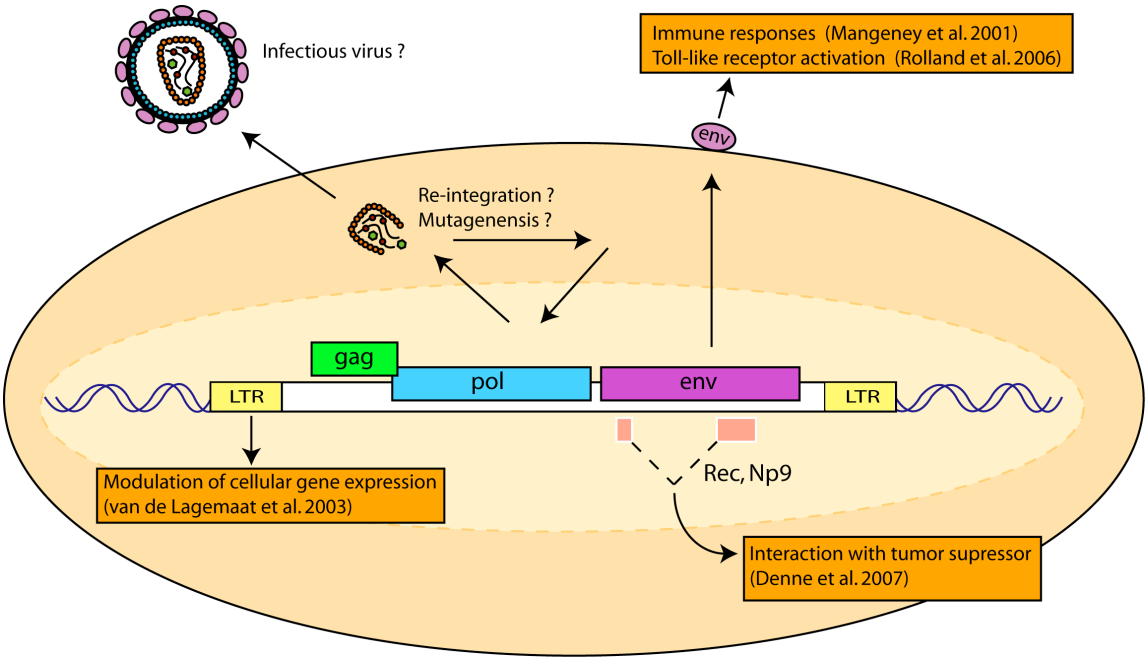


Figure 1.8.: Pathogenic potential of HERVs.
HERVs may be pathogenic by influencing the expression of adjacent genes via LTR sequences or by expression of pathogenic gene products that may modulate immunological functions or interact with cellular transcription factors involved in cancer.

Many HERVs contain functional promoters, enhancer-elements and polyadenylation signals in their LTRs [76]. These regulatory sequences can affect the expression of

neighboring genes. Such modulation could for example lead to an activation of oncogenes or inactivation of tumor suppressor genes. Furthermore, while not forming fully functional infectious virus several HERV-elements code for individual gene products [77] that could be pathological. For example the HERV envelope (*env*) protein is associated with several chronic diseases (autoimmune disease), neurological diseases [78], and suppression or stimulation of the immune response [79,80]. Furthermore some HERV proviruses like HERV-K(HML-2) express a spliced RNA encoding for proteins like Rec or NP9, which have been implicated in oncogenesis [81]. Another controversial issue is the potential of the HERV-K(HML-2) family to produce viral particles, which could infect other cells and thus could cause insertion mutagenesis.

Most HERVs have been silenced by mutations and/or are controlled epigenetically (e.g. methylation of DNA or chromatin inactivation). However, they may be reactivated by environmental conditions such as radiation, chemicals and infectious agents [82,83,84,85] or exogenous viruses [72,73,74,75,86,87,88,89,90,91,92]. The following tables give an overview of reported activation of the expression of ERV sequences in human or mouse cells after infection with different viruses (Table 1.2) or other infectious agents (Table 1.3).

Species	Cell line	Infectious Virus	Activated HERVs identified	References
human	SK-N-MC IMR-32 HBMEC, HCEC	HSV-1	HERV-W	Nellaker et al 2006 Ruprecht et al 2006 Lafon et al 2002
human	CCF-STTG1 239F U937	influenca A/WSN/33	HERV-W	Nellaker et al 2006
human	murine T cells hybridoma cells A20murine B cells lymphoma cells	Epstein-Barr virus (EBV)	HERV-K18	Tai et al 2006, 2008 Hsiao et al 2006, 2009 Sutowski et al 2001, 2004
human	Plasma, PBMC	HIV-1	HERV-K102 HERV-K(HML-2)	Garrison et al 2007 Laderoute et al 2007 Contreras et al 2007
human	Tetracarcinoma cells	HBV (HBx protein)	HERV-K(HML-2)	Löwer (unpublished)
human	PBMC HSB-2 cells	HHV-6A HHV-6B	HERV-K18	Turcanova et al 2009 Tai et al 2009
human	293T	SV40 large T antigen	HERV-I, HERV-T, HERV-H, HERV-K(HML-4)	Zeilfelder et al 2007

Table 1.2.: Overview of known ERV activation after infections with diverse viruses

Species	Cell line	Infectious agent	Activated HERV/ERVs identified	References
human	melanoma cells	5'-aza-2'-deoxycyitidine	HERV-K(HML-2)	Stengel et al 2010
human	SK-N-MC	Toxoplasma gondii	HERV-I, HERV-E, HERV-H, HERV-K(HML-2, HML-3, HML-7), ERV-9, HERV-L	Frank et al 2006
mouse	N2A	prions (scrapie strain RML)	MLV	Stengel et al 2006
mouse	N2A GT1	prions (scrapie strain RML)	IAP-1	Stengel et al 2006
human	HaCat	ultraviolet radiation	several HERVs	Hohenadl et al 1999
human	T47D cells	female steroid hormones	HERV-K	Ono et al 1987

Table 1.3.: Overview of known ERV activation through infectious agents

Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6), for example, have been shown to transactivate expression of a potential HERV-K18 encoded superantigen, which stimulates T-cell activation [88,89,92,93,94]. This process might be crucial for the establishment of long-term infection by EBV and HHV-6 and play a role in the development of associated diseases. Similarly, the expression of HERV-W Gag and Env proteins has been proposed to be induced by Herpesviridae in MS patients and is hypothesized to be linked with MS pathogenesis [87]. Because of the association of HERVs with a multitude of complex diseases, a thorough investigation of the influence of exogenous viruses on the expression of HERVs in human cells is necessary in the context of pathogenic consequences.

Exogenous retroviruses such as HIV are functionally similar in most ways to HERVs with the exception that currently no human lentiviruses are transmitted vertically from the parent germline to offspring genome. Given the similarity of HIV to HERVs and particularly their regulation via the LTRs, it is a plausible and testable hypothesis that infection of cells by HIV and subsequent activation of the HIV provirus could influence the expression of HERV LTRs that rely on similar signals and transcription factors for their own expression. The regulatory proteins Rev and Rec (cORF) of HIV and HERV-K(HML-2), respectively, are structurally and functionally related and Rev

may substitute for Rec in transient transfection assays [61,62,95]. Furthermore, intracellular defense mechanisms that influence HIV are thought to have evolved to control HERV activity [96,97]. There is current evidence that HIV infection can cause the production of anti-HERV-K antibodies [59] and the detection of HERV-K RNA in plasma of HIV-1 infected patients was described [73]. Nixon and colleagues [75] demonstrated that T cells in the human immune system respond to HERV epitopes when a person is infected with HIV. They showed HERV-specific immune responses upon HIV-1 infection, which could be an important factor in controlling HIV infection. Thus, there are multiple levels of interaction between HIV and HERVs that should be explored.

1.6. Aim of the Work

There is strong evidence that infection by exogenous viruses can influence epxression of HERVs. Thus the aim of this work was to investigate the activation of different HERV families by HIV-1. To this end I compared HERV expression profiles in different persistently HIV-1 infected cell-lines (astrocytes, HeLa cells and T-cells) and in uninfected cells. In order to validate a direct connection between exogenous virus infection and HERV expression changes, I investigated the influence of changes in HIV expression levels on HERV expression profiles. Therfore I chose various siRNAs for artifical suppression of HIV expression. To examine the relationship between HIV and HERV expression under more natural conditions, I identified a group of natural host factors that influence HIV production in infected cells (Risp) and investigated the effects of Risp expression on both HIV and HERV expression. Furthermore, I performed studies to elucidate the role of the Risp group in HIV replication.

The results of my study support a direct link between expression of HIV and various HERV sequences.

2. Materials and Methods

2.1. Materials

2.1.1. Buffers

Product	Composition (Manufacturer)
Caesium chloride (5.7 M)	5.7 M CsCl (MW 168.36) 1 mM EDTA, pH 8.0 25 mM Na-citrate, pH 7.0
Hybridization Solution	3 x SSC 0.2 x SDS 50 % Form amide ddH_2O
Lysis buffer (Immunoprecipitation)	5 x TBS 10 % Triton 100 500 mM EDTA 500 mM EGTA 125 mM $Na_4P_2O_7$ 100 mM Na_3VO_4 1.2 % PIM 0.44 % PMSF
Lysis buffer	50 mM Tris-HCl pH 8.0 150 mM NaCl 0.02 % NaN_3 0.1 % SDS 1 % Nonidet P-40 0.5 % Deoxycholat Protease inhibitors: 2 µg/ml Leupeptin 2 µg/ml Aprotinin 1 mM PMSF
Loading buffer for DNA	15 % Ficoll 5 mM EDTA 0.01 % Bromphenolblue 0.01 % Xylenxyanol
MOPS-Buffer	Invitrogen, Karlsruhe
PBS (phosphate buffered saline) pH 7.4	140 mM NaCl 5.4 mM KCl 9.7 mM $Na_2HPO_4 2{\cdot}H_2O$ 2 mM KH_2PO_4
PBS-Tween (0.1%)	PBS + 0.1 % Tween-20

Prehybridization solution	6 x SSC 0.5 % SDS 1 % BSA
Reduction solution ($NaBH_4$-solution)	$NaBH_4$ 1 x PBS 100 % Ethanol
SDS loading buffer	Invitrogen, Karlsruhe
10% SDS solution	100 g Sodiumdodecylsulfate in 1000 ml ddH_2O
20 x SSC	3 M NaCl (MW 58.44) 300 mM Na-Citrate (MW 294.1)
Sodium acetate (3M), pH 5.2	3 M solution, pH was adjusted with acetic acid
TAE (50x)	2 M Tris-Acetate, 100 mM Na_2-EDTA, 10 mM Tris-HCl
TES-buffer	10 mM Tris/HCl, pH 7.5 10 mM EDTA, pH 8.0 0.1 % SDS
Washing solution 1 (DNA chip)	1 x SSC 0.1 % (w/v) SDS
Washing solution 2 (DNA chip)	0.1 x SSC 0.1 % (w/v) SDS
Washing solution 3 (DNA chip)	0.1 x SSC
Western-Blot transfer buffer	Invitrogen, Karlsruhe

2.1.2. Chemicals

Product	**Manufacturer**
2 log DNA Ladder	New England Biolabs, Schwalbach
Ampicillin	Sigma, Deisenhofen
Agar	Invitrogen, Karlsruhe
AMV Reverse Transkriptase	Roche Diagnostics, Mannheim
ß-Mercaptoethanole	Stratagene, Amsterdam, Netherlands
BSA	Sigma, Deisenhofen
Bromphenolblue	Merck, Darmstadt
Calf Intestinal Alkaline Phosphatase	New England Biolabs, Schwalbach

DMEM (Dulbecco's modified eagle's medium) with Glutamax-I (L-Analyl-L-Glutamin) and 4.5 g/l Glucose	Invitrogen, Karlsruhe
dNTPs	Perkin Elmer Cetus, Überlingen
DNase	Promega, Madison, USA
EDTA	Sigma, Deisenhofen
Ethanol	Merck, Darmstadt
Ethidiumbromide (1% w/v)	Serva, Heidelberg
Acetic acid	Merck, Darmstadt
FCS	Biochrom, Berlin
Formamide	Sigma, Deisenhofen
FuGENE^{TM} HD transfection Kit	Roche Diagnostics, Mannheim
G418 / Geniticin	Invitrogen, Karlsruhe
Ionomycin	Merck, Darmstadt
Isopropanol	Merck, Darmstadt
Leptomycin B	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
Paraformaldehyde (used: 2% in PBS)	Sigma, Deisenhofen
Penicillin/Streptomycin solution (P./S.)	Invitrogen, Karlsruhe
PMA	Merck, Darmstadt
Protein G (immobilized)	Perbio Science
Pyromycine	Sigma, Deisenhofen
Restriction enzymes	New England Biolabs, Schwalbach
SeaKem® LE Agarose	FMC Bio Products, Rockland, USA
Sodium borohydride ($NaBH_4$)	Sigma, Deisenhofen
Sodium chloride	Sigma, Deisenhofen
Sodium citrate	Sigma, Deisenhofen
Trypsin-EDTA solution	Invitrogen, Karlsruhe
Tween 20	Sigma, Deisenhofen

2.1.3. Antibodies

Product	Dilution
mouse-anti-GAPDH	1:10000
mouse-anti-humanCD3 (IgG2a)	1 µg/ml
mouse-anti-humanCD28 (IgG1)	5 µg/ml
rabbit-anti-Risp (41-62/282-302/573-593/830-850)	1:1000
rat-anti-16.4.1	1:500
rat-anti-Rev 5C6	1:50
anti-mouse-HRPO	1:10000
anti-mouse-IgG1	2.5 µg/ml
anti-mouse-IgG2a	2.5 µg/ml
anti-rabbit-HRPO	1:10000

2.1.4. Kits

Product	Manufacturer
DNase Treatment Kit	Promega, Madison, USA
ELISA	BD Biosciences, Düren
KAPA 2G Robust	Nippon Genetics Europe GmbH
Light Cycler® 480 System	Roche Diagnostics GmbH, Penzberg
NucleoBond PC 500 Kit	Macherey-Nagel, Düren
NucleoBond PC MINI Kit	Macherey-Nagel, Düren
NucleoSpin Extract II	Macherey-Nagel, Düren
NuPAGE Colloidal-Staining Kit	Invitrogen, Karlsruhe
PARIS™ Kit	Applied Biosystems, Carlsbad, Carlifornia
pGEM®-T Easy Vector System	Promega GmbH, Mannheim
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAshredder	Qiagen, Hilden
RNAiFect Transfection Kit	Qiagen, Hilden
RNEasy Kit	Qiagen, Hilden
Superscript II First strand cDNA synthesis Kit	Invitrogen, Karlsruhe

2.1.5. Consumable materials

Product	Manufacturer
Bis-Tris/Tris-Acetate-Gels (4-12% und 3-8%)	Invitrogen, Karlsruhe
6-, 24- and 96-well plates	Nunc International, Wiesbaden
384 well plates	Genetix Limited, New Milton, United Kingdom
Counting chamber Fast Read 102	Madaus Diagnostics, Cologne
Coverglass Lifter Slip 22x22	Erie Scientific Company, Portsmou^{th,} USA
CSS-100 Silytated Slides	Arraylt-CEL-Associates, Los Angeles, USA
Eppendorf tubes 0,5 ml, 1,5 ml, 2 ml	Eppendorf, Wesseling-Berzdorf
Falcon® EASY GRIP™ Tissue Culture Dish, 35 x 10 mm	Becton Dickinson, USA
Falcon® 15 ml and 50 ml tubes	Becton Dickinson, USA
Fixogum	Marabu, Tamm
Hybridization chamber	Greiner, Nürtingen
Hyperfilm ECL, High performance Chemiluminescence film	Amersham Biosciences, Buckinghamshire
Nitrocellulose membrane	Invitrogen, Karlsruhe
Nunc Cryo Tube™ Vials	Nunc International, Wiesbaden
Nunc Solo Flask 25 cm^2 (Cell Culture Flask)	Nunc International, Wiesbaden
Nunc Solo Flask 80 cm^2 (Cell Culture Flask)	Nunc International, Wiesbaden
Nunc Solo Flask 185 cm^2 (Cell Culture Flask)	Nunc International, Wiebaden
Slide transport box	Greiner, Nürtingen
Super Signal West Pico Chemiluminescent Substrate	Pierce, Rockford
Western-Blot Filterpaper	Invitrogen, Karlsruhe

2.1.6. Equipment

Equipment	Manufacturer
FACScalibur Cytometer ▪ Exitationlaser: 488nm, 15mW ▪ Fluorescence-Detection: o FL-1 (BP 530/30) o FL-2 (BP 585/30) o FL-3 (LP 650)	BD Biosciences, Düren
Sigma 1-15 Microcentrifuge	Sigma GmbH, Oserode
ChemiDoc Quantity One Vers. 2.4.1 Build 008	BioRad, Munich
Hettich Rotanta/TR	Hettich, Tuttlingen
Infors incubator	Infors AG, Switzerland
Master Cycler gradient 5331	Eppendorf, Hamburg
Shimadzu UV-160 A Spektrometer	Shimadzu Corp., Japan
Thermomixer 5435	Eppendorf, Hamburg
Transilluminator 312 nm	Bachofer
KODAK	Kodak, Munich
Horizontal Electrophoresis Systems	Thermo Fisher Scientific, NY, USA
PowerPac 300 Power supply	BioRad, Munich
ChemiDoc Molecular Imager	BioRad, Munich
CSS-100 silylated slides	Telechem, Atlanta, USA
Eppendorf Mastercycler 5333	Eppendorf, Hamburg
Eppendorf Mastercycler gradient 5331	Eppendorf, Hamburg
Special accuracy weighing machine	Scaltec, Heiligenstadt
GMS 418 Arrayer	Affymetrix, Santa Clara, USA
GMS 418 Array-Scanner	Affymetrix, Santa Clara, USA
Herolab Clene Cab	Herolab GmbH Laborgeräte, Wiesloch
Hettich large bench centrifuge Micro Rotanta 460R	Andreas Hettich GmbH, Tuttlingen
Light Cycler (R) 480 System	Roche Diagnostic, Mannheim
Spectrafuge 24 D	Abimed, Langenfeld
Shaking water bath	Köttermann GmbH & Co KG, Uetze/Hänigsen
Thermomixer 5436/compact	Eppendorf, Hamburg
Eppendorf centrifuge 5415R	Eppendorf, Hamburg

Vortex Genie 2	Scientific Industries, New York, USA
Weighing machine SCALTEC SPO51	SCALTEC Instruments GmbH, Göttingen

2.1.7. Oligonucleotides

Mixed oligonucleotide primers (MOP) for multiplex-PCR

Target ID	Source	Orien-tation	Modifi-cation (5')	Oligonucleotide sequence	Degene-rations
Type ABD primer cocktail (MOP-A)					
HML-1	U35102	forward	-	GGAGAAAAAGTACTGCCACAAGGC	-
		reverse	Cy3	GGAGAACAGAATATCATCCATGTA	-
HML-2	M14123	forward	-	GGAGAAAAAGTGTTACCTCAGGGA	-
		reverse	Cy3	GGAGAATAAAATATCATCAATATA	-
HML-3	U35236	forward	-	GGAGAAAAAGTGTTGTCACAGGGC	-
		reverse	Cy3	GGAGAAGTATATATCATCCATATA	-
HML-4	AF020092	forward	-	GGAGAAAAAGTCCTACCACAAGGC	-
		reverse	Cy3	GGAGAAGAGGAGATCATCCATGTA	-
HML-5	U35161	forward	-	GGAGAAGTGCTTCCTGAAGGGATG	-
		reverse	Cy3	GGAGAATAAAATATCATCCATAAA	-
HML-6	U60269	forward	-	GGAGAAAGAGTTTTACCCCAAGGC	-
		reverse	Cy3	GGAGAAAAGAATATCATCCATATA	-
HML-7	AP003171	forward	-	GGAGAAGTTTTACCTCAAGGAATG	-
		reverse	Cy3	GGAGAACAGTATATCATCCATATA	-
HML-8	AL513321	forward	-	GGAGAAGTACTTCCTCAGGGAATG	-
		reverse	Cy3	GGAGAATAAAATATCATCAATATA	-
HML-9	AC025569	forward	-	GGAGAAGTTCTACCCCAAGAGATG	-
		reverse	Cy3	GGAGAACAAAATATCATCCACATA	-
HML-10	U07856	forward	-	GGAGAAAAAGTTTTGCCCCAGGGT	-
		reverse	Cy3	GGAGAATCTACTTTTTGCTGCACA	-
MMTV	M15122	forward	-	GGAGAATAGGTTTTGCCCCAGGGT	-
		reverse	Cy3	GGAGAAAAGAGGATGTCATCCATGTA	-

Target ID	Source	Orien-tation	Modifi-cation (5')	Oligonucleotide sequence	Degene-rations
Type C primer cocktail (MOP-C)					
Type C	Shih et al., 1989	forward	-	GGAGAATGGAAAGTGYTRCCMCARGG	8
		reverse	Cy3	GGAGAACAGCAGSAKGTCATCCAYGTA	8
HERV-I	M92067	forward	-	GGAGAATKKACMSKMYTRCYYCARGGG	1072
HERV-L	G895836	reverse	Cy3	GGAGAAAKMWKRYCATCMAYRTAMTG	8192
HERV-H	AF026252	forward	-	GGAGAAGTACTGCTGCAAAGCTTCA	-
		reverse	Cy3	GGAGAACACACGATCGGCAGGGAGA	-
RGH2	D11078	forward	-	GGAGAAGTAATGCTGCAAGGTTTC	-
		reverse	Cy3	GGAGAAGAAGAACATCACCAATATA	-
HERV-F	AC000378	forward	-	GGAGAARTMCTMCMYCARGGGTT	64
	Z94277	reverse	Cy3	GGAGAAAAGGAGGTCATCTAGATAT	-
HIV-1	K02013	forward	-	GGAGAAGTGCTTCCACAGGGATGG	-
		reverse	Cy3	GGAGAAATACAAATCATCCATGTA	-
Housekeeping gene primer cocktail (MOP- HKG)					
ß-Actin	E01094	forward	-	ATGATGATATCGCCGCGCTCG	-
		reverse	Cy3	CATGTCGTCCCAGTTGGTGACG	-
Ubi-quitin	U49869	forward	-	GTTGGCTTTCTTGGGTGAGCTTG	-
		reverse	Cy3	AAGAGTACGGCCATCTTCCAGCTG	-
RPL19	NM_ 000981	forward	-	CCCGAATGCCAGAGAAGG	-
		reverse	Cy3	CTTCCTTGGTCTTAGACCTG	-
GAPDH	NM_ 002046.1	forward	-	AGTCAACGGATTTGGTCGTATTGGG	-
		reverse	Cy3	ACGTACTCAGCGCCAGCATCG	-
HPRT	NM_ 000194	forward	-	GTGATGATGAACCAGGTTATGACCTTG	-
		reverse	Cy3	CTACAGTCATAGGAATGGATCTATCAC	-

The final concentration of the utilized primer dilutions was 50 pmol/µl. Underlined 5' nucleotide sequences designate so-called "clamp"-sequences which were implemented to improve the binding ability of the primers.

Specific pol*(RT) primers to be combined for detection of non-human retroviruses*

Target ID	Source	Orien-tation	Modifi-cation (5')	Oligonucleotide sequence (5'→3')	Degene-rations
BoEV-1	X99924	forward	-	GGAGAATGGACYCGMYTVCCMCARGG	-
JSRV	A27950	forward	-	GGAGAATAGGTTCTCCCBCAGGG	3
		reverse	Cy3	GGAGAAATATATATATCATCCACATA	-
MLV	J02255	forward	-	GGAGAATAGGTTTACCACAAGGA	-
		reverse	Cy3	GGAGAACAGCAGTAAGTCATCYACGTA	2
OvEV2	X99932	forward	-	GGAGAATGGATTCAACTTCCACAAGG	-
MPMV	M12349	forward	-	GGAGAATAGGTTTTACCACAAGG	-
		reverse	Cy3	GGAGAAAAGAGGATGTCATCCATGTA	-
Mac ERV - ERVK	(Han, K. et al., 2007)	forward	-	§	§
		reverse	Cy3	§	§
GaLV	M26927	forward	-	GGAGAATAGCGGCTACCACAAGGG	-
		reverse	Cy3	GGAGAAACCAAGAGGTCGTCCACATA	-
BaEV	D10032	forward	-	GGAGAATAGCGCCTTCCHCAAGGG	3
		reverse	Cy3	GGAGAACAAGAGGAGGTCATCTACATA	-
PERV	AF038600	forward	-	GGAGAATAGCGACTGCCCCAAGGG	-
		reverse	Cy3	GGAGAACAGAAGCAGGTCATCCACGTA	-
FIV	M59418	forward	-	GGAGAAAGTTTACCACAGGGGTGG	-
		reverse	Cy3	GGAGAAATATATATCATCCATATA	-

The final concentration of the utilized primer dilutions were 50 pmol/µl. Underlined 5' nucleotide sequences designate so-called "clamp"-sequences which were implemented to improve the binding ability of the primers. § = For amplification, the human MOP cocktails were used.

Oligonucleotides for the retrovirus-specific microarray (capture probes)

Retrovirus class	Family/group	RepBase Name [b)]	Genebank-ID	Localization on microarray
Class-I-retroviruses (γ-retrovirus-like)	HERV-I	HERV-I HERVIP10F	HERV-I (M92067) HERV-IP-T47D (U27241) Seq65 (AP000842)	E9 E10 E11
	HERV-S	HERV18	HERV-S (Z84470) Seq77 (AC005040)	E7 E8
	HERV-T	HERVS71	S71pCRTK6 (U12969) S71pCRTK1 (U12970)	F1 F2
	HERV-FRD	MER50I	ERV-FRD (U27240) HS49C23 (Z93019) HERV-Z (Z69907)	F3 F4 F5
	HERV-R	HERV-R	ERV-3 (AC004609)	F6
	HERV-E	HERV-E	E4-1 (M10976) Seq32 (AC010636)	F7 F8
	HERV-H	HERV-H	RGH2 (D11078) HERV-H (AF026252) Seq66 (AL359740)	F9 F10 F11
	HERV-F	HERVH48I HERVFH19I HERVFH21	HERV-F2 (AC002416) HERV-F (Z94277) HERV-Fb (AC00378)	G9 G10 G11
	HERV-W	HERV17	HERV-W (AF009668)	G3
	ERV9	HERV9 HERV17 HERV9 HERFFH19I	Seq64 (AC005253) Seq63 (AC018926) ERV9 (X57147) Seq59 (AC006397) Seq60 (AL135749)	G4 G5 G6 G7 G8
Retrovirus class	**Family/group**	**RepBase Name [b)]**	**Genebank-ID**	**Localization on microarray**
Class-II-retroviruses (β-retrovirus-like)	HML-1	HERVK14I	HML-1 (U35102) Seq29 (S77579)	A8 A9
	HERV-K (HML-2)	HERVK	HERV-K10 (M14123) HERV-K2.HOM (U87592) HERV-K(HP1) (U87588) HERV-K(D1.2) (U87595) HERV-K10 (U39937)	B1 B2 B3 B4 C6

	HML-3	HERVK9I	Seq26 (AC073115) Seq34 (AL592449) HML-3 (U35236) HERV1 (S66676) Seq43 (AF047595)	B5 B6 B7 B8 B9
	HML-4	HERVK13I	Seq10 (AF047591) HERV-K-T47D (AF020092)	B10 B11
	HML-5	HERVK22I	HML-5 (U35161)	B12
	HERV-K (HML-6)	HERVK3I	HML-6 (U60269) Seq38 (AC010328) Seq56 (AC018558)	A10 A11 A12
	HML-7	HERVK11DI	NMWV7 (AP003171)	C12
	HML-8	HERVK11I	NMWV3 (AL513321)	C11
	HML-9	-	NMWV9 (AC025569)	C10
	HERV-K(C4) (=HML-10)	HERVKC4	HERV-KC4 (U07856) Seq31 (AL162734)	C8 C9
Retrovirus class	**Family/group**	**RepBase Name** [b)]	**Genebank-ID**	**Localization on microarray**
Class-III-retroviruses (spumavirus-like)	HERV-L	HERV-L =MLT2	HERV-L (G895836) Seq39 (AC091914) Seq45 (AC006971) Seq51 (AL353741) Seq58 (AL590730)	E2 E3 E4 E5 E6
Human exogenous retroviruses			LAV-1 (K02013) HIV-2 (J04542) HTLV-1 (M81248) HTLV-2 (M10060) HFV (Y07725)	H2 H3 H4 H5 H6
Human housekeeping genes			Ubiquitin (U49869) GAPD (NM_002046.1) RPL19 (NM_000981) ß-Actin (E01094) HPRT (NM_000194)	A2 A3 A4 A5 A6
Localization dots			Cy3 -labelled oligonucleotides	A1, A7, D1, E1, E12, H1

b) RepBase, Genetic Information Research Institute, Sunnyvale, California (http://www.girinst.org).

Control-oligonucleotides for quality assurance of the retrovirus-specific-microarray

Control	Name	Genebank-ID	Oligomer	Localization on microarray
Control	antisense-MMTV	M15122	42-mer	D2
Control	antisense-HML-1	U35102	36-mer	A6
Control	antisense-HML-5	U35161	42-mer	B12
Control	antisense-2HOM	AF298587	36-mer	E5
Control	antisense-HERV-W	AF009668	42-mer	G3

2.1.8. Media

Media	Composition
Storage-Medium for procaryotic cells	2 parts 80 % Glycerin 1 part 10 mM $MgCl_2$
Storage-Medium for eucaryotic cells	DMEM, 20% FCS 1 % Penicillin/Streptomycin 10 % DMSO
LB-Agar	LB-Medium with 15 g/l Agar
LB-Amp Plates	LB-Agar with 100 µg/ml Ampicillin
LB-Medium (after Luria-Bertani)	10 g Trypton 5 g yeast extract 10 g NaCl, Aqua dest. to 1 l, pH 7.0
LB-Amp-Medium	LB-Medium with 100 µg/ml Ampicillin
Medium for eucaryotic celllines	DMEM 10 % FCS 1 % Penicillin/Streptomycin
SOC-Medium (Invitrogen, Karlsruhe)	20 g Trypton 5 g yeastextract 0.5 g NaCl 2.5 mM KCl 20 mM Glucose to 1 l with Aqua dest., pH 7.0

2.1.9.Software

Software	Manufacturer
Adobe Acrobat CS3	Adobe Systems, USA
Adobe Illustrator CS3	Adobe Systems, USA
Adobe Photoshop CS3	Adobe Systems, USA
Apple OSX 10.6	Apple, USA
Gene Construction Kit 3	Textco Inc., USA
MacVector 6.5.1	Accelrys, SanDiego, USA
Microsoft Word and Excel 2004 for MAC	Microsoft Corp., USA
Endnote X	Thomson ISI ResearchSoft
Light Cycler 480 software	Roche Diagnostics, Mannheim
Prism 4.0 b	GraphPad, San Diego, USA

2.1.10. Human cell lines

LC5

Human epithelia cell line from cervix-carcinoma cloned from a HeLa derivative [98] (ATCC No. CRL-7923).

LC5-HIV

Human epithelia cell line (LC5) from cervix-carcinoma, persistently infected with HIV-1IIIb.

LC5-CD4

LC5-CD4, a clonal LC5 cell line transduced with an amphotropic retroviral vector containing the human CD4 receptor gene [99].

85HG66

Human astrocytic cell line [100] originates from a brain tumor.

TH4-7-5

Human astrocytic cell line, 85HG66 [100], persistently infected with HIV-1IIIb

KE37/1

Human T-lymphoma cell line obtained from M. Popovic (Bethesda, USA).

KE37-1/III B

Human T-lymphoma cell line (KE37-1) persistently infected with HIV-1IIIb [101].

U138MG

Human astrocytic glioblastoma cell line. This cell line originates from an astrocytic tumor [102,103,104].

Human primary astrocytes

Human primary astrocytes were purchased from 3H Biomedical AB and cultured according to the vendor's instructions.

HNSC.100 and HNSC.100 derived astrocytes

HNSC.100 cells and generation of enriched astrocyte populations is described in [105] and [106].

HNSC.100 derived neurons

HNSC.100-derived neurons were generated by culturing in neurobasal medium (Invitrogen) supplemented with 1 x B27 supplement (Invitrogen), 10 ng/ml brain-derived neurotrophic factor (BDNF, Tebu-Bio, Offenbach, Germany) and 10 ng/ml platelet derived growth factor (PDGF, Tebu-Bio) for at least 10 days.

2.1.11. Plasmids

pCRispsg143

The plasmid pCRispsg143 contains a human brain cDNA with the entire FAM21C encoding sequence (1320 AA; Accession No. Q9Y4E1) fused in-frame to GFP coding sequences and the CMV promoter for transcriptional control.

pC16.4.1sg143

The plasmid pC16.4.1sg143 was constructed as described in [107].

pLRed(INS)$_2$R

The Rev-reporter plasmid pLRed(INS)$_2$R [107] contains the Rev response element (RRE) and two tandem copies of INS 1 and 2 [108].

pCTat

The pCTat expresses the 86 amino acid-variant of Tat (HXB3) under the control of the CMV promoter with a SV40 polyadenylation signal [39,109].

pCsRevsg143

The sRev amplified from pBsRev was cloned into pFRED143 [110]. In this construct sRev underlies the control of a CMV promoter and is fused to GFP. The GFP coding sequence has no startcodon of his own and can therefore only be expressed together with Rev. (Dr. Neumann, HelmholtzZentrum München)

Y26A and Y47H2

The construction of the Tat-mutated HIV-1 LAI proviral clones was described previously [111]. These plasmids were kindly provided by Ben Berkhout.

2.1.12. Bacteria

TOP10F'

Competent cells for chemical transformation (cfu=1x10^8-5x10^9/µg).

These bacterias overexpress the lac-repressor (*laclq*-gen) for an effective suppression of the lac promoter (Invitrogen, Karlsruhe).

2.1.13. Enzymes

Name	Manufacturer
E. coli RNase H (2 U/µl)	Invitrogen, Karlsruhe
Expand High Fidelity Enzyme mix	Roche Diagnostics GmbH, Mannheim
GoTaq® DNA Polymerase (5 U/µl)	Promega GmbH, Mannheim

2.2. Methods

2.2.1. Cell culture and transfection

2.2.1.1. Cell cultivation and storage

All cells were maintained in Biochrom VLE-RPMI 1640 with stable glutamine and 2,0 g/l $NaHCO_3$ and 10% fetal calf serum (Seromed, Berlin, Germany). If applicable, 100 U/ml penicillin and 100 µg/ml streptomycin was added to the culture. Cells were cultured in an H_2O-saturated atmosphere with 5% CO_2 at 37°C. At a density of about 70-80% cells were splitted. The old medium was removed, cells were washed with 10 ml PBS, and 1 ml trypsin-EDTA was added. After 5 minutes incubation at 37 °C, cells were resuspended in new medium and splitted 1:5 or 1:10 in new medium. Cell lines used were either purchased from the American Type Culture Collection (ATCC) or authentified by the German Collection of Microorganisms and Cell Cultures (DSMZ).

2.2.1.2. Thawing and freezing of cells

Cells stored in nitrogen were thawed in a water bath. Afterwards they were transferred to culture flasks with prewarmed medium. The next day old medium was removed, cells were washed with 10 ml PBS and new medium was added.

For freezing, cells were washed with 10 ml PBS, detached with 0.7 ml Trypsin-EDTA and resuspended in new medium. Cells were transferred to a 15 ml Falcon tube and centrifuged for 10 minutes at 700 rpm at room temperature. The supernatant was removed and storage medium was added. The resuspended cells were transferred into cryotubes (1.0 ml per cryotube) and then stored in a Bicell biofreezing vessel at –80°C.

2.2.1.3. Transfection of eucaryotic cells with FuGENE

All overexpression transfection experiments were performed in 12-well plates. Cells were seeded at a density of 1 x 10^5 cells per well one day prior to transfection and cultured for 72 hours after transfection. Transfection was performed with FuGENE^{TM}HD Transfection Reagent (Roche) using 500 ng plasmid DNA per well.

2.2.1.4. Rev activity assays

Rev activity was determined as described previously [107,109]. Cell transfections were performed with FuGENE^{TM}HD in 6-well plates (2 x 10^5 cells per well) using 1 µg pLRed$(INS)_2$R, 0.4 µg pCTat, 0.3 µg pFRED143 and 0.3 µg pCsRevsg143. Parallel

transfections were performed without pCsRevsg143 to determine reporter gene expression in the absence of Rev.

48 hours after transfection, cells were analyzed by flow cytometry as described in [107]. GFP-positive cells were identified and the percentage of RFP-reporter-positive cells within this transfected cell population determined. The influence of increased Risp expression on Rev activity was evaluated by adding 0.3 µg pCRispsg143 to the transfection mixtures. For Risp knock-down experiments, cells were first transfected with two Risp specific siRNAs according to the manufacturer's protocol (Qiagen). 24 hours after siRNA transfection, the cells were washed, culture media renewed and transfections performed with the plasmids for the Rev activity assay. For quantitative analysis of nucleo-cytoplasmic distribution of reporter RNAs, cells were separated in nuclear and cytoplasmic fractions using the PARIS Kit (Ambion) and total RNA extracted according to the manufacturer's protocol.

2.2.1.5. Stimulation of Jurkat T-cells with PMA/ionomycin and CD3/CD28

For stimulation of Jurkat T-cells, cells in conditional complete RPMI medium were incubated with phorbol-12-myristate-13-acetate (PMA) and ionomycin or antibodies against CD3 and CD28.

Stimulation with PMA/ionomycin: PMA (phorbol-12-myristate-13-acetate) possesses structural similarity to diacylglycerol (DAG) and can therefore activate PKCθ and thus NF-κB in T cells. Ionomycin induces Ca2+ influx from intracellular Ca2+ storage compartments and therefore mainly influences the activation of NFAT. For stimulation of Jurkat T-cells (2 x 10^6 cells in 1 ml), 200 ng/ml PMA and 300 ng/ml ionomycin were added directly to the medium and incubated for the indicated time.

Stimulation through CD3/CD28 antibody ligation: Crosslinking of the co-stimulatory receptor CD28 and the CD3 subunits of the T cell receptor by specific primary and secondary antibodies mimics the receptor aggregation, which is under physiological conditions induced by the T cell/APC (antigen presenting cell) contact. This leads to activation of antigen-receptor specific signaling cascades. The following antibodies were used at the given concentrations to stimulate 2 x 10^6 cells in 300 µl: anti-hCD3 (IgG2a): 1 µg/ml, anti-hCD28 (IgG1): 5 µg/ml, anti IgG1: 2.5 µg/ml and anti IgG2a: 2.5 µg/ml.

After stimulation cells were placed on ice, washed once with cold PBS and lysed immediately with the indicated buffers.

2.2.1.6. RNA interference

Transfections were carried out using RNAiFect transfection reagent (Qiagen) according to the manufacturer's protocol. The day before transfection, 1 x 10^5 target cells per well were seeded in a 12-well plate. 2 µg siRNA per well was used for each transfection. After 24 h, medium was removed, cells were washed with PBS and new medium was added. Gene silencing was monitored by p24 ELISA analysis of the supernatant 72 hours after transfection. RNA of siRNA treated cells was extracted using the Qiagen RNeasy Mini Kit, reverse transcribed using the Superscript II Kit and analyzed using the HERV microarray.

2.2.1.7. Influence of Risp proteins on HIV-1 production

Cells were seeded at a density of 1 x 10^5 cells per well of a 12-well plate one day prior to transfection. To analyze the effect of overexpression of Rispsg143 and 16.4.1sg143, cells were transfected with 500 ng plasmid DNA per well. For Risp knock-down, cells were transfected with two Risp specific siRNAs (siRisp AAGTGGAAGCCAAGTCTATAT and siRisp AAGATGAGGATGACCTCTTTA designed and synthesized by Qiagen). An evaluated non-silencing siRNA (from Qiagen, Hilden, Germany) served as a negative control. siRNAs transfections were performed with RNAiFect transfection reagent (Qiagen) according to the manufacturer's protocol. 24 hours after transfection, culture media was renewed and cells were incubated for another 72 hours. Cell culture supernatants (extracellular sample) were incubated with Triton-X-100 at a final concentration of 0.5%. Cells were lysed in 5% Triton-X-100 (intracellular sample) and lysates diluted with PBS to a final Triton-X-100 concentration of 0.5%. Both samples were centrifuged for 5 minutes at 16.000 g (room temperature) and cleared supernatants were subjected to the HIV-1-p24-Antigen-ELISA assay for quantification of p24 antigen using the manufacturer's protocol (Beckman Coulter, Krefeld, Germany). Late and early HIV-1 transcripts were analyzed using quantitative real-time RT-PCR.

2.2.1.8. Infection of LC5-CD4 cells

LC5-CD4 cells were infected with an HIV-1 patient isolate (Serum 891). Cells were cultured for further three weeks. Then RNA was isolated and further analyzed by microarray analysis (2.2.2.10), real-time RT-PCR (2.2.2.11) or loci identification (2.2.2.12).

Production of Y26A and Y47H2 virus was done as followed: 293T cells were transfected with either of the plasmids together with plasmids for Tat (pCTat) and Rev (pCsRevsg143) production. Cell culture supernatant was used to infect LC5-CD4 cells and cultured for two days.

2.2.2. Molecular biology methods

2.2.2.1. Minipreparation of plasmid DNA

5 ml LB-Amp-medium were inoculated with a single bacteria colony picked from a freshly streaked agar plate. The culture was incubated overnight at 37 °C and ~ 200 rpm. 2 ml of the culture were centrifuged for 5 minutes at 5000 rpm and DNA from the bacteria pellet was isolated after manufacturer's protocol (Macherey-Nagel).

2.2.2.2. Maxipreparation of plasmid DNA

150 ml LB-Amp-Medium was inoculated with 1 ml of a pre-culture. The culture was incubated overnight at 37 °C and ~ 150 rpm. The DNA was extracted using the Nucleobond-Kit (AX500) (Macherey-Nagel).

2.2.2.3. DNA restriction digestion

0,5 µg – 1 µg plasmid DNA was incubated with 10U of the corresponding restriction enzyme for 1 h in the corresponding buffer and at the appropriate temperature.

2.2.2.4. Agarose gel electrophorese

Gels with agarose concentrations between 0.8 % and 2 % were used for electrophorese. The percentage of the agarose content was reduced with increasing height of the DNA-fragment. A 1 % ethidium bromide solution was added in a 1/10000 dilution to the gel (dissolved in 1 x TAE buffer). Electrophoresis was carried out at 80 to 120 V in 1 x TAE buffer. The 2-log DNA ladder from New England Biolabs was used as standard.

2.2.2.5. Isolation of DNA from agarose gels

To isolate DNA from agarose gels the NucleoSpin Extract II Kit from Macherey-Nagel was used after manufacturer's protocol.

2.2.2.6. pGEM®-T Easy Vector System cloning

For cloning PCR DNA fragments into the pGEM®-T Easy vector the following reaction was used:

Fresh PCR product	2 µl
2 x rapid ligation buffer	5 µl
pGEM®-T Easy vector	1 µl
T4 DNA ligase	1 µl
Water	1 µl
end volume	10 µl

The reaction was mixed gently and incubated for 30 minutes at room temperature.
For transformation of the chemo-competent Top10F' cells, 2 µl of the ligation reaction was added to the competent *E.coli* and incubated for 30 minutes on ice. Then the cells were heat-shocked 30 seconds at 42°C without shaking and immediately transferred to ice for another 2 minutes. 250 µl of room temperature SOC medium (Invitrogen) was added and the tubes were shook horizontally (200 rpm) at 37°C for 1 hour. 50 µl from each transformation was spread on a prewarmed ampicillin plate. The plate was incubated at 37°C overnight.

2.2.2.7. RNA isolation

Total RNA was extracted using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol. 1 x 10^6 cells were used for RNA isolation. RNA was eluted with 30 µl of RNase-free water. After measuring the concentration with a spectrophotometer, the RNA was stored at -80 °C.

2.2.2.8. DNase digest

To remove genomic DNA contamination, RNA samples (1µg) were treated with 1 U/µg RNase-free RQ1DNase (Promega, Mannheim).
For DNase digestion the RQ1DNase-KIT from Promega was used.
Digestion Reaction:

RNA	4 µg
RQ1 DNase-buffer	1 µl
RQ1 DNase	1 U/µg RNA
Nuclease free water	variable
end volume	10 µl

The digestion mix was incubated for 30 minutes at 37 °C. Afterwards 1 µl RQ1 DNase Stop solution was added and incubated for another 10 minutes at 65 °C for inactivation.

To assure that all genomic DNA was removed, 25 ng of each mRNA preparation was tested by PCR with mixed oligonucleotide primers (MOP) [70]. Only RNA preparations negative for amplification products were used for subsequent reverse transcription and MOP multiplex PCR. Reaction mixture:

Water	15,375µl
GoTaq Reaction Buffer	2,5µl
dNTPs (10mM)	1µl
MOP-C Primer (20µM)	2µl
MOP-HKG Primer (10µM)	1µl
GoTaq-Polymerase (5U/µl)	0,125µl
Template RNA	3µl
end volume	25µl

PCR program see section 2.2.2.10

2.2.2.9. Reverse transcription PCR (RT-PCR)

Reverse transcription of RNA was generated from 1 µg total RNA with Superscript II performed according to the manufacturer's protocol using random hexamers. Reverse transcriptions were followed by an RNase H digestion step (Promega).

2.2.2.10. Retrovirus-specific microarray

Figure 2.1 shows an application of the DNA-microarray. The so-called capture probes are spotted on the glass slides, which covered by an aldehydsurface.

These capture probes consist of two oligonucleotides, which correspond to a highly conserved region in the *pol* gene of the respective HERV family. The oligos are modified at the 5' prime end with an amino group. This group can bind covalently to the aldehyde surface. Subsequently, a multiplex PCR for the *pol* gene is carried out using degenerate primers. The reverse primers are Cy3 labeled yielding a fluorescently labeled PCR product after amplification. The PCR products are then hybridized to the chip and positive results detected with a microarray scanner.

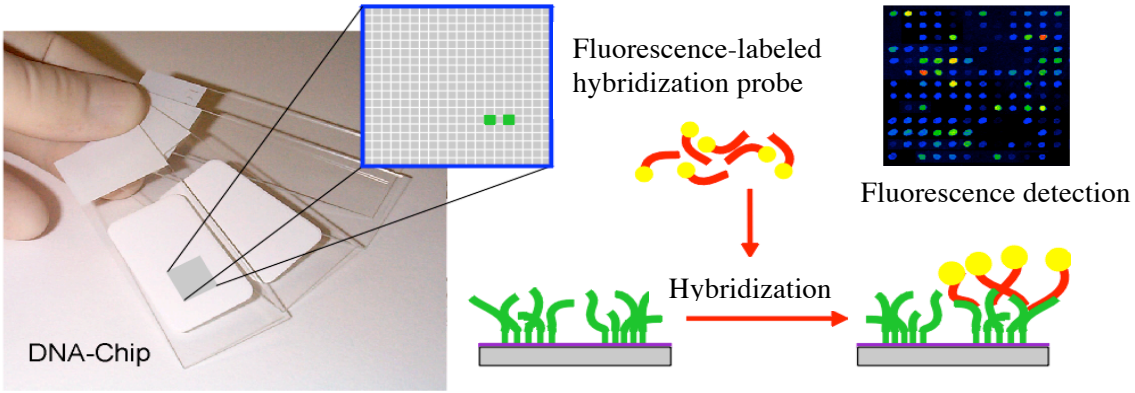


Figure 2.1.: Schematic overview of the DNA chip procedure

Hybridization probe synthesis, MOP multiplex PCR, DNA microarray preparation, hybridization and post-processing of retrovirus-specific microarrays was performed as described in the following sections. Hybridized microarrays were scanned using an Affymetrix Scanner GMS 418 (laser power settings, 100%; gain, 50).

Figure 2.2 illustrates the assortment and arrangement of the retrovirus- and gene-specific oligonucleotides on the microarray. The combination of letters and numbers depicts the exact position of each HERV-family.

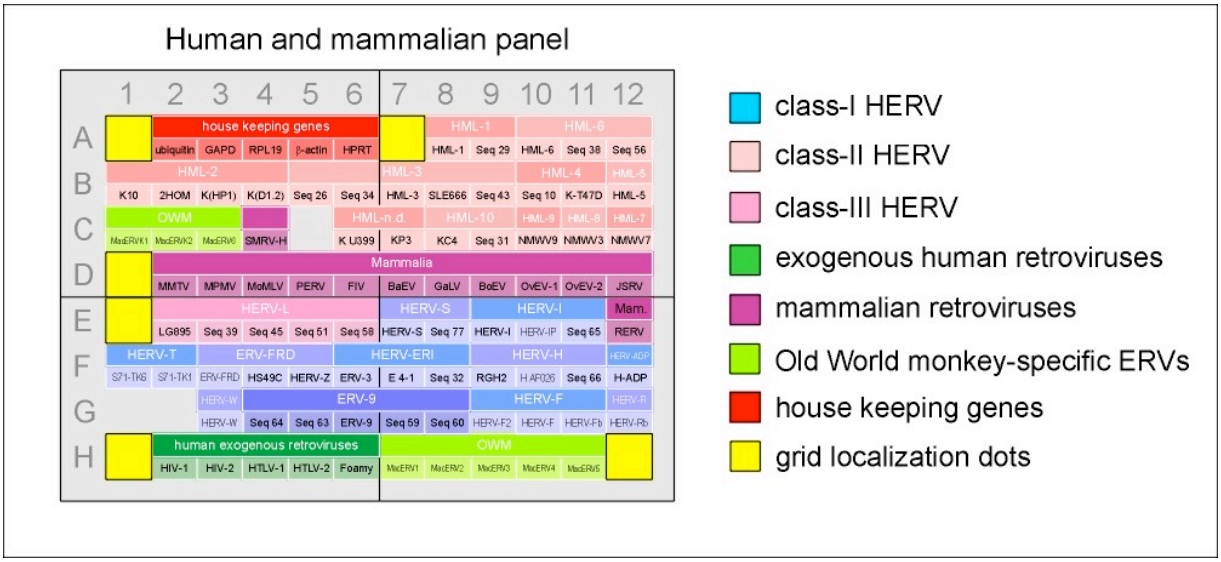


Figure 2.2.: Organization of the oligonucleotides on the DNA chip

2.2.2.10.1. Amplification of hybridization probes by multiplex-PCR

The MOP-C-primer set is based on degenerate primers [112] and permits the amplification of human and vertebrate γ-retroviral RT-sequences and various exogenous virus sequences. The other primer set (MOP-A) enables the amplification of human β-retroviral RT-sequences. The PCR for each primer mixture was performed separately to allow the optimal amplification of the retrovirus-related elements. Oligonucleotides specific for human housekeeping genes (MOP-HKG) served as internal control for the quality of the RNA and the reproducibility of the microarray data [113].

Reaction mixture:

Water	19.8µl
Reaction Buffer	2.5µl
dNTPs (10mM)	1µl
MOP-A / MOP-C	2µl
MOP-HKG	1µl
Polymerase (5U/µl)	0.125µl
Template cDNA	5µl
end volume	50µl

PCR conditions:

	Temperature	**Time**
Initial denaturation	95 °C	1.0 min
Denaturation Annealing Elongation **→ 3 cycles**	94 °C 45 °C 72 °C	0.5 min 3.0 min 2.0 min
Denaturation Annealing Elongation **→ 30 cycles**	94 °C 50 °C 72 °C	0.5 min 2.0 min 2.0 min
Final extension	72 °C 4 °C	7.0 min ∞

2.2.2.11. Quantification of RNA by real-time RT-PCR

For amplification of *pol*(RT) sequences of the HERV families, the following HERV subgroup-specific *pol* primers were used:

HERV subgroup-specific *pol* primers	**Orientation**	**Oligonucleotide sequence (5'→3')**
S71pCRTK-1 (HERV-T)	forward	GTACCCCAGGTAGGAAACTCTGGG
	reverse	CCCCTACCCTTTTTGGGG
E4-1 (HERV-E)	forward	GCTTTCTTTCTGATCCTAGGCTGTG
	reverse	CTTTGGGGAGGCGTTGGCTCGAGACC
ERV-9	forward	CCTCAACTGTTTTAATGTCTTAGGGCGAGG
	reverse	CCCTCATCTGTTTGGTCAGGCCC
Seq59 (ERV-9)	forward	GTGCTGAGGGCCCTGGTTCCTCTGG
	reverse	GTGCTGAGGGCCCTGGTTCCTCTGG
HERV-KC4 (HML-10)	forward	GAATCTCTTCTAATTTGAACCTTTTGAGG
	reverse	GAATCTCTTCTAATTTGAACCTTTTGAGG
HERV-K (HML-2)	forward	GGCCATCAGAGTCTAAACCACG
	reverse	CTGACTTTCTGGGGGTGGCCG
HIV-1	forward	GTTCATAACCCATCCAAAGGAATGGAGG
	reverse	CCAAAGTAGCATGACAAAAATC

In general, HERV-specific primers for LightCycler real-time RT-PCR were designed in such a way that for each HERV one primer matched the capture probe sequences used in the corresponding microarray experiments, whereas the second primer was located 100 to 150 base pairs upstream of the first primer [113]. Quantitative real-time RT-PCR was performed with the Roche LightCycler 480 System, using LC480 DNA Master SYBR Green and standard LightCycler protocol (Roche Diagnostics, Mannheim). Real-time RT-PCR experiments for each gene were performed in triplicate. Cycling conditions on a Roche LightCycler 480 (LC 480) were as follows: initial denaturation step at 95°C for 10 minutes, and then 45 cycles at 95°C for 10 s, 60°C for 5 s, and 72°C for 10s. After 45 cycles, melting curves were generated for the final PCR products by decreasing the temperature to 65°C for 15 s followed by an increase in temperature to 95°C. Fluorescence was measured at 0.2°C increments. RNA-Polymerase II-transcripts (RPII) were analyzed as internal standard, using primers given in [114]. ΔC_T -values are calculated as follows C_T(RPII)-C_T(HERV-element) and were normalized to RPII levels (=15). The x-fold induction of HERV expression in HIV-1 infected cells was calculated by the $2^{-\Delta\Delta C}{}_T$ method [115], with values normalized to RPII and relative to expression in non-infected cells. Furthermore, extensive standardization of PCR reactions was initially performed through melting curve analysis of respective amplicons in order to minimize primer pair formation (data not shown). The relative expression ratio of HIV-1 transcripts and Rev-reporter RNAs was calculated from the real-time RT-PCR efficiencies and the crossing point deviations of the target gene versus the house keeping gene RPII (RNA polymerase II), as described in [116]. The following primers were used for the reporter transcripts: 5' RRE: 5'-CGAGCTCGGTACCCCAAGGCAAAGAGAAGAGT GG-3'; 3' RRE: 5'-CAATAGCCCTCAGCAAATTGTTCTGCTGC-3'). Amplification lead to a 174 bp long product, which represents the first half of the RRE sequence. For the HIV-1 transcripts following primers were used: late HIV-1 transcripts (forward primer 5'-GCCCCTCCCATCAGTGGAC-3'; reverse primer 5'-GCCTTGGTGGGTCGTACTCCTAATGG -3'), early HIV-1 transcripts (forward primer 5'-CTCTATCAAAGCAACCCACCTCCCAA-3'; reverse primer 5'-GCGGTGGTAGCTGAAGAGGCACAGG -3'). All real-time RT-PCR experiments were done as triplicates from three independent RNA preparations.

2.2.2.12. Amplification, cloning, and sequence analysis of HML-2 transcripts.

For the amplification of HML-2 transcripts, PCR was performed using 2 µl of undiluted cDNA from cells infected for three weeks with a HIV-1 patient isolate (LC5-CD4acute) and uninfected cells (LC5-CD4). HERV-K(HML-2) specific primers [117] were used to generate *gag* gene-derived PCR products: gag_plus (5'-GGCCATCAGAGTCTAAACCACG-3') and gag_minus (5'-GCAGCCCTATTTCTTCGGACC-3'). The 50 µl PCR mix contained 1x Expand High Fidelity buffer with $MgCl_2$, deoxynucleotide mix at 0.2 µM each, and 2.6 U expand High Fidelity enzyme mix (Roche Diagnostics, Mannheim). PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 1 min, annealing at 57°C for 45 s, and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. HML-2- specific *gag* PCR products were purified (NucleoSpin Extract II, Macherey-Nagel, Düren) and cloned into the pGEM®-T Easy vector (Promega) and transformed into TOP10F' bacterial cells. Plasmid DNA was isolated from insert-containing colonies according to the manufacturer's protocol (NucleoSpin Plasmid, Macherey-Nagel, Düren). Subsequently, cloned HML-2 cDNAs were analyzed by sequencing (Institute for Immunology und Genetics, Kaiserslautern, Germany) and mapped to their respective genomic loci. For the latter procedure, we used the BLAT tool at the Human Genome Browser database with cloned cDNA sequences as probes to search the March 2006 version of the human genome.

2.2.3. Biochemical methods

2.2.3.1. Western-Blot

Whole cell lysates were prepared with lysis-buffer and separated on precast 4-12% Bis-Tris gradient acryl-amid gels (Invitrogen) and transfered onto nitrocellulose membranes (Biorad, Munich, Germany). Risp peptide antibodies were generated by Peptide Specialty Laboratories. Peptide sequences were selected by the following parameters: hydrophilicity; surface probability; chain flexibility; secondary structure according to Chou-Fasman. Protein bands were detected by an enhanced chemiluminescence system (Perbio Science, Bonn, Germany).

2.2.3.2. p24-antigen analysis

Quantification of p24 antigen was performed using the Coulter HIV-1-p24-Antigen-Assay (Beckman Coulter). Five % Triton-X was added to the whole-cell extracts and the supernatant, centrifuged 5 minutes for 13.000 rpm at room temperature and diluted with PBS to a final concentration of 0.5 % Triton-X. The p24 Elisa was performed according to the manufacturer's protocol.

2.2.3.3. Analysis of brain tissue expression patterns

Frozen brain tissue samples were obtained from the Edinburgh HIV Brain and Tissue Bank (http://www.hivbank.ed.ac.uk/). Tissue samples were derived from the frontal and parietal lobes of two individuals (both age 30) with HIV encephalitis and one HIV negative individual (age 22). Neither of the AIDS subjects had received effective combination therapy, their deaths having occurred before 1995. Brain tissue samples were stored at –80°C from the time of autopsy. Brain tissues were investigated by Western-Blot analysis.

3. Results

Several studies suggest that HIV-1 infection can influence expression of members of the HERV-K family [72,73,74,118,119]. The present study was initiated to determine if HIV-1 infection could influence HERV expression on a more general level. Therefore I investigated the comprehensive HERV expression profile in various HIV-1 infected cell lines using a retrovirus-specific DNA chip based on a highly conserved region within the *pol* gene [113] (described in section 2.2.2.10).

3.1. Several HERV families are activated in persistently HIV-1 infected cells

To investigate the influence of HIV-1 infection on the transcription of human endogenous retroviruses, I used three persistently HIV-1 infected cell-lines TH4-7-5 (astrocytes), LC5-HIV (a HeLa-derived subclone) and KE37/1-IIIB (T-lymphoma cells). I chose these cell lines, because they represent different cellular backgrounds and differ in their levels of HIV productivity.

To analyze the expression profiles of various HERV families in these three HIV infected cell lines I performed microarray analysis. The original uninfected cell lines 85HG66 (astrocytes), LC5 (HeLa cells) and KE37/1 (T-lymphoma cells) corresponding to each infected cell line were used as controls.

3.1.1. HIV-1 production levels of the three persistently infected cell lines

First, the infected cell lines were investigated for their ability to produce viral proteins and virus, respectively. Intracellular (cell lysate) and extracellular (supernatant) samples of the cell lines after 24 h virus production were used to analyze the amount of the capsid protein p24 with a Gagp24 ELISA (Figure 3.1/A-B). Gagp24-protein was detected in all infected cell lines (Figure 3.1/A-B), but not in the uninfected control cell lines. Furthermore, it could be shown that the three cell lines TH4-7-5, LC5-HIV and KE37/1-IIIB differ in their Gagp24 production level. TheTH4-7-5 showed the lowest Gagp24 production. The LC5-HIV and the KE37/1-IIIB showed an approximately 10 to 40-times higher Gagp24 release than TH4-7-5 cells. TH4-7-5 cells were previously shown to contain one provirus copy per cell [39]. Real-time RT-PCR confirmed this result. The KE37/1-IIIB cell line contains about 6 proviral copies per cell (Figure 3.1/C). Less than one provirus per cells was detected in the LC5-HIV cell population, suggesting that not all cells of the examined population contain the HIV-1 provirus.

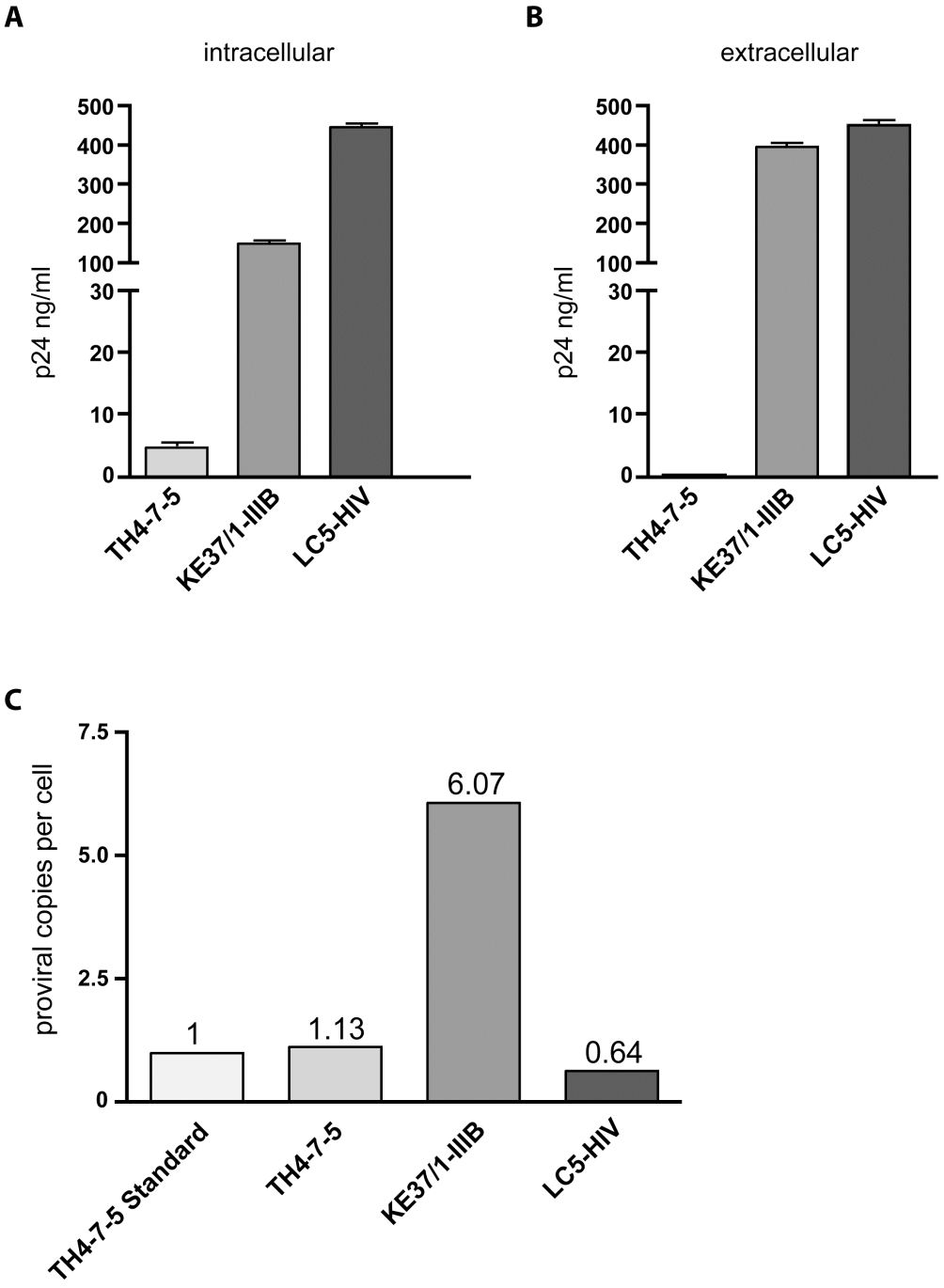
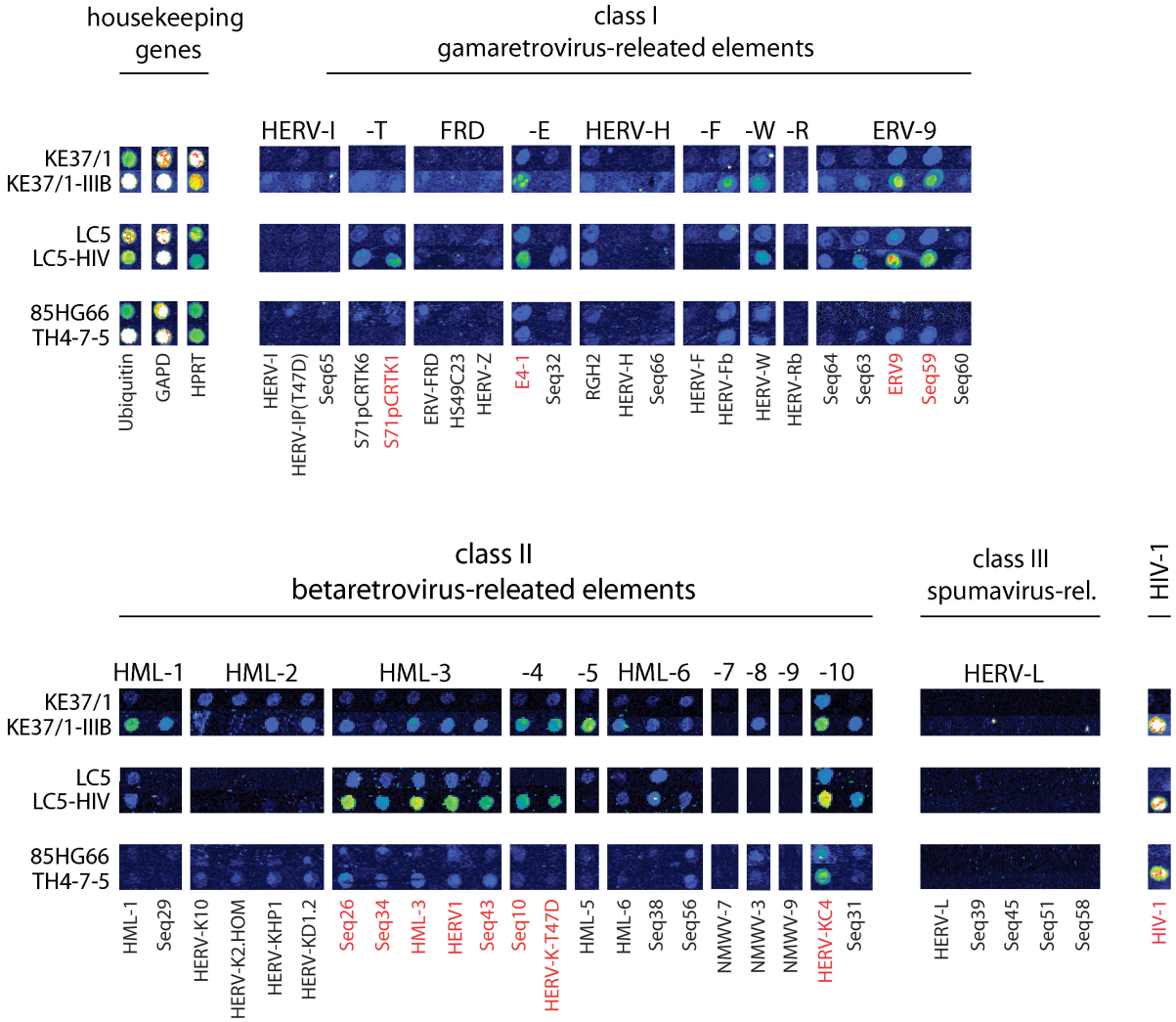


Figure 3.1.: Virus production and provirus copy numbers of different persistently HIV-1 infected human cell lines.
(A) Intracellular Gagp24-production in the infected cell lines TH4-7-5, KE37/1-IIIB and LC5-HIV. (B) Extracellular Gagp24-released by the infected cell lines TH4-7-5, KE37/1-IIIB and LC5-HIV. Gagp24-production was measured using a Gagp24 ELISA. The mean value and standard deviations of triplicate measurements are indicated. (C) The number of proviral DNA copies per cell measured with quantitative real-time RT-PCR.

3.1.2. HERV transcription profiles of three persistently HIV infected cell lines

For microarray analysis HIV-1 infected and uninfected samples were tested in triplicate microarray hybridization assays, according to a standardized chip hybridization protocol [70,120] (see section 2.2.2.10). A digitally processed alignment of a representative data set of infected and uninfected samples is shown in Figure 3.2. The microarray represents all three classes of HERVs (Figure 1.5). It contains 52 representative HERV *pol*(RT)-derived sequences from 18 major HERV families, all of which include at least one full-length provirus in the human genome [44]. Three housekeeping genes served as internal controls for RNA quality. The HIV-1 capture probe served as a positive control to show that all infected cell lines (KE37/1-IIIB, LC5-HIV, TH4-7-5) are HIV-1 positive and all uninfected cells (KE37/1, LC5, 85HG66) are HIV-1 negative. In all infected cell lines several HERV families could be identified that show higher expression levels in infected cells compared to uninfected control cells. Members of at least 6 HERV families, HERV-T, HERV-E, ERV-9, HML-3, HML-4 and HML-10, belonging to class I and II HERVs were found to be upregulated in HIV-1 infected cells (marked red in Figure 3.2). The more ancient class III HERV elements remained unaffected in all cell lines. As expected the HIV-1 capture probe showed positive signals in all three infected cell lines and no signal in the uninfected control cell lines.

Figure 3.2. (next page): Retrovirus specific DNA-microarray to investigate the expression profile of different HERV-families in persistently HIV-1 infected cell lines.
Three HIV-1 infected cell lines were compared with the corresponding uninfected cell line. The housekeeping genes serve as a quality control and consequently as an internal standard. The microarray contains probes for detection of HERV families from all three HERV classes (I, II, III). HIV-1 oligonucleotides are also spotted on the chip as a positive control for HIV-1 expression by HIV-1 infected cells. The members of at least 6 HERV families HERV-T, HERV-E, ERV-9, HML-3, HML-4 and HML-10 (marked in red) were found to be upregulated in persistently HIV-1 infected cell lines. A representative data set from three independent experiments is shown (three independent RNA isolations). False color mapping was used for image visualization.



Interestingly, the microarray analysis suggests differences between the HERV expression profiles of the three infected cell lines (Figure 3.3) Thus LC5-HIV and KE37/1-IIIB cell lines show higher levels of expression of the HERV groups S71pCRTK-1 (HERV-T), E4-1 (HERV-E), ERV9, Seq59 (ERV9), Seq10 (HML-4), HERV-K-T47D (HML-4), HERV-KC4 (HML-10) and the five groups of the HML-3 family, Seq26, Seq34, HML-3, HERV1 and Seq43 than TH4-7-5 cells (marked red in Figure 3.3).

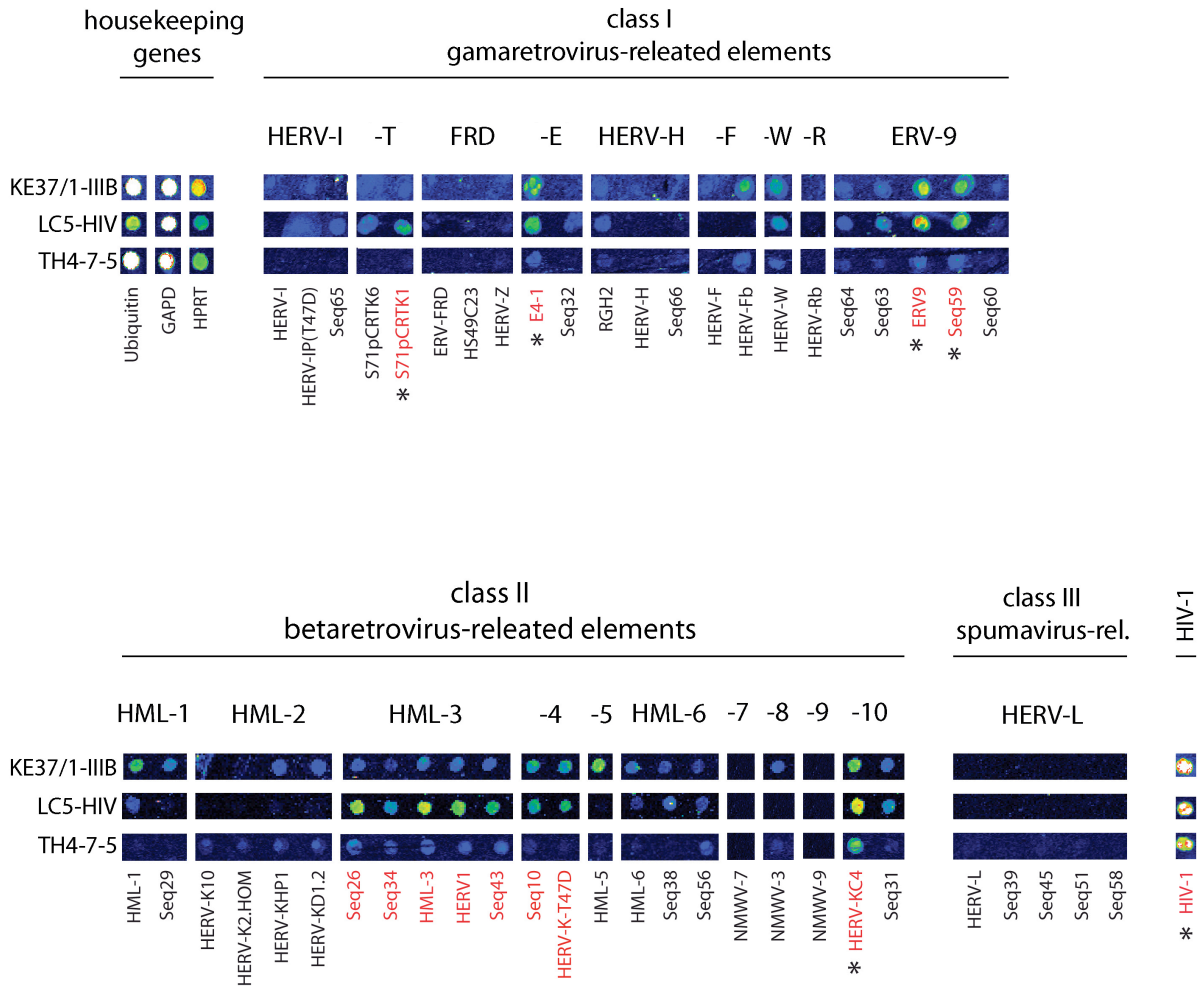


Figure 3.3.: Comparison of the HERV expression profiles of three HIV-1 infected cell lines. Expression of HERV families S71pCRTK-1 (HERV-T), E4-1 (HERV-E), ERV9, Seq59 (ERV9), Seq10 (HML-4), HERV-K-T47D (HML-4), HERV-KC4 (HML-10) and the five groups of the HML-3 family are higher in KE37/1-IIIB and LC5-HIV cells than in TH4-7-5 cells (marked in red). HERV groups validated by real-time RT-PCR are marked with asterisks "*". A representative data set from three independent experiments is shown. The data represent a subset of the data from Figure 3.2. False color mapping was used for image visualization.

3.1.3. Validation of HERV chip data by real-time RT-PCR

Five HERV groups S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4 differentially expressed in infected and uninfected cells as determined by microarry analysis (marked by asterisks * in Figure 3.3) were selected and subsequently analyzed by real-time RT-PCR to confirm the microarray results and to quantify differences in their expression in infected and non-infected cells. Individual primers were designed that bind specifically in the *pol* region of each of the 5 upregulated HERV groups.

These primers are located in a segment of the reverse transcriptase genes that exhibits only marginal homology among HERV-taxa and were selected in a way that the amplicons overlap with the corresponding microarray capture probe sequences. Figure 3.4 shows the relative expression ($2^{-\Delta\Delta C_T}$) of the HERV groups S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4 in the HIV-1 infected cells compared to the uninfected control cells. The data were normalized to RNA Polymerase II (RPII) transcript levels, as this gene has been shown to be stably expressed under a variety of conditions and after exposure to various stimuli [121,122].

HIV-1 served as a positive control. Using HIV-1 specific primers, it could be shown that the infected cell lines produce specific HIV-1 transcripts (Figure 3.4). The TH4-7-5 cells produced lower levels of HIV-1 transcripts than the other persistently infected cell lines, in agreement with the differences in HIV-1 production levels between these cell lines (Figure 3.1). The HERV groups S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4 showed higher expression in the HIV-1 infected cells than in unifected cells, a result consistent with the microarray data (Figure 3.2 - 3.3).

Interestingly, upregulation of expression of several HERVs (S71pCRTK-1, E4-1, ERV9) was more pronounced in the cell lines that produced higher levels of HIV (i.e. LC5-HIV, KE37/1-IIIB) than in the cell line with limited HIV-1 production (TH4-7-5). This suggests that the expression levels of selected HERVs may be related to HIV-1 production levels. This hypothesis is explored in more detail in the next section.

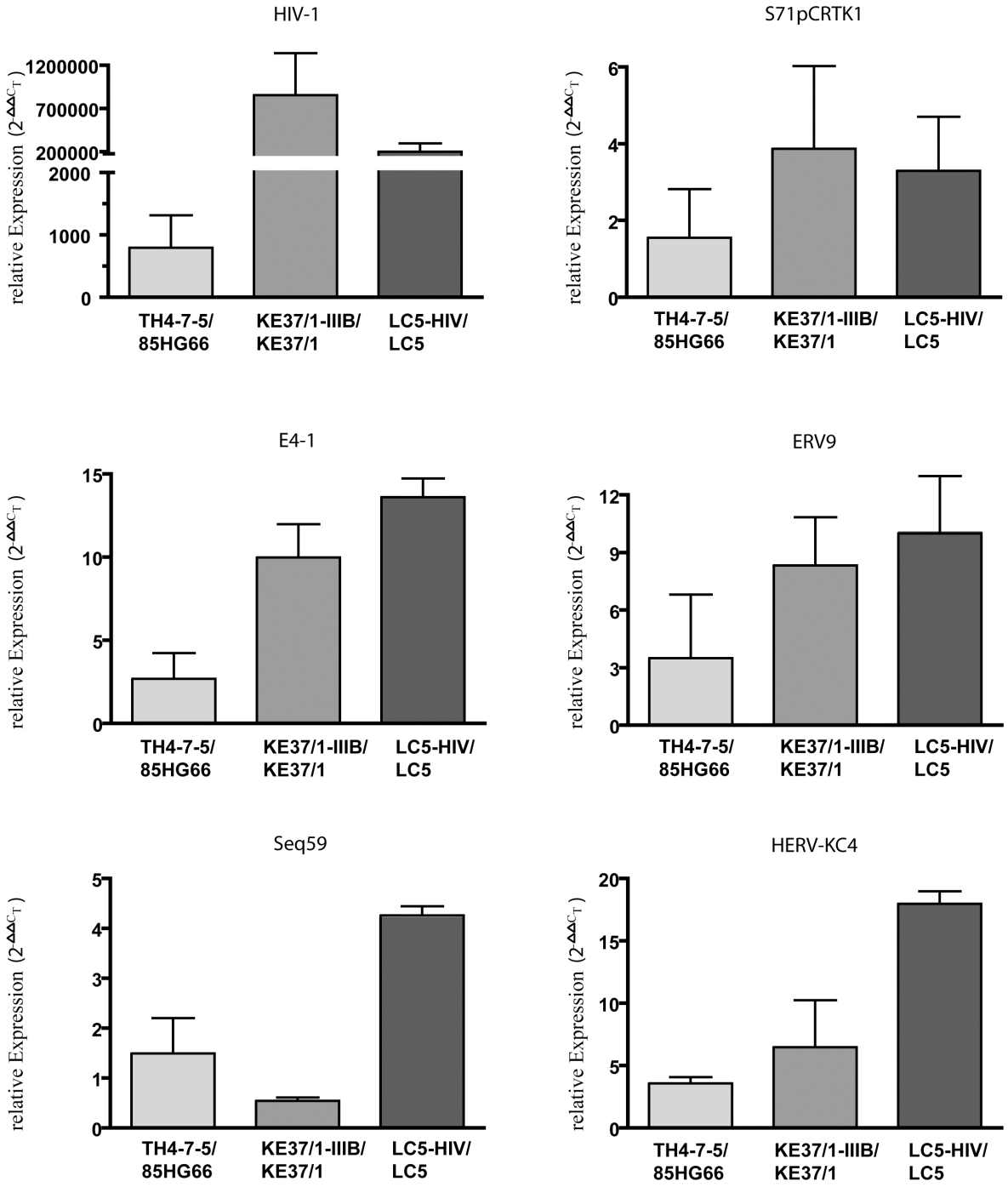


Figure 3.4.: Relative expression of HERV elements and HIV-1 transcripts in the HIV-1 infected cells referred to the uninfected cells.
Quantitative real-time RT-PCR results for HIV-1, S71pCRTK-1 (HERV-T), E4-1 (HERV-E), ERV9, Seq59 (ERV9) and HERV-KC4 (HML-10) are indicated. Retroviral transcripts were normalized to RPII expression levels. The Y-axis shows the x-fold relative expression of the HERV-transcripts (S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4) and HIV-1 transcripts in the infected cells referred to the uninfected cells. The mean values and standard deviations are indicated for triplicate experiments (three independent RNA isolations).

3.2. HIV-1 specific siRNAs reverse HERV upregulation

3.2.1. Experimental proof of the selected siRNAs

To demonstrate that the observed alterations are indeed associated with HIV-1 expression, I investigated if silencing of HIV-1 transcription could reverse the upregulation of HERV expression. I addressed this question using RNA interference assays. The siRNAs against HIV-1 transcripts were selected according to ter Brake and Berkhout [123] and their functionality was tested in initial experiments.

An overview of the HIV-1 transcripts, which are targeted by the chosen siRNAs, is shown in Figure 3.5. The siRNA sigag is predicted to reduce levels of the unspliced *gag* and *pol* transcripts, sitat/rev all transcripts except transcripts encoding for *nef* and four transcripts encoding for *vpu/env* and sienv together with sinef all HIV-1 transcripts. HIV-1 contains three major RNA splice variants encoding for all 15 proteins. These RNAs harbor overlapping sequences regarding the different RNA species (see Figure 3.5) making it impossible to design siRNAs that are unique for knockdown of individual proteins. A non-silencing siRNA was used to control for unspecific effects.

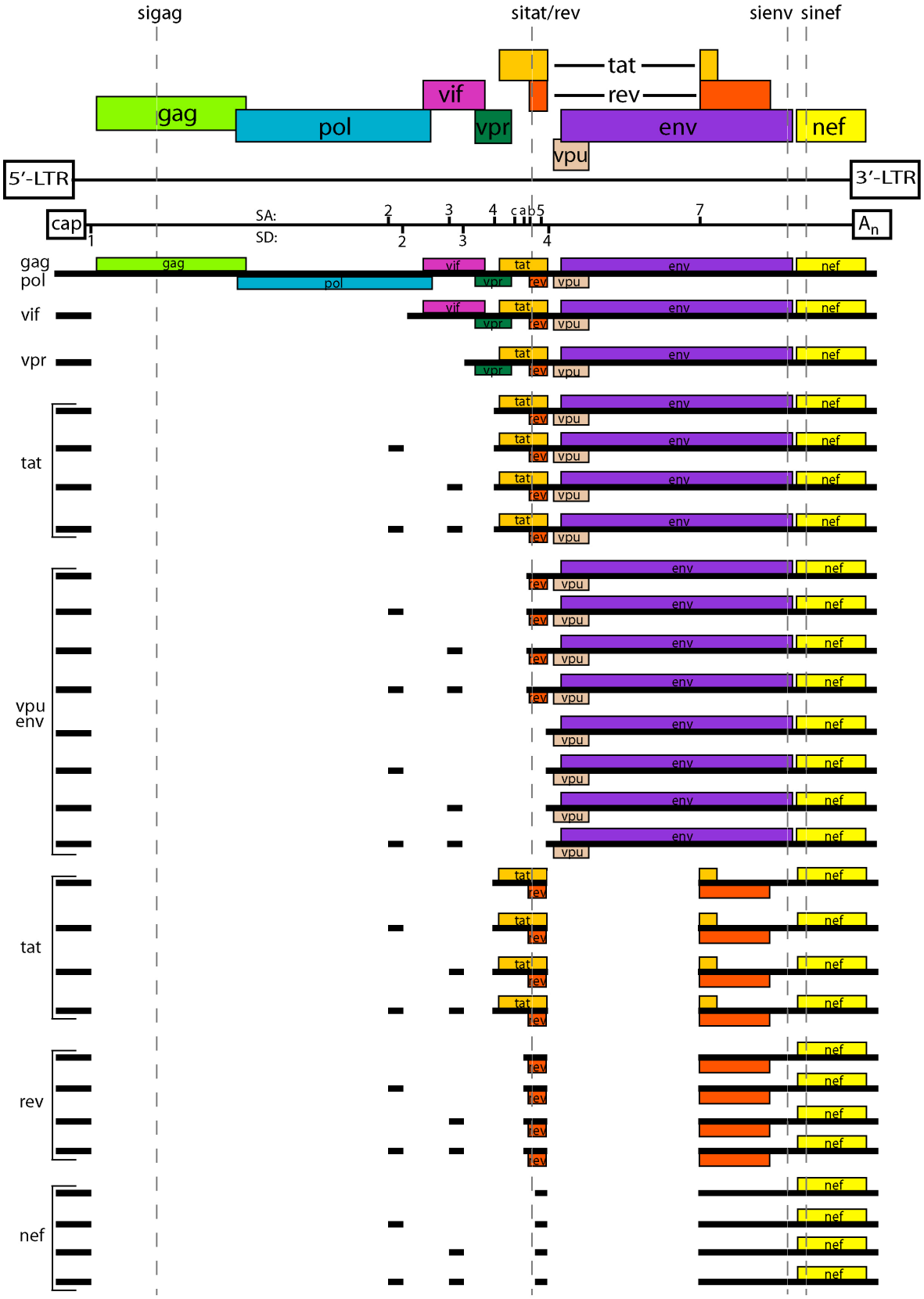


Figure 3.5.: Overview of siRNAs used to inhibit HIV-1 expression.
The Figure illustrates the positions of the sequences targeted by the chosen HIV-1 specific siRNAs in HIV-1 transcripts.

The siRNA functionality was tested in LC5-HIV cells. Levels of the capsid protein Gagp24 were measured in extracellular (supernatant) and intracellular (cell lysate) samples of the cells 72 h after siRNA transfection. Gagp24 levels were substantially lower in all samples of cells treated with siRNAs against HIV-1 transcripts compared to cells treated with the non-silencing control siRNA (Figure 3.6).

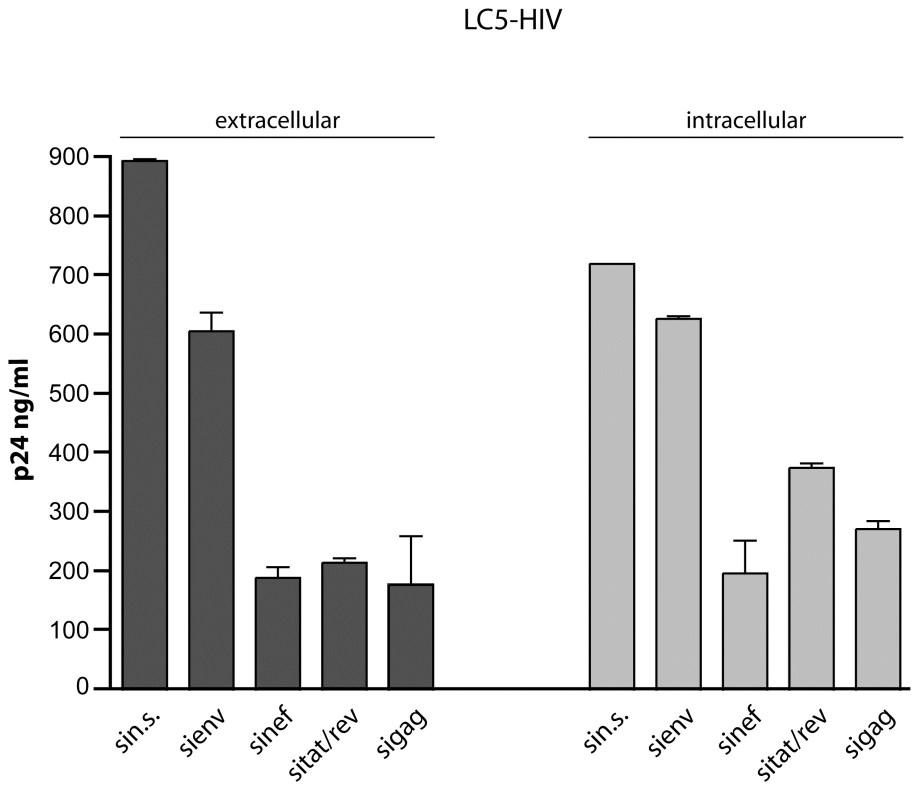


Figure 3.6.: Gagp24 production by persistently HIV-1 infected LC5-HIV cells treated with siRNAs that target various HIV-1 transcripts.
The figure shows the extracellular and intracellular levels of Gagp24 production of LC5-HIV cells treated with specific HIV-1 siRNAs. Gagp24 production was measured using Gagp24 ELISA. The left panel shows the extracellular Gagp24 production, the right panel the intracellular Gagp24 production after treatment with HIV-1 specific siRNAs. Mean values and standard deviations are shown for triplicate experiments.

3.2.2. HERV transcription profiles after HIV-1 knockdown

cDNAs from cells treated with siRNAs were investigated with the HERV DNA chip. Figure 3.7 illustrates the results of the DNA-microarray analyses. A non-silencing siRNA (n.s. siRNA) was used as a control for unspecific silencing effects by siRNAs in general.

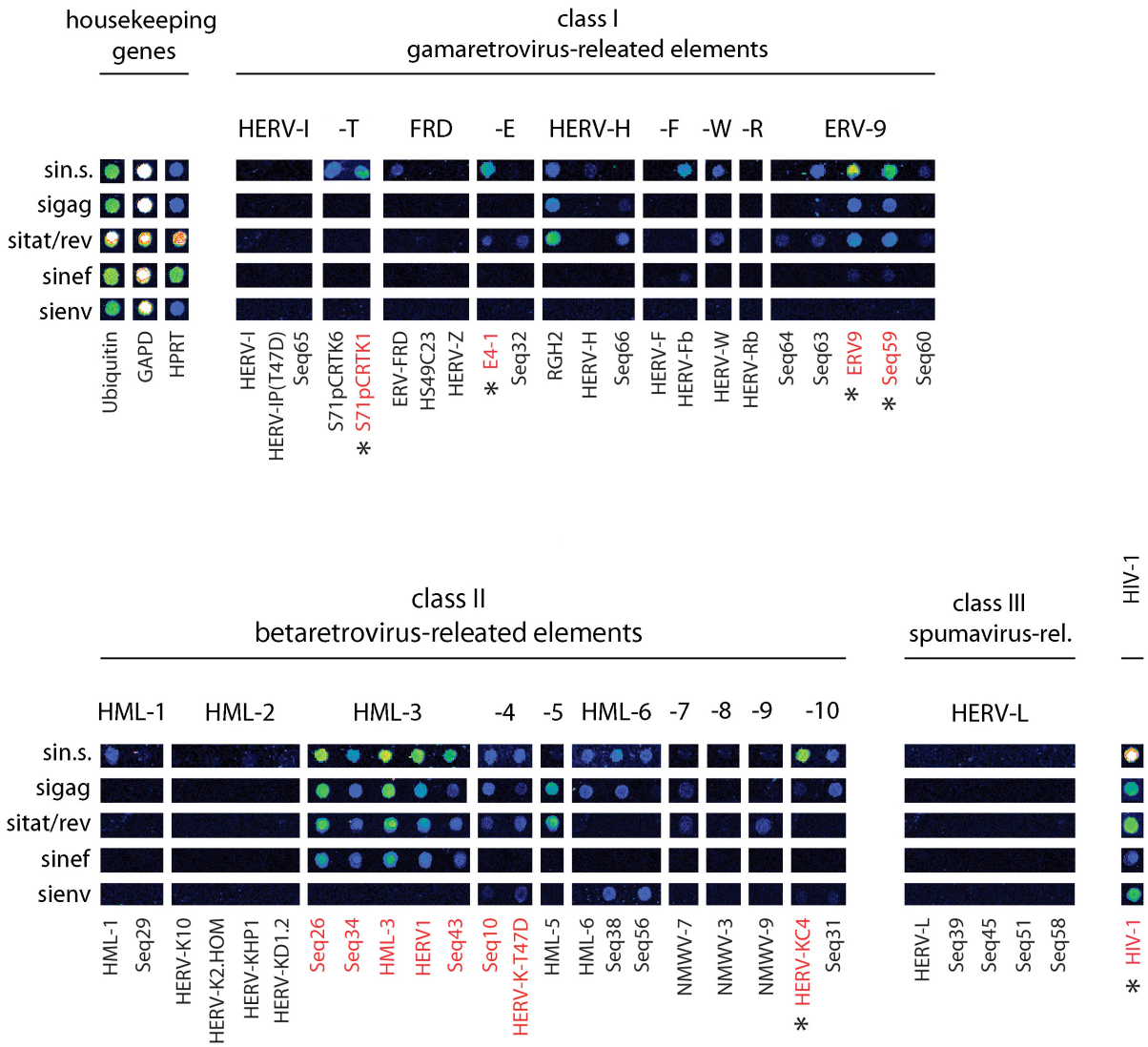
Expression of the 12 HERV groups S71pCRTK-1, E4-1, ERV9, Seq59, Seq26,

Seq34, HML-3, HERV1, Seq43, Seq10, HERV-K-T47D and HERV-KC4 (Figure 3.7 marked in red) was reduced in LC5-HIV cells treated with HIV-specific siRNAs, compared to cells treated with non-silencing siRNAs. Infected cells that were transfected with siRNAs against *gag* or *tat/rev* showed reduced HERV expression for ERV9 and Seq59, both subgroups of the ERV9 family, compared to the non-silencing control. Also the HERV expression for Seq10 and HERV-K-T47D, two subgroups of the HML-4 family was reduced. In the case of E4-1 and HERV-KC4 no HERV expression could be detected in the sigag or sitat/rev treated cells. After siRNA treatment with sinef or sienv no HERV expression was detectable for E4-1, ERV9, Seq59, Seq10, HERV-K-T47D and HERV-KC4. Also five HERV groups of the HML-3 family (Seq26, Seq34, HML-3, HERV1 and Seq43) showed reduced expression after siRNA treatment with sienv.

These results indicate that activation of HERV-groups can be reversed by siRNAs that target HIV-1 transcripts and therefore depends on HIV-1 expression.

Figure 3.7. (next pape): Down-regulation of HIV-1 induced HERV activity by siRNAs targeting HIV-1 transcripts.

LC5-HIV cells were treated with non-silencing siRNAs (sin.s.) or with the siRNA against HIV-1 transcripts (sigag, sitat/rev, sinef and sienv). Cells transfected with the siRNA against HIV-1 showed reduced HIV-production. A diminished HERV expression for S71pCRTK-1, E4-1, ERV9, Seq59, five groups of the HML-3 family, two goups of the HML-4 family and HERV-KC4 was observed compared to cells treated with non-silencing siRNAs (marked in red). HERV groups validated by real-time RT-PCR are marked with asterisks “*”. A representative data set for three independent experiments is shown (three independent RNA isolations). False color mapping was used for image visualization.



3.2.3. Quantification of HERV transcripts after HIV-1 knockdown

To confirm the reduction of HERV expression detected by microarray analysis after reduction of HIV-1 expression, I carried out real-time RT-PCR. Again the 5 HERV groups S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4 were investigated (marked by asterisks * in Figure 3.7). Figure 3.8 shows the relative expression ($2^{-\Delta\Delta C}{}_T$) of the HERV groups in the HIV-1 infected HeLa cells (LC5-HIV) treated with specific siRNAs against *gag*, *tat/rev*, *nef* and *env* compared to the non-silencing control cells. The data were normalized to the housekeeping gene RNA Polymerase II (RPII). HIV-1 served as a positive control to monitor reduction of HIV-1 expression after RNAi. Specific RNAi against the HIV-1 transcripts *gag*, *tat/rev*, *nef* and *env* led to diminished expression of HIV-1 as shown in Figure 3.8. Knockdown of HIV-1 in the LC5-HIV cells by all siRNAs resulted in a reduction of the expression of the HERV-

groups S71pCRTK-1, E4-1, ERV9, Seq59 and HERV-KC4. Only expression of Seq59 elements were less well diminished by siRNAs against *env*. Thus, it could be shown that activation of HERV-elements in HIV-1 infected cells is abrogated after treatment with siRNAs against different HIV-1 transcripts.

All together, these data indicate that activation of expression of several HERV families is linked to HIV-1 expression.

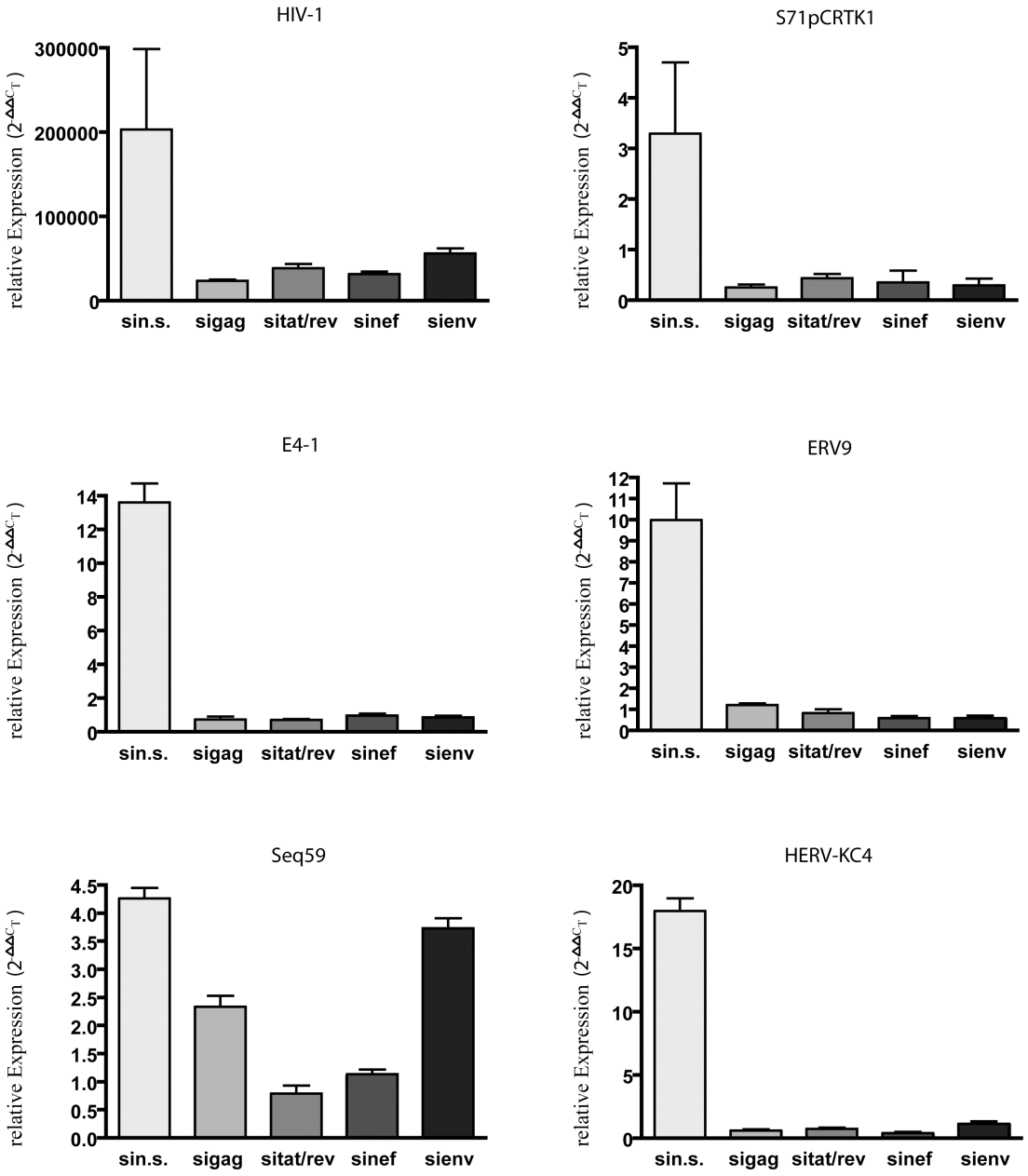


Figure 3.8.: Effects of HIV-1 knockdown on the expression of HIV-1 induced HERVs in HIV-1 infected cells.

HERV transcript levels were quantified by real-time RT-PCR, using RNA polymerase II as standard. The y-axis shows the x-fold relative expression of the HERV-transcripts and HIV-1 transcripts in HIV-1 infected (LC5-HIV) siRNA treated cells referred to the uninfected control cells (LC5) treated with the same siRNA. Mean values and standard deviations of three triplicate experiments are shown (three independent RNA isolations).

3.3. Identification of cellular Rev-interacting HIV suppressors (Risp)

In the previous section I demonstrated that reduction of HIV-1 transcript levels by artificial inhibitors (i.e. siRNAs) could influence HERV expression. Here I set out to analyze whether natural host factors that influence HIV production affect expression of HIV-induced HERVs. To this end, I focused on a protein (16.4.1) encoded by a truncated cDNA that interacts with the HIV-1 Rev protein, the major regulator of HIV replication (elucidated in the Introduction). 16.4.1 was demonstrated to be capable of inhibiting Rev activity, suggesting that it could influence HIV replication.

My aim was to identify native proteins containing the 16.4.1 region, which I called Risp, and to investigate whether they can influence HIV replication levels in persistently HIV-1 infected cells. Finally I examined if modulation of replication efficiencies by Risp also affects HERV expression.

3.3.1. Discovery of native Risp proteins in human brain tissue samples and astrocytes

To identify native proteins with the 16.4.1 region I searched the UniProtKB database and found the 16.4.1 sequence in multiple predicted proteins of the closely related FAM21 family. To further characterize the expression of endogenous Risp proteins, I used antibodies against various Risp peptide sequences located outside the 16.4.1 region in FAM21 proteins (Figure 3.9/A) as well as antibodies against a bacterial recombinant 16.4.1 protein. These pooled Risp peptide antibodies precipitated proteins of different sizes from human cell lysates. Subsequently, the pooled Risp peptide antibodies were used for immuno-detection of Risp proteins in human primary brain tissues (Figure 3.9/B), primary human fetal astrocytes (Figure 3.9/C) and in established brain-derived cell lines (Figure 3.10) by Western blot analysis.

In human brain tissues, expression of Risp proteins was detected in samples from uninfected individuals and from individuals with HIV encephalitis derived from different regions of the brain (Figure 3.9/B). This suggests that Risp proteins are ubiquitously expressed in the human brain. Expression of Risp proteins in human astrocytes was demonstrated for human primary astrocytes (Figure 3.9/C), for astrocytes generated by differentiation of human HNSC.100 neural progenitor cells (Figure 3.10/A), for U138MG astrocytic cells (Figure 3.10/B) and for persistently HIV-1 infected astrocytic cells TH4-7-5 (Figure 3.10/C).

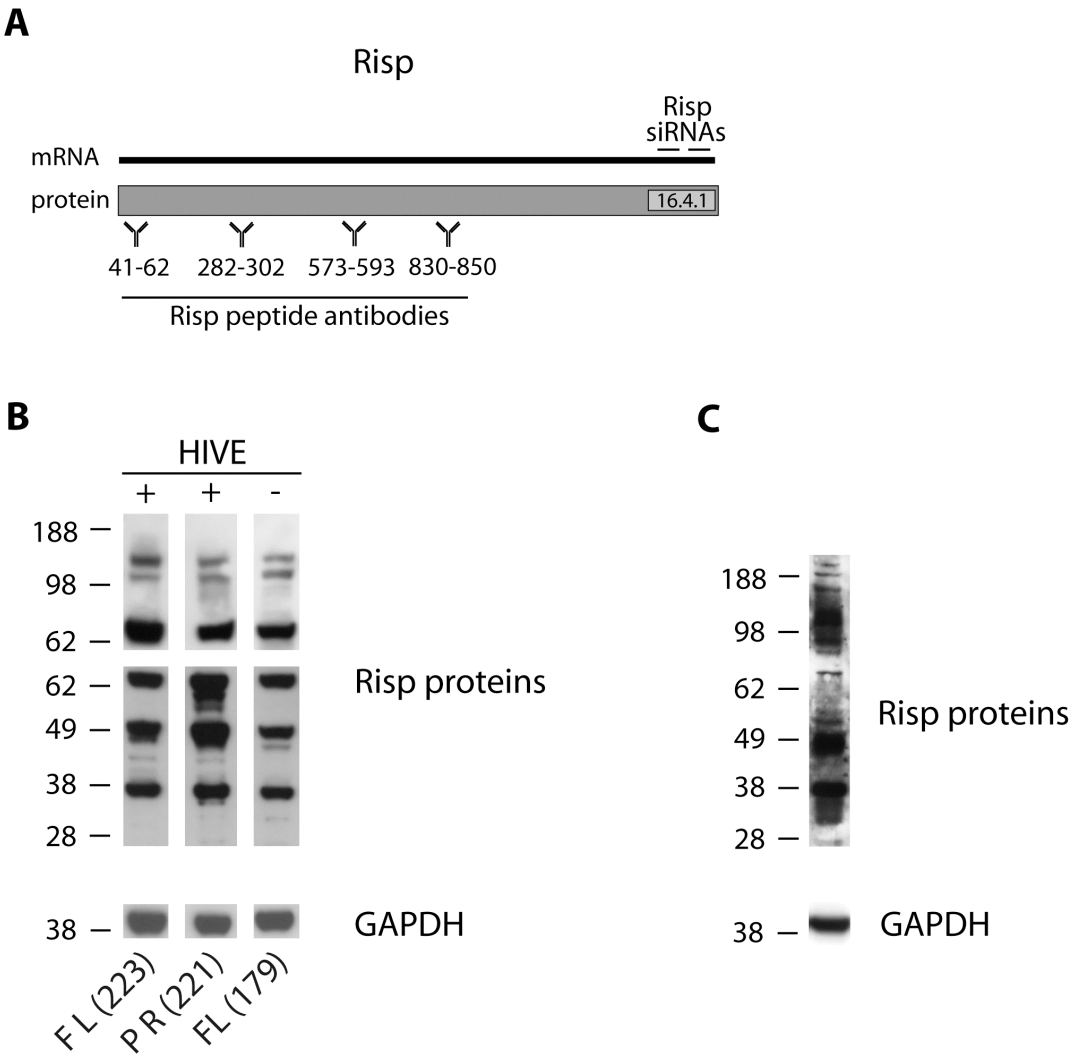


Figure 3.9.: Detection of Risp proteins in human brain tissue samples and in human primary astrocytes.
Antibodies for recognition of Risp proteins were generated by immunizing rabbits with synthetic peptides. Peptide sequences represented four regions of multiple predicted FAM21 proteins in the UniProtKB database. Western blot analyses were performed with pooled antibodies.
(A) Schematic of the location of the peptide sequences and of the Rev-interacting 16.4.1 region.
(B) Detection of Risp in samples from the frontal (FL) or parietal (PR) lobes of brain tissues from two individuals (223, 221) with HIV encephalitis (+HIVE) and from one individual (179) negative for HIV (-HIVE).
(C) Detection of Risp proteins in lysates of human primary fetal astrocytes.

While some variations were observed in the Risp protein expression patterns from different neural tissue/cell sources, a total of 7 protein bands were detected in all brain tissues and astrocyte lysates with the pooled Risp peptide antibodies and anti-16.4.1 antibodies (Table 3.1).

protein sizes detected in Western Blot analysis (kDa)	brain tissue +HIV	primary human astrocytes	U138MG	TH4-7-5
188	–	+	+	+
150	+	+	+	+
110	+	+	+	+
90	–	+	+	+
64	+	+	+	+
49	+	+	+	+
46	–	+	+	+
45	+	+	+	+
40	+	+	–	+
38	+	+	+	+
30	+	+	+	+

Table 3.1.: Overview of Risp proteins detected in all brain tissues and astrocytic cell lysates. Displayed are the eleven Risp proteins detected in all bran tissues and astrocytic cell lysates. Grey shading labels Risp proteins detected with antibodies against Risp peptides and with antibodies against recombinant 16.4.1 protein.

To further confirm that these proteins were *bona fide* Risp proteins, I analyzed the influence of 16.4.1-specific siRNAs on the Risp expression pattern in U138MG cells. As demonstrated in Figure 3.10/B, transfection of U138MG cells with 16.4.1-specific siRNAs diminished production of all Risp proteins, while unspecific siRNAs had no effect on the Risp expression pattern.

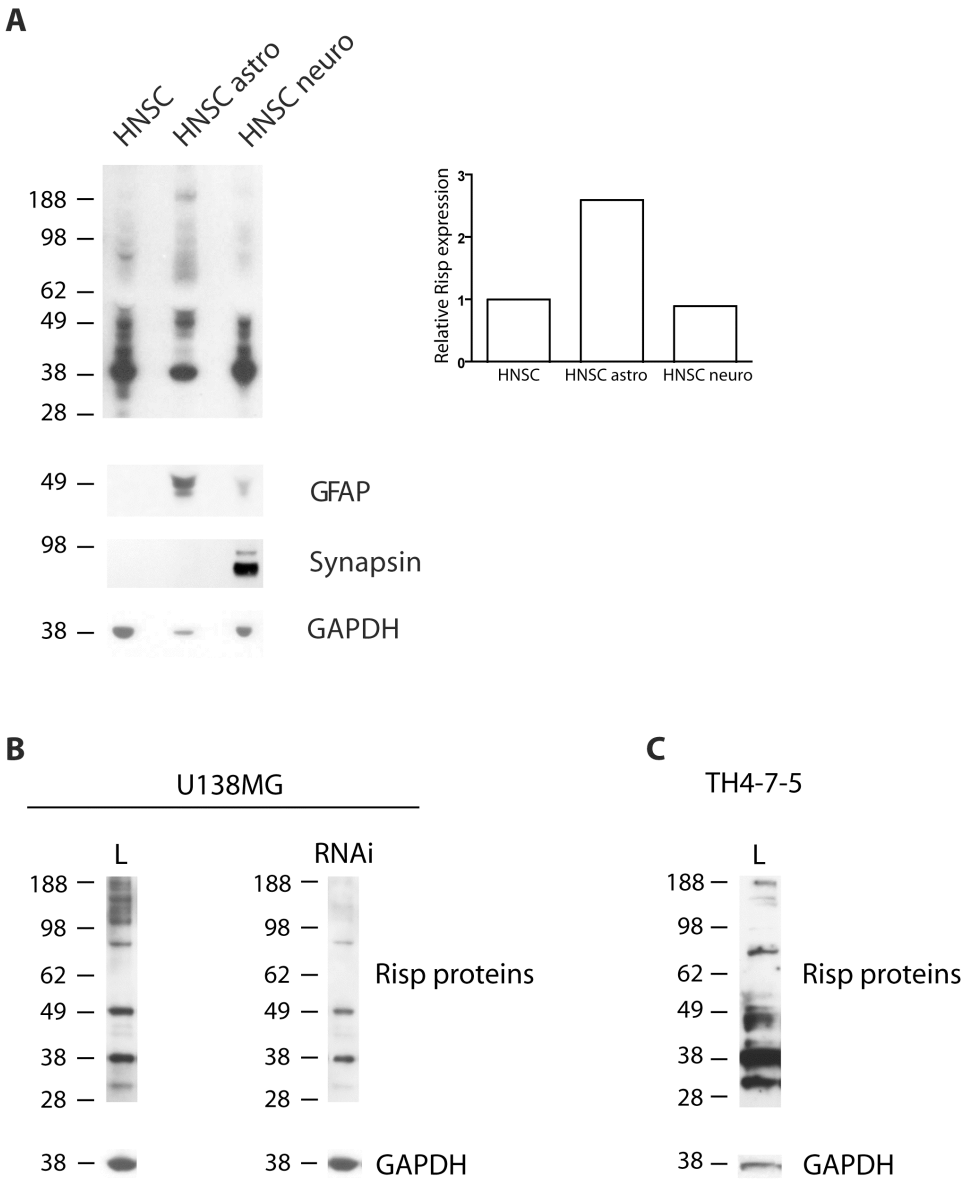


Figure 3.10.: Risp expression patterns in the human brain-derived cell lines (HNSC.100, U138MG and TH4-7-5).
Risp expression was probed by Western blot analysis of cell lysates with pooled Risp peptide antibodies.
(A) Risp expression patterns in cells of the human neural stem cell line HNSC.100 cultured under different conditions. HNSC.100 cells were cultured either as undifferentiated progenitor cells (HNSC) or under conditions that promote differentiation to astrocytes (HNSC astro) or to neurons (HNSC neuro). Upregulated expression of GFAP (glial fibrillary acidic protein) or synapsin confirmed generation of HNSC.100 astrocytes and neurons, respectively. The column graph at the right shows the changes in Risp expression levels in differentiated HNSC.100 cultures relative to undifferentiated progenitor cells (= 1), as quantified by densitometry of Western blot signals. The total intensity value of Risp signals was normalized to the signal intensity of the GAPDH (Glyceraldehyd-3-phosphate dehydrogenase) control protein. Risp expression increased over 2-fold in differentiated cultures enriched for astrocytes, compared to differentiated cultures enriched for neurons. (B) Risp expression in astrocytic U138MG cells. The left panel shows expression of Risp proteins in lysates (L) of U138MG cells treated with non-silencing siRNAs. Treatment of cells with Risp-specific siRNAs that recognize the 16.4.1 region reduced expression of all Risp proteins (right panel). (D) Risp expression pattern in persistently HIV-1 infected astrocytes (TH4-7-5).

3.3.2. Biochemical and functional analysis of interactions of Risp proteins with Rev in human astrocytes (U138MG)

After having confirmed the expression of Risp proteins in human astrocytes, I investigated in collaboration with Dr. Kamyar Hadian whether native Risp proteins are capable of interacting with Rev. To this end an affinity chromatography assay previously established in our laboratory for isolation of Rev-interacting proteins from human cell lysates [124] was used. Cell extracts were applied to *Strep*Tactin-affinity columns containing a bait protein in which Rev was fused to GFP and *Strep*TagII sequences (RevGFP-*Strep*TagII) (Figure 3.11). To control for interaction specificity, parallel assays were performed with a control bait protein lacking the Rev moiety (i.e. GFP-*Strep*TagII). Interactor proteins were eluted from the columns with high-salt buffer and eluted proteins analyzed by Western Blot. Risp peptide antibodies specifically detected four protein bands corresponding to 30, 38, 49 and 64 kDa in elution fractions of assays performed with the Rev-bait protein which were not identified in the elution fractions of the control assays. All 4 bands corresponded to bands consistently detected with the pooled Risp peptide and the 16.4.1 antibodies (Table 3.1). These results support specific interaction of native Risp proteins with Rev.

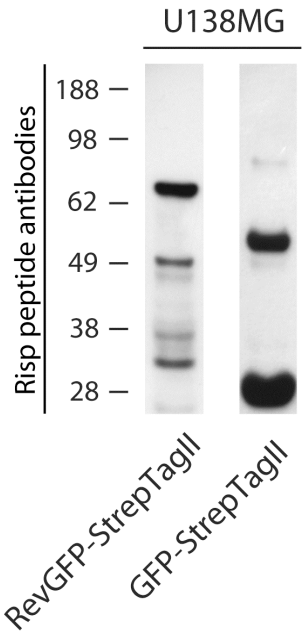
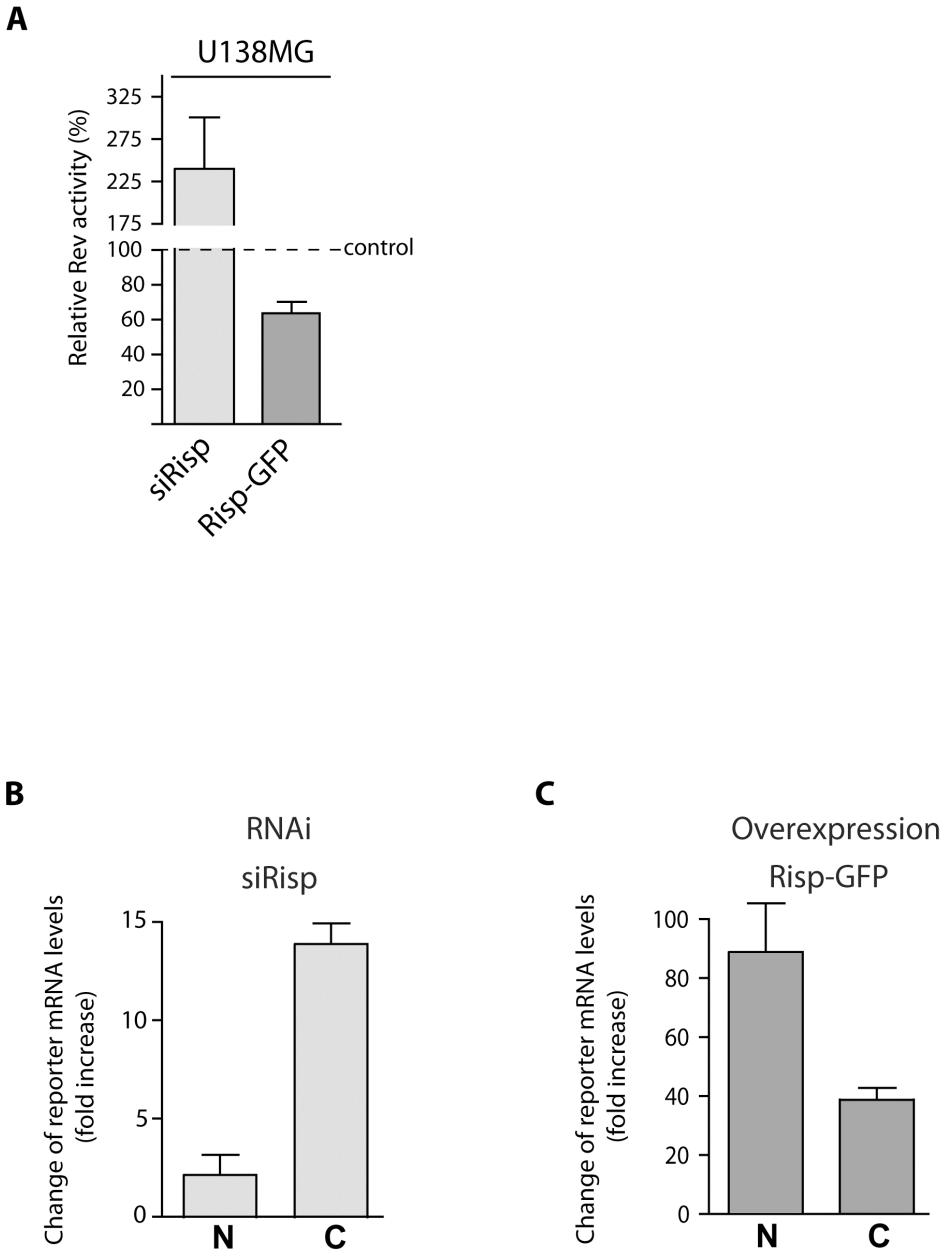


Figure 3.11.: Capture of Risp proteins from U138MG astrocyte cell lysates by affinity chromatography with Rev-bait proteins.
Lysates of U138MG cells were subjected to affinity chromatography, using a bait protein consisting of Rev, Green Fluorescent Protein (GFP) and *Strep*TagII sequences (RevGFP-*Strep*TagII). Bait-interacting proteins were eluted under high-salt conditions and examined for the presence of Risp proteins by Western blot analysis with pooled Risp-peptide antibodies. Parallel affinity chromatography assays were performed with bait proteins lacking the Rev moiety (GFP-*Strep*TagII) to control for specific interactions of native Risp proteins with Rev.

To investigate whether Risp proteins influence Rev activity, a previously established Rev-reporter assay was employed [107]. This assay measures Rev-mediated stimulation of the production of a red fluorescent reporter protein (*Ds*Red) encoded by an unspliced transcript with multiple elements conferring Rev-dependency [108]. Rev-activity is assayed by co-transfecting cells with the reporter plasmid and plasmids for expression of Rev-GFP or GFP (baseline control) and the proportion of *Ds*Red-positive cells in transfected populations is determined by flow cytometry. As demonstrated in Figure 3.12/A, Rev-activity increased by about 250% in U138MG cells transfected with Risp-specific siRNAs, compared to cells transfected with non-specific siRNAs. Conversely, Rev activity diminished by about 40% in cells transfected with Risp-GFP expression plasmids for elevation of endogenous Risp levels. These results indicate a link between expression levels of Risp and Rev activity. In addition to reporter protein production, I investigated the effect of Risp expression on the levels of reporter RNAs in the nuclear and cytoplasmic compartments, respectively. siRNA-mediated knock-down of Risp led to increased reporter RNA levels in the cytoplasm (Figure 3.12/B). Conversely, overexpression of Risp increased levels of reporter RNAs in the nucleus (Figure 3.12/C).

Figure 3.12. (next page): Risp protein levels influence Rev activity in U138MG cells.
The influence of Risp expression levels on Rev activities in U138MG cells was measured with a Rev-reporter assay. Endogenous Risp expression was diminished by transfecting cells with Risp-specific siRNAs (siRisp). Cells transfected with non-silencing siRNAs served as controls. Risp expression was increased by transfecting cells with plasmids for expression of Risp-GFP (i.e. pCRispsg143). Cells transfected with expression plasmids for unfused GFP served as controls.
(A) Influence of Risp expression levels on the Rev-dependent stimulation of production of the red fluorescence reporter protein. Rev activities in cells with changed levels of Risp expression are indicated relative to Rev activities in the respective control assays, which were set at 100%. Columns represent the mean results of three independent experiments and error bars the standard deviation. Reduction of native Risp expression increased the Rev-dependent reporter activity by 250%. Elevation of Risp expression reduced Rev-reporter activity by approximately 40%. (B-C) Influence of altered Risp expression on the levels of Rev-reporter mRNAs in the nucleus (N) and cytoplasm (C) of U138MG cells in the presence of Rev. Relative levels of reporter mRNAs in each subcellular compartment were quantified by real-time RT-PCR according to the method of Pfaffl [116], using RNA polymerase II mRNAs as reference (for details see Materials and Methods). The change of reporter mRNA levels (fold-increase) in each compartment represents relative reporter mRNA levels in the sample with altered Risp expression normalized to the relative reporter mRNA levels in the control sample. Columns represent the mean results of three independent experiments and error bars the standard deviation. Reduction of native Risp expression increased levels of reporter mRNAs in the cytoplasm (B). In contrast, elevation of Risp expression increased reporter RNA levels in the nucleus (C).



3.3.3. Cytoplasmic sequestration of Rev proteins by Risp in astrocytes

Furthermore, the effect of Risp knock-down on the localization of Rev-GFP was examined in the nuclear and cytoplasmic compartments of U138MG cells. To this end, subcellular fractions were prepared from U138MG cells transfected with siRNAs and a Rev-GFP expression plasmid and subjected to Western blot analysis with various antibodies (Figure 3.13). Antibodies against Rev confirmed predominant localization of Rev-GFP in the cytoplasmic compartment of control cells (i.e. transfected with the non-silencing siRNAs). This is in agreement with the previously reported cytoplasmic localization behavior of Rev-GFP in U138MG cells [109]. In

contrast, cells transfected with Risp-specific siRNAs showed increased accumulation of Rev-GFP in the nuclear compartment. Western blot analysis with the Risp peptide antibodies detected Risp proteins exclusively in the cytoplasmic fraction and confirmed specific knock-down of Risp proteins in this compartment by Risp-specific siRNAs. Antibodies against nuclear (histone H2A) and cytoplasmic proteins (GAPDH) confirmed the purity of the subcellular fractions (Figure 3.13).

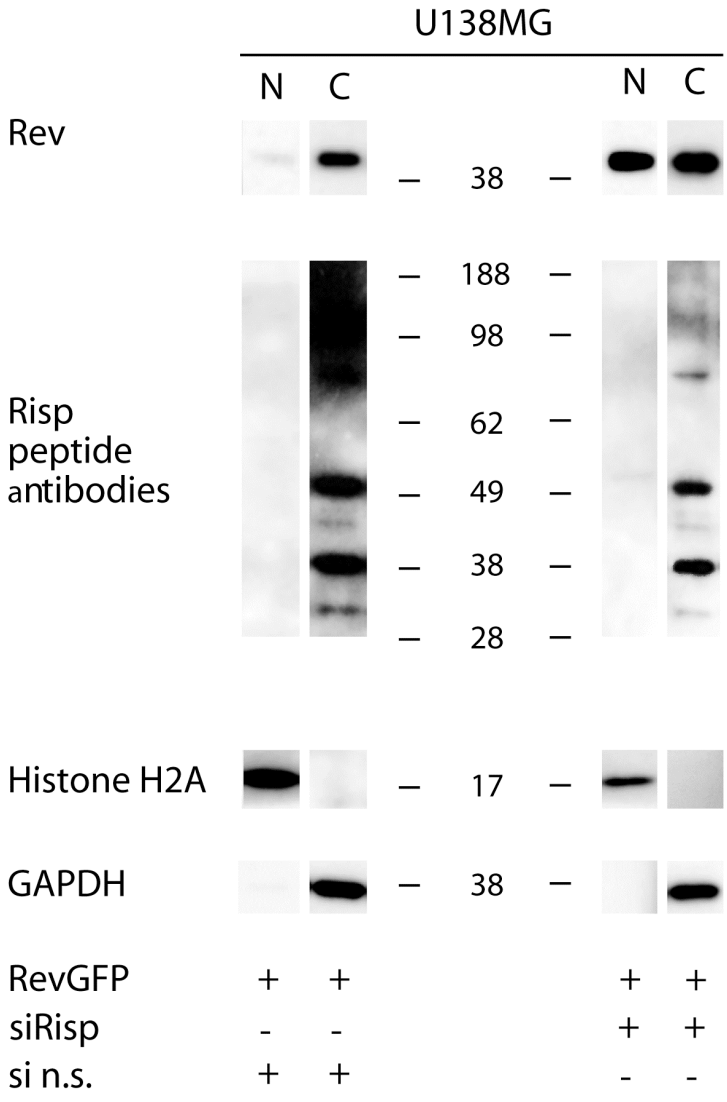


Figure 3.13.: Diminished Risp expression increases nuclear levels of Rev-GFP in U138MG cells. Cells were first transfected with either non-silencing siRNAs (left panels) or with Risp-specific siRNAs (right panels) and then with a plasmid for expression of RevGFP (pCsRevsg143). 48 hours after plasmid transfection, nuclear (N) and cytoplasmic (C) fractions were prepared from cells and subjected to Western blot analysis, using antibodies that recognize Rev, Risp proteins (pooled peptide antibodies), histone H2A (nuclear marker) and GAPDH (cytoplasmic marker). Cells transfected with Risp-specific siRNAs showed increased nuclear levels of Rev-GFP, compared to cells transfected with non-silencing siRNAs. Risp immunodetection showed cytoplasmic localization of Risp proteins and confirmed diminished Risp expression by Risp-specific siRNAs. The purity of the subcellular fractions was confirmed by exclusive immunodetection of Histone H2A in nuclear fractions and GAPDH in cytoplasmic fractions.

Together these data support interaction of native Risp proteins with Rev and suggest Risp proteins as cellular inhibitors of Rev fuction by retaining Rev in the cytoplasm of U138MG cells.

3.3.4. Influence of Risp on HIV production and expression of Rev dependent HIV transcripts in persistently HIV-infected astrocytes

I then assessed the influence of Risp proteins on HIV production in the TH4-7-5 cell line (i.e. persistently HIV-infected astrocytes) [100] that restricts HIV production as shown above. As shown in Figure 3.14/A, transfection of TH4-7-5 cells with Risp-specific siRNAs selectively increased both extra- and intracellular levels of Gag produced by TH4-7-5 cells, compared to transfections with non-silencing siRNAs. Conversely, transfection of TH4-7-5 cells with the Risp-GFP expression plasmid further reduced the Gag production (Figure 3.14/B). The inhibitory effect was recapitulated by expression of the isolated 16.4.1 domain fused to GFP. To investigate the effect of Risp expression levels on the levels of Rev-dependent HIV transcripts, I performed quantitative real-time RT-PCR assays with primers that amplify either late (Rev-dependent) or early (Rev-independent) HIV transcripts. Risp knock-down selectively increased levels of late, but not early HIV transcripts (Figure 3.14/C). An approximately 3-fold increase was observed, compared to cells transfected with non-silencing siRNAs. On the other hand, Risp overexpression selectively reduced levels of late HIV RNAs, whereas levels of early HIV-RNAs increased (Figure 3.14/D). Reduction of late HIV transcripts was recapitulated by expression of 16.4.1-GFP.

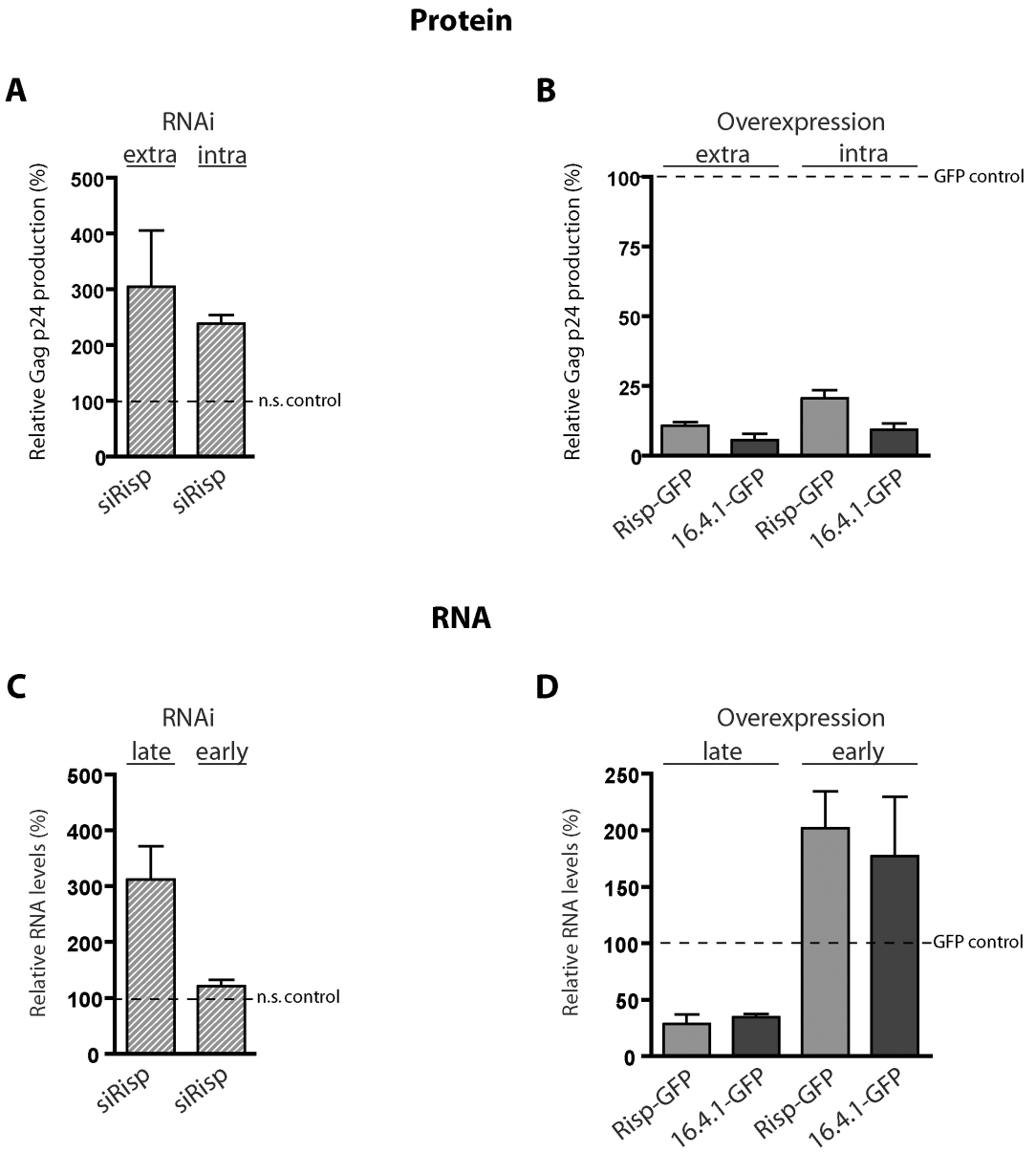


Figure 3.14.: Risp protein levels influence HIV-1 replication in persistently infected astrocytes.
The TH4-7-5 cell line was used to measure the influence of altered Risp expression levels on the production of the HIV-1 Gag p24 (A, B) and on the levels of HIV-1 transcript classes (C, D) in TH4-7-5 cells (i.e.persistently HIV-1 infected astrocytes).
Risp expression levels were diminished by transfecting TH4-7-5 cells with Risp-specific siRNAs (siRisp). Cells transfected with non-silencing siRNAs served as controls (n.s. control). Risp expression levels were increased by transfecting cells with expression plasmids for Risp-GFP or for a fusion protein containing the isolated Rev-interacting domain of Risp (16.4.1) and GFP. Cells transfected with expression plasmids for unfused GFP served as controls. Levels of each indicated HIV-1 parameter in cells with altered Risp expression levels were normalized to the levels of the same parameter in the control cells (i.e. 100%). Columns represent the mean results of three independent experiments and error bars standard deviations. (A) Increased production of extra- and intracellular Gag p24 by TH4-7-5 cells with reduced Risp expression. (B) Reduced production of extra- and intracellular Gag p24 by TH4-7-5 cells expressing Risp-GFP or 16.4.1-GFP. (C) Selectively increased levels of late, Rev-dependent HIV-1 transcripts but not of early, Rev-independent transcripts in TH4-7-5 cells with reduced Risp expression. (D) Selective reduction of levels of late, Rev-dependent HIV-1 transcripts in TH4-7-5 cells expressing Risp-GFP or 16.4.1-GFP.

Together these results show that Risp expression levels influence HIV production and levels of Rev-dependent RNAs in persistently infected astrocytes. Ectopic expression of 16.4.1 has similar effects as Risp overexpression, indicating that the effects of the Risp protein are mediated by the 16.4.1 domain.

3.3.5. Risp overexpression decreases HERV expression

After having established that expression levels of Risp proteins influence HIV-1 production, I investigated whether Risp expression also influences HERV expression as an natural HIV-1 inhibitor compared to the artificial siRNA inhibitors used in section 3.2. Risp proteins were overexpressed in LC5-HIV cells to reduce virus production and HERV expression levels in these cells were investigated by DNA microarray analyses. As expected, Risp overexpression led to a reduced HIV production in LC5-HIV cells (Figure 3.15). Furthermore, the expression of HERV groups S71pCRTK-1, E4-1, ERV9, Seq59, two groups of the HML-4 family, HERV-KC4 and the five groups of the HML-3 family, Seq26, Seq34, HML-3, HERV1 and Seq43 previously shown to be increased in HIV-1 infected cells (Figure 3.2) were nearly completely abolished by overexpression of Risp proteins (marked red in Figure 3.15). To exclude unspecific effects on HERV expression caused by Risp overexpression in an HIV-1 free context uninfected LC5 cells overxpressing Risp proteins were investigated. The HERV expression patterns in uninfected LC5 cells were not upregulated by overexpression of Risp proteins and were similar to those of untreated LC5 cells. These results strengthen the siRNA data (see section 3.2.2, Figure 3.7) and demonstrate again a strong connection between HERV expression and HIV-1 production.

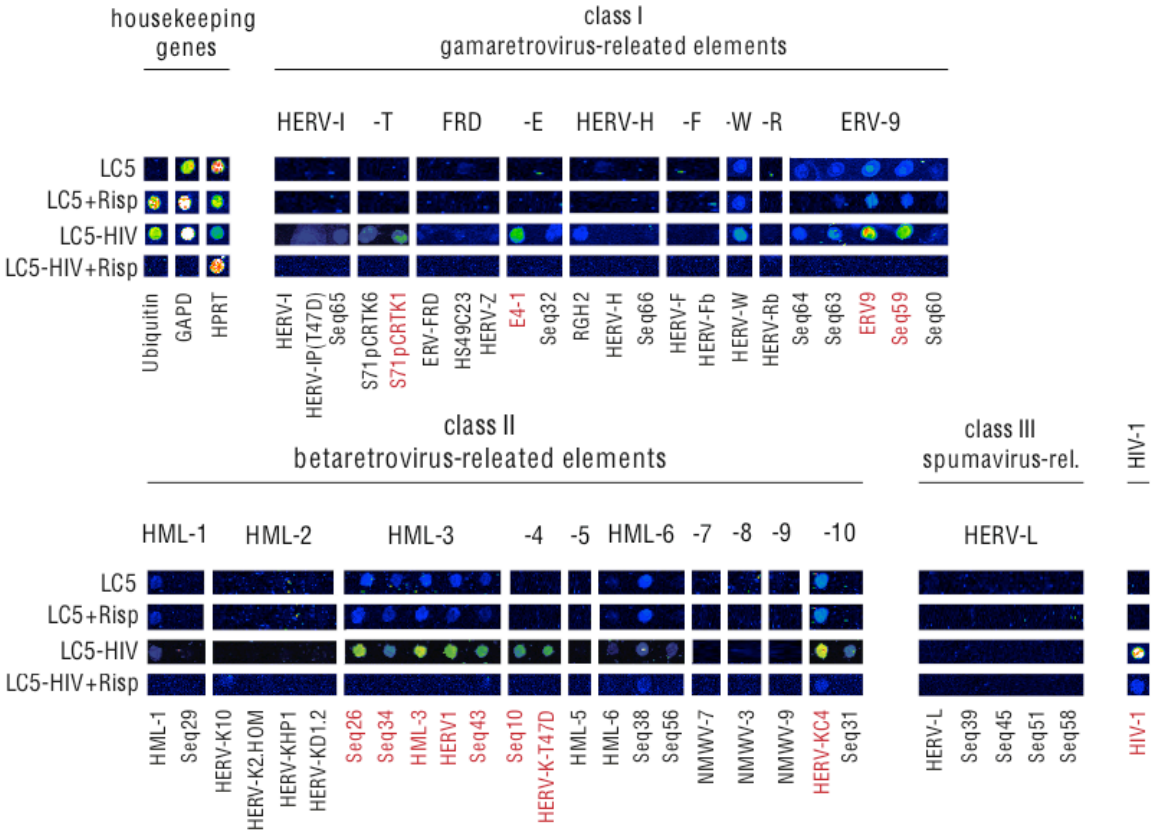


Figure 3.15.: Overexpression of Risp proteins reduces HERV activity.
Risp proteins were overexpressed in LC5-HIV cells. The cells transfected with Risp showed reduced HIV-production. Furthermore there is a diminished HERV expression for S71pCRTK-1, E4-1, ERV9, Seq59, two groups of the HML-4 family, HERV-KC4 and the five groups of the HML-3 family, Seq26, Seq34, HML-3, HERV1 and Seq43 compared to the LC5-HIV cells (marked in red). Uninfected LC5 cells expressing Risp proteins served as a negative control. A representative data set from three independent experiments is shown. False color mapping was used for image visualization.

3.4. Influence of HIV-1 encoded proteins on HERV expression

My next goal was to investigate the effects of individual HIV proteins on the expression of HERV families. It is not possible to selectively knockdown expression of single HIV proteins in HIV-infected cells with the siRNAs used here, as explained in section 3.2.1. Therefore I transfected plasmids encoding single HIV proteins in uninfected LC5 HeLa cells. The effect of the expression of individual selected HIV-1 proteins on HERV expression in these cells was investigated by microarray analysis. Figure 3.16 shows the HERV expression in untreated LC5 cells and LC5 cells ectopically expressing the HIV-1 proteins Tat, Rev and Nef. Cells ectopically expressing Tat and Rev showed only a slight upregulation in HERV expression,

affecting mainly the HERV groups E4-1, ERV-9 and Seq59. In contrast the ectopical expression of the Nef protein resulted in an expression of HERV groups S71pCRTK-1, E4-1, RGH2, ERV9, Seq59, HERV-KC4 and the five groups of the HML-3 family, Seq26, Seq34, HML-3, HERV1. Expression of these HERV groups in the Nef-expressing cells was comparable to the expression of these HERV-groups in HIV-1 infected LC5-HIV cells.

To further exclude the involvement of Tat and Rev in HIV-mediated induction of HERV expression, the following constructs containing the complete HIV-1 viral sequence with Tat and Rev mutations in the *tat* and *rev* sequences (Y47H2 and Y26A) were used [111]. The Y47H2 virus has a partially active Tat, and shows abolished expression of the essential Rev protein, thereby being defective in HIV replication [111]. The Y26A virus expresses a non-active Tat protein due to a single mutation and encodes for a functional Rev protein (Y26A). 293T cells were transfected with plasmids containing the Y47H2 and Y26A genomes and the virus was harvested from the supernatant for further infection studies. LC5-CD4 cells were subsequently infected with the Y47H2 and Y26A viruses. After infection, cells were analyzed for HERV and HIV-1 expression by microarry analysis. As expected, cells infected with Y47H2 or Y26A showed lower HIV-1 expression, than the wildtype virus (LC5-HIV). Both mutants were able to enhance the expression of HERV groups S71pCRTK-1, E4-1, RGH2, HERV-Fb, ERV9, Seq59, Seq10, HML-6, Seq38, NMWV-7, HERV-KC4, Seq31 and the five groups of the HML-3 family Seq26, Seq34, HML-3, HERV1 and Seq43 (Figure 3.16). Therefore the depletion of the Rev protein (Y47H2) and the expression of inactive Tat protein (Y26A) did not prevent upregulation of HERV expression. For that reason, these two proteins don’t seem to be responsible for the upregulation of HERVs. This data validate the results of the overexpression analysis suggesting that HIV-1 Nef is essentially responsible for boosting HERV expression.

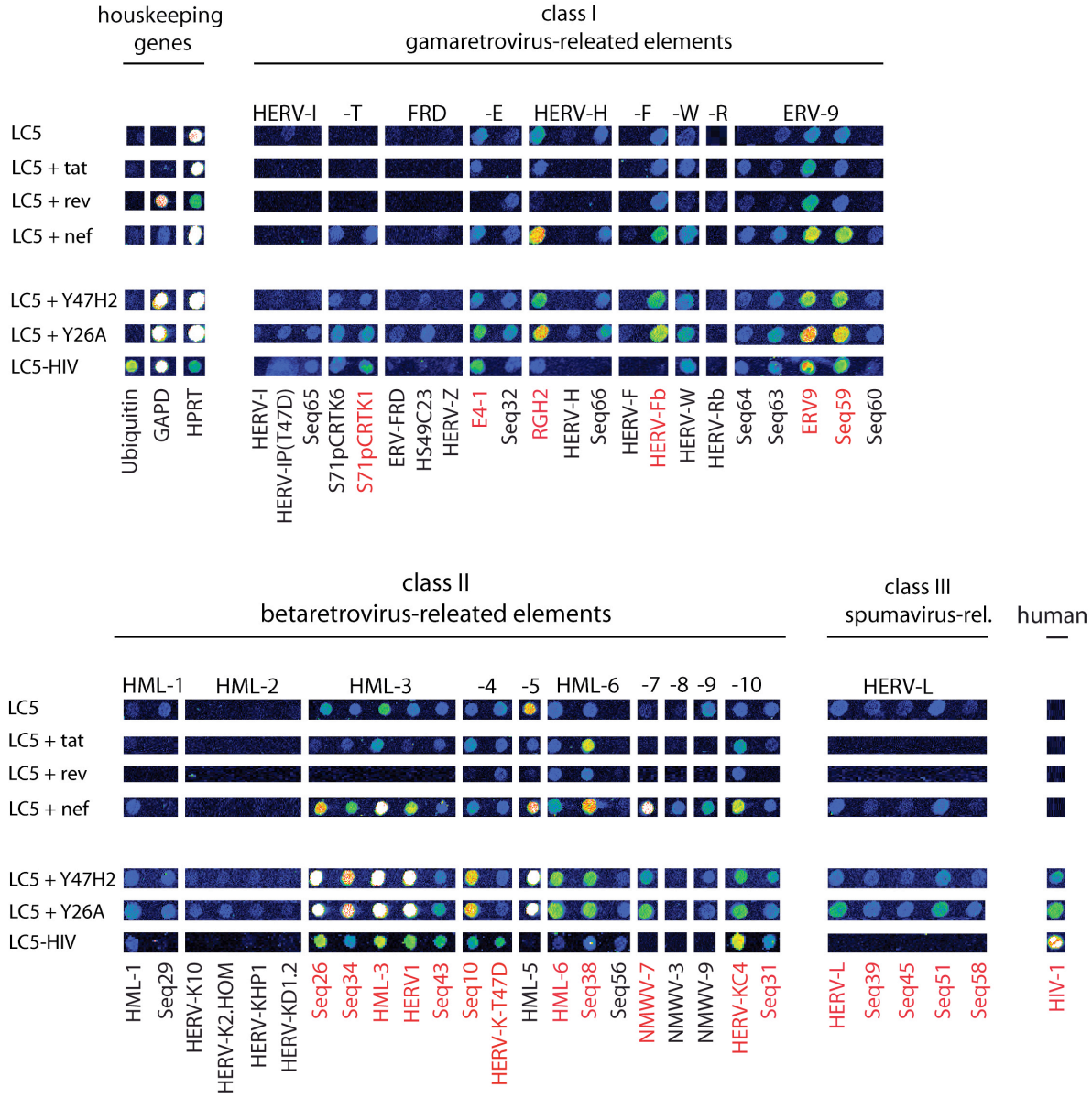


Figure 3.16.: Overexpression of several HIV-1 proteins and infection with Tat and Rev mutated HIV-1 virus.
Displayed is the HERV transcription profile of LC5-cells expressing Tat, Rev or Nef. Uninfected LC5 cells served as a control. Cells expressing Tat or Rev showed similar HERV transcription profiles as the untreated LC5 control cells. Cells expressing Nef showed increased expression of the HERV-groups S71pCRTK-1, E4-1, ERV9, Seq59, HERV-KC4 and the five groups of the HML-3 family (Seq26, Seq34, HML-3 and HERV1) (marked red) similar to LC5-HIV cells. In addition, Nef expressing cells upregulated expression of RGH2 and NMWV-7 (marked in red). Infection of LC5-CD4 cells with virus supernatants from constructs containing the complete HIV-1 viral sequence with Tat and Rev mutations (Y47H2 and Y26A) showed similar HERV expression profiles as cells containing wildtype HIV-1 (LC5-HIV). A representative data set from three independent experiments is shown (three RNA isolations). False color mapping was used for image visualization.

3.5. HERV-K(HML-2) expression is enhanced in cells acutely infected with a primary HIV-1 isolate

3.5.1. HERV expression profiles after acute HIV-1 infection

As HERV-K(HML-2) transcripts has been observed to be expressed in HIV-1 infected Individuals in previous studies [74,119], I wondered why upregulated expression of HERV-K(HML-2) was found only in low to undetectable levels in persistently HIV-1 infected cell lines by microarray analysis (Figure 3.2) and real-time RT-PCR (Figure 3.18). Previously published data [72,74] were collected with infected PBMC cells from HIV-1 infected patients. This raised the question whether there is a difference in HERV expression between persistently infected HIV-1 cells by a laboratory strain and acutely HIV-1 infected cells by a primary isolate. Therefore, LC5-CD4 cells were infected with a primary HIV-1 patient isolate and cultured for two weeks. Subsequently they were subjected to microarray analysis and real-time RT-PCR. Interestingly, the cells acutely infected with a primary patient isolate showed distinct expression of HERV-K(HML-2) (marked red in Figure 3.17), which was not apparent in the non-infected control cell line as well as in cells persistently infected with a laboratory HIV-1 strain. Figure 3.17 shows HERV-K(HML-2) expression assayed by HERV chip analysis. Also representatives of the HERV families HERV-E, ERV-9 and HML-3 (marked red in Figure 3.17) showed higher expression in the cells acutely infected with a primary patient isolate than in the uninfected control cells.

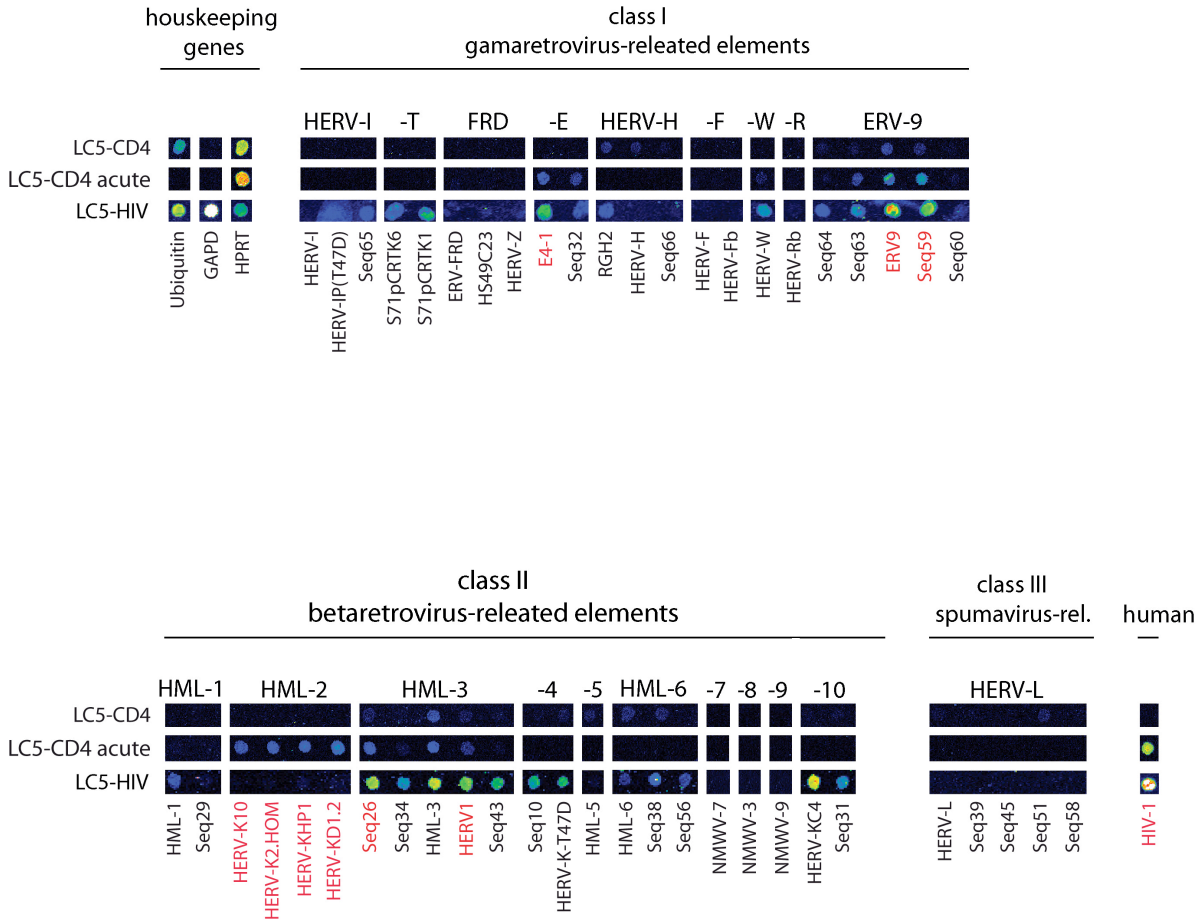


Figure 3.17.: HERV expression after acute infection of LC5-CD4 cells with serum from patient 891.
LC5-CD4 cells were infected for two weeks with HIV-1 serum isolated from patient 891 (LC5-CD4 acute) and showed HIV-productivity compared to the uninfected control cells LC5-CD4. Acute infection resulted in an increase of the HERV families HERV-E, ERV-9, HML-2 and HML-3 (marked in red). A representative data set from three independent experiments is shown (three RNA isolations). False color mapping was used for image visualization.

In addition, real-time RT-PCR analysis (Figure 3.18.) confirmed a higher HERV-K(HML-2) expression in the acutely HIV-1 infected cells compared to the persistently infected cells LC5-HIV.

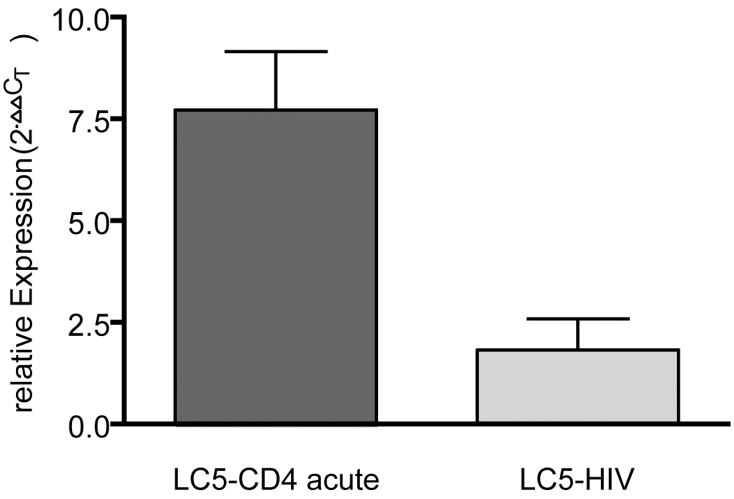


Figure 3.18.: Quantitative real-time RT-PCR for HERV-K(HML-2) in LC5-CD4 cells acutely infected with a primary HIV-1 isolate derived from a patient serum (891) and persistently infected cells.
The y-axis shows the x-fold relative expression of the HERV-K(HML-2) in acutely and persistently HIV-1 infected cells normalized to the uninfected LC5-CD4 or LC5 control cells. HERV-K(HML-2) showed increased expression in acutely HIV-1 infected cells. Mean values and standard deviations are indicated for triplicate experiments (three independent RNA isolations).

3.5.2. Determination of active HERV-K(HML-2) loci

To investigate which of the approximately 60 described HERV-K(HML-2) loci [44] are active in the acutely infected cells I analyzed the sequences of HML-2 transcripts in acutely and persistently infected LC5 cells. To this end I generated cDNAs from RNA isolated from these cells and amplified HML-2 sequences in these cDNAs using primers that bind to the central region of the HML-2 *gag* gene [117]. I then generated molecular clones containing the PCR products. The HML-2 seqeunces were analyzed in 34 clones derived from acutely infected cells and eighteen clones from HML-2 sequences. The sequences in the clones derived from the acutely infected cells mapped to 6 HML-2 proviruses (c1_B, c3_B, c3_C, c3_E, c7_C and c10_B), indicating that six HML-2 proviruses were transcribed in acutely HIV-1 infected cells. In contrast, only one HML-2 locus was found to be transcriptionally active in the uninfected control cell line. Table 3.1 shows the HML-2 proviruses and their chromosomal locations. Comparing our results with published results [117] reveals that three of the active loci (c1_B, c3_B and c3_C) in acutely HIV-1 infected cells can encode a complete Env protein. The proviruses with the chromosomal localization 1q22 (c1_B) and 3q13.2 (c3_B) are described to code for the NP9 protein [125] [126].

acute HIV-1 infected cells

Provirus	Chromosomal localization*	Chromosome band
HML2-c1_B	chr 1: 153870020-153870613	1q22 (+)
HML2-c3_B	chr 3: 114232740-114233333	3q13.2 (+)
HML2-c3_C	chr 3: 127093474-127094067	3q21.2 (+)
HML-2-c3_E	chr 3: 127093474-127094067	3q27.2 (+)
HML2-c7_C	chr 7: 141101009-141101702	7q34 (-)
HML2-c10_B	chr 10: 101576433 -101577026	10q24.2 (-)

non-infected cells

Provirus	Chromosomal localization*	Chromosome band
HML2-c11_B	chr 11: 118103858-118104446	11q23.3 (+)

Table 3.1.: Localization of transcriptionally active HML-2 proviruses in acute infected cells and non-infected cells.
Each provirus was identified by *gag*-derived cDNAs. Provirus designations are given in the first column. Localization data of HML-2 proviral loci were collected from the human genome sequence as given at the Human Genome Browser March 2006 version. *Chromosomal localization of HML-2 proviral portions amplified by RT-PCR.
Cytogenetic localization (Chromosome band) of HML-2 proviruses. The orientation of proviruses on the chromosomes are given in parantheses.

3.6. HERV expression is activated by cellular transcription factors

As transcription factors significantly affect replication or expression of HIV *in vivo* [127,128], they may also influence HERV expression. The HIV-1 LTR consists of three functionally discrete regions (U3, R, and U5). Transcription initiation occurs at the U3-R boundary in the 5`LTR. Several host cell factors like NF-κB, NFAT and AP1 bind to HIV-1 LTRs and influence HIV gene expression [29,129]. Thus these factors could also affect the expression of endogenous retroviruses.

To investigate whether common transcription factors can regulate the expression of HERV families, Jurkat T-cells were stimulated with PMA/Ionomycin or antibodies against CD3/CD28 for 3 hours to activate the transcription factors NF-κB, NFAT and AP1 [130,131,132,133] and the expression profile of several HERVs investigated by DNA-microarray analyses.

To check for successful stimulation of the cells, expression of IL-2 mRNA was

assayed, which is a major target of the transcription factors after stimulation with PMA/Ionomycin or CD3/CD28. Figure 3.19 shows the stimulation-induced expression of IL-2 after PMA/Ionomycin or CD3/CD28 stimulation.

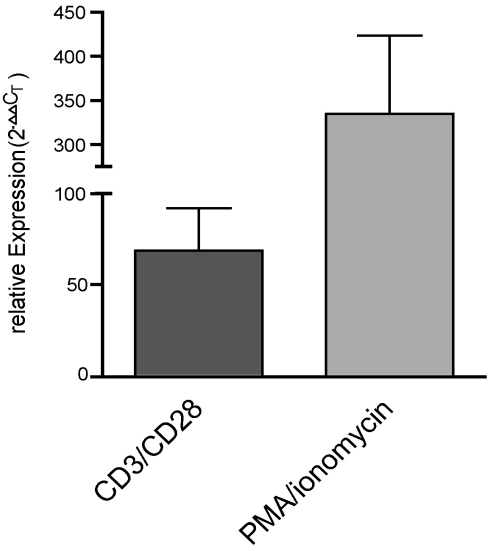


Figure 3.19.: Relative expression of IL-2 by real-time RT-PCR.
The Y-axis shows the x-fold relative expression of IL-2 after PMA/Ionomycin or CD3/CD28 stimulation normalized to unstimulated cells. PMA/Ionomycin or CD3/CD28 stimulation results in a high expression of IL-2. Mean values and standard deviations are indicated for triplicate experiments (three independent RNA isolations).

Figure 3.20 shows the HERV expression pattern in stimulated and unstimulated T-cells. Representatives of the HERV families HERV-T, HERV-E, HERV-F, HERV-W, ERV-9, HML-2, HML-3, HML-4, HML-6, HML-7, HML-9, HML-10 and HERV-L (marked red in Figure 3.20) belonging to class I, II and III HERVs were found to be upregulated in stimulated cells. Both stimuli increased the expression of the HERV-groups S71pCRTK-1, E4-1, ERV9, Seq59, HERV-KC4 and the five groups of the HML-3 family (Seq26, Seq34, HML-3, HERV1 and Seq43) as well as four groups of the HML-2 family (HERV-K10, HERV-K2.HOM, HERV-KHP1 and HERV-KD1.2) that were also upregulated in HIV-1 infected cells (Figure 3.17).

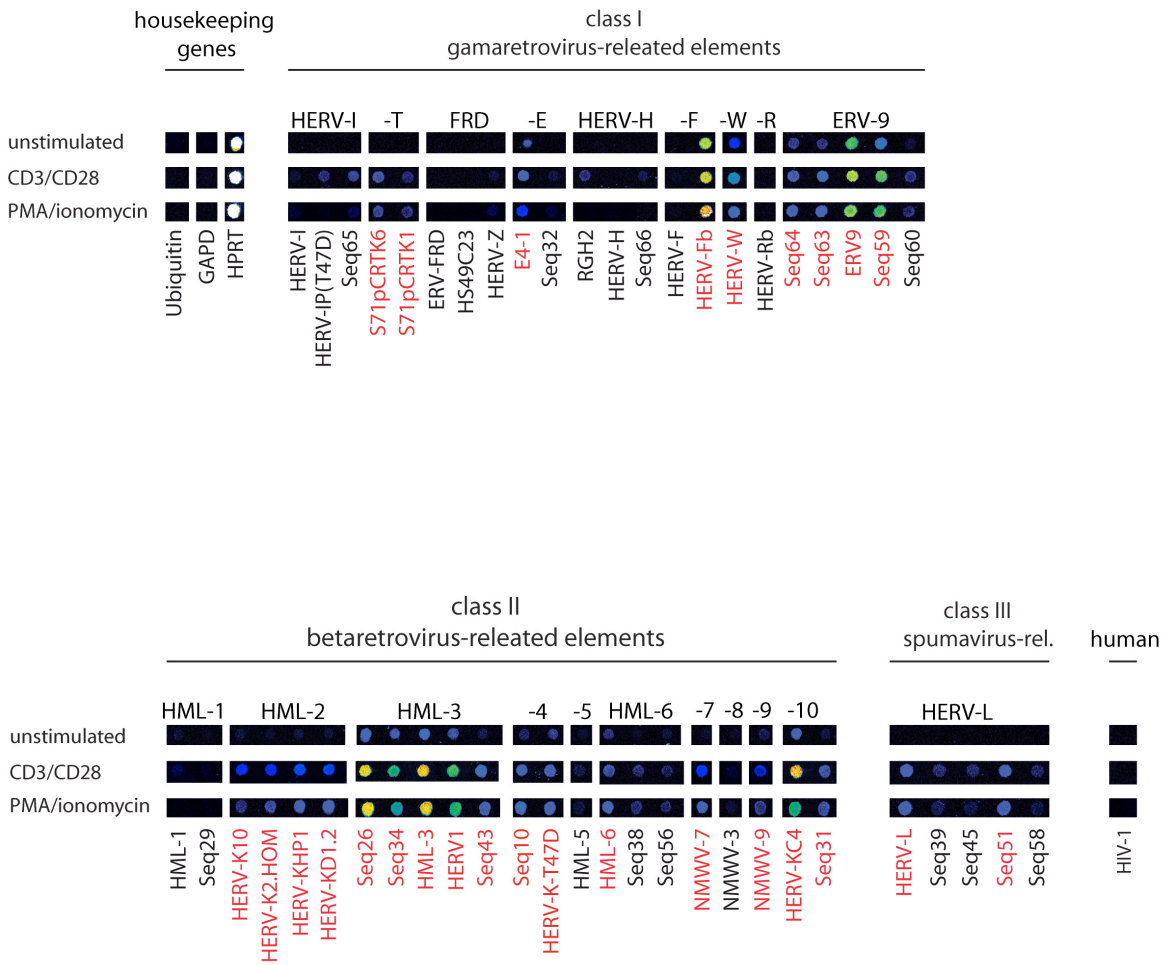


Figure 3.20.: HERV expression profile after stimulation of T-cells to activate cellular transcription factors.
Jurkat T-cells were stimulated with PMA/Ionomycin or CD3/CD28 for 3 hours to activate transcription factors (e.g. NF-κB). Stimulation of Jurkat T-cells resulted in an increase of several HERV groups (marked in red) compared to the unstimulated control cells. A representative data set from three independent experiments is shown (three RNA isolations). False color mapping was used for image visualization.

These data suggest that major transcription factors like NF-κB, NFAT and AP1 could be involved in the activation of HERV expression.

Since activation of transcription factors induced HERV expression, I determined the 5'LTR sequences of the identified HML2 loci (see table 3.1) and analyzed them for transcription factor binding sites using Genomatix bioinformatics software. LTRs of four active HERV-K(HML-2) loci (c1_B; c3_B; c3_C and c3_E) contained two NF-κB transcription factor binding sites whereas NFAT and AP1 sites were not identified (Figure 3.21). The HML-2 locus c11_B found to be active in uninfected cells contains

only one NF-κB transcription factor binding site compared to the NF-κB sites identified in HML-2 proviruses active in acutely HIV infected cells. These results suggest involvement of NF-κB in upregulating the expression of HERV groups found to be overexpressed in HIV-infected cells.

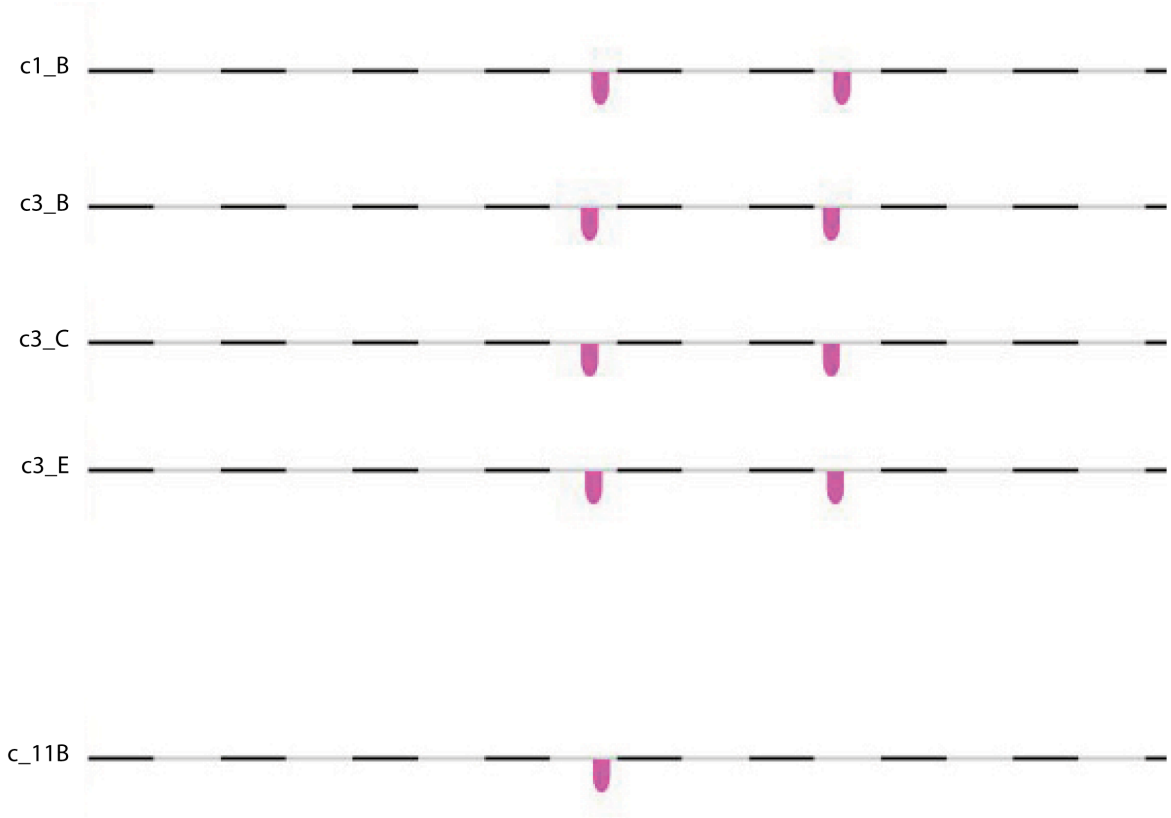


Figure 3.21.: NF-κB transcription factor binding sites in active HERV-K(HML-2) proviruses.
Transciption factor binding sites were investigated with bioinformatic tools (El Dorado software by Genomatix). Several NF-κB transcription factor binding sites could be identified in five active HERV-K(HML-2) proviruses.

4. Discussion

4.1. Upregulated expression of selected HERV-groups in persistently HIV-1 infected cells

Comparison of HERV transcript patterns in three persistently HIV-1 infected cell lines revealed activation of the expression of distinct HERV groups of class I and class II HERV families in the HIV-1 infected cells compared to the uninfected parental cell lines. Four of the upregulated HERV groups belonged to class I families: HERV-T (group S71pCRTK1), HERV-E (group E4-1), and ERV9 (groups ERV9 and Seq59), and 8 groups to class II families: HML-3 (groups Seq26, Seq34, HML-3, HERV1 and Seq43), HML-4 (groups Seq10 and HERV-K-T47D) and HML-10 (group HERV-KC4). Upregulated expression of five HERV groups S71pCRTK1, E4-1, ERV9, Se59 and HERV-KC4 was confirmed by real-time RT-PCR analysis. Manipulation of HIV expression levels directly affected the expression levels of these HERV groups. Thus reduction of HIV expression with specific siRNAs greatly reduced expression of the upregulated HERV groups.

4.2. Risp is a negative regulator of HIV-1 replication leading to decreased HERV expression

In a previous study a cDNA encoding a small (171 As) Rev-interacting protein sequence (16.4.1) was isolated [107]. Database searching showed that the 16.4.1 sequence is highly conserved in a family of proteins encoded by human FAM21 genes (Supplement). The expression of these proteins was previously suggested in large-scale transcriptome and proteome analyses. Only few studies have addressed potential functions of FAM21 proteins. Individual FAM21 proteins were recently proposed to play a role in poxvirus infection [134], have been shown to be part of a mulitprotein complex that associates with endosomes [135,136] and to contain motifs suggesting a role in actin polymerization [137]. However, FAM21 proteins had not been linked to HIV before this study. This work confirms the existence of numerous FAM21 proteins containing the Rev-interacting 16.4.1 region. We called this group of proteins Risp, for Rev-interacting HIV suppressor proteins. I demonstrated that Risp proteins localize exclusively to the cytoplasm of human astrocytes. Furthermore I showed that Risp proteins, could function in the context of HIV replication as negative regulators of Rev-dependent gene expression and HIV production in astrocytes.

Using a Rev-reporter assay, I demonstrated that lowering Risp expression in astrocytes increased Rev-dependent production of the reporter protein as well as levels of reporter mRNAs in the cytoplasm. In addition, I showed that depletion of Risp increases nuclear localization of Rev. These results suggest a model (Figure 4.1) for Risp-mediated regulation of Rev activity in which Rev-Risp interactions promote retention of Rev in the cytoplasm.

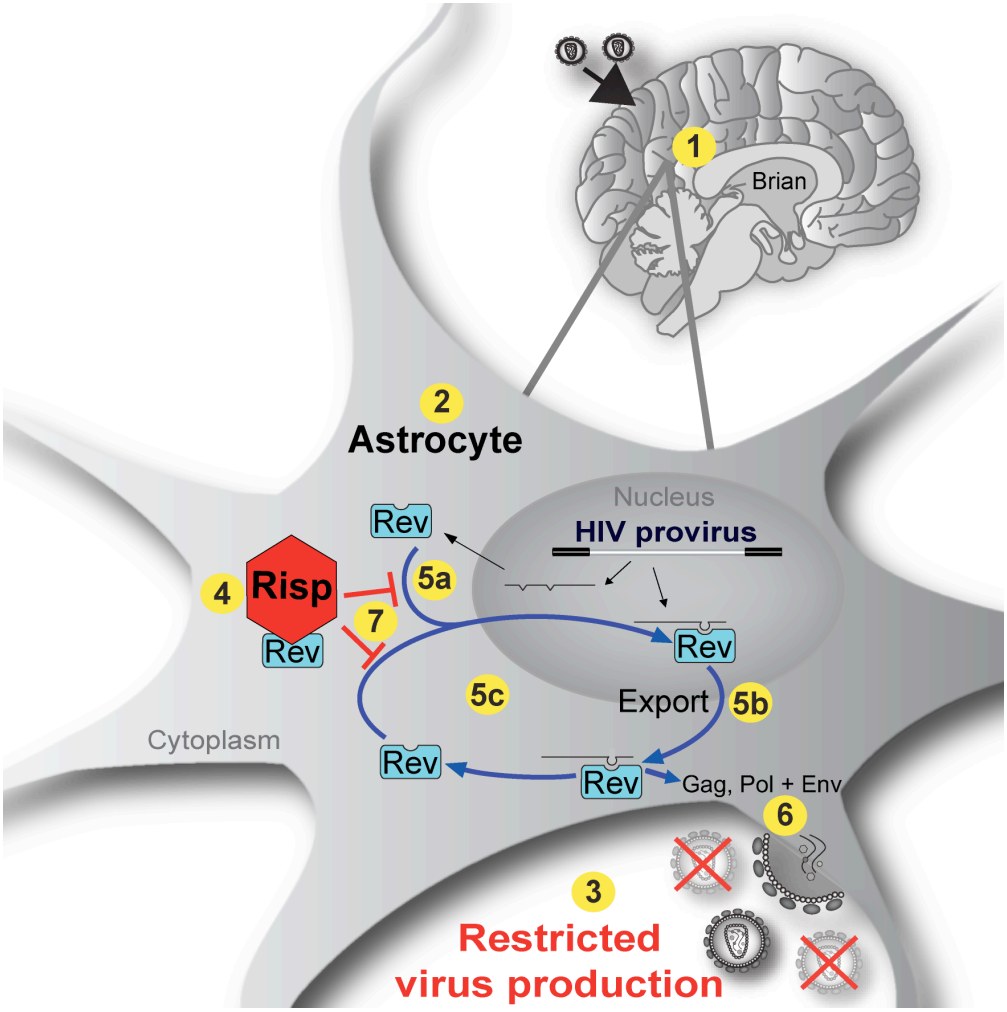


Figure 4.1.: Hypothesis for the regulation of Rev by Risp proteins.
HIV-1 can invade the central nervous system (1) and persist in astrocytes (2) that strongly restrict HIV production (3). Risp proteins are expressed in the cytoplasm and interact with Rev (4). Rev is produced early during HIV replication from multiply-spliced HIV transcripts and continuously shuttles between the nucleus and cytoplasm (5a). In the nucleus, Rev binds to and stabilizes single and unspliced HIV RNAs, and mediates their export to the cytoplasm (5b), after which it can reenter the nucleus (5c). All components for the assembly of new virus particles are produced from Rev-dependent RNAs (6), making Rev activity key to HIV replication. Our data suggest that Risp proteins interfere with Rev (4) by promoting accumulation of Rev in the cytoplasm (7) and thus limit HIV production by persistently infected astrocytes (3).

The resulting depletion of Rev in the nucleus may compromise the stabilization and export of Rev-dependent RNAs to the cytoplasm and ultimately decrease levels of HIV mRNAs in the cytoplasm. This model is supported by several experimental observations. Thus, amino acids located within the NLS of Rev were demonstrated to be essential for interaction of Rev with 16.4.1 [107]. This suggests that interaction of Rev with Risp can result in the masking of the Rev NLS. Furthermore, nuclear uptake of Rev proceeds with delayed kinetics in astrocytes [38], indicating that Rev is prevented from accumulating efficiently in the nucleus of astrocytes during shuttling.

Studies with the TH4-7-5 model cell line indicate that Risp proteins influence the levels of Rev-dependent HIV RNAs and proteins (Gag) during persistent HIV infection of astrocytes. Depletion of Risp increased virus production while ectopic expression of proteins containing either Risp or the isolated 16.4.1 segment diminished low constitutive production of HIV by these cells even further. These results further support the link between Risp and HIV production and also indicate that the 16.4.1 region alone is sufficient to mediate HIV control by Risp proteins. Together these results identify for the first time cellular factors involved in the control of HIV production during persistent HIV infection of astrocytes and show that they target Rev-dependent gene expression.

Furthermore, expression of Risp proteins in human brain tissue samples and in cultured primary human astrocytes was demonstrated in this work. This raises the possibility that Risp proteins may play a role in controlling HIV in astrocytes in infected brain tissues *in vivo*. HIV-infection of astrocytes *in vivo* is well established. Recent data indicate that HIV-infection of astrocytes may occur much more frequently than previously anticipated, especially in the vicinity of blood vessels, which are proposed to be the sites of entry of HIV to the brain [138]. HIV expression markers identified in astrocytes *in vivo* included HIV RNAs and early viral proteins like Nef [139,140,141,142,143,144,145], whereas structural HIV proteins were detected much less frequently [100]. Risp-mediated control of HIV production at the post-transcriptional level may play an important role in protecting the brain from virus-induced damage by infected astrocytes with ongoing HIV transcription. Thus post-transcriptional restriction of HIV replication could limit HIV spread within the brain and protect the brain from the effects of the HIV envelope protein gp120, which has a high potential to induce neurotoxicity [146]. In addition, low Rev activity may promote immune evasion of infected astrocytes as has been suggested for HIV-1 infected

primary T-cells cells [147].
Taken together, Risp proteins may represent a novel group of host cell interaction partners of Rev that are capable of controlling HIV production during persistent infection in astrocytes.

As Risp proteins are host cell factors that can suppress HIV-1 replication, I used these proteins as a tool to investigate the effects of inhibition of HIV-1 replication on HERV expression. This complementary approach was chosen to verify the connection between HIV and HERV expression, which was based on the decrease of HERV expression observed upon siRNA-mediated knockdown of HIV-1 production. Overexpression of a host cell factor to inhibit HIV-1 replication has the great advantage over HIV-1 knockdown by siRNAs that it is much closer to physiological conditions. The RNAi approach has a more drastic inhibitory potential, but remains artificial.
While increased HERV expression patterns were observed in both LC5-HIV (HeLa) and TH4-7-5 (astrocytes) cell lines, upregulation of HERV expression patterns was more pronounced in LC5-HIV cells than in TH4-7-5 cells. HIV production of LC5-HIV cells could be reduced by overexpression of Risp proteins (data not shown), although LC5-HIV cells produced much higher basal levels of HIV than TH4-7-5 cells. This indicates that HIV suppression by the Risp family is not limited to persistently infected astrocytes.
Decreased HIV production by LC5-HIV cells overexpressing Risp proteins also led to substantially lower expression levels of five of the identified HERVs. In contrast manipulation of Risp expression levels in uninfected LC5 cells did not affect expression of HERVs. Hence, these results clearly demonstrate a link between HIV-1 production and the activation of several HERV-groups, including E4-1, S71pCRTK-1, ERV9, Seq59, HERV-KC4 and HML-3 groups.

4.3. HIV-1 Nef increases expression of several HERV groups

Reduction of HIV-1 production with HIV-1 specific siRNAs affects the expression of multiple HIV-1 proteins. Therefore this approach does not allow analyzing the influences of single HIV-1 proteins on HERV expression. I reasoned that early HIV proteins, which are decisive for HIV expression, are also the most probable candidates to influence HERV expression. Therefore I investigated the effects of Nef,

Tat and Rev on HERV expression patterns. The experiments showed that overexpression of Tat and Rev had only a slight influence on the expression of HERVs. In contrast, Nef overexpression resulted in an increase of the same HERV groups also found to be overexpressed in HIV-1 infected cells. Furthermore HeLa cells were infected with full HIV-1 virus supernatants that either produced an inactive Tat protein and an active Rev protein (Y26A) or produced a poorly functional Tat protein and failed to produce Rev (Y47H2) [111]. Interestingly, both virus mutants (Y47H2 and Y26A) enhanced the expression of HERV groups S71pCRTK-1, E4-1, RGH2, HERV-Fb, ERV9, Seq59, Seq10, HML-6, Seq38, NMWV-7, HERV-KC4, Seq31 and the five groups of the HML-3 family Seq26, Seq34, HML-3, HERV1 and Seq43 after infection indicating that Tat and Rev are dispensable for activating HERV expression. Therefore, these data suggest that HIV-1 Nef seems to have a greater impact in boosting HERV expression than Tat and Rev. Future experiments will address this hypothesis by investigating the capacity of Nef-deleted HIV-1 viruses to upregulate expression of HERVs.

Nef is a 27/34 kDa, N-terminal myristoylated accessory protein involved in post integration infection. Nef is found in the viral particle and is one of the first proteins to be produced after invasion of the host cell. Although HIV-1 Nef was originally named "negative factor," it has been shown to have a positive role in viral replication and pathogenesis. Nef is a viral protein that interacts with host cell signal transduction proteins to promote long-term survival of infected T cells [148,149] and for destruction of non-infected T cells by inducing apoptosis. Nef also advances the endocytosis and degradation of cell surface proteins, including CD4 [12,13] and MHC proteins [15]. This action possibly impairs cytotoxic T cell function, thereby helping the virus to evade the host immune response [15]. The multifunctional protein thus helps the virus maintain high levels of viral load and to overcome host immune defenses, contributing to the progression of AIDS. Nef also alters the intracellular signaling pathways in lymphocytic cells, thereby inducing a wide range of effects. In particular, Nef activates both AP-1 [10] and nuclear factor of activated T cell transcription factors [150], as well as the T cell receptor (TCR) chain signaling [151]. Furthermore, Nef activates the calcium dependent signaling in T lymphocytes in a TCR-independent manner [152].

HIV-1 Nef is also associated with the release of inflammatory factors from human macrophages [18]. Treatment of monocytic cells with proinflammatory cytokines,

such as tumor necrosis factor (TNF)-α, has been shown to alter HERV (ERV-3 and HERV-R) gene expression [153,154,155]. Thus it would be possible that the increased HERV expression in HIV-1 infected cells may involve the release of inflammatory factors. Investigating HERV expression patterns and expression of cytokines by monocytes infected with either a Nef defective virus or a wild type virus further validated this data.

4.4. Acute HIV-1 infection induces HERV-K(HML-2) expression

Upregulated HERV-K(HML-2) expression in HIV-1 infected individuals has been observed in previous studies [72,73,74,75,119]. Gontreras-Galindo detected HERV-K(HML-2) viral RNA in the plasma of HIV type 1 infected individuals as well as the increase of HERV-K expression by HIV-1 infection. In my analysis HERV-K(HML-2) transcripts could not be detected in the cells persistently infected with a laboratory HIV-1 strain. Possibly, HERV-K(HML-2) is only activated in HIV-1 infected cell lines for a short period after infection or after infection with a primary HIV-1 strain. Therefore a virus isolated from a HIV-1 infected patient was used to infect LC5-CD4 cells. The cells acutely infected with a primary patient isolate were then investigated with quantitative real-time RT-PCR and the HERV specific microarray. A high expression of HERV-K(HML-2) was detected in these cells acutely infected with a primary HIV-1 strain. Thus, HERV-K(HML-2) expression may be activated only in the acute phase of HIV-1 infection or after infection with a primary HIV-1 strain. After HERV-K(HML-2) detection in the cells acutely infected with a primary HIV-1 patient isolate, the expressed loci of HERV-K(HML-2) transcripts were analyzed and six HML-2 proviruses (c1_B; c3_B; c3_C; c3_E; c7_C; c10_B) located on chromosomes 1, 3, 7 and 10 were found to be active in the HIV-1 infected cells. In the non-infected cells only one transcribed provirus (c11_B) could be identified.

Another aspect that could cause the discrepancy in detection of HERV-K(HML-2) expression levels between my data and the results published in Gallindo et al. 2007 could be that the Gallindo group used HERV-K primers that cannot distinguish between the HERV-K groups HML-2 and HML-3. In contrast, the primers used for real-time RT-PCR analysis in this study specifically bind to HERV-K(HML-2) transcripts. Thus the increased HERK expression found by Gallindo in HIV-infected patients could reflect upregulation of HML-3 groups in addition to or rather than HML-2 groups. I demonstrated upregulation of the HML-3 but not the HML-2 group in

persistently infected cells, whereas members of the HML-2 group are active in cells acute infected with the primary HIV-1 strain. As the Gallindo study cannot distinguish between these two HML-groups, actually the reason for discrepancy between our studies might be the different primers used for HML-2 detection.

High expression of HERV-K proteins has been observed in cancers, autoimmune disease and neurodegenerative disorders, such as HIV encephalopathy (HIVE) [72,73,156,157]. Thus increased expression of HERV-K(HML-2) could be related to AIDS-associated diseases (e.g. HIVE, Kaposis's sarcoma). HERV-K(HML-2) proviruses are present in the genomes of humans, apes and old world monkeys since about 40 million years.

All human HERV-K(HML-2) proviruses described to date have mutations that are lethal for viral replication, but some full-length open reading frames (ORFs) encoding the viral primary translation products, Gag-Pol, Env, and cORF/K-Rev are present in multiple individual proviruses, suggesting that HERV-K(HML-2) might be capable of replicating by complementation and also raising the possibility of recombination among co-packaged HERV-K(HML-2) genomes [58].

In addition to *pol, gag* and *env* encoded proteins two accessory proteins with regulatory functions are produced. HERV-K Type 2 HERV-K(HML-2) members have been shown to encode for a 14 kDa protein within the *env* gene termed Rec [158]. This protein displays striking functional similarities to the HIV-1 Rev protein and may be involved in germ cell tumor genesis. However, compared with the HERV-K proviruses encoding Rev as described in Mayer et al. 2004 [158], none of the active HERV-K(HML-2) loci identified in this study seems to code for a Rec protein. All active loci are members of the HERV-K(HML-2) Type 1. Due to a 292-bp deletion in HERV-K(HML-2) type 1 and the generation of a specific splice donor site, the *env* open reading frame in this provirus type produces a protein called Np9 instead of Rec [64]. The Np9 protein has been linked to tumor genesis, because it is expressed in mammary carcinoma biopsies, germ cell tumor biopsies, and leukemia blood lymphocytes, but not in normal, non transformed cells [66]. Additionally, Np9 was shown to interact with the RING-type E3 ubiquitin ligase LNX (ligand of Numb protein X). The findings point to the possibility that NP9 is involved in the LNX/Notch/Numb pathway and therefore may affect tumor genesis [66]. Three (c1_B; c3_B and c3_C) of the six active HERV-K(HML-2) proviruses identified in the HIV-1 infected cells

have open reading frames for the Env protein, thus these proviruses might also code for the NP9 protein [117]. Two proviruses (c3_B and c1_B) identified in this study with the chromosomal localization 1q22 and 3q13.2 have been described to code for the NP9 protein previously [125,126]. Thus increased expression of HERV-K(HML-2) Np9 could be related to AIDS-associated cancer.

Mechanisms by which HERV-K(HML-2) might also be associated with diseases include the production of viral proteins with biological activities, such as the HERV-K(HML-2) encoded superantigens [159].

Superantigens are a class of antigens, which cause non-specific activation of T cells resulting in polyclonal T cell activation and massive cytokine release [160]. They do this by associating with MHC class II molecules and binding to T cells that express particular T cell receptor β chain variable genes. There are two groups of microorganisms that are known to include superantigens: bacteria and viruses. While a huge number of bacterial superantigens have been well characterized structurally and functionally, only three families of viruses have been associated with superantigen activity: retroviruses, rhabdoviruses and herpesviruses [161].

Superantigens are described as modifying factors in HIV infection [162]. Superantigens have been found to activate T cells and facilitate HIV expression in T cells derived from HIV-1 infected patients [163,164]. CD4 T cells in HIV-1 infected patients are primed to die through apoptosis [165,166,167,168]. The process of apoptotic death of CD4 T cells in HIV-1 infection comprises two steps, the priming of T cells for programmed cell death and stimulation of T cell receptors [165]. For example gp120 is considered as a candidate to prime T cells for programmed cell death [169]. These primed cells can undergo apoptotic death by stimulation through interaction of T cell receptors with specific superantigens [170]. Thus, superantigens represent the second signal for programmed cell death.

Sutkowski et al. identified a superantigen encoded by the envelope gene (*env*) of the human endogenous retrovirus (HERV)-K18 [89]. HERV-K18 is localized to chromosome 1q21.2 - q22 [171] and is normally transcriptionally silent. Since an active HERV-K provirus located on chromosome 1q22 in HIV-1 infected cells was found in this study, it might be possible that the HERV-K18 Env superantigen is presented on the surface of HIV infected cells. Furthermore superantigens have been implicated to autoimmune diseases like rheumatoid arthritis and Kawasaki disease

[172]. As HIV-1 infection is also associated with autoimmune diseases like Kawasaki disease [173,174,175], the HERV-K18 Env superantigen may contribute to the development of such a HIV-1 associated disease.

4.5. HIV-1 induced transcription factor activation (e.g. NF-κB) may be involved in increased HERV expression

As exogenous cofactors can significantly affect replication or expression of HIV in vivo [127,128], they may also affect HERV expression. HIV replication is strongly regulated at the transcriptional level through the specific interaction of viral regulatory proteins, namely Tat and cellular transcription factors binding to a variety of cis-acting DNA sequences in the HIV LTR [176]. One of the main mediators of HIV LTR transcription is the nuclear factor-κB (NF-κB) [177]. Two NF-κB sites in the HIV long terminal repeat (LTR) have been proposed to be involved in viral transcription and replication [129]. Interestingly, it has been reported that HIV infection induces NF-κB activation, which may suppress HIV-induced apoptosis in infected myeloid cells [178].

The transcription factor NF-κB is a critical regulator of many cellular processes including cell survival and inflammation. NF-κB functions as a hetero- or homodimer which can be formed from five NF-κB subunits, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. NF-κB activation involves its nuclear translocation that is initiated by multiple stimuli, such as pro-inflammatory cytokines, pathogens, antigenic peptides, developmental signals or environmental stressors. Activated NF-κB binds to regulatory elements on the DNA and induces expression of target genes involved in immune response, inflammation, survival, proliferation, differentiation or development [179,180].

To investigate if cellular transcription factors (e.g. NF-κB) activate HERV expression, standard stimuli (PMA/Ionomycin or antibodies against CD3/CD28) were used to activate the major transcription factors NF-κB, NFAT and AP1 in T cells [130,131,132,133].

To this end, Jurkat T cells were stimulated with PMA/Ionomycin or antibodies against CD3/CD28 for 3 hours. Several HERV families (HERV-T, HERV-E, HERV-F, HERV-W, ERV-9, HML-2, HML-3, HML-4, HML-6, HML-7, HML-9, HML-10 and HERV-L) showed increased expression in stimulated Jurkat cells. Thus it seems that activation

of cellular transcription factors could be the reason for the HERV upregulation in HIV-1 infected cells.

As HERV-K(HML-2) transcripts showed high expression in stimulated cells, the six HERV-K(HML-2) loci (c1_B; c3_B; c3_C, c3_E, c7_C and c10_B) found to be active in acutely HIV-1 infected cells were investigated for common transcription factor binding sites within their LTRs using bioinformatics tools. LTRs of four active HERV-K(HML-2) loci (c1_B; c3_B; c3_C and c3_E) contained NF-κB transcription factor binding sites, while missing NFAT and AP1 sites. This suggests that HML-2 proviruses may be activated in these cells through NF-κB binding. Provirus c7_C and c10_B lack the 5'LTR [117], suggesting that the transcription of these loci may be directed by unknown flanking promoters. Locus c7_C is located closely downstream of the SSBP1 gene and thus read-through events might have produced c7_C harboring transcripts [117]. As locus c10_B is located within an intron of gene (ABCC2), these alternative splicing events might have produced c10_B harboring transcripts [117]. The HML-2 locus c11_B found to be active in uninfected cells contains only one NF-κB transcription factor binding site with a different location compared to the NF-κB sites identified in HML-2 proviruses active in acutely HIV infected cells.

Several studies showed that HIV-1 production is associated with NF-κB activation [18,19,181,182,183,184,185]. For example in the promonocytic cell line U937, HIV-1 activates the inducible pool of NF-κB as a result of enhanced IκB-α degradation [181,182,183,184]. If LTRs of HERV families HERV-T, HERV-E, ERV-9, HML-3, HML-4 and HML-10 found to be active in persistently HIV-1 infected cells also contain NF-κB transcription factor binding sites, these HERV elements might also be upregulated through the transcription factor NF-κB. This could be analyzed in future studies e.g. by identification of the active loci of HERV-goups S71pCRTK-1, and HERV-KC(4) and the investigation of the existence of NF-κB binding sites within their LTR sequences. The other HERV groups 4-1, ERV-9, Seq59 and the HML-3 groups probably comprise too many proviruses [44] to allow analysis of single active loci. Additionally this hypothesis could be verified by investigation of HERV expression patterns after knockout of the NF-kB binding sites.

Several studies show that HIV-1 Nef activates NF-κB and therefore stimulates NF-κB dependent HIV-1 replication [18,19,185]. Overexpression of HIV-1 Nef in LC5 cells showed that HIV-1 Nef has great impact in boosting HERV expression. A hypothetical model for activation of HERV and HIV expression by Nef is shown in Figure 4.3. Through its N-terminal myristoylation, Nef is targeted to the cell membrane [186,187], where it can interact with a wide number of signaling host molecules. Afterward, Nef starts the retrograde intracellular path by associating with clathrin-coated pits, ultimately accumulating in the endosomal/lysosomal compartment [186,187,188,189]. Nef could interact with the catalytic subunit of the V-ATPase [190,191], inducing its inhibition. The V-ATPase inhibition triggers an intracellular signaling ultimately leading to NF-κB activation [18]. Activated NF-κB dimerizes, translocates into the nucleus where it may bind to the HIV LTR and to HERV LTRs and activate and increase their transcription, respectively.

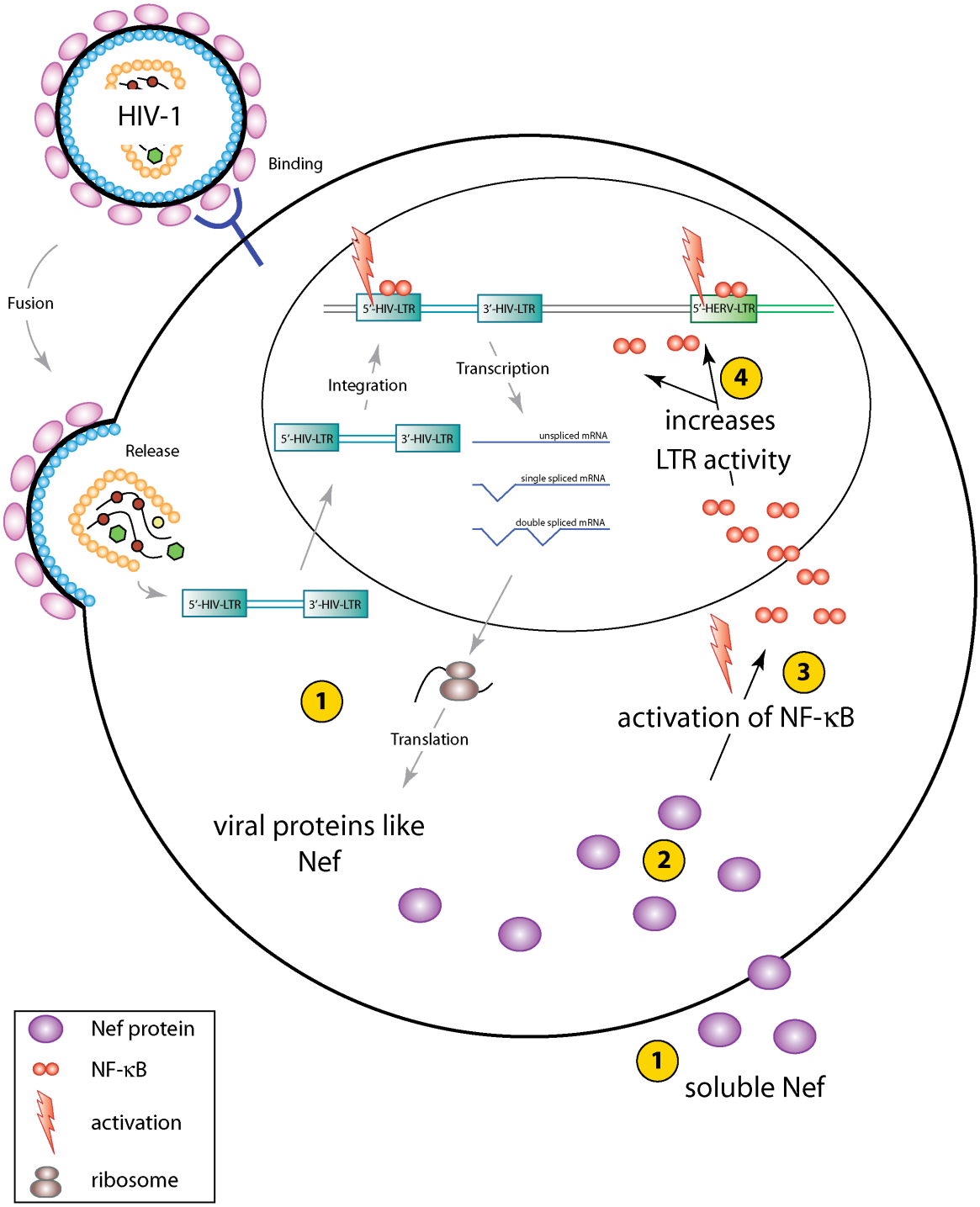


Figure 4.3.: A model for the Nef-induced activation of HERVs.
(1) Nef is produced in the cytoplasm during HIV-1 infection. (2) Furthermore it can be recruited to the cytoplasm through internalization from the extracellular milieu [192] (3) Independent of the origin, Nef molecules interact with the catalytic subunit of the V-ATPase, thereby inducing its inhibition. The V-ATPase inhibition leads to NF-κB activation. (4) Activated NF-κB dimers translocate into the nucleus and activate HIV-LTRs and/or induce HERV activation through binding to the NF-κB transcription factor binding sites within the HERV-LTRs.

4.6. Disease relevance of HIV-1 induced HERV expression

Evans et al 2009 [193] showed in mice that infection with an exogenous retrovirus (MULV) activates epression of endogenous retroviruses.

Retroviral elements that integrate in close proximity of cellular genes may influence normal genome function in their host. Although a *de novo* integration of activated HERV elements has not been described so far, an association of HERV gene transcription with disease has frequently been suggested [68,194,195]. Furthermore, LTRs of activated HERVs may influence the expression of adjacent oncogenes [196].

4.6.1. Expression of various cellular genes by HERV-E elements and possible association with angiogenesis

HERV-E transcription has been demonstrated in ovarian cancer [197] and rheumatoid arthritis [198] and its LTRs serves as promotor for several cellular genes [199,200,201,202]. LTR promoter/enhancers can enhance the transcription of native promoters. An example is the presence of an HERV-E LTR that increases the native promoter activity and expression of apolipoprotein C-I [199]. Such LTR promoter and enhancer functions can influence native promoters over a very long range; distances up to 100 kb have been observed [203].

Previous studies indicate that the LTR of HERV-E acts as an alternative promotor for the endothelin-B receptor [199], a receptor that regulates monocyte activity at sites of inflammation and the production by monocytes of putative neurotoxins such as nitric oxide [204].

Furthermore, in human malignant trophoblasts, HERV-E integration into the growth factor gene pleiotropin (PTN) has generated cell type-specific promoter activity [205]. Kaposis's sarcoma is an angioproliferative disease characterized by intense and aberrant angiogenesis [206,207]. Its appearance and aggressiveness is dramatically increased in HIV infected people. That is why there is a great deal of interest in anti-angiogenic HIV protease inhibitors [208]. As pleiotrophin is associated with angiogenesis [209,210] it would be of interest to determine if the HERV-E loci, where the HERV-E element is integrated upstream of the growth factor gene pleiotropin, is activated in the HIV-1 infected cells and thus is involved in the development of HIV-1 mediated Karposi's Sarcoma. HERV-E showed the highest upregulation in our results. The upregulation of the HERV-E subgroup 4-1 in HIV-1 infected cell lines

suggests a possible role of this HERV-E locus in HIV-1 associated Kaposis's sarcoma. This hypothesis could be clarified in future studies by investigation of the activated loci of HERV-E (4-1) in HIV-1 infected cells, which would be an extended study because HERV-E comprise many proviruses as described in Mager & Medstrand 2003 [44].

4.6.2. HERV-K(C4) expression in HIV-1 infected cells

HERV-K(C4) is located in intron 9 of the complement C4A6 gene but is also found in some C4B genes [211]. Such sequences appear to be principal contributors to the interlocus and interallelic heterogeneity of C4 genes. The occurrence of members of the HERV-K(C4) family in the C4A and C4B genes of the class III region of the human HLA complex has biological and immunological implications. For example, it is plausible that insertions of single or multiple copies of HERV-K(C4) into C4A or C4B sequences could alter important immunologic reactivities, such as complement-dependent cell meditated cytotoxicity, or engender autoimmune reactivities by alterations of HLA antigens. Moreover, regulatory sequences in the LTRs of HERV-K(C4) might affect the expression of C4A, C4B, or HLA genes. Since HIV-1 is capable of activating HERV-K(C4) expression, one could hypothesize that alterations of HLA genes could lead to cellular changes that help the virus to escape the immune system.

4.6.3. Immunsuppressive activity of HERV Env proteins

ERV9 HERVs appear to be severely truncated, although analysis of the 4 kb repetitive sequence revealed the presence of ORFs potentially coding for retrovirus-related *gag, pol* and *env* proteins [212]. Some retroviruses including HERV families such as ERV9 and HERV-E contain an immunosuppressive domain within the TM (Transmembrane) domain of the Env protein [213,214]. Introduction of an infectious murine retrovirus *env* expression vector presenting this domain into cancer cells can, in a mouse model, promote tumor growth by allowing escape from immune surveillance [215]. As ERV9 showed activation in the HIV-1 infected cells it would be of interest if there are proteins expressed from the coding ERV9 related *gag, pol* and *env* genes that might cause an escape from the immune surveillance and thus help HIV-1 to undergo the immune system.

4.6.4 HERVs and their therapeutic potential

Nixon et al 2007 [75] demonstrated that T-cells of the human immune system respond to presented HERV peptides derived from regions of upregulated HERVs in HIV-1 infected persons. The study has identified that HIV-1 infection leads to HERV expression, peptide presentation on MHC class I molecules and thereby stimulation of HERV-specific CD8+ T cell response. They showed HERV-specific immune responses in HIV-1 infection.

One great problem for the immune system to detect HIV-infected cells is that presented viral peptides on infected cells change rapidly over time due to high mutation rates of the virus. In this context it is interesting to speculate that infected cells could use HERVs as a tool to inform the immune system about their infection status. Also in my study several HERV families showed upregulated expression in HIV-1 infected cells. Several of these identified upregulated HERV families have the potential to encode for proteins like NP9 (HERV-K(HML-2)) or superantigens (e.g. HERV-K18 or E4-1). Therefore there might be the possibility that they are presented on HIV-1 infected cells.

If HIV-1 infected cells would produce HERV peptides as described above, HERVs would provide an effective surrogate target for the immune response to eliminate HIV-1-infected cells and could bear the possibility of candidates for inclusion in a new type of HIV-1 vaccine. Thus, these CD8+ T cells would be an important factor in controlling HIV infection.

Furthermore, an antigen-encoding region, CT-RCC, was found to be part of a specific HERV-E locus highly expressed in metastatic renal cell carcinoma (RCC) but not in normal tissue, making them also potential targets for tumor immunotherapy [216,217].

5. Summary

Approximately 8-9% of the human genome is composed of endogenous retroviral elements (HERVs). Although most HERVs are silenced by a variety of mechanisms, they may be reactivated by environmental stimuli like exogenous viruses. As there is evidence that HERVs may contribute to pathogenic conditions such as cancer, autoimmune diseases, and neurological disorders it is important to examine the effect of exogenous viruses such as HIV-1 on the transcriptional activity of HERVs.

HERV expression profiles of three persistently HIV-1 infected cell lines (derived from T-cells, HeLa cells and astrocytes) that differ in their levels of HIV-1 virus production were compared using a retrovirus *pol* specific microarray. Several HERV elements belonging to class I and II HERV families were found to be upregulated in all three persistently HIV-1 infected cell lines. The results were confirmed and quantified using real-time RT-PCR methods. Reduction of HIV-1 transcript levels by artificial inhibitors (i.e. siRNAs) resulted in a decrease in HERV expression. Furthermore a novel family of host factors (ie. Risp) was identified in the course of this study that can modulate HIV-1 production. Overexpression of Risp proteins reduced HIV production and also decreased the expression of the upregulated HERVs. Together these results demonstrate a direct link between HIV production and expression levels of selected HERV families. Studies investigating the influence of the HIV-1 proteins Tat, Rev and Nef on HERV expression indicated that HIV-1 Nef seems to be essential for boosting HERV expression. In addition to investigate the relationship between HIV production and HERV expression during chronic infection, I analyzed HERV expression profiles in cells acutely infected with a primary patient-derived HIV-1 isolate. Interestingly, the acutely HIV-1 infected cells showed distinctly upregulated expression of HERV-K(HML-2), compared to uninfected cells. In contrast, HML-2 expression was not upregulated in the persistently infected cells. Six HML-2 proviruses were found to be transcribed in acutely HIV-1 infected cells, in contrast only one transcriptionally active HML-2 locus was found to be active in the uninfected control cell line. Stimulation with PMA/Ionomycin or antibodies against CD3/CD28 to activate the transcription factors NF-κB, NFAT and AP1 increased the HERV expression pattern of class I, II and III HERVs. Therefore, major transcription factors like NF-κB, NFAT and AP1 seem to be involved in the activation of HERV expression.

These data demonstrate that productive HIV infection can lead to alterations in the transcription patterns of various HERV families in multiple human cell types. The

results indicate the involvement of viral proteins, as well as cellular transcription factors (e.g. NF-κB) in activation of HERV expression. Several HERVs found to be active in HIV-1 infected cells are associated with biological processes like angiogenesis or immunosuppression. This suggests that the upregulation of HERV expression may influence the pathogenicity of HIV-1.

6. Zusammenfassung

Das humane Genom enthält eine Vielzahl an humanen endogenen Retroviren (HERV). Noch vor einigen Jahren ging man davon aus, dass nur etwa 2% des humanen Genoms aus endogenen Retroviren besteht. Inzwischen ist jedoch bekannt, dass der Anteil an HERVs im humanen Genom etwa 8 - 9 % beträgt und HERVs somit eine bedeutende Komponente des menschlichen Genoms darstellen.

Viele HERVs enthalten funktionelle Promotoren, Enhancer-Elemente und Polyadenylierungssignale. Diese regulatorischen Sequenzen können die Expression von benachbarten Genen beeinflussen. Eine solche Modulation könnte zur Aktivierung von Onkogenen oder zur Inaktivierung von Tumorsuppressorgenen führen. HERVs werden daher mit verschiedensten Krankheiten in Zusammenhang gebracht (z.B. Krebs, Autoimmunerkrankungen und neurologischen Erkrankungen).

Fast alle HERVs sind durch epigenetische Kontrollmechanismen inaktiviert worden. HERVs können jedoch durch exogene Viren wieder reaktiviert werden. Daher ist es von Interesse den Einfluss von exogenen Viren wie HIV-1 auf die Expression von HERVs in menschlichen Zellen zu untersuchen.

Drei persistent HIV-1 infizierte Zelllinien (T-Zellen, HeLa-Zellen und Astozyten), welche sich in ihren Levels an HIV-1 Virus Produktion unterscheiden, wurden mittels eines Retroviren *pol* spezifischen Microarrays untersucht. Einige HERV-Elemente der Klasse I und II HERV Familien zeigten eine starke Expressionserhöhung in allen drei HIV-1 infizierten Zelllinien. Diese Ergebnisse konnten auch mittels real-time RT-PCR verifiziert werden. Eine Reduktion der HIV-1 Transkripte mittels artifizieller Inhibitoren (z.B. siRNAs) ergab eine Reduzierung der HERV-Elemente in Ihrer Expression. Des Weiteren wurde in dieser Arbeit eine neue Familie an Proteinen (Risp) gefunden, welche die HIV-1 Produktion beeinflussen. Eine Überexpression dieser Risp Proteine hemmte die HIV-1 Produktion und zeigte zudem eine Reduzierung der HERV Expression. Diese Daten weißen auf einen direkten Zusammenhang zwischen HIV-1 Produktion und der HERV Expression hin. Zudem wurde der Einfluss der einzelnen HIV-1 Proteine Tat, Rev und Nef auf die HERV Expression untersucht. In diesen Untersuchungen schien HIV-1 Nef essentiell für den Anstieg der HERV Expression zu sein. Um zusätzlich das Verhältnis zwischen HIV Produktion und HERV Expression während der chronischen Infektion zu untersuchen, wurde das HERV Expressions-Profil auch in Zellen untersucht, welche mit einem primären HIV-1 Patientenisolat infiziert wurden. Interesannterweise zeigten

diese akut mit einem Patientenisolat infizierten Zellen, einen deutlichen Anstieg an HERV-K(HML-2) Expression verglichen zu den nicht infizierten Zellen. In persistent infizierten Zellen konnte die HERV-K(HML-2) Expressionserhöhung nicht festgestellt werden. Um einen Aufschluss zu bekommen welche Loci in den akut infizierten Zellen für die HERV-K(HML-2) Erhöhung verantwortlich sind, wurden die Zellen auf aktive HERV-K(HML-2) loci untersucht. In den akut infizierten Zellen konnten sechs transkriptionell aktive Proviren gefunden werden, wohingegen in den nicht infizierten Zellen nur ein Provirus aktiv war. Zudem konnte gezeigt werden, dass die Aktivierung der Transkriptionsfaktoren NF-κB, NFAT und AP1, durch Stimulierung von T-Zellen mit PMA/Ionomycin oder Antikörpern gegen CD3/CD28, eine Expressionserhöhung der HERV Familien der Klasse I, II und III zur Folge hatte. Dem zu Folge scheinen bedeutende Transkriptionsfaktoren wie NF-κB, NFAT und AP1 an der Aktivierung von HERVs beteiligt zu sein.

Die Daten zeigen, dass eine Infektion mit HIV-1 in verschiedenen humanen Zelllinien zu Veränderungen des Transkriptionsprofils von verschiedenen HERV Familien führt. Die Ergebnisse deuten auch darauf hin, dass virale Proteine (Nef) als auch zelluläre Transkriptionsfakoren (z.B. NF-κB) an der Aktivierung der HERV Expression beteiligt sind. Mehrere HERVs, welche in den infizierten Zellen eine höhere Expression zeigten als in den nichtinfizierten Kontroll-Zelllinien, sind assoziiert mit biologischen Prozessen wie Angiogenese oder Immunsupression. Dies würde darauf hindeuten, dass die aktivierten HERVs die HIV-1 Pathogenität beeinflussen könnten.

7. Conclusions and Perspectives

These data demonstrate that HIV protein expression leads to alterations in HERV transcription pattern in multiple human cell types. Furthermore the results indicate that transcription factors (e.g. NF-κB) are involved to activate HERV expression.

In this study, I obtained evidence suggesting that Nef mediates overexpression of HERV transcripts (see result 3.4 and discussion 4.3). To further validate this hypothesis one could investigate the HERV expression pattern after infection of LC5 cells with a Nef-deficient virus.

Furthermore, I showed that six HERV-K(HML-2) proviruses (c1_B, c3_B, c3_C, c3_E, c7_C and c10_B) are active in acute HIV-1 infected cells, of which three (c1_B; c3_B and c3_C) can possibly encode for the Env protein, NP9 (see result 3.5.2 and discussion 4.4). As the Np9 protein is associated with AIDS-related cancers, it is of interest if the Np9 protein is in fact expressed in HIV infected cells. To this end, HIV infected cells could be analyzed for Np9 protein production by Western Blot.

Overexpression of HERV-K18 and E4-1 in HIV infected cell lines indicated that these cells might present superantigens on their surface. As these superantigens could serve as a tool to inform the immune system about the HIV infection status of the cell (see discussion 4.4), it would be interesting to investigate the presence of these superantigens on the surface of the LC5-HIV cells.

8. Abbreviations

AP1	Activator protein 1
ARM	Arginin rich motif
ATP	Adenosintriphosphat
bp	base pair
BSA	Bovine Serum Albumine
CD4	Cluster of Differentiation 4
CDK	Cyclin Dependent Kinase
CIP	Calf Intestinal Alkaline Phosphatase
CMV	Cytomegalovirus
CRM1	Exportin1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxynukleotidtriphosphat
E.coli	Escherichia coli
EDTA	Ethylendiamintetraacetat
FACS	Fluorescence activated cell sorter
FCS	Fetal Calf Serum
GDP	Guanosindiphosphat
GFP	Green Fluorescent Protein aus Aequorea victoria
GTP	Guanosintriphosphat
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukemia virus
INS	Instabilitätselement
kb	kilo bases
kDa	kilo Dalton
LTR	Long terminal repeat
MHC	Major Histocompatibility Complex
mRFP	monomeric Red Fluorescent Protein
mRNA	Messenger ribonucleic acid
NES	Nuclear Export Signal
NFAT	Nuclear factor of activated T cells

NF-kB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLS	Nuclear Localization Signal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIM	Protease inhibitor mixture
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonylfluorid
RNA	Ribonucleic acid
RRE	Rev Response element
SDS	Sodiumdodecylsulfat
SV40	Simian Virus 40
Taq	Thermus aquaticus
TAR	Transactivation Response Element
TNFα	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl-)aminomethan
tRNA	Transfer-RNA
U	Units

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10. Supplement

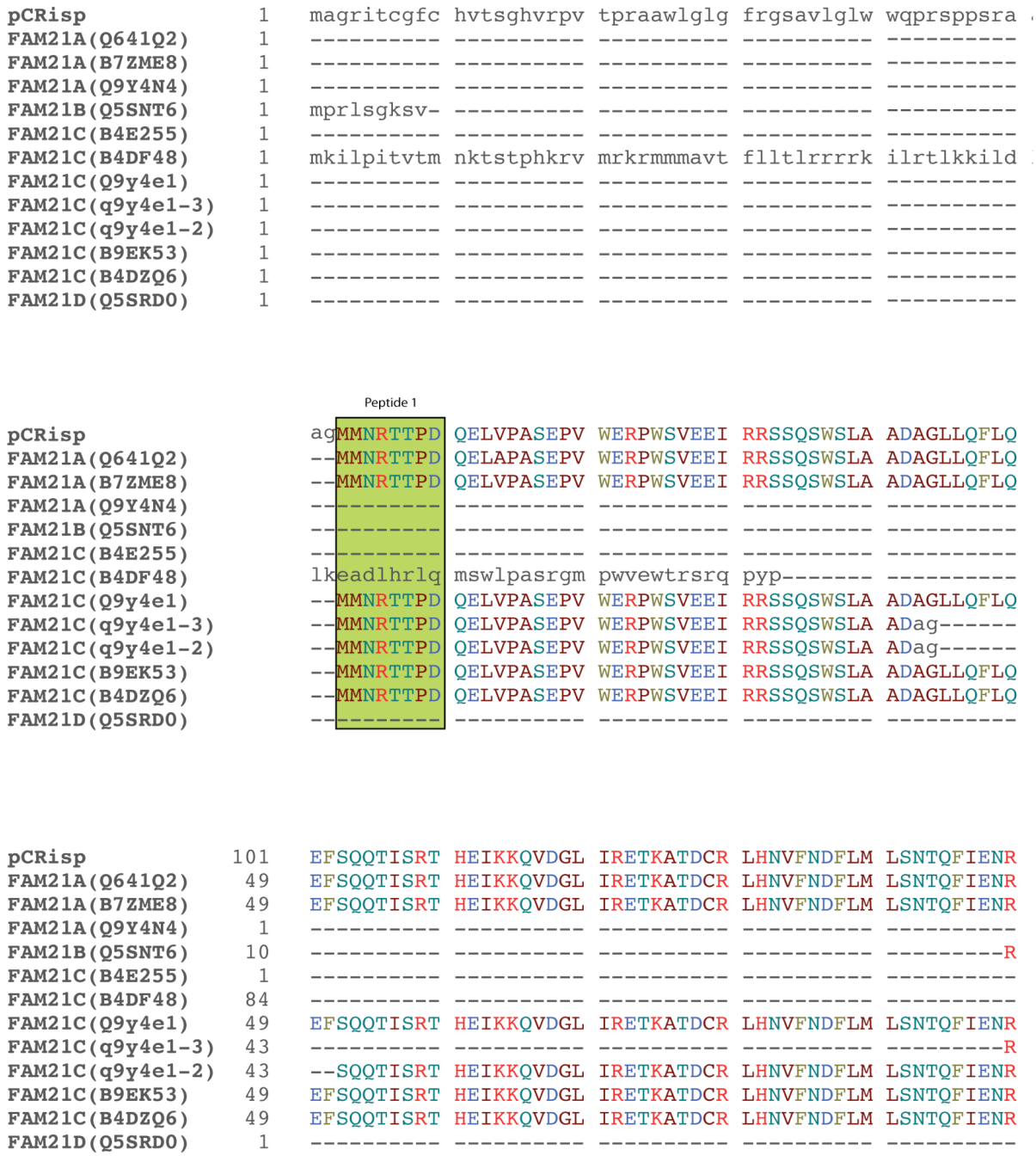
Aligned Sequences:

No.	Sequence Name	Sequence Description	Length
1	pCRisp	pCRisp	1372 aa
2	FAM21A(Q641Q2)	FAM21A(Q641Q2)	1341 aa
3	FAM21A(B7ZME8)	FAM21A(B7ZME8)	1320 aa
4	FAM21A(Q9Y4N4)	FAM21A(Q9Y4N4)	490 aa
5	FAM21B(Q5SNT6)	FAM21B(Q5SNT6)	1253 aa
6	FAM21C(B4E255)	FAM21C(B4E255)	531 aa
7	FAM21C(B4DF48)	FAM21C(B4DF48)	1023 aa
8	FAM21C(Q9y4e1)	FAM21C(Q9y4e1)	1320 aa
9	FAM21C(q9y4e1-3)	FAM21C(q9y4e1-3) splice variant	1265 aa
10	FAM21C(q9y4e1-2)	FAM21C(q9y4e1-2) splice variant	1312 aa
11	FAM21C(B9EK53)	FAM21C(B9EK53)	1279 aa
12	FAM21C(B4DZQ6)	FAM21C(B4DZQ6)	1245 aa
13	FAM21D(Q5SRD0)	FAM21D(Q5SRD0)	308 aa

Alignment (DiAlign format):

Color code is:

- basic amino acids
- nonpolar amino acids
- uncharged polar amino acids
- acidic amino acids
- aromatic amino acid


Peptide 1
pCRisp 1 magritcgfc hvtsghvrpv tpraawlglg frgsavlglw wqprsppsra
FAM21A(Q641Q2) 1 ---------- ---------- ---------- ---------- ----------
FAM21A(B7ZME8) 1 ---------- ---------- ---------- ---------- ----------
FAM21A(Q9Y4N4) 1 ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6) 1 mprlsgksv- ---------- ---------- ---------- ----------
FAM21C(B4E255) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48) 1 mkilpitvtm nktstphkrv mrkrmmmavt flltlrrrrk ilrtlkkild
FAM21C(Q9y4e1) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(q9y4e1-3) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(q9y4e1-2) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(B9EK53) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(B4DZQ6) 1 ---------- ---------- ---------- ---------- ----------
FAM21D(Q5SRD0) 1 ---------- ---------- ---------- ---------- ----------
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FAM21A(Q641Q2) --MMNRTTPD QELAPASEPV WERPWSVEEI RRSSQSWSLA ADAGLLQFLQ
FAM21A(B7ZME8) --MMNRTTPD QELVPASEPV WERPWSVEEI RRSSQSWSLA ADAGLLQFLQ
FAM21A(Q9Y4N4) ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6) ---------- ---------- ---------- ---------- ----------
FAM21C(B4E255) ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48) lkeadlhrlq mswlpasrgm pwvewtrsrq pyp------- ----------
FAM21C(Q9y4e1) --MMNRTTPD QELVPASEPV WERPWSVEEI RRSSQSWSLA ADAGLLQFLQ
FAM21C(q9y4e1-3) --MMNRTTPD QELVPASEPV WERPWSVEEI RRSSQSWSLA ADag------
FAM21C(q9y4e1-2) --MMNRTTPD QELVPASEPV WERPWSVEEI RRSSQSWSLA ADag------
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FAM21C(B4DZQ6) --MMNRTTPD QELVPASEPV WERPWSVEEI RRSSQSWSLA ADAGLLQFLQ
FAM21D(Q5SRD0) ---------- ---------- ---------- ---------- ----------
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FAM21A(Q641Q2) 49 EFSQQTISRT HEIKKQVDGL IRETKATDCR LHNVFNDFLM LSNTQFIENR
FAM21A(B7ZME8) 49 EFSQQTISRT HEIKKQVDGL IRETKATDCR LHNVFNDFLM LSNTQFIENR
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FAM21B(Q5SNT6) 10 ---------- ---------- ---------- ---------- ---------R
FAM21C(B4E255) 1 ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48) 84 ---------- ---------- ---------- ---------- ----------
FAM21C(Q9y4e1) 49 EFSQQTISRT HEIKKQVDGL IRETKATDCR LHNVFNDFLM LSNTQFIENR
FAM21C(q9y4e1-3) 43 ---------- ---------- ---------- ---------- ---------R
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FAM21C(B9EK53) 49 EFSQQTISRT HEIKKQVDGL IRETKATDCR LHNVFNDFLM LSNTQFIENR
FAM21C(B4DZQ6) 49 EFSQQTISRT HEIKKQVDGL IRETKATDCR LHNVFNDFLM LSNTQFIENR
FAM21D(Q5SRD0) 1 ---------- ---------- ---------- ---------- ----------

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FAM21A(B7ZME8)        VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21A(Q9Y4N4)        ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)        VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21C(B4E255)        ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)        ---------- ---------- ---------- ---------- ----------
FAM21C(Q9y4e1)        VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21C(q9y4e1-3)      VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21C(q9y4e1-2)      VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21C(B9EK53)        VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21C(B4DZQ6)        VYDEEVEEPV LKAEAEKTEQ EKTREQKEVD LIPKVQEAVN YGLQVLDSAF
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------

pCRisp            201 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21A(Q641Q2)    149 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21A(B7ZME8)    149 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21A(Q9Y4N4)      1 ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)     61 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21C(B4E255)      1 ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)     84 ---------- ---------- ---------- ---------- ----------
FAM21C(Q9y4e1)    149 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21C(q9y4e1-3)   94 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21C(q9y4e1-2)  141 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21C(B9EK53)    149 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21C(B4DZQ6)    149 EQLDIKAGNS DSEEDDANGR VELILEPKDL YIDRPLPYLI GSKLFMEQED
FAM21D(Q5SRD0)      1 ---------- ---------- ---------- ---------- ----------

                                                          Peptide 2
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FAM21A(B7ZME8)        VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNRHTTQ
FAM21A(Q9Y4N4)        ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)        VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNRHTTQ
FAM21C(B4E255)        ---------- ---------- ---------- ----------
FAM21C(B4DF48)        ---------- ---------- ---------- ----------
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FAM21C(q9y4e1-3)      VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNQHTTQ
FAM21C(q9y4e1-2)      VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNQHTTQ
FAM21C(B9EK53)        VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNRHTTQ
FAM21C(B4DZQ6)        VGLGELSSEE GSVGSDRGSI VDTEEEKEEE ESDEDFAHHS DNEQNQHTTQ
FAM21D(Q5SRD0)        ---------- ---------- ---------- ----------
```

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pCRisp              301   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21A(Q641Q2)      249   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21A(B7ZME8)      249   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21A(Q9Y4N4)        1   ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)      161   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21C(B4E255)        1   ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)       84   ---------- ---------- ---------- ---------- ----------
FAM21C(Q9y4e1)      249   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21C(q9y4e1-3)    194   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21C(q9y4e1-2)    241   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21C(B9EK53)      249   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21C(B4DZQ6)      249   MSDEEEDDDG CDLFADSEKE EEDIEDIEEN TRPKRSRPTS FADELAARIK
FAM21D(Q5SRD0)        1   ---------- ---------- ---------- ---------- ----------

pCRisp                    GDAMGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFAPPKLTD
FAM21A(Q641Q2)            GDAVGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFAPPKLTD
FAM21A(B7ZME8)            GDAVGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFTPPKLTD
FAM21A(Q9Y4N4)            ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)            GDAVGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFTPPKLTD
FAM21C(B4E255)            ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)            ---------- ---------- ---------- ---------- ----------
FAM21C(Q9y4e1)            GDAMGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFAPPKLTD
FAM21C(q9y4e1-3)          GDAMGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFAPPKLTD
FAM21C(q9y4e1-2)          GDAMGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFAPPKLTD
FAM21C(B9EK53)            GDAVGRVDEE PTTLPSGEAK PRKTLKEKKE RRTPSDDEED NLFTPPKLTD
FAM21C(B4DZQ6)            GDAMGRVDEE PTn------- ---------- -------EED NLFAPPKLTD
FAM21D(Q5SRD0)            ---------- ---------- ---------- ---------- ----------

pCRisp              401   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEASQDRQAG ASVKEESSSS
FAM21A(Q641Q2)      349   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEAPQDRQAG ASVKEESSSS
FAM21A(B7ZME8)      349   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEAPQDRQAG ASVKEESSSS
FAM21A(Q9Y4N4)        1   ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)      261   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEAPQDRQAG ASVKEESSSS
FAM21C(B4E255)        1   ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)       84   ---------- ---------- ----QESDLF TEAPQDRQAG ASVKEESSSS
FAM21C(Q9y4e1)      349   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEASQDRQAG ASVKEESSSS
FAM21C(q9y4e1-3)    294   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEASQDRQAG ASVKEESSSS
FAM21C(q9y4e1-2)    341   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEASQDRQAG ASVKEESSSS
FAM21C(B9EK53)      349   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEAPQDRQAG ASVKEESSSS
FAM21C(B4DZQ6)      325   EDFSPFGSGG GLFSGGKGLF DDEDEESDLF TEASQDRQAG ASVKEESSSS
FAM21D(Q5SRD0)        1   ---------- ---------- ---------- ---------- ----------
```

```
pCRisp                KPGKKIPAGA VSVFLGDTDV FGAASVPSLK EPQKPEQPTP RKSPYGPPPT
FAM21A(Q641Q2)        KPGKKIPAGA VSVFLGDTDV FGAASVPSMK EPQKPEQPTP RKSPYGPPPT
FAM21A(B7ZME8)        KPGKKIPAGA VSVFLGDTDV FGAASVPSMK EPQKPEQPTP RKSPYGPPPT
FAM21A(Q9Y4N4)        ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)        KPGKKIPAGA VSVFLGDTDV FGAASVPSMK EPQKPEQPTP RKSPYGPPPT
FAM21C(B4E255)        ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)        KPGKKIPAGA VSVFLGDTDV FGAASVPSLK EPQKPEQPTP RKSPYGPPPT
FAM21C(Q9y4e1)        KPGKKIPAGA VSVFLGDTDV FGAASVPSLK EPQKPEQPTP RKSPYGPPPT
FAM21C(q9y4e1-        KPGKKIPAGA VSVFLGDTDV FGAASVPSLK EPQKPEQPTP RKSPYGPPPT
FAM21C(q9y4e1-        KPGKKIPAGA VSVFLGDTDV FGAASVP LK EPQKPEQPTP RKSPYGPPPT
FAM21C(B9EK53)        KPGKKIPAGA VSVFLGDTDV FGAASVPSMK EPQKPEQPTP RKSPYGPPPT
FAM21C(B4DZQ6)        KPGKKIPAGA VSVFLGDTDV FGAASVPSLK EPQKPEQPTP RKSPYGPPPT
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------

pCRisp            501 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21A(Q641Q2)    449 GLFDDDDGDD DDDFFSAPHS KPSKTGKVQS TADIFGDEEG DLFKEKAVAS
FAM21A(B7ZME8)    449 GLFDDDDGDD DDDFFSAPHS KPSKTGKVQS TADIFGDEEG DLFKEKAVAS
FAM21A(Q9Y4N4)      1 ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)    361 GLFDDDDGDD DDDFFSAPHS KPSKTGKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(B4E255)      1 ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)    160 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(Q9y4e1)    449 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(q9y4e1-3)  394 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(q9y4e1-2)  441 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(B9EK53)    449 GLFDDDDGDD DDDFFSAPHS KPSKTGKVQS TADIFGDEEG DLFKEKAVAS
FAM21C(B4DZQ6)    425 GLFDDDDGDD DDDFFSAPHS KPSKTRKVQS TADIFGDEEG DLFKEKAVAS
FAM21D(Q5SRD0)      1 ---------- ---------- ---------- ---------- ----------

                                                    Peptide 3
pCRisp                PEATVSQTDE NKARAEKKVT LSSSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21A(Q641Q2)        PEATVSQTDE NKARAEKKVT LSSSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21A(B7ZME8)        PEATVSQTDE NKARAEKKVT LSYSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21A(Q9Y4N4)        ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)        PEATVSQTDE NKARAEKKVT LSSSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21C(B4E255)        ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)        PEATVSQTDE NKARAEKK-- ---------- ---------- ---------DLF
FAM21C(Q9y4e1)        PEATVSQTDE NKARAEKKVT LSYSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21C(q9y4e1-3)      PEATVSQTDE NKARAEKKVT LSYSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21C(q9y4e1-2)      PEATVSQTDE NKARAEKKVT LSYSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21C(B9EK53)        PEATVSQTDE NKARAEKKVT LSYSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21C(B4DZQ6)        PEATVSQTDE NKARAEKKVT LSSSKNLKPS SETKTQKGLF SDEEDSEDLF
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------
```

```
pCRisp              601   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21A(Q641Q2)      549   SSQSASKLKG ASLLPGKLPT LVSLFDDEDE EDNLFGGTAA KKQTLCLQAQ
FAM21A(B7ZME8)      549   SSQSASKLKG ASLLPGKLPT LVSLFDDEDE EDNLFGGTAA KKQTLCLQAQ
FAM21A(Q9Y4N4)        1   ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)      461   SSQSASKLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLCLQAQ
FAM21C(B4E255)        1   ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)      231   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21C(Q9y4e1)      549   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21C(q9y4e1-3)    494   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21C(q9y4e1-2)    541   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21C(B9EK53)      549   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21C(B4DZQ6)      525   SSQSASNLKG ASLLPGKLPT SVSLFDDEDE EDNLFGGTAA KKQTLSLQAQ
FAM21D(Q5SRD0)        1   ---------- ---------- ---------- ---------- ----------

pCRisp                    REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21A(Q641Q2)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21A(B7ZME8)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21A(Q9Y4N4)            ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(B4E255)            ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(Q9y4e1)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(q9y4e1-3)          REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(q9y4e1-2)          REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(B9EK53)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21C(B4DZQ6)            REEKAKASEL SKKKASALLF SSDEEDQWNI PASQTHLASD SRSKGEPRDS
FAM21D(Q5SRD0)            ---------- ---------- ---------- ---------- ----------

pCRisp              701   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21A(Q641Q2)      649   GTLQSQEAKA VKKTSLFEED EEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21A(B7ZME8)      649   GTLQSQEAKA VKKTSLFEED EEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21A(Q9Y4N4)        1   ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)      561   GTLQSQEAKA VKKTSLFEED EEDDLFAIAK DSQKKTQRVS LLFEDDVDSR
FAM21C(B4E255)        1   ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)      331   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21C(Q9y4e1)      649   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21C(q9y4e1-3)    594   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21C(q9y4e1-2)    641   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21C(B9EK53)      649   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21C(B4DZQ6)      625   GTLQSQEAKA VKKTSLFEED KEDDLFAIAK DSQKKTQRVS LLFEDDVDSG
FAM21D(Q5SRD0)        1   ---------- ---------- ---------- ---------- ----------
```

```
pCRisp                GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21A(Q641Q2)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21A(B7ZME8)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21A(Q9Y4N4)        ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(B4E255)        ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(Q9y4e1)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(q9y4e1-3)      GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(q9y4e1-2)      GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(B9EK53)        GSLFGSPPTS VPPATKKKET VSEAPPLLFS DEEEKEAQLG VKSVDKKVES
FAM21C(B4DZQ6)        GSLFGSPPTS VPPAT----- ---------- ---------- ----------
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------
```

Peptide 4

```
pCRisp          801   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21A(Q641Q2)  749   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21A(B7ZME8)  749   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21A(Q9Y4N4)    1   ---------- ---------- ---------- ---------- ----------
FAM21B(Q5SNT6)  661   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(B4E255)    1   ---------- ---------- ---------- ---------- ----------
FAM21C(B4DF48)  431   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(Q9y4e1)  749   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(q9y4e1-3) 694  AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(q9y4e1-2) 741  AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(B9EK53)  749   AKESLKFGRT DVAESEKEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21C(B4DZQ6)  690   ---------- ------KEGL LTRSAQETVK HSDLFSSSSP WDKGTKPRTK
FAM21D(Q5SRD0)    1   ---------- ---------- ---------- ---------- ----------
```

```
pCRisp                TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21A(Q641Q2)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21A(B7ZME8)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21A(Q9Y4N4)        ---------- ---------- ---------- --DAHPKSTG VFQDEELLFS
FAM21B(Q5SNT6)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21C(B4E255)        ---------- --MEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21C(B4DF48)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(Q9y4e1)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(q9y4e1-3)      TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(q9y4e1-2)      TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(B9EK53)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(B4DZQ6)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------
```

```
pCRisp                TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21A(Q641Q2)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21A(B7ZME8)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21A(Q9Y4N4)        ---------- ---------- ---------- --DAHPKSTG VFQDEELLFS
FAM21B(Q5SNT6)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21C(B4E255)        ---------- --MEDQNIIQ APQKEVGKGR DPDAHPKSTG VFQDEELLFS
FAM21C(B4DF48)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(Q9y4e1)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(q9y4e1-3)      TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(q9y4e1-2)      TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(B9EK53)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21C(B4DZQ6)        TVLSLFDEEE DKMEDQNIIQ APQKEVGKGC DPDAHPKSTG VFQDEELLFS
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------

pCRisp          901   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21A(Q641Q2)  849   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21A(B7ZME8)  849   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21A(Q9Y4N4)   19   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21B(Q5SNT6)  761   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21C(B4E255)   39   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21C(B4DF48)  531   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL LGDDEDDDLF SSAKSQPLVQ
FAM21C(Q9y4e1)  849   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21C(q9y4e1-3) 794  HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21C(q9y4e1-2) 841  HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21C(B9EK53)  849   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPL--
FAM21C(B4DZQ6)  774   HKLQKDNDPD VDLFAGTKKT KLLEPSVGSL FGDDEDDDLF SSAKSQPLVQ
FAM21D(Q5SRD0)    1   ---------- ---------- ---------- ---------- ----------

pCRisp                EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21A(Q641Q2)        EKKRVVKKDH SVDSFKNQKH PESIQGSKEK GIWKPETPQD SSGLAPFKTK
FAM21A(B7ZME8)        EKKRVVKKDH SVDSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21A(Q9Y4N4)        EKKRVVKKDH SVDSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21B(Q5SNT6)        EKKRVVKKDH SVDSFKNQKH PESIQGSKEK GIWKPETPQD SSGLAPFKTK
FAM21C(B4E255)        EKKRVVKKDH SVDSFKNQKH PESIQGSKEK GIWKPETPQD SSGLAPFKTK
FAM21C(B4DF48)        EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETPQD SSGLAPFKTK
FAM21C(Q9y4e1)        EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21C(q9y4e1-3)      EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21C(q9y4e1-2)      EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21C(B9EK53)        ---------- ---------- ---------- ---------- ----------
FAM21C(B4DZQ6)        EKKRVVKKDH SVNSFKNQKH PESIQGSKEK GIWKPETP-- ----------
FAM21D(Q5SRD0)        ---------- ---------- ---------- ---------- ----------
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```
pCRisp              989   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21A(Q641Q2)      949   EPSTRIGKIQ ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21A(B7ZME8)      937   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21A(Q9Y4N4)      107   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21B(Q5SNT6)      861   EPSTRIGKIQ ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(B4E255)      139   EPSTRIGKIQ ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(B4DF48)      631   EPSTRIGKIQ ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(Q9y4e1)      937   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(q9y4e1-3)    882   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(q9y4e1-2)    929   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(B9EK53)      897   ---------- ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21C(B4DZQ6)      862   ---------Q ANLAINPAAL LPTAASQISE VKPVLPELAF PSSEHRRSHG
FAM21D(Q5SRD0)        1   ---------- ---------- ---------- ---------- ----------

pCRisp                    LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21A(Q641Q2)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21A(B7ZME8)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21A(Q9Y4N4)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21B(Q5SNT6)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(B4E255)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(B4DF48)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(Q9y4e1)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(q9y4e1-3)          LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(q9y4e1-2)          LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(B9EK53)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21C(B4DZQ6)            LESVPVLPGS GEAGVSFDLP AQADTLHSAN KSRVKMRGKR RPQTRAARRL
FAM21D(Q5SRD0)            ---------- ---------- ---------- -----MRGKR RPQTRAARRL

pCRisp              1080  AAQESSEAED MSIPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21A(Q641Q2)      1049  AAQESSETED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21A(B7ZME8)      1028  AAQESSETED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21A(Q9Y4N4)       198  AAQESSETED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21B(Q5SNT6)       961  AAQESSETED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(B4E255)       239  AAQESSETED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(B4DF48)       731  AAQESSEAED MSIPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(Q9y4e1)      1028  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(q9y4e1-3)     973  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(q9y4e1-2)    1020  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(B9EK53)       987  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21C(B4DZQ6)       953  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
FAM21D(Q5SRD0)        16  AAQESSEAED MSVPRGPIAQ WADGAISPNG HRPQLRAASG EDSTEEALAA
```

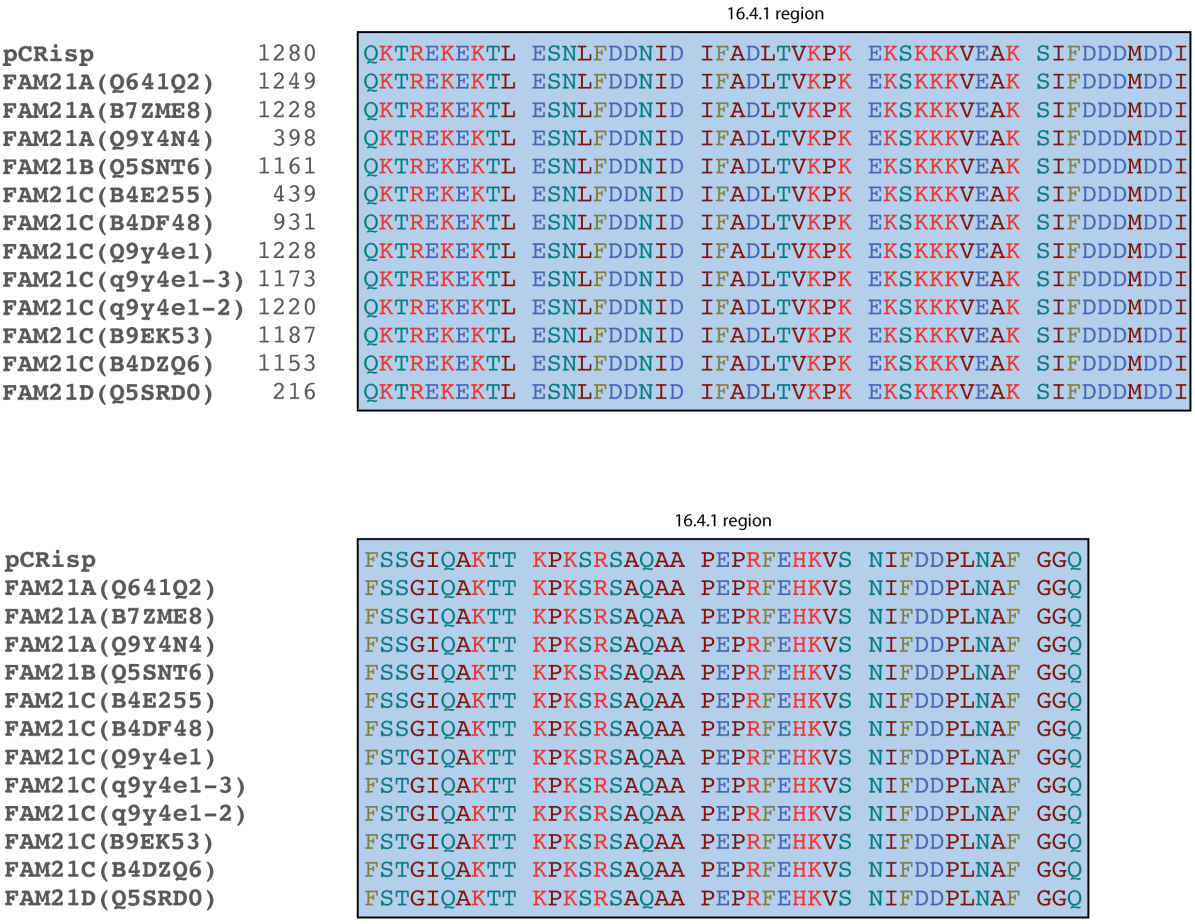
```
pCRisp                AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFSA GTGSQSVERT
FAM21A(Q641Q2)        AAAPWEGGPV PGVDRSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21A(B7ZME8)        AAAPWEGGPV PGVDRSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21A(Q9Y4N4)        AAAPWEGGPV PGVDRSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21B(Q5SNT6)        AAAPWEGGPV PGVDRSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(B4E255)        AAAPWEGGPV PGVDRSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(B4DF48)        AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(Q9y4e1)        AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(q9y4e1-3)      AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(q9y4e1-2)      AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(B9EK53)        AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21C(B4DZQ6)        AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
FAM21D(Q5SRD0)        AAAPWEGGPV PGVDTSPFAK SLGHSRGEAD LFDSGDIFST GTGSQSVERT
```

16.4.1 region

```
pCRisp              1180  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21A(Q641Q2)      1149  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21A(B7ZME8)      1128  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21A(Q9Y4N4)       298  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21B(Q5SNT6)      1061  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNT
FAM21C(B4E255)       339  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(B4DF48)       831  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(Q9y4e1)      1128  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(q9y4e1-3)    1073  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(q9y4e1-2)    1120  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(B9EK53)      1087  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21C(B4DZQ6)      1053  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
FAM21D(Q5SRD0)       116  KPKAKIAENP ANPPVGGKAK SPMFPALGEA SSDDDLFQSA KPKPAKKTNP
```

16.4.1 region

```
pCRisp                FPLLEDEDDL FTDQKVKKNE TKSgSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21A(Q641Q2)        FPLLEDEDDL FTDQKVKKNE TKSNSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21A(B7ZME8)        FPLLEDEDDL FTDQKVKKNE TKSNSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21A(Q9Y4N4)        FPLLEDEDDL FTDQKVKKNE TKSNSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21B(Q5SNT6)        FPLLEDEDDL FTDQKVKKNE TKSNSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(B4E255)        FPLLEDEDDL FTDQKVKKNE TKSNSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(B4DF48)        FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(Q9y4e1)        FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(q9y4e1-3)      FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(q9y4e1-2)      FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(B9EK53)        FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21C(B4DZQ6)        FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
FAM21D(Q5SRD0)        FPLLEDEDDL FTDQKVKKNE TKSSSQQDVI LTTQDIFEDD IFATEAIKPS
```



Supplement (previous pages): High sequence similaritities between members of the Risp protein (i.e. human proteins featuring the 16.4.1 region). Human proteins containing the 16.4.1 region were identified by searching the UniProtKB database (http://www.ebi.ac.uk/uniprot/) with the 16.4.1 amino acid sequence. All proteins identified in this manner belonged to the FAM21 family (gene family with sequence similarity 21). Similarities between these proteins was analyzed with DiAlign, Genomatix Munich. Shown is the multiple alignments of protein sequences, with accession numbers indicated in brackets. pCRisp designates the Risp protein-encoding sequence in the pCRispsg143 expression plasmid. The blue rectangle labels the 16.4.1 region and the green rectangles the locations of the peptides used to generate anti-Risp antibodies.

11. Acknowledgments

Since I would like to say “Thank You” to some people who are not as good in understanding English, I will write this part in German.

Anfangen möchte ich mit meiner Danksagung bei meiner Doktormutter Prof. Dr. Ruth Brack-Werner und meiner inoffiziellen Doktormutter Prof. Dr. Christine Leib-Mösch. Vielen Dank das Ihr mir die Möglichkeit gegeben habt an diesem Projekt zu arbeiten. Ihr habt viel Vertrauen und Zeit in mich investiert und das weiß ich sehr zu schätzen. Danke auch für die anregenden Diskussionen auf der roten Couch, die bedeutend zum Gelingen dieser Arbeit beigetragen haben.

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Danke auch an meine lieben Kollegen für Ihre Unterstützung und das gute Arbeitsklima. Die Zeit mit Euch war wirklich spaßig. Nicht zu vergessen ein großes Dankeschön auch an Frau Ursula Voll, Frau Hildegard Witte, Frau Regina Geiger und das tolle Team der Spülküche: Frau Karin Schalk, Frau Anna Hauptkorn und Frau Ana Grenner (die fleißigen Bienchen).

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Mein allergrößter Dank geht an meinen Freund Kamyar Hadian, der mir zu jeder Zeit sei es privat oder im Labor zur Seite stand. In allen Lebenslagen hat er mir durch seine unendliche Liebe und Geduld besonders viel Kraft gegeben.

12. Curriculum vitae

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Date and place of birth:	01.10.1980, Munich/Germany
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Nationality:	German

Education:

Since 11/2006:	**PhD thesis at the Institute of Virology at the Helmholtz Center Munich, Prof. Dr. Christine Leib-Mösch / Prof. Dr. Ruth Brack-Werner** Topic: „Influence of exogenous viruses on the expression of human endogenous retroviral elements (HERVs) “
10/2001 – 09/2006:	**Studies in Biology at the Technical University Munich and Diploma thesis at the Institute of Virology at the Helmholtz Center Munich, Prof. Dr. Ruth Brack-Werner** Topic: „Identifiaction of a putative Risp-containing KIAA0592-protein “ Achievement of the Degree of a certified Biologist (Diplom-biologe) (Final grade: 2,0)
09/1991 – 06/2001:	School and graduation at the St.-Anna Comprehensive Secondary School in Munich, A-level equivalent (Abitur)

13. Publications

Publications in scientific journals:

Results of this thesis are part of the following publications:

Vincendeau M, Kramer S, Hadian K, Rothenaigner I, Bickel C, Hauck S, Kremmer E, Bell J, Leib-Mösch C, and Brack-Werner R
Identification of cellular Rev-inhibitory factors that restrict HIV production in astrocytes
AIDS, **2010 (Manuscript accepted)**

Vincendeau M, Schreml JMH, Hadian K, Kramer S, Seifarth W, Greenwood A, Brack-Werner R, and Leib-Mösch C
HIV-1 infection increases the expression of several HERV families
Manuscript in preparation

Further publications:

Meyer H, Tripsianes K, **Vincendeau M**, Madl T, Kateb F, Brack-Werner R and Sattler M
Structural basis for homodimerization of the Src-associated during mitosis, 68 kD protein (Sam68) Qua1 domain
Journal of Biological Chemistry, **2010**

Hadian K, **Vincendeau M**, Mäusbacher N, Nagel D, Hauck S, Ueffing M, Loyter A, Werner T,Wolff H and Brack-Werner R
Identification of an hnRNP recognition motif in the HIV-1 Rev protein
Journal of Biological Chemistry, **2009**

Kramer-Hämmerle, S., Ceccherini-Silberstein, F., Bickel,C., Wolff, H., **Vincendeau, M.**, Erfle, V., Werner, T., Brack-Werner, R.
Identification of a novel Rev-interacting cellular protein
BMC Cell Biology, **2005**

Book chapter:

Meggendorfer M, Rothenaigner I, Tigges B, **Vincendeau M** and Ruth Brack-Werner
HIV-1 structural and regulatory proteins and neurotoxicity
The Neurology of AIDS, Oxford University Press (not yet released)

Oral presentations:

Vincendeau M, Kramer S, Hadian K, Rothenaigner I, Bell J, Hauck S, Bickel C, Kremmer E, Leib-Mösch C and Brack-Werner R
A novel family of host-cell factors that control HIV production by targeting Rev
34th annual meeting on Retroviruses, Cold Spring Harbor, 2009

Vincendeau M, Schreml J, Seifarth W, Greenwood A, Frank O, Kramer S, Brack-Werner R and Leib-Mösch C
HIV-1 infection increases human endogenous retrovirus (HERV) expression
Mobile Elements in Mammalian Genomes (FASEB Summer Research Conferences), Snowmass, Colorado, 2009

Selection of poster presentations at scientific conferences :

Vincendeau M., Schreml J., Seifarth W., Frank O., Kramer S., Brack-Werner R., Leib-Mösch C.
HIV-1 infection increases the expression of several HERV families
Frontiers of Retrovirology, Montpellier, France, 2009

Vincendeau M., Kramer S., Hadian K., Kremmer E., Leib-Mösch C., Brack-Werner R.
Risp proteins may contribute to cellular control of HIV-1
German Society of Virology Meeting, Heidelberg, Germany, 2008

Vincendeau M., Kramer S., Kremmer E., Brack-Werner R.
Inhibition of HIV-1 replication by Risp-containing proteins
GFV Meeting 2007, Nuremberg, 2007

Vincendeau M., Kramer S., Kremmer E., Brack-Werner R.
Modulation of HIV-1 production by Risp and identification of Risp-containing proteins in human cells using RNAi
International RNAi-Symposium, Munich, 2006

Vincendeau M., Kramer S., Kremmer E., Brack-Werner R.
Modulation of HIV-1 production by Risp and identification of Risp-containing proteins in human cells
GFV Meeting 2006, Munich, 2006

14. Ehrenwörtliche Versicherung / Erklärung

Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den ....

(Unterschrift)

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Hiermit erkläre ich, *

☐ dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

☐ dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

☐ dass ich mich mit Erfolg der Doktorprüfung im Hauptfach .....
und in den Nebenfächern .....
bei der Fakultät für ..... der .....

(Hochschule/Universität)

unterzogen habe.

☐ dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

München, den ....

(Unterschrift)

*) Nichtzutreffendes streichen