Characteristics and Relevance of the Interaction between Staufen-1 and Rev-related retroviral Proteins

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1 Introduction

1.1 Retroviruses

1.1.1 History

Vilhelm Ellerman and Olaf Bang, first demonstrated that avian sarcoma leukosis virus could be transmitted after cell-free filtration to healthy chickens, inducing leukemia [1] and later Rous Peyton extended Ellerman and Bang's experiments and identified transmissible cancer-causing agents [2].

In 1936, John J. Bittner observed an incidence of mouse mammary tumors which were developed by the mouse mammary tumor virus (MMTV) through the mother's milk [3]. The mouse mammary tumor virus (MMTV) is found as exogenous as well as endogenous part of the germ cell genome in their host species [4].

In 1970 H.M. Temin, S. Mizutani and D. Baltimore discovered the RSV reverse transcriptase enzyme (RNA dependent DNA polymerase) that is essential for the transcription of retroviral single stranded RNA into double stranded DNA provirus [5].

The first human retrovirus was isolated in 1979 by Robert Gallo and his co-workers. This virus was identified as the human T-lymphotropic virus or human T-cell lymphotropic virus (HTLV) that is known to develop a type of cancer called adult T-cell leukemia/lymphoma (ATLL) [6].

In 1983, Robert Gallo isolated a novel retrovirus which infected AIDS patients, and was similar in shape to other human T-lymphotropic viruses (HTLVs) so Gallo called the newly isolated virus HTLV-III [7]. At the same time, Montagnier's group identified a virus from a patient suffering from symptoms of AIDS [8]. The AIDS causing virus discovered by the two separate working groups is called Human Immunodeficiency Virus (HIV).

1.1.2 Classification/Nomenclature

Retroviruses are a large and diverse group of viruses infecting the animals and the humans. The International Committee for Taxonomy on Viruses (ICTV) broadly divides the Retroviridae family into two taxonomic sub-families, Orthoretrovirinae and Spumaretrovirinae. The first sub-family contains the genera Alpha-retrovirus, Beta-retrovirus, Gamma-retrovirus, Delta-retrovirus, Epsilon

retrovirus and Lentivirus while the second contains the single Spumavirus genus (see Fig. 1). The current classification is based on phylogenetic analysis especially in *pol*. This taxonomy correlates with other genetic characteristics: the number and size of virion proteins, structure of the virion, presence of additional genes and the host tRNA used as primer [9].

Moreover, retroviruses are alternatively classified into simple and complex viruses according to genome organization. Simple retroviruses are characterized by the presence of only *gag, pro, pol*, and *env*, while complex retroviruses (e.g. lentiviruses and spumaviruses) encode additional and auxiliary proteins that directly and specifically regulate the gene expression [10] [9].

In addition, retroviruses can be divided into endogenous and exogenous retroviruses. Endogenous retroviruses are normal and usually non-pathogenic genetic elements in the vertebrate DNA germline vertically inherited to offspring and are able to be moved within the genome to act as possible enhancer sequences that stimulate the expression of the adjacent human genes [11] [12]. On the contrary, exogenous retroviruses (e.g. HIV) are horizontally transmitted from an individual to another as infectious RNA viruses that have essential genes for package into virion structure.

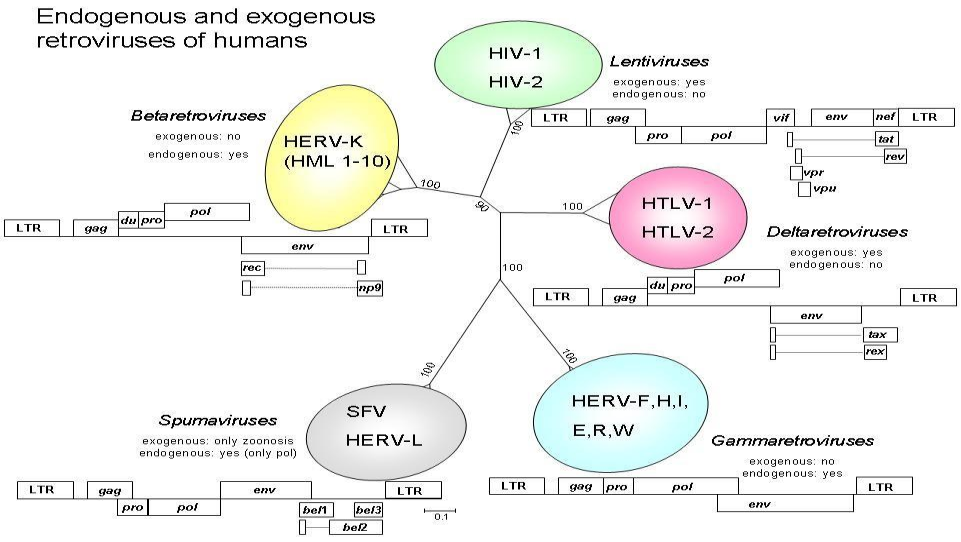


Figure 1: Phylogenetic tree of the Retroviridae. They are classified currently into two sub-families, Orthoretrovirinae and Spumaretrovirinae either exogenous or endogenous retroviruses. Modified from Antoinette, 2011 [13].

1.1.3 Structure

Retrovirus virions consist of characteristic, enveloped particles with a diameter of about 100 nm [14]. These infectious retroviral particles contain two single-stranded positive sense RNA copies differing in length from 7 to 12 Kilo bases (kb) depending on the type of virus as well as they possess a 5' cap structure and 3' polyadenylation signal. The structure of the HIV-1 particle is illustrated by Figure 2.

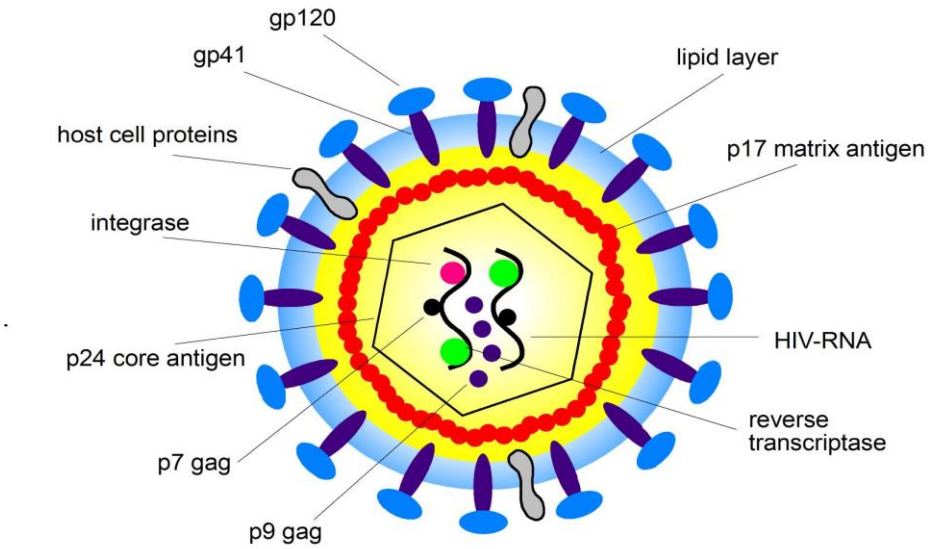


Figure 2: Structure of an HIV virion particle (Andrea Rubbert, 2011) [15].

The detailed explanation of the main virion components and their basic function is mentioned as follows:

I. Membrane Envelope: composed of lipids taken from the plasma membrane of the infected cells during budding process. It is lined by the *gag*-encoded Matrix protein as well as glycoprotein encoded by the *env* gene [16]. The retroviral envelope serves three important functions: protection of the viral RNA genome from the extracellular environment via the lipid bilayer, enabling the retrovirus to enter/exit host cells through endosomal membrane trafficking, and the ability to directly enter cells by fusing with their membranes.

II. *env*-Encoded structural glycoproteins:

1. gp120/gp41 proteins: gp120 is essential for virus penetration into cells because it has a necessary role in binding to specific cell surface receptors [17].gp41/TM (trans-membrane) protein contains peptide which promotes fusion of virion membrane with target cell membrane for the virion entry [18].
2. SU (surface unit): located outside the membrane, non-covalently bound to TM. It binds to specific cell-surface receptors of the host cell, targeting these cells for the virus adsorption and infection [19].

III. Virion core: Structure composed of *gag* (group specific antigen) encoded matrix, capsid and nucleocapsid proteins which protect the core and is the most abundant proteins in the virus particle. Within the core are located:

1. Two viral genomic RNA transcripts, coated by the *gag* encoded Nucleocapsid protein

2. *pol*-encoded enzymes:

   A. Reverse transcriptase enzyme (DNA polymerase) which mediates the retrotranscription of a viral single stranded RNA genome into a double stranded DNA genome serving as a DNA intermediate product during virus replication and incorporating into the host cell genome to form DNA provirus. This RNA to DNA reversion was the first example of a 'backwards' direction of genetic information in living cells [20]. Also, Reverse transcriptase enzyme (RT) has RNase H activity allowing breakdown of RNA from DNA-RNA hybrid strands.

   B. Integrase, an enzyme which inserts the produced retroviral double stranded DNA into host cell genome.

   C. Protease, an enzyme that catalyzes the proteolytic cleavage of Gag and Pol proteins to produce these viral proteins in their mature form.

1.1.4 Morphology

The morphology of retroviruses was examined by thin section and negative stain electron microscopy (Bernhard, 1958). The average size of the retroviral particles is ranging between 100-150 nm. A-type virus possessed a non-enveloped immature intracellular particle which may be resulted from endogenous retrovirus like genetic elements. B-type viruses were extra-cellular with prominent envelope spikes and an electron dense, acentric core, typified by MMTV. The C-type group including most mammalian and avian retroviruses was similar to B-type viruses with a central core but with poorly visible envelope spikes. The D-type virus group is appeared slightly larger, up to 120 nm in size, with less prominent envelope proteins e.g. Mason-Pfizer monkey virus which is currently classified as a Betaretrovirus.

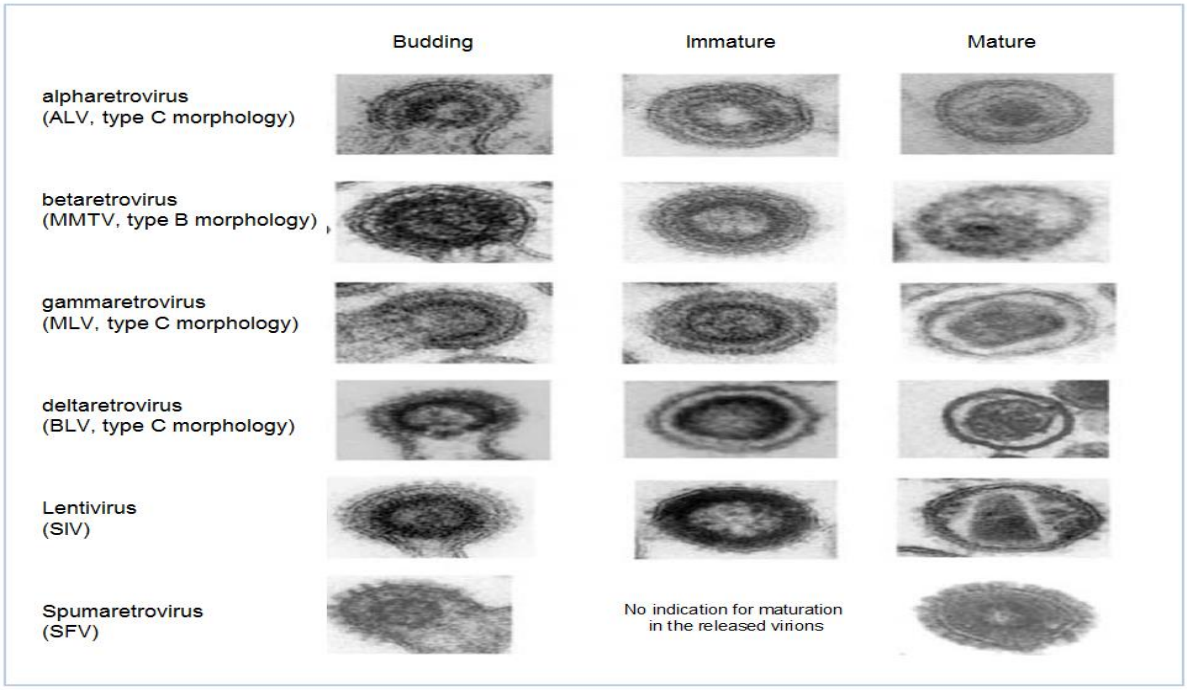


Figure 3: Electron Microscope visualization of prototypic exogenous retroviruses at the budding, the immature and the mature stages (Book "retroviruses" edited by R Kurth and N Bannert [19].

In the immature stage, the centre of the particles is bright and has a dark Gag ring. During particle maturation process, the Gag Precursor protein is cleaved by the viral protease so the appearance of the interior of the immature particles changes clearly except spumaviruses. The mature virions are morphologically distinguished in the centre of various retrovirus strains type A, B, C and D depending on their core structure.

1.1.5 Retrovirus life cycle

The replicative cycle of retroviruses is divided into two well-defined stages: the early stage concerns with the steps of infection from cell surface attachment to the integration of the provirus DNA into the host cell genome, while the late stage starts with the expression of retroviral genes, the budding and maturation of progeny viral particles (as illustrated by Figure 4).

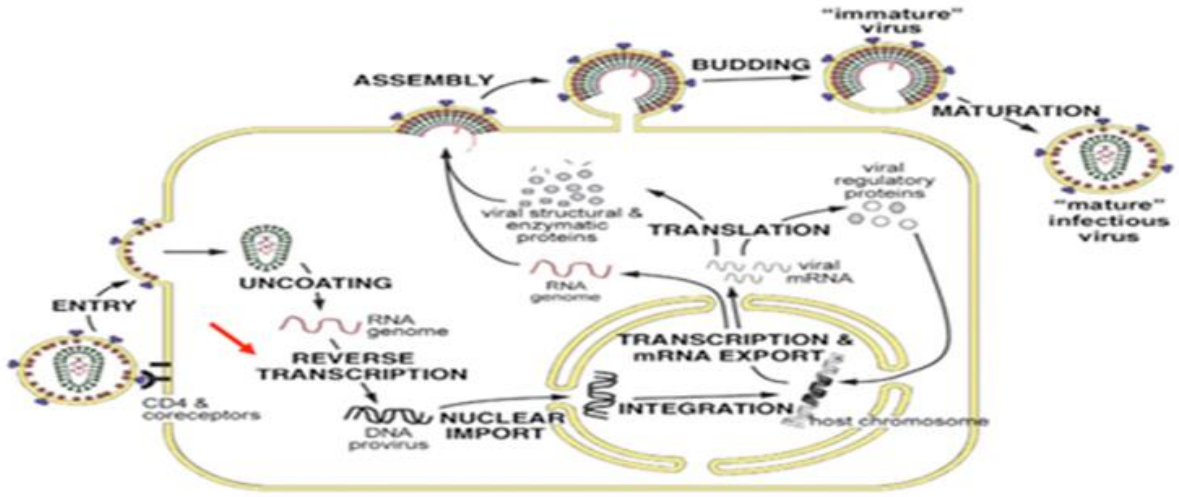


Figure 4: The general features of the retroviral life cycle. A schematic representation of early and late phases of the retroviral replication cycle. The initial step of the retroviral life cycle is the adsorption of viral particles to the target cell surface that leads to fusion of the viral and host cell membranes. The viral enzymes release into the cytoplasm by uncoating process. The initiation of reverse transcription reaction is coupled to the onset uncoating of the nucleocapsid [21]. The viral RNA transcriptase converts the viral RNA into double stranded DNA which is transported as a pre-integration complex (PIC) into the cell nucleus and then it is incorporating into the host cell genome as a provirus by integrase enzyme. If the provirus DNA is transcribed, the various RNA molecules are transported to the cytoplasm and translated. In addition, the viral genomic RNA transcript is produced in an unspliced form in the cytoplasm. Gag proteins are translated and aggregated as virion core while the genomic RNA copies are packaged into virus particles. The virus buds are derived from the host plasma membranes that are bound to the viral Envs. The virus maturation is achieved after the budding process. Nisole *et al.*, 2005 [22].

The retroviral entry involves the specific binding between the viral envelope glycoprotein and the target cell surface receptors. Following the initial interaction step, the virions entry into the host cell is performed by dissociation of gp120 from gp41 and a conformational change, resulting in the insertion of transmembrane peptide into the cell membrane, leading to fusion of the viral and cellular membranes. The viral core is released into the cytoplasm, the capsid is disassembled by a process known as uncoating [23] [24]. Immediately, the viral genomic RNA is reverse transcribed in the cytoplasm by reverse transcription process catalyzed by the virion-packaged reverse transcriptase (RT), producing a linear double-stranded DNA molecule [20]. The generated double stranded DNA molecule is transported as pre-integration complexes (PICs) into the cell nucleus where it integrates into host chromosomal DNA, establishing the provirus [25] [26]. The integrated provirus becomes a permanent genetic material of the cell, may remain latent and is replicated along with the host DNA if the infected cell is undergoing cell division [9]. Depending on the type of the viral strain, 2-30 different mRNA transcripts are formed by splicing machinery [27].

However, expression of the provirus, serves either as viral genomic RNA which is packaged into newly synthesized virus particles or as mRNA for translation into Gag, Pol and Env precursor viral proteins. The Env precursor gp41 transmembrane (TM) and gpl20 surface (SU) proteins are expressed from a singly spliced viral mRNA at the rough endoplasmic reticulum (RER) which is integrated into the virion membrane. The viral full length RNA is unspliced and then encapsidated into the virions by a specific binding to nucleocapsid of the Gag precursor. The matrix protein (MA) is responsible for stimulating the specific interaction between the NC and the viral full length RNA genome [28].

Gag proteins forms virus-like particles (VLPs) that bud from the infected cells, independent of a viral-encoded protease (PR). The assembly and budding processes are controlled principally by viral Gag domains, such as the membrane-targeting (M) domain, the Gag-Gag interaction (I) domain essential for virion formation, and the late assembly (L) domain necessary for the separation of the newly formed immature virus particle from the host cell membrane [29]. Subsequently, the Gag and Gag-Pro-Pol precursors are proteolytically processed by the viral active protease [30]. This process is known as virus maturation involved in formation of infectious virus particles which are able to invade new host cells [31].

1.2 Endogenous retroviruses

Endogenous retroviruses are genetic retroelements characterized by a long terminal repeat (LTR) [32] as a result of retrovirus infection of the ancestors millions of years ago [33]. They are investigated in all vertebrates including humans, integrated into the host genome of the germline (sperms and oocytes) as a non-pathogenic and defective provirus DNA that is vertically transmitted to offspring according to Mendelian mechanisms [34].

Endogenous retroviruses (ERVs) comprise between 5% and 10% of the vertebrate animals genome while these retroviral genetic elements occupy up to 8% of the human genome in different locations [35] [36]. Using bioinformatics technologies, more than 98 000 human ERVs (HERVs) have been estimated [37]. Also, the human genome carries at least 31 different human endogenous retrovirus (HERV) families, each resulting from 31 independent germline infections with retroviruses, the largest of which HERV-H has 1300 full length copies [37].

The LTR containing retroelements can be divided into 6 superfamilies. 8 Class I–III HERVs have limited nucleotide sequence homologies to C-, B- or spumaspumaretroviruses, respectively while the other superfamilies MER4, MST and MLT are ancient retrotransposons that may be not currently active in humans [38].

ERVs are inactivated by recombinational deletions and mutations [39] but the provirus of ERVs may be activated by physical and chemical agents such as X-irradiation, and inflammatory cytokines, and then transcripts are synthesized and expressed as infectious virus particles [40]. Koalas, koala gammaretrovirus (KoRV) can exist as a replication-competent, full-length provirus DNA in the host genome but their sequences are significantly similar to exogenous gibbon ape leukemia virus (GALV) [41] [42].

Some ERVs prove a beneficial role in regulating the human genes [43]. For example, an ERV LTR sequence contributes to the expression of the beta1, 3-galactosyltransferase-5 gene specifically in colorectal tissue [44]. Furthermore, the *env* genes of ERVW express syncytin proteins which participate in the differentiation of syncytiotrophoblast in chorionic villi, helping in normal placental development during pregnancy [45] [46]. Simultaneously, other ERVs, notably ERVK, may be detrimental to the host by its replicative ability to encode viral RNA and proteins. There is no doubt that ERVK is implicated and transcriptionally active in inflammatory

diseases including Rheumatoid Arthritis (RA) [47], Systemic Lupus Erythematosus (SLE) [48], Amyotrophic Lateral Sclerosis (ALS) [49], and multiple types of cancers [50].

There are distinct mechanisms by which the endogenous retroviruses are able to proliferate: re-infection, retrotransposition and complementation in trans as shown in Figure 5. The life cycle of endogenous retroviruses starts with the initial colonization of the germline. Reinfection process refers to the re-entry of the ERV progeny into the host germline and then the provirus is replicating in the somatic cells leading to increasing in the retrovirus fitness. The retroviral functional *gag, pol, env* genes are essential for the reinfection mechanism. Retrotransposition represents an intracellular amplification process of retrotransposons and ERVs within the germline cells in which the virion core containg the viral genomic RNA molecules are biosynthesized. The Complementation of the defective viruses in *trans* is processed by proteins and LTRs promoter derived from other viruses [37]. Therefore, ERVs form infectious exogenous particles that have the ability to reinfect germline cells [33]. Some mutations, such as stop codons and frame shift mutations may be acquired in the new genetic retroelements during reinfection, retrotransposition or during cell division. These implemented mutations can inhibit or reactivate the retrovirus replication [37].

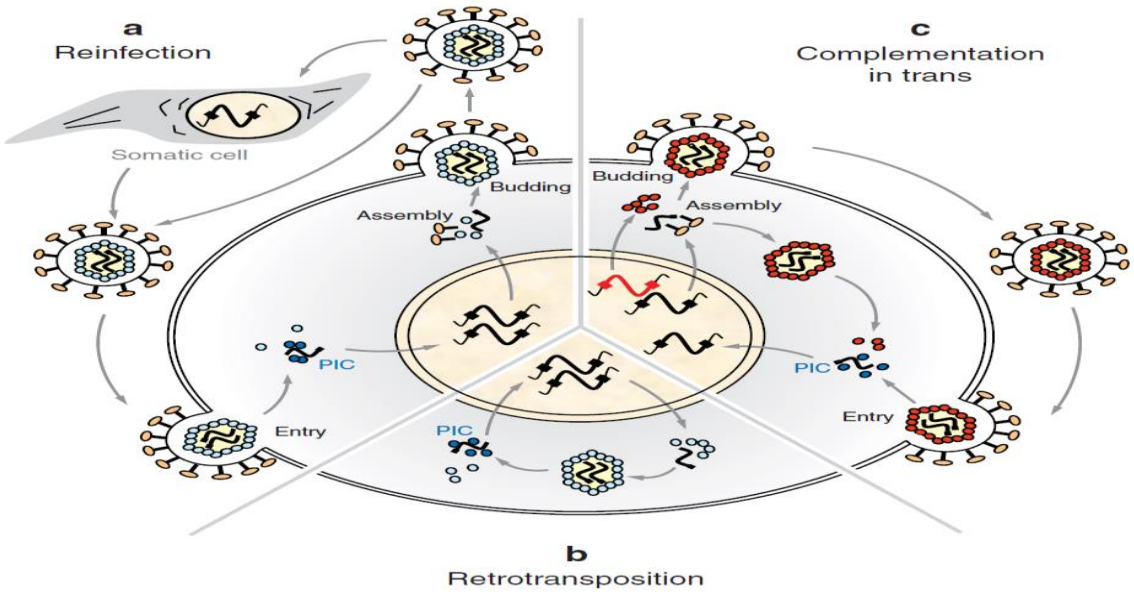


Figure 5: Amplification mechanisms of the endogenous retroviruses. The proliferation processes Reinfection (a), retrotransposition (b) and complementation *in trans* (c) are explained in details in section 1.1.7 (Bannert and Kurth, 2006) [33].

By phylogenetic analysis, the genetic distance between the ERV 5' and 3' LTRs determines the mutation frequency occurred since the integration time and therefore is proportional to the provirus age. Additionally, the comparison of the sequence homology of LTR sequences demonstrates the time of the provirus integration because these sequences are identical at the time of the germline infection and each mutation is achieved after 300,000 years [38]. HERV-KC4, HERV-KHML6.17, and RTVL-Ia are investigated in both old world monkeys (OWMs) and hominoids, which are reported to have last shared a common ancestor over 31 million years ago. In contrast, HERV-K18, RTVL-Ha, and RTVL-Hb are present only in humans, chimpanzees, and gorillas [51] [52] [53]. The last common ancestor of humans and chimpanzees was estimated 5-6 million years ago [51] [52] [53].

1.2.1 HERV-K(HML-2) Family and HERV-K113

HERV families were discovered during analyses of human gene loci (e.g. HERV-H and HERV-I) [54] [55]. Some other HERVs were screened by probes isolated from animal retrovirus genomes that detected HERV proviruses (e.g. HERV-E and HERV-K) [56] [57]. Moreover, oligonucleotides with homology to the retroviral primer binding sites were utilized to assess other HERVs (e.g. HERV-P) [58]. In 1982, HERV-K 1982 was discovered in the human genome due to the similarity to MMTV [59].

A temporary systematic HERVs nomenclature is based on the specificity of the primer binding site to tRNA that serves as a primer to initiate the reverse transcription process. The one-letter code for the specific amino acid is appended to HERV [60]. But, this nomenclature system has limitations when closely related families are sharing homologies in the primer binding site or when cloned proviruses are either without a 5' end or are only partially sequenced. All class II elements identified to have a lysine primer binding site that is recognized by lysine carrying tRNA (e.g. HERV-K use lysine (K) tRNA while HERV-W use the tryptophan (W) tRNA) [4].

HERVs have been divided into two classes according to their similarities to animal retroviruses [61]. Class I families have sequence homology to mammalian retrovirus strain type C. Three families proved homologies in the sequences of the *pol, gag* and *env* genes and they are grouped into a superfamily, the ERI family that closely resemble murine leukemia virus (MuLV) and baboon endogenous virus

(BaEV). Class II families show similarity to mammalian retroviruses type B and D. The Provirus sequences of HERV-K family (HERV-K 10) [62] and of the HERV-K (C4) family[63] exhibit homologies in the *gag, pol,* and *env* genes.

HERV-K(HML-2) is a biologically active family of HERV, one of the most studied and amplified family integrated into the human genome less than 5 million years ago [64]. They were remarkably present and highly conserved in the genome of Old World monkeys ancestor 35 millions of years ago [4].

The HERV-K family has ten groups (HML-1-10) that are closely similar to mouse mammary tumor virus [65]. It has been reported that the human endogenous retrovirus type K (HERV-K) family comprises more than 90 proviral copies and more than 2,500 solitary LTRs per haploid genome in humans resulting from ancestral exogenous retroviral infections [33] [66]. Some proviruses of Betaretrovirus like HERV-K(HML-2) group are displaying intact open reading frames (ORF) with coding capacity for viral particles [65]. The intact HERV-K(HML-2) provirus was specifically distributed on human chromosome 7p22 [67] [68]. More than 20 HERV-K(HML-2) proviruses are [69] expressed in human teratocarcinoma-derived cells and all essential retroviral proteins (e.g. Gag, Pro, Pol, and Env proteins) that are up regulated in normal placental tissues [70], several cancerous tissues such as breast, ovarian tissues, lymphomas, and rheumatoid arthritis [65]. Hence, HERV-K(HML-2) proviruses can generate infectious particles [71]. Therefore, HERV-K(HML-2) activity indeed is linked to malignancies and cancer development.

HML-2 proviruses are grouped according to their LTR sequence [72] or based upon the presence (type 1) or absence (type 2) of a 292 bp deletion within the *pol-env* junction which is important for Rec protein formation [73]. Type 1 proviruses are defective because the 292 bp deletion which occurred after the evolutionary human-chimpanzee split misses the necessary Env protein and inhibits the Rec protein expression but biosynthesizes an alternative protein known as Np9 [74]. Type 1 proviruses are produced in many transformed cells [75]. Type 2 proviruses express the accessory Rec, a nuclear export factor that displays functional homologies to the HIV Rev protein [74]. Only few HML-2 proviruses are characterized by integration polymorphism processes within the human population [32] [76]. For instance, HERVK113, identified on 19p12 [77] had insertionally polymorphic proviruses in the human genomes of some individuals less than 200,000 years ago [78]. HERVK113 is

one of HERV-K type II proviruses and one of the most recently and the best preserved of the HERV-K proviruses.

1.2.2 Proviral structure of HERV-K113

The DNA length of the integrated HERV-K113 provirus is 8.9 kb and is flanked by the two LTRs which regulate the retroviral gene expression. The genomic provirus has open reading frames for all essential retroviral genes therefore; HERV-K113 is active and presumably pathogenic [79]. In Figure 6, it has been shown that the HERV-K113 has the same genomic structure of the characterized exogenous retroviruses, 5'-*gag* - *pro* - *pol* - *env*- 3' [32]. The *gag* gene encodes for the matrix, Capsid and nucleocapsid proteins. The *pro* gene expresses protease which converts the immature Gag and Pol proteins into mature molecules by the proteolysis process. The *pol* gene encodes for enzymes important for the viral replication, namely reverse transcriptase, RNase H and integrase. The *env* gene encodes for the envelope proteins (Env) proteins involved in receptor recognition and membrane fusion.

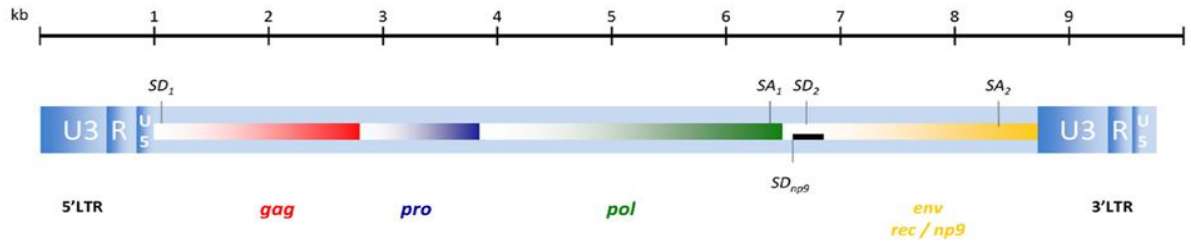


Figure 6: Genomic structural scheme of HERV-K113 provirus. HERV-K113 has a flanking long terminal repeats (the 5´and the 3´ LTR) and a central provirus. LTRs are composed of 3 genetic regions designated U3, R, U5. The provirus contains various genes, *gag*, group specific antigen gene; *pro*, protease gene; *env*, envelope gene; *pol*, polymerase gene which expresses various enzymes including reverse transcriptase and integrase as well as the accessory protein Rec. Modified from Hohn *et al.*, 2013 [80].

In addition to the HERV-K113 genes expressing structural proteins and viral enzymes, there is gene for an accessory protein, known as Rec protein which functionally resembles the HIV-1 Rev and HTLV Rex proteins necessary for the viral mRNA export from nucleus into cytoplasm for their translation [81] [82].

1.3 Lentiviruses

1.3.1 Discovery and origin

SIVs are lentiviruses and able to infect over 40 African non-human primates. The most characteristic feature of these infections is limited immune activation and resistance of the natural hosts to AIDS disease progression therefore this event help researchers understanding HIV infection in humans and to develop vaccine and therapeutic strategies [83] leading to a peaceful coexistence of primate lentiviruses and the immune system of the natural hosts. Based on epidemiological data, the SIVs have been present in monkeys at least 32,000 years ago. Primate lentiviruses have been isolated from most of the African monkeys of the genus Cercopithecus (e.g. SIVgsn, SIVdeb, SIVmus), African green monkeys (Chlorocebus), the mangabeys (SIVrcm), mandrills (SIVmnd) and drills (SIVdrl), colobus monkeys (SIVcol), and a variety of apes, two subspecies of chimpanzees (Pan) (SIVcpz) and gorillas (SIVgor) [84].

SIVs were first studied by AIDS researchers after observation of immunodeficiency in macaques in the California, and Washington primate centers [85]. The virus isolated from macaques originated from sooty mangabey monkeys. In addition, SIVs were transmitted to human population by zoonoses inducing AIDS disease and HIV pandemic [86]. Therefore, SIVmac and SIVsmm infected rhesus macaques are used as a new animal model for studying HIV infection.

In Figure 7, phylogenetic data reveal that cross-species transmissions of two SIVs, chimpanzees SIVcpz and sooty mangabeys SIVsmm, to humans generated HIV-1 and HIV-2 epidemics, respectively [87] [88] [89].

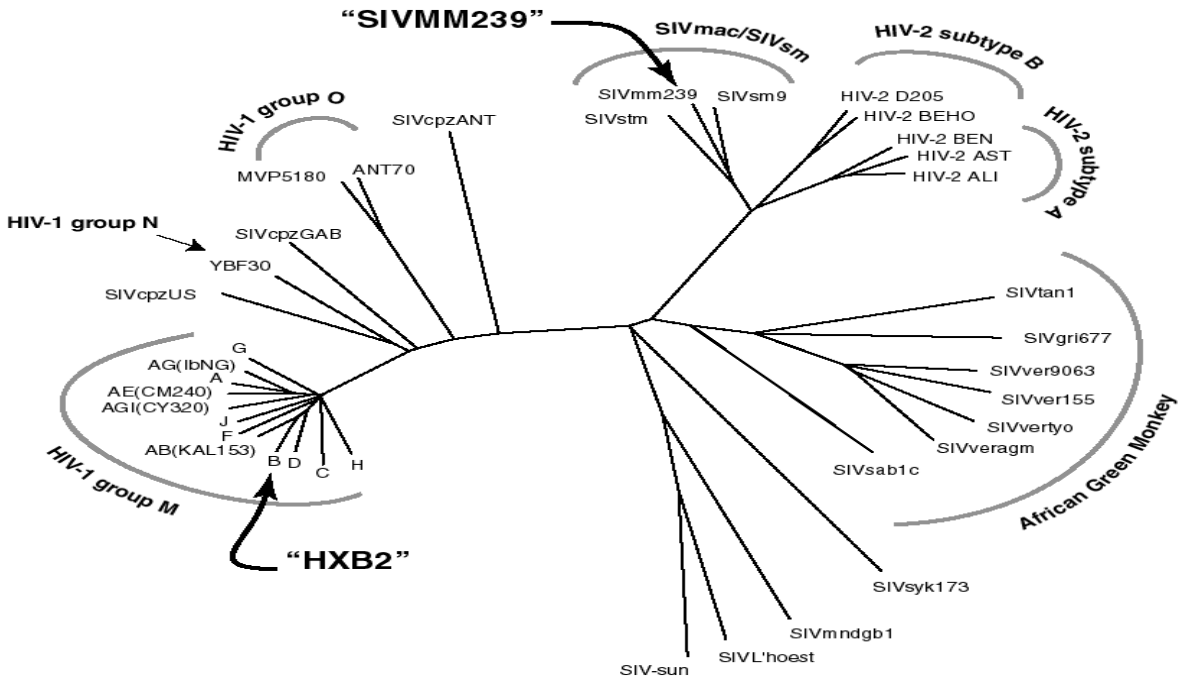


Figure 7: phylogenetic relationships between various primate lentiviruses. Modified from Charles *et al*., 2001 [90].

On the other hand, AIDS was first clinically discovered in 1981 in the United States when it was observed in injecting drug users and gay men suffering from loss of immunity and occurrence of rare opportunistic infections [91]. Afterwards, HIV-1 was isolated from AIDS patients by two separate working groups in 1983 [92], whereas HIV-2 was first detected in West African infected individuals in 1986 [93] [94]. HIV-2 is less pathogenic rate, slower immunodeficiency progression than HIV-1 and worldwide is more restricted in its distribution (rare in North America, Europe and Central or East Africa) [95].

1.3.2 SIV/HIV genomic organization

All lentiviruses including SIVmac239 and HIV carry a common genomic organization encoding the structural and enzymatic proteins 5'*gag*, *pol*, *env*3'. Also, they have several important accessory genes as displayed in Figure 8, such as *tat*, *rev, vif, vpr*, and *nef* genes whose products that mediate the viral gene expression and infectivity [96] [84].

Figure 8: Genomic organization of the SIVmac239. Gray boxes show protein coding regions, while dark lines show the boundaries of the mature viral proteins. Modified from Elizabeth *et al*., 2013 [97].

The *pro-pol* sequence encodes the protease, integrase and reverse transcriptase enzyme with RNase activity. Pol precursor is enzymatically processed into the protease, the reverse transcriptase and the integrase. The reverse transcriptase uses the viral genomic RNA as a template for the generation of cDNA copies. The integrase enzyme inserts the synthesized cDNAs into the genome of the target cell via formation of two characteristic identical LTR genetic segments flanking the viral genome. The *gag* gene expresses the group specific antigens that occupy about 50% of the entire virus particles [98]. During the maturation stage, protease catalyzes the proteolysis of Gag into matrix, capsid and nucleocapsid proteins. Furthermore, protease is essential for separation of Env into the transmembrane protein (gp41) and the surface protein (gp120) subunits [99]. The *env* gene is transcribed into a single spliced mRNA transcript that is translated into the Env precursor (gp160) and then protease enzyme separate the Env precursor into the transmembrane protein (gp41) and the surface protein (gp120) subunits [100].

1.3.3 HIV epidemiology and therapy

HIV is the etiologic agent of acquired immunodeficiency syndrome (AIDS) in humans. The central feature of HIV infection is an obvious increase in activation of the adaptive and the innate immunity. HIV-1 infection results in destroying the activity of cell-mediated immunity by progressive depletion of CD4+ T lymphocytes, macrophages and dendritic cells, resulting in AIDS disease that leads to more susceptibility to cancer development and opportunistic infections such as *Candidiasis* of bronchi, *Cryptococcosis, Kaposi's sarcoma, Pneumocystis carinii pneumonia* and *Kaposi's sarcoma* [101].

The main routes of HIV transmission are sexual transmission (infected semen or vaginal and cervical secretions), infected blood transfusion, infected needles usage, infected breast milk [102]. Worldwide, it has been estimated that 35 million people were living with HIV, nearly 39 million people have died of HIV at the end of 2013 [103] and about 5 million new HIV infections detected per year [104]. HIV-1 is a major health threat worldwide in most countries particularly in sub-Saharan Africa and Southeast Asia [104].

Antiretroviral treatment is the best strategy for inhibition of the viral replication and, subsequently, for reduction of morbidity and mortality rate [105]. However, effective HIV vaccines are complex due to high genetic diversity as well as current drugs do not cure or eradicate HIV infection but when they are taken in combination, they can impair the virus multiplication. Combination of many effective antiretroviral agents referred to as highly active ART (HAART) [106].

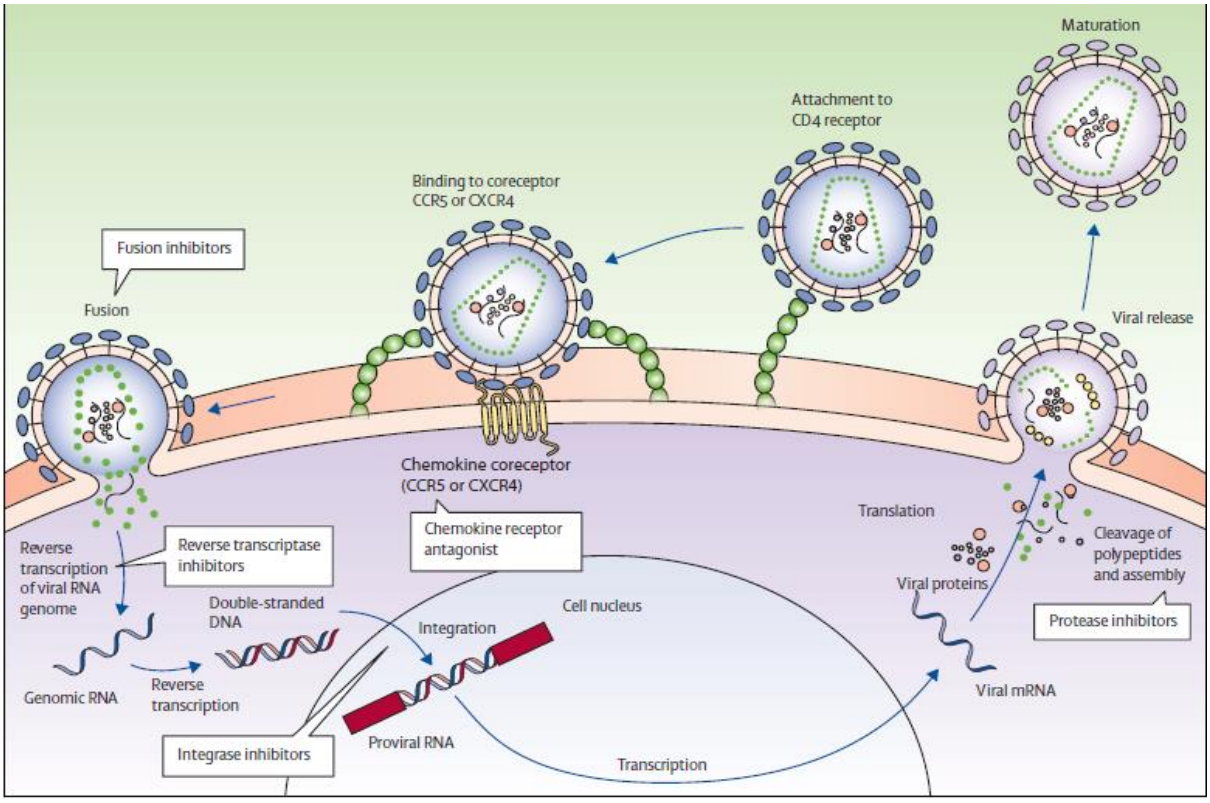


Figure 9: HIV life cycle showing the replication steps against which HAART drugs is effective. Changed from Gary *et al.,* 2014 [105].

The mechanisms of antiretroviral action (Figure 9) depend on targeting the viral envelope gp40 segment (prevention of the cell-virus fusion process before the

virus entry), the viral reverse transcriptase and integrase (viral replication suppression) and viral protease (maturation step inhibition) [106].

1.4 Rev/Rec proteins

The retroviral Rev/Rec (Regulator of Expression of Virions) proteins are a posttranscriptional acting trans-activator which has been shown to be absolutely necessary for regulation of viral gene expression and stimulation of genomic RNA encapsidation 20- to 70-fold [107]. Rev/Rec interact with the Rev/Rec responsive element (RRE), a highly structured RNA sequence located on the unspliced RNA transcript and the single spliced *env* mRNA [108]. RRE appears to be vital for the Rev/Rec function so following binding to the RRE, Rev export the unspliced 9 kb RNA encoding Gag and Gag–Pol, and the five singly 4 kb spliced mRNAs encoding Vif, Vpr, Vpu, and Env via the CRM-1 export pathway from the nucleus into the cytoplasm [109]. Consequently, the Rev protein effectively enhances the cytoplasmic translation of *gag-pol* open reading frames (unspliced viral mRNA) and *env* transcript encoding the viral structural proteins [107].

HIV-1 Rev trans-activator mediates transport of unspliced HIV-1, HIV-2 and SIV mRNA transcripts, whereas the Rev proteins of HIV-2 and SIV were not able to induce the HIV-1 unspliced mRNAs nucleocytoplasmic shuttling and expression [110]. Rev mutagenesis or RRE deletion in HIV-1 vector during viral production significantly decreases the vector titers [107].

As illustrated by Figure 10, Rev/Rec proteins contain distinct functional domains comprising RNA binding domain (RBD) which appears to overlap with short stretches of basic amino acid residues named as NLS (Nuclear localization signal). The NLS/RBD region is arginine-rich motif (ARM) present in the N-terminal domain, and is flanked by two loop or multimerization domains (OLIGO). Moreover, the Rev/Rec proteins have NES (nuclear export signal) domain located within the leucine-rich carboxy-terminal domain. NLS allows Rev/Rec to enter nucleus while NES is involved in the export of viral RNA transcripts from nucleus to cytoplasm for translation so NLS and NES exhibit the biological function of Rev/Rec. Therefore, in the absence of Rev/Rec, the viral mRNAs are retained in the nucleus, preventing their translation [111] [112] [113].

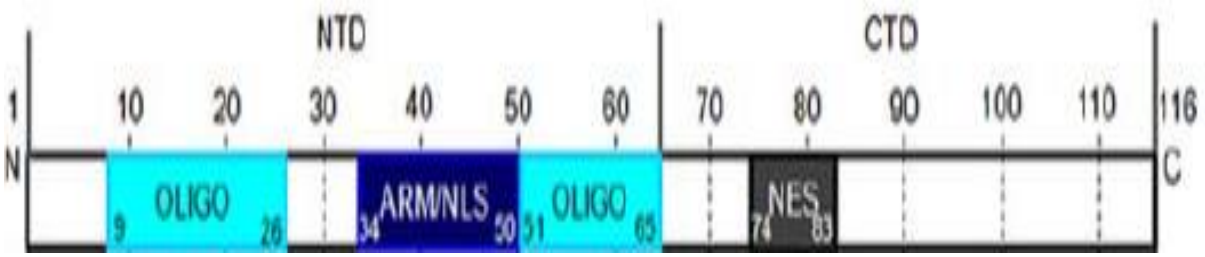


Figure 10: Schematic representation shows the Rev/Rec proteins structure. The Rev/Rec proteins consist of several functional domains: Nuclear localization signal (NLS) which seems to overlap with the RNA binding domain (RBD) in blue. The multimerization region (OLIGO) in green borders the ARM/NLS. Also, Rev/Rec have the nuclear export signal (NLS) in dark gray which is required for transport of the viral RNA from the nucleus into the cytoplasm. Modified from Dimattia *et al*., 2010 [111].

The mechanism of Rev/Rec dependent RNA transport can be achieved via a specific attachment of the RBD region of the Rev/Rec to the RNA target sequence for Rev/Rec, termed as a rev/rec responsive element [114]. RRE is conserved region within the N terminus of gp40 of *env* gene. The HIV-1 RRE is composed of 351 nucleotides [115], whereas the conserved SIVsm RRE is identified between nucleotide 8154 to 8375 [116]. The SIV and HIV-2 RRE sequences shared nearly 90% homology with each other and about 69% with HIV-l [110].

The viral regulatory protein Rev binds to the RRE of the unspliced and single spliced viral mRNAs and shuttles them from the nucleus to the cytoplasm by a cellular export pathway as shown in Figure 11.

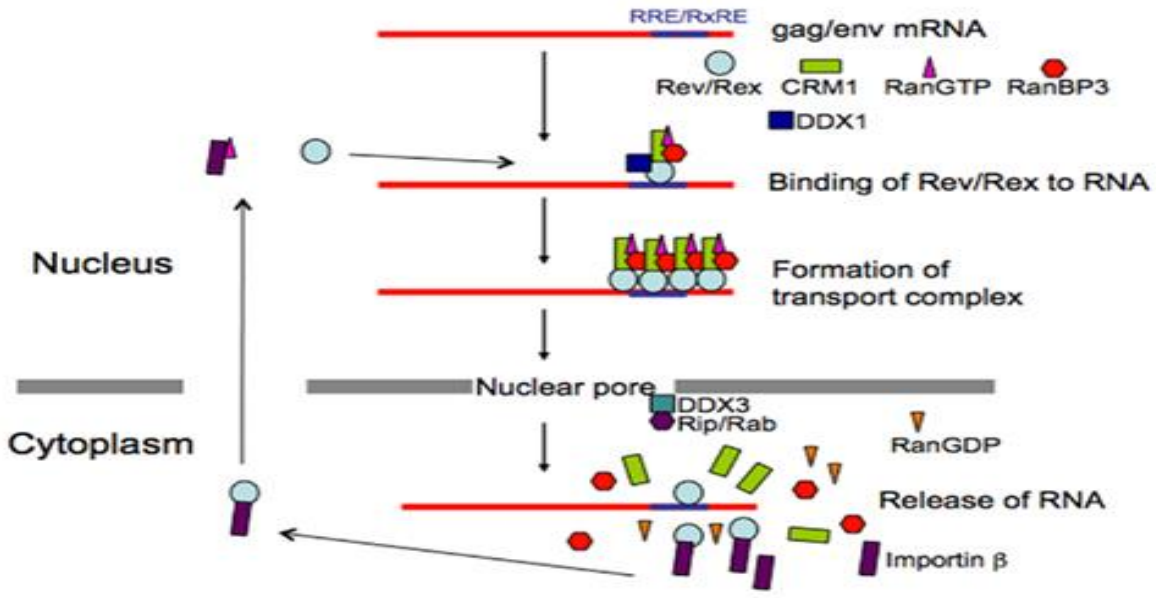


Figure 11: Schematic presentation of Rev/Rex dependent retroviral introns containing RNAs transport pathway. The Rev is transported into the nucleus by importin-β and then it binds to the RRE of the viral RNAs in presence of CRM1, RanGTP and DDX1 forming the transport complex which is exported from the nucleus into the cytoplasm. Modified from Hisatoshi Shida, 2012 [117].

The NLS of Rev/Rex/Rec protein is bound to importin-β to enter the nucleus and initially interacts with single Rev binding site within the RRE. As a consequence, RNA bound to the RRE induces further Rev/Rex/Rec multimerization, which subsequently recruit several hCRM1 molecules [118] [81]. The process is further activated by the DDX1 (an RNA-dependent ATPase) that binds specifically to Rev/Rex/Rec. The NES associates with the cellular CRM1 (chromosome region maintenance 1) in the presence of RanGTP and then the transport ribonucleoprotein complex including viral RNA, Rev/Rex/Rec , CRM1, and other cellular constituents are released via the nuclear pores from the nucleus into the cytoplasm [109]. Afterwards, RanGTP, CRM1 are hydrolyzed leading to separation of Rev/Rex/Rec from the target mRNAs so the NLS of Rev/Rex/Rec is reimported into the nucleus by its functional NLS and importin-β [119] [81].

Interestingly, it has been observed that Rev promotes packaging of the viral RNA into the viral particles to a larger extent than its effect on cytoplasmic RNA amounts therefore, interaction of Rev to the viral RNA during an early nuclear event

is correlated with RNA encapsidation occurring at a late stage of the virus replication cycle [107]; moreover, Rev affect subsequent events in the cytoplasm, including the translation of the cognate mRNA, the movement of Gag proteins to the plasma membrane, and the production of new virus particles [117].

1.5 The Staufen protein

Staufen is a highly conserved double stranded RNA binding protein first identified in Drosophila. There are two types of Staufen proteins named as Staufen-1 and Staufen-2 found in the vertebrates including mammals, fish and birds. Staufen-1 is expressed ubiquitously [120], while expression of Staufen-2 is restricted [121]. The Staufen-1 and Staufen-2 have a molecular weight of 55 kDa and 63/65 kDa, respectively [122]. The human Staufen (hStau) protein displays four copies of functional dsRBDs 2, 3, 4 and 5 (double-stranded RNA-binding domains), which structurally and functionally resemble dsRBD 2 to 5 of the Drosophila melanogaster Staufen (dStau) as illustrated by Figure 12 [123]. Staufen-1 DNA is characterized by absence of dsRBD1 and presence of region between dsRBD4 and dsRBD5 known as tubulin binding domain (TBD) capable of interacting with tubulin [123]. The structure of dsRBD3 shows the highest affinity of a strong binding to RNAs, and protects the short RNA hairpin from the RNases activity [124]. In contrast, dsRBD4 showed a weak binding to RNA [123].

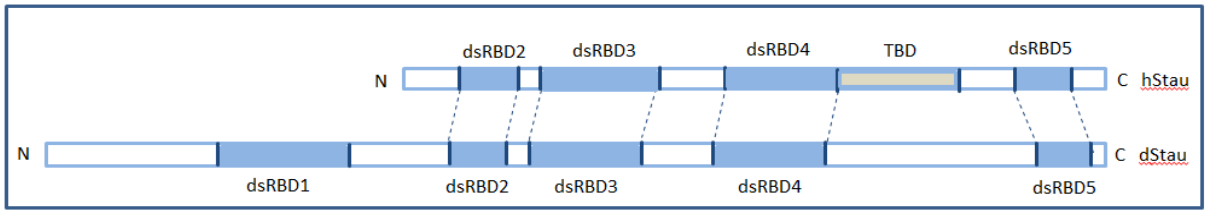


Figure 12: Schematic representation showing the structure of hStau and dStau molecules. Human Staufen is composed of four copies of double-stranded RNA-binding domains (dsRBD2 to dsRBD5), which demonstrated homology to dsRBD2, dsRBD3, dsRBD4 and dsRBD5 belonging to Drosophila melanogaster Staufen. The dStau contains also a dsRBD1 domain, while hStau has a tubulin binding domain (TBD) shown in blue which is located between dsRBD4 and dsRBD5. Modified from Marión *et al*., 1999 [120].

The Staufen-1 and Staufen-2 molecules are expressed and located in the cytoplasm; however these proteins were also detected in the nucleus particularly in the nucleolus [123] because dsRBD3 contains a functional NLS in the carboxyl terminus, by which the Staufen is moved into the nucleus and the nucleolus [125]. The nucleolar trafficking and the cytoplasmic retention termed MOI (modulation of import) process can be carried out by the nuclear localization signal (NLS) with a supporting effect of dsRBD2 and dsRBD4 [125]. Due to lack of NES in the Staufen structure, mammalian Staufen was found to shuttle through the nucleus and exits through exportin-5 and/or CRM1 (Chromosomal Region Maintenance 1)-dependent pathway [126].

Staufen mediates the transport and the translation of the target mRNA, for instance, the Staufen of Drosophila was observed to be correlated with the localization of the mRNAs in oocytes and neuroblasts [127]. Moreover, Staufen molecules appeared to be packaged along with the HIV-1 RNA into virus particles [128]. In addition, it regulates the degradation of mammalian cell mRNAs (Staufen mediated mRNA decay) and the formation of RNA granules [129]. Furthermore, Staufen-1 and Staufen-2 associate to the rough endoplasmic reticulum and the polyosomes and polyribosomal units in the mammalian cytoplasm, and it is examined in the ribonucleoprotein complexes in neurons, therefore, these observations proved the importance of Staufen protein in inducing the RNA transport and the translation event [128]. Staufen is considered as a regulatory component of the stress granules (SGs) containing silenced RNPs (ribonucleoproteins) which are assembled during cellular stress conditions, so Staufen controls the disassembly and formation of the stress granules [129].

1.5.1 Interaction of Staufen-1 with Retroviruses

There are several mechanisms of interaction process is between the Staufen-1 and retroviruses. For example, it has been observed that during HIV-1 replication cycle, the Staufen-1 via its dsRBD3 binds to NC domain of the Gag precursor pr55 through the two zinc finger motifs leading to an enhancement of HIV-1 assembly and an increase in production of new HIV-1 virions by modulating the Gag precursor pr55 multimerization [130] [131] [132]. Interestingly, the Staufen-1 N-terminus domain regulates the Staufen-1 mediated HIV assembly [131]. Also, it has been indicated

that over-expression or depletion of stau1 from cells induced an increase in the viral particles release and the Gag precursor pr55 multimerization [130].

Furthermore, Staufen is responsible for regulation of HIV-1 gene expression by mediating the viral RNA transcripts transport to ribosomes in which Staufen-1 activates the specific mRNA binding to the translation initiation factor known as eIF4E [129]. HIV-1 has the trans-acting responsive (TAR) region located adjacent to the transcription Initiation site at the 5' end of all viral mRNAs and forms a stable secondary RNA stem loop [133]. The RNA TAR structure forms RNA cap structure preventing the accessibility to translation initiation factors leading to inhibition of the translation event [133]. The dsRNA-dependent kinase (PKR) also controls translation of TAR-containing transcripts by attachment of PKR to TAR region resulting in activation of PKR and thus stimulating the phosphorelation of the translation initiation factor 2a (eIF-2a) and inhibition of the translation [134] [135]. In contrast, TAR-RNA binding protein prevents the activation of PKR and therefore, it initiates the translation of TAR bearing RNAs [136]. Staufen-1 shares sequence homology to the TAR-RNA binding protein (TRBP), which binds to TAR sequence so it promotes the translation of TAR-containing RNA transcripts, and increases the generation of viral particles [137]. Additionally, it has been reported that the addition of Staufen-1 caused the up-regulation of reporter activity and the translation process driven by the TAR-carrying RNA. By contrast, Staufen-1 had no influence on translation of translated mRNAs lacking an apparent structured 5' end, indicating that Staufen-1-interaction with the 5' end and ribosomes facilitates the translation initiation [138].

Earlier studies have demonstrated that Staufen-1 is packaged into the HIV-1 and HIV-2 viral particles via its dsRBD3 domain [128]. This suggested that Staufen-1 is necessary for the viral genomic RNA transport, encapsidation for both HIV-1 and HIV-2 because it is bound to the viral RNA genome during the virus assembly and maturation events [139] [132]. Also, overexpression and silencing of Staufen-1 impaired the virus infectivity at several stages such as the reverse transcription, the integration, the level of the packaged genomic RNA and the efficiency of the encapsidation [128] [140]. In addition, the interaction of Staufen-1 with HTLV-1 Rex and HERV-K(HML-2) Rec was studied [141]. It has been observed that overexpression of Staufen-1 induced the nuclear export and/or translation of unspliced HERV-K RNAs, and an increase in the production of new viral particles.

Additional findings revealed that overexpression of Staufen-1 had a positive influence on the assembly of new HIV-1 and HIV-2 virions [142] [143].

In conclusion, Staufen-1 stimulates the generation of the retroviral particles by mediating the translation rate of viral proteins, and binding to Gag and Rev/Rec/Rex and therefore enhancing the nuclear export of the viral RNA transcripts [128] [132] [138].

2 Aims and Objectives of work

The replication of retroviruses relies on interactions between a multitude of cellular factors. The Rec and Gag proteins of HERV-K(HML-2) are known to bind to the cellular protein Staufen-1. Staufen-1 also interacts with the TAR-region HIV-1 RNA and with the HIV-1 Gag protein [137]. Overexpression of Staufen-1 results in a 3-fold increase in the release of HIV-1 from infected cells and a 20-fold enhancement of HERV-K(HML-2) particle production [128] [141]. Moreover, an interaction between Staufen-1 and the HIV-1 Rev protein, a functional homolog of Rec, has also been recently demonstrated [142], but no data are available regarding the potential role of Staufen-1 on SIVmac expression.

The primary objective of this study is therefore to evaluate the impact of Staufen-1 on the expression of SIVmac, including a clarification of the potentially direct interaction between Staufen-1 and SIVmac Rev. The investigation should also provide data on the consequences of Staufen-1 overexpression and downregulation for SIVmac particle production. It should include attempts to describe the underlying molecular mechanisms and to identify the regions on SIVmac Rev and on Staufen-1 involved in the potential modulation of SIVmac expression by Staufen-1.

This analysis of the effect of Staufen-1 on SIVmac should be performed in direct comparison to HIV-1 and HERV-K(HML-2). Should different effects be observed, experiments should be done to shed light on the underlying basis for such differences. The involvement of Staufen-1 in the nucleocytoplasmic transport of viral mRNA should form the focus of this part of the project.

Moreover, within the framework of this study, a HERV-K(HML-2) molecular clone incapable of expressing Rec should be generated. This could be used to investigate the Rec-dependence of HERV-K(HML-2) expression and the ability of Staufen-1 to compensate for Rec. This delta-Rec mutant could also be used to confirm and extend previous knowledge regarding the ability of HIV-1 Rev, HTLV Rex and other retroviral proteins with known or potentially similar functions to substitute for Rec. In this context, the synergistic effect of Staufen-1 should also be clarified

3 Materials and Methods

3.1 Materials

3.1.1 Laboratory Equipment

The machines and supplies used to perform the experiments are shown in Table 1.

Table 1: Laboratory equipment

Model	company
Balance PR803	Mettler Toledo, Giessen, Germany
Balance Ohaus Navigator N2B110	Ohaus, Nänikon, Swiss
Beckman Coulter-Counter Z2	Beckman Coulter, Fullerton, USA
Confocal Microscope LSM 780	Carl Zeiss, Oberkochen, Germany
CPA225D Analytical Balance	Sartorius
CO2 Incubator HeraCell 150	Thermo Scientific, Logan, USA
C24 Incubator Shaker	New Brunswick Scientific, Edison, USA
Dry Block Heater	Grant Instrument Ltd, Cambridgeshire, England
Dialysis membrane	Carl Roth GmbH, Karlsruhe, Germany
Eppendorf Centrifuge 5415D	Eppendorf, Wesseling-Berzdorf, Germany
Eppendorf Centrifuge 5804R	Eppendorf, Wesseling-Berzdorf, Germany
Eppendorf Centrifuge 5810R	Eppendorf, Wesseling-Berzdorf, Germany
Extra Thick Blot Paper	BioRad, Hercules, USA
Film Processing Machine Curix 60	Agfa, Cologne, Germany
Fume Hood	Köttermann GmbH & Co KG, Uetze/Hänigsen, Germany
Gene Pulser XCell + Shock Pod	BioRad, Hercules, USA
Gel Documentation machine 2000	BioRad, Hercules, USA
Heraeus Biofuge Primo R centrifuge	Thermo Scientific
Heraeus multifige 1 S-R	Thermo Fischer Scientific
Hettich Mikro 200R Centrifuge	DJB Labcare Ltd, Buckinghamshire, England
Horizontal Shaker 3016	GFL – Society for Laboratory Technology, Burgwedel, Germany
Incubator Innova 4330	New Brunswick Scientific, Edison, USA
Inverse Light Microscope ID03	Carl Zeiss, Oberkochen, Germany
Microplate Luminometer Centro LB 960	Berthold Technologies, Oak Ridge, USA
Microplate Absorbance Reader Sunrise	Tecan, Männedorf, Swiss

Mini-PROTEAN Tetra Cell Electrophoresis	BioRad, Hercules, California, USA
NanoDrop Spectrophotometer ND-1000	Nanodrop, Wilmingon, USA
Nitrocellulose Membrane (0.45 µm)	BioRad, Hercules, USA
Overhead Rotator Reax 2	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
PCR Thermocycler	BioRad, Hercules, USA
pH-Meter MP220	Mettler Toledo, Giessen, Germany
PowerPAC HC	BioRad, Hercules, USA
PowerPACTM 200	BioRad, Hercules, USA
PVDF Membrane	Carl Roth GmbH, Karlsruhe, Germany
SONIFIER 250	Branson
Sub-Cell GT Agarose Gel Electrophoresis System	BioRad, Hercules, California, USA
Safety cabinet HeraSafe	Thermo Fischer Scientific
Sprout-R Mini-Centrifuge	Heathrow Scientific LLC, Illinois, USA
Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell	BioRad, Hercules, California, USA
UV Transilluminator TFX-20.M	Vilber Lourmat, Paris, France
Water Bath	GFL – Society for Laboratory Technology, Burgwedel, Germany
Whatman® Puradisc 25 syringe Filters (0.2 µm)	Sigma-Aldrich Chemie GmbH, Munich, Germany
X-ray Film	Eastman Kodak Company, Rochester, New York, USA

3.1.2 Chemicals

Chemicals utilized in this study are listed in Table 2.

Table 2: Chemicals

Chemical	Company
Agarose	Peqlab Biotechnologie GmbH, Erlangen, Germany
Ammonium persulfate (w/v) 10% (APS)	Carl Roth GmbH, Karlsruhe, Germany
Acrylamide (30%)	Carl Roth GmbH, Karlsruhe, Germany
Agar-agar	Carl Roth GmbH, Karlsruhe, Germany
BSA (10x)	New England Biolabs Inc., Beverly, USA
β-Mercaptoethanol	Sigma-Aldrich Chemie GmbH, Munich, Germany
Complete protease inhibitor cocktail tablets	Roche Diagnostics GmbH, Mannheim, Germany
Coomassie Brilliant Blue G Concentrate	Sigma-Aldrich Chemie GmbH, Munich, Germany

Dimethylsulfoxide (DMSO) 50%	Sigma-Aldrich Chemie GmbH, Munich, Germany
Deoxynucleotide triphosphates (dNTPs)	Fermentas International Inc., Burlington, Canada
DAPI(4',6-Diamidin-2' phenylindoldihydrochlorid)	Carl Roth GmbH, Karlsruhe, Deutschland
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Deutschland
Ethanol	Carl Roth GmbH, Karlsruhe, Germany
GelRed	Biotium, Hayward, USA
Glycerin (100%)	Serva, Heidelberg, Deutschland
Hydrogen peroxide (30%)	Carl Roth GmbH, Karlsruhe, Germany
Imidazole	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isopropylβ-D-1-thiogalactopyranoside (IPTG)	AppliChem
Isopropanol	Carl Roth GmbH, Karlsruhe, Deutschland
Methanol	MERCK, Darmstadt, Deutschland
Magnesium chloride (MgCl2)	Roche Diagnostics GmbH, Mannheim, Germany
Ni-NTA Agarose	Qiagen GmbH, Hilden, Germany
o-Phenylenediamine dihydrochloride (OPD)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH, Karlsruhe, Germany
Phenylmethanesulfonylfluoride (PMSF)	AppliChem
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Skimmed milk powder	Carl Roth GmbH, Karlsruhe, Germany
Super SignalWest Dura Extended Duration Substrate	Thermo Scientific, Logan, USA
Sulfuric acid, 2.5M	Carl Roth GmbH, Karlsruhe, Germany
Tween-20	Carl Roth GmbH, Karlsruhe, Germany
TrisHCl	Carl Roth GmbH, Karlsruhe, Germany
TEMED	Carl Roth GmbH, Karlsruhe, Germany
Triton-X 100	Carl Roth GmbH, Karlsruhe, Germany

3.1.3 Kits

Kits used in this work are mentioned in Table 3.

Table 3: List of the used kits and their companies.

Kit	Company
Dual-Glo® Luciferase Assay System	Promega Corporation, Madison, USA
Endo-free Plasmid Maxi Kit	Qiagen GmbH, Hilden, Germany
Expand High Fidelity PCR System	Roche Diagnostics GmbH, Mannheim
ECL Plus Western Blot Detection System	GE Healthcare, Fairfield, USA
Luciferase 1000 Assay System	Promega Corporation, Madison, USA
MetafectenePro	Biontex, Munich, Germany
PolyFect Transfection Reagent	Qiagen GmbH, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen GmbH, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen GmbH, Hilden, Germany
QuikChange® Site-Directed Mutagenesis Kit	Agilent Technologies, Inc., Santa Clara, California, USA
QuikChange Multi Site-Directed Mutagenesis Kit	Agilent Technologies, Inc., Santa Clara, California, USA
QIAquick PCR Purification Kit	Qiagen GmbH, Hilden, Germany
Super Signal West Dura Extended Duration Substrate	Thermo Scientific, Logan, USA

3.1.4 Buffers and culture media

All buffers used in the experiments are stated in Table 4.

Table 4: Buffers and culture media

Buffer	Component/Company
Annealing buffer (10x)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Bacterial lysis buffer	250mM HEPES pH 7, 100mM Na2SO4, 400mM (NH4)2SO4, 200mM NaCl, 0.1% Tween-20, 2mM β-mercaptoethanol, 2mM fresh PMSF, protease inhibitor cocktail (one tablet/50ml)
Buffer A	50mM Tris pH 8.0, 250mM NaCl, 400mM (NH4)2SO4, 100mM Na2SO4, 20mM Imidazole, 2mM β-mercaptoethanol
Buffer A+	50mM Tris pH 8.0, 0.1% Tween-20, 2mM NaCl, 400mM (NH4)2SO4, 100mM Na2SO4, 20mM Imidazole, 2mM β-mercaptoethanol

Blocking buffer	5% skimmed milk powder in PBS with 0.1% Tween-20
CutSmart buffer (10x)	New England Biolabs Inc., Beverly, USA
Calcium chloride	Carl Roth GmbH, Karlsruhe, Germany
Carbonate bicarbonate buffer	Sigma-Aldrich Chemie GmbH, Munich, Germany
DNA sample buffer (6x)	10mM tris-acetate, 50mM EDTA, 10% Ficoll-400 (w/v) (Serva), 0.4% Orange- G (w/v) (Sigma) in a double distilled water
DNA polymerase buffer (10x)	Agilent Technologies, Inc., Santa Clara, California, USA
Dulbecco's Modified Eagle Medium (DMEM)	PAA Laboratories GmbH, Pasching,Austria (with 1 g/L glucose and 2mM L-glutamine and 1x Penicillin/Streptomycin)
Expand High Fidelity buffer (10x)	Roche Diagnostics GmbH, Mannheim, Germany
FCS (Fetal calf serum)	Biochrom, Berlin, Germany
2x HBS (HEPES Buffer Saline)	50mM HEPES, 280mM NaCl, 1.5mM disodium phosphate, Double distilled water, pH 7.1
Isoton R II Diluent	Beckman Coulter, Inc., Fullerton, USA
IGEPAL lysis buffer	Sigma-Aldrich Chemie GmbH, Munich, Germany
Lysis buffer	50mM Tris HCL pH 7.5, 150mM NaCl, 5mM EDTA, fresh protease inhibitor tablet (1 tablet/50ml)
Laemmli sample buffer (4x)	200mM Tris-Cl, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 40% (v/v) glycerol, 400mM DTT
LB medium	1% tryptone, 0.5% bacto yeast extract, 1% NaCl, pH 7.5
LB-Agar	LB medium with 20 g/L agar-agar
NEB and EcoRI buffers	New England Biolabs Inc., Beverly, USA
NP-40	New England Biolabs Inc., Beverly, USA
Phosphate buffered saline (PBS)	123mM sodium chloride, 2.7mM potassium chloride, 10mM disodium hydrogen phosphate, 2mM potassium dihydrogen phosphate, pH 7
PM	2% skimmed milk powder in PBS
PMT	2% skimmed milk powder in PBS with 0.05% Tween-20
PBS/Tween	PBS with 0.05% Tween-20
Phosphate citrate buffer 0.05mM, pH 5.0	Sigma-Aldrich Chemie GmbH, Munich, Germany
Resolving gel buffer	BioRad, Hercules, California, USA
Running buffer	BioRad, Hercules, California, USA
RPMI	PAA Laboratories GmbH, Pasching, Austria
Stacking gel buffer	BioRad, Hercules, California, USA

Stripping buffer	62.5 mM Tris, 2%SDS 100 mM ß-Mercaptoethanol, pH 6.7
SOC-Medium	20 g/L bacto tryptone, 5 g/L bacto yeast extract, 4 g/L glucose, 2.5mM KCl, 10mM NaCl, 5mM MgCl, pH 7.0
Tango buffer (10x)	Fermentas International Inc., Burlington, Canada
Transfer buffer	BioRad, Hercules, California, USA (2,5 mM Tris, 19.2 mM Glycin, 20 % Methanol, pH 8.3)
TE (1x)	10 mM Tris/HCl, 1 mM EDTA, pH 8,0
TAE (1x)	40mM Tris, 1mM EDTA, 20mM acetate, pH 8.0
T4 DNA ligase buffer	New England Biolabs Inc., Beverly, USA
Washing buffer	PBS with 0.1% Tween-20

3.1.5 Bacterial Strains

The bacterial strains utilized in this study are displayed in Table 5.

Table 5: The used bacterial strains

Bacterial strains	Company
One Shot® Stbl3 Chemically competent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® Stbl4 Chemically competent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® Stbl3 Electrocompetent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® Stbl4 Electrocompetent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® BL21 (DE3) Chemically Competent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® TOP10 Chemically competent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® TOP10 Electrocompetent *E. coli*	Invitrogen Corporation, Carlsbad, USA

3.1.6 Antibiotics

The antibiotics supplemented in experiments are given in Table 6.

Table 6: The used antibiotics

Antibiotic	Company
Penicillin/Streptomycin (10,000 µg/ml)	Biochrom, Berlin, Germany
Ampicillin (100 µg/ml)	Carl Roth GmbH, Karlsruhe, Germany
Kanamycin (50 µg/ml)	Carl Roth GmbH, Karlsruhe, Germany

3.1.7 Cell lines

The eukaryotic cells used in the current research were Human Embryonic Kidney 293 cells referred to as HEK-293T cells, whereas the virus infection experiments were carried out by using C8166 cells. Cells were obtained from ATCC, LGC Standards GmbH, Wesel, Germany.

3.1.8 Growth Media

HEK 293T cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) comprising 1 g/L glucose, 2 mM L-glutamine and o.5% Penicillin/Streptomycin, 10% FCS while C8166 cells were grown in RPMI medium containing 1 g/L glucose and L-glutamine, supplemented with 10% FCS and 0.5% Penicillin/Streptomycin. The different types of the bacterial cells were grown in LB broth and agar media.

3.1.9 Enzymes

The enzymes included in this work are listed in Table 7.

Table 7: Enzymes

Enzyme	Source
AatII	New England Biolabs Inc., Beverly, USA
ApaI	New England Biolabs Inc., Beverly, USA
BamHI	New England Biolabs Inc., Beverly, USA
BigDye 3. 1.	ABI Terminator Chemie, Applied Biosystems; Foster City
ClaI	New England Biolabs Inc., Beverly, USA
DpnI	New England Biolabs Inc., Beverly, USA
EcoRI	New England Biolabs Inc., Beverly, USA
Expand High Fidelity enzyme mix	Roche Diagnostics GmbH, Mannheim, Germany
HindIII	New England Biolabs Inc., Beverly, USA

Hot Star Taq- Polymerase	Qiagen GmbH, Hilden, Germany
Lysozyme	Carl Roth GmbH, Karlsruhe, Germany
NotI	New England Biolabs Inc., Beverly, USA
PstI	New England Biolabs Inc., Beverly, USA
PfuTurbo DNA Polymerase	Agilent Technologies, Inc., Santa Clara, California, USA
Polynukleotid kinase	New England BioLabs, Ipswich
0.05% Trypsin (pH 7.2), 0.02% EDTA in PBS	Biochrom, Berlin, Germany
SalI	New England Biolabs Inc., Beverly, USA
SpeI	New England Biolabs Inc., Beverly, USA
SacI	New England Biolabs Inc., Beverly, USA
ScaI	New England Biolabs Inc., Beverly, USA
T4 DNA Ligase	Fermentas International Inc., Burlington, Canada
Taq DNA Polymerase	Sigma Life sciences, München
XhoI	New England Biolabs Inc., Beverly, USA
XbaI	New England Biolabs Inc., Beverly, USA
XmaI	New England Biolabs Inc., Beverly, USA
Tth111I	New England Biolabs Inc., Beverly, USA
Benzonase® Nuclease	Novagen, USA

3.1.10 Antibodies

The specific antibodies used in Western blot analysis, ELISA test and Immunofluorescence assay are illustrated by Table 8.

Table 8: Antibodies

Antibody	Origin	Company/Source
AlexaFluor 568 α-Mouse-IgG (1:2000)	Goat	Invitrogen Corporation, Carlsbad, USA
α-FLAG-Tag (1:5000)	Rabbit	Sigma-Aldrich Chemie GmbH, Munich, Germany
β-Actin-HRP (1:50000)	Mouse	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Staufen (1:4000)	Rat	Dr. Kirsten Hanke & Dr. Oliver Hohn
α-HIV/SIV Gag (AG3.0) (1:800)	Mouse	Dr. Stephen Norley
α-V5-Tag (1:5000)	Mouse	AbD Serotec, MorphoSys AG, Martinsried, Deutschland
α-GAPDH (1:1000)	Mouse	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Rat IgG-HRP (1:5000)	Goat	LI-COR, Lincoln, Nebraska, USA
α-V5-HRP (1:5000)	Mouse	Invitrogen Corporation, Carlsbad, USA

α-V5-agarose	Mouse	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Rabbit IgG-HRP (1:5000)	Donkey	LI-COR, Lincoln, Nebraska, USA
α-Human IgG-HRP (1:2000)	Rabbit	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Mouse IgG-HRP (1:5000)	Goat	Sigma-Aldrich Chemie GmbH, Munich, Germany
monoclonal α-V5-Cy3 (1:750)	Mouse	Jackson Immuno Research, Baltimore, USA
α-Mouse-IgG-Cy3 (1:750)	Goat	Jackson Immuno Research, Baltimore, USA
Monoclonal α-HERV-K Gag Herma 7 (1:100)	Rat	PEI, Langen
α-HERV-K capsid (1:5000)	Rat	George *et al*. [144]
HIV plasma pool (1:2000)	Human	Dr. Stephen Norley
α-HERV-K113-Rec (1:1000)	Rat	Hanke *et al.* [141]
α-His-IgG-HRP (1:20000)	Rat	Sigma-Aldrich Chemie GmbH, Munich, Germany
Monoclonal α-Histidine (1:5000)	Mouse	Sigma-Aldrich Chemie GmbH, Munich, Germany

3.1.11 Oligonucleotides

All oligonucleotides were synthesized and lyophilized by Invitrogen Corporation, Carlsbad, USA and Life Technologies GmbH, Darmstadt, Germany. The sequencing primers used are listed in Table 9, while the primers for DNA mutagenesis reactions are mentioned in Table 10.

Table 9: Sequencing primers

Primer name	**Primer sequence** 5'→ 3'
BGH-Rev	TAGAAG GCA CAG TCG AGG
pcDNA3 Seq-For	GTGTACGGTGGGAGGTCT
T3-For	ATTAACCCTCACTAAAGGGA
T7-For	TAATACGACTCACTATAGGG
T7-Rev	GCTAGTTATTGCTCAGCGG
SP6-For	ATTTAGGTGACACTATAG
H1-up Seq-For	TCGCTATGTGTTCTGGGAAA
Seq-pBSK-ori- 9011- F	CACTGGGACATGGTTAGACG
Seq-pBSK-ori- 9556 -R	TCCTGCACCGCCCTTAATCC

Seq-pBSK-ori -8053 -R	CGCACTATTGGCCACACATTC
Seq-pBSK-ori -7451- F	GCACAACTAAAGAAGCTGACG
pCMV-Tag2b-51-F	CATATATGGAGTTCCGCGTTAC
pCMV-Tag2b-687-R	GCTCTTATCGTCGTCATCCTTG
pBSK-ori-RevO	CCAAGCGCGCAATTAACC
pBSK-ori-For2	ATGCCTCTTATCTCAGCTTT
pBSK-ori-For5	AGGCCCCACAACAAACTG
pBSK-ori-For8	TCTATCTTAAGAGGAGACTCAGACTT
pBSK-ori-FO	GGCGATTAAGTTGGGTAACG
pLVTHMShRNA staufen 3084 (Forward)	TCGCTATGTGTTCTGGGAAA
pLVTHMShRNA staufen 3084(Reverse)	ATTTAGGTGACACTATAG

Table 10: Mutagenic oligonucleotide primers

Primer name	**Primer sequence** 5'→ 3'
clon-SIVmac coRev_Δ31-For	CGATATCAAGCTTGCCACCGGAACCGCTAACCAGC
clon-SIVmac coRev_Δ59-For	CGATATCAAGCTTGCCACCAGCTTCCCTGACCCC
mut-SIVmac coRev_M5-For	GGAACCGCTAACCAGgatttaCAGAGGCGCCGACGGTGG
mut-SIVmac coRev_M5-Rev	CCACCGTCGGCGCCTCTGtaaatcCTGGTTAGCGGTTCC
mut-SIVmac coRev_SLT40-For	CCTGGCTGATCGAgaCgACAGCTTCCCTGACC
mut-SIVmac coRev_ SLT40-Rev	GGTCAGGGAAGCTGTcGtcTCGATCAGCCAGG
Mut-oriHERV-SD2- For	CTTGATGATTGTATCAATGGTGGcAAGTCTCCCTATGCCTGCAG
Mut-oriHERV-SD2-Rev	CTGCAGGCATAGGGAGACTTgCCACCATTGATACAATCATCAAG
Mut-oriHERV-SA2-For	CTGTTTTGTCTGTTGTTgGTCTGCAGGTGTACCCAACAG
Mut-oriHERV-SA2-Rev	CTGTTGGGTACACCTGCAGACcAACAACAGACAAAACAG

Del-oriHERV-SD2-For	GCCTTGATGATTGTATCAATTCTCCCTATGC CTGCAGGAG
Del-oriHERV-SD2-Rev	CTCCTGCAGGCATAGGGAGAATTGATACAAT CATCAAGGC

3.1.12 Ladders and Standards

The DNA and protein markers utilized for the DNA gel electrophoresis and Western blot tests are shown in Table 11.

Table 11: Ladders and Standards

Ladder	Company
PageRuler Prestained Protein Ladder	Thermo Scientific, Logan, USA
PageRuler Plus Prestained Protein Ladder	Thermo Scientific, Logan, USA
Generuler 100 bp Ladder	Fermentas International Inc., Burlington, Canada
Generuler 100 bp Plus Ladder	Fermentas International Inc., Burlington, Canada
Generuler 1 kb Ladder	Fermentas International Inc., Burlington, Canada
Generuler 1 kb Plus Ladder	Fermentas International Inc., Burlington, Canada

3.1.13 Vectors

List of vectors used in this work are stated in Table 12.

Table12: Vectors

Vector	Company
pCMV-Tag2b	Agilent Technologies, Inc., Santa Clara, California, USA
pLVTHM	Tronolab, Lausanne
pcDNA4-V5-His/B	Invitrogen Corporation, Carlsbad, USA
pVSV-G	Tronolab, Lausanne
psPAX2	Tronolab, Lausanne

3.1.14 Recombinant Plasmids

The recombinant plasmids utilized in this study are listed in Table13.

Table 13: Recombinant Plasmids

Recombinant Plasmid	Source/Publication
pLVTHM-shStauen_A7	Jula Wamara (Exon 14, GCTGCGCTGAACATCTTAAAG) [145]
pLVTHM-shStauen_A2C4	Jula Wamara (Exon 8/9, GCCACAGACAAGCCCAGAATA) [145]
pLVTHM-shStauen_B3	Jula Wamara (Exon 15, = 3'-UTR, GCAGGGAAGACAACAGAAACA) [145]
pLVTHM-shStauen_C1	Jula Wamara (Exon 6/7, GGAGGTGAATGGAAGAGAATC) [145]
pLVTHM-shStauen_C3	Jula Wamara (Exon 8b, GCCTGCAGTTGAACGAGTAAA) [145]
pLVTHM-shStaufen-3086	In this study (Exon 15, = 3'-UTR, aaatagcacagtttggaaact)
pLVTHM-shCofilin	Jula Wamara [145]
Shuttle	Hanke *et al.*[141]
Shuttle HIV-2RRE	Anna Klara-Amler [143]
pCMV-*Renilla* luciferase	Dr. Kirsten Hanke
pCMV-FLAG-Staufen-1	Hanke *et al.* [141]
pCMV-FLAG-Staufen-1_F135A	Jula Wamara [145]
pCMV-FLAG-Staufen-1_ΔRBD3	Jula Wamara [145]
pCMV-FLAG-Staufen-1_ΔRBD4	Jula Wamara [145]
pCMV-FLAG-Staufen-1_ΔTBD/RBD5	Jula Wamara [145]
pET-16b- V5-Staufen-1	Dr. Kirsten Hanke
pCDNA3-V5-Staufen-1	Hanke *et al.* [141]
pCDNA3-V5-HERV-K113-orico Rec	Hanke *et al.* [141]
pCDNA3-V5-HIV-1-coRev	In this study
pCDNA4-V5-SIVmac-coRev	In this study
pCMV-FLAG- SIVmac-coRev	In this study

pBR_SIVmac239 full length molecular clone	kindly provided by F. Kirchhoff, University Ulm
pNL4-3 HIV-1 molecular clone	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin [146]
pBSK ori HERV-K113 molecular clone	Chudak *et al.* and George *et al*. [147] [144]
ovalbumin expression vector (pTH-OVA)	Dr. Oliver Hohn
pCMV-FLAG-SIVmac-coRevΔ31	In this study
pCMV-FLAG-SIVmac-coRevΔ59	In this study
pCMV-FLAG-SIVmac-coRev-ΔM5	In this study
pCMV-FLAG-SIVmac-coRev-ΔSLT40	In this study
SHIV luciferase reporter gene	Dr. Veronika Lausch
pBSK ori HERV-K113BCD	Prof. Dr. Norbert Bannert
Mut-SD2-ori-BCD	In this study
Mut-SA2-ori-BCD	In this study
Deleted-SD2-ori-BCD	In this study
pBSK ori HERV-K113 ΔRec	In this study
pCDNA4a-V5-np9-5q33-GFP	Dr. S. Stengel
pCDNA4-V5-HTLV-coRex	Dr. Kirsten Hanke

3.1.15 Software

In table 14, Software used is shown.

Table 14: Software

Adobe Photoshop CS5
BioEdit
EditSeq
MicroWIN 2000
MegAlign
Microsoft Office 2010
Nanodrop ND-1000 v3.3
SeqBuilder
Zen2012

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Growth conditions

HEK-293T cells were cultivated in DMEM medium containing 1 g/L glucose and L-glutamine, supplemented with 10% fetal calf serum (FCS) and 0.5% Penicillin/Streptomycin. C8166 cells were grown in RPMI 1860 medium containing 1 g/L glucose and L-glutamine, supplemented with 10% FCS and 0.5% Penicillin/Streptomycin. All cell lines were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

3.2.1.2 Cell splitting

For splitting of HEK-293T cells and C8166 cells, the conditioned growth medium was discarded, cells were washed once with 10mL 1x PBS and Trypsin/EDTA was added and gently mixed around the flask. The trypsin/EDTA treated cells were incubated for 3 min in 37°C CO_2 incubator. Afterwards, cells were rinsed from the culture flask by adding fresh growth medium, transfered to 50ml centrifuge tube and spinned down at 300g for 5 min. The supernatant was discarded and cells were resuspended in fresh growth medium. An appropriate number of cells were dispensed to a new culture flask containing fresh growth medium and placed back into the 37°C CO_2 incubator.

3.2.1.3 Sowing cells

First, cells were washed and treated with trypsin/EDTA as described above (see 3.2.1.2). Suspended cells were counted using the Coulter Counter Z2 and sown into multiwell plates and 100 mm dishes for transfection and infection experiments. Cells were sown in a proper cell number with a given volume of fresh growth medium (see Table 15).

Table 15: Parameters for cell transfection in different reservoirs.

Type of Reservoir	Cell number to sow	Cell yield	Medium volume (ml)
T300 flask (300 cm^2)	15 * 10^6 cells/ml	3.6 * 10^7 cells/ml	200
T150 flask (150 cm^2)	7.5 * 10^6 cells/ml	18.4*10^6 cells/ml	20
100 mm dish	1 * 10^6 cells/ml	4 * 10^6 cells/ml	10
6-well plate	3 * 10^5 cells/ml	2.4 * 10^6 cells/ml	3
12-well plate	6 * 10^4 cells/ml	2.4 * 10^5 cells/ml	1
96-well plate	1 * 10^4 cells/ml	4 * 10^4 cells/ml	0.3

3.2.1.4 Transfection

Cells cultured in 100 mm dishes were transfected by using the calcium phosphate transfection technique. One day before transfection, cells were grown as previously described (see 3.2.1.3). At approximately 80% the cells were transfected with the following calcium phosphate-DNA mixture: 15 – 30 µg DNA was diluted and brought to a final volume of 450 µl with double distilled water. The mixture was gently mixed and then 50 µl 2.5M $CaCl_2$ was added and gently mixed again. Subsequently, drops of the DNA mixture were carefully pipetted onto the surface of 500 µl 2x HEPES in a 1.5ml tube. The DNA mixture was incubated for 30 min at room temperature for complex formation. During incubation period, cells were washed once with 10 ml 1x PBS and a fresh growth medium was added. This transfection mixture was transferred to the cells dropwise and the dish was swirled gently to confirm uniform distribution of the DNA complexes. In the following day, the conditioned growth medium was discarded; cells were washed once with 10 ml 1x PBS and new growth medium was added. Cells grown in 6-well, 12-well or 96-well plates were transfected using PolyFect Reagent which was used according to the manufacturer's instructions.

3.2.1.5 Cell lysis and preparation of supernatant

Before cell lysis 48 h post transfection the supernatant was collected, cell free centrifuged at 1500g for 10 min, aliquoted and stored at -80 °C. For transfection with the pBSK-HERV-K113 molecular clone, cell culture supernatants were centrifuged at 27410g and 4 °C for three hours to pellet the virus. The supernatants were carefully discarded followed by dissolving the virus pellets in 80 µl 0.5% NP-40 or 0.5% IGEPAL lysis buffer. The cells were washed once with ice-cold 1x PBS. After removing PBS, an appropriate volume of 0.5% NP-40 or IGEPAL lysis buffer with fresh protease inhibitor was added. By using a cell scraper, the cells were removed from the dishes or plates, agitated at 4 °C for 30 min to ensure the cell lysis and the lysate was centrifuged at 13400g and 4 °C for 20 min. The supernatant was carefully transferred to a fresh tube and immediately stored at -20 °C until usage.

3.2.1.6 Infection

For the infection with full length SIVmac239 virus the cells at an appropriate density were sown in 100 mm dishes. After one day, 1 ml of the virus stock was added to the cells in 9 ml fresh growth RPMI medium containing 1 g/L glucose and L-glutamine, supplemented with 10% FCS and 0.5% Penicillin/Streptomycin. One day post infection, the old media were discarded and replaced with fresh growth media. Afterwards, the supernatants were collected at 4, 6, and 8 days post incubation, and the concentration of Gag protein was determined by ELISA.

3.2.2 DNA analysis

3.2.2.1 Polymerase Chain Reaction (PCR)

PCR assay was applied to amplify the target DNA segment *in vitro.* This reaction was performed by using specific PCR primers and *Taq* DNA polymerase enzyme as shown in Table 16.

Table 16: The components of PCR reaction

Component	Concentration	Volume	Final concentration
Standard *Taq* reaction buffer/ 25 mM MgCl2	10x	2.5 µl	1x
dNTPs	2.5 mM each	1 µl	0.1 mM each
Forward primer	10 µM	1 µl	400 nM
Reverse primer	10 µM	1 µl	400 nM
Template DNA	-	500 pg - 100 ng	-
Taq polymerase	250 U/µl	0.125 µl	1.25 U/µl
Double distilled water	-	To 25 µl	-

PCR cycling conditions for DNA amplification were given in Table 17. The annealing temperature is variable depending on the PCR primers used in this technique.

Table 17: Thermocycling conditions for PCR assay

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	30 sec	35
Annealing	X °C	20 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

3.2.2.2 Expand High Fidelity PCR

Expand High Fidelity PCR System contains a mixture of Taq (Thermus aquaticus) DNA polymerase and Tgo (Thermococcus gorgonarius) DNA polymerase with proofreading activity for higher DNA yield, synthesis, accuracy and Repair of mismatched primers at 3’ end. Two PCR reaction mixtures were prepared to avoid partial degradation of the primers and the template DNA by the 3’-5’ exonuclease activity of the proofreading polymerase. After setting up the reaction both mixtures were combined. The components of the reaction mixes and thermal profile for Expand High Fidelity PCR are included in Table 18 and Table 19 respectively.

Table 18: The compositions of the reaction mixtures for Expand High Fidelity PCR

Reaction Mixture 1			
Component	**Concentration**	**Volume**	**Final concentration**
dNTPs	10 mM each	1 µl	200 µM each
Forward primer	10 µM	1.5 µl	300 nM
Reverse primer	10 µM	1.5 µl	300 nM
Template DNA	-	50 ng-250 ng	-
Double distilled water	-	To 25 µl	-
Reaction Mixture 2			
Component	**Concentration**	**Volume**	**Final concentration**
Expand High Fidelity buffer/ $Mgcl_2$	10x	5 µl	1x
Expand High Fidelity enzyme mixture	3.5 U/µl	0.75 µl	2.6 U
Double distilled water	-	To 25 µl	-

Table 19: The thermal profile of Expand High Fidelity PCR

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94 °C	2 min	1
Denaturation	94 °C	15 sec	10
Annealing	X °C	30 sec	
Extension	68 °C	8 min	
Denaturation	94 °C	15 sec	20
Annealing	X °C	30 sec	
Extension	68 °C	8 min+5 s	
Final extension	72 °C	7 min	1

3.2.2.3 DNA Mutagenesis

The PCR Site directed mutagenesis method was performed to introduce mutations in the DNA sequence of the desired gene, causing either substitution or deletion of amino acids. The QuikChange Site-Directed Mutagenesis method was carried out according the protocol of the manufacture. The sample reaction is indicated in Table 20, while the cycling parameters for the site-directed mutagenesis PCR is given in Table 21.

Table 20: sample reaction of site directed mutagenesis

Component	Concentration	Volume	Final concentration
Reaction buffer/ 25 mM MgCl2	10x	5 µl	1x
dNTPs	2.5 mM each	1 µl	500 nM each
Forward primer	10 µM	1 µl	200 nM
Reverse primer	10 µM	1 µl	200 nM
Template DNA	-	50 ng	-
PfuTurbo DNA Polymerase	2.5 U/µl	1 µl	0.05 U/µl
Double distilled water	-	To 50 µl	-

Table 21: Cycling parameters for site-directed mutagenesis PCR

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	30 seconds	1
Denaturation	95 °C	30 seconds	18
Annealing	55 °C	1 min	
Extension	68 °C	1 minute/kb of DNA	

Additionally, the QuikChange® Multi Site-Directed Mutagenesis Kit was carried out according to the manufacturer's instructions. The Mutant Strand Synthesis Reaction and the cycling parameters for Multi-Site-Directed Mutagenesis assay are stated in Table 22 and Table 23 respectively.

Table 22: The Mutant Strand Synthesis Reaction

Component	Concentration	Volume	Final concentration
QuickChange Multi Reaction buffer/ 25 mM MgCl2	10x	2.5 µl	1x
QuikSolution	-	0.5 µl	1x
dNTPs	2.5 mM each	1 µl	1 mM each
Forward primer	10 µM	1 µl	400 nM
Reverse primer	10 µM	1 µl	400 nM
Template DNA	-	100 ng	-
PfuTurbo DNA Polymerase	2.5 U/µl	1 µl	0.1 U/µl
Double distilled water	-	To 25 µl	-

Table 23: Cycling parameters for the Multi-site-directed mutagenesis PCR

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	1 min	30
Annealing	X °C	1 min	
Extension	65 °C	2 minute/kb of plasmid length	

Following DpnI digestion, the site-directed mutagenesis PCR products were transformed into bacterial cells (see 3.2.2.8), plasmid DNA was extracted (see 3.2.2.9) and sequenced (see 3.2.2.11). To verify the successful mutation, the original sequences were aligned with the mutated sequences using MegAlign.

3.2.2.4 Restriction Enzyme Digest

Restriction enzymes referred to as digestion endonucleases that cleave double-stranded DNA (dsDNA) at specific sequences within or adjacent to their recognition sites generating ends with 5 'overhang. Each restriction enzyme has its specific optimal reaction buffer which yields 100% activity for the enzyme. In Table 24, the restriction enzyme digest reaction is shown.

Table 24: Components of the restriction digest reaction

Component	Volume for cloning	Volume for detection of the target plasmid
Restriction Enzyme 10X Buffer	3 µl	2 µl
10x BSA	3 µl	2 µl
Restriction Endonuclease	1 µl	0.5 µl
DNA	5 µg	0.5-1 µg
Double distilled water	To 30 µl	To 20 µl

The DNA digestion mixtures were gently mixed by pipetting, and centrifuged for a few seconds in a microcentrifuge and then incubated at the enzyme's optimum temperature (25°C or 37°C) for 2 hours. After incubation, loading buffer to a 1X final

concentration was added and proceeded to gel electrophoresis analysis (see 3.2.2.5).

3.2.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate a population of the mixed DNA molecules by their length in agarose gel matrix. This method was performed by adding an appropriate amount of a powdery agarose (0.8 g to 1.5 g) to 50 ml or 100 ml 1x TAE electrophoresis buffer according to the expected DNA size. Subsequently, agarose gel was completely dissolved in the buffer by heating in a microwave until a clear, transparent solution was observed. Ethidium bromide to a final concentration of 0.5 µg/ml were added to the molten agarose, gently mixed by swirling and poured into a mold containing a suitable comb allowing hardening. Upon solidification, samples mixed with an appropriate quantity of 6x DNA loading buffer were slowly loaded into the slots of the submerged gel. Also, the approximate length of the DNA fragment was identified by loading Generuler 1 kb Ladder, Generuler 1 kb Plus Ladder, Generuler 100 bp Ladder, or Generuler 100 bp Plus Ladder, according to the expected length of the target DNA fragments. Voltage of electrophoresis was applied at 70 to 80V for 60-90 min. After migration of the DNA samples until a sufficient distance, the DNA bands were examined by UV Transilluminator or Gel Documentation machine 2000.

3.2.2.6 Purification of the DNA fragments

3.2.2.6.1 Purification of the PCR product

Purification of PCR products was achieved using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The DNA fragments with a length of 100 bp up to 10 kb were isolated by binding to silica-membrane, followed by washing steps and eluted with double distilled water.

3.2.2.6.2 Gel Extraction

The DNA fragments were separated depending on their sizes using agarose gel electrophoresis (see 3.2.2.5). Upon the DNA fragment separation, the gel was placed on UV Transilluminator, and the target DNA bands were excised with a clean scalpel. The target DNA fragments were purified from agarose gels by applying the QIAquick Gel Extraction Kit according to the manufacturer's instructions by which the extracted gel was dissolved in a binding buffer at 50 °C and then DNA was bound to a silica gel membrane, washed and isolated by elution with 30 µl elution buffer EB or double distilled water.

3.2.2.7 Ligation

Digested DNAs were inserted into a digested DNA vector backbone to create a recombinant vector by T4 DNA ligase, which catalyzes the formation of phosphodiester bonds between the free 3’ hydroxyl end of one DNA terminus with the 5’ phosphate end of another one. The compositions of the ligation mixture are displayed in Table 25.

Table 25: Ligation reaction

Component	Volume
10x ligation Buffer	2 µl
Insert	X µl
Vector	X µl (40 ng – 100 ng)
T4 DNA Ligase	1-2 µl
Double distilled water	To 20 µl

The concentration of the DNA insert used was calculated according to the following equation:

$$\text{mass}_{\text{insert}}\,[\text{ng}] = 3 \text{ to } 5 \times \text{mass}_{\textbf{vector}}\,[40 - 100\ \text{ng}] \times \text{length}_{\textbf{insert}}\,[\text{bp}] / \text{length}_{\textbf{vector}}\,[\text{bp}]$$

The molar ratio of the insert to vector in this experiment was 3-5, while the quantities of the vector used for ligation were in between 40-100 ng. The ligation

mixture was incubated at room temperature/1 h, 4°C or 16 °C/overnight. The DNA ligate was then transformed into electro-competent or chemically-competent bacterial cells, and transferred to the selection LB agar plates.

3.2.2.8 Transformation

The bacterial transformation process was performed by gentle mixing of the ligates (recombinant plasmids) with chemically competent or electrically competent *E.coli* cells. For chemical transformation, the mixture was incubated on ice for 30 min followed by heat shock at 42 °C for 45 seconds and then the mixture was incubated on ice for 2 min. 500 µl SOC medium were added to the mixture and incubated at 37 °C for 1h with gentle agitation. Transformation by electroporation was achieved by adding the DNA to electro-competent cells and transferred to an electroporation cuvette and pulsed at 2.5 kV, 25 µF and 200 Ohm using the Gene Pulser II (BioRad). Subsequently, 500 µL SOC medium were added immediately and the mixture were incubated at 37 °C for 1h. Upon transformation, the transformed cells were plated on LB agar plates containing Ampicillin or Kanamycin for selection of the target transformed bacterial colonies. The LB agar plates were incubated at 37 ºC for 24 hours or room temperature for 48 hours.

3.2.2.9 Plasmid Isolation

Transformed cultured colonies were selected and grown in LB broth media with a selective antibiotic at 37ºC/overnight with shaking. 500 µl bacterial culture were added to 50% sterilized glycerol and stored at - 80 ºC as a glycerol stock. The plasmid DNA was purified using the QIAprep Spin Miniprep Kit or Endo-free Plasmid Maxi Kit according to the manufacturer's instructions. The principle of these protocols is based on the alkaline lysis of bacterial cells. Since bacterial genomic DNA and plasmid DNA are similar in chemical nature, the only way to separate them is by physical mean; upon alkaline lysis of bacterial cell membrane, a bacterial genomic DNA remains attached and is precipitated with other cell debris while plasmid DNA is adsorbed onto silica membrane in presence of high salt. The prepared DNA was dissolved in 50 µl buffer EB by the QIAprep Spin Miniprep Kit or in 200-300 µl using the Endo-free Plasmid Maxi Kit.

3.2.2.10 Colony PCR

This technique is designed to quickly detect the desired plasmid DNA directly from bacterial colonies growing on LB agar plates. This protocol was carried out by choosing the bacterial colonies and resuspending the bacterial cells in 10 µl double distilled water. For the colony PCR assay, 5 µl of the bacterial suspension were used as template DNA, while the rest was stored at 4 ºC for inoculation of overnight cultures. The components of the PCR reaction are listed Table 26.

Table 26: The compositions of the colony PCR assay.

Component	Concentration	Volume	Final concentration
PCR buffer/25 mM MgCl2	10x	2.5 µl	1x
DMSO	50%	2.5 µl	10%
dNTPs	2.5 mM each	1 µl	1 mM each
Forward primer	10 µM	0.5 µl	200 nM
Reverse primer	10 µM	0.5 µl	200 nM
Template DNA	-	5 µl	-
Taq polymerase	250 U/µl	0.125 µl	1.25 µl
Double distilled water	-	To 25 µl	-

Thermocycling conditions for the colony PCR assay are shown in Table 27.

Table 27: Cycling parameters for the colony PCR assay

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	20 sec	35
Annealing	X °C	20 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

To screen the desired DNA insert, part of the PCR products was loaded directly onto an agarose gel using an appropriate DNA ladder.

3.2.2.11 Sequencing PCR

For sequencing reaction, BigDye 3.1 (ABI Terminator Chemie, Applied Biosystems) was utilized which contains AmpliTaq DNA polymerase and dNTPs and fluorescence-labeled 2', 3'-Didesoxynucleotrisphosphats (ddNTPs). Read-out of the sequencing PCR was performed by the RKI sequencing core facility. The analysis of sequence was performed by BioEdit and MegAlign Sequence program. The components and the cycling parameters for the sequencing PCR assay are included in Table 28 and Table 29 respectively.

Table 28: The compositions of the sequencing PCR assay

Component	Concentration	Volume	Final concentration
ABI buffer/25 mM MgCl2	5x	1.5 µl	0.75x
BigDye 3.1	-	1 µl	-
Template DNA	-	X µl (200 ng – 300 ng)	-
Sequencing primer	10 µM	0.5 µl	500 nM
Double distilled water	-	To 10 µl	-

Table 29: The cycling parameters of the sequencing PCR reaction

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	20 sec	35
Annealing	X °C	20 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1

3.2.3 Protein Analysis

3.2.3.1 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was achieved for separation of proteins based on their molecular weight under the effect of an applied electrical field. The electrophoretic analysis was performed in the Mini-Protean II Dual-Slab Cell, (BioRad, USA) using 12% acrylamide for resolving (separating) gel and 5% acrylamide for stacking gel. The components of SDS gels are shown in Table 30.

Table 30: The components of SDS gels

Component	Resolving gel	Stacking gel
1.5M Tris-HCl Buffer pH 8.8	2.5 ml	-
0.5M Tris-HCl Buffer pH 6.8	-	2.5 ml
Rotiphorese® Gel 30 (37.5:1)	4 ml	1.7 ml
10% SDS	100 µl	100 µl
APS	50 µl	50 µl
TEMED	5 µl	10 µl
Double distilled water	3.4 ml	5.7 ml

The SDS-PAGE analysis was performed by dissolving samples in an appropriate volume of 4x Laemmli dissociation buffer and boiled at 95 °C for 5 min. Sample mixtures containing the HIV coRev or SIVmac coRev proteins were denatured at 60 °C for 5 min. The heating step is important for destruction of the protein tertiary structure and conversion of the target proteins into a linear form. The heated samples were carefully loaded under buffer on top of the gel. The electrophoresis was applied under a voltage of 100V for 10 min and later set to 180V for 60 min until the tracking dye was near the bottom of the gel. For determination of the size of the observed proteins, PageRuler Prestained Protein Ladder or PageRuler Plus Prestained Protein Ladder were loaded into the gel. Subsequently, the gels were stained with Coomassie Brilliant Blue G Concentrate or used for Western Blot analysis.

3.2.3.2 Western Blot Analysis

Following the SDS-PAGE assay, Western blot was applied to transfer the separated proteins from the electrophoresis gel to a Polyvinylidene flouride (PVDF) or nitrocellulose membranes. First, the SDS gels were equilibrated in transfer buffer for 20 min. PVDF membranes were activated by methanol for 5 min followed by equilibration in transfer buffer for 10 min, whereas nitrocellulose membranes were directly equilibrated by transfer buffer. Western Blot assay was done based on semi-dry blotting therefore; the gel was sandwiched between two filter papers moistened with transfer buffer. Immunoblotting was carried out at 10 - 20V for 40 min. Next, the membranes were blocked using blocking buffer for one hour at room temperature to inhibit the nonspecific binding of antibodies to the surface of the membrane. The transferred protein was incubated with a diluted primary antibody at 4 °C/overnight or at room temperature for one hour. Excessive washing with a washing buffer three times for 10 min was performed to remove unbound primary antibodies and then the membranes were incubated with the diluted horseradish peroxidase (HRP) conjugated secondary antibody at room temperature for one hour followed by washing of the membranes with a washing buffer three times for 10 min. Afterwards, the Super Signal West Dura Extended Duration Substrate was added to the membranes to detect the bound HRP labelled antibodies because the substrate combines with the enzyme producing light as a byproduct. The light output can be captured by the exposure of the membranes to X-ray film followed by the development of the film by a film processing machine.

3.2.3.3 Protein Co-Immunoprecipitation

Coimmunoprecipitation (co-IP) method was used in this work to study the protein-protein interaction and complex formation of various proteins as shown in Figure 13.

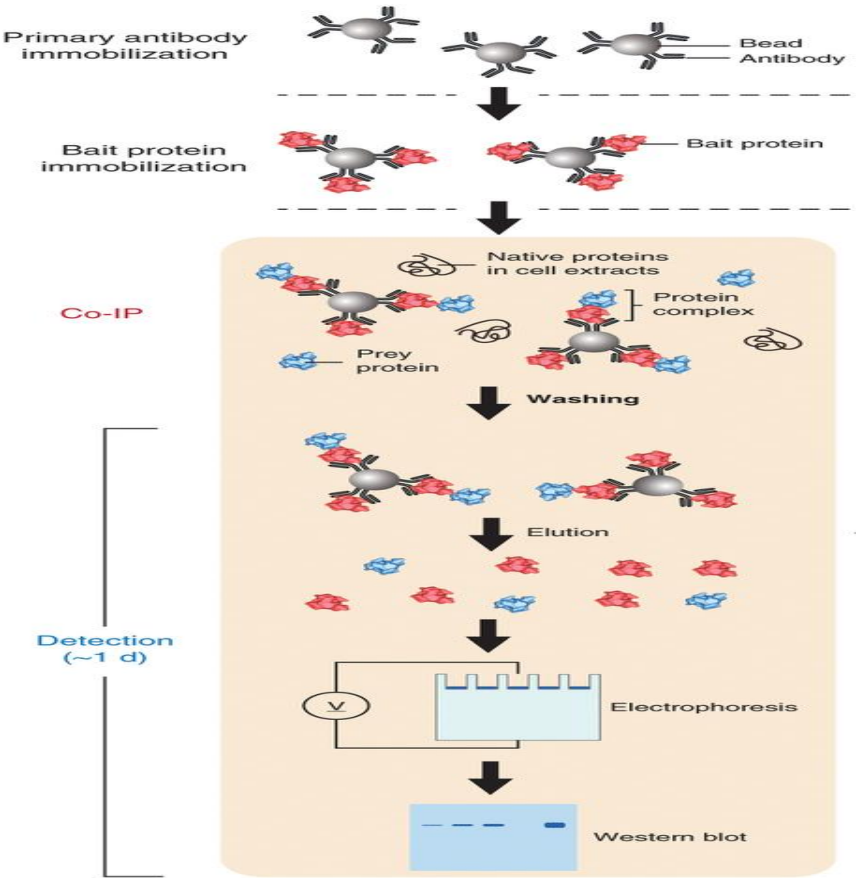


Figure 13: Schematic representation of a conventional co-IP experiment. To assess the interaction between two proteins, cells were transfected with expression constructs for these proteins and after 48 hours, cells were lysed followed by incubation with agarose beads coated with an antibody specific for the first protein (Red color). The beads were precipitated and then washed. Western Blot analysis was achieved using antibody specific for the other protein (Blue color). The positive result indicates the successful binding between these proteins [148].

HEK 293T cells were seeded as previously described (see 3.2.1.3) in 100 mm dishes. The next day, the cells were transfected using calcium phosphate (see 3.2.1.4). 48 hour post transfection, the supernatants were discarded, and 920 µl lysis buffer were added to each dish containing the transfected cells. By using a cell scraper, the cells were removed from the dishes followed by agitation at 4 °C for 30-

45 min for complete lysis of the cells. To purify the cell lysate from the cell debris, centrifugation was performed at 13500g for 20 min at 4 °C and the supernatant was collected. 750 µl cell lysate was mixed with 30 µl of antibody-tagged agarose beads, completed with lysis buffer to a total amount of 1 ml and agitated at 4 °C/ overnight. After overnight incubation, the mixtures of the cell lysates were washed 10 times with lysis buffer. The mixtures in each washing step were centrifuged at 2000g at 4 °C for 5 min. The agarose beads were resuspended in 40 µl 4x Laemmli sample buffer with β-Mercaptoethanol and stored at -20°C until Western Blotting analysis (see 3.2.3.2).

3.2.3.4 Immunofluorescence

Immunofluorescence was applied for examination of the intracellular antigens using fluorescence labeled antibodies. HEK-293T cells were cultured onto cover slips in 6-well plates and transfected as previously described (see 3.2.1.2, 3.2.1.3 and 3.2.1.4). 48 h post transfection, cells were washed with 1x PBS and fixed with 2% paraformaldehyde (PFA) for 30 min at room temperature (RT). The cells were then washed with three times 1x PBS for 5 min to remove the excess of PFA. Afterwards, Cells were permeabilized by treatment with 0.5% Triton-X 100 for 30 min at room temperature and then washed three times with 1x PBS for 5 min. Subsequently, 1% skimmed milk powder in 1x PBS was added to the cells for one hour at RT to prevent nonspecific binding of antibodies. After that, the cells were incubated with the diluted specific primary antibody at 37 °C for one hour followed by washing with 1x PBS three times for 5 min. Thereafter, the diluted fluorescence labeled secondary antibody was added to the cells for one hour at RT. Next, the cells were again washed thoroughly. Cover slips were embedded in Mowiol or Mounting medium with Dapi and stored in dark conditions at 4 °C/overnight. The preparations were analyzed using a Zeiss confocal laser scanning microscope LSM780.

3.2.3.5 CA-Antigen Capture ELISA

The enzyme linked immunosorbent assay was applied for detection of HERV-K113, HIV-1 and SIVmac viruses in cell culture supernatants and cell lysates. ELISA method is highly specific, and was achieved for measurement of the amount of viral Gag p24 and p27 particles. 96-well microtiter plates were coated with monoclonal α-

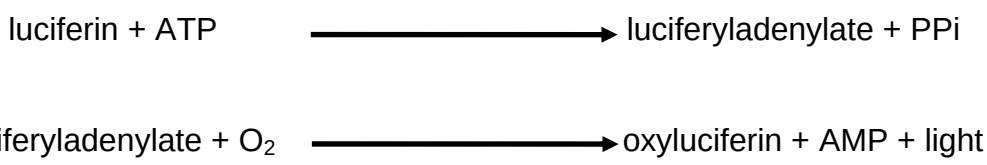
HERV-K Gag Herma 7 (for HERV-K) or monoclonal α-SIV Gag AG3.0 (for HIV-1 or SIVmac) antibody diluted in carbonate bicarbonate buffer followed by incubation at 4 °C/overnight. Each well of antibody coated plates was washed three times with 200 µl PBS/Tween-20/well and then the surface of plate wells were blocked with 200 µl blocking buffer/well at 37 °C for one hour to prevent nonspecific interactions. The samples were inactivated by incubation with Tween-20 in a final concentration of 0.02% at room temperature (RT) for 10 min. Thereafter, the blocking buffer was discarded, and the inactivated samples were added to the ELISA plate wells containing blocking buffer for the titration and incubated at 37 °C for one hour. The sample solutions were removed and the wells were washed three times with 200 µl PBS/Tween-20. The primary antibody was added to each well either 50 µl HIV-1 plasma pool/well diluted in PMT (1:1000) specific for HIV-1 and SIVmac or 50 µl rat α-HERV-K Gag/well diluted in PMT (1:5000) specific for HERV-K113. The plates were incubated at 37 °C for one hour and washed then three times with 200 µl PBS/Tween-20. 50 µl α-Human IgG-HRP diluted in PMT (1:1000) or 50 µl α-Rat IgG-HRP diluted in PMT (1:2500) were transferred to the wells and incubated at 37 °C for one hour followed by the step of washing with 200 µl PBS/Tween-20. For detection of the peroxidase conjugated antibody, substrate was prepared by dissolving 5 mg OPD in 12.4 ml phosphate citrate buffer pH 5.0 mixed with 12 µl H_2O_2.

3.2.3.6 RT Activity Technique

For detection and quantification of the retroviral reverse transcriptase activity in the sample, the Cavidi RT Activity assay was achieved for analysis of the virus infectivity according to the manufacturer's instructions. This experiment is based on two steps, the DNA biosynthesis and the DNA quantifi cation. The RNA template is bound to the bottom of the plate. Next, primer, a nucleotide (BrdUTP) and the sample are added to the plate. The reverse transcriptase enzyme found in the sample will synthesise a DNA-strand. An alkaline phosphatase (AP) conjugated α-BrdU antibody specifically binds to the double stranded DNA/RNA molecule. Addition of a colorimetric AP substrate will result in quantifying the product. The AP activity is proportional to the RT activity in the sample.

3.2.3.7 Luciferase Reporter Assay

The luciferase assay is employed as an indicator to assess the gene expression at the transcriptional level. This technique is depending on the luciferase enzyme which catalyzes the biochemical conversion of luciferin to oxyluciferin leading to light emission (e.g. firefly luciferase found in the firefly *Photinus pyralisis).*



The light output is associated with the amount of RNA exported into the cytoplasm for the luciferase enzyme biosynthesis. The firefly luciferase activity was evaluated by Dual-Glo® Luciferase Assay System or Luciferase 1000 Assay System according to the manufacturer's instructions as follow: One day prior cell transfection, HEK-293T cells were seeded in 96-well plates (see 3.2.1.3). The next day, cells were transfected (see 3.2.1.4) with constructs containing genes of interest, shuttle vectors encoding for firefly luciferase as a reporter gene and with a vector encoding *Renilla* luciferase as internal control for normalization. 48 h post transfection, medium was removed and cells were washed once with 200 µl 1x PBS per well. Following washing, the cells were lysed with 20 µl 5 x passive lysis buffer per well and then the plates were rotated using shaker machine at room temperature for 15 min. After allowing the cell lysis, the cell lysates were transferred onto a white optical 96-well plate (Nunc) and then 100 µl the firefly luciferase reagent (LARII) was added to each well, followed by addition of 100 µl the *Renilla* luciferase reagent and firefly quenching (Stop & Glo buffer). The intensity of bioluminescence was measured using the Berthold Luminometer Centro LB 960.

4 Results

4.1 Effect of Staufen-1 overexpression on the retroviral particles production

Staufen-1 is considered as one of cellular multifunctional proteins because some studies clarified its role in the nucleocytoplasmic RNA shuttling [128] [132], while others demonstrated the significance of Staufen-1 in mediating the rate of translation and viral RNA encapsidation [129] [139]. One of the objectives of this study was therefore to analyze the impact of Staufen-1 overexpression on the expression level of HERV-K113, HIV-1 and SIVmac239.

4.1.1 Staufen-1 promotes the generation of HERV-K(HML-2) particles

The expression level of Gag protein and viral like particles (VLPs) of the native HERV-K113 sequence is very low, causing difficulties in detection. This is attributed to mutations within the proviral DNA generated after the insertion into the human genome [144]. In earlier work, Hanke *et al*. indicated that the overexpressed Staufen-1 elevated the level of oriHERV-K113 particles by an average of 20-fold [141]. Based on this finding, the aim of this experiment was to further investigate the dose-dependent influence of Staufen-1 overexpression on viral particle production derived from the reconstituted oriHERV-K113 sequence. This was performed by transfection of HEK-293T cells with 1µg of the pBSK_oriHERV-K113 molecular clone and different concentrations of pCMV-FLAG-Staufen-1 DNA (0.125 µg, 0.5 µg, 2 µg). Control cells were transfected either with an empty pcDNA4-V5-His/B vector or pCMV-FLAG-Staufen-1 DNA alone as negative controls. Staufen-1 is necessary for viral replication by enhancing viral mRNA export, initiating the translation process and inducing virus packaging and release. The supernatants were therefore collected 48 h post transfection to assess the Staufen-1 activities of RNA transportation and translation; cell lysates were also prepared to analyze the role of Staufen-1 in virus release. A CA-antigen capture ELISA was then carried out to determine the amount of viral Gag protein.

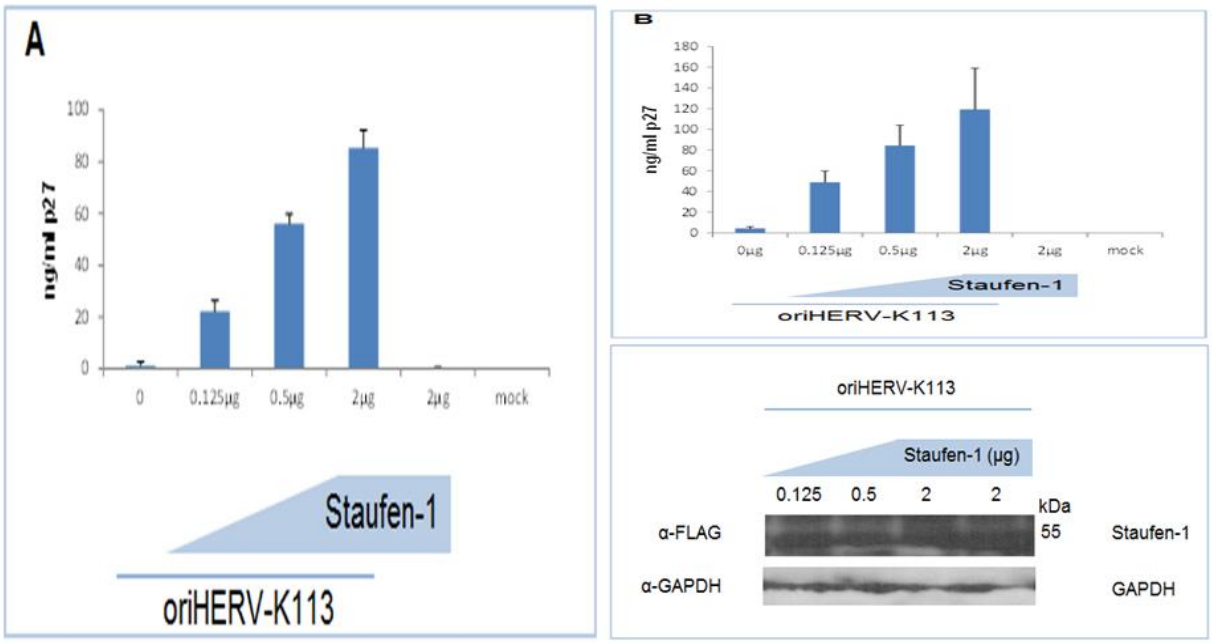


Figure 14: Measurement of the Gag protein of HERV-K(HML-2) in the supernatants and the cell lysates. HEK-293T cells were cotransfected with HERV-K(HML-2) and Staufen-1 at different ratios. A CA-antigen capture ELISA was used to examine the level of viral Gag protein in the supernatants (A) and cell lysates (B). The experiment was repeated 6 times in HEK-293T cells. Data from one representative experiment performed in triplicate are displayed with standard error bars. The expression of ectopic Staufen-1 was demonstrated by Western blot assay using α-FLAG antibody. In addition, GAPDH detection was used as a loading control.

As displayed in Figure 14, the ELISA demonstrates that Staufen-1 overexpression elevates the concentration of the Gag protein in the supernatants (14A) and similarly in the cell lysates (14B). The most effective quantity of Staufen-1 DNA introduced into the cells was 2 µg which showed that the maximum expression level of the viral Gag increase by an average of about 30-fold in the supernatants and nearly 24–fold increase in the cell lysates compared to the expression without Staufen-1. Additionally, no viral Gag protein was detected in the cells serving as a negative control. This result confirmed the critical role of Staufen-1 in oriHERV-K113 propagation, probably through several mechanisms.

For demonstration of the expressed Staufen-1 protein in the cell lysates, Western blot assay was performed using as primary antibody α-FLAG and as secondary antibody α-Rabbit IgG-HRP. In Figure 14, it can be observed that the

density of the positive examined Staufen-1 bands is directly proportional to Staufen-1 DNA used for transfection. To ensure equal amounts of cell lysates loaded into SDS-PAGE, the expression of GAPDH served as a loading control, and was observed using a primary antibody α-GAPDH and a secondary antibody α-Mouse IgG-HRP.

4.1.2 Staufen-1 modulates the production of SIVmac virions

To evaluate whether Staufen-1 positively affects the production of SIVmac particles as well as whether the Staufen-1 effect is dose dependent, HEK-293T cells were cotransfected with 1µg SIVmac molecular clone and pCMV-FLAG-Staufen-1 at different ratios (0.125 µg, 0.5 µg, 2 µg). Cells transfected with either empty vector or Staufen-1 only served as negative controls. The total concentrations of DNA were equalized with pcDNA4-V5-His/B vector to confirm that the variation in the titer of the viral capsid protein is not due to different DNA quantities used for transfection. After 2 days incubation, supernatants were harvested and the cell lysates were prepared (see 3.2.1.5). Afterwards, a CA-antigen capture ELISA was performed to quantify the concentration of SIVmac Gag protein in the supernatants and cell lysates as previously described in 3.2.3.5.

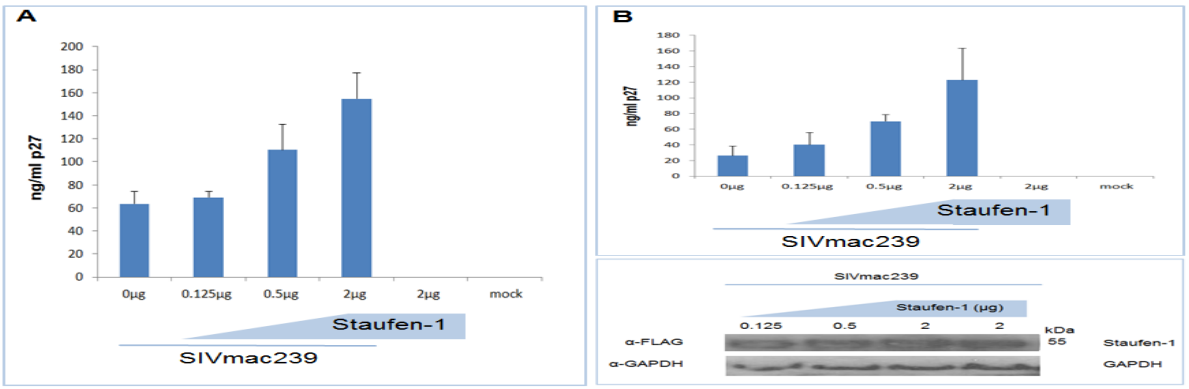


Figure 15: Quantification of SIVmac Gag protein expression in the supernatants and the cell lysates. CA-antigen capture ELISA of the supernatants (A) and the cell lysates (B) was performed to measure the concentration of the viral Gag protein. The experiment was repeated 6 times in HEK-293T cells. Data from one representative experiment done in triplicate are shown with standard error bars. Overexpression of Staufen-1 in samples was shown by Western blotting using an α-FLAG primary antibody. A Western blot for GAPDH is shown from the same lysates as a loading control.

As shown in Figure 15, overexpression of Staufen-1 increases the quantity of the viral Gag protein in the prepared supernatants (Figure 15A) and cell lysates (Figure 15B) and therefore enhances the production of the viral particles. The cells cotransfected with 2 µg of exogenous Staufen-1 DNA showed the highest Gag protein concentration, which is in the supernatants around 2.5-fold higher and in the cell lysates nearly 6-fold higher than in cells with only endogenous levels of Staufen-1. No viral Gag protein was observed in cells encoding only mock DNA or overexpressed Staufen-1. Therefore, this observation proved enhancement of the viral particle production by Staufen-1.

The overexpression of Staufen-1 was confirmed by Western blotting using α-FLAG as primary antibody (1:5000 dilution) due to the N-terminal FLAG tag of Staufen-1 and followed by incubation with a secondary antibody α-Rabbit IgG-HRP (1:5000 dilution). The expected molecular weight of Staufen-1 protein is 55 kDa as indicated in Figure 15. The abundant housekeeping protein GAPDH was used to demonstrate equal amounts of cell lysates loaded and was detected using a primary antibody α-GAPDH (1:1000 dilution) and α-Mouse IgG-HRP (1:5000 dilution) as a secondary antibody. As observed in Figure 15, the intensity of Staufen-1 protein was consistent with the concentration of pCMV-FLAG-Staufen-1 DNA used in the cell transfection assay.

4.1.3 Time-dependent overexpression of ectopic Staufen-1 has a positive effect on the production of SIVmac

The aim of this time-dependent course experiment was to identify whether production of new viral particles is associated with time based Staufen-1 overexpression. HEK-293T cells were cotransfected with 1 µg SIVmac and 0.5 µg pCMV-FLAG-Staufen-1 as seen in Table 31.

Table 31: displaying time dependent transfection.

Sample	Day 1	Day 2	Day 3	Day 4
#1	SIVmac	Staufen-1	Harvesting	
#2	Staufen-1	SIVmac		Harvesting
#3	SIVmac + Staufen-1		Harvesting	

The total concentrations of the transfected DNA were equalized using pcDNA4-V5-His/B vector to correctly assess the influence of the exogenous Staufen-1 in the time-dependent experiment on the SIVmac production. The concentration of the viral Gag protein in the supernatants and in the cell lysates was quantified 48 h after transfection with SIVmac using the CA-antigen capture ELISA.

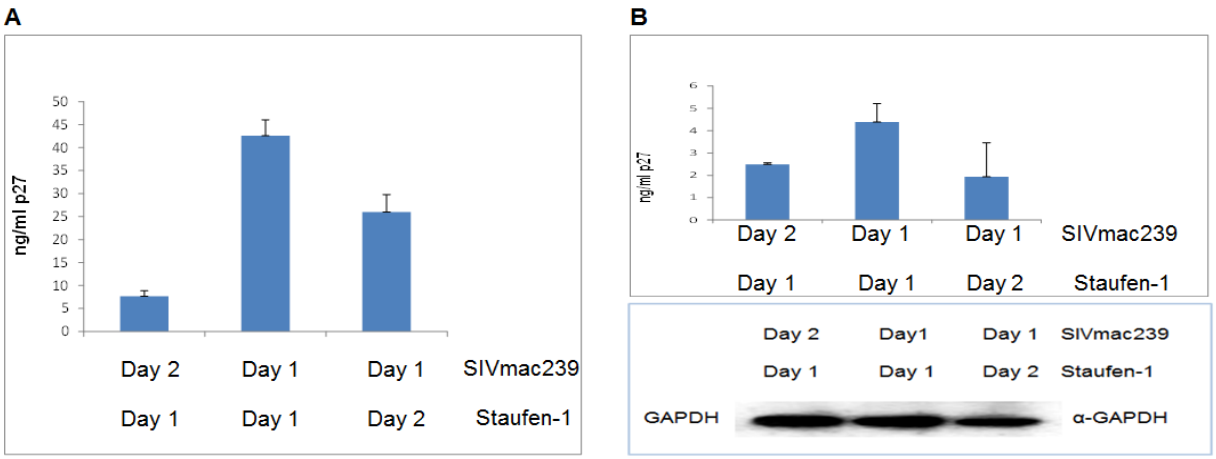


Figure 16: Expression of SIVmac Gag protein after time-dependent Staufen-1 overexpression. The CA-antigen capture ELISA was used to determine the amount of viral Gag protein in the supernatants (A) and cell lysates of cells cotransfected with SIVmac and exogenous Staufen-1 in a time-dependent manner. Data from one representative experiment performed in duplicate are shown with standard error bars. Western blot analysis was applied to examine GAPDH expression in the cell lysates for confirmation that the same quantities were loaded into the gel.

As seen in Figure 16, generation of the viral Gag appears to be directly dependent on the time of cell transfection with Staufen-1. Interestingly, whereas the lowest Gag protein yield was detected in the supernatants of cells cotransfected with Staufen-1 on the first day and SIVmac molecular clone on the second day, cotransfection of the cells with SIVmac and Staufen-1 at the same time induced the largest amount of the viral Gag protein observed in the supernatants (A) and cell lysates (B). Therefore, transfection of SIVmac and Staufen-1 at the same time exhibited the highest positive Staufen-1 effect on the virus production.

To emphasize that equal quantities of the cell lysates were loaded into the gel, expression of GAPDH in cell lysates was evaluated by Western blot. GAPDH protein

was identified by a primary α-GAPDH antibody and a secondary α-Mouse IgG-HRP antibody. Bands corresponding to the protein of interest were displayed based on its size (37 kDa) as shown in Figure 16. The aim of GAPDH detection was to ensure that the differences in the virus production are dependent on the time of cotransfection with Staufen-1 and SIVmac.

4.1.4 Downregulation of endogenous Staufen-1 has a negative effect on SIVmac particle production

Further evaluation of the function of Staufen-1 protein can be performed by downregulation of endogenous Staufen-1 expression. It has been demonstrated that overexpression of ectopic Staufen-1 induces the production of HERV-K113 particles as performed in this study and by Hanke *et al*. [141], HIV-1 (mentioned later) and SIVmac as shown in the current study. In contrast, in earlier work, knockdown of endogenous Staufen-1 decrease the amount of HERV-K113 particles [145] so the aim of the protein downregulation was to analyze whether this process causes an adverse effect on the generation of SIVmac particles. The downregulation mechanism is carried out by gene silencing which is based on RNA mediated impairment of translation using short interfering RNAs (siRNA) consisting of around 20 nucleotides formed by cleavage of long dsRNAs by the RNase III family member, Dicer. siRNAs are incorporated into the RNA Interference Specificity Complex (RISC) followed by binding of RISC to a complementary sequence in the mRNA causing the endonucleolytic degradation of the target mRNA and downregulation of the target protein.

Therefore, four double-stranded 21-bp siRNAs to a selected region of Staufen cDNA were evaluated for Staufen knockdown efficiency in preliminary experiments. The duplex siRNA 3084 (5-AAATAGCACAGTTTGGAAACT-3) was assessed to generate the most significant knockdown of Staufen-1 gene expression [132]. This siRNA was cloned into shRNA vector and then used in this study. Staufen-1 silencing was performed by using Staufen-1 specific shRNA vectors that cleave several independent sites within Staufen-1 mRNA preventing the translation process (see Table 13).

In a previous work, it was shown that downregulation of the endogenous Staufen-1 had a negative impact on production of new HERV-K(HML-2) particles

[145]. Therefore the effect of silencing the endogenous Staufen-1 on SIVmac expression could be evaluated in this study using expression DNA vectors containing Staufen-1 specific shRNA (short hairpin RNAs). The effect of endogenous Staufen-1 downregulation on SIVmac production was tested by cotransfection of HEK-293T cells with equal DNA quantities of six Staufen-1 specific shRNAs. In addition, negative control cells were included in this experiment by transfecting cells with pLVTHM-shCofilin. After one day transfection, the same cells were transfected with 1 µg of the SIVmac molecular clone. The cells were lysed 2 days post transfection. The level of viral Gag protein was measured in cell lysates by CA-antigen capture ELISA assay. Five shRNAs expressing DNA vectors were kindly provided by Jula Wamara, a co-worker in the laboratory.

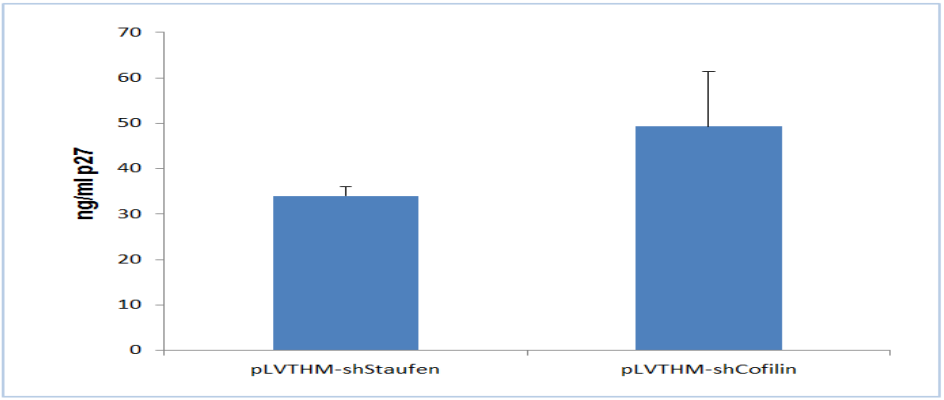


Figure 17: Effect of silencing endogenous Staufen-1 on production of SIVmac Gag. Different types of Staufen-1 specific shRNAs were transfected into HEK-293T cells, and then the effect of Staufen-1 downregulation was evaluated by ELISA. The experiment was done in triplicate in HEK-293T cells and data are shown with standard error bars.

As depicted in Figure 17, the reduced quantity of the viral Gag in the cell lysates of cells cotransfected with Staufen-1 shRNAs mixture was shown to be correlated with downregulation of the endogenous Staufen-1. Gag expression was markedly decreased after silencing endogenous Staufen-1 in cell lysates by factors of 1.5, compared to that observed in cell lysates of cells cotransfected with pLVTHM-shCofilin and the SIVmac molecular clone. Therefore, downregulation of endogenous Staufen-1 results in reduced SIVmac expression.

4.1.5 Staufen-1 has a positive effect on HIV-1 production

Several studies previously described that Staufen-1 increased by 3-4-fold the production of HIV-1 [128] [149]; and another work suggested that Staufen-1 interacted with the nucleocapsid region of $pr55^{Gag}$ resulting in the production of new infectious HIV-1 particles [132]. Consequently, the aim of this experiment was to investigate the dose-dependent impact of Staufen-1 overexpression on the levels of HIV-1 production. For this purpose, HEK-293T cells were cotransfected with 1µg of the HIV-1 pNL4-3 molecular clone and different amounts of pCMV-FLAG-Staufen-1 DNA (0.125 µg, 0.5 µg, 2 µg). Mock-treated cells and cells expressing only the exogenous Staufen-1 DNA served as negative controls. Transfected cells were incubated for 48 h followed by lysis of the cells and harvesting the supernatants. Next, the amount of the viral Gag protein in the supernatants and cell lysates was quantified by CA-antigen capture ELISA.

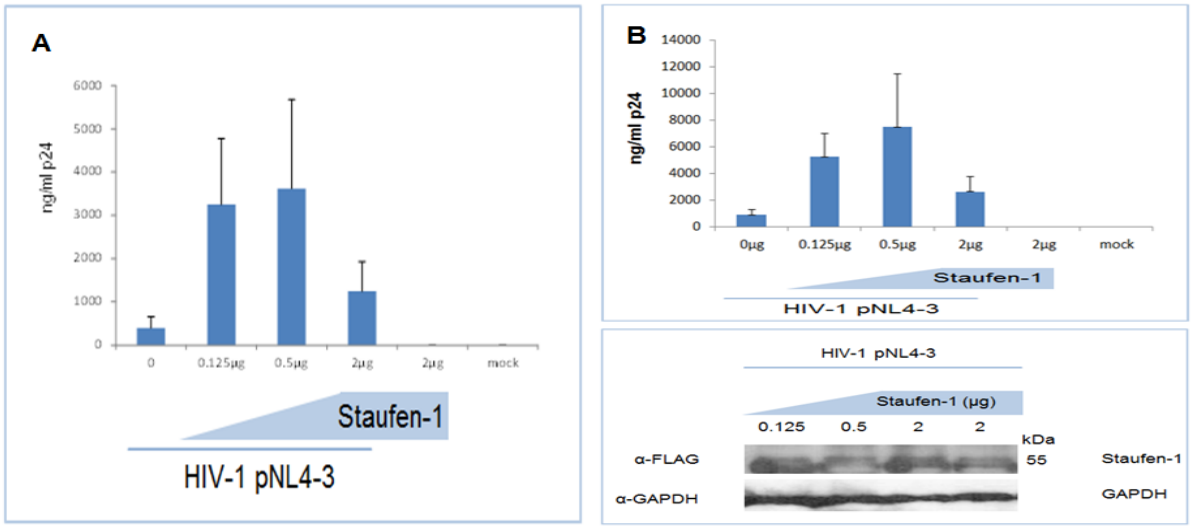


Figure 18: Quantification of HIV-1 Gag protein in the supernatants and the cell lysates. Upon coexpression of HIV-1 and different concentrations of Staufen-1 in transfected HEK-293T cells, the CA-antigen capture ELISA was performed. The experiment was repeated 5 times in HEK-293T cells. Results from one representative experiment carried out in triplicate are shown with standard error bars. Overexpression of Staufen-1 was examined by Western blot using an α-FLAG antibody. GAPDH expression was successfully detected in the same cell lysates.

The ELISA showed an 9-fold and 8.5-fold increase in expression of the viral Gag protein in the supernatants and cell lysates respectively, of cells transfected with 0.5 µg Staufen-1 compared to that without exogenous Staufen-1. No Gag protein was observable in negative control cells transfected with either pcDNA4-V5-His/B or only Staufen-1 DNA. According to these data, a defined range of Staufen-1 overexpression has a positive effect on virus production, whereas in contrast to that observed for SIVmac and oriHERV-K113, a higher concentration of transfected Staufen-1 DNA (2 µg) led to diminished viral expression.

Western blot was used to test the overexpression and the signal intensity of Staufen-1 on whole cell lysates using an α-FLAG and an α-Rabbit IgG-HRP as primary antibody and secondary antibodies, respectively. As displayed in Figure 18, Western blot analysis shows a clear Staufen-1 signal at the expected protein size confirming the success of the cell transfection and expression of the target protein. The intensity of Staufen-1 protein bands appears to be associated with the quantity of pCMV-FLAG-Staufen-1 DNA added to the cells for transfection. For visualization of GAPDH (loading control), a primary α-GAPDH antibody and a secondary α-Mouse IgG-HRP antibody were used.

4.1.6 Lower concentration of transfected HIV-1 DNA results in only slightly higher Staufen-1 enhancement

In this study, Staufen-1 overexpression results in a stronger enhancement of oriHERV-K113 (low virus production after transfection of the full length molecular clone compared to that derived from the HIV-1 pNL4-3 or SIVmac molecular clones) than that measured for HIV-1 and SIVmac; Hanke *et al.* also showed an increase in the production of HERV-K113 particles by a factor of 20 upon Staufen-1 overexpression [141] while other reports demonstrated that Staufen-1 led to a 3-4-fold enhancement for HIV-1 [128] [149]. Therefore, the aim of this experiment was to assess whether the Staufen-1 enhancing effect on HIV-1 expression level is related to lower concentrations of HIV-1 DNA transfected into cells and therefore lower levels of virus production. For this purpose, HEK-293T cells were used for transfection with 0.5 µg pCMV-FLAG-Staufen-1 DNA and full length HIV-1 DNA at different ratios (2000 ng, 666.6 ng, 222.2 ng, 74 ng). Cells were also transfected with HIV-1 DNA alone at the different concentrations mentioned above. Cells transfected with mock

DNA served as a negative control. After incubation of the transfected cells for 48 h, the harvested supernatants were tested by CA-antigen capture ELISA to quantify the amount of viral Gag protein.

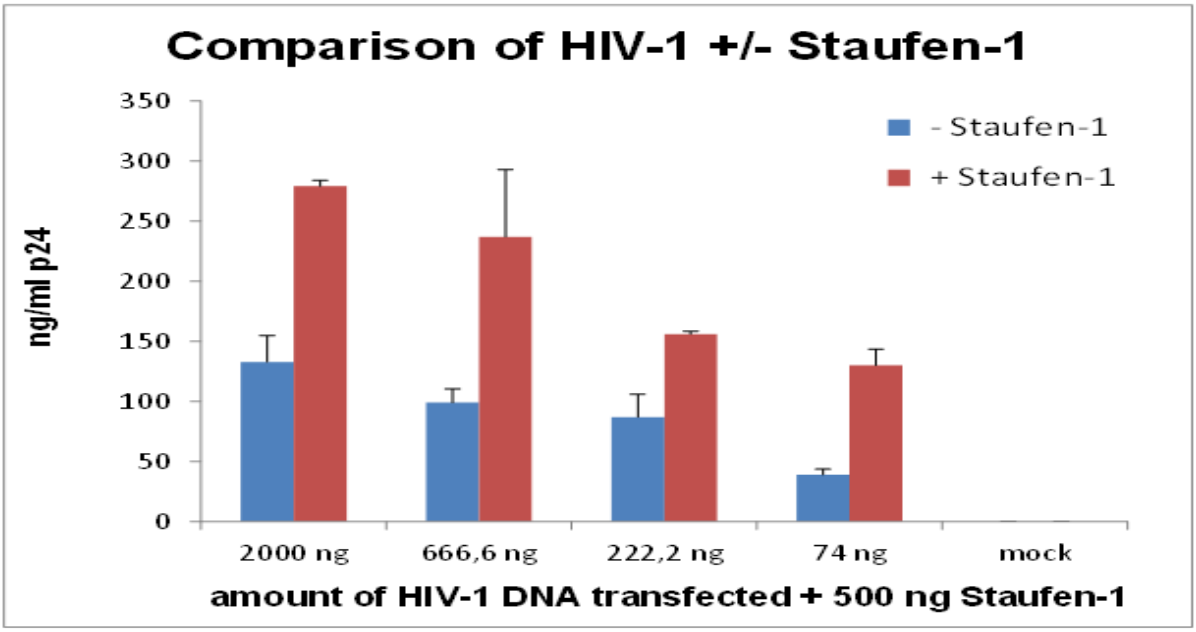


Figure 19: Viral Gag production upon cotransfection with HIV-1 either in the presence or absence of exogenous Staufen-1. HEK-293T cells were cotransfected with 0.5 µg pCMV-FLAG-Staufen-1 DNA and different amounts of full length HIV-1 DNA, and the same amounts of HIV-1 DNA alone were transfected separately into other cells. The experiment was repeated 3 times and data from one representative experiment done in triplicate are shown with standard error bars.

HIV-1 Gag protein was successfully detected in the supernatants from cells transfected either with the virus alone or with the virus plus Staufen-1. Negative control cells (mock) did not produce any viral particles. As depicted in Figure 19, Staufen-1 stimulates an increase in viral particles by an average of approximately 2-fold, 2.4-fold, 2-fold and 3.5-fold for 2000 ng, 666.6 ng, 222.2 ng and 74 ng, respectively, compared to cells not expressing the ectopic Staufen-1. These data indicated that the lowest HIV-1 DNA concentration transfected into the cells allowed a slightly stronger effect of Staufen-1; therefore the lower virus production derived from virus DNA maintains higher Staufen-1 enhancement. Also, these data showed that lower HERV-K(HML-2) expression levels do not explain the stronger Staufen-1 enhancement.

4.1.7 Staufen-1 overexpression stimulates the production of pseudotyped SHIV-luci construct only in the presence of HIV-1 Rev

In this experiment, pSHIV-luci was used, which is composed of the SIVmac239 genome carrying the SalI/XhoI fragment of HIV-1 HXB2 (containing *tat)*; as well as the luciferase gene in place of *env/rev*). This construct can be used as model for a lentivirus with a Rev deletion. In this experiment the dependency of enhanced viral particle production by Staufen-1 overexpression on the HIV-1 Rev protein was investigated and the aim was to analyze how the virus replicates in the presence of Staufen-1 when the *gag-pro-pol* sequence is obtained from SIVmac and the Rev Responsive Element (RRE) used is from HIV-1 as shown in Figure 20.

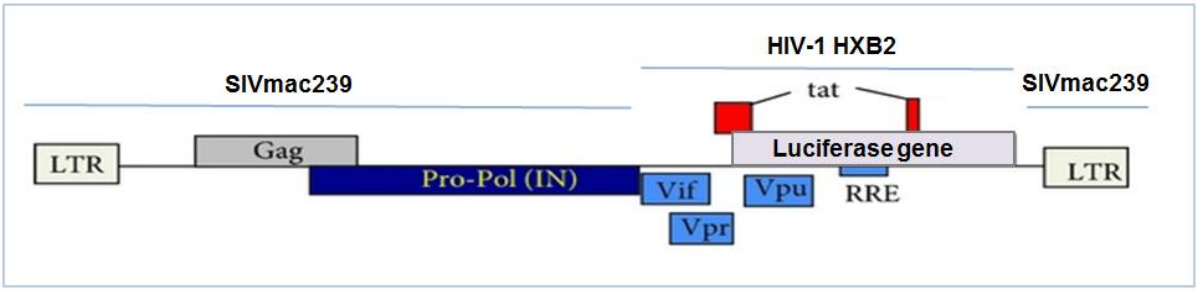


Figure 20: Genomic organization of SHIV-luci construct. The SHIV-luci is composed of two long-terminal repeats (LTR), *gag-pro-pol* genes from SIVmac239 and the accessory genes *vif, vpr, tat, nef* plus, the HIV-1 Rev-response element (RRE) and the luciferase gene in place of *env/rev.*

Therefore, 0.5µg pCMV-FLAG-Staufen-1 DNA and 0.7µg of the SHIV-luci construct plus 0.1 µg pVSV-G either in the presence or absence of 0.2 µg HIV-1 Rev were transfected into HEK-293T cells. Also, cells were transfected with either pcDNA4-V5-His/B or exogenous Staufen-1 DNA only were used as negative controls. After incubation for 48 h, the supernatants were collected and the transfected cells were lysed. Thereafter, the efficiency of Staufen-1 enhancement was assessed by measuring the viral Gag protein in the supernatants and the prepared cell lysates using the CA-antigen capture ELISA.

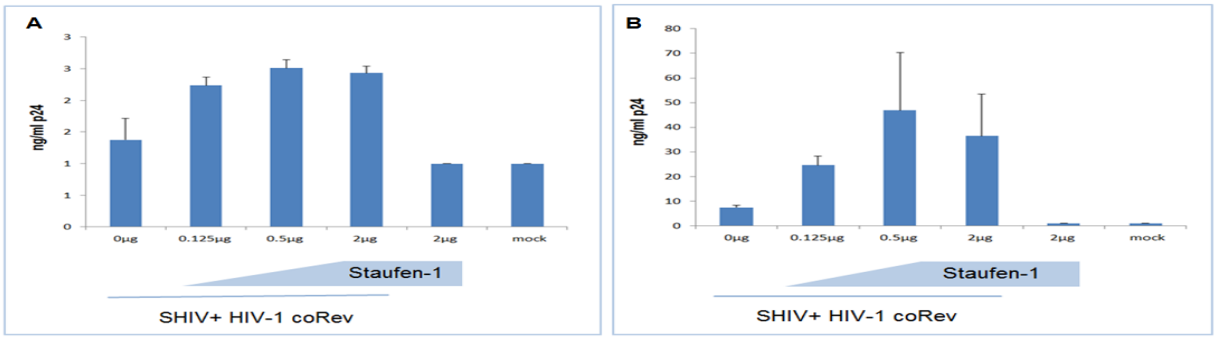


Figure 21: Investigation of the virus production of pseudotyped SHIV-luci upon overexpression of Staufen-1. HEK-293T cells were cotransfected with pCMV-FLAG-Staufen-1 DNA and SHIV-luci construct and pVSV-G either with or without the addition of HIV-1 Rev, followed by analysis in CA-antigen capture ELISA. Data from one representative experiment performed in triplicate are displayed with standard error bars.

As indicated in Figure 21 Staufen-1 contributes strongly to production of new virions by increasing the quantity of viral Gag protein only in cells transfected with HIV-1 Rev DNA, whereas no virus production was observable in cells lacking HIV-1 Rev (data not shown). Also, Figure 21 shows that 0.5 µg Staufen-1 DNA introduced into the cells results in the highest yield viral Gag protein in the supernatants and cell lysates with increases of about 3-fold and 8-fold, respectively, compared to cells not expressing exogenous Staufen-1 DNA. In addition, no viral Gag protein was detected in the control cells.

In conclusion, this result demonstrated that Staufen-1 cannot stimulate the biosynthesis of SHIV-luci Gag protein without HIV-1 Rev. In addition, it indicates that the highest quantity of Staufen-1 DNA (2 µg) caused a reduction in the viral Gag protein production as shown before for the impact of Staufen-1 on HIV-1 production.

4.2 Evaluation of the influence of Rev modulation on virus production with respect to Staufen-1 overexpression

4.2.1 Overexpression of HIV-1 Rev in the presence of exogenous Staufen-1 inhibits the production of virus particles

In this work, it was important to study whether HIV-1 Rev overexpression hinders the expression of viral particles. To achieve this, HEK-293T cells were

cotransfected with 1µg the HIV-1 pNL4-3 molecular clone expressing Rev, 0.5 µg Staufen-1 DNA and different ratios of Rev DNA (0.25 µg, 0.5 µg, 0.75 µg, 1 µg). Other cells were transfected with the HIV-1 pNL4-3 molecular clone alone without additional Rev. The supernatants were harvested 48 post transfection, and the level of Gag protein was determined by CA-antigen capture ELISA and compared for cells cotransfected with HIV-1, Staufen-1 and the highest amounts of Rev DNA and those transfected with the virus, Staufen-1 DNA and the lowest concentration of Rev.

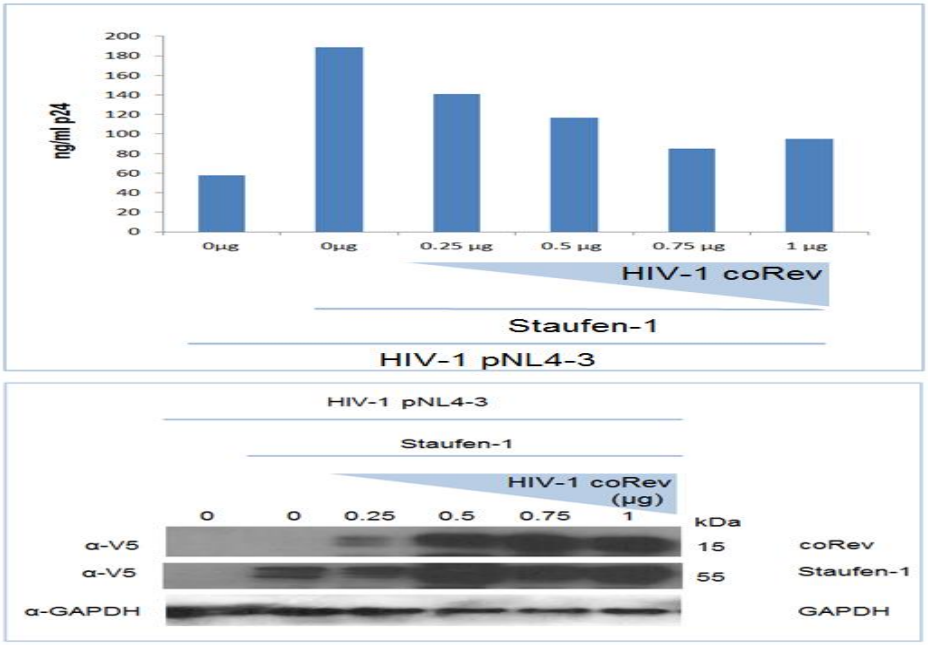


Figure 22: Determination of the effect of HIV-1 Rev and Staufen-1 overexpression on the formation of viral Gag protein. Cells were cotransfected with HIV-1, Staufen-1 and Rev at different concentrations. CA-antigen capture ELISA assay was then carried out to determine the level of viral Gag protein in the supernatants. The experiment was repeated twice in HEK-293T cells. Data from one representative experiment are shown. The α-V5-Tag antibody was used to visualize the Rev and Staufen-1 proteins by Western blotting. Furthermore, α-GAPDH antibody was used to confirm that equal quantities of proteins were loaded.

Notably, the degree of Gag protein expression was reduced by overexpression of Rev as displayed in Figure 22. The lowest amount of Gag protein in the

supernatants was seen with cells transfected with 1 µg and 0.75 µg HIV-1 Rev, with viral particle production impaired by a factor of 2 when compared to that in cells transfected with HIV-1 molecular clone and exogenous Staufen-1 alone. Therefore, cotransfection with HIV-1 Rev had an adverse impact on the generation of new HIV-1 particles.

Expression of HIV-1 Rev and Staufen-1 in the transfected cells was evaluated by Western blotting. As shown in Figure 22, protein bands of 15 kDa, 55 kDa, the expected molecular weight of V5 tagged Rev and V5 tagged Staufen-1 respectively, were visualized by using a primary mouse α-V5-Tag antibody and a secondary α-Mouse IgG-HRP antibody. Additionally, it has been found that higher DNA amounts of pCDNA3-V5-HIV-1-coRev transfected into the cells resulted in an increase in the expression level of Rev protein. Expression of loading control GAPDH as housekeeping protein was assessed using a primary α-GAPDH antibody and a secondary α-Mouse IgG-HRP antibody. It was also shown that transfection of cells with the virus alone resulted in a higher signal than with HIV-1 Rev overexpression.

4.2.2 Overexpression of SIVmac Rev reduces viral particle production

To increase the efficiency of SIVmac251coRev expression, Rev was codon optimized (named SIVmac251 coRev), and cloned into pCMV-FLAG or the pCDNA4-V5 expression vector to produce pCMV-FLAG-SIVmac-coRev or pCDNA4-V5-SIVmac-coRev respectively. The expression of SIVmac coRev protein could then be evaluated using anti-FLAG or anti-V5 antibodies. Based on the significant role of retroviral Rev/Rec protein in the nuclear transportation of unspliced and incompletely spliced RNA transcripts [108] [150], it has been shown by Hanke *et al*. and another work that overexpression of HERV-K(HML-2) Rec appeared to be involved in increasing the yield of virus particles [141] [145]. Therefore, it was vital to analyze the effect of SIVmac Rev overexpression on the virus production. In this regard, HEK-293T cells were cotransfected with 1µg of the SIVmac molecular clone expressing Rev and SIVmac Rev DNA at different ratios (0.125 µg, 0.25 µg, 0.5 µg, 0.75 µg, 1 µg). As control, cells were transfected with SIVmac molecular clone alone without exogenous Rev. The supernatants and the cell lysates were then prepared and subjected to CA-antigen capture ELISA for measuring the amount of SIVmac Gag protein upon SIVmac Rev overexpression.

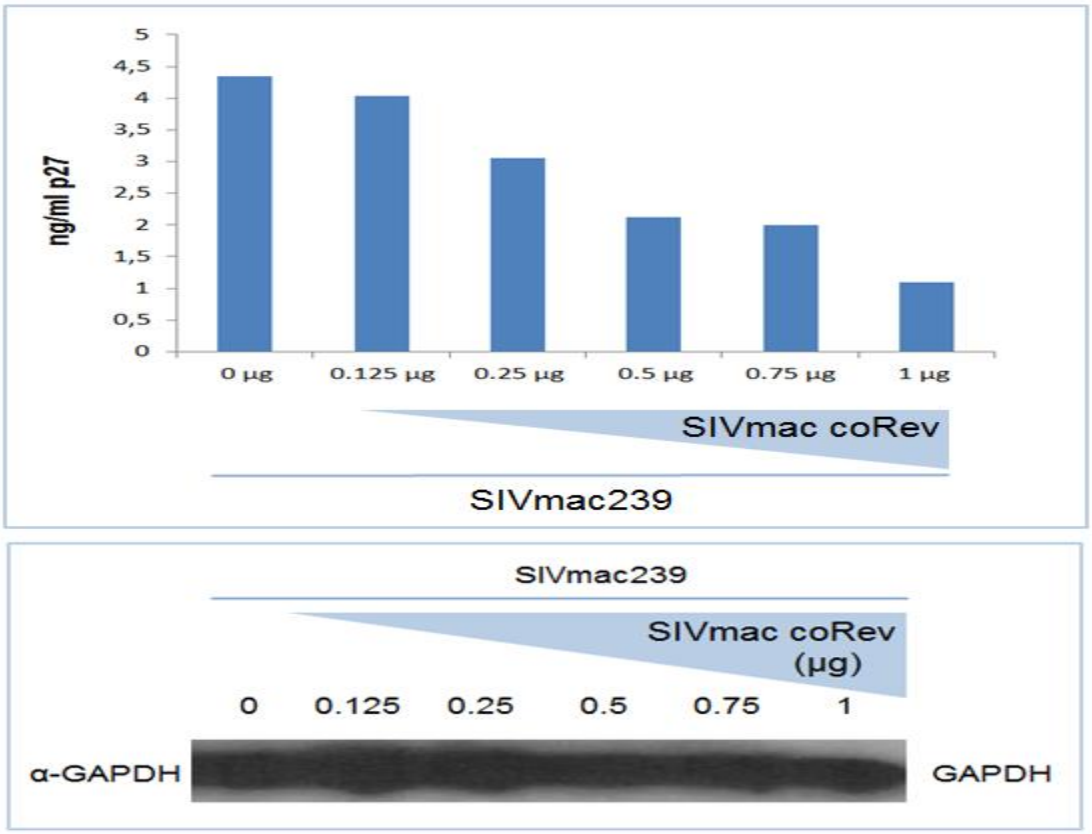


Figure 23: The effect of SIVmac Rev overexpression on viral Gag production. After transfection of the cells with SIVmac and Rev at different concentrations, the amount of the viral Gag protein in the cell lysates was determined using the CA-antigen capture ELISA. The experiment was repeated twice in HEK-293T cells and the results from one representative experiment are shown without standard error bars. Expression of GAPDH was analyzed by Western blotting in lysates from HEK-293T cells transfected with the SIVmac molecular clone and pCDNA4-V5-SIVmac-coRev DNA at different ratios. GAPDH protein used as a loading control was visualized using an α-GAPDH antibody.

The ELISA displayed in Figure 23 suggests that the overexpressed SIVmac Rev has an inhibitory effect on virus production. Moreover, increasing the amount of Rev DNA transfected into the cells leads to a decrease in the yield of viral Gag in the cell lysates. The maximum inhibitory effect of Rev was seen with cotransfection of the cells with the highest amount used of (1µg) Rev DNA.

Expression of GAPDH protein was determined by SDS-PAGE and Western blot analysis of the cell lysates. As a loading control, the blot was incubated with a

primary α-GAPDH antibody and a secondary α-Mouse IgG-HRP antibody. Western blotting detected GAPDH in all cell lysates, confirming that the same quantities of the cell lysates were loaded on to the gel, ensuring that the differences in the quantity of viral Gag protein detected in the ELISA was due to the inhibitory effect of Rev overexpression and did not involve in disturbance of the cellular metabolism.

4.2.3 Overexpression of SIVmac Rev in the presence of exogenous Staufen-1 decreases the production of viral Gag protein

In order to establish whether the SIVmac Rev negatively affects the generation of new SIVmac virions in the presence of exogenous Staufen-1, HEK-293T cells were cotransfected with 1µg of SIVmac molecular clone (which produces the Rev protein), a constant concentration of Staufen-1 (0.5 µg) and different concentrations of Rev DNA. Cells were also transfected with SIVmac and Staufen-1 without coRev. After collecting the supernatants and lysing the cells 48 post transfection, CA-antigen capture ELISA assay was performed to quantify viral Gag protein.

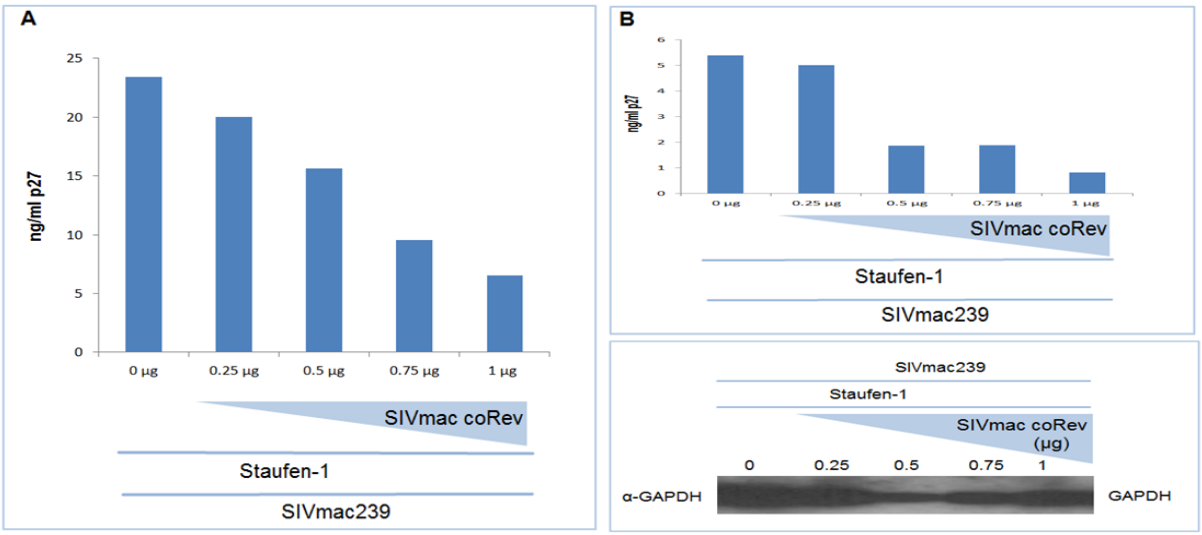


Figure 24: Analysis of the impact of coexpression of SIVmac Rev and Staufen-1 on viral Gag production. Cells were cotransfected with SIVmac, Staufen-1 and Rev at different ratios, followed by ELISA to measure the levels of viral Gag in the supernatants (A) and cell lysates (B). The experiment was repeated and data from one representative experiment are displayed above. To confirm that the amounts of cell lysates loaded were the same, expression of the loading control GAPDH was visualized by Western blot using an α-GAPDH antibody.

The ELISA shown in Figure 24 revealed that the decreased level of viral Gag protein in the supernatants (A) and cell lysates (B) appears to be associated with increasing the amounts of Rev used for transfection. Also, Figure 24 shows that viral particle production upon transfection with 0.25 µg and 0.5 µg Rev with coexpression of exogenous Staufen-1 is higher than that detected after transfection of cells with SIVmac and 0.75 µg or 1 µg Rev. In addition, Figure 24 shows that the highest amount of Gag protein was seen in supernatants and cell lysates of cells transfected with the virus and Staufen-1 alone.

GAPDH Western blotting of cell lysates was performed to ensure that the same amounts were loaded on to the gel. GAPDH expression was detected using a primary α-GAPDH antibody and a secondary α-mouse IgG-HRP antibody.

In conclusion, in contrast to the positive influence of Rec on HERV-K113 production, SIVmac Rev overexpression either with or without expressing exogenous Staufen-1 appears to be associated with the decreased concentration of SIVmac Gag protein.

4.3 Investigation of the interacting functional domains within SIVmac Rev and Staufen-1

4.3.1.1 Site directed mutagenesis of SIVmac Rev

The retroviral Rev trans-activator plays an important role in the regulation of retroviral mRNA expression [151] [152]. To investigate the function of the SIVmac Rev domains necessary for Rev-mediated nucleocytoplasmic RNA shuttling and responsible for binding to Staufen-1, mutations were introduced into the SIVmac Rev DNA sequence. The SIVmac251 Rev amino acid sequence was first aligned with the functional coding sequence of HIV-1 Rev as illustrated in Figure 25. The aim of this alignment was to identify, based on the known domains within HIV Rev, the important domains within SIV Rev to target for mutation.

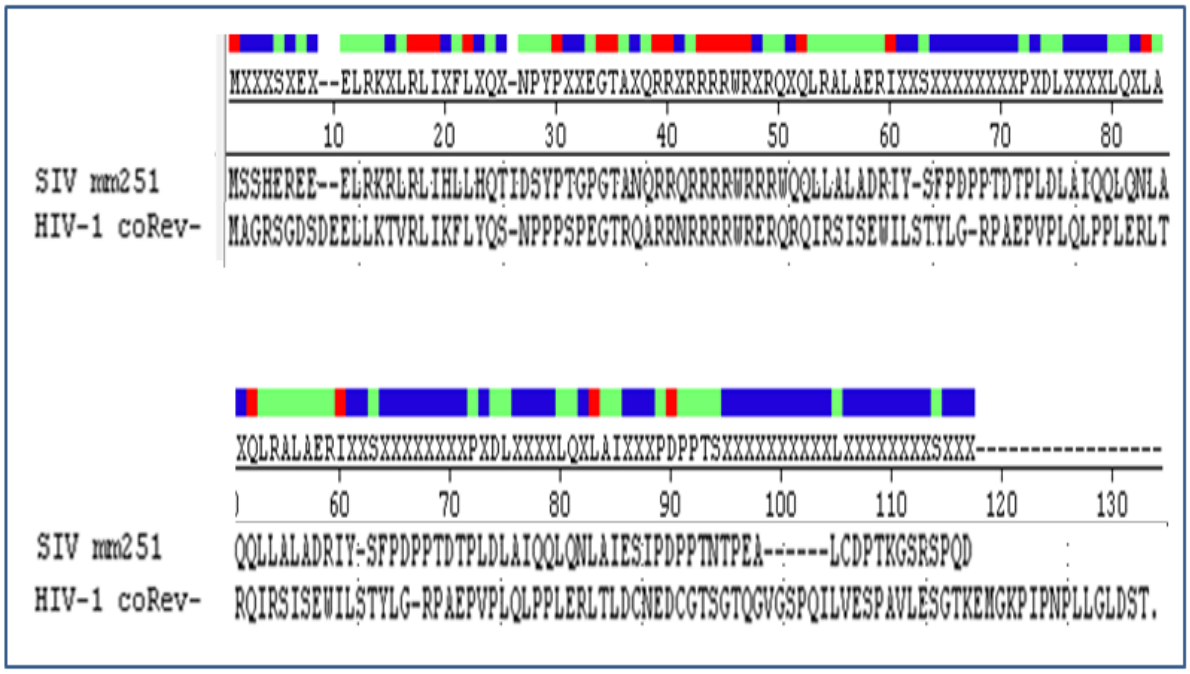


Figure 25: Alignment of SIVmac and HIV-1 Rev sequences. The functional amino acid coding sequence domains of SIVmac Rev were aligned with the amino acid sequences of HIV-1 Rev.

pCMV-FLAG-SIVmac-coRev was subjected to either deletion mutations or substitution mutations. The deletion mutation was created by removal of N-terminal amino acids by PCR using specific primers as shown in Figure 26, and the amplified PCR products were then double digested using particular restriction enzymes. The DNA digests were next ligated with the pCMV-FLAG expression vector. In addition, nucleotide substitution mutations were generated by oligonucleotide site directed mutagenesis (see 3.2.2.3). These constructs were generated to be analogous to known mutants of HIV-1 Rev. For example, HIV-1 Rev M5 and SLT40 fails to transport viral mRNAs from the nucleus into the cytoplasm [142].

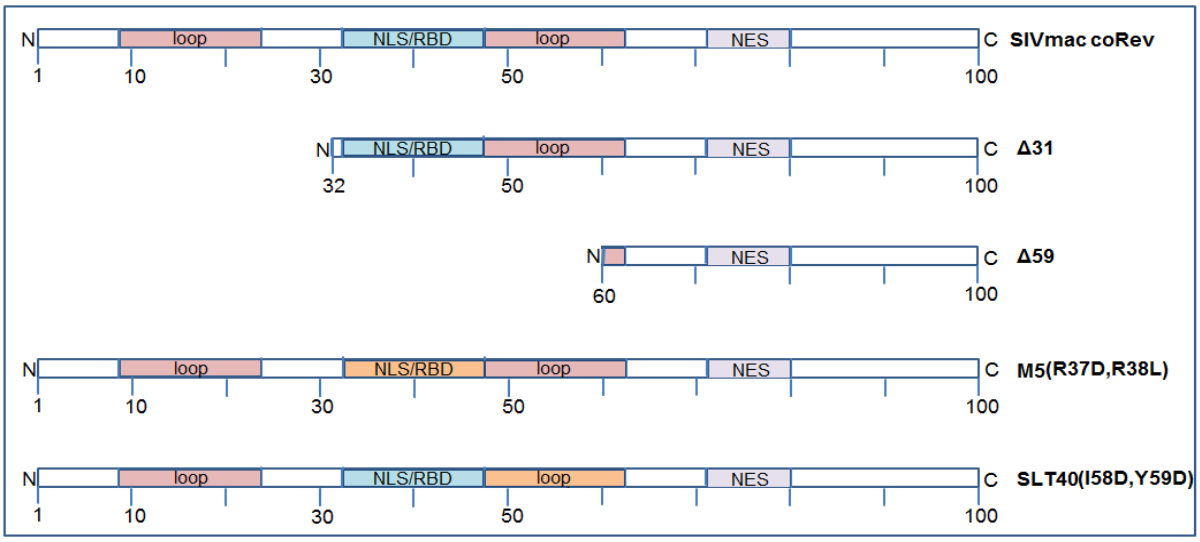


Figure 26: SIVmac Rev mutagenesis by amino acids deletions and substitutions. SIVmac Rev contains 100 amino acids and is composed of many functional domains: Nuclear localization domain (NLS) overlapping with RNA binding domain (RBD) in aquamarine color, and two multimerization domains displayed in red. Rev mutants produced by N-terminal amino acid deletions are Δ31 (1-31), Δ59 (1-59), while mutants with substitution of amino acids M5 (R37D, R38L) and SLT40 (I58D, Y59D) are shown in orange.

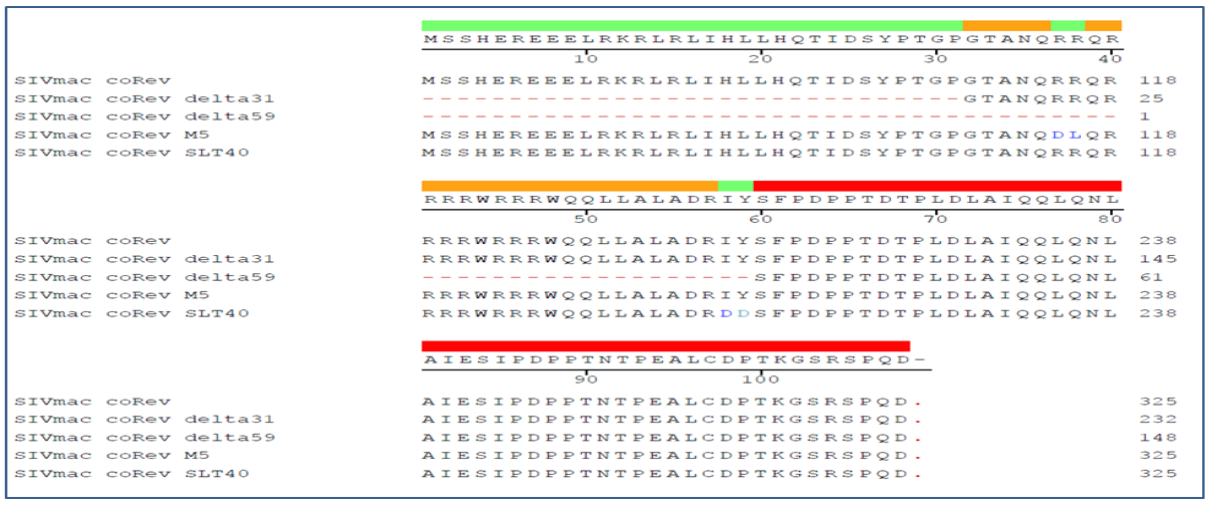


Figure 27: Amino acids alignment of SIVmac Rev with its mutants. The deletion mutants were formed by elimination of 31 or 59 N-terminal amino acids (SIVmac Δ31 and Δ59 respectively), whereas the substitution mutants (SIVmac M5 and SLT40) were created by single amino substitution as indicated in blue and green color. DNASTAR MegAlign was used to produce the alignments.

The SIVmac Rev mutants (see 3.2.2.11) were subsequently sequenced and the success of the mutation process checked by alignment of the amino acid sequences with that of SIVmac251 Rev as displayed in Figure 27.

4.3.1.2 Subcellular localization of SIVmac Rev, HIV-1 Rev, HERV-K(HML-2) Rec and SIVmac Rev mutants

Study of the subcellular localization of retroviral proteins (e.g. Rev) plays a role in examining viral pathogenesis and investigating potential targets for efficient drugs. The Rev protein mediates the nuclear RNA export and is characterized by a cytoplasmic and nuclear subcellular localization in several cell lines [153]. Based on these findings, the expression of SIVmac Rev, as detected by Western blot analysis, was affirmed via immunofluorescence by transfecting HEK-293T cells with 2 µg pCDNA4-V5-SIVmac-coRev. In addition, cells were transfected with pCDNA3-V5-HIV-1-coRev, pCDNA3-V5-HERV-K113-oricoRec or the ovalbumin expression vector (pTH-OVA). The latter was included because it has a high level of expression in the cytoplasm. Naïve cells served as a negative control. After incubation for 48h, the cells were prepared for cLSM analysis as previously described (see 3.2.3.4). Expressed proteins were visualized using the mouse monoclonal α-V5-Cy3 (1:750) and immunofluorescence CLSM imaging.

As seen in Figure 28, the expressed SIVmac Rev appears to be mainly located in the cytoplasm. In addition the expression of HIV-1 Rev by confocal laser scanning microscope was found mostly in the cytoplasm. Also, the immunofluorescence assay demonstrated that ovalbumin and Rec proteins were successfully expressed and mainly found within the cytoplasm. The expression level of all proteins appeared to be similar.

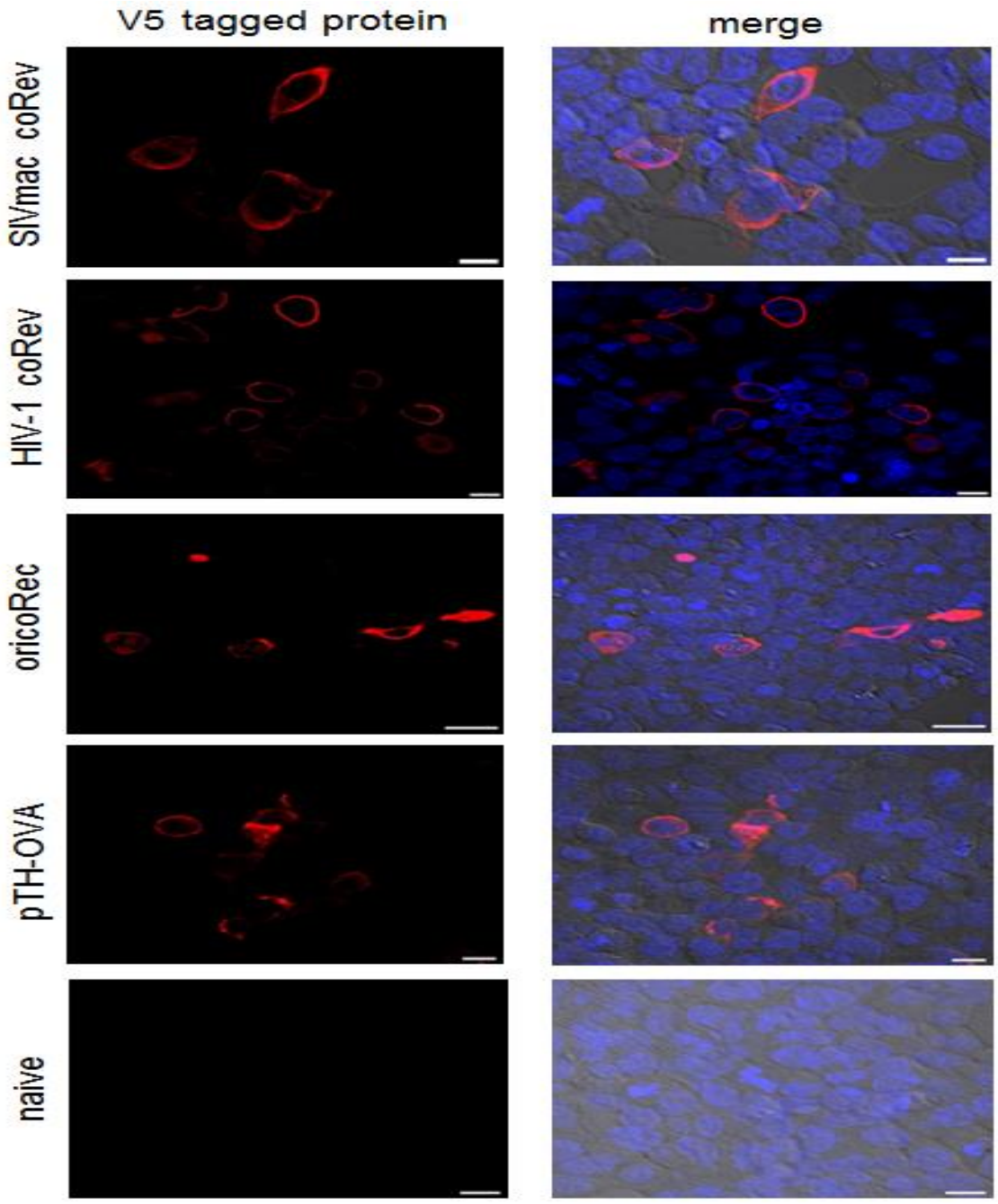


Figure 28: Protein localization and immunofluorescence analysis of SIVmac Rev, HIV-1 Rev and HERV-K(HML-2) Rec. For the localization assay, HEK-293T cells were transfected with plasmids producing either SIVmac Rev, HIV-1 Rev, HERV-K(HML-2) Rec or ovalbumin using 2 µg of each DNA construct. 48 h post transfection, cells were immunostained with a mouse monoclonal α-V5-Cy3. The right hand panel shows the cytoplasmic localization of the expressed proteins (red fluorescence) in the merged images while the blue fluorescence indicated Staining of the nucleus with DAPI dye. Scale bar, 10 µm.

Furthermore, immunofluorescence assays was carried out to determine whether SIVmac Rev mutants were expressed in plasmid treated cells. The cellular and nuclear localizations of the target proteins were investigated using HEK-293T cells transfected either with SIVmac Rev, SIVmac RevΔ31, SIVmac RevΔ59, SIVmac Rev M5, or SIVmac Rev SLT40. At 48 h after cell transfection, cells were subjected to cLSM analysis as previously described (see 3.2.3.4). The expression of SIVmac Rev mutants was shown by incubation of the transfected cells after permeabilization with an α-FLAG-Tag primary antibody and AlexaFluor 568 α-mouse-IgG secondary antibody at a dilution of 1:5000 and 1:2000, respectively. Images were then obtained using confocal laser scanning microscopy.

Notably, confocal laser-scanning microscopy revealed a predominantly cytoplasmic distribution of the SIVmac Rev (green) mutant proteins (Figure 29). The expression of SIVmac RevΔ31 in the cytoplasm was not adversely influenced by deletion of the first loop. In addition, the SIVmac RevΔ59 and SIVmac Rev M5 mutants were expected to be detectable only within the cytoplasm due to deletion of the NLS amino acids (RevΔ59) or inactivation of the NLS by amino acids substitution (Rev M5). Moreover, expression of SIVmac Rev SLT40 mutant within the cytoplasm was confirmed despite substitution of amino acids in the second loop.

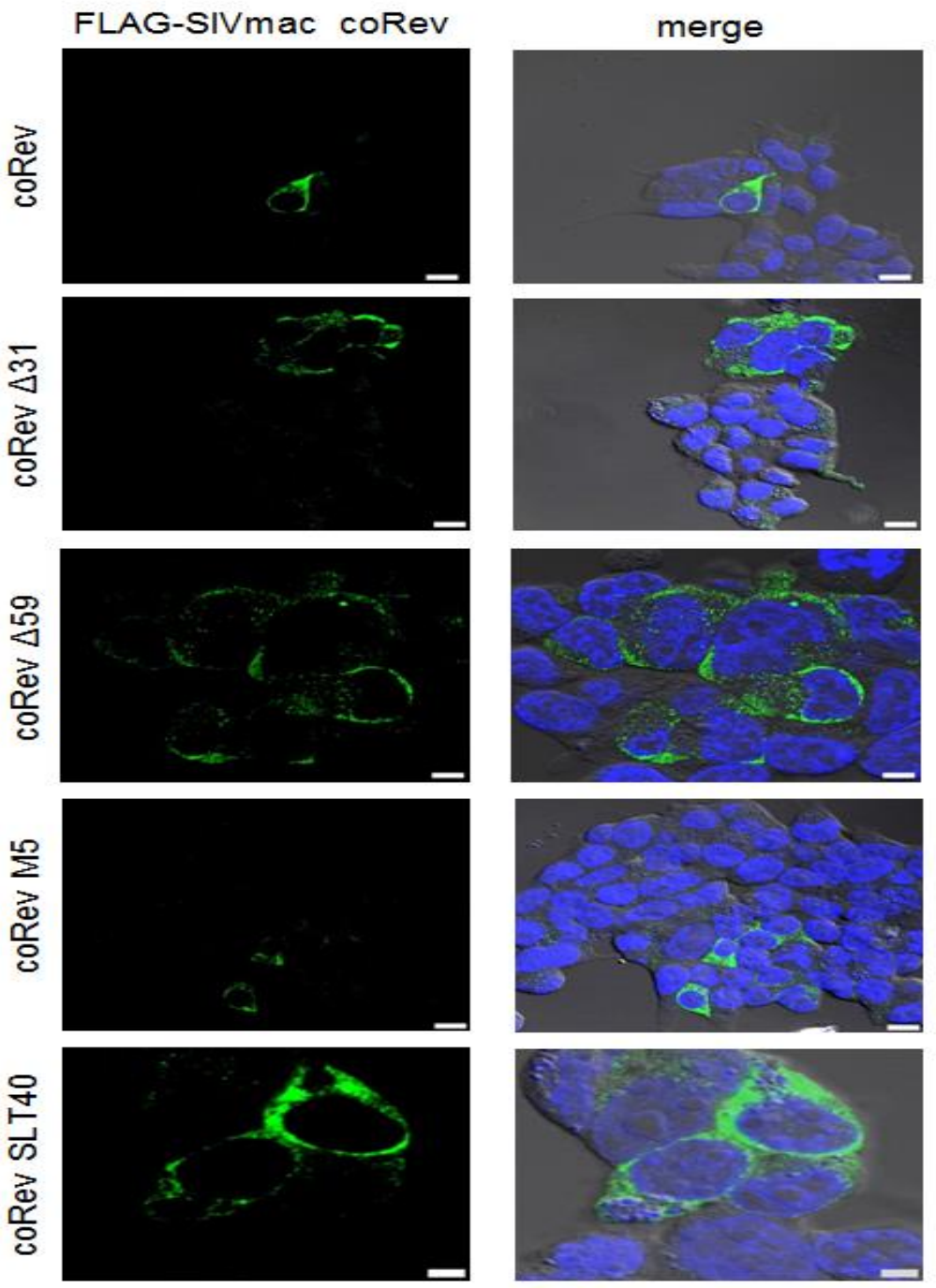


Figure 29: Intracellular localization of SIVmac Rev mutants. Transfection of SIVmac Rev or each Rev mutant (Δ31, Δ59, M5, SLT40) into HEK-293T cells resulted in cytoplasmic staining of the expressed Rev and its mutants using an α-FLAG-Tag primary antibody and AlexaFluor 568 α- mouse-IgG secondary antibody. The right-hand panel shows green fluorescent Rev mutants displayed within the cytoplasm in the merged images, whereas staining of the nucleus with DAPI is indicated in blue. Scale bar, 10 µm.

4.3.1.3 The binding of Staufen-1 to SIVmac Rev or Rev SLT40 is indicated by CoIP and Western blotting while other mutations (RevΔ31, RevΔ59, Rev M5) fail to show interaction with Staufen-1

One of the objectives of this study was to determine which functional domains within SIVmac Rev are responsible for the binding of Rev to Staufen-1. In earlier work, it was shown that the generation of HIV-1 Rev NLS mutants impaired the attachment of Rev to Staufen-1 [142]. For this, the interaction of Staufen-1 and SIVmac Rev mutants was investigated by co-immunoprecipitation experiments and Western blot analysis. In this regard, HEK-293T cells were cotransfected with 15 µg pCDNA3-V5-Staufen-1 and either 15 µg pCMV-FLAG-SIVmac-coRev, RevΔ31, RevΔ59, Rev M5 or Rev SLT40. Western blotting using a primary α-FLAG antibody and a secondary α-rabbit IgG-HRP antibody revealed FLAG-SIVmac-coRev and FLAG-coRev SLT40 bands, corresponding to 15 kDa. The Western blot therefore indicated that deletion of 31 or 59 amino acids (RevΔ31, RevΔ59) and mutation of the NLS domain by amino acid substitution (Rev M5) abrogates Rev-Staufen-1 interaction whereas mutation by amino acid substitution in the multimerization domain (SLT40) does not have this effect. Furthermore, expression of V5-Staufen-1 in the cell lysates was shown by Western blot test using α-V5-HRP.

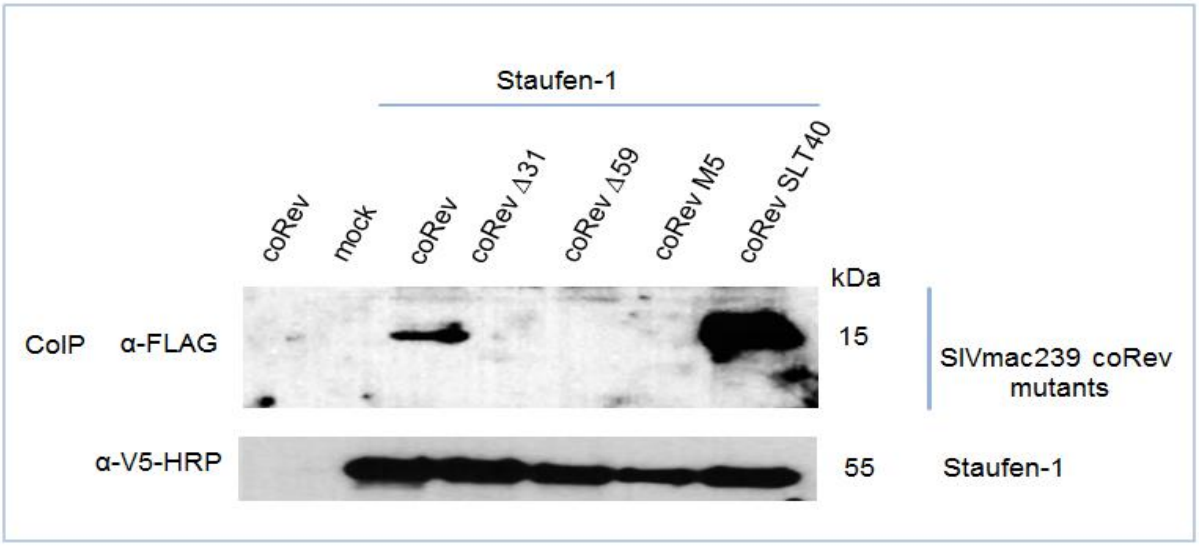


Figure 30: Analysis of the binding between Staufen-1 and SIVmac Rev mutants by CoIP and Western blotting. HEK-293T cells were cotransfected with pCDNA3-V5-Staufen-1 and either pCMV-FLAG-SIVmac-coRev, RevΔ31, RevΔ59, Rev M5 or Rev SLT40. The cell lysates were next treated with anti-V5 tagged agarose beads. Western blot analysis demonstrated a positive interaction between Staufen-1 and Rev/Rev SLT40. V5-Staufen-1 was detected in the cell lysates using α-V5-HRP. Also, cells were cotransfected with an empty pcDNA4-V5-His/B vector alone or in combination with pCMV-FLAG-SIVmac-coRev as a negative control.

In conclusion, these experiments revealed binding of Staufen-1 to SIVmac Rev and, to an enhanced degree, to the SLT40 mutant.

4.3.1.4 Mutation in SIVmac Rev decreases the amount of SIVmac particle production

The results above show that overexpression of SIVmac Rev diminished the yield of viral Gag protein. The effect of overexpression of SIVmac Rev mutants on the virus production was therefore analyzed to determine whether mutation of the functional domains within SIVmac Rev abrogates the negative influence of Rev on viral expression. In recent work, it has been reported that overexpression of HIV-2 Rev mutants did not cause an increase or decrease in the level of the viral Gag protein [143]. Consequently, in this study, HEK-293T cells were cotransfected with 1µg of the SIVmac molecular clone (producing Rev protein) and either 1 µg Rev or

each Rev mutant (Δ31, Δ59, M5, or SLT40). Cells were also transfected with pcDNA4-V5-His/B vector as a negative control or with the virus alone. Supernatants and the cell lysates prepared 48 h post transfection were analyzed for viral Gag protein by CA-antigen capture ELISA.

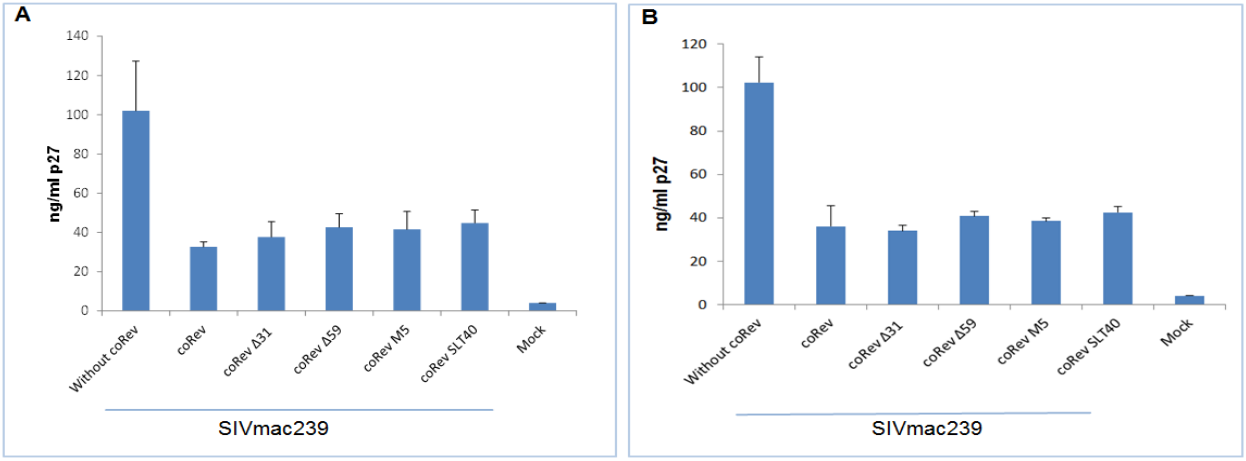


Figure 31: Demonstration of the adverse influence of SIVmac Rev mutant overexpression on the formation of virus particles. Transfection of HEK-293T cells was carried out with SIVmac molecular clone, and 1µg of each Rev DNA mutant. The amount of the viral Gag protein in the supernatants (A) and in the cell lysates (B) was then measured by CA-antigen capture ELISA. Results from one representative experiment performed in triplicate are shown with standard error bars.

As shown in Figure 31, Rev, whether mutated or not, was found to effectively diminish the yield of Gag protein by approximately a factor of approximately 2.5 compared to cells transfected with the SIVmac molecular clone alone.

In conclusion, deletions of the first loop/NLS domain or inactivation of NLS/multimerization domains did not abrogate the inhibitory effect on virus generation seen with non-mutated SIVmac Rev.

4.3.1.5 Overexpression of SIVmac Rev mutants in the presence of an ectopic Staufen-1 DNA impairs the production of new viral particles

In this experiment, the effect of the SIVmac Rev mutants and Staufen-1 was addressed by cotransfection of HEK-293T cells with 1µg of the SIVmac molecular

clone, 0.25 µg of each SIVmac Rev mutant and 0.5 µg Staufen-1 DNA. Furthermore, cells were transfected either with the virus alone or with the virus plus Staufen-1 DNA. Mock-transfected cells served as a negative control. The transfected cells were incubated for 48 hours before lysing the cells. The CA-antigen capture ELISA was then used to determine the concentration of SIVmac Gag in the cell lysates.

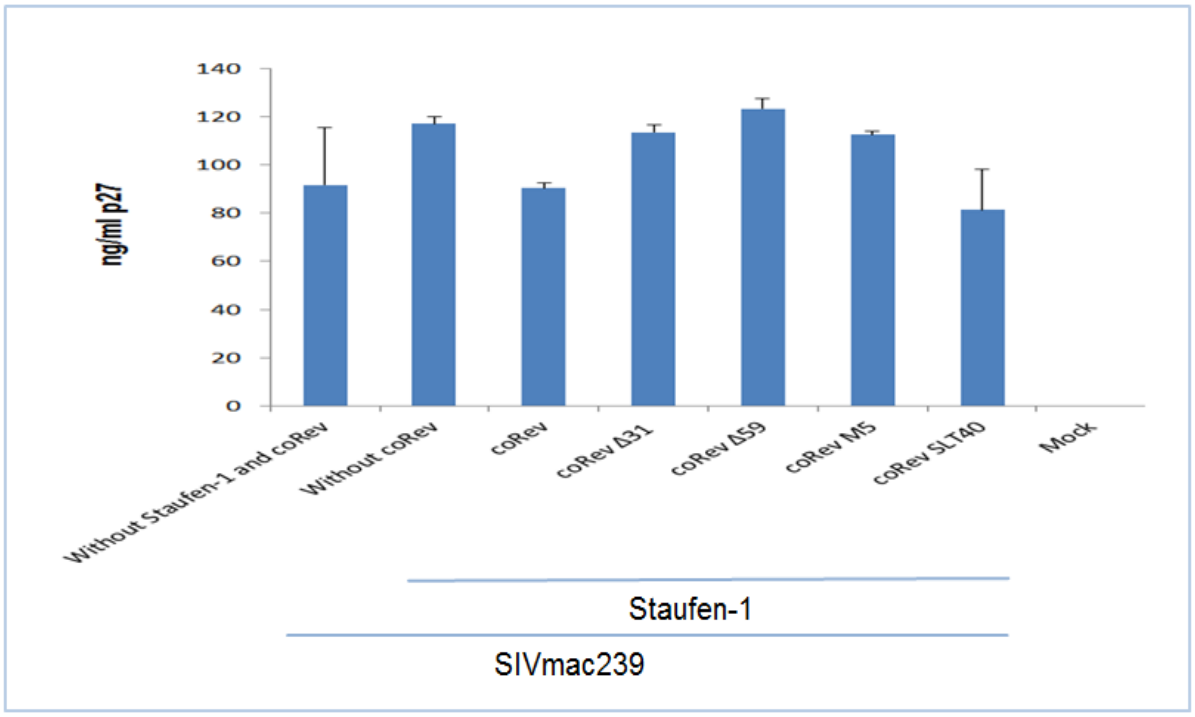


Figure 32: Effect of coexpressing SIVmac Rev mutants and Staufen-1 on the production of the SIVmac Gag protein. The SIVmac molecular clone was cotransfected with each Rev mutant and Staufen-1 DNA into HEK-293T cells and the quantity of Gag protein in the cell lysates subsequently measured by CA-antigen capture ELISA. The experiment was carried out in triplicate and data are shown with standard error bars.

As shown in Figure 32, the levels of the expressed viral Gag protein were diminished in the lysates of cells transfected with the virus, plus Staufen-1 and Rev mutants, compared to that in cells expressing only virus plus Staufen-1, suggesting that the overexpressed SIVmac Rev and SLT40 mutant were responsible for inhibition of the Staufen-1 enhancement, while Δ31, Δ59, M5 do not have a clear negative effect. No viral Gag was detected in the lysate of the negative control cells. Based on this result, cotransfection of Staufen-1 and SIVmac Rev mutants had the same adverse effect on virus production as that seen with non-mutated SIVmac Rev.

In conclusion, mutations in SIVmac Rev did not alter the inhibitory effect of SIVmac Rev, regardless of their ability to interact with Staufen-1 (Rev, SLT40) or not (M5, Δ31, Δ59). This finding demonstrated a reduced negative effect on virus production in the presence of exogenous Staufen-1.

4.3.2.1 Exogenous Staufen-1, Staufen-1_ΔRBD4 and Staufen-1_ΔTBD/RBD5 elevate the quantity of SIVmac Gag protein produced, whereas Staufen-1_F135A/Staufen-1_ΔRBD3 do not

Staufen-1 is composed of several functional double-stranded RNA binding domains (dsRBD2 to dsRBD5) and a tubulin binding domain (TBD) (see Figure 12). Staufen-1 mutants were produced and kindly provided by Jula Wamara, (a co-worker in the laboratory) to determine which domains within Staufen-1 protein are essential for interaction with SIVmac Rev and promotion of virus production. Mutations were introduced by deletion of RBD3, RBD4, or TBD/RBD5 (termed ΔRBD3, ΔRBD4 and ΔTBD/RBD5, respectively) and a Staufen-1_F135A mutant was generated by inactivation of RBD3 via a single point mutation.

A previous study indicated that Staufen-1_F135A and, ΔRBD3 overexpression resulted in lower virus expression levels than those induced by Staufen-1, ΔRBD4 and ΔTBD/RBD5 [143]; Hanke *et al.* also demonstrated that overexpression of Staufen-1 increased the amount of viral Gag protein by a factor 20 [141]. The main purpose of this experiment was therefore to determine the exact domain in Staufen-1 responsible for enhancing the production of SIVmac particles. 1µg SIVmac molecular clone and 0.5µg of either FLAG-Staufen-1, Staufen-1_F135A, Staufen-1_ΔRBD3, Staufen-1_ΔRBD4 or Staufen-1_ΔTBD/RBD5 were cotransfected into HEK-293T cells that were then incubated for 48 h. Cells transfected with pcDNA4-V5-His/B as a mock DNA served as a negative control. Cell lysates were then prepared for measuring the quantities of viral Gag protein using the CA-antigen capture ELISA.

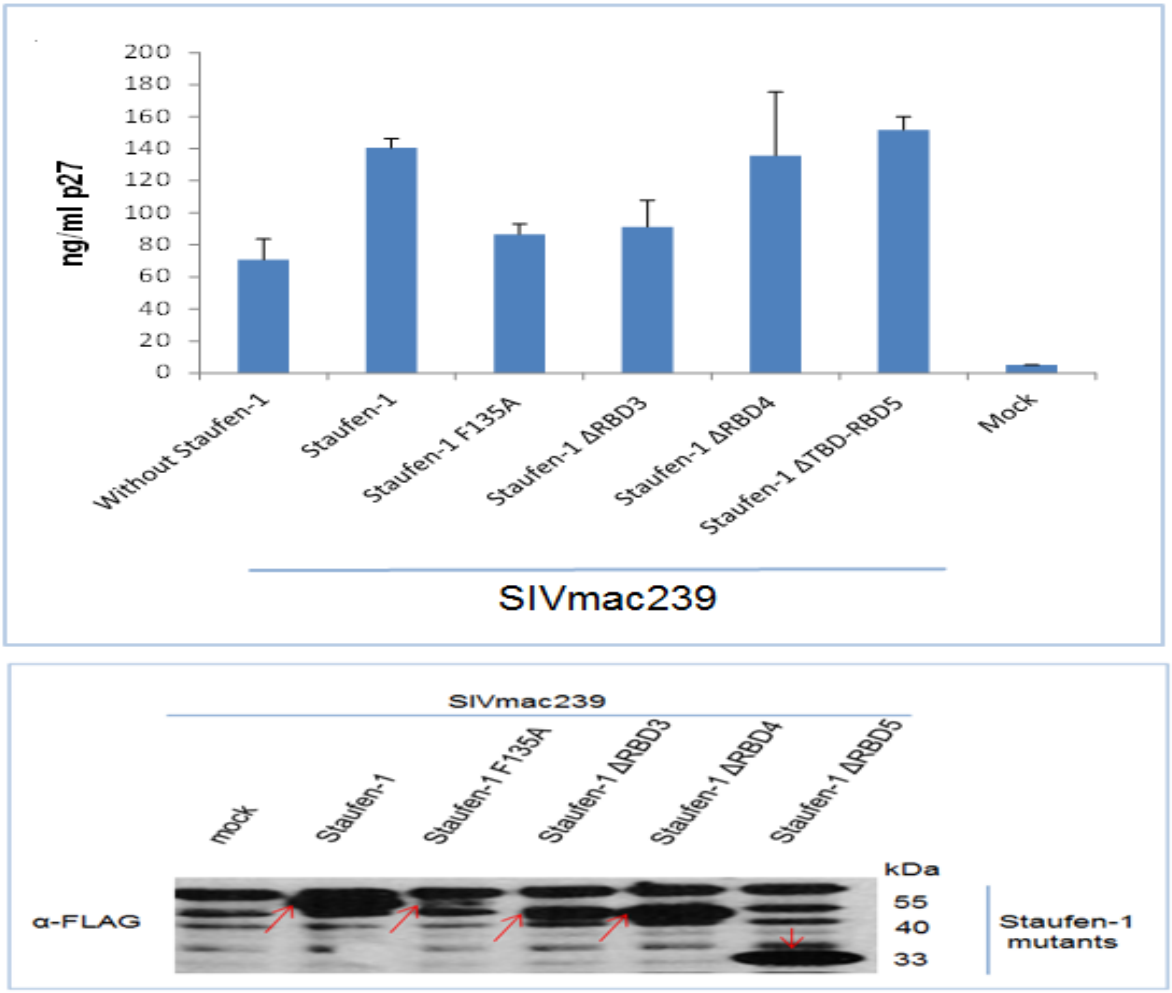


Figure 33: Impact of overexpressing Staufen-1 mutants on the production of SIVmac Gag protein. Lysates of HEK-293T cells cotransfected with SIVmac and each Staufen-1 mutant were subjected to CA-antigen capture ELISA to determine the concentration of Gag protein. Data from one representative experiment done in triplicate are shown with standard error bars. Western blotting as indicated by red arrows confirmed the expression of Staufen-1 mutants at their expected sizes in the cell lysates.

The ELISA shown in Figure 33 demonstrated higher expression levels of the viral Gag protein in lysates of cells overexpressing Staufen-1, Staufen-1_ΔRBD4 or Staufen-1_ΔTBD/RBD5 by factors of 1.5, 2 or 1.5, respectively compared cells cotransfected with only the virus or with the virus plus Staufen-1_F135A/Staufen-1_ΔRBD3. No viral Gag protein was produced in the negative control cells.

Therefore, deletion of the RBD4 or TBD/RBD5 domains did not abolish the generation of SIVmac mediated by Staufen-1, while Staufen-1_F135A/Staufen-1_ΔRBD3 mutants inhibited the positive role of Staufen-1 on viral particle production.

Equal amounts of protein from the cell lysates were subjected to SDS-PAGE and Western blotting to determine the expression of the Staufen-1 mutants. Staufen-1 mutants carrying FLAG tag were detected by a primary α-FLAG antibody and a secondary α-rabbit IgG-HRP antibody. As shown in Figure 33, the 55-kDa band size corresponds to the non-mutated Staufen-1 and Staufen-1_F135A (weak signal indicated with a red arrow between the two dark bands), whereas the Western blot for Staufen-1 ΔRBD3 and Staufen-1 ΔRBD4 confirms their expected molecular masses of 47 kDa. Additionally, expression of Staufen-1 ΔTBD/RBD5 at the expected molecular weight of, approximately 33 kDa was seen by immunoblotting.

4.3.2.2 SIVmac Rev binds to Staufen-1 and its mutants but with lower interaction efficacy to Staufen-1_F135A, ΔRBD3

In a previous publication, Hanke *et al*. confirmed that RBD4 promotes HERV-K(HML-2) Rec/Staufen-1 interaction [141], and in the present study, a co-immunoprecipitation assay was therefore performed to determine which Staufen-1 subdomain plays a significant role in interacting with SIVmac Rev in an RNA-independent manner. HEK-293T cells were therefore cotransfected with pCDNA4-V5-SIVmac-coRev and either pCMV-FLAG-Staufen-1, FLAG-Staufen-1_F135A, ΔRBD3, ΔRBD4 or ΔTBD/RBD5. Cell lysates were prepared 48 h post transfection and 750 µl incubated with anti-V5 agarose beads (see 3.2.3.3). Following co-immunoprecipitation, FLAG-tagged Staufen-1 and its mutants were examined by Western blot using a primary α-FLAG antibody and a secondary α-rabbit IgG-HRP antibody. As shown in Figure 34, the Western blot provides evidence that the expressed FLAG-Staufen-1 (and its mutants) bands co-sedimented with the expressed V5-Rev. This strongly indicates a binding between these proteins but a dramatically with lower efficacy of Rev binding to Staufen-1 ΔRBD3 and Staufen-1 F135A. Western blotting for Staufen-1 and Staufen-1_F135A indicated a molecular weight of 55 kDa, whereas, the size of Staufen-1 ΔRBD3 and Staufen-1 ΔRBD4 was 47 kDa and Staufen-1 ΔTBD/RBD5 was approximately 33 kDa.

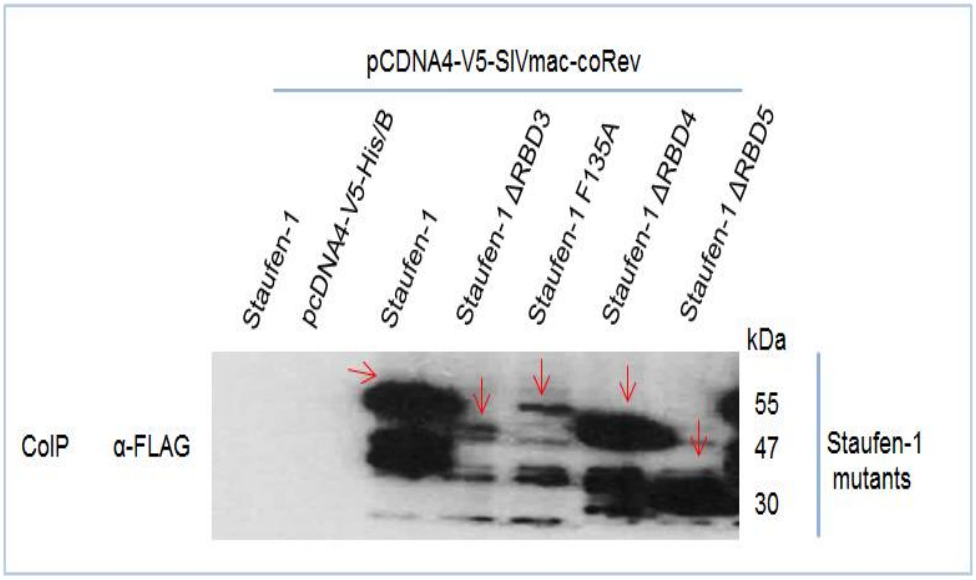


Figure 34: Analysis of the interaction between SIVmac Rev and Staufen-1 mutants by CoIP and Western blot. HEK-293T cells were cotransfected with V5-SIVmac-coRev and either FLAG-Staufen-1, FLAG-Staufen-1_F135A, ΔRBD3, ΔRBD4 or ΔTBD/RBD5. Cell lysates were then analyzed by CoIP using anti-V5 agarose beads followed by Western blotting using anti-FLAG. Interactions between the Rev protein and the Staufen-1 mutants are indicated by red arrows. As a negative control, cells were cotransfected with an empty pcDNA4-V5-His/B vector and either pCDNA4-V5-SIVmac-coRev or pCMV-FLAG-Staufen-1.

This finding demonstrated that inactivation or deletion of dsRBD3 negatively influenced the binding between Staufen-1 and SIVmac Rev.

4.3.3.1 SIVmac Rev and its mutants induce mRNA transport in the presence of HIV-2 RRE while Staufen-1 decreases the efficiency of RNA export

The retroviral Rev/Rec proteins attach to the Rev Responsive Element (RRE) located within the unspliced and incompletely spliced viral mRNAs and then initiate nuclear transportation for protein biosynthesis [154]. The aim of this study was therefore to study SIVmac Rev-dependent transport of viral mRNA, for which two constructs, termed as Shuttle and Shuttle HIV-2 RRE, were used. As shown in Figure 35, each construct is composed of pCDNA4 as a DNA vector backbone, HERV-K113

5´UTR with donor site (SD), a firefly luciferase encoding gene (reporter gene), HERV-K113 Gag and an *env* gene with acceptor site (SA). Four stop codons were introduced into the open reading frame of the *rec* gene to inhibit transcription. To investigate whether the transport of the viral mRNA is dependent on the Rev protein, Shuttle HIV-2 RRE was designed to contain a C-terminal Rev-responsive element (RRE). Shuttle and Shuttle HIV-2 RRE constructs were kindly provided by Anna Klara Amler, a co-worker in the laboratory. HIV-2 RRE was used in this experiment because it is known that both HIV-2 and SIVmac originated from SIVsm and there is therefore a high homology between their DNA sequences. It was shown by Hanke *et al*. that cotransfection of shuttle RcRE and oricoRec increased nucleocytoplasmic shuttling but that when Rec and shuttle lacking RcRE were cotransfected into cells, no luciferase activity was observed [141].

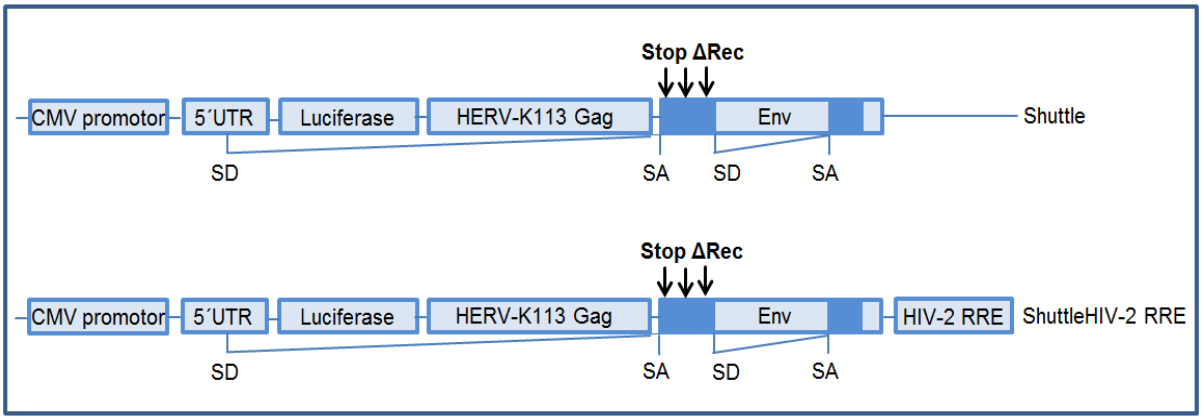


Figure 35: Schematic representation showing the structure of Shuttle and Shuttle HIV-2 RRE constructs. Each DNA construct consists of HERV-K113 5´UTR, a firefly luciferase expressing gene as a reporter gene, HERV-K113 Gag and an *env* gene. To suppress the expression of Rec, four stop codons were introduced into *rec*. To determining transport of only the viral unspliced RNAs, the luciferase gene was introduced between the 5'UTR and the Gag ORF. Additionally, Shuttle HIV-2 RRE comprises an RRE specific for SIVmac and HIV-2 Rev-based RNA transport.

In this regard, the influence of SIVmac Rev and its mutants on the viral RNA transport was assessed by cotransfection of HEK-293T cells (see 3.2.1.4) with Shuttle HIV-2 RRE and SIVmac Rev or each Rev mutant (Δ31, M5, or SLT40) either with or without expressing Staufen-1. Cells were also cotransfected with Shuttle HIV-

2 RRE and HIV-2 Rev as a positive control. To enable normalization of the firefly luciferase data, the cells were cotransfected with a DNA vector encoding *Renilla* luciferase as an internal control. Moreover, cells transfected with either SIVmac Rev or HIV-2 Rev alone were tested. As a negative control, cells were transfected with the pcDNA4-V5-His/B vector. The luciferase reporter assay was then performed (see 3.2.3.7).

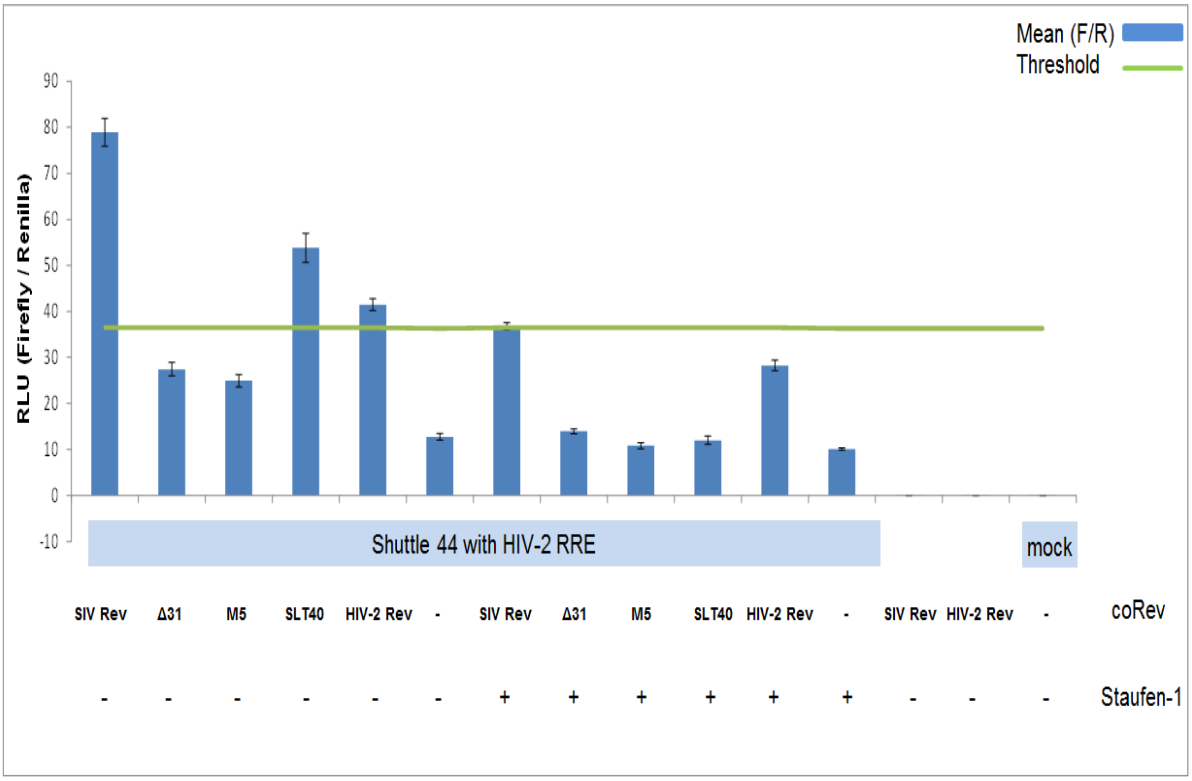


Figure 36: SIVmac Rev-dependent RNA transport in the presence of RRE. Shuttle HIV-2 RRE and SIVmac Rev or Rev mutants were co-expressed in HEK-293T cells. + indicates that the cells express an exogenous Staufen-1. The results were normalized to the activity of the expressed *Renilla* luciferase as an internal control. The green line marks the threshold (average luciferase activity measured for non-specific transport of the construct Shuttle plus three times the standard deviation). The experiment was repeated three times in HEK-293T cells. Data from one representative experiment done with 8 replicates are shown with standard error bars.

As displayed in Figure 36, Shuttle HIV-2 RRE alone induces low luciferase activity in the transfected cells (RLU = 8.36). In addition, SIVmac Rev or Rev mutants and the Shuttle HIV-2 RRE construct cotransfected into cells results a significant

elevation in luciferase activity mediated by SIVmac Rev and Rev-ΔSLT40. In contrast, in cells coding for an exogenous Staufen-1, Shuttle HIV-2 RRE and Rev diminishes the activity of the Rev-dependent RNA export from the nucleus to the cytoplasm compared to that in absence of an ectopic Staufen-1. Also, luciferase activity was observed when cells were cotransfected with Shuttle HIV-2 RRE and HIV-2 Rev, especially without overexpression of Staufen-1. No luciferase activity was detectable in cells transfected with either mock DNA, SIVmac Rev or HIV-2 Rev alone.

In conclusion, SIVmac Rev activates nuclear RNA export and inactivation of the multimerization domain did not lead to inhibition of RNA transport, whereas RevΔ31 and M5 (NLS-deficient) prevented luciferase biosynthesis. Furthermore, SIVmac Rev demonstrated stronger RNA transport activity than that mediated by HIV-2 Re and Staufen-1 impaired Rev-based RNA export.

4.3.3.2 SIVmac Rev, its mutants and Staufen-1 do not affect mRNA export in the absence of HIV-2 RRE

To analyze the non-specific RNA transport without using RRE, HEK-293T cells were cotransfected with equal concentrations of the Shuttle construct, and SIVmac Rev, its mutants (Δ31, M5, or SLT40) or HIV-2 Rev. Cells transfected with a mock DNA vector served as a negative control and cells transfected with SIVmac Rev or HIV-2 Rev alone were also evaluated. As shown in Figure 37, only very low luciferase activity was observed upon transfection of cells with the Shuttle construct lacking RRE, with SIVmac Rev or its mutants, or with HIV-2 Rev, either with or without overexpression of an exogenous Staufen-1. Therefore, coexpression of Staufen-1 and SIVmac Rev mutants had no significant effect on Shuttle reporter expression levels and unspecific Shuttle RNA export from the nucleus into the cytoplasm was not detected with Rev mutants. Also, no luciferase activity was detected when HIV-2 Rev, SIVmac Rev or mock DNA was transfected into the cells.

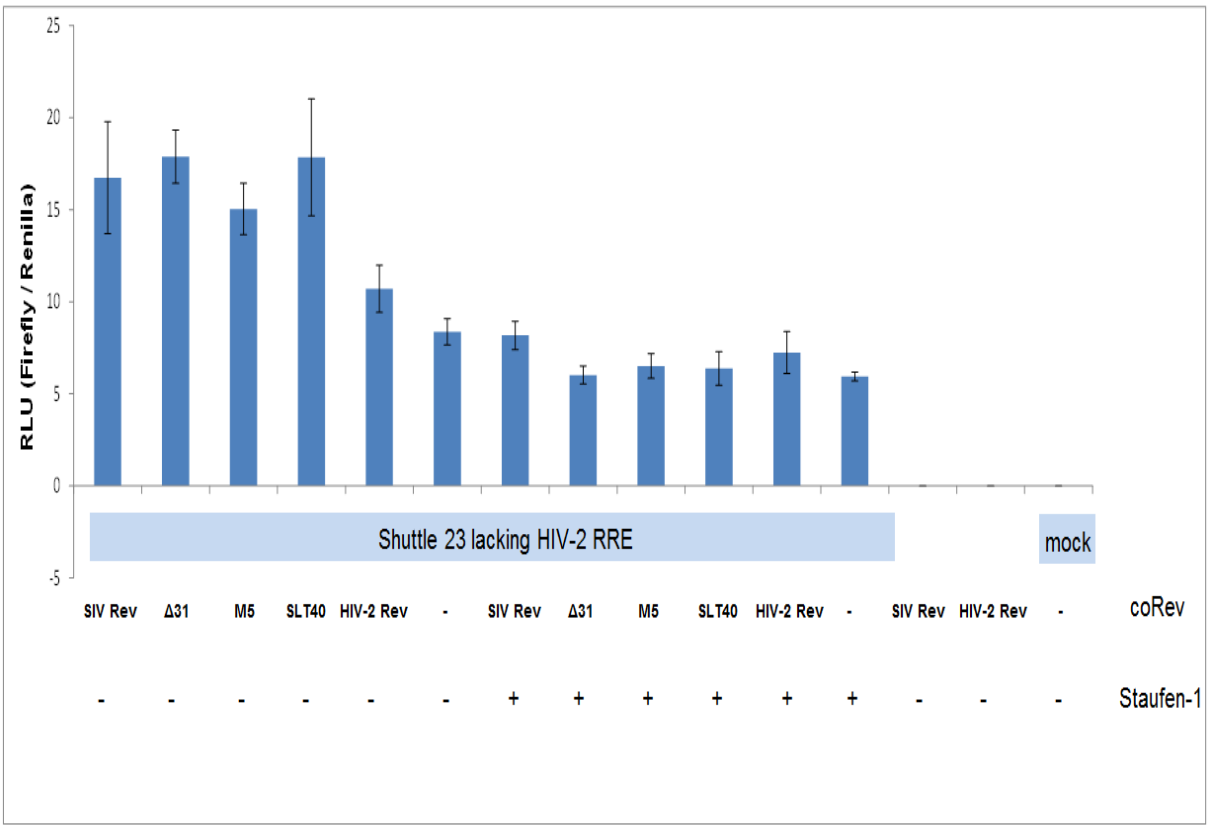


Figure 37: SIVmac-Rev dependent RNA transport in the absence of RRE. To assess non-specific RNA transport using the Shuttle construct lacking RRE, SIVmac Rev, its mutants or HIV-2 Rev plus the Shuttle construct were cotransfected into cells. + indicates coexpression of Staufen-1 in the cells. In addition, values were normalized to the activity of the cotransfected *Renilla* luciferase as an internal control. The experiment was repeated three times in HEK-293T cells. Data from one representative experiment done with eight replicates are displayed with standard error bars.

4.3.3.3 Staufen-1 and particular mutants have an adverse influence on mRNA transport in the presence of RRE

The objective of this experiment was to determine which of the Staufen-1 functional domains cause the inhibitory effect on Rev-dependent RNA transport. Therefore, the impact of Staufen-1 and its mutants (Staufen-1_F135A, Staufen-1_ΔRBD3, Staufen-1_ΔRBD4 or Staufen-1_ΔTBD/RBD5) on Rev based mRNA export was determined by adding equal quantities of Shuttle HIV-2 RRE, SIVmac Rev and Staufen-1 or its mutants to HEK-293T cells for transfection. Also, Shuttle HIV-2 RRE and Staufen-1 or its mutants were transfected into cells to examine the

effect of Staufen-1 and its mutants on Rev independent mRNA transport. As controls, cells were transfected with Shuttle HIV-2 RRE with and without SIVmac Rev. The values from the firefly luciferase assay were normalized by expressing *Renilla* luciferase DNA vector as an internal control in the cotransfected cells.

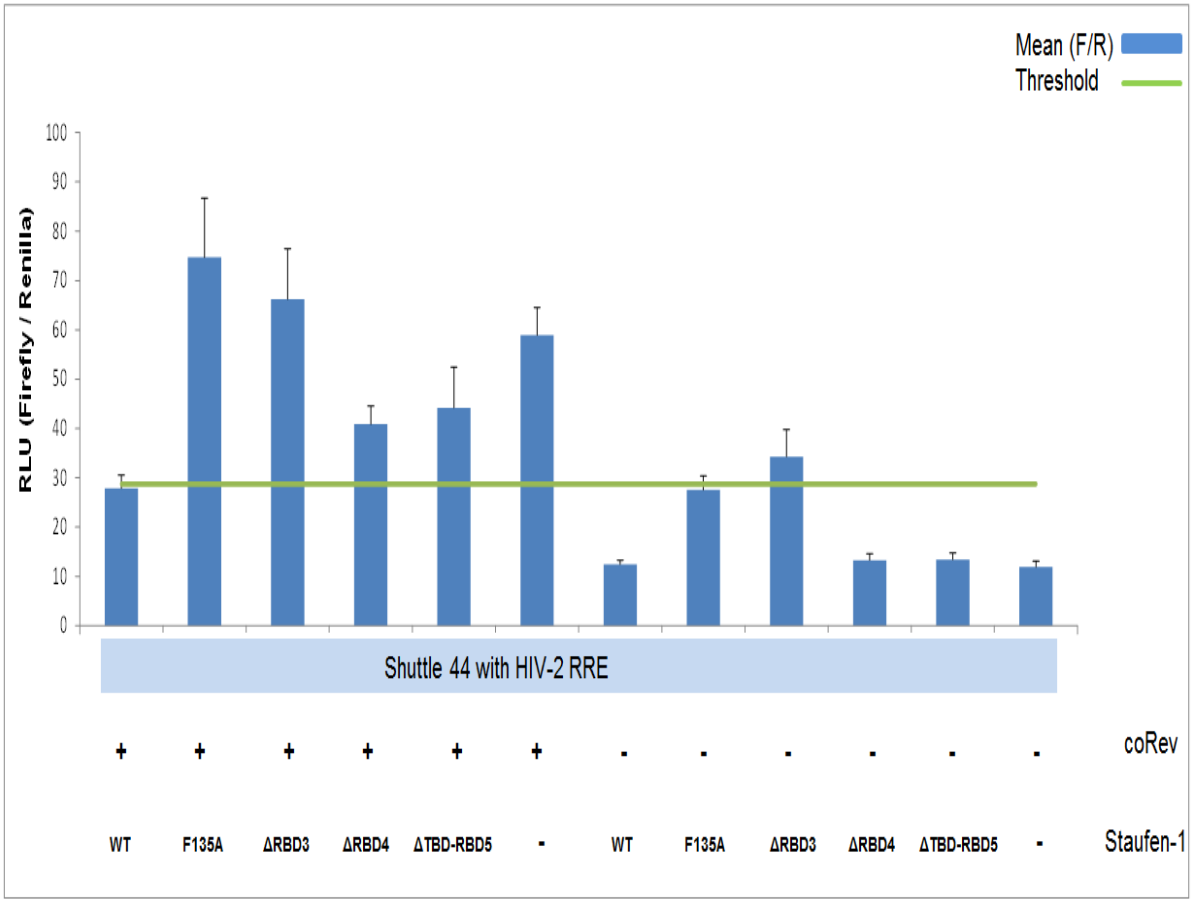


Figure 38: The effectiveness of Staufen-1 and its mutants on mRNA transport using RRE. Cells were cotransfected with Staufen-1 mutants, SIVmac Rev and Shuttle HIV-2 RRE either with or without SIVmac Rev; SIVmac Rev alone and Shuttle HIV-2 RRE were also transfected. The + sign indicates overexpression of Rev in the cells. Values were normalized to the activity of the expressed *Renilla* luciferase as an internal control. The green line refers to the threshold, i.e. the average luciferase activity associated with non-specific transport of the Shuttle construct. Results from one representative experiment performed in quadruplicate are shown with standard error bars.

SIVmac Rev in combination with Shuttle HIV-2 RRE was found to markedly enhance luciferase activity as depicted in Figure 38 compared to activity following

cotransfection of Rev and Staufen-1, which showed no RNA transport activity. Furthermore, luciferase activity was observed upon cotransfection of Rev and Staufen-1_F135A or Staufen-1_ΔRBD3 but was reduced after cotransfection with Rev and Staufen-1_ΔRBD4 or Staufen-1_ΔRBD5. Conversely, only overexpression of Staufen-1 and Shuttle HIV-2 RRE led to a clear reduction in luciferase activity, particularly the lowest promotion of RNA transport was seen after cotransfection of Staufen-1, Staufen-1_ΔRBD4 or Staufen-1_ΔTBD/RBD5. In addition, Shuttle HIV-2 RRE transfected alone resulted in decreased luciferase activity. Therefore, Staufen-1, Staufen-1_ΔRBD4 and ΔTBD/RBD5 reduced Rev-dependent RNA export, whereas Staufen-1_F135A and ΔRBD3 with a non-functional dsRBD3 domain increased the luciferase expression level.

In conclusion, Staufen-1 overexpression prevents Rev-mediated mRNAs transportation from the nucleus into the cytoplasm and the positive influence of Staufen-1 on the virus production is thereby independent of nucleocytoplasmic RNA shuttling.

4.3.3.4 Staufen-1 and its mutants have no impact on mRNA transport in the absence of RRE

To study the effect of Staufen-1 mutants (Staufen-1_F135A, Staufen-1_ΔRBD3, Staufen-1_ΔRBD4 or Staufen-1_ΔTBD/RBD5) on the non-specific RNA transport without using RRE, equal amounts of the Shuttle construct and Staufen-1 or each mutant were cotransfected into HEK-293T cells. Cells were also transfected with equal quantities of the Shuttle construct, SIVmac Rev and each Staufen-1 mutant and cells transfected either with Shuttle construct or with SIVmac Rev alone were examined.

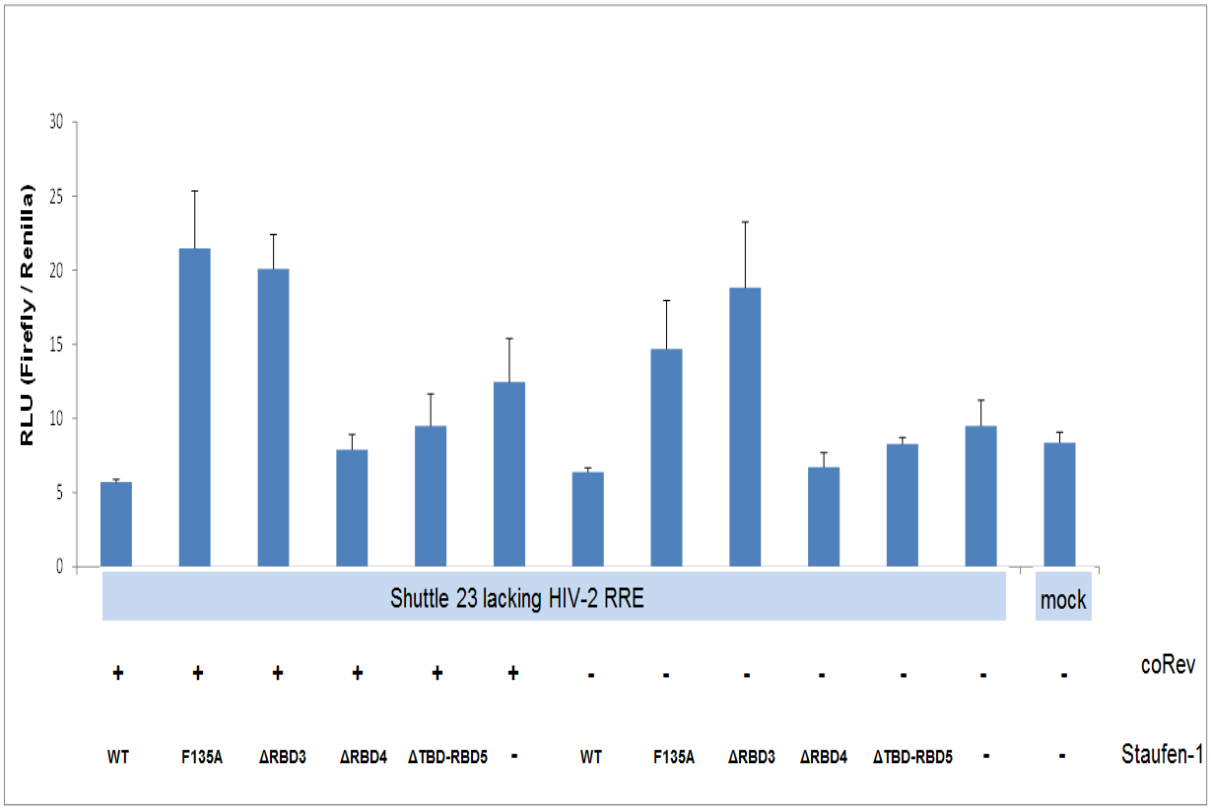


Figure 39: RNA export upon coexpression of Staufen-1 mutants and SIVmac Rev with the Shuttle construct lacking RRE. Cotransfection of cells was performed with the Shuttle construct and with either Staufen-1 mutants alone or with SIVmac Rev together with the Staufen-1 mutants. SIVmac Rev or the Shuttle construct transfected into cells were also included. Cotransfection of the cells with Staufen-1 mutants is indicated with +. Values were normalized to the activity of the expressed *Renilla* luciferase as an internal control. Results from one representative experiment performed in quadruplicate are shown with standard error bars.

Figure 39 demonstrates that cells coexpressing Staufen-1 mutants and SIVmac Rev showed only low levels of luciferase activity due to the lack of an RRE in the Shuttle construct. In addition, Staufen-1 or its mutants overexpressed alone with Shuttle construct resulted in lower levels of luciferase activity than those achieved by using Rev. Furthermore, overexpression of Rev with the Shuttle construct alone appears to slightly increase RNA transport. Finally, transfection of cells with Shuttle construct alone resulted in background luciferase activity.

Luciferase activity was therefore not associated with cotransfection of Staufen-1 mutants and Rev but was also influenced by the presence of an RRE, because

only background levels were seen after cotransfection of only Staufen-1 mutants and Rev.

4.4 Analysis of a Rec-deficient mutant of a reconstituted HERV-K (HML-2)

4.4.1 Generation of pBSK ori HERV-K113 ΔRec

It has been shown that HERV-K(HML-2) genes express an accessory protein, known as Rec which is functionally similar to the HIV-1 Rev and HTLV Rex proteins. The HERV-K113 molecular clone was generated by cloning the entire sequence coding for the reconstituted proteins into the pBSK plasmid vector [144]. This construct expresses Env, Rec and Gag-Pro-Pol. Production from the Gag protein and virus-like particles of the native HERV-K113 sequence in transfected cells is very low causing difficulties in detection due to mutations in the proviral DNA that occurred after insertion into the host's genome. For this purpose, the original envelope protein of HERV-K113 was reconstituted and expressed at high levels. After the insertional mutation, the amino acid sequence of HERV-K113 *gag-pro-pol* region was aligned with those of 10 well-preserved human specific HERV-K(HML-2) viruses [144]. The HERV-K(HML-2) Rec protein is required to transport introns containing partially spliced or unspliced viral mRNA transcripts from the nucleus into the cytoplasm for translation. Several investigators provide evidence that HERV-K(HML-2) Rec overexpression results in an increase in the quantity of the viral Gag protein [141], while overexpression of SIVmac Rev diminishes the viral particle production, similar to the results in the present study. The aim of this study was therefore to evaluate the effect of Rec mutation on the level of viral expression. pBSK ori HERV-K113 ΔRec was generated using the pBSK ori BCD vector which consists of pBSK ori HERV-K113 lacking an 5´ LTR ApaI/SalI fragment (composed of 1221 bp bordered by ApaI/SalI restriction sites). Mutations preventing Rec splicing were introduced into the pBSK ori BCD vector by deletion of nucleutides within the SD2 regions using multi-Site-directed mutagenesis. In the ori HERV-K113 sequence published by Chudak *et al.,* 2013 [147], nine nucleotides at position 6708-6716 (numbered according to the HERV-K113 sequence Genbank Acc. Number AY037928) were deleted without any inhibitory influence on the open reading frame of the *env* gene as indicated in Figure 40. To clone back the mutated Rec into the pBSK ori HERV-K113, the strategy

performed was based on PCR amplification of the mutated Rec sequence within pBSK ori BCD vector followed by another PCR assay in which the amplified fragment was used as primer, and pBSK ori HERV-K113 was employed as a template.

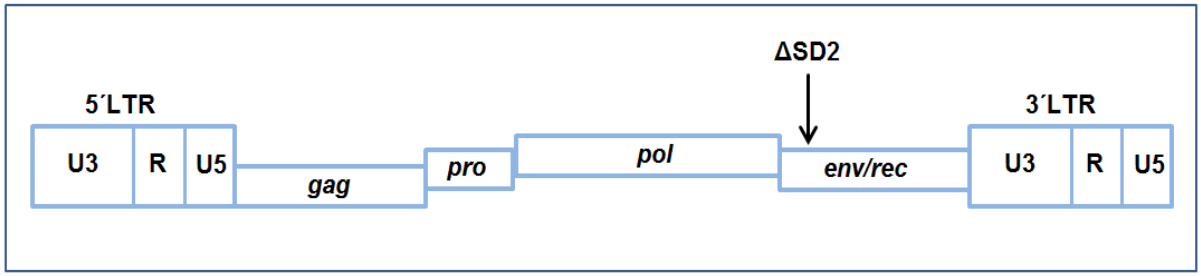


Figure 40: Location of *rec* mutagenesis in pBSK ori HERV-K113 DNA sequence. Site-directed mutagenesis was used to remove nine nucleotides (three amino acids) within the *rec* SD2 region of the pBSK ori BCD vector followed by ligation with a 5´LTR ApaI/SalI fragment using PCR for the formation of the pBSK ori HERV-K113 ΔRec molecular clone.

4.4.2 Deletion of Rec results in undetectable levels of viral Gag protein production

The objective of this experiment was to determine the effect on virus production of preventing pBSK ori HERV-K113 Rec expression. In addition, it could be investigated in this study and earlier work that cotransfection of Staufen-1 enhanced the HERV-K(HML-2) particle production by up to 30-fold [141] so it would be interesting to examine the impact of Staufen-1 modulation on the expression level of ori HERV-K113 ΔRec. For this, HEK-293T cells were cotransfected with 1 µg ori HERV-K113 ΔRec with or without 1.5 µg Staufen-1. Cells were also cotransfected with either 1 µg ori HERV-K113 ΔRec, 0.5 µg oricoRec or a combination together with different amounts of Staufen-1 (0.018 µg, 0.05 µg, 0.16 µg, 0.5 µg, 1.5 µg). In addition, ori HERV-K(HML-2) with or without Staufen-1 was transfected into cells as a control. Cells transfected with the pCDNA4-V5-His/B vector served as a negative control. The supernatants were harvested and cell lysates were prepared 48 h post transfection. The CA-antigen capture ELISA of the supernatants and cell lysates was used to measure the levels of viral Gag protein.

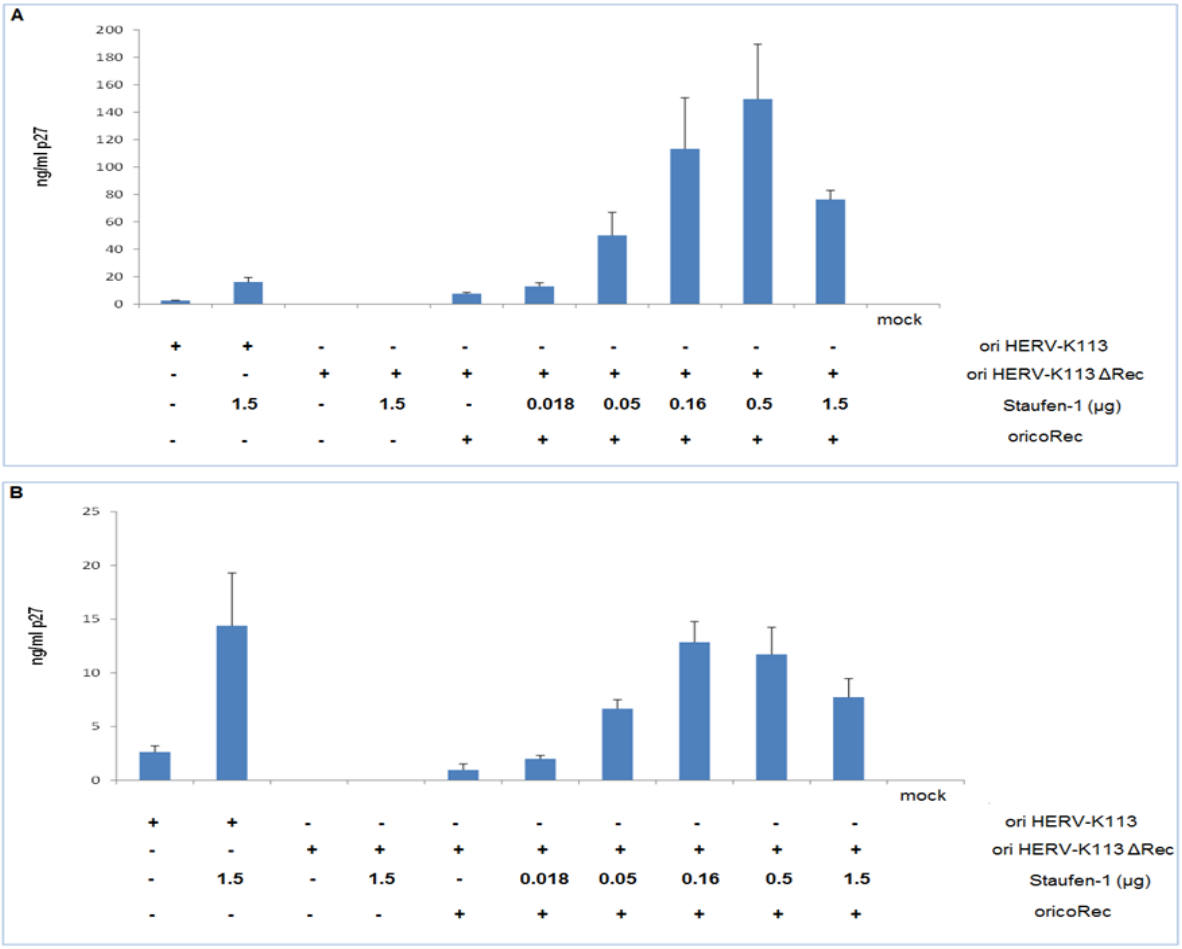


Figure 41: Evaluation of the yield of viral Gag protein after ori HERV-K(HML-2) Rec mutation. The ELISA demonstrated that in the supernatants (A) and cell lysates (B), viral Gag expression was inhibited by blocking Rec production, whereas viral Gag protein could be demonstrated in cells encoding Rec. In addition, level of the viral Gag protein was markedly increased when Staufen-1 was overexpressed. The experiment was repeated four times in HEK-293T cells. Results from one representative experiment carried out in triplicate are indicated with standard error bars.

Figure 41A shows that no viral Gag protein was detectable in the supernatants of cells transfected with ori HERV-K113 ΔRec alone and that Staufen-1 did not induce virus production in the absence of Rec expression. In contrast, viral Gag protein was clearly produced after cotransfection with oricoRec and Staufen-1 overexpression increased viral expression in the presence of Rec. It was observed that higher concentrations of Staufen-1 DNA transfected into cells led to increased production of new virions and the most effective Staufen-1 concentration was 0.5 µg

DNA which enhanced viral Gag expression an approximate factor of 19. Furthermore, cells transfected with ori HERV-K(HML-2) showed virus production that was significantly elevated by Staufen-1 overexpression. In addition, the concentration of viral Gag protein was elevated about 4-fold after cotransfection of 1.5 µg Staufen-1 and ori HERV-K113 ΔRec compared to that for HERV-K(HML-2) and Staufen-1 cotransfection. No virus production was detected by ELISA in the negative control cells.

Similar result was also obtained with the cell lysates as shown in Figure 41B. It has been shown that impairment of Rec expression is linked to inhibition of virus production. In contrast, lysates of cells transfected with ori HERV-K113 ΔRec and Rec produced viral Gag protein in particular the highest level was seen after cotransfection with 0.16 µg Staufen-1 DNA, a 13-fold increase compared to those not expressing ectopic Staufen-1. Moreover, cells transfected with oriHERV-K113 exhibited viral Gag protein production either with or without encoding Staufen-1. Finally, the level of viral Gag protein was increased by about 3-fold after coexpression of 0.166 µg Staufen-1 and ori HERV-K113 ΔRec compared to that for ori HERV-K113 and overexpressed Staufen-1.

In conclusion, inhibiting Rec expression resulted in undetectable levels of viral Gag protein in the supernatants and cell lysates, either in the presence or absence of exogenous Staufen-1. These data also show that an increase in the amount of exogenous Staufen-1 while expressing Rec resulted in higher levels of virus expression.

4.4.3 Effect of Rec titration on HERV-K(HML-2) particle production

The aim of this experiment was to study the influence of increasing the amounts of transfected oricoRec DNA on ori HERV-K113 ΔRec production. For this purpose, 1 µg ori HERV-K113 ΔRec, 0.5 µg Staufen-1 and different quantities of Rec (0.05 µg, 0.166 µg, 0.5 µg, 1.5 µg) were transfected into HEK-293T cells. Cells were also cotransfected with ori HERV-K113 ΔRec and Staufen-1. Cells transfected with mock DNA served as a negative control. The supernatants collected 48 h post transfection were subjected to CA-antigen capture ELISA to quantify the yield of viral Gag protein.

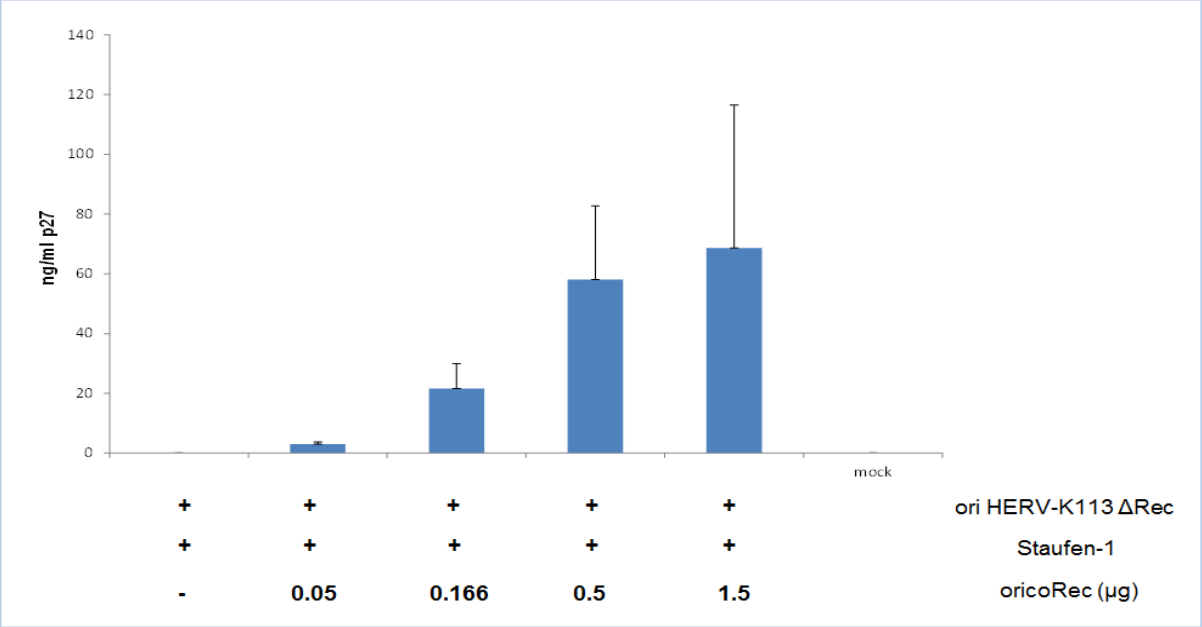


Figure 42: Viral Gag protein production after titration of Rec. The supernatants of cells cotransfected with ori HERV-K113 ΔRec, Staufen-1 and Rec at different ratios were subjected to ELISA analysis. The ELISA demonstrated that Rec expression resulted in remarkably higher levels of the viral particles. The experiment was repeated twice and data from one representative experiment performed in triplicate are shown with standard error bars.

Figure 42 shows that increasing the Rec DNA concentration transfected into cells led to increases in the level of viral expression with a maximum enhancement at 1.5 µg Rec DNA. In contrast, production of new virions was not observed in the supernatants of cells not encoding oricoRec.

Higher concentrations of Rec DNA transfected into the cells therefore have an increasing positive influence on ori HERV-K113 ΔRec production.

4.4.4 Viral Gag protein production in the absence of Rec after transfection with higher quantities of oriHERV-K113 ΔRec

It was previously shown, that ori HERV-K113 ΔRec with or without Staufen-1 produced undetectable levels of Gag protein (see Fig. 41). The viral genes may be expressed after Rec mutation, probably due to cellular mechanisms and/or by the endogenous Rec. Further investigations were therefore performed to evaluate the impact of mutations that prevent Rec splicing on the generation of new infectious viral

particles by increasing the number of transfected cells and the concentrations of oriHERV-K113 Δrec used for the cell transfection. In addition, the effect on the promotion of virus production of adding Rec *in trans* with or without Staufen-1 was analyzed. For this purpose, 80 µg ori HERV-K113 ΔRec/40 µg Rec either with/or without 40 µg ectopic Staufen-1 DNA were cotransfected into cells. Furthermore, 80 µg ori HERV-K113 ΔRec alone (lacking Rec production) was transfected into cells.

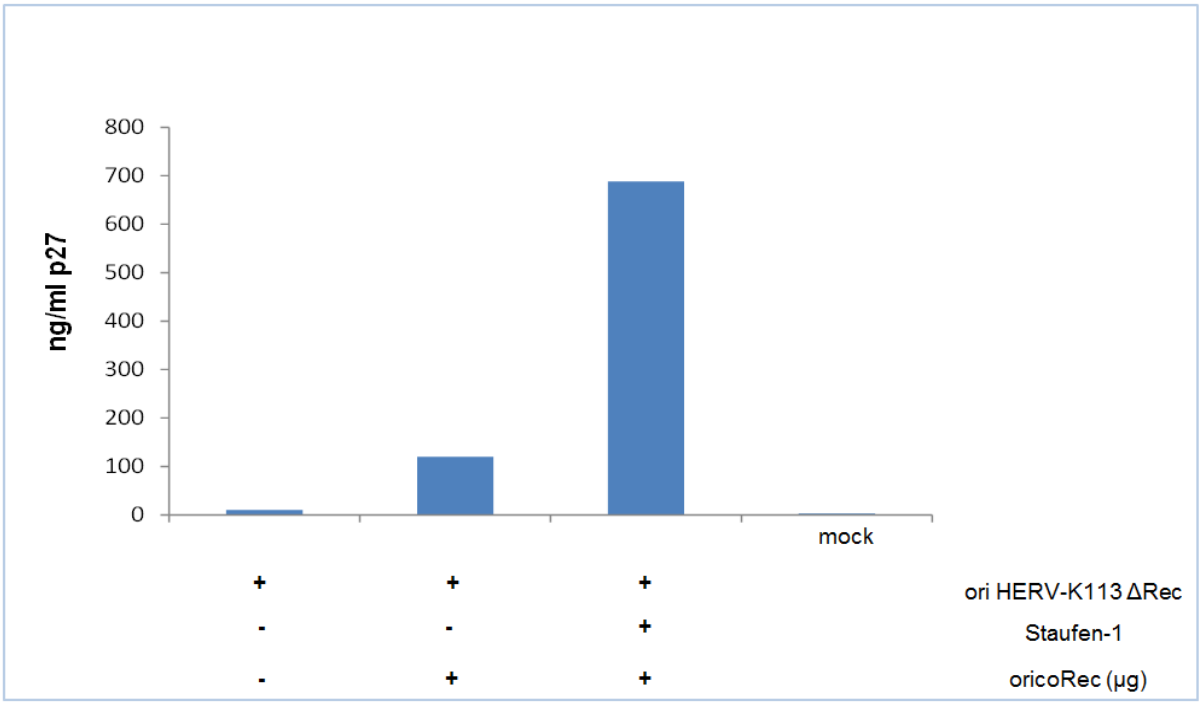


Figure 43: Viral Gag protein after transfection with a large amount of ori HERV-K113 ΔRec alone or ori HERV-K113 ΔRec/Rec/Staufen-1. ELISA of ultracentrifuged supernatants revealed that the Rec mutation significantly diminished the formation of the new virus compared to non-mutated Rec expression. Data of this experiment are shown without standard error bars.

It was shown in Figure 43 that expression of ori HERV-K113 ΔRec and of ori HERV-K113 ΔRec/Rec in HEK-293T cells resulted in remarkable differences in virion production. Substitution of Rec *in trans* resulted in an approximately 11-fold decrease in particle production in cells transfected with ori HERV-K113 ΔRec alone compared to that in cells cotransfected with ori HERV-K113 ΔRec and Rec. Furthermore, an increase of the yield of new virions was observed with the Rec-dependent enhancement of Staufen-1 overexpression by a factor of 6 compared to cells not expressing exogenous Staufen-1.

In conclusion, deletion of Rec nevertheless resulted in the detectable production of viral particles when using very large quantities of ori HERV-K113 ΔRec and increasing the number of the cells transfected. However, providing Rec *in trans* enhanced the level of viral Gag protein 11-fold. Moreover, Staufen-1 overexpression increased the yield of viral Gag in the concentrated supernatants of cells with ori HERV-K113 ΔRec and Rec by a factor of 6 when compared in cells not producing exogenous Staufen-1.

Further studies were performed to evaluate the influence of Rec deletion and residual expression of ori HERV-K113 ΔRec. The quantity of ori HERV-K113 ΔRec DNA cotransfected into the cells was elevated and high numbers of cells were used to aid in detecting very low levels of viral Gag protein. 80 µg ori HERV-K113 ΔRec DNAs were mixed with 40 µg pcDNA4-V5-His/B vector or with 40 µg exogenous Staufen-1 DNA. Negative control cells were transfected with the pcDNA4-V5-His/B vector. The p27 CA-antigen capture ELISA was used to quantify Gag p27 protein.

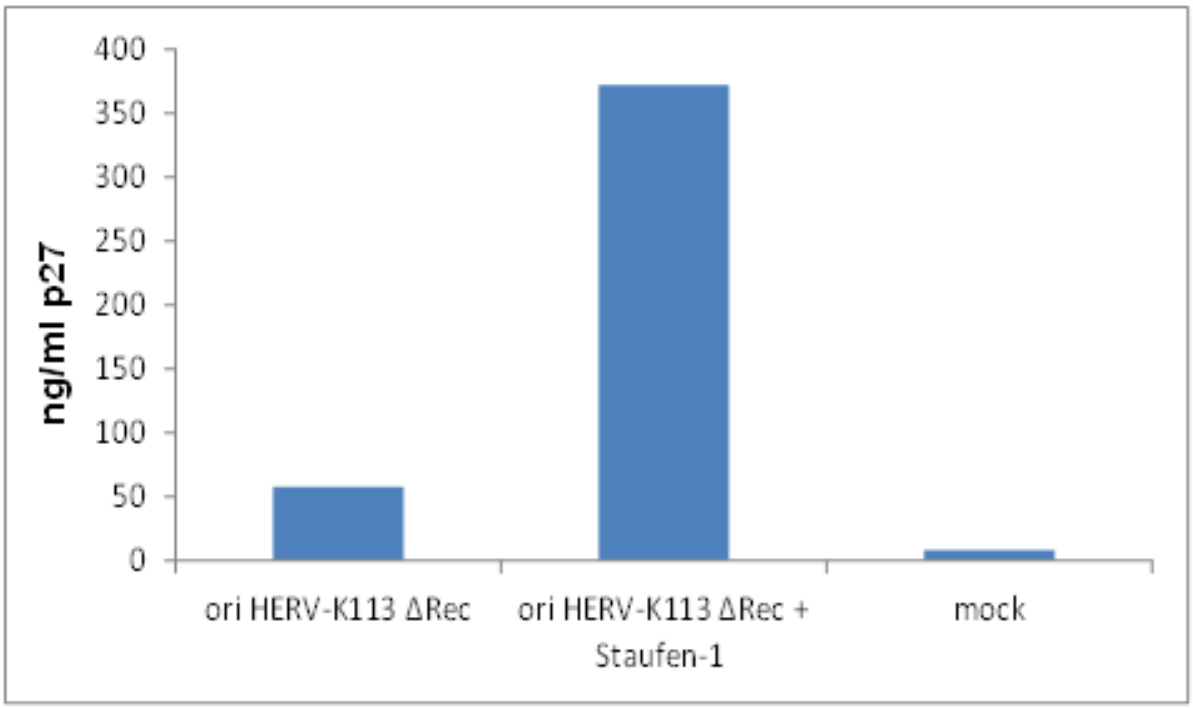


Figure 44: Analysis of the virus production after cotransfection with high quantities of ori HERV-K113 ΔRec and Staufen-1. The supernatants of cells cotransfected with high amounts of ori HERV-K113 ΔRec alone or in combination with Staufen-1 were analyzed by ELISA. Data of this experiment are presented without standard error bars.

In Figure 44, shows that higher amounts of transfected ori HERV-K113 ΔRec DNA clearly resulted in viral expression and that coexpression of Staufen-1 positively enhanced virus production by a factor of approximately six compared to cells not expressing Staufen-1. As expected, viral Gag protein was not detected in the supernatants of negative control cells.

In conclusion, using high quantities of cells, a weak expression of viral Gag protein derived from the ori HERV-K113 ΔRec molecular clone could be detected. Furthermore, cotransfection of Staufen-1 resulted in an approximately 6-fold enhancement of this expression, previously demonstrated.

4.4.5 HIV-1 Rev, SIVmac Rev, HTLV Rex, Np9 positively support the expression of ori HERV-K113 ΔRec whereas NP9 does not have a positive influence

It has been confirmed that HERV-K Rec, which transports unspliced viral mRNAs into the cytoplasm, is functionally similar to HIV-1 Rev, SIVmac Rev and, HTLV Rex. A previous study reported that the HERV-K RNA sequence is recognized by HIV-1 Rev. Also, a notable elevation in the level of HERV-W and HERV-K RNA during HIV-1 infection has been demonstrated [155] and a previous work proved that HIV Rev was able to interact functionally with HERV-K-RRE leading to nuclear RNA export [156]. Consequently, one of our goals in this study was to investigate the effect of expression of HIV-1 Rev, SIVmac Rev, HTLV Rex and Np9 on the formation of new oriHERV-K113 ΔRec particles. For this purpose, HEK-293T cells were cotransfected with ori HERV-K113 ΔRec alone or in combination with Rec, Np9, HIV-1 Rev, SIVmac Rev or HTLV Rex either with or without exogenous Staufen-1. Following incubation for 48 hours, the supernatants were harvested and centrifuged at 27410g and 4°C for three hours to pellet the virus. The supernatants were carefully discarded and the virus pellets resuspended in 40 µl PBS. The Cavidi RT Activity assay was then used to demonstrate that all proteins with the exception of Np9 caused a Staufen-1 mediated increase in viral RT activity (Figure 45).

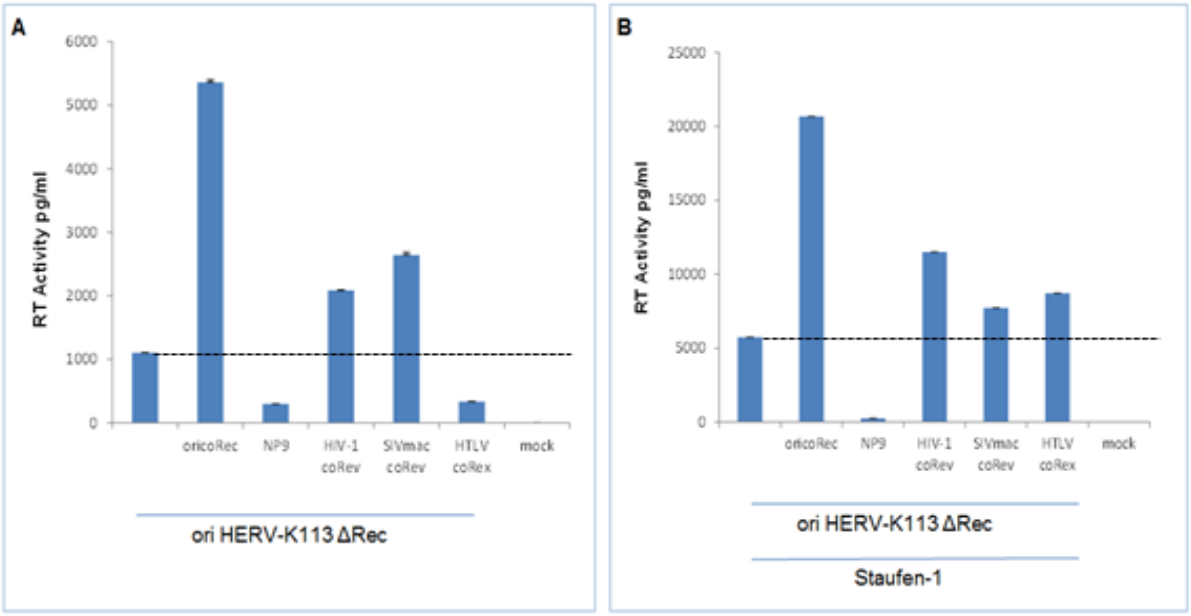


Figure 45: Rescue of particle production by a Rec-deficient HERV-K(HML-2) mutant by Rev and Rex. HEK-293T cells were transfected as indicated (A, without Staufen-1 overexpression, B, with Staufen-1). Particle production was assessed using a sensitive RT-Assay (Cavidi) two days post transfection. The experiment was repeated 6 times in HEK-293T cells. Data from one representative experiment performed in duplicate are displayed with standard error bars.

In the absence of the ectopic Staufen-1 (Figure 45A), cotransfection with Rec, HIV-1 Rev or SIVmac Rev appear to be associated with the highest viral RT activity, whereas expression of Rex and Np9 resulted in viral RT activity lower than that obtained when ori HERV-K113 ΔRec was transfected alone into the cells. Moreover, as shown in Figure 45B, Staufen-1 coexpression with ori HERV-K113 ΔRec alone or in combination with Rec, HIV-1 Rev, SIVmac Rev, or Rex positively contributed to the enhancement of the viral reverse transcriptase activation by factors of 5, 6.5, 5.5, 3, or 26 respectively. Conversely, expression of Np9 showed no a viral RT activity either with or without exogenous Staufen-1.

This study therefore indicates that HIV-1 Rev, SIVmac Rev, HTLV Rex are able to successfully bind to the RcRE of unspliced HERV-K mRNA transcripts leading to nucleocytoplasmic RNA shuttling and thereby significantly increased virion production. Rec coexpression resulted in the most profound enhancement while NP9 cannot functionally substitute Rec and did not facilitate nuclear RNA transportation.

5 Discussions

5.1 The influence of Staufen-1 on retroviral particle production

Human cellular Staufen-1 protein is considered as a double stranded RNA binding protein, first found in *Drosophila* [157]. It is composed of five functional sub domains dsRBD2 to dsRBD5 and a tubulin binding domain (TBD) found between dsRBD4 and dsRBD5 [123]. Mammalian cells have two Staufen homologs, Staufen-1 and Staufen-2, each with multiple isoforms [158]. The interaction between Staufen-1 and retroviruses (HIV-1, HIV-2, and HERV-K113) was studied in our group. The impact of Staufen-1 coexpression on SIVmac239 molecular clone was not evaluated therefore this was analyzed in the current work as well as further investigations regarding the relation between Staufen-1 and HERV-K113/HIV-1 were done. This study describes the aspects of human Staufen-1 in retroviruses pathogenesis. The dose dependent effectiveness of Staufen-1 overexpression on the expression levels of viral Gag protein could be demonstrated by cotransfection of HEK-293T cells with HIV-1 /SIVmac239/or HERV-K113 and Staufen-1 at different ratios. The most influent Staufen-1 concentration exhibited increase of the viral Gag protein yield in the supernatants and the cell lysates for HIV-1 by an average of 9-fold and 8.5-fold respectively. Also, Staufen-1 supported production of the viral SIVmac Gag protein in the supernatants and the cell lysates by a factor of 2.5-fold and 6-fold respectively. Furthermore, overexpression of Staufen-1 positively affected the expression level of HERV-K Gag protein in the supernatants by an average of 30-fold and in the cell lysates by a factor of 24-fold. The different impact of Staufen-1 overexpression on various retroviruses was shown earlier using different kinds of assays. A previous work published by Hanke *et al*. showed that the overexpressed Staufen-1 was involved in increasing the viral particle generation by a factor of 20 [141]. The positive influence of Staufen-1 on HIV-1 and SIVmac239 was the same probably attributed to the similarity of HIV-1 Rev to SIVmac Rev but this Staufen-1 effect was different from that on oriHERV-K113 probably because HERV-K Rec appears to be older than lentiviral Rev.

Previous studies were accomplished to assess the aspects of Staufen protein in the retroviruses propagation. One of these studies revealed that human Staufen-1 interacts with HIV-1 genomic RNA and thereby is involved in promoting the HIV-1

genomic RNA packaging into the new virions by a factor of 3 but it was shown that this process abrogates the virus infectivity due to incorporation of the human Staufen-1 in HIV-1 [128].In addition, Staufen-1 has a positive effect on encapsidation through its association to dimeric viral RNAs [139]. Previous reports demonstrated that overexpression or silencing Staufen-1 adversely affected the virus infectivity during the reverse transcription, the integration step or the efficiency of encapsidation [128] [140].

The retroviral 9-kb mRNA transcripts biosynthesized in the infected cells are unspliced mRNAs, partially spliced mRNAs 4-kb or completely spliced 2-kb mRNAs [159] [160] [161] [162]. The 9-kb mRNAs and singly spliced transcripts 4-kb are transported from the nucleus into the cytoplasm in case of HIV-1 by the viral Rev [163] [164] via interaction of Rev to *ci*s-acting RRE present within all 9-kb and 4-kb mRNAs leading to packaging of the full length viral genome and translation into viral proteins [165] [166]. Thus, in absence of Rev, the viral mRNAs are retained in the nucleus until subjecting to further splicing or degradation. Hence, one of our objectives in the current study was to evaluate the influence of Staufen-1 overexpression on SHIV-luci (a SIV/HIV hybrid virus) (SHIV) which is *re*v deficient because of the insertion of a reporter gene at the site of *en*v gene either HIV-1 coRev is expressed or not. It has been indicated that no viral Gag protein was detectable in the supernatants and the cell lysates of cells not encoding viral Rev but on the other hand, new viral Gag protein could be demonstrated in the supernatants and in the cell lysates when cells expressing HIV-1 coRev. Based on this finding, it has been confirmed in the present study that viral Rev protein is necessary for regulation of the retroviral gene expression.

In light of the positive Staufen-1 effect on the retroviruses production, support for these data is found in previous work done in our group that showed that the overexpressed Staufen-1 induced stronger generation of oriHERV-K113 and HIV-1 particles, respectively [142] [145]. Also, earlier studies indicated that consistent Staufen-1 dependent activation of HIV-1 production [128] [132] [167]. In addition, Staufen-1 enhancement of the retroviral expression level indicated in the present study was in agreement with the positive impact of Staufen-1 on production of HIV-2 particles shown by another Master's thesis [143] who displayed that Staufen-1 cotransfection into HEK-293T cells caused a significant elevation of the quantity of the viral Gag protein by a factor of 6 in the supernatants when compared with that in

cells not encoding the exogenous Staufen-1. The positive impact of Staufen proteins was emphasized by a certain study which suggested that overexpression of Staufen-2 led to increasing in the formation of new HIV-1 particles [149]. Therefore, Staufen-1 effect reflects its vital biological function in the retroviral life cycles. This effect caused by Staufen-1 might be attributed to enhancing Staufen-1 dependent nuclear export of the viral mRNAs for protein biosynthesis by binding to viral Rev/Rec/Rex proteins; also eliciting the translation rate of the viral proteins [128] [132] [141] [138]. This hypothesis was confirmed by previous data investigating that human Staufen-2 protein specifically interacts with HIV-1 Rev protein stimulating the viral unspliced or partially spliced mRNAs transportation form the nucleus to the cytoplasm, thereby mediating the propagation of HIV-1 as well as it has been examined that Staufen-2 expression level is higher in HIV-1 infected human T-lymphocyte and astrocyte cell lines, consequently this proves the importance of human Staufen proteins in the viral replication cycles [149].

Earlier work indicated that Staufen-1 protein is associated with mediating the mRNAs export from the nucleus into the cytoplasm for the efficient translation as well as Staufen-1 particles in previous publication is described as one of the components of the ribonucleoproteins that demonstrated 15-fold enrichment of mRNAs [168] [169]. Additionally, previous finding provided evidence that dmStaufen protein (expressed by *Drosophila melanogaster)* specifically attach to *oskar* mRNA of *Drosophila melanogaster* followed by localization in certain site for gene expression [170]. This was confirmed by another study which suggested by immunofluorescence that human Staufen is colocalized with the rough endoplasmic reticulum as well as human Staufen is linked to ribosomes and polysomes so it transport mRNAs into the translation sites [120].

Moreover, another trial reported that transport of HIV-1 RNA transcripts to ribosomes could be activated by Staufen protein followed by stimulating the viral mRNA binding to the translation initiation factor termed as eIF4E resulting in coding for the viral genes [129].

Also, other investigators suggested that *Drosophila* Staufen has amino acid sequences homology to those of human cellular protein sequence, TRBP [170]. This region can attach specifically to TAR containing viral RNA transcripts and thereby mediating the translation process and producing new virions [137]. In contrast, Staufen-1 did not positively affect the expression of the translated mRNAs lacking an

apparent structured 5' end, so the interaction of Staufen-1 with the 5' end of the mRNA transcripts is essential for their transport into ribosomes [122] and enhancement of the translation process as well as Staufen-1 blocks PKR binding site so no repression of the luciferase translation by activated PKR occurs [138]. Additionally, several proteomic [171] [172] [173] and cell biology [168] [169] [174] [175] [176] studies revealed that Staufen-1 plays a role in the mRNAs export. For examples, Staufen-1 might be involved in mRNA transport in neurons because RNAs were observed within Staufen-1 granules and move in dendrites on microtubules [168] [175]. This finding is supported by decreasing the concentrations of HIV-1 RNA in dendrites of neurons upon silencing of Staufen-1 [171]. On the nuclear level, it has been revealed that Staufen-1 is linked to telomerase and/or telomeric RNA in the nucleolus [177] [178]. In the cytoplasm, the function of Staufen-1 is to stabilize RNAs causing 5-fold elevation in the translation process for biosynthesis of proteins belong to the cell metabolism and/or cell growth. These functions are considered as complementary to its role in RNA transport and translation following localization [179][180]. These reports prove that Staufen-1 is a critical multifunctional protein; therefore, more studies are required for understanding mechanism of Staufen action and its function in synthesizing new infectious virions as well as to analyze the role of host cellular proteins in the replication of retroviruses.

Obviously, it has been proved in this study and another article that the expression level of HERV-K(HML-2) is less than that for HIV-1 [141], it could be found that Staufen-1 enhancement of the generation of HERV-K(HML-2) and HIV-1 particles in the supernatants was 30-fold and 9-fold respectively; also Hanke *et al.*, showed increase in the yield of HERV-K(HML-2) particles by a factor of 20 upon Staufen-1 overexpression [141] while attempts by other investigators revealed that Staufen-1 caused 3-4-fold enhancement for HIV-1 [128] [149]. Thus, based on these observations, one of our aims in this work was to evaluate the correlation between Staufen-1 enhancement and the viral expression level. This was performed by cotransfection of HEK-293T cells with constant concentration of Staufen-1 DNA and different amounts of full length HIV-1 DNA (about 2000 ng, 666.6 ng, 222.2 ng, 74 ng). The results described that lower quantity of HIV-1 DNA introduced into the cells for transfection led to higher Staufen-1 enhancement; consequently these data suggested that Staufen-1 effect is only slightly increased with decreasing in the quantities of the virus DNAs introduced into the cells for transfection. Probably the

viruses with lower expression levels are characterized with lower capabilities of using the cellular replication machines and subsequently this may support the important role of Staufen-1 in the virus propagation.

Furthermore, it would be interesting to study whether Staufen-1 and SIVmac239 are cotransfected on the same time or at different times alters the amounts of the viral Gag protein in the supernatants and the cell lysates. For this regard, HEK-293T cells were cotransfected with Staufen-1 and SIVmac239 on the same time or at various times (see Table 31). The result displayed that the largest amount of the viral Gag protein was detectable when both Staufen-1 and the virus were cotransfected on the same day while the lowest Gag protein quantity in the supernatants was observable when cells were cotransfected first with Staufen-1 then with the virus on the second day. From this finding, it has been assessed that the titer of the viral Gag protein appears to be directly associated with the time course of cell transfection with Staufen-1.

5.2 Effect of Staufen-1 mutants on the virus production

The essentiality of SIVmac251 Rev-Staufen-1 interaction was assessed in this study by using Staufen-1 mutants. Mutations were introduced into Staufen-1 DNA sequence by deletion of dsRBD3, dsRBD4, or dsRBD5/TBD known as ΔRBD3, ΔRBD4 or ΔTBD/RBD5 respectively as well as dsRBD3 domain was mutated and inactivated by single point mutation in dsRBD3 called Staufen-1-F135A which abrogated the double stranded RNA binding efficacy of Staufen-1 as previously reported in a certain work [132]. Examining which Staufen-1 domains mediate the viral Gag protein production was done by transfection of HEK-293T cells with either only SIVmac239 molecular clone or the virus and Staufen-1/or each Staufen-1 mutant. It was shown that generating Staufen-1-F135A and Staufen-1 ΔRBD3 mutants did not support the Staufen-1 dependent enhancement of the expression level of the viral proteins, and thereby the final virus yield, while deletion of dsRBD4 or dsRBD5/TBD domains within Staufen-1 had no a negative influence on Staufen-1 mediated production of SIVmac Gag protein. It was determined that dsRBD3 region is specifically bound to SIVmac251 coRev by CoIP in the present study and indicated by our group for HIV-1 and HIV-2 [142] [143]; therefore, inactivation of this domain inhibits the binding to viral Rev and thereby might reduce the Rev dependent mRNAs

export activity and the virus replication. The negative impact of some Staufen-1 mutations assessed in this study was in agreement with another work which explained that deletion or inactivation of dsRBD3 region strongly contributed to decreasing the concentration of the HIV-2 particles when compared to that displayed in presence of Staufen-1 or the other mutants [143]. Moreover, the significance of dsRBD3 region of Staufen-1 could be checked in a certain trial which confirmed that dsRBD3 facilitate the binding in the nucleus between TAR element of HIV-1 RNAs and Staufen-1 [128] [138]. Additionally, data of our result was consistent with another article which revealed that interaction of Staufen-1 with the nucleocapsid domain of $pr55^{Gag}$ was promoted via dsRBD3 domain leading to the virus assembly and production of new infectious virions [132]. In addition, other trials confirmed that dsRBD3 domain has the ability to strongly bind to RNAs and ribosomal RNA, while dsRBD4 demonstrated a weak RNA binding activity as well as it has been observed by Northern blot analysis that single point mutation of dsRBD3 (F135A) did not interact with RNAs [123] [128]. Furthermore, Laurent Chatel-Chaix and his Co-workers reported that Staufen-1 might be essential for HIV-1 assembly by binding via its dsRBD3 to the nucleocapsid region of Gag protein of HIV-1 resulting in Gag multimerization [130] [131]. Further attempt by another article suggesting that production of human Staufen-2 mutants impaired the direct Rev-Staufen binding and abolished Staufen-2 dependent induction of Rev transport activity; also diminished the titer of the new HIV-1 particles when compared to control cells transfected with HIV-1 alone [149]. These findings described that the importance of Staufen is not only elevating Rev induced viral mRNA transport activity but also Staufen plays an a critical role in the virus packaging. Interestingly, anti-retroviral chemical compounds could be designed based on preventing Staufen/Rev binding process.

5.3 Investigation of the interaction of SIVmac251 coRev with human Staufen-1 and its mutants.

As noticed in the present study, the interaction of SIVmac251 coRev with Staufen-1 and its mutants (Staufen-1, Staufen-1_F135A/ΔRBD3/ΔRBD4/ΔTBD/RBD5) was analyzed by CoIP assay. Because benzonase enzyme was used for degradation of RNA/DNA molecules; the binding process is independent of RNA. Our findings demonstrated that Staufen-1 and its

mutants was physically bound to SIVmac251 coRev exceptionally Staufen-1_F135A/ΔRBD3 mutants which showed lower binding efficiency due to lower expression level of these mutants observable by Western blotting when compared with the intensity of the Staufen-1 and other mutants signals. According to this result, it is speculated that dsRBD3 domain of Staufen-1 could be correlated with binding to SIVmac251 coRev. This data was agreed with results obtained in previous work which investigated that dsRBD3 region played a critical role in attaching Staufen-1 to either HIV-1 coRev or HIV-2 coRev [142] [143]. On the other hand, Hanke *et al.* reported that HERV-K113 Rec was successfully interacted via its dsRBD4 with Staufen-1 [141]. This was confirmed by numerous trials in which pull down binding assay revealed that HIV-1 Rev interacts with human Staufen-2 and that this physical binding up-regulates the Rev dependent RNA transcripts export activity through the nuclear membrane [149]; also, it was identified that dsRBD2 domain of Staufen mediates the posterior localization of the *oskar* mRNA of *Drosophila* oocyte, while actin-dependent *prospero* mRNA localization requires Staufen dsRBD5 [181].

5.4 Analysis of the effectiveness of coRev overexpression on the virus production

Rev is a protein forming multimers in vivo [182] or in vitro [118]. The viral Rev protein carries nuclear localization domain (NLS) and nuclear export domain (NES) which aid in nucleocytoplasmic shuttling. The nuclear import of Rev is activated by interaction of NLS with the cellular receptor importin receptor β [108] [183] [184] [185]. NLS bears several arginine residues known as an argenine rich motif (ARM) [108] which is required for enhancement of Rev binding to Rev responsive element (RRE) of unspliced or partially spliced viral mRNAs followed by RNAs shuttling into the cytoplasm [108] [150]. By contrast, Rev is exported into the cytoplasm via recognition of NES by the cellular receptor CRM1/exportin 1 [186] [187] [188].

It is intriguing in the present study to examine SIVmac Rev overexpression on the virus production. For this regard, HEK-293T cells were cotransfected with SIVmac239 and SIVmac coRev/or each coRev mutants Δ31 (deletion of the first loop), Δ59 (NLS-deficient), M5 (NLS-deficient), or SLT40 (multimerization domain-deficient) either with or without Staufen-1 overexpression. It has been found that cotransfection of SIVmac coRev or its mutants alone or in combination with

exogenous Staufen-1 negatively affected the synthesis of the virus progeny in the supernatants and the cell lysates. Moreover, in this study, coexpression of HIV-2 coRev and Staufen-1 diminished the titer of the viral Gag protein and inhibited the Staufen-1 enhancement. This result might be attributed to inactivation of the nuclear viral RNAs transport by coRev cotransfection, therefore viral Gag protein was not produced. Our report was in agreement with earlier study that indicated that interacting high quantity of Rev protein with exportin receptor CRM1 led to impairment of the cellular mRNA transcripts transportation [189]. Another finding in our group demonstrated that no increase in the yield of the HIV-2 particles after overexpression of HIV-2 coRev or coRev mutants [143]. But, the negative effect of coRev indicated in our study was not matched with data also obtained in our study which provided evidence that the overexpressed HERV-K113 oricoRec was concerned with increasing the quantity of the viral Gag protein when the influence of titeration of oricoRec on formation of ori HERV-K113 ΔRec particles was investigated. In contrast to the inhibitory effect of SIVmac/HIV-1 coRev on the virus production, report of another work confirmed that overexpression of HERV-K113 Rec clearly assisted in the virus propagation [141] [145].

5.5 Influence of Staufen-1 and SIVmac251 coRev on nuclear RNA export

During the retroviral infection, the viral genome produces viral mRNAs by transcription process. Afterwards, the viral mRNA transcripts are either completely, partially spliced or unspliced for biosynthesis of viral proteins to produce new virus particles [108]. The completely spliced viral mRNAs are exported into cytoplasm by the constitutive mRNA nuclear export pathway of the eukaryotic cells [190], whereas the partially spliced and the unspliced viral mRNAs localize in the nuclei of the infected cells until further splicing or degradation. Rev/Rec/Rex proteins bind to Rev/Rec/Rex responsive elements (RRE) present within the unspliced and partially spliced viral mRNAs then Rev binds to CRM1 leading to nuclear exit of the Rev bound mRNA transcripts into cytoplasm for the subsequent translation and formation of new virions [154] [191] [156] [192] [193]. These findings were completely agreed with results documented in the current study in which the cotransfected SIVmac251 coRev showed a remarkable increase in the nuclear export of HIV-2 RRE containing mRNA transcripts and thereby elevation of the expression level of the firefly

luciferase reporter gene in spite of inhibition of the viral Gag protein generation after SIVmac coRev overexpression. Also, to assess the influence of SIVmac coRev mutations on Rev based mRNAs transport, each separate coRev mutant (coRevΔ31, M5 or SLT40) was cotransfected with shuttle construct (with luciferase reporter gene and HIV-2 RRE) into HEK-293T cells. It was investigated that coRev-SLT40 (multimerization domain-deficient) contributed to elevating the nuclear RNA export into the cytoplasm; therefore inactivation of multimerization domain did not cause a negative effect on RNA transport exceptionally coRevΔ31 and M5 (NLS-deficient) that showed no RNA export activity. In another attempt, it was investigated that NLS domain activates the binding of Rev to RRE and mediates the Rev assembly into multimers as well [194] [195] [196]; therefore inactivation of this region by amino acids substitution might cause inhibition of nucleocytoplasmic shuttling. In our data, it has been found that inactivation of multimerization domain might not negatively affect RNA export induced by SLT40 mutant or probably multimerization region was not inhibited by two amino acids substitution. Additionally, our observation was matched with results described by Hoffmann and his Co-workers that suggested that oligomerization-defective Rev mutant was not important for formation of *trans*-activation competent Rev:RRE complexes and nuclear RNA transportation [197]. Moreover, this was affirmed by certain data obtained in our group which indicated that HIV-1 coRev or HIV-2 coRev overexpression elicited the nuclear RNA export and biosynthesis of luciferase enzyme; also it was described that HIV-2 coRev SLT40 mutant increased the luciferase activity similar to that induced by unmutated coRev, whereas NLS-deficient mutants abrogated the RNA export pathway [142] [143] [198]. It has been reported in earlier work that HIV Rev mutants displayed Rev based RNAs shuttling activities similar to unmutated Rev protein [153]. In addition, In contrast to these findings, it was observed that HIV-1 coRev mutants (M5, M10, SLT40) prevented the RNA transport exceptionally coRevΔ32 which exhibited lower RNA shuttle activity [142]. Also, it was displayed in the results presented in this study that no mRNA export activity and luciferase activity were detected upon overexpression of SIVmac coRev and shuttle construct lacking HIV-2 RRE so this confirms that presence of RRE is vital for Rev dependent RNA transport. The significance of viral Rev was further reported in earlier study in which inactivation of Rev´s leucine rich activation domain led to impairment of Rev mediated RNA transcripts shuttling [119]. Moreover, SIVmac coRev overexpression exhibited in this study higher RNA

transport activity that that induced by HIV-2 coRev because probably SIVmac coRev expression level is higher than that for HIV-2 coRev or maybe because the RNA transport activity or the efficiency of binding to RRE by SIVmac coRev is higher than that by HIV-2 coRev. Additionally, it has been reported in our study that Staufen-1 overexpression inactivates the activity of Rev dependent nuclear RNA transportation pathway. Furthermore, the effect of the overexpressed Staufen-1 mutants on RNA transport evaluated in our work displayed that Staufen-1, Staufen-1_ΔRBD4 and ΔTBD/RBD5 also decreased Rev mediated HIV-2 RRE bound RNA export, while Staufen-1_F135A and ΔRBD3 (inactivation or deletion of dsRBD3 domain respectively) supported higher Rev dependent RNA transport activity because probably these mutants are non-functional Staufen-1; also no luciferase activity was remarkably detected after overexpression of Staufen-1 mutants alone, therefore this finding proved the significance of SIVmac coRev in the RNA transportation pathway. The effect of Staufen-1 on Rev mediated RNA export could be assessed in previous work which proved that cotransfection with Staufen-1 had no influence on Rev based RNA transport for HIV-1 and HIV-2 [142] [143]. Based on that information, our hypothesis is that Staufen-1 may mediate the production of the lentiviral capsid particles by using other mechanisms apart from the nuclear RNA export pathway. For instances, Staufen-1 is associated with ribosomes and polysomes so it may induce the translation of mRNAs by enhancing attachment of the viral mRNA transcripts to a translation initiation factor called elF4E [129] [120] [122] as well as it was suggested by other studies that Staufen-1 might interact with nucleocapsid domain of HIV-1 Gag protein causing the virion packaging [130] [131].

In contrast, Hanke *et al*. provided evidence that the overexpressed Staufen-1 strongly promoted the Rec induced RNAs export in presence of RcRE from the nucleus into the cytoplasm [141]. Also, the same study demonstrated that the positive efficacy of Staufen-1 overexpression on the viral particle production is more than that caused by Staufen-1 in nucleocytoplasmic shuttling activity. Thus, there is additional Staufen-1 role, for example interaction of Staufen-1 with HIV-1/HERV-K113 Gag protein causing Gag multimerization [141] [130] [131]. In addition, earlier data by independent studies indicated that CRM1/Exportin-1 binds separately with both Rev and human Staufen-2 activating their RNA export activity [199] [200]. Therefore, it is speculated that viral Rev/Rec proteins and CRM1 may form export complexes which increase the efficiency of the viral mRNAs transportation or/and enhance the nuclear

transport of large viral RNAs [149]. Several previous publications demonstrated successful interaction of viral Rev proteins with some RNA binding host proteins such as human Rev-interacting protein (hRIP) and RNA helicase A (RHA), promoting Rev export activity [201] [202] [203]. Therefore, Rev-mediated RNA exit is required for virus replication, the interference with Rev activity [188] [204], for instance by blocking Rev's capability to self-associate on the RRE, may assist in the development of novel antiretroviral therapies.

5.6 Silencing endogenous Staufen-1

Staufen-1 overexpression was involved in inducing biosynthesis of new retroviral capsid proteins; therefore it was necessary furthermore to analyze the role of human Staufen-1 in the viral replication cycle by investigating the influence of knockdown of the endogenous Staufen-1 expression on generation of SIVmac Gag protein. Downregulation of Staufen-1 expression was carried out by using small interfering RNAs (siRNA) that inhibit the translation of the Staufen-1 mRNA. For this, HEK-293T cells cotransfected with the virus and Staufen-1 specific shRNA vectors (pLVTHM-shStauen_A7, pLVTHM-shStauen_A2C4, pLVTHM-shStauen_B3, pLVTHM-shStauen_C1, pLVTHM-shStauen_C3, and pLVTHM-shStaufen-3086) showed 1.5-fold drop in the amount of the viral Gag protein in the cell lysates when compared to the value obtained by nonspecific shRNA. Thus, this result proved that Staufen-1 silencing is associated with diminishing the yield of the SIVmac Gag protein probably due to either reducing Rev mediated RNA shuttling activity or decreasing the rate of the viral mRNA translation. This observation was matched with other studies which suggested that knockdown of Staufen-1 had a remarkable negative impact on generation of HIV-2 and HERV-K113 Gag proteins [143] [145]. Also, in regard to Staufen-1 silencing, another publication demonstrated that the quantity of RNA in neurons dendrites was decreased after reduction of Staufen-1 expression level [171]. Moreover, another attempt agreed with our finding indicated that silencing of Staufen-1 diminished influenza virus yield by 5-10-fold compared to that in control cells [205]. Conversely, it was indicated in earlier study that the downregulated Staufen-1 caused higher expression level of HIV-1 proteins [130]. In addition, depletion of Staufen-1 notably contributed to diminishing the cellular Gag gene expression [139].

5.7 Assessment of the localization of SIVmac coRev mutants and binding to Staufen-1

The virally encoded Rev trans-activator plays an essential role in the regulation of retroviral mRNA expression [151] [152]. Data involved in subcellular localization of HIV proteins (e.g. Rev) is of importance in assessing the viral pathogenesis and probably relevant for drug and vaccine development. HIV Rev protein is postranscriptional activator of retroviral gene expression. Rev protein is nucleocytoplasmic shuttle protein which is characterized by nuclear subcellular localization in several cell lines [153]. To determine SIVmac251 coRev domains required for Rev mediated nuclear RNA transport and interaction with Staufen-1, Rev mutations were generated either by N-terminal amino acids deletions called coRev Δ31, Δ59 or by Rev functional domain inactivation via amino acid substitution known as M5 (NLS-deficeint), SLT40 (multimerization-deficient). These mutants were produced depending on the high similarity between the amino acids sequences of HIV-1 Rev and SIVmac Rev as previously displayed. In the current study, HERV-K113 oricoRec, HIV-1 coRev and SIVmac251 coRev were expressed in transfected HEK-293T cells. The expressed proteins were examined by immunofluorescent antibodies using Confocal Laser Scanning Microscope (cLSM) and were mainly localized in the cytoplasm in spite of expectations for detection of these proteins in the nucleus and in the cytoplasm due to presence of NLS and NES domains. A previous trial revealed that HIV-1 protein could be expressed and mainly found in the cytoplasm [206] as observed in our result but oricoRec were detected in the cytoplasm and the nucleus so this was in agreement with the result obtained in a Diploma thesis in our group in which it was found that the expressed oricoRec localize in the cytoplasm and the nucleus [145]. Other trials observed localization of HIV-1 coRev and HIV-2 coRev in the nucleus and the cytoplasm [96] [119] [207]. Additionally, all SIVmac251 coRev mutants generated in this study were expressed in transfected cells and detected by cLSM in the cytoplasm especially SIVmac Δ59 and M5 mutants which are possessing nonfunctional nuclear localization signals (NLS) due to deletion or inactivation of this domain respectively; thus, SIVmac coRev mutations did not affect the expression capability of coRev. In regard to localization of coRev mutants, HIV-1 coRev mutants and HIV-2 coRev mutants were also expressed and displayed in our group by cLSM [142] [143].

To check the functionality of SIVmac251 coRev after mutations and to examine which functional domains within SIVmac coRev stimulate the interaction of coRev with Staufen-1, CoIP and Western blotting assays were performed by cotransfection of HEK-293T cells with Staufen-1 and SIVmac251 coRev or one of its mutants (coRevΔ31, Δ59, M5 or SLT40). CoIP demonstrated that SIVmac251 coRev and its mutant called SLT40 (multimerization-deficient) were successfully bound to human Staufen-1, whereas the other mutants failed to interact with Staufen-1. Information reported by Hanke *et al*. proved by CoIP assay that HERV-K113 oricoRec was physically bound to human Staufen-1 [141]. Our data obtained in the present study described that Rev-Staufen-1 interaction process might not be mediated by the inactivated multimerization domain (SLT40); also disability of coRevΔ31 (deletion of the first loop), Δ59 and M5 lacking functional NLS to interact with Staufen-1 by CoIP might prove the influential role of the first loop and NLS domain in binding to Staufen-1. Interestingly, SIVmac coRev SLT40 mutant could be associated with enhancement of the highest RNA transportation activity and inhibitory effect on SIVmac239 production when compared to SIVmac coRev and the other coRev mutants. This data was confirmed by a certain study which provided evidence that nonfunctional NLS of HIV-1 coRev inhibited the interaction of coRev with Staufen-1 [142]. From these findings, determination of the viral Rev domains enhancing the attachment to Staufen-1 may aid in designing effective antiretroviral drugs.

5.8 Rec mutant analysis

Expression of HERV-K Rec protein is essential for the export of intron containing partially spliced or unspliced viral mRNAs from the nucleus into the cytoplasm for protein biosynthesis. Rec protein has several functional regions; Nuclear localization domain (NLS), Nuclear export signal (NES) and multimerization domains [82] [192] [81] [208]. Rec protein binds via its NES to Crm1 export protein forming complex which interacts with the unspliced and partially spliced viral mRNAs and transported from the nucleus into the cytoplasm for translation, then Rec enters back to the nucleus by its NLS. Also, it was found that the Rec protein was expressed and visualized in the nucleus by using immunofluorescence [145] [209] [210].

By many investigators, it was shown that overexpression of Rec could be involved in increase in HERV-K expression level [141] [211]. Thus, in the present study, analysis of the impact of Rec mutation on the virus particle production could be targeted; also the influence of Staufen-1 overexpression on ori HERV-K113 ΔRec expression level could be evaluated. For this purpose, mutation via nine nucleotides deletion was introduced into splice donor 2 (SD2) region to inhibit the expression of Rec. In our results, it could be indicated that Rec deficient mutant showed no detectable viral Gag protein or viral RT activity after transfection of the cells with 1 µg ori HERV-K113 ΔRec either with or without encoding for exogenous Staufen-1. On the other hand, expression *in trans* of oricoRec mediated the viral expression level through induction of the viral RT activity and the biosynthesis of the viral Gag protein; also Staufen-1 cotransfection led to higher RT activity and viral particle production. Furthermore, in the current study, cotransfection of ori HERV-K113 ΔRec and different quantities of oricoRec DNA has exhibited notably higher viral Gag protein level with increase in the amounts of oricoRec DNA introduced into the cells for the transfection. Therefore, our data revealed the significant role of Rec expression in the viral particle production. Our finding was agreed with another attempt that proved that overexpression of Rec could efficiently enhance the production of the viral particles upon cotransfection of Rec and HERV-K Bogota (lacking *po*l, most of the *en*v gene, and the *re*c gene); also it has been shown that viral like particles (VLPs) were produced and infectious when HERV-K Rec and unenveloped HERV-K virus were cotransfected into the cells [71] [211] [212].

Moreover, in this study, it was reported that viral Gag protein could be detected after increasing the concentration of ori HERV-K113 ΔRec transfected into the cells (80 µg) without encoding for oricoRec; this protein level was increased by Staufen-1 overexpression by 6-fold. On the other hand, cotransfection of 80 µg ori HERV-K113 ΔRec in the presence of oricoRec displayed higher viral Gag protein production by a factor of 11 than that in the absence of oricoRec; also Rec mediated Staufen-1 enhancement efficiently supported the expression of the viral proteins by a factor of 6. Thus, Rec mutation negatively affected the virus propagation; consequently, the previous results could prove that deletion of Rec has a significant impact on the Staufen-1 mediated enhancement of particle production. Nevertheless, the impact is not completely abrogated by the lack of Rec indicating an additional, Rec-independent mechanism of HERV-K(HML-2) enhancement by Staufen-1. Other

studies consistent with our conclusion investigated that although most complex retroviruses encode Rev-like proteins, simple retroviruses lacking a Rev depend on the direct recruitment of a host nuclear RNA export factor to the target cis-acting RNA known as a constitutive transport element (CTE) [213]. This factor has been identified as the host protein Tap in the case of the Mason–Pfizer monkey virus (MPMV) CTE [214] [215]. Importantly, CTEyTap dependent nuclear RNA transport is independent of Crm1 function [215] [216] [217].

Interestingly, it has been identified that HERV-K Rec is mechanistically analogous to HERV-K Np9 (involved in the malignancies) [80], HIV-1 Rev, SIVmac Rev, HTLV Rex. Like all these proteins, HERV-K-Rec interacts with both the Crm1 nuclear export factor and with a cis-acting viral RNA target to induce nuclear transportation of unspliced RNAs into the cytoplasm for translation. In a previous study, it was examined that HERV-K RNA sequence is recognized by HIV-1 Rev [156]. These data provide important evidence for an evolutionary link between HIV-1 and endogenous retroviruses that first was integrated into the human genome 30 million years ago [156]. Based on these data, one of our goals in the present study was to assess the influence of expression of HIV-1 coRev, SIVmac251 coRev, HTLV coRex and Np9 on the expression level of ori HERV-K113 ΔRec without expressing oricoRec. By using Cavidi assay, cotransfection of HEK-293T cells with ori HERV-K113 ΔRec alone or in combination with oricoRec, HIV-1 coRev, or SIVmac251 coRev has shown detectable viral RT activity, while expression of Rex and Np9 was associated with viral RT activity lower than observed by cotransfection of ori HERV-K113 ΔRec alone. Although HERV-K Np9 protein as alternative of Rec protein belongs to provirus type II [80], nonetheless did not show viral reverse transcriptase activity. Additionally, this study revealed that coexpression of Staufen-1 with ori HERV-K113 ΔRec alone or in combination with Rec, HIV-1 Rev, SIVmac Rev, or Rex increased the viral reverse transcriptase activity. In contrast, Np9 did not demonstrate a viral RT activation either with or without Staufen-1 overexpression.

According to these findings in this experiment, it is reported that lentiviral Rev and Rex were capable of interacting with HERV-K RcRE and exporting the unspliced mRNAs into the cytoplasm for biosynthesis of the viral proteins demonstrating that HERV-K Rec is biologically similar to HIV-1 Rev, SIVmac Rev and Rex. This experiment therefore has significant implications for assessment of the evolution of present day exogenous retroviruses.

In agreement with our data, a certain attempt has shown that HIV Rev could be able to bind functionally to HERV-K RcRE causing the nucleocytoplasmic RNA shuttling; this confirms the similarity between HERV-K Rec and the lentiviral Rev proteins but the reverse was not done because HERV-K Rec cannot be bound to HIV-1 RRE [156]; also there is report indicated that transmissible units of HERV-K particles containing Bogota transcripts could be induced by overexpression of HIV-1 protein in the presence or absence of HERV-K Rec [211]. Additionally, a previous article proved that HIV-1 Rev stimulated the exit of the unspliced RNA transcripts of HIV-2 and SIVmac [110]. Moreover, Rimsky *et al*. reported that HTLV-1 Rex could function instead of HIV-1 protein [218] as well as it could be demonstrated that a remarkable increase in HERV-W and HERV-K RNA in HIV-1 infected patients [155]. Expression *in trans* of HIV-1 accessory proteins Tat and Vif mediated the production of HERV-K Bogota particles as shown in a certain study [211]. The HIV-1 Tat protein likely stimulates expression of HERV-K viruses via activation of the binding of the transcription factors NF-κB and NFAT to the HERV-K LTR [219] [220], whereas HIV-1 protein Vif participates in the transmissibility of HERV-K virus by inhibition of cytidine deaminases of the APOBEC family; these proteins cause mutations in the viral genome during reverse transcription [221] [222] [223]. These observations are also matched with those from other groups, who have demonstrated increased expression of HERV-K viruses and their structural proteins during HIV-1 infection [156] [224] [225] [226] [227].

6 Summary

Staufen-1 is a cellular protein involved in subcellular RNA transport and the translation and packaging of retroviral RNA into viral particles. In this study, several aspects of the impact of Staufen-1 on SIVmac, HIV-1 and HERV-K(HML-2) particle production were investigated.

The results obtained show that, similar to the situation with HIV-1 and HERV-K(HML-2), overexpression of Staufen-1 also enhances SIVmac particle production. However, the 3-4-fold increase in SIVmac particle production is slightly (2x) lower than that seen with HIV-1 and significantly lower than the 20-30-fold boost of HERV-K(HML-2) production. This enhancement is not dependent on the general level of expression but appears to be an intrinsic property of the virus. As expected, downregulation of endogenous Staufen-1 expression using Staufen-1 specific shRNAs negatively affected SIVmac particle production. On the other hand, an excessive overexpression of Staufen-1 decreases HIV-1, but not HERV-K(HML-2) or SIVmac particle production, indicating that the optimal levels of cellular Staufen-1 are different for these viruses. Using luciferase reporter viruses, differences between the genera were also found with regard to the effect of Staufen-1 on RNA shuttling and translation. Whereas Staufen-1 overexpression enhances HERV-K(HML-2) production at these stages of replication, the opposite has been observed for the two lentiviruses. The positive net effect of Staufen-1 overexpression on HIV-1 and SIVmac particle production therefore appears to result primarily from improved packaging and assembly while, for HERV-K(HML-2), RNA transport and translation are also enhanced. Furthermore, similar to the situation with HIV-1 Rev, Staufen-1 interacts directly with SIVmac Rev, with the Staufen-1 RBD3 domain being involved in this binding.

HERV-K(HML-2) Rec has a significant impact on the Staufen-1 mediated enhancement of particle production. Nevertheless, the fact that this impact is not completely abrogated by the lack of Rec indicates the existence of an additional, Rec-independent mechanism of HERV-K(HML-2) enhancement by Staufen-1. In addition, overexpression of Rec promotes HERV-K(HML-2) production.

Finally, the study confirms previous reports that show a strong boost in particle production by Staufen-1 overexpression and the functional substitution of Rec by

HIV-1 Rev, SIVmac Rev and HTLV Rex proteins. In contrast Np9, the protein produced by type I HERV-K(HML-2), cannot functionally substitute Rec.

7 References

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8. Appendix

8.1 Abbreviations

Abbreviations	Long form
®	Registered Trademark
°C	Degree celcius
AIDS	Acquired Immunodeficiency Syndromes
ALS	Amyotrophic Lateral Sclerosis
AP	alkaline phosphatase
APS	Ammonium persulfate
ARM	Arginine-Rich Motif
ATLL	Adult T-cell Leukemia/Lymphoma
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscope
CoIP	Co-Immuno-Precipitation
DAPI	4’,6-Diamidin-2’ phenylindoldihydrochlorid
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERVs	Endogenous Retroviruses
FCS	Fetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gp	glycoprotein
HAART	Highly Active Anti-retroviral Therapy
HBS	HEPES Buffer Saline
HEK	Human Embryonic Kidney
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HERV	Human Endogenous Retrovirus
HIV	Human Immunodeficiency Virus
HML	Human MMTV-like
HRP	Horseradish Peroxidase
HTLV	Human T-cell Lymphotropic Virus
ICTV	International Committee for Taxonomy on Viruses
IgG	Immunoglobulin G
Kb	Kilo bases
kDa	Kilodalton
LB	Luria-Bertani

LTR	Long Terminal Repeat
min	Minute
ml	Milliliter
mm	millimeter
MMTV	Mouse Mammary Tumor Virus
MP	Matrix Protein
mRNA	messenger RNA
NC	Nucleocapsid
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
nm	Nanometer
nM	Nanomole
OPD	o-Phenylenediamine dihydrochloride
ori	Original
OWMs	Old World Monkeys
PBS	Phosphate Buffured Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PIC	Pre-Integration Complex
PM	Powdery Milk
PMSF	Phenylmethanesulfonylfluoride
PR	Protease
PVDF	Polyvinylidene flouride
RA	Rheumatoid Arthritis
RBD	RNA Binding Domain
RER	Rough Endoplasmic Reticulum
Rev	Regulator of Expression of Virions
RISC	RNA Interference Specificity Complex
RLU	Relative Light Unit
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RRE	Rev/Rec Responsive Element
RSV	Rous Sarcoma Virus
RT	Reverse transcriptase
RT	Room Temperature
SA	Splice Acceptor
SD	Splice Donor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHIV	Simian/Human Immunodeficiency Virus
SiRNA	Short Interfering RNA
SIV	Simian Immunodeficiency Virus
SIVmac	Simian immunodeficiency virus of Rhesus Macaques
SLE	Systemic Lupus Erythematosus
SU	Surface Unit
TAE	Tris-Acetat-EDTA-Buffer
Taq	*Thermophilus aquaticus*
TAR	Trans-Acting Responsive

TBD	Tubulin Binding Domain
TEMED	Tetramethylethylenediamine
TM	Trans-membrane
tRNA	Transfer-Ribonucleic acid
UV	Ultraviolet
V	Volt
VLPs	Virus-Like Particles
VSV	Vesicular Stomatitis Virus
VSV	RNA Interference Specificity Complex

8.2 Publications and conference participations

Publications

Oliver Hohn, Kirsten Hanke, Veronika Lausch, Anja Zimmermann, **Saeed Mostafa** and Norbert Bannert. **CMV-Promoter Driven Codon-Optimized Expression Alters the Assembly Type and Morphology of a Reconstituted HERV-K(HML-2)**. *Viruses* **2014**, *6*(11), 4332-4345.

Oliver Hohn, **Saeed Mostafa,** Stephen Norley and Norbert Bannert. **Development of an antigen-capture ELISA for the detection of the p27-CA protein of HERV-K(HML-2).** *Journal of Virological Methods* (accepted).

Conference Poster presentation

Oliver Hohn, Laura Waldmann, **Saeed Mostafa**, Stephen Norley and Norbert Bannert. **Development of a HERV-K(HML-2) Antigen-Capture ELISA for the Investigation of HERV/HIV-1 Interactions.** 25th Annual Meeting of the Society for Virology (GfV) in Bochum, Germany March, 2015.

Saeed Mostafa, Oliver Hohn, Steve Norley, Kirsten Hanke and Norbert Bannert. **Analysis of a Rec-deficient mutant of a reconstituted HERV-K(HML-2)**. 26th Annual Meeting of the Society for Virology (GfV) in Münster, Germany April, 2016.

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8.4 Declaration

The experimental work of this thesis was achieved at the Robert Koch Institute, Centre of HIV and other Retroviruses, Berlin in the period from October 2013 until March 2016

I hereby declare that this thesis has been composed by myself and the work of which it is a record has been done by myself. It has not been previously submitted for any degree at this or any other university.

Berlin, April 2016

Saeed Mostafa