Impact of human Staufen-1 on the particle production and infectivity of HIV-2

Masterarbeit

zur Erlangung des Hochschulgrades *Master of Science* (*M.Sc.*)

im Masterstudiengang Biotechnologie

vorgelegt von

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am 18.02.2015

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The current dissertation was performed at the Robert Koch-Institute Berlin in the Division 18 HIV and Other Retroviruses.

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Abstract

According to the World Health Organisation, there are 35 million people currently infected with HIV worldwide. Untreated infection with the virus leads to a decline of the $CD4^+$ cell number and weakening of the immune system, resulting in the development of AIDS (acquired immunodeficiency syndrome). This enables opportunistic infections to arise, which may end in death of the infected person. HIV infections are mostly caused by HIV-1, whereas distribution of HIV-2 is more restricted. Due to the fact that viruses are not able to replicate themselves, they are reliant on the host cell and interact with various cellular factors. The human RNA-binding protein Staufen-1 has previously been reported to interact with various proteins of the retroviruses HIV-1, HTLV-1 and HERV-K, affecting production and infectivity of viral particles. Export of viral RNAs, translation of viral proteins as well as assembly of new virions have been named as potential spots for mediating those effects.

Within this thesis, the aim was to investigate the eventual interaction of Staufen-1 and HIV-2, gaining more insights into the impact of Staufen-1 on the retroviral replication cycle. It could be demonstrated that Staufen-1 enhances HIV-2 particle production dose-dependently, while simultaneously impairing the infectious potential of emerging virions. The dsRBD3 domain of Staufen-1 could be determined as the potential domain mediating this effect. Furthermore, downregulation of the expression of endogenous Staufen-1 resulted in impaired particle production. By creating a reporter construct carrying the respective Rev-responsive element (RRE), the Rev/RRE-dependent transport of unspliced RNAs could be investigated, on which Staufen-1 had no influence. As the Rec protein was found to be a major Staufen-1 interacting partner for HERV, the interplay of HIV-2 Rev and Staufen-1 was analyzed more in detail. By means of fluorescence microscopy as well as the generated reporter construct, the function of several HIV-2 Rev mutants was examined, a protein involved in export of unspliced viral RNAs out of the nucleus. In this way, potential

functional domains of HIV-2 Rev were investigated, which have not been defined yet.

Taken together, an impact of Staufen-1 on HIV-2 could be demonstrated for the first time, resulting in outcomes similar to the ones that have been reported for other retroviruses. It remains to be elucidated at which stage of replication the interaction takes place, whether other proteins are involved and by which means the impact of Staufen-1 is important for the virus and the host cell.

Zusammenfassung

Derzeit sind laut World Health Organisation etwa 35 Millionen Menschen weltweit mit HIV infiziert. Eine Infektion mit dem Virus führt unbehandelt zu einer Abnahme der $CD4^+$-Zellzahl und Schwächung des Immunsystems, wodurch AIDS (*acquired immunodeficiency syndrome*) entsteht. Dies ermöglicht das Auftreten opportunistischer Infektionen, die zum Tod des Infizierten führen können. Der Großteil der Infektionen wird durch das HI-Virus Typ 1 verursacht, wohingegen HIV-2 weniger stark verbreitet und dementsprechend weniger untersucht ist. Da Viren sich nicht eigenständig replizieren können, sind sie auf die Wirtszelle angewiesen und interagieren dabei mit verschiedenen zellulären Faktoren. Das humane RNA-bindende Protein Staufen-1 wurde bereits als Interaktionspartner für verschiedene Proteine der Retroviren HIV-1, HTLV-1 und HERV-K beschrieben und kann die Produktion und Infektiosität viraler Partikel beeinflussen. Als mögliche Vermittlungspunkte dieser Effekte wurden der Export viraler RNAs, die Translation viraler Proteine sowie das Assembly neuer Virionen genannt.

In dieser Arbeit sollte die mögliche Interaktion von Staufen-1 mit HIV-2 untersucht werden, um weitere Erkenntnisse zum Einfluss des Proteins auf den retroviralen Replikationszyklus zu gewinnen. Hier konnte gezeigt werden, dass Staufen-1 dosisabhängig zu einer Steigerung der Partikelproduktion von HIV-2 führt, gleichzeitig jedoch die Infektiosität der entstehenden Viren vermindert wird. Als mögliche Interaktionsdomäne von Staufen-1 konnte dabei die dsRBD3-Domäne bestimmt werden. Weiterhin führte eine Herabregulierung der Expression von endogenem Staufen-1 zu einer Verminderung der Partikelproduktion. Durch Herstellung eines Reporterkonstrukts, das das jeweilige *Rev-responsive element* (RRE) besitzt, konnte der Rev/RRE-abhängige Transport ungespleißter RNAs untersucht werden, auf den Staufen-1 keinen Einfluss hatte. Das HIV-2 Rev Protein ist am Export ungespleißter viraler RNAs aus dem Kern beteiligt. Anhand von Fluoreszenzmikroskopaufnahmen sowie

mit Hilfe des generierten Reporterkonstrukts konnten die Funktion von verschiedenen Mutanten und somit mögliche funktionelle Domänen untersucht werden, die für HIV-2 Rev bisher nicht definiert wurden.

Zusammengefasst konnte erstmals ein Einfluss von Staufen-1 auf HIV-2 gezeigt werden, der sich ähnlich wie die bereits beschriebene Interaktion mit anderen Retroviren auswirkt. Wo genau im Laufe des viralen Replikationszyklus die Interaktion stattfindet, ob weitere Proteine eine Rolle spielen und welche Bedeutung Staufen-1 für das Virus und die Zelle hat, ist Gegenstand von weiteren Untersuchungen.

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1 Introduction

1.1 Retroviruses

1.1.1 Preface

The familiy of retroviruses (*Retroviridae*) is made up of morphological and functional different viruses, which all feature two copies of a single-stranded RNA as their genome. One of their striking characteristics is the reverse transcriptase (RT), which allows the virus to transcribe its RNA genome into double-stranded DNA for subsequent integration into the host cell's genome. The term "retrovirus" was also derived from this activity of the RT.

Retroviruses are nowadays classified, following the International Committee on Taxonomy of Viruses (ICTV), into two subfamilies - *Orthoretrovirinae* and *Spumavirinae*. The latter consists only of one genus, the spumaviruses, whereas lentiviruses as well as α-, β-, γ-, δ- and ε-retroviruses are counted among the *Orthoretrovirinae* (see Fig. 1). Criteria for classification comprise specific characteristics of infection, pathology and morphology as well as genetic differences. Furthermore, retroviruses are divided into endogenous and exogenous. Exogenous retroviruses can be transmitted horizontally, because their genome encodes all proteins needed for infection. In contrast, endogenous retroviruses are integrated into every cell's genome of their host and transmitted vertically to the offsprings via the germ cells. Most of these viruses are degenerated, i.e. contain via mutations inactivated or simply lack genetic information essential for replication. They can get activated only under certain conditions, requiring the assistance of a helper virus, which restores the missing functions of the virus.

A further discrimination is made between simple and complex retroviruses. The former hold only the main genes *gag*, *pol* and *env* for the structural proteins and

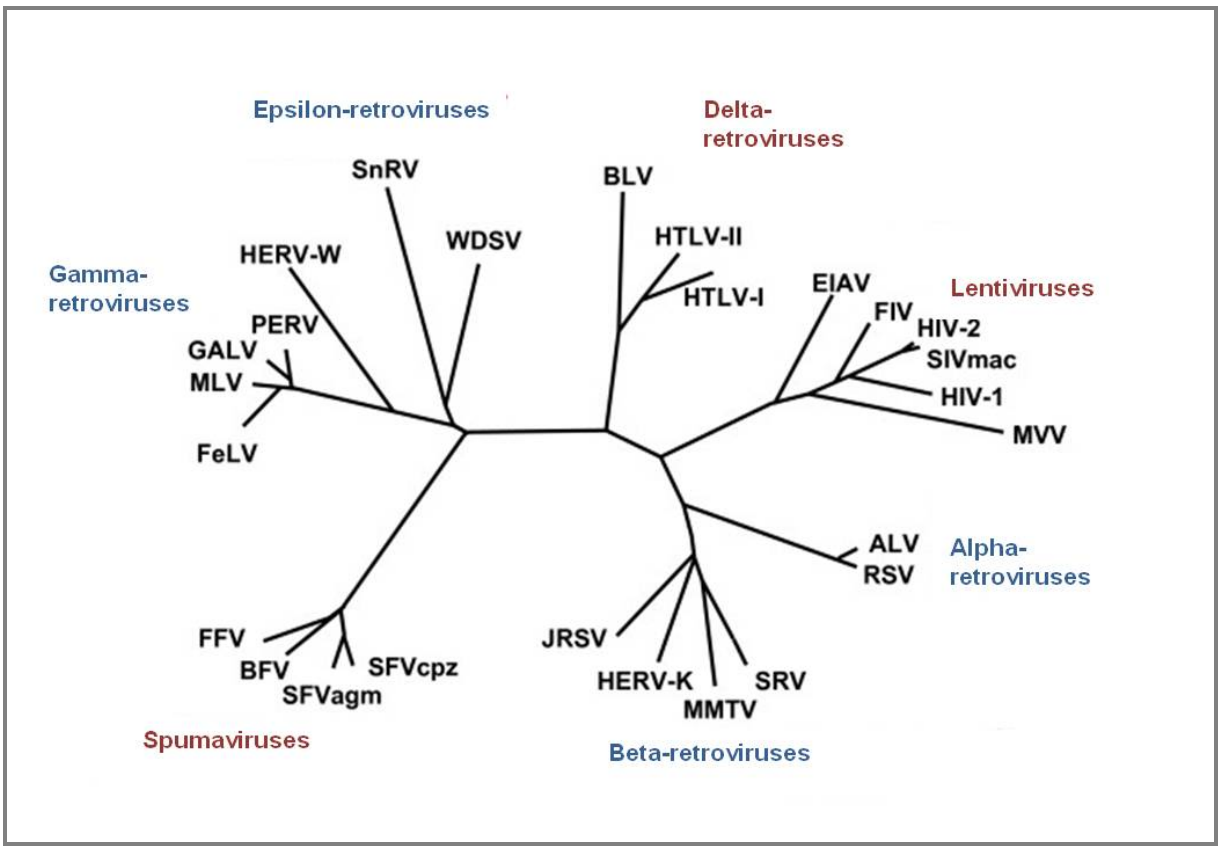

Figure 1: **Phylogenetic tree of the *Retroviridae*.** They are nowadays divided into spuma-, lenti-, α-, β-, γ-, δ- and ε-retroviruses. Simple retroviruses are shown in blue, complex retroviruses are marked red. Modified from Weiss [99].

enzymes, whereas the latter feature also accessory genes regulating the expression of the main genes (reviewed in [99]).

1.1.2 History

Ellerman and Bang were the first ones describing retroviruses in 1908, when they revealed that mouse leukemia can be transmitted to healthy individuals via filtrated tumor extracts [27]. Rous made a similar observation in 1911 regarding chicken tumors [82]. In 1936, Bittner was able to associate the mouse mamma tumor virus (MMTV) with malign mammary gland tumor incidences in mice, which was by that time the first description of retroviruses in mammals [5]. The first human retrovirus

was discovered in 1980 by Poiesz in the lab of Robert Gallo and called the human t-cell lymphotropic virus type-1 (HTLV-1) [76]. Nowadays, most regard is paid to the human immunodeficiency viruses (HIV-1/HIV-2), which are the pathogens causing the acquired immunodeficiency syndrome (AIDS).

1.2 The Human Immunodeficiency Virus

1.2.1 History

The illness AIDS has been known before its causative agent HIV had been identified. AIDS was first described in 1981 for a group of men having sex with men, who were suffering from severe opportunistic infections. Two years later, HIV-1 was successfully isolated from infected lymphocytes of AIDS patients by two independent groups [3] [33]. HIV-2 was first identified in 1986 [15]. Presumably, multiple interspecies transmission have occurred for both viruses. HIV-1 originates from the SIVcpz found in chimpanzees [38], whereas HIV-2 emerged from the SIVsmm of sooty mangabeys [87].

1.2.2 Genomic Organisation and Structure of HIV

The HIV genome contains about 10 kb which include a large number of open reading frames (see Fig. 2). The sequence is flanked by the LTR regions (long terminal repeats), which amongst others functions are needed for integration of the proviral DNA into the host genome, binding of regulatory proteins as well as the production of spliced RNA species. *gag* encodes the group specific antigens and is translated together with *pol* into a Gag/Pro/Pol precursor (160 kDa), which is cleaved into the Gag precursor (p55) and the Pol precursor.

During the course of viral morphogenesis, these polyproteins are further cleaved by the viral protease into the single components. This includes for Gag the matrix protein (p17, MA), the capsid protein (p24, CA), the nucleocapsid protein (p7, NC) and the linker protein (p6, LI), which play a role in budding of the viral particle. The

Pol precursor is processed into the reverse transcriptase (p51/66, RT), the integrase (p32, IN) and the protease (p9, PR). *env* encodes the transmembrane protein (gp41, TM) and the surface protein (gp120, SU) and is translated from a single spliced mRNA as an Env precursor (gp160). Molecular weights of HIV-2 proteins differ slightly from those of HIV-1.

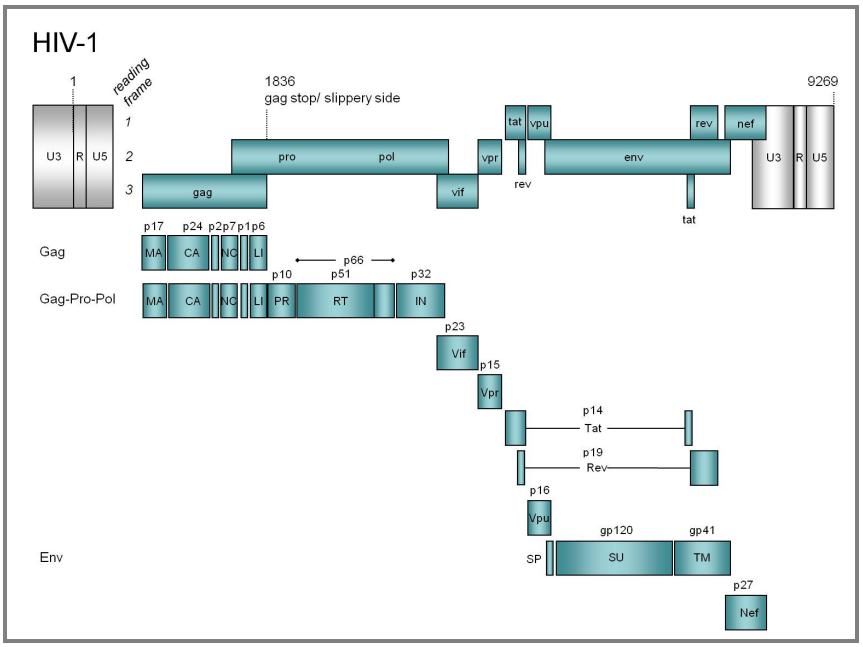

Figure 2: **Schematic representation of the HIV-1 Genome.** Modified from Kurth and Bannert [51].

As HIV is a complex retrovirus, it holds besides these genes several accessory proteins, which are made of single or multiple spliced mRNAs. These proteins are listed together with their functions in Table 1.

The structure of an HIV virus particle is shown in Fig. 3.

The virion has a diameter of 100 to 120 nm and is enveloped in a lipid membrane, which originates from the host. The TM protein is embedded in the membrane and associated with the SU protein via non-covalent bondings. Both glycoproteins constitute the envelope proteins (Env).

Table 1: **HIV accessory proteins and their functions.** Modified from Levy [53].

Protein		Function
Nef	p27	Cell activation, enhanced infectivity
Rev	p19	Regulation of viral mRNA expression
Tat	p14	Transactivation, upregulation of HIV replication
Tev	p26	Tat/Rev activities (only in few HIV-1 isolates)
Vif	p23	Enhanced virus infectivity and transmission
Vpr	p15	Promotes viral replication and transactivation
Vpu	p16	Promotes virus release; HIV-1 only
Vpx	p16	Duplicate of Vpr; HIV-2 only

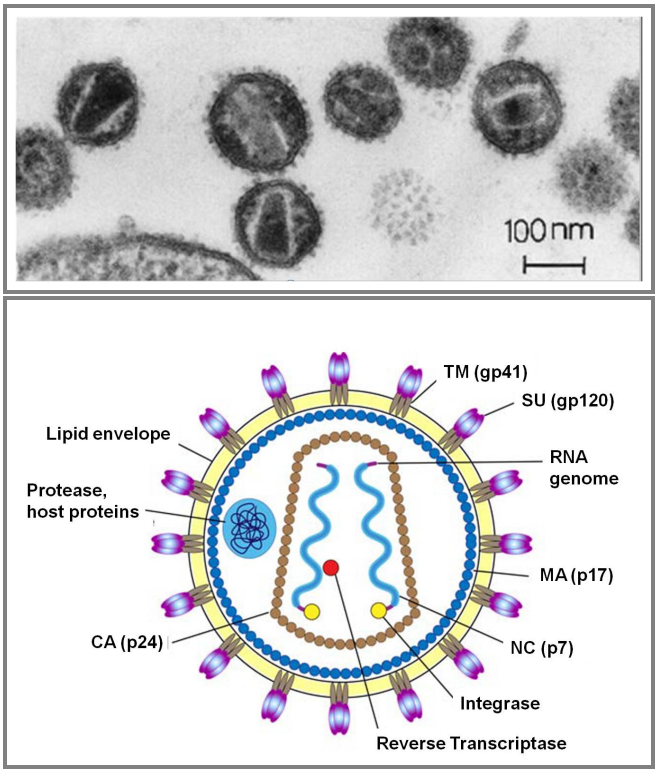

Figure 3: **Top: Electron microscopy .** Modified from Gelderblom *et al.*[35].

Bottom: Schematic representation of an HIV virus particle. Modified from Wikimedia Commons [101].

The MA proteins are bound to the inner face of the envelope membrane. The core is located inside the particle and has a cone-like shape, which is characteristic for lentiviruses. It is composed of the CA protein containing the viral genome. The two identical, positive sense single-stranded RNA molecules form a complex with the NC proteins. MA, CA, NC as well as the LI protein, which connects the capsid to the membrane, belong to the group specific antigens (Gag). The viral core also comprises the enzymes RT, IN and PR.

1.2.3 Replication Cycle

For replication of the virus, the host cell has to get infected (see Fig. 4). The viral particle attaches to the surface via interaction between the SU protein and the cell

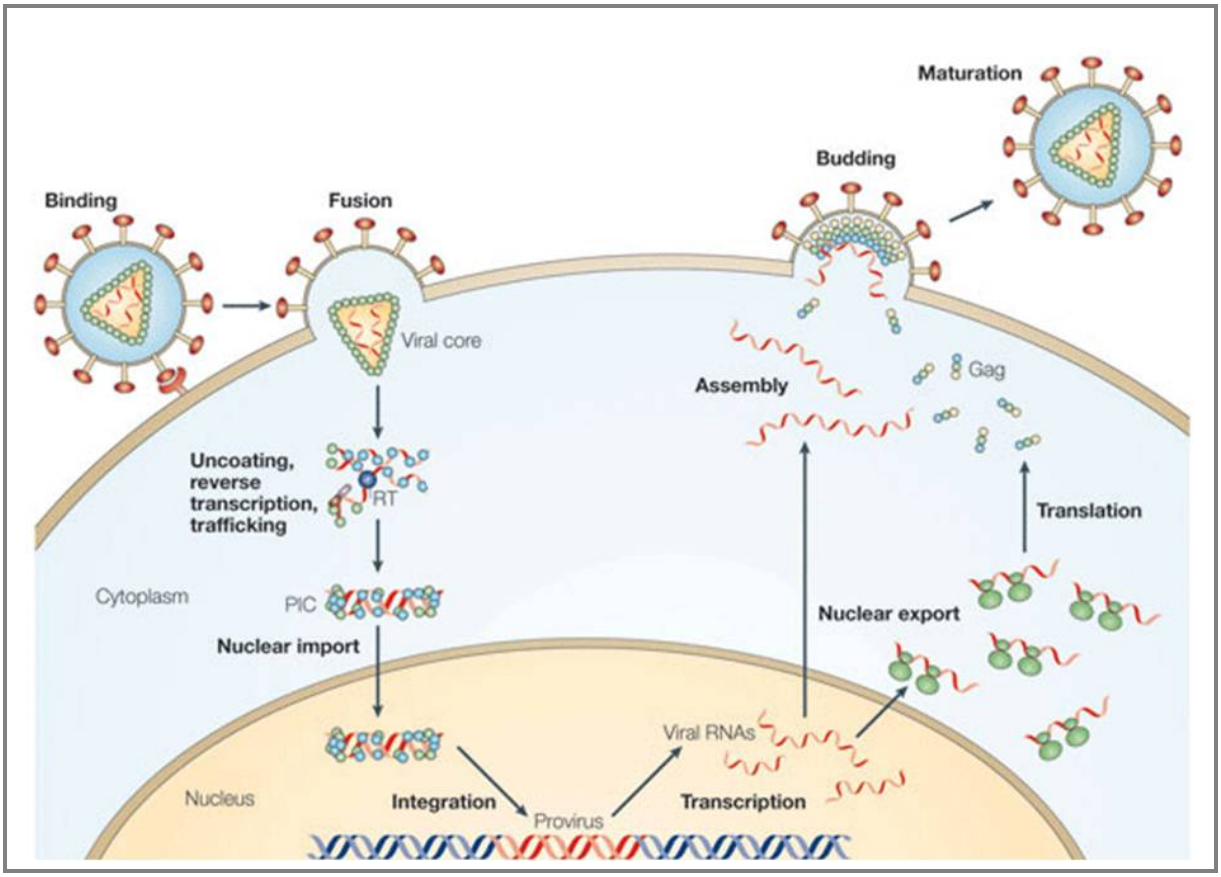

Figure 4: **Replication cycle of HIV.** See text for explanation. Modified from Nisole *et al.*[72].

surface receptor CD4. This leads to conformational changes of both proteins, resulting in the ability for binding to the coreceptor. The TM protein then mediates fusion of the viral envelope with the membrane of the host cell, whereupon the capsid is released into the cytoplasm and becomes uncoated. The reverse transcriptase, which is comprised in the core, copies the single stranded viral RNA genome into double stranded DNA. Because it does not feature a proofreading function, wrong nucleotides are incorporated with a probability of 10^{-3} to 10^{-4}, which results in a high mutation rate. The newly formed DNA associated with the NC proteins is called the *pre integration complex* (PIC).

The PIC is transported into the nucleus, where the viral dsDNA is randomly integrated into the genome of the host cell by the viral integrase and is thereafter called provirus. Lentiviruses are the only retroviruses able to infect resting cells as they encode Vpr and MA, which mediate with the assistance of cellular nuclear import factors the transport of the PIC into the nucleus through the nuclear pores. Subsequently, the virus utilizes the cellular gene expression machinery for the production of new viral particles. In the early stage of infection, multiple spliced mRNAs coding for Tat, Rev and Nef are translocated into the cytoplasm, where the translation takes place. Rev is required for the export of unspliced and incompletely spliced mRNAs, which serve as templates for translation of the Gag/Pro/Pol precursors and for formation of new viral RNA genomes.

The Env proteins are cotranslationally incorporated into the ER (endoplasmic reticulum) membranes of the host cell, where they enter the cellular secretory pathway and are transported to the plasma membrane. Meanwhile they form trimeric complexes and get glycosylated. Gag mediates the assembly of viral particles by association with the viral RNA, the glycoproteins bound to the plasma membrane and other proteins such as the viral protein Vpr. Through eversion of the membrane, budding of viral particles takes place, which is mediated by the host ESCRT (endosomal sorting complexes required for transport) machinery. In the consecutive maturation, precursor proteins are cleaved by the viral protease to fully processed

proteins, which then rearrange to generate the infectious virion with the characteristic cone-shaped core [16] [29] [51] [53] [68] [95].

As viruses are not able to replicate autonomously, they are dependent on this process in the host cell. By this means they have to interact with cellular cofactors, of which several have been identified including their function (reviewed in [32] [94]).

1.2.4 AIDS and Medical Treatment

Referring to the World Health Organisation, there were 35 million people living with HIV worldwide in 2013. In that year, 2.1 million people became newly infected, although the number of new infections declines [103]. The virus targets the cells of the immune system leading to impaired immune function and increased susceptibility to infections, respectively, that people with a healthy immune system can deal with [104]. This results in opportunistic infections including several types of cancer and severe illnesses, for example Kaposi's sarcoma, *Pneumocystis jirovecii* pneumonia, toxoplasmic encephalitis and Non-Hodgkin's lymphoma [53]. As one of these opportunistic infections occurs or the CD4 cell count falls below 200 cells per microliter blood following HIV infection, the disease is defined as AIDS.

Neither a cure nor a vaccine is available yet, due to the fact that HIV features among other things a high genetical variability. Nevertheless, there are various potential approaches to therapies for HIV infection, regarding the HIV virions themselves (by neutralizing antibodies), viral entry (by preventing interaction between and thereby fusion of the virus and the host cell), pre-integration steps (by inhibition of the reverse transcriptase or the viral integrase) and post-integration steps (by inhibition of the viral protease). Nowadays HAART (highly active antiretroviral therapy) is applied, which combines at least three different antiretroviral drugs, resulting in slowing down the progression of the disease.

1.2.5 Comparison Between HIV-1 and HIV-2

HIV-1 and HIV-2 are two closely related viruses both leading to the development of AIDS. In doing so, HIV-2 shows a higher rate of long-time non-progressors and a lower transmission rate compared to HIV-1. Consequently, HIV-1 is distributed worldwide, whereas HIV-2 is restricted to West Africa and countries with links to West Africa, such as Portugal (see Fig. 5) [90]. A dual infection with both viruses is possible, which has been shown for the first time in 1988 [28] [81]. Neither an infection with HIV-2 does protect against infection with HIV-1 nor vice versa. In most of the cases, HIV-1 infection is predominant, whereas HIV-2 has low impact on disease progression. Discrimination of both viruses via ELISA or Western Blot is difficult due to cross-reacting antibodies. However, synthetic peptide based assays and PCR are preferable techniques [90]. HIV-1 is divided into the groups M, N, O and P (partly comprising several subtypes), whereas HIV-2 is classified into groups A-H.

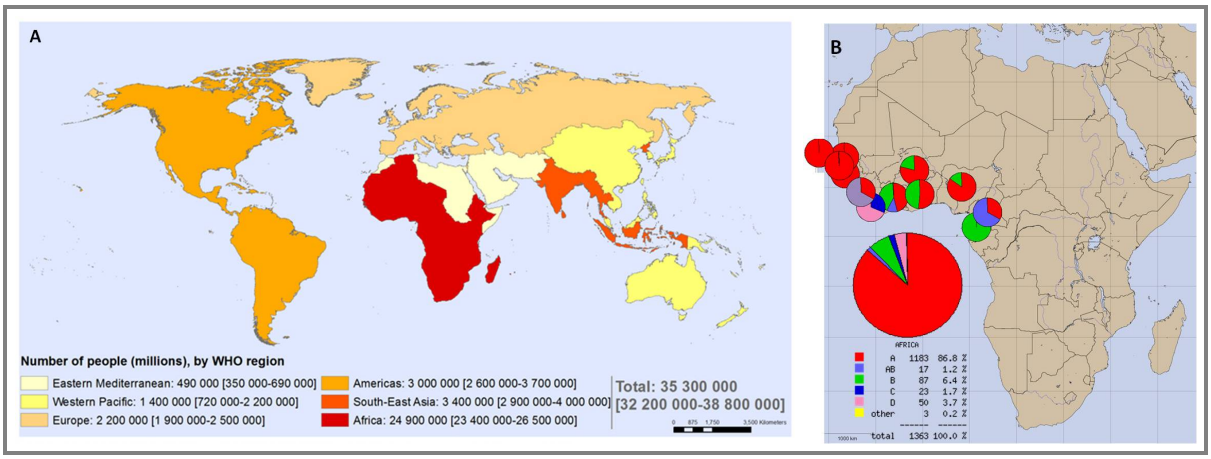

Figure 5: **Geographical distribution of HIV.** A: HIV-1. Modified from WHO [102]
B: HIV-2 groups (subtypes). Based on HIV database (LANL) [55].

HIV-1 and HIV-2 share many similarities such as basic gene arrangement, intracellular mechanisms of replication, clinical consequences and modes of transmission, meaning sexual contacts, transfusion and sharing of contaminated needles [73]. HIV-1 encodes the accessory protein Vpu, which does not exist for HIV-2. Instead, HIV-2 holds Vpx (compare Tab. 1). Additionally, HIV-2 utilizes a broader range of coreceptors than HIV-1 [70] [83] [88] and holds higher mutation rates [91]. Nevertheless, characteristics of an HIV-2 infection are a lower plasma viral load and a higher CD4 cell count compared to HIV-1 infection, which are both factors for successful transmission of the virus [73]. These characteristics are maintained even at later stages of disease progression [56]. Additionally, HIV-2 shows 100-fold lower replication rates *in vitro* [79]. In contrast to this, both viruses show similar infectivity and cytopathicity *in vitro* [85]. Differences do also exist regarding drug susceptibility, leading to distinct requirements in antiretroviral therapy, which impedes treatment of HIV-1/HIV-2 double infections [7] [90].

1.2.6 The Rev Protein

The Rev protein is a posttranscriptional acting transactivator, which is essential for time-wise regulation of the viral gene expression during the replication cycle. Therefore, the term Rev stands for *regulator of expression of virion proteins*. The HIV-1 Rev protein consists of 116 amino acids resulting in an approximate molecular weight of 15 kDa, whereas HIV-2 Rev is made up of 100 amino acids and thus slightly shorter. Translation of the majority of viral proteins, including structural proteins as well as enzymes, is strongly dependent on Rev activity, since it enables export of unspliced (9 kb) and single spliced (4 kb) viral mRNAs from the nucleus into the cytoplasm of the host cell, as mentioned above. Intron-containing, meaning incompletely spliced RNAs are retained within the nucleus under normal conditions, but are required by the virus not only for translation of viral proteins, but also as new viral genomes. Therefore, this function of Rev is essential for generation of new virions. However, Rev itself is translated from the multiple spliced 2 kb viral mRNA, which is found

in the cytoplasm without Rev activity, and thus Rev is counted among the few viral proteins, whose expression is independent of Rev activity and occurs at first in the early stage of infection. Following this, gene expression of HIV can be divided into a Rev-independent and a Rev-dependent phase [47] [78].

The structure of HIV-1 Rev is depicted in Figure 6. The protein features several functional domains, including two loop domains shown in green required for multimerization with other Rev proteins. The arginine-rich region located in the N-terminal domain harbors the overlapping nuclear localization signal (NLS) and RNA-binding domain (RBD), both marked orange. The leucine-rich carboxy-terminal domain contains the nuclear export signal (NES, blue) [4] [23] [39].

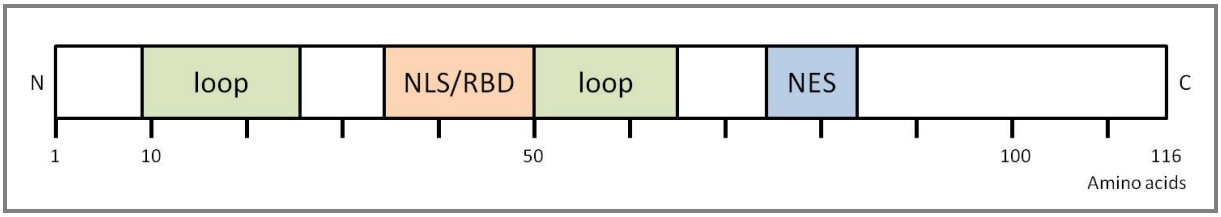

Figure 6: **Schematic representation of the HIV-1 Rev protein.** The overlapping domain of the nuclear localization signal (NLS) and the RNA-binding domain (RBD), shown in orange, is flanked by two multimerization domains ("loop", green). The nuclear export signal (blue) is located in the C-terminus. Modified from DiMattia *et al.* [23].

To allow for Rev-dependent transport, the target mRNA needs to contain the *cis*-acting Rev-responsive element (RRE), which forms a secondary structure the Rev protein specifically binds via its RBD, known for both HIV-1 and HIV-2 [21] [58]. The HIV-1 RRE consists of 351 nucleotides [63] and is located within the *env* gene. Therefore, all unspliced and single spliced viral RNAs feature this element, whereas it is no longer present in completely spliced RNAs encoding among other proteins also Rev. Upon binding to the RRE, Rev induces export of the bound mRNA from the nucleus through the nuclear pores by the cellular export machinery. This mechanism is shown in Figure 7.

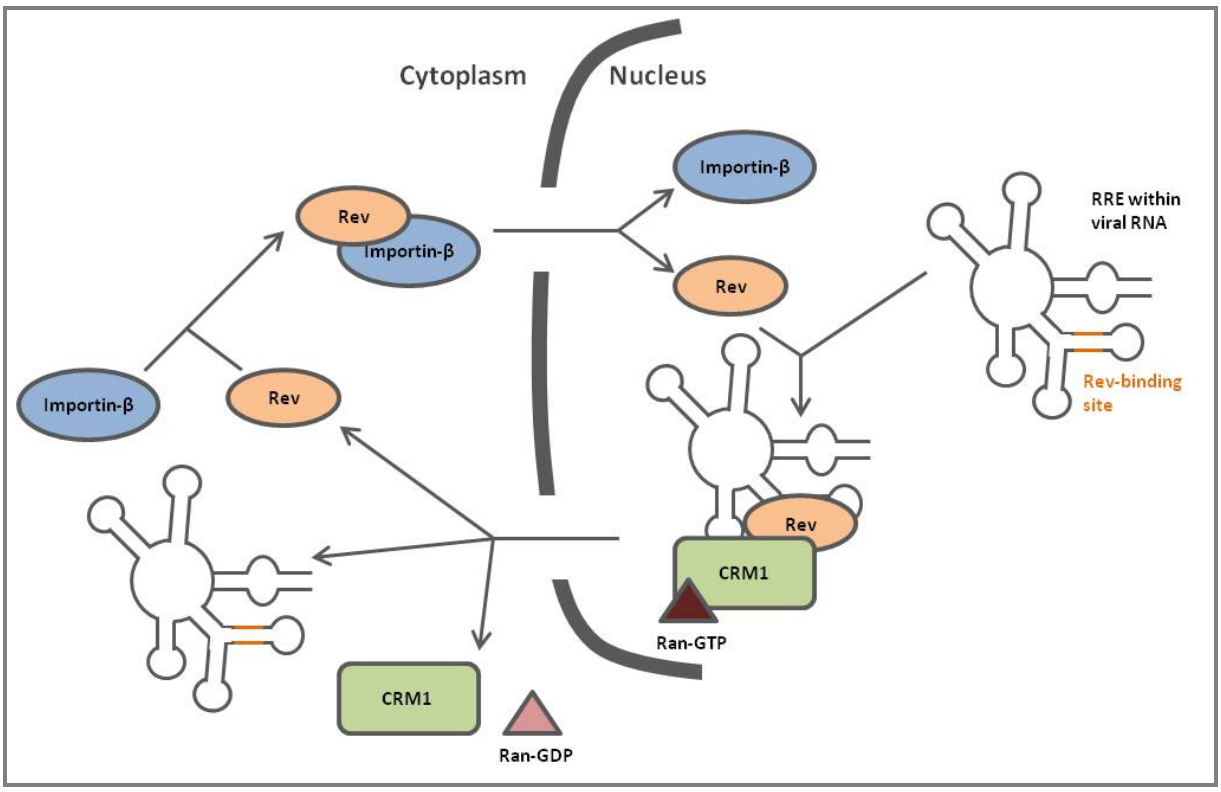

Figure 7: **Mechanism of Rev-dependent RNA export.** The Rev protein is imported into the nucleus by interaction with Importin-β. Here it binds to the Rev-responsive element (RRE) within incompletely spliced mRNA and following multerimerization promotes their export via CRM1, which is RanGTP-dependent. Modified from Pollard and Malim [77] and Cao *et al.* [11].

Rev is imported into the nucleus upon interaction via its NLS with Importin-β. Here, it binds highly specific to incompletely spliced viral RNAs containing the RRE [17] [44] [62]. As the NLS overlaps with the RBD, access to the NLS is not given anymore as long as Rev is bound to the RNA. By this, transport of RNA-bound Rev from the cytoplasm back into the nucleus is prevented. Following binding to the target mRNA, formation of Rev oligomers is mediated by its multimerization domains. For this, protein-protein and protein-RNA interactions are combined [18] [20] [40] [105]. The NES domain then interacts with the cellular protein CRM1 (chromosome region maintenance 1, also referred to as Exportin 1), which mediates export of the

ribonucleoprotein complex through the nuclear pores in a RanGTP-dependent manner [39] [71]. Upon hydrolysis of RanGTP, CRM1 and RanGDP are released in the cytoplasm and Rev dissociates from the mRNA, which is now accessible for the cellular translation machinery. As the NLS of Rev is no longer masked, the protein can be imported into the nucleus again [66].

This mechanism is utilized by all lentiviruses as well as HTLV-1 and HTLV-2 and the endogenous retrovirus HERV, where it is referred to as Rex/RxRE- and Rec/RcRE-dependent RNA transport, respectively [43] [45] [59] [60] [77].

Beside its function in nuclear export of viral RNAs, Rev has been shown to enhance encapsidation of genomic RNA into emerging virions to a larger extent than its effect on cytoplasmic RNA levels [6] [8]. Using lentiviral vectors containing the RRE, Grewe *et al.* were also able to show that enhancement of incorporation of these vectors is independent of whether they have been spliced or not [36]. Furthermore, Rev seems to inhibit integration of the viral genome and thus prevent cellular superinfection (reviewed in [37]).

1.3 The Human Protein Staufen-1

1.3.1 General

The protein Staufen belongs to the family of double-stranded RNA-binding proteins and was initially described in *Drosophila melanogaster* for being involved in the transport of maternal RNA during embryogenesis [30] [92]. It plays a role in various processes, as for instance in *Drosophila* neuroblasts, where it is involved in the localization of mRNA to achieve an asymmetric RNA dispersion during cell division [9] [54].

Vertebrates comprise two homologous genes, Staufen-1 and Staufen-2, which encode several isoforms [64] [100]. The two isoforms of Staufen-1 feature an approximate molecular weight of 55 kDa and 63/65 kDa [57], respectively. Staufen-1 is expressed ubiquitously [64], whereas expression of Staufen-2 is restricted [25].

The human form hStau bears close structural and functional resemblance to the *Drosophila melanogaster* one, dStau, as it features four dsRBDs (double-stranded RNA-binding domain), which are analogous to dsRBD 2 to 5 of dStau (see Fig. 8) [100]. The dsRBD3 holds the highest affinity to bind RNA [97] [100]. Additionally, it exhibits a tubulin-binding domain (TBD) in the carboxyterminus [100]. Down-regulation of the Staufen expression in zebrafish embryos can be compensated by injection of dStau-RNA, indicating a strongly conserved function for this protein in both vertebrates and insects [80].

Within the cell, hStau is mainly localized in the cytoplasm, where large amounts of the protein are found in the perinuclear region [64] [100], but also in the nucleus and by preference nucleolar [97] [100]. This is achieved by the presence of a functional NLS at the C-terminal region of dsRBD3, by which the protein is actively imported into the nucleus [65]. Assistance of other factors can not be excluded. The dsRBD3/NLS also mediates trafficking between nucleus and nucleolus, and cytoplasmic retention, also known as MOI (modulation of import) activity, where dsRBD2 and dsRBD4 fulfill supporting functions. In this context, a competition between dsRBD2 and dsRBD4 for regulating the function of dsRBD3 can not be ruled out [65], as cooperation of dsRBDs has been described for other proteins [93]. Since no NES has been identified yet, hStau might exit the nucleus together with exported

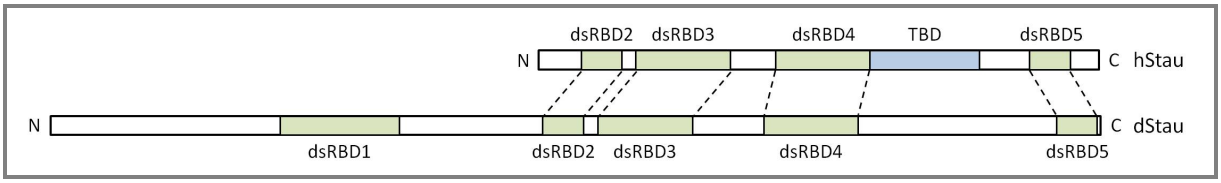

Figure 8: **Schematic representation of hStau and dStau functional domains.** Human Staufen comprises four double-stranded RNA-binding domains (dsRBDs), which are analogous to dsRBD2, dsRBD3, dsRBD4 and dsRBD5 of *Drosophila melanogaster* Staufen. The latter has also a dsRBD1 domain, whereas hStau features a tubulin-binding domain (TBD) shown in blue next to its dsRBD4. Modified from Marión *et al.* [64] .

cofactors. In fact, it is not exported by the CRM1-dependent pathway [65], but its export might occur by interaction with exportin-5 via the dsRBD3 domain, which has been described by Brownawell *et al.* [10]. By these processes, the balance between nuclear import and nucleocytoplasmic export is accomplished, so that hStau is also localized in the cytoplasm despite its NLS [65].

Analogous to its counterpart in *Drosophila melanogaster*, hStau is involved in the transport of RNA, which has been shown in neurons [50]. Additionally to its role in RNA transport, multiple functions have been described for the protein, which are partly contradictory. It is associated with ribosomes [57] [64], polysomes [64] and the rough endoplasmic reticulum [64] [100] and has also been found in ribonucleoprotein complexes in neurons [46]. These findings suggests a function for hStau in the modulation of translation, as has been described by Dugré-Brisson *et al.* [26]. This may be achieved by modification of the ribosomal activity or interaction with cellular chaperons, which stabilize the newly formed protein and by this enhance the translation elongation rate. RNA-containing granules are constituted in the presence of hStau [46] [50], which move along the microtubule network of the cell [50] [100] and might be important for its activity [97]. These RNPs (ribonucleoparticle) complexes are involved in mRNA localization and are continuously modified by binding and releasing of protein partners [65].

Translation of mRNA is being repressed during transport, but becomes derepressed afterwards, which results in localized protein synthesis. Targeted localization of mRNAs is an important process in terms of regulation of gene expression and therefore for the distribution of proteins within the cell. Hereby, hStau is not a general regulator of translation, but instead acts on a specific subset of mRNA targets [26] [48]. For other proteins, it is known that the specificity of RNA-binding is achieved by combination of different dsRBDs and associated proteins [86]. Therefore, association with cofactors, but also posttranslational modifications as well as the site of binding to the mRNA target could influence the nature and the regulation of the process being induced by hStau [26].

hStau binds to ribosomes via both an RNA-binding dependent mechanism as well as protein-protein interaction, which is mediated by dsRBD3 and dsRBD4/TBD, respectively [57]. This presumably leads to enhanced translation by modulation of the ribosomal activity and/or promotion of polysomal loading [26] [57]. The possibility of indirect interaction with the ribosome by binding to the RNA can not be excluded. Due to the fact that ribosomes are associated with the cytoskeleton [74], binding to ribosomes and the cellular microtubule network might take place simultaneously [57].

Concurrently, a role for hStau in promoting degradation of specific mRNAs has been reported which is called *staufen-mediated decay* (SMD) [48] [49]. It is also associated with stress granules (SGs), which are large RNP aggregates in the cytoplasm induced upon cellular stress and contain RNA silenced for the duration of their persistence. Here, hStau promotes SG disassembly and thereby regulates the stress response [97].

Taken together, hStau is involved in the regulation of stability, localization and translation of numerous cellular transcripts with low sequence specificity [97].

1.3.2 Staufen-1 and HIV

Staufen-1 is thought to interact with HIV in various ways. On the one hand, Chatel-Chaix *et al.* have described an interaction between Staufen-1 and the Gag precursor p55, but not with mature Gag proteins [12] [14]. This interaction is mediated by the dsRBD3 domain of Staufen-1 and its C-terminal domain, as well as by the two zinc finger motifs in the NC domain of p55 [13] [14], and may lead to the association of other host factors needed for assembly [13]. p55 features important determinants for multimerization, which can be influenced by Staufen-1 [13]. By this, Staufen-1 is involved in viral assembly [14]. Both overexpression and knockdown of Staufen-1 have the same effect on HIV-1 p55 multimerization, as it leads to an increase in particle release [12]. This process was shown to be dose-dependent [13]. At this, the N-terminal domain of Staufen-1 is responsible for regulating its activity during

HIV assembly [13]. Chatel-Chaix *et al.* were furthermore able to show, that also endogenous Staufen-1 interacts with Gag in an RNA-independent manner [14].

HIV-1 may also utilize Staufen-1 for enhancing the expression of the viral proteins. This may be achieved by transport and positioning of mRNAs to the ribosomes and improving the binding of translation initiation factors such as eIF4E [97]. Alternatively, Staufen-1 may bind to the viral RNA to avoid activation of dsRNA-dependent kinase (PKR), a cellular factor able to bind the TAR RNA sequence [26]. This sequence is present at the 5'end of all HIV-1 transcripts, constituting a stable RNA stem loop, which results in reduced accessibility of the 5'cap und thereby inhibition of translation [75]. As PKR binds to TAR, it gets activated and phosphorylates translation initation factors, thereby leading to general inhibition of translation [26]. By inhibition of the PKR activation, HIV-1 RNA could escape global silencing and facilitate the translation of viral proteins [64]. As Staufen-1 shares sequence similarity with the TAR-RNA binding protein (TRBP) (shown for the highly related *Drosophila melanogaster* in [34]), which enhances the translation of TAR-containing RNAs upon binding [24], it may also itself promote the production of viral particles by binding to the TAR sequence, which has been reported by Dugré-Brisson *et al.* [26].

Furthermore, Staufen-1 is selectively incorporated into viral particles, which has been revealed for both HIV-1 and HIV-2 [69]. This process is mediated by the dsRBD3 domain of Staufen-1 and requires the association of Staufen-1 with the viral RNA, leading to the suggestion that Staufen-1 may play a role in selection and transport of HIV-1 genomic RNA for encapsidation into the viral particle, as it shows no interaction with spliced viral RNA and remains associated with the HIV-1 genome during assembly and maturation of the viral particle [1] [14] [69]. Corresponding to this, a correlation between the level of genomic viral RNA and the incorporation of Staufen-1 into the viral particle has been described [69]. Overexpression of Staufen-1 as well as its knockdown were shown to lead to impaired infectivity of emerging HIV-1 particles [69]. These findings correlate with Shin *et al.*, who stated that viral infectivity can be impaired at multiple stages, including the quantity of

RNA packaged, encapsidation, reverse transcription and integration [89].

Hanke *et al.* stated an interaction of Staufen-1 with HTLV-1 Rex and HERV-K(HML-2) (human endogenous retrovirus) Rec, both related to HIV Rev [41]. They were furthermore able to show that overexpression of Staufen-1 results in an increase in virus production as well as in an enhancement of nuclear export and/or translation of unspliced HERV-K RNAs. Additionally, colocalization of Staufen-1 and Rec could be demonstrated in cells which overexpress both proteins. Similar observations regarding HIV-1 were made by Katharina Fiddeke during her master thesis, in which she investigated the interaction of Staufen-1 with HIV-1 Rev. Besides, she reported an increase in virus production as well as impaired transduction efficiency of pseudotyped SHIV (simian/human immunodeficiency virus) upon overexpression of Staufen-1 [31].

At least in these four ways, Staufen-1 can possibly influence HIV-1 and other retroviruses such as HTLV-1 and HERV-K by increasing the translation rate of viral proteins, modulating its particle production, interacting with Gag and Rev or its homologues and impairing the infectious potential of the virions, respectively [14] [26] [69]. The precise mechanism where these effects of Staufen-1 are mediated remains elusive, but taken all findings together, a model for a cumulative impact of Staufen-1 by targeting export of viral RNAs, translation of viral proteins and assembly of new virions, and simultaneous antiviral activity under stress conditions by silencing of viral RNAs in SGs is promoted.

2 Aim of this Work

The aim of this master thesis is to investigate the impact of the human RNA-binding protein Staufen-1 on the particle production and infectivity of HIV-2, since its interaction with several retroviruses has been demonstrated, but only little research has been done regarding HIV-2.

First, the effect of Staufen-1 on the particle production should be analyzed. For this, the concentration of viral particles in cell supernatants can be determined by usage of a CA-antigen capture ELISA, which cross-reacts with the capsid proteins of both HIV-1 and HIV-2, allowing comparison of the two viruses. Moreover, the expression of the HIV-2 molecular clone pROD should be investigated over a period of seven days with regard to the expression kinetics and therefore effect of Staufen-1. For exploration which functional domain of Staufen-1 is involved in mediating its effect on particle production, Staufen-1 mutants should be employed and their effect on particle release should be determined. As Staufen-1 is endogenously expressed by cells, Staufen-1-specific shRNAs should be used for downregulation of Staufen-1 expression and the impact on particle production should be analyzed.

For addressing the functionality of virions produced in the presence of exaggerated Staufen-1, a real-time PCR assay should be established. Afterwards, this PCR can be employed for exploring the dose-dependent impact of Staufen-1 on the infectivity of emerging virions.

A next objective of this work is to define functional domains of the HIV-2 Rev protein analogous to HIV-1 Rev by alignment of the protein sequences and hence introduce specific mutations into a codon-optimized HIV-2 Rev (coRev) by deletion and substitution of amino acids to knockout these functional domains. Localization of coRev and its mutants as well as colocalization with Staufen-1 should be studied by confocal fluorescence microscopy. Furthermore, the effect of coRev and its mutants on particle production upon Staufen-1 overexpression should be investigated.

The change of nuclear export activity of Rev upon Staufen-1 overexpression should be examined and compared with HIV-1 Rev and HERV-K Rec. For this, a reporter construct containing the HIV-1 and HIV-2 RRE should be created, respectively, based on a HERV-K RcRE shuttle vector to allow comparison of these viruses. The generated construct should also be used for investigating the shuttle function of the coRev mutants.

The results of this project should contribute to the knowledge about the involvement of Staufen-1 in the HIV-2 replication cycle in various aspects and thereby adding some more insights to the similarities or differences in the role of Staufen-1 during retroviral infections.

3 Materials and Methods

3.1 Materials

3.1.1 Laboratory Equipment

Machines and supplies given in Table 2 were exerted in this work.

Table 2: Laboratory equipment

Model	Company
Balance Ohaus Navigator^{TM} N2B110	Ohaus, Nänikon, Swiss
Balance PR803	Mettler Toledo, Giessen, Germany
Beckman Coulter-Counter Z2	Beckman Coulter, Fullerton, USA
Clean Bench Thermo HeraSafe	Kendro, Langenselbold, Germany
Confocal Microscope LSM 780	Carl Zeiss, Oberkochen, Germany
CO_2 Incubator HeraCell 150	Thermo Scientific, Logan, USA
C24 Incubator Shaker	New Brunswick Scientific, Edison, USA
DNA Engine Thermocycler	BioRad, Hercules, USA
Dry Block Heater	Grant Instrument Ltd, Cambridgeshire, England
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 Progression Machine Curix 60	Agfa, Cologne, Germany

Fume Hood	Köttermann GmbH & Co KG, Uetze/Hänigsen, Germany
Gene Pulser XCell + Shock Pod	BioRad, Hercules, USA
GelDoc 2000	BioRad, Hercules, USA
Hettich Mikro 200R Centrifuge	DJB Labcare Ltd, Buckinghamshire, England
Horizontal Shaker 3016	GFL - Gesellschaft für Labortechnik, Burgwedel, Germany
Incubator Innova 4330	New Brunswick Scientific, Edison, USA
Inverse Light Microscope ID03	Carl Zeiss, Oberkochen, Germany
Luminometer Centro LB 960	Berthold Technologies, Oak Ridge, USA
Microplate Reader Sunrise	Tecan, Männedorf, Swiss
Minidizer HB-500 Hybridization Oven	UVP, Upland, USA
Mini-PROTEAN® Tetra Cell Electrophoresis	BioRad, Hercules, California, USA
Multifuge 1S-R	Kendro, Langenselbold, Germany
Mx3000P Multiplex Quantitative PCR System	Stratagene Europe, Amsterdam, Netherlands
NanoDrop Spectrophotometer ND-1000	Nanodrop, Wilmingon, USA
Nitrocellulose Membrane	BioRad, Hercules, USA
Odyssey® Infrared Imaging System	LI-COR, Lincoln, Nebraska, USA
Overhead Rotator Reax 2	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
pH-Meter MP220	Mettler Toledo, Giessen, Germany
PowerPAC^{TM} HC	BioRad, Hercules, USA
PowerPAC^{TM} 200	BioRad, Hercules, USA
PVDF Membrane	Carl Roth GmbH, Karlsruhe, Germany
Sprout® Mini-Centrifuge	Heathrow Scientific LLC, Illinois, USA

Sub-Cell GT Agarose Gel Electrophoresis System	BioRad, Hercules, California, USA
Thermomixer Compact	Eppendorf, Wesseling-Berzdorf, Germany
Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell	BioRad, Hercules, California, USA
UV Transilluminator TFX-20.M	Vilber Lourmat, Paris, France
Water Bath	GFL - Gesellschaft für Labortechnik, Burgwedel, Germany
X-ray Film	Eastman Kodak Company, Rochester, New York, USA

3.1.2 Chemicals

In Table 3, chemicals used for the described experiments are listed.

Table 3: Chemicals

Chemical	Company
30 % Acrylamide	Carl Roth GmbH, Karlsruhe, Germany
Ammonium persulfate (w/v) 10 % (APS)	Carl Roth GmbH, Karlsruhe, Germany
Agar-agar	Carl Roth GmbH, Karlsruhe, Germany
Agarose	Peqlab Biotechnologie GmbH, Erlangen, Germany
BigDye 3.1	ABI Terminator Chemie, Applied Biosystems, Foster City, USA
BSA 100x	New England Biolabs Inc., Beverly, USA
Dimethyl sulfoxide (DMSO) 50 %	Sigma-Aldrich Chemie GmbH, Munich, Germany

Ethanol	Carl Roth GmbH, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Chemie GmbH, Munich, Germany
GelRed	Biotium, Hayward, USA
Hoechst 33528	Roche Diagnostics GmbH, Mannheim, Germany
Skimmed milk powder	Carl Roth GmbH, Karlsruhe, Germany
Magnesium chloride ($MgCl_2$)	Roche Diagnostics GmbH, Mannheim, Germany
Mowiol 4-88	Carl Roth GmbH, Karlsruhe, Germany
Nucleoside triphosphates (dNTPs)	Fermentas International Inc., Burlington, Canada
o-Phenylenediamine dihydrochloride (OPD)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Paraformaldehyde (PFA)	Carl Roth GmbH, Karlsruhe, Germany
Protease inhibitor (tablets)	Roche Diagnostics GmbH, Mannheim, Germany
Sulfuric acid, 2.5 M	Carl Roth GmbH, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Super Signal West Dura Extended Duration Substrate	Thermo Scientific, Logan, USA
TEMED	Carl Roth GmbH, Karlsruhe, Germany
TrisHCl	Carl Roth GmbH, Karlsruhe, Germany
Triton-X 100	Carl Roth GmbH, Karlsruhe, Germany
Tween-20	Carl Roth GmbH, Karlsruhe, Germany
Hydrogen peroxide, 30 %	Carl Roth GmbH, Karlsruhe, Germany

3.1.3 Buffers

Table 4 presents buffers utilized for this work.

Table 4: Buffers

Buffer	Composition
Sample buffer	
6x DNA sample buffer	10 mM tris-acetate, 50 mM EDTA, 10 % Ficoll-400 (w/v) (Serva), 0.4 % Orange-G (w/v) (Sigma) in *Aqua bidest*
4x Laemmli sample buffer	200 mM Tris-Cl, 8 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 40 % (v/v) glycerol, 400 mM DTT
Buffer and media for cell culture	
Calcium chloride	Carl Roth GmbH, Karlsruhe, Germany
Dulbecco's Modified Eagle Medium (DMEM)	PAA Laboratories GmbH, Pasching, Austria
FCS (Fetal calf serum)	Biochrom, Berlin, Germany
2x HBS	50 mM HEPES, 280 mM NaCl, 1.5 mM disodium phosphate, *Aqua bidest*, pH 7.1 (adjusted with NaOH)
Isoton R II Diluent	Beckman Coulter, Inc., Fullerton, USA
RPMI	PAA Laboratories GmbH, Pasching, Austria
NP-40	New England Biolabs Inc., Beverly, USA
Media for culturing of bacterial cells	
LB medium	1 % tryptone, 0.5 % bacto yeast extract, 1 % NaCl, pH 7.5
LB-Agar	LB medium containing 20 $\frac{g}{L}$ agar-agar

SOC-Medium	20 $\frac{g}{L}$ bacto tryptone, 5 $\frac{g}{L}$ bacto yeast extract, 4 $\frac{g}{L}$ glucose, 2.5 mM KCl, 10 mM NaCl, 5 mM MgCl, pH 7.0
SDS-PAGE	
Stacking gel buffer	BioRad, Hercules, California, USA
Resolving gel buffer	BioRad, Hercules, California, USA
Running buffer	BioRad, Hercules, California, USA
Western Blot	
Blocking buffer	5 % skimmed milk powder in PBS with 0.1 % Tween-20
Transfer buffer	BioRad, Hercules, California, USA
Washing buffer	PBS with 0.1 % Tween-20
ELISA	
Carbonate bicarbonate buffer	Sigma-Aldrich Chemie GmbH, Munich, Germany
PBS/Tween	PBS with 0.05 % Tween-20
Phosphate citrate buffer 0.05 mM, pH 5.0	Sigma-Aldrich Chemie GmbH, Munich, Germany
PM	2 % skimmed milk powder in PBS
PMT	2 % skimmed milk powder in PBS with 0.05 % Tween-20
Other buffers	
Phosphate buffered saline (PBS)	137 mM sodium chloride, 2.7 mM potassium chloride, 8.8 mM disodium hydrogen phosphate, 0.7 mM potassium dihydrogen phosphate, pH 7.2
1x TAE	40 mM Tris, 1 mM EDTA, 20 mM acetate, pH 8.0

DNA polymerase buffer	Agilent Technologies, Inc., Santa Clara, California, USA
DNA polymerase buffer	Roche Diagnostics GmbH, Mannheim, Germany
T4 DNA ligase buffer	New England Biolabs Inc., Beverly, USA
TaqMan® Universal PCR Master Mix 2x	Roche Diagnostics GmbH, Mannheim, Germany
NEB buffers	New England Biolabs Inc., Beverly, USA

3.1.4 Kits

Table 5 shows kits used in this work.

Table 5: Kits

Name of Kit	Company
Dual-Luciferase® Reporter Assay System	Promega Corporation, Madison, USA
Effectene Transfection Reagent	Qiagen GmbH, Hilden, Germany
Endo-free Plasmid Maxi Kit	Qiagen GmbH, Hilden, Germany
PolyFect Transfection Reagent	Qiagen GmbH, Hilden, Germany
TOPO® TA Cloning® Kit for Sequencing	Invitrogen Corporation, Carlsbad, USA
QIAamp DNA Blood Mini Kit	Qiagen GmbH, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen GmbH, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen GmbH, Hilden, Germany
QuikChange® Multi Site-Directed Mutagenesis Kit	Stratagene Europe, Amsterdam, Netherlands

3.1.5 Bacterial Strains

Bacterial strains used in this thesis are given in Table 6.

Table 6: Bacterial strains

Strain	Company
One Shot® TOP10 Chemically competent *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® TOP10 Electrocompetent™ *E. coli*	Invitrogen Corporation, Carlsbad, USA
One Shot® Stbl3 Chemically competent *E. coli*	Invitrogen Corporation, Carlsbad, USA

3.1.6 Cell Lines

All eukaryotic cells for described experiments were human embryonic kidney 293T cells (HEK 293T), except for infection experiments, where C8166 cells were used. Cells were obtained from ATCC, LGC Standards GmbH, Wesel, Germany.

3.1.7 Growth Media

HEK 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 1 $\frac{g}{L}$ glucose, 2 mM L-glutamine and 1x Penicillin/Streptomycin. For cultivation of C8166 cells, RPMI was used, which was complemented as for HEK 293T. Bacterial cells were grown in Lysogeny broth (LB) medium.

3.1.8 Antibiotics

In Table 7, antibiotics used in this work are listed.

Table 7: Antibiotics

Antibiotic	Company
Ampicillin	Carl Roth GmbH, Karlsruhe, Germany
Kanamycin	Carl Roth GmbH, Karlsruhe, Germany
Penicillin/Streptomycin	Biochrom, Berlin, Germany

3.1.9 Markers and Standards

Markers and standards listed in Table 8 were used.

Table 8: Markers and standards

Marker/ Standard	Company
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
PageRuler Plus Prestained Protein Ladder	Thermo Scientific, Logan, USA
PageRuler Prestained Protein Ladder	Thermo Scientific, Logan, USA

3.1.10 Enzymes

Enzymes employed in this work are itemized in Table 9.

Table 9: Enzymes

Enzyme	Company
AmpliTaq Gold®	Roche Diagnostics GmbH, Mannheim, Germany
ClaI	New England Biolabs Inc., Beverly, USA
HindIII	New England Biolabs Inc., Beverly, USA
PfuTurbo DNA Polymerase	Agilent Technologies, Inc., Santa Clara, California, USA
0.05 % Trypsin (pH 7.2), 0.02 % EDTA in PBS	Biochrom, Berlin, Germany
T4 DNA Ligase	Fermentas International Inc., Burlington, Canada
XbaI	New England Biolabs Inc., Beverly, USA
XhoI	New England Biolabs Inc., Beverly, USA

3.1.11 Vectors

Table 10 lists vectors used in this work.

Table 10: Vectors

Vector	Company
pcDNA4-V5-His/B	Invitrogen Corporation, Carlsbad, USA
pCMV-Tag2b	Agilent Technologies, Inc., Santa Clara, California, USA
pCR^{TM}4-TOPO® TA Vector	Invitrogen Corporation, Carlsbad, USA

3.1.12 Gene Constructs

In Table 11 listed plamids were used.

Table 11: Gene constructs

Name of construct	Source/ publication
pLVTHM_shCofilin	Dr. Alexander Karlas
HERV-K113 oricoRec	Dr. Kirsten Hanke [42]
pCMV_FLAG-Staufen-1	Hanke *et al.* [41]
pCMV_FLAG-Staufen-1_ΔRBD3	Jula Wamara [98]
pCMV_FLAG-Staufen-1_ΔRBD4	Jula Wamara [98]
pCMV_FLAG-Staufen-1_ΔTBD/RBD5	Jula Wamara [98]
pCMV_FLAG-Staufen-1_F135A	Jula Wamara [98]
pCMV-*Renilla* luciferase	Dr. Kirsten Hanke
pLVTHM_shStau_A7	Jula Wamara [98]
pLVTHM_shStau_C3	Jula Wamara [98]
pLVTHM_shStau_B3	Jula Wamara [98]
pLVTHM_shStau_C1	Jula Wamara [98]
pLVTHM_shStau_A2C4	Jula Wamara [98]
pNL4-3	The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin. [2]
pROD10	The reagent pROD10 was obtained from the Centre for AIDS Reagents, NIBSC and was donated by Dr J-M Bechet, Institute Pasteur, Paris. [15]
Shuttle	Hanke *et al.* [41]
ShuttleRcRE	Hanke *et al.* [41]

3.1.13 Antibodies

Antibodies used for ELISA, immunofluorescence and immunoblotting are itemized in Table 12.

Table 12: Antibodies

Antibody	Origin	Tag	Company/ source
AG3.0 (α-Gag HIV/SIV)	Mouse	-	Dr. Stephen Norley
α-Actin	Mouse	HRP	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-FLAG	Rabbit	-	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Human IgG	Rabbit	HRP	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Mouse IgG	Goat	HRP	Sigma-Aldrich Chemie GmbH, Munich, Germany
α-Mouse IgG	Donkey	IRDye800CW	LI-COR, Lincoln, Nebraska, USA
α-Mouse IgG	Goat	Alexa-568	Invitrogen Corporation, Carlsbad, USA
α-Rabbit IgG	Donkey	IRDye680RD	LI-COR, Lincoln, Nebraska, USA
α-Rabbit IgG	Donkey	Alexa-488	Invitrogen Corporation, Carlsbad, USA
α-Rat IgG	Goat	IRDye680RD	LI-COR, Lincoln, Nebraska, USA
α-Staufen	Rat	-	Dr. Kirsten Hanke & Dr. Oliver Hohn
α-V5 IgG	Mouse	-	AbD Serotec, MorphoSys AG, Martinsried, Germany
HIV plasma pool	Human	-	Dr. Stephen Norley

3.1.14 Oligonucleotides

Oligonucleotides used in this thesis were synthesized by Life Technologies GmbH, Darmstadt, Germany and TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany (only oligonucleotides used for real-time PCR), respectively. Sequencing primers are shown in Table 13, primers and probes used for real-time PCR are listed in Table 14.

Table 13: Sequencing primers

Primer name	Primer sequence 5′ → 3′
3′LTR-For	GAACTGAGGCAATTGCAGGAGTTGCTGATG
BGH-Rev	TAGAAGGCACAGTCGAGG
Env Seq-For	AGAACAGCCCACATGAAGGA
L140-Rev [19]	TCCAACAGGCTCTCTGCTAATCC
M13-For	ACGTTGTAAAACGACGGCCAG
M13-Rev	CAGGAAACAGCTATGACCATGATTAC
pcDNA3 Seq-For	GTGTACGGTGGGAGGTCT
T3-For	ATTAACCCTCACTAAAGGGA
T7-For	TAATACGACTCACTATAGGG
T7-Rev	GCTAGTTATTGCTCAGCGG
U3-For [19]	GGGAGATGGGCGCGAGA

Table 14: Primers and probes used for real-time PCR

Primer name	Primer sequence 5′ → 3′
GAPDH_44-For	GGCGATGCTGGCGCTGAGTAC
GAPDH_45-Rev	TGGTCCACACCCATGACGA
GAPDH-Probe	HEX-CTTCACCACCATGGAGAAGGCTGGG-BHQ1
HIV-2_RT-For	CTCCACGCTTGCTTGCTTAAA
HIV-2_RT-Rev	GGCGCCAAYCTGCTAGG
HIV-2_RT-Probe	6FAM-CATCTCTYCTAGYCGCCGCCTGGTC-BBQ

Oligonucleotides given in Table 15 were used for mutagenesis.

Table 15: Mutagenic oligonucleotide primers

Primer name	Primer sequence 5′ → 3′
Mutagenesis of pcDNA4_HIV-2coRev-V5	
clon-HIV2coRev_d30-For	AAACTTAAGCTTGCCACCATGGGCACTGCATCTCAGCG
clon-HIV2coRev_d46-For	AAACTTAAGCTTGCCACCATGTGGCGACAGATCCTGGC
clon-HIV2coRev_d60-For	AAACTTAAGCTTGCCACCATGCCAGACCCACCTGCAGA
mut-HIV2coRev_M5-For	GCCCGGCACTGCATCTCAGGACCTGAACAGGCGCCGAAGATGG
mut-HIV2coRev_M5-Rev	CCATCTTCGGCGCCTGTTCAGGTCCTGAGATGCAGTGCCGGGC
mut-HIV2coRev_SLT40-For	CCTGGCCCTGGCTGATAGCGACGATACCTTCCCAGACCCACCTGC
mut-HIV2coRev_SLT40-Rev	GCAGGTGGGTCTGGGAAGGTATCGTCGCTATCAGCCAGGGCCAGG
mut-HIV2coRev_M10-For	GGACCAGACCATCCAGCACGACCTAGGGCTGACAATCCAGGAACTGC
mut-HIV2coRev_M10-Rev	GCAGTTCCTGGATTGTCAGCCCTAGGTCGTGCTGGATGGTCTGGTCC
Mutagenesis of shuttle constructs	
clonHXB2_RRE1-BglII_For	ATGCATATCGATAGATCTTCAGACCTGGAGGA
clonHXB2_RRE1-BamHI_Rev	TACGTAATCGATGGATCCGTTCACTAATCGAATG
clonROD_RRE2-Sau3A_For	ATGCATATCGATGATCAATAAAAGACCCAGACAAGC

clonROD_RRE2-Sau3A_Rev	TACGTAATCGATGATCCAGGAGGTTAARTCAA
MutShuttle_3ClaI-For	AAGCACAGCACTTAATCGATTACATTGTCTAT-GATGCC
MutShuttle_3ClaI-Rev	GGCATCATAGACAATGTAATCGATTAAGTGCT-GTGCTT
MutShuttle_5ClaI-For	CAACTCAGAGTTGAATCGATTAAGGGCGGTG-CAGG
MutShuttle_5ClaI-Rev	CCTGCACCGCCCTTAATCGATTCAACTCTGAG-TTG

3.1.15 Software

In Table 16 mentioned software was used.

Table 16: Software

Adobe Photoshop CS5
AmplifX
BioEdit
Cell^F (Olympus Soft Imaging System)
EditSeq
Evince
LI-COR Odyssey v3.0
SeqBuilder
MegAlign
Microsoft Office 2010
MicroWIN 2000
MxPro QPCR
Nanodrop ND-1000 v3.3
TeXnicCenter
Zen2012

3.2 Methods

3.2.1 Cell Culture

3.2.1.1 Growth Conditions

HEK 293T cells were grown in DMEM containing 1 $\frac{g}{L}$ glucose and L-glutamine, supplemented with 10 % FCS and 0.5 % Penicillin/Streptomycin. For culturing of C8166 cells, RPMI 1860 containing 1 $\frac{g}{L}$ glucose and L-glutamine, supplemented with 10 % FCS and 0.5 % Penicillin/Streptomycin was used. Both cell lines were cultivated at 37 °C under humidified air containing 5 % CO_2.

3.2.1.2 Cell Split

For splitting of HEK 293T cells, old growth medium was discarded, cells were washed one time with 10 mL PBS and Trypsin/EDTA was added. After 1 min incubation at room temperature, cells were rinsed from the culture dish by adding fresh growth medium and centrifuged at 453 g for 5 min. C8166 cells were transferred to a falcon tube and centrifuged for 3 min at 201 g. For both cell lines, the supernatant was discarded and cells were resuspended in new medium. According to the cell density, an appropriate number of cells was given back into the culture flask and fresh growth medium was added.

3.2.1.3 Sowing Cells

For transfection and infection, cells were sown into 100 mm dishes or multiwell plates. For this purpose, cells were washed and trypsinized as described above (see 3.2.1.2). Suspended cells were counted using the Coulter Counter and sown in the appropriate cell number with a given volume of fresh growth medium (see Table 17).

Table 17: Specifications for cell culture reservoirs for transfection

	Cell number to sow	Approximate cell yield	Medium volume [mL]
100 mm dish	$1 \cdot 10^6 \frac{\text{cells}}{\text{mL}}$	$4 \cdot 10^6 \frac{\text{cells}}{\text{mL}}$	10
6-well plate	$3 \cdot 10^5 \frac{\text{cells}}{\text{mL}}$	$2.4 \cdot 10^6 \frac{\text{cells}}{\text{mL}}$	2
12-well plate	$6 \cdot 10^4 \frac{\text{cells}}{\text{mL}}$	$2.4 \cdot 10^5 \frac{\text{cells}}{\text{mL}}$	1
96-well plate	$1 \cdot 10^4 \frac{\text{cells}}{\text{mL}}$	$4 \cdot 10^4 \frac{\text{cells}}{\text{mL}}$	0.3

3.2.1.4 Transfection

Cells grown in 100 mm dishes were transfected using the calcium phosphate method. One day prior to transfection, cells were sown as previously described (see 3.2.1.3). 15 to 40 µg DNA were diluted in *Aqua bidest* to a final volume of 450 µL, and 50 µL 2.5 M $CaCl_2$ were added. Little drops of the DNA mixture were carefully pipetted onto the surface of 500 µL 2x HEPES. The mixture was incubated for 30 min at RT (room temperature) for complex formation. During incubation cells were washed once with 4 mL PBS and new growth medium was added. The mixture was transferred to the cells and the dish was swirled gently to ensure uniform distribution of complexes. One day after transfection old growth medium was removed, cells were washed one time with 4 mL PBS and new growth medium was added.

For cells in 6-well, 12-well or 96-well plates, PolyFect was used according to the instructors manual. Transfection of plasmids coding for shRNA was performed using Effectene (both Qiagen GmbH, Hilden, Germany).

3.2.1.5 Cell Lysis

48 to 72 h post transfection, proteins were harvested. For this purpose, the growth medium was either discarded or cell-free centrifuged at 3220 g for 5 min, aliquotted and stored at −80 °C, and PBS was given onto the cells. After centrifugation for 15 min at 290 g, the cell pellet was resuspended in 1 mL PBS and cells were counted using the Coulter Counter. Following another centrifugation step, an appropriate

volume of NP-40 buffer was used for resuspending the cell pellet to ensure the same cell density for every sample. Cells were incubated for 5 min at RT and centrifuged at 15 700 g for 5 min. Supernatant was transferred to a new reaction tube and stored at −20 °C.

3.2.1.6 Infection

Infection was performed by sowing $1 \cdot 10^6$ cells into T25 tissue culture flasks containing 10 mL fresh growth medium, and addition of virus stocks in different dilutions. After incubation for 72 h at normal growth conditions, cells were pelletized at 1811 g for 5 min, washed one time with 2 mL PBS and centrifuged again for 5 min at 1811 g. Supernatant was discarded and cell pellets were stored at −20 °C for further usage.

For single-round infection, cells were pelletized, resuspended and counted according to 3.2.1.3. Cell density was adjusted to $2 \cdot 10^6 \frac{\text{cells}}{\text{mL}}$ and 1 mL of the cell suspension was given into a 15 mL falcon tube. 500 µL virus suspension normalized to p26 concentration were added and cells were incubated in a water bath at 37 °C for 1 h. Afterwards, cells were centrifuged at 805 g for 5 min and washed two times with 1 mL fresh medium with centrifugation between each step. After resuspension in 5 mL fresh medium, cells were transferred into 6-well plates and incubated at normal growth conditions over night.

The next day, cells were transferred into a new 15 mL falcon tube and centrifuged at 1811 g for 5 min. Supernatant was discarded. The cell pellet was washed with 2 mL PBS, centrifuged and resuspended in 1 mL PBS. 10 µL of the suspension were taken for determination of the cell number. Cells were again centrifuged and resuspended in 200 µL PBS.

3.2.2 DNA Analysis

3.2.2.1 Transformation

DNA plasmids were amplified in *E. coli*. For chemically competent cells, the DNA was pipetted into the competent cells and the mixture was incubated on ice for 30 min. Afterwards, a heat shock was performed at 42 °C for 30 s. 250 µL SOC medium were added immediately and cells were incubated at 37 °C for one hour.

Transformation into electrically competent cells was performed using the Gene Pulser II (BioRad). DNA was given into the competent cells, transferred into an electroporation cuvette and pulsed at 2.5 kV, 25 µF and 200 Ω. 250 µL SOC medium were added immediately and cells were incubated at 37 °C for one hour.

Following transformation, cells were plated onto agar plates containing selective antibiotics.

3.2.2.2 DNA Preparation

For amplification of the DNA, single colonies of *E. coli* were picked and grown in LB medium containing an appropriate antibiotic. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit or Endo-free Plasmid Maxi Kit according to the manual. At this, the cells are lyzed and the plasmid DNA is bound to a silica gel membrane and washed several times. The prepared DNA was solved in 50 µl (using the QIAprep Spin Miniprep Kit) and in 200 µl, respectively (using the Endo-free Plasmid Maxi Kit).

3.2.2.3 Polymerase Chain Reaction

For *in vitro* amplification of the desired segment of DNA, the polymerase chain reaction (PCR) was used. In this process, copies of the DNA strand are synthesized by using a polymerase and primers specific for this segment. In Table 18, an example for a PCR reaction is given.

Table 18: PCR reaction

Component	Concentration	Volume/amount	Final concentration
Polymerase buffer	10x	2.5 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	-	10-30 ng	-
AmpliTaq Gold®	250 $\frac{U}{\mu L}$	0.125 µL	1.25 $\frac{U}{\mu L}$
Aqua bidest	-	*ad* 25 µL	-

An exemplary temperature profile is shown in Table 19. Annealing temperature is adjusted to the primers used in the reaction. The time of the elongation step depends on the length of the amplified segment.

Table 19: Temperature profile of the PCR

Temperature	Time	Number of cycles	Step
95 °C	10 min	1	Initial denaturation
95 °C	30 s		Denaturation
X °C	20 s	35	Annealing
72 °C	1 min		Elongation
72 °C	5 min	1	Final elongation

3.2.2.4 Mutagenesis

For deletion and substitution of amino acids, directed mutation of the coding sequence was performed by mutagenesis PCR. Reaction is given in Table 20, the temperature profile can be found in Table 21.

Table 20: Site-directed mutagenesis reaction

Component	Concentration	Volume/amount	Final concentration
Polymerase buffer	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	-	30 ng	-
Pfu Turbo DNA Polymerase	2.5 $\frac{U}{\mu L}$	1 µL	0.05 $\frac{U}{\mu L}$
Aqua bidest	-	*ad* 50 µL	-

Table 21: Temperature profile of the site-directed mutagenesis PCR

Temperature	Time	Number of cycles	Step
95 °C	30 s	1	Initial denaturation
95 °C	30 s		Denaturation
X °C	1 min	18	Annealing
68 °C	11 to 24 min		Elongation

Alternatively, the QuikChange® Multi Site-Directed Mutagenesis Kit was used according to the protocol. The temperature profile utilized for this PCR is shown in Table 22.

Table 22: Temperature profile of the multi site-directed mutagenesis PCR

Temperature	Time	Number of cycles	Step
95 °C	1 min	1	Initial denaturation
95 °C	1 min		Denaturation
X °C	1 min	30	Annealing
65 °C	24 min		Elongation

PCR products were transformed into bacterial cells (see 3.2.2.1), plasmid DNA was isolated (see 3.2.2.2) and sequenced (see 3.2.2.5). By aligning of original and mutated sequences, the products were checked for the right sequence.

3.2.2.5 Sequencing PCR

For Sanger sequencing the Applied Biosystems ABI BigDye® Terminator v3.1 was used, which contains besides the reagents required for standard PCR also fluorescence-labeled dideoxynucleotides (ddNTPs) utilized for sequence analysis. The sequencing reaction composition is listed in Table 23, the PCR program can be taken from Table 24. At this, single-stranded DNA molecules terminated by a ddNTP are generated in varying lengths. The in-house sequencing team determined the base pair order. Interpretation of the sequences was done using BioEdit and MegAlign.

Table 23: Sequencing PCR reaction

Component	Concentration	Volume/amount	Final concentration
Abi buffer	5x	1.5 µL	0.75x
BigDye 3.1	-	1 µL	-
Sequencing primer	10 µM	0.5 µL	500 nM
Template	-	150-300 ng	-
Aqua bidest	-	*ad* 10 µL	-

Table 24: Temperature profile of the sequencing PCR

Temperature	Time	Number of cycles	Step
96 °C	2 min	1	Initial denaturation
96 °C	10 s	25	Denaturation
X °C	10 s		Annealing
60 °C	4 min		Elongation

3.2.2.6 Colony PCR

Colony PCR was used for screening bacteria colonies on agar plates for clones containing the desired plasmid. Therefor, colonies were picked and resuspended in 10 µL *Aqua bidest*. 5 µL of the suspension were used as template in the PCR reaction given in Table 25.

Table 25: Colony PCR reaction

Component	Concentration	Volume/amount	Final concentration
Polymerase buffer	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	-	5 µL	-
AmpliTaq Gold®	250 $\frac{U}{\mu L}$	0.125 µL	1.25 $\frac{U}{\mu L}$
Aqua bidest	-	*ad* 25 µL	-

The temperature profile of a Colony PCR is shown in Table 26. The initial denaturation is extended to 15 min for complete disruption of the bacterial cells, so that the DNA template is released and available for the PCR reaction.

Table 26: Temperature profile of the colony PCR

Temperature	Time	Number of cycles	Step
95 °C	15 min	1	Initial denaturation
95 °C	20 s	35	Denaturation
X °C	20 s		Annealing
72 °C	1 min		Elongation
72 °C	5 min	1	Final elongation

After checking the PCR reaction for amplification of the desired DNA segment via

agarose gel electrophoresis (see 3.2.2.7), the rest of the bacteria suspension could be used for inoculating fresh growth medium.

3.2.2.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed for separation of DNA fragments according to their length. For this, 0.5 to 1.5 g agarose were weighed, filled up to 50 or 100 g with 1x TAE Buffer and heated until agarose was completely dissolved. 1.5 or 3 µL GelRed were added, respectively, and gels were cast. Samples were mixed with an appropriate volume of 6x DNA sample buffer and applied to the gel. Electrophoresis was carried out at 80 to 95 V for 30 to 60 min. For determination of the size of the DNA fragments, Generuler 100 bp Ladder, Generuler 100 bp Plus Ladder, Generuler 1 kb Ladder or Generuler 1 kb Plus Ladder was utilized, depending on the expected size. Bands were detected using the GelDoc 2000.

3.2.2.8 DNA Extraction

For subsequent analysis, cellular DNA was isolated using the QIAamp DNA Blood Mini Kit according to the manual. For this purpose, cells are lyzed, the DNA is bound to a silica gel membrane and washed several times to remove cell debris and other components. Following this, DNA is eluted using 50 µL *Aqua bidest*.

3.2.2.9 Duplex Real-Time PCR

The real-time PCR (qRT) allows simultaneous amplification and detection of a desired DNA segment. In common with the standard PCR, the reaction comprises primers facilitating amplification of the desired segment. Additionally, a sequence specific probe is contained, which is linked to a reporter fluorophore as well as a quencher. The close proximity of the reporter to the quencher suppresses its fluorescence. Following degradation of the probe according to the 5′ to 3′ exonuclease activity of the Taq polymerase, this proximity breaks and in that way allows emission of fluorescence by the reporter, which can be detected after excitation with a laser.

The measurement takes place after every PCR cycle. The intensity of the generated fluorescence correlates with the amount of amplified product and consequently with the originally applied number of copies. The c_T (cycle threshold) value is declared as the cycle, in which the fluorescence signal surpasses the background signal for the first time.

In a duplex PCR, detection of two gene sequences takes places at the same time. By parallel accomplishment of a reaction specific for GAPDH, meaning primers and probe labeled with a different reporter dye, it can be checked that all targeted genes are amplified with similar efficiency. Additionally, this is required as a reference gene for relative quantification using the $2^{-\Delta\Delta c_T}$ method.

Tabel 27 gives the reaction for a duplex real-time PCR, the temperature profile utilized for this is listed in Table 28.

Table 27: Duplex real-time PCR reaction

Component	Concentration	Volume/amount	Final concentration
Taqman Universal Master Mix	2x	12.5 µL	1x
HIV-2_RT-For	10 µM	1.5 µL	600 nM
HIV-2_RT-Rev	10 µM	1.5 µL	600 nM
HIV-2_RT-Probe	10 µM	0.5 µL	200 nM
GAPDH_44-For	10 µM	0.1 µL	40 nM
GAPDH_45-Rev	10 µM	0.1 µL	40 nM
GAPDH-Probe	10 µM	0.1 µL	40 nM
$MgCl_2$	25 µM	1 µL	1 µM
Template	-	420 ng	-
Aqua bidest	-	*ad* 25 µL	-

Table 28: Temperature profile of the duplex real-time PCR

Temperature	Time	Number of cycles	Step
95 °C	10 min	1	Initial denaturation
95 °C	30 s		Denaturation
58 °C	1 min	45	Annealing, Read
72 °C	10 s		Elongation
72 °C	5 min	1	Final Elongation

3.2.2.10 Restriction Digest

For cloning and checking of vectors, DNA was digested using specific bacterial endonucleases. These enzymes recognize their own unique palindrome sequence and thereupon cut the DNA mostly generating overhang bases. Buffers were chosen corresponding to the specification of the used enzymes. An exemplary restriction digest reaction is shown in Table 29. The reaction was carried out at 37 °C for 2 h and subsequently checked on an agarose gel (see 3.2.2.7).

Table 29: Restriction digest reaction

Component	Volume
Restriction endonuclease	0.5 µL
(Restriction endonuclease II)	(0.5 µL if required)
10x Buffer	2 µL
100x BSA	0.2 µL
Template	1 µg
Aqua bidest	*ad* 20 µL

3.2.2.11 Gel Extraction

DNA fragments were separated using agarose gel electrophoresis (see 3.2.2.7). For extraction of particular DNA fragments from agarose gels, the QIAquick Gel Extraction Kit was used according to the protocol. Gels were laid on the UV transilluminator and slices containing the desired fragments were excised using a clean scalpel. Afterwards, the gel is dissolved at 50 °C and DNA is bound to a silica gel membrane, purified by several washing steps and eluted with 40 µL *Aqua bidest*.

3.2.2.12 Ligation

By ligation, DNA fragments can be inserted into cut vectors. In this process, the T4 DNA ligase catalyzes the junction of the free 3′ hydroxyl end of one nucleotide with the 5′ phosphate end of an other by formation of phosphodiester bonds. The composition of a ligation reaction is given in Table 30.

Table 30: Ligation reaction

Component	Volume
T4 DNA Ligase	1 µL
10x Buffer	1 µL
Vector	X µL
Insert	X µL
Aqua bidest	*ad* 10 µL

As standard, 25 ng vector are employed. The applied amount of insert is calculated by the formula

$$\text{mass}_{\text{insert}}[\text{ng}] = 5 \cdot \text{mass}_{\text{vector}}[\text{ng}] \cdot \frac{\text{length}_{\text{insert}}[\text{bp}]}{\text{length}_{\text{vector}}[\text{bp}]}.$$

Reaction was incubated for 1 h at RT. Afterwards the ligase was inactivated at 65 °C for 10 min.

3.2.3 Protein Analysis

3.2.3.1 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was utilized for separation of proteins according to their size. 1.5 mm gels were cast using the BioRad system, containing 12 % acrylamide for resolving gel and 5 % acrylamide for stacking gel, respectively. The composition of gels is given in Table 31.

Table 31: Composition of the SDS gels

Component	Stacking gel	Resolving gel
0.5 M Tris-HCl Buffer pH 6.8	2.5 mL	-
1.5 M Tris-HCl Buffer pH 8.8	-	2.5 mL
Rotiphorese® Gel 30 (37.5:1)	1.7 mL	4 mL
10 % SDS	100 µL	100 µL
TEMED	10 µL	10 µL
APS	50 µL	100 µL
Aqua bidest	5.7 mL	3.4 mL

For preparation samples were mixed with an appropriate volume of 4x Laemmli sample buffer and boiled at 95 °C for 10 min. Samples containing the HIV coRev protein were heated at 60 °C for 5 min. This ensures complete denaturation of the proteins resulting in linearization. SDS binds to positively charged groups in the amino acid sequence of the protein, which causes the same overall negative charge for all proteins. Thereby, proteins are separated according only to their size, because the retention of larger proteins is stronger as they are held back by the net made up of the polyacrylamide. Gels ran at 100 V for 10 min for stacking followed by 180 V for 40 to 50 min for separation of the proteins. Afterwards, gels were transferred into transfer buffer and immediately used for Western Blot analysis.

3.2.3.2 Western Blot

Western Blot is used to transfer the proteins separated during SDS-PAGE from the polyacrylamide gel to a PVDF (Polyvinylidene flouride) or nitrocellulose membrane. Gels were equilibrated in transfer buffer for 15 min, PVDF membranes were activated using ethanol for 3 min followed by equilibration in transfer buffer. In contrast, nitrocellulose membranes just had to be moistened in transfer buffer. Western Blot was carried out by semi-dry blotting. For this, the gel was applied to the membrane as a sandwich between two filter papers soaked with transfer buffer. Blotting was performed at 20 V for 40 min. Afterwards, the membrane was blocked for 1 h at RT using blocking buffer to avoid unspecific binding of antibodies to the membrane. Primary antibody was diluted in blocking buffer and applied to the membrane overnight at 4 °C. For removing excessive antibody, the membrane was washed three times for 10 min with washing buffer. Incubation of the secondary antibody diluted in blocking buffer, either conjugated to a fluorophore or horseradish peroxidase (HRP), was performed at RT for 1 h. The membrane was again washed three times for 10 min using washing buffer. Detection of HRP-conjugated antibodies was accomplished by adding the Super Signal West Dura Extended Duration Substrate. The enzyme catalyzes the oxidation of luminol leading to a simultaneous emission of light. This can be detected by exposition of the membrane to an X-ray film and subsequent development of the film using a film progression machine. Fluorophore-linked antibodies were detected using the Odyssey Infrared Imaging System.

3.2.3.3 Immunofluorescence

By immunofluorescence, the respective antigen can be visualized within the cell using fluorophore-labeled antibodies. For this, cells were sown onto cover slips within 6-well plates and transfected as described above (see 3.2.1.3 and 3.2.1.4). After incubation for 48 h, cells were washed with PBS and fixed with 2 % PFA for 30 min at RT. Residual PFA was removed by washing three times with PBS for 5 min. Cells were permeabilized by incubation with 0.5 % Triton for 30 min at RT to enable de-

tection of intracellular antigens. Following washing three times with PBS for 5 min, unspecific interactions of antibodies were blocked by incubation with 1 % skimmed milk powder in PBS for 1 h at RT. Primary antibody specific for the desired antigen was added in an appropriate dilution in 1 % skimmed milk powder in PBS and incubated at 37 °C for 1 h or at 4 °C overnight. Cells were washed with PBS three times with increasing time ranging from 5 to 15 min. Henceforth all steps were performed protected against light. The fluorophore-linked secondary antibody was diluted in 1 % skimmed milk powder in PBS and given onto the cells. After incubation for 1 h at RT, another three washing steps were done. Nuclei were stained using Hoechst 335258 for 15 min at RT. Excessive Hoechst was removed by washing three times with PBS and one time with *Aqua bidest*. Cover slips were embedded in Mowiol and cured at 4 °C overnight. Samples were analyzed using the confocal laser scanning microscope LSM780 from Zeiss.

3.2.3.4 CA-Antigen Capture ELISA

The p24 CA protein makes up the core of the viral particle and is commonly used for detection of HIV-1. In this work, the capture enzyme-linked immunosorbent assay (ELISA) was utilized for quantification of the viral load in cell supernatants by detecting CA as previously described by Sanders-Beer *et al.* [84]. This assay can also be employed for detection of HIV-2 CA, as used antibodies cross-react with this protein.

96-well microtiter plates were coated with monoclonal AG3.0 antibody diluted in carbonate bicarbonate buffer at 4 °C overnight. Plates were washed three times with PBS/Tween following blocking of the surface with PM for 1 h at 37 °C to avoid unspecific interactions. Samples were inactivated by adding 0.02 % Tween-20 and incubation at RT for 5 to 10 min. After removing the blocking buffer, samples were applied and titrated in PMT, so that 50 µL antigen per well were incubated for 1 h at 37 °C. Plates were washed three times using PBS/Tween and 50 µL per well HIV-1 plasma pool diluted 1:1000 in PMT were added. After incubation at 37 °C for 45 min,

plates were again washed three times with PBS/Tween. 50 µL α-human IgG coupled to peroxidase were diluted in PMT, given to the wells and incubated at 37 °C for 30 min. Substrate was prepared as following: 60 mg OPD were solved in 100 mL phosphate citrate buffer pH 5.0 and 20 µL H_2O_2 were added to 50 mL of this solution. Following a final washing step, 50 µL substrate were added per well and reaction was stopped after 2 to 15 min using 2.5 M sulfuric acid. Optical density of the color intensity was determined using the microplate reader at 492/620 nm. Measurements were furthermore used to normalize virus stocks for infection experiments.

3.2.3.5 Analysis of Shuttle Function/ Luciferase Assay

Cells were transfected with shuttle vectors encoding for firefly luciferase as a reporter gene. Upon transport of the transcribed shuttle vector from the nucleus of the transfected cell into its cytoplasm, the luciferase protein gets synthesized. The reaction of luciferin to oxyluciferin is catalyzed, which results in the emission of light.

$$\text{luciferin} + \text{ATP} \rightarrow \text{luciferyladenylate} + \text{PP}_\text{i}$$

$$\text{luciferyladenylate} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light}$$

The intensity of bioluminescence correlates with the amount of luciferase and thereby with the amount of vector shuttled to the cytoplasm. As an intern control, a vector containing *Renilla* luciferase was transfected.

Cells were sown in 96-well plates (see 3.2.1.3), transfected as described in 3.2.1.4 and incubated for 48 h. Medium was discarded and cells were washed one time with PBS. Activity of both luciferases was determined using the Dual-Luciferase® Reporter Assay System according to the protocol. Measurements were done using the Berthold Luminometer Centro LB 960.

4 Results

4.1 Staufen-1 promotes virus production

The HIV-2 molecular clone pROD and pCMV_FLAG-Staufen-1 were cotransfected at different ratios to investigate whether Staufen-1 has an impact on the particle production of HIV-2, and if so whether this effect is dose-dependent. For comparison, the same experiment was performed with the HIV-1 molecular clone pNL4-3 simultaneously. To ensure differences in particle production are not a result of different DNA quantities, the amounts of DNA were filled up with pcDNA4-V5-His/B as mock DNA. Mock transfected cells as well as cells transfected only with Staufen-1 were used as a control. Cells were grown for 48 h without changing the medium to avoid a loss of virions. Supernatants were collected as described in 3.2.1.5. For quantification of virus particle production, the amount of p24 for HIV-1 and p26 for HIV-2, respectively, in the supernatant of the cells was used as read-out by performing a CA-antigen capture ELISA (see 3.2.3.4). This assay had originally been designed for quantification of the HIV-1 p24 capsid protein, but due to the fact that the antibodies used in the assay cross-react with the HIV-2 p26 capsid protein, it can also be employed for this purpose.

As depicted in Figure 9A, coexpression of Staufen-1 increases the amount of p26 in the supernatant and therefore the production of new virions by factor three on average for HIV-2. This effect is dependent on the amount of *staufen-1* cotransfected with pROD, with a maximum at the ratio pROD:Staufen-1 of 1:0.5. In contrast, no virus particle production was detected in cells expressing only *staufen-1* and mock DNA, respectively. Similar results could be obtained for HIV-1 shown in Figure 9B, whereas p24 amounts were in general approximately fourfold higher compared to HIV-2.

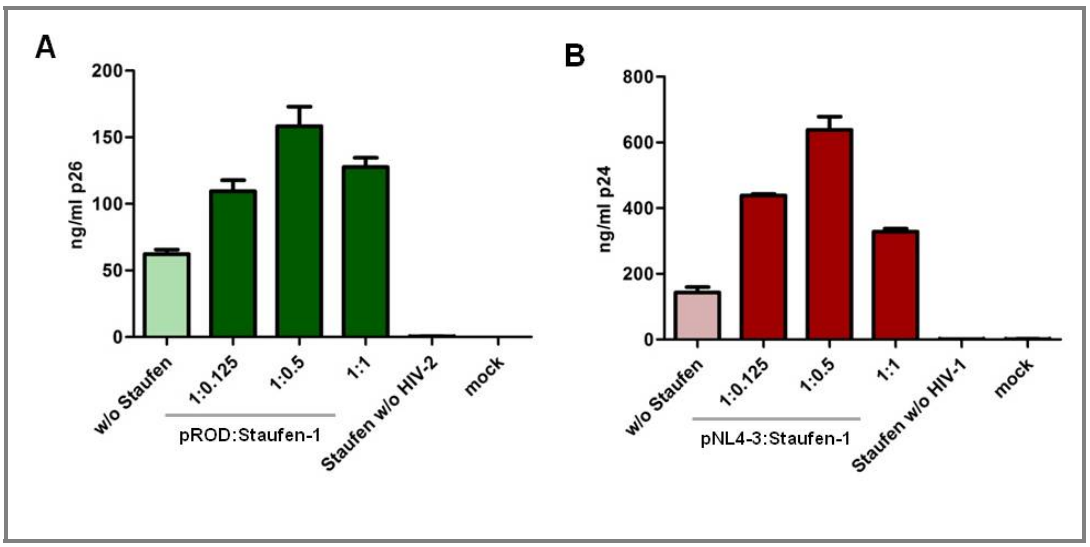

Figure 9: **Concentration of HIV particles in cell supernatants.** Cells were transfected with HIV-2 pROD (A) and HIV-1 pNL4-3 (B), respectively, and *staufen-1* at different ratios. Viral particle production was measured by CA-antigen capture ELISA of the supernatants. Presence of Staufen-1 enhanced particle production in a dose-dependent manner.

4.2 Staufen-1 does not affect HIV-2 expression kinetics

For determination of the point of highest particle production, cells were transfected with HIV-2 pROD and grown for up to 168 h without changing the medium in order to avoid losing virions. The same experiment was performed while cotransfection of pCMV_FLAG-Staufen-1 was done at the ratio of 1:1 to pROD, as this has been shown to clearly increase the amount of viral particles in the supernatant (approximately threefold, see 4.1). Mock transfected as well as naive cells were used as negative control. Discrepancies in the amount of transfected DNA were compensated by addition of mock DNA to the master mix. Cell lysates and supernatants were collected as described in 3.2.1.5 and the amount of p26 in the supernatants was determined by CA-antigen capture ELISA as an indicator for viral particle production.

Expression of FLAG-Staufen-1 was demonstrated by investigation of the cell lysates via SDS-PAGE (see 3.2.3.1) followed by Western Blot using a primary rabbit α-FLAG antibody (1:2000) and a secondary α-rabbit IRDye680RD antibody (1:5000) (see 3.2.3.2), as this protein is fused to a FLAG tag by expression from the pCMV vector. Primary mouse α-actin antibody (1:50000) combined with secondary α-mouse IRDye800CW antibody (1:5000) was used a loading control to ensure equal amounts of cell lysates loaded into the SDS gel. Signals were simultaneously detected using the Odyssey. As shown in Figure 10, expression of Staufen-1 could be verified in all samples transfected with pCMV_FLAG-Staufen-1, whereas no ectopic Staufen-1 could be detected in all other samples. By comparing the intensity of detected bands, the expression maximum of Staufen-1 could be observed at 48 h.

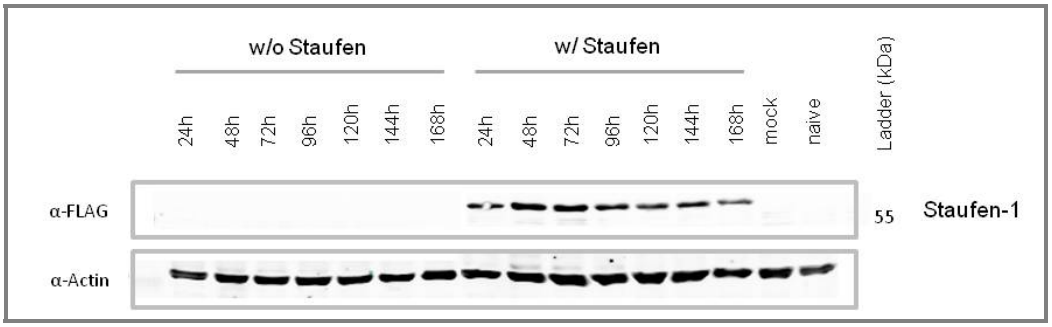

Figure 10: **Expression of Staufen-1 in the kinetics of HIV-2 pROD.** Cells were transfected with HIV-2 pROD, as well as HIV-2 pROD and pCMV_FLAG-Staufen-1. Mock transfected as well as naive cells were used as negative control. Cell lysates and supernatants were collected every 24 h. Expression of ectopical Staufen-1 was confirmed by Western Blot using an α-Flag antibody. α-actin antibody was used as loading control.

As Figure 11 demonstrates, the viral particle release peaks after 48 h incubation, independent of the presence of ectopic Staufen-1. This correlates with the expression maximum of Staufen-1 (compare Fig. 10). There are no conspicuous differences between the kinetics of HIV-2 pROD expression with and without coexpression of Staufen-1, respectively. Again, the increase in the amount of particles by cotransfection of pCMV_FLAG-Staufen-1 could be shown.

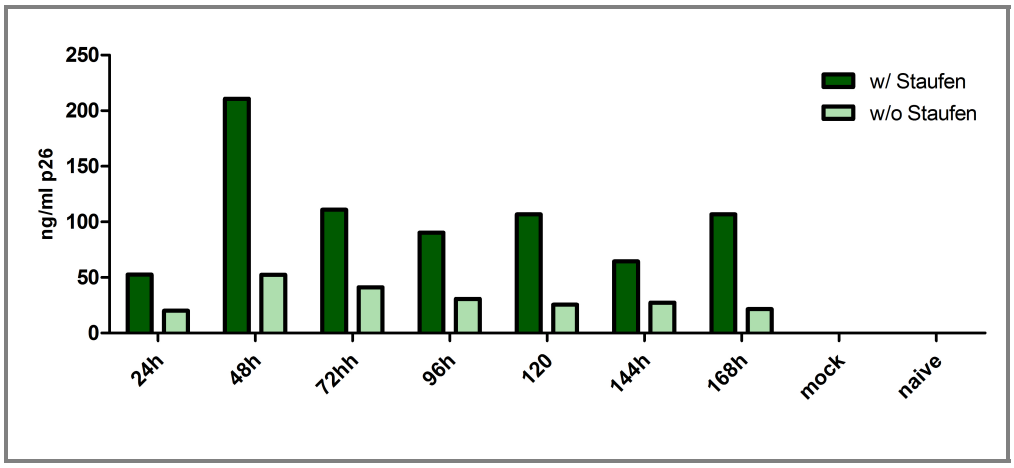

Figure 11: **Expression kinetics of HIV-2 with and without Staufen-1.** Cells were transfected with HIV-2 pROD, as well as HIV-2 pROD and pCMV_FLAG-Staufen-1. Mock transfected as well as naive cells were used as negative control. Cell lysates and supernatants were collected every 24 h. Amount of p26 in supernatants was determined by CA-antigen capture ELISA. Maximum of viral particle production could be shown after 48 h, with no differences in kinetics regarding ectopical Staufen-1 expression. Enhancement of particle production by Staufen-1 could be demonstrated for each individual time point of sampling.

4.3 Staufen-1 dsRBD3 domain is involved in mediating enhancement of HIV-2 particle production

Another aim was to determine the region by which the effect of Staufen-1 on the particle production is mediated. For this purpose, HIV-2 pROD was cotransfected with Staufen-1 mutants at the ratio of 1:1 (see Fig. 34). As negative control, cells transfected with mock DNA as well as naive cells were used. After incubation for 48 h, cells were lyzed and supernatants were collected. The amount of viral particles in the supernatants was quantified using CA-antigen capture ELISA, whereas cell lysates were used for Western Blot analysis. The Staufen-1 mutants plasmids were kindly provided by Jula Wamara, a co-worker in the lab.

Figure 12 depicts the ELISA results, indicating once more that production of viral particles is enhanced by cotransfection of *staufen-1*, leading to a threefold amount of

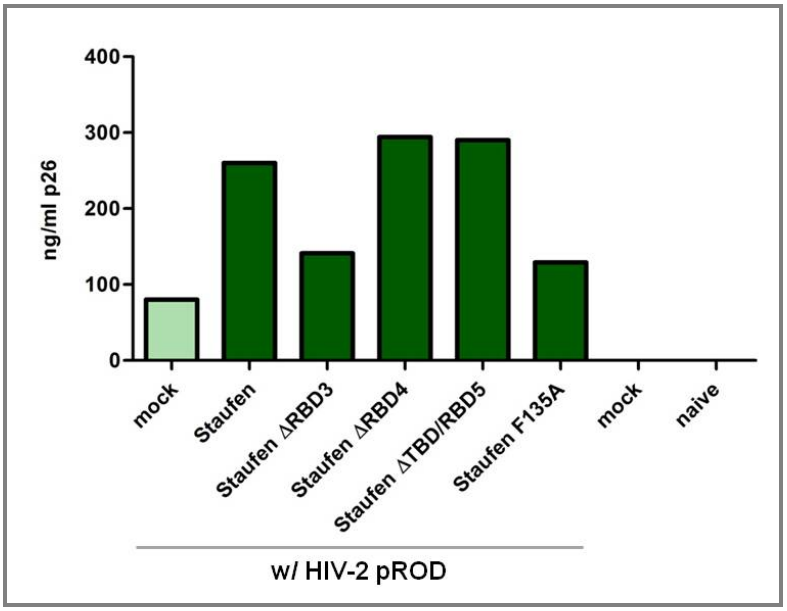

Figure 12: **Viral particle production upon coexpression of HIV-2 pROD and Staufen-1 mutants.** Cells were cotransfected with HIV-2 pROD and Staufen-1 mutants. Transfection of Staufen-1 as well as of the mutants Staufen-1 ΔRBD4 and ΔTBD/RBD5 resulted in the previously reported increase in particle production, whereas coexpression of Staufen-1 ΔRBD3 and F135A impairs this effect.

p26 in the cell supernatant compared to cells cotransfected with HIV-2 pROD and mock DNA. This effect could also be shown for the mutants Staufen-1 ΔRBD4 and Staufen-1 ΔTBD/RBD5. In contrast, the effect is strongly diminished in the presence of Staufen-1 ΔRBD3 and Staufen-1 F135A, both comprising not a functional dsRBD3. No p26 was detected for both mock transfected and naive cells.

Expression of Staufen-1 mutants was confirmed by SDS-PAGE with subsequent Western Blot analysis using a primary rabbit α-FLAG antibody (1:2000) and an appropriate α-rabbit IRDye680RD antibody as secondary antibody (1:5000), as all mutants feature the FLAG tag (see Fig. 13). α-GAPDH/ α-mouse IRDye800CW antibodies were used as loading control (in 1:2000 and 1:5000 dilutions, respectively). Expression level of both Staufen-1 ΔRBD3 and Staufen-1 F135A is lower compared to the other Staufen-1 mutants. Molecular weights, determined by comparison with the ladder carried along, match the theoretical molecular weights modified by the respective deletion.

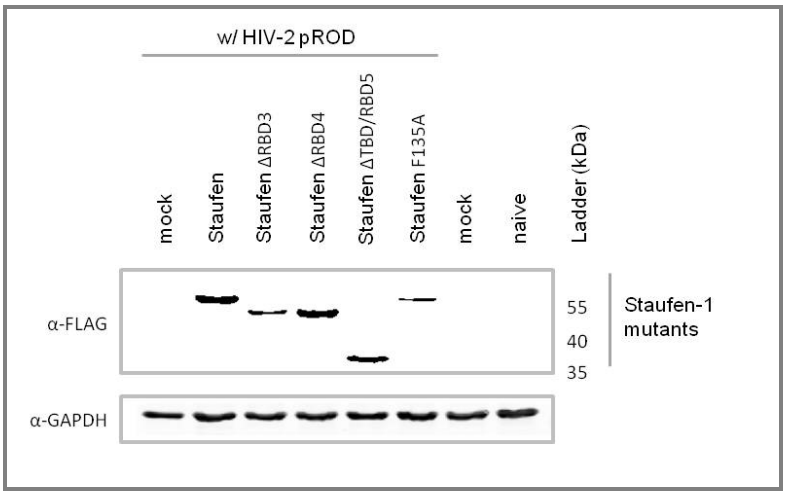

Figure 13: **Western Blot analysis of the coexpression of HIV-2 pROD and Staufen-1 mutants.** Cells were cotransfected with HIV-2 pROD and Staufen-1 mutants. Expression of Staufen-1 mutants was detected in the cell lysates using an α-FLAG antibody. Expression levels of Staufen-1 ΔRBD3 and Staufen-1 F135A were found to be lower than those of the other mutants. Equal amounts of input cell lysates were ascertained by utilization of an α-GAPDH antibody.

4.4 Downregulation of endogenous Staufen-1 impairs virus production

Overexpression of a protein is commonly used to analyze its role. Another possibility for investigating the protein function is to downregulate it, which should usually result in the opposite effect than overexpressing it. As Staufen-1 is already endogenously expressed, it is interesting to see whether downregulating its natural expression also affects the virus production of HIV-2 similar to the effect seen by overexpression of ectopic Staufen-1.

One way to attain downregulation of a protein is gene silencing achieved by RNA interference. Small interfering RNAs (siRNAs) of about 20 nucleotides size are generated by cleavage of double-stranded RNA (dsRNA) done by the RNase Dicer or Drosha. These siRNAs form the RNA-induced silencing complex (RISC) by interaction with a number of proteins and are able to specifically interact with the mRNA featuring the complementary sequence to the particular siRNA, thereby leading to degradation of the mRNA and consequently to downregulation of the encoded protein. Since siRNA is unstable and susceptible to degradation, as well as requires continuous treatment, short hairpin RNAs (shRNA) were chosen for downregulation of Staufen-1 within this work. Upon transfection, shRNAs are stably expressed from the encoding plasmid and subsequently cleaved in siRNAs. This enables constant synthesis of siRNAs, though efficiency of the downregulation is impaired at the same time [67].

Downregulation of endogenous Staufen-1 was performed by transfection of Staufen-1 specific shRNA. For this purpose, five shRNAs were mixed to equal amounts and transfected as an shRNA mix (see Tab. 33). The shRNA-coding plasmids were kindly provided by Jula Wamara. pLVTHM_shCofilin was used as shRNA negative control and mock transfected cells were used as transfection control, respectively. 24 h later, cells previously treated with shRNA were transfected with HIV-2 pROD. 48 h after the first transfection, cells were lyzed and

supernatants were collected. shRNA-mediated downregulation of endogenous Staufen-1 expression was confirmed via Western Blot using a polyclonal α-Staufen rat serum (1:4000) provided by Dr. Kirsten Hanke and Dr. Oliver Hohn (see Fig. 14). Rats had previously been immunized with recombinant Staufen-1 to produce polyclonal antibodies. An α-rat IRDye680RD antibody was used as secondary antibody (1:5000). α-GAPDH/α-mouse IRDye800CW were used as loading control, diluted 1:2000 and 1:5000, respectively. Signals were simultaneously detected using the Odyssey. The smaller band of cells treated with the shRNA Mix indicates that endogenous Staufen-1 is downregulated, whereas non-target shRNA as well as mock DNA does not influence the Staufen-1 expression level.

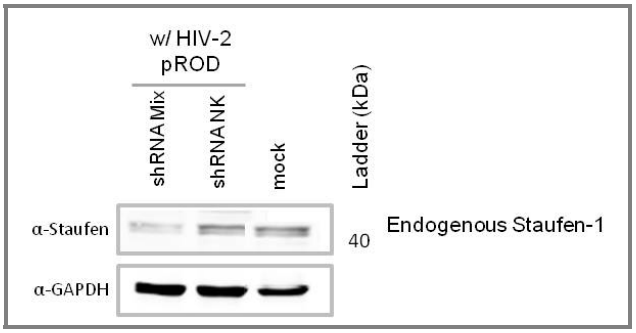

Figure 14: **Downregulation of endogenous Staufen-1 expression.** Cells were transfected with an shRNA mix specific for Staufen-1 as well as pLVTHM_shCofilin as shRNA NK, and 24 h later with HIV-2 pROD. As a negative control, mock DNA was transfected. Less endogenous Staufen-1 could be detected with an α-Staufen rat serum for cells treated with the shRNA mix compared to those transfected with non-target shRNA. Equal amounts of loaded cell lysates were guaranteed by detection of GAPDH.

Figure 15 depicts that downregulation of the endogenous Staufen-1 level results in diminished viral particle production approximately by half compared to transfection with non-target shRNA.

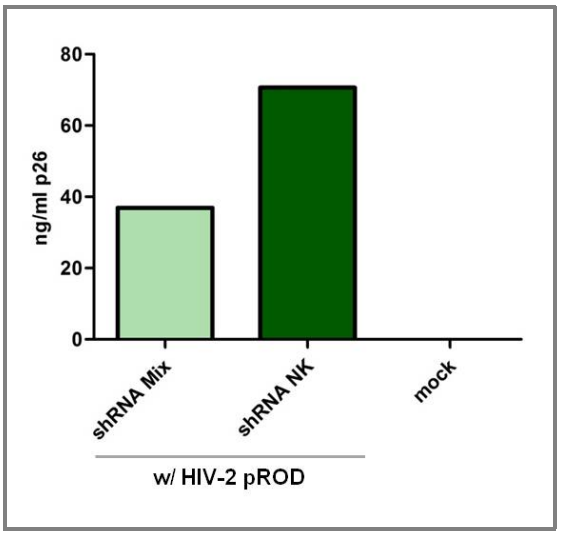

Figure 15: **Virus production upon downregulation of endogenous Staufen-1.** Cells were transfected with a Staufen-1 specific shRNA mix and pLVTHM_shCofilin as shRNA NK, respectively. Transfection of HIV-2 pROD was performed 24 h later. Mock transfected cells were used as negative control. Downregulation of endogenous Staufen-1 by the shRNA mix results in diminished amount of viral particles in the cell supernatant compared to cotransfection of non-target shRNA.

4.5 Impaired viral infectivity results in reduced integration rates

To establish determination of HIV-2 infectivity by detecting provirus integration into the cell genome using real-time PCR, C8166 cells were infected with an HIV-2 virus stock in different dilutions and incubated for 72 h. This virus stock was previously generated by transfection of HIV-2 pROD DNA to 293T cells and collection of supernatants after 72 h incubation according to 3.2.1.5, after which virions were stored in liquid nitrogen to conserve their infectious potential. Following infection, cellular DNA was extracted as described in 3.2.2.8 and a duplex real-time PCR was performed (see 3.2.2.9). DNA previously extracted from PBMCs was used as a calibrator, which were shown to be infected with HIV-2 by CA-antigen capture ELISA. As depicted in Figure 16, variations in the amount of virions and therefore of infectious particles deployed for infection could be detected relative to the calibrator. Following this, the duplex real-time PCR can be used as a read-out for the infectivity of viral particles.

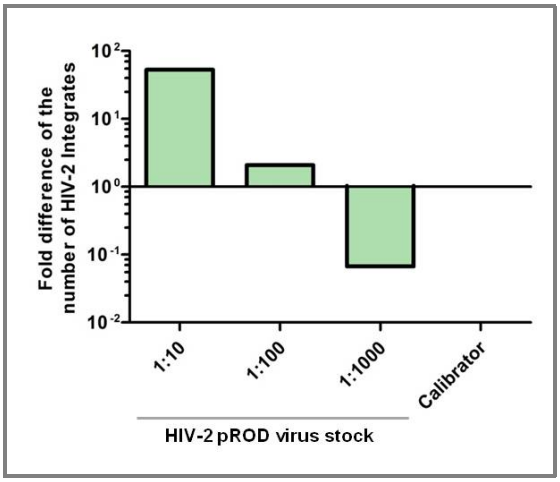

Figure 16: **Detection of diverse infectivities.** C8166 cells were infected with an HIV-2 pROD virus stock in varying dilutions. The differing amounts of infectious viral particles used for infection could be detected by real-time PCR as distinct quantities of proviral integrations into the cell genome referred to a calibrator.

4.6 Staufen-1 increases HIV-2 particle production in a dose-dependent manner, but diminishes virus infectivity

Another point of interest was to examine the influence of the intracellular Staufen-1 level on the viral infectivity as well as a possible correlation between the effect on infectivity and particle production. For this, HIV-2 pROD and pCMV_FLAG-Staufen-1 were cotransfected at different ratios, ranging from 1:0.17 to 1:4 (pROD:Staufen-1). Supernatants were collected and cells were lyzed following incubation for 48 h.

Expression of Staufen-1 was approved by Western Blot (see Fig. 17). Apparently, the signal intensity of Staufen-1, detected by α-FLAG antibody and α-rabbit IRDye680RD antibody (diluted 1:2000 and 1:5000, respectively), correlates with the transfected amount of pCMV_FLAG-Staufen-1. Equal amounts of loaded cell lysates were guaranteed by utilization of a primary mouse α-GAPDH antibody (1:2000) combined with a secondary α-mouse IRDye800CW antibody (1:5000).

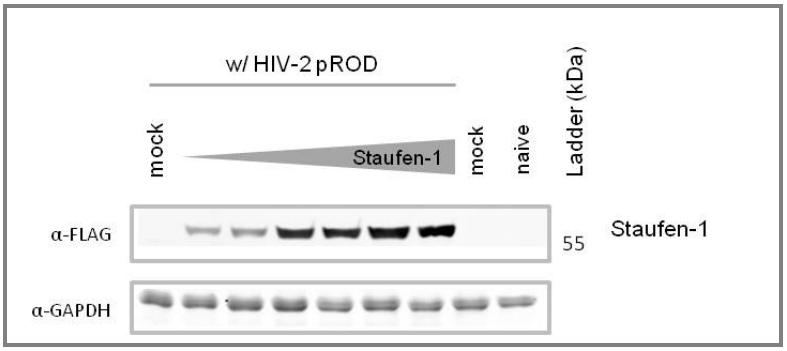

Figure 17: **Western Blot analysis of the coexpression of HIV-2 pROD and Staufen-1.** Cells were cotransfected with HIV-2 pROD and pCMV_FLAG-Staufen-1 at different ratios and lyzed after incubation for 48 h. Intensity of the Staufen-1 band detected by using an α-FLAG antibody is connected to the amount of pCMV_FLAG-Staufen-1 transfected. α-GAPDH antibody was used as loading control to confirm equal amounts of loaded cell lysates.

The amount of p26 in the supernatants was quantified using the CA-antigen capture ELISA (see Fig. 18). In all samples containing HIV-2 pROD cotransfected with Staufen-1, there were more viral particles detected compared to pROD cotransfected with mock DNA. The p26 concentration peaked at the ratio pROD:Staufen-1 1:2 and was declining in both directions.

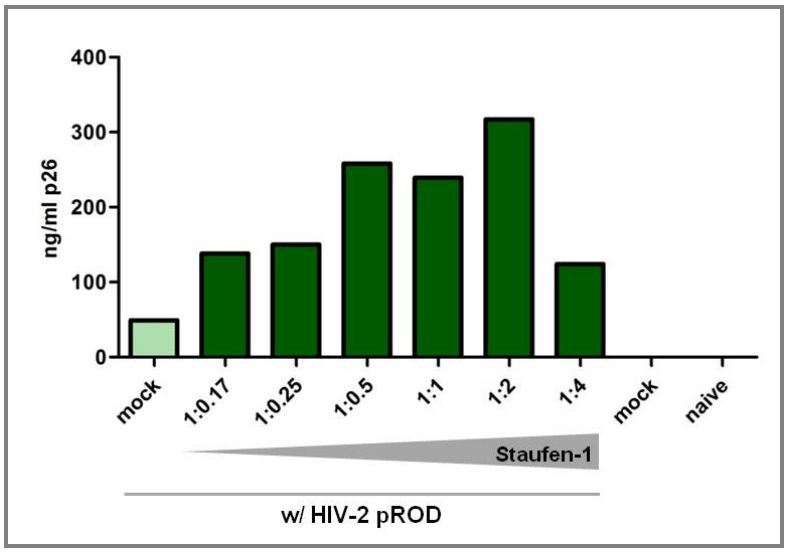

Figure 18: **Virus production upon coexpression of HIV-2 pROD and Staufen-1 transfected at different ratios.** Cells were cotransfected with HIV-2 pROD and pCMV_FLAG-Staufen-1 at different ratios and supernatants were collected after incubation for 48 h. The production of viral particles was quantified by CA-antigen capture ELISA. Coexpression of Staufen-1 increases the p26 concentration in general, while cotransfection of pROD and Staufen-1 at the ratio 1:2 results in maximum p26 amount. Both increase and decrease of the ratio impaired viral particle production.

Following this, C8166 cells were infected in a single-round infection with the produced virus stocks normalized to the previously determined p26 concentration (see 3.2.1.6) and DNA was extracted the next day. To detect the integration of proviral DNA, a duplex real-time PCR was performed using the extracted cellular DNA. The quantity of integrates can be used as a measure for the infectivity of viral parti-

cles used for infection, as has been shown in 4.5. As Figure 19 depicts, the ratio of Staufen-1 does not only influence the viral particle production, but also impairs the viral infectivity in a dose-dependent manner. Here, the virus stock generated by co-transfection of HIV-2 pROD and mock DNA was used as calibrator and set as 100 % infectious virions. The ratio pROD:Staufen-1 1:0.5 had the highest impact on the infectivity of the generated viral particles, leading to a fold difference of the number of HIV-2 integrates and therefore input amount of infectious virions reduced by nearly two log steps.

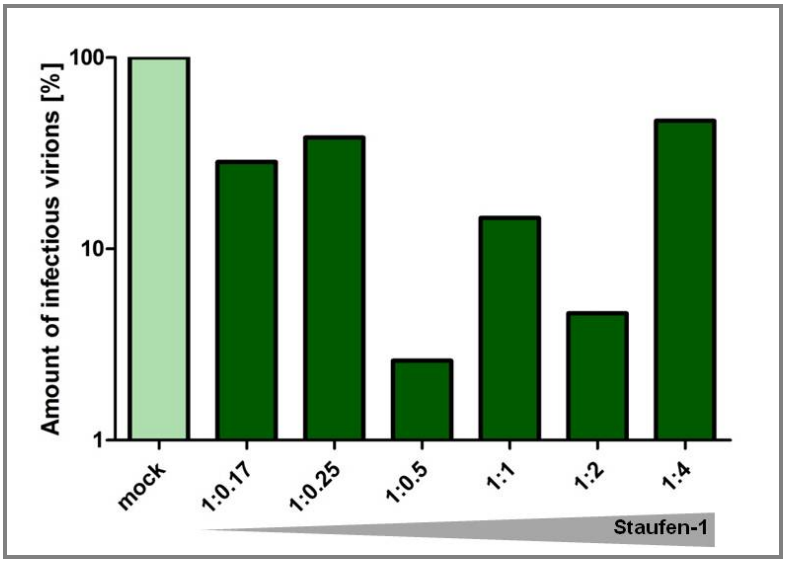

Figure 19: **Impact of Staufen-1 on the infectivity of HIV-2.** C8166 cells were infected in a single-round infection with virus stocks previously produced by cotransfection of HIV-2 pROD and pCMV_FLAG-Staufen-1 at different ratios. Extracted cellular DNA was investigated for integration of proviral DNA via duplex real-time PCR. All virus stocks generated by coexpression of pROD and Staufen-1 showed impaired viral infectivity, while highest reduction was achieved at the ratio pROD:Staufen-1 1:0.5.

4.7 Mutagenesis of HIV-2 coRev-V5

For studying the function of certain domains of a protein, it is a common method to introduce specific mutations into its coding nucleotide sequence. Thereby their functional relevance can be studied in following experiments. As most functional domains of the Rev protein have been revealed for HIV-1, the analogous regions for HIV-2 Rev had to be determined by alignment of the Rev protein sequences of two isolates each for HIV-1, HIV-2 and SIV, as the proteins are highly related (see Fig. 20). Functional domains were defined according to DiMattia *et al.* [23].

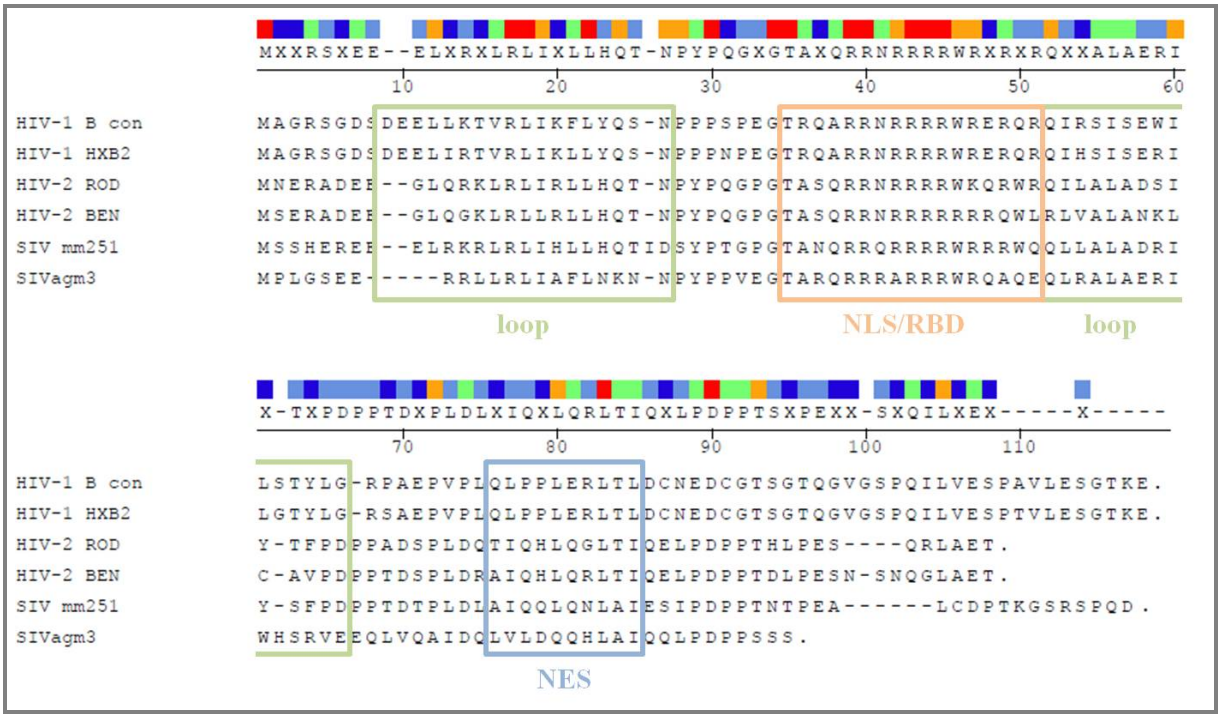

Figure 20: **Alignment of HIV-1, HIV-2 and SIV Rev sequences.** Functional domains of HIV-2 Rev were determined according to DiMattia *et al.* [23] by alignment of the amino acid sequences of HIV-1, HIV-2 and SIV Rev proteins, two isolates each.

First, HIV-2 coRev obtained as synthetic DNA was cloned into pcDNA4-V5-His/B, a CMV promoter driven expression vector, generating pcDNA4_HIV-2coRev-V5. Thus the protein is provided with the V5 tag and can be detected using commercially available antibodies specific for this tag. The term coRev indicates that the

Rev protein is codon usage optimized for enhancing its expression. Following this, mutations were introduced into pcDNA4_HIV-2coRev-V5, which have in parts been described by Malim *et al.* and Thomas *et al.* for HIV-1 coRev previously (see Fig. 21) [61] [96]. Deletion mutants comprising increasing N-terminal amino acid deletions were generated by PCR amplification using specific primers (see Tab. 15; BGH-Rev was employed as reverse primer), restriction digest of the amplified fragments and ligation into the pcDNA4 vector backbone. Furthermore, mutations which have been described to impair certain protein functions were inserted by oligonucleotide-directed mutagenesis (see 3.2.2.4 and Tab. 15 for mutagenesis primers sequences, respectively).

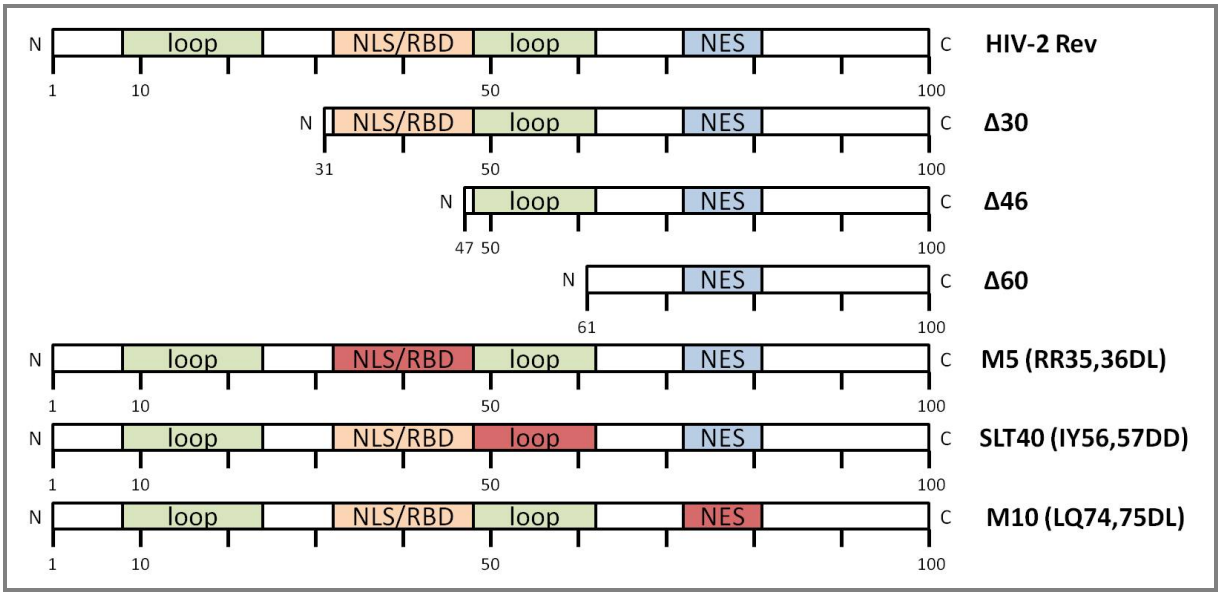

Figure 21: **Location of amino acid deletions and substitutions introduced into HIV-2 Rev.** The wild type Rev protein consists of 100 amino acids and features two multimerization domains ("loop", shown in green), an NLS and RBD (overlapping regions, orange) and an NES (blue). Mutants termed with Δ comprise N-terminal deletions of amino acids 2-30 (Δ30), 2-46 (Δ46) as well as 2-60 (Δ60). Red filled domains indicate inactivated functional domains, caused by substitution of amino acids 35,36 (M5), 56,57 (SLT40) and 74,75 (M10), respectively.

Afterwards, all mutants were sequenced (see 3.2.2.5) and the amino acid sequences were aligned against the coRev-V5 sequence to confirm proper mutagenesis of the protein (see Fig. 22).

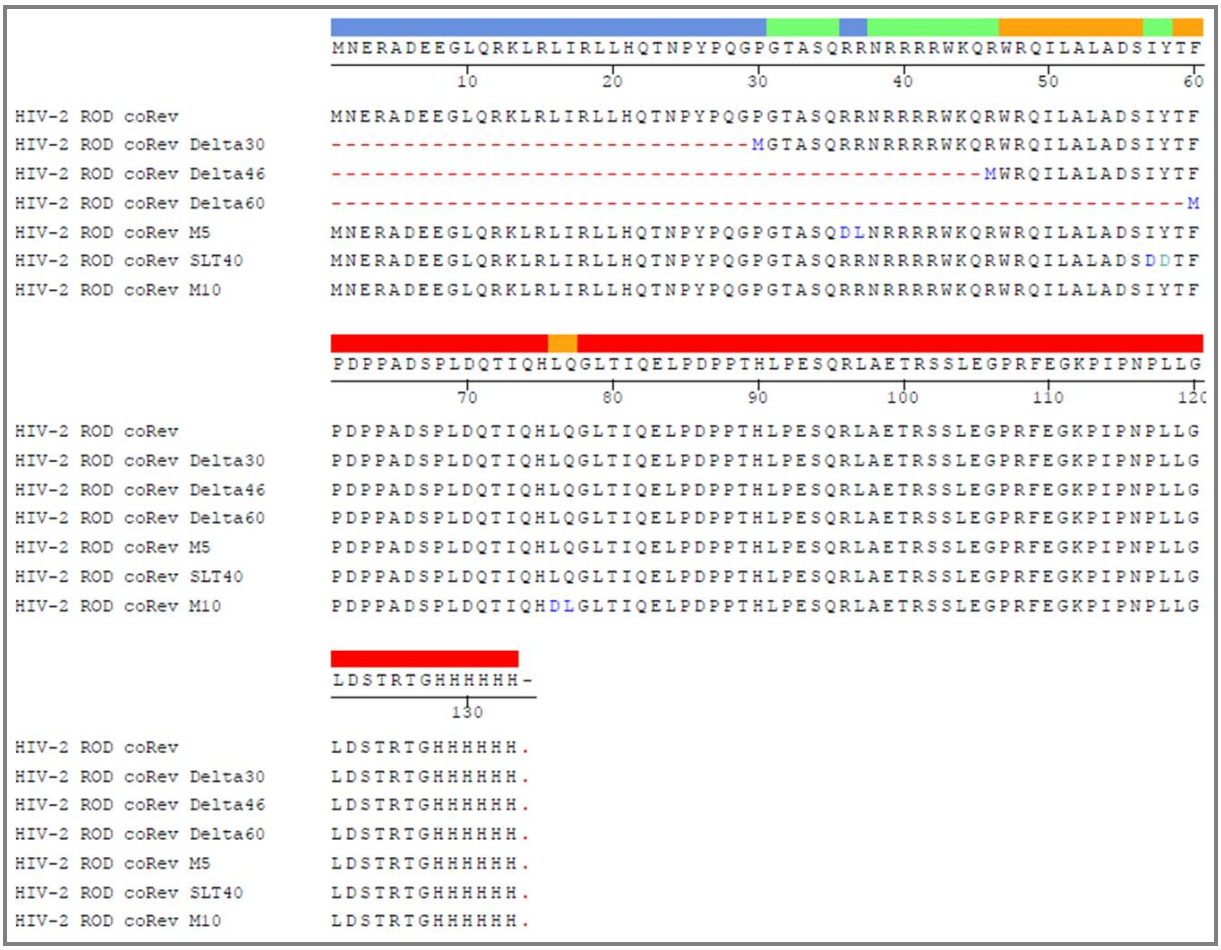

Figure 22: **Amino acid alignment of HIV-2 coRev with generated mutants.** Deletion mutants are indicated with a Δ and comprise increasing deletions of the N-terminus. M5, SLT40 and M10 feature single amino acid substitutions written in blue. Alignments were done using DNASTAR MegAlign.

4.8 Colocalization of coRev and Staufen

To confirm expression of pcDNA4_HIV-2coRev-V5, cells were transfected with the construct, incubated for 48 h and prepared for immunofluorescence microscopy as described in 3.2.3.3. A mouse α-V5 antibody was used as primary antibody, combined with a secondary α-mouse Alexa-568 antibody, for visualization of the pro-

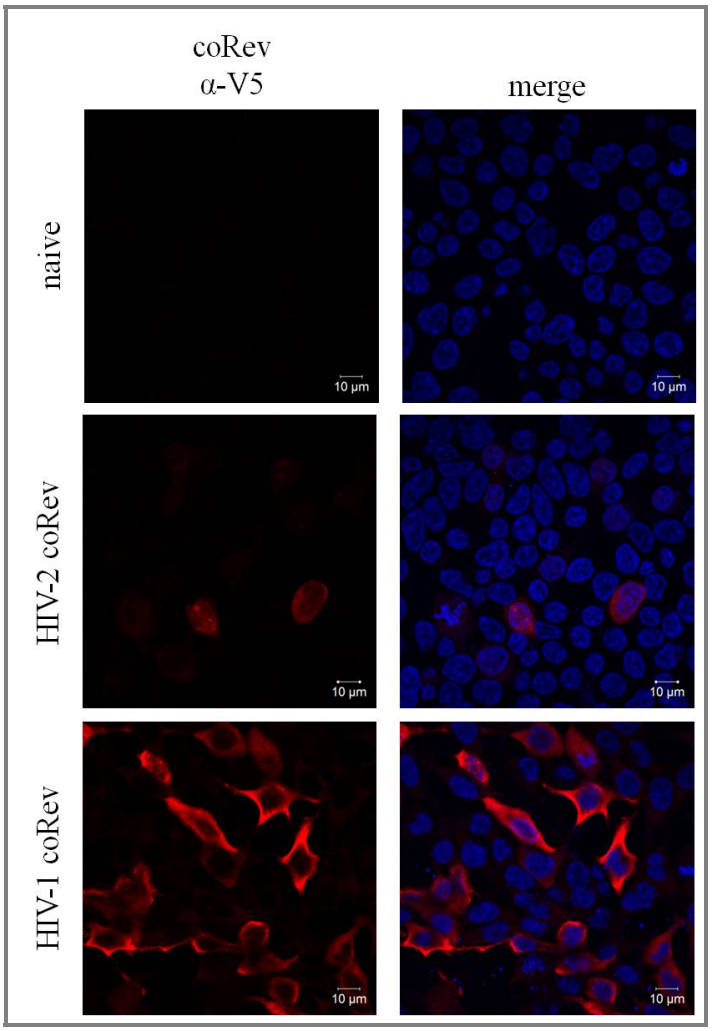

Figure 23: **Comparison of the coRev expression of HIV-1 and HIV-2.** Cells were transfected with plasmids coding for HIV-1 coRev and HIV-2 coRev, respectively. Naive 293T cells were taken as negative control. HIV-1 coRev is mainly found in the cytoplasm, whereas HIV-2 coRev is also located within the nucleus.

teins. Both antibodies were diluted 1:250. Nuclei were stained with Hoechst reagent. HIV-1 coRev was used as positive control, as its expression had been verified before. Naive 293T cells served as negative control. Images were taken using the confocal laser scanning microscope LSM780 from Zeiss.

Figure 23 displays that both HIV-1 coRev and HIV-2 coRev are expressed, although expression level of HIV-2 coRev seems to be lower compared to HIV-1. HIV-2 coRev is located in the nucleus and the cytoplasm as has been expected before, whereas HIV-1 coRev is mainly found within the cytoplasm.

For investigation of colocalization, HIV-2 coRev was cotransfected with Staufen-1. To exclude an eventual effect of the Staufen-1 tag on colocalization, two different plasmids were used coding for FLAG-Staufen-1 and Staufen-1-ECFP. As a control, HIV-1 coRev was cotransfected with pCMV_FLAG-Staufen-1. Protein expression was detected by primary mouse α-V5 and rabbit α-FLAG antibodies, following incubation with appropriate secondary antibodies (α-mouse Alexa-568 and α-rabbit Alexa-488, respectively), all diluted 1:250. Detection of Staufen-1-ECFP did not require the use of an antibody as it is fluorophore-tagged. Results indicate that HIV-2 coRev colocalizes with Staufen-1 in the cytoplasm, while this observation is independent of the Staufen-1 tag. Colocalization in the cytoplasm could also be shown for HIV-1 coRev and FLAG-Staufen-1 (see Fig. 24).

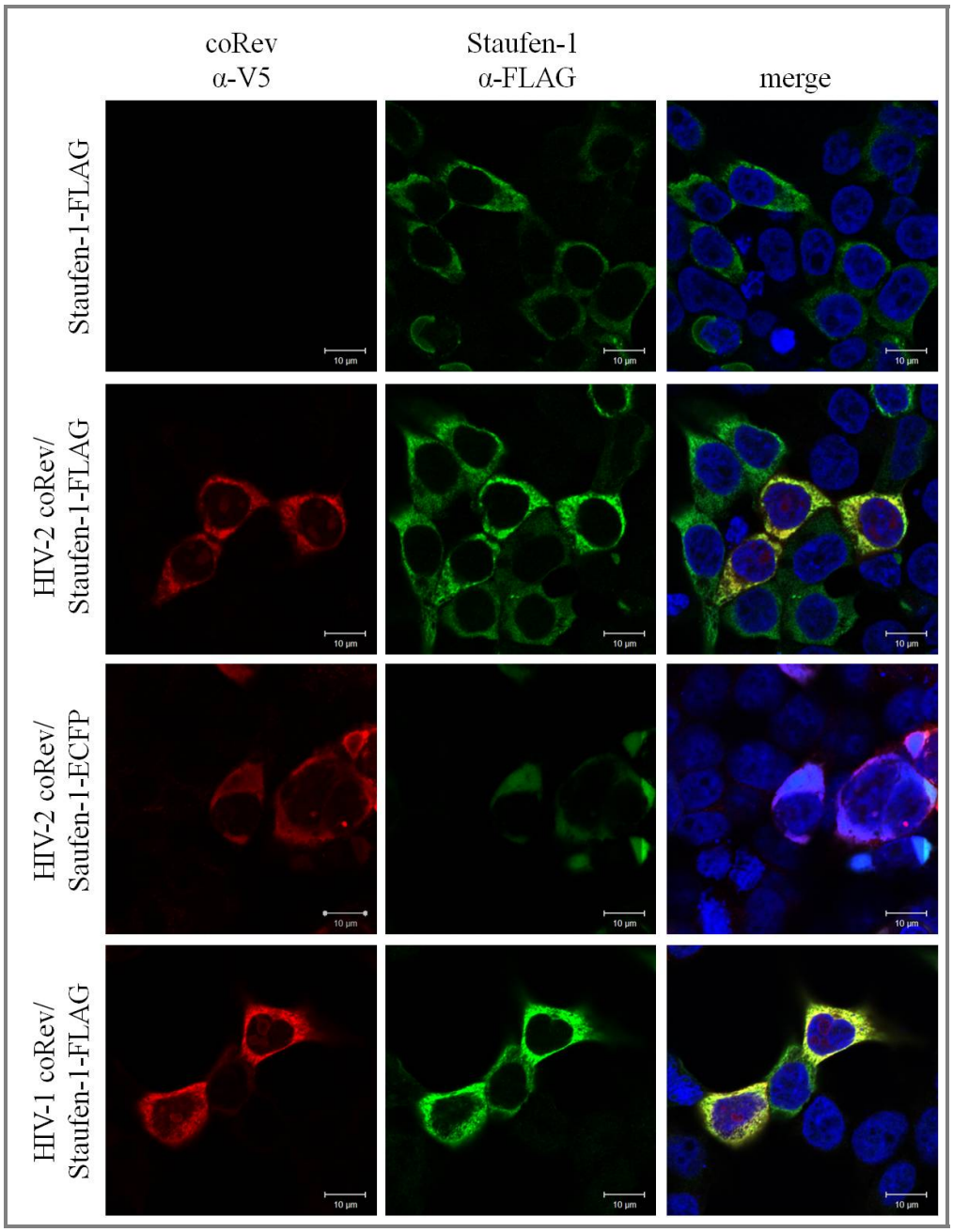

Figure 24: **Colocalization of coRev and Staufen-1.** Cells were cotransfected with plasmids encoding HIV-2 coRev as well as Staufen-1 with two different tags, respectively. HIV-1 coRev cotransfected with FLAG-Staufen-1 was used as a control. Both HIV-1 and HIV-2 coRev were shown to colocalize with Staufen-1 within the cytoplasm, independently of the Staufen-1 tag.

Before the HIV-2 coRev mutants could be used for experiments, their expression had to be ensured. This was done by immunofluorescence as described above. Simultaneously, the mutants were checked for colocalization with ectopic Staufen-1. As some of the mutants bear deletions or substitutions of amino acids in functional domains of the protein, their subcellular localization may differ from the wild type Rev. The mutants Δ46, Δ60 and M5 should not be encountered in the nucleus anymore due to their lack of a functional NLS domain, whereas the M10 mutant was expected to locate only within the nucleus since it has no functional NES domain for nuclear export. As localization of Rev mutants alone did not differ from the one upon coexpression of Staufen-1, only the latter results are shown here.

As Figure 25 and 26 depict, expression of all mutants could be confirmed. However, expression levels of Δ46 and Δ60 mutants appear to be very low. Deletion of the first loop domain did not affect localization of the protein (Δ30), whereas the two other deletion mutants seem to be found only within the cytoplasm, due to the fact that the NLS has been deleted here (see Fig. 25). Spatial distribution of the mutants M5, SLT40 and M10 could be shown as has been described for HIV-1 Rev mutants before [61] [96]. Since the NLS domain has been inactivated by substitution of amino acids 35 and 36, M5 is solely found within the cytoplasm. Substitution of the amino acids 56 and 57 within the second loop domain did not affect localization, as SLT40 is still found both within the nucleus and the cytoplasm. Substitution of amino acids 74 and 75 (M10) inactivates the NES domain, leading to localization of the protein exclusively in the nucleus (see Fig. 26).

All deletion mutants as well as M5 and SLT40 colocalize with Staufen-1 in the cytoplasm. No colocalization could be detected for M10 and Staufen-1, as the green fluorescence signal is spatially separated from the red one.

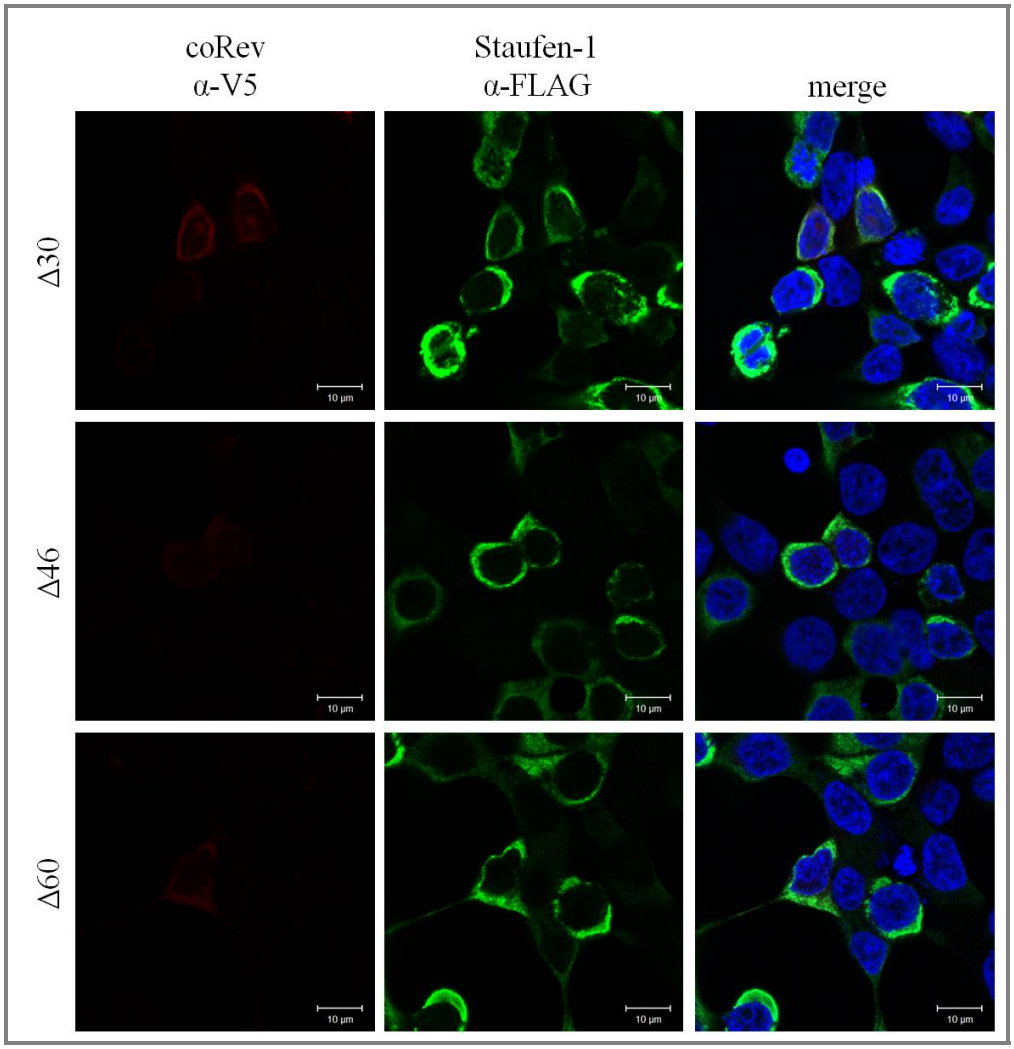

Figure 25: **Localization of Δ30, Δ46 and Δ60 Rev and colocalization with Staufen-1.** Cells were cotransfected with plasmids encoding the HIV-2 coRev mutants Δ30, Δ46 and Δ60 and Staufen-1, respectively. Red fluorescent Rev proteins and green fluorescent Staufen-1 were detected using confocal laser scanning microscopy. Nuclei were stained with Hoechst reagent. Δ30 is located both in the nucleus and the cytoplasm, whereas spatial distribution of Δ46 and Δ60 is restricted to the cytoplasm. Simultaneously, expression levels of these two mutants seem to be lower. All proteins colocalize with Staufen-1 within the cytoplasm. Bar is 10 µm.

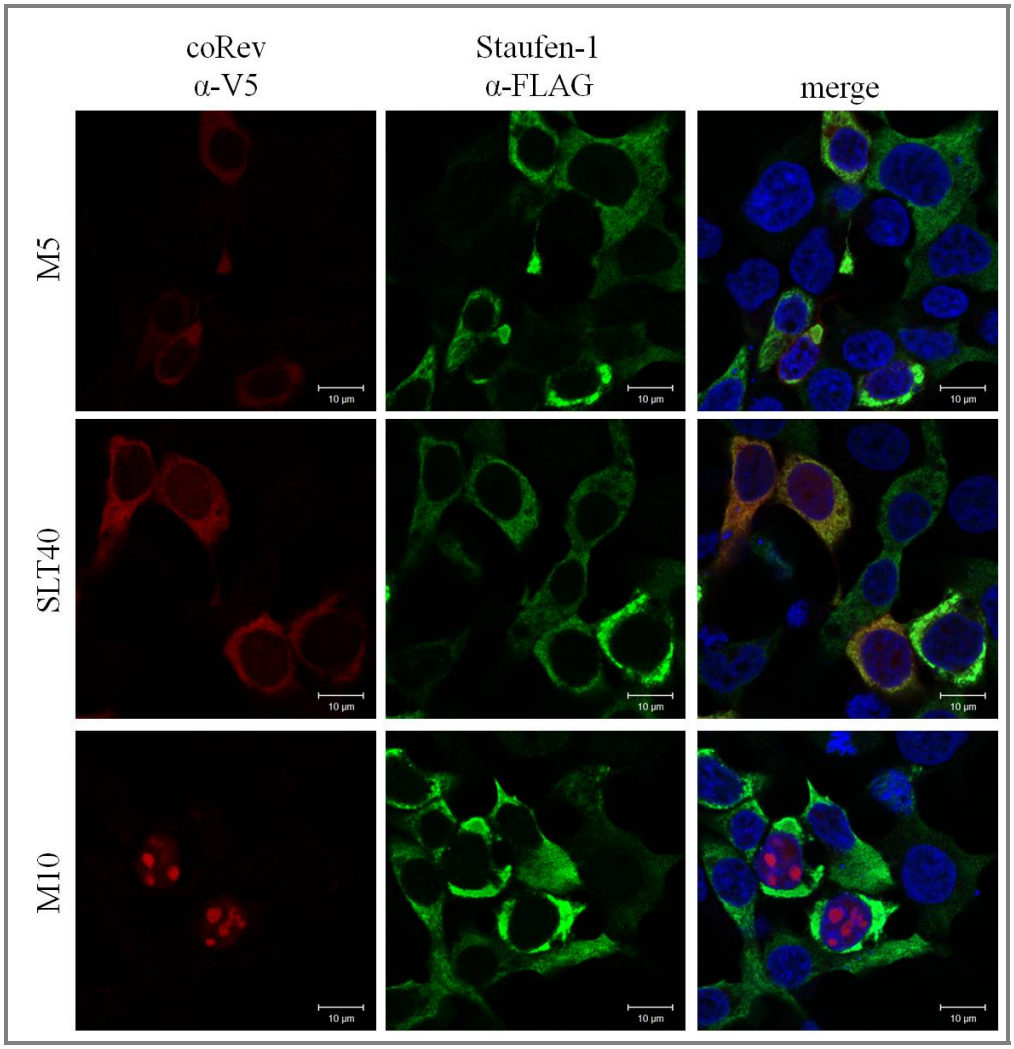

Figure 26: **Localization of M5, SLT40 and M10 Rev and colocalization with Staufen-1.** Cells were cotransfected with plasmids encoding the HIV-2 coRev mutants M5, SLT40 and M10 and Staufen-1, respectively. Red fluorescent Rev proteins and green fluorescent Staufen-1 were detected using confocal laser scanning microscopy. Nuclei were stained with Hoechst reagent. M5 mutant lacking a functional NLS domain was found solely within the cytoplasm. SLT40 mutation did not affect localization of the protein. Both mutants were shown to colocalize with Staufen-1 in the cytoplasm. NES-deficient M10 is exclusively found within the nucleus and does not colocalize with Staufen-1. Bar is 10 µm.

4.9 coRev mutants do not affect the production of viral particles in the presence of Staufen-1

Cotransfection of Rec has been shown to markedly increase the particle production of HERV [41]. For studying the impact of coRev as well as its mutants on the particle production of HIV-2 in the presence of ectopic Staufen-1, cells were transfected with HIV-2 pROD. pCMV_FLAG-Staufen-1 as well as plasmids encoding coRev and its mutants, respectively, were cotransfected. Following incubation for 48 h, supernatants as well as cell lysates were collected as described before (see 3.2.1.5). Supernatants were checked for viral particles by CA-antigen capture ELISA.

Particle production was again increased by cotransfection of pCMV_FLAG-Staufen-1, whereas presence of coRev as well as its mutants did not markedly affect

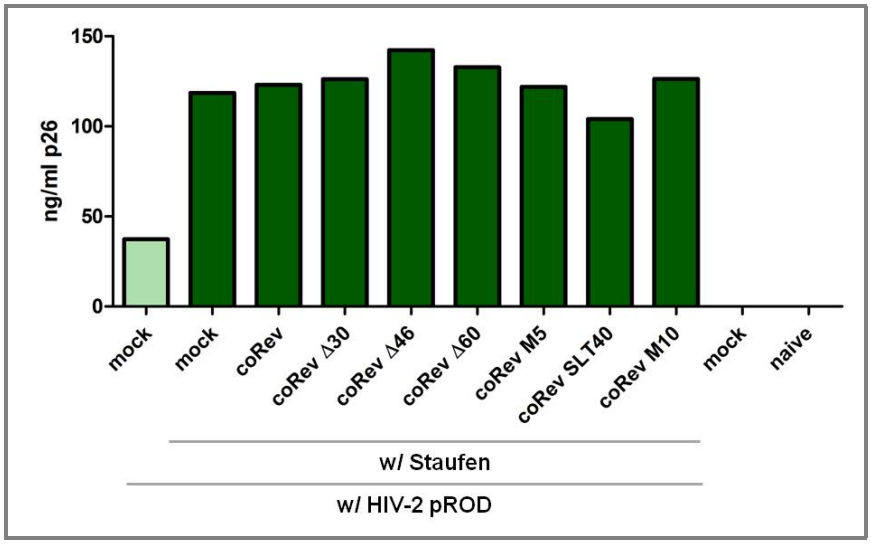

Figure 27: **Viral particle production upon coexpression of Staufen-1 and coRev mutants.** Cells were cotransfected with HIV-2 pROD, pCMV_FLAG-Staufen-1 and plasmids encoding coRev and its mutants. Supernatants were collected after incubation for 48 h and the amount of virions was determined via CA-antigen capture ELISA. Increase in the quantity of viral particles by expression of Staufen-1 was observed once more, while expression of coRev and its mutants had no additional effect on the particle production.

the p26 level (see Fig. 27).

To confirm expression of Staufen-1 as well as coRev, a Western Blot was performed using primary rabbit α-FLAG and mouse α-V5 antibodies diluted 1:2000. α-rabbit IRDye680RD as well as α-mouse IRDye800CW antibodies were used as secondary antibodies (1:5000). Detection of GAPDH using a mouse α-GAPDH antibody combined with secondary α-mouse IRDye800CW served as loading control. Expression could be shown for coRev as well as the mutants Δ30, M5 and SLT40. These findings correspond to the immunofluorescence results, where expression of these proteins could be visualized (see 4.8). As expression level of both Δ46 and Δ60 was found to be very low (see Fig. 25), Western Blot analysis might be not sensitive enough for

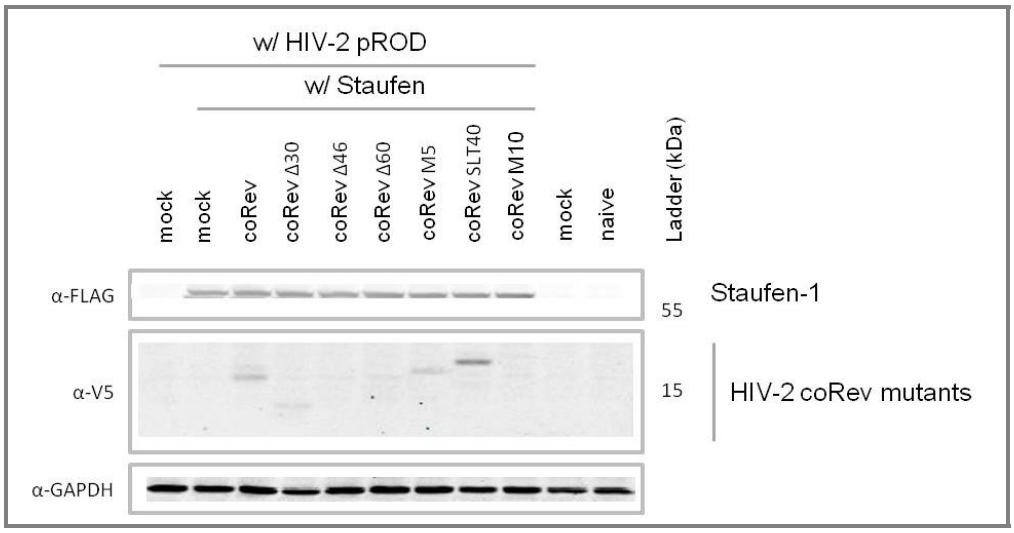

Figure 28: **Western Blot analysis of the coexpression of HIV-2 pROD, Staufen-1 and coRev mutants.** Cells were cotransfected with HIV-2 pROD, pCMV_FLAG-Staufen-1 and plasmids encoding coRev and its mutants. Cells were lyzed after incubation for 48 h and analyzed via SDS-PAGE followed by Western Blot using α-V5 and α-FLAG antibodies. coRev as well as the mutants Δ30, M5 and SLT40 could be detected, whereas no bands were visible for Δ46, Δ60 and M10 proteins. Expression of Staufen-1 was confirmed in all samples transfected with pCMV_FLAG-Staufen-1. GAPDH served as loading control for equal amounts of loaded cell lysates.

detection of these mutants. Since the M10 mutant is found exclusively in the nucleus (see Fig. 26), the cell lysis method applied here might not be sufficient for release of M10 proteins from the nucleus, resulting in no detection of M10 in the Western Blot. Staufen-1 was detected in all cells transfected with pCMV_FLAG-Staufen-1.

4.10 Staufen-1 expression does not enhance Rev/Rec-mediated mRNA transport, but Rev mutants have an impact

To examine whether Staufen-1 modulates Rev-dependent transport of viral RNAs, shuttle constructs were designed analogous to the two published by Hanke *et al.* [41], named Shuttle and ShuttleRcRE (see Fig. 29). These two constructs had originally been designed for examination of Rec-dependent RNA transport and comprise the HERV-K113 5'UTR with the major splice donor site, a gene encoding firefly luciferase as reporter gene as well as the HERV-K113 *env* and *gag* genes, containing a splice acceptor site within *env*. pcDNA4 served as vector backbone. Expression of Rec from these constructs was suppressed by introduction of four stop codons into its open reading frame, labeled as Stop ΔRec. To discriminate between non-specific and Rec-

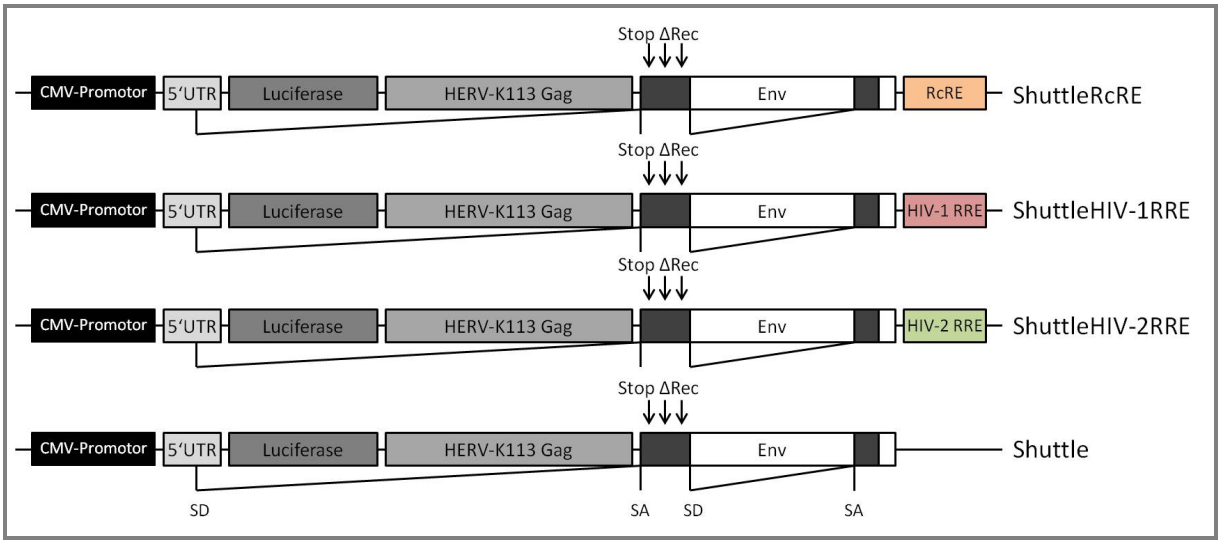

Figure 29: **Schematic overview of the shuttle constructs used in this work.** All constructs feature splice acceptor (SA) and splice donor (SD) sites to achieve measurement of the transport only of unspliced RNAs. Expression of Rec was abolished by introduction of stop codons into its open reading frame. ShuttleRcRE contains the RcRE for Rec-dependent transport, whereas the constructs ShuttleHIV-1RRE and ShuttleHIV-1RRE comprise the RRE sequences specific for HIV-1 Rev and HIV-2 Rev, respectively.

mediated RNA export from the nucleus, only the construct ShuttleRcRE contains the C-terminal Rec-responsive element (RcRE), which was shown to be crucial for Rec-mediated RNA transport [59] [60]. As the constructs include functional splice sites upstream and downstream of the firefly luciferase gene, this allows for exclusive measurement of the transport of unspliced RNAs.

By analogy with this, the RcRE was replaced by HIV-2 Rev RRE. For that, specific restriction sites for ClaI were introduced into the shuttle construct via oligonucleotide-directed mutagenesis (see 3.2.2.4) as well as into the flanking regions of HIV-2 RRE by PCR amplification using specific primers (see 3.2.2.3, Tab. 15 for all used primers). The same procedure was done with HIV-1 RRE to enable comparison of HIV-1 and HIV-2 Rev-dependent RNA transport. After subcloning of the RRE fragments into the pCR^{TM}4-TOPO® TA Vector using the TOPO® TA Cloning® Kit for Sequencing according to the manual, mutated shuttle vector as well as RRE fragments were digested with the ClaI restriction enzyme and ligated using a T4 DNA ligase. The obtained constructs ShuttleHIV-1RRE and ShuttleHIV-2RRE (see Fig. 29) were sequenced and aligned against the target sequence to confirm proper mutagenesis of the constructs (data not shown).

In the following experiments, firefly luciferase values were normalized by a co-transfected *Renilla* luciferase expression construct. Shown data were generated in eight replicates. Cells were grown in 96-well plates and transfected as described above (see 3.2.1.4). Following incubation for 48 h, cells were lyzed using 20 µL passive lysis buffer (Promega). 18 µL of the supernatant were transferred onto a white optical 96-well plate (Nunc) and activities of firefly luciferase and *Renilla* luciferase were sequently determined using the Dual-Luciferase® Reporter Assay System Kit. As mentioned above, the luciferase converts luciferin into oxyluciferin while concurrently emitting light (see 3.2.3.5). This light emission was measured in a Berthold Luminometer. Samples for investigation of Rec-dependent RNA transport served as control, as this has previously been examined by Hanke *et al.* [41].

For determination of non-specific RNA transport in the absence of the RRE/RcRE, the construct Shuttle was cotransfected with Staufen-1 as well as HIV-1 coRev, HIV-2 coRev and HERV-K113 oricoRec encoding plasmids, respectively, in equal amounts. The term orico stands for a **co**don-optimized version of the Rec protein comprising its **ori**ginal amino acid sequence at the time of virus integration into the human germ line. Additionally, cells transfected only with HIV-1 or HIV-2 coRev were investigated. Mock transfected cells were used as negative control. As shown in Figure 30, only a small amount of light is emitted in samples transfected with the construct

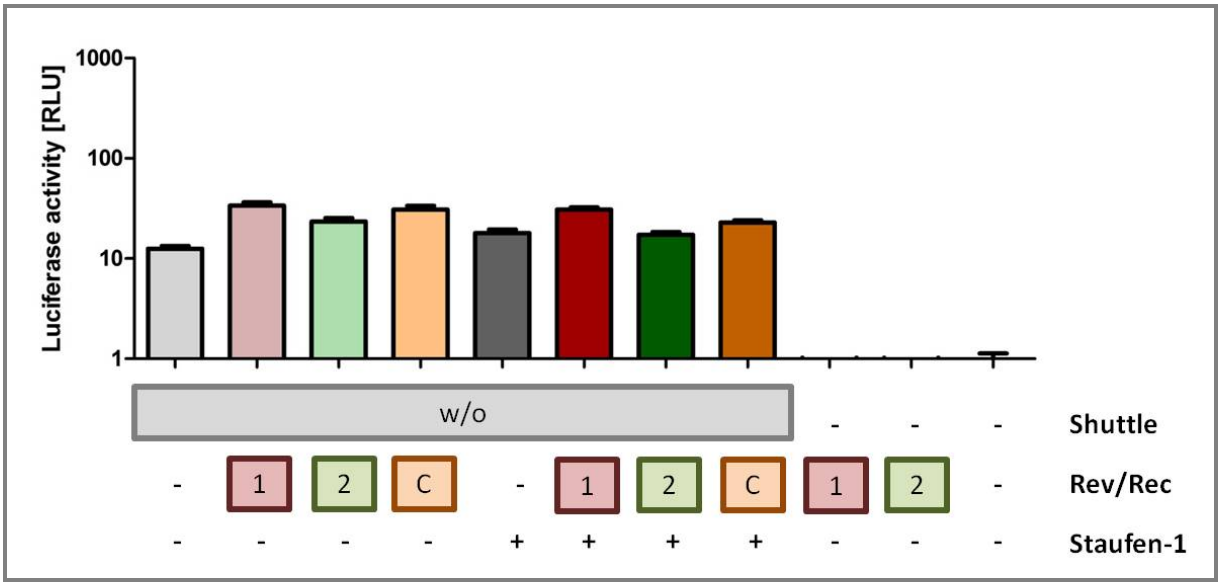

Figure 30: **RNA transport in the absence of RRE/RcRE.** Cells were transfected with the construct Shuttle, as well as HIV-1/HIV-2 coRev or HERV-K113 oricoRec coding plasmids (indicated with 1, 2 and C, respectively). Cotransfection of pCMV_FLAG-Staufen-1 is marked as +. Following incubation for 48 h, cells were lyzed and firefly luciferase activity was determined. Values were normalized to the activity of a cotransfected *Renilla* luciferase. Expression of the Shuttle construct alone caused only a slight emission of light. This was not affected by coexpression neither of Staufen-1 nor of HIV-1 coRev, HIV-2 coRev and HERV-K113 oricoRec. No light was emitted upon separate transfection of HIV-1 coRev, HIV-2 coRev and mock DNA.

Shuttle. Coexpression neither of Staufen-1 nor of HIV-1 coRev, HIV-2 coRev and HERV-K113 oricoRec led to a notable modification of this amount. No or almost no light emission was measured upon transfection of HIV-1 coRev, HIV-2 coRev and mock DNA. Therefore, HIV-1 coRev, HIV-2 coRev, HERV-K113 oricoRec and Staufen-1 do not cause an increase in expression of the reporter construct lacking the RRE/RcRE via unspecific enhancement of export and/or translation.

Data depicted in Figure 31 confirm that these previous observations are also true for the HIV-2 coRev mutants, which were cotransfected with the construct Shuttle as well as mock DNA and pCMV_FLAG-Staufen-1, respectively, resulting in no impact of any given combination of plasmids on the amount of emitted light. Thus, eventual

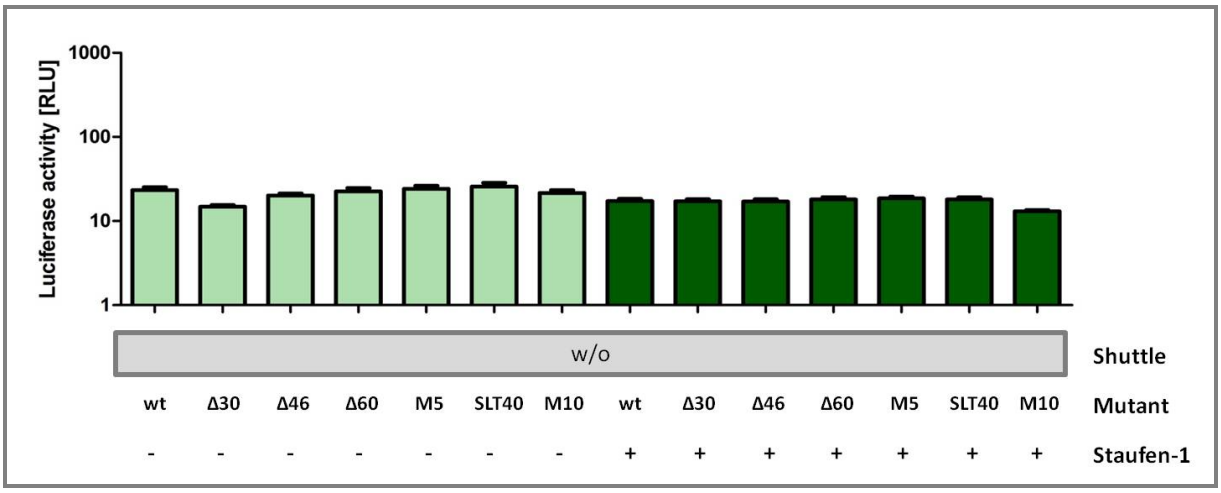

Figure 31: **RNA transport in the absence of HIV-2 RRE upon expression of HIV-2 coRev mutants.** Cells were transfected with the construct Shuttle, as well as DNA encoding HIV-2 coRev mutants. Cotransfection of pCMV_FLAG-Staufen-1 is marked as +. Following incubation for 48 h, cells were lyzed and firefly luciferase activity was determined. Values were normalized to the activity of a cotransfected *Renilla* luciferase. Reporter expression levels of the construct Shuttle were found to be independent of the coexpression of HIV-2 coRev mutants as well as Staufen-1, and therefore the mutants do not cause an unspecific transport of this construct lacking the HIV-2 RRE.

variations of reporter construct expression using the RRE/RcRE-containing shuttle constructs should be due to specific Rev/RRE- and Rec/RcRE-dependent transport.

For determination of Rev/Rec-dependent transport of RNA, the particular shuttle constructs were cotransfected with mock DNA, pCMV_FLAG-Staufen-1 and the respective coRev/oricoRec plasmid. Here, samples had to be diluted 1:20 with PBS

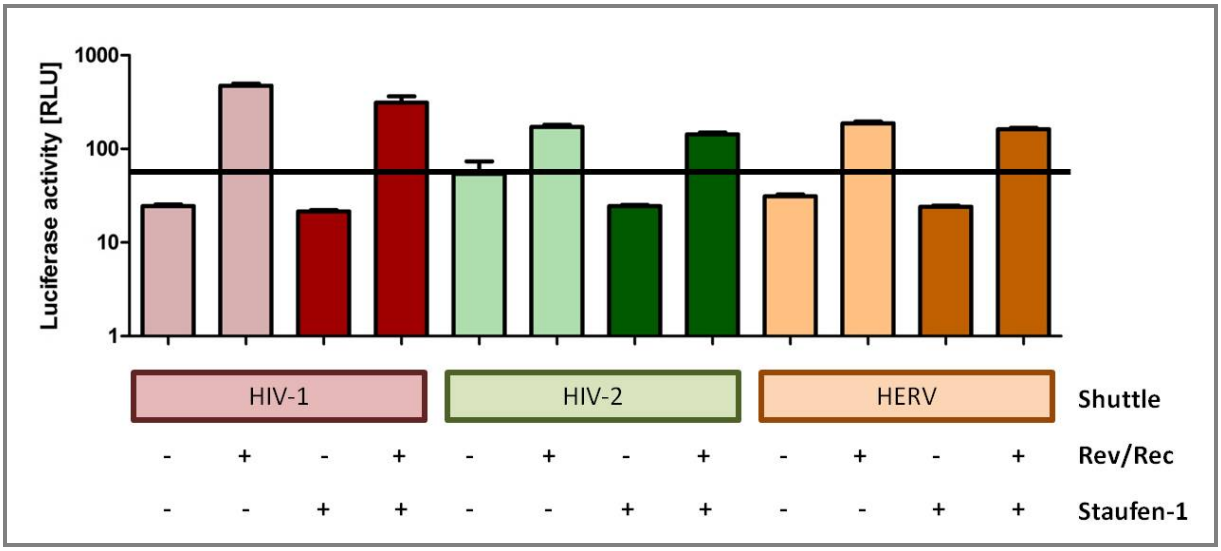

Figure 32: **RNA transport in the presence of RRE/RcRE.** Cells were transfected with the particular shuttle constructs, and HIV-1/HIV-2 coRev or HERV-K113 oricoRec coding plasmids. Cotransfection of pCMV_FLAG-Staufen-1 is marked as +. Following incubation for 48 h, cells were lyzed and firefly luciferase activity was determined. Values were normalized to the activity of a cotransfected *Renilla* luciferase. Black line is threshold, meaning average luciferase activity measured for non-specific transport of the construct Shuttle plus three times the standard deviation (see Fig. 30). Coexpression of coRev/oricoRec markedly increased transport of the reporter constructs, while simultaneous expression of Staufen-1 slightly impaired this effect for all constructs. By contrast, luciferase activity was not altered upon coexpression of Staufen-1 alone. Thus, transport specifically mediated by HIV-1/HIV-2 Rev and HERV-K113 oricoRec, respectively, can be determined using the generated shuttle constructs without being affected by Staufen-1 expression.

prior to luciferase activity determination to obtain a light signal intensity within the detection range of the experiment. Transfection of the shuttle constructs alone as well as with pCMV_FLAG-Staufen-1 did not influence the reporter expression level observed for the particular construct as they remained below the threshold (black line, defined as the mean of all values measured for non-specific RNA transport of the construct Shuttle under equal conditions, shown in Fig. 30, plus three times the standard deviation). However, the light signal was conspicuously increased upon coexpression of coRev/oricoRec, whereas this effect was slightly diminished upon concurrent expression of Staufen-1. These observations were found to be true for HIV-1, HIV-2 as well as HERV-K113 (see Fig. 32). Therefore, the constructs cloned in this work could be shown to be utilizable for investigation of HIV-1/HIV-2 Rev-dependent transport of RRE-containing RNAs. At the same time, no or only little impact of Staufen-1 could be observed.

The same experiment was performed with ShuttleHIV-2RRE and the HIV-2 coRev mutants, cotransfected with pCMV_FLAG-Staufen-1 or mock DNA, to investigate the impact of the respective mutation on the shuttle function of the Rev protein. Results are shown in Figure 33. Here, the threshold was calculated as the average of non-specific transport of the construct Shuttle under equal conditions (see Fig. 31) plus three times the standard deviation, visualized as black line. Only the SLT40 mutant, comprising a non-functional second multimerization domain (see Fig. 21), was able to increase the specific transport and/or translation of the reporter construct ShuttleHIV-2RRE at levels comparable to wild type HIV-2 coRev, while coexpression of Staufen-1 did not visibly affect this resulting light signal enhancement. The Δ30 mutant was only able to enhance reporter expression levels and therefore light emission in the presence of Staufen-1, but not while cotransfected with mock DNA. Coexpression of all other mutants did not affect reporter expression level.

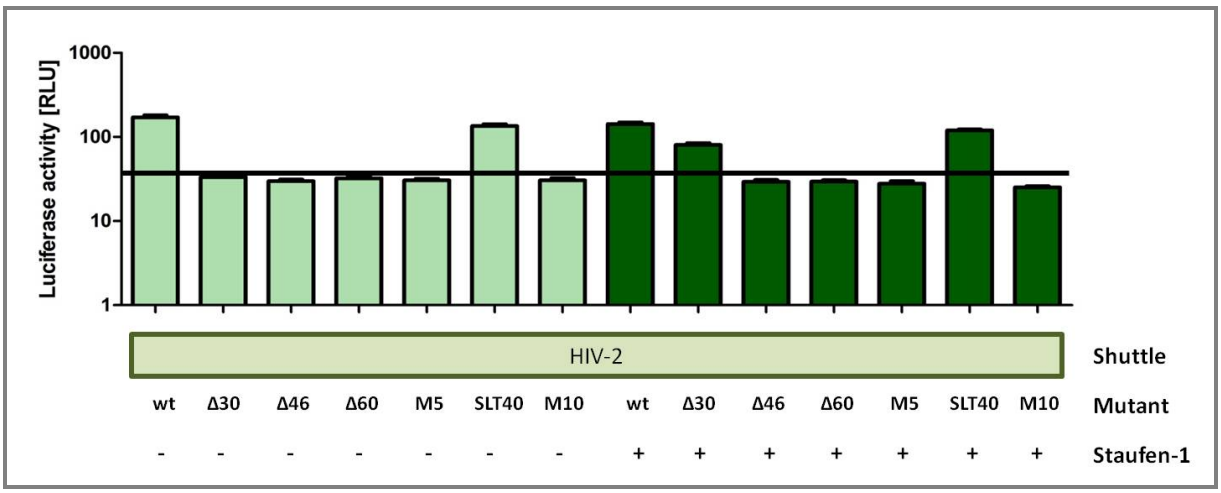

Figure 33: **RNA transport in the presence of HIV-2 RRE upon expression of HIV-2 coRev mutants.** Cells were transfected with ShuttleHIV-2RRE, as well as HIV-2 coRev mutants. Cotransfection of pCMV_FLAG-Staufen-1 is marked as +. Following incubation for 48 h, cells were lyzed and firefly luciferase activity was determined. Values were normalized to the activity of a cotransfected *Renilla* luciferase. Black line is threshold, meaning average luciferase activity measured for non-specific transport of the construct Shuttle, conditions being equal (see Fig. 31), plus three times the standard deviation. Luciferase activity was found to be increased for SLT40 at levels comparable to wild type coRev. Here, Staufen-1 coexpression did not markedly influence this effect, too. For Δ30, an increase in light emission was observed only upon coexpression of Staufen-1, whereas cotransfection with mock DNA did not affect the amount of emitted light.

5 Discussion

5.1 Interaction of Staufen-1 and HIV-1

In the beginning of the 20th century, the first diseases caused by retroviruses and subsequently the viruses themselves were described. Since then, a lot of effort has been made to unravel their pathogenic mechanisms, as they cause several types of cancer and other failures of the immune system in diverse species. Research has especially focused on the human immunodeficiency virus (HIV), causative agent of the acquired immunodeficiency syndrome (AIDS) and being a global problem up to today. Anyhow, development of a vaccine or cure remains to be achieved, as this virus evolves rapidly, and even nowadays only decelerating the progression of the disease is possible. Despite the fact that HIV has been discovered more than 30 years ago, there are still numerous questions to be answered regarding cellular mechanisms and host factors required for replication of the virus.

The human cellular protein Staufen-1 is involved in RNA transport accomplished via the tubulin network of the cell. An interaction with several retroviruses has been reported by our group among others, such as HIV-1 [31], the human t-cell lymphotropic virus type-1 (HTLV-1) [52] and the human endogenous retrovirus K (HERV-K) [41]. Consequently, a hypothesis has been proposed for retroviruses, in which Staufen-1 may target the export of viral RNAs, the translation of viral proteins and the assembly of emerging virions, collectively influencing the production and infectivity of viral particles. At the same time, an antiviral activity under stress conditions has been suggested, as Staufen-1 induces silencing of RNAs in stress granules. However, a possible interaction between Staufen-1 and HIV-2 has not been analyzed yet. This had to be scrutinized within this work, considering the influence of Staufen-1 on the viral particle production and infectivity. These results should shed some more light on the role of Staufen-1 in the retroviral replication cycle.

5.1.1 Impact of Staufen-1 expression level on HIV-2 particle production

A dose-dependent effect of Staufen-1 expression levels on the particle production has been reported for HIV-1 as well as HERV-K before [12] [41] [98]. These findings could be confirmed for HIV-2 within this work by cotransfection of the HIV-2 molecular clone pROD and *staufen-1* at different ratios followed by analysis of cell supernatants via CA-antigen capture ELISA as read-out for viral particle production. Here, Staufen-1 overexpression increased the amount of capsid protein in the supernatants up to sixfold, depending on the amount of *staufen-1* cotransfected (4.1, 4.6). This effect is time-independent, as could be demonstrated by investigation of HIV-2 pROD expression kinetics for seven days both alone and under coexpression of Staufen-1 (4.2).

Compared with Staufen-1 overexpression, downregulation of endogenous Staufen-1 using shRNAs impaired particle production approximately by half. This contrasts the finding of Chatel-Chaix *et al.* for HIV-1, who reported an increase in VLP (virus-like particle) production upon downregulation [12]. However, the impact of downregulation on the particle production was lower compared to overexpression here, too. On the other hand, a similar effect as for HIV-2 has been shown for the less related HERV-K by Jula Wamara during her diploma thesis, where diminished Staufen-1 expression resulted in notable less VLPs [98]. Presumably, these effects might be mediated via different mechanisms and remain unexplained for HIV-1.

Analyzing the particle production upon cotransfection of several Staufen-1 mutants cloned by Jula Wamara (Fig. 34) and HIV-2 pROD, a first hint was given that the dsRBD3 domain of Staufen-1 is required for mediating its enhancing effect (4.3), as this was impaired by coexpression of both mutants lacking a functional dsRBD3 domain (Staufen-1 ΔRBD3 and Staufen-1 F135A), whereas Staufen-1 mutants lacking other functional domains did not influence this effect. Similar results were previously obtained for HIV-1 [13]. Due to the fact that expression levels of both dsRBD-3 deficient mutants were found to be lower, these findings might be somehow debat-

able, although impaired expression of these mutants has been reported before [14] [98]. As the dsRBD3 domain has been proven to bind to and thereby to mediate incorporation of viral RNA into the emerging particle for HIV-1 [69], it would be interesting to investigate this for HIV-2, as Staufen-1 mutants may consequently have an impact on the infectivity.

5.1.2 Impairment of viral infectivity by Staufen-1

Staufen-1 has previously been shown to play a role in transport and packaging of genomic RNA, as it interacts only with unspliced RNA molecules and is found within viral particles, which requires RNA binding capacity [14] [69]. Due to being involved in controlling these processes, it might have an impact on viral infectivity by influencing the content of viral RNAs within viral particles. Both overexpression and depletion of Staufen-1 were reported to impair the infectivity of generated virions for HIV-1 [69].

To study the impact of Staufen-1 on the infectivity of HIV-2, C8166 cells were infected with supernatants normalized to the amount of p26, which had previously been produced by expression of HIV-2 pROD upon different expression levels of Staufen-1. Quantification of the number of HIV-2 integrations into the cells' genome via duplex real-time PCR indicated that the rate of infectious virions is dose-dependently reduced by coexpression of Staufen-1 (4.6). These findings match with data published for HIV-1. Here, Staufen-1 overexpression was also shown to increase the amount of viral RNA within new virions [69]. It remains to be determined whether the reduced infectivity is due to a lower overall number of infectious particles or a diminished infectious potential of the individual particle.

5.1.3 Impact of Staufen-1 on viral RNA export

The Rev proteins and their homologue Rec have been shown to be essential for export of unspliced viral RNAs in HIV-1, HIV-2 and HERV-K, respectively. This process is dependent on the presence of the Rev-responsive element (RRE) and the

Rec-responsive element (RcRE), respectively, within the viral RNA, mediating the binding of Rev/Rec and thus its export into the cytoplasm of the host cell [21] [59] [60]. As the positive effect of Staufen-1 on the amount of produced viral particles could be caused by enhancing this Rev/RRE-dependent export as well as translation of viral RNAs, an eventual impact of Staufen-1 overexpression on this should be analyzed using a reporter construct comprising the RRE, which was generated for both HIV-1 and HIV-2 based on a construct of Kirsten Hanke holding the RcRE.

The need of Rev for export of HIV-2 RRE-containing transcripts could be emphasized as expression levels of the firefly luciferase reporter gene were markedly increased upon coexpression of coRev, which could however not be further enhanced upon additional expression of Staufen-1 (see Fig. 32). This could also be observed for HIV-1 and HERV-K, forming a contrast to the observations of Hanke *et al.*, where Staufen-1 coexpression in the presence of Rec enhanced the export and/or translation of the HERV-K RcRE-containing reporter construct by factor three [41], and the results obtained for HIV-1 by Katharina Fiddeke, where Staufen-1 overexpression rather impaired expression of the reporter gene [31], albeit a different reporter system was employed here, impeding the comparability. Hence, the two reporter constructs made here indeed enable specific analysis of Rev/RRE-dependent RNA transport, as coexpression of Rev and a control vector lacking an RRE did not increase expression levels of the reporter gene normalized to a cotransfected *Renilla* luciferase reference gene (see Fig. 30). According to the findings obtained within this work, the enhancement of viral particle production upon Staufen-1 overexpression is not due to specific effects on the export or translation of viral RNAs. Nevertheless it should be noted that Staufen-1 did non-specifically increase the expression levels of both luciferases (data not shown), resulting in no enhancement of the normalized firefly luciferase signal overall. This might be due to the previously reported general effect of Staufen-1 on translation by interacting with the ribosome [26] [57].

5.2 Investigation of the HIV-2 coRev protein

It has been presumed that the HIV-1 Rev as well as the HERV-K Rec protein are one of the potential targets of Staufen-1 as their interaction has been proven via manifold assays such as coimmunoprecipitation (CoIP), pull-down studies, FRET (fluorescence resonance energy transfer) analysis based on flow cytometry and immunofluorescence for colocalization [31] [41] [98]. By this interaction, Staufen-1 may direct spatial distribution of viral RNAs within the host cell [41].

For HIV-1, this interaction has intensively been studied using several mutants of the codon-optimized coRev protein, each comprising a mutation impairing the function of one of its functional domains [31]. To allow comparison of HIV-2 with this virus, corresponding mutants should be generated. For this purpose, functional domains of the HIV-2 Rev protein had to be defined first due to the absence of publications regarding this. Since the Rev proteins of HIV-1 and SIV, two closely related lentiviruses, feature a similar structure as HIV-2 Rev, the functional domains were assumed to be analogous (see Fig. 20). Based on this, six mutants were generated, containing either a deletion (Δ30, Δ46, Δ60) or a substitution (M5, SLT40, M10) of amino acids (see Fig. 21), supposed to affect the function of Rev.

5.2.1 Localization of coRev mutants and their colocalization with Staufen-1

Localization of the generated HIV-2 coRev mutants as well as their colocalization with overexpressed Staufen-1 were examined by fluorescence microscopy using fluorophore-coupled antibodies. As the Rev protein features both a nuclear localization signal (NLS) and a nuclear export signal (NES), it was expected to be present in the nucleus and the cytoplasm, as has previously been reported for HIV-2 Rev [22] and for HIV-1 Rev as well [16] [66], corresponding to its proposed shuttle function between the nucleus and the cytoplasm. Staufen-1 is mainly found within the cytoplasm under normal conditions, although it comprises a functional NLS located next to the dsRBD3. This is presumably achieved by cytoplasmic retention,

as no NES has been identified yet. Consequently, the function of Staufen-1 within the nucleus is thought to be unrelated to mRNA transport, as its transport into the cytoplasm might rather occur by export of cofactors [65].

Expected localization of both proteins upon separate expression could be confirmed within this work (see Fig. 23 and 24), whereas colocalization was found only within the cytoplasm. Contrasting this, Staufen-1 was detected inside the nucleus in the presence of HIV-1 Rev and HERV-K Rec before, accompanied by evidence for the interaction of Staufen-1 and Rev/Rec [31] [41]. This might indicate that Staufen-1 does not interact with HIV-2 Rev, against expectation due to the highly related functions of the Rev/Rec proteins.

Localization of the six HIV-2 coRev mutants matched the observations for the corresponding HIV-1 Rev mutants (see Fig. 25 and 26, compare [31]), giving rise to the assumption that functional domains had been defined correctly for HIV-2 Rev. All mutants found in the cytoplasm showed colocalization with coexpressed Staufen-1 right there.

5.2.2 Analysis of the coRev mutants function

Another evidence for proper definition of HIV-2 Rev functional domains could be obtained by investigating the functionality of the protein. For this purpose the reporter construct comprising the HIV-2 RRE, already used earlier within this work for analyzing the impact of Staufen-1 on the nuclear export of unspliced viral RNAs, was employed. By this means, an eventual effect of the respective mutation on the presumptive main task of Rev, meaning the transport of RRE-containing RNAs from the nucleus into the cytoplasm, can be examined. Taking account of the affected domain and of the previous findings regarding localization of the mutants, it was presumed that NLS-deficient mutants (Δ46, Δ60, M5) as well as the one lacking a functional NES (M10) could not properly fulfill this function anymore.

This assumption could be verified as none of these mutants was able to enhance reporter gene expression at levels comparable to the wild type coRev, neither alone

nor under coexpression of Staufen-1. Surprisingly, the SLT40 mutant, featuring two amino acid substitutions intended to impair the function of the second multimerization domain, seemed to enable RNA transport similar to the wild type coRev. This allows two possible explanations. On the one hand, the second loop domain might not be essential for the export function of the protein. On the other hand, there is a chance that the substitutions made here did not affect the function of the domain as intended, or the definition of the region might have been wrong. To exclude this, the capacity of the SLT40 for multimerization has to be further investigated. As has been demonstrated for wild type coRev, Staufen-1 had no influence on reporter expression level.

Even more unexpected were the findings regarding the Δ30 mutant, where the first loop domain has been deleted. While the mutant alone did not display any shuttle function, coexpression of Staufen-1 rescued this capacity. Again, there are various considerations matching these observations. Presumably, Staufen-1 binds the Rev/RNA complex inside the nucleus and thereby enables binding of additional Rev molecules, which had been impossible before due to the lacking loop domain. Alternatively, the absence of the functional loop domain might affect stability of the Rev/RNA complex, which might be recovered upon binding of Staufen-1, allowing it to translocate into the cytoplasm. These observations match to some extent the findings of Katharina Fiddeke, where the corresponding Δ32 HIV-1 coRev mutant showed little shuttle function, though the SLT40 mutant was nonfunctional here [31].

Another attempt to examine functionality of the Rev mutants was to check their impact on the viral particle production, carried out by cotransfection of the molecular clone pROD, *staufen-1* and the particular mutant. Analogous experiments regarding HIV-1 and HERV-K showed a distinct increase in the amount of capsid protein released upon additional expression of Rev/Rec [31] [41] [98]. This could not be verified for HIV-2 Rev, as neither the wild type coRev nor one of the mutants were able to enhance particle production (4.9).

5.3 Conclusion

Summarizing the results attained in this work and other studies, it could be demonstrated that the human protein Staufen-1 plays a role in the replication not only of HIV-1, HTLV-1 and HERV-K, but also of HIV-2, as could be shown here. It was reported that Staufen-1 is able to promote production of new viral particles.

However, the mechanism by which this is mediated as well as the importance for the *in vivo* situation remains unclear. Influencing transport of viral RNAs and thereby packaging of viral genomic RNA, translation of viral proteins as well as virion assembly by affecting Gag multimerization, have been proposed for this. Correspondent to the findings observed here, the latter might be more likely as Staufen-1 was not found to specifically enhance transport or translation of viral RNAs. However, differentiation of Staufen-1 effects on nuclear export of viral RNAs and translation, respectively, is not achievable using the reporter system established within this work. Separate investigation of nuclear and cytoplasmic RNA fractions via real-time PCR could allow for discrimination between these two processes.

Concurrently, Staufen-1 gets incorporated into emerging particles via binding to viral RNA [69]. This goes along with an impairment of infectivity, which could be proven to be true also for HIV-2 here. This effect could directly be caused by the presence of Staufen-1 molecules within the viral particle, or alternatively by an increased amount of viral RNA within the particle induced by Staufen-1, thus being indirectly mediated by Staufen-1. For HIV-1, it has previously been shown that downregulation of endogenous Staufen-1 levels impaired viral infectivity, too. This should also be investigated for HIV-2, as only the negative effect of downregulation on particle production has been shown here. At the same time, downregulation should be optimized, for example by utilization of siRNAs, as well as quantified, e.g. using quantitative real-time PCR as has been done before [14].

With these observations in mind, it is likely that a certain level of Staufen-1 within the cell is required for proper function during the retroviral replication cycle. Regarding its function in viral RNA transport, it has already been proposed by Chatel-

Chaix *et al.*, that a particular amount of Staufen-1 molecules enables fine-tuned packaging of exactly two copies of RNA [12] [14].

In addition to this, Staufen-1 interacts with HERV-K Rec, HTLV-1 Rex and HIV-1 Rev. Interaction with HIV-2 Rev remains to be approved, for instance by coimmunoprecipitation, pull-down assays and FRET analysis. Colocalization within the cytoplasm under normal conditions, as demonstrated here, indicates a possible interaction of both proteins only to a limited extent, as they are both usually localized here. Furthermore, colocalization of HIV-2 Rev and Staufen-1 under stress conditions should be demonstrated, as antiviral activity of Staufen-1 by silencing of viral RNAs within stress granules has been reported for the other viruses. If so, the Staufen-1 domain responsible for binding of HIV-2 Rev should be determined, since dsRBD3 has been described for HIV-1 Rev [31], whereas dsRBD4 was proposed to mediate this for HERV-K [41]. The effect of Staufen-1 on particle production and infectivity could to some extent also be caused by interaction with Rev. Further experiments are necessary to provide a basis for discussion.

For confirmation of the functionality of wild type coRev as well as the mutants generated within this work, a Rev-deficient HIV-2 mutant should be employed, which does not express Rev itself. By this, Rev protein function could be investigated, as the cotransfected Rev mutant has to fulfill this function to enable virus production. This would complement the findings made here regarding the impact of additional Rev/mutants expression on the particle production of HIV-2, where no effect was observed. At this, it should be noted that expression levels of all mutants were markedly lower compared with HIV-1 Rev, thereby possibly diminishing their eventual effect (see Fig. 23, 25 and 26, compare [31]). Improving expression of these proteins should be another aim. Taken together, this might eventually indicate whether functional domains have been defined for HIV-2 Rev properly.

Finally, extending the knowledge about molecular mechanisms and cellular cofactors of retroviruses might offer additional targets for antiviral therapy or the development of a vaccine. Therefore, further investigations on cellular cofactors should

be of global interest. It is also easily conceivable that Staufen-1 has an antiretroviral activity, which is abolished upon binding to structural or regulatory viral proteins. Studying the effect of Staufen-1 on other, simple retroviruses might give more insights regarding this.

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A Appendix

A.1 Abbreviations

Table 32: Abbreviations

Abbreviation	Meaning
®	Registered trademark
A	Adenine
AIDS	Acquired immune deficiency syndrome
APS	Ammonium persulfate
Aqua bidest	*Aqua bidestillata*
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degree Celsius
CA	Capsid
CD	Cluster of differentiation
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CoIP	Coimmunoprecipitation
CRM1	Chromosome region maintenance 1
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
ddNTP	Dideoxynucleotides
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
dsDNA	Double-stranded DNA

dsRBD	Double-stranded RNA-binding domain
dsRNA	Double-stranded RNA
dStau	*Drosophila melanogaster* Staufen
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope protein
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FRET	Fluorescence resonance energy transfer
G	Guanine
g	g-force
Gag	Group specific antigen
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
gp	Glykoprotein
h	Hour
HAART	Highly active antiretroviral therapy
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HERV-K	Human endogenous retrovirus K
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
hStau	Human Staufen
HTLV-1	Human t-cell lymphotropic virus type-1
ICTV	International Committee on Taxonomy of Viruses
IgG	Immunglobulin G
IN	Integrase
kb	Kilo-base pair

kD	Kilo Dalton
L	Liter
µ	Mikro
M	Molar
MA	Matrix
min	Minute
MMTV	Mouse mamma tumor virus
mol	Mole, SI unit for an amount of substance
mRNA	Messenger RNA
n	Nano
NC	Nucleocapsid
NES	Nuclear export signal
NLS	Nuclear localization signal
OPD	o-Phenylenediamine dihydrochloride
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PIC	Pre integration complex
PKR	DsRNA-dependent kinase
Pol	Polymerase
PR	Protease
Pro	Protease
PVDF	Polyvinylidene fluoride
RBD	RNA-binding domain
RcRE	Rec-responsive element
Rec	Regulator of Expression encoded by cORF
Rev	Regulator of expression of virion proteins

Rex	Regulator of expression of virion proteins of HTLV
RLU	Relative light unit
RNA	Ribonucleic acid
RNP	Ribonucleoparticle
RRE	Rev-responsive element
RT	Room temperature
RT	Reverse transcriptase
RxRE	Rex-responsive element
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Stress granule
SHIV	Simian/human immunodeficiency virus
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMD	Staufen-mediated decay
SU	Surface
T	Thymine
TBD	Tubulin-binding domain
TEMED	Tetramethylethylenediamine
TM	Transmembrane
TRBP	TAR-RNA binding protein
UV	Ultraviolet
V	Volt
v/v	Volume per volume
VLP	Virus-like particle
w/v	Weight per volume

A.2 Mutated Staufen-1 Proteins

Several Staufen-1 vectors including specific mutations were used in this work (see Fig. 34). These mutants were cloned by Jula Wamara, a co-worker in the lab [98].

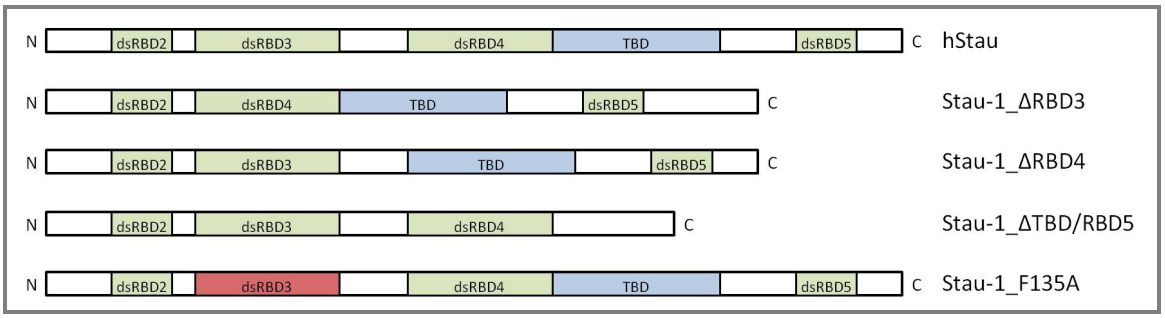

Figure 34: **Amino acid deletions and substitutions of Staufen-1 mutants.** Mutants termed with Δ comprise deletions of functional domains. Red fill indicates a non-functional domain caused by amino acid substitution (F135A).

A.3 shRNA Plasmids for Staufen-1 Silencing

The following shRNA plasmids shown in Table 33 were used for downregulation of endogenous Staufen-1 [98].

Table 33: shRNA plasmids specific for Staufen-1 and their target sequences. Backbone is pLVTHM.

Plasmid for shRNA expression	Target within *staufen-1* (NM_017452), 5′ → 3′
pLVTHM_shStau_A7	Exon 14, GCTGCGCTGAACATCTTAAAG
pLVTHM_shStau_C3	Exon 8b, GCCTGCAGTTGAACGAGTAAA
pLVTHM_shStau_B3	Exon 15, = 3′-UTR, GCTGCGCTGAACATCTTAAAG
pLVTHM_shStau_C1	Exon 6/7, GGAGGTGAATGGAAGAGAATC
pLVTHM_shStau_A2C4	Exon 8/9, GCCACAGACAAGCCCAGAATA

B Acknowledgements

Ich möchte mich bei Dr. Norbert Bannert bedanken für die Möglichkeit, meine Masterarbeit in seiner Arbeitsgruppe anzufertigen und dieses spannende Thema zu bearbeiten, für die Unterstützung während der gesamten Zeit sowie für zahlreiche Anregungen und Kritiken.

Prof. Dr. Roland Lauster und Prof. Dr. Vera Meyer danke ich für die Übernahme der Betreuung und Begutachung meiner Arbeit sowie für die Unterstützung und Motivation während des Studiums.

Mein besonderer Dank gilt Dr. Oliver Hohn für die umfassende und zeitaufwändige Betreuung, zahlreiche Hilfestellungen, Diskussionen und Appelle an mein Durchhaltevermögen sowie seine positive Einstellung, ohne die ich nicht nur ein Mal durchgehangen hätte.

Nicole und Stephen Norley danke ich für die Hilfe bei den ELISAs, Dr. Kazimierz Madela für die Unterstützung und nützliche Hintergrundinformationen bei der Fluoreszenzmikroskopie. Stephen Norley gilt außerdem Dank für die kritische Durchsicht meiner Arbeit.

Danken möchte ich auch allen anderen Mitarbeitern des FG 18, allen voran Katharina Fiddeke, Johanna Best, Nadine Weser und Vanessa Böhmer - mit Lachen und Schokolade sieht die Welt viel schöner aus! Besonderer Dank gilt Nadine Weser auch für die Unterstützung im Labor, sowie Sabina Reichert und Kornelia Gericke für ihren unermüdlichen Einsatz, damit bei uns alles läuft.

Vor allem aber danke ich meiner immer wachsenden Familie. Für die Unterstützung, Möglichkeiten, Motivation, Freude und Liebe, die mir jeder von euch schenkt.

C Declaration/ Eidesstattliche Erklärung

I declare that I am the sole author of this dissertation and that the work presented in it, unless otherwise referenced, is entirely my own. I also declare that the work has not been submitted in whole or in part to any other University as an exercise for a degree or any other qualification.

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und eigenhändig sowie ohne unerlaubte fremde Hilfe und ausschließlich unter Verwendung der aufgeführten Quellen und Hilfsmittel angefertigt habe. Die Arbeit wurde bisher weder einer anderen Prüfungsbehörde vorgelegt, noch veröffentlicht.

Die selbstständige und eigenständige Anfertigung versichert an Eides statt:

Berlin, den 18.02.15

Anna-Klara Amler