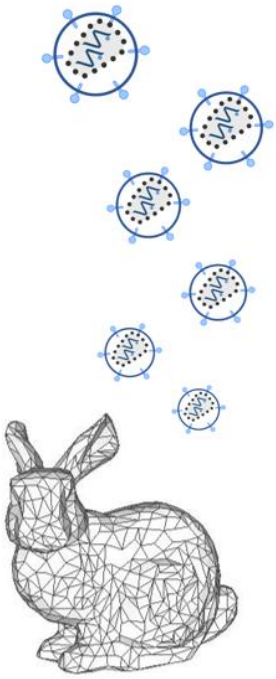
Is the European rabbit (*Oryctolagus cuniculus*) a good animal model to study HIV-1 pathogenesis and virus-host interactions?



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Foreword

The thesis entitled “Is the European rabbit (*Oryctolagus cuniculus*) a good animal model to study HIV-1 pathogenesis and virus-host interactions?” is my own work and I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of any other examination degree.

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Abstract

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). No protective vaccine against HIV is in sight and available pharmacotherapies can only control infection. The development of novel strategies for the prevention or treatment of HIV/AIDS, is greatly facilitated by pre-clinical studies in predictive animal models. A first step for animal model development has been the identification and surmounting of species-specific barriers that HIV encounters along its replication cycle in cells from small animals. Here, we focused on re-evaluating HIV entry into rabbit cells and analysed CD4/CCR5 in an evolutionary perspective and possible interactions with rabbit endogenous viruses. Furthermore we investigated the role of SERINC3/5 as putative restriction factors that reduce HIV infectivity in primary rabbit macrophages.

The first barrier found in rabbit cells is represented by the lack of a functional receptor complex for the interaction with HIV envelope glycoprotein. The 3D structures of these receptors allowed to define small changes sufficient for virus entry. Minimal modifications of rabbit CCR5 revealed that the replacement of the rabbit N-terminal part with the corresponding human sequence and a substitution at position 198 to Ile was sufficient to support virion fusion. With regard to CD4, the European rabbit encodes an extra serine stretch in the D1 domain, whose removal and the substitution of two adjacent amino acids is sufficient to support HIV entry. Evolutionary analyses of lagomorphs' CD4 showed that next to a highly variable D2 domain, the insertion of the serine motif in the D1 domain is not common to all lagomorphs, suggesting a dynamic evolution in rabbits. Although it is tempting to relate this evolutionary events with the presence of endogenized pathogens in rabbits, such as the rabbit endogenous lentivirus K (RELIK) or the rabbit endogenous retrovirus H (RERV-H), there is no clear connection and, despite all the efforts, it was not possible to show that these viruses interacted with CD4 or CCR5 in the past.

The third barrier in rabbit macrophages leads to decreased infectivity of released HIV-1 virions. The human SERINC3/5 proteins were recently described as antiviral proteins that act at a similar step in the replication cycle. The study of SERINC3/5 orthologs showed that, besides being highly conserved in mammals, their antiviral activity is also preserved in rodents and rabbit. Strikingly, the accessory protein HIV Nef as well as murine leukemia virus (MLV) GlycoGag and the S2 protein from equine infectious anemia virus (EIAV) were able to antagonize the antiviral activity of rodent and rabbit SERINC3/5. The results suggest that SERINC3/5 most likely do not cause the infectivity

defect in primary rabbit macrophages, and therefore the third barrier to full HIV-1 replication remains unidentified.

In conclusion, rabbits are a promising model to study HIV, as they express fewer barriers to viral replication when compared to rodents. In rabbit cells, only one cell-specific barrier remains to be solved, while the other blocks were shown to be easily overcome by small changes in the virus itself or in rabbit proteins.

Resumo

O vírus da imunodeficiência humana (HIV) é o agente responsável pelo síndrome da imunodeficiência adquirida (AIDS). Até ao momento, não existe nenhuma vacina contra o HIV e os fármacos disponíveis são apenas capazes de cotrolar a infecção, no entanto, o desenvolvimento de novas estratégias para a prevenção ou tratamento do HIV / AIDS está dependente de estudos pré-clínicos em modelos animais. O desenvolvimento de pequenos modelos animais começa pela identificação e resolução das barreiras que o HIV encontra ao longo de seu ciclo de replicação nestas espécies. Este estudo é focado na reavalição da entrada do HIV em células de coelho, com a análise do CD4/CCR5 numa perspectiva evolutiva e tendo em vista possíveis interações com vírus endógenos encontados no coelho. Além disso, é analisada a função dos SERINC3 e SERINC5 como possíveis fatores de restrição presentes nos macrofagos do coelho.

A primeira barreira encontrada nas células do coelho é representada pela falta de um recetor e co-recetor funcional para a interação com a glicoproteína do envelope do HIV. As estruturas 3D desses receptores permitiram definir quais as pequenas mudanças que seriam suficientes para permitir a entrada do vírus. Modificações mínimas no CCR5 do coelho revelaram que a substituição da parte N-terminal pela correspondente região humana e a substituição para uma Ile na posição 198 foi suficiente para suportar a fusão do virus com as células alvo. Em relação ao CD4, o coelho europeu apresenta uma inserção de quatro serinas no domínio D1, cuja remoção juntamente com a substituição de dois aminoácidos adjacentes é suficiente para permitir a entrada do HIV. As análises evolutivas para o CD4 nos lagomorfos mostraram que, para açém do domínio D2 ser altamente variável, a inserção encontrada no domínio D1 não é comum a todos os lagomorfos, o que sugere uma evolução dinâmica nos coelhos. Embora seja tentador relacionar estes eventos evolutivos com a presença de patógenos endógenos presentes nos coelhos, tal como o vírus endógeno RELIK ou RERV-H, não existe uma ligação clara e, apesar de todos os esforços, não foi possível confirmar se esses vírus interagiram no passado com o CD4 ou o CCR5.

A terceira barreira é especifica dos macrófagos do coelho e traduz-se numa reduzida infecciosidade dos virus de HIV-1 produzidos por estas células. As proteínas humanas SERINC3 e SERINC5 foram recentemente descritas como agentes antivirais e atuam de forma semelhante. O estudo dos ortólogos do SERINC3/5 mostrou que, para além de serem altamente conservados em mamíferos, a sua atividade antiviral também é preservada em roedores e coelhos. Surpreendentemente, a proteína acessória Nef presente no HIV, tal como a proteina GlycoGag do vírus da leucemia murina (MLV) e a proteína S2 do vírus da anemia infecciosa equina (EIAV), foram capazes de antagonizar

a atividade antiviral do SERINC3/5 de roedores e coelhos. Desta forma, os resultados sugerem que o SERINC3 ou SERINC5 não estão na origem da reduzida infectividade registada nos virus produzidos nos macrófagos do coelho e portanto, a terceira barreira à replicação do HIV-1 permanece não identificada.

Em conclusão, o coelho é um modelo promissor no estudo do HIV, sobretudo por expressar menos barreiras à replicação viral em comparação com os roedores. No coelho, a nível celular, apenas uma barreira específica nos macrofagos permanece por resolver, enquanto os outros blocos até agora identificados mostraram poderem ser facilmente superados por pequenas alterações no próprio vírus ou nas proteínas do coelho.

Key words

Small animal model,
Human immunodeficiency virus HIV,
European rabbit,
Lagomorphs,
CD4,
CCR5,
Rabbit endogenous lentivirus type K RELIK,
Rabbit endogenous retrovirus-H RERV-H,
SERINC3,
SERINC5,
Host-virus interaction.

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Abbreviations

AIDS - Acquired Immunodeficiency Syndrome

APOBEC - Apolipoprotein B mRNA-editing Enzyme

BIV - Bovine Immunodeficiency Virus

CA - Capsid

CAEV - Caprine Arthritis-Virus

CCR5 - Chemokine Receptor 5

CD4 - T cell Receptor Cluster of Differentiation 4

CDS - Coding Sequence

CXCR4 - Chemokine Receptor 4

DCs - Dendritic Cells

d_N - Non-synonymous Substitutions

dNTPs - Deoxynucleoside Triphosphate

d_S - Synonymous Substitutions

ECL - Extracellular Loop

EIAV - Equine Infectious Anemia Virus

env - Envelope

ERV - Endogenous Retrovirus

EVEs - Endogenous Viral Elements

FIV - Feline Immunodeficiency Virus

gag - Group-Specific Antigen

GBPs - Guanylate Binding Proteins

HIV - Human Immunodeficiency Virus

IFN - Interferons

IN - Integrase

ISGs - Interferon-Stimulated Genes

JDV - Jembrana Disease Virus

LTR - Long Terminal Repeats

MA - Matrix

MARCH8 - Membrane-Associated Ring-CH 8

MDMs - Monocyte-Derived Macrophages

MHC - Major Histocompatibility Complex

MLV - Murine Leukemia Virus

MUSCLE - Multiple Sequence Comparison by Log-Expectation

Mya - Million Years Ago

MYXV - Myxoma Leporipoxvirus

NC - Nucleocapsid

Nef - Negative Regulator Factor

NK - Natural Killer Cells

OMVV - Maedi-Visna Virus

PBMCs - Primary Blood Mononuclear Cells

PFA - paraformaldehyde

pol - Polymerase

PR - Viral Protease

PSIV - Endogenous Prosimian Virus

RELIK - Rabbit Endogenous Lentivirus K

RERV-H - Rabbit Endogenous Retrovirus H

rev - RNA Splicing-Regulator

RHDV - Rabbit Hemorrhagic Disease Virus

RT - Reverse Transcriptase

SAMHD1 - Sterile Alpha Motif- and HD-domain Containing Protein 1

SERINCs - Serine Incorporators

SIV - Simian Immunodeficiency Virus

stHIV-1 - Simian-tropic HIV-1

SU - Surface Protein

Tat - Transactivator Protein

TLRs - Toll-like Receptors

TM - Transmembrane Protein

TRIM - tripartite motif

Vif - Viral Infectivity Factor

vpr - Virus Protein r

vpu - Virus Protein Unique

vpx - Virus Protein x

VSV - Vesicular Stomatitis Virus

VSV-G - Vesicular Stomatitis Virus Glycoprotein

Chapter I: General introduction

Origins of Human Immunodeficiency virus and pandemics

Acquired immunodeficiency syndrome (AIDS) was first reported in the early 80s in the United States (Hymes et al., 1981, Masur et al., 1981). The disease was associated with an impairment of the immune system and consequent vulnerability to unusual opportunistic infections (Greene, 2007). Most common among gay men, the spread of the disease was first attributed to the life style of this community, being finally characterized as an infectious disease that can be transmitted through body fluids (Greene, 2007). Soon, a new retrovirus was identified (Barresinoussi et al., 1983) and several studies followed to link this virus to AIDS (Gallo et al., 1984, Popovic et al., 1984, Schupbach et al., 1984, Levy et al., 1984) and the causing infectious agent was termed human immunodeficiency virus (HIV) (Coffin et al., 1986).

After the discovery of HIV-1, a second type, named HIV-2, was identified in Western Africa and showed to be closely related to simian immunodeficiency viruses (SIVs) (Clavel et al., 1986). SIVs are species-specific viruses affecting primates, including African green monkeys, sooty mangabeys, mandrills, chimpanzees, among others, however they are in general nonpathogenic in their natural hosts (Sharp and Hahn, 2011). The first evidence for cross-species transmission was given when close simian relatives to HIV-1 and HIV-2 were identified in chimpanzees and sooty mangabeys, respectively (Huet et al., 1990, Hirsch et al., 1989). Independent zoonotic transmissions led to different HIV groups and so far 8 groups (A-H) are recognized for HIV-2 and 4 groups for HIV-1 (M-P) (Sharp and Hahn, 2011). HIV-1 groups M and N originated from independent transmissions of SIV of chimpanzees (SIVcpz), which itself arose from the recombination between the SIV of red-capped mangabeys and great spot-nosed monkeys (Gao et al., 1999, Keele et al., 2006, Bailes et al., 2003). A SIV from gorillas (SIVgor), derived from a SIVcpz ancestor lineage, was the origin of group P, while for group O it is not completely clear if it had its origin directly from SIVcpz or from SIVgor (Sharp and Hahn, 2011, Becerra et al., 2016, Takehisa et al., 2009, Plantier et al., 2009). Although the identification of HIV only occurred in the 80s, evolutionary studies show that group M is likely to be the oldest HIV lineage, existing already since ≈ 1900-1930 (Hemelaar, 2012). This group can be further divided into nine subtypes (A to D, F to H, J and K) that show 17 % - 35 % amino acid variability among them (Korber et al., 2001). Europe and America are mainly affected by subtype B, which seemed to have had its origin in a single strain from Africa, whereas subtype C is more common in Asian countries, and subtype A and D predominates in Africa (Gilbert et al., 2007). Recombination events are also recurrent between the different subtypes, originating

more than 60 circulating recombinant forms (CRFs) (Taylor et al., 2008, Peeters et al., 2014).

The route of transmission to humans is not clear, though hunting and keeping monkeys as pets, combined with cutaneous or mucous membrane exposure to infected blood or body fluids are among the most believed causes (Peeters et al., 2002, Hahn et al., 2000). However, these species diverged for several million years, and the evolution of the host proteins would impose an obstacle to cross-species infection. Therefore, HIV acquired specific mutations that are associated with the infection of human cells (Wain et al., 2007). Furthermore, some viral proteins developed the ability to counteract different host proteins that act as barriers for viral replication (Lim et al., 2010). Each of the HIV-1 groups developed different strategies to overcome human cellular barriers, and the global epidemic reflects how successful this adaptation was (Hemelaar, 2012, Sauter et al., 2009).

HIV-1 group M represents the pandemic form which was first identified, while group O, identified in 1990, only represent 1% of global HIV-1 infections, being restricted to Cameroon, Gabon and neighboring countries (De Leys et al., 1990, Peeters et al., 1997). Group N and group P are restricted to Cameroon and are even less representative, being only identified in 13 and 2 cases, respectively (Simon et al., 1998, Vallari et al., 2010, Plantier et al., 2009, Vallari et al., 2011). HIV-2 has mostly higher rates in Western Africa, however it is declining and giving space for HIV-1 infections (de Silva et al., 2008, van der Loeff et al., 2006). Compared to HIV-1, HIV-2 generally shows lower viral loads which might be related with lower transmission rates and diminished progression to AIDS (Sharp and Hahn, 2011).

In 2016, HIV still affected 36.7 million people worldwide, and until today HIV-related causes lead to more than 35 million deaths all over the world (WHO, 2017). Still in 2016, 1.8 million people become newly infected. Compared to the late 90s, with more than 3 million newly infected people every year (WHO, 2017), there has been a great improvement. Besides a reduction of newly infected individuals, there was also a decrease in mortality by one third (WHO, 2017). Nowadays, Africa is the most affected continent, counting for almost two thirds of the global total new HIV infections, with sex workers, men who have sex with men, drug users and children as the main affected groups (Becerra et al., 2016).

The chronic suppression of HIV through combined antiretroviral therapy (cART) has been a major accomplishment, however the current treatments present several issues, including not being accessible to infected individuals in least developed countries, difficulty in lifelong adherence with the risk of rebound of replicating virus and ultimately

toxicity effects over decades of treatment (Richman et al., 2009). Therefore, HIV is still a public health threat, and a lot of work has to be done for the development of new drugs and a vaccine that completely block infection or reduce viral loads to levels that prevent progression to clinical disease (Richman et al., 2009, Barouch, 2008).

The *Lentivirus* genus

The *Retroviridae* family comprises several genera, including the *Lentivirus* genus. Lentiviruses form a phylogenetically well-supported group and with a distinct morphology and unique capacity to infect non-dividing cells (Yamashita and Emerman, 2006). These viruses show a continuous replication with high error rates and recombination between different viral strains, leading to high diversity shaped by the interaction with the host immune system. This made the use of molecular clock based approaches for time of emergence estimations of the lentivirus genus dubious, and for a long time this group of viruses was thought to be a fairly modern retroviral group (Gifford, 2012). A valuable tool to measure the timescale of lentiviruses are the endogenous viral elements (EVEs). These remain stably integrated in the host genome and escape the high mutation rates associated with viral replication, keeping the original information of the ancestral virus. Therefore, the use of the endogenous fossils, together with temporal and geographical information on the species, is of main importance to understand the evolution of fast evolving pathogens (Gifford, 2012).

To date, circulating lentiviruses were identified in a few mammalian species: simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) in primates; feline immunodeficiency virus (FIV) in felines; maedi-visna virus (OMVV) and caprine arthritis-virus (CAEV) in sheep and goats; Jembrana disease virus (JDV) and bovine immunodeficiency virus (BIV) in the bovine species; and equine infectious anemia virus (EIAV) in the horse (St-Louis et al., 2004) (Figure 1.1). Interestingly, no lentivirus was identified in extensively studied species like mice, rats, dogs or pigs. In primates, lentiviruses were identified only in Old World Monkeys, suggesting that SIV is posterior to the migration that originated New World and Asian primates (Sharp and Hahn, 2010). The discovery of endogenous lentiviruses in Malagasy lemurs constitute evidence that lentiviruses affected primates at least 4.2 million years ago (Gilbert et al., 2009). The lineage that affects felines, FIV, seems to have emerged before the separation of the feline population from Africa to America, which was geographically possible approximately 4.5 million years ago (Mya) due to the last period of low sea level (Troyer

et al., 2008, Gifford, 2012). However, the feline lentivirus was suggested to be older and probably in the origin of the endogenous prosimian virus (PSIV) (Gifford, 2012).

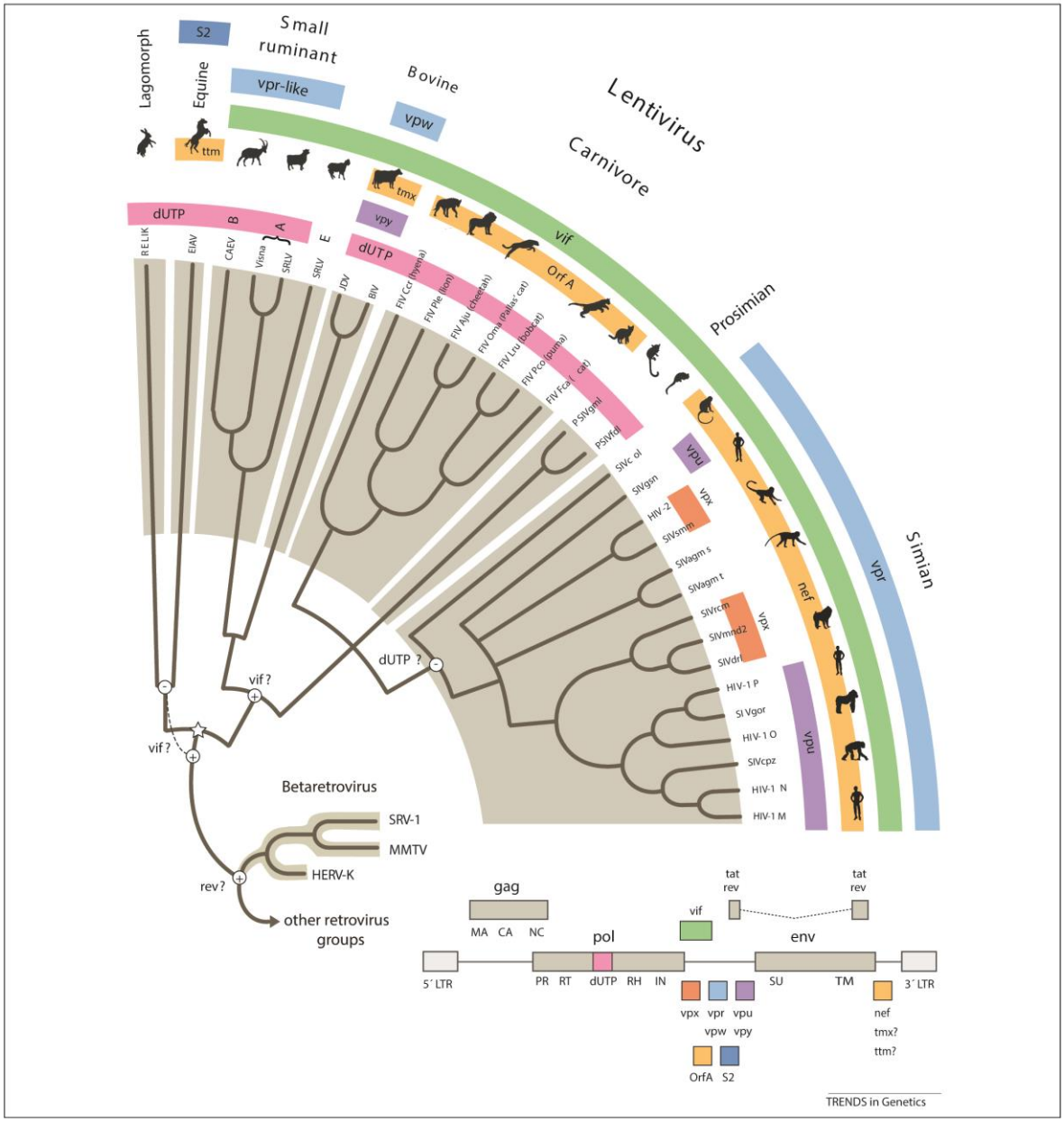

Figure 1.1 - Viral evolution in deep time of lentiviruses and mammals. The evolutionary relationship between different lentiviruses and with other retroviruses is represented. The name of the each lentivirus is represented at the end of the branches, followed by information on accessory proteins expressed and mammalian species affected by each virus. The general genome organization of lentiviruses is also depicted. From Gifford 2012.

Another endogenous lentivirus was identified in rabbits, named rabbit endogenous lentivirus K (RELIK). The Lagomorpha order is divided into two families, *Ochotonidae* and *Leporidae*, that diverged more than 35 Mya. RELIK, however, was specifically found in leporids, placing the endogenization event and a minimum age for the lentivirus of

approximately 12 Mya (van der Loo et al., 2009, Keckesova et al., 2009, Matthee et al., 2004). Nevertheless, it is important to keep in mind that the lentivirus lineage could be older, since the endogenization time does not represents the time of genome invasion by the virus.

The genome organization of lentiviruses is similar, with all lineages sharing the main domains *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope), although, different lineages have distinct accessory proteins. The presence of *rev* (RNA splicing-regulator) in all lentiviruses and *rev* functional homologs in betaretroviruses, suggests that this viral protein has an ancestral origin (Mertz et al., 2005, Gifford et al., 2008). Some viral proteins, such as Tat (transactivator protein) and Vif (viral infectivity factor) , are also present in several lineages, while other proteins, like Nef (negative regulator factor) and OrfA, are specific for some groups (Hong et al., 2010). There are also accessory proteins that are unique for a specific lineage, as for example, Vpx is only present in some SIV clades and HIV-2, and the S2 protein is exclusive for EIAV (Wang et al., 2014, Yu et al., 1991, Tristem et al., 1992).

Vif is one of the proteins present in almost all lineages of circulating lentiviruses, however Vif from distantly related viruses are highly divergent, and even inside the primate lineage it only shows around 30% identity (Oberste and Gonda, 1992). In the region where primate lentiviruses encode for the accessory proteins *vpx* (virus protein x), *vpr* (virus protein r) and *vpu* (virus protein unique), BIV expresses *vpy* and *vpw* genes. However, there is no information on the amino acid or structure similarity with other accessory proteins and their function is poorly understood (Garvey et al., 1990). In a similar way, *tmx* from the bovine lentiviruses and *ttm* from the equine lentivirus are placed in a similar genomic region as the simian lentiviruses' *nef*, yet, once again, their function is still not determined (Snider et al., 1997, Beisel et al., 1993). On the other hand, although *orfA* and *nef* are in different genomic regions, the OrfA protein expressed in the feline lineage was suggested to exert a similar role as Nef in primates, causing the downmodulation of the cellular receptors from the cell membrane in order to control viral infection levels (Hong et al., 2010).

An important feature between these viruses is that they all seem to infect monocytes/macrophages or T cells (Jin et al., 2005, Clapham and McKnight, 2001, Willett and Hosie, 2013, Onuma et al., 1992) (Table 1.1). Besides, FIV and HIV both use CXCR4 (chemokine receptor 4 or fusin) as a co-receptor, and it was shown that BIV and HIV are able to bind to CCR5 (chemokine receptor 5) (Wright et al., 2002, Willett and Hosie, 2013). Strikingly, while there is some sequence similarity in the transmembrane domain of the lentivirus envelope, the surface glycoprotein shares almost no sequence

similarity for most of the viruses (Hotzel and Cheevers, 2000, Pancino et al., 1994, Garvey et al., 1990). Unfortunately, the receptor or the need for co-receptor for the remaining elntiviruses is still unknown.

Table 1.1 – Lentiviruses and their target cells and receptors. Based on (St-Louis et al., 2004) and (Gifford, 2012).

Virus	Host group	Target cells	Receptor	Co-receptor
HIV	primate	T cells / macrophages	CD4	CCR5/CXCR4
FIV	feline	T cells	CD134	CXCR4
BIV	Bovine	macrophages	--	--
OMVV	Small ruminant	macrophages	--	--
EIAV	Equine	macrophages	ELR1	no

HIV structure, life cycle and pathogenesis

HIV is a RNA virus whose genome is composed of two single-stranded copies. The viral genome is flanked by long terminal repeats (LTR) that serve as promoters, and encodes three main coding domains, *gag*, *pol* and *env* (Sundquist and Krausslich, 2012) (Figure 1.2).

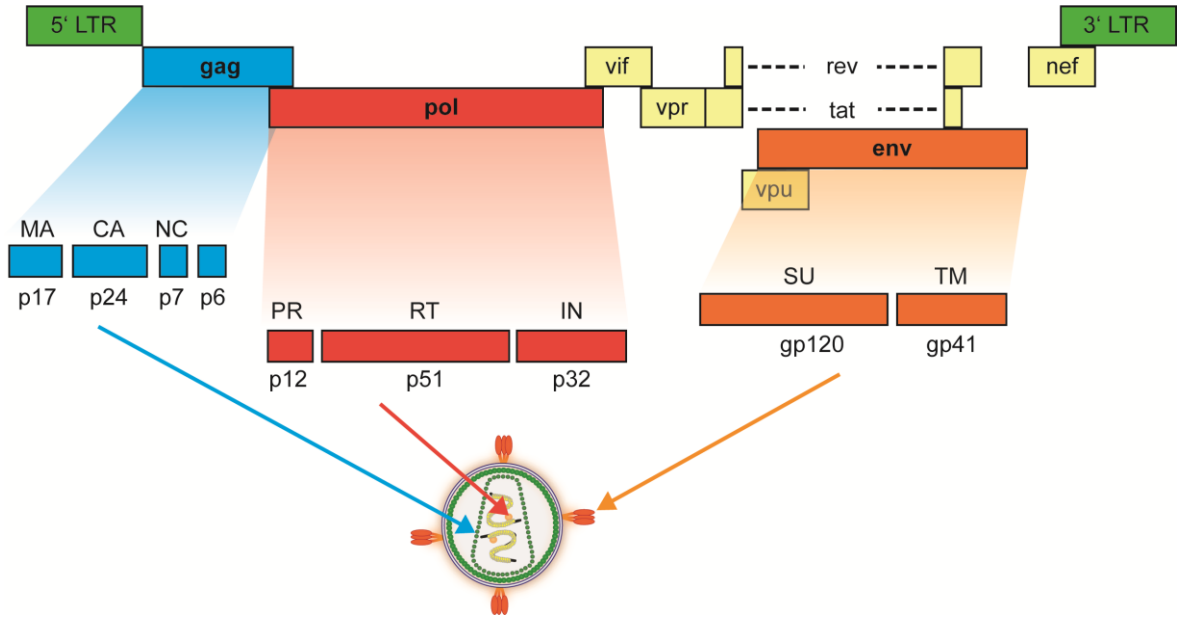

Figure 1.2 - HIV-1 genome organization and structure. The main viral components Gag, Pol and Env are further processed to originate the structural proteins. The genomic location of the accessory proteins Vif, Vpr, Vpu and Nef, Tat and Rev are also represented.

The *gag* domain encodes a polyprotein precursor called $Pr55^{Gag}$ that is cleaved by the viral protease (PR) to originate the structural components of the viral core, the capsid (CA or p24), matrix (MA or p17), nucleocapsid (NC or p7) and the nucleic acid-stabilizing p6 protein. The *pol* domain encodes for the viral protease (PR or p12), reverse transcriptase (RT or p51), integrase (IN or p32) and RNase H (p12). The *env* domain contains the gp160 precursor, which upon cleavage results in the surface protein (SU or gp120) and transmembrane protein (TM or gp41), the glycoproteins responsible for viral attachment and fusion. HIV viral genome encodes additional regulatory proteins, such as Tat and Rev, which play an important role in the initiation of replication, viral transcription and nuclear export (Karn and Stoltzfus, 2012). Nef, Vif, Vpr and Vpu are important for the progression of different steps of the viral replication (Emerman and Malim, 1998). Instead of Vpu, HIV-2 encodes for Vpx (Vicenzi and Poli, 2013).

HIV infects cells by using the human receptor CD4 (T cell receptor cluster of differentiation 4) and the co-receptor CXCR4 or CCR5 (Feng et al., 1996, Clapham and McKnight, 2002, Alkhatib et al., 1996). After nonspecific attachment to the host cell, the C4-domain of the envelope glycoprotein gp120 binds to the D1-domain of CD4, resulting in conformational changes that allow the interaction of the co-receptor with the third hypervariable loop (V3), the β-19 strand and the bridging sheet of gp120 (McDougal et al., 1986, Moore et al., 2004, Klasse, 2012). The V3 loop is the main determinant whether the virus uses CXCR4 (X4-tropic viruses) or CCR5 (R5-tropic viruses) as a co-receptor (Hartley et al., 2005). After binding to the receptor and co-receptor, further conformational changes occur in order to place the highly hydrophobic N-terminal of gp41 in contact with the cell plasma membrane, leading to the fusion of the viral and cell membranes (Pan et al., 2010, Klasse, 2012).

Upon fusion, the viral core is released in the cytoplasm. The core corresponds to the cone-shaped structure formed by capsid multimers that surround the viral genome, nucleocapsid, reverse transcriptase, integrase and some accessory proteins and cell factors (Arhel, 2010). It is still unclear whether uncoating of the viral core occurs before or after the initiation of the viral RNA reverse transcription and where (Bukrinsky, 2004, Suzuki and Craigie, 2007, Warrilow et al., 2009). The viral RNA is reverse transcribed into minus-strand DNA by the viral reverse transcriptase while the RNase H degrades the original RNA strand. Two RNA sequences known as polypurine tract are resistant to degradation and serve as primers for the plus-strand DNA synthesis, generating the double-stranded viral DNA (Hu and Hughes, 2012). The composition of the pre-integration complex (PIC), although not entirely solved, consists of the proviral DNA together with the integrase, the matrix protein and Vpr, to be transported to the cell

nucleus. This complex recruits and binds host proteins to facilitate the nuclear import of the viral DNA through nuclear pores (Bukrinsky, 2004). Once in the nucleus, the proviral DNA is integrated in the host genome by the viral integrase and host enzymes finish the integration by repairing the gaps and ligate the DNA (Craigie, 2012).

After proviral DNA integration replication can cease, and during this latent state the virus remains hidden in the cell. The resting state of memory $CD4^+$ T cells makes them a primary reservoir for latent HIV proviruses, which can resume their replication once these cells become activated (Ruelas and Greene, 2013). HIV replication starts by using the cellular transcriptional machinery, with the first mRNAs produced coding for the accessory proteins Rev and Tat. Tat migrates to the nucleus to activate the viral transcription through the LTRs and Rev helps in the transport of the unspliced and single spliced RNA to the cytoplasm, where all viral components are translated by the host cell translation machinery (Burugu et al., 2014, Ohlmann et al., 2014).

The polyprotein Gag-Pro-Pol starts the assembly process, with each domain of Gag playing a different role. MA domain binds to the plasma membrane and recruits the Env protein, while the CA starts to form the viral shell. The NC domain captures the viral RNA to be packaged and the p6 is responsible for the recruitment of the viral accessory proteins and interacts with host proteins from the ESCRT pathway (Sundquist and Krausslich, 2012). The Env protein is glycosylated on its way to the plasma membrane, assembled into trimeric complexes and processed by the cellular protease furin into the TM and SU domains. Finally, the ESCRT pathway is used by the virus to promote membrane fission and virion release (Sundquist and Krausslich, 2012). Upon budding, the viral protease is activated and cleaves Gag and Gag-Pro-Pol polyproteins. In this maturation step, the processed proteins suffer conformational changes and rearrangements that shape the viral core and stabilize the genomic RNA dimer (Sundquist and Krausslich, 2012). Although several steps are involved in the HIV replication, a replication cycle has the duration of approximately 24 hours (Mohammadi et al., 2013).

HIV targets are typically CD4 positive cells such as T helper cells, dendritic cells, macrophages and astrocytes. The most common route of transmission involves sexual interaction, where HIV in contact with mucous membranes encounters dendritic cells and monocytes or macrophages (Zhang et al., 1999b). These cells work primarily as reservoirs that carry the virus, although R5-tropic viruses can enter and already replicate in macrophages (van't Wout et al., 1994). Once in the blood stream, T helper cells can be infected by both X4- and R5-tropic viruses. A few days after infection, the viral replication induces the release of inflammatory cytokines and chemokines, and an

immune response is mounted during the acute phase, with the production of several neutralizing antibodies against viral antigens and the activation of $CD8^+$ T cells against infected cells (Pincus et al., 2003). However, due to the lack of proofreading activity of the viral reverse transcriptase, one incorrect nucleotide is incorporated on average per reverse transcription round, which creates a flow of quasispecies that can escape immune recognition (Althaus and De Boer, 2008). The viral replication leads to the decline of $CD4^+$ T cells and therefore a deceleration of viral production (Coffin and Swanstrom, 2013). The asymptomatic or latent phase is characterized by a drop of virus titers from 10^5-10^9 to less than 10^2 genome copies/mL (Coffin and Swanstrom, 2013). Despite the drop in viral load, the virus keeps replicating within the host and the depletion of $CD4^+$ T cells continues due to events not entirely known (Doitsh and Greene, 2016). When the $CD4^+$ T lymphocyte counts drop to around 200 cells/mL, the immunodeficiency phase is reached and the individual shows no longer immunity against opportunistic infections (Coffin and Swanstrom, 2013).

Restriction factors and viral antagonism

The innate immune system represents a crucial barrier against pathogens, detecting the presence of foreign molecules at an early stage and starting a set of inflammatory responses. Different pathogen-associated molecular patterns (PAMPs) can be sensed by the cellular pattern recognition receptors (PPRs), which can be found at the cell surface or within endosomes, as for example toll-like receptors (TLRs), or in the cytosol, as for example the RIG-I-like receptors (RLRs) or Nod-like receptors (NLR) (Jensen and Thomsen, 2012). This process leads to the production of type I interferons (IFN), which induce the expression of interferon-stimulated genes (ISGs), including antimicrobial agents. The production of type I interferons also leads to the maturation of dendritic cells (DCs), cytotoxicity natural killer cells (NK) and differentiation of cytotoxic T lymphocytes, thus activating the immune system (Honda et al., 2006, Rustagi and Gale, 2014).
During virus infection, the virus RNA can be recognized in the cytoplasm by the RLRs or in endosomes by TLRs, and after reverse transcription, the viral DNA can still be sensed by the cyclic GMP/AMP synthase (cGAS) or other DNA sensors, such as IFI16, DNA-PK or AIM2 (Sun et al., 2013, Burckstummer et al., 2009, Ferguson et al., 2012, Unterholzner et al., 2010). Different HIV target cells show distinct sensors, some of which the virus can easily escape, while for others the antiviral mechanisms started are enough to inhibit HIV replication. This in part explains why cells like macrophages, dendritic cells or resting T cells are more difficult to infect when compared to $CD4^+$ T activated cells (Silvin and

Manel, 2015, Sattentau and Stevenson, 2016, Bergamaschi and Pancino, 2010, Smed-Sorensen et al., 2005).

Host restriction factors are responsible for inhibit and block pathogens, such as viruses. They can be constitutively expressed or upregulated upon activation of the innate immune response. IFITM proteins were shown to inhibit a broad group of viruses, from influenza A virus to Vesicular Stomatitis virus (VSV) (Brass et al., 2009, Huang et al., 2011, Weidner et al., 2010). IFITM3 specifically was shown to act early on HIV life cycle by inhibiting the entry to the target cells in a cell-to-cell transmission context, however its antiviral mechanism is still not entirely clear (Compton et al., 2014, Tartour et al., 2014). TRIM (tripartite motif) composes a family of proteins with several members that seems to have suffered extended gene expansion and loss along the mammalian lineage (Sawyer et al., 2007, Meroni, 2012). Several TRIMs were shown to have antiretroviral activity, however TRIM5α and TRIM22 are the most prominent members associated with HIV (Kajaste-Rudnitski et al., 2010, Nisole et al., 2005). The mechanism of restriction by TRIM5α starts with binding to the incoming capsid and inducing premature disassembly, which leads to proteasomal degradation of the viral components, and consequently block of the viral replication before the reverse transcription step (Kutluay et al., 2013). TRIM22 acts at a different phase of virus replication by efficiently inhibiting viral gene expression in a LTR-dependent manner, being a strong inhibitor in monocyte-derived macrophages (MDMs) (Tissot and Mechti, 1995, Bouazzaoui et al., 2006, Singh et al., 2011). As for other restriction factors, TRIM22 is able to inhibit several other virus, although using different mechanisms (Gao et al., 2009, Eldin et al., 2009, Kajaste-Rudnitski et al., 2011). The triphosphohydrolase activity of the Sterile alpha motif- and HD-domain containing protein 1 (SAMHD1) is responsible for catalyzing dNTPs (deoxynucleoside triphosphate) into deoxynucleosides and inorganic phosphate, limiting the cellular dNTP pool available during the reverse transcription step, and therefore inhibiting viral replication. SAMHD1 is inducible by type I interferon in monocytes, however is also highly expressed in dendritic cells and in resting $CD4^+$ T cells, playing a major role in the inhibition of HIV-1 replication in these cells (Baldauf et al., 2012, Goldstone et al., 2011, Lahouassa et al., 2012, St Gelais et al., 2012, Diamond et al., 2004). Surprisingly, while HIV-2 and some SIVs encode for Vpx, an accessory protein that is able to counteract SAMHD1, HIV-1 has no mechanism to directly counteract SAMHD1 (Berger et al., 2011, Hrecka et al., 2011, Ahn et al., 2012). In this regard, it was suggested that the reverse transcriptase of HIV-1 is more active and it might be able to work with lower amounts of dNTPs (Post et al., 2003, Lahouassa et al., 2012).

Mx genes are highly conserved in mammals and arose by duplication to give rise to Mx1 (or MxA) and Mx2 (or MxB) (Haller et al., 2015). While Mx1 was shown to be antivirally active against several viruses, Mx2 is able to inhibit HIV-1 (Haller and Kochs, 2011, Kane et al., 2013, Goujon et al., 2013, Liu et al., 2013). Although the exact mechanism through which Mx2 inhibits HIV replication is still not clear, the models so far proposed defects in uncoating, inhibition of nuclear uptake, or even deficiency during integration (Fricke et al., 2014, Goujon et al., 2013, Kane et al., 2013, Liu et al., 2013).

APOBEC (apolipoprotein B mRNA-editing enzyme) constitutes a family of polynucleotide cytidine deaminases that convert cytidine (C) into uridine (U) by deamination. Several members of this family show antiviral activity towards HIV, with APOBEC3G (A3G) being the most potent member, followed by A3F (Holmes et al., 2007). In retroviruses, A3G causes high mutational rates during reverse transcription, characteristically G to A mutations in the negative DNA strand, and impairs a successful replication (Harris et al., 2003, Mangeat et al., 2003, Sheehy et al., 2002). Nevertheless, human A3G is efficiently counteracted by the accessory protein Vif, which interacts and targets A3G for proteasomal degradation (Marin et al., 2003, Sheehy et al., 2003, Stopak et al., 2003, Feng et al., 2014). On the other hand, residual activity of A3G can work in favor of the virus, since minimal mutations can increase virus diversity and potentiate escape from adaptive immunity (Simon et al., 2005, Sadler et al., 2010).

Tetherin (or BST-2) is a restriction factor that acts on HIV budding by interfering with the release of viral particles from the producer cell (Neil et al., 2008, Van Damme et al., 2008). Tetherin's antiviral activity affects several enveloped viruses, mainly because its mode of action does not involve direct viral recognition (Evans et al., 2010). As an integral membrane protein, Tetherin is incorporated in the membrane of the budding virion but keeps part of itself in the cellular membrane, and thus anchors the virion to the cell (Perez-Caballero et al., 2009, Sauter, 2014). Primate lentiviruses use Nef, which promotes its internalization to lysosomal compartments (Zhang et al., 2009). However, HIV Nef is not able to target human Tetherin due to the deletion of the cytoplasmic interacting amino acids. Instead, HIV-2 uses its Env glycoprotein to promote internalization of Tetherin, while HIV-1 uses its accessory protein Vpu which is able to target Tetherin for ubiquitination and consequently degradation (Iwabu et al., 2009, Mangeat et al., 2009, Le Tortorec and Neil, 2009, Sauter, 2014).

Some restriction factors, such as SERINCs (SERine INCorporators), GBPs (Guanylate binding proteins), MARCH8 (Membrane-Associated Ring-CH 8) and 90k (Galectin-3-binding protein), do not reduce viral production or release, but affect viral infectivity. SERINCs constitute a family of proteins composed by five members, and so far,

SERINC3 and SERINC5 were identified as HIV-1 inhibitors (Rosa et al., 2015, Usami et al., 2015). Their expression in the producer cell allows incorporation in the virion and further inhibition at the entry step with the target cells (Rosa et al., 2015, Usami et al., 2015). Although the inhibition mechanism is not well understood, it is known that HIV-1 Nef can counteract SERINC3 and SERINC5 (Sood et al., 2017, Trautz et al., 2017, Gonzalez-Enriquez et al., 2017). Interestingly, SERINC3/5 can restrict other retroviruses, which seem to have similar mechanisms to counteract the action of these proteins (Chande et al., 2016, Ahi et al., 2016).

GBPs are a family of interferon inducible proteins of GTPases, whose role was described as important for the control of intracellular pathogens (Kim et al., 2012). Regarding HIV-1, GBP5 interferes with Env processing and its incorporation into the virion. Its expression in human macrophages was suggested to be important for the susceptibly of these cells to HIV-1 infection (Krapp et al., 2016). Interestingly, defective mutations of HIV-1 Vpu protein seem to be advantageous to overcome GBP5 restriction (Krapp et al., 2016, Hotter et al., 2017).

MARCH8, also expressed in macrophages and dendritic cells, was shown to be a restriction factor to several enveloped viruses, including HIV-1, where its ability to downmodulate transmembrane proteins affects the incorporation of the Env into the virion (Tada et al., 2015). So far, no MARCH8 counteraction mechanism is known.

90k is another restriction factor induced by interferon and expressed at low levels in PBMCs (primary blood mononuclear cells) and $CD4^+$ T cells (Lodermeyer et al., 2013). This protein affects the complete processing of the Env protein and its incorporation in the virion, thus reducing virion infectivity (Lodermeyer et al., 2013). Although no counteraction has been described for the human 90k, the murine ortholog is not able to affect HIV-1 infectivity (Lodermeyer et al., 2013, Lodermeyer et al., 2018).

The viral response does not target exclusively the components of the intrinsic immunity, but it is also used to evade the innate and the adaptive immune system. For example, the ability of Nef to downmodulate host proteins from the cell surface is used by the virus to reduce leukocyte antigen (HLA) expression and avoid antigen recognition by NK cells, or to downmodulate MHC I (major histocompatibility complex) expression and to escape from cytotoxic $CD8^+$ T cells (Cohen et al., 1999, Arhel and Kirchhoff, 2009).

Host virus co-evolution

The interactions between the virus and the host creates a scenario of evolutionary "arms race", with a dynamic co-evolution for the host and the pathogen. Proteins involved in the arms race evolve under the "Red Queen" hypothesis, accumulating an excess of non-synonymous mutations compared to synonymous mutations (Van Valen, 1973). Non-synonymous mutations constitute a nucleotide mutation that alters the amino acid sequence of a protein, while nucleotide mutations that do not alter the amino acid composition are known are synonymous or silent mutations. The ratio between the non-synonymous mutation rate (d_N) to the synonymous mutation rate (d_S), known as d_N/d_S, can be calculated for single residues or across entire proteins, revealing the absence or presence of genetic conflict (Figure 1.3). Usually, an increased selective pressure is visible on the pathogen's components that are related with virulence and host evasion, while for the host, the immune system related genes are the ones under higher pressure to evolve. In that sense, the virus tries to escape the restriction imposed by the host, and by its turn, the host evolves to keep up with the virus and maintain its defense (Little et al., 2010, Compton et al., 2013). Therefore, genetic signatures of positive selection are often found in restriction factors (Duggal and Emerman, 2012).

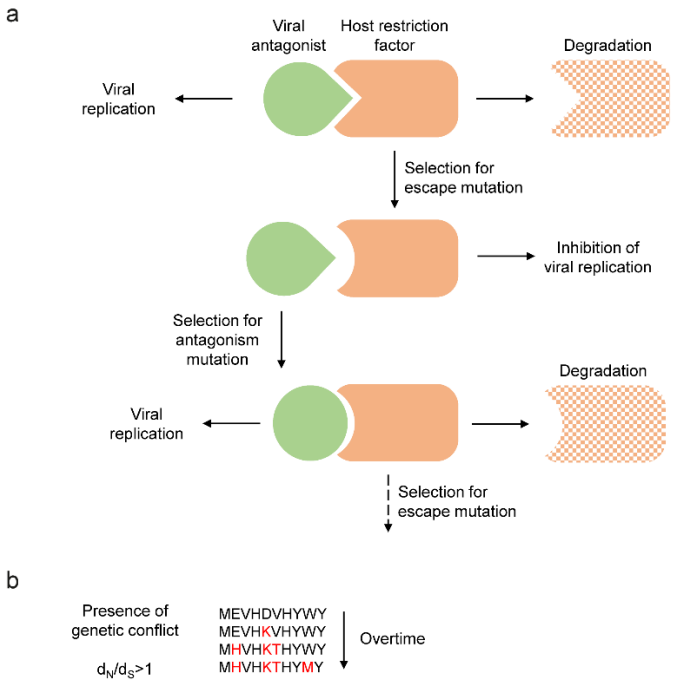

Figure 1.3 - Genetic conflict between pathogen and host. **a.** Virus-host interaction leads to a continuous mutual evolution, with the host evolving to inhibit the replication of the virus, and the virus evolving to keep its counteraction to allow replication. **b.** Virus-host interactions increase the selection pressure on the evolved proteins, which overtime leads to the accumulation of non-synonymous mutations (in red), which is seen in a higher d_N/d_S ratio.

In this arms race, the virus has the advantage of being able to evolve faster, with nucleotide substitution rates around 1000 times higher than those of their hosts (Holmes, 2003, Duggal and Emerman, 2012). However, the virus is limited by its small genome, and as some viral proteins accumulate more than one function, a mutation favorable for one function might thus compromise another. In the host, heterozygosity for a restriction factor might be advantageous in the population, forcing the virus to target multiple alleles. Another advantage of the host is the possibility of gene duplication, which allows to simultaneously explore different evolutionary paths. The duplication of a restriction factor can also generate multiple members that use different ways to target the same pathogen, challenging it to keep multiple defense strategies (Duggal and Emerman, 2012). Therefore, understanding the mechanisms of virus evolution within the host is crucial to predict how similar viruses could evolve.

The reconstruction of a chimeric RELIK capsid showed that, as for contemporary lentiviruses, this ancient endogenous virus required cyclophilin A (CypA) binding for successful viral replication, stating an ancestral origin for the interaction between CypA and lentiviruses (Lin and Emerman, 2006, Goldstone et al., 2010). Lentiviruses also interact with host proteins that act as restriction factors, which is reflected by the positive selection evidenced on these proteins. An example are the members of the APOBEC3 family, which showed to be under positive selection in primates and work as a restriction factor for several lentiviruses and retroviruses (Yamada et al., 2016, Sawyer et al., 2004, Bishop et al., 2004, Etienne et al., 2015). The accessory protein Vif, present in almost all lentiviral genomes, counteracts APOBEC3 antiviral activity (Gifford, 2012, Jonsson and Andresdottir, 2013). However, Vif antagonism is species-specific, i.e. the viral protein is able to counteract the APOBEC from its host, but has no effect on the APOBEC from other species (Mariani et al., 2003). Studies in primates indicated that many of the sites under positive selection in A3G correspond to sites important for the interaction with Vif (Compton and Emerman, 2013, Compton et al., 2012). Particular groups of primates acquired some mutations in these sites, which lead to the disruption of Vif interaction with APOBEC (Compton and Emerman, 2013).

SAMHD1 was also shown to be under positive selection in the primate lineage, particularly the residues that interact with the counteracting proteins Vpr and Vpx (Laguette et al., 2012, Lim et al., 2012). Strikingly, unlike SIV and HIV-2, HIV-1 has no protein that directly counteracts SAMHD1 (Laguette and Benkirane, 2012). SAMHD1 was also reported to restrict other retroviruses besides the lentivirus family. However, for some the counteraction mechanism is not known and it is also not clear if SAMHD1's mode of action is the same for different viruses (Gramberg et al., 2013).

Another restriction factor under strong positive selection is TRIM5. Within primates, the fusion of TRIM5 and CypA by retrotransposition gave origin to the TRIM-CypA fusion protein, representing another host tool for targeting and neutralizing the viral capsid (Malfavon-Borja et al., 2013). Independent fusion events occurred in different groups of New World Monkeys (rhesus monkeys and owl monkeys) (Sayah et al., 2004, Ribeiro et al., 2005), and a previous fusion event was as well identified in Old World Monkeys (Malfavon-Borja et al., 2013). Even though this previous fusion protein has lost its function by accumulation of deleterious mutations, its existence stands for a possible interaction between primates and lentiviruses, or at least retroviruses, that dates back at least 43 million years (Malfavon-Borja et al., 2013, Wilson et al., 2008, Dietrich et al., 2010). Concerning TRIM5α alone, the antiviral properties of this protein have been documented for several species (Sawyer et al., 2005, Si et al., 2006, Ylinen et al., 2006, Schaller et al., 2007). Interestingly, TRIM5α is not expressed in dogs, and rodent TRIM5-like members seem not to be effective in the inhibition of several retroviruses (Sawyer et al., 2007, Tareen et al., 2009). TRIM5α has also been reported to impose species-specific blocks that determine the tropism of a retrovirus, in the sense that the virus is able to overcome the restriction of its host TRIM5α, but is restricted by the TRIM5α of other species (Nakayama and Shioda, 2012). For instance, simian TRIM5α proteins are able to restrict HIV-1 while they cannot restrict their respective SIV. The same happens for human TRIM5α, which does not efficiently inhibit HIV (Stremlau et al., 2004).

As for the other restriction factors, Tetherin did also undergo positive selection in primates (Lim et al., 2010). Antiviral activity was shown from several species and a broad spectrum of enveloped viruses can be inhibited (Jouvenet et al., 2009, Yin et al., 2014, Takeda et al., 2012, Fukuma et al., 2011, Goffinet et al., 2010b). Interestingly, a deletion in the cytoplasmic tail of human Tetherin makes it resistant to Nef, therefore HIV-1 seems to have evolved another mechanism by using Vpu instead of Nef to counteract human Tetherin (Sauter et al., 2009, Neil et al., 2008, Lim et al., 2010). However, it is not clear what originated this deletion during human evolution. It might be possible that selective pressure was exerted by some other virus with similar Nef-like counteracting mechanism (Evans et al., 2010). Regarding other mammalian Tetherins, studies so far published show that HIV-1 Vpu is unable to counteract Tetherin from these species (Yin et al., 2014, Fukuma et al., 2011, Goffinet et al., 2010b), once again highlighting the adaptation of the virus to their host.

A different evidence for virus and host dynamics is displayed by CCR5. An allele frequently found in Northern Europeans carries a deletion (CCR5Δ32) that confers resistance to HIV-1 (Dean et al., 1996). Likewise, similar mutations were identified in

sooty mangabey species, and as a result, their respective SIV has evolved to use another co-receptor (Riddick et al., 2010). Although the origin of these mutations in humans is not clear, they might represent another source of evidence for virus-host co-evolution.

Current animal models of HIV research

HIV-1 transmission, replication and disease progression are widely studied, and advances on therapy and drug response would not be possible without the use of animal models (Hatziioannou and Evans, 2012). The ideal animal model to study HIV should recapitulate similar route of infection, allow a relatively fast progression to disease, have a similar immunological background, be relatively inexpensive, easy to maintain and work with and should involve minimal ethical concerns. While the perfect animal model is still not existing for HIV-1, several models were developed to study different parts of the virus life cycle and infection progression.

Non-human primates constitute an idealized model to study infectious diseases in humans, sharing similar physiology, immune system and infectious agents (Gardner and Luciw, 2008). While HIV-1 can efficiently infect chimpanzees, these primates do not develop AIDS-like symptoms and their use for research involves ethical concerns, high costs, and environmental issues due to their endangered situation, making it not a suitable model (Haigwood, 2009). However, non-human primates are used as a model to study HIV when infected with its close relative SIV. Different African monkeys and apes are endemically infected by their specific SIVs. Yet, due to virus-host co-evolution, these non-pathogenic agents do not lead to disease. For instance, SIVsmm and its natural host, the sooty mangabeys, were vastly studied mainly because this virus was the origin of HIV-2. Viral replication causes acute infections and activation of the immune system, however, it does not lead to chronic infection or causes depletion of mucosal or peripheral $CD4^+$ T cells (Chahroudi et al., 2012). Due to these characteristics, SIV and their natural hosts were mostly used to understand the source of immune protection and what stops disease progression (Pandrea and Apetrei, 2010, Chahroudi et al., 2012). On the other hand, SIV infection of non-natural hosts, such as Asian macaques, leads to mucosal $CD4^+$ T cell depletion, chronic immune activation and disease progression (Chahroudi et al., 2012, Fauci and Desrosiers, 1997, Lackner and Veazey, 2007). The SIV infection of macaque species was therefore recognized as a good model for HIV-1 infection and disease progression studies, and had a key role for the understanding of viral transmission, latency and pathogenesis. However, these species cannot be infected

with HIV-1 due to several replication blocks in their cells, including TRIM5α, APOBEC3 and SAMHD1. Furthermore, differences between SIV and HIV-1 make this model improper for vaccine development or for testing inhibitory drugs against HIV-1 enzymes (Simon et al., 2015, Witvrouw et al., 1999, Witvrouw et al., 2004, Ambrose et al., 2007). Therefore, different recombinant SHIV strains were developed, including SHIVs expressing HIV-1 envelope glycoprotein for vaccine development, and SHIVs containing HIV-1 *pol* gene for the study of drugs targeting HIV-1 enzymes (Joag et al., 1996, Ambrose et al., 2004, Ishimatsu et al., 2007, Smith et al., 2010). Other approaches consisted of adapting HIV-1 to replicate in macaque cells, known as stHIV-1 (simian-tropic HIV-1), and replacing HIV-1 to SIV accessory proteins which are able to overcome the replication blocks in these species (Thippeshappa et al., 2011, Hatziioannou et al., 2009, Igarashi et al., 2007). Unfortunately, the use of non-human primates is far away from being an ideal model, involving high maintenance costs and ethical concerns (Alter et al., 1984, O'Neil et al., 2000).

As for non-human primates and SIV models, cats are infected by FIV, which causes an AIDS-like disease in these animals (Burkhard and Dean, 2003, Elder et al., 2010). Although not infected by HIV, cats were used in the development of different antiretroviral drugs, including Tenofovir, a nucleotide analog reverse-transcriptase inhibitor (Egberink et al., 1990, Savarino et al., 2007, Hartmann et al., 1992, Mohammadi and Bienzle, 2012). However, the different accessory proteins and open reading frames between HIV and FIV impose a major barrier to make this model suitable to study HIV-1 (Sparger, 2006).

Rodents are commonly used as animal models mainly due the advantages that come with their short lifespan, their high reproductive rate, their low costs and the availability of several inbreed strains and associated reagents for these species. However, rodent cells cannot be infected by HIV-1 and several blocks to viral replication were already identified in mice and rats. The first block is represented by the lack of the human receptor and co-receptors, but besides the expression of these cofactors, rat and mouse cells showed further blocks (Bieniasz and Cullen, 2000, Keppler et al., 2002) (Figure 1.4).

Specifically in mouse cells, a post entry defect was identified, and although some studies suggested a block at reverse transcription step or nuclear import (Baumann et al., 2004, Tsurutani et al., 2007), other studies showed that mouse primary T cells and T cell lines do not support HIV integration (Tervo et al., 2008, Zhang et al., 2008a). Rodent cyclin T1 represents another barrier for Tat-mediated transactivation and therefore inefficient transcription (Sun et al., 2006, Goffinet et al., 2007b). Late HIV gene expression is also

reduced in rodent cells, possibly due to oversplicing or decreased nuclear export of unspliced viral RNAs, which has been associated with defective Rev control by the host factor CRM1 (Bieniasz and Cullen, 2000, Winslow and Trono, 1993, Zheng et al., 2003, Okada et al., 2009, Nagai-Fukataki et al., 2011).

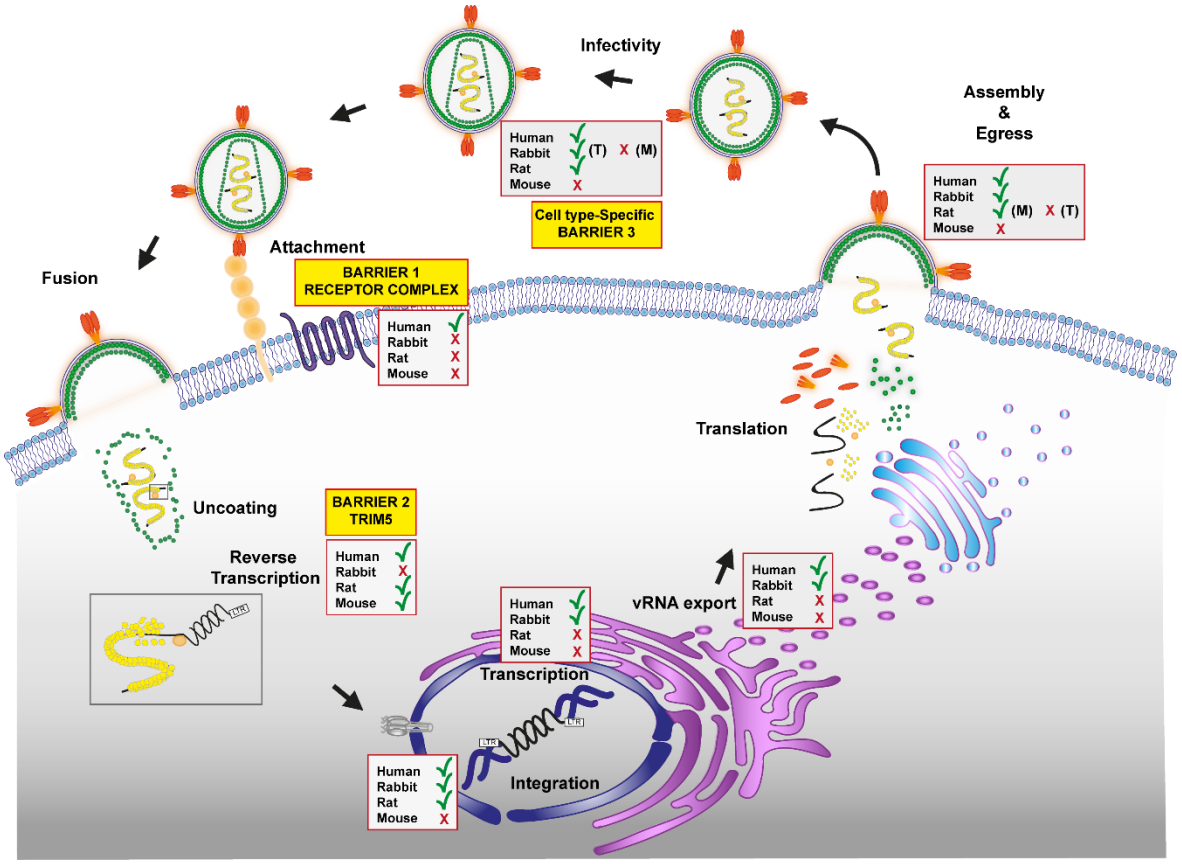

Figure 1.4 - HIV-1 replication cycle and species-specific barriers. HIV enters the target cell via interaction with a human receptor and co-receptor. Inside the cell, the viral genome is reverse transcribed and further integrated in the host genome. Transcription of the viral components is followed by nuclear export, translation and viral assembly. Upon virus release, the particle undergoes maturation and it can finally infect other cells. Rodents and rabbits show barriers at different stages of the virus replication, which are highlighted in the red boxes. Rabbit-specific barriers are further shown in yellow boxes. T = T cells; M = macrophages. Image prepared by Dr. Baldauf.

Rodent cells also affect release of HIV-1 virions. While the accessory protein Vpu is able to counteract the antiviral action of the human Tetherin, the rodent counterparts are resistant to Vpu-mediated degradation (Neil et al., 2008, Goffinet et al., 2009, Goffinet et al., 2010a). Incomplete processing of the Gag was also reported in both mouse and rat cell lines and in rat T cells (Mariani et al., 2000, Zhang et al., 2008a). Murine APOBEC3 impose a further barrier to HIV-1 replication, which cannot be target by the viral protein Vif (Sheehy et al., 2002, Mariani et al., 2003). Considering the amount of barriers for

HIV-1 replication in mouse and rat cells, the latter support more steps and makes it a more promising model (Goffinet et al., 2007a, Goffinet et al., 2007b). However, while the expression of several human co-factors showed to be sufficient for HIV-1 replication in rat macrophages, transgenic rat T cells were still not susceptible to HIV-1 infection (Shida et al., 2017).

Due the several pre-integration barriers in mouse cells, mice expressing HIV transgenes, either individual HIV-1 genes or the full-length provirus, were generated. Still, the expression of the full virus was not entirely successful, mostly due the restriction factors in the mouse cells, and no virus spread was observed even in transgenic mice (Browning et al., 1997, Sawada et al., 1998, Toggas et al., 1994, Corboy et al., 1992, Sun et al., 2006). Hence, humanized mice is so far the most widely used model to study HIV-1. These animals are genetically immunocompromised, and different strategies are used to reconstitute distinct parts of the human immune system in mice. The SCID-hu (combined immune deficiency human) model use human thymocytes and naïve T cells to reconstitute a human system, but lacks a primary immune system and the reconstituted cells do not spread into the periphery. The hu-HSC (human hematopoietic stem cells) model, using peripheral blood lymphocytes, allows a better reconstitution of a nearly complete immune system (Akkina, 2013). Finally, the BLT (bone-liver-thymus) model is a combination of the two previous models, and combines a wide spread of human immune cells with T cells developed in a mimicking human environment (Wege et al., 2008, Dudek et al., 2012). However, is not possible to use these models to access the complete human immune response or the effect of HIV-1 infection in non-hematopoietic tissues. Most of all, these animals have to be generated *de novo* for each experiment, which makes it time and cost consuming, and special conditions are needed due to their immunocompromised state (Karpel et al., 2015).

The lagomorph family and the rabbit as an animal model

Rabbit belong to the lagomorpha order, which can be subdivided in two families, the *Leporidae* and *Ochotonidae*. While *Ochotonidae* is represented by a unique genus, the Ochotona, which include several species of pikas, the family *Leporidae* is subdivided into the hares and rabbits. The hares are composed by the genus *Lepus*. The rabbits comprise ten different genera – *Brachylagus*, *Bunolagus*, *Caprolagus*, *Nesolagus*, *Oryctolagus*, *Pentalagus*, *Poelagus*, *Pronolagus*, *Romerolagus* and *Sylvilagus* (Matthee et al., 2004).

Fossil records suggest that the European rabbit had its origin on the Iberian Peninsula, and the subspecies *Oryctolagus cuniculus cuniculus* has expanded its range until France, where about 1400 years ago the domestication of this species started (Lopez-Martinez, 2008). After that, the European rabbit expansion continued and it is currently found in Europe, North Africa, North and South America, Australia and New Zealand. In the Mediterranean region, wild populations of rabbit and hares are important for the ecosystem, however in other regions the rabbit can represent a pest (Delibes-Mateos et al., 2007, Fenner, 2010). In general, the European rabbit is used for its meat and fur, but also as an important tool for research.

Currently, two viruses, the myxoma leporipoxvirus (MYXV) and the calicivirus rabbit hemorrhagic disease virus (RHDV), affect the rabbit population. The MYXV is a poxvirus that causes harmless fibromas in its natural host, the *Sylvilagus*, however it is lethal for the European rabbit. The virus infects primarily dendritic cells and further spreads to dermally localized macrophages, culminating in the expansion to the draining lymph node. Ultimately, the continuous viral replication leads to a drastic loss of lymphoid cells (MacLachlan, 2017). Nevertheless, co-evolution of the European rabbit with the MYXV gave rise to some resistant populations, but it remains to be studied which host genes participate in the resistance towards this virus (Kerr, 2012, Carmo et al., 2006, van der Loo et al., 2012, de Matos et al., 2014). The European rabbit is also susceptible to RHDV, a lagovirus that causes an acute infection and ultimately destroys the liver and spleen culminating in the death of the rabbits (Abrantes et al., 2012). This virus has decimated the rabbit populations on the Iberian Peninsula, however, the host response and the virulence of this virus is still poorly understood (Abrantes et al., 2012, Abrantes et al., 2013). So far, it was shown that this virus replicates in the hepatocytes, however the receptor used by the virus to enter these cells it is still unknown. Recently, host-cell histo-blood group antigens were identified as putative attachment factors (Prieto et al., 2000, Nystrom et al., 2011, Lopes et al., 2017).

RIG-I (retinoic acid-inducible gene-I), MDA5 (melanoma differentiation associated factor protein 5) and LPG2 (laboratory of genetics and physiology 2) constitute some pattern-recognition receptors involved in viral recognition, which were identified to be under positive selection in Leporids (de Matos et al., 2014). Comparison of the RIG-I in *Oryctolagus* and *Sylvilagus* showed amino acid changes between these species mostly in the repressor domain, which is responsible for viral DNA recognition. In light of this finding, it was suggested that the alterations on RIG-I from *Oryctolagus* and *Sylvilagus* might explain the differences of how these species sense MYXV (Wang et al., 2008). Going into the same direction, differences in rabbit chemokines could be associated with

disease susceptibility. While CXCR4 was shown to be highly conserved in lagomorphs (Abrantes et al., 2008), CCR5 is more diverse. Gene conversion between CCR5 and CCR2 caused the replacement of the QTLKMT motif in the second extracellular loop of CCR5 for the CCR2 motif HTIMRN, exclusively in *Oryctolagus*, *Bunolagus* and *Pentalagus* (Abrantes et al., 2011). Similarly, the CCR5 ligand CCL8 was shown to be pseudogenized in these species and intact in the species where the gene conversion was not observed (van der Loo et al., 2012). Therefore, it was suggested that the absence of this chemokine in *Oryctolagus* might represent an obstacle for leukocyte migration, which is favorable for MYXV infection (van der Loo et al., 2012).

Besides the currently circulating viruses, the presence of the rabbit endogenous lentivirus K (RELIK) in the genome of some leporid species might as well have shaped genes involved in the antiviral response of these species. This lentivirus has a simpler structure compared to other lentiviruses, with only two accessory proteins, Tat and Rev, besides the main coding domains Gag, Pol and Env (Katzourakis et al., 2007). All the main coding domains were defective with several codon stops and frameshift mutations, originated by neutral evolution of the host genome (Katzourakis et al., 2007). The identification of this lentivirus in the genome of other leporids, including hares, indicated that the endogenization event occurred in the ancestral of these species more than 12 million years ago (Keckesova et al., 2009, van der Loo et al., 2009). Although RELIK's infection route and mechanism are still unknown, it was suggested that its presence in rabbits and hares conditioned the evolution of TRIM5α of these species (de Matos et al., 2011). The residues identified under positive selection in the PRYSPRY domain of lagomorphs' TRIM5α, and the ability of hare TRIM5α to inhibit the replication of several retroviruses, suggested that, similar to primates, the selective pressure in these species was exerted by TRIM5-sensitive viruses, such as RELIK (de Matos et al., 2011, Fletcher et al., 2010). Indeed, it was shown that RELIK capsid containing virions are susceptible to TRIM5α-mediated inhibition (Yap and Stoye, 2013). Curiously, the TRIM5α from *Sylvilagus* and *Oryctolagus* inhibit RELIK more efficiently that the TRIM5α from *Ochotona* (which lacks endogenous RELIK sequences) (Yap and Stoye, 2013).

Besides RELIK, other endogenous retroviruses have been described in the genome of lagomorphs. Pika-BERV was an endogenous betaretrovirus identified solely in the genome of pikas (Lemos de Matos et al., 2015). The rabbit endogenous retrovirus H (RERV-H) was initially found in human tissues, however further sequencing of the viral genome and its integration sites revealed that this virus was in fact an endogenous retrovirus (ERV) from rabbits (Griffiths et al., 1997, Griffiths et al., 2002). The first hypothesis was that this virus was present in human tissues due to zoonotic infections

from rabbits. However, the detection of integration sites of rabbit origin and rabbit mitochondrial sequences in the human samples, raised the possibility of contamination, although no clear explanation was presented for the presence of rabbit DNA in human tissues (Griffiths et al., 2002, Forsman et al., 2003a, Forsman et al., 2003b). RERV-H belongs to the genus of betaretroviruses, being also closely related to rodent intracisternal A-particle retrotransposons (IAPs) (Griffiths et al., 1997). Consistent with other members of this genus, RERV-H's genome is composed of LTRs, *gag*, *pol* and *pro* domains, while the *env* domain remained unidentified. This could have been due to its loss, which is common to ERVs, or its detection was hampered by a high degree of polymorphism (Boeke and Stoye, 1997, Griffiths et al., 2002). This endogenous retrovirus was identified in the genome of the European rabbit but not in hares or pikas. So far, the clones retrieved from rabbit samples represented defective proviruses, although, some domains revealed complete ORFs, having the potential to be functionally expressed (Griffiths et al., 2002). It was already demonstrated that RERV-H protease encodes a functional enzyme, which acts like a retroviral aspartic protease, is able to catalyze its own cleavage from the precursor protein and cleaves the RERV-H Gag polyprotein precursor *in vitro* (Voisset et al., 2003). Despite that, is still not clear what the effect was of this endogenous retrovirus on their host species.

Regarding the use of *Oryctolagus cuniculus* as a small animal model, mainly the New Zealand White rabbit breed has been gaining focus in biomedical and pharmaceutical research. Nowadays, their bigger size represents a good alternative to study, for instance, cardiovascular-related diseases (Fan and Watanabe, 2003, Graur et al., 1996, Peng, 2012). In the past, experiments using rabbits contributed extensively to the current knowledge on the immune system (Gilman et al., 1964, Porter, 1959, Nisonoff et al., 1960, Mage et al., 1971). Likewise, rabbits have been applied for the study of human infectious diseases (Peng et al., 2015, Esteves et al., 2018). This small animal model is widely used to study bacterial infections, such as tuberculosis or syphilis, and fungal infections (Chen et al., 2017, Tsenova et al., 2014, Salazar et al., 2007, Krapp et al., 2016). Rabbits have also been used to study viral infections and concomitant disease progression, such as HTLV1 (human T-lymphotropic virus type 1) and the associated T cell leukemia-lymphoma. This RNA virus affects mostly $CD4^+$ activated T cells and the infection is regulated by the interaction between virus replication and immune response (Asquith et al., 2000). Although both rabbits and rats can be infected with HTLV1, different strains of rats show variability in their viral response (Hakata et al., 2001, Kannagi et al., 2000). The rabbit model can be challenged by intravenous injection of a T-cell leukemia line and afterwards it shows similar antibody and hematologic responses

as in humans (Collins et al., 1996, Lydy et al., 1998, Zhao et al., 2005). The use of this animal model supported, for example, detection methods for HTLV1 proviral DNA and the immunization of rabbits with synthetic envelop peptides opened the possibility for a vaccine development (Cockerell et al., 1990, Tanaka et al., 1994). Besides, this model was useful to determine the role of the different viral regulatory proteins (Collins et al., 1998, Bartoe et al., 2000, Silverman et al., 2004, Ye et al., 2003).

Rabbits have also been an important tool for the study of human papillomavirus (HPV) infections. This virus infects cutaneous and mucosal epithelial tissues, and while some strains only causes benign warts, other strains are associated with cutaneous and mucosal cancers, such as cervical cancer (Munoz et al., 2003, zur Hausen, 2002). The creation of an animal model for HPV is greatly impaired by the species-specificity of this virus (Richards et al., 2014). However, the cottontail rabbit papillomavirus (CRPV) is similar to HPV concerning genomic structure and carcinogenesis, making it a good model system to study the development and pathogenesis of HPV (Brandsma, 2005, Breitburd et al., 1997, Christensen, 2005). The rabbit model was also used to determine the importance of the H-*ras* oncogene for carcinoma development (Peng et al., 1993). A mutated form of H-*ras* was frequently found in cervical tumors, and transgenic rabbits with a constitutively activated GTP-bound isoform of H-*ras* (EJ-*ras*) showed accelerated tumorigenesis (Peng et al., 1999, Peng et al., 2001). This model further supports mutagenesis of CRPV, such as the integration of the HPV E7 gene, which maintains virus tumorigenicity (Hu et al., 2006). In this context, a transgenic rabbit model for human MHC I enabled recognition of the E7 epitope and subsequently the evaluation of immunization on viral progression (Hu et al., 2006, Hu et al., 2007, Bounds et al., 2011).

Herpes simplex virus type 1 (HSV1) is a large DNA virus that establishes latent infection in humans (Toma et al., 2008). After infection, the virus can travel to the cornea via the sensory ganglia, where it can cause herpetic stromal keratitis and blindness (Pepose et al., 2006). The use of rabbits to study disease progression is not only advantageous due to their larger eyes compared to other small animal models, but also because they can steadfastly develop herpetic stromal keratitis after inoculation with HSV1 in a comparable way to humans (Webre et al., 2012, Haruta et al., 1987, Naito et al., 2005, Nesburn et al., 2007). The rabbit model clarified, for example, which cells are latently infected and which factors trigger viral reactivation. Furthermore, human MHC I transgenic rabbits were used to test epitopes for the immunization against HSV1 (Nesburn et al., 1972, Kwon et al., 1981, Chentoufi et al., 2010, Shimomura et al., 1983).

Regarding HIV, the first study using small animals revealed no signs of infection for several species including rabbits (Morrow et al., 1987). However, in 1988, a study

published that rabbits could be infected with HIV-1 infected cells after induced peritonitis (Filice et al., 1988). In the same year, another study reported that primary rabbit T cells, T cell lines and some macrophage cell lines were able to support HIV-1 infection, although high titers of cell-free viruses had to be used (Kulaga et al., 1988). The recognition of human CD4 as an entry receptor for HIV-1 lead to a study that suggested no correlation between rabbit CD4 expression and rabbit cell susceptibility to HIV-1 infection (Hague et al., 1992). Furthermore, the authors suggested that rabbit cells expressing rabbit CD4 would use a receptor similar to human CD4, while rabbit cells negative for rabbit CD4 would use a completely different receptor (Hague et al., 1992).

The studies with rabbits as a model for HIV-1 infection continued, but the researchers started to recognize that high viral inoculations were necessary and the virus isolated from rabbit cells showed lower titers and poor infectivity (Reina et al., 1993). In 1995, the first transgenic rabbit model was used for HIV-1 research (Dunn et al., 1995). Rabbits expressing human CD4 on their T lymphocytes were used for peripheral blood lymphocytes (PBLs) isolation and were further infected with HIV-1, showing that transgenic rabbit T lymphocytes were more susceptible to infection than the wild-type rabbit cells (Dunn et al., 1995).

Further studies revealed that rabbit CD4 does not support HIV-1 infection and rabbit cell lines impose a barrier prior to transcription of the viral RNA (Cho et al., 1995). In an attempt to create a more virulent virus for rabbits, blood from HIV-1 infected rabbits were passaged in several animals. Although some signs of disease and death were described, it is not clear if it was due to mutations of the virus or reactivation of any rabbit retrovirus (Simpson et al., 1995).

Expression of human CD4 and human CCR5 were necessary for the infection of rabbit cell lines with R5-tropic viruses (Speck et al., 1998). More importantly, this study showed that the human CD4/CCR5 transgenic rabbit SIRC cells supported Nef, Tat and Rev functions, in terms of CD4 downregulation, transcription initiation, and splicing/nuclear export, respectively (Speck et al., 1998). The authors also showed that primary rabbit PBMCs transfected with proviral DNA produced infectious viral particles (Speck et al., 1998). Yet, further *in vitro* studies identified a block at an early stage of cytoplasmic HIV-1 trafficking (Cutino-Moguel and Fassati, 2006). Interestingly, other retrovirus, such as MLV (murine leukemia virus) or SIV, were not restricted in rabbit cells (Besnier et al., 2002). All in all, the experiments described by different groups were not reproducible and the assays used were not robust enough to detect blocks affecting the different stages of virus replication. Only in 2007, rabbit TRIM5α was identified to inhibit reverse transcription (Schaller et al., 2007). Rabbit TRIM5α targets the viral capsid and

accelerate uncoating or causes steric hindrance for further steps of the virus life cycle (Schaller et al., 2007). Nevertheless, this block in rabbit cells could be easily overcome by using a HIV-1 capsid mutant containing the residues 1 to 149 from SIVmac capsid (Schaller et al., 2007, Owens et al., 2003).

A detailed study in rabbit cell lines and primary rabbit T cells and macrophages confirmed the post-entry block in rabbit cells and the viral escape mutations in the *gag* gene (Tervo and Keppler, 2010). Interestingly, in rodent cells, Tat dependent LTR-transactivation is inhibited by the mutation C261Y in cyclin T1, which is not observed in rabbits (Tervo and Keppler, 2010, Bieniasz et al., 1998, Garber et al., 1998, Kwak et al., 1999). Unlike rodent cells, correct processing of Gag and HIV-1 release was observed in rabbit cells (Figure 1.4). Summarizing this study, both rabbit primary rabbit T cells and macrophages release viral particles to a similar extent to human cells, however, virions released from rabbit macrophages are less infectious (Tervo and Keppler, 2010). In an attempt to explain the reduced infectivity, the authors sequenced the viral DNA recovered from infected cells and detected consistent mutations, which were attributed to the action of rabbit APOBEC1 (Tervo and Keppler, 2010, Ikeda et al., 2008). However, up to date, no clear evidence for APOBEC1's antiviral activity is presented and the mutations and mutation rate are not consistent with APOBEC1's hypermutating activity.

Aims

Small animal models developed for HIV show several unsolved replication defects. Rodents do not support the late phase of HIV-1 replication, while primary T cells and macrophages from rabbits seem to have fewer barriers. As for other species, the first barrier is at the entry stage, which can be overcome by co-expression of the HIV-1 receptor complex comprised of human CD4 and CCR5 with HIV-1 glycoproteins. Nowadays, transgenic animals expressing human proteins can be generated. Yet, is still unknown what prevents the interaction of rabbit CD4 and CCR5. Therefore, the first part of this thesis is dedicated to analyze which amino acids in rabbit CD4 and CCR5 are incompatible to interact with the virus envelope. In parallel, the study of these genes in lagomorphs under an evolutionary perspective might help to understand what shaped their evolution, and more specifically, if the presence of endogenous viruses (RELIK, RERV-H) can be related with the evolutionary events seen in CD4 and CCR5 from lagomorphs and if they could have interacted with the rabbit proteins.

While the block at the reverse transcription step in rabbits was already attributed to the Trim5α, which can be easily overcome by depletion of this protein or modifications in

HIV-1 *gag*, a cell type-specific infectivity defect of HIV-1 virions is still unresolved. This block in rabbit macrophages is the last barrier to full HIV-1 permissivity. Therefore, the second part of this thesis is dedicated to investigate if the recent restriction factors, SERINC3 and SERINC5, are the source for this barrier.

The two main topics of this thesis will focus at different steps of the HIV-1 replication cycle and will hopefully help to resolve the remaining blocks in this species. The evolutionary perspective given to each of the subjects will frame the functional results in the history of lagomorphs, helping to explain why the rabbit represents a good model to study HIV-1 infection.

Chapter II: Evolution of CD4 and CCR5 in lagomorphs and their relation to endogenous viruses

This chapter contains parts of the publications:

de Sousa-Pereira P., Abrantes J., Baldauf HM., Keppler OT., Esteves JP. Evolutionary study of leporid CD4 reveals a hotspot of genetic variability within the D2 domain. Immunogenetics (2016).

de Sousa-Pereira P., Abrantes J., Baldauf HM., Esteves JP. Evolutionary studies on the betaretrovirus RERV-H in the *Leporidae* family reveal an endogenization in the ancestor of *Oryctolagus*, *Bunolagus* and *Pentalagus* at 9 million years ago. Virus Research (2017).

Background

CD4 is a transmembrane glycoprotein expressed at the surface of immune cells that are important for the induction of cell-mediated immunity. Belonging to the immunoglobulin (Ig) superfamily, it has four Ig-like domains, D1 and D3 are variable (V)-like domains, and D2 and D4 are constant (C)-like domains (Wang et al., 2001). In primates, CD4 is under strong positive selection, particularly the surface of D1 domain, where the main binding site for gp120 from primate immunodeficiency viruses is located (Zhang et al., 2008b, Meyerson et al., 2014). In agreement, some of the residues found under positive selection are directly interacting with HIV-1 gp120 (Humes et al., 2012, Kwong et al., 1998, Meier et al., 2012, Ashkenazi et al., 1990) (Figure 2.1a).

The evolutionary studies in primates for the HIV-1 co-receptors CXCR4 and CCR5 showed that they are highly conserved and undergoing negative or purifying selection (Zhang et al., 1999a, Meyerson et al., 2014). While CXCR4 in lagomorphs is also highly conserved, CCR5 underwent gene conversion in some species (Abrantes et al., 2008, Abrantes et al., 2011). Gene conversion between CCR5 and CCR2 caused the replacement of the QTLKMT motif in the second extracellular loop of CCR5 for the CCR2 motif HTIMRN, which can be found in the European rabbit (*Oryctolagus cuniculus*), the Riverine rabbit (*Bunolagus monticularis*) and the Amami rabbit (*Pentalagus furnessi*), but not in other close species, such as the Eastern cottontail rabbit (*Sylvilagus floridanus*) or hares (*Lepus sp.*) (Abrantes et al., 2011, Carmo et al., 2006). Thus, this gene conversion might have been the result of recombination at around 8 million years ago in the ancestral of *Oryctolagus*, *Bunolagus* and *Pentalagus*, which led to its fixation in these species (Pinheiro et al., 2016).

Studies on CCR5 have been of major importance since it represents one of the main co-receptors for SIV and the primary co-receptor in the initial phases of HIV-1 infection (Marx and Chen, 1998, Owen et al., 2000). Although highly conserved, 10% of the European human populations carry the CCR5-Δ32 allele, whose 32 base pairs deletion introduce a stop codon that abolishes CCR5 function as a co-receptor (Galvani and Novembre, 2005). Homozygosity for this allele confers elevated resistance to HIV-1 infection, and even infected heterozygotes show reduced HIV-1 loads and slowed disease progression (Michael et al., 1997, Dean et al., 1996). Besides this natural occurring mutation, CCR5 co-receptor function can be impaired by several mutations at the interaction site with HIV-1 gp120 (Tamamis and Floudas, 2014, Huang et al., 2007, Garcia-Perez et al., 2011) (Figure 2.1b). Due to this phenotype, a lot of work has been done on the development of drugs that target and inhibit CCR5, such as maraviroc (Rao, 2009).

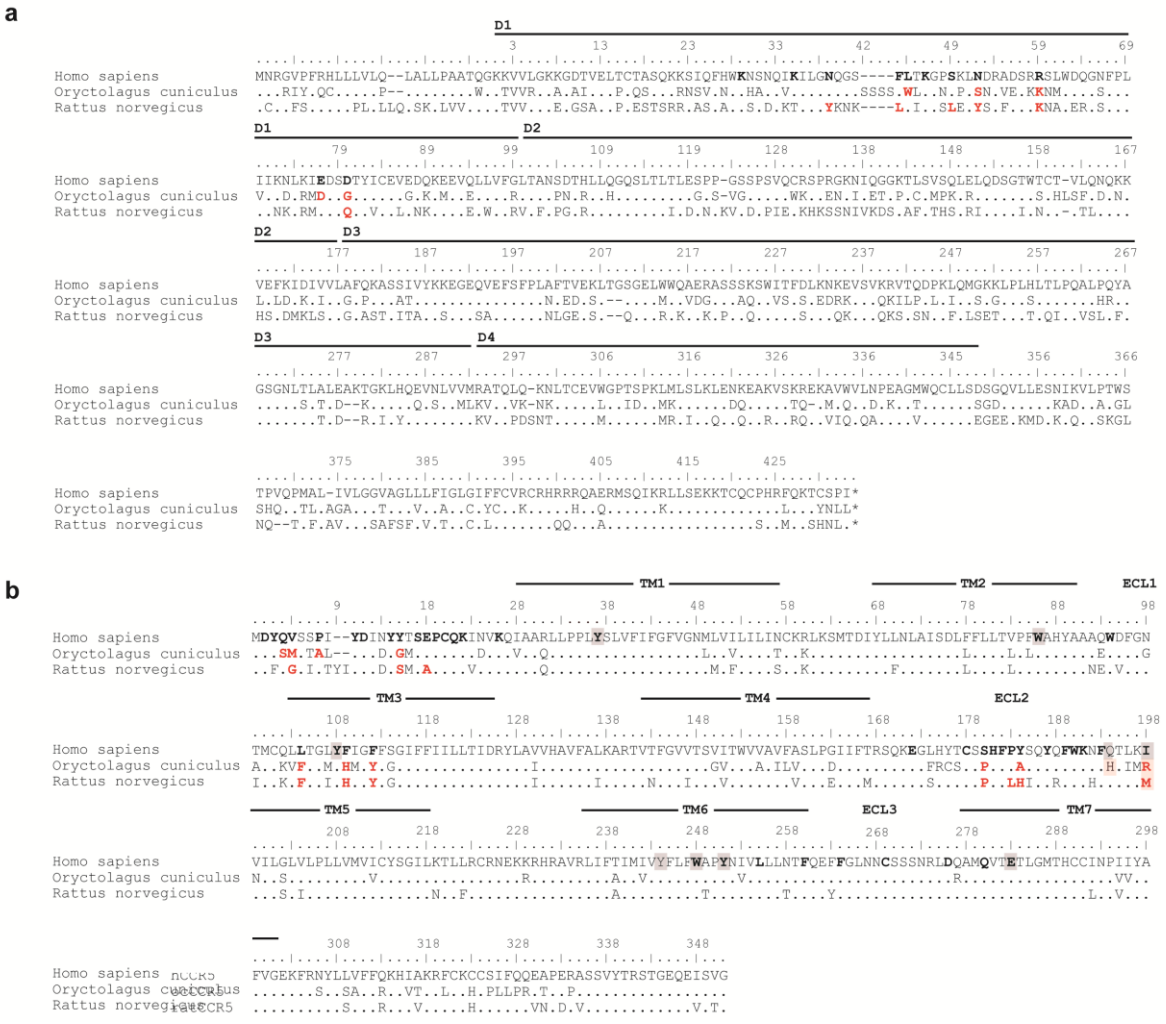

Figure 2.1 - CD4 and CCR5 amino acid sequences reveal important residues for HIV gp120 interaction. **a,** CD4 coding region from *Homo sapiens* (P01730), *Oryctolagus cuniculus* (P46630) and *Rattus norvegicus* (P05540). **b,** CCR5 coding region from *Homo sapiens* (P51681), *Oryctolagus cuniculus* (Q1ZY22) and *Rattus norvegicus* (O08556). Residues shown to be important for the interaction with HIV-1 gp120 envelope protein are presented in bold, and non-synonymous substitutions in rabbit or rat are depicted in red. Amino acids shaded in grey represent important residues for maraviroc binding, and red shaded amino acids reflect non-synonymous substitutions of these residues.

Unlike for CCR5, there is not vast information on the evolution of CD4 in lagomorphs. Therefore, part of this section is intended for the evolutionary analysis of CD4 in lagomorphs to deepen the knowledge of this protein and assess if, as seen for primates, CD4 from lagomorphs is also under selective pressure. Furthermore, the inability of HIV-1 to use non-human CD4 and CCR5 to enter target cells will be evaluated, particularly to identify the residues that impair binding of the envelope protein gp120 to bind to CD4 and CCR5 from small animals, such as rabbit or rat.

Finally, the last part of this section investigates the possibility of leporid endogenous retroviruses as an evolutionary force in these species. RELIK, the endogenous rabbit lentivirus, was identified in different rabbits and hares, being part of the same subfamily as HIV (Keckesova et al., 2009, Katzourakis et al., 2007). Other retroviruses have also been identified in the genome of lagomorphs, such as the RERV-H, the rabbit endogenous gammaretrovirus known to be present in the genome of the European rabbit but absent in hares (Griffiths et al., 2002). The connection between the evolutionary imprints in leporids' CD4 and CCR5 and the presence of retrovirus elements in their genome might help to understand if these viruses shaped the genome of the rabbit, thus making rabbits superior to study lentivirus replication.

Material and methods

Amplification and sequencing of lagomorph CD4

Total RNA was extracted from liver samples of European rabbit (*Oryctolagus cuniculus cuniculus*), Eastern Cottontail (*Sylvilagus floridanus*) and European brown hare (*Lepus europaeus*). Genomic DNA was extracted from tissue samples of European rabbit, Brush rabbit (*Sylvilagus bachmani*), Iberian hare (*Lepus granatensis*), Scrub hare (*Lepus saxatilis*), Cape hare (*Lepus capensis*), Broom hare (*Lepus castroviejoi*), Corsican hare (*Lepus corsicanus*) and American pika (*Ochotona princeps*). The European rabbit, European brown hare and American pika sequences available in GenBank were used for primer design. The primers 5'-ATGAACCGGAGAATCTACTTCC-3' (fw) and 5'-TCACAGGAGATTGTAAGTCTTC-3' (rv) were used to amplify the complete coding region in the Eastern Cottontail. The primer pairs 5'-TGCCGGGAGATAGGAGATG-3' (fw) 5'-AACCCTGAGGATGAGTGCAT-3' (rv), and 5'-TGCGGAGCTAGGAGATCAGA-3' 5'-GCGCACAGCTTGAGAACTG-3', were used to amplify the exon 4 from rabbits/hares and pikas, respectively. PCR amplification was performed using the Phusion high-fidelity PCR master mix (Thermo Fisher Scientific) with 64 ºC annealing temperature for the complete CDS (CoDing Sequence) and 60 ºC annealing temperature for the exon 4. Sequencing was performed on an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems) and PCR products were sequenced in both directions. The sequences can be found in the GenBank under the following accession numbers: KU845555-KU845562.

CD4 evolutionary analysis

The obtained sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) available at http://www.ebi.ac.uk/ (Edgar, 2004) and translated using BioEdit (Hall, 1999). The nucleotide substitution rate variation among different CD4 regions from Primates and Lagomorpha orders was determined by sliding a window along the nucleotide sequence alignment and plotting the differences as averages. For this analysis, following sequences included were: *Homo sapiens* (NM_000616), *Pan troglodytes* (NM_001009043), *Pan paniscus* (XM_003820282), *Gorilla gorilla* (XM_004052582), *Nomascus leucogenys* (XM_004092147), *Papio anubis* (XM_003905871), *Macaca mulatta* (D63347), *Macaca fuscata* (D63348), *Macaca nemestrina* (D63346), *Macaca fascicularis* (D63349), *Callithrix jacchus* (NM_001267772), *Saimiri sciureus* (AF452617), *Oryctolagus cuniculus* (NM_001082313), *Lepus europaeus* (KJ542541), *Sylvilagus floridanus* (KU845555) and *Ochotona princeps* (XM_004596387). The sliding-window analysis was performed using DnaSP version 5.10 (Librado and Rozas, 2009), with a window length of 60 nucleotides and a step size of 12 nucleotides. Synonymous and non-synonymous substitution rates were estimated using the Nei-Gojobori method (Nei and Gojobori, 1986) and the non-synonymous to synonymous substitution ratio (d_N/d_S) was calculated using MEGA 6 (Tamura et al., 2013).

Amplification of the endogenous lentivirus RERV-H

Genomic DNA was extracted from tissue samples of European rabbit (*Oryctolagus cuniculus*), riverine rabbit (*Bunolagus monticularis*), amami rabbit (*Pentalagus furnessi*), brush rabbit (*Sylvilagus bachmani*), pygmy rabbit (*Brachylagus idahoensis*), European brown hare (*Lepus europaeus*) and American pika (*Ochotona princeps*). The sequences obtained from human (GenBank accession number AF480924) and from the European rabbit genome (GenBank accession AF480925) were considered for primer design to amplify different domains of RERV-H. To detect the presence of RERV-H in the samples mentioned above, several primer pairs previously described by Griffiths *et al.* (2002) were used to amplify different regions of the viral genome. The primer pair that amplified the RNaseH fragment was the only combination producing clear PCR results for additional species besides the European rabbit, and was therefore chosen for subsequent analyses. The PCR was performed using the PCR Master Mix (Promega) with 50 ºC annealing temperature for 45 seconds and 1 minute extension at 72 ºC. PCR products were purified (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel, Germany) and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI,

USA). Sixteen clones were selected for each sample. Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) using primers designed on the vector (pUC/M13 sequencing primers from Promega). Sequences were submitted to GenBank under the following accession numbers: MG603347- MG603391.

Cloning of rabbit, hare and rat CD4, CCR5 and CXCR4

CD4, CCR5 or CXCR4 coding regions were amplified from liver cDNA of rabbit, rat or hare, using the Phusion high-fidelity PCR master mix (Thermo Fisher Scientific). Rabbit, rat and hare CD4 were cloned into the pCMV-HA-C plasmid (Clontech) using the restriction enzymes *EcoRI* and *KpnI* (Appendix III). Rabbit and rat CCR5, and rabbit and hare CXCR4, were cloned into the pCMV-myc-C (Clontech) using *EcoRI* and *KpnI* cutting sites. Human CD4 and CCR5 were cloned into the pCMV-HA-C and pCMV-myc-C plasmids, respectively, using the restriction sites for *SalI* and *KpnI*. After ligation with the T4 DNA ligase (New England Biolabs), the constructs were transformed in competent cells and positive colonies were confirmed by sequencing.

Molecular modeling

To resolve the 3D structure of rabbit CCR5, the human CCR5 structure with maraviroc was used (PDB id 4MBS) (Tan et al., 2013). For the structure prediction of rabbit CD4, the complex of human CD4 with the core domain of HIV-1 gp120 was used (PDB 2QAD) (Huang et al., 2007). The complex energy in each model of the rabbit CD4 was estimated with FoldX (http://foldxsuite.crg.eu/) and compared to the complex energy of the model of the human CD4. All the modeling was performed by Dr. Olga Kalinina from the Max Plank Institute for Informatics, Saarbrücken, Germany.

Generation of human/rabbit CD4 and CCR5 chimeras

To generate human/rabbit CD4 and CCR5 chimeras, different strategies were used (Figure S1). For the generation of the hrbhCD4 construct, the human FLT (amino acids 68-70) was replaced by the rabbit segment SSSSFWL, while for rbhrbCD4, rabbit SSSSFLW sequence (amino acids 68-74) was replaced by the human FLT. For that, site directed mutagenesis was applied using the primers hrbh(fw) 5'-TCCTCCTCCTCCTTCTGGCTTAAAGGTCCATCCAAGCTG-3' and hrbh(rv) 5'-GGAGCCCTGATTTCCCAGAATC-3', rbhrb(fw) 5'-TTCTTGACTAAAGGGAACTCCCCGCTGAG-3' and rbhrb(rv) 5'-GGAGCCCTGGTTTCCCAGGATC-3'. The primers were phosphorylated using a T4

Polynucleotide Kinase (Thermo Fisher Scientific) and the remaining protocol was done using the Site Directed Mutagenesis Kit from Thermo Fisher Scientific according to manufacturer's instructions. For the generation of CCR5 chimeras, hArgCCR5 and rbIleCCR5 chimeras were also generated using site directed mutagenesis as described before, using the primers CCR5(rv) 5'-TGGAAATTCTTCCAGAATTG-3', Ile(fw) 5'-CACCATCATGATAAACATCTTGAG-3' and Arg(fw) 5'-GACATTAAAGAGAGTCATCTTGGG-3'. The hrbCCR5, hrbIleCCR5, rbhCCR5 and rbhArgCCR5 chimeras were generated through restriction digestion with *KpnI*, which excised the last 679 nucleotides to be switched (Figure 2.2). All the selected clones were confirmed by sequencing. To linearize the plasmids prior transfection, the constructs were digested with *ScaI* (New England BioLabs) and purified using the PCR clean-up Gel extraction from Macherey-Nagel according to the manufacturer's instruction. The linearized plasmids were necessary to transfect the cells used for the fusion assay to prevent the basal expression of the β-lactamase which confers ampicillin resistance in these plasmids.

Figure 2.2 - Representative illustration of the human/rabbit chimeras for CD4 and CCR5. Human regions are represented in blue and rabbit in red. Highlighted are the single amino acid and small region mutations.

Production of infectious HIV-1 and pseudotyped RELIK lentivirus vectors

For the production of infectious HIV-1, proviral DNA from the lab strains YU-2 (National Institutes of Health AIDS Repository) and 49.5 (Chesebro et al., 1992) were used. HEK293T cells were transfected with 50µg proviral DNA, 16 µg pCMV4-BlaM-Vpr plasmid (Addgene) and 200µL of PEI reagent in plain DMEM. Viruses were purified as

described in Appendix I. Reverse transcriptase activity was measured by SG-PERT (Pizzato et al., 2009), as described in Appendix1.

For lentivirus vector production, the envelope of RELIK was synthetized (Genewiz) according to the published sequence (Katzourakis et al., 2007) with a 3xFLAG tag at the C-terminal. The envelope was subcloned into a pCMV-driven plasmid using the enzymes *EcoRI* and *NotI* (Appendix III). The generated plasmid was sequence verified. pPAX2 (Addgene) or the capsid switch mutant H/SCA (Owens et al., 2004) plasmids were used as packaging vector and pHR'CMV GFP (Addgene) used as transfer vector. HEK293T cells were transfected using 17.2 µg transfer vector, 8.6 µg packaging vector, and 4.3 µg/ 8.6 µg/ 12.9 µg of RELIK envelope or 4.3 µg VSV-G (Vesicular stomatitis virus glycoprotein; pMD2.G), and 90 µL of PEI reagent (Polyethylenimine 25K from Plysciences). The lentiviral particles were purified as described in Appendix I. Reverse transcriptase activity was measured by SG-PERT (Pizzato et al., 2009), as described in Appendix1.

Western blot analysis

Concentrated lentiviral vectors were inactivated with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) containing 2.5 % β-mercaptoethanol at 70°C. The samples were resolved using a NuPAGE Bis-Tris gel system (Thermo Fisher Sceintific). The separated proteins where then blotted on a nitrocellulose membrane, which was first blocked with 5 % milk and further incubated overnight with mouse anti-FLAG M2 antibody (1:1000 of the 5mg/mL; Sigma-Aldrich). The membrane was further incubated with rabbit-anti p24 capsid (1:2000; Sigma). Protein detection was performed using the Clarity Western ECL Blotting Substrate (BioRad) on a Vilber Fusion FX documentation system.

Fusion assay

$1.5x10^5$ feline CRFK cells (ATCC CCL-94) cells were transfected with 1 µg of CD4 and 1 µg of CCR5 linearized plasmids and 6 µL of lipofectamine 2000 (Thermo Fisher Scientific). 48h after transfection half of the cells were harvested and fixed in 4% PFA (paraformaldehyde) for 10 minutes at room temperature. These cells were further permeabilized with Perm/Wash Buffer (BD Biosciences) for 15 minutes and stained with anti-HA-APC and anti-myc-FITC (1:50; Milteny Biotec) for 30 minutes. The other half of transfected cells were used for infection. Drugs inhibiting HIV-1 entry, including AMD3100 (20 µM), T20 (50 µM) and Maraviroc (20 µM) were applied in parallel. The cells were infected with 6 µL of YU-2 BlaM-Vpr or 49.5 BlaM-Vpr (necessary amount to

yield at least 10 % of infected control cells). The fusion assay was performed as previously published and described in Appendix I (Cavrois et al., 2002). After fixation, the cells were analyzed by flow cytometry using a BD FACSVerse (BD Biosciences) and the results processed using the FlowJo 10.1 software.

Lentiviral transduction

$1.5x10^5$ CRFK cells were transfected with 1 µg of rabbit/hare CD4 and 1 µg of rabbit CCR5 or rabbit/hare CXCR4 plasmids and 4 µL of lipofectamine 2000 (Thermo Fisher Scientific). 48h after transfection half of the cells were fixed in 4% PFA for 10 minutes at room temperature. These cells were then permeabilized with Perm/Wash Buffer (BD Biosciences) for 15 minutes, stained using anti-HA-APC and anti-myc-FITC (1:50; Milteny Biotec) for 30 minutes and finally analyzed for receptor expression. The remaining cells were transduced with the RELIK Env or VSV-G pseudotyped lentiviral vectors (5 and 10 µL which correspond to $1.5x10^{11}$ and $3x10^{11}$ pUnits RT). 48h post-transduction, the cells were harvested, fixed in 4% PFA for 90 minutes and analyzed for GFP expression.

$1.5x10^5$ cells of the rabbit B cell line 55D1 (Sethupathi et al., 1994), rabbit T cell line RL-5 (NIH AIDS Reagent Program) or rabbit epithelial cell line SIRC (ATCC CCL-60), were transduced with 5 and 10 µL (corresponding to $1.5x10^{11}$ and $3x10^{11}$ pUnits RT) of the RELIK Env or VSV-G pseudotyped lentiviral vectors with modified capsid (H/SCA). 48 hours post-transduction, the cells were fixed in 4% PFA for 90 minutes at room temperature and GFP expression was measured. All cells were analyzed using the flow cytometer BD FACSVerse (BD Biosciences) and the results were processed using the FlowJo 10.1 software.

Immunoprecipitation

HEK293T cells were transfected with 1µg of rabbit CD4 and 1µg of hare CD4 alone or together with 1µg of RELIK envelope using PEI reagent (Polyethylenimine 25K from Plysciences). 48 hours after transfection, cells were harvested and lysed in RIPA buffer (50 mM Tris-HCL 7.5, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.1 % sodium deoxycholate and supplemented with protease inhibitor (Roche)). After incubation for 1 hour at 4 °C, the cells were centrifuged at 15000 rpm and the supernatant was mixed with protein G magnetic beads (SureBeads, BioRad) and 2 µL of the mouse anti-FLAG M2 antibody (5 mg/mL; Sigma-Aldrich). After overnight incubation at 4 ºC, unbound proteins were washed out and bound proteins were recovered in NuPAGE LDS Sample

Buffer (Thermo Fisher Scientific) at 65°C. 2.5 % β-mercaptoethanol was added and the samples loaded using a NuPAGE Bis-Tris gel system (Thermo Fisher Sceintific). Input lysates were loaded in parallel. The separated proteins were blotted on a nitrocellulose membrane, which was first blocked with 5 % milk and further incubated overnight with mouse anti-FLAG M2 antibody (1:1000 of 5 mg/mL; Sigma-Aldrich) and mouse anti-HA (1:2000 of 1 mg/mL; Biolegend). Proteins were detected using the Clarity Western ECL Blotting Substrate (BioRad) on a Vilber Fusion FX documentation system.

Results

Characterization of lagomorph CD4

The comparative analyses showed that the overall nucleotide diversity along the CD4 coding region is higher in lagomorphs than in primates. This difference is more evident in the D2 region, specifically amino acids 169 to 179, where lagomorphs show a 2.5 fold higher peak for nucleotide diversity than primates (Figure 2.3).

The calculation of non-synonymous to synonymous substitution ratios (d_N/d_S) revealed that values for the D2 region in leporids were slightly higher than those observed for the entire protein (Table 2.1). Interestingly, although the d_N/d_S ratio for the overall CD4 coding region was < 1 for both primates and lagomorphs, indicative of general purifying selection for the protein, the value for the small region between amino acids positions 169 to 179 was 3.2 for lagomorphs, evidencing positive selection in this region. Besides, the nucleotide and amino acid distances were three times higher in the lagomorphs than in primates (Table 2.1). Within this small region, dramatic amino acid changes can be observed among leporid genera. For instance, the European rabbit and the European brown hare sequences differ in nine of the eleven encoded amino acids, which are the result of eleven non-synonymous nucleotide substitutions. Accordingly, major amino acid changes were observed between genera, while minor changes can be found within each genus (Figure 2.4a).

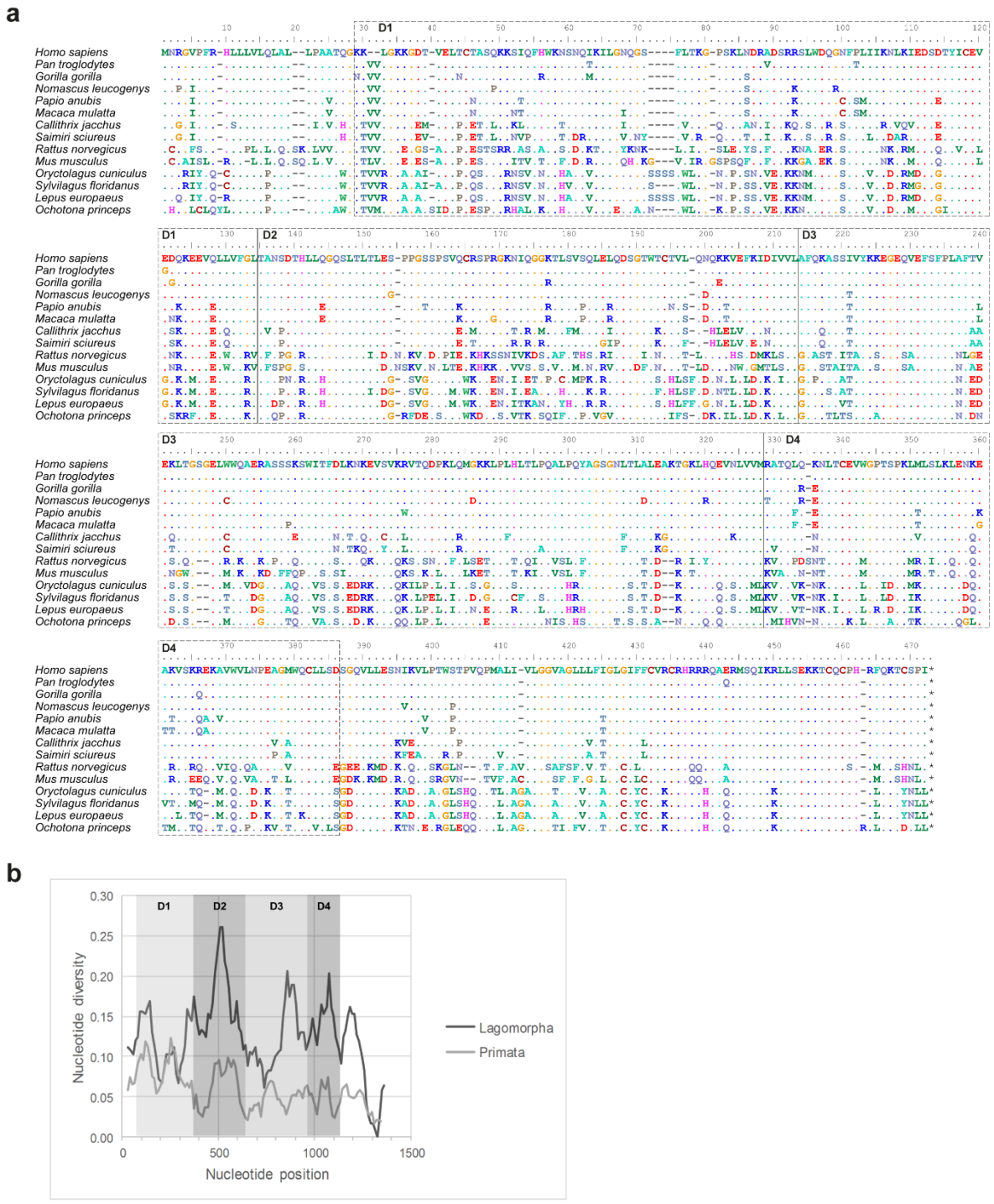

Figure 2.3 - CD4 coding region diversity for primates and lagomorphs. **a,** Translation of the nucleotide sequences of *Homo sapiens* (NM_000616), *Pan troglodytes* (NM_001009043), *Gorilla gorilla* (XM_004052582), *Nomascus leucogenys* (XM_004092147), *Papio anubis* (XM_003905871), Macaca mulatta (D63347), *Callithrix jacchus* (NM_001267772), *Saimiri sciureus* (AF452617), *Rattus norvegicus* (NM_012705), Mus musculus (NM_013488), *Oryctolagus cuniculus* (NM_001082313), *Lepus europaeus* (KJ542541), *Sylvilagus floridanus* (KU845555) and *Ochotona princeps* (XM_004596387). The main CD4 domains are indicated and the D2 domain is shaded. **b,** Sliding window along the CD4 nucleotide sequences listed in **(a)** for primates and lagomorphs showing the nucleotide diversity for these orders. The main CD4 domains are indicated.

Table 2.1 - Summary table for evolutionary distance and synonymous and non-synonymous substitutions for primates and lagomorphs. Shown are the d_N, d_S, ratio d_N/d_S, nucleotide (nt) distance and amino acid (aa) distance values for each of the CD4 domain, complete protein and 11 aa fragment. CDS = Coding Sequence.

		Lagomorphs	Primates
CDS	d_N	0.11	0.05
	d_S	0.25	0.09
	d_N/d_S	0.45	0.60
	nt distance	0.13	0.06
	aa distance	0.20	0.10
D1 (aa 27-131)	d_N	0.09	0.10
	d_S	0.43	0.08
	d_N/d_S	0.20	1.17
	nt distance	0.12	0.08
	aa distance	0.15	0.17
D2 (aa 132-209)	d_N	0.18	0.07
	d_S	0.29	0.08
	d_N/d_S	0.63	0.81
	nt distance	0.15	0.06
	aa distance	0.24	0.13
D3 (aa 210-324)	d_N	0.12	0.03
	d_S	0.24	0.10
	d_N/d_S	0.50	0.29
	nt distance	0.13	0.04
	aa distance	0.20	0.06
D4 (aa 325-381)	d_N	0.14	0.06
	d_S	0.23	0.05
	d_N/d_S	0.63	1.12
	nt distance	0.14	0.05
	aa distance	0.27	0.11
aa 169-179	d_N	0.36	0.11
	d_S	0.11	0.11
	d_N/d_S	3.20	1.00
	nt distance	0.34	0.10
	aa distance	0.71	0.20

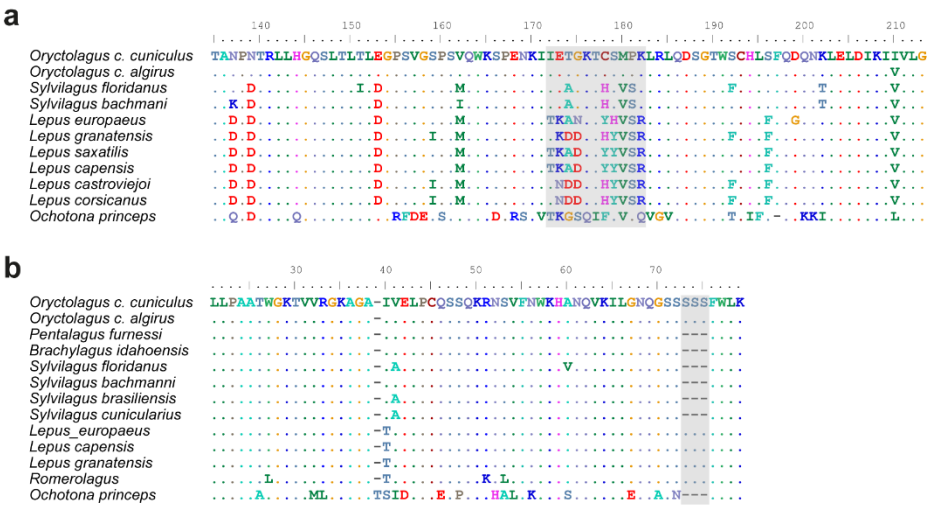

Figure 2.4 - Amino acid alignment for the D2 and part of the D1 domain in lagomorphs reveals high diversity in these regions. **a**, CD4 D2 domain with the highly variable 11 amino acid region shaded; *Oryctolagus cuniculus cuniculus* (NM_001082313), *Oryctolagus cuniculus algirus* (KU845556), *Sylvilagus floridanus* (KU845555), *Sylvilagus bachmani* (KU845557), *Lepus europaeus* (KJ542541), *Lepus granatensis* (KU845558), *Lepus saxatilis* (KU845559), *Lepus capensis* (KU845560), *Lepus castroviejoi* (KU845561), *Lepus corsicanus* (KU845562) and *Ochotona princeps* (XM_004596387). **b**, Part of the D1 domain corresponding to exon 2 of CD4. The region corresponding to the serine motif insertion is shaded.

No significant BLAST matches were obtained for this 33 nucleotide motif, either in the mammalian NCBI database or in the European rabbit genome available on Ensembl, and none of the chromosomally adjacent genes (parathymosin, lymphocyte-activation gene 3, G protein coupled receptor 162 and leprecon-like 2) showed evidence of gene conversion with CD4. Therefore, such event is unlikely to be in the origin of high diversity in this small region in leporids.

The D1 domain, although not highly variable in lagomorphs, showed an insertion of three serine residues in some leporid species (Figure 2.4b). Amplification of this region, corresponding to exon 2, in a variety of lagomorph species showed that this insertion is present in *Oryctolagus*, *Lepus*, and *Romerolagus*, being absent in *Pentalagus*, *Brachylagus*, *Sylvilagus* and *Ochotona*. Considering the phylogenetic tree for lagomorphs, it is unlikely that this motif was independtly inserted into these species (Figure 2.5). Possibly, this serine insertion arose in the ancestral of hares and rabbits, but while it remaining fixed in some rabbits and hares, for unknown reasons, it was posteriorly eliminated in other rabbits.

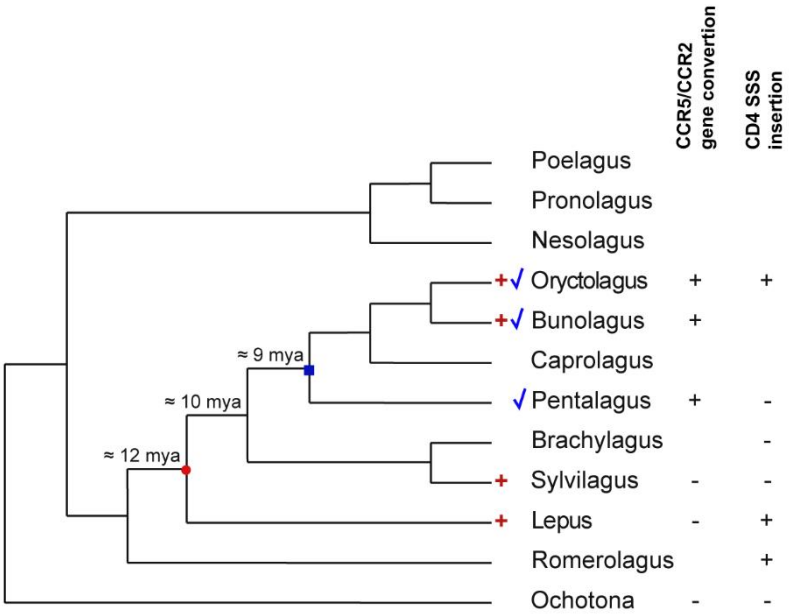

Figure 2.5 - Evolutionary relationships among eleven Leporid genera. The approximate divergence time is shown for relevant branches for this study. The presence or absence of the CCR5/CCR2 recombination and the CD4 serine motif insertion is indicated as (+) or (-) in the species tested. The red cross indicates species where RELIK elements were identified and a red dot marks the common ancestor where endogenization might have occurred. The blue tick indicates species where RERV-H elements were identified and a blue square marks the common ancestor where endogenization might have occurred. Based on Matthee et al., 2004, Abrantes et al., 2011, van der Loo et al., 2009 and van der Loo et al., 2012.

HIV-1 entry with rabbit and human/rabbit chimeric receptor orthologs

The comparison of CD4 and CCR5 between human and rabbit showed that the orthologs show rather high amino acid similarity, with 60.6% and 79.8% similarity, respectively, which is similar with the percentage identity between the human and the mouse or rat orthologs. Nevertheless, the rabbit orthologs do not support binding of the HIV-1 envelope protein gp120. Therefore, in collaboration with Dr. Olga Kalinina, the interaction of human, rabbit and rat CD4 and CCR5 with gp120 was modelled to identify residues that might interfere with or are important for envelope binding (Figure 2.6). The molecular modeling showed that the interaction interface of CD4 with HIV-1 gp120 is generally not well conserved, most of all due to a serine-rich stretch following the position 41 (Figure 2.1a). This rabbit-specific stretch forms a substantial additional volume, which might lead to the formation of non-favorable contacts and potential clashes. Besides, several amino acid substitutions can be observed in the residues considered important for gp120 binding, for both rabbit and rat. For instance, while in rabbit the important residue Phe43 is conserved, the following residue Leu44 is replaced by Trp. The prediction of the interaction energy needed for the gp120 core binding hints that rabbit CD4 without the serine stretch might have a lower binding energy than wild-type CD4. This binding energy

is even lower and comparable to the human CD4 when the serine stretch is removed and the rabbit Trp-Leu (WL) is replaced by Leu-Thr (LT) (Figure 2.6c). Regarding CCR5, unfortunately the first 24 amino acids could not be modelled as it cannot be crystallized. The remaining structure indicated that the interacting residues are mostly conserved in all three species (Figure 2.6b). However, one of the residues known to be important for co-receptor binding, Ile198 (I), is substituted by Arg (R) in rabbits (Figure 2.1b).

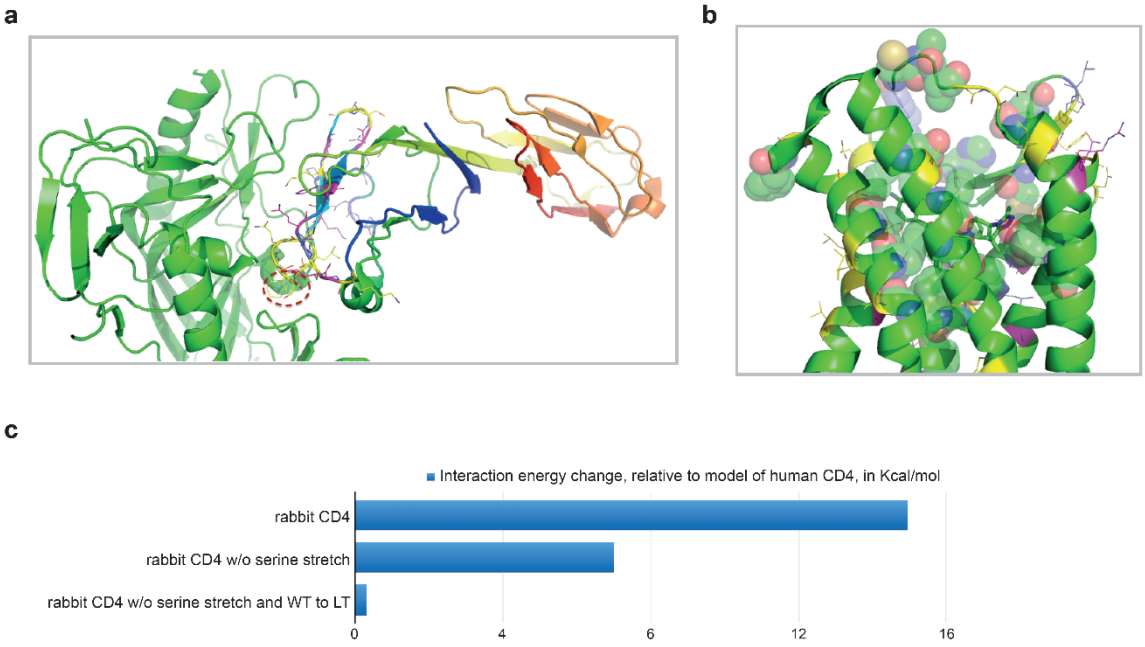

Figure 2.6 - 3D structure prediction identifies important residues in rabbit CD4 and CCR5 for gp120 interaction. **a.** Complex of HIV-1 gp120 core domain (in green) and CD4 (rainbow). The structural location of the corresponding residues to the rabbit serine-rich stretch is marked with a red circle. **b.** View of the CCR5 residues close to the extracellular side of the membrane. Residues involved in HIV-1 co-receptor activity are shown as spheres. Residues conserved in rat and rabbit, but not in human are depicted in red, residues conserved in human and either in rat or rabbit are in yellow, and the residues different in all species are represented in blue. **c.** Estimated complex energy for gp120 core binding in each model of the rabbit CD4. Represented values are relative to the human CD4 model.

The information provided by the molecular modelling limited site-directed mutagenesis approaches in rabbit CD4 and CCR5. For CD4, two chimeras were generated: hrbhCD4 which is basically the human CD4 containing the rabbit amino acids around the serine-rich stretch, and rbhrbCD4 which corresponds to rabbit CD4 without the serine stretch and the WL to LT mutation (Figure 2.2). For CCR5, the generated chimeras included the substitution at position 198 (I to R or vice versa) and the N-terminal part, as the first 24 amino acids could not be modelled and the region until TM3 (Transmembrane domain 3) included several residues reported to be important for gp120 binding (Figure 2.2).

To assess the fusion efficiency of the different wild type and chimeric CD4 and CCR5 orthologs, feline CRFK cells were transfected with different combinations of the receptor and co-receptor. In the first approach, human CD4 was co-transfected with the different CCR5 orthologs and chimeras, and the human CCR5 was co-expressed with different CD4s. Their expression was monitored by flow cytometry, ranging from 22.2 % to 36.7 %, except the combination of human CD4 with rat CCR5 showed only 8.62 % double positive cells (Figure S2). For infection, two different viruses were used: YU-2, which is a CCR5 tropic laboratory-adapted virus strain, and the isogenic virus of NL4-3, known as 49.5. While NL4-3 is a CXCR4-tropic strain, 49.5 carries the V3 loop of Ba-L that makes it CCR5-tropic.

In order to monitor fusion, β-lactamase was incorporated into the virion. Only the cells where fusion occurred contained the enzyme able to cleave the substrate, causing a shift in fluorescence (Cavrois et al., 2002). Here, HIV-1 fusion was successful when human CD4 and human CCR5 were expressed, with 37.7 % and 29.1 % fusion for YU-2 and 49.5, respectively. As expected, the co-expression of the wild-type rabbit and rat CD4 or CCR5 with the human co-receptor or receptor, respectively, did not support HIV-1 fusion. Interestingly, fusion was observed for the combinations hCCR5 / rbhrbCD4, with means of 33.8 % and 27.2 % for YU-2 and 49.5, respectively (Figure 2.7a,b). The combinations hCD4 / hArgCCR5, hCD4 / hrbIleCCR5, hCD4 / rbhCCR5 and hCD4 / rbhArgCCR5 also allowed fusion to different extents. For infections with YU-2, hArgCCR5 was superior among the CCR5 chimeras with 44.5 % fusion efficiency, followed by the rbhCCR5 (31.9 %), hrbIleCCR5 (24.7 %) and finally rbhArgCCR5 (8.1 %). Furthermore, 49.5 infections showed 43.8 % mean fusion efficiencies for hArgCCR5 and comparable fusion ability for rbhCCR5 (28.1 %), hrbIleCCR5 (22.4 %) or rbhArgCCR5 (27.0 %). For both viruses, the fusion inhibitor T20 was able to efficiently block virus entry for all the CD4 / CCR5 combinations. Maraviroc, the negative allosteric modulator of CCR5, efficiently blocked entry for all combination except for the cells expressing hCD4 / hArgCCR5, suggesting that residue 198 is important for the interaction with this antiviral drug. The CXCR4 inhibitor AMD3100 was also applied for YU-2 infections in order to show that addition of this drug would not inhibit fusion of R5-tropic viruses, although statistically significant decrease in fusion, with 1.1- to 1.5-fold, was still observed for the cells expressing some of the CCR5 chimeras.

In a further attempt, cells were transfected with rbhrb CD4 and hrbIle CCR5 in parallel to human CD4 and human CCR5 (Figure 2.7c). The CCR5 chimera contains the first 127 amino acids from human CCR5 and the R198I substitution, while the CD4 construct uniquely lacks the serine stretch and contains the W44L and L45T substitution. As seen

in Figure 2.7c, this receptor and co-receptor combination supported fusion of YU-2 to a similar extent as cells expressing both the human receptor and co-receptor. Equally important, this CCR5 rabbit chimera can be targeted by the CCR5 inhibitor maraviroc, and thereby blocking fusion of the virus.

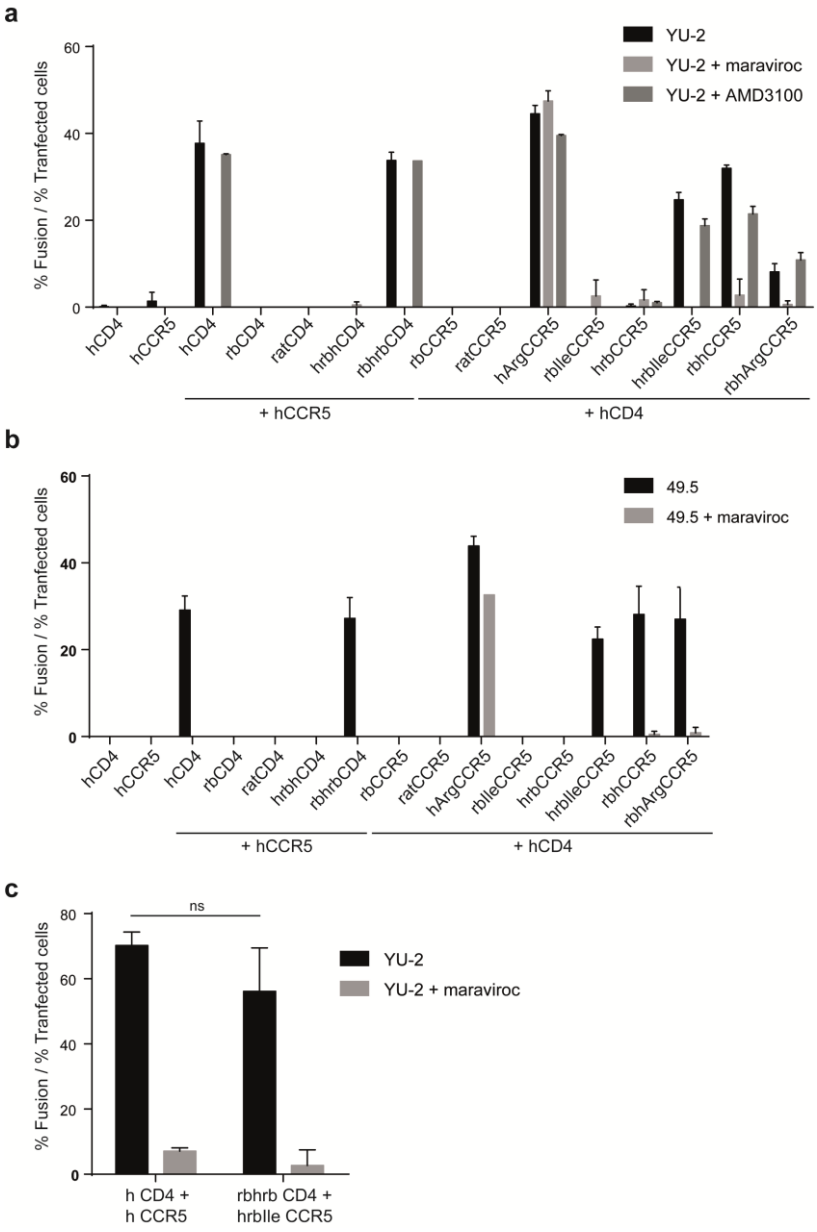

Figure 2.7 - Chimeric CD4 and CCR5 support HIV-1 fusion. CRFK cells were transfected with different combinations of the CD4 and CCR5 chimeras. 48 hours post-transfection the cells were infected and 4 hours post-infection stained for fusion. The % of fusion shown was normalized to the % of transfected CRFK cells for each co-expression scenario. Black bars indicate virus only infection, light grey bars represent infection in the presence of the CCR5 inhibitor maraviroc and dark grey bars show infection in the presence of the CXCR4 inhibitor AMD3100. **a.** infection with YU-2 virus. **b.** Infection with 49.5 virus. **c.** infection with YU-2 of CRFK cells co-expressing human or rabbit chimeric CD4 and CCR5. Shown are the arithmetic means of triplicates from one of two experiments, with depicted standard deviation error bars. h = human, rb = rabbit, ns = not significant.

Hunting for receptor(s) of the endogenous lentivirus RELIK

As selective pressure was observed in rabbit CD4 and CCR5, it was of interest to determine whether they were used by other retroviruses, such as RELIK, for entry into their target cells. The existence of RELIK Env fragments in the genome of leporids and the consensus sequence published by Katzourakis *et al*. (2007) allowed to investigate which receptors were used by the rabbit endogenous lentivirus RELIK. For this purpose, different GFP-expressing lentiviral vectors were produced, pseudotyped with RELIK Env or VSV-G. First, three different combinations were used to produce RELIK Env pseudotyped lentiviruses, using 4:2:1, 4:2:2 and 4:2:3 ratios of transfer : packaging : envelope expression vectors. The western blot analysis showed that RELIK envelope (≈ 98.7 kDa), which could be detected using its 3xFLAG-tag, is incorporated in the virions (Figure 2.8a). CRFK cells transfected with rabbit and hare CD4 and CXCR4 or CCR5 showed 26.1 % to 38.4 % double positive cells (Figure S3), however they were not successfully transduced with the RELIK Env pseudotyped lentiviruses (Figure 2.8c), regardless of the different ratios applied for vector production. The use of the human CD4 alone or together with the rabbit or hare CXCR4 or CCR5 also did not support transduction with the RELIK Env particles (Figure S4). On the other hand, cells were successfully transduced with the VSV-G pseudotyped lentiviruses, although both RELIK Env and VSV-G lentiviruses showed similar reverse transcriptase activity (around $3x10^{10}$ pUnits RT/µL). The use of the fusogenic VSV-G showed that the method worked in principal, yet RELIK envelope simply did not seem to have used rabbit / hare CD4 together with rabbit CCR5 or rabbit / hare CXCR4 to enter the cells. To further confirm these results, immunoprecipitation was performed using an anti-FLAG antibody to pull down the RELIK envelope and possible interaction partners (Figure 2.8b). The input membrane showed that hare CD4 was not strongly expressed, whereas all the other proteins were expressed and correctly detected. The immunoprecipitation was capable to enrich the envelope but no interaction with rabbit CD4 could be observed.

The results indicate that the virus might have used other proteins to enter rabbit cells. As there is no knowledge about these target cells, different rabbit cell lines, including a B cell line, a T cell line and an epithelial cell line, were transduced with RELIK Env or VSV-G pseudotyped lentiviruses. In this case, the H/SCA packaging vector was used to escape the restriction imposed by rabbit TRIM5. Unfortunately, rabbit cells were only successfully transduced with VSV-G pseudotyped but not with RELIK Env pseudotyped lentiviruses, despite similar RT activity of the concentrated lentiviruses ($3x10^{10}$ pUnits RT/µL) (Figure 2.8d). As expected, the rabbit T cell line was in general quite resistant to

transduction, as only 0.33% GFP positive cells were detected when cells were transduced with VSV-G pseudotyped lentiviral vectors.

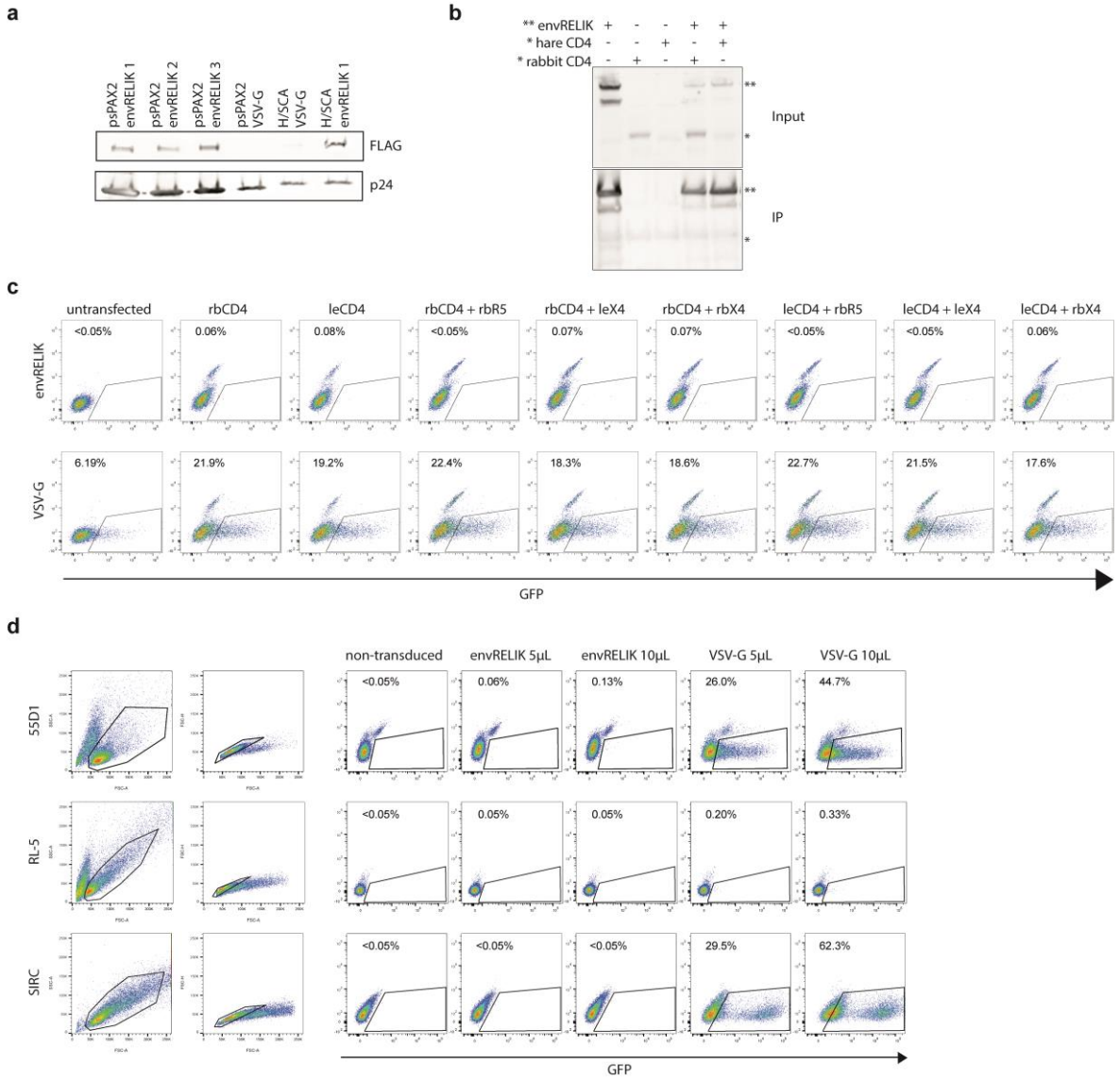

Figure 2.8 - RELIK might not have interacted with rabbit CD4, CCR5 or CXCR4 and rabbit cell lines were resistant to RELIK Env pseudotyped lentiviral vectors **a.** Western blot analysis of the lentiviral particles. RELIK envelop was detected by 3xFLAG-tag at ≈ 98,7 kDa. p24 capsid was used as a loading control. **b.** Immunoprecipitation of RELIK envelope. The top membrane represents the input cell lysate and the lower membrane the enriched proteins detected after immunoprecipitation. * indicates ≈ 98.7 kDa for the envelope protein and ** marks ≈ 50 kDa for CD4 detection. **c.** Flow cytometry analysis of transduced CRFK cells for GFP expression. The combination of receptors expressed is indicated on top. Lentiviral transduction of RELIK envelope (envRELIK) or VSV-G containing virions was measured by GFP expression. **d.** Flow cytometry analysis of transduced rabbit cell lines for GFP expression. The amount of lentivirus used for transduction with RELIK envelope (envRELIK) or VSV-G containing virions is indicated on top, and the kind of cells used is shown on the left side. Transduction efficiency was determined by measuring GFP expression levels using flow cytometry.

As the western blot analyses of RELIK Env pseudotyped lentiviral vectors showed only one band corresponding to the polyprotein, we speculated that it was not properly processed. A BLAST search of the rabbit genome available on the Ensembl database confirmed that the RELIK envelope is fragmented within the genome and contains several deleterious mutations. Lentiviral Env polyproteins are intracellularly processed by the host protease furins. Interestingly, several sequences were found that contain the recognition site for furin processing, R-X-K/R-R (Figure 2.9). In the majority of sequences, including the RELIK envelope sequence previously published and used in this work, the first arginine is mutated, hiding the cutting site for this protease. Whether the envelope from the circulating RELIK virus was actually processed, and if furin was the responsible protease is unknown, but it might explain why the receptor(s) for this ancient virus were not identified.

```
envRELIK                              KPMHKKRDFG
GL018789 937843 to 938445 (-)         KPMLKKRDFG
5 27964 to 28562 (-)                  KPMHKKRDFG
X 30937077 to 30937661 (-)            KPMCKKRDFG
4 89437698 to 89438298 (-)            KLMPQKRDFG
18 16868938 to 16869537 (+)           KPMCKKRDFG
1 11681182 to 11681784 (-)            KLMHKKRDFG
GL019115 110174 to 110773 (+)         KPMRKKRDFG
GL019115 134036 to 134635 (+)         KPMRKKRDFG
GL019206 115011 to 115608 (-)         KPMRKTRDFG
12 10125457 to 10126056 (-)           KPMRKKRDFG
GL018925 265967 to 266567 (-)         KPMHKK:DFG
GL019115 6482 to 7082 (+)             KPMHKKGDFG
GL018870 549692 to 550294 (+)         KPMCKKRDFG
7 42947752 to 42948278 (-)            KPMHKKRDFG
17 59888221 to 59888819 (-)           KPMSKKR:FG
GL018978 322309 to 322904 (+)         KPMRKKR:FG
13 61688829 to 61689425 (+)           KPMRKKRDFG
GL018848 617092 to 617694 (+)         KPMRKKRDFG
GL019054 36554 to 37163 (+)           IKMHKNVQKK
GL018817 580249 to 580827 (+)         KPMHKKKRL
GL018861 111947 to 112558 (-)         :QMHKNVQKK
GL018700 11599871 to 11600474 (-)     KPMHKKKRLW
```

Figure 2.9 - Furin cutting site is found in a few RELIK Env fragments. The first sequence is the one published on Katzourakis et *al.* (2007) and used for envelope synthesis. The remaining sequences were retrieved from the rabbit genome available on Ensembl and their genomic location is indicated on the left side. The residues recognized by the host protease furin (R-X-K/R-R) are highlighted in bolt. The ":" symbol represents out of frame deletions.

RERV-H in the rabbit genome

The Rabbit endogenous retrovirus H (RERV-H) viral genome can be abundantly found in the rabbit genome, however it was not known which rabbit species contained this endogenous virus. Using the reference clone identified in human samples as a query sequence (GenBank accession number AF480924), at least 19 copies of the entire viral genome were retrieved from different European rabbit chromosomes and more than 400 fragments with more than 90% identity are distributed over several chromosomes (data from Ensembl 90 http://aug2017.archive.ensembl.org/index.html). The complete copies are highly conserved and several do not show any deleterious mutation within the main viral components, confirming that the endogenization process did not lead to a loss of function and consequent inactivation of viral replication within the European rabbit. The same BLASTN search was performed for the pika genome but, as expected, no hits were found.

To verify the presence of RERV-H genome in other leporid species, a PCR-based strategy was used. From the tested samples the RNaseH from RERV-H was only amplified in *Oryctolagus cuniculus*, *Pentalagus furnessi* and *Bunolagus monticularis*. Several PCR conditions were tested to ensure that the unsuccessful amplification in other species was not PCR-related but due to the absence of viral elements in these species. Since several copies of this viral region were expected to be present in the genome of *Oryctolagus*, *Pentalagus* and *Bunolagus*, the PCR amplicons were cloned and 16 clones were randomly selected for sequencing. All the sequences found in *Oryctolagus* represented a possible functional enzyme, whereas for *Pentalagus* several sequences showed mutations, including the presence of stop codons that truncated the predicted translation product, and for *Bunolagus* only one sequence was found without any deleterious mutation (Figure 2.10).

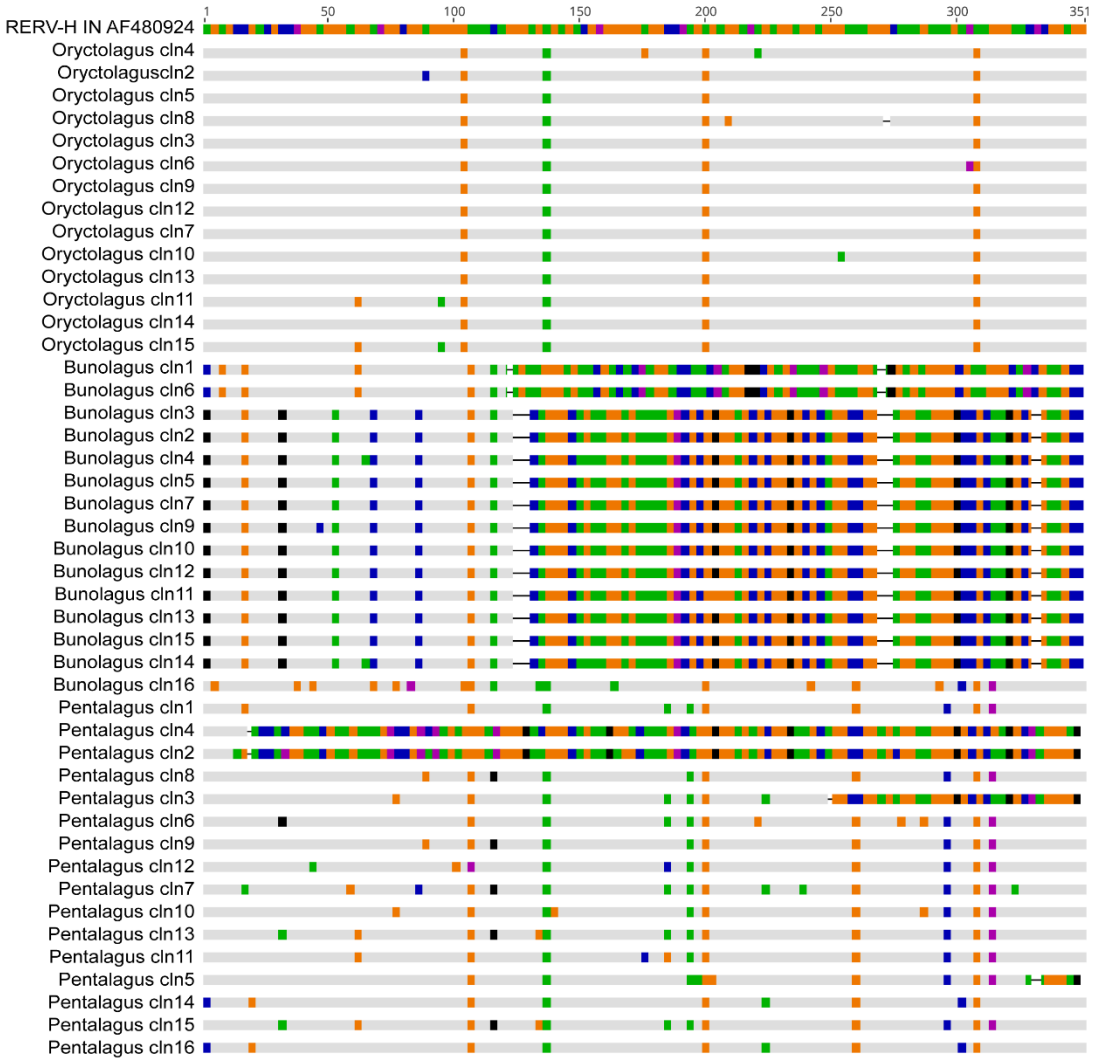

Figure 2.10 - RERV-H RNaseH is present in the genome of *Oryctolagus*, *Pentalagus* and *Bunolagus*. Ambiguous residues are highlighted in color (non-polar in orange, polar unchanged in green, polar acidic in purple and polar basic in blue) and black boxes indicate STOP codons. The sequences used are submitted on GenBank database under the accession numbers MG603347- MG603391.

This analysis was complemented by estimating the average evolutionary divergence between and within species using the Maximum Composite Likelihood model (Table 2.2). The average divergence within species is lower in *Oryctolagus* when compared to *Bunolagus* or *Pentalagus*. Among species, a higher divergence was observed between *Bunolagus* and *Pentalagus* (0.138±0.015) than between *Bunolagus* and *Oryctolagus* (0.121±0.017).

Table 2.2 - Estimates of average evolutionary divergence within and between different RNaseH sequences (sequences depicted in Figure 2.9). The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Standard error estimates were obtained by a bootstrap procedure (150 replicates). Analyses were conducted in MEGA7 using the p-distance model.

	Oryctolagus cuniculus	*Bunolagus monticularis*	*Pentalagus furnessi*
Oryctolagus cuniculus	0.013±0.003		
Bunolagus monticularis	0.121±0.017	0.040±0.005	
Pentalagus furnessi	0.114±0.015	0.138±0.015	0.056±0.006

Discussion

HIV-1 and other primate lentiviruses use CD4 and CCR5 as receptor and co-receptor, respectively. Besides the considerably high amino acid similarity between rabbit and human CD4 or CCR5, rabbit orthologs do not support HIV-1 entry into rabbit cells (Speck et al., 1998). For both CD4 and CCR5, many amino acid exchanges are observed in residues important for HIV-1 gp120 binding (Figure 2.1). Furthermore, reconstruction of the rabbit CD4 and CCR5 3D structure by analogy to the published human structures identified a few amino acid exchanges that could cause problems for virus binding (Figure 2.6). CD4-gp120 interaction is mostly variable and more dependent on electrostatic potential than surface topography, apart from the Phe-43 cavity. This cavity encompasses conserved gp120 residues derived from all variable loops (V1, V2 and V3) which interact with CD4 Phe-43 (Kwong et al., 1998, Kwong et al., 2000). Therefore, residues within the D1 region of CD4, and especially in the vicinity of Phe-43, determine binding to gp120. The mutation of this Phe to Leu in rats was reported to modestly reduce gp120 affinity (Moebius et al., 1992), while the substitution to Val in murine CD4 leads to a drastic decrease in gp120 binding (Siddiqi et al., 1997). Despite the conservation of Phe-43 in rabbits, lagomorphs contain other substitutions in residues shown to be equally essential for gp120 binding, such as the Leu-44 to Trp (Wang et al., 2001). Most of the studies on rat and mouse CD4 used the substitution of large regions, showing the D1 domain as determinant for gp120 binding (Simon et al., 1993, Landau et al., 1988, Davis et al., 1993). Single-site mutagenesis studies on CD4 often used Ala mutations, thus not differentiating to the amino acid substitutions seen in rabbits (Ashkenazi et al., 1990, Wang et al., 2001). Rabbit CD4 additionally has an insertion of four serine residues. According to the 3D structure, this extra serine stretch can block interaction with gp120 due the effect of the bulky volume on the Phe-43 cavity (Figure 2.6a). The estimated energy necessary for CD4-gp120 interaction indicated that rabbit CD4 would need

significantly higher energy than human CD4, however, removal of the serine stretch would be enough to reduce the necessary energy. Additionally, the removal of the serine stretch together with mutations Trp-44-Leu and Leu-45-Thr would be enough to decrease the binding energy to levels comparable to the human CD4-gp120 binding (Figure 2.6c). Regarding CCR5, several residues shown to be essential for gp120 binding are located in the extracellular loops (ECL) or at the extracellular side of the transmembrane domains (Garcia-Perez et al., 2011, Tamamis and Floudas, 2014). The N-terminal domain also comprises several binding residues, most of them mutated in rabbit or rat. In the proximity of the ECL2, the residue Ile-198 was shown to be of major importance for gp120 binding, however it is replaced by an Arg in rabbit and a Met in rat. This residue establishes important contacts with the aromatic residues Phe-109 and Phe-112, which are also not conserved in rabbit and rat, and together with the Trp-190 it promotes proper folding of the ECL2 (Garcia-Perez et al., 2011). Mutagenesis studies done for individual residues showed that the mutation of position 198 to Ala severely decreased gp120 binding, however the substitution by Arg or Met were not addressed. Interestingly, mutation of residue 180 from Ser to Pro, as it is observed in rabbits and rats, was shown to cause distortion of the ECL2 secondary structure and consequently loss of hydrogen bonds between the backbone of this residue and gp120 (Garcia-Perez et al., 2011). Furthermore, mutagenesis studies on murine CCR5 showed the need of several substitutions in the N-terminal domain for efficient gp120 binding (Ross et al., 1998).

The generated chimeras (Figure 2.2) were transiently expressed in CRFK cells, a cat epithelial cell line that does not present any blocks to HIV in the early phase of its life cycle and promotes efficient virus production and release (Munk et al., 2007). Infection of the transfected cells with either YU-2 or 49.5 viruses showed that besides human CD4, also the chimeric rbhrbCD4 is able to support virus entry, corroborating the results from the binding energy estimated with the 3D models (Figure 2.6 and Figure 2.7). Previous mutagenesis studies on CD4 showed that substitutions on residues 48, 50 and 51 to the corresponding murine residues Gly, Pro and Ser, respectively, abolished gp120 binding (Clayton et al., 1988). More importantly, it was shown that mouse CD4 could only support gp120 binding upon several substitutions, including in the residues 39 to 43 and 49 to 53 (Wieder et al., 1996). In rabbits, the residues 39 to 42 and 52 are identical to the human sequence, and the remaining substitutions in rabbit do not seem to affect gp120 binding. Regarding CCR5, the substitution of the Ile-198 to Arg did not affect virus entry. Previous studies showed that the mutation of this residue to Ala abolishes both gp120 and maraviroc binding (Garcia-Perez et al., 2011), however, the substitution to Arg conserved gp120 binding but abolished maraviroc recognition. Interestingly, hrbCCR5 did not

support virus entry, but additional introduction of Ile-198 was sufficient, emphasizing the importance of this hydrophobic amino acid for interaction. In this chimera, residue 180 is a Pro and not a Ser as in humans. Thus, in contrast to published reports, the presence of Pro does not greatly affect virus entry. Contrary to what might be expected, rbhCCR5 and rbhArgCCR5 chimeras supported virus entry. The Arg at position 198 once again did not affect virus entry, but surprisingly, this chimera was targeted by maraviroc, which can possibly be explained by other interactions between the rabbit N-terminal part and maraviroc that do not rely on the residue at position 198. Yet, this scenario is intriguing due the fact that the residues shown to be essential for maraviroc binding are conserved in the rabbit N-terminal. The use of the AMD3100 drug, a CXCR4 antagonist, underlined that the observed results were specific, i.e., the virus entered the cells using only CCR5 as co-receptor. Here, the N-terminal was essential for virus fusion as shown for rodents (Picard et al., 1997).

Combining the best performing CD4 and CCR5 chimeras, the use of the rbhrbCD4 and hrbIleCCR5 was sufficient to support HIV-1 entry in the cells to the same extent as the human proteins. In this scenario, the rabbit CD4 chimera differed only in six amino acids. The CCR5 chimera could still be improved, as more than only the predicted 24 amino acids were changed in the N-terminus.

The evolutionary study of these proteins in primates and lagomorphs was thus a useful tool to understand what shaped their evolution and, in the light of this study, if the rabbit would present any adaptive advantage to support HIV-1 infection. The continuous host-pathogen interaction seemed to have shaped primate CD4, leading to prints of positive selection mainly in the D1 domain of the protein (Zhang et al., 2008b). The importance of this domain for the interaction with the envelope protein of primate lentiviruses, such as HIV-1, suggested a selective pressure caused by such pathogens in this group of mammals (Meyerson et al., 2014). In leporids, a similar evolutionary model is most likely applied for CD4, where high nucleotide diversity was observed in the D2 domain (Figure 2.3). The detailed analysis of this domain revealed high condensed divergence found in leporids within a small region, with up to nine out of eleven amino acid substitutions when comparing rabbit and hare sequences. While lower divergence is observed within each genera, major amino acid changes were noted between *Oryctolagus*, *Sylvilagus* and *Lepus*, suggesting that the selective pressure that shaped CD4 in these species occurred after the speciation event, creating species-specific mutations.

Unlike the high divergence found in the D2 domain, the D1 region, which for human CD4 is known to have the main interacting residues with gp120, is quite similar between the European rabbit and the hare. However, the serine stretch insertion that was shown to

prevent rabbit CD4 to efficiently bind to gp120 is not present in all lagomorphs, with the eastern cottontail rabbit (*Sylvilagus floridanus*), pigmy rabbit (*Brachylagus idahoensis*) and pika (*Ochotona princeps*) showing only one serine (Figure 2.4b). The existence of this serine motif does not follow the phylogeny in lagormorphs, and suggest an ancestral event that remained fixed solely in some species (Figure 2.5). Is not clear if CD4 from these species would allow efficient gp120 binding, but for the first time, it was shown that a mutation in this region is able to affect virus entry. The phenotype originated by the insertion of this serine stretch may have represented an advantage against viruses or other pathogens. However, it is puzzling why it was fixed in some rabbit species and hares and deleted in other rabbits.

Regarding CCR5, the evolutionary story in lagomorphs showed that this gene suffered gene conversion in *Oryctolagus*, *Bunolagus* and *Pentalagus*, while it remained preserved in other leporids (Carmo et al., 2006) (Figure 2.5). Interestingly, this gene conversion appears in the ECL2, a loop that was shown to be important in human CCR5 for gp120 binding (Abayev et al., 2015), and where an Ile insertion was necessary for rabbit CCR5 to support HIV-1 entry. The evolutionary force behind the gene conversion is not clear, but once again, some leporids accumulated mutations in regions that are essential for virus binding and entry. Therefore, endogenous retrovirus found in these species, which were probably exogenous retroviral pathogens in the past, may have exerted selective pressure on CCR5.

The existence of RELIK, an endogenous lentivirus, in the genome of rabbits and hares, and its structural similarity to other lentiviruses, suggested similar interaction with host proteins (Keckesova et al., 2009, Katzourakis et al., 2007). However, not all species where RELIK was identified show the serine stretch insertion on CD4 D1 region or the CCR5/CCR2 gene conversion (see Figure 2.5). Anyway, the surface protein encoded by the *env* gene of RELIK shares high structural similarity with HIV-1 gp120 (Hotzel, 2008), which opens the possibility of an interaction between RELIK envelope and its host CD4 proteins.

In the first approach to investigate if leporids' CD4 together with CCR5 or CXCR4 were used as receptors, CRFK cells transiently expressing these proteins were transduced with HIV-1 based lentivirus particles pseudotyped with RELIK Env VSV-G. The VSV-G pseudotyped particles are a standard for evaluating the efficiency of other pseudotypes (Cronin et al., 2005), and as expected, they successfully transduced the cells. On the other hand, neither rabbit nor hare CD4 and CCR5/CXCR4 combinations mediated RELIK Env-dependent entry (Figure 2.8c). Additionally, immunoprecipitation of RELIK Env was not able to pulldown rabbit or hare CD4, which indicates that there is no

interaction between these proteins (Figure 2.8b). Indeed, other lentiviruses use other receptors, such as CD134, ELR1 or CD206, which could also be the case for RELIK (Larruskain and Jugo, 2013, Shimojima et al., 2004, Zhang et al., 2005).

In the pursue of RELIK target cells, different rabbit cell lines, including the RL-5 T cell line, the 55D1 B cell line and the epithelial cell line SIRC, were transduced with RELIK Env or VSV-G pseudotyped HIV-1 capsid switch lentiviral vectors. Despite these efforts, RELIK Env pseudotyped particles could not successfully transduce any of these cell lines. In a deeper look, it was possible to note that the western blot analysis for RELIK Env showed only one band corresponding to the full size protein (≈ 98,7 kDa). However, many enveloped viruses strongly depend on the processing of their viral envelope glycoproteins by cellular proteases to productively infect their target cells (Pasquato et al., 2013). The envelope of primate lentiviruses are cleaved by furin or a furin-like host protease, and FIV seemed to be cleaved by a protease that recognizes a similar cutting site (Verschoor et al., 1993, Hallenberger et al., 1992). Although Env processing was shown to be necessary for EIAV, is yet not clear which protease is involved (Rice et al., 1990). Proprotein convertases, such as furin, often recognize the amino acid sequence (Arg/Lys)-Xaa-(Arg/Lys)-Arg (Moulard et al., 1998, Apte and Sanders, 2010). The sequence for RELIK Env published by Katzourakis *et al.* (2007) and subsequently used in this study, does not have this recognition cluster, however several fragments of RELIK Env within the rabbit genome contain the possible cutting site Arg-Xaa-Lys-Arg (Figure 2.9). Most of the sequences do not have the first Arg and there are several deleterious mutations in this region, which could have hidden this recognition site in the consensus sequence used to reconstruct RELIK Env. If furin cuts RELIK polyprotein one would expect two fragments with ≈ 70,2 kDa and a ≈ 28,6 kDa. In the light of these results, it is not possible to completely exclude the interaction between rabbit CD4 and RELIK Env. It needs to be clarified whether RELIK Env can be processed by a proprotease, and upon that, if there is any interaction between the envelope and the CD4 of leporids. Furthermore, lentiviruses were reported to frequently infect myeloid cells, such as macrophages (Gifford, 2012), and this type of cells was not tested for RELIK Env lentivirus transduction, being still a possibility to answer which cells were infected by the circulating form and a step forward to identify which receptors were used.

Besides RELIK, other endogenous retroviruses could be in the origin of the evolutionary pressure that shaped leporids CD4 and CCR5. RERV-H was here identified in the genome of three genera that inhabit distant geographical regions - *Oryctolagus* is native from Iberian Peninsula, whereas *Pentalagus* is native to Japan and *Bunolagus* is an endemic species of South Africa (Robinson and Matthee, 2005). According to these

results, RERV-H most likely became endogenized in the common ancestor of *Pentalagus*, *Bunolagus* and *Oryctolagus* in a single endogenization event that must have occurred at ~9 million years ago (Figure 2.5) (Matthee et al., 2004). Interestingly, RERV-H is present only in the leporid species that also harbor the CCR5/CCR2 recombinant receptor (Pinheiro et al., 2016, van der Loo et al., 2016, van der Loo et al., 2012, Abrantes et al., 2011, Carmo et al., 2006). While for *Oryctolagus* other regions were successfully amplified by PCR, RNaseH was the only viral part amplified from the *Bunolagus* and *Pentalagus* samples, either due the loss of other viral domains in these species or due to a high polymorphism level. Similarly, the Env domain could not be retrieved from the rabbit genome, possibly due to its loss, or its detection was hampered by a high degree of polymorphism (Boeke and Stoye, 1997, Griffiths et al., 2002). Either way, the lack of RERV-H Env did not allow any functional studies to access its interaction with CD4 or CCR5 from leporids.

Concluding, the small mutations introduced in the rabbit CD4 and CCR5 proteins necessary for HIV-1 binding and entry were shown to be in regions were evolutionary forces shaped leporid evolution. So far, it was not possible to irrefutably link the evolutionary traits of rabbit CD4 and CCR5 and the presence of a particular endogenous retrovirus. RELIK and RERV-H arose as appealing candidates, however the functional studies performed were not able to clarify their interaction with the rabbit proteins. Both retroviruses, and possibly other unidentified rabbit viruses, could have shaped the evolution of CD4 and CCR5 in leporids in different periods, leading to the complex evolutionary story of these proteins in this group of mammals.

Chapter III: SERINC3/5's antiviral activity and antagonism are highly conserved

This chapter contains parts of the publication:

de Sousa-Pereira P., Abrantes J., Bauernfried S., Pierini V., Esteves JP., Keppler OT., Pizzato M., Hornung V., Fackler OT., Baldauf HM. The antiviral activity of rodent and lagomorph SERINC3 and SERINC5 is counteracted by known viral antagonists. Journal of General Virology (2018).

Background

The lack of a proper animal model to study HIV-1 replication and pathogenesis is a gap on the way to cure HIV. From the small animals explored so far, immunocompetent rabbit seems to have less intracellular blocks to HIV-replication when compared to immunocompetent rodents (Figure 1.4), however the infectivity defect observed in rabbit macrophages is still a major block in this species to full HIV-1 replication (Tervo and Keppler, 2010). In the last years, several restriction factors were identified, some of them affecting HIV infectivity, i.e., these host antiviral proteins allow normal viral production and release but the released particles show reduced infectivity (Rosa et al., 2015, Usami et al., 2015, Krapp et al., 2016, Lodermeyer et al., 2013, Sheehy et al., 2003, Mangeat et al., 2003, Tada et al., 2015). For most of the restriction factors, HIV-1 developed escape mechanisms, however this counteraction is frequently species-specific, which means the virus is able to overcome the human restriction factor but not the orthologs. Classical examples are non-human APOBECs that cannot be counteracted by Vif, or Trim5α protein for which HIV-1 capsid has adapted to escape the human protein but is blocked by the protein from other species (Mariani et al., 2003, Schrofelbauer et al., 2006, Ikeda et al., 2008, de Matos et al., 2011, Nakayama and Shioda, 2012). Rodent Tetherin is also resistant to HIV-1 Vpu (Goffinet et al., 2010b). Therefore, it might be possible that one of these restriction factors play an important role in other species regarding HIV-1 replication.

Chasing for the host restriction factor that made Nef necessary for some cells led to the discovery of the antiviral proteins SERINC3 and SERINC5. In 2015, two groups showed in parallel that the presence of SERINC3 and mainly SERINC5 in producer cells caused reduced infectivity of Nef negative virions (Rosa et al., 2015, Usami et al., 2015). In this context, Nef downmodulated SERINC5 from the cell surface, prevented virion incorporation and restored viral infectivity. Different Envs were shown to have different sensitivities to SERINC5, and vesicular stomatitis virus glycoprotein or Ebolavirus glycoprotein pseudotyped viruses were not susceptible to SERINC5-mediated inhibition (Rosa et al., 2015, Usami et al., 2015). Interestingly, human SERINC5 affected divergent retroviruses, such as MLV and EIAV, and MLV glycogag or the S2 protein from EIAV counteracted SERINC5's antiviral activity in a similar way as Nef (Rosa et al., 2015, Chande et al., 2016, Ahi et al., 2016). The mechanism of how SERINC5 inhibits infectivity is still not completely clear, but the first theory, involving its intrinsic function in sphingolipid biosynthesis and thereby causing alterations in the lipid bilayer detrimental for virus infection, was disproved (Trautz et al., 2017, Inuzuka et al., 2005). The other

proposed mechanism suggests that SERINC5 inhibits the small pore formation with the next target cell (Sood et al., 2017, Tedbury and Sarafianos, 2017). Regarding Nef counteraction, it was shown that SERINC5 intracellular loop 4 is essential and that the viral proteins use clathrin-mediated endocytosis to downmodulate SERINC5, although the complete mechanism of counteraction is still unclear (Dai et al., 2018, Trautz et al., 2016).

Regarding this family of proteins, we were interested to understand if SERINC3 and SERINC5 orthologs were also antivirally active against HIV-1 and whether they could be counteracted by the known antagonists. Therefore, this section deals with the study of rabbit, rat and mouse SERINC3/5, in an evolutionary context as well as in the context of HIV.

Material and methods

SERINCs evolutionary analysis

SERINC1-5 sequences were obtained from the NCBI database and aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) available at http://www.ebi.ac.uk/ (Edgar, 2004). The phylogenetic analyses were performed on MEGA 6 (Tamura et al., 2013). The sequences used are listed in Appendix II (Table S1). The codon-based ML method (CODEML) implemented in PAML v4.4 (Yang, 2007) was used to determine codons under selection. An unrooted neighbor-joining phylogenetic tree was constructed using MEGA 6 with *P*-distance as substitution model and the pairwise deletion option for gaps (Tamura et al., 2013). The site-based models pairs M1a (nearly neutral) and M2a (selection), and M7 (neutral, β) and M8 (selection, β and ω) were compared. M1a and M7 correspond to the null hypothesis while M2 and M8 allow positive selection representing the alternative hypothesis. A likelihood ratio test (LTR) with two degrees of freedom determined which selection model fits best the data (Yang, 2002, Yang et al., 2000). Codons under positive selection were identified using the Bayes Empirical Bayes (BEB) approach with probability > 95%. Using HyPhy software implemented in the DataMonkey web server (Delport et al., 2010, Pond and Frost, 2005), five other methods were used to detect sites under selection: the Single Likelihood Ancestor Counting (SLAC), the Fixed Effect Likelihood (FEL), the Random Effect Likelihood (REL) (Kosakovsky Pond and Frost, 2005), the Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012) and Fast Unbiased Bayesien AppRoximation (FUBAR) (Murrell et al., 2013) methods, with *p*-values < 0.1 for SLAC, FEL and MEME models, Bayes Factor > 50 for REL and a posterior probability > 0.90 for FUBAR. To

avoid false-positive results, only sites identified by at least three of the methods were considered with evidence of selection.

Rabbit, rat and mouse SERINC3 and SERINC5 cloning

SERINC3 and SERINC5 coding regions were amplified from spleen cDNA of rabbit, rat or mouse, using the Phusion high-fidelity PCR master mix (Thermo Fisher Scientific). Rabbit, rat and mouse SERINC3 were cloned into the pCMV-HA-C plasmid (Clontech) using *SalI* and *KpnI* (New England Biolabs), while SERINC5 constructs were cloned using *EcoRI* and *KpnI* (Appendix III). After ligation with the T4 DNA ligase (New England Biolabs), the constructs were transformed into competent cells and positive colonies were confirmed by sequencing. HA-tagged human SERINC3 and SERINC5 were received from Prof. Dr. Pizzato (Rosa et al., 2015). These constructs with the C-terminal HA tag were further subcloned into the pBJ6 using *NotI* and *EcoRI*, and positive colonies confirmed by sequencing.

Generation of SERINC3 and SERINC5 knock out cells

The generation of HEK293T KO cells was performed in collaboration with the group of Prof. Dr. Hornung (Gene Center, Ludwig Maximilian University of Munich). HEK293T cells were transfected with pRZ_BFP_T2A_Cas9 together with GFP-expressing pLKO.1_gRNA plasmids using Lipofectamine 2000. The following guideRNAs were used for SERINC3 (5'GTTTGTTGTTGGCATGATAGGGG3') and for SERINC5 (5'GTAGAGGGCGTACATGAAGCGGG 3'). 2 days later, BFP and GFP double positive cells were FACS-sorted and sub-cloned by limiting dilution. The genomic locus surrounding the gRNA binding site was PCR amplified using the primers SERINC3(fw) 5'-ACACTCTTTCCCTACACGACGctcttccgatctCCTACCAGCATACCACAACCTTG-3', SERINC3(rv) 5'-TGACTGGAGTTCAGCGTGTGctcttccgatctGTACTGCTGGAGACAGAATGTCT-3', SERINC5(fw) 5'-ACACTCTTTCCCTACACGACGctcttccgatctTTTTCTAGCTGGCCTGCTGCTGT-3' and SERINC5(rv) 5'-TGACTGGAGTTCAGCGTGTGctcttccgatctTCATGTTTTTCCCACACACCCTC -3', and subjected to deep sequencing using a MiSeq platform (Illumina) as previously described (Andreeva et al., 2017).

Virus production and infectivity assay

pHIV-1_{NL4-3}ΔNef and pHIV-1_{NL4-3}SF2Nef proviral DNAs were used alone or together with plasmids for SERINC3/5 expression (HA-tagged pcDNA3.1- and pBJ6-based), GlycoGag (HA-tagged pBJ5-based), S2 (HA- tagged pcDNA3.1-based) or VSV-G (pMD2.G) expression plasmids (Fackler et al., 2001, Chande et al., 2016, Pizzato, 2010). pNL4-3/Envfs/Nef_{LAI} was generated by replacing the nucleotide sequence of pNL4-3/Envfs (Pizzato et al., 2007) with the homologous sequence derived from *nef* of HIV-1_{LAI}. The corresponding pNL4-3/Envfs/ΔNefLAI carries a mutation in *nef*. For infectivity experiments, pNL4-3/Envfs/NefLAI and pNL4-3/Envfs/ΔNefLAI were complemented with HXB2 Env (PBJ5) (Rosa et al., 2015). For the initial titration, $1.5x10^5$ HEK293T cells were transfected with 1.5 µg of proviral HIV-1_{NL4-3}ΔNef DNA and 0.1, 0.5 or 1 µg of SERINC3 and SERINC5 plasmids using 6 µL of PEI reagent (Polyethylenimine 25K from Plysciences). For VSV-G pseudotyped viruses, cells were transfected with 1.5 µg of proviral HIV-1_{NL4-3}ΔNef DNA, 0.5 µg of SERINC3 and SERINC5 plasmids and 0.1 µg of VSV-G expression plasmids. pBJ6 SERINC expression plasmids were used to study counteraction by SF2Nef (HIV-1_{NL4-}SF2Nef), LAINef (proviral DNA complemented with 0.2µg HXB2 Env), S2 (0.5 µg with HIV-1_{NL4-3}ΔNef) or GlycoGag (0.8 µg with HIV-1_{NL4-3}ΔNef). SERINC3/5 KO HEK293T cells were used to monitor the impact of endogenous SERINC for the production of HIV-1_{NL4-3}ΔNef in the presence or absence of SERINC overexpression.

Cell supernatants were collected 48 hours post-transfection and $1x10^4$ TZM-bl cells were infected with 25 µL of virus-containing supernatants. 48 hours after infection, cells were lysed (Promega Lysis Buffer) and after a cycle of freeze/thaw, firefly luciferase reporter activity was measured using the Luciferase Assay System (Promega) according to manufacturer's instruction in a TECAN infinite 200. The infectivity data was normalized by the reverse transcriptase activity measured by SG-PERT (Pizzato et al., 2009), as described in Appendix1.

SERINC expression analysis

For flow cytometric analyses, transfected HEK293T cells used for virus production were detached and fixed with 4% PFA for 90 minutes at room temperature. Upon permeabilization with cold Perm Buffer II (BD Biosciences) for 5 minutes at 4°C, cells were stained with anti-HA-FITC (1:50; Milteny Biotec) for 30 minutes and posteriorly analyzed by flow cytometry using a BD FACSVerse (BD Biosciences) and the results processed using the FlowJo 10.1 software.

In parallel, transfected cells were lysed for 5 min at 4°C with 100 mM NaCl, 10 mM HEPES, 1 mM TCEP, 1 % DDM and 2x protease inhibitors. After centrifugation at 10000 rpm, the supernatant was collected and mixed with 2x Laemmli sample buffer with 50mM TCEP and samples were resolved using a Tricine–SDS-PAGE system. The separated proteins were then blotted on a nitrocellulose membrane, which was first blocked with 5% milk and further incubated overnight with mouse anti-HA antibody (1:2000; BioLegend). Vinculin was used as a loading control by incubating with rabbit anti-vinculin (1:1500 Abcam). Proteins were visualized using the Clarity Western ECL Blotting Substrate (BioRad) on a BioRad ChemiDoc gel documentation system.

Capsid ELISA

Nunc Maxisorp plates were coated overnight with mouse anti-p24 capsid (0.5 µg/mL; NIH AIDS Reagent Program hybridoma 183-H12-5C) and blocked with 10 % FCS. Samples and standards where inactivated with Triton X-100 with a final concentration of 0.5% and diluted appropriately in PBS containing 0.05% Tween. After overnight incubation, the primary polyclonal rabbit anti-p24 antibody was added (1:1000; in-house antibody). After incubation with the secondary goat anti-rabbit-peroxidase antibody, the commercial TMB–ready solution (Serva) was added and the reaction stopped with 0.5 M H_2SO_4. The plate was read at 450 nm using 570 nm reference wavelength in a TECAN infinite 200.

Fusion assay

The virions used in the fusion assay were produced in HEK293T cells with 30 µg of proviral DNA, 10 µg of SERINC expression plasmids (CMV or pBJ6-driven), 10 µg of pCMV4-BlaM-Vpr plasmid (Addgene) and 150 µL of PEI reagent (Polyethylenimine 25K from Plysciences). The viruses were purified as described in Appendix I. Reverse transcriptase activity was measured by SG-PERT (Pizzato et al., 2009), as described in Appendix I, and $1x10^5$ TZM-bl cells were infected with 10^{11} RT units of each virus. T20 (50µM) was used in parallel as a fusion inhibitor. The assay was performed as previously published and described in Appendix I (Cavrois et al., 2002). After fixation the cells were analyzed by flow cytometry using a BD FACSVerse (BD Biosciences) and the results processed using the FlowJo 10.1 software.

Results

SERINCs evolution

The evolutionary analysis of *SERINC1-5* showed that these genes are expressed in several eukaryotic species. In the human genome, the five members of this family are found on different chromosomes, and the flanking genes are generally shared with other species, spanning from mammals to fishes (Figure 3.1a). The evolutionary reconstruction of this family in mammals clusters the orthologs together, supporting the idea that these genes arose before the mammalian speciation (Figure 3.1b).

To assess if SERINC1-5 are under positive selection the site models implemented in PAML were used. The models analyzed the codons and tested whether a model that allows for positive selection is a better fit to the data than a null neutral model. The results were obtained from two sets of site models – M1a vs. M2a and M7 vs. M8. The likelihood scores of the null neutral and the selection models were used to perform the likelihood ratio test. The M1a vs. M2a comparison yielded likelihood test ratios that statistically allowed to reject null hypothesis of neutral selection for SERINC3 and SERINC4 (Table 3.1). The selection model (M2a) showed ω (or d_N/d_S) values of 4.8 (*SERINC3*) and 5.5 (*SERINC4*). The *BST2* gene (Tetherin) was used as a comparison parameter for a restriction factor under positive selection. The M2a model was also able to identify sites under positive selection (Table 3.1). The M7 vs. M8 comparison, besides *SERINC3* and *SERINC4*, also identified *SERINC1* under positive selection, however the statistical value to discard the null model was lower (p-value of 0.01) and ω (or d_N/d_S) obtained for the selection test was only 1 ($d_N/d_S < 1$ purifying selection; $d_N/d_S = 1$ neutral selection; $d_N/d_S > 1$ positive selection) (Table 3.1). Besides that, the M8 model (model that allows selection) was not able to identify any sites under positive selection for *SERINC1*. Regarding the other genes, this model was able to identify even more codons under positive selection than the M2a model. In general, the results obtained by both comparisons are similar, although the M1a vs. M2a comparison seems to be more conservative.

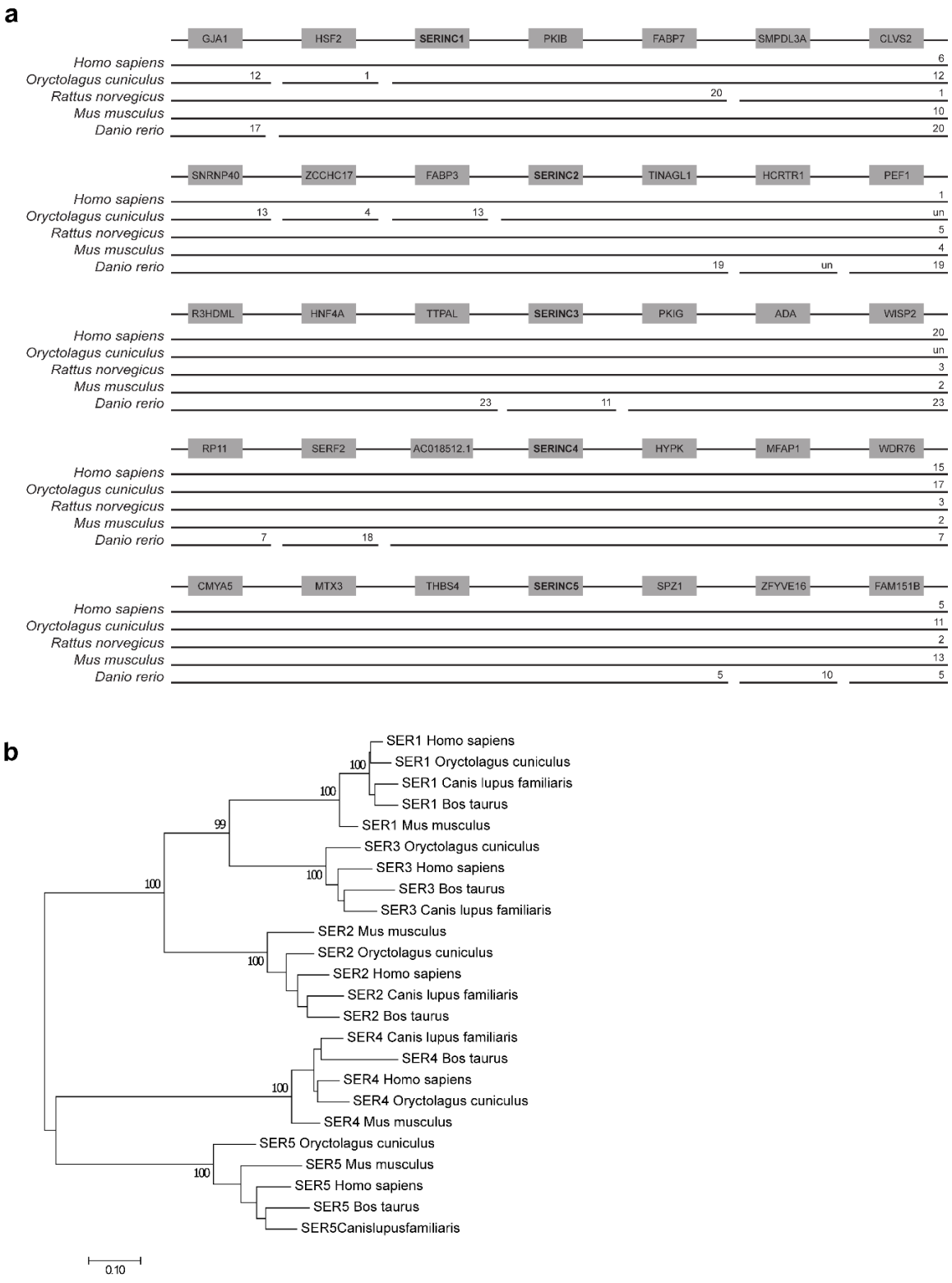

Figure 3.1 - SERINC orthologs are highly conserved. **a**, Synteny blocks representing the genes flanking SERINC1-5 in different species. Data from Ensembl 91 database (http://dec2017.archive.ensembl.org/index.html). **b**, Evolutionary history of SERINC1-5 in mammals inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) with 500 bootstrap replicates. A discrete Gamma distribution was used to model evolutionary rate differences among invariable sites. The analysis was conducted in MEGA 6.

Table 3.1 - SERINC1-5 and Tetherin likelihood ratio tests (2ΔlnL) for PAML M1a vs. M2a and M7 vs. M8 site models. The statistical test was compared to a χ^2 distribution with 2 degrees of freedom and the % of significance is shown. The ω (or dN/dS ratio) is shown for the selection tests significantly supported. Positively selected sites identified using Bayes Empirical Bayes analysis with posterior probability > 95% are shown (numbering and amino acid referring to the human sequence).

Models compared	Gene	2△lnL	p-value	ω	Positive selected sites
M1a vs. M2a	*SERINC1*	0,000	>0.9	--	
	SERINC2	0,000	>0.9	--	369V
	SERINC3	**22,881**	<0,001	4.830	166I; 212A; 335S
	SERINC4	**67,559**	<0,001	5.530	8P; 490W; 502R; 503R; 509I, 510S; 511P; 512D
	SERINC5	0,000	>0.9	--	
	Tetherin	**30,513**	<0,001	2.160	18K; 22L; 122T; 159D; 176S
M7 vs. M8	*SERINC1*	**9,951**	0.01	1.000	
	SERINC2	4,239	0.2	--	369V
	SERINC3	**28,286**	<0,001	2.000	51V; 166I; 212A; 226T; 319V; 335S; 346L
	SERINC4	**76,724**	<0,001	4.763	8P; 15G; 17A; 27L; 53S; 490W; 502R; 503R; 509I, 510S; 511P; 512D
	SERINC5	3,941	0.2	--	4Q
	Tetherin	**45,543**	<0,001	1.784	18K; 22L; 122T; 144L; 159D; 176S

This analysis, considering sequences from several mammals, revealed that from the SERINCs shown to have antiviral activity (SERINC3/5), only *SERINC3* is under positive selection. To increase the strength to find codons under positive selection on *SERINC*' genes, five other models implemented on the DataMonkey web served were used. These models allowed the identification of more sites under positive selection for all SERINCs (Table 3.2 and Figure S5), however when compared to a "classical" restriction factor, the % of sites under positive selection is rather low, even for the genes identified under selection by PAML (*SERINC3* and *SERINC4*).

Table 3.2 - Percentage of sites under positive selection in SERINC1-5 and Tetherin. Residues identified by site models in PAML (M2a and M8) and by DataMonkey models.

Gene	% sites under positive selection	
	PAML	PAML and DataMonkey
SERINC1	0.00%	0.00%
SERINC2	0.22%	1.54%
SERINC3	1.48%	4.02%
SERINC4	2.32%	3.67%
SERINC5	0.22%	0.87%
Tetherin	3.33%	10.56%

SERINC3 and SERINC5 orthologs antiviral activity

To determine if SERINC3 and SERINC5 from rabbit, rat and mouse are as antivirally active as the human counterparts, the coding region for these genes was amplified from spleen RNA of the respective species by PCR and cloned into mammalian expression plasmids containing the HA tag at the C-terminus. Initially, HEK293T were transfected with increasing amounts of pCMV-based SERINC3/5 expression plasmids and HIV-$1_{NL4-3}\Delta$Nef proviral DNA in order to produce Nef negative HIV-1 virus in the presence of SERINC3/5. These viruses were used to infect TZM-bl cells for Tat-induced firefly luciferase reporter gene expression, and the measured luciferase counts were normalized to the amount of reverse transcriptase (RT) activity units determined in the supernatants. All data related to infectivity were normalized to the control HIV-$1_{NL4-3}\Delta$Nef. The first results showed that infectivity of Nef-defective HIV-1 is reduced by all the SERINC3/5 orthologs in a dose-dependent manner (Figure 3.2a). In general, the higher the amount of transfected SERINC3/5 plasmid DNA was, the lower the infectivity, with an 18 - to 151-fold decrease in the presence of SERINC5 and 1.5- to 4.9-fold for SERINC3 orthologs. SERINC3/5 protein expression was monitored by western blot (Figure 3.2a), however these transmembrane proteins were difficult to separate and generated complex patterns, most likely due to different levels of glycosylation. Therefore, the expression levels were also verified by flow cytometry (Figure S6). Both methods showed increasing levels of HA-tagged SERINC3/5 expression for increasing amounts of plasmid DNA transfected. HIV-$1_{NL4-3}\Delta$Nef viruses produced in the presence of equal amounts of SERINC3/5, showed a significant infectivity decrease for all orthologs, with SERINC5 being more potent than SERINC3, as expected (Figure 3.2b). Virus particles pseudotyped with VSV-G did not show any decrease in infectivity when produced in the presence of SERINCs. On the contrary, these viruses were 2.5- to 19-fold more infectious than the control.

The analysis of virus- and cell-associated p24 capsid allowed to calculate the percentage of released particles, which revealed that the expression of SERINC3/5 did not have any significant impact on the release of Nef defective HIV-1 or on VSV-G pseudotyped viruses (Figure 3.2c).

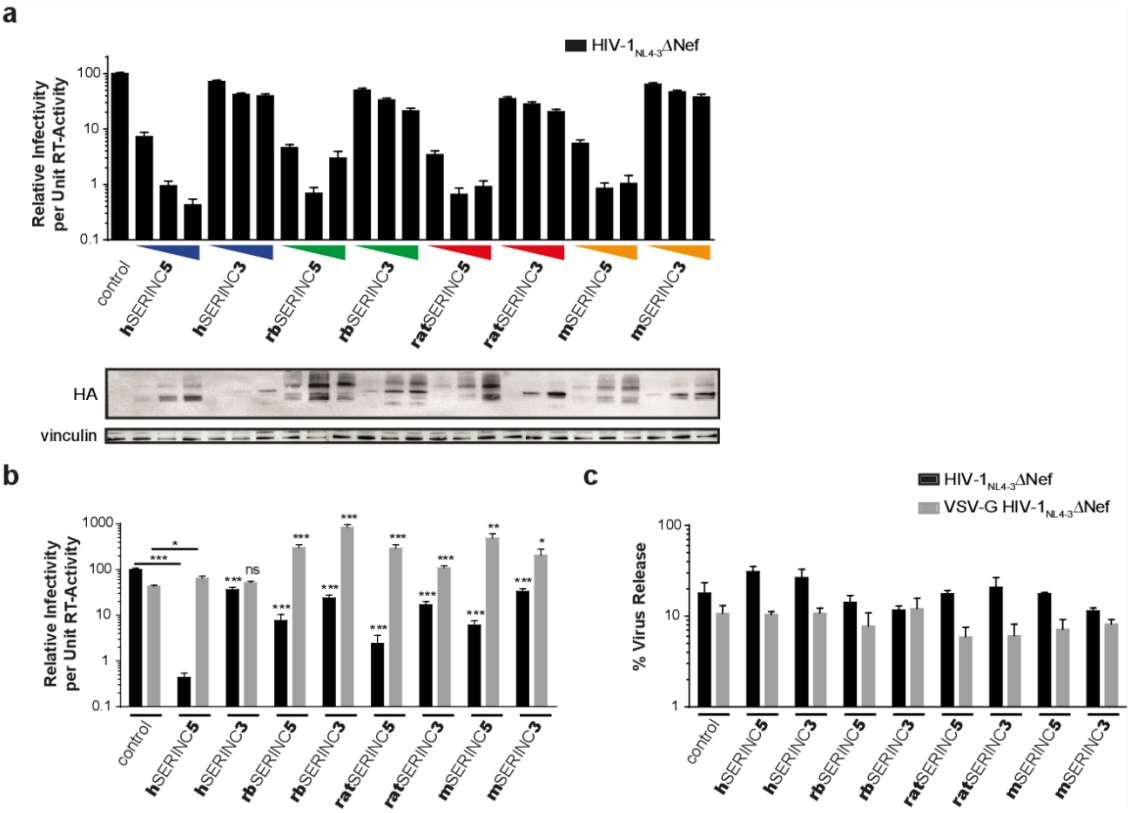

Figure 3.2 - Rodent and rabbit SERINC3/5 orthologs interfere with HIV infectivity in a dose-dependent manner. **a**, HIV-1_{NL4-3} lacking Nef (HIV-1_{NL4-3}ΔNef) produced in the presence of increasing amounts of SERINC3/5 orthologs. Relative HIV-1 infectivity was calculated as a ratio of firefly luciferase counts to RT units and normalized to control (empty expression plasmid). Shown are arithmetic means +/- SEM of three independent experiments. The western blot analysis for SERINC3/5 expression is shown. **b**, HIV-1_{NL4-3}ΔNef virions and HIV-1_{NL4-3}ΔNef pseudotyped with VSV-G virions produced in the presence of the SERINC3/5 orthologs. Shown are arithmetic means +/- SEM of three independent experiments. **c**, HIV-1 release was quantified as the percentage of total (cells plus supernatant) p24 capsid (p24CA) that was secreted as virion-associated p24CA. Shown are arithmetic means +/- SD of two experiments. No significant differences were found between control and SERINC containing samples. * < 0.05; ** < 0.01; *** < 0.001; ns = not significant.

SERINC3/5 orthologs impair HIV at the fusion step

In order to determine the step at which rabbit, rat and mouse SERINC3/5 inhibit Nef defective HIV-1, HIV entry was analyzed. Thus, HIV-1_{NL4-3}ΔNef virions were produced together with BlaM-Vpr in the presence or absence of SERINC3/5. Equal RT Units were used to infect TZM-bl cells. Only cells where fusion occurred contained the β-lactamase able to cleave the subtrate, which caused a shift in fluorescence. Virion fusion measured by flow cytometry revealed that the uninfected control and the fusion inhibitor T20 did not show entry above background. The control viruses supported fusion by 78%, while the presence of SERINC3 and SERINC5 decreased fusion to 6.4- to 27% and 0.2- to 3.6%, respectively (Figure 3.3a).

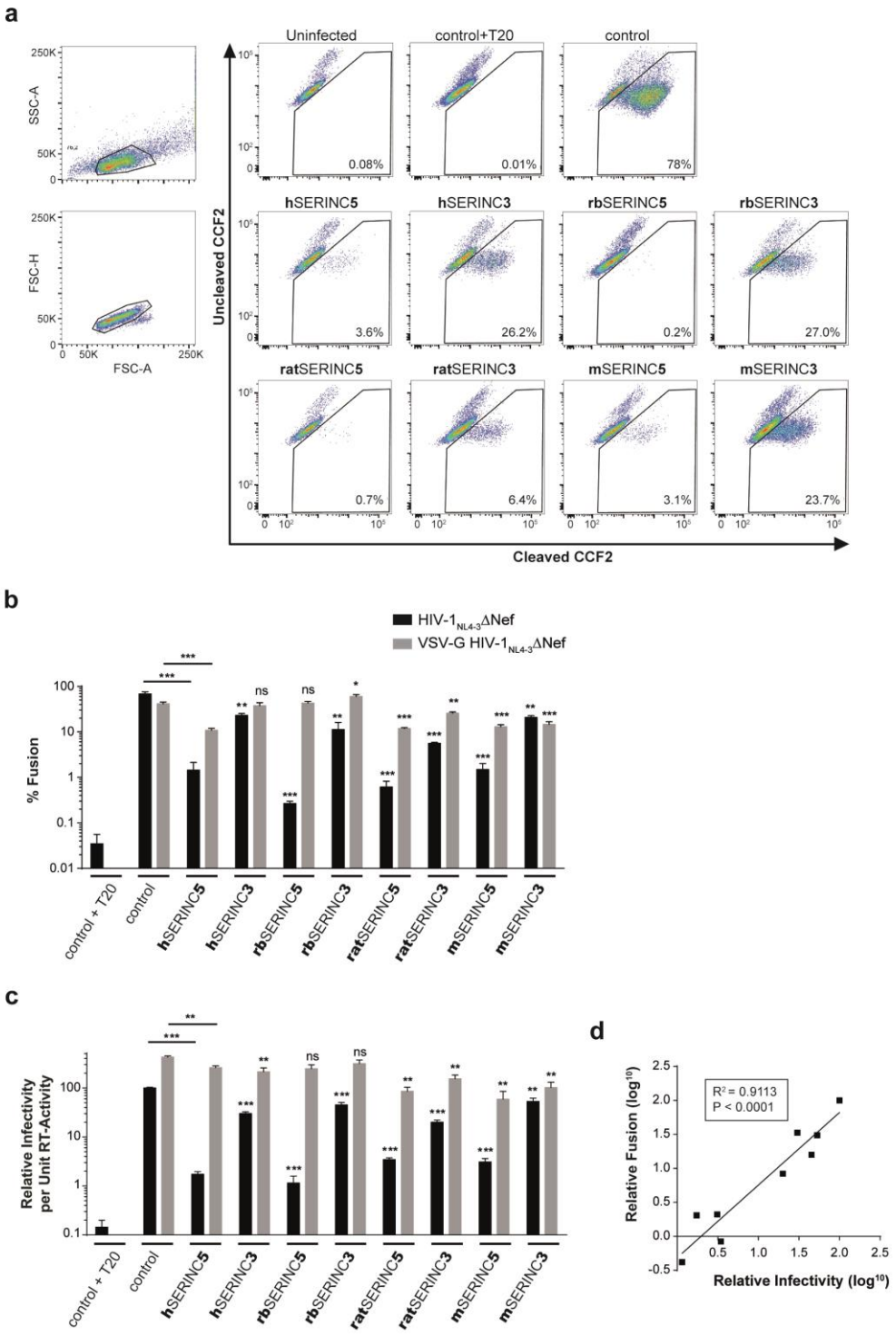

Figure 3.3. - Rodent and rabbit SERINC3/5 orthologs interfere with HIV at the level of virus entry. **a**, HIV-1_{NL4-3}ΔNef incorporating BlaM-Vpr produced in the presence or absence of SERINC3/5 orthologs. Equal RT units were used to infect TZM-bl cells and T20 was used as fusion inhibitor. Shown are representative dot blots of the fusion events. **b**, Graphical representation summarizing the raw data presented in a. Shown are arithmetic means +/- SEM of two independent experiments. **c**, Relative Infectivity data supported by the HIV-1_{NL4-3}ΔNef BlaM-Vpr virions produced in the presence or absence of SERINC3/5 orthologs. Shown are arithmetic means +/- SEM of two independent experiments. **d**, Correlation between fusion events and relative infectivity. * < 0.05; ** < 0.01; *** < 0.001; ns = not significant.

In general, virus entry was significantly reduced in the presence of all SERINC3/5 orthologs, with 3- to 257-fold decrease in fusion efficiency when compared to the control (Figure 3.3b). The infectivity data performed in parallel revealed also a significant decrease for all orthologs (Figure 3.3c). Furthermore, plotting the log10 of relative virion fusion and relative infectivity, showed that both parameters highly correlate with each other, with a R^2 of 0.9113 and $p < 0.0001$ (Figure 3.3d).

Similar experiments with VSV-G pseudotyped HIV-1_{NL4-3}ΔNef BLaM-Vpr, identified a less pronounced, but significant decrease in virion fusion and infectivity for virions produced in the presence of rat and mouse SERINC3 and human, rat and mouse SERINC5 (Figure 3.3b,c). This might be due the presence of the BLam-Vpr fusion protein, which has a negative impact on VSV-G pseudotyped virus, possibly causing steric hindrance of the glycoproteins.

Viral counteraction of SERINC3/5 orthologs

To determine if viral proteins can counteract SERINC3/5 orthologs, pBJ6-driven plasmids were used due to their weaker promoter and thus lower expression levels. As expected, pBJ6-driven SERINC expression was lower than that for the CMV-driven plasmids, making western blot detection nearly impossible. Therefore, flow cytometry was used to monitor the levels of SERINC3/5-HA expression, with 3 % to 17 % expression and similar mean fluorescence intensities for all constructs (Figure S7). Although less pronounced, pBJ6-driven SERINC3/5 significantly reduced virion infectivity of HIV-1_{NL4-3}ΔNef, with 1.3- to 2.9-fold and 2.8- to 9.2-fold for SERINC3 and SERINC5, respectively (Figure 3.4a). The presence of SF2Nef restored infectivity to control levels, and comparison of the plus and minus Nef scenario showed an infectivity increase from 2.6- to 9.2-fold for SERINC5 and just a mild increase (1.1- to 1.9-fold) for SERINC3 in the presence of Nef.

As reported before SF2Nef is virtually inactive against SERINC3, whereas LAINef represents a potent inhibitor (Usami et al., 2015). In contrast to SF2Nef, LAINef counteracted human SERINC5 by only 2-fold and rabbit SERINC5 by 2.8-fold (Figure 3.4b). Unexpectedly, rat and mouse SERINC5 were more sensitive to LAINef-mediated antagonism, showing an infectivity increase of 13.8- and 18.1-fold, respectively. In contrast, LAINef increased virion infectivity by 2.8- to 3.2-fold in the presence of SERINC3 orthologs. Considering the presence or absence of SERINC3/5 in the

presence of LAINef, the fold difference for SERINC3 orthologs (1.2- to 3.7-fold) was smaller in contracts to SERINC5 (1.7- to 5.3-fold).

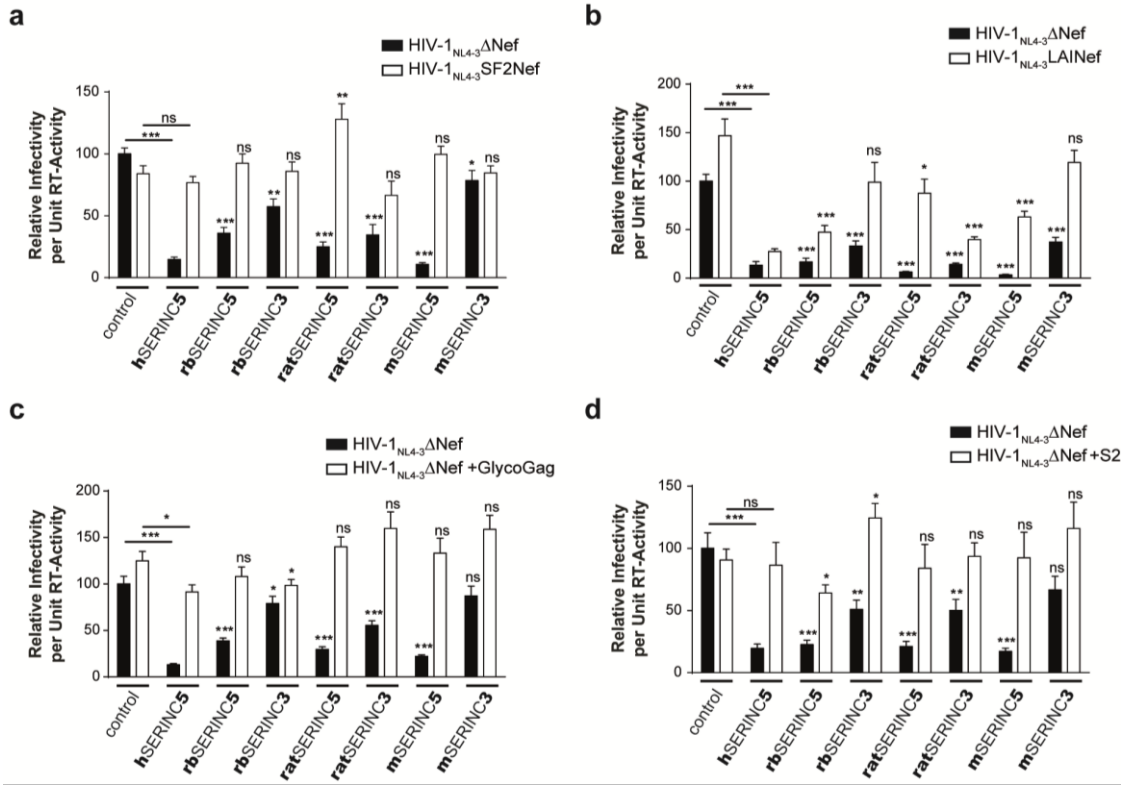

Figure 3.4 - Rodent and rabbit SERINC3/5 orthologs are counteracted by three different viral proteins. **a**, HIV-1_{NL4-3}ΔNef or HIV-1_{NL4-3}SF2 Nef virions produced in the presence or absence of SERINC3/5 orthologs. **b**, HIV-1_{NL4-3}/Envfs/ΔNef or HIV-1_{NL4-3}/Envfs/LAI Nef complemented with HXB2 Env produced in the presence or absence of SERINC3/5 orthologs. **c,** HIV-1_{NL4-3}ΔNef alone or together with a MLV GlycoGag produced in the presence or absence of SERINC3/5 orthologs. **d**, HIV-1_{NL4-3}ΔNef alone or together with the S2 protein from the EIAV produced in the presence or absence of SERINC3/5 orthologs. Relative HIV-1 infectivity was calculated as a ratio of firefly luciferase counts to RT units and normalized to control (HIV-1_{NL4-3}ΔNef control). Shown are arithmetic means +/- SEM of three independent experiments. * < 0.05; ** < 0.01; *** < 0.001; ns = not significant.

Viral proteins from MLV and EIAV were also tested for counteraction. HIV-$_{1NL4-3}$ΔNef produced in the presence of MLV GlycoGag showed comparable infectivities, regardless of SERINC3/5 expression (Figure 3.4c). The presence of GlycoGag increased virion infectivity of SERINC5-containing particles with 2.8- to 7-fold and 1.2- to 2.9-fold for SERINC3. When compared with the control, human SERINC5 and rabbit SERINC3 cause a significant decrease in infectivity despite the presence of GlycoGag, however, the presence of this protein still increases infectivity by 6- and 1.2-fold, respectively. The production of HIV-1_{NL4-3}ΔNef virions in the presence of the EIAV S2 protein revealed that S2 is also able to counteract SERINC3/5 antiviral activity (Figure 3.4d). EIAV S2 significantly increased virion infectivity for SERINC5-containing particles with 2.8- to 5.4-

fold and 1.7- to 2.4-fold for SERINC3-containing particles. Taken all data together, Nef, GlycoGag and S2 can counteract the antiviral activity of SERINC3/5 of the orthologs used in this study.

To reduce the possibility that endogenous SERINCs expressed in HEK293T cells might affect the experiments, SERINC3/5 KO were generated and used to produce HIV-1_{NL4-3}ΔNef virions in the presence of absence of overexpressed SERINC3/5 orthologs. The results demonstrated comparable infectivities data for the WT and the KO cells, discarding the influence of the endogenous SERINC3/5 in HEK293T (Figure 3.5).

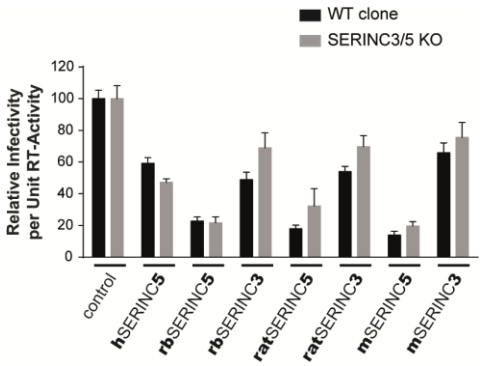

Figure 3.5 - The expression of endogenous of SERINCs in HEK293T cells do not influence the infectivity results. HIV-1_{NL4-3}ΔNef produced in the presence or absence of SERINC3/5 orthologs overexpression in wild-type HEK293T cells and SERINC3/5 KO cells. Relative HIV-1 infectivity was calculated as a ratio of firefly luciferase counts to RT units and normalized to control (HIV-1_{NL4-3}ΔNef control).

Nef antagonism at fusion step

In order to be able to judge at which step Nef counteracts SERINC3/5's antiviral activity, HIV-1_{NL4-3}ΔNef and HIV-1_{NL4-3}SF2Nef virions were produced with the BLam-Vpr in the presence or absence of pBJ6-driven SERINC3/5. Identical RT Units of the purified virions were used to infect TZM-bl cells and virion fusion was measured 4 hours post infection. HIV-1_{NL4-3}ΔNef virions supported around 77% fusion, which was reduced to ≈ 15 to 27 % in the presence of SERINC5 (Figure 3.6a). Using these virions, the presence of SERINC3 did not yield a significant reduction in fusion (Figure 3.6c). The Nef containing virions were not affected by SERINC3/5, with fusion levels compared to the control. On the other hand, Nef negative virions produced in the presence of SERINC3/5 do not show a decrease in infectivity when compared to the control (Figure 3.6b). This could again be due to the pBJ6-driven weak expression of SERINCs and the incorporation of the BLaM-Vpr into the viral particles. Regardless, SF2Nef containing virions showed higher infectivity levels when compared with the Nef negative counterparts, and the correlative analysis of the relative fusion versus relative infectivity established a positive

correlation between both parameters (Figure 3.6d). Summarizing, SERINC3/5 exert their antiviral activity prior virus entry and are counteracted by the SF2Nef prior this step.

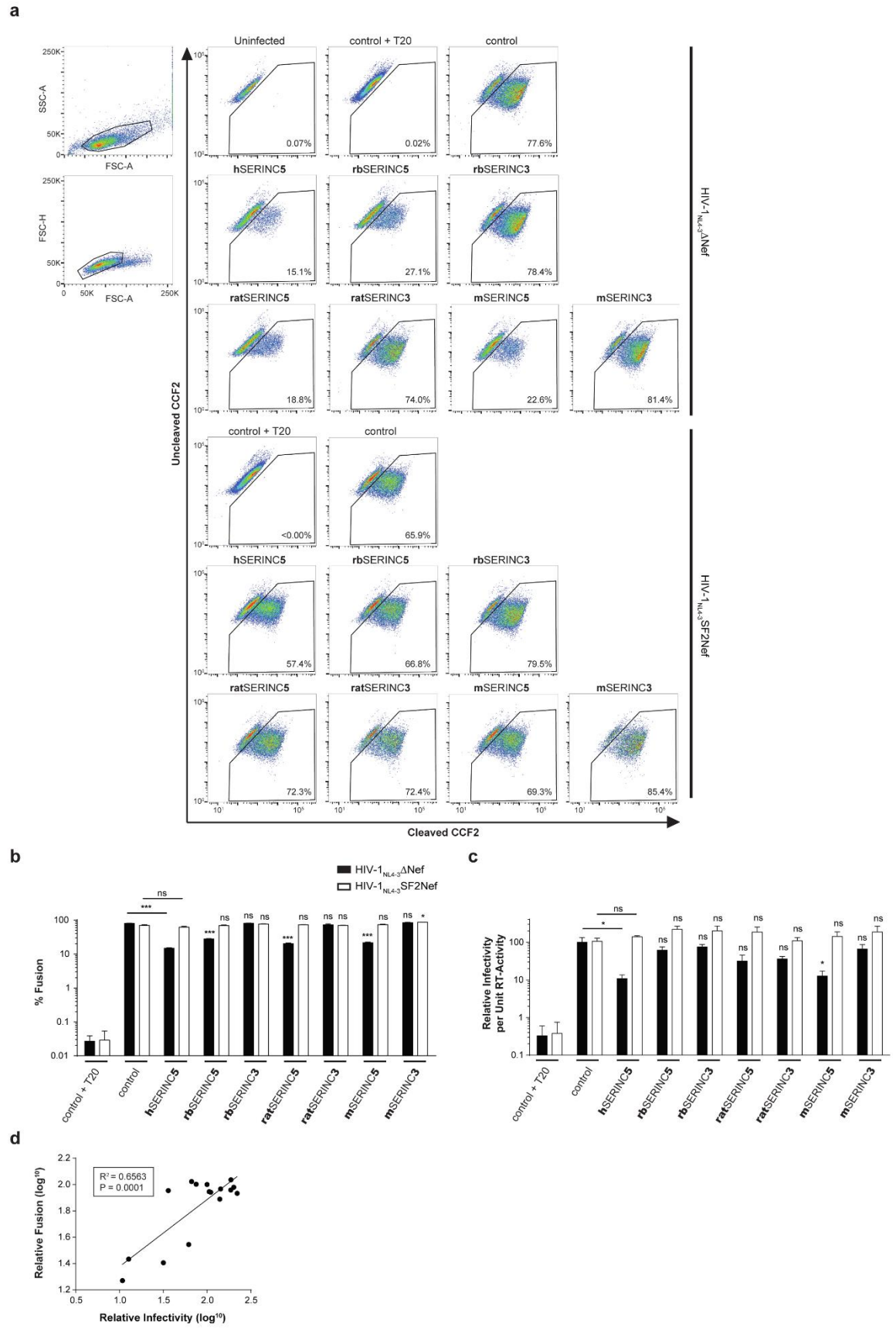

Figure 3.6 - Nef counteracts SERINC prior to virus entry. **a**, HIV-1_{NL4-3}ΔNef or HIV-1_{NL4-3}SF2Nef incorporating BlaM-Vpr produced in the presence or absence of SERINC3/5 orthologs. Equal amounts of RT units were used to infect TZM-bl cells and T20 was used as fusion inhibitor. Shown are representative dot blots of the fusion events. **b**, Graphical representation summarizing the raw data presented in a. Shown are arithmetic means +/- SEM of two independent experiments. **c**, Relative Infectivity data supported by the HIV-1_{NL4-3}ΔNef BlaM-Vpr and HIV-1_{NL4-3}SF2Nef BlaM-Vpr virions produced in the presence or absence of SERINC3/5 orthologs. Shown are arithmetic means +/- SEM of two independent experiments. **d**, Correlation between fusion events and relative infectivity measured in parallel. * < 0.05; ** < 0.01; *** < 0.001; ns = not significant.

Discussion

Analyses with simian orthologs showed that SERINC3 and SERINC5 genes do not exert typical signatures of an arms race with pathogens (Murrell et al., 2016). However, an expanded analysis performed using several mammalian sequences showed that from the SERINC family, SERINC3 and SERINC4 have indications of positive selection. Normally, an arms race between the host and the virus through continuous evolution is manifested through mutations as a result of evolutionary pressure. Although the identification of SERINC3 and SERINC4 being under positive selection, the percentage of sites identified under selection was rather low compared to other classical restriction factors, such as Tetherin (McNatt et al., 2009). More importantly, SERINC5, the main SERINC member with antiviral activity, showed to be highly conserved, suggesting a neutral evolution in mammals. These results, although unexpected, can be explained by evolutionary constraints imposed by the endogenous functions of SERINCs, limiting escape to the viral counteraction. The hypothesis that the antiretroviral activity of some members of this family has a novelty is unlikely, since SERINC3/5 were already shown to counteract different retroviruses, and different accessory proteins antagonize the antiviral activity (Ahi et al., 2016, Chande et al., 2016, Rosa et al., 2015), supporting an ancient interaction.

Despite the high level of conservation of SERINC proteins, is not clear if the antiviral activity for the human SERINC3 and SERINC5 is conserved for other orthologs. In this context, SERINC3 and SERINC5 from rabbit, rat and mouse were analyzed regarding their antiviral activity towards HIV-1. Since SERINC3/5 knockout studies were not possible in rodent or lagomorph cells due to additional restrictions to HIV-1 replication, SERINC3/5 were ectopically expressed in permissive cell lines. Similar to their primate counterparts, rodent or rabbit SERINC3 and SERINC5 are antivirally active in the absence of viral antagonists (Usami et al., 2015, Rosa et al., 2015, Heigele et al., 2016).

Although the observed inhibition is less pronounced for SERINC3, both SERINCs act in a dose-dependent manner. As reported for the human SERINC3/5 proteins, the antiviral activity of rabbit and rodent SERINC3/5 is circumvented when HIV-1 particles are pseudotyped with VSV-G (Usami et al., 2015, Rosa et al., 2015). There is no clear explanation for this phenomenon, however, is important to note that HIV-1 Env and the VSV glycoprotein use a different route to enter the target cell. Besides, higher numbers of VSV glycoproteins are incorporated in the virion compared to the usual low HIV-1 Env composition, which might explain why VSV-G pseudotyped particles are protected from SERINCs antiviral activity (Chojnacki et al., 2012, Thomas et al., 1985). Remarkably, rabbit and rodent SERINC3/5 increased the infectivity of VSV-G pseudotyped virions, which might be due to the presence of both HIV-1 Env and VSV-G simultaneously and some sort of cumulative effect. On the other hand, the VSV-G pseudotyped virions containing Blam-Vpr, showed decreased infectivity and fusion in the presence of SERINC3/5, which might be explained by steric hindrance in the particle due the presence of the Blam-Vpr. Either way, this decrease in infectivity/fusion due to the presence of SERINC3/5 is minor compared to particles lacking the VSV glycoprotein.

The fusion assay showed that, as for the human SERINCs, rabbit and rodent SERINC3/5 antiviral activity is displayed prior to virus entry (Rosa et al., 2015, Trautz et al., 2016). The high correlation between the percentage of fusion and the infectivity do not support an additional post-entry defect, as it was suggested by Rosa *et al*. (2015). To emphasize, this experimental setup involved the overexpression of these proteins, not allowing to evaluate the impact of the endogenous counterparts on virus replication.

It was often described that HIV-1 replication was blocked by other species restriction factors, such as the case of rabbit or rodents APOBEC1, rabbit TRIM5 or rodent Tetherin (Goffinet et al., 2010a, Goffinet et al., 2010b, Ikeda et al., 2008, Schaller et al., 2007, Nakayama and Shioda, 2012). Therefore, it was not completely surprising that these species' SERINC3/5 proteins were also antivirally active, underlining the preservation of the domains responsible for the antiviral activity among the different orthologs. Interestingly, non-human restriction factors were shown to be impervious to known viral antagonists. For instance, rodent APOBEC1 and murine APOBEC3G cannot be counteracted by the virial protein Vif, and the rodent Tetherin is not inhibited by HIV-1 Vpu (Goffinet et al., 2010a, Goffinet et al., 2010b, Ikeda et al., 2008). But surprisingly, SERINC3/5 orthologs were counteracted by different viral proteins, such as HIV-1 Nef, MLV GlycoGag and EIAV S2. As for the antiviral activity expressed by SERINC3/5 orthologs, the counteraction by HIV-1 Nef can be noticed already at the entry step. Previous studies showed that Nef, GlycoGag and S2 protein act in a similar way to inhibit

human SERINC5, using degradation pathways to downmodulate these proteins from the cell surface and avoid their incorporation in the virions (Rosa et al., 2015, Chande et al., 2016, Usami et al., 2015). SERINC3/5 antagonists have in common their localization at the cell membrane, and HIV-1 Nef and EIAV S2 were shown to have similar interacting motifs (Rosa et al., 2015, Chande et al., 2016). However, it is not clear if these viral proteins target similar motives on SERINC3 and SERINC5 or if a direct interaction is necessary for counteraction. Regarding Nef, it was described that SERINC5 intracellular loop 4 is essential for Nef counteraction. For instance, the 6 amino acid substitutions identified in this region in frog SERINC5 make it no longer sensitive to Nef (Dai et al., 2018). Yet, it remains unknown which SERINC regions are important for GlycoGag and S2 counteraction, and most of all, the highly conserved antiviral activity among the SERINC3/5 orthologs here in study did not allow to delimit which regions are essential for viral inhibition. Besides that, the exact mechanism through which SERINC3/5 inhibits HIV-1 replication is still unresolved (Trautz et al., 2017, Sood et al., 2017).

Regarding the development of a small animal model to study HIV-1 replication, on the first glance, SERINC3/5 orthologs would not impose a barrier in rabbits, rats or mice, since wild-type HIV-1 expresses the antagonist to overcome the restriction. However, it is important to keep in mind that both the antiviral activity and the counteraction is dependent on the amount of SERINC3/5 expressed in the producer cells. High levels of SERINC5 expression lead to an almost completely abolished infectivity and with such high expression levels, the viral proteins are not able to counteract efficiently. As shown before, the presence of Nef is not sufficient to prevent SERINC5 virion incorporation at high expression levels, and the rescue effect is very dim (Trautz et al., 2016). Therefore is important to determine the levels of endogenous SERINC3/5 in T cells and macrophages from rabbits to definitely determine if these proteins play a role in the reduced infectivity seen in HIV virions produced by rabbit macrophages.

Chapter IV: General discussion

General discussion

Small animal models have been used to better understand human diseases, with the ideal animal model replicating the human disease phenotype and its underlying causality. In the late years, rodents have been mainly used as animal models, mostly due to several advantages of these species, such as low costs, several existing inbred strains, short reproducible cycle and easy breeding with high numbers of progeny. Besides that, several immunological reagents have been developed for rodent species, just as the existence of many knock-out and transgenic models (Webb, 2014). However, particularly for HIV-1 infection, rodents do not support viral replication due to the lack of essential host proteins or the existence of restriction factors that cannot be overcome by the virus (Bieniasz and Cullen, 2000). On the other hand, humanized mice are still a good alternative to study virus replication, but have several disadvantages, such as the high costs and ethical concerns (Karpel et al., 2015).

Rabbits have been an alternative to rodent models, with a bigger size that allows greater access to blood, cells and tissues, and an immune system that seems more similar to that of humans (Graur et al., 1996, Neves et al., 2015). As for other species, rabbits show different blocks to HIV-1 replication (Figure 1.4). The first block lies in the lack of a compatible entry receptor complex. The existing technologies of transgenesis allowed the creation of genetically modified animals, and human CD4 and CCR5 transgenic mice were shown to support HIV-1 entry (Seay et al., 2013). Like for rat cells, *in vitro* studies showed that human$CD4^+/CCR5^+$ rabbit cells supported HIV-1 entry (Tervo and Keppler, 2010, Goffinet et al., 2007b). Despite the importance of these results, it remained unclear why the rabbit receptor complex orthologs do not support gp120 binding. Studies involving chimeric CD4 and CCR5 proteins allowed to pinpoint protein domains essential for gp120 binding (Landau et al., 1988, Simon et al., 1993, Moebius et al., 1992, Platt et al., 2015, Dragic, 2001, Picard et al., 1997, Davis et al., 1993), while the mutagenesis of isolated amino acids, although mostly to Ala residues, allowed to identify key residues (Wang et al., 2001, Garcia-Perez et al., 2011, Ashkenazi et al., 1990, Siddiqi et al., 1997, Wieder et al., 1996, Doranz et al., 1997). This work showed that only a few as six amino acids in rabbit CD4 impair virus entry, while for CCR5, besides the need for the human N-terminus of the protein, only a single mutation is required. Therefore, some amino acid substitutions prevalent in rabbit CD4 and CCR5, which were previously identified to be essential for gp120 binding, seem to be irrelevant. Unlike the studies for rodent CD4 and CCR5, where larger fragments were mutated, or several residues had to be substituted to support gp120 binding (Davis et al., 1993, Landau et al., 1988, Simon et al., 1993,

Wieder et al., 1996, Picard et al., 1997), this study showed that small mutations in the rabbit proteins make them functional to interact with HIV gp120.

Remarkably, the regions mutated in rabbit CD4 and CCR5 correspond to spots where evolutionary events were identified in lagomorphs. The serine stretch insertion observed on CD4 from rabbits (*Oryctolagus cuniculus*) is not common to all lagomorphs, being absent in pika (*Ochotona princeps*) and in the eastern cottontail rabbit (*Sylvilagus floridanus*). For CCR5, the point mutation necessary for virus entry is in the region where the Δ32 mutation was reported in humans and in leporids this region is mutated due to gene conversion (Galvani and Novembre, 2005, Carmo et al., 2006). For the first time, the evolutionary history of CD4 and CCR5 in lagomorphs was directly linked to a phenotype that affects viral entry. Besides that, the human/rabbit CD4 and CCR5 chimeras open the possibility to use knock-in technologies that allow to modify a few amino acids in the endogenous rabbit orthologs, replacing the need to overexpress the human receptor complex in rabbits to study HIV-1. Similar strategies have been used to create and improve animal models to study human diseases, from the introduction of a single-nucleotide polymorphism (SNP) in a mouse model to evaluate its pathological effect, to the introduction or larger fragments to achieve gene replacement (Birling et al., 2017).

The link between the evolutionary history of a gene in leporids and its viral function was previously shown. RELIK, the endogenous virus found in rabbits' and hares' genome, was shown to be susceptible to TRIM5α antiviral activity, which in turn is under strong positive selection in these species (Yap and Stoye, 2013, Fletcher et al., 2010, de Matos et al., 2011). Therefore, it is conceivable that viruses were the origin of the selective pressure behind CD4 and CCR5 evolution in leporids. There is no clear connection between the presence of a virus, circulating or endogenous, with the mutations found in different leporid species for CD4 or CCR5, which suggests the participation of different pathogens as the evolutionary force for these genes. For example, the endogenous virus RERV-H was found precisely in the genome of leporids that also possess the CCR5 gene conversion. On the other hand, no correlation with the CD4 serine stretch insertion can be made, since this virus did not seem to affect hares which have the CD4 insertion (Figure 2.5). Besides that, no functional assays for the interaction of CCR5 or CD4 are currently possible with RERV-H Env, as its structural domain remains to be identified (Griffiths et al., 2002). Similarly, there is no clear connection between RELIK's presence and the CD4 or CCR5 substitutions, i.e. while RELIK is found in rabbits and hares, the CD4 insertion was not observed in all rabbits and the CCR5 gene conversion did not occur in hares. Regardless, this virus still had the potential to have shaped the evolution

of these genes, and unlike for the RERV-H, parts of the envelope domain of RELIK were retrieved from the rabbit genome and a complete consensus sequence was previously published (Katzourakis et al., 2007). Unfortunately, the functional studies using this envelope sequence showed no interaction with rabbit (*Oryctolagus cuniculus*) or hare (*Lepus europaeus*) CD4 alone, neither together with the chemokine receptors CXCR4 or CCR5. A deeper look revealed that the sequence previously published for RELIK Env might lack an important cleavage site. This was found in a few envelope fragments within the rabbit genome but it might have been masked in the consensus sequence. Without the proper processing, the envelop might not be fully functional, and therefore no interaction could be observed. Thus, RELIK still might have used CD4 or the chemokine receptors to enter its target cells, yet due to the technical issues we were until now not able to uncover it. To sum up, the human/rabbit chimeras for CD4 and CCR5 allowed to create an interesting link between a rabbit genotype and a phenotype that clearly affects viral replication, but beyond that, this study showed that small mutations in the rabbit CD4 and CCR5 are enough to support virus entry.

The second barrier for HIV-1 replication imposed by the rabbit TRIM5α was already shown to be easily overcome by a small modification on the virus capsid (Schaller et al., 2007, Owens et al., 2003, Tervo and Keppler, 2010). For the next steps, from genome integration to virus assembly and release, rodents showed several blocks while rabbit cells supported subsequent steps (Baumann et al., 2004, Bieniasz and Cullen, 2000, Goffinet et al., 2009, Keppler et al., 2002, Nagai-Fukataki et al., 2011, Neil et al., 2008, Tervo et al., 2008, Tsurutani et al., 2007, Zhang et al., 2008a). Regarding the last steps of the viral life cycle, all species seem to have rather cell-specific limitations. Transgenic mice, expressing an array of human proteins to overcome earlier blocks, do not show high levels of HIV-1 infection and virus spread, mostly due to post-translational defects of the viral components in $CD4^+$ T cells (Seay et al., 2013). While rabbit T cells released as infectious HIV-1 particles as human cells, the virions produced in rabbit macrophages were rather uninfectious (Tervo and Keppler, 2010). This cell-specific block is the third one found in the rabbit cells, however, the cause of this block is still unknown.

Recently, two proteins from the same family, SERINC3 and SERINC5, were identified as potent antiviral proteins (Rosa et al., 2015, Usami et al., 2015). They were reported to affect the infectivity of the virions produced in their presence, without changing virus release or processing of the structural components (Rosa et al., 2015, Usami et al., 2015). The similarity between the restriction imposed by SERINC3/5 and the observed infectivity defect in HIV-1 virions produced in rabbit macrophages, suggested the possibility that rabbit SERINC3/5 might the causative restriction factor. Although the first

studies showed that the human SERINC3/5 inhibition could be counteracted by the viral protein Nef, it was not clear if the rabbit proteins were antivirally active and antagonized by Nef. Quite frequently reported, the antagonism is species-specific, i.e. the virus is able to counteract the antiviral action imposed by a factor from its host, but it is unable to act on other species' restriction factor. Classic examples are HIV-1 Vif, which counteracts human APOBECs but not its orthologs, or the Vpu counteraction of human Tetherin but not of its murine counterpart (Ikeda et al., 2008, Mariani et al., 2003, Goffinet et al., 2010b, McNatt et al., 2009). *In vitro* studies conducted with rabbit, rat and mouse SERINC3/5 showed that these proteins are highly conserved, with similar antiviral activity to the human orthologs. Surprisingly, all orthologs could be antagonized by Nef or other viral components from different retroviruses, such as the GlycoGag from MLV or the S2 protein from the EIAV. At first glance, the results suggested that rabbit SERINC3/5 could not represent the block seen in rabbit macrophages, since the virus used in that study possessed the viral protein Nef (Tervo and Keppler, 2010). However, the antiviral activity as well as the counteraction by viral proteins is dependent on the SERINC3/5 expression levels. With a strong overexpression of these proteins, HIV-1 infectivity is reduced to baseline levels and the Nef antagonism is hardly seen (Trautz et al., 2016). Only a mild overexpression of SERINC3/5 allowed to appreciate the Nef antagonism. Therefore, until the level of SERINC3/5 expression in rabbit macrophages are determined, it is not possible to completely discard these proteins as the main source for the third block seen in the rabbit model. Unfortunately, there are no antibodies available for human, rodent or rabbit SERINCs, being necessary the development of other means to evaluate the level of SERINC3/5 protein expression in rabbit cells. Besides SERINC3/5, other restriction factors were shown to cause reduced infectivity of the viral particles, such as GBP5, MARCH8 or 90k (Hotter et al., 2017, Krapp et al., 2016, Lodermeyer et al., 2018, Lodermeyer et al., 2013, Tada et al., 2015). These proteins affect the virus by compromising in general Env processing and incorporation, representing strong candidates for the origin of the third block seen in rabbit macrophages.

In conclusion, the work presented allowed to point out the exact amino acids that constrain gp120 binding to the rabbit CD4 and CCR5 receptor complex, delimiting the first barrier in the rabbit model. This information can be a useful tool in the development of genetically modified animals, as it opens the door for genome editing instead of inserting human counterparts. The work done on the third block observed in rabbit macrophages, which is so far the only unresolved block in the rabbit cells, gave insights in the antiviral activity of two proteins, SERINC3 and SERINC5. Although it was not

possible to confirm SERINC3/5 as the reason behind this block, the work done helped to understand the effect of these rabbit proteins on HIV-1 life cycle. A lot of work has still to be done to move towards a rabbit model of HIV infection, as only *in vitro* studies were conducted so far. Regardless, the fewer blocks encountered in rabbit cells and the simplicity by which some of this barriers seemed to be overcome is a great advantage over rodents. The resolution of the third block to HIV-1 replication in rabbits will hopefully raise the possibility to create a genetically modified rabbit, via knock-in and knockout technology or transgenesis that would fully support HIV-1 replication.

Future perspectives

The work presented here contributed to the understanding of rabbits as a tool to study HIV-1 replication, but several questions were raised and still not answered. The first part of the work showed that simple mutations on rabbit CD4 and CCR5 would be enough to allow virus fusion into the cells, however it was not possible to make it clear if the presence of viruses in leporids shaped the evolution of these proteins and represented an advantage, or disadvantage, for HIV-1 replication in rabbit cells. In this context, a lot of effort was put into the investigation of a possible interaction between RELIK envelope and the leporid proteins. RELIK, as HIV-1, belongs to the *lentivirus* genus, and therefore can represent a strong candidate for this interaction. Besides that, it is of great interest to find which cells were infected by the circulating form of this virus and which proteins are used as receptors. To do so, the next steps would involve the reconstitution of the furin cleavage site in RELIK envelope and evaluate its processing and further interaction with CD4, CXCR4 and CCR5 from leporids. Facing the possibility that none of these proteins interact with RELIK envelope and allow viral entry into the cells, cDNA library screen with the surface part of RELIK envelope could be performed to identify putative interactors. In order to identify which cells were infected by this lentivirus, more rabbit cell lines can be tested for transduction with lentiviral vectors pseudotyped with RELIK envelope, and more importantly, primary rabbit T cells and macrophages should be included, since these are the main target cell types for other lentiviruses. The final results from this project would help to reconstruct the evolutionary history of host proteins involved in the interaction with RELIK.

For the second part of this work, focused on the third block for HIV-1 replication in rabbit cells, the next steps would be centered in the determination of the expression levels of endogenous SERINC3/5 in rabbit macrophages. The generation of specific antibodies has been shown difficult to achieve, however the expression levels can be monitored by

real-time PCR, and for a more precise protein expression determination, new technologies could be used, such as CRISPR-Cas9-mediated gene tagging. As it was done here for SERINC3/5, other possible restriction factors such as MARCH8, GBP5 or 90k could be investigated. Another strategies could also be employed to uncover this cell-specific block: the comparison of the transcriptome of rabbit T cells and rabbit macrophages to reveal the differential mRNA expression in this cell types; proteomic analysis of the virions produced in rabbit T cells and in rabbit macrophages for the identification of possible incorporated proteins. The hits obtained by these analysis could be functionally tested for their antiviral activity as it was done for SERINC3/5, and finally validated in rabbit primary cells using to knockout or knockdown strategies.

The continuity of these projects would allow to unveil the factor behind the third block to HIV-1 replication in rabbits and hopefully make the link between the effect of a virus in its host and the advantages or disadvantages for its use as an animal model on the study of other viruses.

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Appendices

Appendix I – General methods

DNA and RNA extraction

Genomic DNA was extracted using the EasySpin Genomic DNA Minipreps Tissue Kit (Citomed, Torun, Poland) according to manufacturer's instructions. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, followed by first-strand complementary cDNA synthesis with the SuperScriptTM III Reverse Transcriptase Kit (Invitrogen).

Transformation of bacteria and large scale amplification and purification of plasmid DNA

To amplify plasmid DNA, 30-50 µL competent cells (*E.coli* Stabl II) were thawn for 20 minutes on ice and 0.5-2 µg plasmid DNA added. Transformation was achieved by heat shock at 42 °C for 2 minutes and bacteria were subsequently cooled down on ice for 5 minutes. After 30 minutes pre-incubation in LB-medium (Carl Roth) at 37 ºC, bacteria were plated on LB-agar plates (Carl Roth) containing 100 µg/mL ampicillin and incubated overnight at 37 °C. Single colonies were transferred into TB-medium (Carl Roth) with ampicillin for approximately 8 h pre-culture at 37 ºC. The pre-cultures were then added to 200 mL of TB-medium and grown overnight at 37 ºC with 180 rpm. For low copy plasmids, such as pBJ6, chloramphenicol (25 µg/mL) was added for the last 12 to 16 incubation hours to increase plasmid yield. Plasmid DNA was purified according to the manufacturer's instructions using the Nucleobond Xtra Midi EF 50 Kit from Macherey-Nagel.

Cultivation of eukaryotic cells

All cells used in this study were cultivated at 37°C with a humid atmosphere of 95% and 5% CO_2. Adherent cell lines, such as the human epithelial cell line HEK293T, the HeLa derivate cell line TZM-bl, the cat epithelial cell line CRFK and the rabbit epithelial cell line SIRC, were generally grown as a monolayer in DMEM (Dulbecco's Modified Eagle Medium from Thermo Fisher Scientific) complemented with 10% FCS (Fetal Calf Serum) and 1% penicillin/streptomycin (Merck). Every third day, these cells were detached with 0.25% Trypsin/EDTA (Biochrom) and splitted at a ratio of 1:10. Suspension cells, such as the rabbit B cell line 55D1 and the rabbit T cell line RL-5 were cultivated in RPMI (Thermo Fisher Scientific) complemented with 10% FCS and 1% penicillin/streptomycin (Merck) and kept at a density of $5x10^5$ to $1x10^6$ cells/ml.

Virus and lentiviral vector production and purification

For lentiviral vector and virus production, HEK293T cells were seeded 24 h before transfection in 15 cm^2 dishes with a density of 4-6x10^6 cells/dish. The cells were transfected using 1 mg/ml PEI reagent (Polyethylenimine 25K from Plyscieneces). After 48h to 72h, the cell supernatants were cleared off from cell debris using 0.45 µm PVDF filters and the virus concentrated using a 25 % sucrose cushion by ultracentrifugation at 24000 rpm for 2 hours at 4ºC. After centrifugation, supernatants were discarded and the pellets dissolved in PBS (50-100 µl/tube), aliquoted and stored at -80 °C.

Reverse transcriptase activity measurement

Measurement of the reverse transcriptase activity was previously described (Pizzato et al., 2009). In this SYBR green I-based real time PCR-enhanced reverse transcriptase assay (SG-PERT), the samples provide the reverse transcriptase while the RNA is part of the reaction mix. In the first step, the samples were lysed in the 2× lysis buffer containing 0.25% Triton X-100, 50 mM KCl, 100 mM Tris HCl pH 7.4, 40% glycerol and 0.4 U/µL of the RNase inhibitor RiboLock (Thermo Fisher Scientific). The lysed samples were then mixed with the 2x reaction mix, which contains 10 mM $(NH_4)_2SO_4$, 40 mM KCl, 40 mM Tris HCl pH 8.0, 10 mM $MgCl_2$, 0.2 mg/ml BSA, 1/10,000 SYBR Green I (Invitrogen), 0.4 mM of each dNTP, 1 µM forward primer (5'-TAGTTGTTGGGCTTCGCTTT-3'), 1 µM reverse primer (5'-TTGTCGGCTTTACCTGCTTT-3'), 8 ng/µL MS2 RNA (Roche) and 0.05 U/µL GoTaq Hot Start DNA Polymerase (Promega). The reaction was performed using the CFX96 Real-Time PCR Detection System (BioRad) with following conditions: 42°C for 20 min for reverse transcriptase reactivation, 95°C for 2 min for *Taq* activation, and 40 cycles of 95°C for 5 sec, 60°C for 5 sec, 72°C for 15 sec and 80°C for 7 sec for data acquisition. A melt curve analysis was performed at the end of the run from 70 ºC to 95 ºC with 10 seconds per step, in order to assess if the qPCR produced single and specific products. The results were analyzed using the CFX Manager 3.1 (BioRad).

Fusion Assay

The fusion assay was previously described (Cavrois et al., 2002). In this study, TZM-bl cells or CRFK cells were seeded the day prior infection. 4 hours post-infection, the cells were detached with 0.25% Trypsin/EDTA (Biochrom), washed and stained with 2 µL/mL of CCF2 and 8 µL/mL of solution B from the LiveBLAzer FRET-B/G Loading Kit (Thermo

Fisher Scientific) in CO_2 independent medium (Thermo Fisher Scientific) supplemented with 10% FCS and 2.5 mM probenecid to maximize dye retention in the cells (Cavrois et al., 2002). After overnight staining, the cells were fixed in 4 % PFA for 90 minutes at room temperature.

Appendix II – Supplementary material

Table S1 - Accession numbers of the nucleotide sequences for SERINC1-5 and Tetherin used on the evolutionary analysis.

	SERINC1	SERINC2	SERINC3	SERINC4	SERINC5	Tetherin
Homo sapiens	NM_020755	NM_178865	NM_006811	NM_001258031	NM_001174072	NM_004335
Pan troglodytes	XM_518719	XM_016957919	---	XM_003952645	XM_001136963	NM_009434956
Pan paniscus	XM_003827596	XM_008953469	XM_003825928	XM_003814957	XM_003822274	XM_008970959
Gorilla gorilla	XM_019029109	XM_004025342	XM_004062198	XM_019010483	XM_019013565	XM_019014326
Pongo abelii	NM_001132252	---	NM_001135261	XM_002825393	XM_024247377	NM_001172587
Nomascus leucogenys	XM_003255646	XM_003276346	XM_012511824	XM_003266833	XM_003261521	XM_004093042
Macaca mulatta	NM_001257550	XM_015133730	XR_001447470	XM_001102469	XM_015140335	HM775182
Macaca fascicularis	XM_005551702	XM_005544155	XM_005569083	XM_015452760	XM_015451395	XM_005588381
Papio anubis	XM_009206190	XM_003891478	---	XM_009210045	XM_009208684	XM_003915138
Chlorocebus sabaeus	XM_008007194	XM_007979660	XM_008016540	XM_008016806	XM_007976640	XM_007995721
Rhinopithecus roxellana	XM_010375417	XM_010353706	XM_010384274	---	XM_010386010	XM_010361448
Callithrix jacchus	XM_002746920	XM_017974510	XM_002747577	XM_002753390	XM_008991895	XM_002761884
Saimiri boliviensis	XM_003932370	XM_003937586	M_003936404	XM_003928933	XM_003920804	XM_003942267
Rattus norvegicus	NM_182951	NM_001031656	NM_001008312	XM_006234853	NM_133395	NM_198134
Mus musculus	NM_019760	NM_172702	NM_012032	NM_001025371	NM_172588	NM_198095
Cricetulus griseus	XM_007644745	XM_007639366	XM_003504565	---	XM_007636204	NM_001244115
Microtus ochrogaster	XM_005363640	XM_005353159	XM_005362954	---	XM_005356499	---
Cavia porcellus	XM_003479407	XM_003471227	XM_003467673	---	XM_013143471	XM_003464252;XM_013146524
Octodon degus	XM_004630237	---	XM_004631014	---	XM_023700960	XM_023707620; XM_004647411
Chinchilla lanigera	XM_005389150	XM_013519725	XM_005392472	---	XM_013513014	---
Fukomys damarensis	---	---	XM_010631460	---	XM_010643034	---
Ochotona princeps	XM_004587212	XM_004591646	XM_012927253	---	XM_004586309	XM_012929913
Oryctolagus cuniculus	XM_002714795	XM_008273789	XM_002721072	XM_008269084	XM_017344548	---
Felis catus	XM_003986516	XM_006934521	XM_011280708	---	XM_011284262	NM_001243085
Acinonyx jubatus	XM_015068270	XM_015080618	XM_015065407	---	---	XM_015065666
Panthera tigris	XM_007078674	XM_015542219	XM_007077750	---	---	XM_015540423
Canis lupus familiaris	XM_533483	---	XM_534427	XM_014109485	XM_536311	XM_860510
Odobenus rosmarus divergens	XM_004403619	XM_004406395	XM_004414099	---	XM_004405629	XM_004404714
Ailuropoda melanoleuca	XM_002919685	XM_002923974	XM_002917367	XM_019801637	---	XM_011221978
Ursus maritimus	XM_008691155	---	XM_008698079	XM_008702154	---	XM_008703958

Mustela putorius furo	XM_004740105	XM_004740950	XM_004746378	---	XM_013063699	---
Leptonychotes weddellii	XM_006727937	---	XM_006734485	---	XM_006731895	---
Enhydra lutris kenyoni	XM_022503633	XM_022497069	XM_022494530	---	XM_022504950	---
Capra hircus	XM_005684515	XM_018057400	XM_005688580	XM_005695308	XM_018053667	XM_018051379
Ovis aries	XM_004011156	---	XM_015099893	XM_012130053	XM_012180719	---
Pantholops hodgsonii	---	---	---	XM_005956457	---	---
Bubalus bubalis	XM_006047580	---	XM_006059226	---	XM_006053984	---
Bos taurus	NM_001035427	NM_001035285	NM_001083412	---	XM_002690460	XM_002688577
Bison bison	---	---	XM_010840987	XM_010836162	XM_010834583	---
Sus scrofa	---	NM_001244148	XM_013985391	---	XM_013994946	NM_001161755
Vicugna pacos	---	---	---	XM_015237211	---	---
Camelus dromedarius	XM_010979173	---	XM_010977726	XM_014554314	XM_010975043	---
Equus caballus	XM_001504205	XM_023634619	XM_001917430	XM_023651579	XM_023618093	---
Myotis davidii	XM_015557817	---	XM_015563933	XM_015557552	XM_015571635	XM_006768454
Myotis brandtii	XM_014533756	---	XM_005871236	XM_014548751	XM_014550173	---
Myotis lucifugus	XM_006089223	XM_014468258	XM_006091419	XM_023757594	XM_006081273	---
Eptesicus fuscus	XM_008138745	XM_008147957	XM_008158687	XM_008143234	XM_008161983	---
Rousettus aegyptiacus	XM_016153927	XM_016150386	XM_016152423	XM_016145016	XM_016118865	---
Pteropus alecto	XM_006926133	XM_015598770	XM_006921835	---	XM_006913182	XM_006904279XM_015587122
Pteropus vampyrus	XM_011358765	XM_011357808	XM_011369365	XM_011355019	---	XM_011366834XM_011366835

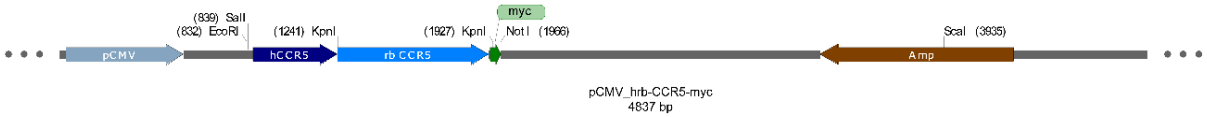


Figure S1 - Representative illustration of the pCMV-myc-C multiple cloning region containing the chimeric hrbCCR5. All the restriction enzymes used for cloning are represented. Grey = MCV promoter; Dark blue= human CCR5 fragment; Light blue= rabbit CCR5fragment; Green=HA tag; Brown= ampicillin resistance gene.

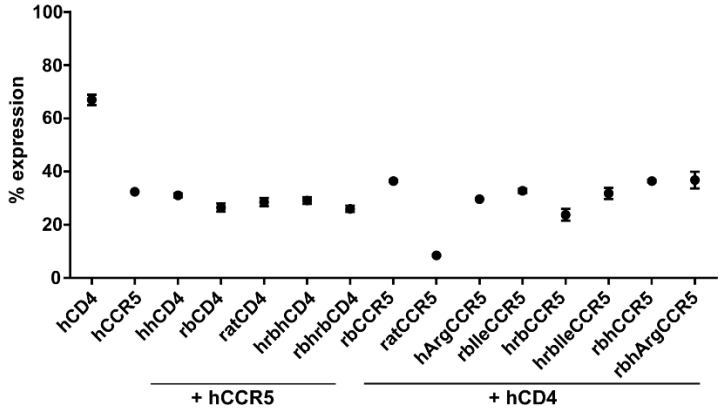


Figure S2 – Similar overexpression of CD4 and CCR5 constructs in CRFK cells. CRFK cells were transfected with different combinations of CD4 and CCR5 and stained 48 hours post-transfection. Graphic representation summarizing the % of CD4 and CCR5 double-expression. Shown are arithmetic means +/- SD of two independent experiments. h=human; rb=rabbit.

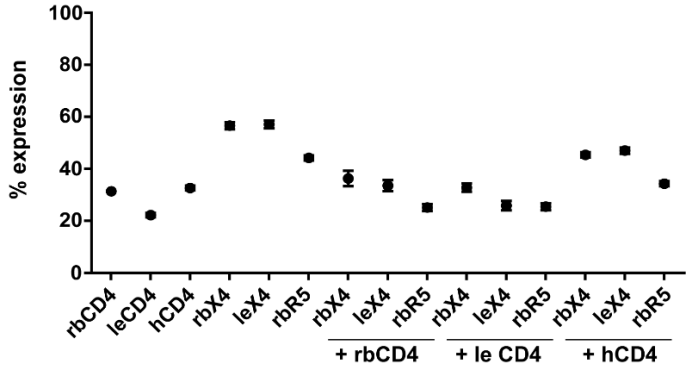


Figure S3 - Similar overexpression of CD4, CCR5 and CXCR4 constructs in CRFK cells. CRFK cells were transfected with different combinations of CD4 and CCR5 or CD4 and CXCR4 and stained 48 hours post-transfection. Graphic representation summarizing the % of CD4 and CCR5 or CD4 and CXCR4 expression. Shown are arithmetic means +/- SD of two independent experiments. h=human; rb=rabbit; le=hare; X4=CXCR4; R5=CCR5.

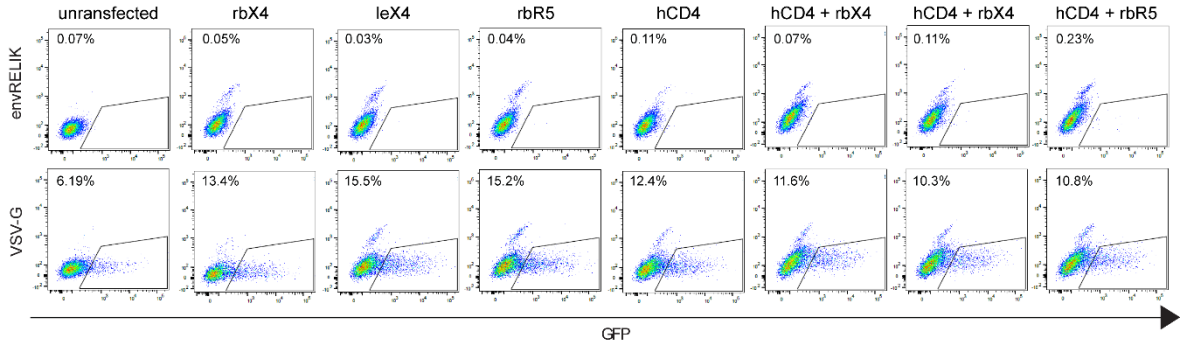
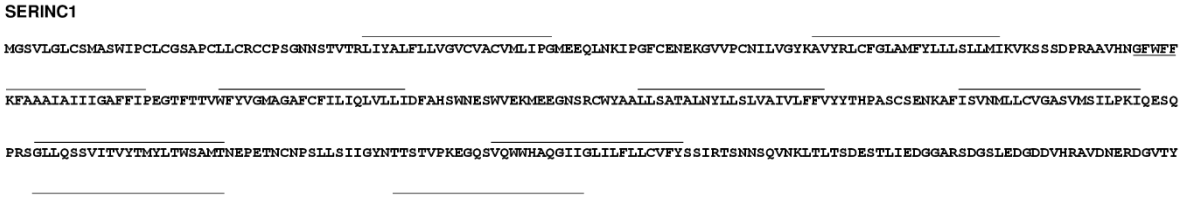


Figure S4 - RELIK might not have interacted with rabbit CD4, CCR5 or CXCR4. Flow cytometry analysis of transduced CRFK cells for GFP expression. The combination of receptors expressed is indicated on top. Lentiviral transduction of RELIK envelope (envRELIK) or VSV-G containing virions was measured by GFP expression.

SERINC1



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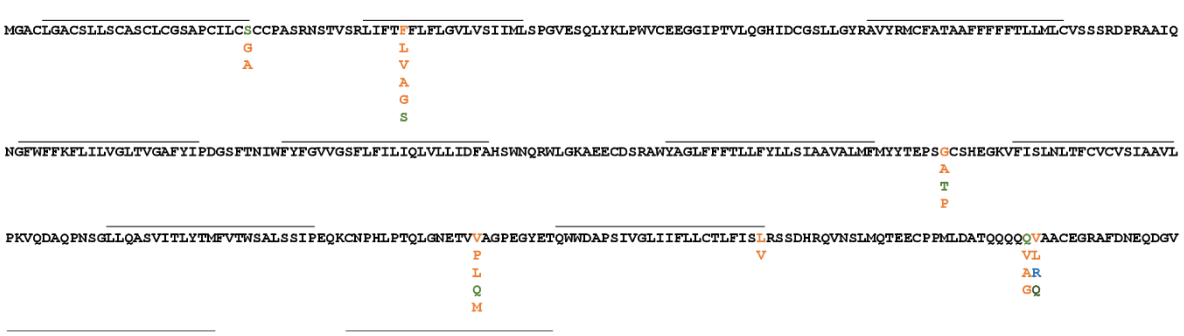

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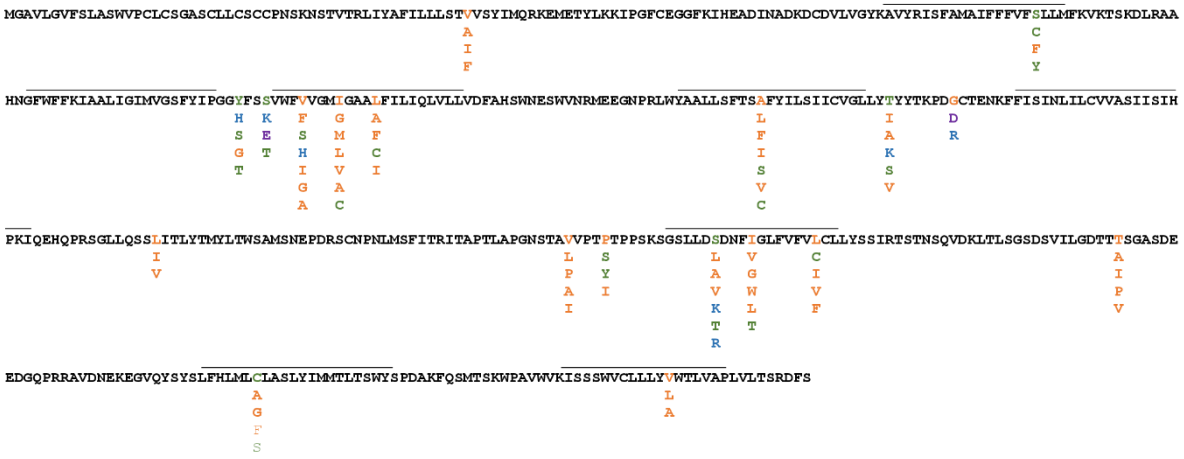

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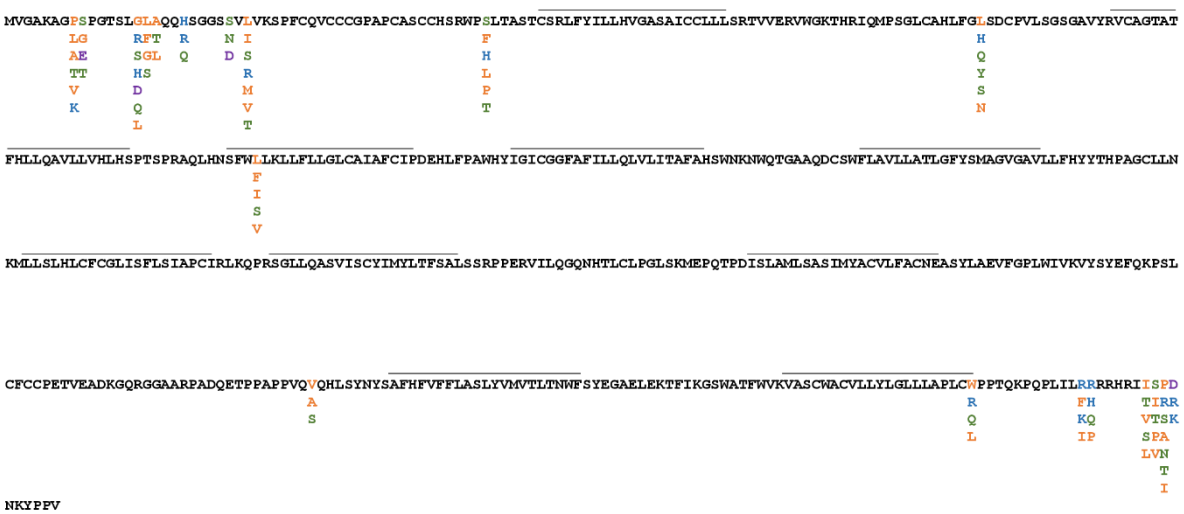

Figure S5 - Human SERINC1-5 sequences with residues under positive selection marked in color and other amino acids for these positions displayed below. Data from PAML and DataMonkey methods. Non-polar amino acids are marked in orange, polar unchanged amino acids are marked in green, polar acidic amino acids are marked in purple and polar basic amino acids are marked in blue. Black lines identify transmembrane domains.

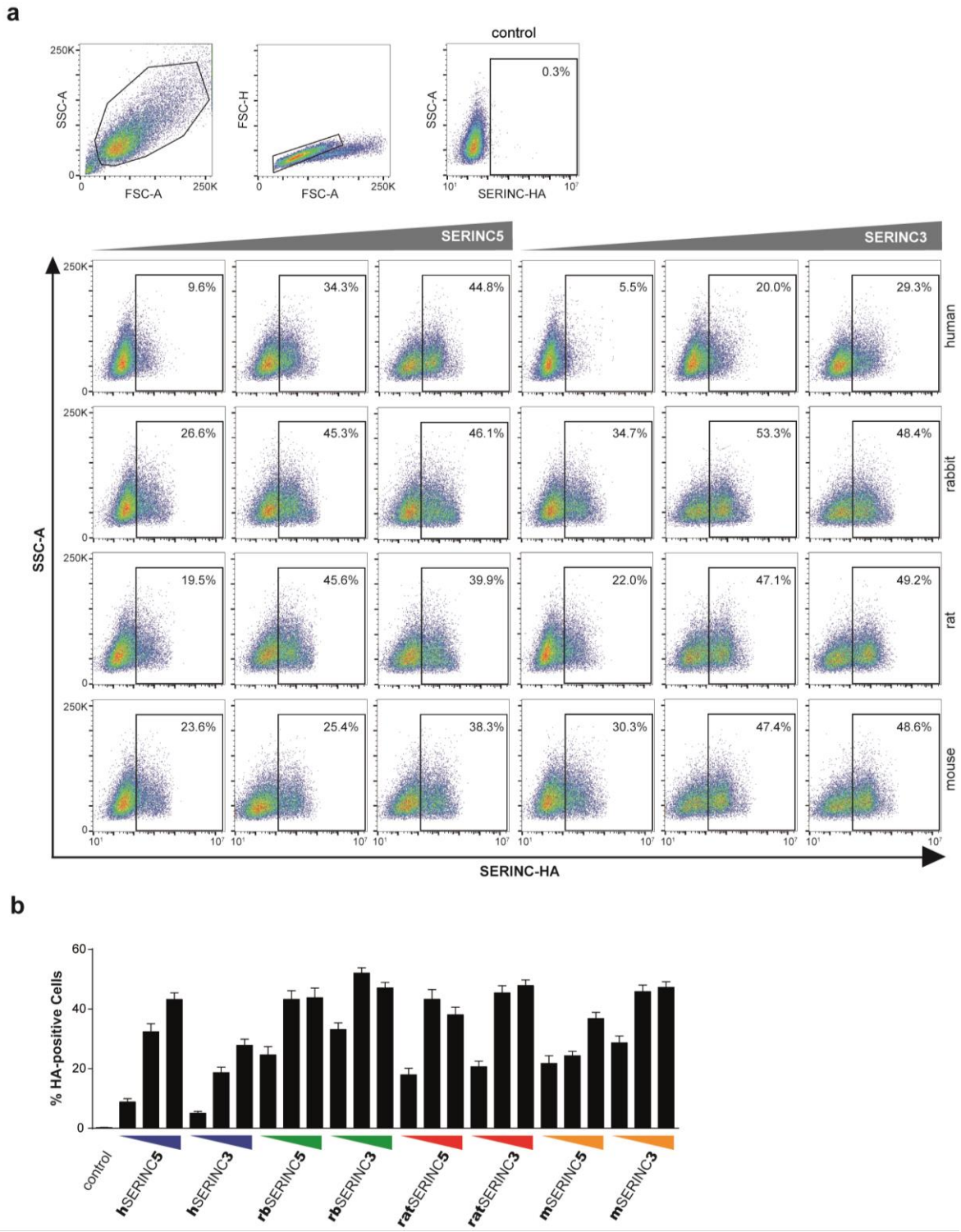

Figure S6 – Transient overexpression of SERINC3/5 can be measured by flow cytometry. **a,** HEK293T cells were transfected with proviral HIV-1_{NL4-3}ΔNef plasmid DNA and increasing amounts of expression plasmids encoding for the different SERINC3/5 orthologs (CMV-promoter). 48 hours post-transfection, the cells were collected, fixed for 90 minutes in 4 % PFA, permeabilized and stained with anti-HA antibody. Shown are representative images for gating strategy (upper row) and HA-staining for increasing amount of SERINC3/5 orthologs expression. **b,** Graphical representation summarizing the raw data presented in **(a)**. Shown are arithmetic means +/- SD of two independent experiments.

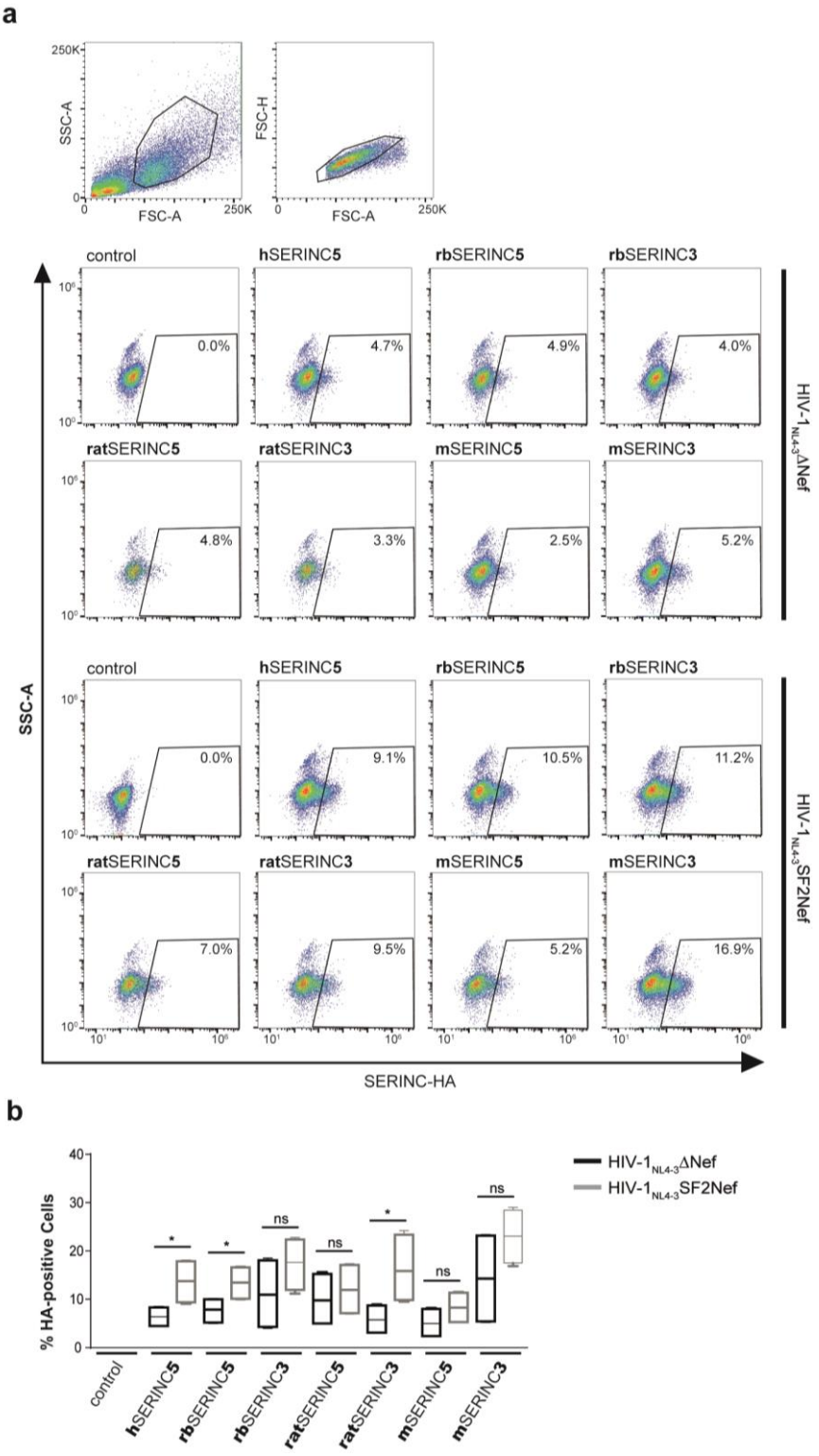

Figure S7 – Transient overexpression of SERINC5 and SERINC3 orthologs in HEK293T cells. **a,** HEK293T cells were transfected with proviral HIV-1_{NL4-3}ΔNef or HIV-1_{NL4-3}SF2 Nef plasmid DNA and pBJ6-based expression plasmids encoding for the different SERINC3/5 orthologs (0.5 µg). 48 hours post-transfection, the cells were harvested, fixed for 90 minutes in 4 % PFA and stained with anti-HA antibody. **b,** Graphic representation summarizing the raw data represented in **(a)** Shown are box plots with median and quartiles of two independent experiments. *<0.05; **<0.01; *<0.001; ns= not significant.

Appendix III – Generated plasmids

All plasmids were generated using the backbones pCMV-HA-C (Clontech), pCMV-myc-C (Clontech) or pBJ6. The coding sequence is indicated below with the restriction sites used for insertion indicated in lowercase letters. Start and stop codons are highlighted in bold and the sequence corresponding to the tag is underlined.

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CAGATTACGCT**TAA**

>rbSERINC5-HA (pCMV)
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>ratSERINC3-HA (pCMV)
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>mSERINC3-HA (pCMV)
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>rbSERINC3-HA (pBJ6)
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>ratSERINC3-HA (pBJ6)
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>ratSERINC5-HA (pBJ6)
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>mSERINC5-HA (pBJ6)
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GAGCTCTCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>rbIleCCR5-myc (pCMV)
gaattcTA**ATG**GATTATTCAATGTCCACTGCCCTCTACGACATCGATTATGGGATGTCAGAGCCCTGCCAAAAAATCGA
CGTGAAGCAGGTGGCAGCCCAGCTCCTGCCCCCACTGTACTCGCTTGTGTTCATTTTCGGCTTCGTGGGCAACCT
GCTGGTCGTCCTCATCCTGATCACCTGCAAGAAGCTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATC
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TGAGCCTGGTCCTGCCGCTGCTGGTCATGGTCGTGTGCTACTCGGGGATCCTGAAAACCCTGCTGCGGTGTCGGA
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CCTCCCGCGGGAGACTCCGGAGCCGGCGAGCTCAGTGTACACCCGATCCACCGGGGAGCAGGAGATCTCTGTTG
GCCTGGCTCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>hrbCCR5-myc (pCMV)
gtcgacACTAGTGGATCCACC**ATG**GATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCT
GCCAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTGTTCATCTTTGGTTTT
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CCCGCTCCTCCCGCGGGAGACTCCGGAGCCGGCGAGCTCAGTGTACACCCGATCCACCGGGGAGCAGGAGATCT
CTGTTGGCCTGGCTCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>hrbIleCCR5-myc (pCMV)
gtcgacACTAGTGGATCCACC**ATG**GATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCT
GCCAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTGTTCATCTTTGGTTTT
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GGAAATACAATGTGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATCCTCCTG
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CAAGCGGGGTCACCTGGGTGGCGGCCATCTTGGTGTCTCTCCCAGACATCATCTTTACCAGATCTCAAAAAGAAGG
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CCCGCTCCTCCCGCGGGAGACTCCGGAGCCGGCGAGCTCAGTGTACACCCGATCCACCGGGGAGCAGGAGATCT
CTGTTGGCCTGGCTCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>rbhCCR5-myc (pCMV)

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TCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>rbhArgCCR5-myc (pCMV)
gaattcTA**ATG**GATTATTCAATGTCCACTGCCCTCTACGACATCGATTATGGGATGTCAGAGCCCTGCCAAAAAATCGA
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GTTCAGAAACTACCTCTTAGTCTTCTTCCAAAAGCACATTGCCAAACGCTTCTGCAAATGCTGTTCTATTTTCCAGCA
AGAGGCTCCCGAGCGAGCAAGCTCAGTTTACACCCGATCCACTGGGGAGCAGGAAATATCTGTGGGCTTGAGCTC
TCGAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>rbCXCR4-myc (pCMV)
gaattcTA**ATG**GAAGGGATCAGTATTTACACTTCAGACAACTACACGGAGGAATTGGGCTCAGGGGACTATGACTCCA
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CTTGACTGGCATAGTGGGCAATGGGCTGGTCATCTTGGTCATGGGTTACCAGAAGAAGCAGAGAAGCATGACAGA
CAAGTACAGGCTGCACCTGTCGGTGGCCGATCTCCTGTTTGTCATCACGCTTCCCTTCTGGGCAGTGGATGCTGTG
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CATCCTGGCCTTCATCAGTCTGGATCGGTACCTGGCCATCGTCCATGCCACTAACAGTCAGAAGCCAAGGAAGCTA
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GAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>leCXCR4-myc (pCMV)
gaattcTA**ATG**GAAGGGATCAGTATTTACACTTCAGACAACTACACAGAGGAATTGGGCTCAGGGGACTATGACTCCA
TGAAGGAGCCCTGCTTCCGGGAAGAAAACGCCCATTTCAACCGCATCTTCCTGCCCACCATCTACTCCATCATCTT
CTTGACTGGCATAGTGGGCAATGGGCTGGTCATCTTGGTCATGGGTTACCAGAAGAAGCTGAGAAGCATGACGGA
CAAGTACAGGCTGCACCTGTCGGTGGCCGATCTCCTGTTTGTCATCACGCTTCCCTTCTGGGCAGTGGATGCTGTG
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TGTCAGAGAGGCAGAGGGGAGGTACATCTGTGACCGCTTCTACCCCAGTGACCTGTGGGTGGTTGTGTTCCAGTT
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GGCTGCCCTACTACATTGGGATCAGCATCGACTCCTTCATCCTCCTGGAAGTCATCAAGCAAGGATGTGAGTTTGA
GAACACTGTGCACAAATGGATTTCTATCACCGAGGCCCTAGCCTTTTTCCACTGTTGTCTCAATCCCATCCTGTATG
CCTTCTTCGGGGCCAAATTTAAAACCTCTGCTCAGCACACACTCACCTCTGTGAGCAGAGGGTCCAGCCTCAAGAT
TCTCTCCAAAGGAAAGCGGGGTGGACATTCTTCTGTTTCAACCGAGTCCGAATCTTCAAGTTTTCACTCCAGCGCTC
GAggtaccGGCGAGCAGAAGCTGATCTCAGAGGAGGACCTG**TAA**

>RELIK-Env-3xFLAG (pCMV)
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GCACTTAAACAATTAAGTGAGGAAGAAAATCCACCAGTTGTAAACCCTTTTGAGGGATTGCCCGAGGACCAACAAG
ATGAATTAGCAGCCCGACAACAAGCACATCTACAACAGGTAAAAGAAGAATTAAAGAGATGGGACCCAGATAAAGG
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GAAGGGATACCTAGCCCTCCGAAAGGAACCCAGCCCACCAGAAATAGAAGAAGAAGAAGAAACCCCGAATCT
GGAAATCAATCCAGGATATGCTCAAGAATGTTCATCCCCTTGGCCCAGCGGCTCAACGGGCTTTACAGGGCTAGAC
TACAAAGACCATGACGGTGATTAT<u>AAAGATCATGACATCGACTACAAGGATGACGATGACAAG</u>**TAG**gcggccgc

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