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"Discovery and characterization of novel Nef-like infectivity factors"

Tutor

Prof. Massimo PIZZATO

CIBIO - University of Trento

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Emilia Cristiana CUCCURULLO

CIBIO - University of Trento

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Declaration

I, Emilia Cristiana Cuccurullo, confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

X Cuccurullo Emilia Cristiana

Abstract

The SERINC family is a highly conserved group of genes which in the human genome comprises 5 members, encoding five homologous multipass transmembrane proteins. SERINC5, and to a lesser extent SERINC3, are powerful inhibitors of Human immunodeficiency virus 1 (HIV-1). SERINC5, expressed in virus-producing cells, is incorporated into the envelope of newly formed retroviral particles and inhibits an early stage of the virus infection process of the target cell, by preventing the delivery of the retroviral core into the target cell cytoplasm.
Nef, an accessory protein of HIV-1, counteracts the antiretroviral activity of SERINC5 by promoting its endocytosis, which results in its removal from the cell surface, preventing its incorporation into retroviral particles.

SERINC5 inhibits not only HIV-1, but also other divergent retroviruses, such as Murine leukemia virus (MLV). During my Ph.D. studies I demonstrated that the S2 auxiliary protein from Equine infectious anemia virus (EIAV) functionally resembles Nef and MLV glycoGag and counteracts SERINC5 with a similar mechanism.

While the inhibitory effect of SERINC5 has been established on retroviruses, nothing is yet known about its effect on other viruses. Here I describe evidence which indicates the possible inhibitory activity of the SERINC gene family against other RNA viruses.

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List of abbreviation

AIDS: acquired immune deficiency syndrome
ALV: Avian leukosis virus
AMV: Alfalfa mosaic virus
AP-1/2: adaptor protein complex-1/2
APOBEC: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
BBB: blood–brain barrier
BFV: Barmah forest virus
BIV: Bovine immunodeficiency virus
BST2: Bone marrow stromal antigen 2
CCR5: C-C chemokine receptor type 5
CD4: cluster of differentiation 4
CD8: cluster of differentiation 8
CNS: central nervous system
CoV: Coronavirus
CRISPR: clustered regularly interspaced short palindromic repeats
CYP-A: Cyclophilin A
DDM: n-dodecyl β-D-maltoside
DENV: Dengue virus
DTT: dithiothreitol
EBOV: Ebola virus
EIAV: Equine infectious anemia virus
ELR1: Equine lentivirus receptor 1
ER: endoplasmic reticulum
ERV: endogenous retrovirus
FBS: fetal bovine serum
FCS: fetal calf serum
FeLV: Feline leukemia virus
FFV: Feline foamy virus
FIV: Feline immunodeficiency virus
FTLV: Feline T-lymphotropic virus
FuSV: Fujinami avian sarcoma virus
GALV: Gibbon ape leukemia virus
GFP: green fluorescent protein
GPI: glycophosphatidylinositol
HA: hemagglutinin
hA3G: human APOBEC3G
HERV-W: Human endogenous retrovirus type W
HIV-1/2: Human immunodeficiency virus 1/2
HTLV-1/2: Human T-lymphotropic virus 1/2
IU: infectious unit

JSRV: Jaagsiekte sheep retrovirus
KDa: KiloDalton
KO: knock out
KoRV: Koala retrovirus
LTR: long terminal repeat
M1: Influenza matrix protein 1
mA3: mouse APOBEC3
MARV: Marburg virus
MDM: monocyte-derived macrophages
MHC-1: major histocompatibility complex-1
MLV: Murine leukemia virus
MMTV: Mouse mammary tumor virus
mU: microunit
NA: neuraminidase
NMR: nuclear magnetic resonance
NPC: nuclear Pore protein Complex
ORF: open reading frame
PBS: phosphate buffered saline
PBS: primer binding site
PC: phosphatidylcholine
PCR: polymerase chain reaction
PERV: Porcine endogenous retrovirus
PFA: paraformaldehyde
PIC: pre-integration complex
PLK1: polo-like kinase 1
PPT: polypurine tract
RFP: red fluorescent protein
RSV: Respiratory syncytial virus
RT: reverse transcriptase
RTC: reverse transcription complex
SAMHD1: SAM domain and HD domain-containing protein 1
SARS: severe acute respiratory syndrome
SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SERINC: serine incorporator
SFV: Simian foamy virus
SG-PERT: one-step SYBR Green I-based product-enhanced reverse transcriptase assay
SH3: SRC homology 3 Domain
SILAC: stable isotope labelling by/with amino acids in cell culture
SIV: Simian immunodeficiency virus
SLX4: structure-specific endonuclease subunit
SM: sphingomyelin
SMRV: Squirrel monkey retrovirus

SNAT: serotonin N-acetyltransferase
SNV: Spleen necrosis virus
SSE: separase
STEM: short-time series expression miner technique
TCEP: tris(2-carbossietil)fosfina
TCR: T-cell receptor
TM: transmembrane
TNF: tumor necrosis factor
UNG-2: uracil DNA glycosylase-2
UTR: untranslated region
VSV-G: Vesicular stomatitis virus

Chapter 1: Introduction

The Retroviridae family of viruses

1970 was the year in which the central dogma of molecular biology was questioned by David Baltimore and Howard Temin; before then, the direction of the chain of events that leads to proteins from DNA, going through an RNA intermediate, had never been doubted. With the discovery of the reverse transcriptase enzyme, that was awarded the Nobel prize for Medicine and Physiology, a new world had opened[1].

Reverse transcriptase is a viral gene encoded by Retroviridae, that gave this family of viruses the name. We can distinguish three genera: Oncovirinae, which comprises Alpharetroviruses (ALV, RSV, AMV, FuSV), Betaretroviruses (MMTV, JSRV, SMRV), Deltaretroviruses (HTLV-1, HTLV-2, BLV), Epsilonretroviruses (WDSV), Gammaretroviruses (MLV, FeLV, GALV, XMRV); Lentiviridae (HIV-1, HIV-2, SIV, BIV, FIV, EIAV) and Spumaviridae represented by Foamy viruses (FFV, BFV, SFV) (Figure 1.1). This classification is based on genetic similarity within *pol* genes, even if other features are taken in consideration, such as the presence or absence of additional viral genes. Another possible classification is based on the type of pathologies caused by the retroviral infection; they could be recapitulated in:

- Oncogenesis (HTLV, BLV)
- Neurological diseases (HTLV, HIV-1)

- Immunodeficiencies (HIV-1, FIV, SIV)

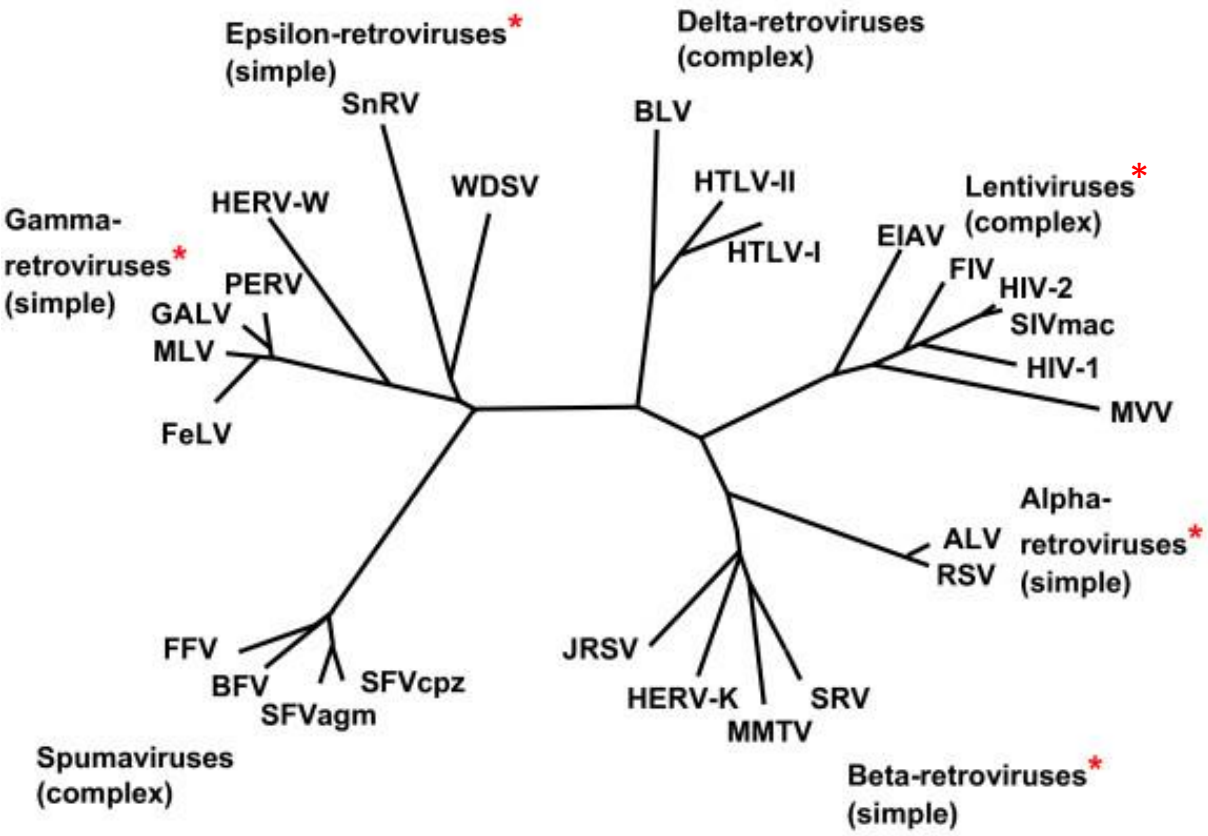


Figure 1.1
Phylogeny of retroviruses.
Genera that include endogenous genomes are marked with an asterisk.
Modified from Retrovirology20063:67DOI: 10.1186/1742-4690-3-67Weiss. 2006

- Other retroviruses-induced diseases (lentivirus-induced equine anemia, lentiviral infections of sheep and goats, wasting induced by avian retroviruses, anemia induced by FeLV-C, osteoporosis induced by ALV and MAV).

Association between cancer and retrovirus infection was hypothesized in the early 1900, when Peyton Rous studied virus coming from chicken fibrosarcoma[2]. Later, sarcomas, hematopoietic tumours and some examples of carcinoma were found to be caused by viruses which belong to this viral family.

In the genome of some retroviruses it is possible to find additional factors called viral oncogenes (*v-onc* genes), originated from recombination with cellular proto-oncogenes. The discovery of these additional viral proteins helped to clarify not only their role during carcinogenesis but also their physiological function. Retroviruses therefore provided a unique tool to elucidate molecular mechanisms involved in tumour onset and progression in particular, and in host cellular biology in general.

In most of the cases, insertion of *v-onc* could hyper-activate cellular pathways controlling crucial events of the cell cycle leading to misregulated proliferation; although extremely rare insertional mutagenesis could also result in inactivation of a tumour suppressor or in expression of aberrant chimeric proteins[3].

Different neurological central nervous system diseases could be associated with retroviruses in human and in animals (loss of neuronal function due to HTLV-1 and HIV-1, amyotrophic lateral sclerosis)[3]. How viruses can enter the

CNS remains still debated, but it seems evident that in the case of HIV-1, the virus crosses the blood-brain barrier (BBB) almost immediately after infection. One possibility is the so-called “Trojan horse” hypothesis which suggests that the virus hides in $CD4^+$ T lymphocytes or monocytes to reach the CNS. Once there, accumulation of cytokines and toxic products caused by activation of an immune response leads to a progressive neurodegeneration[4].

Many retroviruses display tropism for cells involved in the immune response that once infected are eliminated, causing immune suppression. This phenomenon was more or less ignored until the dramatic diffusion of AIDS and the discovery of HIV-1. Immunodeficiencies could be found in cats and non-human primates as a result of FIV and SIV retroviruses and are sometimes referred to as feline and simian AIDS, respectively[3].

As already mentioned, one of the peculiarities amenable to retroviruses, is the integration of their genetic information in the genome of the infected host; if the event occurs in germ line cells, this modification becomes a heritable trait vertically transmitted through generations. It has been calculated that up to 8% of the human DNA is composed by no longer active retro-elements named endogenous retroviruses (ERV). This phenomenon originated millions of years ago and was quite common during evolution with at least 98.000 calculated insertions found to be present in all vertebrate genome investigated[5].

Usually considered as “junk material”, the product of accumulation of a great number of mutations, frameshifts and deletions, and no longer able to produce infectious particles, their reactivation is possible and a variety of

autoimmune diseases are suggested to be associated with this phenomenon (lupus erythematosus, rheumatoid arthritis, multiple sclerosis)[6,7].

In some rare cases they developed a role in helping the host organism with physiological events, such as the placenta formation. This is the case of HERV-W encoding for syncytin[8].

Genetic structure

In each virion there are two RNA molecules each ranging in size from 7 to 13 kb depending on the genera. Thanks to the diploid nature of the genetic information, a high-rate of recombination can be found in this particular viral family.

Different modifications are added to the viral RNA with the insertion of a cap and a poly-A at the 5' and 3' ends, making the transcript similar to the host mRNA. It is possible to recognize two different populations of newly formed RNA: one that remains unspliced, and acts as genomic RNA and as mRNA for translation of Gag and Pol. The other which is processed and spliced to generate the envelope protein. The proper ratio of spliced and unspliced RNA is fundamental to achieve efficient replication.

In viral assembly, the genomic RNA is identified as the one to be included in the progeny virus by virtue of a complex sequence in the leader region, called ψ (from the original work on MLV) or E (for encapsidation, in the Spleen necrosis virus system). In the mature virion, the diploid RNA genomes are

held together by a sequence called the “dimer linkage,” which is in the leader region or in some cases in *gag*.

In all retroviruses, reverse transcription starts with association between the tRNA primer and the complementary “primer-binding site” (PBS) in the viral sequence.

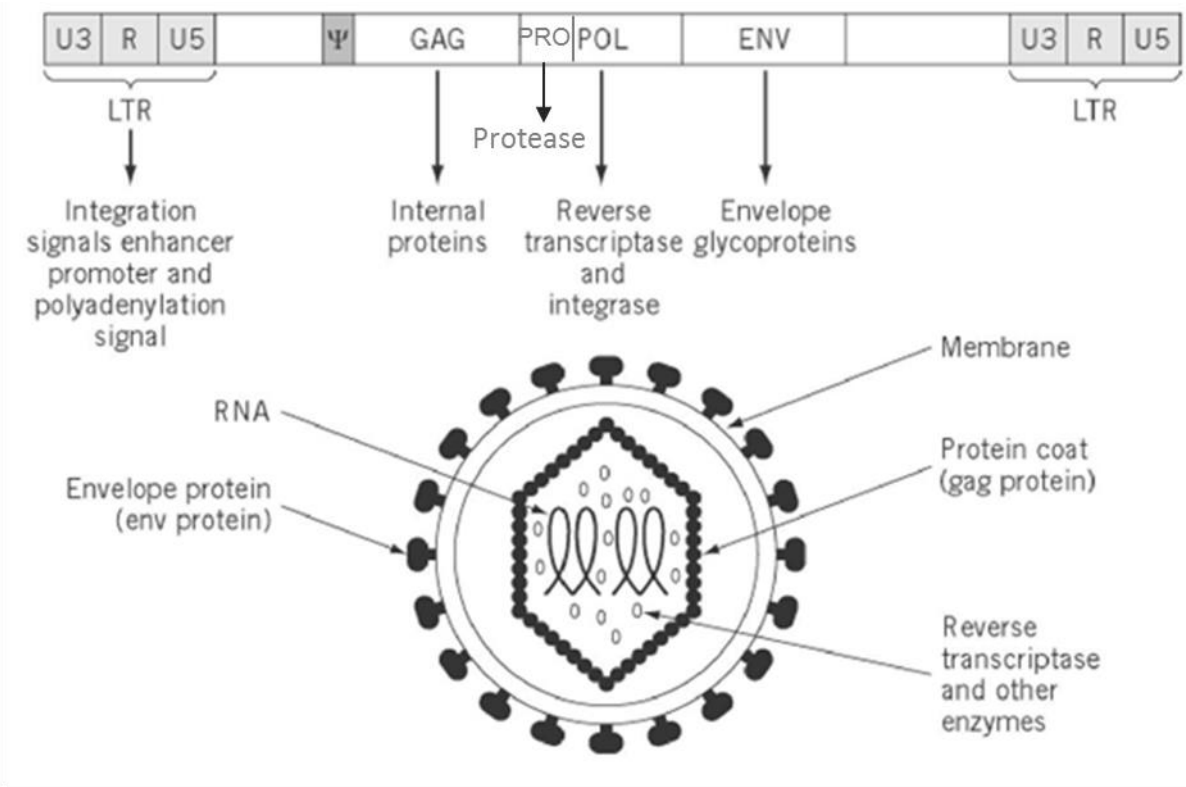


Figure 1.2
Retroviruses genome and viral particle.
Modified from www.tutorhelpdesk.com

Different retroviruses evolved to use different tRNAs as primers ($tRNA^{Lys}$ for HIV-1, $tRNA^{Pro}$ for MLV). As a tRNA is used as primer for synthesis of the "minus" strand, an RNA fragment derived from the polypurine tract (PPT) is used as primer for the "plus" strand.

Interestingly, in retroviral particles it is also possible to detect a small amount of DNA: in HIV representing about 0.1% of the amount of genomic RNA (early products of reverse transcription). In Spumaviruses the situation changes with a large proportion of reverse transcripts present in the virion. Accordingly, the reverse transcriptase sequence of this viral genus is most distant from all other retroviral genera.

From the reverse transcription process, viral DNA is generated and integrated in the cellular genome leading to the formation of the so called "provirus". Differences between virus and provirus genomes are attributable to two identical long terminal repeats (LTR), added during the reverse-transcription and resulting from the duplication of U3 and U5. Each LTR is formed by U3, R and U5. The LTR in 5' drives the production of viral RNA[3] (Figure 1.2).

The Retroviridae life cycle

In the Retroviridae family, genetic information is encoded in two identical single-stranded positive-sense RNA molecules. With its function as RNA-dependent DNA polymerase, reverse transcriptase is able, once in the cytoplasm of the infected cell, to convert viral RNA into DNA which will be integrated into the genome of the host cell and translated together with

cellular genes, allowing the production of proteins necessary for the assembly of viral particles.

The enzyme is encoded by the *pol* gene (driving the expression of a polyprotein that includes even integrase and protease), which along with *gag* (encoding for structural proteins) and *env,* is considered a fundamental component of the minimal genome present in all Retroviridae family members.

Reverse transcription and integration are only two of many steps occurring during the journey that culminates with the formation of a new virus particle. To briefly have an overview, the events occurring during the viral life cycle can be recapitulated in:

- Binding of viral envelope glycoproteins to cellular surface receptors.

This interaction triggers a conformational change allowing the physical contact between the two membranes and allowing the formation of a fusion pore that drives the entry of the virus inside the cell cytosol[3]. Alternatively, for example in the case of ALV or EIAV, the fusogenic property of the envelope is dispatched only under low pH conditions of the endosomal compartment. In this situation the retroviral core is internalized and directly delivered within the cell[9,10]. Micropinocytosis could mediate the entry process for amphotropic MLV and for Ebolavirus[11,12]. Rafts microdomains have been demonstrated to be the preferential site on the cell membrane for viral entry, with accumulation of both the envelope glycoproteins and the host receptors[13].

- Uncoating of the viral capsid and reverse transcription.

Once the viral RNA is released in the cell cytoplasm, dissociation of capsid and reverse transcription occurs concurrently with the formation of the reverse transcription complex (RTCs) and the pre-integration complex (PICs).
The reverse transcription of the viral genome is a key step taking place at an early phase of the viral life cycle for all retroviruses studied.

- Nuclear import and integration.

Nuclear import is governed essentially by the size of the nuclear pores and the interaction between nucleoproteins (NUPs) and specific carrier proteins.
This is the main reason why some retroviruses are able to reach the nucleus only during mitosis when the nuclear membrane is dissolved (for example MLV)[14].
Integration happens thanks to the integrase enzyme that inserts a double-strand break in the host DNA sequence, and promotes the formation of a new phosphodiester bond between viral DNA and the cellular genome. Nicks are filled by the host DNA repair machinery.
The specific site in which the process occurs seems not to be random, but affected by chromatin architecture[15].

- Transcription, export and translation of viral RNA.

All these events take advantage of the already existing cellular machinery (controlling for example trafficking or splicing) which process the integrated viral gene as a part of the host genome.

- Assembly, budding and maturation.

According to the different retroviral origins, assembly take place in the

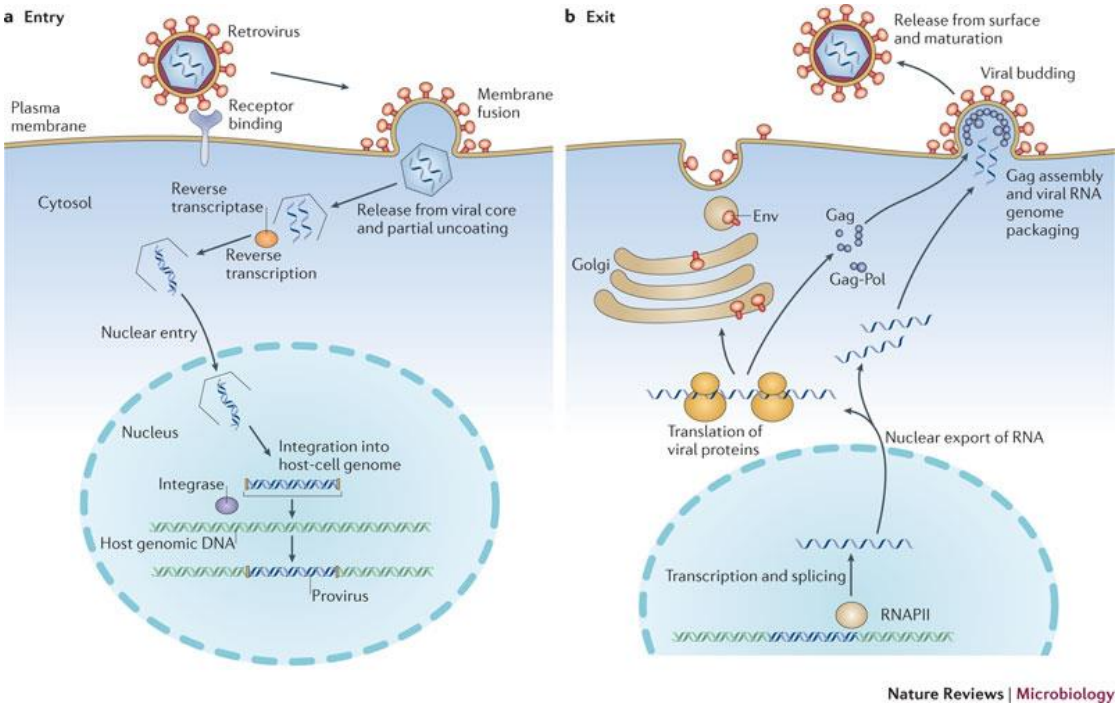


Figure 1.3

Different events in the life cycle of retroviruses are illustrated.

Viral entry into cells involves the following steps: binding to a specific receptor on the cell surface; membrane fusion either at the plasma membrane or from endosomes (not shown); release of the viral core and partial uncoating; reverse transcription; transit through the cytoplasm and nuclear entry; and integration into cellular DNA to give a provirus. b) Viral exit involves the following steps: transcription by RNA polymerase II (RNAPII); splicing and nuclear export of viral RNA; translation of viral proteins, Gag assembly and RNA packaging; budding through the cell membrane; and release from the cell surface and virus maturation. Stoye, J. P., Nature Reviews Microbiology 10, 395-406 (2012)

cell cytoplasm (for example MMTV) or on the cell surface (for example MLV) and it is mediated by the Gag and Gag-Pol polyproteins. During the budding process, lipids from the host cell plasma membrane will be incorporated in the viral envelope becoming an integral part of the virus[16].

After assembly, the immature morphology of the viral particle is redefined by the viral protease that gives the virion the final shape after or during budding [3](Figure 1.3).

Due to the complexity of the events involved, the majority of the retrovirus family members developed a number of regulatory and accessory proteins, along with Gag, Pol and Env, with the function of performing additional activities in the virus life cycle and to modify the local environment within the infected cells to ensure viral persistence, replication, dissemination and transmission. These factors are often needed to overcome both non-specific cellular barriers (cell membrane, actin cortex, nuclear envelope) and specific antiviral host factors.

HIV-1

HIV-1 and HIV-2 originated through cross species transmission of simian retroviruses from monkeys to human, which occurred several times over decades.

HIV-1 includes four groups: M, N, O and P; with M recognized as the cause of the pandemic diffusion of AIDS (almost 90% of the infected patients) (Figure 1.4).

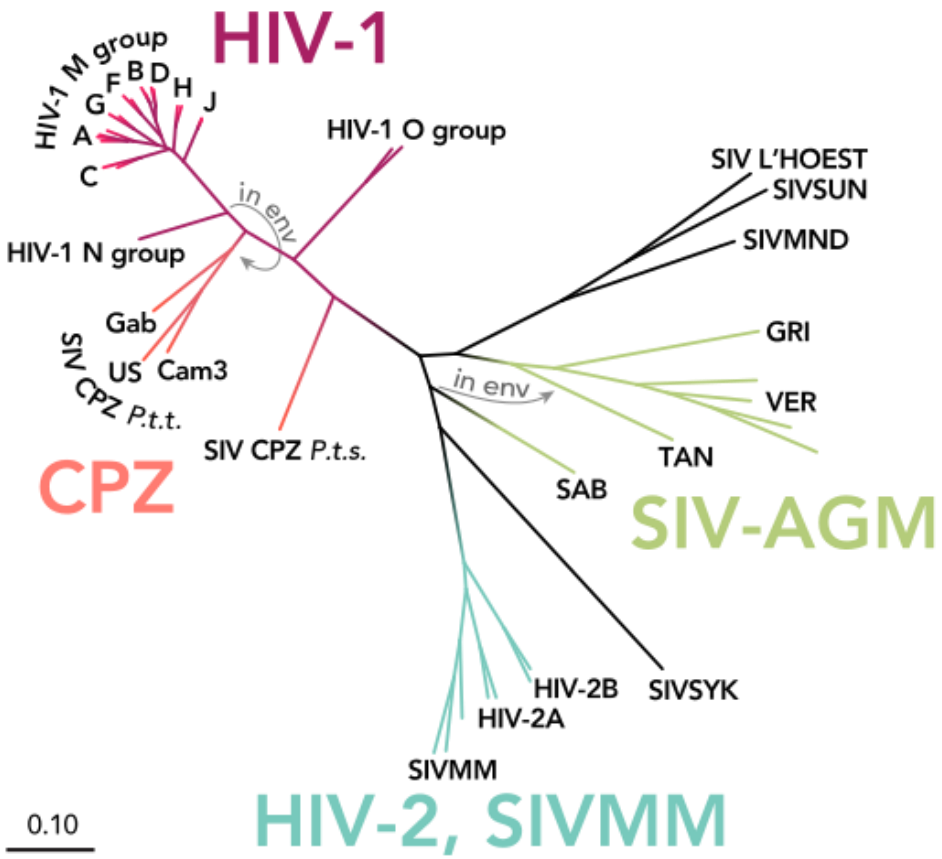


Figure 1.4

Phylogenetic Tree of the SIV and HIV.

Viruses Depicted: Human HIV-1 M (Main) group, including reference strains from subtypes A-J. Group M is responsible for the pandemic. HIV-1 O (Outlier) group, most commonly found in West Africa HIV-1 N (Not-M, Not-O) group, found in a very small number of individuals in West Africa HIV-1 M group reference strains: A_UG.U455, A_KE.Q2317, B_US.JRFL, B_US.WEAU160, C_ET.ETH2220, C_IN.21068, D_ZR.NDK, D_ZR.ELI, F_FI.FIN6393, F_BE.VI850, G_SE.SE6165, G_BE.DRCBL, H_CF.90CF056, H_BE.VI997, J_SE.SE91733, J_SE.SE92809, and CRF01 AE_CF.90CF402 and AE_TH.CM240, which are subtype A in pol. HIV-1 N group: N_CM.YBF30 HIV-1 O group: O_CM.ANT70, O_CM.MVP5180 HIV-2 subtypes A and B: H2A_DE.BEN, H2A_SN.ST, H2B_GH.D205, and H2B_CI.EHO Simian SIVcpz from chimpanzee Pan troglodytes troglodytes (P.t.t.): SIVcpz.GAB, SIVcpz.US, and SIVcpz.Cam3 SIVcpz from chimpanzee Pan troglodytes shweinfuthii (P.t.s.): SIVcpz.ANT SIV African Green Monkey (SIVagm): Tantalus (TAN): SIVagm.TAN1 Vervet (VER): SIVagm.VERTYO, SIVagm.VERAGM3, SIVagm.VER9063, SIVagm.VER155 Grivet (GRI): SIVagm.GRI677 Sabaeus (SAB): SIVagm.SAB1C SIV Sooty Mangaby (SIVsm) (also found in captive macaques): SIVsm.mac251, SIVsm.smm9 SIV L'hoest: SIV.LHOEST SIV Mandrill: SIV.MNDGB1 SIV Sun: SIV.SUN

Based on work by the Theoretical Biology and Biophysics Group, Los Alamos National Laboratory. This image belong to Thomas Splettstoesse

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus belonging to the lentivirus genus. It is probably the best-known virus of the entire family since it is the etiological agent of acquired immune deficiency syndrome (AIDS), which caused 39 million deaths from 1983, when it was first identified by Montagnier's group. The disease is characterized by the progressive decay of the immune system leading to death of the infected patients caused by opportunistic infections or cancer.

Natural cellular targets of the virus are lymphoid cells (CD4+ T cells, macrophages, dendritic cells), expressing the CD4 receptor, along with CCR5 and CXCR4 co-receptors, used for entry. Once HIV-1 integrates in the cellular genome, it undergoes a latency phase during which the immune system is unable to detect the infection. During this phase, that can proceed for a long time, patients are infectious but do not show any symptom of the disease. Only when the CD4+ lymphocyte count reaches a level <200 per ml it can be defined as AIDS[17,18] (Figure 1.5).

During HIV-1 progression, a dramatic decrease of lymphocytes is due to the effect of infection. In addition, the chronic immune response activation leads to an increase in number of activated memory CD4+ T cells, that become available for viral infection[19].

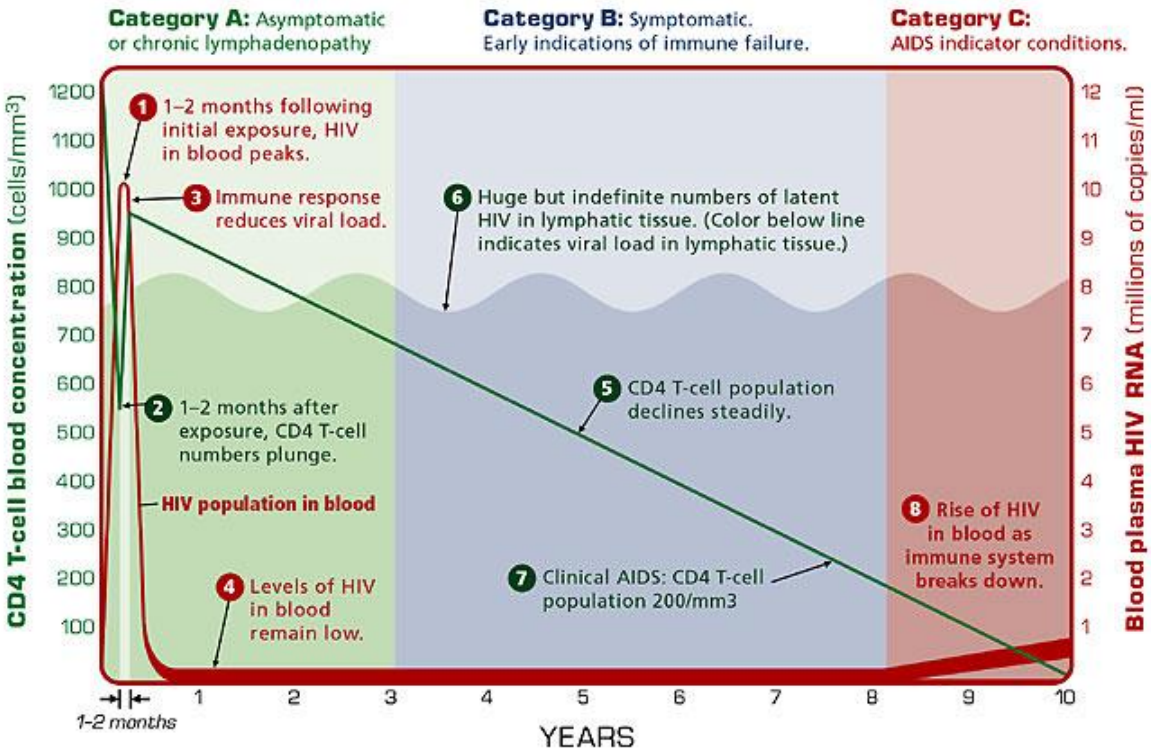


Figure 1.5
Graph of the typical progression of HIV infection and AIDS.
https://www.learner.org/courses/biology/archive/images/1925.html

HIV genome and accessory proteins

As for all retroviruses, the genome is composed by the three canonical open reading frames, *gag, pol and env*, flanked by several accessory proteins with the function of increasing viral spreading and propagation. Apart from Tat and Rev, classified as regulatory proteins, (trans-activator of viral-genome transcription, Tat, and regulator of splicing, Rev) we can find four additional regulatory genes, *vif, vpr, vpu* and *nef*.

The RNA genome is surrounded by capsid and nucleocapsid proteins together with essential viral enzymes (reverse transcriptase, integrase, and protease); capsid is enclosed in a matrix layer, while the virus is encircled by cellular lipids and viral glycoproteins (gp140; gp120) (Table 1).

Vif (virion infectivity factor) is a cytoplasmic protein of 192 aa, found to be required for replication of the virus in non-permissive cells, CD4+ T cells and monocyte-derived macrophages (MDMs), and throughout natural infection[20,21]. For long time the role of Vif remained an unresolved question, until 2002, when Malim's group identified a human gene able to inhibit HIV-1 infection. APOBEC3G, apolipoprotein B messenger RNA–editing enzyme catalytic polypeptide-like 3[22]. This protein is a member of an evolutionarily maintained family, that in humans includes 7 members[23]. APOBEC3G is a single-strand DNA deaminase incorporated in viral particles and acting at the level of target cells. It is able to strongly inhibit replication causing hypermutations within the viral genome, and to produce accumulation of random G to A transitions. Once reverse transcription occurs, cells are able to sense these anomalous products and to drive them to degradation. In

addition, insertion of stop codons and missense mutations leads to the formation of a virus no longer capable of progressing through the infection process[24,25]. Vif interferes with this mechanism by excluding APOBEC3G from the viral particles of the progeny viruses, and mediating its degradation thought direct recruitment of the Cullin 5-based E3 ubiquitin ligase complex[26,27]. Viral factors counteracting the APOBEC family members could be found in retroviruses able to infect different species: MLV-glycoGag counteracts APOBEC3 in mice[28,29]; Bet, an accessory protein of Spumaviruses, counteracts the same protein during mammalian infection in a degradation-independent manner that needs to be elucidated[30].

Vpr (viral protein R) is a 96 aa protein required for viral replication in non-dividing cells. It is conserved among human and simian immunodeficiency viruses[31]. Vpr has been shown to be involved in the cell cycle, mediating arrest during the G2/M transition[32]. It interacts with SLX4 and the structure-specific endonucleases (SSEs). This complex is strictly regulated during the cell cycle; up-regulation derived from PLK1 phosphorylation leads to genomic instability and to an establishment of a steady state during cell cycle progression. Since PLK1 directly cooperates with Vpr, the viral protein allows a physical interaction between PLK1 and the SLX4-SSEs complex; PLK1-mediated aberrant activation of the endonuclease complex culminates with misregulation of cell cycle progression and degradation of cDNA coming from reverse transcription of the viral genome. At this point the model proposed is that the reduction in copy number of RT products makes the host cell unable to sense viral infection, failing to activate the interferon-mediated immune response and facilitating virus dissemination[33].

A role for Vpr in facilitating shuttling of the viral c-DNA between the cytosol and the nucleus has been proposed by several groups. This hypothesis is supported by the observation that along with the amino acid sequence of the viral protein, we can recognize two non-canonical nuclear localization signals and one nuclear export signal. In addition, experimental data suggesting interaction with the RTC/PIC complex were published[34–36].

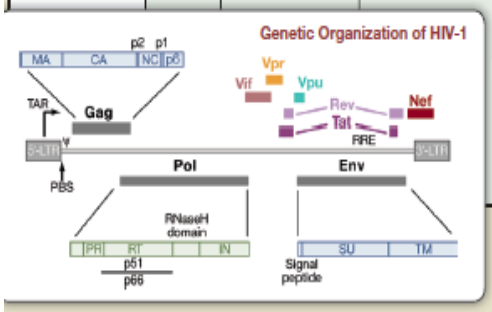
Lastly, Vpr is responsible for the incorporation of UNG2 uracil glycosylase[37,38] in the viral particles, but the role of this interaction needs to be clarified.

Retroviruses from HIV-2/SIVsm, SIVrmc, SIVmnd2 have in their genome another accessory protein named Vpx[39,40]. Interestingly, Vpx is the product of recombination and duplication of the Vpr ancestral gene[41,42]. Vpx is able to neutralize the effect of SAMHD1, a cellular phosphohydrolase involved in the catabolism of dNTP[43–45] which has also an exonuclease activity on ss-RNA[46]. Both events have a clear negative effect on the viral reverse transcription process. As already discussed for Vif, Vpx is able to recruit DCA1-DDB1-CUL4-RBX1 machinery and target SAMHD1 for proteasome degradation[47].

Vpu (viral protein unique) is present in HIV-1 and some SIV isolates. This protein is able to prevent virus accumulation on the cell membrane after budding of the new-born viral particles. Virus released is hindered by BST2, also called tetherin[48]. Tetherin is a type-2 transmembrane protein anchored to the cell lipid bilayer through a GPI linker[49]. The evidence that viruses that don't encode for

Table 1: HIV-1 proteins and genomic organization

Virus Protein	# Copies/ Virion	Interactions with Other Viral Factors	Virus Protein Function	Cellular Partners	Cellular Partner Functions; Results of Interaction with Viral Proteins
Matrix, MA ($p17^{Gag}$)	~5000	Transmembrane glycoprotein (TM)	Plasma membrane targeting of Gag for virion assembly; Env incorporation; post-entry events	Phosphatidylinositol (PI) 4,5-bisphosphate [PI(4,5)P2]	Mediates Gag interaction with plasma membrane
				TIP47	Promotes Env incorporation into virions
Capsid, CA ($p24^{Gag}$)	~5000 (see Briggs et al., 2004)		Virion core structure and assembly	Cyclophilin A	Modulates sensitivity to TRIM5α; suppressed by cyclosporin A
				TRIM5α	Post-entry inhibitor of infection
Nucleocapsid, NC ($p7^{Gag}$)	~5000	RNA genome (gRNA) of virus	Virion packaging of genome RNA; RNA chaperone; virion assembly	HP68/ABCE1	Promotes virion assembly
				APOBEC3G, APOBEC3F	Packaged into virions with RNA; inhibits infection; G-to-A hypermutation
				$tRNA^{Pro}$	Primer for reverse transcription
				7SL RNA, other cellular RNAs	Unknown
$p6^{Gag}$	~5000	Vpr	Promotes virion budding	TSG101	Recruit ESCRT machinery to promote virion budding
				ALIX	
Protease, PR	~250	Gag, Pol	Proteolytic processing of Gag and Gag-Pol polyproteins	PR may cleave many cellular proteins	
Reverse Transcriptase, RT	~250	gRNA, IN	cDNA synthesis; RNaseH domain degrades RNA	$tRNA^{Pro}$	Primer for reverse transcription
Integrase, IN	~250	Viral cDNA, RT	Covalent insertion of virus cDNA into cellular DNA	LEDGF/p75	cDNA integration; targeting to active genes
				INI1	Virion assembly; reverse transcription/integration
				UNG2	DNA repair enzyme; enhances replication fidelity
Surface Glycoprotein, SU ($gp120^{Env}$)	4 to 35 trimers	TM	Binds cell-surface receptors; mediates virus attachment and entry	CD4	CD4 plus CCR5/CXCR4 mediate virion entry; major determinants of viral tropism
				Chemokine receptors (CCR5 and CXCR4)	
				C-type lectin receptors (DC-SIGN, Langerin)	Virion capture; viral transmission from dendritic cells to T cells
Transmembrane Glycoprotein, TM ($gp41^{Env}$)	4 to 35 trimers	SU, MA	Contains fusion peptide; mediates membrane fusion and virus entry	TIP47	Env incorporation into virions
				Clathrin sorting machinery (AP-1, AP-2)	Env downregulation from cell surface
Virion Infectivity Factor, Vif	1 to 150		Suppresses APOBEC3G/ APOBEC3F, host factors that inhibit infection	APOBEC3G, APOBEC3F	Vif recruits Cullin5-ElonginB/C-Rbx E3 ubiquitin ligase to APOBEC3G, APOBEC3F; degradation of APOBEC3G and APOBEC3F
				ElonginC, Cullin5	
Viral Protein R, Vpr	~700	p6	Moderate enhancer of post-entry infectivity; G2/M cell-cycle arrest	DCAF1/VprBP	Bridges Vpr and unknown substrates to Cullin4A-DDB1-Rbx E3 ubiquitin ligase
				nucleoporins (various)	Post-entry nuclear import
				UNG2	DNA repair enzyme; enhances replication fidelity
				CDC25C	G2 cell-cycle arrest
trans-Activator of Transcription, Tat	No	Viral RNA via trans-acting response (TAR) element	Potent activator of viral transcription elongation	Cyclin T1	Cyclin T with CDK9 forms p-TEFb, which promotes viral transcription
				Importin-β/Karyopherin-β1	Nuclear import receptor
Regulator of Expression of Virion Proteins, Rev	No	Intron-containing viral RNAs via Rev response element (RRE)	Induces nuclear export of intron-containing viral RNAs	CRM1/Exportin-1	Nuclear export receptor; transport of Rev and intron-containing viral RNAs to cytoplasm
				Importin-β/Karyopherin-β1	Nuclear import receptor
Viral Protein U, Vpu	No		CD4/MHC downregulation; induces virion release from host cell surface	CD4	Vpu recruits Cullin1-$SCF^{\beta TrCP}$ E3 ubiquitin ligase to CD4 resulting in CD4 degradation
				βTrCP	
				Tetherin/BST-2/CD317	Blocks virion release from host cell surface
Negative Factor, Nef	Yes, cleaved by PR		CD4/MHC downregulation; T-cell activation; moderate enhancer of viral infectivity; blocks apoptosis; pathogenicity determinant	CD4, CD28, MHC-I, MHC-II, TCR-CD3ζ, other cell-surface proteins	Nef connects immunologically important host surface proteins to clathrin-dependent and -independent sorting pathways to regulate trafficking, degradation, and immune recognition
				AP-1, AP-2, AP-3, β-COP, vacuolar ATPase, PACS-SRC family kinase-PI3K complex	
				Several kinases, including PAK2, LCK, ASK1	Roles in signal transduction, host cell activation, blocking apoptosis, stimulating viral replication
				Dynamin-2	Enhances virion infectivity



MA, CA, NC, p6 synthesized as the $p55^{Gag}$ polyprotein (Gag, group-specific antigen), which is cleaved by viral PR after particle assembly and during maturation to yield these four proteins and the p1 and p2 spacer peptides. PR, RT, and IN synthesized as a 160 kDa Gag-Pol polyprotein (Pol, polymerase), which is cleaved by PR to yield these three enzymes including the 51 kDa and 66 kDa subunits of the RT dimer (as well as Gag proteins).

Synthesis of the 160 kDa envelope (Env) glycoprotein precursor is followed by removal of signal peptide in the ER, extensive posttranslational modification and cleavage by a furin-like protease into SU and TM, which are further assembled into Env trimers.

TRIM5α variants in the natural host species for different HIV and SIV strains are inactive against those "cognate" viruses. For example, human TRIM5α blocks infection by SIVagm from African green monkeys but not HIV-1; African green monkey TRIM5α blocks infection by HIV-1 but not SIVagm.

APOBEC3G/F of the natural hosts of HIV and SIV strains are inhibited by the Vifs of those viruses to preserve viral infectivity. For example, human APOBEC3G is inhibited by HIV-1 Vif but not by SIVagm Vif.

Most Nefs of SIVs and HIV-2 downregulate TCR-CD3, which correlates with reduced viral pathogenicity in natural hosts; HIV-1 Nef does not downregulate human TCR-CD3.

742 Cell 133, May 16, 2008 ©2008 Elsevier Inc. DOI 10.1016/j.cell.2008.05.005

See online version for legend and references.

this gene have evolved other unrelated proteins to counteract the same cellular factor (Nef for the majority of SIV isolates[50] and envelope proteins for some primate lentiviruses[51]) suggests how fundamental this activity is.

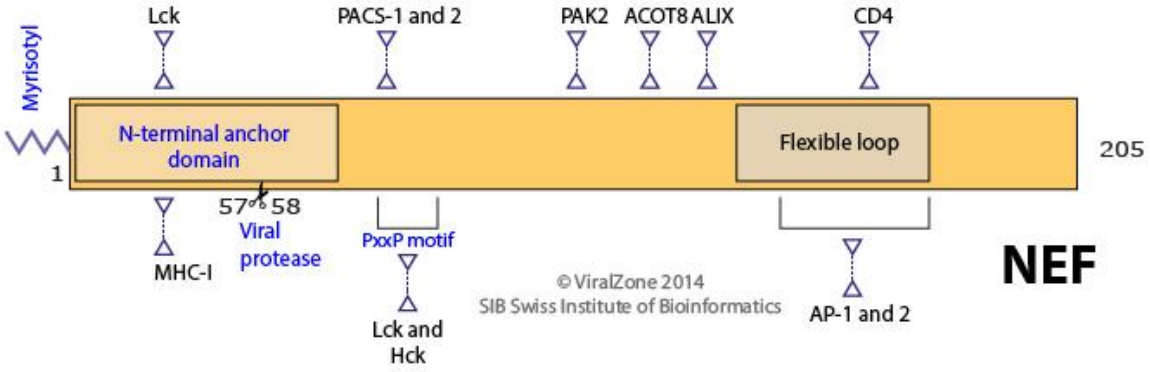
Interestingly, Vpu is also involved in CD4 downregulation from the ER surface by interaction with cullin1-Skp1 ubiquitin ligase complex[52,53].

Both tetherin and CD4 could be targeted for degradation also through recruitment of b-TrCP complex[54] with a molecular mechanism which needs to be elucidated.

The multifunctional activity of Nef

Nef (Negative factor) is a 27 KDa myristoylated protein encoded only by primate lentiviruses; this post–translational modification, along with a repetition of basic amino acids present at the N-terminal of the molecule, enables its interaction with the inner face of the plasma membrane. The complete understanding of all the different mechanisms in which the protein is involved and all the cellular interactors are still controversial (Figure 1.6). Accordingly, the name of the factor is derived from a controversial observation. The first evidence of the expression of a *nef* ORF, partially overlapping with the 3' UTR region, arrived in 1986 when at least two different groups were able to detect antibody targeting this factor during the course of natural infection[55,56]. A study reported that its overexpression causes attenuation in viral transcription and virus replication[57]; only three

a)



b)

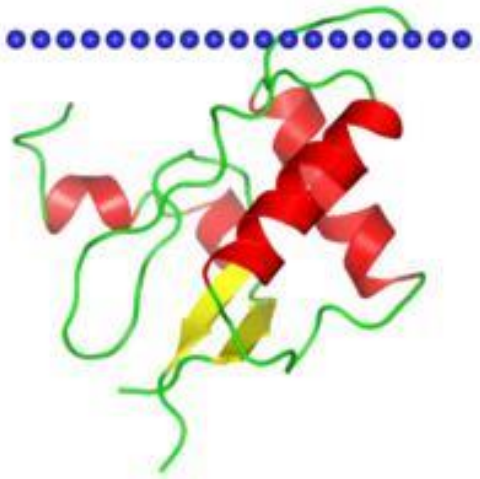


Figure 1.6
Nef protein.
a) Nef motifs; b) Nef secondary structure.
viralzone.expasy.org
opm.phar.umich.edu

years later, in 1989, it became clear that the observed negative effect was due to promotor competition between the LTR in the vector expressing Nef and those present in the HIV-1 genome[58].

Nef, as for other HIV-1 accessory proteins, can be dispensable for virus replication *in vitro* but required for efficient viral propagation *in vivo*[59]. In addition, patients infected with HIV strains depleted of Nef do not progress from the symptomless phase of the disease that remains quiescent basically all through life (long-term non-progressor patients)[60]. On the other hand, expression of the Nef protein alone in transgenic mice, causes depletion of CD4+ T lymphocytes that resembles that observed during the course of AIDS pathogenesis[61].

From these studies stem the idea that we are in the presence of a factor essential for virus replication and for the disease progression. The structure of the protein, solved through crystallography in 1996[62] and confirmed by NMR [63], is well established: a central globular core with two disordered loops at both the N-terminal and C-terminal.

We can mention at least three fundamental and unrelated Nef activities:

- Modulation of T-cell activation
- Modulation of cell surface molecule abundance
- Modulation of retrovirus infectivity

The effect of Nef on primary T cell activation was initially linked to a PxxP motif able to interact in a specific manner with the SH3 domain of two Src tyrosine kinase family members: Hck and Lyn[64]. The outcome of this interaction is the modulation of the TCR signalling pathway to create an

optimal host cell environment to allow virus replication and to avoid suppression of infected cells. In addition, to facilitate trafficking of the assembled virus-cell machinery inside the cell, Nef mediates rearrangement of the cytoskeleton and the actin cortex[65]. Cellular proteins involved in this process are Vav, (Rho GTPase exchange factor) and PAK (serine-threonine kinases) again through a PxxP/SH3 binding. In particular, it seems conceivable that Nef could inactivate cofillin allowing actin turn-over and induction of cell motility[66,67]

Regulation of a number of receptor molecules exposed on the host cell surface is a crucial activity which the virus uses to escape the immune response and obtain an optimal infection level. With its ability to retrieve and recruit the host clathrin adaptor protein complex[68], Nef is able to achieve both these results. Crucial in this case are the ExxxLL[69] and Yxxϕ[70] motifs, a di-acidic EE at the C-terminal loop[71] and an acidic EEEE stretch at the N-terminal[72].

Nef is well known to down regulate and promote CD4 degradation[73] *via* the endosome/lysosome pathway. This activity could be important to avoid super-infection[74]. In addition, Nef prevents the formation of cytoplasmic CD4/gp120 complexes which interfere with the incorporation of the viral envelope glycoproteins in the new virus particles, as suggested by the evidence that the degradation of intracellular CD4 receptors results in an increasing number of envelope proteins available for virogenesis[75,76]. The mentioned process occurs constantly throughout disease progression. Nef also mediates down regulation of MHC-I during the acute infection phase[77]. The molecular basis of this mechanism is not yet well understood. One

possibility is that Nef recruits the AP-1 complex to address MHC-I molecules recycled from trans-Golgi to the endosomal compartment[78]. Alternatively it is possible to hypothesize the involvement of the Src-family of kinases, mediating the discharge of MHC-I molecules from their natural localization to endocytic vesicles[79] (Figure 1.7).

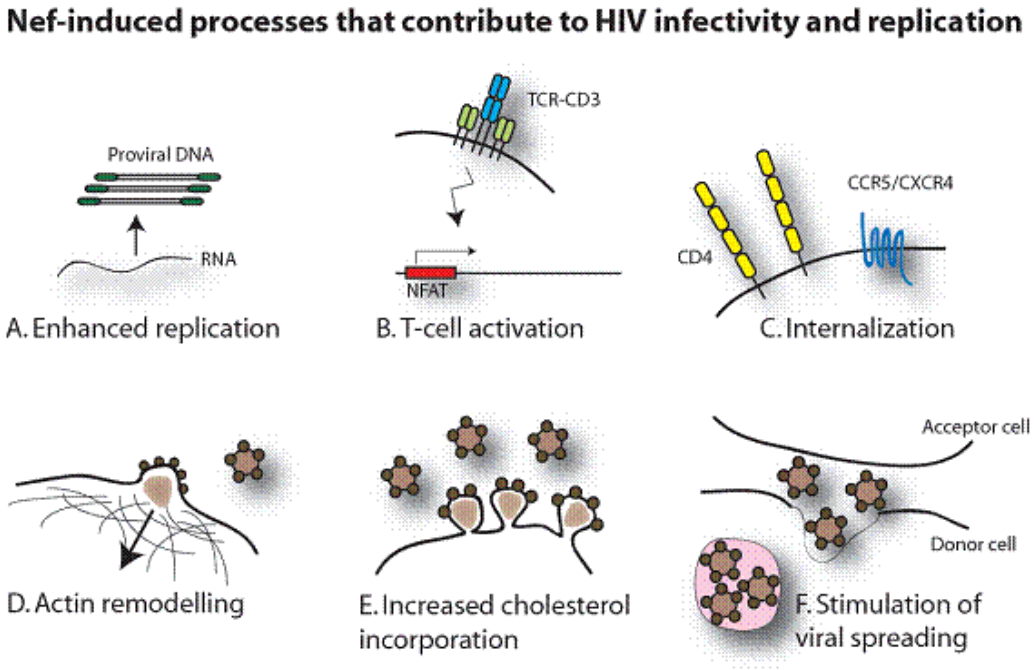


Figure 1.7
Nef induces processs that contribute to HIV-1 infectivity and replication.
www.inef.ugent.be

Nef as infectivity factor

The AP-2 adaptor complex along with clathrin and dynamin[80] is involved in the Nef enhancement of HIV-1 infectivity. The first observation of this phenomenon came from Guatelli's lab in 1994, which observed that optimal infectivity *in vitro* of HIV-1 requires an intact *nef* gene[81]. Then Pizzato lab demonstrated that the Nef requirement is restricted particularly to the lymphoid lineage[82] (primary T cells, macrophages prior to activation). In addition, this effect is visible at the level of producer cells but not at the level of target cells[83]. For several years, the mechanism of the Nef effect on infectivity remained unknown. However another fundamental clue was added later: HIV-1 Nef is not the only retroviral factor with such activity. GlycoGag, an unrelated protein of MLV, is able to recapitulate the Nef phenotype[82] (see below).

With the information available at that time, two different hypothesis could explain the infectivity impairment observed in the Nef-defective virus (Figure 1.8):

- The packaging of a retroviral inhibitor into virions
- The exclusion of a host cofactor crucial for infectivity

Trying to solve the problem, at the same time and with the same purpose, my current research lab headed by Massimo Pizzato and the Gottlinger's group started to look for a host factor able to interact with Nef, whichcould explain

the observed effect on virion infectivity. With two different approaches the two groups found the same putative candidates: SERINC5 and SERINC3.

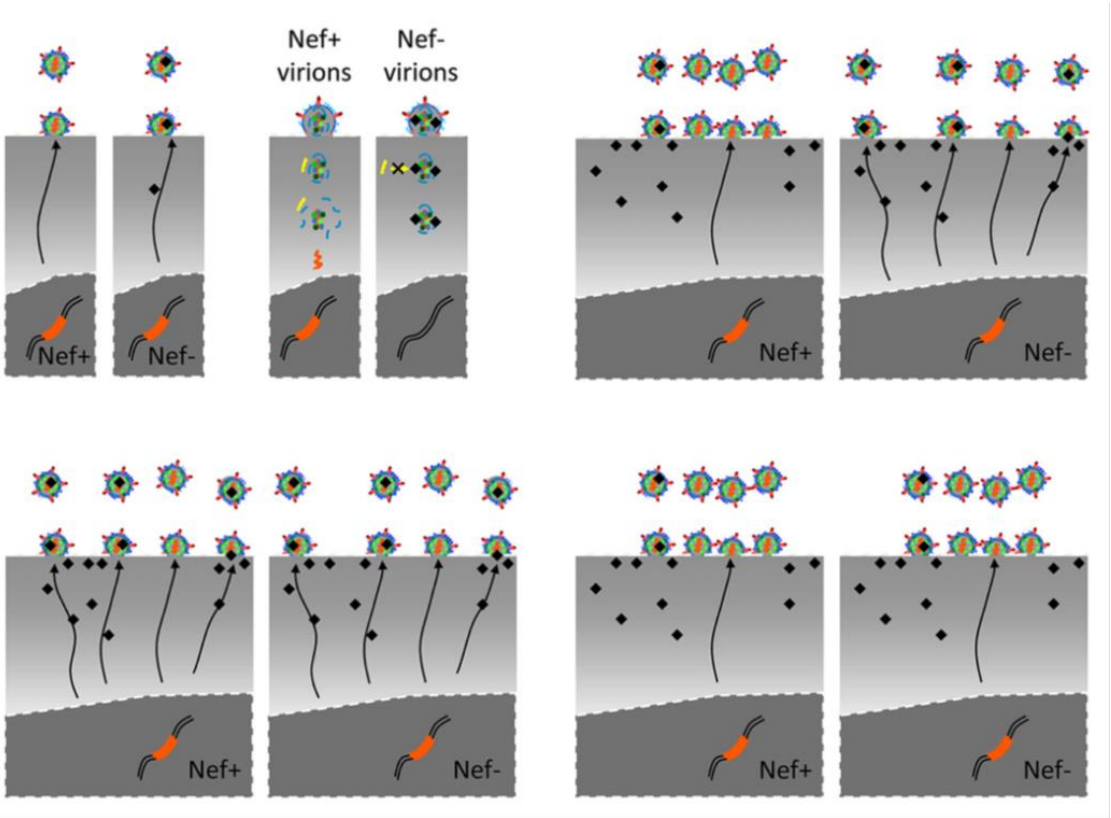


FIGURE 1.8

Possible mechanisms responsible for the differential infectivity of Nef+ and Nef− viruses. (a), Nef− virions may acquire a defect during biogenesis which could be either the packaging of an inhibitor into virions (black shape), or the exclusion of a cofactor (not shown). This defect might then prevent the recruitment of cofactor (yellow shape) or be the target of an inhibitor (not shown) in target cells. (b) The effect of Nef on virus infectivity is evident when virus is produced from Nef-responsive cells, in which Nef regulates such inhibitor or promote virus assembly to subcellular locations where the defect is not acquired (b). (d) In contrast, Nef-non-responsive producer cells generate Nef+ and Nef− viruses with similar infectivities (f). Two possibilities may explain this phenotype: Nef fails to protect the virus from the defect [d (1), Virions have suboptimal infectivity even in the presence of Nef]; alternatively, producer cells lack the cause of the defect or target virus assembly away from inhibitors [e,f(2), Nef- virus already has optimal infectivity].

Basmasciogullari, S., Pizzato, M., Front Microbiol. 2014; 5: 232 (2014).

SERINC5/3 as Nef-sensitive restriction factors

HIV-1 produced in different cell lines displays different Nef dependence forproducing an optimal infection level. The range of the effect on infectivity varies between 2 and 40 fold, with all lymphoid lineages showing highest level of Nef requirement (Jurkat TAg, Ramos, CEM-X, Bl41), consistent with the lymphotropic nature of HIV-1[82].

Starting from this evidence, Rosa et al. performed RNA sequencing on transcriptomes of 8 high Nef-responsive cell lines, and 7 low Nef-responsive cell lines. This allowed to identify SERINC5 as a cellular retrovirus inhibitor counteracted by Nef.

Until this discovery, that assigned SERINC5 the role of antiretroviral restriction factor, the protein was poorly investigated. SERINC5 is a protein with 9 or 10 putative transmembrane insertions (according to the splice isoforms)[84]. This factor is a member of a family which in humans includes 5 genes, and which is highly conserved between different animal species (Figure 1.9). One report proposed a role of serine incorporator in cellular membranes, a function suggested by experiments of overexpression in yeast, *E. coli* or kidney fibroblasts from African green monkey[84].

SERINC5 expression was found to perfectly correlate with Nef requirement: high Nef requirement is reflected by high expression of the *SERINC5* gene indicating anti-HIV-1 activity counteracted by Nef.

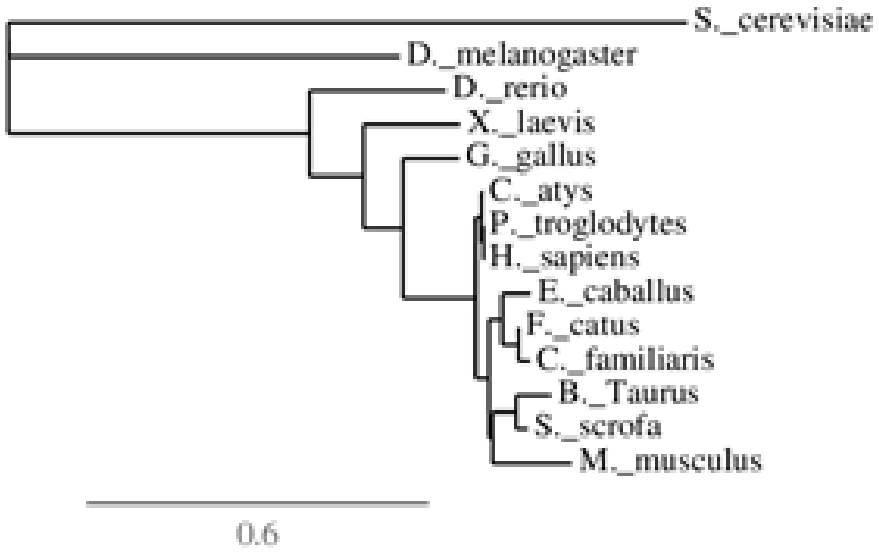


Figure 1.9
Phylogenetic tree showing SERINC5 divergence between different selected animal species.

Indeed, SERINC5 and to a lesser extent SERINC3 were found to be active against HIV-1.

Recruitment of the the AP-2 adaptor complex is crucial for the ability of Nef to counteract SERINC5. Immunofluorescence experiments showed a re-localization of the cellular protein from the plasma membrane to the endosomal compartment in the presence of Nef; SERINC5 incorporation in the newly formed viral particles was found to prevent a productive fusion process[85]. Nef counteracts SERINC5 by excluding it from the virions (Figure 1.10).

Approaching the question from a different point of view, Gottlinger's group performed a proteomic analysis of the virion composition in the presence and absence of Nef. This experimental procedure confirmed that SERINC3 and SERINC5 are incorporated in viral particles only in Nef-depleted virus, consistent with what was observed by Pizzato's group[86].

Later, an independent study analysed cellular plasma membrane proteins down regulated during HIV-1 infection using SILAC and the Short Time Series Expression Miner technique (STEM).

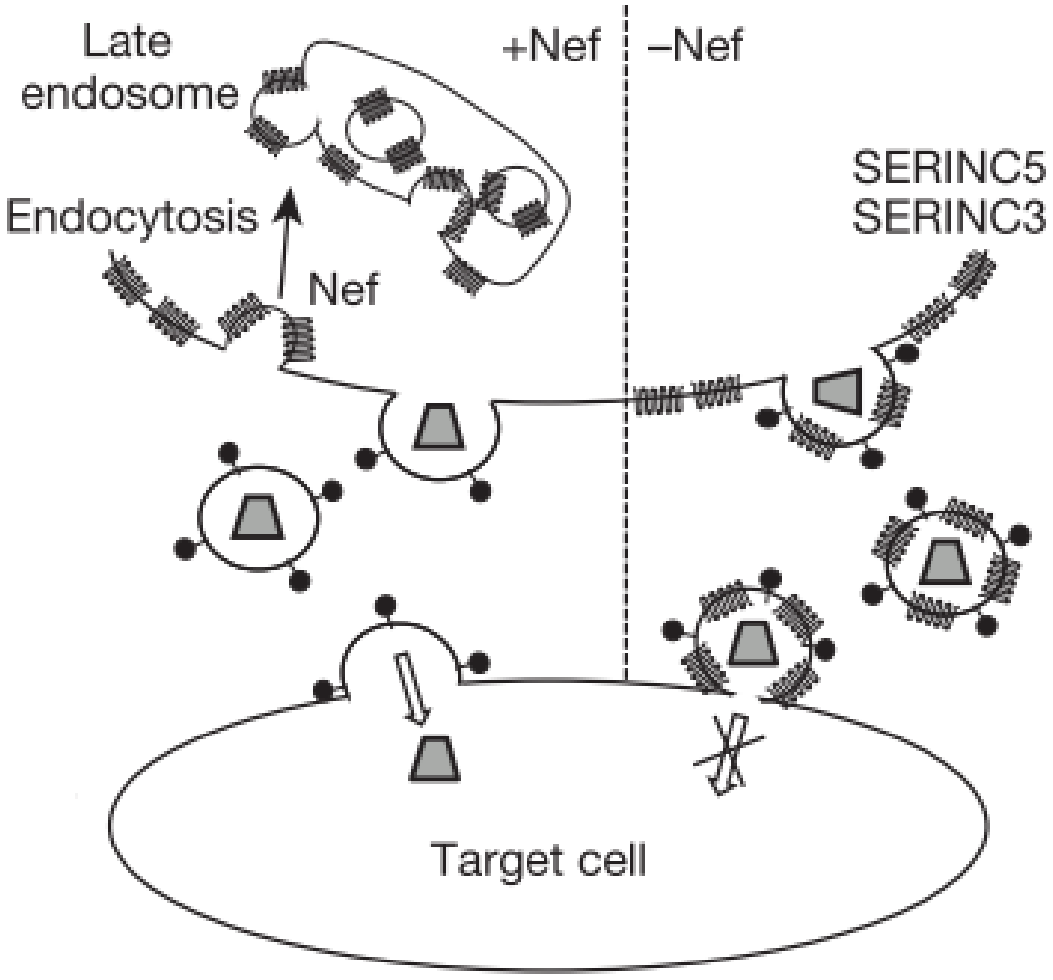


Figure 1.10
Schematic showing the activity of SERINC5 on HIV-1.
Rosa, A. et al. Nature 526, 212–217 (2015).

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Down regulation of SERINC5 and SERINC3 by Nef was observed, confirming the results obtained by the Pizzato and Gottlinger labs.[87]

To the classical role of Nef as a modulator of proteins exposed on the viral particle surface, we need to add the information that even the lipid composition is affected by the viral protein, as reported by Fackler's group. In particular, an increase of sphingomyelin and a decrease of phosphatidylcholine was detected in the viral envelope in the presence of Nef[88]. In contrast to previous studies[89], cholesterol abundance was found unaffected. The impact of the effect of Nef on lipid composition of the viral envelope remains controversial and needs to be further investigated.

To complete this portrait, the Nef effect on infectivity was found to be isolate-dependent. Nef-responsiveness was found to depend on residues in the V1/V2 and V3 regions of gp120[86]. Finally, HIV-1 pseudotyped with envelope proteins which lead to a pH-dependent fusion process (see for example VSV-G), are able to achieve optimal infection level even in the absence of Nef[90,91].

MLV glycoGag

Gammaretroviruses are able to infect different vertebrates and can be found as exogenous or endogenous viruses.

Their genome is classified as “simple”, generally lacking ORFs additional to *gag, pol* and *env*. Since the early 1970s it is evident that *gag* contains a CUG start codon upstream to the canonical AUG, which allows the production of a longer Gag protein, named GlycoGag[92].

In the case of MLV, the protein is incremented by 88 amino acids at the N-terminal and acquires a type-II transmembrane topology. Once inserted in the plasma membrane it is subjected to proteolytic cleavage that releases the C-terminal half, while the rest of the molecule remains associated to the cell membrane and incorporated into the virion[93]. GlycoGag could therefore be considered as an accessory protein of MLV.

GlycoGag was found to exert a role in counteracting the anti-viral activity of mouse APOBEC3 (mA3). Stavrou and colleagues provided evidences that glycoGag prevents access of mA3to the RTC both in virions and infected cells. Furthermore, glycoGag was found to reduce the ability of an yet unknown cytosolic sensor of viral infection to respond to MLV infection; finally, glycoGag was found to enhance the stability of viral cores/capsids[28].

The effect of hA3G and mA3 on MLV was compared. hA3G was found to induce a significantly more severe inhibition compared to mA3. In contrast, mA3 and hA3G were found to inhibit HIV-1^{Vif-} with a similar magnitude. MLV therefore appears to be partially resistant to mA3. It is possible to reconstruct a scenario in which MLV exhibits partial resistance to mA3 by partially excluding it from nascent virions; however, incorporation of mA3 in MLV particles was found to occur as efficiently as for hA3G[94]. Therefore, the mechanism by which glycoGag confers MLV resistance resistance to mA3 remains enigmatic.

In 2010 Pizzato reported GlycoGag as a "Nef-like" infectivity factor able to precisely resemble the Nef protein by rescuing infectivity of HIV-1 Nef-depleted virus. Similarly, both proteins are dispensable for replication *in vitro* but not *in vivo*[95], they are sensitive to different envelope pseudotyping and their action is restricted mainly to lymphoid lineage producer cells. In addition, they share the same intracellular localization[82] and the ability to interact with AP-2[96]. Finally, and most importantly, they are able to similarly re-localize and prevent inclusion of SERINC5/3 in viral particles[85,86].

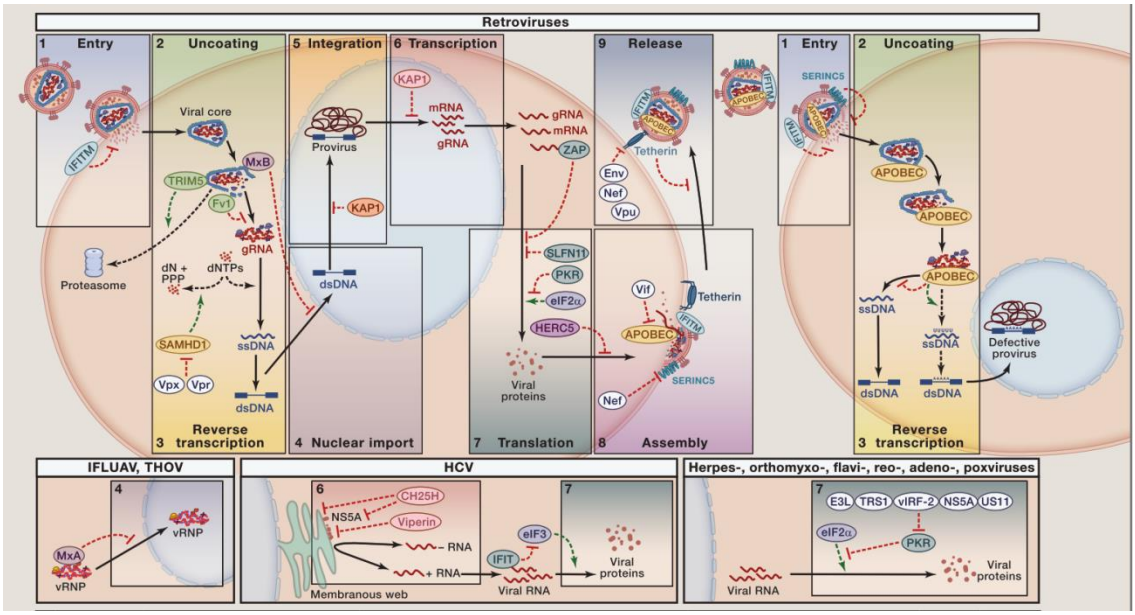
We can conclude that the effect of Nef is a fundamental property of the virus, genetically separated from other functions. Viruses belonging to different retroviral genera (lentiviruses, y-retroviruses) developed, probably through a mechanism of convergent evolution, two unrelated proteins to solve the same problem of neutralizing the inhibitory effect of SERINC proteins.

This is the result of the host and parasite to a co-evolution over time. On one side restriction factors were developed by host cells to protect themselves from invasion, and on the other accessory proteins were evolved by viruses to avoid the block, and create a suitable cellular environment to achieve a productive infectious process.

APOBEC3 and tetherin evolved the ability to inhibit not only retroviruses from different families, but also different virus genera: APOBEC works even against Hepadnaviruses and tetherin shows a very broad spectrum since it is able to restrict Retro-, Flavi-, Filo-, Rhabdo-, Herpes-, Corona-, Paramyxo-, Arena-, Toga-, and Hepadnaviruses[97] (Figure 1.11).

After the identification of SERINC5/3 as a HIV-1 restriction factors a great number of open questions remains and will certainly be investigated by research groups around the world in the future.

With my Ph.D. thesis I will try to understand how powerful the SERINC5 activity is and how many viruses were forced to independently evolve “Nef-like” infectivity factors to win the battle.



	Restriction factor	Virus(es) targeted	Mechanism(s) of restriction	Viral antagonist(s) or evasion mechanism(s)	IFN-inducible	Positive selection
1	IFITM family	Retro-, orthomyxo-, flavi-, filo-, corona-, rhabdo-, bunya-, reoviruses	Inhibits membrane fusion, modification of lipid components, or membrane fluidity	None known	Some	Some
	SERINC3, 5	Retroviruses (HIV, SIV, MLV, EIAV)	Reduces membrane fusion	Nef (HIV, SIV), Glyco-Gag (MLV), S2 (EIAV)	N	N
	CH25H	Flavi-, retro-, filo-, bunya-, rhabdo-, herpesviruses	Inhibits membrane fusion by generating 25-hydroxycholesterol	None known	Y	Unknown
2	TRIM5α, TRIM-Cyp	Retroviruses (HIV, SIV, MLV, EIAV)	Accelerates uncoating, thereby inhibiting reverse transcription	Capsid mutation	Y	Y
	Fv1	MLV	Targets the viral capsid protein and interferes with uncoating	Capsid mutation	N	Y
3	APOBEC3 family	Hepadna-, retroviruses	Induces hypermutation by deamination, inhibits reverse transcription of HIV by binding to RNA and suppressing tRNA3Lys priming	Vif (lentiviruses), Bet (spumaviruses), Gag (gammaretroviruses)	Some	Some
	SAMHD1	Retroviruses	Hydrolyzes cellular dTNP and degrades viral RNA	Vpx (HIV-2, some SIV), Vpr (some SIV)	Y	Y
4	MxA	IFLUAV, THOV	Inhibits vRNP nuclear import	Nucleoprotein mutations (pandemic IFLUAV)	Y	Y
	MxB	HIV, SIV	Prevents integration of proviral DNA by inhibiting uncoating, nuclear uptake, and/or integrity/stability of the PIC	Capsid mutation	Y	Y
5	KAP1/TRIM28	HIV-1	Induces deacetylation of HIV integrase	None known	N	N
6	KAP1/TRIM28	Herpes-, retroviruses	Silences transcription and induces latency	vPK (KSHV)	N	N
	Viperin/RSAD2	HCV, DENV	Inhibits formation of the HCV replicon complex by sequestration of hVAP-33 and interaction with NS5A, interacts with NS3 (DENV)	None known	Y	Y
	CH25H	HCV	Inhibits membranous web formation and NS5A dimerization	None known	Y	Unknown
	IFI16	HPV, HCMV, HSV1	Accumulates on the viral genome and prevents association of transcriptional activators, induces heterochromatin formation	pUL97, pUL83 (HCMV)	Y	Y
	MxA	Bunyaviruses (LACV, RVFV, BUNV)	Sequesters newly synthesized viral N protein into perinuclear complexes	None known	Y	Y
	RNaseL (+OAS1)	Picorna-, flavi-, toga-, corona-, reo-, pox-, orthomyxo-, paramyxo-, herpes-, retro-, rhabdo-, hepadna-, polyomaviruses	Degrades viral (m)RNA, RNaseL is activated by 2′-5′-linked oligoadenylates produced by OAS1	NS1 (IFLUAV), E3L, D9, D10 (VACV), σ3 (ReoV), Tat (HIV), ns2 (murine hepatitis virus), VP3 (RotaV), L* (Theiler's virus), hairpin RNA structure (poliovirus), genome adaptation (HCV)	Y	Y
	SAMHD1	Arteri-, pox-, herpesviruses	Hydrolyzes cellular dNTP and degrades viral RNA	None known	Y	Y
	APOBEC3 family	Herpes-, papillomaviruses	Induces hypermutation by deamination	None known	Some	Some
7	PKR	Herpes-, orthomyxo-, retro-, flavi-, reo-, adeno-, poxviruses	Inhibits mRNA translation by eIF2α phosphorylation	NS1 (IFLUAV), E2, NS5A (HCV), TRS1, IRS1 (HCMV), K3L, E3L (VACV), US11 (HSV1), vIRF-2, LANA2 (KSHV), NSs (RVFV), σ3, σ4 (ReoV), SM, EBER-1 (EBV), Tat (HIV), VAI RNAs (AV), C8L, K3L (Swinepoxv), Nsp3 (RotaV), γ(1)34.5 (HSV-1)	Y	Y
	SLFN11	HIV, other retroviruses	Inhibits viral protein synthesis by altering tRNA function	None known	Y	Y
	ZAP	Retro-, filo-, hepadna-, togaviruses	Recruits RNA exosome complex to degrade viral RNA	None known	Y	Y
	IFIT family	Flavi-, bunya-, rhabdo-, orthomyxo-, picorna-, coronaviruses	Inhibits cap- and IRES-dependent translation by binding to eIF3 (HCV), binding and degradation of PPP-RNA (RVFV, VSV, IFLUAV) and RNA lacking 2′-O methylation (WNV, JEV)	2′-O methylation of viral RNA (WNV, SARS-CoV, VACV), hairpin structures near the 5′ ends of viral RNA (VEEV), masking of the 5′ end by Vpg (EMCV)	Y	Y
8	HERC5 (+ISG15)	HIV, MLV, HPV, IFLUAV	Inhibits HIV and MLV assembly by ISGylation of Gag, ISGylation of IFLUAV NS1 and HPV L1 capsid reduces infectious virus yield	NS1 (?) (IFLUAV)	Y	Y
9	Tetherin/BST2/CD317	Retro-, flavi-, filo-, rhabdo-, herpes-, corona-, paramyxo-, arena-, toga-, hepadnaviruses	Prevents virus release by tethering budding progeny virions to the plasma membrane of the infected cell	Vpu (HIV-1 M/N, SIVgsn/mon/mus), Nef (most SIV, HIV-1 O), K5 (KSHV), Env (HIV-2, EBOV, MARV, SIVagm), Nsp1 (CHIKV), gM (HSV-1), HA/NA (pandemic IFLUAV), F/HN (SeV), HBs (HBV)	Y	Y

Figure 1.11
Antiviral restriction factors.
Kluge, S. F. et al. Cell 163, 774-774.e1 (2015).

Aim of the thesis

Nef, an accessory protein of HIV-1, counteracts the antiretroviral activity of SERINC5 by promoting its endocytosis, which results in its removal from the cell surface preventing its incorporation into retroviral particles. SERINC5 inhibits not only HIV-1 but also other divergent retroviruses, such as MLV. MLV glycoGag can rescues the activity of Nef-defective HIV-1, further indicating that the two (Nef-like) retroviral factors, genetically unrelated, originated by convergent evolution. HIV and MLV are both lymphotropic retroviruses. Did other lymphotropic retroviruses evolve additional Nef-like infectivity factors? Do different Nef-like factors modulate retrovirus infectivity using the same mechanism?

The aim of my project is to investigate the presence and understand the function of Nef-like factors across different retrovirus species.

During the first few months in the lab, I have identified a factor (S2) encoded by EIAV (Equine infectious anemia virus) which can also functionally complement the infectivity of Nef-defective HIV-1. This further suggests that Nef-like factors are a previously over-looked class of retrovirus infectivity factors derived by convergent evolution. Given that the genomes of many retroviruses remain to be fully explored I continued to search for similar factors in other retroviruses (such as BIV and FIV).

Chapter 2: The Nef-like activity on infectivity across different retroviruses

Introduction

Nef is an accessory lentiviral protein only present in the genome of primate lentiviruses. GlycoGag of MLV was found to act as an infectivity factor functionally related to Nef, able to rescue the infectivity of Nef-defective HIV-1[82]. Translation of glycoGag in the MoMLV genome starts from a non-canonical CUG inititation codon[92]. I have analyzed the sequences of different gammaretroviruses, both endogenous and exogenous, and found that a similar initiation codon, which allows translation of a glycoGag molecule, is present in all species considered (Figure 2.1).

Because the GALV genome sequences available in the NCBI database lack a functional glycoGag ORF, we have cloned and sequenced the 5' region of GALV from infected cells. We found that two nucleotides missing in the sequences from the database restore a functional glycoGag ORF starting from a CUG embedded in a perfect Kozak context. The predicted amino acid sequence shares similarity with glycoGag of retroviruses from non-rodent mammals (Figure 2.2) and provides initial evidence that the effect on retrovirus infectivity could be a conserved feature of glycoGag from divergent gammaretroviruses.

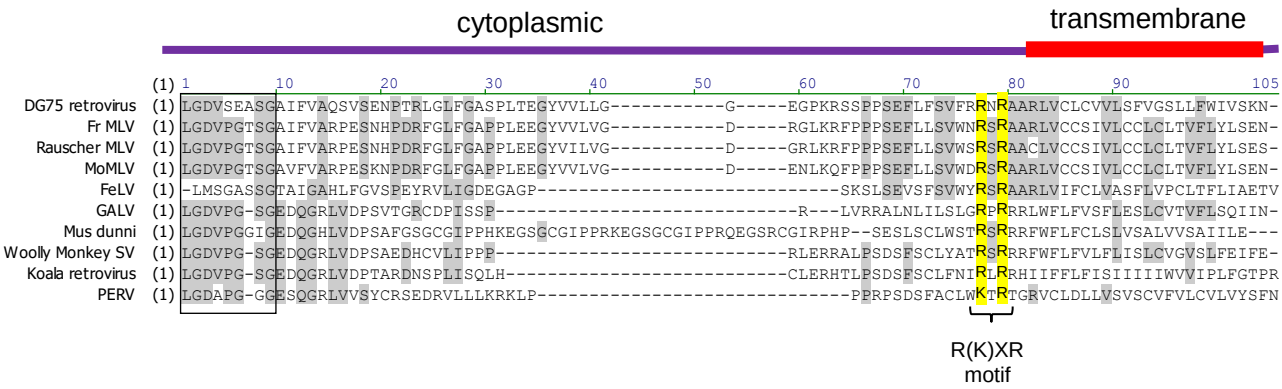


Figure 2.1

Alignment of glycoGag amin oacid sequences from different gammaretrovirus species.

Alignment of the cytoplasmic and transmembrane domains of glycoGag molecules derived from members of gammaretrovirus species with a preserved ORF. The boxed region at the N-terminal represents the most conserved stretch of amino acids. Highlighted in yellow is a conserved R(K)XR motif investigated.

Figure 2.2
GALV-X *glycoGag* alignment.
Alignment of the nucleotide sequence of the 5' *glycoGag* region of GALV-X using the sequence available in Genebank and the sequence determined in our laboratory from virions released by chronically infected cells.

Noteworthy, no report exists to suggest that FeLV is indeed capable of encoding a glycoGag molecule. From analyzing the nucleotide sequence available, a *glycoGag* potential ORF in FeLV was observed starting from a canonical AUG. If FeLV indeed synthesizes glycoGag, then such start codon must be used by the ribosome with suboptimal efficiency, to allow translation of Gag starting from a downstream AUG.

All putative glycoGag molecules listed in Figure 2.1 were analyzed using the transmembrane topology prediction application TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0), confirming the type II transmembrane topology in all cases.

An alignment of the predicted glycoGag amino acid sequences derived from the gammaretroviruses available in the laboratory indicates that the region upstream of the transmembrane domain is highly variable, defining three distinct groups (MLV, GALV-KoRV-PERV and FeLV) which recapitulates the phylogenesis of the viruses analyzed.

In this chapter, I describe the attempts to understand 1) which molecular features of glycoGag are crucial for the activity and 2) whether the activity on infectivity is a conserved attribute of the different alleles.

Results

Understanding the determinants important for the activity of glycoGag

The transmembrane region

Previous results have established that most of the extracellular region of glycoGag is dispensable for the activity of the molecule on infectivity[82]. Accordingly, a 96aa glycoGag molecule, predicted to retain only 14 aa downstream of the transmembrane domain, retains considerable activity[82]. Given the presence of the transmembrane domain, it is impossible to further delete the molecule without imparing its proper topology. To investigate whether the specific transmembrane domain (TM) sequence of glycoGag is important, I replaced the 23aa contained in the predicted TM helix of glycoGag with those derived from influenza neuraminidase (NA), a heterologous type II transmembrane protein. The construct containing such substitution retained most of the ability to rescue HIV-1 infectivity (Figure 2.3.), indicating that a glycoGag-specific transmembrane sequence is not absolutely required for the activity.

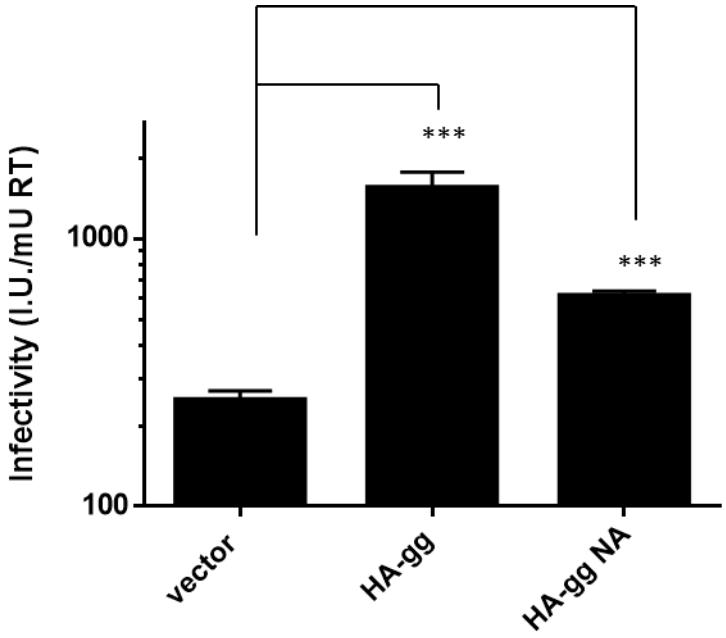


Figure 2.3

The glycoGag-specific transmembrane sequence is not required for its activity on infectivity.

Infectivity of Nef-defective HIV-1 alone or in the presence of HA-glycoGag (HA-gg) wt or HA-glycoGag NA (HA-gg NA) obtained by electroporation of JTAg used as producer cells. Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3-derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, HA-glycoGag/HA-glycoGag NA or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values are the average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed *t-test* (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

The N-terminal region

The alignment of glycoGag molecules reveals that the region of glycoGag upstream the *gag* AUG is relatively poorly conserved. However, the first 10 aa appear to be highly conserved (with the only exception of FeLV). To test whether the N-terminal of the protein plays an important role and verify whether the first 10aa of MoMLV glycoGag are crucial for the activity on infectivity, two increasing N-terminal deletions (23 aa and 56 aa) were introduced in the context of a minimal glycoGag molecule, which lacks most of the extracellular region (gg189). To facilitate detection in Western blotting, an HA tag was fused at the N-terminal, since it was previously found to be compatibile with the activity of MoMLV glycoGag [82].

The ability of these deletion mutants to rescue the infectivity of HIV-1 was tested by expressing the mutated constructs together with a two-part HIV-1 NL4-3 vector (*env*-defective HIV-1^{NL4-3} complemented with *env* derived from HIV-1^{HXB2}) in JTAg cells. JTAg cells were chosen because among the cell lines that have been tested in our laboratory, they are those which depend most on Nef for the production of fully infectious HIV-1. Viruses obtained were used to infect a TZM-bl reporter cell line, engineered to contain a HIV-1 LTR-driven cassette for Tat-dependent expression of nlsZsGreen (TZM-GFP), which allows scoring infection events using an automated fluorescent microscope for High Content Screening (Operetta PE). After normalization of progeny virus based on the RT-activity of the inocula using SG-PERT, virus infectivity was calculated. Results show that deletion of the first 26 aa do not affect the ability of the molecule to rescue HIV-1, indicating that the most

conserved N-terminal region (the first 10aa) is not required for the activity. Larger deletions totally impaired glycoGag function. Western blotting analyses revealed that glycoGag expression was majorly and increasingly affected by the extent of the deletion introduced. Since deletions spanning over the first N-terminal 26aa are crucial for the correct expression, the role of the downstream aa remains to be established (Figure 2.4).

The potential presence of sorting signals

One additional conserved feature which was observed among glycoGag molecules from different retrovirus species is a R(K)XR motif adjacent to the transmembrane helix.

A similar motif of basic amino acids located near the transmembrane domain resembles the stop transfer signal for translocation of type-II proteins through the lipid bi-layer[98]. However, I have tested an exhaustive dataset of Type II transmembrane proteins (http://ccb.imb.uq.edu.au/golgi/documents/Training_Set.html) and rarely found an R(K)XR motif in close proximity of the transmembrane domain. Considering the high variability of the glycoGag region surrounding this motif, we think that the conservation of the R(K)XR sequence could have another functional meaning. Interestingly, an RXR motif within the cytoplasmic tail of type II proteins is known to function as an endoplasmic reticulum retention signal[99–101]. Despite some evidence indicating that RXR signals are preferentially found distantly from the membrane[98], given the perinuclear

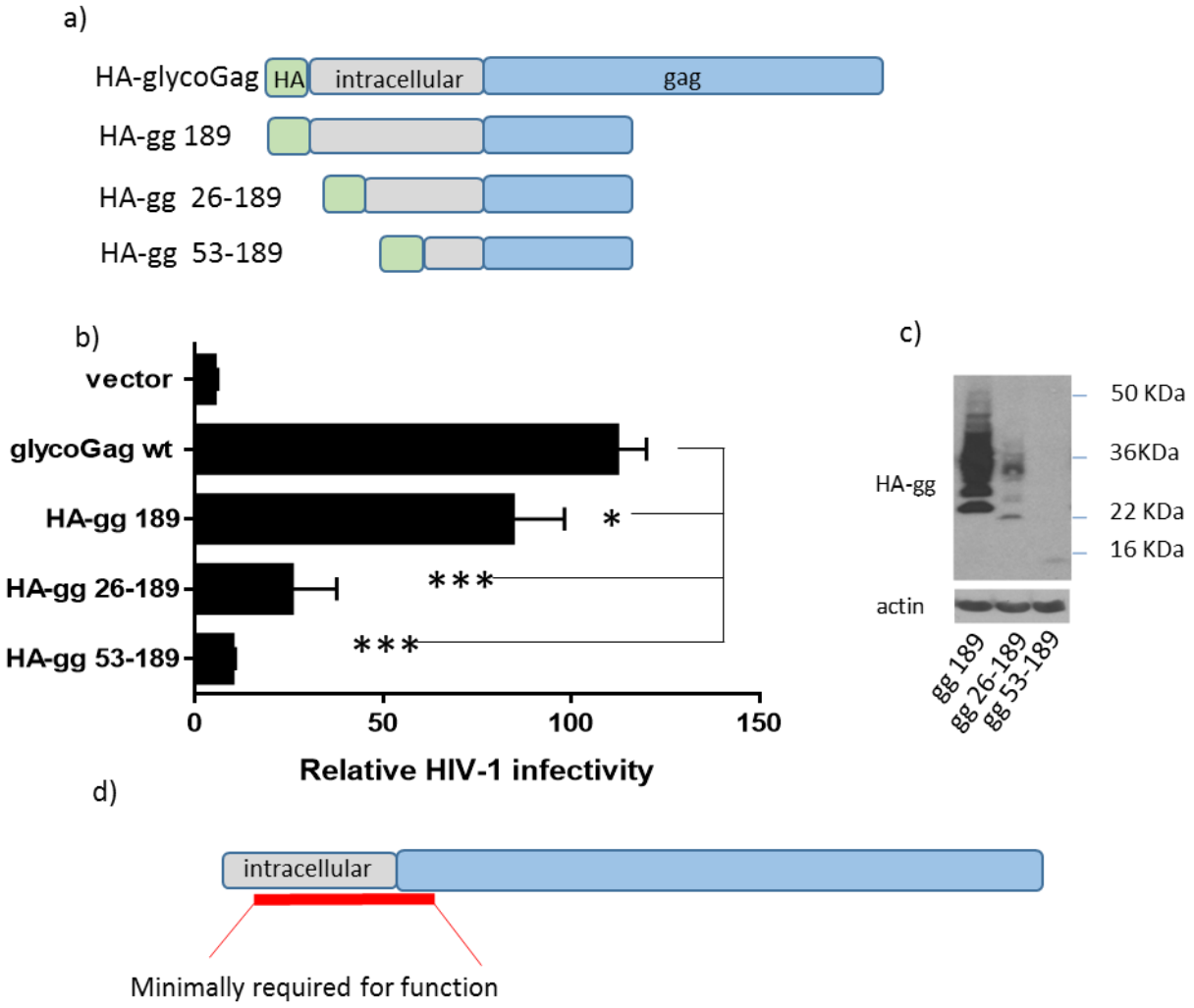


Figure 2.4

The N-terminal 26 aa of MoMLV glycoGag are not required for activity.

Schematic (a) and activity on infectivity (b) of N-terminal deletions introduced.

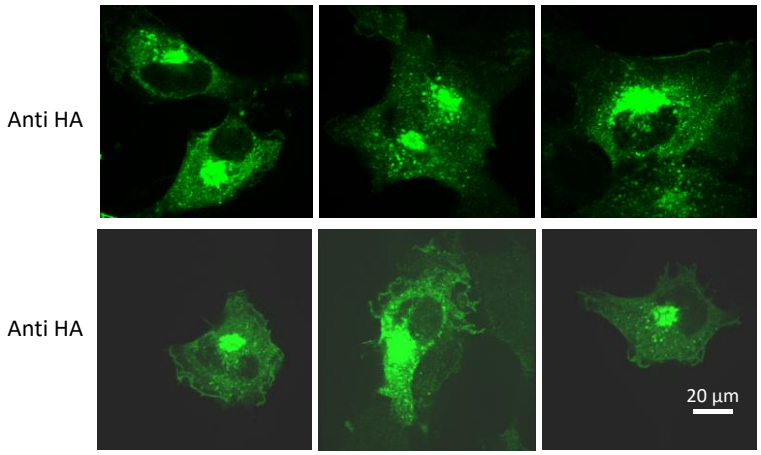
(c) Western blot analysis of glycoGag deletion mutants expression in corresponding cell lysates. (d), schematic illustrating the minimal glycoGag region required for activity on infectivity.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, HA-glycoGag wt/HA-glycoGag 189 mutant/ HA-glycoGag 26-189 mutant/HA-glycoGag 53-189 mutant or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy.

Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB.

Infectivity values represent the average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

a)



b)

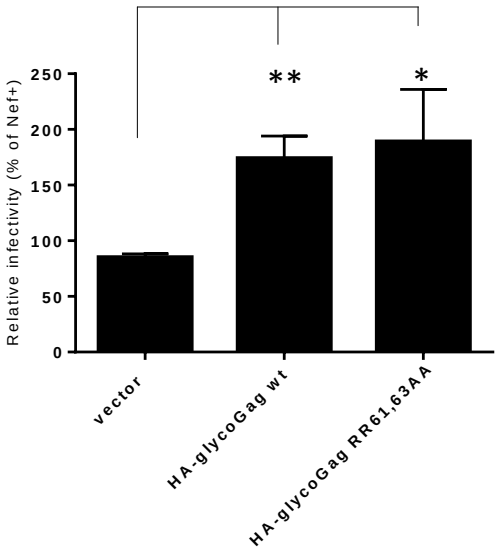


Figure 2.5

The RXR motif of MoMLV glycoGag is not required for activity.

(a) Immunofluorescence microscopy on COS-7 cells transfected to allow the expression of HA-glycoGag wt or HA-glycoGag RR61,63AA.

(b) Activity on infectivity of the indicated glycoGag constructs.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, HA-glycoGag wt/HA-glicoGag RR61,63AA or empty vector and *env* was used. Supernatant of JTAG producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values represent the average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

localization of glycoGag observed in immunofluorescence microscopy, I tested whether the R(K)XR sequence plays a role in subcellular targeting of glycoGag and is important for its activity.

The RXR motif of gg189 (derived from MoMLV) was converted into AXA using site-directed mutagenesis. The effect of the mutation was tested on the subcellular localization of the protein and its ability to rescue Nef-defective HIV-1 infectivity. As shown in Figure 2.5 a, the intracellular localization of the AXA variant in transfected COS-7 cells was identical to that of the wt protein, denoting a strong accumulation in perinuclear compartments. Similarly, the mutant protein could rescue HIV-1 infectivity as much as the wt protein (Figure 2.5 b). The RXR motif, therefore, although highly conserved, plays no role on glycoGag function.

The importance of endocytosis for glycoGag function

The activity of Nef on infectivity is known to depend on the ability of the protein to interact with important players of the biogenesis of endocytic vesicles such as AP2 and dynamin 2. In addition, it has been demonstrated that a functional endocytic machinery is required for the effect on infectivity[80]. It has not been so far demonstrated that this was also the case for glycoGag.

To investigate the role of endocytosis in glycoGag activity, transdominant-negative inhibitors of clathrin-mediated vesicle biogenesis were expressed in virus producing cells and their effect on the ability of glycoGag to increase

Nef-defective HIV-1 infectivity was evaluated. Both, dominant-negative dynamin 2 and a C-terminal fragment of the clathrin adaptor protein AP180 were used, since they were previously found to efficiently inhibit Nef[80]. Similarly, both inhibitors strongly impaired the ability of glycoGag to alter HIV-1 infectivity, suggesting that, like in the case of Nef, clathrin-mediated endocytosis plays a crucial role (Figure 2.6). This indicates that, similarly to Nef, glycoGag could recruit components of the endocytic machinery. Accordingly, while we were attempting to identify intracellular sorting signals within the aa sequence of glycoGag, the Gottlinger's laboratory reported that a YxxL motif in MLV glycoGag, predicted to recruit AP-2, is crucial for the activity on infectivity[96]. Interestingly, such motif is not conserved among glycoGag alleles from different retroviruses, raising the possibility that also the activity on infectivity may not be conserved.

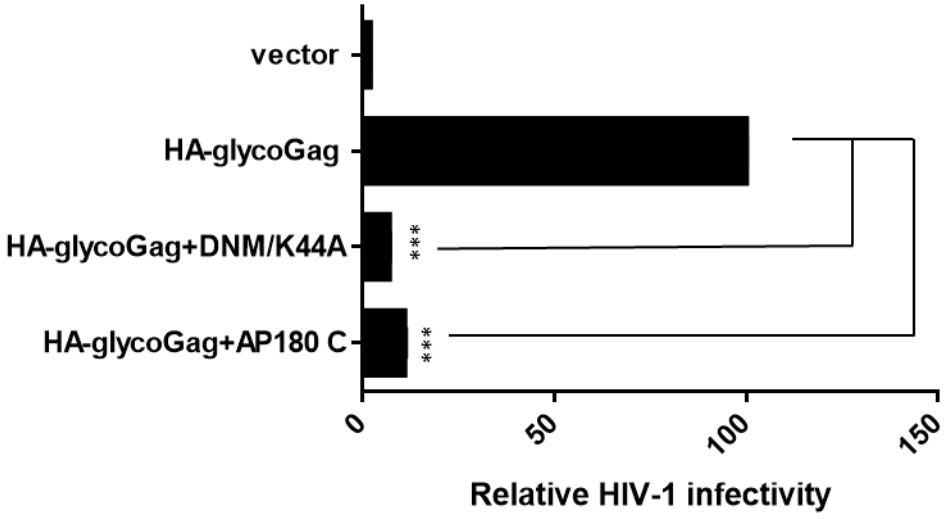


Figure 2.6

The importance of endocytosis for glycoGag function.

Expression of dominant negative dynamin 2 (DNM2/K44A) and a transdominant negative fragment of AP-180 (AP180 C) in virus producing cells inhibits the activity of MoMLV glycoGag on the infectivity of Nef-defective HIV-1.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2} *env*. 4:4:1 ratio between viral backbone, HA-glycoGag or empty vector and *env* was used. 1:1.5 ratio between glycoGag and each endocytosis inhibitor tested separately was used.

Supernatant of JTAG producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods, and analysed through automatized microscopy. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed *t*-test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Shown is a representative experiment out of 3 repetitions.

The activity of glycoGag alleles from different gammaretroviruses on HIV-1 infectivity is not conserved

The ability of FeLV to produce a glycoGag molecule has never been established. We therefore started with testing whether the FeLV genome is capable of providing a Nef-like activity on HIV-1 infectivity. We produced HIV-1 in JTAg cells in the presence or absence of the full FeLV-A provirus. As expected, the absence of Nef introduced a 7-fold defect in HIV-1 infectivity. Co-transfection with a FeLV-A genome resulted in a 3-fold rescue of the infectivity of Nef-defective HIV-1 (Figure 2.7). While this increase of infectivity appears to be partial, this result was reproducible in several experiments, suggesting that the FeLV-A genome could encode a Nef-like infectivity factor.

Indication that GALV encodes for a Nef-like factor came previously with the observation that HIV-1 derived from the lymphoid cell line A3.01/F7[102], which was found to be contaminated with GALV-X, does not require Nef for infectivity. In contrast, HIV-1 derived from the parental, non-contaminated, A3.01 cells depends on Nef for optimal infectivity[82].

In order to test and compare the activity of glycoGag alleles isolated from different retrovirus species, we isolated and cloned each sequence in a pbj5 expression vector, under the control of the same SRalpha promoter. GlycoGag sequences were cloned from FeLV-A, KoRV, GALV-X and PERV-A. The glycoGag from GALV was derived from viral RNA isolated from retrovirus particles pelleted from the supernatant of A3.01/F7 cells.

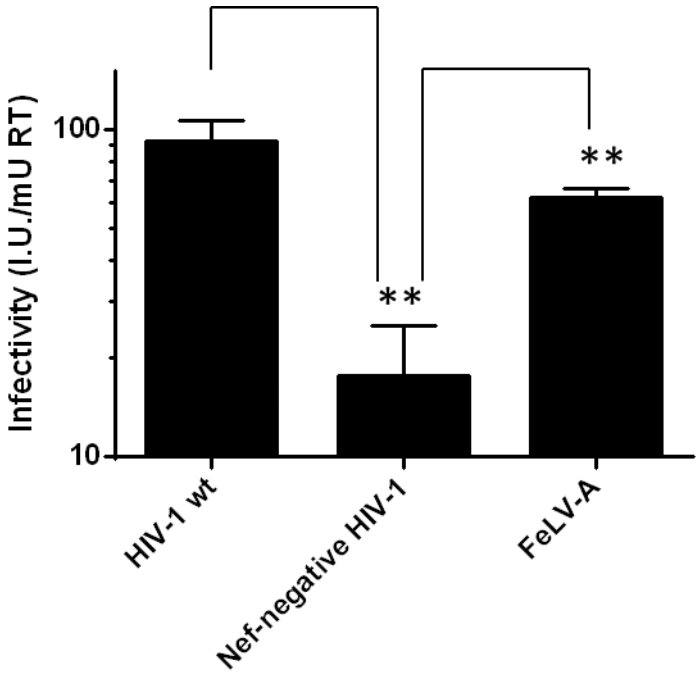


Figure 2.7

FeLV-A activity on infectivity.

Infectivity of HIV-1 wt or Nef-negative produced by electroporation of JTAg cells, in the presence and in the absence of FeLV-A molecular clone.

HIV-1 wt or Nef-negative is obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:8:1 ratio between viral backbone, FeLV molecular clone or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

Since it was previously demonstrated that the activity on infectivity maps within the intracellular domain of glycoGag, only the 5' 288 nt were cloned and used to replace the homologous region within MoMLV HA-gg196, up to a stretch of highly conserved amino acids, which include a AflII restriction site in the MoMLV genetic sequence. (See materials and methods section for cloning strategy)

To allow detection in Western blot assays, all genes were cloned by fusing an HA-tag at the N-terminal, which was already shown to be compatible with the activity of MoMLV glycoGag[82]. Accordingly, after transfecting JTAG cells with the constructs encoding the different glycoGag alleles, all molecules could be detected at the expected molecular size.

Each construct expressing glycoGag was co-transfected, along with *Nef*-defective HIV-1 provirus constructs, into JTAg cells. (Figure 2.8). Infectivity of progeny viruses measured on TZM-GFP cells revealed that glycoGag from GALV can partially rescue the infectivity of HIV-1, which could be fully rescued by MoMLV glycoGag. This confirms the indication originated from A3.01 cells, that GALV has the ability to repair the infectivity of Nef-defective HIV-1. In contrast, the molecules cloned from PERV, KoRV and FeLV-A did not affect HIV-1 infectivity, suggesting that, at least in this experimental setting, the intracellular portion of glycoGag derived from these retroviruses cannot replace the activity of Nef. These results indicate that the ability to increase retrovirus infectivity is variable and might not represent a conserved property of gammaretrovirus glycoGag alleles. Unexpectedly, expression of FeLV-derived HA-glycoGag did not confirm the effect on HIV infectivity obtained by transfecting the full-length retroviral molecular clone. Further experiments

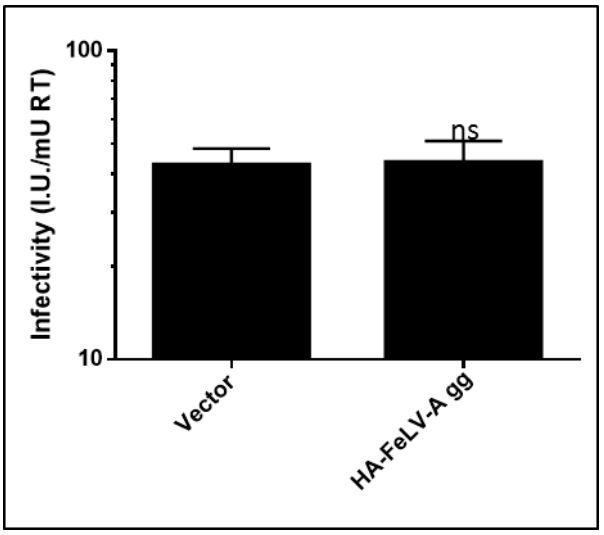
would be at this stage required to understand whether this is due to suboptimal expression levels of the HA-glycoGag molecule, or to a requirement FeLV glycoGag sequences at the C-terminus of the molecule.

The ability of glycoGag alleles to target SERINC5

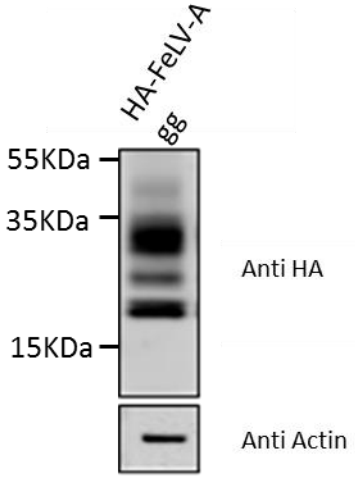
While I was carrying out this investigation on glycoGag alleles, a parallel project in the lab identified a host transmembrane protein (SERINC5) which explains the effect of Nef and glycoGag on retrovirus infectivity. SERINC5 acts as an inhibitor of the retrovirus particle and is counteracted by both retrovirus molecules. As observed by immunofluorescence microscopy, Nef and glycoGag target SERINC5 and downregulate its surface expression level by promoting its accumulation into a late endosomal compartment. We investigated whether the variable activity of glycoGag molecules observed on virus infectivity correlates with a variability on their ability to promote endocytosis and intracellular accumulation of SERINC5.

To assess the ability of glycoGag molecules to target SERINC5 we transfected JTAg cells with constructs expressing HA-tagged gg189 genes along with a plasmid expressing SERINC5-GFP. As revealed by HA immunostaining, similarly to MoMLV glycoGag, despite the absence of a reproducible effect on HIV-1, FeLV-A, PERV and KoRV-derived glycoGag could to various extent produce a noticeable SERINC5 intracellular accumulation. This indicates that all molecules are capable of targeting the host factor, but this is not sufficient to rescue the infectivity of HIV-1(Figure 2.9).

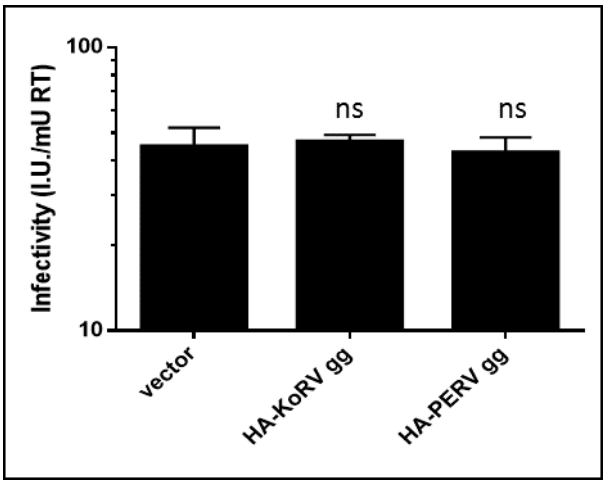
a)


Infectivity (I.U./mU RT)
100
10
ns
Vector
HA-FeLV-A gg

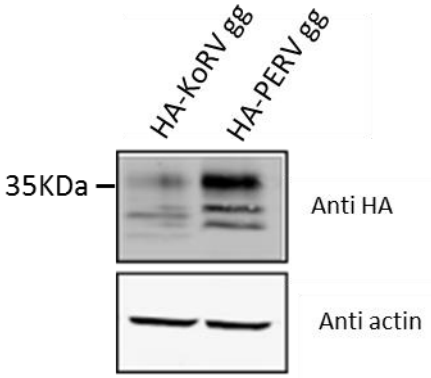
b)


HA-FeLV-A gg
55KDa
35KDa
15KDa
Anti HA
Anti Actin

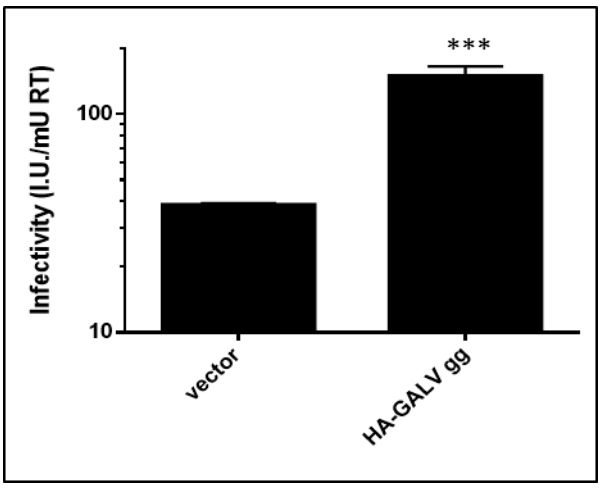
c)


Infectivity (I.U./mU RT)
100
10
ns
ns
vector
HA-KoRV gg
HA-PERV gg

d)


HA-KoRV gg
HA-PERV gg
35KDa
Anti HA
Anti actin

e)


Infectivity (I.U./mU RT)
100
10
***
vector
HA-GALV gg

f)

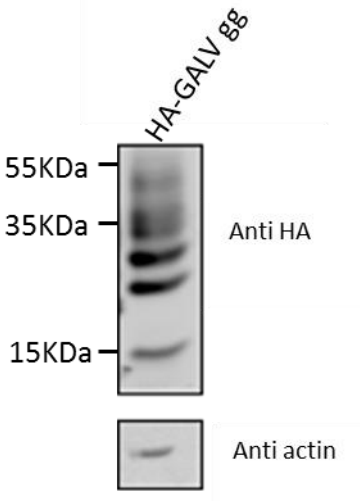

HA-GALV gg
55KDa
35KDa
15KDa
Anti HA
Anti actin

Figure 2.8

Overexpression of glygoGag (gg) from different species do not rescue infectivity of HIV-1 Nef defective virus.

Infectivity (a, c, e) and expression levels in corresponding cell lysates (b, d, f) of HA-FeLV-A , HA-KORV, HA-PERV and HA-GALV gg transfected in the presence of Nef-negative HIV-1 in JTAg producer cells.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, each glycoGag or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

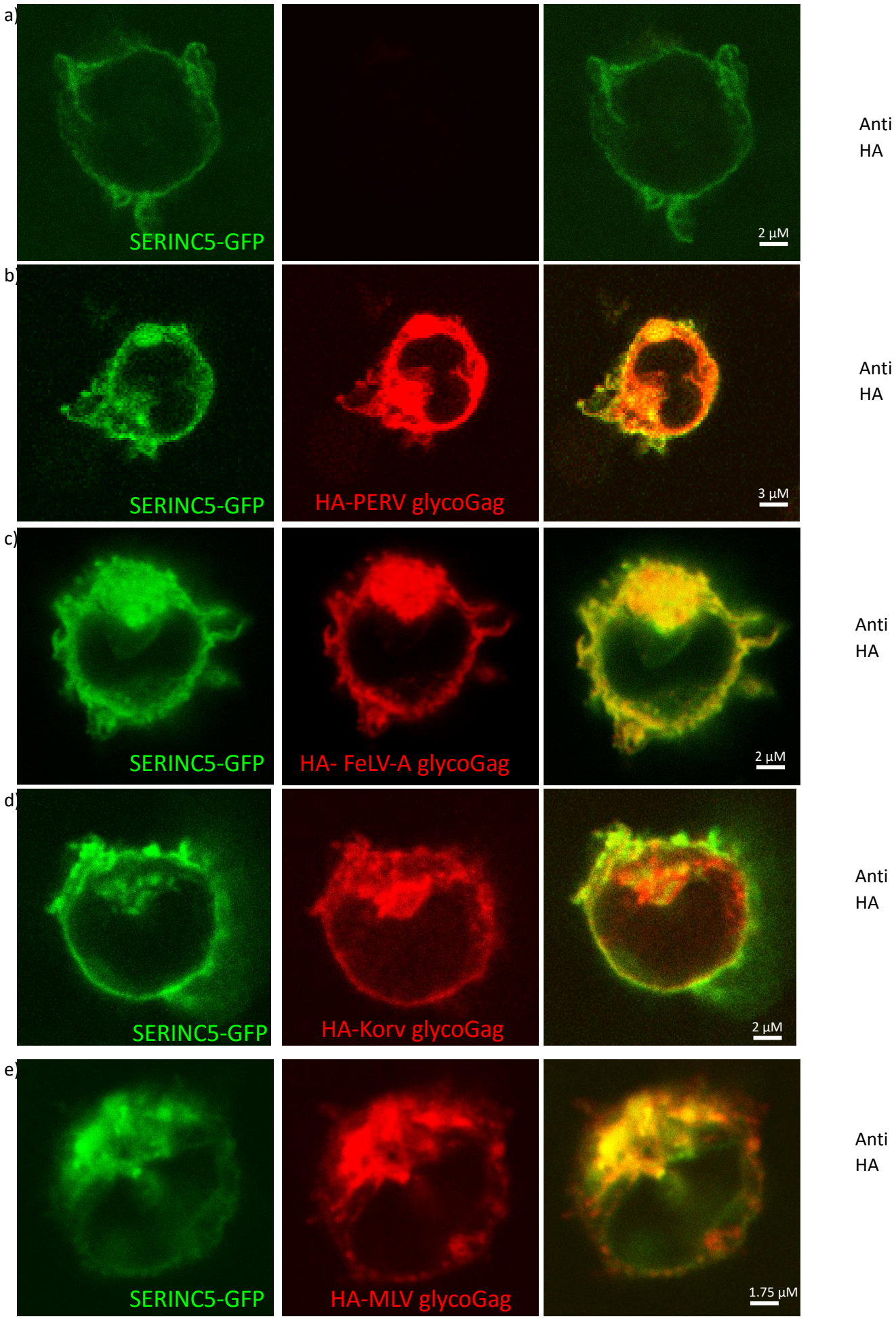

a)
SERINC5-GFP
2 µM
Anti HA
b)
SERINC5-GFP
HA-PERV glycoGag
3 µM
Anti HA
c)
SERINC5-GFP
HA- FeLV-A glycoGag
2 µM
Anti HA
d)
SERINC5-GFP
HA-Korv glycoGag
2 µM
Anti HA
e)
SERINC5-GFP
HA-MLV glycoGag
1.75 µM
Anti HA

Figure 2.9

The ability of glycoGag alleles to target SERINC5.

Confocal microscopy of JTAg cells transfected to express SERINC GFP alone (a), or along with HA-glycoGag molecules derived from PERV (b), FeLV (c), KoRV (d) and MLV (e).
Representative experiment out of 3 repetitions.

Discovery and characterization of novel Nef-like infectivity factors

Research in our lab has demonstrated that Nef from primate lentiviruses and glycoGag from gammaretroviruses have independently evolved functionally related activities which modulate retrovirus infectivity. We investigated whether retroviruses other than primate lentiviruses and gammaretroviruses have evolved Nef-like factors. We restricted our investigation to FIV, WDSV, BIV and EIAV, because their genome encode for yet not fully functionally characterized accessory proteins.

1) Feline Immunodeficiency Virus causes a pathology which is very similar to human AIDS and, like HIV, is a T-cell tropic lentivirus. We focused our attention to the accessory protein ORF-A which was reported to be required for optimal intrinsic infectivity of FIV produced from T-lymphocytes[103] and, like Nef, promotes the downregulation of the receptor (CD134)[104].

2) The activity of both glycoGag (a transmembrane protein) and Nef (myristoylated) requires the ability of these accessory proteins to associate with the cell membrane. We searched, among different retroviruses, for those capable of encoding transmembrane accessory proteins. Bovine immunodeficiency virus, another lentivirus, expresses a transmembrane protein (TMX) from an ORF located, like *nef* in HIV-1, at the 3' of the genome. Since TMX remains currently orphan of a molecular function, we decided to investigate whether it could have evolved a Nef-like function.

3) Another common feature between Nef and glycoGag is the presence of motifs (E/DxxxLL and YxxLL respectively) predicted to interact with AP2. While searching from retrovirus factors which contain putative AP2 binding motifs, we noticed that both an E/DxxxLL and YxxLL motifs are present in the accessory protein ORF2 from Walleye Dermal Sarcoma, indicating its possible role in modulating endocytosis.

4) Finally, our attention was focused on Equine infectious anemia virus, which expresses a small accessory protein (S2) required for disease progression *in vivo*, which, like Nef, also contains a ExxxL motif.

To investigate whether Orf-A is a Nef-like factor, the gene from the Petaluma isolate fused to a HA-Tag at its C-terminal was overexpressed in JTAg cells producing Nef-defective HIV-1. We observed no effect on HIV-1 infectivity in conditions where Nef caused a 10-fold increase of infectivity. However, with immunoblotting with anti-HA, no signal was visible from cell lysates (not shown). Given the low molecular weight of ORF-A (6KDa), detection could be difficult and might require maximization of expression. We therefore synthesized an ORF optimized for the human codon usage. Because a tag fused to such a small protein could have deleterious effects, both an untagged and a FLAG-tagged versions were generated. While the tagged protein could be readily visualized in the lysates of virus producing cells, again no effect was observed on HIV-1 infectivity (Figure 2.10) in the absence of Nef. Similarly, the untagged protein did not alter HIV-1 (not shown).

Genes expressing TMX from BIV (accession number: AAA64394.1) and ORF-2 from WDSV (accession number: NP_045940) were also synthesized with

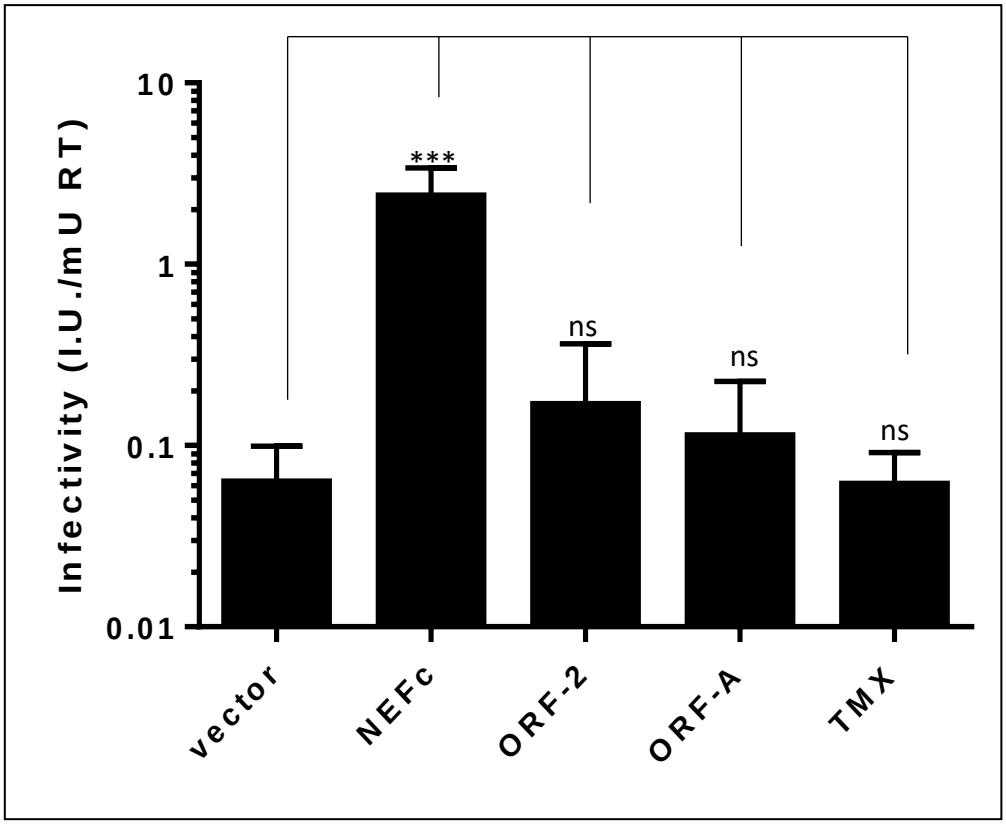
optimized human codon usage and with a HA tag fused at the C- terminal, based on the sequence of reference genomes. Both proteins were readily visible by immunoblotting in the lysates of HIV-1 producing cells. However no effect could be observed on HIV-1 infectivity (Figure 2.10).

These results indicated that, in these experimental conditions, ORF-A, TMX and ORF-2 do not function as a Nef-like protein for HIV-1 infectivity.

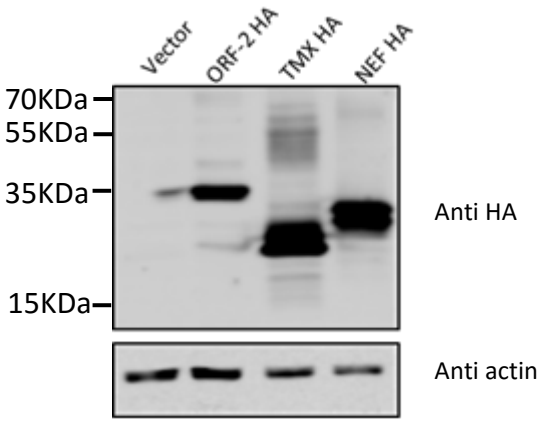
In contrast, the infectivity of Nef-defective HIV-1 was rescued by expression of the accessory protein S2 from the P19/Wenv 16 EIAV isolate.

The molecular and functional characterization of S2 is described in the next chapter.

a)


Infectivity (I.U./mU RT)
10
1
0.1
0.01
***
ns
ns
ns
vector
NEFc
ORF-2
ORF-A
TMX

b)


Vector
ORF-2 HA
TMX HA
NEF HA
70KDa
55KDa
35KDa
15KDa
Anti HA
Anti actin

c)

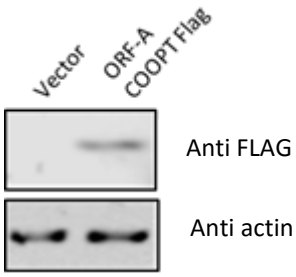

Vector
ORF-A COOPT Flag
Anti FLAG
Anti actin

Figure 2.10

WEHV-ORF2, FIV-ORF-A and BIV-TMX don't show significant Nef-like activity.

a) Infectivity data of Nef clade C used as a positive control, ORF2, ORF-A and TMX transfected in the presence of HIV-1 Nef deficient virus in JTAg producer cells.

b,c) Expression levels in corresponding cell lysates.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, Nef c /ORF2/ORF-A/TMX or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB.
Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

Discussion

An ORF encoding glycoGag is present in most gammaretrovirus genomes that we could retrieve in the Genbank database. The sequence of the predicted cytoplasmic and transmembrane domains appears to be poorly conserved among different retroviruses. Initially, we assumed that all glycoGag alleles would be active on retrovirus infectivity. We therefore studied whether the same features that are most common between the alleles are also those required for the activity. Surprisingly, the two most conserved features, i.e. the highly conserved N-terminal region and a R(K)xR motif present in all glycoGag alleles, are not required. We therefore investigated whether indeed all glycoGag molecules function as Nef-like infectivity factors on HIV-1. Our results suggest that the activity on HIV-1 infectivity might not be a conserved feature of glycoGag. However, further studies should address this possibility. While all our investigations with glycoGag alleles isolated from different gammaretroviruses species were performed using HIV-1 as a model, it would be now essential to test their activity on the infectivity of the retrovirus from which each molecule was derived. However, technical difficulties make this approach challenging. First, an infectivity assay for all different gammaretroviruses considered here is not readily available, making difficult to generate the molecular tools required to assess the effect of glycoGag on infectivity. Second, the sequence encoding glycoGag overlaps with the packaging signal of gammaretroviruses. This implies that the overexpression of glycoGag *in trans* in a system producing a gammaretrovirus vector would negatively impact on the infectivity of the retroviral particles because the

glycoGag RNA sequence overexpressed would likely compete with the packaging signal of the retroviral genome, excluding the latter from virions. The effect of each glycoGag would therefore have to be studied by mutating its sequence *in cis* within each retrovirus genome.

Our laboratory has established that Nef and glycoGag act by counteracting the host factor SERINC5. We have performed all our experiment using human cell lines as producer cells, therefore testing only the ability of the different glycoGag molecules to counteract the human host factor. A possibility remains that retroviruses which evolved in different animal species adapted to antagonize SERINC5 in a species-specific manner. While our lab has established that MoMLV glycoGag can efficiently counteract human SERINC5, it remains possible that other glycoGag, such as those from PERV and KoRV, are active against the porcine and koala host factors rather than the human counterpart. To this end, SERINC5 from all these different species should be cloned and expressed in order to verify whether such species-specificity exists. The same considerations should be extended to my attempt to identify Nef-like factors in the genome of other retroviruses (FIV, WDSV and BIV), which could have evolved host species-specificity against SERINC5.

While this research was in progress, the Gottlinger group identified a YxxL motif, predicted to recruit AP2, as essential for the activity. This is consistent with our result which shows that clathrin-mediated endocytosis is also required for the effect on infectivity. Accordingly, we also observed the presence of a YxxL in FeLV glycoGag, and a ExxxLL motif in the PERV molecule. It is therefore possible that the ability to interact with AP2 is required but not sufficient for the glycoGag effect on infectivity. In line with

this hypothesis, we observed that, irrespectively of the effect documented on HIV-1 infectivity, all glycoGag molecules indeed can cause the intracellular accumulation of SERINC5, suggesting that the ability to perturb intracellular vesicle trafficking is present. Interestingly, our laboratory identified a *nef* allele, from a subtype H isolate, which, like the glycoGags described here, does not affect HIV-1 infectivity, despite maintaining the ability to promote intracellular accumulation of SERINC5 (unpublished). Altogether, this is reminiscent of the activity of Vpu against the restriction factor BST2[105]. While Vpu was found to re-route BST2 from the plasma membrane *via* an endocytic pathway, naturally occurring alleles were identified which fail to counteract the antiviral activity of BST2 despite retaining the ability to promote BST2 dowregulation. Like in the case of Vpu, therefore, the mechanism by which Nef and glycoGag counteract SERINC5 remains to be fully established.

The gammaretroviruses analyzed in this thesis are both exogenous and endogenous retroviruses. It is interesting to notice that, while an effect of glycoGag from exogenous retroviruses (MoMLV, FeLV-A and GALV) on HIV-1 infectivity was observed (though weaker with FeLV and GALV), the glycoGag molecules from the PERV and from KoRV, which is currently undergoing the process of endogenization, display no activity on HIV-1 infectivity, It is therefore tempting to speculate that the process of endogenization might require the loss of glycoGag ability to counteract SERINC5. To this end it will be interesting to analyze glycoGag alleles from more exogenous and endogenous retroviruses, to verify this hypothesis.

Chapter 3: The Equine infectious anemia virus S2 accessory protein is a factor which promotes retrovirus infectivity

Introduction

EIAV is a lentivirus with macrophage-tropism causing a chronic disease in equids. The disease associated with EIAV was identified in 1843 and was the first pathology demonstrated to be caused by an infectious agent. It was also the first disease proven to be caused by a virus ("a filterable" agent) in 1904. However, the identification and characterization of the etiological agent causing such disease occurred only in the 1970.

EIAV infection occurs through blood-feeding horse flies and results in three clinical stages: acute, chronic and inapparent. The acute and chronic stages are symptomatic and characterized by fever, anemia, thrombocytopenia, edema and lethargy. One year after infection, the animals typically progress to a life-long inapparent stage, displaying non recognizable clinical signs of disease. During this period of steady state viral replication, monocyte-rich tissues become a reservoir of infection that could be re-activated by both immune suppression and stress conditions. During this phase of the infection, the animals are carriers and transmit the virus to other animals[106].

During the last twenty years, the characterization of cellular receptors used by retroviruses has been well established for different lymphotropic lentiviruses such as human, simian and feline immunodeficiency viruses. Their study helped to underline a common theme for entry of these viruses that share the peculiarity to bind sequentially two cellular coreceptors. In contrast EIAV lentiviruses use a single receptor, ELR1, belonging to the family of TNF receptor proteins. This family of receptors mediate the infectious process for B, D and E avian leukosis virus subtypes and for feline leukimia virus as well[107].

EIAV is a complex retrovirus, with the genes *tat, rev* and *S2* expressed in addition to *gag*, *pol* and *env.* (Figure 3.1a). While the function of all EIAV genes is relatively well understood and recapitulates the functions already discussed for other retroviruses of the Retroviridae family, the role of S2 remains enigmatic.

S2 is a small (7KDa), poorly studied accessory factor. Neither its position in the genome nor its amino acid sequence were found to be similar to those of other viral or cellular proteins. The information available can be summarized as follows:

- S2 is not required for optimal virus release, as *in vitro* studies have shown that EIAV lacking S2 is produced as efficiently as wild-type EIAV[108].
- No evident defect could be observed in the ability of EIAV to infect target cells, including monocytes, in the absence of S2.[109]

The pathology associated with S2-defective viruses is less severe than that caused by the wt virus. The removal of S2 from the EIAV genome was found to associate with lower viral load in animals, and with absence of clinical

symptoms[110]. Given the crucial pathogenic role of S2, a S2-defective EIAV vaccine was also studied, as it was found to confer 100% protection[111].

This observation prompted us to investigate the potential of S2 as a Nef-like infectivity factor. The structure of S2 has never been investigated. Computational studies, however, predict a putative SH3-binding motif and a controversial putative myristoylation signal. Accordingly, myristoylation of S2 was suggested, but not experimentally proven, by a report[112] (Figure 3.1b). Interestingly, we also observed that a ExxxLL motif is present in S2 and could be predicted to function as the binding site for AP2.

As mentioned in the previous chapter, during a preliminary screening to investigate the presence of Nef-like infectivity factors in different retrovirus species, S2 was found capable of increasing the infectivity of Nef-defective HIV-1. Here I therefore describe an in depth functional and molecular characterization of EIAV S2.

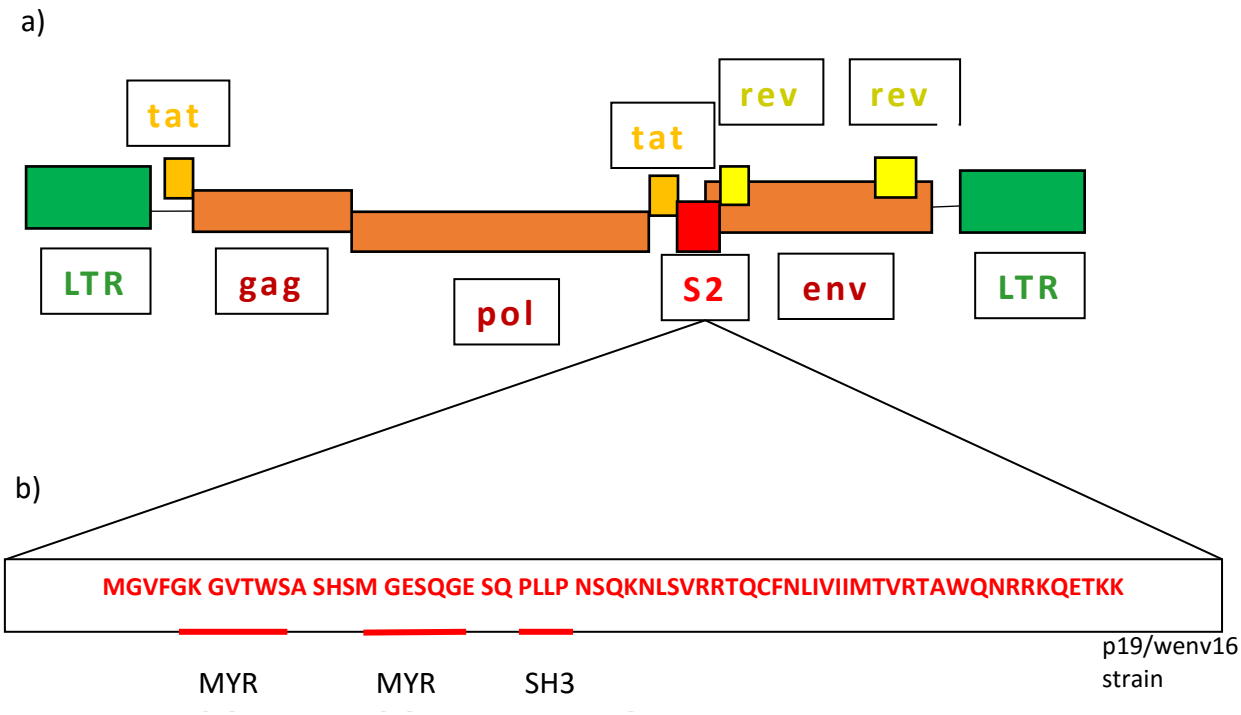


Figure 3.1

Genome of Equine infectious anemia virus and the S2 amino acid sequence.

a) Location of all genes is indicated. LTR: long terminal repeat; tat: transactivator; pol: polimerase; rev: regulator of virion expression; env: envelope. b) S2 amino acid sequence and predicted functional motifs.

Results

S2 is an infectivity factor for the EIAV retrovirus

S2 rescues the infectivity of Nef-defective HIV-1

To investigate the putative role of S2 in the enhancement of viral infectivity, we tested whether S2 is able to rescue the defective infectivity of HIV-1 in the absence of Nef. JTag cells where used as producer cells in these experiments, because they are highly Nef responsive for the effect on HIV-1 infectivity.

Virus capable of only one round of replication (wt and Nef-defective HIV-1) was produced by transfecting JTAg cells, with *env*-defective molecular clones supplemented with a plasmid encoding HIV-1 Env and a vector expressing S2, derived from the p19/wenv 16 EIAV strain. While the absence of Nef caused an 80% reduction of virus infectivity, S2 expressed in producer cells rescued such defect (Figure 3.2). Expression of S2 did not alter significantly the amount of HIV-1 produced (not shown) indicating that the EIAV protein affects exclusively the intrinsic infectivity of virus particles. In contrast, no significant enhancement was documented on the infectivity of HIV-1 in the presence of Nef, indicating that there is no synergistic effect between Nef and S2 and suggesting that the two proteins complement the same defect.
Having tested the activity of S2 derived from the highly virulent p19/wenv 16 strain, we tested whether the ability to affect HIV-1 infectivity was shared with S2 from a different EIAV strain. We therefore synthesized S2 based on the sequence from the Wyoming EIAV strain, which differs for 2 aa.

Such S2 alleles were found to exert a similar activity on the infectivity of Nef-defective HIV-1 produced in Jurkat TAg cells. (Figure3.3)
Altogether, these results suggest that S2 functions as a Nef-like molecule.

The Nef effect on HIV-1 is strongly dependent on the producer cells type[82]. We therefore investigated whether the same is true for the activity of S2.
We used CEMX-174, which is a cell line which is not responsive to Nef. When HIV-1 was produced in this cell line in the presence of a vector expressing S2, the EIAV protein did not affect virus infectivity, confirming the Nef-like character of its activity (Figure 3.4).

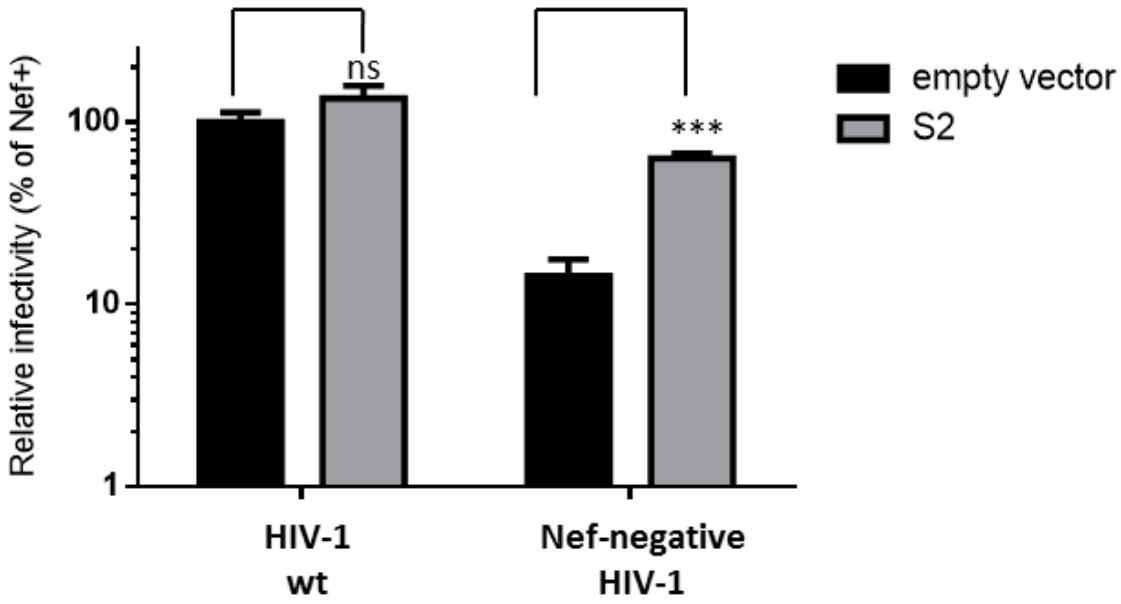


Figure 3.2

EIAV S2 rescues the infectivity of Nef-negative HIV-1 virus.

Relative infectivity (% of the control Nef+ sample) of HIV-1 wt or Nef-negative, obtained by JTAg electroporation in the presence or in the absence of EIAV S2 provided *in trans*.

HIV-1 wt or Nef-negative was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2 or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

a)

MG**V**FGKGVTWSASHSMG**G**SQGESQPLLPNSQKNLSVRRTQCFNLIVIIMTVRTAWQNRRKQETKKYPYDVPDYA

Wyoming strain

b)

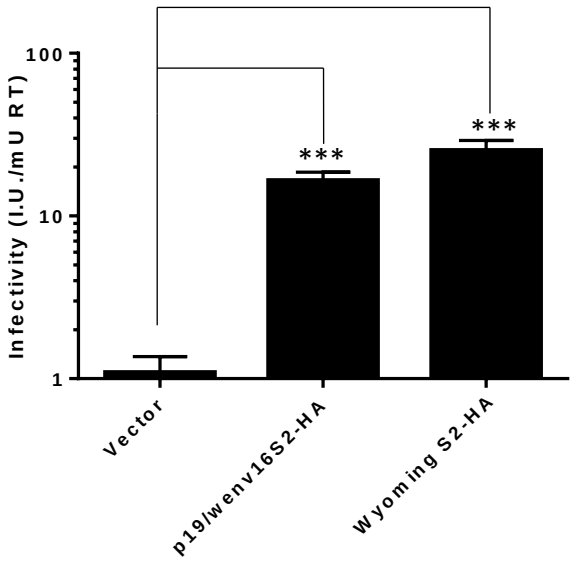


Figure 3.3

EIAV S2 from the Wyoming strain rescues infectivity of HIV-1^{nef-} virus.

a) Schematic representation of S2 Wyoming amino acid sequence.

b) Infectivity of Nef-negative HIV-1, obtained by JTAg electroporation in the presence or in the absence of p19/wenv 16 EIAV S2-HA or Wyoming EIAV S2-HA provided *in trans*.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1HXB2env. 4:4:1 ratio between viral backbone, p19/wenv 16 EIAV S2-HA / Wyoming EIAV S2-HA or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

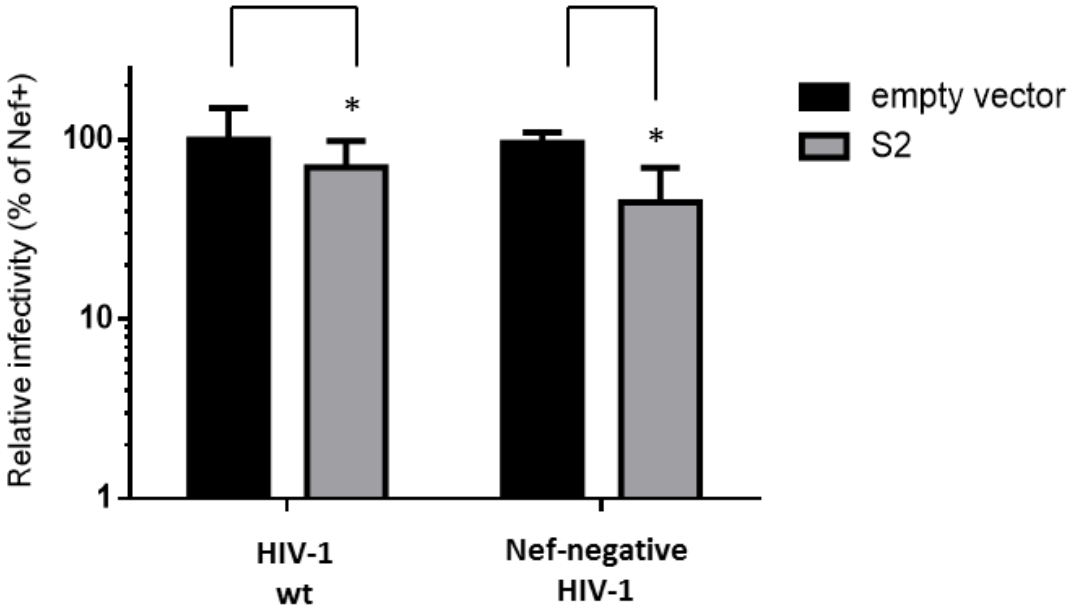


Figure 3.4

S2 does not affect the infectivity of HIV-1 produced in CEMX174 cells.

Relative infectivity (% of the control Nef+ sample) of HIV-1 wt or Nef-negative, obtained by electroporation of Nef non responsive CEMX174 cells line, in the presence or in the absence of EIAV S2 provided *in trans*.

HIV-1 wt or Nef-negative was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2 or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

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S2 does not affect MLV infectivity

As HIV requires Nef for infectivity, MLV requires glycoGag[82]. We therefore tested whether S2 can replace the activity of glycoGag for MLV infectivity. A MoMLV which expresses GFP in place of *env* was pseudotyped with the envelope glycoprotein derived from the xenotropic NZB isolate by transfecting JTAg cells. Wt or glycoGag-negative virus was produced in the presence or absence of the S2-expressing construct. The level of infection of TE671 target cells was scored in flow cytometry. As shown in Figure 3.5, the absence of glycoGag introduces a 12-fold defect on infectivity. Co-transfection of the S2 expressing plasmid, however, did not rescue virus infectivity, suggesting that S2 does not functionally replace glycoGag (Figure 3.5). This is reminiscent of a result reported previously, which indicates that while glycoGag can rescue the infectivity of Nef-defective HIV-1, Nef cannot rescue the infectivity of the glycoGag-defective MLV[82].

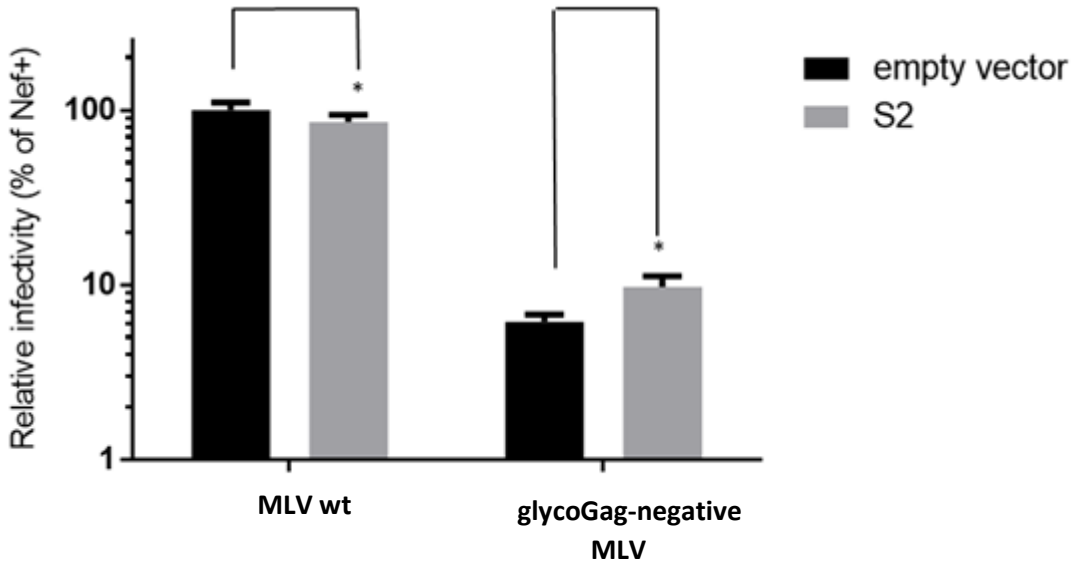


Figure 3.5

EIAV S2 does not rescue the infectivity of glycoGag-negative MLV.

Relative infectivity of MLV wt or glycoGag-negative viruses, produced in JTAg cells in the presence or in the absence of EIAV S2 provided *in trans*.

MLV wt or glycoGag-negative MLV are complemented with and MLV-NZB-9-1 envelope. 4:4:1 ratio between viral backbone, S2 or empty vector and env was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TE671 cells as described in materials and methods and analysed through FACS.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$) Representative experiment out of 3 repetitions.

Immuno-detection of S2

S2 is a 6 KDa protein and the low molecular weight makes it difficult to detect in Western blotting. In addition, no commercial antibody is available. We therefore decided to increase gene expression by constructing an ORF optimized for human codon usage. To facilitate its detection, an HA-tag was added either at the N-terminus and at the C-terminus. The protein expressed by the codon-optimized gene was readily detected in immunoblotting experiments, however, the N-terminal addition of the HA tag appeared to impair expression and functionality of the protein. Codon-optimized S2 with a C-terminal tag, in contrast, showed the same level of rescue of Nef-defective HIV-1 infectivity as untagged S2 expressed from the native cDNA sequence. Having observed that the codon-optimized construct achieves the same effect as S2 expressed from the native sequence, but allows better S2 immune-detection, this was used for all the experiments we performed thereafter (Figure 3.6).

Constructs expressing S2 fused with both C-terminal GFP or RFP were also produced, but the proteins were non-functional and poorly detected and in our assay. (data not shown).

Similarly to the activity of Nef, the ability of S2 to increase HIV-1 infectivity is envelope glycoprotein-dependent.

Retroviruses can incorporate in their lipid envelope heterologous cell surface proteins. Accordingly, envelope pseudotyping is used to modify the tropism of retroviruses. The requirement of Nef for HIV-1 infectivity is known to

depend on the nature of the envelope glycoprotein used to pseudotype the virus[85,113,114]. HIV-1^{nef-} infectivity was tested using different envelope pseudotypes in the absence and in the presence of S2 expression. When HIV-1 is pseudotyped with the envelope glycoproteins derived from VSV-G and from the HIV-1 JR-FL isolate, the virus was found to be not Nef-responsive[85]. Similarly, expression of S2 in producer cells did not affect the infectivity of these virus particles, further suggesting that Nef and S2 target the same infectivity defect (Figure 3.7).

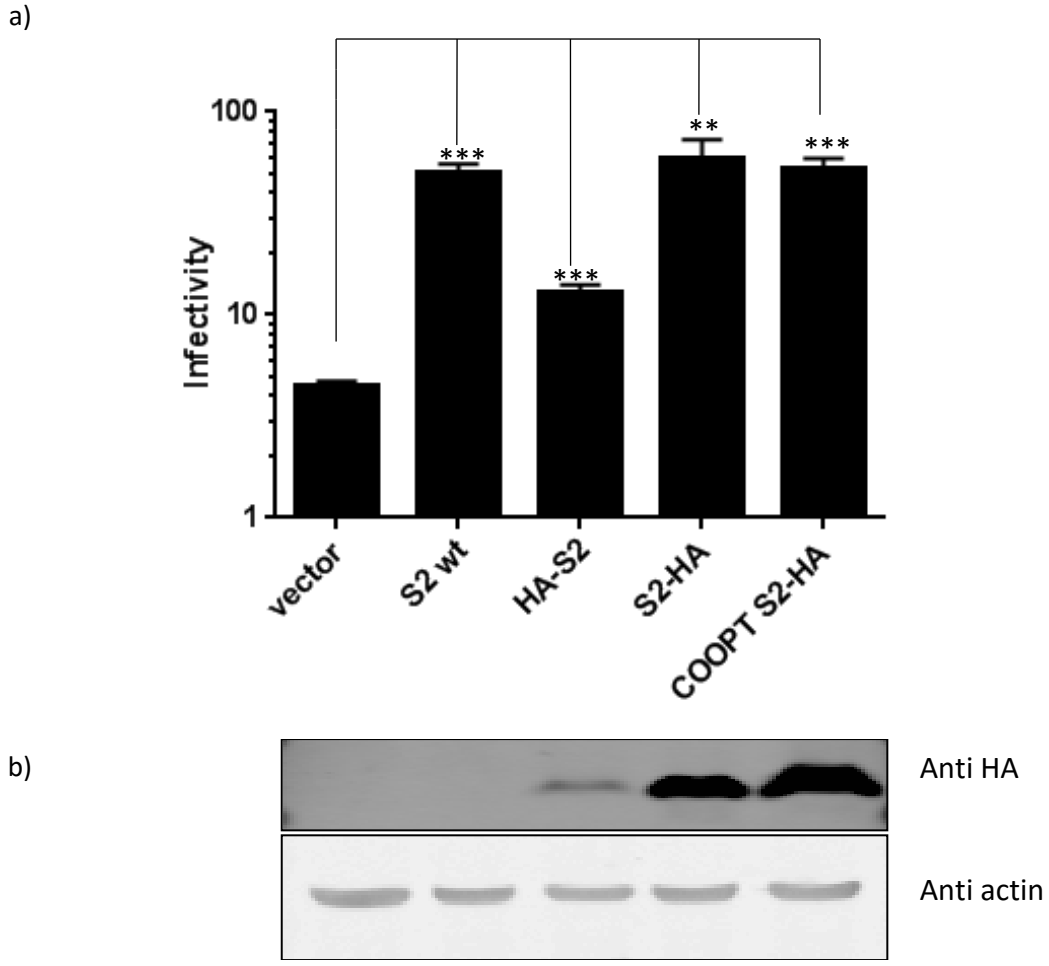


Figure 3.6

Effect of S2 codon optimization and HA-tag addition on gene expression and function.

a) Infectivity of Nef-defective HIV-1, obtained by JTAg electroporation , in the presence or in the absence of S2 wt, HA-S2, S2-HA, codon optimized S2-HA (S2 COOPT).

b) WB of corresponding cell lysates to evaluate the expression level of each S2 expression plasmid.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2 wt/N-terminal HA tagged S2/C-terminal HA tagged S2/codon optimized C-terminal HA tagged S2/ or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Corresponding cell lysates were resolved by SDS-PAGE and analysed by WB. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

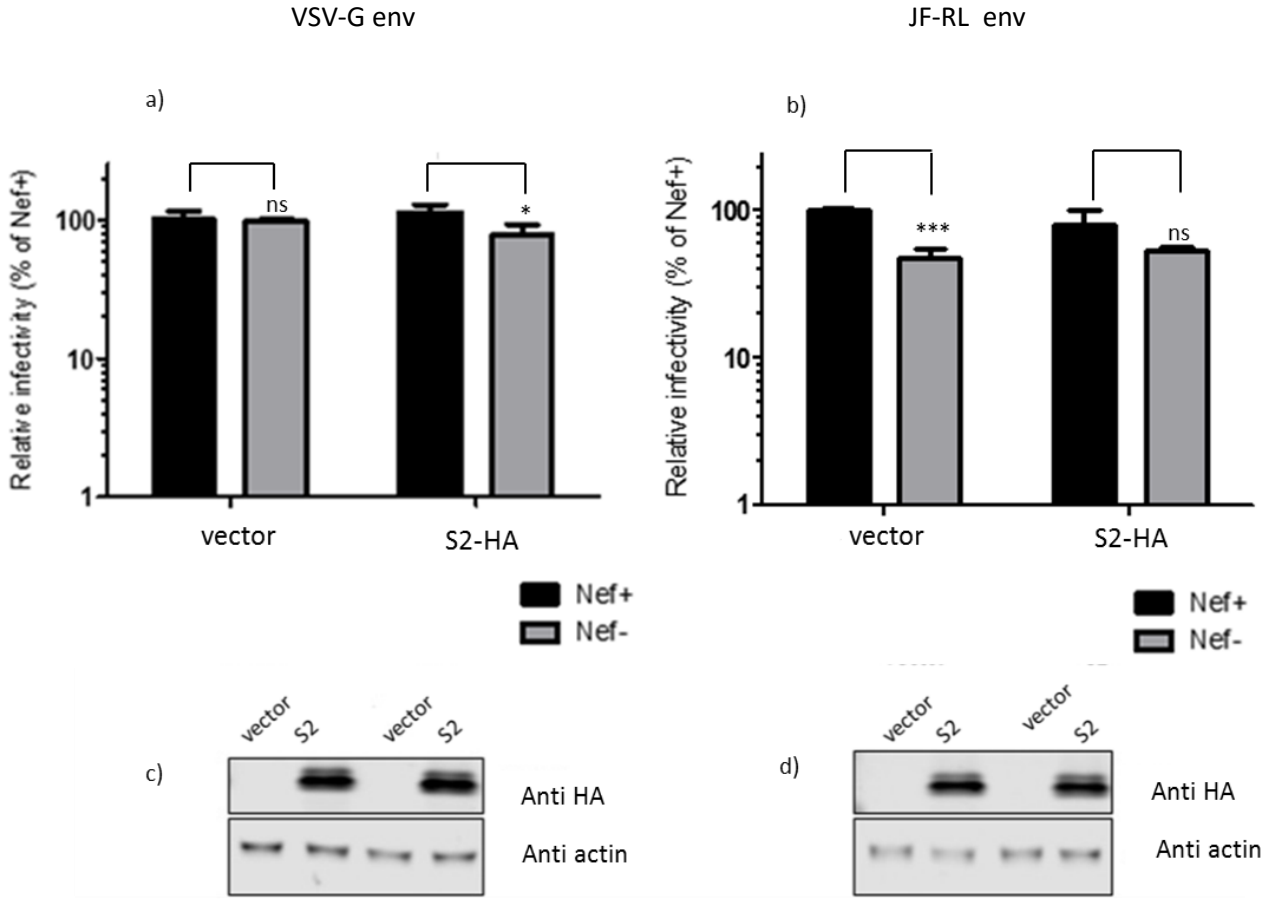


Figure 3.7

Envelope pseudotype dependence.

a,b) Relative infectivity (% of the control Nef+ sample) of HIV-1 wt or Nef-negative pseudotyped with VSV-G or JR-FL isolate envelope, in the presence or in the absence of EIAV S2-HA provided *in trans*.

c,d) Evaluation of expression level of S2 expression plasmid.

HIV-1 wt or Nef-negative is obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and for *env* and *nef* and complemented with pbj5 expressing VSV-G or HIV1$^{JF\text{-}RL}$*env*. 4:4:1 ratio between viral backbone, S2-HA or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

The involvement of endocytosis in S2 activity

A conserved ExxxLL motif, potentially interacting with AP2, is present within the S2 aa sequence. To investigate its relevance for S2 activity on infectivity, the ExxxLL motif was mutated to ExxxAA and tested in our infectivity assay using Nef-defective HIV-1 (Figure 3.8a). As shown in Figure 3.9b, the mutant protein loses its ability to increase HIV-1 infectivity, despite the mutation not affecting protein expression in the producer cell lysates, demonstrating the functional importance of the LL motif.

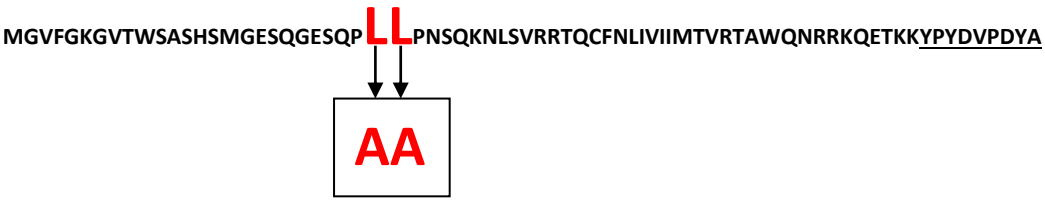
A potential SH3 binding PxxP motif is also present in S2 and adds another element of similarity with Nef. The PxxP motif is overlapping with the ExxxLL motif (ESQPLLP). We therefore investigated whether PxxP plays any role in S2 activity by mutating both prolines into alanines (AxxA). The mutant protein resulted as active as the wt protein excluding a role of such motif in the infectivity effect (Figure 3.9). This is in line with the evidence that the similar PxxP sequence does not contribute to the effect of Nef on infectivity. The crucial role of the ExxxLL motif prompted further experiments to investigate the involvement of the endocytic machinery in the activity of S2.

First, we investigated the intracellular localization of S2. Immunofluorescence microscopy was performed on JTAg cells transfected with the construct expressing S2-HA, stained with anti-HA antibody. Images revealed that the protein is localized at the plasma membrane and also present in a perinuclear compartment, reminiscent of the localization of Nef and glycoGag[82]. Indeed, when S2-HA was co-expressed together with Nef-GFP, the two proteins

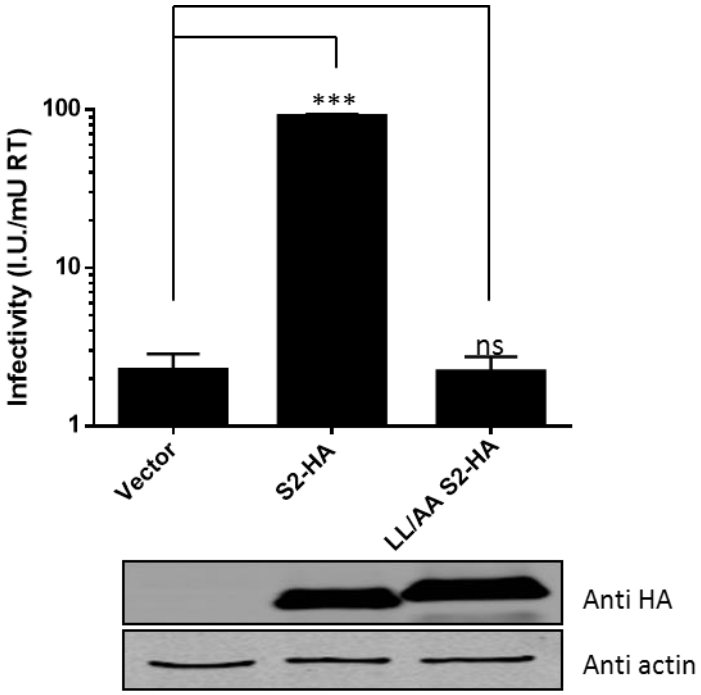
colocalized, suggesting that, as demonstrated with Nef and glycoGag, S2 associates with a late endocytic intracellular compartment (Figure 3.10).

Then, we investigated whether clathrin-mediated endocytosis is required for S2 activity. We tested whether the effect of S2 is sensitive to inhibitors of the endocytosis machinery. The C-terminal fragment of AP-180 and the dynamin2 K44A transdominant negative mutant potently inhibit clathrin mediated vesicle biogenesis and were reported to inhibit the Nef effect[80,115]. In the presence of both inhibitors S2 failed to recover HIV-1^{nef-} infectivity, indicating that a functional endocytic machinery is required for the ability of S2 to affect retrovirus infectivity. (Figure 3.8 c and d).

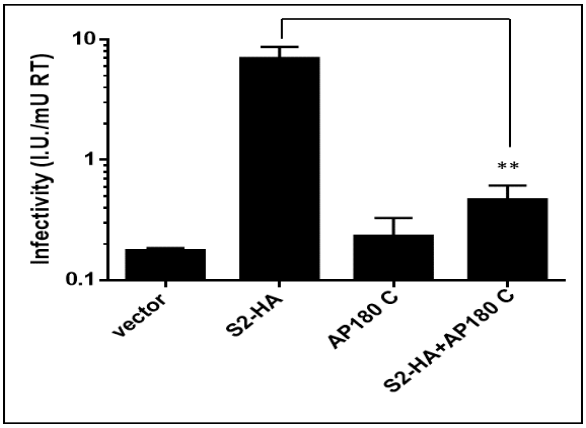
a)


MGVFGKGVTWSASHSMGESQGESQP LL PNSQKNLSVRRTQCFNLIVIIMTVRTAWQNRRKQETKKYPYDVPDYA
AA

b)


Infectivity (I.U./mU RT)
100
10
1
***
ns
Vector
S2-HA
LL/AA S2-HA
Anti HA
Anti actin

c)


Infectivity (I.U./mU RT)
10
1
0.1
**
vector
S2-HA
AP180 C
S2-HA+AP180 C

d)

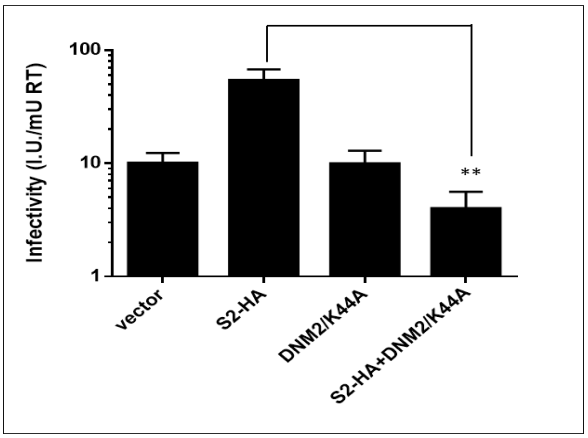

Infectivity (I.U./mU RT)
100
10
1
**
vector
S2-HA
DNM2/K44A
S2-HA+DNM2/K44A

Figure 3.8

Involvement of the EXXXLL motif on S2 activity.

a) Schematic representation of amino acid sequence of S2-HA LL/AA mutant.

b) Infectivity values and corresponding expression levels in cell lysates of Nef defective HIV-1 alone and together with S2-HA wt or LL/AA S2 –HA produced in JTAg cells

c), d) Nef-negative HIV-1 obtained by transfection of JTAg cells in the presence or in the absence of S2-HA and two different endocytosis inhibitors separately tested: AP180 C-terminal and Dynamin K44A.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2-HA or empty vector and *env* was used. 1:1.5 ratio between S2-HA and each endocytosis inhibitor tested separately was provided. Supernatant of JTAG producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. .Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

a)

b)

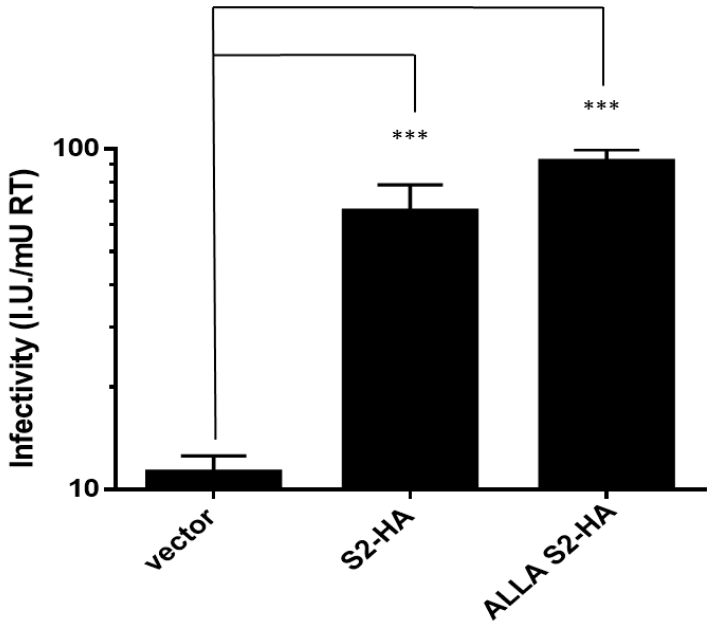


Figure 3.9

Involvement of SH3 binding domain on S2 activity.

a) Schematic representation of amino acid sequence of S2-HA ALLA mutant.

b) Infectivity values of Nef-defective HIV-1 alone and together with S2-HA wt or ALLA S2–HA mutant produced in JTAg cells.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2-HA/ALLA S2-HA or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

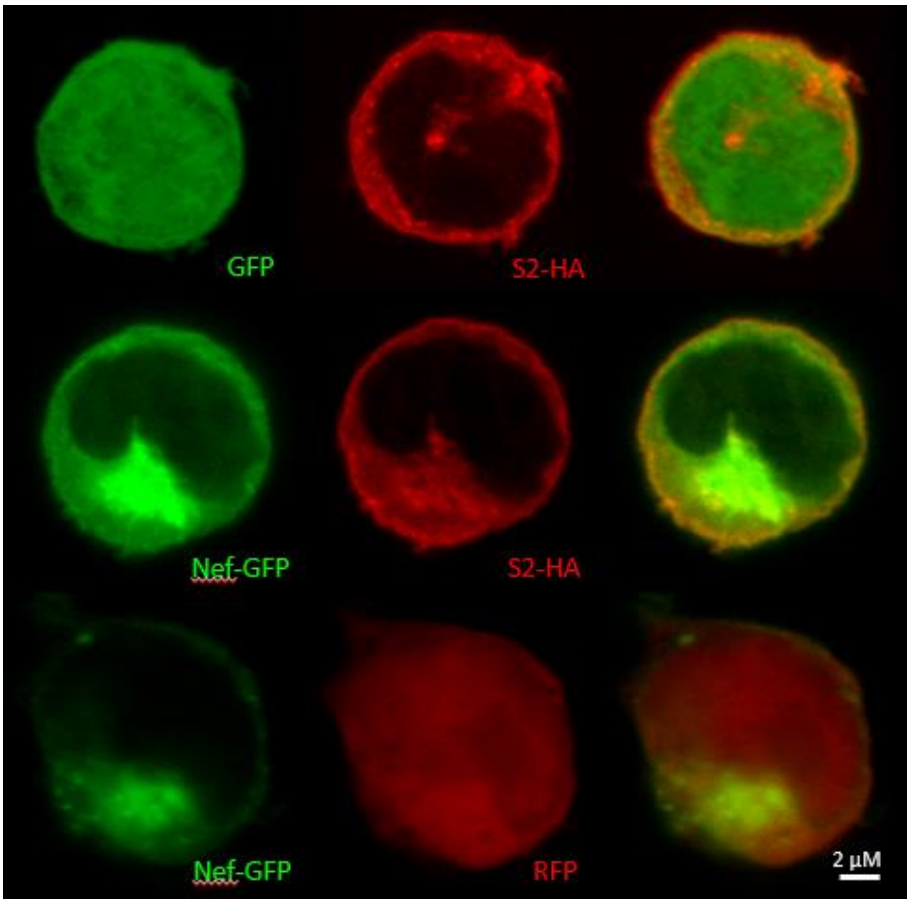


Figure 3.10
EIAV S2 intracellular localization.
a) Confocal microscopy of JTAg cells transfected to drive the expression of S2-HA along with eGFP used as control.
b) Confocal microscopy of JTAg cells transfected with S2-HA along with NefGFP to compare cellular localization.
c) Confocal microscopy of JTAg cells transfected to drive the expression of Nef-GFP along with RFP used as control.

As already discussed, Nef is a myristoylated protein; on the other hand glycoGag is a type II transmembrane protein. In both cases, the ability to interact with the plasma membrane is a requirement for their effect on infectivity. We therefore investigated whether S2 presents some features which allow an interaction with the membrane.

Using the transmembrane topology predictor TMpred, we evaluated whether S2 contains a transmembrane helix. The TMpred program functions using an algorithm based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight-matrices for scoring[116]. A putative TM domain was found by TMpred within the S2 hydrophobic region, between aa in position 33 and 55; we therefore tested whether this sequence is required for S2 activity by inserting several substitutions (Figure 3.11a) which disrupt the hydrophobic motif (Figure 3.11c). Accordingly, the mutated protein was no longer predicted by TMpred to contain a transmembrane helix. However, this modified S2 retained the ability to increase the infectivity of Nef-defective HIV-1, similarly to the wt protein, suggesting that the predicted transmembrane domain plays no functional role.

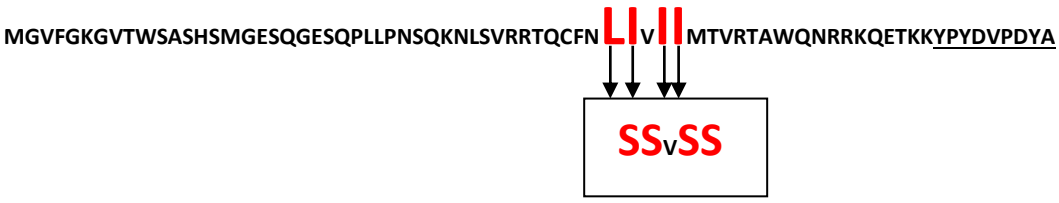
Another mechanism which mediates association of proteins to the plasma membrane is *via* N-terminal myristoylation. Myristoylation is a co-translational or post-translational modification derived from covalent addition of a myristic acid moiety at the N-terminal glycine residue of the protein. We therefore explored the possibility that S2, similarly to Nef, is myristoylated.

Interestingly, Yoon et al.[112], suggested that S2 contains a putative myristoylation signal. While the aa sequence of myristoylation signals is not well defined, a glycine at the N-terminal is required[117]. Interestingly, a N-terminal glycine in S2 is highly conserved. To investigate the role of such residue, a G to A mutation was inserted to generate an S2 variant that would not be myristoylated. Expressed in JTAg HIV-1 producer cells, the G2A mutant, expressed at a level similar to the wt protein, did not affect virus infectivity (Figure 3.12), indicating that the N-terminal glycine plays a crucial functional role, and supporting the hypothesis that this could be the substrate of myristic acid addition by the N-myristoyl transferase.

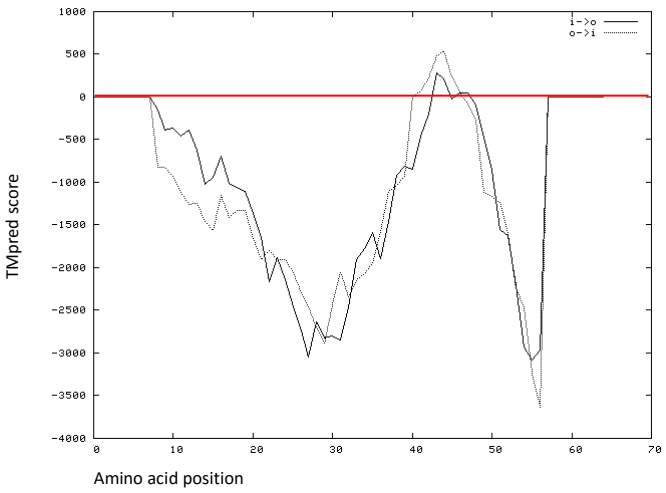
To directly assess whether S2 is myristoylated *in vivo*, a CLICK chemistry-based assay[118] was used. HEK293T cells were transfected to drive the expression of S2-HA wt or the G2A mutant and cultured in the presence of azide-labelled myristic acid. Myristoylated proteins were then captured in cell lysates by alkyne-coupled beads. The presence of myristoylated S2 in the pull-down was verified by Western blot analysis using an anti-HA antibody.

As shown in Figure 3.13, a signal was detected when S2 wt, but not the G2A mutant, was expressed. Confirming the significance of the assay, a similar result was obtained with Nef-HA used as a positive control. No signal was detected with Cyclophilin A-HA, a protein that is not myristoylated, or with Nef G2A mutant, both used as a negative controls.

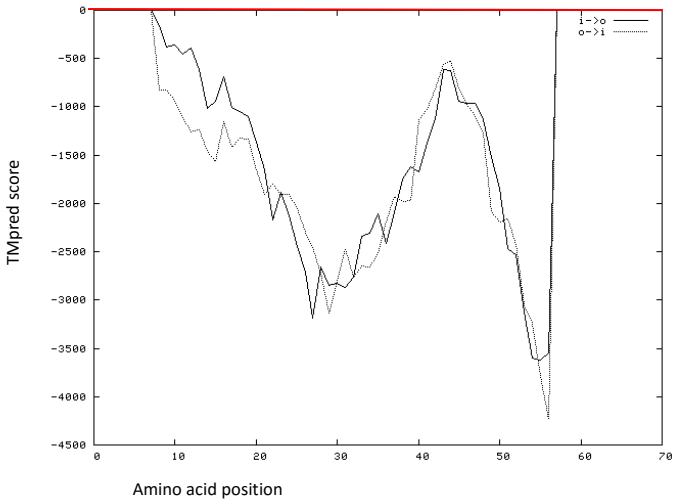
a)


MGVFGKGVTWSASHSMGESQGESQPLLPNSQKNLSVRRTQCFN LI V II MTVRTAWQNRRKQETKKYPYDVPDYA
SSvSS

b)


TMpred score
Amino acid position
i->o
o->i

c)


TMpred score
Amino acid position
i->o
o->i

d)

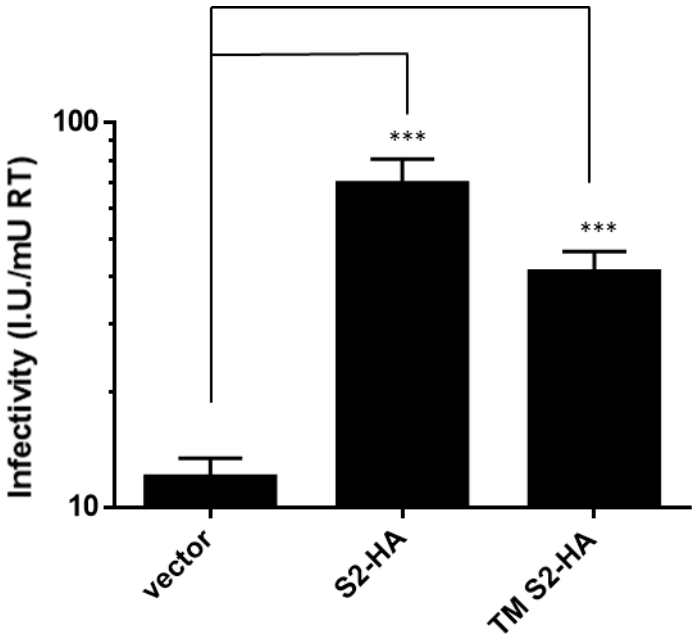

Infectivity (I.U./mU RT)
100
10
***
***
vector
S2-HA
TM S2-HA

Figure 3.11

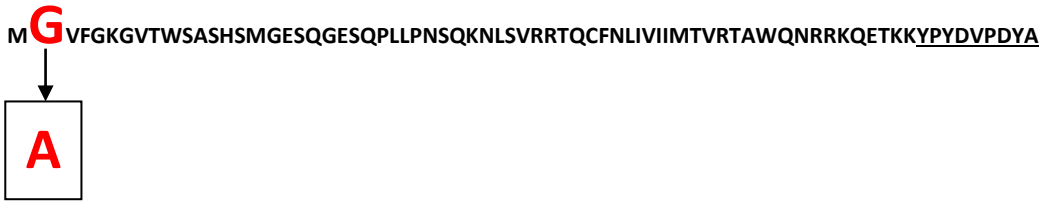
S2 protein topology investigation.

a) Schematic representation of amino acid sequence of S2-HA TM mutant.

b), c) bioinformatic prediction of putative transmembrane domains for S2 wt and S2 mutant respectively. Red lines on figures indicate the threshold background.

d) Infectivity values of Nef-defective HIV-1 alone and together with S2-HA wt or S2–HA transmembrane mutant. Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2-HA wt/S2-HA TM mutant or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values represent the average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

a)



b)

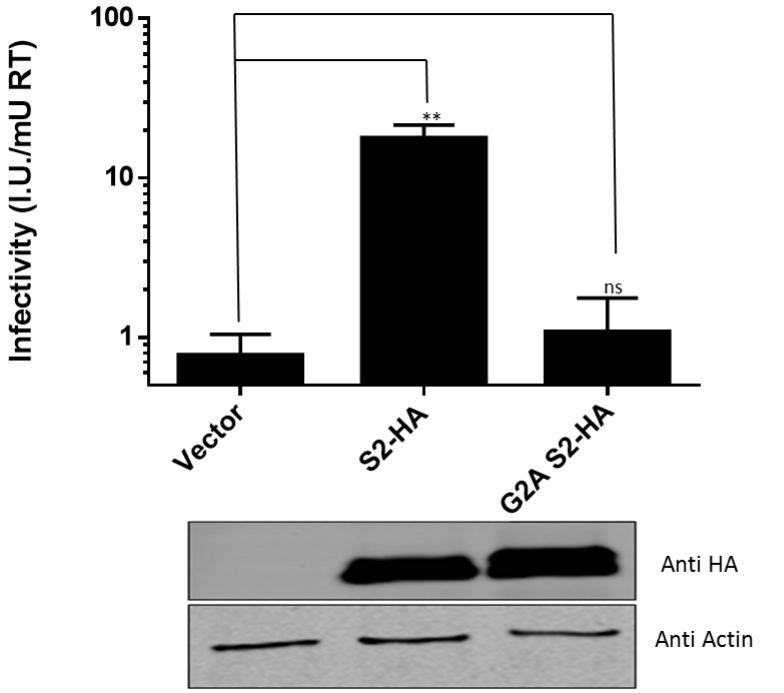


Figure 3.12

Importance of the N-terminal glycine for S2 activity.

a) Schematic representation of amino acid sequence of S2-HA G2A mutant.

b) Infectivity values and S2-HA and G2A S2-HA expression levels in corresponding cell lysates.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. Electroporations are performed in JTAg used as producer cells. 4:4:1 ratio between viral backbone, S2-HA/ G2A S2-HA or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Corresponding JTAg cell lysates are resolved by SDS-PAGE and analysed by WB.

Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

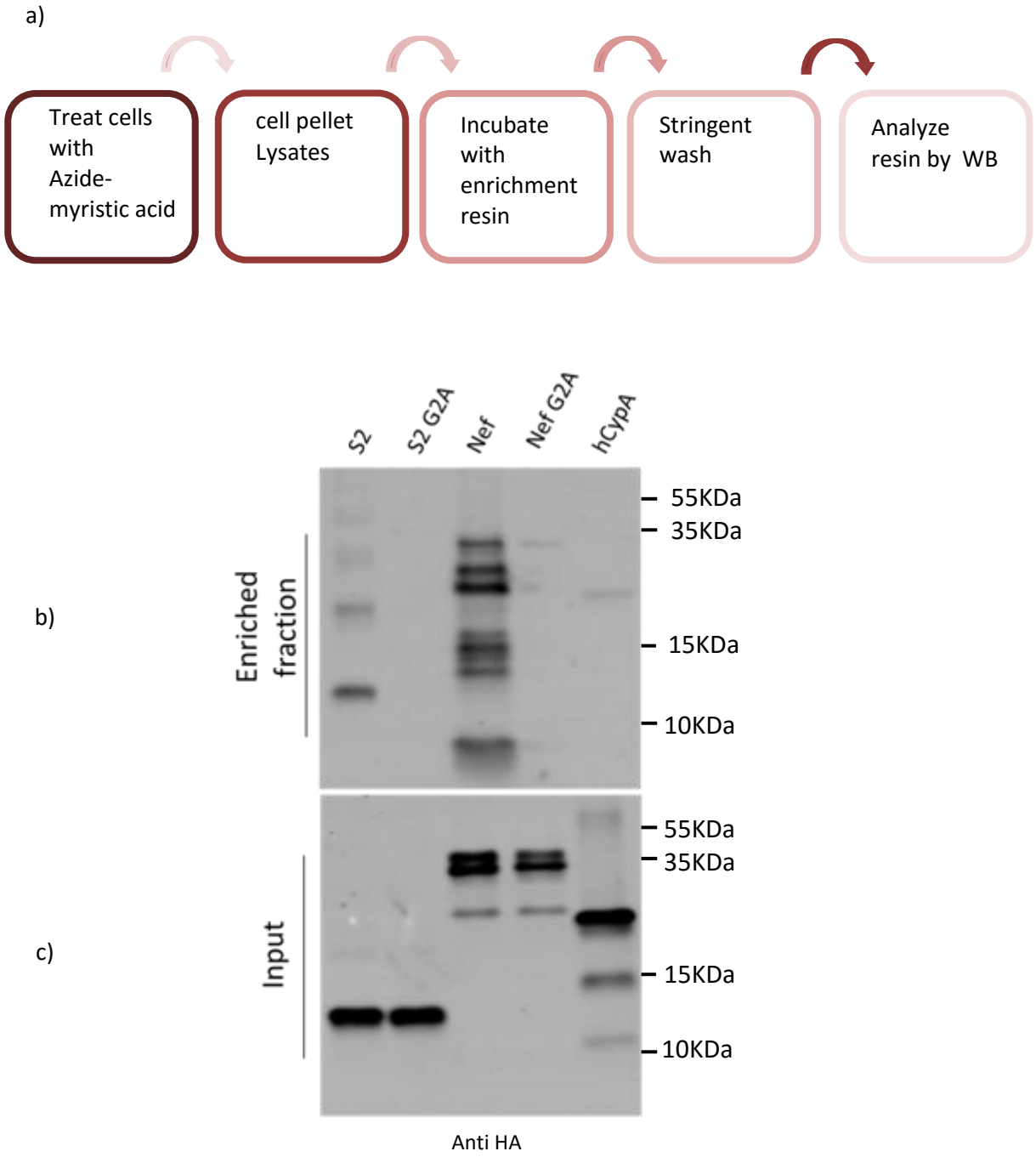


Figure 3.13

Detection of myristoylated S2.

a) Click-IT chemistry methodology (for further information see materials and methods section). WB analysis of input (c) and enriched fraction (b) for detection of HA-tagged S2 wt, S2 G2A, Nef and Nef G2A mutant used as positive controls and hCyp used as negative control.

The S2 activity against SERINC5

While investigating the role of S2 in infectivity, SERINC5 was identified by our laboratory as the retrovirus inhibitor counteracted by Nef and glycoGag[85].

Given that all the experiments in our hands demonstrated that S2 is functionally related to Nef and glycoGag, its ability to counteract SERINC5 was therefore investigated next.

To this end, two strategies were followed:

- Testing whether the activity of S2 on HIV-1 infectivity in JTAg cells requires SERINC5 and SERINC3. This was achieved using a JTAg$^{SERINC5-/-}$ and the double knock out JTAg$^{SERINC5-/-\ SERINC3-/-}$ cell lines developed in our laboratory using a CRISPR-Cas9 vector.
- Testing the ability of S2 to counteract exogenously expressed SERINC5 in a cell line which naturally lacks its expression.

S2 counteracts SERINC5 and SERINC3

Two JTAg-based cell lines were established in our lab using the PX458 CRISPR-Cas9 vectors which express simultaneously the nuclease together with the specific sgRNA. The two cell lines where established sequentially by first targeting SERINC5 (JTAg$^{SERINC5-/-}$) gene sequence and subsequently by targeting SERINC3 on the same cell line (JTAg$^{SERINC5-/-\ SERINC3-/-}$)[85].

As already reported, compared with the infectivity of HIV-1 produced in JTAg wt cells, Nef-defective HIV-1 from JTAg$^{SERINC5-/-}$ cells was 6-fold more infectious while the same virus from the double KO was 18-fold more infectious. This inversely correlated with the effect of S2 on infectivity of the Nef-defective virus which was 9-fold for virus produced in WT JTAg, 3-fold from virus derived from JTAg$^{SERINC5-/-}$ and none for virus derived from the double knock out cells. This mirrors the effect of Nef on the same viruses and demonstrate that the effect of S2 requires SERINC5 and SERINC3 (Figure 3.14).

Furthermore, the effect of S2 was tested on ectopically expressed SERINC5. When Nef-defective HIV-1 was produced in HEK293T cells, which do not naturally express high levels of SERINC5[85], increasing ectopic expression of SERINC5 resulted in an increasing inhibition of the virus only in the absence of S2. In contrast, when S2 was present in the producer cells, the infectivity of the virus was preserved, irrespectively of the amount of SERINC5 being transfected, further demonstrating that S2 acts on HIV-1 infectivity by counteracting the host factor (Figure 3.15a).

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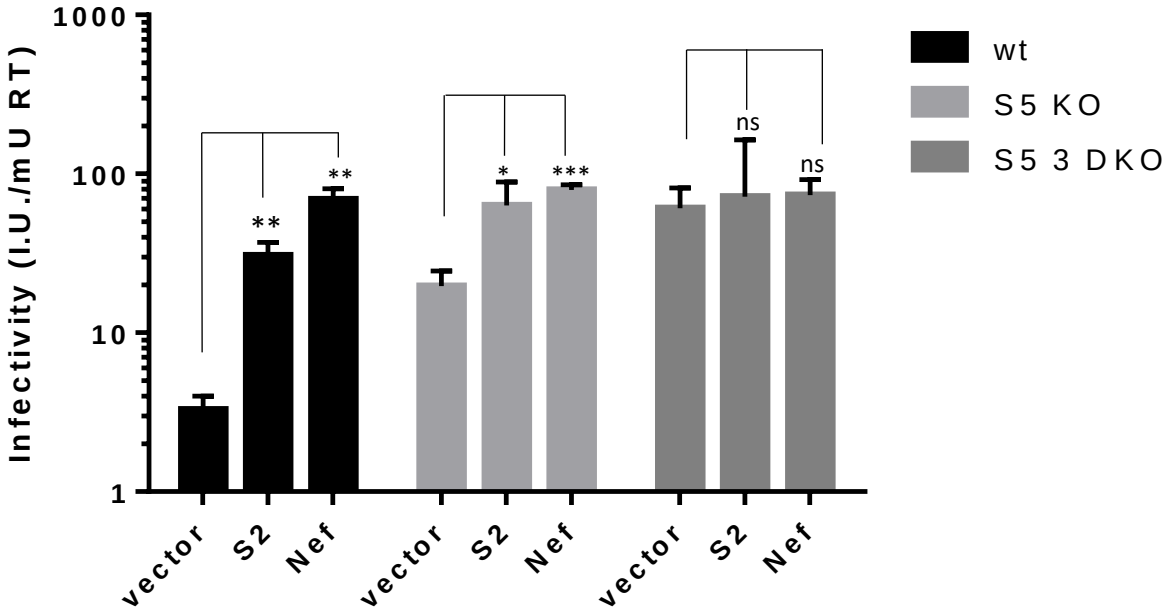
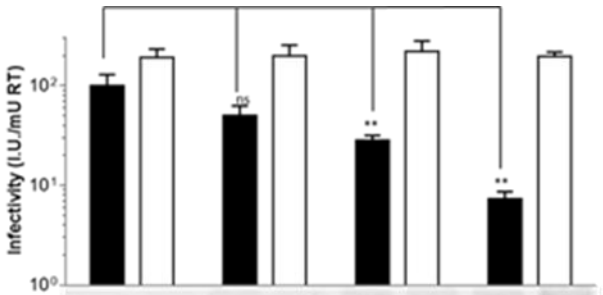


Figure 3.14

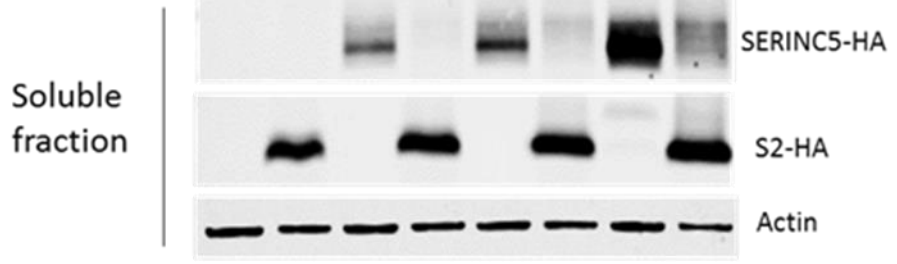
EIAV–S2 effect on HIV-1 produced in SERINC5 and SERINC3 DKO cells.

Infectivity of Nef-negative HIV-1 obtained by electroporation of JTAg SERINC5 KO or SERINC5 and SERINC3 DKO, in the presence or in the absence of EIAV S2 or Nef used as positive control.
Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4:4:1 ratio between viral backbone, S2 or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect JTAG, JTAg SERINC5 KO or SERINC5 and SERINC3 DKO as described in materials and methods and analysed through FACS. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

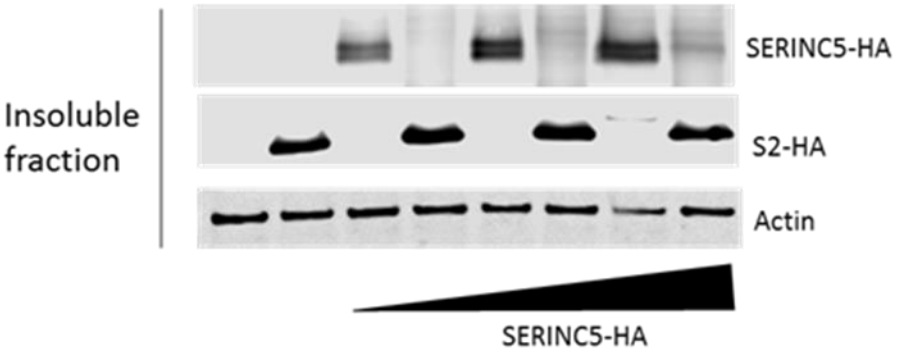
a)


Infectivity (I.U./mU RT)
10^2
10^1
10^0
ns
**
**

b)


Soluble fraction
SERINC5-HA
S2-HA
Actin

c)


Insoluble fraction
SERINC5-HA
S2-HA
Actin
SERINC5-HA

d)

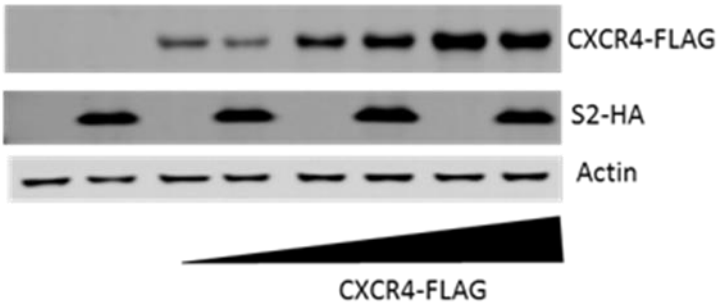

CXCR4-FLAG
S2-HA
Actin
CXCR4-FLAG

Figure 3.15

EIAV–S2 effect in overexpressing condition of increasing amount of SERINC5 in Nef low responsive HEK293T cell line.

a) Infectivity of HIV-1^{nef-} virus produced in HEK293T cells in the presence of S2-HA and 0.125, 0.25 and 0.5 µg of PBJ6 SERINC5-HA.

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. 4::4:1 ratio between viral backbone, S2 or empty vector and *env* was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

Corresponding cell pellets were collected from both the soluble b) and the insoluble fraction c) resolved by SDS-PAGE and analysed by WB. d) S2-mediated degradation of 0.125, 0.250, 0.5 µg of CXCR4 receptor was also tested as negative control.

S2 promotes SERINC5 endocytosis

As we have already demonstrated, clathrin-mediated endocytosis and a putative AP2 binding motif is crucial for the activity of S2 on infectivity. As it has been demonstrated that Nef and glycoGag cause SERINC5 accumulation from the cell membrane into the late endosomal compartment, we verified, using immunofluorescence microscopy, whether S2 could achieve the same effect. While JTAg transfected to express SERINC5-GFP together with a control vector expressing TagRFP, the host factor was almost exclusively localized on the cell surface. When cells were transfected to express S2-HA, on the other hand, the host factor appeared to accumulate, together with S2-HA, into a perinuclear compartment. When vectors expressing S2-HA and SERINC5-GFP were co-transfected into JTAg cells together with a plasmid expressing RAB7-RFP, the host factor appeared to co-localize with the marker for late endosomes, indicating that, like Nef and glycoGag, S2 promotes SERINC5 endocytosis. (Figure 3.16)

S2 prevents SERINC5 incorporation into virions

We next assessed whether, in analogy with Nef and glycoGag, the effect of S2 on SERINC5 intracellular localization reflects on the level of incorporation of the host factor into virus particles. Western blotting of pelleted Nef-defective HIV-1 produced by transfecting HEK293T cells also expressing SERINC5 revealed that, while the host factor is readily detected when S2 is absent,

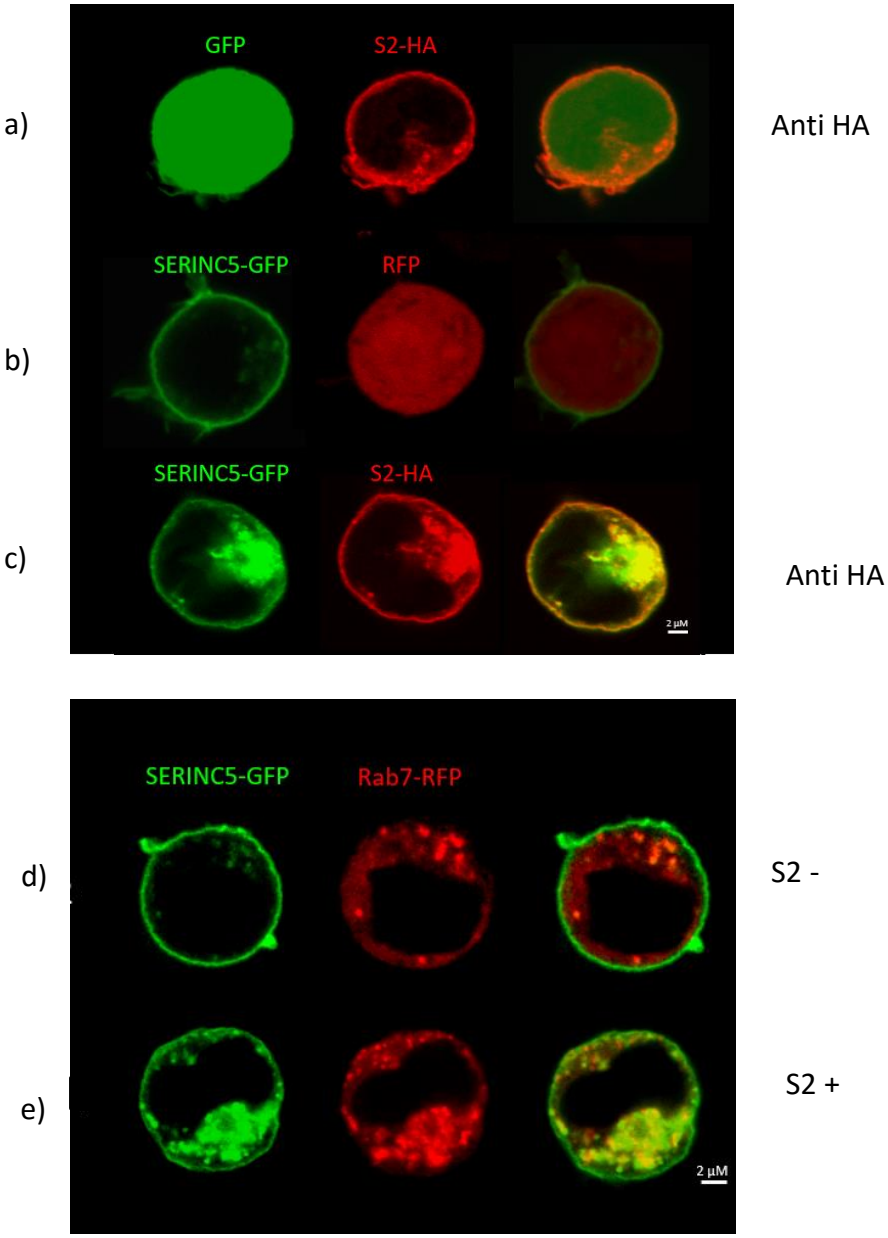


Figure 3.16

EIAV S2 mediates SERINC5 re-localization into a late endosomal compartment.

Confocal microscopy of JTAg cells transfected to drive the expression of S2-HA a), SERINC5-GFP b) or S2-HA along with SERINC5-GFP c). GFP and RFP are used as control respectively for panel a) and b)

Confocal microscopy of JTAg cells transfected with SERINC5-GFP together with Rab7-RFP, in the presence d) or in the absence of S2 e). (S2 is not shown in panel d and e)

Representative experiment out of 3 repetitions.

when the EIAV factor is expressed, SERINC5 is mostly excluded from virus particles. Like Nef and glycoGag, therefore, S2 prevents virus incorporation of SERINC5 (Figure 3.17).

Western blotting of producer cell lysates revealed that the level of steady state expression level of SERINC5 was markedly decreased when S2 was co-expressed. This evidence, which could indicate that S2 promotes degradation of SERINC5, was further investigated.

S2 affects the steady-state expression level of SERINC5

HEK293T cells were cotransfected with pcDNA3.1 S2-HA along with increasing amounts of a pcDNA3.1 SERNIC5-HA. To take into account possible effects deriving from promoter competition, the total amount of plasmid transfected was equalized among samples by adding empty pcDNA3.1 vector. Western blot analyses from the soluble fraction of lysates produced by lysing cells with a DDM-based buffer revealed that while SERINC5 was readily detected in the absence of S2 expression, the presence of the EIAV accessory protein resulted in a drastic decrease of SERINC5 expression, irrespectively of the amount of SERINC5-HA plasmid transfected.

The same result was observed when the insoluble fraction of the cell lysates was analysed. This excludes the possibility that failed SERINC5 detection in the lysate could be explained by the formation of SERINC5 insoluble aggregates caused by S2, and lost when the cell lysate is clarified by centrifugation.

To further investigate whether this effect is SERINC5-specific, we investigated whether S2 affects steady state expression level of another multispanning transmembrane protein (CXCR4-Flag) expressed from the same vector (pcDNA3.1). Results show unequivocally that S2 does not affect CXCR4-FLAG expression, indicating that its effect on SERINC5 is specific and suggesting that the EIAV protein could promote a fast degradation of the host restriction factor (Figure 3.15).

S2 is incorporated into retrovirus particles

Figure 3.17 indicates that S2 is associated into retroviral particles. This is reminiscent of the evidence that Nef, which is myristoylated, is also found incorporated into HIV-1 virions. Nef is thought to be taken up passively into viral particles, as a consequence of its propensity to localize with the cell membrane. We further investigated whether the presence of S2 depends on the myristoylation of the EIAV protein or on its putative ability to interact with AP2. Western blotting of HIV-1 producer HEK293T cells expressing S2-HA or the G2A and ExxxAA mutants pelleted through a sucrose cushion revealed that in all three cases the protein is equally and abundantly associated with viral particles, indicating that virus incorporation of S2 is independent of the protein myristoylation or its possible association with AP2. Together with the evidence already discussed, that both mutants are defective for the ability to increase virus infectivity, this result also suggests that the activity of S2 on infectivity does not depend on its ability to associate with virion particles. (Figure 3.18)

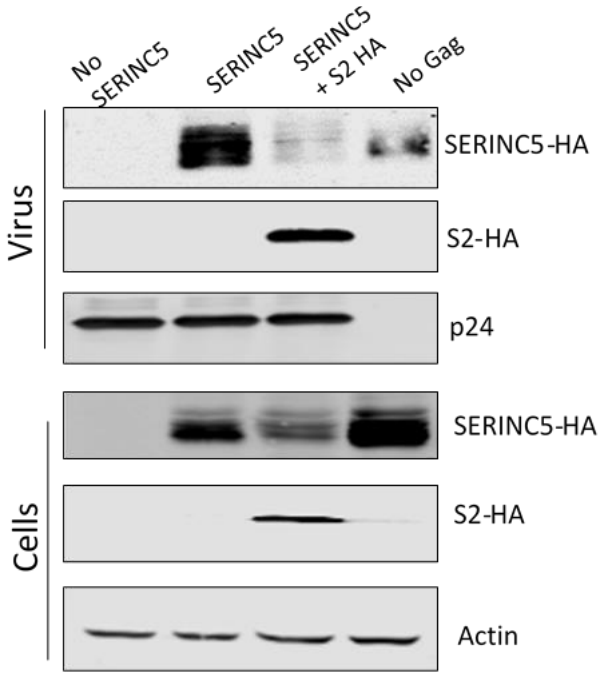


Figure 3.17

EIAV–S2 decreases the amount of SERINC5 both incorporated into virion and in cell lysates.

Viral particles were isolated by ultracentrifugation of supernatant of HEK293T producer cells, transfected with HIV-1^{nef-} virus alone or along with SERINC5 and SERINC5 plus S2-HA. The obtained virions and corresponding lysates were resolved by SDS-PAGE and analysed by WB. Representative experiment out of 3 repetitions.

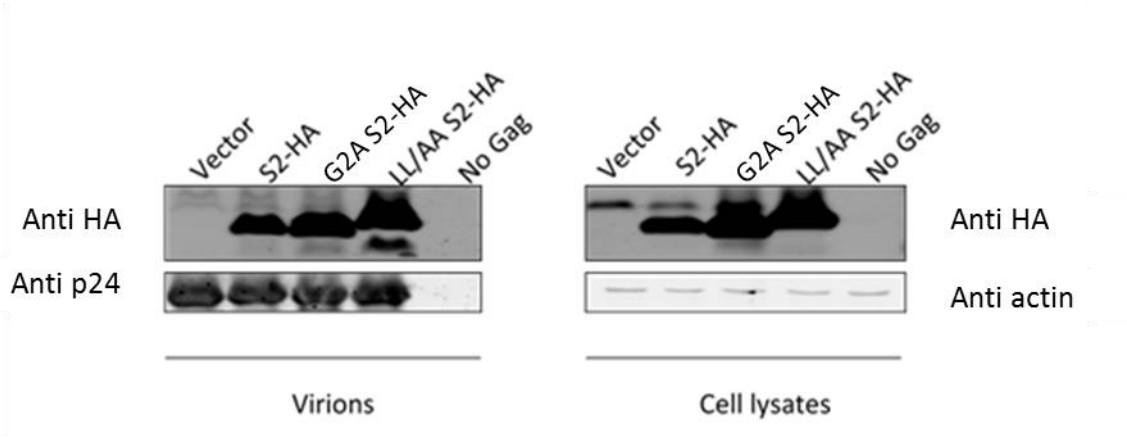


Figure 3.18

S2 incorporation into viral particles.

Viral particles were isolated by ultracentrifugation of the supernatant of JTAg producer cells, transfected with HIV-1^{nef-} virus alone or along with S2-HA, G2A, S2-HA LL/AA S2-HA. The obtained virions and corresponding lysates were resolved by SDS-PAGE and analysed by WB.
Representative experiment out of 3 repetitions.

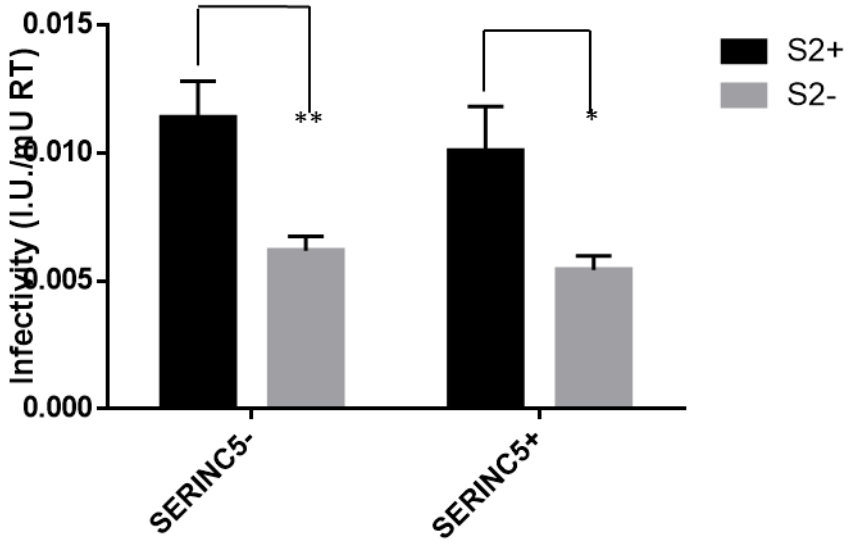
The effects of SERINC5 and S2 on EIAV infectivity

Most of the experiments to investigate the activity of S2 on retrovirus infectivity were conducted using HIV-1 as a model system. This mainly because reagents and assays to study HIV-1 infection are readily available and robust. The activity of EIAV LTR is low in most cell lines, making it difficult to use the entire provirus to study EIAV infection. Systems to produce recombinant EIAV in human cells exist. However, beside the fact that EIAV capsid is targeted by human TRIM5, these vectors are commonly and exclusively used as VSV-G pseudotypes, which are not suitable for our experiments. For our purposes, we obtained a plasmid expressing EIAV Env derived from the pSPEIAV19 isolate, which allowed us to pseudotype EIAV particles derived from a three-part vector system, packaging a GFP reporter gene. A construct encoding the EIAV packaging functions was mutated in order to disrupt the S2 ORF to produce S2-defetive vectors.

A retroviral vector encoding the EIAV receptor, ELR1, was also obtained, which we used to generate EIAV permissive cell lines stably expressing the receptor. Two stable lines expressing ELR1 were generated, based on TZM-bl and JTAg. EIAV vectors were produced in the presence and absence of SERINC5 and S2 by transfecting HEK293T cells. Infectivity of virus particles was measured by flow cytometry on both permissive cell lines. In contrast to results from the previous experiments with HIV-1 vectors, in conditions which results in a 10 to 50-fold effect on infectivity, the effect of SERINC5 on EIAV vector infectivity was negligible. While the infectivity of EIAV vectors was not

affected by SERINC5 when TZM-bl or Jurkat-ELR1 cells were used as target cells. Accordingly, S2 had little effect on the infectivity of the virus. This result suggests that EIAV vectors are resistant to the inhibition by SERINC5 independently on the presence of S2 (Figure 3.19). Previous results in the lab suggest that heterologous envelope glycoproteins promoting retrovirus fusion into a endocytic vesicle render the virus particle resistant to SERINC5. Interestingly, EIAV Env was reported to mediate infection via a pH-dependent entry pathway[9,10]. We therefore investigated whether EIAV Env is capable of making retrovirus particles resistant to SERINC5[85]. To this end, HIV-1 was pseudotyped with EIAV Env and produced in the presence or absence of SERINC5. The infectivity of the virus was marginally affected (3-fold) in the presence of SERINC5, while in the same conditions, HIV-1 bearing native Env was inhibited 16-fold, indicating that EIAV Env confers at least partial resistance to SERINC5. The presence of S2 was sufficient to rescue the infectivity of the virus regardless of Env used for pseudotyping (Figure 3.20).

a)



b)

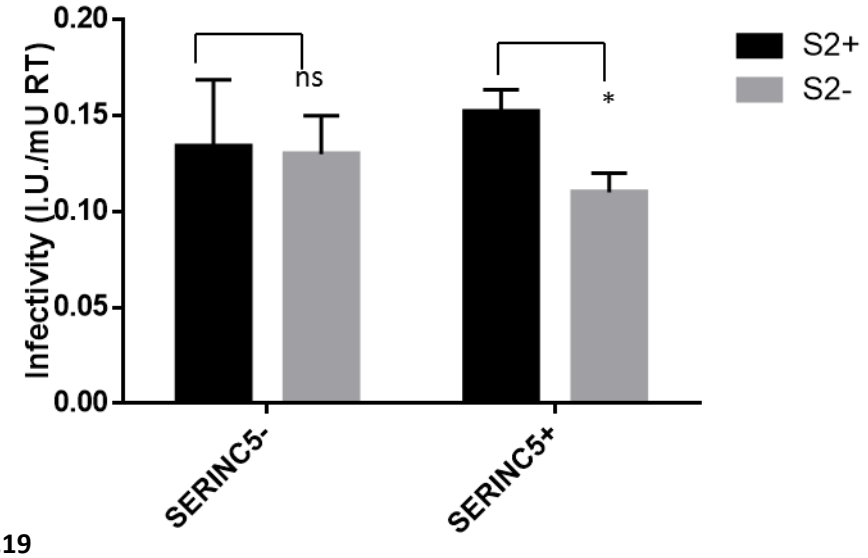


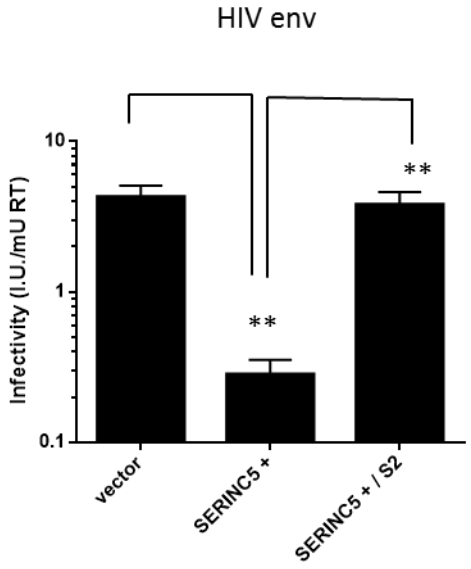
Figure 3.19

Importance of SERINC5 and S2 for EIAV infectivity using different target cell lines.

Infectivity of S2-negative EIAV , produced in HEK293T cells in the presence or in the absence of SERINC5 and S2 provided *in trans*.

S2-negative EIAV was obtained with a three-vector system composed by pEIAV53D, pw sin6.1 and pLG338/30 vectors with a 2:2:1 ratio. S2 and SERINC5 are also provided with 1.5:1 ratio. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-bl ELR1 (a) or JTAg ELR1 (b) as described in materials and methods and analysed through FACS. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

a)



b)

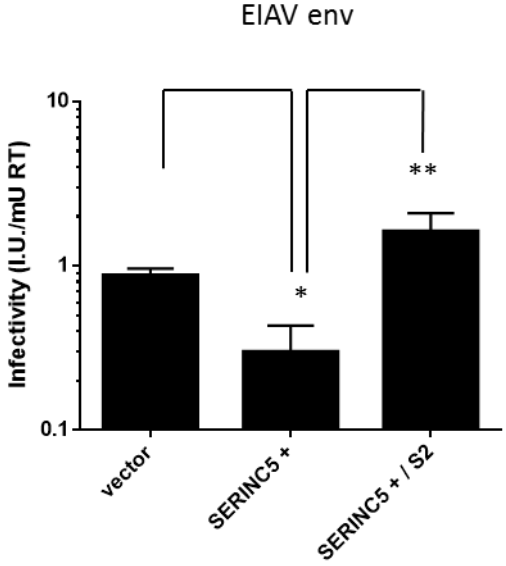


Figure 3.20

EIAV Env acts synergistically together with S2.

Infectivity of Nef-negative HIV-1 produced in HEK293T cells in the presence or in the absence of SERINC5 or SERINC5 plus S2 HA supplemented *in trans* and pseudotyped with HIV-1 a) or EIAV envelope b).

Nef-negative HIV-1 was obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for env and nef and complemented with pbj5 expressing HIV-1^{HXB2} env or pLG338/30 EIAV *env* 4:4:1 ratio between viral backbone, S2 or empty vector and env was used. Supernatant of producer cells was collected 48h post-transfection and used to infect TZM-GFP reporter cells, as described in materials and methods and analysed through automatized microscopy. Infectivity values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* $P<0.05$, ** $p<0.01$, *** $p<0.001$). Representative experiment out of 3 repetitions.

Discussion

Our research has identified S2 as a Nef-like infectivity factor. While my research was developing, SERINC5 was identified as the host restriction factor targeted by Nef and glycoGag. I here showed that S2 is also capable of counteracting the anti-viral activity of SERINC5. Altogether, this highlights the pressure posed by the host factor on diverse retroviruses, which evolved counteracting measures independently. In fact the three retrovirus factors are encoded from different locations of the retrovirus genome and they share no sequence homology. The evidence that viruses adapted to humans, rodents and ungulates are all capable of acting similarly on human SERINC5 denotes a broad spectrum of their antagonizing activity. This is quite unusual if compared to other antagonizing factors. For example, EIAV Env was found to be the counteracting factor of BST2. But while it strongly antagonizes the activity of the equine allele, EIAV Env has no ability to counteract human BST2. Similarly, while glycoGag from MLV was recently uncovered to counteract also the antiviral factor APOBEC3[29], its antagonizing activity is restricted to the murine allele, since glycoGag has no effect against human APOBEC3G. The absence of species-specificity in the case of SERINC5 could be the consequence of its high degree of conservation among different species compared, for example, with BST2. Assuming that the counteracting factor interact directly with SERINC5, one possibility could be that all antagonizing factors recognize a similar moiety of the host factors, which remains the same because it is required for an unidentified conserved cellular activity. Unveiling the molecular features of the interaction with SERINC5 will be

required to comprehend the low species-specificity barrier behind the counteracting activity of S2, glycoGag and Nef.

similarly to Nef and glycoGag, S2 was found to induce the relocalization and prevent virion incorporation of SERINC5. However in respect to Nef and glycoGag, S2 also greatly affects the steady state level of SERINC5. This effect specifically targets SERINC5, since another multipass transmembrane protein (CXCR4) was not affected by S2. This may indicate the ability of S2 to promote SERINC5 degradation, but further studies will be required to fully prove this hypothesis. Generally, transmembrane proteins are degraded by being routed into the lysosomal compartment, in particular by being degraded into multivesicular bodies. This implies that, like a protein degraded by the proteasome, the transmembrane protein is ubiquitinated. Future studies should therefore investigate whether SERINC5 is ubiquitinated in the presence of S2 and whether inhibitors of the lysosomal maturation affect the ability of S2 to decrease SERINC5 expression levels.

S2 was found incorporated into retrovirus particles, an evidence which was never reported before. The meaning and the mechanism of S2 virion incorporation remain unknown and is reminiscent of the ability of Nef to associate not only with HIV-1 but also with heterologous virus particles[119]. Like in the case of Nef, the ability of S2 to associate with virions seems unlikely to play a role on infectivity, since mutations impairing putative AP2 binding and the N-terminal myristoylation, both of which ablate the function on infectivity, do not affect S2 virion incorporation.

While most experiments to characterize the activity of S2 against SERINC5 were performed in the context of HIV-1, my attempts to study the role of S2

in the context of EIAV revealed that the equine lentivirus vector particles we used are substantially refractory to inhibition by SERINC5. A possibility we did not verify is that EIAV could be sensitive to equine SERINC5 but resistant to the human allele. However, we found that the EIAV Env glycoprotein we used has the inherent ability to render also HIV-1 more resistant to SERINC5. This is similar to what has been observed with some Env glycoproteins of HIV-1 and MLV. HIV-1 Env from some primary isolates, such as for example JR-FL, were shown to make the virus unresponsive to Nef by making it resistant to SERINC5. Similarly, Env from MoMLV was observed to confer unresponsiveness to glycoGag by making MLV resistant to SERINC5. Interestingly, EIAV Env was shown to promote virus particle fusion via a pH-dependent pathway, which makes it similar, in this regard, to the Env glycoprotein of ALV, VSV, Ebola and influenza HA. As all of these glycoproteins share the ability to confer resistance to SERINC5, it is possible that fusion *via* an endosomal route could automatically provide a SERINC5-resistant entry pathway. As shown with VSV-G and Ebola-GP, in fact, these glycoproteins do not act by preventing SERINC5 incorporation into virions but rather make virus particles non-sensitive to the effect of SERINC5 in the particles[85]. It will be interesting to verify whether this is the mechanism by which also EIAV Env works. In addition, having tested the effect of only one EIAV *env* allele, it would be interesting to investigate whether this is a general property and verify if, like in the case of HIV-1, Env glycoproteins from different EIAV isolates differ for their ability to confer SERINC5 resistance. In any case, a question arises: why did EIAV (like HIV and MoMLV) evolve a SERINC5-counteracting gene if Env alone can confer SERINC5 resistance? Assuming this is not the consequence of the limitation of our experimental

setting (it is possible that, for example, a defective infectivity would be visible using different cells as targets, such as, in the case of EIAV, primary equine monocytes which are the natural target *in vivo*) one simple explanation would be the need of viruses to develop redundant mechanism to ensure efficient counteraction. An insightful precedent could, for example, be the ability of HIV-1 to target CD4 with both Nef and Vpu to ensure efficient downregulation of the receptor. One additional possibility is that the anti-viral activity of SERINC5 is not limited to targeting the virus particle infectivity, but extends to another, yet unidentified, aspect of virus replication. By analogy with BST2, one suggestive hypothesis is that SERINC5 could also play a role in the anti-retroviral innate immunity by providing one additional mechanism to sense the retroviral pathogen.

Chapter 4: Assessing the role of the SERINC gene family in influenza virus infection

Introduction

Influenza A is an enveloped virus belonging to the Orthomyxoviridae family. Its genome is composed by eight segments of ssRNA able to drive the synthesis of new influenza virus particles.

After entry, negative sense RNA is reverted to positive by an RNA-dependent

RNA-polymerase[120], which is then used as a template for viral proteins production (Figure 4.1).

The virus originally infects aquatic birds but acquires the ability to infect other species thanks to its high genetic flexibility. A central role in the adaptation process is played by intermediate hosts[121].

The *HA* and *NA* genes encode for the viral proteins inserted into the influenza A envelope. They are highly variables in sequence and allow the classification of the virus according to their subtype. It has been estimated that there have been at least 14 pandemics in the last 500 years, of which the last five were well documented[122]. The H3N2 Hong Kong pandemic emerged during 1968 as product of triple recombination between the previous seasonal H2N2 and the avian PB1 and HA subtype 3 genes. In 2009, after a triple recombination between classical swine H1N1, avian H1N1 and seasonal H3N2 a H1N1

pandemic virus evolved which caused more than 200000 deaths[123] (Figure 4.2).

The A/Victoria/3/75 and the A/England/195/2009 I used for my studies derived from epidemic H3N2 and pandemic H1N1 respectively.

Influenza A virus is restricted by IFN-inducible transmembrane proteins (IFTM); IFITM1, 2, 3, act at the level of virus entry, inhibiting fusion between virus end cell membranes. This family inhibits replication of multiple pathogenic viruses including DENV, SARS, CoV, EBOV, MARV and HIV-1[124]. IFTMs represent an example of host factors having activity across several viral families.

While the inhibitory effect of SERINC5 has been established on retroviruses, nothing is yet known about its effect on viruses belonging to other families. Given that SERINC gene products are transmembrane proteins and are likely to gain access to the virus particle via association with the virus lipid envelope, it is conceivable to hypothesize that SERINC proteins could potentially be found associated with enveloped viruses and therefore act as potential inhibitors of viruses not belonging to the Retroviridae family. If this is the case, it is also conceivable that, as observed with different retroviruses, other viruses could have developed the ability to counteract these inhibitors.

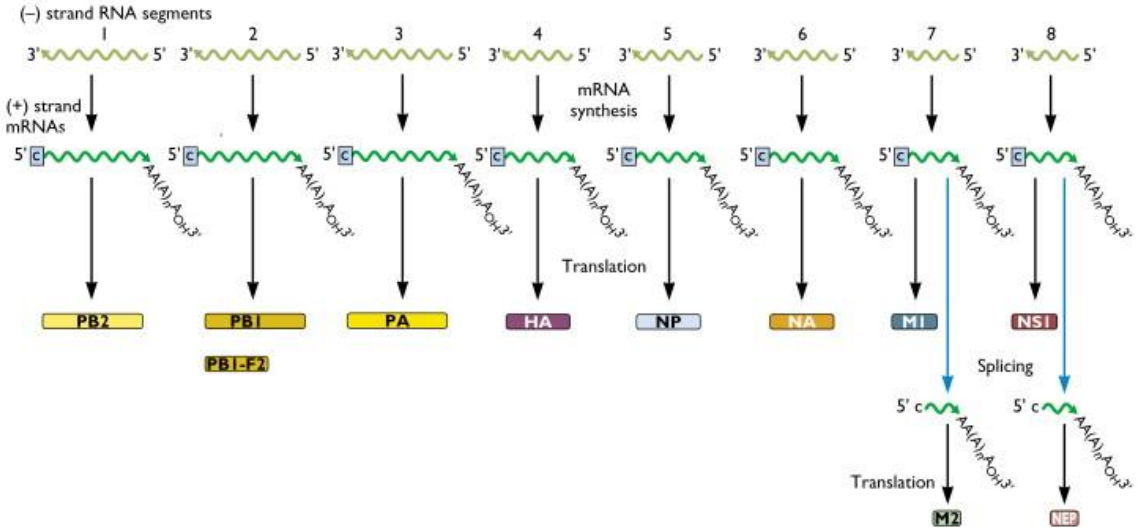


Figure 4.1

Influenza A genome organization.

http://www.virology.ws/2009/05/01/influenza-virus-rna-genome/

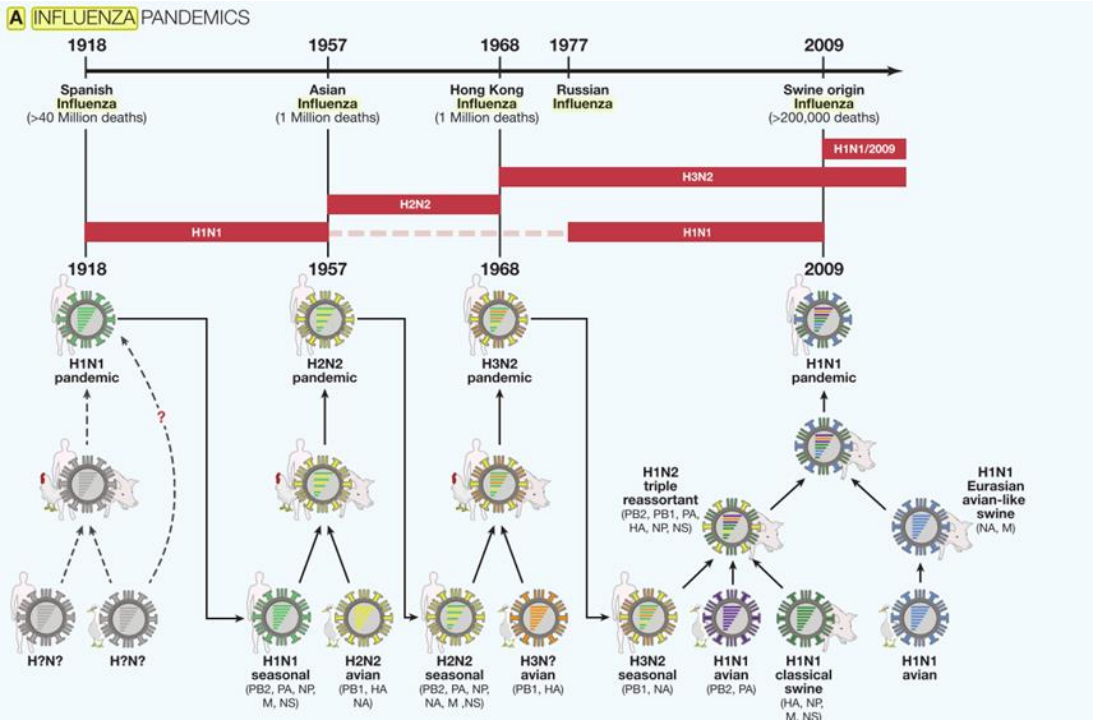


Figure 4.2

Evolution of Human Influenza A viruses.

Wendel, I., Matrosovich, M., Klenk, H. D., Cell Host Microbe. ; 17(3):416.e1, (2015).

Results

Expression of SERINC5 impairs production of infectious influenza A

To investigate whether SERINC5 affects the infectivity of influenza virus, HEK293T cells already transfected to express increasing amount of the host retrovirus restriction factor, were infected with influenza A. Progeny virus particles were used to infect MDCK target cells and infection level measured by a plaque assay.

The plaque assay on target cells infected by serially diluted viral inocula is based on the assumption that each plaque formed is representative of one infective virus particle (Figure 4.3).

Ectopic expression of SERINC5 in producer cells resulted in a strong inhibition of both England195 and Victoria75 influenza A viruses. This experiment suggests that SERINC5 is able to modulate infectivity of the virus in a dose-dependent manner, with a 50-fold reduction at the highest amount of the SERINC5 expression plasmid we used (Figure 4.3).

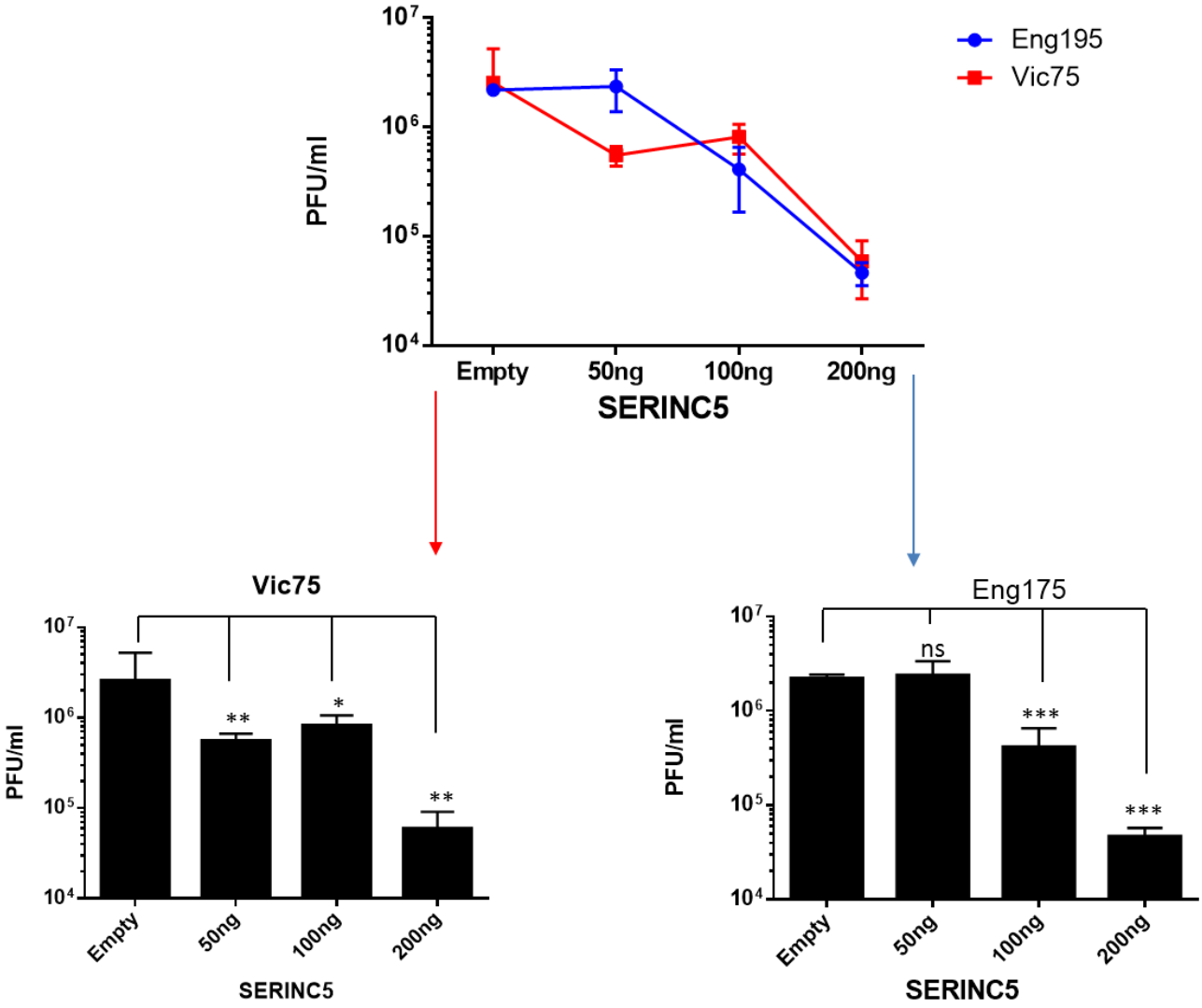


Figure 4.3

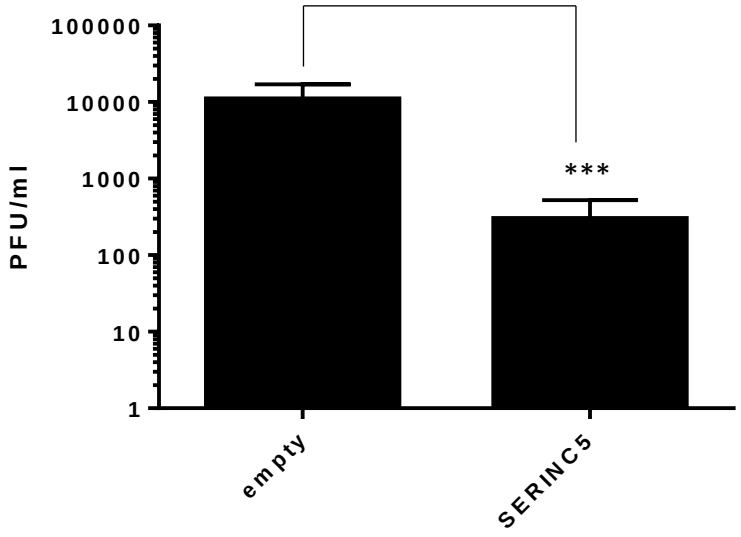
Victoria and England strains of infuenza A are inhibited by SERINC5.

Overexpession of increasing amounts of SERINC5 in HEK293T producer cells leads to a dose – dependent reduction in infectivity of both England and Victoria strains of human influenza A virus. HEK 293T cells were transfected with 50/100/200 ng of SERINC5 PBJ6 and infected the day after respectively with $3.1x10^7$ MOI and $3.9x10^7$ MOI of Eng195 or Vic75 strain of influenza A. Viruses are collected 24h post infection and used to perform a plaque assay. PFU/ml values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

The effect of SERINC5 does not target the intrinsic infectivity of influenza virus

Having observed an inhibitory effect of SERINC5 on the production of infectious influenza A, we next investigated whether the host factor, like in the case of HIV-1, impaired intrinsic infectivity. As described above, virus was produced by infecting HEK293T cells expressing SERINC5 or an empty vector control. Virus titre measured by plaque assay was inhibited 50-fold by the expression of SERINC5. The amount of virus released was evaluated by quantifying the virus RNA encoding for the *matrix* gene in the supernatant of producer cells, using qRT-PCR. Results show that the supernatant from SERINC5 expressing cells contains 100-fold less influenza virus RNA than the control sample. Altogether these data suggest that SERINC5 does not affect the intrinsic infectivity of influenza A virus (Figure 4.4). It is therefore possible that the host factor expressed in HEK293T cells acts by inhibiting influenza A production/release. However, because in our experimental setting, influenza A was produced by infecting HEK293T cells already over-expressing SERINC5, the possibility remains that the host factor acted upstream by preventing infection of these cells, therefore functioning as influenza inhibitor in target cells. Unfortunately, I could not investigate further to discriminate between these two possibilities as I had limited time available in Wendy Barclay's Influenza Laboratory.

a)



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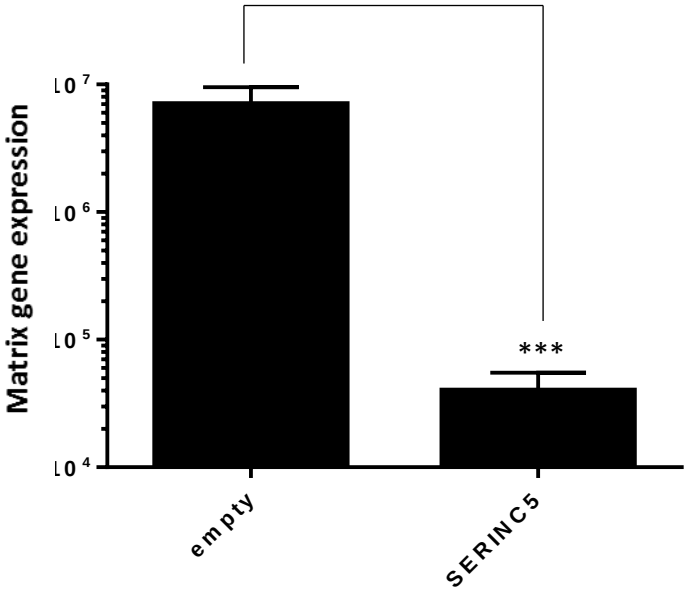

Figure 4.4

The effect of SERINC5 does not target the intrinsic infectivity of influenza A.

a) Plaque assay and corresponding quantification b) of influenza A virus produced in the presence and in the absence of SERINC5.

HEK 293T cells were transfected with 100 ng of SERINC5 PBJ6 and infected the day after with $3.1x10^7$ MOI of Eng195 strain of influenza A. Viruses are collected 24h post infection and used to perform a plaque assay. In parallel, same viruses were retro-transcribed and used to perform a Real Time PCR to quantify viral particles present in the supernatant of the infected cells in the presence or in the absence of SERINC5. PFU/ml values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions.

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SERINC3 and SERINC2 are also able to inhibit human influenza A

As already reported, not only SERINC5, but also SERINC3 acts as retrovirus inhibitor. We therefore investigated whether any other *SERINC* gene is able to inhibit the infection of human influenza A virus. The effects of SERINC5, SERINC3 and SERINC2 on the England 195 influenza A strain was therefore tested.

Results show that all *SERINC* genes tested inhibit influenza A with an efficiency similar to IFTM3, a well-established inhibitor of orthomyxoviruses. Interestingly, SERINC2, which in our laboratory was shown to have no activity against HIV-1 (unpublished), has instead a marked effect on influenza A (Figure 4.5).

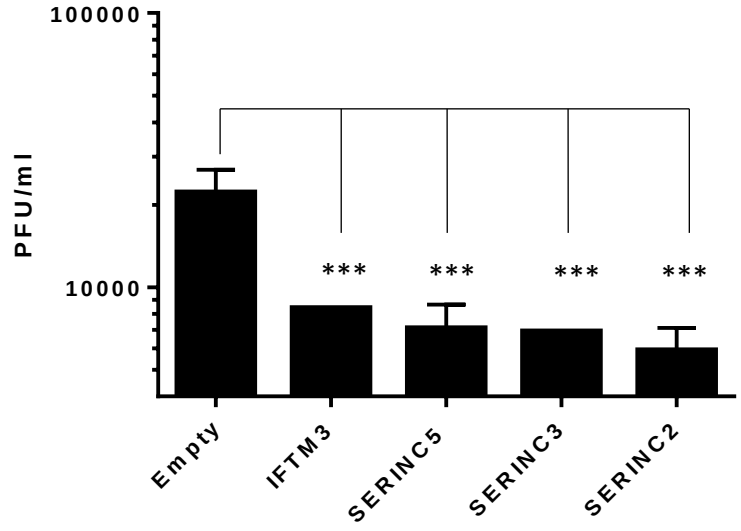


Figure 4.5

Not only SERINC5, but also SERINC3 and SERINC2 inhibit influenza A.

Overexpression of SERINC5, SERINC3 or SERINC2 in HEK293T producer cells inhibit infectivity of England strain of influenza A virus.

IFTIM3, established restriction factor of influenza A virus, was used as a positive control**.** HEK 293T cells were transfected with 100 ng of SERINC5/3/2 PBJ6 and infected the day after with $3.9x10^7$ MOI of Vic75 strain of influenza A. Viruses are collected 24h post infection and used to perform a plaque assay. PFU/ml values are average of 4 independent transfections (biological quadruplicates). Error bars represent standard deviation of the mean. Statistical significance was calculated by unpaired two-tailed test (* P<0.05, ** p<0.01, *** p<0.001). Representative experiment out of 3 repetitions

Discussion

This chapter describes a preliminary work performed during my one month internship in Professor Barclay's lab at Imperial College, London.

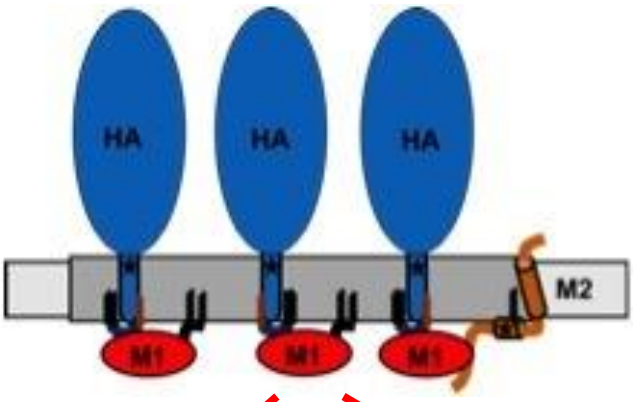
Being the SERINC proteins transmembrane, it is plausible to hypothesize that not only retroviruses but other enveloped viruses would also incorporate them during the virion budding process. Indeed, it would be interesting to verify whether SERINC5 or SERINC3 are found associated with other enveloped viruses. In any case, my preliminary experiments indicate that overexpression of SERINC2, 3 and 5 in virus producing cells all impair influenza A, at a step which remains to be identified. However, unlike the effect of SERINC5 and SERINC3 on HIV-1, my experiments show that the host factors do not impair intrinsic virus infectivity, pointing to an effect at the level of virus production, which could include virus release or virus gene expression. Since the virus for these experiments was produced by infecting cells already overexpressing the SERINC genes, the possibility also exists that the host factors interfere with influenza A entry. While we are at this moment unable to discriminate between these possibilities, the result obtained so far is encouraging because it describes a large dose-dependent effect, it was reproduced in 3 independent experiments, it was similarly obtained with three SERINC genes (SERINC5, SERINC3 and SERINC2), and observed using two different strains of human influenza A: England195 and Victoria75.

While SERINC5 is predominantly expressed in blood cells and in the CNS, other SERINCs, such as SERINC3, are express ubiquitously, including in airway

epithelial cells, the natural target of human influenza A virus, indicating that our preliminary observation could be relevant to the *in vivo* infection.

If SERINC proteins are able to inhibit influenza, then it is possible that the virus has evolved a mechanism to minimize or escape from the control of the host restriction. The evolution of our study will therefore include understanding whether the influenza A virus has developed a factor capable of counteracting the antiviral activity of SERINC genes.

By analogy with Nef, glycoGag and S2, the protein encoded by this gene would require the ability to interact with membranes and to recruit the endocytic machinery. After investigating the amino acid sequence of all proteins encoded by human influenza A virus, a YxxL and E/DxxxLL motifs were found in M1 and M2, both of which have the ability to associate (M1) or interact (M2 is a transmembrane protein) with the membrane. (Figure 4.6). Future experiments will address these possibilities.



MSLLTEVETYVLSIVPSGPLKAEIAQRLEDVFAGKNTDLEALMEWLKTRPILSPLT
KGILGFVFTLTVPSERGLQRRRFVQNALNGNGDPNNMDRAVKLYRKLKREITF
HGAKEIALSYSAGALASCMGLIYNRMGAVTTESAFGLICATCEQIADSQHKSHR
QMVTTTNPLIRHENRMVLASTTAKAMEQMAGSSEQAAEAMEVASQARQM
VQAMRAIGTHPSSSTGLKNDLLENLQAYQKRMGVQMQRFK

Figure 4.6
M1 protein localization and amino acid sequence.
Adapted from

http://www.vetmed.fuberlin.de/einrichtungen/institute/we05/arbeitsgruppen/zellbiologie/inhalt/index.html
(Accession number: DQ415350.1)

Chapter 5: General conclusions

During the long co-evolution between host and parasite, cells developed restriction factors to avoid virus replication and dissemination. These proteins are a first line of defence against viral pathogens and include a plethora of factors with a no-univocal structure that may act at every level of the viral life cycle. Restriction factors were developed to act against conserved viral components, (viral genomes; membranes) and in some cases evolved to be effective against different viral families. The SERINC proteins are the latest addition to the growing list of restriction factors. After our lab demonstrated that primate lentiviruses and gammaretroviruses have evolved SERINC antagonizing factors, with my research I have demonstrated that a counteracting factor was also evolved by EIAV, identifying a third event of convergent evolution leading to anti-SERINC activity.

Convergent evolution is “the process whereby organisms not closely related (not monophyletic), independently evolve similar traits as a result of having to adapt to similar environments or ecological niches” [125](Figure 5.1). SERINC proteins provide an important element in the environment, to which at least three different virus groups had to adapt. This underlines a fundamental importance of the ability to counteract SERINC5. Accordingly, Nef, glycoGag and S2 are all similarly crucial pathogenic determinants[126].

There is yet no cure and eradication strategies for diseases caused by retroviruses, a problem particularly evident in the case of the HIV pandemic. By highlighting the importance of the SERINC5-Nef-like factors, my work

indicates a potential focus for novel antiviral strategies, needed to complement the current therapies.

Nef and other Nef-like infectivity factors have no enzymatic activity, making it difficult to develop effective drugs targeting their activities. Once future studies will have elucidated the molecular interaction between the Nef-like factors and SERINC proteins, molecules interfering with such interaction could be designed. While the development of clinically active drugs to target protein-protein interaction remains problematic, promising progresses were made in recent years with the development of molecules able to target Nef[127,128] and Vif[129] activities and providing important proofs of principle.

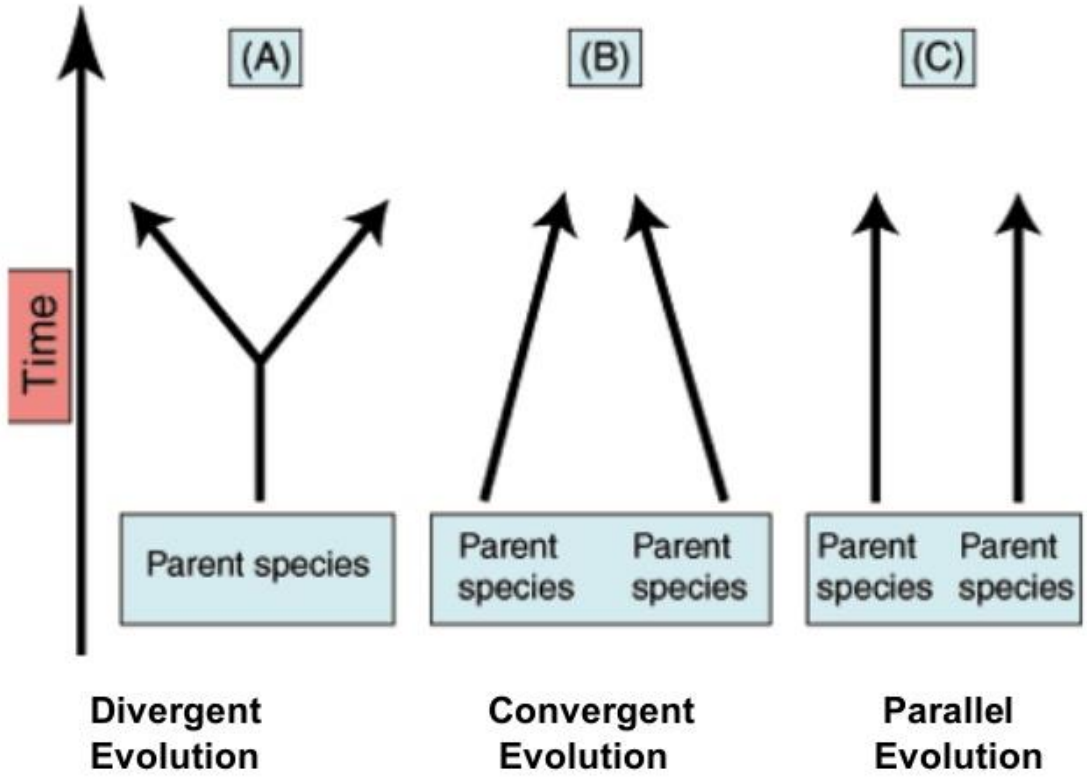


Figure 5.1
Convergent evolution schematic representation.
http://www.sparknotes.com/biology/evolution/patternsofevolution/section1.rhtml

Chapter 6: Materials and methods

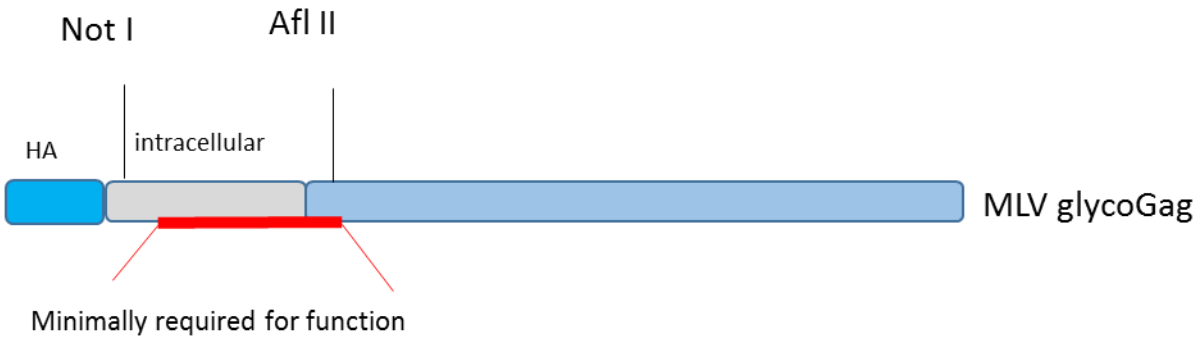
Plasmids

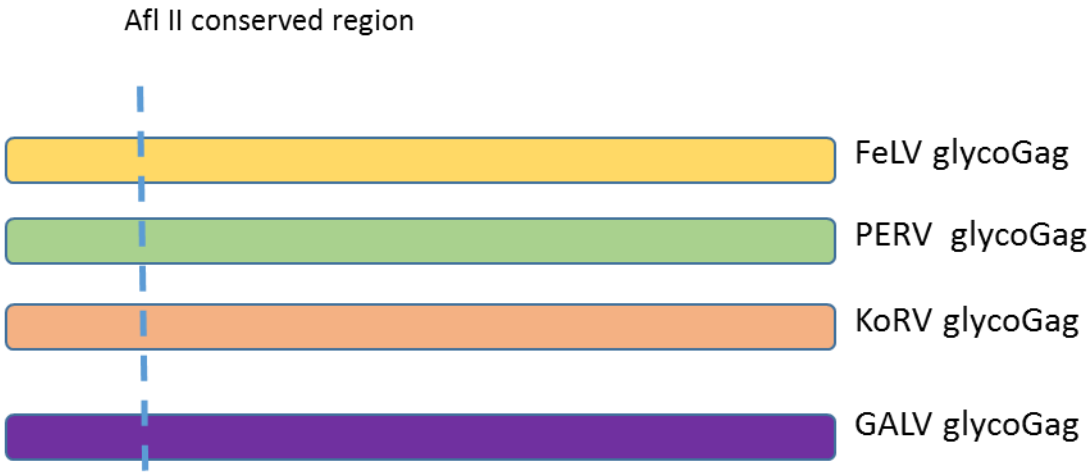
For all experiments performed with HIV-1, NL4-3 derived plasmids deficient for *env* and/or *nef* were used as described in Pizzato *et al*.[80] Env defective viruses were complemented with pbj5 expressing HIV-1^{HXB2}*env*. HA-GlycoGag NA, and the RXR/AXA mutant were obtained by site-directed mutagenesis.

FeLV-A gg and KoRV gg were isolated from the corresponding FeLV-A and KoRV522 molecular clones[130] kindly provided by Prof Takayuky Miyhazawa, (Institute for Virus Research, Kyoto University), PERV glycoGag was amplified from PERV-A molecular clone kindly provided by Dr Yasuhiro Takeuchi (UCL, London). GALV gg was obtained by retrotranscribing with specific primers RNA extract from the A3.01/F7 cells line, recognized to be permanently infected with GALV. The 5' 288nt of all glycolGag alleles were cloned and used to replace the homologous region within MoMLV glycoGag (HA-gg196) up to a stretch of highly conserved amino acids, which include a AflII restriction site in the MoMLV genetic sequence. An HA N-terminal tag was also added to facilitate the detection (Figure 6.1). Synthetic fragments (GeneArt, Life Technologies) encoding for codon optimized BIV TMX (AAA64394.1) and WDSV ORF2 (NP_045940) fused to a HA C-term tag, were cloned and inserted in a pbj5 expression vector using the in-fusion strategy (Clontech) according to the manufacturer guidelines. pcDNA based Orf-A from the Petaluma strain fused to an HA tag was a gift from Prof. Mauro Pistello (University of Pisa, Italy). EIAV S2 with and without C-terminal HA tag

was amplified from p19/wenv16 EIAV molecular clone (af028231.1) and inserted into the pbj5 expression vector between EcoRI and NotI restriction sites. Codon-optimized version of S2-HA was obtained through a seven-step PCR, which allowed the sequential substitution of the required triplets according to the human codon usage. Codon optimized S2-HA was used as a template to generate Wyoming S2-HA (m87581), by inserting point mutations at positions 3 (V->L) and 18 (E->G); again, was chosen as expression vector. The same strategy already mentioned for S2 was used to codon optimize Orf-A HA.

MLV and glycoGag-negative MLV have been modified to express GFP instead of *env* and supplemented by MLV-NZB-9-1 envelope glycoproteins expressed with a pCDNA-based vector. A plasmid encoding the vescicular stomatitis virus G protein (pMD.G) was used as already described, as well as *env* from the HIV-1 JRFL isolate[131]. All S2 mutants ($L_{27}L_{28}$->AA, G_2->A myristoylation mutant, $P_{26}P_{29}$->AA) were generated by PCR-based mutagenesis. The hydrophobic region mutant was obtained by mutating LIVII at positions 45 to 49, into SSVSS. pBJ6 vector was used to drive low expression levels of SERINC5-HA as already described[85]. SERINC5-GFP was previously generated in our lab and used accordingly[85]. pEIAV53D and pw sin6.1 cgfpw (M87581,Addgene) were used to generate EIAV vectors. The first is a plasmid encoding EIAV packaging functions from Wyoming strain genome, with a deletion in the LTR sequence, a deletion of the viral packaging signal, a deletion of the first exon of *tat* and an extensive deletion of the *env* gene. The second is a lentiviral vector driving the expression of GFP.





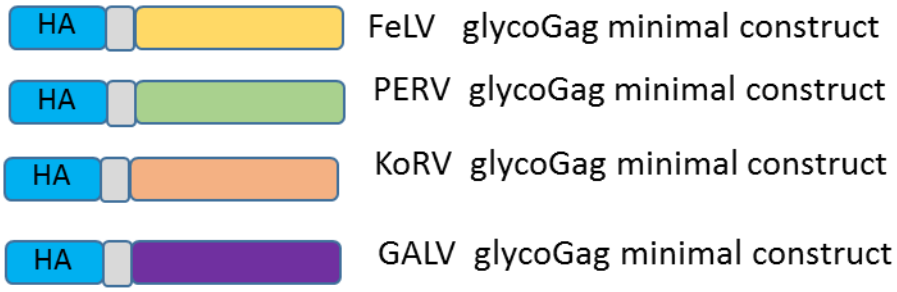


Figure 6.1
GGs cloning strategy.

A pEIAV43D (packaging construct) mutated in S2 sequence was obtained by enzymatic digestion with BamHI followed by a fill-in and ligation to insert frameshift disrupting the S2 ORF. EIAV codon optimized envelope pglSUTM was kindly provided by Susan Carpenter, (Washington State University). e-GFP, RFP, Rab7-RFP, AP180 C-terminal transdominant mutant, K44A dynamin2 mutant, Nef-HA G2A mutant, hCypA, Nef-HA were used following previous reports.[80,82,85,115] All constructs were confirmed by sequencing.

Growing condition of EIAV envelope expression plasmid

Codon-optimized EIAV envelope inserted in pLG338/30 vector was amplified in *E. coli* Stbl2 strain at 30°C for 20 hours. The combined effect of codon optimization and amplification in a low copy number plasmid resulted in an increase in the stability of the EIAV *env,* as reported[132]*.*

Generation of stable cell lines

To generate ELR1 stable cell lines, retroviral vectors encoding the equine receptor were obtained by co-transfection of MLV based pcg-*gag-pol*, pFB ELR1 transfer vector, and VSV-G *env* in HEK293T by calcium phosphate transfection. Vectors were collected 48h post-transfection and used to transduce TZM-bl cells spinoculated for 2h at 3000rpm. TZM-bl single cell cloning strategy was applied to obtain a cell line expressing ELR1

homogenously. A similar protocol was adopted for generation of JTAg stable expressing ELR1. JTAg SERINC5-KO and SERINC5/SERINC3 double KO were previously generated in our lab with a CRISPR-Cas9 technology[85].

Cell lines and culture conditions

JTAg, CEMX174, JTAg SERINC5 KO, SERINC5/3 DKO and JTAg ELR1 were cultured in suspension. Life Techonologies RPMI 1640 + 10% of heat inactivated fetal bovine serum FBS supplemented with 2mM L-Glutamine was used as a growth medium. The adherent TZM-GFP and TZM-bl ELR1, TE671, HEK293T and COS-7 cell lines were grown in Life Techonologies DMEM supplemented with 10% FBS and 2mM L-Glutamine.

Madin-Darby canine kidney (MDCK) cells (ATCC) were maintained in cell culture media (Dulbecco's modified Eagle's medium (DMEM; Life Techonologies) supplemented with 10% fetal calf serum (FCS) (Biosera) and with 1% Penicillin-Streptomycin (Life Techonologies).

Cultures were maintained in humidified incubators at 37°C and 5% $C0_2$. All cells lines resulted negative when routinely screened for possible mycoplasma contamination.

Retroviruses production and infectivity assays

All retroviruses infectivity data were obtained producing single round replication viruses by transfection. CEMX174 and JTAg were electroporated in

0.2cm gap electroporation cuvettes (Biorad) using 140V and 1000uF with exponential decay. Fresh medium was added to the cells the day before transfection; 10^6 cells/sample and 20µg of total DNA were used. HEK293T and TE671 were transfected by the calcium phosphate co-precipitation method. Cells were seeded the day before transfection in 10 cm plates to be 50% confluent at transfection. Culture supernatants containing HIV-1 or Nef-deficient HIV-1, were obtained with a two-vector system composed by a NL4-3 derived plasmid deficient for *env* or for *env* and *nef* and complemented with pbj5 expressing HIV-1^{HXB2}*env*. A 4:4:1 ratio between viral backbone, the tested infectivity factor and envelope expressor respectively was used. 48h after transfection, viruses are collected, clarified by centrifugation at 300g for 5 min and passed through filters with 0.45-µm pores. Viruses produced from quadruplicate transfections were then quantified using the SG-PERT reverse transcription assay (described in the following section), diluted serially (3 fold) and used to infect target cells. TZM-GFP were seed 5000 cells/well in 96 well plates the day before transfection. Infection of reporter cells was scored using the nuclei with Hoechst 33342 for each virus dilution. Those values falling into a linear dilution range were used to calculate infectivity. Infectivity was calculated by dividing the number of infected cells in a well for the amount of reverse transcriptase activity associated to the virus inoculum, measured in mU[85].

MLV was obtained by electroporation of JTAg cells and quantified by SG-PERT assay. MLV and glycoGag-negative MLV molecular clones, S2 and MLV-NZB-9-1 envelope glycoproteins were used with 4:4:1 ratio, with a total amount of 20µg of DNA. 48 hours after transfection viruses were collected and used to

infect TE671 cells. To increase infectivity of the virus final concentration of 8 µg/ul of Polybrene was added to each well during infection. Four days later, cells were washed twice with PBS-EDTA 5mM and fixed in 2% PFA. Percentage of infected cells were measured by FACS and infectivity obtained with the same calculation mentioned described earlier.

EIAV vector was produced in HEK293T cells by calcium phosphate co-precipitation method, using a three-vector system composed by pEIAV53D, pw sin6.1 and pLG338/30 vectors with a 2:2:1 ratio and a total amount of 30µg of DNA. Virus collection, infection of target cells, detection of infected cells 4 days post infection and calculation to obtain infectivity values are made with the same experimental procedures described for MLV.

In all experiments involving transfection of SERINC5/3/2, 1:1.5 ratio between SERINC and the tested infectivity factor was used.

In experiments in figures 2.6 and 3.8, where AP-180 C or K44A dynamin 2 mutant were used, 1:1.5 ratio between the tested infectivity factor and each endocytosis inhibitors separately tested was used.

SG-PERT[133]

5 µl of 2x lysis buffer (0.25% Triton X-100, 50 mM KCl, 100 mM Tris HCl pH 7.4, 40% Glycerol, 80U of Human Placenta RNase inhibitor/100 (New England Biolabs), mixed with 5 µlof virus suspension or controls. After 10 minutes of incubation at RT, 90 µl of 1X core buffer (50 mM $(NH_4)_2SO_4$, 20 mM KCl, 200 mM Tris-HCl pH 8.3) was added. 10 µl aliquots were mixed with 10 µl of 2x

reaction buffer (5 mM $(NH_4)_2SO_4$, 2 mM KCl, 20 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 0.2 mg/ml BSA, 1/10000 SYBR Green-I, 400 µM dNTPs, 1µM forward primer, 1µM reverse primer, 7 picomoles/ml MS2 RNA, 5U HotstartTaq (Thermo Scientific)) for RealTime PCR. RT activity for each sample was compared with a standard curve using viruses of known concentrations expressed in functional units[133] (Figure 6.2).

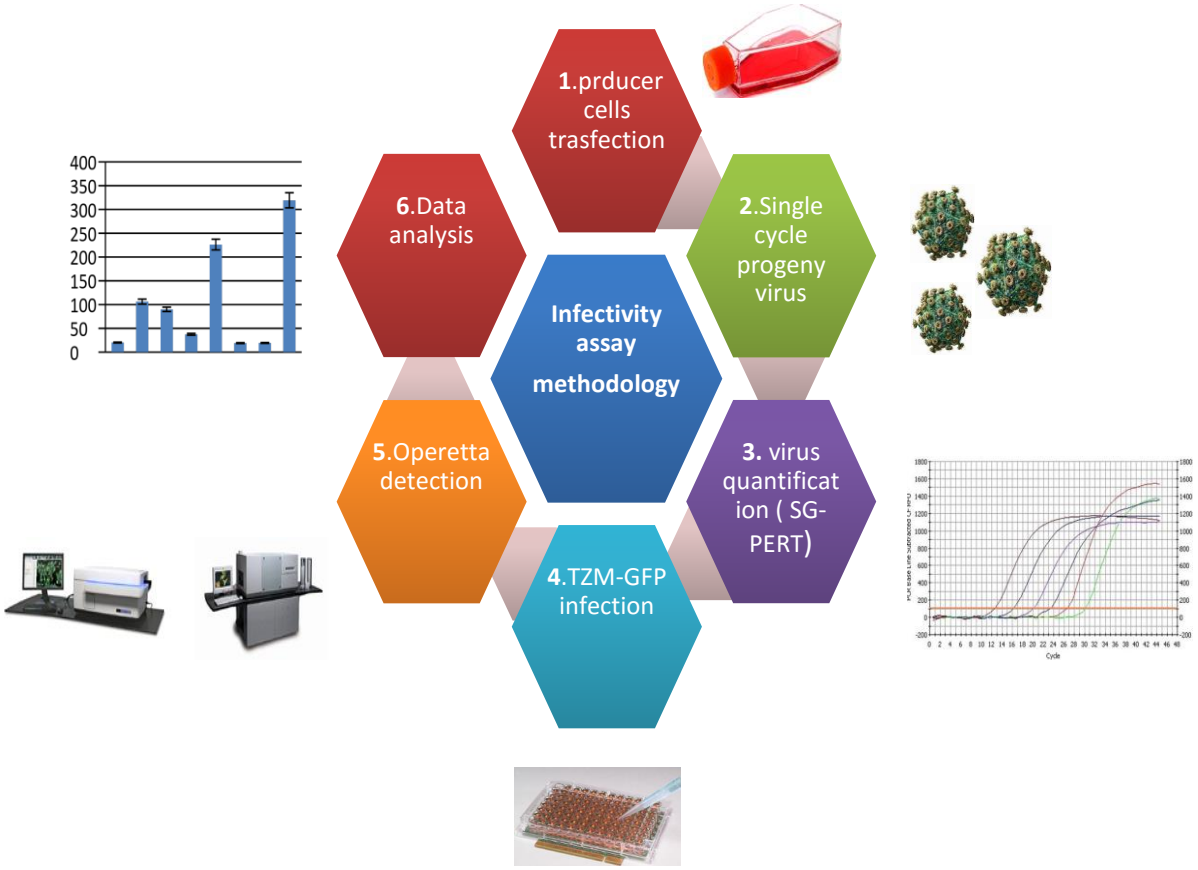


Figure 6.2
Infectivity assay methodology.

Immunofluorescence assay

5 million JTAg cells per sample were electroporated with 20 µg of total DNA using the same conditions previously described. Transfected cells were collected after 48h and seeded on poly-L-lysine coated coverslip; COS-7 cells were seeded the day before transfection in 6 well plates to be 50% confluent at transfection, carried out by the calcium phosphate method using 5 µg of total DNA. 2 days later, cells were fixed in 4% PFA and permeabilised for 15 minutes with BD Perm/Wash buffer. At this point, cells were first stained with a mouse anti-HA antibody diluted 1:1000 (HA.11, Covance) and then with a secondary antibody (Alexa 633, Life Technologies) diluted 1:500. After each antibody staining, 3 washes were performed with BD Perm/Wash buffer. The coverslips were mounted on slides using Pro Long Diamond Antifade Mountant (Life Thecnologies). The Images were acquired with a Leica TCS SP5 confocal microscope.

Western blotting

Cell lysates and virion pellets were analyzed by SDS-PAGE and Western blotting. Viral particles were collected 48h after transfection, centrifuged at 300g to remove cells debris and filtered through a 0.45 µm pore size filter. The clarified supernatants were overlaid on 25% sucrose cushion and concentrated at 100,000g for 2h. The pellets were resuspended directly in

Laemmli buffer supplemented with 50mM TCEP pH 7.0, normalized by reverse transcriptase assay (in case of virion pellets) and resolved by SDS-PAGE. Samples were loaded on 12.5% acrylamide gel with 5mM TCEP after 5-pulse sonication. After the electrophoretic separation (10mA constant current), proteins were electro-transferred on Immobilon-FL PVDF membrane (Millipore) with semi-dry transfer apparatus (TE22 Mighty Small Transphor Unit – Hoefer), for 75 minutes at 155mA, 20 Volt maximum. Odyssey Blocking Buffer (Li-COR) diluted 1:1 in TBS was used for blocking. Probing was performed using a mouse anti-HA antibody (HA.11, clone 16B12, Covance) or a mouse or rabbit beta-actin antibody (Li-COR) or an anti-HIV-1 p55/p24 antibody (National Biological Standards Board); secondary antibody used were IRDye 680 and IRDye 800 (Li-COR). After each antibody staining, 3 washes were performed using TBS-Tween 0,01%. Blot were imaged using a Li-COR Odyssey infrared imaging System (Li-COR). Antibody dilutions were chosen according to the manufacturer recomendation[85].

Click chemistry

Click assay was performed with Invitrogen Click iT Metabolic Labeling Reagents for Proteins, modified to our need the suggested protocol. Briefly 40 µM of myristic acid-azide was added to cell medium 24h post-transfection and incubated O/N. The following day the cell pellet for each sample was lysed with urea lysis buffer provided with the kit supplemented with protease inhibitors (Roche) and 100 units of Benzonase (Sigma). After a 5 minutes centrifugation at 10000 rcf, lysates were added to 2x catalyst solution and to the resuspended resin; this preparation was kept rotating end-over-end at

room temperature over-night. The day after, the reaction was centrifuged for 1 minute at 1000g to separate the resin from the supernatant. After a wash with water, SDS wash buffer supplemented with 10 mM DTT was added to the resin, followed by a step of heating at 70°C and cooling at room temperature for 15 minutes. Then resin was centrifuged again, resuspended in water and allowed to pass through a column. After 5 washing with washing buffer supplemented with 1M Urea, one last wash was performed with 8M urea, 100 mM Tris pH8.

At this point, resin was collected and directly resuspended in 2x Laemmli buffer to perform a SDS-PAGE.

Human influenza A virus production

Reverse genetics systems for the following influenza virus strains were used in this study. Virus stocks were generated in MDCK cells with infection media (serum free DMEM) supplemented with 1% Penicillin-Streptomycin and 1 µg/ml TPCK-treated trypsin (Lorne Labs) and incubated at 37 °C. Clinical isolate A/Victoria/3/75 and the A/England/195/2009 (Public Health England) was propagated in MDCK cells with infection media. Aliquots of infectious virus were stored at −80 °C. Infectious titres were determined by plaque assay on MDCK cells.

Influenza A virus infection

HEK293T cells were seeded the day before in 24 well plates to be 70–90% confluent at transfection, and then transfected with SERINC 5/3/2 HA PBJ6 or

IFITM3, using 1.5 μg of total DNA according to the Lipofectamine 3000 manufacturer recommendation (Invitrogen). After 20 h infection with virus diluted in serum-free DMEM for 1 h at 33 °C or 37 °C, medium was replaced with DMEM supplemented with 0.1% FCS and 1 μg/ml TPCK trypsin (Worthington-Biochemical) (for infectious virus titres). $3.1x10^7$ MOI and $3.9x10^7$ MOI were used respectively for A/Victoria/3/75 and A/England/195/2009. Infected cell lysates and cell supernatants were harvested at 24h post-infection. Infectious titres were determined by plaque assay on MDCK cells.

Plaque assay

Six different 10-fold serial dilutions of each virus obtained in triplicates, were used to infect in duplicate 90% confluent MCDK cells previously washed twice in PBS. Viruses were kept incubating with cells for 1 hour at 37°C, and then they were removed and replaced with 1 ml of overlay media (DMEM high glucose with 4 to 6 mM glutamine and 2% fetal bovine serum, 2.3 μg/ml flu trypsin) mixed with an appropriate volume of oxoid high purified 2% agarose solution. Plates were left in a humidified incubator at 37°C and 5% CO_2 for three days to allow the plaques formation (Figure 6.3).

Real-Time PCR

Viral RNA was extracted from the supernatant of cells infected with A/England/195/2009 in the presence of SERINC5 or pcDNA 3.1 negative

control using QIAamp Viral RNA extraction Kit according to the manufacturer instructions (QIAGEN). 1 µl of the obtained viral RNA was used as template for a retro-transcription reaction using SuperScript III First-Strand Synthesis System for RT-PCR kit (Thermo Fisher) and oligo(dT) primer.

Equal volumes of cDNA for each sample were used to set-up a SYBR green based qPCR. Matrix gene from the genome of influenza was used to normalize the viral particle release.

Safety/biosecurity

All experiments with infectious agents were conducted in biosafety level 2+ facilities, approved by the Health and Safety Executive of the UK and in accordance with local rules, at Imperial College London, UK.

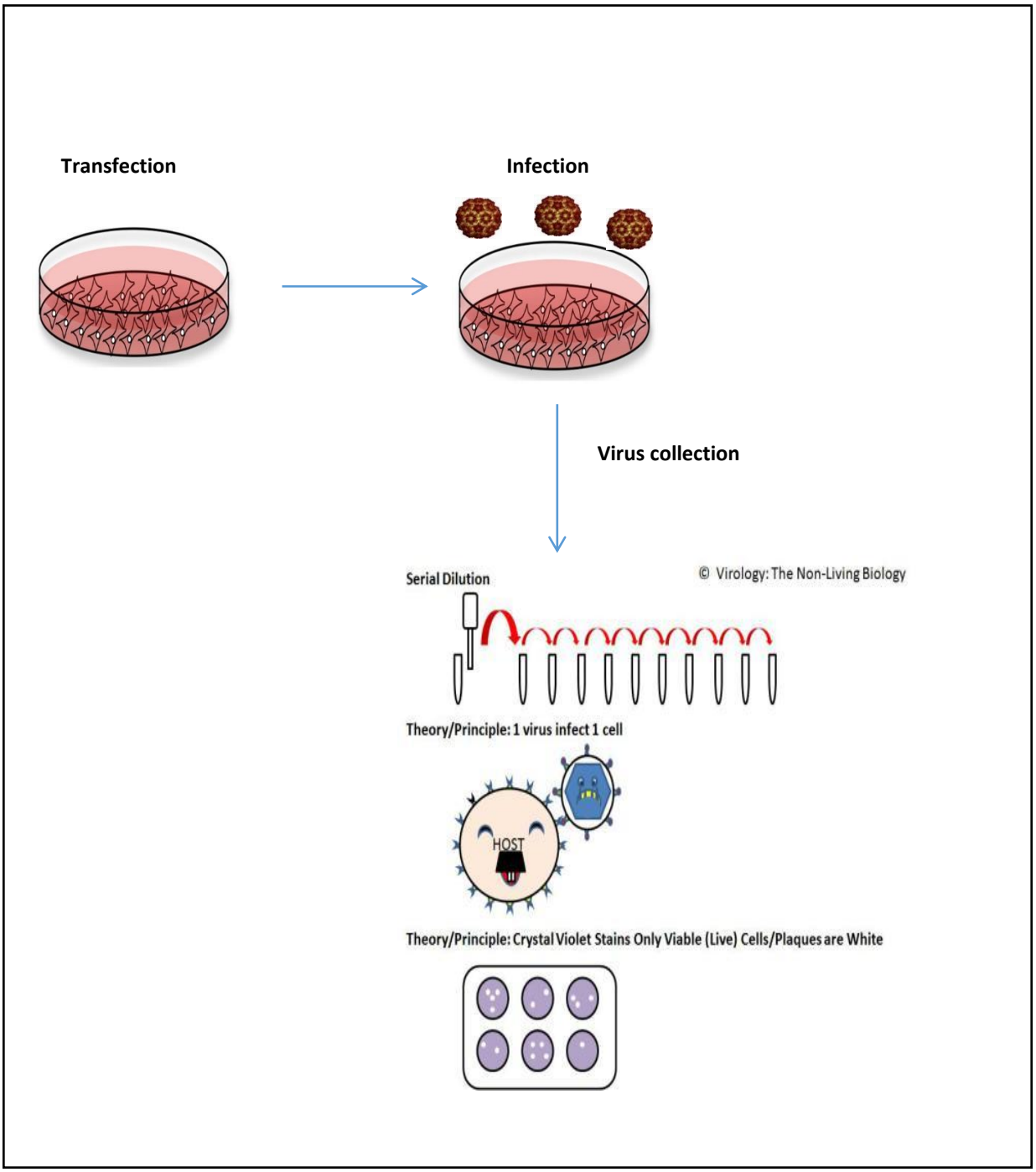


Figure 6.3
Plaque assay methodology.

Table 2: Primers for generation of chimeric glycoGag constructs

Name	Sequence
PERV Fw NotI	ATATATGCGGCCGCAACCATGGGAGACGTCCCAGGGACTT
PERV rev AflII	GGTCAAACTTAAGGGGGTAGTCACTGTCTGTCC
FeLV fw NotI	TTACGCTGCGGCCGCAACCATGTCTGGAGCCTCTAGTG
FeLV rev AflII	GGTGAGGCTTAAGGGGGTAGTTATAGTTTGGCC
GALV fw NotI	AAAAAAGCGGCCGCCATGACCCGATTTCATCGCCCGTCTGG
GALV rev AflII	ATATATCTTAAGGGGGCAGAAGTACCTTGTCC
KoRV fw NotI	AGGGAGACCCAAGCTGGCGGCCGCCGCCATGGGAGACGTCCCAGGA
KoRV rev AflII	GTCAAACTTAAGGGGGTCGACTCACCCTGTC

Table 3: Primers for codon optimization and mutagenesis

Name	Sequence
S2 V3L co fw	CGAGGCCACCATGGGGCTCTTCGGCAAGGGAGTGACTTGG
S2 V3L co rev	CCAAGTCACTCCCTTGCCGAAGAGCCCCATGGTGGCCTCG
S2 E18G fw	GCCAGCCACAGCATGGGAGGATCCCAGGGCGAAAGCCAG
S2 E18G fw	CTGGCTTTCGCCCTGGGATCCTCCCATGCTGTGGCTGGC
S2 Co fw	ATATATCTCGAGGCCACCATGGGCGTGTTCGGCAAGGGAGTGACTTGGAGCGCCAGCC
MCS-S2 rev	ACACGCCCATGGTGGCCTCGAGCTCGAGTCTAGAGGGCCCGTTTAAAC
S2 Co rev 1	GTGGCTGGCGCTCCAAGTCACTCCCTTGCCGAACACGCCCATGGTGGCCTCGAG
S2 Co rev 2	GGGCAGCAGGGGCTGGCTTTCGCCCTGGCTCTCTCCCATGCTGTGGCTGGCGCTCCAAG
S2 Co rev 3	GGTTGAAGCACTGGGTCCGCCGCACGCTCAGGTTTTTCTGGCTGTTGGGCAGCAGGGGC
S2 Co rev 4	CCGCCGGTTCTGCCAGGCGGTCCGCACGGTCATGATGATCACGATCAGGTTGAAGCACT
S2 Co rev 5	CGTCGTAGGGGTATTTCTTTGTTTCCTGCTTCCGCCGGTTCTGCC
S2 Co rev 6	GTGGTGGAATTCAGGCGTAGTCGGGCACG
S2 Co LLAA mut fw	GGGCGAAAGCCAGCCGGCCGCGCCCAACAGCCAG

S2 Co LLAA mut rev	CTGGCTGTTGGGCGCGGCCGGCTGGCTTTCGCCC
S2 Co GA mut fw	CTCGAGGCCACCATGGCCGTCTTCGGCAAGGGAGTG
S2 Co GA mut rev	CACTCCCTTGCCGAAGACGGCCATGGTGGCCTCGAG
S2 Co PPAA mut fw	CAGGGCGAAAGCCAGGCCTTACTGGCCAACAGCCAGA
S2 Co PPAA mut rev	TCTGGCTGTTGGCCAGTAAGGCCTGGCTTTCGCCCTG
S2 Co TM mut fw	GACCCAGTGCTTCAACTCTAGCGTCTCATCAATGACCGTGC GGACCG
S2 Co TM mut rev	CGGTCCGCACGGTCATTGATGAGACGCTAGAGTTGAAGC ACTGGGTC
OrfA CO fw	TCTAGACTCGAGCCACCATGG
OrfA R 01	GGTCACGCGGTTGAACAGCACGATGATATCTTCCATGGTG GCTCGAGTCTAGAGGGCCC
OrfA R 02	AGCACGAAGATCCGGATGGCCAGCTCTTTTTCCAGCTTCTC GGTCACGCGGTTGAACAG
OrfA R 03	GCAGCAGTCTGATGGCCTTGTCCCGTTCCAGCTGGTGGGC CAGCACGAAGATCCGGATG
OrfA R 04	CACCCGGGGTTTCTTGAATCTGTACCGCCAGAACAGGCCC TGCAGCAGTCTGATGGCCT
OrfA R 05	CAGTAGTAGAACTTGCAGCACCACCAGCACAGGCAGTAG TCCACCCGGGGTTTCTTGAA
OrfA R 06	GTGATGCTCAGGGTGCTCTGCAGCTGCCAGTAGTAGAACT TGCAG
OrfA R 07	CGTCCTTGTAGTCGGCGGTGGTGATGCTCAGGGTGCT

OrfA R 08 FLAG	ATATATGAATTCTCACTTGTCGTCGTCGTCCTTGTAGTCGG CG

Table 4: Cell lines description

Cell line	Cell type	Source
Jurkat TAg (JTAg)	T Lymphocyte, Acute T Cell Leukemia	Heinrich Gottlinger, DFCI, Harvard University
CEMX174	Limphocytes, fusion between a Bcell line and a human T cell line	NIH AIDS Research and reference Reagent program
HEK293T	Epithelial, embryonic Kidney	ECACC
TE671	Rhabdomyosarcoma	Yasuhiro Takeuki, UCL, London
TZM-bl	HeLa cell line generated from JC.53 cells by introducing separate integrated copies of the luciferase and ß-galactosidase genes under control of the HIV-1 promoter.	NIH AIDS Research and reference Reagent program
COS-7	Cercopithecus aethiops, Kidney	ATTC
MDCK	Madin Darby Canine Kidney	ATTC

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