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"The cellular and molecular basis of the Nef requirement for HIV-1 infectivity"

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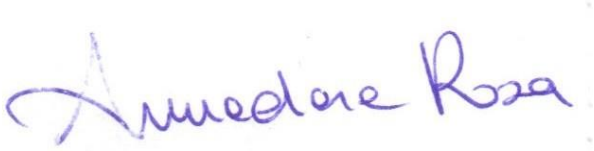
Declaration

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



Abstract

Nef is an HIV -1 accessory protein with a fundamental role for virus replication in vivo and for the development of AIDS. Among its several activities, Nef is essential for full HIV-1 infectivity, a function highly prominent in lymphoid cells. So far, the mechanism by which Nef promotes HIV-1 infectivity has remained elusive. Over the course of 3 years, my PhD research activity has led to the identification of the host transmembrane protein SERINC5, and to a lesser extent SERINC3, as potent inhibitors of HIV-1 infectivity counteracted by the viral protein Nef [Rosa et al., 2015].

SERINC5 is predominantly localized on the plasma membrane where it is efficiently incorporated into budding HIV-1 virions and impairs subsequent virion penetration of susceptible target cells. Nef relocalizes SERINC5 to an endosomal compartment preventing its incorporation into HIV-1 particles.

The ability to counteract SERINC5 is conserved in Nef proteins encoded by different primate immunodeficiency viruses, as well as in the structurally unrelated glycosylated Gag from murine leukaemia virus (MLV). These examples of functional conservation and convergent evolution emphasize the fundamental importance of SERINC5 in the interaction of the host with retroviral pathogens.

Remarkably, SERINC5 potently inhibits HIV-1 even in the presence of Nef in a dose-dependent manner, suggesting that this cellular factor might be exploited as an anti-HIV-1 therapeutic gene.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
Ad	Adenovirus
ALV	Avian Leukosis Virus
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like
bNAbs	brodly Neutralizing Antibodies
CA	Capsid
CAT-1	Carnitin acyltransferase 1
CCR5	C-C chemokine receptor type 5
CD4	Cluster of Differentiation 4
cPPT	central PolyPurine Tract
CPSF6	Cleavage and polyadenylation specificity factor subunit 6
CRM1	Chromosome region maintenance 1
CsA	Cyclosporine A
CTL	Cytotoxic T cells respones
CTS	Central Termination Sequence
CXCR4	Chemokine (C-X-C Motif) Receptor 4
CypA	Cyclophillin A
DNA	Deoxyribonucleic acid
EIAV	Equine Infectious Anemia Virus
env	*envelope*
ER	Endoplasmic Reticulum
ERV	Endogenous Retroviruses
FIV	Feline immunodeficiency virus
gag	*group-specific antigen*
GaLV	Gibbon ape leukemia virus
GLUT-1	Glucose transporter 1
gp120	HIV surface glycoprotein
gp41	HIV transmembrane glycoprotein
GTP	Guanosine-5'-triphosphate
HA	Hemagglutinin
HERV	Human Endogenous Retroviruses
HIV-1	Human Immunodeficiency Virus 1
HIV-2	Human Immunodeficiency Virus 2
HMG-I (Y)	high mobility group proteins
HTLV	Human T-lymphotropic virus
IFN	Interferon
IFTM	interferon induced transmembrane protein
IN	Integrase
ISG	Interferon Stimulated Gnes
LEDGF	Lens Epithelium-Derived Growth Factor
LFA1	Lymphocyte function-associated antigen 1

LTR	Long Terminal Repeats
LV	Lentiviral Vectors
MA	Matrix
MLV	Murine Leukemia Virus
MoMLV	Moloney Murine Leukemia Virus
MX2	Myxovirus Resistance 2
NA	Neuroaminidase
NAbs	Neutralizing Antibodies
NC	Nucleocapsid
NEF	Negative Factor
NPC	Nuclear Pore Complex
NUP	Nucleoporine
ORF	Open Reading Frame
PBMCs	Pheripheral Blood Mononuclear Cells
PBS	Primer Binding Site
PCR	Polymerase Chani Reaction
PFV	Prootype Foamy Virus
PIC	Pre-Integration Complex
pol-pro	*protease-polymerase*
PPT	PolyPurine Tract
PR	Protease
$Pr160^{gag\text{-}pol}$	Gag polyprotein precursor 160
$Pr55^{gag}$	Gag polyprotein precursor 55
R	Repeated region
Ran-GTP	RAs-related Nuclear protein binding GTP
RER	Rough Endoplasmic Reticulum
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RRE	Rev Responsive Element
RSV	Respiratory Syncytial Virus
RT	Reverse Transcriptase
RTC	reverse-transcription complex
SA	splicing acceptor site
SD	splicing donor site
SEC	Siize Exclusion Chromatography
SIV	Simian Immunodeficiency Virus
SLFN11	Schlafen 11
SU	Surface domain
TAT	Transactivating Protein
TM	Transmembrane domain
TNPO3	Transportin 3
TRIM5α	Tripartite motif-containing protein 5 alpha isoform
tRNA	transfer RNA
U3	unique sequence 3' end
U5	unique sequence 5' end
VIF	Viral Infectivity Factor
VPR	Viral Protein R

VPU	Viral Protein Unique
VSV	Vesicular Stomatitis Virus
VSV-G	G glycoprotein from Vesicular Stomatitis Virus

1. INTRODUCTION

1.1 The importance of studying Retroviruses

The term “Retroviruses” defines a large family of enveloped viruses with single-stranded positive-sense RNA genomes. [Coffin et al., 1997]. This virus family owes its name to the reverse transcriptase enzyme discovered in 1970 by D. Baltimore and H. M. Temin, who independently showed that retroviral RNA genomes are copied into DNA [Baltimore, 1970; Temin and Mizutani, 1970]. Turning over the dogmatic direction of genetic information [Crick, 1970], the discovery of reverse transcriptase impacted different fields of biology and caused an explosion of attention on research into Retroviruses. The importance of Temin and Baltimore’s work led them to receive the Nobel Prize for Physiology and Medicine in 1975.

Retroviruses infect a wide range of vertebrates ranging from fish to humans [Robertson, 2012]. Being human and animal pathogens, Retroviruses therefore arose the research interest from biomedical, agricultural and economic point of view. They are in fact responsible for a wide range of human and veterinary diseases including different kinds of cancer, immunological disorders and neurological conditions [Coffin et al, 1997].

As obligatory intracellular parasites, Retroviruses co-opt host molecular pathways to achieve a proper replication. Retroviral infection is the result of a constant compromise between supportive and inhibitory factors in the target cells. Supportive factors positively regulate retroviral replication, while on the other hand host restriction factors play an inhibitory role targeting different steps of replication. Due to their unique replication strategy and intricate life cycle, a better understanding of the molecular mechanisms behind host–pathogen interactions can provide novel insights into host cell biology. A more detailed description about viral and host factors relationship will be provided later in this dissertation.

The fast evolution rate of retroviruses [Combe and Sanjuán, 2014; Sanjuán et al, 2010] together with the physical and phylogenetic proximity between their different hosts make events of cross-species infection not unlikely [Hayward et al, 2013; Parrish et al, 2008]. Epidemics are indeed the outcome of viral adaptation into new species. Remarkably, the transfer of an animal pathogen to a human host is a process defined as zoonosis. Numerous zoonoses have involved the human population since long time and possibly the more devastating example is represented by HIV, which causes the Acquired Immune Deficiency Syndrome (AIDS). Therefore, a deeper knowledge of retroviral biology is important to prepare a prompt response to the risk of zoonosis or recombination between different viruses [Locatelli and Peeters, 2012; Hahn et al, 2000

Retroviral transmission could be horizontal, meaning via cell-free virus or infected cells, or vertical occurring from parents to the offspring. Retroviruses can in fact occasionally integrate into the germ cells DNA, becoming Endogenous Retroviruses (ERV), as discovered in the late 1960s. The inheritance as Mendelian elements turned out to be the smartest and most cost-effective modality of viral transmission [revieweb by Stoye, 2012]. Phylogenetic studies report that genomes of all vertebrates species analyzed underwent multiple waves of “invasion” by retroviruses. The endogenous proviral genetic elements may be referred as "fossil" retroviral genomes, giving rise to an emerging field of evolutionary biology known as Paleovirology [reviewed by Stoye, 2012].

ERVs represent about 8% of the human DNA, most of which are widely incorporated in old world monkeys, as proof of an early acquisition in primate evolution. In some animal species, a few ERV proviruses produce replication-competent viruses able to infect the same species or other species [reviewed by Stoye, 2012; and Weiss, 2013]. On the other hand, ERVs in humans appear also to be parasitic DNA sequences, exploited by the host as protection against further retrovirus infection. Their integration in the human genome could represent a potential risk of damage due to insertional mutagenesis and homologous recombination, but examples of disorders caused by an endogenous retroviral insertion into a gene reported in literature are rare [Villesen et al, 2004; reviewed by Stoye, 2012]. Most of human endogenous retroviruses (HERV) contains mutations preventing the production of infectious viral particles. However, a few open reading frames are preserved and some viral proteins may be produced. In 1995, Venables and colleagues reported the identification of a conserved human endogenous retroviral *env* gene expressed as glycoprotein in the human placenta differentiation [Mi et al, 2000]. HERV have been proposed as etiological co-factors in various diseases due to their enhanced expression in pathological conditions. Retroviral research is now focused on the possible HERV effects on host cell biology with particular regards in finding how HERV might contribute to diseases such as some kind of cancers and multiple sclerosis [reviewed by Stoye, 2012; Dolei et al, 2014; reviewed by Tugnet, 2013; reviewed by Bhardwaj and Coffin, 2014].

The ability of Retroviruses to be fixed and to cause a permanent modification into host cell genome can be exploited for therapeutic purposes. Retroviruses are promising tools to deliver and insert genetic information for replacement therapy in inherited disorders. The main concern for their use in therapy is the potential effect of insertional mutagenesis and the non-specific target cell recognition. The viral vectors can be properly engineered in order to avoid side effects and to optimize the desired target cell transduction. Apart from applications in gene therapy, viral vectors have also become important molecular biology tool to investigate gene functions [Bouard et al, 2009]. Over the last two decades, retroviral vectors have been extensively studied in order to improve their safety and efficacy. Remarkably, lentiviral vectors (LV) derived from infectious human retroviral isolates HIV-1 and HIV-2 (Human Immunodeficiency Virus 1 and 2 respectively) have been accepted as the most promising gene delivery vehicles [reviewed by Naldini, 2015].

1.2 Retroviral classification and structure

Retroviridae is a family of enveloped viruses, divided into two subfamilies *Orthoretroviridae* and *Spumaretrovirinae*. The former subfamily contains six genera known as *α-retrovirus, β-retrovirus, γ-retrovirus, δ-retrovirus, ε-retrovirus* and *lentivirus*; while the latter consists of only *spumavirus*. This classification is based on the genetic relatedness of the Reverse Transcriptase ORF (Table 1) [reviewed by Voisset *et al*, 2008].

Table 1 Classification of Retroviruses. a Refers to exogenous retrovirus only. From Voisset et al, 2008, Microbiol Mol Biol Rev 72, 157-196, table of contents.

Subfamily and genus[a]	Previous nomenclature	Species infected	Example(s)
Orthoretrovirinae			
Alpharetrovirus	Avian C-type oncoretrovirus	Birds	Avian leukosis viruses, Rous sarcoma virus
Betaretrovirus	B-type oncoretrovirus	Mice	MMTV
	D-type oncoretrovirus	Primates	MPMV
		Sheep	JSRV
Gammaretrovirus	Mammalian C-type oncoretrovirus	Mice	MLVs
		Cats	Feline leukemia viruses
		Primates	Gibbon ape leukemia virus
		Birds	Reticuloendotheliosis virus
Deltaretrovirus	C-type oncoretrovirus	Cattle	Bovine leukemia virus
		Primates	Human T-lymphotropic viruses
Epsilonretrovirus	None	Fish	Walleye dermal sarcoma virus
Lentivirus	Lentivirus	Primates	HIV and SIV
		Sheep	Maedi/visna virus
		Cats	Feline leukemia virus
		Horses	Equine infectious anemia virus
Spumaretrovirinae			
Spumavirus	Foamy virus	Primates	HFV and SFV
		Cats	Feline foamy virus
		Cattle	Bovine foamy virus

In addition, Retroviruses are broadly clustered into two groups according to their genome organization: “simple retroviruses” (*α-retrovirus, β-retrovirus, γ-retrovirus* and *ε-retroviruses*) and “complex retroviruses” (*lentiviruses, δ-retroviruses* and *spumaviruses*). Simple retroviruses contain just only three main structural genes (*gag, pol-pro and env*), while “complex” retroviruses express additional sets of genes with regulatory roles. A schematic representation of murine leukemia Virus (MLV) and human immunodeficiency virus (HIV) proviral genomes organization, as example of simple and complex retroviruses respectively, is reported in Figure 1. With reference to ERV, only those having a simple genome have become endogenous in their hosts (marked with a red asterisk in Figure 2) [Weiss, 2006].

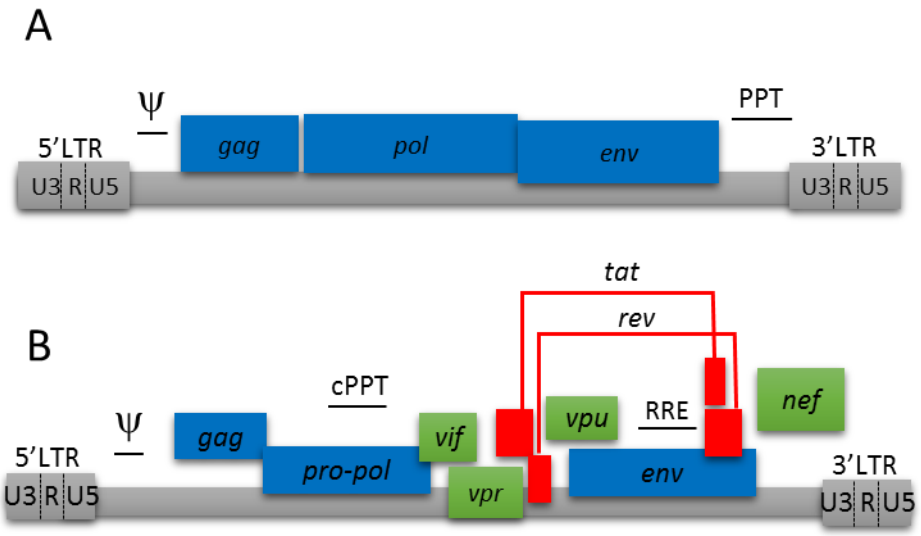

Figure 1. Retroviral genomes. Schematic representation of (A) MLV and (B) HIV-1 proviral genomes as examples of simple and complex retrovirus, respectively. Three main ORFs are reported in blue, the regulatory genes in red, the auxiliary genes in green and the regulatory regions in grey.

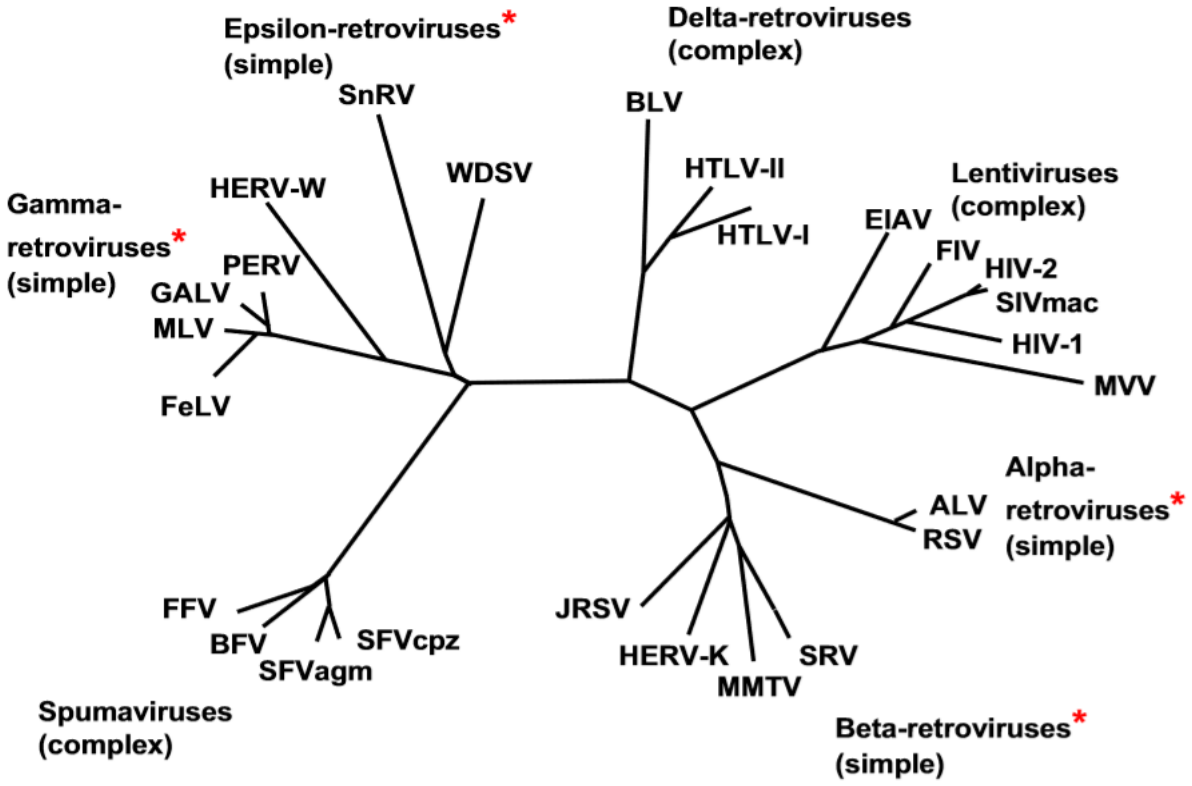

Figure 2. Phylogeny of Retroviruses. Asterisks mark genera including endogenous genomes. From Weiss, Retrovirology, 2006, 3:67

The retroviral genome consists of a single-stranded positive-sense RNA molecule, typically ranging between 7 to 12 kilobases in length, containing at least three main open reading frames (ORFs) (Figure 1):

- *group-specific antigen (gag)* for core and structural proteins production, such as matrix (MA), capsid (CA) and nucleocapsid (NC) proteins (Figure 3);
- *protease-polymerase (pro-pol)* expressing the viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN) (Figure 3);
- *envelope (env)* encoding the surface (SU) and transmembrane (TM) domains of retroviral coat glycoproteins (Figure 3).

In addition to these three genes, complex retroviruses encode accessory genes. For instance, the lentivirus HIV-1 encodes six accessory genes: vif, vpr, tat, vpu, rev and nef (Figure 1).

Each viral particles has a diameter in the range of 80 to 120 nm determined by scanning transmission electron microscopy [Vogt & Simon, 1999]. A representation of the structure of a generalized retrovirus particle is reported in Figure 4. All the retroviruses show common virion components despite their differences in morphology and biology [Coffin et al, 1997]. The retroviral lipid envelope is reported to be enriched in sphingomyelin and cholesterol, suggesting that the viral budding occurs in correspondence of cholesterol-rich rafts at the cell plasma membrane [reviewed by Waheed and Freed, 2010].

Each virus particle is diploid, meaning that it contains two unspliced genome copies held together at the 5' ends. Each RNA molecule is capped at the 5' end and polyadenylated at the 3'-end. A complex splicing pattern ensures the expression of all retroviral ORFs. A splice donor (SD) and a splice acceptor (SA) sites are generally upstream of *gag* and *env* genes respectively (Figure 3). However, some retroviruses have additional splice sites. The RNA genome contains noncoding regions as well, important for its replication (Figure 3). At each end of the viral genome, there is a short repeated sequence known as R region, which is necessary to ensure correct end-to-end transfer in the growing chain during the reverse transcription. The repeated regions (R) are followed by "unique sequences" U5 and U3, at 5' and 3' ends respectively. Once converted into double stranded DNA, R, U5 and U3 regions form the long terminal repeats (LTRs), essential for provirus integration into the host. Another cis-acting sequence is the Primer binding site (PBS), where the annealing of a specific cellular transfer RNA (tRNA) takes place, to prime reverse transcription

(Figure 3) . Each retrovirus needs a specific tRNA as primer for reverse transcription, for instance HIV-1 adopts $tRNA^{Lys}$ while MLV uses $tRNA^{Pro}$.

The 5' LTR is followed by a region, called Ψ, acting as a signal for genome packaging into budding virions. Immediately upstream the U3 sequence there is a very important region for the synthesis of the positive-strand DNA in the reverse transcription process, called polypurine tract (PPT) (Figure 3) [reviewed by Vogt, 1997]. Lentiviruses and some Spumaviruses contain an additional polypurine tract at the center of their genome (cPPT) together with a central termination sequence (CTS). In the reverse transcription process, this peculiarity results in the formation of a three stranded DNA structure called the central "DNA Flap", that is of controversial functional significance [reviewed by Basu et al, 2008]. Further details about the reverse transcription process will be provided in the next paragraphs.

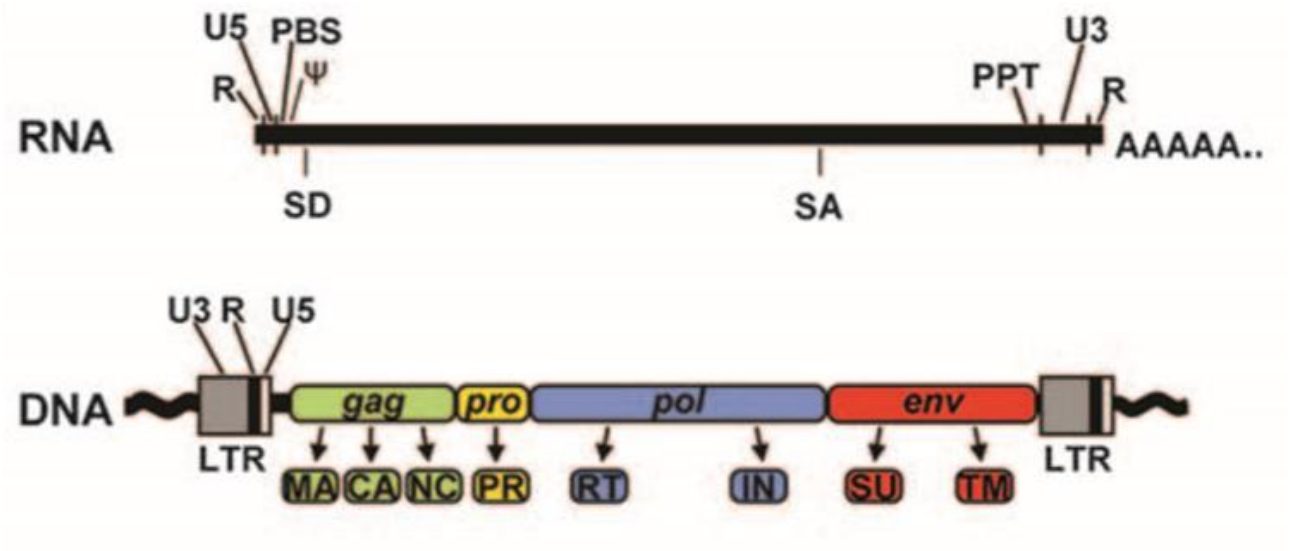

Figure 3 Retroviral genome organization of its RNA and DNA forms. Abbreviations: R - repeated region at the ends of RNA genome; U5 and U3, unique elements close to the 5' and 3' ends respectively; PBS, primer binding site used for initiation of reverse transcription; Ψ - encapsidation signal; PPT – polypurine tract; SD - Splice donor site; SA - splice acceptor site. During reverse transcription, the LTR is formed, which contains gene promoter and enhancer elements. Four main genes are present in all infectious retroviruses,gag, pro-pol, and env. Retroviral proteins are synthesized as large polyprotein precursors and later cleaved into the mature viral proteins: matrix (MA), capsid (CA), nucleocapsid (NC), protease (PR), reverse transcriptase (RT), integrase (IN) and into-the-surface (SU) and transmembrane (TM) envelope glycoproteins. Specific retroviruses encode additional proteins with specialized functions in the viral life cycle or pathogenesis. Adapted from Voisset et al, 2008, Microbiol Mol Biol Rev 72, 157-196, table of contents.

All retroviral proteins are synthesized as large polyprotein precursors, later cleaved into mature viral proteins by both viral (PR) and cellular proteases.

Matrix (MA) proteins are associated with the lipid envelope through their N-terminal myristoylation; a post-translational modification important for assembly of retroviral particles [Bryant and Ratner, 1990; Göttlinger *et al*, 1989]. Not all retroviral MA proteins are myristoylated, suggesting that additional factors are involved in its membrane targeting [Inlora et al, 2011]. Matrix proteins also interact with the TM domain of retroviral Envelope proteins during the budding step, an interaction which may promote Env incorporation into virions [Murakami and Freed, 2000; Wyma et al, 2000] but it is not essential [Briggs et al, 2003]. The capsid proteins (CA) form the core containing the viral genome associated with nucleocapsid proteins (NC). Mutations in a conserved cysteine and histidine motif of NC impair the packaging of the murine leukemia (MLV) genome into the nascent viral particles [reviewed by Rein, 2011]. Furthermore, depending on the type of retrovirus, other mature proteins may be produced from *gag*. For instance, murine leukemia virus (MLV) *gag* encodes an additional protein, p12, which seems to important for core stability and for tethering the pre-integration complexes to mitotic chromosomes [Wight et al., 2014; Elis et al., 2012].

The protease enzyme (PR) converts the Gag-Pol polyprotein into functional proteins during the viral assembly and maturation steps. Reverse transcriptase (RT) is a RNA-dependent DNA polymerase, which uses the viral genomic RNA as template to synthesize a copy of the minus strand DNA. RT has also a ribonuclease H activity (RNase H), degrading the RNA strand in the newly synthesised hybrid RNA:DNA [Telesnitsky and Goff, 1997]. The plus strand DNA synthesis is carried out by the same enzyme using the undigested PPT (polypurine tract) as primer. Once synthesised the proviral DNA is delivered to the nucleus, where the viral integrase (IN) promotes its integration into the host genome.

The proteins encoded by the *env* gene contain an N-terminal signal sequence allowing their delivery to the ER and subsequently to Golgi, where they are glycosylated and then cleaved by cellular proteases into two subunits: surface (SU) and transmembrane (TM). These subunits oligomerise into heterodimers, whose 3D structure determines the spikes on the viral envelope [reviewed by Eckert and Kim, 2001]. Being localized on the viral surface, the Env proteins establish the first virus-cell interactions and the entry of the virus into the host [Coffin, 1997].

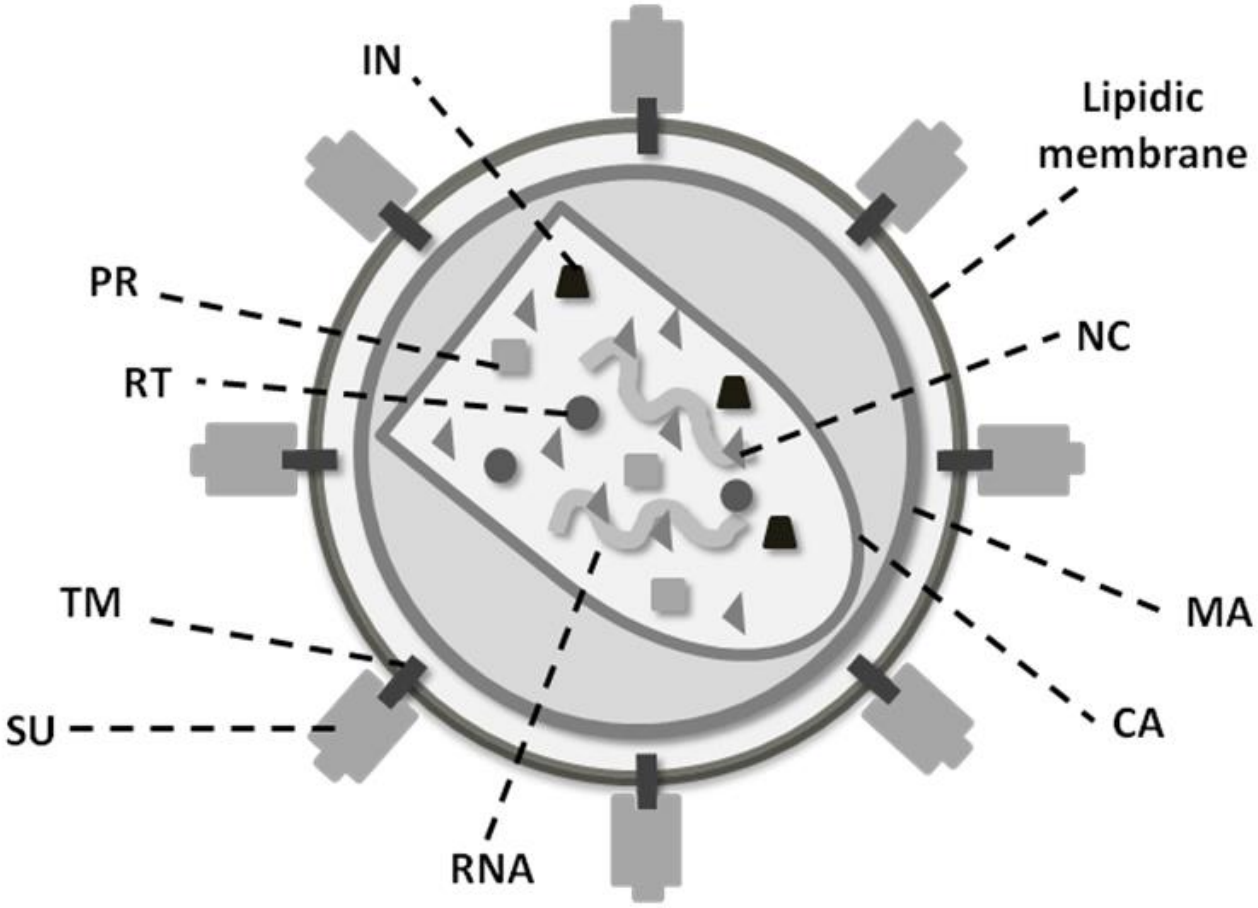

Figure 4. Retrovirus particle structure. Abbreviations: NC – nucleopcapsid; MA – matrix; CA – capsid; SU – surface subunit of Env protein; TM – transmembrane subunit of Env protein; RT – reverse transcriptase; PR – protease; IN – integrase**. F**rom Thomás et al., 2013, Gene Therapy - Tools and Potential Applications, Chapter 12 - Lentiviral Gene Therapy Vectors: Challenges and Future Directions. DOI: 10.5772/52534.

1.3 Retrovirus replicative cycle

As obligatory intracellular parasites, Retroviruses need a host cell to carry out their life cycle. They indeed can orchestrate host molecular mechanisms to achieve a proper replication. Since most of them are human and animal pathogen, a deep knowledge of their replication cycle is therefore needed for the development of proper antiviral therapies.

The life cycle of retroviruses can be simply divided into two major distinct phases: the early phase going from the viral entry to the integration into the host cell genome, and the late phase covering the steps from the expression of viral genes to the release of mature progeny viral particles (Figure 5).

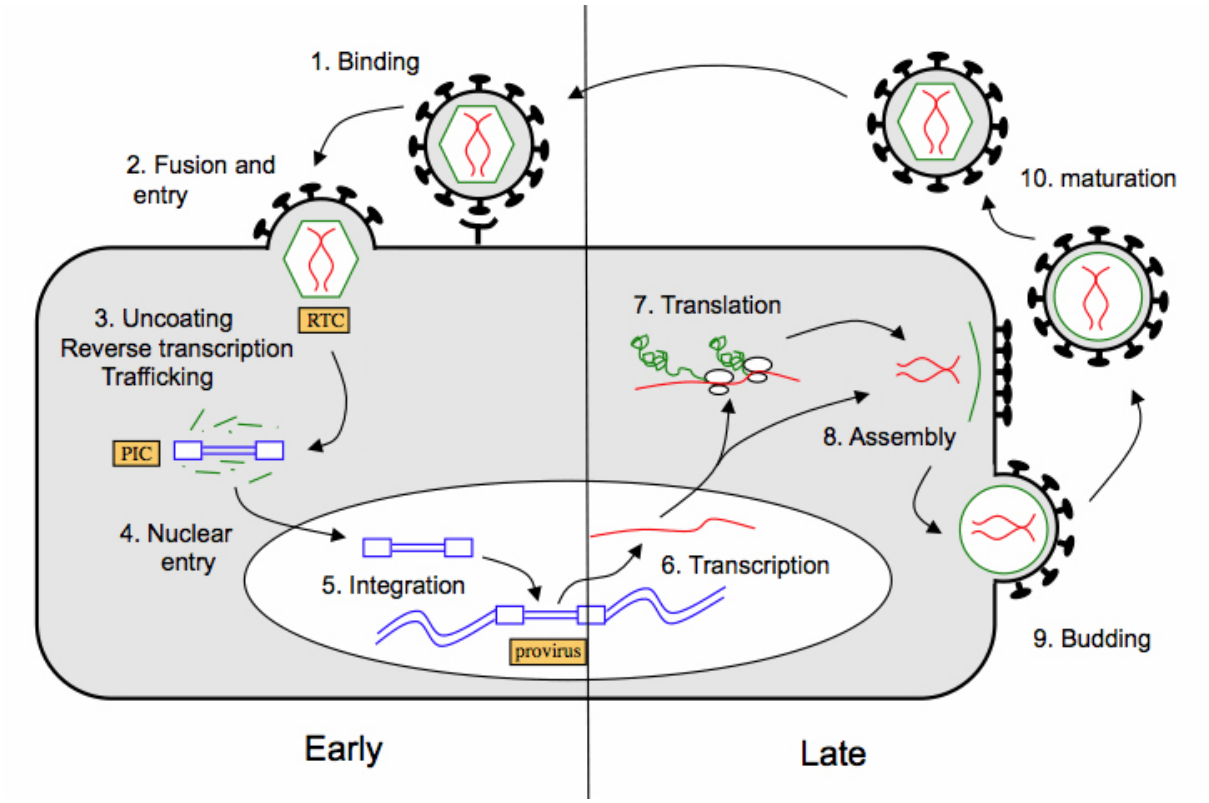

Figure 5. Steps in retroviral life cycle. The early phase of retroviral life cycle consists of: the binding of the virus to the target cell (1); the subsequent fusion between the host cell and the viral particle (2), the release of the viral content in the cytoplasm (3. Uncoating) and the simultaneous reverse transcription converting the viral single stranded RNA genome into double stranded DNA; the import of viral genome into the nucleus (4) and viral integration into the host genome(5). The late steps of retroviral life cycle include the expression of viral genome (6. Transcription) and the production of viral proteins (7. Translation), the subsequent assembly of viral particles (8) and their budding from the host cell (9). Once the virions are released from the host cells, they need to go through a further step known as maturation (10) to be properly infectious.

1.3.1 Binding

The viral entry starts with the virus adsorption to the cell surface, mediated by viral glycoprotein and specific cell surface molecules interaction. The attachment process was thought to be dominated by a viral receptor, expressed by the host cell, recognizing viral glycoproteins. Conversely, other interactions came out to be important for virus binding [Sharma *et al*, 2000]. Reports about MLV and HIV-1 binding to target cells have shown the dispensable role of a specific receptor-Env interaction [Pizzato *et al*, 1999; reviewed by Ugolini *et al*, 1999], suggesting the involvement of other cell surface molecules like heparan sulfate proteoglycan [Mondor *et al*, 1998], LFA-1 [Fortin *et al*, 1998] and nucleolin [Nisole *et al*, 1999]. These additional attachment factors may be useful to concentrate the virus at the target cell surface in order to facilitate the subsequent specific receptor recognition [Pinon *et al*, 2003; Walker *et al*, 2002;]. Env or receptor-independent binding of HIV do not promote a productive infection [Schaeffer et al, 2004], which strictly requires Env binding to its cognate receptor. However, HIV can exploit its envelope glycoproteins interaction with lectins exposed on dendritic cells surface to get carried by these cells from peripheral sites of infection to lymph nodes. This way the infection *in trans* of target cells provided with the appropriate receptor can occur [Sewald et al., 2015]. This strategy is shared by many other viruses [reviewed by van Kooyk and Geijtenbeek, 2003] and by non-viral pathogens like *Mycobacterium tuberculosis* [reviewed by Montellaro et al, 2009].

1.3.2 Entry

The cell membrane represents at the same time the major obstacle and the strategic way to access the target cell for the infecting viruses. Retroviruses are able to employ a wide range of cell surface molecules to get the infection started. Membrane transport proteins such as CAT-1 for ecotropic MLV [Kim *et al*, 1991; Wang *et al,* 1991], GLUT-1 for HTLV, PIT-1 and PIT-2 for GaLV and amphotropic MLV respectively, and the T-cell surface marker CD4 for HIV [reviewed by Manel et al, 2005] are just few examples. This step of viral lifecycle is well characterized for HIV, which still represents a significant public threat. Initially, the interaction between envelope glycoprotein surface subunit gp120 and CD4 receptor takes place, leading to conformational changes and the subsequent recruitment of coreceptors belonging to the chemokine receptor family (CXCR4 and CCR5) [reviewed by Berger *et al*, 1999]. The envelope glycoprotein gp120 then associates to the coreceptors and performs a further conformational shift [Kwon et al, 2014]. This set of structural modifications causes the dissociation of the surface subunit gp120 from gp41 and the subsequent exposure of the fusion peptide that accounts for the fusion of viral and cellular membranes [Gallo et al, 2003; Moore et al, 2003]. This process is energetically unfavourable and requires the destabilization of the membrane microenvironment [Cohen and Melikyan, 2004]. As a result, the formation of a fusion pore allows the viral core to be released into the cytosol. [Gallo et al, 2003; Moore et al, 2003].

The direct fusion with the host cell plasma membrane is the entry route observed for many retroviruses like HIV (Figure 6 B) [McClure *et al*, 1988]. However, more recent studies have shown that HIV infection occurs after internalization into intracellular vesicles [Miyauchi et al, 2009; Carter et al; 2011; de la Vega et al 2011; Maréchal et al, 2001]. Taken together, these reports suggest that HIV particles may be internalized into the target cell by endocytosis and their release into the host cytoplasm is pH independent, as it takes place at early endosomes without requiring endosome acidification (Figure 7A). The acidic late endosome can actually activate proteases degrading the viral particles. A receptor-independent binding of HIV virions may promote its entry via endocytosis as well [Schaeffer et al, 2004]. In this case HIV entry is pH dependent, as the virus has to compete with degrading proteases into the late endosome to reach the cytoplasm (Figure 7B) [Yoshii et al, 2011]. However, CD4-independent entry pathway leads to a less productive infection compared to the CD4-dependent one [Fredericksen et al, 2015].

Since the degradation of the endocytosed components into acidic intracellular compartments is part of a cellular innate immune response to external threats, the CD4-dependent HIV entry might be evolved from the CD4-independet one, in order to overcome the late endosome degradation. The virus adaptation to use cellular immune reactions to get into the cells has revealed a great strategy to avoid host immune defence.

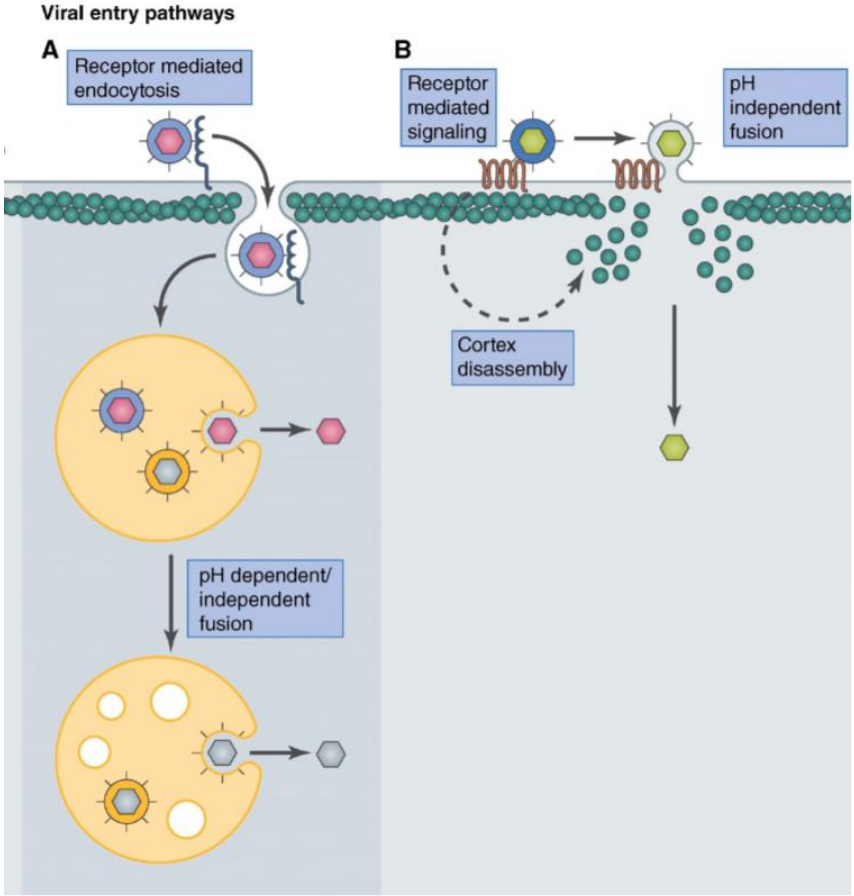

Figure 6. Virus entry strategies. Viruses have evolved various strategies to overcome the barriers imposed by the target cell, such as receptor-mediated endocytosis followed by pH-dependent/ independent fusion from endocytic compartments (A) or pH-independent fusion at the plasma membrane, coupled with receptor-mediated signaling and coordinated disassembly of the actin cortex (B). Non-enveloped viruses use similar strategies, although the mechanisms of action are different. Adapted from Grove and Marsh, 2011, JBC.

Some other retroviruses follow the endocytosis pathway to infect the target cells although through different mechanisms. For instance, avian leukosis virus (ALV) and equine infectious anemia virus (EIAV) require clathrin-mediated endocytosis [Brindley et al, 2005; Jin et al, 2005]; while amphotropic MLV and Ebolavirus have been recently identified to entry the target cells via macropinocytosis [Rasmussen and Vilhardt, 2014]. Retrovirus are actually able to exploit different internalization pathways for productive infection, like phagocytosis, macropinocytosis or clathrine-mediated endocytosis. However, the type of target cells may determine the viral preferential entry pathway and the eventual requirement of endosome acidification as well.

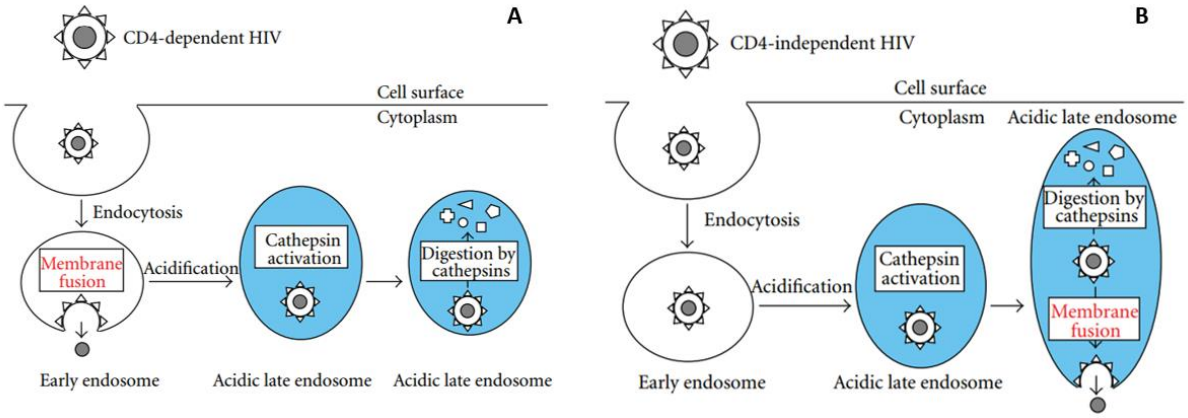

Figure 7. Entry pathway of CD4-dependent (panel A) and CD4-independent (panel B) HIV. Blue area indicates acidic conditions. Adapted from Kubo et al, 2012, Advances in Virology, 640894

Retroviruses may enter the host cell through specific membrane microdomains known as “rafts”. They are made up of sphingolipids and cholesterol packed together in ordered, rigid and mobile structures [Brown and London, 2000; Lee, 2001]. and they may work as docking sites for proteins involved in processes like signalling or endocytosis [Alonso and Millan, 2001; reviewed by Simons and Toomre, 2001; Simons and Ehehalt, 2002]. Experimental data, arguing for a role of lipid rafts in retroviral entry process, show the clustering of both viral envelope glycoproteins [Bavari et al, 2002; Pickl et al, 2001] and cellular receptors in these membrane domains [Popik et al, 2002].

The retargeting of either envelope glycoproteins [Rousso et al, 2000] or cellular receptors to non-raft membranes may impair viral entry [Del Real et al, 2002]. Moreover, lipid components of cell membrane rafts, like some glycosphingolipids, may interact with viral envelope glycoprotein acting as alternative entry co-factors [Hug et al, 2000]. In addition, cholesterol depletion in target cells prevents HIV-1 infection and syncytium formation [Manes et al, 2000; Liao et al, 2003]. On the other hand, controversial reports question the actual role of lipids in retroviral entry. For instance, some studies have demonstrated that HIV entry is not affected by CD4 and CCR5 localization into non-rafts membrane domains [Percherancier et al, 2002; Popik and Alce, 2004]. Additionally, the effect of cholesterol depletion on HIV-1 infectivity may be rescued by an increasing expression of CD4 and CXCR4 molecules [Viard et al, 2002].

In summary, it remains unclear whether membrane rafts act as concentrating platforms of factors modulating the entry process and further studies are required to better characterize and clarify lipid domains implications into retroviral biology.

1.3.4 Uncoating

Upon the fusion between the viral and cellular membranes, the viral core is released into the cytoplasm where it starts disassembling into subviral particles, called reverse-transcription complexes (RTCs) and pre-integration complexes (PICs).

The uncoating is a gradual multi-steps process, starting with the initial break of HIV-1 capsid [Yu et al, 2013] followed by its complete dissociation [Jun et al., 2011, Xu et al., 2013). Recent studies have demonstrated that capsid stability is required for reverse transcription process, supporting the idea that the two processes are mechanistically coupled [reviewed by Ambrose and Aiken, 2014]. CA reduced stability is often linked to a reduced reverse transcription and decreased infection [Rihn et al., 2013; von Schwedler et al., 2003].

Recent in vivo imaging show the uncoating as a process taking place just before the end of reverse transcription [Hulme et al., 2011; Xu et al., 2013] suggesting that the DNA synthesis and the uncoating are simultaneous events. However, the uncoating steps is necessary to allow some cellular factors to take part to the retrotrascription process [Warren et al., 2012; Warrilow et al., 2009]. In 2011, Hulme and colleagues supported the hypothesis of the induction of the uncoating by reverse transcription itself, as inhibition of reverse transcription turns into a delay in core disassembling as consequence of the increased core stability [Yang et al, 2012].

HIV-1 uncoating is a strictly monitored process based on a proper capsid stability and interaction with multiple host factors. Premature or delayed dissociation of capsid may affect reverse transcription and nuclear import resulting in abortive infection. The incoming viral particles have evolved specific mechanisms to interact with host molecular partners in order to facilitate their journey through the cytoplasm with nuclear destination. In particular, cellular cytoskeleton proteins seem to play an important role for the uncoating step of different viruses [Ploubidou and Way; 2001]. Another host protein fundamental for viral uncoating is cyclophillin A (CypA), which directly binds the capsid and gets incorporated into nascent viral particles [Luban *et al*, 1993; Thali *et al*, 1994; reviewed by Ambrose, 2014]. Lacking of CypA or impaired binding between CypA and capsid upon the treatment with cyclosporine A (CsA), result in a defective disassembly of the HIV-1 core [Braaten *et al*, 2001]. Actually, CypA role consists of protecting HIV-1 capsid by the human restriction factor TRIM5α action [Towers *et al*, 2003; Hatziioannou et al., 2004) Stremlau et al, 2004], and by the innate sensing [Rasaiyaah et al., 2013], thus increasing HIV-1 infectivity.

Among the host protein interacting with capsid there are CPSF6 [Henning et al., 2013], which is involved in mRNA processing and shuttles between nucleus and cytoplasm, the transportin TNPO3 and the nucleoporines NUP358 and NUP153 [Price et al, 2012] which allow the delivery of PICs through the nuclear membrane. The association of CA ultrastructures with NPCs and the nuclear envelope reported by Arhel and colleagues in 2007, suggests that uncoating takes place mainly at the nuclear periphery upon completion of reverse transcription. Although the role of host factors during the uncoating step is still poorly understood, it is possible that the process takes place in proximity of nuclear pore just to promote a safe delivery of viral contents into the nucleus of the infected cell [Rasaiyaah et al., 2013].

1.3.5 Reverse transcription

Although there is evidence showing DNA synthesis in virions before infection [Lori *et al*, 1992; reviewed by Coffin et al, 1997; Davis et al, 2008] and during the uncoating step [Zhang *et al*, 2000], reverse transcription usually takes place in the cytoplasm in parallel with the release of the viral core. Upon fusion with the target cell, the virus starts reverse transcribing its genome inside a ribonucleoparticle complex known as reverse transcription complex (RTC) [Fassati and Goff, 2001].

The only exception is represented by Foamyvirus virions, in which the retroviral genome is already found almost completely reverse transcribed [Zamborlini et al, 2010]. In all other cases studied so far, the viral RNA is reverse transcribed in the target cell cytoplasm by the virion-packaged reverse transcriptase (RT). This process might be stimulated by the high concentration of deoxyribonucleotides in the cytoplasm [reviewed by Goff, 2001].

The viral RT enzyme starts the polymerization of a minus-sense DNA strand using the RNA genome as template and the 3' end of a cellular tRNA as primer annealed at the primer binding site (PBS) (Figure 8-A). Each retrovirus uses a specific tRNA, for instance HIV-1 adopts $tRNA^{Lys}$ while MLV uses $tRNA^{Pro}$. Once the 5' end of the viral RNA template is reached (Figure 8-B), the RT uses its ribonuclease H activity (RNase H) to degrade the genomic RNA strand in the newly synthesised hybrid RNA:DNA (Figure 8-C). The correct end-to-end transfer of the growing minus-strand DNA chain is governed by the repeated region (R) matching (Figure 8-D), so that RT can carry on the synthesis of the minus-strand DNA and the progressive digestion of the RNA template (Figure 8-E). The polypurine tract region (PPT) of the viral RNA genome is resistant to RNase H degradation, and therefore is used as primer for the plus strand DNA synthesis (Figure 8-F). The complete polymerization of the plus-strand DNA until the portion of the tRNA primer allows its removal (Figure 8-G). The plus-strand DNA is then transferred to the opposite site of the minus-strand DNA template to allow the annealing of PBS regions (Figure 8-H). At this point the synthesis of both strands is completed, resulting in a linear double stranded DNA molecule flanked by two long terminal repeats (LTR) (Figure 8-I) [Telesnitsky and Goff, 1997].

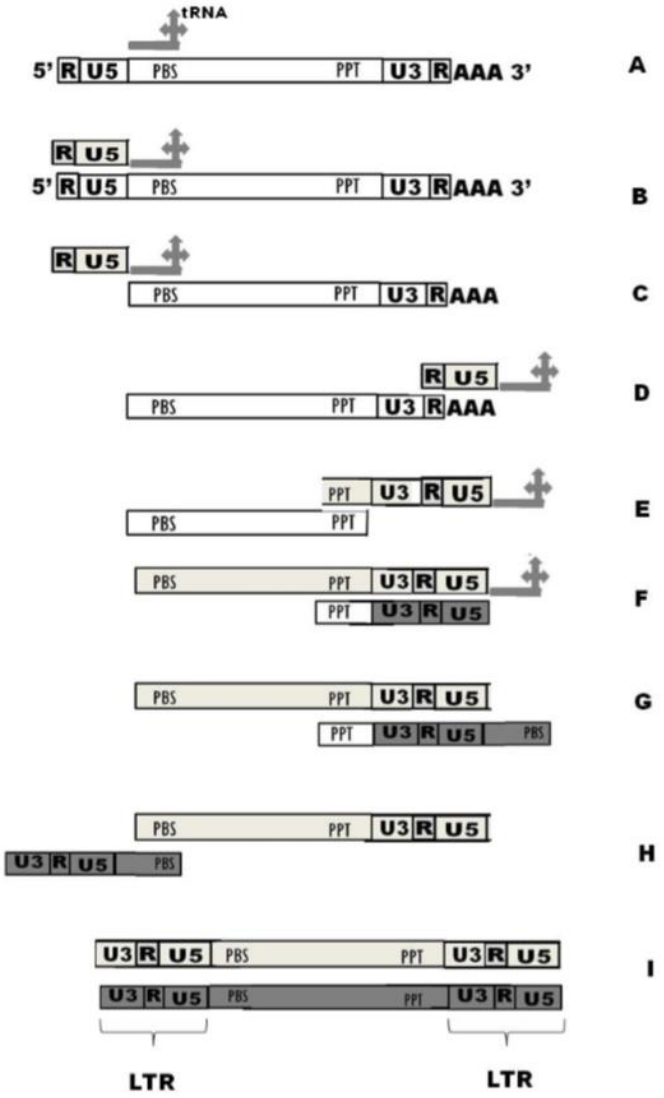

Figure 8. The reverse transcription process. A detailed description of the process is provided in the text.

Lentiviruses and some Spumaviruses contain an additional polypurine tract in the center of their genome (cPPT) together with a central termination sequence (CTS). During reverse transcription, the positive DNA strand is produced into two halves initiated from the two different polypurine tracts respectively, resulting in the formation of an unusual three stranded DNA structure known as "DNA Flap" in Lentiviruses and "DNA gap" in Spumaviruses (Figure 9) [Zennou *et al*, 2000].

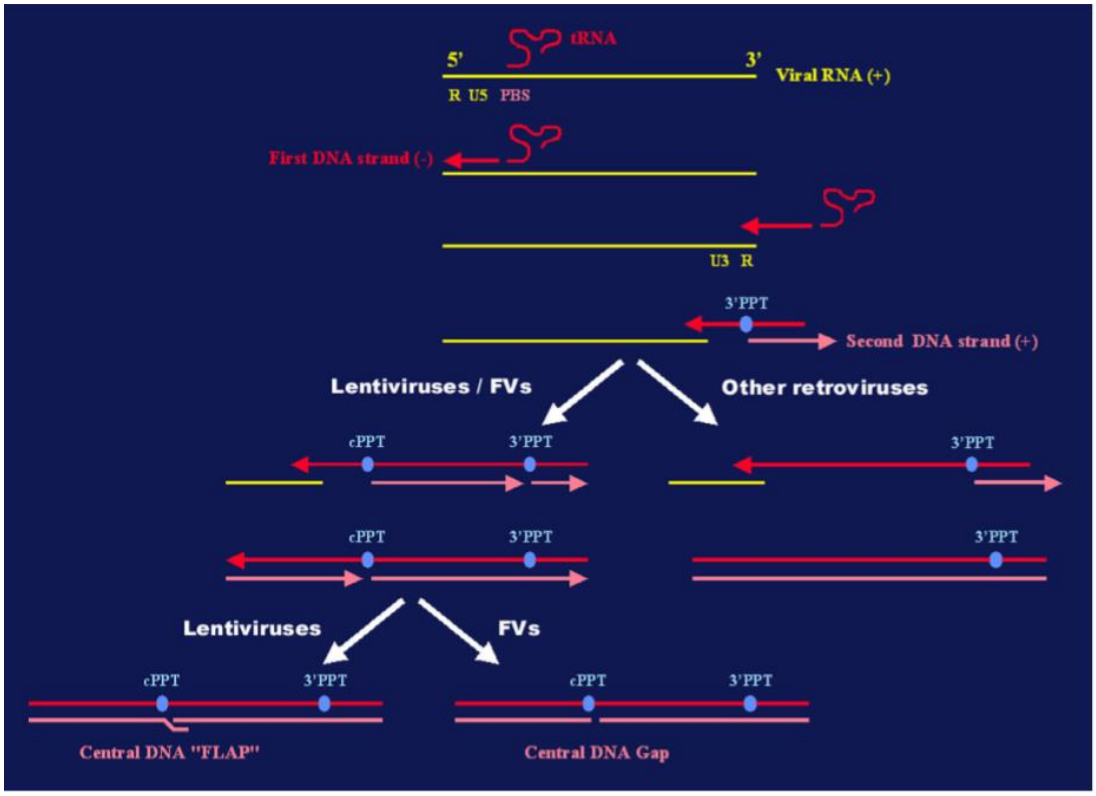

Figure 9. The central DNA FLAP generated by HIV-1 reverse transcription. Taken from Nisole and Saib, 2004, Retrovirology.

DNA plus-strand extension from the cPPT primer may slow down reverse transcription rate inside the cell [Riviere et al, 2010; Skasko and Kim, 2008]. Thus resulting into a kinetic advantage conferred by cPPT since the viral single stranded DNA is less exposed to the in inhibitory activity of APOBEC3 cytosine deaminase restriction factors [Hu et al, 2010], and the RTC/PIC are more protected from other host defense proteins action[Poeschla et al, 2013].

1.3.6 Nuclear Import

Once synthesised, the viral DNA is associated with some viral proteins forming PICs (pre-integration complexes). These complexes need to overcome the nuclear membrane barrier in order to get the viral DNA integrated into host genome. For most retroviruses, like MLV, PICs may access the host genome only during cellular mitosis, due to their inability to enter intact nuclei [Roe et al, 1993; Lewis and Emerman, 1994]. These retroviruses are therefore unable to replicate in non-dividing cells. On the other hand, PICs form Lentiviruses are able to cross the nuclear membrane [Bukrinski et al, 1992] resulting in infection of non-proliferating cells, like macrophages or quiescent T-lymphocytes [Gartner et al, 1986; Weinberg et al, 1991]; whereas Foamyviruses are able to replicate in both dividing and non-dividing cells [Bieniasz et al, 1995; Saib et al, 1997]. In order to cross the nuclear membrane, lentiviral PICs need to interact with the nuclear pore complexes (NPCs), made up of 30 different nucleoproteins (NUPs) crossing the nuclear membrane and connecting cytoplasm and nucleoplasm [Hoelz et al, 2011]. The transport through the nuclear membrane is governed by the size of the NPCs and by the protein-protein interactions between NUPs and specific carrier proteins [Conti et al, 2006; Terry et al, 2009]. The directionality of trans-nuclear trafficking is regulated by the gradient of Ran (Ras related nuclear) GTPase proteins, which binding protein carrier with different affinity according to their association with GTP/GDP (guanosine triphosphate/ guanosine diphosphate) [Nemergut et al, 2001; Saitoh et al, 1997]. Usually, the RTCs/PICs (reverse transcription/pre-integration complexes) observed in the cytoplasm are larger than the nuclear pore diameter [Lelek et al, 2012; McDonald et al, 2002]. The core of the PIC driving integration is made up of double-stranded reverse-transcribed viral DNA and a tetramer of IN proteins and it is known as intasome [Li et al, 2006; Hare et al, 2010]

With reference to HIV, the viral nucleoprotein complexes are made up by integrase (IN), matrix (MA), Vpr, NC, RT, PR proteins and the viral DNA [Iordansky et al, 2006, Bukrinsky et al, 1993; Gallary et al, 1997; Heinzinger et al, 1994]. Recent microscopy reports have identified also CA as part of cytoplasmic nucleoprotein complex [Lelek et al, 2012; McDonald et al, 2002]. Indeed, CA viral protein was found to be a master regulator of HIV-1 nuclear import. Chimeric HIV-1 viruses expressing MLV CA were no more able to replicate in non-dividing cells [Yamashita and Emerman, 2004]. Interestingly, some CA mutants impaired in infectivity showed a post-entry defect [Yamashita

et al, 2007] while others proved a defect at the level of nuclear import itself upon a delayed uncoating [Dismuke et al, 2006].

A set of shuttling receptors known as importins [Gallay et al, 1997; Ao et al, 2010], mediate the nuclear import through the recognition of a nuclear localization signal (NLS), a short amino acidic sequence that marks the protein for nuclear delivery [reviewed by Fried and Kutay, 2003]. Recent findings have shown a direct interaction between IN and transportin 3 (TNPO3) [Larue et al, 2012; Christ et al, 2008] and to nucleoporins like NUP153 [Woodward et al, 2009], thus avoiding the involvement of a molecular adaptors. While PICs protein components show different kinds of NLS mediating their interaction with NPCs elements with differential strenght [reviewed by Sherman and Greene, 2002], a crucial role for HIV-1 PIC nuclear import could also be played by the viral DNA structure itself.

As mentioned previously, in Lentiviruses the reverse transcription leads to the formation of a particular DNA structure known as “DNA flap” (Figure 9). This structure has been defined as a *cis*-determinant of its own efficient nuclear import [Zennou et al, 2000]. Interestingly, DNA flap structure was observed to play a role in proviral integration as well [Van Maele et al, 2003]. Although this structure could be dispensable for integration [Marsden et al, 2007], some recent findings have shown its positive effect on viral replication [De Rijck et al, 2006; Riviere et al, 2010; Ao et al, 2004].

So far, microscopy-based approaches have provided important tools to detect HIV-1 nuclear import and integration [Lelek et al, 2012; Christ et al, 2008; Di Primio et al, 2013] but live-cell tracking strategies for nuclear import are still missing.

1.3.7 Integration

Following nuclear import, the viral DNA integrates as a provirus, flanked by the long terminal repeat (LTR). As part of the host genome, the viral DNA may be passed on to progeny cells. This singular feature of retroviruses allows them to keep the genetic information life-long in the cell-genome.

The integrase (IN) enzyme is the key factor mediating this step. It not only accounts for the viral DNA integration itself, but it also determines the site of integration, together with cellular partners taking part to the process. IN cleaves the 3' ends of each LTR of viral DNA, thanks to its nuclease activity, then it catalyses the formation of phosphodiester bonds between the target cellular DNA and the viral DNA through its DNA binding and transesterification activities. These events turn the RTC into PIC complex [reviewed by Krishnan and Engelman, 2012]. Conventionally cellular proteins involved in the DNA damage response fills in the nicks and gaps flanking the viral DNA [reviewed by Smith and Daniel, 2006]. The integration and the consequent expression of viral DNA might be affected by the chromatin architecture of the flanking cellular sequences [Pryciak *et al*, 1992]. For instance, MLV integration targets actively transcribed genes [reviewed by Craigie, 1992]; mainly in the proximity of the gene transcriptional start sites and CpG islands [Moalic et al, 2006; Wu et al, 2003]. HIV and SIV integration occurs in highly expressed genes as well, but unlike MLV it is found along the transcriptional unit [Wu et al, 2003; Schroder et al, 2002]. On the other hand, ALV does not integrate in transcriptionally active regions. The integration site choice might also rely on host cell proteins interacting with viral IN and PICs factors. For HIV, several host IN binding partners have been identified to take part to the integration process. LEDGF (lens epithelium-derived growth factor) and HMG-I (Y) (high mobility group proteins) are two examples of host proteins that seem to stimulate HIV DNA integration through their direct interaction with IN [Maertens et al, 2003; Cherepanov et al, 2003; Farnet and Bushman, 1997; Li et al, 2000].

1.3.8 Viral RNA synthesis and processing

After the integration, the viral DNA mimics a cellular gene and its expression is carried out by the host-cell transcriptional machinery including RNA polymerase II and its transcription factors. The transcription process is orchestrated by the viral *cis*-acting elements, localized in the viral DNA non-coding regions (Figure 3). The long terminal repeats (LTRs) contain an enhancer sequence (U3), binding sites for host transcription factors and regulating transcriptional initiation by cellular RNA polymerase II [Speck and Baltimore, 1987]. Moreover, the promoter in the U3 region contains the TATA element upstream the transcription start site [Wilson et al, 2003]. Each retrovirus employs a specific repertoire of transcription factors depending on the target cell. The complex retroviruses (like HIV and HTLV) express their own transcriptional activators and regulators (Tat and Tax respectively), which provide the virus with additional gene expression control. The *cis*-acting elements in 3' LTR are involved in the post-transcriptional processing of the RNA products. This step is carried out by host the RNA processing machinery, which provides each RNA molecule with a 5' end cap and a polyadenylated tail at the 3' end. During retroviral replication, part of the full-length viral RNA transcripts are subsequently packaged into budding virions as genomic RNA, while subgenomic-sized RNA molecules may be produced by alternative RNA splicing. Both genomic- and subgenomic-sized RNAs may function as mRNA for viral proteins production [Rabson and Graves, 1997].

1.3.9 Nuclear export

Retroviruses have to face the critical task of exporting their unspliced RNAs from the nucleus to the cytoplasm. In simple retroviruses this step relies on *cis*-acting sequences, while in complex retroviruses the presence of accessory proteins can provide for nuclear export. Gag proteins from RSV, FIV, and PFV interact with the cellular CRM1 protein for nuclear export [Kemler et al, 2012; Renault et al, 2011]. In Moloney MLV (MoMLV) the packaging signal ψ can account for this function [Smagulova *et al,* 2005], while in HIV-1 the regulatory protein Rev can act as adaptor between the viral RNA and the nuclear export protein [reviewed by Polland and Malim, 1998]. Rev protein multimerize on the RNA molecules and recruits cellular cofactors like h CRM [[Daugherty et al, 2010; Chaytania and Belasco, 2001]. Rev and the RRE-containing viral are transcripts both found into the nucleolus, suggesting a possible nucleolar step of the Rev-RRE complex necessary for nuclear export [Michienzi et al, 2000; Buonomo et al, 1999; Fischer et al, 1999]. Recent reports have shown a co-localization of Rev with HIV-1 RNA at the transcription sites, supporting the idea that Rev binds the transcripts co-transcriptionally [Kula et al, 2013]. HIV-1 RNA export takes place thanks to the presence of nuclear export signals in Rev proteins mediating its interaction mainly with CRM1/RanGTP export complex and a subset other cellular proteins involved [Kula et al, 2013; Naji et al 2012; Nekhai and Jeang, 2006; Hadian et al, 2009]. Interestingly, the nuclear matrix-associated protein Matrin 3 is reported to be an important co-factor for HIV-1 RNA export. Matrin 3 binds HIV-1 RNA cotranscriptionally and it generally increases cellular mRNA stability; however the mechanism by which facilitates Rev-mediated nuclear export of unspliced RNA is still poorly understood [Kula et al, 2013; Yedavalli et al, 2011; Salton et al 2011]. Recent reports have shown how nuclear matrix components form tubular pathways connecting genomic DNA and nuclear pore complexes for nuclear export of transcripts [Malecki and Malecki, 2012]. Being a nuclear matrix-associated protein, Matrin 3 might act as a bridge between Rev and active HIV-1 RNA transcription sites, recruiting all the cellular factors needed like CRM1 nuclear export machiner [Yedavalli et al, 2011; Kula et al, 2013]. Other complex retroviruses encode Rev-like proteins that can account this role [Indik et al, 2005; Mertz et al., 2005].

1.3.10 Assembly

Assembly is a highly dynamic process and follows different routes for different retroviruses. For lentiviruses, α- and γ-retroviruses assembly takes place at the plasma membrane where all viral components are delivered, whereas β- and δ–retroviral cores are assembled in the cytoplasm and subsequently move to the cell membrane proximity [Swantstrom and Willis, 1997].

Lipid rafts in the plasma membrane work as a meeting point for the transmembrane and core components to assemble the viral particle. As a result, viral particles incorporate both the envelope components as well as constituents of host cell lipid rafts, like gangliosides, cholesterol and lipid-anchored proteins. Many viruses are reported to assemble in correspondence of specific rafts domains, which associate with the specific budding site of the viruses. For instance, the apical targeting of influenza virus hemagglutinin (HA) and neuroaminidase (NA) envelope glycoproteins correlates with the apical budding of the virus itself [Scheiffele et al, 1997; Simons and Ikonen, 1997]. The same has been reported for vesicular stomatitis virus (VSV), whose G glycoprotein expression in rafts has a basolateral localization corresponding with its preferential budding site [Pickl et al, 2001]. The assembly of measles virus within membrane rafts has been demonstrated too [Manie et al, 2000; Vincent et al, 2000]. Additionally, HIV-1 lipidic envelope composition strongly support its assembly and subsequent budding from lipid rafts [Briggs *et al*, 2003; Ono and Freed, 2005].

For HIV-1, Gag polyprotein production strategy orchestrates the assembly process, since it facilitates the targeting of each components to the assembly site. In the cytoplasm, the ribosomes synthesize the Gag polyprotein precursors $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ (Figure 10), from the same unspliced viral RNA. These protein precursors are cleaved by the viral protease during or after assembly of the viral particle, leading to the maturation of an infectious virion. On the other hand, spliced RNA transcripts are used to synthetize Env proteins on the membrane of the RER (rough endoplasmic reticulum), where they are firstly produced as a single polypeptide (gp160), glycosylated and then cleaved by a cellular protease into surface (gp120) and transmembrane (gp41) subunits. Vesicular transport drives the viral glycoproteins through the Golgi apparatus to the plasma membrane. Once on the cell surface they move towards the budding site for viral core packaging [Swantstrom and Willis, 1997], clustering all together in membrane rafts [Bavari et al, 2002; Manie et al, 2000; Pickl et al, 2001]. The plasma membrane localization and the recruitment of Envelope glycorpoteins are regulated by MA (Figure 10) [Bryant and Ratner, 1990; Göttlinger *et al*, 1989; Rein *et al*, 1986]. CA is

the Gag protein central domain and it mediates interactions among proteins required to create the viral protein shell. Interestingly, CA C-terminal domain is conserved across different retroviruses confirming its particular importance in immature particle assembly [von Schwedler al, 2003]. The Gag basic C-terminal domain NC contains two zinc finger motifs used to capture the viral RNA dimer during assembly. Finally, an additional C-terminal protein (p6) binds several protein partners, including the accessory viral protein Vpr [reviewed by Sundquist and Krausslich, 2012]. Furthermore, HIV-1 accessory protein Nef is targeted to the inner leaflet of the membrane rafts through its N-terminal myrystoylation, although it is not a structural protein per se. Nef therefore gets incorporated into the viral particles [Welker et al, 1998], but whether its incorporation plays any role remains unclear [Wang et al, 2000; Zheng et al, 2003]. A model for HIV-1 assembly and budding through membrane rafts is reported in figure 11.

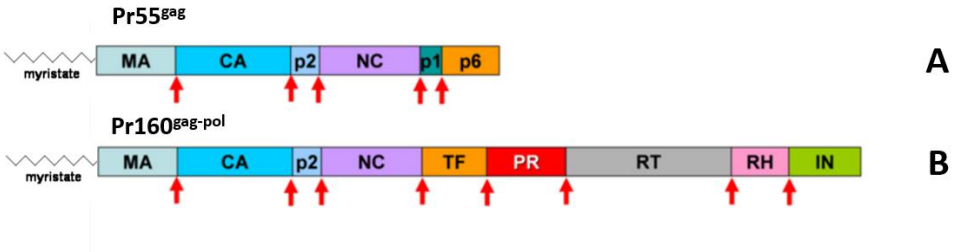

Figure 10. Linear organization of the HIV-1 Gag polyprotein precursors. The myristylated matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains are codified by $Pr55^{gag}$ precursor (A). Protease (PR), Reverse transcriptase (RT), RNaseH (RH), Integrase (IN) domains are expressed by $Pr160^{gag\text{-}pol}$ precursor (B). Red arrows indicate sites of PR processing. PR cleavage produces p2 and p1 residues in $Pr55^{gag}$ precursor, whereas Transframe peptide (TF) in $Pr160^{gag\text{-}pol}$ precursor. Adapted from Tözsér, 2010, Viruses, 2, 147-165

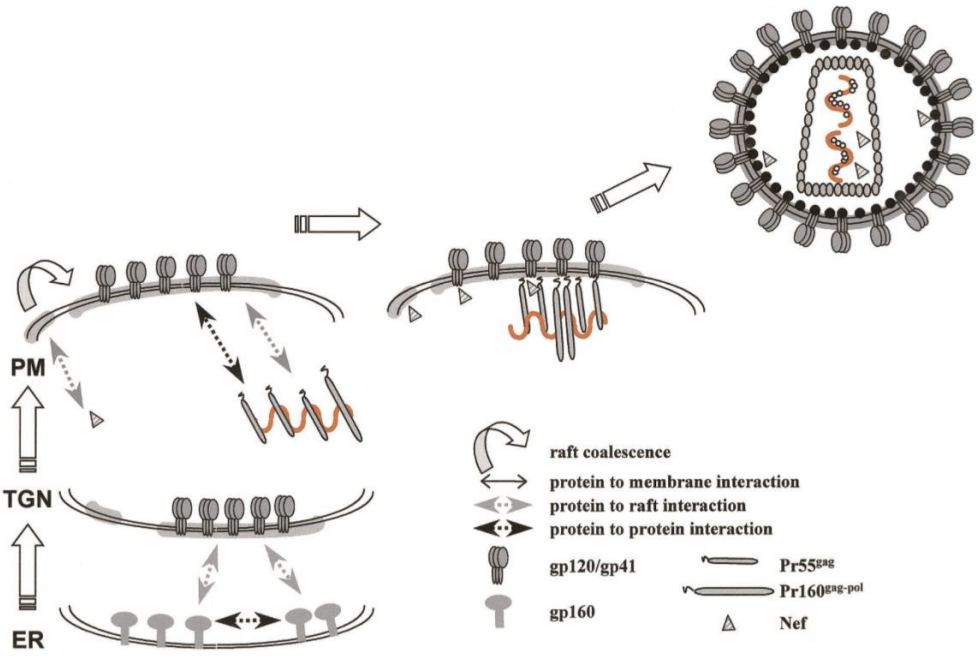

Figure 11. HIV-1 assembly and budding through membrane rafts. gp160 trimerizes inside the ER, then it gets associated to the rafts and migrates to the plasma membrane. Pr55gag and Pr160gag-pol oligomerize around two genomic RNAs and are anchored to the plasma membrane rafts due to the myristoylation of the MA domain. This allows the binding of MA to the cytoplasmic tail of glycoproteins. The cytoplasmic Nef protein, after palmitoylation, associates with the inner leaflet of the plasma membrane raft resulting in Nef incorporation into HIV-1 particles and in the enrichment of the envelope in lipid rafts. Then HIV-1 virion buds from the plasma membrane rafts and undergoes to maturation following the of Gag precursors. Once incorporated into the viral particle, Nef is partly cleaved off by the viral protease into a soluble domain, which is thought to bind to the RNP. Membrane rafts are reported in grey regions within the lipid bilayer.From Chazal and Gerlier, 2003, Microbiol. Mol. Biol. Rev, 67, 226-237.

1.3.11 Virions budding and maturation

The budding steps consist in the release of the virions from the plasma membrane [Garroff et al, 1998]. Assembly of the immature viral core induces a local curvature of the membrane adopting a dome-shaped structure. This event is followed by the formation of a lipid stalk and its final fission. The association of lipid rafts in the assembly step suggests their possible involvement in the budding process of different retroviruses like influenza and HIV-1 [reviewed by Chazal and Gerlier, 2003]. Moreover, lipid analysis of viral envelopes shows an enrichment of raft-lipids, supporting their budding from raft domains [Takeda et al, 2003; Brugger et al, 2005; Nguyen, 2000].

HIV-1 and many other enveloped viruses, like Ebola virus, VSV and rabies [Freed, 2002; reviewed by Chazal and Gerlier, 2003], exploit the host ESCRT (endosomal sorting complexes required for transport) machinery to catalyse virions release. ESCRT factors are able to facilitate membrane fission, in fact they are employed by the cells in mechanisms like endosomal vesicles release or cytokinesis [Morita and Sundquist, 2004; Beniasz, 2009; Carlton and Martin-Serrano, 2009; Usami et al, 2009; Hurley and Hanson, 2010; Peel et al, 2011]. HIV-1 Gag polyprotein precursor $Pr55^{gag}$ plays a leading role in this phase too, since its p6 domain recruits motifs ESCRT factors [Pornillos et al, 2003]. The internal appearance of all immature retroviral particles is fundamentally the same and characterized by spherical structures with an electron-lucent center.

Viral maturation takes place almost simultaneously with the budding process. PR is activated and cleaves the Gag polyprotein precursors $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ into their fully processed constituent proteins [Swanstrom and Wills, 1997; Hill et al, 2005]. The events triggered by Gag proteolysis include the condensing of viral RNA genome and the rearrangement of the capsid into a canonical shape. These processes mark the conversion of an immature non-infectious virion into a mature one, ready to enter and replicate in a new host cell. Foamyviruses represent an exception, since they retain an immature morphology [Swanstrom and Wills, 1997].

1.4 Retroviruses and human diseases

Retroviruses include many human pathogens able to cause a wide variety of malignant, immunological and neurological diseases [Coffin et al, 1997; Goff, 2000]. Human T-lymphotropic virus type 1 (HTLV-I) was the first to be discovered in 1980 [Poiesz et al, 1980]. It is reported to cause cancer and neurodegeneration [Barmak et al, 2003]. Few years later, another subtype of human T-cell leukemia virus (HTLV-II) was discovered to be associated with a variant T-cell leukemia [Kalyanaraman et al, 1982]. A particular scientific attention has been focusing on the causative agents of the pandemic acquired immunodeficiency syndrome (AIDS): the human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) identified in 1983 and 1986, respectively [Barr'e-Sinoussi et al., 1983; Clavel et al., 1986].

1.5 HIV

1.5.1 Origins

Molecular phylogeny studies have demonstrated that HIV-1 and HIV-2 are the result of cross-species transmissions of lentiviruses infecting African non-human primates. These viruses are known as Simian Immunodeficiency Viruses (SIVs) which naturally infect their host primate specie without inducing pathogenesis. Interestingly, in 1987 a study reported the evolutionary relationship between HIV-2 and SIVsm, a non pathogenic virus in sooty mangabeys [Chakrabarti et al, 1987; Guyader et al, 1987] (Figure 12).

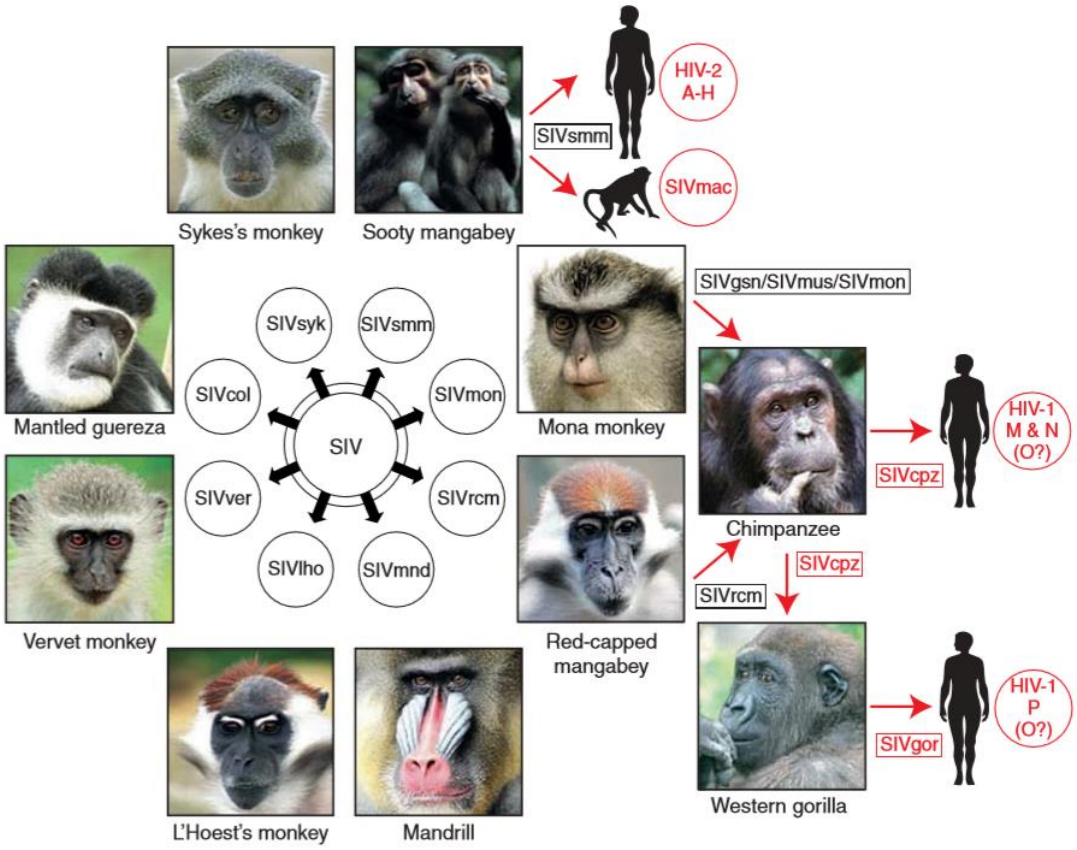

Figure12 .Origins of human AIDS viruses. From Sharp and Hahn, 2011, Cold Spring Harbour Perspect Medicine; 1

Further studies identified a close phylogenetic correlation between HIV-1 and SIVcpz [Huet et al, 1990] and later it was demonstrated that SIVmac, causing immunodeficiency in macaques, had been actually generated by a zoonotic transfer of SIVsm [Apertei et al, 2005]. Taken together these results suggested that cross-infections events of lentiviruses from different primate species had led to the development of AIDS in both humans and macaques [Sharp et al, 1994]

HIV-1 and HIV-2 are both complex Retroviruses belonging to the subfamily of Lentiviruses. A schematic representation of HIV life cycle is reported in Figure 13.

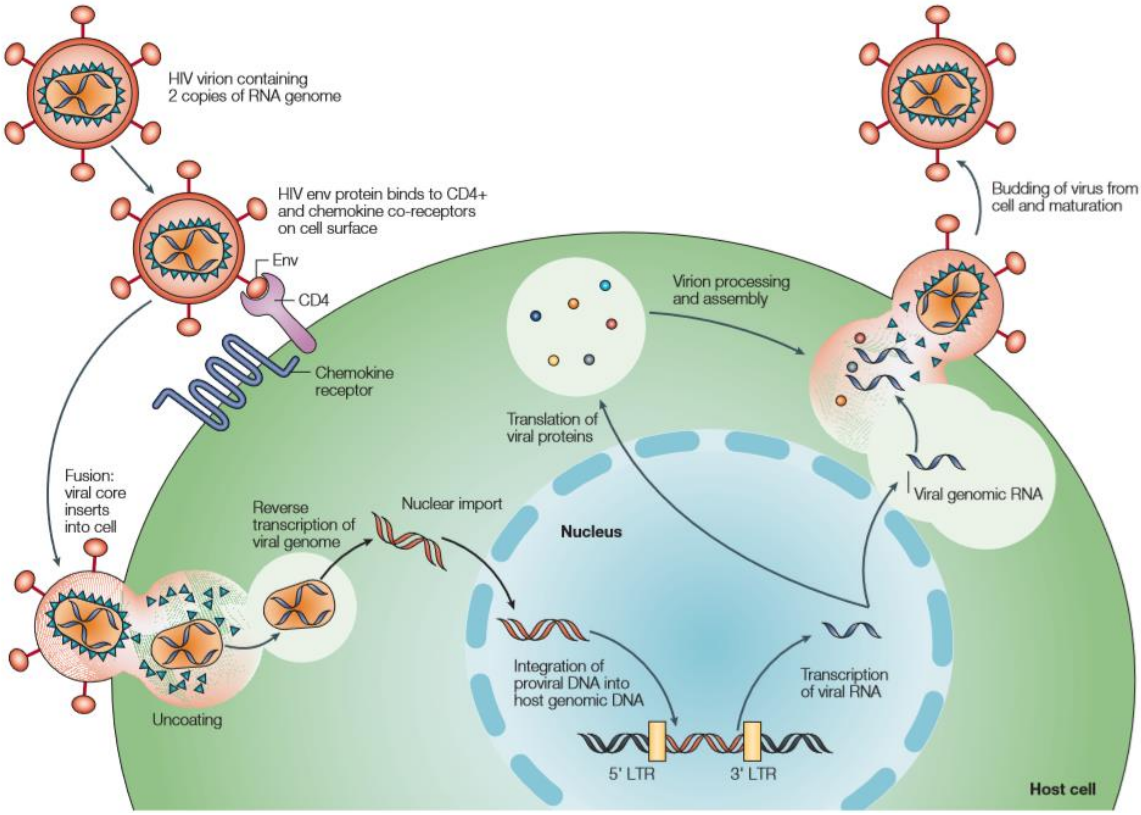

Figure 13. HIV-1 life cycle. From Rambaut et al.,2004, Nature Reviews Genetics, 5, 52-61

1.5.2 AIDS Pathogenesis

If not treated, HIV-1 infection leads to a pathological condition known as AIDS (Acquired Immune Deficiency Syndrome), characterized by a massive decrease of $CD4^+$ T cells number with the consequent instauration of many lethal opportunistic infections. The fast HIV-1 evolution within the host has conferred a pandemic trait to the disease, which currently still affects 36.9 million people in the world [2014 global statistics UNAIDS]. Currently, there is no region of the world unaffected by this pandemic (figure 14). However, the remarkable amount of information collected in about 30 years of research studies has provided the AIDS patients with better life expectancy and quality despite the lack of an eradication therapy.

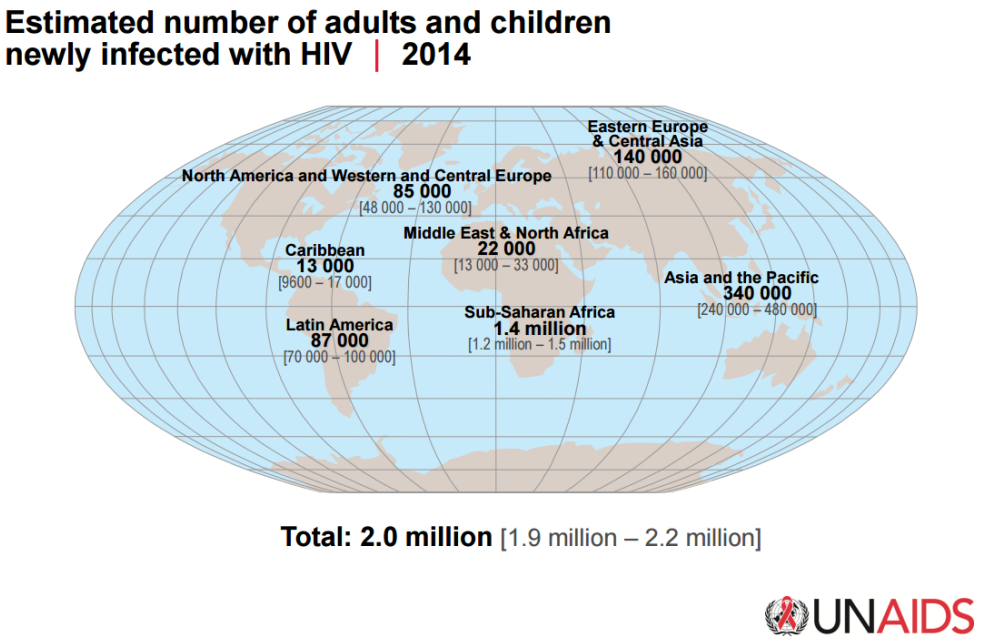

Figure 14. Global estimation of HIV infected people worldwide. *From UNAIDS, July 2015, Core Epidemiology Slide*.

The infection process (Figure 15) starts with the virus entry into the target host cell, from which it keeps spreading and replicating without causing any visible symptoms or immune reaction for the first 2 weeks after the first infection event. This step is then followed by an acute phase characterized by high levels of viraemia, activated immune response and the manifestation of "flu-like" symptoms. At the end of this acute phase, the level of viraemia declines dramatically because of the partial control by the immune system and the decline in the numbers of $CD4^+$ T cells available for infection. The following stage is known as "clinical latency", a time of variable length (from 1 to 20 years) where the patients are asymptomatic and usually unaware of their condition. On the other hand, the virus is not latent since it keeps infecting new $CD4^+$ T cells constantly and slowly. When the number of $CD4^+$ T cells declines over a threshold value (about 200 cells/µl), the host immune system is unable to face external threats and opportunistic infections become out of control [Lackner et al. 2011]. The level of viremia rises as well, becoming lethal for the infected individuals. Among all infectious disease, AIDS is therefore the one with the highest mortality rate (over 95%) [Coffin and Swanstrom, 2013].

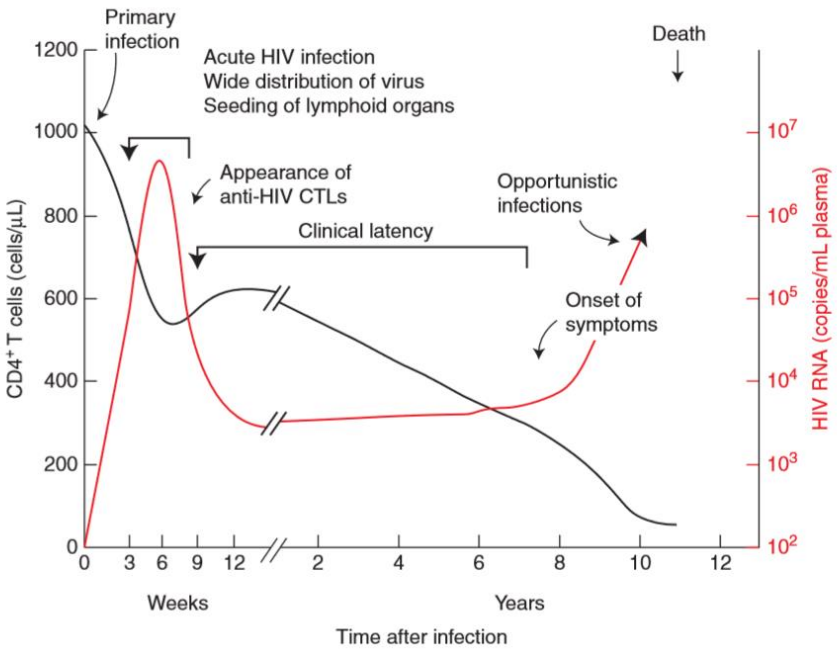

Figure 15. Time course of HIV-1 infection. *From Fauci and Desrosiers, 1997. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY)*

The availability of sensitive and accurate tools to detect viraemia is important not only for diagnosis but also for prognosis purposes. Available assays are based on the detection of HIV-1 genomic RNA in blood samples by PCR and on the detection of HIV-specific antibodies. The level of virus detected in the blood indicates the damage rate of host immune system by viral infection [Mellors et al, 1996] and it is predictive of transmission risk [Quinn et al, 2000; Wawer et al, 2005]. Moreover, these kind of clinical assays are also indicative of the efficiency of a specific antiviral therapy. Remarkably, the current treatment strategies [Arts and Hazuda, 2011] have revealed a kind of viral persistence after a prolonged therapy, showing the necessity for lifelong therapy. Indeed, as soon as the therapy is suspendend, the level of viraemia rapidly increases, despite the prolonged suppressive treatment [Palmer et al, 2008]. Since the level of viraemia reflects the number of virus-producing cells [Coffin, 1995], this phenomenon was explained through the identification of a pool of cells acting as viral reservoir and insensitive to the treatment [Siciliano and Green, 2011]. This pool of latently infected cells is characterized by a longer lifetime and by the ability to produce viral particles after *ex vivo* stimulation. However, they represent just only a small fraction compared to the population of productively infected cells in chronic infection [reviewed by Coffin and Swanstrom, 2013]. The "protection" of these cells from the antiviral treatment could be explained by the less accessibility of the virus as confined to anatomic sites, defined as "sanctuaries" [Sharkey and Stevenson 2001]. Altogether, this evidence excludes the possibility to eradicate infection through antiviral therapy alone [Maldarelli 2011; Siliciano and Green 2011]. In addition, even if accessible to anti-retroviral treatment, latently infected cells represent a safe and stable hideout for the virus, capable of refuelling virus replication.

Recent studies have reported that IFN treatment can help suppressing initial viral transmission. HIV-1 infected patients treated with pegylated-IFNα for 12 weeks were found immunologically stable after therapy withdrawal [Asmuth et al, 2010]. IFNα treatment drastically reduced the viral load at the beginning, and then followed by a little increase of HIV-1 viremia before treatment stop. The decrease of viremia has been linked to the increased expression of ISG, like MX2, in patients' peripheral blood mononuclear cells (PBMCs) [Hubbard et al, 2012; Azzoni et al, 2013]. However, it is important to contextualize the IFN responsiveness according to the patient, since the extent of ISG upregulation is highly variable. Al together these reports suggest a potential application of IFN treatment to hit HIV-1 infected cellular reservoir as potential step towards HIV-1 cure [Barouch and Deeks, 2014]

The way in which HIV-1 exerts its pathogenicity is still a matter of discussion. Based on the reports collected so far, it is possible to assess that the infected cells die from the effects of infection alone, but their lifetime is somehow affected by the cytotoxic T cells response $CD8^+$ (CTL), specifically when virus is being produced. Therefore, $CD8^+$ mediated response may modulate infection without eliminating it. The chronic immune response activation might play a role in favour of viral replication, since the production of activated memory $CD4^+$ T cells increases the number of available target cells leading to the immune system collapse [Coffin and Swanstrom, 2013].

1.5.3 Epidemiology and prevention

AIDS represents the biggest public-health crisis of our time. Available antiretroviral treatment has reduced the morbidity and mortality of the disease, turning it into a chronic, manageable condition. Unfortunately, therapeutic approaches are not always easily available in the developing countries, where AIDS is highly prevalent. Indeed, sub-Saharian Africa remains the epicentre of the pandemic with the highest rates of new HIV-1 infections [Hayes and Weiss, 2006], accounting for 66% of the worldwide infections only in 2014 [UNAIDS, 2015]. The broad implementation of suitable infrastructures in these regions is therefore a scientific, economic and political imperative.

Sexual route is still the main way of transmission [Hladik and McElrath, 2008; Cohen et al, 2011]. Another major way of HIV-1 transmission is the injecting drug use, particularly spread in Eastern Europe and central and Southeast Asia [UNAIDS 2013]. In the last 20 years, a burst on HIV-1 infections has affected women [Quinn and Overbaugh, 2005] representing almost 48% of the infected individuals in the world, with the consequent implication of mother-to-child transmission [UNAIDS, 2014]. As a result, about 2.6 million of children below 15 years are currently affected by AIDS worldwide [UNAIDS, 2014]. HIV-1 prevalence may be increased by different factors within a population, like sexual practices and behaviour, the presence of other sex-linked diseases [Siegfried et al., 2003; Aral et al, 2005; Rottingen et al, 2005], migration waves [Bloom et al, 2002; Nunn et al, 1995; Lurie et al, 2003], and finally drug and alcohol use [Buchbinder et al, 2005]. Remarkably, the co-infections with other sexually transmitted diseases in asymptomatic HIV-1 infected people can increase viraemia to levels observed in the acute phase of infection [Galvin et al, 2004]. Therefore, sexually transmitted diseases could enhance the rates of HIV-1 trasmission and at the same time may help to the identification of the pathological condition in asymptomatic patients [Cohen and Pilcher, 2005].

The immediate treatment of recently infected people is an important preventing strategy to reduce HIV-1 transmission. Furthermore, encouraging safer sex practices through the use of condom has revealed a powerful preventing strategy [reviewed by Cates, 2005]. The increasing number of countries reporting declines in AIDS prevalence could be attributed to an efficient social awareness of preventive behavioural approaches like postponement of sexual debut, reduction in casual relationships and more consistent condom use in casual relationships [UNAIDS, 2006]. Prevention of mother-to-child transmission has been achieved by lowering intra-partum and breast feeding transmission [McIntyre, 2006; Newell, 2006; Magoni et al, 2005; Tuomala et al, 2002]. Other

preventing interventions in resource-constrained countries include male circumcision [Patteron et al, 2002; Szabo and Short, 2000] and chemoprophylactic treatment of other sexual transmitted infections like herpes simplex virus-2 (HSV-2), bacterial vaginosis, candidosis and trichomonas vaginalis [Corey et al, 2004; Schwebke, 2005].

In the absence of a protective vaccine or a cure, prevention and availability of antiretroviral treatments are the best possibilities to reduce the HIV-1 pandemic. The constantly evolving HIV-1 viral diversity makes the development of any preventive or therapeutic intervention very challenging [Korber et al, 2001]. Moreover, co-infections of different HIV-1 isolates may occur in the same indivual with the consequent implication of recombination events between the viral genomes [Thomson and Najera, 2005; Blackard et al, 2002], leading to the production of new drug resistant strains and to a faster clinical progression to AIDS [Gottlieb et al, 2004].

1.5.4 Treatment

Although a set of highly active antiretroviral therapies (HAART) can increase the AIDS patients' life expectancies, they do not provide a definitive eradication of HIV yet, as consequence patients must take the drugs daily for life. In addition, they show some adverse side effects in terms of costs and toxicity [Schackman et al, 2006; Chen et al., 2006]. A wide range of aspects and principles needs to be considered for the production of an effective anti-HIV vaccine as well as a deep knowledge of HIV interaction with the immune system is required. Very powerful immunogens are necessary for cross-clade neutralizing antibodies production (NAbs) and an efficient T-cell response induction. In order to be eligible as potential HIV vaccine, the candidate molecules must provide a constant and long-term protection against future challenges during the clinical trials. In addition, safety and potential side effects are other important parameters that need to be considered. HIV structural [Asmuth et al, 2010; Sacha et al, 2007], regulatory [Bellino et al, 2009] and accessory proteins [Ayyavoo et al, 2010] are eligible as immunogenic molecules for vaccine development.

Viral vectors [Draper and Heeney, 2010; Rerks-Ngarm et al, 2009], like adenovirus (Ad5) and modified vaccinia virus Ankara (MVA), and plasmid DNA vectors [Scheid et al., 2009] have been properly engineered to express HIV recombinant proteins as antigenic factors [reviewed by Schiavone et al., 2008] (Fig 16). The main limit of the employment of such vectors relies on the pre-existance of antibodies against them inside the organism, leading to their rapid removal [reviewed by Chhatbar et al, 2011]. In the STEP trials, alternative human serotypes vectors with lower seroprevalence have shown a better advantage. In 2010 Kibuuka et al. reported a safer and more efficient multiclade DNA vaccine primed with a replication-defective rAd5 boost. Therefore, the combination of a DNA-based vector priming followed by an MVA boosting strategy induces a strong but temporary immune response.

Virus-like particles (VLPs) (Fig 16) and liposome has been exploited as a safe delivery systems for immunogens like the envelope spikes in their native conformation [reviewed by Chhatbar et al, 2011], as they do not contain the viral genome [Jennings and Bachmann, 2008]. The negative aspects about their employment consists in the possibile stimulation of antibodies against other membrane proteins and in the failure of exposing a functional trimeric Env [Crooks et al, 2007].

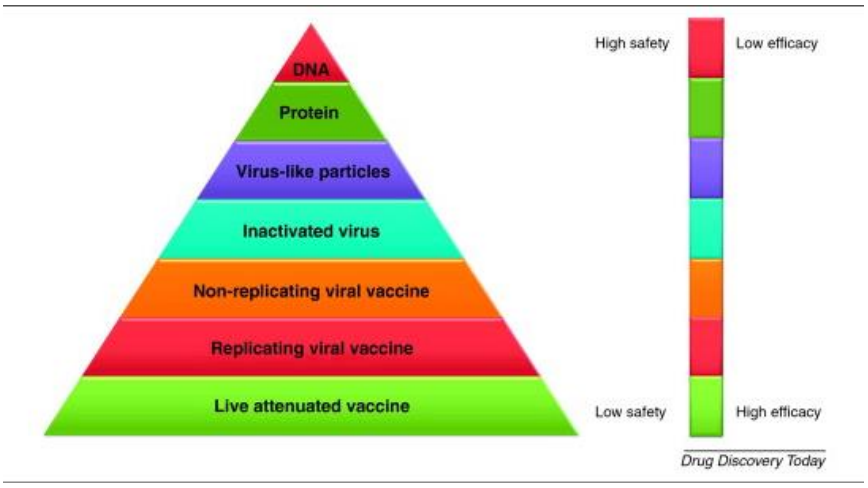

Figure 16. Diagrammatic representation of safety and efficacy issues related to vaccine. *From Drug Discovery Today 16, 948-956*

Live attenuated or heat killed viruses (Fig 16) are considered highly efficient immunogenic factors for vaccines against HIV and SIV, but the possible generation of recombinant viruses poses a big safety issue [Reynolds et al, 2008]. In addition, once in the cells the protein expressed by live viral vectors have to compete with the exposed ones for antigen presentation [Harrington et al, 2002].

The isolation of broadly neutralizing antibodies (bNAbs) [Burton et al., 2005] from AIDS patients has led to a re-evaluation of an antibody-based approach for therapeutic purposes. The interaction of two somatic IgG variants VRC01 and VRC02 with CD4 binding region of gp120 mimics the interaction of CD4 and gp120 itself. Interestingly, VRC01, VRC02, VRC03 are able to neutralize 90% of circulating HIV-1 isolates [Wu et al.,2010]. Therefore, they are known as broadly neutralizing antibody and they are neither common nor of long duration [McElrath et al, 2010] and require time to be developed [Stamatatos et al, 2009; Haynes et al, 2010].

In order to completely eradicate the disease, the latent $CD4^+$ T cells reservoir has to be destroyed. One of the latest clinical studies has reported a smart approach to push the latent viruses out from the cell and leading to the elimination of the cell itself. The strategy is named “shock and kill” (Fig 17). The histone deacetylase inhibitor Vorinostat, a therapeutic molecule for cancer treatment, has been tested for anti-latency activity. Purified resting memory $CD4^+$ T cells from patients were exposed to gradually increasing doses of the molecule and at the end of the treatment they found a considerable increase of HIV RNA in the resting cells. A combined action of immune system and antiretroviral therapy could therefore lead to the complete elimination of HIV latent reservoir

[Archin et al, 2012]. This approach opens the way to novel strategies to straightaway attack and eradicate latent HIV infection.

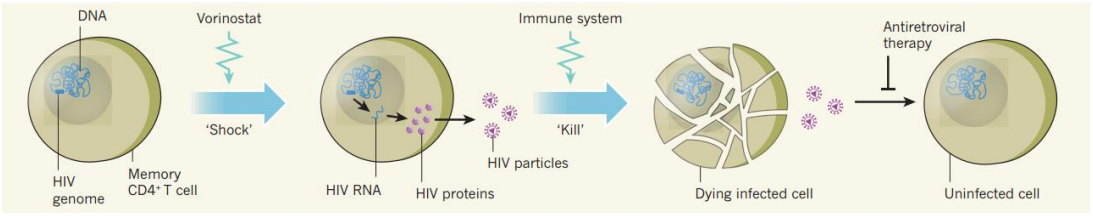

Figure 17 Shock and kill approach From Deeks, 2012. Nature 487, 439-440

Recently, another approach aimed to directly target the pool of resting cells containing the virus reservoir has been proposed. It consists in the *in vitro* excision of the proviral DNA following the expression of a site-specific recombinase (Tre) within a lentiviral self-inactivating vector in HIV-1 infected cells. Tre recombinase recognizes a specific 34 bp sequence inside the proviral LTR regions, leading to the excision of the proviral DNA from the infected cell genome [Sarkar et al, 2007]. The additional evidences *in vivo* systems supports the Tre-recombinase strategy as an eligible approach for therapeutic purposes [Hauber et al., 2013]. This kind of strategy could be used in combination to different antiviral approaches based on drugs interfering with viral life cycle, immunogenic molecules and antibodies.

1.6 NEF

Nef is a 27-32 kDa accessory protein expressed only by primate lentiviruses (HIV-1, HIV-2 and SIV). It was firstly identified in 1985 as 3' ORF, partially overlapping with the 3' LTR in the HIV-1 genome [Ratener et al, 1985; Sanchez-Pescador et al, 1985]. After many initial controversial reports [Luciw et al., 1987; Ahmad and Venkatesan, 1988; Niederman et al., 1989; Hammes et al., 1989; Kim et al., 1989] which contributed to its definition as Negative Factor, hence the currently Nef name, evidence of its actual positive role on infectivity *in vivo* finally came out. Studies on both $SIVmac_{239}$ infected Rhesus macaques [Kestier et al., 1991] and HIV-1 infected patients [Deacon et al., 1995; Kirchhoff et al., 1995] supported the requirement of the Nef protein for viral replication and the timely development of immunodeficiency. Evidence *in vitro* of the Nef effect on HIV-1 replication in primary cell culture subsequently emerged [Terwilliger et al., 1991; de Ronde et al., 1992; Zazopoulos and Haseltine, 1993; Miller et al., 1994; Spina et al., 1994].

Crystal and NMR structure studies reported Nef as a globular protein flanked by an N-terminal flexible arm and a C-terminal disordered loop [Lee et al., 1996; Arold et al., 1997; Grzesiek et al., 1996 and 1997] (Figure 16). The myristoyl group at the N-terminus, followed by a cluster of basic aminoacidic residues [Bentham et al., 2006], targets Nef to the inner leaflet of the membrane [Wang et al, 2000; Zheng et al, 2001], and to perinuclear compartments [Kohleisen et al., 1992; Fujii et al., 1996; Greenberg et al., 1997]. Although Nef does not show any enzymatic activity, it is able to take actively part to important host cell mechanisms. A di-leucine motif in the disordered C-terminal loop, together with a cluster of acidic residues in the disordered loop and in the core domain, mediate Nef interactions with a wide range of host factors involved in intracellular trafficking [Aiken et al., 1994; Piguet et al., 1999 and 2000], representing a crucial feature allowing its multi-functionality.

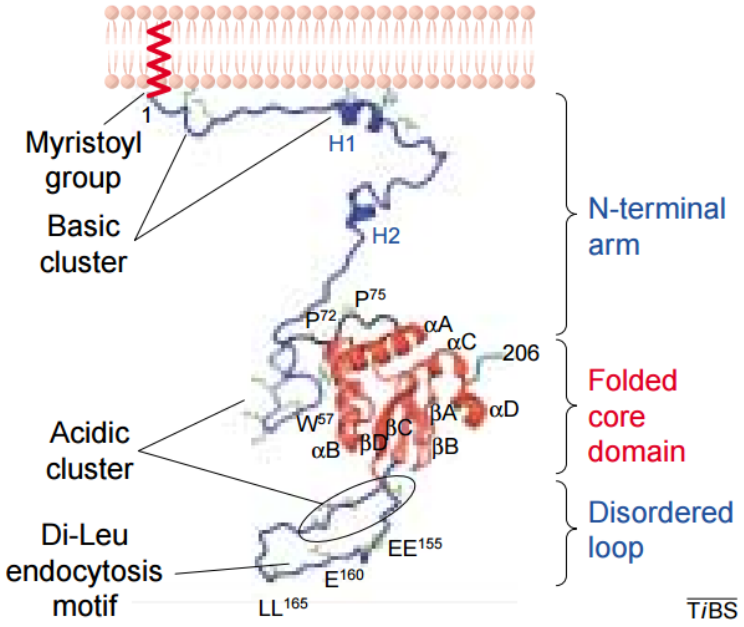

Figure 18. A model of full-length Nef, anchored to the cellular membrane. From Arold and Baur, 2001, TRENDS in Biochemical Science 6-26

Nef modulates expression of different cell surface molecules [Landi et al., 2011], like the CD4 receptor [Garcia and Miller, 1991] and the major histocompatibility complex-I MHC-I [Schwatz et al., 1996], although with different mechanisms. CD4 receptor is internalized through clathrin-mediated endocytosis [Aiken et al., 1994; Chowers et al., 1994; Rhee and Marsh, 1994; Schwatz et al., 1995a; Bresnahan et al., 1998; Craig et al., 1998; Piguet et al., 1998 and 1999; Janvier et al, 2001.; Faure et al., 2004] in order to prevent a toxic super-infection of the target cells [Benson et al, 1993; Little et al, 1994] and to avoid its interaction with envelope glycoproteins. This way, Nef promotes viral dissemination [Benson et al., 1993; Little et al., 1994] and envelope glycoproteins incorporation into the viral membrane [Lama et al., 1999; Cortes et al., 2002; Arganaraz et al., 2003; Lundquisit et al., 2004; Schiavoni et al., 2004]. The mechanism by which Nef performs the downmodulation of MHC-I is still debated, nevertheless it seems to be AP2/clathrin independent and to involve PAC (phosphofurin acid cluster sorting) proteins [Piguet et al., 2000; Blagoveshchenskaya et al., 2002; Williams et al., 2002, 2005; Larsen et al., 2004; Roeth et al., 2004; Lubben et al., 2007; Noviello et al., 2008; Dikeakos et al., 2012]. Nef is also able to impair MHC-II functions [Mu et al., 2003] and to induce FasL expression, leading to the apoptosis of bystander cytotoxic cells [Xu et al, 1997; 1999]. Altogether, these mechanisms represent a Nef strategic system to protect infected cells by the cytotoxic action of immune T cells mediated response [Collins et al., 1998]. Remarkably, Necf

modulation of the cell surface molecules described so far is conserved among all primate lentiviruses (HIV-1, HIV-2 and SIV) [Schindler et al., 2003] and maintained throughout disease progression [Carl et al., 2001]. In the end, Nef impairs T-cell receptor (TCR) activity through CD28 down regulation in HIV [Bell et al., 2001; Swigut et al., 2001], and TCR/CD3 complex down modulation by SIV Nef alleles [Schindler et al., 2006; Schaefer et al., 2000; Munch et al., 2002].

Nef plays an important role in modulating cell signaling. Its structural features include the proline-rich motif [Saksela et al., 1995], an amphipatic α-helix in the N-terminal arm [Baur et al., 1994] and an hydrophobic stretch within the C-loop [Agopian et al., 2006], implicated in interactions with different kinds of cellular kinases [Saksela et al., 1995; Sawai et al., 1994, 1996; Khan et al., 1998; Renkema et al., 1999; Agopian et al., 2006] and adaptors [Fackler et al., 1999; Rauch et al., 2007] involved in T-cells activation. This net of interactions affects the T-cells transcriptional regulation, modulating their activation status and creating a suitable environment for viral replication.

Taken together these reports suggest that Nef works as an adaptor between intravescicular trafficking and signaling pathways thanks to its ability to bind a wide range of interactors inside the host cells through its multiple binding motifs. By mediating the interaction between cellular protein and factors from the endocyotis machinery, Nef is able to modulate their intracellular trafficking. Apart from CD4 downmodulation, Nef is able to re-route peripheral membrane protein like Src family kinases too. Nef involvement in cytoskeletal remodelling and cell motility creates a favorable environment for cell to cell virus transfer. Moreover, Nef induces the secretion of chemockines (like MIP1- α/β) inducing the migration of non infected cells towards the infected ones [reviewed by Laguette et al, 2010; Fackler et al, 2007; Geyer et al, 2001; Roeth and Collins, 2006]; Muratori et al, 2009]. Nef exists also as soluble form, as secreted protein by infected cells, affecting the viability of bystander T cells and inducing B cells dysfunctions [Lenassi et al, 2009; Moir and Fauci, 2009]

Evidence *in vivo* strongly confirmed Nef requirement for viral replication [Kestier et al, 1991; Deacon et al, 1995; Kirchhoff et al, 1995], while controversial reports about its role *in vitro* have left the question open. A strong Nef effect on viral replication was reported in macrophages and in unstimulated T-cells *in vitro* [Miller et al., 1994; Spina et al., 1994]. Conversely, in activated primary human T-cells cultures only a marginal Nef effect was reported on viral replication in both cell-free viruses and cell-associated viruses mediating cell-to-cell spreading infection [Haller et al., 2011; Malbec et al, 2013].

1.6.1 Nef role in infectivity

The effect of Nef on virion infectivity was confirmed by several research groups in different contexts [Chowers et al., 1994; Aiken and Trono 1995; Goldsmith et al., 1995; Miller et al., 1994; Tokunaga et al., 1998; Khan et al., 2001; Tobiume et al., 2001; Papkalla et al., 2002]. The mechanism behind the Nef requirement for HIV-1 infectivity has remained poorly understood since the first evidence reported by Chowers and colleagues in 1994. None of the Nef functions described so far seems to correlate with the still elusive role of Nef in the infectivity enhancement. Firstly, Nef is able to increase infectivity when HIV is produced in CD4 negative cells [Chowers et al., 1995; Goldsmith et al.,1995] and in the context of virions pseudotyped with envelopes not interacting with CD4 [Miller et al, 1995; Pizzato et al., 2008], suggesting that the infectivity increase is not a consequence of the effect of Nef on the surface level of CD4 in producer cells. Moreover, Nef increases cell-free virus infectivity also in non-T-cell systems [Miller et al., 1994], indicating that the sought Nef activity does not depend on the activation status of T-cells. Despite having a clear effect on infectivity, Nef only marginally affects the viral replication *in vitro* [Haller et al., 2011]. Interestingly, the molecular features of Nef important for its involvement in vesicular trafficking, seem to be fundamental for its activity on infectivity, providing an indication of a functional link between the two activities. Importantly, Nef activity on infectivity is phylogenetically conserved among diverse HIV and SIV isolates [Munch et al., 2007] and is maintained throughout the disease progression [Carl et al., 2001].

The first insight about the mechanism behind Nef effect on infectivity came by demonstrating that the the viral protein requires to be expressed in virus producer cells rather than in target cells [Aiken and Trono, 1995]. Indeed, the infectivity impairment shown by Nef-depleted viruses could be rescued by expressing Nef *in trans* in producer cells rather than target cells [Aiken and Trono, 1995]. Further studies have proved that the extent by which Nef affects HIV-1 infectivity is strictly dependent on the producer cell line [Pizzato et al., 2010]. However, Nef seems to be particularly required when the viruses are produced by lymphoid cell lines [Pizzato et al., 2010]. Interestingly, impairments of Nef interaction with clathrin-mediated endocytosis factors, like dynamin and AP2, strongly affect virions infectivity [Chowers et al., 1994; Pizzato et al., 2007], suggesting that intracellular trafficking in virus producing cells may play a crucial role in the infectivity modulation.

Nef association with membrane rafts [Wang et al., 2000] allows its incorporation into viral particles, where it is found both in association with viral envelope and partially in a soluble form due the

cleavage by viral proteases during the maturation step [Pandori et al., 1996; Welker et al., 1996, 1998; Bukovsky et al., 1997]. Thus, it was proposed that Nef takes part to the formation of the budding scaffold in the producer cells and due to its ability to bind both reverse transcriptase and integrase enzymes [Ciuffi et al., 2004] could affects early steps of infection in the target cells. However, site-direct mutagenesis experiments affecting Nef incorporation and maturation into virions confirmed no correlation with infectivity enhancement [Chen et al., 1998; Miller et al., 1997; Welker et al., 1998]. Additionally, Nef can be also incorporated into MLV particles without affecting their infectivity [Bukovsky et al., 1997]. Taken together, all these reports collectively exclude the requirement of Nef incorporation into viral particles to enhance the infectivity, suggesting that the progeny viruses acquire a modification in the producer cells that enhances their infectious potential.

Two issues have therefore remained unsolved: the type of modification acquired by the viral particles affecting the infectivity and the step of the virus biogenesis affected by Nef (Figure 19). Two reports have tracked the Nef effect on infectivity to the biogenesis of the viral particles, demonstrating that the infectivity enhancement requires Nef association with the virus assembly complex [Qi and Aiken, 2008; Laguette et al., 2009]. Thus, the virions seem to acquire a Nef-dependent modification during the late events of lifecycle.

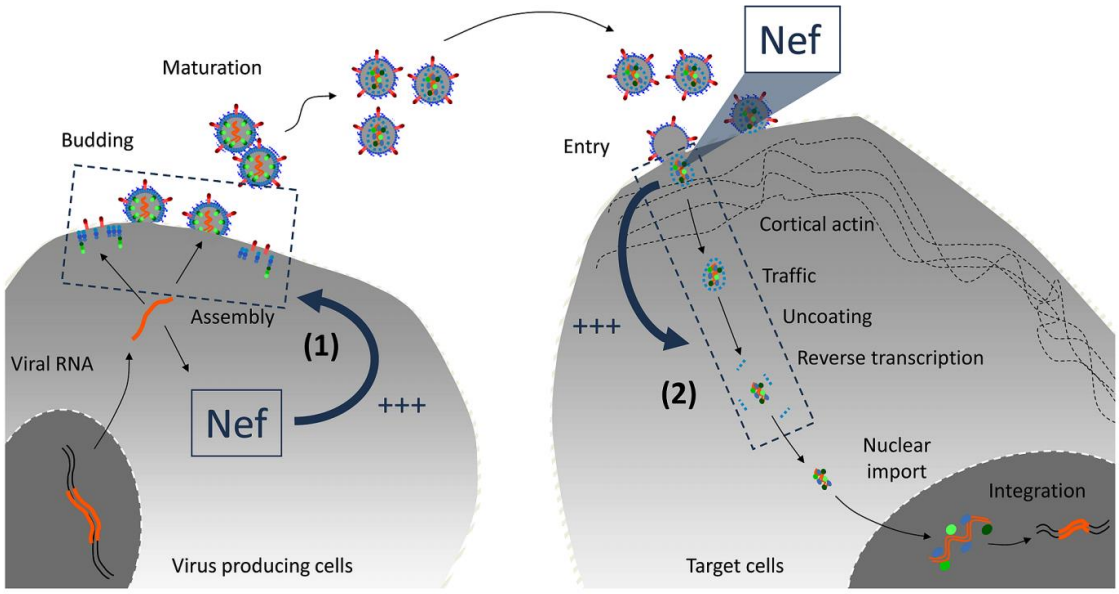

Figure 19. Localization of Nef activity on virus infectivity. Nef effect on infectivity is acquired in the producer cells at the level of viral biogenesis **(1)** and it is visible during the early steps of infection at the level of target cells **(2)**. From Basmaciogullari and Pizzato (2014), Frontiers in Microbiology, 5-232.

Altogether, available data support the hypothesis that the Nef-mediated modification acquired by the nascent virus particle could consist of a preferential incorporation or exclusion of one or more cellular factors. Nef was shown to affect the lipid composition of viral membrane [Brugger et al, 2007; Zheng et al, 2003 and 2001]. In particular, Nef was observed to increase cholesterol synthesis and transport to lipid rafts enhancing its incorporation into the viral membrane. As consequence, Nef defective virions show a lower content in cholesterol compared to wild type (Figure 16) [Zheng et al, 2003]. However, this result was not confirmed by another study [Brugger et al., 2007]. Although other differences have been traced by proteomic analysis in WT and Nef-defective virions [Bregnard et al, 2013], none was able to fully explain Nef requirement for infectivity. Overall, these reported modifications in the viral particles conferred by Nef, represent a proof of its effect on virus biogenesis.

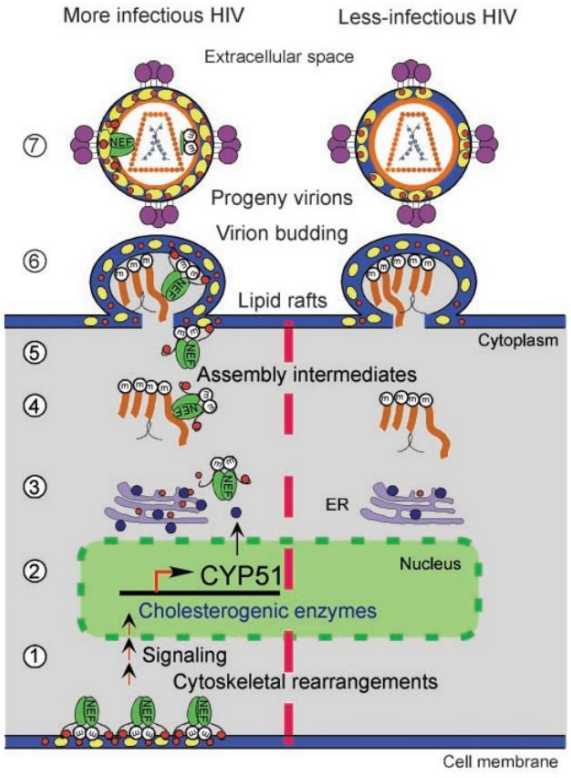

Figure 20. A model for the effects of Nef on viral infectivity. Nef is expressed as an oligomer abundantly before viral structural proteins.It activates cellular signaling cascades and causes cytoskeletal rearrangements (step 1). These lead to increased transcription of at least one cholesterogenic enzyme, CYP51 (step 2). Increased CYP51 activity (blue circles) increases synthesis of cholesterol (red circles) in the endoplasmic reticulum (step 3). The model shows the transport of Nef in viral assembly intermediates (step 4) to lipid rafts (step 5). Nef and newly synthesized cholesterol are incorporated into DRM and virions (step 6). More infectious viral particles are released into extracellular space (step 7). Nef is also cleaved by the viral protease in virions (step 7). From Zheng et al., 2003, PNAS 100-14.

At the level of target cells, the Nef effect on infectivity results in an optimization of post-entry events required to efficiently synthesize the viral DNA [Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al, 1995]. Indeed, viral particles containing Nef yeald a higher amount of early reverse transcription products than Nef depleted virions [Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al, 1995]. Therefore, Nef ability to enhance infectivity might be exerted at the fusion step between the virus and cell membrane and/or at an immediately subsequent level [Schaeffer et al., 2001; Campbell et al., 2004]. It was suggested that when viral entry takes place *via* endocytosis, Nef becomes dispensable. However, it was more recently established that Nef responsiveness is strictly determined by the nature of the envelope glycoprotein [Lai et al., 2011; Usami and Göttlinger, 2013] and is not dependent on the viral entry route [Miyauchi et al., 2009; van Wilgenburg et al, 2014]. With reference to fusion, controversial reports have been collected so far. Nef enhancement of cytoplasmic delivery of virions together with stimulation of intravirion fusion have been proposed [Schaeffer et al., 2001; Zhou and Aiken, 2001], in contrast with other reports showing no Nef effect of the fusion step [Miller et al., 1995; Tobiume et al., 2003; Cavrois et al., 2004]. Using the same assay as the previous studies, another report revealed a mild effect of Nef in enhancing virus fusion with the cells [Delay et al., 2004]. Altogether, these contradictory reports are the result of a lack of a robust and sensitive fusion assay.

In 2010 a protein with a Nef-like activity was discovered in a gammaretrovirus (MLV) [Pizzato, 2010]. Most gammaretroviruses express and accessory protein whose translation from unspliced RNA starts from an alternative CUG initiation codon, localized upstream and in frame with the Gag protein coding sequence[Edwards and Fan, 1979; Evans et al., 1977; Neil et al., 1980; Schultz et al., 1979; Prats et al., 1989]. As a result, gammaretroviruses express a leader sequence at the N-terminus of the conventional Gag, and the resulting protein, termed glycoGag, is targeted to the cell membrane. This protein shows a tipe II transmembrane topology and it is glycosylated. Glycogag from MLV seems to be involved in viral replication *in vivo* with a still undefinied mechanism [Schwartzberg et al., 1983; Goff and Lobel, 1987; Chun and Fan, 1994; Fan et al., 1983; Corbin et al., 1994; Fujksawa et al., 1998; Munk et al., 2003]. Ectopic expression of Glycogag in cells producing Nef-depleted HIV rescues virus infectivity [Pizzato, 2010] despite sharing no sequence homology with Nef. The requirement of glycogag for infectivity is Env-dependent and determined by the virus producer cells [Pizzato, 2010; Usami and Göttlinger, 2010]. Nef and Glycogag colocalize [Pizzato, 2010] and both have been reported to facilitate virus release through lipoid rafts [Zheng et al., 2000; Nitta et al., 2010]. The defect in infectivity acquired by MLV and HIV in the absence of Glycogag and

Nef, is reflected in an equally early step of the infectious process [Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al, 1995; Pizzato, 2010]. Altogether, these indications suggest that Nef and glycoGag are te result of convergent evolution.

Viral auxiliary proteins play an important role in infectivity of lentiviruses, due to their involvement in the neutralization of the so-called cellular “restriction factors”. The host cells are provided with intrinsic mechanisms to protect themselves from viral infections. The ability to counteract restrictive cellular activities have been identified for Vif, VpU and Vpx. We have been hypothesizing that the Nef activity on infectivity could also be explained by its ability to counteract a restriction factor. My PhD research project has provided important insights about cellular and molecular basis of Nef requirement for HIV-1 infectivity, which will be discussed further in this dissertation.

1.7 Intrinsic antiviral immunity: HIV restriction factors

Vertebrates have evolved a versatile and complex protein-based antiviral immunity, shaped by the constant exposure to many pathogens. They express a wide range of pattern-recognition receptors (PRRs), recognizing molecular patterns of viruses and other pathogens and triggering antiviral interferon and proinflammatory response afterwards [Iwasaki and Medzhitov, 2010]. Their inhibition of viral infection relies on the activation of signaling cascades resulting in the transcriptional activation of antiviral factors. Proteins detecting viral nucleic acids inside the host cell like Toll-like receptors (TLRs), RIG-I (cytosolic retinoic acid inducible gene I) like receptors (RLRs), Node-like receptors (NLRs) and C-type lectin receptors are some example of mammalian PPRs.

An early line of defense against the viral threat is represented by the intrinsic antiviral immunity, which is considered part of innate immunity and is conferred by restrictive cellular activities. Overall, these inhibitory elements are known as restriction factors, due to their antiviral action [Bieniasz, 2004; Goff, 2004]. Despite the absence of an unambiguous definition of these antiviral proteins [Doyle et al., 2015], they share some specific features. The restriction factors are able to interact with specific viral antagonists blocking the viral replication straightaway, often before the stimulation of interferon response. Their expression is generally constitutive, allowing their immediate action during viral invasion, but it can be upregulated by interferon upon the detection of viral infection. Remarkably, they are able to exert the antiviral activity as single genes, without the involvement of any cellular cofactors. A positive selective pressure drives the rapid diversification of both restriction factors and viral antagonists throughout the evolution. The exposure of a host restriction factor to iterative rounds of viral conflicts may lead to the selection of variations across the restriction factor sequence that make it resistant to the viral counteraction. Over the evolution, this result in an overabundance of amino acid non-synonymous substitutions (dN) in correspondence of the domains mainly involved in the virus restriction. This positive selection signature is shared by most of restriction factors identified so far [reviewed by Duggal and Emerman, 2010]. Since the amino acid sequence of each restriction factors has been finely tuned by many previous host-pathogen conflicts, the host antiviral elements may define the viral-host range, mediating a powerful specie-specific suppression and limiting cross-species transmissions. For instance, HIV-1 has adapted to overcome the restriction in its natural target cells (human cells) but it is sensitive to the restrictive activities present in other hosts. Therefore, the presence of

restriction factors determines the susceptibility of the cells to the viral invasion. The investigation of the differential susceptibility displayed by diverse target cells, combined with comparative transcriptomic studies has led to the discovery of important HIV-1 antiviral factors such as APOBEC3G [Sheehy et al., 2002], TRIM5α [Stremlau et al., 2004], BST-2 [Neil et al., 2008; Van Damme et al., 2008] and SAMHD1 [Laguette et al., 2011; Hrecka et al., 2011].

1.7.1 APOBEC3G

APOBEC3G is one of the first HIV-1 restriction factors identified. The approach followed was based on the investigation of the variable responsiveness to the 23 kDa accessory protein Vif (Virion Infectivity Factor) observed in different virus-producing cell lines [Sheehy et al., 2002]. ABOBEC3G is a host protein belonging to a family of polynucleotide cytidine deaminase expressed in several human tissues and in particular in hematopoietic cells [Koning et al., 2009; Refsland et al., 2010]. In absence of Vif, APOBEC3G is incorporated into the HIV-1 virions through its interaction with the nucleocapsid (NC) region of Gag [Bogerd and Cullen, 2008]. Its restrictive activity is exerted during the viral reverse-transcription step (Figure 19). Thanks to its association with the viral reverse transcriptase complex (RTC), APOBEC3G mediates the editing of cytidine residues to uridines during the first DNA strand synthesis [Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003; Yu et al., 2004] in a sequence specific manner [Harris et al., 2003; Yu et al, 2004]. This kind of modification results in guanosine-to-adenosine hypermutation of the HIV DNA genome, often determining the formation of premature stop codons in the viral DNA sequence. Interestingly, the presence of guanosine-to-adenosine mutations has been detected in the viral DNA isolated from AIDS patients [Vartanian et al., 1991], although the modulation of APOBEC proteins in the context of natural HIV-1 infection is not well defined yet. Evidence about the further inhibiting activity of the host factor in the reverse transcription and integration steps [Iwatani et al., 2007; Bishop et al., 2008; Mbisa et al., 2007] still lacks of a mechanistic explanation. HIV-1 accessory protein Vif counteracts APOBEC3G restrictive activity by promoting its polyubiquitylation and subsequent proteasomal degradation in the producer cells, in order to prevent its incorporation into nascent viral particles [Sheehy et al., 2003; Marin et al., 2003; Stopak et al., 2003; Yu et al., 2003]. Remarkably, the polyubiquitylation step defines a critical and direct interaction between Vif and APOBEC3G which poses a barrier to inter-species transmission. Mutation in amino acidic residues localized in APOBEC3G domains involved in the recognition by Vif, modulate the sensitivity of the protein to different Vif alleles from

different species. For instance, APOBEC3G from African green monkey is recognized by SIV_{AGM} Vif but is resistant to HIV-1 Vif counteraction [Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004]. Therefore, the interaction between Vif and APOBEC3G is specie-specific. In addition to APOBEC3G, primates encode six more APOBEC genes, of which APOBEC3D, APOBEC3F and APOBEC3H show restrictive activity against HIV-1 [Hultquisit, 2011]; although their restrictive activity is less powerful than the one exerted by APOBEC3G [Holmes et al., 2007; Miyagi et al., 2010]. This difference in the magnitude of restriction might be reflected by their lower expression compared to APOBEC3G [Koning et al., 2009; Refsalnd et al., 2010]. All APOBEC proteins have diversified under strong positive selection during the evolution of primates [Sawyer et al., 2004] and their expression is induced by type I IFNs [Koning et al., 2009; Refsalnd et al., 2010]. This family of proteins display all the features shared by HIV-1 restriction factors.

1.7.2 TRIM5α and TRIMCYp

TRIM5α is a cytoplasmic protein able to bind retroviral capsid [Stremlau et al., 2006]. It belongs to the family of "tripartite motif (TRIM)-containing proteins, whose members share the same domain organization [Nisole et al., 2005]. Its restrictive activity has been identified expressing a library of cDNA from rhesus macaques in human cells [Stremlau et al., 2004].The bock exerted by TRIM5α might be tracked back to the uncoating step (Figure 19) [Stremlau et al., 2006] despite its restriction mechanism is not well defined yet. The capsid binding domain, known as PRYSPRY or B30.2 domain, is localized at the C-terminus of the protein and it is important for TRIM5α restrictive activity. Being directly involved in the interaction with the viral antagonist, PRYSPRY domain shows a high rate of non-synonymous mutations accumulated throughout the evolution [Sawyer et al., 2005; Song et al., 2005; Johnson and Sawyer, 2009]. This fast accumulation of non-synonymous substitutions is a hallmark of a positive selective pressure, as result of multiple exposures to retroviral threats over the evolution. The capsid binding domain determines the specie tropism of retroviruses. As consequence of virus-host adaptation, a TRIM5α protein found in a host species usually inhibits retroviruses infecting other target species [reviewed by Malim and Bieniasz, 2012]. For instance, HIV-1 is resistant to human TRIM5 whereas N-MLV and EIAV are inhibited [Hatziioannou et al., 2004; Keckesova et al., 2004, Perron et al., 2004; Yu et al., 2004]. TRIM5α is therefore considered an example of cross-species barrier mediated by a restriction factor [Hatziioannou et al., 2006].

On the other hand, Cyclophillin A (CypA) is a host factor that helps HIV-1 to evade TRIM5 α restriction through its binding to lentiviral capsid [Berthoux et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006]. A chimeric gene *TRIMCyp*, produced by the fusion of CypA cDNA with TRIM5 locus, is naturally generated in owl monkeys and in some macaques as result of retrotrasposition events [Sayah et al., 2004; Liao et al., 2007]. The chimeric protein produced contains the CypA capsid-binding domain replacing the PRYSPRY domain of TRIM5α, resulting in a powerful lentiviral inhibitor [reviewed by Malim and Bieniasz, 2012]. A model proposed for both TRIM5α and TRIMCyp mediated restriction arose from the evidence of an accelerated capsid fragmentation after the binding of TRIM5α or TRIMCyp [Stremlau et al., 2006], implying the consequent RTC disruption and the block of reverse transcription. However, in addition to capsid binding, only the TRIM5α multimerization activity seems to be necessary for viral inhibition. Conversely, proteasome activity, accelerated capsid fragmentation and inhibition of reverse transcription do not seem to be absolutely required [reviewed by Malim and Bieniasz, 2012]. Mutations in viral capsid represent the strategy adopted by the viruses to evade the TRIM5α and TRIMCyp restrictions. Interestingly, apart from antiviral restrictive activity, TRIM5α has a more general role in antiviral signalling. It may act as pattern recognition receptor recognizing the capsid of many retroviruses (like MLV, HIV and SIV) and thus triggering an antiviral immune response [Tareen et al., 2011; Pertel et al., 2011]

1.7.3 Tetherin

Tetherin, known also as BST-2 or CD317, is an IFN induced membrane protein able to block the virions release from an infected cell [Neil et al., 2008; Van Damme et al., 2008]. The viral particle are trapped on the cell surface from which they are subsequently internalized into endosome [Neil et al., 2006]. Thanks to its particular structure, Tetherin may act as a bridge between the virus and the infected cell. It is actually provided of a transmembrane anchor at the N-terminus by which it infiltrates the virion envelope, and a lipid anchor at the C-terminal region which remains in the plasma membrane [Kupzig et al., 2003]. As consequence, it can restrict enveloped viruses belonging to diferent virus families since no specific recognition is required for its function [Le Tortorec et al., 2011]. HIV-1 counteracts Tetherin action through its accessory protein Vpu (Figure 19) [Neil et al., 2008; Van Damme et al., 2008], while SIVs use Nef protein [Jia e al., 2009; Zhang et al., 2009] and in some other primate lentiviruses Env proteins may be also employed as tetherin antagonists [Gupta et al., 2009; Le Tortorec and Neil, 2009]. However, the counteraction mechanisms of Tetherin

mediated by the different antagonists in primate lentiviruses are still needs to be clarified. The lack of conservation in the viral antagonism of Tetherin between different primate lentiviruses is the result of past cross species infections that gave rise to the present HIV-1 and HIV-2. The instauration of zoonosis between SIVsm and SIVcpz and a different host (humans) have induced the virus to evolve a way to counteract human Tetherin, which is resistant to SIV Nef. Therefore, the relatively "recent" HIV-1 and HIV-2 have faced the need to develop a human Tetherin antagonistic activity played by Vpu and Env proteins respectively.

1.8 Intrinsic antiviral immunity: HIV resistance factors

The features just described are not shared by all the antiviral factors identified so far. A new category of antiviral proteins without any identified viral antagonism has been recently defined as “resistance factors” [Doyle et al., 2015]. They are all interferon stimulated cellular activities causing a post-entry block of HIV-1 [Goujon and Malim, 2010; Cheney and McKnight, 2010].

Myxovirus resistance 2 (MX2) was one of the first resistance factors to be discovered as a powerful HIV-1 nuclear import and integration inhibitor induced by IFN. It is a Dynamin-like GTPase with both nuclear and cytoplasmic localization able to block the nuclear entrance and the integration of different HIV-1 strains. Although the mechanism of its antiviral activity is still poorly understood, MX2 is less powerful against SIVs strains, while it does not restrict other retroviruses like MLV. This evidence might suggest a possible mechanism linked to CA protein binding [Goujon et al, 2013; Kane et al, 2013; Melen et al, 1996; Goujon et al, 2014; Fricke et al, 2014; Fribourgh et al, 2014].

A set of interferon induced transmembrane protein (IFTM1, 2 and 3) are expressed on cell membranes and get incorporated into viral particles. Being expressed on viral particles and target cells surfaces, they affect the entry step by disturbing the fusion between the virions with the membranes of target cells [Compton et al, 2014; Tartour et al, 2014; Lu et al, 2011].

Schlafen 11 (SLFN11) is an RNA binding protein blocking the translation of HIV-1 proteins, due to the different codon usage of HIV-1 transcripts. However, SLFN1 mechanism of action still remains elusive [Li et al, 2012].

A summarizing representation of the host restriction mechanisms and the viral antagonism is described is reported in Figure 21.

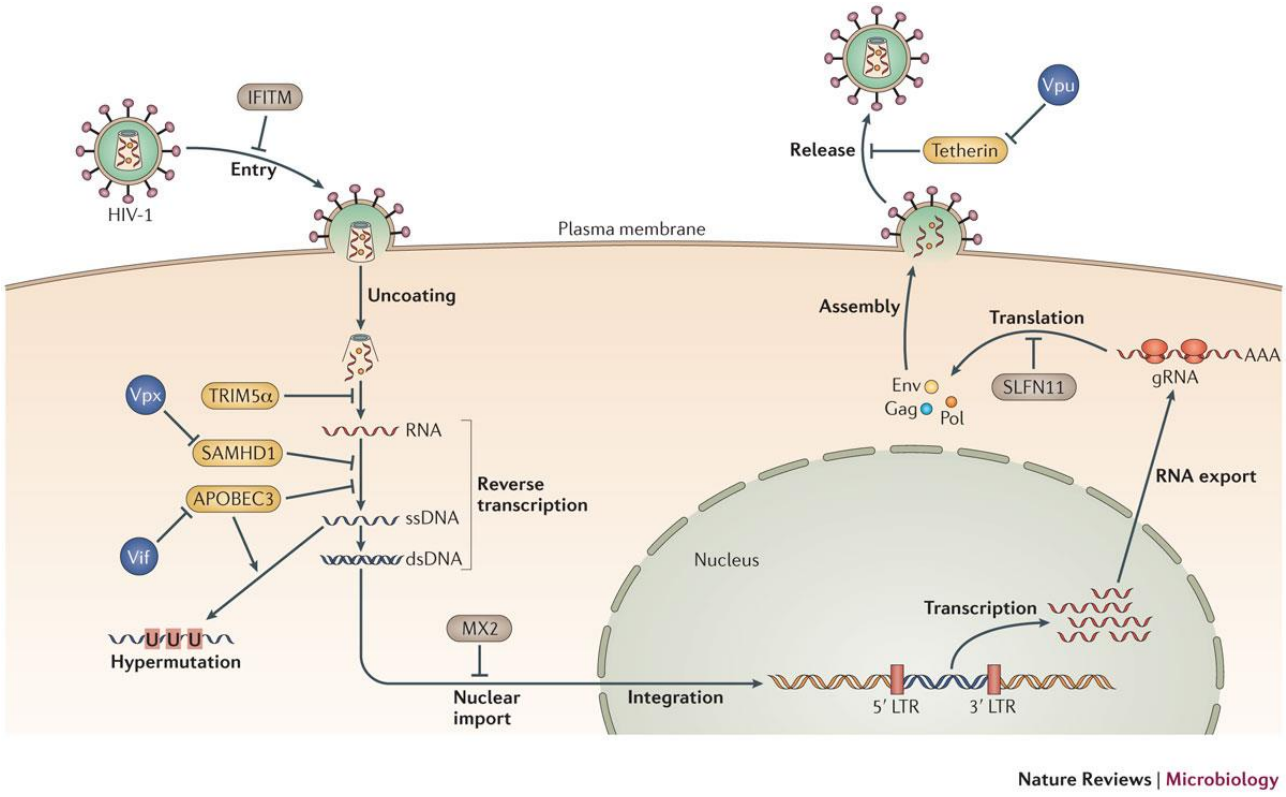

Figure 21. Host restriction and viral antagonism mechanisms. *From Doyle et al., 2015,* Nature Reviews Microbiology 13, 403–413

Genome wide screening approaches may represent useful tools for the discovery of new antiviral factors in the context of other viruses. The further investigation of the molecular mechanisms behind the antiviral factors action and their viral antagonism counteraction represents the current challenge. The insights gained by these studies will be employed for the development of future antiviral therapies and will broaden the knowledge of virus and host cell biology as well.

1.9 Aims

The aim of this research project is to understand the mechanism by which Nef enhances HIV-1 infectivity through the following objectives:

- Studying the character of the Nef requirement for HIV-1 infectivity.

Since Nef requirement for HIV-1 infectivity is reported to be highly dependent on the virions producer cell type [Aiken and Trono, 1995; Pizzato, 2010], several human cell lines of different histological origin have been used as HIV-1 producing cells in order to quantify the extent to which Nef is required to enhance virion infectivity. Due to the high variability of the requirement of the lentiviral accessory protein, I have tried to investigate whether Nef increases HIV-1 infectivity by counteracting a cellular inhibitor or by promoting a cellular activity. To this purpose, I have produced Nef-positive and Nef-negative HIV-1 virions from heterokaryons derived from the fusion of two different cell lines with opposite Nef responsiveness. The high Nef-dependence of HIV-1 produced by heterokaryons has pointed put the presence of a cellular inhibitor mainly expressed in highly Nef responsive cell lines, which makes Nef activity necessary for its counteraction.

- Identifying the cellular factor responsible for such requirement.

Gene expression profiles of cell lines with marked difference in Nef responsiveness have been analysed and compared to identify differentially expressed genes, which correlate with Nef responsiveness. Based on the correlation analysis, SERINC5 emerged as the gene whose expression correlated best with the requirement of Nef for HIV-1 infectivity. A functional study consisting in gene silencing (candidate gene knockout) and ectopic expression of the identified factor in virus producer cells with different Nef responsiveness has validated its involvement in HIV-1 infectivity enhancement. An extension of this kind of study to the other 4 genes belonging to the human SERINC family [Inuzuka et al, 2005] has revealed SERINC3 as another HIV-1 inhibitor although less powerful than SERINC5.

- Identifying the pathway employed by Nef to enhance HIV-1 infectivity.

According to what is reported in literature, Nef requires to be myristoylated and to interact with clathrin-mediated endocytosis machinery to enhance HIV-1infectivity [Craig et al., 1998; Pizzato et al., 1997]. Using gene-silencing approach (AP2 knockdown) together with a screening of a panel of Nef mutants impaired in different functions, I have found that the cellular pathway exploited by Nef to counteract SERINC5 is the clathrin-mediated endocytosis. Nef mutants impaired in the interaction with molecular partners from the endocytosis machinery as well as those affecting its membrane-anchored localization (myristoylation) are not able to counteract SERINC5 inhibitory activity. Immunostaining techniques have been used to show the differential localization of SERINC5 in presence of Nef. Nef-mediated internalization of SERINC5 is perfectly in agreement with the data supporting the involvement of clathrin-mediated endocytosis pathway, providing important insights about Nef molecular mechanism. Western blotting analyses have been used to check the amount of SERINC5 that gets incorporated into viral particles as well as the level of expression of the protein in virus producer cells.

- Identifying the step of the HIV-1 life cycle where Nef protein is required

Previous reports showed that in the absence of Nef protein the infection process is blocked at an early stage of the HIV-1 life cycle [Aiken and Trono, 1995]. Fully or almost completely reverse transcribed HIV-1 genomes can be measured by qPCR using a couple of primers matching on the upstream LTR and on the region downstream the primer binding site. The viral DNA detected is a late product of reverse transcription since it is produced upon the second template switch step. This kind of assay is known as Late RT assay [Butler et al, 2001] and it has been used to show how Nef defective HIV-1 virions fail to accumulate products of reverse transcription in target cells. Due to a lack of a sensitive and reliable fusion assay, controversial evidence about the eventual role of Nef in the fusion between the virions and the target cells are reported in literature [Campbell et al, 2004; Schaeffer et al, 2001; Zhou and Aiken, 2001; Tobiume et al, 2003; Cavrois et al, 2004; Day et al, 2004]. Thanks to the development of a novel a more sensitive fusion assay by my collaborators [Rosa et al, 2015], I was able to collect some indications about the defect acquired by the virus in presence of SERINC5 in the delivery of its core into the cytoplasm.

2. MATERIALS AND METHODS

2.1 Nef counteracts a retrovirus inhibitor

2.1.1 Cells and transfection methods

The cells, the transfection methods and the infectivity assay are described in Methods section from Rosa et al, 2015.

2.1.2 Transfection Plasmids

Plasmids used to transiently express RNAseH enzyme in HT1080 and HEK293T cells are reported in table2

Table 2. Transfection plasmids

Name	Gene	Reference
pcDNA RNaseH A subunit	**RNaseH A**	A kind gift from Dr C. Reinhard
pcDNA RNaseH B subunit	**RNaseH B**	A kind gift from Dr C. Reinhard
pcDNA RNaseH C subunit	**RNaseH C**	A kind gift from Dr C. Reinhard

2.2 SERINC5 does not evolve under positive selection pressure

The human SERINC5 gene produces four different splicing isoforms, whose protein products differ for about 40 amino acid residues clustered at the C-terminus of the protein (Figure 22). The positive selection analysis was performed on the human SERINC5 splicing isoform number 3 (QU86VE9-3), which is the one used to perform all my experiments.

Figure 22. SERINC5 human splicing isoforms. Alignment of human SERINC5 splicing isoforms shows differences at the C-terminus of the protein produced.

Firstly, a virtual transcript for each primate sequence analyzed has been created, by aligning each SERINC5 ortholog sequence with the human SERINC5 gene sequence using blastn (basic logical alignment search tool nuceotide) and splicing together the exons identified. In order to understand the variation in the human SERINC5 exons, its coding sequence has been compared with those virtually produced in the other species through a multiple alignment (**MU**ltiple **S**equence **C**omparison by **L**og- **E**xpectation), in which the human reading frame has been kept as reference. The gaps inserted by the alignment, due to the nucleotides removed throughout the evolution, were removed in the analysis in order to maintain the reading frame. To construct a phylogeny based on the alignment produced, the evolution model is a necessary parameter. Therefore, a statistical selection of best-fit models of the nucleotide substitutions detected in the alignment produced has been performed using JmodelTest2. This tool provides a ranking of possible evolution models fitting with the alignment that is used as input. The parameters of the best ranked model, according to the statistical score, were used to construct a phylogenetic tree based on the alignment of the coding sequences of the species analyzed (MrBayes software). The phylogeny constructed was in good agreement with the accepted primate phylogeny, indicating the fidelity of the alignment. Maximum likelihood analysis was performed using CODEML, part of the PAML 3.14 software package [Yang, 1997]. Likelihood Ratio tests [Yang, 1998] were used to verify if a positive selection model fits with the evolution model of the input sequences. The LRT was used to compare the likelihood of different models of evolution (M1 vs M2 and M7 vs M8). A resulting p-value lower than the chosen threshold indicates that the sequence can be under the effect of positive selection.

2.3 Optimization of a purification protocol for SERINC5 protein

2.3.1 Cell culture methods

HEK293T cell were grow in DMEM supplemented with 10% FBS at 37°C with 5% CO_2.

2.3.2 Construction design for SERINC5 recombinant protein

Human SERINC5 cDNA expressing a recombinant protein fused with a 3C protease recognition site followed by a FLAG and STREP tags in tandem at the C-terminus was created by PCR and cloned in a pcDNA3.1 expression vector (Life Technologies) using primers MP1082, MP1083 and MP1084 (table 3).

Another version of human SERINC5 cDNA fused at the C-terminus to a 3C protease recognition site followed by two STREP tags in tandem has been cloned in a pESG-IBA 103 vector (Stargate) using primers PC1001 and PC1002 (table 3).

Table 3. List of primers to produce recombinant SERINC5

PRIMER	SEQUENCE 5'->3'	COMMENT
MP1082	GCGCCCAAGGTCACCCGGGCCCTGAAACAGCACTTCCAGTCCGGACACAGAGAACTC	SERINC5-3C-STREP-FLAG in pcDNA vector
MP1083	TTTATAATCTTTTTCAAACTGCGGATGGCTCCACGCGCGCCCAAGGTCACCCGG	SERINC5-3C-STREP-FLAG in pcDNA vector
MP1084	GTGGTGGAATTCAGTTATTTATCATCATCATCTTTATAATCTTTTTCAAACTGC	SERINC5-3C-STREP-FLAG in pcDNA vector
PC1001	TCATGTATTCCATTGCCACTGGAGG	SERINC5-3C-2XSTREP in pESG vector
PC1002	CTTATTTCTCGAACTGCGGGTGGCTCCACG	SERINC5-3C-2XSTREP in pESG vector

2.3.3 Cell transfection

The day prior to transfection, cells were seeded in 15 cm^2 dishes at a density of 80-90% of confluence without antibiotics.

30 µg DNA per dish was diluted in 9 ml Optimem serum-free medium (Gibco) together with 1 ml of 0,1% linear PEI (Sigma-Aldrich). The mixture was incubated at room temperature for 15 minutes and then added to the cells.

2.3.4 FLAG purification protocol

Cell were collected, washed in PBS and lysed in lysis buffer (table 2) supplemented with 1% of detergent. A list of screened detergents is reported in table 3. The cell lysate was left rotating at 4°C for 2 hours. To remove cell debris, the lysates were ultracentrifuged at 31000rpm at 4°C for 1 hour. The supernatant was pre-cleared upon the incubation at 4°C for 30 minutes with GST-Sepharose 4 fast flow beads (GE Healthcare Life Science) pre-equilibrated with lysis buffer. The samples were separated from the beads through a centrifugation step and then incubated with EZview red ANTI-FLAG M2 beads (Sigma-Aldrich) pre-equilibrated with lysis buffer. After 2hrs incubation at 4°C, the supernatant was applied onto a gravity flow column. Unbound material was removed by washing 5 times with washing buffer (table 2). The protein has been eluted by adding 0,5mg/ml FLAG peptide in washing buffer solution.

2.3.5 STREP purification protocol

Cell were collected, washed in PBS and lysed in lysis buffer (table 2) supplemented with 10% of detergent. A list of screened detergents is reported in table 3. The cell lysate was left rotating at 4°C for 2 hours. To remove cell debris, the samples were ultracentrifuged at 31000rpm at 4°C for 1 hour. The supernatant was pre-cleared upon the incubation at 4°C for 30 minutes with GST-Sepharose 4 fast flow beads (GE Healthcare Life Science) beads pre-equilibrated with lysis buffer. The sample was separated from the beads through a centrifugation step and then incubated with Strep-Tactin Sepharose resin (iba) pre-equilibrated with lysis buffer. After overnight incubation at 4°C, the supernatant was applied onto a gravity flow column. Unbound material was removed by washing 5 times with washing buffer (table 2). The protein has been eluted by adding 1mg/ml D-desthiobiotine (Sigma-Aldrich) in washing buffer.

Table 4. Buffers

Lysis buffer	300mM KCl 10mM Hepes pH7.5 1 mM TCEP 2 mM DTT 1X protease inhibitor
Washing buffer	300 KCl 10mM Hepes pH7.5 0.1% DDM 1 mM TCEP 2mM DTT
Size Exclusion Chromatography Buffer	300 KCl 10mM Hepes pH7.5 0.03% DDM 1 mM TCEP 2mM DTT

Table 5. List of the screened detergents and their abbreviations

Detergent	Abbreviation
Amidosulfobetaine-14	ASB-14
3-(4-Heptyl)phenyl-3-hydroxypropyl-dimethylammoniopropanesulfonate	C7BzO
CHAPS	CHAPS
n-Dodecyl β–D-maltoside	DDM
Octyl β–D-glucopyranoside	OGP
Octyl β–D-1-thioglucopyranoside	OTP
3-(Decyldimethylammonio)propanesulfonate	SB-13
Triton X-100	Triton-X100
Octyl Glucose Neopentyl Glycol	NPG-OG
Lauryl Maltose Neopentyl Glycol	NPG-DDM

2.3.6 Western Blotting

A small aliquot of lysate from each step of both FLAG and Strep purification protocols have been resuspended in Laemmli buffer supplemented with 50mM TCEP pH7 (Sigma) and resolved by 12,5% SDS-PAGE. The primary antibodies used for recombinant SERINC5 detection in Western Blotting are reported in Table 5.

Table 5. Primary antibodies used for Western Blotting

Name	animal source	Dilution	Reference
ANTI-FLAG M2 PEROXIDASE (HRP)	mouse, monoclonal	1:3000 (Western Blotting)	Sigma
ANTI-STREP-TAG-II (ab76949)	rabbit polyclonal	1:3000 (Western Blotting)	abcam

2.3.7 Size Exclusion Chromatography

Eluted fractions were resolved by SDS-PAGE and SERINC5 containing flow-through fractions were combined and applied to a HiLoad 10/300 Superdex 200 pre-equilibrated with Size Exclusion Chromatography Buffer (table 2). The gel filtration column was operated on an AKTA purifier system at room temperature with a flow rate of 1ml/min. Separation efficiency and final purity were evaluated by SDS-PAGE analysis. SERINC5 containig fractions were pulled together, concentrated using an ultrafiltration device with a 100 kDa cutoff and shock frozen in liquid nitrogen for log-term storage at -80°C.

3.RESULTS

3.1 Nef counteracts a retrovirus inhibitor

During the first year of my PhD I have been focused on studying the character of Nef requirement for HIV-1 infectivity. I have contributed to the optimization of the heterokaryon assay providing the evidence of the presence of a restrictive cellular activity counteracted by Nef, and to the identification of SERINC5 preparing RNA samples for sequencing (Figure 1 **b**, 1 **c** and 1 **d**, Extended Data Figure 1 **e**; Rosa et al., 2015).

Before focusing completely on SERINC5, I have started a preliminary functional study on RNase H2B, another candidate gene proposed by the transcriptome analysis performed as potential HIV-1 inhibitor (Figure 23).

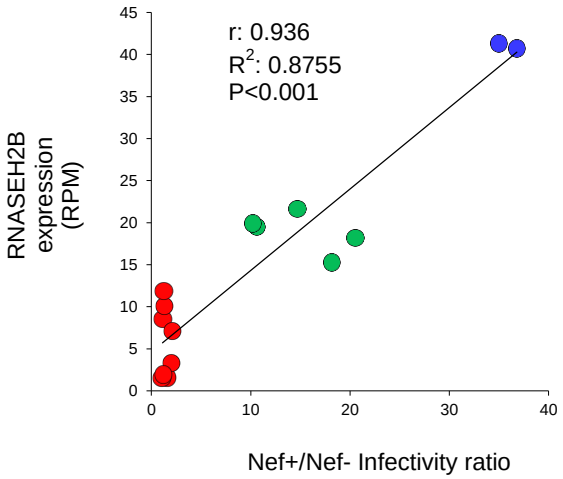

Figure 23. Correlation of RnaseH2B expression in producer cells and Nef requirement for infectivity. The colors indicate the cell lines reported in Figure 1 **a** Rosa et al., 2015. Trendline indicates linear regression. (Pearson correlation, two-tailed, P<0,0001). RPM, reads per million

I have performed some experiments aimed to check the inhibitory action of RNase H2B on HIV-1 through its ectopically expression in cells with minimal Nef-dependence like HT1080 and HEK293T cells. Since RNase H2B is not the catalytic subunit of the enzyme RNase H2, the two other subunits of RNase H (A and C) have been co-expressed to have a fully functional protein. The preliminary results obtained (Figure 22) failed to show a significant effect on HIV-1 infectivity. Since the preliminary functional data of SERINC5 revealed clearly and immediately its involvement in the effect of Nef on HIV-1 infectivity, the investigation involving RNAseH2B was therefore no further pursued.

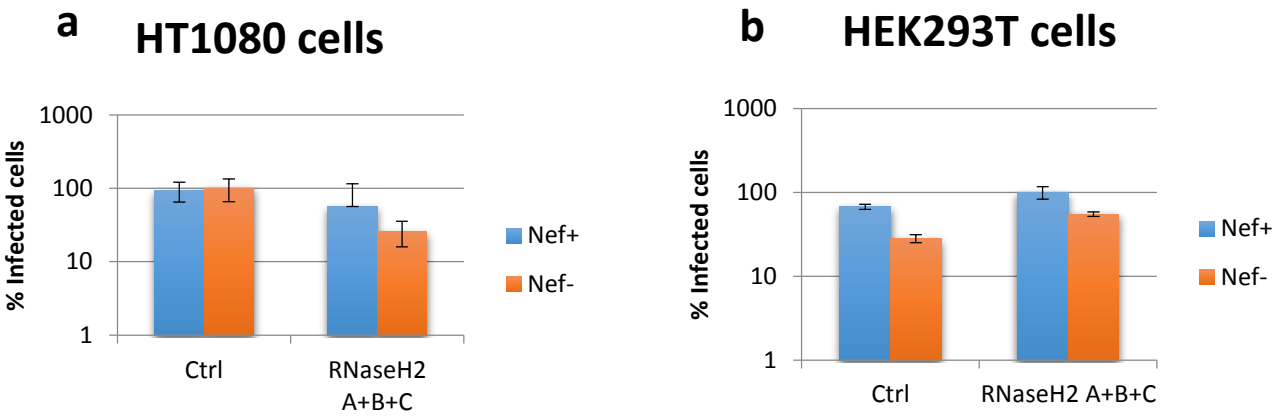

Figure 24. Infectivity of HIV-1 produced by HT1080 (a) and HEK293T cells (b) transiently expressing RNaseH2 enzyme. Virions limited to a single round of replication were produced transfecting HT1080 and HEK293T cells with pro-viral constructs together with plasmids expressing Env glycoproteins, Nef and the 3 different subunits of RNaseH. The infectivity of the viruses produced in presence and in absence of Nef has been tested on TZM-GFP reporter cells as described in Rosa et al, 2015.

3.2 SERINC5 and SERINC3 inhibit HIV-1

In the second year of my PhD I have investigated the functional role of SERINC5 in infectivity and the molecular mechanism of Nef counteraction.

My contribution consisted in the establishment of a *SERINC5* knock out stable cell line, in the knock out of the gene in primary cultures (Figure 2 **a**, **b** and **d**, Extended data Figure 1 **a**, **b**; Rosa et al., 2015), and in knocking out all the other *SERINC* genes in Jurkat cells (Figure 2 **h** and **I**; Rosa et al., 2015). I have also contributed in the developing of molecular constructs to allow a differential and gradual expression of SERINC5 (Figure 2 **g**; Rosa et al., 2015).

3.3 Determinants of Nef activity against SERINC5 and conservation across different retroviruses

I have tested the ability to counteract SERINC5 of different *nef* alleles, demonstrating the conservation of Nef activity among different primate lentiviruses (Figure 3 **d**; Rosa et al., 2015). I have also contributed to provide evidence of SERINC5 retrictive activity against MLV, which is evolutionary distant from HIV (Figure 3 **e**, **f** and **g** Rosa et al., 2015).

3.4 Nef and glycoGag promote relocalization of SERINC5 to an endosomal compartment and prevent its incorporation into virions

I have contributed to the evidence that the conservation of Nef activity among different primate lentiviruses results in the exclusion of SERINC5 from the viral particle (Figure 4 **b** and Extended Data Figure 2 **a**; Rosa et al., 2015). I have done also the part of the experiments supporting the indication that the exclusion of SERINC5 from the viral particles is performed also by glycoGag from MLV (Figure 4 **c**; Rosa et al., 2015).

3.5 SERINC5 inhibits an early step of virus infection

During the third year of my PhD I was focused on the effect of SERINC5 in the target cells. I had the possibility to investigate this aspect thanks to the development of a new and more sensitive fusion assay in my laboratory. I had the chance to work both on the validation of the assay itself (Extended Data Figure 3 b; Rosa et al., 2015), together with my colleagues, and on its application to detect SERINC5 effect (Figure 5 **e**, Extended Data Figure 3 **e**; Rosa et al., 2015).

3.6 SERINC5 does not evolve under positive selection pressure

The diversification under positive selection is a peculiarity of most restriction factors identified so far [reviewed by Duggal and Emerman, 2012]. In the last part of my PhD I tried to investigate the type of selective pressure exerted on SERINC5 gene evolution using a codon analysis method based on the comparison of the rates of non-synonymous (dN) (that alter the encoded amino acid) and synonymous (dS) DNA changes between different primate species has been used [Hurst, 2002].

The ratio between the global synonymous changes per site (dS) and replacement changes per site (dN) for the tree (Figure 25) were calculated by a free-ratio model, which allows dN/dS to vary along different branches. The presence of positive selection is indicated by values grater than 1. In all the branches of the primate phylogeny constructed we found no evidence of positive selection on SERINC5 evolution (defined as dN/dS < 1.0).

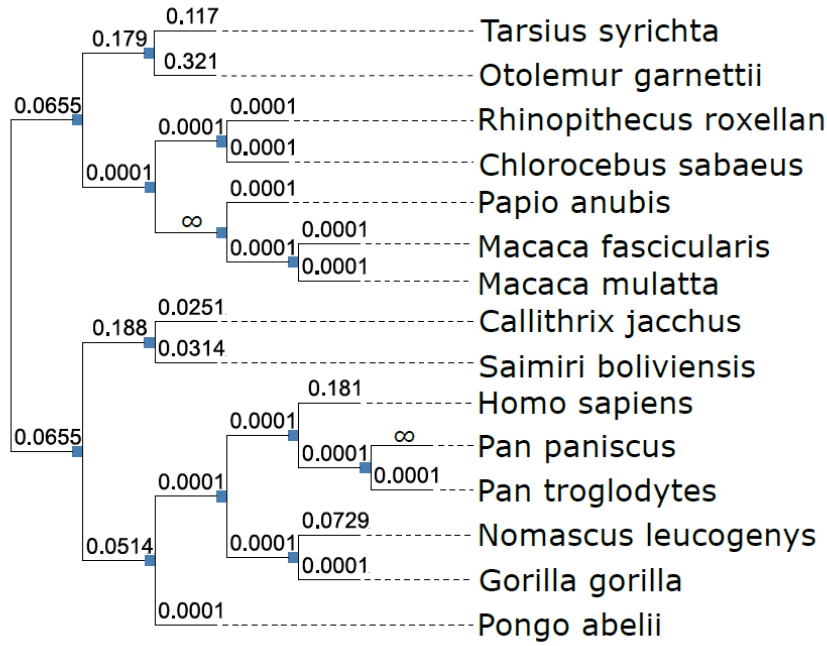

Figure 25 SERINC5 does not evolve under positive selection pressure. Codon analysis methods, based on comparing patterns of synonymous and nonsynonymous changes in protein coding sequences, were used to detect positive selection. The ratio between number of non synonymous substitutions and the number of synonymous substitutions (dN/dS) greater than 1 suggests that positive selection has acted along that lineage. The analysis was performed by CodeML software, which is part of PAML (Phylogenetic Analysis by Maximum Likelihood) suite. The phylogenetic tree was produced using MrBayes software.

3.7 Optimization of a purification protocol for SERINC5 protein

The overexpression of human membrane proteins for purification purposes represents a great challenge. During this study, SERINC5 turned out to be a problematic protein to work with whenever its visualization by Western blotting was required. In a series of trial and error tests, we pinpointed the difficulties down to two prominent features of the protein: its hydrophobicity and its unusually high content of cysteines. Both such features contributed to form protein aggregates which were either lost during the process of protein extraction or failed to enter the SDS-PAGE. During the last three months of my PhD, I have been working in collaboration with Prof. Peter Cherepanov (Cancer Research UK) in order to develop and optimize a protocol to purify sufficient amount of soluble SERINC5 to undertake biochemical studies on the protein.

Since mammalian cells provide a better tool for producing properly folded membrane proteins [reviewed by Tate, 2001], HEK293T cell line has been chosen as expression system for SERINC5.

Human SERINC5 cDNA expressing a recombinant protein with a 3C protease recognition site followed by a FLAG and STREP tags in tandem fused at the C-terminus, was created by PCR and cloned in a pcDNA3.1 expression vector. Efficiency of expression in HEK293T cells transfected by PEI was assessed by Western blotting (Figure 26).

In order to be purified, a membrane protein must be extracted from the membrane and maintained in a soluble form. To determine the suitable solubilization conditions, a small scale screen of detergent was performed [Chaudhary et al., 2011 and 2012]. The detergents tested are reported in Table 2. Detergents able to increase the ratio of the solubilized materials, indicated as “after spin” samples, relative to the total protein produced, reported as “before spin” samples, have been identified. DDM, NPG-OG and NPG-DDM detergents provided a higher % of solubilization of the protein compared to the other detergents (Figure 27) and they were selected for FLAG affinity purification experiments.

Size exclusion chromatography has been used to assess the quality and the quantity of the solubilized protein. All the selected detergents showed the same chromatographic profile for the solubilized protein, with a monodisperse, included volume peak (Abs 280) revealing the presence of SERINC5. DDM clearly provided a larger yield of solubilized protein compared to the other

detergents screened (Figure 26), therefore it was chosen for the next FLAG affinity purification experiments.

The FLAG purification protocol developed retrieved a modest amount of protein, which was lost in the different steps of the procedure, strongly affecting the final yield (Figure 28). This reduced capability of the α-FLAG antibody to recognize SERINC5 tag might be due to the strong reducing conditions in which the purification has been performed in the attempt to reduce the high amount of cysteines of the protein. Such conditions ended up reducing, and therefore inactivating, the anti-FLAG antibody. An alternative purification strategy without the use of antibodies has therefore been considered. Taking advantage of the STREP-tag present in addition to the FLAG tag, a parallel STREP purification was performed in the same samples. However, this strategy did not work, probably due to the reduced accessibility of the STREP tag in this construct.

Another construct expressing SERINC5 with a double STREP-tag at the C-terminus has been produced and tested. STREP purification of SERINC5 was more efficient compared to the FLAG-Tag, showing a good depletion of the protein in the steps before elution and allowing the use of strong reducing conditions throughout the protocol (Figure 30).

The sample was then injected for size exclusion chromatography and fractions of the desired peak were pooled together to concentrate the protein using an ultrafiltration device with a 100 kDa cutoff (Figure 31).

So far, using this method, a sufficient amount of protein has been purified for biochemical characterization, whereas for structural studies a large scale production of the protein is still required. The establishment of a cell line constitutively expressing SERINC5 would be a less laborious and cost-effective approach for crystallization purposes.

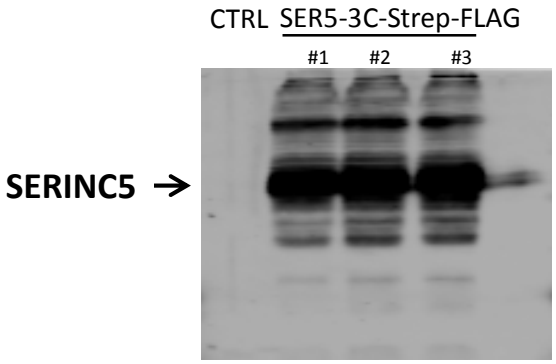

Figure 26. Expression levels of recombinant SERINC5. HEK293T transfected using PEI with three different molecular clones of human SERINC5-3C-Strep-FLAG in pcDNA3.1 vector were lysed in Laemmli buffer supplemented with 50mM TCEP pH7 and resolved by 12,5% SDS-PAGE after 5 pulses of sonication. SERINC5 was immunoblotted using mouse monoclonal anti-FLAG M2 PEROXIDASE HRP conjugated antibody.

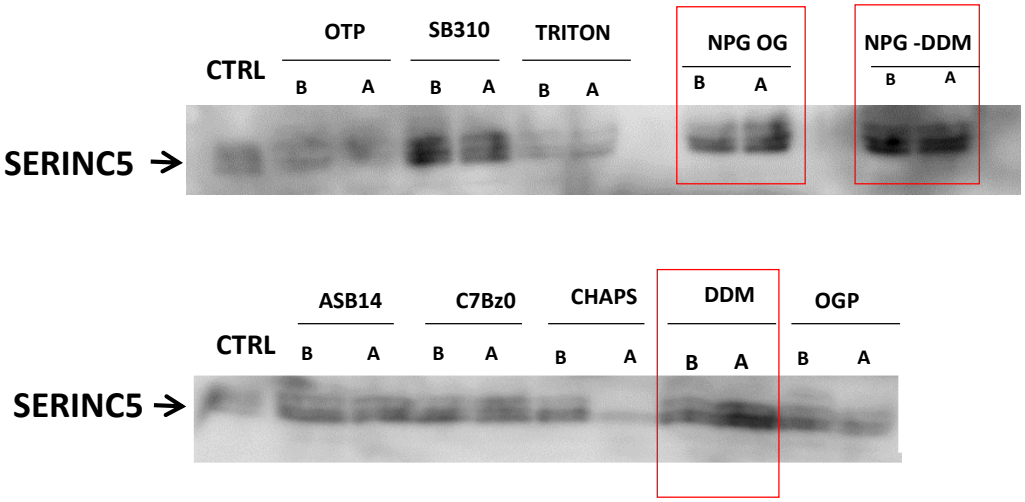

Figure 27 Western blotting analysis of small-scale, whole cell solubilized SERINC5

Human SERINC5-3C-Strep-FLAG recombinant protein has been solubilized from HEK293T cells transfected using PEI. Cells were resuspended in lysis buffer (see table 4) supplemented with 1% of each tested detergent (see table 5). The lysates were resuspended in Laemmli buffer supplemented with 50 mM TCEP pH7 and resolved by 12,5% SDS-PAGE. SERINC5 was immunoblotted using mouse monoclonal anti-FLAG M2 PEROXIDASE HRP conjugated antibody. For each detergent screened, the total amount of produced SERINC5 protein is reported in lanes B, while the amount of solubilized SERINC5 protein recovered after ultracentrifugation of the lysates is reported in lanes A. The CTRL lanes report whole cell lysates processed without detergents. DDM, NPG-OG and NPG-DDM detergents provided a higher % of solubilization of the protein compared to the other detergents.

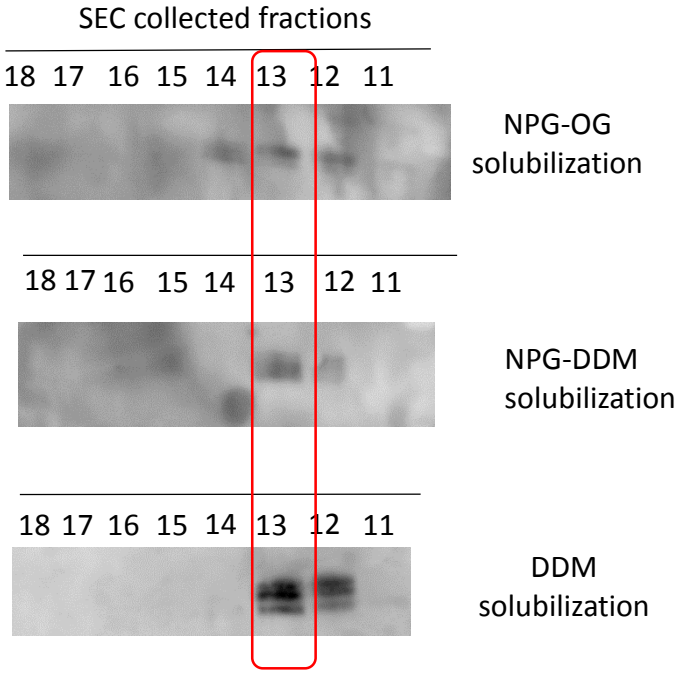

Figure 28. Western blotting analysis on Size Exclusion Chromatography (SEC) fractions collected after SERINC5 solubilization with the indicated detergents. Fractions collected during the Size Exclusion Chromatography of solubilized human SERINC5 protein from HEK293T cells transfected using PEI have been resuspended in Laemmli buffer supplemented with 50 mM TCEP pH7 and resolved by 12,5% SDS-PAGE. SERINC5 was immunoblotted using mouse monoclonal anti-FLAG M2 PEROXIDASE HRP conjugated antibody. Lanes are labeled with the elution volumes of the analyzed aliquots (11-18).

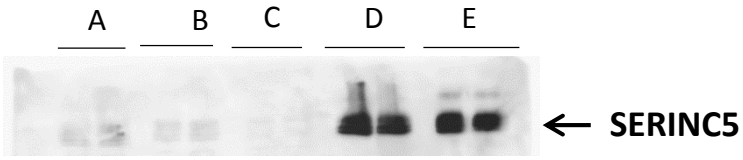

Figure 29. Western blotting analysis to detect recombinant SERINC5 in different steps of FLAG purification protocol. Human SERINC5-3C-Strep-FLAG recombinant protein has been solubilized from HEK293T cells transfected using PEI. Cells were resuspended in lysis buffer (see table 4) supplemented with 1% of DDM (see table 5). Cell lysates collected after clarification via ultracentrifugation (lanes A), incubation with GST-sepharose beads (lanes B) and incubation with anti-FLAG M2 beads (lanes C) were resuspended in Laemmli buffer supplemented with 50 mM TCEP pH7 and resolved by 12,5% SDS-PAGE. Unbound material removed through washing steps of anti-FLAG M2 beads (lanes D) and eluted SERINC5 recombinant protein (1:20 diluted) from anti-FLAG M2 beads were resolved by SDS-PAGE accordingly. SERINC5 was immunoblotted using mouse monoclonal anti-FLAG M2 PEROXIDASE HRP conjugated antibody. Each sample was loaded twice.

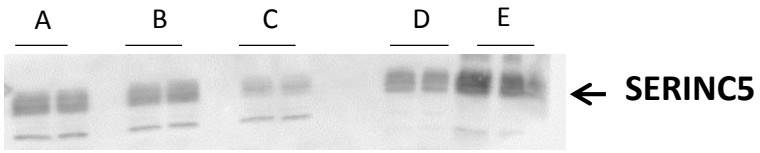

Figure 30. Western blotting analysis to detect recombinant SERINC5 in different steps of STREP purification protocol. Human SERINC5-3C-Strep-FLAG recombinant protein has been solubilized from HEK293T cells transfected using PEI. Cells were resuspended in lysis buffer (see table 4) supplemented with 1% of DDM (see table 5). Cell lysates collected after clarification via ultracentrifugation (lanes A), incubation with GST-sepharose beads (lanes B) and incubation with Strep-tactin sepharose beads (lanes C) were resuspended in Laemmli buffer supplemented with 50 mM TCEP pH7 and resolved by 12,5% SDS-PAGE. Unbound material removed through washing steps of Strep-tactin sepharose beads (lanes D) and eluted SERINC5 recombinant protein (1:20 diluted) from Strep-tactin sepharose beads were resolved by SDS-PAGE accordingly. SERINC5 was immunoblotted using mouse monoclonal anti-FLAG M2 PEROXIDASE HRP conjugated antibody. Each sample was loaded twice.

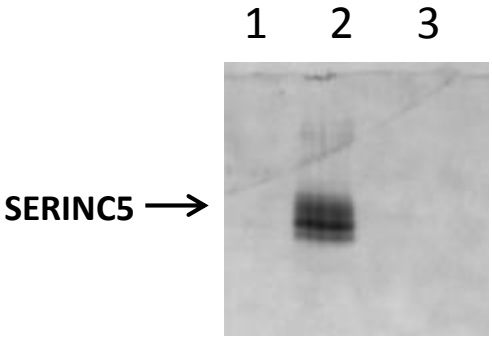

Figure 31. Coomassie blue staining on purified SERINC5 recombinant protein. Selected fractions of the desired peak collected during the Size Exclusion Chromatography of purified human SERINC5-3C-Strep-FLAG recombinant protein from HEK293T cells have been pooled together and concentrated using an ultrafiltration device with a 100 kDa cutoff. Sample before concentration (lane 1), after concentration (lane 2) and flowthrough (lane 3) were resuspended in Laemmli buffer supplemented with 50 mM TCEP pH7, resolved by 12,5% SDS-PAGE and stained with Coomassie blue (Instant blue Expedeon).

4. DISCUSSION

A first step towards the understanding of the mechanism behind the Nef requirement for optimal HIV infectivity dates back to the mid-1990s, when Nef was reported to mediate HIV-1 infectivity enhancement upon its expression in producer cells rather than in target ones [Aiken and Trono, 1995]. In particular, Nef expression is particularly important in lymphoid cell lines to produce fully infectious viruses [Pizzato, 2010], suggesting a cell-type dependency of the Nef requirement for infectivity. Starting from these observations, a quantification of the extent to which Nef affects the infectivity of HIV-1 virions produced from 31 human cell lines of different histological origin has been performed (Figure 1 **a** and Table 1, Rosa et al., 2015). The Nef effect shows high variability, ranging from 2 to 40-fold depending on the producer cell type. Interestingly, in some lymphoid cell lines such as CEMX174, MT4 and DAUDI, Nef seems to be dispensable for the production of optimally infectious virions. Given the marked cell-type specificity, it appeared conceivable that the observed variability could be due to a differential expression of one or more cellular components, providing two different possibilities: Nef could either counteract a dominant cellular HIV-1 inhibitor expressed in cell lines with high Nef requirement or conversely, Nef could promote the expression of a cellular co-factor missing in those cells and require Nef for optimal HIV-1 infectivity. To understand the nature of the sought host factor a heterokaryon assay has been established (Figure 1 **b,** Rosa et al., 2015). This kind of assays has been used in previous studies as complementation assay to identify the character of specific phenotypes shown by different cell types [Mehle and Doudna, 2008; Varthakavi et al., 2003; Simon et al, 1998; Dragic et al., 1992; Madani and Kabat, 1998]. Thus, heterokaryons derived from cell lines with opposite Nef-requirements have been used to produce Nef-positive and Nef-negative HIV-1 virions. The resulting high dependence on Nef of HIV-1 virions produced by heterokaryons (Figure 1 **c,** Rosa et al., 2015) indicated the presence of a trans-dominant cellular inhibitor of HIV-1 infectivity expressed in cells with high Nef-responsiveness. This therefore revealed that Nef is required to counteract a potential restrictive cellular activity in those cells. The subsequent quantification of the global transcriptome in a panel of cell lines characterized by different Nef dependence, has revealed the best correlation (r= 0,945) between the requirement of Nef and SERINC5 expression (Figure 1 **d**, Rosa et al., 2015). The expression of other candidate genes such as RNase H2B (r=0,935) and ZNF643 (r=0,938) has shown a good correlation as well (Figure 30). However, despite the similarly good degree of correlation, only expression of SERINC5 could predict Nef responsiveness with no exception. In contrast, other genes (such as RNaseH2B and ZNF643) were found to be equally expressed in some Nef responsive as well in some Nef-non responsive cell lines. RNase H2B is a subunit of RNaseH2, an endonuclease that

specifically degrades the RNA of DNA:RNA hybrids. Interestingly, RNaseH2 was found implicated in the Aicardi Goutièrs Syndrome [Crow and Rehwinkel, 2009] together with SAMHD1 [Hrecka et al., 2011; Laguette et al., 2011], a retroviral restriction factor, and TREX1, which was found to target retroviral cDNA in the cytoplasm [Yan et al., 2010]. Due to this possible functional relation, the ability of RNase H2 to inhibit HIV-1 infectivity was investigated. The preliminary results obtained (Figure 22 Result chapter) failed to show a significant effect of RNAse H2B overexpression on infectivity. In contrast, the early experiments in which SERINC5 was knocked out or overexpressed revealed clearly and immediately its involvement in the effect of Nef. The investigation involving RNAseH2B was therefore no further pursued.

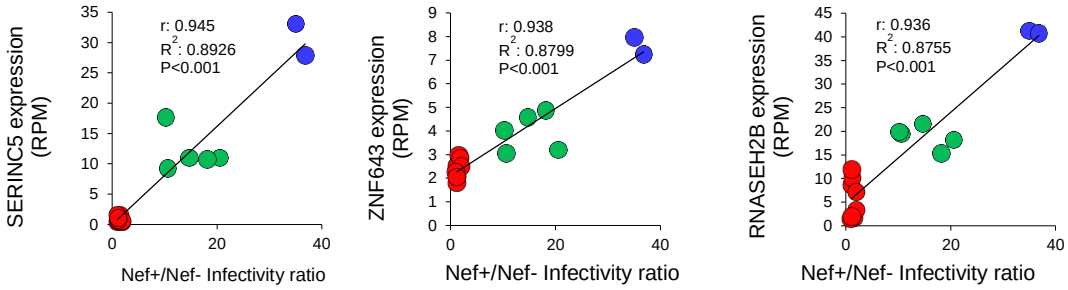

Figura 32. Correlation of SERINC5 , ZNF643 and RNaseH2B in producer cells and Nef requirement. The colors indicate the cell lines reported in Figure 1 a from Rosa et al., 2015. Trendline indicates linear regression. (Pearson correlation, two tailed, P>0,0001). RPM, reads per million.

SERINC5 is highly expressed in cells with strong Nef responsiveness, like the lymphoid Jurkat cell line (Figure 1 d, Rosa et al., 2015). A comparable level of gene expression is detectable also in primary blood cells from three different donors (PBMC) (Extended data Figure 1 **c**, Rosa et al., 2015), suggesting SERINC5 activity in the primary targets of HIV infection *in vivo*. Functional studies have then confirmed SERINC5 as a powerful host inhibitor of HIV-1 infectivity counteracted by Nef (Figure 2 **a – g**, Extended figure 1 **a - d**, Rosa et al., 2015).

SERINC5 belongs to a unique family of gene made up of five members in *H. sapiens* [Grossman et al., 2000; Xu et al., 2003]. It was suggested that SERINC proteins mediate the incorporation of serine into membrane lipids [Inuzuka et al, 2005], hence the name “Serine Incorporators”, but their function is still unknown. The family is conserved in eukaryotes with a predictive membrane topology containing 10 putative transmembrane helices [Inuzuka et al, 2005; Xu et al., 2003]. The five members of the human SERINC family share more than 17% amino acid identity and a similar predictive membrane topology (Figure 31).

In addition to SERINC5, other members of SERINC gene family are expressed in Nef responsive cell lines, like the lymphoid Jurkat cell line (Extended Figure 1 **e,** Rosa et al., 2015). Therefore, the functional study in the context of HIV-1 infectivity has been extended also to the the other members of SERINC gene family, leading to the identification of SERINC3 as another HIV-1 inhibitor counteracted by Nef. However, SERINC3 effect on virions infectivity is not as powerful as the one exerted by SERINC5 (Figure 2 **h – i,** Rosa et al., 2015).

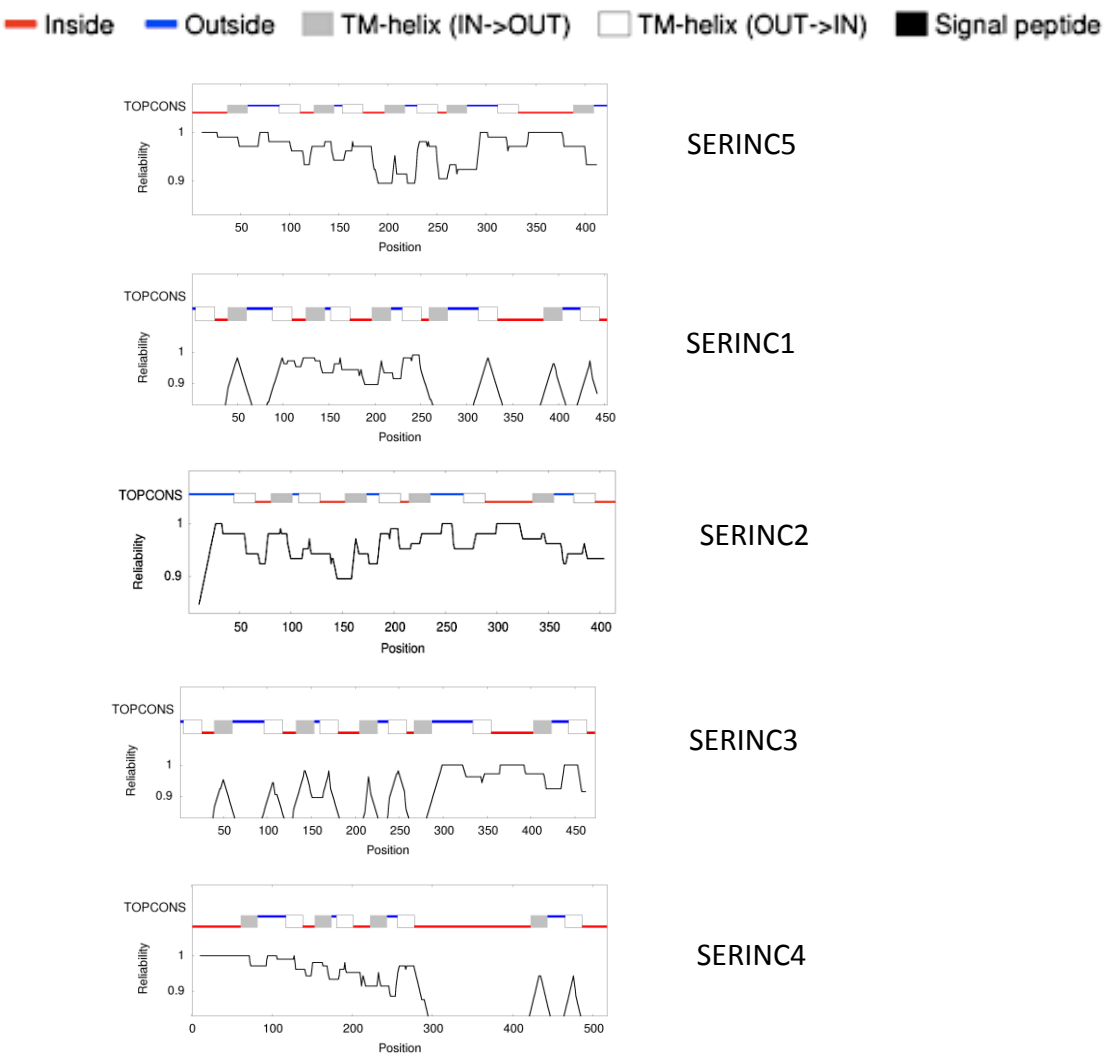

Figure 33. Predictive membrane topology of SERINC family members.
The analysis has been performed using TOPCONS tool.

In order to counteract the SERINC5 restrictive activity, Nef needs specific molecular determinants. Mutations affecting Nef myristoylation (G2A) impair its ability to target SERINC5 (Figure 3 **a,** Rosa et al., 2015), suggesting that the importance of Nef ability to interact with membranes is important for the enhancement of HIV-1 infectivity. Moreover, Nef interaction with factors involved in clathrin-dependent intravesicular trafficking like dynamin 2 and AP2, reported to be required for HIV-1 infectivity enhancement [Pizzato et al., 2007; Craig et al., 1998], are necessary for SERINC5 counteraction as well (Figure 3 **a** and 4 **f,** Rosa et al., 2015). Immunofluorescence microscopy data show that SERINC5 is predominantly localized on the plasma membrane (Figure 4 **g,** Rosa et al., 2015) and is relocalized in late endosomes by Nef (Figure 4 **h,** Rosa et al., 2015), explaining the dependence of Nef activity on the endocytosis machinery. By decreasing SERINC5 cell surface levels, Nef prevents its incorporation into the nascent viral particles (Figure 4 **a,** Rosa et al., 2015). Nef mutants unable to interact with members of the endocytosis pathway fails to exclude SERINC5 from virions (Figure 4 **a,** Rosa et al., 2015), underlying the crucial role of intracellular trafficking for Nef activity on viral infectivity. Interestingly, among the Nef mutants analysed, those which fail to counteract SERINC5 are also known to be defective for CD4 down modulation (G2A, LL165,166AA, D123A), (Figure 3 **a,** Rosa et al., 2015), allowing us to speculate that the molecular mechanism employed by Nef to internalize both CD4 and SERINC5 could be the same.

Another important determinant affecting the magnitude of the Nef requirement for optimal HIV-1 infectivity is the nature of the envelope glycoprotein expressed on the viral surface [Pizzato, 2010; Miller et al., 1995; Aiken, 1997; Chazal et al, 2001; Pizzato et al., 2008; Luo et al., 1998]. HIV-1 pseudotyping with glycoproteins able to relieve the need for Nef in the infection process, like VSV and EBOV, makes the virus resistant to SERINC5 (Figure 3 **b,** Rosa et al., 2015). The absence of infectivity inhibition, despite SERINC5 being incorporated into viral pseudotyped particles (Figure 4 **d** and **e,** Rosa et al., 2015), suggests that these glycoproteins offer an alternative mechanism to antagonize the SERINC5 inhibitory effect. One possibility is that the entry step mediated by those envelope glycoproteins may help the virus to override the SERINC5 block, explaining the dispensable role of Nef.

The dependence of the Nef activity on the nature of the envelope glycoprotein has been reported also when HIV-1 is pseudotyped with Env from HIV-1 naturally occurring strains [Usami and Göttlinger, 2013; Lai et al., 2011]. A recent report has shown a relatively weak Nef responsiveness of some primary Envs compared to Env glycoproteins of laboratory-adapted strains [Usami and

Göttlinger, 2013]. Interestingly, some HIV-1 envelope glycoproteins from clinical isolates appeared to modulate the susceptibility of the virus to SERINC5 inhibition (Figure 3 **c,** Rosa et al., 2015); suggesting the possibility that in some isolates HIV-1 might use Env in addition to Nef to antagonize SERINC5.

The Nef requirement for the early stages of infection [Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al, 1995] is mirrored by the inhibitory action of SERINC5 soon during the infection process. The presence of SERINC5 in HIV-1 virions impairs their ability to accumulate reverse transcription products in the target cells (Figure 5 **a,** Rosa et al., 2015), suggesting an early block. The potential effect of Nef on viral entry has been widely debated, remaining unsolved so far [Day et al., 2004; Cavrois et al., 2004; Tobiume et al., 2003]. The development of a novel and sensitive fusion assay, based on the detection of the Cre protein delivered to the nucleus of the target cells independently of productive infection (Figure 5 **b,** Extended Data Figure 3 **a** and **b,** Rosa et al., 2015), has addressed this question. SERINC5 incorporation into virions impairs the ability of Cre to access the target cells. We have consolidated this observation also using another assay (delivery of Vpr-BLAM) which has been used in previous reports [Cavrois et al., 2002; Cavrois et al., 2004; Day et al., 2004] (Extended Data Figure 3 **d,** Rosa et al., 2015). However, the magnitude of this defect is ten-fold lower compared to the powerful inhibition of infectivity mediated by SERINC5 (Figure 5 **c** and **e**, Extended data 5 **f,** Rosa et al., 2015). Considering that Cre is a small protein (~40 kDa), it is possible to hypothesize that SERINC5 exerts a bigger effect in the translocation of a larger protein complex like the viral core (60-120 nm) into the target cells. Therefore, since fusion between the viral particle and the target cells remains detectable in the presence of SERINC5, the host inhibitor might target a post-fusion event required for the translocation of the viral core into the target cells. Membrane fusion is a multi-step process, where the expansion of the fusion pore is reversible and represents the highest energy requiring stage [Chanturiya et al., 1997; Cohen and Melikyan, 2004]. Once formed, the fusion pore may close again or expand further until the expansion becomes irreversible (Figure 32). While fusogenic proteins might promote the enlargement of a fusion pore by reducing the energy barrier required, membrane proteins, such as SERINC5, may inhibit fusion by increase the same barrier [Chanturiya et al., 1997].

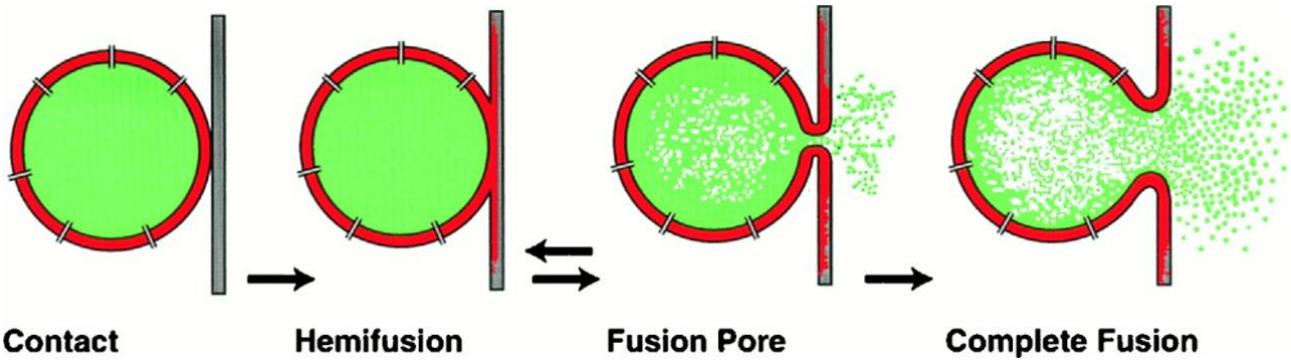

Figure 34 Sequential steps in phospholipid membrane fusion. From Chanturiya et al. PNAS 1997;94:14423-14428

The lipid composition also sets the e nergy barrier necessary to enlarge the fusion pore [Ciechonska et al, 2014; Razinkov et al, 2000; Chanturiya et al., 1997]. It is therefore possible to speculate that the effect on fusion pore enlargement could be due to the influence of SERINC5 on the membrane composition rather than to the presence of the protein itself. The envelope glycoproteins may actually reduce the energy barrier required for the enlargement of fusion pore to allow the translocation of the viral content in the target cells. Indeed, HIV-1 pseudotyped with envelope glycoproteins like VSV-G and EBOV GP, are able to perform productive infection without preventing SERINC5 incorporation into viral particles (Figure 3 **b**, 5 **d**, 4 **d** and **e**, Rosa et al., 2015), in line with the dispensable effect of Nef on the infectivity of such pseudotypes. However, the presence of proteins altering the rigidity and the curvature of the lipid bilayer might also affect the expansion of the fusion pore [Chen et al., 2008], including the possibility that the incorporation of SERINC5 into the viral particles may account for this effect as well.

On the other hand, it has been suggested that the envelope glycoproteins which render HIV high Nef responsive require a high potential energy to mediate fusion [Medjahed et al, 2013; Usami and Göttlinger, 2013]. The amount of such energy is associated with the clustering of Env trimers [Brandenberg et al., 2015; Usami and Göttlinger, 2013] mapped on HIV-1 surface and at virus-cell contact regions [Chojnacki et al., 2012; Sougrat et al., 2007]. The clustering of Envelope trimers might therefore be disturbed by either the presence of SERINC5 itself on the viral surface or by the altered lipid composition. These speculations are also consistent with the proposed role of Nef in enhancing the cytoplasmic delivery of the viral core [Schaeffer et al., 2001]. At the moment it remains unknown whether SERINC5 alters the lipid composition of the viral envelope leaving the question open.

SERINC5 is expressed in HIV-1 target cells *in vivo* (Extended Figure 1 c and Extended Figure 4, Rosa et al., 2015), where the cell-to-cell transmission mechanism could be important [Jolly et al., 2004].

Cell-to-cell transmission of HIV-1 throughout a culture is extremely efficient compared to transmission *via* cell-free virus [Jolly et al., 2004; Sourisseau et al., 2007]. The positive effect of Nef on viral replication *in vivo* has been confirmed by different reports [Kim et al., 1989; Jamienson et al., 1994; Deacon et al, 1995], while its effect on spreading infection *in vitro* is still unclear. Recent studies have reported only a marginal effect of Nef in cell-to-cell mediated HIV-1 transmission without affecting the formation of contact sites between cells known as virological synapses [Malbec et al., 2013; Haller et al., 2011].

Because of a comparable expression of SERINC5 in PBMCs and in the cell line like Jurkat, a similar potential in restricting viral replication could be expected. The investigation of SERINC5 and SERINC3 effect on HIV-1 spreading in Jurkat cell lines has confirmed their restrictive activity, even if the magnitude of the effect observed does not mirror the powerful block on infectivity [Usami et al., 2015]. Considering the strong requirement of Nef for viral replication *in vivo*, it would be worth to assess the SERINC5 and SERINC3 restrictive role in the context of spreading infection of human primary cultures. This information is still missing since obtaining a stable SERINC5 $^{-/-}$ primary culture to assess spreading infection is quite challenging. Further work is needed to fully understand whether cell-to-cell spread of HIV-1 is susceptible to SERINC5 mediated restriction in the absence of Nef. However albeit modest, the Nef ability to enhance viral cell-to-cell transfer was reported to be conserved among primate lentiviruses [Malbec et al., 2013; Münch et al., 2007] like the counteraction of SERINC5 restrictive activity (Figure 3 **d**, 4 **b**, Extended data Figure 2, Rosa et al., 2015). A potential effect of SERINC5 cell-to-cell transfer and therefore in viral replication *in vivo* is plausible.

SERINC5 might be involved in the induction of an intracellular innate response in the target cells, as observed for other host antiviral factors such as BST-2 and TRIM5α [Pertel et al., 2011, Galão et al., 2012]. SERINC5 may also work as a pathogen recognition molecule detected on viral particles by specific cellular components, which in turn may trigger a proinflammatory response. This hypothesis implies the existence of a possible “cellular receptor” recognizing SERINC5 on the viral surface.

SERINC5 and SERINC3 have been listed as restriction factors [Kluge et al., 2015], although they show features that do not reflect the classic hallmarks of the antiviral factors identified so far.

In contrast to most antiviral factors reported in literature, SERINC5 and SERINC3 expression in primary CD4$^+$ T cells or dendritic cells is not upregulated neither by type I interferon nor by an interferon inducing agent (Extended data Figure 4, Rosa et al., 2015). Thus, SERINC5 and SERINC3 are examples of constitutively expressed intrinsic restriction factors.

Another peculiarity of most restriction factors is their diversification under strong positive selection [Duggal and Emerman, 2012]. Throughout the evolution, the exposure of a host restriction factor to a viral threat and the consequent interaction with the viral antagonist result in a positive selection signature on the host gene sequence and on the viral counterpart as well (Figure 35 **a**) [Duggal and Emerman, 2012]. This kind of selective pressure is characterized by an excess rate of non-synonymous mutations (dN) compared with synonymous mutations (dS), as consequence the dN/dS ratio is generally high at the level of single amino acid residues directly involved in the viral restriction and across the entire protein coding sequence (Figure 35 **b**). A method based on the estimation of the dN/dS ratio has been used to evaluate a potential positive selective pressure on SERINC5 evolution. In line with SERINC5 high degree of conservation in eukaryotes [Grossman et al., 2000; Inuzuka et al., 2005], its alleles in different primate species taken into consideration do not show any positive selection in the coding sequence (Figure 23). This represents another peculiar feature in contrast with other anti-retroviral factors.

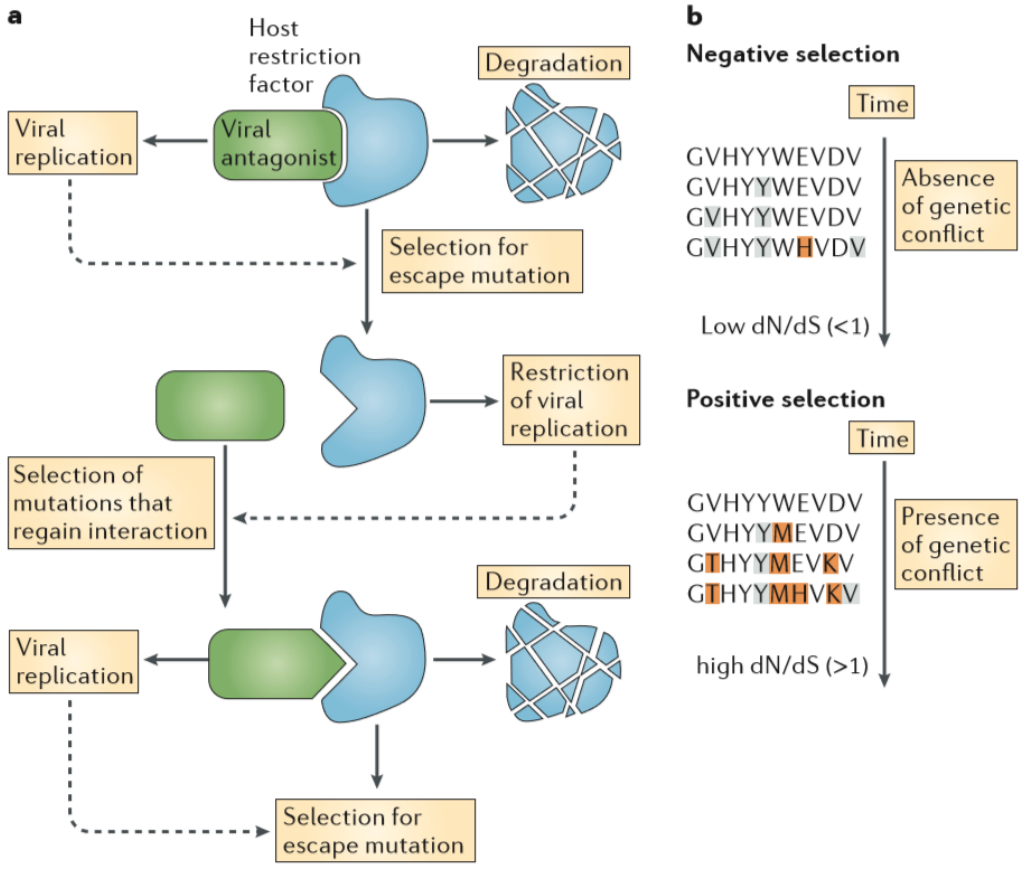

Figure 35. Genetic conflict between virus and host. *From Duggal and Emerman, 2012, Nature Reviews – Immunology, 12, 686-695*

The SERINC gene family is highly conserved, since an ortholog is present also in yeast. A core biological function of SERINC5, however, remain unclear. Furthermore, the presence of five SERINC genes in human species is likely the result of gene duplication, which often implies subfunctionalization, in which only one gene retains the essential cellular function while the paralogues might develop further functions [Duggal and Emerman, 2012]. Therefore, SERINC5 and SERINC3 antiviral activity might represent just an additional role of this family of proteins in the cell biology. On the other hand, an attractive hypothesis is that the increase of SERINC gene copy number might be a strategy of the host to restrict different viruses. Since primate restriction factors have been reported to prevent cross-species transmission events *in vivo* [Kirchhoff, 2010], it would be interesting to check the susceptibility of HIV-1 and other retroviruses to SERINC5 from different eukaryotic species in order to test whether the antiviral activity is conserved and whether it shows specie-specificity.

The evidence that the SERINC5 counteracting activity is conserved among *nef* alleles of different primate lentiviruses (Figure 3 **d**, 4 **b**, Extended data Figure 2, Rosa et al., 2015) and shared with glycoGag from a retrovirus (MLV) evolutionary distant from HIV (Figure 3 **e – g**; Figure 4 **c**; Extended Data figure 2 **b**, Rosa et al., 2015), highlights a fundamental role in the virus-host interaction. Given this example of convergent evolution of factors antagonizing the same restrictive cellular activity, it is possible that other viruses may be targeted by SERINC5 and have in turn evolved a counteracting agent.

The massive HIV-1 inhibition exerted by SERINC5 when ectopically expressed despite the presence of Nef (Figure 2 **g,** Rosa et al., 2015), indicates its potential exploitation as an anti-HIV gene therapy factor. Interfering with the ability of Nef to downregulate SERINC5 could also represent a potential strategy to fight AIDS. For this purpose, a better understanding of the mechanism by which Nef downregulates SERINC5 is required. It remains to be established whether the two proteins interact directly. Having failed to detect positive selection within SERINC5, it could imply that no specific domains of the protein are directly involved in Nef interaction, suggesting the implication of other cellular factors. I have developed a purification protocol for SERINC5 which represents the first important step required to understand whether SERINC5 and Nef interact directly, and, if so, to identify the an interface that can be targeted by novel antiviral drugs.

5. REFERENCES

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6. PUBLICATIONS

ARTICLE

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HIV-1 Nef promotes infection by excluding SERINC5 from virion incorporation

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HIV-1 Nef, a protein important for the development of AIDS, has well-characterized effects on host membrane trafficking and receptor downregulation. By an unidentified mechanism, Nef increases the intrinsic infectivity of HIV-1 virions in a host-cell-dependent manner. Here we identify the host transmembrane protein SERINC5, and to a lesser extent SERINC3, as a potent inhibitor of HIV-1 particle infectivity that is counteracted by Nef. SERINC5 localizes to the plasma membrane, where it is efficiently incorporated into budding HIV-1 virions and impairs subsequent virion penetration of susceptible target cells. Nef redirects SERINC5 to a Rab7-positive endosomal compartment and thereby excludes it from HIV-1 particles. The ability to counteract SERINC5 was conserved in Nef encoded by diverse primate immunodeficiency viruses, as well as in the structurally unrelated glycosylated Gag from murine leukaemia virus. These examples of functional conservation and convergent evolution emphasize the fundamental importance of SERINC5 as a potent anti-retroviral factor.

Nef is a 27–32-kilodalton (kDa) protein expressed uniquely by primate lentiviruses that has a fundamental role in virus replication and the development of AIDS[1–3]. It is a multifunctional factor that performs a plethora of activities within the cell, among which is the ability to downregulate crucial cell surface molecules (including CD4, MHC-I and T-cell receptor) via interaction with vesicular trafficking machinery[4]. Other activities of Nef include the ability to alter the activation state of T cells and macrophages[5–8] and to perturb the actin cytoskeleton[9] by engaging with cellular kinases. These relatively well-characterized activities, however, do not explain another function of Nef that was reported 20 years ago[10], that is, its ability to enhance the infectivity of the virion. The latter activity seems to be important for HIV-1 pathogenesis because it is phylogenetically conserved among widely divergent primate lentiviruses[11] and maintained under strong selective pressure during disease progression[12]. Such enhancement of virion infectivity depends on *nef* being expressed from within virus-producing cells[13], but it is manifest at an early stage in the subsequent infection of susceptible target cells[13–15], indicating a yet unknown modification of progeny virus particles.

Although Nef is unique to HIV and SIV, glycosylated Gag from an unrelated gammaretrovirus (Moloney murine leukaemia (MLV)) fully substitutes for the activity of Nef on HIV-1 infectivity[16]. Despite the lack of any sequence homology, Nef and glycosylated Gag share a remarkable functional similarity, as they both require host cell endocytosis machinery to boost virion infectivity[17]. A Nef-like activity promoting retrovirus infectivity has therefore arisen by convergent evolution within an unrelated family of retroviruses. However, the molecular mechanism underlying the requirement of Nef and glycosylated Gag for retrovirus infectivity has so far remained elusive.

Nef counteracts a retrovirus inhibitor

We investigated to what extent the Nef requirement for virion infectivity is producer cell-type dependent, by comparing the infectivity of wild-type HIV-1 to its Nef-defective counterpart produced from 31 different human cell lines (Fig. 1a and Extended Data Table 1). Varying with the producer cell type, the effect of Nef ranged from 2- to 40-fold, arguing in favour of the presence of a cellular inhibitor of HIV-1 counteracted by Nef. We then investigated whether this Nef responsiveness is a dominant feature in producer cells by generating Nef-positive and Nef-negative HIV-1 virions from heterokaryons derived from cell lines with opposite Nef-responsiveness (Fig. 1b). When lymphoid cells (high Nef responsive) were fused with fibrosarcoma cells (low Nef responsive), HIV-1 produced by heterokaryons displayed relatively high dependence on Nef (Fig. 1c), indicating the presence of a transdominant cellular inhibitor of HIV-1 infectivity counteracted by Nef.

To identify such a putative host factor, the global transcriptome of high and low Nef-responsive cells was examined to pinpoint differentially expressed genes that correlate with Nef responsiveness. Transcriptomes from seven highly Nef-responsive cell lines (Nef effect ranging from 10- to 40-fold) and eight low Nef-responsive cell lines (Nef effect lower than fourfold) were subjected to RNA-sequencing (RNA-seq). On the basis of correlation analysis, SERINC5 emerged as the gene whose expression correlated best with the requirement of Nef for HIV-1 infectivity (Fig. 1d).

SERINC5 inhibits HIV-1 and MLV

To validate functionally the effect on virion infectivity, the *SERINC5* genomic sequence was disrupted in the cell line with the highest Nef responsiveness (Jurkat TAg or JTAg) using a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 lentiviral vector (Extended Data Fig. 1a). SERINC5 knockout cells produced a 20–30-fold increase in the infectivity of the Nef-defective HIV-1, whereas the Nef-positive virus was only affected 2–3-fold, thus reducing the Nef effect from 50- to 3-fold (Fig. 2a, b). This result was reproduced targeting three different regions of the *SERINC5* gene (Extended Data Fig. 1b). When haemagglutinin (HA)-tagged SERINC5 was expressed from a complementary DNA non-targetable by the CRISPR-Cas9 vector, the high Nef-dependent phenotype was

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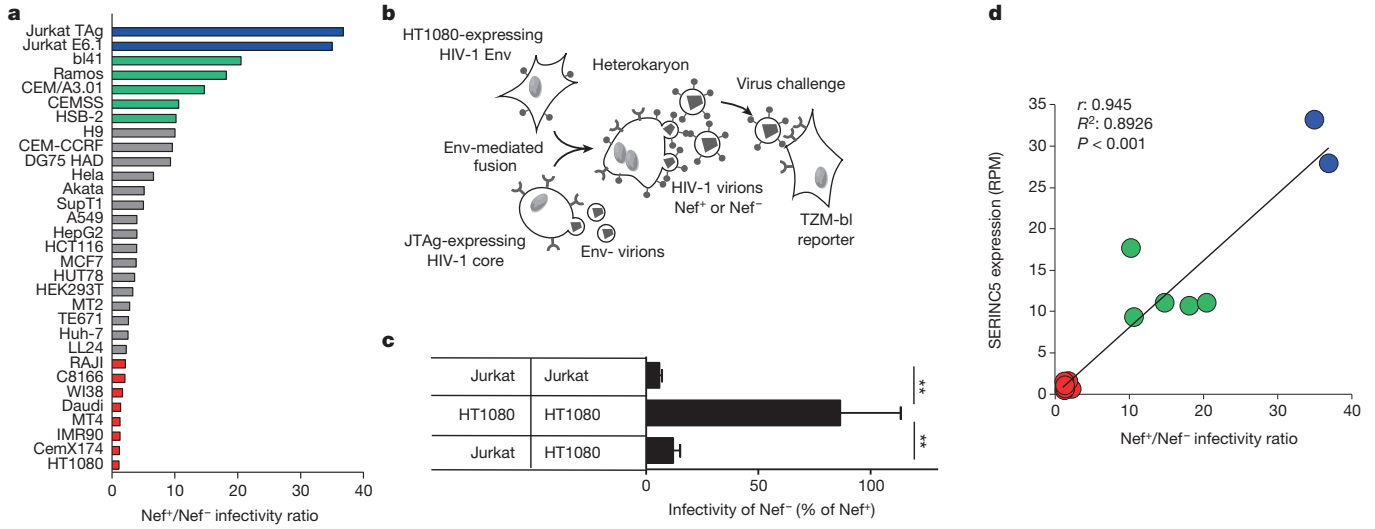

Figure 1 | Nef counteracts an HIV-1 inhibitor. **a**, Ratio of the infectivity of NL4-3 and NL4-3^{Nef-} produced from the indicated cell lines and measured on TZM-bl reporter cells. **b**, The schematic of the heterokaryon assay. **c**, Infectivity of HIV-1 derived from heterokaryons generated by the indicated cell lines ($n = 3$, mean ± s.d., unpaired t-test, $^{**}P < 0.01$.). **d**, Correlation of SERINC5 expression in producer cells and Nef requirement for infectivity. Colours in **a** and **d** represent the same cell lines. Trendline indicates linear regression. (Pearson correlation, two-tailed, $P < 0.0001$). RPM, reads per million.

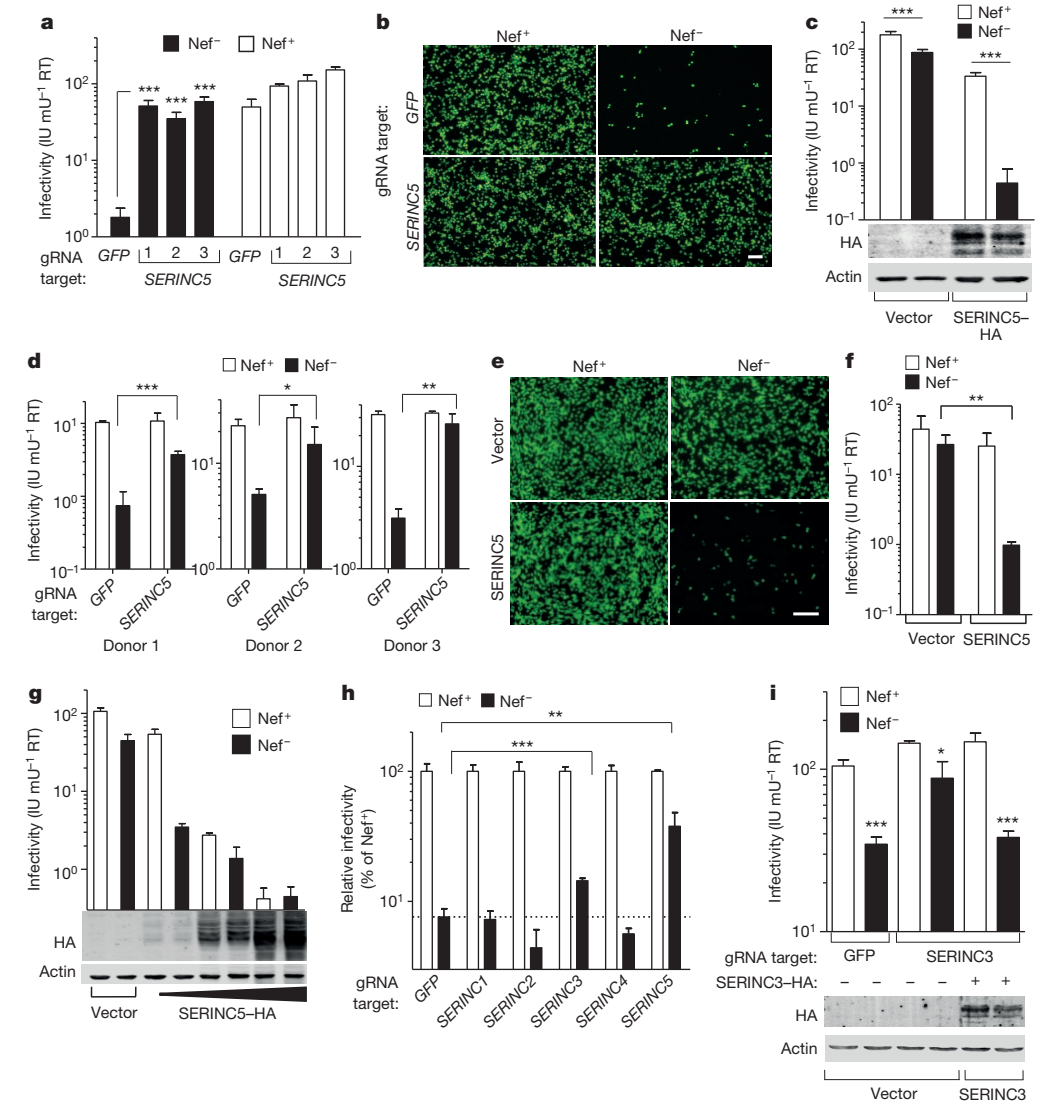

Figure 2 | SERINC5 and SERINC3 inhibit HIV-1. **a**, Infectivity of HIV-1 from cells stably transduced with lentiCRISPR ($n = 4$). gRNA, guide RNA; RT, reverse transcriptase. **b**, Fluorescence microscopy of reporter cells from **a**. **c**, Infectivity of HIV-1 from JTAg *SERINC5*$^{-/-}$ and immunoblotting of producer cells. **d**, Infectivity of HIV-1 from PBMC, co-transfected with CRISPR-Cas9 vectors ($n = 3$, one experiment performed per donor). **e**–**g**, Infectivity of HIV-1 from HEK293T expressing SERINC5–HA. Reporter cells infected with HIV-1 from HEK293T expressing SERINC5–HA ($n = 4$). **h**, **i**, Infectivity of HIV-1 produced from JTAg (**h**) and JTAg *SERINC5*$^{-/-}$ (**i**) transfected with CRISPR-Cas9 vectors targeting the indicated *SERINC* genes ($n = 4$). In **i**, immunodetection of SERINC3–HA in producer cells. Mean ± s.d., unpaired two-tailed t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars, 100 μm.

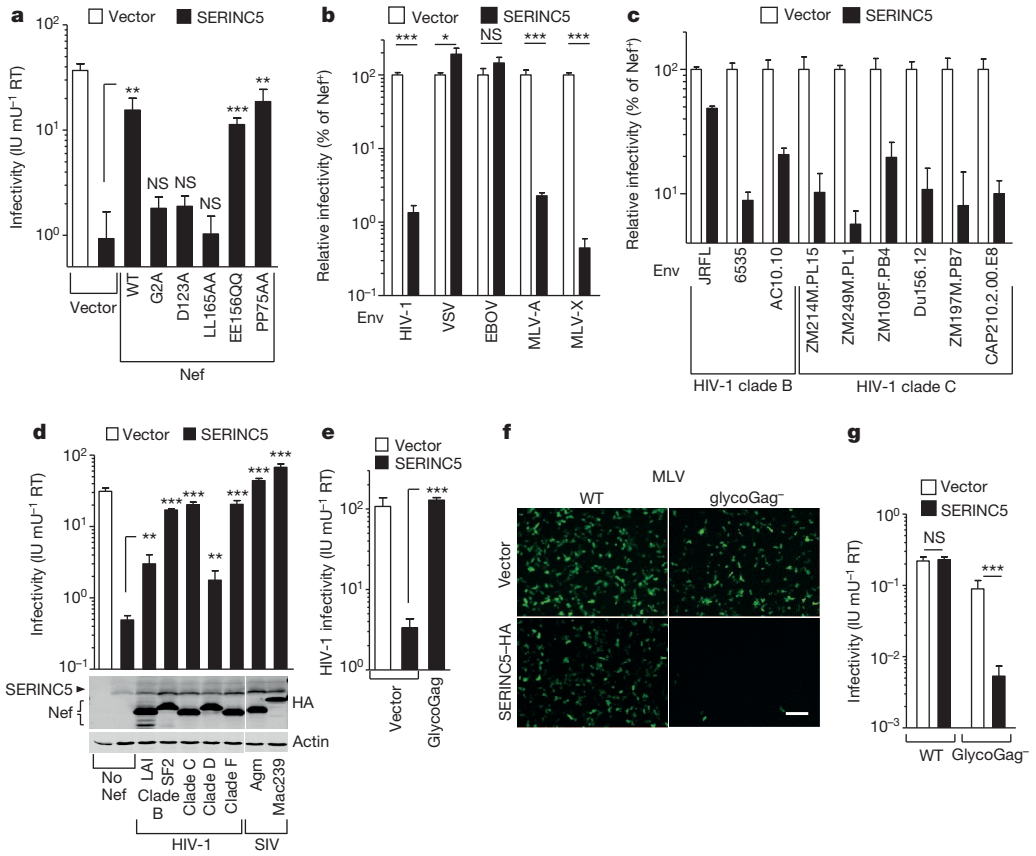

Figure 3 | Determinants of Nef activity against SERINC5 and conservation across different retroviruses. **a**, The ability of Nef mutants to counteract SERINC5 inhibition of HIV-1 infectivity ($n = 4$). **b**, **c**, Susceptibility of viral pseudotypes to inhibition of infectivity by SERINC5 ($n = 4$). **d**, **e**, Counteraction of SERINC5 by *nef* alleles and immunoblot from producer cells (**d**) and glycoGag (**e**) on HIV-1. **f**, **g**, Infectivity of wild-type and glycoGag-defective MLV from HEK293T expressing SERINC5 ($n = 4$). Mean ± s.d., unpaired two-tailed *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; NS, not significant. Scale bar, 100 μm.

restored (Fig. 2c), and the infectivity of the Nef-defective HIV-1 was reduced 197-fold versus a fivefold only reduction of the Nef-positive counterpart. SERINC5 was found to be expressed in primary blood cells from three different donors to a level comparable to that observed in Jurkat cells (Extended Data Fig. 1c). Accordingly, CRISPR-Cas9 vector-mediated SERINC5 knockout cells increased specifically the infectivity of Nef-defective HIV-1 produced in cultured peripheral blood mononuclear cells (PBMC) derived from three different individuals (Fig. 2d), demonstrating that SERINC5 inhibits HIV-1 produced in primary human blood cells.

Ectopic expression of SERINC5 in cells with minimal Nef-dependence (Fig. 2e, f and Extended Data Fig. 1d), resulted in a 10–40-fold selective inhibition of Nef-defective HIV-1. SERINC5 is therefore not only required, but also sufficient to inhibit HIV-1 infectivity and to confer Nef responsiveness. While inhibition of HIV-1 infection by SERINC5 is dose-dependent (Fig. 2g), the ability of Nef to preserve the infectivity of the virus particle is abolished with increasing expression of SERINC5, suggesting that the ability of Nef to counteract SERINC5 is saturable (Fig. 2g). At the highest SERINC5 expression level, virion infectivity was reduced 256-fold, regardless of Nef expression.

SERINC5 belongs to a unique gene family present in all eukaryotes and contains 10 putative transmembrane helices[18,19]. While it was suggested that SERINC proteins mediate incorporation of serine into membrane lipids[20], their function is unknown. The five members of the human SERINC family share more than 17% amino acid identity and a similarly predicted membrane topology. We observed that virus produced in JTAg *SERINC5*$^{-/-}$ cells retains a 2–3-fold responsiveness to Nef (Fig. 2a). Our transcriptome analysis indicated that JTAg cells express other *SERINC* genes in addition to *SERINC5* (Extended Data Fig. 1e). We therefore explored the possibility that other SERINC family members have anti-HIV-1 activity by knocking out the five *SERINC* genes individually. Targeting *SERINC3* in JTAg *SERINC5*$^{-/-}$ cells resulted in a 2–3-fold rescue of Nef-defective virus infectivity (Fig. 2i), thus further reducing the residual Nef responsiveness to 1.6-fold (Fig. 2i). Ectopic expression of SERINC3 resulted in threefold inhibition of Nef-defective HIV-1 (Fig. 2i), confirming that SERINC3 can also inhibit HIV-1 infectivity.

The Nef activity against SERINC5

The effect of Nef on infectivity requires Nef myristoylation and interaction with clathrin-mediated endocytosis[21,22] (AP2 and dynamin2). Accordingly, the ability to counteract SERINC5 was impaired by *nef* mutations that abolish Nef amino-terminal myristoylation (G2A), disrupt a di-leucine-based sorting signal (LL165AA) necessary for AP2 interaction[21], or prevent binding to dynamin 2 (D123A, Fig. 3a)[22]. By contrast, mutations abrogating either a proline-rich SH3 binding domain (PP75AA)[23], or di-acidic motif required for CD4 downregulation (EE156QQ)[24], do not affect the ability to counteract SERINC5 (Fig. 3a). The molecular features of Nef already known to be crucial for the effect on infectivity are therefore required for counteracting SERINC5.

It has been reported that the effect of Nef on infectivity depends on the nature of the envelope glycoprotein[16,25–29]. Accordingly, pseudotyping HIV-1 with vesicular stomatitis virus G protein (VSV-G) and with the Ebola virus glycoprotein (EBOV GP), but not with MLV-A nor MLV-X Env, makes HIV-1 resistant to SERINC5 (Fig. 3b). The magnitude of the effect of Nef on infectivity was also reported to vary when HIV-1 is pseudotyped with envelope glycoproteins derived from different HIV-1 isolates[30,31]. Accordingly, virions carrying Env derived from a panel of HIV-1 primary isolates were variably affected by SERINC5 (Fig. 3c), indicating that naturally occurring isolates are inhibited by the host factor to different extents.

The activity of Nef on infectivity is highly conserved among primate lentiviruses[11]. We therefore tested whether the ability to counteract SERINC5 is shared among *nef* alleles. Nef proteins derived from subtypes B, C, D and F clinical isolates could counteract ectopically

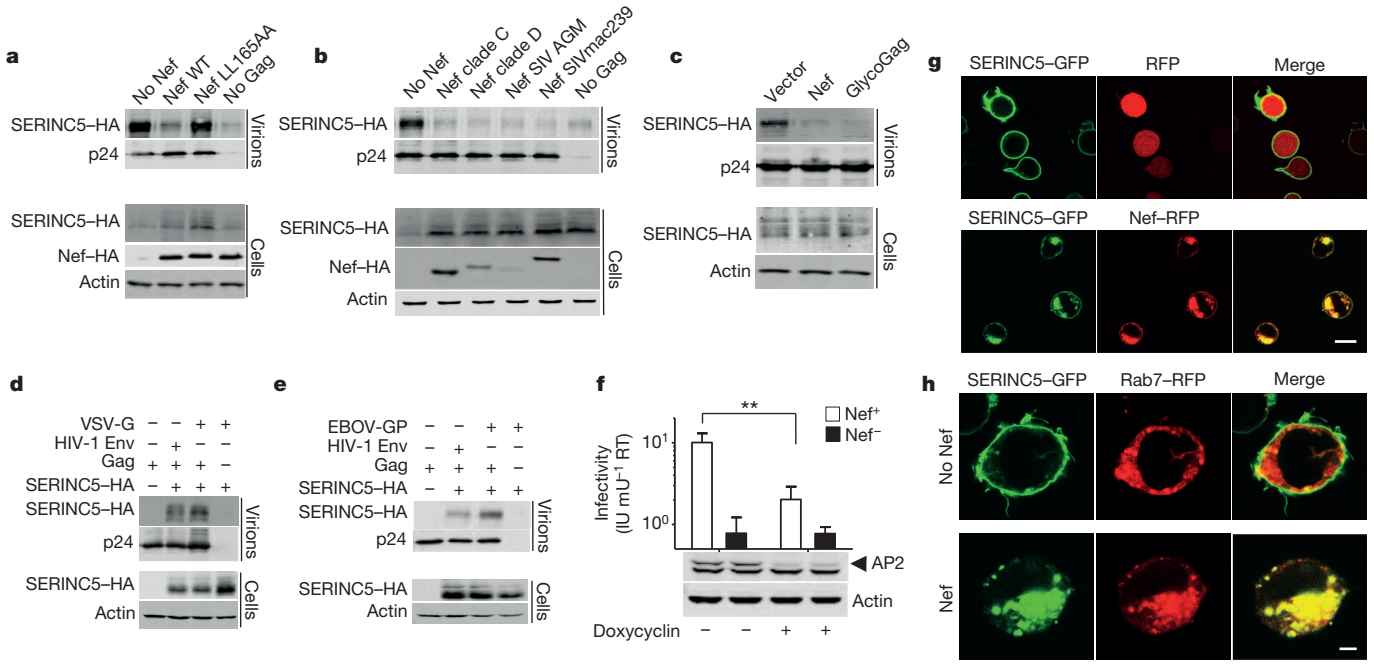

Figure 4 | Nef and glycoGag promote relocalization of SERINC5 to an endosomal compartment and prevent its incorporation into virions. **a**–**e**, Immunoblots on viral particles and corresponding cell lysates from *nef*-defective NL4-3 complemented with plasmids encoding Nef proteins as indicated (**a** and **b**), MLV glycoGag (**c**), VSV-G (**d**) EBOV-GP (**e**) and a vector expressing SERINC5–HA. **f**, Infectivity of HIV-1 from HEK293T cells stably expressing a doxycycline-inducible shRNA targeting *AP2* and transfected with PBJ6-SERINC5. Western blot: AP2 in cell lysates derived from producer cells (mean ± s.d., $n = 4$ unpaired two-tailed *t*-test, $^{**}P < 0.01$, experiment replicated twice). **g**, **h**, Confocal microscopy of JTAg cells transfected to express SERINC5–GFP with RFP, Nef–RFP (**g**) or Rab7–RFP (**h**). Scale bars, 10 μm (**g**) and 2 μm (**h**).

expressed SERINC5–HA with a potency 5–10-fold higher than that observed with Nef derived from a laboratory adapted strain (HIV-1_{LAI}, Fig. 3d). Similarly, Nef from two divergent SIV lineages (SIVmac239 and SIVagm) also counteracted SERINC5 with tenfold higher efficacy than HIV-1_{LAI} (Fig. 3d). The ability to counteract SERINC5 is therefore a prominent feature of Nef, conserved across different primate lentivirus species.

We next tested whether SERINC5 can target retroviruses other than lentiviruses. We have shown that glycosylated Gag (glycoGag) from MLV is capable of rescuing the infectivity of Nef-defective HIV-1 (ref. 16), despite sharing no sequence homology with Nef. Indeed, glycoGag efficiently rescues the infectivity of HIV-1 (Fig. 3e) by counteracting SERINC5, suggesting that SERINC5 has an important role also in the context of infection with gammaretroviruses. Accordingly, SERINC5 expression in producer cells potently inhibited infectivity of MLV only in the absence of glycoGag (Fig. 3f, g). Therefore, while SERINC5 targets divergent retroviruses, factors capable of overcoming its inhibitory activity on infectivity have evolved independently.

Incorporation of SERINC5 into virions

The ability of SERINC5 to be incorporated into the lipid envelope of HIV-1 virions was tested next. HIV-1 was produced in JTAg *SERINC5*$^{-/-}$ expressing SERINC5–HA. Despite being barely detectable in cells in the absence of Nef, SERINC5–HA was readily visualized in Nef-defective virions and was largely excluded from virions generated in the presence of Nef (Fig. 4a) but not in the presence of the Nef mutant lacking the AP2 binding site (LL165AA, Fig. 4a). The ability to prevent virion incorporation of SERINC5 was readily observed with Nef alleles from HIV-1 and SIV (Fig. 4b) and with MLV glycoGag (Fig. 4c), suggesting that association with virions is crucial for the effect on infectivity and is tightly controlled by both primate lentiviral and gammaretroviral factors. The effect of Nef on SERINC5 association with virions did not alter the amount of incorporated Env (Extended Data Fig. 2a), in line with previous observations that failed to observe any effect of Nef on virion Env abundance[16,25,31]. By contrast, the amount of SERINC5 incorporated into HIV particles was not reduced by VSV-G (Fig. 4d) nor by EBOV GP (Fig. 4e), despite the infectivity of VSV-G and EBOV GP pseudotypes being resistant to the host factor (Fig. 3b). Therefore, while Nef and glycoGag seem to counteract SERINC5 by preventing its incorporation into virions, VSV-G and EBOV GP must antagonize its effect by a different mechanism.

The ability of Nef to counteract SERINC5 was significantly reduced by silencing AP2 (Fig. 4f), confirming the crucial involvement of clathrin-dependent intravesicular trafficking. Using immunofluorescence microscopy, green fluorescent protein (GFP)-tagged SERINC5 (Fig. 4g) was observed to localize almost exclusively to the plasma membrane. By contrast, the expression of HIV-1 Nef caused SERINC5 to relocalize together with Nef into perinuclear vesicles identified as late endosomes (RAB7-positive, Fig. 4h). SERINC5 was similarly efficiently retargeted into perinuclear vesicles by SIV Nef and by MLV glycoGag (Extended Data Fig. 2b), indicating a common ability of the retroviral factors to relocalize SERINC5, which is removed from the plasma membrane, and prevented from accessing nascent virions.

The anti-HIV-1 activity of SERINC5

Which step of the HIV-1 life cycle is blocked by SERINC5 was investigated next. HIV-1 produced in the presence of SERINC5–HA failed to accumulate products of reverse transcription in target cells, confirming previous reports that in the absence of Nef the infection process is halted at an early stage of the HIV-1 life cycle (Fig. 5a). Whether Nef affects fusion between the virion particle and the target cell membrane has remained questionable[25,32–37]. We therefore developed a novel protein transduction assay in which the bacteriophage Cre recombinase fused to a nuclear localization signal flanked by HIV-1 protease cleavage sites (Fig. 5b) is packaged as part of the Gag polyprotein into HIV-1 particles (Extended Data Fig. 3a). Cre, delivered into the cell after fusion, activates expression of a reporter

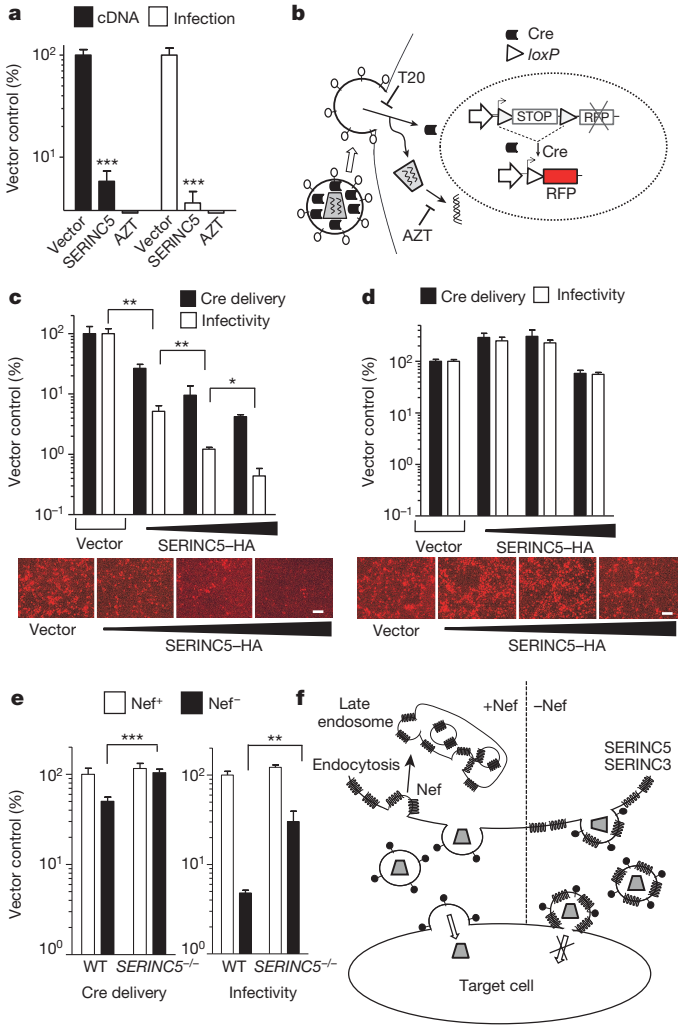

Figure 5 | SERINC5 inhibits an early step of virus infection. **a**, Effect of SERINC5 on the generation of HIV-1 late reverse transcription products ($n = 3$; experiments replicated twice) and the corresponding effect on infectivity. **b**, Schematic of the nlsCre delivery assay. **c**, **d**, Effect of SERINC5 on Cre-delivery by HIV-1 (**c**) and HIV-1 pseudotyped with VSV-G (**d**). **e**, Cre delivery and infectivity by HIV-1-derived from JTAg or JTAg *SERINC5*$^{-/-}$. **f**, Schematic showing the activity of SERINC5 on HIV-1 infectivity and the counteracting mechanism by Nef. Mean ± s.d., $n = 4$, unpaired two-tailed *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Scale bars, 100 μm.

gene (nlsRFP) following *loxP* recombination. Confirming the ability to detect cytoplasmic delivery of Cre independently of productive infection, Cre-mediated reporter activation was not blocked by the reverse transcriptase inhibitor azidothymidine (AZT) but was blocked by the fusion inhibitor T20 (Extended Data Fig. 3b).

Increasing expression of SERINC5 in producer cells did not affect the amount of Cre associated with virions (Extended Data Fig. 3c), but resulted in a gradually increased inhibition of Cre-mediated activation of the reporter gene in target cells by Nef-defective HIV-1 (Fig. 5c), with a 25-fold inhibition observed at the highest SERINC5 expression level, which in turn inhibited infectivity by 250-fold (Fig. 5c). This observation was reproducible also using a fusion assay based on the viral incorporation and cytoplasmic delivery of a BLAM-VpR chimaeric gene[38] (Extended Data Fig. 3d). By contrast, Cre-delivery from Nef-defective HIV-1 pseudotyped with VSV-G (Fig. 5d) or with EBOV GP (Extended Data Fig. 3e) was not inhibited by SERINC5, consistent with the intrinsic resistance of these pseudotypes to the inhibition by the host factor (Fig. 3b).

When the host factor was expressed at a level which introduced a 20-fold effect on infectivity, SERINC5 resulted in a 2–3-fold inhibition of Cre delivery, fully counteracted by Nef (Extended Data Fig. 3f). Similarly, Nef-defective HIV-1 derived from wild-type JTAg cells delivered Cre to target cells with a 2–3-fold lower efficiency than Nef-positive virions in the presence of endogenously expressed SERINC5, in spite of a 20-fold lower infectivity (Fig. 5e).

Altogether, these results suggest that SERINC5 perturbs the ability of the viral particle to translocate its content to the cytoplasm.

Discussion

Here we demonstrate that SERINC5, and to a lesser extent SERINC3, are responsible for the long-sought anti-HIV-1 activity that is overcome by Nef. These cellular proteins join a growing list of host factors that inhibit retrovirus infection and are referred to as restriction factors. However, SERINC5 and SERINC3 have features that distinguish them from other known retroviral restriction factors. For example, SERINC5 expression in primary CD4$^+$ T cells or dendritic cells is not upregulated by type I interferon or by an interferon-inducing agent such as lipopolysaccharide (LPS; Extended Data Fig. 4). SERINC5 and SERINC3 therefore appear to be examples of constitutively expressed intrinsic restriction factors.

Human SERINC5 shares 28% identity at the amino acid level with the *Saccharomyces cerevisiae* orthologue, *TMS1* (ref. 18). Such a degree of conservation suggests a yet unidentified core biological function in cells and represents another peculiar feature compared with other antiretroviral restriction factors (for example, TRIM5 could be traced back only to teleosts[39]), which diversified under strong positive selection[40]. Remarkably, Nef from HIV-1 and SIV, as well as glycoGag from MLV, are all capable of counteracting human SERINC5, denoting an unusual low species-specificity between the host factor and the viral antagonist.

We provided evidence that SERINC5 perturbs the ability of small intravirion proteins, such as Cre and BLAM-VpR (less than 40 kDa), to access the target cell. However, inhibition of infectivity by SERINC5 is tenfold higher, suggesting that infection is blocked despite detectable fusion. The effect on infectivity, which requires the delivery of the 60–120-nm viral core[41], is therefore unlikely to be explained only by an effect of SERINC5 on the initial membrane fusion event. The host protein could therefore affect a step after the fusion pore generation, required for the translocation of the viral core (Fig. 5f). After the initial membrane fusion triggered by fusogenic glycoproteins, the formation of a fusion pore is followed by its expansion, the highest energy requiring step in the fusion process[42]. This event is known to be affected by the lipid membrane composition[43,44] and the presence of proteins altering the rigidity and the curvature of the lipid bilayer[45]. How SERINC5 would affect this step of HIV-1 infection remains to be established. By contrast, VSV-G or EBOV GP may override such inhibition by intrinsically promoting more efficient expansion of the fusion pore. Interestingly, some HIV-1 Env glycoproteins from clinical isolates appear also to modulate the susceptibility of the virus to SERINC5 inhibition (Fig. 3c), suggesting the possibility that HIV-1 uses Env in addition to Nef to overcome such a powerful block.

In conclusion, the ability to target evolutionary distant retroviruses (HIV and MLV) and the convergent evolution of antagonistic retroviral factors (Nef and glycoGag) indicate that SERINC5 has a fundamental role in the interaction of the host with retroviral pathogens. Interestingly, ectopic expression of SERINC5 potently inhibits HIV-1, even in the presence of Nef (Fig. 2g), suggesting that this cellular antiviral factor might be exploited as an anti-HIV-1 therapeutic gene.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information RNA-seq fatsq data have been deposited in NCBI Sequence Read Archive (SRA) under accession code SRP062444. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.P. (massimo.pizzato@unitn.it).

METHODS

Plasmids. *Env*-defective and *nef*-defective HIV-1^{NL4-3} have been described previously[22]. *Env*-defective and *glycoGag*-defective MLV were engineered to express GFP in place of Env. Unless otherwise indicated, single round HIV-1 Env-defective HIV-1 (NL4-3) was complemented with Env derived from HIV-1^{HXB2} expressed with the vector PBJ5 (ref. 22). Constructs for expression of other viral factors include: plasmids encoding Env from primary HIV-1 isolates (obtained from NIH AIDS Reagent Program); plasmids encoding wild-type and mutated HA-tagged Nef from HIV-1^{LAI} [22] and Nef from primary HIV-1 isolates belonging to subtypes C, D, F; plasmids for expression of HA-tagged Nef from SIV^{mac} and SIV^{agm} [22]; plasmids encoding untagged or HA-tagged MLV glycoGag truncated at residue 189 (ref. 16), pCAGGS expressing codon optimized Zaire Ebola virus glycoprotein (GenBank accession number KJ660346.2); pMDG[46] encoding VSV-G.

DNA encoding SERINC5 with or without the HA-tag were amplified from cDNA derived from JTAg cells. DNA sequence was confirmed to match the reference sequence with accession number NM_001174072.2. DNA encoding SERINC3 (reference sequence NM_006811) was custom synthesized (GeneWiz). For expression in mammalian cells, DNAs were cloned into expression vectors PCDNA3.1 (Life Technologies), PBJ5, PBJ6 (derived from PBJ5 by removing the SV40 origin of replication from the SV40-HTLV-1 hybrid promoter region), and pEGFPN1 (Clontech).

Increasing amount of SERINC5–HA expression in HEK293T cells was obtained by transfecting cells with PBJ6-, PBJ5-, and PCDNA3.1-based vector in increasing order (PBJ6<PBJ5<PCDNA3.1).

mRFP–Rab7 was a gift from A. Helenius (Addgene plasmid 14436). TagRFP657 was fused at the C terminus of Nef to generate pNef-Tag-RFP.

Cell lines. Cell lines used (also described in Extended Data Table 1 together with the source) were all tested for possible contamination with mycoplasma and tested negative. Cell line TE671 (Fig. 1a) is listed in the ICLAC database of commonly misidentified cell lines. However, for our purposes the nature of the cell line does not influence the outcome of the research which was only meant at investigating a correlation between the Nef requirement with gene expression. In addition to cell lines listed in Extended Data Table 1, TZM-bl indicator cells were obtained from the NIH AIDS Research and Reference Reagent program.

CRISPR-Cas9 knockout. Stable cell lines knocked out for SERINC5 were generated by transduction with LentiCRISPR (a gift from F. Zhang, Addgene plasmid 49535) after puromycin selection and, where indicated, clonal expansion. PX330 CRISPR-Cas9 (a gift from F. Zhang, Addgene plasmid 42230) was used for generating knockout by transient transfection, targeting simultaneously two different exons of the same gene. The following target sequences were used: 5′-GCTGAGGGACTGCCGAATCC-3′ (SERINC5-1, exon 2), 5′-GACGGCTCCCACATAGCGCC-3′ (SERINC5-2, exon 6), 5′-GGCGTACCACAGCTTGTTAC-3′ (SERINC5-3, exon 8), 5′-GCATCGGCATAGCAAACACG-3′ and 5′-CTATGCCGATGCTGTCCTAG-3′ (SERINC1), 5′-CCGCATGTGCTTCGCCACGG-3′ and 5′-ATCCTGGTGGGCCTCACCGT-3′ (SERINC2), 5′-ATAAATGAGGCGAGTCACCG-3′ and 5′-CTCCGAGCGGCAGTACACAA-3′ (SERINC3), 5′-TGATGACAGAAGCTTGTAGG-3′ and 5′-GGTTCCATTTTACTCAGGCC-3′ (SERINC4), 5′-GTGAACCGCATCGAGCTGAA-3′ (GFP).

To verify the occurrence of indels and the disruption of the SERINC5 open-reading frame (ORF) in clonal populations of JTAg cells stably transduced with the LentiCRISPR vector targeting SERINC5 exon 2 (using SERINC5-1 gRNA), genomic DNA was extracted from cells, a 228-nucleotide fragment encompassing exon 2 was amplified by PCR using primers 5′-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**-*TAAGCAGATGCCTTCTGTTCCTT*-3′ and 5′-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**-*AATAGGACGAGCTGAACACGG*-3′ (in which italic denotes the locus-specific sequence, and bold denotes the overhang adapters). A subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Normalized and pooled libraries were, then, sequenced on the Illumina MiSeq system using v2 reagents (2×250-nucleotide paired-end reads).

Viruses and infectivity assay. Cell lines in Fig. 1a were infected with NL4-3 and NL4-3^{Nef-} produced by transfection of HEK293T cells and transiently pseudotyped with VSV-G. Virus supernatant was collected 48 h after infection and inoculated onto TZM-bl cells in the presence of the protease inhibitor Saquinavir (10 μM) to limit infection to a single round of replication.

For all other experiments, virions limited to a single round of replication were used and were produced by transfection. JTAg and 174XCEM cells were transfected using electroporation, HT1080 using Mirus TransIT-2020, HEK293T cells by the calcium phosphate co-precipitation method. PBMC were transfected by nucleofection 48 h after stimulation with phytohaemagglutinin (PHA) and interleukin-2 (IL-2). As indicated, virus constructs were co-transfected together with other plasmids expressing Env glycoproteins, Nef, glycoGag, SERINC5, or PX330-based CRISPR-Cas9 vectors. Virus-containing culture supernatants were collected 48 h after transfection, clarified by centrifugation at 300*g* for 5 min and passed through filters with 0.45-μm pores. Virus prepared in quadruplicate were then quantified using the SG-PERT reverse transcription assay[47], diluted three- or fivefold in a series of six steps and used to infect TZM-GFP reporter cells seeded one day before infection in 96-well plates. TZM-GFP is a modified version of TZM-bl containing an integrated nlsGFP reporter gene under the transcriptional control of the HIV-1 long terminal repeat. Infection of reporter cells was scored using the High Content Imaging System Operetta (Perkin Elmer) after counterstaining nuclei with Hoechst 33342 for each virus dilution. Those values falling into a linear dilution range (normally below 20% of infected cells) 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[47].

Heterokaryons. Heterokaryons were produced following a strategy previously reported[48]. Production of single round virions infectious only upon heterokaryon formation was obtained by transfecting one fusion partner with *env*-defective/*nef*-defective HIV-1^{NL4-3} and the other with PBJ5-HXB2-Env, PBJ5-Nef^{LAI} or the empty control vector PBJ5. To promote efficient fusion mediated by HIV-1 Env, plasmids encoding for CD4 and CXCR4 were co-transfected together with the *env*-defective provirus construct. Then 24 h after transfection, cells were co-cultured and progeny viruses collected 24 h later.

Preparation of RNA-seq libraries and sequencing. Five micrograms of total RNA extracted from seven highly Nef-dependent cell lines (JTAg, Jurkat E6.1, bl41, Ramos, CEM A301, CEM SS and HSB2) and eight low Nef-dependent cell lines (MT4, HT1080, RAJI, DAUDI, C8166, IMR90, CEMX174 and WI38) was subjected to rRNA depletion using Ribo-Zero Magnetic Gold Kit (Epicentre). RNA-seq libraries were prepared from the rRNA depleted RNAs extracted from the 15 cell lines (Fig. 1a) using a modified protocol of the Illumina TruSeq RNA Sample Prep Kit. Libraries were sequenced on the Illumina HiSeq 2000 using paired-end sequencing 2 × 100 bp. Raw reads were mapped against the human (hg19) genome reference using tophat2 (ref. 49). RPM[50] values were estimated for each transcript in each sample with a custom pipeline. Genes were ranked according to Pearson correlation between their relative expression (RPM) in cell line and the corresponding Nef^{+}/Nef^{-} infectivity ratio (Fig. 1a). The computations were performed at the Vital-IT Center (http://www.vital-it.ch) for high-performance computing of the SIB Swiss Institute of Bioinformatics in Geneva.

Microscopy. JTAg cells were electroporated with constructs expressing Nef-TagRFP657 or the control TagRFP657, Nef–HA, HA–glycoMa, SERINC5–GFP and Rab7–RFP as indicated. Then 48 h after transfection, cells were overlaid on poly-L-lysine coated glass slides, fixed with 4% paraformaldehyde and permeabilzed with 0.1% Triton X-100. The HA tag was detected by staining with mouse anti-HA (HA.11, Covance) and the secondary antibody Alexa 633 (Life Technologies). Images were acquired using a Leica TCS SP5 confocal microscope.

Western blotting. Cell lysates and virion pellets were analysed by SDS–PAGE and western blotting. In brief, viral particles were collected 48 h after transfection, centrifuged at 300*g* to remove cell debris and filtered. The clarified supernatants were overlaid on 25% sucrose cushion and concentrated at 100,000*g*. The pellets were resuspended directly in Laemmli buffer (supplemented with 50 mM TCEP), normalized by reverse transcriptase assay and resolved by SDS–PAGE. After observing that SERINC5 and SERINC3 form aggregates that are lost while clarifying the cell lysate or fail to enter the separating gel, cells were lysed directly in Laemmli buffer containing TCEP (Sigma, final concentration 50 mM, pH 7.0) and avoiding boiling. Samples were loaded on gel after a 5-pulse sonication. Having failed to find a commercially available antibody capable of detecting the endogenous protein, probing was performed using mouse anti-HA (HA.11, Clone 16B12, Covance), mouse or rabbit anti-β-actin (Li-COR), anti-HIV-1 p55/p24 (National Biological Standards Board), anti-gp41 Chessie-8 (obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from G. Lewis), mouse anti-Cre (Mab3120, Chemicon) and secondary antibodies IRDye 680 and IRDye 800 (Li-COR). Blots were imaged using an Odyssey Imager system (Li-COR).

nlsCre delivery assay. A packaging vector based on p8.9 lentiviral gag-pol expressing plasmid[46] (8.9-Cre) was generated to carry an insertion of nlsCre between MA and CA flanked by native HIV-protease cleavage sites for processing and release of proteins from Gag.

A Cre-responsive nuclear RFP-expressing lentiviral vector (p-lenti LoxP-Blasti-mRFP) was created. It consists of a nls-mRFP sequence lacking the translation initiation codon and preceded by a sequence encoding the blasticidin antibiotic resistance (Bla) between two *loxP* sites. The nlsRFP is translationally inactive unless Cre-mediated recombination of *loxP* and excision of Bla occurs, providing an authentic translation initiation for mRFP. A TZM-bl-GFP derivative cell line (TZM-GFP) stably transduced with p-lenti LoxP-Blasti mRFP was generated (TZM-GFP-LoxP-RFP) to detect delivery of nlsCre, and Tat-driven expression of nlsGFP.

To package nlsCre in retrovirus particles, HIV-1 was produced by mixing 8.9-Cre together with the *env*-defective (and *nef*-defective where applicable) NL4-3 provirus at a ratio of 1:2. Virus was produced by cotransfecting HEK293T cells with the viral constructs together with PBJ5-HXB2-env or vectors for expression of VSV-G and Ebola glycoprotein, and plasmids encoding SERINC5 or the empty vector. To achieve increasing level of expression, SERINC5 was expressed from vector PBJ6, PBJ5 and PCDNA3.1 (in increasing order). Progeny virus was inoculated onto TZM-GFP-LoxP-RFP and red and green fluorescence quantified 48 h later using the High Content Imaging System Operetta (Perkin Elmer) after counterstaining nuclei with Hoechst 33342, following the method described for infectivity.

BLAM-VpR assay. Virus was produced by transfection of HEK293T with the calcium phosphate method in 10 cm tissue culture plates with 10 μg of NL4-3 Envfs/Neffs (bearing a frameshift) together with 2 μg HIV-1 Env expressor, 5 μg of BLAM-VpR vector[38] and 5 μg of SERINC5 expression vectors or the empty vector control.

Target cells (TZM-bl) were seeded in clear bottom 96-well plates (Optiplates, Perkin Elmer) at a density of 25,000 cells per well in phenol-Red-free medium one day before assay. Virus samples were normalized for reverse transcriptase activity content and added to wells (200 μl) serially diluted as described for infectivity. Cells were spin-infected for 2 h at 4 °C at 1,550*g*, virus was removed, cells washed twice with complete medium and incubated for 90 min at 37 °C. Medium was then replaced with GeneBlazer substrate loading solution containing 2 μM CCF2AM (GeneBLAzer *In vivo* Detection Kit, Life Technologies) and 2.5 mM Probenecid (Sigma). Cells were incubated overnight at 11 °C, fixed with 2% paraformaldehyde and plates analysed using the Operetta imaging system for blue and green fluorescence to reveal the number of blue positive cells. Transduction units were derived from the number of blue positive cells divided per reverse transcriptase activity associated to the virus inocula as described for infectivity.

Quantification of HIV-1 reverse transcription products. NL4-3 normalized based on reverse transcriptase activity was incubated with target cells (NP2-CD4-CXCR4). Cell-free virions were normalized by reverse transcriptase activity and incubated with target cells in 6-well plates for 12 h. For each virus, infections were also performed in the presence of 40 μM AZT, to control for contamination of plasmid DNA in the PCR reaction. Cells were collected and washed extensively with PBS. Total DNA was extracted (Qiagen, Qiamp DNA mini kit), quantified, and subjected to real-time PCR with a Biorad CFX96 cycler. cDNA was detected with SYBR-Green I based reactions using 100 ng template DNA and 320 nM of each primer pair (5′-ACAAGCTAGTACCAGTTGAGCCAGATAAG-3′ and 5′-GCCGTGCGCGCTTCAGCAAGC-3′) in 20 mM Tris-Cl, pH 8.3, 5 mM $(NH_4)_2SO_4$, 20mM KCl, 5 mM $MgCl_2$, 0.1 mg ml^{-1} BSA, 1/20,000 SYBR Green I (Sigma), and 200 μM dNTPs. The PCR was programmed for 40 cycles of denaturation at 95 °C for 5 s, annealing 55 °C for 5 s, extension at 72 °C for 20 s and acquisition at 80 °C for 5 s. Relative quantification of retroviral cDNA sequences was obtained with respect to standard curves prepared from serial dilutions of DNA derived from the cell culture with the highest infection, diluted in DNA extracted from non-infected cells.

PBMC. Buffy coats obtained from anonymous blood donors were provided by the Department of Immunotransfusion, Padova University Hospital, for experiments involving virus production, or purchased from the New York Blood Center, for interferon induction studies. PBMC were isolated using Ficoll-Paque Plus (GE Healthcare).

Isolation, stimulation and treatment of dendritic cells. $CD14^+$ monocytes were enriched from PBMC by positive selection using CD14 MicroBeads following the manufacturer's protocol (Miltenyi Biotec). $CD14^+$-enriched cell populations were counted, centrifuged at 200*g* for 10 min, and resuspended at 2×10^6 cells ml^{-1} in RPMI-1640 supplemented with 5% human AB+ serum, 1 × MEM non-essential amino acids (NEAA), 20 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. To induce differentiation of monocytes into dendritic cells, cells were cultured for 5 days in GM-CSF (50 ng ml^{-1}) and IL-4 (25 ng ml^{-1}), both cytokines from R&D Systems. Dendritic cells were treated with LPS (100 ng ml^{-1}, LPS-EK Ultrapure, Invivogen) or IFN-β (37 ng ml^{-1}, PBL Assay Science). Cells were collected at various time points ($t = 0$ h, 2 h, 6 h, 24 h) after the LPS and IFN-β treatments for RNA extraction and subsequent RT–PCR analysis.

Isolation, stimulation and treatment of $CD4^+$ T cells. $CD4^+$ T cells were isolated from CD14-depleted PBMCs by positive selection using CD4 magnetic microbeads (Miltenyi Biotec) and plated at 2×10^6 cells ml^{-1} in RPMI-1640, supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, 1× MEM NEAA, and 1× GlutaMAX (Life Technologies). In one experiment, $CD4^+$ T cells were treated directly with LPS (100 ng ml^{-1}) or IFN-β (37 ng ml^{-1}, PBL Assay Science). Separately, $CD4^+$ T cells from the same donors were stimulated with 4 μg ml^{-1} of PHA-M for 48 h, 20 IU ml^{-1} IL-2 was added, and cells were stimulated with LPS or IFN-β. Cells were collected at various time points ($t = 0$ h, 2 h, 6 h, 24 h) after the LPS and IFN-β treatments for RNA extraction and subsequent RT–PCR analysis. Jurkat T cells were cultured and stimulated similarly.

RNA isolation and qRT–PCR. RNA was isolated using RNeasy Plus Mini Kit (Qiagen 74134) with additional on column DNase treatment (Qiagen 79254) and reverse transcribed with SuperScript VILO Master Mix (Invitrogen 11755050). Gene expression was assayed on a Biorad CFX96 Real-Time PCR detection system.

For quantification of SERINC5 transcripts in cell lines and PBMC (Extended Data Fig. 2c), the SYBR-Green-based real-time PCR method was used with the following primers 5′-TAAGCAGATGCCTTCTGTTCCTT-3′ and 5′-AATAG GACGAGCTGAACACGG-3′ (for *SERINC5*) and 5′-GACAGGATGCAGAAG GAGATTACTG-3′ and 5′-CTCAGGAGGAGCAATGATCTTGAT-3′ (for β-actin used as normalization control).

For Extended Data Fig. 4, gene expression was measured using TaqMan Gene Expression Master Mix (Life Technologies 4369016) and the following TaqMan probes and primers sets: SERINC5 (Hs00968169_m1, Life Technologies 4351372) and SERINC3 (Hs01566572_m1), CXCL10 (Hs00171042_m1) and, as a normalization control, OAZ1 (Hs00427923_m1, Life Technologies 4331182).

Statistics. Statistical tests were performed using GraphPad Prism. Given the nature of the experiments and the type of samples, significance of differences was assessed with unpaired two-tailed Student's *t*-test. Variance was estimated by calculating the standard deviation in each group, as represented by error bars. Variances between groups of samples were compared using the F-test function integrated in GraphPad. No statistical methods were used to predetermine sample size. Unless otherwise specified in figure legends, all experiments were performed independently at least three times and '*n*' indicates technical replicates, with a representative experiment being shown. Experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

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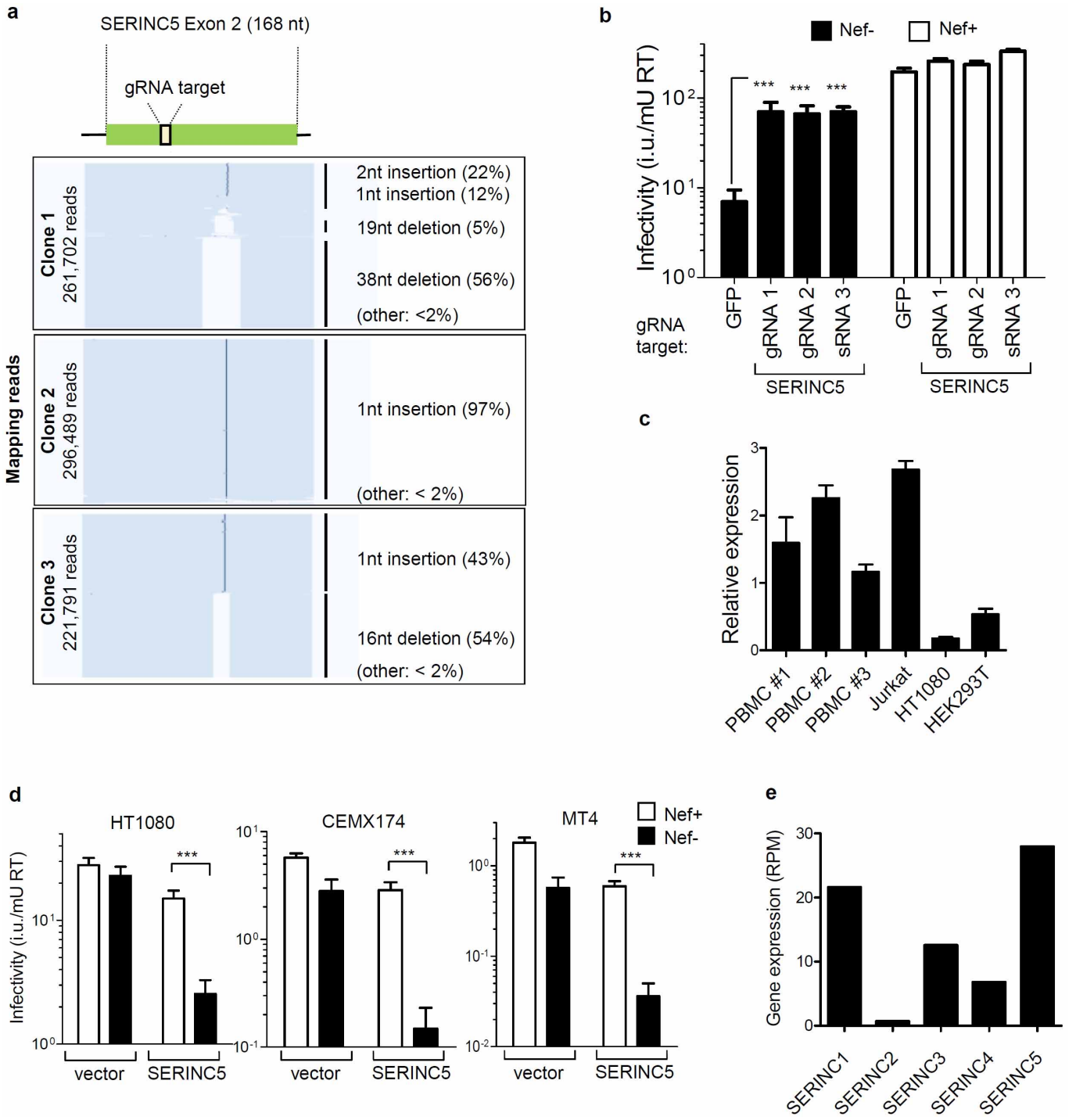
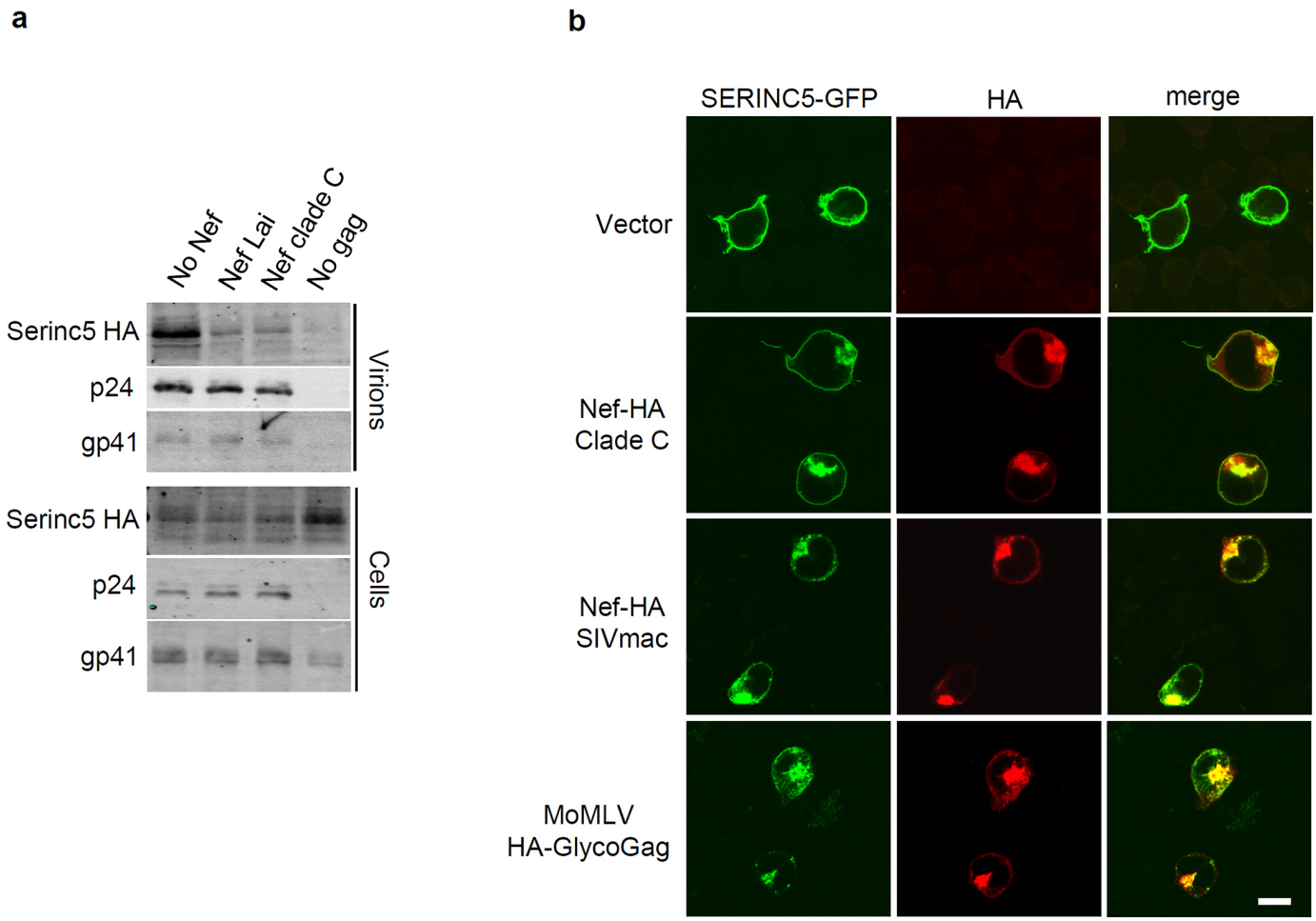
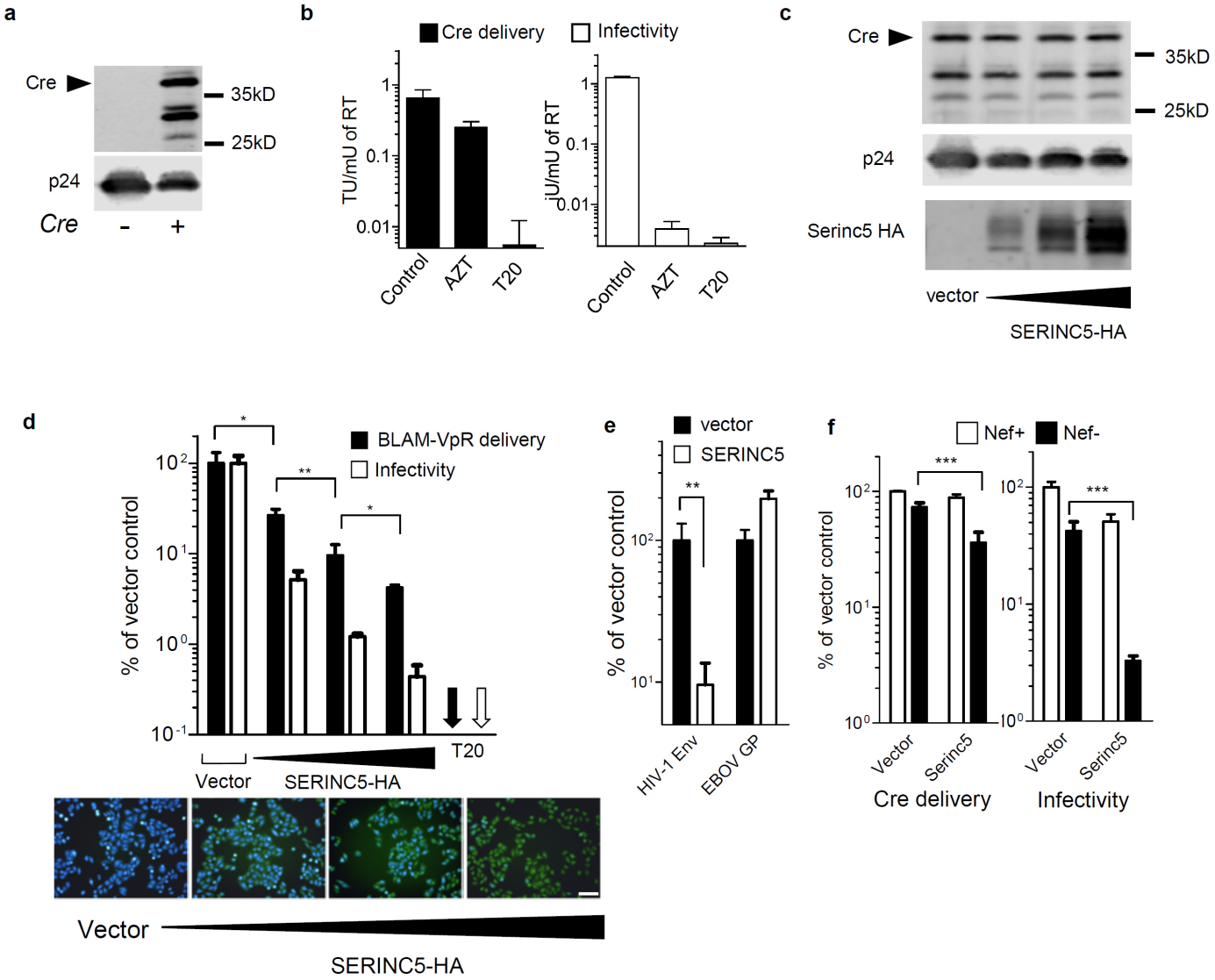


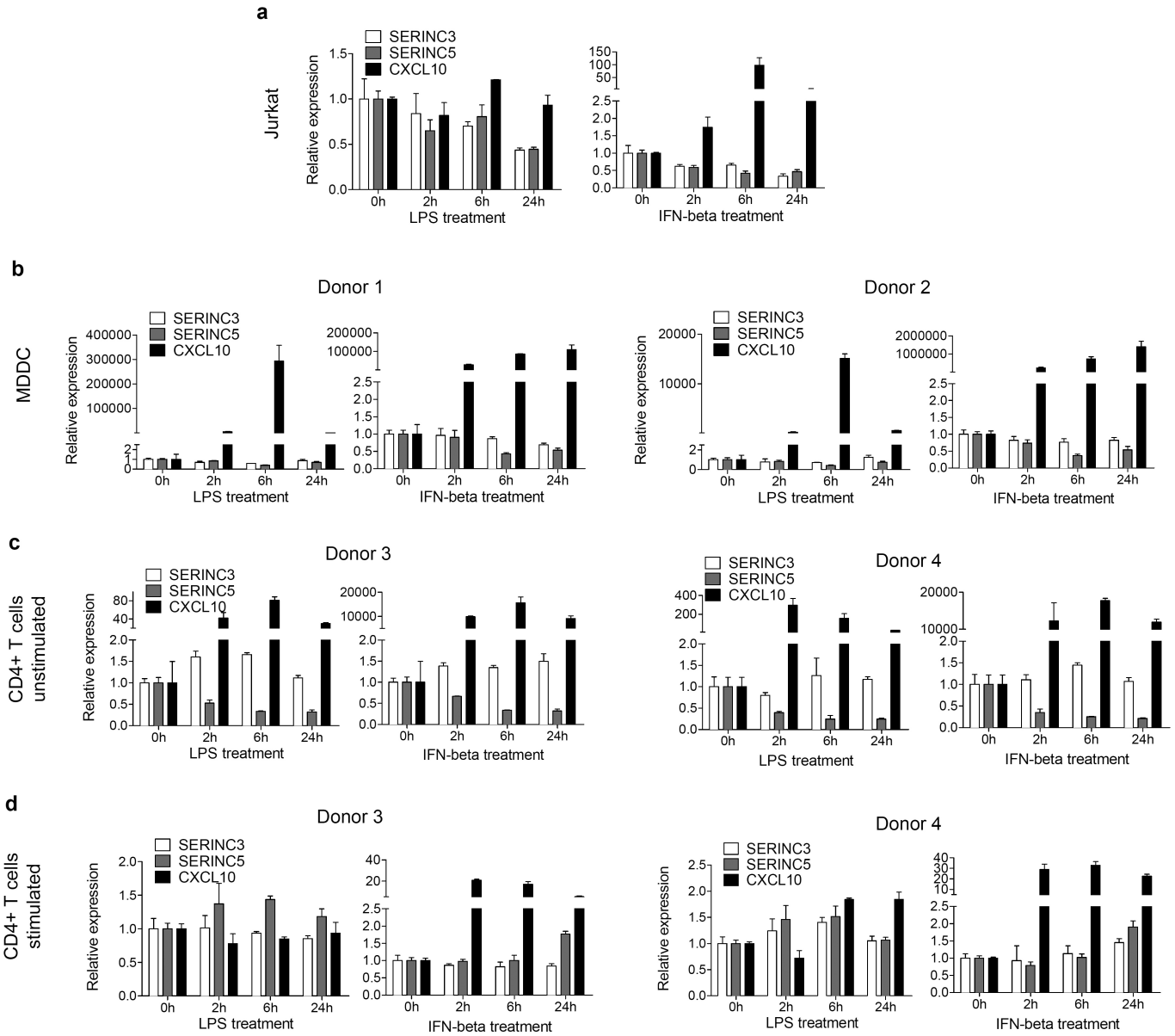
Extended Data Figure 1 | SERINC5 is an inhibitor of HIV-1 infectivity. **a**, Mapping of the INDELS in the genomic locus spanning SERINC5 exon 2 in JTAg cell clonal populations from Fig. 2a. **b**, Infectivity of HIV-1 from JTAg cells stably transduced with lentiCRISPR targeting GFP or SERINC5 in three different exons ($n = 4$, experiment replicated twice). **c**, Relative expression of *SERINC5* in primary cells and in cell lines measured by qPCR normalized by expression of *ACTB* ($n = 3$). **d**, Infectivity of HIV-1 from the indicated cell lines expressing SERINC5 ($n = 4$, experiments were replicated twice). Mean ± s.d., unpaired two-tailed *t*-test, $^{***}P < 0.001$ **e**, Expression levels of the five *SERINC* genes in JTAg cells obtained from RNA-seq.



Extended Data Figure 2 | Nef and glycoGag expression result in relocalization of SERINC5 to an endosomal compartment and prevent its incorporation into virions. **a**, Single round Nef-defective NL4-3 produced by cotransfection of HEK293T cells with plasmids expressing Nef proteins or the empty vector control, and PBJ6-SERINC5–HA: immunoblotting of virions and cell lysates from producer cells. **b**, Immunofluorescence staining of JTAg cells transfected to express SERINC5–GFP, Nef–HA from HIV-1 isolate 97ZA012 (clade C), from SIV^{mac239}, HA–glycoGag or an empty vector control. Scale bar, 10 μm.



Extended Data Figure 3 | SERINC5 inhibits cytoplasmic delivery of virion content. **a**, Immunodetection of Cre-recombinase (38 kDa) and p24 in HIV-1 particles. **b**, Effect of 1 μM AZT or 100 nM T20 on Cre-delivery and virus infectivity (TU, transducing units). **c**, Immunoblotting of HIV-1 virus particles produced from HEK293T expressing increasing levels of SERINC5–HA. **d**, Effect of SERINC5 on virus fusion measured with BLAM assay T20 served as a negative control. ($n = 4$, experiment replicated twice). **e**, Cre delivery by EBOV-GP pseudotyped HIV-1 particles. **f**, Inhibition of Cre delivery and counteraction by Nef on HIV-1 from HEK293T expressing SERINC5. Mean ± s.d., $n = 4$, unpaired two-tailed t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar, 100 μm.



Extended Data Figure 4 | SERINC3 and SERINC5 expression is not induced by interferon nor LPS treatments. **a**–**d**, Relative gene expression levels of *SERINC3*, *SERINC5* and *CXCL10* in response to treatment with IFN-β and LPS in Jurkat (**a**), monocyte-derived dendritic cells from two donors (MDDC, **b**), $CD4^+$ primary T cells unstimulated (**c**) or stimulated with PHA (**d**) from two donors. Expression of the housekeeping gene *OAZ1* was used as a normalization control. Mean ± s.d., $n = 3$.

Extended Data Table 1 | Description of the cells lines used in Fig. 1a

Cell Line	Cell Type	Source
Jurkat E6.1	T Lymphocyte, Acute T Cell Leukemia	ATCC
Jurkat TAg	T Lymphocyte, Acute T Cell Leukemia. Derivative of Jurkat E6.1, contains Sv40 LargeT antigen	Heinrich Gottlinger, DFCI, Harvard University
bl41	B-Lymphocyte, Burkitt's lymphoma	Paul Farell, Imperial College London
Ramos	B-Lymphocyte, Burkitt's lymphoma	ATCC
CEM-CCRF	T-Lymphocyte, Acute Lymphoblastic Leukemia	ATCC
CEM/A3.01	T-Lymphocyte, Acute Lymphoblastic Leukemia Derivative of CEM-CCRF	NIH AIDS Reagent Program
CEMSS	T-Lymphocyte, Acute Lymphoblastic Leukemia.	NIH AIDS Reagent Program
HSB-2	T- Lymphocyte, Acute Lymphoblastic Leukemia, CD4-	NIH AIDS Reagent Program
H9	T-Lymphocyte, human cutaneous T cell lymphoma. Derivative of HUT-78	NIH AIDS Reagent Program
DG7 HAD	B-Lymphocyte, Burkitt's lymphoma	Sidney Grossberg, University of Wisconsin
Hela	Epithelial, cervix adenocarcinoma	ATCC
Akata	B-Lymphocyte, Burkitt's lymphoma	Paul Farell, Imperial College London
SupT1	T-Lymphocyte, T-Cell Lymphoblastic Lymphoma	NIH AIDS Reagent Program
A549	Epithelial, lung carcinoma	ATCC
HepG2	Hepatocellular Carcinoma	ATCC
HCT116	Epithelial, colorectal carcinoma	ATCC
MCF7	Epithelial, adenocarcinoma	ATCC
HUT78	T-Lymphocyte, human cutaneous T cell lymphoma	NIH AIDS Reagent Program
293T	Epithelial, embryonic Kidney	ECACC
MT2	T Lymphocyte, T-cell leukemia	NIH AIDS Reagent Program
TE671	Rhabdomyosarcoma	Yasuhiro Takeuchi, UCL, London
Huh-7	Hepatocellular Carcinoma	Michel Strubin, University of Geneva
LL24	Lung Fibroblast	ATCC
RAJI	B-Lymphocyte, Burkitt's lymphoma	ATCC
C8166	T lymphocytes, T cell leukaemia	NIH AIDS Reagent Program
WI38	Lung fibroblast	ATCC
Daudi	B-Lymphocyte, Burkitt's lymphoma	Paul Farell, Imperial College London
MT4	T lymphocytes, T cell leukaemia	NIH AIDS Reagent Program
IMR-90	Lung fibroblast	ATCC
CEMX174	Lymphocytes, Fusion between a B cell line and a human T cell line	NIH AIDS Reagent Program
HT1080	Epithelial, fibrosarcoma	Yasuhiro Takeuchi, UCL, London