JOURNAL OF GENERAL VIROLOGY

RESEARCH ARTICLE

de Sousa-Pereira *et al.*, *Journal of General Virology* 2019;100:278–288

DOI 10.1099/jgv.0.001201

MICROBIOLOGY SOCIETY

The antiviral activity of rodent and lagomorph SERINC3 and SERINC5 is counteracted by known viral antagonists

Patrícia de Sousa-Pereira,[1,2,3,4,5] Joana Abrantes,[1] Stefan Bauernfried,[6] Virginia Pierini,[7] Pedro José Esteves,[1,2,8] Oliver T. Keppler,[3,4,5] Massimo Pizzato,[9] Veit Hornung,[6] Oliver T. Fackler[7] and Hanna-Mari Baldauf[3,4,5,*]

Abstract

A first step towards the development of a human immunodeficiency virus (HIV) animal model has been the identification and surmounting of species-specific barriers encountered by HIV along its replication cycle in cells from small animals. Serine incorporator proteins 3 (SERINC3) and 5 (SERINC5) were recently identified as restriction factors that reduce HIV-1 infectivity. Here, we compared the antiviral activity of SERINC3 and SERINC5 among mice, rats and rabbits, and their susceptibility to viral counteraction to their human counterparts. In the absence of viral antagonists, rodent and lagomorph SERINC3 and SERINC5 displayed anti-HIV activity in a similar range to human controls. Vesicular stomatitis virus G protein (VSV-G) pseudotyped virions were considerably less sensitive to restriction by all SERINC3/5 orthologs. Interestingly, HIV-1 Nef, murine leukemia virus (MLV) GlycoGag and equine infectious anemia virus (EIAV) S2 counteracted the antiviral activity of all SERINC3/5 orthologs with similar efficiency. Our results demonstrate that the antiviral activity of SERINC3/5 proteins is conserved in rodents and rabbits, and can be overcome by all three previously reported viral antagonists.

INTRODUCTION

The generation of immunocompetent human immunodeficiency virus (HIV)-permissive animal models has been hampered by the fact that HIV encounters replication barriers in rodents and lagomorphs, most of which remain uncharacterized or have not been overcome [1–5]. These barriers are, in some instances, due to missing or incompatible cellular co-factors. This is especially the case for CD4, CCR5 and CXCR4. Here, HIV entry is supported only by the human orthologs [2, 6, 7]. In addition, a single species-specific amino acid change or variant (C261Y) in rodent CyclinT1 abrogates Tat-mediated transcription elongation [8, 9]. Sherer *et al.* identified motifs in human CRM1, which are unique to monkeys and humans and important for Rev-regulated nuclear export of unspliced and singly spliced viral mRNA [10]. The expression of human CyclinT1 and/or human CRM1 enhanced HIV gene expression in T cells and macrophages from transgenic rats [4, 11], yet further undefined limitations exist in the late phase of HIV replication in T cells from hCD4/hCCR5/hCyclin T1 transgenic rats [4].

On the other hand, HIV encounters barriers that are due to the presence of intrinsic innate immunity factors, so-called restriction factors. HIV has evolved accessory proteins that counteract these factors to facilitate successful replication in human cells. HIV reverse transcription is affected by lagomorph TRIM5 proteins whose restriction can be evaded by exchanging the first 149 amino acids of the HIV capsid by the simian immunodeficiency virus (SIV) capsid [5, 12, 13]. Human SAMHD1 restricts HIV replication at the level of reverse transcription by reducing intracellular dNTP pools and is degraded by lentiviral Vpx proteins [14, 15], yet the antiretroviral activity of murine SAMHD1 cannot be antagonized by Vpx [16]. Human CD317/BST-2/Tetherin tethers mature virions at the plasma membrane, and its antiviral

Received 26 September 2018; Accepted 26 November 2018; Published 19 December 2018

Author affiliations: [1]CIBIO/InBIO- Research Network in Biodiversity and Evolutionary Biology, Campus de Vairão, University of Porto, Vairão, Portugal; [2]Department of Biology, Faculty of Sciences, University of Porto, Porto, Portugal; [3]Institute of Medical Virology, University Hospital Frankfurt, Frankfurt, Germany; [4]Max von Pettenkofer Institute & Gene Center, Virology, National Reference Center for Retroviruses, Faculty of Medicine, LMU München, Munich, Germany; [5]Institute of Virology, Technische Universität München/Helmholtz Zentrum, Munich, Germany; [6]Gene Center and Department of Biochemistry, LMU München, Munich, Germany; [7]Department of Infectious Diseases, Integrative Virology, University Hospital Heidelberg, Heidelberg, Germany; [8]CITS - Centro de Investigação em Tecnologias de Saúde, CESPU, Gandra, Portugal; [9]University of Trento, Centre for Integrative Biology, Trento, Italy.

***Correspondence:** Hanna-Mari Baldauf, baldauf@mvp.lmu.de

Keywords: HIV-1; SERINC; restriction factor; species-specific antagonism.

Abbreviations: SERINC, serine incorporator proteins; VSV-G, vesicular stomatitis virus G protein; Nef, negative factor protein; MLV, murineleukemia virus; EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; dNTPs, deoxyribonucleotide triphosphates; RT, reverse transcriptase; SG-PERT, SYBR Green I-based PCR-enhanced reverse transcriptaseassay; RLU, relative light units.

Four supplemantary figures are available with the online version of this article.

activity is counteracted by HIV-1 Vpu/HIV-2 Env [17, 18]. However, rodent CD317 proteins are resistant to all currently known antagonists [19, 20] and MLV appears to be devoid of a murine CD317 antagonist [21]. Members of the human APOBEC cytidine deaminase family are incorporated into budding virions and trigger G-to-A hypermutations during HIV reverse transcription in the next round of infection. Virion inclusion is prevented by lentiviral Vif proteins. However, rat APOBEC1, mouse APOBEC3 and rabbit APOBEC1 are still incorporated in the presence of Vif [22–24]. Thus, species-specific comparisons may not only add valuable information to the mode of action and interacting motifs, but also identify factors that are critical for building HIV-permissive small animal models.

Here, we are interested in a species-specific comparison of rodent and lagomorph orthologs of SERINC3/5, two newly described antiviral factors. The antiviral phenotype of hSERINC3/5 resembles in part the infectivity defect recently reported in primary rabbit macrophages, which manifests itself by 20-fold decreased HIV infectivity compared to human macrophages [5].

Human SERINC3 and SERINC5 were recently found to reduce virion infectivity [25, 26]. They have been proposed to belong to a family of serine incorporators [27], yet they are not involved in lipid biosynthesis [28]. SERINC3/5 are highly conserved in terms of amino acid sequences among eukaryotes, and primate SERINC3/5 show no signatures of positive selection or difference in their antiviral activity [29, 30]. Their exact mode of action is still under debate, yet fusion to the next target cell is impaired. Recently, Sood and colleagues demonstrated that virion-associated hSERINC3/5 interfere with HIV entry prior to small pore formation with the new target cell [31]. Furthermore, hSERINC5 prevents the delivery of the viral core into the cytoplasm [32]. These steps are critical within the HIV entry process. First, HIV envelope molecules attach to the cell membrane and bind to its major receptor hCD4 and subsequently to its co-receptors hCXCR4 or hCCR5. Second, co-receptor binding induces exposure and insertion of the gp41 fusion peptide into the host cell membrane. Small pore formation is finally induced by gp41 pre-bundles, which culminate in the formation of six-helix bundles (reviewed in [33]). Those six-helix bundles most likely stabilize and expand the opening pores (reviewed in [33]).

Importantly, viruses have developed strategies to evade the inhibitory effect of restriction factors. HIV/SIV Nef proteins counteract human, primate, mouse, frog and zebrafish SERINC3/5 and increase virus particle infectivity [25, 26, 29, 34–36]. MLV GlycoGag increases virion infectivity of Nef-deficient HIV and GlycoGag-deficient xenotropic MLV particles in the presence of human and mouse SERINC3/5 [25, 26, 29, 37]. EIAV S2 proteins antagonize human and equine SERINC3/5 and rescue GlycoGag-deficient xenotropic MLV [37, 38].

The way in which this antagonism works remains unclear, but it was suggested that both virion exclusion of cellular SERINC5 and inactivation of virion-associated SERINC5 is driven by HIV-1 Nef [39, 40]. An interplay of GlycoGag and Envelope seems to be important in overcoming SERINC5 antiviral activity against MLV [37]. In addition, equine SERINC5 also inhibits EIAV in an Env-dependent manner and EIAV S2 uses similar motives to those of HIV Nef in regard to counteraction [38]. Recent evidence also points to HIV envelopes as determinants for SERINC5 restriction [31, 40–42].

In our current work, we find that SERINC3/5 antiviral activity is highly conserved also among rat and rabbit orthologs. Our results indicate that rodent and lagomorph SERINC3/5 most likely act prior to virion fusion. Interestingly, we are the first to show that rodent and lagomorph SERINC3/5 are counteracted by all three antagonists, HIV-1 Nef, MLV GlycoGag and EIAV S2 proteins, and thus may not cause any problems in regard to animal model development.

METHODS

Evolutionary analysis

The sequences obtained from NCBI were aligned using ClustalW [43] on BioEdit [44].

Cells and reagents

Human HEK293T and TZM-bl cells were cultivated as recently reported [45]. Generation of HEK293T SERINC3/5 double-KO cells was, in principle, performed as recently described [46, 47]. The following guideRNAs were used for *SERINC3* (5'GTTTGTTGTTGGCATGATAGGGG3') and for *SERINC5* (5'GTAGAGGGCGTACATGAAGCGGG 3'). Briefly, HEK293T cells were transfected with pRZ_BFP_T2A_Cas9 together with GFP-expressing pLKO.1_gRNA plasmids using Lipofectamine 2000. Two days later, BFP and GFP double-positive cells were FACS-sorted and subcloned by limiting dilution. The genomic locus surrounding the gRNA binding site was PCR-amplified (*SERINC3* forward primer: 5′ ACACTCTTTCCCTACACGACGctcttccgatctCCTACCAGCATACCACAACC TTG 3′; *SERINC3* reverse primer: 5′ TGACTGGAG TTCAGCGTGTGctcttccgatctGTACTGCTGGAGACAGAA TGTCT 3′; *SERINC5* forward primer: 5′ ACACTCTTTCCC TACACGACGctcttccgatctTTTTCTAGCTGGCCTGCTGC TGT; *SERINC5* reverse primer: 5′ TGACTGGAG TTCAGCGTGTGctcttccgatctTCATGTTTTTCCCACA-CACCCTC 3′) and subjected to deep sequencing using a MiSeq platform (Illumina) as previously described [47]. Polyethylenimine 25K was purchased from Plysciences, Inc.

HIV-1 proviral constructs and expression plasmids

Proviral DNAs pHIV-1_{NL4-3}ΔNef and pHIV-1_{NL4-3}SF2Nef [48], as well as human pcDNA3.1- and pBJ6-based expression plasmids for HA-tagged hSERINC5 and hSERINC3, were recently described [25]. We generated pNL4-3/Envfs/Nef_{LAI} by replacing the nucleotide sequence of pNL4-3/

EnvFs [49], between the *BsmBI* and *BspEI* restriction sites (the latter located in the 3' LTR), with the homologous sequence derived from *nef* of HIV-1_{LAI}. The corresponding pNL4-3/Envfs/Neffs carries a mutation at the unique *XhoI* site in *nef*. For infectivity experiments, pNL4-3/Envfs/Nef_{LAI} and pNL4-3/Envfs/Neffs were complemented by the recently described HXB2 Env (pBJ5) [25]. The coding sequences (CDS) for rabbit, rat and mouse SERINC5 and SERINC3 were amplified from spleen cDNAs using the Phusion Flash High-Fidelity PCR Master Mix according to the manufacturer's protocol (Thermo Fisher Scientific). The PCR products were cloned in the pCMV-HA-C plasmid (Clonetech) using the restriction sites *EcoRI* and *KpnI* or *EcoRI* and *SaII* for SERINC5 and SERINC3, respectively. SERINC5 and SERINC3 orthologs with the HA-tag at the C-terminus were subsequently cloned in the pBJ6 plasmid using *EcoRI* and *NotI* restriction sites. All constructs generated were validated by sequencing.

Expression plasmids for HXB2 Env (pBJ5-based), HA-tagged GlycoGag (pBJ5-based) and HA-tagged S2 proteins (pcDNA3.1-based) were described previously [25, 38, 50]. For pseudotyping and virion fusion assays, VSV-G (pMD2.G) and BlaM-Vpr (pCMV4-based) expression plasmids were used [45].

Virus production

For viral production, HEK293T cells were co-transfected with 1.5 µg proviral DNA and 0.5 µg SERINC3/5 expression plasmids using PEI (3 : 1 of total DNA). For S2 and GlycoGag co-expression, 0.5 or 0.8 µg of plasmid DNA was added, respectively. For experiments with LAINef (Nef derived from HIV_{LAI} strain [51]), 0.2 µg of HXB2 Env were co-transfected. The supernatant was collected 48 h after transfection and passed through 0.45 µm filters. The virus-containing supernatant was stored for further usage at −80 °C. Only supernatants used for the fusion assay were first purified through a sucrose cushion as previously described [45].

The number of particles in the supernatant was measured by quantification of virion-associated reverse transcriptase (RT) activity (SG-PERT) [52]. The level of physical particles in the supernatant and associated with the producer cells was determined by p24CA ELISA [53].

HIV-1 infectivity assay

TZM-bl cells were infected with the same volume of HIV-containing supernatant produced in the presence or absence of SERINCs. Forty-eight hours after infection, cells were lysed with cell culture lysis reagent (Promega) and Firefly luciferase activity was measured with a TECAN infinite 200, using the luciferase assay system from Promega according to the manufacturer's instructions. Relative infectivity per unit RT activity was calculated by normalization of relative light units (RLU) and RT units measured by SG-PERT for each virus to HIV-$1_{NL4\text{-}3}$ΔNef control.

Viral fusion assay

TZM-bl cells were infected with the same RT units of HIV produced in the presence or absence of SERINC3/5. The total of RT units (10^{11} RT units per 100 000 cells) used was chosen to ensure that fusion would be detected by flow cytometry for every virus produced in this assay. Four hours after infection, cells were washed with CO_2-independent medium complemented with 10 % FCS and loaded with CCF2 dye as described previously [45]. After overnight staining, the cells were detached, fixed with 4 % PFA and analyzed by flow cytometry using a BD FACSVERSE.

Flow cytometry

HEK293T cells used for virus production in the presence or absence of SERINCs were detached and fixed with 4 % PFA. The cells were permeabilized using Perm Buffer II (BD Biosciences) according to the manufacturer's instructions and stained using 1 : 40 HA-FITC (Miltenyi Biotec). The samples were analyzed by flow cytometry using a BD FACSVERSE (BD Biosciences).

Western blot analysis

HEK293T cells used for virus production in the presence or absence of SERINC3/5 were detached and lysed for 5 min at 4 °C (100 mM NaCl, 10 mM HEPES, 1 mM TCEP, 1 % DDM and 2× protease inhibitors). After centrifugation, the supernatant was mixed with 2× Laemmli sample buffer with 50 mM TCEP. Samples were resolved using a Tricine-SDS-PAGE system and blotted on nitrocellulose membranes. SERINC expression was detected using mouse anti-HA (1 : 2000; BioLegend) and Vinculin using a rabbit polyclonal antibody (1 : 1500; Abcam).

RESULTS

Rodent and rabbit SERINC3/5 orthologs are highly conserved

To assess the breadth of amino acid sequence conservation, we aligned mouse, rat, rabbit and human SERINC3/5 orthologs using ClustalW [43]. SERINC3 (Fig. 1a) and SERINC5 (Fig. 1b) orthologs are highly conserved, sharing 78–93 and 81–91% amino acid identity, respectively. In addition, putative transmembrane domains, highlighted in grey, are well preserved.

Rodent and rabbit SERINC3/5 orthologs interfere with HIV infectivity in a dose-dependent manner

All experiments were performed as shown in Fig. 2a. HEK293T cells were co-transfected with proviral DNA, together with expression plasmids encoding for mouse, rat, rabbit or human SERINC3/5 or a vector control. Two days post transfection, supernatants were filtered and quantified for RT activity and p24 capsid (CA) content of released virions. Infectivity was then quantified in a TZM-bl reporter cell assay.

Based on the high level of amino acid conservation among SERINC3/5 orthologs, we asked whether these would also

Fig. 1. SERINC3 and SERINC5 are highly conserved. (a, b) Translation of the nucleotide sequences for *Homo sapiens* (NM_006811 and NM_001174072), *Oryctolagus cuniculus* (XM_002721072 and XM_008261873), *Rattus norvegicus* (NM_001008312 and NM_133395) and *Mus musculus* (NM_012032 and NM_172588) SERINC3 (a) and SERINC5 (b). Highlighted in grey are the transmembrane domains predicted using the web tool PredictProtein [65] and in accordance with [27]. The default setting of BioEdit colours amino acids sharing similar properties as follows: turquoise (A, Y, F), ochre (G), dark brown (P), dark blue (S, T), red (D, E), light green (W), pink (H), navy blue (K, R), dark green (I, L, M, V), light blue (N, Q), dark red (C). '-' or '~' indicate gaps, '.' denotes identical amino acids as in the reference sequence, and '*' denotes stop codons.

be capable of reducing HIVΔNef infectivity as recently published for human SERINC3/5 and simian and mouse SERINC5 [25, 26, 29, 34, 35]. HEK293T cells were thus transfected with increasing levels of pCMV-based SERINC3/5 expression plasmids, and analyses were performed as described above. Luciferase counts were normalized to the level of RT Units measured in the supernatant to quantify particle infectivity. Nef-defective HIV-1 was inhibited by SERINC3/5 orthologs in a dose-dependent manner (Fig. 2b). The reduction imposed by SERINC5 ranged between 14- and 232-fold, whereas SERINC3 was less potent (1.4- to 4.9-fold). SERINC5 reduced virion infectivity to a maximum of 0.43–0.85 % and SERINC3 only to 20.32–39.67 %. The protein abundance of SERINC3/5 was determined in parallel by Western blotting (Fig. 2b) and flow cytometry (Fig. S1, available in the online version of this article). For both analyses and all orthologs, increasing levels of HA-tagged SERINC3/5 were detected with increasing levels of plasmid DNA. Nevertheless, as seen in Fig. 2b, HA-tagged SERINC3 orthologs showed only one or two

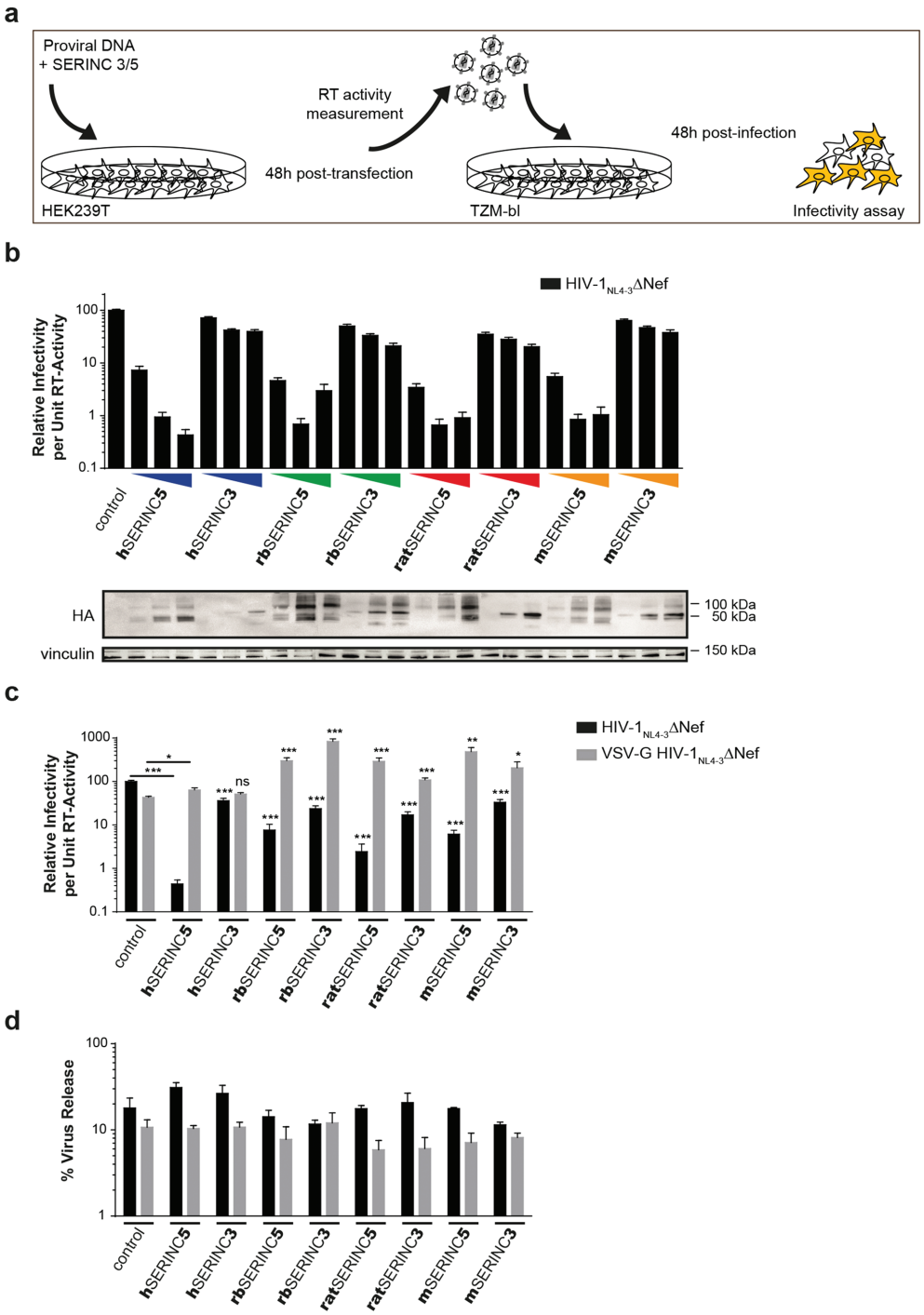

Fig. 2. Rodent and rabbit SERINC3/5 orthologs interfere with HIV infectivity in a dose-dependent manner. (a) HEK293T cells were transfected with proviral HIV-1_{NL4-3} plasmid DNA lacking Nef (HIV-1_{NL4-3}ΔNef) and increasing amounts of expression plasmids encoding for the various SERINC3/5 orthologs (0.1–1 µg). Supernatants were collected 48 h post-transfection and analyzed for RT activity of released viral particles using SG-PERT. In parallel, TZM-bl cells were inoculated with harvested supernatants and Firefly luciferase activity measured 48 h post inoculation. (b) Relative HIV-1 infectivity was calculated as a ratio of Firefly luciferase counts to RT units and normalized to control (empty expression plasmid). Shown are arithmetic means±SEM of three independent experiments. (c) HEK293T cells were transfected with proviral HIV-1_{NL4-3}ΔNef plasmid DNA and expression plasmids for the various SERINC3/5 orthologs (0.5 µg) in the presence or absence of a VSV-G expression plasmid. Supernatants were essentially harvested as described above and relative infectivities calculated. Shown are arithmetic means±SEM of three independent experiments. (d) HIV-1 release was quantified as the percentage of total (cells plus supernatant) p24 capsid (p24CA) secreted as virion-associated p24CA. Shown are arithmetic

means±SD of two experiments. No significant differences were found between control and SERINC-containing samples. Statistical analyses were performed using unpaired two-tailed Student's *t*-test. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

prominent bands at the expected size of ~53 kDA. The expression pattern for SERINC5, in contrast, is probably due either to different levels of glycosylation [54] or translation initiation at internal start codons (AUGs) in an overexpression context [55, 56]. Even though SERINC3 expression levels are lower than those for SERINC5 its antiviral activity is still weaker, ranging from 5- to 55-fold when normalizing for comparable expression levels of SERINC3 and SERINC5 from the same species (Fig. 2b). This finding is also in line with the current literature [25, 26].

In concordance with previous reports, the impact of SERINC3/5 on HIV-1ΔNef infectivity was abolished when virions were pseudotyped with VSV-G (Fig. 2c, grey bars). Strikingly, VSV-G pseudotyped virions were 2.4- to 19-fold more infectious when rabbit, rat or mouse SERINC3/5 were co-transfected. In order to rule out the possibility that SERINC3/5 expression affects HIV-1 particle release, we measured in parallel virus- and cell-associated p24CA and calculated the percentages of released particles. As seen in Fig. 2(d), SERINC3/5 expression did not have a negative impact on HIV-1ΔNef release and no significant differences were found between control and SERINC-containing particles.

Thus, rodent and rabbit SERINC3/5 proteins reduce HIV-1ΔNef infectivity in a dose-dependent manner.

Rodent and rabbit SERINC3/5 orthologs interfere with HIV prior to virus entry

To determine the step in the HIV replication cycle where SERINC3/5 orthologs act as restriction factors, we assessed the fusion capacity of HIV-1ΔNef virions produced in the presence or absence of individual SERINC proteins, by performing a BlaM-Vpr fusion assay as established by Cavrois *et al.* [57]. Identical RT Units were applied to TZM-bl cells, and virion fusion was measured by flow cytometry [45]. Fig. 3(a) depicts representative dot plots of fusion events in TZM-bl cells. Uninfected and fusion inhibitor T20 control-treated, infected TZM-bl cells did not display infection levels above background. Fusion of particles produced in the presence of SERINC5 and SERINC3 in control viruses was reduced from 78 % to 0.2–3.6 % and 6.4–27 %, respectively (Fig. 3a).

Over a range of multiple experiments, virus entry was significantly reduced (from 3- to 257-fold) when HIV-1ΔNef virions were produced in the presence of SERINC3/5 orthologs (Fig. 3b). This major effect of SERINC3/5 on virion fusion is in part due to marked overexpression of SERINC3/5 using transient transfection of a CMV IE promoter construct in virion producer cells, in contrast to the studies performed by Rosa *et al.* and Usami *et al.*, which employed weaker promoters [25, 26].

We then performed similar experiments with VSV-G pseudotyped HIV-1ΔNef BlaM-Vpr-incorporated virions. Here, primary dot plots (Fig. S2a) and a bar chart (Fig. S2b) revealed a less pronounced, but significant, decrease in virion fusion (Fig. S2a,b) and infectivity (Fig. S2c) for virions with incorporated rodent SERINC3/5 and human SERINC5.

Summarizing our findings, SERINC3/5 orthologs most probably exert antiviral activity prior to virus entry, primarily in an HIV envelope-dependent manner.

Rodent and rabbit SERINC3/5 orthologs are counteracted by three different viral proteins

To assess whether SERINC3/5 orthologs can be antagonized by all known viral counteractors of SERINC proteins, HIV-1ΔNef and SF2Nef virions were produced in the presence or absence of SERINC expressed from pBJ6-based plasmids. Because SERINC5 has a relatively strong antiviral activity, SF2Nef was chosen as being among the strongest SERINC5 antagonists of HIV-1 [25]. The expression of pBJ6-driven plasmids is much weaker than that of CMV-driven ones [25, 39], which further facilitates Nef antagonism studies. For this reason, SERINC3/5 protein levels could not be detected by Western blotting yet their expression was verified by measuring HA-tag levels using flow cytometry (Fig. S3). First, we titrated the amount of pBJ6-driven SERINC3/5 required to obtain the optimal dose showing maximal inhibition of HIV-1ΔNef and best rescues by SF2Nef virions (data not shown). We then repeated the experiment with HIV-1ΔNef using a HEK293T SERINC3/5 double-knockout clone and obtained similar results, indicating that low endogenous SERINC3 and SERINC5 expression levels in HEK239T cells had not altered our results (Fig. S4).

As expected, and in line with the literature, the impact of pBJ6-driven SERINC3/5 on virion infectivity was less pronounced than with CMV-driven expression plasmids. Nevertheless, individual SERINC5 proteins reduced the infectivity of HIV-1ΔNef virions by 2.8- to 9.2-fold whereas SERINC3 had only a marginal effect (1.3- to 2.9-fold) effect (Fig. 4a). As the effects of SERINC3 orthologs were found to be rather modest, we investigated SERINC3 incorporation into HIV-1 particles by Western blotting: pBJ6-driven SERINC3 expression levels were undetectable, even in cell lysates (data not shown).

In the presence of SF2Nef, the antiviral activity of SERINC3/5 was antagonized. Specifically, SF2Nef significantly increased the virion infectivity of SERINC5-containing particles from 2.6- to 9.2-fold. This effect was less pronounced for SERINC3-containing particles (1.1- to 1.9-fold), and no significant effect was detected for murine SERINC3.

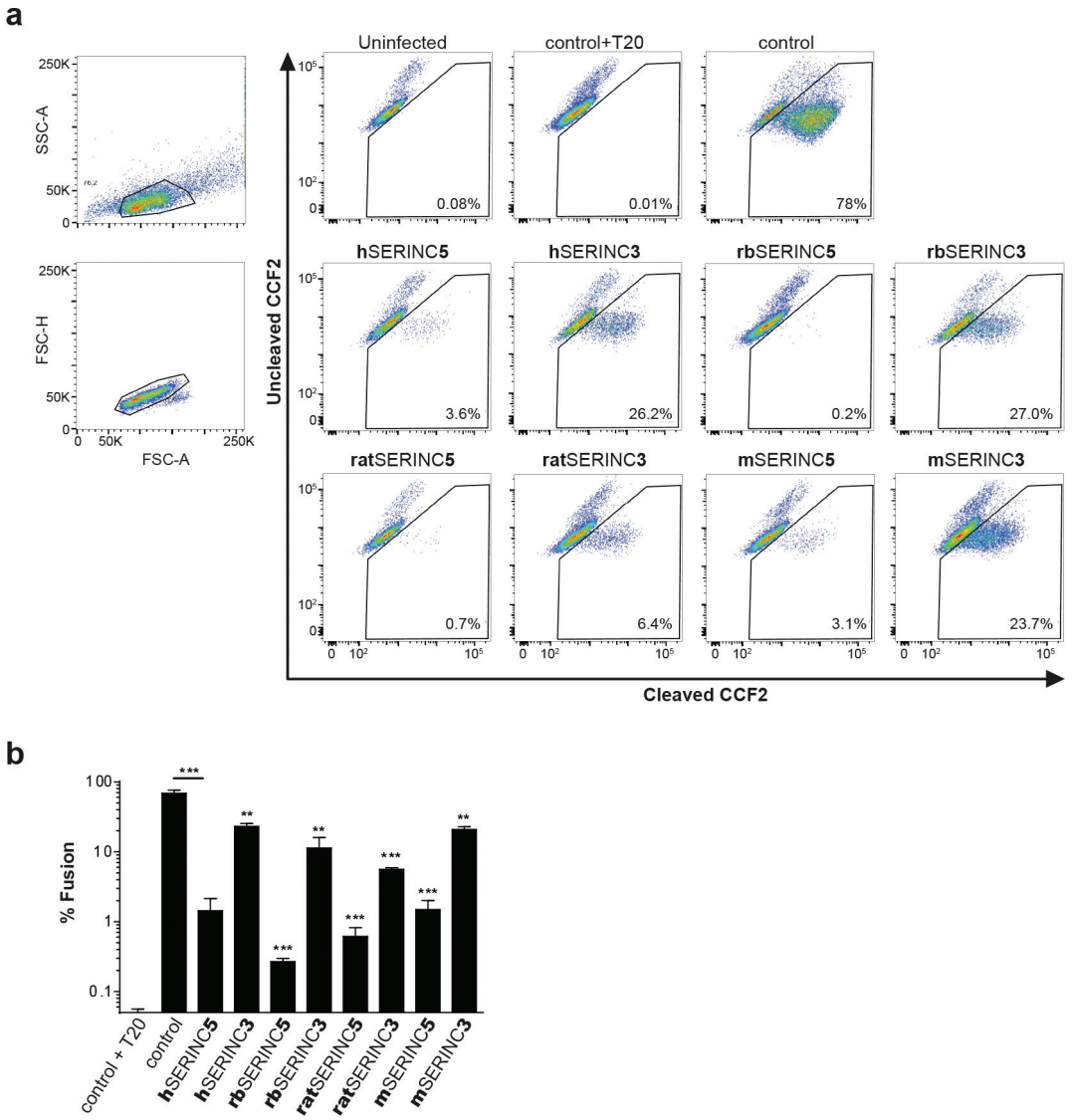

Fig. 3. Rodent and rabbit SERINC3/5 orthologs interfere with HIV prior to virus entry. (a) HEK293T cells were transfected with proviral HIV-1_{NL4-3}ΔNef plasmid DNA, a BlaM-Vpr expression plasmid, together with expression plasmids encoding for the SERINC3/5 orthologs (0.5 µg). Viral particles were harvested 48 h post transfection and pelleted via sucrose cushion. Equal numbers of RT units (determined by SG-PERT) were used to infect TZM-bl cells. Fusion was analyzed 4 h post infection via flow cytometry, measuring the change in fluorescence caused by cleavage of CCF2 following cellular entry. T20 was used as fusion inhibitor. Shown are representative dot blots. (b) Graphical representation summarizing the raw data presented in (a). Shown are arithmetic means±SEM of two independent experiments. Statistical analyses were performed using unpaired two-tailed Student's *t*-test. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

Because SF2Nef is virtually inactive in regard to inhibiting SERINC3 incorporation into virions [26], we repeated the foregoing experiments in the presence or absence of LAI-Nef, which was previously shown to antagonize SERINC3 incorporation [26]. Here, SERINC5 and SERINC3 reduced the virion infectivity of HIV-1ΔNef by 6.0- to 28.8-fold and 2.7- to 7.1-fold, respectively (Fig. 4b). In contrast to SF2Nef, LAINef counteracted human SERINC5 by only twofold, as expected [26]. LAINef-mediated antagonism to rabbit SERINC5 was within a similar range (2.8-fold). Unexpectedly, rat and mouse SERINC5 were more sensitive to LAINef-mediated antagonism, as virion infectivity increased by 13.8- and 18.1-fold, respectively. LAINef increased virion infectivity in the presence of SERINC3 orthologs by 2.8- to 3.2-fold. However, considering the fold difference for HIV-1 LAINef in the presence or absence of SERINC3/5, that for SERINC3 orthologs was lower (1.2- to 3.7-fold), in contrast to SERINC5 (1.7- to 5.3-fold). In addition, rabbit and mouse SERINC3-containing particles were equally infectious as control HIV-1 LAINef virions.

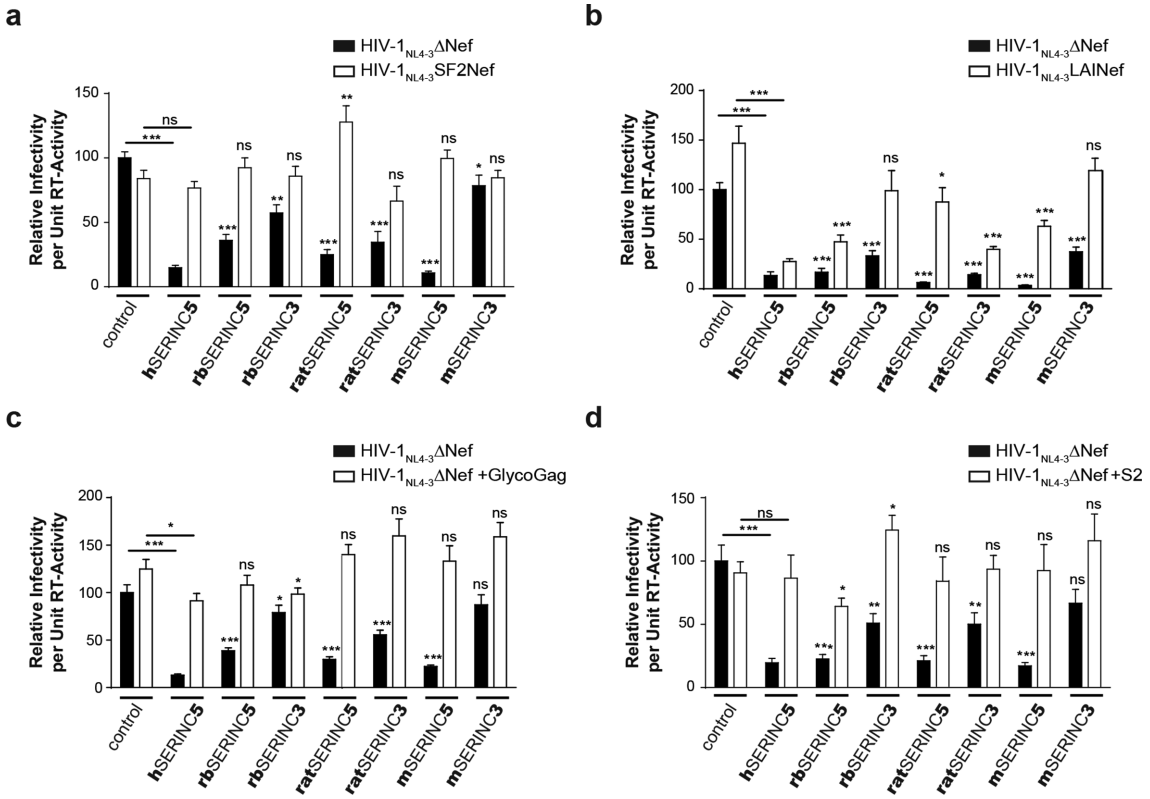

Fig. 4. Rodent and rabbit SERINC3/5 orthologs are counteracted by three different viral proteins. (a) HEK293T cells were transfected with plasmids encoding for SERINCs (0.5 µg) and proviral HIV-1_{NL4-3}ΔNef or HIV-1_{NL4-3}SF2 Nef plasmid DNA. (b) Proviral NL4-3/Envfs/Nef_{LAI} and NL4-3/Envfs/Neffs complemented with the HXB2 Env expression plasmid. (c) Proviral HIV-1_{NL4-3}ΔNef plasmid DNA in the presence or absence of a murine leukemia virus (MLV) GlycoGag expression plasmid. (d) Proviral HIV-1_{NL4-3}ΔNef plasmid DNA in the presence or absence of an equine infectious anemia virus (EIAV) S2 expression plasmid. Relative infectivity values were calculated as described previously. Shown are arithmetic means±SEM of three independent experiments. Statistical analyses were performed using unpaired two-tailed Student's *t*-test. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

In addition to HIV-1 Nef, MLV GlycoGag was shown to antagonize SERINC5 [25, 26]. Thus, we produced HIV-1ΔNef virions in the presence or absence of pBJ6-based SERINC3/5 and MLV GlycoGag. Regardless of which SERINC3/5 orthologs were used during virus production, antiviral activity was antagonized by GlycoGag (Fig. 4c). Here, GlycoGag significantly increased the virion infectivity of SERINC5-containing particles, by 2.8- to 7-fold. As already seen for SF2Nef, infectivity enhancement regarding SERINC3-containing particles was less pronounced (1.2- to 2.9-fold), without being significant for rabbit SERINC3.

In a final approach, we produced HIV-1ΔNef virions in the presence or absence of pBJ6-based SERINC3/5 and EIAV S2. Here, we also detected no antiviral activity for SERINC3/5 when S2 was present during virion production (Fig. 4d). More importantly, EIAV S2 significantly increased the virion infectivity of SERINC5-containing particles, by 2.8- to 5.4-fold, and the infectivity of SERINC3-containing particles was enhanced by only 1.7- to 2.4-fold in the presence of EIAV S2, without being significant for murine SERINC3.

In summary, the antiviral activity of rodent and rabbit SERINC3/5 orthologs is counteracted by all known antagonists – HIV-1 Nef, MLV GlycoGag and EIAV S2.

DISCUSSION

Typical animal models used to study human diseases include rodents and lagomorphs [58–60], although rodent and lagomorph cells do not support HIV replication. To create permissive small animal models, it is important to study the antiviral activity of HIV restriction factor orthologs and their counteraction. Here, we studied the ectopic expression of rodent and lagomorph orthologs of SERINC3 and SERINC5 in permissive human cell lines, to delineate whether SERINC3/5 pose a barrier to HIV animal model development. Studies in SERINC3/5 knockout rodent and lagomorph cell lines are unfortunately not possible due to additional restrictions regarding HIV replication. Human, rodent and lagomorph SERINC3 and SERINC5 orthologs are conserved in regard to their amino acid sequences. Analyses with simian orthologs showed that these genes do not exert the typical signatures of 'an arms race' with pathogens

[30]. Normally, as a consequence of 'an arms race' between host and virus and through continuous evolution, mutations manifest themselves within the host and/or the virus as a result of evolutionary pressure. This was recently shown for human restriction factors including APOBEC3G and BST-2, in contrast to human SERINC3 and SERINC5 [30]. Rodent and rabbit SERINC3 and SERINC5 orthologs have not encountered HIV, yet other retro- or lentiviruses, such as MLV or rabbit endogenous lentivirus K (RELIK) [61], may have imposed pressure on those genes.

We observed that rodent and lagomorph SERINC3 and SERINC5 are as active antivirally as human and simian orthologs in the absence of viral antagonists [25, 26, 29]. Both act in a dose-dependent manner, whereas the magnitude of inhibition is less pronounced for SERINC3 orthologs. The antiviral activity of SERINC3/5 orthologs was bypassed when virions were pseudotyped with VSV-G [25, 26]. The reason for this finding may be either the different entry route used by HIV and VSV or the number of glycoprotein molecules displayed on the cell surface due to which SERINC can no longer exert its antiviral activity [62, 63].

In the absence of a viral antagonist, SERINC3/5's antiviral activity is most likely displayed prior to virus entry, which is consistent with other reports [25, 26, 39]. Because we measured the change in fluorescence emission following the release of BlaM-Vpr proteins from incoming virions into the cytoplasm, virion fusion assay cannot discriminate whether virion attachment to, or virion fusion with, the plasma membrane is primarily affected. Our data do not indicate the additional post-entry defect induced by SERINC3/5 proposed by Rosa *et al.* [25], suggesting that this may depend on the experimental system regarding whether the antiviral activity of SERINC3/5 is evident prior to virus entry only, or also afterwards. Moreover, a second post-entry restriction imposed by SERINC3/5 would not be in line with the model proposed by Sood *et al.* and Schulte *et al.* [31, 32]. In addition, endogenous SERINC3/5 may have only a minor effect on virus entry yet a greater impact post-entry, as recently suggested [25, 26], which we cannot exclude based on our experimental set-up.

Studying the cross-reactivity of restriction factors and their viral counteracting elements is important in the process of developing permissive small animal models for HIV. This is the first study to analyze the antiviral activity of all known viral antagonists using a species comparison approach. To date, counteraction activities have been demonstrated mainly for human SERINC3/5, and only two viral proteins have been studied in connection with mouse SERINC5 [25, 26, 29, 34–38]. Contrary to expectation, the three known antiviral factors – HIV-1 Nef, MLV GlycoGag and EIAV S2 [25, 26, 38] – were able to counteract all SERINC3 and SERINC5 orthologs used in the present study. Normally, although HIV restriction factor orthologs are antivirally active, the block induced by these factors cannot be surmounted by the viral antagonists. Rodent CD317/BST-2/Tetherin inhibits the release of HIV-1, HIV-2, SIV and MLV, while viral antagonists including HIV-$2_{ROD\text{-}10}$ Env, Ebola GP, KSHV K5 and HIV-1 Vpu were unable to rescue infection [19, 20]. Likewise, HIV-1 Vif did not prevent the incorporation of murine APOBEC3G into budding virions [22]. Furthermore, rodent and rabbit APOBEC1 also have antiviral activity against HIV, SIV and MLV, with no counteraction by Vif proteins [24]. Thus, our results show that the domains responsible for antiviral activity are highly conserved among SERINC3/5 orthologs. In addition, the interaction domains necessary for counteraction have to be preserved to a very high degree so that these can be counteracted by viral antagonists expressed by complex lentiviruses (HIV, EIAV) and a simple gamma retrovirus (MLV). However, this does not mean that all three viral proteins target similar motives or antagonize via direct interaction. The possibility of direct interactions, or those in the same complex of SERINC3/5 orthologs and these counteracting viral proteins, could be investigated by pull-down assay in combination with mass spectrometric analyses or by FRET/BRET-based assay. In a subsequent step, if technically accessible, structural studies of SERINC3/5 orthologs, in complex with HIV-1 Nef, MLV GlycoGag or EIAV S2, could be used to identify interacting amino acids, possibly shedding light also on the inhibitory mode of action. The proteins HIV-1 Nef, MLV GlygoGag and EIAV S2 most likely evolved independently to counteract SERINC3/5 proteins. The proteins HIV-1 Nef and EIAV S2 share similar interacting motifs [25, 38] and, together with MLV GlycoGag, localize to cellular membranes. Schulte *et al.* recently identified two separate domains in SERINC5 responsible for incorporation and restriction, respectively [32]. Using SERINC2–SERINC5 chimeras, SERINC5 incorporation was mapped to loop5 (L5), transmembrane domain 6 (TM6) and loop6 (L6) [32]. Within this region, human, rodent and rabbit SERINC5 differ in only 5–10 amino acids (Fig. 1b). Furthermore, the determinants to restrict HIV were found in the TM7-L7 region [32]. Here, human, rodent and rabbit SERINC5 differ only in 3–5 amino acids (Fig. 1b). This indicates that regions important for both SERINC5 incorporation and SERINC5-mediated restriction are highly conserved among rodent and lagomorph orthologus.

Furthermore, Dai *et al.* identified the intracellular loop 4 of SERINC5, specifically amino acids 9–26, as being responsible for confering sensitivity to Nef [35]. Within this stretch, amino acid sequences among human, rodent and rabbit SERINC5 present between one to three amino acid differences (Fig. 1b), in contrast to six to seven for frog and zebrafish SERINC5 [35]. Although this may explain the high conservation of Nef antagonism, little is known about the key domains in SERINC3/5 orthologs and those essential for counteraction by the proteins MLV GlycoGag or EIAV S2. Thus, the underlying interactions need to be determined. Future experiments extending the strategies of Schulte *et al.* and Dai *et al.* could

also identify key motifs for the proteins MLV GlycoGag and EIAV S2 [32, 35]. Li *et al.* recently reported residues in MLV GlycoGag that are important for re-localization and downregulation of murine SERINC5 from the plasma membrane [64].

Interestingly, LAINef was shown to be a more potent antagonist for human SERINC3 and relatively inactive against the incorporation of human SERINC5 into virions [26]. However, our data show that the capacity of LAINef's capacity to counteract SERINC5 may be dependent on the ortholog present, as rat and mouse SERINC5 were counteracted by up to 18.1-fold (Fig. 4b). This result indicates that generalized statements about the potency of individual Nef alleles to counteract SERINC3/5 cannot be made, and must be evaluated in each experimental set-up.

In summary, rodent and lagomorph SERINC3/5 orthologs restrict HIV infectivity and can be counteracted by the proteins HIV-1 Nef, MLV GlycoGag and EIAV S2. Our key conclusion is that rodent and lagomorph SERINC3/5 orthologs, in contrast to other known restriction factors, do not pose any barrier to HIV animal model development.

Funding information
P.P. is supported by a FCT PhD grant (ref. PD/BD/52602/2014) within the Doctoral Programme Biodiversity, Genetics and Evolution (BIODIV). J.A. is supported by a FCT Investigator grant (ref. IF/01396/2013). O.T. F. acknowledges funding from the Deutsche Forschungsgemeinschaft (SPP1923). This work was supported by the programme Frankfurter Forschungsförderung (FFF) of the Faculty of Medicine of Johann Wolfgang von Goethe University, Frankfurt, and by the LMU Munich's Institutional Strategy LMUexcellent within the framework of the German Excellence Initiative.

Acknowledgements
We are grateful to Ulrike Protzer for support and access to biosafety laboratory level 3 (BSL3) at the Technische Universität Munich (TUM). We thank Christine Goffinet for critical reading of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Bieniasz PD, Cullen BR. Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. *J Virol* 2000;74:9868–9877.
2. Keppler OT, Welte FJ, Ngo TA, Chin PS, Patton KS *et al.* Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J Exp Med* 2002;195:719–736.
3. Zhang JX, Diehl GE, Littman DR. Relief of preintegration inhibition and characterization of additional blocks for HIV replication in primary mouse T cells. *PLoS One* 2008;3:e2035.
4. Michel N, Goffinet C, Ganter K, Allespach I, Kewalramani VN *et al.* Human cyclin T1 expression ameliorates a T-cell-specific transcriptional limitation for HIV in transgenic rats, but is not sufficient for a spreading infection of prototypic R5 HIV-1 strains ex vivo. *Retrovirology* 2009;6:2.
5. Tervo HM, Keppler OT. High natural permissivity of primary rabbit cells for HIV-1, with a virion infectivity defect in macrophages as the final replication barrier. *J Virol* 2010;84:12300–12314.
6. Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA *et al.* The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 1986;47:333–348.
7. Browning J, Horner JW, Pettoello-Mantovani M, Raker C, Yurasov S *et al.* Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc Natl Acad Sci USA* 1997;94:14637–14641.
8. Bieniasz PD, Grdina TA, Bogerd HP, Cullen BR. Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. *EMBO J* 1998;17:7056–7065.
9. Chen D, Fong Y, Zhou Q. Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription. *Proc Natl Acad Sci USA* 1999;96:2728–2733.
10. Sherer NM, Swanson CM, Hué S, Roberts RG, Bergeron JR *et al.* Evolution of a species-specific determinant within human CRM1 that regulates the post-transcriptional phases of HIV-1 replication. *PLoS Pathog* 2011;7:e1002395.
11. Okada H, Zhang X, Ben Fofana I, Nagai M, Suzuki H *et al.* Synergistic effect of human CycT1 and CRM1 on HIV-1 propagation in rat T cells and macrophages. *Retrovirology* 2009;6:43.
12. Schaller T, Hué S, Towers GJ. An active TRIM5 protein in rabbits indicates a common antiviral ancestor for mammalian TRIM5 proteins. *J Virol* 2007;81:11713–11721.
13. Fletcher AJ, Hué S, Schaller T, Pillay D, Towers GJ. Hare TRIM5α restricts divergent retroviruses and exhibits significant sequence variation from closely related lagomorpha TRIM5 genes. *J Virol* 2010;84:12463–12468.
14. Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M *et al.* Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* 2011;474:658–661.
15. Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C *et al.* SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* 2011;474:654–657.
16. Rehwinkel J, Maelfait J, Bridgeman A, Rigby R, Hayward B *et al.* SAMHD1-dependent retroviral control and escape in mice. *EMBO J* 2013;32:2454–2462.
17. Neil SJ, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 2008;451:425–430.
18. van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R *et al.* The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 2008;3:245–252.
19. Goffinet C, Allespach I, Homann S, Tervo HM, Habermann A *et al.* HIV-1 antagonism of CD317 is species specific and involves Vpu-mediated proteasomal degradation of the restriction factor. *Cell Host Microbe* 2009;5:285–297.
20. Goffinet C, Schmidt S, Kern C, Oberbremer L, Keppler OT. Endogenous CD317/Tetherin limits replication of HIV-1 and murine leukemia virus in rodent cells and is resistant to antagonists from primate viruses. *J Virol* 2010;84:11374–11384.
21. Liberatore RA, Bieniasz PD. Tetherin is a key effector of the antiretroviral activity of type I interferon in vitro and in vivo. *Proc Natl Acad Sci USA* 2011;108:18097–18101.
22. Mariani R, Chen D, Schröfelbauer B, Navarro F, König R *et al.* Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 2003;114:21–31.
23. Bishop KN, Holmes RK, Sheehy AM, Davidson NO, Cho SJ *et al.* Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Curr Biol* 2004;14:1392–1396.
24. Ikeda T, Ohsugi T, Kimura T, Matsushita S, Maeda Y *et al.* The antiretroviral potency of APOBEC1 deaminase from small animal species. *Nucleic Acids Res* 2008;36:6859–6871.
25. Rosa A, Chande A, Ziglio S, de Sanctis V, Bertorelli R *et al.* HIV-1 Nef promotes infection by excluding SERINC5 from virion incorporation. *Nature* 2015;526:212–217.
26. Usami Y, Wu Y, Göttlinger HG. SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef. *Nature* 2015;526:218–223.

27. Inuzuka M, Hayakawa M, Ingi T. Serinc, an activity-regulated protein family, incorporates serine into membrane lipid synthesis. *J Biol Chem* 2005;280:35776–35783.
28. Trautz B, Wiedemann H, Lüchtenborg C, Pierini V, Kranich J *et al.* The host-cell restriction factor SERINC5 restricts HIV-1 infectivity without altering the lipid composition and organization of viral particles. *J Biol Chem* 2017;292:13702–13713.
29. Heigele A, Kmiec D, Regensburger K, Langer S, Peiffer L *et al.* The Potency of Nef-Mediated SERINC5 Antagonism Correlates with the Prevalence of Primate Lentiviruses in the Wild. *Cell Host Microbe* 2016;20:381–391.
30. Murrell B, Vollbrecht T, Guatelli J, Wertheim JO. The Evolutionary Histories of Antiretroviral Proteins SERINC3 and SERINC5 Do Not Support an Evolutionary Arms Race in Primates. *J Virol* 2016;90: 8085–8089.
31. Sood C, Marin M, Chande A, Pizzato M, Melikyan GB. SERINC5 protein inhibits HIV-1 fusion pore formation by promoting functional inactivation of envelope glycoproteins. *J Biol Chem* 2017; 292:6014–6026.
32. Schulte B, Selyutina A, Opp S, Herschhorn A, Sodroski JG *et al.* Localization to detergent-resistant membranes and HIV-1 core entry inhibition correlate with HIV-1 restriction by SERINC5. *Virology* 2018;515:52–65.
33. Melikyan GB. Common principles and intermediates of viral protein-mediated fusion: the HIV-1 paradigm. *Retrovirology* 2008;5: 111.
34. Zhang X, Zhou T, Yang J, Lin Y, Shi J *et al.* Identification of SERINC5-001 as the Predominant Spliced Isoform for HIV-1 Restriction. *J Virol* 2017;91.
35. Dai W, Usami Y, Wu Y, Göttlinger H. A Long Cytoplasmic Loop Governs the Sensitivity of the Anti-viral Host Protein SERINC5 to HIV-1 Nef. *Cell Rep* 2018;22:869–875.
36. Kmiec D, Akbil B, Ananth S, Hotter D, Sparrer KMJ *et al.* SIVcol Nef counteracts SERINC5 by promoting its proteasomal degradation but does not efficiently enhance HIV-1 replication in human CD4+ T cells and lymphoid tissue. *PLoS Pathog* 2018;14:e1007269.
37. Ahi YS, Zhang S, Thappeta Y, Denman A, Feizpour A *et al.* Functional iterplay between murine leukemia virus glycogag, serinc5, and surface glycoprotein governs virus entry, with opposite effects on gammaretroviral and ebolavirus glycoproteins. *MBio* 2016;7.
38. Chande A, Cuccurullo EC, Rosa A, Ziglio S, Carpenter S *et al.* S2 from equine infectious anemia virus is an infectivity factor which counteracts the retroviral inhibitors SERINC5 and SERINC3. *Proc Natl Acad Sci USA* 2016;113:13197–13202.
39. Trautz B, Pierini V, Wombacher R, Stolp B, Chase AJ *et al.* The Antagonism of HIV-1 Nef to SERINC5 Particle Infectivity Restriction Involves the Counteraction of Virion-Associated Pools of the Restriction Factor. *J Virol* 2016;90:10915–10927.
40. Shi J, Xiong R, Zhou T, Su P, Zhang X *et al.* HIV-1 Nef Antagonizes SERINC5 Restriction by Downregulation of SERINC5 via the Endosome/Lysosome System. *J Virol* 2018;92.
41. Usami Y, Göttlinger H. HIV-1 Nef responsiveness is determined by Env variable regions involved in trimer association and correlates with neutralization sensitivity. *Cell Rep* 2013;5:802–812.
42. Beitari S, Ding S, Pan Q, Finzi A, Liang C. Effect of HIV-1 Env on SERINC5 Antagonism. *J Virol* 2017;91.
43. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680.
44. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 1999;41:95–98.
45. Geuenich S, Goffinet C, Venzke S, Nolkemper S, Baumann I *et al.* Aqueous extracts from peppermint, sage and lemon balm leaves display potent anti-HIV-1 activity by increasing the virion density. *Retrovirology* 2008;5:27.
46. Wittmann S, Behrendt R, Eissmann K, Volkmann B, Thomas D *et al.* Phosphorylation of murine SAMHD1 regulates its antiretroviral activity. *Retrovirology* 2015;12:103.
47. Andreeva L, Hiller B, Kostrewa D, Lässig C, de Oliveira Mann CC *et al.* cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein-DNA ladders. *Nature* 2017;549:394–398.
48. Fackler OT, D'Aloja P, Baur AS, Federico M, Peterlin BM. Nef from human immunodeficiency virus type 1(F12) inhibits viral production and infectivity. *J Virol* 2001;75:6601–6608.
49. Pizzato M, Helander A, Popova E, Calistri A, Zamborlini A *et al.* Dynamin 2 is required for the enhancement of HIV-1 infectivity by Nef. *Proc Natl Acad Sci USA* 2007;104:6812–6817.
50. Pizzato M. MLV glycosylated-Gag is an infectivity factor that rescues Nef-deficient HIV-1. *Proc Natl Acad Sci USA* 2010;107:9364–9369.
51. Wain-Hobson S, Vartanian JP, Henry M, Chenciner N, Cheynier R *et al.* LAV revisited: origins of the early HIV-1 isolates from Institut Pasteur. *Science* 1991;252:961–965.
52. Vermeire J, Naessens E, Vanderstraeten H, Landi A, Iannucci V *et al.* Quantification of reverse transcriptase activity by real-time PCR as a fast and accurate method for titration of HIV, lenti- and retroviral vectors. *PLoS One* 2012;7:e50859.
53. Keppler OT, Allespach I, Schüller L, Fenard D, Greene WC *et al.* Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor Nef. *J Virol* 2005;79:1655–1665.
54. Sharma S, Lewinski MK, Guatelli J. An N-Glycosylated Form of SERINC5 Is Specifically Incorporated into HIV-1 Virions. *J Virol* 2018;92.
55. Liu CC, Simonsen CC, Levinson AD. Initiation of translation at internal AUG codons in mammalian cells. *Nature* 1984;309:82–85.
56. Peri S, Pandey A. A reassessment of the translation initiation codon in vertebrates. *Trends Genet* 2001;17:685–687.
57. Cavrois M, de Noronha C, Greene WC. A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat Biotechnol* 2002;20:1151–1154.
58. Eppig JT, Blake JA, Bult CJ, Kadin JA,Richardson JE. The mouse genome database (MGD): facilitating mouse as a model for human biology and disease. *Nucleic Acids Res* 2015;43: D726–D736.
59. Shimoyama M, Laulederkind SJ, de Pons J, Nigam R, Smith JR *et al.* Exploring human disease using the Rat Genome Database. *Dis Model Mech* 2016;9:1089–1095.
60. Esteves PJ, Abrantes J, Baldauf HM, Benmohamed L, Chen Y *et al.* The wide utility of rabbits as models of human diseases. *Exp Mol Med* 2018;50:66.
61. Katzourakis A, Tristem M, Pybus OG, Gifford RJ. Discovery and analysis of the first endogenous lentivirus. *Proc Natl Acad Sci USA* 2007;104:6261–6265.
62. Thomas D, Newcomb WW, Brown JC, Wall JS, Hainfeld JF *et al.* Mass and molecular composition of vesicular stomatitis virus: a scanning transmission electron microscopy analysis. *J Virol* 1985; 54:598–607.
63. Chojnacki J, Staudt T, Glass B, Bingen P, Engelhardt J *et al.* Maturation-dependent HIV-1 surface protein redistribution revealed by fluorescence nanoscopy. *Science* 2012;338:524–528.
64. Li S, Ahmad I, Shi J, Wang B, Yu C *et al.* Murine leukemia virus glycosylated Gag reduces murine SERINC5 protein expression at steady-state levels via endosome/lysosome pathway to counteract the SERINC5 antiretroviral activity. *J Virol* 2018.
65. Yachdav G, Kloppmann E, Kajan L, Hecht M, Goldberg T *et al.* PredictProtein-an open resource for online prediction of protein structural and functional features. *Nucleic Acids Res* 2014;42: W337–W343.