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SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef

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HIV-1 Nef and the unrelated mouse leukaemia virus glycosylated Gag (glycoGag) strongly enhance the infectivity of HIV-1 virions produced in certain cell types in a clathrin-dependent manner. Here we show that Nef and glycoGag prevent the incorporation of the multipass transmembrane proteins serine incorporator 3 (SERINC3) and SERINC5 into HIV-1 virions to an extent that correlates with infectivity enhancement. Silencing of both SERINC3 and SERINC5 precisely phenocopied the effects of Nef and glycoGag on HIV-1 infectivity. The infectivity of *nef*-deficient virions increased more than 100-fold when produced in double-knockout human CD4$^+$ T cells that lack both *SERINC3* and *SERINC5*, and re-expression experiments confirmed that the absence of SERINC3 and SERINC5 accounted for the infectivity enhancement. Furthermore, SERINC3 and SERINC5 together restricted HIV-1 replication, and this restriction was evaded by Nef. SERINC3 and SERINC5 are highly expressed in primary human HIV-1 target cells, and inhibiting their downregulation by Nef is a potential strategy to combat HIV/AIDS.

Nef is an accessory protein encoded by HIV-1 and other primate lentiviruses. *In vivo*, Nef is a major pathogenicity determinant that is required for high virus loads[1–3]. Although not essential for virus replication in cell culture, Nef enhances virus spreading in primary CD4$^+$ T cells, particularly when such cells are infected before mitogenic stimulation[4–6]. Nef robustly downregulates the viral entry receptor CD4 from the surface of virus-producing cells by inducing its clathrin-dependent endocytosis and subsequent lysosomal degradation[7–10]. A recent study suggests that an important physiological function of CD4 downregulation by Nef is to prevent the CD4-induced exposure of epitopes in HIV-1 envelope (Env) that make infected cells susceptible to antibody-dependent cell-mediated cytotoxicity[11]. Apart from CD4, Nef downregulates several other cell surface proteins[12]. The selective down-modulation of HLA-A and HLA-B but not of HLA-C by Nef serves to protect infected cells both from cytotoxic T cells and from natural killer cells[13–15]. The Nef proteins of most primate lentiviruses also down-modulate the T cell receptor complex, which is thought to protect infected T cells from activation-induced cell death in non-pathogenic natural SIV infections[16]. This function of Nef was lost in HIV-1 and closely related viruses, which may contribute to the pathogenicity of HIV-1 in humans[16].

One of the most conserved yet poorly understood functions of Nef is the enhancement of progeny virion infectivity[17,18]. Although Nef exerts its effect on HIV-1 infectivity in virus producer cells, it does not detectably affect virus morphogenesis or maturation[19–22]. Nevertheless, progeny virions produced in the absence of Nef do not efficiently reverse transcribe their genome in target cells[19,20]. It has been reported that high levels of cell-surface CD4 inhibit the release or infectivity of HIV-1 progeny virions, and that Nef relieves these effects[23,24]. However, the enhancement of HIV-1 infectivity depends on residues within Nef that are dispensable for its ability to downregulate CD4 (ref. 25). Furthermore, Nef enhances HIV-1 progeny virion infectivity even when CD4 is not expressed or cannot be downregulated[17,19,20]. Finally, the glycoGag protein of Moloney murine leukaemia virus (MLV) closely mimics the effect of Nef on HIV-1 infectivity, even though glycoGag does not downregulate CD4 (ref. 26). MLV glycoGag is an accessory protein whose translation begins at an inefficient CTG start codon upstream and in-frame with the *gag* gene[27]. The resulting product is a type II transmembrane protein with an amino-terminal cytosolic non-Gag portion and an extracellular Gag domain[28]. The potent Nef-like activity of glycoGag on HIV-1 infectivity resides entirely in its cytosolic domain, which is unrelated to Nef[29]. Nevertheless, the effects of Nef and glycoGag on HIV-1 infectivity appear mechanistically related. Both are similarly dependent on the producer cell type[26], are similarly determined by variable regions of HIV-1 Env[30], and exhibit a similar reliance on clathrin-mediated endocytosis[29,31,32]. However, the molecular basis for these similarities remains unknown.

Nef inhibits the incorporation of SERINC proteins

Because of the essential role of the endocytic machinery in the enhancement of HIV-1 infectivity by Nef or glycoGag, we examined the possibility that both proteins downregulate a restriction factor that gets incorporated into assembling virions in their absence. To identify factors whose incorporation is prevented by both Nef and glycoGag, we conducted a proteomic analysis of OptiPrep gradient-purified virions produced by T lymphoid cells infected with wild-type (Nef$^+$) or Nef$^-$ HIV-1$_{NL43}$, or with a version that encodes a fully active minimal glycoGag (termed glycoMA[30]) instead of Nef (Extended Data Fig. 1a). The only host protein that could reproducibly be identified in Nef$^-$ virion samples in independent experiments but was not identified in any Nef$^+$ or glycoMA virion sample was SERINC3, a member of a family of putative carrier proteins with at least 10 transmembrane domains[33] (Extended Data Fig. 1b). In one experiment, STOM and PFKP were also identified in Nef$^-$ but not in Nef$^+$ or glycoMA virion samples (Extended Data Fig. 1b). However, in another experiment, STOM was identified in all virions samples, and PFKP was not identified in any sample. Thus, STOM and PFKP were not further pursued. Immunoblotting of virion samples confirmed that the incorporation of haemagglutinin (HA)-tagged SERINC3 is strongly inhibited by the Nef proteins of several laboratory-adapted and primary HIV-1 isolates from different clades

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(Fig. 1a) and by glycoMA (Extended Data Fig. 2a). Furthermore, the effects of glycoMA truncation mutants on the incorporation of SERINC3–HA (Extended Data Fig. 2a) correlated closely with their abilities to enhance HIV-1 infectivity[29]. Two of the Nef proteins tested did not inhibit the incorporation of SERINC3–HA (Fig. 1a), and one of these ($Nef_{90CF056}$) also had no effect on HIV-1 infectivity (Fig. 1c). Because the other (Nef_{SF2}) did enhance HIV-1 infectivity (Fig. 1c), we examined its effect on the incorporation of other human SERINC family members. Although Nef_{SF2} did not affect the incorporation of SERINC3–HA (Fig. 1a), it strongly inhibited the incorporation of SERINC5–HA (Fig. 1b). Among the primary Nef proteins examined, those that were most active in enhancing HIV-1 infectivity ($Nef_{97ZA012}$ and $Nef_{93BR020}$) strongly inhibited the incorporation of both SERINC3 and SERINC5, the less active $Nef_{94UG114}$ was a less effective inhibitor particularly of SERINC5 incorporation, and the inactive $Nef_{90CF056}$ inhibited neither SERINC3 nor SERINC5 incorporation (Fig. 1a–c). Like the most active Nef proteins, wild-type glycoMA, which enhances HIV-1 infectivity at least as potently[30], also strongly inhibited the incorporation of both SERINC3 and SERINC5 (Extended Data Fig. 2a, b). Furthermore, the effects of glycoMA truncation mutants on SERINC5 incorporation (Extended Data Fig. 2b), like those on SERINC3 incorporation (Extended Data Fig. 2a), correlated with their effects on HIV-1 infectivity enhancement[29].

Subcellular localization of SERINC5

SERINC5–mCherry clearly localized to the plasma membrane and to filopodia-like protrusions when expressed alone, but accumulated in perinuclear vesicles when co-expressed with Nef or glycoGag (Extended Data Fig. 3a and data not shown). Furthermore, SERINC5(iHA), which contains an internal HA tag next to a conserved consensus glycosylation site within a proposed extracellular loop[34], could be readily detected on the surface of transfected Jurkat TAg (JTAg) T lymphoid cells by flow cytometry, and its surface expression was greatly reduced when either Nef_{SF2} or glycoGag were co-expressed (Extended Data Fig. 3b). We infer that Nef and glycoGag decrease the virion-association of SERINC5 by decreasing its cell surface levels.

Effects of exogenous SERINCs on HIV infectivity

HIV-1 virions produced in Jurkat T lymphoid cells are more dependent on Nef for optimal infectivity than virions produced in 293T cells[26], which in turn are more dependent on Nef than virions produced in exceptionally permissive MT4 cells[35]. Interestingly, the relative requirement for Nef correlates with *SERINC5* messenger RNA levels, which are high in Jurkat cells, lower in 293T cells, and lower yet in MT4 cells (Extended Data Fig. 4a, b). *SERINC5* mRNA levels in unstimulated or stimulated human peripheral blood mononuclear cells (PBMC) were even higher than in Jurkat cells, and were not further increased by treatment with interferon-α (INF-α) (Extended Data Fig. 4b, c).

In single cycle replication assays, exogenous SERINC5 reduced the specific infectivity of Nef^- HIV-1 virions produced in 293T cells for TZM-bl indicator target cells >100-fold, even when as little as 100 ng of the relatively weak pBJ5-based SERINC5 expression vector was used (Fig. 2a). Under the same conditions, exogenous SERINC3 reduced progeny virus infectivity only two- to threefold (Fig. 2a). However, although endogenous *SERINC5* mRNA levels in 293T cells are low, these cells have relatively high endogenous *SERINC3* mRNA levels (Extended Data Fig. 4b). Even at 500 ng, the vectors expressing SERINC3 or SERINC5 did not affect virus particle production, Gag processing, or HIV-1 Env incorporation (Fig. 2b). However, late reverse transcriptase products in target cells exposed to Nef^- HIV-1 virions produced in 293T cells transfected with 500 ng of the SERINC5 expression vector were reduced >100-fold (Fig. 2c).

To examine whether SERINC5 affects HIV-1 virion fusion with target cells, we co-expressed a chimaeric β-lactamase-Vpr (BlaM-Vpr) protein that is taken up into virions[36]. Fusion was then quantified based on the cleavage of a fluorescent substrate after the transfer of BlaM-Vpr from virions into target cells. We initially used 1 μg of the SERINC5 expression vector to compensate for potential competition by the strong promoter driving BlaM-Vpr expression, and found that the ability of Nef^- HIV-1 progeny virions to fuse with target cells was largely abolished (Extended Data Fig. 5). However, 100 ng of the SERINC5 expression vector, which reduced the infectivity of Nef^- HIV-1 virions ~20-fold when co-transfected with the BlaM-Vpr expression vector, caused only an ~4-fold reduction in the ability to fuse with target cells (Extended Data Fig. 5).

The effects on HIV-1 infectivity were specific, because 500 ng of the vectors expressing SERINC3 or SERINC5 had at most modest effects on the infectivities of Env^- HIV-1 particles pseudotyped with the vesicular stomatitis virus G protein (VSV-G) (Fig. 2d), which do not require Nef or glycoGag for optimal infectivity[26,37,38]. Surprisingly, the incorporation of HA-tagged SERINC5 into Env^- HIV-1 particles was reduced in the presence of VSV-G (Fig. 2e). Thus, reduced incorporation may have contributed to the relative resistance of VSV-G-pseudotyped HIV-1 to exogenous SERINC5. Crucially, the effect of exogenous SERINC5 on Nef^- HIV-1 was counteracted by Nef_{SF2} or glycoGag expressed *in trans* (Fig. 2f). Indeed, exogenous SERINC5 had no effect whatsoever in the presence of glycoGag (Fig. 2f). In two independent experiments performed with cells from different donors, exogenous SERINC5 also greatly reduced the infectivity of Nef^- HIV-1 virions produced in 293T cells for primary human target cells (Extended Data Fig. 6).

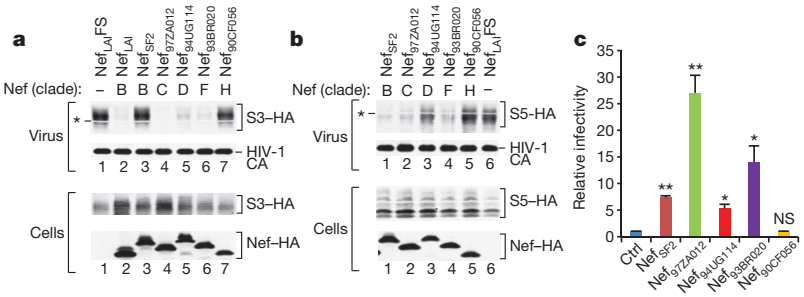

Figure 1 | Inhibition of incorporation of SERINC proteins into HIV-1 virions by Nef correlates with infectivity enhancement. **a**, **b**, Western blots showing the effects of Nef proteins from various HIV-1 clades on the incorporation of SERINC3–HA (S3–HA) (**a**) or SERINC5–HA (S5–HA) (**b**) into Nef^- HIV-1 virions. The white bands marked by asterisks are caused by co-migrating HIV-1 $Pr55^{gag}$. The experiment shown in **a** was performed twice. Supplementary Information contains full scans for **a** and **b**. **c**, Ability of Nef proteins from different HIV-1 clades to enhance HIV-1 infectivity. Env^-/Nef^- $HIV\text{-}1_{HXB2}$ particles *trans*-complemented with Env_{HXB2} were produced in JTAg cells in the absence or presence of the indicated Nef proteins, and infectivities normalized for p24 antigen were determined using TZM-bl indicator target cells ($n = 3$). Data are mean and s.d. $^*P < 0.05$, $^{**}P < 0.01$, NS, not significant ($P > 0.05$) (two-tailed unpaired *t*-test with Welch's correction in case of unequal variance; *F*-test, $\alpha = 0.025$). Ctrl, control.

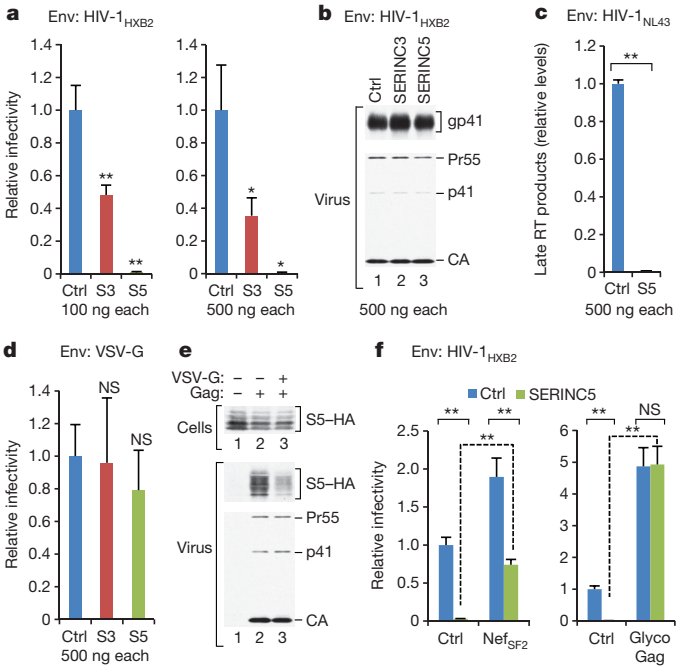

Figure 2 | Effects of exogenous SERINCs on HIV-1 infectivity. **a**, Overexpression of SERINC5 in virus producer cells dramatically reduces Nef$^-$ HIV-1 progeny virus single-round infectivity. The effects of exogenous SERINC3 (S3) and SERINC5 (S5) were measured using TZM-bl indicator cells ($n = 3$). **b**, Western blots showing that virus production, Gag processing, and gp41 (Env) incorporation were unaffected. **c**, Nef$^-$ HIV-1 progeny virions produced in the presence of exogenous SERINC5 are defective in the synthesis of late reverse transcriptase (RT) products ($n = 2$). **d**, Effects of exogenous SERINCs on the single-round infectivity of VSV-G-pseudotyped Nef$^-$ HIV-1 virions measured as in **a** ($n = 3$). **e**, VSV-G reduces the association of SERINC5–HA with Env$^-$ HIV-1 virions. The HIV-1 proviral plasmid in lane 1 has a disrupted *gag* gene. This experiment was repeated twice. Supplementary Information contains full scans for **b** and **e**. **f**, Nef_{SF2} and glycoGag expressed *in trans* in virus producer cells counteract the effect of exogenous SERINC5 on Nef$^-$ HIV-1 progeny virion infectivity ($n = 3$). Data are mean and s.d. $^*P < 0.05$, $^{**}P < 0.01$ (two-tailed unpaired *t*-test with Welch's correction in case of unequal variance).

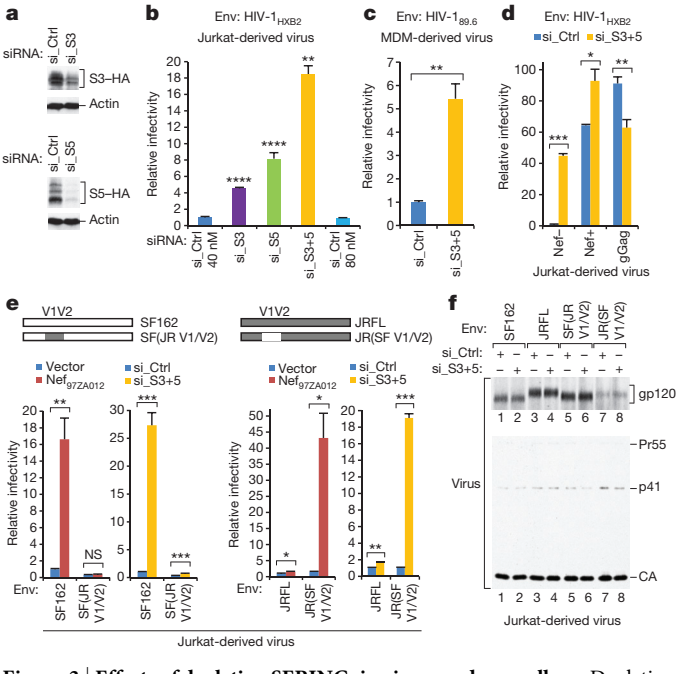

Figure 3 | Effects of depleting SERINCs in virus producer cells. **a**, Depletion of HA-tagged SERINCs in JTAg cells by specific siRNAs. si_Ctrl, non-targeting siRNA control; si_S3, siRNA targeting *SERINC3*; si_S5, siRNA targeting *SERINC5*. **b**, Single-round infectivities of Nef$^-$ HIV-1 virions produced in JTAg cells ($n = 3$) subjected to non-targeting siRNA or to siRNAs targeting *SERINC3* (si_S3), *SERINC5* (si_S5), or both (si_S3 + 5). **c**, Single-round infectivities of Nef$^-$ HIV-1 virions produced in primary monocyte-derived macrophages (MDM) subjected to siRNAs ($n = 3$). **d**, Simultaneous depletion of SERINC3 and SERINC5 has negligible effects on Nef$^-$ HIV-1 progeny virion infectivity when Nef or glycoGag are provided *in trans* ($n = 3$). **e**, The effects of depleting SERINC3 together with SERINC5 on virus infectivity are governed by the same determinants in gp120 V1/V2 that govern Nef-responsiveness ($n = 3$). **f**, Western blots showing that the combined siRNAs targeting *SERINC3* and *SERINC5* did not affect particle production, Gag processing, or Env incorporation. Supplementary Information contains full scans for **a** and **f**. Data are mean and s.d. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, (two-tailed unpaired *t*-test with Welch's correction in case of unequal variance). The experiments shown in **a** and **b** were performed twice.

Effects of SERINC depletion on HIV infectivity

JTAg T lymphoid cells express both *SERINC3* and *SERINC5* at relatively high levels (Extended Data Fig. 4b). Short interfering RNAs (siRNAs) that knocked down HA-tagged SERINC3 or SERINC5 (Fig. 3a) enhanced the specific infectivity of Nef$^-$, Env_{HXB2}-pseudotyped HIV-1 particles produced in JTAg cells by >4- or >8-fold, respectively (Fig. 3b). In five independent experiments, both siRNAs together enhanced the specific infectivity of Nef$^-$ progeny virions 18- to 45-fold (Fig. 3b, d and data not shown). The siRNAs against SERINC3 and SERINC5 together also significantly enhanced the infectivity of Nef$^-$, $Env_{89.6}$-bearing HIV-1 virions produced by infected primary macrophages (Fig. 3c). However, they had little effect on the already high specific infectivity of Nef$^-$ progeny virions produced in JTAg cells that was observed when $Nef_{97ZA012}$ or glycoGag were expressed *in trans* (Fig. 3d).

The Env proteins of the R5-tropic primary HIV-1 isolates SF162 and JRFL differ considerably in their responsiveness to Nef or glycoGag, which is determined by variable regions 1 and 2 (V1/V2) of gp120 (ref. 30). Remarkably, the siRNAs against SERINC3 and SERINC5 together precisely phenocopied the differential effects of $Nef_{97ZA012}$ on the specific infectivities of Nef$^-$ HIV-1 progeny particles bearing Env_{SF162} or Env_{JRFL} (Fig. 3e). Furthermore, responsiveness to $Nef_{97ZA012}$ and to the siRNAs targeting SERINC3 and SERINC5 could be switched simultaneously by exchanging the V1/V2 regions of Env_{SF162} and Env_{JRFL} (Fig. 3e). Nef$^-$ HIV-1 virions bearing Env proteins that differed profoundly in their responsiveness to Nef or to SERINC depletion incorporated comparable amounts of SERINC5–HA (Extended Data Fig. 7). Furthermore, $Nef_{97ZA012}$ largely prevented the incorporation of SERINC5–HA in the presence of both Env proteins (Extended Data Fig. 7). Importantly, the simultaneous depletion of SERINC3 and SERINC5 in virus producer cells affected neither particle morphogenesis nor Env incorporation (Fig. 3f). Collectively, these data indicate that SERINC3 and SERINC5 together account for the effects of Nef and glycoGag on HIV-1 infectivity.

HIV infectivity in SERINC knockout cells

Next, we knocked out the *SERINC3* and *SERINC5* genes in JTAg cells using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system[39] (Extended Data Fig. 8). Nef$^-$, Env_{HXB2}-pseudotyped HIV-1 particles produced in JTAg clones lacking either *SERINC3* or *SERINC5* were ~5-fold or 13–20-fold more infectious, respectively, than particles produced in the parental cells (Fig. 4a). Notably, the specific infectivity of particles produced in double-knockout cells lacking *SERINC3* and *SERINC5* was >100-fold higher (Fig. 4a). Furthermore, the markedly increased specific infectivity of viral particles produced in double-knockout cells could be confirmed by visualizing green fluorescence protein (GFP)-positive cells after exposure to recombinant HIV-1 expressing GFP (Fig. 4b). Nef and glycoGag potently enhanced the specific infectivity of particles produced in parental JTAg cells as expected, but had no significant effects

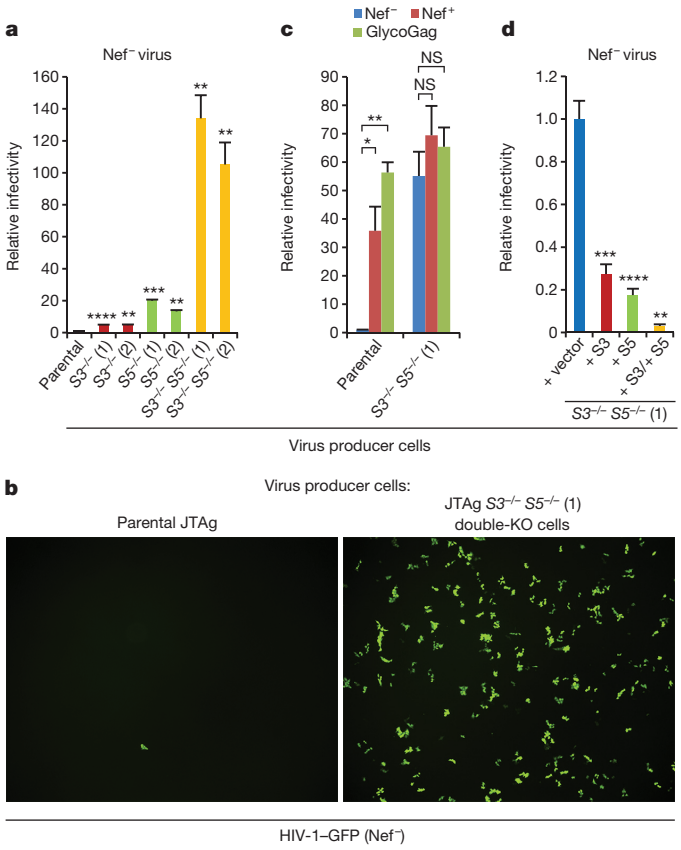

Figure 4 | Effects of *SERINC* knockout and reconstitution on HIV-1 infectivity. **a**, Single-round infectivities of Nef$^-$ HIV-1 progeny virions produced in parental or in knockout JTAg cells lacking *SERINC3* ($S3^{-/-}$), *SERINC5* ($S5^{-/-}$) or both ($S3^{-/-}$ $S5^{-/-}$) ($n = 3$). Numbers in parentheses denote clone numbers. **b**, TZM-bl cells were incubated with equal amounts of single-cycle Nef$^-$ HIV-1-GFP produced in parental or double-knockout (KO) cells lacking *SERIN3* and *SERINC5*. Infected TZM-bl cells expressing GFP were detected by fluorescence microscopy. **c**, Effects of Nef and glycoGag provided *in trans* on the single-round infectivities of Nef$^-$ HIV-1 virions produced in parental or double-knockout cells ($n = 3$). **d**, Effects of introducing expression cassettes for SERINC3, SERINC5 or both into the double-knockout cells on Nef$^-$ HIV-1 progeny virus infectivities ($n = 3$). Data are mean and s.d. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (two-tailed unpaired *t*-test with Welch's correction in case of unequal variance). The experiments shown in **a** and **b** were repeated three times.

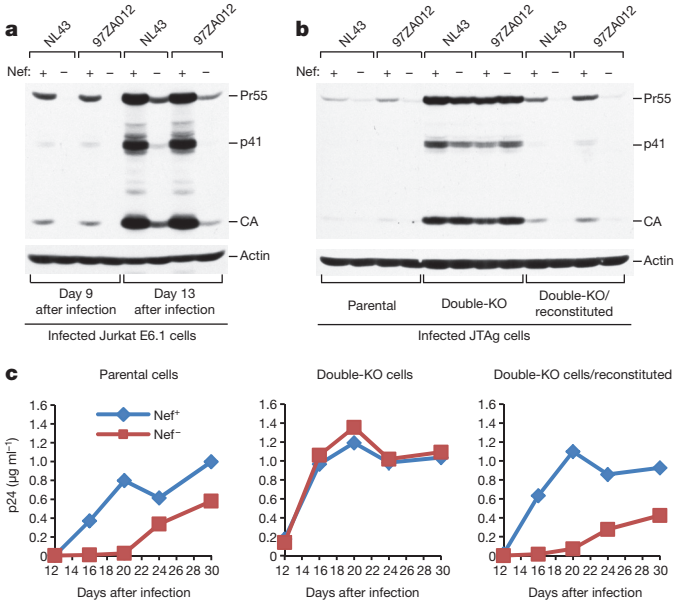

Figure 5 | Nef counteracts inhibition of HIV-1 replication by SERINC3 and SERINC5. **a**, Effect of Nef on HIV-1 spreading in Jurkat E6.1 cells infected at a low input virus concentration (100 pg ml^{-1} p24). Gag protein expression in the cultures 9 and 13 days after infection was examined by western blotting as a measure of virus replication. **b**, **c**, Effects of Nef on virus spreading in parental JTAg cells, double-knockout cells lacking *SERINC3* and *SERINC5*, and SERINC3+SERINC5-reconstituted double-knockout cells. The spreading of HIV-1_{NL43}-based viruses encoding either wild-type or disrupted versions of Nef_{NL43} or $Nef_{97ZA012}$ was examined by western blotting of cell lysates with anti-CA antibody 9 days after infection with 20 ng ml^{-1} p24 (**b**), or by monitoring p24 accumulation in the supernatants after infection with 4 ng ml^{-1} p24 (**c**). Relatively high input virus concentrations were used to compensate for low CD4 levels on JTAg cells. The experiment shown in **b** was repeated twice. Supplementary Information contains full scans for **a** and **b**.

on the already highly infectious particles produced in double-knockout cells (Fig. 4c). The introduction of expression cassettes for SERINC3, for SERINC5, and for both, into the double-knockout cells via retroviral transduction led to 3.6-fold, 5.7-fold, and 32-fold reductions, respectively, in the specific infectivities of Nef$^-$ HIV-1 particles produced in these cells (Fig. 4d). These data confirm that SERINC3 and SERINC5 synergistically restrict HIV-1 infectivity in the absence of Nef.

The effects of Nef on HIV-1 replication in cell lines have generally been more modest than in primary lymphocytes[4,6]. However, apart from Jurkat cells, T cell lines often express only low levels of *SERINC5* mRNA (Extended Data Fig. 4 and data not shown). We observed that at low input virus concentrations, Nef_{NL43} and $Nef_{97ZA012}$ robustly enhanced HIV-1 spreading in highly permissive Jurkat E6.1 cells (Fig. 5a). Nef_{NL43} and $Nef_{97ZA012}$ also enhanced HIV-1 replication in JTAg cells, as judged from Gag protein expression levels in the infected cells and from the release of p24 antigen over time (Fig. 5b, c). In marked contrast, the Nef$^+$ and Nef$^-$ viruses replicated with similar kinetics in double-knockout JTAg cells lacking *SERINC3* and *SERINC5*, which were generally more permissive than the parental cells (Fig. 5b, c). Crucially, the requirement for Nef was restored in double-knockout cells reconstituted with SERINC3 and SERINC5 expression cassettes (Fig. 5b, c). While the levels of SERINC3 and SERINC5 in the reconstituted cells were higher than in the parental cells (Extended Data Fig. 9), they were comparable to those in human PBMC (Extended Data Fig. 4).

Although endogenous CD4 levels in JTAg cells are low, similar results were obtained with more permissive CD4^{high} versions generated by retroviral transduction. In the presence of extra CD4, Nef again clearly enhanced virus replication in parental JTAg cells, but was entirely dispensable in double-knockout cells lacking *SERINC3* and *SERINC5* (Extended Data Fig. 10). Furthermore, the role of Nef in virus replication was restored after reconstitution of SERINC3 and SERINC5 expression in the double-knockout cells (Extended Data Fig. 10). These results demonstrate that SERINC3 and SERINC5 together restrict HIV-1 replication, and that Nef antagonizes this restriction.

Discussion

Our findings reveal that HIV-1 Nef and MLV glycoGag efficiently downregulate SERINC3 and SERINC5 from the cell surface, which prevents their incorporation into HIV-1 virions and consequently counteracts their inhibitory effect on HIV-1 infectivity. Importantly, these findings offer an explanation for why the enhancement of HIV-1 infectivity by Nef and glycoGag is highly dependent on dynamin 2, clathrin and the AP-2 clathrin adaptor complex[29,31]. SERINC family members are present in all eukaryotes, but their functions remain largely unknown. SERINC proteins reportedly enhance the incorporation of serine into phosphatidylserine and sphingolipids[33]. In principle, this activity could affect the lipid composition of the viral envelope, which is considered crucial for virion infectivity[40]. Our data demonstrate that

SERINC3 and SERINC5 together account for most if not all of the effects of Nef on HIV-1 infectivity and on HIV-1 replication in JTAg cells. Notably, Nef enhances HIV-1 infectivity and stimulates HIV-1 replication in human PBMC[4–6,18,41], whose *SERINC3* and *SERINC5* mRNA levels exceed those of Jurkat cells (Extended Data Fig. 4).

The ability of virions produced in the absence of Nef to reverse transcribe their genome in target cells is impaired[17,19,20]. Consistent with these observations, we find that SERINC5 in virus producer cells strongly inhibits the ability of Nef$^-$ HIV-1 virions to complete reverse transcription. We also find that SERINC5 can in principle abolish the ability of progeny HIV-1 virions to fuse with target cells. However, lower levels of SERINC5 inhibited the fusion step to a lesser extent than the ability of progeny virions to productively infect target cells. Although there is controversy about the effect of Nef on HIV-1 entry[35,42,43], a twofold reduction in the ability of Nef$^-$ HIV-1 virions to fuse with target cells was noted in one study[35]. In all of these studies, virus was produced in 293T cells, whose endogenous *SERINC5* mRNA levels are low (Extended Data Fig. 4). It is conceivable that relatively low levels of virion-associated SERINC5 impair primarily fusion pore enlargement, which poses a higher energy barrier to overcome than pore formation[44]. This would be expected to impair passage of the viral core but not necessarily of the much smaller BlaM-Vpr fusion indicator into target cells. Consistent with a role in entry, Nef enhances the cytoplasmic delivery of viral cores[45]. Further, the requirement for Nef is determined by HIV-1 Env[30].

Interestingly, the Env proteins of HIV-1_{NL43} and HIV-1_{SF162}, which are highly responsive to Nef and glycoGag, require a higher number of Env trimers to complete entry than the poorly Nef/glycoGag-responsive Env_{JRFL}[30,46]. Furthermore, the naturally occurring Asn160Lys mutation in the V2 loop of HIV-1 Env, which results in the loss of a glycosylation site, can increase both the responsiveness to Nef and glycoGag and the stoichiometry of entry[30,46]. Mechanistically, a link between Nef/glycoGag responsiveness and the stoichiometry of entry could be due to an inhibitory effect of virion-associated SERINCs on the clustering of Env trimers. Notably, such clusters have been visualized on the surface of mature HIV-1 virions and, most prominently, at virus-cell contact zones[47,48]. Alternatively, SERINCs embedded in the virion membrane could increase the energy barrier for fusion pore expansion. In both scenarios, differences in Nef/glycoGag-responsiveness among HIV-1 Envs, as well as differences in SERINC-sensitivity, may ultimately be due to differences in the amount of energy that these Envs provide towards fusion. Regardless of the mechanism, our observation that viruses as distant as HIV-1 and MLV have evolved to counteract SERINC3 and SERINC5 raises the possibility that these proteins have a broader role in innate antiviral immunity.

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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Supplementary Information is available in the online version of the paper.

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Author Contributions Y.U., Y.W. and H.G.G. designed the experiments and analysed the data. Y.U. carried out the analysis of virions and the SERINC overexpression and depletion experiments. Y.W. generated and characterized the *SERINC* knockout cells, carried out all experiments involving knockout cells and primary cells, and performed the qRT–PCR experiments and the BlaM-Vpr-based fusion assays. H.G.G. wrote the manuscript.

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METHODS

No statistical methods were used to predetermine sample size. Investigators were not blinded to allocation during experiments and outcome assessment, and experiments were not randomized.

Cells. JTAg, 293T, MT4, A549 and U2-OS cells were gifts from G. Crabtree, D. Baltimore, W. Haseltine, M. Bujny and A. Brass, respectively. Jurkat E6.1 and MOLT-3 cells were obtained from the ATCC. TZM-bl cells were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. PBMC were isolated from the blood of healthy donors by Ficoll-Hypaque density gradient centrifugation. The MycoSensor PCR assay system (Agilent) was used to check all cell lines for mycoplasma. The cell lines were not authenticated for this study.

HIV-1 proviral constructs. NL4-3/Nefstop, the *nef*-deficient variant of the prototypic HIV-1_{NL4-3} used in this study, has *nef* codons 31–33 replaced by three consecutive premature termination codons. NL-$nef_{97ZA012}$ is a version of HIV-1_{NL4-3} that has the *nef* gene precisely replaced by that of p97ZA012.1 (GenBank accession AF286227), a near full-length molecular clone of a primary subtype C HIV-1 isolate[49]. NL-$nef_{97ZA012}$FS is a *nef*-deficient variant of NL-$nef_{97ZA012}$ owing to a frameshift at a unique XhoI site in *nef*. HXB/89.6_{ecto}-GFP is a macrophage-tropic variant of the infectious HIV-1 molecular clone HXBH10 that encodes GFP within the *nef* region, and that has a KpnI-BamHI fragment (nucleotides 6351–8475 of K03455) encoding the Env ectodomain replaced by the corresponding fragment from p89.6, a biologically active molecular clone of the primary HIV-$1_{89.6}$ isolate[50]. The proviral plasmids NL4-3/glycoMA, HXB/Env^-/Nef^+, HXB/Env^-/Nef^-, and HXBH10-gag^- have been described[29,51,52].

Expression plasmids. The pBJ5-based Nef expression vectors used, the *nef*-deficient control vector pNef$_{LAI}$FS, and the pBJ5-based expression vectors for glycoGag–HA, for wild-type glycoMA–HA, and for its truncation mutants have been described[29,31] The latter plasmids were used as templates to amplify fragments encoding carboxy-terminally Flag-tagged versions of wild-type glycoMA and of its mutants, which were also cloned into the mammalian expression vector pBJ5. The HIV-1 Env expression vectors used have also been described[30]. The coding sequences for *SERINC3* and *SERINC5* without or with a C-terminal HA-tag were amplified from BC006088 (*SERINC3*) and from BC101281 and AW005635 (*SERINC5*) (GE Healthcare). The primers included a Kozak sequence and XhoI and NotI cloning sites for insertion into pBJ5. The vectors expressing SERINC5(iHA) and SERINC5–mCherry are also pBJ5-based. SERINC5(iHA) has an HA tag inserted between residues 290 and 291 of SERINC5. SERINC5–mCherry has a Thr-Gly-Ala-Gly linker inserted between SERINC5 and mCherry.

Retroviral vectors. The human *SERINC3* and *SERINC5* coding sequences preceded by a Kozak sequence were inserted into pMSCVhyg and pMSCVpuro, respectively (Clontech). The human *CD4* coding sequence was inserted into the retroviral vector pCXbsr[53].

Protein identification. For the identification of virus-associated host proteins, virions released by chronically infected T-lymphoid MOLT-3 cells were pelleted through sucrose, resuspended in PBS, and further purified in OptiPrep velocity gradients as described[54]. OptiPrep gradient fractions were collected from the top and diluted with PBS. Viral particles were harvested from the fractions by ultracentrifugation and lysed in SDS–PAGE loading buffer (60 mM Tris-HCl, pH 6.8, 1% SDS, 10% (v/v) glycerol, 0.005% bromophenol blue, 5% (v/v) 2-mercaptoethanol). Virus-containing fractions were then identified by western blotting with antibody 183-H12-5C against HIV-1 capsid (CA)[55].

For mass spectrometry, virion-associated proteins were briefly run into an SDS–PAGE gel to allow removal of SDS. After in-gel digestion with trypsin, peptides were separated on a NanoAcquity (Waters) UPLC and analysed with a Q Exactive hybrid mass spectrometer (Thermo). The run conditions followed the 'sensitive' settings recommended for optimizing the Q Exactive for low abundance proteins[56]. Raw data files were peak processed with Proteome Discoverer (version 1.3, Thermo) before searching with Mascot Server (version 2.4) against the SwissProt database. Search results were then loaded into the Scaffold Viewer (Proteome Software, Inc.).

Viral particle analysis. To examine the incorporation of SERINCs, 293T cells were co-transfected with NL4-3/Nefstop, vectors expressing HA-tagged SERINCs, and vectors expressing various epitope-tagged Nef or glycoGag proteins, or the appropriate control vectors. To examine whether VSV-G affects SERINC5 incorporation, 293T cells were co-transfected with 1 μg HXBH10-gag^- (a control HIV-1 proviral construct unable to express Gag) or HXB/Env^-/Nef^-, 100 ng of a plasmid expressing VSV-G or control vector, and 500 ng of a plasmid expressing SERINC5–HA. Virions released into the medium were pelleted through sucrose, and virus- and cell-associated proteins were detected by western blotting as described previously[57]. Samples used for the detection of SERINCs were maximally heated to 37 °C, because SERINC proteins are highly aggregation-prone at higher temperatures (data not shown). In some cases, 25 mM TCEP was used as the reducing agent. The antibodies used were 183-H12-5C against HIV-1 CA, HA.11 (Covance) against the HA epitope, M2 against the Flag epitope (Sigma-Aldrich), and AC-40 (Sigma-Aldrich) against actin. To examine Env incorporation, virions produced by transiently transfected 293T or JTAg cells were pelleted through 20% sucrose cushions by ultracentrifugation, and examined by western blotting using the anti-gp41 monoclonal antibody Chessie 8 (ref. 58), an anti-gp120 polyclonal antibody (20-HG81; Fitzgerald), and the anti-CA monoclonal antibody 183-H12-5C.

SERINC overexpression experiments. Pseudovirions capable of a single round of replication were produced by transfecting 293T cells in triplicate using a calcium phosphate precipitation method. The cells were co-transfected with 1 μg HXB/Env^-/Nef^-, 100 ng of a plasmid expressing Env_{HXB2} or VSV-G, and 100 or 500 ng of plasmids expressing SERINC3 or SERINC5, or with equimolar amounts of the empty vector. To examine the effects of Nef or glycoGag, 293T cells were co-transfected with 1 μg HXB/Env^-/Nef^-, a plasmid expressing Env_{HXB2} (100 ng), a plasmid expressing SERINC5 (100 ng) or the empty vector, and plasmids expressing Nef_{SF2} (2 μg) or glycoGag (200 ng) or the empty vector. Supernatants containing progeny virions were collected two days after transfection, clarified by low-speed centrifugation, filtered through 0.45-μm pore filters, and then used immediately to infect TZM-bl indicator cells in T25 flasks. Aliquots of the filtered virus stocks were frozen for HIV-1 CA (p24) antigen quantitation by a standard ELISA. Three to five days after infection, the indicator cells were lysed in reporter lysis buffer (Promega), and β-galactosidase activity was determined as a measure of infection using a kit (E2000; Promega) according to the manufacturer's instructions.

To examine the effects of exogenous SERINC5 on the single-cycle infectivity of *nef*-deficient HIV-1 for primary target cells, viral stocks were obtained by co-transfecting 293T cells with HXB/Env^-/Nef^-, a plasmid expressing Env_{HXB2}, a plasmid expressing SERINC5 or the empty vector, and an HIV-1-based lentiviral vector expressing GFP. Filtered viral stocks normalized for p24 antigen were used to infect human PBMC, and infected cells expressing GFP were quantified by flow cytometry.

SERINC depletion experiments. To obtain pseudovirions capable of a single round of replication, Lipofectamine 2000 (Invitrogen) was used to transfect JTAg cells in triplicate with 1 μg HXB/Env^-/Nef^-, 100 ng of an HIV-1 Env expression plasmid, and siRNAs (40 nM each). Additionally, 500 ng of a plasmid expressing $Nef_{97ZA012}$, or 200 ng of a plasmid expressing glycoGag, or the empty pBJ5 expression plasmid, were co-transfected in some experiments. The siRNAs targeting *SERINC3* (Hs_TDE1_2; target sequence: 5′-CACGGTGACTCGCCT CATTTA-3′) or *SERINC5* (Hs_C5orf12_3; target sequence: 5′-CACCGTCT ACATCTACTCCTA-3′), and AllStars negative control siRNA were purchased from Qiagen. As a control for experiments in which the siRNAs targeting *SERINC3* and *SERINC5* were co-transfected, the concentration of the control siRNA was doubled. The infectivities of JTAg-derived virus stocks normalized for p24 antigen content were determined as above using TZM-bl indicator cells.

To examine the effects of SERINC proteins on the infectivity of HIV-1 progeny virions produced in primary cells, monocyte-derived macrophages were infected with replication-competent, dual-tropic HXB/89.6_{ecto}-GFP. On day 5 after infection, Lipofectamine 2000 was used to simultaneously transfect the monocyte-derived macrophages with the siRNAs targeting *SERINC3* and *SERINC5* (240 nM each), or with the negative control siRNA. The cells were washed 5 h later to remove the transfection agent. Virus-containing culture medium was collected on day 3 after transfection, and infectivities normalized for p24 antigen were determined using TZM-bl indicator cells. Indinavir (2 μM) was added to the TZM-bl cells together with virus to limit replication to a single cycle, and AMD3100 (5 μM) and maraviroc (50 nM) were added the next day to prevent Env-induced cell–cell fusion.

Generation and use of knockout cells. Expression plasmids for single-guide RNAs (sgRNAs) targeting exons within the *SERINC3* and *SERINC5* genes were transiently transfected into JTAg cells by nucleofection, along with a plasmid expressing Cas9. The sites targeted by the sgRNAs are depicted in Extended Data Fig. 8. Whereas the two sgRNAs targeting the *SERINC3* gene were expressed individually, the two sgRNAs targeting the *SERINC5* gene were expressed together. To obtain double-knockout cells, JTAg *S3*$^{-/-}$ (2) cells were co-transfected with the two sgRNAs targeting the *SERINC5* gene and the Cas9 expression plasmid. Nine days after transfection, gene editing in the bulk cultures was confirmed by PCR amplification of the targeted regions of the genome, followed by digestion of the PCR products with appropriate restriction enzymes (NcoI and BtsCI for target sites A and B within the *SERINC3* gene, respectively; BsoBI for target site B within the *SERINC5* gene). Clones were then obtained by limiting dilution in 96-well flat-bottomed culture plates. Whenever possible, the clones were pre-screened by PCR amplification of the targeted

regions of the genome and restriction analysis. Furthermore, the PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen/Life Technologies), and up to 10 independent clones were sequenced in each case. The primer pairs used for PCR amplification of the sgRNA target sites were: 5′-CCATAGTCAGTCTTG CAGTTG-3′ and 5′-GTACGTAGTATCTAGCATAGTGC-3′ (*SERINC3* target site A), 5′-CTTCTAGGCTAATGTTGTCC-3′ and 5′-GTGAGTTGCAGGTA CTAAGTC-3′ (*SERINC3* target site B), 5′-CACACGATCCATTTCCACAG-3′ and 5′-CGCATCATGGTACCAGGTG-3′ (*SERINC5* target site A), and 5′-GATCATTGGCAGGTAAGAGC-3′ and 5′-CACACCGCAAACACAAGC-3′ (*SERINC5* target site B). Deletions between *SERINC5* target sites A and B were identified using primers 5′-CACACGATCCATTTCCACAG-3′ and 5′-CACAC CGCAAACACAAGC-3′ for PCR amplification and direct sequencing of the products. An inversion between *SERINC5* target sites A and B was characterized using primer pair 5′-CACACGATCCATTTCCACAG-3′ and 5′-GATCATTGG CAGGTAAGAGC-3′, and primer pair 5′-CGCATCATGGTACCAGGTG-3′ and 5′-CACACCGCAAACACAAGC-3′. Ectopic SERINC expression cassettes were introduced into the double-knockout cells by retroviral transduction with MSCVhygSERINC3 and/or MSCVpuroSERINC5, followed by selection with hygromycin and/or puromycin. SERINC3 expression was examined by western blotting with a rabbit anti-TDE1 (SERINC3) antibody (GTX115512; GeneTex).

To determine the effects of SERINC proteins on HIV-1 infectivity in the absence of Nef, parental, knockout, double-knockout, and gene-reconstituted double-knockout JTAg cells were co-transfected in triplicate with a plasmid expressing Env_{HXB2} and HXB/Env^{-}/Nef^{-}. To determine the effects of Nef and glycoGag in cells lacking *SERINC* genes, parental and double-knockout JTAg cells were co-transfected with a plasmid expressing Env_{HXB2} and HXB/Env^{-}/Nef^{-}, HXB/Env^{-}/Nef^{+} or HXB/Env^{-}/Nef^{-} together with a plasmid expressing glycoGag. Progeny virus infectivities normalized for p24 antigen were determined using TZM-bl indicator cells. Alternatively, the HIV-1 vector HIVec2.GFP was co-transfected together with HXB/Env^{-}/Nef^{-} and the Env expression plasmid. After exposure to equal amounts of virus, infected TZM-bl cells were then identified based on GFP expression.

For virus replication studies, replication-competent HIV-1 was produced by transiently transfecting 293T cells with NL4-3, NL4-3/Nefstop, NL-$nef_{97ZA012}$ or NL-$nef_{97ZA012}$FS. Virus-containing supernatants were passed through 0.45-μm filters, normalized for p24 antigen, and used to infect parental, double-knockout and gene-reconstituted double-knockout JTAg cells, or $CD4^{high}$ versions obtained by retroviral transduction with pCXbsrCD4 and selection with blasticidin.

Analysis of mRNA expression. Total cellular RNA was extracted from cell lines and PBMC using an RNeasy mini kit (Qiagen) and treated with RNase-free DNase (Qiagen). The $A_{260\ nm}/A_{280\ nm}$ ratio was >2.0 for all samples analysed. Quantitative reverse transcription PCR (qRT–PCR) was performed in triplicate for each biological sample using a LightCycler 96 real-time PCR system (Roche) and a Kapa SYBR FAST One-Step qRT–PCR Universal kit (Kapa Biosystems) according to the manufacturer's instructions. Threshold cycle values were normalized for those obtained for *GAPDH*, and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[59]. The primer pairs used were: *SERINC3*, 5′-AATTCAGGAACACCAGCCTC-3′ and 5′-GGTTGGGATTGCAGGAAC GA-3′; *SERINC5*, 5′-ATCGAGTTCTGACGCTCTGC-3′ and 5′-GCTCTTC AGTGTCCTCTCCAC-3′; *GAPDH*, 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′.

Analysis of late reverse transcriptase products. Virions were produced by co-transfecting 293T cells with NL4-3/Nefstop (1.5 μg) and the pBJ5-based vector expressing SERINC5 (500 ng) or an equimolar amount of empty pBJ5. Cell-free virions were treated with RNase-free DNase I (Roche), and used to infect A549/CD4/CXCR4 cells in duplicate in T25 flasks for 14 h in the absence or presence of a cocktail of reverse transcriptase inhibitors. Genomic DNA was extracted with DNAzol (Life Technologies), and 100 ng of each template DNA was used for quantitative PCR using a LightCycler 96 real-time qPCR system (Roche) and a Kapa SYBR FAST Universal qPCR kit (Kapa Biosystems) according to the manufacturer's instructions. The primers used to quantify late reverse transcriptase products were J1 forward 5′-ACAAGCTAGTACCAGTTGAGCCAGATAAG-3′, and J2 reverse 5′-GCCGTGCGCGCTTCAGCAAGC-3′. The J1 forward primer exploits differences between the 5′ and 3′ long terminal repeats of pNL4-3 to help distinguish between late reverse transcriptase products and contaminating plasmid DNA. Standard curves were obtained from tenfold serial dilutions of DNA extracted from cells infected with virions produced in the absence of exogenous SERINC5. Quantitative PCR results were normalized for input virus based on p24 antigen quantifications. A549/CD4/CXCR4 target cells were generated by transduction with retroviral vectors expressing CD4 (pMSCVpuroCD4) and CXCR4 (pCXbsrCXCR4).

Virion fusion assay. Virions containing BlaM-Vpr were produced by transfecting 293T cells with HXB/Env^{-}/Nef^{-} (2.5 μg), a vector expressing the Env protein of HIV-1_{HXB2} or a frameshifted version unable to express Env (200 ng), the BlaM-Vpr expression vector pMM310 (1 μg), and a pBJ5-based vector expressing SERINC5 (1 μg or 100 ng) or an equimolar amount of empty pBJ5 (0.7 μg or 70 ng). Cell-free virions were normalized for p24 antigen and incubated with 2×10^5 TZM-bl or A549/CD4/CXCR4 cells in 6-well plates for 4 h at 37 °C. After washing with PBS, 1 ml CCF4-AM dye solution in phenol-free DMEM/2% FBS was added to the cells. The CCF4-AM dye solution was prepared using a LiveBLAzer FRET-B/G loading kit (Life Technologies) according to the alternative protocol recommended by the manufacturer. After incubation for 12–14 h at 11 °C in an ECHOterm chilling incubator (Torrey Pines Scientific), the cells were washed 3 × with PBS, detached with Versene (Life Technologies), fixed in 2% paraformaldehyde/PBS, and analysed on a Becton Dickinson LSR II flow cytometer. Samples were excited with a 405-nm violet laser, and fluorescence emission was measured in the Pacific Blue channel (450/50-nm filter) and in the AmCyan channel (525/20-nm filter).

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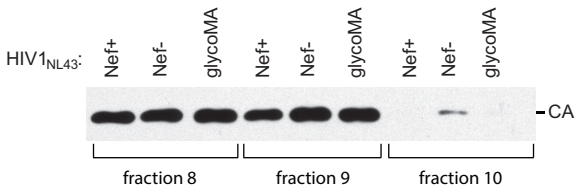
a



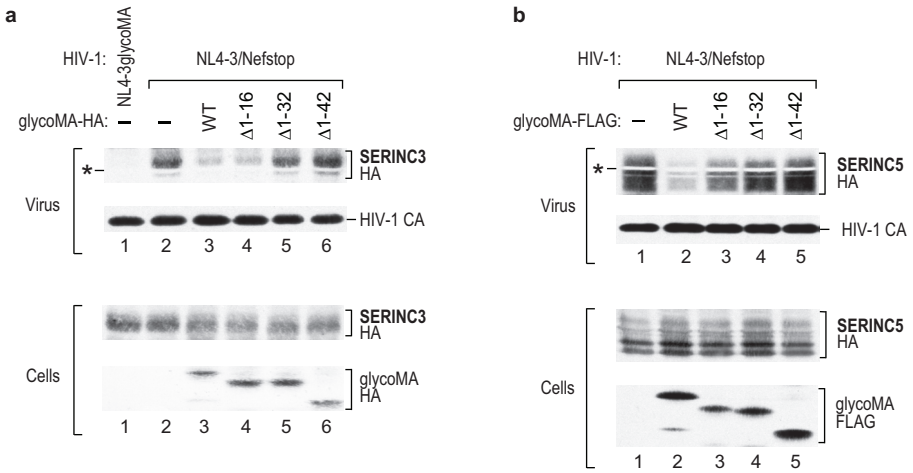
b

Proteins identified only in Nef- virions[a]

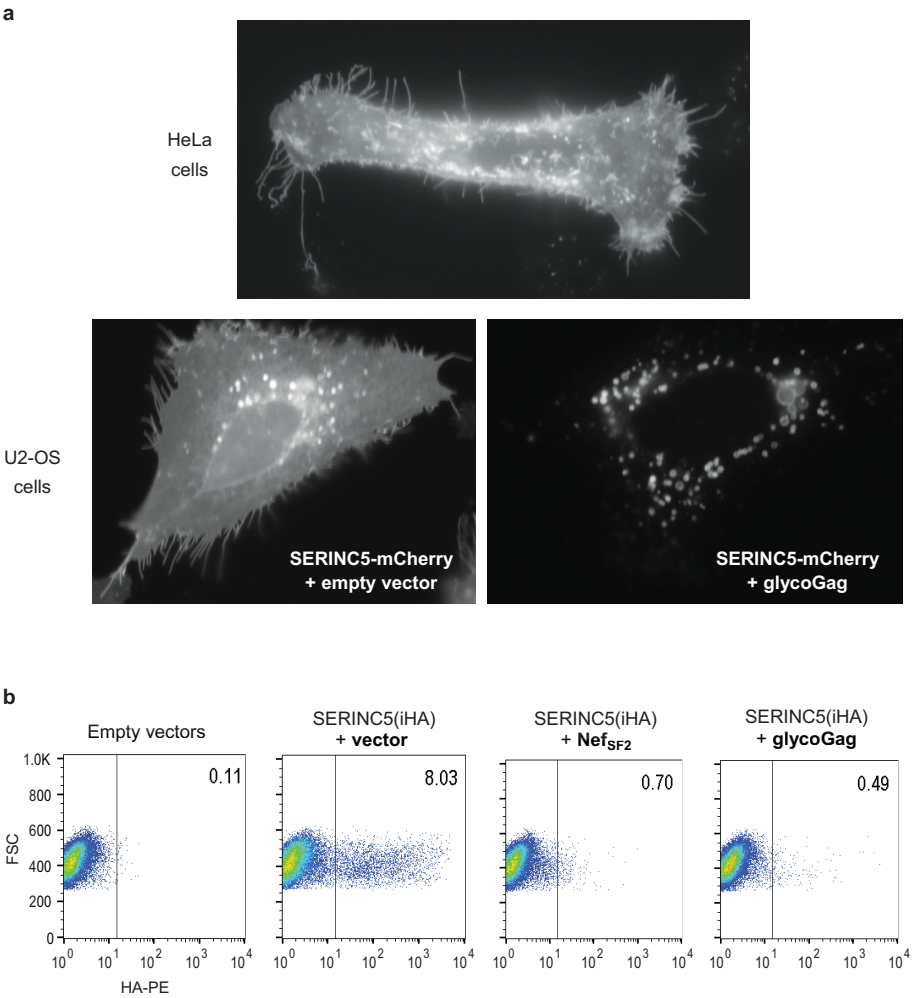
	Protein Symbol	Gradient Fraction	Total Spectrum Count	% Coverage
Experiment 1	SERINC3	8	7	15
		9	11	16
	STOM	8	2	7
		9	6	16
	PFKP	8	5	5
		9	10	8
Experiment 2	SERINC3	8	5	9
		9	9	9

[a]Proteins identified with at least 5% coverage both in fraction 8 and in fraction 9 are shown

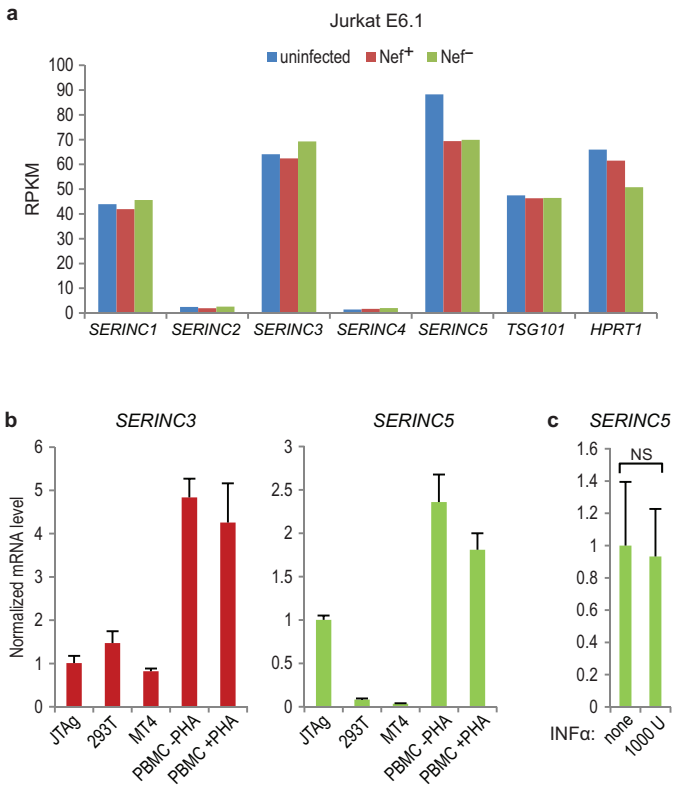
Extended Data Figure 1 | Identification of SERINC3 as a candidate target of Nef and glycoGag. **a**, Anti-HIV-1 CA immunoblot of Nef^{+}, Nef^{-} and $glycoMA^{+}$ HIV-1 virions collected from the indicated fractions of OptiPrep gradients. **b**, Proteins identified by mass spectrometry in Nef^{-} but not in Nef^{+} or $glycoMA^{+}$ virion lysates. The data are from two independent experiments.



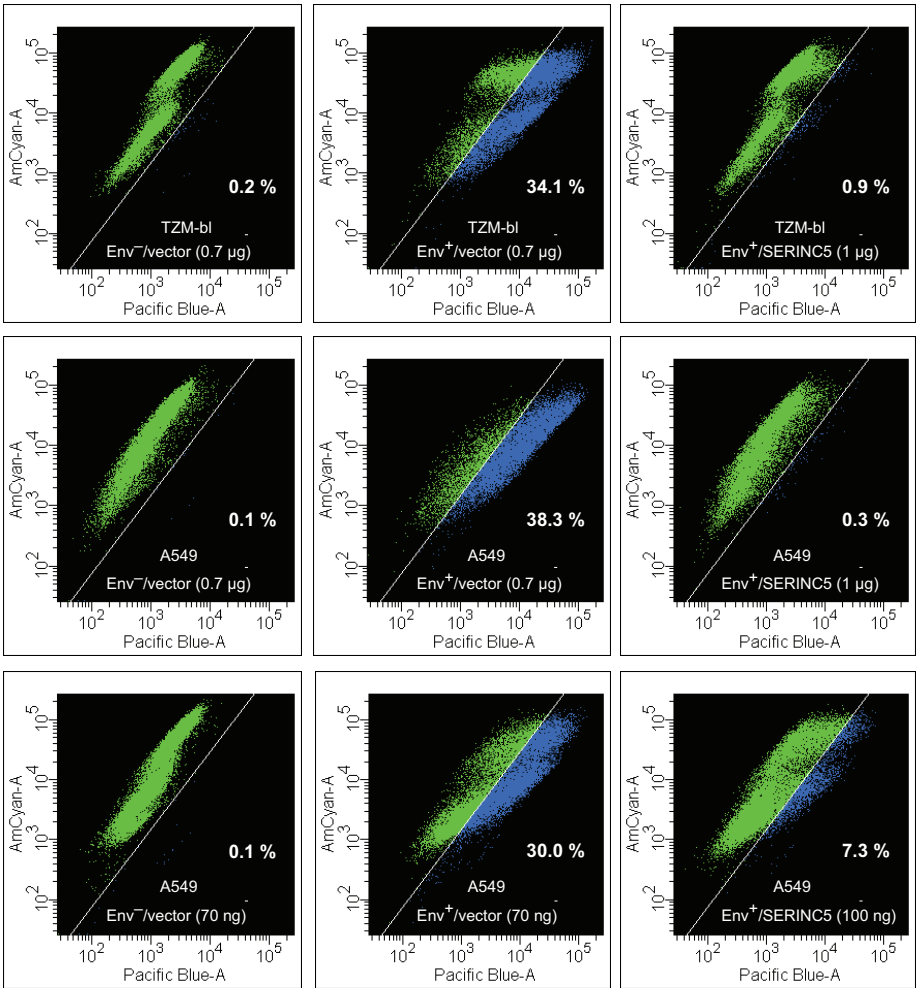
Extended Data Figure 2 | MLV glycoGag inhibits the incorporation of SERINC3 and SERINC5 into HIV-1 virions. **a**, **b**, Western blots showing the effects of wild-type or mutant glycoMA on the incorporation of SERINC3–HA (**a**) or SERINC5–HA (**b**) into Nef^{-} HIV-1 virions. The NL4-3/glycoMA proviral construct expresses untagged glycoMA *in cis*. In all other cases, HA-tagged (**a**) or Flag-tagged (**b**) glycoMA proteins were expressed *in trans*. The white bands marked by asterisks are caused by co-migrating HIV-1 $Pr55^{gag}$. Both experiments were performed twice.



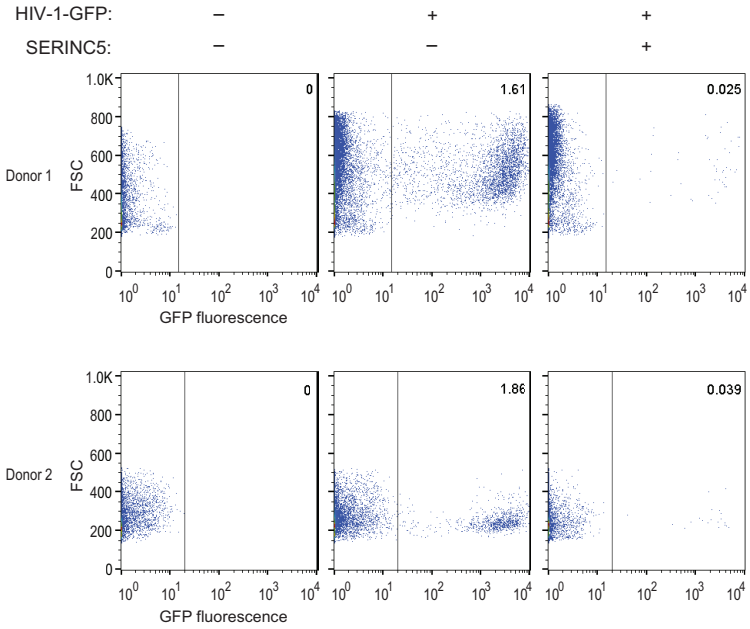
Extended Data Figure 3 | Nef and glycoGag downregulate SERINC5 from the cell surface. **a**, SERINC5 re-localizes from the plasma membrane to perinuclear vesicles in the presence of glycoGag. HeLa or U2-OS cells transiently expressing SERINC5–mCherry alone or together with glycoGag were examined by live-cell fluorescence microscopy. **b**, Nef and glycoGag both downregulate SERINC5. JTAg cells transiently expressing SERINC5(iHA), either alone or together with Nef_{SF2} or glycoGag, were surface-stained with anti-HA antibody and analysed by flow cytometry. Per cent fractions of cells expressing SERINC5(iHA) on the surface are indicated. This experiment was performed twice.



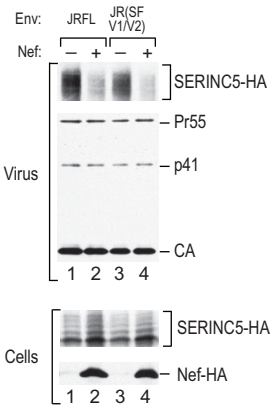
Extended Data Figure 4 | *SERINC* mRNA expression levels. **a**, Expression of *SERINC* family members in uninfected and HIV-infected Jurkat E6.1 cells. RNA was extracted at the peak of infection with wild-type (Nef^+) or Nef^- HIV-1_{NL43}, and gene expression was quantified by RNA-seq as reads per kilobase of coding sequence per million reads (RPKM) ($n = 1$). The HIV-1 budding factor *TSG101* and the housekeeping gene *HPRT1* are included for comparison. **b**, Levels of *SERINC3* and *SERINC5* mRNA (arbitrary units) in cell lines and primary cells, as measured by qRT–PCR ($n = 3$). PBMC were left unstimulated or stimulated with 0.5 μg ml^{-1} phytohemagglutinin (PHA) and 20 U ml^{-1} IL-2 for 2 days. **c**, *SERINC5* mRNA expression is not induced by INF-α. PBMC were left untreated or treated with 1,000 U ml^{-1} human INF-α 2a (PBL Assay Science) for 14 h ($n = 2$). Data are mean and s.d. NS, not significant ($P > 0.05$) two-tailed unpaired *t*-test.



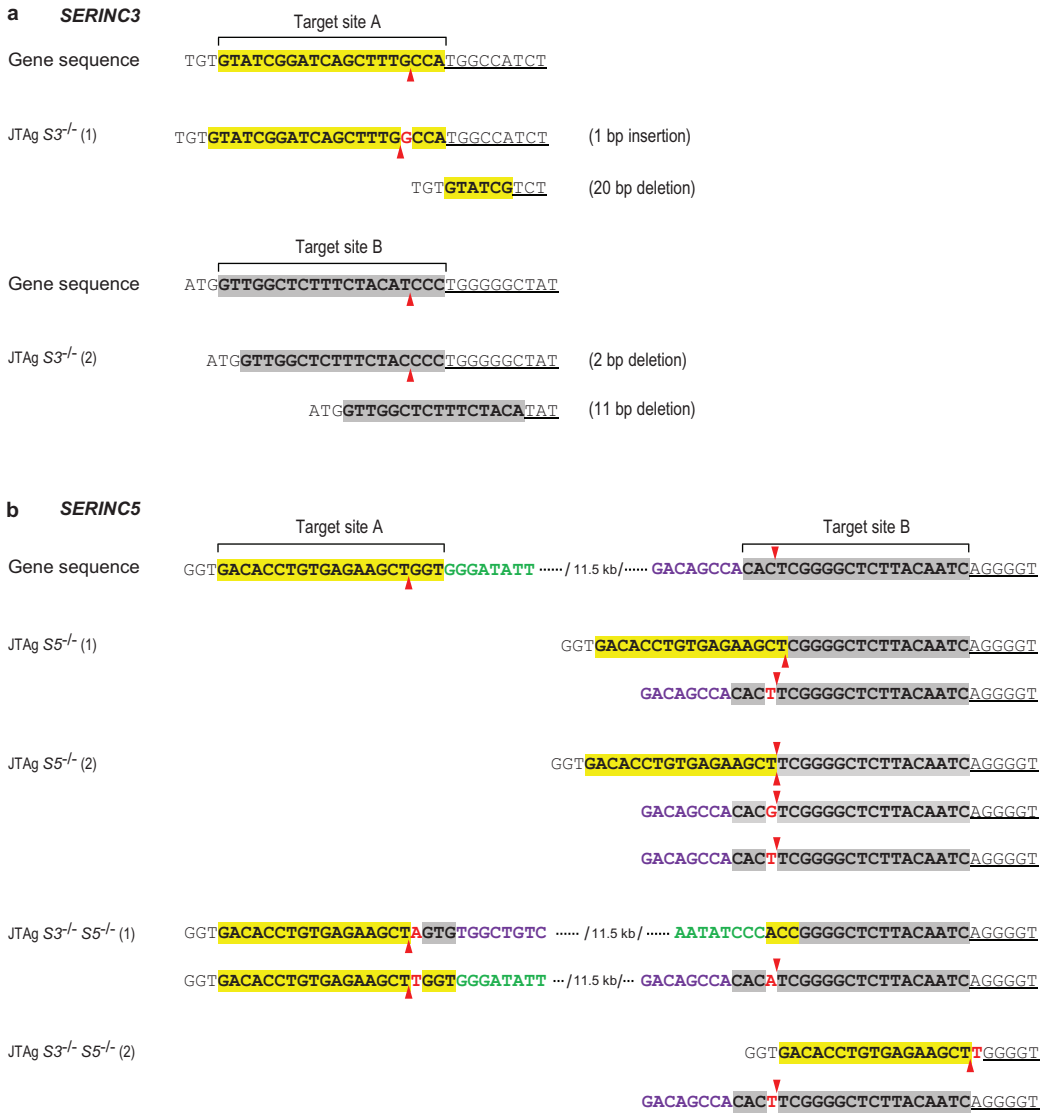
Extended Data Figure 5 | Exogenous SERINC5 inhibits the fusion of progeny virions with target cells. TZM-bl or A549/CD4/CXCR4 cells were exposed to equal amounts of virus containing BlaM-Vpr, and fusion was analysed by measuring the Env-dependent increase in blue fluorescence using multiparameter flow cytometry. Virions were produced in 293T cells transfected with an Env^- HIV-1 provirus, a vector expressing Env_{HXB2} (Env^+) or a frameshift mutant (Env^-), a vector expressing BlaM-Vpr, and a vector expressing SERINC5 (1 µg or 100 ng) or an equimolar amount of the empty vector (0.7 µg or 70 ng). The percentage of cells displaying increased blue fluorescence is indicated.



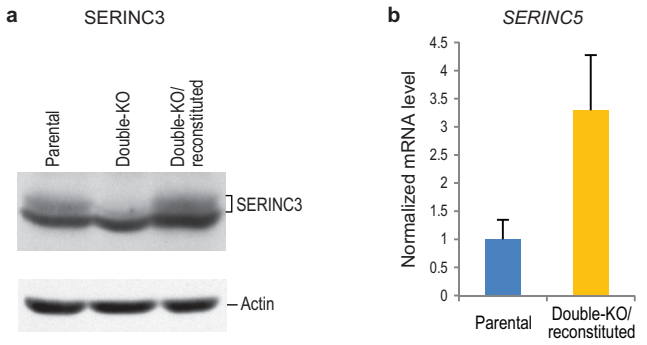
Extended Data Figure 6 | Exogenous SERINC5 reduces the infectivity of Nef⁻ HIV-1 progeny virions for primary target cells. In two independent experiments, PHA-stimulated PBMC from different donors were infected with equal amounts of single-cycle GFP–HIV-1 virions produced in 293T cells in the absence or presence of exogenous SERINC5. Per cent fractions of infected (GFP-positive) cells are indicated.



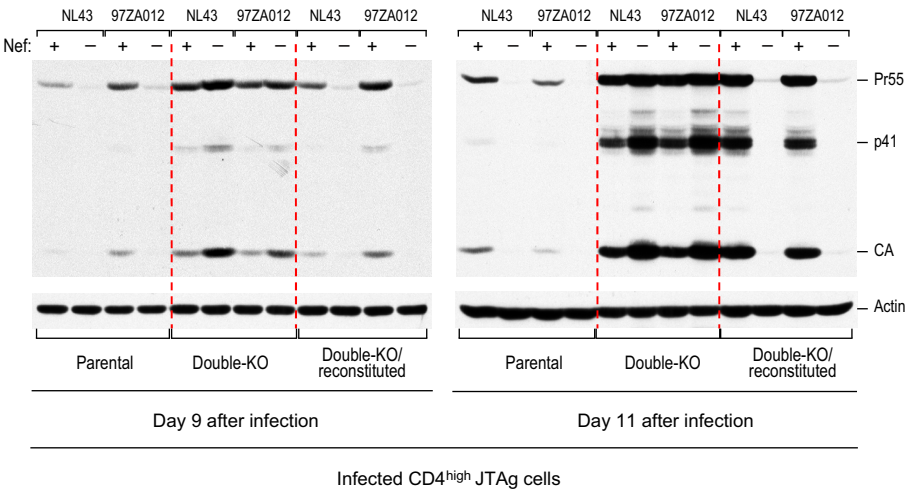
Extended Data Figure 7 | SERINC5 incorporation into HIV-1 virions that differ in Nef responsiveness. Recombinant virions were produced in 293T cells co-transfected with the HXB/Env^{-}/Nef^{-} provirus and vectors expressing the poorly Nef-responsive Env_{JRFL} or the highly Nef-responsive JR(SF V1/V2) Env chimaera, along with a vector expressing SERINC5–HA. Empty pBJ5 vector or a version expressing HA-tagged $Nef_{97ZA012}$ was also co-transfected. SERINC5–HA in purified virions was detected by western blotting. This experiment was performed twice.



Extended Data Figure 8 | Characterization of JTAg knockout cells. **a**, Mutant *SERINC3* alleles identified in *SERINC3* knockout clones. **b**, Mutant *SERINC5* alleles identified in *SERINC5* knockout and *SERINC3/5* double-knockout clones. The single-guide RNA (sgRNA) target sites are highlighted, and the predicted Cas9 target sites are indicated by arrowheads. Inserted nucleotides are in red. One of the two mutated *SERINC5* alleles in JTAg $S3^{-/-}$ $S5^{-/-}$ (1) cells has an inversion between sgRNA target sites A and B. JTAg $S5^{-/-}$ (2) cells contain three mutated *SERINC5* alleles. All mutations cause frameshifts and/or large deletions of coding sequence. No wild-type alleles were detected in any of the knockout clones.



Extended Data Figure 9 | SERINC3 and SERINC5 expression levels in reconstituted double-knockout cells. a, SERINC3 protein levels in parental, double-knockout, and reconstituted double-knockout JTAg cells were compared by western blotting. SERINC3 migrated close to a prominent background band that was also recognized by the anti-SERINC3 antibody. **b**, *SERINC5* mRNA levels in parental and reconstituted double-knockout JTAg cells were compared by qRT–PCR ($n = 3$).



Extended Data Figure 10 | Effects of *SERINC* knockout and reconstitution on HIV-1 replication. Parental, double-knockout and SERINC3+SERINC5-reconstituted double-knockout $CD4^{high}$ JTAg cells were analysed by immunoblotting with anti-HIV CA at days 9 and 11 after infection with equal amounts (2 ng ml^{-1} p24) of $HIV\text{-}1_{NL43}$ encoding either wild-type or disrupted versions of Nef_{NL43} or $Nef_{97ZA012}$.