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Steven W. Jin
Simon Fraser University

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Graphical Abstract
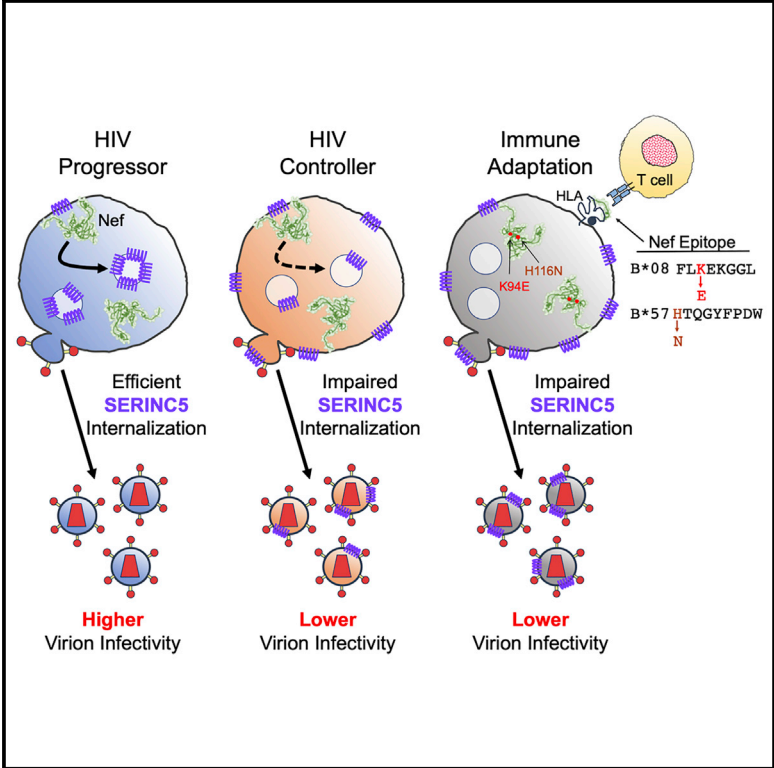
Authors

Steven W. Jin, Nirmin Alsahafi, Xiaomei T. Kuang, ..., Andrés Finzi, Zabrina L. Brumme, Mark A. Brockman

Correspondence
mark_brockman@sfu.ca

In Brief

HIV-1 Nef counteracts the cellular restriction factor SERINC5, but the importance of this for pathogenesis is unclear. Jin et al. show that Nef clones isolated from HIV controllers display lower ability to antagonize SERINC5, in part because of viral mutations that are selected to evade host T cells.

Highlights

- HIV-1 Nef clones from elite controllers display impaired SERINC5 antagonism

- Nef polymorphisms associated with variable SERINC5 antagonism are identified

- HLA B*57 and B*08-associated Nef mutations reduce SERINC5 antagonism

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Report

Natural HIV-1 Nef Polymorphisms Impair SERINC5 Downregulation Activity

Steven W. Jin,[1] Nirmin Alsahafi,[2,3] Xiaomei T. Kuang,[4] Shayda A. Swann,[1] Mako Toyoda,[5] Heinrich Göttlinger,[6] Bruce D. Walker,[7,8] Takamasa Ueno,[5] Andrés Finzi,[2,3,9] Zabrina L. Brumme,[1,10] and Mark A. Brockman[1,4,10,11,*]

[1]Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada
[2]Centre de Recherche du CHUM, Montreal, QC, Canada
[3]Department of Microbiology and Immunology, McGill University, Montreal, QC, Canada
[4]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada
[5]Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[6]Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA, USA
[7]Ragon Institute of MGH, MIT, and Harvard, Cambridge, MA, USA
[8]Howard Hughes Medical Institute, Cambridge, MA, USA
[9]Department of Microbiologie, Infectiologie et Immunologie, Université de Montréal, Montreal, QC, Canada
[10]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada
[11]Lead Contact
*Correspondence: mark_brockman@sfu.ca
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SUMMARY

HIV-1 Nef enhances virion infectivity by counteracting host restriction factor SERINC5; however, the impact of natural Nef polymorphisms on this function is largely unknown. We characterize SERINC5 downregulation activity of 91 primary HIV-1 subtype B *nef* alleles, including isolates from 45 elite controllers and 46 chronic progressors. Controller-derived Nef clones display lower ability to downregulate SERINC5 (median 80% activity) compared with progressor-derived clones (median 96% activity) (p = 0.0005). We identify 18 Nef polymorphisms associated with differential function, including two CTL escape mutations that contribute to lower SERINC5 downregulation: K94E, driven by HLA-B*08, and H116N, driven by the protective allele HLA-B*57. HIV-1 strains encoding Nef K94E and/or H116N display lower infectivity and replication capacity in the presence of SERINC5. Our results demonstrate that natural polymorphisms in HIV-1 Nef can impair its ability to internalize SERINC5, indicating that variation in this recently described function may contribute to differences in viral pathogenesis.

INTRODUCTION

The HIV-1 Nef protein is crucial for viral pathogenesis (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995), serving to modulate diverse cellular events related to vesicular transport, signal transduction and actin cytoskeletal remodeling that collectively enhance viral infectivity and replication (Abraham and Fackler, 2012; Cheng-Mayer et al., 1989;

Chowers et al., 1994; Landi et al., 2011; Miller et al., 1994; Pereira and daSilva, 2016; Terwilliger et al., 1986; Tokarev and Guatelli, 2011). Nef's well-characterized abilities to internalize CD4 and HLA class I from the infected cell surface also allow HIV-1 to evade antibody-dependent cellular cytotoxicity (ADCC) and cytotoxic T lymphocytes (CTLs) (Aiken et al., 1994; Alsahafi et al., 2015, 2017; Collins et al., 1998; Garcia and Miller, 1991; Schwartz et al., 1996).

Nef-mediated enhancement of viral infectivity was recently shown to be due in part to its ability to counteract members of the serine incorporator (SERINC) family of host restriction factors, of which SERINC5 is most potent (Rosa et al., 2015; Usami et al., 2015). SERINC5 enters into the membrane of progeny virions and inhibits fusion with target cells (Sood et al., 2017). Nef prevents this by internalizing SERINC5 from the cell surface and trafficking it to lysosomes via an endosomal route that is similar to that used to downregulate CD4 (Shi et al., 2018). Several Nef mutations are reported to impair its ability to antagonize SERINC5, including G2A, D123A, and LL165AA, which block myristoylation, dimerization, and interaction with AP-2 trafficking complexes, respectively (Foster et al., 2011); however, while HIV-1 *nef* exhibits extensive genetic diversity (Brumme et al., 2007; Foster et al., 2001), these mutations are rare in circulating viral strains (all >99% conserved; HIV Sequence Database, https://www.hiv.lanl.gov). Studies to examine the impact of naturally occurring HIV-1 Nef polymorphisms on its ability to counteract SERINC5 have not been conducted. In a prior study, we demonstrated that Nef clones isolated from HIV-1 elite controllers, who spontaneously suppress plasma viremia without therapy (Deeks and Walker, 2007), displayed functional impairments despite the absence of obvious genetic defects (Mwimanzi et al., 2013). Rather, reduced function was linked to natural variation in *nef* sequences, including mutations selected by the protective HLA allele B*57, indicating that viral adaptation to host immune selection pressure contributed to attenuation in at least some cases.
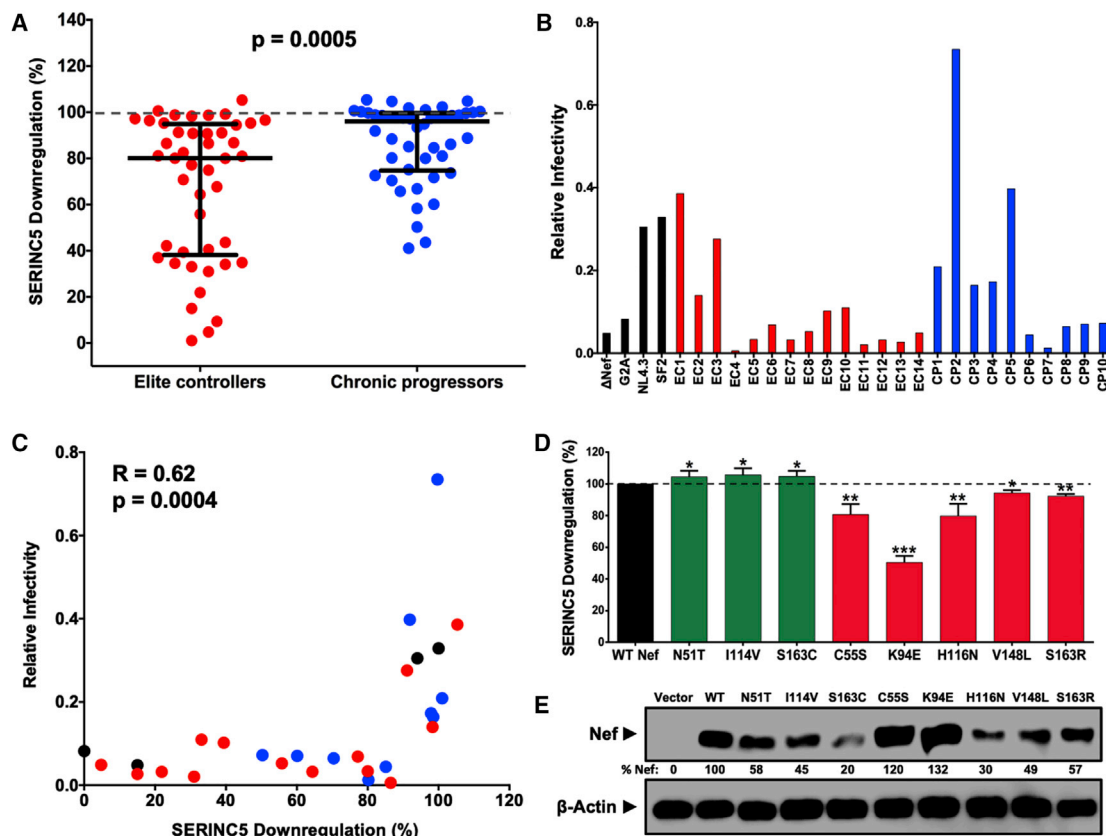
Figure 1. Lower SERINC5 Downregulation by Nef Isolates from HIV Controllers

(A) SERINC5 downregulation function was compared between Nef clones from 45 elite controllers (EC; red) and 46 chronic progressors (CP; blue) (p < 0.001, Mann-Whitney U-test) using flow cytometry (see Figure S1; STAR Methods). Mean function of each clone (relative to Nef SF2 strain) is reported, on the basis of triplicate data from three independent experiments. Bars represent median (± interquartile range) of all EC or CP Nef clones.

(B) Relative infectivity of NL4.3 strains encoding 24 *nef* alleles (14 elite controllers, red; 10 chronic progressors, blue) and 4 controls (G2A, ΔNef, NL4.3 Nef, and SF2 Nef, black) was quantified as the quotient of infectivity for each virus generated in the presence of SERINC5 divided by that of the same virus generated in the absence of SERINC5. All viruses were tested at least twice in independent experiments. Mean results based on triplicate data from one representative experiment are shown.

(C) Correlation between SERINC5 downregulation and viral infectivity is shown for data described in (A) and (B) (Spearman R = 0.62, p = 0.0004).

(D) Eight Nef polymorphisms (see Table 1) were confirmed by mutagenesis. Results for mutants that were anticipated to increase (green) or decrease (red) SERINC5 downregulation function are reported as mean (±SD), on the basis of at least three independent experiments. Significant differences compared with NL4.3 Nef (100%) are indicated by asterisks (unpaired Student's t test): *p < 0.05, **p < 0.01, and ***p < 0.001.

(E) Nef expression was assessed by western blot, and percentage relative to NL4.3 Nef is indicated.

RESULTS

Primary HIV-1 Nef Alleles Display Variable Abilities to Internalize SERINC5

To assess whether SERINC5 antagonism differs among circulating HIV-1 strains, we characterized the ability of 91 primary subtype B *nef* alleles (representative isolates collected from 45 elite controllers and 46 chronic progressors during untreated infection) to downregulate SERINC5 using a transfection-based assay (Figure S1; STAR Methods). The function of each Nef clone was normalized to that of a control subtype B Nef isolate (SF2 strain), such that activity better or worse than SF2 Nef is reported as >100% or <100%, respectively. Empty vector and Nef G2A mutant were included as negative controls. We observed that controller-derived clones displayed lower SERINC5 down-

regulation activity (median 80%, interquartile range [IQR] 38%–95%) compared with progressor-derived clones (median 96%, IQR 75%–100%) (p = 0.0005, Mann-Whitney test; Figure 1A; Table S1). To confirm this, we engineered HIV-1 subtype B reference strain NL4.3 to encode each of 24 *nef* alleles that were selected to display a range of SERINC5 downregulation phenotypes, as well as control *nef* sequences, and then produced virions in the absence or presence of SERINC5. We observed that relative viral infectivity, calculated as the ratio of infectivity for each strain produced in the presence versus absence of SERINC5, was variable (Figure 1B). Consistent with prior studies showing that internalization of SERINC5 is a key mediator of Nef's ability to counteract this restriction factor (Rosa et al., 2015), a strong correlation was found between SERINC5 downregulation function and viral infectivity

Table 1. Nef Polymorphisms Associated with SERINC5 Downregulation (N ≥ 5, p < 0.05, q < 0.35)

Codon[a]	Amino Acid (AA)	Median Nef Activity		Number of Individuals		Impact[b]	p Value	q Value
		With AA	Without AA	With AA	Without AA			
11	A	100.5	86.5	6	75	+14	0.005	0.22
19	K	100.7	85.6	9	82	+15	0.011	0.25
28	D	85.1	93.4	47	44	−8	0.047	0.34
43*	V	39.4	88.4	5	85	−49	0.025	0.33
51	T	95.3	80	43	45	+15	0.004	0.22
51	N	80	96	42	46	−16	0.005	0.22
55	C	90.8	73.7	82	9	+17	0.03	0.33
65	E	84.9	98.3	84	7	−13	0.01	0.25
94*	E	50.3	88.6	5	86	−38	0.015	0.28
94	K	88.4	55.2	83	8	+33	0.047	0.34
114	V	89.8	74.9	70	21	+15	0.037	0.34
116	H	95.3	80.6	59	32	+15	0.012	0.25
116*	N	80.9	95.3	31	60	−14	0.02	0.3
148	V	89.8	66.8	78	13	+23	0.041	0.34
163	C	97.2	83.6	27	64	+14	0.028	0.33
163	R	39.4	87.6	7	84	−48	0.033	0.33
170	L	81	91.1	55	36	−10	0.049	0.34
182	Q	97.9	85.1	10	81	+13	0.021	0.3

[a]HXB2-aligned residues; Nef polymorphisms associated with evasion from CD8+ T cells are indicated by asterisks (Brumme et al., 2007).
[b]Median Nef activity with AA − median Nef activity without AA, rounded to nearest whole number.

(Spearman R = 0.62, p = 0.0004; Figure 1C). These data also indicated that ~80% normalized SERINC5 downregulation function was necessary to enhance infectivity in our assays, suggesting that Nef must maintain relatively high SERINC5 internalization activity to be effective and, conversely, that relatively modest impairment of this function may be biologically relevant. While additional studies are needed to assess the activity of each primary Nef allele against a wider range of SERINC5 expression levels, we noted that more than half of controller-derived clones displayed SERINC5 downregulation activity below 80%, compared with one-quarter of progressor-derived clones (p = 0.02, Mann-Whitney test). Together, these results demonstrate that the ability of Nef to internalize SERINC5 varies among circulating isolates and that this function is impaired in clones isolated from elite controllers.

Nef Polymorphisms Are Associated with SERINC5 Downregulation Function

Analysis of our linked genotype-phenotype dataset (see STAR Methods) identified 18 naturally occurring Nef polymorphisms, located at 14 codons, that were associated with differential SERINC5 downregulation activity (p < 0.05 for all, Mann-Whitney test) (Table 1). The most statistically significant correlation was observed at Nef codon 51, where clones encoding threonine (n = 43) displayed higher activity (median 95.3%) compared with clones that did not (n = 45, typically asparagine) (81%) (p = 0.004). We validated eight polymorphisms by introducing the mutation into a subtype B Nef isolate (NL4.3 strain), each of which resulted in the expected change in function (p < 0.05 for all, Student's t test) (Figure 1D). Specifically, downregulation

function was increased modestly for N51T, I114V, and S163C mutants, each of which displayed ~5% higher activity compared with parental Nef; whereas downregulation was impaired for C55S, K94E, H116N, V148L, and S163R mutants, each of which displayed 5%–50% lower activity compared with parental Nef. Of note, Nef codon 51 is associated with viral infectivity (Carl et al., 2001). Furthermore, Nef codon 163 is located adjacent to the dileucine motif that is critical for SERINC5 antagonism (Rosa et al., 2015; Shi et al., 2018) and has been shown previously to affect Nef binding to clathrin adaptor protein complexes (Coleman et al., 2006). While steady-state expression of these Nef mutants was variable (Figure 1E), SERINC5 downregulation activity did not correlate with our ability to detect Nef by western blot, indicating that changes in Nef function were unlikely to be due to differences in protein stability.

SERINC5 Downregulation Correlates with CD4 Internalization

The mechanism of Nef-mediated SERINC5 downregulation shares several features with that of CD4 downregulation, including reliance on Nef's dileucine motif (LL165) (Rosa et al., 2015; Shi et al., 2018) and trafficking of the internalized receptors to lysosomes through endosomal compartments (Shi et al., 2018). To explore potential associations between Nef functions, we compared the SERINC5 downregulation activity of our 91 Nef clones with prior results for CD4 and HLA downregulation as well as protein stability, reported by Mwimanzi et al. (2013) (Figure 2; Table S1). Notably, SERINC5 downregulation correlated more strongly with the ability of Nef clones to internalize CD4 (Spearman R = 0.55, p < 0.0001) compared with HLA
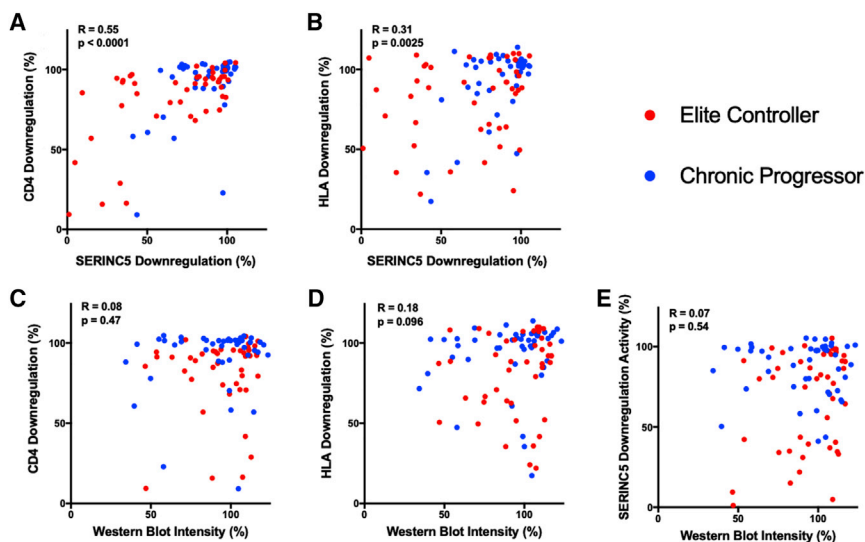
Figure 2. Associations between Nef Functions and Protein Stability

(A and B) The ability of each elite controller (red) or chronic progressor (blue) Nef clone to downregulate SERINC5 was compared with that for CD4 (A) and HLA class I (A*02) (B).

(C–E) Next, the ability of each Nef clone to downregulate CD4 (C), HLA class I (D), or SERINC5 (E) was compared with its steady-state protein expression as detected by western blot.

Correlations were assessed using Spearman rank test. Data for CD4 and HLA downregulation and western blot were reported in Mwimanzi et al., (2013).

(R = 0.31, p = 0.0025). In addition, polymorphisms at S163 that are adjacent to the dileucine motif were similarly associated with differential ability of Nef to downregulate CD4 (Mwimanzi et al., 2013). We observed no correlation between Nef expression levels detected by western blot and downregulation of SERINC5, CD4, or HLA, indicating that associations between these functions were not due simply to differences in protein stability; however, additional studies will be needed to address this. These results suggest that similar polymorphisms in circulating *nef* alleles contribute to variation in SERINC5 and CD4 downregulation functions, while effects on HLA downregulation activity may be more independent.

Viral Adaptation to T Lymphocytes Contributes to Variability in SERINC5 Downregulation Activity

Notably, three Nef polymorphisms that were associated with a substantial reduction in SERINC5 downregulation function, I43V (−49% activity), K94E (−38%), and H116N (−14%) (Table 1), are viral "escape mutations" selected in CD8+ T cell epitopes restricted by HLA-C*03, B*08, and B*57, respectively (Altfeld et al., 2006; Brumme et al., 2008). This indicates that immune pressure on Nef may attenuate its ability to antagonize SERINC5. Notably, H116N is often selected early following infection in individuals expressing the protective B*57 allele (Brumme et al., 2008), and it is the predominant variant observed for the HW9 epitope (at position 1 of HTQGYFPDW). Thus, we speculate that reduced Nef-mediated SERINC5 downregulation may contribute to lower viremia in these cases. In contrast, K94E is selected more slowly in individuals expressing the non-protective B*08 allele (Brumme et al., 2008) and 94E is not the dominant variant observed for the FL8 epitope (at position 5 of FLKEKGGL) (rather K92R). This suggests that Nef can adapt to B*08-restricted T cell pressure without enduring the detrimental impact of this polymorphism on SERINC5 downregulation. Additional studies will be needed to explore these links between host immune pressure and Nef function in greater detail.

Viral Infectivity and Replication Capacity Are Impaired by K94E and H116N

To assess the impact of K94E and H116N on viral phenotypes, we generated HIV-1 NL4.3-derived strains encoding these mutations in the absence or presence of SERINC5. Consistent with downregulation results, we observed lower SERINC5 surface expression on HEK293T cells producing wild-type NL4.3 compared with cells producing viruses that lacked Nef (ΔNef) or those that encoded Nef mutations that were anticipated to be detrimental to this function (G2A, K94E, H116N, or K94E/H116N) (Figure 3A). Moreover, while viral infectivity was comparable for all NL4.3-derived strains generated in the absence of SERINC5 (Figure 3B, white bars), differences in infectivity were seen among viruses generated in the presence of SERINC5. Specifically, ΔNef virus was 41-fold less infectious compared with wild-type NL4.3, while G2A, K94E, H116N, and double-mutation viruses were 11-, 4-, 3-, and 5.5-fold less infectious than NL4.3, respectively (Figure 3B, black bars) (p < 0.05 for all, unpaired Student's t test). Infectivity of the ΔNef mutant was modestly impaired in the absence of SERINC5 (1.7-fold lower compared with NL4.3), which may be due to SERINC3 expression by HEK293T cells (Usami et al., 2015). Notably, NL4.3-derived viruses encoding Nef K94E, H116N, or the double mutation also displayed reduced infectivity when they passaged using primary peripheral blood mononuclear cells (PBMCs) (Figure 3C), indicating that these polymorphisms altered viral phenotypes in the presence of endogenous levels of SERINC5. Next, we examined the impact of these Nef mutations on viral replication using Jurkat-derived GFP reporter T cells, which express high endogenous levels of SERINC5 (Rosa et al., 2015). The results of these studies were consistent with those obtained for SERINC5 downregulation and viral infectivity enhancement by each mutant. Representative data from one experiment are shown in Figure 3D. In repeated assays, we observed statistically significant delays in viral replication (measured as the slope of viral cell-to-cell spread over time) for viruses encoding Nef K94E, H116N, or the double mutation compared with wild-type NL4.3 (p < 0.05 for all). To confirm that these differences were due to SERINC5, we generated a SERINC5 knockout (KO) clone of the Jurkat-GFP reporter T cell line (Figure S2)
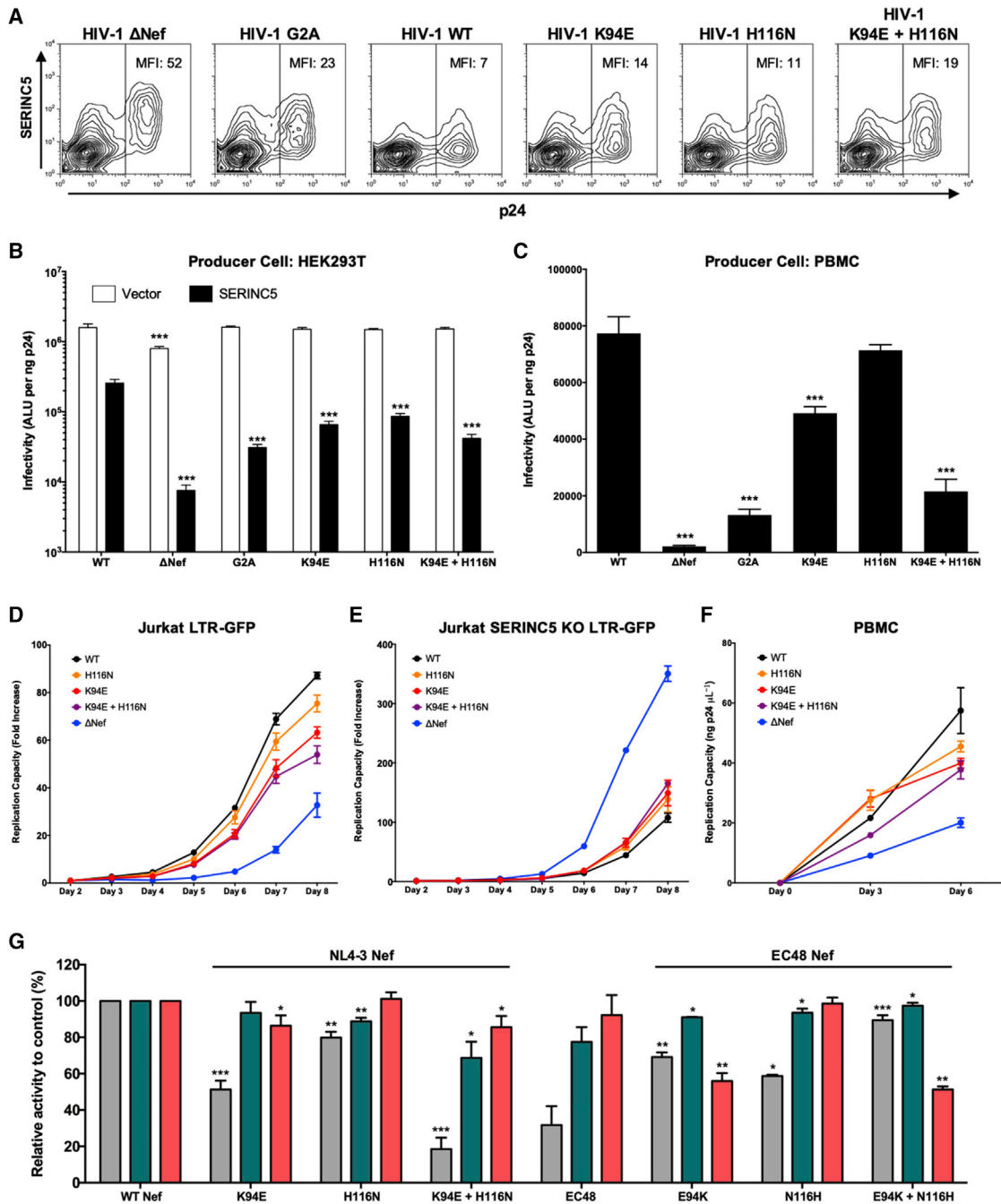
Figure 3. Impact of K94E and H116N Mutants on Nef Function

(A) Representative flow cytometry plots display SERINC5 surface expression on cells co-transfected with pSERINC5-iHA and pNL4.3. MFI of SERINC5 in the p24+ gate is indicated.

(B and C) Infectivity of NL4.3-derived viruses produced in the absence (white) or presence (black) of SERINC5-iHA (B) or viruses passaged once in healthy donor PBMCs (C) is shown. Results reflect luminescence (absolute light units [ALUs]) following incubation of TZM-bl reporter cells with a normalized amount of virus. Data are representative of two independent experiments (B) or PBMCs from three independent donors (C). Unpaired Student's t test was used to compare each mutant to NL4.3 (***$p < 0.001$).

(D and E) Viral replication was examined using Jurkat LTR-GFP R5 cells (D) or those in which SERINC5 was knocked out using CRISPR/Cas9 (Figure S2; STAR Methods) (E). Viral spread was monitored by flow cytometry. The mean (±SD) fold increase in percentage GFP+ cells (from day 2) is reported for each mutant, on the basis of triplicate infections. Results are representative of three experiments.

(legend continued on next page)

and examined the impact of Nef mutations on viral replication using this line. Representative data from one experiment are shown in Figure 3E. In repeated assays, we observed no significant differences in replication capacity between NL4.3-derived viruses encoding wild-type Nef versus Nef K94E, H116N, or the double mutation in cells lacking SERINC5 (p > 0.05 for all). Unexpectedly, NL4.3 viruses encoding Nef mutants displayed a modest enhancement of replication in our SERINC5 KO cell line, which was significant for the virus lacking Nef (ΔNef) (p < 0.01). We confirmed that this strain lacked Nef (Figure S2) and also obtained similar results using a second independent SERINC5 KO clone (data not shown). While the mechanism for this is unclear, similar results have been observed for some Nef mutant viruses in other studies (Wu et al., 2019), suggesting that other Nef functions modulate viral replication and that these may differ in part depending on cell type (Li et al., 2019; Wu et al., 2019). Finally, to further validate our observations, we performed viral replication assays in PHA-stimulated PBMCs infected with equivalent amounts of each virus (5 ng p24) and measured viral Gag p24 in culture supernatants at days 0, 3, and 6. Representative data from one experiment are shown in Figure 3F. Results from repeated assays were consistent with our prior observations, indicating that viruses encoding Nef K94E, H116N, or the double mutation displayed significantly lower replication capacity in primary cells compared with wild-type NL4.3 (p < 0.01 for all). Together, these studies demonstrate that K94E and H116N impair viral infectivity and replication capacity in the presence of SERINC5.

K94E and H116N Selectively Impair Nef-Mediated SERINC5 Downregulation Function

To determine if the effect of K94E or H116N on SERINC5 downregulation was selective, we next tested the ability of Nef mutants encoding these substitutions to internalize CD4 and HLA class I (Figure 3G, left side). Compared with parental NL4.3 Nef, K94E reduced HLA downregulation activity modestly (to 87%) (p < 0.05, unpaired Student's t test) but it had no effect on CD4 internalization. Conversely, H116N reduced CD4 downregulation activity modestly (to 89%) (p < 0.05), but it had no effect on HLA internalization. All three Nef-mediated downregulation functions were impaired in the mutant encoding both K94E and H116N, indicating that the impact of these polymorphisms can be additive. In particular, SERINC5 downregulation activity was reduced markedly in the Nef double mutant (to 19%) (p < 0.001), while more moderate effects were seen for downregulation of CD4 (69%) and HLA (87%) (p < 0.05 for both). To explore this further, we identified an elite controller-derived Nef clone (EC48) encoding E94 and N116 that displayed poor SERINC5 downregulation activity (32%) but retained moderate CD4 and HLA internalization functions (78% and 92%, respectively). To confirm that E94 and/or N116 were determinants of impaired SERINC5 downregulation function by this pri-

mary Nef clone, we reverted these polymorphisms to the consensus residue (Figure 3G, right side). Consistent with results for NL4.3 Nef, reversion of E94K or N116H alone partially rescued SERINC5 downregulation activity (to 69% and 59%, respectively) (p < 0.05 for both); while reversion of both polymorphisms enhanced this activity further (to 89%) (p < 0.001). In contrast to results obtained using NL4.3 Nef, the E94K and double reversion mutant displayed lower HLA downregulation activity compared with the primary Nef clone (56% and 51%, respectively) (p < 0.01), suggesting that unidentified compensatory polymorphisms in the EC48 nef sequence contributed to maintain this function. Together, these results demonstrate that K94E and H116N reduce Nef's ability to downregulate SERINC5 to a greater extent compared with CD4 or HLA class I and further indicate that their impact on all three Nef functions may be modulated by other sequence variation present in primary nef alleles.

DISCUSSION

We have demonstrated that circulating HIV-1 subtype B nef alleles exhibit substantial variability in SERINC5 downregulation function. Nef clones isolated from elite controllers displayed lower in vitro function compared with those from chronic progressors, suggesting that impaired SERINC5 antagonism may contribute to lower plasma viremia and slower disease progression in this rare group of individuals. Consistent with the observation that even limited amounts of SERINC5 incorporation into viral particles reduces infectivity (Trautz et al., 2016), we found that Nef-mediated infectivity enhancement required ∼80% normalized SERINC5 downregulation activity in our assays, a threshold that was not met by 51% of elite controller-derived Nef clones.

While a prior study examined the ability of diverse HIV and SIV Nef isolates to antagonize SERINC5 (Heigele et al., 2016), it assessed relatively few (∼15) HIV-1 group M clones. Our analysis of 91 HIV-1 subtype B Nef clones offers greater power to explore functional variation among patient-derived sequences. Here, we identified 18 polymorphisms that were associated with differential SERINC5 downregulation activity. We validated 8 of these polymorphisms, including 2 well-characterized CD8+ T cell escape mutations, K94E and H116N, using site-directed mutagenesis. While mechanisms responsible for impaired SERINC5 internalization by primary nef alleles or mutants have not been defined in this study, it is intriguing that K94E and H116N had only modest effects on Nef's ability to downregulate CD4 or HLA class I, suggesting that K94 and H116 lie within domains of Nef that are particularly important to antagonize SERINC5 rather than to internalize CD4 or HLA.

Our results indicate that Nef polymorphisms can impair viral infectivity and replication in the presence of SERINC5;

(F) Replication was also assessed following infection of PHA-stimulated PBMCs. Supernatant was harvested on days 0, 3, and 6 and viral p24 quantified using ELISA. Results are representative of PBMCs from three donors.
(G) The ability of NL4.3 Nef mutants encoding K94E and/or H116N or EC48 Nef reversion mutants encoding E94K and/or N116H to downregulate SERINC5, CD4, and HLA is shown. Bars represent the mean (±SD) function, normalized to control, in at least three experiments. Unpaired Student's t test was used to compare each mutant with control (*p < 0.05, **p < 0.01, and ***p < 0.001).

however, additional work is needed to assess the importance of Nef-mediated SERINC5 antagonism for HIV-1 pathogenesis. Our ability to address this question is hindered by an incomplete understanding of SERINC5 restriction mechanisms and by Nef's continuously evolving role as a mediator of viral infectivity (Li et al., 2019; Wu et al., 2019). A limitation of this study is that we did not directly measure SERINC5 incorporation into the virions of strains encoding Nef mutants. Strong correlations between SERINC5 downregulation activity and viral infectivity suggest that internalization of SERINC5 is the most important contribution of Nef in this setting, but we cannot rule out a role for other known (or unknown) Nef functions. In addition, HIV-1 Envelope modulates viral sensitivity to SERINC5 (Beitari et al., 2017), but its relative impact compared with Nef has not been elucidated, and potential functional interactions between primary *nef* and *env* alleles have not been studied. Furthermore, Nef's ability to counteract SERINC5 is dependent on the expression levels of both proteins (Schulte et al., 2018; Trautz et al., 2016), which may not be recapitulated fully by our *in vitro* assays. While our results using primary cells are consistent with data collected using T cell lines, validation using other cell types is needed. Despite these limitations, our study provides strong evidence that natural variation in Nef-mediated SERINC5 antagonism function contributes to clinical outcomes following HIV-1 infection. Our data highlight the constraints placed on Nef by the interplay between adaptive host immunity and intrinsic host restriction mechanisms, which may result in viral attenuation during the course of natural infection.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.007.

AUTHOR CONTRIBUTIONS

Conceptualization, S.W.J., A.F., and M.A.B.; Reagents and Specimens, H.G., B.D.W., T.U., Z.L.B., and M.A.B.; Data Collection, S.W.J., N.A., X.T.K., S.A.S., and M.T.; Manuscript Writing, S.W.J. and M.A.B.; Manuscript Revisions, S.W.J., H.G., B.D.W., T.U., A.F., Z.L.B., and M.A.B.; Supervision, T.U., A.F., Z.L.B., and M.A.B.; Funding Acquisition, Z.L.B. and M.A.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Beta-Actin, Mouse clone AC-15	Sigma-Aldrich	Cat # A1978; RRID: AB_476692
CD4, Mouse Clone RPA-T4 (APC)	BD Biosciences	Cat # 555349; RRID: AB_398593
HA.11 Epitope Tag antibody (Alexa Fluor® 647)	BioLegend	Cat # 682404; RRID:AB_2566616
HIV-1 Core Antigen, Mouse clone KC57 (RD1)	Beckman Coulter	Cat # 6604667
Human HLA-A2 Antibody, Mouse clone BB7.2 (R-PE)	BD Biosciences	Cat # 558570; RRID: AB_647220
Mouse IgG, Goat polyclonal, minimal x-reactivity (HRP)	BioLegend	Cat # 405306; RRID: AB_315009
Nef, Rabbit polyclonal	NIH AIDS Reagents Program, gift of R Swanstrom (Shugars et al., 1993)	Cat # 2949
Rabbit IgG, Donkey polyclonal, minimal x-reactivity (HRP)	BioLegend	Cat # 406401; RRID: AB_2099368
SERINC5, Rabbit polyclonal	Abcam	Cat # ab204400
Bacterial and Virus Strains		
E. cloni 10G SOLOs Chemically Competent Cells	Lucigen	Cat # 0106-2
One Shot Stbl3 Chemically Competent *E. coli*	Invitrogen	Cat # C737303
Biological Samples		
Human Peripheral Blood Mononuclear Cells, Frozen	Stem Cell Technologies	Cat # 70025
HIV-1 subtype B Nef clones	Mwimanzi et al., 2013	N/A
Chemicals, Peptides, and Recombinant Proteins		
Lectin from Phaseolus vulgaris Erythrohemagglutinin (PHA-E)	Sigma-Aldrich	Cat # L8629
Human Recombinant IL-2	StemCell Technologies, Inc.	Cat # 78036
Fixation/Permeabilization Solution Kit	BD Biosciences	Cat # 554714
DNAfectin 2100 Transfection Reagent	Applied Biological Materials	Cat # G2100
TrypLE Express Enzyme (1X), phenol red	GIBCO	Cat # 12605010
Critical Commercial Assays		
Gene Pulser MXcell Electroporation System	Bio-Rad	Cat # 165-2670
96-Well Electroporation Plates	Bio-Rad	Cat # 1652681
HIV-1 p24 ELISA Kit	XpressBio	Cat # XB-1000
Steady-Glo® Luciferase Assay System	Promega	Cat # E2550
Deposited Data		
HIV-1 subtype B Nef sequences	Mwimanzi et al., 2013	GenBank accession JX171199-JX171243 (controllers) and JX440926-JX440971 (progressors)
Experimental Models: Cell Lines		
CEM-A*02, human T cell	Anmole et al., 2015	N/A
HEK293T, human kidney cell	American Type Culture Collection	Cat # CRL-3216 RRID: CVCL_0063
Jurkat LTR-GFP CCR5+ (JLTRG-R5), human T cell	NIH AIDS Reagents Program, gift of O Kutsch (Kutsch et al., 2004)	Cat #11586
Jurkat LTR-GFP CCR5+ (JLTRG-R5) SERINC5 knockout, human T cell	This paper	N/A
TZM-bl, human carcinoma cell, derived from HeLa	NIH AIDS Reagents Program, gift of JC Kappes and X Wu (Derdeyn et al., 2000; Platt et al., 1998; Wei et al., 2002)	Cat # 8129-442; RRID: CVCL_B478

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Nef_Forward Primer (AGAGCACCGG CGCGCCTCCA CATACCTASA AGAATMAGAC ARG)	This paper	N/A
Nef_Reverse Primer (GCCTCCGCGG ATCGATCAGG CCACRCCTCC CTGGAAASKC CC)	This paper	N/A
Recombinant DNA		
pHEF-VSVG	NIH AIDS Reagent Program, gift of L-J Chang (Chang et al., 1999)	Cat # 4693
pMaxFP-Green-N	Amaxa	Cat # VDF-1012
pNL4-3, infectious HIV-1 molecular clone	NIH AIDS Reagents Program, gift of M Martin (Adachi et al., 1986)	Cat # 114
pSELECT-GFPzeo-mcs	InvivoGen	Cat # psetgz-mcs
pSELECT-ΔGFP-zeo-mcs	This paper	N/A
pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene, provided by F Zhang (Cong et al., 2013; Ran et al., 2013)	Cat # 42230; RRID: Addgene_42230
Software and Algorithms		
FlowJo 9.9.5	FlowJo, LLC	FlowJo
Prism 7	Graphpad	Prism

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mark Brockman (mark_brockman@sfu.ca).

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Human subjects

All HIV-infected participants were recruited from the Boston (USA) area and provided written informed consent. None were receiving antiretroviral therapy at the time of specimen collection. Elite controllers (EC) were defined as having an untreated HIV-1 plasma viral load (pVL) of less than 50 RNA copies/mL for at least 2 years. Chronic progressors (CP) were defined as having an untreated pVL of at least 10,000 RNA copies/mL. The EC cohort (N = 45) displayed a median pVL of 2 RNA copies/mL (interquartile range [IQR] 0.2 – 14) and a median CD4 count of 811 cells per mm^3 (IQR 612 – 1022). The CP cohort (N = 46) displayed a median pVL of 80,500 RNA copies per mL (IQR 25,121 – 221,250) and a median CD4 count of 293 cells per mm^3 (IQR 73 – 440). Information regarding the sex of EC participants (84% male) is indicated in Table S1. Information regarding the sex of CP participants and the age of all participants was not recorded at the time of specimen collection, and therefore this data is unavailable. This study was approved by the Research Ethics Boards at the Massachusetts General Hospital (Boston, MA USA) and Simon Fraser University (Burnaby, BC Canada).

Primary Cells

Human peripheral blood mononuclear cells (PBMC) from healthy HIV-uninfected donors were purchased from StemCell Technologies and maintained in RPMI-1640 media supplemented with 10% FBS, 2mM L-glutamine, 1000 U/mL Penicillin, and 1 mg/mL Streptomycin (all from Sigma-Aldrich), plus 50 U/mL human recombinant IL-2 (NIH AIDS Reagents Program) at 37°C with 5% CO_2. All donors were male.

Cell lines

CEM-A*02 cells were derived from CEM (a female human acute lymphoblastic leukemia T cell line) by stably transduction of HLA-A*02:01 using a retroviral vector (murine stem cell virus; Clontech), and maintained in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 1000 U/mL Penicillin and 1 mg/mL Streptomycin (all from Sigma-Aldrich) at 37°C with 5% CO_2. Jurkat LTR-GFP CCR5+ reporter cells (JLTRG-R5), which are derived from Jurkat (a male human acute T cell leukemia cell line), were maintained using the same conditions. HEK293T cells (a human embryonic kidney cell line) and TZM-bl reporter cells (a female human carcinoma cell line, derived from HeLa) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1000 U/mL Penicillin and 1 mg/mL Streptomycin.

METHOD DETAILS

Reagents

The following materials were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 NL4-3 infectious molecular clone (pNL4-3), from Dr. Malcolm Martin (Adachi et al., 1986); Jurkat LTR-GFP CCR5+ cells (JLTRG-R5), from Dr. Olaf Kutsch (Kutsch et al., 2004; Ochsenbauer-Jambor et al., 2006); and TZM-bl cells from Dr. John C. Kappes, and Dr. Xiaoyun Wu (Derdeyn et al., 2000; Platt et al., 2009; Platt et al., 1998; Takeuchi et al., 2008; Wei et al., 2002).

Generation of HIV-1 nef expression and proviral constructs

HIV-1 subtype-B *nef* alleles were amplified from plasma viral RNA. A single phylogenetically representative *nef* clone was isolated from each participant in a previous study (Mwimanzi et al., 2013). *Nef* alleles were transferred into pSELECT-GFPzeo (InvivoGen), which features a composite hEF1-HTLV promoter driving *nef* and an independent CMV promoter driving expression of GFP. To do this, we modified the multiple cloning site in pSELECT-GFPzeo to incorporate unique Asc I and Sac II sites. Each *nef* gene was amplified by PCR using degenerate primers incorporating these restriction sites (Fwd: 5′-AGAGCACC**GG CGCGCC**TCCA CAT ACCTASA AGAATMAGAC ARG-3′, HXB2 nt 8746-8772 underlined, Asc I site in bold; Rev: 5′-GCCT**CCGCGG** ATCGATCAGG CCACRCCTCC CTGGAAASKC CC-3′, HXB2 nt 9474-9449 underlined, SacII site bolded). The same strategy was used to clone *nef* from HIV-1 subtype B reference strains SF2 and NL4.3, which served as positive controls. Point mutations were introduced into *nef* sequences using overlap extension PCR, including G2A substitutions in both reference clones that served as negative controls. All *nef* clones and mutations were validated by Sanger sequencing.

Nef sequences and mutants were introduced into the HIV-1 subtype B NL4.3 reference strain backbone as described previously (Mwimanzi et al., 2013). Wild-type NL4.3 served as a positive control, while strains encoding G2A or premature stop codons at positions 31 and 33 (referred to as ΔNef) were used as negative controls for infectivity and replication assays.

Nef polymorphisms are reported using HXB2 numbering convention (Korber et al., 1998). Sequences were pairwise-aligned to the reference strain HXB2 (GenBank accession number K03055) and insertions with respect to HXB2 were removed using an in-house alignment algorithm based on the HyPhy platform (Pond et al., 2005).

Generation of SERINC5 knockout Jurkat LTR-GFP reporter cells

Jurkat T cell lines express high endogenous SERINC5 (Rosa et al., 2015). CRISPR/Cas9 methods were used to disrupt the SERINC5 gene in Jurkat LTR-GFP R5 cells. To do this, cells were co-transfected with px330-based plasmids (Cong et al., 2013; Ran et al., 2013) encoding target sequences described by Rosa et al. (2015) along with pMAX-GFP for use as a transfection control. Following transfection, GFP+ cells were isolated as single cells into R20+ media (RPMI-1640 supplemented with 2 mM L-glutamine, 1000 U/ml Penicillin, 1 mg/ml Streptomycin and 20% vol/vol FBS, all from Sigma-Aldrich) and expanded in 96-well flat bottom plates. Stable SERINC5 knockout (KO) clones were validated by western blot using a rabbit polyclonal anti-SERINC5 antiserum (Abcam).

SERINC5 downregulation assays

To assess Nef-mediated internalization of SERINC5 from the cell surface, 1×10^6 CEM-A*02 T cells were co-transfected with 1 μg of pSELECT-GFPzeo encoding *nef* and 5 μg of pSELECT-SERINC5-internal HA tag (iHA)-ΔGFP (sub-cloned from pBJ5-SERINC5(iHA)) (Usami et al., 2015) by electroporation in 150 μL OPTI-mem medium (Thermo Fisher Scientific) using a BioRad GenePulser MXCell™ instrument (square wave protocol: 250 V, 2000 μF, infinite Ω, 25 ms single pulse). Cultures were recovered for 20 hours with 350 μl of R10+ medium (RPMI-1640 supplemented with 2 mM L-glutamine, 1000 U/ml Penicillin, 1 mg/ml Streptomycin and 10% vol/vol FBS, all from Sigma-Aldrich) at 37°C plus 5% CO_2. Following this, 2.5×10^5 cells were stained with 0.5 μg of Alexa Fluor® 647 anti-HA.11 (BioLegend) and analyzed by flow cytometry for GFP expression (as a marker for transfected cells) and cell surface HA staining (as an indicator of SERINC5 expression) using a Millipore Guava 8HT instrument. A minimum of 25,000 cells were assessed in each case. The median fluorescence intensity (MFI) values of SERINC5 for each Nef clone were normalized to the positive (pSELECT-*nef*$_{WT}$-GFPzeo) and negative (pSELECT-Δ*nef*) controls using the formula: $(MFI_{\Delta Nef} - MFI_{CLONE})/(MFI_{\Delta Nef} - MFI_{WT}) \times 100$, such that Nef function less than or greater than wild-type Nef is represented by values of < 100% or > 100%, respectively. The activity of each primary Nef clone was normalized to Nef (SF2 strain) to be consistent with our previous studies. Since point mutations were introduced into Nef (NL4.3 strain), that strain was used to normalize results from those studies. The SERINC5 downregulation activity of each Nef clone is reported as the mean ± SD based on at least three independent transfection experiments.

CD4 and HLA class I downregulation assays

Selected primary Nef clones and mutants were evaluated for their ability to internalize CD4 and HLA-A*02 (as a representative class I HLA molecule) as described previously (Mwimanzi et al., 2013). Briefly, 2.5×10^5 transfected CEM-A*02 CD4 T cells from the same pool of transfected cells described above were stained with anti-CD4-APC and anti-HLA-A*02-PE (BD Biosciences). The MFI of surface CD4 and HLA-A*02 expression levels in the GFP+ cell subsets were determined by flow cytometry and normalized using the same formula as described for SERINC5. The CD4 or HLA downregulation activity of each Nef clone or mutant is reported as the mean ± SD based on at least three independent transfection experiments.

Viral infectivity assays

Virus stocks were generated by co-transfecting 8×10^5 HEK293T cells seeded in one well of a 6-well plate with 2.5 μg of pNL4.3 and either 30 ng of pSELECT-*SERINC5(iHA)*-ΔGFP or empty pSELECT-ΔGFP vector using DNAfectin 2100 (Applied Biological Materials). Culture supernatants were harvested 48 hours post-transfection, aliquoted and stored at −80°C prior to use. To assess SERINC5 surface expression on virus-producing cells, the transfected cells were collected using Trypsin/EDTA (Sigma-Aldrich) and dissociated cells were prepared for analysis by flow cytometry. Briefly, cells were stained with 0.5 μg of Alexa Fluor® 647 anti-HA.11 (BioLegend), washed to remove unbound antibody, treated with Fix/Perm solution (BD Biosciences), and then stained with PE anti-Gag/p24 (KC57; Beckman Coulter). A minimum of 10,000 cells was assessed in each case. Virus particles in the supernatant were quantified by p24 ELISA (XpressBio) and viral infectivity was determined by exposing 1×10^4 TZM-bl reporter cells to a standardized amount of each virus (1 or 5 ng p24) on a 96-well flat bottom plate. Luminescence activity (absolute light units, ALU) of TZM-bl cells was measured 48 hours later using the Steady-Glo® Luciferase Assay (Promega) and a Tecan Infinite M200 PRO plate reader. The relative infectivity of each viral strain (i.e., the difference between viruses generated in the presence versus absence of SERINC5) was calculated using the following formula: (ALU of virus produced by SERINC5-expressing HEK293T cells) / (ALU of virus produced by SERINC5-negative HEK293T). The infectivity of each viral strain produced by HEK293T cells was assessed in at least three independent experiments.

To determine the infectivity of viruses produced by primary cells, VSV-g pseudotyped viruses were generated using HEK293T cells in the absence of SERINC5, as described above and in Kinloch et al. (2018), and then used to infect PBMC isolated from HIV-uninfected donors. Briefly, 5×10^5 activated PBMC (generated by pre-stimulation with 5 μg/ml PHA for 72 hours) were incubated with 5 ng p24 of each virus in 96-well U bottom plates by spinoculation (800 x g for 1 hour at room temperature). PBMC were subsequently incubated for 8 hours at 37°C and unbound virus was removed by washing with PBS. Cells were then resuspended in R10+ media supplemented with 100 U/mL human IL-2 (NIH AIDS Reagent Program). Culture supernatants were collected at 48 and 72 hours post-infection and newly produced viruses were quantified using p24 ELISA. Viral infectivity was measured by exposing TZM-bl reporter cells to a standardized amount of each virus, as described above. The infectivity of each viral strain produced by PBMC was assessed in at least two independent experiments.

Viral replication capacity assays

Replication capacity was determined using viruses produced in the absence of SERINC5 by infecting 1×10^6 Jurkat LTR-GFP CCR5+ and Jurkat SERINC5 KO LTR-GFP CCR5+ cells at a multiplicity of infection (MOI) of 0.003. The proportion of GFP+ cells in culture was measured on days 2 to 8 and results are shown as the fold-increase in %GFP+ cells based on day 2 values. Replication capacity of each viral strain was assessed in at least three (Jurkat LTR-GFP R5) or two (SERINC5 KO cells) independent experiments. To examine potential differences in replication more robustly, we calculated the natural log slope of viral spread during the exponential phase of growth for each virus, as described in Brockman et al. (2006). The resulting slope values from independent experiments were then compared to wild-type NL4.3 using the unpaired Student's t test to identify statistically significant differences.

To examine replication capacity in primary cells, VSV-g pseudotyped viruses produced using HEK293T cells in the absence of SERINC5, as described above and in Kinloch et al. (2018), were used to infect PBMC isolated from HIV-uninfected donors. Briefly, 5×10^5 activated PBMC (generated by pre-stimulation with 5 μg/ml PHA for 72 hours) were incubated with 5 ng p24 of each virus in 96-well U bottom plates by spinoculation (800 x g for 1 hour at room temperature). PBMC were subsequently cultured for 8 hours at 37°C in R10+ media and unbound virus was removed by washing cells with PBS. Cells were then resuspended in R10+ media supplemented with 100 U/mL human IL-2 (NIH AIDS Reagent Program). Culture supernatants were collected on days 0, 3, 6, and 9, and newly produced viruses were quantified using p24 ELISA. The replication capacity of each viral strain in PBMC was assessed in three independent donors. To examine potential differences in replication more robustly, we calculated the natural log slope of viral spread during the exponential phase of growth for each virus, as described in Brockman et al. (2006). The resulting slope values from independent experiments were then compared to wild-type NL4.3 using the unpaired Student's t test to identify statistically significant differences.

Western blot

To assess steady-state protein expression of primary Nef alleles and mutants, 5×10^6 CEM-A*02 CD4 T cells were transfected with 10 μg of pSELECT-*nef*-GFPzeo alone via the electroporation settings as described above. After 24 hours, cells were pelleted, lysed and prepared as described previously (Mwimanzi et al., 2013). Nef was labeled using a polyclonal rabbit serum (NIH AIDS Reagent Program) (1:2,000) followed by staining with donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare) (1:30,000). To validate CRISPR/Cas9-mediated knockout of SERINC5 in the Jurkat LTR-GFP cells, 2×10^6 parental or KO cells were pelleted and lysed. SERINC5 protein expression was detected using rabbit polyclonal anti-SERINC5 antibody (Abcam ab204400) at a 1:300 dilution, followed by staining with donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare) (1:30,000). Proteins were detected using Clarity Western ECL substrate (Bio-Rad) and visualized on an ImageQuant LAS 4000 imager (GE healthcare).

QUANTIFICATION AND STATISTICAL ANALYSIS

Nef polymorphisms associated with differential *in vitro* SERINC5 downregulation function were identified using a custom Perl script. For every Nef polymorphism present at > 5% in our dataset (i.e., 5 or more unique HXB-aligned sequences), clones were repeatedly grouped according to the presence versus absence of the observed variant and the nonparametric Mann-Whitney U-test was then used to compare median SERINC5 downregulation function between groups. Multiple comparisons were addressed using q-values, the p value analog of the false discovery rate (FDR). The FDR is the expected proportion of false positives among results deemed significant at a given p value threshold (e.g., at a q $\leq$ 0.2, we expect 20% of identified associations to be false positives).

All other statistical analyses were performed using Prism v.7 (Graphpad). Results of two-tailed tests were considered significant if the p value was less than 0.05. The nonparametric Mann-Whitney U test was used to compare differences in median Nef function between EC and CP cohorts. The parametric Students t test was used to compare differences in the mean Nef function of viral mutants. For parametric tests, normality of the data was assessed using the Kolmogorov-Smirnov test. Multiple comparisons were addressed using q-values, the p value analog of the false discovery rate (FDR). The FDR is the expected proportion of false positives among results deemed significant at a given p value threshold (e.g., at a q $\leq$ 0.2, we expect 20% of identified associations to be false positives). Following normalization of data positive and negative controls, we used the unpaired t test to determine if the observed function of each Nef mutant was significantly different from that of the wild-type Nef.

DATA AND CODE AVAILABILITY

The Nef downregulation data that support this study are provided in Table S1. *Nef* sequences are available at GenBank (accession numbers JX171199-JX171243 for elite controllers; JX440926-JX440971 for chronic progressors). The code for scripts that were used to align *nef* sequences and to analyze the linked Nef sequence-function dataset is available by contacting the Lead Author.