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Genetic determinants of HIV-1 subtype C Nef-mediated SERINC3 down-regulation

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Abstract

Background Nef-mediated down-regulation of the host restriction factors SERINC3 and SERINC5 significantly enhances HIV-1 infectivity. Natural Nef polymorphisms that affect SERINC3 down-regulation are not as well-characterised as those that affect SERINC5 down-regulation, particularly in HIV-1 subtype C infection. We therefore aimed to identify genetic determinants of SERINC3 down-regulation by subtype C Nef. In addition, we investigated the role of SERINC3 down-regulation activity in disease progression and its contribution to overall Nef function, using Nef fitness model-derived E values as a proxy for overall Nef function in vivo.

Methods SERINC3 down-regulation activity of 107 participant-derived Nef clones was measured using a flow cytometry-based assay in a T cell line. The relationship between SERINC3 down-regulation activity and viral load set point or rate of CD4 + T cell decline during untreated HIV infection was analysed by linear regression. Quantile regression was used to assess the contribution of SERINC3 down-regulation activity to overall Nef function. Individual Nef amino acids associated with a significantly altered SERINC3 down-regulation activity were identified using codon-by-codon Mann Whitney U tests.

Results SERINC3 down-regulation activity was not a significant predictor of viral load set point nor rate of CD4 + T cell decline. SERINC3 down-regulation activity was a significant predictor of estimated Nef fitness (E values) in univariate analysis ($p < 0.0001$) and remained significant in multivariate analyses adjusting for other Nef functions that were measured for the same Nef clones ($p < 0.02$). A total of 30 amino acids were identified to be associated with differential Nef-mediated ability to down-regulate SERINC3 ($p < 0.05$ and $q < 0.3$), with 63% of these residues being in the N-terminal domain.

Conclusion Although SERINC3 down-regulation did not associate significantly with markers of HIV disease progression, our results nevertheless suggest that SERINC3 down-regulation contributes significantly to overall Nef function and fitness. The identification of Nef amino acids associated with differential SERINC3 down-regulation ability may be useful for rational design of therapeutics and vaccines targeting the Nef region.

Keywords HIV-1, Nef, HIV-1 subtype C, SERINC3 down-regulation, SERINC5 down-regulation

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Background

Nef is an accessory protein encoded by HIV-1 that down-regulates molecules from the infected cell surface (including CD4 and HLA-I) and alters cellular signalling, thereby playing a significant role in viral pathogenesis and disease progression [1]. These Nef activities promote viral replication and allow HIV to evade host immune responses. Animal model studies suggest that Nef-mediated CD4 down-regulation and CD4-independent enhancement of virion infectivity are likely major contributors to the enhancement of HIV-1 pathogenicity by Nef [2, 3], although the relative importance of different Nef activities is incompletely understood.

Nef-mediated enhancement of HIV infectivity is in part due to its ability to internalise the host restriction factors serine incorporator 3 and 5 (SERINC3/5) from the infected cell surface [4, 5], by mechanisms similar to that of CD4 down-regulation [6]. SERINC3 and SERINC5 are incorporated into budding virions and subsequently inhibit viral fusion with uninfected cells [7]. This antiviral activity is more potent for SERINC5 than SERINC3, although these proteins act synergistically to impair virion infectivity [5]. Given the more potent effect of SERINC5 than SERINC3 on virion infectivity, most studies investigating Nef sequence determinants of Nef-mediated SERINC down-regulation have focussed on SERINC5, while SERINC3 remains understudied. It is known that Nef residues 2G, 109I, 112L, 115W and 121F are required for Nef interaction with SERINC5 [6], and several Nef motifs (CAW (57–59), RR (105–106), LL (164–165), and E/DD (174–175)) are required for both SERINC5 and CD4 down-regulation [6, 8]. However, these residues are rarely mutated in natural sequences. Several studies have described the effect of natural Nef polymorphisms on SERINC5 and SERINC3 down-regulation ability in subtype B infection [9–11], and on SERINC5 down-regulation ability in subtype C infection [12, 13], but less is known about the effect of natural variation in subtype C Nef on SERINC3 down-regulation function [13].

To identify genetic determinants of subtype C Nef-mediated SERINC3 down-regulation, the SERINC3 down-regulation activities of 107 Nef clones derived from early subtype C infection were measured in a T cell line using a flow cytometry-based assay, followed by a Nef sequence-function analysis. Given that Nef-mediated SERINC down-regulation enhances virion infectivity, we also aimed to determine whether SERINC3 down-regulation ability is a significant determinant of HIV-1 subtype C disease progression by exploring associations between this function and both viral load set point and rate of CD4 + T cell decline during untreated HIV infection. Finally, since multiple Nef functions have been measured for the same Nef clones [12, 14, 15], we also assessed the relative contribution of SERINC3 down-regulation activity to overall Nef function, as previously [12, 16], using a computationally predicted measure of overall Nef function.

Methods

Nef clones

Single representative Nef clones from 107 antiretroviral naïve individuals living with HIV-1 subtype C, that were isolated during early infection, were previously cloned into a pSELECT green fluorescent protein (GFP) reporter expression plasmid, and characterised for HLA-I and CD4 down-regulation [14], alteration of T-cell receptor (TCR) signalling [15], and SERINC5 down-regulation [12] abilities. The individuals from whom the Nef sequences were derived were from the Tshedimoso cohort in Botswana [17], the HIV Pathogenesis Programme (HPP) Acute Infection Cohort in Durban, South Africa [18, 19], and the Tenofovir Gel Research for AIDS Prevention Science (TRAPS) Cohort in KwaZulu-Natal, South Africa (including only those from the placebo arm of the clinical trial) [20]. Nef clone sequences are available under GenBank accession numbers KF208819, KF208821-3, KF208825-8, KF208831-4, KF208836, KF208838-9, KF208842-3, KF208845, KF208847-53, KF208855, KF208857-61, KF208863-5, KF208867, KF208870, KF208872-3, KF208878-9, KF208886, KF208889, KF208893-5, KM262907-23, and KM262925-68.

SERINC3 down-regulation assay

SERINC3 down-regulation activity was measured for each Nef clone in an HLA-A*02-expressing CEM-derived CD4 + T cell line as previously described [13]. One million cells were co-transfected with 2 µg Nef clone and 3.5 µg HA-tagged SERINC3 expression plasmid in 400 µl of Megacell medium (Sigma-Aldrich), using a BioRad Gene Pulser Xcell electroporator (exponential protocol: 250 V, 950 µF, and infinite Ω). Electroporated cells were mixed with 500 µl of R10 medium (RPMI-1640 medium supplemented with 10% foetal bovine serum (Gibco), 10 mM HEPES buffer (Gibco), 2 mM L-glutamine (Sigma) and 50 U/ml penicillin–streptomycin (Gibco)), divided evenly into 2 tubes, and incubated for 20–24 h to allow for the expression of Nef and SERINC3. Cells in each tube were stained with 0.375 µg of Alexa Fluor 647 anti-HA.11 Epitope Tag antibody to detect SERINC3 (BioLegend). This was followed by flow cytometry, using an LSR Fortessa (BD Biosciences), to detect the median fluorescence intensity (MFI) of Alexa Fluor (SERINC3-HA cell-surface expression) in GFP-positive cells (cells transfected with Nef clones). The SERINC3

down-regulation activity of each participant-derived Nef clone was calculated by normalising the MFI of SERINC3 expression in Nef-transfected cells to the results obtained for the negative control (a mutant G2A Nef clone that had no SERINC3 down-regulation activity) and positive control (NL4-3 Nef, which represented 100% SERINC3 down-regulation activity) as follows: [(G2A $_{\text{MFI SERINC3}}$−Nef clone $_{\text{MFI SERINC3}}$)/(G2A $_{\text{MFI SERINC3}}$−NL4-3 $_{\text{MFI SERINC3}}$)] X 100. Experiments were performed in duplicate independently and results were averaged.

Site-directed mutagenesis

Selected mutations were introduced into Nef clones encoding the consensus C Nef sequence (2004 consensus sequence obtained from www.hiv.lanl.gov) and two subtype C participant-derived Nef sequences, SK-68 Nef (GenBank accession: KC906737) and AS02-0802 B2 Nef (GenBank accession: KM262916). These sequences were first cloned into a TOPO 2.1 expression plasmid (Invitrogen) and then mutated using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent) and custom-designed primers. The mutant Nef sequences were confirmed by Sanger sequencing (ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems) and then cloned into a pSELECT GFP reporter expression plasmid, as described previously [21], to allow measurement of SERINC3 down-regulation activity.

Statistical analysis

Nef amino acids associated with differential SERINC3 down-regulation activity ($p < 0.05$ and $q < 0.3$) were identified using codon-by-codon Mann–Whitney U tests [22]. In this analysis, multiple comparisons were addressed using a false-discovery rate (q-value) approach [23], where results with $p < 0.05$ and $q < 0.3$ were considered statistically significant. Differences in SERINC3 down-regulation activity between Nef mutants and their respective wild-type sequences were assessed using Analysis of variance (ANOVA) with Tukey post-hoc tests, where a corrected $p < 0.05$ was considered statistically significant.

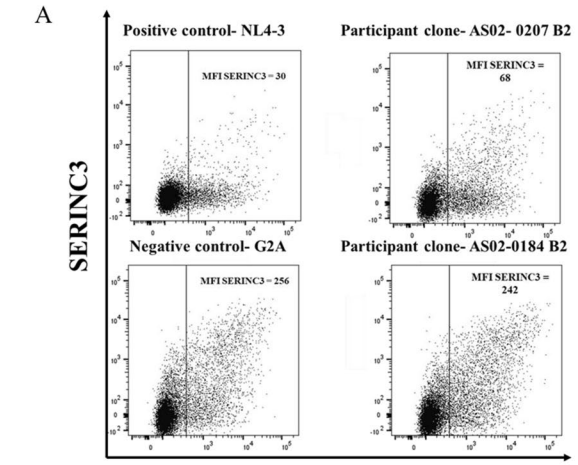
Linear regression was used to assess the relationship between the SERINC3 down-regulation activity of the 107 Nef clones and clinical markers of disease progression during untreated HIV infection, namely, viral load set point and rate of $CD4^+$ T cell decline. Viral load set point was defined as the average viral load from 3 to 12 months post-infection. Rates of CD4 T cell decline were previously calculated by simple linear regression where the estimated slope of the fitted regression line defines the rate of CD4 + T cell decline [15]. Multivariable analysis was performed including potential confounding variables if their inclusion (in bivariate analyses) resulted in a 10% or greater change in the co-efficient of SERINC3 down-regulation. Potential confounding variables that were considered included cohort, follow-up time, and baseline CD4 + T cell count.

The overall Nef fitness measure (E value) for each subtype C Nef clone was previously derived from a sequence-based computational analysis of amino acid covariation using 6469 subtype C Nef sequences from the Los Alamos HIV sequence database [16]. E values that were derived from a model that considers only consensus versus mutant amino acids at each codon (all non-consensus amino acids are counted as the same mutant type) ("Ising model") were termed dE0 values, while E values that were derived from a model that considers all amino acid variants at each codon ("Potts model") were termed dE90 values. A high dE0 or dE90 value indicates low in vivo Nef fitness (or a high fitness cost), while a low dE0 or dE90 value indicates high in vivo Nef fitness. The contribution of SERINC3 down-regulation activity to the dE0 and dE90 values was assessed and compared to that of other Nef functions using quantile regression (since the E values have a skewed distribution with outliers). To allow for comparability of co-efficients, all Nef functions were first standardized by deducting the mean and dividing by the standard deviation to convert them to z-scores. Potential confounding variables were included in multivariable analyses if they resulted in a 10% or greater change in the co-efficient of SERINC3 down-regulation, and collinearity was investigated through estimation of variance inflation factors with a cutoff of 5 applied [24]. Cohort was not considered as a potential confounder in the E value models, since these models did not take into account clinical markers in the participants from which the Nef sequences were derived and because neither dE0 nor dE90 value distributions differed significantly by cohort. Further, the Nef sequences from the different cohorts intermingled in a phylogenetic tree [14], indicating no major genetic differences in Nef by cohort. Stata 15.0 was used for all regression analyses and $p < 0.05$ was considered statistically significant.

Results

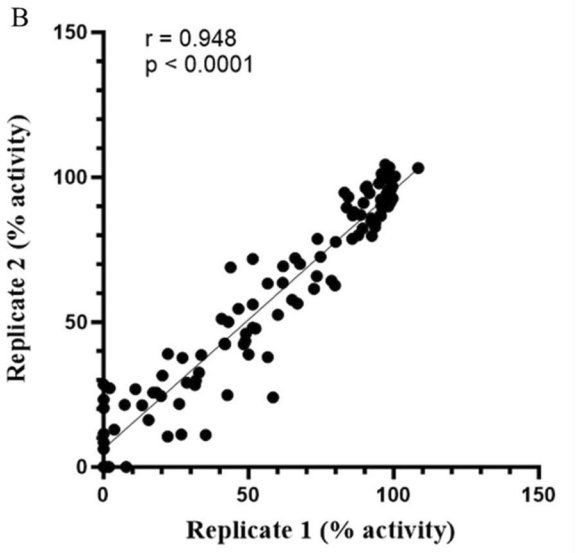
SERINC3 down-regulation activities of Nef clones

Nef-mediated SERINC3 down-regulation was measured for 107 Nef clones derived from early HIV-1 subtype C infection that were characterised previously [12, 14, 15]. Down-regulation of SERINC3 was assessed by co-transfection of a T cell line with an HA-tagged SERINC3 expression plasmid along with the participant-derived Nef clone, after which SERINC3 cell-surface expression was quantified by flow cytometry (Fig. 1A). SERINC3 down-regulation activity of each participant-derived Nef clone was expressed relative to that of NL4-3



$$\%\ \text{SERINC3 down-regulation activity} = [(\text{G2A}_{\text{MFI SERINC3}} - \text{Nef clone}_{\text{MFI SERINC3}}) / (\text{G2A}_{\text{MFI SERINC3}} - \text{NL4-3}_{\text{MFI SERINC3}})] \times 100$$

Sample:	MFI SERINC3 (GFP+ subset)	% Activity
NL4-3	30	
G2A	256	
AS02-0207 B2	68	83
AS02-0184 B2	242	6



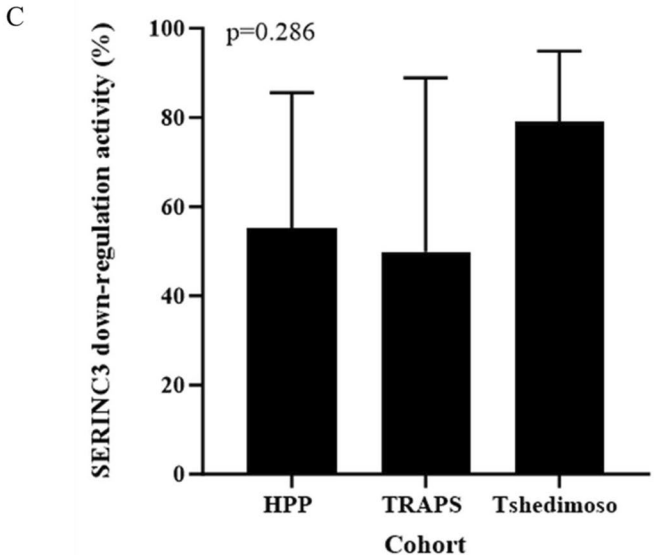


Fig. 1 SERINC3 down-regulation activities of participant-derived Nef clones. **A** Flow cytometry plots showing SERINC3 expression (y axis) in Nef-transfected cells expressing green fluorescent protein (GFP) (x axis) for the positive control (NL4-3 Nef), negative control (G2A Nef) and participant-derived Nef clones (AS02-0207 B2 and AS02-0184 B2). SERINC3 down-regulation activities (% activity) of participant-derived Nef clones were calculated by normalising the median fluorescence intensity (MFI) of SERINC3 expression in Nef-transfected cells (GFP + subset) to the results obtained for the positive (NL4-3 Nef, 100% activity) and negative (G2A Nef, 0% activity) controls. **B** The correlation (Spearman's) between assay replicates is shown. **C** SERINC3 down-regulation activities did not differ between the HPP, TRAPS and Tshedimoso cohorts. The Kruskal–Wallis *p* value is shown. Bars indicate the medians with inter-quartile ranges

Nef and G2A Nef, which were the positive (100% activity) and negative (0% activity) controls, respectively. The assay was reproducible, with replicate values correlating strongly (Spearman's correlation, r = 0.948 and $p < 0.001$) (Fig. 1B). In addition, transfection efficiency did not influence SERINC3 down-regulation activity (Spearman's correlation, r = 0.12 and $p = 0.21$) (additional Fig. 1). Participant-derived clones showed variable abilities to down-regulate SERINC3, with a median activity of 58% (interquartile range [IQR] 26–89.1%). Since there were no significant differences in SERINC3 down-regulation activities between cohorts (Kruskal–Wallis, $p = 0.292$) (Fig. 1C), the data from all 3 cohorts were combined for further analysis.

The Nef clones studied here for SERINC3 down-regulation were previously characterized for CD4 and HLA-I down-regulation [14], alteration of TCR signalling [15] and SERINC5 down-regulation [12] (data shown in additional Table 1). SERINC3 down-regulation activity was modestly and positively correlated with SERINC5

down-regulation activity (Spearman's, $p < 0.0001$) and CD4 down-regulation activity (Spearman's, $p < 0.0001$), but was not correlated with HLA-I down-regulation and alteration of TCR signalling activities (data shown in additional Fig. 2).

Sequence-function analysis

To determine which amino acid variants significantly affect SERINC3 down-regulation activity, a codon-by-codon sequence-function analysis was performed, that included all amino acids observed at least five times in the sequence dataset at a given Nef position. In total, we identified 30 amino acids that were significantly associated with altered SERINC3 down-regulation activity at our predefined statistical threshold of $p < 0.05$; $q < 0.3$, where 63% of these lay in Nef's N-terminal domain (residues 1–57) (Table 1). The associations at codons 3–12 overall tended to have lower p-values when compared with the rest of the associations (Table 1), suggesting that this region has

Table 1 Amino acid variants significantly associated with altered SERINC3 down-regulation function

Codon [a]	Amino Acid [b]	ConsC [c]	Relative Nef function (%) [d]		Number of Samples [e]		Impact[f]	*p* value	q value
			with AA	without AA	with AA	without AA			
3	N	G	18	71	13	94	−53	0.000	0.02
3	G	G	76	32	73	34	44	0.000	0.03
5	W	W	68	34	98	9	34	0.041	0.27
8	S	S	71	22	91	13	49	0.001	0.04
8	R	S	31	66	7	97	−35	0.016	0.21
9	S	S	76	34	86	7	42	0.018	0.21
10	I	I	78	33	78	14	44	0.005	0.14
10	K	I	25	76	6	86	−51	0.005	0.14
11	V	V	79	36	79	13	43	0.001	0.04
12	G	G	75	34	89	7	41	0.008	0.15
12	E	G	34	75	7	89	−41	0.008	0.15
20	I	I	77	43	50	57	34	0.021	0.24
20	M	I	42	74	41	66	−32	0.027	0.25
32	A	A	70	42	87	20	28	0.014	0.21
32	T	A	36	63	10	97	−27	0.027	0.25
38	D	D	75	35	77	30	40	0.005	0.14
49	P	A	42	64	29	78	−22	0.036	0.25
51	T	N	86	47	25	82	38	0.008	0.15
51	N	N	48	80	79	28	−32	0.016	0.21
64	G	E	89	55	5	102	34	0.037	0.25
81	F	Y	88	55	15	92	33	0.018	0.21
81	Y	Y	55	87	91	16	−32	0.035	0.25
101	V	I	89	56	5	102	33	0.045	0.28
108	E	E	75	38	62	45	37	0.030	0.25
108	D	E	38	75	45	62	−37	0.030	0.25
116	N	H	48	74	53	54	−27	0.033	0.25
116	H	H	74	48	54	53	27	0.033	0.25
188	L	S	17	61	6	101	−44	0.026	0.25
205	D	D	62	26	98	8	37	0.028	0.25
205	N	D	26	62	6	100	−37	0.047	0.28

[a] Codon numbered with reference to the HIV-1 subtype B genomic reference sequence HXB2

[b] Amino acids (AA) associated with altered SERINC3 down-regulation activity in participant-derived sequences, with $p < 0.05$, $q < 0.3$. Only amino acids observed at least 5 times at a given position were included in the analysis

[c] The consensus amino acid according to the 2021 consensus C Nef sequence (www.hiv.lanl.gov)

[d] The median SERINC3 down-regulation activity in Nef sequences harbouring the amino acid at that position

[e] The number of sequences with (+) and without (–) the amino acid

[f] Impact of the amino acid on Nef function, calculated as the difference in function between those expressing and not expressing the amino acid in question

a notable influence on SERINC3 down-regulation. To validate the sequence-function analysis, two mutations in this region were selected for site-directed mutagenesis to confirm their functional effects. A previous study that evaluated Nef sequences from subtypes A, B, C and D (where the subtype C sequences were independent from the present study) confirmed that Nef substitutions 8R and 11G negatively impacted SERINC3 down-regulation activity [13], so it was notable that the present analysis also identified 8R as being associated with significantly lower activity and 11V to be associated with significantly higher activity. In the present study however, we wished to identify mutations that selectively affect SERINC3 down-regulation and which have not previously been described to alter SERINC3 down-regulation by subtype C Nef. To identify candidates, we compared our amino acid "hits" to those from prior SERINC5 studies [9, 11–13], and selected mutations 5L and 12E for testing as these were not previously associated with altered SERINC5 down-regulation yet are both expected to reduce Nef-mediated SERINC3 down-regulation activity (Table 1). The mutation 5L is indirectly expected to have a negative effect on SERINC3 down-regulation since the consensus 5W was associated with increased SERINC3 down-regulation (Table 1), and 5L was selected due to it being the most common mutation at this codon in our dataset. While 12G was previously reported to increase infectivity in the presence of SERINC3 in subtype B [11], the effect of the mutation 12E on SERINC3 down-regulation by subtype C Nef has not previously been described. As a control, we included 8R which was already known to selectively decrease SERINC3 down-regulation activity [12–14].

Mutations were first introduced into consensus C as a "representative" Nef sequence. However, since this is not a natural Nef sequence, mutations were also introduced into a participant-derived sequence that was highly similar to consensus C (SK68). For further confirmation of the functional effect of the selected mutations, a clone (AS02-0802) with poor SERINC3 down-regulation activity (31%) that harboured two of the mutations of interest (8R and 12E), was mutated to the wild-type residues at these positions. AS02-0802 was also previously reported to have poor SERINC5 down-regulation activity but had high CD4 and HLA-I down-regulation ability [12].

Consistent with the sequence-function analysis, introducing the 5L, 8R and 12E substitutions into the consensus C and SK68 Nef sequences decreased SERINC3 down-regulation activity compared to the parental clones, with the results for 8R and 12E achieving statistical significance (ANOVA with Tukey post-hoc tests, $p < 0.01$) (Fig. 2A and B). While 8R reduced SERINC3 down-regulation activity to 60% and 56% of the activity of the consensus C and SK68 parental clones, respectively, 12E resulted in a larger decrease in activity to 55% and 28%. Consistent with a detrimental impact of these substitutions on Nef-mediated SERINC3 down-regulation activity, reversion of these substitutions back to consensus (8S and 12G, respectively) in participant AS02-0802's Nef clone increased Nef activity in both cases, though only 8S reached statistical significance (Fig. 2C). Of note, introduction of 8S enhanced SERINC3 down-regulation by twofold in this isolate. The

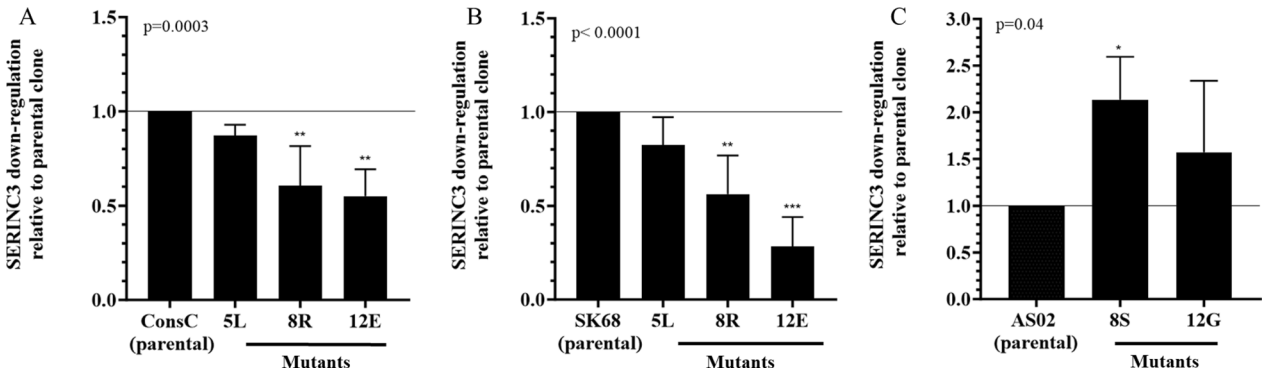


Fig. 2 SERINC3 down-regulation function of mutant Nef clones. Substitutions 5L, 8R, 12E and 51T were introduced into Nef consensus C (panel **A**) and the Nef clone isolated from participant SK68 (panel **B**). For the clone from participant AS02-0802, which naturally encoded 8R and 12E, we reverted these residues to the consensus 8S and 12G (panel **C**). Here, the activity of each mutant is shown relative to its parental clone, whose activity is set to 1.0 (for context, the NL4-3-normalized activities of the consensus C, SK68 and AS02-0802 parental Nef sequences were 93.5%, 90.5% and 30.4%, respectively). The ANOVA *p* values are shown, and asterisks indicate significant differences between the mutants and their respective wild-types: $p < 0.05$ (*), $p < 0.001$ (***) after multiple comparisons correction. Bars represent the mean activity of three independent experiments with standard deviations shown

negative effect of mutations 5L, 8R and 12E on SERINC3 down-regulation was further confirmed to be specific to SERINC3, as these had no significant effects on SERINC5 down-regulation activity (ANOVA, $p > 0.28$) (data shown in additional Fig. 3).

SERINC3 down-regulation did not associate with markers of disease progression

While SERINC3 down-regulation by Nef enhances virion infectivity [4, 25], though to a lesser extent than SERINC5 down-regulation, is not known to what extent this Nef function contributes to HIV-1 disease progression. We therefore analyzed the relationship between Nef-mediated ability to down-regulate SERINC3 in early infection and two well-established markers of HIV disease progression, namely viral load set point and rate of CD4 + T cell decline. Univariable linear regression was used to assess the relationship between SERINC3 down-regulation and viral load set point, revealing no significant relationship ($p = 0.622$). Similarly, SERINC3 down-regulation was not significantly associated with rate of CD4 + T cell decline (Table 2).

SERINC3 down-regulation is a significant contributor to overall Nef function

The Nef clones were previously characterized for CD4 and HLA-I down-regulation [14], alteration of TCR signalling [15] and SERINC5 down-regulation [12]. Computationally predicted E values had also been previously assigned to all of these Nef clones as a measure of overall in vivo Nef fitness, and a previous exploration of the relative contribution of these various in vitro Nef functions to each sequence's E-value had identified CD4 down-regulation and SERINC5 down-regulation as the most significant contributors [12, 16]. Building on previous work [12], we investigated the contribution of SERINC3 down-regulation, relative to other Nef functions, to Nef E values.

Nef E-values are calculated in two ways, termed dE0 and dE90. The dE0 values were derived from a model that considers only consensus versus mutant amino acids at each codon, while the dE90 values were derived from a model that considers all amino acid variants at each codon. The relationship between each standardized Nef function (SERINC3, SERINC5, HLA-I and CD4 downregulation, as well as TCR signalling alteration) and dE0 is presented in Table 3. Univariable analyses revealed that SERINC3 down-regulation ($p < 0.0001$), SERINC5 down-regulation ($p = 0.038$), and CD4 down-regulation ($p = 0.046$) were significantly, and negatively, associated with dE0 values. A negative coefficient indicates that an increase in that Nef function leads to a lower dE0 value (i.e. an increase in overall Nef fitness). Bivariate analyses indicated that the effect of SERINC3 down-regulation on dE0 was modified by HLA-I down-regulation and SERINC5 down-regulation, as these Nef functions changed the co-efficient of SERINC3 down-regulation by more than 10% (not shown). When adjusting for the effect of these Nef functions in a multivariable model (Table 3), only the association of SERINC3 down-regulation with dE0 remained significant ($p = 0.002$).

Consistent with the dE0 analysis, SERINC3 down-regulation ($p = 0.0001$), SERINC5 down-regulation ($p = 0.003$) and CD4 down-regulation ($p = 0.020$) were all significantly and negatively associated with the dE90 values in univariable analyses (Table 3). When adjusting for Nef functions that modified the co-efficient of SERINC3 down-regulation by more than 10% (HLA-I down-regulation and SERINC5 down-regulation), SERINC3 down-regulation remained a statistically significant driver of dE90 ($p = 0.009$) (Table 3).

Overall, SERINC3, SERINC5 and CD4 down-regulation were significant predictors of Nef E values, and this was consistent for both the dE0 and dE90 models.

Table 2 SERINC3 down-regulation and CD4 + T cell decline

Univariable (n = 102)				Multivariable (n = 102) [a]			
Variable	**Coefficient**	**Standard error**	***P* value**	**Variable**	**Coefficient**	**Standard error**	***P* value**
SERINC3 [b]	2.455	2.956	0.408	SERINC3 [b]	4.242	2.612	0.108
				Follow up time (days)	0.009	0.002	< 0.0001
				Baseline CD4 (cells/mm^3)	−0.196	0.005	< 0.0001

CD4 + T cell decline values (cells/mm^3 per month) were limited to the range of − 50 to 50 cells/mm^3 per month to exclude outliers (n = 5)

[a] Multivariable analysis controlled for potential confounders that altered the co-efficient of SERINC3 down-regulation by more than 10% in bivariate analyses

[b] SERINC3 down-regulation activity is expressed relative to NL4-3 Nef as a proportion (where NL4-3 function is 1)

Table 3 Quantile regression models showing the relationship between each Nef function and E values

Nef function [a]	Univariable analysis		Multivariable analysis	
	Coefficient	*P* value	Coefficient	*P* value
dE0[b]				
SERINC3 down-regulation	−4.041	0.0001	−3.548	0.002
SERINC5 down-regulation	−2.229	0.038	−0.835	0.464
HLA-I downregulation	−1.647	0.145	−1.920	0.046
CD4 downregulation	−2.225	0.046		
Alteration of TCR signalling	0 .458	0.691		
dE90[b]				
SERINC3 downregulation	−6.094	0.0001	−4.678	0.009
SERINC5 downregulation	−4.883	0.003	−2.786	0.123
HLA-I downregulation	−2.096	0.190	−0.701	0.641
CD4 downregulation	−3.634	0.020		
Alteration of TCR signalling	−0.425	0.798		

[a] Each Nef function was standardised by subtracting the mean and dividing by the standard deviation to calculate a z-score

[b] The dE0 and dE90 values assigned to each Nef clone were derived from the Ising and Potts models respectively and represent Nef fitness in vivo [16]. The Ising model distinguishes between consensus and non-consensus residues at each codon. The Potts model accounts for the diversity of amino acids present at each codon

Discussion

Here we studied 107 Nef isolates derived from early HIV-1 subtype C infection in order to explore the Nef sequence determinants of SERINC3 down-regulation and assess the contribution of SERINC3 down-regulation activity to viral load set point and rate of CD4+T cell decline. We also explored the contribution of SERINC3 downregulation to overall Nef fitness, estimated for each sequence using a previously-described computational model [16].

We undertook a Nef sequence-function analysis revealing 30 naturally occurring polymorphisms at 15 different Nef codons that were associated with differential ability to down-regulate SERINC3. Consistent with previous work on Nef clones from HIV subtypes A, B, C and D [13], most of the natural polymorphisms affecting SERINC3 internalisation function were located in the N-terminal domain. This pattern was not previously observed for SERINC5 down-regulation, where the residues associated with altered activity were more evenly distributed throughout the protein [12, 13]. Within the N-terminal domain, the strongest associations with altered SERINC3 down-regulation activity were located in the first 12 codons, suggesting that the SERINC3 down-regulation of Nef may be particularly affected by mutations in this region. Some non-consensus substitutions within this region (specifically at codons 5, 8 and 12) negatively affected SERINC3 but not SERINC5 down-regulation, as further confirmed by the mutagenesis experiments. On the other hand, non-consensus substitutions at codons 3, 9, and 10, which were linked to lower SERINC3 down-regulation activity here, have been previously associated with negative effects on both SERINC3 and SERINC5 down-regulation [12, 13]. Conflicting results exist for codon 11 in terms of selective or common effects on SERINC3 and SERINC5 down-regulation, where the consensus 11V is associated with higher SERINC3 down-regulation activity here and was previously associated with increased SERINC5 down-regulation activity in the same subtype C Nef clones [12], while non-consensus substitutions at codon 11 selectively negatively affected SERINC3 but not SERINC5 down-regulation in another study that included subtype A, B, C and D Nef clones [13]. Interestingly, conservation at residues 8–12 has been linked to lower HLA-I down-regulation ability [21], which suggests a possible trade-off between SERINC3 and HLA-I down-regulation functions or opposing effects of conservation in this region on these two Nef functions. Within the N-terminal domain, residues 32–39 have previously been reported to be critical determinants of CD4 down-regulation and SERINC5 down-regulation as well as key to viral infectivity and spread [26]. Here, we observed that substitutions at codons 32 and 38 negatively affect SERINC3 down-regulation, which together with the previous report [26] supports that mutations within residues 32–39 negatively affect multiple functions of Nef.

We also investigated the overlap in Nef activities, and observed that SERINC3 down-regulation activity correlated modestly and positively with both SERINC5 and CD4 down-regulation activities, supporting that there is overlap in the genetic determinants of these Nef activities. This was not surprising as they share similar down-regulation mechanisms and there are shared motifs that are reported to be critical for both SERINC5 and CD4 down-regulation [6].

Interestingly, these 3 overlapping Nef activities—down-regulation of SERINC3, SERINC5 and CD4—were each individually significant predictors of a computationally predicted measure of overall Nef function (the E value), while down-regulation of HLA-I down-regulation and alteration of TCR signalling were not. This is in line with a previous animal model study showing that Nef activities dependent on the SH3-binding domain ($_{72}$PxxPxR$_{77}$), for example HLA-I down-regulation and alteration of cellular signalling, were not as important for Nef-mediated enhancement of viral pathogenesis as those activities that did not require this domain, in particular CD4 down-regulation [3]. That study did not directly assess the SERINC protein down-regulation function of Nef, which is also not reliant on the SH3-binding domain [6].

While there is an overall congruency between our study and the previous work of the relative importance of different Nef functions, it was surprising that SERINC3 down-regulation activity was a stronger predictor of the E value when compared with SERINC5 down-regulation activity since SERINC5 is known to inhibit viral infectivity to a significantly greater extent than SERINC3 [5]. It could be speculated, however, that the association between SERINC3 down-regulation and E values may not be solely mediated by SERINC3's effect on viral infectivity, or that there may be relationship between SERINC3 down-regulation and another unknown/unmeasured Nef function that partly contributes to the association, which is a hypothesis requiring further investigation.

Previous work suggests that virion infectivity is a significant contributor to HIV-1 disease progression [2, 27]. Since SERINC3 and SERINC5 antagonism have a synergistic effect on virion infectivity [5], we investigated the role of SERINC3 down-regulation by subtype C Nef on HIV-1 disease progression. However, it was found that, as previously reported for SERINC5 down-regulation activity measured for the same subtype C Nef clones [12], SERINC3 down-regulation activity was not significantly associated with markers of disease progression. Somewhat consistent with this finding, Kruize et al. (2021) previously reported that the majority of Nef mutations that affect SERINC3/5 down-regulation do not significantly affect pathogenesis [11]. This lack of association between SERINC3/5 down-regulation and disease progression despite the significant relationship between SERINC3/5 down-regulation and predicted overall Nef function (E values), may be partly explained by the dilution of Nef's effect on disease progression by the contribution of many other viral and host factors to the rate of HIV-1 disease progression [28] (indeed E values correlated weakly with markers of disease progression in the same cohort [12]) and/or that E values (while still meaningful) have some limitations as a proxy for overall Nef function. Limitations of the study methods may also play a role in partly explaining the lack of significant association between SERINC3/5 down-regulation and markers of disease progression:

First, the in vitro assay does not fully assess all mechanisms whereby HIV may antagonize SERINC proteins. For example, HIV envelope plays a role in determining resistance to SERINC independently of Nef [29] and it was also reported that Nef can antagonise SERINC proteins by means other than down-regulation [30]. In addition, the level of expression of SERINC proteins and Nef could influence the extent of HIV restriction [8], nuances that may not have been captured in the present co-transfection assay. Although the present SERINC3/5 down-regulation assay does correlate with viral infectivity in vitro (yet notably only for SERINC5 down-regulation when both SERINC3 and SERINC5 proteins are expressed) [13], viral infectivity can also be enhanced through other mechanisms: CD4 down-regulation, performed by Nef and Vpu, is also a determinant of virion infectivity (indeed, CD4 down-regulation activity in the present cohort correlated with viral load set point [14]) and in certain cell lines, Nef may also enhance virus infectivity independently of SERINC3/5 [31, 32]. Secondly, our use of a CD4 + T-cell line, while providing the advantage of consistency, may not be fully representative of primary cells [32]; ideally, findings in cell lines should be validated by producing Nef-encoding viruses in primary cells with endogenous SERINC3/5 expression [9]. Finally, we isolated only one clone per participant, which may not fully capture within-host Nef sequence diversity (though genetic diversity would likely be limited during early infection, when the clones were isolated).

Conclusion

Our findings highlight SERINC3 down-regulation as a significant contributor to the overall function of the Nef protein. In conjunction with previous studies of Nef sequence and function, this work advances our understanding of the naturally occurring polymorphisms in Nef that impact its multiple activities. This information may be useful for attenuation-based vaccine design that seeks to attenuate multiple functions of Nef simultaneously.

Abbreviations

HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency virus
CD4	Cluster of differentiation 4
HLA-I	Human leukocyte antigen class I
SERINC3	Serine incorporator protein 3
SERINC5	Serine incorporator protein 5
TCR	T cell receptor
ANOVA	Analysis of variance

Supplementary Information

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Supplementary material 1: Figure 1. Transfection efficiency and SERINC3 down-regulation activity of Nef clones. The transfection efficiency of Nef clones, as measured by the percentage of cells expressing green fluorescent protein (GFP), did not correlate (Spearman's) with the SERINC3 down-regulation activity of clones. On average, 2344 GFP positive events were measured per clone.

Supplementary material 2: Figure 2. Relationships between SERINC3 down-regulation and other Nef functions. Spearman's correlation was used to test for significant relationships between SERINC3 down-regulation and other Nef functions, namely (A) CD4 down-regulation, (B) SERINC5 down-regulation, (C) HLA-1 down-regulation and (D) alteration of TCR signalling. All Nef functions are expressed as a percentage of the Nef control clones included in these assays.

Supplementary material 3: Figure 3. SERINC5 down-regulation function of mutant Nef clones. Substitutions 5L, 8R, 12E and 51T were introduced into Nef consensus C (panel A) and the Nef clone isolated from participant SK68 (panel B). For the clone from participant AS02-0802, which naturally encoded 8R and 12E, we reverted these residues to the consensus 8S and 12G (panel C). Here, the activity of each mutant is shown relative to its parental clone, whose activity is set to 1.0 (for context, the NL4.3-normalized activities of the consensus C, SK68 and AS02-0802 parental Nef sequences were 100%, 81.5% and 25.1%, respectively). The ANOVA p values are shown. Bars represent the mean activity of three independent experiments with standard deviations shown.

Supplementary material 4: Table 1. In vitro Nef functional measurements and E values of Nef clones obtained from early HIV-1 subtype C infection. Nef functions were expressed relative to SF2 Nef (SF2 Nef function = 1) for all Nef functions except for SERINC3 down-regulation which was normalised to NL4-3 Nef (NL4-3 Nef function = 1). The table shows E values (dE0 and dE90), which are a computationally predicted measure of overall Nef function that were previously assigned to each Nef clone. Clinical information and Nef clone sequences are included.

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Author contributions
NS performed the experiments and analysed the data. TR performed regression analyses. NI supervised processing of participant samples. MAB and ZLB provided plasmids and developed the assay protocol. TN provided resources. JKM conceptualized the study and supervised experiments. JKM and NS obtained funding. NS, ZLB and JKM wrote the manuscript. All authors reviewed the manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its additional files, with the exception of the Nef sequences (GenBank accession numbers are provided).

Declarations

Ethics approval and consent to participate
This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (ref: BREC/00005610/2023). Written informed consent was obtained from all study participants at the time of recruitment and participant identity remained confidential.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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