UNIVERSITY OF CALIFORNIA, SAN DIEGO

Investigating transmitted/founder HIV-1 *nef* and *env* effects on SERINC5 inhibition of infectivity

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by

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The Thesis of Jasmine Jane Chau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This thesis is in dedication to my parents, who have always supported me unconditionally in all my endeavors. I would also like to thank my two sisters for all the love and laughter they bring into my life. I would not be where I am today without all their love and support.

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ABSTRACT OF THE THESIS

Investigating transmitted/founder HIV-1 *nef* and *env* effects on SERINC5 inhibition of infectivity

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Professor John Guatelli, Chair
Professor Michael David, Co-Chair

HIV has killed millions of people since its discovery in the 1980s and continues to be a costly disease that affects millions worldwide. The advent of antiretroviral drug-therapy made this once deadly disease manageable through a daily regimen of drugs. However, there is still no cure or preventative vaccine, largely due to the virus's genetic

diversity and ability to evolve to escape the host immune system. More information about HIV's infection mechanisms and accessory proteins is needed to advance further towards a cure. This research study focused on two HIV proteins, Env and Nef, and their relationship with a human host cell protein, SERINC5. Env is essential for viral infectivity and is responsible for fusing virus particles into target cells, but its activity seems to be counteracted by SERINC5. Nef antagonizes the SERINC-effect. Envs and Nefs from 10 transmitted/founder (T/F) patient clones were analyzed with and without SERINC5 using infectivity and ELISA assays to determine how infectivity varies with these different proteins. The Env expression constructs were not evaluable, but the Nef expression constructs showed expected decreased infectivity in the presence of overexpressed SERINC5, as well as varying effectiveness in enhancing infectivity and counteracting SERINC5. Sequence alignments of the 10 T/F Nefs with the consensus sequence Nef revealed variations in several Nef sequence motifs that might explain Nef's varying effectiveness. This study indicates that Nefs from different infected patients have varying abilities to counteract SERINC5, increasing our understanding about this HIV accessory protein's role in viral infectivity, and its potential roles in viral transmission and replication.

INTRODUCTION

Barriers to developing a cure for HIV

Human Immunodeficiency Virus, or HIV, is a lentivirus that infects the human immune system (primarily $CD4^+$ T cells) and eventually causes the chronic illness Acquired Immunodeficiency Syndrome (AIDS). Over 36 million people worldwide are infected with HIV [1], and while the infection can be managed through antiretroviral drug-therapy, this is a lifelong condition with no current cure. Furthermore, antiretroviral therapy requires daily adherence to multiple drugs, expensive regimens that are not accessible to many people. Antiretroviral therapy can extend the lifespan of an HIV patient to nearly that of an uninfected person, but it comes at the cost of toxic side effects. A cure or preventative vaccine must be discovered to fully stop this epidemic. However, one of the main barriers to finding a curative solution to HIV is the virus's genetic diversity and ability to rapidly evolve to escape the host's immune system [2]. More information about the virus's infection mechanisms and its accessory proteins that allow HIV to evade the immune system is needed to advance further towards a cure.

Transmitted/founder viruses

When HIV infects a new host, a single or very few transmitted/founder (T/F) viruses are transmitted, which then evolve to escape the host's immune system [3]. Such T/F viruses can be isolated from patients living with HIV shortly after their initial infection (before the immune system can drive much evolution of the virus), sequenced,

and copy-DNA assembled to create the infectious molecular clones [3, 4]. These T/F viruses are thought to be the most similar to the virus that first infected the original host, and they are a subject of great interest in many research studies on HIV transmission [5]. Some studies have shown that T/F viruses are more infectious and express more HIV viral envelope than viruses from chronically infected people [6]; therefore, T/F viruses are often used in research studies as they are expected to be highly infectious and more transmittable. Furthermore, these T/F viruses are derived from human patients rather than lab strains of HIV, ideally making experiments conducted with T/F viruses more closely related to how the virus acts inside a human host. Because T/F viruses represent the viruses most similar to the original infecting virion, they can be used to study how various HIV gene-activities are genetically linked as well as to investigate the selective pressures that drive the rapid evolution of the virus.

Env and Nef proteins affect the infectivity of HIV

The *env* gene encodes the glycoprotein for the envelope of HIV, which allows the virus to fuse into target cells and continue transmission. The *env* gene codes for the gp160 precursor protein, which is cleaved into the gp120 and gp41 polypeptides. The gp120 protein resides on the outer portion of the viral envelope and interacts with the CD4 receptor on target cells, which are typically helper T cells from the human immune system. Gp41 fuses the viral lipid envelope with that of the target cell [7]. Because of Env's importance to viral infection of target cells, it has been the subject of many research studies.

The expression product of *rev*, a regulatory protein, is needed for expression of Env. Rev regulates the accumulation of spliced and unspliced transcripts of the virus inside the cytoplasm of infected cells, which leads to the expression of Env [8]. Rev binds to the Rev-responsive element (RRE) in Env mRNA, which exports HIV mRNA before being fully spliced [8]. Studies have shown that transfected cells lack Env expression when *rev* is deleted, and that *rev* regulates the export of *env* messenger RNA from the nucleus [9]. Preliminary experiments for this study were conducted to create an *env* expression construct. Despite the constructs containing the entirety of the *env* gene, proper Env expression was not detected, even when Rev was provided in *trans* on a separate plasmid. Therefore, in this research study, Env constructs were made to include the entirety of the *rev* gene, sequences upstream of the coding region found to be essential for optimal expression, as well as the *env* gene in order to enable expression of Env.

The *nef* gene is a peripheral membrane protein that mediates protein interactions that affect membrane-tracking and sorting as well as cell signaling pathways [10]. It increases the infectivity of HIV, although its exact mechanism is not completely understood. High levels of CD4 on the surface of infected cells greatly reduces infectivity because CD4 sequesters Env; however, Nef antagonizes this effect by downregulating CD4 and targeting it for degradation in lysosomes [10, 11]. HIV-1 that expresses Nef has higher infectivity compared to HIV-1 that lacks Nef expression [12].

Nef's ability to enhance infectivity increases when there is low expression of Env in virus producer cells or low expression of CD4 in the target cells [13]. Env partly determines the Nef requirement for optimal infectivity [14] and Nef's ability to increase

infectivity varies with different Envs [15]. Different HIV strains are thus differentially reliant on Nef to increase infectivity. When HIV-1 particles are pseudotyped with other viral envelope glycoproteins that require low pH exposure for fusion into target cells, Nef is no longer required [14]. These studies suggest that Nef and Env co-evolve in a way to that optimizes the virus's infectivity.

SERINC5 is a potent inhibitor of HIV infectivity

Recently, a transmembrane protein in human host cells called SERINC5 was found to be a potent inhibitor of HIV-1 infectivity [16, 17]. SERINC5 is a member of a family of five SERINC proteins (SERINC1-5), which are multi-pass transmembrane proteins whose function is to incorporate serine into membrane lipids [18]. SERINC3 and SERINC5 in particular have been found to inhibit HIV-1 infectivity, and they are antagonized by Nef, explaining how Nef enhances infectivity independently of its effects on CD4 [16, 17]. This research study focused on SERINC5 because of its ability to strongly inhibit viral infection. While the mechanism by which SERINC5 inhibits infectivity is still under investigation, it incorporates into budding virions and seems to interfere with Env activity, preventing viral fusion into target cells [16, 17, 19]. However, Nef somehow protects Env from SERINC5 activity, and this might be by modulating endosomal trafficking to remove SERINC5 from the plasma membrane of the host cell and prevent it from incorporating into budding virions [16, 17]. Very importantly to our research plans here, not all HIV-1 Env proteins are susceptible to inhibition by SERINC5 [15, 20]. Why some Envs are sensitive to SERINC5 and others are not, and whether Nef

proteins differ in their antagonism of SERINC5 depending on the Envs with which they are linked genetically, is unclear.

Goals and hypothesis of this research study

This project aimed to study how the Envs and Nefs from 10 different patient-derived viral genomes are affected by SERINC5. The primary samples are from 10 transmitted/founder viral DNAs, because these seemed likely to represent viruses that are optimally infectious and transmittable. The T/F viruses may also give valuable information about the selective pressures acting on the virus and give clues to the evolutionary mechanism of HIV-1 as it infects and replicates in the host. Our overarching hypothesis is that *env* and *nef* co-evolve, such that Nef maintains activity as a SERINC5 antagonist only when the linked Env is susceptible to SERINC5.

Our original intention was to study how Envs and Nefs from each sample were phenotypically linked in response to SERINC5. However, during the initial experiments, I found that *env*-negative constructs were not well *trans*-complemented (i.e., my Env-expression constructs did not rescue the infectivity of a *env*-negative genome), thus producing unreliable data in which the measured infectivities were too low. The experiments with the Env constructs were postponed for future projects. Nef *trans*-complemented the *nef*-negative genome relatively well, so the study then focused on the how different Nefs rescued the inhibition of infectivity by SERINC5.

MATERIALS AND METHODS

Preliminary experiments: making the *env* expression construct with upstream sequences

Cloning the env *gene*

The *env* gene from NL4-3, a lab strain of HIV, was isolated and inserted into the expression vector pcDNA3.1(-) (Invitrogen) to create the Env expression construct (Figure 1). The pcDNA3.1(-) plasmid vector was digested at 37°C overnight with restriction enzymes EcoRV and NotI in 10X NEBuffer 3.1 (New England Biolabs). The *env* gene from NL4-3 was amplified with an Advantage 2 Genomic polymerase chain reaction (PCR) kit (Clontech Laboratories). In the original Env construct, the primers were designed to include only the *env* gene without the entire *rev* gene or other upstream sequences (Figure 1A). When this design did not produce proper Env expression, a second Env expression construct was made that included the *env* gene with upstream *rev* included in its entirety as well as other upstream sequences (Figure 1B). The primers used for the second plan were JCRev-Env-F1 (GCCCTCTAGACTCGAGCCTTAGGCA TCTCCTATGGCAGGAAGAA) as the forward primer and JC-R (TGGTGGAATTCTG CACCACTTGCCACCCATBTTATAGCA) as the reverse primer. The PCR product and linearized vector backbone were then purified with a Zymoclean Gel DNA Recovery Kit (Zymo Research) according to manufacturer's instructions. The *env* gene and linearized pcDNA3.1(-) vector were ligated together and cloned into One Shot® Chemically Competent TOP10 *E. coli* using a Seamless Cloning and Assembly kit (GeneArt)

according to manufacturer's instructions. After the gene was cloned into the vector backbone, it was grown up in Luria Broth (LB) with ampicillin and the plasmid DNA was extracted with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) according to manufacturer's instructions. The plasmid was run on a 1% agarose/TBE/ethidium bromide gel to check that the plasmid was the expected size.

Transfecting the env *expression construct to check for surface expression and protein expression of* env

HEK293T cells were plated with 8 x 10^5 cells per well. They were then transfected with pcDNA3.1(-) alone as an empty vector, a construct expressing Rev and GFP (pRev-IRES-GFP) with my Env construct that included only the *env* coding region (pcDNA-NL4-3-Env), or pRev-IRES-GFP with my Env-expressing construct that included upstream sequences including *rev* and *vpu* (pcDNA-NL4-3-Rev-Env). After incubating overnight, cells were harvested for fluorescence-activated cell sorting (FACS) and Western Blot analysis.

FACS analysis for surface expression of env

Transfected HEK293T cells were harvested and stained for Env with 2G12, an anti-Env antibody, and DαHu-AF647, Jackson Alexa Fluor 647-conjugated donkey anti-human IgG secondary antibody. Samples were then analyzed for surface expression of Env using a BD Accuri™ C6 Cytometer (BD Biosciences) after gating around live cells.

Transmitted/Founder Clone Samples

A panel of 10 transmitted/founder (T/F) HIV-1 infectious molecular clones were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH:

Panel of full-length transmitted/founder (T/F) HIV-1 Infectious Molecular Clones (Cat #11919) from Dr. John Kappes [3, 21-23] and are denoted as: pCH040-C(2625), pCH058-C(2960), pCH077-T(2627), pCH106-C(2633), pREJO-C(2864), pRHPA-C(2635), pSUMA-C(2821), pTHRO-C(2626), pTRJO-C(2851), and pWITO-C(2474).

Cloning of *env* and *nef* genes from transmitted/founder clones

To express the Env and Nef proteins of each of the transmitted/founder clones, each sample's *env* and *nef* gene were cloned into the expression vectors pcDNA3.1(-) (Invitrogen) and pCI-neo (Promega), respectively, to create Env- and Nef-expressing constructs (Figure 3). pcDNA3.1(-) was digested with restriction enzymes NotI and EcoRV in 10X NEBuffer 3.1 (New England Biolabs) and pCI-neo was digested with restriction enzymes NheI-HF and EcoRI-HF in 10X CutSmart Buffer (New England Biolabs) and incubated overnight at 37°C. The linearized plasmids were then purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research) according to manufacturer's instructions. The *env* and n*ef* genes were amplified from each of the 10 transmitted/founder clones with an Advantage 2 Genomic polymerase chain reaction (PCR) kit (Clontech Laboratories). The primers used to amplify each of the transmitted/founder clones are shown in Table 1 for the *env* gene and in Table 2 for the *nef* gene.

The PCR products were purified with a Zymoclean Gel DNA Recovery Kit (Zymo Research) and then cloned into the linearized expression vector (pcDNA3.1(-) for Env and pCI-neo for Nef) using an In-Fusion® HD Cloning Kit (Clontech Laboratories) according to manufacturer's instructions (Figure 3). Once the constructs were cloned into

the vector backbone, they were grown up in Luria Broth with ampicillin and the plasmid DNA was extracted with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega) according to manufacturer's instructions. Constructs were verified through sequencing (GENEWIZ, Inc.), again grown up in Luria Broth/ampicillin, and the plasmid DNA extracted with the QIAGEN Plasmid Midi Kit (QIAGEN) according to manufacturer's instructions.

Cell Culture

HEK293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and penicillin-streptomycin. HeLa P4-R5 cells (acquired from the NIH AIDS Reagent Program from Dr. Nathaniel Landau) [24-26] were grown in DMEM with 10% fetal bovine serum (FBS), penicillin-streptomycin, and 1 ug/ml puromycin.

Transfection

HEK293 cells, were plated in 6-well plates with 8 x 10^5 cells per well. They were then transfected with 2 µg of the Env or Nef expression construct and 2 µg of a lab clone of HIV that lacks Env (NL4-3 ΔEnv) or lacks Nef (NL4-3 ΔNef) using the transfection reagent Lipofectamine 2000 (Invitrogen) (Figure 5). Three controls were used for the Nef expression constructs. The first, NL4-3, is a proviral lab strain of HIV-1 with Nef in *cis* that was used as a positive control. The second was a negative control, NL4-3 ΔNef, which was pNL4-3 lacking *nef*. The third control was NL4-3 ΔNef with pCINL. pCINL is a construct in which the *nef* gene from pNL4-3 was subcloned into the expression

vector pCI-neo by PCR [27]. Adding pCINL to NL4-3 ΔNef adds *nef* back in *trans* to the *nef*-negative pNL4-3 (Figure 4). The experimental samples were the *nef*-negative pNL4-3 ΔNef vector with Nef added in *trans* from each of the T/F clones. Each sample was also transfected with 75 ng of a plasmid expressing SERINC5 with an internal HA-tag (pBJ5-SERINC5(iHA) [17], a gift from Heinrich Gottlinger) or an empty vector (pBJ5) to observe how the infectivity mediated by each Env and Nef is affected by SERINC5 activity. The cells were incubated at 37°C to produce virus particles and the cell culture supernatant was harvested 36-48 hours later. The virus particles were pelleted through a 20% sucrose cushion at 23,500 x g for 1 hour at 4°C. After pelleting through sucrose, the supernatant was aspirated off and the virion pellets were resuspended in DMEM-C in preparation for the infectivity assay and ELISA assay. The remaining HEK293 cells were harvested for Western Blot analysis in Laemmli sample buffer.

Infectivity Assay

HeLa P4-R5 cells (which express beta-galactosidase following infection) were infected with the virion preparations in 48-well plates with 2.5 x 10^4 cells per well in triplicate. The remaining supernatant was diluted for a p24 ELISA assay to determine a physical measurement of the amount of virions in the preparation. 48 hours later, the infected P4-R5 cells were fixed and stained to reveal the infected cells (Figure 7). The number of infectious centers was counted using an image analysis program, nicknamed after its originator, the "Romanizer," [28] and normalized to the amount of virus particles produced, which was measured using the ELISA assay.

ELISA Assay

The HEK293 supernatant that was harvested after transfection was diluted 1:5000 and 1:10000 in p24 buffer (0.5% Triton-X in diH_2O) and analyzed with a HIV-1 p24 capsid protein ELISA assay (Advanced Biosciences Laboratories) to determine the amount of virus particles produced. p24 data from ELISA assay was determined by comparison to a standard curve using p24 provided by the manufacturer.

Western Blots

Cell lysates were analyzed by immunoblot for Nef and tubulin (loading control). Samples were added to Laemmli sample buffer and heated to boiling point for 10 minutes prior to loading. 1 mm SDS-page gels (12% resolving gel and 5% stacking gel) were loaded with Pageruler Prestained Protein ladder (Thermo Fisher Scientific) and each condition, run in 1X SDS Running Buffer at 140 volts, then transferred to PVC membrane in Towbin Buffer using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). After blocking the blots in 5% milk/PBS-T (Phosphate-Buffered Saline with Tween 20), the primary antibodies were added. The primary antibody used to immunoblot for Nef was sheep α-Nef (1:500) and the primary antibody used to immunoblot for tubulin was mouse α-tubulin (1:1000). After incubating in the primary antibody solutions overnight, the Western blots were washed with PBS-T and the secondary antibodies were added. The secondary antibody used to immunoblot for Nef was goat α-mouse HRP (1:2000) and the secondary antibody used to immunoblot for tubulin was rabbit α-sheep HRP (1:2000). The blots were then washed in PBS-T. The Nef immunoblot was incubated for 5 minutes in SuperSignal® West Femto Maximum

Sensitivity Substrate (Thermo Fisher Scientific) and the tubulin immunoblot was incubated for 1 minute in Clarity™ Western ECL Blotting Substrates (Bio-Rad Laboratories), and then both were imaged using the ChemiDoc™ Imager (Bio-Rad Laboratories).

Cell lysates from select transfected Env constructs were analyzed by immunoblot for Env and GAPDH (loading control) using the same methods as described above (pg. 11-12).

Statistical Analysis

Normalization of infectivity assay data to ELISA assay data and generation of graphs and error bars was done in Microsoft Excel 2016.

RESULTS

Preliminary experiments making an Env expression construct

The original Env expression construct used primers that amplified the Env gene, starting shortly before its start codon (Figure 1A). After analyzing the data using FACS, Env expression was not strongly detected (Figure 2B) and a new plan was created to include upstream sequences in the construct. Previous studies have shown that *rev* and other upstream sequences are needed for translation of *env* [29], so the next experiments utilized primers that were designed to include the entirety of the *rev* gene along with *env* (Figure 1B).

Cells were transfected with three experimental conditions: an empty vector as a negative control; a construct expressing Rev and GFP with my initial attempt at creating a Env-expression construct, in which the HIV sequence begins just upstream of the Env start codon; and my Rev-Env expressing clone with the Rev-GFP construct. Cell lysates were harvested and stained with an anti-Env antibody and secondary antibody for FACS analysis (Figure 2). The empty vector showed no expression of GFP or Env, as expected (Figure 2A). The initial Env construct did not show strong Env expression, but did show GFP expression as expected (Figure 2B). The Env clone that now included Rev also showed expression of Env, and this was further increased in cells also expressing GFP from the Rev-GFP construct (Figure 2C), indicating that this construct properly expressed Env.

Transfection of Env and Nef expression constructs and measuring infectivity

The *env* and *nef* genes were successfully amplified via PCR from 10 transmitted/founder DNA clones and inserted into plasmid vector backbones to create Env and Nef expression constructs (Figure 3). Constructs were verified by sequencing, then transfected into HEK293 cells. The viral supernatant was used to infect HeLa P4-R5 cells (Figure 5). These are HeLa cells that express CD4 and contain in their genome a viral LTR-driven β-galactosidase expression cassette. After fixing and staining the cells for β-galactosidase activity, the blue infectious-centers, usually individual cells, were counted using an image analysis program named the Romanizer (Figure 7). The number of infectious centers was normalized to the amount of virus particles in the preparation determined by an ELISA assay for the viral p24 capsid antigen (Figure 5). After analyzing the infectivity data obtained using Env-expression vectors, we determined that the Env constructs did not sufficiently rescue the infectivity of NL4-3 ΔEnv (data not shown) even though there was detection of Env by immunoblot (Figure 6). The reason for this technical problem was not clear, but because of this, the experiments with Env were not further pursued and were postponed for future projects. This research study herein moved forward by studying only the Nef expression constructs.

In all experimental conditions, infectivity levels dropped with the presence of overexpressed SERINC5 compared to when SERINC5 was absent (Figure 8). As expected, the positive control (NL4-3) had high levels of infectivity compared to the other experimental samples. The negative control (NL4-3 ΔNef) had very low levels of infectivity. The other control (NL4-3 ΔNef with pCINL) showed some rescue of infectivity when Nef was added back in *trans*, although the infectivity did not reach the

same levels as the positive control in which Nef was expressed in *cis*. The 4 selected T/F clones shown in Figure 8 showed varying levels of infectivity-enhancement, with SUMA having the highest, and notably being more infectious than NL4-3 ΔNef with pCINL. The other 3 T/F clones showed low levels of infectivity, similar to that of the negative control.

After normalizing each sample by dividing the number of infectious units from the infectivity assay data by the nanograms of p24 protein from the ELISA assay data, the fold *nef*-effect was calculated by taking the ratio of each sample in which Nef was expressed to the infectivity of ΔNef (Figure 9). This allowed for comparison of the samples to the controls as well as for comparison of each sample without over-expressed SERINC5 to each sample with SERINC5. Importantly, HEK293 cells express low levels of SERINC3 and SERINC5 RNA (data not shown), so *nef* does contribute to optimal infectivity even in the absence of over-expressed SERINC5. Nonetheless, we expected the *nef*-phenotype with respect to infectivity to be greater when SERINC5 was over-expressed, provided that the Nef being tested was indeed an effective SERINC5-antagonist.

In all three controls, the *nef*-phenotype was greater when SERINC5 was over-expressed compared to when SERINC5 was not. This trend proved to be similar for most of the T/F clones, with the exception of clones CH077, CH106, and RHPA, where the overexpression of SERINC5 had a lesser *nef*-effect than when SERINC5 was not overexpressed, possibly due to these clones having weaker Nefs than the others. Furthermore, varying *nef*-effects were observed among the 10 T/F clones. CH040, REJO, SUMA, and WITO all had *nef*-effects above 10 when SERINC5 was overexpressed, with SUMA having the greatest *nef*-effect at 38.8 when SERINC5 was present (Figure 9).

SUMA surpassed the NL4-3 positive control *nef*-effect in this aspect. Other T/F clones had smaller *nef*-effects, with CH106 and RHPA having *nef*-effects below 1 both when SERINC5 is overexpressed and when it is not.

Each control and sample were run in duplicate, and the standard deviation of the *nef*-effect is represented as error bars in Figure 9.

Detecting Nef in T/F clones using Western Blot

To check for expression of Nef in our T/F samples, a western blot was run probing for Nef and for tubulin as a loading control (Figure 10). Tubulin was detected in all samples at approximately 55 kDa, assuring that loading was performed successfully and with relatively minor variation. Nef was detected in NL4-3 and NL4-3 ΔNef + pCINL, which was expected of the positive controls, and not detected in NL4-3 ΔNef, which was expected of the negative control. For the T/F clones, an immuno-reactive band was detected strongly in CH040, CH058, CH077, REJO, SUMA, and WITO, and weakly detected in THRO and TRJO. Nef was not detected in CH106 and RHPA. The Nef proteins were varying sizes, but all were between 25 kDa and 35 kDa. Non-specific bands were detected slightly above the 35 kDa band across all samples.

Comparing amino acid sequences of T/F Nefs to consensus sequence

Nucleic acid sequences of each *nef* from the 10 T/F clones were translated to the amino acid sequences in A plasmid Editor (ApE). The 10 amino acid sequences were aligned using ClustalW Multiple alignment in BioEdit to a consensus of HIV-1 Clade B sequences, and NL4-3 sequence (Figure 11A). In Figure 11A, the different colored letters

each represent an amino acid, and when the aligned sequence has an amino acid matching the one in the consensus of consensus sequence, it is represented as a dot. When the amino acid in the aligned sequence is different from the one in the consensus of consensus sequence, the differing amino acid is represented as its letter abbreviation.

The aligned sequences were then compared to a table of conserved sequence motifs of HIV-1 Nef [30]. Differences from the consensus sequence in a conserved region were highlighted with a red box in Figure 11A. Variations in the T/F Nef sequences were found in the region coding for the HIV-1 protease-cleavage sites (Figure 11B) starting at base pair 55 of the consensus sequence. There were several differences found in many the T/F Nef sequences in the PACS-1 region, the binding site for cellular proteins PACS-1 which targets MHC-1 for downregulation [31], starting at base pair 62 of the consensus sequence (Figure 11C). Many of these changes were glutamic acid (E) to aspartic acid (D), which is a conservative change. The SH3 binding domain for the Src family kinases (Figure 11D) had two variations from the consensus, both of which were valines (V) instead of leucine (L), again a conservative change. The four variations found in the β-COP binding region, the binding site for β-COP which may mediate Nef's trafficking in infected cells [32], were from glutamic acid (E) in the consensus sequence to lysine (K) in the T/F clone (Figure 11E); this is non-conservative variation that replaces a negatively charged side chain with a positively charged side chain. The region that binds the clathrin adaptor protein complexes AP-1/2/3 also had several variations from the consensus sequence (Figure 11F).

DISCUSSION

Preliminary experiments making an Env expression construct show a requirement for Rev and upstream sequences

The original plan to create an Env expression plan (Figure 1A) included just the *env* gene but did not properly express Env when analyzed using FACS (Figure 2B). Thus, new primers were designed to include the upstream *rev* gene in its entirety along with the *env* gene (Figure 1B) since previous studies indicated that sequences upstream of the *rev* gene is needed for *env* expression [29]. Our experiments seem to support these findings, as the new Env expression construct showed Env detection when analyzed by FACS (Figure 2C). In future studies involving Env expression, it should be noted that Rev and sequences upstream of it, as should be included in *cis* in the construct to see proper expression of Env.

Different Nef alleles have varying effects on HIV-1 infectivity

In all experimental conditions and using all Nef clones, the infectivity levels were lower when SERINC5 was over-expressed than when it was not (Figure 9). This was as expected, since SERINC5 has been shown to inhibit infectivity of HIV-1 and it can overwhelm Nef when over-expressed [16, 17]. The infectivity was significantly lower in the negative control (NL4-3 ΔNef) compared to the positive control (NL4-3) because the negative control lacked Nef, which enhances infectivity. For the Nef-in *trans* control (NL4-3 ΔNef + pCINL), Nef was added back into the *nef*-negative NL4-3, and Nef

rescued infectivity, although the infectivity levels were not as great as when Nef was encoded in *cis* (in NL43).

The T/F clones had varying Nef effects; some T/F Nefs increased the virus's ability to infect, while others were not as effective. Some Nef constructs rendered the virus less affected by SERINC5, as shown when the number of infected cells did not significantly drop when SERINC5 was overexpressed, resulting in an unusually large *nef*-effect, at least relative to NL4-3. Notably, SUMA had a fold *nef*-effect almost double that of the positive control NL4-3, indicating the possibility that this particular clone has a very effective Nef variant. CH040 and WITO also had large *nef*-effects. However, other Nefs were much less effective at preserving infectivity when SERINC5 was overexpressed, as indicated by the large drop in infectivity when SERINC5 was added. These less effective Nefs, such as CH077, CH106, and RHPA, had very low *nef*-effect, some below 1. Theoretically, the *nef*-effect ratio should not be below 1, as that would suggest that these clones have less infectivity with Nefs present than when Nef is absent in the negative control. This anomaly in the data is possibly due to variations in measurement, which could be addressed in future experiments by having more replicates of each sample's data. Overall, Figure 4 suggests that different Nefs have varying abilities to effectively antagonize the SERINC5 effect, and thus have varying effects on enhancing HIV-1 infectivity.

Western Blot analysis shows variations in Nef detection

A western blot was run to probe for Nef in the experimental samples (Figure 10). Nef was strongly detected in the NL4-3 and NL4-3 ΔNef + pCINL controls, while not

detected in the NL4-3 ΔNef negative control as expected. In most of the T/F clones that showed *nef*-effects similar to that of the negative control or better, Nef was detected in the immunoblot. However, CH106 and RHPA, both relatively weaker Nefs, were not detected in the blot, possibly explaining the lack of a robust Nef effect. Conversely, THRO, which had a Nef effect almost as great as that of NL4-3 ΔNef + pCINL, had very faint detection in the blot compared to the robust detection of Nef in NL4-3 ΔNef + pCINL. This could be explained by a limitation of our immunoblot; the antibody used to probe for Nef may not bind to all Nef variants. To compensate for this limitation in the future, an HA or another epitope tag should be incorporated into the Nef expression constructs during the cloning process so an antibody detecting the epitope tag could be used for the immunoblot.

Sequence alignment of T/F Nefs reveal variations in conserved Nef motifs

Aligned sequences of the 10 T/F clones to the consensus and consensus of HIV-1 Clade B sequences revealed several variations in conserved Nef motifs. In Figure 11B, four of the T/F clones had variations from the consensus in the region encoding a site recognized by the HIV-1 protease. HIV-1 protease cleaves Nef at a very conserved region between Trp57 and Leu58, although the biological relevance of this is unclear [33]. CH106 contains a threonine (T) instead of the conserved alanine (A), which is a notable change since threonine is a polar side chain and alanine has a hydrophobic side chain. This variation might explain why CH106's Nef was not very effective in enhancing infectivity. Conversely, REJO, SUMA, and WITO, which all had relatively active Nefs, had variations in this region as well. SUMA and WITO did not have the conserved

cysteine (C) amino acid at the beginning of the motif, but rather had valine (V) and serine (S) respectively. It is possible that the change from cysteine to other amino acids played a role in their Nefs having increased infectivities, particularly SUMA since it showed a *nef*-effect greater than that of the positive control (Figure 9).

There were several variations from the consensus in the region coding for PACS-1 (Figure 11C), many of which were changes from glutamic acid (E) to aspartic acid (D). PACS-1 is a cellular protein that is bound by Nef to downregulate class I major histocompatibility complexes (MHC-1) [31]. It is possible that certain variations in this motif may affect the effectiveness of different Nefs, but it is unclear how since there are variations in most of the T/F clones.

A proline-rich sequence starting at base pair 72 of NL4-3 binds to the SH3 domains of a subset of Src kinases and are needed for the efficient growth of viruses encoding Nef but are not required for CD4 downregulation [34]. Two of the T/F viruses had valines (V) instead of leucine (L) in this motif, CH077 and WITO (Figure 11D); however, it is not clear whether or how these variations would affect Nef's ability to enhance infectivity as CH077 had a weaker Nef while WITO seemed to have a relatively stronger Nef. Nevertheless, this variation is possibly worth investigating in the future.

All four T/F clone variations found in the conserved EE region that encodes for binding to β-COP were from glutamic acid (E) to lysine (K) (Figure 11E). β-COP is a major component of non-clathrin vesicles coats and may mediate Nef's trafficking in infected cells [32]. Out of the four T/F clones with this variation, two of them (SUMA and WITO) have relatively effective Nefs and the other two (CH077 and TRJO) have relatively weaker Nefs. Thus, it is unclear if this variation has an effect on infectivity,

especially after noting that glutamic acid and lysine have oppositely charged amino acid side chains.

Six out of ten of the T/F clones had variations in the sequence that encodes for the binding of Nef to the clathrin adaptor proteins AP-1/2/3 (Figure 11F). Nef binding to adaptor proteins seems to be required for CD4 downregulation [27, 35], and variations in this region may be a point of interest in future research studies because of its role in downregulating CD4. Moreover, this region was previously described as important for Nef-mediated enhancement of infectivity, and it was more recently described as specifically important for the antagonism of SERINC5. A possible way to test how these variations in Nef sequence motifs affect infectivity would be to construct point mutations at various locations in each of these motifs.

General conclusions

This research study indicates that Nefs from different infected patients have varying abilities to counteract SERINC5. This could be significant for future studies, because if certain variances from the consensus sequence is found to result in a weaker Nef-effect, this could identify regions in Nef that could be potential therapeutic targets. Future studies could also look at the Envs from different T/F viruses to see how they vary in sensitivity to SERINC5 and test the hypothesis that this phenotype is correlated with Nef-activity, thus confirming or rejecting that the phenotypes of Env and Nef with respect to SERINC5 are genetically linked in a given virus. For example, if this hypothesis is correct, then the Envs of T/F viruses CH077, CH106, and RHPA are predicted to be relatively resistant to SERINC5, since the Nef proteins of these viruses

were poorly active as SERINC5 antagonists. Other experiments could mutate the different Nefs in the conserved regions to see if certain mutations inhibit Nef's ability to increase the infectivity of HIV-1.

Overall, this research study expands our knowledge of Nef and its ability to counteract SERINC5 by showing that this activity is quite variable in HIV-1 clones that likely reflect biologically relevant viruses. Whether this variability reflects an interplay between the activity of Nef as a SERINC5 antagonist and the susceptibility of the Env with which it is linked to SERINC5, or whether SERINC5 is not a substantial host defense against transmitted HIV-1, remains to be determined.

FIGURES AND TABLES

Table 1: Sequences of forward and reverse primers used to isolate *env* gene from T/F DNA samples.

T/F Clone	Forward primer	Reverse primer
pCH040-C(2625)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	CH040-077-REJO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCCACTTGCCACCCATCTTATAGCA-3')
pCH058-C(2960)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	CH058-RevEnv-R (5'-TGGTGGAATTCTGCAGATCTTATAGTAAAGCTCTTTCTAAGCCCTGT -3')
pCH077-T(2627)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	CH040-077-REJO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCCACTTGCCACCCATCTTATAGCA-3')
pCH106-C(2633)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	CH106-RevEnv-R (5'-TGGTGGAATTCTGCAGATTTTATAGTAAAAGCCTCTCAAGGCCTTGTC -3')
pREJO-C(2864)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	CH040-077-REJO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCCACTTGCCACCCATCTTATAGCA-3')
pRHPA-C(2635)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	RHPA-RevEnv-R (5'-TGGTGGAATTCTGCAGATCTTATTGCAATGCCCTTTCCAAGCC-3')
pSUMA-C(2821)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	SUMA-RevEnv-R (5'-TGGTGGAATTCTGCAGATTTTATAGTAAAGCCCTTTCCAAGCCCTG-3')
pTHRO-C(2626)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	THRO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCTTATAGCAAAGCTCTTTCAAGGCCC-3')
pTRJO-C(2851)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	TRJO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCTTATTGCAAAGCCCTTTCTGCGCC-3')
pWITO-C(2474)	JCEnv2-F (5'-GCCCTCTAGACTCGAGCCTTAGGCATCTCCTATGGCAGGAAGAA-3')	WITO-RevEnv-R (5'-TGGTGGAATTCTGCAGATCTTATAGTAAAGCCCTTTCGAAGCCCT-3')

Table 2: Sequences of forward and reverse primers used to isolate *nef* gene from T/F DNA samples.

T/F Clone	Forward primer	Reverse primer
pCH040-C(2625)	CH040-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAATGTAG-3')	CH040-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTTCTTGTAGTACTCCGGAT-3')
pCH058-C(2960)	CH058-RHPA-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAACGTA-3')	CH058-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGTTCTTGTAGTATTCCGGATACAGC-3')
pCH077-T(2627)	CH077-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAATTTGCT-3')	CH077-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAACAGTCCTTGTAAAACTCCGGATGT-3')
pCH106-C(2633)	CH106-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAAAATAAGTTTG-3')	CH106-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTCTTTGTAGAACTCCGGAT-3')
pREJO-C(2864)	REJO-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCCAAAAGTA-3')	REJO-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTCCTTGAAGTACTCCGGA-3')
pRHPA-C(2635)	CH058-RHPA-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAACGTA-3')	RHPA-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTTCTTGTAGTAGTCCGGA-3')
pSUMA-C(2821)	SUMA-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAAAGTAG-3')	SUMA-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTCCTTGTAGTACTCCGGA-3')
pTHRO-C(2626)	THRO-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAATGGTCAAAACGTAGT-3')	THRO-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGTCCTTGTAGAACTCCGGGTGT-3')
pTRJO-C(2851)	TRJO-Nef-F (5'-TACGACTCACTATAGGATGGGTGGCAAGTGGTCAAAAAGGA-3')	TRJO-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTCCTTGTAGTAATCCGGA-3')
pWITO-C(2474)	WITO-Nef-F (5'-TACGACTCACTATAGGATGGGGGGCAAGTGGTCAAAAAGTT-3')	WITO-Nef-R (5'-AGAGGTACCACGCGTGAATTTCAGCAGTCTTTGTAAAACTCCGGATG-3')

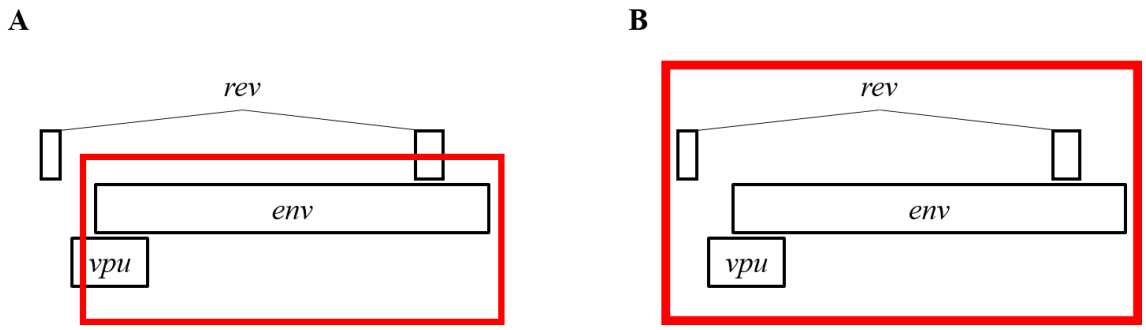

Figure 1: Schematic of plans for making Env expression constructs. (A) Original plan to create the Env expression construct. The primers were designed to amplify the *env* gene starting shortly before its start codon. This plan did not allow for proper Env expression. **(B)** Revised plan where the primers were redesigned to include the upstream elements *rev* and *vpu*. This plan ultimately enabled proper expression of Env and was used to create the Env expression constructs.

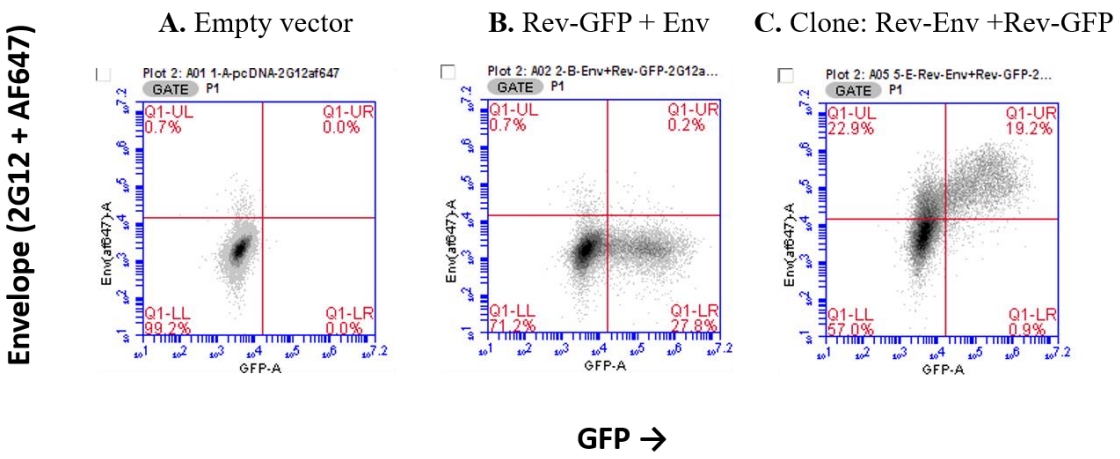

Figure 2: FACS data showing cell surface expression of Env during preliminary experiments of making and characterizing Env expression vectors. Transfected cells were harvested and stained with an anti-Env antibody and Alexa Fluor 647-conjugated donkey anti-human IgG secondary antibody. The x-axis shows GFP expression and the y-axis shows Env expression. **(A)** Empty vector negative control that shows no GFP or Env expression. **(B)** Rev + GFP and the initial Env plasmid that lacked upstream *rev* and *vpu* sequences. **(C)** Experimental Rev-Env clone with Rev + GFP showing both GFP and Env expression.

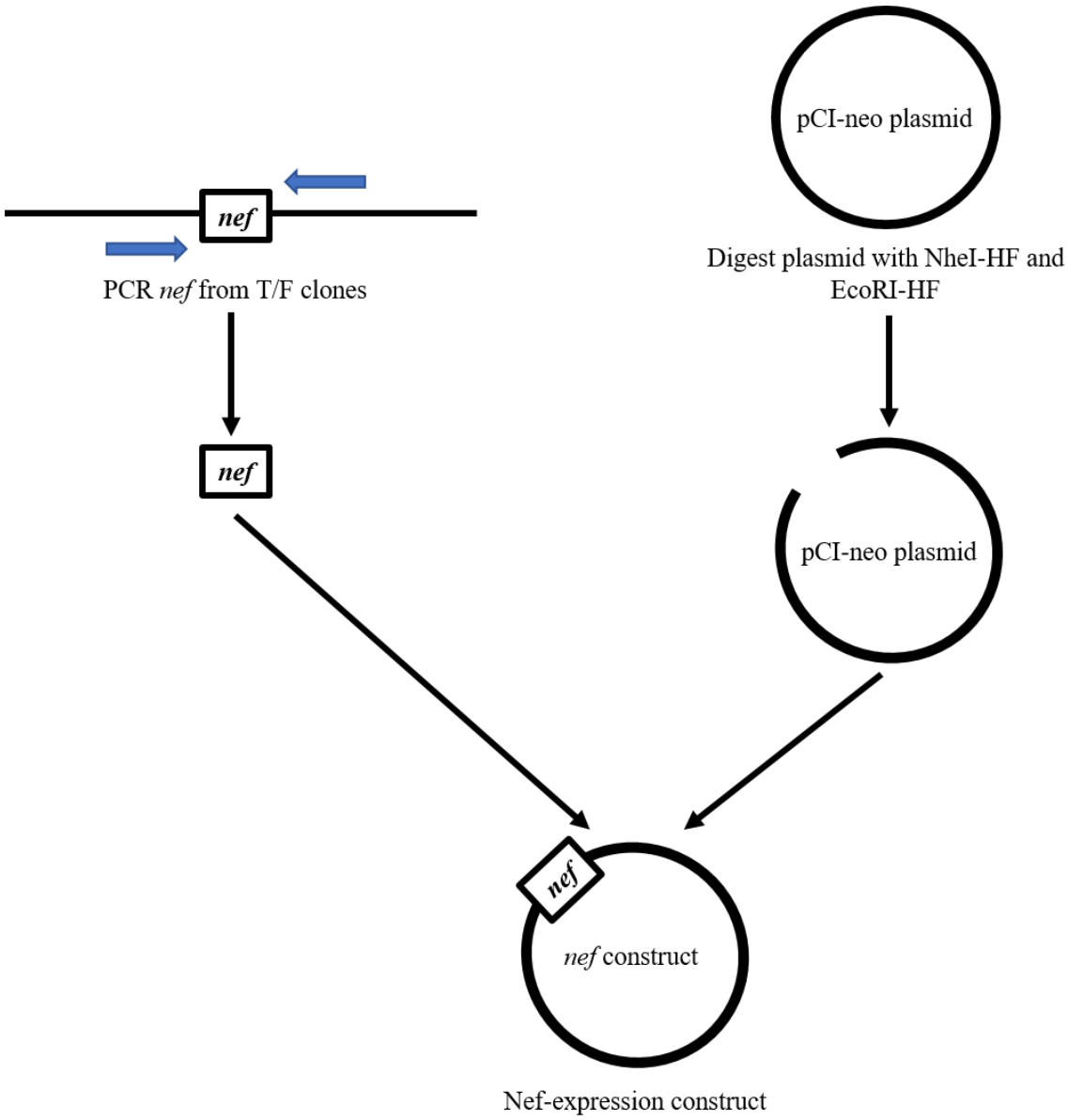

Figure 3: Workflow of making Nef expression vector. PCR was used to isolate and amplify *nef* gene from each of the 10 DNA T/F clones. The *nef* gene insert and the pCI-neo plasmid were both digested with NheI-HF and EcoRI-HF restriction enzymes, then cloned together using the In-Fusion® HD Cloning Kit. Constructs were verified to have the correct insert by sequencing before continuing with the rest of the project. Env expression constructs were made with the same protocol.

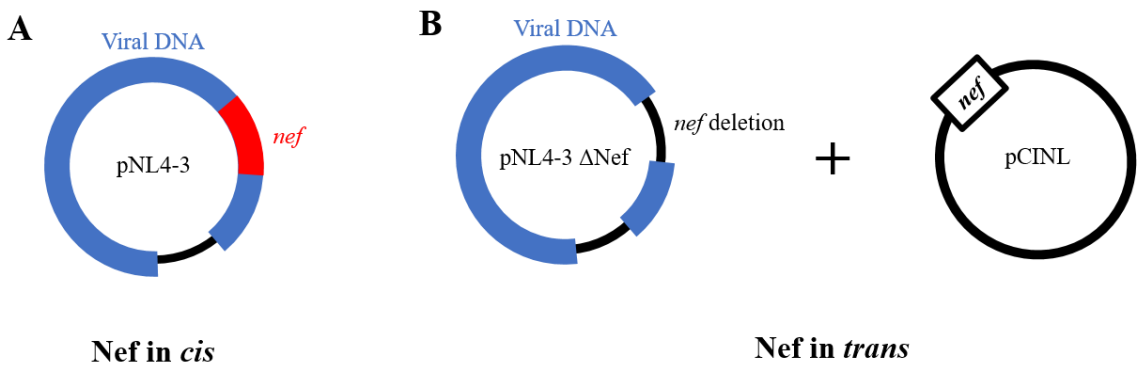

Figure 4: Schematic of *nef* in *cis* and in *trans* relative to the rest of the HIV-1 genome. (A) Picture of plasmid expression vector pNL4-3. Most of the plasmid is the viral DNA of HIV-1, including the *nef* gene in *cis*. **(B)** Picture of plasmid expression vector pNL4-3 ΔNef, which is pNL4-3 with *nef* deleted, with pCINL, which is a plasmid expressing *nef*. Both are expressed in cells, providing the *nef* gene in *trans*.

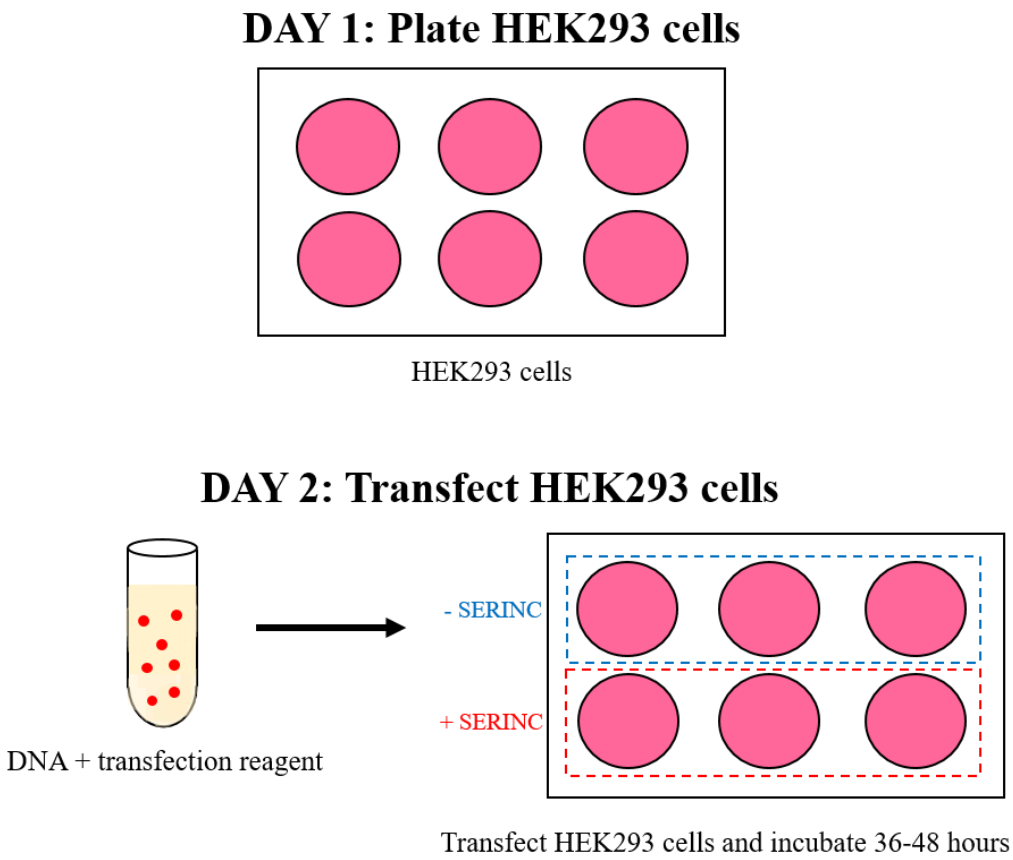

Figure 5: Schematic of transfection and infectivity assay. Day 1: HEK293 cells plated in 6-well plates. Day 2: DNA samples including proviral plasmid DNA together with a Nef-expression plasmid (or not), as well as either an empty vector or a plasmid expressing SERINC5 were transfected into HEK293 cells. Day 3: HeLa P4-R5 cells plated in 48-well plate.

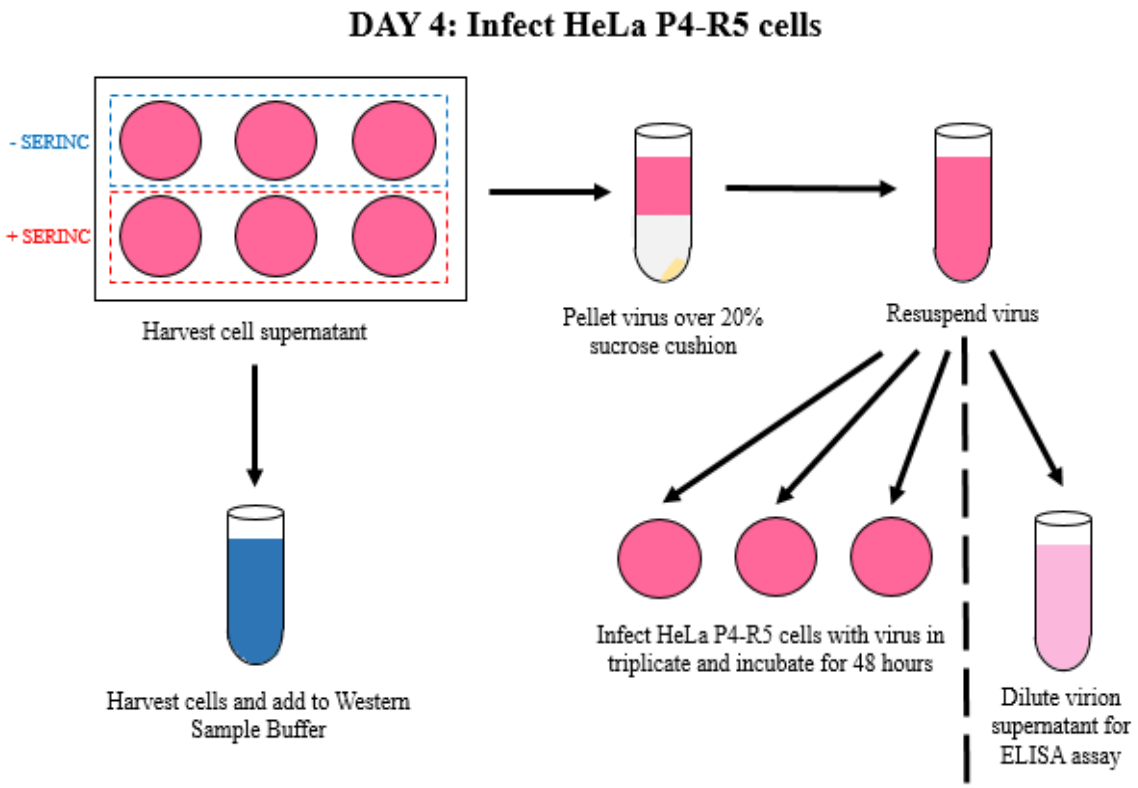

Figure 5 continued. Day 4: HEK293 cell supernatant harvested and pelleted over a 20% sucrose cushion. Virion supernatant was used to infect HeLa P4-R5 cells in triplicate and was also diluted in p24 lysis buffer for ELISA assay. Remaining HEK293 cells were harvested for Western blot. Day 6: fix and stain to reveal infected cells. Blue infectious centers were counted using the Romanizer image analysis program and normalized to the p24 ELISA assay data to correct for variations in the amount of virions produced.

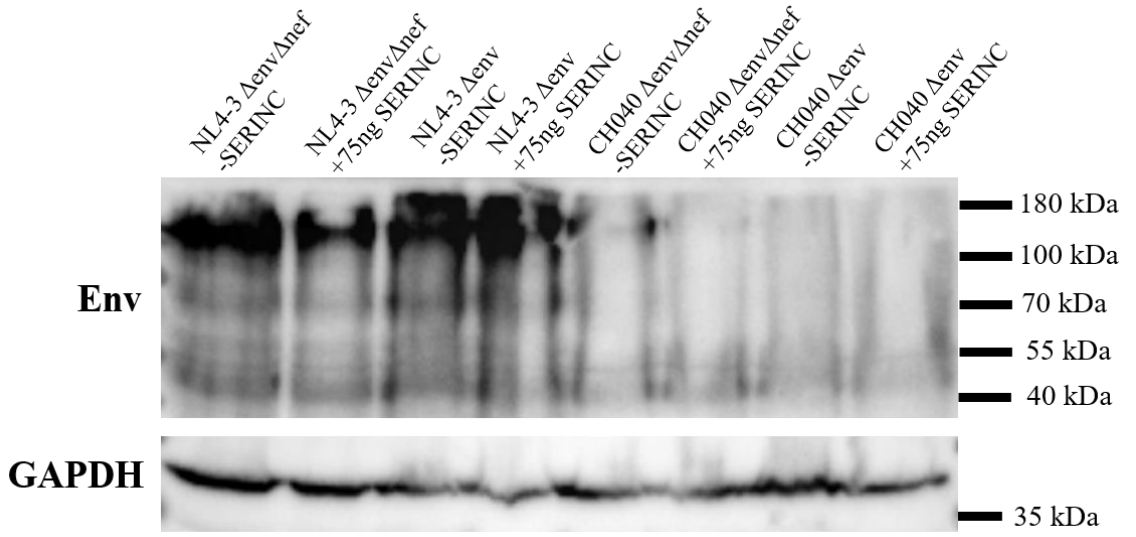

Figure 6: Western blot probing for Env and GAPDH. Samples used were harvested from cells that were transfected with *env*-negative NL4-3 plasmids with Env added back in with T/F Env expression constructs. GAPDH (bottom) was used as a loading control and detected in all samples. Env (top) presented as large smears in the blot that were present in all samples. NL4-3 ΔΔ is an expression plasmid lacking both *env* and *nef*, and CH040 ΔΔ similarly lacks *env* and *nef* genes.

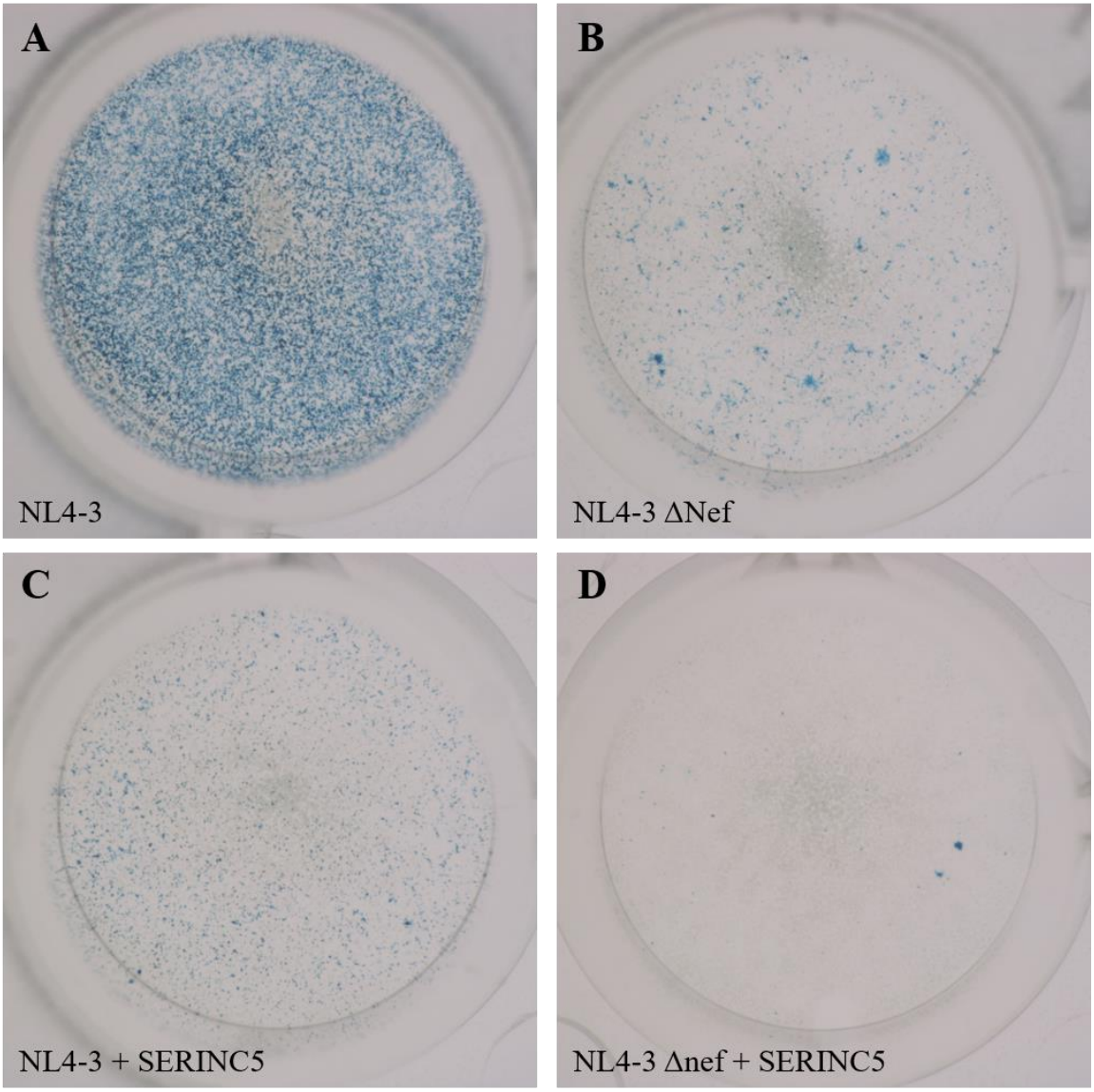

Figure 7: Infected HeLa P4-R5 cells stained blue as seen in the "Romanizer" image analysis program. (A) Picture of cells infected with NL4-3. Cells that are infected turn blue via the action of a β-galactosidase that is activated upon infection. **(B)** Picture of cells infected with NL4-3 ΔNef. **(C)** Picture of cells infected with NL4-3 produced from cells with overexpressed SERINC5. **(D)** Picture of cells infected with NL4-3 ΔNef produced from cells with overexpressed SERINC5.

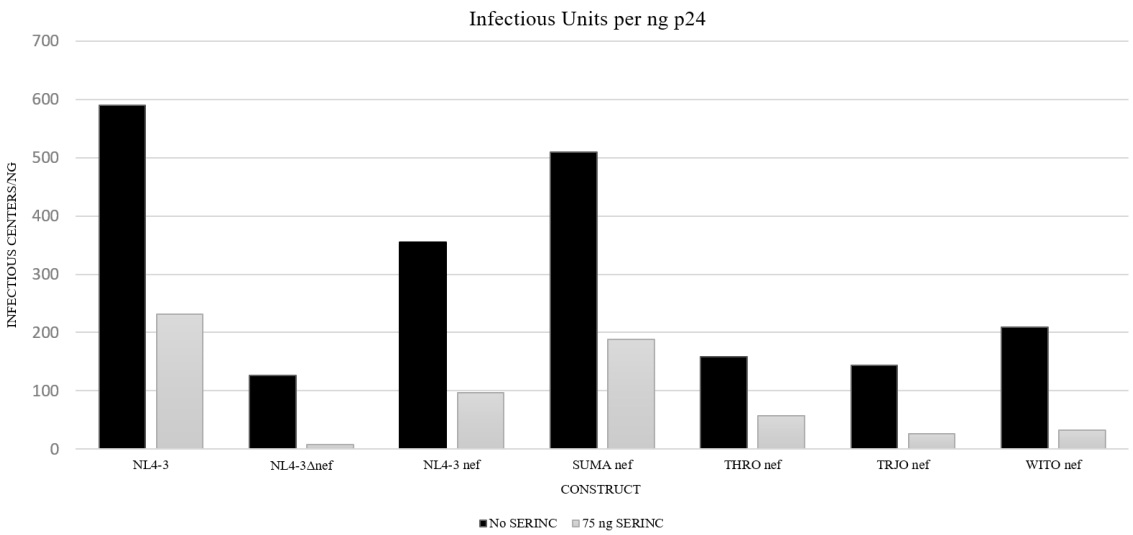

Figure 8: Graph of infectious units per nanogram p24 of controls (*nef*-negative and NL4-3 *nef* in *cis* and in *trans)* and selected T/F *nef* clones provided in *trans* to a *nef*-negative HIV-1 genome. Each experimental sample was tested with either no additional SERINC5 or with overexpressed SERINC5. "NL4-3" is a complete proviral plasmid encoding Nef and was used as a positive control (Nef in *cis*). "NL4-3 Nef" is another control where an exogenous Nef from pCINL was added to NL4-3 ΔNef in *trans*. All other samples are the *nef*-negative version of NL4-3 (NL4-3 ΔNef) to which Nef from the indicated clone has been provided in *trans* at the time of virus production. This graph shows infectivity data for all 3 controls and 4 of the 10 T/F clones. The number of infectious cells counted was normalized by the amount of virus particles determined by the ELISA assay for all samples.

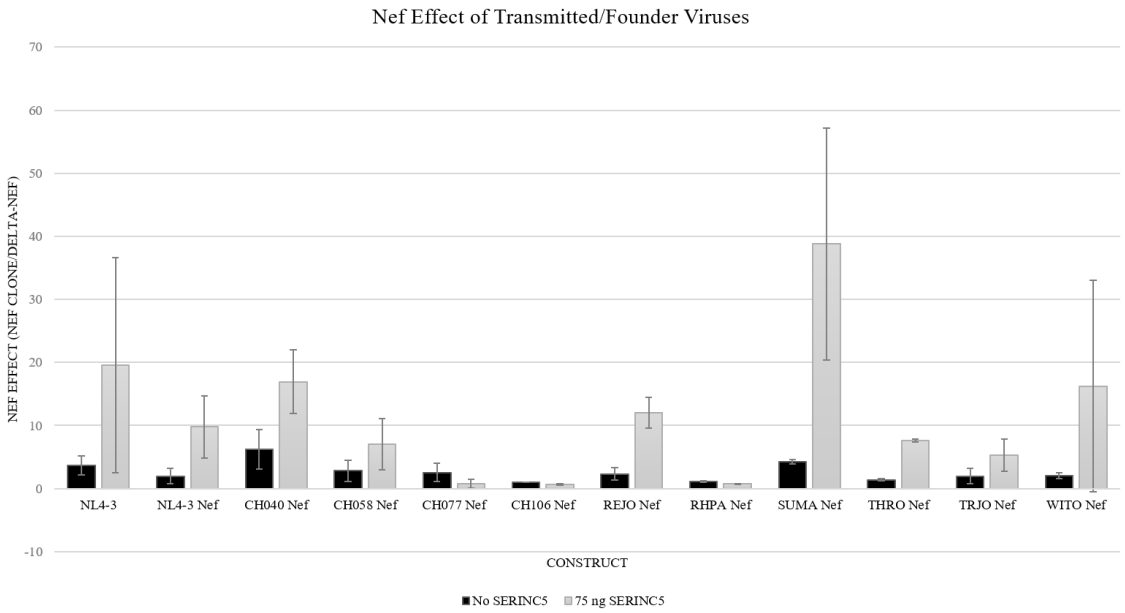

Figure 9: Graph of *nef*-effect of controls and 10 T/F clones. The number of infectious centers was normalized to nanograms of p24 as seen in Figure 8. The *nef*-effect was then calculated by taking the ratio of each sample's normalized infectious cells to the normalized infectious cells of NL4-3 ΔNef, as shown in this graph. All 3 controls and 10 T/F clones are represented in this graph. Error bars represent the standard deviation of duplicates of each sample.

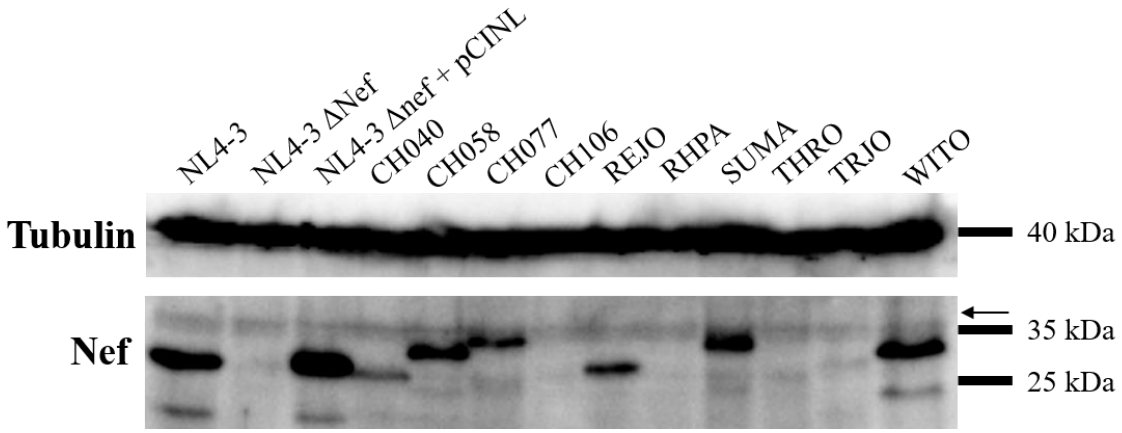

Figure 10: Western blot probing for Tubulin and Nef. Samples analyzed were harvested from cells that were transfected with *nef*-negative NL4-3 plasmid with Nef added back in *trans* using the T/F Nef expression constructs. Tubulin (top) was used as a loading control and was detected in all samples. Nef (bottom) was present in the positive controls (NL4-3 and NL4-3 ΔNef + pCINL) and not present in the negative control (NL4-3 ΔNef). Nef was detected in most of the T/F clones except for CH106 and RHPA, although the bands for THRO and TRJO are very weak. Arrow shows non-specific bands above the Nef bands.

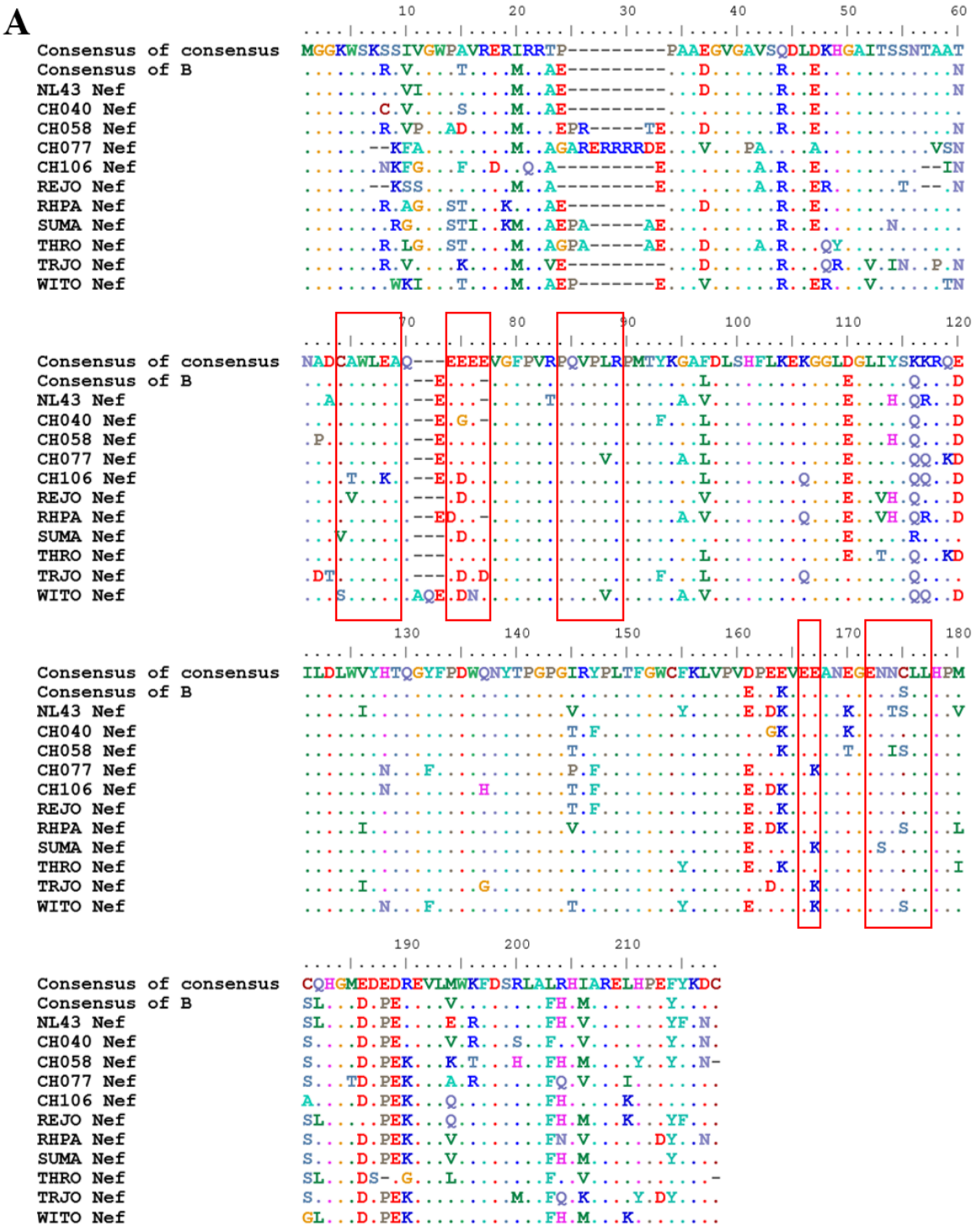

Figure 11: Amino acid sequence alignment of T/F Nef alleles compared to a consensus of Clade B sequences and NL4-3. (A) Entire Nef allele sequence alignment. Red boxes indicate differences in sequence motifs of HIV-1 Nef from the consensus sequence.

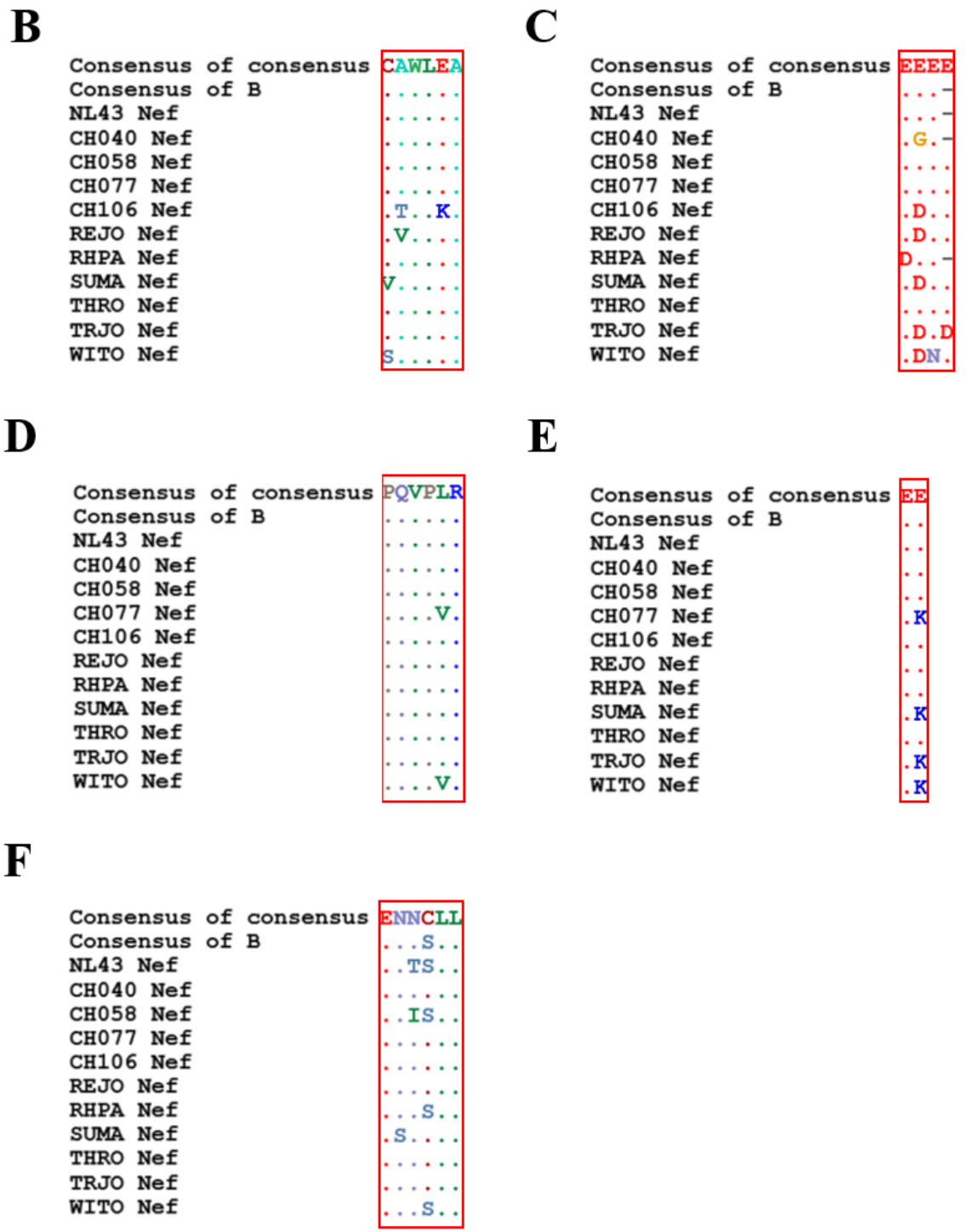

Figure 11 continued. (B) Close-up of sequence alignment at position 55 of consensus sequence where the motif for the HIV-1 protease is located. **(C)** Close-up of sequence alignment where the motif for PACS-1 is located. **(D)** Close-up of sequence alignment where the motif for the SH3 domains of Src family kinases is located. **(E)** Close-up of sequence alignment where the motif for β-COP is located. **(F)** Close-up of sequence alignment where the motif for adaptor proteins AP-1/2/3 is located.

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