HIV-1 NEF-SRC FAMILY KINASE INTERACTION: A NOVEL TARGET FOR THE INHIBITION OF HIV-1 PATHOGENESIS

by

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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February 27, 2012

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Human immunodeficiency virus-1 (HIV-1) is a lentivirus responsible for development of AIDS. In addition to typical retroviral proteins (Gag, Pol and Env), primate lentiviruses like HIV-1 encode two regulatory (Tat and Rev) and four accessory proteins (Vif, Vpu, Vpr and Nef). The HIV-1 accessory factor Nef is essential for high-titer viral replication and AIDS progression. Nef function requires interaction with many host cell proteins, including specific members of the Src kinase family. In this dissertation project, I explored whether Src-family kinase (SFK) activation is a conserved property of *nef* alleles from a wide range of primary HIV-1 isolates and its sensitivity to selective pharmacological inhibitors. Representative Nef proteins from the major HIV-1 subtypes A1, A2, B, C, F1, F2, G, H, J and K strongly activated Hck and Lyn as well as c-Src to a lesser extent, demonstrating for the first time that SFK activation is a highly conserved property of primary M-group HIV-1 Nef isolates. Moreover, patient-derived Nef proteins also strongly activated Hck. Recently, our group identified 4-amino diphenylfuranopyrimidines (DFPs) and diphenylpyrazolyldiazene (PPD-B9) compounds that selectively inhibit Nef-dependent SFK activation as well as HIV replication. To determine whether these novel compounds exhibit broad-spectrum Nef-dependent antiretroviral activity against HIV-1, I first constructed chimeric forms of the viral strain NL4-3 expressing the same 10 primary *nef* alleles. The infectivity and replication of these Nef chimeras was indistinguishable from that of wild-type in three distinct cell lines (MT2, U87MG and CEM-T4). Importantly, the 4-aminopropanol and 4-aminobutanol derivatives of DFP as well as PPD-B9

potently inhibited the replication of all chimeric forms of HIV-1 in both U87MG and CEM-T4 cells in a Nef-dependent manner. The effects of these compounds against HIV replication correlated with inhibition of Nef-dependent activation of endogenous SFKs. My results demonstrate that the activation of Hck, Lyn and c-Src by Nef is highly conserved among all major clades of HIV-1 and that selective targeting of this pathway uniformly inhibits HIV-1 replication. My results have strong public health significance for developing therapeutics against current drug resistant variants of HIV-1.

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AKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor and dissertation committee chairman Dr. Thomas E. Smithgall for his constant encouragement, guidance and nurturing my growth as a researcher. It was an invaluable and wonderful experience working with him. I am also thankful to my dissertation committee members: Dr. Todd A. Reinhart, Dr. Fred L. Homa and Dr. Tianyi Wang for their helpful guidance and suggestions for my research project. I am thankful to our collaborators Dr. John Engen (Northeastern University), Dr. Joanna Yeh (University of Pittsburgh) and Dr. Gabuzda (Harvard University) for their help in my research projects.

Thanks to wonderful environment created by past and present Smithgall Lab members. I am thankful to Dr. Lori Emert-Sedlak and Dr. Ronald Trible for their helpful guidance as well as for developing valuable assays in our lab. I appreciate and thankful to Shoghag B. Panjarian for her invaluable help in my experiments. Thank you to current lab members Dr. Linda O'Reilly, Dr. Jerrod Poe, Dr. Sabine Hellwig, Dr. Sherry Shu, Dr. M. Patricia George, Dr. John Jeff Alvarado, Xiong Zhang, Jamie Moroco, Sreya Tarafdar, Sean Kellner, Prerna Grover, Disha Joshi and past lab members Dr. Malcolm A. Meyn, Dr. Jonathan Shaffer, Dr. Dora Pene-Dumitrescu for their help and providing enjoyable work environment. I would like to thank the department of Infectious Diseases and Microbiology as well as Microbiology and Molecular Genetics and all the students, faculty and staff that made it such a pleasure to work and learn here.

My heartfelt thanks to my friends Nitin, Himanshu, Jessica, Radhika, Nidhi, Shashank, Pradeep, Amarjit, Vishal, Yaddu, Yogi, Jay, Parvez, Poonam and Amar. I am also thankful to my friends from India: Dhanu, Yogesh, Mahesh, Sambhaji, Kiran, Ajit and Nitin. Thank you for being my friends.

I owe my deep sense of gratitude to my family members, Aai, Aaba, my sister Anita and brother Harishchandra for emotional support and encouragement to pursue my education in United States.

1.0 INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) is an enveloped RNA virus belonging to the genus *Lentivirus* of the *Retroviridae* family and is the causative agent responsible for acquired immune deficiency syndrome (AIDS). After infection, the HIV-1 RNA genome is reverse transcribed to DNA and can integrate into host cell chromosome, which in turn can become a continuous source of viral progeny throughout the life of host cell (61,271). There are three major groups of HIV-1 sequences: M (*major*), O (*outliers*) and N (*non* M, *non* O). Among these, the HIV-1 M group is diversified into nine subtypes/clades (*A to K*) as well as several circulating recombinant forms (*CRF*) and are mainly responsible for global AIDS pandemic (174).

The first report of a mysterious disorder affecting five gay men in California occurred in 1981, yet there is still no curative treatment or vaccine for AIDS (1). These 30 years of worldwide epidemic of AIDS has caused the death of more than 25 million people. It is very difficult to develop a globally effective vaccine for HIV-1/AIDS due high variability of the HIV genome as well as its high mutation rate. Several different vaccine approaches have been tried but still there is no effective vaccine available. The development of highly active anti-retroviral therapy (HAART) during the mid-1990's substantially reduced the mortality associated with HIV/AIDS, effectively turning the disease from a death sentence into a chronic disease for those with access to antiretroviral drugs. However, as with the vaccine approach, the high mutation rate of HIV-1 continues to make HAART therapy less effective in controlling new infections

over time. Most anti-retroviral drugs currently used for AIDS treatment target HIV-1 proteins (e.g., reverse transcriptase, protease) which selects for drug resistance. This reality underscores the strong need for developing new drugs by targeting essential host-viral protein interactions in the hope of reducing the chances of developing drug resistant mutant. Our group is actively pursuing this approach, and has discovered several new classes of inhibitors of HIV-1 replication that target the interaction of HIV-1 Nef with the host cell kinase, Hck.

As these newly discovered anti-retroviral compounds are potential leads for AIDS therapeutics, the present study evaluated their broad-specificity against all major clades of HIV-1. In the course of these studies, we have shown for the first time that the activation of a subset of Src family kinases (SFKs) is a highly conserved property among all major clades of HIV-1 Nef. Then we showed that these newly discovered compounds are broadly active against Hck following activation by each of these Nef variants. Moreover, we showed for first time that targeting this cell signaling pathway broadly inhibited HIV-1 replication in a Nef-dependent manner in two different host cell systems. These discoveries set the stage for further development of these compounds as a new approach to antiretroviral therapy.

1.1 HUMAN IMMUNODEFICEINCY VIRUS

The Human Immunodeficiency Virus (HIV) belongs to the genus *lentivirus* (slow growing virus) of the Retroviridae family and is the causative agent of Acquired Immune Deficiency Syndrome (AIDS) (61,271). The HIV virion consists of a diploid genome containing two copies of positive-sense 5' capped RNA enclosed within a capsid and a lipid envelope. After docking on host cells by binding of HIV envelope proteins to host cell surface receptor CD4 as a primary receptor and either CXCR4 or CCR5 as a co-receptor; the virus enters into the cells. The choice of co-receptor determines the tropism of HIV-1. The macrophage tropic (M-tropic) strains also known as non-syncitia-inducing strains (NSI) enter by binding to the β-chemokine receptor CCR5 while the T cell tropic (T-tropic) strains also known as syncitia-inducing strains (SI) enter by binding to α-chemokine receptor CXCR4. The dual-tropic viruses are able to use both CCR5 and CXCR4 as co-receptors for entry into host cells (49,59). Following infection, viral proteins and the RNA genome are released into the cytoplasm. The single-stranded HIV RNA genome undergoes reverse transcription into double-stranded DNA by the viral reverse transcriptase, followed by transport into the nucleus where it integrates into host chromosomal DNA. Upon availability of specific host cell transcription factors, the viral genome is transcribed to produce various structural and regulatory proteins as well as full length copies of the RNA genome for packaging. Then new virions are assembled at the cellular membrane and released from the cell via budding [reviewed in (44,60,158)].

HIV-1 is mainly transmitted through homosexual or heterosexual intercourse, transfusion of infectious blood and from mother-to-child, but not every exposure leads to infection and not

every infection progresses to AIDS [reviewed in (84)]. Depending upon virus and host genetics, HIV infection eventually causes AIDS which is characterized by a drop in $CD4^+$ T cell counts and susceptibility to opportunistic infections such as *Pneumocystis pneumonia*, esophageal candidiasis, or toxoplasmosis, as well as certain AIDS-associated cancers, such as non-Hodgkins lymphoma and Kaposi's sarcoma [reviewed in (158,182,235,271)].

Although 30 years have passed since the first report of AIDS by the CDC, no curative treatment or vaccine has been developed. Though several candidate vaccines have been evaluated in clinical trials, the high rate of HIV mutation, variations in the host immune response and the development of quasispecies within the same patient make it challenging to develop vaccines against HIV-1 (51,126). The current therapeutic regimen, HAART based on targeting viral proteins, has extended the life of many people suffering from AIDS or HIV-1 infection. However, development of drug resistance, non-compliance due to the severe toxicity associated with chronic drug therapy as well as the cost of the treatment have made HAART therapy ineffective in controlling this pandemic (26,118,218). Therefore, there is strong need for developing new therapeutics. Anti-HIV drugs targeting virus-host protein-protein interactions represent an underexplored strategy for AIDS therapy.

1.1.1 The HIV-1 Virion

Unlike other cone shaped retroviruses, HIV virions are roughly spherical particles of about 120 nm in diameter spiked with protein tufts of about 200 Å per particle, as determined by atomic force microscopy (AFM) (150,258). Similar to other lentiviruses, the HIV virion is enveloped with a lipid bilayer originated while budding from host cells [Figure 1 and (258,264)]. Since this lipid bilayer is derived from the host cell, it contains several cellular membrane proteins

including major histocompatibility antigens, actin and ubiquitin (17). Additionally, the viral envelope also contains about 72 copies of two major viral glycoproteins derived from Env (Figure 1). Each envelope protein is composed of three copies of the heavily glycosylated surface protein (SU), gp120, and the transmembrane protein (TM), gp41. These proteins are derived from gp160 after proteolytic processing and have role in viral entry as well as syncytia formation (76,173).

The 40-60 nm conical core is composed of about 2000 copies of capsid protein (CA, p24) and is surrounded by a matrix shell comprised of approximately 2000 copies of the matrix protein (MA, p17) on the inner surface of the viral membrane. Two copies of the RNA genome are stabilized with about 2000 copies of nucleocapsid protein (NC, p7) as a ribonucleoprotein complex inside the core. The capsid also encapsulates viral enzymes (Protease, Reverse transcriptase, Integrase) as well as accessory proteins (Nef, Vif, Vpr) [reviewed in (258)].

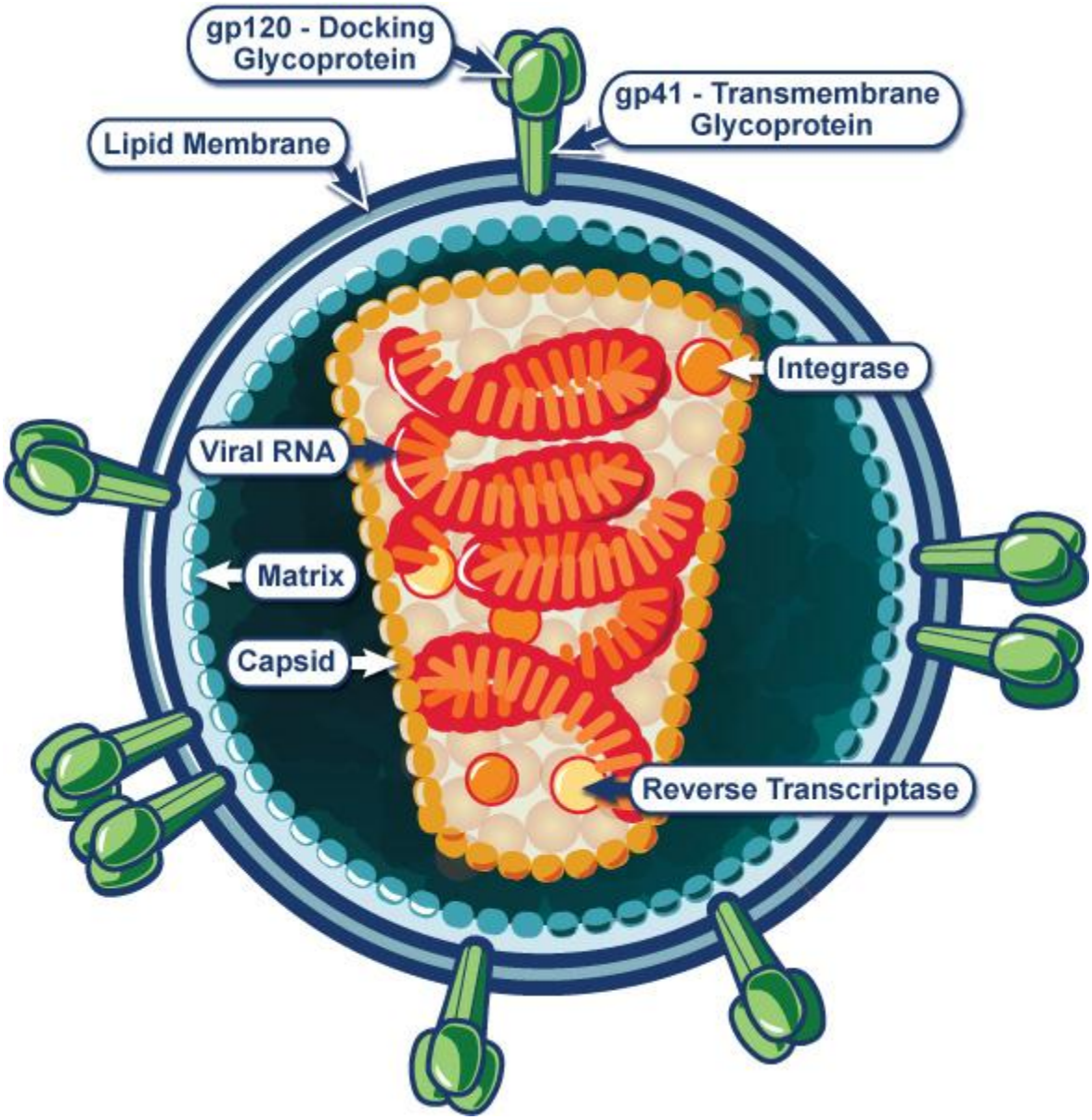

Figure 1. Structure of HIV-1 Virion.

This cartoon depicts a typical HIV-1 virion, with the major structural proteins indicated. (Image was reproduced from a publicly available source, the National Institute of Allergy and Infectious Diseases.)

1.1.2 HIV-1 Genome and Proteins

The HIV-1 provirus is approximately 9.8 kilobases in length and flanked by repeated sequences know as long terminal repeats (LTRs). The HIV-1 genome encodes 9 open reading frames (ORF) and gives rise to 15 proteins which include common retroviral major structural proteins; Gag (group-specific antigen), Pol (polymerase) and Env (envelope), two regulatory (Tat and Rev) as well as four accessory proteins (Vif, Vpr, Vpu and Nef) [Figure 2 (74,78,183)].

Gag

HIV-1 *gag* gene encodes a 55 kDa myristoylated Gag polyprotein that self assembles at the membrane to form immature virion or virus-like particles (VLP) in the absence of other viral components (38,82). During assembly, the Gag protein recruits two copies of unspliced, full-length genomic RNA as well as viral and cellular proteins and triggers budding of immature viral particles. After budding and during the process of maturation, p55 is proteolytically processed by the virally coded protease into MA (17 kDa), CA (24 kDa), NC (7 kDa) as well as low molecular weight products like p1, p2 and p6 (89).

The myristoylated MA polypeptide stabilizes each viral particle at the membrane during assembly as well as transports the viral genome into the nucleus with the help of the nuclear transport machinery after viral entry. The p24 CA protein forms the conical viral core and incorporates Cyclophilin A (CypA) which is critical for viral replication. The NC polypeptide recognizes and binds the packaging signal of the HIV-1 genomic RNA and stabilizes the HIV-1 genome during viral assembly. Moreover, NC facilitates reverse transcription after viral entry into host cell. A conserved LXXLF sequence within the p6 is required for Vpr packaging. The p6 polypeptide is also plays important role in viral budding [reviewed in (74,115,258)].

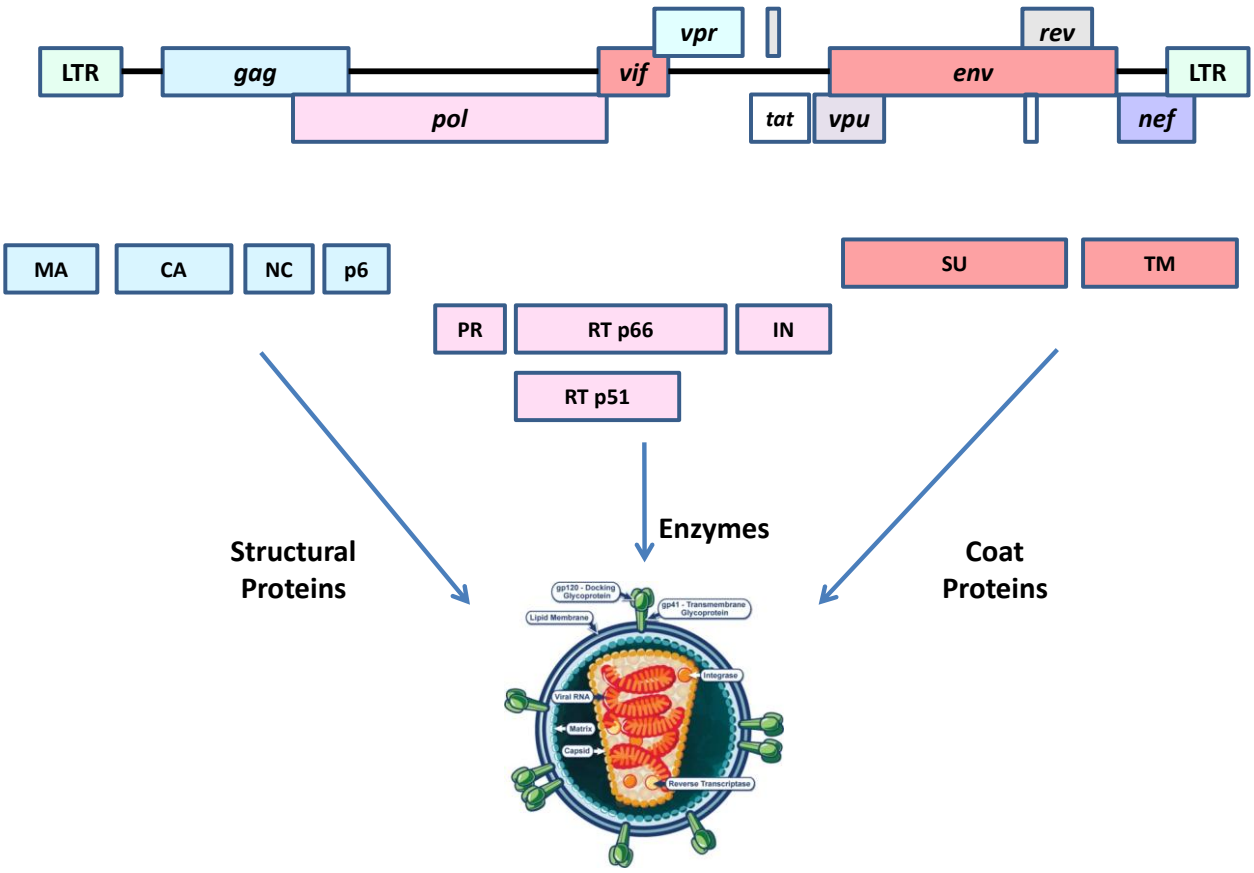

Figure 2. Organization of HIV-1 Genome.

The HIV-1 proviral genome is flanked by two long terminal repeats (LTR) which contain the promoter elements necessary for viral gene transcription. The *gag* gene encodes structural proteins, *pol* encodes the essential viral enzymes reverse transcriptase, protease, and integrase, and the *env* gene encodes the envelope glycoproteins required for HIV-1 virion assembly. [This figure is adapted from (78)].

Pol

HIV-1 *pol* gene encodes viral enzymes as Gag-Pol fusion proteins through ribosomal frame-shifting during translation involving a specific RNA motif in unspliced HIV-1 mRNA (122,197). The Gag-Pol polyprotein is autocatalytically processed and separates the Pol polypeptide from Gag. The Pol protein is further processed into protease (p10), RT (p50), RT-RNase H (p65), RNase H (p15), and integrase (p31).

As mentioned above, protease is required for processing of the Gag-Pol polyprotein during viral maturation. RT has RNA-dependent and DNA-dependent DNA polymerase activity and is responsible for the generation of the double-stranded DNA copy of the viral genome with help of RNase H before integration into the host cell genome. As RT has no proof-reading activity, quasispecies of HIV-1 arise even in the same patient which can lead to drug resistance. After reverse transcription, the double-stranded viral genome is integrated into the host cell genome by integrase, which possesses exonuclease and ligase activity. As all of these enzymes play critical roles in HIV-1 replication, they are natural targets for anti-HIV-1 drugs [reviewed in (115,258)].

Env

The singly spliced *env* mRNA encodes a 160 kDa glycoprotein. This protein undergoes extensive N-glycosylation at specific Asn residues in the Golgi compartment, followed by cellular protease cleavage into the gp41 (TM) and gp120 (SU) proteins which exists as trimers on mature viral particles. The 515-residue SU protein binds to the CD4 receptor on subsets of T cells and macrophages and initiates viral entry. V3, one of the five hypervariable regions on the SU protein interacts with either the CXCR4 or CCR5 co-receptors and determines viral tropism for

T-cells versus macrophages, respectively. The SU protein also interacts with DC-SIGN which can facilitate mucosal transmission by transporting HIV to lymphoid tissues. The TM protein has an N-terminal fusogenic domain that mediates the fusion of the viral and cellular membranes for viral entry into host cell. This domain is also a promising target for anti-HIV drugs that block viral entry [reviewed in (74,115,258)].

Tat

Tat, the transcriptional transactivator of HIV, is expressed from a multiply spliced mRNA early in the viral life cycle. Transcription from the integrated HIV-1 proviral genome initiates from the 5' LTR with the help of cellular transcription factors like RNA Pol II, NFκB, Sp1, and TBP but can terminate prematurely due to abrogative elongation. Tat enhances transcriptional elongation by binding to a stem-loop structure on the nascent viral RNA, known as TAR (*trans*-activating response element) as a Tat-cyclin T-Cdk9 complex. Cdk9 then stimulates transcriptional elongation by phosphorylating the RNA polymerase II transcription complex [reviewed in (74,115,258)].

Rev

Rev is a 13 kDa protein expressed very early in the HIV-1 life cycle from multiply spliced mRNA. Rev has an arginine-rich RNA binding domain, a multimerization domain and an effector domain with a nuclear export signal. Rev binds specifically to RRE (rev response element) sequences within singly spliced and unspliced transcripts containing the *env* gene sequence and transports them to the cytoplasm. HIV-1 proviruses with Rev mutations are transcriptionally active but do not produce virions [reviewed in (74,115,258)].

Vif

Vif is a 23 kDa protein important for production of highly infectious virus. Sheehy *et al*. discovered the HIV-1 replication inhibitor, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), which is targeted for proteosomal degradation by Vif (237). In the absence of Vif, APOBEC3G is efficiently encapsidated in progeny virions and responsible for G to A mutations in newly synthesized cDNA by RT. These mutations arise from the APOBEC3G-mediated deamination of cytosine nucleotides, and this uracil-containing DNA is then degraded before integration. In addition, the modified proviral DNA that does integrate cannot be effectively translated due to multiple stop codons. In the case of wild-type HIV-1, Vif directly interacts with APOBEC3G and targets this restriction factor to an elongin/cullin/suppressor of cytokine signaling (SOCS) box (ECS) E3 ligase complex, resulting in APOBEC3G polyubiquitination and subsequent degradation [reviewed in (74,189)]. We have recently reported that similar to HIV-1 Nef, Vif also binds and activates SFKs in yeast-based and *in vitro* kinase assays but the significance of this interaction to HIV-1 pathogenesis is currently unknown (168).

Vpr

Vpr is a 14 kDa protein packaged into virions by direct interaction with the p6 domain of the Gag protein. Vpr plays a role in ability of HIV to infect non-dividing cells like monocyte-derived macrophages by transporting the viral preintegration complex (PIC) to the nucleus through an intact nuclear membrane in the absence of mitosis. In addition, Vpr is involved in modulation of transcription of the viral genome, induction of apoptosis, disruption of cell-cycle control,

induction of defects in mitosis, facilitation of reverse transcription, suppression of immune activation and reduction of the HIV mutation rate [reviewed in (77,221)].

Vpu

Vpu is a 16 kDa protein expressed exclusively in HIV-1 and is expressed from the *env* transcript. Vpu is an integral membrane protein which is phosphorylated by casein kinase II, and is localized to the internal membranes of the cell. Vpu triggers the ubiquitin-mediated degradation of CD4 molecules complexed with Env and thus prevents the interference of virus assembly due to formation of intracellular Env-CD4 complexes. Additionally, Vpu enhances viral budding and viral load through the formation of ion channels which modify the electrical potential of the plasma membrane. The function of enhancement of viral budding by Vpu is executed by the Env proteins in HIV-2 and SIV, which lack Vpu [reviewed in (27,115)].

Nef

Nef is a 27 kDa myristoylated protein expressed early in the HIV-1 life cycle. Initially described as a *ne*gative *f*actor for HIV-1 replication, later studies revealed that this protein has *n*umerous *e*ffector *f*unctions (See section 1.2). Although, Nef is not necessary for HIV-1 replication in some continuous cell lines *in vitro*, it is essential for high titer replication *in vivo* as well as AIDS. Nef is involved in enhancement of HIV-1 pathogenesis as well as immune evasion by down-regulating cell surface receptors including CD4, CXCR4, CCR5 and MHC class I molecules. Moreover, Nef alters cell signaling pathways involved in cell proliferation, cellular activation, immune recognition and survival by interacting with diverse number of host-cell proteins (See section 1.2). Many lines of study have revealed the importance of Nef and

especially its interaction with SFKs in HIV-1 pathogenesis and progression to AIDS (58,80,102,104,132,134,141,145,228). The evaluation of this interaction and its implications for new anti-HIV drug discovery is the main focus of my dissertation research.

1.1.3 HIV-1 Life Cycle

The mature HIV-1 virion infects subsets of T lymphocytes and macrophages through interaction of SU (gp120) with CD4 and chemokine receptors (either CXCR4 or CCR5). After initial docking on cell surface, the fusogenic peptide of TM (gp41) undergoes a conformational change that triggers fusion of the viral envelope with the host cell plasma membrane to allow entry of the viral core. The core is then uncoated in the presence of CypA and the viral genomic RNA is reverse transcribed by RT, resulting in the formation of the pre-integration complex (PIC). With the help of Vpr and other cellular factors the PIC is then transported to the nucleus. The viral double-stranded DNA is then integrated into host chromosomes as a provirus with the help of the viral IN enzyme. After cellular activation, and in the presence of host transcription factors, RNA Pol II initiates transcription from the 5'LTR of the HIV-1 provirus. This transcription is enhanced by Tat binding to specific regions on newly synthesized mRNA (TAR) and results in production of several copies of differntially spliced genomic and sub-genomic mRNA. The viral Rev protein is responsible for transport of these mRNAs from nucleus to cytoplasm by binding the RRE in the mRNAs. After translation, the Gag and Gag-Pol polyproteins localize to the plasma membrane while Env proteins are translated in the ER and glycosylated in the Golgi apparatus. The core particles are assembled at the membrane from Gag and Gag-Pol polyproteins, Vif, Vpr, Nef, and the genomic RNA, and an immature virion begins to bud from the cell surface. Vpu assists in the assembly of SU and TM on the viral envelope by preventing

interaction between Env proteins and CD4. Moreover, Nef promotes endocytosis and degradation of cell-surface CD4 and helps in the release of immature virus from producer cells. After budding, the virion undergoes maturation which involves proteolytic processing of Gag and Gag-Pol polyproteins. These mature virions are then ready to infect new cells by binding to CD4 and chemokine receptors [reviewed in (74)].

1.1.4 HIV-1 Vaccines and Therapeutics

The development of a globally effective and safe vaccine which induces durable immunity is the best approach to prevent new infections as well as to boost immunity in infected individuals. One major obstacle for development of a useful HIV-1 vaccine relates to global HIV-1 diversity. Among the three groups of HIV-1 sequences, the M group is responsible for more than 90% of HIV-1 infections associated with the global HIV/AIDS pandemic (111,194,205). The M group is further classified into nine subtypes or clades (A, B, C, D, F, G, H, J and K) which display more than 25% sequence variation in the Env and Gag regions (200,265). These subtypes also show significant diversity in global distribution. The B clade is most prevalent in America and Europe, while the C clade is mostly in the Indian subcontinent, China and Africa. Other clades (A, F, G, H, J and K) are also prevalent in Africa (Figure 3).

Over the last 30 years, more than 40 candidate vaccines have been evaluated in 80 phase I/II clinical trials. These vaccine trials have including live attenuated, recombinant DNA, and subunit vaccines as well as various prime-boost strategies but no effective vaccine has been developed [reviewed in (51,84,85,170,224)]. Among the four vaccines evaluated in human clinical trials III and IIb; including Vaxgen's gp120, Merck's rAd5, and ALVAC + gp120 (RV144), only RV144 showed modest efficacy in preventing HIV-1 infection. Inter- and

intraclade amino acid divergence in the HIV-1 glycoprotein as well as numerous CRF allows selection of variants which escape from host immunity and thus is the major obstacle for the development of effective global HIV-1 vaccine [Figure 3 and (84)].

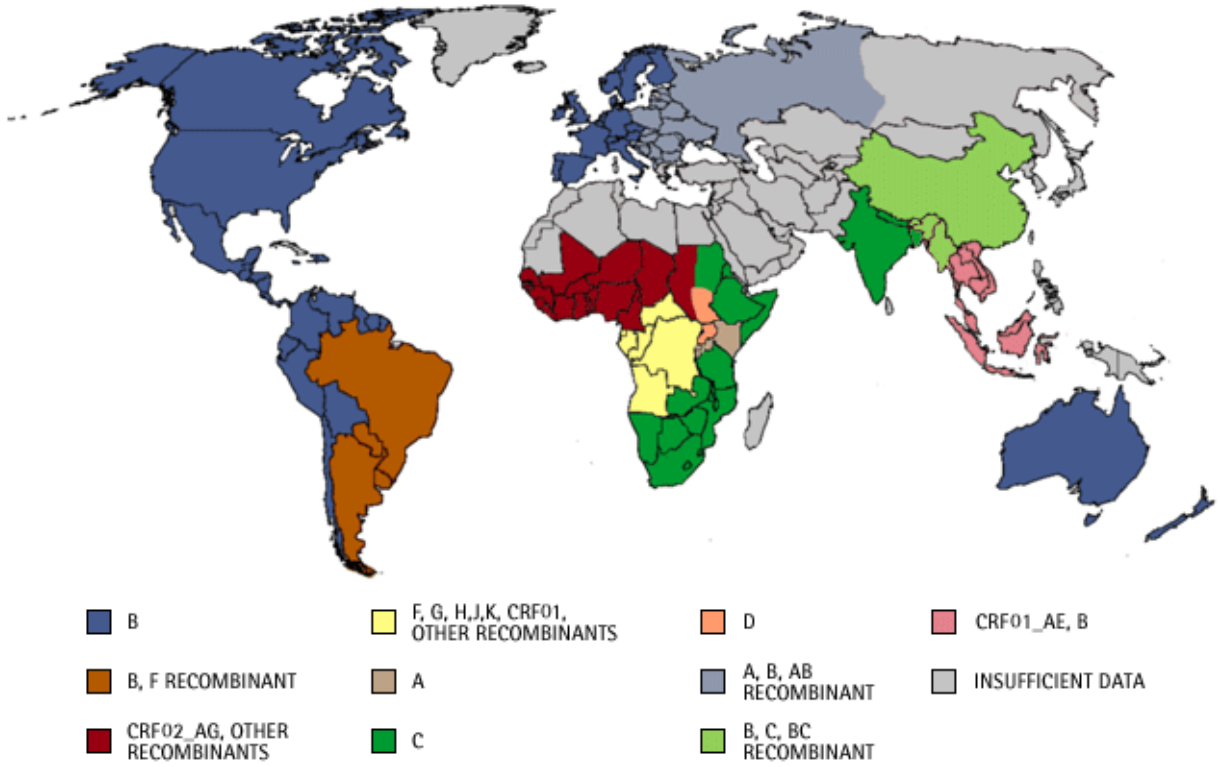

Figure 3. HIV-1 global distribution and diversity.

This map shows the global distribution and genetic diversity of the nine major HIV-1 subtypes, known as "clades," as well as major recombinants. [This figure was reproduced from a publicly available source, the IAVI Report, August 2003 (2)].

A live attenuated vaccine was initially produced by deleting the *nef* gene from SIV. While initially very promising, infected macaques ultimately progressed to SAIDS (see section 1.2.2). Similarly, HIV-1 vaccines based on virus-like particles (VLPs) with various modifications of glycoproteins and adjuvant combinations also failed to elicit neutralizing antibodies. Moreover, live vector or recombinant DNA vaccines expressing individual or subunit viral antigens failed to produce effective and protective immune responses, and even enhanced viral pathogenesis in some cases [reviewed in (42,84)].

As it is very difficult to develop an effective vaccine for HIV/AIDS, we must rely on antiretroviral drugs to treat the HIV-1 infected population. The development of highly active antiretroviral therapy (HAART) has been remarkably effective in controlling viral load and prolonging life as well as lowering the number of new infections (4). HAART is composed of a combination of three or more drugs targeting different stages of the HIV-1 life cycle. However, because HIV-1 remains dormant as the integrated provirus, patients must remain on these drugs throughout their lifetime to avoid relapse. This lifelong treatment results in several toxicities as well as development of drug-resistant viruses (11,26,149,177,190,270).

Currently, the U.S. Food and Drug Administration (FDA) has approved about 30 drugs which targets different stages of the HIV-1 life cycle, including virus-cell fusion, reverse transcription, integration and proteolytic cleavage (maturation) (260). These includes 2 multi-class combination products (Atripla, Complera), 13 nucleoside reverse transcriptase inhibitors (Combivir, Emtriva, Epivir, Hivid, Retrovir, Trizivir, Truvada, Videx EC, Videx, Viread, Zerit, Ziagen), 6 nonnucleoside reverse transcriptase inhibitors (Edurant, Intelence, Rescriptor, Sustiva, Viramune, Viramune XR), 11 Protease Inhibitors (Agenerase, Aptivus, Crixivan, Fortovase, Invirase, Kaletra, Lexiva, Norvir, Prezista, Reyataz, Viracept), 1 Fusion Inhibitor (Fuzeon), 1

Entry Inhibitor (Selzentry) and 1 HIV integrase strand transfer inhibitor (Isentress) (260). In addition to these, therapy based on siRNA/shRNA targeting HIV-1 and/or host genes looks like a promising new approach to combat AIDS [reviewed in (290)].

As described in detail in the next section, many studies have identified the *nef* gene as a critical virulence factor required for AIDS progression. In particular, patients infected with Nef-defective HIV-1 took more than 15 years to develop AIDS supporting Nef as a target for drug development. Considering the fact that HIV replication is highly mutable, new drugs targeting viral accessory factors such as Nef may also select for drug-resistant HIV-1 variants. One approach to circumvent this problem may be to target the interaction of the viral protein with host cell effector proteins, or inhibit critical downstream signaling pathways triggered by these factors. In this study, we showed that interaction of the HIV-1 Nef with the host signaling molecule Hck is highly conserved among all major clades of HIV-1. Moreover, we demonstrated that inhibitors of Nef-induced Hck activation are broadly active against Nef-dependent HIV-1 replication in cell culture.

1.2 HIV-1 NEF

The HIV-1 *nef* gene encodes a 27 kDa myristoylated protein expressed early in the HIV-1 life cycle (15,81,136). Initially described as a negative factor for HIV-1 replication, later studies showed that Nef has either no effect or a positive effect on HIV-1 replication depending on the host cell under study (10,106,135). More importantly, Nef is required for SIV-induced AIDS in Rhesus macaques, while defective or mutated forms of Nef have been detected in long-term non-progressors infected with HIV-1 (24,58,141). Nef exerts its effects by interacting with myriad host cell proteins involved in cellular activation, immune recognition and survival (114,216). Many protein-protein interaction surfaces have been mapped on Nef which have helped to validate important Nef functions, such as the down-regulation of CD4 and MHC-1 molecules and activation of host cell signaling pathways (Figure 4).

	Residues	Sequence	Binding partner / function	Reference(s)
a	1-6	MGxxxS(T)	Consensus sequence for myristoylation - essental for CD4, MHCI, MHCII modulation	[Harris 1995]
b	6	S	Phosphorylation, subcellular localization	[Wolf et al. 2008]
c	4-7	KxxK	Lipid raft targeting	[Giese et al. 2006]
d	17-22	RxRxRR	Basic cluster for membrane targeting	[Bentham et al. 2006]
e	20	M	MHC I recycling and p53 interaction	[Akari et al. 2000; Blagoveshchenskaya et al. 2002; Greenway et al. 2002]
f	55-60	CAWLEA	HIV-1 protease cleavage site (between WL)	[Freund et al. 1994]
g	57-9, 95-7, 106, 110	WLE,GGL, R,L	CD4 binding	[Grzesiek et al. 1996]
h	62-65	EEEE	PACS binding; MHC-1 retention in trans Golgi network	[Piguet et al. 2000]
i	72-75	PxxP	SH3 binding; Src family kinase binding; TCRζ binding; induction of Fas ligand	[Saksela et al. 1995; Xu et al. 1999]
j	105,106	RR	PAK 1/2 binding; Nef dimerization; apoptosis signaling	[Gross et al. 1999; Renkema et al. 1999; Fackler et al. 2000]
k	121-123	FDP	thioesterase binding; Nef dimerization	[Liu et al. 2000]
l	154,155	EE	beta-COP binding; lysosomal targeting	[Piguet et al. 1999]
m	160-165	ExxxLL	CD4/MHC modulation via AP 1/2/3 binding; clatherin coated pit targeting; CD28 downmodulation	[Bell et al. 2001; Swigut et al. 2001; Coleman et al. 2006]
n	174,175	DD	Vacuolar ATPase binding; CD4 downregulation; AP2 binding	[Geyer et al. 2002; Lindwasser et al. 2007]
o	174-179	DDPxxE	c-RAF1 kinase binding	[Hodge et al. 1998]

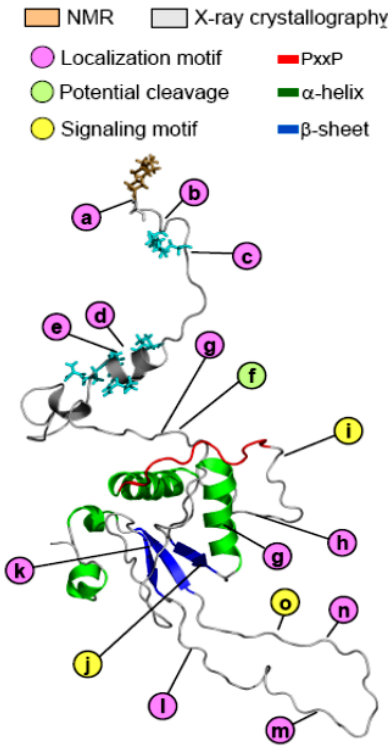

Figure 4. Structure Function relationship of HIV-1 Nef.

Different functional motifs and domains are mapped onto a molecular model of HIV-1 Nef as shown. Major functions associated with of each of these motifs are described in the table as well as in text (Courtesy of Dr. John Engen, Northeastern University).

1.2.1 Nef Structure-Function relationships

Nef has no known enzymatic activity, and appears to exert its effects by interacting with a diverse group of host cell proteins. The highly conserved HIV-1 Nef protein contains numerous well-defined structural motifs, many of which localize to the stable Nef core and are involved in binding to cellular proteins (Figure 4). X-ray crystallography as well as nuclear magnetic resonance (NMR) has shown that Nef consists of a globular core domain (amino acids 54-205) and flexible N- and C-terminal loops (13,75,96,155). The comparison of HIV-1 Nef sequences from 186 different HIV-1 strains showed that many structural motifs are highly conserved. Important examples include sequences for myristoylation MGxxx(S/T), the diluecine motif L164L, the acidic motif E62EEE, the proline-rich motif P72xxP, and a diarginine motif R105R. The functional significance of each of these motifs is detailed in the following sections. While HIV-1 Nef has been described to interact with a diverse group of host-cell proteins, the relevance of these interactions to HIV-1 pathogenesis has not been established in all cases (22,25,52,69,83,95,97,226,249,277).

Myristyolation motif

The flexible N-term domain of HIV-1 Nef contains the sequence MGxxx(S/T), which induces post-translational modification by N-myristoyl transferase (31,81). The covalent amide linkage of the 14 carbon saturated fatty acid to N-term Glycine at the G2 position targets Nef to the inner leaflet of the plasma membrane (217). This myristoylation motif is highly conserved among different strains of HIV-1 which highlights its importance in HIV-1 pathogenesis [reviewed in

(81) and (105)]. In addition to myristoylation of Nef, four N-terminal arginines between residues 17 and 22 as well as two lysines at positions 4 and 7 are required for its membrane targeting (22).

Mutational analysis revealed that N-terminal myristoylation of Nef is critical for HIV-1 replication. A Nef mutant defective for this post-translational modification completely blocked its ability to induce MHC-1 and CD4 down-regulation (108,203). The myristoylation of HIV-1 Nef is also required for its interaction with actin and its subcellular localization in human B and T cells (68). The Gly to Ala (G2A) mutation of the myristoylation site completely abolished the ability of Nef to drive AIDS-like disease development as well as depletion of $CD4^+/CD8^+$ thymocytes in transgenic mice. Thus the myristoylation and other regions at the N-terminus of Nef (amino acids 25-35 and 57-66) are critical for mediating severe T-cell phenotypes and organ disease, although residues 8-17 are dispensable for these Nef functions (103). The G2A mutant of Nef also failed to associate with PAK2 kinase as well as development of disease of the non-lymphoid organs (kidneys and lungs) (263). The myristoylation of Nef is also required for *in vitro* activation of STAT2, IRF3 as well as phosphorylation of α and β subunits of the IκB kinase complex and of JNK, ERK1/2, and p38 mitogen-activated protein kinase family members in human monocyte-derived macrophages (MDMs) (167). Nef myristoylation is essential for high affinity binding to calmodulin suggesting its role in calcium signaling (171). Myristoylated Nef also plays an important role in Nef-mediated activation of NFAT, NFκB, and induction of IL-2 expression. The Nef-driven pathways enhance transcription from the viral LTR as well as HIV-1 replication in T cells stimulated by CD3 and CD28 (269,288). Thus myristoylation of HIV-1 Nef is a very important post-translational modification critical for HIV-1 pathogenesis.

SH3 domain-binding motif (PxxP)

One important group of Nef-interacting proteins includes members of the Src protein-tyrosine kinase family (64). Nef directly bind and activates a subset of SFKs, namely Hck, Lyn and c-Src (154,156,226,256). Nef interacts with SFKs by binding to their SH3 domains through its conserved PxxPxR sequence. HIV-1 Nef is a relatively high affinity ligand for the Hck SH3 with affinity constants of in the nM to low μM range, depending upon the allelic variant (see Chapter 3 for more details). Structural studies of the Nef-SH3 interface reveals that the interaction involves not only the conserved PxxPxR motif on Nef but also a hydrophobic pocket formed by the three-dimensional fold of the Nef core region (96,155). This pocket accommodates an Ile residue on the SH3 domain RT loop, and is found only in the SH3 domains of Hck and the related Src-family member, Lyn. Subsequent studies revealed that Nef binding induces constitutive activation of Hck and other SFKs by displacing the SH3 domain from its negative regulatory interaction with the SH2-kinase linker on the back of the kinase domain (35,156,180). Activation requires both the Nef PxxPxR motif and hydrophobic pocket, and may produce a unique activated conformation of the partner kinase (45,156,201). In addition to these highly conserved Nef regions directly involved in SH3 engagement, studies of Nef alleles derived from long-term non-progressors have shown that residues at a distance from the SH3 binding site also impact Nef-dependent Hck activation, possibly through an allosteric mechanism [Figure 6 and reference (257)]. Nef-induced AIDS-like disease is completely reversed in mice expressing a Nef PxxP mutant unable to bind to Hck SH3 domain underscoring the importance of this motif in HIV-1 pathogenesis (102,104). The PxxPxR motif of Nef is not required for down-regulation of cell surface CD4 receptors but vital for enhancement of HIV-1 replication in infected peripheral blood mononuclear cells (PBMC) as well as SIV replication in macaques

(132,226). However, the ability of HIV-1 Nef to downregulate MHC class I receptors, a key function in immune evasion of HIV-infected cells, requires an interaction between the Nef PxxP motif and SFK SH3 domain (25,40,91,119). Similarly, the SH3-binding motif of Nef is required for down-regulation of the MHC class Ib protein HFE, which regulates iron homeostasis in macrophages (62). Additionally, the SH3-binding motif of Nef in conjunction with the myristoylation signal sequence is required for constitutive Hck-dependent STAT3 activation and proliferation of a myeloid cell line (34). The intact SH3-binding motif of Nef is also required for intracellular Lck accumulation as well as for its ability to interfere with the functions of the immunological synapse (99).

The dileucine motif:

One of the important functions of HIV-1 Nef is down-regulation of the cell surface receptor CD4 to help viral infection and spread as well as to block super-infection (21,56,161,163,225). Unlike SIV Nef where a tyrosine-based motif is involved (152,207), HIV-1 Nef has a highly conserved dileucine motif which interacts with components of the host cell protein trafficking machinery and act as an internalization signal for CD4 down-regulation (9,30). The conserved dileucine motif (L164L) sequence is localized to the carboxy-terminal disordered loop of HIV-1 Nef (90). The dileucine motif and tyrosine-based motif are two well characterized signals that direct cellular proteins to clathrin-coated vesicles (139). The L164L sequence of Nef binds directly and specifically to the beta-adaptin subunit of the clathrin adaptor complexes AP-1 and AP-2. Mutation of the dileucine motif impairs the ability of Nef to localize into clathrin-coated vesicles as well as to down-regulate CD4 without affecting down-regulation of MHC class I molecules (30,90).

The acidic motif:

HIV-1 Nef facilitates the replication of HIV-1 in the face of host immunity by down-regulating MHC class I molecules of the infected cells. HIV-1 Nef triggers the down-regulation of MHC class I molecules by binding to the trafficking adaptor protein PACS2 through a mechanism that requires a conserved N-terminal acidic motif (E62EEE). This interaction targets Nef to the trans-Golgi compartment where it assembles a multi-kinase complex (SFK/ZAP-70/PI3K) to internalize MHC I molecules (18,25,91,119). Sequence alignment of HIV-1 Nef derived from 186 different strains revealed that the acidic motif (E62EEE) is highly conserved with the variation is the insertion of fifth acidic residue (E or D) and non-conservative insertion of a Gly only in eight sequences [reviewed in (81)].

The diarginine motif:

HIV-1 Nef interacts with several cell signaling molecules and activates T cells to facilitate HIV-1 replication. Nef interact with and activates the cellular serine/threonine p21-activated kinase, Pak2 (16,69,214). In addition to the SH3-binding motif, a highly conserved diarginine motif (R105K/R) of Nef is required for Nef-mediated Pak2 activation (69,70,227). This motif is present at the N-terminus of helix α4 of Nef with the first Arg absolutely conserved and the second replaced with Lys or Gln. The presence of these two positively charged amino acids have a strong impact on helix formation as well as protein stability [reviewed in (81)]. Nef-mediated Pak2 activation is important for its antiapoptotic effects, induction of LTR activity and cytoskeletal rearrangement (70,278). Nef-mediated activation of Pak2 is important in progression of SAIDS in macaques as well as for AIDS-like disease development in HIV-1 transgenic mice (102,104,229).

1.2.2 Nef in HIV-1 Pathogenesis

Nef, an accessory protein only present in primate lentiviruses, is not required for HIV-1 replication *in vitro* (253) but is essential for high titer viral replication *in vivo* (8,103,174,222,241) and AIDS pathogenesis (58,132,133,146). HIV-1 and SIV both weaken the immune system by causing depletion of $CD4^+$ lymphocytes resulting in acquired immunodeficiency syndrome (AIDS) in humans and macaques respectively (29,159,172,175,176).

Nef is required for progression of simian AIDS, which resembles human AIDS in terms of depletion of $CD4^+$ lymphocytes, muscle wasting, and mortality (131). In this AIDS model, Nef-deleted SIV replicated very poorly compare to wild-type SIV, and macaques infected with Nef-defective SIV failed to develop SAIDS (132). Furthermore, a live attenuated SIV vaccine lacking the Nef gene completely protected macaques from challenge by intravenous inoculation of live, pathogenic SIV. Interestingly, when macaques were infected with live SIV having a Nef gene with a premature stop codon, the Nef reading frame was eventually restored, suggesting strong selective pressure for restoration of Nef expression in the context of SAIDS pathogenesis (57,132). The importance of Nef in AIDS progression became further evident from cohort studies of long-term non progressors (LNTPs) infected with Nef-defective HIV-1. These individuals remained asymptomatic for long periods of time and took more than 15 years to develop reduced $CD4^+$ lymphocyte counts (24,58,87,141). Moreover, Hanna *et al*. showed that the expression of Nef alone under the control of a hybrid CD4 promoter in the HIV-1 target cell population ($CD4^+$ lymphocyte and macrophages) is sufficient to cause AIDS-like disease development in transgenic mice. (102,103). Along similar lines, Nef is required for high titer HIV-1 replication as well as $CD4^+$ lymphocyte depletion in SCID-hu and hu-PBL-SCID mice

(98,123,124). While the specific mechanisms responsible for the impact of Nef on HIV disease are not clear, Nef has several important functions related to HIV-1 replication and T-cell activation that are likely to play a major role. These are described in more detail below.HIV-1 infectivity in both lymphoid and myeloid cell lines is enhanced 100- to 10,000-fold by Nef-competent HIV-1 (48). In addition, Nef enhanced both reverse transcription as well as p24 capsid production irrespective of cell line used (233). Moreover, infection of mitogen-activated PBMCs also showed the enhancement of HIV-1 replication by Nef. This effect was more pronounced when unstimulated PBMCs were infected with Nef $^{(-)}$ versus Nef $^{(+)}$ viruses followed by mitogen activation, suggesting that those Nef-defective viruses required much more time to replicate to appreciable levels. These studies also demonstrated a positive effect of Nef on HIV-1 replication in macrophages (178,245). This positive effect of Nef on HIV-1 replication could be due to Nef-mediated enhancement of proviral DNA synthesis (10) and an increase in secondary viral spread in PBMC cultures (178). Nef also up-regulates the DC-SIGN receptor on dendritic cells (DC) which contributes to viral spread from DCs to T-lymphocytes [reviewed in (12)]. Nef is also involved in cytoskeleton reorganization by interacting with Vav1, a guanine nucleotide exchange factor for Rho family GTPases. This pathway may contribute to Nef-induced activation of Pak2, which is required for virion fusion, trafficking of the viral core as well as efficient reverse transcription (39,70,160,215,234) and [reviewed in (12)]. Similarly, Nef increases the lipid content in progeny virions by directing them to lipid rafts enriched in sphingolopids and cholesterol, which may also help to enhance viral infectivity [reviewed in (12)].

Another important function of Nef is to create an optimal environment for HIV-1 replication by activating T cells. Nef induces T cell activation by short-circuiting pathways involved in TCR signaling as well as the secretion of cytokines by macrophages which has a

paracrine effect on T cell activation (20,71,143,204,231,241,242,250,251). Gene-expression profiling in Jurkat cells showed that Nef expression triggers a transcriptional program which mimics anti-CD3 T activation. This study also showed that Nef downregulates IL-16 and YY1, which are negative regulators of HIV-1 transcription, while upregulating factors that, promote transcription, including Tat-SF1, U1 SNRNP, and IRF-2 (241).

Thus, Nef indeed has numerous effector functions that enhance HIV-1 replication and contribute to AIDS progression, and therefore represents a promising target for therapeutic intervention in AIDS. In this dissertation research project, we first validated the interaction of Nef and host cell signaling molecules, focusing on the SFKs because of their potential as targets for drug discovery. The basic structure-function relationship governing Src-family kinase activity and the mechanisms by which this is influenced by Nef is described in the next section.

1.3 SRC FAMILY KINASES

The Src-family of protein-tyrosine kinases (SFKs) consists of eight related kinases in mammals: c-Src, Fyn, c-Yes, Hck, Fgr, Lck, Lyn and Blk. Three of these (Src, Yes and Fyn) are expressed ubiquitously while the others exhibit expression patterns more restricted to hematopoietic cells (37,255). Hck and Fgr are expressed in macrophages (274,291) while Lck and Fyn are expressed in T cells (195); both of these cell types represent major host cells for HIV-1. SFKs are involved in regulating cell growth, survival, differentiation, cellular adhesion, migration, invasion, synaptic transmission as well as both acquired and innate immune responses (37,198,199). As SFKs are key regulators of cell signaling, the loss of regulation of SFK activity has been associated with several pathological states, including AIDS (43,104,121,144).

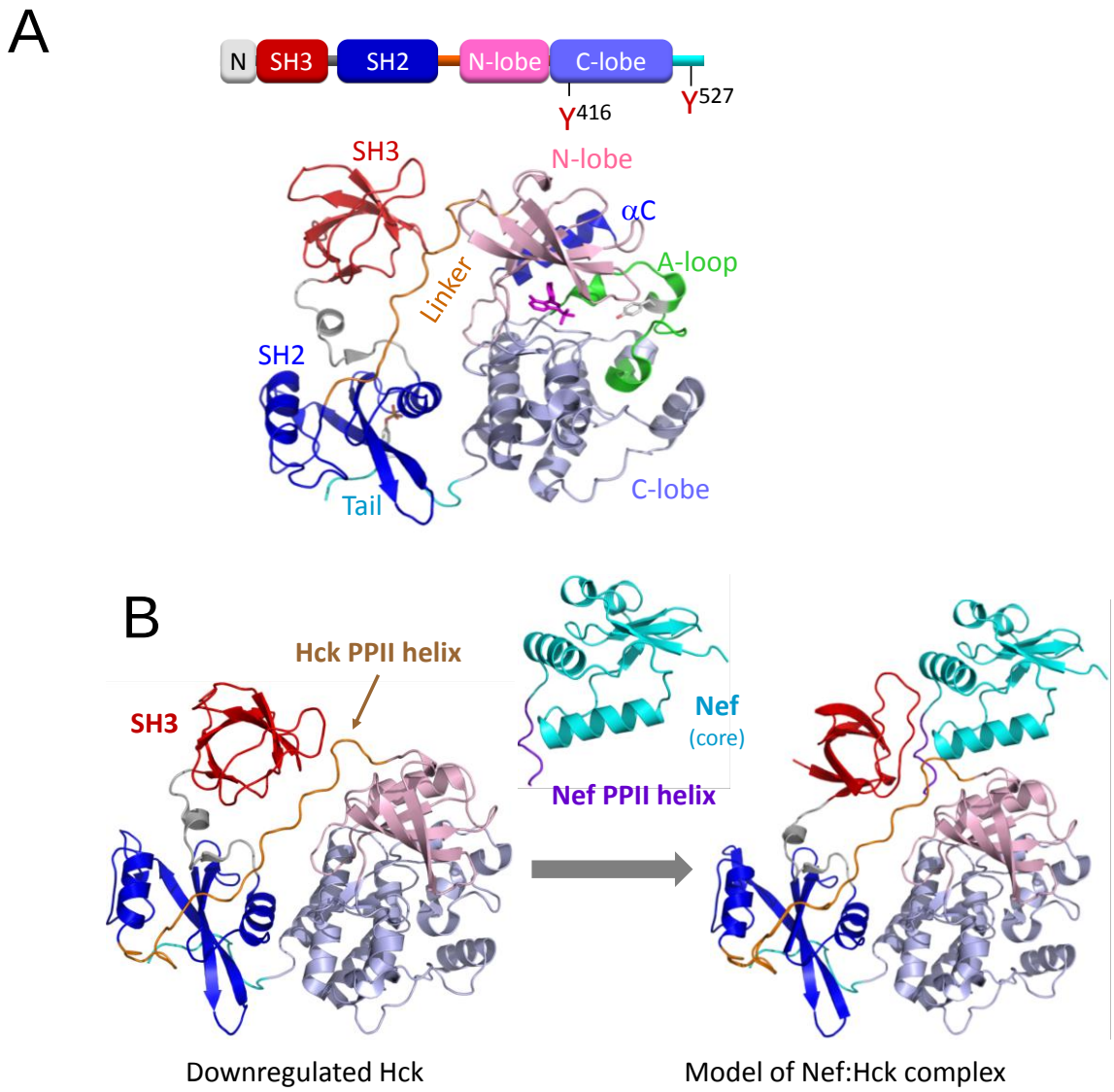

Figure 5. Crystal structure of the downregulated form of the macrophage Src-family member, Hck, and its activation by Nef binding.
A) The linear arrangement of the Hck domains is shown at the top, and corresponding colors are mapped onto the crystal structure below it. Shown are the unique N terminal (N; not in crystal structure), SH3, SH2, and kinase domains (N- and C-lobes) as well as the negative regulatory tail. The SH2-kinase linker is shown in orange. The location of the activation loop is indicated (A-loop), and tyrosine phosphorylation sites (loop and tail) are shown in red. B) Hck, like other members of the Src-kinase family, is held in the downregulated conformation by intramolecular interactions of the SH2 domain (blue) with the C-terminal tail (cyan) as well as the SH3 domain (red) with the SH2-kinase linker, which forms the PPII helix shown (orange). The HIV Nef core engages the Hck SH3 domain via a conserved PPII helix (purple), causing SH3 displacement from the linker and constitutive kinase activation. *(PDB coordinates used in this model: Downregulated Hck: 1QCF; Nef core: 1EFN; Hck: Nef complex: 1QCF, 1EFN, and 3NHN. Alignment and modeling via PyMol; model of Hck: Nef complex, courtesy of John Jeff Alvarado, Department of Structural biology, University of Pittsburgh School of Medicine*).

1.3.1 Src Family Kinase Structure

The determination of the crystal structures of near-full length c-Src as well as Hck in their downregulated conformations has provided critical insights into our understanding of the three-dimensional arrangement of their structural domains and their contribution to regulation of kinase activity (55,273,281). All SFKs contain a similar N- to C-terminal arrangement of structural domains (Figure 5). The myristylated N-terminal unique region (Src-homology 4 domain) is followed by a Src-homology 3 (SH3) domain, a Src-homology 2 (SH2) domain, a regulatory SH2-kinase linker, a bi-lobed tyrosine kinase domain (Src-homology 1 domain), and a C-terminal negative regulatory tail [reviewed in (64)].

Unique Domain (SH4 Domain)

The amino terminal unique domains (SH4 domain) are typically 50-80 residues in length and contain sequences for myristoylation and in most cases palmitylation that direct SFKs for membrane attachment (239). This membrane targeting of SFK is essential for full functional activity and signal transduction (37,128,286). SH4 domains are the only non-conserved regions within the Src kinase family and may determine specificity for downstream targets in some cases (195). For example, a dicysteine motif in the unique domain of Lck determines its ability to bind to the TCR co-receptors CD4 and CD8α. In another example, switching the unique domain of c-Yes for that of c-Src blocked c-Src-mediated activation of PI-3 kinase, phosphorylation of c-Raf and Akt and downregulation of RhoA-GTP, which is involved in dynamic regulation of actin. (247,259).

SH3 Domain

Src-family kinase SH3 domains are about 55-70 amino acids in length and have small hydrophobic grooves on their outer surface suitable for binding target peptides rich in prolines (50,148,289). These proline-rich target sequences generally form a left-handed polyproline type II (PPII) helix which associates with the hydrophobic surface of the SH3 domain (148,186). The central proline in this PPII helix interacts with a key tryptophan located within one of the hydrophobic grooves on the SH3 binding surface (66,232). In addition to interacting with signaling partners, the SH3 domain also regulates the kinase activity by binding to the linker region between SH2 and kinase (210). This SH2-kinase linker forms a PPII helix in the structure of the downregulated kinase.

SH2 Domain

SH2 domains are about 100 amino acids in length and bind to short peptide sequences containing a phosphotyrosine residue (50,148,267). The canonical high-affinity peptide motif for interaction with SFK SH2 domains is pTyr-Glu-Glu-Ile (pYEEI). This sequence forms a structure similar to a two-pronged plug which binds to a two-holed socket formed by the structure of the SH2 domain. One hole in the SH2 domain accommodates the phosphorylated tyrosine while the other shallower hole recognizes a hydrophobic isoleucine residue [(243,244,267) and reviewed in (64)]. All SH2 domains contain an invariant arginine residue (Arg174 in c-Src) essential for interaction with the phosphorylated tyrosine (266). The SH2 domains bind only to phosphorylated tyrosine target sequences (148).

Kinase Domain

The bi-lobed kinase domains (SH1) of SFKs are responsible for recognition of and catalytic transfer of a phosphate group to the target peptide sequence. The catalytic site is surrounded by key amino acid residues integral to the binding of both the substrate sequence and ATP (142). This region is highly conserved among SFKs, and the kinase domain structures of c-Src, Fyn, and Lck can be interchanged without loss of functional activity (28,138,282). The structure of the kinase domain is highly conserved within the members of the protein tyrosine kinase family including insulin receptor kinase, C-terminal Src kinase (Csk) and Abl kinase (116,117,151,188,191). The Asp386 and Asn391 within the kinase domain are critical for coordination of magnesium ions involved in ATP binding. Similarly, Asp404 of the conserved DFG motif (amino acids 404-406), coordinates ATP-Mg++, while the conserved Lys295 and Glu310, form an ionic interaction critical to maintaining the active conformation [Note: residue numbering is based on chicken c-Src] (28,100,222). This active site is formed by the space between the small and large lobes of the kinase domain, and access to this site is regulated in part by a highly mobile element known as the activation loop, which contains an auto-phosphorylation site (Tyr416). Phosphorylation of the activation loop tyrosine is associated with an increase in kinase activity (54,210). In the unphosphorylated state, Tyr416forms hydrogen bonds with Arg385 and Asp386 and effectively blocks the opening of catalytic site, whereas the phosphorylation of Tyr416 disrupts these interactions, causing displacement of the activation loop and opening of the catalytic site (5,55,230,280,282). Phosphorylation of Tyr416 leads to extended conformation that turns the activation loop to the “on” position that is believed to favor entry of ATP and peptide substrate into the catalytic cleft (109).

C-terminal tail

The carboxy-terminal tails of SFKs are about 15-17 amino acids in length and contain a suboptimal tyrosine-based SH2-binding motif (243). The C-terminal tail is critical for the negative regulation of kinase activity. The conserved tail tyrosine residues of SFKs are phosphorylated by a distinct regulatory kinase known as Csk (for C-terminal Src kinase) as well as a closely related kinase known as Chk (47,187). The phosphorylation of this inhibitory tyrosine (Tyr527 in c-Src) induces intramolecular binding of the tail to the SH2 domain, which helps to stabilize the downregulated conformation (37). One mechanism of SFK activation involves dephosphorylation of Tyr527 by cellular phosphatases. For example, activation of Lck in the context of TCR signlaing involves tail dephosphorylation by the the transmembrane phosphatase CD45, which releases intramolecular binding of SH2 to phosphotyrosine of the tail and promotes kinase activation (113,211,223,254).

1.3.2 Intra-molecular Regulation

As described in the preceding sections, the SH2 domains of SFKs interact with the tyrosine-based motif in the C-terminal tail, while the SH3 domains interact with PPII helices formed by the linker joining the SH2 and kinase domains. These two interactions are critical in kinase regulation by locking the kinase in the inactive conformation revealed in the X-ray crystal structures of c-Src and Hck (210,240,273,281).

The crystal structures of c-Src and Hck revealed the interaction of the SH3 domain with a suboptimal SH3-binding sequence linking the SH2 and kinase domains (240,273,281). The SH3-binding motif of the SH2-kinase linker is not well conserved among the SFK family and poorly resembles the canonical PxxP SH3-binding motif typical of high affinity PPII-based SH3 ligands

(6). This interaction alone is critical to downregulation of kinase activity. For instance, replacement of the two prolines in the Hck linker with alanines is sufficient to induce activation of the kinase in rodent fibroblasts via SH3 domain release (36). Conversely, substitution of the Hck linker lysines with prolines strongly enhances the binding of the linker to the SH3 domain, demonstrating that the wild-type linker region indeed mediates suboptimal SH3 binding (157). As described in detail above, Nef encodes a conserved proline-rich SH3-binding motif which has a high affinity for the SH3 domains of Hck and Lyn. This Nef motif therefore displaces the Hck SH3 domain from its regulatory position on the SH2-kinase linker and activates Hck through an SH3-domain displacement mechanism (154,180).

The interaction between the SH2 domain and the tyrosine-based SH2-recognition motif of the C-terminal tail is another key component of intra-molecular regulation of SFK activity. The high-resolution X-ray crystal structures of the inactive forms of c-Src and Hck revealed that the SH2-tail interaction holds SFKs in the down-regulated state (230,240,273,280,281). Replacement of the native Hck tail sequence with the high-affinity SH2-binding sequence YEEI yields a protein that adopts an auto-downregulated conformation (230). The Hck-YEEI molecule does not require Csk to adopt the inhibitory conformation, suggesting that a low level of autophosphorylation at the tail is sufficient to drive the molecule into a stable downregulated structure (164). The tight SH2-tail connection generated with the YEEI sequence is, unlike the wild-type sequence, refractory to release in the presence of an SH2-binding peptide (210). Even in the presence of SH3-linker contact, disruption of the SH2-tail interaction via mutation of Tyr527 causes a marked increase in kinase activity (157). Moreover, the activation of SFK by growth factor receptor tyrosine kinases involve SH2-dependent recruitment to the activated,

autophosphorylated form of the receptor; causing displacement of SH2-negative regulatory tail interaction (198).

1.3.3 Binding and Activation of SFK by HIV-1 Nef

SFK activity is regulated by the abrogation of intra-molecular interactions of SH3 and/or SH2 domains of SFKs can stimulate kinase activity via displacement of the inhibitory regulatory modules. Our laboratory and others have shown that HIV-1 Nef specifically binds and activates subsets of SFK namely Hck, Lyn and c-Src through displacement of SH3-linker interaction (35,154,156,226,256). The mechanism by which Nef induced Hck activation is modeled in Figure 5.

The Hck tyrosine kinase is strongly expressed in macrophages and dendritic cells (213,291), important target cells for HIV-1 infection. The SH3 domain of Hck recognizes the PPII motif of the Nef core domain, and Hck:Nef binding is blocked by proline-to-alanine mutations in Nef (45,226). Additionally, the hydrophobic pocket within the Nef core domain binds a key isoleucine residue in Hck, Ile96, and mutation of either Ile96 or key residues within the Nef pocket, such as Trp90, abrogates Nef binding to Hck (45,154). Other residues within the Nef hydrophobic pocket, such as Tyr120, have also been found to be critical for Nef-mediated binding and activation of Hck [Figure 6 (45)]. The HIV-1 Nef-Hck interaction is strongly implicated in AIDS progression. Suppression of Hck expression with antisense oligonucleotides dramatically inhibits M-tropic HIV-1 replication in primary human macrophages (145). In brain-derived microglial cells, HIV-1 infection induces Nef-dependent Hck activation; suppression of Nef-Hck activity via expression of dominant-negative Hck or CD45 phosphatase inhibits HIV replication (134).

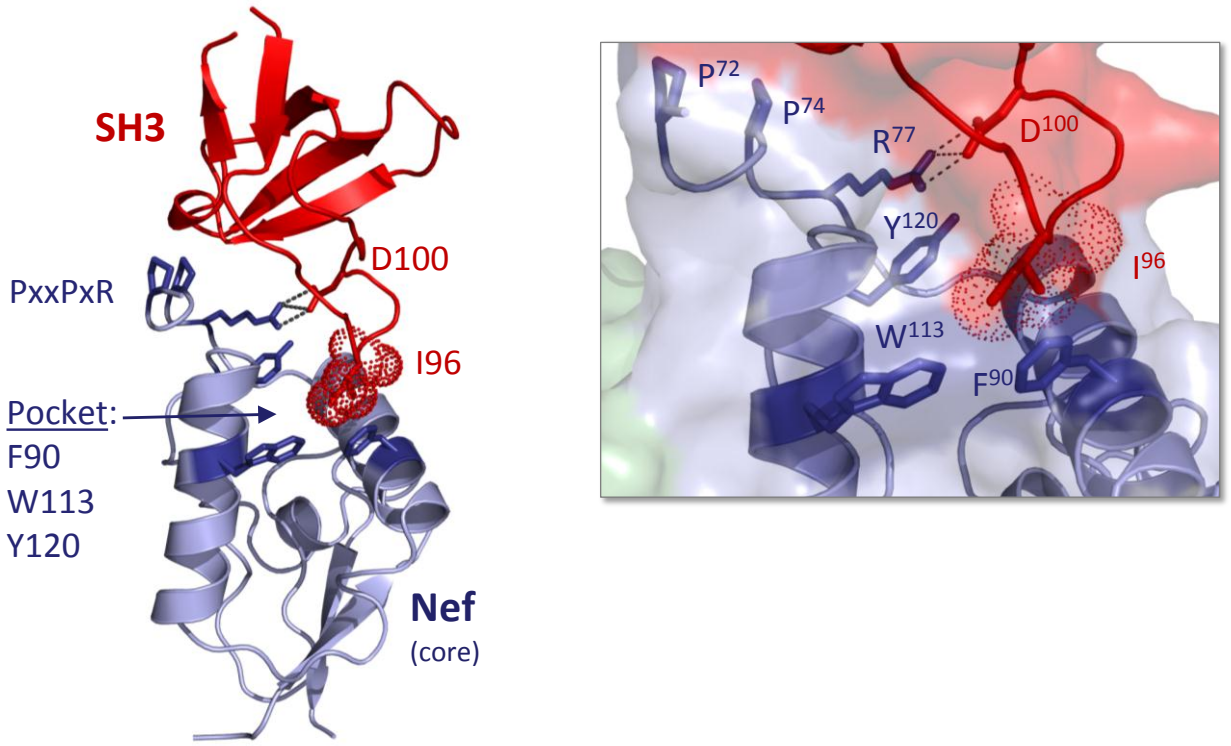

Figure 6. Structural basis of Nef: SFK interaction.

Nef interacts with the SH3 domains of Src family kinases (SFKs) via a highly conserved PxxPxR motif as well as hydrophobic pocket residues F90, W113, and Y120. A close-up of the Nef: SH3 domain interface is shown on the right, and also includes a polar contact between Nef R77 and SH3 domain RT loop residue D100. (Model based on the crystal coordinates of Lee *et al*. [PDB: 1EFN; (155).]

The Lyn tyrosine kinase is expressed in myeloid cells, as well as in brain and B cells (283). Similar to Hck, Lyn is the only other SFK that contains the SH3 domain Ile96 residue important for binding to the Nef hydrophobic pocket (45,154). Several studies have reported the ability of Nef to bind the Lyn SH3 domain, full-length Lyn as well as the Nef-mediation activation of Lyn (33,35,41,157,226).

c-Src is the proto-typical member of the Src kinase family and exhibits broader expression patterns including all HIV target cell types. Unlike Hck and Lyn, c-Src lacks the Ile96 residue implicated in high-affinity binding to the Hck-SH3 domain to Nef, and instead encodes an arginine at this position (154,155,273). Nef binds the SH3 domain of c-Src, though with a lower affinity than the Hck SH3 domain as well as full-length c-Src (14,33,45). Though Nef does not induce c-Src activation in a fibroblast transformation assay, Nef was shown to activate c-Src using a yeast-based kinase assay as well as in a model of HIV-associated nephropathy utilizing glomerular podocytes from HIV-transgenic mice (33,110,256).

Other than Hck, Lyn and c-Src, no evidence for direct association or activation of other Src-family members present in HIV-1 target cells has been reported. A major goal of this dissertation project, therefore, was to use a well-defined system to evaluate the range of SFKs that are HIV-1 Nef targets. Furthermore, we sought to establish whether the activation of Hck, Lyn and c-Src was a general property of representative Nef proteins derived from all of the major subtypes of HIV-1. These studies were critical to establish the profile of SFKs that represent general Nef effectors, an important step in target validation for novel antiretroviral drug discovery.

2.0 STATEMENT OF HYPOTHESIS AND SPECIFIC AIMS

2.1 HYPOTHESIS

Nef is a small myristoylated protein expressed early in the HIV-1 replication cycle that is essential for high titer viral replication and AIDS progression. Because Nef lacks enzymatic activity, it exerts its effects by interacting with various host cell proteins involved in cellular activation, immune recognition and survival. In particular, Nef interacts with the SH3 domains of Hck and other SFKs through its highly conserved proline-rich (PxxPxR) motif and hydrophobic pocket. Nef alone is sufficient to produce AIDS-like symptoms in mice and is required for SIV-induced AIDS in monkeys. Nef-induced AIDS-like disease is delayed in the absence of Hck in the transgenic mouse model. Our laboratory has shown that HIV-1 Nef specifically activates Hck, Lyn and c-Src.

We hypothesize that Nef mediated activation of specific SFKs is a general property of Nef and is required for HIV-1 replication in macrophages and T cells and therefore represents a novel therapeutic target for drug intervention.

To test this hypothesis we propose the following two specific aims:

1) Evaluate the ability of representative Nef alleles from all HIV-1 clades to activate SFKs and

2) Evaluation of inhibition of HIV-1 replication with novel small molecule inhibitors of Nef signaling.

2.2 SPECIFIC AIMS

Aim 1: Evaluate the ability of representative *nef* alleles from all HIV-1 clades to activate specific SFKs:

Nef proteins of all M-group HIV-1 clades (A, B, C, D, F, G, H, J and K) have a conserved PxxPxR polyproline type II helix responsible for binding to the SH3 domain of SFKs. Our laboratory has shown that Nef-induced Hck activation requires not only the PxxPxR motif, but also a hydrophobic pocket that interacts with the RT-loop of the SH3 domain. In this aim, I evaluated the interactions of Nef proteins representative of all major non-recombinant HIV-1 clades with Src-family members expressed in HIV-1 target cells (Hck, Lck, Lyn, Fyn, Fgr, and c-Src). The binding of HIV-1 Nef to the SH3 domain of Hck was evaluated using GST pull-down assays as well as surface plasmon resonance spectroscopy. Nef-mediated SFK activation was evaluated using *in vitro* kinase assays with recombinant proteins as well as a yeast-based system previously developed in our laboratory. These studies showed that HIV-1 Nef selectively activates the Src-family members Hck, Lyn and c-Src by binding to their SH3 domains. Furthermore, patient-derived Nef alleles from brain and peripheral organs also demonstrated strong Hck activation in the yeast-based assay. Together, these results demonstrate that SFK activation is a general property of HIV-1 Nef, helping to validate Nef: SFK complexes as therapeutic targets.

Aim 2: Evaluation of inhibition of HIV-1 replication with novel small molecule inhibitors of Nef signaling:

Studies in transgenic mice have shown that CD4 promoter-directed expression of Nef alone can cause AIDS-like disease in mice. This phenotype is partially reversed in Hck-null animals and is

completely abrogated with a PxxPxR mutant of Nef that can no longer bind to SH3 domains. These findings strongly indicate that inhibition of the Nef-SFK signaling pathway may inhibit HIV-1 pathogenesis. Our group has recently identified several novel classes of Nef-dependent antiretroviral compounds using high-throughput screening assays for inhibitors of Nef-induced Hck activation. In this Aim, I screened these compounds to test their ability to inhibit Hck activation by recombinant primary Nef proteins *in vitro*. Similarly, the broad specificity of these compounds was evaluated for the inhibition of Nef-dependent HIV-1 replication in U87MG and CEM-T4 cells using HIV-1 NL4-3 chimeras expressing the major *nef* alleles clade set. Replication of all HIV-1 Nef chimeras was potently inhibited by two distinct classes of small molecules, one that targets Nef directly (the phenylpyrazolyldiazine analog PPD-B9) and a second that selectively inhibits SFKs when Nef is bound (diphenylfuropyrimidine analogs). These results validate the Nef-Hck signaling axis as a viable target for development of broad-based inhibitors as a new approach to anti-retroviral therapeutics.

3.0 ACTIVATION OF HCK, LYN AND C-SRC IS A GENERAL PROPERTY OF HIV-1 NEF ALLELES FROM ALL M-GROUP HIV-1 CLADES*

*Some of the work presented in this chapter has been published in PLoS ONE: 2012 **7(2)**:e32561.

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Narute, P.S. and Smithgall, T.E. 2012 Nef alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor-sensitive manner. PLoS ONE **7(2)**:e32561.

3.1 ABSTRACT

The HIV-1 accessory factor Nef is important for high-titer viral replication and AIDS progression. Nef function requires interaction with many host cell proteins, including specific members of the Src kinase family. Our laboratory and others have shown that Nef specifically binds and activates subsets of SFKs namely Hck, Lyn and c-Src. All of these previous studies have used common laboratory alleles of Nef, including SF2 and NL4-3. It is necessary to evaluate this property of Nef using primary Nef alleles in order to target this interaction for therapeutic intervention. Nef proteins from HIV-1 clades (A, B, C, D, F, G, H, J and K) have a conserved PxxPxR polyproline type II helix which is responsible for binding to the SH3 domain of SFKs. Our laboratory has shown that Nef-induced Hck activation requires not only PxxPxR motif, but also a hydrophobic pocket that interacts with the RT-loop of the SH3 domain. The amino acid residues at a distance from the SH3 binding domain affected Nef-Hck complex formation and subsequent kinase activation. These observations strongly suggest that sequence alignments of *nef* alleles alone may not be predictive of Nef and SFKs interactions. In this aim, we first evaluated the ability of recombinant purified primary Nef proteins to bind Hck SH3 domain using GST pull-down assay as well as Surface Plasmon Resonance. Next, we evaluated the ability of these Nef proteins to activate SFKs expressed in HIV-1 target cells. In summary, we demonstrate that Nef proteins derived from all of the major subtypes of HIV-1 responsible for the global pandemic specifically bind and activate the same subset of SFKs. These results are significant because they establish for the first time that host cell SFK signaling is activated by all

variants of HIV-1 Nef, and that inhibition of this pathway may represent a broadly useful strategy to combat HIV/AIDS.

3.2 INTRODUCTION

The HIV-1 *nef* gene encodes a small myristoylated protein expressed early in the HIV-1 life cycle (15,81,136). Nef, one of several accessory factors unique to primate lentiviruses, is not required for HIV-1 replication *in vitro* but is essential for high-titer replication *in vivo* (10,107,178,226,245,253). Nef is also an important factor for HIV-1 pathogenesis and AIDS progression (67,127,206). For instance, introduction of a premature stop codon in the *nef* gene of SIV delayed simian AIDS progression and reduced viral load. Moreover, macaques immunized using this strategy, were protected from intravenous challenge with pathogenic SIV (57,132). However, some animals infected with non-pathogenic, *nef*-mutant SIV showed *in vivo* repair of the mutation and progression to AIDS-like disease, suggesting strong pressure to maintain Nef function (132,228,272). Similarly, defective or mutated forms of Nef have been detected in long term non-progressors infected with HIV-1 underscoring the importance of Nef in AIDS pathogenesis (58,80,140,141,153,169). Finally, the CD4 promoter-directed expression of Nef alone to HIV target cells in transgenic mice is sufficient for the development of an AIDS-like syndrome characterized by $CD4^+$ T cell depletion, diarrhea, wasting, and 100% mortality (102), supporting a major role for Nef in HIV-1 pathogenicity.

Nef has no known enzymatic activity and instead enhances viral replication and pathogenesis by interacting with various host cell proteins and altering cellular signaling pathways. HIV-1 Nef interacts with diverse number of host-cell proteins involved in cellular activation, immune recognition and survival (73,125,212,279). Many protein-protein interaction

surfaces of Nef have been mapped which reveal key functions such as the down-regulation of CD4 and MHC-1 molecules and activation of cell signaling pathways (Figure 4).

One important group of Nef-interacting proteins includes members of the Src protein-tyrosine kinase family. Earlier studies revealed that Nef interacts with the isolated Src homology 3 (SH3) domains from Src family members expressed in HIV target cells, including Fyn, Hck, Lck, Lyn and c-Src (13,14,32,45,92,93,226). Nef directly binds and activates a subset of SFKs, namely Hck, Lyn and c-Src (35,154,156,226,256). Of these, Hck is strongly expressed in macrophages and dendritic cells, while c-Src and Lyn exhibit broader expression patterns including all HIV target cell types.

Structural studies of the Nef-SH3 interface reveals that the interaction involves a highly conserved PxxPxR motif on Nef, which forms the polyproline type II helix typical of SH3 ligands as well as Ile96 within the RT-loop of the Hck SH3 domain, which fits into a hydrophobic pocket formed by the three-dimensional fold of the Nef core region (96,155). Both of these interactions are required but not sufficient for high affinity binding of Nef to Hck SH3 domain (35,45). Subsequent studies revealed that Nef binding induces constitutive activation of Hck and other SFKs *in vitro* and in cell-based systems including fibroblasts, myeloid cell lines, yeast and HIV-infected primary macrophages (34,35,145,180,256,287). In the down-regulated conformation of Hck, its SH3 domain associates with the polyproline type II helix formed by the linker connecting the SH2 and kinase domains (230,240). This interaction is further stabilized by the interaction of the SH2 domain with the C-terminal tail, which is phosphorylated on a conserved tyrosine residue by the negative regulatory kinases Csk and Chk (47,273,280,281). HIV-1 Nef activates Hck and other SFKs by displacing the SH3 domain from its negative regulatory interaction with the SH2-kinase linker on the back of the kinase domain (35,156,180).

Nef-mediated activation of Hck requires both the Nef PxxPxR motif and hydrophobic pocket, and does not require tail dephosphorylation or displacement from the SH2 domain suggesting this interaction may produce a unique activated conformation of the partner kinase (45,156). In addition to these highly conserved Nef regions directly involved in SH3 engagement, studies of Nef alleles derived from long-term non-progressors have shown that residues at a distance from the SH3 binding site also impact Nef-dependent Hck activation, possibly through an allosteric mechanism (257). Thus sequence alignments alone cannot necessarily predict the ability of primary Nef sequences to induce kinase activation.

Several studies have reported the importance of Nef-mediated Hck activation in HIV-1 pathogenesis and AIDS progression. For Instance, there is strong positive correlation between Hck expression and high-titer replication of macrophage-tropic HIV-1 in macrophages. Suppression of Hck expression with antisense oligonucleotides dramatically inhibits M-tropic HIV-1 replication in primary human macrophages (145). Moreover, Kim *et al.* showed that HIV-1 infection induces Nef-dependent Hck activation in brain-derived microglial cells. Suppression of this Nef-mediated Hck activation by expressing of dominant-negative Hck or CD45 phosphatase inhibits HIV replication (134). As mentioned above, Hanna *et al.* showed that targeted expression of Nef alone in HIV-1 target cells (T cells and macrophages) in transgenic mice results in AIDS-like disease development (101,102). However, mice expressing a mutant form of Nef lacking the PxxPxR motif essential for SH3 binding failed to develop this AIDS-like phenotype (104). Additionally, Nef-mediated AIDS like disease development is delayed in Hck-null mice (104). Taken together, these results underscore the importance of Nef-SFK interaction in HIV replication and AIDS pathogenesis.

Most previous studies investigating the Nef mediated SFK activation, have used laboratory alleles such as Nef SF2, NL4-3, and Bru-Lai (35,45,156,226,256). This led us to question whether SFK activation, particularly of Hck, was a general function of primary HIV-1 Nef variants from major clades of HIV-1. In the present study, we directly evaluated whether Hck SH3 domain binding and kinase activation *in vitro* are indeed properties common to Nef alleles representative of all major HIV-1 subtypes. In addition, we used our established yeast-based kinase assay to evaluate Nef-mediated activation of SFKs in a defined cellular context (256,257). The Nef proteins used in this study were derived from all major HIV-1 clades (A, B, C, D, F, G, H, J and K) share the conserved PxxPxR motif and hydrophobic pocket residues essential for SH3 binding. Using GST pull-down and *in vitro* kinase assays, we first showed that these Nef proteins from primary isolates bind to the Hck SH3 domain and activate recombinant, downregulated Hck *in vitro*. Next, using the system, we showed that all of the primary Nef proteins also activated Hck and Lyn (and c-Src to a lesser extent) in cells. However, Nef had no detectable effect on other SFKs expressed in HIV-1 target cells, namely Lck, Fyn and Fgr. These results support for the first time that Nef-mediated activation of a subset of Src-family kinases is broadly conserved among all major HIV-1 clades, validating complexes of Nef with Hck, Lyn and c-Src as broad-based targets for anti-HIV drug discovery.

In related work, we evaluated the effect of AIDS patient-derived Nef alleles on SFK activation to determine whether selection occurs for enhanced SFK activation during disease progression within a single patient. Previous studies have shown that HIV-1 infects macrophages and microglia cells in the brain and about 10-30% of AIDS patients eventually develop HIV-1 associated dementia (HAD), in which Nef plays an important role (86,261). Remarkably, Nef alleles derived from brain showed significant divergence in sequence and function compared to

those derived from lymphoid tissues within the same patients suffering from AIDS/HAD (7,8,193). This could be due to positive selective pressure for replication capacity of HIV-1 in macrophages and microglia cells (86,88). Moreover, Nef expression in macrophages enhances HIV-1 replication and results in secretion of cytokines which lead to paracrine activation of T cells as well as enhancement of transcription from the viral LTR (165,251,261). Nef alone in the absence of other viral proteins is able to induce monocyte/macrophage recruitment, expression of TNF-alpha, and astrogliosis as well as cognitive deficits in a murine model (181). Similarly, Hck activation in macrophages enhances HIV-1 replication and pathogenesis by enhancing proviral transcription. Moreover, Hck activation leads to proliferation of HIV-1 infected microglia and dysregulation of the immune response by altering macrophage phagocytosis and chemotaxis (65,134,246). These observations point to a connection between Nef-mediated Hck activation in the development of HAD. Previous studies by Gabuzda *et al*. have shown that *nef* alleles isolated from brain have adaptively evolved to enhance specific Nef functions compare to those isolated from peripheral organs from same patients (8,193). In the second part of this study, we evaluated the impact of adaptive evolution of *nef* sequences from four HAD patients on Hck and Fyn T activation using our yeast-based kinase assay. These patient-derived Nefs strongly activated Hck, but failed to activate Fyn T. Moreover, using a yeast growth suppression assay as well as quantitative western blotting we showed that brain-derived Nef positively evolved to activate Hck more strongly than Nef from peripheral lymphoid organs within the same patients. This study suggests that evolution of HIV-1 associated with HAD involves selection of Nef sequences with an enhanced ability to activate Hck.

The two main analytical methods used in the first part of this study involve the co-expression of Nef and SFK proteins in a yeast system, as well as an *in vitro* kinase assay with

purified recombinant Nef and SFKs. Each of these systems, and the rationale for their use, are described briefly below.

Yeast-based kinase assay: We have used a yeast cell-based kinase assay to evaluate the effect of primary HIV-1 Nef proteins on the activity of SFKs expressed in HIV-1 target cells. Our laboratory has previously shown that yeast is a good model system to study the effect of Nef on SFKs due to the absence of intrinsic tyrosine kinases (256,257). Moreover, similar to mammalian cells, yeast also provide post-translational modification of expressed proteins such as myristoylation of SFKs and HIV-1 Nef (147,256). When ectopically expressed in yeast, SFKs phosphorylate endogenous yeast proteins and cause growth suppression (72,219). However, the mechanism by which cellular SFK activation in yeast leads to growth suppression is unknown. Previous studies reported that activation of v-Src leads to phosphorylation and activation of cyclin-dependent kinase Cdc28 that negatively regulates DNA replication during mitosis and meiosis and causes growth suppression (72,219). In this way, HIV-1 Nef-mediated activation of SFKs can be measured by either evaluating tyrosine phosphorylation of endogenous proteins from lysed yeast cells or evaluating growth of yeast cells on solid culture medium. In this way, this system allows us to analyze not just the physical binding but the functional consequences of Nef-SFK interaction. Moreover, with the help of this system we were able to express functionally active full-length SFKs without adding epitope tags in a cellular context free from endogenous tyrosine kinases and regulators of SFK activity. Similarly, we were able to evaluate the effect of primary *nef* alleles from all clades on SFK regulation which was not possible using the *in vitro* kinase assay, because some of the Nef proteins could not be produced in soluble recombinant form. In the present study, we used yeast strain *Saccharomyces cerevisiae* YPH499

(Stratagene) and yeast expression plasmids pESC-TRP (Stratagene), pESC-URA (Stratagene) and pYC2/CT (Invitrogen). The genes of interest are cloned under the control of GAL promoters present in these vectors, so expression is induced only in the absence of glucose and in the presence of galactose as the sole carbon source on defined medium. In addition, the SFKs are expressed with modified "YEEI" tails so that they adopt the downregulated conformation in the absence of the negative regulatory kinase, Csk. This change simplifies the system while avoiding growth suppression when expressed in the absence of Nef or other activating factors. Our previously published studies have shown that Nef interacts with Hck in this system through an SH3-dependent mechanism previously defined *in vitro* and in mammalian cells (256,257). Thus, yeast provides a well-defined, experimentally tractable and relevant model for rapid analysis of Nef interactions with SFKs.

Z'-lyte kinase assay: The Z'-lyte kinase assay (Invitrogen) is a FRET (Fluorescence Resonance Energy Transfer)-based assay which employs a coupled-enzyme format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage (Figure 7). In the primary reaction, the kinase transfers the gamma-phosphate of ATP to a single tyrosine residue in a synthetic FRET-peptide substrate. In the secondary reaction, a site-specific protease recognizes and cleaves non-phosphorylated FRET-peptides. Cleavage disrupts FRET between the donor (i.e., coumarin) and acceptor (i.e., fluorescein) fluorophores on the FRET-peptide, whereas uncleaved, phosphorylated FRET-peptides maintain FRET (63,220). Phosphorylation of the FRET-peptides is measured by ratiometric analysis of both fluorescent emissions (coumarin 445 nm/FRET 520 nm). Our previous work has shown that Nef induces recombinant Hck-YEEI activation in this system, and that inhibitors of this reaction discovered

using this assay have activity against Nef-dependent HIV-1 replication (63,256). This approach provides an important *in vitro* complement to the yeast assay, as it shows that Nef is sufficient to induce kinase activation in the absence of other cellular factors in most cases. Moreover, this *in vitro* assay has an excellent Z-factor (~0.8) which allowed our laboratory to screen more than 300,000 compounds for inhibition of Nef-mediated Hck activation to date.

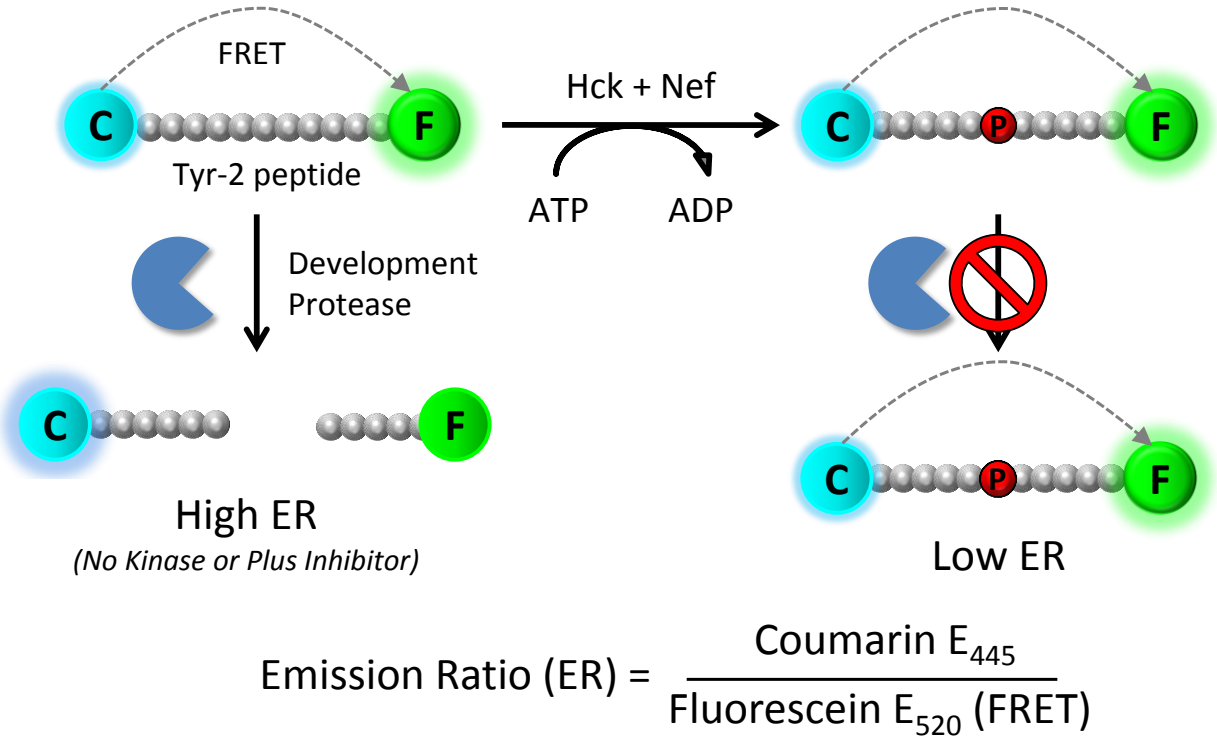


Figure 7. Z'-lyte kinase assay principle.

The kinase transfers the γ-phosphate of ATP to a single tyrosine residue in a synthetic FRET-peptide substrate (Tyr-2 peptide). Phosphorylation of the FRET-peptide suppresses cleavage by a protease in the Development Reagent. Cleavage disrupts FRET between the coumarin donor and fluorescein acceptor fluorophores on the FRET-peptide, whereas uncleaved, phosphorylated FRET-peptides maintain FRET. This is a ratiometric assay, in which kinase activity is reported from each well as an emission ratio of coumarin fluorescence to the FRET signal. In this way, the coumarin signal provides an internal standard for substrate concentration and reduces well-to-well variation (63,220).

3.3 MATERIALS AND METHODS

Yeast and bacterial expression vectors

Nucleotide sequences encoding representative Nef proteins from each of the HIV-1 M-group subtypes (A1, A2, B, C, F1, F2, G, H, J and K) were synthesized commercially (DNA2.0). The Nef sequences were PCR amplified and subcloned into the bacterial expression vectors pET14b and pET21a (Novagen/EMD Biosciences). Construction of similar bacterial expression vectors for Nef-SF2 (lab allele, B-clade) and Nef-ELI (D-clade) has been described (257). For yeast studies, the Nef sequences from DNA2.0 plasmids were subcloned into the yeast expression vectors pESC-Trp or pESC-Trp-Csk under the control of the GAL10 promoter as described previously (256). Similar pESC and pYC2/CT-based vectors for yeast expression of Hck, Lyn, Lck, Fyn, Fgr, and c-Src as well as the Herpesvirus saimiri Tip protein have been reported previously by our group (179,256,257).

Antibodies

Antibodies used in this study were obtained from the NIH AIDS Research and Reference Reagent Program (Nef, 2949), Santa Cruz Biotechnology (pY99, sc-7020; Hck, sc-72; Lyn, sc-15; Fyn, sc-16; Lck, sc-13; c-Src, sc-18; Csk, sc-286; Fgr, sc-17; His-tag, sc-803; GST, sc-138), Sigma-Aldrich (Flag-tag, A2220), and Millipore (actin, MAB 1501).

Nef and Hck protein purification

E. coli BL21 Rosetta (Novagen) or ArcticExpress (Stratagene) strains were transformed with pET14b or pET21a Nef expression plasmids and protein expression was induced with 0.5 mM IPTG at 28 °C for 4 h (Rosetta) or at 11 °C for 24 h (ArcticExpress). The bacterial cells were collected by centrifugation and lysed by sonication in either His-lysis buffer (20 mM Tris-HCl, pH 8.3, 500 mM NaCl, 20 mM imidazole, 10% glycerol and 5 mM β-mercaptoethanol) or Q-lysis buffer (20 mM Tris-HCl, pH 8.3, 10% glycerol and 5 mM β-mercaptoethanol). Lysates were clarified by centrifugation at 30,000 g at 4 °C for 30 min. His-tagged Nef proteins were purified by Ni-NTA affinity chromatography while untagged Nef proteins were purified by anion exchange chromatography using an ÄKTAexplorer automated chromatography system in both cases (GE Healthcare). Lysates containing His-tagged Nef were loaded on a Ni-NTA HiTrap Chelating HP column (5 ml; GE Healthcare) pre-equilibrated with lysis buffer. After washing with 2 column volumes (CV) of lysis buffer, proteins were eluted in His-elution buffer (20 mM Tris-HCl, pH 8.3, 1 M imidazole, 10% glycerol and 5 mM β-mercaptoethanol) with a linear gradient of 20 mM to 1 M imidazole over 6 CV. Lysates containing untagged Nef were loaded onto a HiPrep Q FF 16/10 column (20 ml; GE Healthcare) pre-equilibrated with lysis buffer. After washing with 2 CV of lysis buffer, proteins were eluted in Q-elution buffer (20 mM Tris-HCl, pH 8.3, 1 M NaCl, 10% Glycerol and 5 mM β-mercaptoethanol) using a linear gradient of 0 to 500 mM NaCl over 3 CV. Both the His- tagged and untagged proteins were further purified by gel filtration chromatography on a HiLoad 26/60 Superdex 75 column (GE Healthcare) with 20 mM Tris-HCl, pH 8.3, 100 mM NaCl and 3 mM DTT as mobile phase. Recombinant near full-length Hck kinase was purified in its downregulated conformation from Sf-9 insect cells as described in detail elsewhere (63,256).

Nef-SH3 domain binding assay

GST-tagged Hck SH3 domain fusion proteins (wild-type and W93A mutant) as well as GST alone were expressed in *E. coli* BL21 Rosetta cells and immobilized on glutathione-agarose beads according to our published protocol (232). For Nef-SH3 binding assays, each recombinant purified Nef protein (1 µg) was incubated with an equimolar amount of immobilized GST or the GST-SH3 fusion proteins in 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM Mg_2Cl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors at 4 °C for 2 h. Binding reactions were then washed by resuspending the beads 4 times in 1 ml RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) supplemented with protease inhibitors. Associated Nef proteins were eluted in SDS-PAGE sample buffer at 95 °C for 10 min, separated by SDS-PAGE, and visualized by immunoblotting with antibodies against the N-terminal His tag.

Surface Plasmon Resonance (SPR)

For SPR analysis of Hck-YEEI and untagged primary Nef proteins, buffer-exchange and concentration was first conducted using HBS-EPD buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005 % v/v P20 surfactant, 1 mM DTT) and an Amicon Ultra 15 ml 10 kDa molecular weight cutoff spin concentrator. For SPR studies with the Hck SH3 domain alone, proteins were buffer-exchanged and concentrated as above but using HBS-EPD buffer containing 0.05% (v/v) P20 surfactant in order to reduce non-specific binding to the biosensor chip. SPR analysis was performed on a BIAcore 3000 instrument (GE Healthcare) using a four channel CM5 biosensor chip with Hck-YEEI or Hck SH3 covalently attached as ligand via standard amine coupling chemistry (185). Nef proteins (as analyte) were flowed past the

immobilized Hck proteins on the biosensor chip at a flow rate of 10 µl/min for 5 min using Nef protein concentrations above and below the K_D (see Table 1). The initial binding reaction was followed by dissociation for 5 min, and the chip surface was regenerated using HBS-EPD buffer at a flow rate of 30 µl/min for 10 min. Binding curves recorded at each analyte concentration were assessed in triplicate, corrected for buffer effects, and fit to either a 1:1 Langmuir binding model or a steady-state affinity model using the BIAevaluation 4.1 software suite (GE Healthcare).

Table 1. Concentrations of recombinant HIV-1 Nef proteins.

Nef Protein	Nef Type	[Nef] used for Hck-YEEI SPR (μM)	[Nef] used for Hck SH3 SPR (μM)
SF2	Laboratory (B-clade)	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0	0, 0.007, 0.02, 0.062, 0.19, 0.56, 1.67, 5.0
Consensus	Laboratory (B-clade)	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0	0.02, 0.062, 0.19, 0.56, 1.67, 5.0
A2	Primary	0, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0, 30.0	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0
B	Primary	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0
F1	Primary	0, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0, 50.0	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0
F2	Primary	0, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0, 40.0	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0
J	Primary	0, 5.0, 10.0, 20.0, 40.0, 80.0, 100.0, 160.0	0, 5.0, 10.0, 20.0, 60.0
K	Primary	0, 0.062, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0	0, 0.19, 0.56, 1.67, 5.0, 10.0, 20.0

Concentrations of recombinant HIV-1 Nef proteins used to determine equilibrium dissociation constants with Hck-YEEI and the Hck SH3 domain. SPR analysis was performed with recombinant Hck-YEEI or the isolated Hck SH3 domain covalently attached to the biosensor chip as described under Materials and Methods. Untagged, purified Nef proteins were flowed past the immobilized Hck proteins at each of the concentrations shown, and binding response curves were fit to a 1:1 Langmuir binding model or steady-state affinity model using the BIAevaluation 4.1 software suite to determine the equilibrium dissociation constants shown.

In vitro kinase assay

Nef-mediated activation of Hck tyrosine kinase activity was assayed using the Z'-Lyte method with the Tyr2 peptide substrate (Invitrogen/Life Technologies). The principle of this FRET-based assay is described in detail elsewhere (63,256) and in Figure 7. Briefly, reactions (10 µl) were conducted in 384-well plates. Recombinant Hck (25 ng) was incubated with a 10-fold molar excess of each recombinant Nef protein in kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM MgCl2, and 1 mM EGTA, 0.01% Brij-35) for 1 h at room temperature. For experiments with Hck in the absence of Nef, higher input kinase was used (75 ng) to achieve equivalent levels of substrate phosphorylation to that observed in the presence of Nef. Development reagent was then added to the reaction for an additional 60 min at room temperature, followed by the stop reagent. Fluorescence was assessed at an excitation wavelength of 400 nm; coumarin fluorescence and the fluorescein FRET signal were monitored at 445 and 520 nm, respectively. Reactions run in the absence of ATP served as the 0% phosphorylation control, whereas stoichiometrically phosphorylated Tyr2 peptide was used as the 100% phosphorylation control. Raw fluorescence values were corrected for background, and reaction endpoints calculated as emission ratios of coumarin fluorescence divided by the fluorescein FRET signal. These ratios were then normalized to the ratio obtained with the 100% phosphorylation control peptide. Each condition was assayed in quadruplicate, and results are presented as the mean ± S.D.

Yeast assay for SFK activation

The yeast-based assay for analysis of SFK activation by Nef and other viral proteins is described in detail elsewhere (168,179,256,257). Briefly, *Saccharomyces cerevisiae* strain YPH 499 (Stratagene) was co-transformed with pESC-Ura (or pYC2/CT-Ura) and pESC-Trp plasmids

containing the genes of interest via electroporation (Bio-Rad GenePulser II). Transformed colonies were selected on synthetic drop-out medium lacking uracil and tryptophan with glucose as sole carbon source for 3 d at 30 °C. Colonies were cultured in galactose-containing medium lacking uracil and tryptophan for 18 h at 30 °C to induce gene expression. Cell densities were normalized to an OD600 of 0.2 in water and lysed with 0.1N NaOH for 5 min at room temperature. Aliquots of each lysate were separated via SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and probed for protein phosphotyrosine content by immunoblotting with anti-phosphotyrosine antibodies. Replicate blots were probed for expression of each SFK, Nef and actin as a loading control.

3.4 RESULTS AND DISCUSSION

3.4.1 Alignment of Primary Nef Proteins: SFK SH3-binding sequences are highly conserved across primary HIV-1 Nef isolates

One of the major objectives of this study was to address whether SH3 domain-mediated activation of SFKs is conserved across primary Nef alleles derived from all M-group clades of HIV-1. To address this question, we assembled a set of Nef cDNA clones representative of all major non-recombinant HIV-1 subtypes. We first queried the NIH HIV-1 sequence database (3) to obtain sequences of Nef alleles from patient isolates of HIV-1 clades A1, A2, B, C, D, F1, F2, G, H, J and K. These clades are representative of the major subgroup of HIV-1 strains responsible for more than 90% of HIV-1 infections associated with the global HIV/AIDS pandemic (111,194,205). Each of these sequences was translated *in silico* and those that encoded truncated Nef proteins were eliminated. Of the remaining sequences, one from each clade was aligned together with the laboratory alleles Nef-SF2 (clade B) and Nef-ELI (clade D). These two Nef variants have been studied extensively by our group in terms of Nef-mediated SFK activation, and serve as useful controls (45,156,256). The alignment revealed strong conservation of known residues and motifs essential for SFK SH3 domain binding and kinase activation, including the PxxPxR motif and the hydrophobic pocket residues F90, W113, and Y/F120 (Figure 3.2).

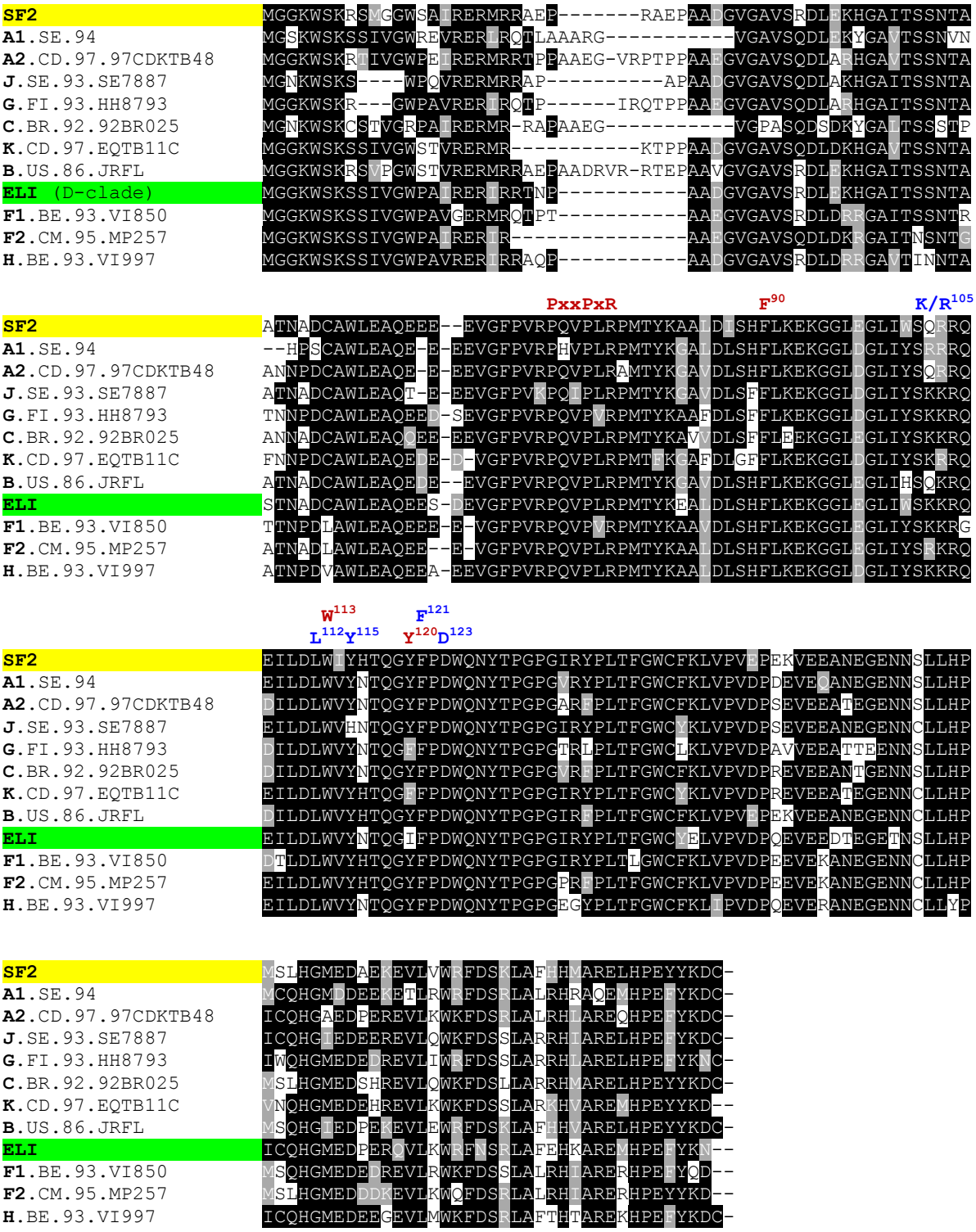

PxxPxR
F90
K/R105
W113
L112Y115
F121
Y120D123

Figure 8. Sequence alignment of primary and laboratory Nef alleles used in this study.

Nef cDNA clones representative of all major non-recombinant HIV-1 clades were selected from the NIH HIV-1 sequence database. The sequences with truncated Nef proteins were eliminated and representative sequences, one from each clade, were chosen and aligned. The first letter in each clone ID indicates the subgroup assignment. This Clustal W alignment also includes Nef-SF2 (top), which has been studied extensively in terms of Hck activation, as well as Nef-ELI, which fails to activate Hck or other SFKs. Key residues in the SF2 sequence that are essential for SH3 binding are shown in red, and include the PxxPxR motif and hydrophobic pocket residues F90, W113, and Y120. Key residues involved in Nef dimerization interface include K/R105, L112, Y115, F121, and D123 and are shown in blue.

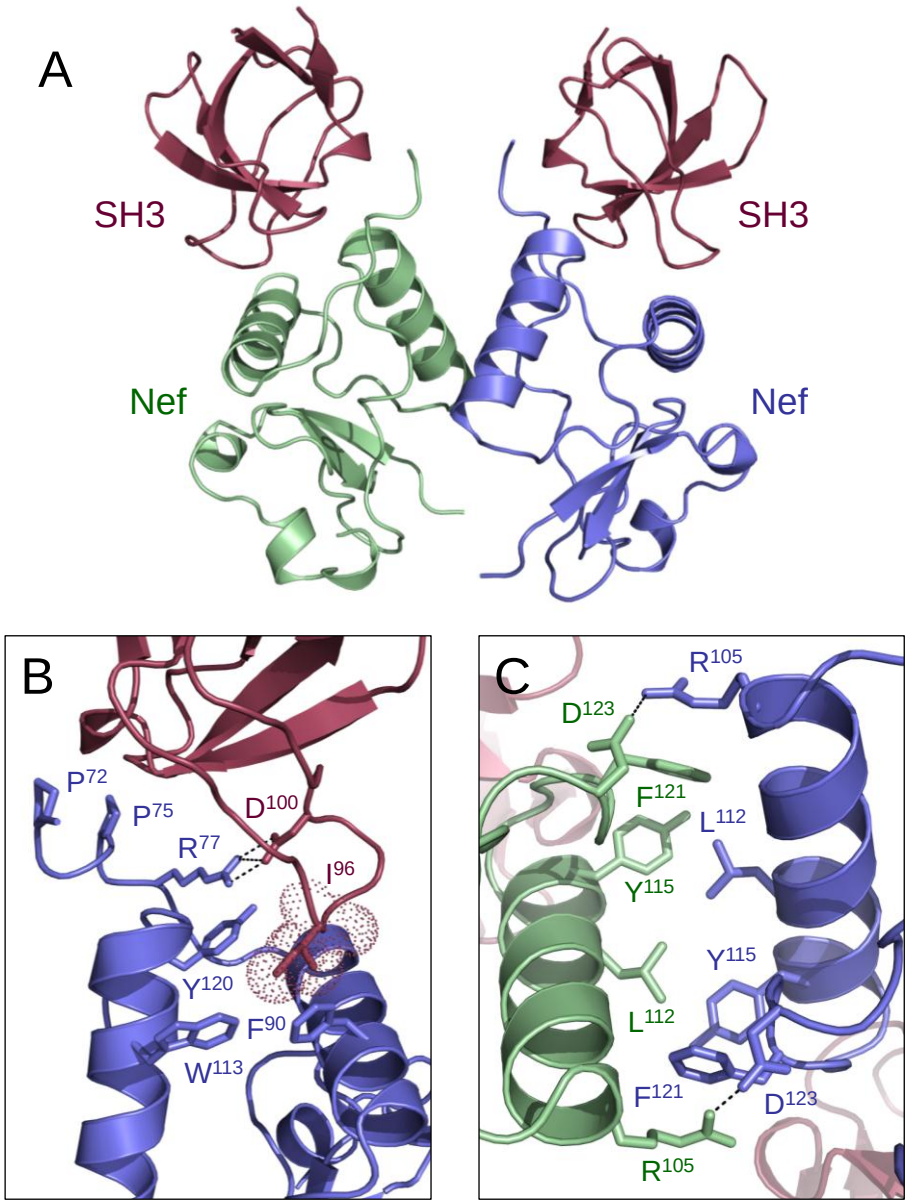


Figure 9. Structurral features of the Nef: SH3 and Nef dimerization interfaces.
A) Overview of the Nef:SH3 dimer X-ray crystal structure, which is modeled on the crystal coordinates of Lee *et al*. [PDB: 1EFN; (155)]. The monomeric Nef subunits are modeled in blue and green respectively; the SH3 domains are shown in red. B) Close-up view of the Nef:SH3 interface. Nef residues P72 and P75 define a polyproline type II helix that meshes with the hydrophobic grooves of the SH3 surface, and is oriented and stabilized by an ionic interaction between Nef R77 and SH3 D100. High affinity interaction also requires a hydrophobic pocket formed in part by Nef residues F90, W113, and Y120. This pocket engages SH3 domain RT loop I96 (Van der Waals surface shown as dots). Mutagenesis of either Nef Y120 or SH3 I96 is sufficient to disrupt Nef:SH3 interaction (45,154). C) Close-up view of the Nef dimerization interface. Dimer packing involves hydrophobic interactions of side chains of the αB helices (L112, Y115, F121) which are orthogonally opposed. This hydrophobic core is capped on both ends by ionic interactions involving D123 and R105.

A model of the contributions of each of these residues to the Nef:SH3 interface, based on the crystal structure of Lee et al., is shown in Figure 9 (155). Note that while substitution of Y120 with isoleucine in Nef-ELI (clade D) prevents it from binding and activating Hck *in vitro* and *in vivo* (45), alignments of a larger group of primary Nef D-clade alleles revealed that this substitution is quite rare (data not shown). In addition to the SH3 domain binding site, there is also strong conservation of residues that form the Nef dimerization interface (K/R105, L112, Y115, F121, and D123). Dimerization is critical to many Nef functions, including support of HIV-1 replication and SFK activation (209,268,287). Figure 9 shows a model of Nef:SH3 dimers, as well as close-up views of the Nef:SH3 and Nef:Nef interfaces.

3.4.2 Recombinant Nef proteins representative of the HIV-1 major clades bind to the Hck SH3 domain

To evaluate the functional properties of our M-group HIV-1 Nef clade set, we first expressed the recombinant Nef proteins in *E. coli* and purified them to homogeneity. As shown in Figure 10 A, most of these primary Nef sequences yielded soluble recombinant proteins, with the exceptions of Nef-C and Nef-H. All of the recombinant Nef proteins eluted as single symmetrical peaks by gel filtration, and their molecular weights were confirmed by mass spectrometry (data not shown).

We first evaluated the binding of purified Nef proteins to the Hck SH3 domain. Because SH3 interaction requires the proper three-dimensional fold of the Nef core (96,155), these studies provided an assessment of whether our recombinant purified Nef proteins folded correctly in bacteria. The wild-type Hck SH3 domain, as well as a mutant known to be defective in SH3 binding [W93A; (232)], were expressed in bacteria as GST fusion proteins and purified using

glutathione-agarose beads. Wild-type GST-SH3, the GST-SH3-W93A mutant control, as well as GST alone were incubated with purified recombinant Nef proteins, followed by extensive washing. Bound Nef was then visualized by immunoblot analysis. As shown in Figure 10 B, all primary Nef proteins showed robust binding to the wild-type Hck SH3 domain, while no binding was observed with the W93A mutant or with GST alone. As an additional control, we also prepared recombinant Nef-ELI, the rare D-clade allele that lacks the hydrophobic pocket residue Y120 and thus fails to activate Hck (256). As expected, Nef-ELI failed to bind to the Hck SH3 domain in this assay. Taken together, these data show that Hck SH3 domain binding is a conserved property of Nef proteins derived from M-group HIV-1 isolates.

While the results shown in Figure 10B establish the potential for Nef: SH3 interaction *in vitro*, they do not provide information about the binding affinity of this crucial interaction for the SH3 domain or in the context of the full-length kinase. To pursue this question in more detail, we turned to surface plasmon resonance analysis. For these studies, either recombinant downregulated near full-length Hck (Hck-YEEI) or the isolated Hck SH3 domain was immobilized on the biosensor chip. Recombinant Nef proteins were flowed over the chip using a range of concentrations, and the resulting sensorgrams were analyzed to determine the dissociation constants for each Nef isoform. As shown in Table 2, the interactions of Hck-YEEI with the laboratory Nef proteins Nef-SF2 and Nef-Consensus were the strongest, with K_D values of 0.80 μM and 1.77 μM, respectively. The primary Nef proteins also yielded K_D values with Hck-YEEI in the low micromolar range, with the exception of Nef-J (K_D = 108.60 μM). The K_D values for Nef interaction with the isolated Hck SH3 domain were somewhat more variable, with Nef-SF2 showing the highest affinity (K_D = 0.07 μM) and primary Nef-A2 the lowest (K_D = 21.10 μM). Interestingly, for six of the eight Nef proteins tested, the ratios of the K_D values for

near full-length Hck versus the isolated SH3 domain were greater than one, suggesting that in most cases Nef must compete with the regulatory intramolecular SH3-linker interaction within Hck in order to bind. However, in the case of Nef-A2 and Nef-F1, this ratio was less than one, raising the interesting possibility that additional Nef: Hck contacts may contribute to destabilization of the Hck SH3: linker interaction required for activation in some cases.

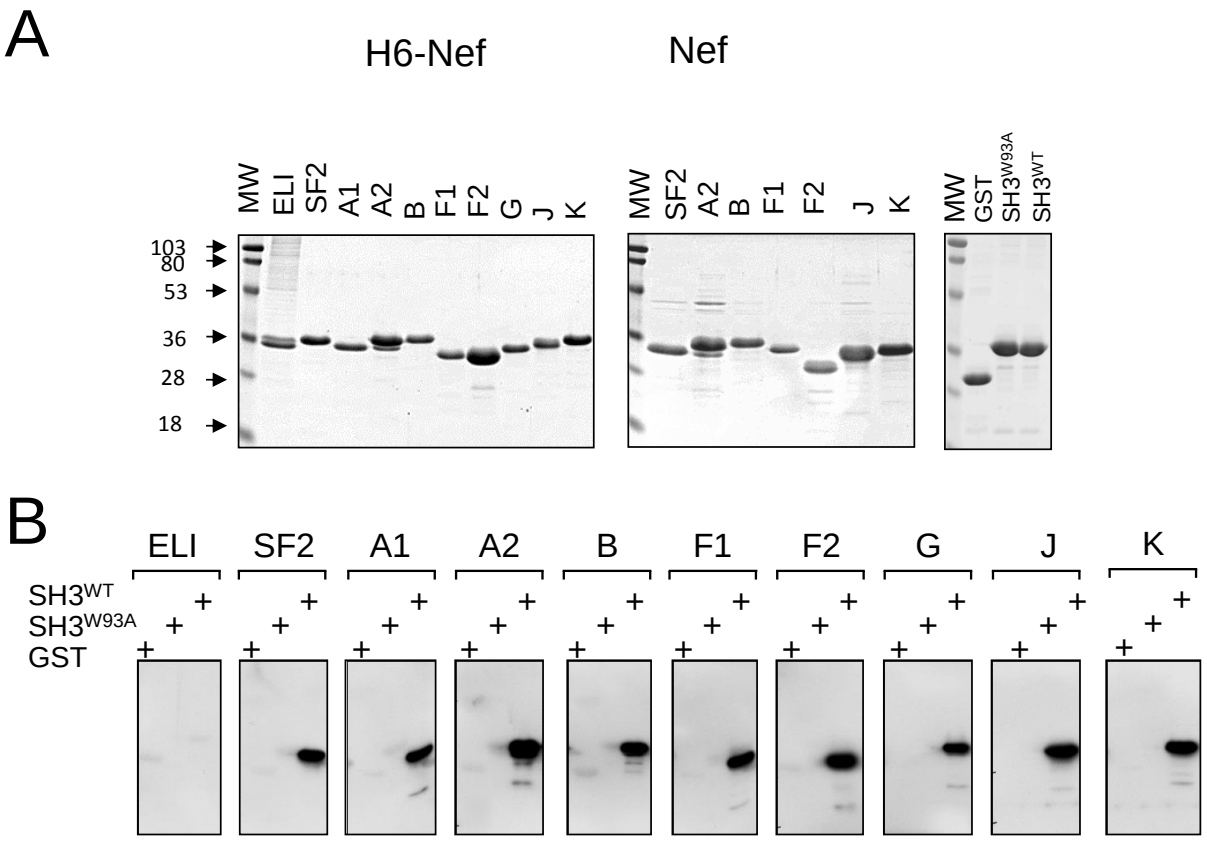


Figure 10. Purified primary Nef proteins bind to the Hck SH3 domain.

A) SDS-PAGE of recombinant purified Nef proteins. Each of the indicated Nef subtypes was expressed in bacteria and purified either with N-terminal His-tags (left) or left untagged (center) as described in the text. The untagged Nef proteins were used for SPR experiments (see Table 2). GST and GST fusion proteins with the Hck SH3 domain (wild-type and W93A mutant) were also expressed in bacteria and immobilized on glutathione-agarose beads (right). Aliquots of each protein were resolved on 15% SDS-PAGE and stained with Coomassie blue. B) Nef-SH3 binding assay. Recombinant His-tagged Nef proteins (1 µg) were incubated with equimolar amounts of immobilized GST and GST-SH3 fusion proteins. The agarose beads were then washed, and associated Nef proteins were detected by immunoblotting using antibodies to the His-tags.

Table 2. Evaluation of Nef binding to Hck-YEEI by Surface Plasmon Resonance (SPR).

Nef protein	Nef type	Hck-YEEI, K_D (μM)	Hck SH3, K_D (μM)	YEEI/SH3 K_D ratio
SF2	Laboratory (B-clade)	0.80 ± 0.15	0.07 ± 0.01	11.43
Consensus	Laboratory (B-clade*)	1.77 ± 0.04	0.12 ± 0.01	14.75
A2	Primary	12.80 ± 1.64	21.10 ± 1.41	0.61
B	Primary	6.05 ± 0.78	3.82 ± 0.10	1.58
F1	Primary	2.77 ± 0.70	3.41 ± 0.16	0.81
F2	Primary	4.71 ± 0.44	1.31 ± 0.42	3.60
J	Primary	108.60 ± 8.80	13.41 ± 3.10	8.10
K	Primary	11.90 ± 2.68	3.93 ± 0.09	3.03

SPR analyses were performed with recombinant purified Hck-YEEI or the isolated Hck SH3 domain covalently attached to the biosensor chip, as described under Materials and Methods. The analyte in the mobile phase was the untagged, purified Nef protein while the ligand immobilized on the surface was the Hck protein (Hck-YEEI or SH3 alone). Each Nef protein was serially injected and interaction monitored over a range of Nef concentrations as shown in Table 1. The kinetics and affinities of binding were calculated by fitting the data to a 1:1 Langmuir binding model or, for interactions exhibiting fast rates of association, the measurements were repeated under steady-state conditions. The dissociation constants shown were generated using the BIAevaluation 4.1 software suite. These data were produced in collaboration with John Jeff Alvarado, Haibin Shi, and Joanne Yeh, Department of Structural Biology, University of Pittsburgh School of Medicine.

*Consensus Nef is based on a sequence alignment of multiple B-clade Nef proteins (238).

3.4.3 Recombinant Nef proteins representative of the HIV-1 major clades activate the Hck *in vitro*

Previous studies have established that Nef activates Hck (and other Src-family members) through displacement of the SH3 domain from its regulatory interaction with the SH2-kinase linker (180,256). Thus, Nef must have sufficient binding affinity to compete for the internal SH3-linker interaction present in the downregulated form of Src-family kinases (64). To determine whether the SH3-binding activity of our primary Nef proteins was sufficient to induce Hck activation, we turned to the FRET-based Z'-Lyte kinase assay which we have used previously to demonstrate Nef-mediated Hck activation *in vitro* (see section 3.2). Purified, downregulated Hck (Hck-YEEI) was incubated in the absence or presence of a 10-fold molar excess of each Nef protein prior to assay. As shown in Figure 11, all of the primary Nef proteins activated Hck in the Z'-Lyte assay, demonstrating for the first time that Hck activation is a highly conserved property of M-group Nef alleles despite some variation in the affinity of the individual Nef proteins for the Hck SH3 domain.

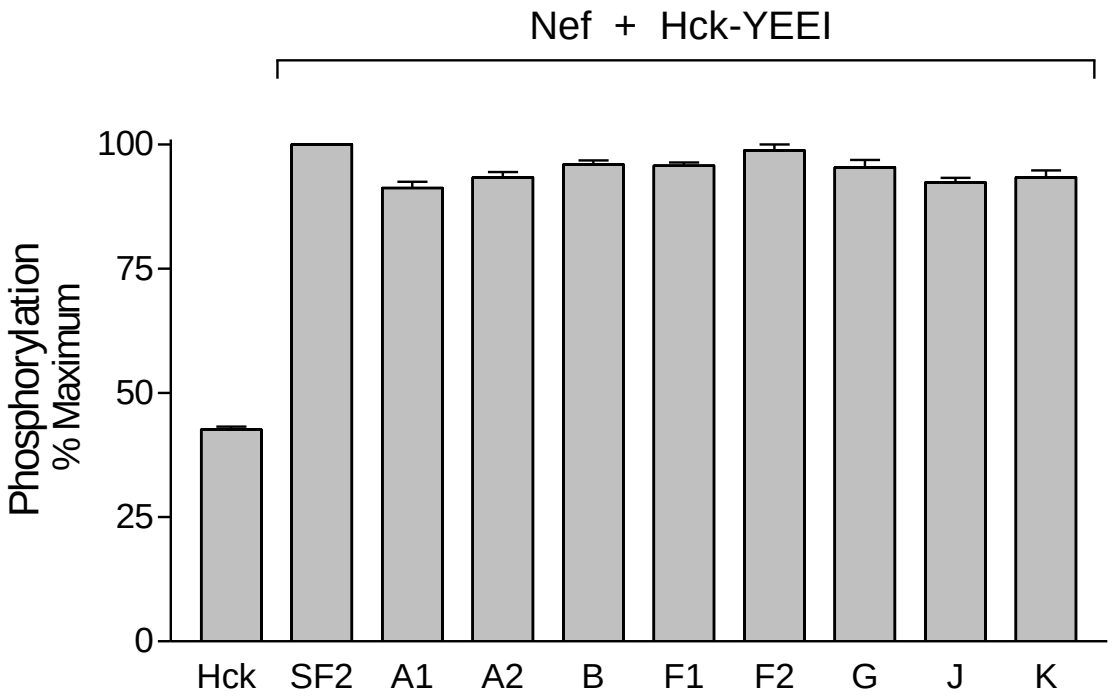


Figure 11. Purified primary Nef proteins activate downregulated Hck *in vitro*.

Recombinant downregulated Hck [Hck-YEEI; (63,256,257)] kinase activity was determined in the absence or presence of the indicated Nef proteins using the Z-Lyte assay as described in the text. Results are expressed as the mean percent of maximum substrate phosphorylation ± S.D.; this experiment was repeated twice with comparable results.

3.4.4 HIV-1 Nef proteins from all major clades strongly activate Hck, Lyn and c-Src in a cell-based assay

Studies with recombinant Nef proteins derived from primary HIV-1 sequences all exhibited SH3-binding and Hck activation (Table 2 and Figure 10B and 11). However, we were not able to assess the activity of Nef-C and Nef-H, because soluble recombinant proteins could not be obtained from these sequences. To assess the interaction of these Nef proteins with Hck, and to expand our study to other members of the Src kinase family in a cell-based assay, we turned to a yeast system previously developed by our group (256,257). In this assay, Hck and other Src-family kinases are expressed with modified "YEEI" tails so that they adopt the downregulated conformation in the absence of the negative regulatory kinase, Csk. This point is important because active Src-family kinases cause growth arrest in yeast (256).

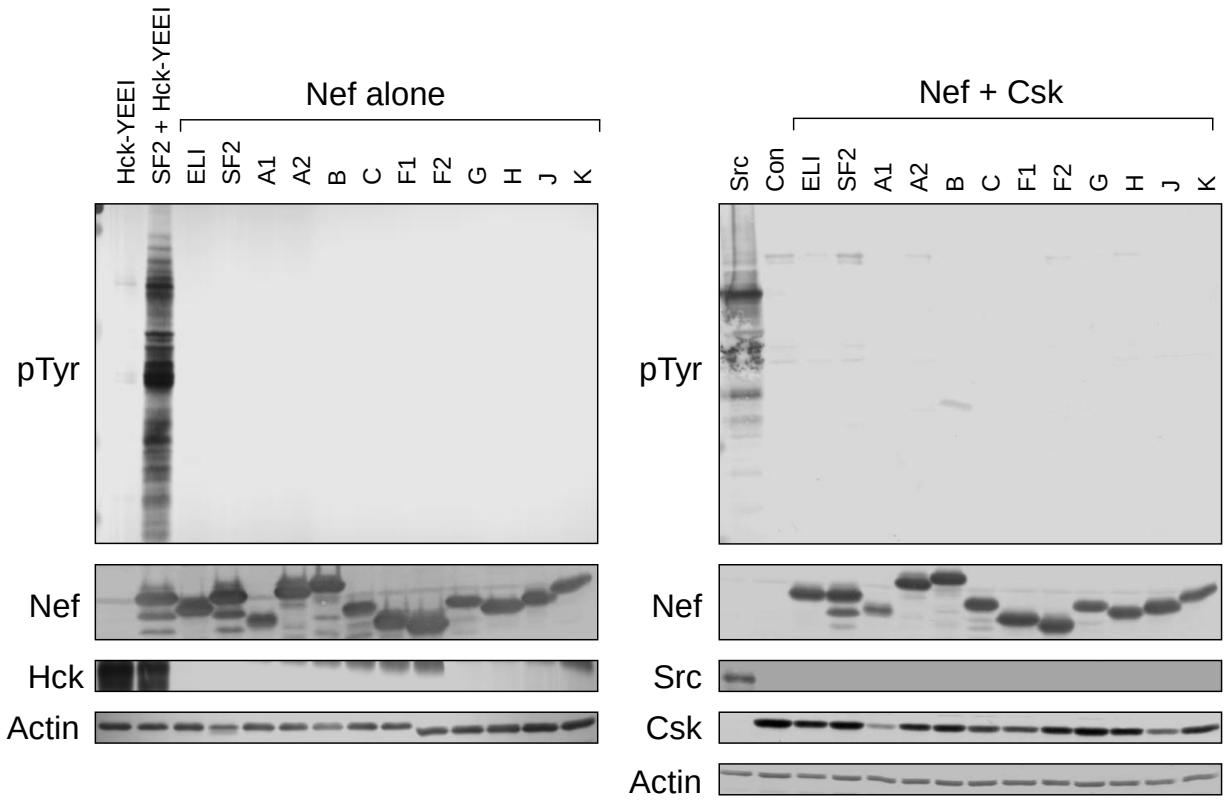


Figure 12. Expression of Nef alone or Nef with Csk has no effect on phosphorylation of yeast proteins.

Yeast (*S. cerevisiae*) strain YPH499 was co-transformed with Nef expression plasmids either alone (A) or in the presence of Csk (B). Colonies were selected on synthetic drop-out (SD) medium in the absence of uracil and tryptophan (-Ura/-Trp) with dextrose as sole carbon source at 30^0 C. Colonies were picked and expanded in liquid SD -Ura/-Trp medium plus dextrose to suppress Nef and SFK expression. Protein expression was induced by transferring the cultures to galactose-containing SD -Ura/-Trp medium for 16 h at 30°C. Yeast lysates were analyzed for phosphorylation of yeast proteins by anti-phosphotyrosine immunoblotting (pTyr). Co-expression of Nef-SF2 with Hck-YEEI (A) and active c-Src (B) served as positive controls for phosphorylation of yeast proteins. Note that none of Nef alleles alone or in presence of Csk have any effect on phosphorylation of yeast proteins.

Before evaluating the effect of the primary Nef protein expression on SFK activity in yeast, we first performed control experiments to determine whether Nef alone (or Nef with the SFK regulator, Csk) had any effect on tyrosine phosphorylation of yeast proteins. Yeast cultures expressing each primary Nef protein as well as Nef-SF2 and Nef-ELI were grown in defined medium with galactose as the sole carbon source to induce gene expression. Yeast protein-tyrosine phosphorylation was evaluated by immunoblotting of yeast cell lysates. As shown in Figure 12, none of the Nef proteins induced tyrosine phosphorylation in the absence of a co-expressed SFK. Co-expression of Nef-SF2 with Hck is included as a positive control. Co-expression of Nef with Csk, the negative regulator of SFKs, also did not induce yeast protein-tyrosine phosphorylation (Figure 12). In contrast, expression of c-Src (in the absence of Csk) led to strong tyrosine phosphorylation of yeast proteins. These results indicate that expression of Nef alone or Nef with Csk does not induce tyrosine phosphorylation signals, consistent with the lack of endogenous SFK orthologs in yeast.

To assess primary Nef-induced SFK activation in this system, yeast cultures were co-transformed with expression plasmids for each primary Nef sequence and Src family member, and colonies were selected on glucose agar to repress protein expression from the Gal promoter. Transformed colonies were then grown in liquid medium with galactose as sole carbon source to induce Nef and SFK expression. The cultures were lysed, and SFK activation was evaluated by immunoblotting with anti-phosphotyrosine antibodies. Nef-SF2 and Nef-ELI served as positive and negative controls, respectively. As shown in Figure 13, Nef-SF2 strongly activated Hck while Nef- ELI failed to do so, consistent with our previous data (256). All of the primary *nef* alleles tested also induced dramatic Hck activation, as reflected by the strong phosphorylation of yeast cell proteins in comparison to downregulated Hck-YEEI alone. Similar results were

obtained with Lyn, although tyrosine phosphorylation of yeast cell proteins was less extensive for this Src family member and may reflect more stringent substrate protein selection relative to Hck. Interestingly, Lyn also appeared to strongly phosphorylate selective Nef isoforms, including A2, B, C, F2, G, H, and K, as well as SF2 (Figure 14). This observation raises the possibility that tyrosine phosphorylation of Nef may influence its function or interaction with other binding partners.

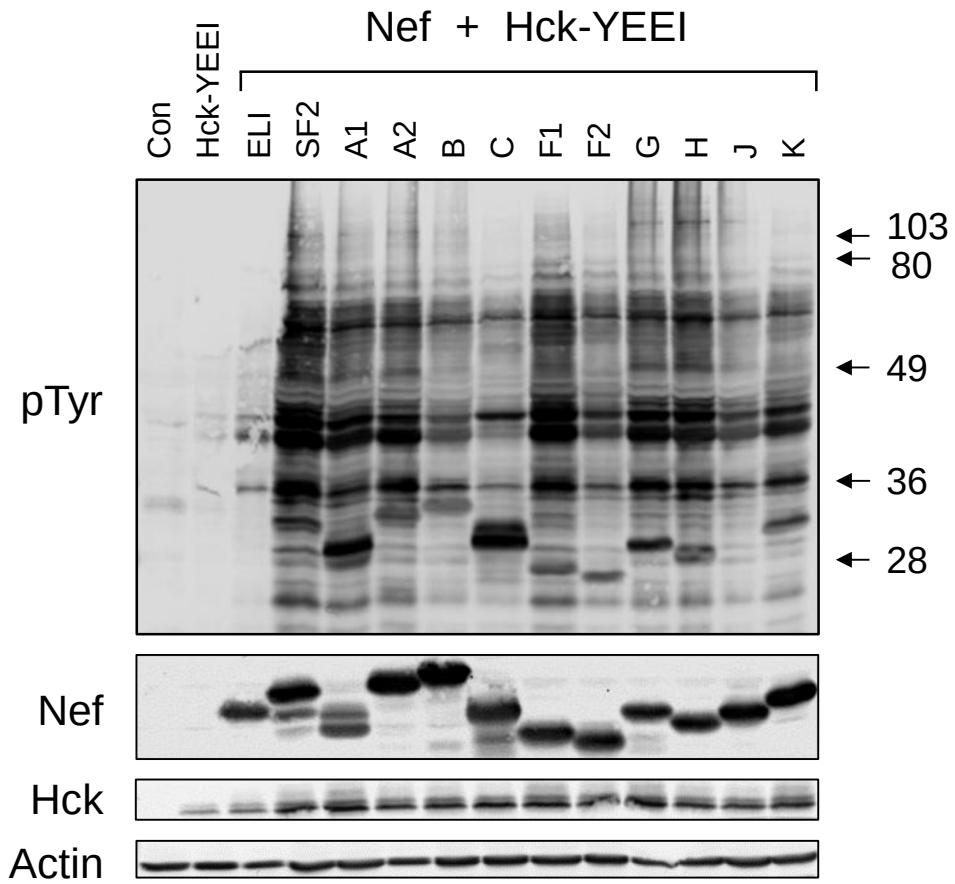


Figure 13. Primary M-group HIV-1 Nef proteins strongly activate Hck in cells.

Yeast cultures were transformed with a galactose-inducible expression plasmid for Hck-YEEI, which carries a modified C-terminal tail that enables downregulation in the absence of Csk (257), or the empty expression plasmid as a negative control (Con). Where indicated, Hck-YEEI cells were co-transformed with galactose-inducible vectors for Nef-ELI, Nef-SF2, and 10 primary *nef* alleles (A1, A2, B, C, F1, F2, G, H, J, and K). Transformed cells were grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Hck and actin as a loading control. Nef-ELI is unable to interact with Hck and serves as an additional negative control (257). This experiment was repeated three times with comparable results.

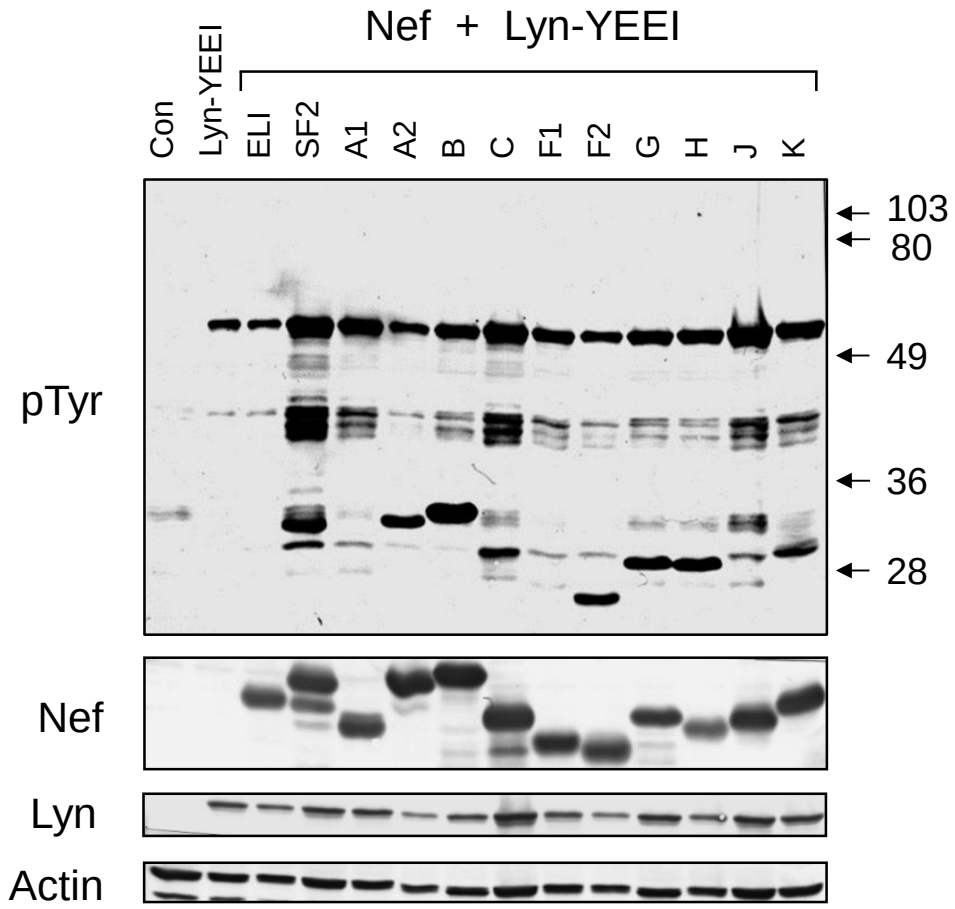


Figure 14. Primary M-group HIV-1 Nef proteins strongly activate Lyn in cells.

Yeast cultures were transformed with a galactose-inducible expression plasmid for Lyn-YEEI, which carries a modified C-terminal tail that enables downregulation in the absence of Csk (257), or the empty expression plasmid as a negative control (Con). Where indicated, Lyn-YEEI cells were co-transformed with galactose-inducible vectors for Nef-ELI, Nef-SF2, and 10 primary Nef alleles (A1, A2, B, C, F1, F2, G, H, J, and K). Transformed cells were grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Lyn and actin as a loading control. Nef-ELI is unable to interact with Lyn and serves as an additional negative control (257). This experiment was repeated three times with comparable results.

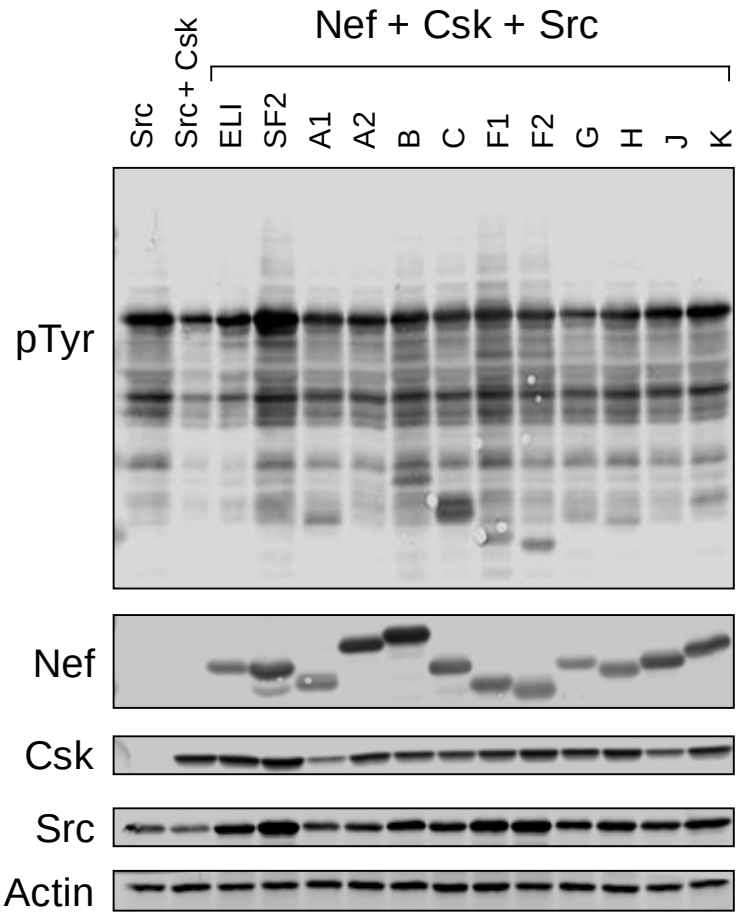


Figure 15. Primary M-group HIV-1 Nef proteins induce modest activation of c-Src in cells.

Yeast cultures were transformed with galactose-inducible expression plasmids for c-Src, Csk, and the indicated Nef alleles (ELI, SF2, A1, A2, B, C, F1, F2, G, H, J, and K). Transformed cells were grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Csk, c-Src and actin as a loading control.

In addition to Hck and Lyn, we also evaluated the effect of the primary Nef alleles on c-Src. In case of c-Src, unlike the other SFKs used in the yeast studies, modification of c-Src tail with a YEEI tail did not result in effective downregulation in the absence of Csk. Therefore we decided to downregulate c-Src by co-expression of Csk as described previously (256). Yeast cultures were co-transformed with c-Src, Csk, and Nef expression plasmids, and protein expression was induced in galactose medium as described under Materials and Methods. Protein-tyrosine phosphorylation was then evaluated by anti-phosphotyrosine immunoblotting. Expression of c-Src alone induced strong phosphorylation of yeast proteins that was downregulated in the presence of Csk. As shown in Figure 15, co-expression of Nef-SF2 overcame Csk-mediated c-Src inhibition, while Nef-ELI failed to activate c-Src, consistent with our previous report (256). Similarly, all of the primary Nef proteins also activated Csk-downregulated c-Src kinase, albeit to a lesser extent compared to Nef-SF2.

To look for additional evidence of Nef-mediated c-Src activation, we also monitored yeast cell growth. Previous work has shown that ectopic expression of active SFKs induces growth arrest in yeast, providing another measure of Nef-SFK interaction in this system (157,256,257). For these experiments, aliquots of each transformed yeast culture shown were spotted over a series of dilutions on galactose-agar plates, and the yeast patches appear as dark circles in the resulting scanned images of the plates. Expression of c-Src alone induced growth suppression compared to control cultures, while expression of c-Src with Csk reversed this effect. As shown in Figure 16, and consistent with our previous work, expression of Nef-SF2 overcame the inhibition of c-Src by Csk, leading to growth suppression, while the non-interacting allele Nef-ELI failed to do so (256). Co-expression of primary Nef proteins with c-Src also induced growth suppression in the presence of Csk, with the exceptions of Nef-F2 and

Nef-H. These data provide additional evidence that primary Nef proteins from almost all major HIV-1 clades activate c-Src in this system. To control for culture plating density, replicate dilutions of each culture were also spotted on glucose-agar plates. Because glucose represses protein expression from the GAL promoter, c-Src, Csk, and Nef are not expressed and all cultures grow to the same extent.

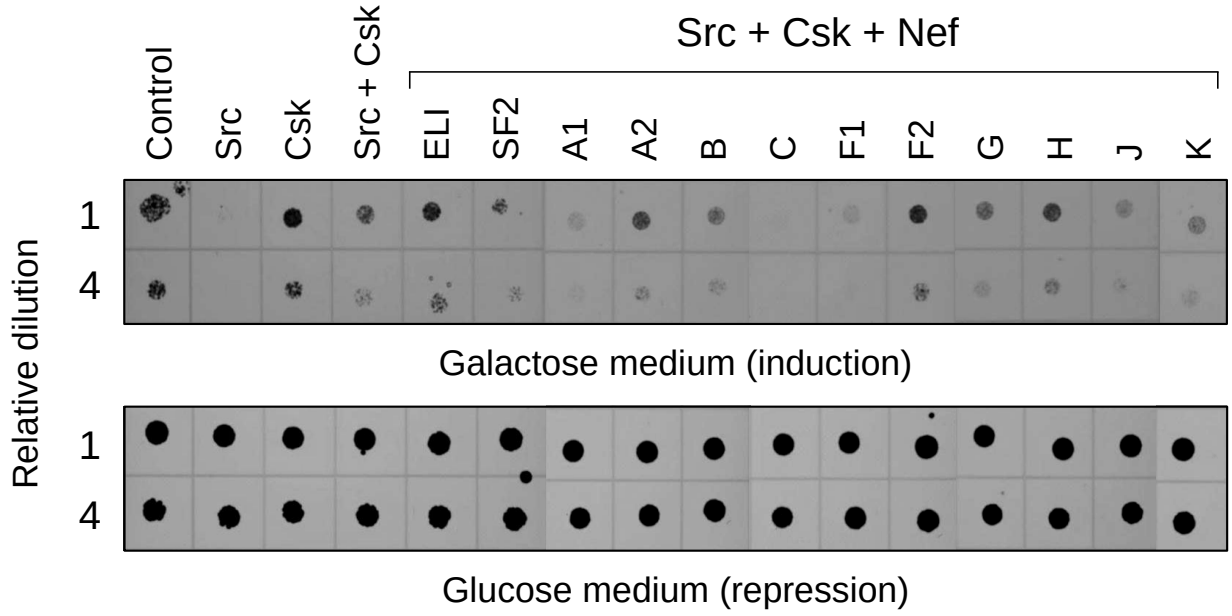


Figure 16. Primary M-group HIV-1 Nef proteins induce modest activation of c-Src in cells: Growth reversion assay.

Yeast cultures as in Figure 15 were grown in SD (-Ura/-Trp) medium containing dextrose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar plates containing galactose as the sole carbon source and incubated for 3 days at 30 °C to induce gene expression. Cultures were spotted in 4-fold dilutions to enhance visualization of the growth-suppressive phenotype. Plates were scanned, and yeast patches appear as dark circles. Note that expression of active c-Src causes the growth inhibition. This experiment was repeated three times with comparable results.

3.4.5 HIV-1 Nef proteins from all major clades failed to activate Lck, Fyn and Fgr in a cell-based assay

We next examined whether primary Nef proteins can activate other SFKs expressed in HIV-1 target cells using the yeast assay. Primary Nef proteins were co-expressed with the down-regulated (YEEI) forms of Lck, Fyn and Fgr, followed by anti-phosphotyrosine immunoblotting of yeast cell lysates. As shown in Figure 17, co-expression with Nef had no effect on Lck, Fyn or Fgr despite strong expression of the Nef proteins and each SFK. As a positive control, we co-expressed Lck-YEEI with the herpesvirus Tip protein, which led to strong Lck activation as observed previously (179). Fyn and Fgr were also expressed as their wild-type forms; in the absence of Csk, these SFKs induced strong yeast tyrosine phosphorylation as observed previously (256).

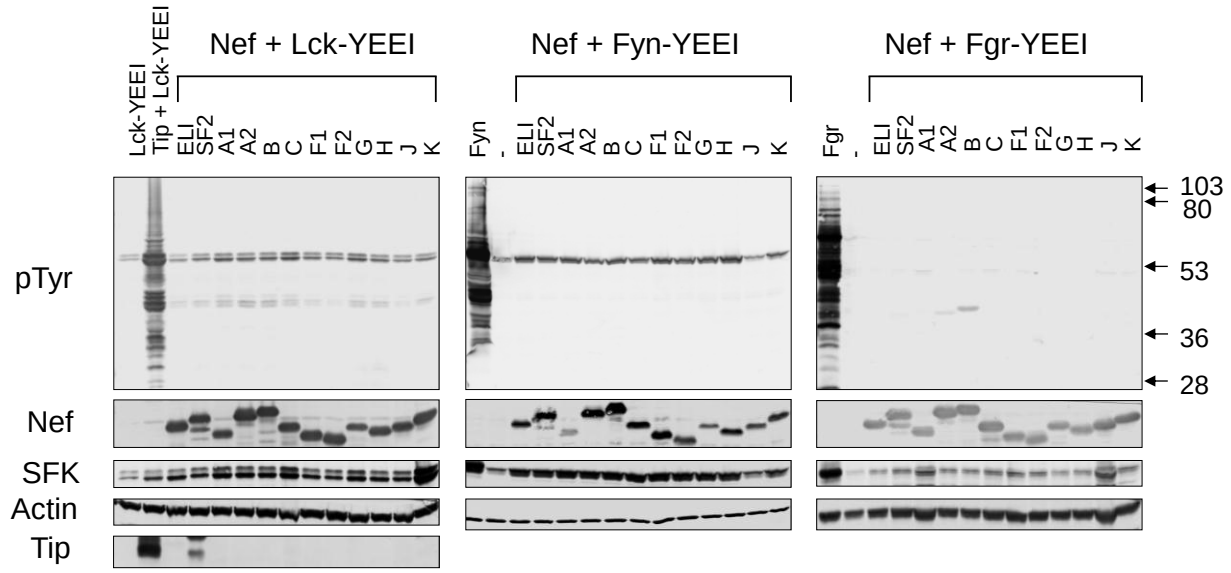
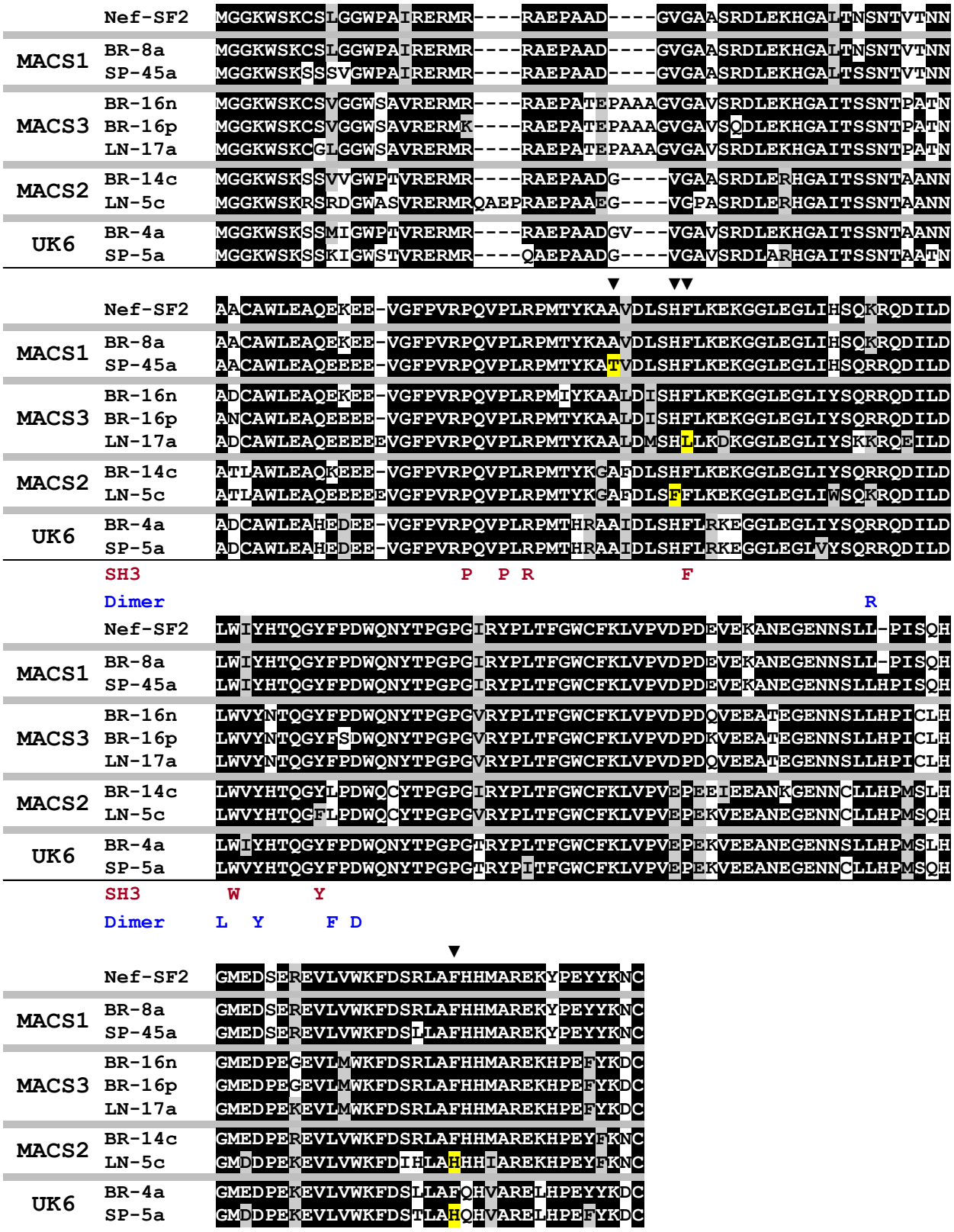


Figure 17. Primary M-group HIV-1 Nef proteins do not activate Lck, Fyn or Fgr in cells.

Yeast cultures were transformed with a galactose-inducible expression plasmid for Lck-YEEI, Fyn-YEEI, Fgr-YEEI, which carries a modified C-terminal tail that enables downregulation in the absence of Csk (257), or the empty expression plasmid as a negative control (Con). Where indicated, these cells were co-transformed with galactose-inducible vectors for Nef-ELI, Nef-SF2, and 10 primary *nef* alleles (A1, A2, B, C, F1, F2, G, H, J, and K). Transformed cells were grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Tip, Lck, mFgr and actin as a loading control. Wild-type Fyn, mFgr and co-expression of Tip with Lck YEEI served as positive control for respective kinase activation. This experiment was repeated three times with comparable results.

3.4.6 Alignment of patient-derived Nef proteins

We next evaluated the effect of divergent evolution of the *nef* gene in HIV-1 isolates from brain versus lymphoid organs from same patient on SFK (Hck and Fyn T) activation. For these studies, full-length *nef* sequences were amplified from genomic DNA from autopsy brain and lymphoid organs from four AIDS patients suffering with HAD (7,8,193). The predicted Nef amino acid sequences were determined and the alignment is shown in Figure 8, together with the sequence of the laboratory allele Nef-SF2. The alignment revealed strong conservation of known residues and motifs essential for SFK SH3 domain binding and kinase activation, including the PxxPxR motif and the hydrophobic pocket residues F90, W113, and Y/F120 (Figure 8). In addition to the SH3 domain binding site, there is also strong conservation of residues that form the Nef dimerization interface (K/R105, L112, Y115, F121, and D123). Dimerization is critical to many Nef functions, including support of HIV-1 replication and SFK activation (209,268,287).


Nef-SF2 MGGKWSKCSLGGWPAIRERMR----RAEPAAD----GVGAASRDLEKHGALTNSNTVTNN
MACS1 BR-8a MGGKWSKCSLGGWPAIRERMR----RAEPAAD----GVGAASRDLEKHGALTNSNTVTNN
SP-45a MGGKWSKSSSVGWPAIRERMR----RAEPAAD----GVGAASRDLEKHGALTSSNTVTNN
MACS3 BR-16n MGGKWSKCSVGGWSAVRERMR----RAEPATEPAAAGVGAVSRDLEKHGAITSSNTPATN
BR-16p MGGKWSKCSVGGWSAVRERMK----RAEPATEPAAAGVGAVSQDLEKHGAITSSNTPATN
LN-17a MGGKWSKCGLGGWSAVRERMR----RAEPATEPAAAGVGAVSRDLEKHGAITSSNTPATN
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LN-5c MGGKWSKRSRDGWASVRERMRQAEPRAEPAAEG----VGPASRDLERHGAITSSNTAANN
UK6 BR-4a MGGKWSKSSMIGWPTVRERMR----RAEPAADGV---VGAVSRDLEKHGAITSSNTAANN
SP-5a MGGKWSKSSKIGWSTVRERMR----QAEPAADG----VGAVSRDLARHGAITSSNTAATN
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SP-45a AACAWLEAQEEEE-VGFPVRPQVPLRPMTYKATVDLSHFLKEKGGLEGLIHSQRRQDILD
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Dimer R
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MACS1 BR-8a LWIYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVDPDEVEKANEGENNSLL-PISQH
SP-45a LWIYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVDPDEVEKANEGENNSLLHPISQH
MACS3 BR-16n LWVYNTQGYFPDWQNYTPGPGVRYPLTFGWCFKLVPVDPDQVEEATEGENNSLLHPICLH
BR-16p LWVYNTQGYFSDWQNYTPGPGVRYPLTFGWCFKLVPVDPDKVEEATEGENNSLLHPICLH
LN-17a LWVYNTQGYFPDWQNYTPGPGVRYPLTFGWCFKLVPVDPDQVEEATEGENNSLLHPICLH
MACS2 BR-14c LWVYHTQGYLPDWQCYTPGPGIRYPLTFGWCFKLVPVEPEEIEEANKGENNCLLHPMSLH
LN-5c LWVYHTQGFLPDWQCYTPGPGVRYPLTFGWCFKLVPVEPEKVEEANEGENNCLLHPMSQH
UK6 BR-4a LWIYHTQGYFPDWQNYTPGPGTRYPLTFGWCFKLVPVEPEKVEEANEGENNSLLHPMSLH
SP-5a LWVYHTQGYFPDWQNYTPGPGTRYPITFGWCFKLVPVEPEKVEEANEGENNCLLHPMSQH
SH3 W Y
Dimer L Y F D
Nef-SF2 GMEDSERFVLVWKFDSRLAFHHMARFKYPEYYKNC
MACS1 BR-8a GMEDSERFVLVWKFDSRLAFHHMARFKYPEYYKNC
SP-45a GMEDSERFVLVWKFDSLLAFHHMARFKYPEYYKNC
MACS3 BR-16n GMEDPEGFVLMWKFDSRLAFHHMARFKHPEFYKDC
BR-16p GMEDPEGFVLMWKFDSRLAFHHMARFKHPEFYKDC
LN-17a GMEDPEKFVLMWKFDSRLAFHHMARFKHPEFYKDC
MACS2 BR-14c GMEDPERFVLVWKFDSRLAFHHMARFKHPEYFKNC
LN-5c GMDDPEKFVLVWKFDIHLAHHHIARFKHPEYFKNC
UK6 BR-4a GMEDPEKFVLVWKFDSLLAFQHVARFLHPEYYKDC
SP-5a GMDDPEKFVLVWKFDSTLAHQHVARFLHPEFYKDC

Figure 18. Sequence alignment of patient-derived Nef alleles.

The Nef amino acid sequences shown were derived from four AIDS patients, designated MACS1, MACS2, MACS3, and UK6. The corresponding cDNAs were isolated directly from patient autopsy brains (BR), lymph nodes (LN), and spleens (SP), with the exception of MACS2 LN-5C, which was cloned from a lymph node primary viral isolate. Cloning and tissue origins of these Nef alleles and their functional characteristics are described in detail elsewhere (7,8,193). The sequence of the laboratory allele Nef-SF2 is included at the top for reference. Residues in the Nef-SF2 sequence essential for SH3 binding are shown in red, and include the PxxPxR motif and hydrophobic pocket residues F/L90, W113, and Y120. Conserved residues that form the Nef dimerization interface include K/R105, L112, Y115, F121, and D123 and are shown in blue. Positions of amino acid differences between peripheral (LN, SP) and brain-derived sequences derived from the same patient that may account for enhanced activation of Hck are indicated by the arrowheads, with the residues in question highlighted in yellow.

3.4.7 Patient-derived Nef alleles differentially activate Hck in a cell-based assay

To assess patient-derived Nef-induced Hck activation, we used the yeast growth assay. For these experiments, yeast cultures were co-transformed with expression plasmids for Nef-SF2, Nef-ELI as well as the nine organ-derived Nef sequences from four patients [MACS1 (Br-8a, SP-45a); MACS2 (Br-14c, LN-5c); MACS3 (Br-16n, Br-16p, LN-17a); UK6 (Br-4a, SP-5a)]. These Nef sequences were expressed with Hck-YEEI, and colonies were selected on glucose agar to repress protein expression from the Gal promoter. As described in section 3.4.4, aliquots of each transformed yeast culture were then spotted over a series of dilutions on galactose-agar plates to induce protein expression, and the yeast patches appear as dark circles in the resulting scanned images of the plates. As shown in Figure 19 A, expression of downregulated Hck alone, as well as the patient-derived Nef alleles in the absence of Hck, did not affect yeast growth. Co-expression Nef-SF2 with Hck-YEEI resulted in strong growth suppression while the non-interacting allele Nef-ELI failed to do so (256). Similar to Nef-SF2, co-expression of all nine patient-derived Nef alleles with Hck-YEEI resulted in strong growth suppression (Figure 19 B). Surprisingly, the extent of growth suppression, which is directly related to Hck activation, was different for Nef proteins from the same patients. In three out of four patients (MACS1, MACS3 and UK6), the brain-derived Nef proteins showed enhanced Hck activation compare to lymph node or spleen-derived Nef within same patient.

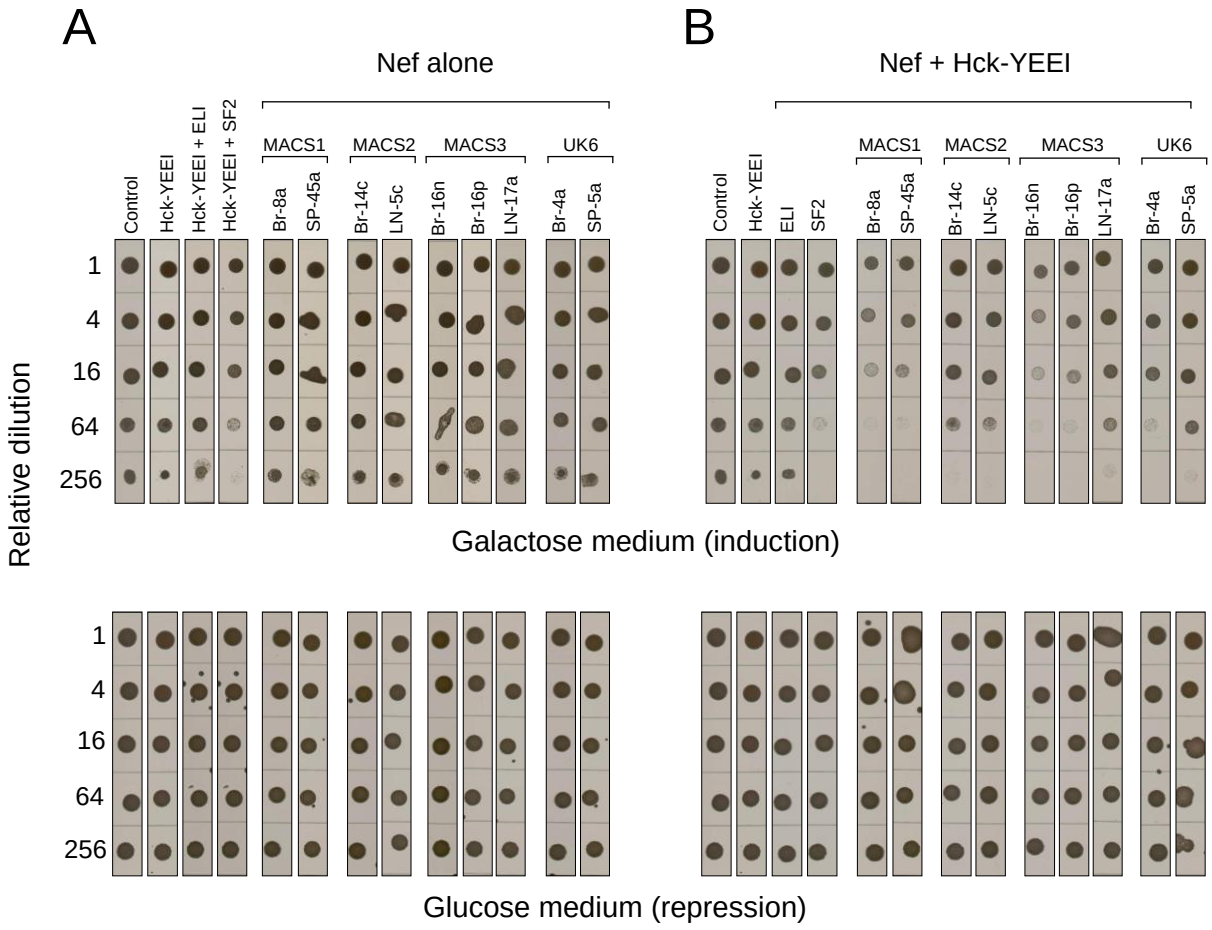

Figure 19. Enhanced activation of Hck by brain-derived versus peripheral HIV-1 Nef proteins from the same patient: Growth reversion assay.

Yeast cultures were transformed either with a galactose-inducible expression plasmid for Nef alone (A) or in the presence of Hck-YEEI (B), which carries a modified C-terminal tail that enables downregulation in the absence of Csk (257), or the empty expression plasmid as a negative control (Con). Where indicated, Hck-YEEI cells were co-transformed with galactose-inducible expression vectors for Nef-ELI, Nef-SF2, and nine patient-derived Nef alleles [MACS1 (Br-8a, SP-45a); MACS2 (Br-14c, LN-5c); MACS3 (Br-16n, Br-16p, LN-17a); UK6 (Br-4a, SP-5a)]. Transformed cells were grown in liquid culture in SD (-Ura/-Trp) medium containing dextrose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar plates containing galactose as the sole carbon source and incubated for 3 days at 30 °C to induce gene expression. Cultures were spotted in 4-fold dilutions to enhance visualization of the growth-suppressive phenotype. Plates were scanned, and yeast patches appear as dark circles. Note that expression of active Hck causes growth inhibition. Nef-ELI is unable to interact with Hck and serves as an additional negative control (257). This experiment was repeated two times with comparable results.

To control for culture plating density, replicate dilutions of each culture were also spotted on glucose-agar plates. Because glucose represses protein expression from the GAL promoter, Hck-YEEI and Nef are not expressed and all cultures grow to the same extent.

All of these Nef proteins have the conserved PxxPxR motif as well as the hydrophobic pocket residues required for SH3 binding and activation. Comparison of the amino acid sequences reveals a few changes that might account for differential Hck activation in brain versus peripheral organs (these residues highlighted in yellow in Figure 18). For example, spleen-derived Nef from patient MACS1 has a polar Thr84 instead of nonpolar Ala84 just after the PxxP motif. This threonine residue has the potential to form H-binds and may also undergo phosphorylation which might hinder interaction with SH3 domain. In the case of patient MACS3, lymph node-derived Nef has Leu90 instead of the critical Phe90 in the hydrophobic pocket, while in patient UK6, spleen-derived Nef has a charged histidine residue at this position instead of phenylalanine. These mutations seem likely to influence the ability of Nef to bind productively to the SH3 domain as required for Hck activation. This hypothesis could be tested in future work using SPR with purified primary Nef proteins as described in Section 3.4.2. In addition, Hck and other SFK activation could be validated using either the yeast-based approach or Z-lyte assay with the purified proteins.

To look for additional evidence of enhanced Hck activation by brain-derived patient-derived *nef* alleles, transformed colonies were grown in liquid medium with galactose as the sole carbon source to induce Nef and Hck expression. The cultures were lysed, and Hck activation was evaluated by immunoblotting with anti-phosphotyrosine antibodies. Nef-SF2 and Nef-ELI served as positive and negative controls, respectively. As shown in Figure 20, Nef-SF2 strongly activated Hck while Nef-ELI failed to do so, consistent with our previous data (256). None of

the Nef proteins induced tyrosine phosphorylation in the absence of a co-expressed Hck. All of the patient derived Nef alleles tested also induced dramatic Hck activation, as reflected by the strong phosphorylation of yeast cell proteins in comparison to downregulated Hck-YEEI alone. Similar to yeast growth assay, brain-derived Nef proteins from MACS1 (Br-8a, SP-45a); MACS3 (Br-16n, Br-16p, LN-17a) and UK6 (Br-4a, SP-5a) have showed stronger activation of Hck than lymph node or spleen-derived Nef.

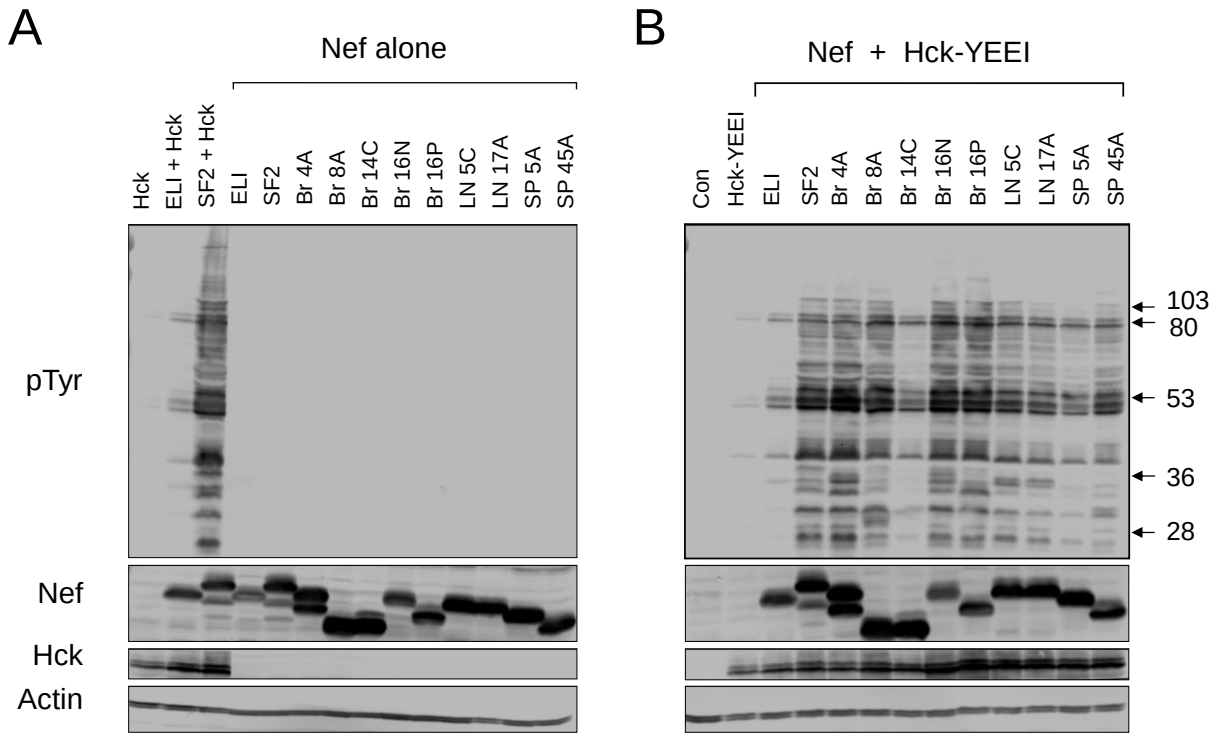

Figure 20. Enhanced activation of Hck by brain-derived versus peripheral HIV-1 Nef proteins from the same patients: immunoblot assay.

Yeast cultures were transformed with the expression plasmids described in Figure 19 and grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Hck and actin as a loading control. Nef-ELI is unable to interact with Hck and serves as an additional negative control (257). This experiment was repeated two times with comparable results.

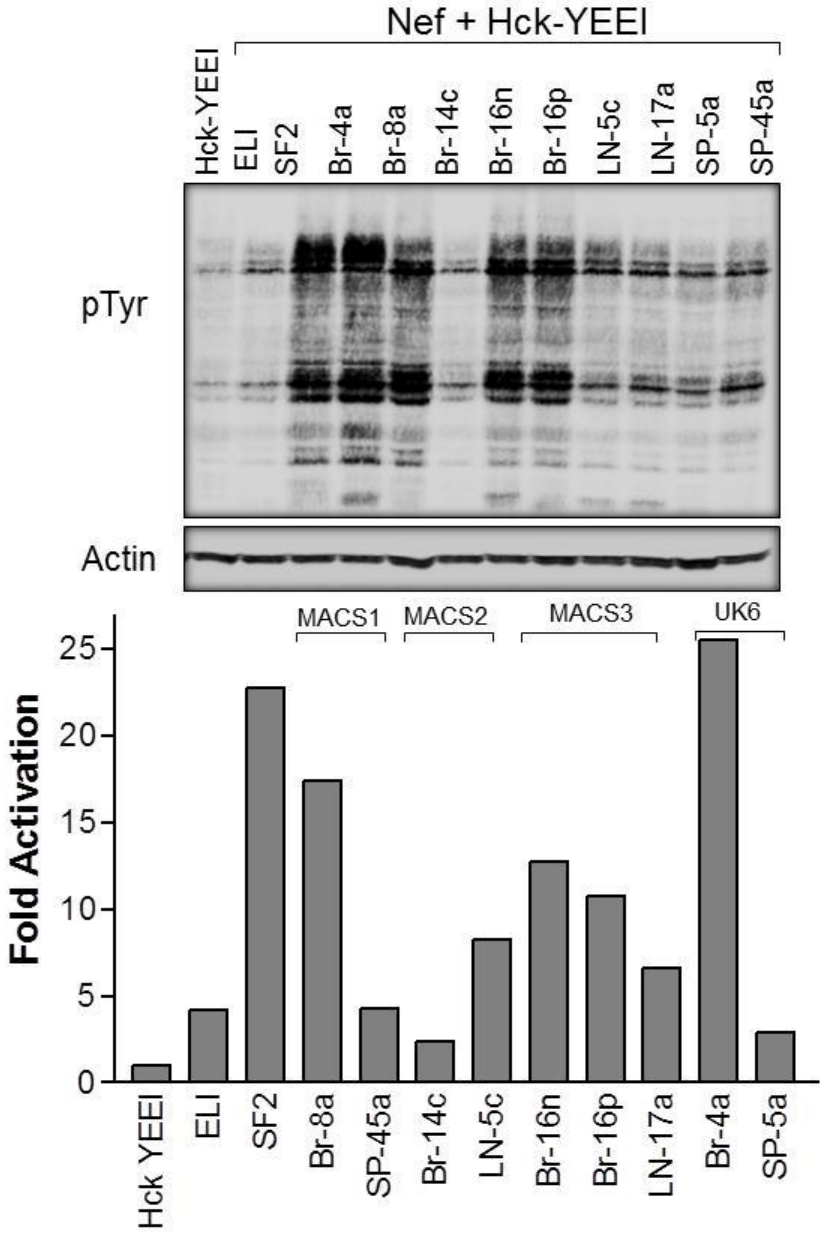

Figure 21. Enhanced activation of Hck by brain-derived versus peripheral HIV-1 Nef proteins from the same patient: quantitative immunoblot assay.

Yeast cultures were transformed with the expression plasmids described in Figure 19 and grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for actin as a loading control. The blots were scanned and quantitated for *pTyr* bands using an Odyssey infrared imager (LI-COR Biosciences). This experiment was repeated two times with comparable results.

Results from the growth suppression assay and antiphosphotyrosine immunoblots provide clear evidence of enhanced Hck activation by brain versus peripheral HIV-1 Nef proteins from three separate patients. To evaluate this result in a more quantitative way, antiphosphotyrosine immunoblots were repeated using an Odyssey infrared imager (LI-COR Biosciences) with respect to loading control actin. As shown in Figure 21, the brain-derived Nef proteins from patients MACS1, MACS3 and UK6 showed enhanced Hck activation. Several studies have reported the importance of the *nef* gene in HAD through the activation of macrophages (165,192). Our results demonstrate for the first time that the *nef* gene undergoes evolution in the brain to enhance Hck activation. The enhanced Hck activation by brain-derived *nef* alleles could increase HIV-1 replication in brain, inducing dysregulation of macrophages and microglia during the progression to HAD. Thus, these results suggest that enhanced Hck activation by Nef evolved in brain has role in development of HAD.

3.4.8 Patient-derived Nef alleles failed to activate Fyn in a cell-based assay

Previous studies have shown that Nef associates with Fyn in the presence of the TCR ζ chain where TCR ζ stabilizes the polyproline type II helix on Nef (13,137). Therefore, we decided to explore the evolutionary effect of the specialized organ-derived Nef alleles on Fyn. We examined whether patient-derived Nef proteins can activate Fyn T (Fyn T is a Fyn isoform specifically expressed in T cells) using the yeast assay. Patient-derived Nef proteins were co-expressed with the down-regulated (YEEI) forms of Fyn T, followed by anti-phosphotyrosine immunoblotting of yeast cell lysates. As shown in Figure 22, co-expression with Nef had no effect on Fyn T despite strong expression of the Nef proteins and Fyn T. Fyn T was also expressed as wild-type form; in the absence of Csk, Fyn T induced strong yeast tyrosine

phosphorylation as observed previously (256). Though the patient-derived Nef alleles had no effect on Fyn T regulation alone, it would be interesting to re-evaluate this interaction in the presence of the TCR ζ chain using the yeast-based system. The presence of TCR ζ chain might influence Nef-Fyn T interaction.

(The patient-derived *nef* alleles used in this study were provided by Kevin Olivieri and Dana Gabuzda, Dana-Farber Cancer Institute, Harvard Medical School.)

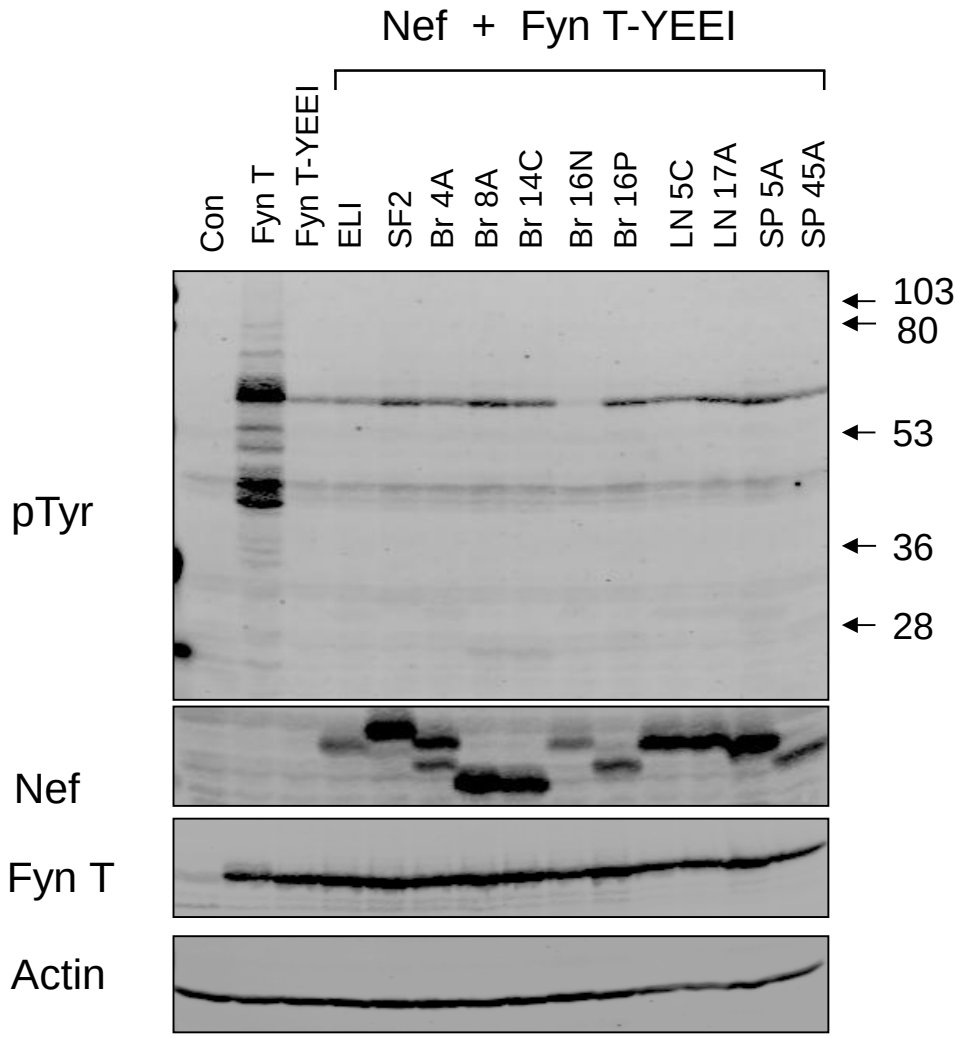

Figure 22. Patient-derived M-group HIV-1 Nef proteins do not activate Fyn T.

Yeast cultures were transformed with a galactose-inducible expression plasmid for Fyn-YEEI, which carries a modified C-terminal tail that enables downregulation in the absence of Csk (257), or the empty expression plasmid as a negative control (Con). Where indicated, Fyn-YEEI cells were co-transformed with galactose-inducible vectors for Nef-ELI, Nef-SF2, and nine patient-derived Nef alleles [MACS1 (Br-8a, SP-45a); MACS2 (Br-14c, LN-5c); MACS3 (Br-16n, Br-16p, LN-17a); UK6 (Br-4a, SP-5a)]. Transformed cells were grown in liquid culture in the presence of galactose at 30 °C for 18 h. Protein extracts were separated via SDS-PAGE and immunoblotted for tyrosine-phosphorylated proteins (*pTyr*) as well as for Nef, Fyn and actin as a loading control. Wild-type Fyn served as positive control for respective kinase activation. This experiment was repeated two times with comparable results.

3.5 SIGNIFICANCE AND CONCLUSIONS

Almost all previous studies of the interaction of HIV-1 Nef with SFKs involved the use of laboratory alleles. As these interactions are important in HIV-1 pathogenesis and may represent possible therapeutic targets, it was necessary to expand these studies to a larger more heterogeneous population of Nef variants derived from the major HIV-1 subtypes responsible for the AIDS pandemic. The present study represents the first systematic analysis to address the generality of Nef-induced activation of SFKs by employing Nef alleles representative of all major HIV-1 clades. Our study demonstrates for the first time that Src-family kinase binding and activation are highly conserved properties of HIV-1 Nef isolates representative of all major HIV-1 subtypes. As observed previously for Nef proteins derived from laboratory strains of HIV-1 such as SF2 (45,256), SFK activation is limited to Hck and Lyn, with some activation apparent for c-Src. In contrast, Fyn, Lck, and Fgr, which are also expressed in HIV host cells, do not appear to be direct Nef effectors. Furthermore, we extended this observation with patient-derived Nef proteins which also strongly activated Hck but had no effect on Fyn T kinase. Furthermore, in three of four patients, brain-derived Nef sequences showed enhanced activation of Hck, which has previously been implicated in HAD. These observations support the formation of specific Nef-SFK complexes in all HIV target cell types.

4.0 NEF ALLELES FROM ALL MAJOR HIV-1 CLADES ENHANCE HIV-1 REPLICATION IN AN INHIBITOR-SENSITIVE MANNER*

*Some of the work presented in this chapter has been published in PLoS ONE: 2012 **7(2)**:e32561.

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Narute, P.S. and Smithgall, T.E. 2012 Nef alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor-sensitive manner. PLoS ONE **7(2)**:e32561.

4.1 ABSTRACT

HIV-1 Nef is a 27 kDa myristylated protein expressed early in the HIV-1 replication cycle and is essential for high titer viral replication. Because Nef lacks intrinsic enzymatic activity, it exerts its effects by interacting with various host cell proteins involved in cellular activation, immune recognition and survival. In particular, Nef interacts with the SH3 domain of Hck and other Src-family protein-tyrosine kinases (SFKs) through its highly conserved proline-rich (PxxPxR) motif and hydrophobic pocket. Nef alone is sufficient to produce AIDS-like symptoms in mice and is required for SIV-induced AIDS in monkeys. Moreover, defective or mutated forms of Nef have been detected in long term non-progressors patients infected with HIV-1. Nef-induced AIDS-like disease was delayed in the absence of Hck kinase in the transgenic mouse model underscoring the importance of *nef* gene in HIV-1 pathogenesis. Our laboratory has shown that HIV-1 Nef specifically activates Hck, Lyn and Src. In addition to this, we also showed that this property of Nef is highly conserved among all major clades of HIV-1. Recently, our lab identified 4-amino substituted diphenylfuranopyrimidines (DFPs) and diphenylpyrazolyldiazene (PPD-B9) that selectively inhibit Nef-dependent HIV replication. To determine whether these compounds exhibit broad-spectrum Nef inhibition, we first evaluated effects on primary Nef proteins-mediated Hck activation in *in vitro* assay. Then to evaluate broad-spectrum Nef-dependent antiretroviral activity against HIV-1, we constructed chimeric forms of the HIV-1 strain NL4-3 expressing the same 10 primary *nef* alleles. The infectivity and replication of these Nef- chimeric HIV-1 was indistinguishable from that of wild-type in three distinct cell lines (MT2, U87MG

and CEM-T4). Importantly, the 4-aminopropanol and 4-aminobutanol derivatives of DFP as well as PPD-B9 potently inhibited the replication of all 10 chimeric forms of HIV-1 in cell lines where HIV-1 replication is Nef-dependent. Moreover, we showed that the inhibition of HIV-1 replication is due to direct inhibition of HIV-1 Nef-mediated activation of endogenous SFKs in CEM-T4 cells. Thus our results validate for the first time that Nef-mediated SFK activation is broad-based target for anti-HIV drug discovery.

4.2 INTRODUCTION

HIV-1 Nef is a 27 kDa myristylated protein expressed early in the HIV-1 replication cycle and is essential for high titer viral replication (107,136,178,226,245). Nef, an accessory protein only present in primate lentiviruses, is not required for HIV-1 replication *in vitro* but required for high titer replication and AIDS pathogenesis *in vivo* (58,107,132,133,146,178,245,253). Nef is required for SIV induced AIDS in monkeys and defective or mutated forms of Nef have been detected in long term non-progressors, patients infected with HIV-1 but fail to develop AIDS over many years (58,141).

As detailed in the Introduction, Nef lacks intrinsic enzymatic activity, and interacts with many host proteins and exploits cellular signaling pathways to optimize viral replication and promote AIDS progression (67,94,127,208,216). One group of key molecular targets for Nef are the Src-family kinases (SFKs) which are involved in cellular activation, immune recognition and survival (34,35,94,199,216,255,256). As shown in Chapter 3, Nef interacts with and activates a subset of SFKs through displacement of regulatory SH3-linker interactions which are required for SFK downregulation (35,45,226,256). Moreover, Saksela *et al.* showed that mutation of the PxxP motif of Nef required for SFK SH3 binding significantly hampered Nef-mediated SFK activation as well as HIV-1 replication in primary blood cells although there was no effect on Nef-mediated CD4 receptor down-regulation (226). Similarly, Nef-mediated activation of Hck is required for STAT3 activation and proliferation of myeloid cell line (34). Moreover, Komuro *et al.* showed that suppression of Hck expression with antisense oligonucleotides dramatically inhibits M-tropic HIV-1 replication in primary human macrophages (145). In addition, Nef can

interrupt macrophage colony-stimulating factor signaling by activating Hck, suggesting that Nef: Hck association may contribute to macrophage dysfunction in HIV-infected cells (248).

The highly conserved nature of Nef-SFK activation and its importance to HIV-1 replication and pathogenesis has raised interest in this signaling pathway as a therapeutic target. In the light of this evidence, our laboratory developed a high-throughput screening assay for inhibitors of Nef-mediated Hck activation *in vitro* as well as yeast-based kinase assay. This effort led to the discovery of 4-amino-substituted diphenylfuropyrimidine (DFP), and diphenylpyrazolyldiazene (PPD-B9) compounds with low micromolar activity against both Nef-mediated Hck activation and Nef-dependent HIV-1 replication in cell culture [(63) and L. Emert-Sedlak, P. Narute, and T. Smithgall, *et al*., manuscript in preparation]. These prior studies were conducted with two laboratory strains of HIV-1, SF2 and NL4-3, once again raising the question of the broader applicability of these compounds against the many allelic variants of HIV-1 and Nef. Therefore, we first investigated the effect of the DFP analogs on primary Nef-mediated Hck activation and then evaluated the effect of DFP and PPD-B9 on replication of HIV-1 NL4-3 Nef chimeras that express each primary Nef protein.

In the present study, we describe the evaluation of the broad specificity of these novel inhibitors of Nef function against HIV-1 replication. First, we first evaluated the activity of 4-amino-substituted DFPs against primary Nef -mediated Hck activation using an *in vitro* kinase assay. Next, we successfully developed chimeric HIV-1_{NL4-3} expressing each individual primary Nef protein and then evaluated the antiretroviral activity of these novel inhibitors in cell culture. Active analogs of DFP identified in our previous work potently inhibited primary Nef-mediated Hck activation *in vitro* as well as replication of all of the HIV-1 Nef chimeras. Similarly, PPD-B9 also broadly inhibited HIV-1 replication in cell culture. These results validate the Nef-Hck

signaling axis as a viable target for development of broad-based inhibitors as a new approach to anti-retroviral therapeutics.

4.3 MATERIALS AND METHODS

Cell lines and antibodies

Human 293T cells were obtained from the ATCC and grown in Dulbecco's Modified Eagle's Medium/high glucose (Invitrogen) supplemented with 10% fetal bovine serum. U87MG-$CD4^+$/ $CXCR4^+$, MT2 and CEM-T4 cells were obtained from the NIH AIDS Research and Reference Reagent Program. U87MG-$CD4^+$/$CXCR4^+$ cells were grown in Dulbecco's Modified Eagle's Medium/high glucose supplemented with 25 mM HEPES, pH 7.4, 10% fetal bovine serum, G418 (400 µg/ml) and puromycin (0.5 µg/ml); G418 and puromycin are required to maintain expression of ectopically expressed CD4 and CXCR4 in this cell line. MT2 and CEM-T4 cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

Antibodies used in this study were obtained from the NIH AIDS Research and Reference Reagent Program (Nef, 2949; p24, 4121), Santa Cruz Biotechnology (Hck, sc-72; Lyn, sc-15; Fyn, sc-16; Lck, sc-13; c-Src, sc-18; Fgr, sc-17; pan-specific SFK, sc-5266), Abcam (Yes, Ab13954), Invitrogen (pY418, 44660G), and Millipore (actin, MAB 1501).

In vitro kinase assay

Nef-mediated activation of Hck tyrosine kinase activity was assayed using the Z'-Lyte method with the Tyr2 peptide substrate (Invitrogen/Life Technologies). The principle of this FRET-based assay is described in detail elsewhere (63,256) and in Figure 3.1. The assay conditions are described in Chapter 3.

HIV-1 Nef chimera construction and viral replication assay

To generate the HIV-1 Nef chimeras, unique *Cla* I restriction sites flanking the Nef ORF were introduced into pUC18 carrying the complete HIV-1 NL4-3 proviral DNA sequence (pUC18-NL4-3) (63,209). The coding sequences for the primary Nef genes as well as Nef-SF2 were PCR-amplified with *Cla* I linkers and used to replace the NL4-3 Nef sequence in pUC18-NL4-3. Control and chimeric viruses were generated by transfection of 293T cells, followed by amplification of the viral supernatants in the T cell line, MT2. To evaluate the infectivity, MT2 cells were infected with 500pg/ml of each chimeric virus for 4 days in 6-well plate and imaged on a microscope (Nikon). HIV-1 replication was determined in cell culture supernatants using p24 ELISA.

For HIV-1 replication assays, U87MG-$CD4^+$/$CXCR4^+$ and CEM-T4 cells were incubated overnight in the absence or presence of the DFP analogs or PPD-B9 in a final concentration of 0.1% DMSO as carrier solvent. U87MG-$CD4^+$/$CXCR4^+$ cells were then infected with 200 pg p24/ml of each chimeric virus for 5 d while CEM-T4 cells were infected with 62.5 pg p24/ml of each chimeric virus for 10 d in 96-well plates. HIV-1 levels in the culture supernatants were then determined by p24 ELISA as described (63).

Activation of endogenous SFKs by HIV-1 Nef chimeras

CEM-T4 cells (1×10^5 per T25 flask) were infected with 50 pg p24 equivalents/ml of wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in a final culture volume of 10 ml in the absence or presence of 3 μM DFP-4A, DFP-4AP, DFP-4AB, or 1 μM PPD-B9 or the carrier solvent (DMSO) alone as a control (Con). The infected cells were lysed eight days later in RIPA buffer and SFK proteins were immunoprecipitated with a pan-specific

antibody against SFK and protein-G sepharose beads as described elsewhere (36,45). SFK activation was assessed by immunoblotting each immunoprecipitate with a phosphospecific antibody against the activation loop phosphotyrosine residue common to all Src family members (pY418; (232)). Control blots were performed on cell lysates for HIV-1 Gag proteins (p55, p40, and p24), Nef, as well as actin as a loading control.

Src-family kinase expression profiling

CEM-T4 cells were lysed in RIPA buffer (202) and SFK protein levels were assessed by immunoblotting with antibodies specific for the individual Src family members.

4.4 RESULTS AND DISCUSSION

4.4.1 Small molecule inhibitors of Nef-induced Hck activation are broadly effective against Hck activation by primary Nef proteins

Recently we described a screening assay for small molecule inhibitors of Nef-dependent Hck activation *in vitro* (63). Using this assay, our laboratory identified a series of 4-amino substituted DFPs and PPD-B9 as potent inhibitors of both Nef-SF2-mediated Hck activation and Nef-dependent HIV-1 replication. Because these compounds represent potential leads for Nef-directed HIV-1/AIDS therapeutics, we next examined whether these compounds inhibit Hck following activation by each of the recombinant primary Nef proteins using Z'-Lyte kinase assay. As shown in Figure 23, both the 4-aminopropanol and 4-aminobutanol derivatives of DFP inhibited primary Nef-induced Hck activation by more than 50% at 10 μM. Remarkably, the inhibitory action of both compounds was more pronounced with the primary Nef proteins than with Nef-SF2, the laboratory allele originally used to develop the screen. In contrast, the unsubstituted 4-amino DFP pharmacophore was without effect, consistent with our previous results (63). These results demonstrate that 4-amino substituted derivatives of DFP are broadly active against the Hck: Nef complex independent of the Nef isolate used.

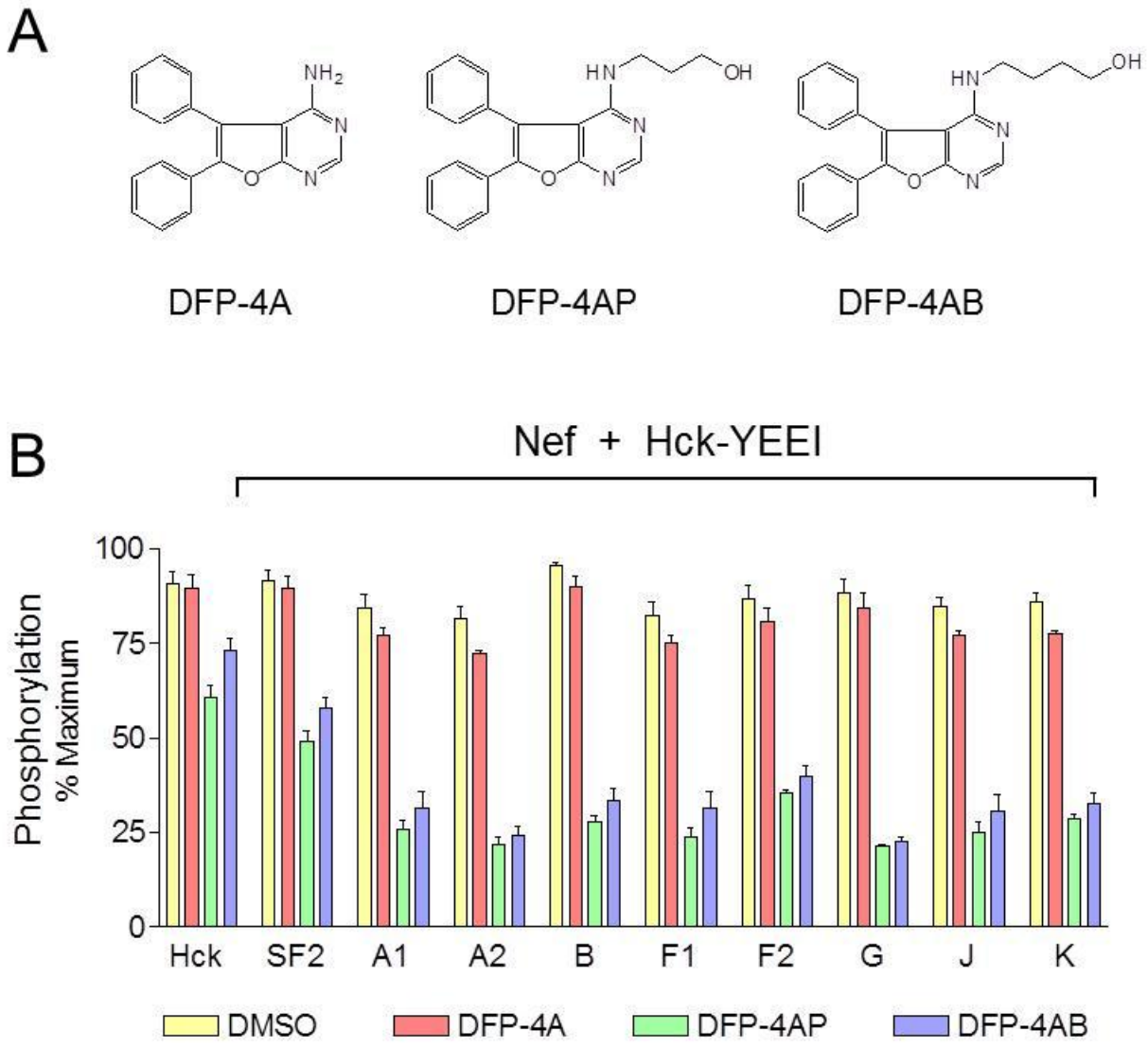

Figure 23. Diphenylfuropyrimidine (DFP) analogs are broadly active against primary Nef-mediated Hck activation.

A) Structures of 4-amino DFP (DFP-4A) and the corresponding 4-aminopropanol (DFP-4AP) and 4-aminobutanol (DFP-4AB) analogs. B) Recombinant downregulated Hck [Hck-YEEI; (63,256,257)] activity was determined either alone or in the presence of the indicated Nef proteins as described in the text. Reactions were run in the presence of 10 µM concentrations of DFP-4A, DFP-4AP, DFP-4AB, or the DMSO carrier solvent as negative control. Results are expressed as the mean percent of maximum substrate phosphorylation ± S.D.; this experiment was repeated twice with comparable results.

In addition to 4-amino substituted diphenylfuranopyrimidines (DFPs), our lab has discovered a substituted diphenylpyrazolyldiazene (PPD-B9) in a high-throughput screening campaign for inhibitors of Nef-mediated Hck activation. This compound also blocks Nef-dependent Hck activation as well as HIV-1 replication with submicromolar potency. Moreover, structural and computational studies using SPR and Autodock Vina respectively, suggest that PPD-B9 directly binds to two sites on the Nef surface. The binding of PPD-B9 to one site may induce conformational changes in Nef for high affinity binding of PPD-B9 to the second site, which is localized by the docking studies to the Nef dimer interface. In contrast to the DFP compounds, PPD-B9 appears to represent a direct inhibitor of Nef activity (L. Emert-Sedlak, P. Narute, T. Smithgall, *et al*., manuscript in preparation). Since PPD-B9 was discovered using the same screening approach used for DFPs, we decided to evaluate its broad specificity against chimeric HIV-1 replication.

4.4.2 Generation of chimeric HIV-1 and evaluation of replication and pathogenicity in MT2 cells

In order to evaluate the broad specificity of recently discovered DFP compounds, we generated HIV-1_{NL43} chimeras expressing Nef alleles from all of the major clades. We first characterized the infectivity of each chimeric Nef virus in MT2 cells. As shown in Figure 24A, all of the HIV-1_{NL43} Nef chimeras replicated as efficiently as wild-type HIV-1_{NL43} in MT2 cells where HIV-1 replication is Nef-independent. Furthermore, as shown in Figure 24B, each chimeric form of HIV-1 produced Nef as well as other viral proteins, as detected by immunoblotting. Additionally we evaluated the cytopathic effect of these viruses in MT2 cells. As shown in Figure 25, all of

the chimeric viruses produced cytopathic effects similar to wild type HIV-1 in MT2 cells. Overall, these results strongly indicate that all chimeric viruses are as infectious and replication-competent as wild type HIV-1 $_{NL43}$.

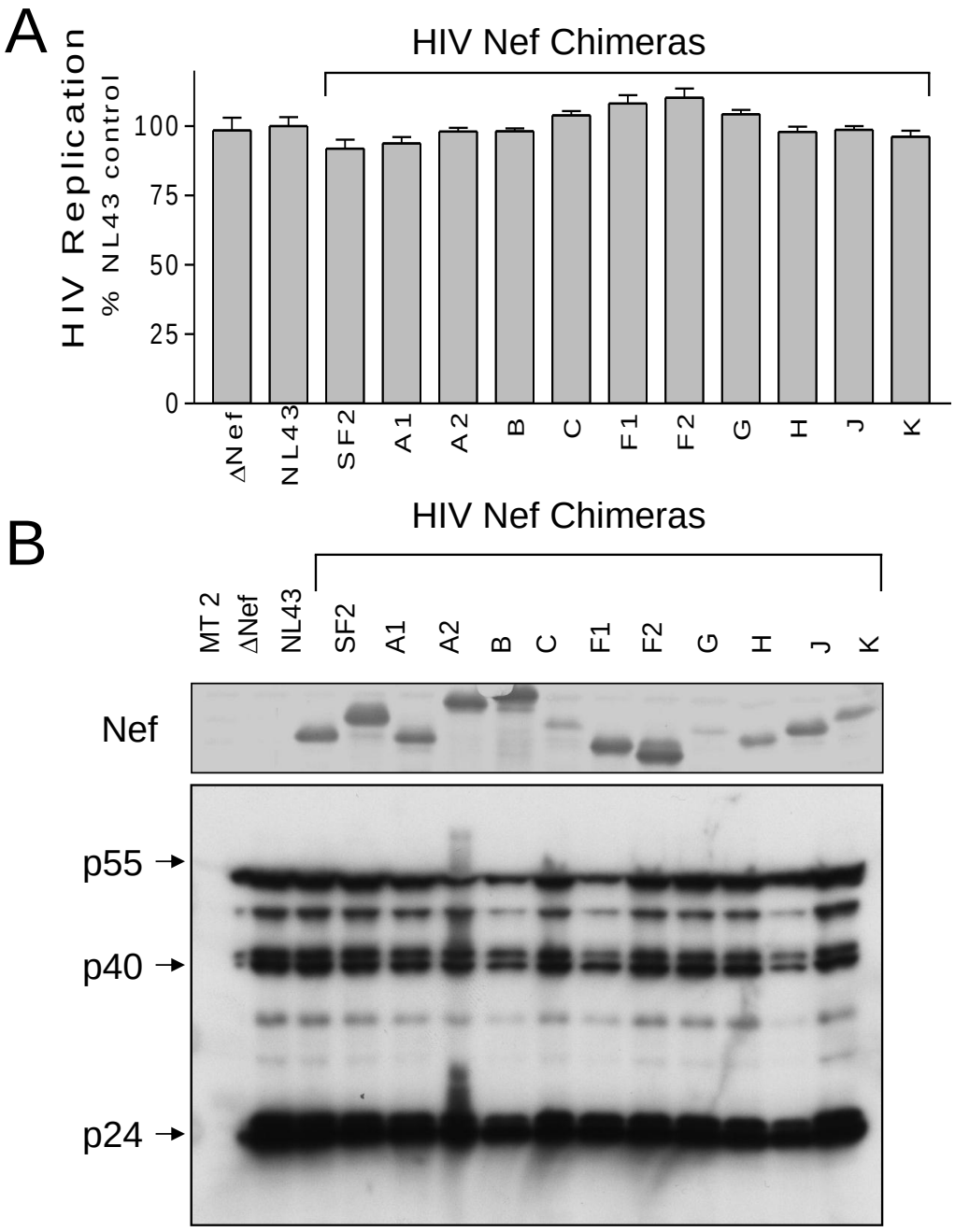

Figure 24. Replication of HIV-1 Nef chimeras in MT2 cells.
A) MT2 cells (1 x 10^4 per well of a 96-well plate) were infected with 500 pg p24 equivalents/ml of wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in a final culture volume of 200 µl. HIV p24 levels were determined by ELISA 4 days later. Data are presented as percent of p24 release observed relative to the HIV-1 NL4-3 control ± S.D. B) U87MG-$CD4^+CXCR4^+$ cells (1 x 10^5 per T-12.5 flask) were infected with 500 pg p24 equivalents/ml of the same panel of viruses as in part A. Viral Nef and Gag protein expression was verified by immunoblotting lysates of infected cells 4 days later. Lysates from uninfected cells are included as a negative control (MT2; far left lane).

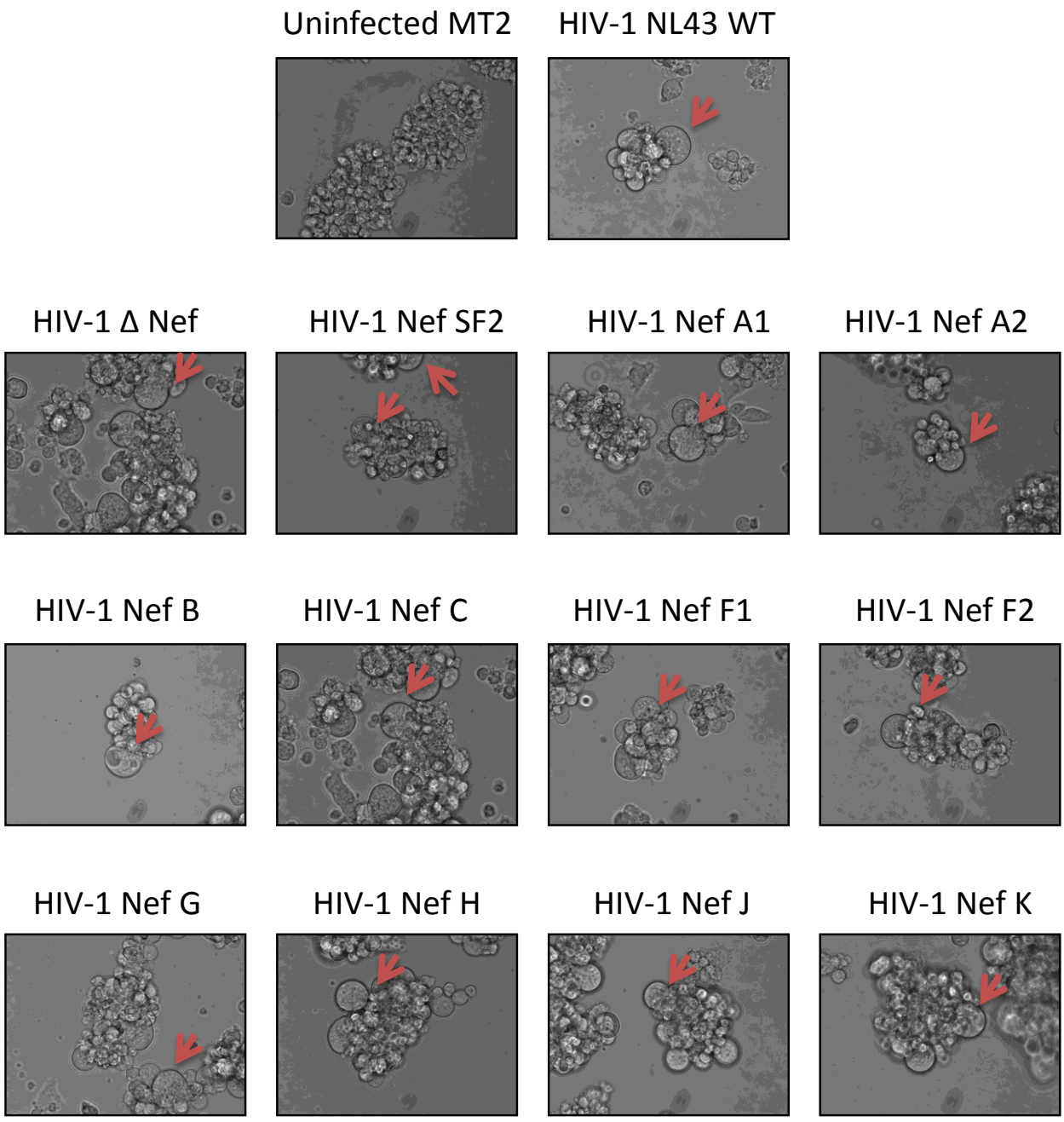

Figure 25. HIV-1 Nef chimeras are as infectious and cytopathic as wild-type HIV-1 NL43. MT2 cells (1 x 10^5 cells in T 12.5 flask) were infected with 500 pg/ml of the viral strain and incubated at 37 °C for 4 days and observed for the cytopathic effect of viral infection. Note that syncytia formation by chimeric HIV-1 expressing the primary Nef proteins is very similar to that observed with wild-type HIV-1 NL43. Red arrows indicate the presence of syncytia in each of the infected cultures.

4.4.3 HIV-1 replication is Nef-dependent in U87MG and CEM-T4 cells

Previous work from our group and others has established that Nef expression is essential for optimal replication of HIV-1 in the astroglioma cell line U87MG which has been engineered to express CD4 and the co-receptor CXCR4 and in the T-cell line, CEM-T4 (48,63,196). Replication of HIV-1 strain NL4-3 is potently inhibited by 4-amino substituted DFP compounds in the U87MG model system, and this inhibitory effect is dependent upon the expression of Nef (63). In order to investigate the broader utility of the DFP compounds against M-group HIV-1 isolates, we first needed to demonstrate functional enhancement of HIV-1 replication by each of our primary *nef* alleles in these cell lines. We then evaluated the ability of each primary *nef* allele to support HIV-1 replication in U87MG- $CD4^+/CXCR4^+$ cells. As observed previously, Nef-defective HIV-1 replicated very poorly in this cell line (Figure 26A). However, replication of each of the Nef chimeras was indistinguishable from wild-type HIV-1 NL4-3, demonstrating that these Nef proteins function to support HIV replication. Immunoblots demonstrate that each Nef variant is expressed in infected cells, along with the major capsid proteins (Figure 26B). Similarly, replication of Nef-defective HIV-1 replicated to only 10% of wild-type levels in CEM-T4 cells, and this replication defect was completely rescued by *cis*-complementation with all of the primary Nef alleles tested (Figure 27A). Immunoblotting verified Nef expression in CEM-T4 cells infected with wild-type HIV-1 and each of the chimeras, as well as capsid proteins (Figure 27B). These results demonstrate that HIV-1 replication is Nef-dependent in both the U87MG-$CD4^+/CXCR4^+$ and CEM-T4 cell lines, providing an important new tool to interrogate the broad efficacy of small molecule inhibitors of Nef function on M-group HIV-1 replication.

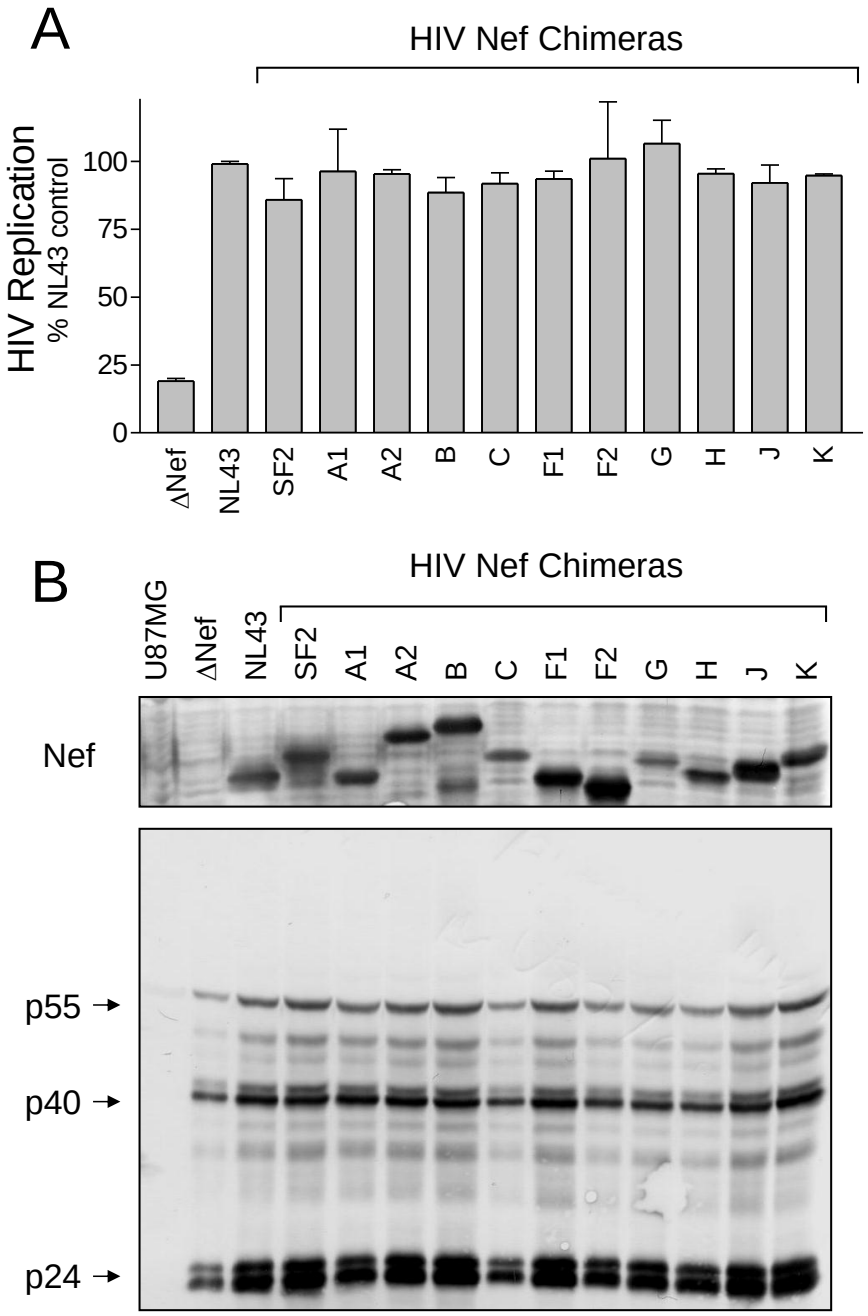

Figure 26. Replication of HIV-1 Nef chimeras in U87MG-CD4$^+$/CXCR4$^+$ cells.
A) U87MG- CD4$^+$/CXCR4$^+$ cells (2 x 10^4 per well of a 96-well plate) were infected with 200 pg p24 equivalents/ml of wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in a final culture volume of 200 µl. HIV p24 levels were determined by ELISA 5 days later. Data are presented as percent of p24 release observed relative to the HIV-1 NL4-3 control ± S.D. B) U87MG-CD4$^+$CXCR4$^+$ cells (1 x 10^5 per well of a 6-well plate) were infected with 1 ng p24 equivalents/ml of the same panel of viruses as in part A. Viral Nef and Gag protein expression was verified by immunoblotting lysates of infected cells 4 days later. Lysates from uninfected cells are included as a negative control (U87MG; far left lane).

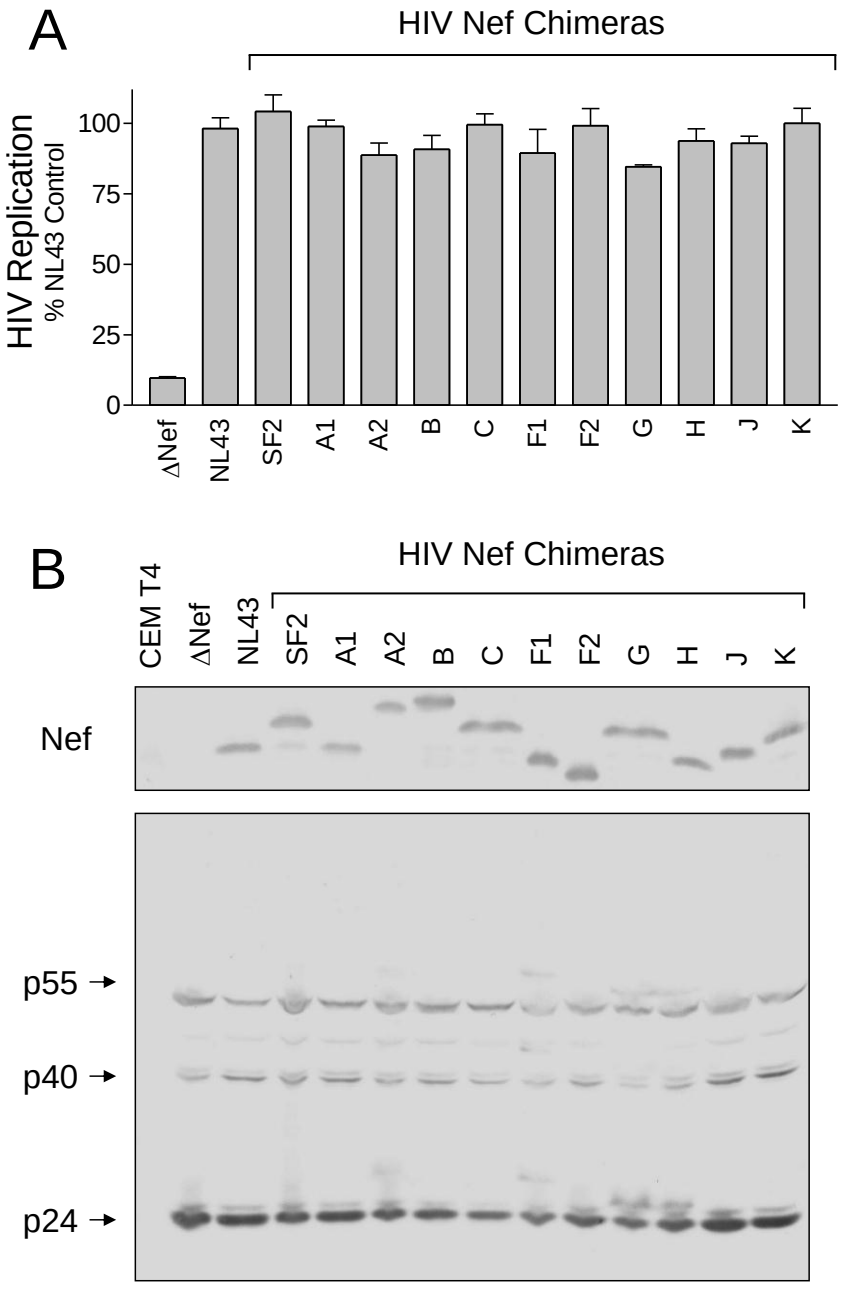

Figure 27. Replication of HIV-1 Nef chimeras in CEM-T4 cells.
A) CEM-T4 cells (1 x 10^4 per well of a 96-well plate) were infected with 62.5 pg p24 equivalents/ml of wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in a final culture volume of 200 µl. HIV p24 levels were determined by ELISA 10 days later. Data are presented as percent of p24 release observed relative to the NL4-3 control ± S.D. B) CEM-T4 cells (5 x 10^5 per well of a 6-well plate) were infected with 1 ng p24 equivalent/ml of the same panel of viruses as in part A. Viral Nef and gag protein expression was verified by immunoblotting lysates of infected cells 6 days later. Lysates from uninfected cells are included as a negative control (CEM-T4; far left lane).

4.4.4 Small molecule inhibitors of Nef-induced Hck activation are broadly active inhibitors of HIV-1 Nef chimera replication

As described in the preceding sections, 4-amino substituted DFP analogs are potent inhibitors of both Nef-dependent SFK activation and HIV replication (63). Two DFP derivatives, DFP-4-AP and DFP-4-AB, showed potent inhibition of Hck activation by all of the recombinant M-group Nef proteins tested (Figure 23), suggesting that they may inhibit replication of the HIV-1 chimeras expressing each of these primary Nef alleles as well. To test this possibility, we infected both U87MG-$CD4^+$/$CXCR4^+$ and CEM-T4 cells with wild-type HIV-1 NL4-3, the Nef-defective mutant and the 10 Nef chimeras in the absence or presence of DFP-4-AP or DFP-4-AB. As shown in Figure 28, both 4-amino DFP analogs inhibited replication of wild-type HIV-1, as well as all of the Nef chimeras, by more than 75% at a concentration of 3 μM in both cell lines. In contrast, the compounds had no impact on the replication of Nef-defective HIV-1, demonstrating that antiretroviral activity of the DFP analogs requires the expression of Nef. As with the *in vitro* kinase assays, the unsubstituted 4-amino DFP pharmacophore was completely inactive, consistent with our original observations (63).

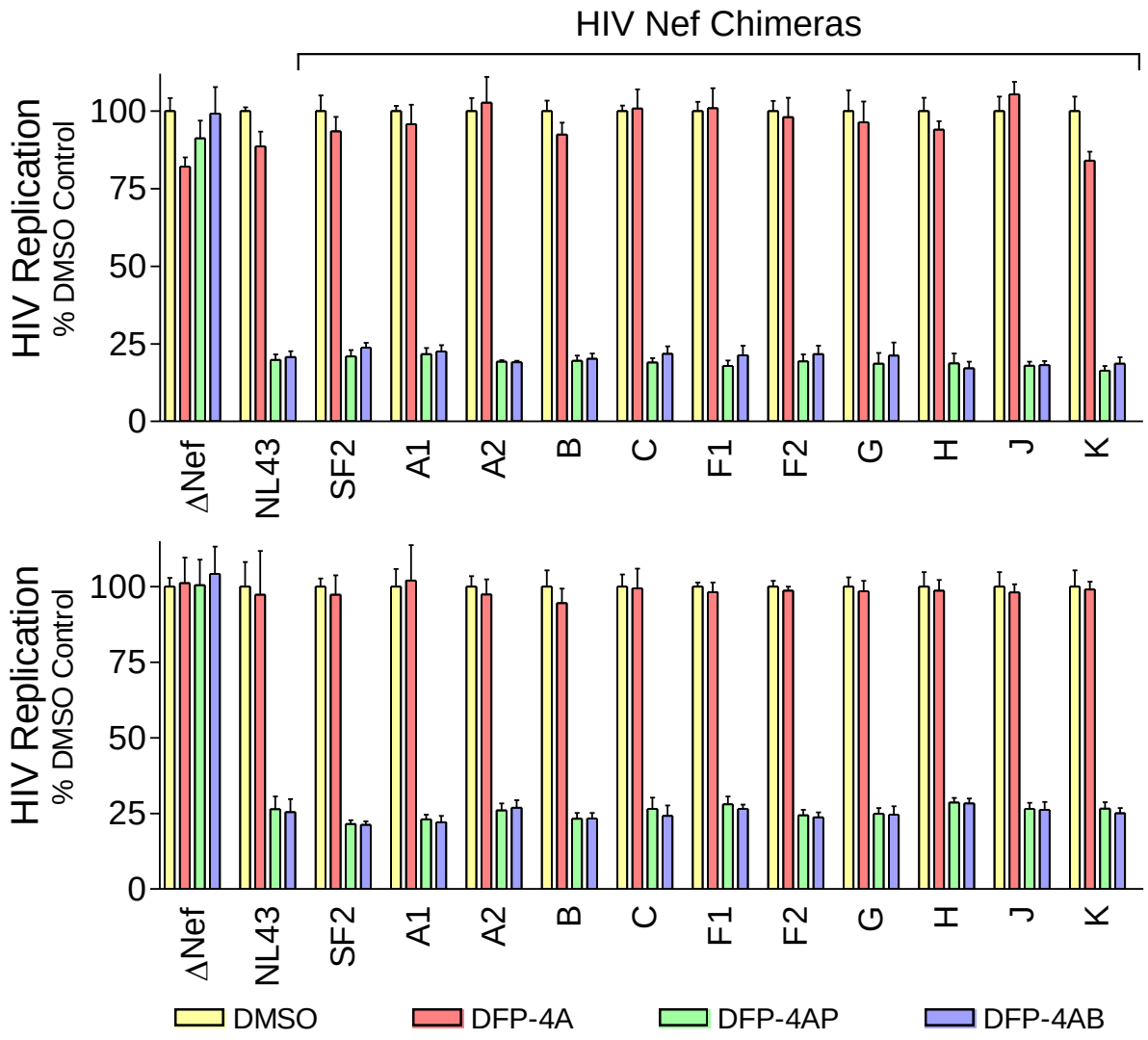

Figure 28. Inhibition of HIV-1 Nef chimeras by 4-amino DFP analogs.
U87MG-CD4^{+}/CXCR4^{+} cells (upper panel) and CEM-T4 cells (lower panel) were infected with wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras as described in the legends to Figures 26 and 27. DFP-4A, DFP-4AP, or DFP-4AB were added to the cultures to a final concentration of 3 µM, and viral replication was determined by p24 ELISA 5 days (U87MG) or 10 days (CEM-T4) later. Data are expressed as the mean percent of HIV-1 replication observed in control cultures incubated with the carrier solvent (0.1 % DMSO) ± S.D. (n=6).

To obtain more insight into the mechanism by which these compounds inhibited the HIV-1 replication in CEM-T4 cells, we investigated the effect of HIV-1 infection on endogenous SFK activation in CEM-T4 T cells, as well as the impact of the DFP-based inhibitors on HIV-mediated kinase activation. For these studies, CEM-T4 cells were infected with wild-type HIV NL4-3, the Nef-defective mutant, as well as each of the 11 Nef chimeras. Infected cells were lysed and SFK activity was monitored with a phosphospecific antibody that recognizes the phosphotyrosine residue in the activation loop of active SFKs (pY416). As shown in Figure 29 B, HIV infection resulted in a Nef-dependent increase in endogenous Src-family kinase activation in every case. Immunoblots with SFK isoform-specific antibodies revealed that CEM-T4 cells express the direct Nef targets c-Src and Hck (Figure 29 A), which are most likely the SFKs activated by Nef in this system. Furthermore, when cells were treated with DFP-4AB or DFP-4AP, both of which block Nef-dependent HIV-1 replication (Figure 28), SFK activation was completely inhibited. In contrast, the inactive DFP analog, DFP-4A, had no effect on kinase activity.

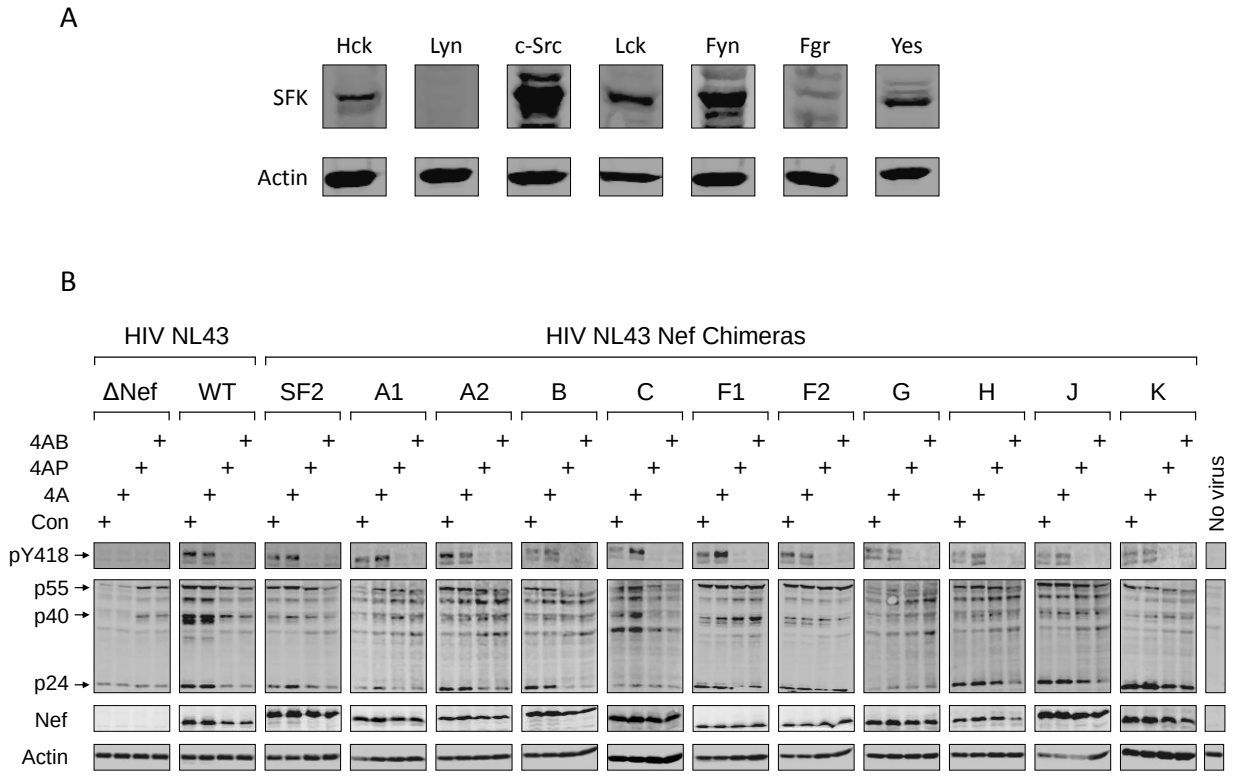

Figure 29. Inhibition of HIV-1 Nef chimera-mediated activation of endogenous SFKs in CEM-T4 cells by 4-amino DFP analogs.

A) CEM-T4 cells were lysed in RIPA buffer (202) and SFK protein levels were assessed by immunoblotting with antibodies specific for the individual Src family members shown. Lysates were also blotted with actin antibodies as a loading control. This expression profile shows that the CEM-T4 lymphoblasts express Hck, Lyn, c-Src, Lck, Fyn and Yes. B) CEM-T4 cells were infected with wild-type HIV-1 NL4-3 (WT), a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in the absence or presence of 3 μM DFP-4A, DFP-4AP, DFP-4AB, or the carrier solvent (DMSO) alone as a control (Con). The infected cells were lysed and SFK proteins were immunoprecipitated with a pan-specific antibody against SFK and protein G-sepharose beads. SFK activation was assessed by immunoblotting with a phosphospecific antibody against the activation loop phosphotyrosine residue common to all Src family members (pY418). Control blots were performed on cell lysates for HIV-1 Gag proteins (p55, p40, and p24), Nef, as well as actin as a loading control. Results from uninfected cells are shown in the far right lane (no virus control). This experiment was repeated twice with comparable results.

Next, we evaluated the effect of PPD-B9 on replication of HIV-1 Nef chimeras in CEM-T4 cells. As shown in Figure 30, PPD-B9 inhibited replication of wild-type HIV-1, as well as all of the Nef chimeras, in a dose-dependent manner in CEM T4 cells compared to the DMSO control. PPD-B9 demonstrated more potent activity than DFPs; it inhibited HIV-1 replication by more than 75% at a concentration of 1 μM. Similar to DFPs, PPD-B9 also had no impact on the replication of Nef-defective HIV-1, demonstrating that antiretroviral activity requires the expression of Nef. Furthermore, we evaluated the impact of the PPD-B9 on HIV-mediated endogenous SFK activation in CEM T4 cells. As shown in Figure 31, HIV infection resulted in a Nef-dependent increase in endogenous Src-family kinase activation in every case. When these cells were treated with PPD-B9, there was strong inhibition of endogenous SFK activation. This result suggests that PPD-B9 also inhibits HIV-1 replication by inhibiting Nef-mediated SFK activation. These data provide important new evidence that HIV-1 infection results in sustained SFK activation in a Nef-dependent manner. This function is shared by *nef* alleles derived from all major HIV-1 clades in the context of HIV-1 replication. Furthermore, this pathway is sensitive to the DFP compounds as well as PPD-B9, strongly supporting inhibition of this pathway as their primary mechanism of action. These results support the conclusion that therapeutic targeting of Nef-dependent SFK activation might represent a broadly useful strategy against HIV-1 replication.

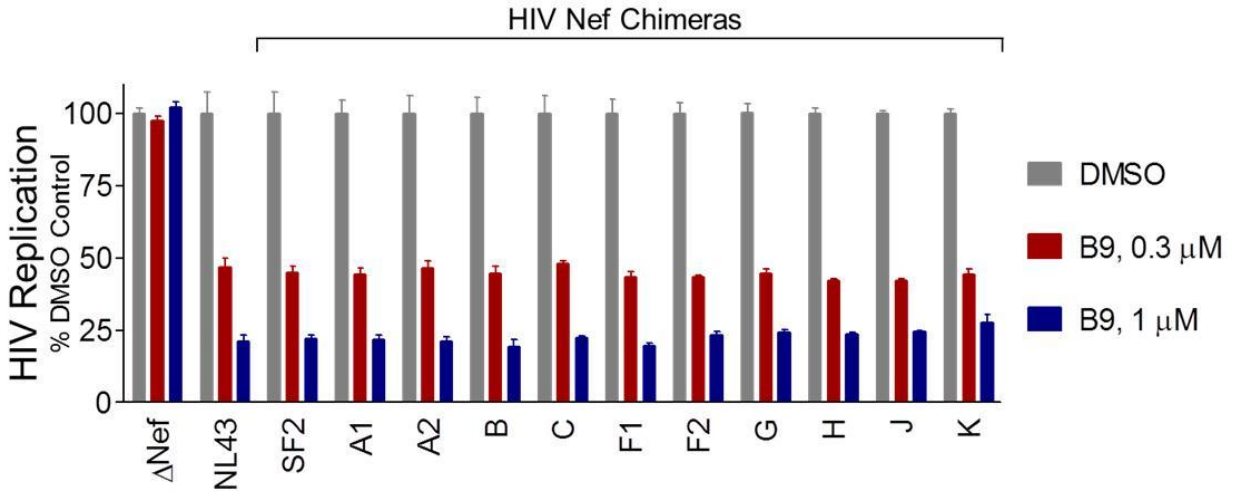

Figure 30. Inhibition of HIV-1 Nef chimeras by PPD-B9.
CEM-T4 cells were infected with wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras as described in the legends to Figures 26 and 27. The direct Nef antagonist PPD-B9 was added to the cultures to final concentrations of 0.3 and 1.0 μM, and viral replication was determined by p24 ELISA 10 days later. Data are expressed as the mean percent of HIV-1 replication observed in control cultures incubated with the carrier solvent (0.1 % DMSO) ± S.D. (n=6).

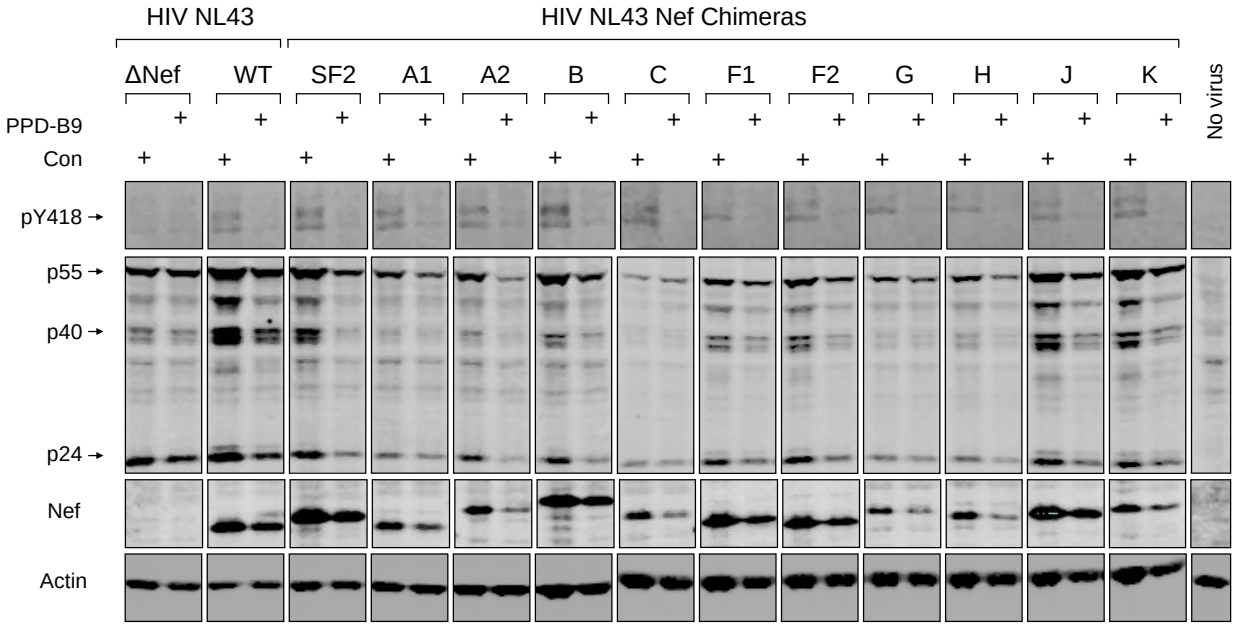

Figure 31. Inhibition of HIV-1 Nef chimera-mediated activation of endogenous SFKs in CEM-T4 cells by PPD-B9.

CEM-T4 cells (1 x 10^5 per T25 flask) were infected with 50 pg p24 equivalents/ml of wild-type HIV-1 NL4-3, a Nef-defective mutant (ΔNef), or the indicated Nef chimeras in a final culture volume of 10 ml in the absence or presence of 1 μM PPD-B9 or the carrier solvent (DMSO) alone as a control (Con). The infected cells were lysed and Src-family kinase proteins were immunoprecipitated with a pan-specific antibody and G-sepharose beads. The SFK activation state was assessed by immunoblotting each immunoprecipitate with a phosphospecific antibody against the activation loop phosphotyrosine residue common to all Src-family members (pY418). Control blots were performed on cell lysates for HIV-1 Gag proteins (p55, p40, and p24), Nef, as well as actin as a loading control. Results from uninfected cells are shown in the far right lane (No virus control). This experiment was repeated twice with comparable results.

4.5 SIGNIFICANCE AND CONCLUSION

In spite of more than 30 years of research in the field of HIV-1 since the first report of AIDS, a vaccine is not available. The current therapeutic regimen used is ineffective and contraindicated in some cases. HIV-1 eventually develops resistance against current anti-HIV drugs due to error-prone replication of the genome. Thus, a strong need exists for the development of new drugs to inhibit critical steps in HIV-1 pathogenesis. HIV-1 Nef is an essential virulence factor for development of AIDS, and has long been considered as a viable drug target. Here we demonstrate that 4-amino substituted DFP analogs as well as PPD-B9 exhibit a broad spectrum of activity against Nef:Hck complexes in cells. Importantly, both the DFP-based compounds and PPD-B9 showed equipotent inhibition of Nef-dependent HIV-1 replication using a panel of HIV-1 Nef chimeras representative of all major HIV-1 clades. Inhibition of viral replication correlated with inhibition of Nef-dependent activation of endogenous SFKs in the infected cells. These observations strongly support the development of new antiretroviral agents that target the interaction of this key HIV-1 virulence factor with SFKs and other host cell signaling proteins.

5.0 OVERALL DISCUSSION

5.1 SUMMARY OF MAJOR FINDINGS

My PhD research involves evaluation of HIV-1 Nef interactions with Src Family Kinases (SFKs) for the purpose of therapeutic intervention. In this project, we explored whether SFK activation is a conserved property of *nef* alleles from a wide range of primary HIV-1 isolates. Representative Nef proteins from the HIV-1 major subgroups A1, A2, B, C, F1, F2, G, H, J and K strongly activated Hck and Lyn as well as c-Src to a lesser extent, demonstrating for the first time that SFK activation is a highly conserved property of HIV-1 Nef isolates. To evaluate this functional property of the M-group HIV-1 Nef clade set, we first evaluated the binding of purified recombinant Nef proteins with Hck SH3 domain. All primary Nef proteins showed robust binding to the wild-type Hck SH3 domain, while no binding was observed with the Hck SH3 W93A mutant or with GST alone consistent with previous reports with laboratory Nef alleles. Moreover, using SPR, we showed that similar to Nef-SF2, most of the primary Nef proteins have high affinity for Hck SH3 domain. We next showed that all primary Nef proteins can activate recombinant Hck in the FRET-based, Z'-Lyte kinase assay. In addition to this, using our well established yeast-based kinase assay, we showed that all of the primary Nef alleles strongly activated Hck, Lyn and c-Src to a lesser extent among all SFKs expressed HIV-1 target cells. None of the *nef* alleles had measurable effects on other SFKs including Lck, Fyn and Fgr,

consistent with previous reports from our group with common B-clade laboratory alleles. Furthermore, we also showed that Nef-mediated Hck activation is highly conserved in *nef* alleles derived from AIDS patients.

As detailed in the Introduction, Nef induces a unique active Hck conformation by displacing SH2-kinase linker interaction with the SH3 domain without affecting SH2-tail interaction. We believe that this unique conformation might drive differential signaling pathways in HIV-1 infected cells and thus allow discovery of selective small molecule inhibitors of Nef-mediated Hck activation without affecting physiological Hck signaling. This hypothesis needs to be evaluated in detail to get an idea about specific signaling pathways involved in Nef-Hck mediated enhancement of HIV-1 pathogenesis. This can be evaluated by comparing global protein expression, phosphorylation, and gene expression profiles from cells (especially U87MG, U937 and CEM-T4 cells) expressing an active form of Hck (Hck-YF) versus co-expression of Nef and Hck. Once candidate signaling pathways are identified, these can be validated using specific promoter-reporter assay system.

Moreover, we also observed that Nef-mediated activation of Lyn leads to robust phosphorylation of the Nef protein itself in the yeast screen, which is less evident in case of Hck. This difference could be due to a saturation effect caused by less stringent substrate specificity of Hck in yeast compared to Lyn. Interestingly, the tyrosine phosphorylation of Nef by these SFKs raises the possibility that this may influence the interaction of Nef with other signaling molecules containing SH2 domains. Bioinformatics studies have identified 115 proteins with SH2 domains encoded by human genome (162). Whether or not tyrosine phosphorylation of Nef drives its interaction with SH2-containing targets could be addressed by affinity screening of tyrosine

phosphorylated Nef with a phage-display library containing a collection of all human SH2 domains as described recently for screening of Nef with human SH3 domains (130).

In the present study we have not seen direct interaction of Nef with the T cell specific SFK, Lck, despite the fact that several previous studies reported the physical and functional interaction of Nef with Lck (14,19,53,256,275). The yeast system used for these studies directly evaluates the interaction of HIV-1 Nef with SFKs (See Chapter 3) and therefore clearly rule out the direct interaction of Nef with Lck. In contrast, these other studies have used mammalian cell systems, raising the possibility that adaptor proteins are also involved in Nef-Lck interaction. Moreover, Collette *et al.* showed that both the PxxP motif as well as tyrosine phosphorylation of Nef are necessary for this interaction (53). As mentioned before, tyrosine phosphorylation of Nef by other SFKs might affect the interaction of Nef with other kinases. Similar to Lck, we have not seen a measurable effect of Nef on Fyn, another SFK involved in TCR signaling. As detailed in Chapter 3, studies have shown that Nef interacts with a Fyn isoform expressed in T cells and this interaction is stabilized by TCR CD3 ζ chain. The functional consequences of this multi-protein complex (Nef-Fyn- CD3 ζ chain) should be further evaluated using yeast-based system.

Despite of development of HAART therapy, it is difficult to completely cure individuals suffering with HIV-1 as well as to prevent new infection which underscores the strong need for new drugs. Nef represents an important potential drug target because of its important role in HIV-1 pathogenesis and interactions with host cell signaling molecules. Recently, our lab identified several unique classes of small molecule inhibitors of Nef-mediated Hck activation and HIV-1 replication by screening a chemical library of more than 220,000 compounds. Here I extended these findings by showing that representatives of two compound classes, the DFP analogs and PPD-B9, also showed efficacy against all primary Nef proteins in HIV-1 replication

assays. To accomplish this goal, I first generated a series of HIV-1 NL4-3 chimeras expressing the representative *nef* alleles from each of the major clades by substitution of the endogenous Nef ORF in the wild-type NL4-3 provirus. These HIV-1 Nef chimeras replicated equally in MT2, U87MG CD4+/CXCR4+ and CEM-T4 cell lines. The DFP analogs inhibited replication of wild-type HIV-1, as well as all of the Nef- chimeras, by more than 75% at 3 μM in U87MG CD4+/CXCR4+ and CEM-T4 cell lines. Similar to DFPs, PPD-B9 also broadly inhibited replication of wild-type as well as each of Nef chimeras. PPD-B9 demonstrated even more potent activity than DFPs, inhibiting HIV-1 replication by more than 75% at a cellular concentration of just 1 μM. We next showed that HIV-1 infection results in activation of endogenous SFKs in CEM T4 cells in Nef-dependent manner. In addition to this, we also showed that both DFPs and PPD-B9 inhibited HIV-1 replication by blocking HIV-1-mediated activation of endogenous SFKS. These results support the conclusion that therapeutic targeting of Nef-dependent Src-family kinase activation may represent a broadly useful strategy against HIV-1.

The discovery of broadly active Nef-directed antiretroviral compounds such as the DFPs and PPD-B9 has great public health significance in terms of salvage therapy for people infected with current drug resistant HIV-1. Unlike other drugs which directly target the essential viral enzymes such as RT and protease, we have targeted a virus-host cell protein interaction which inherently reduces the chances of development of drug resistance. HIV-1 Nef not only enhances HIV-1 replication but also disturbs the host immune system. We expect that treatment with Nef-directed drugs will blunt HIV-1 pathogenesis, resulting in a response similar to LNTPs or macaques infected with Nef-defective viruses. My work strongly suggests that these compounds inhibit HIV-1 replication by blocking Nef-mediated SFK activation, further supporting the role

of this interaction in AIDS pathogenesis. These compounds should be further evaluated in terms of other Nef-mediated immune defects including downregulation of MHC class I molecules, chemokine co-receptors as well as other functions as described in next section. We expect that global blockade of Nef function by these inhibitors will be highly beneficial if added to HAART in terms of a better immune response to HIV-1 infection as well as reducing the risk of drug resistance to HAART.

Model of Nef-SFK signaling in HIV-1 pathogenesis

As described in detail in the Introduction, Nef is versatile adaptor protein that interacts with diverse cellular proteins. In particular, the PxxP motif of Nef is involved in binding and activation of SFKs. Saksela *et al*. were the first to demonstrate the importance of the PxxP motif in enhancement of HIV-1 replication in PBMCs (226). Among all cellular Nef-interacting partners with SH3 domains, Hck remains as the highest affinity binding partner for Nef (155,226).

In terms of downstream signaling pathways for Nef-SFK complexes, Nef expression in murine and human macrophages enhanced AP-1 DNA binding activity through activation of MAPK signaling pathway in PxxP-dependent manner. This activation of MAPK pathway was further associated with increased levels of c-fos and increased expression of the AP-1 responsive gene, tissue inhibitor of metalloproteinase-1 (TIMP-1) (23). This Nef-mediated activation of the MAPK pathway was blocked by expression of a dominant negative form of Hck, underscoring the importance of Nef-mediated Hck activation (23). Several studies have shown a role for the AP-1 signaling pathway in the induction of pro-inflammatory cytokines as well as enhanced expression from the HIV-1 LTR (112,166,262,284,285). Moreover, Nef expression in human

macrophages promotes survival through activation of STAT3 (34), although a role for Hck in this pathway has not been established. The Erk MAPK pathway is also activated by Nef in T cells in a Lck-dependent manner (275), although my work suggests that Lck must serve an indirect role as it does not interact with Nef directly. Moreover, Nef-mediated enhancement of HIV-1 transcription in T cells is also dependent on Lck activity (275,276). Finally, Nef expression in T cells causes transcriptional downregulation of the HIV-1 genome through induction of inhibitory factors like IL-2, IL-16 and the transcriptional regulator, YY-1 (241).

Nef has also been implicated in the high proliferation rate and loss of differentiation markers in podocytes in a murine model of HIV-1 associated nephropathy (HIVAN) (102,120,129). This renal phenotype was abolished by expressing the PxxP mutant of Nef but not by Hck knock out (104). Biochemical studies revealed that Nef activates c-Src and the STAT3 transcription factor in this system. Moreover, this study also showed that Nef activates the Ras-Raf-MAPK signaling cascade. This study revealed that the STAT3 and MAPK pathways are both activated in podocytes from the murine model of HIVAN as well as HIVAN patients. This effect is reversed by expression of dominant negative form of c-Src (110).

In conclusion, Nef-mediated SFK activation leads to activation of STAT3, AP-1, and MAPK signaling downstream which is ultimately responsible for enhancement of HIV-1 replication. Our study demonstrated discovery broadly active Nef inhibitors DFPs and PPD-B9 which inhibited HIV-1 replication in two different cell lines. Furthermore, we also demonstrated that these inhibitors block the HIV-1 mediated activation of endogenous SFKs in a Nef-dependent manner. These results suggest that both compounds inhibit Nef-proximal signaling pathways i.e. Nef-mediated SFK activation, which in turn leads to the activation of multiple signlaing pathways downstream that contribute to HIV-1 pathogenesis. This hypothesis needs to

be investigated in more detail by evaluating impact of these compounds on the downstream signaling pathways described above. These experiments will enable us to evaluate differential effects of DFPs versus PPD-B9 on Nef signaling. As described in Chapter 4, in addition to inhibiting Nef-mediated Hck activation, PPD-B9 is also a potential direct inhibitor of Nef oligomerization. Because it interacts directly with Nef, we predict that PPD-B9 will show broader specificity against Nef-mediated activation of cell signaling pathways compared to DFPs.

5.2 FUTURE DIRECTIONS

5.2.1 Evaluation of the activity of newly discovered Nef inhibitors in PBMCs

The present study reported the discovery of broadly specific HIV-1 Nef inhibitors which inhibited HIV-1 replication in cell lines (U87MG and CEM-T4) where HIV-1 replication is Nef-dependent. Similar to these continuous cell lines (U87MG and CEM-T4), others have reported that HIV-1 replication is Nef-dependent in peripheral blood mononuclear cells (PBMCs) as well as co-culture of T cells with macrophages or endothelial cells (46,178,236,245). This system may be closer to natural HIV-1 infection in the host where antigen presenting cells (APCs) have major role in activation of naïve T cells and enhancing HIV-1 replication. Several studies have shown the importance of *nef* gene in APC-mediated activation of T cells as well as enhancement of HIV-1 infection in $CD4^+$ cells (250,251). Therefore, it will be important to evaluate the effect of DFPs and PPD-B9 on HIV-1 replication in this system. Moreover, the development of DFP- and PPD-B9-resistant HIV-1 mutants will help us to understand the impact of evolutionary mutations in Nef on SFK signaling.

5.2.2 Evaluation of the efficacy of Nef inhibitors in animal model

In the present study we have shown that both DFP analogs and PPD-B9 are broadly active against HIV-1 expressing Nef from all major clades of HIV-1. This makes them potential candidates for therapeutic intervention for AIDS patients. To move forward in this direction, we

need to evaluate the pharmacokinetics (PK) and pharmacodynamics (PD) of these compounds in order to determine plasma half-life, dose as well as toxicity in a suitable animal model. The chimpanzee, cats (FIV) and macaques (SIV) are the closest animal models for AIDS in humans (79). In addition to these expensive animal models, Hanna *et al.* described HIV-1 and HIV-1 Nef-transgenic mouse model characterized by AIDS-like disease development (101,102). In addition to PK/PD study, these transgenic mice are suitable to evaluate the efficacy of these novel inhibitors in vivo. The efficacy of these compounds should be evaluated for interference with progression of AIDS-like disease by blocking Nef function before moving forward to non-human primate (SIV) models.

As mentioned in the Introduction, the present therapeutic regimen for AIDS is HAART which is composed of a combination of three or more drugs targeting different stages of the HIV-1 life cycle. HIV-1 develops drug-resistance due to its rapid replication rate (10^{10} virions produced per day) and high mutation rate (3×10^{-5} per nucleotide base per cycle). Our results showed that DFPs and PPD-B9 inhibited HIV-1 replication by more than 75% at low micromolar concentrations by targeting host cell signaling. It would be interesting to evaluate the impact of addition of these inhibitors to HAART on development of drug resistance in the simian AIDS model. I expect that inhibition of HIV-1 replication by Nef inhibitors will help to overcome the development of drug resistance to HAART. Similarly, a live attenuated vaccine produced by deleting the *nef* gene from SIV was very promising in controlling simian AIDS. So it would be interesting to evaluate the effect of these Nef inhibitors as chemical adjuvants in development of vaccines for AIDS.

5.2.3 Insights towards mechanism of action

The experiments presented in this study clearly indicate that DFPs and PPD-B9 inhibitors of Nef blocked HIV-1-mediated activation of endogenous SFKs as well as HIV-1 replication in a Nef-dependent manner. This observation leads to the new hypothesis that DFPs and PPD-B9-mediated inhibition of endogenous SFKs causes inhibition of HIV-1 replication. One way to validate this hypothesis is to use anti-sense or small interfering RNA specific for Hck and/or c-Src with the assay described in section 4.4.4. Additionally, one could express a constitutively active form of Hck and/or c-Src in HIV-1 target cells and evaluate the effect of these Nef inhibitors on HIV-1 replication. As Nef-SFK signaling has a major impact on signaling pathways in infected cells, the specific cell signaling pathways affected by these inhibitors is unknown. Several studies have shown that Nef expression results in secretion of cytokines by macrophages and activation of T cells (71,143,204,231,241,250,251) as well as enhancement of transcription from the LTR promoter (10,184,241,277). Similarly, studies have reported that activation of SFKs (Hck in macrophages, Lck/Fyn in T cells) is involved in macrophage (252,292) and T cell activation (195). Based on this information, the Nef-mediated gene expression profiling in the absence and presence of these inhibitors, especially in U87MG and CEM-T4 cells where HIV replication is Nef-dependent, will help us to obtain insights into which signaling pathways are affected by these novel inhibitors of Nef. Alternatively, a phosphoproteomics approach in a similar setting may reveal in broader terms the kinase pathways affected by these compounds.

Finally, Swingler *et al*. (251) described an assay to evaluate the paracrine effects of macrophages on T cell chemotaxis and activation as well as enhancement on HIV-1 replication in T cells. Using a similar system, the paracrine effect of culture supernatants from macrophages expressing Nef (in the absence and presence of these inhibitors) could be evaluated by assessing

the activation of T cells, chemotaxis of T cells, HIV-1 replication and transcription from the LTR. The results from these experiments will give us a clearer idea about the impact of these compounds on macrophage-mediated enhancement of HIV-1 replication in T cells.

5.3 CLOSING REMARKS

HIV-1, the human retrovirus responsible for AIDS, encodes several small "accessory factors" essential for viral growth and disease progression in infected individuals. One of these factors, termed Nef, promotes viral infection of HIV target cells, enhances viral replication, and allows HIV-infected cells to escape recognition by the host immune system. Nef works in part by binding to a variety of host cell signaling molecules and disrupting their regulation. The focus of my dissertation is a group of Nef-interacting partner proteins known as Src-family kinases (SFKs), which we have previously shown to be activated by Nef proteins derived from laboratory strains of HIV-1. In this study, we demonstrate that Nef proteins derived from all of the major subtypes of HIV-1 responsible for the global pandemic specifically bind and activate the same subset of SFKs. Moreover, we found that pharmacological inhibition of Nef-mediated SFK activation blocks the enhancement of HIV-1 replication supported by all of the Nef subtypes tested. These results are significant because they establish for the first time that host cell SFK signaling is activated by Nef variants examined from all clades of HIV-1, and that inhibition of this pathway may represent a broadly useful strategy to combat HIV/AIDS.

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