BIMOLECULAR FLUORESCENCE COMPLEMENTATION REVEALS THAT HIV-1 NEF OLIGOMERIZATION IS ESSENTIAL FOR CD4 DOWNREGULATION AND VIRAL REPLICATION

by

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HIV-1 Nef is a small myristoylated protein capable of interaction with a diverse array of host cell signaling molecules. Multi-faceted in its function, Nef is a critical accessory factor, essential for high-titer viral replication and AIDS progression. Despite its essential role, the molecular mechanisms of Nef-mediated HIV pathogenicity are not fully understood. Previous biochemical and structural studies have suggested that Nef may form homodimers and higher order oligomers in HIV-infected cells. The studies summarized below investigated the oligomeric status of the HIV-1 *nef* gene product and its role relative to Nef-mediated function.

We explored the formation of Nef oligomers in live cells by adapting a bimolecular fluorescence complementation (BiFC) assay, a well-defined system in which dimeric protein interactions are observed in live cells. Using this assay, we provided the first direct evidence for Nef oligomerization in vivo. We then assessed the generality of oligomerization by a group of Nef alleles broadly representative of all major HIV-1 subtypes and found oligomerization was highly conserved across all subtypes examined.

We then used our BiFC system to define residues previously suggested via X-ray crystallographic studies to comprise the Nef dimerization interface. Using a systematic strategy for the mutagenic profiling of the oligomerization interface, we discovered two classes of residues were critical to Nef oligomerization. BiFC was completely abolished when either all

four key hydrophobic interactions were simultaneously removed or when ionic interactions mediated by D123 and R105 were disrupted.

Finally, we utilized Nef mutants identified in the mutagenic profiling of the oligomerization interface to explore the effects of oligomeric disruption on Nef function. Screening a panel of Nef mutants with varying degrees of oligomeric disruption we discovered, surprisingly, despite the varying effects on oligomerization, all of these mutants were shown to dramatically disrupt Nef-induced CD4 downregulation and viral replication. Taken together, the studies presented in this dissertation advance the field of HIV research by furthering our understanding of the regulation of Nef-mediated downregulation of CD4 and enhancement of HIV replication as well as validating the Nef oligomerization interface as a potential target for anti-retroviral drug design.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

For more than a quarter of a century, a virus belonging to the genus *Lentivirus* in the *Retroviridae* family has plagued our world. Perhaps best recognized simply as the etiological agent of acquired immunodeficiency syndrome (AIDS), multiple names were suggested for the AIDS-virus: human T-lymphotropic virus-III (HTLV-III), lymphadenopathy-associated virus (LAV), and AIDS-associated retrovirus (ARV) (38). By 1986, the International Committee on Taxonomy of Viruses recommended giving the AIDS-virus its separate name: the human immunodeficiency virus, HIV(37). Since the initial report describing five young men suffering from a mysterious illness (1), over 60 million people have contracted HIV globally, of which over a third have since died from their illness (63). To date, over 270,000 HIV/AIDS-related reports have been published in the NIH National Library of Medicine PubMed database. Such reports describe the alterations of numerous intracellular pathways and host interactions upon infection with the HIV virus, all in order to promote survival of the virus and disease progression. Indeed, continued research into the molecular mechanisms of HIV-mediated disease and the discovery of solutions to inhibit those mechanisms is globally at the forefront of scientific investment.

1.1.1 Description of HIV: Virion Structure

HIV is a member of the lentiviral group of retroviruses, and as such maintains the general characteristics of all retroviruses with the exception of its cone-shaped core (Figure 1). Comprised of the viral p24 Gag capsid (CA) protein, the core measures 40-60 nm at its widest point and approximately 20 nm at its narrowest end (127). Within the core, the highly basic nucleocapsid (NC) protein, p7, interacts with the viral genomic RNA in a non-specific manner to provide protection from nucleases and compactness within the viral core. The myristoylated matrix (MA) core protein, p17, lies between the capsid and the envelope, providing structure to the inner portion of the viral membrane (81,82). The viral envelope, a bilayer membrane comprised of specific lipid domains derived from the host cell (8), contains two major viral glycoproteins, the heavily glycosylated surface (SU) protein, gp120 and the transmembrane (TM) protein, gp41. Derived from the precursor glycoprotein gp160, gp120 and gp41 mediate the initial stages of viral entry and in later stages, syncytium formation (70,152).

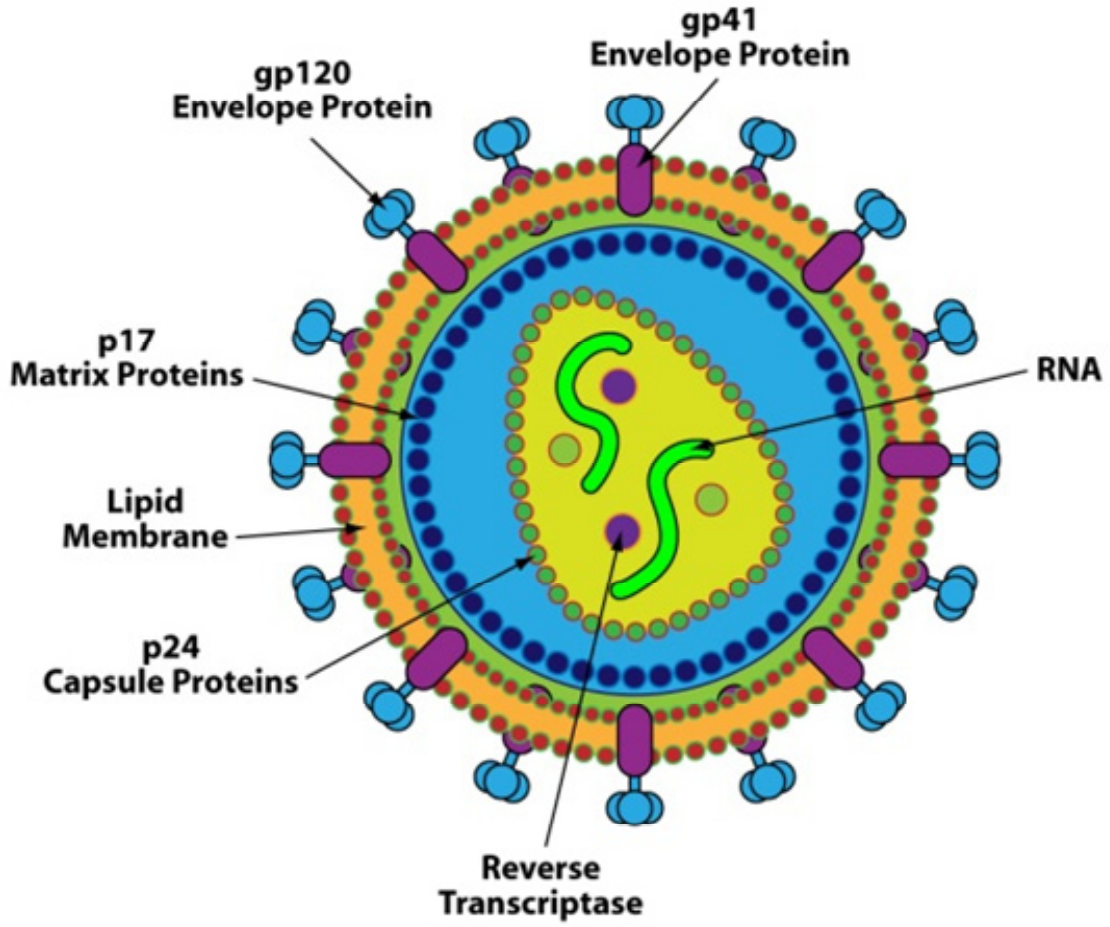


Figure 1. Virion Structure

An HIV virion with the structural proteins identified. The exact location of Nef in association with the core has not been well established. The abbreviated viral protein designations are those recommended (136).

1.1.2 Description of HIV: Entry & Replication

Viral entry requires the successful interaction of HIV with receptors on the host cell surface (Figure 2). Interaction of the viral glycoprotein gp120 with the cell surface receptor CD4 and a chemokine receptor (CCR5 or CXCR4) mediates viral attachment to the host cell and determines viral tropism. The interaction of these proteins induces the displacement of gp120,

leading to the uncovering of domains present on gp41 which then interact with phospholipid moieties promoting the process of viral fusion and entry (162,228). Subsequently, the viral capsid is uncoated and the viral genome and its associated proteins are injected into the cytoplasm where reverse transcription converts the viral RNA into a double-stranded complementary DNA (cDNA) molecule. Once transported to the nucleus, the newly synthesized viral cDNA is integrated into the host chromosome, a process involving the HIV integrase (IN) and other host cell factors (50,134). Upon initiation of cellular activation signals, the provirus is transcribed to create an array of viral transcripts, including the full-length viral RNA genome. Following splicing by RNAse H, viral transcripts are translated and processed to create functional viral proteins. Viral assembly concludes at the cellular membrane where immature virions are released from the cell via budding.

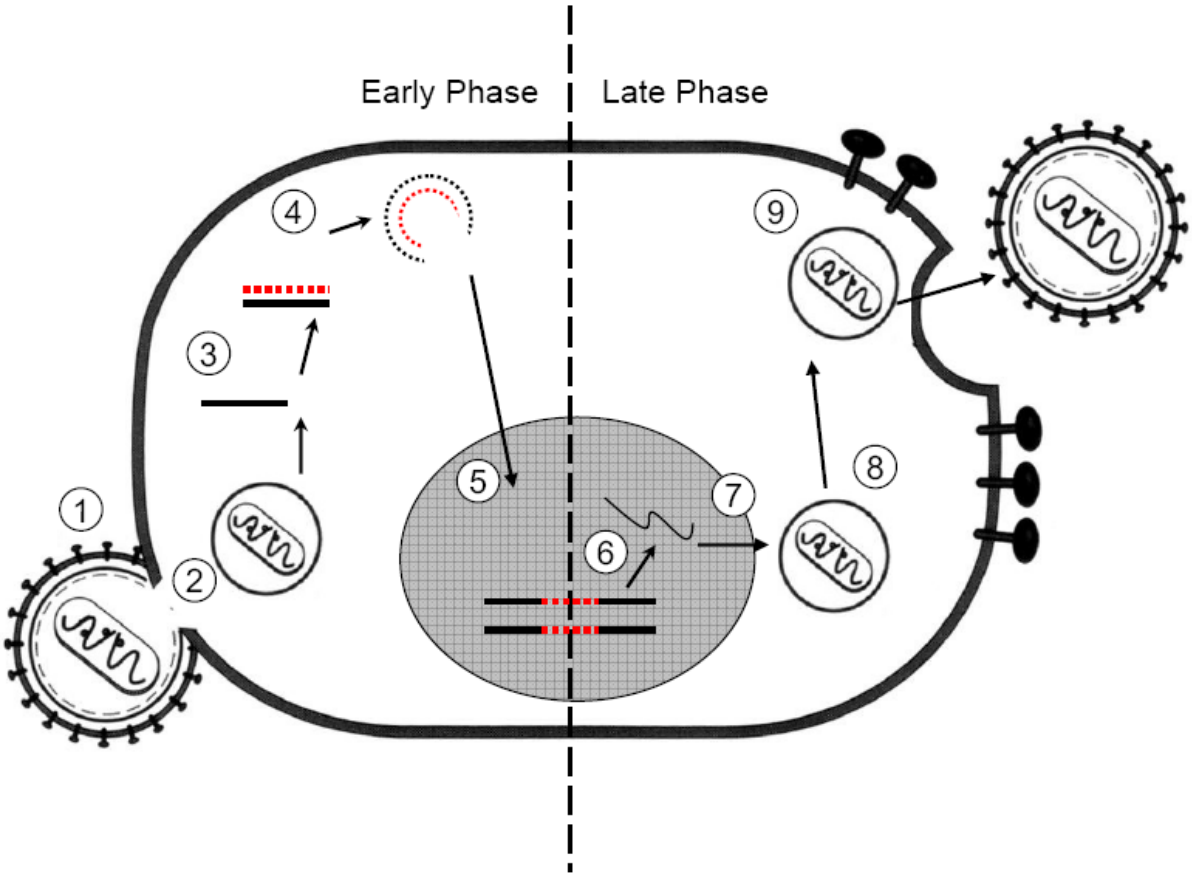


Figure 2. HIV Entry and Replication

The phases of the HIV-1 replication cycle may be generally divided into nine stages. (1) The early phase begins with viral attachment, quickly followed by fusion and entry (2) Uncoating of the viral core (3) Reverse transcription converts the single-stranded viral RNA genome into double-stranded DNA (4) Circularization, the preintegration complex (PIC) of viral and cellular proteins is transported to the nucleus (5) Integration of viral DNA into the host chromosomal DNA. (6) In the late phase of the cycle, transcription is initiated by RNA Pol II forming spliced and unspliced mRNA templates. (7) Translation produces accessory proteins and polyproteins (Gag and Gag-Pol) encoding structural and enzymatic proteins (8) Core particle assembly is initiated and (9) Final assembly and release of progeny virions. Figure adapted from (137).

1.1.3 Description of HIV: Genome

There are two forms of HIV, designated HIV-1 and HIV-2, of which HIV-2 shares 40-60% homology with HIV-1, the most prevalent strain. As a primate lentivirus, the genome of HIV is more complex than other retroviruses in that it encodes six auxiliary genes in addition to the three common lentiviral genes *gag* (group-specific core antigen), *pol* (polymerase) and *env* (envelope). These three genes, which are present in all replication competent retroviruses, encode well-characterized structural and enzymatic protein components essential for proper virus processing and assembly. The accessory genes of HIV-1 consist of two regulatory genes, *tat* (transactivation of transcription) and *rev* (regulator of viral protein expression) and four accessory genes *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein U) and *nef* (originally an acronym for "negative factor" but perhaps more appropriately "numerous effector functions;" see below). HIV-2 lacks the *vpu* gene, but contains a related gene, *vpx* (viral protein X). Thus, the HIV genome consists of nine overlapping genes, spanning approximately 10 kb, with open reading frames coding for each of these viral proteins (Figure 3, Table 1).

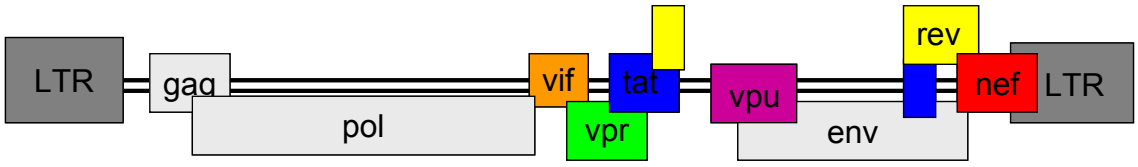


Figure 3. HIV Genome

The viral genome is flanked by two long-terminal repeats (LTRs) that contain promoter elements necessary for viral transcription. The auxiliary genes are highlighted. The studies presented in this dissertation focus on the pathogenic role of the *nef* gene, located at the 3' end of the genome (red). This figure is adapted from (72).

Proteins[1]	Designation(s)[2] and Size[3]	Function
Gag	p24	Capsid (CA), structural protein
	p17	Matrix (MA) protein, myristoylated
	p7	Nucleocapsid (NC)
	p6	Role in budding
Polymerase (Pol)	p66, p51	Reverse Transcription (RT): RNAse H
Protease (PR)	p10	Posttranslational processing of viral proteins
Integrase (IN)	p32	Viral cDNA integration
Envelope (Env)	gp120	Envelope surface (SU) protein
	gp41	Envelope transmembrane (TM) protein
Tat	p14	Transactivation
Rev	p19	Regulation of viral mRNA expression
Nef	p27	Pleiotropic, can increase or decrease virus replication
Vif	p23	Increases virus infectivity
Vpr	p15	Virus replication, transactivation
Vpu	p16	Virus release

Table 1. HIV Proteins

[1]See Figure 3 for location of viral genes on the HIV genome

[2]See Figure 1 for location of structural proteins within virion

[3]Numbers in designations are sizes, in kilodaltons (kDa)

This figure is adapted from (137).

1.1.3.1 HIV-1 Structural Proteins

As noted above, the primary morphology of HIV is determined by three main structural genes, *gag*, *pol* and *env*. Each of the three structural genes undergoes a proteolytic cleavage event, generating multiple viral proteins. The primary transcript of HIV is a full-length viral mRNA, from which the Gag and Gag-Pol polyprotein precursors are derived. Within the nascent viral particle, the Gag precursor gives rise to p24 (CA), p17 (MA), p7 (NC), p6 (Proline-rich) and two smaller spacer polypeptides p2 and p1. Approximately 2000 Gag precursors are packaged within the virion to ensure that a sufficient quantity of structural proteins are present during assembly (81,159). The Pol region of the precursor encodes three essential viral enzymes, protease (PR), reverse transcriptase (RT), and integrase (IN). The *pol*-encoded enzymes of Gag-Pol are made in relatively small quantities compared to the structural proteins of Gag. The Gag and Gag-Pol products are synthesized in a ratio of about 20:1 to ensure controlled activation and proper localization within newly formed virions (116). Prior to viral assembly, the surface and transmembrane envelope proteins, gp120 and gp41 are made from the singly spliced Env precursor gp160. Proteolytic cleavage of gp160 is mediated by the cell host enzyme furin, an endoprotease (97).

1.1.3.2 HIV-1 Auxiliary Proteins

As a complex retrovirus, HIV also encodes a variety of regulatory and accessory proteins. Although the regulatory proteins are essential for viral replication, the accessory proteins were initially considered dispensable for replication (48,115). However, studies have since shown that

accessory proteins contribute to the maintenance and even enhancement of efficient viral replication and pathogenesis in vivo (57,86).

The regulatory protein, Tat, is a potent transcriptional activator of the 3' portion of the viral long terminal repeat (LTR) promoter element and is involved in up-regulating HIV replication from the provirus—the integrated form of the virus. Tat acts mainly at the level of transcriptional elongation (46,47). The second regulatory protein, Rev, is a sequence specific nuclear export factor. Rev, in association with other cellular host proteins, facilitates the entry of unspliced mRNA into the cytoplasm where production of full-length viral proteins needed for virion formation occurs (51,64,201). Thus, both Tat and Rev are RNA binding proteins that interact with cellular factors to influence and optimize viral replication.

The HIV accessory proteins affect numerous stages of viral production ranging from cell activation, assembly, cell cycling, budding and infectivity. Vif influences viral infectivity and proviral DNA synthesis in infected cells (212). Recent reports have suggested Vif enhances infectivity through its interaction with and degradation of the antiviral cytidine deaminase protein, APOBEC3G (153,196). In the absence of Vif, the incorporation of APOBEC within viral particles inactivates viral replication during the post-entry phase of infection by causing extensive editing of the newly synthesized viral genome. Produced late in virus replication, Vpr mediates the nuclear transport of the preintegration complex (PIC). Vpr also arrests cell proliferation in the G2 stage of the cell cycle where it transactivates the LTR of the integrated viral DNA and consequently increases virus production (66,80,167). Vpu enhances virus replication at the level of virion release from infected cells. Vpu lowers the surface expression of CD4 by initiating the rapid degradation of CD4 following its synthesis in the endoplasmic reticulum. Continual expression of CD4 on the cell surface, in the absence of Vpu, can reduce

viral particle formation up to five-fold (23,219). Finally, Nef is perhaps the most multi-faceted accessory factor, capable of interactions with a diverse array of host cell signaling molecules. Ranging from the establishment of infection upon viral entry to AIDS progression, the role of Nef in HIV pathogenesis will be described later in more detail (see section 1.3).

1.1.4 HIV Subtypes

Sequence comparisons among HIV-1 and HIV-2 isolates revealed extreme envelope diversity, as well as striking similarities among viruses. Based on full-length viral genome sequencing there are currently three designated HIV-1 groups: M (main), O (outlier) and N (non M or O). Eight HIV-2 groups have also been identified (173). The most widespread of all HIV groupings, nine HIV-1 subtypes (clades) have been identified within the M group and are designated A, B, C, D, F, G, H, J and K (163,178). As an independent clade, each subtype differs in amino acid composition by at least 20% in the envelope region and 15% in the Gag region. Altogether, the groups display greater than 25% nucleotide sequence variation in the Env and Gag regions and, as defined, are approximately equidistant from each other (Figure 4). The HIV-1 M groups also distinguish the prevalence of infection on a global scale. Whereas subtype B is found primarily in North and South America and Europe, subtypes A, C, D and E are most common in the developing world (Figure 5). However, recombinant viruses emerge frequently in populations where multiple clades are present. Over time and with growing prevalence, these recombinant forms (CRFs) are recognized as an epidemiologically important lineage. Currently, there are 16 established CRFs derived from the group M HIV-1 isolates (11,163,178).

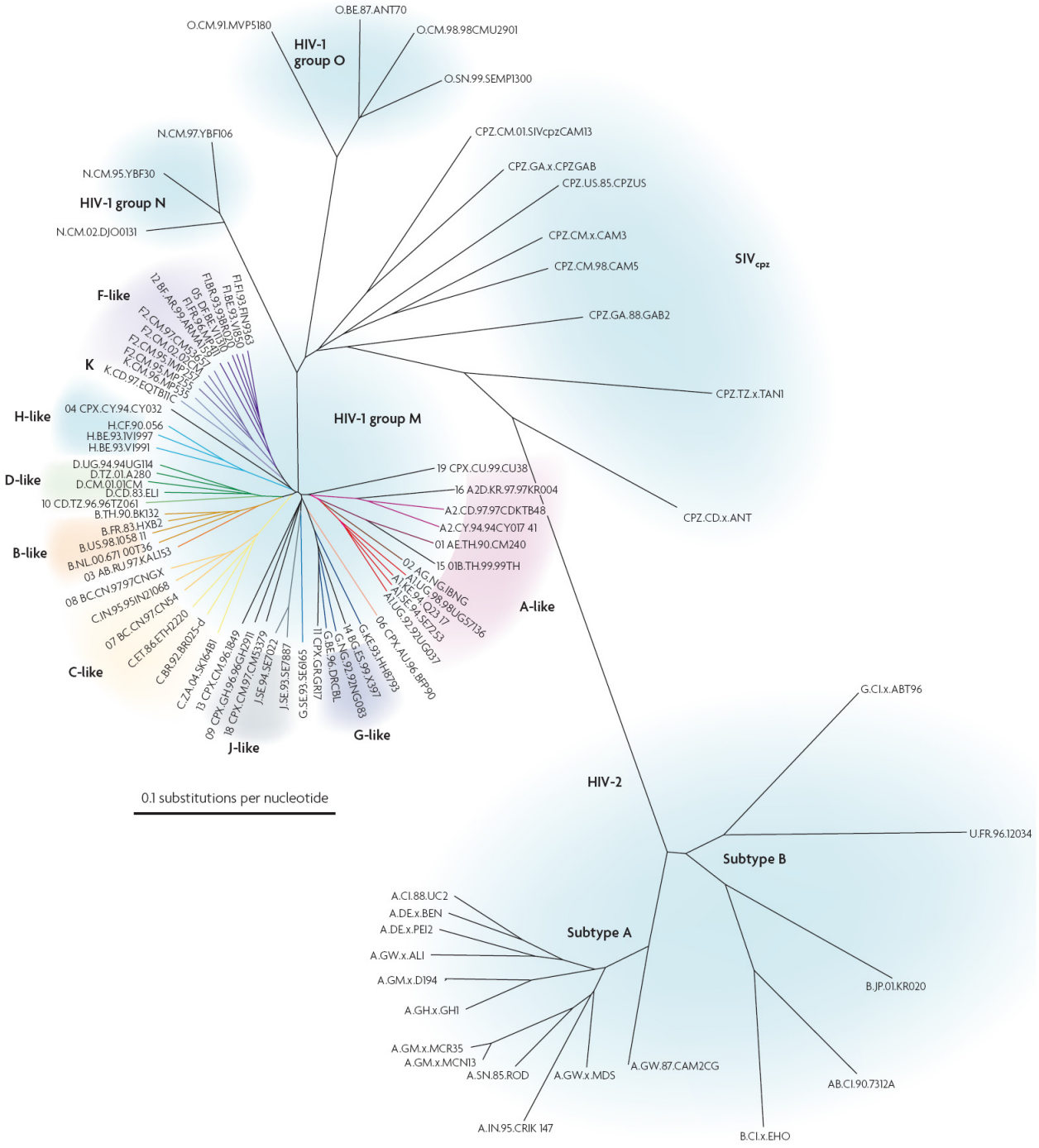


Figure 4. Phylogenetic Tree of Human and Simian Lentiviruses

The genetic similarity between different HIV and simian immunodeficiency virus (SIV) strains was compared by aligning the full genome sequences. HIV-2 and HIV-1 share only 50–60% sequence identity and cluster at distinct locations on the phylogenetic tree whereas SIVcpz branches out from the root of the HIV-1 groups. The distances between main (M) group HIV-1 subtypes are highlighted as shown. This figure was adapted from (11)

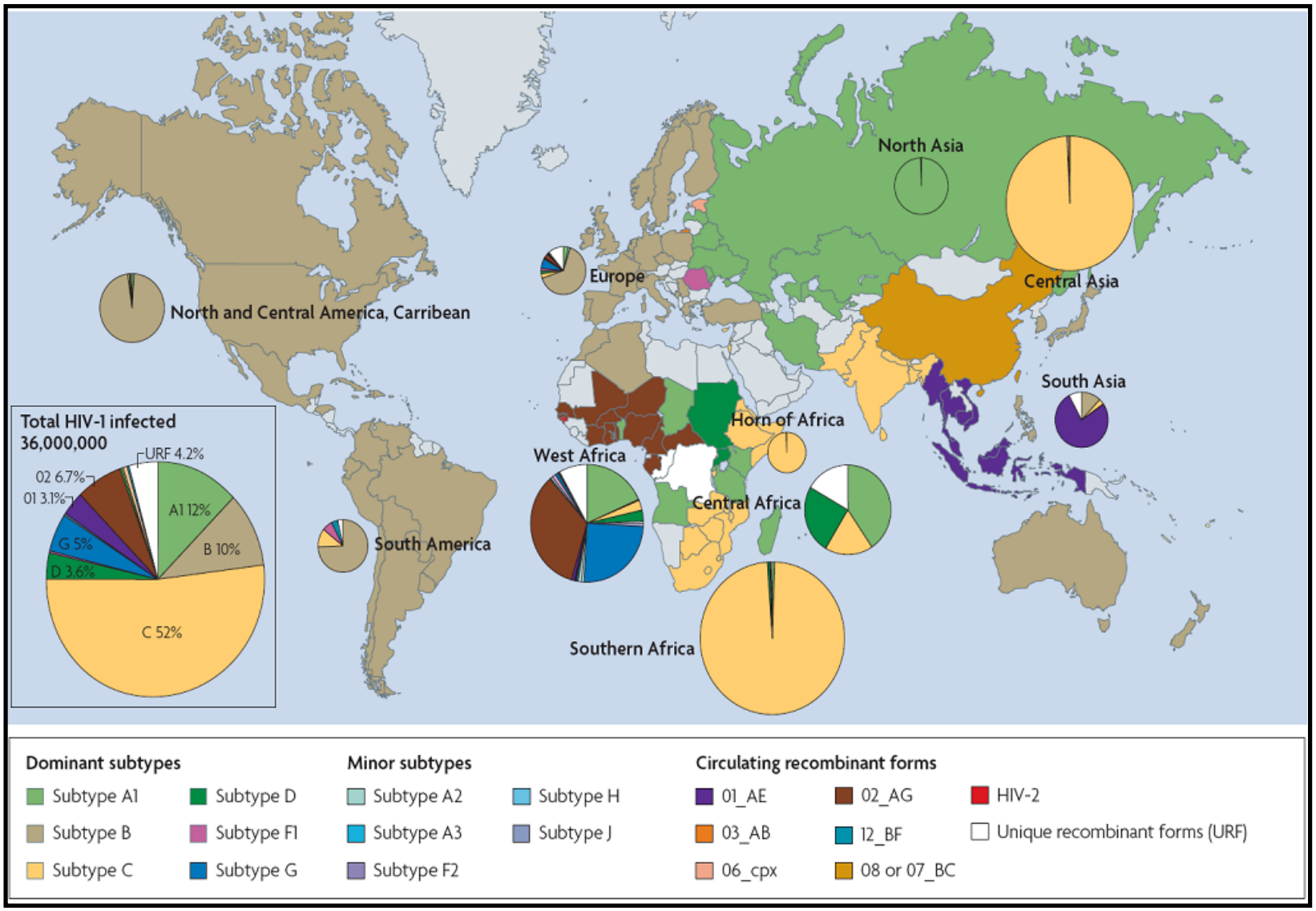


Figure 5. World Map of HIV Diversity

The countries are color-coded based on the dominant HIV-1 group main (M) subtype. The pie charts depict the proportion of each subtype or recombinant form in each geographical region. The size of the pies is proportional to the number of HIV-1 infected individuals in that particular region. This figure was adapted from (11)

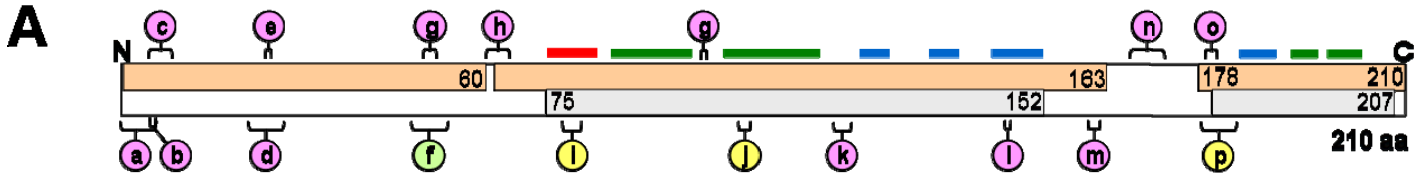
1.2 HIV-1 NEF CHARACTERISTICS

The HIV-1 *nef* gene product is a ~ 27 kDa protein (the largest of the auxiliary factors) responsible for numerous critical functions during viral infection and pathogenesis (7,49,53,89). Located at the 3' end of the *env* gene and partially overlapping the U3 region of the 3' LTR, *nef* is abundantly expressed during the onset of infection and localizes to the plasma membrane via myristoylation at its N-terminus (103,118). In addition, a cholesterol recognition motif at its C-terminus may also target Nef to cholesterol-rich regions of the plasma membrane, including lipid rafts (233). Nef may also be present in the cytosol, trans-Golgi network (TGN), and nucleus and is incorporated into the virion of newly formed viral particles [reviewed by (69,76)]. In particular, the *nef* gene product is responsible for an ever-growing list of viral functions essential for HIV pathogenesis.

1.2.1 Key Recognition Motifs

Nef lacks intrinsic enzymatic activity, and it is therefore presumed to exert its effects via binding to multiple host cellular proteins. Well conserved among HIV-1 subtypes, the *nef* gene encodes an accessory protein containing numerous well-defined recognition motifs (Figure 6). Perhaps not surprisingly, most of the numerous motifs critical for binding to various cellular proteins are located within the stable core domain of Nef. Sequence alignments of the HIV-1 Nef protein from a total of 186 different strains from varying subtypes revealed an unexpectedly high conservation (77). The degree of sequence conservation also revealed that the Nef protein contains several conserved, well-described sequence motifs implicated in binding to distinct

intracellular targets: the myristoylation motif, $MGxxxS_1$; the acidic region, $E_{62}EEE$ (PACS-1); the dileucine motif, $L_{164}L$ (CD4, AP-1/2/3); the proline-rich motif, $P_{72}xxP$ (SH3 domains from multiple targets); and the diarginine motif $R_{105}R$ (PAK1/2) (Figure 6) (15,77). Indeed, the *nef* gene product is capable of binding to an ever-growing list of cellular targets within the host-infected cell (53,68).



B

	Residues	Sequence	Binding partner / function	Reference(s)
a	1-6	MGxxxS(T)	Consensus sequence for myristoylation - essential for CD4, MHCI, MHCII modulation	[Harris 1995]
b	6	S	Phosphorylation, subcellular localization	[Wolf et al. 2006]
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	FPD	thioesterase binding; Nef dimerization	[Liu et al. 2000]
l	144	K	CD4 downregulation via ubiquitin	[Jin et al. 2006]
m	154,155	EE	beta-COP binding; lysosomal targeting	[Piguet et al. 1999]
n	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]
o	174,175	DD	Vacuolar ATPase binding; CD4 downregulation; AP2 binding	[Geyer et al. 2002; Lindwasser et al. 2007]
p	174-179	DDPxxE	c-RAF1 kinase binding	[Hodge et al. 1998]

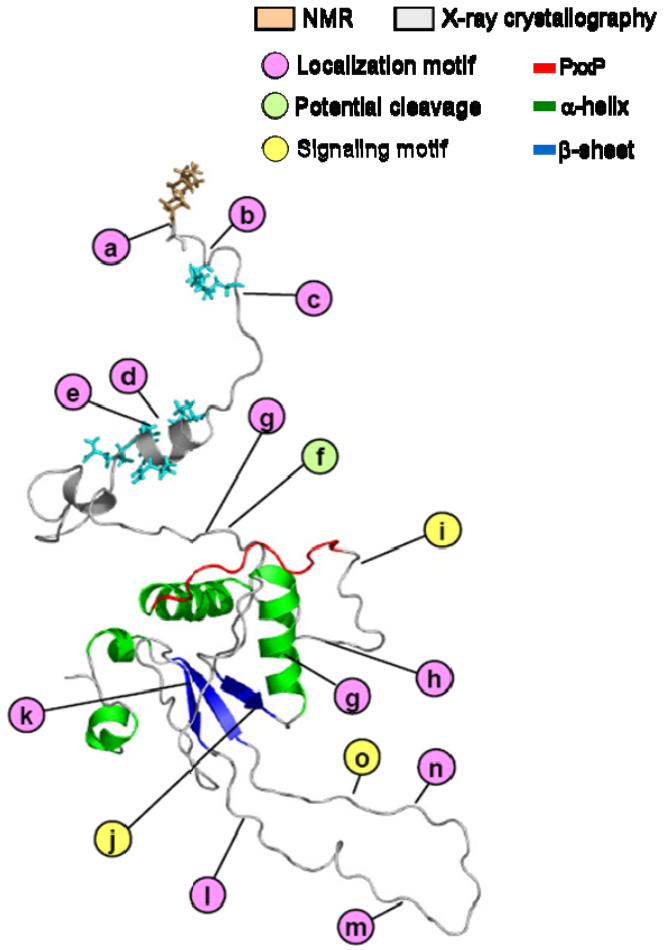


Figure 6. Key Recognition Motifs of Nef

(A) A linear representation of the Nef coding region summarizing the regions of Nef examined by various biochemical and structural assays (color-coded). (B) Summary table of Nef recognition motifs corresponding to the regions highlighted on the crystal structure (right) and the numerous interactions and functions of Nef that have been previously identified. Figure courtesy of Dr. John Engen, Northeastern University.

1.2.2 Structural Biology of Nef

Although it has not been possible to crystallize the full-length Nef protein, structural information on key Nef regions has been obtained through NMR spectroscopy and X-ray crystallography. Crystallization of the HIV-1 Nef core domain, complexed with the Src homology 3 (SH3) domain of the Src family kinase Lyn, revealed that the core region of Nef is highly stable, with several well-conserved motifs accessible on the outer surface of the protein (12,133). Using magnetic resonance (NMR) spectroscopy, the solution structure examined the N- and C- terminal domains and confirmed the stability and modeling of the Nef core in association with various binding partners (93-95). Data obtained from these studies show that the N-terminal region of Nef consists of a type II polyproline helix (aa 70-77), which contains the main binding site for the SH3 domain of Src family kinases. Following this domain are two anti-parallel α helices (aa 81-120, designated αA and αB respectively), which are kept in close proximity by a four-stranded anti-parallel β sheet (aa 121-186). The flexible C-terminal tail region consists of two short α helices (aa 187-203). Forming flexible solvent-exposed loops are regions within the N-terminal lobe (aa 60-71) and C-terminus (aa 149-177). The structural characteristics of the Nef protein may prove advantageous for eliciting multiple functional associations, as will be discussed later in more detail.

1.2.3 Myristoylation

Post-translationally modified by the irreversible attachment of the 14 carbon saturated fatty acid myristic acid, myristoylation targets Nef to the plasma membrane (96,118). Nef contains a highly

conserved Met-Gly-X-X-X-Ser/Thr consensus sequence, where covalent linkage of myristic acid occurs via an amide bond to the N-terminal glycine at the Gly 2 position. Once the initial methionine is cotranslationally removed by methionine amino-peptidase, the soluble enzyme N-myristoyltransferase (NMT) mediates the addition of myristic acid. This process, however, is not unique to Nef as this posttranslational modification is common in both viral and cellular proteins also found in the plasma membrane. This includes all members of the Src kinase family, which are also important binding partners for Nef [reviewed by (175,176)].

Mutation of the N-terminal glycine to alanine greatly reduced or prevented membrane binding, indicating the requirement of Gly 2 for myristoylation-mediated membrane association (102,175). Nef mutants lacking this posttranslational modification were unable to bind critical membrane and intracellular proteins such as the HIV receptor, CD4 (105); a component of non-clathrin coated vesicles, β-COP; the Src family kinase, Lck (102); the cytoskeletal protein, actin(60) and numerous other intracellular proteins (104). These studies indicate that myristoylation is not only critical for membrane targeting of Nef but is also for interaction with multiple partner proteins. Further, the addition of a myristoyl group to the amino terminus of Nef is critical for virtually all of its biological activities (4,35,187) as will be discussed later (see section 1.3).

1.2.4 Oligomerization

At a time when the biological importance and molecular mechanisms of Nef-mediated effects were still ambiguous, multiple studies examined the biochemical and biophysical properties of the Nef protein in an attempt to reveal structural characteristics or associations that may be

significant for its function. Examination of the amide composition of Nef initially suggested a conserved leucine zipper-like repeat may be present in the core region (185). Later expressed in bacteria and eukaryotic cells, studies with the recombinant protein suggested that Nef was able to form homo-oligomers, on the order of dimers, trimers and tetramers, via intermolecular disulfide bonds between cysteine residues of Nef (122). NMR data from the same study also suggested non-covalent interactions between Nef oligomers, stabilized mainly by polar or electrostatic interactions, can occur even under strong reducing conditions. Subsequent studies by Fujii et al. further supported the presence of non-covalently associated Nef oligomers at both the cell surface and cytoplasm of infected CD4+ HeLa cells (71). A detailed description of the secondary and tertiary structure of Nef, via NMR, further supported homo-dimerization of the Nef core domain (94). However, counter to previous studies, Nef oligomers were observed under both reducing and non-reducing conditions, suggesting the formation of an oligomeric Nef complex occurs without the stabilization of disulfide bonds. In support of the presence of Nef oligomers, crystallization of the Nef core further revealed a prominent solvent-accessible hydrophobic crevice suggestive of a binding target which, if disrupted, may prevent interaction with other molecular partners, including additional Nef proteins (133). In support, the crystal structure of the Nef core solved by Arold et al. demonstrates an interface buried between adjacent αB helices of Nef molecules may provide the structural basis for oligomerization (12).

In perhaps the most extensive study on the molecular basis of Nef oligomerization, Arold et al. further analyzed the self-associating properties of HIV-1 Nef by chemical cross-linking, dynamic light scattering, equilibrium analytical ultracentrifugation and NMR spectroscopy (13). Using a Nef core mutant Δ206, where C206 is the only solvent-accessible cysteine, chemical cross-linking experiments supported the notion that the formation of Nef oligomers did not

required disulfide bridges. These studies also revealed that the oligomeric state of Nef is concentration dependent, with the most prevalent forms being monomers and dimers, while less frequently observed were trimeric complexes. In both the dimeric and trimeric arrangements, the principal homologous contacts are established by: R105, D108, I109, L112, Y115, H116, F121, P122 and D123. Highly conserved among HIV-1 isolates, these residues comprise a hydrophobic core, centralized around Y115 and F121 (I109-P122), flanked by electrostatic interactions between R015 and D123 (Figure 7). Further, the oligomeric configuration of Nef did not interfere with the recruitment of the SH3 domains (12,133), CD4 (95), or MHC-I (85), suggesting a structure-function role for Nef oligomerization in vivo. In support, studies by Liu et al. suggest that mutation of the highly conserved residue D123 not only disrupted Nef dimerization, but also Nef-mediated interactions with various cellular proteins (139). However, the biological significance of Nef oligomerization within HIV infected host cells remains to be determined. Indeed, as suggested by the X-ray crystal structures, the intermolecular contacts between the αB helices comprise the Nef dimer interface. This region was a major focus of my dissertation as a requisite for Nef function.

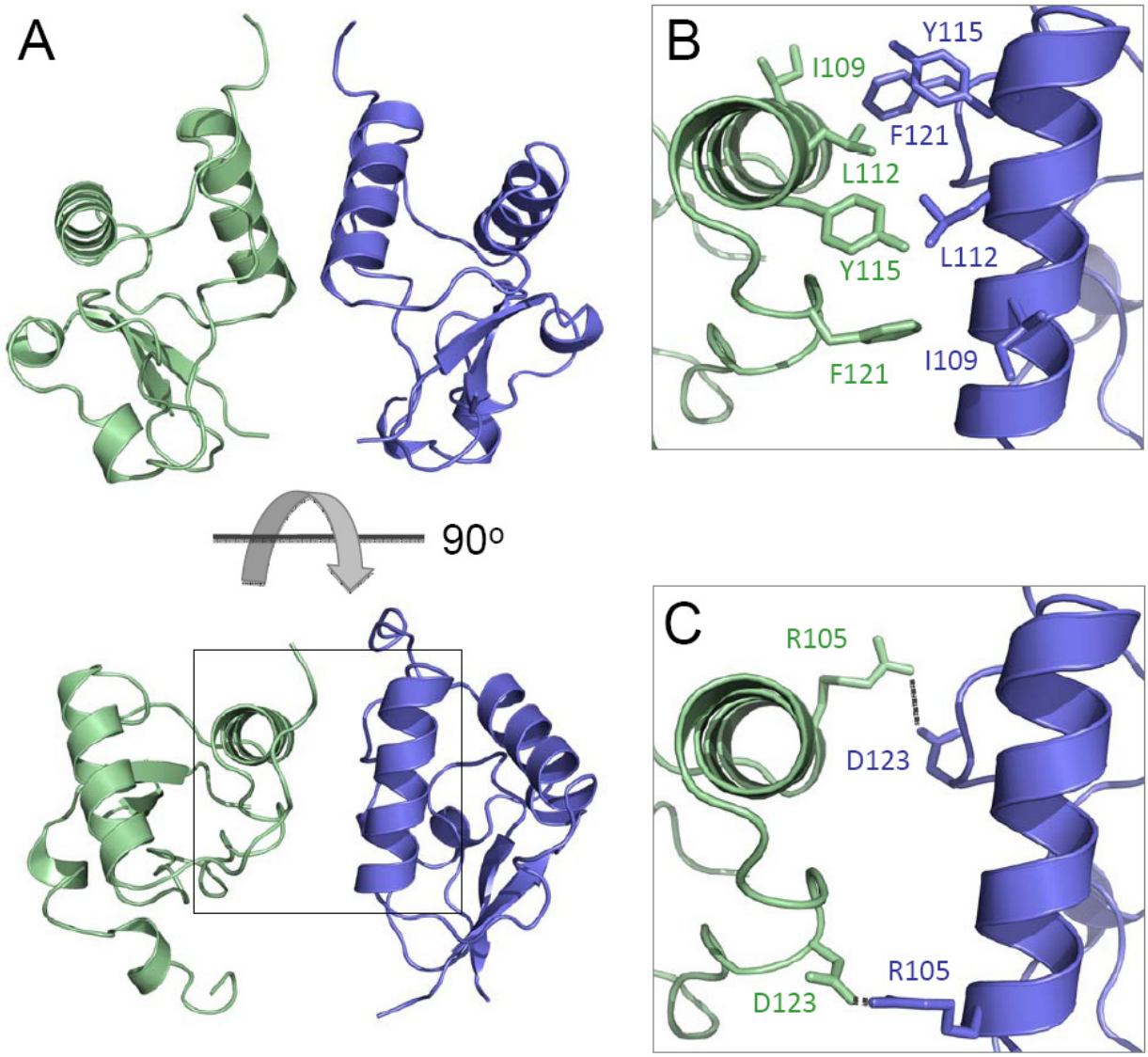


Figure 7. Crystal Structure of the Nef Dimerization Interface

Two views of the crystal structure of the dimeric Nef core. (A) The dimerization interface between the αB helices of two Nef proteins. The boxed region at the bottom indicates the orientation of the enlarged dimerization interface. Close-up views of the dimer interface formed by the αB helix, showing key (B) hydrophobic residues and (C) the reciprocal ionic interactions between Asp 123 and Arg 105. These models were produced using the X-ray crystal coordinates of Lee, et al. (PDB 1EFN) (133).

1.3 ROLE OF NEF IN HIV INFECTION

1.3.1 Initial Findings

To date, the characterization of the functional roles of Nef has expanded into numerous aspects of viral pathogenesis. Initially, mutation or deletion of the coding region of *nef* indicated that the 3' ORF protein product was an inhibitor of viral pathogenesis that decreased viral transcription and replication in culture (2,65,143,204). In a complementary approach, mutation of this region resulted in a significant increase in viral replication further suggesting an inhibitory role, leading to the name *ne*gative *f*actor, or Nef (72). However, subsequent studies soon after failed to confirm the alleged negative effects, and a significant amount of evidence has since accumulated that suggests a strongly positive effect of Nef on viral replication and infectivity (78,98,123,154,156). Among these studies, three key pieces of evidence have emerged to support a role for Nef as a critical determinant in AIDS pathogenesis. These are described in more detail in the next section.

1.3.2 Evidence of a Positive Effect

Primates

Utilizing the SIV rhesus macaque model and molecular clones of SIV containing either wild-type or mutated *nef* sequences, a positive effect of *nef* on disease progression was documented.

In these studies, Kestler et al. demonstrated macaques infected with SIV present with an AIDS-like phenotype that mimics human disease, including key features such as profound CD4+ cell depletion, opportunistic infections, wasting and early death (120). In contrast, macaques infected with a *nef*-deleted strain of SIV demonstrated 1000-fold lower viral loads and, most importantly, failed to develop disease (121). Multiple studies have since confirmed that the targeted disruption of the SIV *nef* gene, in the context of an otherwise intact virus, renders the virus non-pathogenic in rhesus macaques (52,226,227). Interestingly, mutant clones of SIV carrying a premature stop codon in the *nef* gene were shown to have reverted, in vivo, to the wild-type functional coding sequence (31,121,218). The restoration of both function and viral pathogenicity indicates a strong selective pressure for maintaining a functional *nef* gene and further supports the initial evidence of Nef as a critical factor in disease progression.

Mice

In a model system established by Hanna et al. transgenic (Tg) mice were engineered to express the entire HIV-1 coding sequence under the control of a hybrid human-mouse CD4 (CD4C) promoter. This promoter targets the expression of viral genes to cells susceptible to HIV infection, namely immature CD4+ T cells and cells of the monocyte/macrophages lineage (99). Analogous to results reported by Kestler et al. (120) using the monkey model, these Tg mice also developed a severe AIDS-like disease with immunodeficiency, loss of peripheral CD4+ T-lymphocytes, weight loss, wasting and early death. Confirming the results of the rhesus macaque model, Hanna et al. revealed that disruption of the *nef* ORF, in the context of an otherwise intact virus, also resulted in a non-pathogenic infection in Tg mice (100). Moreover, in a reciprocal experiment, the same disease phenotype was elicited in Tg mice by expressing only the *nef* gene,

thus suggesting that the mouse model provides strong evidence that Nef alone is a major determinant for HIV-1 pathogenicity.

Humans

In the years following the initial reports describing what would later become known as HIV/AIDS, a subset of HIV-infected patients were documented as having been asymptomatic for a period of greater than 10 years—the average time span in which most untreated patients succumb to AIDS (182). Referred to as long-term non-progressors (LTNPs) and comprising less than .00003% of seropositive (HIV+) individuals, these patients show no signs of HIV-related disease or decline in CD4+ T cell counts despite >10 years of documented HIV infection (28). Interestingly, viruses isolated from some patients with long-term nonprogressive infection were shown to contain defective forms of Nef (54,124). Numerous reports have since described viruses recovered from LTNPs that contain deletions or mutations with the *nef* gene (74,131,148,206,216). However, recent reports indicate some of the earliest-described LTNPs are beginning to show signs of disease, suggesting that *nef* deletions are not completely protective against the progression of HIV disease (36,87,131).

1.3.3 Nef-Mediated Cell Survival Promotes HIV Propagation

Programmed cell death (apoptosis) has been recognized for many years as a strategy employed by the host immune system to defend against viruses. Successful viral replication is therefore dependent on the ability of HIV to evade immune surveillance and circumvent host self-

destruction. Nef enhances cell survival by specifically blocking or subverting multiple cellular apoptotic pathways within the infected cell. Specifically, Nef prevents p53-mediated apoptosis through direct interactions with this tumor suppressor protein, thereby resulting in its destabilization and subsequent degradation (90). Subsequent studies also showed Nef blocks Bad-mediated mitochondrial apoptotic signaling (223). Induced upon HIV infection, Bad is a pro-apoptotic member of the Bcl-2 protein family comprised of pro- and anti-apoptotic heterodimers (92). Phosphorylation of the Bad protein, initiated via Nef-mediated activation of phosphatidylinositol 3-kinase (PI3K) and the Nef-associated kinase, PAK, results in its inhibition and consequently an increase in anti-apoptotic signaling (223). Nef also induces a survival signal through the Erk/MAPK pathway in macrophage lineages, resulting in increased expression of the anti-apoptotic factor Bcl-X_L (34). In T cells, Nef binds and inhibits apoptosis signal regulatory kinase (ASK1), a key intermediate of both the CD95/CD95L (Fas/FasL) and the tumor necrosis factor-alpha (TNF-alpha) pro-apoptotic pathways (75).

In order to promote adequate viral replication, Nef also enhances host survival by protecting the infected cell from external factors through multiple cellular mechanisms. Nef induces upregulation of the death factor FasL on the surface of the infected cell (230). FasL then binds to Fas (CD95) receptors prevalent on nearby immune cells, including HIV-specific cytotoxic T cells (CTL), and induces their apoptosis (157). In the absence of an apoptotic induction, activated CD8+ T cells (CTL) bind infected cells expressing viral antigen presented on major histocompatibility complex (MHC-I) receptors, inducing apoptotic signaling via the targeted release of lytic granules and/or the induction of the Fas/FasL pathway, as described above. However, HIV-1 infected cells are protected against CTL-mediated targeting, a phenomenon dependent on the presence of the Nef protein (40). Further, a 40% reduction in

surface expression of MHC-I was found to render CEM-E5 cells less susceptible to recognition and subsequent killing by CTLs (189). Although the protective effects of Nef-mediated MHC-I downregulation is clear, the precise mechanism and host interactions required to elicit and maintain a protective response remains controversial. In a series of experiments, Nef promoted only retrograde transport of MHC-I molecules, via a PACS-1 and PI3K dependent pathway, away from the cell surface and to the trans-Golgi network (21,194). Contrary to previous reports, work by Collins et al. suggests that Nef-mediated MHC-I downregulation is based on the ability of Nef to target newly synthesized MHC-I from the TGN to lysosomes, a process independent of PI3K but rather dependent on interactions with the μ1 subunit of adaptor protein (AP-1) (179,220). Disputing the latter evidence, Hung et al. note that many T cell lines, including those used in the previous study, lack a functional PIP_3 phosphatase (PTEN), resulting in the inability to constitute the Nef/PACS-1/PI3K pathway required for the removal of MHC-I from the cell surface (111). Despite the uncertainty of specific Nef-host cell interactions required for MHC downregulation, further support for a Nef-mediated protective effect was provided by comparison of MHC-I levels of HIV+ individuals with varying CTL responses. Data obtained revealed the MHC-I downregulatory function of Nef was directly proportional to the breadth of CTL response (138). However, the downregulation of MHC-I is not the only strategy elicited by the HIV-1 Nef protein to ensure host survival.

Nef also promotes the downregulation of CD4, a cell surface transmembrane glycoprotein expressed on the surface of helper and regulatory T lymphocytes, thymocytes and macrophage/monocyte lineage cells. Loss of CD4 surface expression is a highly conserved attribute of Nef, as observed in natural isolates of both HIV and SIV Nef proteins (9,19,73,149). As the primary cellular receptor for HIV and SIV, Nef downregulates CD4 by directly binding

and linking it to both the AP-2 sorting protein and the COP-1 lysosomal targeting protein (18,55,84,164). Indeed, numerous assays support the formation of Nef-CD4 complexes with endocytic machinery via direct interactions (95,105,171,181). Specifically, the N-terminal region proximal to the core of Nef (residues 57-59) binds to the dileucine motif located in the cytoplasmic tail of CD4 (4,95). Once internalized, CD4 molecules accumulate in early endosomes until they are subsequently degraded in late/acidic compartments (4,195). Failure to adequately lower CD4 surface expression during the course of HIV infection may induce proapoptotic signals delivered through CD4, trigger the inhibition of viral transcription, or even elicit an HIV-specific immune response (117,128). Nef-induced removal of CD4 from the cell surface, perhaps most importantly, ensures survival by preventing the detrimental cytopathic effects of superinfection by other HIV virions (19,32). Furthermore, the protective effect of Nef via CD4 downmodulation augments viral replication. As CD4 binds the HIV Env receptor gp120, removal of CD4 from the surface of an infected cell prevents the sequestering of budding progeny virus thus increasing viral egress and HIV replication (23,168,180). Cell lines engineered to overexpress a modified CD4 receptor resistant to Nef-mediated downregulation show reduced support of HIV replication, further demonstrating the importance of CD4 downregulation (41,150). Additionally, the targeted degradation of CD4 by Nef enhances viral infectivity by altering the composition of newly formed virions, increasing the availability and incorporation of Env protein receptors required for host-entry (129,180).

1.3.4 Nef Optimizes the Cellular Host Environment for Replication

In addition to enhancing survival of the infected cell, Nef optimizes the host cellular environment to promote adequate viral replication and infectivity. To this end, a large and growing number of studies suggest Nef manipulates multiple signaling pathways, inducing a series of biochemical events ultimately resulting in cellular activation. Activation promotes gene expression within the host cell, increasing the availability of essential host cell factors utilized by HIV for highly efficient viral replication. Thus, as more virus particles are being produced and released, viral infectivity is accordingly increased. As a requirement for efficient viral replication within the host cell (232), increased activation of T cells has been shown to be predictive of disease progression (107). As such, Nef plays a role in lowering the threshold for T cell activation, which may promote viral integration and the initiation of early transcription (191,225). The implication of Nef-associated cellular activation to promote viral production is further supported by Nef-mediated upregulation of IL-2 and subsequent autocrine stimulation, both hallmarks of T cell activation (177). Accordingly, expression of HIV/SIV Nef in infected T cells derived from macaques stimulated IL-2 production, resulting in an 8-100 fold enhancement in viral replication (6). These results were further recapitulated in an oncogenic T cell line, demonstrating Nef induces a cellular gene expression profile remarkably similar to the activation profile induced by TCR engagement in anti-CD3 signaling (200). Nef also induces the activation of nuclear factor of activated T cell (NFAT), a key transcriptional mediator of IL-2 gene expression, via a TCR-independent, ionositol triphosphate receptor (IP3R1) dependent, calcium/calcineurin-mediated pathway (146). Nef further supports T cell activation by stimulating the cellular ERK/MAPK pathway, a key mediator of both T cell activation and HIV

function (190,191). Taken together, Nef-mediated T cell activation is regulated via numerous signaling pathways and multiple host factors essential for propelling HIV infection.

1.3.5 Nef Enhances Viral Infectivity

The importance of the *nef* gene for AIDS pathogenesis is highlighted by the observations in animal models and the long-term non-progressor cohort of HIV-infected individuals, as described above (see section 1.3.2). Viral infectivity is further augmented by Nef, as evidenced by severe replication deficiencies in macrophages and primary blood lymphocytes infected with Nef-mutated virions (154). Single-round infection assays demonstrated Nef-deleted viruses are less infectious than wild-type Nef+ viruses (35,154,202). Further, Nef+ viruses productively infected 5-20 fold more cells compared to an equivalent titer of Nef-deleted HIV (88,160) Subsequent studies revealed the low infectivity of Nef-deleted viral particles can be complemented via expression of Nef in *trans* during virus production whereas no effect was seen resulting from ectopic expression in target cells (5,193). Additionally, Nef does not improve viral infectivity by either enhancing the fusion of HIV virions or affecting the stability or structure of the viral core or matrix proteins (29,56,67,161,205). Instead, Nef may mediate the infectivity of new viruses in part by altering the composition of the cell membrane at the site of virion binding. Recent studies suggest interactions between Nef and cytoskeleton-associated proteins induce reorganization of the cortical actin barrier, a dense network of actin filaments located directly beneath the plasma membrane known to impede the entry of intracellular pathogens (29,141). Nef-mediated cytoskeletal rearrangement thus eases the post-fusion movement of the HIV

genome into the cytoplasm resulting in an increase in viral infectivity. More specifically, Nef-directed reorganization of actin filaments was observed to enhance viral replication through the binding and subsequent activation of Vav, a guanine nucleotide exchange factor for Rho-family GTPases linked to F-actin reorganization (61). The mechanism of Nef effects on viral infectivity may also involve its ability to optimize the composition of the cell membrane at the site of virion budding. Nef stimulates additional cholesterol synthesis in the host cell and selectively directs its intracellular distribution to detergent-resistant membranes (DRMs), also known as lipid rafts, the preferred site of HIV budding and release (158,233). The selective transport of cholesterol to the plasma membrane in Nef expressing cells correlated with increased virion infectivity and Nef incorporation into viral particles (233).

2.0 HYPOTHESIS & SPECIFIC AIMS

2.1 HYPOTHESIS

HIV-1 Nef is a small myristoylated protein capable of interaction with a diverse array of host cell signaling molecules. While previous biochemical and structural studies have suggested that Nef may form homodimers and possibly even higher order oligomers in HIV-infected cells, the significance of oligomerization for Nef function and its role in HIV pathogenesis is largely unknown. Using a novel fluorescence-based approach, I demonstrated for the first time that Nef forms oligomers in live cells. Moreover, our laboratory reported previously that enforced oligomerization greatly enhanced Nef-induced Hck activation in vivo (231). Based on the observed results, I became curious as to the significance of a unique structure-function relationship in Nef-mediated disease progression. I therefore hypothesized that **Nef oligomerization is a critical and conserved function of all Nef alleles, which, if disrupted, may prevent the known effects of Nef in HIV pathogenesis.**

To address this hypothesis I have set forth three aims: (1) to identify residues within the dimeric Nef core that are required for oligomerization in vivo; (2) to investigate whether Nef oligomerization is required for (a) the downregulation of CD4 and CXCR4 receptors and (b) HIV replication; (3) to test whether oligomerization is a general property of HIV-1 Nef alleles.

2.2 SPECIFIC AIMS

Aim 1: Identify residues within the dimeric Nef core that are required for oligomerization in vivo.

Previous biochemical and structural studies strongly suggest that Nef may form homodimers and higher order oligomers in HIV-infected cells. These studies suggest multiple homologous points of contact are essential for maintaining the dimerization interface. Using site-directed mutagenesis and a novel live-cell assay for protein-protein interaction known as Bimolecular Fluorescence Complementation (BiFC; see below for details), I examined the stability of the oligomerization interface and identified residues critical for oligomerization. Developing the BiFC assay further, I was able to confirm the formation of oligomers within key subcellular locations previously shown to be critical for Nef function as well as in multiple cell lines under conditions relevant to HIV infection.

Aim 2: Test the hypothesis that Nef oligomerization is required for CD4/CXCR4 down-regulation, and HIV replication.

In perhaps the best-characterized function of Nef, the removal of CD4 from the surface of infected cells is essential for host survival and viral propagation. However, the requirement of Nef oligomerization for receptor downregulation is largely unknown. To address this aim, I generated stable cell lines expressing oligomerization-defective Nef mutants from Aim 1 in order to examine cell-surface expression of CD4 and the CXCR4 co-receptor via FACS analysis.

Moreover, an SH3 binding assay was used to investigate the correct folding of the Nef core of select mutants observed to be oligomerization defective. To investigate the importance of Nef oligomerization for HIV-1 replication, I used the U87MG-CD4/CXCR4 cell line as a host for the replication assay, in which a functional *nef* gene is required for efficient HIV replication.

Aim 3: Test the hypothesis that oligomerization is a general property of HIV-1 Nef alleles.

Most of the work from our laboratory has focused on a single allele of Nef (SF2), both in terms of Hck activation and oligomerization. However, many other HIV-1 Nef subtypes exist (subgroups A-K), each containing unique Nef sequences. Experiments completed in this Aim, therefore, addressed the generality of oligomerization by a group of Nef alleles representative of all major HIV-1 subgroups. To address whether oligomerization is a general property of HIV alleles, representatives of M (major)-group HIV-1 Nef alleles were tested via BiFC. In light of the function data obtained in Aim 2, demonstrating that oligomerization is a common structural feature of a wide range of active Nef isolates, helped to validate the Nef oligomerization interface as a new target for drug development in the fight against HIV/AIDS.

3.0 CHARACTERIZATION OF THE HIV-1 NEF OLIGOMERIZATION INTERFACE IN VIVO THROUGH BIMOLECULAR FLUORESCENCE COMPLEMENTATION

3.1 SUMMARY

HIV-1 Nef is a small myristoylated protein capable of interaction with a diverse array of host cell signaling molecules. Previous biochemical and structural studies have suggested that Nef may form homodimers and higher order oligomers in HIV-infected cells, which may be required for both immune and viral receptor down-regulation as well as viral replication. Using a bimolecular fluorescence complementation (BiFC) assay, we provide the first direct evidence for Nef oligomers in multiple cell lines, including 293T epithelial, SupT1 T-lymphoma, and U87MG astroglioma cells. The Nef oligomers localized to the plasma membrane as well as the *trans*-Golgi network, two subcellular localizations essential for Nef function. Mutation of the N-terminal myristoylation signal sequence resulted in predominantly cytoplasmic fluorescence, suggesting Nef oligomerization is independent of membrane localization. Moreover, BiFC analysis of Nef alleles from multiple HIV-1 subtypes strongly suggests that oligomerization is a highly conserved property of Nef. Using the BiFC assay, we also probed the Nef dimerization interface defined by the X-ray crystal structure using site-directed mutagenesis. Single residue

substitutions within the hydrophobic interface (Y115D, L112A) reduced but did not abolish the Nef BiFC signal. However, substitution of all hydrophobic interface residues (Ile-109, Leu-112, Tyr-115, Phe-121) with Asp completely abolished BiFC. Similarly, disruption of an intermolecular ionic interaction flanking the hydrophobic interface also abolished BiFC. Taken together, these results suggest that the interface previously defined by the crystal structure is important for Nef oligomerization in vivo.

3.2 INTRODUCTION

HIV-1 Nef is a small myristoylated HIV accessory protein essential for AIDS pathogenesis. Highly conserved among primate lentiviruses, Nef is a critical determinant of both high-titer viral replication and AIDS progression (121). In a transgenic mouse model, expression of Nef alone was sufficient to induce profound immunodeficiency and CD4+ T-cell depletion, identifying this accessory protein as a key determinant of HIV pathogenesis (22,100). Conversely, HIV strains with defective *nef* alleles have also been isolated from patients with long-term, non-progressive HIV infection, further implicating Nef as a critical virulence factor for AIDS (54,124).

Nef serves as an adaptor protein, and multiple sequence motifs have been previously identified that are critical for altering numerous cellular activation and signaling pathways (53,69). While regions within the flexible amino-terminal arm and central loop have been well

characterized (5,12,76,133), the biological relevance of the structured core has not been fully investigated, especially in terms of its role in homo-oligomeric Nef interactions. Structural studies from several groups indicate multiple contact points of highly conserved amino acids between Nef molecules (13,133). The principal homologous contacts are established by residues R105, I109, L112, Y115, F121, and D123 within the αB helices of the Nef core (numbering based on the crystal coordinates of Lee et al., 1996) (Figure 8). These residues comprise a hydrophobic interface (residues I109 through F121) flanked by pairs of electrostatic interactions (residues R105 and D123). All of these residues are highly conserved among HIV-1 isolates, strongly suggesting an essential function for oligomerization in vivo. Indeed, mutation of the highly conserved residue D123 not only disrupted dimerization in vitro, but also Nef-mediated interactions with various cellular proteins (139,220), although the impact of this mutation on Nef oligomerization in HIV target cells is currently unknown.

Although a functional *nef* gene product has been identified in HIV-1/HIV-2 (and SIV) subtypes, a wide genetic variation exists within these multiple alleles. Based on full-length viral genome sequencing there are currently three designated HIV-1 groups: M (main), O (outlier) and N (non M or O). Eight HIV-2 groups have also been identified (172). The most widespread of all HIV groupings, nine HIV-1 subtypes (clades) have been identified within the M group and are designated A, B, C, D, F, G, H, J and K (163,178). The HIV-1 M group also distinguishes the prevalence of infection on a global scale. Whereas subtype B is found primarily in North and South America and Europe, subtypes A, C, D and E are most common in the developing world. Perhaps the most widely characterized and most often used lab strains, SF2 and NL4-3 are both derivations of subtype B. To date, all biochemical approaches employed to examine Nef oligomerization have been done so with regards to only SF2 or NL4-3. Therefore, demonstrating

the commonality of oligomerization might suggest a strong selective pressure exists across multiple subtypes that Nef must preserve for its function.

In this study, we provide direct evidence in support of oligomerization and identify residues critical for maintaining this interaction. Further, we demonstrate oligomerization is a highly conserved property of Nef subtypes. Using a unique fluorescence-based approach known as bimolecular fluorescence complementation (BiFC) (231), we identified the structural requirements for Nef oligomerization and the subcellular localization within HIV host cells. We found that Nef oligomerization in vivo is highly sensitive to mutations targeting the hydrophobic and ion pair residues within the interface predicted by the crystal structure, but is independent of both membrane association and the highly conserved protein-protein interaction motif, PxxPxR. BiFC analysis also revealed that Nef oligomers localize to the plasma membrane as well as the *trans*-Golgi network, subcellular sites essential for Nef function (21,111). Further, we found that Nef oligomerization was highly conserved across multiple HIV subtypes (A1 through K). Taken together, our findings are in accordance with previous structural and cellular localization studies of Nef, confirming the formation of Nef oligomers in live cells.

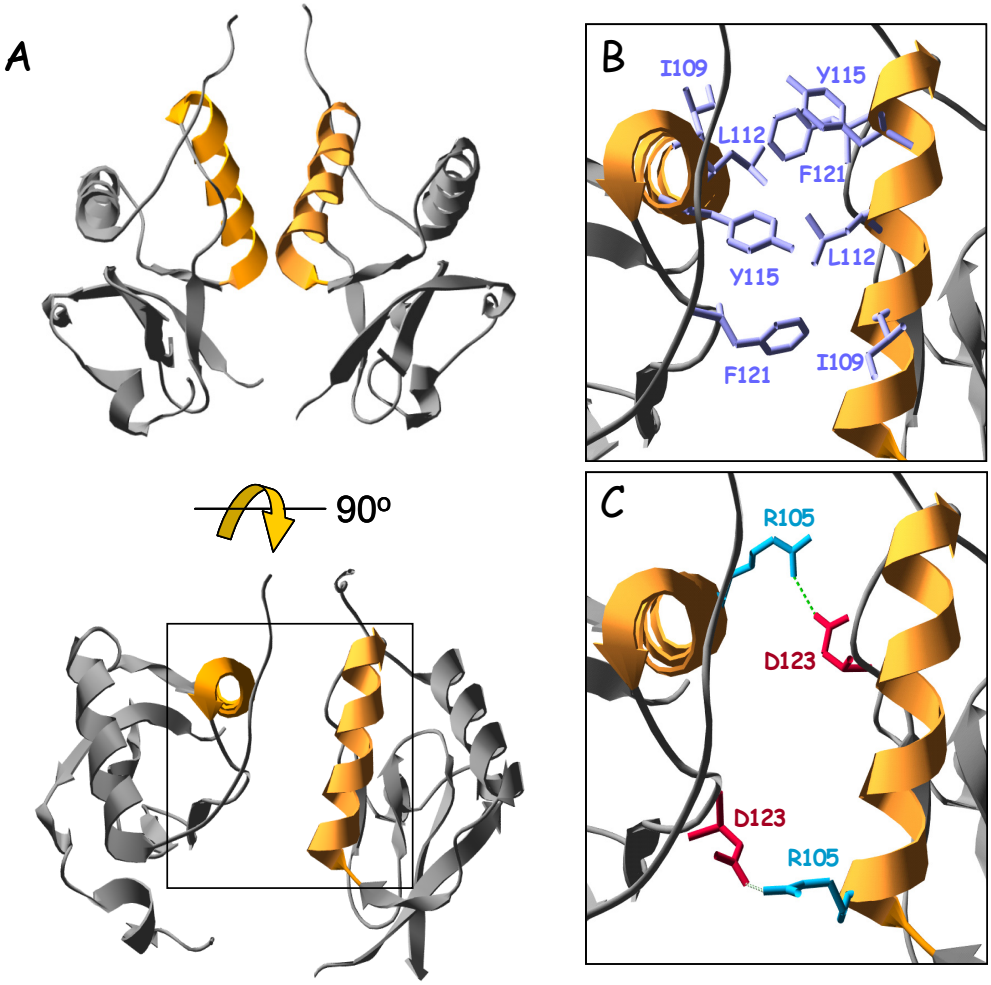


Figure 8. Crystal Structure of the Nef Dimerization Interface

Two views of the crystal structure of the dimeric Nef core. (A) The oligomerization interface with the αB helix highlighted in gold. The boxed region at the bottom indicates the orientation of the enlarged oligomerization interface later targeted for mutagenesis shown in B. Close-up views of the dimer interface formed by the αB helix, showing key (B) hydrophobic residues and (C) the reciprocal ionic interactions between Asp 123 and Arg 105. These models were produced using the X-ray crystal coordinates of Lee, et al. (PDB 1EFN) (133).

3.3 RESULTS

3.3.1 Detection of Nef Oligomers in vivo

In previous work, we provided evidence for Nef oligomerization in live cells using the fluorescence imaging technique known as bimolecular fluorescence complementation (BiFC) (231). For these experiments, two expression vectors were created by fusing non-fluorescent N-terminal (YN_{1-154}) or C-terminal ($YC_{154-238}$) fragments of YFP to the C-terminus of Nef (SF2 allele). The YFP fragments were fused to the C-terminal end of Nef in order to preserve the native N-terminal myristoylation signal sequence. Direct Nef-Nef interactions then brings the two non-fluorescent complementary fragments within close proximity, reconstituting a functional YFP structure, as schematically represented in Figure 9. Co-expression of the Nef-YN and Nef-YC fragments in 293T cells results in a strong fluorescent signal as detected by confocal microscopy (Figure 10, top right). In contrast, the Nef-YN and Nef-YC fragments alone produced no YFP fluorescence, despite strong expression of these Nef fusion proteins as assessed by immunofluorescent staining with Nef antibodies (Figure 10).

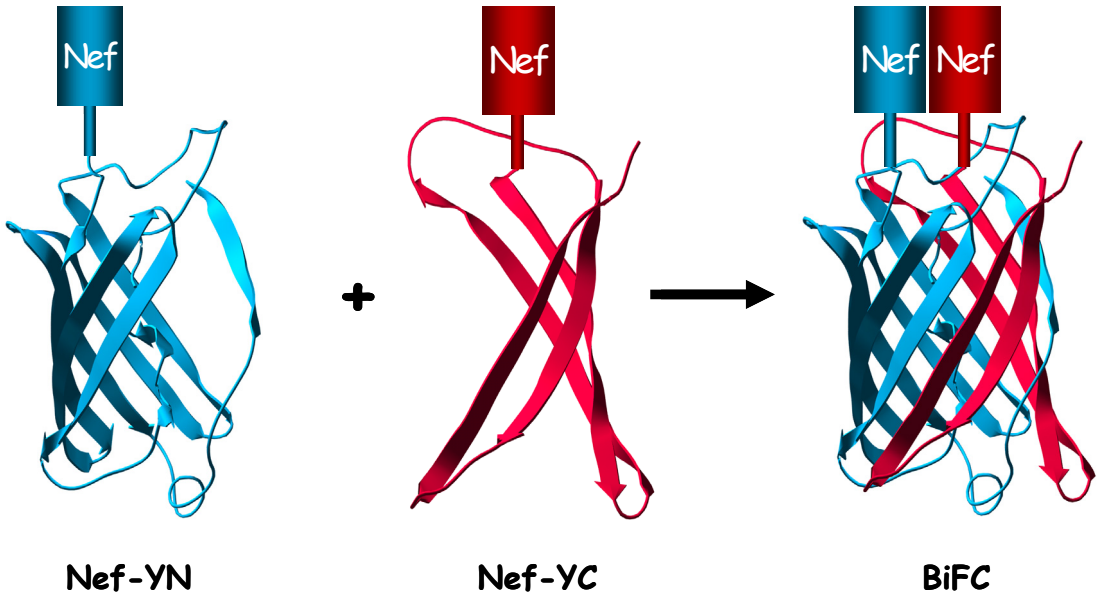


Figure 9. Bimolecular Fluorescence Complementation

Illustration of the BiFC principle. Non-fluorescent fragments of YFP (YN and YC) are fused to interacting proteins (Nef in this case). When the Nef-YN and Nef-YC fusion proteins are co-expressed in the same cell, Nef:Nef interaction brings the YFP fragments into close proximity, resulting in functional complementation and fluorescence.

To establish that the BiFC signals observed were dependent upon Nef oligomerization, control BiFC expression plasmids were generated using glutathione *S*-transferase (GST). GST was chosen as it does not interact with Nef, yet is a dimeric protein in its own right and can serve as an internal positive control. GST-based BIFC expression vectors were thus paired with the complementary Nef-BiFC counterparts (e.g. GST-YN + Nef-YC and GST-YC + Nef-YN) and co-expressed in 293T cells. As shown in Figure 10 (top row, middle columns), co-expression of the GST and Nef BiFC pairs did not produce a detectable BiFC signal. In contrast, co-expression of GST-YN + GST-YC produced a strong BiFC signal consistent with GST dimerization (Figure

10 top row, outside columns). Protein expression and localization was verified via immunofluorescent staining with monoclonal antibodies against either Nef or GST (Figure 10 bottom panels). Despite high-level co-expression, the mere presence of the YN and YC fragments was not sufficient to drive fluorescence complementation. Taken together, these data support the conclusion that the Nef BiFC signal is strictly dependent upon Nef homo-oligomerization in vivo. [Note: While the BiFC assay indicates the formation of dimeric complexes, it does not indicate the possible formation of higher-order oligomeric complexes (see section 5.1.1). Therefore, Nef *oligomerization* will be used to refer to the potential formation of undetected multimeric Nef complexes in vivo, while Nef *dimerization* will refer specifically to the interface defined by the crystal structure].

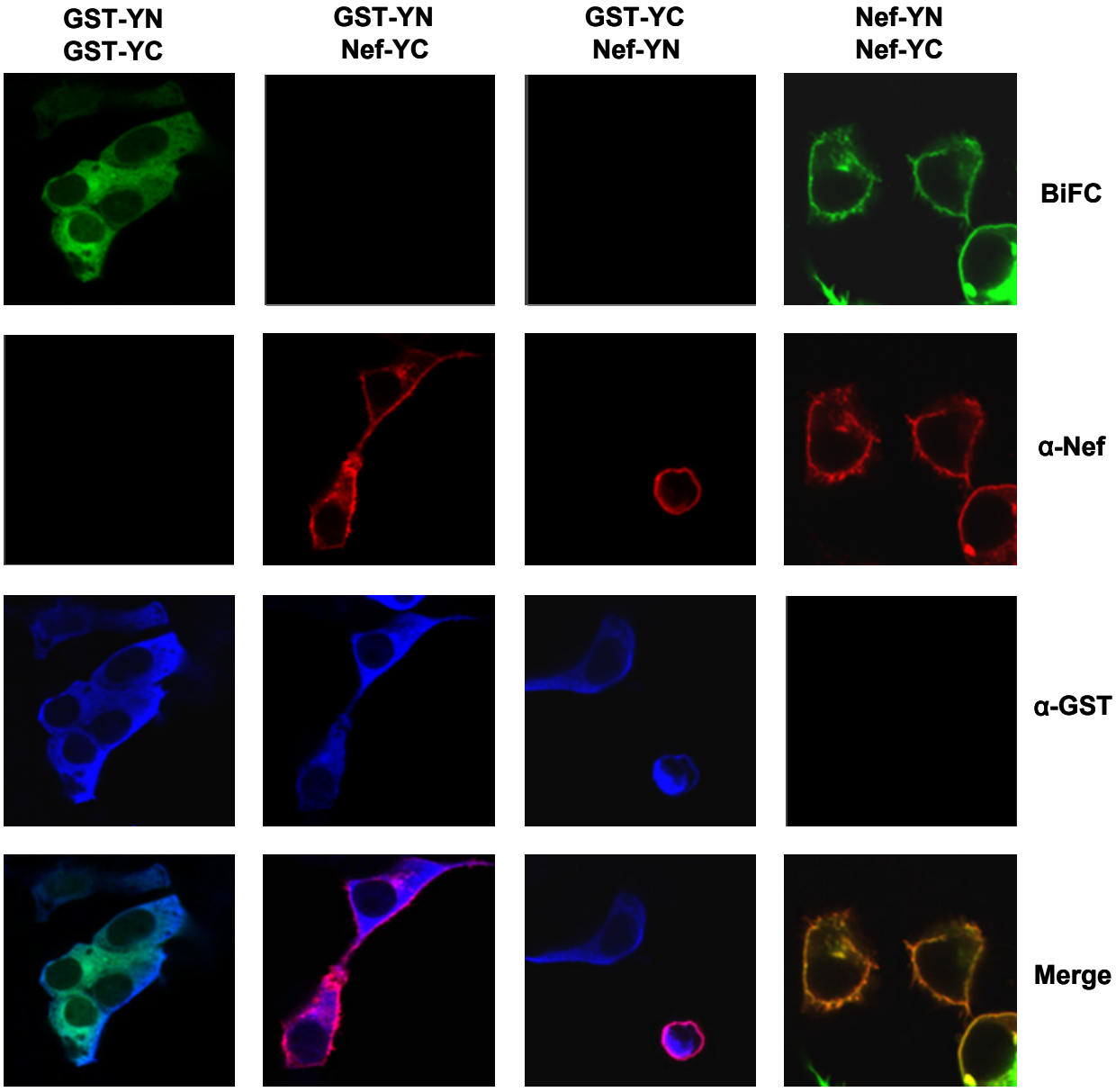


Figure 10. Nef Oligomerization Drives BiFC in 293T Cells

BiFC expression constructs were created for HIV-1 Nef (Nef-YN and Nef-YC) as well as for GST as a control (GST-YN and GST-YC) as described under Experimental Procedures. All four possible combinations of fusion proteins were co-expressed in 293T cells, and BiFC was assessed by confocal microscopy (BiFC; green; top row). Protein expression was assessed by immunostaining with anti-Nef (second row; red) and anti-GST (blue; third row) antibodies. A merged image is shown in the bottom row.

We next expanded our study of Nef oligomerization using cell lines that support HIV replication, including the U87MG astroglioma and SupT1 T-lymphoblast cell lines. However, unlike the 293T cells previously used in the experiments described above, these cell lines did not tolerate the ~3 hour incubation at room temperature required to promote maturation of the YFP fluorophore. To overcome this problem, we used the newly described GFP variant, Venus, which yields a BiFC signal without a requirement for room temperature incubation (197). The Nef coding sequence was therefore fused to Venus N- and C-terminal fragments analogous to those used for the YFP fusions, and the resulting Nef-VN and Nef-VC BiFC partners were introduced into each of these cell lines using retroviral vectors. BiFC signals were readily observed in each case (Figure 11), demonstrating that Nef oligomerizes in a wide variety of host cell environments and supports the conclusion that oligomerization is an intrinsic property of Nef.

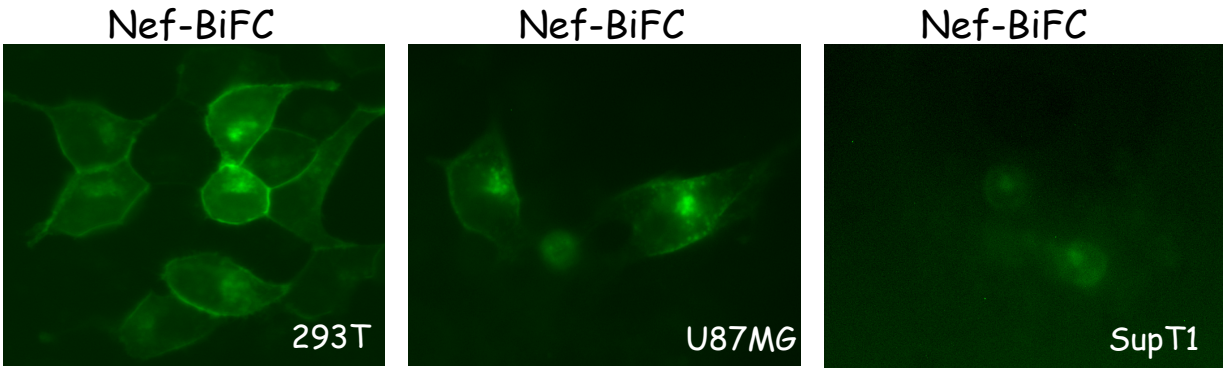


Figure 11. Demonstration of Nef Oligomerization in Multiple Cell Lines

U87MG and SupT1 cells, both of which support HIV-1 replication, were co-infected with Nef-YN and Nef-YC retroviruses and examined for BiFC by fluorescence microscopy. 293T cells co-transfected with Nef-YN and Nef-YC expression constructs were included as a positive control.

3.3.2 Subcellular Localization of Nef Oligomers

Nef is targeted to cellular membranes via post-translational attachment of myristic acid to its N-terminus through a conserved Met-Gly-X-X-X-Ser/Thr motif (25,77). This finding suggests that localization to the plasma membrane and clustering in lipid rafts may be required to promote Nef oligomerization. To test this idea, a point mutation (G2A) was introduced into the Nef myristoylation signal sequence. BiFC expression vectors were then generated from the resulting Nef-G2A mutant (G2A-YN + G2A-YC), expressed in 293T cells and assayed for oligomerization. As shown in Figure 12A, Nef-G2A produced a strong fluorescent BiFC signal, indicative of oligomerization. However, the G2A mutant displayed exclusively cytoplasmic fluorescence, and lacked the membrane localization observed with wild-type Nef. These results show that Nef oligmomerization is independent of membrane localization.

Nef has been shown to bind with high affinity to the SH3 domains of Src-family kinases and other proteins via its conserved PxxPxR motif (14,93,133). The Nef mutant 2PA, in which the key proline residues within this motif were replaced with alanines, was used to examine the requirement for SH3 partner proteins in Nef oligomerization. As shown in Figure 12A, disruption of the Nef SH3-binding motif had no effect on the BiFC signal or subcellular localization as compared to wild-type Nef. This finding shows that Nef oligomerization does not require interactions with other proteins through the PxxPxR motif, despite the presence of the SH3 domain in the X-ray crystal structures (12,133).

In addition to the plasma membrane, other studies have indicated that Nef also persists in the cytoplasm and trans-Golgi network (TGN) where it interacts with multiple trafficking and signaling proteins (166,221). We observed a consistent subpopulation of Nef BiFC-positive cells with peri-nuclear fluorescence, suggesting that Nef oligomers localize to the TGN. To determine

if this was the case, cells were counterstained with antibodies to TGN-46, a marker for this subcellular compartment. As shown in Figure 12B, strong co-localization of the Nef BiFC signal and TGN-46 staining was observed, indicating that Nef oligomers localize to the TGN in 293T cells. A similar peri-nuclear Nef-BiFC signal was also observed in U87MG and SupT1 cells, suggesting that localization to the TGN is a general characteristic of Nef oligomers and not specific to a particular cell type (Figure 11). These results suggest that Nef oligomerization may play a role in assembly of the multi-kinase complex recently linked to Nef-induced downregulation of MHC-1 (111).

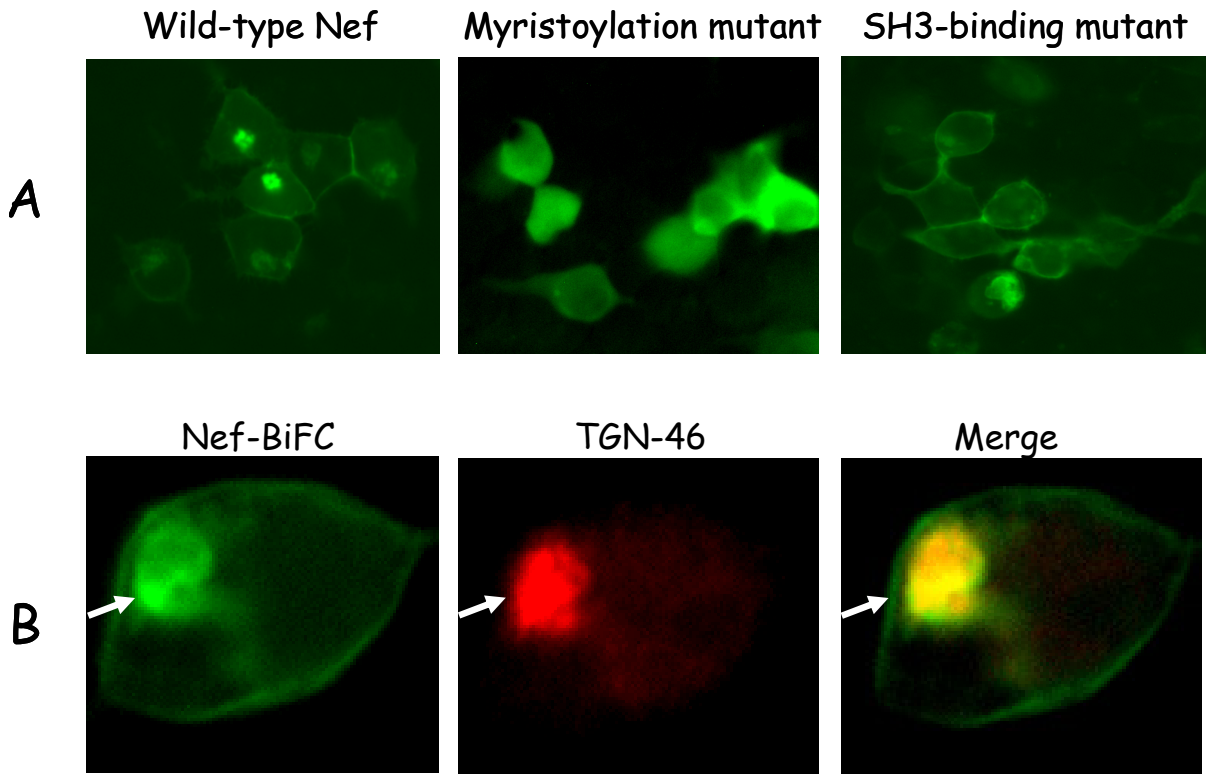


Figure 12. Characterization of Nef Oligomerization by BiFC

Nef oligomerization does not require myristoylation or interaction with SH3-binding proteins. (A) 293T cells were co-transfected with wild-type Nef-YN and Nef-YC expression constructs, with constructs bearing a Gly to Ala substitution in the N-terminal myristoylation signal sequence, or Ala for Pro substitutions in the SH3-binding motif, PxxPxR. BiFC was analyzed by fluorescence microscopy 48 h later. (B) Nef oligomers localize to the trans-Golgi network (TGN). 293T cells co-expressing Nef-YN and Nef-YC were stained with antibodies to the TGN marker, TGN-46, and a secondary antibody conjugated to Texas red. The merged image shows co-localization of the perinuclear BiFC signal and the TGN marker (arrows).

One of the principal advantages of using BiFC is the ability to observe the formation and localization of oligomeric complexes in live cells. To ensure that the fusion of the YFP fragments (-YN and –YC) did not alter or produce an artificial subcellular localization, the

expression and distribution of Nef within 293T cells transfected with unfused wild-type Nef were confirmed via Nef-directed immunofluorescence. As shown in Figure 13, despite fusion of YFP to the C-terminus of Nef, similar staining patterns were observed (localizing to the plasma membrane, TGN and cytoplasm) suggesting BiFC accurately depicts the localization of Nef and Nef dimers within 293T cells.

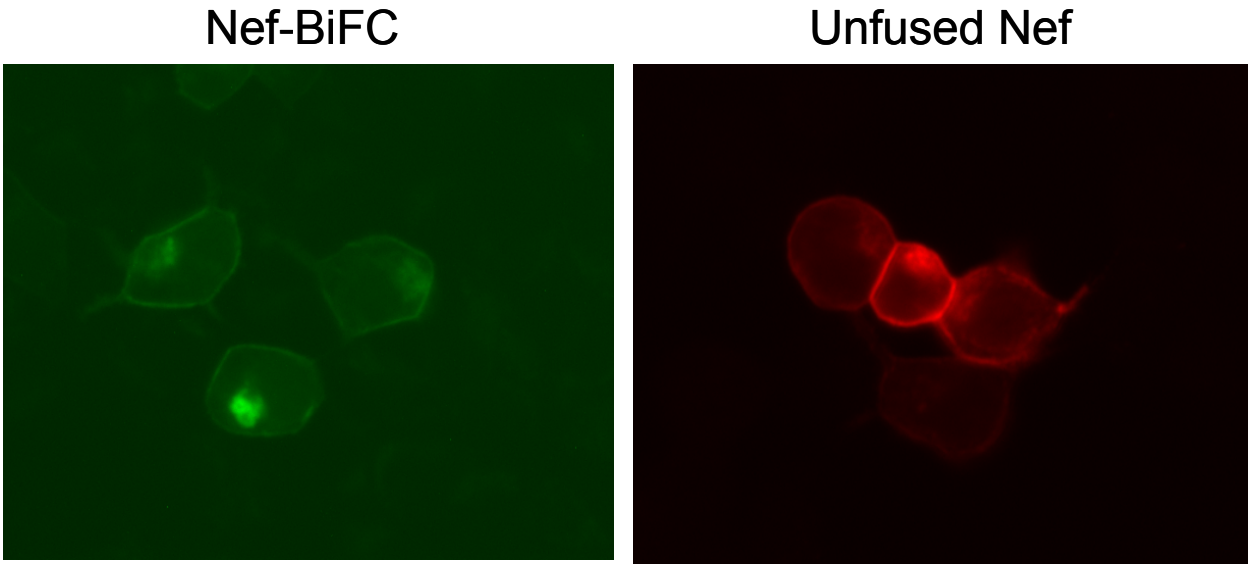


Figure 13. BiFC Fragments Do Not Interfere with Nef Trafficking

293T cells expressing either complementing wild-type Nef BiFC fusion constructs or unfused wild-type Nef. Nef expression as monitored via BiFC (green) or by direct immunofluorescence using the Nef mAb Hyb6.2 (red).

3.3.3 Efficiency of Nef Oligomerization

As a prelude to mutagenesis of the Nef oligomerization interface, we next examined the efficiency with which Nef oligomerization can be detected using the BiFC approach. For this study, Nef expression levels were detected within the same Nef-BiFC cell population using indirect immunofluorescence (IF) with a Nef-specific antibody. This approach permitted a direct correlation between the number of cells expressing Nef (IF) and the number of cells in which Nef oligomerization had occurred (BiFC). As shown in Figure 14, detection of Nef oligomerization using BiFC is highly efficient, with a positive BiFC signal present in virtually every cell positive for Nef expression by IF. Localization of Nef oligomers to the plasma membrane and the TGN was also consistently observed. Taken together, these data demonstrate that BiFC provides a highly efficient method to detect Nef oligomerization in cells, and provides an excellent model system to probe the dimer interface by site-directed mutagenesis as described in the next section.

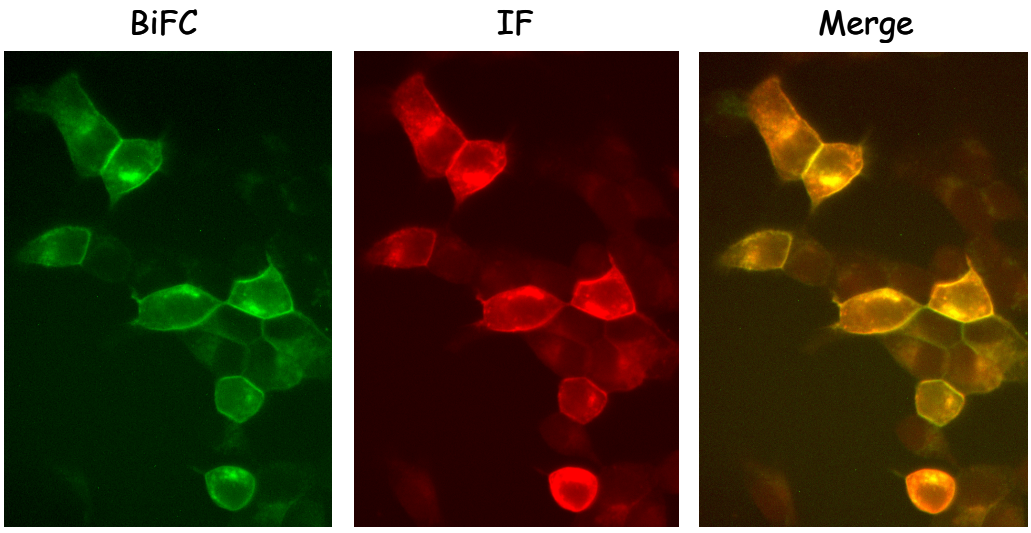


Figure 14. Efficiency of Nef Oligomerization

Nef-YN and Nef-YC were co-expressed in 293T cells. Forty-eight hours later, cells were fixed and stained with a monoclonal antibody to the Nef protein and a secondary antibody Texas-red conjugate. A strong BiFC signal (green) was observed in virtually all cells found to express Nef by immunofluorescence (IF; red). Both the Nef BiFC and IF signals exhibit the same subcellular localization pattern (Merge).

3.3.4 Mutagenesis of the Nef Dimerization Interface

Previous structural studies indicate that multiple contact points of highly conserved amino acids define the Nef dimerization interface (133). These contacts are principally established between the αB helices, forming a hydrophobic core (residues I190 through F121) flanked on either end by ion pairs (residues R105 and D123) as depicted in Figure 8. Inspection of the Nef crystal structure suggests that disruption of either the hydrophobic core or ion pairs may interfere with Nef oligomerization in vivo. To test this idea, hydrophobic and ionic interactions that form the interface were systematically disrupted by site-directed mutagenesis and the effects of the

mutations on oligomerization assayed via BiFC. In order to scan the hydrophobic interface (Figure 8B), changes were introduced with single (L112A, F121A, L112D, Y115D), double (L112D/Y115D) or quadruple (I109D/L112D/Y115D/F121D) mutations. Similar single and double mutations were created in the ion pairs flanking the hydrophobic core (D123A, D123N, D123V, R105E, and R105E/R106E; Figure 8C). The Nef mutants were transfected as pair-wise BiFC constructs into 293T cells and then counterstained with the anti-Nef antibody to verify expression. The transfected cell populations were then imaged for oligomerization (BiFC) and Nef expression (IF) and the BiFC:IF ratio was calculated using the Metamorph software package. A complete set of representative images are presented in Figures 15, 16 and 17, and the results of the image analysis are summarized in Table 2. Single amino acid changes designed to disrupt hydrophobic interactions within the helical dimer interface significantly reduced but did not abolish the Nef BiFC signal. However, substitution of all four hydrophobic interface residues (Ile-109, Leu-112, Tyr-115, Phe-121) with aspartate (Nef-4D mutant) completely abolished BiFC, demonstrating that an intact hydrophobic interface is essential for oligomerization in vivo. Similarly, disruption of the intermolecular ionic interaction between Asp-123 and Arg-105 by substitution of Asp-123 with asparagine completely abolished BiFC, identifying this residue as a key modulator of oligomerization. However, substitution of Asp-123 with Ala or Val reduced but did not abolish BiFC, suggesting that these mutants may form destabilized, non-functional oligomers (see Chapter 4.0).

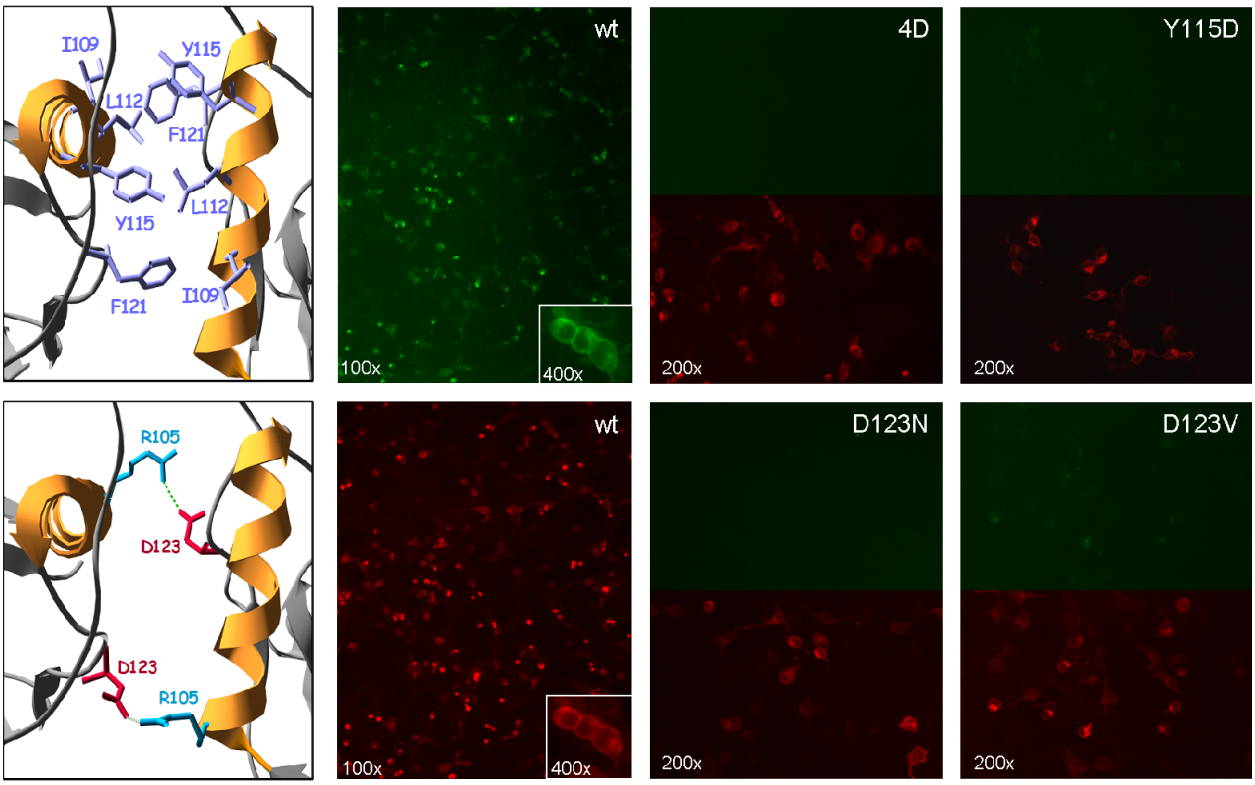


Figure 15. Mapping the Nef Oligomerization Interface by BiFC

Site-directed mutagenesis was used to disrupt hydrophobic core residues (I109, L112, Y115, F121; top panels) or the ion pair (D123-R105; bottom panels) within the Nef oligomerization interface. 293T cells were transfected with wild-type (WT) Nef-YN and Nef-YC expression vectors, counterstained with anti-Nef antibodies and visualized with secondary antibodies conjugated to Texas-red. Similarly, representative of the varying degrees of oligomeric disruption, the Y115D mutation in the hydrophobic core, or Asp substitutions of all four hydrophobic residues (4D) are shown. Cells were also transfected with BiFC pairs in which D123 was replaced with either Asn (D123N) or Val (D123V). BiFC was assessed by fluorescence microscopy 48 h later (green).

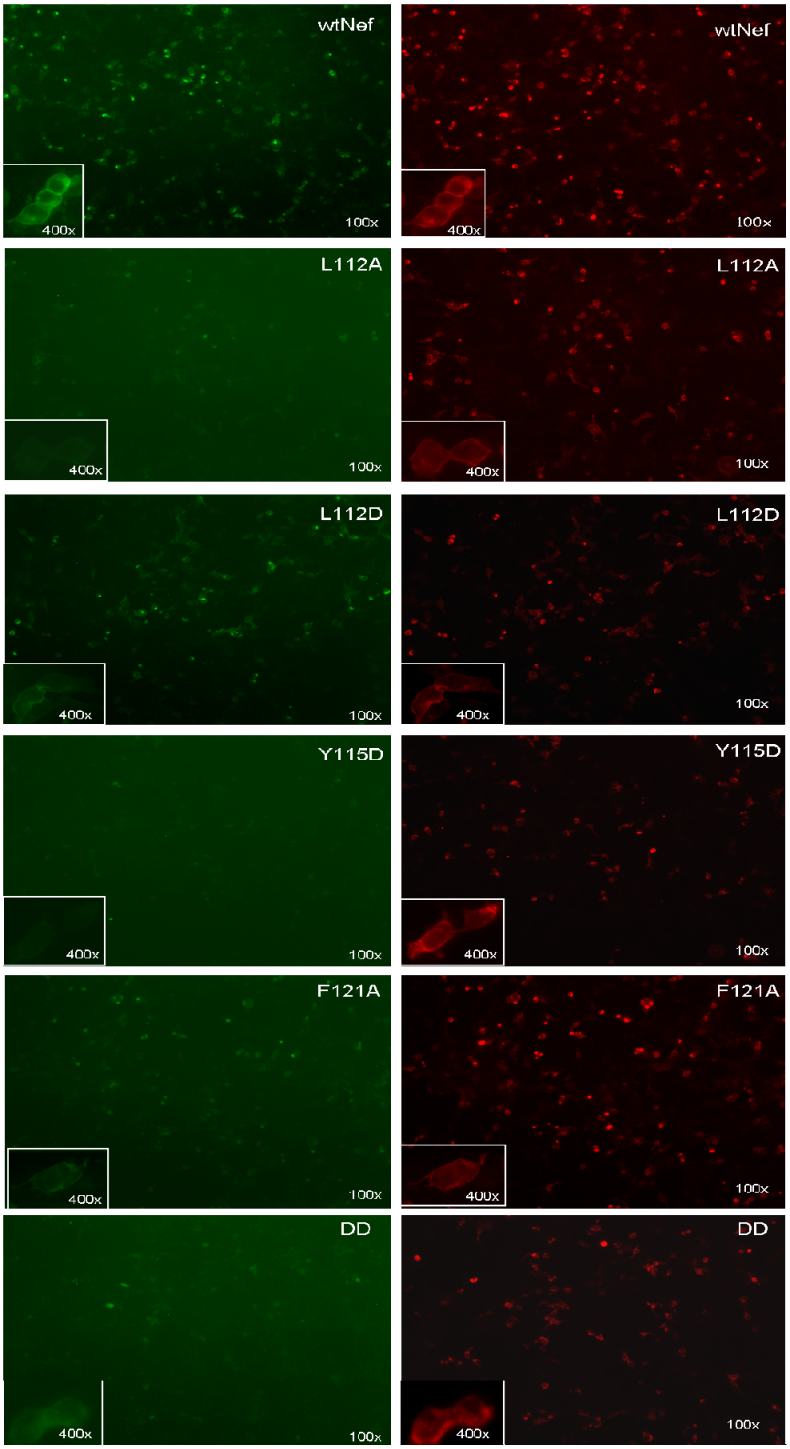


Figure 16. Representative Images of BiFC Results with Hydrophobic Nef Mutants

Nef mutants were expressed as pair-wise BiFC constructs (green) and counterstained with anti-Nef antibodies and visualized with secondary antibodies conjugated to Texas-Red (red). BiFC images were quantified using MetaMorph software (Table 2).

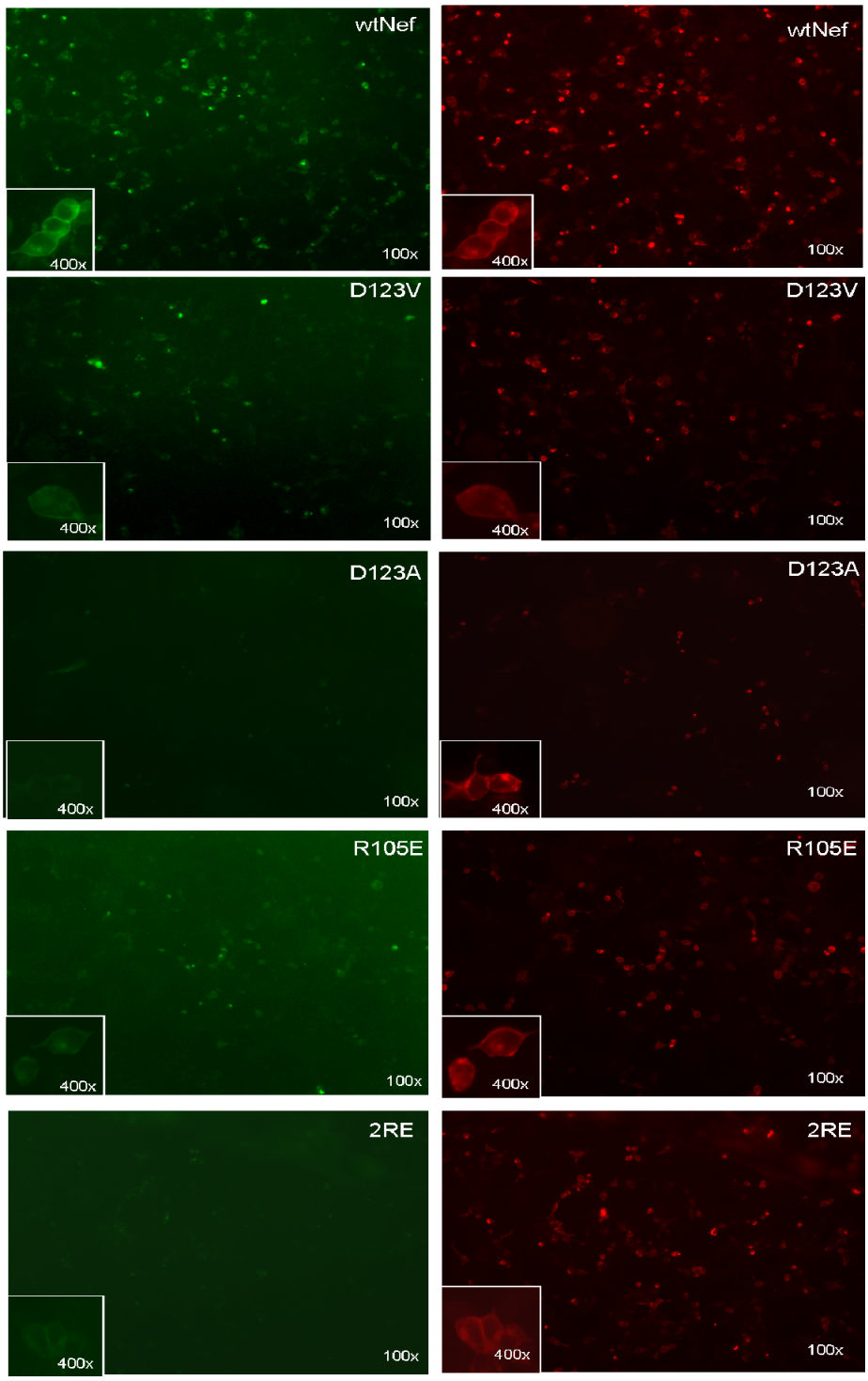


Figure 17. Representative Images of BiFC Results with Ion Pair Nef Mutants

Nef mutants were expressed as pair-wise BiFC constructs (green) and counterstained with anti-Nef antibodies and visualized with secondary antibodies conjugated to Texas-Red (red). BiFC images were quantified using MetaMorph software (Table 2).

	Nef Mutant	% Decrease [100- (BiFC:IF)]
Hydrophobic	L112A	52.80
	F121A	33.04
	L112D	47.55
	Y115D	59.13
	L112D/Y115D (DD)	86.74
	I109D/L112D/Y115D/F121D (4D)	**99.99**
Ion Pairs	D123A	69.60
	D123N	**97.11**
	D123V	52.95
	R105E	49.94
	R105E/R106E (2RE)	71.12
	G2A	0.00
	2PA	0.00

Table 2. Nef Mutagenesis Summary

3.3.5 Oligomerization is a Highly Conserved Property of Nef Subtypes

In the previous sections, the evaluation of Nef oligomerization has been focused on a single Nef allele, SF2. However, many other HIV-1 Nef subtypes exist (subgroups A-K), each containing unique Nef sequences. While the impact of the observed sequence variation on oligomerization remains to be elucidated, alignment of Nef sequences revealed a region of high conservation between residues Gln104 and Asp123. As shown in Table 3 this region contains many of the conserved residues that establish the principal contacts within the Nef-SF2 dimer. For this reason, I assessed the generality of oligomerization by a group of Nef alleles representative of all

major (M-group) HIV-1 subgroups via BiFC. As described previously, the Venus fragments were fused to the C-terminal end of Nef to preserve the native N-terminal myristoylation signal sequence. $Nef_{(A-K)}$-VN and $Nef_{(A-K)}$-VC were expressed in 293T cells, and the resulting fluorescent images are shown in Figure 18. Remarkably, all Nef subtypes showed strong fluorescence, with notable localization to the cell periphery, trans-Golgi network (TGN) and cytoplasm. As an internal control, the subtype B mutant chosen was neither SF2 nor NL4-3 (both classified as B subtypes), yet displayed virtually identical fluorescence as compared to previous BiFC experiments. Taken together, these results provide the first direct evidence of Nef oligomerization in vivo across a broadly representative group of HIV subtypes.

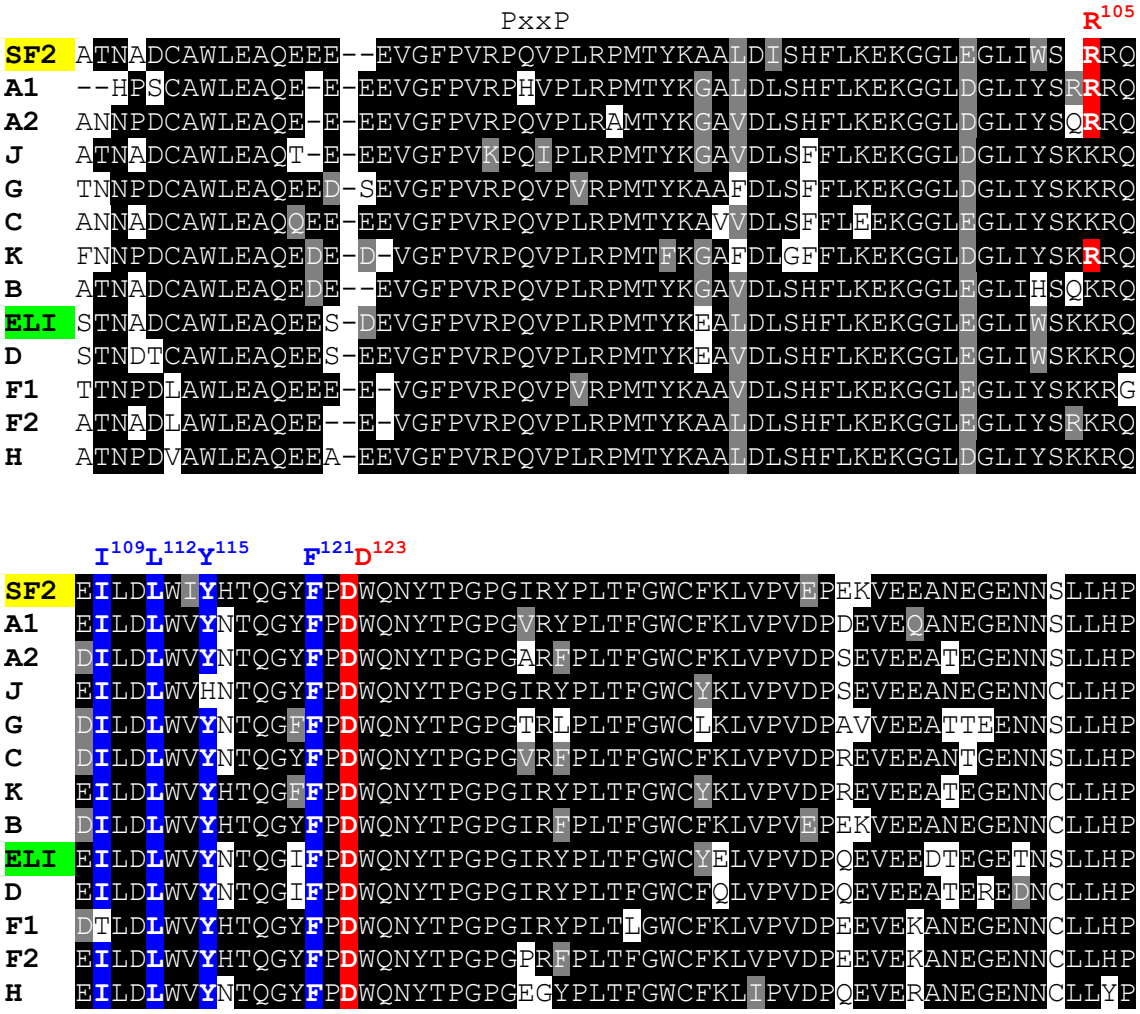


Table 3. Alignment of HIV-1 Nef Subtypes Amino Acid Sequences

The HIV-1 Nef sequences shown below are broadly representative of the major non-recombinant HIV-1 subtypes A through K. The Nef subtype reference alignment was obtained from the NIH HIV Sequence Database (http://hiv-web.lanl.gov/content/index); The alignment also includes SF2 Nef (highlighted in yellow; top), which we have studied extensively in terms of Hck activation and oligomerization, as well as ELI Nef (green) which fails to activate Hck. The SF2 allele will be included as controls in all experiments. Hydrophobic (I109, L112, Y115 and F121) and charged residues (R105 and D123) that contribute to the dimer interface in the crystal structure are highlighted in blue and red, respectively.

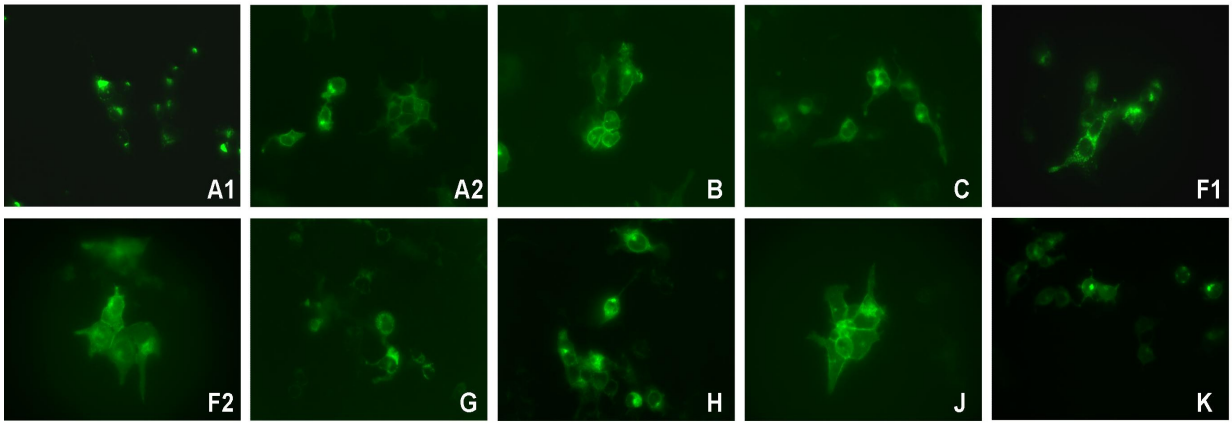


Figure 18. Oligomerization is Conserved Across Nef Subtypes

293T cells expressing Venus-BiFC constructs for each representative HIV-1 Nef subtype (A-K) were examined for oligomerization. Fluorescence was observed in every case indicating the formation of Nef oligomers.

3.4 DISCUSSION

In this study, we used BiFC of Nef-YFP constructs to identify the structural features of Nef essential for oligomerization in live cells. Using this approach, we identified critical residues present in the X-ray crystal structure of the Nef dimerization interface as essential for maintaining this interaction. Further, we identified the subcellular locations of Nef oligomers. The commonality of Nef oligomerization was also demonstrated using a panel of Nef alleles broadly representative of all M-group (main) subtypes.

In the first part of this study, we confirmed that fluorescence complementation was dependent upon Nef:Nef interaction, and not driven by the YFP fragments themselves. This was accomplished by fusing the non-fluorescent YFP fragments to the GST protein, which is itself a

native dimer but does not interact with Nef (33). Importantly, BiFC was not observed when these control GST BiFC fusion proteins were co-expressed with complementary Nef BiFC partners (e.g., Nef-YN + GST-YC and Nef-YC + GST-YN). The control GST BiFC fusion proteins also served as an internal control for the assay, as co-expression of GST-YN with GST-YC resulted in a strong BiFC signal. The subcellular localization of the GST and Nef oligomers, as reported by BiFC, were also quite distinct, with GST dimers localizing to the cytoplasm and Nef oligomers localizing to the plasma membrane and TGN. These findings demonstrate that BiFC accurately reflects the subcellular localization of Nef oligomers within the cellular environment, consistent with recent reports that applied this technique to study of other oligomeric proteins (24,91,112,155).

Our observation that Nef forms oligomers in multiple cell lines, all of which support HIV replication (U87MG and SupT1), suggests that oligomerization is a general property of the HIV accessory protein. However, the efficiency of the BiFC assay for the detection of Nef oligomers varied among the cell lines. In 293T cells, which can be transfected with high efficiency and express relatively high levels of the Nef BiFC fusion proteins, both the efficiency and strength of the BiFC signals were quite high (Figure 14). This observation made the use of 293T cells ideal for analyzing the dimerization interface mutants. However, Nef BiFC was reduced in U87MG and SupT1 cells with respect to both efficiency and signal intensity relative to 293T cells. This difference was most likely due to the requirement for co-infection of these cell lines with recombinant BiFC retroviruses from which Nef-YN and Nef-YC expression is driven by a weaker promoter. Nevertheless, Nef-BiFC signals were readily observed in these cell lines despite the lower levels of expression, indicating that oligomerization is an inherent attribute of Nef independent of the cell type.

The BiFC assay provided a means for the analysis of Nef oligomerization in live cells, allowing us to examine the structural requirements for Nef oligomerization. Previous studies have clearly shown the significance of membrane localization for Nef function (20,76,151). These findings led us to examine if membrane localization was required for oligomerization, possibly due to Nef protein clustering in lipid rafts (215). However, we observed that a myristoylation-defective mutant of Nef produced a diffuse cytoplasmic BiFC signal, indicating that membrane attachment is not required for the formation of Nef oligomers. These findings are consist with previous studies of Arold et al., who demonstrated that a purified recombinant Nef core domain formed homo-dimers in vitro (13).

Nef is known to interact with multiple binding partners in HIV infected cells, suggesting that binding of host cell proteins may influence Nef oligomerization in vivo. The highly conserved PxxPxR motif is well known to mediate Nef interactions with partners proteins that have SH3 domains, including Src-family kinases and guanine nucleotide exchange factors, as well as other signaling proteins (39,61,76,174,183,187). However, mutation of the the essential prolines in this motif (Nef-2PA) did not diminish the BiFC signal nor did it influence the subcellular localization of Nef. This observation suggests that interactions with other factors, at least through the PxxPxR motif, are not essential for Nef oligomerization. This finding is consistent with the observation that the Nef SH3-binding motif is spatially separated from the dimerization interface in the crystal structure (Figure 8A)

In addition to providing information on protein-protein interaction in cells, BiFC also reports on the subcellular localization of the oligomers. Our experiments revealed consistent localization of Nef BiFC signals to the plasma membrane and perinuclear region in all three cell types examined. The peri-nuclear localization of the Nef oligomers was coincident with

immunofluorsecent staining for the TGN marker protein, TGN-46 (Figure 3C). These observations are consistent with previous studies showing that localization of Nef to the TGN is essential for MHC downregulation (111,166), and suggests that Nef oligomerization may be essential for MHC downregulation as well. Indeed, previous work by Liu et al. shows that mutation of Asp 123, shown here to completely quench the Nef-BiFC signal (Figure 15), interferes with Nef-induced MHC downregulation (139).

Previous structural studies have identified residues within the Nef core that define a dimerization interface in vitro (133). Highly conserved among isolates of both HIV and SIV Nef, these residues contribute to dimerization through a combination of hydrophobic and electrostatic interactions. Using the BiFC assay and site-directed mutagenesis, we show for the first time that the dimerization interface defined by crystallography is indeed essential for Nef oligomerization in cells. Both an intact hydrophobic core and the flanking ionic interactions are essential for maintaining Nef oligomerization and function in vivo. A complete loss of the Nef-BiFC signal was observed when the four key residues that form the hydrophobic core were replaced with charged residues (Nef-4D) or when Asp 123, which ion pairs with Arg 105, was replaced with Asn. Interestingly, replacement of Asp 123 with the nonpolar residues Val or Ala reduced but did not abolish BiFC, suggesting that these mutants still retained the capacity to oligomerize. Thus, Nef Asp-123 may be a major regulator not only of oligomerization but perhaps also of the overall conformation of Nef.

To date, all of the work performed by our laboratory, with respect to Nef oligomerization, has involved the allele, SF2. However, the degree of genetic variation among HIV subtypes suggests that Nef oligomerization may not be conserved. Therefore, in order to assess the commonality of oligomerization, Nef alleles broadly representative of all major subtypes were

assayed via BiFC. Remarkably, despite known sequence variations, all of the subtypes were BiFC positive, a result indicative of oligomerization. Alignment of Nef sequences revealed a region of high conservation between residues Gln104 and Asp123 (Table 3). Interestingly, this region comprises the oligomerization interface suggested by biochemical and structural studies and includes the conserved hydrophobic (I109, L112, Y115 and F121) and ionic (R105 and D123) residues shown to impact oligomerization of Nef-SF2 (Figure 8 and Table 3). Taken together, this suggests the contacts established within the interface defined by the crystal structure as indeed vital for oligomerization in vivo. In addition, this finding suggests that the greater sequence variation observed both N- and C-terminal to the structure core of Nef, as well as within the internal flexible loop, does not impact the oligomerization of Nef in vivo. In summary, our findings of Nef oligomerization across multiple subtypes (Figure 18) and cell lines (Figure 11) strongly advocates oligomerization is a common attribute of all active Nef isolates.

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Cell Culture and Reagents

The cell lines used in this study were obtained from the ATCC and include 293T, U87MG, and SupT1. All cells were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 5% fetal bovine serum (FBS; Atlanta Biological) and treated with antibiotic-antimycotic (Invitrogen). U87MG cells were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS and 50 µg/mL gentamycin (Invitrogen). U87MG-CD4/CXCR4 cells (generous gift of Dr. Toshiaki Kodama, University of Pittsburgh School of Medicine) were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS, 300 µg/mL G418 and 0.5 µg/mL puromycin. SupT1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and antibiotic-antimycotic. 293T cells were transfected using standard calcium phosphate techniques as described elsewhere (26).

3.5.2 Bimolecular Fluorescence Complementation Expression Vectors

Two Nef-BiFC expression vectors were created in which the N-terminal coding sequence of YFP (residues Val2-Ala154) was PCR-amplified from the plasmid vector pEYFP-C1 (Clontech) and ligated into the mammalian expression vector pcDNA3.1(-) (Invitrogen) containing Nef via a unique Acc III restriction site found in close proximity to the Nef C-terminal coding region. A similar approach was used to fuse the C-terminal coding region for YFP (residues Ala154-

Lys238) to Nef. In both instances, C-terminal fusions were used to preserve the native N-terminal myristoylation signal sequence of Nef. The resulting N- and C-terminal Nef-YFP fusions used for BiFC are referred to as Nef-YN and Nef-YC, respectively. Mutagenesis of the N-terminal myristoylation signal sequence, the conserved PxxPxR SH3-binding motif, and the Nef dimerization interface were performed using either the QuikChange II site-directed mutagenesis kit (Stratagene) or using standard PCR-based techniques.

For the Venus-based BiFC expression vectors, PCR was used to amplify the Venus N-terminal coding sequence for residues Val2-Asp173 and the C-terminal coding region for Ala154-Lys238. The resulting N- and C-terminal Venus coding regions were fused to Nef in pcDNA3.1(-) as described above for the YFP BiFC constructs, and are referred to as Nef-VN and Nef-VC. The Venus template was a kind gift of Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan.

To create the control BiFC-GST fusion constructs, the identical N- and C-terminal coding sequences of YFP were PCR-amplified as described above and subcloned into pcDNA3.1(+) (Invitrogen) via unique NheI/HindIII restriction sites. Two sequential repeats of the coding sequence for the HA epitope tag (YPYDVPDYA) were added to the C-terminus of the BiFC fragments thus generating YN-HA-HA and YC-HA-HA fusions, respectively. The coding sequence of GST was then PCR-amplified from pGEX-2T (GE Life Sciences) and ligated in-frame with YN and YC coding regions to generate pcDNA3.1(+)YN-GST-HA-HA and pcDNA3.1(+)YC-GST-HA-HA fusion plasmids. Protein expression was verified via monoclonal antibodies raised against either Nef or GST as described below.

3.5.3 Immunoflourescence Microscopy and Fluorescence Imaging

The antibodies used for immunofluorescence experiments include anti-Nef (NIH AIDS Research & Reference Reagent Program; mAb Hyb 6.2; 1:500), anti-GST (Santa Cruz Biotechnology; sc-459; 1:1000), and anti-human *trans*-Golgi network (Serotec; TGN46; 1:500). Transfected 293T cells were grown on coverslips and fixed with 4% paraformaldehyde in PBS for 10 min. Cells were washed with PBS, treated with 0.2% Triton X-100 in PBS for 15 min, and washed again with PBS. Cells were then blocked with PBS containing 2% BSA for 30 min and incubated for 45 min at room temperature with the primary antibodies diluted as described above, followed by additional washing with PBS. Immunostained cells were then visualized with secondary antibodies conjugated to Cy3/Cy5 (Chemicon; 1:1000) or Texas Red (Southern Biotech; 1:750). BiFC and immunofluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT cooled CCD high-resolution digital camera and software (Diagnostic Instruments). Confocal multi-color images (YFP, Cy3, Cy5) were obtained using a Zeiss Meta 510 confocal microscope at the Center for Biological Imaging (CBI) at the University of Pittsburgh School of Medicine.

To quantify the effects of mutations on BiFC, same culture fluorescent cell images (both IF and BiFC) were quantitatively analyzed using the Metamorph software suite (Molecular Devices). In order to remove any background, lower threshold fluorescence intensities for all BiFC images were standardized using BiFC control plasmids (above), as per protocol (108) (http://people.pharmacy.purdue.edu/~cdhu/protocols.html). Upper threshold fluorescence intensities were then standardized to wildtype Nef-BIFC and the corresponding IF image such that the final output was based on total threshold area and staining intensity at individual pixel levels. Mutant Nef-BiFC levels were then measured relative to the standards established above.

3.5.4 Recombinant Retroviruses for BiFC Assay

Retroviral expression vectors were used to generate high-titer retroviral stocks in 293T cells by cotransfection with an amphotropic packaging vector as described elsewhere (231). Nef-Venus fusion constructs were subcloned into the retroviral expression vector pSRαMSVtkneo, which carries a G418 resistance marker to enforce expression of Nef-BiFC proteins by antibiotic selection. Retroviral stocks were supplemented with Polybrene (Sigma) to 4 μg/mL and added to U87MG and SupT1 cells in 6-well plates (2.5 x 10^5 cells/well). The plates were centrifuged at 1000*g* for 4 h at 18 °C to enhance infection efficiency. Because the BiFC system requires the co-expression of two Nef fusion proteins (Nef-VN and Nef-VC), cells were super-infected with the second retrovirus 24 h later. Cultures were screened for BiFC 72 h later and images recorded using a Nikon TE300 inverted microscope as described above.

3.5.5 Nef Subtypes and BiFC Expression Vectors

For the Venus-based BiFC fragment expression vectors, PCR was used to amplify the Venus N-terminal coding sequence for residues Val2-Asp173 and the C-terminal coding region for Ala154-Lys238. Each of VN and VC fragment 5' forward primers contained a unique BamHI restriction site upstream of Val2 or Ala154, respectively. The resulting N- and C-terminal Venus coding regions were cloned into pcDNA3.1(-) via BamHI/HindIII sites and are referred to as pcDNA3.1(-)VN and pcDNA3.1(-)VC. Nef genes were synthesized such that silent mutations were introduced (DNA2.0 Menlo Park, CA) within the coding regions of all Nef subtypes (A1,

A2, B, C, F1, F2, G, H, J, K) in order to maintain single restriction sites. Specifically, EcoRI and BamHI sites were introduced directly upstream of both the start and stop codons. To generate BiFC fusion constructs, each Nef subtypes was then inserted, in-frame, with the parent pcDNA-based Venus fragment vectors via EcoRI/BamHI.

3.6 FOOTNOTES

*This work was supported by NIH Grants AI057083 and CA081398 as well as the Pittsburgh AIDS Research Training (PART) Grant NIH/NIAID T32 AI065380. A kind thanks to Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan for providing the Venus template; Dr. Huihui Ye for generating some of the Nef mutants; Dr. Jonathan Shaffer for generating the GST-BiFC control plasmid used in this study and Dr. Simon Watkins and the Center for Biological Imagining at the University of Pittsburgh School of Medicine for their help with confocal imaging.

4.0 HIV-1 NEF OLIGOMERIZATION IS ESSENTIAL FOR CD4 DOWNREGULATION AND VIRAL REPLICATION

4.1 SUMMARY

HIV-1 Nef is a small myristoylated protein capable of interaction with a diverse array of host cell signaling molecules. Previous biochemical and structural studies have suggested that Nef may form homodimers and higher order oligomers in HIV-infected cells, which may be required for both immune and viral receptor down-regulation as well as viral replication. In Chapter 3, I demonstrated Nef oligomerization in multiple cell lines that support HIV replication and across numerous HIV-1 subtypes using bimolecular fluorescence complementation (BiFC). Using BiFC, I was also able to identify residues critical for Nef oligomerization in live cells. In this Chapter, I examine the functional consequences of oligomeric disruption within the context of Nef-induced receptor downregulation, interactions with the host cell binding partner Hck, and Nef-dependent HIV replication. Interestingly, despite varying effects on oligomerization, all of these mutants were shown to dramatically disrupt Nef-induced CD4 down-regulation via FACS analysis. Despite varying degrees of oligomeric disruption, all Nef mutants tested bound the Hck SH3 domain to the same extent as wild-type Nef. Finally, and in all cases, disruption of Nef

oligomerization significantly impaired HIV replication to levels comparable to that of cells infected with HIV that fails to express Nef. Taken together, these results suggest that while multiple residues contribute to Nef oligomerization in live cells, the formation of functional Nef oligomers is very sensitive to any modifications within the dimer interface. This observation provides the first evidence that oligomerization is critical for both Nef-mediated downregulation of CD4 and enhancement of HIV replication and identifies the Nef oligomerization interface as a potential target for anti-retroviral drug design.

4.2 INTRODUCTION

HIV-1 Nef is a small oligomeric protein capable of interacting with a diverse array of host cell signaling molecules and is an essential factor in AIDS progression (59,76). The importance of Nef in the maintenance of high viral loads and subsequent development of AIDS-like disease has been well established in both rhesus monkeys and transgenic mice (100,101,121). Interestingly, HIV strains with defective *nef* alleles have been isolated from long-term survivors of HIV infection (54,124). Although these and other studies implicate Nef as a major factor of disease progression, the molecular mechanism of Nef action in HIV replication and disease progression has not been fully elucidated.

Despite the lack of catalytic activity, the Nef protein influences numerous signaling pathways within the infected host cell. Nef enhances viral replication and disease progression by altering the threshold of T-cell activation (6,59,200); mediating transcriptional and cellular

activation (17,100,113,183); enhancing virion infectivity (3,35,154,195) and allowing escape of infected cells from immune surveillance through downregulation of cell surface MHC-I molecules (21,164,165). Perhaps the most widely characterized function of Nef is its ability to drastically reduce the steady state level of CD4 from the cell surface (9,19,73,149). The rapid downregulation of CD4 prevents viral superinfection while also enabling viral particle release by preventing the sequestration of viral progeny (129,180). Removal of CD4 from the cell surface may also contribute to the production of infectious HIV-1 particles (10,144). Indeed, HIV replicates poorly in cell lines engineered to overexpress CD4 molecules that are insensitive to Nef-mediated downregulation (41,150).

Nef serves as an adaptor protein, and multiple sequence motifs have been identified that are critical for altering the cellular activation and signaling pathways described above [reviewed by (53,69)]. While regions within the flexible amino-terminal arm and central loops of Nef have been well characterized (5,12,76,133), the biological relevance of the structured core, especially in terms of its role in oligomerization, has yet to be fully investigated. Previous work from our laboratory has shown that enforced Nef dimerization significantly enhanced the activation of the Src-family kinase and Nef partner protein Hck in a Rat-2 fibroblast transformation assay, providing important evidence that homotypic Nef:Nef interactions are critical to the regulation of downstream signaling (231). As defined by the crystal structure, the dimerization interface is comprised by residues forming a hydrophobic interface (residues I109 through F121) flanked by pairs of electrostatic interactions (formed by residues R105 and D123) (described in section 1.2.4, Figure 7). All of these residues are highly conserved among HIV-1 Nef isolates, strongly suggesting an essential function for oligomerization in vivo. Indeed, mutation of the highly conserved residue D123 has been shown to affect Nef-induced kinase activation and receptor

downregulation (139,220), although the impact of this mutation on Nef oligomerization in HIV target cells is currently unknown.

In this study, we provide direct evidence in support of oligomerization as a critical determinant of multiple Nef functions. Recently, we demonstrated for the first time that Nef forms oligomers in live cells using a novel fluorescence-based approach known as bimolecular fluorescence complementation (BiFC) (231). Using this assay, we identified critical residues in the Nef dimerization interface essential for oligomerization in vivo (Chapter 3). Here we used these mutants to examine the requirements of oligomerization for Nef-mediated function ranging from the well-characterized downregulation of CD4, the high affinity engagement of the Hck SH3 domain, to overall HIV replication. Partial or complete disruption of the oligomerization interface by mutagenesis completely prevented Nef-induced CD4 downregulation in every case, suggesting that a precise conformation of the Nef oligomer is required for this essential Nef function. Despite the introduction of single or multiple point mutations within the dimerization interface, all of the Nef mutants tested bound the Hck SH3 domain to the same extent as wild-type Nef, suggesting that mutations in the Nef oligomerization interface do not influence the overall fold of the Nef core. Furthermore, disruption of Nef oligomerization significantly impaired HIV replication to levels comparable to that of cells infected with HIV that fails to express Nef. This observation provides the first evidence that oligomerization is critical to Nef-dependent enhancement of HIV replication and identifies the Nef oligomerization interface as a potential target for anti-retroviral drug design.

4.3 RESULTS

4.3.1 An Intact Oligomerization Interface is Required for Nef-induced CD4 Downregulation

The rapid removal of CD4, the primary receptor for HIV, is arguably the most extensively studied property of Nef. CD4 downregulation from the cell surface prevents superinfection (19) and enhances viral replication (45,144). Based on previous observations that some Nef residues critical for CD4 downregulation are located outside of the dimerization interface defined by the Nef crystal structure (139), we explored the requirement for Nef oligomerization in CD4 downmodulation using our panel of BiFC-defective mutants (described in the section 3.3.4). The T-lymphoblast cell line, SupT1 was infected with recombinant retroviruses carrying wild-type or oligomerization-defective Nef mutants, and cell-surface CD4 levels were assessed via flow cytometry. As shown in Figure 19A, SupT1 cells expressing wild-type Nef strongly downregulated CD4 relative to the uninfected control cell population. In contrast, cells expressing the BiFC-negative (oligomerization-defective) Nef mutants 4D and D123N completely failed to downregulate CD4 from the cell surface. Surprisingly, all mutations of the oligomerization interface completely prevented Nef-induced CD4 downregulation, despite partial oligomerization of these Nef mutants as judged by BiFC (Figure 19B and Table 2). These data provide the first direct correlation between Nef oligomerization in cells and CD4 downregulation, and suggests that a highly specific oligomeric conformation of Nef is required to induce CD4 downregulation. Expression of wild-type and mutant Nef protein expression in

the transduced SupT1 population was verified via immunoblotting (Figure 19C). Equivalent levels of Nef expression were observed, with the exception of the Nef-4D mutant which was somewhat reduced. Expression of the 4D mutant was previously and consistently verified in cells via immunofluorescent staining (section 3.3.4, Figure 15) as well as purified for in vitro studies (see below, section 4.3.2) suggesting this difference is most likely due to disruption of the epitope for antibody recognition, which partially overlaps with the site of these mutations.

The Nef-G2A mutant served as a control for the extent of Nef-induced CD4 downregulation, as previous studies have established that myristoylation and membrane targeting are essential for this Nef function (20). As shown in Figure 19B, the reduction of CD4 downregulation by the oligomerization-defective Nef mutants was comparable to that observed with Nef-G2A despite membrane localization of all Nef mutants as confirmed by IF staining. These results demonstrate that localization to the plasma membrane alone is insufficient for this Nef function, and implicate oligomerization as a critical property of Nef required for the removal of CD4 from the surface of HIV-infected cells.

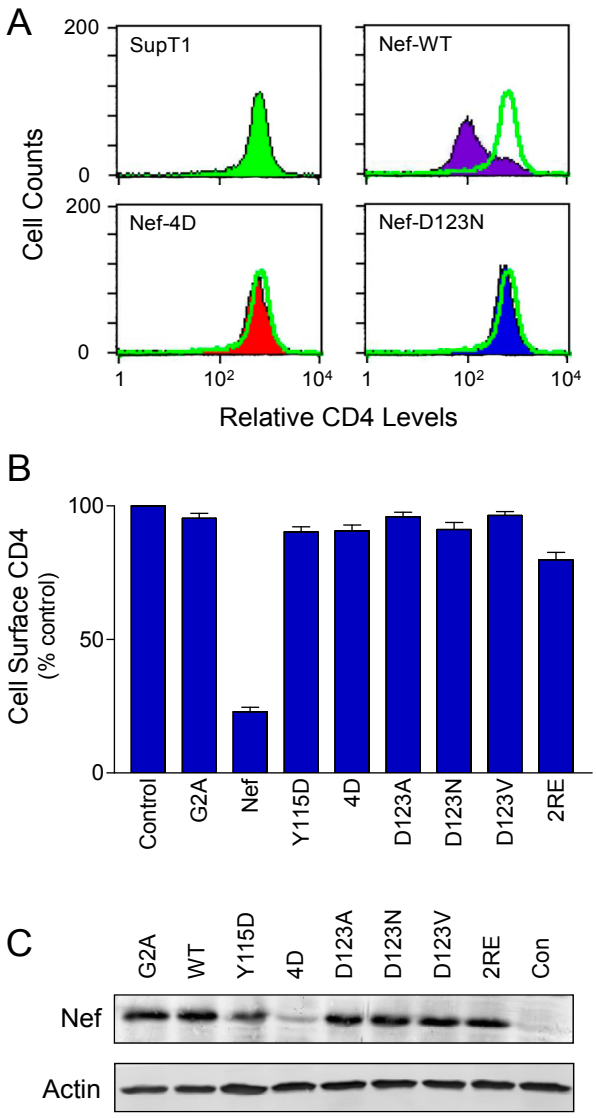


Figure 19. Nef Oligomerization is Required for CD4 Downregulation

(A) SupT1 cells (green) were infected with a wild-type Nef (purple) retrovirus or with retroviruses carrying the oligomerization-defective Nef mutants 4D (red) and D123N (blue). Cell populations were selected with G418, and cell surface levels of CD4 were assessed by flow cytometry as described under Experimental Procedures. (B) Cell-surface expression of CD4 was determined by flow cytometry in SupT1 cells expressing myristoylation-defective Nef (G2A), wild-type Nef (WT), and the Nef dimerization interface mutants Y115D, 4D, D123A, D123N, D123V, and 2RE. Data are expressed as percent downregulation relative to cell-surface CD4 levels observed in uninfected control cells (data as mean ± SD; n=3). (C) Expression of wild-type and mutant forms of Nef was confirmed in the SupT1 cell populations by immunoblotting of cell lysates with anti-Nef antibodies. Actin blots were also performed as loading controls.

4.3.2 Mutations within the Oligomerization Interface do not Affect Engagement of the Hck SH3 Domain

In order to ensure that the loss of CD4 downregulation was not due to misfolding of the Nef core as a result of the mutations, an SH3 domain-binding assay was used to assess the functional activities of several of the oligomerization-defective mutants. Engagement of Nef by the SH3 domain of the Src-family kinase Hck requires not only an intact PxxPxR motif, but also is dependent upon the proper three-dimensional fold of the Nef core region which creates a binding pocket for the RT loop of the SH3 domain (33,132). Indeed, SH3 binding by a polypeptide encompassing only the Nef PxxPxR motif was more than 300-fold weaker in comparison to the Nef core protein (132). To determine the binding activity of the Hck SH3 domain towards the Nef mutants, recombinant immobilized GST-SH3 fusion proteins were incubated with purified recombinant wild-type and mutant Nef proteins containing an N-terminal hexa-histidine tag. A W93A mutant of the Hck SH3 domain served as a negative control for nonspecific binding as it was previously shown to prevent SH3 function (106,192). As shown in Figure 20, all of the oligomerization-defective Nef mutants tested bound the Hck SH3 domain to the same extent as wild-type Nef, suggesting that mutations in the Nef oligomerization interface do not influence the overall fold of the Nef core. No binding was observed with the SH3 W93A mutant.

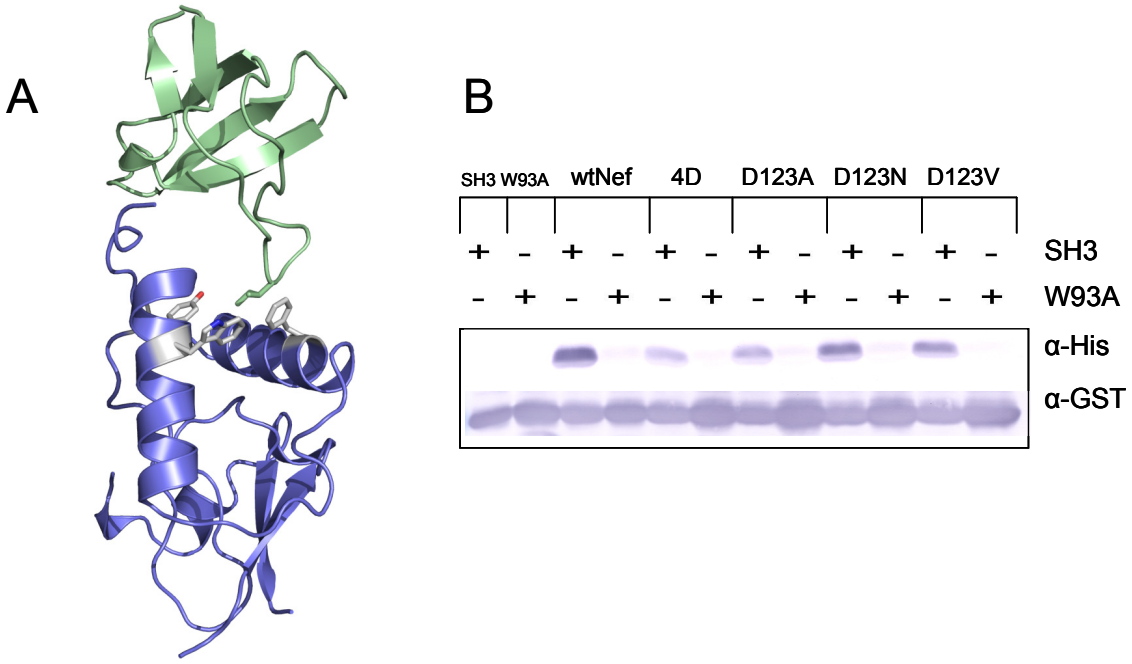


Figure 20. Mutations within the Oligomerization Interface do not Prevent Binding of the Hck SH3 Domain

An SH3 domain-binding assay was used to assess the functional activities of several of the oligomerization-defective mutants. (A) Crystal structure of the Hck SH3 domain (green) bound to Nef (blue) via interaction with the PxxPxR motif and hydrophobic pocket. Side chains of conserved Nef pocket residues that interact with the SH3 domain RT loop are shown (F90, W113, Y120). (B) Recombinant immobilized GST-SH3 fusion proteins were incubated with purified recombinant wild-type and mutant His-tagged Nef proteins. All of the oligomerization-defective Nef mutants tested bound the Hck SH3 domain to the same extent as wild-type Nef. A W93A mutant of the Hck SH3 domain served as a negative control for nonspecific binding.

4.3.3 Nef Oligomerization is Required for High Titer HIV Replication

Results presented in the previous section demonstrate that Nef oligomerization is required for CD4 downregulation. Because removal of CD4 from the host cell surface is critical for optimal HIV replication and pathogenesis (79,144), we next investigated the requirement for Nef oligomerization in HIV replication. For these experiments, each of the Nef mutants evaluated for oligomerization in the BiFC assay were introduced into the Nef coding region of the HIV_{NL43} provirus. The resulting NL43/SF2 hybrid contains a 188 bp region of SF2, a segment that encompasses the entire dimerization interface, with minimal perturbation to the NL4-3 amino composition (Table 4). BiFC was then used to confirm that introduction of this SF2-derived segment did not alter oligomerization, as compared to wild-type Nef-NL43. Two expression vectors were generated for each Nef construct and complementary Venus fragments ($Nef_{NL43/SF2}$-VN + $Nef_{NL43/SF2}$-VC) were co-expressed in 293T cells and examined for complementation. As shown in Figure 21A, strong and virtually identical BiFC fluorescence was observed with both the wild-type NL43 and NL43/SF2 hybrid Nef proteins, supporting the formation of Nef oligomers in each case. To confirm that the NL43/SF2 hybrid did not affect HIV production or replication, 293T cells were transfected with wild-type NL4-3 and NL4-3/SF2 hybrid proviral plasmids. Viral supernatants were collected 48 hour post-transfection, amplified in SupT1 cells and viral titers determined by p24 ELISA. U87MG cells were then infected with equivalent viral titers and the extent of Nef-dependent viral replication monitored by p24 ELISA 3-5 days post-infection, as described in detail below. As shown in Figure 21B, the formation of syncytia, a phenotype indicative of HIV replication was readily observed relative to the HIV_{NL43} provirus in SupT1 cells. Moreover, both Nef proviruses exhibited comparable levels of p24 production

(Figure 21C), further supporting the use of the hybrid $HIV_{NL43/SF2}$ provirus in our replication assays.

NL43	MGGKWSKSSVIGWPAVRERMRRAEPAADGVGAVSRDLEKHGAIT
NL43/SF2	MGGKWSKSSVIGWPAVRERMRRAEPAADGVGAVSRDLEKHGAIT
NL43	SSNTAANNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH
NL43/SF2	SSNTAANNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAALDISH
NL43	FLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVR
NL43/SF2	FLKEKGGLEGLIWSQRRQEILDLWIYHTQGYFPDWQNYTPGPGIR
NL43	YPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPERE
NL43/SF2	YPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPERE
NL43	VLEWRFDSRLAFHHVARELHPEYFKNC
NL43/SF2	VLEWRFDSRLAFHHVARELHPEYFKNC

Table 4. Amino Acid Alignment of Nef-NL43 and NL43/SF2

A 188 bp region of Nef-SF2 was inserted in-frame with NL43 (highlighted). Comparison of the amino acid sequence of Nef NL43 and the NL43/SF2 hybrid reveals a minimal perturbation (red) to the NL43 sequence within this region. The inserted segment encompasses the region containing all of the individual residues previously targeted for mutagenesis in SF2 with respect to oligomerization (underlined). Note that this region is completely conserved between the NL4-3 and SF2 Nef sequences.

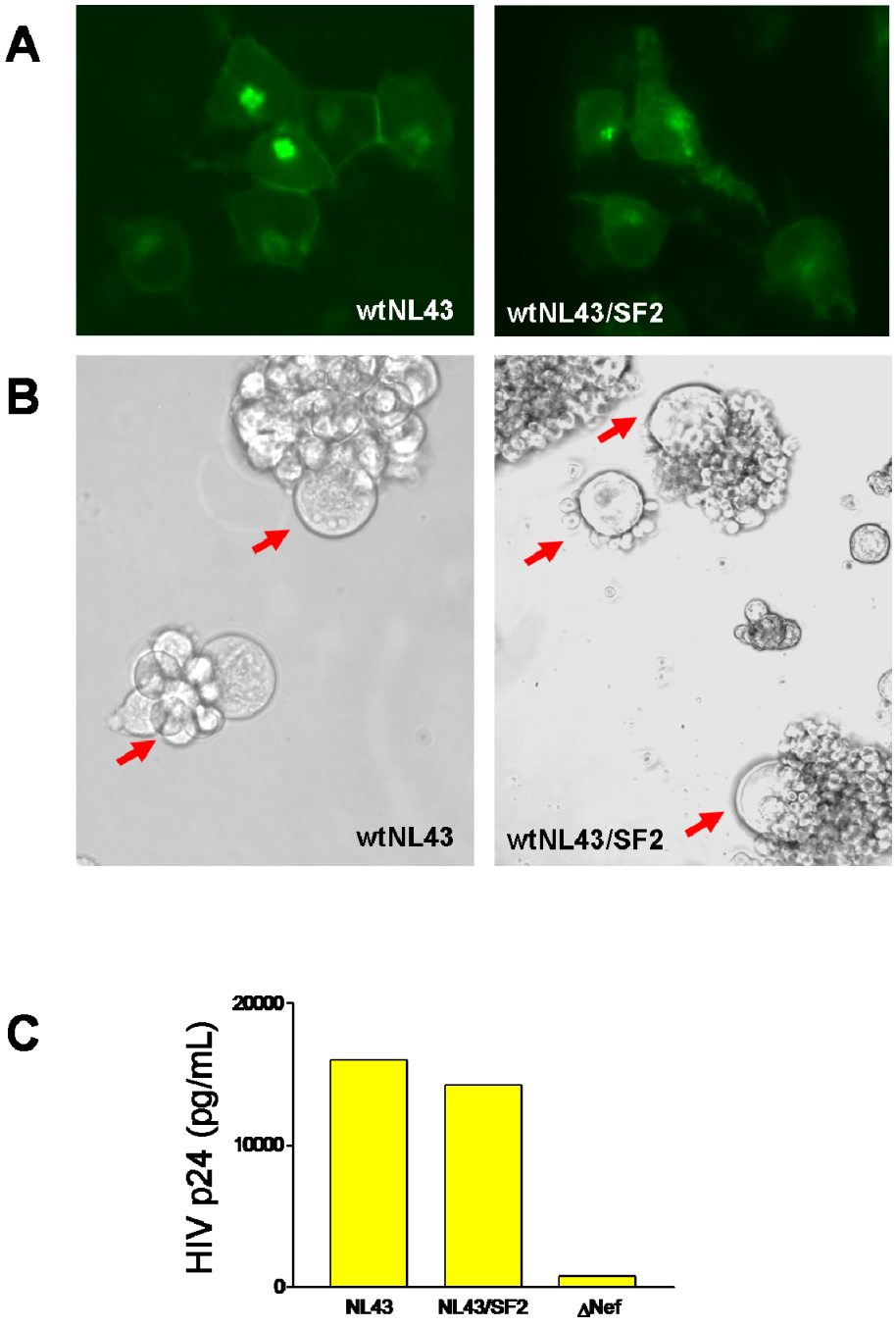


Figure 21. Oligomerization and Replication of Nef-NL43/SF2

The Nef NL43/SF2 hybrid was assayed for oligomerization and viral replication relative to wild-type NL43. (A) BiFC fusion plasmids were generated for NL43 and NL43/SF2 and expressed in 293T cells. A positive BiFC signal was observed in both cases, with fluorescence localizing to the plasma membrane and TGN. (B) SupT1 cells were infected with equivalent viral titers generated via expression of each provirus in 293T cells. Syncytia formation was equally observed 3-5 days post-infections (arrows) and (C) the extent of Nef-dependent viral replication in U87MG-CD4/CXCR4 cells monitored by p24 ELISA 3-5 days post-infection.

Viral replication was then investigated for each of the oligomerization-defective Nef mutants using the astroglioma cell line U87MG, which has been modified to express CD4 and the co-receptor CXCR4. U87MG cells have been used to investigate co-receptor requirements for HIV replication in several previous studies (83,184,208). These cells also provide a useful in vitro system for analysis of Nef-dependent HIV replication, as deletion of Nef from the virus reduces replication by more than 80% (Figure 22).

As described above, the corresponding 188 bp segment of each of the dimerization domain Nef mutants was then used to replace the analogous region in the NL4-3 provirus. The complete coding sequence of each recombinant Nef mutant was then confirmed within the proviral sequence, prior to the initiation of viral production in 293T cells, as described above. U87MG-CXCR4/CD4 cells were then infected with equivalent titers of wild-type or mutant Nef viruses, and the extent of viral replication was monitored by p24 ELISA 3-5 days later. As shown in Figure 22, disruption of the Nef oligomerization interface substantially reduced HIV replication in every case, with an observed 75-85% decrease of replication relative to the wild-type virus. Moreover, replication of HIV carrying the oligomerization-defective Nef mutants was reduced to an equivalent extent as the virus lacking Nef (ΔNef). Taken together, these data show for the first time that a wild-type Nef dimerization interface is a structural feature critical for HIV replication.

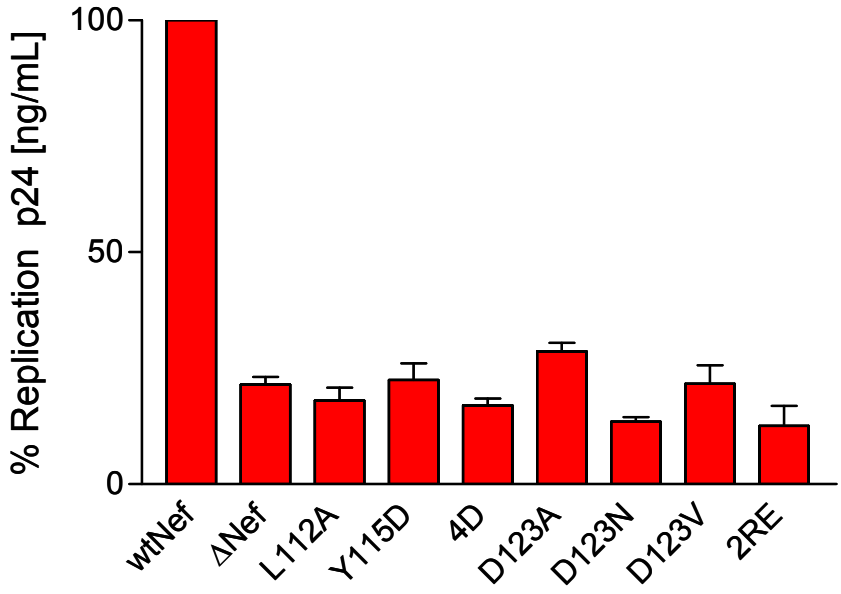


Figure 22. Nef Oligomerization is Required for HIV Replication

U87MG-CD4/CXCR4 cells were infected with equivalent titers of wild-type Nef, ΔNef and the dimerization interface mutants L112A, Y115D, 4D, D123A, D123N, D123V and 2RE. Viral replication was monitored 3-5 days post-infection by quantification of $p24^{CA}$ levels in culture lysates by ELISA.

4.4 DISCUSSION

In this study, we examined the functional consequences of oligomeric disruption on Nef-mediated CD4 downregulation and viral replication. Previous structural studies have identified residues within the Nef core that define a dimerization interface in vitro (133). Highly conserved among isolates of both HIV and SIV Nef, these residues contribute to dimerization through a combination of hydrophobic and electrostatic interactions. Using the BiFC assay, these

interactions were shown in Chapter 3 as essential for maintaining Nef oligomerization, suggesting a similar requirement may be essential for function in vivo. Using these oligomerization-defective Nef mutants, we demonstrate that Nef oligomerization as essential for both CD4 downregulation and Nef-dependent HIV-1 replication. Most unexpected were the profound effects of Nef dimerization interface mutations on HIV replication, identifying this structural feature of Nef as an unexplored target for anti-retroviral therapy.

Defining regions of Nef critical for the induction of CD4 downregulation have been the subject of previous studies and coincidently involved mutagenesis of residues near or within the dimerization interface defined by the X-ray crystal structures (95,139). Results presented here demonstrate a direct correlation between residues critical for CD4 downregulation and those essential for oligomerization in vivo. Without exception, our data reveals that all mutations within the dimerization interface that reduce or abolish oligomerization in cells also interfered with Nef-induced CD4 downmodulation. Notably, the complete loss of oligomerization previously observed when the four key residues that form the hydrophobic core were replaced with charged residues (Nef-4D) or when Asp 123, which ion pairs with Arg 105, was replaced with Asn, resulted in a complete loss of this Nef-mediated function. Interestingly, replacement of Asp 123 with the nonpolar residues Val or Ala reduced but did not abolish dimerization as judged by BiFC, suggesting that these mutants still retained the capacity to oligomerize, albeit with severe functional consequences in terms of CD4 downregulation (see Table 2). Thus, Nef Asp-123 may be a major regulator not only of oligomerization but also of the overall conformation essential for Nef function. Our observation that oligomerization was disrupted to varying degrees in our panel of Nef mutants yet CD4 downregulation was completely lost

suggests that full inhibition of Nef oligomerization is not required to induce a loss of this Nef function.

The loss of Nef-mediated CD4 downregulation in all Nef mutants whether exhibiting partial or complete oligomeric disruption suggested mutations within the oligomerization interface might disturb the proper tertiary structure required for function. Therefore, an SH3 domain-binding assay was used to assess the binding activity of the Hck SH3 domain towards the Nef mutants. A representative group of Nef mutants was chosen to assess the effects of altering the hydrophobic or electrostatic interactions within the oligomerization interface on the overall Nef fold. Although several Nef mutants possess mutations within the hydrophobic interface (L112A, F121A, L112D, Y115D, DD), the quadruple Nef mutant 4D (I109D/L112D/Y115D/F121D) was chosen as it not only includes all of the single residue mutations but was also predicted to have the greatest effect on folding. Further, Nef mutants contributing to the ion pairs within the dimerization interface were represented by mutants D123A, D123N, D123V. The varying degrees of oligomeric disruption previously observed (Table 2), suggested the D123 residue was a major contributor to the overall confirmation and may produce non-functional dimers. Despite the presence of these mutations, each of these recombinant Nef proteins retained the ability to interact with the SH3 domain (Figure 20). As binding of the SH3 domain is dependent upon the proper three-dimensional fold of the Nef core region, retention of Nef:SH3 interaction by these mutants suggests that mutations in the Nef oligomerization interface do not influence the overall folding of Nef.

In a final series of experiments, we examined the impact of oligomeric interference on HIV replication. Our data demonstrate for the first time that Nef oligomerization is essential for active viral replication in cell culture. In conjunction with the loss of CD4 downregulation,

comparable loss of replication between viruses that fail to express Nef ($HIV_{NL43\Delta Nef}$) and those expressing each of the oligomerization-defective Nef mutants, suggest that Nef oligomerization is essential for HIV replication, at least in the U87MG system where viral replication in Nef-dependent. Interestingly, a functional link between Nef-induced CD4 downregulation and HIV replication has been reported (45,62,144), suggesting that the observed effects of oligomerization interface mutations on CD4 and HIV replication may be related. Further, the loss-of-function phenotypes observed independent of single (L112A, Y115D, D123A, D123N, D123V) or multiple (4D and 2RE) point mutations suggest a wild-type Nef conformation is essential to Nef function.

The results presented here may help to explain the rationale behind the previously observed loss of function phenotype associated with various Nef mutants. Our data provide the first cell-based evidence that mutations in the Nef dimerization interface that correlate with the loss of these Nef functions do indeed affect Nef oligomerization. Our results further support previous reports that downmodulation of CD4 by Nef may positively impact HIV replication (79,144). We establish for the first time that disruption of a single target, the Nef dimerization interface, inhibits multiple functional attributes of Nef. Accordingly, despite the highly stable core region of Nef, as evidenced via BiFC and SH3 binding assays, the overall impact of modest oligomeric interference within this region further suggests a highly conserved and attractive target for antiretroviral therapy.

4.5 EXPERIMENTAL PROCEDURES

4.5.1 Cell Culture and Reagents

The cell lines used in this study were obtained from the ATCC and include 293T, U87MG, and SupT1. All cells were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 5% fetal bovine serum (FBS; Atlanta Biological) and treated with antibiotic-antimycotic (Invitrogen). U87MG cells were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS and 50 µg/mL gentamycin (Invitrogen). U87MG-CD4/CXCR4 cells (generous gift of Dr. Toshiaki Kodama, University of Pittsburgh School of Medicine) were cultured in DMEM supplemented with 25 mM HEPES, 10% FBS, 300 µg/mL G418 and 0.5 µg/mL puromycin. SupT1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS and antibiotic-antimycotic. 293T cells were transfected using either standard calcium phosphate techniques as described elsewhere (26) or Fugene 6 (Roche).

4.5.2 Retroviral Transduction of Nef Mutants

Nef-mutant constructs were subcloned into the retroviral expression vector pSRαMSVtkneo, which carries a G418 resistance marker to enforce expression of Nef-mutant proteins by antibiotic selection. High-titer retroviral stocks were produced in 293T cells by cotransfection with Nef-encoding retroviral and amphotropic packaging vectors as described elsewhere (231).

Retroviral stocks were supplemented with Polybrene (Sigma) to 4 μg/mL and added to SupT1 cells in 6-well plates (2.5 x 10^5 cells/well). The plates were centrifuged at 1000*g* for 4 h at 18 °C to enhance infection efficiency. Two days later, cells were resuspended in 5 mL selection media containing G418 (800 μg/mL). Expression of Nef mutants was verified via immunoblotting.

4.5.3 Immunoblotting

To detect the expression of Nef mutants, cells were lysed in 0.5 mL Hck lysis buffer (50mmTris-HCl (pH 7.4), 50mM NaCl, 1mM EDTA, 10mM $MgCl_2$, 1% (v/v) Triton X-100) containing 2 mM PMSF, 25 μg/mL leupeptin, 10 μg/mL aprotinin, 25 nM NaF, and 2 mM Na_3VO_4 and incubated on ice for approximately one hour. Lysates were heated in equivalent volumes of SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane and probed for Nef by immunoblotting with anti-Nef antibodies. Nef antibodies (monoclonal EH1 and Hyb 6.2) were obtained from the NIH AIDS Research and Reference Reagent Program.

4.5.4 Flow Cytometric Analysis

SupT1 cells were infected with the recombinant retroviruses and selected with G418 (800 μg/mL) for two weeks and Nef protein expression was confirmed by immunoblotting. Cells

were stained for cell-surface CD4 in FACS medium (2% fetal bovine serum in PBS) containing an allophycocyanin (APC)-conjugated anti-human CD4 monoclonal antibody (clone RPA-T4; BD Pharmingen). Cells were analyzed on a FACSCalibur flow cytometer using CellQuest Pro software (BD Pharmingen).

4.5.5 Expression of GST-SH3 Fusion Proteins and Nef-SH3 Binding Assay

For GST-SH3 fusion protein expression, PCR fragments encoding the SH3 domains of wild-type and mutant Hck (W93A) were subcloned into the bacterial expression vector pGEX-2T (Amersham Biosciences). Wild-type and mutant forms of HIV-1 Nef-SF2 were subcloned into the bacterial expression vector pET14b. GST-SH3 fusion proteins were expressed in *E. coli* strain DH5α and immobilized on GSH-agarose beads as described elsewhere (192). Nef proteins were expressed in BL21(DE3)pLysS cells via N-terminal His-tags as described (207). Equimolar amounts of Nef (2μg) and immobilized GST-SH3 proteins were incubated in 500 μL incubation buffer (2% BSA+ 50mmTris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% (v/v) Triton X-100) and rotated at 4°C for 2 hours. Nef-SH3 complexes were pelleted by centrifugation, washed extensively in RIPA buffer (50mmTris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% SDS and 1% sodium deoxycholate) and resolved by 12% SDS-PAGE and complexes detected by immunoblotting.

4.5.6 Bimolecular Fluorescence Complementation Expression Vectors

The coding sequence for Nef was excised from the NL4-3 provirus (in pUC18) and subcloned into pGL3-Basic (Promega) via the unique restriction sites BamHI/NcoI. A 188 bp Bsu36I/EcoRV fragment was cut from the coding region of wild-type and mutant forms of Nef used in the BiFC experiments (SF2 strain) and subcloned into the pGL3-Basic/Nef vector to generate hybrid NL4-3/SF2 Nef alleles. The coding regions of these Nef hybrids were then amplified by PCR and subcloned cloned into pcDNA3.1(-) for construction of BiFC fusion plasmids. Two Nef-BiFC expression vectors were created in which the N-terminal coding sequence of the YFP-variant Venus (residues Val2-Asp173) was PCR-amplified from the plasmid vector pCS2-Venus and ligated into the mammalian expression vector pcDNA3.1(-) (Invitrogen) containing Nef via a unique Acc III restriction site found in close proximity to the Nef C-terminal coding region. A similar approach was used to fuse the C-terminal coding region for Venus (residues Ala154-Lys238) to Nef. In both instances, C-terminal fusions were used to preserve the native N-terminal myristoylation signal sequence of Nef. The Venus template was a kind gift of Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan.

4.5.7 Generation of Mutant Proviruses and HIV Replication Assays

To create Nef mutant proviruses, the coding regions of the Nef-NL4-3/SF2 hybrid (see section 4.5.7) was subcloned back into the NL4-3 provirus via BamHI/NcoI. The sequence of the mutant *nef* alleles as well as flanking regions was confirmed within the context of the proviral

DNA. The wild-type HIV-1_{NL4-3} and HIV-$1_{Nef-NL4-3/SF2}$ proviral constructs were then transfected into 293T cells using FuGENE6 (Roche) using the manufacturer's protocol and viral supernatants collected 48 h post-transfection. SupT1 cells (5 x 10^5) were infected with 200 μL viral supernatant, and incubated for 2 h at 37 °C. Infected cells were washed with serum-free RPMI and centrifuged at 1500 x *g* for 4 min at room temperature and resuspended in 6 mL of RPMI supplemented with 10% FBS. Upon syncytia formation (3-5 days post-infection), amplified viral supernatants were clarified by centrifugation at 1500 x *g* for 4 min and viral titers were determined by p24 ELISA (SAIC-Frederick, Inc). Viral replication was monitored in U87MG-CD4/CXCR4 cells by infection with a serial dilution of each viral preparation with a starting titer of 250 ng p24/ml. Replication of Nef oligomerization mutant proviruses was measured relative to wild-type virus and quantified via p24 ELISA. All ELISA plates were read on a Multiscan MCC341 Microplate Reader (Finstruments) and analyzed using Spectrosoft software.

4.5.8 Microscopy and Fluorescence Imaging

The 293T cells used for the Nef-NL4-3/SF2 BiFC studies were imaged 24 to 48 hours post-transfection, while bright-field images of syncytia formation representative of 72-96 hours post-infection. All images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT cooled CCD high-resolution digital camera and software (Diagnostic Instruments).

4.6 FOOTNOTES

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5.0 OVERALL DISCUSSION

5.1 SUMMARY OF MAJOR FINDINGS

For this thesis project, I set forth to build upon previous biochemical and structural evidence that suggested Nef may form oligomers via homotypic interactions in HIV-infected cells. For this study, I adapted a bimolecular fluorescence complementation (BiFC) assay in which dimeric protein interactions are observed in live cells by reconstituting a functional YFP (or any GFP variant) structure (109,110). Using this assay, I provided the first direct evidence for Nef oligomerization in live cells. I then expanded the study of Nef oligomerization using cell lines that support HIV replication using the U87MG astroglioma and SupT1 T-lymphoblast cell lines, in which positive BiFC signals were readily observed. I next demonstrated the generality of oligomerization (via BiFC) by a group of Nef alleles broadly representative of all major (M-group) HIV-1 subgroups, A through K. Taken together, these findings provide the first evidence of Nef oligomerization in a wide variety of host cell environments and support the conclusion that oligomerization is an intrinsic property of the HIV-1 *nef* gene product.

One of the principal advantages of the BiFC assay is the ability to observe the formation of oligomeric complexes within a subcellular context. Previous localization studies have indicated that Nef is targeted to the cellular membrane via the post-translational attachment of myristic acid to its N-terminus and also persists in the cytoplasm and trans-Golgi network (TGN)

(25,77,166,221). Therefore, I introduced a point mutation (G2A) into the Nef myristoylation signaling motif Met-Gly-X-X-X-Ser/Thr, consequently removing Nef from the plasma membrane. Nef has also been shown to bind with high affinity to the SH3 domains of Src-family kinases and other proteins via its conserved PxxPxR motif. Therefore, in a separate experiment, I also replaced the key proline residues with alanine (2PA) to prevent additional proteins from influencing or possibly driving Nef-BiFC. Using the BiFC assay, I discovered that Nef oligomerization is independent of membrane localization and does not require interactions with other proteins through the PxxPxR motif. In addition to the plasma membrane, I observed a consistent localization of Nef BiFC signals to the perinuclear region in all three cell types examined, suggesting Nef oligomers also localize to the TGN. Coincident with immunofluorescent staining for the TGN marker protein, TGN-46, I discovered Nef oligomers were present in the TGN, a localization essential for Nef-mediated MHC downregulation (21,111,166). Thus, the BiFC assay is a useful assay for the both the identification and localization of protein:protein interactions in vivo.

Previous X-ray crystallographic studies indicate that multiple contact points of highly conserved amino acids comprise the Nef dimerization interface (12,133). To define directly these residues in vivo using the BiFC assay, I devised a systematic strategy for the mutagenic profiling of the oligomerization interface. Coupled with Nef-directed immunofluorescence as positive control for expression of mutant BiFC pairs, I found that detection of Nef oligomerization using BiFC is highly efficient, with a positive BiFC signal present in virtually every cell expressing Nef. Using this approach to screen Nef mutants, I discovered two possible classes of residues were critical to Nef oligomerization in vivo. First, although changes designed to disrupt hydrophobic interactions within the helical dimer interface significantly reduced but did not

abolish the Nef BiFC signal (L112A, L112D, Y115D, F121A and DD), BiFC was completely abolished once all four key hydrophobic residues were simultaneously removed (Nef-4D). Secondly, disruption of electrostatic interactions mediated by D123 and R105 via substitution of Asp-123 with asparagine was sufficient to also abolish BiFC. However, I found that substitution of Asp-123 with alanine or valine reduced but did not abolish BiFC, suggesting that these mutants may form destabilized oligomers. Taken together, using the BiFC assay and site-directed mutagenesis, I show for the first time that the dimerization interface defined by X-ray crystallography is indeed essential for Nef oligomerization in cells.

Utilizing the Nef mutants identified in the mutagenic profiling of the oligomerization interface, I expanded our studies to explore the effects of oligomeric disruption on Nef function. As perhaps the best characterized and most essential functions of Nef, I explored Nef-mediated enhancement of CD4 downregulation as well as Nef-dependent replication of HIV. Using Sup T1 lymphoblasts as a model system, I found that wild-type Nef strongly downmodulated CD4 as observed in many other HIV host cells. However, SupT1 cells expressing BiFC-negative (oligomerization-defective) mutants completely failed to downregulate CD4 from the cell surface. Surprisingly, despite varying effects on oligomerization, all Nef mutants failed to downregulate CD4, suggesting a highly specific oligomeric conformation of Nef may be required to induce CD4 downregulation. This is the first report to provide a direct correlation between Nef oligomerization and the removal of CD4 from the cell surface.

Using a cell line where HIV replication is dependent upon Nef (U87MG astroglioma cells), I also evaluated the effects of oligomeric disruption on viral replication. Virtually identical to the results obtained from the CD4 assay, disruption of the Nef oligomerization interface dramatically reduced HIV replication in every case. Interestingly, a functional link between Nef-

induced CD4 downregulation and HIV replication has been reported (45,62,144), suggesting that the observed effects of the oligomerization interface mutants on CD4 and HIV replication shown here may be related. Evidence for this relationship is supported by the observance that viral egress is inversely proportional to the surface expression of CD4 (23,168,180). Thus, an increase in virion dissemination leads to a higher incident of infection and levels of viral replication. Further, as Nef independently binds both CD4 and the p6 region of GagPol, failure to remove CD4 from the cell surface may inhibit the latter interaction through competitive inhibition. Nef-mediated downregulation of CD4 may thus promote Nef:GagPol interactions which is important for retention of Nef in the virion which facilitates viral budding (42). Moreover, I found that replication of HIV variants carrying oligomerization-defective Nef mutants were reduced to an equivalent extent as virus that fails to express Nef. Taken together, it is likely that oligomerization may be essential for other Nef functions during the course of HIV infection, which will be addressed in future work (section 5.2.1).

5.1.1 Implications of BiFC Assay

Prior to this thesis, and despite numerous biochemical and structural studies (12,13,71,93-95,133), the oligomeric status of the HIV-1 *nef* gene product, in vivo, remained unknown. Using the BiFC assay I was able to demonstrate for the first time that Nef forms *at least* dimers in live cells. While the BiFC assay is a powerful tool for the in vivo analysis of protein:protein interactions (24,91,109,110,112,155), it does not indicate the possible formation of higher-order oligomeric complexes. However, the very nature of the assay affords the possibility of detecting

trimeric complexes by coupling BiFC with FRET, as recently described (198). Although the BiFC-FRET assay presents a unique strategy for the visualization of oligomeric complexes, the homotypic nature of Nef interactions presents a unique challenge as each BiFC fusion protein is competing with the expression of its own non-complementing fragment. Interestingly, the biochemical and structural data supporting the formation of Nef trimerization (13), albeit at lower frequencies, identifies the same residues that comprise the dimerization interface as involved in establishing the trimer interface. Therefore, the mutational studies presented here relative to the dimerization interface are predicted to elicit the same oligomeric disruption of at least some multimeric Nef complexes.

The partial BiFC signal observed for several Nef mutants (L112A, L112A, Y115D, F121A, DD, D123A, D123V, R105E, 2RE) coupled with the loss of function in CD4 downregulation and HIV replication suggests the formation of non-functional dimers. Partial BiFC responses are somewhat difficult to interpret. One thought is that the mutants cause a destabilization in the interaction interface, and lower the Kd for Nef:Nef interaction. However, because YFP is completely stable once reconstituted, this may provide a driving force to "pull" weakly interacting (and possibly non-functional) dimers together, leading to the low levels of BiFC fluorescence observed with some of the mutants. Hydrogen exchange/mass spectrometry may offer clues as to the mechanism of action resulting in the loss of function observed (213). For these in vitro studies, the pCDFDuet-1 expression system (Novagen) will be used for the simultaneous expression of the Nef-BiFC proteins. An N-terminally labeled hexa-histidine tag of one of the BiFC pairings (H6-Nef-YN + Nef-YC) favors the direct purification of Nef dimers. Comparison of wild-type and oligomeric-defective Nef mutants via hydrogen exchange may be

useful in determining the dynamic or structural changes within the dimerization interface of non-functional yet oligomeric Nef mutants.

5.1.2 Implications of Nef Oligomerization

Although the role of Nef as a critical determinant of HIV pathogenesis has been well documented (52,54,99,100,120,121,226,227) and numerous motifs identified as essential for function (Figure 6), a single unifying element has yet to be identified within the Nef protein as essential for its functions. As Nef oligomerization was shown to be essential for the downregulation of CD4 and HIV replication, the work presented here suggests a new mechanism for the regulation of Nef function. Oligomerization is a common biological regulatory mechanism and signaling is often regulated by the dimerization of signaling proteins (125). Interestingly, multiple cellular targets of Nef are dimeric when activated, including CD4 and MHC-I (30,224). As binding energies are additive, the oligomerization of Nef may increase its affinity for a dimeric partner, leading to more efficient interactions required for its subsequent removal from the cell surface. Additionally, Nef oligomerization may also increase the association of protein complexes, effectively driving the interactions required to initiate specific functions. For instance, the internalization of cell-surface CD4 occurs through an endosomal/lysosomal pathway, a process mediated by clathrin-pit formation via the intracellular host proteins AP-2,/NBP1 and β-Cop (164,188,229). As Nef directly associates with each of these proteins (43,44,58,84,142), Nef oligomerization may enhance the removal of CD4 from the

cell surface by recruiting and activating each of these cellular components required for initiating the degradation pathway.

The effects of Nef oligomerization are not limited to CD4 downregulation. As reported here, the expression of oligomerization-defective Nef mutants resulted in a dramatic reduction in the overall efficiency of viral replication suggesting Nef oligomerization may influence numerous viral pathways. A recent computational analysis has suggested that Nef may interact with over 300 cellular host factors (203); whether each of these interactions is essential for Nef function and is dependent upon the oligomeric Nef structure will be a major area of investigation in the future. The commonality of Nef oligomerization across a broadly representative group of HIV subtypes further suggests oligomerization is highly conserved attribute utilized by Nef to promote disease progression in HIV infected cells. Although a comprehensive examination of all specific interactions dependent upon Nef oligomerization for the enhancement of function remains to be fully investigated, the work presented here suggests the formation of functional oligomers is very sensitive to any modifications within the oligomerization interface, identifying this region of as a structurally unique target for anti-retroviral drug discovery.

5.2 FUTURE DIRECTIONS

5.2.1 Identification of Oligomerization-Mediated Nef Functions

Now that Nef oligomerization has been demonstrated in vivo, the formation of higher-order oligomeric complexes needs to be investigated. Although the principal contacts within the dimerization interface of Nef are maintained within the modeling of a trimeric complex (13), elucidating the true oligomeric status of Nef is essential for understanding the mechanism of interactions relative to its function. Recently published, a FRET-based BiFC assay for the identification of trimeric complexes is now possible (199). Nef-BiFC-FRET plasmid constructs could be generated using the GFP variants CFP and YFP as previously demonstrated (199,231). Further, using multi-color BiFC additional binding partners can be identified within the context of HIV infection. Multi-color BiFC permits the detection of multiple Nef binding partners as each specific interaction examined is visualized with a distinct GFP variant (110). Of particular significance are the proteins associated with the promotion of disease progression within HIV infected cells. Such proteins of interest include those associated with increased viral infectivity, cell signaling, immune surveillance and T-cell activation: Vav (29,61,141), Src family kinases (101,126), MHC-I (21,179,194,220), and IL-2 (6,177), respectively. Utilizing the oligomerization-defective Nef mutants identified in this study, the requirement for oligomerization can then be expanded to proteins identified via multi-color BiFC.

The precise subcellular location of Nef oligomers also alludes to possible sites of Nef:target protein interactions. Having identified Nef oligomers as present in the TGN, a subcellular site of kinase and major histocompatibility complex class (MHC) trafficking (16,21,111,130,186), Nef oligomerization may play a role in the activation or retention of these

proteins, respectively. Indeed, enforced dimerization of Nef significantly enhanced the activation of the Src family kinase, Hck (231), an interaction which has subsequently been linked to MHC retention in the TGN. Given my findings regarding the requirement of oligomerization for CD4 downregulation, a similar requirement may be predicted to elicit the activation or removal of these signaling proteins. However, relative to the intracellular concentration of Nef that down-modulates surface CD4, an order of magnitude or greater increase in Nef expression was required for a comparable downmodulation of MHC-I (140,214). In order to achieve adequate Nef expression, an adenoviral infection, rather than the current retroviral system may be required. Further, infection with HIV carrying wild-type vs. oligomerization-defective Nef mutants may provide the most meaningful analysis of the requirement for this Nef property in the removal of both CD4 and MHC-I from the cell surface, rather than use of more artificial over-expression systems that use Nef alone. The identification of specific Nef interactions and the requirement of oligomerization for their various functions would only further substantiate the role of Nef oligomerization in disease progression.

5.2.2 HIV Replication

Additional studies investigating the effects of oligomeric disruption on Nef-mediated enhancement of viral replication in primary cell types need to be explored. Examination of viral replication in physiologically relevant cell lines such as primary $CD4^+$ T cells and monocyte-derived macrophages (MDM) would provide conditions more closely related to that of an infected host, as described previously (27,135,144). Although the requirement for

oligomerization in primary cell types remains unknown, reports have shown that Nef-mediated CD4 downregulation stimulates HIV production (180) and infectivity (41,129). Accordingly, since my work showed that oligomerization was required for CD4 downregulation, one might predict that HIV replication and infectivity would be drastically reduced in primary $CD4^+$ T cells. To test this hypothesis, Nef-mutant viral stocks generated in this study can be used to infect primary cells and the effects of oligomeric-disruption monitored relative to levels of replication via $p24^{CA}$ ELISA as well as CD4, co-receptor and MHC downregulation via FACS analysis.

Perhaps a more ambitious goal is to generate transgenic (Tg) mice expressing the complete coding sequences of HIV-1, including those sequences containing oligomerization-defective Nef mutants, in $CD4^+$ T cells and in cells of the macrophage/dendritic lineages, as described previously (99-101). Use of Tg mice would generate a viable animal model of disease progression relative to Nef oligomerization. In addition to the effects on viral replication and receptor downregulation, a Tg model would allow the observance of several phenotypes remarkably similar to those observed in HIV-1 infected individuals, as described previously (99,100,119,169,170,217). Similarly, an SIV model of Nef oligomerization-defective mutants in rhesus monkeys could be generated as previously described (120,121). More specifically an SHIV model (114,147), in which the coding regions of SIV and HIV Nef are interchanged, would more closely model human disease progression relative to the Nef mutants described here.

5.2.3 Nef-Directed High-Content Screening (HCS)

The results presented in this thesis suggest that disrupting Nef oligomerization with chemical compounds may dramatically inhibit HIV replication. By adapting the cell-based BiFC assay to a high-throughput format, chemical libraries can be rapidly screened for Nef-directed compounds that block oligomerization. Using ArrayScan technology (145,209-211,222), cells could be transfected with wild-type Nef BiFC constructs and plated in a 384-well format such that chemical hits can be identified as a loss of BiFC fluorescence. However, one of the disadvantages of the BiFC assay is the irreversible reconstitution of a functional YFP protein. Thus, Nef oligomers that form prior to the introduction of compounds will likely remain fluorescent. To avoid false negatives, cells may be incubated with compounds prior to transfection with BiFC expression vectors. However, the irreversibility of BiFC would limit the screen to the identification high-affinity chemical compounds, as only tightly bound compounds would be sufficient to block Nef oligomerization and consequently prevent the reconstitution of YFP. Positive hits should also be tested against the GST-BiFC control plasmids to ensure the compounds are directed towards the oligomerization interface of Nef and not the interface of the BiFC fragments. To ensure the compounds used in this study do not interfere with protein expression or enhance its degradation, cells will be counterstained with anti-Nef antibodies and compared to untreated cells. The identification of inhibitors of Nef oligomerization are likely to affect CD4, CXCR4/CCR5 and MHC downregulation, SFK kinase activation and viral replication, and will be further verified in the Nef-dependent U87MG viral replication assay as described in Chapter 4. The positive identification of inhibitors of Nef oligomerization will then be tested against the panel of broad representatives of all major Nef subtypes with respect to both oligomerization and viral replication. Completion of these preclinical experiments will provide a

sound foundation for animal testing in either the Nef-Tg mouse model or ultimately in the Macaque model of HIV disease.

5.3 CLOSING REMARKS

Since the identification of the HIV-1 *nef* gene product as a critical determinant of AIDS progression, researchers have spent countless hours identifying, modifying, rejecting and confirming the molecular mysteries behind this intriguing auxiliary protein of HIV. Multi-faceted in its function, Nef alters numerous signaling pathways in order to mediate cell survival and the enhancement of viral infectivity and replication. Although biochemical and structural analysis of the Nef protein have supported the formation of oligomeric complexes, a structural characteristic perhaps relevant to its function, both have remained largely undefined.

However, the advent of a new fluorescent assay (BiFC) provided a new strategy for the examination of oligomeric proteins in live cells. In this dissertation, I have attempted to advance the understanding of the human immunodeficiency virus by first identifying and then exploring the requirement of a structurally distinct feature of the HIV-1 Nef protein for its role in disease progression. In the process, I have provided the first direct evidence for Nef oligomerization and its respective subcellular locations in live cells as well as its commonality across Nef subtypes, identified residues required for maintaining the dimerization interface, and discovered that oligomerization is required for the Nef-mediated functions of CD4 downregulation and HIV replication.

2009 marks the 10th anniversary of the paradigm shift in Nef-related research. No longer identified as an inhibitor of HIV transcription, it is now evident Nef plays a much greater role in the enhancement of disease progression in HIV infected individuals. It is my hope that the hours spent and the work presented in this thesis will contribute to the field of HIV research and possibly to the opening of new avenues for the development of HIV therapeutics for those suffering from HIV/AIDS.

* In light of the evidence presented in this dissertation and that of many researchers before me, perhaps it is time to rename the improperly named negative factor, Nef. In accordance with previous nomenclature and its re-defined pleiotropic nature, I put forth a new term: positive effector, Poe.

Reference List

1. 1981 Pneumocystis pneumonia--Los Angeles *MMWR Morb. Mortal. Wkly. Rep.* 30, 250-252

2. **Ahmad, N. and Venkatesan, S.** 1988 Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR *Science* 241, 1481-1485

3. **Aiken, C.** 1997 Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A *J. Virol.* 71, 5871-5877

4. **Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E., and Trono, D.** 1994 Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain *Cell* 76, 853-864

5. **Aiken, C. and Trono, D.** 1995 Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis *J. Virol.* 69, 5048-5056

6. **Alexander, L., Du, Z., Rosenzweig, M., Jung, J. U., and Desrosiers, R. C.** 1997 A role for natural simian immunodeficiency virus and human immunodeficiency virus type 1 Nef alleles in lymphocyte activation *J. Virol.* 71, 6094-6099

7. **Allan, J. S., Coligan, J. E., Lee, T. H., McLane, M. F., Kanki, P. J., Groopman, J. E., and Essex, M.** 1985 A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients *Science* 230, 810-813

8. **Aloia, R. C., Tian, H., and Jensen, F. C.** 1993 Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes *Proc. Natl. Acad. Sci. U. S. A* 90, 5181-5185

9. **Anderson, S., Shugars, D. C., Swanstrom, R., and Garcia, J. V.** 1993 Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells *J. Virol.* 67, 4923-4931

10. **Arganaraz, E. R., Schindler, M., Kirchhoff, F., Cortes, M. J., and Lama, J.** 2003 Enhanced CD4 down-modulation by late stage HIV-1 nef alleles is associated with increased Env incorporation and viral replication *J. Biol. Chem.* 278, 33912-33919

11. **Arien, K. K., Vanham, G., and Arts, E. J.** 2007 Is HIV-1 evolving to a less virulent form in humans? *Nat. Rev. Microbiol.* 5, 141-151

12. **Arold, S., Franken, P., Strub, M. P., Hoh, F., Benichou, S., Benarous, R., and Dumas, C.** 1997 The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3

domain suggests a role for this complex in altered T cell receptor signaling *Structure.* 5, 1361-1372

13. **Arold, S., Hoh, F., Domergue, S., Birck, C., Delsuc, M. A., Jullien, M., and Dumas, C.** 2000 Characterization and molecular basis of the oligomeric structure of HIV-1 nef protein *Protein Sci.* 9, 1137-1148

14. **Arold, S., O'Brien, R., Franken, P., Strub, M. P., Hoh, F., Dumas, C., and Ladbury, J. E.** 1998 RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef *Biochemistry* 37, 14683-14691

15. **Arold, S. T. and Baur, A. S.** 2001 Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein *Trends Biochem. Sci.* 26, 356-363

16. **Atkins, K. M., Thomas, L., Youker, R. T., Harriff, M. J., Pissani, F., You, H., and Thomas, G.** 2008 HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: analysis using short interfering RNA and knock-out mice *J. Biol. Chem.* 283, 11772-11784

17. **Baur, A. S., Sawai, E. T., Dazin, P., Fantl, W. J., Cheng-Mayer, C., and Peterlin, B. M.** 1994 HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization *Immunity.* 1, 373-384

18. **Benichou, S., Bomsel, M., Bodeus, M., Durand, H., Doute, M., Letourneur, F., Camonis, J., and Benarous, R.** 1994 Physical interaction of the HIV-1 Nef protein with beta-COP, a component of non-clathrin-coated vesicles essential for membrane traffic *J. Biol. Chem.* 269, 30073-30076

19. **Benson, R. E., Sanfridson, A., Ottinger, J. S., Doyle, C., and Cullen, B. R.** 1993 Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection *J. Exp. Med.* 177, 1561-1566

20. **Bentham, M., Mazaleyrat, S., and Harris, M.** 2006 Role of myristoylation and N-terminal basic residues in membrane association of the human immunodeficiency virus type 1 Nef protein *J. Gen. Virol.* 87, 563-571

21. **Blagoveshchenskaya, A. D., Thomas, L., Feliciangeli, S. F., Hung, C. H., and Thomas, G.** 2002 HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway *Cell* 111, 853-866

22. **Bonyhadi, M. L., Rabin, L., Salimi, S., Brown, D. A., Kosek, J., McCune, J. M., and Kaneshima, H.** 1993 HIV induces thymus depletion in vivo *Nature* 363, 728-732

23. **Bour, S., Perrin, C., and Strebel, K.** 1999 Cell surface CD4 inhibits HIV-1 particle release by interfering with Vpu activity *J. Biol. Chem.* 274, 33800-33806

24. **Bracha-Drori, K., Shichrur, K., Katz, A., Oliva, M., Angelovici, R., Yalovsky, S., and Ohad, N.** 2004 Detection of protein-protein interactions in plants using bimolecular fluorescence complementation *Plant J.* 40, 419-427

25. **Breuer, S., Gerlach, H., Kolaric, B., Urbanke, C., Opitz, N., and Geyer, M.** 2006 Biochemical indication for myristoylation-dependent conformational changes in HIV-1 Nef *Biochemistry* 45, 2339-2349

26. **Briggs, S. D., Scholtz, B., Jacque, J. M., Swingler, S., Stevenson, M., and Smithgall, T. E.** 2001 HIV-1 Nef promotes survival of myeloid cells by a Stat3-dependent pathway *J. Biol. Chem.* 276, 25605-25611

27. **Brown, A., Moghaddam, S., Kawano, T., and Cheng-Mayer, C.** 2004 Multiple human immunodeficiency virus type 1 Nef functions contribute to efficient replication in primary human macrophages *J. Gen. Virol.* 85, 1463-1469

28. **Buchbinder, S. P., Katz, M. H., Hessol, N. A., O'Malley, P. M., and Holmberg, S. D.** 1994 Long-term HIV-1 infection without immunologic progression *AIDS* 8, 1123-1128

29. **Campbell, E. M., Nunez, R., and Hope, T. J.** 2004 Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity *J. Virol.* 78, 5745-5755

30. **Capps, G. G., Robinson, B. E., Lewis, K. D., and Zuniga, M. C.** 1993 In vivo dimeric association of class I MHC heavy chains. Possible relationship to class I MHC heavy chain-beta 2-microglobulin dissociation *J. Immunol.* 151, 159-169

31. **Chakrabarti, L. A., Metzner, K. J., Ivanovic, T., Cheng, H., Louis-Virelizier, J., Connor, R. I., and Cheng-Mayer, C.** 2003 A truncated form of Nef selected during pathogenic reversion of simian immunodeficiency virus SIVmac239Deltanef increases viral replication *J. Virol.* 77, 1245-1256

32. **Cheng, H., Hoxie, J. P., and Parks, W. P.** 1999 The conserved core of human immunodeficiency virus type 1 Nef is essential for association with Lck and for enhanced viral replication in T-lymphocytes *Virology* 264, 5-15

33. **Choi, H. J. and Smithgall, T. E.** 2004 Conserved residues in the HIV-1 Nef hydrophobic pocket are essential for recruitment and activation of the Hck tyrosine kinase *J. Mol. Biol.* 343, 1255-1268

34. **Choi, H.-J. and Smithgall, T. E.** 2004 HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-X_L expression in an Erk-dependent manner *J. Biol. Chem.* 279, 51668-51696

35. **Chowers, M. Y., Spina, C. A., Kwoh, T. J., Fitch, N. J., Richman, D. D., and Guatelli, J. C.** 1994 Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene *J. Virol.* 68, 2906-2914

36. **Churchill, M., Sterjovski, J., Gray, L., Cowley, D., Chatfield, C., Learmont, J., Sullivan, J. S., Crowe, S. M., Mills, J., Brew, B. J., Wesselingh, S. L., McPhee, D. A., and Gorry, P. R.** 2004 Longitudinal analysis of nef/long terminal repeat-deleted HIV-1 in blood and cerebrospinal fluid of a long-term survivor who developed HIV-associated dementia *J. Infect. Dis.* 190, 2181-2186

37. **Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and .** 1986 Human immunodeficiency viruses *Science* 232, 697

38. **Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and .** 1986 What to call the AIDS virus? *Nature* 321, 10

39. **Collette, Y., Dutartre, H., Benziane, A., Ramos-Morales, F., Benarous, R., Harris, M., and Olive, D.** 1996 Physical and functional interaction of *Nef* with Lck - HIV-1 Nef-induced T-cell signaling defects *J. Biol. Chem.* 271, 6333-6341

40. **Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D., and Baltimore, D.** 1998 HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes *Nature* 391, 397-401

41. **Cortes, M. J., Wong-Staal, F., and Lama, J.** 2002 Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells *J. Biol. Chem.* 277, 1770-1779

42. **Costa, L. J., Zheng, Y. H., Sabotic, J., Mak, J., Fackler, O. T., and Peterlin, B. M.** 2004 Nef binds p6* in GagPol during replication of human immunodeficiency virus type 1 *J. Virol.* 78, 5311-5323

43. **Craig, H. M., Pandori, M. W., and Guatelli, J. C.** 1998 Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity *Proc. Natl. Acad. Sci. U. S. A* 95, 11229-11234

44. **Craig, H. M., Reddy, T. R., Riggs, N. L., Dao, P. P., and Guatelli, J. C.** 2000 Interactions of HIV-1 nef with the mu subunits of adaptor protein complexes 1, 2, and 3: role of the dileucine-based sorting motif *Virology* 271, 9-17

45. **Crotti, A., Neri, F., Corti, D., Ghezzi, S., Heltai, S., Baur, A., Poli, G., Santagostino, E., and Vicenzi, E.** 2006 Nef alleles from human immunodeficiency virus type 1-infected long-term-nonprogressor hemophiliacs with or without late disease progression are defective in enhancing virus replication and CD4 down-regulation *J. Virol.* 80, 10663-10674

46. **Cullen, B. R.** 1990 The HIV-1 Tat protein: an RNA sequence-specific processivity factor? *Cell* 63, 655-657

47. **Cullen, B. R.** 1991 Regulation of HIV-1 gene expression *FASEB J.* 5, 2361-2368

48. **Cullen, B. R.** 1998 HIV-1 auxiliary proteins: making connections in a dying cell *Cell* 93, 685-692

49. **Cullen, B. R.** 1999 HIV-1 Nef protein: An invitation to a kill *Nat. Med.* 5, 985-986

50. **Cullen, B. R.** 2001 Journey to the center of the cell *Cell* 105, 697-700

51. **Cullen, B. R. and Malim, M. H.** 1991 The HIV-1 Rev protein: prototype of a novel class of eukaryotic post-transcriptional regulators *Trends Biochem. Sci.* 16, 346-350

52. **Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C.** 1992 Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene *Science* 258, 1938-1941

53. **Das, S. R. and Jameel, S.** 2005 Biology of the HIV Nef protein *Indian J. Med. Res.* 121, 315-332

54. **Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C., Lawson, V. A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J. S., Cunningham, A., Dwyer, D., Dowton, D., and Mills, J.** 1995 Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients *Science* 270, 988-991

55. **Doms, R. W. and Trono, D.** 2000 The plasma membrane as a combat zone in the HIV battlefield *Genes Dev.* 14, 2677-2688

56. **Dorfman, T., Popova, E., Pizzato, M., and Gottlinger, H. G.** 2002 Nef enhances human immunodeficiency virus type 1 infectivity in the absence of matrix *J. Virol.* 76, 6857-6862

57. **Emerman, M. and Malim, M. H.** 1998 HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology *Science* 280, 1880-1884

58. **Erdtmann, L., Janvier, K., Raposo, G., Craig, H. M., Benaroch, P., Berlioz-Torrent, C., Guatelli, J. C., Benarous, R., and Benichou, S.** 2000 Two independent regions of HIV-1 Nef are required for connection with the endocytic pathway through binding to the mu 1 chain of AP1 complex *Traffic.* 1, 871-883

59. **Fackler, O. T. and Baur, A. S.** 2002 Live and let die: Nef functions beyond HIV replication *Immunity.* 16, 493-497

60. **Fackler, O. T., Kienzle, N., Kremmer, E., Boese, A., Schramm, B., Klimkait, T., Kucherer, C., and Mueller-Lantzsch, N.** 1997 Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization *Eur. J. Biochem.* 247, 843-851

61. **Fackler, O. T., Luo, W., Geyer, M., Alberts, A. S., and Peterlin, B. M.** 1999 Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions *Mol. Cell* 3, 729-739

62. **Fackler, O. T., Moris, A., Tibroni, N., Giese, S. I., Glass, B., Schwartz, O., and Krausslich, H. G.** 2006 Functional characterization of HIV-1 Nef mutants in the context of viral infection *Virology* 351, 322-339

63. **Fauci, A. S.** 2003 HIV and AIDS: 20 years of science *Nat. Med.* 9, 839-843

64. **Feinberg, M. B., Jarrett, R. F., Aldovini, A., Gallo, R. C., and Wong-Staal, F.** 1986 HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA *Cell* 46, 807-817

65. **Fisher, A. G., Ratner, L., Mitsuya, H., Marselle, L. M., Harper, M. E., Broder, S., Gallo, R. C., and Wong-Staal, F.** 1986 Infectious mutants of HTLV-III with changes in the 3' region and markedly reduced cytopathic effects *Science* 233, 655-659

66. **Fletcher, T. M., III, Brichacek, B., Sharova, N., Newman, M. A., Stivahtis, G., Sharp, P. M., Emerman, M., Hahn, B. H., and Stevenson, M.** 1996 Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM) *EMBO J.* 15, 6155-6165

67. **Forshey, B. M. and Aiken, C.** 2003 Disassembly of human immunodeficiency virus type 1 cores in vitro reveals association of Nef with the subviral ribonucleoprotein complex *J. Virol.* 77, 4409-4414

68. **Foster, J. L. and Garcia, J. V.** 2007 Role of Nef in HIV-1 replication and pathogenesis *Adv. Pharmacol.* 55, 389-409

69. **Foster, J. L. and Garcia, J. V.** 2008 HIV-1 Nef: at the crossroads *Retrovirology.* 5, 84

70. **Freed, E. O., Englund, G., and Martin, M. A.** 1995 Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection *J. Virol.* 69, 3949-3954

71. **Fujii, Y., Otake, K., Fujita, Y., Yamamoto, N., Nagai, Y., Tashiro, M., and Adachi, A.** 1996 Clustered localization of oligomeric Nef protein of human immunodeficiency virus type 1 on the cell surface *FEBS Lett.* 395, 257-261

72. **Gallo, R., Wong-Staal, F., Montagnier, L., Haseltine, W. A., and Yoshida, M.** 1988 HIV/HTLV gene nomenclature *Nature* 333, 504

73. **Garcia, J. V. and Miller, A. D.** 1991 Serine phosphorylation-independent downregulation of cell-surface CD4 by nef *Nature* 350, 508-511

74. **Geffin, R., Wolf, D., Muller, R., Hill, M. D., Stellwag, E., Freitag, M., Sass, G., Scott, G. B., and Baur, A. S.** 2000 Functional and structural defects in HIV type 1 nef genes derived from pediatric long-term survivors *AIDS Res. Hum. Retroviruses* 16, 1855-1868

75. **Geleziunas, R., Xu, W., Takeda, K., Ichijo, H., and Greene, W. C.** 2001 HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell *Nature* 410, 834-838

76. **Geyer, M., Fackler, O. T., and Peterlin, B. M.** 2001 Structure--function relationships in HIV-1 Nef *EMBO Rep.* 2, 580-585

77. **Geyer, M. and Peterlin, B. M.** 2001 Domain assembly, surface accessibility and sequence conservation in full length HIV-1 Nef *FEBS Lett.* 496, 91-95

78. **Glushakova, S., Grivel, J. C., Suryanarayana, K., Meylan, P., Lifson, J. D., Desrosiers, R., and Margolis, L.** 1999 Nef enhances human immunodeficiency virus replication and responsiveness to interleukin-2 in human lymphoid tissue ex vivo *J. Virol.* 73, 3968-3974

79. **Glushakova, S., Munch, J., Carl, S., Greenough, T. C., Sullivan, J. L., Margolis, L., and Kirchhoff, F.** 2001 CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with CD4(+) T-cell depletion in human lymphoid tissue ex vivo *J. Virol.* 75, 10113-10117

80. **Goh, W. C., Rogel, M. E., Kinsey, C. M., Michael, S. F., Fultz, P. N., Nowak, M. A., Hahn, B. H., and Emerman, M.** 1998 HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo *Nat. Med.* 4, 65-71

81. **Gomez, C. and Hope, T. J.** 2005 The ins and outs of HIV replication *Cell Microbiol.* 7, 621-626

82. **Gomez-Cambronero, J., Colasanto, J. M., Huang, C.-K., and Sha'afi, R. I.** 1993 Direct stimulation by tyrosine phosphorylation of microtubule- associated protein (MAP) kinase activity by granulocyte- macrophage colony-stimulating factor in human neutrophils *Biochem. J.* 291, 211-217

83. **Green, L. A., Liu, Y., and He, J. J.** 2009 Inhibition of HIV-1 infection and replication by enhancing viral incorporation of innate anti-HIV-1 protein A3G: a non-pathogenic Nef mutant-based anti-HIV strategy *J. Biol. Chem.* 284, 13363-13372

84. **Greenberg, M. E., Bronson, S., Lock, M., Neumann, M., Pavlakis, G. N., and Skowronski, J.** 1997 Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation *EMBO J.* 16, 6964-6976

85. **Greenberg, M. E., Iafrate, A. J., and Skowronski, J.** 1998 The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes *EMBO J.* 17, 2777-2789

86. **Greene, W. C. and Peterlin, B. M.** 2002 Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy *Nat. Med.* 8, 673-680

87. **Greenough, T. C., Sullivan, J. L., and Desrosiers, R. C.** 1999 Declining CD4 T-cell counts in a person infected with nef-deleted HIV-1 *N. Engl. J. Med.* 340, 236-237

88. **Greenway, A. and McPhee, D.** 1997 HIV1 Nef: the Machiavelli of cellular activation *Res. Virol.* 148, 58-64

89. **Greenway, A. L., Holloway, G., McPhee, D. A., Ellis, P., Cornall, A., and Lidman, M.** 2003 HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication *J. Biosci.* 28, 323-335

90. **Greenway, A. L., McPhee, D. A., Allen, K., Johnstone, R., Holloway, G., Mills, J., Azad, A., Sankovich, S., and Lambert, P.** 2002 Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis *J. Virol.* 76, 2692-2702

91. **Grinberg, A. V., Hu, C. D., and Kerppola, T. K.** 2004 Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells *Mol. Cell Biol.* 24, 4294-4308

92. **Gross, A., McDonnell, J. M., and Korsmeyer, S. J.** 1999 BCL-2 family members and the mitochondria in apoptosis *Genes Dev.* 13, 1899-1911

93. **Grzesiek, S., Bax, A., Clore, G. M., Gronenborn, A. M., Hu, J.-S., Kaufman, J., Palmer, I., Stahl, S. J., and Wingfield, P. T.** 1996 The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase *Nature Struct. Biol.* 3, 340-345

94. **Grzesiek, S., Bax, A., Hu, J. S., Kaufman, J., Palmer, I., Stahl, S. J., Tjandra, N., and Wingfield, P. T.** 1997 Refined solution structure and backbone dynamics of HIV-1 Nef *Protein Sci.* 6, 1248-1263

95. **Grzesiek, S., Stahl, S. J., Wingfield, P. T., and Bax, A.** 1996 The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR *Biochemistry* 35, 10256-10261

96. **Guy, B., Kieny, M. P., Riviere, Y., Le, P. C., Dott, K., Girard, M., Montagnier, L., and Lecocq, J. P.** 1987 HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product *Nature* 330, 266-269

97. **Hallenberger, S., Bosch, V., Angliker, H., Shaw, E., Klenk, H. D., and Garten, W.** 1992 Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160 *Nature* 360, 358-361

98. **Hammes, S. R., Dixon, E. P., Malim, M. H., Cullen, B. R., and Greene, W. C.** 1989 Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor *Proc. Natl. Acad. Sci. U. S. A* 86, 9549-9553

99. **Hanna, Z., Kay, D. G., Cool, M., Jothy, S., Rebai, N., and Jolicoeur, P.** 1998 Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease *J. Virol.* 72, 121-132

100. **Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S., and Jolicoeur, P.** 1998 Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice *Cell* 95, 163-175

101. **Hanna, Z., Weng, X., Kay, D. G., Poudrier, J., Lowell, C., and Jolicoeur, P.** 2001 The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck *J. Virol.* 75, 9378-9392

102. **Harris, M.** 1995 The role of myristoylation in the interactions between human immunodeficiency virus type I Nef and cellular proteins *Biochem. Soc. Trans.* 23, 557-561

103. **Harris, M.** 1996 From negative factor to a critical role in virus pathogenesis: the changing fortunes of Nef *J. Gen. Virol.* 77 (Pt 10), 2379-2392

104. **Harris, M. and Coates, K.** 1993 Identification of cellular proteins that bind to the human immunodeficiency virus type 1 nef gene product in vitro: a role for myristylation *J. Gen. Virol.* 74 (Pt 8), 1581-1589

105. **Harris, M. P. and Neil, J. C.** 1994 Myristoylation-dependent binding of HIV-1 Nef to CD4 *J. Mol. Biol.* 241, 136-142

106. **Hassaine, G., Courcoul, M., Bessou, G., Barthalay, Y., Picard, C., Olive, D., Collette, Y., Vigne, R., and Decroly, E.** 2001 The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral vif protein *J. Biol. Chem.* 276, 16885-16893

107. **Hazenberg, M. D., Otto, S. A., van Benthem, B. H., Roos, M. T., Coutinho, R. A., Lange, J. M., Hamann, D., Prins, M., and Miedema, F.** 2003 Persistent immune activation in HIV-1 infection is associated with progression to AIDS *AIDS* 17, 1881-1888

108. **Hiatt, S. M., Shyu, Y. J., Duren, H. M., and Hu, C. D.** 2008 Bimolecular fluorescence complementation (BiFC) analysis of protein interactions in Caenorhabditis elegans *Methods* 45, 185-191

109. **Hu, C. D., Chinenov, Y., and Kerppola, T. K.** 2002 Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation *Mol. Cell* 9, 789-798

110. **Hu, C. D. and Kerppola, T. K.** 2003 Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis *Nat. Biotechnol.* 21, 539-545

111. **Hung, C. H., Thomas, L., Ruby, C. E., Atkins, K. M., Morris, N. P., Knight, Z. A., Scholz, I., Barklis, E., Weinberg, A. D., Shokat, K. M., and Thomas, G.** 2007 HIV-1 Nef assembles a Src family kinase-ZAP-70/Syk-PI3K cascade to downregulate cell-surface MHC-I *Cell Host & Microbe* 1, 121-133

112. **Hynes, T. R., Tang, L., Mervine, S. M., Sabo, J. L., Yost, E. A., Devreotes, P. N., and Berlot, C. H.** 2004 Visualization of G protein betagamma dimers using bimolecular fluorescence complementation demonstrates roles for both beta and gamma in subcellular targeting *J. Biol. Chem.* 279, 30279-30286

113. **Iafrate, A. J., Bronson, S., and Skowronski, J.** 1997 Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling *EMBO J.* 16, 673-684

114. **Igarashi, T., Kuwata, T., Yamamoto, H., Moriyama, H., Ui, M., Miyazaki, Y., and Hayami, M.** 1998 Infectivity and immunogenicity of SIVmac/HIV-1 chimeric viruses (SHIVs) with deletions in two or three genes (vpr, nef and vpx) *Microbiol. Immunol.* 42, 71-74

115. **Ikuta, K., Kameoka, M., and Luftig, R. B.** 1997 AIDS pathogenesis: the role of accessory gene mutations, leading to formation of long-lived persistently infected cells and/or apoptosis-inducing HIV-1 particles *Virus Res.* 52, 145-156

116. **Jacks, T., Power, M. D., Masiarz, F. R., Luciw, P. A., Barr, P. J., and Varmus, H. E.** 1988 Characterization of ribosomal frameshifting in HIV-1 gag-pol expression *Nature* 331, 280-283

117. **Joseph, A. M., Kumar, M., and Mitra, D.** 2005 Nef: "necessary and enforcing factor" in HIV infection *Curr. HIV. Res.* 3, 87-94

118. **Kaminchik, J., Bashan, N., Itach, A., Sarver, N., Gorecki, M., and Panet, A.** 1991 Genetic characterization of human immunodeficiency virus type 1 nef gene products translated in vitro and expressed in mammalian cells *J. Virol.* 65, 583-588

119. **Kay, D. G., Yue, P., Hanna, Z., Jothy, S., Tremblay, E., and Jolicoeur, P.** 2002 Cardiac disease in transgenic mice expressing human immunodeficiency virus-1 nef in cells of the immune system *Am. J. Pathol.* 161, 321-335

120. **Kestler, H., Kodama, T., Ringler, D., Marthas, M., Pedersen, N., Lackner, A., Regier, D., Sehgal, P., Daniel, M., King, N., and .** 1990 Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus *Science* 248, 1109-1112

121. **Kestler, H., Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D., and Desrosiers, R. C.** 1991 Importance of the nef gene for maintenance of high viral loads and for development of AIDS *Cell* 65, 651-662

122. **Kienzle, N., Freund, J., Kalbitzer, H. R., and Mueller-Lantzch, N.** 1993 Oligomerization of the Nef protein from human immunodeficiency virus (HIV) type 1 *Eur. J. Biochem.* 214, 451-457

123. **Kim, S., Ikeuchi, K., Byrn, R., Groopman, J., and Baltimore, D.** 1989 Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1 *Proc. Natl. Acad. Sci. U. S. A* 86, 9544-9548

124. **Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L., and Desrosiers, R. C.** 1995 Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection *N. Engl. J. Med.* 332, 228-232

125. **Klemsz, M. J., McKercher, S. R., and Maki, R. A.** 1987 Nucleotide sequence of the mouse *hck* gene *Nuc. Acids Res.* 15, 9600

126. **Komuro, I., Yokota, Y., Yasuda, S., Iwamoto, A., and Kagawa, K. S.** 2003 CSF-induced and HIV-1-mediated distinct regulation of Hck and C/EBPbeta represent a heterogeneous susceptibility of monocyte-derived macrophages to M-tropic HIV-1 infection *J. Exp. Med.* 198, 443-453

127. **Kuznetsov, Y. G., Victoria, J. G., Robinson, W. E., Jr., and McPherson, A.** 2003 Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes *J. Virol.* 77, 11896-11909

128. **Lama, J.** 2003 The physiological relevance of CD4 receptor down-modulation during HIV infection *Curr. HIV. Res.* 1, 167-184

129. **Lama, J., Mangasarian, A., and Trono, D.** 1999 Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner *Curr. Biol.* 9, 622-631

130. **Larsen, J. E., Massol, R. H., Nieland, T. J., and Kirchhausen, T.** 2004 HIV Nef-mediated major histocompatibility complex class I down-modulation is independent of Arf6 activity *Mol. Biol. Cell* 15, 323-331

131. **Learmont, J. C., Geczy, A. F., Mills, J., Ashton, L. J., Raynes-Greenow, C. H., Garsia, R. J., Dyer, W. B., McIntyre, L., Oelrichs, R. B., Rhodes, D. I., Deacon, N. J., and Sullivan, J. S.** 1999 Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort *N. Engl. J. Med.* 340, 1715-1722

132. **Lee, C.-H., Leung, B., Lemmon, M. A., Zheng, J., Cowburn, D., Kuriyan, J., and Saksela, K.** 1995 A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein *EMBO J.* 14, 5006-5015

133. **Lee, C.-H., Saksela, K., Mirza, U. A., Chait, B. T., and Kuriyan, J.** 1996 Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain *Cell* 85, 931-942

134. **Lehmann-Che, J. and Saib, A.** 2004 Early stages of HIV replication: how to hijack cellular functions for a successful infection *AIDS Rev.* 6, 199-207

135. **Leiherer, A., Ludwig, C., and Wagner, R.** 2009 Influence of extended mutations of the HIV-1 transframe protein p6 on Nef-dependent viral replication and infectivity in vitro *Virology* 387, 200-210

136. **Leis, J., Baltimore, D., Bishop, J. M., Coffin, J., Fleissner, E., Goff, S. P., Oroszlan, S., Robinson, H., Skalka, A. M., Temin, H. M., and .** 1988 Standardized and simplified nomenclature for proteins common to all retroviruses *J. Virol.* 62, 1808-1809

137. Levy, J. A. (2007) *HIV and the pathogenesis of AIDS*, Third Ed., ASM Press, Washington, DC

138. **Lewis, M. J., Balamurugan, A., Ohno, A., Kilpatrick, S., Ng, H. L., and Yang, O. O.** 2008 Functional adaptation of Nef to the immune milieu of HIV-1 infection in vivo *J. Immunol.* 180, 4075-4081

139. **Liu, L. X., Heveker, N., Fackler, O. T., Arold, S., Le Gall, S., Janvier, K., Peterlin, B. M., Dumas, C., Schwartz, O., Benichou, S., and Benarous, R.** 2000 Mutation of a conserved residue (D123) required for oligomerization of human immunodeficiency virus type 1 Nef protein abolishes interaction with human thioesterase and results in impairment of Nef biological functions *J. Virol.* 74, 5310-5319

140. **Liu, X., Schrager, J. A., Lange, G. D., and Marsh, J. W.** 2001 HIV Nef-mediated cellular phenotypes are differentially expressed as a function of intracellular Nef concentrations *J. Biol. Chem.* 276, 32763-32770

141. **Lu, T. C., He, J. C., Wang, Z. H., Feng, X., Fukumi-Tominaga, T., Chen, N., Xu, J., Iyengar, R., and Klotman, P. E.** 2008 HIV-1 Nef disrupts the podocyte actin cytoskeleton by interacting with diaphanous interacting protein *J. Biol. Chem.* 283, 8173-8182

142. **Lu, X., Yu, H., Liu, S. H., Brodsky, F. M., and Peterlin, B. M.** 1998 Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4 *Immunity.* 8, 647-656

143. **Luciw, P. A., Cheng-Mayer, C., and Levy, J. A.** 1987 Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication *Proc. Natl. Acad. Sci. U. S. A* 84, 1434-1438

144. **Lundquist, C. A., Tobiume, M., Zhou, J., Unutmaz, D., and Aiken, C.** 2002 Nef-mediated downregulation of CD4 enhances human immunodeficiency virus type 1 replication in primary T lymphocytes *J. Virol.* 76, 4625-4633

145. **Madiraju, C., Edler, M. C., Hamel, E., Raccor, B. S., Balachandran, R., Zhu, G., Giuliano, K. A., Vogt, A., Shin, Y., Fournier, J. H., Fukui, Y., Bruckner, A. M.,**

Curran, D. P., and Day, B. W. 2005 Tubulin assembly, taxoid site binding, and cellular effects of the microtubule-stabilizing agent dictyostatin *Biochemistry* 44, 15053-15063

146. **Manninen, A. and Saksela, K.** 2002 HIV-1 Nef interacts with inositol trisphosphate receptor to activate calcium signaling in T cells *J. Exp. Med.* 195, 1023-1032

147. **Marecki, J. C., Cool, C. D., Parr, J. E., Beckey, V. E., Luciw, P. A., Tarantal, A. F., Carville, A., Shannon, R. P., Cota-Gomez, A., Tuder, R. M., Voelkel, N. F., and Flores, S. C.** 2006 HIV-1 Nef is associated with complex pulmonary vascular lesions in SHIV-nef-infected macaques *Am. J. Respir. Crit Care Med.* 174, 437-445

148. **Mariani, R., Kirchhoff, F., Greenough, T. C., Sullivan, J. L., Desrosiers, R. C., and Skowronski, J.** 1996 High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection *J. Virol.* 70, 7752-7764

149. **Mariani, R. and Skowronski, J.** 1993 CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals *Proc. Natl. Acad. Sci. U. S. A* 90, 5549-5553

150. **Marshall, W. L., Diamond, D. C., Kowalski, M. M., and Finberg, R. W.** 1992 High level of surface CD4 prevents stable human immunodeficiency virus infection of T-cell transfectants *J. Virol.* 66, 5492-5499

151. **Matsubara, M., Jing, T., Kawamura, K., Shimojo, N., Titani, K., Hashimoto, K., and Hayashi, N.** 2005 Myristoyl moiety of HIV Nef is involved in regulation of the interaction with calmodulin in vivo *Protein Sci.* 14, 494-503

152. **McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieberman, M., Kosek, J. C., Reyes, G. R., and Weissman, I. L.** 1988 Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus *Cell* 53, 55-67

153. **Miller, J. H., Presnyak, V., and Smith, H. C.** 2007 The dimerization domain of HIV-1 viral infectivity factor Vif is required to block virion incorporation of APOBEC3G *Retrovirology.* 4, 81

154. **Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C., and Feinberg, M. B.** 1994 The human immunodeficiency virus-1 nef gene product: A positive factor for viral infection and replication in primary lymphocytes and macrophages *J. Exp. Med.* 179, 101-113

155. **Morell, M., Espargaro, A., Aviles, F. X., and Ventura, S.** 2007 Detection of transient protein-protein interactions by bimolecular fluorescence complementation: the Abl-SH3 case *Proteomics.* 7, 1023-1036

156. **Murphy, K. M., Sweet, M. J., Ross, I. L., and Hume, D. A.** 1993 Effects of the tat and nef gene products of human immunodeficiency virus type 1 (HIV-1) on transcription controlled by the HIV-1 long terminal repeat and on cell growth in macrophages *J. Virol.* 67, 6956-6964

157. **Nagata, S. and Golstein, P.** 1995 The Fas death factor *Science* 267, 1449-1456

158. **Ono, A. and Freed, E. O.** 2001 Plasma membrane rafts play a critical role in HIV-1 assembly and release *Proc. Natl. Acad. Sci. U. S. A* 98, 13925-13930

159. **Oroszlan, S. and Luftig, R. B.** 1990 Retroviral proteinases *Curr. Top. Microbiol. Immunol.* 157, 153-185

160. **Pandori, M. W., Fitch, N. J., Craig, H. M., Richman, D. D., Spina, C. A., and Guatelli, J. C.** 1996 Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein *J. Virol.* 70, 4283-4290

161. **Papkalla, A., Munch, J., Otto, C., and Kirchhoff, F.** 2002 Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism *J. Virol.* 76, 8455-8459

162. **Pascual, R., Moreno, M. R., and Villalain, J.** 2005 A peptide pertaining to the loop segment of human immunodeficiency virus gp41 binds and interacts with model biomembranes: implications for the fusion mechanism *J. Virol.* 79, 5142-5152

163. **Peeters, M., Toure-Kane, C., and Nkengasong, J. N.** 2003 Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials *AIDS* 17, 2547-2560

164. **Piguet, V., Gu, F., Foti, M., Demaurex, N., Gruenberg, J., Carpentier, J. L., and Trono, D.** 1999 Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes *Cell* 97, 63-73

165. **Piguet, V. and Trono, D.** 2001 Living in oblivion: HIV immune evasion *Semin. Immunol.* 13, 51-57

166. **Piguet, V., Wan, L., Borel, C., Mangasarian, A., Demaurex, N., Thomas, G., and Trono, D.** 2000 HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes *Nat. Cell Biol.* 2, 163-167

167. **Planelles, V., Jowett, J. B., Li, Q. X., Xie, Y., Hahn, B., and Chen, I. S.** 1996 Vpr-induced cell cycle arrest is conserved among primate lentiviruses *J. Virol.* 70, 2516-2524

168. **Potash, M. J. and Volsky, D. J.** 1998 Viral interference in HIV-1 infected cells *Rev. Med. Virol.* 8, 203-211

169. **Poudrier, J., Weng, X., Kay, D. G., Hanna, Z., and Jolicoeur, P.** 2003 The AIDS-like disease of CD4C/human immunodeficiency virus transgenic mice is associated with accumulation of immature CD11bHi dendritic cells *J. Virol.* 77, 11733-11744

170. **Poudrier, J., Weng, X., Kay, D. G., Pare, G., Calvo, E. L., Hanna, Z., Kosco-Vilbois, M. H., and Jolicoeur, P.** 2001 The AIDS disease of CD4C/HIV transgenic mice shows

impaired germinal centers and autoantibodies and develops in the absence of IFN-gamma and IL-6 *Immunity.* 15, 173-185

171. **Preusser, A., Briese, L., Baur, A. S., and Willbold, D.** 2001 Direct in vitro binding of full-length human immunodeficiency virus type 1 Nef protein to CD4 cytoplasmic domain *J. Virol.* 75, 3960-3964

172. **Reeves, J. D. and Doms, R. W.** 2002 Human immunodeficiency virus type 2 *J. Gen. Virol.* 83, 1253-1265

173. **Reeves, J. D. and Doms, R. W.** 2002 Human immunodeficiency virus type 2 *J. Gen. Virol.* 83, 1253-1265

174. **Renkema, G. H. and Saksela, K.** 2000 Interactions of HIV-1 NEF with cellular signal transducing proteins *Front Biosci.* 5, D268-D283

175. **Resh, M. D.** 1994 Myristylation and palmitylation of Src family members: The fats of the matter *Cell* 76, 411-413

176. **Resh, M. D.** 1999 Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins *Biochim. Biophys. Acta* 1451, 1-16

177. **Rhee, S. S. and Marsh, J. W.** 1994 HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement *J. Immunol.* 152, 5128-5134

178. **Rodriguez, M., Pavelko, K. D., Njenga, M. K., Logan, W. C., and Wettstein, P. J.** 1996 The balance between persistent virus infection and immune cells determines demyelination *J. Immunol.* 157, 5699-5709

179. **Roeth, J. F., Williams, M., Kasper, M. R., Filzen, T. M., and Collins, K. L.** 2004 HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail *J. Cell Biol.* 167, 903-913

180. **Ross, T. M., Oran, A. E., and Cullen, B. R.** 1999 Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein *Curr. Biol.* 9, 613-621

181. **Rossi, F., Gallina, A., and Milanesi, G.** 1996 Nef-CD4 physical interaction sensed with the yeast two-hybrid system *Virology* 217, 397-403

182. **Rutherford, G. W., Lifson, A. R., Hessol, N. A., Darrow, W. W., O'Malley, P. M., Buchbinder, S. P., Barnhart, J. L., Bodecker, T. W., Cannon, L., Doll, L. S., and .** 1990 Course of HIV-I infection in a cohort of homosexual and bisexual men: an 11 year follow up study *BMJ* 301, 1183-1188

183. **Saksela, K., Cheng, G., and Baltimore, D.** 1995 Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef^+ viruses but not for down-regulation of CD4 *EMBO J.* 14, 484-491

184. **Salvatori, F. and Scarlatti, G.** 2001 HIV type 1 chemokine receptor usage in mother-to-child transmission *AIDS Res. Hum. Retroviruses* 17, 925-935

185. **Samuel, K. P., Hodge, D. R., Chen, Y. M., and Papas, T. S.** 1991 Nef proteins of the human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV) are structurally similar to leucine zipper transcriptional activation factors *AIDS Res. Hum. Retroviruses* 7, 697-706

186. **Sato, I., Obata, Y., Kasahara, K., Nakayama, Y., Fukumoto, Y., Yamasaki, T., Yokoyama, K. K., Saito, T., and Yamaguchi, N.** 2009 Differential trafficking of Src, Lyn, Yes and Fyn is specified by the state of palmitoylation in the SH4 domain *J. Cell Sci.* 122, 965-975

187. **Sawai, E. T., Baur, A. S., Peterlin, B. M., Levy, J. A., and Cheng-Mayer, C.** 1995 A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity *J. Biol. Chem.* 270, 15307-15314

188. **Schaefer, M. R., Wonderlich, E. R., Roeth, J. F., Leonard, J. A., and Collins, K. L.** 2008 HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells *PLoS. Pathog.* 4, e1000131

189. **Scheppler, J. A., Nicholson, J. K., Swan, D. C., hmed-Ansari, A., and McDougal, J. S.** 1989 Down-modulation of MHC-I in a CD4+ T cell line, CEM-E5, after HIV-1 infection *J. Immunol.* 143, 2858-2866

190. **Schrager, J. A., Der, M., V, and Marsh, J. W.** 2002 HIV Nef increases T cell ERK MAP kinase activity *J. Biol. Chem.* 277, 6137-6142

191. **Schrager, J. A. and Marsh, J. W.** 1999 HIV-1 Nef increases T cell activation in a stimulus-dependent manner *Proc. Natl. Acad. Sci. U. S. A* 96, 8167-8172

192. **Schreiner, S. J., Schiavone, A. P., and Smithgall, T. E.** 2002 Activation of Stat3 by the Src family kinase Hck requires a functional SH3 domain *J. Biol. Chem.* 277, 45680-45687

193. **Schwartz, O., Marechal, V., Danos, O., and Heard, J. M.** 1995 Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell *J. Virol.* 69, 4053-4059

194. **Schwartz, O., Marechal, V., Le, G. S., Lemonnier, F., and Heard, J. M.** 1996 Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein *Nat. Med.* 2, 338-342

195. **Schwartz, O., utry-Varsat, A., Goud, B., Marechal, V., Subtil, A., Heard, J. M., and Danos, O.** 1995 Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes *J. Virol.* 69, 528-533

196. **Sheehy, A. M., Gaddis, N. C., Choi, J. D., and Malim, M. H.** 2002 Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein *Nature* 418, 646-650

197. **Shyu, Y. J., Liu, H., Deng, X., and Hu, C. D.** 2006 Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions *Biotechniques* 40, 61-66

198. **Shyu, Y. J., Suarez, C. D., and Hu, C. D.** 2008 Visualization of ternary complexes in living cells by using a BiFC-based FRET assay *Nat. Protoc.* 3, 1693-1702

199. **Shyu, Y. J., Suarez, C. D., and Hu, C. D.** 2008 Visualization of ternary complexes in living cells by using a BiFC-based FRET assay *Nat. Protoc.* 3, 1693-1702

200. **Simmons, A., Aluvihare, V., and McMichael, A.** 2001 Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators *Immunity.* 14, 763-777

201. **Sodroski, J., Goh, W. C., Rosen, C., Dayton, A., Terwilliger, E., and Haseltine, W.** 1986 A second post-transcriptional trans-activator gene required for HTLV-III replication *Nature* 321, 412-417

202. **Spina, C., Kwoh, T. J., Chowers, M. Y., Guatelli, J. C., and Richman, D. D.** 1994 The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes *J. Exp. Med.* 179, 115-123

203. **Tastan, O., Qi, Y., Carbonell, J. G., and Klein-Seetharaman, J.** 2009 Prediction of interactions between HIV-1 and human proteins by information integration *Pac. Symp. Biocomput.* 516-527

204. **Terwilliger, E., Sodroski, J. G., Rosen, C. A., and Haseltine, W. A.** 1986 Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity *J. Virol.* 60, 754-760

205. **Tobiume, M., Lineberger, J. E., Lundquist, C. A., Miller, M. D., and Aiken, C.** 2003 Nef does not affect the efficiency of human immunodeficiency virus type 1 fusion with target cells *J. Virol.* 77, 10645-10650

206. **Tolstrup, M., Laursen, A. L., Gerstoft, J., Pedersen, F. S., Ostergaard, L., and Duch, M.** 2006 Cysteine 138 mutation in HIV-1 Nef from patients with delayed disease progression *Sex Health* 3, 281-286

207. **Trible, R. P., Emert-Sedlak, L., Wales, T. E., Ayyavoo, V., Engen, J. R., and Smithgall, T. E.** 2007 Allosteric loss-of-function mutations in HIV-1 Nef from a long-term non-progressor *J. Mol. Biol.* 374, 121-129

208. **Trkola, A., Ketas, T., Kewalramani, V. N., Endorf, F., Binley, J. M., Katinger, H., Robinson, J., Littman, D. R., and Moore, J. P.** 1998 Neutralization sensitivity of

human immunodeficiency virus type 1 primary isolates to antibodies and CD4-based reagents is independent of coreceptor usage *J. Virol.* 72, 1876-1885

209. **Vogt, A., Cooley, K. A., Brisson, M., Tarpley, M. G., Wipf, P., and Lazo, J. S.** 2003 Cell-Active Dual Specificity Phosphatase Inhibitors Identified by High-Content Screening *Chem. Biol.* 10, 733-742

210. **Vogt, A., Kalb, E. N., and Lazo, J. S.** 2004 A scalable high-content cytotoxicity assay insensitive to changes in mitochondrial metabolic activity *Oncol. Res.* 14, 305-314

211. **Vogt, A. and Lazo, J. S.** 2005 Chemical complementation: A definitive phenotypic strategy for identifying small molecule inhibitors of elusive cellular targets *Pharmacol. Ther.* 107, 212-215

212. **von, S. U., Song, J., Aiken, C., and Trono, D.** 1993 Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells *J. Virol.* 67, 4945-4955

213. **Wales, T. E. and Engen, J. R.** 2006 Hydrogen exchange mass spectrometry for the analysis of protein dynamics *Mass Spectrom. Rev.* 25, 158-170

214. **Walk, S. F., Alexander, M., Maier, B., Hammarskjold, M. L., Rekosh, D. M., and Ravichandran, K. S.** 2001 Design and use of an inducibly activated human immunodeficiency virus type 1 Nef to study immune modulation *J. Virol.* 75, 834-843

215. **Wang, J. K., Kiyokawa, E., Verdin, E., and Trono, D.** 2000 The Nef protein of HIV-1 associates with rafts and primes T cells for activation *Proc. Natl. Acad. Sci. U. S. A* 97, 394-399

216. **Wang, S. H., Xing, H., He, X., Zhu, F. X., Meng, Z. F., Ruan, Y. H., and Shao, Y. M.** 2008 Nef mutations in long-term non-progressors from former plasma donors infected with HIV-1 subtype B in China *Biomed. Environ. Sci.* 21, 485-491

217. **Weng, X., Priceputu, E., Chrobak, P., Poudrier, J., Kay, D. G., Hanna, Z., Mak, T. W., and Jolicoeur, P.** 2004 CD4+ T cells from CD4C/HIVNef transgenic mice show enhanced activation in vivo with impaired proliferation in vitro but are dispensable for the development of a severe AIDS-like organ disease *J. Virol.* 78, 5244-5257

218. **Whatmore, A. M., Cook, N., Hall, G. A., Sharpe, S., Rud, E. W., and Cranage, M. P.** 1995 Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence *J. Virol.* 69, 5117-5123

219. **Willey, R. L., Maldarelli, F., Martin, M. A., and Strebel, K.** 1992 Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4 *J. Virol.* 66, 7193-7200

220. **Williams, M., Roeth, J. F., Kasper, M. R., Filzen, T. M., and Collins, K. L.** 2005 Human immunodeficiency virus type 1 Nef domains required for disruption of major

histocompatibility complex class I trafficking are also necessary for coprecipitation of Nef with HLA-A2 *J. Virol.* 79, 632-636

221. **Williams, M., Roeth, J. F., Kasper, M. R., Fleis, R. I., Przybycin, C. G., and Collins, K. L.** 2002 Direct binding of human immunodeficiency virus type 1 Nef to the major histocompatibility complex class I (MHC-I) cytoplasmic tail disrupts MHC-I trafficking *J. Virol.* 76, 12173-12184

222. **Wipf, P., Graham, T. H., Vogt, A., Sikorski, R. P., Ducruet, A. P., and Lazo, J. S.** 2006 Cellular analysis of disorazole C1 and structure-activity relationship of analogs of the natural product *Chem. Biol. Drug Des.* 67, 66-73

223. **Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d'Aloja, P., Schurmann, A., and Baur, A. S.** 2001 HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals *Nat. Med.* 7, 1217-1224

224. **Wu, H., Kwong, P. D., and Hendrickson, W. A.** 1997 Dimeric association and segmental variability in the structure of human CD4 *Nature* 387, 527-530

225. **Wu, Y. and Marsh, J. W.** 2001 Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA *Science* 293, 1503-1506

226. **Wyand, M. S., Manson, K. H., Garcia-Moll, M., Montefiori, D., and Desrosiers, R. C.** 1996 Vaccine protection by a triple deletion mutant of simian immunodeficiency virus *J. Virol.* 70, 3724-3733

227. **Wyand, M. S., Manson, K. H., Lackner, A. A., and Desrosiers, R. C.** 1997 Resistance of neonatal monkeys to live attenuated vaccine strains of simian immunodeficiency virus *Nat. Med.* 3, 32-36

228. **Wyatt, R. and Sodroski, J.** 1998 The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens *Science* 280, 1884-1888

229. **Xu, T., Vasilyeva, E., and Forgac, M.** 1999 Subunit interactions in the clathrin-coated vesicle vacuolar (H(+))-ATPase complex *J. Biol. Chem.* 274, 28909-28915

230. **Xu, X. N., Screaton, G. R., Gotch, F. M., Dong, T., Tan, R., Almond, N., Walker, B., Stebbings, R., Kent, K., Nagata, S., Stott, J. E., and McMichael, A. J.** 1997 Evasion of cytotoxic T lymphocyte (CTL) responses by nef-dependent induction of Fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells *J. Exp. Med.* 186, 7-16

231. **Ye, H., Choi, H. J., Poe, J., and Smithgall, T. E.** 2004 Oligomerization is required for HIV-1 Nef-induced activation of the Src family protein-tyrosine kinase, Hck *Biochemistry* 43, 15775-15784

232. **Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A., and Chen, I. S.** 1990 HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure *Cell* 61, 213-222

233. **Zheng, Y. H., Plemenitas, A., Fielding, C. J., and Peterlin, B. M.** 2003 Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions *Proc. Natl. Acad. Sci. U. S. A* 100, 8460-8465