

**INTERACTIONS OF THE HIV-1 NEF VIRULENCE FACTOR WITH HOST CELL
TYROSINE KINASES OF THE SRC AND TEC FAMILIES**

by

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For my Maa & Baba

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Sreya Tarafdar, PhD

University of Pittsburgh, 2014

ABSTRACT

Current antiretroviral therapies effectively slow AIDS progression and lengthen the life of AIDS patients but sadly, cannot completely cure HIV-positive individuals. The development of drug-resistance in HIV often renders the current anti-HIV therapeutic regimens ineffective and even contraindicated in some cases. Thus there exists an urgent need to identify alternate targets for the discovery and development of newer anti-HIV drugs. A promising approach lies in targeting an underexplored yet critical accessory factor in HIV pathogenesis – Nef, which promotes AIDS progression by binding to a plethora of host cell factors leading to altered cell signaling. Identifying novel host factors that are direct effectors for HIV-1 Nef will enable future drug discovery directed against this key HIV virulence factor.

In the first part of my dissertation study, I developed a novel, cell-based approach to explore the scope of Nef-SH3 interactions. Particularly, I explored the interaction of Nef with Tec-family kinases and their relevance to HIV biology. This assay allowed direct visualization of protein-protein interactions between Nef and three Tec family members – Bmx, Btk and Itk in live cells. Interaction occurred between the SH3 domains of the kinases and a conserved polyproline motif on Nef. Allelic variants of Nef representing all the M-group HIV-1 subtypes interacted strongly with Itk demonstrating the highly conserved nature of this interaction. Interaction with Nef induced Itk activation which was reversed by treatment with an Itk inhibitor that also potently blocked Nef-dependent HIV replication. These results provide the first

evidence that Nef interacts with cytoplasmic tyrosine kinases of the Tec family, and suggest that Nef provides a mechanistic link between HIV-1 and Itk signaling in the viral life cycle.

In the second part of this study, I validated the biological relevance of a newly determined high resolution crystal structure of Nef in complex with its best characterized kinase binding partner, Hck. Using human and yeast cell-based systems, I have shown by mutagenesis studies that the newly recognized intercomplex contact between Nef R105 and E93 in the RT loop of the SH3 domain is critical to complex formation and function. These results renew our perception of the Nef:Hck binding interface by offering new insight into possible conformations for the active Nef:Hck complex, which is essential for Nef function and further establishes it as a valid druggable target for HIV-1.

Taken together, the studies presented in this dissertation deepen our understanding of the interaction between the HIV-1 virulence factor Nef and the Src family kinase, Hck; identify additional novel cytoplasmic tyrosine kinases that are direct SH3-based effectors of HIV-1 Nef and validate a novel virus:host cell interaction as a potential target for therapeutic intervention. Thus, my results not only have a strong public health significance and advance the field of HIV research, but also offer a step forward in our combat against what remains as one of the most relevant public health menaces of today – HIV/AIDS.

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“Not everything that can be counted counts, and not everything that counts can be counted”

- William Bruce Cameron, 1963.

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complete me. Thank you for being there beside me at every step. On this journey of togetherness, with more dreams to fulfill and joys to behold - I certainly look forward to our days together ahead in life.

- Sreya Tarafdar

1.0 INTRODUCTION

1.1 HUMAN IMMUNODEFICIENCY VIRUS

1.1.1 AIDS AND HIV

In 1981 the Centers for Disease Control and Prevention (CDC) reported the occurrence of *Pneumocystis carinii* pneumonia (PCP) in five previously healthy gay individuals [1, 3]. The unexplained incidence of this cellular-immune disorder in men with no prior history of immunodeficiency, was indeed the first report of what, a year after came to be known as acquired immunodeficiency syndrome (AIDS). The etiological agent of AIDS in humans was discovered in the laboratories of Drs. Robert Gallo and Luc Montagnier in 1983 [30, 117]. Barre-Sinoussi and co-workers recovered a retrovirus from the lymph node of an individual suffering from lymphadenopathy syndrome (LAS), a condition that was believed to be associated with AIDS at that time. This retrovirus exhibited characteristics similar to that of Human T-cell Leukemia virus (HTLV). Gallo and his group concurred on finding HTLV isolates from AIDS patients during the same time. However, the unlikeliness of HTLV being the etiologic agent of AIDS was supported by the fact that this virus was lymphotropic, had high-titer replication in CD4+ cells yet was cytopathic and resulted in a characteristic loss of the CD4+ target population. Besides, hemophiliacs were also reported to have AIDS [2]. Further studies

corroborated the fact that this new human retrovirus was similar to HTLV in infecting CD4+ lymphocytes but had very distinct properties in being cytolytic - killing its target cells instead of producing immortalized stable cell lines as is characteristic of a typical HTLV [116, 210, 232]. Initially known by multiple names including Human T-Lymphotropic virus III (HTLV-III), Lymphadenopathy-associated virus (LAV) and AIDS-associated retrovirus (ARV), the retrovirus was ultimately renamed by the International Committee on Taxonomy of Viruses as human immunodeficiency virus (HIV) in 1986 [69, 70]. The HIV/AIDS pandemic has expanded ever since, and thirty-three years later, continues to baffle the scientific community and remain as a major global public health issue.

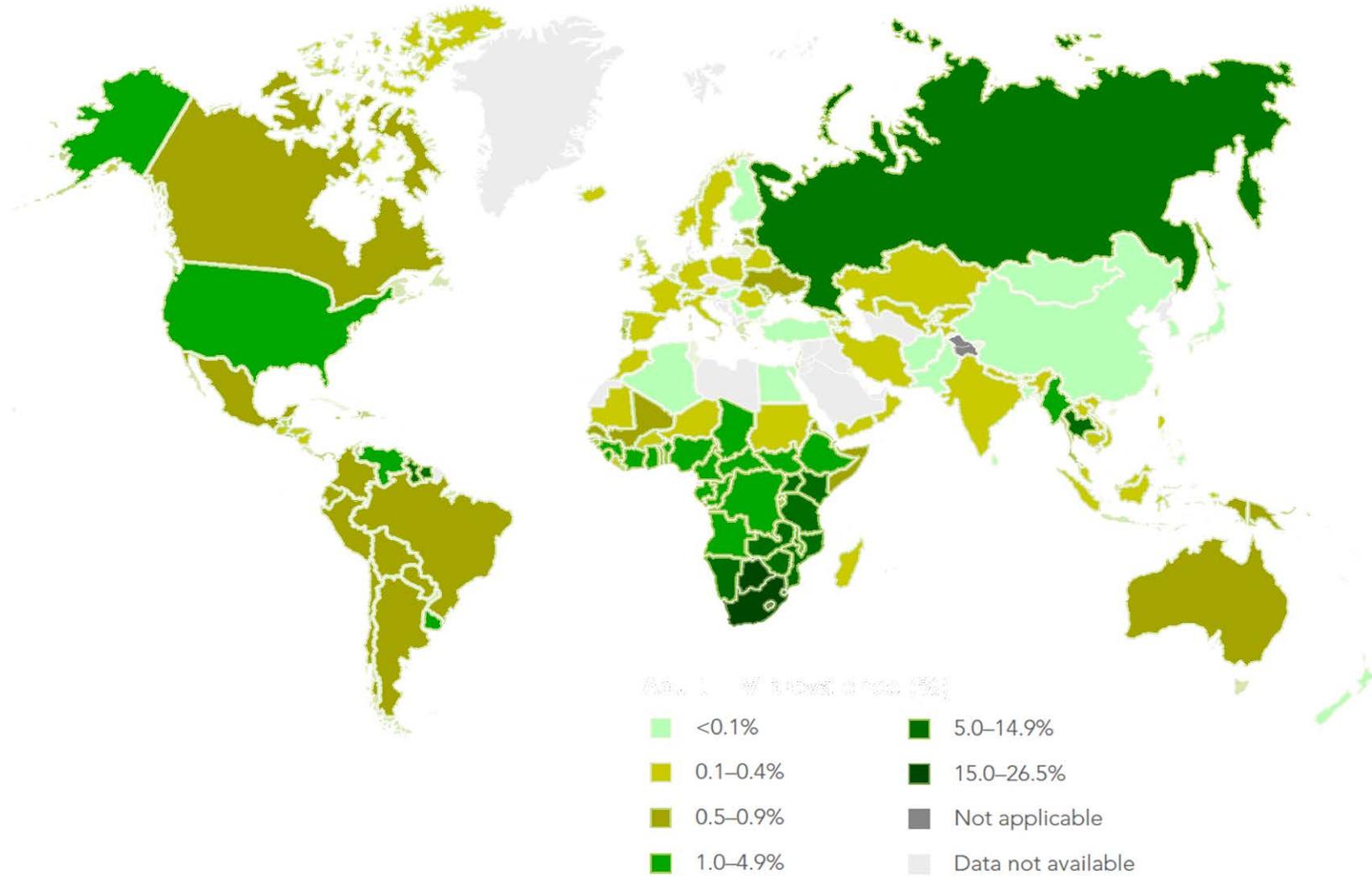
In these three decades, according to the World Health Organization (WHO), HIV/AIDS has claimed nearly 36 million lives. The most recent epidemiological estimates of 2013 from UNAIDS/WHO show that 35.3 million people worldwide are living with HIV/AIDS (Table 1.1). During 2012 alone, nearly 2.3 million adults and children were newly infected with HIV while 1.6 million estimated individuals died of AIDS [4]. Sadly, sub-Saharan Africa happens to be the most affected region in the world, where nearly 1 in every 20 adults is infected with HIV. Overall, 69% of all people worldwide living with HIV resides in this region (Figure 1.1).

Table 1.1. Global HIV and AIDS statistics (regional estimates), 2012.

	Individuals living with HIV (millions)*	Individuals newly infected with HIV (millions)*	Adult Prevalence (%)*	Deaths due to AIDS (thousands)*
Sub-Saharan Africa	25.0	1.6	4.7	1200
South and South-East Asia	3.9	0.27	0.3	220
East Europe and Central Asia	1.3	0.13	0.7	91
Latin America	1.5	0.86	0.4	52
North America	1.3	0.048	0.5	20
East Asia	0.88	0.081	<0.1	41
Middle East and North Africa	0.26	0.032	0.1	17
Caribbean	0.25	0.012	1	11
Western and Central Europe	0.86	0.029	0.2	7.6
Oceania	0.051	0.0021	0.2	1.2
<u>TOTAL</u>	35.3	2.3	0.8	1600

Adapted from [4].

* The numbers reported here are estimates, based on the best information available.



Adapted from [4]

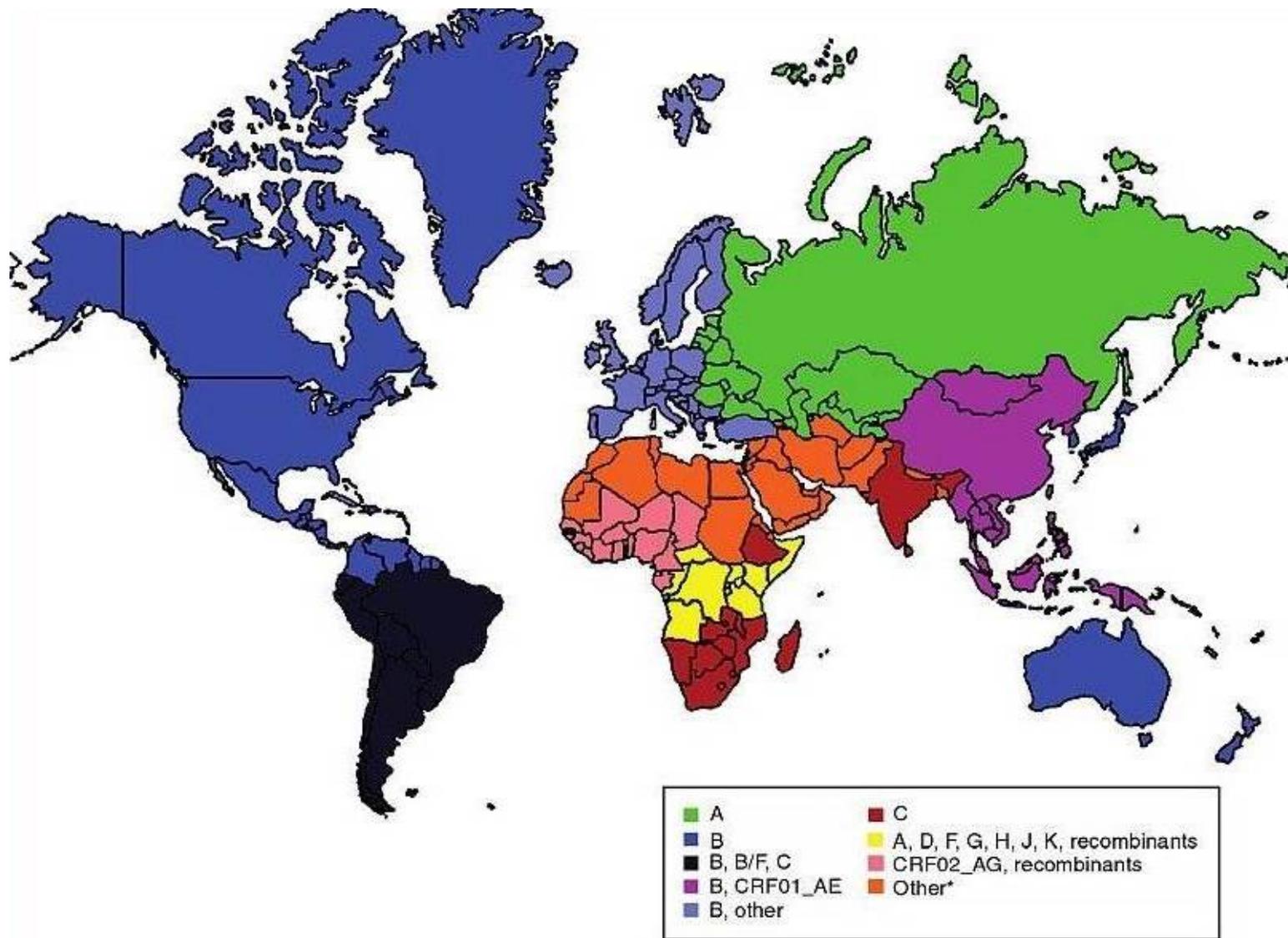
Figure 1.1. HIV prevalence among adults in 2012

1.1.2 SIV AND HIV

The history of simian immunodeficiency virus (SIV) dates back to as early as 1969 when housed rhesus macaques (*Macaca mulatta*) at the California Regional Primate Research Center reportedly had an outbreak of lymphomas. This apparently non-infectious condition was found to be accompanied with immunodeficiency in these animals and eventually led to the onset of opportunistic infections. Several years later the first prototypic isolates of SIV, the causative agent of what later came to be known as simian AIDS (sAIDS), were isolated [119, 312]. HIV and SIV both belong to the Lentivirus genus of the subfamily Orthoretrovirinae and family Retroviridae. Both of these viruses infect primates. Primate lentiviruses are subdivided into five distinct groups based on their sequence homology – HIV-1/SIV_{cpz}, HIV-2/SIV_{mac}/SIV_{sm}, SIV_{agm}, SIV_{syk}, SIV_{rh}/SIV_{mnd} [343]. Alignment of full genome sequences of HIVs and SIVs reveal genetic similarity as well as differences amongst all members. Based on molecular epidemiologic data and the high sequence homology, it is believed that the most common type of HIV that infects humans, HIV-1, was derived from SIV_{cpz}, the strain that infects *Pan troglodytes troglodytes* subspecies of chimpanzee. Similarly, HIV-2 was derived from the other form of SIV found in sooty mangabeys (SIV_{sm}) (*Cercocebus atys*). HIV subtypes 1 and 2 share merely 50-60% sequence homology and thus lie on distinctly separate branches on the phylogenetic tree [17]. In fact HIV-2 shares greater similarity with SIV than with HIV-1. HIV-2 is predominantly found in the regions of Guinea-Bissau and Senegal in West Africa [187]. All of these viruses commonly infect CD4+ cells including macrophages and lymphocytes. In natural infections, SIV unlike HIV-1 does not cause illness but supports high-titer viral replication. However, rhesus macaques experimentally infected with SIV have been reported to develop AIDS-like disease.

Taking advantage of this finding, these experimentally infected monkeys have proved to be useful models for infection and disease progression in humans [52, 181].

Based on alignment of full-length genome sequences, HIV-1 is subdivided into four main groups – M (*major*), N (*neither M nor O*), O (*outlier*) and P (*pending identification of further human cases*). It is likely that an independent cross-species transmission event gave rise to each of these groups. The M group constitutes the pandemic form of HIV-1 that likely emerged as a consequence of multiple cross-species transmission of SIV_{cpz} to humans during the first score of the 20th century. Group M is further subdivided into nine subtypes or clades: A, B, C, D, F, G, H, J, and K, which are widespread and unique with respect to their global distribution (Figure 1.2). Clade B is mostly prevalent in America, Europe and Oceania while clade C is the predominant subtype in South Africa, Indian subcontinent and China. Clade A is mostly circulating in North Asia, Western and Central Africa [17, 53, 157]. In regions where multiple clades co-exist, circulating recombinant forms (CRFs) of HIV-1 are generated. Through 2011, a total of 48 CRFs have been described. Epidemiological data report that subtype C infections account for nearly 48% of all global infections, while subtypes A (12%), B (11%), CRF02_AG (8%), G (5%) and D (2%) follow closely. All the clades of M group taken together, exhibit more than 25% variation in their amino acid sequences in the Env and Gag regions [251, 336]. HIV-1 Group N was discovered much later in 1998 and to date only 13 cases have been reported from Cameroon. Group O represents less than 1% of global infections. It was discovered before group N in 1990 and to date has been prevalent only in Cameroon, Gabon, and the neighboring regions. The most recently identified (2009) group P was found to have greater homology in its sequence with SIV_{gor} (found in wild gorillas) than SIV_{cpz} and has been documented for only two cases in Cameroon [187].



Adapted from [53]

Figure 1.2 Global distribution and diversity of HIV-1 M group clades

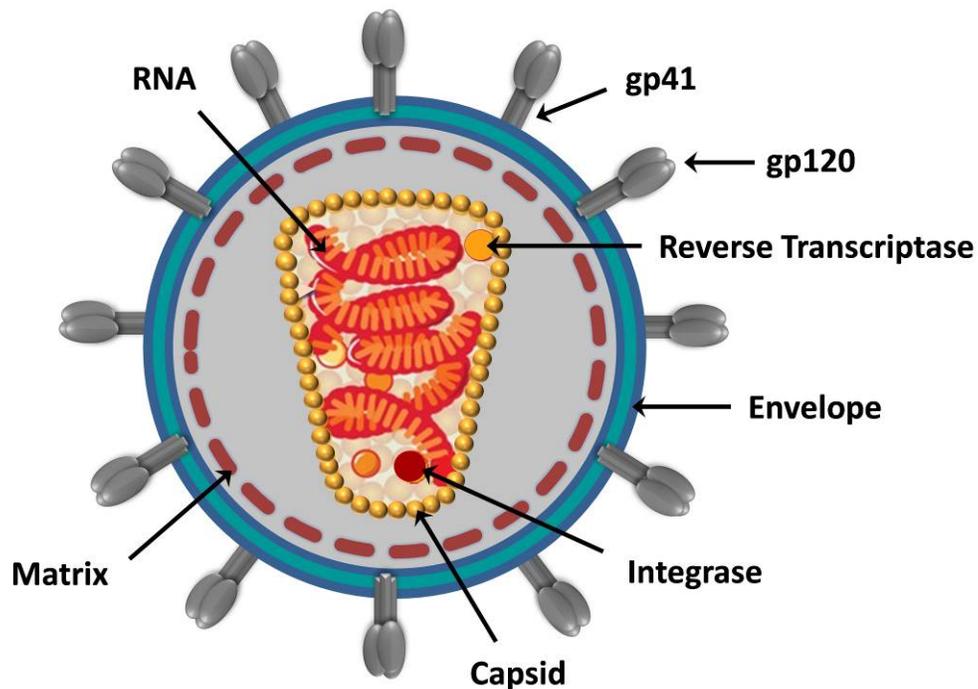
1.1.3 HIV-1: Structure, Genome and Proteins

HIV-1, the causative agent of AIDS, is a member of the exogenous Group VI viruses (ssRNA-RT) since it uses its virally encoded reverse transcriptase enzyme to produce DNA from its single-stranded RNA genome and fuses with the host genome thereafter for replication and transcription by the host machinery.

Virion Structure

Unlike other retroviruses that are cone shaped, HIV is roughly spherical in shape with a diameter of about 120 nm (Figure 1.3). It is around 60 times smaller than the size of a red blood corpuscle. HIV also differs from the other retroviruses within its family in housing a conical capsid that has a diameter of 40-60 nm at its broader end and nearly 20 nm towards the narrower end [194]. The conical capsid is comprised of 250 hexameric subunits with 9.6 nm hexamer-hexamer spacing and exactly 12 pentamers, 5 at the narrow and 7 at the broader end [187]. The core is comprised of about 2000 copies of the viral Gag capsid (CA) protein, p24. The matrix shell that surrounds the capsid is comprised of the myristoylated matrix (MA) protein, p17 [329]. The matrix protein layer lies between the outer envelope and the inner capsid core thereby imparting a definite rigid structure to the outer viral membrane. The outermost viral envelope is derived from the host cell via budding and thus consists of a lipid bilayer enriched with cellular proteins like beta-2-microglobulin, major histocompatibility antigens, actin, HLA-DR and ubiquitin [11, 22]. The viral envelope has 72 surface projections or spikes containing two major viral glycoproteins derived from the precursor gp160 by proteolytic cleavage [113]. These glycoproteins stick out of

the viral membrane as spiked protein tufts of about 200 Å per particle. They help in viral entry and syncytia formation. Each of these glycoprotein spikes is comprised of three copies of the glycosylated surface (SU) protein, gp120, and a stem comprised of three copies of the transmembrane (TM) protein, gp41 [227]. The central capsid core houses a ribonucleoprotein complex comprised of two copies of the viral RNA genome bound to thousands of the highly basic nucleocapsid (NC) protein, p7. Inside the core, there are also several copies of viral enzymes including protease, integrase, reverse transcriptase and some of the accessory proteins like Nef, Vif and Vpr.



The structure of a typical HIV-1 virion showing the major structural and enzymatic proteins. *Adapted and redrawn from a publicly available figure from the webpage of the NIAID, NIH, US Dept. of Health and Human Services.*

Figure 1.3. HIV virion structure

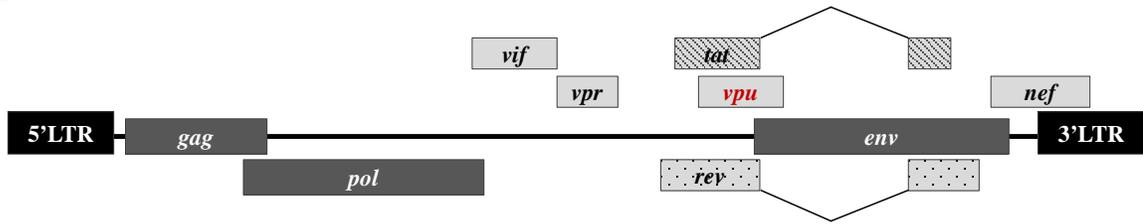
Genome

The HIV genome spans more than 9.8 kilobases comprising nine overlapping genes that are flanked by long repetitive sequences at the both ends known as long terminal repeats (LTR) (Figure 1.4). The nine open reading frames (ORFs) encode 15 proteins including structural, enzymatic and ancillary viral proteins [111]. The three common lentiviral genes of HIV are the *gag* (group-specific core antigen), *pol* (polymerase) and *env* (envelope) genes that encode for structural and enzymatic proteins essential for the viral life cycle. The HIV genome is comparatively more complex than the other retroviral genomes within its family, in that it includes six auxiliary genes - *tat* (transactivator) and *rev* (regulator of viral protein expression) that serve regulatory roles and *vif*, *vpu*, *vpr* and *nef* that serve accessory yet indispensable roles in viral infectivity, replication, egress and pathogenesis *in vivo* [115, 209]. However, HIV-2 harbors another gene, *vpx* in lieu of *vpu* (Figure 1.4).

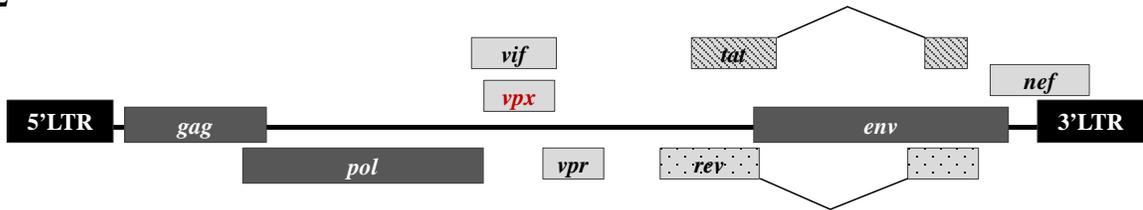
Structural Proteins

The three main lentiviral genes – *gag*, *pol* and *env* that determine the primary structure of the virus, undergo post-translational proteolytic processing to yield multiple structural and enzymatic proteins (Table 1.2). The primary unspliced HIV-1 mRNA transcript produces the Gag protein along with a Gag-Pol fusion protein in a ratio of 20:1. The Gag-Pol fusion polyprotein results from a ribosomal frameshifting during translation within the HIV-1 *gag-pol* overlapping region [169]. Autocatalytic processing and cleavage of the fusion polypeptide yields the Gag and the Pol precursor proteins.

HIV-1



HIV-2



Schematic representation of the primate lentiviral HIV RNA genome which includes 9 ORFs. These encode structural (*gag*, *pol*, *env*), regulatory (*tat*, *rev*) and accessory (*vif*, *vpu*, *vpr*, *nef*) genes that give rise to fifteen proteins. To note, HIV-2 encodes for a different accessory protein, Vpx, in lieu of Vpu in HIV-1. Diagram not to scale.

Figure 1.4. HIV genome organization

The 55kDa N-terminally myristoylated Gag precursor polyprotein (p55), encoded by the *gag* gene, self-assembles at the host cell membrane and recruits the viral genomic RNA (2 copies), viral and other cellular proteins thereby triggering viral budding. During the maturation step, the p55 protein is proteolytically cleaved into the capsid (CA, p24), matrix (MA, p17), nucleocapsid (NC, p7) proteins and other smaller spacer polypeptides (p1, p2, p6) [128, 170]. The myristoylated MA is derived from the N-terminal end of p55. The MA molecules largely associate with the inner layer of the viral envelope. They also assist in transporting the viral genome to the host nucleus after uncoating of the virion particle post-entry [114]. The nuclear import machinery of the host recognizes a karyophilic signal on the MA protein which allows

the viral genome to enter the host nucleus, thereby allowing HIV to infect nondividing cells [211]. The viral core CA protein helps incorporate Cyclophilin A (CypA) into the virion particle which is critical for replication [110]. The viral RNA houses a packaging signal that consists of four stem loop structures towards its 5' end. The RNA-binding protein, NC recognizes and binds to the viral genome at the site of packaging signal via two zinc finger motifs, thereby stabilizing it during viral assembly and also during reverse transcription [152, 201, 329].

The Pol polypeptide is cleaved by the virally encoded protease into 4 different enzymes – protease (PR, p10), integrase (IN, p31), RNase H (p15) and reverse transcriptase (RT, p50). Sometimes, inefficient cleavage results in the RNase H being linked to the RT as a single polypeptide (p65). RT exhibits RNA- and DNA-dependent DNA polymerase activity and along with RNase H, is responsible for the generation of the viral cDNA. RT lacks proof-reading activity, leading to error-prone replication and generation of HIV-1 quasispecies within the same host. After reverse transcription, IN mediates the integration of the HIV proviral DNA into the host cell genomic DNA. The multifaceted IN includes an exonuclease activity (that trims the linear viral cDNA at its ends), an endonuclease activity (that cleaves the host DNA) and a ligase activity (that creates covalent linkages at the ends of the proviral cDNA). Host cellular enzymes then repair and integrate the viral and the host DNA. The aspartyl protease p10 is required for cleaving the Gag-Pol polyprotein during viral maturation [162].

The envelope precursor protein, Env (gp160) is synthesized in the ER from the singly spliced viral mRNA and then undergoes glycosylation on specific asparagine residues while migrating through the Golgi complex. Env glycosylation is essential for viral infectivity. A cellular protease cleaves Env (gp160) into a transmembrane domain (TM, gp41) and a surface protein (SU, gp120). The Env protein exists as a trimer of gp120 in noncovalent interactions with

gp41 [111, 329]. The SU protein has nine highly conserved disulfide bonds and five hypervariable regions that are responsible for recognizing, binding and interacting with the viral receptors on the host cell surface. The third loop, V3 of the hypervariable regions binds to the HIV co-receptors CXCR4 and/or CCR5, thereby determining the preferential tropism of the virus for either T cells or primary macrophages, respectively [168]. The gp120 protein also interacts with DC-SIGN on the surface of dendritic cells. This results in infection of CD4+ T cells more efficiently and also potentially facilitates mucosal transmission of HIV to lymphoid tissues. After attachment of the virus onto the host cell, the TM protein, via its N-terminal fusogenic domain, helps in the fusion of the viral and cellular membranes thereby releasing the viral inner components into the cytosol of the infected cell [122].

Regulatory Proteins

Unlike conventional transcription factors that bind DNA, the regulatory proteins of HIV – Rev and Tat - are sequence-specific RNA binding proteins (Table 1.2). They are mainly found in the nuclei and nucleoli of infected cells.

The 19 kDa Rev protein (p19) binds to a 240 base region of a complex secondary structure of RNA, known as the Rev response element (RRE). Specifically Rev binds at the Rev high affinity binding site on stem loop 2 of the RRE, which forms a non-Watson-Crick G-G base pair [31]. Rev mediates the transport of unspliced and/or incompletely spliced viral mRNA from the nucleus to the cytosol. Rev-mediated export of the viral mRNA out of the nucleus occurs through the interaction of the Rev effector domain (that contains a nuclear export signal, NES) with its cognate NES receptor, CRM1. Essentially the role of Rev is indispensable for HIV-1

replication since it induces the transition from the early to the late phase of HIV gene expression. Proviruses lacking Rev function have been found to be transcriptionally active but fail to express the viral late genes and thus also fail to produce infectious mature virions.

The 14 kDa Tat protein is a transcriptional transactivator that binds to the transactivation response element (TAR), a short stem-loop structure located at the 5'-end of HIV RNAs [108]. The binding of Tat to TAR enhances transcription from the LTR regions of the HIV genome. More specifically, Tat stimulates the elongation phase of HIV transcription in an effort to allow more time to produce full-length transcripts rather than shorter ones [107]. Tat essentially recruits a serine kinase, CDK9 that phosphorylates the RNA polymerase II at its C'-terminal domain. Tat has also been found to regulate the expression of cellular genes like TNF- β , TGF- β , and MIP-1 α [162].

Auxiliary Proteins

The accessory proteins of HIV are not absolutely indispensable for viral replication *in vitro*, yet serve as critical virulence factors *in vivo* (Table 1.2). Vpr, Vpu, and Vif are translated from incompletely spliced mRNA and thus are expressed during the late phase of viral infection which is Rev-dependent. On the other hand, Nef is expressed from a multiply spliced mRNA and is thus Rev-independent.

The smallest of the accessory proteins is Vpr. Approximately 100 copies of this 15 kDa protein get incorporated into each viral particle during viral budding and egress with the help of p6 protein [71]. Vpr helps HIV infect non-dividing cells by abetting nuclear import of the preintegration complex (PIC) [156]. Vpr likely tethers the viral genome directly to the nuclear pore rather than involving any additional nuclear localization signals to the PIC. Studies have

reported that expression of Vpr in cells blocks the activation of p34cdc2/cyclin B complex, thereby preventing cells from entering into the mitosis phase during cell division cycle [154].

Vpu is a 16 kDa polypeptide that is expressed from the same mRNA that encodes for Env. This integral membrane phosphoprotein primarily has two roles. First, it triggers the ubiquitin-mediated degradation of CD4 molecules complexed with Env. This helps separate Env molecules from being bound to CD4 molecules that get trapped within the ER during virion assembly [345]. Vpu also enhances the release of mature virions from the infected host cell surface. Previous studies suggest that in absence of Vpu, mature virions are prevented from being released from the host cell and mainly tend to remain attached to the host cell surface [188].

Vif plays an essential role in the replication of HIV in PBMCs and subsequent production of infectious progeny viruses. This 23 kDa protein interacts with the HIV restriction factor, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) which is a cytidine deaminase. Vif targets APOBEC3G for proteosomal degradation via an elongin-cullin-suppressor of cytokine signaling (SOCS) box (ECS) E3 ligase complex [240]. In the absence of Vif, this host antiviral factor gets incorporated in progeny virions, resulting in deamination of cytosine nucleotides during the cDNA synthesis step. These deoxycytidine to deoxyuridine transitions cause G to A hypermutations in the proviral DNA, resulting in reduced coding and replicative capacity of the virus. Vif has also recently been found to interact with and activate Src family kinases but the relevance of this interaction to HIV-1 pathogenesis yet remains to be determined [224].

Nef is expressed in the early steps of the HIV life cycle and is typically the first viral protein to be produced to detectable levels in an infected cell [9, 86, 101]. This 27 kDa protein is

myristoylated at its N terminal end which leads to it being targeted to the membrane of the cell post translation [148]. Nef has been shown to have multiple functions, like the downregulation of the expression of viral receptors and coreceptors (CD4, CXCR4, CCR5) and also MHC-I on the infected cell's surface, the perturbation of T cell activation, and enhancement of HIV infectivity. Nef supports high-titer viral replication in vivo and is essential for HIV pathogenesis and AIDS progression. Also, Nef functions by interacting with a multitude of host cellular factors resulting in alteration of cell signaling pathways leading to cell proliferation, regulation of transcription factors, immune recognition and cell survival [270]. Since the current dissertation revolves around Nef and its interactions with host cellular factors, its structure, key motifs instrumental in mediating its functions and its role in viral pathogenesis and disease progression are discussed in detail in section 1.2.

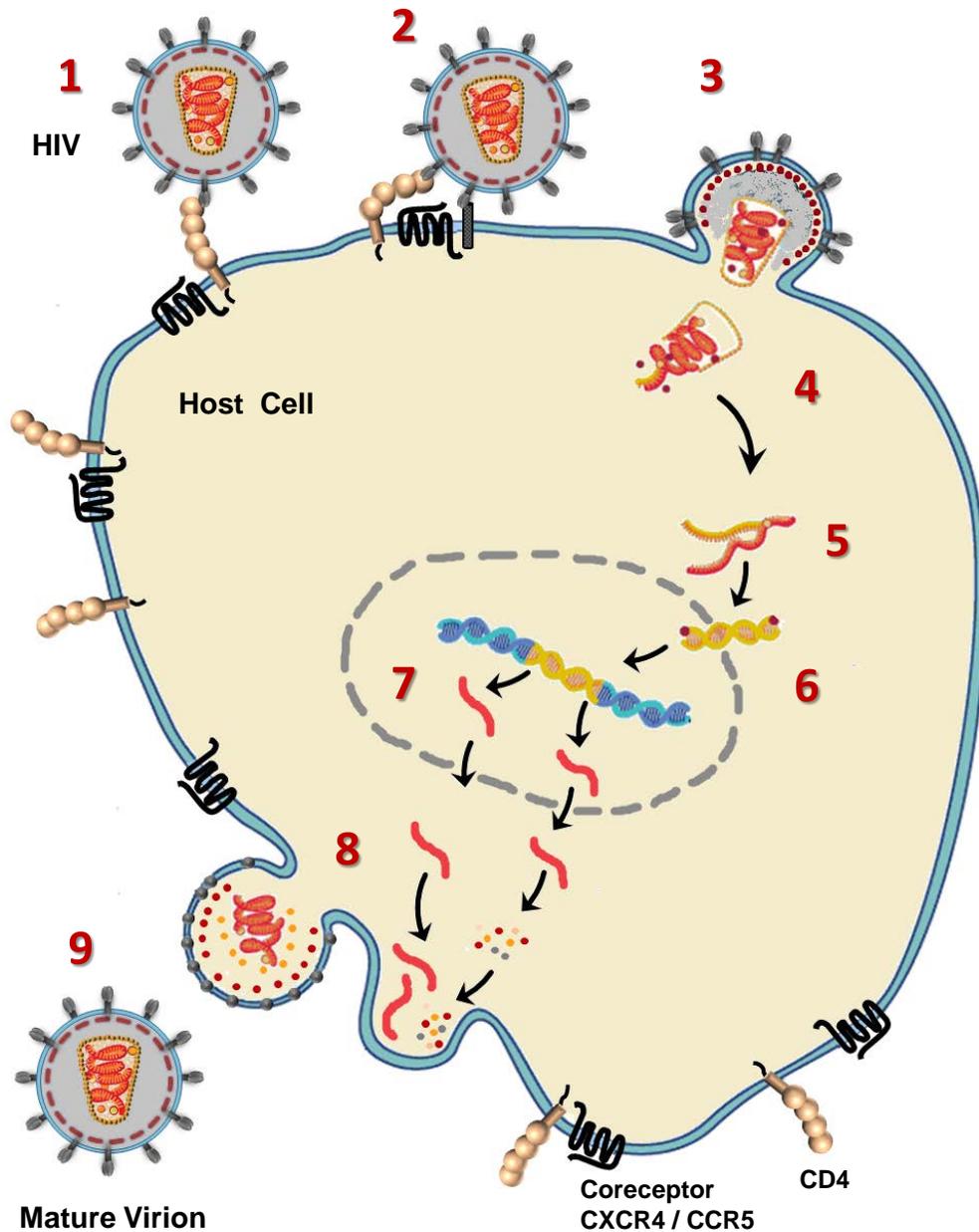
Table 1.2. HIV proteins

<i>Gene</i>	<i>Name of Protein</i>		<i>Protein Size</i>		<i>Function</i>
	<i>Precursor</i>	<i>Products</i>	<i>Precursor</i>	<i>Products</i>	
<i>gag</i>	Gag		p55		Gag precursor protein
		CA		p24	Capsid structural protein
		MA		p17	Viral matrix protein
		NC		p7	RNA binding protein
				p6	Role in viral budding
<i>pol</i>	Pol		Pr160 ^{gag-pol}		Pol precursor protein
		RT		p66/51	Reverse Transcriptase polymerase; RNase H
		PR		p15	Protease: processes viral proteins post translation
		IN		p11	Integrase: integrates viral cDNA into host genome
<i>env</i>	Env		gp160		Envelope precursor protein
		SU		gp120	Envelope surface protein
		TM		gp41	Transmembrane protein
<i>tat</i>		Tat		p14	Transactivates viral gene expression
<i>rev</i>		Rev		p19	Regulates viral mRNA transport
<i>nef</i>		Nef		p27	Enhances infectivity, replication and immune escape
<i>vif</i>		Vif		p23	Enhances viral infectivity
<i>vpu</i>		Vpu		p16	Viral release and egress
<i>vpr</i>		Vpr		p15	Virus replication, transactivation

Adapted from [209, 310]

1.1.4 HIV-1 life cycle

HIV-1 infects and replicates in CD4⁺ T lymphocytes and macrophages. A pictorial schematic of the life cycle of an HIV-1 virion is represented in Figure 1.5 with all the steps numbered 1 through 9. A mature virion enters into a host cell through interaction of the viral surface gp120 protein with the CD4 receptor on the surface of the target cell (*step 1*). This interaction exposes the coreceptor binding site leading to the engagement of host cell CCR5 or CXCR4 chemokine coreceptors (*step 2*). At this stage the transmembrane gp41 protein undergoes conformational changes at its HR1 and HR2 helical regions and with the help of the fusogenic peptide leads to the fusion of the viral envelope and the host cell plasma membrane (*step 3*). This creates a fusion pore through which the viral core enters the cell [130]. Inside the cell, the viral core uncoats in the presence of CypA to release the 2 copies of *positive sense* single-stranded RNA into the cytosol (*step 4*). Next, the reverse transcriptase (RT) enzyme binds to the viral genomic RNA and reverse transcribes the single stranded RNA to synthesize linear double-stranded cDNA resulting in the formation of the pre-integration complex (PIC). The viral accessory protein Vpr and other cellular factors then help the PIC enter the host cell nucleus (*step 5-6*). The viral integrase (IN) enzyme then integrates the proviral DNA into the host cell genomic DNA where staggered nascent cDNA 3' ends covalently attach to the 5' open ends of the chromosomal DNA. Host transcription factors drive RNA Pol II mediated transcription from the 5' LTR of the HIV-1 provirus. The viral Tat protein binds to TAR on the transcribed mRNAs to produce differentially spliced variants of the viral genomic mRNA. The mRNAs are transported to the cytosol by the Rev protein bound to the RRE on the mRNAs (*steps 6-7*). The genomic RNA serves as mRNA for translation of Gag and Gag-Pol precursor proteins. The singly and multiply spliced mRNA transcripts upon translation produce Env and the other auxiliary proteins, respectively. The Env



Adapted and redrawn from a publicly available figure from the webpage of the NIAID, NIH, US Dept. of Health and Human Services.

Schematic diagram representing the entry, replication and egress of the virus in and out of a host cell. The main steps involve (1) viral binding to receptor, (2) engagement of coreceptor, (3) fusion and entry, (4) uncoating of viral core, (5) reverse transcription, (6) entry of PIC into nucleus and integration into host chromosomal DNA, (7) transcription and splicing to generate spliced and unspliced mRNA transcripts (8) translation, post-translational modification and core particle assembly at host cell membrane and (9) release and post-release maturation of progeny virions. For details of every stage, see text section 1.1.4.

Figure 1.5. HIV life cycle

proteins undergo post-translational glycosylation in the Golgi complex. The Env, Gag, Gag-Pol proteins are targeted to the plasma membrane of the infected cell where they cluster into detergent-resistant membrane microdomains. The viral envelope gets enriched in lipids from the host plasma membrane. The viral core gets assembled at the host cell membrane from Env, Gag, Gag-Pol, Vpr, Nef, Vif and 2 copies of capped polyadenylated full-length viral genomic RNA (*step 8*) [43, 177]. Post assembly, virions bud off from the host cell. Nef plays an essential role in viral egress releasing non-infectious immature virions from host cells. Viral maturation begins along with, or immediately following virion budding (*step 9*). The viral protease cleaves and processes the Gag and Gag-Pol precursor proteins at specific sites, thereby separating the functional domains [111]. Viral maturation involves production of the fully processed components MA, CA, NC, p6, protease, reverse transcriptase, and integrase proteins, which rearrange to form mature infectious virions.

1.1.5 HIV-1 pathogenesis

Cell tropism and Receptors

The entry of HIV into a host cell is primarily triggered by the interaction of the HIV envelope glycoproteins with CD4 receptor on the surface of the host cell. Besides the CD4 receptor, seven-pass transmembrane fusion-mediating coreceptors from the chemokine receptor family are also required for HIV to infect cells. The chemokine receptors and coreceptors include the CXC family (CXCR1- 5) and the CC family (CCR1- 9). CCR5 and CXCR4 are the major co-receptors and nearly all HIV-1 isolates can use either or both. The T-tropic HIV strains (X4 viruses) selectively interact with the CXCR4 chemokine coreceptor to infect lymphocytes [68]. The M-tropic HIV strains (R5 virus) interact with the CCR5 chemokine coreceptor, and also in some instances CCR2 and CCR3, to infect macrophages and dendritic cells. CCR8 permits infection by both X4 and R5 viruses. The selectivity of different HIV strains in infecting cells depends on the differences in chemokine coreceptors present on the target cell. The gp120 V3 loop region determines which of the host cell CCR5 or CXCR4 chemokine coreceptors will be engaged [68]. HIV target cells with CD4 receptors commonly include mononuclear phagocytes like blood monocytes and tissue macrophages, as well as T lymphocytes, natural killer (NK) cells, hematopoietic stromal cells, dendritic cells (follicular dendritic cells in lymph nodes and epithelial Langerhans cells), and microglial cells in brain [68, 209]. Galactosylceramide expressed by immature dendritic cells derived from human monocytes as well as those isolated from blood and mucosal tissues harbor a mucosal epithelial receptor for gp41 on HIV [218]. The R5 virus more readily infects dendritic cells and macrophages, has a high rate of replication, and is less visible to cytotoxic lymphocytes. Thus the CCR5 tropic virus generally predominates

early in HIV infection. Over time, mutations in HIV lead to the emergence of CCR5/CXCR4 dual tropic virus, and finally the more cytopathic CXCR4 tropic strain predominates. Polymorphisms and mutations in the chemokine receptor genes explain the phenomenon of resistance to HIV infection in some individuals. The four commonly reported mutations in the co-receptors that confers resistance to HIV-1 infection include CCR5- Δ 32, CCR5-m303, CCR2-64I and the ligand for CXCR4, SDF1-3'A [282]. The most prominent polymorphism is the defective CCR5 product with a 32 base pair deletion (CCR5- Δ 32). The resistance to HIV infection in individuals homozygous for CCR5- Δ 32 highlights the importance of CCR5 receptor during transmission and pathogenesis [88, 202].

Infection and transmission

After the initial entry into host cells and establishment of infection, progeny virions either bud off from infected cells, or are released into the systemic circulation following lysis of the host cell. This burst of viremia can be easily detected in peripheral blood in mononuclear cells and plasma. Mature virions are carried via lymphatic vessels to regional lymph nodes. Neurons, astrocytes, and microglia in the central nervous system also express CCR5 and thus are susceptible to infection. Mononuclear phagocytes in the spleen, liver, and bone marrow can then become infected with HIV. Besides lymph nodes, the gut associated lymphoid tissue in gastrointestinal submucosa represents a considerable reservoir for HIV [187, 209].

The major routes of HIV transmission were originally identified as sexual contact, contaminated blood and mother-to-child. HIV infection can occur through oropharyngeal, cervical, vaginal, and gastrointestinal mucosal surfaces, even without mucosal disruption. Dendritic, epithelial, and microfold cells pave the gateway for viral entry into mucosal lamina

propria. Dendritic cells bind HIV through a type C lectin, squeeze between the tight junctions in the epithelium and deliver it to the underlying layer of T cells resulting in dissemination to lymphoid organs. HIV-infected macrophages can transmigrate across fetal oral and intestinal epithelia as well [187].

Disease progression and Immunological response to HIV infection

Most HIV infections likely begin from a single "founder" virus, from which subsequent clones develop. On average, it takes about 8 to 10 years for an initial infection to progress to clinical AIDS in untreated adults, yet this period varies. In some individuals AIDS may be manifested in 2 to 3 years following infection while for some the onset of AIDS may be delayed beyond 10 years. The time from mucosal infection to viremia is about 4 to 11 days. Individuals with primary HIV infection, also known as acute retroviral syndrome, may develop symptoms in 2 to 6 weeks from the time of exposure. These symptoms often include fever, fatigue, nausea, myalgia, lymphadenopathy, acute meningoencephalitis, pharyngitis, diffuse erythematous macular, weight loss, mucocutaneous ulcerations, and diarrhea [209].

The *acute* phase of HIV infection is marked by lymphocytopenia and/or thrombopenia in the peripheral blood. During this time, there is active viral replication particularly in CD4 lymphocytes (typically with a doubling time of around 20 hrs), marked HIV viremia (~50,000 copies/mL) and seroconversion accompanied by a steady decline in CD4+ lymphocyte counts. During this time, cytotoxic CD8 lymphocyte counts increase, as symptoms subside and viremia decreases. However, atypical lymphocytes are absent [263, 331]. Both humoral and cell-mediated immune responses play a role during this stage. Generally, within 1-3 months following initial infection with HIV, as the viremia decreases, levels of CD4 lymphocytes

rebound after their primary phase of depletion, but fail to attain pre-infection levels. The final stage of progression to AIDS is typically preceded by five initial stages – an eclipse period followed by the respective appearance in blood of HIV-1 RNA, p24 antigen, positive EIA and finally the p31 band in positive immunoblots [74].

Following the acute phase, the infection next becomes clinically *latent* when little or no virus can be detected in peripheral blood mononuclear cells yet active viral replication continues in lymphoid tissues [247]. The CD4 T cell count and the immune response remain moderately low. In this stage, HIV is found either in the extracellular region of the follicular dendritic cells comprising the germinal centers in lymphoid tissues or as latent or replicating virions inside mononuclear cells. A typical immune response consisting of virus-specific CD4 lymphocytes is lacking in HIV infections [208]. However, a handful of seropositive patients on antiretroviral therapy have been reported to lodge a persistent polyclonal CD4 lymphocyte mediated immune response leading to a cytokine response with elaboration of interferon gamma and beta chemokines. With progression to disease, the actively replicating virus cripples the innate immune system which in turn can allow opportunistic bacterial infections to set in. Mononuclear phagocytic cells of the innate immune system (monocytes and macrophages) serve as reservoir for infection. During the latent phase of infection, the immune system deteriorates, primarily through depletion of CD4 lymphocytes, cytotoxic CD8 cells (leading to exhaustion of controlling T cell responses) and cytokines like IL-2 (that regulate lymphoid proliferation) [187, 209]. HIV also affects thymic activity in curbing its capacity to produce new T lymphocytes, which can be detected by measuring levels of T-cell receptor excision circle (TREC) that results from differential splicing of the variable (V), diversity (D) and joining (J) chains of the TCR gene [273].

The stage of *clinical AIDS* is defined and marked by a total CD4 lymphocyte count dipping below 200 - 500 cells per μL , the emergence of syncytia-forming (SI) variants of HIV, derived from non-syncytia-forming (NSI) variants and subsequent development and contraction of typical opportunistic infections and/or neoplasms diagnostically associated with AIDS [179]. Other markers that indicate progression to AIDS are HIV-1 RNA levels in peripheral blood, p24 antigenemia, elevated serum levels of beta2-microglobulin ($\beta\text{2-M}$), IgA, and high levels of neopterin in serum, CSF and urine.

HIV-infected individuals can be grouped as typical progressors, rapid progressors, nonprogressors, and elite controllers. The typical progressors average 8 to 10 years of “latent” infection before the appearance of clinical AIDS while rapid progressors progress to AIDS in 2-3 years. Genetic factors play a deciding role in the speed of progression of HIV infection. Individuals with HLA alleles B*5701, B*5703, B27, and B51 or the 801le variant of the Bw4 motif, a ligand for the immunoglobulin-like receptors KIR3DS1 and KIR3DL1 (that control NK cell function) exhibit slower progression to AIDS [229]. About 10% of HIV positive individuals are nonprogressors, or “long-term survivors,” in whom the immune system does not seem to demonstrate any significant decline for more than 10 years. These patients typically have a stable CD4 lymphocyte count, are seronegative and host a strong virus-inhibitory CD8⁺ T-lymphocyte response. The “elite controllers” are able to subdue their viremia levels even below the detectable limit (< 50 copies/ml) without treatment. They, like the typical progressors have an initial viremia after the primary infection with HIV, but soon after their immune system stands up with minimal loss of CD4 lymphocytes, and a robust response from cytolytic NK cells, polyfunctional CD4 and CD8 cytotoxic lymphocytic responses including efficient granzyme-B-mediated cytotoxicity [29, 242, 248].

1.1.6 Vaccines and current treatment options

Design and development of a safe and universally efficacious vaccine against HIV is undoubtedly the most desired armament in our combat against HIV/AIDS. Vaccine therapy is imperative since it will not only provide long-lasting immunity to protect healthy uninfected individuals but also boost immunity in seropositive individuals as well (generally referred to as post-infection immunization). Unfortunately no such standard vaccine therapy is available yet. Amongst the major obstructions in the development of an effective vaccine are HIV epitope variability, ability of HIV to evade host immune surveillance during cell to cell transmission, lack of an effective neutralizing immune response, and the induction of adverse immune reactions to endogenous human proteins leading to pathogenicity and non-effectiveness of engineered viral strains that have been generated as candidate vaccines [228].

Several initial studies used the HIV gp120 or 160 envelope protein to induce a humoral response, or lipopeptides where synthetically produced palmitoylated peptide fragments of viral proteins were used to enhance the cell-mediated immunogenic response [84, 92]. Another approach was to use recombinant vaccinia poxvirus along with HIV envelope proteins that could induce mucosal immunity. Parallel studies generated gene disrupted HIV-1 strains that could provide protection against the wild type pathogenic strains[228, 338]. Findings like SIVmac239 with a partial deletion in the *nef* gene or even carrying multiple gene deletions, protected rhesus macaques effectively against the wild type intact SIV infection were exciting [85, 351, 352]. However the observation that the disrupted *nef* gene can revert to a fully functional form over the course of viral replication represented a major setback to this approach [50, 285]. These early studies highlighted the importance of the *nef* gene for the viral replication process. In other contemporary relevant studies, it was found that a secondary deletion in the *vpr* gene of

SIVmac239, alongside *nef*, offered protection against a related strain (SIVmac251) but failed to protect against challenge with a non-homologous SIVsmE660 strain [351]. Taken together, these studies suggested that heightened immune responses against one viral strain may not be effective against infection with a non-homologous viral strain. Inability to protect against infection with heterologous strains was observed in humans as well. Patients mounting a strong immune response against a particular HIV clade were able to control their initial HIV infection, but eventually became superinfected with a different clade of HIV [264]. Thus, a single HIV vaccine may not be able to provide effective protection against other heterologous strains [209].

In recent years, only three candidate HIV vaccines have completed clinical efficacy trials. These include (1) a recombinant protein of the HIV-1 envelope (AIDSVAX); (2) a nonreplicating adenovirus serotype-5 vector expressing an internal HIV-1 protein (Gag); and (3) (RV144), a combination of AIDSVAX and a canarypox vector expressing HIV-1 immunogens (ALVAC). The first two candidate vaccines failed their efficacy trials. The third candidate vaccine, RV144 appeared to be short-lived, although it showed a modest 31.2% efficacy [27].

Presently the most efficacious treatment option available is ‘highly active antiretroviral therapy’ (HAART) which slows down the progress of the disease, yet cannot completely eliminate HIV from infected persons. A variety of pharmacologic agents have been developed to treat HIV infection. HAART comprises of a cocktail of at least three medications belonging to at least two different classes of antiviral agents. The antiviral agents are classified into four groups based on the mechanism of their action (1) nucleoside reverse transcriptase inhibitors, (2) non-nucleoside reverse transcriptase inhibitors, (3) protease inhibitors, and (4) others like entry, integrase and fusion inhibitors.

The first effective antiretroviral agent was zidovudine (ZDV) which is a nucleoside analog that interferes with the function of reverse transcriptase (RT). This *nucleoside reverse transcriptase inhibitor (NRTI)*, initially called azidothymidine (3'-azido-3'-deoxythymidine) is an analog of thymidine which is phosphorylated to an active triphosphate form by cellular enzymes. Incorporation of the phosphorylated ZDV results in the termination of chain elongation and cDNA formation which is otherwise essential for subsequent viral replication [340, 350]. Some other examples of NRTIs in the clinic are Stavudine (d4T), Abacavir, Tenofovir and Lamivudine (3TC). *Non-nucleoside reverse transcriptase inhibitors (NNRTI)* also block the action of the RT. However, these drugs bind to an allosteric site close to the active site leading to a conformational change and loss of RT activity [259, 340]. Examples of NNRTIs currently on the market are Efavirenz, Nevirapine and Etravirine. NNRTIs can be used in combination with NRTIs to increase the efficacy of antiviral therapy.

Protease inhibitors (PIs) are an important class of anti-HIV drugs that block the generation of mature virions from immature precursor proteins. PIs are synthetic analogues of the regions of HIV proteins that bind to the active site of the protease enzymes to block activity. A chief problem in the development of this class of drugs was finding an effective, specific inhibitor of HIV protease that did not also interfere with normal cellular proteases. Examples of FDA approved drugs include saquinavir (saquinavir mesylate), ritonavir, indinavir (indinavir sulfate), nelfinavir, and amprenavir. Protease inhibitors are often most effective at high dosages, but myriad side effects including nausea, vomiting and diarrhea and general gastric intolerance and adverse reactions at high doses limit their use [341].

Other prominent classes of antiretroviral drugs include the *entry inhibitors*, *fusion inhibitors* and *integrase inhibitors*. Entry inhibitors prevent HIV entry into the host cells and are

particularly effective in the early stages of viral infection. Entry inhibitors like vicriviroc and maraviroc are CCR5 receptor antagonists that compete with viral gp120 binding to CD4+ co-receptors on T cells [146]. The fusion inhibitor, enfuvirtide blocks HIV gp41-mediated membrane fusion. It is a synthetic peptide corresponding to 36 amino acids within the C-terminal heptad repeat region (HR1) of HIV gp41 protein. It has a low genetic barrier to resistance and must be used in combination with other drugs [197]. Integrase inhibitors, also known as Integrase Strand Transfer Inhibitors (ISTIs), inhibit the action of the viral integrase enzyme. Integrase inhibition prevents the incorporation of the proviral DNA copy to the host cell DNA. Raltegravir and elvitegravir are ISTIs that specifically inhibit the final step of the three steps involved in the DNA transfer process [288].

The HAART approach combines several anti-HIV agents belonging to different classes. The standard recommended ART regimens have various combinations including (2NRTI + NNRTI); (NtRTI + NRTI); (NtRTI + NNRTI); (2NRTI + PI) OR (2NRTI + 2PI) [323]. Fixed dose combinations with different anti-viral agents have been combined into single medication pills to enhance patient adherence and effectiveness of HAART. For example a combination of emtricitabine and tenofovir (both NRTIs) with Rilpivirine (second generation NNRTI) named Complera (manufactured by Gilead Sciences and Johnson and Johnson) was approved by the FDA in 2011 [5, 34, 105] . However due to the high error rate of the HIV reverse transcriptase and poor patient commitment to treatment regimens, mutant viruses are rapidly generated and selected over non-mutant strains.

The field of HIV vaccine development is challenging however the rewards of a safe and globally effective vaccine will be tremendous. In parallel, ART is useful in prolonging the lives of treated patients by elevating CD4 T-cell counts and partially suppressing HIV replication

resulting in the decrease of the frequency and severity of opportunistic infections. However, drug intolerance, cross reactivity, and toxic side effects of anti-viral agents, particularly in high doses are significant problems for all drugs used to treat HIV infection and thus can severely limit the use and benefit of HAART in patients. This necessitates continued research to identify newer alternative targets for therapeutic intervention as well as the design and development of improved antivirals for the inhibition of viral spread to curtail new infections. Most importantly, a continuing pipeline of new agents is needed to increase the lifespan of infected individuals, especially those that are refractory to existing HAART regimens.

The accessory proteins (Vpr, Vpu, Vif, and Nef) encoded by the HIV genome play critical roles in viral pathogenicity and thus represent alternative targets for drug discovery [10, 134, 138, 165, 219]. Owing to its multifaceted nature, and essential role in promoting immune escape, HIV-1 Nef is a very attractive target for anti-retroviral drug discovery. HIV-1 Nef stands at a very central position in my dissertation research, wherein I have explored the scope of interaction of this HIV-1 protein with host cell kinases and characterized their interactions. Thus, a more detailed discussion will follow in the subsequent sections and chapters leading to the rationalization of Nef:host cell factor interactions as a promising target for future antiretroviral drug targeting.

1.2 HIV-1 NEF

1.2.1 AIDS and HIV-1 Nef: role in viral pathogenesis

Despite our enormous efforts to combat the most prevalent contemporary social menace, HIV still remains an elusive target for eradicating cure or effective immunization. The key to its elusive nature is the ability of the virus to mutate spontaneously, interact and hijack host cellular and transcriptional machinery, alter host cell trafficking and signaling, evade host immune system and persist comfortably in latent reservoirs. The multifaceted accessory protein of HIV and SIV, Nef plays a key role in mediating most, if not all, of the above listed viral survival strategies to its own advantage. Nef is a multifunctional protein that lacks intrinsic catalytic activity, and thus functions by interacting with host cellular factors involved in cellular activation, immune recognition and survival [270] and in a way optimizes the cellular environment for viral replication and release.

Early studies identified Nef as a *negative* factor based on findings that it decreased viral replication and transcription, resulting in inhibition of viral pathogenesis [7, 115, 321]. However, soon after and in hundreds of studies since, Nef has been firmly established as a positive effector of HIV pathogenesis. Nef, now re-identified as an accessory HIV protein with *numerous* *effector* *functions*, is required for high titer viral replication and AIDS pathogenesis *in vivo* [9, 101, 143, 149, 150, 182, 183, 230, 311].

Some of the earliest and most powerful evidence for Nef as an essential gene for disease progression *in vivo* comes from Kestler et al., who showed that rhesus monkeys infected with *nef*-deleted SIV failed to develop AIDS-like disease [182]. Further, while some groups reported that interruption of the unique portion of the *nef* gene from SIV rendered the virus non-

pathogenic [85, 182, 351], some described the phenomenon of natural reversion of mutations in SIV nef genes to restore functional Nef protein and the pathogenicity of the virus [50, 51, 285, 342]. The existence of strong selective pressure to drive the reversion of Nef mutations in order to regain SIV virulence confirmed the vital role of Nef in the induction of viral pathogenicity.

In 1998, Hanna et al. showed that in transgenic mice, human CD4 promoter-directed expression of the full HIV-1 genome targeted the expression of viral genes to CD4⁺ T cells, macrophages and dendritic cells, leading to the development of AIDS-like disease. These mice exhibited AIDS-associated symptoms including depletion of CD4⁺ T cells, thymic atrophy, immunodeficiency and premature death [142]. Follow-up studies showed that targeted expression of Nef alone to T-cells and macrophages is sufficient for development of an AIDS-like syndrome in transgenic mice [143].

A more recent study has demonstrated an essential role for Nef in HIV infection in vivo. Using mice reconstituted with the human immune system, Zhou et al. showed that wild-type HIV-1 infection in these mice resulted in rapid depletion of CD4⁺ T-cells while infection with Nef-defective virus failed to deplete CD4⁺ T-cells. This observation supports a direct role for Nef in thymocyte killing that complements the results with Nef-transgenic mice [367].

Moreover, further studies have reported detection of mutated Nef alleles in long term non-progressors, defined as a subset of HIV-infected individuals who defy the normal progression curve of the disease, maintain normal levels of CD4⁺ T cells and fail to progress to AIDS after a decade or more in the absence of antiretroviral therapy [89, 121, 186, 225, 326]. These findings support an essential role for Nef in HIV infection, disease progression and AIDS development in humans.

1.2.2 HIV-1 Nef: structure and key recognition motifs

Nef is a polymorphic, 27-35 kDa accessory protein unique to primate lentiviruses and is encoded by a single exon located towards the 3' end of the *env* gene and extends into the U3 region of the 3' LTR of the viral genome. Nef is one of the early proteins of HIV that is expressed even before the integration of the viral genome into the host chromosome. It is the first viral protein to be expressed to detectable levels in a cell right after HIV-1 infection.

Nuclear magnetic resonance (NMR) and X-ray crystallography studies have shown that Nef consists of flexible N- and C-terminal loops and a globular core domain (a.a. 54-205) [112, 205]. The largest of the six accessory proteins of HIV/SIV, Nef lacks any known biochemical activity and thus executes its functional effects by interacting with various host cellular factors. Although the amino acid sequence and length of HIV-1 Nef are variable, several distinct functional domains have been identified. Comparison of aligned sequences from Nef allelic variants reveals conserved structural motifs with defined roles in binding specific intracellular proteins. Figure 1.6 summarizes many of these Nef motifs and the most prominent ones are discussed in greater detail below.

N-terminal myristoylation

HIV-1 Nef contains the sequence MGxxx(S/T) at its flexible amino-terminal domain. Nef undergoes post-translational modification at this motif by a ubiquitously distributed enzyme N-myristoyl transferase, wherein a myristoyl group is covalently linked to the glycine residue at the G2 position. The myristoylation at G2 along with two lysine residues (K₄xxK₇), and four arginines (R₁₇xRxRR₂₂) near the N-terminus of the protein targets the protein to the inner leaflet

of the cell membrane [33, 41, 126, 148]. Some of the validated binding partners of Nef include CD4, MHC-I, PAK2 kinase, actin, and calmodulin [102, 226, 283]. The highly conserved N-terminal myristoylation motif of Nef is critical not only for interaction with these binding partners but also for induction of MHC-1 and CD4 down-regulation and in a holistic sense, for HIV-1 replication [151, 253].

Mutagenesis studies have shown that the myristoylation site is indispensable for the ability of Nef to induce depletion of CD4⁺/CD8⁺ thymocytes and subsequent development of AIDS-like disease in transgenic mice [144]. Myristoylation also plays a vital role in Nef-mediated activation of NFAT, NFκB, and induction of IL-2 expression leading to enhanced transcription from the viral LTR and HIV-1 replication in T cells stimulated by CD3 and CD28 [339]. Thus the conserved myristoylation motif plays a crucial role in many Nef functions critical for HIV-1 pathogenesis.

The acidic motif

HIV-1 Nef has a conserved N-terminal acidic motif (E₆₂EEE) which is required for the binding and interaction of Nef with trafficking adaptor protein PACS-2. This interaction targets Nef to the trans-Golgi region where it assembles a multi-kinase complex (SFK/ZAP-70/PI3K) to internalize MHC I molecules. HIV-1 Nef triggers the down-regulation of MHC class I molecules and in a way facilitates the viral replication by evading host immunity and NK cell mediated lysis of the infected cell as described in the previous section. Sequence alignment from 186 different strains revealed that the Nef acidic motif is highly conserved. Rare exceptions include the insertion of a fifth acidic residue (E or D) and sometimes a non-conservative insertion of a Gly only in eight sequences [24, 36, 126, 166].

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

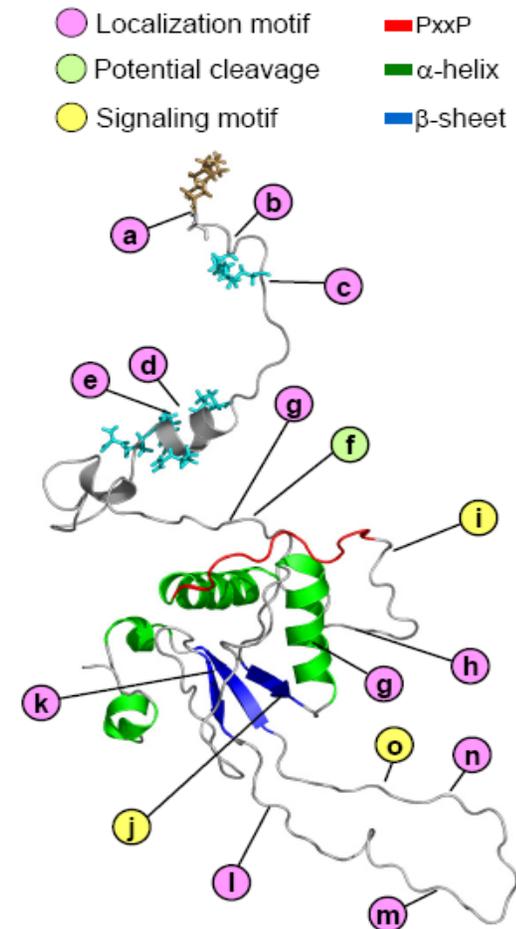


Figure courtesy of Dr. John Engen, Northeastern University, Boston, USA.

Figure 1.6. Structure-function relationships in Nef

The dileucine motif

HIV-1 Nef has a highly conserved dileucine (L₁₆₄L₁₆₅) sequence motif localized to its carboxy-terminal loop which functions as an endocytosis signal. SIV Nef has a tyrosine-based motif instead. Both the dileucine motif and tyrosine-based motif act as well characterized internalization signals for CD4 down-regulation that directs cellular proteins to clathrin-coated vesicles. Mutation of the dileucine motif impairs the ability of Nef to down-regulate CD4 [40, 133, 256]. This motif on Nef is involved in binding the clathrin adaptor AP-2 through the alpha and sigma (α - σ 2) subunits hemicomplex. The Nef:AP-2 complex interacts with the cytoplasmic tail of CD4, to form a tripartite Nef:AP-2:CD4 complex [57] which triggers the formation of clathrin-coated pits. This results in rapid internalization of CD4, delivering it to lysosomes via the multivesicular body pathway [87]. Several other studies have reported the requirement for the dileucine motif on Nef for binding to the AP-1 and AP-3 adaptor complexes via their medium (μ) subunits [75, 83, 124, 171].

The diarginine motif

Nef encodes a highly conserved diarginine motif (R₁₀₅K/R) at the N-terminus of the α 4 helix of the folded core. The first Arg is absolutely conserved while the second can be replaced with Lys or Gln. This conserved motif, together with the SH3-binding motif, is required for Nef-mediated activation of the cellular serine/threonine p21-activated kinase (PAK2) leading to T- cell activation, induction of LTR activity, cytoskeletal rearrangement and subsequent facilitation of HIV-1 replication. Nef-induced activation of PAK2 is also essential in progression of simian AIDS. Moreover, this activation and binding of PAK2 is conserved and required but not

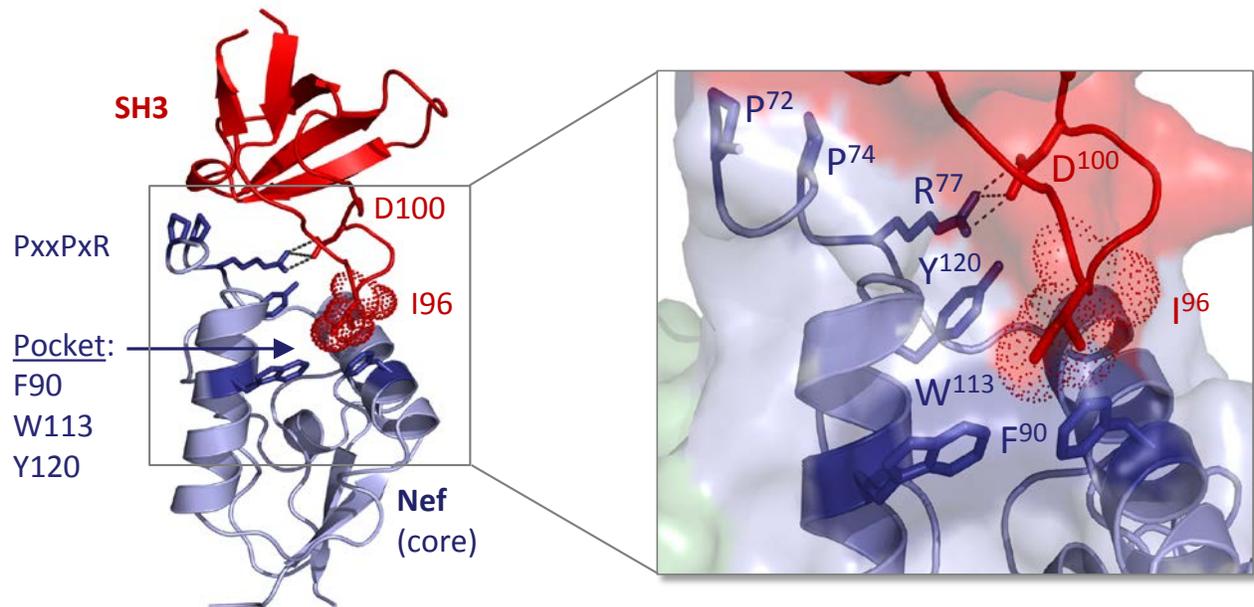
sufficient, for induction of multi-organ AIDS-like disease in transgenic mice [103, 104, 143, 269, 284, 286, 334].

The SH3-binding motif (PxxPxR)

Nef has a highly conserved proline-rich motif (PxxPxR) that binds to the SH3 domains of host cell proteins [270, 281, 325], most notably members of Src family of non-receptor protein-tyrosine kinases (SFKs) and causes constitutive activation [46, 281, 325]. The PxxPxR motif is highly conserved across all major clades of HIV-1, implying an essential role in Nef function. X-ray crystallography of a Nef-SH3 complex (PDB:1EFN) (Figure 1.7) reveals that the conserved proline residues form a typical polyproline type II helix that contacts the hydrophobic surface of the SH3 domain [137, 205]. This interaction is stabilized by an ionic interaction between SH3 Asp100 and the conserved Arg in the Nef PxxPxR sequence. The crystal structure and other mutagenesis studies revealed other essential determinants of high-affinity Nef-SH3 interaction in addition to the PxxPxR motif. These include residues that form a hydrophobic pocket (Y120, F90, and W113) in the three dimensional structure of the folded core that contacts Ile96 in the RT loop of the Hck SH3 domain [61, 205]. HIV-1 Nef binds to Hck SH3 with relatively high affinity in the nM to low μ M range, depending upon the allelic variant. The laboratory B-clade SF2 allele has the highest reported affinity for isolated Hck SH3 domain ($K_D = 70$ nM) while Nef from the primary HIV-1 subtype A2 has the lowest ($K_D = 21.10$ μ M) [239].

The interaction of Nef with an SFK SH3 domain via its PxxPxR motif is required for Nef-induced down-regulation of MHC class I receptors, a key function in immune evasion of HIV-infected cells [36, 54, 166]. The intact SH3-binding motif of Nef has been shown to be essential for intracellular Lck accumulation and also for interference with the functions of the

immunological synapse [140]. Although the polyproline motif on Nef is dispensable for the down-regulation of cell surface CD4 receptors, it is critical for augmentation of HIV-1 replication in infected peripheral blood mononuclear cells (PBMC) as well as SIV replication in macaques [182, 281] and development of the AIDS-like syndrome in transgenic mice [145].



Crystal structure of Nef interacting with a Src family kinase SH3 domain (*PDB: 1EFN*). Key determinants of the high affinity binding of Nef (blue-gray) to SH3 domain (red) involve contacts between not only the SH3 hydrophobic surface and the conserved PxxPxR motif on Nef but also the I96 residue on the RT loop of SH3 and the hydrophobic pocket formed by Nef residues Y120, F90, and W113. Numbering of residues in the figure is based on the crystal coordinates from [205].

Figure 1.7. Crystal structure of Nef-SH3 complex

1.2.3 Implications of Nef:Host cell interactions in viral pathogenesis

Nef protects the infected cell, ensures its survival and enhances primate lentiviral persistence mainly by (1) facilitating viral immune evasion and (2) supporting viral replication. The non-enzymatic Nef protein functions through interactions with a multitude of host proteins and these interactions form the mechanistic basis for almost all of Nef's pleiotropic effects on an HIV infected cell. Based on current reports to date, Nef may interact directly with as many as 60 cellular factors and affect the function of more than 180 cellular proteins. An extensive list of all cellular factors that have been reported to bind to Nef or whose function is affected by Nef has been summarized at <http://www.ncbi.nlm.nih.gov/projects/RefSeq/HIVInteractions/nef.html>.

In the section that follows, I have summarized the interactions and functions of Nef that facilitate immune evasion and ones that directly support viral replication and spread. It might be advantageous for the reader (in an effort to refrain from developing any misconceptions) to bear in mind that these functions of Nef are not spatio-temporally exclusive from one another. Instead the Nef:host cell interactions mutually complement one another and cooperatively ensure the maintenance of a favorable cellular environment that facilitates viral persistence and spread.

Interactions facilitating viral immune evasion

Nef enables the virus to escape recognition by the host immune system in several different ways. One of the most prominent functions of Nef involves its ability to down-modulate cell surface expression of major histocompatibility-I (MHC-I) molecules by targeting them to the *trans*-Golgi network (TGN), followed by TGN-associated endosomal compartments and finally to lysosomes for degradation. Nef may either accelerate endocytosis of expressed MHC-I from the

cell membrane or prevent the normal anterograde transport of MHC-I molecules post translation from the TGN to the cell surface [277]. In this process Nef recruits and interacts with the phosphofurin acidic cluster sorting protein-1 (PACS-1); the μ 1 subunit of the clathrin adaptor complex AP1 and subsequently β -COP – all of which are implicated in endosomal trafficking. The downregulation of Class I MHC helps the HIV-infected cell to avoid host immune surveillance by CD8+ cytotoxic T lymphocytes and thus decreases the efficiency of the killing of HIV infected cells by cytotoxic T cells [36, 78, 287, 293].

MHC-I HLA-C and –E are recognized by inhibitory NK cell receptors (iNKRs) to prevent NK cell cytotoxicity under normal physiological conditions. Non-selective down-modulation of MHC-I results in reduced iNKR engagement and induces NK cell cytolysis. Nef therefore selectively interacts with the cytoplasmic tails of HLA-A and HLA-B but not HLA-C or –E [72, 203]. This prevents NK cell-mediated lysis of infected cells despite reduced levels of MHC-I surface molecules. Besides hiding from surveilling NK and CTLs, Nef also assists in cell survival by directly killing attacking bystander CD8+ CTLs by upregulating expression of FasL death factor on the infected cell surface [355]. Nef interacts directly with the HLA class II histocompatibility antigen gamma/invariant chain (Ii or CD74). Nef up-regulates surface expression of Ii which in turn caps the MHC-II peptide binding site during endosomal trafficking, resulting in restricted MHC-II-antigen presentation leading to impaired helper T cell responses [324].

One of the most extensively studied roles of Nef involves downregulation of the cell surface expression of CD4 leading to perturbation of T cell activation, and enhancement of HIV infectivity [118]. Nef directs the endocytosis of CD4 by linking it to both the AP-2 clathrin adaptor complex and COP-1 lysosomal targeting protein culminating in its subsequent lysosomal

degradation. Nef binds the cytoplasmic tail of CD4. This interaction involves a dileucine repeat sequence in the membrane proximal region of CD4 and also the dileucine motif, two diacidic motifs and a hydrophobic pocket on Nef. Reduced expression of CD4 on the cell surface appears to be advantageous to the virus for several reasons: 1) avoiding superinfection of the host cell by newly released progeny virions; 2) enhancement of virion budding by preventing CD4 interaction with Env; and 3) reducing CD4-mediated inhibition of HIV transcription by releasing the CD4-associated Lck signaling molecule, which may promote T cell activation and enhanced viral replication [8, 118, 198, 199].

Nef has been reported to bind to the cytoplasmic tails of the co-stimulatory molecules, CD28 in T cells and B7 (CD80/CD86) in APCs and induce their endocytosis via the AP-2 clathrin adaptor pathway and an actin-based endocytic pathway (involving activation of c-Src and Rac) respectively. By down-regulating surface expression of the co-stimulatory molecules, Nef induces antigen-specific tolerance and T cell anergy, thereby suppressing an effective immune response against the virus [16, 55, 56, 315].

Interactions facilitating viral infectivity, replication and spread

Nef modulates the activation status of the infected T cells and enhances viral infectivity of progeny virions to promote replication and persistence. In addition to down-regulating cell surface expression of CD4 and CD28, Nef also interacts with numerous signaling proteins and increases the responsiveness of infected T cells to stimulation. This results in constitutive activation of downstream signaling pathways that activate transcription factors. The virus benefits from this, since activation of transcription factors would result in increased expression of HIV provirus.

It is well established that Nef interacts with SH3 domains of host cellular proteins via its conserved polyproline (PxxPxR) motif. Members of the Src family of non-receptor protein tyrosine kinases (SFKs) are among the best studied binding partners for Nef, and are involved in different Nef-mediated functions like MHC-I downregulation, viral infectivity and replication [61, 91, 98, 239, 281, 325]. Nef has also been reported to interact with the SH3 domain of VAV, a Rac1 guanine nucleotide exchange factor (GEF), probably to trigger the JNK/SAPK signaling pathway and to induce cytoskeletal rearrangements [104].

Nef interacts with serine and threonine kinases as well, most notably the p21-activated serine–threonine kinase 2 (PAK-2). Association of Nef with PAK-2 reportedly plays a crucial role in viral replication, apoptosis, T cell activation, and disease progression [66, 103, 270]. Interactions of Nef with additional effectors that lead to subversion of T cell signaling pathways include protein kinase C (PKC), and interactions with the Ras-Raf-MAP kinase pathway [161, 348]. Nef alters T cell signaling by exerting pleomorphic effects on T cell activation depending on where it is localized in the cell. Early studies showed that expression of Nef in Jurkat T cells prevented antigen receptor-mediated induction of interleukin 2 mRNA expression and also inhibited induction of transcription factor NF-kappa β DNA-binding activity by T-cell mitogens [216, 241]. Expression of a CD8-Nef chimeric molecule in Jurkat cells led to either inhibition or stimulation of T-cell receptor signaling when the chimeric molecule localized to the cytosol or the cell membrane respectively [32]. Further studies reported that Nef led to elevation of T cell signaling in Nef transgenic mice [304].

In T cells, Nef blocks the Fas/FasL and tumor necrosis factor-alpha (TNFalpha) apoptotic pathways through the apoptosis signal-regulating kinase-1 (ASK-1) [123]. Similarly, through an association with PAK and PI3-kinases, Nef stimulates Bad-phosphorylation resulting in the

induction of anti-apoptotic signaling [349]. In macrophages, Nef increases expression of the anti-apoptotic factor Bcl-X_L through the Erk/MAPK pathway [62]. In addition, Nef also has been shown to promote macrophage cell survival through activation of the signal transducer and activator of transcription, STAT-1 and -3 [45, 106, 254]. By redirecting the infected cell from normal cellular apoptotic pathways to cell survival, Nef enables the infected cell to survive, thus providing a favorable cellular environment for its reproduction and persistence.

In an effort to augment the expression of its own genome, HIV Nef induces transcription factors like NFAT, NFκB, IRF-1/2, c-fos, Jun-D that transactivate the HIV-1 LTR promoter. It also induces the expression of cellular co-factors like CDK9, elongation factor Tat-SF1 and cytokines like IL-2, TGFβ that help in viral replication [15, 303].

Nef is incorporated into progeny virions during assembly, which is then cleaved by the viral protease upon maturation. Recent studies have implicated the interaction of Nef with the GTPase Dynamin-2 (Dyn-2) in the enhancement of virion infectivity [257]. By binding to Dyn-2, which is an essential regulator of clathrin-dependent endocytosis, Nef increases clathrin-coated pit formation [230]. Thus, Nef-mediated enhancement of virion infectivity depends on both, Nef binding to Dyn-2 and clathrin-coated pit formation.

Besides manipulating the infected host cells, Nef also alters the cellular environment to promote viral spread. It has been reported that Nef induces secretion of the two CC-chemokines, macrophage inflammatory proteins 1α and 1β (MIP-1α/β) by HIV-1-infected macrophages. These secreted chemokines in turn stimulate chemotaxis of resting T-cells towards sites of virion release from infected macrophages, contributing to viral spread [317]. Further studies reported that Nef can also stimulate the production of soluble paracrine factors, sCD23 and sICAM from HIV-1 infected macrophages which in turn upregulate B-cell receptors leading to resting T-

lymphocyte stimulation making them permissive for productive HIV-1 infection [316]. Extracellular Nef has also been found to expedite the spread of T-cell-tropic HIV variants and facilitate a switch in dominant replicating viral strains from R5 to X4 viruses [28]. Taken together, these and other studies indicate that Nef has evolved complex strategies to manipulate the cross-talk between different cell types to ensure viral persistence, spread and the expansion of the cellular reservoir of HIV-1 within the host.

1.2.4 Nef as a drug target for HIV/AIDS

The high rate of mutations resulting from the error-prone activity of HIV-1 reverse transcriptase leads to the incidence of new resistance mutations in patients on chronic antiretroviral therapy. Failure to adhere to prescribed drug regimens also threatens the emergence of resistance leading to limited range of subsequent treatment options, higher rates of disease progression and poorer clinical outcomes. The gradual development of resistance to ART regimen drugs points to the continual need for the discovery and development of new antiretrovirals against alternate targets. As discussed in section 1.1.6, Nef and its downstream signaling partners represent very attractive targets for antiviral drugs. For example, Nef binds to SH3 domains of Hck, Lyn and Src via its proline-rich motif [281, 325] and a hydrophobic pocket [61] causing constitutive kinase activation. Other studies have reported that transgenic mice that expressed Nef-2PA, a mutant in which the conserved PxxP motif is mutated to AxxA never developed AIDS-like disease [145]. These studies strongly suggest that Nef-SH3 interaction is a crucial step essential for viral pathogenesis and thus pose as an exciting alternative target for rational drug discovery and design. Several virtual, computational and in vitro screening approaches have begun to identify novel antagonists for Nef [307]. These studies are described in more detail below.

Early studies identified a phenoxyacetamido benzoic acid derivative (D1) and an analog (DLC27), that interfered with not only Nef:SH3 interaction in a mammalian cell-based protein-protein interaction assay but also partially blocked Nef-induced MHC-I downregulation [35]. Similarly, a derivative of the *Streptomyces* metabolite UCS15A, called '2c' was found to interact with the SH3-domain binding site on Nef as well as the ridge in between the central β -sheet and the C-terminal α -helices of Nef. This compound rescued down-regulation of MHC-I expression in HIV-1 infected primary CD4+ T-cells by blocking the formation of the Nef-mediated multi-kinase (SFK/ZAP-70/PI3K) complex and subsequent Src-family kinase activation. [67, 91]. However the low potency and poor physiochemical properties of these compounds limits their further development as antiretroviral drug leads.

Recent work from our group has established that selective inhibitors of Nef-mediated Src-family kinase activation also block Nef-dependent HIV replication and infectivity. An in vitro screen of a kinase inhibitor-biased library (2500 compounds) identified a group of 4-amino-diphenylfuranopyrimidine (DFP) compounds that selectively blocked Nef-mediated Hck activation. These compounds also inhibited Nef-dependent HIV-1 replication in cells infected with HIV-1 chimeras carrying *nef* alleles representative of all of the major clades of HIV-1 [97, 239]. Subsequent studies used the same Nef-Hck assay in an automated screen of a much larger, chemically diverse library of more than 200,000 compounds. This screen identified a novel diphenylpyrazolodiazene compound (B9) that preferentially inhibited Nef-dependent activation of Hck. This compound also potently inhibited Nef-dependent replication of wild-type HIV-1 NL4-3 chimeras expressing *nef* alleles representative of all M-group HIV-1 clades, with IC₅₀ values in the nanomolar range [98]. Our group also recently identified another small molecule Nef antagonist, a dihydrobenzo-1,4-dioxin-substituted analog of N-(3-aminoquinoxalin-2-yl)-4-

chlorobenzenesulfonamide (DQBS). This compound was identified in a yeast-based phenotypic screen for compounds that rescue the inhibitory effect of ectopic Nef-mediated Hck activation in yeast. DQBS was shown to potently inhibit Nef-dependent HIV-1 replication and MHC-I downregulation in T-cells [327]. Computational docking studies, as well as direct interaction assays (surface plasmon resonance; differential scanning fluorimetry), demonstrate that both B9 and DQBS interact directly with Nef to block Hck activation.

Taken together, these studies highlight the promise of pharmacologically targeting this virulence factor as a new approach towards AIDS treatment. Discovering and developing antagonistic molecules that can interfere with the critical functions of HIV-1 Nef may lead to alternate therapeutic strategies that can work synergistically with the existing antiretroviral therapies.

1.3 PROTEIN TYROSINE KINASES

Literature reviewed in the preceding sections illustrates the critical role for host protein kinases as binding partners and effectors for HIV-1 Nef. In the succeeding sections that follow, I will first write about the background on general strategies by which viruses exploit host cell kinases to their own advantage. I will then discuss the structure and function of the Src kinase family in detail, because of its importance to HIV-1 Nef function. This will then be followed by a review of the related Tec kinase family - the interaction of Tec family kinases with HIV Nef being the main focus of this dissertation project.

1.3.1 Role of Kinases in virus:host interactions

The kinome, defined as the kinase complement of the human genome, constitutes around 1.7% of all human genes. It consists of 518 kinases, amongst which 90 belong to the superfamily of protein tyrosine kinases (PTKs) where every active protein kinase phosphorylates a tyrosine on a distinct set of substrates in a regulated manner [220]. The PTKs principally function to mediate signal transduction in eukaryotes and thus are involved in the regulation of diverse cellular processes including growth, differentiation, metabolism, transcription, cell cycle progression, adhesion, and motility. Serving as key regulators of cell signaling, the loss of regulation of PTK activity has often been associated with development of several pathological states including diabetes, cancer, congenital syndromes and even viral infections [220, 245, 276]. Viruses, for the sake of their progression and spread, often hijack host cell protein-tyrosine kinase signaling pathways. A few examples are described in more detail below.

In a recent study, the cytoplasmic tyrosine kinase, focal adhesion kinase (FAK) has been found to link early activation of phosphatidylinositol-3-kinase (PI3K) and actin reorganization to aid in influenza A virus entry. Inhibition of FAK signaling was found to not only disrupt the actin network leading to sequestration of the virus at the cellular boundary but also impede viral RNA replication [96].

The host tyrosine kinases often can play dual roles, interacting with viral proteins to alter cellular signaling cascades but also signal to activate the innate and humoral immune arsenal against the pathogen. For instance, a comprehensive genome-wide high-throughput cDNA screen has recently identified a novel antiviral tyrosine kinase (TNK1) that induces IFN-stimulated genes, and phosphorylates STAT1 leading to the induction of JAK-STAT signaling during Hepatitis C virus (HCV) infection [244].

The Src family of non-receptor tyrosine kinases (SFKs) has also recently emerged as cellular factors that are often exploited by viruses to their own benefit. Commonly, viral proteins bind to specific motifs on these kinases, leading to phosphorylation of viral and cellular proteins and triggering of aberrant signaling cascades. Some of the different viral proteins that interact with host cell SFKs are tabulated in Table 1.3. Some of the earliest studies reported the interaction of the polyoma virus middle T (mT) antigen with the kinase (SH1) domains of the SFKs, Src and Fyn. The mT protein, which is expressed early in the viral lytic cycle and is responsible for the onset of multiple tumors, is a substrate for SFKs and also interacts with molecules essential for downstream oncogenic signaling (e.g. PLC γ , PI3K and Shc) [81, 129, 178].

The nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) has been shown to bind to the recombinant SH3 domains of Hck, Lck, Lyn and Fyn in vitro, but not Src, down-

modulating the activity of Hck, Lck and Lyn but stimulating that of Fyn [217]. In B cells, it directly binds to the Src homology 2 (SH2) and 3 (SH3) domains of Fyn and activates it. Observations like upregulation of HCV replication following siRNA targeted knock-down of Fyn suggests that NS5A possibly negatively regulates HCV replication by activating Fyn kinase for persistent infection [238, 295]. However, in HCV-infected hepatocytes c-Src directly interacts with the viral RNA-dependent RNA polymerase (NS5B) via its SH3 domain and with the nonstructural phosphoprotein NS5A via its SH2 domain. These interactions are essential to maintain the protein-protein interaction of NS5A and NS5B, an event which is required for viral replication, and whereby NS5A mediated Fyn activation remains dispensable [255]. The VP11/12 protein of herpes simplex virus (HSV-1) mimics an activated growth factor receptor in binding to the SH2 domain of and activating the SFK Lck via its tyrosine-based motifs (YETV and YEEI), resulting in the stimulation of PI3K/Akt signaling [313, 335].

Recent reports implicate the interaction of the neuraminidase protein (NA) of influenza A virus with carcinoembryonic antigen-related cell adhesion molecule 6/cluster of differentiation 66c (C6) in enhancing host cell survival during productive viral infection and replication. The NA:C6 interaction activates the Src/Akt signaling cascades via elevated phosphorylation levels of Src, FAK, Akt, GSK3 β , and Bcl-2 [120].

Table 1.3. Different viral proteins that interact with host SFKs

Virus	Viral Protein	Src family kinase
Polyomavirus	Polyoma middle-T antigen (mT)	Src and Fyn
Avian Influenza virus	Non-structural protein 1	Src
Epstein-Barr virus	Latent membrane protein 2A	Lyn
Human T-cell leukemia virus type 1	Accessory protein p13	SFKs
Herpes simplex virus	Tegument protein VP11/12	Lck
Herpesvirus saimiri	Tyrosine kinase interacting protein	Lck
Hepatitis C virus	Non-structural protein 5A (NS5A)	Src
	RNA-dependent RNA polymerase non-structural protein 5B (NS5B)	Src
SIV/HIV	Accessory viral protein X (Vpx)	Fyn
HIV-1	Accessory viral protein Nef	Hck, Lyn, c-Src

Adapted from [245].

1.3.2 Src family of non-receptor protein tyrosine kinases (SFK)

The Src-family kinases (SFKs) are a group of non-receptor protein tyrosine kinases that transduce signals for cell adhesion, migration, growth, differentiation and survival in response to growth factors, cell surface ligands and cytokines [49, 249, 250]. Of the 11 members that comprise the Src-family kinase complement of the human genome, 8 have been well characterized, namely c-Src, Lck, Hck, Lyn, Fyn, c-Yes, Blk, and Fgr [99, 300]. Of these, Src, Yes and Fyn are ubiquitously expressed while Blk, Fgr, Lck, Hck and Lyn are found mainly in hematopoietic cells. Lck and Fyn are found in T-cells while Hck and Fgr are restricted almost exclusively to macrophages and other myeloid leukocytes. Blk and Lyn are expressed primarily in B-cells [80, 246, 322, 366].

1.3.2.1 SFK structure

Elucidation of the crystal structures of the downregulated conformations of Hck and c-Src have advanced our understanding of the three dimensional spatial arrangement of the structural domains of SFKs [82, 301, 346, 354]. As shown in Figure 1.8, the general domain organization of all SFKs consists of an N-terminal myristoylation signal sequence for membrane localization followed by a unique region, a Src-homology 3 domain (SH3), a Src-homology 2 domain (SH2), and a tyrosine kinase domain (Src-homology 1 domain) and a C-terminal tail [33, 99, 271, 300].

Unique region

The N-terminus of each Src family member contains a consensus sequence for myristoylation ($M_1GxxxST_7$) which contributes to membrane localization [271, 272]. Membrane targeting of SFK is critical for their complete functional activity and signal transduction [49]. This sequence is followed by a stretch of about 100 amino acids unique to each family member (Figure 1.8). This region is also the site of palmitoylation in most cases, another acyl modification important for membrane localization. This unique region may represent a key determinant in the selectivity of these closely related kinases for their downstream targets [246]. Indeed, in chicken embryo fibroblasts replacement of the c-Src unique domain with that of its closest phylogenetic relative, c-Yes not only blocked c-Src-mediated activation of PI3K, phosphorylation of c-Raf and Akt and downregulation of RhoA-GTP which is involved in dynamic regulation of actin, but also compromised the ability of Src to alter cell morphology and stimulate motility and invasive potential [314].

Src-homology 3 (SH3) domain

The SH3 domain is about 55-70 amino acids long, lacks catalytic activity and thus exerts its effects through protein-protein interactions. Small hydrophobic grooves on the outer surface of the domain are suitable for binding polyproline sequences on a target peptide (Figure 1.8)[73, 362]. The proline-rich (PxxP) sequence on the target protein typically forms a left-handed polyproline type II (PPII) helix in which every residue is oriented 120° from each other [191, 192, 233]. This results in the two conserved Pro residues pointing in the same direction towards the SH3 domain. The first proline in this PPII helix interacts with a key Trp located within one of the hydrophobic pockets on the SH3 binding surface [100, 292]. In the downregulated crystal structures of inactive c-Src and Hck, the “linker” region between SH2 and kinase domain forms a PPII helix to which the SH3 binds in an intramolecular fashion. The SH3 domain regulates the kinase activity by binding to the SH2-kinase linker.

Src-homology 2 (SH2) domain

The SH2 domain is larger in size than the SH3 domain, and is about 100 amino acids in length. It recognizes and binds to specific phosphotyrosine sequences, with pTyr-Glu-Glu-Ile (pYEEI) the preferred recognition sequence for Src-family kinase SH2 domains (Figure 1.8). The two pockets for ligand binding in the SH2 domain accommodate the phosphorylated tyrosine in the first pocket followed by a hydrophobic isoleucine residue in the second shallower pocket [73, 99, 308, 309, 337]. However, all SH2 domains contain an invariant arginine residue (Arg174 in c-Src) in the first binding pocket that is essential for interaction with the phosphorylated tyrosine [337]. The second binding pocket and surrounding surface residues may differ amongst SH2

domains to confer specificity to the SH2-binding motif. In the context of downregulated SFKs, the SH2 domain binds to the tyrosine-phosphorylated tail, providing a second contact important for repression of kinase activity.

Kinase (SH1) domain

The kinase domain of SFKs mediates the catalytic activity of the protein (Figure 1.8) and thus, is responsible for the catalytic transfer of the γ -phosphate group from ATP to tyrosine in the substrate. In between the two lobes of the kinase domain rests a catalytic cleft, which is surrounded by key amino acids integral to the binding of both the substrate sequence and ATP [189]. The key residues within the catalytic site which coordinate the ATP-Mg⁺⁺ complex during catalysis include Asp386 and Asn391 (residues are numbered according to chicken c-Src). Conserved Lys295 and Glu310 form a salt bridge critical to the catalytic step, while Asp404 of the conserved DFG motif (amino acids 404-406) coordinates association of ATP-Mg²⁺ [39, 141, 278]. The catalytic site can switch between “open” and “closed” states during activation or inhibition of the kinase respectively and this is regulated by an activation loop tyrosine residue (Y416) (Figure 1.8). Phosphorylation on Y416 opens the catalytic site, allowing access to the ATP and substrate into the cleft [79, 353]. When unphosphorylated, Y416 forms hydrogen bonds with R385 and D386, effectively blocking the opening of catalytic site [6, 82, 289]. The sequence and structural homology of the kinase domain is high not only amongst family members (c-Src, Lck, Fyn) within the Src family but also with members of other tyrosine kinase families (like Csk and Abl kinase) [39, 185, 200, 237, 243].

C-terminal tail

The carboxy-terminal tail is relatively short, about 15-17 amino acids long and mainly functions to negatively regulate kinase activity via a weak tyrosine-based SH2-binding motif (Figure 1.8) [215, 308]. The conserved Tyr (Y527 in c-Src) is phosphorylated by negative regulatory kinases, C-terminal Src kinase (Csk) or Csk-homologous kinase (CHK) (Figure 1.8), allowing the C-terminal tail to bind intramolecularly to the SH2 domain [49, 64, 65, 234, 235]. Dephosphorylation of Tyr527 by cellular phosphatases releases the tail from the SH2 domain, leading to activation of the kinase domain [278, 279]. In the activated state, the unphosphorylated Y527 stacks onto a conserved Pro residue (P529) thereby holding the tail in a pocket within the C-lobe of the kinase domain [82].

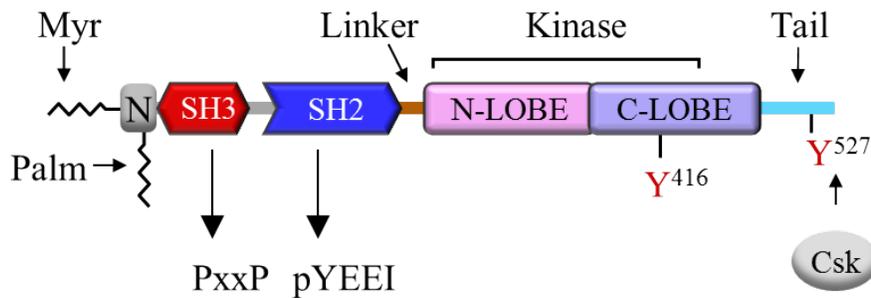


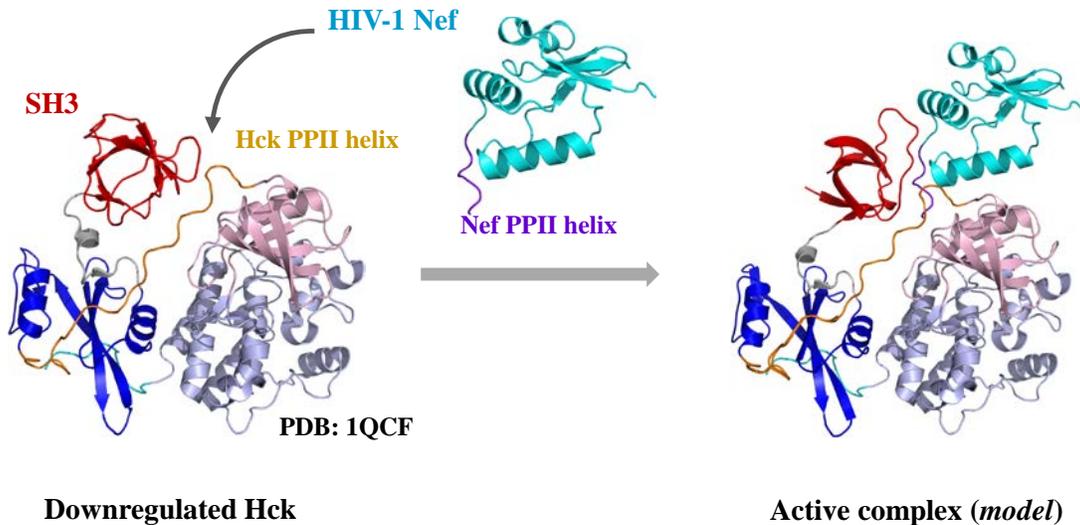
Figure 1.8. Structure of Src Family Kinases

1.3.2.2 Regulation of activity of SFKs

Intra- and inter- molecular interactions in SFKs directly or indirectly regulate their activity. As discussed above, intramolecular interaction of the SH2 domain with the C-terminal tail Tyr-based motif (SH2-tail interaction) and binding of the SH3 domain with the SH2-kinase linker (SH3-linker interaction) are critical to kinase regulation (Figure 1.9). Substitution of wild type Hck tail with the high-affinity SH2-binding sequence YEEI rendered the kinase refractory to activation by competing high affinity SH2-binding ligands [260, 289]. Release of the SH2-tail association is sufficient to activate the kinase, in spite of an intact SH3-linker contact. Previous work from our lab has shown that mutation of Y527 increases kinase activity by releasing the SH2-tail association, even in the presence of a mutationally enhanced SH3-linker contact [207].

X-ray crystal structures of c-Src and Hck provided critical insights into the role of the interaction of the SH3 domain with the linker connecting the kinase and the SH2 domains, in regulation of kinase activity [260, 301, 346, 354]. The SH3 domain engages a PPII helix formed by a weak SH3-binding motif on the linker, which is not well conserved among the SFK family and poorly resembles the established PxxP sequence typical of high affinity SH3 ligands. Mutagenesis studies from our group have shown substituting the Hck linker prolines with alanines is sufficient to induce SH3 domain release from the linker, resulting in activation of the kinase in rodent fibroblasts [46, 47]. Follow-up studies from our group reported that switching the Hck linker lysines for prolines strengthened the binding of the linker to the SH3 domain [207]. Taken together, these findings indicate that the wild-type linker regions in SFKs indeed mediate a suboptimal SH3 binding which may allow for external high-affinity SH3 ligands to compete with the linker to bind to the SH3 domain and induce kinase activation. In case of a

high-affinity SH3 ligand like Nef, SH3 engagement results in displacement of the linker thus relieving its negative regulatory influence on the kinase domain (Figure 1.9) [46, 47, 206, 231].



Regulatory intramolecular interactions in SFKs critical to kinase regulation include the interactions of SH2 domain with the C-terminal tail Tyr-based motif (SH2-tail interaction) and also binding of the SH3 domain with the SH2-kinase linker (SH3-linker interaction). A high-affinity SH3 ligand like Nef binds to and engages the SH3 domain resulting in displacement of the linker thus relieving its negative regulatory influence on the kinase domain.

Figure 1.9. Nef binding to Hck-SH3 domain causes constitutive kinase activation.

Thus, activation of SFKs can result from inter-molecular interactions of the protein with either SH2 or SH3 ligands that can release the kinase from its negative regulatory intramolecular interactions. In the presence of Nef, kinase activity reaches a higher level than that achieved with tail release alone, suggesting that maximal activation of the kinase requires release of both the intramolecular engagements or that the SH3-linker interaction is the more dominant regulator of activity of Src kinases. As described in detail above, besides these two levels of regulation, a direct regulatory mechanism lies in the catalytic cleft of the kinase domain where

phosphorylation of the activation loop tyrosine residue switches the catalytic site from a ‘closed’ to an ‘open’ state and vice versa.

1.3.2.3 Binding and Activation of SFKs by Nef

As discussed in section 1.2.2, Nef contains a conserved proline-rich SH3-binding motif P₇₂QVP₇₅LR that can bind with high affinity to SH3 domains on host proteins including SFKs [46, 270, 281, 325]. Previous in vitro and cell-based studies have shown that Nef binds to SH3 domains of Hck, Lyn, Fyn, Lck and Src via its PxxPxR motif, although high affinity binding is only observed with the SH3 domains of Hck and Lyn in vitro. In the case of Hck, Lyn and c-Src, Nef binding displaces the SH3-linker interaction, leading to constitutive kinase activation [42, 61, 281, 325].

A few studies have reported binding of Nef to the Fyn SH3 domain [18] or full-length Fyn [58], albeit in both cases the binding affinity was relatively low (two-digit micromolar range). However, switching the Fyn-SH3 Arg96 residue to Ile (as in the sequence of Hck) raised the affinity of Nef for Fyn almost 100-fold [$K_b(\text{Fyn}) > 20 \mu\text{M}$ versus $K_b(\text{FynR96I}) = 380 \text{ nM}$] [204] [205]. Yet, several other studies reportedly failed to establish evidence of Nef binding to Fyn [44, 61, 76, 281]. Thus, whether Nef actually binds to Fyn in a cellular environment remains controversial.

Interaction of Nef with Lck has also been controversial. There are reports of Nef binding to SH3 domain, SH2 domain and full length Lck, though the interaction is quite weak [42, 58, 77, 93]. HIV-1 Nef, instead of activating, reportedly inhibits Lck activity both in vitro and in cultured cells [77, 135, 136], although other studies failed to show any effect of Nef on Lck in defined expression systems (fibroblasts and yeast).

The prototype SFK, c-Src, is ubiquitously expressed in all HIV target cell types. Nef binds to the full-length as well as the SH3 domain of c-Src, albeit with a lower affinity than the Hck SH3 domain [19, 44, 61]. Contrary to Hck and Lyn, c-Src has an Arg in lieu of the critical Ile96 residue, implicated in high-affinity binding of Nef to the Hck-SH3 domain [204, 205, 346]. Nef has been reported to be able to activate c-Src in a yeast based kinase assay [325] and in a model of HIV-associated nephropathy utilizing glomerular podocytes from HIV-transgenic mice [155] but not in a fibroblast transformation assay [44]. This difference may be due to the requirement for additional cellular factors in the activating mechanism.

The Lyn tyrosine kinase is expressed in myeloid cells, as well as in brain and B cells (283). Lyn is the only other SFK that contains an Ile residue at position 96 in its SH3 domain like Hck [61]. Several studies have shown that Nef binds to the SH3 domain of Lyn and full-length Lyn as well. Nef has also shown to activate Lyn [46, 207, 239, 281, 325].

As is common to the regulation of SFK activity, Hck in its inactive conformation, involves intramolecular association of the SH3 domain with the SH2-kinase linker region [99]. The SH3 domain engages a PPII helix formed by a weak SH3-binding motif on the linker (Figure 1.8). The conserved Nef core has been found to have a particularly high affinity for the Hck SH3 domain. This polyproline motif on Nef binds to the Hck SH3 domain and displaces it from its regulatory position from the back of the kinase domain and activates Hck through an SH3-domain displacement mechanism (Figure 1.8) [46, 231]. Substitution of the Pro residues on Nef to Ala, blocks Nef:Hck interaction and kinase activation. Besides the PxxP motif, conserved residues Phe90, Trp113 and Tyr120 in the hydrophobic pocket within the Nef core domain are essential for the recruitment and activation of Hck [61, 281]. Nef-mediated Hck activation has

been implicated in many of the critical functions of Nef including downregulation of MHC-I and enhancement of viral replication as well as infectivity [24, 91, 98, 166, 327].

1.3.2.4 Hck and HIV-1 AIDS

Hck is chiefly expressed in macrophages and dendritic cells that are important HIV-1 target cells. Several studies from our group as well as other laboratories have provided a strong body of evidence supporting a role for Hck as a direct effector of HIV-1 Nef and is exploited by this virulence factor to mediate its critical functions towards the establishment of a productive infection and disease progression. Cell-based and *in vitro* studies have shown that HIV-1 Nef binds to Hck SH3 domain via its PxxPxR motif [281, 325] and a hydrophobic pocket [61] leading to constitutive kinase activation. Transgenic mice expressing Nef-2PA, a mutant in which the conserved PxxP motif is mutated to AxxA, never developed AIDS-like disease [145]. Also, Nef-induced AIDS-like disease development was delayed in Hck-null mice [145]. M-tropic HIV replication is blocked upon knockdown of Hck expression with antisense oligonucleotides in primary human macrophages, an important site of expression of this Src-family member [190]. Suppression of Nef-dependent Hck activation in brain-derived microglial cells, by expressing dominant-negative Hck or CD45 phosphatase, inhibits HIV replication [184]. As already described above, ongoing work from our group has established that selective inhibitors of Nef-mediated Hck activation also block Nef-dependent HIV-1 replication and infectivity, suggesting that the Nef-Hck interaction may be essential for efficient viral growth in

vivo [91, 97, 98, 239, 252, 327]. Thus these studies highlight the importance of the Nef-Hck signaling pathway, and more generally of Nef interactions with cellular tyrosine kinases, in HIV-1 infection, pathogenesis and disease progression.

1.3.3 Tec family of non-receptor protein tyrosine kinases (TFK)

The Tec family of non-receptor protein tyrosine kinases (TFKs) comprises the second largest family of cytoplasmic PTKs and includes five members – Bmx, Btk, Itk, Tec and Txk [306]. These proteins share amino acid sequence homology within the family and also have a similar structural domain organization as the SFKs. Information on other synonymous names for each member of this family and their chromosomal locations, molecular sizes, expression profile, and other properties are tabulated in table 1.4.

In 1990, Mano et al. in an effort to isolate novel PTKs, screened a mouse liver cDNA library with a probe encoding the v-Fps tyrosine kinase domain and identified the coding sequence of a previously unknown kinase [222]. This resulted in the identification of the first member of the family, Tec. In the following years, several other groups reported the identification of the four other related kinases – Itk, Btk, Txk and Bmx (in the chronology of their discovery) that comprised the entire Tec kinases family [139, 302, 319, 328, 332]. In mammals, these kinases are expressed predominantly in hematopoietic cells including T lymphocytes and other HIV target cell types. Btk is expressed primarily in B cells whereas T cells express Itk, Tec and Txk. Bmx is expressed both in B-cells and macrophages [359]. The members of this family share a common domain organization with Src family kinases (e.g., an SH3-SH2-kinase core) with subtle and comparable differences in relevance with their functional

regulations, although the mode of regulation is less clear in case of TFKs. The protein structure of the Tec kinases will be discussed in greater detail in section 1.3.3.1.

Table 1.4 Overview of Tec family Kinases

Family Members	Bmx	Btk	Itk	Tec	Txk
Other names	PSCTK3, Etk	PSCTK1, ATK, BPK	PSCTK2, Emt, Tsk, LYK	PSCTK4, Dsrc28C	PSCTK5, Rlk
Chromosomal positions	Xp22.2	Xq21.33-q22	5q31-32	4p12*	4p12*
Gene accession number	X83107	X58957	D13720.1	D29767	L27071
Protein Size (a.a)	675	659	620	631	502; 527
Molecular wt (kDa)	78	76.3	71.8	73.6	55; 58
Tissue distribution	Ubiquitous: epithelial cells, keratinocytes, granulocytes, monocytes	Myeloid and B cells, mast cells	Myeloid and T cells, mast cells, NK and NK-T cells	Myeloid, B and T cells, melanocytes, embryonic limb, hepatocytes	T cells, mast cells
Phosphorylation sites	Y ²¹⁵ (auto); Y ²²³ ; Y ⁵⁶⁶	Y ²²³ (auto); Y ⁵⁵¹	Y ¹⁸⁰ (auto); Y ⁵¹¹	Y ²⁰⁶ (auto); Y ⁵¹⁹	Y ⁹¹ (auto); Y ⁴²⁰
Function	Inhibits p53; activates STAT3; antiapoptotic.	BCR driven; activates PLCγ1,γ2, PKC; B-cell maturation; cell adhesion; apoptosis	TCR driven; Th2 responses; activates PLCγ1; actin reorganization; thymic selection	Activates AP1, NFAT, transcription from IL-2 promoter	TCR driven; Th1 responses; activates transcription from IFNγ promoter; activates PLCγ1

Adapted from [306, 359]

Bmx (bone marrow tyrosine kinase gene in chromosome X), also known as Etk (epithelial and endothelial tyrosine kinase) is expressed in the endothelium of large arteries, endocardium, bone marrow, myeloid cell lines, lung, and prostate cell lines. Bmx has the highest amino acid sequence homology to Btk and is localized on the same chromosome (X), suggesting that they were generated during evolution by gene duplication [319]. This ~80 kDa protein kinase has been implicated in the activation of Rho and serum response factor in response to heterotrimeric G proteins stimulated by extracellular stimuli like hormones and neurotransmitters. In addition, Bmx is phosphorylated by SFKs and is responsible for Src-induced activation of STAT3 in the context of cellular transformation.

Btk (Bruton's tyrosine kinase) is a ~77 kDa protein kinase expressed chiefly in myeloid and B-cells. Also known as **Emb** (expressed in mast cells, myeloid cells and B-lymphocytes), inactivating mutations in this kinase cause X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice. XLA is an inherited immunodeficiency characterized by a complete absence of the humoral immune response resulting from a severe deficit of B-lymphocytes, plasma cells and profound hypogammaglobulinemia. Consistent with the deficient expression of Btk mRNA, protein, and kinase activity in XLA pre-B and B cell lines, it was originally termed as **Bpk** (B-cell progenitor kinase) [167, 328].

Itk (IL-2 inducible T-cell kinase) or **Tsk** (T-cell-specific kinase) or **Emt** (expressed in mast cells, myeloid cells and T lymphocytes), as the names suggest is the highest expressed family member in naïve T cells and thymocytes [159, 302, 357]. This 72 kDa kinase is expressed in spleen, lymph nodes, and natural killer T cells. The localization of the *itk* gene to chromosome 5q31-32 makes it unique since the other members of the family are believed to have been generated in evolution by gene duplication. Itk plays a major role in regulating downstream

signaling from the T-cell receptor, in T-cell development and function, all of which shall be discussed in greater detail in subsequent sections.

As described above, this family of kinases was named after **Tec** (Tyrosine kinase expressed in hepatocellular carcinoma), the first member discovered. Alternative splice sites on mRNA and alternate start sites yield multiple isoforms (ranging from 58 to 73.6 kDa) in humans. Tec is expressed in bone marrow, spleen, thymus, T-cells, B-cells, myeloid cells and even in hepatocarcinoma. Chromosomal mapping and detailed analysis reveals that the *tec* and *txk* genes are both localized on the same locus 4p12 of chromosome 4 in humans and are just 1.5 kbp apart from each other. Both of these kinase genes likely arose from a common ancestor through gene duplication.

Txk/Rlk (resting lymphocyte kinase) is found in thymocytes (mast and T-cells) at several-fold lower levels than Itk, and is an atypical member of this family. Txk lacks the Pleckstrin homology (PH) domain and the Btk motif in its amino-terminal region, the structural hallmarks of the other TFKs. Instead, it contains a cysteine-rich motif in its unique region that upon being palmitoylated can subsequently target the kinase to the cell membrane. Smallest in size (~61 kDa) of all the family members, Txk is commonly expressed as two isoforms that arise by alternative initiation of translation from the same cDNA. The shorter isoform lacks the cysteine string motif and in the absence of any fatty acid modification localizes to the nucleus while the larger isoform is cytosolic. However, Txk is similar to the other members of the family, in being phosphorylated and activated by SFKs, supporting a role for this kinase in signaling downstream of Src kinases in T cell activation [90, 221].

1.3.3.1 Tec Family Kinase structure

The Tec kinases share a common domain organization like Src family kinases that includes SH3 and SH2 domains, followed by the kinase domain. However, Tec kinases lack N-terminal myristoylation sequences and C-terminal regulatory tyrosines as are found in SFKs. Instead the Tec kinases possess an amino-terminal pleckstrin homology (PH) domain that binds inositol phosphates and acts as a membrane anchor [359]. Also, Tec kinases possess a Tec homology (TH) region, just upstream of the SH3 domain, consisting of a proline-rich motif engaged in intramolecular interaction with the SH3 domain and thus kinase regulation [13].

Pleckstrin Homology (PH) domain

The Pleckstrin Homology (PH) domain, about 100 amino acids long, forms a β -barrel from two β -sheets and a C-terminal α -helix and is a characteristic feature present only in the Tec family of kinases, amidst all non-receptor protein tyrosine kinases known. It serves to tether the Tec kinases to the cell membrane, as does the myristoylation signal in SFKs. The positive charge on one end of its structure renders the domain with a strong affinity for phospholipids and phosphatidylinositols (PIs) [147, 267]. The PH domains of Btk, Tec and Itk have all been reported to have higher binding affinities for phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P₃] than phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂], phosphatidylinositol-4-phosphate [PI(4)P] or inositol-1,3,4,5-tetrakisphosphate (IP₄) [296]. Thus, the PH domain acts as a conditional switch that recruits the Tec kinases to the cell membrane in a PI3-K activity-dependent manner. This is supported by the finding that addition of PI3-K inhibitors blocked membrane targeting and subsequent activation of Itk [25, 60]. Substitution of the entire Itk PH domain with a myristoylation sequence targeted the kinase constitutively to the membrane but

failed to activate it, indicating that mere localization of these kinases to the membrane is not sufficient for their activation. The PH domain upon binding the PI3-K product, PIP₃ localizes the kinases to the cell membrane in close proximity of their cognate activating kinases (typically SFKs). Also, the PH domains serve as binding modules for other membrane-associated proteins that possibly regulate Tec kinase activities. For instance, the Btk PH domain has been shown to bind to G_α subunits of heterotrimeric G- proteins and protein kinase C β1 as well [173, 360].

Tec Homology (TH) domain

The 80 residue long TH domain contains a conserved Btk motif (a globular core consisting of 27 amino acids) at the amino-terminus of the domain followed by a proline-rich (PxxP) region in the carboxy-terminal half of the domain. The Btk motif contains characteristic conserved Cys and His residues forming a zinc binding fold. Along with the C-terminal end of the PH domain, the Btk motif constitutes a binding site for active G_α subunits of heterotrimeric G proteins. The PxxP motif assumes a PPII helix constituting a binding site for SH3 domains on other proteins, typically SFKs. Btk and Tec contains two such PxxP motifs while Bmx, Itk, and Txk have only one. Except for having the PxxP motif, Bmx has a ‘TH-like’ domain instead of a classical one. The TH domain is integral to the activity of these kinases since its intramolecular association with the adjacent SH3 domain via its PxxP motif locks the kinase in an autoinhibited state [221, 306, 333].

Src Homology 3 (SH3) domain

The SH3 domain in TFKs (except for Bmx, which lacks a classical SH3 domain) is related to those of SFKs and shows a high degree of conservation of sequence. Bmx carries a truncated

SH3-like domain. As in SFKs, the SH3 domain binds to polyproline motifs and is involved in inter- and intra-molecular associations, thereby holding a key to the regulation of the kinase' activity. Besides binding to the PxxP motif, the Itk SH3 domain was also shown to bind specifically to the Itk SH2 domain, which does not contain a canonical PxxP consensus sequence [38]. A handful of proteins that reportedly bind to the SH3 domain of TFKs include Wiskott-Aldrich syndrome protein (WASP), Sam68, SLP-76, Vav1, c-Cbl and hnRNP-K [221]. Studies that report constitutive activation of Tec following truncation of its SH3 domain and development of XLA following a deletion in the Btk SH3 domain support a critical role for this domain in regulation of kinase' activity [358, 365].

Src Homology 2 (SH2) domain

Like the SH3 domain, TFK SH2 domains also bear high sequence homology to SFK SH2 domains. As is reminiscent of SFKs, the SH2 domain in TFKs binds to phosphotyrosine motifs on other proteins. Some of the host cell proteins identified to bind to SH2 domain of Btk include phosphorylated adaptor protein BLNK/SLP-65 in B cells; while Itk has been found to bind to SLP-76, Vav1, LAT and PLC γ 1 via its SH2 domain. These interactions are presumably needed for downstream PLC γ activation. The Tec SH2 domain essentially binds to and phosphorylates the docking protein Dok-1 and BRDG1 as well as PI-3K subunits p85b and p55PIK [59, 131, 318]. Intermolecular association of TFKs with their cognate binding partners via their SH2 domains may result in activation or inactivation of the kinase. For instance, interaction of Itk SH2 domain with the prolyl-isomerase cyclophilin A (CypA) inactivates the kinase, while interaction with Cyclosporin A leads to Itk activation [132].

Kinase (SH1) domain

The catalytic domains of Tec kinases are highly conserved and include a conserved activation loop tyrosine residue that is transphosphorylated by an SFK for the initial activation of the kinase. X-ray crystal structures of Itk kinase domain in both active and inactive states reveal that phosphorylation of the activation loop tyrosine residue does not induce any conformational change in the kinase domain, indicating that the activation of the kinase may not involve a phosphorylation-dependent conformational switch [48]. In contrast to many other tyrosine kinase domains, the isolated kinase domains of TFKs are kinetically inactive, indicating the possible role of other domains in regulating kinase activity [153]. Besides their enzymatic activity, the kinase domains of TFKs contain a highly conserved caveolin-binding motif. Both Bmx and Btk have been reported to associate with caveolin (a principal component of caveolae membranes that play critical roles in several cellular signaling cascades) *in vivo* thereby inhibiting tyrosine phosphorylation on Btk [330]. The interaction of Tec SH2 domain with p85b and p55PIK subunits of PI3K is also dependent on the Tec kinase domain [318].

1.3.3.2 TFK: Function and Regulation

Functionally Tec kinases play a role in modulating antigen receptor signaling by participating in the phosphorylation of phospholipase C- γ (PLC γ), regulate the actin cytoskeleton and serve as critical mediators of immune responses in both B and T cells. In T cells, Tec kinases contribute to T helper cell differentiation and lineage commitment [306]. Tec kinases bind to SFKs via their TH domain Pro-rich motif and act downstream of SFK-induced intracellular signaling pathways [223].

In B- and T-cells, BCR and TCR stimulation and activation unfolds a complex orchestration of events downstream which involves activation of TFKs. Downstream consequences include mobilization of intracellular Ca^{2+} , Erk activation, gene transcription, cytokine release and actin reorganization. The common upstream regulators of Itk include the TCR/CD3 signaling complex, chemokine receptors, and heterotrimeric GPCRs. Antigen receptor stimulation leads to phosphorylation of the CD3 immunoreceptor tyrosine-based activation motifs (ITAMs) by activated Lck which can then bind the ZAP-70 kinase (zeta-associated protein of 70 kilodaltons). ZAP-70 activation by Lck in turn activates the linker for activated T cells (LAT) and the SH2 domain-containing leukocyte phosphoprotein of 76 kilodaltons (SLP-76). At this point a signalosome complex consisting of LAT-SLP-76-PLC γ -Grb2-GADS-SOS is formed in T cells while parallel activation of PI3K and accumulation of its product, PIP₃ helps recruit Itk to the cell membrane. Here Itk gets phosphorylated by Lck and autophosphorylates itself to interact with the signalosome. Itk then binds to and directly phosphorylates PLC γ and activates it, which in turn catalyzes the hydrolysis of PIP₂ to IP₃ and DAG. IP₃ functions as a major mediator of Ca^{2+} mobilization from cytoplasmic storage vesicles while DAG leads to activation of MAPK pathways and protein kinase C isozymes. Similarly in B-cells, the signalosome complex consists of BLNK/SLP-65, PLC γ , Vav, Grb2 and SOS and leads to the ultimate downstream effects of activation of transcription factors [14, 132, 265].

As discussed in detail earlier (section 1.3.2.1), SFKs are regulated via intramolecular and/or intermolecular interactions. In the downregulated state, the SFK SH2 domain binds to the regulatory phosphotyrosine on the C-term tail, the SH3 domain binds to the SH2:kinase linker and the unphosphorylated activation loop tyrosine covers the catalytic site between the two lobes of the kinase domain. However, a mechanistic understanding of the regulation of activity in

TFKs remains somewhat obscure, since Itk and other Tec family members have remained refractory to crystallization in their full-length forms.

Unlike SFKs, TFKs lack a conserved C-terminal inhibitory tyrosine residue. Instead, TFKs possess two principal tyrosine residues, one in the activation loop of the kinase domain and one in the SH3 domain that play regulatory roles. Like other non-receptor PTKs, Tec kinases also are involved in intramolecular and intermolecular interactions. Requirements for the activation of Tec kinases typically involve four events: (1) interaction of the PH domain with phosphorylated inositol lipids (that are products of PI3-K activation) required for membrane association; (2) phosphorylation of the regulatory activation loop tyrosine residue by SFKs; (3) disruption of intramolecular engagements and (4) intermolecular interactions with other proteins via the SH2 and kinase domains [14, 265]. Results from several studies show that in TFKs, the activation loop tyrosine residue in the kinase domain (Y511 in Itk – corresponding Tyr residues for each of the TFK members are tabulated in table 1.4) is phosphorylated by SFKs, leading to partial activation of the kinase. This is followed by subsequent autophosphorylation of the SH3 domain tyrosine residue (Y180 in Itk). Phosphorylation of both regulatory tyrosine residues is required for the full activation of the protein [160, 344]. Recruitment of the kinases to the plasma membrane via their PH domain brings the kinase to close proximity to its cognate upstream kinase (e.g. a Src-family member) that can phosphorylate the kinase domain tyrosine residue - this allows for the disruption of inhibitory intramolecular associations, allowing the regulatory domains to bind their substrates.

Andreotti et al. showed by multidimensional NMR that the Itk TH and SH3 domains engage in an intramolecular regulatory association that prevents the Itk SH3 domain and the TH proline-rich region from interacting with their ligands. This intramolecular complex formation

may hold the kinase in a closed, autoinhibited conformation [13]. In a follow up study, they found that Txk-TH domain does not interact with its own SH3 domain. This opposing mode of self-association within the otherwise closely related Txk and Itk proteins may be attributed to the difference in the lengths of their TH-SH3 linker regions. Itk has five more residues in its linker region than Txk, which likely imparts the flexibility to the kinase to fold and self-associate intramolecularly [196]. In a further study, the Andreotti group found that dual SH3-SH2 domain-containing fragments of Itk could self-associate intermolecularly in solution indicating the presence of a possible intramolecular association between the SH3 and SH2 domains of Itk. This may provide a plausible mechanism for the displacement of the intramolecular regulatory sequence, a step that might be required for full activation of the kinase [38]. Unlike Itk, the Btk and Tec-TH domains possess two proline-rich sequences which can undergo asymmetric homodimerization with their SH3 domains. With accumulating concentrations of the kinase at the cell membrane, these kinases tend to form dimers [195, 261].

Bmx and Txk are the atypical members in this family. Bmx lacks a classical SH3 domain, and has a PxxP motif surrounded by non-conserved residues while Txk lacks a TH domain. Moreover, the SH3-like domain in Bmx has two potential sites of tyrosine autophosphorylation. Yet, Bmx activation requires the two major activation steps of membrane association and phosphorylation of the regulatory tyrosine residue in the kinase domain [95, 262]

Termination of Tec family kinase signaling can occur in two different ways – either by the activity of a phosphatase or by interaction with other proteins. Expression of activated phosphatases like PTEN in T cells or SHIP and PTEN in B cells reduces PIP₃ levels and thus prevents further recruitment and activation of Tec kinases [294]. Several proteins have also been implicated in the inactivation of Tec kinases. Btk activity is regulated by IBtk, PKC β , Pin1

(protein interacting with NIMA1) [214] while Itk is negatively regulated by a peptidyl prolyl isomerase, cyclophilin A (CypA) [175].

1.3.3.3 Itk and HIV-1 AIDS

Given its critical role in CXCR4- and TCR-mediated signaling and cytoskeletal regulation in T cells, Itk has very recently emerged as a potential target for anti-HIV therapeutic intervention. In resting human CD4+ T cells the endogenous level of Itk expression is extremely low [176]. Stimulation of TCR leading to subsequent activation of CD4+ cells enhances transcription of the Itk gene – which is key to a productive infection of HIV in these cells. Readinger et al. showed that use of either a selective Itk chemical inhibitor or Itk SH2 mutants to block Itk function, leads to inhibition of actin-dependent HIV-1 entry, viral replication and transcription. Use of an siRNA directed against Itk to block kinase expression, also reduced F-actin polarization in response to HIV gp120 and HIV replication in primary human CD4+ T lymphocytes and Jurkat T cells [266]. In a follow up study, this group went on to show that Itk impacts HIV-1 replication and viral egress in T cells by co-localizing with the HIV-1 Gag protein at sites of F-actin accumulation and membrane lipid rafts at the plasma membrane [290].

Other work has shown that proteasome inhibitors reduced the steady state levels of TFKs in hematopoietic cells [363]. Based on the finding that reduction of Itk activity can affect HIV replication, Yu et al. evaluated the effects of proteasome inhibitors on HIV infection and/or replication. HIV replication was significantly blocked in activated PBMCs treated with the clinically approved proteasome inhibitor Bortezomib (Velcade) [364].

Mice that are homozygous null for Itk (Itk^{-/-}) have delayed responses to viral infection yet can clear all viral infections tested to date [23]. However in these mice the major *in-vivo* defects include impaired responses to pathogens and Th2-stimulants, indicating that compensatory pathways are less efficient in the absence of Itk [26]. However, these results suggest that it would be fair to believe that antagonists targeting Itk would not impair all immune functions in T cells, and may represent a reasonable approach to HIV therapy. All these studies taken together present a justifiable body of evidence for the implication of Itk and other Tec family kinases in HIV/AIDS, and therefore represent an attractive drug target for the treatment of AIDS.

2.0 STATEMENT OF HYPOTHESES AND SPECIFIC AIMS

The HIV-1 accessory protein Nef supports high-titer viral replication in vivo and is essential for HIV pathogenesis and AIDS progression [101, 126, 143, 182]. Nef functions by interacting with multiple host cell effectors involved in cellular activation, immune recognition and survival [158]. Nef binds to the SH3 domains of host cell proteins, most notably members of Src family of non-receptor protein-tyrosine kinases (SFKs) like Hck, Lyn and Src [46, 281, 325] via its conserved proline-rich motif (PxxPxR) and hydrophobic pocket [61] causing constitutive kinase activation. Selective inhibitors of Nef-induced SFK activation block Nef-dependent HIV replication [97] suggesting that the Nef-SFK pathway may be essential for efficient viral growth in cell culture. These studies strongly suggest that Nef-SH3 interaction is a common early event in HIV-infected cells that generates important downstream signals essential for viral pathogenesis. Thus, Nef-SH3 interactions represent a rational target for anti-HIV drug development and it is important to elucidate all these Nef-directed signaling pathways for rational drug design. The importance of Nef:SH3 interaction demands a systematic cell-based analysis of the Nef-SH3 interactions for a better understanding of Nef-directed signaling in the HIV-1 infected cell.

2.1 HYPOTHESIS I

Previous work has established that Nef binds the SH3 domains and activates SFKs in vitro, in defined cellular systems (fibroblasts, yeast) and in HIV target cells. Besides SFKs, Tec kinases (Bmx, Btk, Itk, Tec, Txk) are also expressed in HIV target cells and possess SH3 domains important to their regulation. Tec kinases modulate antigen receptor signaling in lymphocytes and contribute to T_H cell differentiation, lineage commitment and their activation downstream of antigen receptors involves SFKs. Recent studies have implicated the Tec family member Itk in HIV infectivity and replication [266], although the mechanism by which HIV infection is linked to Itk is unknown. The possibility of Nef interaction with Tec-family kinases through their SH3 domains has not been investigated. However the predominant expression of this family of kinases in HIV target cells supports an investigation of this possibility. In light of the importance of SH3 interaction in Nef function, I tested the hypothesis that ***HIV-1 Nef preferentially binds to a specific subset of SH3 domain-containing proteins (kinases) and that these interactions are indispensable for Nef-mediated downstream signaling in support of viral replication.***

To address this hypothesis, I developed a novel, cell-based approach and explored the scope of interaction of HIV-1 Nef with Tec family kinases and the relevance of this interaction to HIV biology with four specific aims as outlined in the following section.

2.1.1 SPECIFIC AIMS

Aim 1: Test the ability of SH3-domain containing Tec family kinases to bind to HIV-1 Nef in cells and characterize the interaction mechanism.

In this Aim, I probed the interaction of HIV-1 Nef with Tec-family kinases using a cell-based bimolecular fluorescence complementation (BiFC) assay. First I developed and validated this assay using the Src-family kinase Hck, which is a well characterized binding partner for Nef. Using this approach, I found that full-length Bmx, Btk, and Itk interacted strongly with HIV-1 Nef at the plasma membrane, while Tec and Txk did not. Parallel BiFC experiments with shorter constructs consisting of only SH3 domains (along with the N-terminal regions required for membrane localization) were carried out, which supported interaction through the SH3 domain.

To characterize the interaction further, I carried out mutagenesis studies to evaluate the importance of residues that contribute to interaction of Nef with the Tec family kinases through their SH3 domains. Replacement of the SH3 domain RT-loop Ile critical for interaction with the Nef hydrophobic pocket with Arg compromised interaction of Hck, Btk and Itk with Nef, thereby supporting a role for the SH3 RT-loop in Nef recognition. Replacement of the Ala residue in the RT loop of the Tec SH3 domain with Ile restored interaction with Nef by BiFC, supporting the idea that Tec and Src family kinases interact with Nef through a similar structural mechanism.

Aim 2: Test the hypothesis that interaction with the Tec kinases is a conserved property of Nef allelic variants.

In this Aim, I used primary *nef* alleles representative of the major HIV-1 subgroups A1, A2, B, C, F1, F2, G, H, J, K and tested for their interaction with full length Itk, using the cell-based

BiFC assay. Allelic variants of Nef representative of all M-group HIV-1 clades interacted strongly with Itk in the BiFC assay, suggesting Nef-Itk interaction is highly conserved across all major HIV-1 subtypes. These findings are important because they address for the first time whether or not HIV Nef variants retain a conserved pattern of SH3 recognition and also address the generality Nef-Itk interaction.

Aim 3: Investigate the functional relevance of Nef interaction with Tec family kinases in the context of HIV infection.

Selective inhibitors of Nef-induced SFK activation block Nef-dependent HIV replication [97]. Previous studies have shown that a small molecule inhibitor of Itk, BMS-509744, blocks HIV-1 infectivity and replication in vitro [266]. To investigate the importance of Nef:Itk interaction in HIV-infected cells, I tested the effect of BMS-509744 on wild-type vs. Nef-defective HIV infectivity in TZM-bl cells as well as replication in CEM-T4 and Jurkat cells. I found that this Itk inhibitor blocks Nef-dependent wild-type HIV infectivity and replication, but not that of a Nef-defective mutant. These results suggest that Nef provides a mechanistic link between HIV and Itk signaling in the viral life cycle.

Aim 4: Evaluate the effect of Nef:SH3-kinase interactions on the activity of Itk.

To address this aim, I turned to immunoprecipitation assays. Itk expressed alone or in combination with Nef in mammalian cells was immunoprecipitated and subsequently analyzed for levels of tyrosine phosphorylation by immunoblotting. Results from this aim indicated that Nef induces autophosphorylation and thus activation of Itk which was found to be sensitive to the Itk inhibitor, BMS-509744.

2.2 HYPOTHESIS II

Elucidation of high-resolution structures of Nef:SFK complexes is critical to our understanding of Nef:SFK signaling and rational drug design. Our lab recently determined the crystal structure of a recombinant HIV-1 Nef core protein in complex with the tandem SH3-SH2 regulatory region of human Src-family kinase Hck (Hck32). Novel Nef:SFK interactions were observed in the crystal structure. The complex crystallized as a dimer of Nef:Hck32 complexes, with the expected interaction between the conserved Nef PxxPxR motif and the Hck SH3 domain observed in earlier structures of Nef with SH3 only. However, the Nef dimer interface was found to be completely reoriented and much more compact relative to these previous structures, with an intercomplex contact observed between Nef R105 and SH3 E93. Based on these findings, I hypothesized that *interaction of Nef R105 with Hck SH3 E93 is required for complex formation with full-length Hck and kinase activation by Nef.*

In an effort to validate the biological significance of the new X-ray crystal structure of the HIV-1 Nef:Hck32 complex, I completed two specific aims as follows below.

2.2.1 SPECIFIC AIMS

Aim 1: Investigate the role of the Hck-SH3 RT loop E93 in stabilizing Hck binding to the Nef dimer.

In order to evaluate the functional importance of this interaction to complex formation, I addressed this aim by mutagenesis studies and compared the mutant versus wild type proteins in a cell-based BiFC assay. The Hck-SH3 RT loop E93 was mutated to Ala. Mutant and wild type full-length Hck and corresponding shorter constructs (consisting of SH3 and SH3-SH2 domains

only, including the N-terminus in each case) were tested for their ability to bind to Nef in the BiFC assay. Interaction of mutant full-length Hck with Nef was significantly weaker than wild type Hck. This finding indicated that this additional SH3 domain contact is important for stable interaction of full-length Hck with Nef in cells, providing important validation for the new crystal structure of Nef in complex with the Hck SH3-SH2 unit.

Aim 2: Investigate whether Hck-SH3 RT loop residue E93 is important to the activation of Hck by Nef.

To evaluate the effect of the Hck SH3 domain E93 mutation on Nef-mediated kinase activation, I took advantage of a yeast-based kinase assay previously developed in our laboratory to study Nef-mediated activation of Src-family kinases without interference from mammalian host cell kinases [239, 325]. Wild type full length Hck was found to be constitutively activated by Nef in yeast, consistent with our previous studies. However, Nef failed to activate mutant full length Hck E93A, in agreement with its inability to interact with Nef by BiFC. In parallel experiments, I evaluated by immunoblot analyses the extent of tyrosine phosphorylation on yeast cell proteins as a result Nef-mediated Hck activation. Levels of overall tyrosine phosphorylation were significantly reduced in cells co-expressing Nef and mutant Hck E93A, compared to cells expressing wild type Hck. These results support an essential role for Hck SH3 E93 in the formation of the active Nef-Hck complex.

3.0 THE INTERACTION OF HIV-1 NEF WITH TEC FAMILY KINASES AND ITS RELEVANCE TO HIV BIOLOGY[§]

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With kind permission from JBC:

Tarafdar, S.^{1,2}, J. A. Poe², and T. E. Smithgall². The Accessory Factor Nef Links HIV-1 to Tec/Btk Kinases in an SH3 Domain-dependent Manner. *J Biol Chem.* 2014 May 30; 289(22):15718-15728. Epub 2014 Apr 10.

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3.1 SUMMARY

The non-enzymatic HIV-1 Nef virulence factor functions by interacting with diverse host cell signaling proteins. Nef particularly binds to the SH3 domains of Src-family kinases via its conserved polyproline motif, resulting in kinase activation important to viral infectivity, replication, and MHC-I downregulation. In addition to SFKs, Itk and other Tec-family kinases are also present in HIV target cells and possess SH3 domains important to their regulation. Recently, Itk has been implicated in HIV-1 infectivity and replication. However, the molecular mechanism linking Itk to HIV-1 is not clearly known. In this study, we explored the interaction of Nef with Tec-family kinases using a cell-based bimolecular fluorescence complementation (BiFC) assay. In this approach, interaction of Nef with a partner kinase juxtaposes non-fluorescent YFP fragments fused to the C-terminus of each protein, resulting in YFP complementation and a bright fluorescent signal. Using BiFC, we observed that Nef interacts with the Tec family members Bmx, Btk, and Itk but not Tec or Txk. Interaction with Nef occurs through the kinase SH3 domains, and localizes to the plasma membrane. Allelic variants of Nef from all major HIV-1 subtypes interacted strongly with Itk in this assay, demonstrating the highly conserved nature of this interaction. A selective small molecule inhibitor of Itk kinase activity (BMS-509744) potently blocked wild-type HIV-1 infectivity and replication in a Nef-dependent manner. We also showed that Nef induced Itk activation in transfected cells that was sensitive to inhibitor treatment. Taken together, these results provide the first evidence that Nef interacts with cytoplasmic tyrosine kinases of the Tec family, and suggest that Nef provides a mechanistic link between HIV-1 and Itk signaling in the viral life cycle.

3.2 INTRODUCTION

The HIV-1 accessory factor Nef is a 27 kDa protein unique to the primate lentiviruses HIV and SIV. Nef is expressed early in the viral life cycle [20, 126] and is required for high-titer viral replication and disease progression in vivo [101, 143, 182, 311]. Previous studies have shown that non-human primates infected with Nef-deleted SIV failed to develop AIDS-like disease [182]. Defective *nef* alleles have also been detected in HIV sequences recovered from long-term non-progressors [89, 186, 225, 367], individuals infected with HIV that do not or only very slowly develop AIDS despite many years without antiretroviral therapy. Moreover, targeted expression of Nef in CD4⁺ T-cells and macrophages induces an AIDS-like syndrome in transgenic mice even in the absence of other HIV-1 gene expression [143]. More recent studies with HIV-1-infected humanized mice show that viral load and CD4⁺ T-cell loss are also dependent on Nef [367]. Taken together, these studies support an essential role for Nef in HIV pathogenesis and AIDS progression.

Non-catalytic in nature, Nef acts by interacting with a multitude of host cell proteins involved in cellular activation, protein trafficking, immune recognition and survival [158]. Nef selectively binds to the SH3 domains of several classes of host cell proteins [280], including members of Src family of non-receptor protein-tyrosine kinases. Of the Src-related kinases in the human kinome, Nef has been shown to preferentially interact with Hck, Lyn and c-Src via their SH3 domains. Structural studies have shown that Nef interacts with Src-family kinase SH3 domains through a highly conserved polyproline (PxxPxR) motif as well as a hydrophobic pocket formed by the three-dimensional fold of the Nef core [61]. The Nef PxxPxR motif adopts a polyproline type II helix, which engages the hydrophobic grooves on the SH3 domain surface, while the hydrophobic pocket engages an isoleucine residue on the RT-loop of the SH3 domain.

Nef activates Src-family kinases constitutively by disrupting and displacing the inhibitory engagement of the SH3 domain with the SH2-kinase linker region and releasing it from its regulatory position on the back of the kinase domain [46, 231].

Several lines of evidence support a critical role for the Nef PxxPxR motif in the various functions of this multifaceted virulence factor, Nef. Development of AIDS-like disease in Nef transgenic mice requires the PxxPxR motif and is delayed in mice that are homozygous-null for Hck [145]. The PxxPxR motif is also required for Nef-mediated MHC-I [91] and CCR5/CXCR4 downregulation [305], functions critical to escape of HIV-infected cells from host immune surveillance. M-tropic HIV replication is blocked upon knockdown of Hck expression in primary human macrophages, an important site of expression of this Src-family member [190]. Our laboratory has recently established that selective inhibitors of Nef-induced SFK activation block Nef-dependent HIV infectivity and replication in cell culture [97, 98, 239]. These studies support the idea that interaction of Nef with SH3-containing kinases and other proteins is an early event in HIV-infected cells that generates important downstream signals essential for a productive infection and viral pathogenesis.

In addition to Src-family kinases, members of the Tec kinase family are also expressed in HIV target cells. Like Src-family members, Tec kinases also have SH2 and SH3 domains that control kinase activity and function [14, 265]. These kinases are expressed predominantly in hematopoietic cells in mammals. The Tec family consists of five members: Btk, which is expressed primarily in B cells; Itk, Tec and Txk, which are expressed in T cells; and Bmx, which is expressed both in B-cells and macrophages. Tec kinases modulate antigen receptor signaling and serve as critical mediators of immune responses in both B and T cells. In T cells, Tec kinases contribute to T helper cell differentiation and lineage commitment [265]. Interestingly,

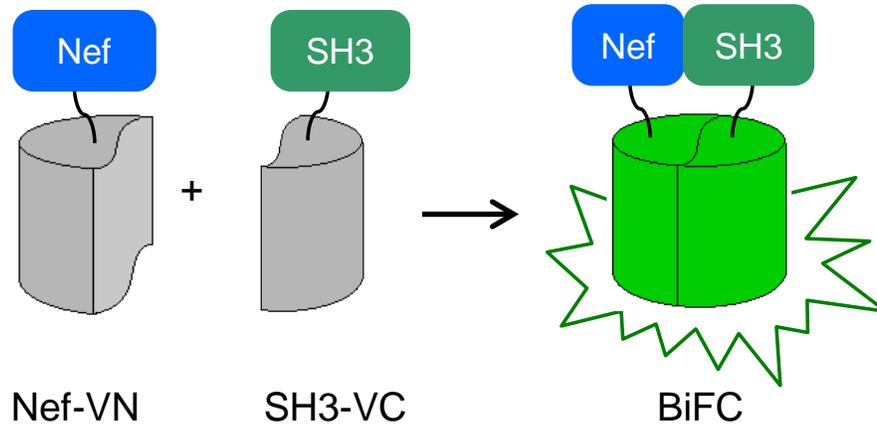
Tec kinases are activated by direct phosphorylation by Src-family kinases downstream of the T-cell receptor, leading to intracellular calcium mobilization as well as phospholipase C γ and MAPK activation [14].

Recent studies have implicated the Tec family kinase Itk in the HIV-1 life cycle. Using selective pharmacological inhibitors and siRNA targeting Itk, Readinger et al. showed that loss of Itk activity compromised viral transcription, particle assembly, and viral spread [266]. However, the molecular mechanism linking HIV-1 to this T cell kinase was not reported. The well-known connection of HIV-1 Nef to Src-family kinase activation, the close relationship of Src and Tec family kinases in T cells, and the requirement for Itk activity in HIV replication suggested a possible link between Nef and Tec family kinases in HIV target cells. In this study, we developed a novel, cell-based approach to investigate the direct interactions of HIV-1 Nef with Tec-family kinases. Using a bimolecular fluorescence complementation (BiFC) assay, we demonstrate that three members of the Tec kinase family interact directly with Nef through their SH3 domains. We also found that Nef induces activation of Itk which is sensitive to the selective Itk inhibitor, BMS-509744. Our experiments also confirmed that the interaction of Nef with Itk kinase is a conserved property of Nef allelic variants representative of ten distinct M-group HIV-1 subtypes. Using BMS-509744, we show that Itk kinase activity is required for wild-type HIV infectivity and replication, but not that of a Nef-defective mutant. Taken together, these results show that Nef provides a mechanistic link between HIV-1 and Itk signaling in the viral life cycle, and support further exploration of this signaling pathway as a potential target for anti-retroviral drug development.

3.2.1 Bimolecular Fluorescence Complementation (BiFC) Assay

The bimolecular fluorescence complementation (BiFC) assay has emerged as a relatively straightforward and sensitive assay for direct visualization of protein:protein interaction in living cells. BiFC also enables visualization of the subcellular localization of the interacting protein complexes. Application of this assay to mammalian cells was first described by Hu et al., in their 2002 study of interactions between dimeric transcription factors of the Myc-Mad-Max family [163]. The technique involves fusing two interacting proteins to non-fluorescent fragments of the yellow fluorescent protein (YFP; yellow shifted variant of GFP) such that when the two proteins interact, the YFP fragments are brought into close proximity. As a result, the YFP structure reassembles, leading to a fluorescent signal (Figure 3.1) [164, 180].

One practical limitation of using YFP for BiFC assays is the requirement for incubation at room temperature to allow maturation of the chromophore. This potentially limits its application in studies involving living cells that do not tolerate non-physiological conditions. To circumvent this problem, fluorescent protein fragments derived from Venus, a variant of YFP, are often used [299]. The Venus protein, first developed by Miyawaki and co-workers in 2002, encodes a novel mutation (F46L) which under physiological conditions accelerates chromophore oxidation thereby allowing fast and efficient maturation at 37 °C [236, 268].



Non-fluorescent N- and C-terminal fragments of the YFP variant Venus are fused to Nef (Nef-VN) and the SH3 domain of Hck (SH3-VC). Co-expression and interaction of the two proteins inside cells juxtaposes the Venus fragments resulting in structural complementation and green fluorescence.

Figure 3.1. Bimolecular Fluorescence Complementation (BiFC) assay principle

3.3 MATERIALS AND METHODS

3.3.1 Cell culture, Reagents and Antibodies

Human 293T cells were obtained from the American Type Culture Collection (ATCC). TZM-bl reporter cells and the T lymphoblast cell lines CEM-T4, MT2 and Jurkat (Clone E6-1) were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bl and 293T cells were cultured in Dulbecco's Modified Eagle's Medium/high glucose (DMEM) (Gibco Life Technologies) supplemented with 10% fetal bovine serum (heat inactivated, 3x 0.1 µm sterile filtered) (Gemini Bio-Products). CEM-T4 and MT2 cells were passaged in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum and 2mM L-glutamine (200 mM; 100x; Gibco Life Technologies). All cells were maintained at 37⁰C in a 5% CO₂ water-jacketed incubator (Forma Scientific).

Primary antibodies used in this study were obtained from Santa Cruz Biotechnology (Hck rabbit polyclonal N-30, sc-72; Itk mouse monoclonal clone 2F12, sc-23902; Itk rabbit polyclonal M-109, sc-1697), Abcam (V5 tag mouse monoclonal, ab27671), Millipore (V5 tag rabbit polyclonal, ab3792; Phosphotyrosine mouse monoclonal, clone 4G10, 05-321), the National Institutes of Health AIDS Research and Reference Reagent Program (HIV-1 JR-CSF Nef monoclonal, 1539; HIV-1 Nef monoclonal EH1, 3689; Nef Antiserum polyclonal, 2949) and the National Cancer Institute-Frederick AIDS and Cancer Virus program (HIV-1 p24). Secondary antibodies used in this study were obtained from Southern Biotech [Goat Anti-Rabbit IgG (H+L) Mouse/Human ads-TXRD, 4050-07; Goat Anti-Mouse IgG (H+L), Human ads-TXRD, 1031-07; Goat Anti-Mouse IgG (H+L), Human ads-AP, 1031-04], LiCor [Donkey Anti-Mouse IgG (H+L) IRDye 800CW, 926-32212], Invitrogen Molecular Probes [Pacific Blue Goat Anti-Mouse IgG

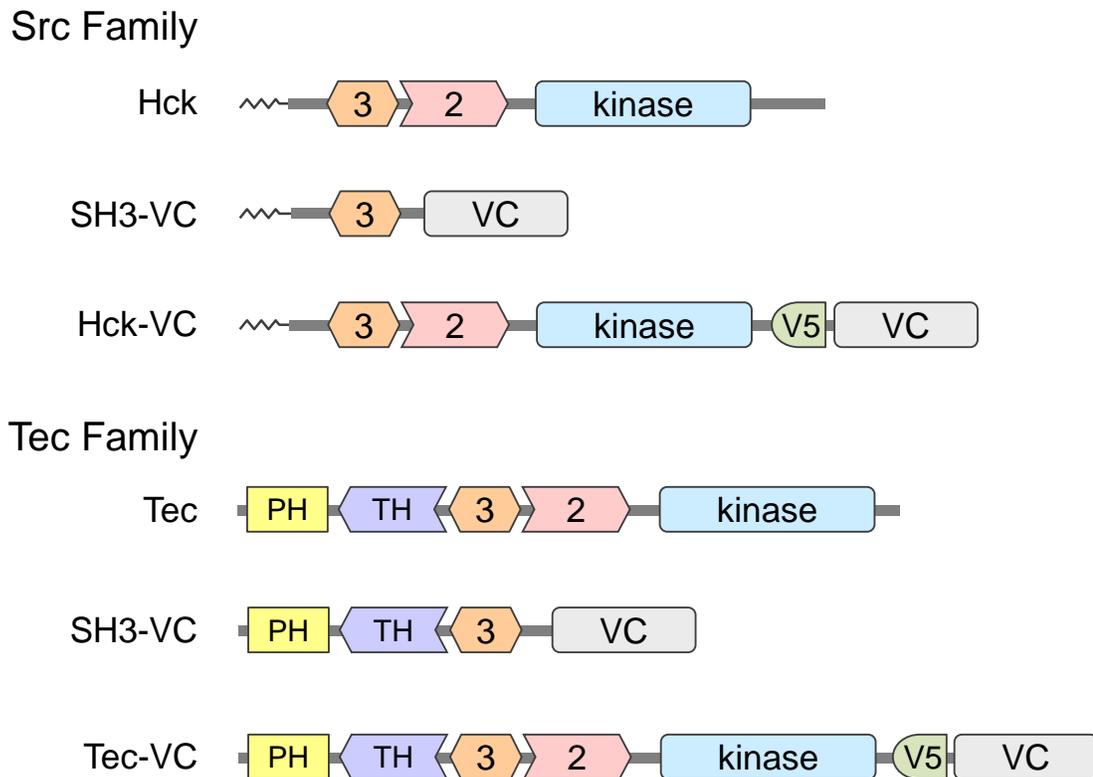
(H+L), P31582; Pacific Blue Goat Anti-Rabbit IgG (H+L), P10994] and the NCI-Frederick AIDS and Cancer Virus program [Goat anti-Rabbit IgG (H+L)-HRP].

3.3.2 BiFC assay expression vectors

Tec-family kinase cDNA clones were obtained from the Dana-Farber/Harvard Cancer Center PlasmID DNA Resource Core (Bmx, HsCD00327726; Btk, HsCD00346954; Itk, HsCD00021352; Tec, HsCD00341367; and Txk, HsCD00294897). The coding regions for wild-type, full length Hck and Tec family kinases were amplified by PCR and fused in-frame with a V5 epitope tag at their C-termini (Figure 3.2). The reverse primers for each individual kinase was designed to include the V5 tag sequence and as well introduce the unique KpnI or AccIII restriction sites at the 3'-end. The C-terminal coding sequence of the Venus protein (residues Ala154-Lys238) was amplified by PCR with an introduction of a unique KpnI or AccIII restriction site at its 5'-end. The PCR amplified products were then subcloned into the mammalian expression vector pcDNA3.1(-) via the unique sites to create the BiFC fusion full length wild type (WT) constructs. Truncated shorter BiFC constructs were created with the Venus C- fragment fused to the SH3 domains of each kinase including the N-terminal myristoylation sequence and unique domain for Hck and N-terminal Pleckstrin and Tec Homology domains for the Tec kinases to maintain the natural lipid modification and spacing from the membrane (Figure 3.2).

For the BiFC expression construct, Nef-VN (SF2 allele; B clade), was created as described elsewhere [258]. A similar BiFC construct with a PxxPxR mutant of Nef-SF2 was prepared for use as a negative control. The N-terminal sequence of the Venus protein (encoding residues Val2-Asp173) was PCR-amplified and subcloned via a unique BamHI restriction site to

the C-terminal end of the coding sequences of representative Nef proteins from the HIV-1 M-group subtypes A1, A2, B, C, F1, F2, G, H, J and K in the mammalian expression vector pcDNA3.1(-). The Nef-2PA, Hck-I96R, Btk-M96R, Itk-N96R and Tec-A96I mutants were created via site-directed mutagenesis (QuikChange[®] II XL Site-Directed Mutagenesis Kit, Stratagene). The Venus template was a generous gift from Dr. Atsushi Miyawaki, RIKEN Brain Science Institute, Saitama, Japan.



The Src and Tec family kinases share a core of SH3 and SH2 domains followed by a kinase domain. Truncated expression constructs were created for BiFC experiments (SH3-VC) in which sequences N-terminal to the SH2 domain from Hck and all five Tec family members were fused to the C-terminal fragment of the Venus form of YFP (VC). Full-length BiFC expression constructs were created in a similar manner, with a V5 epitope tag inserted between the C-terminal end of each kinase and the VC fragment to enable immunostaining for kinase expression.

Figure 3.2. Src and Tec family kinase domain organization and BiFC expression constructs

3.3.3 BiFC assay and Immunofluorescence

Human 293T cells were plated on glass bottom microwell dishes (MatTek, P35G-1.5-14-C) and allowed to attach overnight. The cells were transfected with complementary pairs of BiFC expression vectors using standard calcium phosphate techniques as described elsewhere [45]. Thirty hours post transfection, the cells were fixed with freshly prepared 4% paraformaldehyde (stock 16% solution; Electron Microscopy Sciences), permeabilized with 0.2% Triton X-100 and blocked with 2% BSA (Sigma) in PBS overnight. Cells were stained with primary antibodies against Hck (1:1000), the V5 tag (1:750) or Nef (1:1000) for 1 hour at room temperature at their respective dilutions (in parentheses). Immunostained cells were visualized with secondary antibodies conjugated to Texas Red or Pacific Blue at a dilution of 1:1000 and 1:500 respectively. Two-color imaging was done at constant exposure times for each channel using a Nikon Eclipse TE300 inverted microscope and a SPOT RT slider CCD high resolution digital camera and software (Diagnostic Instruments). Three-color BiFC and IF images were recorded using confocal microscopy (Fluoview FV-1000, Olympus). Image analysis was performed with the Java-based image processing program, ImageJ (version 1.48s), to determine the BiFC and immunofluorescence (IF) intensities of individual cells. Mean pixel densities were calculated for the BiFC and IF channels for a minimum of 100 cells and data are expressed as BiFC to IF signal ratios.

3.3.4 Cytotoxicity Assay

The Itk inhibitor BMS-509744 was the generous gift of Dr. Jian-Kang (Jack) Jiang of the National Center for Advancing Translational Sciences, NIH. CEM-T4, Jurkat and TZM-bl cells

were incubated at 37°C in absence or presence of the Itk inhibitor BMS-509744 over a concentration range of 10nM - 10µM in DMSO as a carrier solvent at a final concentration of 0.5%. Cytotoxicity was assessed for TZM-bl cells after 48 hours and CEM-T4 and Jurkat cells after 10 days using the Cell Titer Blue reagent (Promega) and the manufacturer's protocol.

3.3.5 HIV assays

Wild-type (WT) and Nef-defective (Δ Nef) HIV-1 proviral genomes (NL4-3 strain) in the plasmid vector pUC18 were transfected into 293T cells with XtremeGENE 9 transfection Reagent (Roche) according to the manufacturer's protocol. The viral supernatant was collected 48 h post-transfection and amplified by infecting MT2 cells with 500pg/ml of virus for 4 days in a 6-well plate. Titers of the amplified stocks were determined by HIV-1 p24 Antigen Capture ELISA kit (NCI-Frederick) as per the manufacturer's protocol.

For HIV replication assays, CEM-T4 and Jurkat cells were incubated overnight in the absence or presence of the Itk inhibitor BMS-509744 in a final concentration of 0.5% DMSO as carrier solvent. Cells were then infected with the WT and Δ Nef viruses, and replication was assessed 10 days later by p24 Gag protein levels in the culture supernatant by standard HIV-1 p24 Antigen Capture ELISA kit (NCI-Frederick) using manufacturer's protocol. ELISA plates were read on Biotek.

For infectivity assays, the reporter cell line TZM-bl was pre-incubated with BMS-509744 overnight in a final concentration of 0.5% DMSO as carrier solvent. The WT and Δ Nef viruses were also pre-incubated with the inhibitor for 4 hours prior to infecting the cells. Viral infectivity was determined using the Luciferase Assay system (Promega) as described elsewhere [98].

3.3.6 Immunoprecipitation and Blotting

For inhibitor experiments, 293T cells were pre-incubated with the Itk inhibitor BMS-509744 or the DMSO carrier solvent (at a final concentration of 0.5%) overnight prior to transfection. Human 293T cells were transfected with wild-type full-length Itk and HIV-1 Nef (SF2 allele) either alone or in combination as described above. Thirty hours post-transfection, the cells were lysed by sonication in lysis buffer (50 mM Tris-hydrochloride, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, and 1 mM EDTA) supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 U/ml Benzonase (Novagen), and Protease Inhibitor Cocktail Set III (Calbiochem). Lysate protein concentrations were determined using Pierce Coomassie Plus assay reagent (Thermo Fisher). Itk was immunoprecipitated from 1 mg of each cell lysate with 1 μ g anti-Itk rabbit polyclonal antibody and protein G-Sepharose (Invitrogen) for 2 hours at 4 °C, followed by washing with lysis buffer. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF or nitrocellulose membranes, and probed with mouse monoclonal antibodies to phosphotyrosine (4G10), Itk and Nef.

3.3.7 Statistical Analyses

Quantitative analyses of all BiFC data are reported as the mean value \pm standard error of mean (SEM) from three independent experiments (n=3). Data from the cytotoxicity assays and the viral infectivity and replication assays are presented as the mean value \pm SEM from three independent experiments (n=3), each performed in triplicate wells.

3.4 RESULTS AND DISCUSSION

3.4.1 Src and Tec kinases share a common structural domain organization

Previous work has established that Nef binds to the SH3 domains of the Src-family members Hck, Lyn and c-Src, leading to kinase activation in vitro [231, 325], in defined cellular systems such as fibroblasts and yeast [46, 206, 325] and in HIV-1 target cells [98, 239]. Members of the Tec family of non-receptor protein tyrosine kinases also possess SH3 domains important to their regulation [37]. Germane to our investigation, we compared the domain organization of the Src and the Tec kinase families. Figure 3.2 illustrates the overall domain organization of these two kinase families. A closer look at their general structure revealed that both the families share a core of SH3 and SH2 domains, followed by the kinase domain. In addition, Src-family kinases have an N-terminal myristoylation signal sequence for membrane localization [271, 275], followed by an N-terminal sequence unique to each family member. SFKs also have a C-terminal tail with a phosphotyrosine residue critical to negative regulation of kinase activity [65]. Structural studies have shown that the SH3 domain engages a PPII helix formed by the SH2-kinase linker to hold the kinase in the downregulated state [289, 300, 301, 353, 354]. Binding of Nef to the SH3 domain displaces the linker, relieving its negative regulatory influence on the kinase domain [46, 206, 231]. In contrast to Src-family kinases, each Tec family kinase possesses an N-terminal pleckstrin homology (PH) domain that binds to lipid phosphoinositides and acts as a membrane anchor [37] (Figure 3.2). Tec kinases also possess a Tec homology (TH) domain N-terminal to the SH3 domain, consisting of a proline-rich motif engaged in intramolecular interaction with the SH3 domain that may also contribute to regulation of kinase

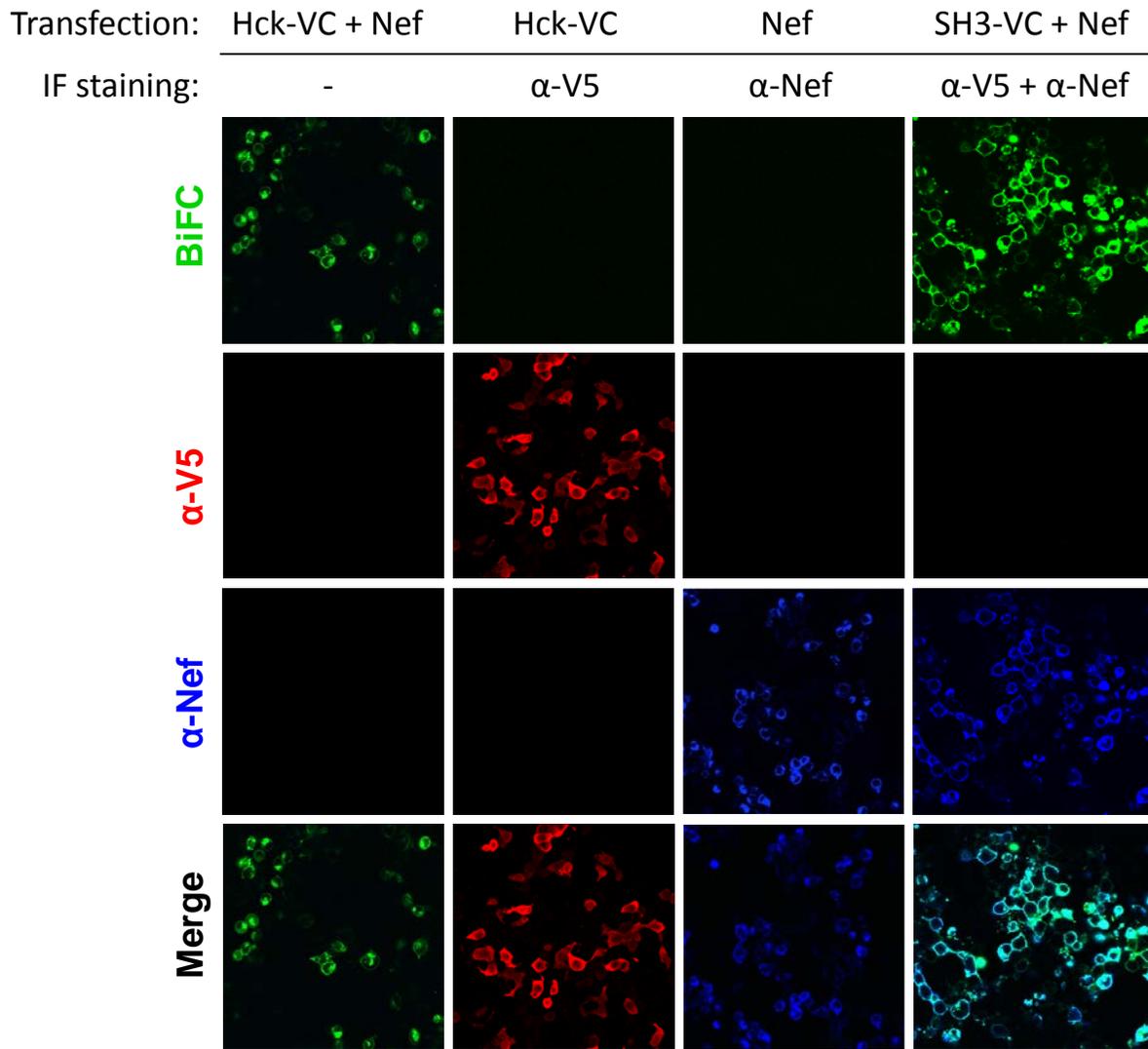
activity [13]. The similarity in the domain architecture of these two kinases families led us to explore the possibility that Nef may interact with Tec kinases through their SH3 domains.

3.4.2 Development of a cell-based BiFC assay for Nef:SH3 interaction

Previous studies of Nef interactions with the SH3 domains of Src-family kinases have relied on solution-based approaches with recombinant purified proteins that do not account for the influence of membrane localization on the interacting partners. In addition, sequences N-terminal to the SH3 domain (Figure 3.2) impact the spatial relationship between the SH3 domain and Nef at the membrane surface. To address these issues, we were interested in developing a cell-based BiFC assay to investigate the interaction of Nef with SH3 domain containing Tec and Src-family kinases. In this assay, the two interacting proteins (Nef and the SH3-kinase, in our case) are fused to non-fluorescent fragments of the yellow fluorescent protein (YFP; yellow-shifted variant of GFP). If and when the two proteins of interest interact, juxtaposition of the YFP fragments would result in structural complementation of the fluorophore, leading to a bright fluorescent signal (Figure 3.1) [180].

To develop a working BiFC assay for Nef interactions with SH3 domains, we first chose to examine the interaction of Nef with the Src-family kinase, Hck, which has been studied in detail in many other systems (see Introduction). The readout of the BiFC assay involves multicolor confocal microscopy and relies on the simultaneous detection of green YFP fluorescence (indicative of interaction) and immunofluorescent staining of the partner proteins as normalizing controls for protein expression. Therefore assay development first necessitated control experiments to ensure the specificity of the antibodies and to rule out the possibility of bleed-through fluorescence between the different channels.

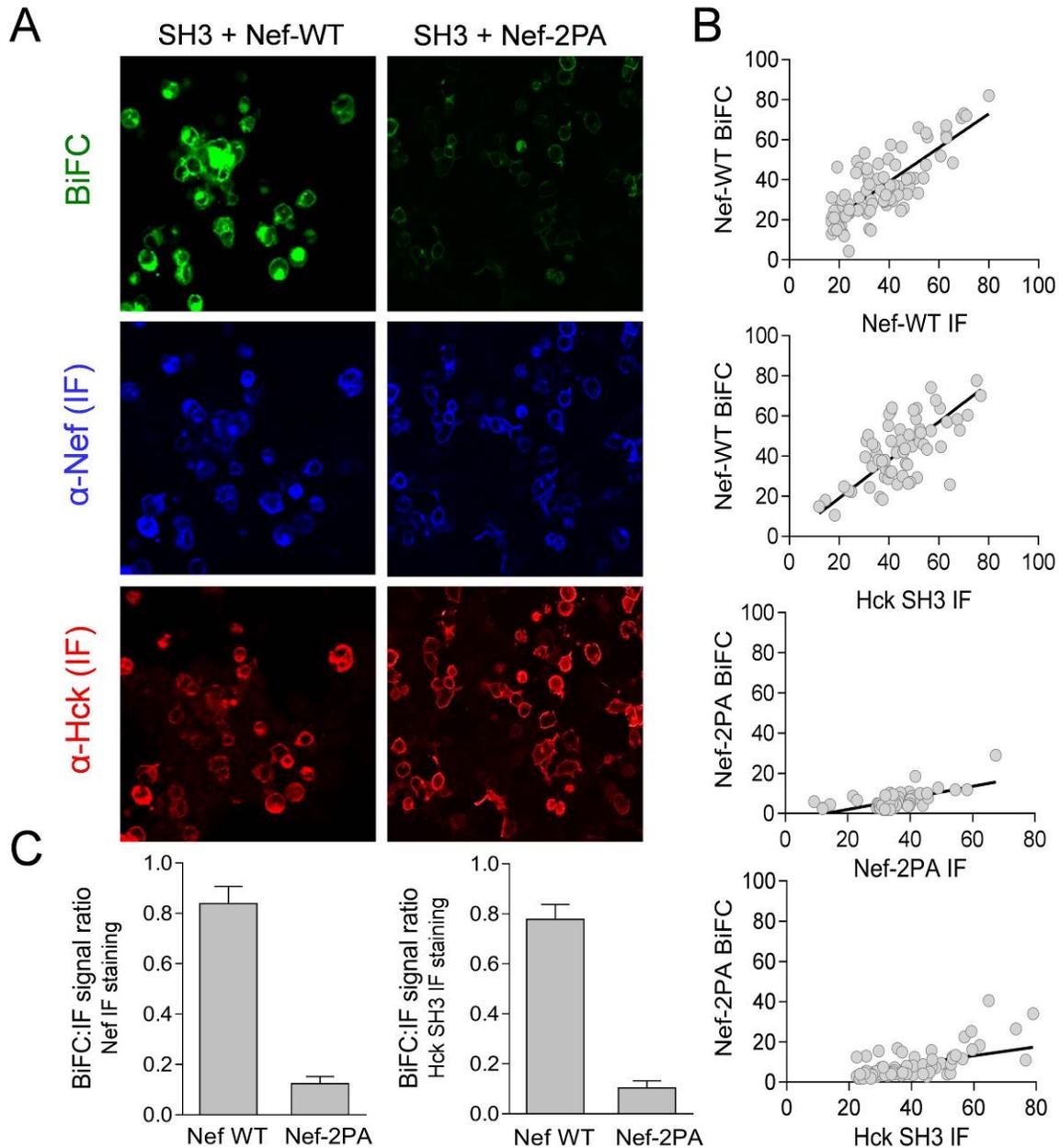
We first expressed the full-length or SH3 domain of Hck as a fusion protein with the C-terminal fragment of Venus [268]. The Hck BiFC fusion proteins (Hck-VC and SH3-VC, see Figure 3.2) also retained the natural N-terminal myristoylation signal and unique domain to enable membrane targeting and proper spacing from the membrane. Similarly, to preserve N-terminal myristoylation and membrane localization of Nef, the complementary N-terminal fragment of Venus (VN) was fused to the C-terminal end of HIV-1 Nef (SF2 allele) to make the Nef BiFC fusion protein (Nef-VN). To test for interaction of Nef with the Hck by BiFC, the Hck-VC and SH3-VC proteins were expressed either alone or in combination with wild type Nef-VN in 293T cells. Transfected cells were fixed, immunostained with antibodies against Nef or the kinase epitope tag, V5, and imaged by confocal microscopy using the ‘sequential mode’ with every image resulting from an average of three Kalman frames. The ‘sequential’ mode of imaging allows light of each wavelength to pass through its corresponding color filter sequentially, thereby eliminating the possibility of the colors bleeding through parallel open channels. As shown in Figure 3.3, expression of full-length Hck or Nef alone, showed up either in the red (Texas Red) or blue (Pacific blue) channels, respectively (Figure 3.3; column 2 and 3). Co-expression of Nef with Hck (full-length and truncated after the SH3 domain) resulted in strong fluorescence in the green (GFP) channel specifically (Figure 3.3; columns 1 and 4), indicative of interaction. Staining of the SH3-VC construct with anti-V5 antibody failed to produce red fluorescence, thereby confirming the specificity of the V5 antibody (Figure 3.3; column 4). Results from this experiment serve as an important control for our subsequent imaging analyses and confirmed the specificity of the individual antibodies, and the selectivity of the color filters on the microscope thereby excluding the possibility of false positive signals.



Confocal microscopy IF staining controls validate the three color channels and antibody specificity. N-terminal and full-length Hck BiFC expression constructs (SH3-VC & Hck-VC; see Figure 3.2) and Nef-VN were expressed alone or in combination in 293T cells. Cells were fixed, immunostained with antibodies against the V5 epitope and/or Nef as indicated above each column panel, and imaged by confocal microscopy for interaction (BiFC) and protein expression by immunofluorescence 30 h later.

Figure 3.3. Confocal microscopy IF staining controls

Next, we wanted to validate that the emergence of green fluorescence signal was dependent on Nef:SH3 interaction. As shown in Figure 3.4A, co-expression of Hck-SH3-VC with wild type Nef-VN resulted in a strong BiFC signal, consistent with interaction between Nef and the SH3 domain of Hck. In contrast, co-expression of the Hck-SH3-VC with a Nef mutant in which the PxxPxR motif essential for SH3 binding was replaced with AxxAxR (Nef-2PA-VN) dramatically reduced the BiFC signal (Figure 3.4A). Immunofluorescence (IF) staining with anti-Nef and anti-Hck antibodies showed a comparable level of protein expression in each transfected cell population. Image analysis was performed to determine the relative fluorescence intensities for BiFC and IF from each of the transfected cell populations. Mean pixel intensities of the BiFC and IF signals were determined for approximately 100 individual cells, and are plotted in Figure 3.4B. This approach showed a strong linear correlation of Nef/SH3 interaction (BiFC signal) as a function of protein expression level relative to both Nef and the Hck SH3 domain. BiFC to IF ratios of approximately 1.0 were obtained for cells transfected with wild-type Nef, regardless of whether the Nef or SH3 IF signal was used as the normalizer. This ratio decreased by nearly 10-fold for cells expressing the Nef-2PA mutant. We also calculated BiFC to IF ratios from the mean pixel intensities of the entire image fields obtained from three independent experiments (Figure 3.4C). This approach produced BiFC to IF ratios ~ 0.8 for wild-type Nef that was reduced to nearly 0.1 with the Nef-2PA mutant. This finding strongly argues for the importance of the conserved PxxPxR motif on Nef for a strong interaction with the SH3 domains of other proteins, and provides an important control for the BiFC assay in that it demonstrates that fluorescence complementation is dependent upon Nef-SH3 domain interaction. These results validated the use of the BiFC approach to explore the interactions of Nef with Tec family kinases in a cell-based assay.

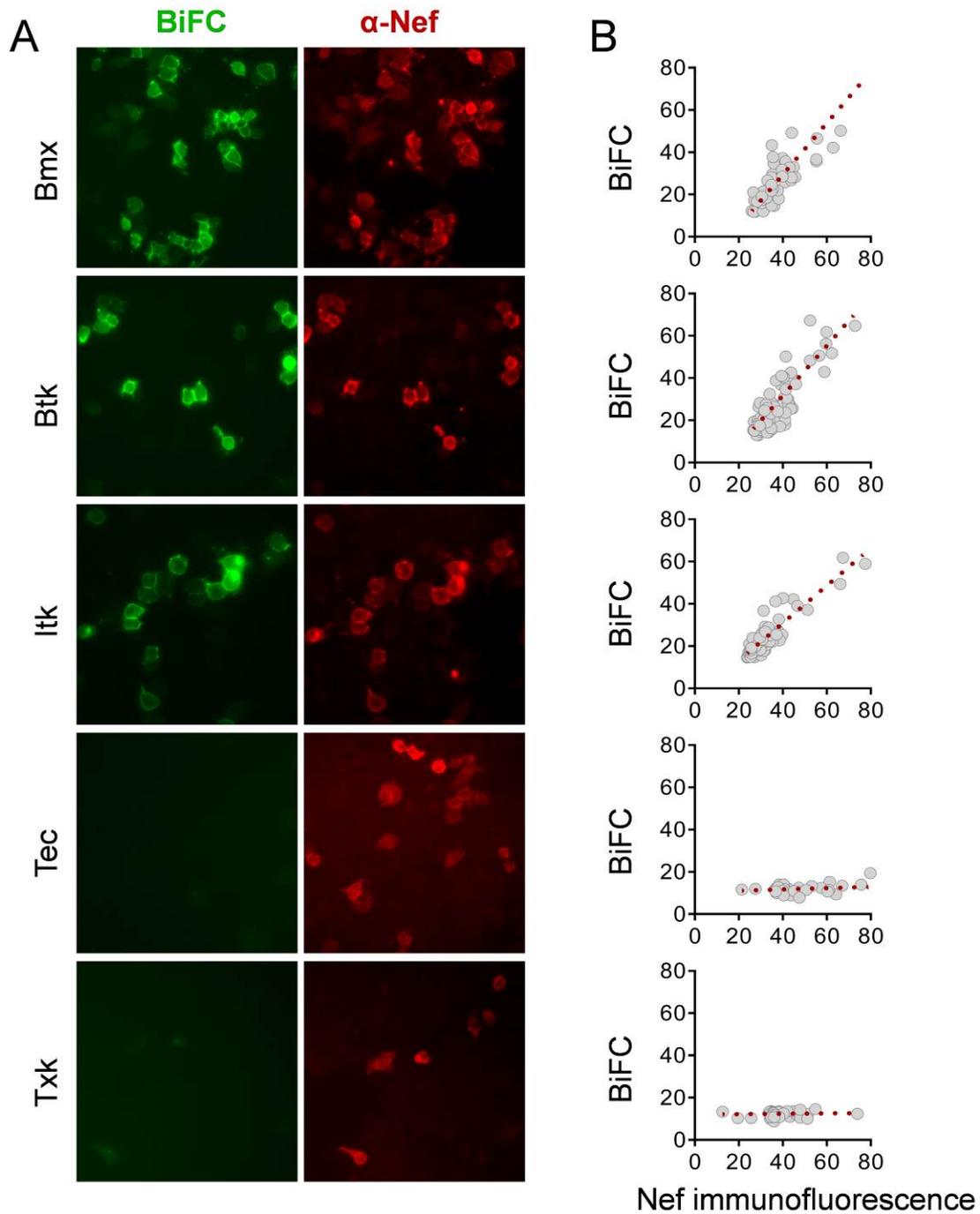


(A) Nef interaction with the Hck SH3 domain drives BiFC in transfected 293T cells. The Hck-SH3-VC fusion protein was co-expressed with the wild-type Nef (Nef-VN) or an interaction-defective mutant (Nef-2PA-VN). Transfected cells were fixed and immunostained with anti-Nef and anti-Hck antibodies, followed by confocal microscopy. Representative BiFC and Nef immunofluorescence (IF) images are shown. (B) Single-cell image analysis. Mean pixel intensities for the Nef/Hck-SH3 BiFC signal are plotted against the Nef and Hck IF signal intensities for ~100 cells. Results with wild-type (WT) and mutant (2PA) Nef are shown. The linear regression lines are also included on the plots. (C) BiFC to Nef-IF and Hck SH3-IF signal ratios were determined from the average pixel intensities of whole image fields from three independent experiments and are presented as the mean ratio value \pm SEM. Results with wild-type (WT) and mutant (2PA) Nef are shown. The mean BiFC/IF ratios obtained for the Nef-2PA mutant are significantly lower compared to wild-type Nef when normalized to either Nef or Hck SH3 immunofluorescence ($p < 0.0001$ in a two-tailed unpaired t-test).

Figure 3.4. Cell-based BiFC assay for Nef/SH3 domain complex formation

3.4.3 Nef interacts with a subset of Tec family kinases

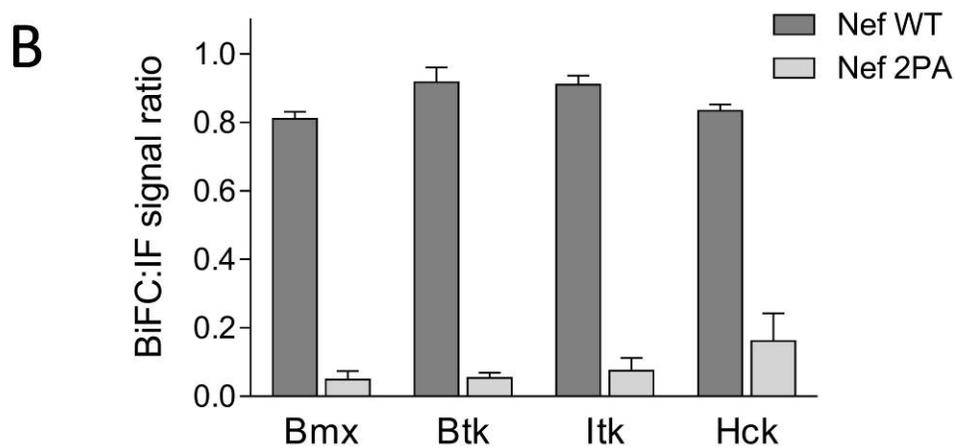
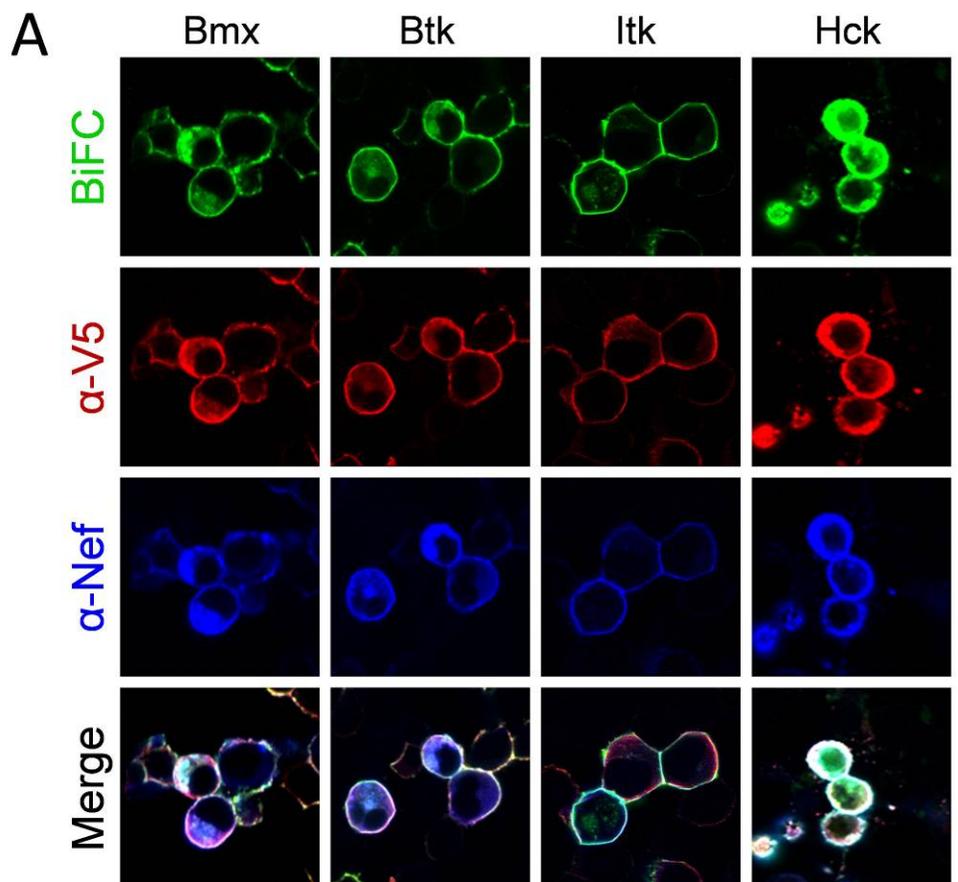
Using the BiFC assay, we next investigated the interaction of HIV-1 Nef with Tec family kinases through their SH3 domains. First, we fused the SH3 domains of Btk, Itk, Tec and Txk to the C-terminal fragment of Venus. These SH3-VC constructs also included the N-terminal PH and TH domains to maintain proper membrane targeting and spacing from the membrane (Figure 3.2). Because Bmx lacks clear SH3 domain sequence homology (see Figure 3.7), we created an analogous VC construct using the Bmx sequence encoding the region starting from the N-terminal end until a couple of residues upstream of the SH2 domain start site. Each of the Tec kinase SH3-VC constructs was then co-expressed with Nef-VN in 293T cells (0.5 μ g of each complementary construct), followed by immunofluorescent staining for Nef as before. As shown in Figure 3.5A, co-expression with Nef-VN yielded a strong, membrane-localized BiFC signal with the SH3-VC proteins from Bmx, Btk and Itk, but not for Tec or Txk. Nef expression was assessed by immunostaining with an anti-Nef-antibody. Single-cell image analysis yielded linear correlations for BiFC vs. IF with the Bmx, Btk and Itk SH3 domains, with BiFC to IF ratios of approximately 1.0. For the Tec and Txk SH3 domains, this ratio was less than 0.05, consistent with a lack of interaction (Figure 3.5B). Comparing the mean pixel densities of BiFC and IF signals of the whole image fields yielded a BiFC to IF ratio of approximately 1.0 for cells transfected with Bmx, Btk or Itk and \sim 0.2 for Tec and Txk. These results support the idea that HIV-1 Nef selectively interacts with Bmx, Btk, and Itk through their SH3 domains.



(A) Tec family kinase N-terminal BiFC expression constructs (SH3-VC; see Figure 3.2) were co-expressed with Nef-VN in 293T cells. Cells were fixed, immunostained with Nef antibodies, and imaged by epifluorescence microscopy for interaction (BiFC) and protein expression by IF 30 hours later. (B) Mean pixel densities of the BiFC and Nef IF signal intensities were measured for ~100 cells and plotted as shown. The linear regression lines are also included on the plots.

Figure 3.5. BiFC reveals novel interactions between Nef and a subset of Tec-family kinases

BiFC results presented in Figure 3.5 show that Nef interacts with the N-terminal regions of Bmx, Btk and Itk, most likely through their SH3 domains. However, the absence of the kinase domain in these constructs may expose an SH3 surface not normally available for interaction with Nef. To address this issue, we next performed BiFC experiments with full-length Bmx, Btk and Itk as well as full-length Hck as a positive control. In each case, the C-terminal fragment of Venus (VC) was fused to the C-terminal end of the kinase (Figure 3.2). A V5 epitope tag was inserted between the C-terminus of the kinase and the VC fragment to enable immunofluorescent staining for kinase expression. Each of these kinase-VC proteins was co-expressed in 293T cells with Nef-VN as before, and the cells were fixed and immunostained for Nef as well as kinase expression. The cells were then imaged by 3-color confocal microscopy for BiFC as well as Nef and kinase expression by immunofluorescence. As shown in Figure 3.6A, a strong BiFC signal was observed with all four kinases, providing direct evidence that Nef interacts with full-length Bmx, Btk, and Itk as well as Hck in cells. The BiFC signal observed with each of the Tec family kinases showed striking localization to the cell periphery, consistent with the plasma membrane localization of both interacting partners. To verify that the BiFC signals observed with the full-length Tec kinases were dependent on the interaction of Nef with their SH3 domains, parallel experiments were performed with the Nef-2PA-VN construct which is defective for SH3 binding as described above. Co-expression of Nef-2PA with Bmx, Btk and Itk significantly reduced the BiFC signals thereby lowering the BiFC to IF ratio (Figure 3.6B), supporting a requirement for the Nef PxxPxR motif for interaction with Tec family kinases through their SH3 domains.



BiFC constructs for the full-length Tec-family members Bmx, Btk and Itk as well as the Src-family kinase Hck (see Figure 3.2) were co-expressed with wild-type Nef -VN or a Nef mutant defective for SH3 binding (Nef-2PA-VN) in 293T cells. Cells were fixed, immunostained for Nef and kinase expression (V5 epitope) 30 hours later, and analyzed by confocal microscopy. (A) Representative images of wild-type Nef interaction with each kinase by BiFC (green), Nef expression (blue), partner kinase expression (red), along with a merged image. (B) Quantitative comparison of the BiFC to IF ratios for interactions of the full length kinases with wild-type versus mutant forms of Nef. The BiFC to Nef IF signal ratios were determined from the average pixel intensities of the whole image fields from three independent experiments using ImageJ. Data are plotted as the mean ratio \pm SEM.

Figure 3.6. Nef interacts with full-length Tec-family kinases

3.4.4 Tec and Src family kinase SH3 domains interact with Nef in a similar manner

BiFC results presented above provide strong evidence for the interaction of Nef with a subset of Tec family kinases, and point to the SH3 domain as the site of Nef binding. Structural studies have shown that the Nef PxxPxR motif forms a polyproline type II helix that contacts the hydrophobic surface of Src-family kinase SH3 domains [137, 205]. This interaction is stabilized by a salt bridge between SH3 Asp100 and the conserved arginine in the Nef PxxPxR sequence. Conserved residues in the Nef hydrophobic pocket (Tyr120, Phe90, and Trp113) contact Ile96 in the RT loop of the SH3 domain, and the importance of this interaction has been verified in mutagenesis studies [61, 205]. A sequence alignment of the Tec family kinase SH3 domains with that of the Src-family kinase Hck is presented in Figure 3.7A, and shows the clear amino acid sequence homology of the Btk, Itk, Tec, and Txk SH3 domains with that of Hck. Interestingly, the corresponding Bmx region is quite divergent in terms of sequence conservation (Figure 3.7B). Nevertheless, this Bmx region interacts strongly with Nef in a PxxP-dependent manner (Figure 3.6), suggesting that it adopts an SH3-like fold that is competent for Nef binding.



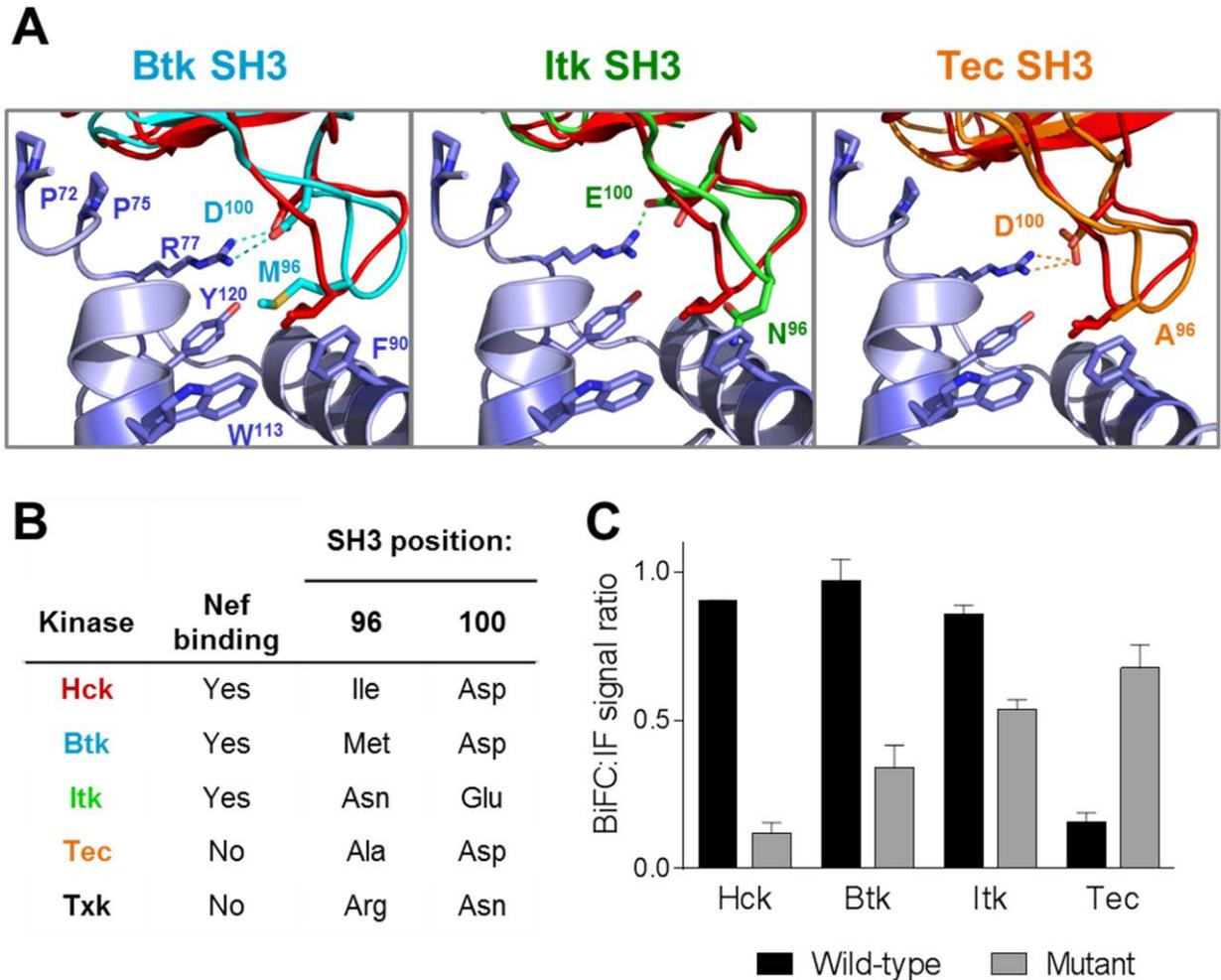
(A) The SH3 domain amino acid sequence of the Src-family kinase Hck is aligned with SH3 sequences of the Tec family members Btk, Itk, Tec, and Txk. The positions of Hck residues essential for Nef interaction are highlighted in red (Ile96 and Asp100). (B) Alignment of the Btk SH3 domain amino acid sequence with the corresponding region of Bmx. Despite low sequence homology, this region of Bmx is sufficient for Nef binding in a PxxP-dependent manner (see text)

Figure 3.7. SFK and TFK SH3 domain sequence alignment

To explore the structural basis of Tec-family kinase SH3 domain interactions with Nef, we aligned the NMR structures of the Btk, Itk, and Tec SH3 domains with the X-ray crystal structure of Nef in complex with a Src-family kinase SH3 domain (PDB: 1EFN). As shown in Figure 3.8A, an acidic residue is positioned in the Btk, Itk and Tec SH3 domains for a possible ionic contact with Nef Arg77 in a manner analogous to Asp100 in the Src-family kinase SH3 domain. The Txk SH3 domain, however, has an unfavorable asparagine (Asn) in this position. Amino acid position 96 in the RT-loops of the Btk and Itk SH3 domains is occupied by methionine and asparagine, respectively. These substitutions may be tolerated in place of the isoleucine found in the RT loop of the Src-family kinase SH3 domain, as a strong BiFC signal was observed with both of these kinases and Nef (Figures 3.5 and 3.6). In contrast, alanine and arginine are present in this position in the Tec and Txk SH3 domains, respectively. These

differences may compromise interaction with the Nef hydrophobic pocket, as no BiFC signal was detected with these SH3 domains (Figure 3.5). SH3 domain residues that occupy RT-loop positions 96 and 100 in the Tec versus Src-family kinases are summarized in Figure 3.8B; their positions in the overall SH3 sequences are highlighted in the sequence alignment presented in Figure 3.7.

SH3 domain structural alignments described above suggest that the amino acid occupying the RT-loop position analogous to Ile96 in the Hck SH3 domain may be an important determinant of Nef binding to Tec family kinases via their SH3 domains. To test this possibility, we substituted RT-loop residue 96 in the SH3 domains of full-length Hck, Btk and Itk (all Nef binders) with arginine, which is present at this position in the Txk SH3 domain (a non-binder). In all three cases, arginine substitution compromised interaction with Nef as visualized by BiFC (Figure 3.8C). This reduction was stronger with Hck and Btk, while the effect was less pronounced with Itk, suggesting that other regions of Itk may contribute to Nef recruitment. In a complementary experiment, we substituted RT-loop Ala96 in the Tec SH3 domain with Ile, and found that this change dramatically enhanced interaction with Nef by BiFC (Figure 3.8C). These results support a key role for the SH3 domain in the interaction of Nef with Tec family kinases in cells, and suggest that Nef interacts with Src and Tec family kinases through a similar mechanism.

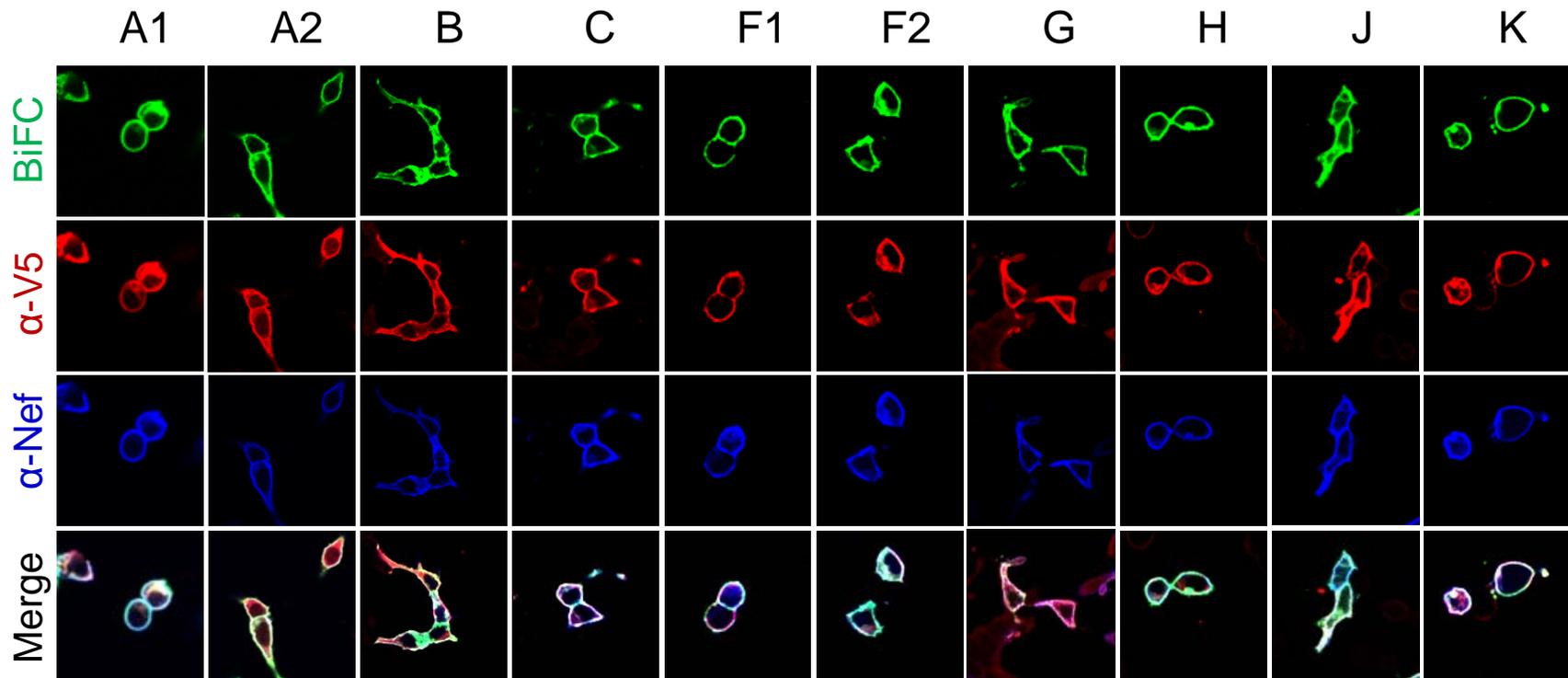


(A) Structural alignment of Tec family kinase SH3 domains with a Nef-SH3 complex. The X-ray crystal structure of Nef (purple) bound to a Src family kinase SH3 domain (red) was aligned with the NMR structures of the Btk (cyan), Itk (green), and Tec (orange) SH3 domains. An acidic residue is present at RT-loop position 100 in all three Tec family SH3 domains for potential polar contact with Nef Arg77 in a manner analogous to Asp100 in the Hck SH3 domain. In Hck, RT-loop Ile96 makes hydrophobic contacts with conserved Nef residues Phe90, Trp113, and Tyr120. Btk and Itk have Met and Asn at position 96, respectively, while human Tec has Ala at this position. The side chains of the conserved Nef PxxP motif are also shown (Pro72 and Pro75). Note that in the mouse Tec SH3 structure used for the model, position 96 is occupied by threonine; this residue was replaced with alanine to match the human sequence for illustrative purposes. The PDB codes for the structures used in this model are: Nef/SH3 complex, 1EFN; Btk SH3, 1AWX; Itk SH3, 2RNA; Tec SH3, 1GL5. Models of X-ray and NMR structures were produced using PyMOL. (B) Comparison of Hck SH3 domain residues (RT-loop positions 96 and 100) essential for Nef binding and kinase activation with the analogous positions in Tec-family kinases. (C) Mutagenesis of RT-loop residue 96 impacts Hck, Btk, Itk, and Tec interaction with Nef as assayed by BiFC. The BiFC to Nef-IF signal ratios were determined from whole-field analyses of three independent transfected cultures and are plotted as the mean ratio value \pm SEM.

Figure 3.8. Tec family kinase SH3 domain RT-loops control interactions with Nef

3.4.5 Interaction with Itk is a highly conserved property of all M-group HIV-1 Nef alleles

Previous studies have reported that selective targeting of Itk with siRNA or kinase inhibitors blocks crucial steps in the HIV-1 life cycle [266, 290]. Fluorescence complementation studies presented above show that Nef interacts with Itk via its SH3 domain, suggesting a mechanistic link between HIV-1 and Itk activity in the host cell. We therefore explored whether interaction with Itk is conserved across a wide variety of HIV-1 subtypes. For these studies, we created BiFC expression constructs for primary *nef* alleles representative of all M-group HIV-1 subtypes (A1, A2, B, C, , F1, F2, G, H, J, K), and co-expressed them with the complementary expression vector for full-length Itk. Sequence information for these alleles has been reported elsewhere [239]. As shown in Figure 3.9, co-expression of full-length Itk-VC in combination with the VN fusions of each of the Nef alleles yielded a strong BiFC signal that localized to the cell membrane. Expression of both interacting partners was confirmed in individual cells by immunostaining with anti-Nef and anti-V5 (epitope tag for Itk) antibodies, and merged images show co-localization. This result demonstrates that interaction of Nef with Itk is a highly conserved property of a diverse set of HIV-1 subtypes.



Nef clones representative of each of the M-group HIV-1 clades shown (A1, A2, B, C, F1, F2, G, H, J, and K) were fused to the N-terminal fragment of Venus (VN) and co-expressed with the complementary full-length Itk-VC construct in 293T cells. Cells were fixed and immunostained for Nef and kinase protein expression (anti-V5 antibody) 30 h later. Cultures were analyzed by confocal microscopy for Nef:Itk interaction by BiFC (green), Nef expression (blue) and Itk expression (red). Representative images are shown along with a merged image in the bottom panel.

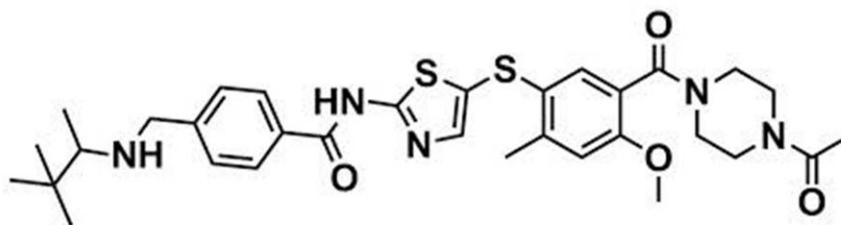
Figure 3.9. Interaction with Itk is conserved across Nef alleles

3.4.6 Assessment of the cytotoxicity of the Itk inhibitor, BMS-509744, to human cell lines

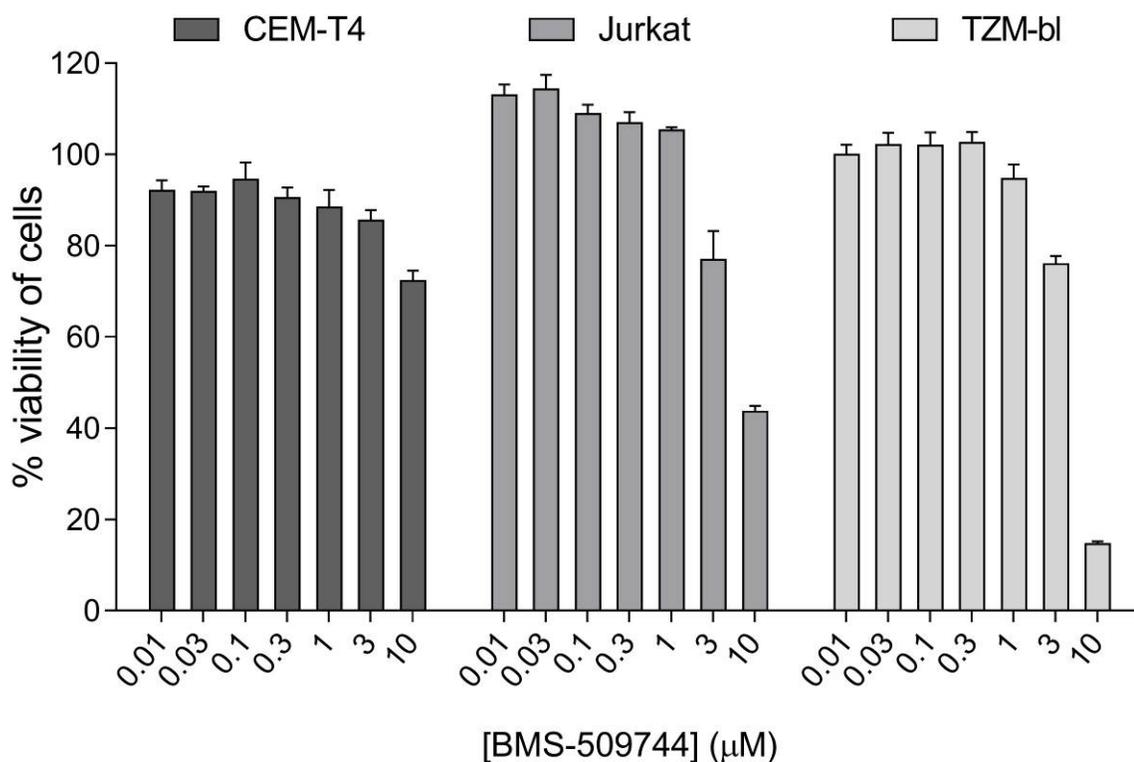
BMS-509744 was first described by Lin et al. as a highly potent and selective inhibitor of Itk that can reduce lung inflammation in an ovalbumin induced allergy (asthma) mouse model [212]. The X-ray crystal structure of Itk in complex with BMS-509744 revealed that this aminothioaryl-thiazolo compound (Figure 3.10A) potently inhibits kinase activity in an ATP-competitive manner by binding to the kinase domain and stabilizing the activation loop (amino acids 503-520) in a substrate-blocking inactive conformation [193]. This cell-permeable compound is extremely selective for Itk with an IC_{50} as low as 19 nM and exhibits little or no activity against 14 other related kinases ($IC_{50} \geq 1.1 \mu\text{M}$).

Prior to using the Itk inhibitor, BMS-509744 in HIV replication experiments, we performed independent experiments to verify whether BMS-509744 exhibited cytotoxicity in the human cells that we would use for viral assays. The HIV host cell lines Jurkat, CEM-T4 and TZM-bl cells were cultured in presence of increasing concentrations of BMS-509744 ranging from 0.01 μM to 10 μM . Cell viability was determined after 2 days for TZM-bl cells and after 10 days for Jurkat and CEM-T4 cells in accordance with the length of treatment anticipated for infectivity and replication experiments in each cell line. Little or no cytotoxicity was observed in any of the cell lines up to a concentration of 3 μM (Figure 3.10B). At the highest concentration tested (10 μM) the CEM-T4 cells were the most tolerant while only 10% of the TZM-bl cells were viable in the culture. Subsequent experiments with this compound were therefore performed at different concentrations as noted in the experiments with the maximum concentration tested at 3.0 μM .

A



B

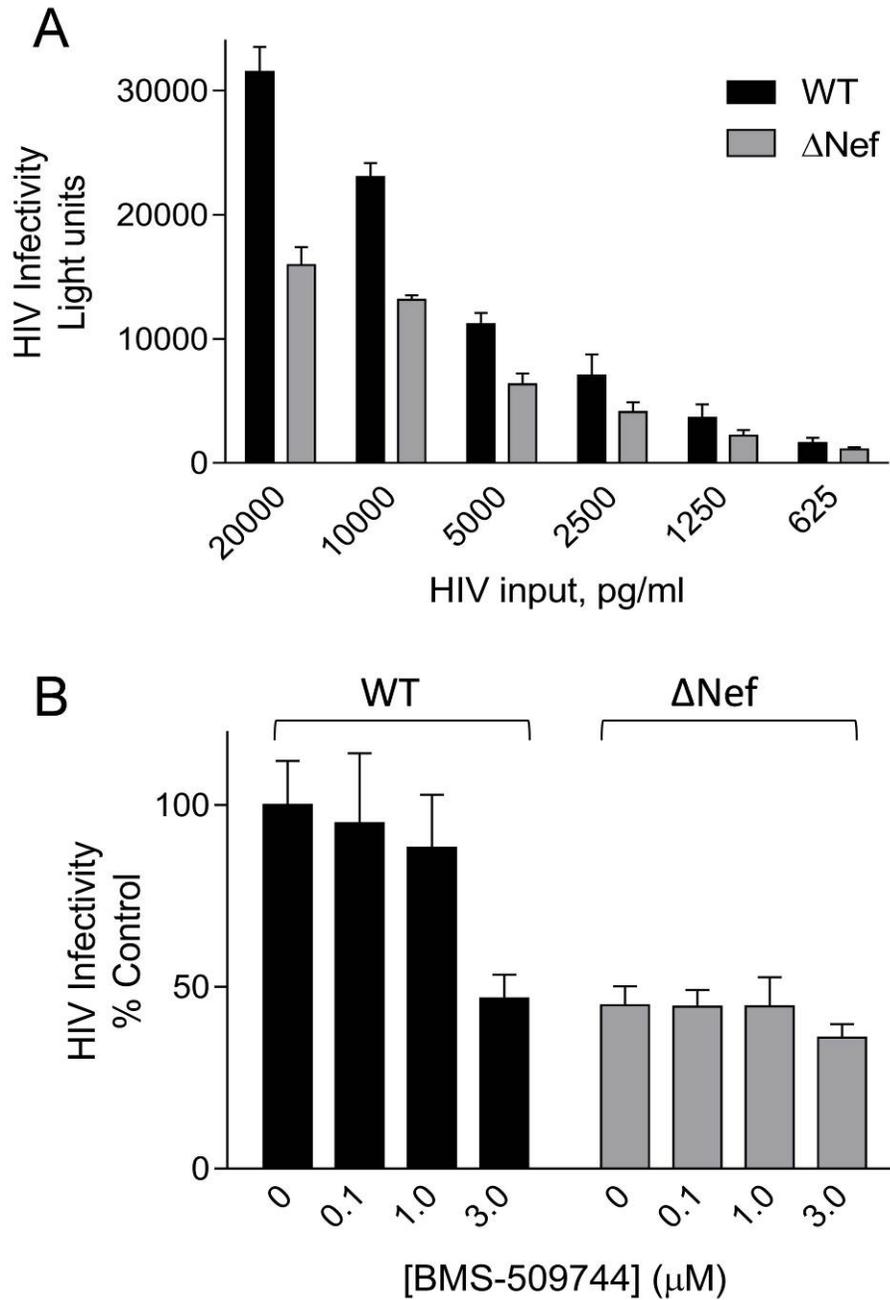


(A) Chemical structure of the Itk inhibitor BMS-509744, [N-(5-((3-((4-Acetylpiperazin-1-yl)carbonyl)-4-methyl-6-methoxyphenyl)thio)thiazol-2-yl)-4-(N-1,2-dimethylpropylaminomethyl)benzamide]. (B) To evaluate the cytotoxicity of BMS509744, each of the cell lines shown was incubated with the Itk inhibitor over the range of concentrations indicated. Cell viability was determined by Cell Titer Blue assay (Promega) after 48 h in TZM-bl cells and after 10 days in Jurkat and CEM-T4 cells. These time points were selected to match the time course of the HIV assays performed in the respective cell lines. Results are presented as mean percent of cell viability observed in the presence of the DMSO carrier solvent alone \pm SEM (n=3).

Figure 3.10. Cell viability in presence of the Itk inhibitor, BMS509744

3.4.7 A selective Itk Inhibitor blocks HIV infectivity and replication in a Nef-dependent manner

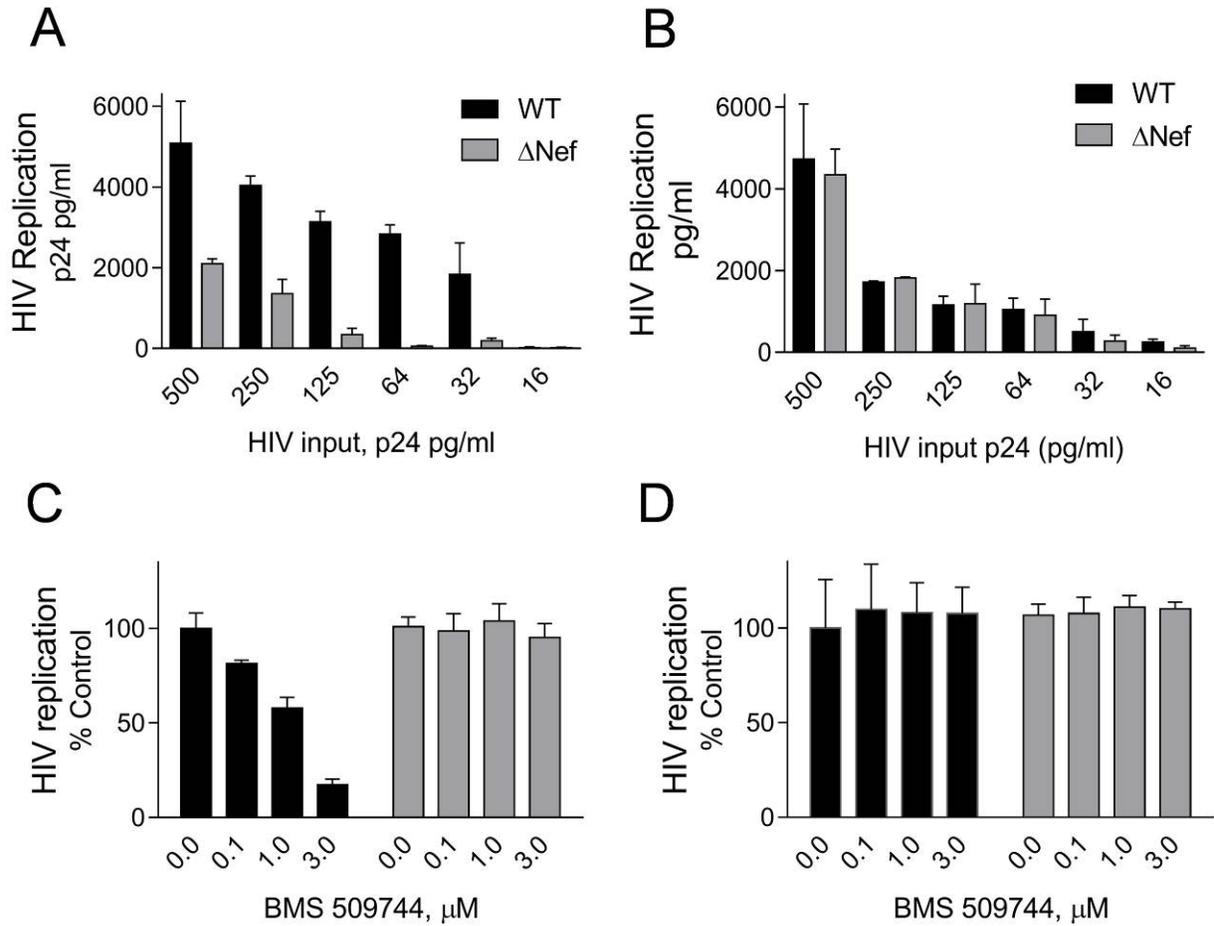
Previous work has shown that HIV infectivity and replication are sensitive to the selective Itk inhibitor, BMS-509744 [266]. However, the molecular mechanism linking HIV-1 infection to activation of Itk is still unclear. In the present study so far, we have shown that Nef interacts with Itk and this interaction is conserved amongst all Nef alleles representative of M-group HIV-1 subtypes. Thus our next aim was to deduce the relevance of this interaction to HIV biology. To explore the possible role of Nef in the activation of Itk in the context of HIV-1 infection, we first evaluated Nef-dependent HIV-1 infectivity in presence or absence of BMS-509744 in the TZM-bl reporter cell line [125]. In this system, infectivity is measured by stimulation of a luciferase reporter gene which is driven by the HIV-1 LTR following infection with wild-type or Nef-deleted (Δ Nef) HIV-1. As shown in Figure 3.11 A, HIV-1 infectivity was reduced by almost 50% in the absence of Nef in the TZM-bl cells, consistent with prior studies [98]. Incubation of cells with BMS-509744 at a concentration of 3 μ M suppressed wild-type HIV-1 infectivity to the same level as Nef-defective HIV (Figure 3.11 B). By contrast, this Itk inhibitor had no effect on infectivity of Nef-defective HIV-1, supporting a requirement for Nef expression in its mechanism of action.



(A) HIV-1 infectivity is Nef-dependent. TZM-bl cells were infected with wild-type and Nef-deleted (Δ Nef) HIV NL4-3, and viral infectivity was assessed 2 d later as luciferase activity. Results are plotted as relative light units \pm SEM (n=3). B) Inhibition of HIV infectivity by BMS-509744 is Nef-dependent. TZM-bl cells were pre-incubated overnight with BMS-509744 over the range of concentrations shown, followed by infection with wild-type and Δ Nef HIV NL4-3. Infectivity was assessed 2 days later as luciferase activity. Results are presented as mean percent of HIV-1 infectivity observed in the presence of the DMSO carrier solvent alone \pm SEM (n=3).

Figure 3.11. Inhibition of HIV infectivity by the Itk inhibitor BMS-509744 requires Nef

To assess the role of Nef-dependent Itk activity in the context of HIV-1 replication, we turned to CEM-T4 lymphoblasts, which support viral replication in a Nef-dependent manner [98, 239]. As shown in Figure 3.12A, wild-type HIV-1 replicated more efficiently than the Nef-deleted virus over a wide range of viral inputs in CEM-T4 cells. Addition of BMS-509744 blocked wild-type virus replication in a concentration-dependent manner, with a reduction of almost 80% at the highest concentration tested (3 μ M; Figure 3.12C). Remarkably, this Itk inhibitor had no effect on the replication of Nef-defective HIV-1 over the same concentration range, suggesting that Nef couples HIV-1 to Itk signaling in T-cell hosts. Note that Nef-deleted HIV-1 input was 10-fold higher than wild-type for these experiments (500 vs. 50 pg/ml p24 equivalents) to compensate for the reduced infectivity and replication of the mutant. In addition to CEM-T4 cells, we also tested the effect of BMS-509744 on HIV-1 replication in Jurkat T-cells. Unlike CEM-T4 cells, HIV-1 replication is Nef-independent in this cell line (Figure 3.12B) [97]. As shown in Figure 3.12D, treatment of Jurkat cells with BMS-509744 had no effect on either wild-type or Nef-defective HIV-1 replication, supporting a requirement for Nef in the mechanism of action of this compound on viral replication.

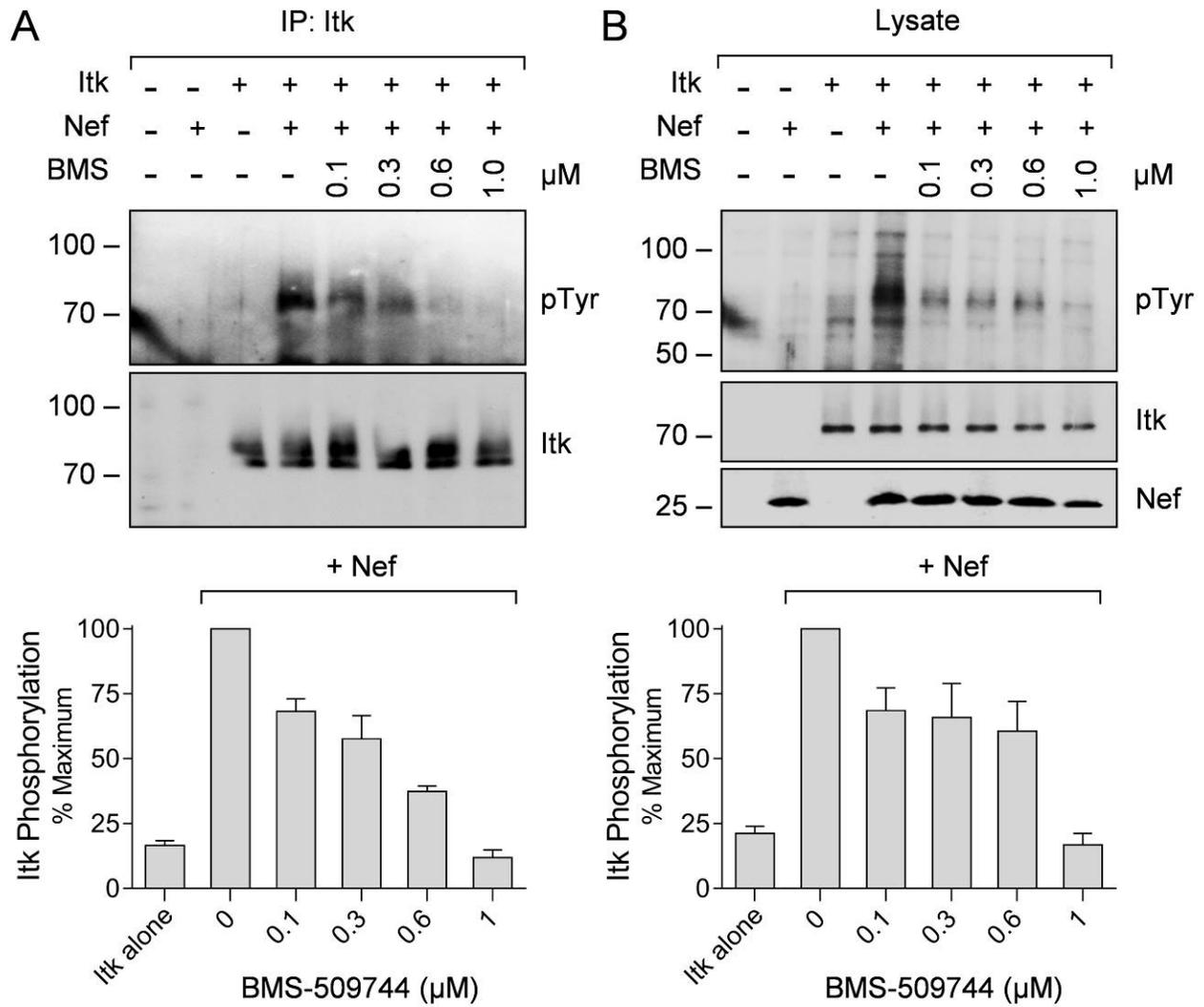


(A) HIV-1 replication in CEM-T4 cells is enhanced by Nef. CEM-T4 cells were infected with wild-type and Nef-deleted (Δ Nef) HIV NL4-3 and viral replication was assessed 10 d later by p24 Gag ELISA. Results are plotted as mean p24 levels \pm SEM (n=3). (B) HIV-1 replication is independent of Nef in Jurkat T cells. Jurkat cells were infected with wild-type and Δ Nef HIV NL4-3 and viral replication was assessed 10 d later by p24 Gag ELISA. Results are plotted as mean p24 levels \pm SEM (n=3). (C) Inhibition of HIV-1 replication by BMS-509744 requires Nef in CEM-T4 cells. Following overnight pre-incubation with BMS-509744, cells were infected with the wild-type and Δ Nef viruses. Input of Δ Nef HIV was increased by 10-fold relative to the wild-type virus to compensate for the reduced replication of the mutant virus. HIV replication was determined by p24 ELISA 10 days later, and is expressed as mean percent of replication observed with the DMSO-treated controls \pm SEM (n=3). (D) BMS-509744 has no effect on HIV-1 replication in Jurkat T cells. Jurkat cells were pre-incubated overnight with BMS-509744 before infection with the wild type or Δ Nef virus. HIV replication was determined by p24 ELISA 10 days later, and is expressed as mean percent of replication observed with the DMSO-treated controls \pm SEM (n=3).

Figure 3.12. Inhibition of HIV replication by the Itk inhibitor BMS-509744 requires Nef

3.4.8 Nef stimulates Itk kinase activity

Results presented so far demonstrate that HIV-1 Nef interacts with Itk at the plasma membrane, and that the enhancement of HIV-1 infectivity and replication by Nef are sensitive to a selective Itk kinase inhibitor. These observations imply that interaction with Nef stimulates Itk kinase activity. To test this possibility directly, we transfected 293T cells with full-length Itk and Nef either alone or in combination. Itk was then immuno-precipitated from the transfected cell lysates and probed with anti-phosphotyrosine antibodies. As shown in Figure 3.13A, Itk was not detectably autophosphorylated when expressed alone in 293T cells, consistent with its low intrinsic kinase activity as reported elsewhere [176]. However, co-expression of Itk with Nef resulted in a dramatic increase in Itk phosphotyrosine content, consistent with Nef-dependent enhancement of kinase activity. We then repeated this experiment in the presence of the Itk inhibitor, BMS-509744, and observed a concentration-dependent decrease in Itk phosphotyrosine content. Nef-dependent Itk tyrosine phosphorylation was almost completely blocked at an inhibitor concentration of 1.0 μM (Figure 3.13A), which agrees with the concentration of this compound required to inhibit Nef-dependent HIV replication and infectivity. Very similar results were obtained following immunoblot analyses of the transfected cell lysates with antiphosphotyrosine antibodies (Figure 3.13B). In this case, a strong tyrosine-phosphorylated band was observed in cells co-expressing Nef and Itk but not in cells expressing either protein alone. Tyrosine phosphorylation of this band, which migrates at ~ 72 kDa, is also potently inhibited by BMS-509744, and therefore is likely to represent autophosphorylated Itk.



Full-length Itk kinase and Nef were expressed in 293T cells either alone or in combination in absence or presence of increasing concentrations of the Itk Inhibitor BMS-509744. A) Cells were lysed 30 hours later, and Itk immunoprecipitates were analyzed by immunoblotting for phosphotyrosine content (pTyr) as well as Itk protein expression. Representative blots are shown at the top. This experiment was repeated in triplicate, and the relative phospho-Itk and Itk protein levels were quantitated using the LiCOR Odyssey infrared imaging system. Relative signal intensities were corrected for background and used to calculate ratios of the phospho-Itk to Itk levels. The resulting ratios were then normalized to the highest value (phospho-Itk in presence of Nef without inhibitor) and are presented in the bar graph as the mean normalized ratio \pm SEM (n=3). B) Lysates from the same cell cultures in part A were immunoblotted directly with antibodies to phosphotyrosine (pTyr) as well as Itk and Nef. A representative blot is shown at the top, and the data were quantitated and processed as per part A.

Figure 3.13. Co-expression with Nef induces Itk activation that is sensitive to BMS 509744

3.5 SIGNIFICANCE AND CONCLUSION

Most current anti-HIV combinational therapeutic regimens target viral enzymes like reverse transcriptase, integrase, protease, as well as viral fusion and entry proteins. Growing incidences of multiple and cross resistance against approved HAART drugs necessitate identification of newer molecular targets in an infected cell. In an effort to widen the arena of cytoplasmic tyrosine kinases with SH3 domains that are direct effectors for HIV-1 Nef, we used a novel, cell-based approach to explore the scope of Nef:SH3 interactions. Using a BiFC approach, we discovered for the first time that Nef via its conserved PxxP motif directly binds to the SH3 domain of a specific subset of Tec family kinases. Binding of Nef to Itk is highly conserved among all major clades of HIV-1 and selective pharmacological targeting of Nef-induced Itk activation inhibits Nef-dependent HIV-1 infectivity and replication. Taken together, our findings open the doors to a handful of novel interactions that take place within an infected cell. These results validate the Nef-Itk interaction as a potential target for future anti-HIV drug development.

**4.0 THE BIOLOGICAL SIGNIFICANCE OF A NEW X-RAY CRYSTAL
STRUCTURE* OF HIV-1 NEF IN COMPLEX WITH THE TANDEM SH3-SH2 REGION
OF THE SRC-FAMILY KINASE, HCK^{§§}**

* The X-ray crystal structure was determined by Dr. John Jeff Alvarado.

^{§§} Work presented in this chapter has been published in the *Journal of Biological Chemistry*, 2014. Details of the new Nef structure in complex with the Hck SH3-SH2 region are described in introductory section 4.2.

With kind permission from JBC:

Alvarado, J. J.^{1,2}, S. Tarafdar^{1,3}, J. I. Yeh², and T. E. Smithgall¹. Interaction with the SH3-SH2 region of the Src-family kinase Hck structures the HIV-1 Nef dimer for kinase activation and effector recruitment. *J Biol Chem*. 2014 Aug 13. [Epub ahead of print]

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4.1 SUMMARY

HIV-1 Nef represents an important potential drug target by virtue of its interactions with host cell signaling molecules and its critical role in HIV-1 pathogenesis and AIDS progression. Nef supports high-titer viral replication in vivo and functions by interacting with multiple host cell effectors, including Hck and other Src-family kinases. We have recently determined the high resolution X-ray crystal structure of Nef in complex with the Hck SH3-SH2 regulatory regions. The complex crystallized as a dimer of Nef:SH3-SH2 complexes. Elucidation of structural details of the dimeric complex not only confirmed previously recognized contacts, but also identified new contacts at the Nef:Hck interface and revealed a novel Nef dimer interface. In this present study we have performed human and yeast cell-based studies to validate the biological significance of one novel inter-complex contact between Nef and the Hck SH3 domain (involving E93 on the Hck RT-loop and Nef R105) identified in this new structure. Mutagenesis of Hck SH3 E93 interfered with Nef:Hck complex formation by BiFC and kinase activation supporting a role for this previously unrecognized contact in stable complex formation between Nef and full-length Hck in cells.

4.2 INTRODUCTION

As has been already discussed earlier in this document, the lentiviral virulence factor Nef is essential for the high-titer replication of HIV and SIV *in vivo* and is required for the development of AIDS-like disease in monkeys [20, 126, 158, 182]. Targeted expression of Nef in CD4+ T-cells and macrophages causes AIDS-like disease in transgenic mice [143, 174]. Viral strains with mutated Nef alleles have been recovered from long-term non-progressors, implicating Nef as a critical virulence factor for human AIDS [89, 186].

Lacking any biochemical activity, Nef mediates its functional effects by interacting with multiple cellular factors, like immune (MHC-I, TCR) and viral (CD4, CXCR4, CCR5) receptors, trafficking proteins, and protein kinases [280], most notably members of the Src kinase family. Nef binds to the SH3 domains on the SFKs via its PxxPxR motif which is highly conserved among primary HIV isolates [127, 205, 298]. Nef proteins derived from all major and minor subtypes of HIV-1 have been shown to bind to and activate endogenous Src-family kinase activity in HIV-infected cells. Inhibition of this pathway blocks Nef-dependent enhancement of HIV replication, infectivity [98, 239] and MHC-I downregulation [91].

Early structural studies of HIV-1 Nef shed light upon the Nef dimer interface and also the mechanism of SFK-SH3 domain binding. However, structural details of the N-terminal anchor domain and the internal flexible loop were not reported. A more recent structure of full-length Nef bound to an MHC-I peptide in complex with the clathrin adaptor AP-1 μ 1 subunit, revealed a larger portion of the Nef N-terminal anchor domain. The putative N-terminal amphipathic α -helix and the acidic cluster on Nef were shown to interact with the second helix in the Nef core domain and μ 1 subunit, respectively [172]. These observations highlight the principle that interaction with larger protein ligands provides additional stabilizing contacts for flexible Nef

regions. In an effort to better understand the mechanism of Nef-dependent SFK activation and also identify additional regions of contact between Nef and Hck, we recently determined the X-ray crystal structure of HIV-1 Nef in complex with the SH3-SH2 tandem regulatory domains of human Hck to 2.0 Å resolution, which is described in detail in the following section. Remarkably, the Nef complex structure reveals previously unrecognized contacts at the Nef:SH3 interface, contacts between Nef and the SH2 domain, and a novel Nef dimer interface. We performed cell-based studies to validate the newly identified contacts in the structure and our results demonstrate that these novel interactions are essential for stable association of Nef with full-length Hck in cells and kinase activation. Our findings suggest that Nef interaction with Src-family kinases not only leads to kinase activation, but also results in structural remodeling of Nef consistent with recruitment of the AP-1 machinery essential for MHC-I downregulation.

4.2.1 Structure of the HIV-1 Nef core in complex with the Hck tandem SH3-SH2 domains (Hck32)

The Nef:Hck32 complex corresponds to the structured core of Nef (residues 58-205; residue numbering is based on the crystal structure of Nef NL4-3 [205]) bound to the Hck SH3-SH2 region. The Nef:Hck32 complex crystallized as a dimer of the two complexes with a total buried surface area of 10,520 Å² (Figure 4.1). Superposition of the individual Nef protein, SH3 and SH2 domain structures from each half of the dimer reveal that they are almost identical with root-mean-square-deviations of 0.41 Å, 0.17 Å, and 0.59 Å respectively. The new structure of the dimer complex is consistent with the proposed mechanism of Nef-induced Hck activation by disruption of the inhibitory interaction between the SH3 domain and the SH2-kinase linker. Superposition of the Nef:Hck32 complexes onto the SH2 domain of downregulated, near full-

length Hck reveals that in the dimer complex, the Nef-bound SH3 domains are significantly displaced from the linker, supporting the SH3 domain displacement model of Hck activation [231].

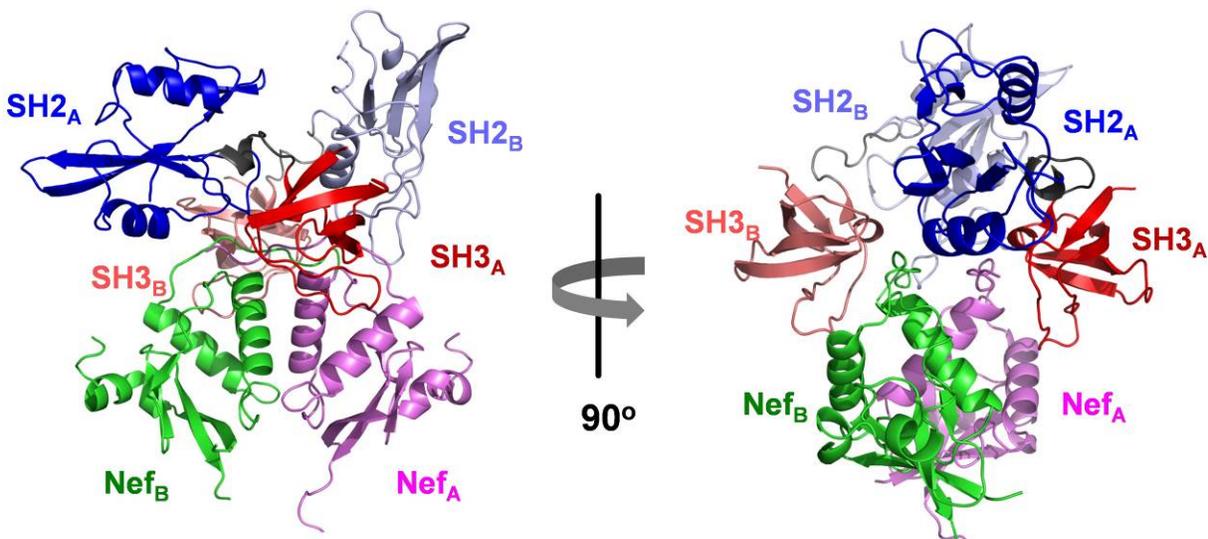


Figure Courtesy of Dr. John Jeff Alvarado, University of Pittsburgh.

The dimer of two Nef:Hck32 complexes A and B, oriented in a fashion resembling a 'handshake' conformation. For complex A, Nef is colored light purple, the SH3 domain is red, and the SH2 domain is blue; in complex B, Nef is rendered in green, the SH3 domain is pink, and the SH2 domain is light blue.

Figure 4.1. Overview of the dimeric Nef:Hck32 complex structure

Typically in all SFKs, the SH3 and SH2 domains are joined by a connector region comprised of eight residues that form an N-terminal β -turn followed by a 3_{10} -helix [12, 21, 94, 301, 346, 353]. While the structures of the individual Nef, SH3, and SH2 proteins making up the dimeric Nef:Hck32 complex are nearly identical, the relative orientation of the SH2 domains in each of

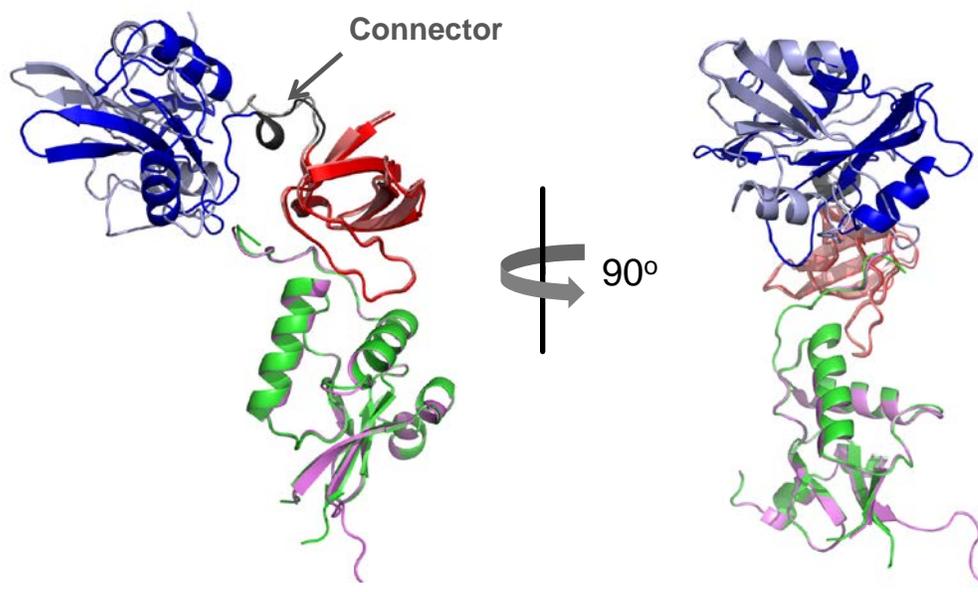


Figure Courtesy of Dr. John Jeff Alvarado, University of Pittsburgh.

Superposition of the individual Nef structures in the complexes A and B superimposes the SH3 domain perfectly. However, it reveals alternative positioning of the SH2 domains which are redirected 116° away from each other. The positions of the SH3-SH2 connector regions (gray) are indicated in the superposition on the left. The color of individual Nef, SH3 and SH2 proteins are similar to those in Fig. 4.1.

Figure 4.2. Distinct relative orientation of SH2 domains in the Nef:Hck32 dimer complex

the two hemi-complexes are distinct, such that they are oriented 116° away from each other based on the angle of the axes passing through the center of mass of each domain (Figure 4.2). The difference in orientation arises from the absence of the conserved 3_{10} -helix in the SH3-SH2 connector region in one of the hemi-complexes, where Hck residues 143-146 compulsively adopt an extended non-helical conformation due to lack of main-chain hydrogen bonding. In this dimer complex, residues in the loops connecting the central β -sheets and α -helices of each SH2 domain engages in extensive Van der Waals contacts with Nef residues from the distal end of the N-terminal anchor domain and α B helix.

Consistent with previous crystal structures of Nef:SH3 dimer complexes (Figure 4.3A) [18, 205], the Nef α B-helices form the dimer interface in our new Nef:Hck32 dimeric complex

as well (Figure 4.3B). However, the Nef monomers in the Nef:Hck32 complex are significantly reoriented relative to the previous Nef:SH3 complex. As shown in Figure 4.3C, superposition of one Nef monomer from each of the dimer complexes (our Nef:Hck32 dimer versus previous Nef:SH3 dimer; PDB: 1EFN) reveals that the other Nef monomers in each dimer complex are rotated 87° around the y-axis and 82° around the z-axis from the center of mass resulting in a much more compact structure. The relatively reoriented Nef dimer observed in our Nef:Hck32 complex structure is stabilized by four distinct dimer interfaces – three hydrophobic interfaces involving the N-terminal residues and α B-helix residues the Nef and a unique fourth interface that involves hydrophobic interactions as well as hydrogen-bonding contacts encompassing the Nef α A-helix C-terminus, the α B-helix N-terminus, and the α A- α B loop. Contrary to the Nef:SH3 structures where an ionic contact between the side chains of Nef R105 and D123 stabilizes the dimer interface (Figures 4.3 D) [18, 204], these residues do not contribute to the Nef dimer interface in the Nef:Hck32 complex. Instead, as shown in Figure 4.3E, they are now repositioned 22 Å away from each other leading the Nef residue R105 to engage in novel intermolecular interactions while D123 is solvent exposed and potentially available to participate in interactions with other proteins, such as the AP-1 μ 1 clathrin adaptor protein critical to MHC-I downregulation [172, 213].

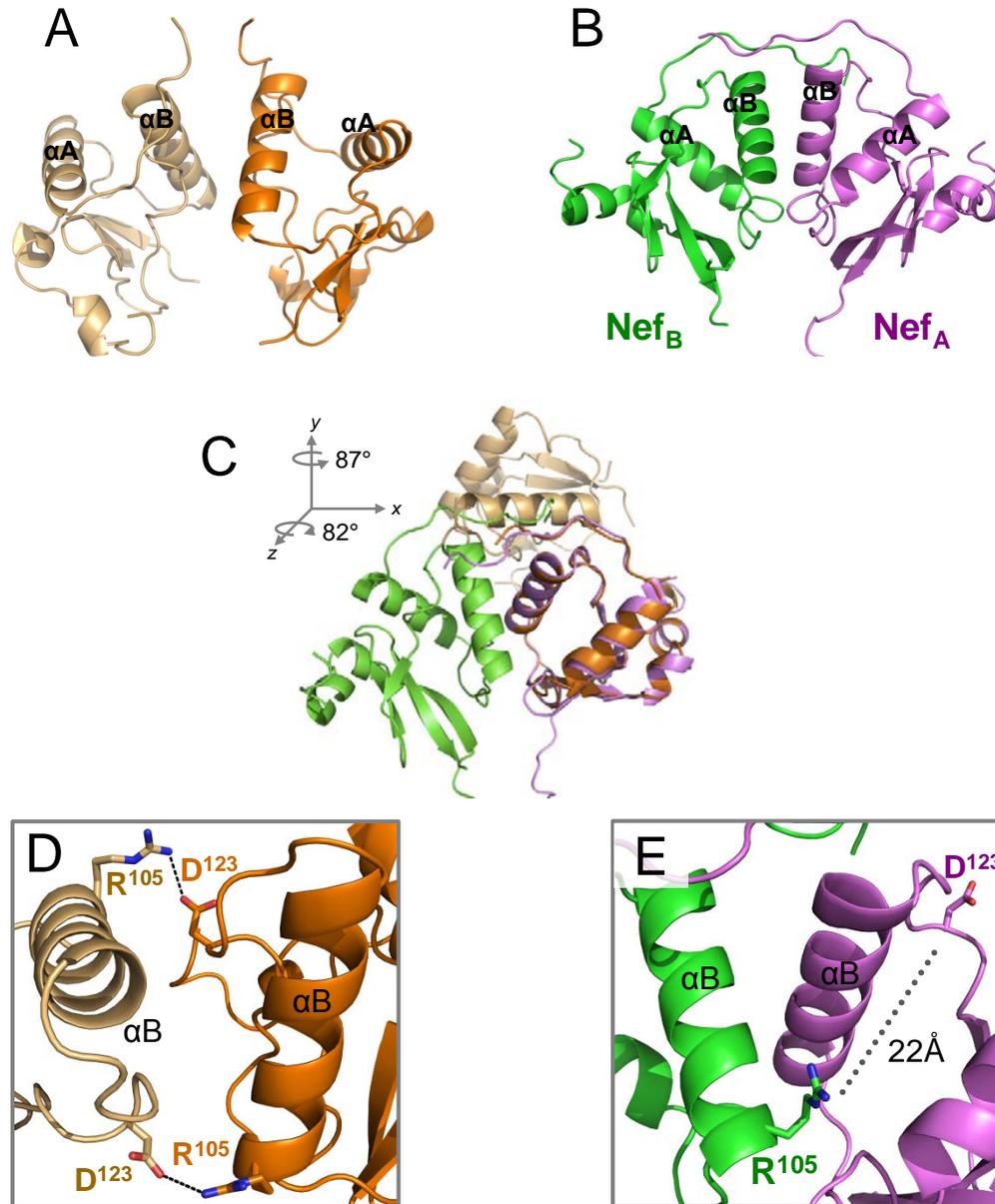


Figure Courtesy of Dr. John Jeff Alvarado, University of Pittsburgh.

(A) Nef dimer from a previous Nef:SH3 complex (Nef monomers are light and dark orange; PDB: 1EFN). (B) Nef dimer in the Nef:Hck32 complex (Nef monomers are green and purple). Nef α B helices form the dimer interface in both the structures. (C) Superposition of Nef monomers from the structures in A and B. The resulting rotations along the y and z axes for the non-superimposed Nef monomer are shown. (D) Close up view of the dimer interface from a complex of Nef with an SH3 domain showing the role of D123:R105 ionic interaction in dimerization. (E) Nef D123 is solvent exposed due to being repositioned away from the dimer interface more than 22 Å away from Nef R105 in the Nef:Hck32 structure.

Figure 4.3. Hck SH3-SH2 binding stabilizes a compact Nef dimer

4.2.2 Unique intermolecular Nef:SH3 interactions in the HIV-1 Nef:Hck32 complex

Previous structural and functional studies have implicated two HIV-1 Nef regions in SFK-SH3 domain engagement and kinase activation – the highly conserved P₇₂xxP₇₅xR₇₇ motif and a hydrophobic pocket formed by the first two α -helices of the Nef core [18, 46, 61, 204, 205, 231, 281]. As has been discussed in detail in the preceding chapters, the polyproline motif interacts with the RT- and N-Src-loops of the SH3 domain while conserved residues in a Nef hydrophobic pocket engage a single RT-loop isoleucine residue unique to the Hck and Lyn SH3 domains. Both of these interactions are present in the structure of the Nef:Hck32 complex and involve all previously identified residues on both Nef and the kinase proteins.

Besides these known Nef:SH3 contacts, a unique intermolecular/intercomplex interaction was identified in the new dimeric Nef:Hck32 structure. As described in the previous section 4.2.1 the relatively reoriented Nef dimer interface lacks the ionic contact between the side chains of Nef residues R105 and D123 in the Nef:Hck32 dimer complex. While the repositioned D123 residue faces the solvent, the R105 residue on one complex engages in a previously unrecognized intercomplex salt-bridge with the SH3 domain RT-loop residue E93 from the opposite complex (Figure 4.4). Two such reciprocal intercomplex R105:E93 salt bridges are observed within the dimeric Nef:Hck32 complex structure. This novel interaction serves as an additional intermolecular contact between Nef and the kinase-SH3 domain near the previously identified critical I95 (corresponds to I96 as referred in [61, 204, 320]) residue, supporting a stabilizing influence in the context of full-length Hck. Studies described below provide evidence that this previously unrecognized interaction is important for Nef:Hck complex stabilization and subsequent kinase activation.

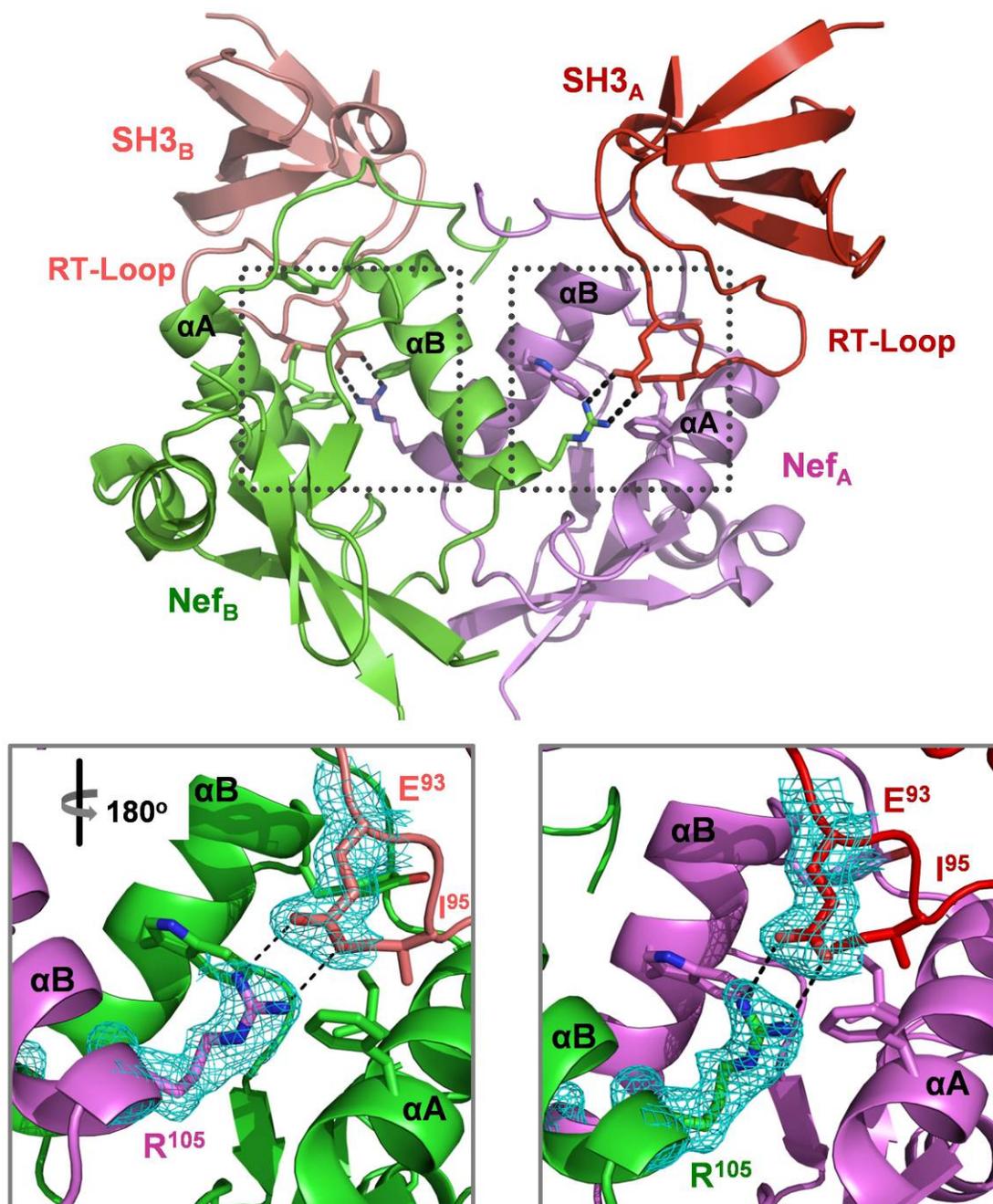


Figure Courtesy of Dr. John Jeff Alvarado, University of Pittsburgh.

The dimer of Nef:SH3 complexes A and B is shown at the top with the coloring of individual proteins as per Figure 4.1. The unique intercomplex ionic contacts between Nef R105 and SH3 E93 are illustrated as sticks. Close-up view of each ionic interaction is enlarged below and shows a well-ordered $2F_o - F_c$ electron density (cyan mesh; contoured at 1σ). The expanded view on the left is rotated 180° with respect to the overall view above in order to maintain the same orientation as the view on the right.

Figure 4.4. Unique Nef:SH3 interactions in the Nef:Hck32 complex

4.3 MATERIALS AND METHODS

4.3.1 Cell culture, Reagents and Antibodies

Human 293T cells were obtained from the ATCC and were cultured in Dulbecco's Modified Eagle's Medium/high glucose (DMEM) (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gemini Bio-Products). All cells were maintained at 37⁰C in a 5% CO₂ water-jacketed incubator (Forma Scientific).

Primary antibodies used in this study were obtained from Santa Cruz Biotechnology (Phosphotyrosine mouse monoclonal, pY99, sc-7020; Csk rabbit polyclonal C-20, sc-286; Hck rabbit polyclonal N-30, sc-72), Millipore (Actin mouse monoclonal, clone C4, MAB1501) and the National Institutes of Health AIDS Research and Reference Reagent Program (HIV-1 JR-CSF Nef monoclonal, 1539; HIV-1 Nef monoclonal EH1, 3689). Secondary antibodies used in this study were obtained from Southern Biotech [Goat Anti-Rabbit IgG (H+L) Mouse/Human ads-TXRD, 4050-07; Goat Anti-Mouse IgG (H+L), Human ads-AP, 1031-04; Goat Anti-Rabbit IgG (H+L), Human ads-AP, 4010-04], LiCor [Donkey Anti-Mouse IgG (H+L) IRDye 800CW, 926-32212; Donkey Anti-Rabbit IgG (H+L) IRDye 680RD 926-68073], and Invitrogen Molecular Probes [Pacific Blue Goat Anti-Mouse IgG (H+L), P31582].

4.3.2 Mammalian Expression Vectors for BiFC

The coding regions of wild-type human Hck SH3 and SH3-SH2 domains (including the N-terminal myristoylation sequence and unique domain to maintain the natural lipid modification and spacing from the membrane) was PCR-amplified using primers: FWD

5'(CGCgaattcGCCACCATGGGGTG)3'; REV_{SH3} 5'(GCGggtaccCTCTGTCTCCAGAGAGTCAAC)3'; REV_{SH3-SH2} 5'(GCGggtaccCATGCAGGGCACCGACAGTTTCTGG)3'. The reverse primers were designed to introduce the unique KpnI restriction site at the 3'-end. The C-terminal coding sequence of the Venus variant of the YFP protein (residues Ala154-Lys238) was amplified by PCR with an introduction of a unique KpnI restriction site at its 5'-end using the primers: FWD 5'(GCGggtaccGCCGACAAGCAGAAGAACGG)3'; REV 5'(GCCgaagctTCACTTGACAGCTCG)3' and subcloned into the mammalian expression vector, pcDNA3.1(-) (Invitrogen). The coding regions of wild-type human Hck SH3 domain, Hck SH3-SH2 domains as well as full-length p59 Hck were then subcloned upstream and in frame with this Venus fragment for expression of the SH3-VC, 32-VC and Hck-VC fusion proteins, respectively. The SH3 domain mutation (E93A) was then introduced into these vectors via site-directed mutagenesis (QuikChange[®] II XL Site-Directed Mutagenesis Kit, Stratagene) using the primers: Sense 5'(GCCCTGTATGATTACGcGGCCATTCACCACGAA)3'; Antisense 5'(TTCGTGGTGAATGGCCgCGTAATCATAACAGGGC)3'. Construction of the complementary BiFC expression vector for HIV-1 Nef SF2 (Nef-VN) has been described elsewhere [258].

4.3.3 Yeast Expression Vectors

The coding sequence for human wild-type Hck (p59 isoform) with either a wild type (YQQQ) or a constitutively downregulated (YEEI) C-terminal tail were PCR amplified with the introduction of unique restriction sites EcoRI and NotI at their 5' and 3'-ends respectively. The primers used were: FWD 5'(CGCgaattcGCAATAATGGGGTGCATGAAGTCCAAG)3'; REV_{YQQQ} 5'(GCCGgcgccgcTCATGGCTGCTGTTGGTACTGGCT)3' and REV_{YEEI} 5'(GCCGgcgccgcTCATGGATCTCTTCGTACTGGCTCTCTGTGGCCGT)3'. The 5' primers were designed to

introduce the yeast translation initiation sequence (AATA) immediately upstream of the 5' ATG start codon. Hck and HIV-1 Nef (SF2 allele) were subcloned downstream of the Gal1 and Gal10 promoters in the yeast expression vectors pYC2/CT-Ura (Invitrogen) and pESC-Trp (Stratagene), respectively. The pYC2/CT vector houses the CEN6/ARSH4 sequence for low-copy replication. Hck SH3 domain mutants E93Q and E93A were created via site-directed mutagenesis using the QuikChange[®] II XL site-directed mutagenesis kit and the manufacturer's protocol (Stratagene). The primers used were: Sense_(E93Q) 5'(GTTGCCCTGTATGAT TACcAGGCCATTCACCACG)3'; Antisense_(E93Q) 5'(CGTGGTGAATGGCCTgGTAATCAT ACAGGGCAAC)3'; Sense_(E93A) 5'(GCCCTGTATGATTACGcGGCCATTCACCACGAA)3'; Antisense_(E93A) 5'(TTCGTGGTGAATGGCCgCGTAATCATAACAGGGC)3'. Construction of the yeast expression vectors pESC-Trp-Csk and pESC-Trp-Csk/Nef has been described by our group previously elsewhere [325].

4.3.4 Bimolecular Fluorescence Complementation (BiFC) Assay

Human 293T cells were plated on glass bottom microwell dishes (MatTek, P35G-1.5-14-C) and allowed to attach overnight. Cells were transfected with complementary BiFC expression vectors - wild type(WT) and mutant (E93A) forms of SH3-VC, SH3-SH2-VC, Hck-VC and Nef-VN using standard calcium phosphate techniques as described elsewhere [45]. Eighteen hours post transfection, cells were fixed with freshly prepared 4% paraformaldehyde (stock 16% solution; Electron Microscopy Sciences), permeabilized with 0.2% Triton X-100 and blocked with 2% BSA (Sigma) in PBS overnight. Cells were immunostained with primary antibodies against Hck (1:1000), and Nef (1:1000) for 1 hour at room temperature at their respective dilutions (in

parentheses). Immunostained cells were visualized with secondary antibodies conjugated to Texas Red or Pacific Blue at a dilution of 1:1000 and 1:250 respectively. Three-color BiFC and IF images were recorded using confocal microscopy (Fluoview FV-1000, Olympus). Image analysis was performed with the Java-based image processing program, ImageJ (version 1.48s), to determine the BiFC and immunofluorescence (IF) intensities of individual cells for a minimum of 100 cells. Mean pixel densities were calculated for the BiFC and IF channels and (mean \pm SEM) of the BiFC:IF signal ratios were computed from independent experiments (n=3).

4.3.5 Yeast assay for Nef-mediated Hck activation

Growth Suppression assay

Saccharomyces cerevisiae strain YPH 499 (Stratagene) was transformed (Bio-Rad GenePulser II) with pESC-Trp and pYC2/CT-Ura expression plasmids for Hck, Csk and Nef by electroporation (at 1.5 kV, 25 μ F, 200 W). Transformed colonies were grown for 3 days at 30 °C on synthetic drop-out agar plates lacking uracil and tryptophan (SD/-U-T) with glucose as the sole carbon source to repress protein expression. Colonies were then cultured in synthetic drop-out liquid medium with glucose for 18 hours at 30 °C. Culture densities were normalized to OD₆₀₀ of 0.2 (A₆₀₀=0.2) and spotted onto SD/-U-T agar plates containing galactose as the sole carbon source to induce protein expression. Yeast cultures were spotted in 4 fold dilutions starting at an A₆₀₀=0.2. Plates were incubated for 4 days at 30 °C and imaged on a scanner. Growing yeast colonies appear as dark spots against the translucent agar background. Representative image of one from three independent experiments is shown.

Immunoblot analysis of Nef-Mediated Hck activation

Yeast cultures from transformed colonies that were used for the growth suppression assay were cultured in galactose-containing liquid dropout medium lacking uracil and tryptophan (SD/-U-T) for 18 hours at 30 °C to induce protein expression. Optical densities of cultures were normalized to an OD₆₀₀ of 0.2, and cells were pelleted and lysed with 0.1 N sodium hydroxide for 5 min at room temperature. Lysates were separated via SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes or nitrocellulose membranes, and probed with antibodies to phosphotyrosine, Csk, Hck, and Nef. Actin levels were also probed as a loading control.

4.3.6 Statistical Analyses

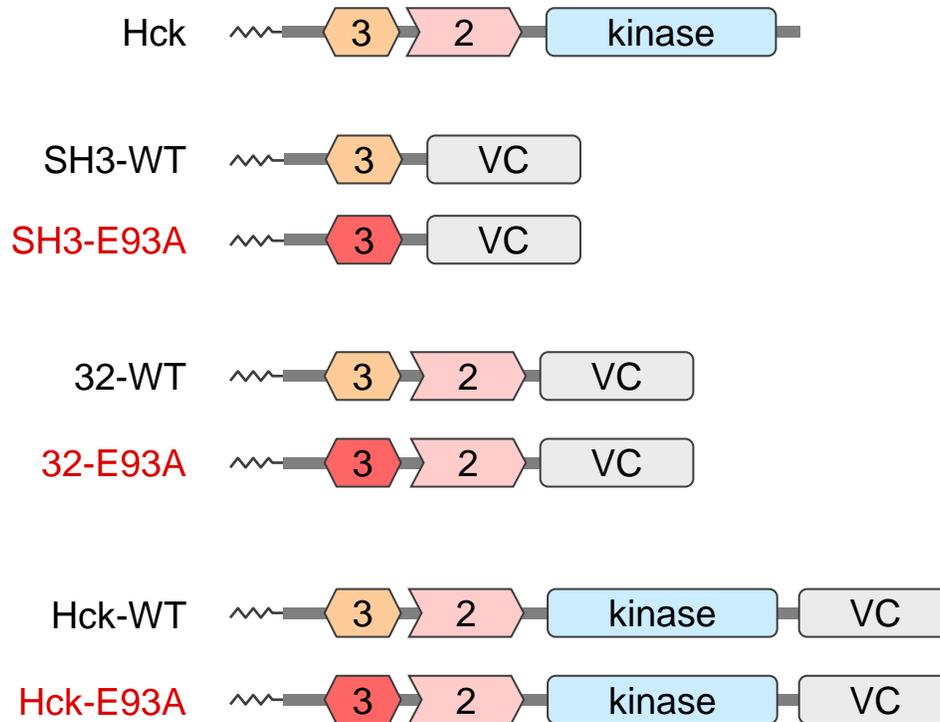
Quantitative analyses of the data are reported as the mean value \pm standard error of mean (SEM) from three independent experiments (n=3).

4.4 RESULTS AND DISCUSSION

4.4.1 Hck-SH3 RT loop residue E93 is involved in stabilizing Hck binding to Nef dimer

As detailed in the introduction (section 4.2.2), the recently determined high resolution structure of the dimeric Nef:Hck32 complex from our group revealed novel contacts between Nef and the Hck SH3 domain, likely providing additional stability to the dimer complex structure. To evaluate the functional importance of the newly identified interaction of Nef R105 with the Hck-SH3 RT Loop Glu93 to complex formation, we turned to the cell-based bimolecular fluorescence complementation (BiFC) assay [180, 320]. The BiFC assay, as we have discussed in the previous chapter, is a relatively straight-forward technique that relies on the association between two non-fluorescent fragments of YFP when brought into close proximity by an interaction between proteins fused to the fragments.

For these studies, a non-fluorescent N-terminal fragment of Venus (a YFP variant that permits fluorophore maturation under cell culture conditions [268]) was fused to the C-terminal end of HIV-1 Nef (SF2 allele) to preserve N-terminal myristoylation and membrane localization (Nef-VN). On the other hand, we expressed the full-length, the SH3 domain, and the SH3-SH2 domain of Hck as fusion proteins with the C-terminal fragment of Venus to create the wild type SH3-WT, 32-WT and Hck-WT constructs. The Hck BiFC fusion proteins included the natural N-terminal myristoylation signal and unique domain to enable membrane targeting and proper spacing from the membrane (Figure 4.5).

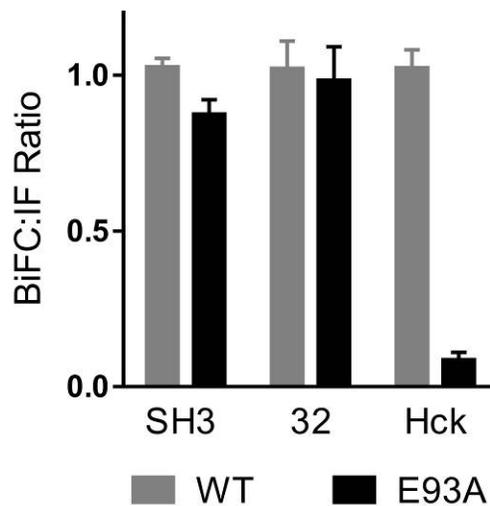
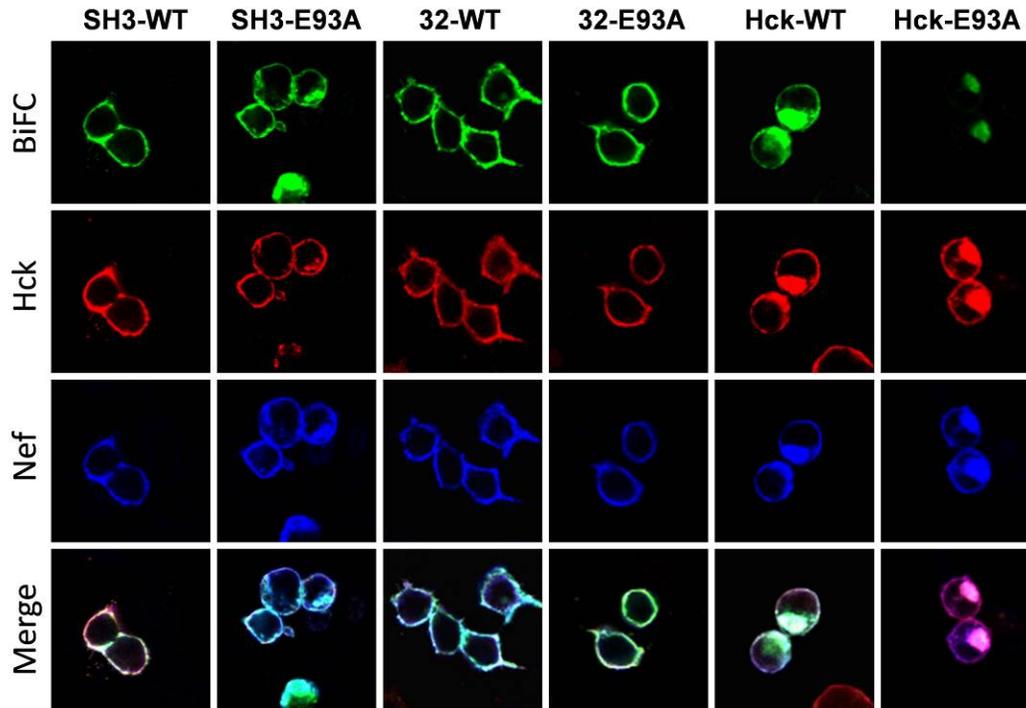


Truncated and full-length expression constructs were created for BiFC experiments (SH3-WT; 32-WT; Hck-WT) by fusing the proteins to the C-terminal fragment of the Venus form of YFP (VC). The truncated shorter constructs included sequences N-terminal to either the SH2 domain (SH3-WT) or kinase domain (32-WT) from wild type Hck. The glutamic acid (E) residue in the Hck SH3 RT-loop was mutated to Alanine (A) in the wild type expression constructs to generate the corresponding mutant BiFC constructs (SH3-E93A; 32-E93A; Hck-E93A).

Figure 4.5. Mammalian expression vectors for BiFC fusion constructs

Co-expression of Nef-VN with wild type SH3-WT, 32-WT and full-length Hck-WT in 293T cells yielded a bright fluorescent signal (18 hours post transfection) that localized to the membrane, indicative of Nef interaction with both the truncated and full-length Hck proteins and subsequent complementation of the Venus fluorophore (Figure 4.6, *top panel*). When this experiment was repeated using the SH3-E93A and 32-E93A constructs (in which SH3 E93 was substituted with alanine), Nef interacted with the truncated mutant constructs resulting in a

comparable level of BiFC signal as seen with their wild type counterparts (Figure 4.6, *bottom panel*). The observation that the E93A substitution did not impact the interaction of Nef with the shorter Hck constructs consisting of only the unique, SH3 and SH2 domains is consistent with previous Nef:SH3 crystal structures in which the SH3 E93:Nef R105 contact is not present (18,54). However, when Nef-VN was expressed with the full-length Hck-VC, the BiFC signal was reduced by more than 90% (Figure 4.6, *bottom panel*), indicating that this additional SH3 domain contact is important for stable interaction of full-length Hck with Nef in cells.



(Top) Bimolecular fluorescence complementation (BiFC) analysis of Nef:Hck interaction. Human 293T cells were transfected with expression plasmids for Nef fused to N-terminal fragment Venus, and either the N-terminal region of Hck including the SH3 domain, SH3-SH2 domain or full-length Hck fused to the complementary C-terminal Venus fragment. Parallel experiments were conducted with wild-type (WT) and SH3 domain mutants in which E93 was replaced with alanine (E93A). Eighteen hours after transfection, cells were fixed and stained with antibodies to the Hck N-terminal region and Nef. Three-color confocal images were recorded for BiFC (green), which is indicative of Nef:Hck interaction, as well as Hck and Nef protein expression by immunofluorescent staining. (Bottom) Mean pixel intensities of the BiFC and immunofluorescent (IF) signals were determined for at least 100 cells per condition using ImageJ. BiFC:IF signal ratios were calculated and are presented below as mean ratio \pm S.E.M.

Figure 4.6. Interaction of Nef R105 with Hck SH3 E93 is required for complex formation

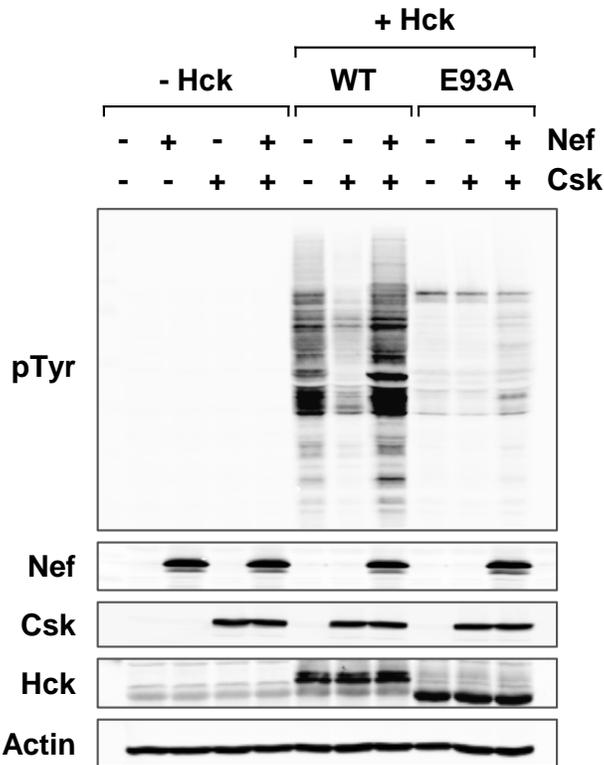
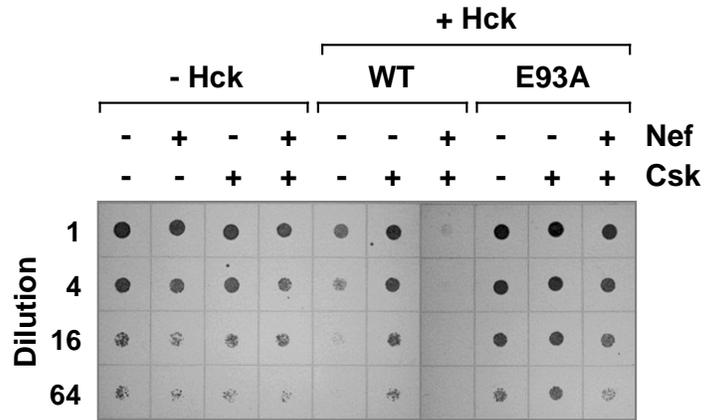
4.4.2 Hck-SH3 RT loop E93 is important to the activation of Hck by Nef

To evaluate the effect of the Hck SH3 domain E93 mutation on Nef-mediated kinase activation, we turned to a yeast-based co-expression system previously used in our laboratory to study Nef-mediated activation of Src-family kinases. Yeast cells do not intrinsically express orthologs of mammalian tyrosine kinases providing an ideal background for study of Src-family kinase interactions with partner proteins. However, yeast do perform post-translational modifications including myristoylation, which is critical to SFK and HIV-1 Nef function.

Because yeast cells lack Csk, the master regulator of Src-family kinase activity, ectopically expressed SFKs (Hck, in our case) are highly active and phosphorylate endogenous yeast proteins and cause growth suppression [109, 274]. Co-expression of Hck with Csk causes downregulation of Hck kinase activity and rescues growth. However, our previous work has shown that expression of Nef with Csk-downregulated Hck causes constitutive activation of Hck in the same manner as observed in HIV-infected cells, leading to growth arrest [325]. HIV-1 Nef-induced activation of SFKs can be measured by both monitoring yeast cell growth on solid culture medium and by evaluating levels of tyrosine phosphorylation of endogenous proteins from cell lysates. Thus yeast cells provide a well-defined, experimentally amenable and relevant model system to study the effect of Nef interactions with Src family kinases [325, 326].

We first expressed functionally active wild type and mutant full-length Hck in yeast expression vectors. The genes of interest were cloned using GAL-inducible expression vectors, in order to control protein expression with galactose as the sole carbon source. To use this system to explore the effect of the SH3 E93 mutant on Nef-induced activation, the wild-type, and E93A forms of Hck were expressed alone, in the presence of Csk, or in the presence of Csk and Nef. The cultures were grown to equal densities, and then plated on galactose agar over a

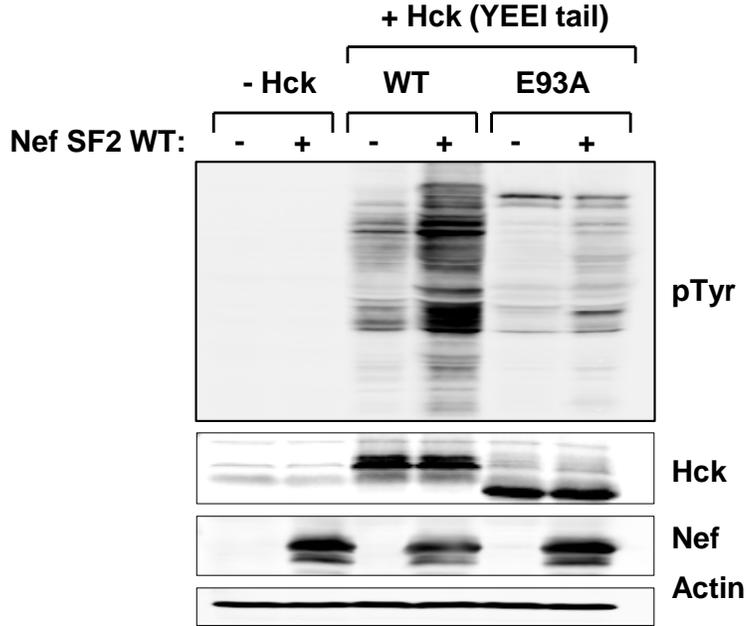
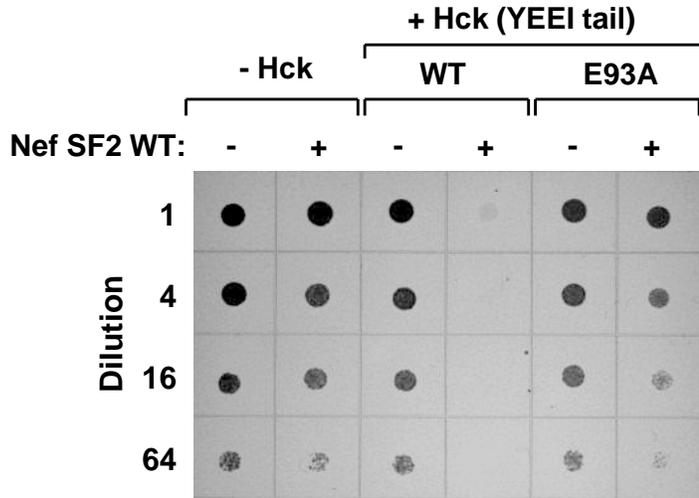
range of dilutions to induce protein expression. Parallel cultures were grown in liquid medium containing galactose, and cell lysates were prepared and immunoblotted for Hck, Nef and Csk protein expression as well as protein-tyrosine phosphorylation of yeast cell proteins. As shown in Figure 4.7 (*top panel*), expression of the wild type Hck alone caused growth arrest of yeast cells which can be rescued with the co-expression of Csk. Co-expression of the wild-type Hck with Nef caused, as expected, almost complete growth arrest in yeast, even in the presence of Csk. However, the Hck-E93A mutant was completely refractory to Nef-mediated activation, consistent with its inability to interact with Nef by BiFC. These effects correlated well with strong phosphorylation of cellular proteins on tyrosine Figure 4.7 (*bottom panel*), consistent with our previous work [239, 325]. These results taken together suggest that the inter-complex interaction of Nef R105 with SH3 domain E93 is required for kinase activation



Saccharomyces cerevisiae was transformed with galactose inducible expression plasmids for wild-type Hck (WT), the SH3 domain mutant (E93A), the regulatory kinase Csk and HIV-1 Nef in the combinations shown. Transformed colonies were cultured in glucose medium to equal densities and spotted onto agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for 4 days at 30°C and imaged on a scanner. Growing yeast colonies appear as dark spots against the translucent agar background (top panel). Transformed yeast colonies were also cultured in liquid medium plus galactose to induce protein expression. Cell lysates were separated via SDS-PAGE followed by immunoblotting for protein phosphotyrosine content (pTyr) as well as Nef, Csk, and Hck expression along with actin as a loading control (bottom panel).

Figure 4.7. SH3 domain E93 is important to the activation of Hck by Nef

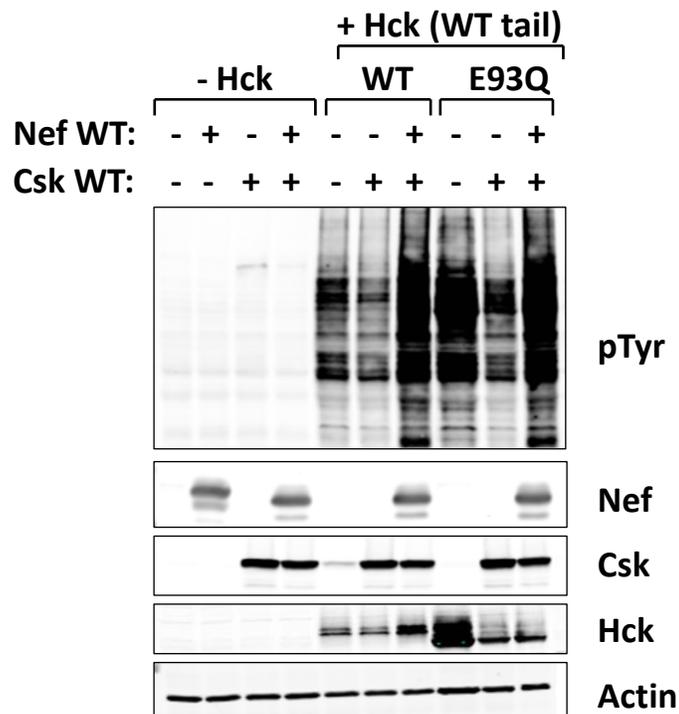
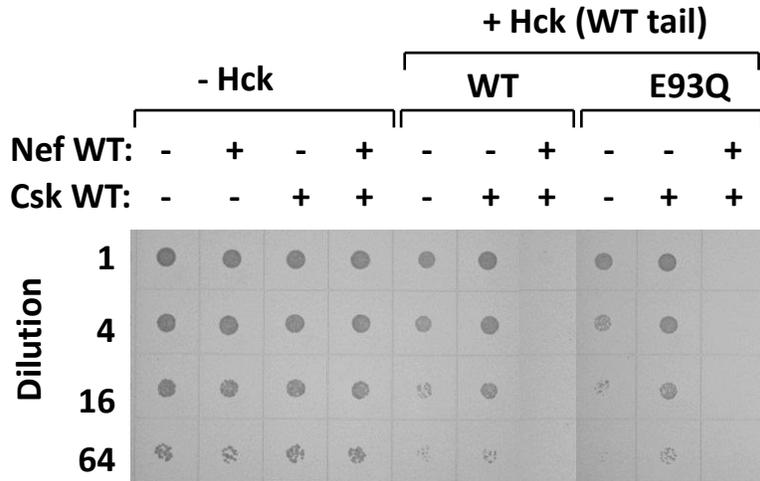
In similar experiments, we expressed full length wild-type and mutant (E93A) Hck with the modified “YEEI” tail. This modification enhances tail interaction with the SH2 domain and controls Hck activity in the absence of the negative regulatory ‘tail kinase’ Csk, so that the kinase adopts a constitutively downregulated conformation even in the absence of Csk. When these downregulated forms of wild-type and mutant Hck were expressed alone, they failed to arrest yeast cell growth, indicative of their inactive states and consistent with our recently published work Figure 4.8 (*top panel*) [327]. In presence of Nef, the wild-type Hck-YEEI caused complete growth arrest in the yeast cells, suggesting that Nef interacts with Hck in this system and activates it through an SH3-dependent mechanism, previously defined *in vitro* and in mammalian cells [325, 326]. In contrast, co-expression of Nef with the Hck-YEEI E93A mutant resulted in only a partial induction of growth arrest and a modest increase in phosphorylation of yeast cell proteins. As was observed in the growth suppression assay, the differential effects of Nef on the activation of downregulated wild-type and mutant Hck was also seen in the immunoblot analyses of tyrosine phosphorylation of cellular proteins Figure 4.8 (*bottom panel*). These results taken together support an essential role for Hck SH3 E93 in the activation of Hck by Nef.



Saccharomyces cerevisiae was transformed with galactose inducible expression plasmids for constitutively downregulated wild-type and mutant Hck (WT and E93A) carrying an YEEI tail, and HIV-1 Nef in the combinations shown. Transformed colonies were cultured in glucose medium to equal densities and spotted onto agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for 4 days at 30°C and imaged on a scanner. Growing yeast colonies appear as dark spots against the translucent agar background (top panel). Transformed yeast colonies were also cultured in liquid medium plus galactose to induce protein expression. Cell lysates were separated via SDS-PAGE followed by immunoblotting for protein phosphotyrosine content (pTyr) as well as Nef, Hck and actin expression (bottom panel).

Figure 4.8. Nef-mediated Hck activation by SH3 domain displacement requires SH3 domain E93 residue

As a control, we also expressed and tested a mutant Hck wherein the Glu93 was replaced by Glutamine (Gln, Q) – that is not expected to disrupt the salt bridge. As shown in Figure 4.9 (*top panel*), expression of wild-type and mutant (E93Q) Hck alone suppressed yeast cell growth indicative of their activity, which can be rescued with the co-expression of Csk. Expectedly, co-expression of both the WT and E93Q Hck with Nef arrested yeast cell growth completely even in the presence of Csk. The levels of strong phosphorylation of cellular proteins on tyrosine Figure 4.9 (*bottom panel*), agree with the growth suppression assay observation. Thus these control experiment results confirm that the formation of intercomplex salt bridge between residue 105 on Nef and residue 93 on the Hck SH3 domain is critical for Nef-mediated activation of Hck.



Saccharomyces cerevisiae was transformed with galactose-inducible expression plasmids for wild-type Hck (WT), the SH3 domain mutant (E93Q), the regulatory kinase Csk and HIV-1 Nef in the combinations as shown. Transformed colonies were cultured in glucose medium to equal densities and spotted onto agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for 4 days at 30°C and imaged on a scanner. Growing yeast colonies appear as dark spots against the translucent agar background (*top panel*). Transformed yeast colonies were also cultured in liquid medium plus galactose to induce protein expression. Cell lysates were separated via SDS-PAGE followed by immunoblotting for protein phosphotyrosine content (pTyr) as well as Nef, Csk, and Hck expression along with actin as a loading control (*bottom panel*).

Figure 4.9. Substitution of Glu with Gln does not affect Nef-induced Hck activation

4.5 SUMMARY AND CONCLUSIONS

Better comprehension and analysis of critical contacts by which Nef binds to cellular kinases and thus mediates its functions form the basis of identifying vulnerable targets for rational drug design and development. The structure of Nef in complex with the tandem SH3-SH2 region of Hck revealed a new interaction at the Nef:SH3 interface not identified in previously reported structures of Nef with the SH3 domain alone. Here we validate the biological significance of the new X-ray crystal structure of HIV-1 Nef in complex with the Hck SH3-SH2 region, and demonstrate in human and yeast cell-based systems, that the novel interaction of Nef R105 with Hck-SH3 E93 discovered in this structure is essential for complex formation with full-length Hck and kinase activation. Mutating the Glu93 residue to Ala on Hck not only compromised interaction between Nef and the full-length kinase in a cell-based BiFC assay but also inhibited Nef-induced Hck activation. These findings strongly suggest that Hck SH3 Glu93 residue is critical to Nef signaling through this kinase pathway. The observation that disruption of this contact leads to inhibition of Nef-mediated SFK activation and subsequent downstream signaling underscores the validity of the Nef:SH3 interface as a rational target for structure-based design and development of new antiretroviral agents.

5.0 DISCUSSION

5.1 SUMMARY AND DISCUSSION OF MAJOR FINDINGS

For my dissertation project, I set forth to develop a novel, cell-based approach to explore the scope of Nef-SH3 interactions. In particular, I planned to focus on the ability of Nef to bind to SH3 domains of a broad range of tyrosine kinases that are predominantly expressed in HIV-1 target cells. The rationale for the focus on kinases related to their key role in HIV target cell activation, and our recent proof of concept studies demonstrating that inhibitors of Nef-induced Src-family kinase activation block Nef-dependent HIV-1 replication.

For this study, I adapted a BiFC assay previously developed in our lab to study Nef homodimerization to probe the interaction of Nef with Tec-family kinases through their SH3 domains in a cellular context. The BiFC assay as has been discussed in chapter 3 is a relatively straightforward assay that allows direct visualization of protein-protein interactions and their subcellular localization within the live cells. However, one disadvantage of this assay relates to the irreversible nature of the reconstituted YFP fluorophore. Since the assay relies on the reconstitution of the functional structure of YFP from juxtaposition of its two non-fluorescent halves, it is often susceptible to false positive signals resulting from very weak interacting partners or even from non-interacting proteins that happen to come close at a distance sufficient

for structural complementation of the YFP. For this reason it was very important to validate the assay using a well-known interaction prior to extending this approach to other kinases.

I validated the reproducibility and reliability of the assay using the Src-family kinase Hck, one of the best characterized SH3 binding partners for Nef. The Hck SH3 domain and full-length Hck both interacted strongly with Nef by BiFC, providing assay validation as well as new evidence for this interaction at the cellular level. In order to verify whether the observed BiFC signal was solely dependent on Nef:SH3 interaction, a mutant of Nef defective for SH3 binding was used. The Nef-2PA mutant interacted with the Hck SH3 domain very weakly, almost 10-fold lower than wild type Nef. It is to be noted that substitution of the conserved Nef proline residues to alanine did not completely kill interaction of Nef with the SH3 domain completely. This observation is consistent with previous findings that besides the polyproline motif, other key determinants and critical motifs on Nef are essential for SH3 domain binding.

Using the BiFC approach, next I went on to show that Nef also interacts with the Tec-family kinases Bmx, Btk, and Itk but not Tec or Txk. Experiments with truncated forms of Bmx, Btk, and Itk lacking the SH2 and kinase domains also produced bright, membrane-localized BiFC signals in presence of Nef, supporting interaction through the SH3 domain. Sequence and structural alignment revealed that Tec and Txk have alanine and arginine, respectively, in place of the Hck SH3 domain RT-loop isoleucine critical for interaction with the Nef hydrophobic pocket, suggesting a possible explanation for their failure to interact. Replacement of this alanine in the Tec SH3 domain with isoleucine restored Nef binding, supporting a role for the RT-loop residue 96 in Nef recognition, and suggesting that Src and Tec-family kinases interact with Nef through very similar mechanisms. The corresponding residues of Hck, Bmx, Btk and Itk at position 96 were mutated to arginine to match the residue on Txk (a non-binder) and even

Fyn, since the structure of Nef in complex with an SFK-SH3 domain was first determined with Fyn carrying a R96I mutation [204]. Replacement of Ile96 to Arg in Hck almost completely blocked interaction with Nef, reminiscent of previous findings where the highly homologous wild type Fyn SH3 (carrying R96) domain had very weak affinity ($K_D > 20 \mu\text{M}$) as opposed to wild type Hck SH3 (carrying I96) domain bound to Nef with the highest affinity reported for an SH3-mediated interaction at that time (K_D 250 nM) [204]. However, replacement of the Btk and Itk residue 96 with Arg interrupted their interaction with Nef but did not completely block it. This finding suggests that other residues in the SH3 domains of these two kinases play an essential role in Nef binding. Nef allelic variants representing all M-group HIV-1 clades interacted strongly with Itk in this assay, demonstrating that Nef-Itk interaction is highly conserved all major HIV-1 subtypes. My findings report for the first time that co-expression with Nef induces Itk autophosphorylation, which is sensitive to inhibition by BMS-509744, a highly selective small molecule inhibitor of Itk activity. BMS-509744 also inhibited both HIV-1 infectivity and replication in a Nef-dependent manner, supporting a functional link between Nef and Itk in the HIV-1 life cycle. Consistent with these observations, this inhibitor had no effect on HIV replication in Jurkat T-cells, which support HIV replication in a Nef-independent manner. These results suggest that Nef directly bridges HIV-1 infection with Itk signaling in T cells.

In the second part of my project, I set out to build upon and validate the biological significance of our recently determined X-ray crystal structure of HIV-1 Nef in complex with the regulatory apparatus (tandem SH3-SH2 unit) of its binding partner and kinase effector, Hck. While key elements of previous Nef structures with isolated SH3 domains are present in our new dimeric Nef:Hck32 complex, the addition of the SH2 domain and SH3-SH2 connector in the

crystal structure revealed remarkable differences. These included a completely reoriented Nef dimer interface with the Hck SH2 domains impinging on the N-terminal region of Nef to stabilize a dimer conformation that exposes Asp123, a residue critical in multiple Nef functions including CD4 and MHC-I downregulation as well as enhancement of viral replication [172, 213, 258]. The Nef:SH2 interactions likely help to position the PxxPxR motif for interaction with the SH3 domain, and also stabilize a functionally important Nef dimer conformation. A novel intercomplex salt bridge between Nef R105 and E93 in the RT loop of the SH3 domain was also identified.

I performed mutagenesis studies to test whether this new contact between Nef and the kinase is required for complex formation and activation of Hck by Nef. Replacement of the glutamic acid residue with alanine (E93A) compromised Nef interaction with full length Hck but not the shorter truncated constructs consisting of SH3 domain alone or SH3-SH2 domains. While the full-length Hck-E93A protein failed to interact with Nef by BiFC signal at eighteen hours post transfection, a partially positive BiFC signal was observed with this mutant thirty-six to forty hours post transfection. The gradual incidence of a BiFC signal with the mutant Hck may be interpreted in two alternate ways. First, it may be so that the mutation of this crucial new contact residue does not abrogate interaction completely, but weakens and delays the interaction between Nef and Hck significantly. Secondly, it is likely that the interaction actually may never take place. The observation of the BiFC signal after long hours of incubation, may be an artifact resulting from the irreversible nature of the assay. However, overall these results indicate that the salt bridge is critical for the complex formation of Nef with full-length Hck.

In the yeast-based assay, the disruption of this newly identified salt bridge by mutagenesis resulted in failure of Nef to activate the full length Hck. However, the basal level of

Hck activity being unequal in case of the wild type and mutant kinases complicates interpretations of the results. It apparently becomes difficult to compare the extent of Nef-induced change in phosphorylation levels of Hck if the basal levels of phosphorylation vary. To address this problem, we switched to constitutively downregulated forms of Hck proteins that carry a YEEI tail and therefore do not require co-expression of Csk for kinase inactivation. This change simplifies the system while avoiding growth suppression when expressed in the absence of Nef. Our previously published studies have shown that Nef interacts with Hck-YEEI in this system through an SH3-dependent mechanism and the structural basis of Hck-YEEI activation by Nef in yeast is indistinguishable from that observed in mammalian cells [327]. Thus failure of Nef to activate mutant Hck-E93A-YEEI clearly implies that Nef is unable to displace and release the SH3:linker interaction which is consistent with its inability bind full-length mutant in Hck the BiFC studies. Taken together, our recently determined Nef:Hck32 structure and results from my study to validate the new intermolecular contact between Nef and Hck strongly support the existence of multiple active conformations for Nef that dependent upon the host cell binding partner to which they are bound.

5.2 OVERALL DISCUSSION

The biological consequences of most of the interactions of Nef with host cell proteins are still poorly understood. Overall, however, our current understanding suggests that Nef binding recruits host signaling proteins to the inner cell membrane and lipid rafts thereby, bringing them into close proximity with other signaling proteins that modulate their catalytic activity. In this way, Nef may prime T cells for activation without any external stimuli. In fact, several studies have shown that Nef alone can trigger T cell activation signaling pathways, inducing a transcriptional program that mimics conditions resulting from T cell activation by an anti-CD3 antibody.

As I discussed in detail in section 1.3.3.2, stimulation of the antigen receptor on an uninfected T-cell leads to downstream effects involving activation of Itk (Figure 5.1 *upper panel*; figure not to scale), culminating in the activation of transcription factors that drive gene expression. Physiological activation of Itk requires three conditions: disruption of autoinhibitory interactions within the kinase; recruitment and localization of Itk molecules at the cell membrane; and transphosphorylation of its activation loop tyrosine (Y511) by an SFK, typically Lck. In an uninfected T-cell, stimulation of the TCR by an antigenic peptide bound to an MHC molecule on an APC, engages CD4 leading to activation of Lck. Activated Lck phosphorylates ITAMs on the TCR and the coreceptor CD3, which in turn recruits ZAP-70 to the ITAMs via their SH2 domains and leads to ZAP-70 activation. Lck also phosphorylates Itk that is recruited to the cell membrane following the activation of PI3K.

As has been discussed previously, Nef binds and activates Hck and other Src-family kinases by SH3 domain displacement from its autoinhibitory interaction with the SH2:kinase

linker region. The observation that Nef induces activation of Itk in the absence of exogenous stimuli raises interesting questions about the mechanism of Itk kinase activation.

Previous work from our group and others has shown that Nef forms dimers in cells, and that dimerization is important for Src-family kinase activation by Nef as well as other Nef functions [98, 213, 258, 361]. These observations suggest that a single Nef dimer may recruit two kinase molecules, resulting in juxtaposition of their kinase domains and subsequent activation by trans-phosphorylation of their kinase domain activation loops. In theory, a Nef dimer could recruit two heterologous kinases, i.e., a Src-family kinase and a Tec family member, like Itk as is in our study. Nef-dependent formation of such a heterodimer could lead to stimulation of both kinases through a similar mechanism. Indeed, physiological activation of Itk requires, in part, trans-phosphorylation by a Src-family kinase in the context of T cell receptor activation as discussed above [14]. Thus it can be reasonably speculated that in a HIV-infected T-cell, in absence of any external stimuli, Nef possibly recruits Itk to the inner cell membrane and allows transphosphorylation of the two bound heterologous kinases resulting in their activation (Figure 5.1 *lower panel*; figure not to scale). In parallel, the Nef-activated SFK may also phosphorylate the CD3 ITAMs and ZAP-70 in an effort to unleash the subsequent downstream signaling steps. Indeed, other recent work from our group has shown that Nef induces activation of ZAP70 in H9 T-cells [327]. Thus in the absence of any external stimuli, HIV-1 Nef may trigger multiple T-cell signaling pathways, including Itk, leading to transcription of viral genes from the HIV-1 LTR. My results also complement the recent observations of Schiralli Lester, *et al.*, which show co-localization of Itk and HIV-1 Gag proteins to the plasma membrane at sites of F-actin accumulation and lipid rafts in HIV-1 infected T cells [290]. They also reported that Itk inhibitors disrupt Itk co-localization with viral Gag as well as virus-like

particle release. Taken together, these results suggest that Nef may play a role in the recruitment of Itk to this subcellular compartment, resulting in kinase activation, which in turn contributes to viral replication and egress.

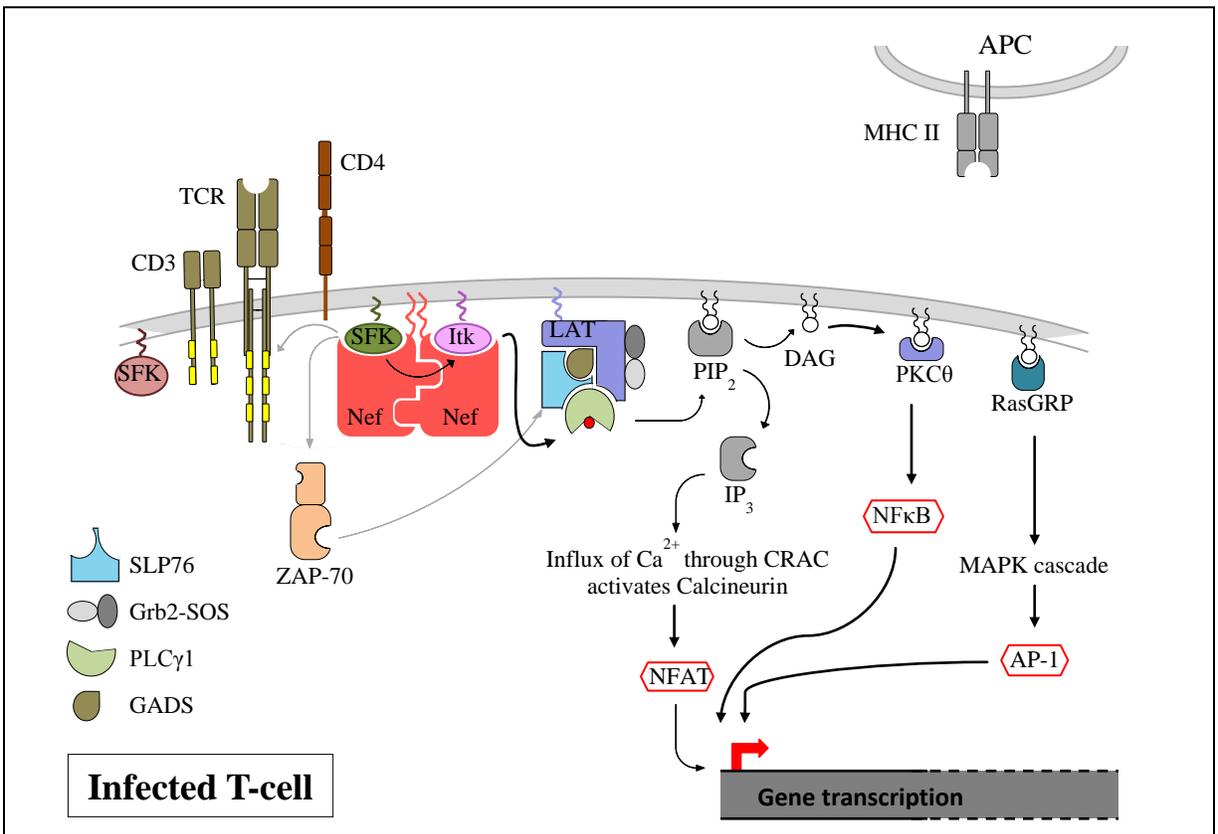
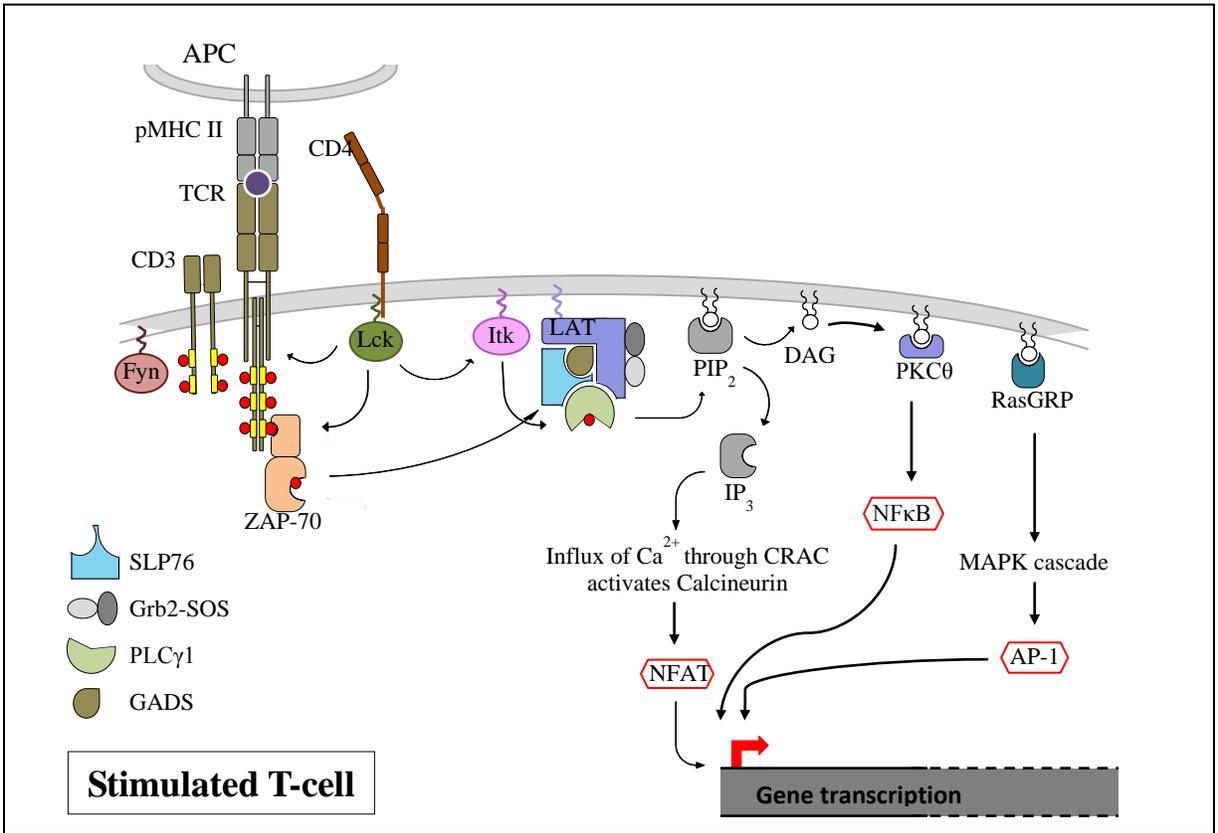


Figure 5.1. Comparison of T-cell signaling pathway in an uninfected stimulated versus HIV-infected cell

5.3 FUTURE DIRECTIONS

Evaluation of the effect of inhibition of Nef-induced Itk activation on Nef-dependent HIV-1 replication in more relevant settings

My current study presents the first evidence that Nef interacts with the Tec family kinase, Itk and that pharmacological inhibition of this interaction blocks HIV-1 replication in a Nef-dependent manner. For my study I used the human 293T cells and the T lymphoblast cell lines CEM-T4 and Jurkat to evaluate the effect of inhibiting Nef-induced Itk activation on the viral life cycle. HIV-1 replication is also Nef-dependent in peripheral blood mononuclear cells (PBMCs) as well as co-culture of T cells with macrophages or endothelial cells [63, 230, 311]. Since these cells when infected with HIV-1 represent a “closer to the real infection” milieu including other natural host cellular players, it would be interesting to evaluate the effect of inhibition of Nef-induced Itk activation in these systems. This can be achieved either by using the Itk inhibitor or siRNA directed against Itk followed by subsequent complementation/rescue studies. Further, it will be even more interesting to evaluate the same in the transgenic mouse model characterized by AIDS-like disease development. This will also allow us to evaluate the efficacy of the Itk inhibitor in vivo, which is an essential finding before trying this inhibitor in higher animal models including non-human primates.

Determination of high resolution crystal structure of Nef in complex with Bmx, Btk and Itk

Cell-based BiFC data from my studies have shown that Nef binds to Bmx, Btk and Itk through their SH3 domains. Further, mutagenesis studies have shown that the residue at position 96

(which corresponds to Ile 96 in Hck) is a critical determinant for Btk and Itk in their ability to bind to Nef. However, replacement of Met96 and Asn96 to Arg on Btk and Itk respectively did not abolish their interaction with Nef completely, indicating that other intermolecular contacts may contribute to the interaction. In light of the importance of Nef:Itk signaling in the viral life cycle, it would be very interesting to determine the high resolution X-ray crystal structure of Nef in complex with either of these kinases. This will not only provide significant insight into the Nef:Tec-family kinase binding interface and help identify additional crucial contacts for kinase activation but will also help delineate the mechanism of Nef-induced Tec-family kinase activation, which is not completely clear even under physiological circumstances.

Bmx is an atypical member of the Tec family of kinases, in that it lacks sequence homology with classical SH3 domains yet possesses an “SH3-like” domain for Nef binding. Surprisingly, despite the poor sequence homology of Bmx with the other members of the Tec family in this region (Figure 3.7), my work shows that there must exist a PxxP/Nef recognition element in Bmx that allows it to bind strongly to Nef. This is reminiscent of the SH2 and phosphotyrosine-binding (PTB) domains – which, in spite of having no structural relationship/similarity, bind specifically to phosphotyrosine-containing peptides, albeit with subtle differences in their selectivity towards recognizing residues adjacent to the binding sites on their targets [291, 297, 356]. Thus, determination of the crystal structure of Nef with the shorter construct of Bmx lacking the SH2 and kinase domains would be really interesting. This, I believe, will highlight novel recognition motifs on the kinase that Nef can bind to even in absence of a classical SH3 domain. Also, this will exemplify and corroborate the idea that two very distinct and dissimilar sequences indeed can fold and form the same functional domain.

Elucidation of mechanism of Nef-induced Itk activation and signaling

Previous studies have reported that Nef-mediated activation of the Erk MAPK pathway and enhancement of HIV-1 transcription in T cells depends on Lck activity [347]. While some studies report that Nef binds to Lck directly, some others suggest an indirect role for Lck [42, 58, 77, 93, 239, 325]. Since Itk is activated through phosphorylation of its activation loop tyrosine Y511 by Lck under normal conditions (e.g., T-cell receptor activation), Nef-mediated binding and recruitment of Itk to the plasma membrane hints at the underlying link between Lck activity and Nef effector functions. This possibility may be explored further in greater detail by using RNAi knockdown studies followed by complementation or rescue experiments.

Based on our logical thought that a single Nef dimer may recruit two different kinase molecules bringing them to close proximity to allow transphosphorylation and subsequent kinase activation, it may be inferred that such a phenomenon, if true, may drive differential signaling pathways simultaneously in an HIV infected cell. Whether such a dimer of heterocomplexes indeed forms in an infected cell can be determined by coupling FRET to BiFC assays. If such dimeric heterocomplexes indeed exist, it will help us delineate specific downstream signaling cascades and identify additional Nef effectors that can be targeted for pharmacological inhibition. Expression of Nef leads to activation of T cells and enhancement of transcription from the LTR promoter. To evaluate the downstream effects of Nef-induced Itk activation, Nef-mediated gene expression profiling may be done in the presence or absence of the Itk inhibitor in cell lines that exhibit Nef-dependent viral replication. This approach will help us identify signaling pathways that are up-/down-regulated by the Nef:Itk interaction.

5.4 CLOSING REMARKS

The year 2014 marks the thirty third anniversary of the HIV/AIDS pandemic – and sadly, despite of intensive research efforts worldwide, this devastating disease cannot be cured. While current therapeutic strategies have transformed HIV infection from a terminal to a chronic illness for many patients, lack of an effective vaccine and emergence of drug-resistant strains mark HIV/AIDS as one of the most challenging public health threats even today. This underscores the urgency in finding additional targets for development of newer drugs. Nef represents an exciting potential drug target because of its important role in HIV-1 pathogenesis and interactions with host cell signaling molecules. In an effort to identify novel interactions of Nef within an infected cell, in my dissertation I have provided the first direct evidence for Nef interacting with three cytoplasmic tyrosine kinases that are critical to HIV target cell signaling. In particular, I have shown that pharmacological inhibition of Nef-mediated Itk activation blocks Nef-dependent enhancement of HIV-1 replication and infectivity in human cells. Based on my current study and observations, I believe, targeting the Nef:Itk signaling axis in an infected cell may represent a broadly useful strategy to combat HIV/AIDS. I earnestly hope, my findings will contribute to the field of HIV research, advance our understanding of the varied viral strategies that are undertaken by the virus to ensure a productive infection and also possibly lead us towards newer avenues of HIV therapeutics.

ABBREVIATIONS

AIDS.....	Acquired immunodeficiency syndrome
AP-1/2.....	Activator protein 1/2
APC.....	Antigen presenting cell
BiFC.....	Bimolecular fluorescence complementation
BMS.....	Bristol-Myers Squibb
BSA.....	Bovine Serum Albumin
C-.....	Carboxy
CA.....	Capsid
CCR/CXCR.....	Chemokine receptors
CD4.....	Cluster of differentiation 4
cDNA.....	complementary DNA
COP-1.....	Coat protein 1
Csk.....	c-Src tyrosine kinase
CypA.....	Cyclophilin A
DFP.....	Diphenylfuranopyrimidine
DMSO.....	Dimethyl sulfoxide
DQBS.....	Diaminoquinoxaline benzenesulfonamide
ER.....	Endoplasmic reticulum
FAK.....	Focal adhesion kinase
FWD.....	Forward
GEF.....	GTP exchange factor
GFP.....	Green fluorescent protein
GSK3 β	Glycogen synthase kinase-3 beta
HAART.....	Highly active antiretroviral therapy
HIV.....	Human immunodeficiency virus
HLA.....	Human leucocyte antigen
HTLV.....	Human T-lymphotrophic virus
IC ₅₀	half maximal inhibitory concentration
IF.....	Immunofluorescence
IFN.....	Interferon
IgG.....	Immunoglobulin G
IL-2.....	Interleukin-2
IN.....	Integrase

ITAM..... Immunoreceptor tyrosine-based activation motif
 JAK..... Janus kinase
 JNK..... c-Jun N terminal kinase
 kb..... kilobase
 K_D dissociation constant
 kDa..... kilo-daltons
 LAT..... Linker of activated T cells
 LTR..... Long terminal repeat
 M..... Molar
 MA..... Matrix
 MAPK..... Mitogen-activated protein kinase
 MHC..... Major histocompatibility complex
 MIP-1 α Macrophage inflammatory protein-1 alpha
 ml..... milliliter
 mM..... millimolar
 mRNA..... messenger RNA
 N..... Normal
 N-..... Amino
 N/NRTI..... non-/nucleoside reverse transcriptase inhibitor
 NC..... Nucleocapsid
 NFAT..... Nuclear factor of activated T cells
 NF κ B..... Nuclear factor kappa-light-chain-enhancer of activated B cells
 NK..... Natural killer
 nm..... nanometer
 nM..... nanomolar
 ORF..... open reading frame
 P..... Proline
 PACS-2..... Phosphofurin acidic cluster sorting protein 2
 PAK2..... p21 protein- activated kinase 2
 PBMC..... Peripheral blood mononuclear cells
 Pg..... picograms
 PH..... Pleckstrin homology
 PI..... protease inhibitor
 PI3K..... phosphoinositide 3- kinase
 PIC..... preintegration complex
 PIP3..... phosphatidylinositol (3,4,5) triphosphate
 PKC..... Protein kinase C
 PLC γ Phospholipase C gamma
 PPII..... polyproline type II
 PR..... Protease
 PTK..... protein tyrosine kinase
 pTyr/pY..... phosphotyrosine
 R..... Arginine

REV..... Reverse
 RT..... Reverse transcriptase
 sAIDS..... simian AIDS
 SEM..... Standard error of mean
 SFK..... Src family kinase
 SH3..... Src-homology 3
 SIV..... Simian immunodeficiency virus
 ssRNA..... single stranded RNA
 STAT..... Signal transducer and activator of transcription
 SU..... Surface
 TCR..... T cell receptor
 TGN..... trans-Golgi network
 TH..... Tec homology
 TM..... transmembrane
 U..... Units
 VC..... C-terminal fragment of Venus
 VN..... N-terminal fragment of Venus
 vs..... versus
 WT..... wild type
 XLA..... X-linked agammaglobulinemia
 YFP..... Yellow fluorescence protein
 α- anti
 ΔNef..... delta Nef/defective Nef
 32..... SH3-SH2 domain
 μg..... microgram
 μl..... microliter
 μM..... micromolar
 Å..... angstrom
 °C..... degree Celsius

BIBLIOGRAPHY

1. Pneumocystis pneumonia--Los Angeles. *MMWR Morb Mortal Wkly Rep* 1981; **30(21)**:250-252.
2. Prevention of acquired immune deficiency syndrome (AIDS): report of inter-agency recommendations. *MMWR Morb Mortal Wkly Rep* 1983; **32(8)**:101-103.
3. Pneumocystis pneumonia--Los Angeles. 1981. *MMWR Morb Mortal Wkly Rep* 1996; **45(34)**:729-733.
4. Global Report: UNAIDS report on the global AIDS epidemic 2013. In: 2013.
5. U.S. FDA Approves Gilead's Once-Daily Single Tablet HIV-1 Regimen Complera® for Patients Switching from a Stable Regimen. (<http://www.gilead.com/news/press-releases/2013/12/us-fda-approves-gileads-oncedaily-single-tablet-hiv1-regimen-complera-for-patients-switching-from-a-stable-regimen>). In: 2013.
6. Adams JA. Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? *Biochemistry* 2003; **42(3)**:601-607.
7. Ahmad N, Venkatesan S. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. *Science* 1988; **241(4872)**:1481-1485.
8. Aiken C, Konner J, Landau NR, Lenburg ME, Trono D. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* 1994; **76(5)**:853-864.
9. Aiken C, Trono D. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Virol* 1995; **69(8)**:5048-5056.
10. Ali A, Wang J, Nathans RS, Cao H, Sharova N, Stevenson M, *et al.* Synthesis and structure-activity relationship studies of HIV-1 virion infectivity factor (Vif) inhibitors that block viral replication. *ChemMedChem* 2012; **7(7)**:1217-1229.
11. Aloia RC, Tian H, Jensen FC. Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. *Proc Natl Acad Sci U S A* 1993; **90(11)**:5181-5185.

12. Alvarado JJ, Betts L, Moroco JA, Smithgall TE, Yeh JI. Crystal structure of the Src-family kinase Hck SH3-SH2-linker regulatory region supports an SH3-dominant activation mechanism. *J Biol Chem* 2010; **285**:35455-35461.
13. Andreotti AH, Bunnell SC, Feng S, Berg LJ, Schreiber SL. Regulatory intramolecular association in a tyrosine kinase of the Tec family. *Nature* 1997; **385**:93-97.
14. Andreotti AH, Schwartzberg PL, Joseph RE, Berg LJ. T-cell signaling regulated by the Tec family kinase, Itk. *Cold Spring Harb Perspect Biol* 2010; **2(7)**:a002287.
15. Arendt CW, Littman DR. HIV: master of the host cell. *Genome Biol* 2001; **2(11)**:REVIEWS1030.
16. Arganaraz ER, Schindler M, Kirchhoff F, Cortes MJ, Lama J. Enhanced CD4 down-modulation by late stage HIV-1 nef alleles is associated with increased Env incorporation and viral replication. *J Biol Chem* 2003; **278(36)**:33912-33919.
17. Arien KK, Vanham G, Arts EJ. Is HIV-1 evolving to a less virulent form in humans? *Nat Rev Microbiol* 2007; **5(2)**:141-151.
18. Arold S, Franken P, Strub MP, Hoh F, Benichou S, Benarous R, *et al.* 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* 1997; **5(10)**:1361-1372.
19. Arold S, O'Brien R, Franken P, Strub MP, Hoh F, Dumas C, *et al.* RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef. *Biochemistry* 1998; **37(42)**:14683-14691.
20. Arold ST, Baur AS. Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein. *Trends Biochem Sci* 2001; **26(6)**:356-363.
21. Arold ST, Ulmer TS, Mulhern TD, Werner JM, Ladbury JE, Campbell ID, *et al.* The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases. *J Biol Chem* 2001; **276(20)**:17199-17205.
22. Arthur LO, Bess JW, Jr., Sowder RC, Benveniste RE, Mann DL, Chermann JC, *et al.* Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* 1992; **258(5090)**:1935-1938.
23. Atherly LO, Brehm MA, Welsh RM, Berg LJ. Tec kinases Itk and Rlk are required for CD8+ T cell responses to virus infection independent of their role in CD4+ T cell help. *J Immunol* 2006; **176(3)**:1571-1581.
24. Atkins KM, Thomas L, Youker RT, Harriff MJ, Pissani F, You H, *et al.* 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* 2008; **283(17)**:11772-11784.

25. August A, Sadra A, Dupont B, Hanafusa H. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. *Proc Natl Acad Sci U S A* 1997; **94(21)**:11227-11232.
26. Bachmann MF, Littman DR, Liao XC. Antiviral immune responses in Itk-deficient mice. *J Virol* 1997; **71(10)**:7253-7257.
27. Baden LR, Dolin R. The road to an effective HIV vaccine. *N Engl J Med* 2012; **366(14)**:1343-1344.
28. Balog K, Minarovits J. Nef: a pleiotropic modulator of primate lentivirus infectivity and pathogenesis. *Acta Microbiol Immunol Hung* 2006; **53(1)**:51-75.
29. Barker E, Mackewicz CE, Reyes-Teran G, Sato A, Stranford SA, Fujimura SH, *et al.* Virological and immunological features of long-term human immunodeficiency virus-infected individuals who have remained asymptomatic compared with those who have progressed to acquired immunodeficiency syndrome. *Blood* 1998; **92(9)**:3105-3114.
30. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983; **220(4599)**:868-871.
31. Bartel DP, Zapp ML, Green MR, Szostak JW. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. *Cell* 1991; **67(3)**:529-536.
32. Baur AS, Sawai ET, Dazin P, Fantl WJ, Cheng-Mayer C, Peterlin BM. HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity* 1994; **1(5)**:373-384.
33. Bentham M, Mazaleyrat S, Harris M. Role of myristoylation and N-terminal basic residues in membrane association of the human immunodeficiency virus type 1 Nef protein. *J Gen Virol* 2006; **87(Pt 3)**:563-571.
34. Bernardini C, Maggiolo F. Triple-combination rilpivirine, emtricitabine, and tenofovir (Complera/Eviplera) in the treatment of HIV infection. *Patient Prefer Adherence* 2013; **7**:531-542.
35. Betzi S, Restouin A, Opi S, Arold ST, Parrot I, Guerlesquin F, *et al.* Protein protein interaction inhibition (2P2I) combining high throughput and virtual screening: Application to the HIV-1 Nef protein. *Proc Natl Acad Sci U S A* 2007; **104(49)**:19256-19261.

36. Blagoveshchenskaya AD, Thomas L, Feliciangeli SF, Hung CH, Thomas G. HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. *Cell* 2002; **111(6)**:853-866.
37. Bradshaw JM. The Src, Syk, and Tec family kinases: distinct types of molecular switches. *Cell Signal* 2010; **22(8)**:1175-1184.
38. Brazin KN, Fulton DB, Andreotti AH. A specific intermolecular association between the regulatory domains of a Tec family kinase. *J Mol Biol* 2000; **302(3)**:607-623.
39. Breitenlechner CB, Kairies NA, Honold K, Scheiblich S, Koll H, Greiter E, *et al.* Crystal structures of active SRC kinase domain complexes. *J Mol Biol* 2005; **353(2)**:222-231.
40. Bresnahan PA, Yonemoto W, Ferrell S, Williams-Herman D, Geleziunas R, Greene WC. A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. *Curr Biol* 1998; **8(22)**:1235-1238.
41. Breuer S, Gerlach H, Kolaric B, Urbanke C, Opitz N, Geyer M. Biochemical indication for myristoylation-dependent conformational changes in HIV-1 Nef. *Biochemistry* 2006; **45(7)**:2339-2349.
42. Briese L, Preusser A, Willbold D. Mapping the binding site of full length HIV-1 Nef on human Lck SH3 by NMR spectroscopy. *J Biomed Sci* 2005; **12(3)**:451-456.
43. Briggs JA, Krausslich HG. The molecular architecture of HIV. *J Mol Biol* 2011; **410(4)**:491-500.
44. Briggs SD, Lerner EC, Smithgall TE. Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo. *Biochemistry* 2000; **39**:489-495.
45. Briggs SD, Scholtz B, Jacque JM, Swingler S, Stevenson M, Smithgall TE. HIV-1 Nef promotes survival of myeloid cells by a Stat3-dependent pathway. *J Biol Chem* 2001; **276(27)**:25605-25611.
46. Briggs SD, Sharkey M, Stevenson M, Smithgall TE. SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1. *J Biol Chem* 1997; **272**:17899-17902.
47. Briggs SD, Smithgall TE. SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in rat-2 fibroblasts. *J Biol Chem* 1999; **274**:26579-26583.
48. Brown K, Long JM, Vial SC, Dedi N, Dunster NJ, Renwick SB, *et al.* Crystal structures of interleukin-2 tyrosine kinase and their implications for the design of selective inhibitors. *J Biol Chem* 2004; **279(18)**:18727-18732.

49. Brown MT, Cooper JA. Regulation, substrates, and functions of Src. *Biochim Biophys Acta* 1996; **1287**:121-149.
50. Carl S, Daniels R, Iafraite AJ, Easterbrook P, Greenough TC, Skowronski J, *et al.* Partial "repair" of defective NEF genes in a long-term nonprogressor with human immunodeficiency virus type 1 infection. *J Infect Dis* 2000; **181(1)**:132-140.
51. Chakrabarti LA, Metzner KJ, Ivanovic T, Cheng H, Louis-Virelizier J, Connor RI, *et al.* A truncated form of Nef selected during pathogenic reversion of simian immunodeficiency virus SIVmac239Deltanef increases viral replication. *J Virol* 2003; **77(2)**:1245-1256.
52. Chalifoux LV, Ringler DJ, King NW, Sehgal PK, Desrosiers RC, Daniel MD, *et al.* Lymphadenopathy in macaques experimentally infected with the simian immunodeficiency virus (SIV). *Am J Pathol* 1987; **128(1)**:104-110.
53. Chan PA, Kantor R. Transmitted drug resistance in nonsubtype B HIV-1 infection. *HIV Ther* 2009; **3(5)**:447-465.
54. Chang AH, O'Shaughnessy MV, Jirik FR. Hck SH3 domain-dependent abrogation of Nef-induced class 1 MHC down-regulation. *Eur J Immunol* 2001; **31(8)**:2382-2387.
55. Chaudhry A, Das SR, Hussain A, Mayor S, George A, Bal V, *et al.* The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. *J Immunol* 2005; **175(7)**:4566-4574.
56. Chaudhry A, Das SR, Jameel S, George A, Bal V, Mayor S, *et al.* HIV-1 Nef induces a Rab11-dependent routing of endocytosed immune costimulatory proteins CD80 and CD86 to the Golgi. *Traffic* 2008; **9(11)**:1925-1935.
57. Chaudhuri R, Mattera R, Lindwasser OW, Robinson MS, Bonifacino JS. A basic patch on alpha-adaptin is required for binding of human immunodeficiency virus type 1 Nef and cooperative assembly of a CD4-Nef-AP-2 complex. *J Virol* 2009; **83(6)**:2518-2530.
58. Cheng H, Hoxie JP, Parks WP. 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* 1999; **264(1)**:5-15.
59. Ching KA, Grasis JA, Tailor P, Kawakami Y, Kawakami T, Tsoukas CD. TCR/CD3-Induced activation and binding of Emt/Itk to linker of activated T cell complexes: requirement for the Src homology 2 domain. *J Immunol* 2000; **165(1)**:256-262.
60. Ching KA, Kawakami Y, Kawakami T, Tsoukas CD. Emt/Itk associates with activated TCR complexes: role of the pleckstrin homology domain. *J Immunol* 1999; **163(11)**:6006-6013.

61. Choi HJ, Smithgall TE. Conserved residues in the HIV-1 Nef hydrophobic pocket are essential for recruitment and activation of the Hck tyrosine kinase. *J Mol Biol* 2004; **343(5)**:1255-1268.
62. Choi H-J, Smithgall TE. HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-X_L expression in an Erk-dependent manner. *J Biol Chem* 2004; **279**:51668-51696.
63. Choi J, Walker J, Boichuk S, Kirkiles-Smith N, Torpey N, Pober JS, *et al.* Human endothelial cells enhance human immunodeficiency virus type 1 replication in CD4⁺ T cells in a Nef-dependent manner in vitro and in vivo. *J Virol* 2005; **79(1)**:264-276.
64. Chong YP, Chan AS, Chan KC, Williamson NA, Lerner EC, Smithgall TE, *et al.* C-terminal Src kinase-homologous kinase (CHK), a unique inhibitor inactivating multiple active conformations of Src family tyrosine kinases. *J Biol Chem* 2006; **281(44)**:32988-32999.
65. Chong YP, Ia KK, Mulhern TD, Cheng HC. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. *Biochim Biophys Acta* 2005; **1754(1-2)**:210-220.
66. Chu PC, Wu J, Liao XC, Pardo J, Zhao H, Li C, *et al.* A novel role for p21-activated protein kinase 2 in T cell activation. *J Immunol* 2004; **172(12)**:7324-7334.
67. Chutiwitoonchai N, Hiyoshi M, Mwimanzi P, Ueno T, Adachi A, Ode H, *et al.* The identification of a small molecule compound that reduces HIV-1 Nef-mediated viral infectivity enhancement. *PLoS One* 2011; **6(11)**:e27696.
68. Clapham PR, McKnight A. HIV-1 receptors and cell tropism. *Br Med Bull* 2001; **58**:43-59.
69. Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, *et al.* Human immunodeficiency viruses. *Science* 1986; **232(4751)**:697.
70. Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, *et al.* What to call the AIDS virus? *Nature* 1986; **321(6065)**:10.
71. Cohen EA, Dehni G, Sodroski JG, Haseltine WA. Human immunodeficiency virus vpr product is a virion-associated regulatory protein. *J Virol* 1990; **64(6)**:3097-3099.
72. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, *et al.* The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 1999; **10(6)**:661-671.
73. Cohen GB, Ren R, Baltimore D. Modular binding domains in signal transduction proteins. *Cell* 1995; **80**:237-248.

74. Cohen MS, Gay CL, Busch MP, Hecht FM. The detection of acute HIV infection. *J Infect Dis* 2010; **202 Suppl 2**:S270-S277.
75. Coleman SH, Hitchin D, Noviello CM, Guatelli JC. HIV-1 Nef stabilizes AP-1 on membranes without inducing ARF1-independent de novo attachment. *Virology* 2006; **345(1)**:148-155.
76. Collette Y, Arold S, Picard C, Janvier K, Benichou S, Benarous R, *et al.* HIV-2 and SIV nef proteins target different Src family SH3 domains than does HIV-1 Nef because of a triple amino acid substitution. *J Biol Chem* 2000; **275(6)**:4171-4176.
77. Collette Y, Dutartre H, Benziane A, Ramos-Morales F, Benarous R, Harris M, *et al.* Physical and functional interaction of *Nef* with Lck - HIV-1 Nef-induced T-cell signaling defects. *J Biol Chem* 1996; **271**:6333-6341.
78. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 1998; **391(6665)**:397-401.
79. Cooper JA, Howell B. The when and how of *src* regulation. *Cell* 1993; **73**:1051-1054.
80. Corey SJ, Anderson SM. Src-related protein tyrosine kinases in hematopoiesis. *Blood* 1999; **93(1)**:1-14.
81. Courtneidge SA, Smith AE. Polyoma virus transforming protein associates with the product of the c-src cellular gene. *Nature* 1983; **303(5916)**:435-439.
82. Cowan-Jacob SW, Fendrich G, Manley PW, Jahnke W, Fabbro D, Liebetanz J, *et al.* The Crystal Structure of a c-Src Complex in an Active Conformation Suggests Possible Steps in c-Src Activation. *Structure (Camb)* 2005; **13(6)**:861-871.
83. Craig HM, Reddy TR, Riggs NL, Dao PP, Guatelli JC. 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* 2000; **271(1)**:9-17.
84. D'Souza MP, Cairns JS, Plaeger SF. Current evidence and future directions for targeting HIV entry: therapeutic and prophylactic strategies. *JAMA* 2000; **284(2)**:215-222.
85. Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 1992; **258(5090)**:1938-1941.
86. Das SR, Jameel S. Biology of the HIV Nef protein. *Indian J Med Res* 2005; **121(4)**:315-332.
87. daSilva LL, Sougrat R, Burgos PV, Janvier K, Mattera R, Bonifacino JS. Human immunodeficiency virus type 1 Nef protein targets CD4 to the multivesicular body pathway. *J Virol* 2009; **83(13)**:6578-6590.

88. de SE, Stumpf MP. HIV and the CCR5-Delta32 resistance allele. *FEMS Microbiol Lett* 2004; **241(1)**:1-12.
89. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, *et al.* Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 1995; **270**:988-991.
90. Debnath J, Chamorro M, Czar MJ, Schaeffer EM, Lenardo MJ, Varmus HE, *et al.* rtk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. *Mol Cell Biol* 1999; **19(2)**:1498-1507.
91. Dikeakos JD, Atkins KM, Thomas L, Emert-Sedlak L, Byeon IJ, Jung J, *et al.* Small molecule inhibition of HIV-1-induced MHC-I down-regulation identifies a temporally regulated switch in Nef action. *Mol Biol Cell* 2010; **21(19)**:3279-3292.
92. Durier C, Launay O, Meiffredy V, Saidi Y, Salmon D, Levy Y, *et al.* Clinical safety of HIV lipopeptides used as vaccines in healthy volunteers and HIV-infected adults. *AIDS* 2006; **20(7)**:1039-1049.
93. Dutartre H, Harris M, Olive D, Collette Y. The human immunodeficiency virus type 1 Nef protein binds the Src- related tyrosine kinase Lck SH2 domain through a novel phosphotyrosine independent mechanism. *Virology* 1998; **247(2)**:200-211.
94. Eck MJ, Atwell SK, Shoelson SE, Harrison SC. Structure of the regulatory domains of the Src-family tyrosine kinase Lck. *Nature* 1994; **368**:764-769.
95. Ekman N, Arighi E, Rajantie I, Saharinen P, Ristimaki A, Silvennoinen O, *et al.* The Bmx tyrosine kinase is activated by IL-3 and G-CSF in a PI-3K dependent manner. *Oncogene* 2000; **19(36)**:4151-4158.
96. Elbahesh H, Cline T, Baranovich T, Govorkova EA, Schultz-Cherry S, Russell CJ. Novel Roles of Focal Adhesion Kinase in Cytoplasmic Entry and Replication of Influenza A Viruses. *J Virol* 2014.
97. Emert-Sedlak L, Kodama T, Lerner EC, Dai W, Foster C, Day BW, *et al.* Chemical library screens targeting an HIV-1 accessory factor/host cell kinase complex identify novel antiretroviral compounds. *ACS Chem Biol* 2009; **4(11)**:939-947.
98. Emert-Sedlak LA, Narute P, Shu ST, Poe JA, Shi H, Yanamala N, *et al.* Effector Kinase Coupling Enables High-Throughput Screens for Direct HIV-1 Nef Antagonists with Antiretroviral Activity. *Chem Biol* 2013; **20(1)**:82-91.
99. Engen JR, Wales TE, Hochrein JM, Meyn MA, III, Banu OS, Bahar I, *et al.* Structure and dynamic regulation of Src-family kinases. *Cell Mol Life Sci* 2008; **65**:3058-3073.
100. Erpel T, Superti-Furga G, Courtneidge SA. Mutational analysis of the Src SH3 domain: The same residues of the ligand binding surface are important for intra- and intermolecular interactions. *EMBO J* 1995; **14**:963-975.

101. Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity* 2002; **16(4)**:493-497.
102. Fackler OT, Kienzle N, Kremmer E, Boese A, Schramm B, Klimkait T, *et al.* Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization. *Eur J Biochem* 1997; **247(3)**:843-851.
103. Fackler OT, Lu X, Frost JA, Geyer M, Jiang B, Luo W, *et al.* p21-activated kinase 1 plays a critical role in cellular activation by Nef. *Mol Cell Biol* 2000; **20(7)**:2619-2627.
104. Fackler OT, Luo W, Geyer M, Alberts AS, Peterlin BM. Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. *Mol Cell* 1999; **3(6)**:729-739.
105. FDA Center for Drug Evaluation and Research (CDER). Complera (Emtricitabine, Rilpivirine, Tenofovir Disoproxil Fumarate) Tablets (<http://www.fda.gov/safety/medwatch/safetyinformation/ucm318602.htm>). In: 2013.
106. Federico M, Percario Z, Olivetta E, Fiorucci G, Muratori C, Micheli A, *et al.* HIV-1 Nef activates STAT1 in human monocytes/macrophages through the release of soluble factors. *Blood* 2001; **98(9)**:2752-2761.
107. Feinberg MB, Baltimore D, Frankel AD. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc Natl Acad Sci U S A* 1991; **88(9)**:4045-4049.
108. Feng S, Holland EC. HIV-1 tat trans-activation requires the loop sequence within tar. *Nature* 1988; **334(6178)**:165-167.
109. Florio M, Wilson LK, Trager JB, Thorner J, Martin GS. Aberrant protein phosphorylation at tyrosine is responsible for the growth-inhibitory action of pp60v-src expressed in the yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* 1994; **5(3)**:283-296.
110. Franke EK, Yuan HE, Luban J. Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* 1994; **372(6504)**:359-362.
111. Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* 1998; **67**:1-25.
112. Franken P, Arold S, Padilla A, Bodeus M, Hoh F, Strub MP, *et al.* HIV-1 Nef protein: purification, crystallizations, and preliminary X-ray diffraction studies. *Protein Sci* 1997; **6(12)**:2681-2683.

113. Freed EO, Englund G, Martin MA. Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. *J Virol* 1995; **69(6)**:3949-3954.
114. Gallay P, Swingler S, Song J, Bushman F, Trono D. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* 1995; **83(4)**:569-576.
115. Gallo R, Wong-Staal F, Montagnier L, Haseltine WA, Yoshida M. HIV/HTLV gene nomenclature. *Nature* 1988; **333(6173)**:504.
116. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 1984; **224(4648)**:500-503.
117. Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, *et al.* Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* 1983; **220(4599)**:865-867.
118. Garcia JV, Miller AD. Downregulation of cell surface CD4 by nef. *Res Virol* 1992; **143(1)**:52-55.
119. Gardner MB. The history of simian AIDS. *J Med Primatol* 1996; **25(3)**:148-157.
120. Gaur P, Ranjan P, Sharma S, Patel JR, Bowzard JB, Rahman SK, *et al.* Influenza A virus neuraminidase protein enhances cell survival through interaction with carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) protein. *J Biol Chem* 2012; **287(18)**:15109-15117.
121. Geffin R, Wolf D, Muller R, Hill MD, Stellwag E, Freitag M, *et al.* Functional and structural defects in HIV type 1 nef genes derived from pediatric long-term survivors. *AIDS Res Hum Retroviruses* 2000; **16(17)**:1855-1868.
122. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 2000; **100(5)**:587-597.
123. Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC. HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* 2001; **410(6830)**:834-838.
124. Georgiev VS, Western KA, McGowan JJ. *National Institute of Allergy and Infectious Diseases, NIH*. New York: Humana Press; 2008.
125. Gervaix A, West D, Leoni LM, Richman DD, Wong-Staal F, Corbeil J. A new reporter cell line to monitor HIV infection and drug susceptibility in vitro. *Proc Natl Acad Sci U S A* 1997; **94(9)**:4653-4658.

126. Geyer M, Fackler OT, Peterlin BM. Structure--function relationships in HIV-1 Nef. *EMBO Rep* 2001; **2(7)**:580-585.
127. Geyer M, Peterlin BM. Domain assembly, surface accessibility and sequence conservation in full length HIV-1 Nef. *FEBS Lett* 2001; **496(2-3)**:91-95.
128. Gheysen D, Jacobs E, de FF, Thiriart C, Francotte M, Thines D, *et al.* Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* 1989; **59(1)**:103-112.
129. Glover HR, Brewster CE, Dilworth SM. Association between src-kinases and the polyoma virus oncogene middle T-antigen requires PP2A and a specific sequence motif. *Oncogene* 1999; **18(30)**:4364-4370.
130. Gomez C, Hope TJ. The ins and outs of HIV replication. *Cell Microbiol* 2005; **7(5)**:621-626.
131. Grasis JA, Guimond DM, Cam NR, Herman K, Magotti P, Lambris JD, *et al.* In vivo significance of ITK-SLP-76 interaction in cytokine production. *Mol Cell Biol* 2010; **30(14)**:3596-3609.
132. Grasis JA, Tsoukas CD. Itk: the rheostat of the T cell response. *J Signal Transduct* 2011; **2011**:297868.
133. Greenberg M, DeTulleo L, Rapoport I, Skowronski J, Kirchhausen T. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. *Curr Biol* 1998; **8(22)**:1239-1242.
134. Greene WC, Debyser Z, Ikeda Y, Freed EO, Stephens E, Yonemoto W, *et al.* Novel targets for HIV therapy. *Antiviral Res* 2008; **80(3)**:251-265.
135. Greenway A, Azad A, McPhee D. Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines. *J Virol* 1995; **69(3)**:1842-1850.
136. Greenway A, Azad A, Mills J, McPhee D. Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. *J Virol* 1996; **70**:6701-6708.
137. Grzesiek S, Bax A, Clore GM, Gronenborn AM, Hu J-S, Kaufman J, *et al.* 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* 1996; **3**:340-345.
138. Hagiwara K, Murakami T, Xue G, Shimizu Y, Takeda E, Hashimoto Y, *et al.* Identification of a novel Vpr-binding compound that inhibits HIV-1 multiplication in macrophages by chemical array. *Biochem Biophys Res Commun* 2010; **403(1)**:40-45.

139. Haire RN, Ohta Y, Lewis JE, Fu SM, Kroisel P, Litman GW. TXK, a novel human tyrosine kinase expressed in T cells shares sequence identity with Tec family kinases and maps to 4p12. *Hum Mol Genet* 1994; **3(6)**:897-901.
140. Haller C, Rauch S, Fackler OT. HIV-1 Nef employs two distinct mechanisms to modulate Lck subcellular localization and TCR induced actin remodeling. *PLoS One* 2007; **2(11)**:e1212.
141. Hanks SK, Quinn AM, Hunter T. The protein kinase family: Conserved features and deduced phylogeny of the catalytic domain. *Science* 1988; **241**:42-52.
142. Hanna Z, Kay DG, Cool M, Jothy S, Rebai N, Jolicoeur P. Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease. *J Virol* 1998; **72(1)**:121-132.
143. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* 1998; **95(2)**:163-175.
144. Hanna Z, Priceputu E, Kay DG, Poudrier J, Chrobak P, Jolicoeur P. In vivo mutational analysis of the N-terminal region of HIV-1 Nef reveals critical motifs for the development of an AIDS-like disease in CD4C/HIV transgenic mice. *Virology* 2004; **327(2)**:273-286.
145. Hanna Z, Weng X, Kay DG, Poudrier J, Lowell C, Jolicoeur P. 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* 2001; **75(19)**:9378-9392.
146. Haqqani AA, Tilton JC. Entry inhibitors and their use in the treatment of HIV-1 infection. *Antiviral Res* 2013; **98(2)**:158-170.
147. Harlan JE, Hajduk PJ, Yoon HS, Fesik SW. Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature* 1994; **371(6493)**:168-170.
148. Harris M. The role of myristoylation in the interactions between human immunodeficiency virus type I Nef and cellular proteins. *Biochem Soc Trans* 1995; **23(3)**:557-561.
149. Harris M. From negative factor to a critical role in virus pathogenesis: the changing fortunes of Nef. *J Gen Virol* 1996; **77 (Pt 10)**:2379-2392.
150. Harris M. HIV: a new role for Nef in the spread of HIV. *Curr Biol* 1999; **9(12)**:R459-R461.
151. Harris MP, Neil JC. Myristoylation-dependent binding of HIV-1 Nef to CD4. *J Mol Biol* 1994; **241(2)**:136-142.

152. Harrison GP, Lever AM. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. *J Virol* 1992; **66**(7):4144-4153.
153. Hawkins J, Marcy A. Characterization of Itk tyrosine kinase: contribution of noncatalytic domains to enzymatic activity. *Protein Expr Purif* 2001; **22**(2):211-219.
154. He J, Choe S, Walker R, Di MP, Morgan DO, Landau NR. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J Virol* 1995; **69**(11):6705-6711.
155. He JC, Husain M, Sunamoto M, D'Agati VD, Klotman ME, Iyengar R, *et al.* Nef stimulates proliferation of glomerular podocytes through activation of Src-dependent Stat3 and MAPK1,2 pathways. *J Clin Invest* 2004; **114**(5):643-651.
156. Heinzinger NK, Bukinsky MI, Haggerty SA, Ragland AM, Kewalramani V, Lee MA, *et al.* The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* 1994; **91**(15):7311-7315.
157. Hemelaar J, Gouws E, Ghys PD, Osmanov S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* 2006; **20**(16):W13-W23.
158. Herna RG, Saksela K. Interactions of HIV-1 NEF with cellular signal transducing proteins. *Front Biosci* 2000; **5**:D268-D283.
159. Heyeck SD, Berg LJ. Developmental regulation of a murine T-cell-specific tyrosine kinase gene, Tsk. *Proc Natl Acad Sci U S A* 1993; **90**(2):669-673.
160. Heyeck SD, Wilcox HM, Bunnell SC, Berg LJ. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J Biol Chem* 1997; **272**(40):25401-25408.
161. Hodge DR, Dunn KJ, Pei GK, Chakrabarty MK, Heidecker G, Lautenberger JA, *et al.* Binding of c-Raf1 kinase to a conserved acidic sequence within the carboxyl-terminal region of the HIV-1 Nef protein. *J Biol Chem* 1998; **273**(25):15727-15733.
162. Hope T, Trono D. Structure, Expression and Regulation of the HIV genome (<http://hivinsite.ucsf.edu/InSite-KB-ref.jsp?page=kb-02-01-02&rf=12>). In: HIV InSite; UCSF Medical Center; 2000.
163. Hu CD, Chinenov Y, Kerppola TK. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol Cell* 2002; **9**(4):789-798.

164. Hu CD, Grinberg AV, Kerppola TK. Visualization of protein interactions in living cells using bimolecular fluorescence complementation (BiFC) analysis. *Curr Protoc Cell Biol* 2006; **Chapter 21**:Unit.
165. Huang W, Zuo T, Luo X, Jin H, Liu Z, Yang Z, *et al.* Indolizine Derivatives as HIV-1 VIF-ElonginC Interaction Inhibitors. *Chem Biol Drug Des* 2013; **81(6)**:730-741.
166. Hung CH, Thomas L, Ruby CE, Atkins KM, Morris NP, Knight ZA, *et al.* HIV-1 Nef assembles a Src family kinase-ZAP-70/Syk-PI3K cascade to downregulate cell-surface MHC-I. *Cell Host & Microbe* 2007; **1**:121-133.
167. Hussain A, Yu L, Faryal R, Mohammad DK, Mohamed AJ, Smith CI. TEC family kinases in health and disease - loss-of-function of BTK and ITK and the gain-of-function fusions ITK-SYK and BTK-SYK. *FEBS J* 2011.
168. Hwang SS, Boyle TJ, Lyerly HK, Cullen BR. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* 1991; **253(5015)**:71-74.
169. Jacks T, Power MD, Masiarz FR, Luciw PA, Barr PJ, Varmus HE. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. *Nature* 1988; **331(6153)**:280-283.
170. Jacobs E, Gheysen D, Thines D, Francotte M, De WM. The HIV-1 Gag precursor Pr55gag synthesized in yeast is myristoylated and targeted to the plasma membrane. *Gene* 1989; **79(1)**:71-81.
171. Janvier K, Craig H, Hitchin D, Madrid R, Sol-Foulon N, Renault L, *et al.* HIV-1 Nef stabilizes the association of adaptor protein complexes with membranes. *J Biol Chem* 2003; **278(10)**:8725-8732.
172. Jia X, Singh R, Homann S, Yang H, Guatelli J, Xiong Y. Structural basis of evasion of cellular adaptive immunity by HIV-1 Nef. *Nat Struct Mol Biol* 2012; **19(7)**:701-706.
173. Jiang Y, Ma W, Wan Y, Kozasa T, Hattori S, Huang XY. The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. *Nature* 1998; **395(6704)**:808-813.
174. Jolicoeur P. The CD4C/HIV(Nef)transgenic model of AIDS. *Curr HIV Res* 2011; **9(7)**:524-530.
175. Joseph RE, Andreotti AH. Conformational snapshots of Tec kinases during signaling. *Immunol Rev* 2009; **228(1)**:74-92.
176. Joseph RE, Kleino I, Wales TE, Xie Q, Fulton DB, Engen JR, *et al.* Activation loop dynamics determine the different catalytic efficiencies of B cell- and T cell-specific tec kinases. *Sci Signal* 2013; **6(290)**:ra76.

177. Jouvenet N, Simon SM, Bieniasz PD. Visualizing HIV-1 assembly. *J Mol Biol* 2011; **410(4)**:501-511.
178. Kaplan DR, Pallas DC, Morgan W, Schaffhausen B, Roberts TM. Mechanisms of transformation by polyoma virus middle T antigen. *Biochim Biophys Acta* 1989; **948(3)**:345-364.
179. Kaplan JE, Benson C, Holmes KK, Brooks JT, Pau A, Masur H. Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. *MMWR Recomm Rep* 2009; **58(RR-4)**:1-207.
180. Kerppola TK. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annu Rev Biophys* 2008; **37**:465-487.
181. Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, *et al.* Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. *Science* 1990; **248(4959)**:1109-1112.
182. Kestler H, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, *et al.* Importance of the nef gene for maintenance of high viral loads and for development of AIDS. *Cell* 1991; **65**:651-662.
183. Khan M, Garcia-Barrio M, Powell MD. Restoration of wild-type infectivity to human immunodeficiency virus type 1 strains lacking nef by intravirion reverse transcription. *J Virol* 2001; **75(24)**:12081-12087.
184. Kim MO, Suh HS, Si Q, Terman BI, Lee SC. Anti-CD45RO suppresses human immunodeficiency virus type 1 replication in microglia: role of Hck tyrosine kinase and implications for AIDS dementia. *J Virol* 2006; **80(1)**:62-72.
185. Kinoshita T, Matsubara M, Ishiguro H, Okita K, Tada T. Structure of human Fyn kinase domain complexed with staurosporine. *Biochem Biophys Res Commun* 2006; **346(3)**:840-844.
186. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med* 1995; **332**:228-232.
187. Klatt EC. *Pathology of AIDS; Version 25*. Mercer University, Savannah; 2014.
188. Klimkait T, Strebel K, Hoggan MD, Martin MA, Orenstein JM. The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release. *J Virol* 1990; **64(2)**:621-629.

189. Knighton DR, Zheng JH, Ten Eyck LF, Ashford VA, Xuong NH, Taylor SS, *et al.* Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* 1991; **253(5018)**:407-414.
190. Komuro I, Yokota Y, Yasuda S, Iwamoto A, Kagawa KS. 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* 2003; **198(3)**:443-453.
191. Kuriyan J, Cowburn D. Structures of SH2 and SH3 domains. *Current Opinion in Structural Biology* 1993; **3**:828-837.
192. Kuriyan J, Cowburn D. Modular peptide recognition domains in eukaryotic signaling. *Annu Rev Biophys Biomol Struct* 1997; **26**:259-288.
193. Kutach AK, Villasenor AG, Lam D, Belunis C, Janson C, Lok S, *et al.* Crystal structures of IL-2-inducible T cell kinase complexed with inhibitors: insights into rational drug design and activity regulation. *Chem Biol Drug Des* 2010; **76(2)**:154-163.
194. Kuznetsov YG, Victoria JG, Robinson WE, Jr., McPherson A. Atomic force microscopy investigation of human immunodeficiency virus (HIV) and HIV-infected lymphocytes. *J Virol* 2003; **77(22)**:11896-11909.
195. Laederach A, Cradic KW, Brazin KN, Zamoon J, Fulton DB, Huang XY, *et al.* Competing modes of self-association in the regulatory domains of Bruton's tyrosine kinase: intramolecular contact versus asymmetric homodimerization. *Protein Sci* 2002; **11(1)**:36-45.
196. Laederach A, Cradic KW, Fulton DB, Andreotti AH. Determinants of intra versus intermolecular self-association within the regulatory domains of Rlk and Itk. *J Mol Biol* 2003; **329(5)**:1011-1020.
197. Lalezari JP, Henry K, O'Hearn M, Montaner JS, Piliero PJ, Trottier B, *et al.* Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. *N Engl J Med* 2003; **348(22)**:2175-2185.
198. Lama J. The physiological relevance of CD4 receptor down-modulation during HIV infection. *Curr HIV Res* 2003; **1(2)**:167-184.
199. Lama J, Mangasarian A, Trono D. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Curr Biol* 1999; **9(12)**:622-631.
200. Lamers MB, Antson AA, Hubbard RE, Scott RK, Williams DH. Structure of the protein tyrosine kinase domain of C-terminal Src kinase (CSK) in complex with staurosporine. *J Mol Biol* 1999; **285(2)**:713-725.

201. Lapadat-Tapolsky M, De RH, Van GD, Roques B, Plasterk R, Darlix JL. Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. *Nucleic Acids Res* 1993; **21(4)**:831-839.
202. Laurichesse JJ, Persoz A, Theodorou I, Rouzioux C, Delfraissy JF, Meyer L. Improved virological response to highly active antiretroviral therapy in HIV-1-infected patients carrying the CCR5 Delta32 deletion. *HIV Med* 2007; **8(4)**:213-219.
203. Le GS, Erdtmann L, Benichou S, Berlioz-Torrent C, Liu L, Benarous R, *et al.* Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity* 1998; **8(4)**:483-495.
204. Lee CH, Leung B, Lemmon MA, Zheng J, Cowburn D, Kuriyan J, *et al.* 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* 1995; **14(20)**:5006-5015.
205. Lee C-H, Saksela K, Mirza UA, Chait BT, Kuriyan J. Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell* 1996; **85**:931-942.
206. Lerner EC, Smithgall TE. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. *Nat Struct Biol* 2002; **9**:365-369.
207. Lerner EC, Tribble RP, Schiavone AP, Hochrein JM, Engen JR, Smithgall TE. Activation of the Src Family Kinase Hck without SH3-Linker Release. *J Biol Chem* 2005; **280(49)**:40832-40837.
208. Levy JA. Pathogenesis of human immunodeficiency virus infection. *Microbiol Rev* 1993; **57(1)**:183-289.
209. Levy JA. *HIV and the pathogenesis of AIDS*. Washington, DC: ASM Press; 2007.
210. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM, Oshiro LS. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 1984; **225(4664)**:840-842.
211. Lewis P, Hensel M, Emerman M. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J* 1992; **11(8)**:3053-3058.
212. Lin TA, McIntyre KW, Das J, Liu C, O'Day KD, Penhallow B, *et al.* Selective Itk inhibitors block T-cell activation and murine lung inflammation. *Biochemistry* 2004; **43(34)**:11056-11062.
213. Liu LX, Heveker N, Fackler OT, Arold S, Le Gall S, Janvier K, *et al.* 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* 2000; **74(11)**:5310-5319.
214. Liu W, Quinto I, Chen X, Palmieri C, Rabin RL, Schwartz OM, *et al.* Direct inhibition of Bruton's tyrosine kinase by IBtk, a Btk-binding protein. *Nat Immunol* 2001; **2(10)**:939-946.
 215. Liu X, Brodeur SR, Gish G, Songyang Z, Cantley LC, Laudano AP, *et al.* Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene* 1993; **8(5)**:1119-1126.
 216. Luria S, Chambers I, Berg P. Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. *Proc Natl Acad Sci U S A* 1991; **88(12)**:5326-5330.
 217. Macdonald A, Crowder K, Street A, McCormick C, Harris M. The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. *J Gen Virol* 2004; **85(Pt 3)**:721-729.
 218. Magerus-Chatinet A, Yu H, Garcia S, Ducloux E, Terris B, Bomsel M. Galactosyl ceramide expressed on dendritic cells can mediate HIV-1 transfer from monocyte derived dendritic cells to autologous T cells. *Virology* 2007; **362(1)**:67-74.
 219. Malim MH, Emerman M. HIV-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host Microbe* 2008; **3(6)**:388-398.
 220. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002; **298(5600)**:1912-1934.
 221. Mano H. Tec family of protein-tyrosine kinases: an overview of their structure and function. *Cytokine Growth Factor Rev* 1999; **10(3-4)**:267-280.
 222. Mano H, Ishikawa F, Nishida J, Hirai H, Takaku F. A novel protein-tyrosine kinase, *tec*, is preferentially expressed in liver. *Cell Growth and Differentiation* 1990; **5**:1781-1786.
 223. Mano H, Yamashita Y, Miyazato A, Miura Y, Ozawa K. Tec protein-tyrosine kinase is an effector molecule of Lyn protein-tyrosine kinase. *FASEB J* 1996; **10**:637-642.
 224. Marcsisin SR, Narute PS, Emert-Sedlak LA, Kloczewiak M, Smithgall TE, Engen JR. On the solution conformation and dynamics of the HIV-1 viral infectivity factor. *J Mol Biol* 2011; **410(5)**:1008-1022.
 225. Mariani R, Kirchhoff F, Greenough TC, Sullivan JL, Desrosiers RC, Skowronski J. High frequency of defective nef alleles in a long-term survivor with nonprogressive human immunodeficiency virus type 1 infection. *J Virol* 1996; **70(11)**:7752-7764.

226. Matsubara M, Jing T, Kawamura K, Shimojo N, Titani K, Hashimoto K, *et al.* Myristoyl moiety of HIV Nef is involved in regulation of the interaction with calmodulin in vivo. *Protein Sci* 2005; **14**(2):494-503.
227. McCune JM, Rabin LB, Feinberg MB, Lieberman M, Kosek JC, Reyes GR, *et al.* Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* 1988; **53**(1):55-67.
228. McMichael AJ. HIV vaccines. *Annu Rev Immunol* 2006; **24**:227-255.
229. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* 2010; **10**(1):11-23.
230. Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB. The human immunodeficiency virus-1 nef gene product: A positive factor for viral infection and replication in primary lymphocytes and macrophages. *J Exp Med* 1994; **179**:101-113.
231. Moarefi I, LaFevre-Bernt M, Sicheri F, Huse M, Lee C-H, Kuriyan J, *et al.* Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. *Nature* 1997; **385**:650-653.
232. Montagnier L, Chermann JC, Barre-Sinoussi F, Chamaret S, Gruest J, Nugeyre MT, *et al.* A new human T-lymphotropic retrovirus: characterization and possible role in lymphadenopathy and acquired immune deficiency syndromes. In: *Human T-cell leukemia/lymphoma virus*. Gallo RC, Essex ME, Gross L (editors). N.Y.: Cold Spring Harbor Laboratory; 1984. pp. 363-379.
233. Musacchio A, Saraste M, Wilmanns M. High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. *Nat Struct Biol* 1994; **1**(8):546-551.
234. Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of pp60^{c-src}. *Nature* 1991; **351**:69-72.
235. Nada S, Yagi T, Takeda H, Tokunaga T, Nakagawa H, Ikawa Y, *et al.* Constitutive activation of Src family kinases in mouse embryos that lack Csk. *Cell* 1993; **73**:1125-1135.
236. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol* 2002; **20**(1):87-90.
237. Nagar B, Hantschel O, Young MA, Scheffzek K, Veach D, Bornmann W, *et al.* Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* 2003; **112**(6):859-871.

238. Nakashima K, Takeuchi K, Chihara K, Horiguchi T, Sun X, Deng L, *et al.* HCV NS5A protein containing potential ligands for both Src homology 2 and 3 domains enhances autophosphorylation of Src family kinase Fyn in B cells. *PLoS One* 2012; **7(10)**:e46634.
239. Narute PS, Smithgall TE. Nef alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor-sensitive manner. *PLoS One* 2012; **7(2)**:e32561.
240. Navarro F, Landau NR. Recent insights into HIV-1 Vif. *Curr Opin Immunol* 2004; **16(4)**:477-482.
241. Niederman TM, Garcia JV, Hastings WR, Luria S, Ratner L. Human immunodeficiency virus type 1 Nef protein inhibits NF-kappa B induction in human T cells. *J Virol* 1992; **66(10)**:6213-6219.
242. O'Connell KA, Bailey JR, Blankson JN. Elucidating the elite: mechanisms of control in HIV-1 infection. *Trends Pharmacol Sci* 2009; **30(12)**:631-637.
243. Ogawa A, Takayama Y, Sakai H, Chong KT, Takeuchi S, Nakagawa A, *et al.* Structure of the carboxyl-terminal Src kinase, Csk. *J Biol Chem* 2002; **277(17)**:14351-14354.
244. Ooi EL, Chan ST, Cho NE, Wilkins C, Woodward J, Li M, *et al.* Novel antiviral host factor, TNK1, regulates IFN signaling through serine phosphorylation of STAT1. *Proc Natl Acad Sci U S A* 2014; **111(5)**:1909-1914.
245. Pagano MA, Tibaldi E, Palu G, Brunati AM. Viral proteins and Src family kinases: Mechanisms of pathogenicity from a "liaison dangereuse". *World J Virol* 2013; **2(2)**:71-78.
246. Palacios EH, Weiss A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* 2004; **23(48)**:7990-8000.
247. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, *et al.* HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* 1993; **362(6418)**:355-358.
248. Pantaleo G, Menzo S, Vaccarezza M, Graziosi C, Cohen OJ, Demarest JF, *et al.* Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* 1995; **332(4)**:209-216.
249. Parsons JT, Parsons SJ. Src family protein tyrosine kinases: Cooperating with growth factor and adhesion signaling pathways. *Curr Opin Cell Biol* 1997; **9**:187-192.
250. Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene* 2004; **23(48)**:7906-7909.

251. Peeters M, Toure-Kane C, Nkengasong JN. Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials. *AIDS* 2003; **17(18)**:2547-2560.
252. Pene-Dumitrescu T, Shu ST, Wales TE, Alvarado JJ, Shi H, Narute P, *et al.* HIV-1 Nef interaction influences the ATP-binding site of the Src-family kinase, Hck. *BMC Chem Biol* 2012; **12**:1.
253. Peng B, Robert-Guroff M. Deletion of N-terminal myristoylation site of HIV Nef abrogates both MHC-1 and CD4 down-regulation. *Immunol Lett* 2001; **78(3)**:195-200.
254. Percario Z, Olivetta E, Fiorucci G, Mangino G, Peretti S, Romeo G, *et al.* Human immunodeficiency virus type 1 (HIV-1) Nef activates STAT3 in primary human monocyte/macrophages through the release of soluble factors: involvement of Nef domains interacting with the cell endocytotic machinery. *J Leukoc Biol* 2003; **74(5)**:821-832.
255. Pfannkuche A, Buther K, Karthe J, Poenisch M, Bartenschlager R, Trilling M, *et al.* c-Src is required for complex formation between the hepatitis C virus-encoded proteins NS5A and NS5B: a prerequisite for replication. *Hepatology* 2011; **53(4)**:1127-1136.
256. Piguet V, Chen YL, Mangasarian A, Foti M, Carpentier JL, Trono D. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. *EMBO J* 1998; **17(9)**:2472-2481.
257. Pizzato M, Helander A, Popova E, Calistri A, Zamborlini A, Palu G, *et al.* Dynamin 2 is required for the enhancement of HIV-1 infectivity by Nef. *Proc Natl Acad Sci U S A* 2007; **104(16)**:6812-6817.
258. Poe JA, Smithgall TE. HIV-1 Nef dimerization is required for Nef-mediated receptor downregulation and viral replication. *J Mol Biol* 2009; **394(2)**:329-342.
259. Pomerantz RJ, Horn DL. Twenty years of therapy for HIV-1 infection. *Nat Med* 2003; **9(7)**:867-873.
260. Porter M, Schindler T, Kuriyan J, Miller WT. Reciprocal regulation of Hck activity by phosphorylation of Tyr(527) and Tyr(416). Effect of introducing a high affinity intramolecular SH2 ligand. *J Biol Chem* 2000; **275(4)**:2721-2726.
261. Pursglove SE, Mulhern TD, Mackay JP, Hinds MG, Booker GW. The solution structure and intramolecular associations of the Tec kinase SRC homology 3 domain. *J Biol Chem* 2002; **277(1)**:755-762.
262. Qiu Y, Robinson D, Pretlow TG, Kung HJ. Etk/Bmx, a tyrosine kinase with a pleckstrin-homology domain, is an effector of phosphatidylinositol 3'-kinase and is involved

in interleukin 6-induced neuroendocrine differentiation of prostate cancer cells. *Proc Natl Acad Sci U S A* 1998; **95**(7):3644-3649.

263. Quinn TC. Acute primary HIV infection. *JAMA* 1997; **278**(1):58-62.
264. Ramos A, Hu DJ, Nguyen L, Phan KO, Vanichseni S, Promadej N, *et al.* Intersubtype human immunodeficiency virus type 1 superinfection following seroconversion to primary infection in two injection drug users. *J Virol* 2002; **76**(15):7444-7452.
265. Readinger JA, Mueller KL, Venegas AM, Horai R, Schwartzberg PL. Tec kinases regulate T-lymphocyte development and function: new insights into the roles of Itk and Rlk/Txk. *Immunol Rev* 2009; **228**(1):93-114.
266. Readinger JA, Schiralli GM, Jiang JK, Thomas CJ, August A, Henderson AJ, *et al.* Selective targeting of ITK blocks multiple steps of HIV replication. *Proc Natl Acad Sci U S A* 2008; **105**(18):6684-6689.
267. Rebecchi MJ, Scarlata S. Pleckstrin homology domains: a common fold with diverse functions. *Annu Rev Biophys Biomol Struct* 1998; **27**:503-528.
268. Rekas A, Alattia JR, Nagai T, Miyawaki A, Ikura M. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. *J Biol Chem* 2002; **277**(52):50573-50578.
269. Renkema GH, Manninen A, Mann DA, Harris M, Saksela K. Identification of the Nef-associated kinase as p21-activated kinase 2. *Curr Biol* 1999; **9**(23):1407-1410.
270. Renkema GH, Saksela K. Interactions of HIV-1 NEF with cellular signal transducing proteins. *Front Biosci* 2000; **5**:D268-D283.
271. Resh MD. Myristylation and palmitoylation of Src family members: The fats of the matter. *Cell* 1994; **76**:411-413.
272. Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta* 1999; **1451**(1):1-16.
273. Ribeiro RM. Dynamics of CD4+ T cells in HIV-1 infection. *Immunol Cell Biol* 2007; **85**(4):287-294.
274. Rice LM, Plakas C, Nickels JT, Jr. Loss of meiotic rereplication block in *Saccharomyces cerevisiae* cells defective in Cdc28p regulation. *Eukaryot Cell* 2005; **4**(1):55-62.
275. Robbins SM, Quintrell NA, Bishop JM. Myristoylation and differential palmitoylation of the *HCK* protein-tyrosine kinases govern their attachment to membranes and association with caveolae. *Mol Cell Biol* 1995; **15**:3507-3515.

276. Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. *Oncogene* 2000; **19(49)**:5548-5557.
277. Roeth JF, Williams M, Kasper MR, Filzen TM, Collins KL. HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail. *J Cell Biol* 2004; **167(5)**:903-913.
278. Roskoski R, Jr. Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun* 2004; **324(4)**:1155-1164.
279. Roskoski R, Jr. Src kinase regulation by phosphorylation and dephosphorylation. *Biochem Biophys Res Commun* 2005; **331(1)**:1-14.
280. Saksela K. Interactions of the HIV/SIV pathogenicity factor Nef with SH3 domain-containing host cell proteins. *Curr HIV Res* 2011; **9(7)**:531-542.
281. Saksela K, Cheng G, Baltimore D. 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* 1995; **14(3)**:484-491.
282. Salem AH, Farid E, Fadel R, Abu-Hijleh M, Almawi W, Han K, *et al.* Distribution of four HIV type 1-resistance polymorphisms (CCR5-Delta32, CCR5-m303, CCR2-64I, and SDF1-3'A) in the Bahraini population. *AIDS Res Hum Retroviruses* 2009; **25(10)**:973-977.
283. Sawai ET, Baur A, Struble H, Peterlin BM, Levy JA, Cheng-Mayer C. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc Natl Acad Sci U S A* 1994; **91(4)**:1539-1543.
284. Sawai ET, Baur AS, Peterlin BM, Levy JA, Cheng-Mayer C. 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* 1995; **270(25)**:15307-15314.
285. Sawai ET, Hamza MS, Ye M, Shaw KE, Luciw PA. Pathogenic conversion of live attenuated simian immunodeficiency virus vaccines is associated with expression of truncated Nef. *J Virol* 2000; **74(4)**:2038-2045.
286. Sawai ET, Khan IH, Montbriand PM, Peterlin BM, Cheng-Mayer C, Luciw PA. Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques. *Curr Biol* 1996; **6(11)**:1519-1527.
287. Schaefer MR, Wonderlich ER, Roeth JF, Leonard JA, Collins KL. HIV-1 Nef targets MHC-I and CD4 for degradation via a final common beta-COP-dependent pathway in T cells. *PLoS Pathog* 2008; **4(8)**:e1000131.
288. Schafer JJ, Squires KE. Integrase inhibitors: a novel class of antiretroviral agents. *Ann Pharmacother* 2010; **44(1)**:145-156.

289. Schindler T, Sicheri F, Pico A, Gazit A, Levitzki A, Kuriyan J. Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. *Mol Cell* 1999; **3(5)**:639-648.
290. Schiralli Lester GM, Akiyama H, Evans E, Singh J, Gummuluru S, Henderson AJ. Interleukin 2-inducible T cell kinase (ITK) facilitates efficient egress of HIV-1 by coordinating Gag distribution and actin organization. *Virology* 2013; **436(1)**:235-243.
291. Schlessinger J, Lemmon MA. SH2 and PTB domains in tyrosine kinase signaling. *Sci STKE* 2003; **2003(191)**:RE12.
292. Schreiner SJ, Schiavone AP, Smithgall TE. Activation of Stat3 by the Src family kinase Hck requires a functional SH3 domain. *J Biol Chem* 2002; **277**:45680-45687.
293. Schwartz O, Marechal V, Le GS, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med* 1996; **2(3)**:338-342.
294. Shan X, Czar MJ, Bunnell SC, Liu P, Liu Y, Schwartzberg PL, *et al.* Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol Cell Biol* 2000; **20(18)**:6945-6957.
295. Shelton H, Harris M. Hepatitis C virus NS5A protein binds the SH3 domain of the Fyn tyrosine kinase with high affinity: mutagenic analysis of residues within the SH3 domain that contribute to the interaction. *Virol J* 2008; **5**:24.
296. Shirai T, Tanaka K, Terada Y, Sawada T, Shirai R, Hashimoto Y, *et al.* Specific detection of phosphatidylinositol 3,4,5-trisphosphate binding proteins by the PIP3 analogue beads: an application for rapid purification of the PIP3 binding proteins. *Biochim Biophys Acta* 1998; **1402(3)**:292-302.
297. Shoelson SE. SH2 and PTB domain interactions in tyrosine kinase signal transduction. *Curr Opin Chem Biol* 1997; **1(2)**:227-234.
298. Shugars DC, Smith MS, Glueck DH, Nantermet PV, Seillier-Moiseiwitsch F, Swanstrom R. Analysis of human immunodeficiency virus type 1 *nef* gene sequences present in vivo. *J Virol* 1993; **67**:4639-4650.
299. Shyu YJ, Liu H, Deng X, Hu CD. Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *Biotechniques* 2006; **40(1)**:61-66.
300. Sicheri F, Kuriyan J. Structures of Src-family tyrosine kinases. *Curr Opin Struct Biol* 1997; **7(6)**:777-785.

301. Sicheri F, Moarefi I, Kuriyan J. Crystal structure of the Src family tyrosine kinase Hck. *Nature* 1997; **385**:602-609.
302. Siliciano JD, Morrow TA, Desiderio SV. itk, a T-cell-specific tyrosine kinase gene inducible by interleukin 2. *Proc Natl Acad Sci U S A* 1992; **89(23)**:11194-11198.
303. Simmons A, Aluvihare V, McMichael A. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* 2001; **14(6)**:763-777.
304. Skowronski J, Parks D, Mariani R. Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *EMBO J* 1993; **12(2)**:703-713.
305. Sloan RD, Donahue DA, Kuhl BD, Bar-Magen T, Wainberg MA. Expression of Nef from unintegrated HIV-1 DNA downregulates cell surface CXCR4 and CCR5 on T-lymphocytes. *Retrovirology* 2010; **7**:44.
306. Smith CI, Islam TC, Mattsson PT, Mohamed AJ, Nore BF, Vihinen M. The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. *BioEssays* 2001; **23(5)**:436-446.
307. Smithgall TE, Thomas G. Small molecule inhibitors of the HIV-1 virulence factor, Nef. *Drug Discov Today: Technol* 2013; **10**:523-529.
308. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, *et al.* SH2 domains recognize specific phosphopeptide sequences. *Cell* 1993; **72**:767-778.
309. Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, *et al.* Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* 1994; **14**:2777-2785.
310. Spach DH, Hooton TM. *The HIV manual a guide to diagnosis and treatment*. New York: Oxford University Press; 1996.
311. Spina C, Kwoh TJ, Chowder MY, Guatelli JC, Richman DD. The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *J Exp Med* 1994; **179**:115-123.
312. Stowell RE, Smith EK, Espana C, Nelson VG. Outbreak of malignant lymphoma in rhesus monkeys. *Lab Invest* 1971; **25(5)**:476-479.
313. Strunk U, Saffran HA, Wu FW, Smiley JR. Role of herpes simplex virus VP11/12 tyrosine-based motifs in binding and activation of the Src family kinase Lck and recruitment of p85, Grb2, and Shc. *J Virol* 2013; **87(20)**:11276-11286.
314. Summy JM, Qian Y, Jiang BH, Guappone-Koay A, Gatesman A, Shi X, *et al.* The SH4-Unique-SH3-SH2 domains dictate specificity in signaling that differentiate c-Yes from c-Src. *J Cell Sci* 2003; **116(Pt 12)**:2585-2598.

315. Swigut T, Shohdy N, Skowronski J. Mechanism for down-regulation of CD28 by Nef. *EMBO J* 2001; **20(7)**:1593-1604.
316. Swingler S, Brichacek B, Jacque JM, Ulich C, Zhou J, Stevenson M. HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. *Nature* 2003; **424(6945)**:213-219.
317. Swingler S, Mann A, Jacque J, Brichacek B, Sasseville VG, Williams K, *et al.* HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nat Med* 1999; **5(9)**:997-103.
318. Takahashi-Tezuka M, Hibi M, Fujitani Y, Fukada T, Yamaguchi T, Hirano T. Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. *Oncogene* 1997; **14(19)**:2273-2282.
319. Tamagnone L, Lahtinen I, Mustonen T, Virtaneva K, Francis F, Muscatelli F, *et al.* BMX, a novel nonreceptor tyrosine kinase gene of the BTK/ITK/TEC/TKK family located in chromosome Xp22.2. *Oncogene* 1994; **9(12)**:3683-3688.
320. Tarafdar S, Poe JA, Smithgall TE. The Accessory Factor Nef Links HIV-1 to Tec/Btk Kinases in an SH3 Domain-dependent Manner. *J Biol Chem* 2014; **289(22)**:15718-15728.
321. Terwilliger E, Sodroski JG, Rosen CA, Haseltine WA. 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* 1986; **60(2)**:754-760.
322. Thomas SM, Brugge JS. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 1997; **13**:513-609.
323. Thompson MA, Aberg JA, Cahn P, Montaner JS, Rizzardini G, Telenti A, *et al.* Antiretroviral treatment of adult HIV infection: 2010 recommendations of the International AIDS Society-USA panel. *JAMA* 2010; **304(3)**:321-333.
324. Toussaint H, Gobert FX, Schindler M, Banning C, Kozik P, Jouve M, *et al.* Human immunodeficiency virus type 1 nef expression prevents AP-2-mediated internalization of the major histocompatibility complex class II-associated invariant chain. *J Virol* 2008; **82(17)**:8373-8382.
325. Tribble RP, Emert-Sedlak L, Smithgall TE. HIV-1 Nef selectively activates SRC family kinases HCK, LYN, and c-SRC through direct SH3 domain interaction. *J Biol Chem* 2006; **281**:27029-27038.
326. Tribble RP, Emert-Sedlak L, Wales TE, Ayyavoo V, Engen JR, Smithgall TE. Allosteric loss-of-function mutations in HIV-1 Nef from a long-term non-progressor. *J Mol Biol* 2007; **374(1)**:121-129.

327. Tribble RP, Narute P, Emert-Sedlak LA, Alvarado JJ, Atkins K, Thomas L, *et al.* Discovery of a diaminoquinoxaline benzenesulfonamide antagonist of HIV-1 Nef function using a yeast-based phenotypic screen. *Retrovirology* 2013; **10**:135.
328. Tsukada S, Saffran DC, Rawlings DJ, Parolini O, Allen RC, Klisak I, *et al.* Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 1993; **72(2)**:279-290.
329. Turner BG, Summers MF. Structural biology of HIV. *J Mol Biol* 1999; **285(1)**:1-32.
330. Vargas L, Nore BF, Berglof A, Heinonen JE, Mattsson PT, Smith CI, *et al.* Functional interaction of caveolin-1 with Bruton's tyrosine kinase and Bmx. *J Biol Chem* 2002; **277(11)**:9351-9357.
331. Vergis EN, Mellors JW. Natural history of HIV-1 infection. *Infect Dis Clin North Am* 2000; **14(4)**:809-8vi.
332. Vetrie D, Vorechovsky I, Sideras P, Holland J, Davies A, Flinter F, *et al.* The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* 1993; **361(6409)**:226-233.
333. Vihinen M, Nilsson L, Smith CI. Tec homology (TH) adjacent to the PH domain. *FEBS Lett* 1994; **350(2-3)**:263-265.
334. Vincent P, Priceputu E, Kay D, Saksela K, Jolicoeur P, Hanna Z. Activation of p21-activated kinase 2 and its association with Nef are conserved in murine cells but are not sufficient to induce an AIDS-like disease in CD4C/HIV transgenic mice. *J Biol Chem* 2006; **281(11)**:6940-6954.
335. Wagner MJ, Smiley JR. Herpes simplex virus requires VP11/12 to activate Src family kinase-phosphoinositide 3-kinase-Akt signaling. *J Virol* 2011; **85(6)**:2803-2812.
336. Wainberg MA. HIV-1 subtype distribution and the problem of drug resistance. *AIDS* 2004; **18 Suppl 3**:S63-S68.
337. Waksman G, Shoelson SE, Pant N, Cowburn D, Kuriyan J. Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: Crystal structures of the complexed and peptide-free forms. *Cell* 1993; **72**:779-790.
338. Walker BD, Burton DR. Toward an AIDS vaccine. *Science* 2008; **320(5877)**:760-764.
339. Wang JK, Kiyokawa E, Verdin E, Trono D. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc Natl Acad Sci U S A* 2000; **97(1)**:394-399.
340. Warnke D, Barreto J, Temesgen Z. Antiretroviral drugs. *J Clin Pharmacol* 2007; **47(12)**:1570-1579.

341. Wensing AM, van Maarseveen NM, Nijhuis M. Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance. *Antiviral Res* 2010; **85(1)**:59-74.
342. Whatmore AM, Cook N, Hall GA, Sharpe S, Rud EW, Cranage MP. Repair and evolution of nef in vivo modulates simian immunodeficiency virus virulence. *J Virol* 1995; **69(8)**:5117-5123.
343. Whetter LE, Ojukwu IC, Novembre FJ, Dewhurst S. Pathogenesis of simian immunodeficiency virus infection. *J Gen Virol* 1999; **80 (Pt 7)**:1557-1568.
344. Wilcox HM, Berg LJ. Itk phosphorylation sites are required for functional activity in primary T cells. *J Biol Chem* 2003; **278(39)**:37112-37121.
345. Willey RL, Maldarelli F, Martin MA, Strebel K. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *J Virol* 1992; **66(12)**:7193-7200.
346. Williams JC, Weijland A, Gonfloni S, Thompson A, Courtneidge SA, Superti-Furga G, *et al.* The 2.35 Å crystal structure of the inactivated form of chicken Src: A dynamic molecule with multiple regulatory interactions. *J Mol Biol* 1997; **274**:757-775.
347. Witte V, Laffert B, Gintschel P, Krautkramer E, Blume K, Fackler OT, *et al.* Induction of HIV transcription by Nef involves Lck activation and protein kinase Ctheta raft recruitment leading to activation of ERK1/2 but not NFkappaB. *J Immunol* 2008; **181(12)**:8425-8432.
348. Wolf D, Giese SI, Witte V, Krautkramer E, Trapp S, Sass G, *et al.* Novel (n)PKC kinases phosphorylate Nef for increased HIV transcription, replication and perinuclear targeting. *Virology* 2008; **370(1)**:45-54.
349. Wolf D, Witte V, Laffert B, Blume K, Stromer E, Trapp S, *et al.* HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. *Nat Med* 2001; **7(11)**:1217-1224.
350. Wright K. AIDS therapy. First tentative signs of therapeutic promise. *Nature* 1986; **323(6086)**:283.
351. Wyand MS, Manson K, Montefiori DC, Lifson JD, Johnson RP, Desrosiers RC. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. *J Virol* 1999; **73(10)**:8356-8363.
352. Wyand MS, Manson KH, Garcia-Moll M, Montefiori D, Desrosiers RC. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. *J Virol* 1996; **70(6)**:3724-3733.
353. Xu W, Doshi A, Lei M, Eck MJ, Harrison SC. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell* 1999; **3(5)**:629-638.

354. Xu W, Harrison SC, Eck MJ. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* 1997; **385**:595-602.
355. Xu XN, Sreaton GR, Gotch FM, Dong T, Tan R, Almond N, *et al.* 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* 1997; **186**(1):7-16.
356. Yaffe MB. Phosphotyrosine-binding domains in signal transduction. *Nat Rev Mol Cell Biol* 2002; **3**(3):177-186.
357. Yamada N, Kawakami Y, Kimura H, Fukamachi H, Baier G, Altman A, *et al.* Structure and expression of novel protein-tyrosine kinases, Emb and Emt, in hematopoietic cells. *Biochem Biophys Res Commun* 1993; **192**(1):231-240.
358. Yamashita Y, Miyazato A, Ohya K, Ikeda U, Shimada K, Miura Y, *et al.* Deletion of Src homology 3 domain results in constitutive activation of Tec protein-tyrosine kinase. *Jpn J Cancer Res* 1996; **87**(11):1106-1110.
359. Yang WC, Collette Y, Nunes JA, Olive D. Tec kinases: a family with multiple roles in immunity. *Immunity* 2000; **12**(4):373-382.
360. Yao L, Kawakami Y, Kawakami T. The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc Natl Acad Sci U S A* 1994; **91**(19):9175-9179.
361. Ye H, Choi HJ, Poe J, Smithgall TE. Oligomerization is required for HIV-1 Nef-induced activation of the Src family protein-tyrosine kinase, Hck. *Biochemistry* 2004; **43**(50):15775-15784.
362. Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL. Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* 1994; **76**:933-945.
363. Yu L, Mohamed AJ, Simonson OE, Vargas L, Blomberg KE, Bjorkstrand B, *et al.* Proteasome-dependent autoregulation of Bruton tyrosine kinase (Btk) promoter via NF-kappaB. *Blood* 2008; **111**(9):4617-4626.
364. Yu L, Mohanram V, Simonson OE, Smith CI, Spetz AL, Mohamed AJ. Proteasome inhibitors block HIV-1 replication by affecting both cellular and viral targets. *Biochem Biophys Res Commun* 2009; **385**(1):100-105.
365. Zhu Q, Zhang M, Rawlings DJ, Vihinen M, Hagemann T, Saffran DC, *et al.* Deletion within the Src homology domain 3 of Bruton's tyrosine kinase resulting in X-linked agammaglobulinemia (XLA). *J Exp Med* 1994; **180**(2):461-470.
366. Ziegler SF, Marth JD, Lewis DB, Perlmutter RM. Novel protein-tyrosine kinase gene (*hck*) preferentially expressed in cells of hematopoietic origin. *Mol Cell Biol* 1987; **7**:2276-2285.

367. Zou W, Denton PW, Watkins RL, Krisko JF, Nochi T, Foster JL, *et al.* Nef functions in BLT mice to enhance HIV-1 replication and deplete CD4+CD8+ thymocytes. *Retrovirology* 2012; **9**:44.