SELECTIVE ACTIVATION OF SRC FAMILY KINASES BY THE HIV-1 NEF PROTEIN

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Nef is a critical HIV-1 accessory factor shown to promote viral pathogenesis by altering host cell signaling pathways. Nef has been shown to bind several members of the Src family of protein-tyrosine kinases, and these interactions have been implicated in the pathogenesis of HIV/AIDS. The studies summarized below investigated this key interaction between virus and host cell proteins.

We explored the direct effect of Nef interaction on Src family kinases (SFKs) using *Saccharomyces cerevisiae*, a well-defined system in which c-Src expression arrests yeast cell growth in a kinase-dependent manner. The seven SFKs found in HIV target cells wre expressed in yeast; each was found to be active alone, but repressed by co-expression of the negative regulatory kinase Csk. We then co-expressed each SFK with both Csk and HIV-1 Nef and found that Nef selectively activated Hck, Lyn, and c-Src among SFKs.

We then used our yeast-based system to identify small molecule inhibitors of the active Nef:Hck complex using the auto-dowregulated Hck-YEEI molecule. Yeast expressing the Nef:Hck-YEEI complex were used to screen a library of small heterocyclic compounds based on their ability to rescue growth inhibition. Two compounds identified in this screen potently blocked Nef-dependent HIV replication, indicating Nef:SFK complexes as valid targets for anti-HIV drug therapy.

Finally, we used the yeast assay to identify novel mechanisms of Nef:SFK interactions. We screened a panel of primary Nef alleles containing the known SH3-binding elements and discovered four alleles whose proteins demonstrated altered activation of SFKs. Sequence examination revealed the existence of amino acid changes in regions not previously suspected to be involved in SH3-mediated interaction. Particularly intriguing are residues in a large unstructured loop that projects from the Nef core. These findings suggest that critical residues outside of the known SH3-binding motifs may affect SFK binding and activation.

Together, the results presented here advance the field of HIV research by furthering our understanding of the interaction between the HIV-1 Nef virulence factor and the Src kinase family, as well as validating this virus:host cell interaction as a rational target for anti-HIV drug discovery.

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1.0 OVERALL INTRODUCTION

2006 marks the 25th anniversary of a report describing five young men suffering from a mysterious new illness that later came to be known as acquired immunodeficiency syndrome (AIDS) (1). Since then, the global health community has put forth an extraordinary effort to investigate and combat AIDS and its etiologic agent, human immunodeficiency virus (HIV) (283). To date, over 221,000 HIV/AIDS-related reports have been published in the NIH National Library of Medicine PubMed database. This work has led to the discovery and production of twenty antiviral agents approved by the U. S. Food and Drug Administration that have made HIV, at least in the developed world, a chronic illness and not an inevitably fatal disease (103,283).

However, despite the millions of dollars and decades spent researching HIV, we still have only a cursory understanding of the pathogenic mechanisms employed by the virus during host infection. More importantly, we still lack an HIV vaccine, and the antiviral drugs currently in use are ineffective or contraindicated in many patients who have developed or acquired drug-resistant viral strains (157,230,355). Throughout this time, an estimated 60 million people have contracted HIV, over a third of whom have since died from their illnesses (103). Continued research into the molecular mechanisms of HIV-mediated disease, and the discovery of drugs that inhibit those mechanisms, is imperative to extend the lives of those individuals currently infected with HIV and to prevent the spread of HIV to future generations.

1.1 DESCRIPTION OF HIV

HIV is a member of the lentiviral group of retroviruses, a family of viruses that utilizes an RNA genome to encode viral proteins (reviewed by (58,91,131)). Two copies of the HIV RNA genome are packaged within an enveloped viral capsid. Upon binding and fusion of HIV to its target cell, the viral genome and associated proteins are released into the cytoplasm and undergo reverse transcription to form a double-stranded cDNA molecule. Viral cDNA is integrated into the host chromosome, and, upon initiation of cellular activation signals, the genome is transcribed to create a full array of viral transcripts, including the full-length viral RNA genome. Viral transcripts are then spliced, translated, and processed to create functional viral proteins. New virions are assembled at the cellular membrane and released from the cell via budding.

The HIV genome contains nine overlapping genes (*gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env*, and *nef*) that encode at least fifteen viral proteins (Figure 1-1). Two long-terminal repeats (LTRs) flank the genome and contain viral promoters that are induced by host activation factors, such as NF-κB. The *gag* and *env* genes encode for structural proteins; the *gag* core and matrix proteins make up the viral capsid, and the *env* glycoproteins gp120 and gp41 mediate virion-cell binding and fusion. The *pol* gene products are the workhorses of the virus and include the viral reverse transcriptase, protease, and integrase enzymes. *tat* and *rev* are regulatory proteins that drive transcription of viral gene products and viral replication. Finally, *vif*, *vpr*, *vpu*, and *nef* are viral accessory proteins that enhance viral infectivity, replication, and viability. In particular, the *nef* gene product is responsible for an ever-growing list of viral functions that will be described later in more detail (see section 1.3).

There are two forms of HIV, HIV-1 and HIV-2, which have each evolved from different primate carriers (334). HIV-1 originated from simian immunodeficiency virus (SIV) in chimpanzees and is the most prevelant strain of the virus. HIV-2 shares 40-60% homology with HIV-1 and has been traced to an SIV strain found in the sooty mangabey. The primate hosts do not suffer from effects of the disease and serve as carriers. Infection of non-natural hosts, however, like humans or other primate species, results in rapid disease progression (324,334).

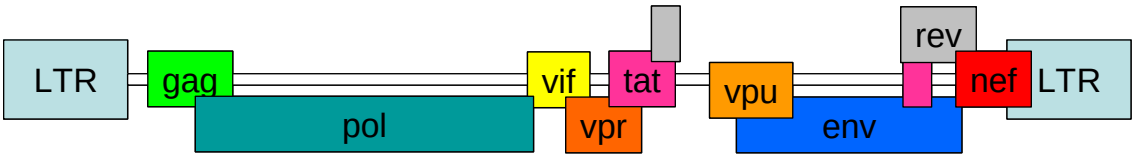

Figure 1-1. The HIV-1 genome consists of nine overlapping genes.

The viral genome is bookended by two long-terminal repeats (LTRs) that contain promoters necessary for viral transcription. The studies presented in this dissertation focus on the pathogenic role of the *nef* gene, located at the 3' end of the genome. This figure is adapted from (112).

1.2 PATHOGENESIS OF HIV

To better study HIV:host interactions, it is helpful to understand the dynamics and pathogenesis of viral infection. Previous theories about the course of HIV infection claimed that, following the initial wave of attack by HIV virions, a prolonged and intensive steady-state battle ensued. This molecular struggle was believed to pit the killing of virions by a CD4+ T cell-mediated immune response against the killing of CD4+ T cells by the virus, ultimately leading to a slow, steady decline in host immunity (71,162,373). However, it is now clear that HIV gains an irrevocable advantage very early during infection, that the immune response is unable to overcome (139,278). In addition, during the course of infection, damage secondary to the host's unrelenting immune response, and not direct virus-mediated killing, appears responsible for the bulk of T cell depletion (139,140,186,243). Indeed, the ability to repopulate the pool of short-lived effector memory T cells correlates better with rapid SIV disease progression in rhesus macaques than the level of virus in the plasma (277). Further, SIV-infected mangabeys do not progress to disease, and this phenomenon is attributed to the diminished immune response they generate to the virus (325). Finally, constitutive expression of the activation molecule CD70 in a mouse model demonstrates that generation of chronic immune stimulation is sufficient to induce an immunodeficient phenotype similar to that caused by HIV infection (348). Though the human host engineers a strong immune response to HIV infection, the virus appears to use this response to its own advantage to weaken the host defense system and enhance viral survival.

1.2.1 Normal Immune Response to Virus

Upon infection with a viral pathogen, the host normally mounts an immediate and strong immune response in an attempt to rid the body of the infection. This immune reaction relies on a coordinated effort from several cell types that participate in a process of activation, differentiation, targeting, and, upon conclusion of the attack, shutdown of the response (reviewed in (2)).

The first cells to encounter an infecting virus are usually macrophages and dendritic cells (DCs), both phagocytic cells that reside within tissues and mucosal surfaces. DCs in the periphery are immature and are devoted phagocytes. However, after taking up antigen, DCs migrate to nearby lymph tissue (lymph nodes, spleen, or Peyer's patches) where they mature into antigen-presenting cells (APCs) and present viral antigen to naïve T cells for activation (24,25). Macrophages typically remain in the periphery and function as general phagocytes until they are exposed to pathogen, after which they express cytokines and co-stimulatory molecules that enable them to recruit and present antigen to naïve and memory lymphocytes (22).

After APCs take up a virus, viral antigens are processed and presented on surface major histocompatibility complex (MHC) molecules. Viruses that enter the cell through direct infection and reside in the cytosol have their antigens presented on MHC type I molecules, while endocytosed viral antigens are presented on MHC class II molecules. A naïve T cell is primed for activation through the binding of its T cell receptor (TCR) to the MHC/antigen complex on the surface of the APC. In addition, co-stimulation is required for proper activation, which occurs by binding of CD28 ligands on the T cell to B7 molecules on the APC. TCR-mediated signaling induces transcription of the proliferation factor IL-2, while CD28 signaling stabilizes IL-2 transcripts. Both signals are necessary to induce production and release of the IL-2 cytokine. Released IL-2 then binds to IL-2 receptors on the T cell surface to induce cell proliferation and differentiation into activated effector T cells capable of eliminating virus-infected cells (2).

After several days of proliferation and differentiation in the lymph tissue, activated effector T cells are released into the blood, where they will migrate to the sites of infection. Activated CTLs (CD8+ T cells) bind infected cells expressing viral antigen on MHC-I molecules and kill them either through targeted release of lytic granules and/or through the induction of the FasL/Fas pathway; both pathways result in programmed cell death (apoptosis) of the infected cell. Activated CD4+ T cells bind infected cells expressing antigen on MHC-II molecules and, through cytokine release and engagement of co-stimulatory molecules, induce the activation of macrophages to kill phagocytosed pathogens (T_H1 effector CD4+ T cells), or activate B cells to produce virus-neutralizing antibodies (T_H2 effector CD4+ T cells) (2).

These effector T cells are short-lived and will die via activation-induced cell death (AICD) soon after they are produced. This is an active process that involves a concerted

"instructional process" of cytokine and receptor-mediated apoptosis (333). The immune response is self-limiting to prevent excessive stress to the immune system, including the unnecessary release of harmful inflammatory cytokines and clogging of the lymph tissue with redundant circulating effector cells (2,333).

As the infection resolves, a small population of effector T cells escape AICD and survive to become memory T cells (168,315). The memory phenotype may be created during the waning stages of infection when a suboptimal level of antigen is available for APC presentation (333). Memory cells are separated into two groups – central (or inductor) memory T cells, that reside in the lymph tissues and specialize in producing more effector cells; and effector memory T cells, that reside in peripheral tissues, such as the lamina propria of the gut, and are prepared to quickly engage previously encountered pathogens (139,236,291). In this way, the body can maintain a strong immunologic defense against re-introduction of a pathogen. However, HIV infection does not allow for the proper assembly of this immunological defense network, working instead to force the host immune system into a chronic, and ultimately overwhelming, inflammatory response.

1.2.2 HIV Infection

Acute Phase

Recent studies have shown that shortly after infection of humans by HIV, or infection of rhesus macaques by the SIV, CD4+CCR5+ effector memory T cells are massively depleted from the gut lymphoid tissue, the site of approximately 60% of the total lymphocytes in the body (39,144,186,238,362). These effector memory CD4+ T cells are an optimal target for infection by newly introduced virus for several reasons: (1) they are present in high concentrations within the mucosal lining; (2) they contain the preferred cellular surface receptors (CD4 and CCR5) for infection by early HIV particles; and (3) they are readily replenished by newly-induced HIV-specific activated effector CD4+ T cells, providing, at least initially, a renewable source of HIV target cells (278). In fact, HIV-specific CCR5+CD4+ activated effector T cells produced during the immune reaction are preferentially infected by HIV (92).

The inflammatory response that accompanies HIV infection leads to substantial activation bursts of T cells in the lymph nodes (139). As would occur with any viral infection, naïve and memory T cells differentiate into activated, short-lived HIV-specific effector T cells; however, in the case of HIV, this response is much greater than normal. As a result, massive numbers of T cells are produced and rapidly turned over, either via direct virus-mediated killing or by AICD, the host's natural method for regulating the duration of such inflammatory responses.

DCs and macrophages capture and present HIV antigens to naïve and memory lymphocytes (336,378). Both of these cell types express the CD4 and CCR5 surface receptors and can be productively infected by HIV (130,239,378). In addition, both cells express C-type lectins, such as DC-SIGN, that bind and internalize HIV particles (248,357). After migrating to lymph nodes, DCs can release internalized virions that still maintain full pathogenicity, allowing them to infect the rich sources of newly produced and activated HIV-specific CCR5+CD4+ T cells (130,209). Macrophages not only internalize HIV virions, but support viral replication within their endosomes (317,336). Thus, in addition to priming the immune system to combat HIV infection, APCs contribute directly to the spread of the virus.

Chronic phase

Once the infection is established, HIV relies on antigen-driven activation bursts for the production of HIV-specific CD4+ target cells (139). During the continuous rounds of virus-induced T cell activation, the pool of both naïve and central memory T cells are drastically reduced. As stable, long-lived naïve and memory T cells are induced to differentiate into fast-replicating, short-lived effector cells, two things appear to occur: 1) central memory T cells are not sufficiently replenished, and 2) the conditions of high-level immune activation produce "collateral damage", as the inflammatory environment created is both toxic to bystander (non-HIV-specific) T cells and destructive to the immune architecture of the lymph tissue (139,336). Both of these factors greatly reduce the ability of the immune system to produce and maintain an effective response to pathogens.

Toward the end of the chronic phase, viral variants emerge that preferentially infect CXCR4+ cells, such as the CXCR4+CD4+ central memory T cells (78,93,139,246). This switch in tropism offers a much larger pool of target cells for the virus, and CXCR4 viruses

productively infect lymphocytes better than CCR5 strains (138). The emergence of CXCR4 viruses is also accompanied by a rapid depletion in remaining CD4+ T cells and progression to AIDS (78), though it is unclear whether the co-receptor tropism switch is the cause or the effect of this final stage of immune depletion (246).

Immunodeficiency

Years of persistent immune system activation and inflammation-mediated destruction of lymphatic architecture combine to leave the body unable to produce sufficient T cells to provide even the most rudimentary level of protection against invading microbes (139). The host becomes susceptible to opportunistic infections (OIs), such as *Pneumocystis* pneumonia, esophageal candidiasis, or toxoplasmosis, or certain AIDS-associated cancers, such as non-Hodgkins lymphoma or Kaposi's sarcoma (247). Presentation of any of these or related afflictions, or a drop in CD4+ T cell count below 200 cells/mm^3, is an AIDS-defining event.

During this final stage of the disease, in the wake of a nearly defunct T cell population, macrophages survive as the primary host cell for HIV and continue to support virus production (174). The co-infection of OIs augments viral production in infected macrophages, accelerating end-stage disease and offering a strong indication to strictly control OIs during this critical phase of HIV infection (265). Fortunately, the percentage of HIV patients developing AIDS, and the accompanying incidence of OIs, has dropped greatly since the introduction of highly active antiretroviral therapy (HAART), though certain OIs remain a concern for patients struggling to suppress their viral loads (181).

1.2.3 Viral Survival

Once the virus establishes residence within the host, how is it able to survive amidst an immune system that is fiercely targeting its destruction? As discussed in the previous section, HIV initially puts the immune system at a disadvantage by destroying the bulk of the mucosal CD4+ effector memory T cells, essentially neutralizing much of the body's front line defenses. These sentry cells are necessary to alert the immune system of a recognized pathogen and initiate the immune response against it. Recent data suggests that this subset of effector CD4+ memory T cells within the gut mucosa never recovers to pre-infection levels, even after prolonged virus-

suppressive therapy (238), striking a serious blow to the host's defense system (278). Besides its direct attack on the immune system, HIV utilizes several other mechanisms to ensure its survival within the host. One example is altering the host cell environment to evade immune recognition, prevent apoptosis, and create an optimal setting for viral replication. Many of these mechanisms are mediated by the viral Nef protein and will be discussed in more detail later (see subsection 1.3.2). In addition to tailoring its surroundings to meet its needs, HIV adapts to its host in other ways, such as by selecting for survival mutations and establishing long-lived reservoirs for the maintenance of prolonged, low-level replication.

Survival Mutations

During the high levels of HIV replication characteristic of the acute phase of infection, 10^{10} virions are produced each day (273). The viral transcription machinery is notoriously error-prone, and it has been estimated that, during these times of high virus production, every possible mutation in the viral genome will occur thousands of times a day, with a sizeable fraction of all possible *double* mutations also occurring each day (71,271). As a result, variant strains are inevitably produced that enable the virus to help evade the host immune attack and adapt to changing conditions over the course of the infection (11,217). For instance, mutations in the outer viral envelope protein provide a mechanism of escape from host neutralizing antibodies (299,372). Mutations within viral epitopes presented on the infected cell surface MHC-I molecules have been shown to render HIV-specific CTL responses ineffective (9). Later in the infection, as the pool of CCR5+CD4+ T cells is depleted, viral variants with mutations in the envelope proteins emerge and change the tropism of the virus. These new particles recognize the CXCR4 co-receptor and are able to infect a much larger cellular pool, including the CXCR4+CD4+ central memory T cells (78,140,246).

Reservoirs and Viral dynamics

HIV establishes long-lived reservoirs within the host, posing a challenge for virus eradication. Treatment with HAART has given researchers unique insight into the viral dynamics of HIV infection and provided clues as to those cells serving as reservoirs for HIV particles. By blocking HIV replication with HAART, measurement of viral decay over time allows for the determination of the half-lives of those cells harboring live HIV virions (Figure 1-

2). Since the half-life of HIV in blood is only about 6 hours (273), detectable virus in the plasma must be newly released from cellular reservoirs. Measurement of viral RNA during treatment with HAART reveals a multi-phasic pattern of viral decay and suggests several cell types utilized by HIV as viral reservoirs (33,271,313,336).

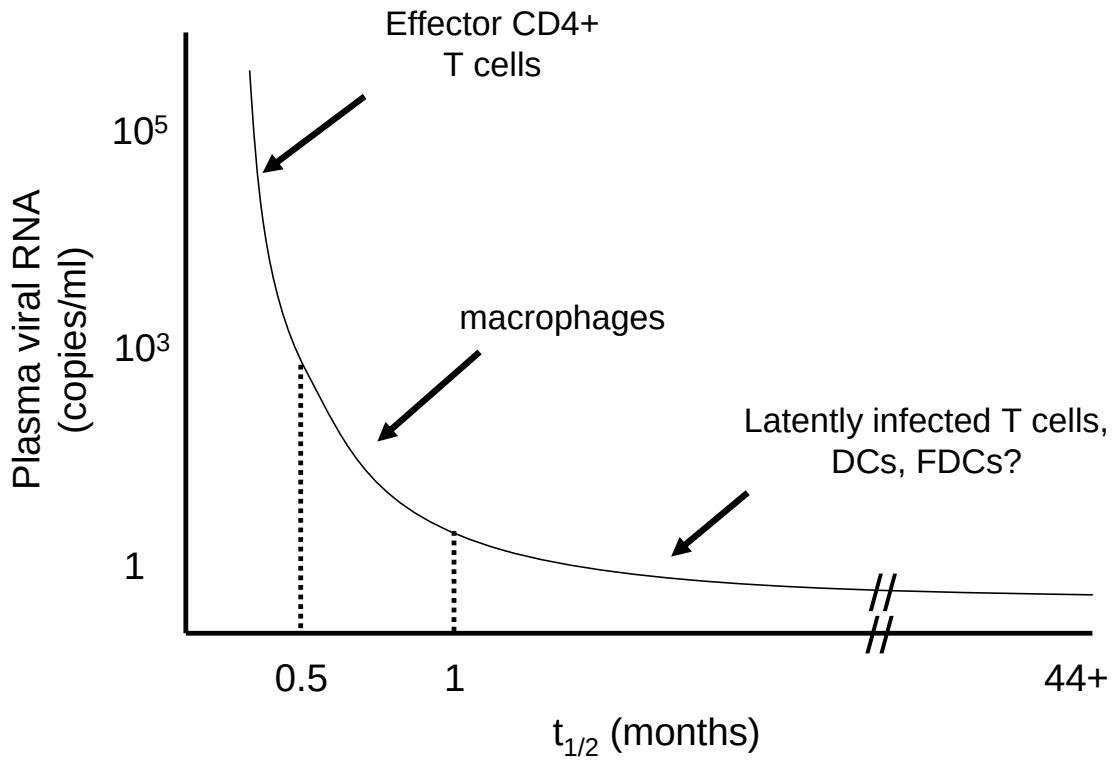

Figure 1-2. HIV-1 viral decay following HAART.

Following the administration of HAART, virus replication is halted and measurement of plasma viral RNA over time suggests the presence of three major cellular compartments for HIV. The first compartment of cells has a half-life of 1-2 days and almost certainly represents the loss of virus contained within effector CD4+ T cells. The second phase has a half-life of 2 weeks and most closely relates to the half-life of macrophages, indicating these cells to be the major viral compartment at this stage of therapy. The final stage includes the longest-lived reservoirs for HIV, likely DCs and latently infected memory T cells.

Following a block in HIV replication, a dramatic and immediate two-log drop in detectable virus is noted after 24-48 hours. This first phase of viral decay represents loss of virus contained within the short-lived CCR5+CD4+ activated effector T cells, that have a half-life of between one and two days during HIV infection (33,162,273,373). The substantial (99%) decrease in viral load indicates that effector T cells comprise the primary viral target cell, however, the significant viral load fraction remaining demonstrates the existence of longer-lived viral stores.

A less robust phase of decay (~ 1 log decrease) then follows over the next two weeks. The second phase represents a minor percentage of virus-infected cells that not only have longer half-lives, but also are more resistant to killing by HIV (336). These cells are likely to be macrophages, which have a half-life of about 2 weeks, represent about 10% of all HIV-infected cells during acute infection, and are more resistant to the cytopathic effects of the virus (33,272,363). Furthermore, HIV-infected macrophages have been shown to release HIV virions for weeks (13,117,174,317). Some longer-lived effector memory T cells may also be reflected in this fraction of infected cells (139,272,336).

A third phase of decay occurs over the next six months, lasts for many years, and correlates best with the very long-lived populations of resting and latently-infected memory T cells and DCs. These are the cells utilized by HIV as viral reservoirs to maintain a lifelong presence in the host. The existence of viral reservoirs was first suspected when a subset of surviving HIV-infected CEM T cells was able to be induced to produce virions (284), then more firmly realized when replication-competent virus could be recovered from patients even after years of successful viral suppression with HAART (106,384,404). The primary cellular reservoir appears to be latently-infected memory T cells (33,95,313) that have a notably long half-life of about 44 months (7 years) (106,322). These T cells are likely infected as active effector T cells prior to reverting to a resting state to survive as long-lived memory cells (323).

It soon became clear that, besides T cells, other cell types likely serve as viral reservoirs (67). Most notably, follicular dendritic cells (FDCs) have been found to facilitate the survival of HIV virions for prolonged periods of time (49). Unlike DCs, FDCs are not infected by HIV (292), but they have been shown to bind and harbor infectious HIV particles for at least 9 months (328), even in the presence of virus-neutralizing antibodies (156). FDCs may even serve as long-lived reservoirs for HIV, though there are conflicting views regarding this point (336,378).

1.2.4 Macrophages are Targets for HIV

Since they were first described as a target for HIV infection (117), macrophages, and related cells of the monocyte lineage, have been increasingly recognized as an important and persistent source of HIV throughout disease progression (77,239,317). HIV-infected macrophages show marked differences from HIV-infected T cells, which will be explored below. Some of these key differences include: resistance to HIV cytotoxicity, methods for incorporation of HIV particles, and the ability to disseminate HIV virions and disease.

Increased survival of HIV-infected macrophages

Unlike effector T cells, that are rapidly depleted during HIV infection, macrophages are relatively resistant to the cytotoxic effects of the virus (117,120,266), and may actually be protected from apoptotic death by HIV (81). Macrophages are not depleted during the course of HIV infection; in fact, while early in the course of HIV infection macrophages make up about 10% of infected cells (33,272,405), by the end of the disease process macrophages represent the predominantly infected cell type (174,265). Similarly, blood monocytes, the circulating precursor of tissue macrophages, demonstrate long-lived infection by HIV, even in the presence of HAART (207,331).

One mechanism for macrophage survival during HIV infection may be the HIV-induced expression of nerve growth factor (NGF), which induces an autocrine survival signal in macrophages (114). The role of NGF is particularly evident in the survival of HIV-infected cells in the brain, offering a rationale for how HIV replication can persist in the CNS for many years without killing its host cell (113,363). In addition, the Nef protein of HIV induces a survival signal in macrophage-like cells in culture (42), a process that involves the activation of the anti-apoptotic factor Bcl-X_L (60). Finally, infected macrophages also impart increased drug-resistance on internalized HIV particles as compared with T cells, offering a mechanism to protect the virus from external anti-HIV factors (129).

Capture of HIV particles

Macrophages localize to the genital mucosae, so they are exposed to HIV from the initial stages of the infection and likely are, along with dendritic cells, among the first cells infected

(209,239). Macrophages are infected preferentially by CCR5-tropic viruses following binding of viral gp120 to the CD4 and CCR5 receptors (138). However, low levels of CXCR4 coreceptors are expressed on macrophages, and some groups have shown infection of macrophages by CXCR4-tropic viruses (363,399).

Besides being infected by HIV, macrophages can also capture whole HIV virions and later present these infectious particles to other immune cells weeks after their initial uptake (77,117,317,363). Orenstein et al. demonstrated initially that macrophages internalize whole HIV particles within intracellular structures, now known to be late endosomal compartments (290), in contrast with infected T cells that feature virions scattered around the outer membrane surface (266). Some particles may be internalized into macrophages by the uptake of opsonized (antibody-coated) HIV particles via Fc receptors or by complement-coated virions via complement receptors (239,363). HIV virions can also bind to macrophage surface receptors, including C-type lectins or the macrophage mannose receptor (MMR) (56,257,357,363). MMRs have been shown to be responsible for up to 60% of all macrophage-bound HIV particles (257). Additionally, macrophages engage in macropinocytosis, a process of non-specifically internalizing extracellular fluid and antigens, including HIV particles (233). It is likely that HIV, via gp120 envelope proteins, binds initially to syndecan or other heparan sulfate proteoglycans on the surface of macrophages, allowing for close association with the cell membrane prior to being internalized into macropinosomes (34). Internalized virions can be released following fusion of the virus-containing endosomes or macropinosomes with the plasma membrane, allowing for the infection of nearby immune cells (233,290). This method of release is distinct from T cell virion budding and may account for some of the differences in membrane markers between macrophage- and T cell-derived viruses (363).

Dissemination of disease throughout immune system

HIV viruses within macrophages engage in numerous alterations to signaling pathways to promote viral dissemination. Notably, the HIV Nef protein induces the secretion of the chemokines MIP-1α and MIP-1β that attract T cell targets for subsequent infection (344). In addition, Nef induces the release of soluble CD23 and ICAM molecules that stimulate nearby APCs to activate resting T cells, producing a T cell environment favorable for HIV replication (343).

Macrophages appear well suited for the ability to help deplete nearby T cells through a variety of other mechanisms. One of the earliest known mechanisms described for macrophage-induced T cell death is the finding that infected macrophages can fuse with adjacent uninfected CD4+ cells to form lethal syncytia (85). Similarly, infected macrophages have been shown to kill CD8+ T cells in a gp120/TNF-mediated fashion (158). Uninfected macrophages can mediate apoptosis in nearby T cells from HIV-infected individuals through the engagement of FasL and TNF-alpha apoptotic pathways (21). MMRs play a key role in the transfer of macrophage-bound virions to nearby T cells, as 80% of the virions transferred from macrophages to T cells in culture are blocked by MMR inhibitors (257). Finally, during the late stages of disease, the co-existence of opportunistic infections further augment macrophage-mediated HIV production (265). This may represent an additional mechanism for the maintenance of high viral loads in the absence of CD4+ T cells during the final stage of HIV infection. By exploiting normal macrophage function, and inducing alterations to other physiologic signals, HIV commandeers macrophages to assist in its efforts to infect and destroy the immune system.

Role in CNS disease

Besides their role in HIV-induced damage to host defense, macrophages have long been implicated in the development of HIV-associated dementia in AIDS patients (197,377). It is now well understood that monocytes/macrophages are the primary, if not exclusive, mechanism for delivery of HIV into the central nervous system (CNS) (116,183). Monocytic infection in the brain plays a critical role in HIV-mediated neurotoxicity and the establishment of a protected anatomical HIV reservoir (313,332,363). Once in the CNS, macrophages are believed to spread the virus to microglial cells (115,183), the resident monocyte-derived immune cells of the brain (145,365). Together these two cell types make up the only resident brain cells capable of supporting productive HIV infection (82,220). HIV-infected macrophages and microglia both exhibit dysfunctional signaling, which has been postulated to contribute to neuronal degeneration and toxicity, leading to HIV dementia (115,183,332). One example of perturbed signaling is the HIV gp120-induced release of inflammatory cytokines from microglia/macrophages, which leads to apoptosis in neurons (126,184). Also, the release of Fas ligand from HIV-infected macrophages induces apoptosis of nearby astrocytes (14), similar to macrophage-induced killing of CD4+ T cells in HIV-infected individuals (21).

HIV utilizes T cells to establish infection and for mass replication during the acute and chronic stages of the disease. In contrast, HIV seems to exploit the mobility and longevity of macrophages, as well as their resistance to the cytotoxic effects of HIV, to enhance viral dissemination and develop persistent infection. The unique role of macrophages in HIV disease, and their differential response from T cells to current antiviral agents (15), highlights these cells as important targets for anti-viral drug therapy.

1.2.5 Current Therapeutic Options

Currently, twenty antiviral agents are approved for treatment of HIV in the United States (358). These drugs are grouped into four major categories: (1) nucleoside reverse transcriptase inhibitors, (2) non-nucleoside reverse transcriptase inhibitors, (3) protease inhibitors, and (4) fusion inhibitors.

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)

After being unpackaged and released into the host cell, the single-stranded viral RNA genome is reverse transcribed into double stranded viral cDNA by the *pol* gene product, reverse transcriptase (RT) (58,131). NRTIs, most of which are nucleoside analogs, act at this step in the viral replication cycle by competing with native nucleotide molecules for addition to the growing nucleotide chain (298). NRTIs lack a critical 3' hydroxyl group required for nucleotide chain extension, such that once an NRTI is incorporated into the cDNA chain, the transcriptional process is halted (298). AZT (zidovudine), the first drug approved for treatment of HIV, is a potent NRTI (99,283). Whereas nucleo*s*ide inhibitors require tri-phosphorylation by cellular kinases to function properly, nucleo*t*ide inhibitors, such as the anti-HIV agent tenofovir, do not require the initial phosphorylation step and may have greater activity across infected cells as a result (354).

Non-nucleoside reverse transcription inhibitors (NNRTIs)

NNRTIs target the same reverse transcriptase enzyme as NRTIs, though their mechanism of action differs. Rather than competing with cellular nucleotides to block cDNA chain

extension, NNRTIs bind the RT enzyme at sites distant from the active site and induce conformational changes that interfere with catalytic activity (283). RT mutations that confer resistance to NRTIs do not necessarily block the activity of NNRTIs, and NNRTIs can be used in combination with NRTIs to synergistically block HIV activity (89). Only three NNRTIs are currently approved for HIV therapy: delavirdine, efavirenz, and nevirapine (358).

Protease inhibitors (PIs)

Following reverse transcription, viral cDNA integrates into the host chromosome, where it awaits proper host signals to begin the production of viral mRNAs. Several viral gene products require post-translational proteolytic processing, a function that is carried out by the viral protease protein. PIs target the active site of the viral protease, to prevent the production of mature, infectious viral particles (283). The appearance of PIs marked a celebrated advancement in the treatment of HIV, as these drugs were quickly realized to be among the most powerful anti-HIV agents available. However, the price paid for the enhanced efficacy of PIs is a myriad of toxic side effects, including serious metabolic and lypodystrophic complications that routinely limit the utility of this drug class (283).

Fusion inhibitors

The outer surface of the HIV particle contains the *env* gene products: gp120, a surface molecule that binds the CD4 and chemokine receptors found on HIV target cells, and its associated subunit gp41, a transmembrane protein that mediates viral fusion and entry into the target cell (54,91). After gp120 binds to host cell receptors, the trimeric coiled-coil gp41 molecule undergoes a conformational change to reveal hidden fusion domains that attach to the target cell plasma membrane (54,131). Subsequent fusion of the viral and cellular membranes leads to injection of the viral core into the host cell (54). Enfuvirtide, the only fusion inhibitor in clinical use, acts by binding directly to gp41 and preventing the conformational change necessary for virus:cell fusion (205).

Highly active antiretroviral therapy (HAART)

Due to the high error rate of the RT machinery (271), drug-resistant viral mutants are rapidly selected. For this reason, patients are treated with combination therapy, or HAART, that

involves the use of several anti-HIV agents taken from different classes of viral inhibitors. A typical starting regimen consists of two NRTIs and either an NNRTI or a PI (111). Recently, three such drugs, emtricitabine and tenofovir (both NRTIs) plus efavirenz (an NNRTI), have been combined into a once-a-day pill that should greatly enhance patient adherence (359). However, resistance mutations continually develop, either naturally over time or due to poor patient commitment to the treatment regimen (298). These mutant viruses are stored in host cellular reservoirs likely for the life of the host (106). In addition, resistance mutations to one inhibitor frequently confer cross-resistance to the rest of the drugs in its class; a prime example is the K103N mutation of the RT enzyme, which confers resistance to all three members of the NNRTI class of anti-HIV agents (111). Acquisition of cross-resistance mutations, along with the toxicities associated with many of these antiviral agents, can severely limit the therapeutic options for some HIV-infected patients over time, necessitating the need for new drug discovery and vaccine development (298). Development of new anti-HIV agents, in turn, requires a better understanding of the molecular mechanisms of HIV pathogenesis.

1.2.6 Vaccine Outlook

The development of a safe and effective HIV vaccine is imperative to halt the continued spread of this incurable disease. However, despite over 25 years of HIV research, no effective vaccine has been discovered. The most notable vaccine trial to date is the VaxGen trial that utilized the HIV gp120 envelope protein in an effort to elicit sufficient antibody response to prevent HIV infection. Unfortunately, the results of this first and only completed Phase III HIV vaccine trial showed that the vaccine provided no protective effect against HIV infection (125).

Early studies in rhesus macaques offered hope that viral strains with targeted gene disruptions could be used to vaccinate against fully-pathogenic strains. In particular, a strain of SIVmac239 with a deletion in part of the *nef* gene was found to effectively vaccinate against challenge with intact SIVmac239 in macaques (87), as were strains of SIV carrying multiple gene deletions (386,387). However, the excitement over these findings was quickly tempered due to subsequent findings that a disrupted *nef* gene could be repaired over the course of multiple viral replication cycles to form a functional gene (50,308,375). These reports initiated concerns

over the use of a *nef*-disrupted HIV strain as a human vaccine. In addition, these findings offered early insight into the importance of a functional *nef* gene for viral pathogenesis.

Even when *nef*-disrupted SIV strains did not undergo repair to become pathogenic, these attenuated viruses did not always protect against challenge. A combination *nef*- and *vpr*-deleted strain of SIVmac239, that protects against infection with the SIVmac239-related SIVmac251 strain, protected poorly against challenge with a non-homologous SIVsmE660 strain (386). Also, immunization of monkeys with a modified vaccinia virus engineered to express SIV proteins was unable to offer protection against challenge with wild-type SIVmac239 (167). These reports suggest that strong antibody responses to one viral strain may be ineffective against infection with other non-homologous viruses. This problem of a lack of protection against heterologous strains has recently become apparent in humans as well. Patients have been described who, despite controlling their initial HIV infection, became superinfected with HIV from a different clade (177,289). Even more daunting to the prospect of developing an HIV vaccine, Altfeld et al. report a patient infected with a second HIV clade B virus, despite having strong CD8+ T-cell responses to 25 different epitopes from his original B strain (10).

While researchers continue to search for a much-needed HIV vaccine, it is imperative that other groups continue to develop new treatment options, not only for those already infected with HIV, but for those who will undoubtedly be infected in the future. One method for identifying novel therapeutic agents is to study those virulence factors that interact with host proteins to mediate viral pathogenesis. One potential target for pharmacologic intervention is the *nef* gene product, a critical HIV virulence factor.

1.3 HIV-1 NEF CHARACTERISTICS

The HIV-1 *nef* gene product is a 27 kDa protein that performs numerous critical functions during viral infection and pathogenesis (8,86,122,137), many of which will be described below. Nef lacks intrinsic enzymatic activity, and it is presumed to exert its effects via binding to host proteins. Examination of *nef* sequence alignments reveals that the protein product contains several conserved, well-described sequence motifs implicated in binding to distinct intracellular targets: the acidic region, E_{62}EEE (PACS-1); the dileucine motif, L_{164}L (AP-1/2/3); the proline-rich motif, P_{72}xxP (SH3 targets like Vav and Src kinases); and the diarginine motif, R_{105}R (PAK1/2) (18,124). (Nef numbering based on Shugars et al. Consensus sequence (319).) Crystallization of the HIV-1 Nef core domain and the flexible N-terminal region, in addition to molecular modeling of the C-terminus, reveal that Nef has a stable core domain with several conserved motifs accessible on the outer surface of the protein (Figure 1-3) (123,124,143). Nef is myristoylated at its N-terminus and targeted to the plasma membrane (180). In addition, a cholesterol recognition motif at its C-terminus may also target Nef to cholesterol-rich regions of the plasma membrane, including lipid rafts (406).

1.3.1 Importance of *nef* for viral pathogenesis in vivo

Primates

The first evidence of the critical nature of *nef* in viral pathogenesis came from studies using the SIV rhesus macaque model. Macaques infected with SIV present with an AIDS-like phenotype that mimics human disease, including key features such as: depletion of CD4+ T cells, opportunistic infections, wasting, and early death (187). The hallmark finding demonstrating the positive effect of *nef* on HIV pathogenesis occurred when Kestler et al. described macaques infected with a *nef*-deleted strain of SIV that failed to develop disease (188). Since this finding, several other groups have confirmed that the targeted disruption of the unique portion of the SIV *nef* gene, in the context of an otherwise fully-intact virus, renders the virus non-pathogenic in

rhesus macaques (87,188,387,388). Interestingly, numerous reports describe the existence of strong selective pressures that drive the reversion of some mutated SIV *nef* genes to functional coding sequences capable of restoring both *nef* function and viral pathogenicity (53,188,249,308,375).

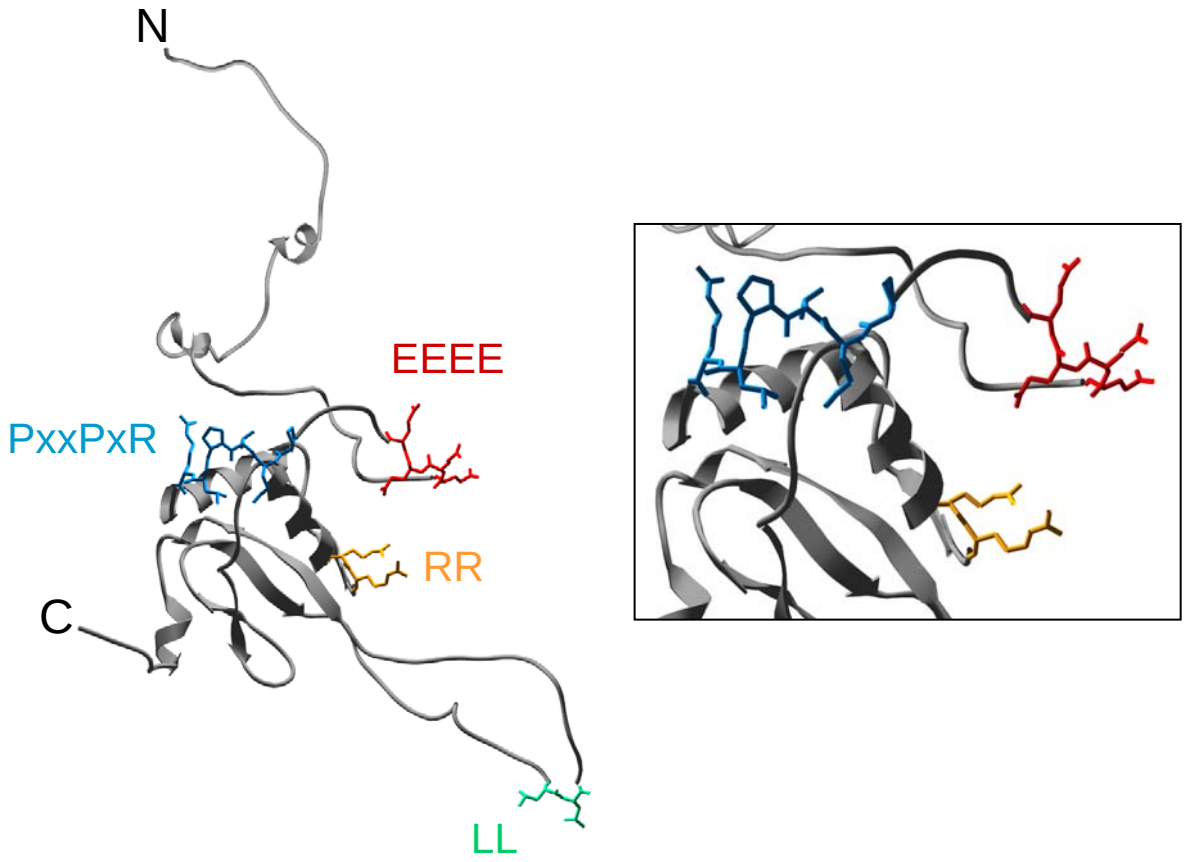

Figure 1-3. Molecular model of HIV-1 Nef.

The Nef structure consists of a long unstructured N-terminal region, a central core, a flexible internal loop, and a short C-terminus. The internal loop of the central core was deleted to solve the crystal structure, so its structure is unknown. Cellular binding motifs are color-coded: the SH3-binding motif, PxxPxR (blue); the acidic motif, EEEE (red), the diarginine motif, RR (gold); and the dileucine motif, LL (green). Inset: Close-up of the core domain. Highlighted residues correspond to the labeled motifs indicated in the full structure. This model is based on that assembled by (124).

Mice

No known retroviral pathogen naturally infects mice in a manner similar to HIV in humans or SIV in monkeys, likely because mice lack the necessary CD4-like viral co-receptors for entry into cells. However, AIDS-like disease can be closely simulated in mice by transgenically expressing the HIV-1 coding sequence under control of the human CD4 (CD4C) promoter gene regulatory sequences (147). This transgenic method targets the expression of viral genes to those cells susceptible to HIV infection, namely CD4+ T cells, macrophages, and DCs. Mice expressing the full HIV genome suffer from AIDS-related effects including immunodeficiency, CD4+ T cell lymphopenia, thymic atrophy, wasting, and early death (147). Follow-up studies showed that the same disease phenotype can be elicited by expressing just the HIV *nef* gene alone behind the CD4C promoter (148). Thus, the mouse model provides strong evidence that Nef alone is a major factor in the induction of HIV pathogenicity.

Humans

Human studies indicate the clinical importance of a functional *nef* gene in the context of HIV infection. Whereas most untreated HIV patients succumb to AIDS within ten years of initial infection, a subset of HIV-infected patients, referred to as long-term nonprogressors (LTNPs), maintain normal CD4+ T cell counts and remain disease-free for ten years or more. Numerous reports describe viruses recovered from LTNPs that contain mutations or deletions within the *nef* gene (90,118,192,211,234), though these findings demonstrate solely an association between *nef* mutations and lack of disease progression and not a direct *nef*-mediated cause for progression. Recently, many of the earliest-described LTNPs have been reported to show signs of disease, suggesting that *nef* deletions are not completely protective against the advancement of HIV disease (68,132,211). These findings correlate with macaque studies showing that *nef*-deleted SIV delivered at high titers can cause disease, though progression is markedly delayed compared with wild-type virus (20,165,388). *nef* sequence variability and function has been correlated with HIV disease progression throughout the course of infection (51,191), and at least one mutational study details how *nef* can evolve compensatory mechanisms to preserve critical functions (259). Furthermore, Carl et al. describe a *nef*-disrupted strain of HIV-1 that underwent repair of its 36-bp deletion and partial restoration of its function,

indicating a selective pressure for functional Nef in human infection (50). While disruption of the *nef* gene alone in humans appears insufficient to completely abrogate disease progression, it seems clear that *nef* plays an important role in the pathogenesis of HIV.

1.3.2 Cellular functions of the *nef* gene product

Analyses of the functions of HIV-1 Nef have revealed its roles in numerous aspects of viral pathogenesis. Initially, it was thought that the *nef* gene product was an inhibitor of viral pathogenesis, since it was shown to decrease viral transcription and replication in culture (7,227,347), and was thus named *ne*gative *f*actor (112). However, since these early findings, much evidence has been accumulated that demonstrates the strongly positive effect of *nef* on HIV pathogenesis. The reason for this conflicting data may be due in part to the expression-dependent effects of Nef within cells (221). One clue to the importance of Nef in viral pathogenesis is that *nef* is transcribed very early in the HIV life cycle, even before integration of the viral DNA into the host chromosome (385). In addition, Nef is packaged into newly-produced virions, prompting suggestions that Nef plays a role in virus budding and/or the establishment of infection in the next target cell (268,374). However, a recent report challenges the notion that the inclusion of Nef in new virions is essential for viral pathogenicity (102).

Nef has been found to have several critical roles in the establishment and maintenance of HIV infection within the cell. First, Nef protects and enhances the survival of the infected cell until viral replication can occur, by promoting both immune and viral evasion and by blocking apoptotic signaling. Second, Nef optimizes the cellular environment for viral replication by inducing cellular activation and altering the content of the plasma membrane for optimal virion release. Finally, Nef is involved in numerous other cellular signaling and trafficking pathways, though the rationales for some of these effects are not fully understood. Each of these functions is considered in more detail below.

Enhancing infected cell survival

As discussed previously, CD8+ cytotoxic T lymphocytes (CTLs) detect and destroy virus-infected cells by recognizing cell surface major histocompatibility–I (MHC-I) molecules

displaying viral antigens. Yet HIV-1 infected cells are protected against CTL-mediated killing, a phenomenon dependent on the presence of the *nef* gene (76). Specifically, the HIV-1 Nef protein downregulates surface expression of the MHC-I/antigen complex by targeting MHC-I, via PACS-1, away from the surface and to the *trans*-Golgi network (32,314). A 40% reduction in surface expression of MHC-I molecules was found to render CEM-E5 T cells less susceptible to killing by CTLs (309). This is a highly conserved function of Nef early in infection as demonstrated by functional analysis of allelic variants over the course of infection (50,51). In addition to hiding from surveilling CTLs, Nef promotes cell survival by directly killing approaching CTLs. Nef induces upregulation of the death factor FasL on the infected cell surface (392). FasL binds to Fas (CD95) receptors prevalent on nearby immune cells, including HIV-specific CTLs, and induces their apoptosis (255).

Nef also promotes the downregulation of the CD4 surface receptor by directly binding CD4 and linking it to both the AP-2 sorting protein and the COP-1 lysosomal targeting protein (91,151,279). Lowering the surface expression of CD4 may be advantageous for the infected cell for several reasons: averting superinfection by other HIV virions, including those virus particles newly released from the infected cell (286); increasing the infectivity of newly produced virions (206,301); reducing CD4-mediated inhibition of HIV transcription (28); and by releasing the CD4-associated Lck signaling molecule, which may promote T cell activation and enhanced viral replication (280).

Recent reports describe a less direct mechanism of Nef-mediated protection of the infected cell – inhibition of B cell immunoglobulin class switching (287). HIV greatly diminishes host IgA and IgG responses, and this effect appears to be Nef-mediated. Qiao et al. demonstrate that soluble Nef, known to be released by HIV-infected cells (110), penetrates and activates negative feedback pathways in B cells that interfere with CD4+ T cell-governed B cell class switching (287). As a result, HIV can suppress the host humoral response to the virus, providing yet another level of protection against immune attack.

Finally, Nef enhances cell survival by specifically blocking or subverting a multitude of cellular apoptotic pathways within the infected cell. In T cells, Nef binds and inhibits apoptosis signal-regulating kinase-1 (ASK-1), a common mediator of both the Fas/FasL and tumor necrosis factor-alpha (TNFalpha) apopototic pathways (119). Similarly, through an association with phosphatidylinositol 3-kinase and p21-activated kinase, Nef blocks Bad-mediated apoptotic

signaling (383). Nef has been shown to reduce the surface levels of CD28, a co-stimulatory molecule that signals the stabilization of IL-2 transcripts and may play a role in activation-induced apoptosis (2,27,342). By lowering CD28 levels, the infected cell is able to induce activation to promote viral replication without inducing IL-2-mediated cell proliferation, which would sequester resources needed for viral replication. Further, without full CD28 signaling, the cell cannot be maximally stimulated, a condition that would likely result in activation-induced apoptosis (137). Along these lines, Nef also induces the endosomal accumulation of the TCR and Lck signaling molecules in T cells, a maneuver that may block activation-induced apoptosis, while also liberating signaling pathways for HIV-mediated exploitation (350). In macrophages, Nef induces a survival signal through the Erk/MAPK pathway, resulting in increased expression of the anti-apoptotic factor Bcl-X_L (60). In addition, Nef also has been shown to activate signal transducer and activator of transcription (STAT) 1 (104) and 3 (270), and Nef-associated STAT3 activation has been shown to correlate with macrophage cell survival (42). By interfering with normal cellular apoptotic pathways and promoting cell survival, Nef enables the infected cell to bypass host attempts to self-destruct and allow the virus sufficient opportunity to reproduce and maintain infection.

Optimizing host environment for viral replication and infectivity

Besides enhancing survival of the infected cell, Nef optimizes the cellular environment for viral replication and infectivity. One requirement for efficient viral replication is the activation of the host cell (402), and increased activation of T cells has been shown to be predictive of HIV disease progression (154). Nef plays a role in helping to lower the threshold for T cell activation, which may assist the virus with integration and early transcription (312,385). Accordingly, in T cells, Nef activates the cellular Erk/MAPK pathway, a key mediator of both T cell activation and HIV function (311). Nef also induces the activation of NFAT, a key T cell transcription factor involved in IL-2 gene expression, via a TCR-independent, calcium-mediated mechanism (232). Further, Simmons et al. report that Nef induces a cellular gene expression profile in an oncogenic T cell line that closely resembles the activation profile induced by anti-CD3 signaling (326). Directly following TCR/CD3 engagement, the Src family kinases Lck and Fyn are activated, which in turn phosphorylate and

activate TCR-ζ and ZAP-70 kinase (169). Nef requires TCR-ζ and ZAP-70 to yield its full expression profile (326) and has been shown to bind TCR-ζ directly (391).

In addition to direct manipulation of the T cell environment, Nef in HIV-infected macrophages can modulate the activity of nearby T cells to prime them for infection. Specifically, Nef induces the release of the chemokines MIP-1α and MIP-1β that attract T cells to the site of the infected macrophage (344). Further, mimicking CD40L signaling, Nef induces NF-κB-mediated release of soluble CD23 and ICAM, which in turn upregulate co-stimulatory receptors on nearby B cells (343). These stimulated B cells then interact with and prime neighboring CD4+ T cells, inducing a state amenable to HIV entry and productive infection (343).

Finally, Nef mediates the infectivity of new viruses in part by optimizing the makeup of the cell membrane at the site of virion budding. The presence of Nef at the cell membrane has been reported to be critical for the production of infectious viral particles (66). The mechanism of Nef effects on infectivity may involve its ability to increase cholesterol synthesis in the host cell and target the cholesterol to detergent-resistant membranes (DRMs), the preferred site of HIV budding and release (263,406). DRMs, also known as lipid rafts or glycolipid-enriched membrane domains (GEMs), are regions of the plasma membrane enriched in cholesterol and glycolipids (327). The effect of Nef on cholesterol targeting correlates with increased delivery of Nef into viral particles and an increase in virion infectivity (406).

Other Effects of Nef on Cellular signaling

Nef has been shown to be involved in the alteration of numerous other cellular signaling pathways, though the functional consequences of many of these signaling perturbations are unknown. In T cells for instance, Nef has been shown to bind the key T cell signaling molecule Vav, known to mediate cytoskeletal rearrangements (101). Also, Nef induces the phosphorylation of the proto-oncogene c-Cbl (396), though the mechanism and implication of this effect is not well understood.

Much work has centered upon the association between Nef and a cellular serine kinase termed Nef-activated kinase (NAK) (26), identified recently as p21-activated kinase 2 (PAK2) (293). Several groups have shown that Nef can bind and activate PAK2 (6,45,293,366) and that this interaction is highly conserved (118,193). The functional consequences of the Nef-PAK2

complex are not fully understood, but some reports are beginning to delineate the importance of this interaction. For instance, the Nef-NAK complex may be involved in augmenting HIV infectivity in mononuclear cells (382). Recently, Nef-PAK2 was found to inhibit dendritic cell maturation and MHC-I/antigen presentation, though the reasons for these functions are unclear (231).

Another major group of signaling proteins known to interact with Nef is the Src family of tyrosine kinases. Src kinases have been implicated in numerous cancers and have established roles in cell growth, differentiation, and survival (31,222,269,349). Over the last decade, much work has focused on the binding of Nef with Src kinases, though little effort has been directed toward investigating the ability of Nef to activate these key signaling proteins. Further, there has been much controversy in the field regarding the role of Src kinases in Nef signaling, in part due to assay differences and the use of Src protein fragments instead of full-length kinases. Below I will give a description of the structure and regulation of Src kinases, followed by an overview of the current understanding of the interactions between Nef and the family of Src kinases.

1.4 STRUCTURE AND REGULATION OF SRC FAMILY KINASES

The Src family of non-receptor tyrosine kinases consists of eight members in humans, as grouped by sequence homology and shared structural domain characteristics: Blk, Fgr, Fyn, Hck, Lck, Lyn, c-Src, and c-Yes (47,349). Three of these kinases (Fyn, c-Src, and c-Yes) are expressed across most cell types, while the remaining family members have more restricted expression patterns, predominantly to hematopoietic cells (349). A ninth SFK, Yrk, is found in chickens but not humans (337). Four other related tyrosine kinases initially classified as Src kinase members, Brk, Frk, Srm, and Src42A, have recently been re-classified into the Brk family of tyrosine kinases (316) and will not be discussed further in this document.

1.4.1 Overall Structure

The crystal structures of c-Src and Hck clearly delineate the distinct modular structural arrangement shared by this kinase family (Figure 1-4) (83,321,379,390). SFKs exhibit a myristylated N-terminal unique region (Src-homology 4 domain), a Src-homology 3 (SH3) domain, a Src-homology 2 (SH2) domain, an SH2-kinase linker, a bi-lobed tyrosine kinase domain (Src-homology 1 domain), and a C-terminal tail sequence featuring a tyrosine-based motif.

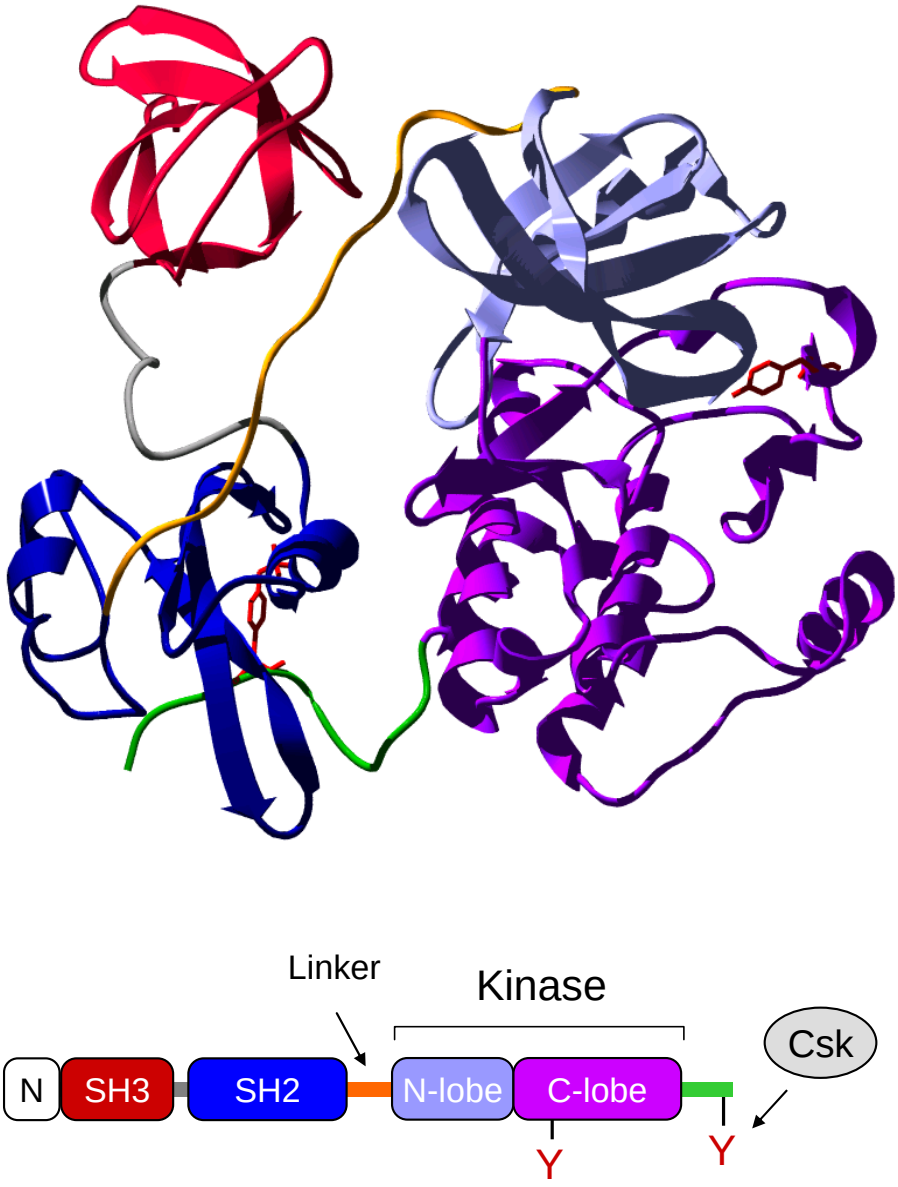

Figure 1-4. Structure of the inactive conformation of an SFK.

Domains in the structure are color-coded and correspond with the schematic below. The N-terminus was deleted prior to crystallization and is omitted from this model. Phosphotyrosine residues pY416 (on kinase lobe) and pY527 (on C-terminal tail) are colored red in the structure and indicated on the schematic diagram. Note the intramolecular contacts between the SH3 domain (red) and the SH2-kinase linker (gold), as well as between the SH2 domain (blue) and the phosphorylated C-terminus (green). Csk phosphorylates the inhibitory residue Tyr527 (see section 1.4.7). The image presented is derived from the crystal structure of Hck-YEEI reported by Schindler et al. (310).

1.4.2 Unique domain

The unique domain of Src kinases lacks a defined structural motif and, likely due to high flexibility, has proven refractory to crystallization. All SFKs contain a consensus N-terminal myristoylation sequence (Met-Gly-X-X-X-Ser/Thr) that targets them to the cell membrane (295,296). Membrane targeting has been shown to be essential for the full function of Src kinases (47,179,397). In addition, all SFK members, except c-Src and Blk, contain a palmitoylation motif at their N-termini (296) that maintains membrane targeting of the protein and partitions them into specialized lipid microdomains involved in enhancing the initiation and transduction of receptor-mediated signaling (46,169,179,210). Recently it has been proposed that the myristoylated end of c-Src kinase, when not bound to the membrane, could associate with a hydrophobic recess within the C-terminal lobe of its own kinase domain and induce the formation of a downregulated structure (83). A similar mechanism was first reported to occur in the inactive conformation of the Src-related kinase Abl (254).

As the name implies, the N-terminal unique domains differ among Src kinases and could indicate a mechanism for the selectivity of these closely related proteins for their distinct sets of downstream targets (267). For instance, a di-cysteine motif in the N-terminus of Lck determines its ability to specifically bind the TCR co-receptors CD4 and CD8α (356). Also, exchange of the unique domain of c-Yes for that of c-Src blocks c-Src signaling, indicating N-terminal-mediated signal specificity that cannot necessarily be substituted by replacing the N-terminus of one SFK for that of another (338).

1.4.3 Modular binding domains

The SH3 and SH2 regions fold into well-defined structural modules, and their combination in the same SFK molecule gives them the appearance of beads on a string. These domains lack catalytic activity and, instead, exert their effects via protein-protein interactions. Each domain is capable of independent functioning (72,285); the SH3 domain associates with

polyproline helical structures (401), and the SH2 domain binds phosphotyrosine-based motifs (368). Domains from different proteins present differential target selectivity due in part to subtle sequence variations within their binding surfaces.

SH3 domain

The SH3 domain is a small module of about 55-70 amino acids that contains small hydrophobic grooves on the outer surface of the domain suitable for binding appropriately positioned prolines on target peptides (72,201,401). The core of these target peptides contains the canonical PxxP motif that directly binds the SH3 hydrophobic pockets, as well as residues that serve to provide stability for the peptide and confer specificity for the SH3 target (401). The SH3 recognition motif adopts the unique structure of a left-handed polyproline type II (PPII) helix (5,72). This helix is oriented such that a complete turn is made after three residues. In this way, both of the conserved prolines can point in exactly the same direction – toward the SH3 domain. The residues within this PxxP motif, where "x" is any residue, are referenced to the initial proline. Thus, the first proline position is designated P_0 and the second proline position is P_{+3}. The central proline in this motif (P_0) interacts with, among other residues, a key tryptophan located within one of the hydrophobic pockets in the SH3 domain (98).

SH2 domain

The SH2 domain is a structural binding module of about 100 amino acids that recognizes phosphotyrosine-based motifs (72,201,367). Similar to SH3 domains, SH2 domains contain two regions for ligand binding. One pocket in the domain accommodates the phosphorylated tyrosine while the other pocket recognizes a hydrophobic residue three residues C-terminal to the Tyr. This binding arrangement has been compared to a two-pronged plug binding a socket (368). All SH2 domains contain an invariant arginine residue (Arg174 in c-Src (367)) within the first hole of the socket that complexes with the phosphorylated tyrosine. The second binding cavity and surrounding surface residues vary among SH2 domains to confer specificity to the SH2-binding motif. Unlike the helical nature of SH3-binding peptides, SH2-binding sequences form extended conformations that lie across the SH2 domain (72,201).

1.4.4 Tyrosine kinase domain

The kinase domain of SFKs represents the core of the protein machinery. This region of the molecule is responsible for recognition of, and catalytic transfer of a phosphate group to, the target sequence. The phosphorylation event occurs at the catalytic site, a wedge of space between the kinase lobes containing carefully coordinated residues. The catalytic site is surrounded by key amino acid residues integral to the binding of both the substrate sequence and ATP, the latter of which provides the phosphate and the energy to power the phosphotransfer reaction (196).

The sequence of the catalytic region is highly conserved among SFKs, and known SFK kinase domain structures – c-Src (38), Fyn (190), and Lck (394) – are essentially transposable. Further, the kinase domain shares high structural homology with other members of the protein tyrosine kinase family, such as insulin receptor kinase (171,172), C-terminal Src kinase (Csk) (208,260), and Abl kinase (254), demonstrating structural conversation of this key signaling device. Key residues within the c-Src catalytic site that mediate kinase activity have been identified and include (chicken c-Src numbering is used throughout for all SFKs): Asp386 and Asn391, which coordinate the magnesium ion required for catalysis; the DFG motif (amino acids 404-406), the Asp404 of which coordinates the magnesium ion of ATP; and Lys295 and Glu310, which form a salt bridge that governs the association of ATP with the tyrosyl substrate in the active conformation (38,300).

The catalytic site is guarded by an activation loop "switch" that opens to allow ATP and substrate binding during activation and closes upon inhibition of the kinase (152). Phosphorylation of the activation loop tyrosine, Tyr416, enhances activation of the kinase and is required for full kinase activation (285). This occurs through phosphorylation-mediated displacement of the activation loop from the catalytic site opening (3). When unphosphorylated, Tyr416 creates hydrogen bonds with Arg385 and Asp386, located in the center of the catalytic cleft, effectively blocking the opening (310,389). Phosphorylation of Tyr416 however, disrupts the association of Tyr-416 with catalytic site residues and, instead, induces the loop to adopt an extended conformation, allowing for contacts with Arg385/363 and Arg409/387 along the outside of the cleft (83,394). Phosphorylation of Tyr416 thus removes the inhibitory action of

the activation loop and throws the activation loop "switch" to the "on" position to allow for entry of ATP and substrate into the catalytic cleft (152).

Movement of the activation loop is partnered with rotation of a key helical structure within the kinase domain, helix αC. In the active conformation, Glu310 of helix αC coordinates with Lys295 in the catalytic cleft to form a salt bridge important for efficient enzyme function (310,389). When the activation loop is closed, however, the αC loop is forced to rotate outside of the cleft, breaking the salt bridge.

Flexibility is critical for proper action of the kinase (38,152). Without the ability of the catalytic site to rotate and flex, the protein would be unable to accept ATP and release ADP, as well as to bind and release the substrate to be phosphorylated. Any alterations to the molecule that prohibit motion within the kinase domain would thus suppress kinase activity.

1.4.5 C-terminal tail

The C-terminal sequence of SFKs contains a weak tyrosine-based SH2-binding motif as compared with preferred SFK SH2 binding peptide sequence (330). Upon phosphorylation of the inhibitory tyrosine (Tyr527) by a negative regulatory kinase, such as C-terminal Src kinase (Csk) or Csk-homologous kinase (CHK) (see section 1.4.7), the tail sequence binds intramolecularly to the SH2 domain, internally locking this binding module (47). Dephosphorylation of Tyr527 promotes release from the SH2 and contributes to activation of the molecule.

During activation, the unphosphorylated tail of c-Src has been shown to fold back onto itself, via the stacking of Tyr527 with Pro529, and tuck into a recess within the C-lobe of the kinase domain (83). Most SFKs share a proline at position 529 and are thus predicted to mimic this c-Src fold-and-tuck maneuver during the active state. Hence, during activation, the tail can be sequestered from its inhibitory association with the SH2 domain by binding to the recess within the kinase lobe. However, during inhibition, the tail releases from the C-terminal kinase lobe, possibly replaced by the myristoylated N-terminus, and once again binds intramolecularly to the SH2 domain.

1.4.6 Intramolecular regulation

The presence of two sets of binding partners, SH3 plus the PPII motif and SH2 plus the tyrosine-based motif, in the same molecule predicts the possibility of cooperative intramolecular interactions within these kinases. Indeed, crystal structures have revealed that both sets of interactions exist in the downregulated (inactive) conformation of the kinase (Figure 1-4) (321,379,390). These intramolecular associations offer unique mechanisms for regulation of kinase activity, in addition to roles for protein-protein *inter*molecular interactions in the kinase activation.

Multiple regulatory mechanisms exist to maintain control over the activities of these potent kinases, thereby allowing the kinases to operate as cellular "rheostats" instead of oncogenic activators (222). The role of the activation loop tyrosine in directly governing kinase regulation was described earlier. In addition to direct action at the catalytic site, SFK activity can also be regulated indirectly through at least three specific mechanisms of intramolecular communication: binding of the SH3 domain with the SH2-kinase linker (SH3-linker interaction), binding of the SH2 domain with the C-terminal Tyr-based motif (SH2-tail interaction), and the coordinated influence of the SH3-SH2 (SH32) "snap-lock" mechanism (400).

SH3-linker interaction

The interaction of the SH3 domain with the region linking the SH2 and kinase domains was not suspected until after c-Src and Hck were crystallized (321,379,390). Only then was this SH2-kinase linker noted to contain a PPII helix formed from a suboptimal SH3-binding sequence (401). The surprising nature of this SH3-linker contact offered new insights into the molecular mechanisms of Src regulation.

The SH3-binding motif of the SH2-kinase linker is oriented in a reverse direction and associates with corresponding binding pockets in the SH3 domain. Across the Src family, the linker sequences are not well conserved and poorly resemble the canonical PxxP SH3-binding motif typical of PPII regions (5). For instance, c-Src contains only one proline in its linker region (at position P_0), while in Hck the two prolines are each shifted over one spot (to P_{-1} and P_{+2}) and replaced by lysines at P_0 and P_{+3} instead. Yet the crystal structures of both

downregulated molecules reveal that the SH2-kinase linkers clearly associate intramolecularly with their respective SH3 domains (321,379,390).

Despite their unique assignment within the helix, the Hck PPII prolines have been found to help mediate the SH3-linker interaction. Substitution of alanines for the Hck linker prolines is sufficient to induce activation of the kinase in rodent fibroblasts via SH3 domain release (44). Mutation of the PPII lysines to prolines in Hck strongly enhances the binding of the linker to the SH3 domain, demonstrating that the wild-type linker region mediates suboptimal SH3 binding (216).

None of the SFK linker regions match the high-affinity SH3-binding motif identified in a combinatorial peptide screen, R-X-L-P-P-L-P-R-X (401). The less-than-optimal SH3-binding motifs in the SFK PPII helices are important, however, to allow for external high-affinity SH3 binders to compete the SH3 domain off of the linker and induce kinase activation. For instance, addition of purified HIV-1 Nef to inactive Hck leads to Hck-SH3 domain displacement and kinase activation (244). Nef contains the conserved proline-rich SH3-binding motif P-V-X-P_{72}-Q-V-P_{75}-L-R-P, which, in the context of the conserved Nef core, has a particularly high affinity for the SH3 domain of Hck (212).

Two other residues on the SH2-kinase linker are also critical for SFK regulation. In the inactive conformation of c-Src, the linker residue Leu255 (or Trp254 in Hck) is directed toward a hydrophobic pocket in the N-terminal kinase lobe (321,379,390). Mutation of Leu255 activates c-Src, suggesting a role for this residue in SH3-mediated inhibition of kinase activity by helping to stabilize the kinase domain (127). In addition, Trp260, located where the SH2-kinase linker meets the N-terminal end of the kinase domain, appears to coordinate with Asn312 on the αC helix, helping to stabilize the helix in its outwardly-rotated, inactive conformation (320,321). Substitution of alanine for Trp260 augments basal Hck activity and destabilizes the intramolecular binding of the SH3 and SH2 domains (128,203). This small region of the molecule may act as a key switching mechanism for long-range regulation of the kinase by the distant SH3 and SH2 modular domains (23).

SH2-tail interaction

The second site of intramolecular regulatory contact is between the SH2 domain and the tyrosine-based SH2-recognition motif of the C-terminal tail. Similar to variations in the linker

proline motif, SFK members have differing C-terminal tail sequences, yet none of these match the canonical SFK SH2 domain binding motif as predicted by phosphopeptide library screening (Table 1-1) (330). The presence of a suboptimal sequence in the native C-terminal tail may promote efficient on-off switching and allow for greater responsiveness to upstream SH2-based signals. Songyang et al. determined that the optimal binding motif for the Fyn, Lck, Fgr, and c-Src SH2 domains is the four amino-acid sequence Tyr-Glu-Glu-Ile (YEEI). Replacement of the native Hck tail sequence with YEEI yields a protein that adopts an auto-downregulated conformation (Figure 1-4) (310). The YEEI molecule does not require Csk to adopt the inhibitory conformation, suggesting that a low level of autophosphorylation at the tail is sufficient to drive the molecule into a stable downregulated structure (229). The tight SH2-tail connection generated with the YEEI sequence is, unlike the wild-type sequence, refractory to release in the presence of an SH2-binding peptide (285).

Table 1-1. SFK C-terminal tail sequences aligned by the inhibitory tyrosine, Tyr527.

Fgr	**P-Q-Y-Q-P-G-D-Q-T**
Fyn	**P-Q-Y-Q-P-G-E-N-L**
Hck	**S-Q-Y-Q-Q-Q-P**
Lck	**G-Q-Y-Q-P-Q-P**
Lyn	**G-Q-Y-Q-Q-Q-P**
c-Src	**P-Q-Y-Q-P-G-E-N-L**
c-Yes	**P-Q-Y-Q-P-G-E-N-L**

Disruption of the SH2-tail association is sufficient to activate the kinase, even when the SH3-linker contact remains intact. Our lab has shown that, in the presence of tight engagement of the SH3 domain with the linker, release of the Hck SH2 domain from its C-terminal tail via mutation of Tyr-527 causes a marked increase in kinase activity (216). Addition of Nef to tail-released Hck further activates the kinase above the level attained with tail release alone (216,244). This suggests either that maximal kinase activity may require both tail release and SH3-linker disengagement (216), or that SH3-linker association is the more dominant regulator of Src kinase activity (320).

These findings indicate that disruption of just one of the two primary modular regulatory contacts is necessary and sufficient to enable activation of Src kinases. This is consistent with the circuit theory logic of "OR" gate switching, where release of either modular domain structure is sufficient to activate the kinase (218). Furthermore, since engagement of one modular domain leaves the other available for binding to downstream targets, it is likely that the particular method of SFK activation may determine downstream effector function (215,216).

SH32 tandem

Together, the SH3 and SH2 domains serve to "clamp" down the back of the kinase domain and block its ability to catalyze the phosphotransfer reaction (152,400). Crystal structures of the tandem SH32 domains have shown that most SFK tandems have a restricted SH3-SH2 connector that allows for limited mobility (19). In addition, molecular dynamics of the SH3 and SH2 regions in inactive Hck have been shown to be tightly coupled, in that the domains tend to move together "en bloc" (400). For instance, binding of SH2 to the Tyr527 phosphorylated tail was shown to restrict motion in both the SH2 and SH3 domains, however upon C-terminal tail dephosphorylation this correlation was lost and mobility of the SH3 domain increased. Kuriyan and co-workers depict this tandem relationship as an "inducible snap-lock", borrowed from the description of similar behavior in zinc finger proteins (204). In the inactive state SH3 and SH2 are together rigidly bound to the kinase, yet upon tail release the connector "breaks" to allow displacement of the individual domains. Indeed, mutation of connector residues to highly flexible glycines is sufficient to block phospho-tail regulation of kinase activity (400).

Further study into the SH32 tandem has revealed that communication between SFK SH3 and SH2 domains involves more than just the connector region. Engen and colleagues have showed using a purified SH32 region that ligand binding to one domain does not alter molecular dynamics within the other domain (163). These results suggest that there is no direct "crosstalk" between the domains via the SH32 connector, but rather their coupled motions depend on other structural influences from the protein.

The structure of active c-Src has offered further insight into the tandem interactions between the SH3 and SH3 domains and their putative role in kinase activation (83). Surprisingly, in the active form, the SH3 domain appears bound to the end of the SH2-kinase linker. As a result, the SH32 tandem is bent back over the linker sequence, maintaining its "clamped" position (152). The entire SH32-linker structure is rotated about 130º away from the kinase region as compared to the inactive form, freeing the kinase domain from being backed against the rigid SH32 tandem (83). This structure confirms our findings that the SH3 domain need not be released from the linker to allow for kinase activation (216).

Several structural mechanisms have been described for how SH32 tandem binding to the kinase lobes can inhibit catalytic activity on the opposite surface of the kinase domain (35,173,300). Upon SH32 binding to the kinase domain, the alpha C helix is rotated such that the Glu310 residue, which faces the interior of the active site during catalysis, is re-positioned to face outwardly. In this position, Glu310 is unable to bind Lys295 to form the salt bridge critical for coordinating the phosphotransfer reaction (310,389). Also, the activation loop is induced to adopt an α-helical conformation that blocks the active site to substrate binding and sequesters Tyr-416 from phosphorylation. Finally, the presence of the locked SH32 tandem rigidly abutting the kinase lobes may decrease the overall flexibility of the kinase lobes otherwise important for enzyme function (38,152).

1.4.7 Negative regulators of SFKs

Csk

C-terminal Src kinase, or Csk, was the first protein found to phosphorylate the inhibitory tail tyrosine on c-Src (252). Sequence homology reveals that Csk shares 46% homology with c-Src (252), and structural studies confirm that, like c-Src, Csk contains modular SH3, SH2 and

tyrosine kinase domains (208,260). Contrary to c-Src, though, Csk lacks the N-terminal unique region and the regulatory tyrosines on the activation loop and C-terminal tail. As a result, the regulation of Csk is much different than that of SFKs. Instead, it appears that the structural units of Csk are involved in intramolecular *activation* of the kinase. Addition of the Csk SH3 domain, but not Csk SH2 or other SH3 domains, to purified Csk kinase domain led to a partial increase in kinase activity (329). Later structural studies demonstrated that the SH3-SH2 linker region is vital for Csk activation (260,318). Other studies showed that engagement of SH2 by the Csk-binding protein (Cbp/PAG) further enhances kinase activity (346). Cbp/PAG is a transmembrane protein that binds and recruits the non-myristoylated Csk protein to the membrane to associate with SFK targets (37,185,261). Finally, investigation into the activation loop, which is vital to proper kinase regulation in SFKs, suggests that in Csk this structure is expendable for full kinase activity (219).

The interaction between Csk and SFKs is not well understood. A Csk:SFK complex has not been demonstrated (302), though a non-modular region on the outer surface of the C-lobe of the Csk kinase domain has been implicated in binding to c-Src (214). The corresponding binding site on c-Src is unknown, however unpublished results from our laboratory suggest a similar region on the outer surface of the SFK kinase domain may be involved in Csk interaction (unpublished data). Evidence also exists that c-Src dimerization may enhance Csk-mediated inhibition (371).

Finally, the essential role of Csk in cellular function as a regulator of Src kinase activity comes from a variety of studies. Knockout studies reveal that mice lacking Csk fail to develop beyond early embryogenesis and display a massive increase in Src kinase activity (175,253). Csk negatively regulates signaling through the T cell receptor in a manner dependent upon the SH3 and SH2 domains of Csk (64,69,361). By downregulating Src activity in colon adenocarcinoma, overexpression of Csk has been shown to suppress tumor metastasis in mice (256). Csk is also necessary to regulate Src-mediated signaling related to the formation of actin stress fibers (226).

CHK

Following the discovery of Csk, a second inhibitor of SFKs, Csk-homologous kinase (CHK, also BatK (200), Ctk (195), Hyl (303), Lsk (237), Matk (29), Ntk (65)) was described

(88). CHK shares sequence homology and structural motifs with Csk, as well as the ability to phosphorylate the tail tyrosine and downregulate the activity of SFKs (62).

Some notable differences exist between CHK and Csk. CHK-knockout mice show no obvious abnormalities in the development of their hematopoietic systems, suggesting the ability of Csk to compensate for the lack of CHK (62,146,307). In addition, CHK binds an array of transmembrane receptor tyrosine kinases for membrane targeting, but not Cbp/PAG. Finally, recent evidence suggests that CHK may inhibit SFK activity in part via a kinase-independent mechanism (63), whereas kinase-inactivating mutations are known to abrogate Csk activity (166).

1.5 BINDING AND ACTIVATION OF SFKS BY HIV-1 NEF

Proteins known to bind the SH3 and/or SH2 domains of SFKs can stimulate kinase activity via displacement of the inhibitory regulatory modules. However, few studies have examined the interaction of Nef with other Src family members (Tables 1-2 and 1-3). Below I will summarize the extent of our knowledge with respect to the ability of Nef to bind and activate SFKs.

1.5.1 Hck

Hematopoietic cell kinase (Hck) is found primarily in cells of the monocytic lineage, as well as megakaryocytes, microglia, and B cells, but not T cells (288,407). Hck has been implicated in numerous macrophage signaling pathways, including: LPS-mediated TNFα production (96), IL-2 signaling (36), phagocytosis (225), β1 integrin- and Cbl-mediated spreading and migration (52,240), and podosome formation (282). Constitutively activate Hck induces an exaggerated innate immune response in the lungs of transgenic mice, due in part to the release of the inflammatory molecule TNF-α and matrix metalloproteinases (97). Interestingly, Hck expression is augmented following macrophage activation, making it a readily available target for Nef in HIV-infected cells (96).

Nef binds and activates full-length Hck (43,135,285), and several critical points of contact have been described (Figure 1-5). The SH3 domain of Hck recognizes the PPII motif of the Nef core domain, and Hck:Nef binding is blocked by proline-to-alanine mutations in Nef (59,304). In addition, the hydrophobic pocket within the Nef core domain binds a key isoleucine residue in Hck, Ile96, and mutation of either Ile96 or key residues within the Nef pocket, such as Trp90, abrogates Nef binding to Hck (59,212). Other residues within the Nef hydrophobic pocket, such as Tyr120, have also been found to be critical for Nef-mediated binding and activation of Hck (59).

Table 1-2. **Interactions of different HIV-1 Nef proteins with SFK SH3 domains.**

	Cons	*ELI*	*LAI*	*NL4-3*	*SF2*
Fyn	Y(59)	N(59)	Y(16,17,59); N(73)	N(212,304)	Y(59)
Hck	Y(59)	N(59)	Y(17,59,73,94,182)	Y(135,212,304)	Y(59)
Lck	n/a	n/a	Y(75); N(73,75,94)	Y(57,134,135); N(304)	Y(40)
Lyn	Y(59)	N(59)	Y(59)	Y(304)	Y(59)
Src	Y(59)	Y(59)	Y(17,59); N(73)	n/a	Y(59)

Y = binding observed; N = no binding observed; n/a = not tested. References listed in parentheses.

Table 1-3. Interactions of different HIV-1 Nef proteins with full-length SFKs.

	Cons	*ELI*	*LAI*	*NL4-3*	*SF2*
Fyn	N(59)	N(59)	N(59)	Y(57)	N(41,59)
Hck	Y(59)	N(59)	Y(59)	Y(57,135,285,304)	Y(41,43,59)
Lck	n/a	n/a	n/a	Y(57,134,135)	N(26,41)
Lyn	Y(59)	N(59)	Y(59)	Y(57)	Y(41,43,59)
Src	Y(59)	N(59)	Y(59)	n/a	Y(41,59)

Y = binding observed; N = no binding observed; n/a = not tested. References listed in parentheses.

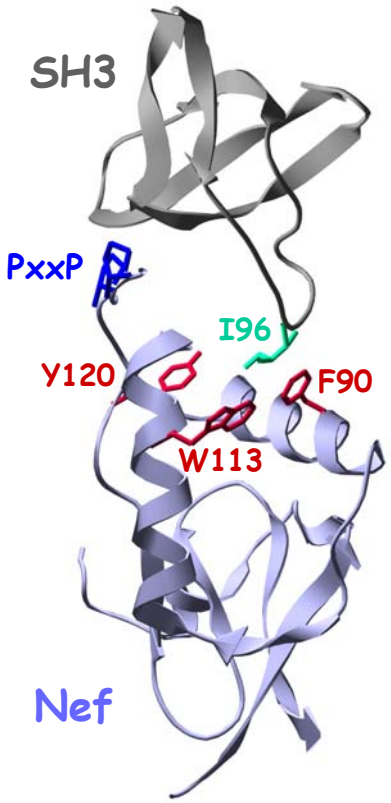

Figure 1-5. Structure of SFK SH3 domain bound to HIV-1 Nef conserved core.

The SFK SH3 domain is shown in grey, the Nef core is in violet. Residues known to be involved in the Nef:SFK interaction are highlighted; PxxP motif in blue, hydrophobic pocket residues (F90, W113, and Y120) in red. The SH3 residue Ile-96 (green) interacts with the hydrophobic pocket of Nef. This model is based on the original crystal structure solved by Lee et al. (213).

The Hck-Nef interaction is strongly implicated as having a role in HIV disease progression. When Nef is transgenically expressed in CD4+ cells, mice exhibit an AIDS-like phenotype, featuring T cell depletion, wasting, and early death (148,149). However, when Nef is similarly expressed in $Hck^{-/-}$ mice, AIDS-like disease progression is delayed and some animals live a normal lifespan (150). Further evidence for the role of Hck in HIV disease comes from

cellular studies that show the level of Hck expression correlates with the ability of macrophage-tropic (CCR5-tropic) HIV to infect primary monocyte-derived macrophages (MDMs) (198). This study also demonstrates that the viral replication in these MDMs is blocked by treatment with anti-sense RNA to suppress Hck expression.

1.5.2 Fyn

Fyn is expressed as one of two isoforms – FynT, found primarily in T cells, and FynB, found mostly in the brain – though both isoforms are broadly expressed in other cells (79,276). Fyn is the only Src kinase found to associate directly with the TCR complex (306) and, in turn, is involved in the transduction of signals through the TCR (267,403). Though dispensable for normal mouse development (12,335), Fyn shares overlapping functions with Lck and can substitute for Lck for some aspects of thymocyte development (141,360).

The ability of Nef to bind Fyn is controversial. Several studies have tried but failed to co-precipitate Nef with either full-length Fyn (41,59) or the Fyn SH3 domain (73,212,304). Lee et al. have shown that a single amino acid change in the Fyn SH3 domain of Arg96 to Ile, as found in the Hck SH3 domain, is sufficient to impart strong affinity for Nef [K_D(Fyn) > 20 uM; K_D (FynR96I) = 380 nM] (212). In fact, this modified FynR96I SH3 domain was used to solve the first crystal structure of a Src SH3 domain complexed to the Nef core (213). In contrast, two groups report binding of Nef to either Fyn SH3 (16) or full-length Fyn (57), albeit in both cases binding was greatly reduced compared with Hck. Interestingly, though Lee et al. found the Nef:Fyn SH3 interaction to be too weak to determine a binding constant (212), Arold et al. solved a crystal structure of Fyn SH3 (wild-type) bound to the Nef core (16). One reason for the conflicting reports could be the different Nef alleles used by each group; the former group used an Nef NL4-3 allele, containing an introduced T71R substitution to model the Consensus sequence, while the latter group used the Nef LAI allele.

To date, no reports have indicated the ability of Nef to activate Fyn. Previous work in our laboratory found that Nef failed to activate Fyn in a fibroblast transformation assay (43).

1.5.3 Lck

Lck is a 56 kDa protein expressed exclusively in lymphocytes, mostly in T cells (274). Interestingly, Lck appears to be closely related functionally with the Fyn tyrosine kinase. During development, Lck is essential for the early stages of thymocyte differentiation and expansion (245). However, most other developmental roles for Lck appear to be compensated for by Fyn (141,403). Along with Fyn, Lck is intimately associated with the TCR signaling complex. Specifically, Lck associates with the CD4 and CD8 coreceptors via cysteine motifs in its N-terminal unique domain (356), and has been found to play a key role in TCR signal transduction (267,403). In addition, Lck may be involved in the activation of Fyn following TCR engagement (105).

Nef has been shown to bind full-length Lck (57,75,134,135), the Lck SH3 domain (57,75,134), and, interestingly, the Lck SH2 domain (57,75,94), though many of these results are in conflict (Tables 1-2 and 1-3). Binding studies have shown that Nef binds to the Lck SH3 domain with a similar affinity as Src and Fyn SH3 domains, but not as tightly as Hck (17). In addition, an NMR study of full-length HIV-1 Nef with the Lck SH3 domain demonstrates that Nef:Lck binding relies on the classically described SH3-based interaction (40).

The impact of Nef on Lck activity appears to differ from the effect of Nef on other SFKs. HIV-1 Nef has been reported to inhibit Lck activity, both in vitro (75,134) and in cultured cells (133). In infected T cells, HIV inhibits the targeting of Lck to the immunological synapse in a *nef*-dependent manner, instead directing Lck to endosomal compartments (350). In addition, Nef-mediated phosphorylation of the multi-functioning c-Cbl proto-oncoprotein requires Lck, though the functional consequences of this event are not well understood (396).

1.5.4 Lyn

The Lyn tyrosine kinase is expressed in myeloid cells, as well as in brain and B cells, but not T cells (395). Unlike most SFKs, which are involved in propagating activation signals, Lyn appears to function primarily by attenuating receptor-driven signals, in particular, signals emanating from the B cell receptor and the FcεRI receptor (393). $Lyn^{-/-}$ mice have B cells that are hyperresponsive to BCR engagement and fail to downregulate activation signals, leading to

lethal autoimmune disease in the animals (160,258). Lyn phosphorylates immunoreceptor tyrosine-based inhibitory motifs on B cell (and other) receptors, which allows for the binding of phosphatases that downregulate receptor activity (159,393). However, Lyn does retain the ability to activate B cell receptor pathways (222), indicating a dual role for Lyn in the regulation of signaling pathways.

With regard to Nef binding, Lyn is most similar to Hck in that these are the only two SFKs that contain the Ile96 residue shown to be important for binding to the Nef hydrophobic pocket (59,212). Several reports have demonstrated the ability of Nef to bind either the Lyn SH3 domain (304) or full-length Lyn (41,43,57). However no studies have yet demonstrated an ability of Nef to activate Lyn.

1.5.5 c-Src

c-Src is the proto-typical member of the Src kinase family and has been studied extensively in the thirty years since its discovery. c-Src has been clearly implicated in a multitude of normal and oncogenic signaling processes too numerous to be described here (47,349). However, its role in HIV pathogenesis is not well understood. c-Src is found in DCs but is not well expressed in T cells and macrophages (222). Nef binds the SH3 domain of c-Src (59), though with a one-log lower affinity than the Hck SH3 domain (17), as well as full-length c-Src (41,59). Though Nef does not induce c-Src activation in a fibroblast transformation assay (41), Nef was shown to activate c-Src in a model of HIV-associated nephropathy utilizing glomerular podocytes from HIV-transgenic mice (155).

Src lacks the Ile 96 residue shown to be important for binding of the Hck-SH3 domain to Nef, instead containing an arginine at this position. Arold et al. argue, using the Nef:Fyn SH3 crystal structure for support (16), that the Arg 96 residue present in c-Src should be sufficient to interact with the Nef hydrophobic pocket and suggest instead that other residues confer Nef binding specificity (17). Further investigation is required to better understand the mechanism and implications of the interplay between Nef and c-Src.

1.5.6 Fgr and c-Yes

The Fgr tyrosine kinase is predominantly expressed in monocytes in later stages of development (380). This kinase has been best studied in knockout mouse models, in which Fgr has been shown have a role in directing eosinophils to the lung during inflammatory allergic response (364). However, other functional roles for Fgr may be masked in the knockout model by the presence of Hck, which appears to share multiple functional redundancies with Fgr in macrophages (223-225).

The c-Yes tyrosine kinase is closely related to c-Src, and the two kinases share several key features, including sequence homology (80%), ubiquitous expression, and similar upstream activators (47,70). While many of its functions parallel that of c-Src, c-Yes is also uniquely involved in the maintenance of tight junctions and the transcytosis of immunoglobulin A across cells (70,339).

To date, there have been no published reports of the interaction of Nef with either Fgr or c-Yes.

Much of the controversy regarding the ability of Nef to bind and/or activate SFKs may stem from the different alleles of Nef used or from the variations between using full-length versus SH3 domain fragments of the kinases. Our laboratory recently performed a comprehensive binding study to examine the ability of several allelic variants of Nef to bind to full-length and SH3 domains of different SFKs (59). In this report, three commonly used laboratory Nef alleles, Consensus, LAI and SF2, were all found to bind to both the full-length versions and the SH3 domains of Hck, Lyn, and c-Src. However, while the Fyn SH3 domain bound to these three Nef alleles, full-length Fyn bound to none of them. A fourth Nef allele, ELI, was unable to bind to any SFK protein with the sole exception of the c-Src SH3 domain. The lack of ELI binding is attributed to a mutation of Tyr120 within the hydrophobic pocket of the Nef core domain previously suggested to be important for the binding of Nef to SFKs (213). In an effort to better understand the direct structural relationship between Nef and SFKs, a major part of my thesis research has focused on investigating the ability of a panel of Nef alleles to activate different members of the Src kinase family. These results are presented in Chapter 4.

1.6 HYPOTHESIS AND SPECIFIC AIMS

1.6.1 Hypothesis

The *nef* gene product of HIV is firmly established as a critical virulence factor for HIV disease progression. Recent evidence has implicated Src family kinases (SFKs) in Nef-mediated HIV pathogenesis. However, less is known about the interaction of Nef and SFKs, in particular regarding the mechanism of SFK activation by Nef. I was interested in investigating the scope and mechanisms of Nef-mediated Src family kinase activation to identify new targets for drug discovery and improve our understanding of the role of Nef in HIV pathogenesis. Therefore, I hypothesized that **HIV-1 Nef preferentially activates select members of the Src family of non-receptor protein-tyrosine kinases, and that these protein complexes serve as relevant targets for the discovery of small molecule inhibitors of HIV function**. To address this hypothesis, I have set forth three aims: (1) to determine whether HIV-1 Nef selectively activates a subset of SFKs; (2) to investigate whether small molecule inhibitors of Nef-mediated SFK activation impair mechanisms of HIV pathogenesis; and (3) to test whether SFKs are differentially activated by allelic variants of HIV-1 Nef.

1.6.2 Specific Aims

Aim 1: To determine whether HIV-1 Nef selectively activates a subset of SFKs

The direct effect of Nef interaction on SFK regulation and activity has not been systematically addressed. I explored this issue by developing a yeast growth suppression assay to examine the ability of Nef to activate those SFKs expressed in HIV target cells. SFKs activated by Nef were further analyzed for SH3-dependence of this interaction by co-expressing the SFKs with a Nef construct containing a mutated SH3-binding motif.

Aim 2: To investigate whether small molecule inhibitors of Nef-mediated SFK activation impair mechanisms of HIV pathogenesis

Nef lacks intrinsic kinase activity and utilizes cellular signaling proteins and pathways, including SFKs, to promote viral pathogenicity. Thus, inhibitors of Nef:SFK interaction may block HIV function. To address this aim, I used the yeast assay developed in Aim 1 to discover small molecule inhibitors of Nef:Hck-induced growth suppression. These compounds were then tested in an HIV replication assay to determine if inhibitors of Nef-mediated SFK activation can also serve as anti-HIV agents.

Aim 3 – To test whether SFKs are differentially activated by allelic variants of HIV-1 Nef

The binding of Nef to SFKs is based primarily on an SH3-mediated interaction involving a highly conserved PxxP motif located on the Nef core domain. However, we recently demonstrated that residues outside of this canonical SH3 binding motif are critical for Nef:SFK association. In this Aim we explored additional components of this interaction by screening primary Nef alleles for altered SFK activation. Those alleles that demonstrated modified abilities to activate SFKs were analyzed for amino acid variations that may indicate additional mechanisms of Nef-induced SFK activation.

2.0 CHAPTER 2

HIV-1 NEF SELECTIVELY ACTIVATES SRC FAMILY KINASES HCK, LYN, AND c-SRC THROUGH DIRECT SH3 DOMAIN INTERACTION

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*The work presented in this chapter has been accepted for publication (352).

2.1 ABSTRACT

Nef is an HIV-1 virulence factor that promotes viral pathogenicity by altering host cell signaling pathways. Nef binds several members of the Src kinase family, and these interactions have been implicated in the pathogenesis of HIV/AIDS. However, the direct effect of Nef interaction on Src family kinase (SFK) regulation and activity has not been systematically addressed. We explored this issue using Saccharomyces cerevisiae, a well-defined model system for the study of SFK regulation. Previous studies have shown that ectopic expression of c-Src arrests yeast cell growth in a kinase-dependent manner. We expressed Fgr, Fyn, Hck, Lck, Lyn, and Yes as well as c-Src in yeast and found that each kinase was active and induced growth suppression. Co-expression of the negative regulatory kinase Csk suppressed SFK activity and reversed the growth-inhibitory effect. We then co-expressed each SFK with HIV-1 Nef in the presence of Csk. Nef strongly activated Hck, Lyn, and c-Src, but did not detectably affect Fgr, Fyn, Lck, or Yes. Mutagenesis of the Nef PxxP motif essential for SH3 domain binding greatly reduced the effect of Nef on Hck, Lyn, and c-Src, suggesting that Nef activates these Src family members through allosteric displacement of intramolecular SH3-linker interactions. These data show that Nef selectively activates Hck, Lyn and c-Src among SFKs, identifying these kinases as proximal effectors of Nef signaling and potential targets for anti-HIV drug discovery.

2.2 INTRODUCTION

Nef is an accessory protein encoded by the human (HIV-1, HIV-2) and simian (SIV) immunodeficiency viruses and is an essential mediator of viral pathogenicity (100,176,275). Experimental deletion within the SIV *nef* gene reduces viral load, delays the onset of AIDS-like disease and offers immune protection against challenge with pathogenic SIV in rhesus macaques (87,188). Strong selective pressure has been demonstrated for a functional *nef* gene, as some animals infected with non-pathogenic, *nef*-mutant SIV show in vivo repair of the mutation and progression to AIDS-like disease (188,308,375). In addition, some HIV-positive individuals that fail to develop AIDS exhibit *nef* mutations or deletions (90,118,191,192,211), supporting the hypothesis that *nef* is essential for efficient disease progression.

Nef has no known catalytic function and is believed to promote viral pathogenicity by altering signaling pathways in infected cells through its interactions with cellular proteins. Nef affects several distinct classes of host cell proteins, including immune receptors, protein kinases, trafficking proteins, and guanine nucleotide exchange factors (18,122,294). Through interactions with these and other signaling proteins, Nef can affect multiple cellular processes leading to enhancement of viral replication, immune evasion, and enhanced survival in T-cells and macrophages (42,60,100,280).

Protein kinases are a major class of Nef effector proteins, and members of the Src family of non-receptor protein-tyrosine kinases have been strongly linked to Nef function. Numerous reports have demonstrated that Nef interacts with the isolated Src homology 3 (SH3) domains from Src family members expressed in HIV target cells, including Fyn, Hck, Lck, Lyn and c-Src itself (16,17,40,59,134,135,304). Among these, the interaction of Nef with Hck has been studied in great detail at the cellular and molecular levels. Hck is strongly expressed in cells of the monocyte/macrophage lineage (288,407), which are essential HIV-1 target cells and viral reservoirs (84,235,264). X-ray crystallography demonstrates that Nef interacts with the Hck SH3 domain via a bipartite mechanism dependent upon the three-dimensional fold of Nef (213). These contacts include a highly conserved Nef PxxPxR motif, which forms the polyproline type II helix typical of SH3 ligands. In addition, Ile-96 within the RT-loop of the Hck SH3 domain

fits into a pocket within the Nef core that is lined with several highly conserved hydrophobic residues. Both of these interactions are necessary, but not independently sufficient, for high affinity binding of Nef to the Hck SH3 domain (43,59).

Nef-SH3 domain interaction leads to constitutive Hck activation in vitro (244) and in cell-based systems including fibroblasts (43), myeloid cell lines (42,398), and HIV-infected primary macrophages (198). Nef binding is believed to disrupt the normal role of the Hck SH3 domain in suppression of kinase activity (43). When Hck is downregulated, its SH3 domain associates with the polyproline type II helix formed by the linker connecting the SH2 and kinase domains (310,321). This interaction is stabilized by the interaction of the SH2 domain with the C-terminal tail, which is phosphorylated on a conserved tyrosine residue by the negative regulatory kinases Csk and Chk (61,195,262). Binding of Nef to the SH3 domain causes linker displacement (244), resulting in a conformational shift in the kinase domain permissive for ATP binding, target protein access, and phosphotransfer (3). Nef-induced Hck activation does not require tail dephosphorylation or displacement from the SH2 domain, suggesting that Nef may induce a novel signaling conformation of Hck (215).

While the functional consequences of the Nef-Hck pathway are still under investigation, mounting evidence suggests a key role for this interaction in AIDS progression. In monocyte-derived macrophages, Komuro et al. established a strong positive correlation of high-titer replication of macrophage-tropic HIV-1 with Hck expression (198). In addition, they showed that HIV replication is blocked following suppression of Hck with anti-sense oligonucleotides. At the whole-animal level, targeted expression of Nef to the T-cell and macrophage compartments in transgenic mice induces an AIDS-like phenotype, characterized by CD4+ T cell depletion, diarrhea, wasting, and uniform mortality (148). In contrast, mice expressing a mutant form of Nef lacking the PxxPxR motif essential for SH3 binding show no evidence of the AIDS-like phenotype (150). Interestingly, when transgenic mice expressing wild-type Nef were crossed into a *hck*-null background, appearance of the AIDS-like phenotype was delayed with a significant proportion of the mice living normal lifespans (150). The observation that the Nef-induced AIDS-like syndrome is reduced but not eliminated in the absence of Hck suggests that other SFKs may contribute to Nef signaling in this system.

Less is known about the functional interaction of Nef with other Src family members. Lyn is the only Src family member other than Hck with an Ile residue in the SH3 domain RT

loop. While the Lyn SH3 domain appears to bind as tightly to Nef as the Hck SH3 domain in vitro (59,304), the effect of Nef on Lyn kinase activity has not been reported. Several reports suggest that HIV-1 Nef binds to Lck and downregulates its kinase activity (75,133,135). However, other work failed to detect a direct interaction of Nef with Lck or provide evidence for an effect on kinase activity in vivo (26,41). Conflicting reports exist regarding the interaction of Nef with Fyn and its SH3 domain (41,57,59,73,212,304), while the direct effect of Nef on Fyn kinase activity is unknown. Nef also interacts with both full-length c-Src and its SH3 domain (59), although the direct effect of Nef on c-Src activity is not clear (41,155). Finally, while Fgr and Yes are present in HIV target cells, neither has been tested for interactions with Nef.

One explanation for the conflicting literature regarding the impact of Nef on SFK activity relates to the use of diverse systems for analysis. In addition, Nef is likely to activate multiple kinases in HIV target cells, obscuring its direct effects on individual Src family members. Identification of those SFKs that are directly activated by Nef is the first step towards validation of these kinases as drug discovery targets. Here we address this important issue using a yeast-based expression system, originally developed for the study of c-Src regulation (48,80,199). Yeast represent a useful model for the study of SFK regulation because they do not express orthologs of c-Src or other mammalian protein-tyrosine kinases. In addition, ectopic expression of c-Src and other mammalian protein-tyrosine kinases has been shown to induce kinase-dependent growth arrest in yeast, providing a convenient end-point for structure-function analysis (108,251,340,345,370). In the case of c-Src, co-expression of the regulatory kinase Csk reverses the growth-inhibitory effect through phosphorylation of the negative regulatory tail, modeling the natural mechanism of downregulation in mammalian cells (251,252,340).

In this report, we first show that regulation of Hck kinase activity by Nef can be faithfully reconstituted in yeast. When expressed alone, Hck was highly active and produced a strong growth-suppressive phenotype. Hck activity and growth suppression were reversed upon co-expression of the negative regulatory kinase, Csk. Introduction of Nef led to re-activation of Hck despite the presence of Csk, closely modeling previous reports in mammalian cells types (42,43). We then extended the study to include all other SFKs expressed in HIV target cells: Fgr, Fyn, Lck, Lyn, c-Src, and Yes. Like Hck, all of these kinases suppressed yeast cell growth when active, and this phenotype was reversed upon co-expression with Csk. Introduction of Nef led to clear activation of Lyn and c-Src in addition to Hck. In all three cases, activation involved

the PxxPxR motif of Nef, suggesting a common SH3-linker displace-ment mechanism previously described for Hck. In contrast, Nef did not affect Lck or Fyn activity, despite previous reports of Nef binding to these kinases (16,26,57,73,75,134). Nef also failed to affect Yes or Fgr activity. These data provide the first complete analysis of direct HIV-1 Nef-SFK interaction in living cells, and identify the complexes of Nef with Hck, Lyn and c-Src as unique targets for anti-HIV drug discovery.

2.3 MATERIALS AND METHODS

Yeast expression vectors - Coding sequences for human Csk, c-Src, Fyn, Hck, Lck, and Lyn as well as murine Fgr and Yes were amplified by PCR from existing templates to introduce a yeast translation initiation sequence (AATA) immediately 5' to the ATG start codon. The cDNA clones for HIV-1 Nef (SF2 strain) and the *Herpesvirus saimiri* Tip protein (a.a. 1-187) were similarly amplified and modified. A FLAG epitope tag was added to the N-terminus of the Tip coding sequence. All SFK cDNA clones were subcloned downstream of either the Gal1 or Gal10 promoter in the yeast expression vector pESC-Ura (Stratagene). Hck was also subcloned downstream of the Gal10 promoter in the pYC2/CT vector (Invitrogen), which carries the CEN6/ARSH4 sequence for low-copy replication. The Csk, Nef and Tip cDNAs were subcloned downstream of either the Gal 1 or Gal10 promoter in pESC-Trp (Stratagene). cDNA clones for kinase-defective Hck (Hck-K269D), tail-activated Hck (Hck-Y501F) and kinase-defective Csk (Csk-K222D) were created via site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene). The Nef-2PA mutant, in which prolines 72 and 75 are replaced with alanines, has been described elsewhere (43).

Yeast growth suppression assay - *S. cerevisiae* strain YPH 499 (Stratagene) was co-transformed with pESC-Ura (or pYC2/CT) and pESC-Trp plasmids containing the genes of interest via electroporation (BioRad Gene Pulser II). Yeast were selected for three days at 30° C on standard synthetic drop-out plates lacking uracil and tryptophan (SD/-U-T) with glucose as the sole carbon source to repress protein expression. Positive transformants were grown in liquid SD/-U-T medium plus glucose, normalized to $OD_{600} = 0.2$ in water, and then spotted in four-fold dilutions onto SD/-U-T agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for three days at 30° C and imaged on a flatbed scanner. Yeast patches appear as dark spots against the translucent agar background. All growth suppression assays were repeated at least three times starting with randomly selected independent transformed clones and produced comparable results; representative examples are shown.

Immunoblotting - Aliquots of the yeast cultures used for the spot assay were grown in SD/-U-T medium plus galactose for 18 h. Cells were pelleted, treated with 0.1 N NaOH for 5 min at room temperature (202), and normalized with SDS-PAGE sample buffer to 0.02 OD_{600} units per μl. Aliquots of each lysate (0.2 OD_{600} units) were separated via SDS-PAGE, transferred to PVDF membranes, and probed for protein phosphotyrosine content with a combination of the anti-phosphotyrosine antibodies PY99 (Santa Cruz Biotechnology) and PY20 (Transduction Laboratories). Protein expression was verified by immunoblotting with antibodies to Csk (C-20; Santa Cruz), Fgr (C1; Santa Cruz), FLAG (M2; Sigma), Fyn (FYN3; Santa Cruz), Hck (N-30; Santa Cruz), Lck (2102; Santa Cruz), Lyn (44; Santa Cruz), Src (N-16; Santa Cruz) and Yes (3; Santa Cruz). Nef antibodies (monoclonal EH1 and Hyb 6.2) were obtained from the NIH AIDS Research and Reference Reagent Program.

Expression and purification of recombinant SFKs and Nef - Human Hck, Lyn and c-Src clones were modified on their C-terminal tails to encode the sequence Tyr-Glu-Glu-Ile-Pro. This modification promotes autophosphorylation of the tail and permits high-yield purification of the downregulated form of each kinase without the need for co-expression of Csk (310). The N-terminal unique domain of each kinase was replaced with a hexa-histidine tag, and each construct was used to produce a recombinant baculovirus in Sf9 insect cells using Baculogold DNA and the manufacturer's protocol (BD-Pharmingen). Recombinant SFKs were purified from 1 L of infected Sf9 cell culture using a combination of ion-exchange and affinity chromatography as originally described by Schindler et al. for Hck (310). The purity and concentration of each kinase preparation were confirmed by SDS-PAGE and densitometry. The SF2 allele of HIV-1 Nef was similarly expressed and purified with an N-terminal hexa-histidine tag.

In vitro kinase assays - Tyrosine kinase assays were performed in 384-well plates using the FRET-based Z'-lyte kinase assay system and the Tyr 2 peptide substrate (Invitrogen). Reactions (10 μl) were conducted in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% BRIJ-35). Assay conditions were first optimized to determine the amount of each kinase and the incubation time necessary to phosphorylate 20-30% of the Tyr2 peptide in the absence of Nef. To assess the effect of Nef on SFK activity, Hck (20 ng), Lyn (50 ng), and Src (50 ng) were incubated at room temperature for 5 min with a 5- or 10-fold molar excess of Nef. ATP (50 μM final) and Tyr2 substrate (2 μM final) were then added to the reaction followed by

a 1 h incubation (45 min for Hck). Development reagent, containing a protease that digests non-phosphorylated peptide, was then added to the reaction for an additional 60 min at room temperature, at which time the reaction was terminated with the proprietary stop reagent. Fluorescence was assessed at an excitation wavelength of 400 nm; coumarin fluorescence and the fluorescein FRET signal were monitored at 445 nm and 520 nm, respectively. The coumarin emission excites fluorescein by FRET in the phosphorylated (uncleaved) substrate peptide only. Reactions containing unphosphorylated peptide and kinase in the absence of ATP served as 0% phosphorylation control, while a stoichio-metrically phosphorylated peptide was used as a 100% phosphorylation control. Raw fluorescence values were corrected for background and reaction endpoints were calculated as emission ratios of coumarin fluorescence divided by the fluorescein FRET signal. These ratios were then normalized to the ratio obtained with the 100% phosphorylation control. Each condition was assayed in quadruplicate, and results are presented as the mean ± S.D. The entire experiment was repeated twice with comparable results.

2.4 RESULTS

2.4.1 Active Hck suppresses yeast growth in a kinase-dependent manner

Before reconstituting the interaction of Hck with HIV-1 Nef in yeast, we first determined whether Hck produced a similar growth-suppressive phenotype as described previously for c-Src (108,251,340). To accomplish this, we used a plate-based assay to visualize the effects of Hck expression on yeast cell growth. Yeast cultures transformed with galactose-inducible Hck and Csk expression vectors were spotted as a dilution series on galactose-agar plates. As shown in Figure 2-1, expression of wild-type Hck induced growth suppression relative to control cultures transformed with empty expression plasmids. Growth suppression was reversed when Hck was co-expressed with wild-type Csk, the kinase responsible for downregulation of SFKs in mammalian cells (62). Kinase-dead Csk (Csk-K222D) was unable to reverse Hck-induced growth suppression, consistent with negative regulation of Hck by Csk-mediated tail tyrosine phosphorylation as observed previously in fibroblasts (215).

To determine whether growth suppression induced by Hck correlated with Hck kinase activity, yeast cell lysates were probed with anti-phosphotyrosine antibodies. Expression of Hck alone correlated with strong phosphorylation of many yeast proteins (Figure 2-1). Co-expression with Csk led to a marked decrease in protein phosphotyrosine content, consistent with downregulation of Hck kinase activity. Kinase-dead Csk was unable to inhibit Hck activity, establishing the role of Csk kinase activity in the control of Hck. Expression of Csk alone produced no growth suppressive effect or yeast protein-tyrosine phosphorylation, demonstrating the exquisite specificity of Csk activity for the tail region of Hck. These findings show that co-expression with Csk is sufficient to downregulate Hck in yeast, consistent with previous observations for c-Src (251,252,340).

Immunoblots of yeast lysates with Hck antibodies revealed that Hck consistently migrated as three distinct bands (Figure 2-1). Interestingly, this banding pattern changed when Hck was co-expressed with wild-type but not kinase-dead Csk, with three bands shifting to a single high mobility band. This effect of Csk suggests that the variation in Hck mobility reflects

different activation states, with the highest mobility form representing the downregulated conformation.

To demonstrate that growth suppression is dependent upon Hck kinase activity, we transformed yeast with a kinase-dead form of Hck, Hck-K269D. Hck-K269D failed to induce either growth suppression or tyrosine phosphorylation of yeast proteins (Figure 2-1). Co-expression with Csk led to tyrosine phosphorylation of Hck-K269D, presumably on the tail tyrosine residue. In addition, Hck-K269D runs as a single high-mobility band on the anti-Hck immunoblot, providing further evidence that the high-mobility form of Hck corresponds to the inactive conformation.

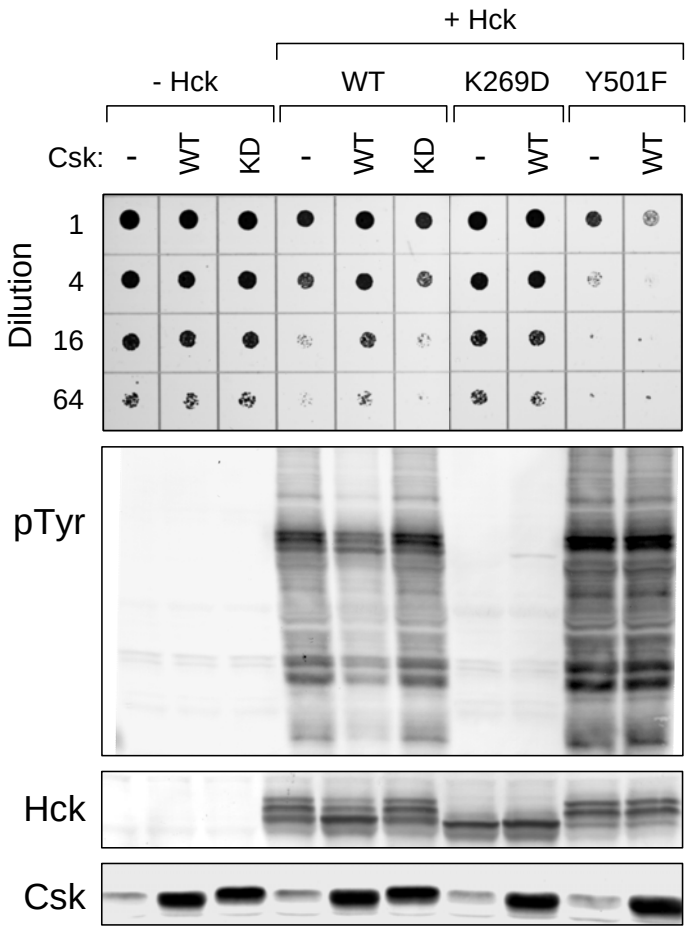


Figure 2-1. Hck induces yeast growth suppression in a kinase-dependent manner.

Yeast cultures were transformed with galactose-inducible expression plasmids for wild-type Hck (WT), a kinase-dead mutant (K269D), a mutant lacking the C-terminal Csk phosphorylation site (Y501F), or the empty expression plasmid (- Hck). Cells were co-transformed with galactose-inducible vectors for wild-type (WT) or kinase-dead (KD) Csk as indicated or with the empty vector as a negative control (-). Top: Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. Plates were scanned and yeast patches appear as dark circles. Lower panels: Immunoblots from cultures shown at the top. Transformed cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for Hck and Csk.

Csk downregulates Hck activity in mammalian cells by phosphorylating Tyr-501 on the C-terminal tail (215). To confirm this mechanism of Csk-induced downregulation of Hck in yeast, we co-expressed a Hck mutant lacking the regulatory tail tyrosine (Hck-Y501F) in the presence or absence of Csk. As shown in Figure 2-1, Hck-Y501F markedly suppressed yeast growth and heavily phosphorylated yeast proteins. Csk was unable to alleviate the growth suppression or the protein-tyrosine phosphorylation induced by Hck-Y501F. Interestingly, Hck-Y501F migrated as the two lower mobility bands on anti-Hck immunoblots, and co-expression with Csk had no effect on this pattern. These results show that inhibition of Hck by Csk in yeast requires both Csk kinase activity and the Hck tail tyrosine residue (Y501), thus faithfully modeling the mechanism in mammalian cells.

2.4.2 Nef activates Hck in a PxxP-dependent manner

Previous studies have established that HIV-1 Nef binds tightly to the SH3 domain of Hck, leading to constitutive kinase activation both in vitro and in mammalian cells (43,215,244). To determine if Nef activates Hck in yeast through a similar mechanism, we first co-expressed Nef with Hck in the absence of Csk (Figure 2-2). Interestingly, Hck-induced growth arrest and protein-tyrosine phosphorylation were both markedly increased in the presence of Nef. We then repeated the experiment in the presence of Csk, and found that Nef completely reversed the inhibitory effect of Csk, leading to growth suppression and protein-tyrosine phosphorylation very similar to that observed when Hck is co-expressed with Nef in the absence of Csk. These observations suggest that Nef may generate a unique highly active conformation of the kinase (see Discussion). Control cultures show that Nef, either alone or when co-expressed with Csk, has no effect on cell growth or protein-phosphotyrosine content.

To determine if Nef-induced Hck activation is dependent upon the Nef SH3-binding function, we mutated the PxxP motif of Nef to AxxA (Nef-2PA). As shown in Figure 2-2, Nef-2PA failed to enhance growth suppression by Hck or increase protein-tyrosine phosphorylation in the presence or absence of Csk. These results support an allosteric mechanism in which Nef-induced activation of Hck requires SH3 binding and displacement of the SH2-kinase linker.

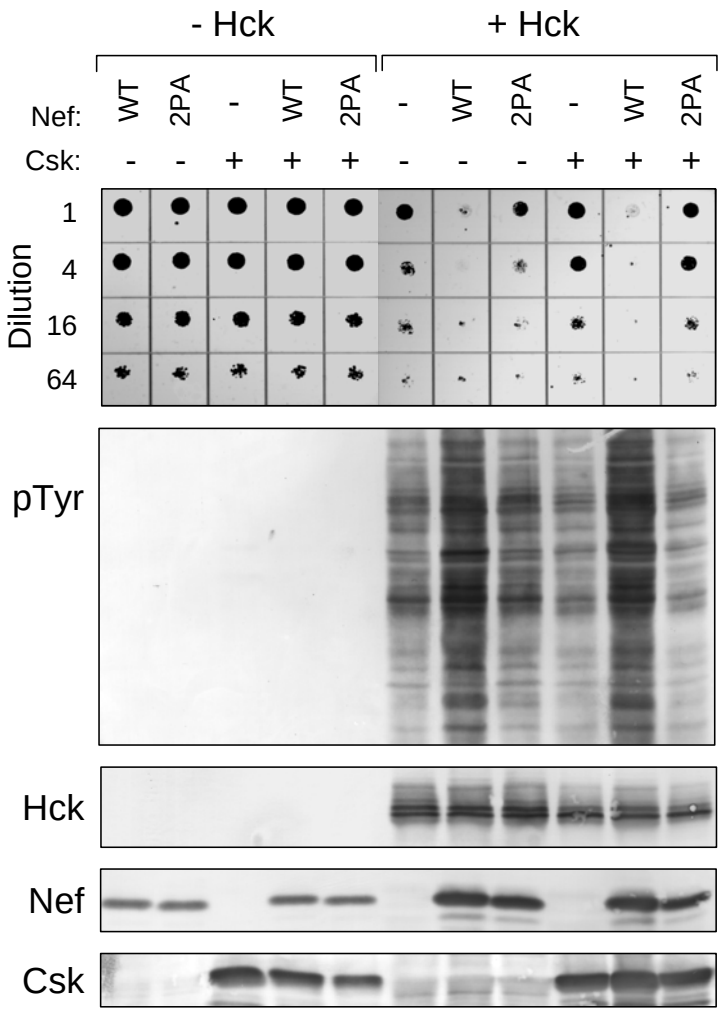


Figure 2-2. HIV-1 Nef activates Hck in a PxxP-dependent manner.

Yeast cultures were transformed with galactose-inducible expression plasmids for wild-type Hck (+ Hck) in the absence (-) or presence of wild-type (WT) or PxxP mutant (2PA) forms of HIV-1 Nef. Cells were co-transformed with galactose-inducible expression vectors for Csk or the corresponding empty vector as indicated. Control cultures without Hck are shown on the left (- Hck). Top: Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. Plates were scanned and yeast patches appear as dark circles. Lower panels: Immunoblots from cultures shown at the top. Transformed cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for Hck, Nef and Csk.

2.4.3 Suppression of yeast cell growth is a shared property of SFK

Before evaluating the effect of Nef expression on other members of the Src kinase family, we first determined whether other SFKs produced the same growth suppressive effect as c-Src and Hck. Fgr, Fyn, Lck, Lyn and Yes were each expressed in yeast with or without Csk and spot assays performed to measure growth suppression. Hck and c-Src were also included for comparison. As shown in Figure 2-3, all seven SFKs suppressed yeast growth and co-expression of Csk reversed the growth-inhibitory phenotype.

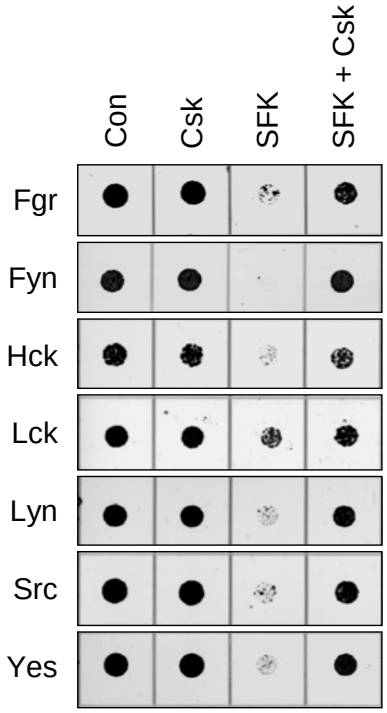


Figure 2-3. Csk reverses growth suppression of yeast by SFKs.

Yeast cultures were transformed with the SFKs indicated on the left either alone (SFK) or in the presence of Csk (SFK + Csk). Cells transformed with empty vectors (Con) or with Csk alone (Csk) were included as controls in each experiment. Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Plates were scanned and yeast patches appear as dark circles. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. The dilutions showing the clearest differences in growth in the presence and absence of Csk are presented.

Cell lysates from each of the cultures in Figure 2-3 were then immunoblotted for tyrosine-phosphorylated proteins, as well as for expression of each SFK and Csk (Figure 2-4). In each case, co-expression with Csk resulted in a decrease in the intensity of the anti-phosphotyrosine signal. These results show that all SFKs are constitutively active following ectopic expression in yeast, and provide direct evidence that Csk alone is sufficient to downregulate the activity of each member of the Src kinase family.

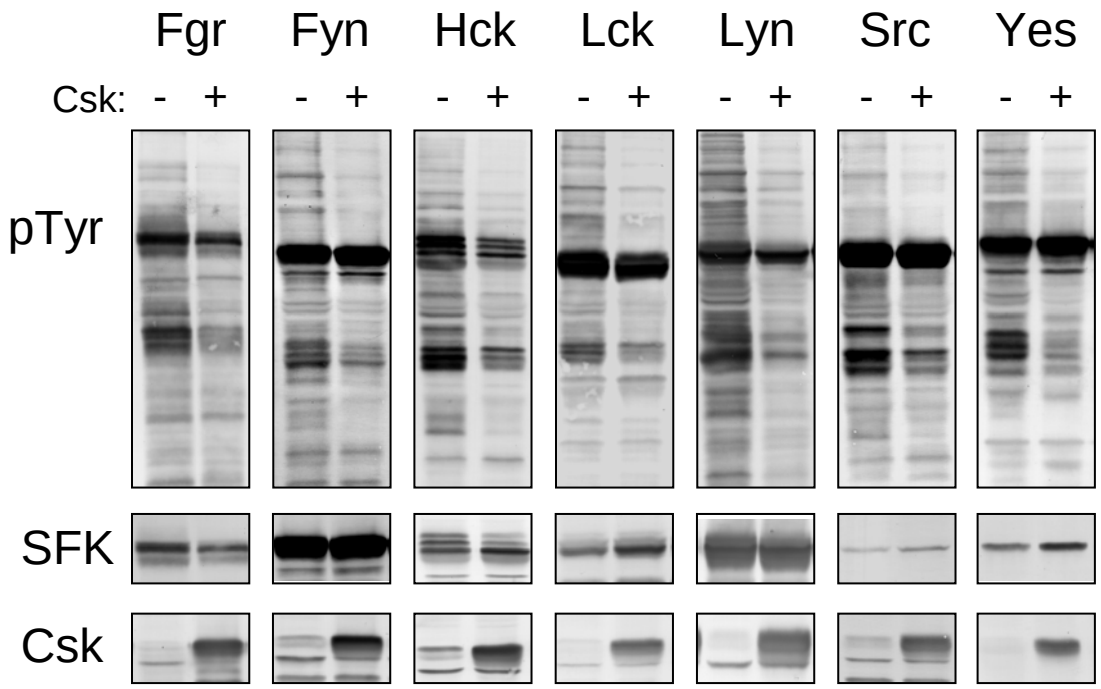


Figure 2-4. Csk suppresses SFK activity in yeast.

Yeast cultures were transformed with expression vectors for each of the SFKs shown at the top in the presence (+) or absence (-) of Csk. Cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for each Src family member (SFK) and Csk.

2.4.4 Nef selectively activates a subset of Src family kinases

We next investigated whether co-expression of HIV-1 Nef was sufficient to activate SFKs other than Hck in the yeast model system. The SFKs were also co-expressed with Csk to determine whether interaction with Nef is sufficient to activate the downregulated form of each kinase. Growth suppression data for each SFK in the presence or absence of Csk and Nef are shown in Figure 2-5. In the absence of Csk, Nef enhanced the growth suppression observed with Hck but did not affect growth suppression by the other SFKs. However, Nef readily restored growth suppression by Csk-downregulated c-Src and Lyn in addition to Hck. Csk-downregulated Fyn, Fgr, Lck, and Yes were not affected by Nef.

To evaluate the effects of Nef on SFK activity, lysates were prepared from each of the transformed cultures shown in Figure 2-5 and immunoblotted with anti-phosphotyrosine antibodies. As shown in Figure 2-6, expression of each SFK alone induced strong tyrosine phosphorylation of multiple yeast cell proteins, and this effect was markedly dampened upon co-expression with Csk. Co-expression of Nef with Hck in the absence of Csk led to an even greater degree of protein-tyrosine phosphorylation, consistent with the effect of Nef on Hck-induced growth suppression (Figure 2-5). Nef reversed downregulation of Hck, Lyn and c-Src kinase activity by Csk, consistent with the growth suppression obtained with Nef and these three Csk-downregulated SFKs (Figure 2-5). In contrast, no detectable changes in the protein-tyrosine phosphorylation patterns or signal intensity were observed upon co-expression of Nef with Fgr, Fyn, Lck, or Yes in the presence or absence of Csk, providing strong evidence that these Src family members are not direct targets for HIV-1 Nef in vivo.

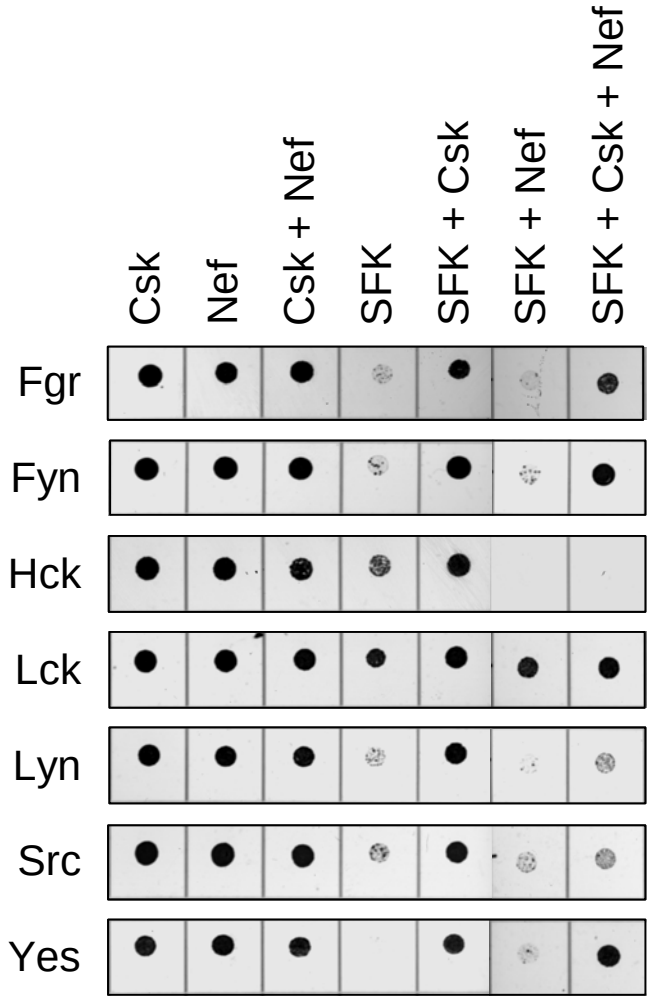


Figure 2-5. HIV-1 Nef selectively induces growth suppression in yeast co-expressing downregulated forms of Hck, Lyn, and c-Src.

Yeast cultures were transformed with the SFKs indicated on the left either alone (SFK) or in the presence of Csk (SFK + Csk), HIV-1 Nef (SFK + Nef), or both (SFK + Csk + Nef). Cells transformed with Csk alone (Csk), Nef alone (Nef) or both (Csk + Nef) were included as controls in each experiment. Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Plates were scanned and yeast patches appear as dark circles. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. The dilutions showing the greatest differences in growth are presented.

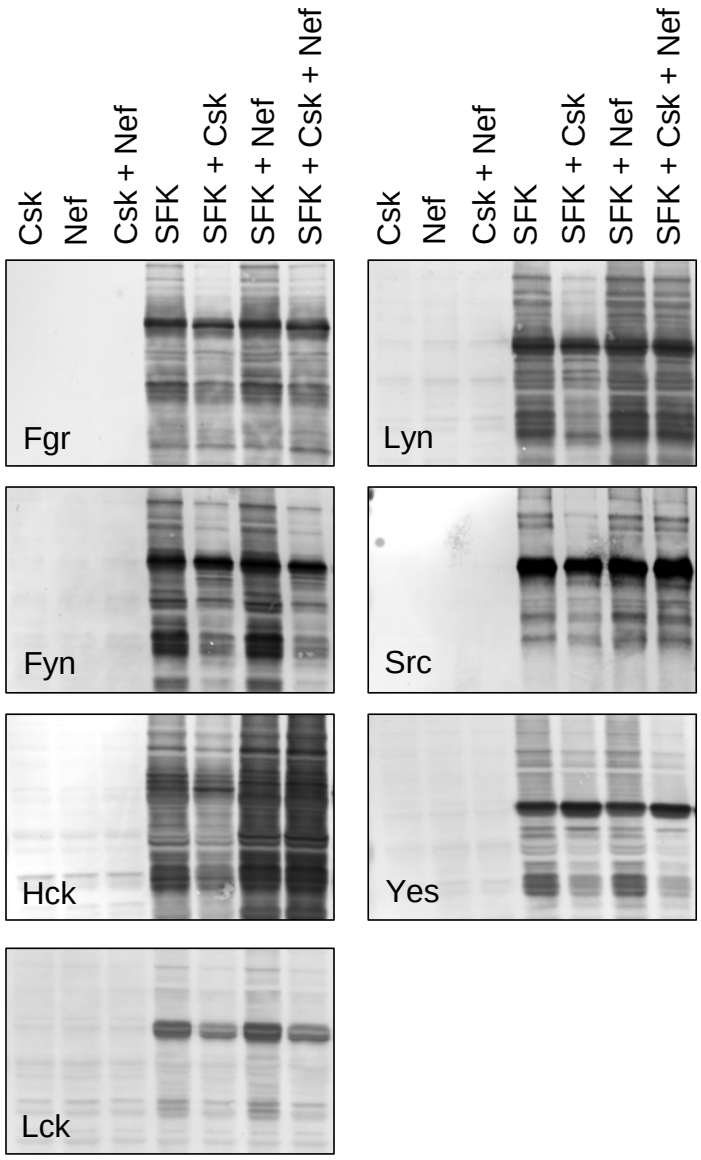


Figure 2-6. HIV-1 Nef selectively activates Hck, Lyn, and c-Src in yeast.

Yeast cultures were transformed with expression vectors for each of the SFKs indicated either alone (SFK) or in the presence of Csk (SFK + Csk), HIV-1 Nef (SFK + Nef), or both (SFK + Csk + Nef). Cells transformed with Csk alone (Csk), Nef alone (Nef) or both (Csk + Nef) were included as negative controls. Cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr). Control immunoblots confirmed expression of each SFK, Csk, and Nef (data not shown).

2.4.5 Lck is activated by Herpesvirus saimiri Tip but not HIV-1 Nef in yeast

Data presented above show that Lck exhibited relatively low basal kinase activity in yeast and induced weak growth suppression as a consequence. Neither parameter was influenced by Nef, suggesting that these proteins fail to interact in vivo. As an additional control to support this conclusion, we performed an experiment with Tip, a Herpesvirus saimiri protein shown previously to bind and activate Lck (30,153,194,228,376). As shown in Figure 2-7, co-expression with Tip led to very strong activation of Lck, inducing marked growth suppression that correlated with enhanced protein-tyrosine phosphorylation. In contrast, co-expression of Lck with Nef did not affect yeast growth or enhance basal kinase activity, consistent with the results presented in Figures 2-5 and 2-6. These data show that co-expression with a known activator enhances Lck kinase activity and induces growth suppression in yeast, thus validating the negative result with Nef.

2.4.6 Nef-mediated activation of Lyn and c-Src is PxxP-dependent

We next investigated whether Nef-mediated activation of Lyn and c-Src employs the SH3-binding function of Nef, as is the case with Hck (Figure 2-2). For these experiments, we again employed the Nef-2PA mutant, in which proline residues in the conserved PxxPxR motif critical for SH3 engagement are replaced with alanines. Lyn and c-Src were co-expressed with Csk and either wild-type Nef or the Nef-2PA mutant. While wild-type Nef induced strong activation of Lyn and c-Src in terms of growth suppression and protein-tyrosine phosphorylation, both effects were nearly reversed with Nef-2PA (Figure 2-8). These results indicate that activation of Lyn and c-Src by HIV-1 Nef requires interaction with the SH3 domains of these kinases, identifying SH3-linker displacement as a common mechanism of SFK activation by Nef.

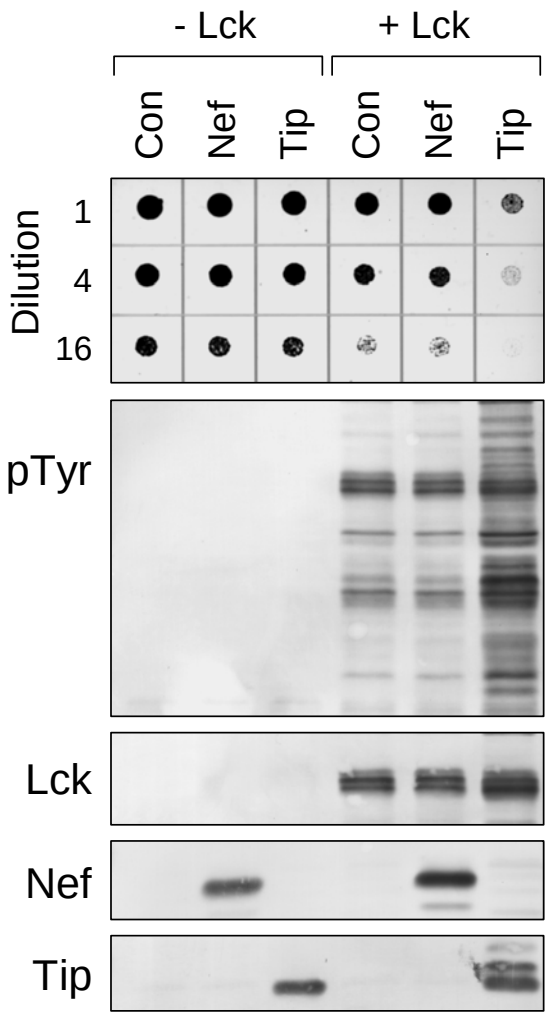


Figure 2-7. Lck is activated by Herpesvirus saimiri Tip but not HIV-1 Nef in yeast.

Yeast cultures were transformed with galactose-inducible expression plasmids for Lck (+ Lck) or the empty expression plasmid as negative control (- Lck). Cells were co-transformed with galactose-inducible vectors for HIV-1 Nef or FLAG-tagged Herpervirus saimiri Tip as indicated or with the empty vector (Con). Top: Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. Plates were scanned and yeast patches appear as dark circles. Lower panels: Immunoblots from cultures shown at the top. Transformed cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for Lck, Nef, and Tip. Tip was visualized using an anti-FLAG antibody and runs as multiple bands in the presence of Lck due to tyrosine phosphorylation.

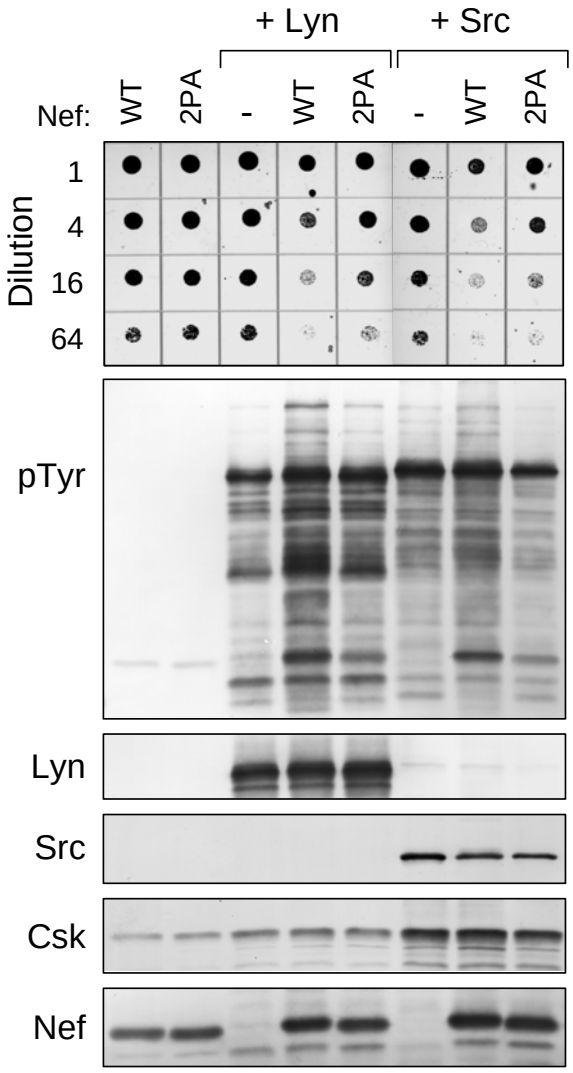


Figure 2-8. HIV-1 Nef-mediated activation of Lyn and c-Src is PxxP-dependent.

Yeast cultures were transformed with galactose-inducible expression plasmids for Lyn (+ Lyn) and c-Src (+ Src) in the absence (-) or presence of wild-type (WT) or PxxP mutant (2PA) forms of HIV-1 Nef. Cells expressing the Nef proteins alone were included as a negative control. Top: Liquid cultures were grown with glucose as the sole carbon source to repress protein expression and normalized to equal densities. Cells were then spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30º C. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. Plates were scanned and yeast patches appear as dark circles. Lower panels: Immunoblots from cultures shown at the top. Transformed cells were grown in liquid culture in the presence of galactose at 30º C for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for Lyn, Src, Csk, and Nef.

2.4.7 Nef activates Hck and Lyn but not c-Src in vitro

In a final series of studies, we investigated whether the presence of Nef was sufficient for SFK activation, or whether co-expression in a cell-based system is essential. To accomplish this, Hck, Lyn and c-Src, the three SFKs activated by Nef in yeast, were purified to homogeneity in their inactive forms (see Materials and Methods). Each of the kinases was then assayed in vitro with a peptide substrate either alone or in the presence of a 5- or 10-fold molar excess of purified recombinant Nef. As shown in Figure 2-9, Hck was strongly activated by Nef under these conditions, supporting the idea that SH3-linker displacement is sufficient for Hck activation as described in previous studies both in vitro and in vivo (215,244). Similar to Hck, Lyn was also strongly stimulated by Nef in this system, suggesting that SH3-linker displacement is also sufficient for Lyn activation. In contrast to Hck and Lyn, Nef failed to activate c-Src under these conditions. This observation suggests that binding of Nef to the Src SH3 domain is not sufficient for kinase activation in vitro, and that myristylation and localization to the plasma membrane may also have a role in the activation mechanism observed in vivo (see Discussion).

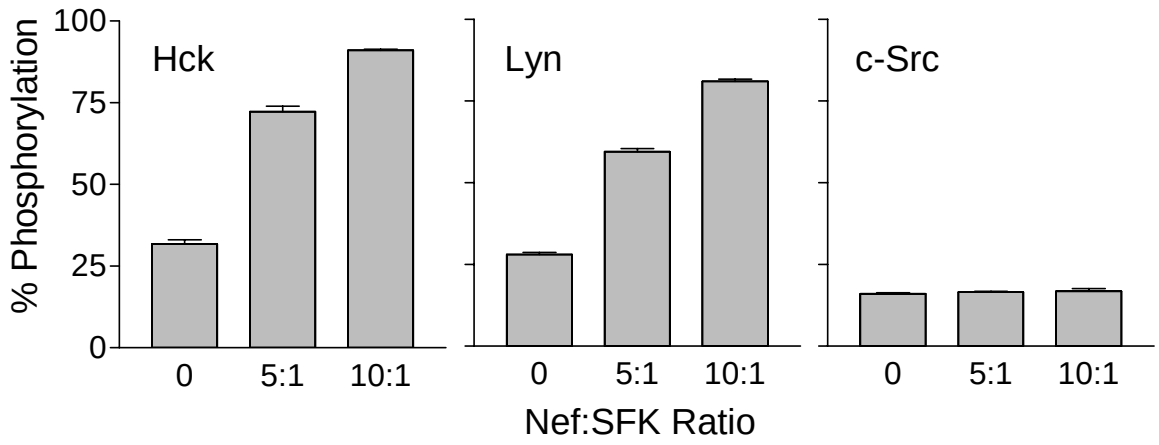


Figure 2-9. Activation of SFKs by HIV-1 Nef in vitro.

Recombinant Hck, Lyn and c-Src were purified from Sf9 insect cells in their downregulated forms, and assayed for kinase activity with a peptide substrate in vitro either alone or in the presence of a 5- or 10-fold molar excess of purified recombinant Nef. Details of the FRET-based tyrosine kinase assay used for this experiment can be found under Materials and Methods. Each condition was repeated in quadruplicate, and the extent of phosphorylation is expressed as mean percent phosphorylation relative to a control phosphopeptide ± S.D. The overall experiment was repeated twice with comparable results. This work was performed by L. Emert-Sedlak.

2.5 DISCUSSION

A growing body of evidence identifies SFKs as important targets for HIV-1 Nef in vivo. Some of the strongest evidence exists for the macrophage Src family member Hck, which is constitutively activated by Nef (43,244) and has been implicated in disease progression in a mouse model of AIDS (148,150). At the molecular level, Nef has been shown to bind to isolated SFK SH3 domains and the corresponding full-length kinase proteins in some cases (see Introduction). However, no comparative study of the impact of Nef on full-length SFK activity has been conducted. Here we provide the first complete analysis of the direct effects of Nef on the activities of all SFKs expressed in HIV target cells (T cells and macrophages). Using a yeast-based expression system, we show for the first time that c-Src and Lyn, in addition to Hck, are directly activated by HIV-1 Nef in vivo. Activation occurs in the presence of the physiological SFK regulator, Csk. In contrast, Fgr, Fyn, Lck and Yes are not activated by Nef, despite previous reports describing the interaction of Nef with several of these kinases or their SH3 domains in vitro (see below). Our observation that Nef selectively activates Hck, Lyn and c-Src among SFKs may explain why wild-type Nef induces a partial AIDS-like phenotype in Hck-null mice, while Nef lacking the PxxPxR motif essential for SFK binding fail to develop AIDS-like disease (150). In the former case, activation of c-Src and Lyn by Nef may functionally compensate for the lack of Hck. Indeed, upregulation of Lyn kinase activity has been reported in Hck-null macrophages, consistent with a possible compensatory mechanism (225). In contrast, Nef-induced activation of Hck, Lyn, and c-Src requires the PxxPxR motif, supporting the idea that Nef signaling through all three of these Src family members may be essential for development of AIDS-like disease in this model.

We validated our yeast system by demonstrating appropriate regulation of Hck kinase activity by Csk and Nef as reported previously in mammalian cells (42,43,215). Yeast provide a very useful tool for the study of protein-protein interactions in SFK regulation in vivo because they lack orthologs of mammalian protein-tyrosine kinases. Here we show that wild-type Hck, when expressed alone, exerts a growth-suppressive effect in yeast consistent with previous reports for c-Src (48,199,251). Co-expression with Csk markedly suppressed Hck kinase activity

and reversed the growth inhibitory effect. Suppression of Hck activity required catalytically active Csk as well as an intact Hck tail tyrosine residue, previously established as an essential site for downregulation of Hck in mammalian cells (215). HIV-1 Nef strongly activated downregulated Hck, and this effect required the Nef PxxPxR motif responsible for Hck SH3 binding (43,213). These data support a model in which Nef engages Hck through its SH3 domain and displaces its negative regulatory interaction with the SH2-kinase linker. Demonstration that Nef can overcome Csk-induced downregulation of Hck in yeast strongly supports a direct activating effect of Nef on Hck in vivo, and supports the use of yeast to faithfully model this interaction.

We next extended our study to other members of the Src kinase family expressed in HIV target cells. Fgr, Fyn, Lck, Lyn, and Yes were all active in yeast and induced growth suppression in a manner analogous to c-Src and Hck. Co-expression with Csk reversed growth suppression and reduced endogenous protein-tyrosine phosphorylation, providing direct evidence that Csk alone is sufficient to induce downregulation of each SFK tested. Introduction of Nef into this system revealed for the first time that Lyn and c-Src are direct targets for Nef-induced activation in vivo. In contrast, Nef had no apparent effect on the activity of Fgr, Fyn, Lck or Yes. These results are surprising in light of previous reports of Nef interaction with Lck and Fyn, which are discussed in more detail below.

Lck is selectively expressed in T-lymphocytes and plays an essential role in T-cell receptor signal transduction and in thymocyte development (267). Numerous studies have reported that Nef can interact with Lck through its SH2 and SH3 domains (57,75,94,134,135,304), and may repress Lck kinase activity and signaling (74,133,134). In contrast, our work suggests that although Nef-Lck interactions can be demonstrated in vitro, these proteins might not interact directly in cells. Unlike Hck, co-expression with Nef had no impact on Lck kinase activity in yeast, in terms of either activation or inhibition. Interestingly, Lck did not suppress yeast growth as strongly as the other SFKs tested, which is most likely a reflection of its relatively low basal kinase activity. To provide a positive control for Lck activation, we co-expressed Lck with the *Herpesvirus saimiri* Tip protein, which has been previously established as an Lck-specific binding protein and activator (30,153,194,228,376). In contrast to Nef, Tip readily activated Lck and induced strong growth arrest and tyrosine phosphorylation of yeast cell proteins.

Like Lck, Fyn is also important in T-lymphocyte antigen responsiveness and development, although its expression pattern is more broad than that of Lck (267). Because of its functional role in T-cells, Fyn has attracted attention as an HIV-1 Nef target protein. The Fyn SH3 domain has been shown to interact with Nef in vitro, although with lower affinity than the Hck and Lyn SH3 domains (17). The structural basis for this difference has been attributed to the lack of an Ile residue in the Fyn SH3 domain RT loop (212,213). Indeed, substitution of the Arg residue at this position with Ile converts the Fyn SH3 domain from a low to a high affinity binding partner for Nef (212). The ability of full-length Fyn to interact with Nef is more controversial, and may reflect the different experimental approaches used for evaluation of the interaction (57,59). Here we show that Nef failed to activate Fyn in yeast, both in the growth suppression assay and by anti-phosphotyrosine immunoblotting. Together, these data suggest that while Nef may interact with the isolated SH3 domain of Fyn in vitro, Nef does not directly affect Fyn kinase activity in vivo.

Like Hck, Lyn is also expressed in macrophages, an important HIV target cell and viral reservoir. At the structural level, only Lyn and Hck have the SH3 domain Ile residue essential for high-affinity Nef binding (212). This Ile residue interacts with a hydrophobic pocket within the Nef core (213) and together with the conserved PxxPxR motif is essential for Nef-induced activation of Hck in a rodent fibroblast model (43,59). Here we show for the first time that Nef activates Lyn by a similar PxxPxR-dependent mechanism, and can overcome the negative regulatory influence of Csk in doing so. This finding implies that Lyn is regulated by a similar mechanism as Hck, with the SH2-kinase linker engaging the SH3 domain in the downregulated conformation of the kinase (35). High-affinity binding of Nef to the SH3 domain may be sufficient to displace the linker, relieving its inhibitory effect on the kinase domain. Our observation that Nef can drive Lyn activation in vitro also supports this mechanism (Figure 2-9). Although Nef readily activates Lyn in vitro and in yeast, we did not observe activation of Lyn by Nef in a previous study using a fibroblast transformation model (41). This difference may relate to localization of Lyn and Nef to different subcellular compartments in fibroblasts, where a portion of the Lyn molecules may localize to the nucleus (189). Alternatively, additional cellular factors may be present in fibroblasts that interfere with downstream signaling by the Nef:Lyn complex.

Although c-Src lacks the SH3 domain Ile residue essential for high-affinity Nef binding, we and others have observed Nef interaction with the isolated Src SH3 domain as well as full-length c-Src, although with lower relative affinity than Hck (17,59). Here we show that co-expression of Nef is sufficient to overcome Csk-induced down-regulation of c-Src in the yeast model system. Both growth suppression and protein-tyrosine phosphorylation by c-Src were dependent upon the Nef PxxPxR motif, strongly suggesting an SH3-based activation mechanism. Consistent with our findings, He et al. recently showed that Nef augments c-Src kinase activity and induces proliferation in immortalized podocytes in a PxxPxR-dependent manner (155). Activation of c-Src by Nef led to activation of Stat3 and Erk signaling downstream, as previously observed in other cell types (42,60). Nef-Src interaction may contribute to HIV-1 associated nephropathy, the most common cause of chronic renal failure in HIV-seropositive patients (155).

Although Nef was able to activate c-Src in yeast and mammalian cells, we found that Nef alone is not sufficient to induce c-Src activation in an in vitro kinase assay under conditions which led to strong Hck and Lyn activation (Figure 2-9). The most likely explanation for this difference is the lower affinity of Nef for the c-Src SH3 domain. Arold et al. (17) determined the equilibrium dissociation constants for Nef interaction with various SFK SH3 domains by isothermal titration calorimetry, and found that Nef bound the Hck SH3 domain 10- to 20-times more strongly than those of c-Src, Fyn, or Lck. They attributed this difference to the sequence of the Hck RT loop, which in addition to having the optimal Ile residue is more flexible and able to adopt a conformation favorable for binding to the Nef hydrophobic pocket. The c-Src RT loop, on the other hand, is more constrained by hydrogen bonds and may be less able to adopt a conformation compatible with Nef binding. Although interaction between the Src SH3 domain and Nef may not be sufficient to activate c-Src in solution, this lower affinity interaction may be enhanced when the two proteins co-localize to the plasma membrane in cells. Another possibility is that the myristoyl group of native Nef may contribute to Src binding via its myristic acid binding pocket (83); note that none of the recombinant proteins tested in Figure 2-9 were myristoylated. Future studies will examine the mechanism of Src SH3:Nef interaction in more detail.

The final two SFKs examined in our study were Fgr and Yes, which have not been previously examined with respect to Nef binding or kinase activation. Fgr is strongly expressed in macrophages, and knock-out experiments suggest significant functional overlap with Hck in

this cell type (225). Yes expression is more broadly distributed, often mirroring the pattern observed with c-Src (121). Nef did not activate either kinase in yeast, suggesting that these SFKs are not direct targets for Nef in HIV-infected cells. Consistent with this observation, neither the Yes nor the Fgr SH3 domain contains an RT-loop Ile residue, suggesting that the lack of kinase activation may be due to low SH3 affinity as observed for Fyn and Lck.

In summary, our results show that HIV-1 Nef selectively activates Hck, Lyn, and c-Src among the various SFK isoforms expressed in HIV target cells. The mechanism of activation requires binding of Nef via its PxxPxR motif to the SFK SH3 domain, consistent with the SH3-linker displacement mechanism previously described for Hck. The essential role for the Nef PxxPxR motif in the murine AIDS model, together with the finding that Hck knockout mice are only partially protected from Nef-induced pathogenesis, strongly suggest that redundant activation of Hck, Lyn and c-Src occurs in HIV-infected cells (148,150). Future work will address whether small molecule inhibitors targeted to the Nef-SFK complexes affect HIV replication and AIDS progression.

2.6 FOOTNOTES

*This work was supported by NIH Grants AI057083 and CA81398. The authors wish to thank Dr. Bart Sefton of the Salk Institute for providing the Tip cDNA. We also acknowledge the NIH AIDS Research and Reference Reagent Program for providing antibodies to Nef.

3.0 CHAPTER 3

DISCOVERY OF NOVEL HIV REPLICATION INHIBITORS USING A YEAST-BASED SCREEN TARGETING THE HIV NEF:HCK PROTEIN-TYROSINE KINASE COMPLEX

Ronald P. Trible, Toshi Kodama, Caleb Foster, John S. Lazo, and
Thomas E. Smithgall

3.1 ABSTRACT

The HIV-1 Nef protein is a viral accessory factor critical for the progression of HIV/AIDS. Nef lacks intrinsic catalytic activity and targets multiple host cell signaling proteins, including Hck and other members of the Src kinase family. Nef interacts with Src family kinases through their SH3 domains, leading to constitutive kinase activation that has been implicated in AIDS progression. In this study, we developed a yeast-based system to identify small molecule inhibitors of the active Nef:Hck complex. We show that Nef:Hck interaction can be faithfully reconstituted in yeast, resulting in constitutive kinase activation and growth arrest. Yeast expressing the active Nef:Hck complex were used to screen a library of small heterocyclic compounds based on their ability to rescue growth inhibition. Two compounds identified in the screen potently blocked Nef-dependent HIV replication, validating Nef:Src family kinase complexes as rational targets for anti-HIV drug discovery.

3.2 INTRODUCTION

HIV-1 *nef* encodes a small myristoylated protein required for optimal viral replication and AIDS pathogenesis (100,122). Deletion of *nef* from the HIV-related simian immunodeficiency virus prevents AIDS-like disease progression in rhesus macaques (188). In addition, expression of the *nef* gene alone is sufficient to induce an AIDS-like syndrome in transgenic mice very similar to that observed upon expression of the complete HIV-1 provirus (147,148). In humans, *nef* sequence variability and function correlate with HIV disease progression over the course of infection (51,191). Indeed, long-term non-progressive HIV infection has been associated with *nef*-defective strains of HIV in some cases (90,192). These and other studies identify the HIV-1 Nef accessory protein as a key molecular determinant of AIDS.

Nef lacks a known intrinsic enzymatic or biochemical function and instead exploits numerous host cell signaling pathways to optimize conditions for viral replication and AIDS progression (100,137,176,280,294). Mounting evidence identifies the Src family kinases (SFKs), a group of non-receptor protein-tyrosine kinases that control cell growth, differentiation, and survival (269,349), as key molecular targets for Nef (137,294). One striking example is Hck, a Src family member expressed in macrophages that binds strongly to Nef via an SH3-mediated interaction (17,212). Several studies support a role for Nef:Hck interaction in HIV pathogenesis. Productive infection of primary human macrophages by M-tropic HIV requires both Hck expression and kinase activity (198). In addition, Nef can interrupt macrophage colony-stimulating factor signaling by activating Hck, suggesting that Nef:Hck association may contribute to macrophage dysfunction in HIV-infected cells (341). Finally, Nef-transgenic mice, which rapidly succumb to AIDS-like disease, show delayed disease progression and decreased mortality when bred into a Hck-null background (150).

In this report, we describe the development of a yeast-based screen to identify inhibitors of Nef:Hck signaling. First, we established that co-expression with Nef leads to constitutive activation of Hck in yeast by the same biochemical mechanism observed in mammalian cells. The active Nef:Hck complex induced growth arrest in yeast that was reversed with a known SFK

inhibitor, providing a basis for a simple yet powerful screen for novel compounds. Using this system, we screened a small library of kinase-biased heterocycles and isolated two compounds that potently blocked Nef-dependent HIV replication in vitro. This study identifies complexes of Nef with Hck and other SFKs as rational targets for anti-HIV drug discovery.

3.3 MATERIALS AND METHODS

Yeast expression vectors. Coding sequences for human Csk and Hck as well as HIV-1 Nef (SF2 strain) were modified by PCR to introduce a yeast translation initiation sequence (AATA) immediately 5' to the ATG start codon. The coding sequence for Hck was subcloned downstream of the Gal10 promoter in the pYC2/CT vector (Invitrogen), which carries the CEN6/ARSH4 sequence for low-copy replication. The Csk and Nef coding sequences were subcloned downstream of the Gal1 and Gal10 promoters, respectively, in the yeast expression vector pESC-Trp (Stratagene). The coding sequence of the wild-type Hck tail (YQQQP) was modified by PCR to encode the high-affinity SH2-binding sequence, YEEIP, as described elsewhere (215,310). The Nef-PA mutant, in which prolines 72 and 75 are replaced with alanines, has also been described elsewhere (43).

Yeast growth suppression assay. *S. cerevisiae* strain YPH 499 (Stratagene) was co-transformed with pESC-Ura (or pYC2/CT) and pESC-Trp plasmids containing the genes of interest via electroporation (BioRad Gene Pulser II). Yeast were selected for three days at 30° C on standard synthetic drop-out plates lacking uracil and tryptophan (SD/-U-T) with glucose as the sole carbon source to repress protein expression. Positive transformants were grown in liquid SD/-U-T medium plus glucose, normalized to OD_{600nm} = 0.2 in water, and then spotted in four-fold dilutions onto SD/-U-T agar plates containing galactose as the sole carbon source to induce protein expression. Duplicate plates containing glucose were also prepared to control for yeast loading (data not shown). Plates were incubated for three days at 30° C and imaged on a flatbed scanner. Yeast patches appear as dark spots against the translucent agar background. All growth suppression assays were repeated at least three times starting with randomly selected independent transformed clones and produced comparable results; representative examples are shown. For the liquid growth assay, yeast strain W303a (gift of Dr. Frank Boschelli, Wyeth Pharmaceuticals) was co-transformed with the required plasmids, seeded at an initial density of OD_{600nm} = 0.05 units in SD/-U-T medium, and incubated for 21 h at 30 ºC. The control inhibitor A-419259 was added with DMSO as carrier solvent to a final concentration of 0.1%.

Immunoblotting. Aliquots of the yeast cultures used for the spot assay were grown in SD/-U-T medium plus galactose for 18 h. Cells were pelleted, treated with 0.1 N NaOH for 5 min at room temperature (202), and normalized with SDS-PAGE sample buffer to 0.02 OD_{600nm} units per μl. Aliquots of each lysate (0.2 OD_{600nm} units) were separated via SDS-PAGE, transferred to PVDF membranes, and probed for protein phosphotyrosine content with a combination of the anti-phosphotyrosine antibodies PY99 (Santa Cruz Biotechnology) and PY20 (Transduction Laboratories). Immunoblots were also performed with antibodies to Csk (C-20; Santa Cruz), Hck (N-30; Santa Cruz), actin (MAB1501; Chemicon International) and Nef (monoclonal Hyb 6.2; NIH AIDS Research and Reference Reagent Program).

Yeast drug screen. Yeast strain W303a was co-transformed with Hck-YEEI and Nef expression plasmids and grown to an OD_{600nm} of 0.05. Cells (100 μl) were plated in duplicate wells of a 96-well plate in the presence of each compound from the ChemDiv kinase-biased inhibitor library (ChemDiv, San Diego, CA). All compounds were initially screened at 10 μM with 0.5% DMSO as carrier solvent. Control wells contained 0.5% DMSO to define the extent of growth arrest as well as cells transformed with Hck-YEEI plus the Nef-2PA mutant to define maximum outgrowth. Each plate also contained wells with 5 μM A-419259 as a positive control for drug-mediated growth reversion. Cultures were incubated at 30ºC, and the OD_{600nm} was measured at 0 and 22 h. Those compounds which induced a 10% or greater increase in yeast growth relative to the DMSO control were further assayed in triplicate and compared against A-419259-mediated growth reversion. Compounds from this secondary screen which recovered yeast growth to at least 25% of A-419259-induced recovery were obtained in powder form, then assayed a third time in triplicate at 1, 3, 10, and 30 μM in comparison with 5 μM A-419259.

HIV replication assay. HIV-1 replication assays were conducted using HIV-1 strain NL4-3. Note that NL4-3 Nef is very similar in sequence to the SF2 allele used in the yeast assays and strongly activates Hck-YEEI (R. Trible and T. Smithgall, manuscript in preparation). Virus stocks were prepared by transfection of the recombinant viral genome into 293T cells. Viral replication was monitored in the U87MG astroglioma cell line expressing CD4 and CXCR4 (U87MG/CD4-X4 cells). U87MG cells have been a useful model system for

investigation of co-receptor requirements for HIV replication in several previous studies (305,353). Viral replication was monitored by measuring p24 gag protein levels in the culture supernatant 4 and 5 days after infection by standard ELISA-based techniques. Test compounds were added to the culture 30 min prior to infection with HIV, and DMSO was used as the carrier solvent at a final concentration of 0.1%.

3.4 RESULTS

3.4.1 Hck-YEEI models Csk-downregulated Hck in yeast

Previous work in our laboratory has shown that HIV-1 Nef binds to Hck and induces its constitutive activation in yeast and mammalian cells (42,43,59,352). We have also shown that binding of Nef to Hck creates a unique active kinase conformation (215,216), which represents an attractive target for small molecule inhibitor discovery. To identify inhibitors of this complex, we required a cell-based assay in which Nef:Hck signaling drives a simple read-out amenable to high-throughput screening. For this assay we turned to yeast, where expression of active c-Src is well known to induce growth arrest in a kinase-dependent manner (48,108,199,251). Co-expression of C-terminal Src kinase (Csk), a negative regulator of SFKs (252), reverses Src-mediated yeast growth suppression by phosphorylating the c-Src negative regulatory tail and inducing repression of its kinase activity (251,253,340,352). Using a similar yeast-based system, we have recently shown that Nef induces the reactivation of several Csk-inhibited Src kinases, namely Hck, Lyn, and c-Src, resulting in growth arrest (352). These observations suggested that the yeast system may be ideal for an inhibitor screen, as compounds which block Nef-induced SFK signaling should rescue cell growth.

To simplify the assay and make it more amenable to high-throughput screening, we mutated the Hck tail sequence to the high-affinity SH2-binding motif YEEI. Previous work has shown that this substitution redirects autophosphorylation from the activation loop to the tail, leading to intramolecular engagement of the SH2 domain and downregulation of kinase activity in the absence of Csk (310). In addition, the X-ray crystal structure of this modified form of Hck (Hck-YEEI) is nearly identical to that of native Hck that has been down-regulated by Csk (310,321). To determine whether the YEEI substitution was sufficient to downregulate Hck in yeast, wild-type Hck and Hck-YEEI were expressed in the presence and absence of Csk. As shown in Figure 3-1, Hck-YEEI failed to suppress yeast growth, and showed reduced kinase activity compared with wild-type Hck on anti-phosphotyrosine immunoblots of yeast cell lysates. Co-expression of Csk reduced wild-type Hck kinase activity and reversed growth suppression,

but had no additional effect on Hck-YEEI auto-downregulation. These results show that Hck-YEEI effectively models the behavior of Csk-downregulated wild-type Hck in yeast.

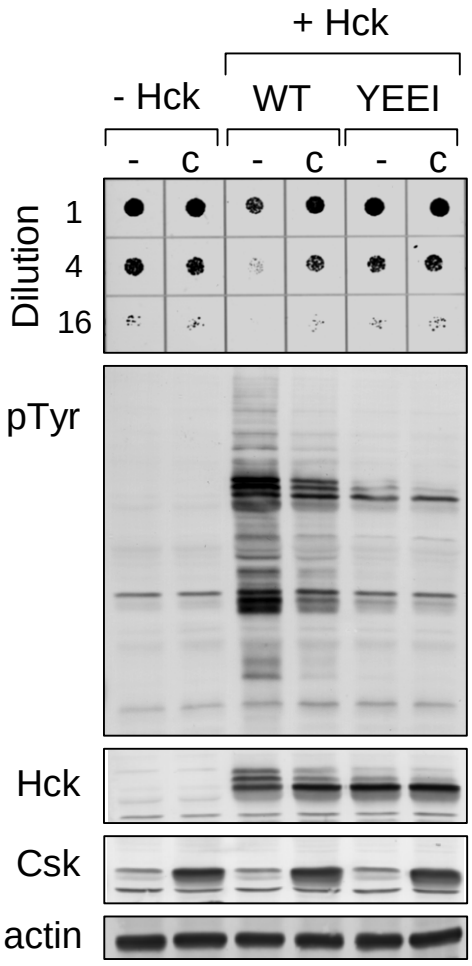


Figure 3-1. Hck-YEEI models Csk-downregulated Hck in yeast.

Yeast cultures were transformed with expression plasmids for wild-type Hck (WT), Hck-YEEI (YEEI) or the empty expression plasmid (- Hck). Cells were co-transformed with expression vectors for Csk (C) or the corresponding empty vector (-) as indicated. Top: Cells were spotted onto agar selection plates containing galactose as the sole carbon source and incubated for 3 days at 30ºC. Cultures were spotted in four-fold dilutions to enhance visualization of the growth suppressive phenotype. Plates were scanned and yeast patches appear as dark circles. Lower panels: Immunoblots from cultures shown at the top. Transformed cells were grown in liquid culture in the presence of galactose at 30ºC for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) as well as for Hck, Csk, and actin as a loading control.

3.4.2 Nef activates Hck-YEEI in yeast by the same mechanism observed in mammalian cells

Our recent work has shown that HIV-1 Nef can activate Csk-downregulated wild-type Hck in yeast, leading to growth suppression (352). To determine whether Nef could similarly activate auto-inhibited Hck-YEEI, yeast were transformed with plasmids encoding wild-type Hck or Hck-YEEI in the presence or absence of Csk and Nef. Csk and Nef expression had no effect on yeast growth in the absence of Hck (Figure 3-2*a*, columns 1-3). Wild-type Hck suppressed yeast growth, and this effect was reversed upon co-expression of Csk (columns 4 and 5). Nef strongly enhanced Hck-mediated growth suppression independent of Csk (columns 6 and 7) as observed previously (352). Importantly, co-expression of Nef with Hck-YEEI also induced a strong growth suppressive effect which was unaffected by Csk (columns 8-11). Co-expression of Nef with wild-type Hck resulted in much stronger tyrosine phosphorylation of yeast proteins than observed with Hck alone or in the presence of Csk (Figure 3-2*b*, lanes 4-7). Nef produced a similar increase in the kinase activity of Hck-YEEI (lanes 8 and 10). The effects of Nef on wild-type Hck and Hck-YEEI activities were unaffected by the presence of Csk (lanes 7 and 11). In all cases, a strong inverse correlation was observed between yeast protein-tyrosine phosphorylation and yeast growth. These data establish that Nef strongly activates Hck-YEEI and induces a growth-suppressive phenotype very similar to that observed with wild-type Hck.

Before initiating the chemical library screen, we also investigated whether the key structural determinants of Nef-induced Hck activation were functional in the yeast system. Nef activates Hck by binding tightly to its SH3 domain and disrupting a negative regulatory interaction with the SH2-kinase linker on the non-catalytic face of the kinase domain (35). To determine if Nef activates Hck-YEEI via SH3 domain engagement in yeast, we mutated the Nef PxxP motif essential for SH3 engagement (Figure 3-3*a*) and co-expressed this mutant (Nef-PA) with Hck-YEEI. In contrast to wild-type Nef, the Nef-PA mutant failed to activate Hck-YEEI and induce growth suppression (Figure 3-3*b*).

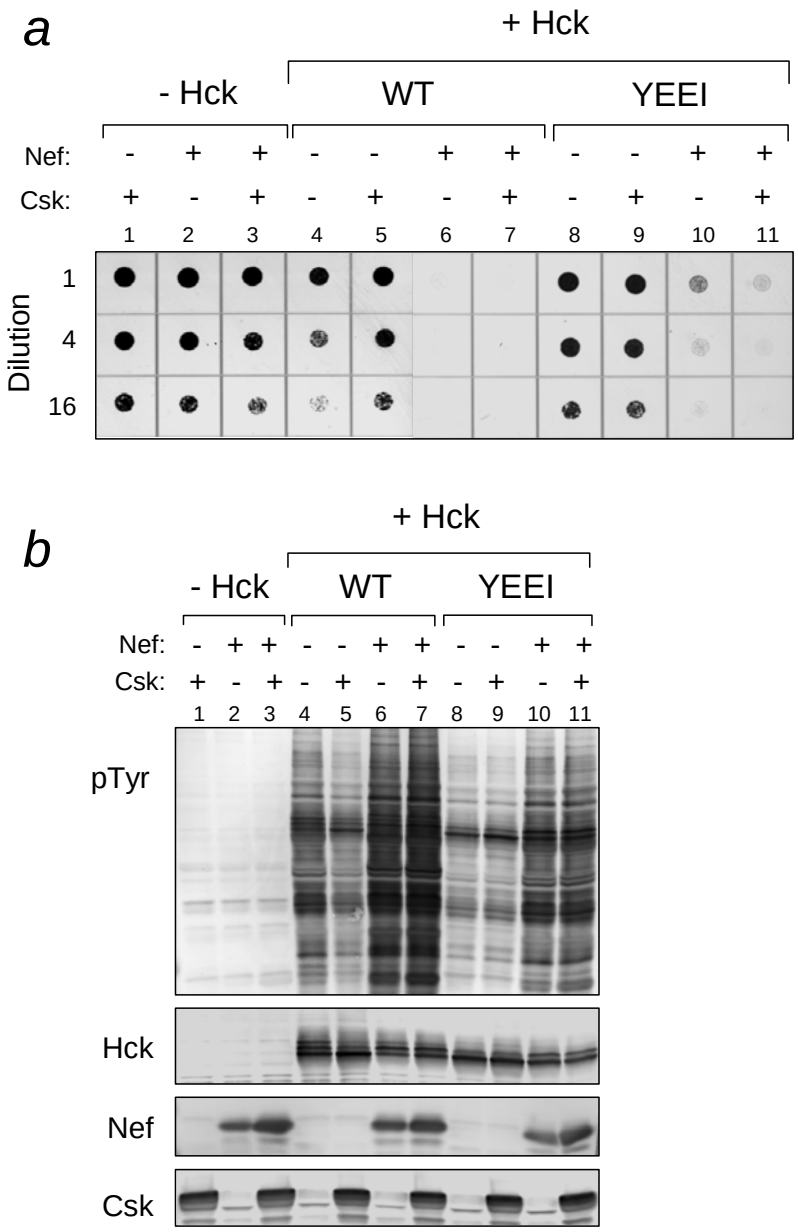


Figure 3-2. Nef activates Hck-YEEI in yeast.

Yeast cultures were co-transformed with expression plasmids for wild-type Hck, Hck-YEEI, Csk, and Nef in the combinations shown. ***a***) Cultures were grown on galactose-agar plates and scanned as described in the legend to Figure 3-1. ***b***) Immunoblots from cultures shown in panel *a*. Transformed cells were grown in liquid culture in the presence of galactose at 30ºC for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr) and for Hck, Csk, and HIV Nef. Note that the numbers in *a* correspond with the lanes in ***b***.

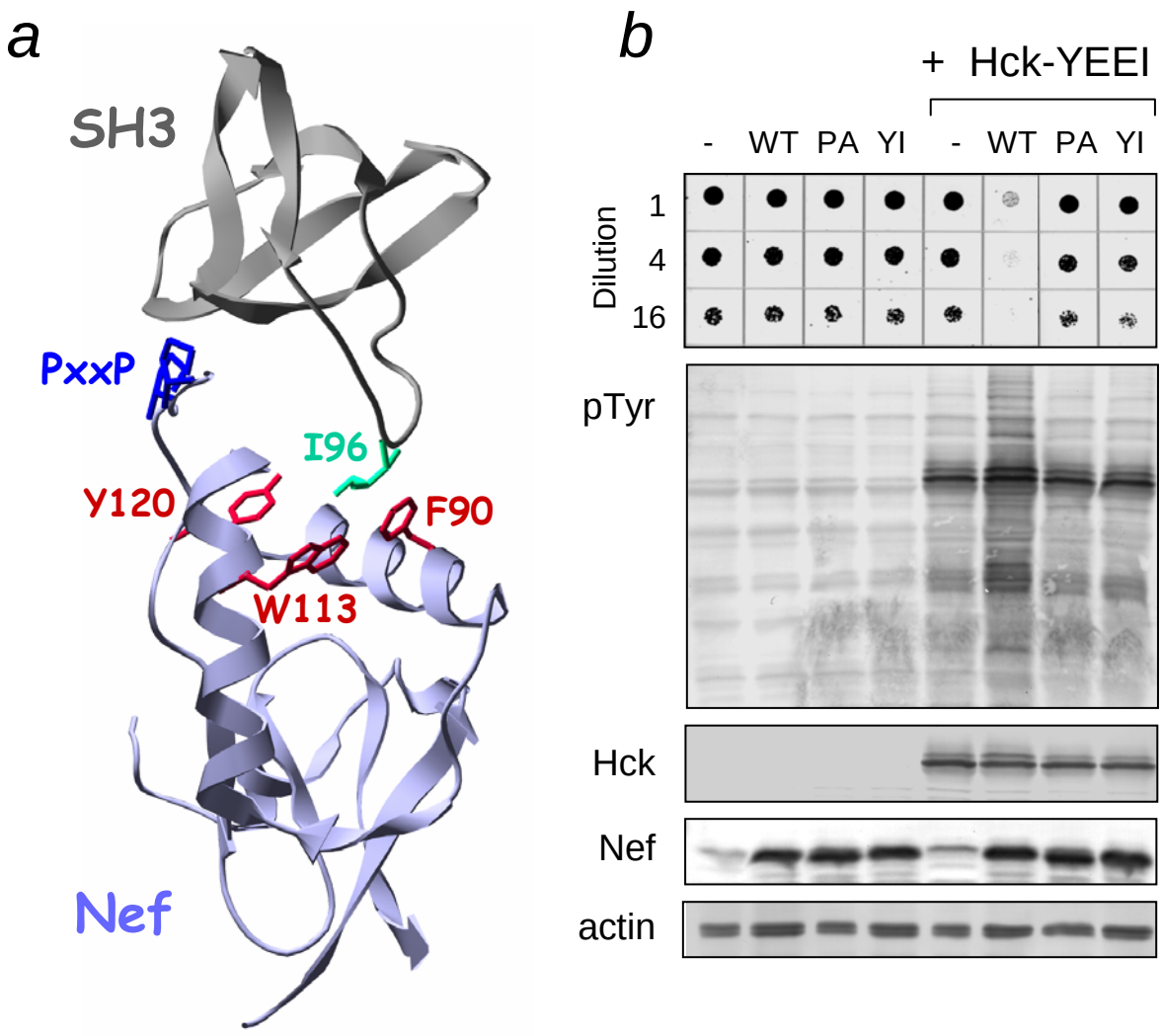
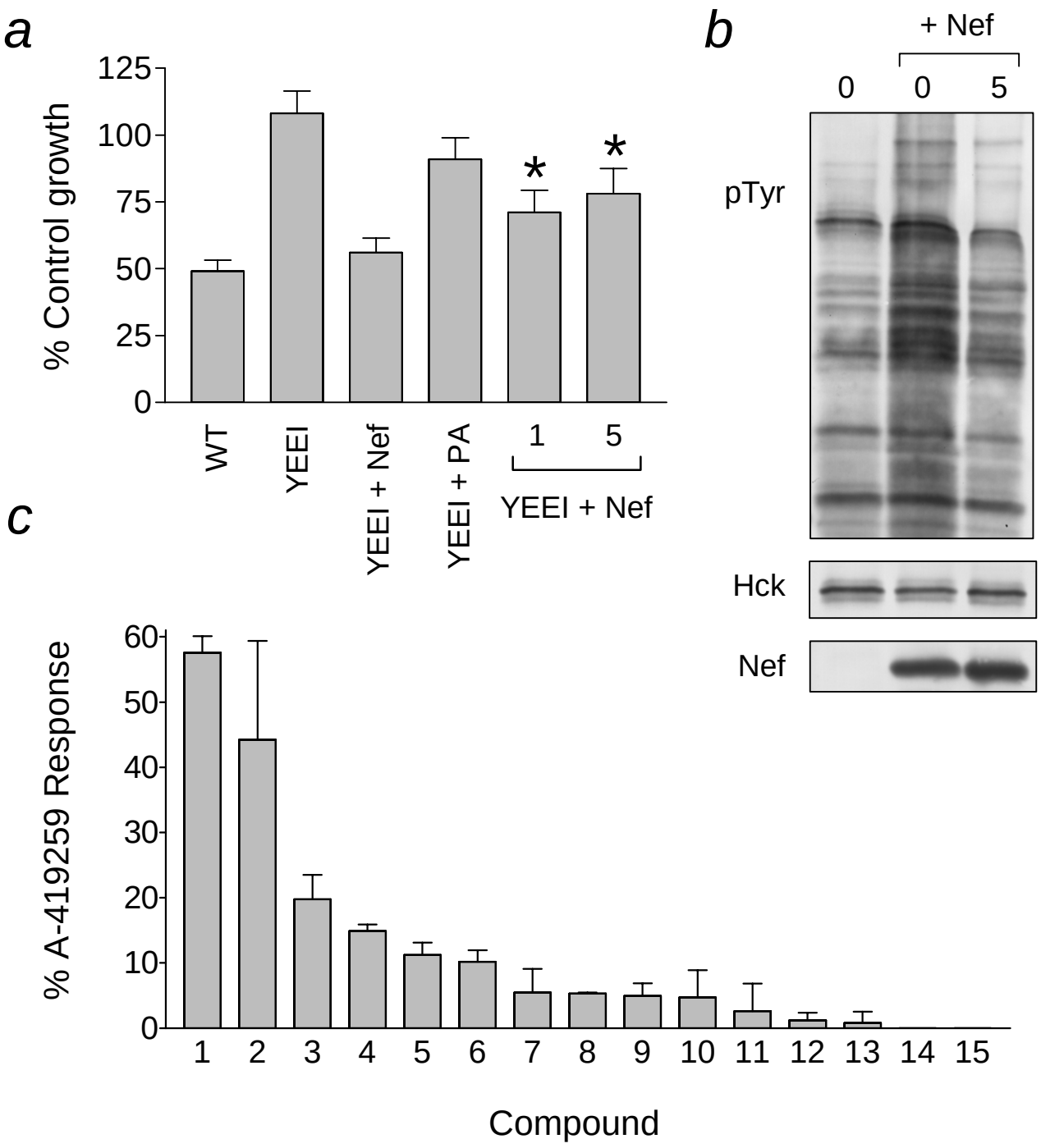


Figure 3-3. Activation of Hck-YEEI in yeast depends on an intact Nef-PxxP motif and hydrophobic pocket.
a) Structure of the Nef:SH3 complex. The SH3 domain is shown in grey, while Nef is colored violet. Side chains of conserved prolines in the Nef N-terminal region that contact the SH3 hydrophobic surface are shown (PxxP; blue). The SH3 RT loop Ile reside (I96; green) interacts with several conserved hydrophobic residues that extend from the intersection of the αA and αB helices to form a hydrophobic pocket (F90, W113, Y120; red). This view was produced using the crystallographic coordinates of Lee, et al. (213) ***b***) Upper panel shows growth of yeast cultures expressing Hck-YEEI either alone (-) or together with wild-type Nef (WT), the Nef-PA mutant in which the PxxP motif is replaced by AxxA (PA), or the Nef hydrophobic pocket mutant Y120I (YI). Cultures were spotted and scanned as per the legend to Figure 3-1. Lower panels: Lysates of the yeast cultures shown in the top panel were immunoblotted with anti-phosphotyrosine antibodies (pTyr) as well as Hck, Nef and actin antibodies as described in the legends to Figures 3-1 and 3-2.

The structure of the Nef-SH3 complex revealed that a hydrophobic pocket formed by several conserved non-polar side chains in the Nef core interact with Ile-93, a residue unique to the RT loops of the Lyn and Hck SH3 domains (213) (Figure 3-3*a*). Recently, we showed that substitution of Tyr-120 within this Nef hydrophobic pocket with isoleucine (Nef-Y120I) disrupts Nef-mediated Hck activation in rodent fibroblasts (59). Similarly, Nef-Y120I was unable to activate Hck-YEEI in yeast, failing to produce growth suppression (Figure 3-3*b*). These data show that Nef recognizes and activates Hck-YEEI in yeast through the same mechanism observed in mammalian cells.

3.4.3 Chemical inhibition of Nef:Hck-YEEI activity restores yeast growth

Because the active Nef:Hck-YEEI complex causes growth arrest, we predicted that inhibitors of this complex should restore growth, thus providing the basis for an inhibitor screen. We tested this idea with A-419259, a potent inhibitor of Hck and other SFKs (241,381). Liquid cultures of yeast co-expressing Hck-YEEI and Nef were grown in the presence or absence of A-419259, and growth was monitored as the change in optical density at 600 nm. As shown in Figure 3-4*a*, A-419259 significantly reversed the growth suppression induced by the Nef:Hck-YEEI complex at both 1 and 5 μM in comparison to untreated cultures expressing Nef and Hck-YEEI. At 5 μM, A-419259 treatment was nearly as effective as mutation of the Nef PxxP motif essential for SH3 binding in terms of reversing the growth arrest. This effect of A-419259 correlated with a decrease in tyrosine phosphorylation of yeast proteins to control levels in the inhibitor-treated cultures (Figure 3-4*b*). The ability of A-419259 to rescue growth from the suppressive effects of the Nef:Hck-YEEI complex supported the broader use of the yeast-based system to identify selective inhibitors of Nef:SFK signaling.


a
% Control growth
125
100
75
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25
0
WT
YEEI
YEEI + Nef
YEEI + PA
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YEEI + Nef
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+ Nef
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pTyr
Hck
Nef
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60
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30
20
10
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1
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6
7
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11
12
13
14
15
Compound

Figure 3-4. Identification of inhibitors of Nef:Hck-YEEI signaling in yeast.

a) Assay validation. Liquid cultures of yeast expressing wild-type Hck (WT), Hck-YEEI (YEEI), and Hck-YEEI plus wild-type or mutant (PA) Nef were grown in 96-well plates for 22 h at 30 ºC. Cultures expressing Hck-YEEI and Nef were also grown in the presence of the broad-spectrum SFK inhibitor A-419259 at 1 and 5 μM under the same conditions. Growth was recorded as change in optical density at 600 nm, and data are normalized to the percentage of growth observed relative to cells transformed with the empty expression plasmids. Each condition was repeated in triplicate, and the bargraph shows the mean percentage ± S.D. The statistical significance of the values obtained with Hck-YEEI plus Nef alone was compared to the same cultures grown in the presence of 1 or 5 μM A-419259; * indicates statistical significance ($p = 0.01$ in each case). ***b***) Yeast cultures expressing Hck-YEEI alone or Hck-YEEI plus Nef in the presence or absence of 5 μM A-419259 were grown in liquid medium in the presence of galactose at 30 ºC for 18 h. Protein extracts were separated via SDS-PAGE, and immunoblotted for tyrosine-phosphorylated proteins (pTyr), Hck and HIV Nef. ***c***) Fifteen initial hits from the chemical library screen were retested over a range of concentrations against A-419259 (5 μM). The plot shows a ranking of the results as a percentage of the growth reversion observed with A-419259. Optimal concentrations varied between compounds, which most likely reflects an effect on the Nef:Hck target vs. cytotoxic effects at higher concentrations for some compounds. Data shown were obtained at 30 μM with the exception of compounds 3, and 10 (10 μM), 4 and 6 (3 μM), and 9 (1 μM). The library compounds in this assay were obtained from ChemDiv, Inc. and prepared by C. Foster and J. Lazo. Powder compounds used in (c) were purchased from Chembridge Research Laboratories, Inc.

Yeast cultures expressing the Nef:Hck-YEEI complex were then used to screen a chemical library of 2496 discrete heterocyclic compounds to find small molecules that reversed the growth suppressive effect of Nef-induced Hck-YEEI activation. In the first pass, each compound was tested in duplicate for its ability to increase yeast growth over Nef:Hck-YEEI cultures incubated with the carrier solvent alone. From this primary screen, 170 compounds were observed to restore at least 10% of growth to Nef:Hck-YEEI cultures compared with control cultures. These compounds, taken from the original library plates, were then re-screened at approximately 10 uM concentration and evaluated against 5 μM A-419259. Of these, fifteen compounds were observed to rescue growth to at least 25% of A-419259-induced recovery. Each of these compounds was then obtained in powder form and re-tested a third time over a range of concentrations to verify growth recovery of Nef:Hck-YEEI cultures compared with A-419259. Figure 3-4*c* shows the resulting rank order of these compounds. Though the activities

of these compounds were noticeably lower than those taken from the original library, the relative activities of these compounds were similar to that seen in the secondary screen.

3.4.4 Hits from Nef:Hck-YEEI yeast screen block Nef-dependent HIV replication

We next evaluated the top five compounds from the yeast screen for activity in a Nef-dependent HIV replication assay. For these experiments, we used U87MG astroglioma cells engineered to express the HIV-1 co-receptors CD4 and CXCR4. Replication of HIV-1 NL4-3 is dependent upon an intact viral *nef* gene in these cells, and they express the Nef-responsive Src family kinases Lyn and c-Src (R. Trible, T. Kodama, and T. Smithgall, unpublished data). U87MG cells were infected with HIV-1 in the presence of the top five compounds identified in the yeast screen (Figure 3-4*c*) and HIV replication was monitored as p24 release into the culture supernatant 4 and 5 days later. As shown in Figure 3-5*a*, compounds [2] and [3] showed marked inhibition of HIV replication in this assay at 5 μM. Subsequent dose-response studies revealed that compound [2] blocked HIV replication with an IC_{50} value of approximately 130 nM in this system (Figure 3-5*b*). Neither of these compounds is cytotoxic to U87MG cells, as judged by Alamar Blue (resazurin) cell viability assay, up to 50 μM, indicating that the inhibition of HIV replication is not due to non-specific effects on cell growth (data not shown). To our knowledge, this is the first report describing the discovery of novel small molecules targeted to an HIV accessory protein:host protein kinase complex that exhibit anti-HIV activity.

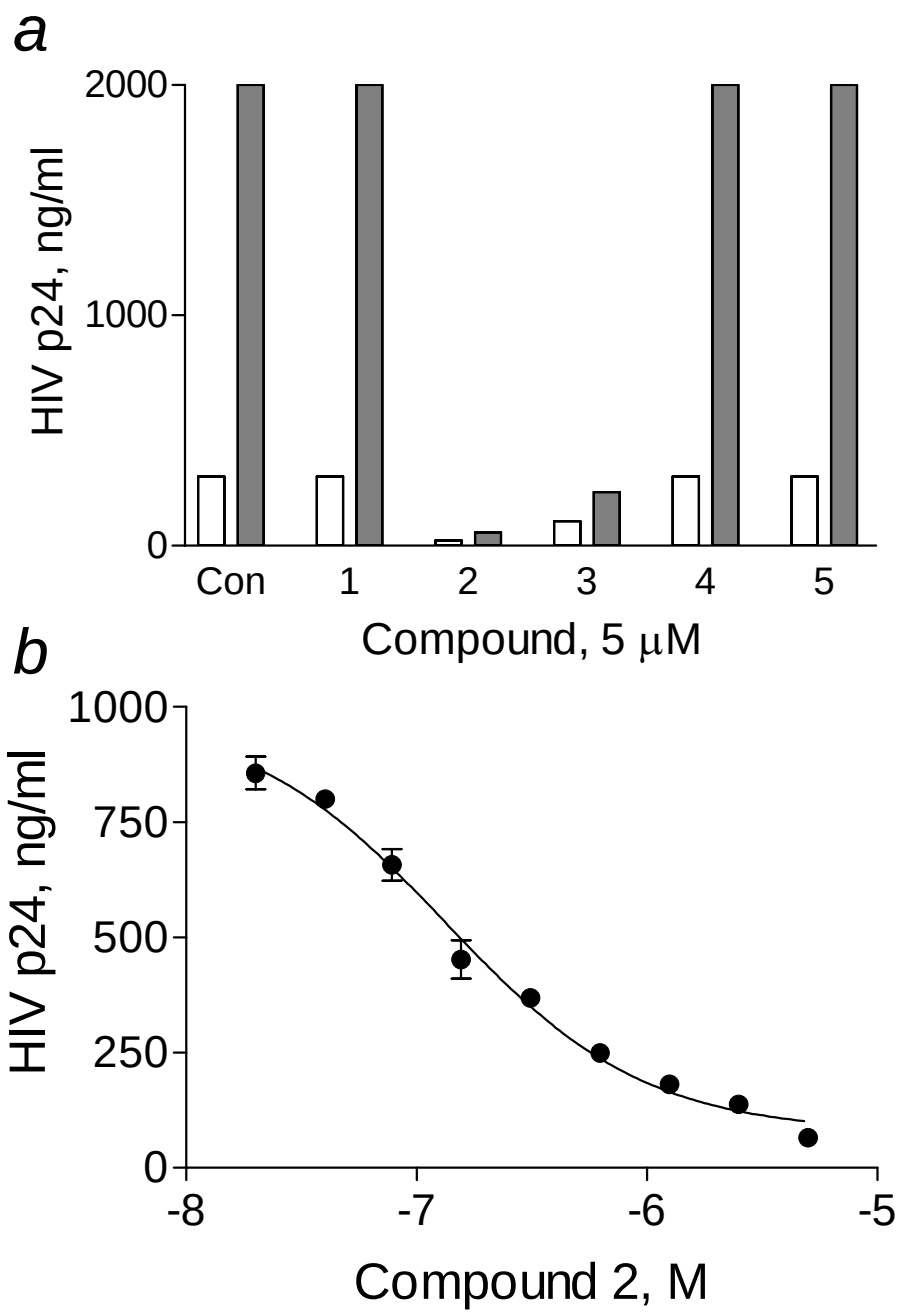

Figure 3-5. Hits from the yeast-based Nef:Hck screen block HIV replication.

a) U87MG/CD4-X4 cells were infected with HIV strain NL4-3 for 4 (white bars) or 5 (gray bars) days in the presence of the top five compounds selected from the Nef:Hck-YEEI yeast screen shown in Figure 3-4*c*. Release of viral p24 was determined by ELISA 4 and 5 days post-infection. Note that the upper limit of p24 detection in this assay is 2000 ng/ml. ***b***) Dose response curve for compound 2 from part *a*. Non-linear curve fitting was used to estimate an IC_{50} value of 130 nM. This work was performed by T. Kodama.

3.5 DISCUSSION

In this report we describe the discovery of two novel anti-HIV agents identified by targeting the complex of the HIV-1 accessory protein Nef with one of its host cell targets, the Src family kinase Hck. The yeast-based screening assay was developed so that activation of Hck-YEEI, a modified form of Hck capable of auto-downregulation, requires Nef binding for activity and subsequent growth arrest. In this way, Hck kinase activation serves as a useful reporter for Nef function, which lacks direct catalytic activity amenable to HTS. Yeast provided a useful system for lead compound identification, because hits rescue Nef:Hck-mediated growth suppression. In contrast, cytotoxic compounds cannot rescue growth and therefore do not score as false positives. Remarkably, two of the top three compounds identified in this screen were found to potently block Nef-dependent HIV-1 replication in vitro.

The anti-HIV effects of the compounds reported here may be due to several mechanisms. Because the compounds were isolated from a chemical library biased towards heterocyclic structures that resemble protein kinase inhibitors, one possibility is that they target the ATP-binding site of Nef-activated SFKs. Using an in vitro kinase assay and recombinant purified Hck and Lyn, we were unable to detect inhibition of kinase activity against a peptide substrate by either of the compounds that exhibited anti-HIV activity (data not shown). However, this result does not rule out the possibility that the compound may target another region of the kinase and affect cellular substrate recruitment essential for Nef:SFK signaling in HIV-infected cells. A precedent for this idea is provided by the recent work of Adrian, et al. (4), who describe a potent new class of anti-CML compounds that target the Bcr-Abl kinase through its myristic acid binding pocket rather than the active site. Interestingly, these compounds were also discovered in a cell-based assay, and failed to block the activity of the Abl kinase domain in a more conventional in vitro kinase assay. Another possibility is that our compounds target HIV Nef directly and affect its ability to signal through SFKs and other Nef binding proteins. This possibility is supported by the observation that these compounds are potent inhibitors of HIV replication in the U87MG system, where viral replication depends upon Nef. Possible Nef-directed targets for these compounds may include the Nef hydrophobic pocket, which is essential

for SFK recruitment and activation (59) (Figure 3-3). Effects on Nef dimerization and localization to the plasma membrane are also possible, which are critical for activation of SFKs and other Nef functions (398). Future work will address the specific mechanism of action of these compounds in HIV-infected cells.

With the exception of the viral fusion inhibitor enfuvirtide, most clinically useful anti-HIV agents target either the viral reverse transcriptase or protease enzymes (358). Here we provide the first proof-of-concept that the HIV-1 accessory protein Nef, together with its host cell kinase binding partners, represents an additional target for small molecule anti-HIV drug discovery. In a related study, Murukami et al. used a yeast two-hybrid screen to identify inhibitors of Nef:Hck complex formation (250), limiting its utility as a drug lead. However, this approach yielded a compound known to non-specifically inhibit RNA synthesis. In contrast, our compounds blocked the ability of Nef to activate downregulated Hck in yeast and, more importantly, arrest Nef-dependent viral replication in HIV-infected cells. The growing number of HIV strains resistant to conventional anti-retroviral therapy (230,355) combined with the lack of an HIV vaccine underscore the continued need for new anti-HIV drugs. Work presented here validates HIV-1 Nef as a target for small molecule anti-HIV therapy, and demonstrates the potential of a screen based on the complex of an HIV-1 accessory protein with a host cell factor as a route to their discovery.

4.0 CHAPTER 4

INVESTIGATION OF PRIMARY HIV-1 NEF ALLELES SUGGESTS ADDITIONAL MECHANISMS OF NEF-INDUCED SRC FAMILY KINASE ACTIVATION

Ronald P. Trible, Lori Emert-Sedlak, Jayanth Venkatachari, Velpandi Ayyavoo, and Thomas E. Smithgall

4.1 ABSTRACT

Activation of Src family kinases (SFKs) by HIV-1 Nef plays an important role in the pathogenesis of HIV/AIDS. The binding of Nef to SFKs is based primarily on an SH3-mediated interaction with a highly conserved PxxP motif in the Nef core domain. However, we recently showed that the PxxP sequence alone is not sufficient for high-affinity SFK binding, as other residues were found to be critical for the association. Here, we explored other facets of Nef:SFK interactions outside of the canonical SH3-binding motif. We used a yeast growth suppression assay, previously utilized to identify SFK targets of Nef, to screen a panel of patient-derived primary Nef alleles for altered SFK activation. We identified two alleles whose proteins failed to activate the SFKs Hck and Lyn. We also discovered two proteins which activated SFKs markedly better than previously studied Nef proteins. Sequence analysis revealed amino acid changes in regions not previously suspected to be involved in SH3-mediated interaction, including a long flexible loop not present in the x-ray crystal structure. Together, these findings suggest that previously unrecognized residues outside of the Nef PxxP binding motif directly affect SFK activation in vivo.

4.2 INTRODUCTION

The HIV-1 Nef protein has been well documented as a critical factor for the pathogenesis of AIDS (100,136,149,176,188). The role of Nef in HIV has gained particular interest since it was noted that numerous HIV-positive individuals who survived ten years or more without requiring therapy, termed long-term nonprogressors (LTNPs), showed mutations or deletions within the *nef* genes of their infecting viruses (90,192,234). While many other factors may be responsible for the remarkable delay in disease onset in LTNPs (170,242), in some cases disease progression can be correlated with changes in *nef* sequence and function (51,191).

Recently, the interactions between HIV-1 Nef and the Src family of nonreceptor protein-tyrosine kinases (SFKs) have been found to be important for disease progression. For instance, monocyte-derived macrophages show a correlation between HIV infectivity and Hck expression (198). Further, in an HIV mouse model, Nef alone is sufficient to drive an AIDS-like phenotype (148), and this progression is greatly delayed when the SFK member Hck is deleted by gene targeting (150). Finally, Nef-mediated c-Src activation has been found to be critical in the formation of HIV-associated nephropathy (155).

SFKs share a homologous set of modular domains, including SH3 and SH2 domains, a bi-lobed tyrosine kinase domain, and a C-terminal tail containing a tyrosine-based SH2-binding motif (47,349). In their inactive states, SFKs feature two sites of intramolecular binding which modulate the downregulated conformation – SH2 binding to the phosphorylated C-terminal tail and SH3 binding to a polyproline type II helix located in the linker sequence between the SH2 and kinase domains (321,379,390). Release of either of these intramolecular contacts induces conformational changes within the catalytic site to permit kinase activation (35,215,216). In fact, we and others have demonstrated that Nef can activate downregulated SFKs via engagement of the SH3 domain and its concomitant release from the linker (43,215,244).

The structure of an SFK SH3 domain bound to the structured core domain of Nef has been solved and indicates two regions of Nef important for association with SFKs – a proline-rich motif (PxxP) and a hydrophobic pocket that engages in Ile96 of the SH3 domain RT loop (213). The PxxP motif is highly conserved among Nef alleles and is generally regarded as the

predominant factor for Nef binding to SFK SH3 domains (102,191,259). However, we recently confirmed the critical nature of the hydrophobic pocket, finding that substitution of a single tyrosine in this region is sufficient to disrupt Hck binding and activation, even in the presence of an intact PxxP motif (59). Further, we have also discovered that, despite the high structural homology among the SH3 domains of Src kinases, there is considerable variation in the ability of Nef to bind and activate different Src family members (59,352). Finally, we have also demonstrated that binding of Nef to SFK SH3 domains in vitro does not necessarily predict Nef-mediated kinase activation in vivo (41). One such example is that Nef SF2 binds the SH3 domain of Fyn, yet fails to activate the full-length kinase in a fibroblasts. Together, these findings suggest that sequence homology is insufficient to predict protein binding, and that binding studies are insufficient to predict kinase activation. Further, these data imply that additional Nef motifs may contribute to SFK activation.

In the present study, we investigated the ability of a wide variety of Nef alleles to activate SFKs to better understand the nature of Nef:SFK interactions and their role in disease pathogenesis. We utilized a yeast growth suppression assay, recently established as a model for investigating the regulation of Src kinases and their activation by Nef (48,199,251). All of the Nef alleles used in our study contain the conserved PxxP motif and hydrophobic pocket residues, offering us the chance to investigate other regions of the molecule that may be involved in Src kinase activation. We found that commonly used laboratory Nef alleles, with the exception of Nef ELI, activated Hck in our yeast assay in a manner consistent with what we have seen previously in a fibroblast transformation assay (59). Furthermore, the primary Nef proteins screened here demonstrated unexpected variability in their ability to activate SFKs, indicating that residues outside of known SH3-binding regions contribute to Nef-mediated SFK activation.

4.3 MATERIALS AND METHODS

Yeast constructs: Coding sequences for human Csk, Hck, and Lyn were amplified by PCR from existing templates to introduce a yeast translation initiation sequence (AATA) immediately 5' to the ATG start codon. The cDNA clones for HIV-1 Nef laboratory- and patient-derived strains were similarly amplified and modified. A FLAG tag was added to the C-terminus of each Nef coding sequence to provide a conserved epitope for immunoblotting. The Lyn cDNA clone was subcloned downstream of either the Gal10 promoter in the yeast expression vector pESC-Ura (Stratagene). Hck was subcloned downstream of the Gal10 promoter in the pYC2/CT vector (Invitrogen), which carries the CEN6/ARSH4 sequence for low-copy replication. The Csk and Nef cDNAs were subcloned downstream of either the Gal 1 or Gal10 promoter in pESC-Trp (Stratagene). The Nef-2PA mutant, in which prolines 72 and 75 are replaced with alanines, has been described elsewhere (43).

Yeast growth suppression assay: Yeast were prepared as described before (352). Briefly, *S. cerevisiae* strain YPH 499 (Stratagene) cells were electoporated (BioRad GenePulser II) with pESC-Ura (or pYC2/CT) and pESC-Trp plasmids containing the genes of interest. Co-transformed yeast were selected by nutritional selection (SD/-Ura/-Trp) for three days at 30° C on glucose agar plates, which repress gene expression. Positive transformants were grown in selection liquid containing glucose, normalized to OD_{600} = 0.2 in water, and then spotted in dilutions onto SD/-Ura/-Trp agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for three days at 30° C and imaged on a flatbed scanner, where yeast patches appear as dark spots against the translucent agar background. All growth suppression assays were repeated at least three times from independent transformed clones, of which representative examples are shown.

Immunoblotting: Remaining liquid yeast cultures used for the spot assay were incubated in SD/-Ura-/Trp medium plus galactose for 18 h. Cells were pelleted, treated with 0.1 N NaOH for 5 min at room temperature (202), and resuspended with SDS-PAGE sample buffer

to 0.02 OD_{600} units per μl. Lysates (0.2 OD_{600} units) were separated via SDS-PAGE, transferred to PVDF membranes, and probed for protein phosphotyrosine content with a combination of the anti-phosphotyrosine antibodies PY99 (Santa Cruz Biotechnology) and PY20 (Transduction Laboratories). Protein expression was verified by immunoblotting with antibodies to Csk (C-20; Santa Cruz), FLAG (M2; Sigma), Hck (N-30; Santa Cruz), and Lyn (44; Santa Cruz). Nef antibodies (Hyb 6.2) were obtained from the NIH AIDS Research and Reference Reagent Program.

Purification of Nef-va04:

The Nef-va04 allele was expressed in Sf9 insect cells and purified as described previously for Nef-SF2 (352). Briefly, a hexahistidine tag was added to the N-terminus of va04 by PCR and the cDNA subcloned into the pVL1393 baculovirus expression plasmid. Protein was expressed in Sf9 insect cells using Baculogold DNA according to the manufacturer's protocol (BD-Pharmingen). Recombinant proteins were purified using metal chelating affinity chromatography. Purity and concentration were determined by SDS-PAGE and densitometry, as well as via Bradford assay (Pierce, Coomassie Plus Protein Assay Reagent).

In vitro kinase assay: Tyrosine kinase assays were performed using the FRET-based Z'-lyte kinase assay system as described before (352). Briefly, Hck (20 ng) and Lyn (50 ng) were incubated at room temperature for 5 min with a 5- or 10-fold molar excess of Nef-SF2 or Nef-va04, ATP (50 μM final) and Tyr2 substrate (2 μM final) were added to the reaction, then incubated for 45 min (Hck) or 1 h (Lyn). Development reagent, containing a protease that digests non-phosphorylated peptide, was then added, the reaction incubated another 60 min, then stopped with the proprietary stop reagent. Fluorescence was assessed at an excitation wavelength of 400 nm; coumarin fluorescence and the fluorescein FRET signal were monitored at 445 nm and 520 nm, respectively. Reactions containing unphosphorylated peptide and kinase without ATP served as 0% phosphorylation control, while a stoichiometrically-phosphorylated peptide was used as a 100% phosphorylation control. Reaction endpoints were calculated as emission ratios of coumarin fluorescence divided by the fluorescein FRET signal, then normalized to the ratio obtained with the 100% phosphorylation control. Each condition was

assayed in quadruplicate, and the entire experiment was performed one to three times; results are presented as the mean ± S.D.

4.4 RESULTS

4.4.1 Activation of Hck by multiple HIV-1 Nef alleles

We and others have previously shown that SFKs are active upon expression in *S. cerevisiae*, as defined by growth suppression and increased tyrosine phosphorylation of yeast proteins (48,199,352). SFK activity is inhibited by co-expression of the negative regulator Csk, resulting in restored yeast growth and diminished phosphorylation of yeast cell proteins (352). This system utilizes the alterations in yeast growth as an easily-readable indicator of Src kinase activity.

To evaluate the ability of different HIV-1 Nef alleles to activate Csk-downregulated Hck, we co-expressed Nef isolates from six common laboratory strains of HIV-1 (Consensus (319), ELI, LAI, NL4-3, SF2, and YU-2) with Hck and Csk in yeast and evaluated growth and tyrosine kinase activity. Each of these Nef alleles contains the conserved PxxP SH3-binding motif and lacks any obvious deletions or predicted to interfere with Hck binding and activation. As shown in Figure 4-1, expression of Hck alone induces growth suppression, which is reversed with the co-expression of Csk. However, co-expression of Hck and Csk with Consensus, LAI, NL4-3, SF2, and YU-2 Nef proteins induced rigorous growth suppression (Figure 4-1, top) and markedly increased tyrosine phosphorylation of yeast proteins (Figure 4-1, middle), as compared with Hck and Csk alone. In contrast, the Nef-ELI protein had no effect on the growth of yeast when co-expressed with Csk and Hck, and failed to increase Hck kinase activity. This result is consistent with our previous findings in mammalian cells, and can be attributed to replacement of Tyr-120 with Ile in the ELI hydrophobic binding pocket (59).

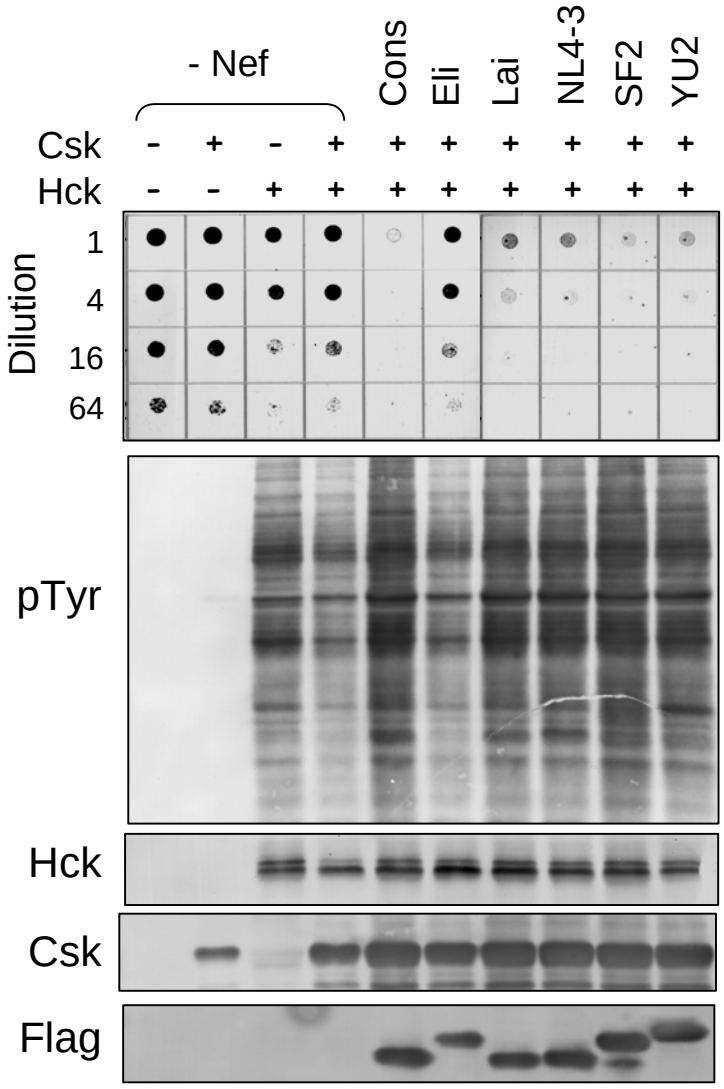

Figure 4-1. Activation of Csk-downregulated Hck by laboratory-derived Nef alleles.

Top: Yeast cultures were co-transformed with constructs expressing Hck, Csk, and either Consensus, ELI, LAI, NL4-3, SF2, or YU-2 FLAG-tagged Nef alleles, normalized to cell density, then spotted onto galactose agar plates at increasing dilutions, incubated for 3 days at 30ºC, then scanned. Yeast patches appear as dark circles against the translucent agar background. Bottom: Lysates from these same cultures were separated via SDS-PAGE and immunoblotted for phosphotyrosine (pTyr), Hck, Csk, or Nef (FLAG) as indicated. Each laboratory-derived Nef allele was expressed individually and shown to have no effect on yeast growth or tyrosine phosphorylation (data not shown).

4.4.2 Laboratory-derived HIV-1 Nef alleles activate Hck-YEEI

In order to simplify our assay for the screening of larger samples of Nef alleles, we tested the ability of the laboratory Nef alleles to activate an auto-downregulated Hck molecule, Hck-YEEI. Hck-YEEI contains a substitution of the C-terminal tail of wild-type Hck with the high-affinity SH2-binding motif, YEEI (310,330). We have previously shown that Hck-YEEI is inactive in yeast and faithfully models Csk-downregulated Hck (see Section 3.4.1). In this same study, we also demonstrated that the Nef-SF2 allele activates Hck-YEEI in an SH3-dependent manner.

Each of the laboratory alleles of Nef was co-expressed with Hck-YEEI in yeast. All of these Nef isoforms were also able to activate Hck-YEEI, with the exception of ELI, as demonstrated by enhanced growth suppression and yeast tyrosine phosphorylation (Figure 4-2). These findings show that Hck is a target for multiple Nef variants and agree with the results obtained using Csk-downregulated Hck (Figure 4-1). Further, these data validate the yeast growth suppression assay as a useful system to screen Nef alleles for the ability to activate SFKs.

4.4.3 Analysis of primary Nef alleles in yeast

To explore whether SFK activation is a general feature of HIV Nef, we next screened a panel of primary *nef* gene products for their effects on Hck function. These Nef cDNAs were obtained from the Johns Hopkins AIDS Consortium and were all derived from LTNPs. Sequence analysis of the primary alleles used in this study reveal no obvious deletions or truncations, and the known Hck-binding regions, including the consensus PxxP motif and core hydrophobic pocket residues, are intact (Figure 4-3).

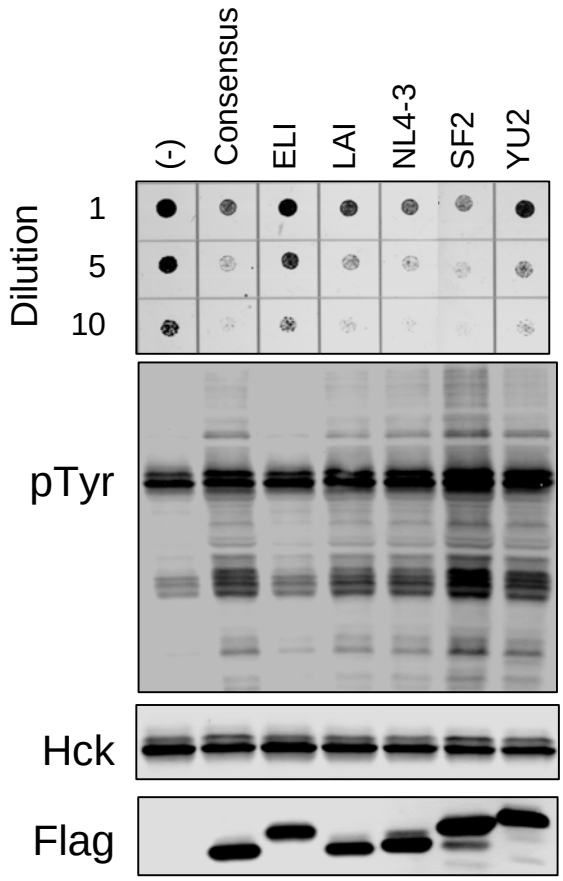

Figure 4-2. Activation of Hck-YEEI by laboratory-derived Nef alleles.

Yeast were co-transformed with constructs expressing Hck-YEEI and either empty vector (-) or the indicated laboratory Nef allele. Cultures were spotted onto galactose agar at increasing dilutions to observe changes in growth suppression. Lysates from the cultures used in the growth assay were immunoblotted for phosphotyrosine (pTyr) to observe changes in kinase activity, or Hck or Nef (FLAG) to check protein expression.

```
Consensus     1 MGGKWSKRSVSGWPAVRERMR----------RAEPAAEGVGAVSRDLEKHGAITSSNTAA
SF2           1 MGGKWSKRSMGGWSAIRERMRRAEP------RAEPAADGVGAVSRDLEKHGAITSSNTAA
LAI           1 MGGKWSKSSVVGWPTVRERMR----------RAEPAADGVGAASRDLEKHGAITSSNTAA
NL4-3         1 MGGKWSKSSVIGWPAVRERMR----------RAEPAADGVGAVSRDLEKHGAITSSNTAA
YU2           1 MGGKWSKRSMAGWPTVRERMRRAEPAAERMRRAEPAADGVGAVSRDLERHGAITSSNTAA
ELI           1 MGGKWSKSSIVGWPAIRERIRRTN----------PAADGVGAVSRDLEKHGAITSSNTAS
va05          1 MGGKWSKRSTTGWSNVRDKMRR----------AEPAADGVGAASRDLEKHGALTSSNTAA
va11          1 MGGKWSKRSMGGWSVVREKMRQAK-------PAEPAADGVGAASRDLEKYGALTNSNTAA
va04          1 MGGKWSKRSGVGWPRVRERMHR----------AEPAADGVGAASRDLEKYGAITSN-TAA
va02          1 MGGKWSKSSLVGWPNVRERMR----------RAEPAADGVGAASRDLEKHGAITSSNTAA
va12          1 MGGKWSKCSVVGWPAVRERMKR----------AEPAAEGVGAVSRDLEKHGAITSSNTAA
va01          1 MGGKWSKSSMFGWPAIRERMR----------RAEPAADGVGAASRDLEKHGALTSSNTAT
va03          1 MGSKGSKC--IGWPAVRERMKRAEP-------AEPAADGVGAVSRDLEKYGAVTSSNTAA
va15          1 MGNKGSKC--IGWPAVRERMKRAEP-------AEPAADGVGAVSRDLEKYGAVTSSNTAA

                                                                        *
Consensus    51 TNAACAWLEAQEE-EEVGFPVRPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIYSQKRQDI
SF2          55 TNADCAWLEAQEE-EEVGFPVRPQVPLRPMTYKAALDISHFLKEKGGLEGLIWSQRRQEI
LAI          51 TNAACAWLEAQEE-EEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDI
NL4-3        51 NNAACAWLEAQEE-EEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDI
YU2          61 TNADCAWLEAQEE-EEVGFPVRPQVPLRPMTHKAAMDLSHFLKEKGGLEGLIHSQQRQDI
ELI          51 TNADCAWLEAQEESDEVGFPVRPQVPLRPMTYKEALDLSHFLKEKGGLEGLIWSKKRQEI
va05         57 TNADCAWLEAQEE-EEVGFPVRPQVPLRPMTYKAAVDLSHFLKEKGGLEGLVYSQKRRDI
va11         60 TNADCAWLEAQED-EEVGFPVRPQVPLRPMTYKAAVDLSHFLKEKGGLEGLVYSQKRQDI
va04         56 NNADCAWLEAQEG-EEVGFPVRPQVPLRPMTYKGALDLSHFLREKGGLEGLVYSQKRQDI
va02         57 NNAACAWLEAQED-EEVGFPVKPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQKRQDI
va12         57 TNADCAWLEAQED-EEVGFPVKPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDI
va01         57 NNAACAWLEAQEE-EEVGFPVRPQVPSRPMTYKAAVDLSHFLKEKGGLEGLIHSQKRQDI
va03         58 NNAACAWLEAQEE-EEVGFPVRPQVPLRPMTYKAALDLSHFLKEKGGLEGLIYSSKRQEI
va15         58 NNAACAWLEAQEE-EEVGFPVRPQVPLRPMTYKSALDLSHFLKEKGGLEGLIYSSKRQEI

                                                            *
Consensus   110 LDLWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEKVEEANEGENNCLLHPMS
SF2         114 LDLWIYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEKVEEANEGENNSLLHPMS
LAI         110 LDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVS
NL4-3       110 LDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVS
YU2         120 LDLWVYHTQGYFPDWQNYTPG-GTRWPLTFGWCFKLVPVEPEKIEEANAGENNCLLHPMS
ELI         111 LDLWVYNTQGIFPDWQNYTPGPGIRYPLTFGWCYELVPVDPQEVEEDTEGETNSLLHPIC
va05        117 LDLWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEKIEEANEGENNSLLHPMS
va11        120 LDLWVYHTQGYFPDWSNYTPGPGIRYPLTFGWCFKLVPVDPQQVEEANEGENNSLLHPMS
va04        116 LDLWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEKVEEINEGENNCLLHPIS
va02        117 LDLWIYHTQGYFPDWQNYTPGPGTRWPLTFGWCFKLVPVEPEKIEEANEGENRSLLHPMS
va12        117 LDLWIYHTQGYFPDWQNYTPGPGIRYPLTFGWCFKLVPVETEQVEEANEGENNSLLHPMS
va01        117 LDLWVYHTQGYFPDWQNYTPGPGIRYPLTFGWCYKLVPVEPEQVEKANEGENNCLLHPMS
va03        118 LDLWVYHTQGFFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEQIEEANKGENNCLLHPMS
va15        118 LDLWVYHTQGFFPDWQNYTPGPGIRYPLTFGWCFKLVPVEPEQIEEANKGENNCLLHPMS

                 *                    *
Consensus   170 QHGMDDPEKEVLVWKFDSKLAFHHMARELHPEYYKDC
SF2         174 LHGMEDAEKEVLVWRFDSKLAFHHMARELHPEYYKDC
LAI         170 LHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC
NL4-3       170 LHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNC
YU2         179 QHGMDDPEREGLEWRFDSRLAFHHVARELHPEYYKN-
ELI         171 QHGMEDPERQVLKWRFNSRLAFEHKAREMHPEFYKN-
va05        177 LHGMEDPEKEVLEWKFDSRLAFHHMARELHPEYFKN-
va11        180 LHGMEDPEKEVLVWRFDSRLAFHHVAREKHPEFYKN-
va04        176 LHGMDDPEREVLVWKFDSRLALHHMARELHPEYYKNC
va02        177 LHGMEDPEREVLVWKFDSRLAFHHMARELHPEYYKDC
va12        177 LHGIEDPEREVLRWKFDSHLAFRHMAREMHPEYYKDC
va01        177 QHGMDDPEKEVLVWKFDSRLAFHHMARELHPEYYKD-
va03        178 QCGMDDPEKEVLQWKFDSHLAFRHMARELHPEYYKDC
va15        178 QCGMDDPEKEVLQWKFDSHLAFRHMARELHPEYYKDC
```

Figure 4-3. Sequence alignment of laboratory and primary HIV-1 Nef alleles.

Commonly used laboratory Nef alleles (Consensus, ELI, LAI, NL4-3, SF2, and YU2) and Nef alleles derived directly from patients (va01, va02, va03, va04, va05, va11, va12, and va15) were aligned using the Multiple Sequence Alignment tool (ClustalW, Kyoto University Bioinformatics Center). Those residues known to be involved in SFK SH3 binding are highlighted in blue, and include the P_{72}xxPxR motif and the hydrophobic residues F90, W113, and Y120. Note that ELI contains an isoleucine at position 120, which has been shown to contribute to its inability to bind and activate Hck. The stars indicate residues unique to va03 and va15 (green) or va04 (red). Numbering is based on the crystal structure of NL4-3 by Lee et al. (213).

Hck-YEEI was co-expressed with empty vector, Nef SF2 as a positive control, each of the primary Nef alleles, or a Nef SF2 construct with a mutation within the PxxP motif as a negative control (PA) (43,244,304). Six of eight primary Nef proteins (va01, va02, va03, va05, va12, and va15) induced Hck-mediated growth suppression at least equivalent to Nef-SF2, indicative of primary Nef-mediated Hck activation (Figure 4-4, top). Interestingly, despite the conservation of all known Hck-binding sequence motifs, the va04 protein failed to induce growth suppression in Hck-YEEI cultures. The co-expression of another primary allele, va11, with Hck-YEEI also showed reproducible growth inhibition, though this reduction was not as pronounced as that seen with va04. To our surprise, two primary Nef proteins, va03 and va15, mediated a Hck-YEEI growth suppressive effect much stronger than Nef SF2. As expected, the PA mutant failed to activate Hck-mediated growth suppression (352). The results of the growth suppression assay suggest that the primary Nef va03 and va15 molecules augment, and va04 decreases, Hck kinase activity. We analyze yeast proteins for changes in phosphotyrosine signal, and noted a slight decrease in intensity for va04-containing cultures (Figure 4-4, bottom). However, we were unable to detect any increase in signal for yeast cultures expressing va03 or va15.

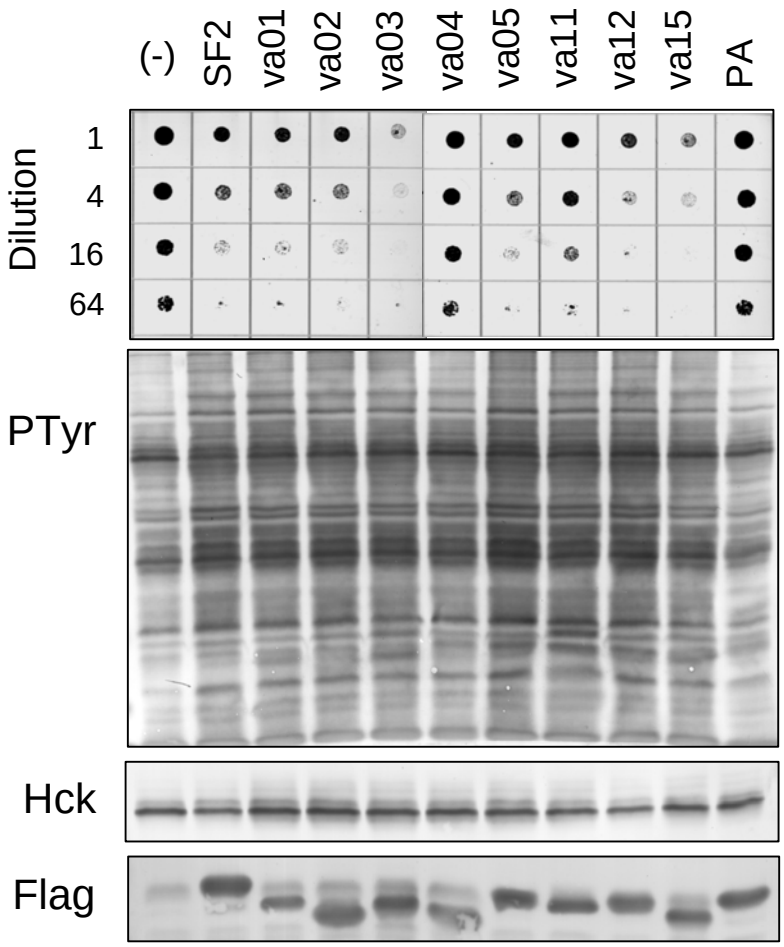

Figure 4-4. Activation of Hck-YEEI by patient-derived Nef alleles.

Yeast were co-transformed with constructs expressing Hck-YEEI with either empty vector (-), the LD Nef control allele SF2, the indicated patient-derived Nef alleles, or a mutant SF2 allele containing a PxxP to AxxA substitution which fails to activate Hck-YEEI (PA). Cultures were spotted onto galactose agar at increasing dilutions, or lysed and immunoblotted as described in Figure 4-1. Nef alleles were expressed individually and shown to have no effect on yeast growth or tyrosine phosphorylation (data not shown).

We have previously shown that, in addition to Hck, Nef SF2 can also activate the Src family kinase Lyn (352). To evaluate the effects of the primary Nef proteins on the activity of Lyn, we first developed a YEEI tail-modified version of the kinase and tested it for auto-downregulation in the yeast assay. Wild-type and YEEI-substituted forms of Lyn were expressed in yeast with or without Csk. Lyn-YEEI failed to induce growth suppression and exhibited reduced kinase activity (Figure 4-5*a*), comparable to wild-type Lyn upon co-expression of Csk. Thus, Lyn-YEEI appears to effectively model Csk-mediated wild-type kinase inhibition as observed previously for Hck.

We then tested our panel of primary Nef alleles for their abilities to influence Lyn-YEEI-mediated growth suppression in yeast. While the growth effects of the primary Nef proteins on Lyn-YEEI were not as pronounced as those seen with Hck-YEEI (Figure 4-3), several Nef molecules failed to activate Lyn-YEEI, including va04 (Figure 4-5*b*). In striking contrast, the va03 and va15 proteins appear to activate Lyn noticeably more than other Nef proteins tested.

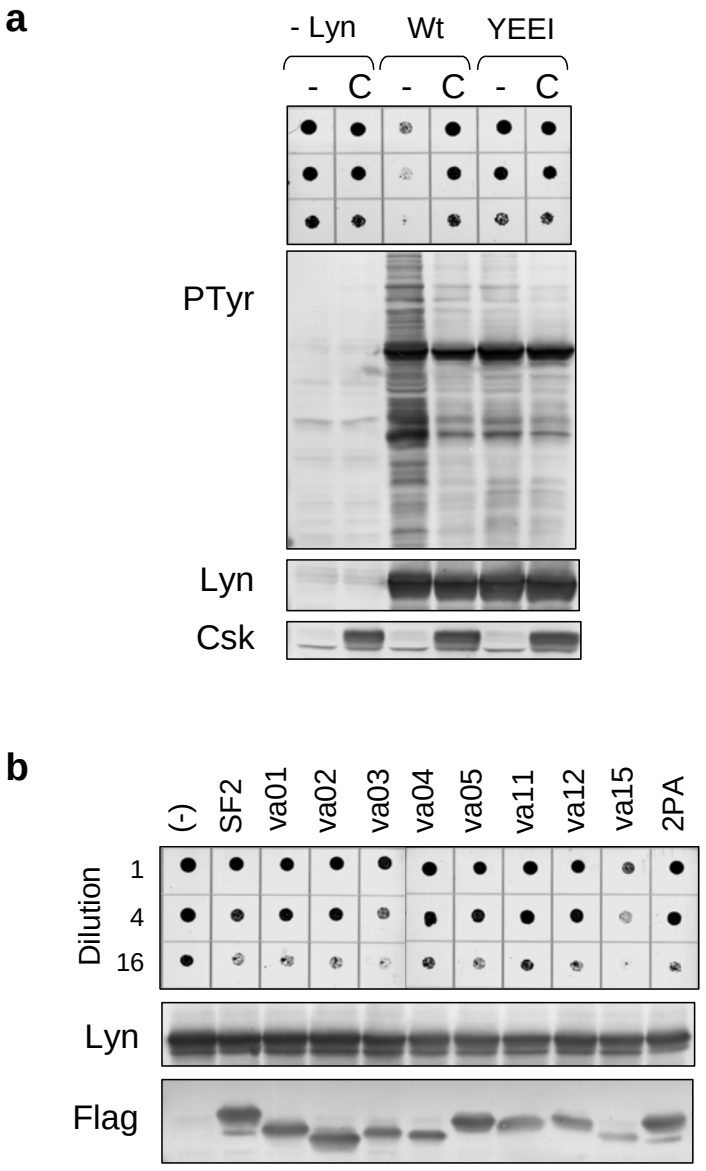

Figure 4-5. Activation of Lyn-YEEI by primary Nef alleles.

a, Wild-type Lyn (Wt) or YEEI-modified Lyn (YEEI) were co-expressed in yeast with or without Csk (C). Cultures were spotted onto galactose agar at increasing dilutions or lysed and immunoblotted as described previously. **b**, Lyn-YEEI was co-expressed in yeast with Nef SF2 for a positive control, primary HIV-1 Nef alleles as indicated or with Nef-PA mutant (PA) for a negative control. Cultures were spotted on galactose agar at increasing dilutions as described previously, or lysed and immunoblotted for Lyn or Nef (FLAG). Note: The data in columns 3 and 4 of (a) were originally presented in (352) and reproduced here for comparison.

4.4.4 The va04 Nef protein fails to induce Hck and Lyn kinase activity in vitro

Nef va04 failed to produce growth suppression upon co-expression with Hck and Lyn in yeast. This observation suggests that Nef va04 is unable to activate these SFKs, despite the presence of conserved SH3-binding motifs. To confirm this idea, Nef va04 was expressed in Sf9 insect cells, purified, and tested for its ability to activate Hck and Lyn. As shown in Figure 4-6, Nef va04 failed to activate Hck or Lyn at molar ratios resulting in strong activation by Nef SF2, as reported previously (352). These observations suggest that regions outside of the canonical SH3-binding functions contribute to SFK activation by Nef (see Discussion).

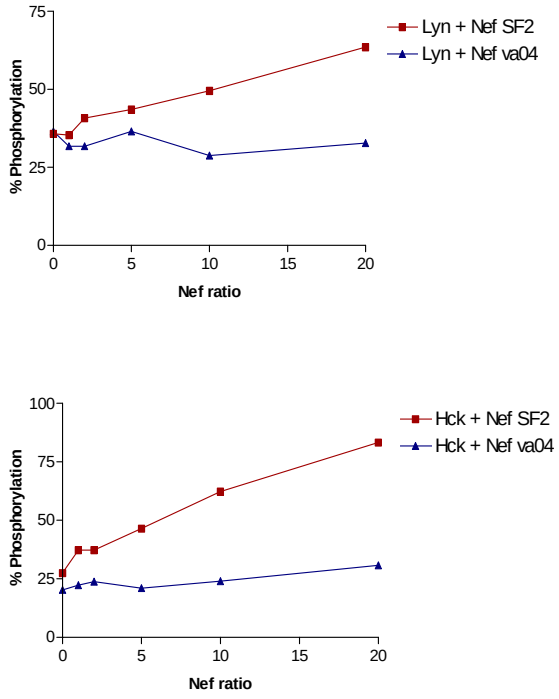

Figure 4-6. Purified Nef va04 fails to activate Hck or Lyn in vitro.
Recombinant Hck and Lyn were purified in their downregulated forms and assayed for kinase activity in the presence of increasing molar ratios of recombinant Nef SF2 or Nef va04 using a FRET-based assay. Each data point represents quadruplicate repeats and a representative experiment is shown. This work was performed by L. Emert-Sedlak.

4.5 DISCUSSION

In this report, we evaluated several common laboratory and unique primary Nef proteins for the ability to activate the SFKs Hck and Lyn in a defined cellular system. We demonstrated using a yeast growth suppression assay that Hck tyrosine kinase is a target for multiple commonly used Nef variants. We then used the yeast assay to screen a panel of primary Nef alleles for differential activation of Src kinases and identified two proteins, va04 and va11, which poorly activated Hck, as well as Lyn. We also found two alleles, va03 and va15, which activated Hck and Lyn to a greater extent than familiar Nef alleles. Finally, we expressed a recombinant Nef va04 protein and showed that it was unable to induce Hck or Lyn activation in vitro.

Previous work in our laboratory utilizing a rodent fibroblast transformation assay has demonstrated that the commonly used Nef proteins Consensus, LAI, and SF2, but not the variant ELI, are each able to bind and activate Hck kinase (59). In the present study, we showed that Consensus, LAI, and SF2, along with two closely related Nef proteins, NL4-3 and YU2, all induce Hck-YEEI activation in yeast, agreeing with previous findings in fibroblasts. Also in accordance with the earlier results, ELI failed to activate Hck-YEEI in our yeast assay. Our findings in yeast are consistent with previous findings in fibroblasts and validate our assay for the screening of Nef alleles for the ability to activate SFKs.

Just as the failure of ELI to activate Hck enlightened us to the importance of the hydrophobic pocket for Nef:SFK binding (59), we hypothesized that the investigation of other Nef alleles in a SFK activation assay would allow us to identify critical, previously unexplored mechanisms of Nef-mediated SFK activation. Therefore, we examined a panel of primary Nef alleles, each containing all of the known SH3-binding residues (Figure 4-3). We identified several Nef variants which demonstrate differential activation of SFKs. Remarkably, one Nef protein, derived from the va04 allele, failed to activate Hck and Lyn, even though this allele contains an intact PxxP motif and completely conserved hydrophobic pocket residues Phe90, Trp113, and Tyr120. Several notable sequence variations unique to va04 are apparent, including a deletion of Ser-46, and several residue changes: E63G, A156I, and F191L. None of these residues are positioned for direct SFK SH3 binding, as predicted from the co-crystal structure of the Nef core with a Src SH3 domain (213). Of note, the Phe191 mutation has been reported to be critical for the Nef-mediated PAK2 activation, which is also an SH3-based interaction (109).

Phe191 resides within the core domain of Nef and may be involved in altering the tertiary structure of the Nef SH3-binding region (Figure 4-7). Ala156 maps to a large flexible loop which is not present in the Nef crystal structure (124). It is conceivable that this loop may make secondary contact with SFKs. Glu63 lies within the EEEE acidic motif known to mediate binding to PACS-1, so it is unlikely this change affects SFK binding (122,281). Of note, the E63G mutation may disrupt PACS-1-mediated MHC-I downregulation, a key Nef function shown to be lost frequently among long-term nonprogressors (50,51).

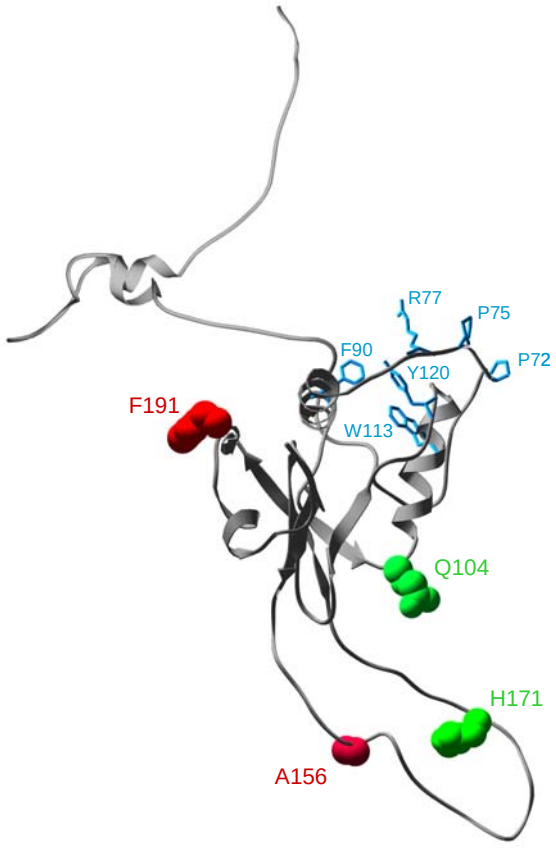

Figure 4-7. Molecular model of HIV Nef.

The conserved PxxPxR motif and hydrophobic pocket residues involved in SFK SH3 binding are highlighted in blue. Residues unique to primary Nef alleles that show gain of function towards SFK activation are highlighted in green (Q104S and H171C); residues associated with loss of function are highlighted in red (F191L; A156I). This model was compiled using the PDB coordinates of Geyer and Peterlin 2001 (124).

Similarly, two primary Nef alleles, va03 and va15, induced Hck and Lyn growth suppression more strongly than Nef SF2 in yeast. Notable sequence alterations unique to these alleles include: G3S/N, W5G, Q104S, Y120F, and H171C. The first two mutations are located adjacent to the myristoylation site of Nef. However, these residues have been shown to be unimportant for recognition by N-myristoyl transferase (296); in addition, mutations to this region would be expected to downregulate, not upregulate, Nef functional effects (122). Since Tyr120 is believed to be involved in nonpolar interactions with the SFK Ile96 residue, the Y120F change likely has minimal effect on Nef function. However, the other two amino acid alterations map to regions which lack clearly ascribed functions; Q104S maps to the outer edge of the Nef core, and H171C is located along the flexible internal loop (Figure 4-7). The localization of H171C supports the possibility that the flexible loop may make direct contact with the kinase domain.

Some insight into the relevance of the observed sequence variations in these primary Nef alleles can be obtained by examination of a comprehensive collection of Nef sequences compiled by O'Neill et al. (259). In their report, Foster and colleagues analyzed 1,643 clade B Nef sequences to determine the preferred residue at each position. HIV-1 clade B is the most common subgroup in North America (178). Comparison of the va04 sequence reveals that the E63G (19.3, 0.7), A156I (5.0, 0.4) and F191L (6.3, 2.5) are all relatively rare observances, suggesting these are not well tolerated by the virus. (The first number is the percentage of total variant residues at that position, the second number is the percentage of alleles containing the indicated amino acid change.) A similar comparison with the va03 and va15 alleles shows that while the Y120F (10.5, 10.0) is not uncommon, neither the Q104S (5.5, 0) nor H171C (0.9, 0) alterations were observed in any of the 1,643 sequences analyzed.

We have utilized a yeast growth suppression system, which we have previously used to evaluate selective activation of Src kinases by Nef (352), to investigate novel mechanisms of Nef-mediated SFK activation. By using primary HIV-1 Nef alleles pre-screened for the presence of known SH3-binding residues, we identified several residues not previously studied which appear to alter the ability of Nef to activate SFKs. These residues may mark additional sites of contact between Hck and Nef, or they may be involved in altering the tertiary structure of Nef in a way that impacts Nef:SFK binding and activation. Other evidence for indirect conformational effects of residue changes comes from examination of the H116N mutation in ELI, which may

impact the spatial position of the PxxP motif relative to SH3 (59). F191L, discovered on the va04 protein which fails to activate SFKs, is in such a position to alter the conformation of the Nef core domain. Similarly, Q104S may stabilize a conformation of Nef that enhances SFK activation. Also, for the first time, we offer evidence that the large flexible loop of Nef may contribute to SFK activation. In particular, residues Ala156 and His171 appear to influence the ability of Nef to bind and/or activate Hck and Lyn in opposing ways (Figure 4-7). Further investigation of these potentially critical residues will improve our understanding of the mechanism of Nef-mediated SFK activation, and may offer novel drug targets for the inhibition of Nef-mediated activation of Src kinases.

5.0 OVERALL DISCUSSION

5.1.1 Summary of Major Findings

For this thesis project, I undertook a comprehensive analysis of the effects of HIV-1 Nef on the activities of all SFKs expressed in the major HIV target cell types (T cells, macrophages, and dendritic cells). For this study, I developed a yeast-based assay in which activation of Src kinases yields a growth suppressive phenotype. Using this assay, I found that Nef (SF2 allele) directly activated Hck, Lyn, and c-Src in an SH3-dependent manner. This is the first report describing the ability of Nef to directly activate Lyn and c-Src. Nef did not activate Lck, consistent with previous studies showing that Nef NL4-3 and Nef LAI may instead inhibit Lck (75,134). Nef also did not activate Fyn, which is not unexpected considering that the ability of Nef to bind Fyn is controversial. Finally, Nef failed to activate Fgr and c-Yes, despite their functional redundancies with Hck and c-Src, respectively.

Since SFKs are active in yeast, these kinases needed to be downregulated to observe any activating effects of Nef. I therefore co-expressed SFKs with their physiologic negative regulator, Csk, and observed that Csk downregulated the activities of all SFKs tested. Interestingly, there is substantial variation in the C-terminal tail sequence among SFKs that does not seem to effect Csk recognition or function (Table 1-1). I later substituted the C-terminal tail region of each SFK with the YEEI sequence predicted by phosphopeptide screening to be a high-affinity SFK SH2-binding motif (330). I discovered that the YEEI substitution was sufficient to induce downregulation of all seven SFKs without the need for Csk co-expression. Crystal structure and phosphopeptide mapping studies both confirm that YEEI-modified SFKs are capable of autophosphorylation at the C-terminal tyrosine (Tyr527 in c-Src) (215,310). This suggests that YEEI-altered SFKs in yeast retain a low level of autophosphorylation that is re-directed from the activation loop tyrosine to the inhibitory tail tyrosine. Importantly, despite the

apparent tight binding that occurs between the YEEI tail and the SH2 domain, SFK-YEEI molecules were still activated by disengagement of the SH3 domain from the SH2-kinase linker by Nef. Thus, YEEI-modified SFKs are useful models for the study of Nef:SFK interactions, as well as other SH3-based SFK activators.

Secondly, I utilized the SFK-YEEI yeast growth suppression system to explore inhibitors of the Nef:SFK activation complex. I identified five compounds that substantially and reproducibly recovered yeast growth in Nef:Hck-YEEI cultures when compared with the known general SFK inhibitor A-419259. These five molecules were then tested in an HIV replication assay in a microglial model cell line, in which two compounds were found to markedly block HIV replication. Hence, I isolated two small-molecule inhibitors of HIV replication identified by targeting the complex of HIV-1 Nef and the Src family kinase Hck. This is the first report of an anti-HIV agent selected by targeting the function of an HIV accessory protein.

Finally, I expanded our studies to examine primary Nef alleles for the ability to activate SFKs. Previous studies have failed to adequately consider if structural elements outside of the immediate SH3-binding region have any bearing on Nef:SFK interaction and activation. To determine if other molecular determinants are involved in Nef-mediated SFK activation, I obtained a panel of primary HIV-1 Nef alleles, each of which contains known SH3-binding residues, and co-expressed them with Hck-YEEI to observe changes in growth suppression. Inferring kinase activity from the results of the growth suppression assay, two primary Nef proteins, va04 and va11, poorly activated Hck-YEEI, and two Nef proteins, va03 and va15, activated Hck-YEEI better than commonly-used laboratory Nef alleles. In vitro kinase assays confirmed the failure of va04 to activate Hck-YEEI or Lyn-YEEI.

Sequence examination of these four primary Nef alleles indicated several residues that may directly impact Nef:SFK interaction. The Phe191 residue, which was found to be mutated to leucine in the va04 allele, is located within the conserved core of Nef. Mutation of Phe191 could induce changes to the tertiary conformation of Nef, interfering with the ability of the core region to bind the SH3 domain. Indeed, substitution of Phe191 was found to block the ability of Nef to activate PAK2, suggesting this substitution impacts SH3-mediated binding (109). Another core domain residue noted to alter Nef:SFK activation was Gln104, though its role in Nef function is currently unknown. Based on recent hydrogen exchange data for full-length Nef, both Gln104 and Phe191 are found to be within regions of poor hydrogen exchange, indicating

that these residues are likely buried within their respective helical secondary structures (164). Substitution of these residues, as seen in the primary Nef alleles identified here, may confer disruption to both secondary and tertiary structures sufficient to block SH3 binding. Interestingly, two other residues identified in this screen, Ala156 and His171, are both located on the highly flexible internal loop region of Nef (124,164). The structure of this internal loop is poorly understood, as this region was excised prior to crystallization (142), but has been shown by hydrogen exchange to be highly solvent exposed, indicating it is likely unstructured (164). However, it is conceivable that this loop could fold in such a way as to either enhance or disrupt SFK binding and/or activation. Future work will address these important questions.

5.1.2 Implications of Nef:SFK Interactions

Nef and Hck:

Though the specific cellular functions of both Nef and Hck are still being elucidated, it seems evident from work in *nef/hck*$^{-/-}$ transgenic mice that the interaction of Nef and Hck plays a crucial role in the advancement of HIV disease (150). In addition, work from Komuro et al. demonstrate the importance of Hck expression and activity for HIV infection of macrophages (198). Since macrophages are one of the earliest infected cell types, and as coordinators of immune responses are in prime position to disseminate the virus, it is plausible that macrophages are critical for the establishment of HIV infection within the host. The interaction of Nef, the earliest transcribed viral gene, and Hck, a key mediator of macrophage signaling pathways, may be important for Nef to prepare a newly infected macrophage for optimal virus production and release. The increased expression of Hck following HIV infection (198), and the well-described activation of Hck by Nef, lends support to the role of this signaling kinase in the maintenance of viral infection within this HIV reservoir cell type.

Nef and Lyn:

The similarities between Hck and Lyn with respect to their activation by Nef suggest that these two kinases could overlap viral functions during HIV infection. The fact that Nef-mediated disease progression in the transgenic mouse model is delayed in the absence of Hck, but not eliminated, strongly suggests that other factors are involved in maintaining the disease

process. It is possible that Lyn expression substitutes for the loss of Hck in these *nef/hck*$^{-/-}$ transgenic mice. In addition, many reports on the normal cellular function of Lyn suggest that this kinase mediates both activating and inhibitory pathways, indicating a role for Lyn as a moderator of cellular signaling processes (222). As it has been determined that HIV requires sufficient activation to induce viral replication, but not enough to trigger AICD and other apoptotic pathways, it is conceivable that activation of Lyn by Nef serves to maintain the proper balance of controlled activation for optimal HIV functioning.

Nef and Fyn/Lck:

The lack of Nef-mediated activation of Fyn or Lck in my hands suggests that, regardless of the nature of the binding, these SFKs are likely not involved in Nef-mediated stimulatory functions. These results are somewhat surprising considering the numerous reports describing the binding of Nef with these proteins. However, my findings are consistent with all available functional data, which suggest that Nef fails to activate, or inhibits, Fyn and Lck (see sections 1.5.2 and 1.5.3). We do not observe inhibition of Lck by Nef in our system, as occurs with its natural negative regulator Csk. However, wild-type Lck gives a minimal activation phenotype in yeast making it difficult to ascertain inhibitory effects.

It is interesting that Nef activates TCR-signaling pathways, yet the two primary T cell signaling SFKs, Lck and Fyn, do not seem to be activated by Nef. It is possible that Nef can activate these kinases in a strict context-dependent fashion. For instance, following TCR engagement, both Lck and Fyn are recuited to the TCR-signaling comples located within lipid rafts (267). Nef is similarly recruited to lipid rafts due to its role in the cholesterol-enrichment of these membrane domains (406). Perhaps given a high enough local concentration of these molecules, Nef is able to induce Fyn and/or Lck activation. More likely, however, is that Nef is not involved in signaling through SFKs in T cells. Instead, Nef may utilize other cellular proteins, such as PAK2, to exert its signaling effects in T cells.

5.1.3 Yeast Growth Suppression System

Much of the work presented in this dissertation involved the establishment and validation of a yeast assay for the investigation of Nef-mediated SFK activity. The unique phenotype of Src kinase-mediated growth suppression, along with the lack of endogenous SFKs or SFK regulators, makes this system highly valuable for the study of Src kinase activity. In fact, this system can be adapted to study the activation of SFKs by other upstream targets, as we showed with Tip and Lck, or SFK inhibition, as I demonstrated with Csk throughout this document. Much of the power of this system comes from the fact that I was able to assess the functional consequences of protein:protein interactions, as opposed to just binding. While SFKs can likely serve as adaptor proteins due to the presence of two distinct modular binding domains, the vast majority of reported SFK-mediated effects involve its kinase activity, and the yeast assay presented here offers a valid system for the study of SFK regulation.

In addition, using our yeast system, I was able to investigate the effects of Nef on full-length SFKs free from epitope tags or other artificial modifications. I evaluated the contributions of all domains and regions working in concert, including the N-terminal myristoylated domain, which is frequently removed during purification for in vitro studies. Yeast provide the enzymes for myristoylation of SFKs (199,351), as well as a cellular context free from endogenous regulators of SFK activity. In addition, I was able to screen a panel of Nef alleles for their effects on SFK activation, demonstrating the utility of this yeast assay for large-scale analysis of viral Nef alleles on SFK function.

Several groups have previously studied the action of c-Src on yeast growth, though no conclusive mechanism of SFK-induced growth suppression has ever been described. v-Src, the viral form of c-Src that lacks the inhibitory C-terminal tail, has been shown in yeast to phosphorylate and induce the activation of Cdc28p, a cyclin-dependent kinase that negatively regulates DNA replication during mitosis and meiosis (297). The increase in Cdc28p kinase activity correlates with yeast growth suppression (108), however, the mechanism of Cdc28p-mediated growth suppression in the presence of Src kinase activity is not well understood.

5.1.4 Future Directions

Downstream effects of Nef:SFK interactions

Additional studies investigating the downstream effects of Nef-mediated Src activation in primary cell types need to be investigated. However, primary cells and most common mammalian cell lines express multiple Src kinases, making it difficult to examine the effects of Nef-induced activation of individual SFKs. Nef functions vary depending on its concentration within the cell, so overexpression of Nef with or without individual SFKs is not preferable (221). However, other techniques could be utilized to better elucidate the downstream effects of Nef-induced SFK activation.

Komuro et al. have demonstrated the utility of anti-sense RNA technology in primary cells to show that a block in Hck expression prevents productive HIV infection of monocyte-derived macrophages (198). Similar experiments using anti-sense RNA or small interfering RNA to suppress Lyn (macrophages) and c-Src (dendritic cells) expression would be useful given my findings. In addition to observing effects on viral function, one could also assess changes in pathways utilized by Nef-mediated SFK activation. For instance, Nef activates the Erk/MAPK pathway in T cells (311), and one could evaluate the effects of Nef activity on this pathway in the context of Lyn or c-Src transcriptional repression. Gene arrays, or other comprehensive analyses of gene expression similar to that described in Simmons et al. (326), could then be used to identify the outputs of global signaling pathways stimulated by Nef-induced activation of specific Src kinases.

Further information could be gained about Nef:Lyn effects by utilizing knockout mouse models. Expression of Nef in *lyn*$^{-/-}$ or *lyn*$^{-/-}$/*hck*$^{-/-}$ primary mouse monocytic cells may offer insight into the relative roles of Lyn and Hck in Nef-mediated cellular functions (55,107,161). Furthermore, it would be interesting to cross the Nef-transgenic HIV mouse model (148) with *lyn*$^{-/-}$ or *lyn*$^{-/-}$/*hck*$^{-/-}$ mice, similar to what was done previously using *hck*$^{-/-}$ mice (150), to examine effects of these additional SFK deletions on the AIDS-like phenotype.

Utility of Nef-Hck Inhibitors

Now that two potent HIV replication inhibitors have been identified, the mechanism(s) of action of these compounds needs to be elucidated. The yeast system is amenable for studying

the effects of these molecules on different wild-type SFKs, Nef-mediated activation of different SFK-YEEI proteins, and possibly other related protein tyrosine kinases, to determine the specificity of these inhibitors. Outside of yeast, one can evaluate the effects of these inhibitors on the in vitro binding of recombinant Nef and SFK proteins to give clues as to whether the drugs block binding, kinase activity, or both. A more ambitious goal is to solve a crystal structure of the inhibitor bound to the Nef:Hck complex, which would provide the most direct method for identifying the site of action. If a crystal suitable for x-ray diffraction cannot be formed, hydrogen exchange/mass spectrometry may be useful in determining changes in the dynamics of SFK modular domains in the presence of compounds, and offer clues as to the mechanism of action (369).

Besides its effects on Nef:SFK function, these molecules have been shown to block a functional effect of HIV replication. Further studies should investigate the point along the viral life cycle that is interrupted by these compounds. In addition, these inhibitors can be used in more limited cell culture assays to determine if they interfere with any of the numerous cellular functions of Nef, such as receptor down-modulation and stimulation of cellular activation pathways.

Pursuing alternate mechanisms of Nef-mediated SFK binding and activation

My work has suggested that the mechanism of Nef-mediated SFK activation may be more intricate than previously believed. Further evaluation of the primary *nef* alleles isolated during my screen must be performed to elucidate the mechanisms involved in the aberrant activation of SFKs. For instance, the ability of these alleles to activate other SFK proteins should be examined in yeast. Also, site-directed mutagenesis of the notable primary allele substitutions into known activating laboratory Nef alleles, similar to what we have presented before (59), will help define the relative importance of the individual residues involved in Nef:SFK activation.

Purification of the remaining primary Nef proteins identified here will enable a variety of experiments to be performed to help better determine the specific nature of the binding and activation events occurring between Nef and SFK proteins. For instance, kinetics of the interactions can be assessed using the Z'-lyte in vitro kinase assay as described in my studies. At this point, examination of SH3 binding to all SFKs would be appropriate to help further define

the mechanism of activation. Binding experiments could be performed using co-precipitation procedures on the benchtop, or attempted using surface plasmon resonance (SPR) to identify subtle variations in SFK SH3 domain binding to the different Nef alleles.

The presence of purified proteins would allow for a more detailed look at the structural interaction between the different Nef and SFK proteins. Hydrogen exchange/mass spectrometry technology can help indicate which residue alterations in Nef confer dynamic or structural changes to the molecule during Nef:SFK interaction (369). Ultimately, crystal structures of the different Nef:SFK combinations, especially of the higher activating va03 and va15 proteins with Hck and Lyn, could be useful in the delineation of additional aspects of the activation mechanism. For example, the Gln104 residue, that is mutated to serine in these highly SFK-activating Nef proteins to serine, is located just beneath the Nef core domain and points outwardly, such that it may complex with the surface of the bound SFK molecule. Also, the flexible loop domain may fold over to secure the SFK molecule in its Nef-bound position, and the residues identified in this report may enhance (H171C) or disrupt (A156I) this interaction.

In addition to examining the structural interactions with SFKs, other functions of these unique Nef alleles should be investigated to determine whether the respective amino acid changes affect solely SFK activation. In particular, well established Nef functions should be evaluated, such as binding to PAK2 and PACS-1, downmodulation of the cell surface receptors CD4 and MHC-I, and augmentation of HIV replication and infectivity. These latter experiments are best studied by introducing these primary *nef* alleles into the context of a wild-type virus. Such follow-up experiments will help determine if the *nef* mutations involved in altered SFK activation are also responsible for more global functional effects in HIV pathogenesis.

5.1.5 Closing Remarks

HIV has transformed the clinical and scientific realms unlike any other pathogen of this era. Throughout the twenty-five year investigation of HIV/AIDS, physicians have tracked the unrelenting, immune-ravaging course of the disease, while researchers have furiously worked to uncover many of the molecular mysteries of this intriguing virus. As a result of our increased knowledge of the inner workings of HIV, the scientific community has been able to offer a host

of new therapeutic agents, which have enabled clinicians to transform a previously fatal disease into a manageable, chronic illness.

However, the molecular mechanisms of HIV have proven to be complex beyond the predictions of most in the scientific field. For this reason, the study of HIV pathogenesis is far from complete. In this dissertation, I have attempted to advance the understanding of HIV by exploring the interaction between the virus and a family of host cellular kinases. In the process, I have identified two previously unknown targets of Nef-induced activation, discovered two inhibitors of HIV replication that block the Nef:SFK activation complex, and offered evidence for additional mechanisms involved in the activation of SFKs by Nef. I hope that some of these findings will help further the field of HIV research and possibly contribute to the development of better treatment options for those persons suffering from HIV/AIDS.

BIBLIOGRAPHY

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

2. 1999. T-Cell Mediated Immunity, p. 263-305. *In* C. A. Janeway, P. Travers, M. Walport, and J. D. Capra (ed.), Immunobiology: The Immune System in Health and Disease. Elsevier Science Ltd/Garland Publishing, London.

3. **Adams, J. A.** 2003. Activation loop phosphorylation and catalysis in protein kinases: Is there functional evidence for the autoinhibitor model? Biochemistry **42**:601-607.

4. **Adrian, F. J., Q. Ding, T. Sim, A. Velentza, C. Sloan, Y. Liu, G. Zhang, W. Hur, S. Ding, P. Manley, J. Mestan, D. Fabbro, and N. S. Gray**. 2006. Allosteric inhibitors of Bcr-abl-dependent cell proliferation. Nat.Chem.Biol. **2**:95-102.

5. **Adzhubei, A. A. and M. J. Sternberg**. 1993. Left-handed polyproline II helices commonly occur in globular proteins. J.Mol.Biol. **229**:472-493.

6. **Agopian, K., B. L. Wei, J. V. Garcia, and D. Gabuzda**. 2006. A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2. J.Virol. **80**:3050-3061.

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

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

9. **Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins**. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **407**:386-390.

10. **Altfeld, M., T. M. Allen, X. G. Yu, M. N. Johnston, D. Agrawal, B. T. Korber, D. C. Montefiori, D. H. O'Connor, B. T. Davis, P. K. Lee, E. L. Maier, J. Harlow, P. J. Goulder, C. Brander, E. S. Rosenberg, and B. D. Walker**. 2002. HIV-1 superinfection

despite broad CD8+ T-cell responses containing replication of the primary virus. Nature **420**:434-439.

11. **Altman, J. D. and M. B. Feinberg**. 2004. HIV escape: there and back again. Nat.Med. **10**:229-230.

12. **Appleby, M. W., J. A. Gross, M. P. Cooke, S. D. Levin, X. Qian, and R. M. Perlmutter**. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. Cell **70**:751-763.

13. **Aquaro, S., P. Bagnarelli, T. Guenci, A. De Luca, M. Clementi, E. Balestra, R. Calio, and C. F. Perno**. 2002. Long-term survival and virus production in human primary macrophages infected by human immunodeficiency virus. J.Med.Virol. **68**:479-488.

14. **Aquaro, S., S. Panti, M. C. Caroleo, E. Balestra, A. Cenci, F. Forbici, G. Ippolito, A. Mastino, R. Testi, V. Mollace, R. Calio, and C. F. Perno**. 2000. Primary macrophages infected by human immunodeficiency virus trigger CD95-mediated apoptosis of uninfected astrocytes. J.Leukoc.Biol. **68**:429-435.

15. **Aquaro, S., C. F. Perno, E. Balestra, J. Balzarini, A. Cenci, M. Francesconi, S. Panti, F. Serra, N. Villani, and R. Calio**. 1997. Inhibition of replication of HIV in primary monocyte/macrophages by different antiviral drugs and comparative efficacy in lymphocytes. J.Leukoc.Biol. **62**:138-143.

16. **Arold, S., P. Franken, M. P. Strub, F. Hoh, S. Benichou, R. Benarous, and C. Dumas**. 1997. The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling. Structure **5**:1361-1372.

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

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

19. **Arold, S. T., T. S. Ulmer, T. D. Mulhern, J. M. Werner, J. E. Ladbury, I. D. Campbell, and M. E. Noble**. 2001. The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases. J.Biol.Chem. **276**:17199-17205.

20. **Baba, T. W., V. Liska, A. H. Khimani, N. B. Ray, P. J. Dailey, D. Penninck, R. Bronson, M. F. Greene, H. M. McClure, L. N. Martin, and R. M. Ruprecht**. 1999. Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. Nat.Med. **5**:194-203.

21. **Badley, A. D., D. Dockrell, M. Simpson, R. Schut, D. H. Lynch, P. Leibson, and C. V. Paya**. 1997. Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-

infected individuals is mediated by FasL and tumor necrosis factor. J.Exp.Med. **185**:55-64.

22. **Balter, M.** 1996. HIV's other immune-system targets: macrophages. Science **274**:1464-1465.

23. **Banavali, N. K. and B. Roux**. 2005. The N-terminal end of the catalytic domain of SRC kinase Hck is a conformational switch implicated in long-range allosteric regulation. Structure (Camb.) **13**:1715-1723.

24. **Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka**. 2000. Immunobiology of dendritic cells. Annu.Rev.Immunol. **18**:767-811.

25. **Banchereau, J. and R. M. Steinman**. 1998. Dendritic cells and the control of immunity. Nature **392**:245-252.

26. **Baur, A. S., G. Sass, B. Laffert, D. Willbold, C. ChengMayer, and B. M. Peterlin**. 1997. The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase. Immunity **6**:283-291.

27. **Bell, I., T. M. Schaefer, R. P. Trible, A. Amedee, and T. A. Reinhart**. 2001. Down-modulation of the costimulatory molecule, CD28, is a conserved activity of multiple SIV Nefs and is dependent on histidine 196 of Nef. Virology **283**:148-158.

28. **Benkirane, M., H. Schmid-Antomarchi, D. R. Littman, M. Hirn, B. Rossi, and C. Devaux**. 1995. The cytoplasmic tail of CD4 is required for inhibition of human immunodeficiency virus type 1 replication by antibodies that bind to the immunoglobulin CDR3-like region in domain 1 of CD4. J.Virol. **69**:6904-6910.

29. **Bennett, B. D., S. Cowley, S. Jiang, R. London, B. Deng, J. Grabarek, J. E. Groopman, D. V. Goeddel, and H. Avraham**. 1994. Identification and characterization of a novel tyrosine kinase from megakaryocytes. J.Biol.Chem. **269**:1068-1074.

30. **Biesinger, B., A. Y. Tsygankov, H. Fickenscher, F. Emmrich, B. Fleckenstein, J. B. Bolen, and B. M. Broker**. 1995. The product of the Herpesvirus saimiri open reading frame 1 (tip) interacts with T cell-specific kinase p56lck in transformed cells. J.Biol.Chem. **270**:4729-4734.

31. **Bjorge, J. D., A. Jakymiw, and D. J. Fujita**. 2000. Selected glimpses into the activation and function of Src kinase. Oncogene **19**:5620-5635.

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

33. **Blankson, J. N., D. Persaud, and R. F. Siliciano**. 2002. The challenge of viral reservoirs in HIV-1 infection. Annu.Rev.Med. **53**:557-593.

34. **Bobardt, M. D., A. C. Saphire, H. C. Hung, X. Yu, S. B. Van der, Z. Zhang, G. David, and P. A. Gallay**. 2003. Syndecan captures, protects, and transmits HIV to T lymphocytes. Immunity **18**:27-39.

35. **Boggon, T. J. and M. J. Eck**. 2004. Structure and regulation of Src family kinases. Oncogene **23**:7918-7927.

36. **Bosco, M. C., R. E. Curiel, A. H. Zea, M. G. Malabarba, J. R. Ortaldo, and I. Espinoza-Delgado**. 2000. IL-2 signaling in human monocytes involves the phosphorylation and activation of p59hck. J.Immunol. **164**:4575-4585.

37. **Brdicka, T., D. Pavlistova, A. Leo, E. Bruyns, V. Korinek, P. Angelisova, J. Scherer, A. Shevchenko, I. Hilgert, J. Cerny, K. Drbal, Y. Kuramitsu, B. Kornacker, V. Horejsi, and B. Schraven**. 2000. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J.Exp.Med. **191**:1591-1604.

38. **Breitenlechner, C. B., N. A. Kairies, K. Honold, S. Scheiblich, H. Koll, E. Greiter, S. Koch, W. Schafer, R. Huber, and R. A. Engh**. 2005. Crystal structures of active SRC kinase domain complexes. J.Mol.Biol. **353**:222-231.

39. **Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek**. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J.Exp.Med. **200**:749-759.

40. **Briese, L., A. Preusser, and D. Willbold**. 2005. Mapping the binding site of full length HIV-1 Nef on human Lck SH3 by NMR spectroscopy. J.Biomed.Sci. **12**:451-456.

41. **Briggs, S. D., E. C. Lerner, and T. E. Smithgall**. 2000. Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo. Biochemistry **39**:489-495.

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

43. **Briggs, S. D., M. Sharkey, M. Stevenson, and T. E. Smithgall**. 1997. SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1. J.Biol.Chem. **272**:17899-17902.

44. **Briggs, S. D. and T. E. Smithgall**. 1999. SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in Rat-2 fibroblasts. J.Biol.Chem. **274**:26579-26583.

45. **Brown, A., X. Wang, E. Sawai, and C. Cheng-Mayer**. 1999. Activation of the PAK-related kinase by human immunodeficiency virus type 1 Nef in primary human peripheral

blood lymphocytes and macrophages leads to phosphorylation of a PIX-p95 complex. J.Virol. **73**:9899-9907.

46. **Brown, D. A. and E. London**. 1998. Functions of lipid rafts in biological membranes. Annu.Rev.Cell Dev.Biol. **14**:111-136.

47. **Brown, M. T. and J. A. Cooper**. 1996. Regulation, substrates and functions of src. Biochim.Biophys.Acta **1287**:121-149.

48. **Brugge, J. S., G. Jarosik, J. Andersen, A. Queral-Lustig, M. Fedor-Chaiken, and J. R. Broach**. 1987. Expression of Rous sarcoma virus transforming protein pp60v-src in Saccharomyces cerevisiae cells. Mol.Cell Biol. **7**:2180-2187.

49. **Burton, G. F., B. F. Keele, J. D. Estes, T. C. Thacker, and S. Gartner**. 2002. Follicular dendritic cell contributions to HIV pathogenesis. Semin.Immunol. **14**:275-284.

50. **Carl, S., R. Daniels, A. J. Iafrate, P. Easterbrook, T. C. Greenough, J. Skowronski, and F. Kirchhoff**. 2000. Partial "repair" of defective NEF genes in a long-term nonprogressor with human immunodeficiency virus type 1 infection. J.Infect.Dis. **181**:132-140.

51. **Carl, S., T. C. Greenough, M. Krumbiegel, M. Greenberg, J. Skowronski, J. L. Sullivan, and F. Kirchhoff**. 2001. Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS. J.Virol. **75**:3657-3665.

52. **Caveggion, E., S. Continolo, F. J. Pixley, E. R. Stanley, D. D. Bowtell, C. A. Lowell, and G. Berton**. 2003. Expression and tyrosine phosphorylation of Cbl regulates macrophage chemokinetic and chemotactic movement. J.Cell Physiol **195**:276-289.

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

54. **Chan, D. C. and P. S. Kim**. 1998. HIV entry and its inhibition. Cell **93**:681-684.

55. **Chan, V. W., F. Meng, P. Soriano, A. L. DeFranco, and C. A. Lowell**. 1997. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. Immunity **7**:69-81.

56. **Chehimi, J., Q. Luo, L. Azzoni, L. Shawver, N. Ngoubilly, R. June, G. Jerandi, M. Farabaugh, and L. J. Montaner**. 2003. HIV-1 transmission and cytokine-induced expression of DC-SIGN in human monocyte-derived macrophages. J.Leukoc.Biol. **74**:757-763.

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

58. **Chinen, J. and W. T. Shearer**. 2002. Molecular virology and immunology of HIV infection. J.Allergy Clin.Immunol. **110**:189-198.

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

60. **Choi, H. J. and T. E. Smithgall**. 2004. HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-X-L expression in an extracellular signal-regulated kinase-dependent manner. J.Biol.Chem. **279**:51688-51696.

61. **Chong, Y. P., K. K. Ia, T. D. Mulhern, and H. C. Cheng**. 2005. Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. Biochim.Biophys.Acta.

62. **Chong, Y. P., T. D. Mulhern, and H. C. Cheng**. 2005. C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)--endogenous negative regulators of Src-family protein kinases. Growth Factors **23**:233-244.

63. **Chong, Y. P., T. D. Mulhern, H. J. Zhu, D. J. Fujita, J. D. Bjorge, J. P. Tantiongco, N. Sotirellis, D. S. Lio, G. Scholz, and H. C. Cheng**. 2004. A novel non-catalytic mechanism employed by the C-terminal Src-homologous kinase to inhibit Src-family kinase activity. J.Biol.Chem. **279**:20752-20766.

64. **Chow, L. M., M. Fournel, D. Davidson, and A. Veillette**. 1993. Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk. Nature **365**:156-160.

65. **Chow, L. M., C. Jarvis, Q. Hu, S. H. Nye, F. G. Gervais, A. Veillette, and L. A. Matis**. 1994. Ntk: a Csk-related protein-tyrosine kinase expressed in brain and T lymphocytes. Proc.Natl.Acad.Sci.U.S.A **91**:4975-4979.

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

67. **Chun, T. W., R. T. Davey, Jr., M. Ostrowski, J. J. Shawn, D. Engel, J. I. Mullins, and A. S. Fauci**. 2000. Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. Nat.Med. **6**:757-761.

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

69. **Cloutier, J. F., L. M. Chow, and A. Veillette**. 1995. Requirement of the SH3 and SH2 domains for the inhibitory function of tyrosine protein kinase p50csk in T lymphocytes. Mol.Cell Biol. **15**:5937-5944.

70. **Clump, D. A., I. H. Qazi, M. Sudol, and D. C. Flynn**. 2005. c-Yes response to growth factor activation. Growth Factors **23**:263-272.

71. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267**:483-489.

72. **Cohen, G. B., R. Ren, and D. Baltimore**. 1995. Modular binding domains in signal transduction proteins. Cell **80**:237-248.

73. **Collette, Y., S. Arold, C. Picard, K. Janvier, S. Benichou, R. Benarous, D. Olive, and C. Dumas**. 2000. 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. **275**:4171-4176.

74. **Collette, Y., H. Dutartre, A. Benziane, and D. Olive**. 1997. The role of HIV1 Nef in T-cell activation: Nef impairs induction of Th1 cytokines and interacts with the Src family tyrosine kinase Lck. Res.Virol. **148**:52-58.

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

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

77. **Collman, R. G., C. F. Perno, S. M. Crowe, M. Stevenson, and L. J. Montaner**. 2003. HIV and cells of macrophage/dendritic lineage and other non-T cell reservoirs: new answers yield new questions. J.Leukoc.Biol. **74**:631-634.

78. **Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau**. 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J.Exp.Med. **185**:621-628.

79. **Cooke, M. P. and R. M. Perlmutter**. 1989. Expression of a novel form of the fyn proto-oncogene in hematopoietic cells. New Biol. **1**:66-74.

80. **Cooper, J. A. and K. Runge**. 1987. Avian pp60c-src is more active when expressed in yeast than in vertebrate fibroblasts. Oncogene Res. **1**:297-310.

81. **Cosenza, M. A., M. L. Zhao, and S. C. Lee**. 2004. HIV-1 expression protects macrophages and microglia from apoptotic death. Neuropathol.Appl.Neurobiol. **30**:478-490.

82. **Cosenza, M. A., M. L. Zhao, Q. Si, and S. C. Lee**. 2002. Human brain parenchymal microglia express CD14 and CD45 and are productively infected by HIV-1 in HIV-1 encephalitis. Brain Pathol. **12**:442-455.

83. **Cowan-Jacob, S. W., G. Fendrich, P. W. Manley, W. Jahnke, D. Fabbro, J. Liebetanz, and T. Meyer**. 2005. The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure.(Camb.) **13**:861-871.

84. **Crowe, S., T. Zhu, and W. A. Muller**. 2003. The contribution of monocyte infection and trafficking to viral persistence, and maintenance of the viral reservoir in HIV infection. J.Leukoc.Biol. **74**:635-641.

85. **Crowe, S. M., J. Mills, T. Elbeik, J. D. Lifson, J. Kosek, J. A. Marshall, E. G. Engleman, and M. S. McGrath**. 1992. Human immunodeficiency virus-infected monocyte-derived macrophages express surface gp120 and fuse with CD4 lymphoid cells in vitro: a possible mechanism of T lymphocyte depletion in vivo. Clin.Immunol.Immunopathol. **65**:143-151.

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

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

88. **Davidson, D., L. M. L. Chow, and A. Veillette**. 1997. Chk, a Csk family tyrosine protein kinase, exhibits Csk-like activity in fibroblasts, but not in an antigen-specific T-cell line. J.Biol.Chem. **272**:1355-1362.

89. **De Clercq, E.** 1998. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. Antiviral Res. **38**:153-179.

90. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, and .** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270**:988-991.

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

92. **Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup**. 2002. HIV preferentially infects HIV-specific CD4+ T cells. Nature **417**:95-98.

93. **Douek, D. C., L. J. Picker, and R. A. Koup**. 2003. T cell dynamics in HIV-1 infection. Annu.Rev.Immunol. **21**:265-304.

94. **Dutartre, H., M. Harris, D. Olive, and Y. Collette**. 1998. The human immunodeficiency virus type 1 Nef protein binds the Src-related tyrosine kinase Lck SH2 domain through a novel phosphotyrosine independent mechanism. Virology **247**:200-211.

95. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase**. 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature **362**:359-362.

96. **English, B. K., J. N. Ihle, A. Myracle, and T. Yi**. 1993. Hck tyrosine kinase activity modulates tumor necrosis factor production by murine macrophages. J.Exp.Med. **178**:1017-1022.

97. **Ernst, M., M. Inglese, G. M. Scholz, K. W. Harder, F. J. Clay, S. Bozinovski, P. Waring, R. Darwiche, T. Kay, P. Sly, R. Collins, D. Turner, M. L. Hibbs, G. P. Anderson, and A. R. Dunn**. 2002. Constitutive activation of the SRC family kinase Hck results in spontaneous pulmonary inflammation and an enhanced innate immune response. J.Exp.Med. **196**:589-604.

98. **Erpel, T., G. Superti-Furga, and S. A. Courtneidge**. 1995. Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J. **14**:963-975.

99. **Ezzell, C.** 1987. AZT given the green light for clinical treatment of AIDS. Nature **326**:430.

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

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

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

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

104. **Federico, M., Z. Percario, E. Olivetta, G. Fiorucci, C. Muratori, A. Micheli, G. Romeo, and E. Affabris**. 2001. HIV-1 Nef activates STAT1 in human monocytes/macrophages through the release of soluble factors. Blood **98**:2752-2761.

105. **Filipp, D., J. Zhang, B. L. Leung, A. Shaw, S. D. Levin, A. Veillette, and M. Julius**. 2003. Regulation of Fyn through translocation of activated Lck into lipid rafts. J.Exp.Med. **197**:1221-1227.

106. **Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano**. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278**:1295-1300.

107. **Fitzer-Attas, C. J., M. Lowry, M. T. Crowley, A. J. Finn, F. Meng, A. L. DeFranco, and C. A. Lowell**. 2000. Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J.Exp.Med. **191**:669-682.

108. **Florio, M., L. K. Wilson, J. B. Trager, J. Thorner, and G. S. Martin**. 1994. Aberrant protein phosphorylation at tyrosine is responsible for the growth-inhibitory action of pp60v-src expressed in the yeast Saccharomyces cerevisiae. Mol.Biol.Cell **5**:283-296.

109. **Foster, J. L., R. P. Molina, T. Luo, V. K. Arora, Y. Huang, D. D. Ho, and J. V. Garcia**. 2001. Genetic and functional diversity of human immunodeficiency virus type 1 subtype B Nef primary isolates. J.Virol. **75**:1672-1680.

110. **Fujii, Y., K. Otake, M. Tashiro, and A. Adachi**. 1996. Soluble Nef antigen of HIV-1 is cytotoxic for human CD4+ T cells. FEBS Lett. **393**:93-96.

111. **Gallant, J. E.** 2000. Strategies for long-term success in the treatment of HIV infection. JAMA **283**:1329-1334.

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

113. **Garaci, E., S. Aquaro, C. Lapenta, A. Amendola, M. Spada, S. Covaceuszach, C. F. Perno, and F. Belardelli**. 2003. Anti-nerve growth factor Ab abrogates macrophage-mediated HIV-1 infection and depletion of CD4+ T lymphocytes in hu-SCID mice. Proc.Natl.Acad.Sci.U.S.A **100**:8927-8932.

114. **Garaci, E., M. C. Caroleo, L. Aloe, S. Aquaro, M. Piacentini, N. Costa, A. Amendola, A. Micera, R. Calio, C. F. Perno, and R. Levi-Montalcini**. 1999. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. Proc.Natl.Acad.Sci.U.S.A **96**:14013-14018.

115. **Garden, G. A.** 2002. Microglia in human immunodeficiency virus-associated neurodegeneration. Glia **40**:240-251.

116. **Gartner, S.** 2000. HIV infection and dementia. Science **287**:602-604.

117. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic**. 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233**:215-219.

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

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

120. **Gendelman, H. E., J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke, and .** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J.Exp.Med. **167**:1428-1441.

121. **Gessler, M. and A. Barnekow**. 1984. Differential expression of the cellular oncogenes c-src and c-yes in embryonal and adult chicken tissues. Biosci.Rep. **4**:757-770.

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

123. **Geyer, M., C. E. Munte, J. Schorr, R. Kellner, and H. R. Kalbitzer**. 1999. Structure of the anchor-domain of myristoylated and non-myristoylated HIV-1 Nef protein. J.Mol.Biol. **289**:123-138.

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

125. **Gilbert, P. B., M. L. Peterson, D. Follmann, M. G. Hudgens, D. P. Francis, M. Gurwith, W. L. Heyward, D. V. Jobes, V. Popovic, S. G. Self, F. Sinangil, D. Burke, and P. W. Berman**. 2005. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J.Infect.Dis. **191**:666-677.

126. **Giulian, D., E. Wendt, K. Vaca, and C. A. Noonan**. 1993. The envelope glycoprotein of human immunodeficiency virus type 1 stimulates release of neurotoxins from monocytes. Proc.Natl.Acad.Sci.U.S.A **90**:2769-2773.

127. **Gonfloni, S., F. Frischknecht, M. Way, and G. Superti-Furga**. 1999. Leucine 255 of Src couples intramolecular interactions to inhibition of catalysis. Nat.Struct.Biol. **6**:760-764.

128. **Gonfloni, S., J. C. Williams, K. Hattula, A. Weijland, R. K. Wierenga, and G. Superti-Furga**. 1997. The role of the linker between the SH2 domain and catalytic domain in the regulation and function of Src. EMBO J. **16**:7261-7271.

129. **Goodenow, M. M., S. L. Rose, D. L. Tuttle, and J. W. Sleasman**. 2003. HIV-1 fitness and macrophages. J.Leukoc.Biol. **74**:657-666.

130. **Granelli-Piperno, A., E. Delgado, V. Finkel, W. Paxton, and R. M. Steinman**. 1998. Immature dendritic cells selectively replicate macrophagetropic (M-tropic) human immunodeficiency virus type 1, while mature cells efficiently transmit both M- and T-tropic virus to T cells. J.Virol. **72**:2733-2737.

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

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

133. **Greenway, A., A. Azad, and D. McPhee**. 1995. Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines. J.Virol. **69**:1842-1850.

134. **Greenway, A., A. Azad, J. Mills, and D. McPhee**. 1996. Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity. J.Virol. **70**:6701-6708.

135. **Greenway, A. L., H. Dutartre, k. Allen, D. A. McPhee, D. Olive, and Y. Collette**. 1999. Simian immunodeficiency virus and human immunodeficiency virus type 1 nef proteins show distinct patterns and mechanisms of Src kinase activation. J.Virol. **73**:6152-6158.

136. **Greenway, A. L., G. Holloway, and D. A. McPhee**. 2000. HIV-1 Nef: a critical factor in viral-induced pathogenesis. Adv.Pharmacol. **48**:299-343.

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

138. **Grivel, J. C., M. L. Penn, D. A. Eckstein, B. Schramm, R. F. Speck, N. W. Abbey, B. Herndier, L. Margolis, and M. A. Goldsmith**. 2000. Human immunodeficiency virus type 1 coreceptor preferences determine target T-cell depletion and cellular tropism in human lymphoid tissue. J.Virol. **74**:5347-5351.

139. **Grossman, Z., M. Meier-Schellersheim, W. E. Paul, and L. J. Picker**. 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. Nat.Med. **12**:289-295.

140. **Grossman, Z., M. Meier-Schellersheim, A. E. Sousa, R. M. Victorino, and W. E. Paul**. 2002. CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? Nat.Med. **8**:319-323.

141. **Groves, T., P. Smiley, M. P. Cooke, K. Forbush, R. M. Perlmutter, and C. J. Guidos**. 1996. Fyn can partially substitute for Lck in T lymphocyte development. Immunity **5**:417-428.

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

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

144. **Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar**. 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. J.Virol. **77**:11708-11717.

145. **Guillemin, G. J. and B. J. Brew**. 2004. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. J.Leukoc.Biol. **75**:388-397.

146. **Hamaguchi, I., N. Yamaguchi, J. Suda, A. Iwama, A. Hirao, M. Hashiyama, S. Aizawa, and T. Suda**. 1996. Analysis of CSK homologous kinase (CHK/HYL) in hematopoiesis by utilizing gene knockout mice. Biochem.Biophys.Res.Commun. **224**:172-179.

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

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

149. **Hanna, Z., E. Priceputu, D. G. Kay, J. Poudrier, P. Chrobak, and P. Jolicoeur**. 2004. 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 **327**:273-286.

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

151. **Harris, M.** 1999. HIV: a new role for Nef in the spread of HIV. Curr.Biol. **9**:R459-R461.

152. **Harrison, S. C.** 2003. Variation on an Src-like theme. Cell **112**:737-740.

153. **Hartley, D. A., T. R. Hurley, J. S. Hardwick, T. C. Lund, P. G. Medveczky, and B. M. Sefton**. 1999. Activation of the lck tyrosine-protein kinase by the binding of the tip protein of herpesvirus saimiri in the absence of regulatory tyrosine phosphorylation. J.Biol.Chem. **274**:20056-20059.

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

155. **He, J. C., M. Husain, M. Sunamoto, V. D. D'Agati, M. E. Klotman, R. Iyengar, and P. E. Klotman**. 2004. Nef stimulates proliferation of glomerular podocytes through activation of Src-dependent Stat3 and MAPK1,2 pathways. J.Clin.Invest **114**:643-651.

156. **Heath, S. L., J. G. Tew, J. G. Tew, A. K. Szakal, and G. F. Burton**. 1995. Follicular dendritic cells and human immunodeficiency virus infectivity. Nature **377**:740-744.

157. **Hecht, F. M., R. M. Grant, C. J. Petropoulos, B. Dillon, M. A. Chesney, H. Tian, N. S. Hellmann, N. I. Bandrapalli, L. Digilio, B. Branson, and J. O. Kahn**. 1998. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. N.Engl.J.Med. **339**:307-311.

158. **Herbein, G., U. Mahlknecht, F. Batliwalla, P. Gregersen, T. Pappas, J. Butler, W. A. O'Brien, and E. Verdin**. 1998. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. Nature **395**:189-194.

159. **Hibbs, M. L. and K. W. Harder**. 2006. The duplicitous nature of the Lyn tyrosine kinase in growth factor signaling. Growth Factors **24**:137-149.

160. **Hibbs, M. L., D. M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S. A. Stacker, and A. R. Dunn**. 1995. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell **83**:301-311.

161. **Hibbs, M. L., D. M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S. A. Stacker, and A. R. Dunn**. 1995. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell **83**:301-311.

162. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz**. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373**:123-126.

163. **Hochrein, J. M., E. C. Lerner, A. P. Schiavone, T. E. Smithgall, and J. R. Engen**. 2006. An examination of dynamics crosstalk between SH2 and SH3 domains by hydrogen/deuterium exchange and mass spectrometry. Protein Sci. **15**:65-73.

164. **Hochrein, J. M., T. E. Wales, E. C. Lerner, A. P. Schiavone, T. E. Smithgall, and J. R. Engen**. 2006. Conformational features of the full-length HIV and SIV Nef proteins determined by mass spectrometry. Biochemistry **45**:7733-7739.

165. **Hofmann-Lehmann, R., J. Vlasak, A. L. Williams, A. L. Chenine, H. M. McClure, D. C. Anderson, S. O'Neil, and R. M. Ruprecht**. 2003. Live attenuated, nef-deleted SIV is pathogenic in most adult macaques after prolonged observation. AIDS **17**:157-166.

166. **Honda, Z., T. Suzuki, N. Hirose, M. Aihara, T. Shimizu, S. Nada, M. Okada, C. Ra, Y. Morita, and K. Ito**. 1997. Roles of C-terminal Src kinase in the initiation and the termination of the high affinity IgE receptor-mediated signaling. J.Biol.Chem. **272**:25753-25760.

167. **Horton, H., T. U. Vogel, D. K. Carter, K. Vielhuber, D. H. Fuller, T. Shipley, J. T. Fuller, K. J. Kunstman, G. Sutter, D. C. Montefiori, V. Erfle, R. C. Desrosiers, N. Wilson, L. J. Picker, S. M. Wolinsky, C. Wang, D. B. Allison, and D. I. Watkins**.

2002. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J.Virol. **76**:7187-7202.

168. **Hu, H., G. Huston, D. Duso, N. Lepak, E. Roman, and S. L. Swain**. 2001. CD4(+) T cell effectors can become memory cells with high efficiency and without further division. Nat.Immunol. **2**:705-710.

169. **Huang, Y. and R. L. Wange**. 2004. T cell receptor signaling: beyond complex complexes. J.Biol.Chem. **279**:28827-28830.

170. **Huang, Y., L. Zhang, and D. D. Ho**. 1995. Characterization of nef sequences in long-term survivors of human immunodeficiency virus type 1 infection. J.Virol. **69**:93-100.

171. **Hubbard, S. R.** 1997. Crystal structure of the activated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog. EMBO J. **16**:5572-5581.

172. **Hubbard, S. R., L. Wei, L. Ellis, and W. A. Hendrickson**. 1994. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature **372**:746-754.

173. **Huse, M. and J. Kuriyan**. 2002. The conformational plasticity of protein kinases. Cell **109**:275-282.

174. **Igarashi, T., C. R. Brown, Y. Endo, A. Buckler-White, R. Plishka, N. Bischofberger, V. Hirsch, and M. A. Martin**. 2001. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. Proc.Natl.Acad.Sci.U.S.A **98**:658-663.

175. **Imamoto, A. and P. Soriano**. 1993. Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell **73**:1117-1124.

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

177. **Jost, S., M. C. Bernard, L. Kaiser, S. Yerly, B. Hirschel, A. Samri, B. Autran, L. E. Goh, and L. Perrin**. 2002. A patient with HIV-1 superinfection. N.Engl.J.Med. **347**:731-736.

178. **Julg, B. and F. D. Goebel**. 2005. HIV genetic diversity: any implications for drug resistance? Infection **33**:299-301.

179. **Kabouridis, P. S., A. I. Magee, and S. C. Ley**. 1997. S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes. EMBO J. **16**:4983-4998.

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

181. **Kaplan, J. E., D. Hanson, M. S. Dworkin, T. Frederick, J. Bertolli, M. L. Lindegren, S. Holmberg, and J. L. Jones**. 2000. Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. Clin.Infect.Dis. **30 Suppl 1**:S5-14.

182. **Karn, T., B. Hock, U. Holtrich, M. Adamski, K. Strebhardt, and H. Rubsamen-Waigmann**. 1998. Nef proteins of distinct HIV-1 or -2 isolates differ in their binding properties for HCK: isolation of a novel Nef binding factor with characteristics of an adaptor protein. Virology **246**:45-52.

183. **Kaul, M., G. A. Garden, and S. A. Lipton**. 2001. Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature **410**:988-994.

184. **Kaul, M. and S. A. Lipton**. 1999. Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. Proc.Natl.Acad.Sci.U.S.A **96**:8212-8216.

185. **Kawabuchi, M., Y. Satomi, T. Takao, Y. Shimonishi, S. Nada, K. Nagai, A. Tarakhovsky, and M. Okada**. 2000. Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature **404**:999-1003.

186. **Kelleher, A. D. and J. J. Zaunders**. 2006. Decimated or missing in action: CD4+ T cells as targets and effectors in the pathogenesis of primary HIV infection. Curr.HIV/AIDS Rep. **3**:5-12.

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

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

189. **Kharbanda, S., A. Saleem, Z. M. Yuan, S. Kraeft, R. Weichselbaum, L. B. Chen, and D. Kufe**. 1996. Nuclear signaling induced by ionizing radiation involves colocalization of the activated p56/p53lyn tyrosine kinase with p34cdc2. Cancer Res. **56**:3617-3621.

190. **Kinoshita, T., M. Matsubara, H. Ishiguro, K. Okita, and T. Tada**. 2006. Structure of human Fyn kinase domain complexed with staurosporine. Biochem.Biophys.Res.Commun. **346**:840-844.

191. **Kirchhoff, F., P. J. Easterbrook, N. Douglas, M. Troop, T. C. Greenough, J. Weber, S. Carl, J. L. Sullivan, and R. S. Daniels**. 1999. Sequence variations in human

immunodeficiency virus type 1 Nef are associated with different stages of disease. J.Virol. **73**:5497-5508.

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

193. **Kirchhoff, F., M. Schindler, N. Bailer, G. H. Renkema, K. Saksela, V. Knoop, M. C. Muller-Trutwin, M. L. Santiago, F. Bibollet-Ruche, M. T. Dittmar, J. L. Heeney, B. H. Hahn, and J. Munch**. 2004. Nef proteins from simian immunodeficiency virus-infected chimpanzees interact with p21-activated kinase 2 and modulate cell surface expression of various human receptors. J.Virol. **78**:6864-6874.

194. **Kjellen, P., K. Amdjadi, T. C. Lund, P. G. Medveczky, and B. M. Sefton**. 2002. The herpesvirus saimiri tip484 and tip488 proteins both stimulate lck tyrosine protein kinase activity in vivo and in vitro. Virology **297**:281-288.

195. **Klages, S., D. Adam, K. Class, J. Fargnoli, J. B. Bolen, and R. C. Penhallow**. 1994. Ctk: a protein-tyrosine kinase related to Csk that defines an enzyme family. Proc.Natl.Acad.Sci.U.S.A **91**:2597-2601.

196. **Knighton, D. R., J. H. Zheng, L. F. Ten Eyck, V. A. Ashford, N. H. Xuong, S. S. Taylor, and J. M. Sowadski**. 1991. Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253**:407-414.

197. **Koenig, S., H. E. Gendelman, J. M. Orenstein, M. C. Dal Canto, G. H. Pezeshkpour, M. Yungbluth, F. Janotta, A. Aksamit, M. A. Martin, and A. S. Fauci**. 1986. Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. Science **233**:1089-1093.

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

199. **Kornbluth, S., R. Jove, and H. Hanafusa**. 1987. Characterization of avian and viral p60src proteins expressed in yeast. Proc.Natl.Acad.Sci.U.S.A **84**:4455-4459.

200. **Kuo, S. S., P. Moran, J. Gripp, M. Armanini, H. S. Phillips, A. Goddard, and I. W. Caras**. 1994. Identification and characterization of Batk, a predominantly brain-specific non-receptor protein tyrosine kinase related to Csk. J.Neurosci.Res. **38**:705-715.

201. **Kuriyan, J. and D. Cowburn**. 1997. Modular peptide recognition domains in eukaryotic signaling. Annu.Rev.Biophys.Biomol.Struct. **26**:259-288.

202. **Kushnirov, V. V.** 2000. Rapid and reliable protein extraction from yeast. Yeast **16**:857-860.

203. **LaFevre-Bernt, M., F. Sicheri, A. Pico, M. Porter, J. Kuriyan, and W. T. Miller**. 1998. Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue. J.Biol.Chem. **273**:32129-32134.

204. **Laity, J. H., H. J. Dyson, and P. E. Wright**. 2000. DNA-induced alpha-helix capping in conserved linker sequences is a determinant of binding affinity in Cys(2)-His(2) zinc fingers. J.Mol.Biol. **295**:719-727.

205. **Lalezari, J. P., K. Henry, M. O'Hearn, J. S. Montaner, P. J. Piliero, B. Trottier, S. Walmsley, C. Cohen, D. R. Kuritzkes, J. J. Eron, Jr., J. Chung, R. Demasi, L. Donatacci, C. Drobnes, J. Delehanty, and M. Salgo**. 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N.Engl.J.Med. **348**:2175-2185.

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

207. **Lambotte, O., Y. Taoufik, M. G. de Goer, C. Wallon, C. Goujard, and J. F. Delfraissy**. 2000. Detection of infectious HIV in circulating monocytes from patients on prolonged highly active antiretroviral therapy. J.Acquir.Immune.Defic.Syndr. **23**:114-119.

208. **Lamers, M. B., A. A. Antson, R. E. Hubbard, R. K. Scott, and D. H. Williams**. 1999. Structure of the protein tyrosine kinase domain of C-terminal Src kinase (CSK) in complex with staurosporine. J.Mol.Biol. **285**:713-725.

209. **Larsson, M.** 2005. HIV-1 and the hijacking of dendritic cells: a tug of war. Springer Semin.Immunopathol. **26**:309-328.

210. **Latour, S. and A. Veillette**. 2001. Proximal protein tyrosine kinases in immunoreceptor signaling. Curr.Opin.Immunol. **13**:299-306.

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

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

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

214. **Lee, S., X. Lin, N. H. Nam, K. Parang, and G. Sun**. 2003. Determination of the substrate-docking site of protein tyrosine kinase C-terminal Src kinase. Proc.Natl.Acad.Sci.U.S.A **100**:14707-14712.

215. **Lerner, E. C. and T. E. Smithgall**. 2002. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat.Struct.Biol. **9**:365-369.

216. **Lerner, E. C., R. P. Trible, A. P. Schiavone, J. M. Hochrein, J. R. Engen, and T. E. Smithgall**. 2005. Activation of the Src family kinase Hck without SH3-linker release. J.Biol.Chem. **280**:40832-40837.

217. **Letvin, N. L. and B. D. Walker**. 2003. Immunopathogenesis and immunotherapy in AIDS virus infections. Nat.Med. **9**:861-866.

218. **Lim, W. A.** 2002. The modular logic of signaling proteins: building allosteric switches from simple binding domains. Curr.Opin.Struct.Biol. **12**:61-68.

219. **Lin, X., S. Lee, and G. Sun**. 2003. Functions of the activation loop in Csk protein-tyrosine kinase. J.Biol.Chem. **278**:24072-24077.

220. **Lipton, S. A. and H. E. Gendelman**. 1995. Seminars in medicine of the Beth Israel Hospital, Boston. Dementia associated with the acquired immunodeficiency syndrome. N.Engl.J.Med. **332**:934-940.

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

222. **Lowell, C. A.** 2004. Src-family kinases: rheostats of immune cell signaling. Mol.Immunol. **41**:631-643.

223. **Lowell, C. A. and G. Berton**. 1998. Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. Proc.Natl.Acad.Sci.U.S.A **95**:7580-7584.

224. **Lowell, C. A., L. Fumagalli, and G. Berton**. 1996. Deficiency of Src family kinases p59/61hck and p58c-fgr results in defective adhesion-dependent neutrophil functions. J.Cell Biol. **133**:895-910.

225. **Lowell, C. A., P. Soriano, and H. E. Varmus**. 1994. Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. Genes Dev. **8**:387-398.

226. **Lowry, W. E., J. Huang, Y. C. Ma, S. Ali, D. Wang, D. M. Williams, M. Okada, P. A. Cole, and X. Y. Huang**. 2002. Csk, a critical link of g protein signals to actin cytoskeletal reorganization. Dev.Cell **2**:733-744.

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

228. **Lund, T., M. M. Medveczky, and P. G. Medveczky**. 1997. Herpesvirus saimiri Tip-484 membrane protein markedly increases p56lck activity in T cells. J.Virol. **71**:378-382.

229. **MacAuley, A., M. Okada, S. Nada, H. Nakagawa, and J. A. Cooper**. 1993. Phosphorylation of Src mutants at Tyr 527 in fibroblasts does not correlate with in vitro phosphorylation by CSK. Oncogene **8**:117-124.

230. **Machouf, N., R. Thomas, V. K. Nguyen, B. Trottier, M. R. Boulassel, M. A. Wainberg, and J. P. Routy**. 2006. Effects of drug resistance on viral load in patients failing antiretroviral therapy. J.Med.Virol. **78**:608-613.

231. **Mann, J., C. N. Patrick, M. S. Cragg, J. Honeychurch, D. A. Mann, and M. Harris**. 2005. Functional analysis of HIV type 1 Nef reveals a role for PAK2 as a regulator of cell phenotype and function in the murine dendritic cell line, DC2.4. J.Immunol. **175**:6560-6569.

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

233. **Marechal, V., M. C. Prevost, C. Petit, E. Perret, J. M. Heard, and O. Schwartz**. 2001. Human immunodeficiency virus type 1 entry into macrophages mediated by macropinocytosis. J.Virol. **75**:11166-11177.

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

235. **Martin, J. C. and J. C. Bandres**. 1999. Cells of the monocyte-macrophage lineage and pathogenesis of HIV-1 infection. J.Acquir.Immune.Defic.Syndr. **22**:413-429.

236. **Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois**. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. Science **291**:2413-2417.

237. **McVicar, D. W., B. K. Lal, A. Lloyd, M. Kawamura, Y. Q. Chen, X. Zhang, J. E. Staples, J. R. Ortaldo, and J. J. O'Shea**. 1994. Molecular cloning of lsk, a carboxyl-terminal src kinase (csk) related gene, expressed in leukocytes. Oncogene **9**:2037-2044.

238. **Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz**. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J.Exp.Med. **200**:761-770.

239. **Meltzer, M. S., D. R. Skillman, P. J. Gomatos, D. C. Kalter, and H. E. Gendelman**. 1990. Role of mononuclear phagocytes in the pathogenesis of human immunodeficiency virus infection. Annu.Rev.Immunol. **8**:169-194.

240. **Meng, F. and C. A. Lowell**. 1998. A beta 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. EMBO J. **17**:4391-4403.

241. **Meyn, M. A., III, S. J. Schreiner, T. P. Dumitrescu, G. J. Nau, and T. E. Smithgall**. 2005. SRC family kinase activity is required for murine embryonic stem cell growth and differentiation. Mol.Pharmacol. **68**:1320-1330.

242. **Michael, N. L., G. Chang, L. A. D'Arcy, C. J. Tseng, D. L. Birx, and H. W. Sheppard**. 1995. Functional characterization of human immunodeficiency virus type 1 nef genes in patients with divergent rates of disease progression. J.Virol. **69**:6758-6769.

243. **Moanna, A., R. Dunham, M. Paiardini, and G. Silvestri**. 2005. CD4+ T-cell depletion in HIV infection: killed by friendly fire? Curr.HIV/AIDS Rep. **2**:16-23.

244. **Moarefi, I., M. LaFevre-Bernt, F. Sicheri, M. Huse, C. H. Lee, J. Kuriyan, and W. T. Miller**. 1997. Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature **385**:650-653.

245. **Molina, T. J., K. Kishihara, D. P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C. J. Paige, K. U. Hartmann, A. Veillette, D. Davidson, and T. W. Mak**. 1992. Profound block in thymocyte development in mice lacking p56lck. Nature **357**:161-164.

246. **Moore, J. P., S. G. Kitchen, P. Pugach, and J. A. Zack**. 2004. The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res.Hum.Retroviruses **20**:111-126.

247. **Moylett, E. H. and W. T. Shearer**. 2002. HIV: clinical manifestations. J.Allergy Clin.Immunol. **110**:3-16.

248. **Mummidi, S., G. Catano, L. Lam, A. Hoefle, V. Telles, K. Begum, F. Jimenez, S. S. Ahuja, and S. K. Ahuja**. 2001. Extensive repertoire of membrane-bound and soluble dendritic cell-specific ICAM-3-grabbing nonintegrin 1 (DC-SIGN1) and DC-SIGN2 isoforms. Inter-individual variation in expression of DC-SIGN transcripts. J.Biol.Chem. **276**:33196-33212.

249. **Munch, J., N. Stolte, D. Fuchs, C. Stahl-Hennig, and F. Kirchhoff**. 2001. Efficient class I major histocompatibility complex down-regulation by simian immunodeficiency virus Nef is associated with a strong selective advantage in infected rhesus macaques. J.Virol. **75**:10532-10536.

250. **Murakami, Y., H. Fukazawa, T. Kobatake, S. Yamagoe, Y. Takebe, M. Tobiume, M. Matsuda, and Y. Uehara**. 2002. A mammalian two-hybrid screening system for

inhibitors of interaction between HIV Nef and the cellular tyrosine kinase Hck. Antiviral Res. **55**:161-168.

251. **Murphy, S. M., M. Bergman, and D. O. Morgan**. 1993. Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with Saccharomyces cerevisiae. Mol.Cell Biol. **13**:5290-5300.

252. **Nada, S., M. Okada, A. MacAuley, J. A. Cooper, and H. Nakagawa**. 1991. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature **351**:69-72.

253. **Nada, S., T. Yagi, H. Takeda, T. Tokunaga, H. Nakagawa, Y. Ikawa, M. Okada, and S. Aizawa**. 1993. Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell **73**:1125-1135.

254. **Nagar, B., O. Hantschel, M. A. Young, K. Scheffzek, D. Veach, W. Bornmann, B. Clarkson, G. Superti-Furga, and J. Kuriyan**. 2003. Structural basis for the autoinhibition of c-Abl tyrosine kinase. Cell **112**:859-871.

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

256. **Nakagawa, T., S. Tanaka, H. Suzuki, H. Takayanagi, T. Miyazaki, K. Nakamura, and T. Tsuruo**. 2000. Overexpression of the csk gene suppresses tumor metastasis in vivo. Int.J.Cancer **88**:384-391.

257. **Nguyen, D. G. and J. E. Hildreth**. 2003. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. Eur.J.Immunol. **33**:483-493.

258. **Nishizumi, H., I. Taniuchi, Y. Yamanashi, D. Kitamura, D. Ilic, S. Mori, T. Watanabe, and T. Yamamoto**. 1995. Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. Immunity **3**:549-560.

259. **O'Neill, E., L. S. Kuo, J. F. Krisko, D. R. Tomchick, J. V. Garcia, and J. L. Foster**. 2006. Dynamic evolution of the human immunodeficiency virus type 1 pathogenic factor, Nef. J.Virol. **80**:1311-1320.

260. **Ogawa, A., Y. Takayama, H. Sakai, K. T. Chong, S. Takeuchi, A. Nakagawa, S. Nada, M. Okada, and T. Tsukihara**. 2002. Structure of the carboxyl-terminal Src kinase, Csk. J.Biol.Chem. **277**:14351-14354.

261. **Ohtake, H., N. Ichikawa, M. Okada, and T. Yamashita**. 2002. Cutting Edge: Transmembrane phosphoprotein Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains as a negative feedback regulator of mast cell signaling through the FcepsilonRI. J.Immunol. **168**:2087-2090.

262. **Okada, M., S. Nada, Y. Yamanashi, T. Yamamoto, and H. Nakagawa**. 1991. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J.Biol.Chem. **266**:24249-24252.

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

264. **Orenstein, J. M.** 2001. The macrophage in HIV infection. Immunobiology **204**:598-602.

265. **Orenstein, J. M., C. Fox, and S. M. Wahl**. 1997. Macrophages as a source of HIV during opportunistic infections. Science **276**:1857-1861.

266. **Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman**. 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study. J.Virol. **62**:2578-2586.

267. **Palacios, E. H. and A. Weiss**. 2004. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene **23**:7990-8000.

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

269. **Parsons, S. J. and J. T. Parsons**. 2004. Src family kinases, key regulators of signal transduction. Oncogene **23**:7906-7909.

270. **Percario, Z., E. Olivetta, G. Fiorucci, G. Mangino, S. Peretti, G. Romeo, E. Affabris, and M. Federico**. 2003. 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. **74**:821-832.

271. **Perelson, A. S.** 2002. Modelling viral and immune system dynamics. Nat.Rev.Immunol. **2**:28-36.

272. **Perelson, A. S., P. Essunger, Y. Cao, M. Vesanen, A. Hurley, K. Saksela, M. Markowitz, and D. D. Ho**. 1997. Decay characteristics of HIV-1-infected compartments during combination therapy. Nature **387**:188-191.

273. **Perelson, A. S., A. U. Neumann, M. Markowitz, J. M. Leonard, and D. D. Ho**. 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science **271**:1582-1586.

274. **Perlmutter, R. M., J. D. Marth, D. B. Lewis, R. Peet, S. F. Ziegler, and C. B. Wilson**. 1988. Structure and expression of lck transcripts in human lymphoid cells. J.Cell Biochem. **38**:117-126.

275. **Peter, F.** 1998. HIV nef: The mother of all evil? Immunity **9**:433-437.

276. **Picard, C., J. Gabert, D. Olive, and Y. Collette**. 2004. Altered splicing in hematological malignancies reveals a tissue-specific translational block of the Src-family tyrosine kinase fyn brain isoform expression. Leukemia **18**:1737-1739.

277. **Picker, L. J., S. I. Hagen, R. Lum, E. F. Reed-Inderbitzin, L. M. Daly, A. W. Sylwester, J. M. Walker, D. C. Siess, M. Piatak, Jr., C. Wang, D. B. Allison, V. C. Maino, J. D. Lifson, T. Kodama, and M. K. Axthelm**. 2004. Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. J.Exp.Med. **200**:1299-1314.

278. **Picker, L. J. and D. I. Watkins**. 2005. HIV pathogenesis: the first cut is the deepest. Nat.Immunol. **6**:430-432.

279. **Piguet, V., O. Schwartz, S. Le Gall, and D. Trono**. 1999. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol.Rev. **168**:51-63.

280. **Piguet, V. and D. Trono**. 1999. The Nef protein of primate lentiviruses. Rev.Med.Virol. **9**:111-120.

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

282. **Poincloux, R., C. Vincent, A. Labrousse, J. Castandet, M. Rigo, C. Cougoule, C. Bordier, C. Le, V, and I. Maridonneau-Parini**. 2006. Re-arrangements of podosome structures are observed when Hck is activated in myeloid cells. Eur.J.Cell Biol. **85**:327-332.

283. **Pomerantz, R. J. and D. L. Horn**. 2003. Twenty years of therapy for HIV-1 infection. Nat.Med. **9**:867-873.

284. **Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore**. 1990. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell **61**:1271-1276.

285. **Porter, M., T. Schindler, J. Kuriyan, and W. T. Miller**. 2000. 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. **275**:2721-2726.

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

287. **Qiao, X., B. He, A. Chiu, D. M. Knowles, A. Chadburn, and A. Cerutti**. 2006. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. Nat.Immunol. **7**:302-310.

288. **Quintrell, N., R. Lebo, H. Varmus, J. M. Bishop, M. J. Pettenati, M. M. Le Beau, M. O. Diaz, and J. D. Rowley**. 1987. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. Mol.Cell Biol. **7**:2267-2275.

289. **Ramos, A., D. J. Hu, L. Nguyen, K. O. Phan, S. Vanichseni, N. Promadej, K. Choopanya, M. Callahan, N. L. Young, J. McNicholl, T. D. Mastro, T. M. Folks, and S. Subbarao**. 2002. Intersubtype human immunodeficiency virus type 1 superinfection following seroconversion to primary infection in two injection drug users. J.Virol. **76**:7444-7452.

290. **Raposo, G., M. Moore, D. Innes, R. Leijendekker, A. Leigh-Brown, P. Benaroch, and H. Geuze**. 2002. Human macrophages accumulate HIV-1 particles in MHC II compartments. Traffic. **3**:718-729.

291. **Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell, and M. K. Jenkins**. 2001. Visualizing the generation of memory CD4 T cells in the whole body. Nature **410**:101-105.

292. **Reinhart, T. A., M. J. Rogan, G. A. Viglianti, D. M. Rausch, L. E. Eiden, and A. T. Haase**. 1997. A new approach to investigating the relationship between productive infection and cytopathicity in vivo. Nat.Med. **3**:218-221.

293. **Renkema, G. H., A. Manninen, D. A. Mann, M. Harris, and K. Saksela**. 1999. Identification of the Nef-associated kinase as p21-activated kinase 2. Curr.Biol. **9**:1407-1410.

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

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

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

297. **Rice, L. M., C. Plakas, and J. T. Nickels, Jr.** 2005. Loss of meiotic rereplication block in Saccharomyces cerevisiae cells defective in Cdc28p regulation. Eukaryot.Cell **4**:55-62.

298. **Richman, D. D.** 2001. HIV chemotherapy. Nature **410**:995-1001.

299. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos**. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc.Natl.Acad.Sci.U.S.A **100**:4144-4149.

300. **Roskoski, R., Jr.** 2004. Src protein-tyrosine kinase structure and regulation. Biochem.Biophys.Res.Commun. **324**:1155-1164.

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

302. **Sabe, H., B. Knudsen, M. Okada, S. Nada, H. Nakagawa, and H. Hanafusa**. 1992. Molecular cloning and expression of chicken C-terminal Src kinase: lack of stable association with c-Src protein. Proc.Natl.Acad.Sci.U.S.A **89**:2190-2194.

303. **Sakano, S., A. Iwama, J. Inazawa, T. Ariyama, M. Ohno, and T. Suda**. 1994. Molecular cloning of a novel non-receptor tyrosine kinase, HYL (hematopoietic consensus tyrosine-lacking kinase). Oncogene **9**:1155-1161.

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

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

306. **Samelson, L. E., A. F. Phillips, E. T. Luong, and R. D. Klausner**. 1990. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. Proc.Natl.Acad.Sci.U.S.A **87**:4358-4362.

307. **Samokhvalov, I., J. Hendrikx, J. Visser, A. Belyavsky, D. Sotiropolous, and H. Gu**. 1997. Mice lacking a functional chk gene have no apparent defects in the hematopoietic system. Biochem.Mol.Biol.Int. **43**:115-122.

308. **Sawai, E. T., M. S. Hamza, M. Ye, K. E. Shaw, and P. A. Luciw**. 2000. Pathogenic conversion of live attenuated simian immunodeficiency virus vaccines is associated with expression of truncated Nef. J.Virol. **74**:2038-2045.

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

310. **Schindler, T., F. Sicheri, A. Pico, A. Gazit, A. Levitzki, and J. Kuriyan**. 1999. Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. Mol.Cell **3**:639-648.

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

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

313. **Schrager, L. K. and M. P. D'Souza**. 1998. Cellular and anatomical reservoirs of HIV-1 in patients receiving potent antiretroviral combination therapy. JAMA **280**:67-71.

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

315. **Seder, R. A. and R. Ahmed**. 2003. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat.Immunol. **4**:835-842.

316. **Serfas, M. S. and A. L. Tyner**. 2003. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. Oncol.Res. **13**:409-419.

317. **Sharova, N., C. Swingler, M. Sharkey, and M. Stevenson**. 2005. Macrophages archive HIV-1 virions for dissemination in trans. EMBO J. **24**:2481-2489.

318. **Shekhtman, A., R. Ghose, D. Wang, P. A. Cole, and D. Cowburn**. 2001. Novel mechanism of regulation of the non-receptor protein tyrosine kinase Csk: insights from NMR mapping studies and site-directed mutagenesis. J.Mol.Biol. **314**:129-138.

319. **Shugars, D. C., M. S. Smith, D. H. Glueck, P. V. Nantermet, F. Seillier-Moiseiwitsch, and R. Swanstrom**. 1993. Analysis of human immunodeficiency virus type 1 nef gene sequences present in vivo. J.Virol. **67**:4639-4650.

320. **Sicheri, F. and J. Kuriyan**. 1997. Structures of Src-family tyrosine kinases. Curr.Opin.Struct.Biol. **7**:777-785.

321. **Sicheri, F., I. Moarefi, and J. Kuriyan**. 1997. Crystal structure of the Src family tyrosine kinase Hck. Nature **385**:602-609.

322. **Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick, C. Kovacs, S. J. Gange, and R. F. Siliciano**. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. Nat.Med. **9**:727-728.

323. **Siliciano, J. D. and R. F. Siliciano**. 2004. A long-term latent reservoir for HIV-1: discovery and clinical implications. J.Antimicrob.Chemother. **54**:6-9.

324. **Silvestri, G.** 2005. Naturally SIV-infected sooty mangabeys: are we closer to understanding why they do not develop AIDS? J.Med.Primatol. **34**:243-252.

325. **Silvestri, G., A. Fedanov, S. Germon, N. Kozyr, W. J. Kaiser, D. A. Garber, H. McClure, M. B. Feinberg, and S. I. Staprans**. 2005. Divergent host responses during primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. J.Virol. **79**:4043-4054.

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

327. **Simons, K. and E. Ikonen**. 1997. Functional rafts in cell membranes. Nature **387**:569-572.

328. **Smith, B. A., S. Gartner, Y. Liu, A. S. Perelson, N. I. Stilianakis, B. F. Keele, T. M. Kerkering, A. Ferreira-Gonzalez, A. K. Szakal, J. G. Tew, and G. F. Burton**. 2001. Persistence of infectious HIV on follicular dendritic cells. J.Immunol. **166**:690-696.

329. **Sondhi, D. and P. A. Cole**. 1999. Domain interactions in protein tyrosine kinase Csk. Biochemistry **38**:11147-11155.

330. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley**. 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72**:767-778.

331. **Sonza, S., H. P. Mutimer, R. Oelrichs, D. Jardine, K. Harvey, A. Dunne, D. F. Purcell, C. Birch, and S. M. Crowe**. 2001. Monocytes harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. AIDS **15**:17-22.

332. **Speth, C., M. P. Dierich, and S. Sopper**. 2005. HIV-infection of the central nervous system: the tightrope walk of innate immunity. Mol.Immunol. **42**:213-228.

333. **Sprent, J. and C. D. Surh**. 2002. T cell memory. Annu.Rev.Immunol. **20**:551-579.

334. **Stebbing, J., B. Gazzard, and D. C. Douek**. 2004. Where does HIV live? N.Engl.J.Med. **350**:1872-1880.

335. **Stein, P. L., H. M. Lee, S. Rich, and P. Soriano**. 1992. pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. Cell **70**:741-750.

336. **Stevenson, M.** 2003. HIV-1 pathogenesis. Nat.Med. **9**:853-860.

337. **Sudol, M., H. Greulich, L. Newman, A. Sarkar, J. Sukegawa, and T. Yamamoto**. 1993. A novel Yes-related kinase, Yrk, is expressed at elevated levels in neural and hematopoietic tissues. Oncogene **8**:823-831.

338. **Summy, J. M., Y. Qian, B. H. Jiang, A. Guappone-Koay, A. Gatesman, X. Shi, and D. C. Flynn**. 2003. The SH4-Unique-SH3-SH2 domains dictate specificity in signaling that differentiate c-Yes from c-Src. J.Cell Sci. **116**:2585-2598.

339. **Summy, J. M., M. Sudol, M. J. Eck, A. N. Monteiro, A. Gatesman, and D. C. Flynn**. 2003. Specificity in signaling by c-Yes. Front Biosci. **8**:s185-s205.

340. **Superti-Furga, G., S. Fumagalli, M. Koegl, S. A. Courtneidge, and G. Draetta**. 1993. Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. EMBO J. **12**:2625-2634.

341. **Suzu, S., H. Harada, T. Matsumoto, and S. Okada**. 2005. HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and inhibits M-CSF bioactivities. Blood **105**:3230-3237.

342. **Swigut, T., N. Shohdy, and J. Skowronski**. 2001. Mechanism for down-regulation of CD28 by Nef. EMBO J. **20**:1593-1604.

343. **Swingler, S., B. Brichacek, J. M. Jacque, C. Ulich, J. Zhou, and M. Stevenson**. 2003. HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. Nature **424**:213-219.

344. **Swingler, S., A. Mann, J. Jacque, B. Brichacek, V. G. Sasseville, K. Williams, A. A. Lackner, E. N. Janoff, R. Wang, D. Fisher, and M. Stevenson**. 1999. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. Nat.Med. **5**:997-103.

345. **Takashima, Y., F. J. Delfino, J. R. Engen, G. Superti-Furga, and T. E. Smithgall**. 2003. Regulation of c-Fes tyrosine kinase activity by coiled-coil and SH2 domains: Analysis with Saccharomyces cerevisiae. Biochemistry **42**:3567-3574.

346. **Takeuchi, S., Y. Takayama, A. Ogawa, K. Tamura, and M. Okada**. 2000. Transmembrane phosphoprotein Cbp positively regulates the activity of the carboxyl-terminal Src kinase, Csk. J.Biol.Chem. **275**:29183-29186.

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

348. **Tesselaar, K., R. Arens, G. M. van Schijndel, P. A. Baars, M. A. van der Valk, J. Borst, M. H. van Oers, and R. A. van Lier**. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. Nat.Immunol. **4**:49-54.

349. **Thomas, S. M. and J. S. Brugge**. 1997. Cellular functions regulated by Src family kinases. Annu.Rev.Cell Dev.Biol. **13**:513-609.

350. **Thoulouze, M. I., N. Sol-Foulon, F. Blanchet, A. Dautry-Varsat, O. Schwartz, and A. Alcover**. 2006. Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. Immunity **24**:547-561.

351. **Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon**. 1987. Purification and characterization of yeast myristoyl CoA:protein N-myristoyltransferase. Proc.Natl.Acad.Sci.U.S.A **84**:2708-2712.

352. **Trible, R. P., L. Emert-Sedlak, and T. E. Smithgall**. 2006. HIV-1 Nef selectively activates SRC family kinases HCK, LYN, and c-SRC through direct SH3 domain interaction. J.Biol.Chem. **In press**.

353. **Trkola, A., T. Ketas, V. N. Kewalramani, F. Endorf, J. M. Binley, H. Katinger, J. Robinson, D. R. Littman, and J. P. Moore**. 1998. Neutralization sensitivity of human immunodeficiency virus type 1 primary isolates to antibodies and CD4-based reagents is independent of coreceptor usage. J.Virol. **72**:1876-1885.

354. **Tsai, C. C., K. E. Follis, A. Sabo, T. W. Beck, R. F. Grant, N. Bischofberger, R. E. Benveniste, and R. Black**. 1995. Prevention of SIV infection in macaques by (R)-9-(2-phosphonylmethoxypropyl)adenine. Science **270**:1197-1199.

355. **Turner, D. and M. A. Wainberg**. 2006. HIV transmission and primary drug resistance. AIDS Rev. **8**:17-23.

356. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman**. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60**:755-765.

357. **Turville, S., J. Wilkinson, P. Cameron, J. Dable, and A. L. Cunningham**. 2003. The role of dendritic cell C-type lectin receptors in HIV pathogenesis. J.Leukoc.Biol. **74**:710-718.

358. **U.S.Food and Drug Administration**. 2006. Drugs Used in the Treatment of HIV Infection. http://www.fda.gov/oashi/aids/virals.html.

359. **U.S.Food and Drug Administration**. 2006. FDA Approves the First Once-a-Day Three-Drug Combination Tablet for Treatment of HIV-1: Atripla is a Landmark Achievement of Three Cooperating Companies. http://www.fda.gov/bbs/topics/NEWS/2006/NEW01408.html.

360. **van Oers, N. S., B. Lowin-Kropf, D. Finlay, K. Connolly, and A. Weiss**. 1996. Alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity **5**:429-436.

361. **Vang, T., K. M. Torgersen, V. Sundvold, M. Saxena, F. O. Levy, B. S. Skalhegg, V. Hansson, T. Mustelin, and K. Tasken**. 2001. Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. J.Exp.Med. **193**:497-507.

362. **Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner**. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science **280**:427-431.

363. **Verani, A., G. Gras, and G. Pancino**. 2005. Macrophages and HIV-1: dangerous liaisons. Mol.Immunol. **42**:195-212.

364. **Vicentini, L., P. Mazzi, E. Caveggion, S. Continolo, L. Fumagalli, J. A. Lapinet-Vera, C. A. Lowell, and G. Berton**. 2002. Fgr deficiency results in defective eosinophil recruitment to the lung during allergic airway inflammation. J.Immunol. **168**:6446-6454.

365. **Vilhardt, F.** 2005. Microglia: phagocyte and glia cell. Int.J.Biochem.Cell Biol. **37**:17-21.

366. **Vincent, P., E. Priceputu, D. Kay, K. Saksela, P. Jolicoeur, and Z. Hanna**. 2006. 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. **281**:6940-6954.

367. **Waksman, G., D. Kominos, S. C. Robertson, N. Pant, D. Baltimore, R. B. Birge, D. Cowburn, H. Hanafusa, B. J. Mayer, M. Overduin, M. D. Resh, C. B. Rios, L. Silverman, and J. Kuriyan**. 1992. Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. Nature **358**:646-653.

368. **Waksman, G., S. E. Shoelson, N. Pant, D. Cowburn, and J. Kuriyan**. 1993. Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell **72**:779-790.

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

370. **Walkenhorst, J., A. Goga, O. N. Witte, and G. Superti-Furga**. 1996. Analysis of human c-Abl tyrosine kinase activity and regulation in S. pombe. Oncogene **12**:1513-1520.

371. **Wang, D., X. Y. Huang, and P. A. Cole**. 2001. Molecular determinants for Csk-catalyzed tyrosine phosphorylation of the Src tail. Biochemistry **40**:2004-2010.

372. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw**. 2003. Antibody neutralization and escape by HIV-1. Nature **422**:307-312.

373. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw**. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373**:117-122.

374. **Welker, R., H. Kottler, H. R. Kalbitzer, and H. G. Krausslich**. 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology **219**:228-236.

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

376. **Wiese, N., A. Y. Tsygankov, U. Klauenberg, J. B. Bolen, B. Fleischer, and B. M. Broker**. 1996. Selective activation of T cell kinase p56lck by Herpesvirus saimiri protein tip. J.Biol.Chem. **271**:847-852.

377. **Wiley, C. A., R. D. Schrier, J. A. Nelson, P. W. Lampert, and M. B. Oldstone**. 1986. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proc.Natl.Acad.Sci.U.S.A **83**:7089-7093.

378. **Wilflingseder, D., Z. Banki, M. P. Dierich, and H. Stoiber**. 2005. Mechanisms promoting dendritic cell-mediated transmission of HIV. Mol.Immunol. **42**:229-237.

379. **Williams, J. C., A. Weijland, S. Gonfloni, A. Thompson, S. A. Courtneidge, G. Superti-Furga, and R. K. Wierenga**. 1997. The 2.35 A crystal structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions. J.Mol.Biol. **274**:757-775.

380. **Willman, C. L., C. C. Stewart, J. K. Griffith, S. J. Stewart, and T. B. Tomasi**. 1987. Differential expression and regulation of the c-src and c-fgr protooncogenes in myelomonocytic cells. Proc.Natl.Acad.Sci.U.S.A **84**:4480-4484.

381. **Wilson, M. B., S. J. Schreiner, H. J. Choi, J. Kamens, and T. E. Smithgall**. 2002. Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis. Oncogene **21**:8075-8088.

382. **Wiskerchen, M. and C. Cheng-Mayer**. 1996. HIV-1 Nef association with cellular serine kinase correlates with enhanced virion infectivity and efficient proviral DNA synthesis. Virology **224**:292-301.

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

384. **Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman**. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278**:1291-1295.

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

386. **Wyand, M. S., K. Manson, D. C. Montefiori, J. D. Lifson, R. P. Johnson, and R. C. Desrosiers**. 1999. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J.Virol. **73**:8356-8363.

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

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

389. **Xu, W., A. Doshi, M. Lei, M. J. Eck, and S. C. Harrison**. 1999. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol.Cell **3**:629-638.

390. **Xu, W., S. C. Harrison, and M. J. Eck**. 1997. Three-dimensional structure of the tyrosine kinase c-Src. Nature **385**:595-602.

391. **Xu, X. N., B. Laffert, G. R. Screaton, M. Kraft, D. Wolf, W. Kolanus, J. Mongkolsapay, A. J. McMichael, and A. S. Baur**. 1999. Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain. J.Exp.Med. **189**:1489-1496.

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

393. **Xu, Y., K. W. Harder, N. D. Huntington, M. L. Hibbs, and D. M. Tarlinton**. 2005. Lyn tyrosine kinase: accentuating the positive and the negative. Immunity **22**:9-18.

394. **Yamaguchi, H. and W. A. Hendrickson**. 1996. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature **384**:484-489.

395. **Yamanashi, Y., S. Fukushige, K. Semba, J. Sukegawa, N. Miyajima, K. Matsubara, T. Yamamoto, and K. Toyoshima**. 1987. The yes-related cellular gene lyn encodes a possible tyrosine kinase similar to p56lck. Mol.Cell Biol. **7**:237-243.

396. **Yang, P. and A. J. Henderson**. 2005. Nef enhances c-Cbl phosphorylation in HIV-infected CD4+ T lymphocytes. Virology **336**:219-228.

397. **Yasuda, K., A. Kosugi, F. Hayashi, S. Saitoh, M. Nagafuku, Y. Mori, M. Ogata, and T. Hamaoka**. 2000. Serine 6 of Lck tyrosine kinase: a critical site for Lck myristoylation, membrane localization, and function in T lymphocytes. J.Immunol. **165**:3226-3231.

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

399. **Yi, Y., W. Chen, I. Frank, J. Cutilli, A. Singh, L. Starr-Spires, J. Sulcove, D. L. Kolson, and R. G. Collman**. 2003. An unusual syncytia-inducing human immunodeficiency virus type 1 primary isolate from the central nervous system that is restricted to CXCR4, replicates efficiently in macrophages, and induces neuronal apoptosis. J.Neurovirol. **9**:432-441.

400. **Young, M. A., S. Gonfloni, G. Superti-Furga, B. Roux, and J. Kuriyan**. 2001. Dynamic coupling between the SH2 and SH3 domains of c-Src and hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell **105**:115-126.

401. **Yu, H., J. K. Chen, S. Feng, D. C. Dalgarno, A. W. Brauer, and S. L. Schreiber**. 1994. Structural basis for the binding of proline-rich peptides to SH3 domains. Cell **76**:933-945.

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

403. **Zamoyska, R., A. Basson, A. Filby, G. Legname, M. Lovatt, and B. Seddon**. 2003. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. Immunol.Rev. **191**:107-118.

404. **Zhang, L., C. Chung, B. S. Hu, T. He, Y. Guo, A. J. Kim, E. Skulsky, X. Jin, A. Hurley, B. Ramratnam, M. Markowitz, and D. D. Ho**. 2000. Genetic characterization of rebounding HIV-1 after cessation of highly active antiretroviral therapy. J.Clin.Invest **106**:839-845.

405. **Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, R. S. Veazey, D. Notermans, S. Little, S. A. Danner, D. D. Richman, D. Havlir, J. Wong, H. L. Jordan, T. W. Schacker, P. Racz, K. Tenner-Racz, N. L. Letvin, S. Wolinsky, and A. T. Haase**. 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science **286**:1353-1357.

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

407. **Ziegler, S. F., J. D. Marth, D. B. Lewis, and R. M. Perlmutter**. 1987. Novel protein-tyrosine kinase gene (hck) preferentially expressed in cells of hematopoietic origin. Mol.Cell Biol. **7**:2276-2285.