IDENTIFICATION AND

CHARACTERIZATION OF A NEF-ASSOCIATED KINASE

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Should this work contribute to the betterment of any individual,

then this work is dedicated to him.

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by

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The *nef* gene encoded by primate lentiviruses is a major determinant of virulence in vivo. It is expressed early in the viral life cycle; its importance likely stems from its ability to prime the host environment for efficient viral replication. Reasonable models by which cellular phenotypes associated with Nef expression could enhance viral replication in vivo have been proposed. The molecular mechanisms by which Nef executes its functions, however, are poorly understood. The work presented here investigates the regulation of cellular proteins by Nef. I first identified a previously described Nef-associated kinase as the cellular kinase p21 activated kinase 2 (Pak2). This was done using proteolytic digestion of the Nef-associated kinase in multiple systems as well as by demonstrating the presence of active, ectopically expressed Pak2 associated with Nef in a cellular expression system. I further demonstrated that Nef induces Pak2 activation in vivo using multiple systems. First, Nef dependent activation of ectopically expressed tagged Pak2 was demonstrated in vivo. Second, an in gel renaturable kinase activity assay showed in cell extracts a Nef dependent kinase activity I subsequently demonstrated to be Pak2 by proteolytic digestion. Third, I showed that in vivo Nef expression induces the phosphorylation of Merlin at S518, a known and specific Pak2 substrate. The mechanism by which Nef leads to Pak2

activation was also addressed. Rho family GTPases are well-described endogenous activators of Pak2. Inhibition of Rho family GTPase activity in vivo also blocked Nef mediated activation of Pak2 as did mutation of the Rho GTPase binding site in Pak2. Thus, Nef induced Pak2 activation is dependent on endogenous Rho family GTPases. No Nef dependent effect on Rho GTPase activity levels, however, was detected. Instead, biochemical separation and cellular localization suggested that Nef mediates Pak2 activation by recruiting Pak2 to membranes where it encounters high concentrations of constituitively active Rho family GTPases. In summary, this work conclusively demonstrated that Pak2 associates with Nef and is activated by Nef via a endogenous Rho GTPase. Lastly, the potential roles of these molecular events in mediating Nef's pathogenic effects are discussed.

Table of Contents

Publications

Arora VK. Fredericksen BL. Garcia JV. Nef: Agent of cell subversion. *Microbes & Infection*. 4(2):188-99, 2002 Feb.

Foster JL. Molina RP. Luo T. Arora VK. Huan Y. Ho DD. Garcia JV. Genetic and functional diversity of human immunodeficiency virus type 1 subtype B Nef primary isolates. *Journal of Virology*. 74(4):1672-80, 2001 Feb.

Arora VK. Molina RP. Foster JL. Blakemore JL. Chernoff J. Fredericksen BL. Garcia JV. Lentivirus Nef specifically activates Pak2. *Journal of Virology*. 74(23):11081-7, 2000 Dec.

Baude EJ. Arora VK. Yu S. Garbers DL. Wedel BJ. The cloning of a Caenorhabditis elegans guanylyl cyclase and the construction of a ligand-sensitive mammalian/nematode chimeric receptor. *Journal of Biological Chemistry*. 272(25):16035-9, 1997 June 20.

Ulloa-Aguirre A. Stanislaus D. Arora V. Vaananen J. Brothers S. Janovick JA. Conn PM. The third-intracellular loop of the rat gonadotropin-releasing hormone receptor couples the Gs- and G(q/11)-mediated signal transduction pathways: evidence from loop fragment transfection in GGH3 cells. *Endocrinology*. 139(5):2472-8, 1998 May.

Stanislaus D. Arora V. Awara WM. Conn PM. Biphasic action of cyclic adenosine 3',5'-monophosphate in gonadotropin-releasing hormone (GnRH) analog-stimulated hormone release form GH3 cells stably transfected with GnRH receptor complementary deoxyribonucleic acid. *Endocrinology*. 137(3):1025-31, 1996 Mar.

List of Figures & Tables

List of Abbreviations

AIDS – Acquired Immunodeficiency Syndrome

ATP – Adenosine Triphosphate

CIP – Calf Intestinal Phosphatase

CTL – Cytotoxic T Lymphocyte

DMEM – Dulbecco's Modified Eagle Medium

DTT - Dithiothreitol

GAP – GTPase Activating Protein

GEF – Guanine Nucleotide Exchange Factor

GFP – Green Fluorescent Protein

GST – Glutathione-S-Transferase

GTP – Guanosine Triphosphate

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HRP – Horseradish Peroxidase

MHC – Major Histocompatability Complex

NBP1 – Nef Binding Protein 1

NMR – Nuclear Magnetic Resonance

Nak – Nef Associated Kinase

NEO – Neomycin Phosphotransferase Gene

NFAT – Nuclear Factor of Activated T Cells

Pak – p21 Activated Kianse

PBD – p21 Binding Domain

PHA – Phytohaemagglutanin

SCID – Severe Combined Immodeficiency

SIV – Simian Immunodeficiency Virus

TCR – T Cell Receptor

Chapter 1: Background

Since the first case reports of Acquired Immunodeficiency Disease Syndrome (AIDS) over twenty years ago, great strides have been made in our understanding of the basic molecular mechanisms of its etiological agent, human immunodeficiency virus (HIV). The enormous effort to understand HIV has been complemented by investigation of other closely related primate lentiviruses. Like all retroviruses, the genomes of primate lentiviruses contain *gag*, *pol*, and *env* genes that encode well-characterized structural and enzymatic proteins essential for proper virus processing and assembly. In addition, primate lentiviruses encode up to six accessory genes. The two best characterized accessory genes, *tat* and *rev*, encode essential regulators of viral gene expression (1). In contrast, the remaining accessory genes play less well-defined roles in viral replication. Much ambiguity, for example, remains about the function of the *nef* accessory gene encoded by HIV-1,-2, and simian immunodeficiency virus (SIV).

Nef consists of a single open reading frame (ORF) that overlaps with the 3' long terminal repeat (LTR) of HIV-1,-2, and SIV (2). Along with Tat and Rev, Nef is expressed early after infection from a multiply spliced mRNA; much higher levels of *nef* mRNAs are found then for either *tat* or *rev* (3). Limited evidence indicates that some *nef* transcription begins before provirus integration (4). Large deletions in *nef* are associated with greatly attenuated viral pathogenicity (5). Moreover, *nef* can enhance viral replication in vivo and in vitro (5-7). However, it is not absolutely necessary for viral replication in either setting. Studies in many in vitro systems, furthermore, indicate *nef* has no effect on viral replication (8;9). Consistent with these observations, no essential role for Nef in the viral life cycle has been clearly established. Rather, Nef expression is

associated with a number of cellular phenotypes that early in the viral life cycle may prime the host cell for efficient viral replication in vivo and in limited contexts in vitro (10). Elucidation of how Nef regulates host factors, thus, is critical to understanding virus subversion of its host.

Nef in vivo: Nef expression is associated with enhanced pathogenicity in human HIV infection. Median time of progression to AIDS after HIV infection in untreated patients has been reported to be 7.2 years (11). Less than 5 percent of patients, however, remain asymptomatic for greater then 10 years with stable CD4+ lymphocyte counts (12). Infection with virus containing a deletion in the Nef gene and overlapping 3' LTR is associated with such delayed disease progression. The Sydney Blood Bank cohort is the best studied group of patients infected with Nef deleted virus and consists of 1 donor and 8 transfusion recipients all infected before 1985 (11;13;14). One member of the cohort died of a non-HIV related illness 5 years following infection. The remaining 8 members of the cohort were reported to be asymptomatic 10 to 14 years following infection with extremely low viral load and no immunological signs of disease progression (13). Other isolated slow progressors infected both by sexual and parenteral routes are also reported to have been infected with virus containing Nef deletions (15-17). These deletions affect the 3' LTR in regions distinct from that of the Sydney cohort virus, arguing against a role for the 3' LTR in the attenuated phenotype. Follow-up studies of the Sydney Cohort suggest that *nef* deleted virus can cause disease. Three members of the cohort have had clear declines in CD4 counts and two have begun antiretroviral therapy (11;14). Three other members, however, remain asymptomatic with normal CD4 counts as of the last

published follow-up in 2001 (14). The other two members included in the initial report of the cohort have died; in neither case was death clearly related to AIDS (11;14). Thus, *nef* deleted virus is capable of producing disease, but there is a correlation between infection with *nef* defective HIV and dramatically decreased rate of disease progression.

Work in SIV using isogenic viruses substantiates the importance of *nef* for lentivirus pathogenicity (5). Kestler et. al. infected adult macaques with SIV_{mac239}, a viral isolate that contains a naturally occurring premature termination codon in the *nef* ORF. Macaques were infected with 'wild-type' virus, a virus with a corrected *nef* ORF, or a virus with a large deletion in the *nef* ORF. After infection with the 'wild-type' virus or the virus with a corrected *nef* ORF, macaques developed AIDS-like symptoms. In contrast, the virus with a deletion in *nef* was not pathogenic and replicated poorly *in vivo* throughout the one-year study. The data obtained with the 'wild-type' virus was unexpected as it does not encode a functional Nef. However, this result was explained by analyzing the *nef* sequences of virus isolated from the macaques soon after the infection. Within a few weeks, only virus with mutations that re-established the *nef* ORF could be isolated from these macaques. These results showed that Nef is important for virus replication in vivo and that there is a significant pressure for SIV to encode a functional *nef*. However, some of the adult monkeys infected with *nef* deleted virus did eventually develop disease. Moreover, others showed that infection with Nef deleted SIV can induce an AIDS-like illness in neonatal macaques (18). Thus, as with HIV Nef, SIV Nef is not essential for disease development, but clearly enhances virulence.

Primate lentivirus do not replicate in mouse cells. Mouse based models that circumvent this limitation, however, highlight the contribution of *nef* to pathogenicity.

HIV-1_{NL4-3} infection of SCID-hu mice transplanted with human thymus leads to the massive depletion of double positive thymocytes (19). In this system, viruses with an intact *nef* ORF replicate faster, achieve a higher titer, and deplete thymocytes better than a *nef* deleted counterpart. Interestingly, Duus et. al. reported that in SCID-hu infection with $HIV_{HXB/LW}$ virus, Nef does not enhance virus replication, yet still increases double positive thymocyte depletion (20). Therefore, Nef may have pathogenic effects distinct from its ability to enhance viral replication. Results from a transgenic mouse model expressing HIV genes under the regulation of CD4 promoter/enhancer elements also suggest Nef directly mediates some of the pathogenic properties of HIV and SIV. In this system, expression of Nef, but none of the other HIV proteins, induces profound immunodeficiency and $CD4^+$ lymphocyte depletion (21). Thus, the mechanisms by which Nef optimizes viral replication in vivo may themselves contribute to pathogenicity.

Nef in vitro: The importance of Nef for replication in vivo is not always recapitulated in vitro. Nef was originally described as a negative regulator of HIV replication and was thus named NEgative Factor (22). As would occur with many subsequent observations about Nef, however, these initial findings were later refuted (8;9). Although some sill suggest that an unusual Nef isolate may have a negative effect (23), most investigators find that Nef actually has either no effect or a positive effect on viral replication in vitro. The ability of Nef to enhance viral replication is system dependent. For example, Nef generally has little or no effect on viral replication in activated peripheral blood mononuclear cells (PBMC) (5;24), dividing CD4 expressing T cell lines (25), or mature dendritic cell-T cell co-cultures (26). On the other hand, a significant role for Nef in viral

replication has been found in post-infection stimulated PBMC or lymphoid cultures (27;28), quiescent T cells (25), immature dendritic cell-T cell co-cultures (26), and an ex vivo tonsillar culture system (29).

Numerous cellular and viral phenotypes have been associated with expression of the Nef gene and may explain the ability of Nef to enhance virus replication in limited contexts (Fig. 1). Nef has been shown to down-regulate cell surface levels of CD4 (30) and MHC class I antigens (31), to mediate cellular signaling and activation (32), and to alter the susceptibility of cells to apoptosis. Nef has also been shown to reduce surface expression levels of CD28 on T cells and to up-regulate DC-SIGN on dendritic cells. Lastly, Nef has been shown to enhance virus infectivity (7), perhaps through effecting membrane trafficking events during viral budding. How these effects may translate into enhanced replication in limited contexts and mechanisms by which Nef may execute them will be discussed for each putative function.

CD4 Down-modulation: The most extensively characterized of the Nef functions is its ability to reduce dramatically steady state levels of CD4 on the cell surface (30). CD4 is down-modulated by almost all Nef isolates, in all mammalian cell types tested, and under nearly all experimental conditions tested (33-36). At least two benefits to virus replication are proposed to result from down-modulation of CD4, the primary receptor for both HIV and SIV. First, Benson and colleagues have demonstrated that SIV Nef expression renders human T cell lines resistant to HIV infection, suggesting Nef prevents disadvantageous super-infection of the host cell (34). A second role for Nef may be in overcoming the detrimental effects of high cellular CD4 expression that has been shown

both to inhibit progeny release and to decrease viral infectivity by sequestration of viral Env (37;38). (It should be noted that Nef also enhances infectivity of virus produced by transfection of CD4 negative cells. Thus, viral infectivity enhancement is generally considered an independent property of Nef - see below.) Two proteins expressed late in infection, Env and Vpu, also reduce CD4 surface expression. Such redundancy attests to the importance of CD4 down-modulation in the HIV life cycle, but may also limit the advantage conferred to HIV by Nef in vitro (1). On the other hand, in vivo where the virus is under stronger evolutionary pressure due to host immune factors, redundancy for CD4 down-modulation may be more important.

Nef-induced CD4 down-modulation involves the internalization of surface CD4 followed by degradation via the endosomal/lysosomal pathway. In support, Nef increases the number of CD4 containing clathrin coated pits (39). Moreover, Nef induced CD4 down-modulation is blocked by molecular (40) and pharmacological inhibitors (41) of clathrin coated pit mediated endocytosis. Inhibition of lysosomal acidification blocks Nef induced CD4 degradation, without restoring CD4 surface expression (42-44). Most, but not all, data indicates that Nef does not effect transport of newly synthesized CD4 to the cell surface (45;46). In the steady state, Nef expression also increases the percent of CD4 endocytosed from the cell surface per unit time (47). From this data it has been argued that Nef enhances the rate of CD4 endocytosis (47). However, the percent of CD4 internalized per unit time is not a rate and reflects the significantly lower total CD4 levels on the cell surface found in the steady state of Nef expressing cells. Nef likely does induce an increase in the rate of CD4 endocytosis before a new steady state is achieved, but this has not been proven (46).

Although much work has aimed at elucidating the molecular mediators of Nef induced CD4 down-modulation, conclusive evidence upon which a definitive model can be built is lacking. It is clear that, unlike CD4 down-modulation by phorbol esters, Nef-induced down-modulation is independent of phosphorylation of serine residues on the CD4 cytoplasmic tail (30). Instead, current data suggests that Nef acts as a connector between CD4 and elements of the cell's endocytic machinery (46). Unfortunately, a CD4/Nef complex is yet to be demonstrated in mammalian cells. In support of this model, however, an interaction has been demonstrated in insect cells (48), by yeast two hybrid (49), and by NMR analysis (50;51). Further evidence supporting Nef's role as a connector comes from mutational and chimeric analysis. The cytoplasmic domain of CD4 is both necessary and sufficient for Nef-induced CD4 down-regulation (35;52). While SIV Nef and HIV Nef utilize distinct residues in the membrane proximal cytoplasmic tail of CD4, both rely on an overlapping region containing a dileucine motif (53). NMR and yeast two hybrid analysis, furthermore, indicates that the CD4 dileucine motif is necessary for its interaction with Nef (49;50). Nef residues W57 and L58 in the HIV R7 clone (corresponds to W58 and L59 in $Nef_{D.Con}$, Fig. 2) predicted by NMR to be critical in this interaction (50) have also been functionally demonstrated to be important for CD4 down-modulation (54).

How Nef connects to the endocytic machinery is unclear. AP-2 adaptor complexes are known to initiate clathrin coated pit formation at the plasma membrane and may play a role in Nef induced CD4 endocytosis. This theory is supported by studies showing that Nef co-localizes with AP-2 on the cytoplasmic face of the plasma membrane (55). Moreover, a highly conserved leucine based sorting motif (E/DXXXLϕ, where ϕ is a

hydrophobic residue with a bulky side chain, residues 162-167 in $Nef_{D.Con}$ depicted in Fig. 2) in the C-terminal flexible loop of Nef has been shown to be important for CD4 down-modulation and for AP-2 co-localization (56-58). The relationship between Nef and AP-2, however, is not clear. Most data suggests that HIV Nef only weakly interacts with AP-2 (59). SIV Nef, in contrast, shows more striking interaction with the □ chain of AP-2 (57;60;61). A possible explanation is that SIV has evolved functionally redundant mechanisms to interact with the endocytic machinery. Indeed N-terminal regions in SIV Nef, but not HIV Nef, have been identified as independent AP-2 interaction domains (57;60). Surprisingly, mutations in SIV Nef have been characterized that greatly reduce AP-2 binding but, in most reports, have little effect on CD4 down-modulation (57;60;61). Thus, the role of AP-2 binding in Nef induced CD4 down-modulation is unresolved.

Nef may also interact with the endocytic machinery via Nef Binding Protein 1 (NBP1). NBP1 is a subunit of the vacuolar membrane ATPase complex known to be a part of clathrin coated vesicles (62). Lu et. al. identified NBP1 using a yeast two hybrid screen for Nef binding partners (63). They further showed in a cellular over-expression system that NBP1 interacts with both HIV Nef and SIV Nef (63). Nefs from both species utilize a C-terminal flexible loop diacidic motif for this interaction (63;64). Mutation of the diacidic motif in HIV or SIV Nef also impairs Nef-induced CD4 down-modulation (63;64). Interestingly, the C-terminal leucine based sorting motif in SIV Nef has also recently been suggested to be important for the NBP1 interaction (64); however, the role of the HIV Nef leucine motif in this interaction has not been reported. Thus, a possible scenario emerges in which the C-terminal flexible loops of SIV Nef and HIV Nef interact

with the endocytic machinery via NBP1. In addition, N-terminal regions of SIV Nef may facilitate the interaction via binding to AP-2.

Other cellular proteins have also been suggested to play a role in Nef mediated CD4 trafficking. The □chains of both AP-1, known to be involved in sorting events between the trans-golgi and endosomal system, and AP-3, known to be involved in trafficking to the lysosomes, have been suggested to bind to the C-terminal loop of Nef (59;65). The role, if any, of these adaptor complexes in CD4 trafficking has yet to be defined. □-Cop, a coatamer protein, has also been shown to interact with Nef (66;67). Mutation of a C-terminal EE motif in HIV Nef (corresponding to residues 156 and 157 in $Nef_{D.Con}$, Fig. 2) to QQ was originally shown to abolish the interaction between Nef and β-Cop and to impair the ability of Nef to target CD4 to lysosomes (66). The EE motif, thus, was postulated to target Nef to lysosomes via β-Cop (66). However, the role of the EE motif is not obvious since the primary isolate Nef_{233} has an EK at this position, but is fully functional for CD4 down-modulation (68;69). Furthermore, Janvier et. al. have recently shown that the mutations EE-QQ, EE-AA and EE-GG have no effect on Nef's ability to bind □-cop or to down-modulate CD4 (70). Thus, the EE motif is not necessary for CD4 down-modulation and the contribution of β-cop to CD4 trafficking needs to be re-evaluated. Nef also has been shown to bind to a novel human thioesterase (human thioesterase II) identified in a yeast two hybrid screen (71). Residues identified in Nef as important for thioesterase interaction are also important for CD4 down-modulation, suggesting that the thioesterase may play a role in Nef mediated CD4 trafficking (71;72). However, this prediction was refuted by the observation that the HIV Nef_{SF2} isolate that is fully functional for CD4 down-modulation (69) does not bind thioesterase (72).

MHC class I down-modulation: Nef induces the down-modulation of cell surface MHC class I antigens. Consistent with a more appreciable role for Nef in vivo than in vitro, this likely helps infected cells evade immune surveillance. In support, Collins et. al. demonstrated that Nef expression reduces the susceptibility of HIV infected cells to cytotoxic T lymphocyte (CTL) mediated lysis in vitro (73). Yang. et. al. confirmed this conclusion and further showed that Nef's protective effect is dependent on HLA antigen presentation in the target cell. These investigators introduce a CD4-TCRζ hybrid molecule into a CTL line. The CD4 moiety of the chimera recognizes the Env protein, gp120, found on the cell surface of HIV infected cells, thereby leading to CTL activation via the TCRζ moiety. In this system killing of infected targets is therefore independent of antigen presentation via MHC class I. These CTLs display antiviral effects in vitro that are unaffected by Nef expression. Importantly, HLA down-modulation does not enhance the susceptibility of infected cells to NK cells. HIV Nef and SIV Nef selectively down-regulate the MHC class I molecules HLA-A and HLA-B. This selectivity is conferred by a cytoplasmic tyrosine residue unique to these MHC class I molecules (74-76). HLA-A and HLA-B molecules present antigen to CTLs, but are not inhibitory to NK cell mediated lysis. The HLA-C and HLA-E molecules, in contrast, inhibit natural killer (NK) cell mediated lysis and are not down-modulated by Nef. Nef, thus, reduces the efficiency of CTL mediated lysis of infected cells without increasing their susceptibility to NK cell mediated killing. However, it should be noted that Nef does not render cells completely protected from immune surveillance as there is a measurable CTL response to HIV antigens including Nef itself (77).

Residues in the C-terminal flexible loop of Nef implicated in CD4 down-modulation are not important for HLA down-modulation (Fig. 2) (56;78). Thus, Nef induced CD4 and HLA down-modulation occur via at least partially distinct mechanisms. However, our understanding of how Nef does induce HLA down-modulation is incomplete. Initially it was proposed that Nef induces both endocytosis and lysosomal degradation of HLA molecules. Moreover, HLA biosynthesis up to the cis golgi was shown not to be effected by Nef (31). More recent work, however, indicates that Nef only enhances the rate of HLA endocytosis to a minor extent (79). Two groups have further shown that over-expression of a dynamin mutant known to inhibit clathrin coated pit mediated endocytosis blocks Nef induced CD4 down-modulation, but not HLA down-modulation (40;79). Thus, if Nef does effect HLA endocytosis it likely does so by a clathrin independent mechanism. Nef also has been shown to induce the accumulation of HLA in the trans golgi and perhaps other organelles (79;80). The mechanism by which this occurs, however, is not clear. Some data suggests that Nef promotes the retrieval of HLA to the trans golgi (80). An acidic stretch (amino acids 62-65 in HIV-1 isolate NA7; corresponds to 64-67 in $Nef_{D.\ Con}$, Fig. 2) has been implicated in this process (80) and perhaps functions by directly interacting with the cellular protein PACS-1 (81), which also retrieves Furin to the trans golgi (82). Other data, however, indicates that Nef prevents the delivery of HLA to the cell surface (79). Moreover, it was shown that inhibition of PI-3 kinase activity with wortmannin inhibits Nef's ability to mediate intracellular retention of HLA molecules, but has no effect on Nef's down-regulation of CD4 (79). Lastly, it is no longer clear if Nef increases HLA trafficking to the lysosome. It was initially reported that Nef stimulates the degradation of HLA molecules and that

the normal half-life of HLA molecules was restored in the presence of an inhibitor of lysosomal degradation (31). More recent reports, in contrast, suggest an insignificant decrease in HLA half-life in the presence of Nef (79). Thus the mechanism by which Nef regulates HLA down-modulation has still not been fully deciphered.

Other surface receptors: Nef does not lead to a general reduction in cell surface receptor expression levels. However, the ability of Nef to regulate cell surface expression of receptors is not limited to CD4 and MHC class I molecules. SIV and HIV-2 Nefs associate with the ζ chain of the CD3 T cell receptor signaling complex and induce CD3 down-modulation (83). Two groups, moreover, have shown that SIV Nef down-modulates cell surface levels of CD28, the T cell co-stimulatory receptor (84;85). The HIV isolate NA7 also weakly down-modulates CD28 (84). The significance of CD3 and CD28 down-modulation is not known, but implicates Nef in the regulation of T cell signaling cascades. However, a specific relevance of these phenomena to virus replication has not been shown.

Nef has also been shown to up-regulate DC-SIGN expression on dendritic cells (86). DC-SIGN is a dendritic cell specific lectin that binds to HIV envelope. It is thought to promote viral spread by first capturing virus particles in peripheral organs and then delivering them to lymphoid organs (87). Thus, up-regulation of DC-SIGN by Nef has been proposed to enhance viral spread by dendritic cells in vivo (86). However, it is difficult to envision how this could happen since there is little evidence suggesting significant dendritic cell infection in vivo. Physiologic DC-SIGN endocytosis requires a DC-SIGN cytoplasmic dileucine sorting motif similar to the dileucine sorting motif in

Nef (86). Thus, high level Nef expression in dendritic cells would likely competitively inhibit binding of endocytic machinery to the DC-SIGN cytoplasmic tail and prevent normal turnover of DC-SIGN. Thus, DC-SIGN up-regulation by Nef may not represent a highly specific Nef function.

Cellular activation and signaling: A large body of work has investigated Nef mediated perturbations in cellular signaling. Although conflicting findings have been reported, much evidence implicates Nef in cellular activation. T cell activation dramatically enhances viral replication. Therefore, Nef mediated cellular activation may enhance replication in vivo and in un-stimulated primary cell culture systems, but not in activated primary cells or transformed cells. Some of the evidence supporting a role for Nef in T cell activation comes from experiments using T cells derived from a macaque infected with herpesvirus Saimiri. Infection of these cells with SIV expressing either SIV or HIV Nef not only induces IL-2 production, but also enhances virus replication 8-100 fold (25). Recent micro-array analysis using Jurkat cells further demonstrates that Nef activates T cells (32). Simmons et. al. showed that induction of Nef expression up-regulates genes that are 98% identical to those up-regulated following anti-CD3 simulation. A number of the Nef up-regulated genes express products that are known to activate the HIV LTR (32). In some cases, the up-regulated genes express secreted products that have been shown to enhance in vitro HIV replication when added to culture media (32). Thus the paracrine effects of Nef induced factors may be sufficient to enhance replication. Similar findings have been reported with human macrophages. Supernatants from macrophages transduced with adenovirus vector expressing Nef can facilitate HIV replication in resting

lymphoid cultures (88). Interestingly, Nef also up-regulates expression of cellular proteins that facilitate the actions of the Tat viral protein (32). These findings suggest that Nef, which is expressed early in the virus life cycle, may play an important role in optimizing the cellular gene expression profile for virus replication.

Nef also may perturb T cell receptor (TCR) function. Some reports have suggested that Nef inhibits TCR signaling (89). This view is supported by the observation that SIV Nef down-regulates the major components of the antigen induced T cell stimulation machinery, namely CD3, CD4, and CD28. Other literature, however, favors the view that Nef potentiates T cell receptor initiated signaling, leading to modest Nef dependent enhancement of IL-2 production following stimulation with αCD3 and αCD28 (90;91). T cells from Nef transgenic mice, moreover, show increased LAT and MAPK phosphorylation, following TCR stimulation (21). However, it is not clear if any of these perturbations are of physiological relevance, as Page et. al. observed no Nef dependent alterations in cytokine production following antigen specific T cell stimulation with low and medium dose antigen (92). Lastly, the short half-life of an HIV infected T cell makes it difficult to envision a functional consequence of any effects on T cell receptor function, per se. Nonetheless, these findings implicate Nef as a regulator of signaling pathways activated by the T cell receptor. Moreover, Nef dependent alterations in TCR function have been observed in HIV infected resting T cells in which provirus integration is not thought to have occurred. Thus, Nef expressed before provirus integration may be sufficient to mediate cellular activation pathways (90).

A complete signaling pathway directly linking Nef to its reported effects on transcription and cellular activation has not been demonstrated. Mannien et. al. have

shown Nef enhances Nuclear Factor of Activated T cells (NFAT) transcriptional activity in Jurkat T cells. In their system, transient expression of Nef alone had no effect on NFAT activity. However, in the presence of PMA, a variety of Nef alleles enhance NFAT reporter activity 13-142 fold (93). As NFAT has been implicated in Nef induced up-regulation of activation associated genes (32), Nef may effect gene transcription via NFAT. It is unclear, however, why no effect on NFAT activity was observed in the absence of PMA, when Nef expression alone was reported to be sufficient to lead to the up-regulation of NFAT dependent genes. Moreover, older studies using stable Nef expressing T cell clones actually suggested that Nef may inhibit NFAT activation and IL-2 transcription in response to T cell stimulation (94). Thus, the true nature of regulation of NFAT by Nef is not clear.

Apoptosis: A limited body of evidence suggests a role for HIV Nef in regulating apoptotic pathways. For example, a role for Nef in protecting infected cells from Fas mediated apoptosis has been suggested (95;96). Nef has been proposed to inhibit the cellular apoptotic protein Ask1 to regulate this process (96). Other reports suggest that Nef up-regulates FasL, thereby leading to bystander killing of neighboring cells including perhaps HIV specific CTLs (97). Presumably, the infected cell itself would be protected from *cis* activation of Fas by Ask1 inhibition. Thus, Nef dependent replication enhancement could be envisioned to involve FasL mediated killing of virus specific CTL while protecting the infected cell from Fas mediated apoptosis. However, it is difficult to make such conclusions with the limited existing evidence. Moreover, Nef also may enhance the susceptibility of cells to apoptosis in response to a number of pro-apoptotic

stimuli that, unlike Fas, induce apoptosis via a mitochondria dependent pathway (98). Consistent with this observation, Nef expressing CEM T cells have been shown to down-regulate bcl-2 expression (98). However, this function of Nef has recently been challenged by the observation that SIV nef up-regulates bcl-2 expression in Jurkat cells (99). Moreover, a benefit to virus replication provided by increasing the infected cells susceptibility to apoptosis is hard to envision.

Infectivity enhancement. Single round infectivity assays consistently show a Nef dependent increase in productive target cell infection (7;100). Why infectivity enhancement does not translate into enhanced viral replication in all systems in not clear. Nonetheless, infectivity enhancement is observed with nearly all Nef isolates tested, with the magnitude being Nef allele dependent (68;69). The effect is dependent upon Nef expression in the virus producer cell and can be elicited by *trans* complementation of *nef* defective provirus (100;101). At least two lines of evidence suggest that Nef enhances the efficiency of an early event in the viral life cycle. First, Nef may increase proviral DNA synthesis without effecting the activity of Reverse Transcriptase (102-104). Secondly, enhancement of infectivity has been shown to be dependent on the mode of viral entry. Productive HIV infection is thought to occur via direct fusion of the virus and plasma membranes followed by cytoplasmic delivery of viral content. Nef enhances infectivity of HIV particles pseudotyped with various viral envelope proteins that fuse at neutral pH (102;105). However, HIV pseudotyped with viral envelope proteins that mediate entry via the endocytic pathway show no Nef dependent enhancement of infectivity (105-107).

The precise mechanism of infectivity enhancement is poorly understood. Initial reports indicated that Nef has no effect on viral entry (100;103). However, more recent work challenges this view. Schaeffer et. al. showed that the majority of viral particles enter CD4 expressing Hela cells through an endocytic route that presumably results in non-productive infection. They confirm that Nef does not appreciably change total virus entry, but suggest that it increases cytoplasmic delivery of viral particles (108). Thus Nef could enhance fusion of HIV/SIV at the plasma membrane or perhaps in early endosomes. Nef may exert its positive effects on infectivity via modification of envelope composition during viral morphogenesis (109). Consistent with this view, Nef has been suggested to target viral budding to lipid rafts (110). However, some of these data argue against the hypothesis that Nef enhances fusion with the target cell. Indirect evidence indicates that Nef does not enhance fusion of viral particles with the HIV envelope to other particles displaying CD4 and co-receptor (109). The effect of Nef on viral fusion at the more dynamic plasma membrane, however, is more difficult to assess and is uncertain. The cellular mediators of infectivity enhancement are also almost completely unknown, although regions in the C-terminus of Nef important for CD4 down-modulation have been implicated in infectivity enhancement as well (Fig. 2) (58;111). This region of Nef is thought to connect Nef to the host trafficking machinery (59;63;64). Current data, thus, best supports a model in which Nef enhances infectivity by regulating trafficking of components of the viral envelope in the producer cell. Nef dependent modification of virus envelope components could enhance fusion of virus to the target cell or facilitate an early, post-fusion event.

Regulation of Nef function: Although essential to our understanding of Nef function, much remains to be learned about the regulation of Nef and functional interactions that Nef makes with cellular proteins. Nef is a 27-34 kDa myristolated phosphoprotein with the exact size varying with Nef isolate. Myristolation has been shown to be important for most of Nef's reported cellular functions (7;47;112). Biochemical fractionation studies have demonstrated that myristolation leads to the association of Nef with cell membranes and cytoskeleton (113-115). Phosphorylation of Nef enhances CD4 down-modulation (116); however, the role of Nef phosphorylation in other functions is not known. Mutations in the Nef protein that effect only a subset of Nef's known functions suggest the existence of multiple functional domains in the protein. In addition to putative functional domains already described, a notable motif is a highly conserved proline rich region, $P(XXP)_{3,}$ in HIV Nef (corresponds to residues 71-80 in Nef D. Con, Fig. 2) and $(XXP)_3$ in SIV Nef. The first proline in HIV Nef lacks an analogous counterpart in SIV Nef. In vitro, the polyproline region has been shown to be a strong ligand for SH3 domains (117). Structural analysis suggests that only the central prolines of the region in HIV Nef are important for this interaction (118). Mutation of the central prolines has also been reported to impair infectivity enhancement and MHC class I down-modulation (54;80;101). However, others have challenged this view attributing the defects to impaired Nef stability (119). Moreover, mutation of corresponding prolines in SIV Nef has no effect on these functions. Thus, the importance of the proline region to specific cellular functions of Nef is not known.

Over 20 cellular Nef binding partners have been suggested (68). In some cases, Nef binding partners have been implicated in Nef induced cellular phenotypes. However,

data demonstrating a functional interaction between Nef and putative binding partners at endogenous expression levels in cells is almost entirely absent. One exception is with the myeloid lineage specific tyrosine kinase, Hck. Co-expression of Nef and Hck in Rat-2 fibroblasts leads to cellular transformation (120). Nef, moreover, tightly binds the Hck SH3 domain in vitro and activates Hck (121). Thus, Nef may directly activate Hck in cells. Nef has also recently been shown to modestly mediate activation of endogenous Hck and, in turn, the Stat3 transcription factor (122). Interestingly, Hck activation is the only cellular activity of Nef known to not require Nef myristolation (122). As none of Nef's other functions have been shown to be myristolation independent or myeloid specific, Hck activation does not account for any of Nef's known cellular phenotypes.

The Nef associated kinase (Nak): The most well documented in vivo Nef binding partner is Nak which was first described by Sawai et. al. and subsequently observed by our laboratory and numerous others (123-125). Nak is detected by incubating cell extracts from Nef (HIV or SIV) producing cells with anti-Nef serum, adsorbing the immunocomplex to Protein A beads, and incubating the washed beads with □[^{32}P]ATP and either Mg++ or Mn++. Phosphate is incorporated into multiple proteins with the predominate species being 62,000 daltons. This protein became known as Nak. The incorporated phosphate has been determined to be on serine residues. The strength and specificity of the Nef/Nak interaction is indicated by the fact that washing the immunoadsorbed Nef/Nak complex with 1 M $MgCl_2$ does not disrupt the association. The Nef/Nak complex is observed with transfection protocols which produce high levels of Nef expression and in retrovirally transduced cell lines that produce relatively low

levels of Nef. Moreover, Nak appears to have very wide tissue distribution since expressing Nef in many different cell lines produces the complex.

Nak controversies: Early work suggested that Nak may belong to the p21 activated kinase (Pak) family of cellular kinases (Fig. 3) (125). This conclusion was based on limited immuno-crossreactivity of Nak with anti-Pak antibodies and increased Nak phosphorylation when Nef was co-expressed with active forms of Cdc42 and Rac1, endogenous activators of Pak kinases. However, the exact identity of Nak was not proposed until Renkema et. al. identified Nak as Pak2 (126). These investigators co-transfected 293T cells with Nef NL4-3 and $CDC42_{V12}$, a constitutively active CDC42 mutant. They then performed in vitro kinase assays on Nef immunoprecipitates and identified Nak as Pak2 by 're-ip' experiments following Nak assays using non-specific antibody to Pak2 and by proteolytic mapping. These investigators were unable to detect Nak phosphorylation in the absence of co-transfected $CDC42_{V12}$, making it unclear how their system compared to that of ours and others who observe robust Nak activity with Nef expression alone. Following Renkema's report, Fackler et. al. used a CD8-Nef_{SF2} chimera expressed in Jurkat T cells to identify Nak as Pak1 (127). Their conclusions were based on experiments done with either an antibody or a peptide inhibitor suggested to be specific for Pak1. These conflicting reports led to the suggestion that Nak may represent distinct cellular kinases, with the exact identity depending upon the Nef allele expressed or the cell type used (127). The role of Nef in mediating Nak activation has also remained contentious. As active Pak kinases autophosphorylate in vitro, Nef was hypothesized to associate with an active Pak. While some argued that Nef mediates Nak

activation (128;129), others suggested that Nef preferentially binds to already active Nak, but does not mediate Nak activation (130).

The function of Nak: The importance of Nak association is suggested by its specificity and conservation amongst Nef isolates of different species. However, no Nef induced cellular phenotypes have been conclusively attributed to Nak association. The potential role of Nak in mediating Nef functions in light of recent data from our laboratory is considered in Discussion. When the work presented here was started, our laboratory had reported that a primary isolate of HIV Nef, Nef $_{233}$, is defective for Nak association, but functional for CD4 down-modulation and infectivity enhancement (69;124). Others had shown that mutation of critical determinants of the Nef SH3 binding surface also abolish Nak association (Fig. 2) (131). While mutations in the central prolines of the HIV polyproline tract are also defective for MHC class I down-modulation and infectivity enhancement, these mutations also destabilize Nef (119). Other SH3 binding surface mutations that do not appear to lower steady state levels of Nef expression also ablate Nak association, but have not been tested for the other functions (131). Interestingly, mutation of the first proline of the HIV Nef polyproline tract has no apparent effect of steady state Nef levels, but ablates Nak association (131). This residue is not important for SH3 binding in vitro and has no SIV counterpart. Manninen et. al. who reported Nef mediates NFAT activity showed a correlation in the ability of various Nef isolates or mutants to associate with Nak and activate NFAT, raising the possibility that Pak2 may play a role in Nef's effects on T cell activation (93). However, whether NFAT lies

downstream of Nak has not been determined. Thus no clear association exists between Nak and Nef induced cellular phenotypes. The work presented here was based on the premise that the identification of Nak is essential for understanding Nak's role in Nef function at the cellular level and eventually in vivo. Moreover, it is hoped that a better understanding of the relationship between Nef and Nak will provide insight into the biochemical processes initiated by Nef, paving the way for the identification of Nef initiated signaling cascades and, therefore, potential therapeutic targets.

Overview of work: Herein, I investigate the identity of Nak as well as the biochemical relationship between Nef and Nak. Moreover, I begin to address the biochemical and cellular consequences of Nef's interaction with Nak. In chapter 3, I show that Nak is the p21 activated kinase 2 (Pak2). In chapter 4 , I extend upon this observation and show that Nef mediates the activation of Pak2 in cells. In chapters 5 and 6, I address the mechanism of Nef mediated Pak2 activation and suggest that Nef recruits Pak2 to cellular membranes were it encounters active forms of Pak activating GTPases (e.g. Rac or CDC42). In chapter 7, I show that Nef expression leads to phosphorylation of a newly identified Pak2 substrate, Merlin. Lastly, in chapter 8, I consider the possible in vivo significance of the phenomena I describe in light of our laboratories recent data and the literature.

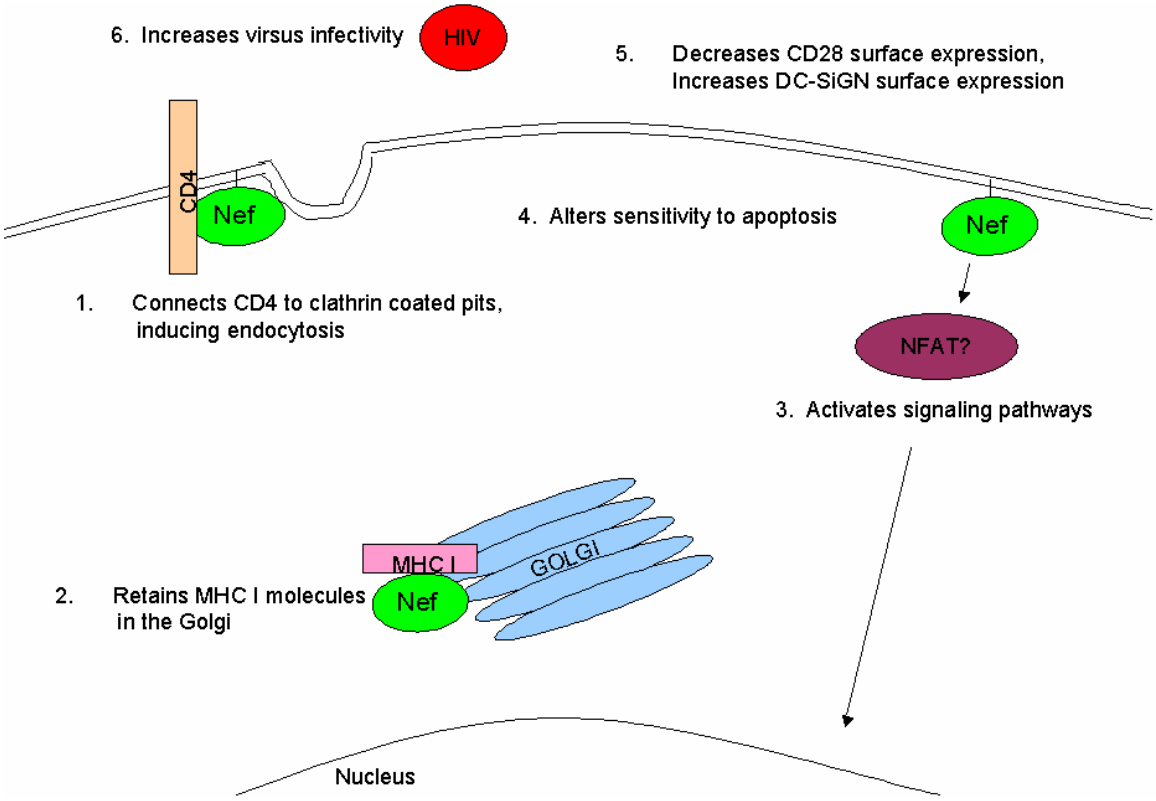
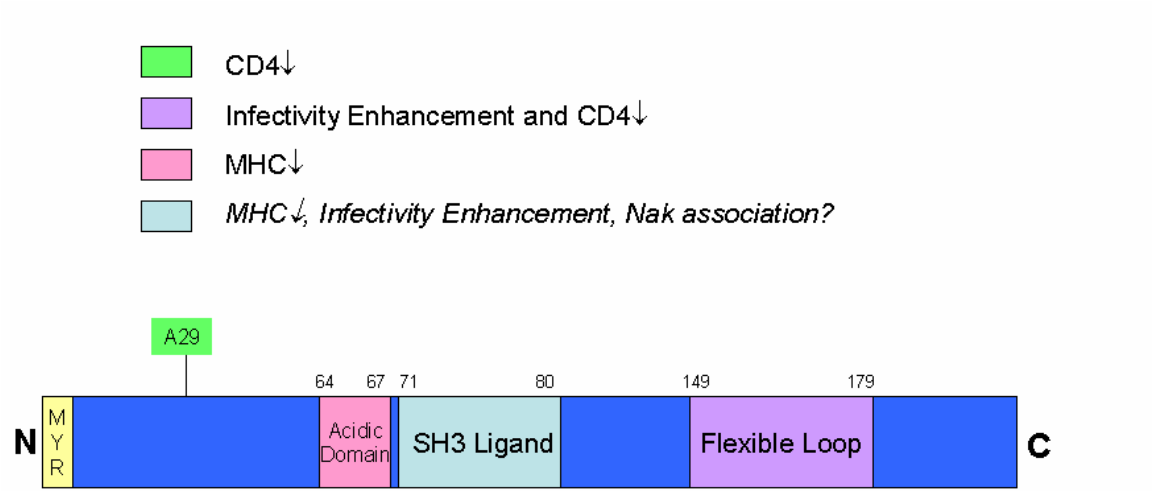


Figure 1: Multiple Functions of Nef. Multiple cellular and viral functions of Nef have been reported (1-6) and are discussed in detail in text.

A



B

1 16 31
MGGKWSKSSIVGWPA VRERMRRARAEPAAD GVGAASRDLEKHGAI

46 61 76
TNSNTAANNADCAWL EAQEEEEVGFPVRPQ VPLRPMTYKGALDLS

91 106 121
HFLKEKGGLEGLIYS QKRQDILDLWVYHTQ GYFPDWQNYTPGPGT

136 151 166
RYPLTFGWCFKLVPV EPEKVEEANAGENNC LLHPMSLHGMDDPEK

181 196
EVLQWKFDSRLAFHH MARELHPEYYKDC

Figure 2: Functional Domains of Nef. Cartoon representation (A) and sequence (B) of $Nef_{D.Con}$, a consensus Nef sequence created in our laboratory (64).

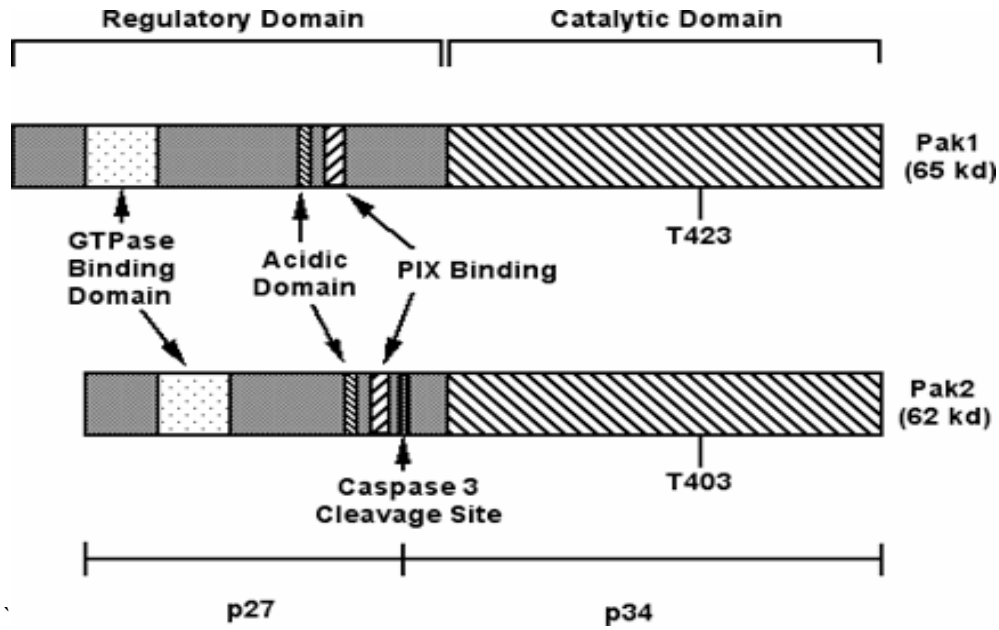


Figure 3: Diagrammatic representation of human Pak1 and Pak2. The two highly homologous kinases contain a N-terminal regulatory region and a C-terminal catalytic domain. Threonine phosphorylation of either residue 423 in Pak1 or 403 in Pak2 activates the kinases and primes them for autophosphorylation *in vitro*. Of particular interest for this work is the Caspase 3 cleavage site found in Pak2, but not in Pak1. Cleavage at this site generates a 27 kDa N-terminal fragment and a 34 kDa C-terminal fragment. Serine phosphorylation of the 27 kDa N-terminal fragment increases its apparent molecular weight to 32 kDa {781}. Also important for this work are the p21 GTPase binding domain and the PIX binding domain also indicated.

Chapter 2: Materials and Methods

Cell culture conditions. 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies) supplemented with 10% fetal bovine serum, 50 IU penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Human HuT-78, CEM, and Jurkat T-cells as well as U937 and THP-1 human monocytic cells were cultured in RPMI 1640 medium supplemented with either 10% heat inactivated (Hut 78) or non-heat inactivated (CEM, Jurkat, THP-1, and U937) fetal bovine serum (Hyclone, Logan, UT), 50 IU penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cell lines were maintained at 37 ^{o}C in a humidified incubator with 5% $C0_2$.

DNA Plasmids. Plasmid DNA constructs expressing Pak1 and Pak2 HA-tagged at their amino terminus were kindly provided by J. Chernoff (Fox Chase Cancer Center; Philadelphia, PA) (132) and were used to subclone both *paks* into pCDNA1/AMP (Invitrogen, Carlsbad CA). Mutagenesis of *pak2* was carried out using the QuickChange kit (Stratagene; La Jolla, CA) according to the manufacturers protocol. The *pak2* $83,86_{HH\text{-}LL}$ mutant was generated by first subcloning the BamHI/EcoRI fragment into pUG cloning vector which was then used as a template. Reactions were carried out using the following primers: 5'CATCTGATTTTGAGCTCACCATCCTTGTTGGCTTTGATGC3' and 3'GCATCAAAGCCAACAAGGATGGTGAGCTCAAAATCAGATG5'. Mutations were confirmed by sequencing and the BamHI/EcoRI fragment was cloned into pCDNA1HAPak2.

Nef plasmids were generated as follows. $Nef_{D.Con}$ was constructed by Dr. John Foster as a combination of $nef_{D85\text{-}11E160A}$ and $nef_{D88\text{-}11}$ obtained from David Ho (68;133). Both of these primary isolate *nef* clones contain a second XhoI site 5' to the site in Nef_{SF2}. This second site was mutated in $nef_{D.Con}$. $nef_{D.Con}$ as well as the other *nefs* were cloned into pLXSN (30) for the generation of transduced CEM cells. Nef_{SF2}, $Nef_{NL4\text{-}3}$, $Nef_{D90\text{-}7}$, $Nef_{D88\text{-}11}$, and SIV Nef_{mac239} alleles were also cloned into pcDNA1/AMP.

Myc-tagged $CDC42_{G12V}$ cloned into pCMV6 was kindly provided by M. Cobb (UTSW; Dallas, TX) (134). pcDNA3CDC42Q61LHA and pCGN-HA-RacQ61L were kindly provided by J. Chernoff (149). pGEXRhoGDIα was kindly provided by P. Sternweiss (UTSW; Dallas, TX, unpublished). RhoGDIα was subcloned into pCDNAI/AMP as a BamHI/EcoRI fragment downstream of an HA tag using the BamHI/EcoRI generated backbone of pCDNAI/AMP HA-Pak1. pCDNAMerlin and pCDNAMerlinA518 were kindly provided by J. Testa (Fox Chase Cancer Center; Philadelphia, PA) (149). pN1GFP (Clonetech) was kindly provided by Deepak Nijhawan (UTSW; Dallas, TX).

Transfection experiments: 293T cells were transfected using the calcium phosphate method (Gibco Life Technologies; Rockville, MD). 6 well plates were seeded with 300,000-500,000 cells the day before transfection. On the day of transfection, a total of 10 μg of DNA was adjusted to 150 μl using water. 21 μl of 2 M $CaCl_2$ were then added to each reaction tube. Precipitation reactions were carried out with the dropwise addition of 150 μl 2X Hepes Phosphate Buffered Saline (pH 7.05-7.12) while vortexing. After 25 minutes, precipitates were added to cells. 16-24 hrs later, media was replaced with

complete (Figs. 2,3, and 6-8) or serum free DMEM (Figs. 14-16 and 21-22). Cells were harvested for analysis 36-40 hrs post transfection.

Transduction experiments: Cells were transduced to express only the neomycin phosphotransferase gene (neo^r) or Nef and neo^r using a murine retroviral transduction system (135). Nef isolates were cloned into the retrovirus vector pLXSN as EcoRI fragments as previously described (30). LXSN and pEQPAM plasmids were co-transfected into 293T cells by the calcium phosphate method to transiently generate amphotropic vectors. At 36 to 48 h after transfection, vector-containing media were collected for transduction of cells. 24 well plates were coated with 200 ml Retronectin (5 μg/ml for 2 hrs; Takara Bio Inc.), followed by 500 μl 2% BSA in phosphate buffered saline (PBS; Gibco Life Technologies; Rockville, MD) for 30 minute at RT. Wells were then rinsed with serum free RPMI and incubated with 500 μl viral supernatants twice for 1 hr at 37˚ C. Target cells (300,000) in complete RPMI were then added to each well. The next morning an additional 500 μl of viral supernatant was added to each well. 24 hr later, cells were pelleted and resuspended in cRPMI with 1.5 mg/ml G418 for selection.

Production and purification of recombinant Caspase 3. Bacteria expressing $histidine_{(6)}$-tagged Caspase 3 were generously provided by Lilly Lee(136). Bacterial cultures were grown at 37˚ C to an optical density (A_{600}) of 0.6. Isopropyl-1-thio-beta-D-galactopyranoside was then added to a final concentration of 2 mM. After a 2 hr induction, bacteria were pelleted and lysed by sonication in buffer A (20 mM Hepes-KOH pH7.4, 10 mM KCl, 1.0 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM

dithiothreitol (DTT), and 0.1 mM PMSF). After centrifugation, supernatants were loaded onto a 2 ml nickel-Sepharose column (Qiagen; Los Angeles, CA) equilibrated with buffer A. The column was then washed with 10 ml of buffer A, 10 ml of buffer A containing 1 M NaCl, and then rinsed with 10 ml of buffer A. Caspase 3 was then eluted (1 ml fractions) with buffer A containing 250 mM imidazole. Relative activity of fractions was determined using a colorometric substrate (Caspase 3 Substrate I Calbiochem; La Jolla, CA).

Western blot analysis. HIV and SIV Nef expression was determined with sheep polyclonal anti-HIV or anti-SIV Nef serum (1:4000 and 1:2000 dilution, respectively), followed by horseradish peroxidase (HRP) conjugated anti-sheep IgG (1:20,000; Chemicon Int; Temecula, CA). Mouse anti-HA tag (Roche; Indianapolis, IN) monoclonal antibodies were used to detect Paks, Merlin, $CDC42_{Q61L}$, and Rac_{Q61L}. Mouse anti-myc tag (Invitrogen; Carlsbad, CA) was used to detect $CDC42_{G12V}$ expression. Nef Western blot analysis following immunoprecipitation with sheep polyclonal antibodies were performed using EH1 mouse monoclonal anti-Nef antibody (1:2500; kindly provided by J. Hoxie (UPenn; Philadelphia, PA). Mouse primary antibodies were detected by HRP conjugated anti-mouse IgG (1:5,000-10,000; Zymed; South San Francisco, CA). HRP conjugates were visualized using enhanced chemiluminescence (Amersham; Quebec, Canada).

***In vitro* kinase assay.** The assay for the cellular kinase activity associated with Nef was performed essentially as described by Sawai et. al. (123) with the addition of a 1 M

$MgCl_2$ wash prior to the kinase assay (124). Cell extracts were made with kinase assay lysis buffer (50 mM Tris 8.0, 10% Glycerol, .5% IGEPAL CA-630 (Sigma; St. Louis, MO), 1mM EDTA, 100 mM NaCl, 2 mM $NaVO_4$, 25 mM NaF, 1 mM phenyl methyl sulfonyl fluoride (PMSF; Sigma), 20 mM β̃glycerophosphate, 25 mM Benzamidine, protease inhibitor cocktail (Roche; Indianapolis, IN)). Protein determinations of lysates were performed using Bio-Rad protein assay (Bio-Rad; Hercules, CA). Immunoprecipitations were carried out for 3-16 hrs using anti-Nef (5 μl sheep polyclonal per 300 μg protein lysate) or anti-HA (1.6 μg mouse monoclonal per 300 μg protein lysate) antibodies as indicated in the text. 40 μl of a 1:1 Protein A slurry were used per 5 μl Nef antibody or 1.6 μg HA antibody. Precipitates were washed twice with kinase assay lysis buffer, once with 1 M $MgCl_2$, and twice with kinase buffer (50 mM Tris 8.0, 5 mM $MgCl_2$, 100 mM NaCl, and 1% Triton X – 100 (Sigma)). Protein A beads were then resuspended in 50 μl kinase buffer per 300 μg lysate. Kinase reactions were carried out for 10 min at room temperature (RT) with the addition of 3 μl $\gamma[32^P]$ATP (NEN, 3000 Cu/mmol). Reactions were stopped by addition of EDTA to a final concentration of 33 mM. All lanes represent immunoprecipitates from 250-300 μg of cell lysate unless otherwise indicated.

Caspase 3 treatment of in vitro kinase assay: Nine hundred μg of Nef containing lysates were immunoprecipitated. Following the kinase assay, reactions were then placed on ice and the Protein A beads washed twice with ice cold Caspase 3 buffer (50 mM Hepes pH 7.5, 100 mM sodium chloride, 0.1% Triton X-100, 5 mM DTT, 20 mM sodium fluoride, 2 mM sodium vanadate, 20 mM β-glycerophosphate). During the last wash, the

samples were divided into three aliquots. The immunoprecipitates were then mock treated, treated with Caspase 3, or treated with Caspase 3 plus ZVAD (40 μm; Calbiochem), a Caspase 3 inhibitor, and incubated for 30 min at 37˚ C. Reactions were stopped by the addition of 1.5X Laemmli protein loading buffer, and the proteins were resolved by sodium dodecyl sulfate poylacrylamide gel electrophoresis (10% SDS-PAGE). Dried gels were then exposed to a phosphorimager screen (Packard; Meriden, CT) and/or to film (Kodak).

Immunodepletion experiments. Nef/Pak2 cell lysates (250 μg) were first immunoprecipitated with 5 μl sheep anti-Nef or 1.6 μg mouse anti-HA antibodies as described above. Protein A beads were then pelleted and supernatants removed. A second immunoprecipitation with the same antibody (5 μl anti-Nef or 1.6 μg anti-HA) was then performed to ensure complete depletion of the immunoprecipitated protein. A third immunoprecipitation was then carried out using the complementary antibody. All immunoprecipitations were carried out for 4-12 hours at 4^0C and kept on ice during subsequent serial immunoprecipitations. Samples were then washed for kinase assay. During the last wash step, one-fifth of the protein A was removed from each sample and pelleted separately for direct elution into Laemmli protein loading buffer and subsequent Western blot analysis.

Cellular Fractionation: CEM cells (20 million) were washed once in PBS and resuspended in 1 ml hypotonic buffer (10mM Tris 8.0, 1 mM $MgCl_2$, 0.5mM Beta-mercaptoethanol (BME), 2 mM Sodium Orthovanadate, 2.5 mM Sodium Fluoride, 10

mM beta-glycerophosphate). After 20 minutes, cells were passed 25 times through a 25 gauge needle with a 1 ml syringe. Nuclei and unbroken cells were pelleted at 1000*g* for 5 min (Ependorf 5417R). The supernatant was removed and centrifuged at 20,000*g* for 30 min (Ependorf 5417R). The 20,000*g* pellet was designated membrane fraction and the 20,000*g* supernatant was designated the cytosol. The membrane pellet was then resuspended in either hypotonic buffer or kinase assay lysis buffer for subsequent protein determination and analysis.

In gel kinase assay: Fractionated protein lysates from either control or Nef expressing CEM cells were heated to 95˚ C for 5 min in Laemmli buffer. For experiments using cells fractionated into membrane and cytosolic fractions, 30 μg of protein were used for each sample. For gradient experiments, 8 μl of each fraction were used for each sample. Samples were then loaded onto 10% polyacrylamide minigels that were polymerized in the presence of a total of 2.5 mg of Histone H4 (Fluka; Ronkonkoma, NY) or Myelin Basic Protein (MBP) (Sigma; St. Louis, MO). Proteins were resolved at 100-120V. Gels were then incubated at RT twice for 1 hr in 20% isopropanol fix (20% Isopropanol, 50 mM Tris pH 8.0, 5 mM BME) followed by 1 hr at RT in equilibration buffer (50mM Tris pH 8.0, 5mM β-mercaptoethanol). Fixed proteins were then denatured by incubating gels twice for 1hr at RT in denaturation buffer (50 mM Tris pH 8.0, 7 M Guanidinium HCl, 5 mM β-mercaptoethanol). Gels were then incubated in renaturation buffer (50 mM Tris pH 8.0, 5 mM β-mercaptoethanol, .04% Tween-20) for a total of 19-21 hrs at 4˚ C with five buffer changes at the following time intervals: 5 min, 2 hr, 13-15 hr, 2 hr, and 2hr. Gels were then incubated at RT in kinase buffer (50 mM Tris pH 7.5 and 5 mM

βmercaptoethanol) first for 10 min followed by 1 hr in fresh kinase buffer. Kinase reactions were then performed for 1 hr at RT in kinase buffer with the addition of 5 mM $MgCl_2$ and 50 μM ATP containing 300 μCuries of $\gamma P^{32}ATP$ (NEN, 3000 Cu/mmol). After the kinase reaction, gels were incubated for ten minutes in fresh kinase buffer (without ATP) and then in background reduction buffer (5% TCA, 1% Sodium Pyrophosphate, 1 μM ATP) for a total of 16-22 hr with 5 buffer changes at the following time intervals: 1 hr, 1 hr, 10-16 hr, 2hr, and 2 hr. Gels were then rinsed twice in water, dried, and signal was visualized by autoradiography and/or by phosphorimager.

Caspase 3 treatments for in gel kinase assay: Membrane pellets were resuspended in modified kinase assay lysis buffer (same as lysis buffer, but with 400 mM NaCl, 50 mM Tris pH 7.5 instead of Tris pH 8.0, and 5 mM DTT). Protein (30 μg) was either mock treated, treated with 25 units recombinant Caspase 3, or with 25 units of recombinant Caspase 3 in the presence of 40 μM zVAD (Calbiochem; San Diego, CA) at 37˚C for 30 minutes. Reactions were stopped by the addition of 5X Laemmli loading buffer and then heated to 95˚ C. Samples were then loaded onto a 10% polyacrylamide gel for in gel kinase assay as indicated above.

FACS: Cells (500,000) were pelleted at 1500 RPM in a Jouan CR422 centrifuge, washed once with PBS, and fixed with 3% paraformaldehyde (Electron Microscopy Sciences; Ft. Washington, PA) in Ca++ and Mg++ free phosphate buffered saline (PBS; Gibco Life Technologies; Rockville, MD) for 20 minutes at RT. Fixation was quenched by washing the cells once in 4 ml 50 mM NH_4Cl in PBS. Cells were then permeablized

using permeabilization buffer (0.01% saponin, 0.25% gelatin, 0.02% NaN_3 in PBS) for 20-30 minutes at room temperature. For staining, cells were incubated with appropriate antibodies at 1:100 dilution or peptide at 100 μg/ml in permeabilization buffer. Between each staining step, cells were washed 2 times with 4 ml permeabilization buffer. For detection of active Rac/CDC42, cells were stained with GST-PBD (100 μg/ml), followed by 1:100 anti-GST antibody (Molecular Probes; Eugene, OR), followed by 1:100 CY5 conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA). Following the last wash step after staining with secondary antibody, cells were fixed with 1% PFA in PBS. 50,000 events were collected using a FACScan (Becton Dickinson; San Jose, CA) and analyzed with Cell Quest software. GST-PBD positive staining was determined relative to cells stained with GST peptide.

Indirect Intracellular Immunofluorescence Microscopy: For staining of suspension cells, cover slips were treated overnight at 4 degrees with Poly-D-Lysine (0.1 ml /ml; MW 30,000-70,000; Sigma; St. Louis, MO). Cover slips were washed once in PBS, and 200,000 cells were adhered to the coverslip for 10 minutes at 37˚ C. Adherent cells were washed once with PBS. Cells were fixed with 3% paraformaldehyde (Electron Microscopy Sciences; Ft. Washington, PA) in Ca++ and Mg++ free phosphate buffered saline (PBS; Gibco Life Technologies; Rockville, MD) for 20 minutes at RT. Fixation was quenched by washing cells three times (3 minutes each) with 50 mM NH_4Cl in PBS. Cells were then permeablized using permeabilization buffer (0.01% saponin, 0.25% gelatin, 0.02% NaN_3 in PBS) for 20-30 minutes at RT. For staining, cells were incubated with appropriate antibodies (all dilutions 1:100) or peptide at 100 μg/ml in

permeabilization buffer at RT. Between each staining step, cells were washed 5 times (3-5 minutes each) with permeabilization buffer. For Nef staining, cells were stained with anti-Nef mouse monoclonal antibodies (EH-1, J. Hoxie) followed by FITC conjugated anti-mouse antibody. For visualization of HA tagged proteins, cells were stained with mouse anti-HA antibody (Babco; Berkley, CA) followed by FITC conjugated anti-mouse antibody (Southern Biotechnology Associates Inc.; Birminigham, AL). For detection of active Rac/CDC42, cells were stained with GST-PBD, followed by anti-GST (Molecular Probes; Eugene, OR), followed by CY3 conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA). For double labeling experiments, staining with the two primary antibodies was performed simultaneously; after washing, staining with the two secondary antibodies was also done together. Coverslips were mounted in Mowiol and allowed to dry overnight before viewing with a Zeiss Axiovert 100M confocal microscope and Zeiss software Figures were generated by importing the images into Adobe Photoshop (Adobe Systems Incorporated; San Jose, CA). HA antibody was determined to be specific by the lack of staining in cells not expressing HA fusion proteins. The Nef antibody was determined to be specific by the lack of staining in non-Nef expressing cells.

Expression of GST-PBD peptide: A 10 ml overnight culture (LB 50 microgram/ml Ampicillin LB) was inoculated with pGEXGST-PBD transformants (gift from P. Sternweis). Starter cultures were expanded to 500 ml and grown for approx. 2.5 hrs to an $O.D._{600}$ of 1.00. Expression was then induced with 0.3mM IPTG and cultures were grown for 2.5 hrs at 30 degrees. Bacteria were pelleted at 5000 RPM using a Beckman

JA-20 rotor in a Beckman J2MC centrifuge, lysed in 20 ml of bacterial lysis buffer (50 mM Tris 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1% Triton X-100, protease inhibitor cocktail (Roche; Indianapolis, IN)), and sonicated 6 times for 20 seconds at two minute intervals at level 4 using the Sonic Dismembrator 550 from Fisher Scientific (Pittsburgh, PA). Lysates were then cleared by centrifugation at 12,000g (Beckman JA-20 rotor in a Beckman J2MC centrifuge) for 10 minutes at 4˚ C. Lysates were then incubated with 1 ml Glutathione immobilized on cross-linked 4% beaded agarose (Sigma; St. Louis, MO) (pre-washed in bacterial lysis buffer) for 1-3 hr. Beads were then washed 3 times in bacterial lysis buffer followed by 3 times in bacterial lysis buffer without detergent. For 'pull-down' experiments, beads were frozen in aliquots at –80^{o}C. For staining experiments, GST-PBD was eluted into 1 ml fractions with detergent free bacterial lysis buffer with 5 mM reduced glutathione. The first two 1 ml fractions were concentrated on a 2 ml Amicon 10 column to a final concentration of 4 μg/ml.

Isolation of low density membranes by floatation through a discontinuous sucrose gradient: Control or Nef expressing CEM cells (300 million) were homogenized in 3.6 ml isotonic homogenization buffer (10 mM Tris 7.5, 5 mM NaF, 2mM Na_3VO_4, 10 mM Beta-glycerophosphate, 2 mM EDTA, 0.25 M Sucrose, and protease inhibitor cocktail (Roche; Indianapolis IN)) by passing 25 times through a 25 gauge needle using a 5 ml syringe. Homogenate was adjusted to 1.4 M sucrose by the addition of 2 volumes of homogenization buffer containing 2M sucrose. The discontinuous sucrose gradient was then prepared consisting of a 1.6M sucrose layer (4.2 ml), a 1.4M sucrose layer (loading zone), a 1.2M sucrose layer (12.6 ml), and a 0.8M sucrose layer (6.3 ml). The gradient

was then centrifuged at 110,000*g* in a SW28 rotor using a Beckmen Optima LE-80-R ultracentrifuge at 4˚ C for 30 minutes. Fractions (1.5 ml) were collected starting at the top. Purified low density membranes were recovered at the 0.8M/1.2M interface (Fractions 3, 4 and 5). These fractions were determined by western blot analysis to be negative for the ER protein BIP78, the endosomal protein EEA1, or the mitochondrial protein Cyt C (not shown). In contrast, the fractions were positive for the Golgi Marker Syntaxin 6. We have not yet determined the location of plasma membrane on our gradient.

Calf intestinal phosphatase (CIP) treatments: Equal volumes of lysates from cells co-transfected with HA-Merlin and either control or Nef expressing plasmids were immunoprecipitated with 5 □l of anti-HA antibodies (Babco; Berkley, CA) followed by 40 μl of a 1:1 protein A slurry. Beads were washed 3 times in phosphatase buffer (100 mM NaCl, 1 mM DTT, 50 mM Tris 8.0, and 10 mM $MgCl_2$). During the last wash, beads were split in two and resuspended in 80 μl phosphatase buffer. Samples were then mock treated or treated with 10 Units CIP (New England Biolabs; Beverly, MA) for 10 minutes at 37˚ C. Reactions were stopped by the addition 20 μl of 5X Laemmli loading buffer.

Chapter 3: The identification of Nak as Pak2.

Overview: In this section, I address the hypothesis that the identity of Nak may vary based on Nef allele expressed or cell type used. I utilized a Caspase 3 cleavage site found in Pak2, but not other Paks, to distinguish Pak2 and Pak1 (126;137;138). I first confirmed that Pak2, but not Pak1 is cleaved by Caspase 3 in our *in vitro* system and then demonstrated that Nak is Caspase 3 sensitive, regardless of Nef allele or cell type used. I tested three lentivirus Nef alleles (SF2, NL4-3, and SIVmac239) and used multiple cell lines of myeloid, lymphoid, and non-hematopoietic origin to evaluate the identity of Nak. I also demonstrated that ectopically expressed Pak2 successfully substitutes for Nak, while ectopically expressed Pak1 does not.

Caspase 3 catalyzed cleavage serves as a diagnostic test to differentiate between Pak1 and Pak2. Despite the extensive similarity of Pak family members, only Pak2 has a consensus Caspase 3 cleavage site (between residues D212 and G213)(Fig. 3). Pak2 is cleaved by Caspase 3 into a N-terminal 27 kDa fragment that contains most of the kinase regulatory domain and a C-terminal 34 kDa fragment containing the catalytic domain (Fig. 3) (138;139). In order to confirm that under my experimental conditions Pak2, but not Pak1, is cleaved by Caspase 3 and that this specific cleavage event can be used as a diagnostic test to clearly differentiate the two proteins, the following experiment was performed. 293T cells were transiently transfected with either HA tagged Pak1 or Pak2 expression constructs and a Myc tagged $CDC42_{G12V}$ construct that expresses a constitutive activator of Paks (142). Cells were harvested 40 hr post transfection, Pak and $CDC42_{G12V}$ expression was verified by Western blot analysis (Fig. 4A), and *in vitro*

kinase assays were performed using anti-HA immunoprecipitates. Reactions were stopped with EDTA and beads were washed twice with Caspase 3 buffer. During the last wash, samples were divided into three aliquots and then either mock treated, treated with Caspase 3, or treated with Caspase 3 plus zVAD, a specific peptide inhibitor of Caspase 3 mediated proteolysis (139). As shown in Fig. 4B, both Pak1 and Pak2 were active in the presence of $CDC42_{G12V}$ and Pak1 migrated slower than Pak2 in agreement with their respective molecular weights. Caspase 3 only cleaved Pak2, producing the expected p27 N-terminal fragment. zVAD efficiently blocked the cleavage of Pak2, confirming the specificity of the protease activity. These results confirm that in our experimental system active Pak1 and Pak2 migrate with distinct electrophoretic mobilities and that sensitivity to Caspase 3 mediated cleavage clearly differentiates Pak1 and Pak2.

The identity of Nak as Pak2 is *nef* allele independent. Fackler et. al. hypothesized that different nef *alleles* could bind preferentially to Pak1 or Pak2 (127). To test this hypothesis, I performed transient transfections on 293T cells using the HIV *nef* alleles NL4-3 and SF2. I also transfected the SIV *nef* allele mac239, which is functional *in vivo* (5) and was not used by this group or by Renkema et. al. (126). As shown in Figure 5A, all three Nef proteins were expressed in 293T cells as determined by Western blot analysis. *In vitro* protein kinase assays were then performed on Nef immunoprecipitates from extracts of cells expressing the different Nef alleles. All three Nef proteins associated with Nak (Fig. 5B). Consistent with previous results, Nef_{NL4-3} associated with less Nak activity than Nef_{SF2} or $SNef_{mac239}$ (69). To distinguish whether the kinase autophosphorylation activity present in the immunoprecipitates corresponded to either

Pak1 or Pak2, we investigated its sensitivity to Caspase 3. In all three cases, the band corresponding to Nak was found to be susceptible to cleavage by Caspase 3 under conditions that failed to cleave Pak1 (Fig. 5B). The specificity of the Caspase 3 digestion of Nak was further confirmed by addition of zVAD. Addition of this inhibitor completely blocked the cleavage of Nak associated with SF2, NL4-3 and SIVmac239 Nef (Fig. 5B). In agreement with Renkema et. al. (126) these results demonstrate that in 293T cells, HIV-1 and SIV Nefs associate with a Caspase 3 sensitive kinase suggesting that in all three cases Nak is Pak2, and not Pak1.

The identity of Nef_{SF2} associated Nak is cell type independent. I also investigated the hypothesis that the identity of Nak may be cell type dependent (127). To address this question, I stably transduced three T cell lines (CEM, Hut78, and Jurkat) and two monocytic cell lines (U937 and THP-1) with LXSN expressing Nef_{SF2} (30), the Nef isolate originally used to identify Nak and more recently to determine that Nak is Pak1 (140). Expression of Nef_{SF2} in these cell lines was monitored by Western blot analysis (Fig. 6A and 7A). *In vitro* kinase assays confirmed that Nef associates with Nak in both T cells (Fig. 6B) and monocytic cells (Fig. 7B). To determine whether the active kinase bound to Nef was Pak1 or Pak2, I tested its sensitivity to Caspase 3 mediated proteolysis. The results show that Nak activity is susceptible to cleavage by Caspase 3 in both cell types and that cleavage of Nak by Caspase 3 is specifically inhibited by zVAD. Thus, in three different T cell lines and two different monocytic cell lines, the Nef_{SF2}-associated kinase activity is Pak2.

Ectopically expressed Pak2, but not Pak1, efficiently substitutes for Nak. In order to demonstrate directly that Nak is Pak1 or Pak2, 293T cells were co-transfected with expression constructs for Nef_{SF2} and either HA-tagged Pak1 or Pak2. Expression of Nef, HA-Pak1 and HA-Pak2 was confirmed by Western blot analysis (Fig. 8A and B). Cell extracts were immunoprecipitated with anti-Nef antibodies and in vitro kinase assays were performed. Immunoprecipitates from extracts of cells where Nef_{SF2} was expressed alone showed typical Nak activity (Fig. 8C, lane 2). Due to the presence of the HA tag which slightly increases the size of the ectopically expressed Pak1 and Pak2, a shift in mobility would be expected if ectopic Pak1 or Pak2 could efficiently substitute for Nak. Co-expression of tagged Pak1 with Nef_{SF2} did not produce a shift in the apparent mobility of Nak (Fig. 8C, lane 3). Moreover, it is noteworthy that ectopic Pak1 expression reproducibly inhibited endogenous Nak activity (Fig. 8C, lane 3). The significance of this will be considered in discussion. In contrast, *in vitro* kinase assays on extracts from cells co-expressing Pak2 and Nef_{SF2} consistently showed high levels of phosphorylation of a protein that migrated with a slightly higher mobility than endogenous Nak and that corresponded to the mobility of activated HA-tagged Pak2 (Fig. 8C, lane 4). These results indicate that only ectopically expressed Pak2 can efficiently substitute for endogenous Nak.

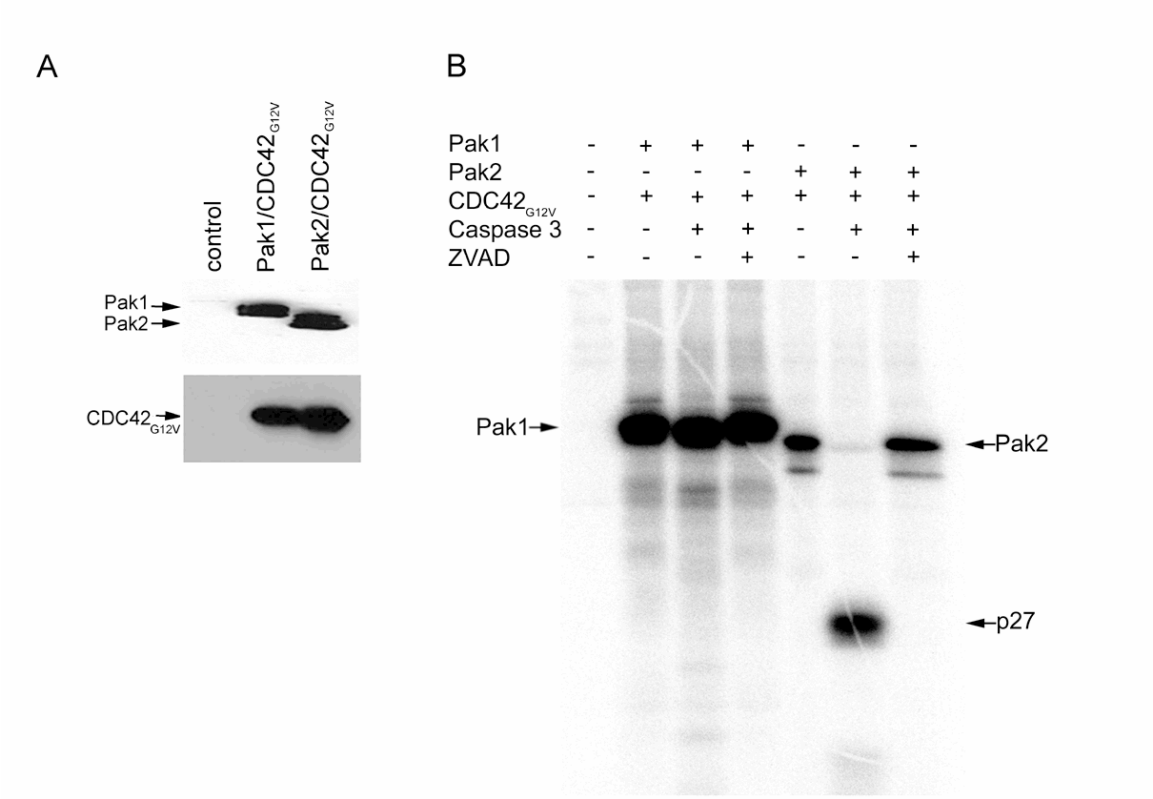


Figure 4: Caspase 3 cleaves Pak2, but not Pak1. A) Western blot analysis confirming appropriate expression of HA tagged Pak1 or Pak2 in 293T lysates (top) and the Pak activator CDC42G12V (bottom). CDC42G12V is myc tagged and was detected using a myc antibody. B) In vitro kinase assay performed using anti-HA immunoprecipitates of lysates used in A). Both Pak1 and Pak2 are active as evidenced by autophosphorylation activity. Note that treatment of Pak1 immunoprecipitates after in vitro kinase assay with Caspase 3 has no effect. In contrast, treatment of Pak2 immunoprecipitates with Caspase 3 following kinase assay generates a fragment (p27) consistent with the region N-terminal to the Caspase cleavage site. This fragment contains the serine residues autophosphorylated by active Pak2. Cleavage does not occur when treatments are done in the presence of ZVAD, a Caspase inhibitor. Also note that Pak1 has a distinctly slower apparent electrophoretic mobility than Pak2.

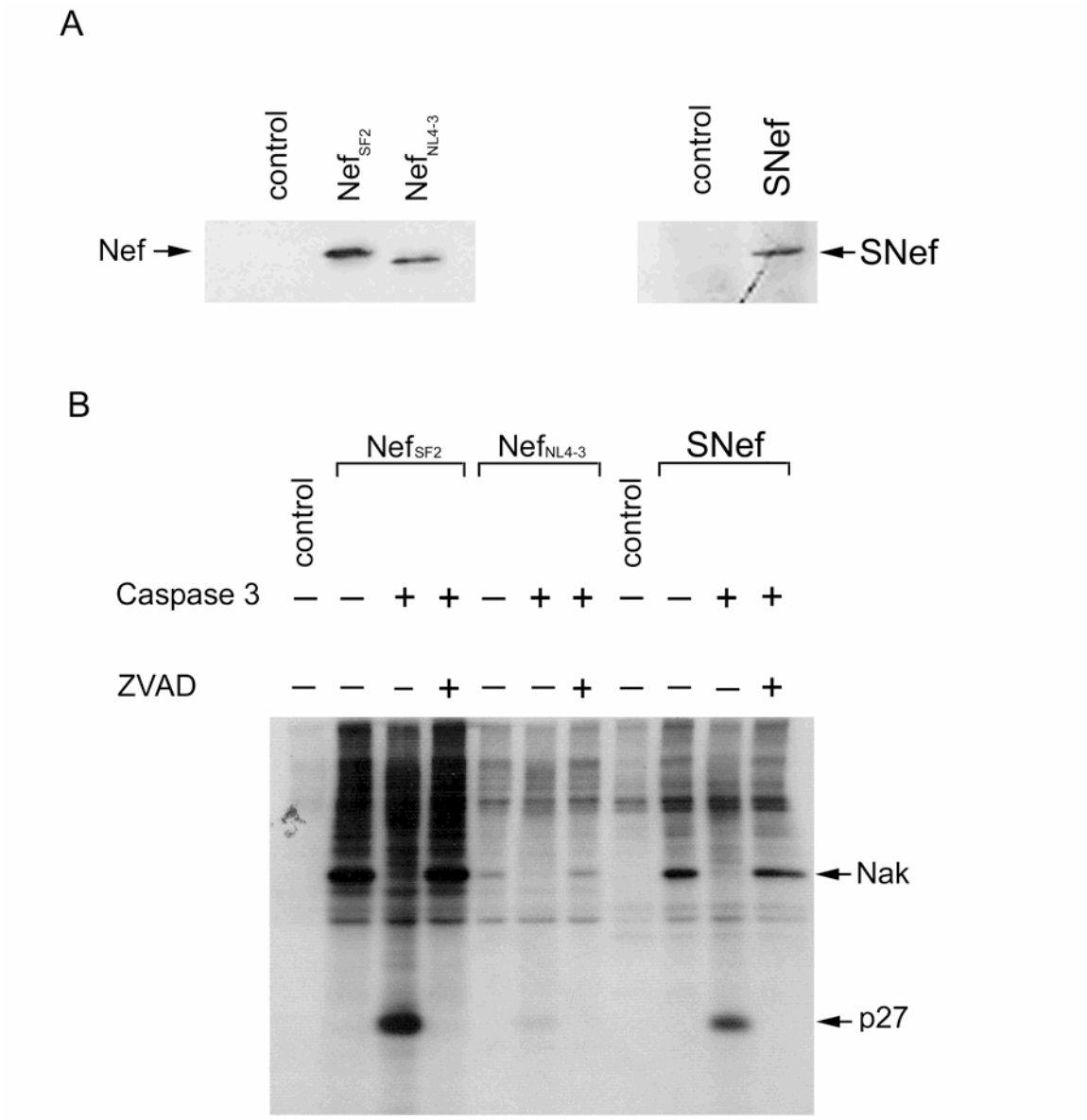


Figure 5: The Caspase 3 mediated cleavage of the Nef associated kinase is *nef* allele independent. A) Western blot analysis for HIV-1 and SIV Nef expression in lysates of 293T transfected cells. B) *In vitro* kinase assay and Caspase 3 treatments of lysates from (A). Note that all three Nefs associate with a Caspase 3 sensitive Nak.

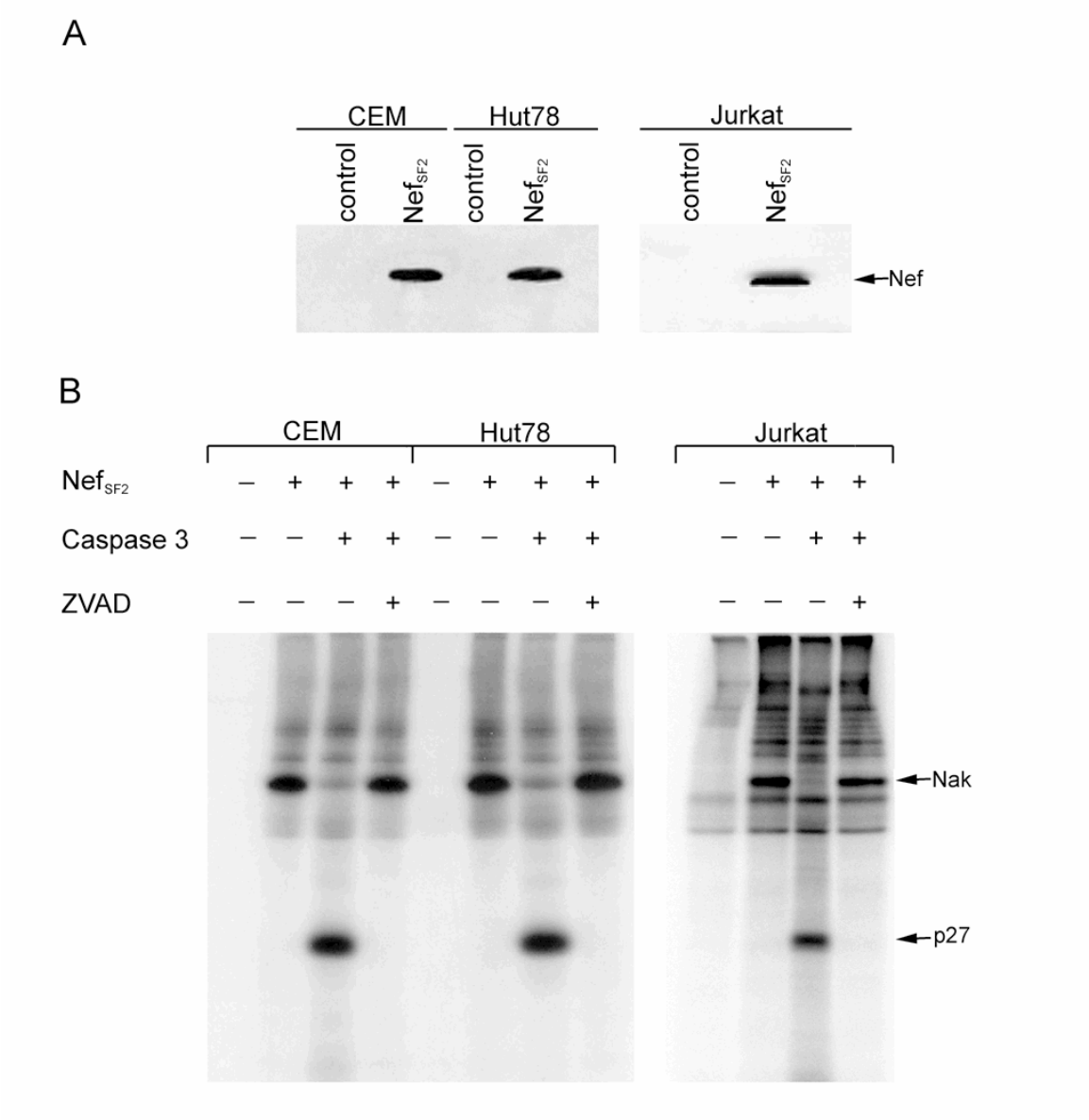


Figure 6: Nef binds Pak2 in human T cells. A) Western blot analysis for Nef_{SF2} expression in three different human T cell lines: CEM, Hut78 and Jurkat. B) *In vitro* kinase assay followed by Capase 3 cleavage of anti-Nef immunoprecipitates from cell lysates obtained from each cell line in (A). Note that in all three cases the Nak immunoprecipitated is sensitive to Caspase 3 treatment and that this cleavage was inhibited by ZVAD.

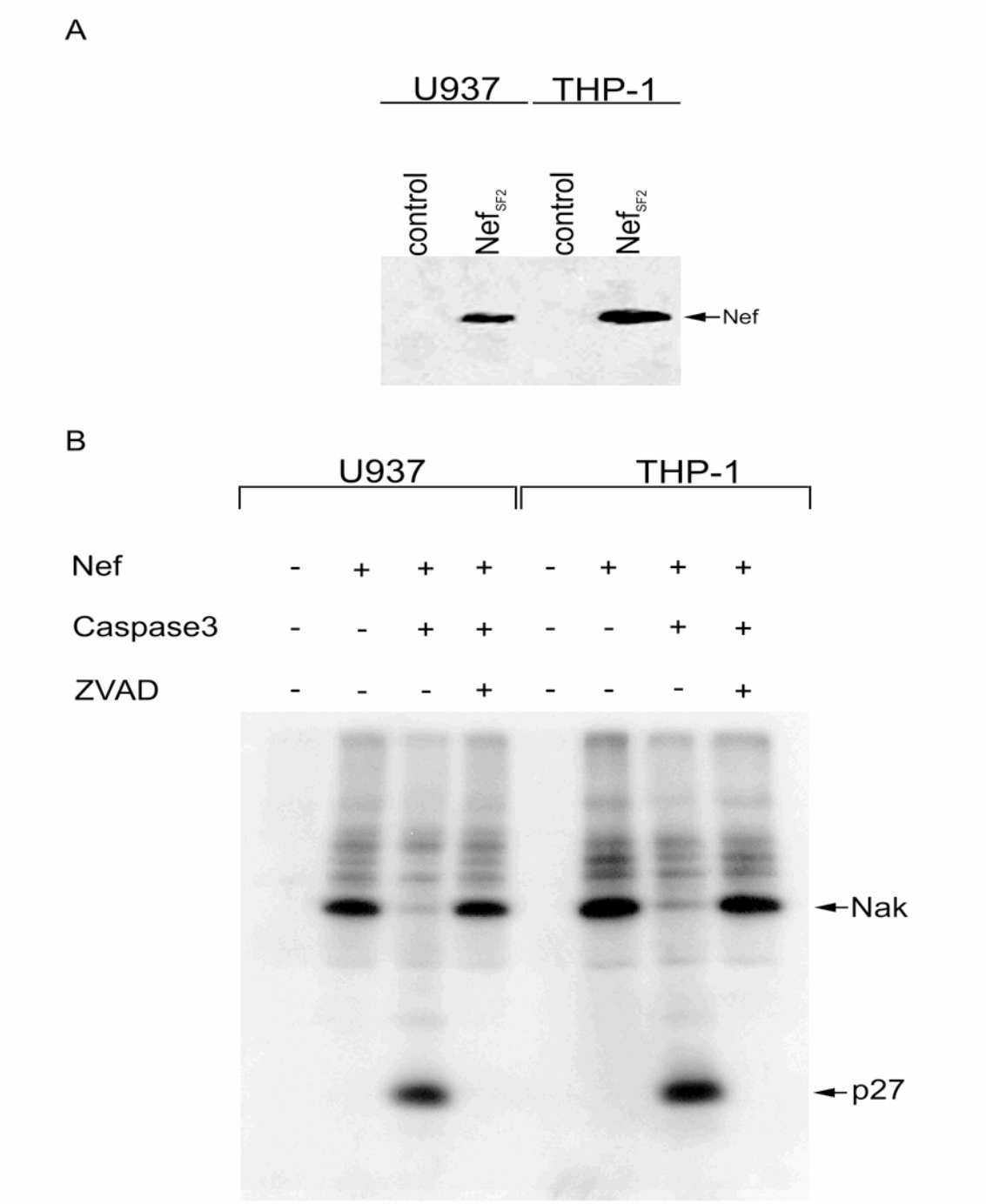


Figure 7: Nef binds to Pak2 in human monocytic cells. A) Western blot analysis for Nef_{SF2} expression in two different human monocytic cell lines: U937 and THP-1. B) *In vitro* kinase assay followed by Caspase 3 treatment of Nef immunoprecipitates from cell lysates obtained from each cell line. Nak was found to be Caspase 3 sensitive in both monocytic cell lines.

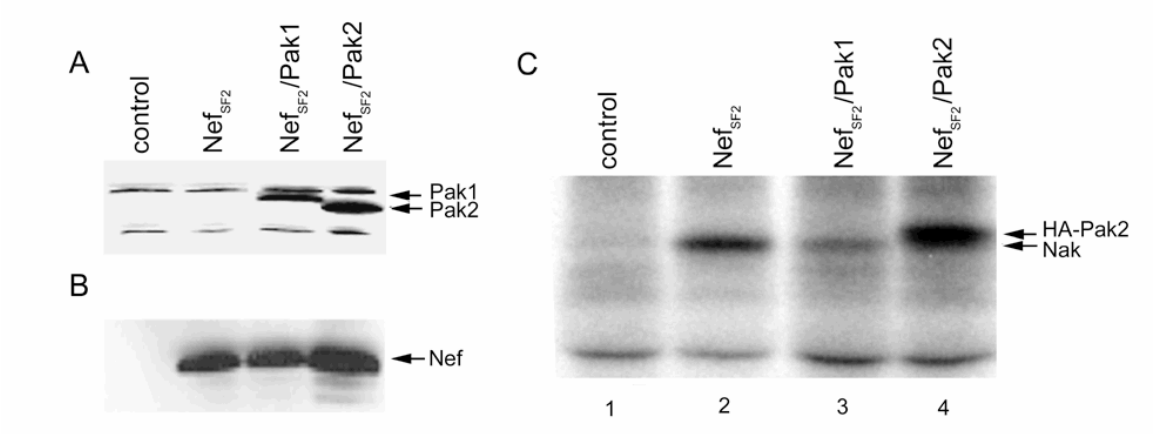


Figure 8: Ectopically expressed Pak2 substitutes for endogenous Nak. A) Western blot analysis with mouse anti-HA demonstrating expression of both Pak1 and Pak2 (top and bottom arrows respectively) in 293T transfected cells. B) Western blot analysis demonstrating Nef expression in the transfected cells from panel A. C) *In vitro* kinase assay of anti-Nef immunoprecipitates from the 293T cells transfected with Nef_{SF2} and either Pak1 or Pak2 in (A) and (B). The position of the phosphorylated HA-tagged Pak2 and endogenous Nak are indicated on the right in panel C (top and bottom arrows respectively).

Chapter 4: Nef mediates the activation of Pak2 on cellular membranes.

Overview: Phosphorylation of T403 of Pak2 renders the kinase 'active' and able to efficiently phosphorylate substrate (Fig. 3). Active Pak kinases also autophosphorylate both in vivo and in vitro. It is therefore likely that Nef associates with active Pak2 that autophosphorylates in in vitro kinase assays. At least two models could explain the presence of active Pak2 in Nef immunoprecipitates. One is that Nef forms a complex with already active Pak2. Another is that Nef mediates Pak2 activation in a Nef/Pak2 complex. To distinguish these two possibilities I assessed total cellular Pak2 activation levels in the presence or absence of Nef. In this section I demonstrate that Nef mediates Pak2 activation consistent with second model. I showed this by in vitro kinase assay using immunoprecipitates of ectopically expressed Pak2. I further showed using this system that, in cells, most of the Pak2 activated in the presence of Nef is found in a low abundance complex with Nef. I also analyzed endogenous Pak2 activation levels in T cells in the presence or absence of Nef. To do this, I utilized an in gel kinase assay protocol created by Kameshita and Fujisawa and modified by Dr. John Foster (141). This protocol allows for the assessment of total Pak activity in protein extracts. I performed the in gel kinase assay using cellular extracts fractionated into cytosolic and membrane components. I showed that Nef mediates the activation of endogenous Pak2. The Nef activated Pak2 co-fractionates with Nef to membranes. Interestingly, I also showed that the vast majority of Pak2 in the cell detectable by western blot localizes to the cytosolic fraction. Together, these data indicate that Nef induces the activation of a small Pak2

subpopulation that localizes to membranes. Most of the active Pak2, moreover, is in a complex with Nef.

Nef_{SF2} activates Pak2, but not Pak1. In order to resolve whether Nef mediates the activation of Pak2 or if Nef simply binds to an already active pool of Pak2, we first transfected cells with Pak expression constructs and either a control plasmid or a Nef_{SF2} expression plasmid. Expression of Paks and Nef_{SF2} was confirmed by Western blot analysis (Fig. 9A and B). Because we did not include an active form of p21 in our co-transfection, we were able to directly assess the effect of Nef on Pak activation by performing *in vitro* kinase assays on anti-HA immunoprecipitates. As expected, in the absence of Nef or $CDC42_{G12V}$ no active Pak2 was detected (Fig. 9, lane 2). Also as expected, anti-HA immunoprecipitates of cells transfected with Nef alone did not show kinase activity (Fig. 9, lane 3). However, in the HA-immunoprecipitates, the presence of Nef clearly caused robust activation of HA-tagged Pak2 (>90 fold) (Fig. 9C, lane 4). In contrast, the presence of Nef consistently had no significant effect on Pak1 activation (Fig. 9C, lanes 5 and 6). These results demonstrate that Nef expression results in Pak2 activation. Moreover, the lack of activation of Pak1 by Nef further confirms that Nak is Pak2.

Nef-activated Pak2 is found mostly in a Nef/Pak2 complex, but a small fraction of active Pak2 is not bound to Nef. In order to further characterize the active Pak2 complex, I investigated whether all the Nef activated Pak2 is found associated to Nef. 293T cells were transfected with control plasmids or co-transfected with Nef_{SF2} and Pak2

expressing plasmids and expression was confirmed by Western blot analysis (Fig. 10A). Successive immuno-depletions followed by in vitro kinase assays were then performed to determine if anti-Nef antibodies could deplete all of the active HA-Pak2 (Fig. 10B, top). In addition, during the last wash step of the protein A beads prior to the *in vitro* kinase assay, one-fifth of the beads was removed for Nef western blot analysis (Fig. 10B, bottom). *In vitro* kinase assays performed on anti-Nef immunoprecipitates from co-transfected cells produced the expected activity corresponding mostly to HA-Pak2, but also some endogenous Pak2 (Fig. 10B lane 2, top). A second immunoprecipitation of the supernatant from above with anti-Nef antibodies confirmed the near complete depletion of Nef bound active Pak2 as well as Nef (Fig. 10B lane 3, top and bottom). Anti-HA immunoprecipitation of the supernatant from the second immunodepletion was then performed. As shown in the top panel of lane 4, a small amount of active HA-Pak2 remained, despite the depletion of the Nef-bound active Pak2. These results indicate that active Pak2 is found both free of and bound to Nef, although the majority of the activity associates with Nef. Anti-HA immunoprecipitations performed on lysates from cells co-expressing Nef and Pak2 effectively depletes the HA-Pak2 activity (Fig. 10B lanes 7 and 8). Anti-Nef immunoprecipitations of the resulting supernatant contains a residual amount of mostly endogenous Pak2 activity (Fig. 10B lane 9), clearly showing that anti-HA immunoprecipitation depletes the majority of the Nef-associated Pak2 activity found in lysates of co-transfected cells. In contrast, all the detectable Nef remained in the supernatant (Fig. 10B lanes 7, 8, and 9, bottom). Thus, relative to total Nef expression levels, the Nef/Pak2 complex is of extremely low abundance even when Pak2 is over-expressed, indicating the complex contains other factors limiting its abundance.

Nef mediates the activation of an endogenous 62 kDa histone H4 kinase that is detectable by in gel kinase assay and is enriched on cellular membranes. Pak kinase activity is detectable by in gel kinase assays. If Nak activates Pak2 phoshorylation of protein substrate, it should be specifically observed in this assay. Thus, I utilized an in gel kinase assay protocol to assess Pak2 activity levels in control and Nef expressing cells as described in Materials and Methods. The details of the protocol are described in Materials and Methods.

In gel kinase assays were performed on cell extracts generated from CEM cells stably transduced with either control vector or Nef expressing vectors. Two Nef expressing cell lines were used, one that expresses the nef_{SF2} allele and one that expresses the $nef_{D.Con.}$ allele, an artificial consensus Nef sequence (68). Cells were fractionated into cytosolic and membrane fractions as described in Material and Methods and equal amounts of protein from each fraction were then analyzed by in gel kinase assay and Western blot. As shown in Figure 11 (top), the SF2 and D.Con extracts contain a 62 kDa activity that is highly enriched on membranes and not present in the control fractions. Western blot analysis shows that this activity co-fractionates with Nef to cellular membranes. Thus, Nef mediates the activation of a cellular kinase of 62 kDa that is detectable by in gel kinase assay and co-localizes with Nef to membranes. These findings are consistent with Nef mediating the activation of endogenous Pak2 in T cells.

The Nef dependent kinase detected after renaturation in gel is Caspase 3 sensitive: In order to determine if the Nef dependent histone H4 renaturable kinase is Pak2, I

assessed its susceptibility to Caspase 3 mediated cleavage. Membrane pellets obtained from control and D.Con Nef expressing CEM cells were solubilized and then mock treated, treated with Caspase 3, or treated with Caspase 3 in the presence of the Caspase 3 inhibitor ZVAD. Samples were then analyzed by in gel kinase assay. As shown in figure 12, a Nef dependent 62 kDa activity is apparent in mock treated membranes. When the membrane samples are treated with Caspase 3, the activity is cleaved (>50%) into a 34 kDa fragment. This is consistent with the kinase domain containing fragment predicted after Caspase cleavage of Pak2 (Fig 3). The Caspase 3 mediated cleavage is inhibited by zVAD, attesting to the specificity of the cleavage event. We conclude that Pak2 is a Nef activated renaturable H4 kinase. Thus, Nef increases cellular Pak2 activity levels. As this assay analyzes Pak activity after resolution of lysates by SDS-PAGE, these findings are consistent with Nef inducing a post-translational modification of Pak2 that renders the kinase active. Furthermore, as Pak1 activated by $CDC42_{G12V}$ can be detected in our in gel system at a distinct electrophoretic mobility from Pak2 (not shown), this assay confirms that Nef does not mediate endogenous Pak1 activation in T cells.

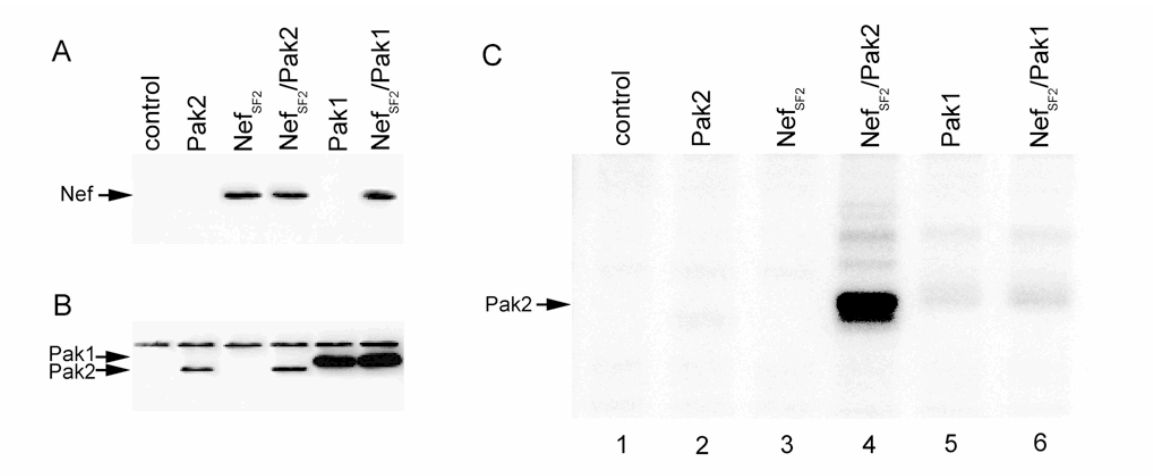


Figure 9. Specific activation of Pak2 by Nef in 293T cells. A and B) Western blot analysis for Nef and Pak1/Pak2 expression, respectively, in transfected 293T cells. C) *In vitro* kinase assays on anti-HA immunoprecipitates from the transfected cells in panels A and B. Note that Pak2 activity was clearly increased in the presence of Nef_{SF2} (compare lanes 2 and 4). In contrast, Nef had not significant effect on basal Pak1 activity (lanes 5 and 6).

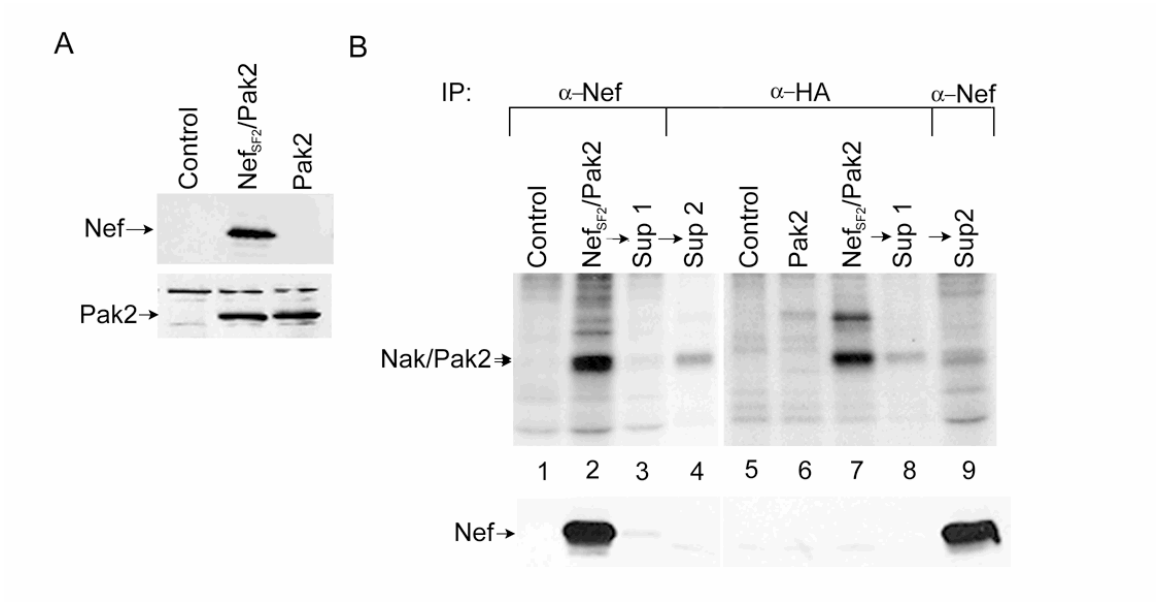


Figure 10. Nef activated Pak2 is found mostly in a low abundance Nef/Pak2 complex, but a fraction of active Pak2 is not bound to Nef. A) Western blot analysis to confirm Nef (top) and Pak2 (bottom) expression in transfected 293T cells. B) *In vitro* kinase assays (top) and Nef Western blot analysis (bottom) of immunoprecipitates from Nef/Pak2 (or control) transfections using antibodies indicated above. Samples in lanes 2, 3, and 4 represent successive immunoprecipitaions of the same lysate. Samples in lanes 7, 8, and 9 also represent successive immunoprecipitations of the same lysate. Note in lanes 2 and 3 (top) that immunoprecipitation with anti-Nef depletes all of the Nef associated Pak2 and most, but not all, of the total active Pak2 (lane 4, anti-HA immunoprecipitate). Also note that depletion of HA-tagged Pak2 (lanes 7 and 8 top) depletes the majority of Nak but does not deplete endogenous PAK2 (Lane 9, anti-Nef immunoprecipitate). Interestingly, HA-Pak2 immunodepletion did not remove an appreciable amount of the total Nef in the lysates (lanes 7, 8, and 9, bottom), indicating that only a small amount of Nef interacts with Pak2 even in the presence of overexpressed Pak2.

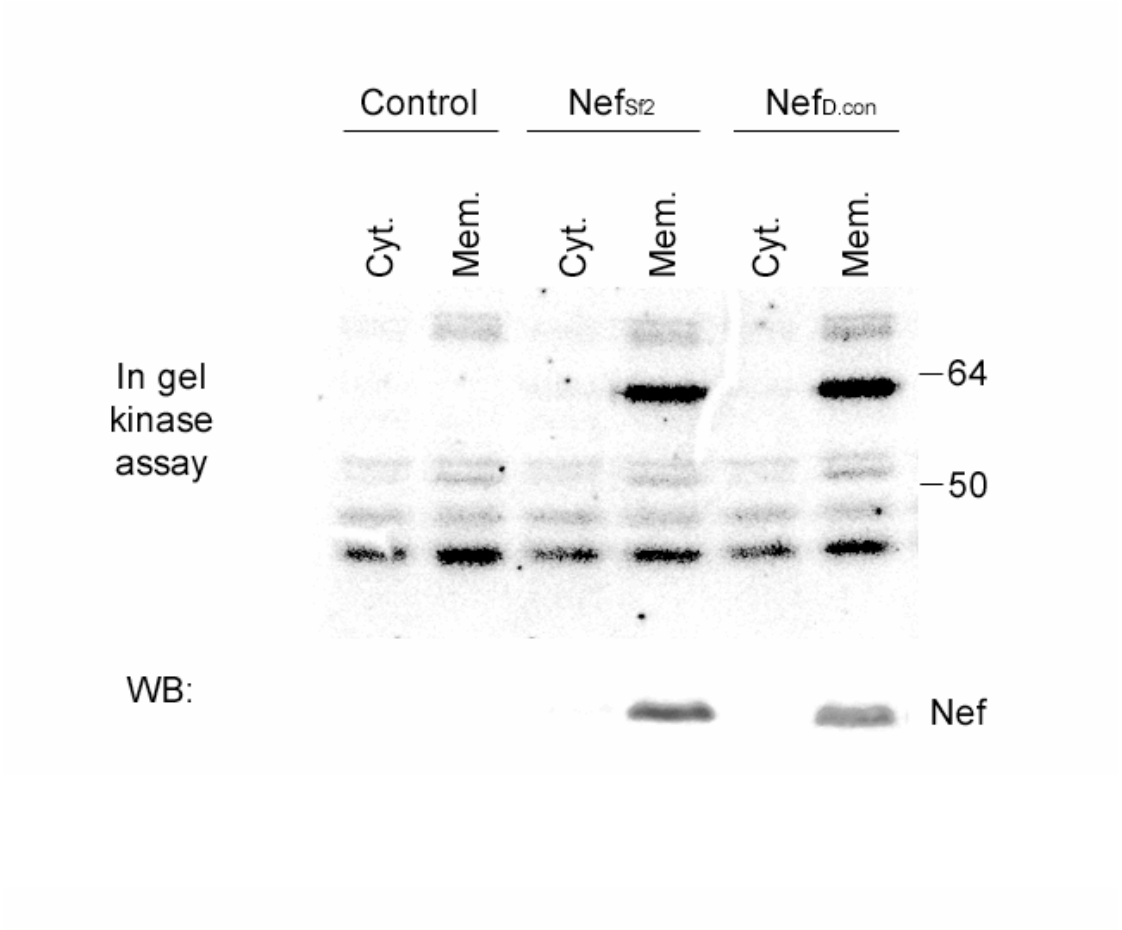


Figure 11: Nef activates a 62 kDa renaturable H4 kinase that co-localizes with Nef to membranes. Lysates from control, Nef SF2, $Nef_{D.Con}$ expressing CEM cells were fractionated into cytosol and membranes. Fractions were denatured by SDS and boiling and then resolved by PAGE using equal amounts of protein per lane. Top: Gels were co-polymerized with Histone H4 and analyzed by in gel kinase assay. Note the 62 kDa Nef dependent activity enriched in membrane fractions. Bottom: Nef western blot analysis of the same fractions. Note that Nef is also enriched on membranes.

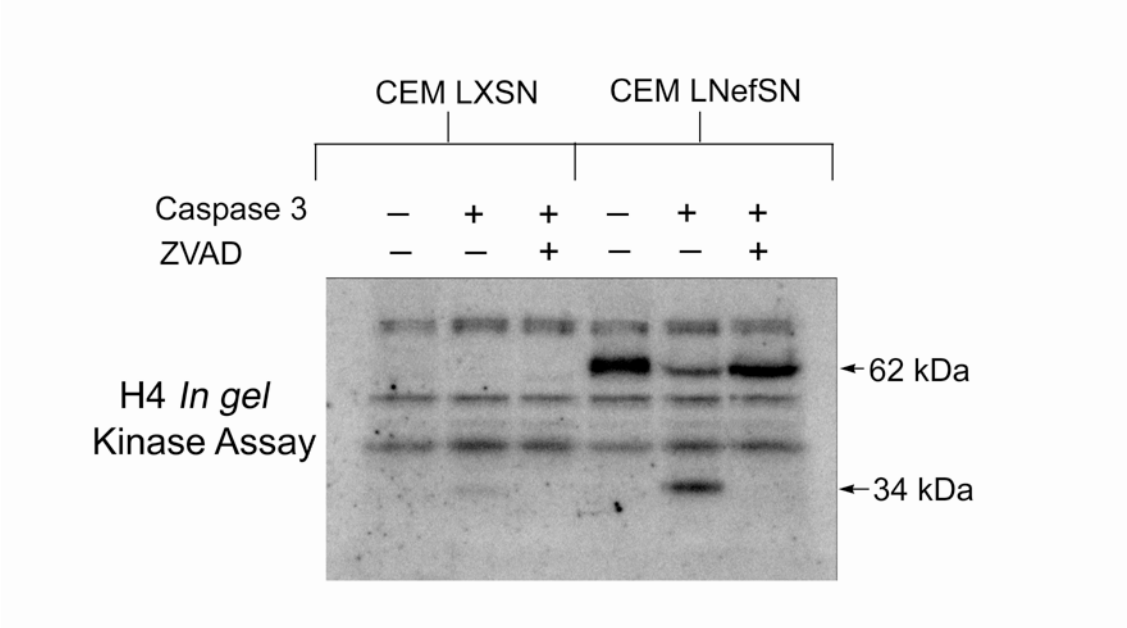


Figure 12: Caspase 3 cleaves the Nef actviated H4 renaturable kinase. Membrane fraction from control (LXSN) and Nef (LnefSN) expressing CEM cells were either mock treated, treated with Caspase 3, or treated with Caspase 3 in the presence of ZVAD. Samples were then denatured by SDS and boiling and analyzed by in gel kinase assay. Note the Nef dependent activity is cleaved by Caspase 3 into a 34 kDa fragment consitent with the kinase domain containing fragment expected following cleavage of Pak2 with Caspase 3.

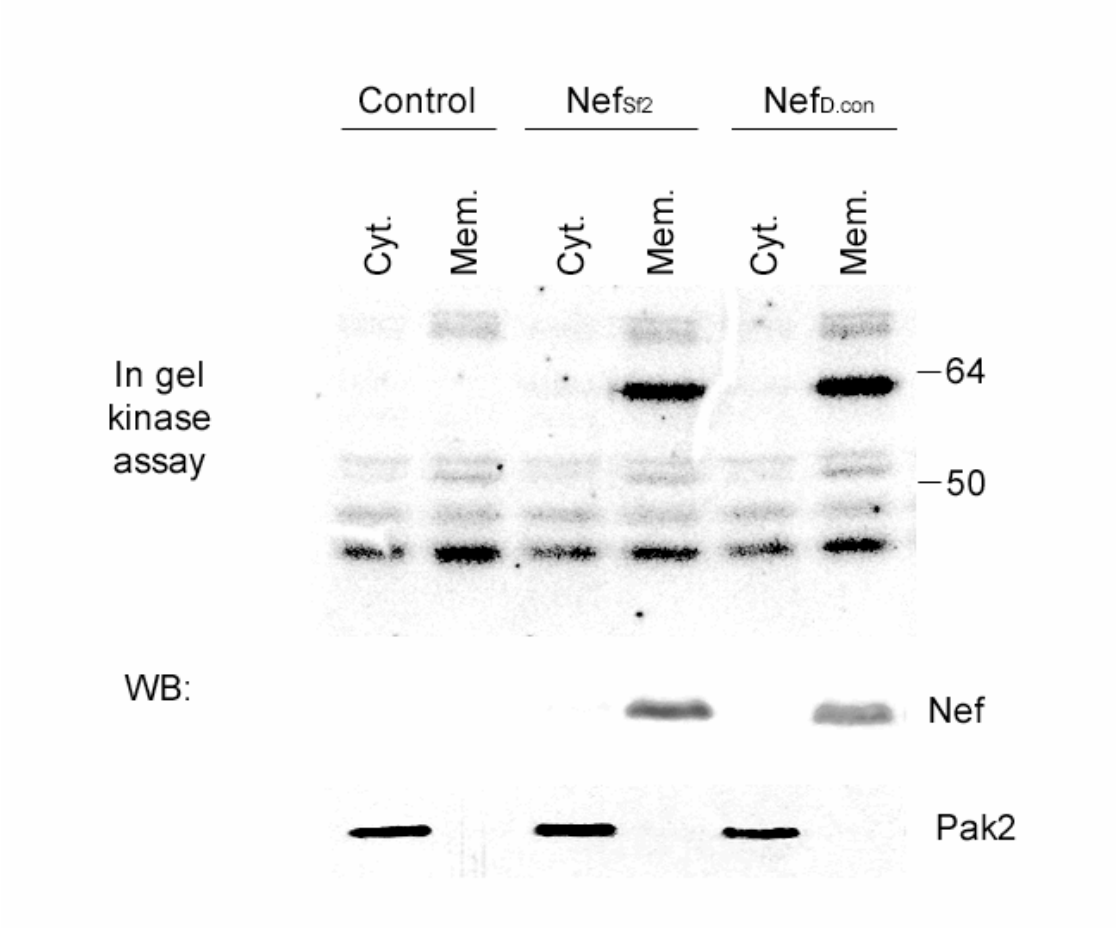


Figure 13. Most of the cellular Pak2 is cytsolic and inactive. In gel kinase assay (top) and Nef western blot (middle) are the same as form Figure 11. Equal amounts of protein from each fraction where also analyzed for total Pak2 by western blot analysis. Note that no Pak2 was detectable in the membrane fraction by western blot. However, all of the active Pak2 in these fractions localizes to the membranes.

Chapter 5: Pak2 activation is dependent on Rho family GTPases.

Overview: In the 'inactive' state, the Pak2 kinase and regulatory domains interact in an autoinhibitory fashion (142). The best understood endogenous activators of Paks are members of the Rho family of GTPases (142). In vivo and in vitro, active (GTP bound) forms of Rac1, Rac2, and CDC42 bind to the GTPase binding domain in the regulatory region of Pak2 (Fig. 3)(142). This binding relieves autoinhibition and allows for phosphorylation of T403, resulting in kinase activation. In this section, I address the role of endogenous Rho family GTPases in Nef mediated Pak2 activation. First, I evaluated whether endogenous Rho family GTPase activity is necessary for Nef mediated Pak2 activation. To do this, I utilized RhoGDIα, a Rho family GTPase regulatory protein that when over-expressed has been shown to be an effective inhibitor of Rac and CDC42 activity (143). RhoGDIα likely inhibits by at least two different mechanisms. RhoGDIα binds to GTPases in the GDP bound state and inhibits nucleotide exchange; furthermore, RhoGDIα sequesters GTPases in the cytosol, preventing the accumulation of high concentrations of active GTPases at cell membranes where they execute effector functions (144;145). Using in vitro kinase assays, I showed that RhoGDIα expression blocks Nef's activation of Pak2. Secondly, I evaluated the ability of Nef to mediate the activation of a mutant Pak2 that cannot bind GTPases due to well described substitutions in the GTPase binding domain. I then showed using in vitro kinase assay that Nef does not mediate the activation of this mutant Pak2. These results indicate that Nef requires endogenous GTPase activity and that the GTPases function by binding directly to Pak2.

Thus Nef likely facilitates the binding of endogenous active GTPases to Pak2, thereby inducing Pak2 activation.

Nef mediated activation of endogenous Pak2 requires endogenous Rho family GTPase activity: When over-expressed, RhoGDIα has been shown to be an effective inhibitor of certain Rho family GTPases including Rac and CDC42 (143). In order to assess the role of active GTPases in Nef associated Pak2 activity, I co-transfected 293T cells with increasing amounts of RhoGDIα expressing plasmid in the presence of a fixed amount of Nef_{SF2} expressing plasmid. Expression levels of both proteins were confirmed by western blot analysis (Fig. 14A bottom 2 panels). In vitro kinase assays were then performed on anti-Nef immmunoprecipitates from each sample. Immunoprecipitations were carried out using saturating amounts of anti-Nef antibody. Pak2 autophosphorylation decreases as RhoGDIα expression increases (Fig. 14A top; Fig. 14B, quantitative analysis of two experiments). Thus, Nef associated Pak2 activity is dependent on endogenous Rho family GTPase function.

Activation of Pak2 by Nef occurs via the Pak2 GTPase binding domain. I next determined if endogenous Rho family GTPases bind directly to Pak2 during Nef mediated Pak2 activation. I utilized a Pak2 mutant, $Pak2_{HH\text{-}LL}$, that has well described substitutions in the GTPase binding domain (132). These substitutions abolish functional interactions of this domain with active GTPases (146). $Pak2_{wt}$ and $Pak2_{HH\text{-}LL}$ were expressed as N-terminally tagged HA fusion proteins. $Pak2_{wt}$ or $Pak2_{HH\text{-}LL}$ expressing

plasmids were transfected in 293T cells alone, with Nef, or with the constitutively active $CDC42_{G12V}$. Expression of all proteins was confirmed by western blot (Figure 15, bottom).

Pak activity was first determined by in vitro kinase assay using anti-HA immunoprecipitates of lysates from transfected cells. As shown in figure 15 (top, lanes 2 and 3), the Pak molecules had little appreciable autophosphorylation activity when expressed alone. As expected, $CDC42_{G12V}$ activates $Pak2_{wt}$, but not $Pak2_{HH-LL}$ (lanes 6 and 7). Co-expression of Nef with $Pak2_{wt}$ leads to an increase in Pak2 autophosphorylation (lane 4). In contrast, $Pak2_{HH-LL}$ activity is unaffected by Nef (lane 5). In vitro kinase assays were also performed on Nef immunoprecipitates. When performed with lysates of cells in which Nef was expressed alone, autophosphorylation of endogenous Pak2 was detected (Figure 15 lane 9). Kinase assays performed with anti-Nef immunoprecipitates from lysates with ectopically expressed $Pak2_{wt}$ showed active endogenous Pak2 as well as autophosphorylation of the slower migrating HA tagged protein. When Nef was expressed with $Pak2_{HH-LL}$, however, only autophosphorylation of endogenous Pak2 was detected (Figure 15 lanes 10 and 11). Thus, these findings indicate that Nef mediated Pak2 activation is dependent on the GTPase binding domain of Pak2. This observation along with experiments with RhoGDIα indicate that Nef mediated Pak2 activation occurs via the binding of active endogenous GTPases to the Pak2 GTPases binding domain. The GTPase(s) involved could be Rac1, Rac2, CDC42, and/or an undescribed Pak activating GTPase.

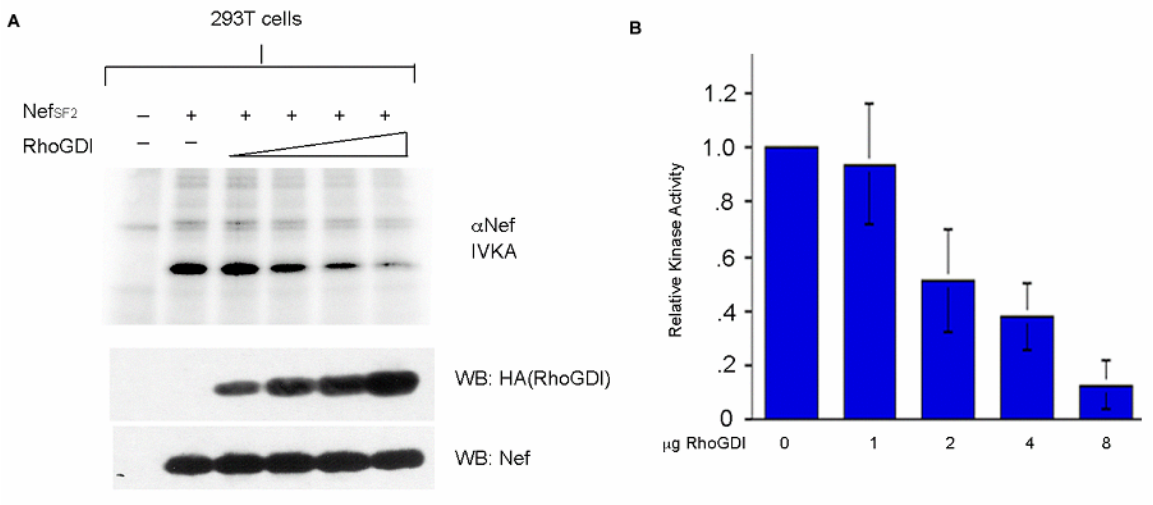


Figure 14: RhoGDI expression inversely correlates with Nef associated Pak2 activity. A) 293T cells were transfected with a fixed amount of Nef expressing plamid alone or with increasing amounts of RhoGDI expressing plasmid. After cells were harvested, In vitro kianse assays were then performed using Nef immunoprecipitates. Nef associated Pak2 autophosphorylation activity decreased as a function of RhoGDI expression (top). Appropriate expression was confirmed by HA or Nef western blot (bottom two panels). B) Quantitative analysis of Pak2 activity from duplicate experiments performed as described in A. Relative kinase activity is indicated by the Y axis. Micrograms of RhoGDI plasmid used for transfection are indicated on the X axis. Standard error at each point is also indicated.

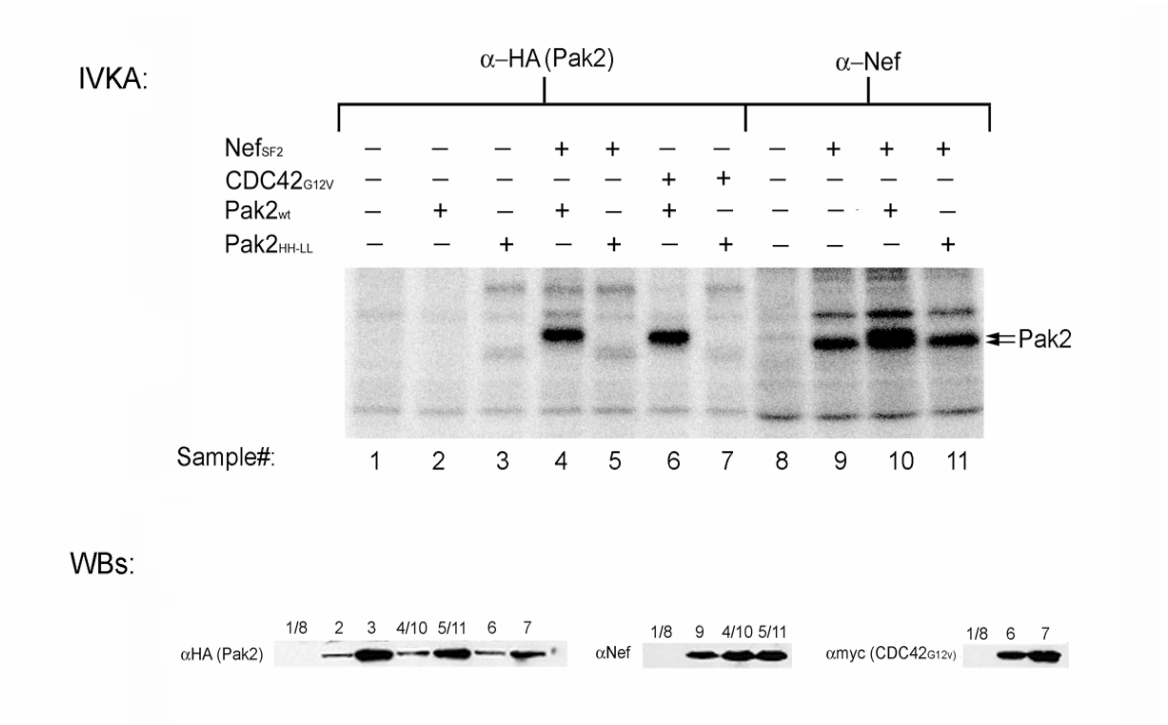


Figure 15: Nef mediated Pak2 activation requires the Pak2 GTPase binding domain. Plasmids expresing HA tagged wild type Pak2 or Pak2 HH-LL were co-transfected into 239T cells with control, Nef, or CDC42$_{G12V}$ plasmids. Top: In vitro kinase assays were performed on lysates using HA (1-7) or Nef (8-11) immunoprecipitates. Note that Nef expression induces Pak2 autophosphorylation (compare lanes 2 and 4), but does not affect activity of the mutant Pak2 (compare lanes 3 and 5). As expected, CDC42G12V activates wild type but not mutant Pak2 (lanes 6 and 7). Nef immunoprecipitates from cells expressing only Nef contain endogenous Pak2 activity (lane 9). When Nef is co-expressed with HA-tagged Pak2, a doublet is observed corresponding to endogenous as well as ectopically expressed tagged Pak2 (lane 10). In contrast, mutant Pak2 activity is not found associated with Nef. Bottom: Expression of appropriate proteins was confirmed by western blot for each lysate used for in vitro kinase assay.

Chapter 6: Mechanism by which Nef induces Pak2 activation via Rho family GTPases.

Overview: The implication of Rho family GTPases in Nef mediated Pak2 activation suggests that Nef regulates RhoGTPase function. The work in this section addressed the mechanism by which Nef leads to the activation of Pak2 via endogenous GTPases. I first investigated the hypothesis that Nef activates endogenous GTPases that in turn activate Pak2. I used a novel cell based assay to assess the effects of Nef expression on the activity levels of Pak activating GTPases. I began by generating a protein that contains the Pak1/Pak2 GTPase binding domain fused to the C-terminus of Glutathione-S-Transferase (GST). It has been shown that this fusion protein, GST-PBD (p21 binding domain), binds to Rac and CDC42 in the active state in vitro and does not bind to GDP bound forms of these GTPases (147). Having established the importance of the Pak2 GTPase binding domain in Nef mediated activation, I used this peptide as a probe to detect active GTPases utilized by Nef to activate Pak2. Fixed and permeabilized cells were stained with GST-PBD, and indirect immunofluoresence was then performed. Staining was first analyzed by FACS which provides a quantitative measure of GST-PBD binding. I show that this approach is able to detect active GTPases in cells and that there is detectable constitutive PBD binding activity in control cells. Nef, moreover, does not appreciably affect levels of active GTPases that target Pak2. I then visualized similarly stained cells by confocal microscopy. I confirm that active GTPases, but not inactive GTPases, are detected by this method and then show that in CEM T cells, Nef and PBD binding GTPases co-localize to the peri-nuclear region. I hypothesized, thus, that Nef mediates Pak2 activation on endomembranes.

Biochemical fractionation using a discontinuous sucrose gradient was carried out to elucidate which endomembrane system is involved. I showed that Nef induces Pak2 activity on low density membranes and that the activity is highly enriched in this compartment. The low density membrane compartment is highly enriched for golgi membranes, but not other endomembrane systems. Thus, it is possible that Nef activates Pak2 on golgi membranes, though plasma membrane has not been excluded. I have not localized plasma membranes on our gradient. Thus, I cannot use the biochemical fractionation data to confirm or deny that Nef induced Pak2 activity does not localize to plasma membrane. I also used an established GST-PBD 'pull-down' approach to show the low density fractions also contain active Rac1 and Rac2 (147). I speculated in section II that Nef recruits Pak2 to membranes. The data presented here is consistent with a model in which Nef recruits Pak2 to peri-nuclear membranes were it encounters active GTPases.

Nef expression does not significantly affect Pak2 activating GTPase levels. Published protocols for assessing activity levels of Rac and CDC42 utilize the GST-PBD peptide conjugated to glutathione beads to 'pull down' active form of these GTPases. After washing and eluting the beads, active GTPase are then detected by western blot analysis using antibodies specific for the GTPase of interest (i.e. Rac or CDC42). This method has been shown to be an effective and specific way of detecting GTP bound GTPases (147) and was used for experiments described below. These experiments, as well as others (not shown), did not reveal any Nef induced differences in GTPase activity levels. However, a major limitation of this system is that it only allows for the analysis of known

Pak activating GTPases with good reagents for their detection. As our CDC42 antibody performed poorly, we were not able to compare CDC42 activity in control and Nef expressing cells using this method. Moreover, GTP hydrolysis due to GTPase activating protein (GAP) activity in solution was a concern due to the absence of useful GAP inhibitors. Thus, we sought to develop a novel approach to assess Pak activating GTPase levels that would overcome these limitations. Cannon et. al. recently demonstrated that the p21 binding domain of WASP, a CDC42 effector, can be used as a specific probe for active GTPases in fixed and permeablized cells (148). They used indirect fluorescence to detect their tagged WASP p21 binding domain peptide. They then localized active GTPases by confocal microscopy. We reasoned that a similar approach could be utilized for staining active forms of Pak activating GTPases in cells and, moreover, that GTPase activity levels could be quantified by FACS.

To detect active forms of Pak activating GTPases we first generated a GST tagged Pak1/2 GTPase binding domain, GST-PBD. We then transfected 293T cells with limiting amounts of a Green Fluorescent Protein (GFP) expression plasmid either alone, with an active CDC42 mutant, an inactive CDC42 mutant, or a Nef_{SF2} expressing plasmids. We then fixed cells, permeabilized them, and stained them with GST-PBD. A no GST-PBD control was also included to assess background fluorescence. Indirect immunofluoresence was then performed on all cells using a rabbit anti-GST primary antibody and Cy5 conjugated anti-rabbit secondary antibody. Cells were then analyzed by FACS. Transfected cells were identified by GFP expression. As cells were transfected with a limiting amount of GFP plasmid (<1/75 the amount of the co-transfected plasmid), it was assumed that all GFP positive cells were also transfected with

the plasmid present in vast excess. The GFP positive gate (Figure 16A) was then analyzed for PBD staining in FL4 (Figure 16B). A sample in which GST-PBD was not included in the staining procedure was used to determine background staining. Average data from 2 experiments is shown. In cells transfected with GFP only, PBD staining significantly exceeded background (>5 fold). This finding is consistent with constitutive GTPase activity. CDC42Q61L (a constitutively active CDC42 mutant) transfected cells stained brightly with PBD (>40 fold over background). In contrast, staining of the CDC42 N17 mutant, which is defective in GTP binding, was not significantly greater then PBD staining in GFP only cells. To assess the role of Nef on Pak activating GTPases, I also expressed Nef_{SF2}. I did not detect appreciable difference in GTPase levels between control cells and Nef expressing cells. While I cannot rule out subtle GTPase activation by Nef, I was unable to detect any activation. The data, however, is consistent with the presence of constitutively active GTPases in these cells. Lastly, a caveat to these interpretations is the lack of a control experiment staining with a GST-PBD_{HH-LL} peptide. Such experiments would be needed to definitively show that the putatively active GTPases detected are indeed binding specifically to the PBD.

GST-PBD can be used to localize active GTPases in cells. To demonstrate that, as reported for the WASP PBD (148), the Pak PBD can be used to localize active GTPases in cells, I transfected 239T cells with HA tagged $CDC42_{Q61L}$ or $CDC42_{N17}$. I then fixed and permeabilized the cells using the same reagents used for FACS. Importantly, I permeablized the cells using saponin. Michaelson et. al. have shown that the permeabilization method affects the observed localization of Rho GTPases in cells (144).

It was determined that the localization observed in saponin permeabilized fixed cells closely recapitulated the localization patterns observed for the same proteins in live cells (144). Thus, GST-PBD staining in saponin permeabilized cells should accurately reflect the localization of active GTPases in living cells. I co-stained the transfected 293T cells for the HA tagged CDC42 molecules and for active GTPases. Cells were first stained with GST-PBD. After washing, cells were then incubated with rabbit anti-GST antibody and anti-HA antibody. After washing again, secondary antibody staining was performed with anti-mouse FITC and anti-rabbit conjugated Cy3. Staining was then visualized by confocal microscopy. As show in figure 17, PBD and $CDC42_{Q61L}$ staining show a high degree of overlap. In contrast, $CDC42_{N17}$ was not detected by GST-PBD using similar microscope settings (Fig. 18). These findings indicate that GST-PBD can be used to specifically localize active GTPases in cells.

GST-PBD and Nef staining co-localizes to the peri-nuclear region of T cells: We next determined if Nef co-localizes with PBD staining in T cell lines. We used retrovirally transduced CEM control or Nef expressing cells. Cells were stained as described above, but with mouse anti-Nef in place of mouse anti-HA. As shown in figure 19 (upper right), control cells did not stain with anti-Nef antibody, attesting to the specificity of the antibody. These cells also show a peri-nuclear distribution of PBD staining, suggestive of constitutively active GTPases. In Nef expressing cells, Nef and GST-PBD staining localized to an overlapping peri-nuclear region (Fig. 20). I have biochemically shown Nef to be highly enriched on membranes. Thus, Nef and Pak activating GTPases likely co-localize to peri-nuclear membranes. As Pak2 activation

occurs in a complex with Nef, recruitment of Pak2 to membranes, likely juxtanuclear membranes, by Nef would expose Pak2 to high concentrations of active forms of GTPases that activate Pak.

Nef and Nef activated Pak2 co-localize with low density membranes on a discontinuous sucrose gradient. Biochemical fractionation was used to elucidate the membrane compartment in which Nef induced Pak2 activity is found. Low density membranes were purified by floatation on a discontinuous sucrose gradient. As described in methods, low density membranes are enriched for golgi membranes, but not other endomembranes. The presence of plasma membrane in these low density membrane fractions has not been determined. Samples obtained from the sucrose gradient were analyzed by commassie stain to qualitatively assess enrichment. As shown in figure 21A, Nef is enriched in low density fractions (Fr. 3,4 and 5). Enrichment of the golgi marker Syn6 is also indicated (Figure 21A). In gel kinase assay on these fractions was also performed to localize active Pak2 (Figure 21B). The in gel kinase was selected over an in vitro kinase assay to analyze Pak2 activation for two reasons. First, it requires minimal sample preparation time, minimizing the chance of losing kinase activity due to phosphatase activity or protein degradation. This is a particular concern in the highly purified fractions which have very low protein concentrations. Secondly, the in gel kinase assay allows for assessment of Pak2 activity in membrane samples solubilized with SDS. Thus, activity in detergent resistant compartments is more likely to be detected by this method than by in vitro kinase assay which is performed after solubilization with a non-ionic detergent. Crude membrane preparations from Nef

expressing CEM cells were included in the assay to demonstrate consistent kinase reaction efficiency in each gel (Figure 21B, indicated by +). Like Nef, active Pak2 was enriched in the low density fractions (Figure 21B). Nef and active Pak2, however, were also found in higher density fractions. Thus, we cannot rule out the presence of Nef and Pak2 in other membrane compartments. However, as a significant portion of Syn6, a golgi specific marker also localized to these fractions, the presence of Nef and active Pak2 in the non-low density compartments could simply reflect presence of Golgi membranes in these samples. Thus the observations shown here along with the peri-nuclear staining pattern observed for Nef and active forms of Pak activating GTPases, are consistent with Pak2 activation by Nef occurring on golgi membranes.

Active Rac1 and Rac2, are found in the low density membrane fractions: To localize biochemically Pak activating GTPases, we utilized a GST-PBD pull-down method to detect active GTPases in the post nuclear supernatant (PNS) as well as the low density membranes fractions. We analyzed both Nef expressing and control samples. Samples were incubated with glutathione conjugated beads pre-bound with GST-PBD. Precipitates were then washed and analyzed by western blot for Rac1, Rac2, and CDC42. Presence of these proteins in the GST-PBD pull-down indicates that they are in the active, GTP-bound state. A GST only pull-down was also included to demonstrate that detected proteins specifically bound to the Pak GTPase binding domain. As shown in Figure 22, no Rac1 or Rac2 was detectable in the precipitates using GST alone. In contrast, Rac1 and Rac2 were detected in the PNS samples. Consistent with a lack of a role for Nef in regulating GTPase activation, no significant differences in Rac1 or Rac2

activity levels were found in control and Nef expressing cells when total PNS was analyzed. Equivalent inputs from Nef and control lysates was confirmed by Syn6 western blot (bottom). The presence of active GTPases in the low density membrane compartment was also analyzed. We found significant amounts of active GTPases in these fractions. Thus, Nef could utilize these active GTPases to mediate Pak2 activation in the low density membrane compartment. We also analyzed CDC42, but were unable to detect any active CDC42. While it is possible that there is no active CDC42 in these cells, the poor sensitivity of commercially available antibodies makes us reluctant to make this conclusion.

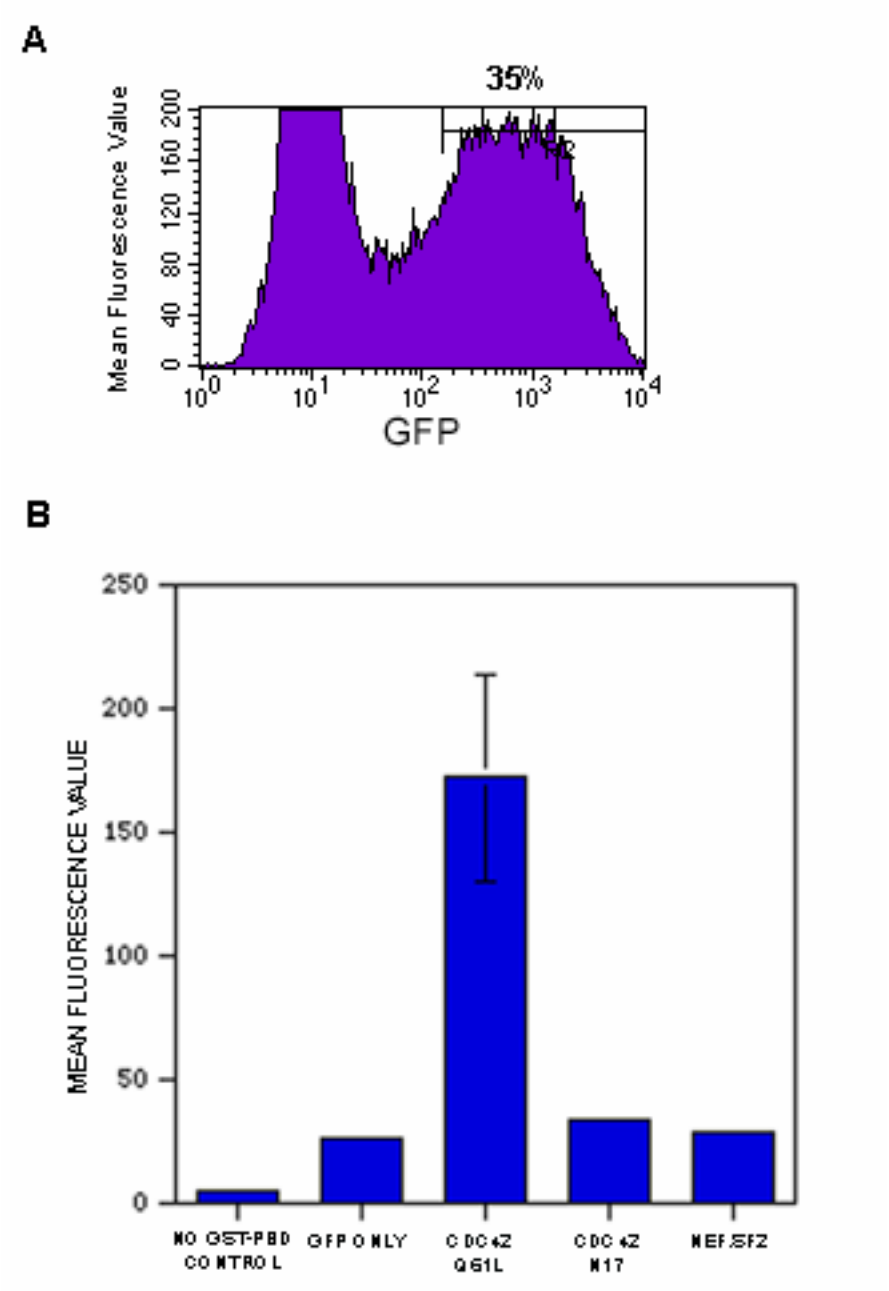


Figure 16. Analysis of PBD staining in transfected 293T cells. 293T cells were transfected with limiting amounts of GFP plasmid plus indicated plasmids. PBD staining was performed first with rabbit anti-GST primary antibody followed by Cy5 conjugated anti-rabbit antibody. A) Transfected cells were identified by GFP. Data from the GFP only sample of one experiment is shown. B) PBD staining in transfected (GFP+) cells was then analyzed. Averages from 2 experiments with SEMs are indciated. A mock PBD stain was also included in which the GST-PBD peptide was not included (first column).

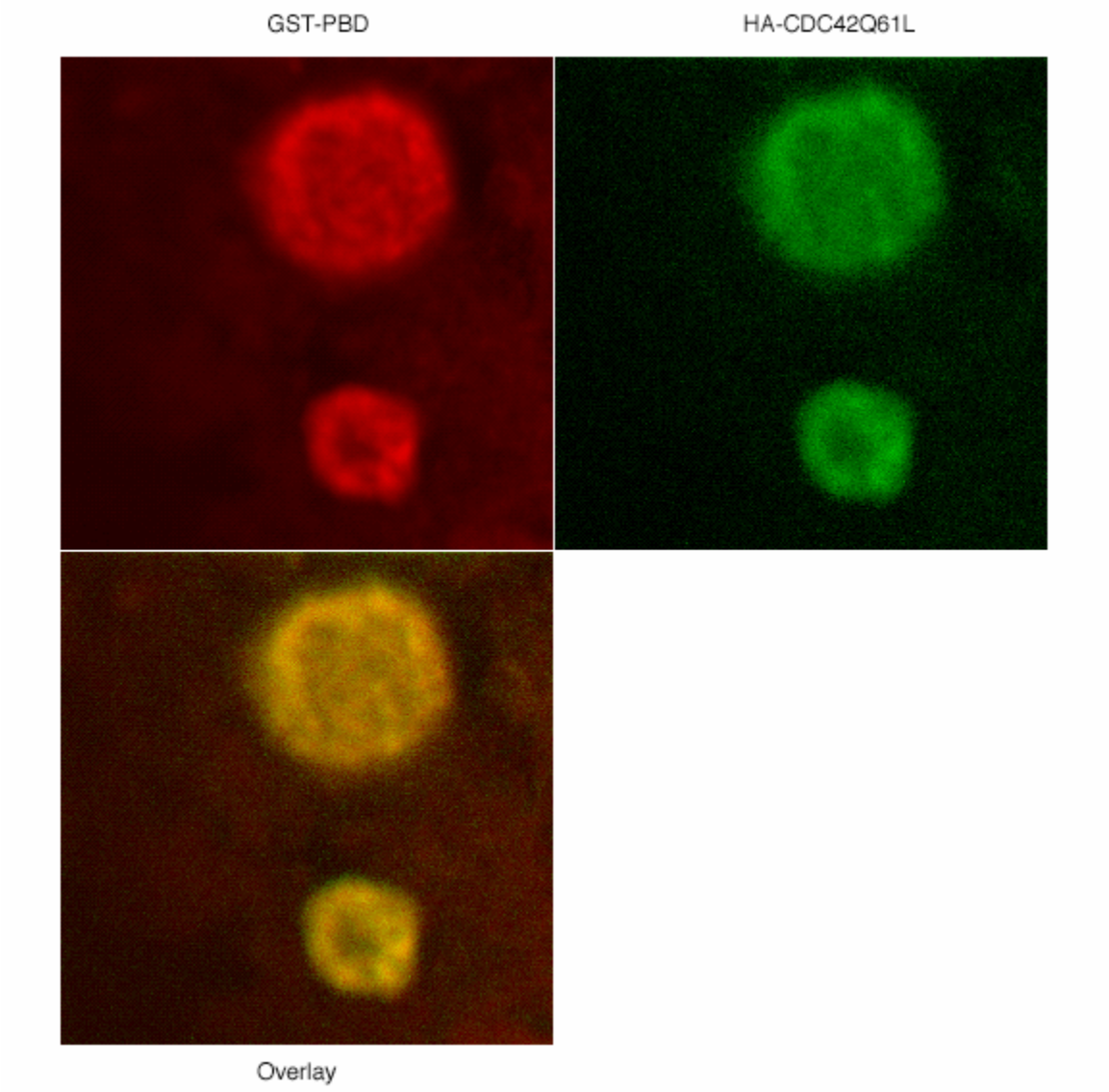


Figure 17: GST-PBD and active CDC42 show similar distribution in cells. 293T cells were transfected with HA-$CDC42_{Q61L}$ expressing plasmid. $CDC42_{Q61L}$ is constituitively active. Cells were stained with GST-PBD and for HA. GST-PBD staining was visualized by staining with rabbit anti-GST primary antibody followed by Cy3 conjugated anti-rabbit secondary antibody. Upper left: cell visualized with a Cy3 filter. HA was visualized by staining with anti-HA primary antibody followed by FITC conjugated anti-mouse secondary antibody. Upper right: cell visualized with an FITC filter. Bottom left: overlay of the other two images. Note that the staining patterns extensively overlap.

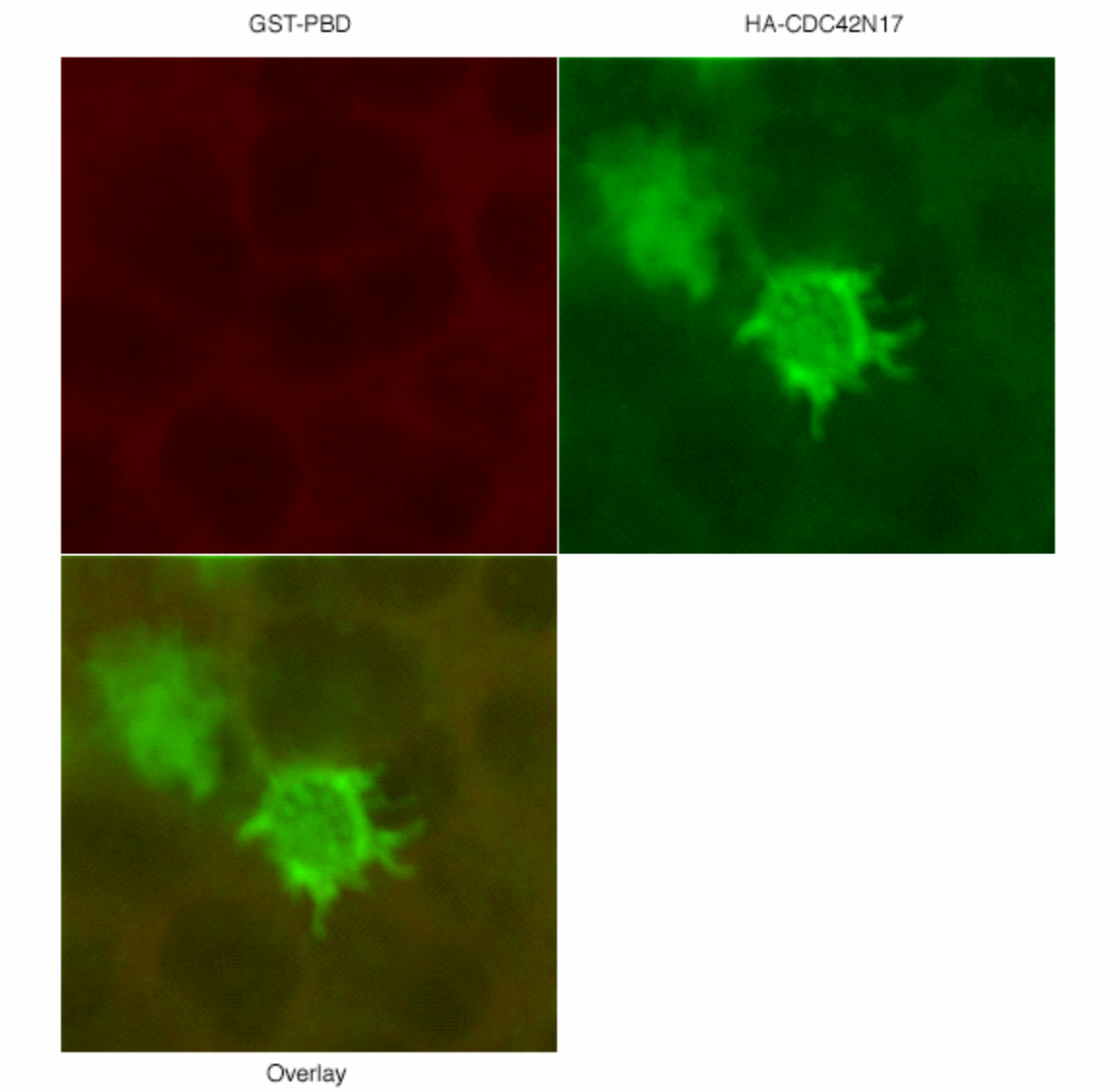


Figure 18. GST-PBD does not bind inactive CDC42 in cells. 293T cells were transfected with HA-$CDC42_{Q61L}$ expressing plasmid. $CDC42_{Q61L}$ is constituitively active. Cells were stained with GST-PBD and for HA. GST-PBD staining was visualized by staining with rabbit anti-GST primary antibody followed by Cy3 conjugated anti-rabbit secondary antibody. Upper left: cell visualized with a Cy3 filter. HA was visualized by staining with anti-HA primary antibody followed by FITC conjugated anti-mouse secondary antibody. Upper right: cell visualized with an FITC filter. Bottom left: overlay of the other two images. Note that no GST-PBD staining is observed. This experiment was done under similar conditions to those used for Figure 15.

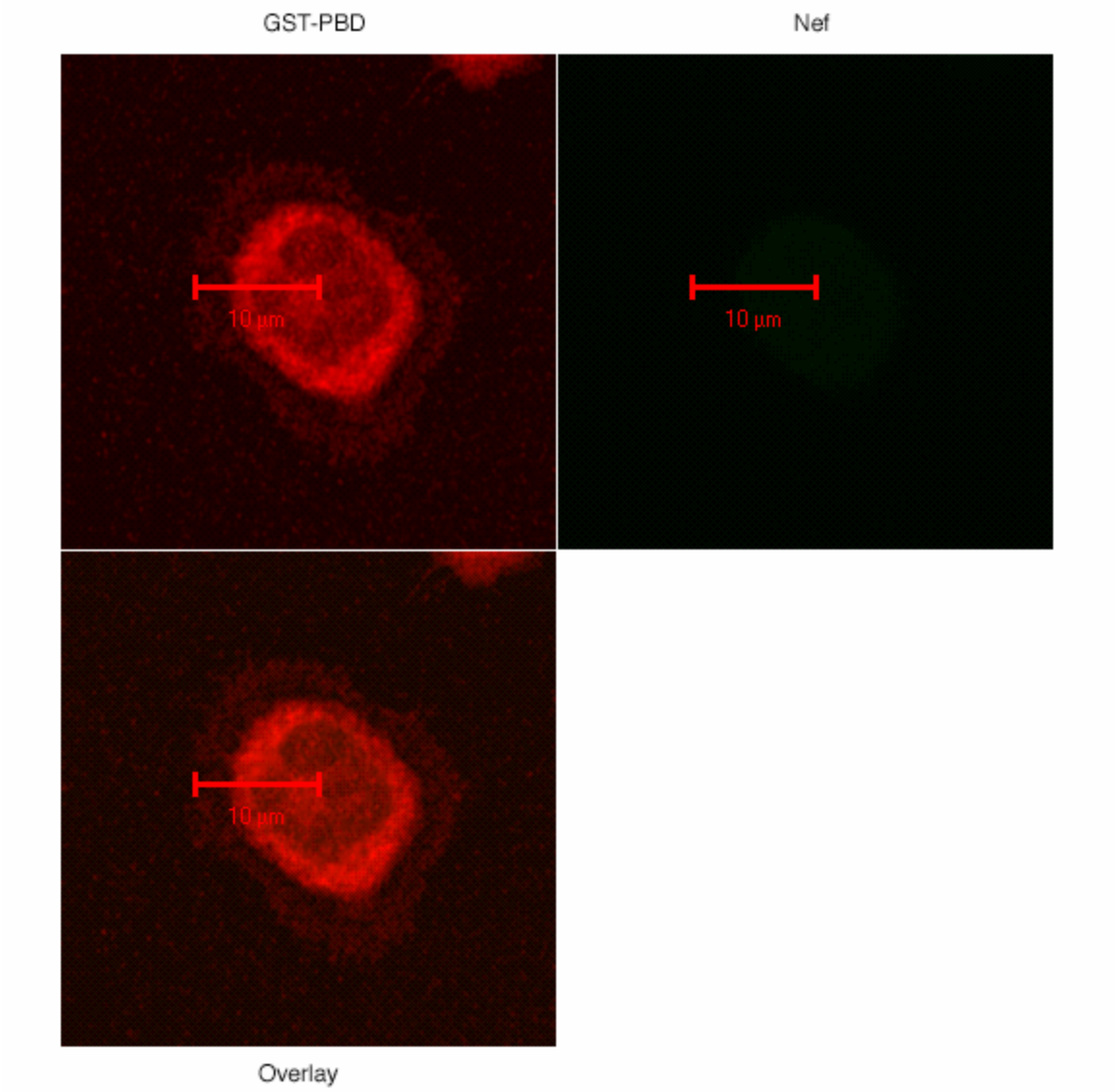


Figure 19. Perinuclear staining of active forms of Pak activating GTPases in T cells. Control CEM cells were stained with GST-PBD and anti-Nef. GST-PBD staining was visualized by staining with rabbit anti-GST primary antibody followed by Cy3 conjugated anti-rabbit secondary antibody. Upper left: cell visualized with a Cy3 filter. Nef was visualized by staining with anti-Nef primary antibody followed by FITC conjugated anti-mouse secondary antibody. Upper right: cell visualized with an FITC filter. Bottom left: overlay of the other two images. Note the perinuclear staining pattern of the GST-PBD. Also note that no Nef staining is observed attesting to the specificity of the antibody.

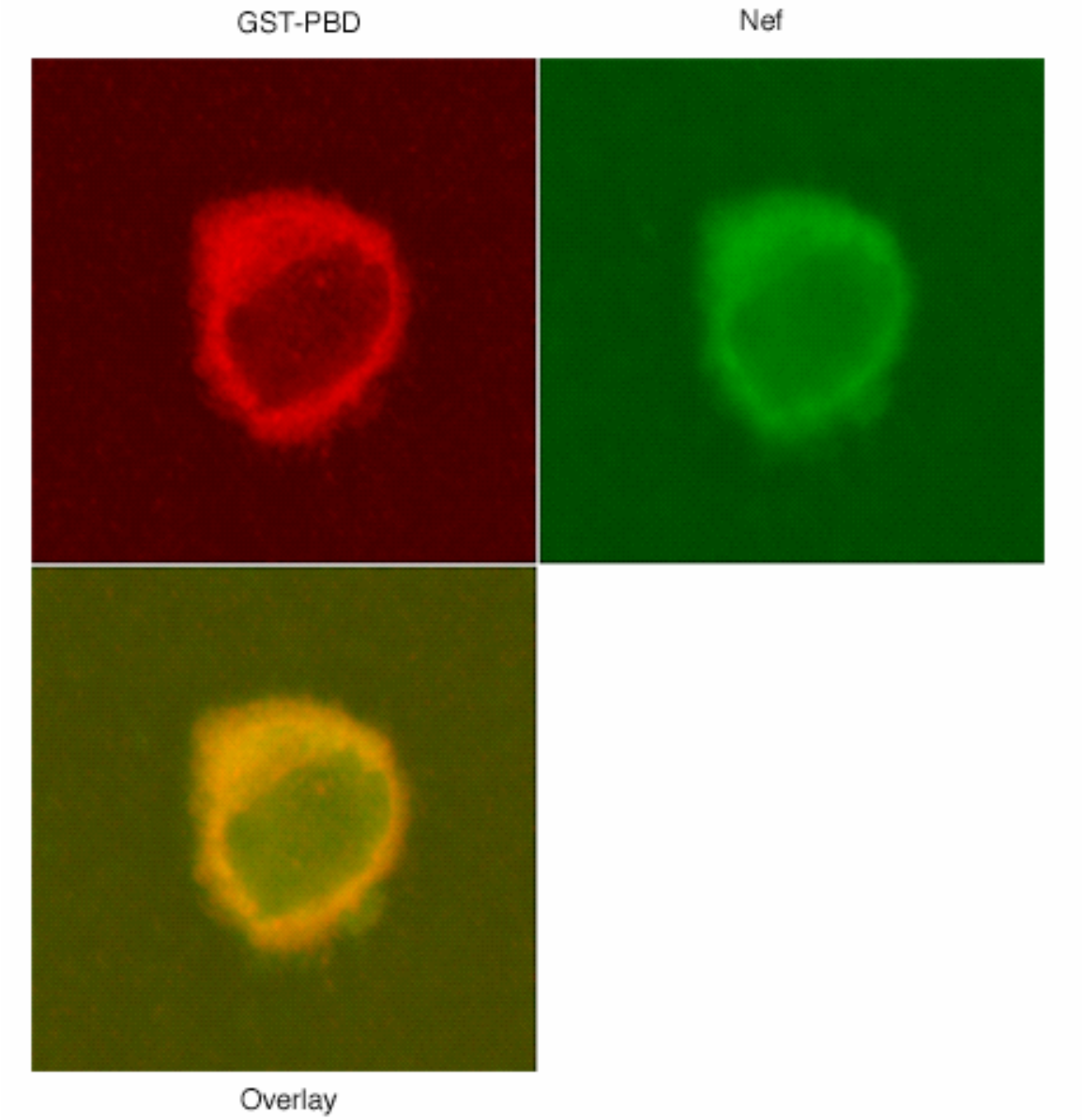


Figure 20. GST-PBD and Nef overlap in CEM Nef expressing cells. Nef expressing CEM cells were stained with GST-PBD and anti-Nef. GST-PBD staining was visualized by staining with rabbit anti-GST primary antibody followed by Cy3 conjugated anti-rabbit secondary antibody. Upper left: cell visualized with a Cy3 filter. Nef was visualized by staining with anti-Nef primary antibody followed by FITC conjugated anti-mouse secondary antibody. Upper right: cell visualized with an FITC filter. Bottom left: overlay of the other two images. Note the extensively overlapping perinuclear distribution of GST-PBD and Nef.

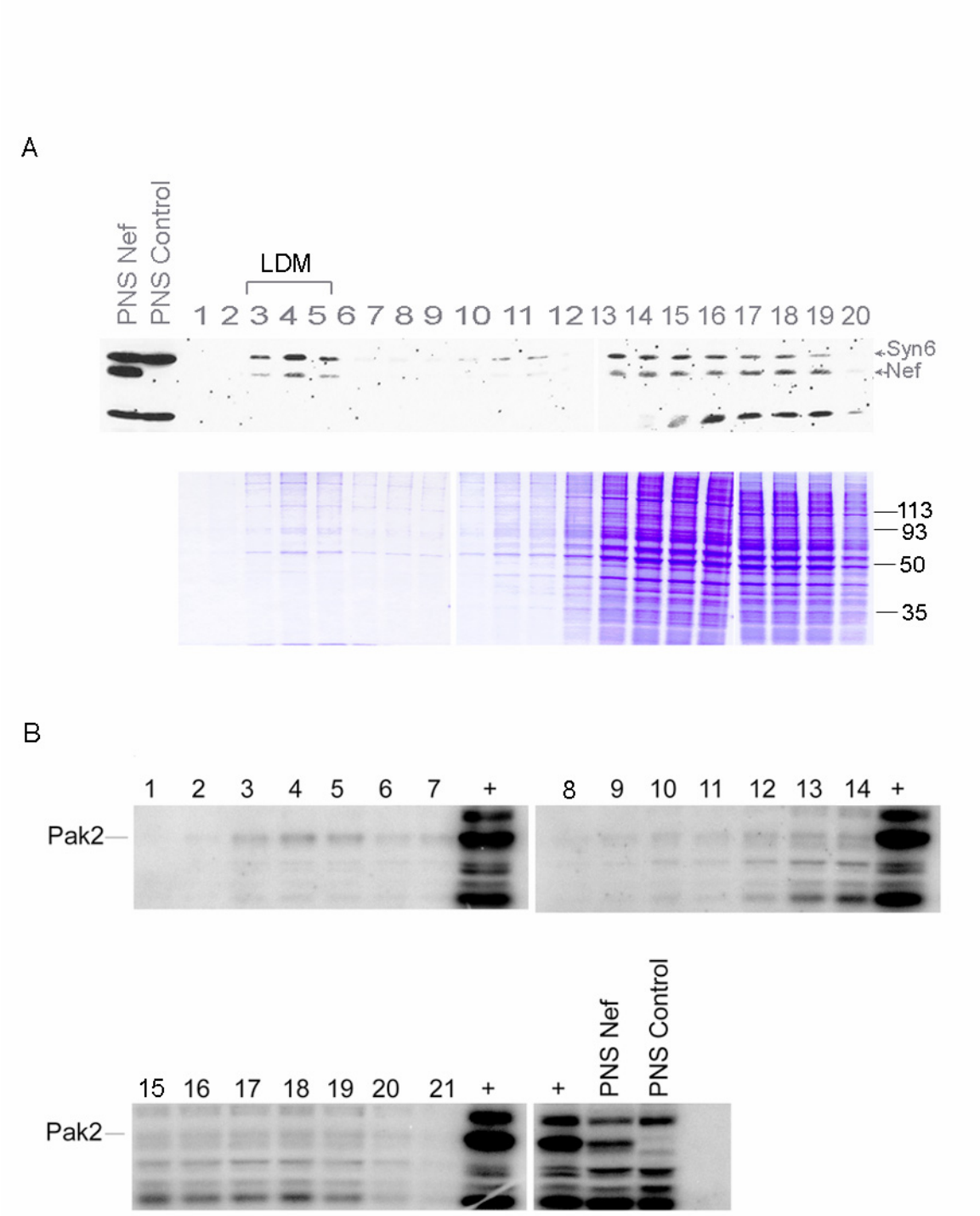


Figure 21. Nef and active Pak2 are highly enriched in low density membranes. Low density membranes (LDM) were isolated by sucrose gradient as described in Methods. A: Fractions were analyzed by western blot analysis with antibody to the golgi marker Sytanxin 6 or Nef. Samples were also analyzed by comassie blue staining to qualitatively assess enrichment. Note the co-fractionation of the golgi marker Syntaxin 6 and Nef in low density membranes (Fr 3,4, and 5). B: Samples were also analyzed by in gel kinase assay. To confirm equal efficiency of kinase reactions, equivalent positive control samples of crude membranes from Nef expressing CEM cells were included on each gel (indicated

by +). As expected, PNS from Nef expressing cells shows Pak2 activity, while PNS from control cells does not. Note that Pak2 activity co-fractionates with Nef and the golgi marker Syn6 to fractions 3, 4, and 5.

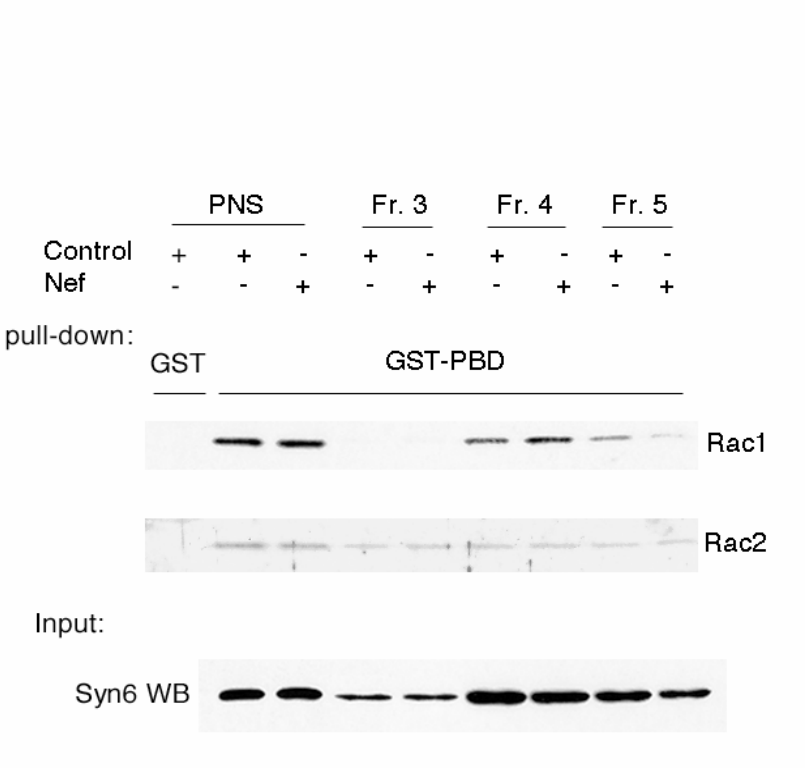


Figure 22: Active Rac1 and Rac2 are detected in golgi fractions. GST-PBD 'pull down' assays were performed on PNS and fractions 3,4, and 5 of the discontinuous sucrose gradient. As a control, a 'pull down' using the control PNS was also performed with GST alone. 'Pull downs' were then analyzed for Rac1 and Rac2 by western blot. Note that neither protein precipitates with GST alone. Equal amounts of active Rac1 and Rac2 are detected in the PNS of control and Nef expressing cells. Moreover, active GTPases are detected in low density membrane fractions. Again, the total active Rac1 or Rac2 in Nef expressing and control cells does not significantly differ. Attempts do detect CDC42 failed, likely due to the poor sensitivity of the antibody used. Syn6 levels in each fraction were also determined to confirm that equivalent amounts of lysate from Nef expressing and control cells were used for pull-downs.

Chapter 7: Nef induces the phosphorylation of Merlin on S518.

In this section we test the ability of Nef to induce phosphorylation of Merlin, the product of the neurofibromatosis 2 gene. Co-expression of Merlin and active Rac1 or CDC42 has been shown to lead to Merlin phosphorylation at residue S518 (149). The phosphorylation can be detected by a decrease in electrophorteic mobility of Merlin. Two groups have shown that Rac and CDC42 modulate Merlin phosphorylation via endogenous Pak molecules (149;150). Although multiple Pak family members have been shown to lead to Merlin phosphorylation when over-expressed, endogenous Pak2 has been shown to be the primary Pak family member responsible for Merlin S518 phosphorylation (149;150). Moreover, in vitro, Pak2 directly phosphorylates Merlin S518 (150). We show here that, in vivo, Nef expression leads to increased phosphorylation of Merlin on residue S518. Using transient transfection of 293T cells, we demonstrate that Nef_{SF2} increases the apparent electrophoretic mobility of ectopically expressed HA tagged Merlin. In contrast, Nef does not affect the mobility of Merlin containing a S518A mutation. Moreover, $Nef_{D88\text{-}11}$, a Nef primary isolate that is not functional for Pak2 activation, does not effect Merlin phosphorylation. The Nef_{SF2} induced increase in electrophoretic mobility of wild type Merlin is reversed by phosphatase treatment. Thus, these data indicate that Nef initiates Pak2 signaling cascades in cells.

Expression of active Rac or Nef_{SF2} leads to modification of Merlin at residue S518. To explore the effect of Nef on Merlin phosphorylation, I first co-transfected either an

HA tagged wild type Merlin expression plasmid or HA tagged mutant $Merlin_{S518A}$ with either a control plasmid, Rac_{Q61L}, Nef_{SF2} or Nef_{D88-11}. Nef_{SF2} is fully functional for Pak2 activation, while Nef_{D88-11} is defective for this function (68). Modification of Merlin was detected by HA western blot analysis (Fig. 23, top panel). When wild type Merlin is expressed alone, two forms are detected: a predominant faster migrating species and a less abundant slower migrating species. In contrast, only one form of $Merlin_{S518A}$ is detected, suggesting that in 293T cells there is some phosphorylated Merlin. When wild-type Merlin was co-expressed with active Rac_{Q61L}, only the slower migrating form is detected. As expected, $Merlin_{S518A}$ was not affected by Rac_{Q61L} expression. Co-expression of Nef_{SF2} produces a similar result; wild-type Merlin is found only in the slower migrating form, while $Merlin_{S518A}$ is not affected. Nef_{D88-11}, in contrast does not significantly effect migration of Merlin. Thus, these findings are consistent with Nef inducing phosphorylation of Merlin at site S518 via Pak2. Expression of RacQ61L and Nef was confirmed by western blot analysis (Fig. 23, bottom panels).

Phosphatase treatment reverses the effect of Nef expression on Merlin. In order to confirm that the modification of Merlin induced by Nef is phosphorylation, the effects of phosphatase on Nef induced modification of Merlin were tested. 293T cells were co-transfected with HA-Merlin and either a control or Nef expressing plasmid. HA immunoprecipitations were then performed and precipitates were either mock treated or treated with CIP. Samples were then resolved by SDS-PAGE and Merlin was detected by western blot analysis. As shown in figure 24, control lysates contain two species of Merlin with the predominant form having a faster electrophoretic mobility. After

phosphatase treatment, only the faster migrating species is detected. As expected, the mock treated lysates from Nef expressing cells contain only the slower migrating Merlin species. When immunoprecipitates from these lysates were CIP treated, however, the electrophoretic mobility of Merlin increased. Thus, the Nef induced modification of Merlin is phosphorylation. I conclude that Nef induces phosphorylation of Merlin at residue S518 via Pak2.

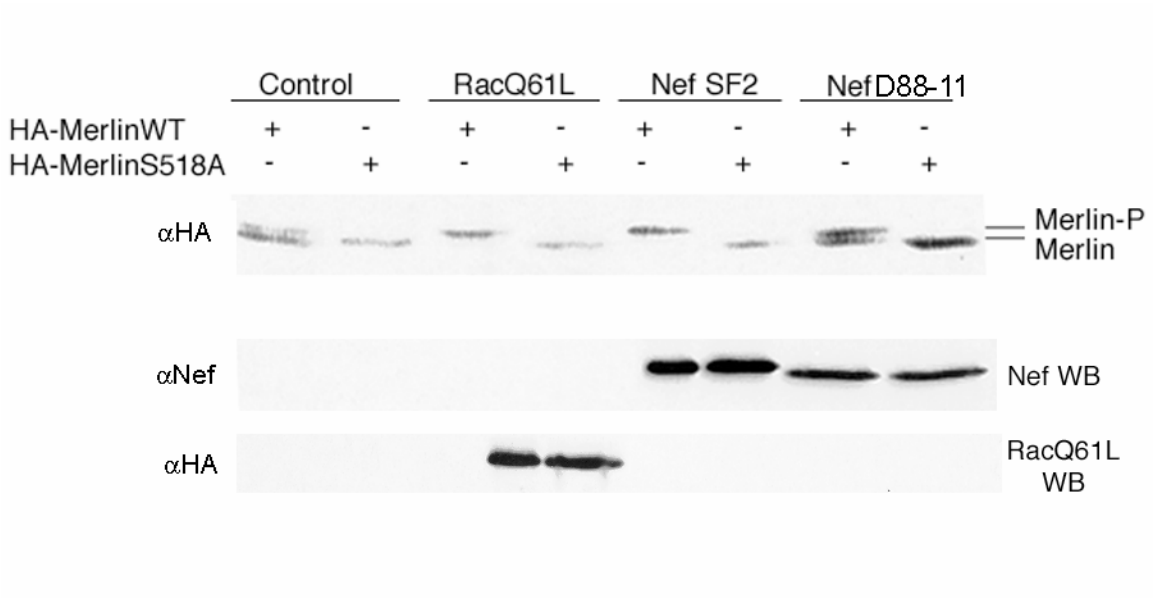


Figure 23: Nef SF2 induces a modification at Merlin S518. 239T cells were co-transfected with plasmid expressing HA tagged wild-type Merlin or Merlin S518A and either control, active Rac(Q61L), Nef_{SF2}, or $Nef_{88\text{-}11}$ expressing plasmid. Top: Wild-type Merlin expressed alone is detected as a doublet with the primary species having a faster mobility. Active Rac and Nef_{SF2} expression shifts all of the wild-type Merlin to the slower migrating form. Mobility of mutant Merlin is not affected suggesting that the alteration induced by Nef occurs at residue 518. $Nef_{D88\text{-}11}$, which has not detectable Pak2 activation function, does not significantly change the mobility of Merlin. Bottom: Expression of Nef's and RacQ61L was confirmed by western blot.

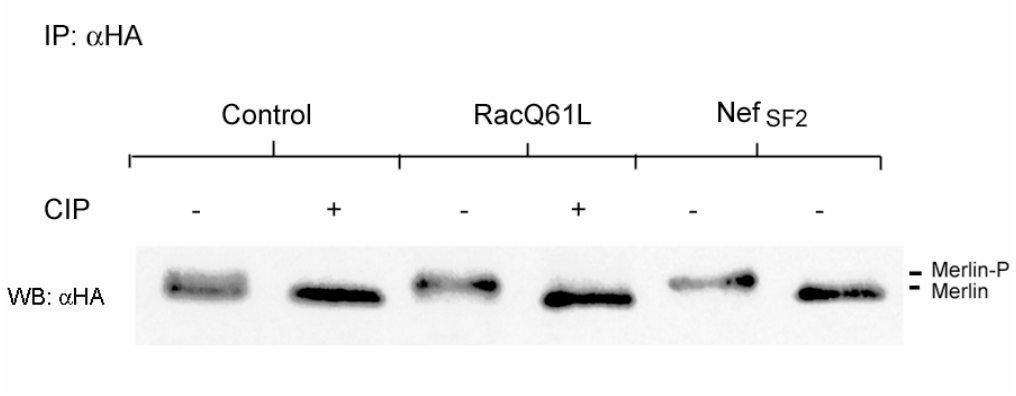


Figure 24. Nef modification of Merlin is reversed by phosphatase treatment. Lysates of 293T cells transfected with either HA-Merlin alone or HA-Merlin and Nef_{SF2} were immunoprecipitated with anti-HA. Precipitates were either mock treated or treated with calf intestine phosphatase (CIP). Precipitates were then analyzed by HA western blot. Note that mock treated wild type Merlin when expressed alone is detected as doublet with the predominant species migrating with a faster mobility. CIP treatment shifts all the detected Merlin to the faster mobility. When expressed with Nef, mock treated Merlin is detected only as a slower migrating form, but after CIP treatment the apparent mobility increases.

Chapter 8: Discussion

In the work presented here, a number of issues regarding a previously well documented kinase activity associated with Nef are addressed. The existence of Nak was first revealed by Sawai et al. who detected the 62 kDa kinase by in vitro kinase assay using Nef immunoprecipitates (123). Subsequent work from this laboratory and others demonstrated Nak association with Nef to be a highly conserved property of Nef demonstrable in a number of experimental systems (69;125). As primate lentiviruses replicate actively and mutate rapidly, the demonstration of Nak association with Nefs from distantly related primate lentiviruses is suggestive of Nak playing an important role in the execution of a function for which Nef evolved. (151). I reasoned that identifying and characterizing Nak was thus a worthwhile endeavor that would pave the way to better understanding cellular pathways critical to efficient HIV replication in vivo.

I first explored Nak's identity. Both Pak1 and Pak2 proteins had been previously identified as Nak (126;127). Here, I provided conclusive evidence that Nak is Pak2. The high degree of structural similarity shared by the Pak family members is likely the cause of discrepancies regarding the identity of Nak. Commercial antibodies used to identify Nak as Pak1 have indeed been demonstrated to be cross reactive with Pak2 (126). Peptide inhibitors of Pak1 have also been used to identify Nak as Pak1 (127). While it has been argued, based on sequence comparison, that these peptides specifically inhibit Pak1, no experimental evidence to support this claim has been offered. Given the extensive homology of the Pak1 and Pak2 catalytic domains, it is necessary to demonstrate inhibitory specificities of these proteins. In light of the extensive homology

between Pak1 and Pak2, it is also reasonable to speculate that under certain *in vitro* conditions both Pak1 and Pak2 may associate with Nef. However, my results show that Nef does not activate Pak1 in the cell, even under over-expressed conditions.

An important difference between my results and those of Renkema et. al. (126) who had identified Nak as Pak2 prior to me is that I did not need to co-express a constitutively active form of Rac or CDC42 to observe robust Pak2 autophosphorylation. The most likely explanation for this difference is allelic variation between Nef_{NL4-3}, which they used for their experiments, and Nef_{SF2} used for most of our experiments. I confirmed our laboratory's published observation that, in the presence of similar amounts of Nef, Nef_{SF2} produces significantly greater Nak activity than Nef_{NL4-3}(69). Activation of endogenous Pak2 by $CDC42_{G12V}$ could have certainly facilitated the detection of active Pak2 in their experiments, but also raises the possibility that the activity observed in those experiments might be distinct from that observed in the absence of activated p21. The work presented here, however, refutes that possibility. The need for co-expression of active CDC42 to detect Pak2 in their system, moreover, led Renkema et al. to conclude that Nef does not mediate Pak2 activation, but instead associates with an already active Pak2 sub-population. Since over-expressed constitutively active p21s are sufficient to activate Pak2, their experiments could not accurately assess whether Nef mediates Pak2 activation. I first showed here that Nef indeed mediates the activation of ectopically expressed Pak2 in 293T cells. I also investigated the ability of Nef to mediate Pak2 activation in a system more relevant to HIV infection. To do this, I used a modified version of an in gel kinase assay protocal originally developed by Dr. John Foster. This assay was used to determine cellular Pak2 activation levels in a T cell line in the presence

or absence of Nef and demonstrated that Nef expression results in Pak2 activation, confirming the functional regulation of Pak2 by Nef.

Current models of Pak activation were used to begin elucidating a mechanism. Membrane targeting of Pak family members is likely critical for Pak function. For example, the cellular Nck adaptor protein is thought to mediate Pak activation by binding and recruiting Pak1 to the membranes in response to tyrosine phosphorylation of surface receptors (152). The addition of membrane localization sequences to Pak1, moreover, has been shown to be sufficient to induce its activation (152). At the cell membrane, Paks encounter high concentrations of direct activators. For example, pools of active forms of CDC42/Rac localize to membranes due to lipid modifications (152). Recruitment of Paks to membranes, thus, would expose Paks to concentrated pools of active GTPases, facilitating the binding of GTPases to the Pak regulatory region. Consistent with this model, active GTPases have been shown to be important for the activation of membrane localized Pak (143). GTPase independent mechanisms of Pak activation may also exist at membranes. This conclusion is based on the observation that Pak1 mutated at the GTPase binding domain retains a minor ability to be activated by membrane localization (146). It is speculated that this effect is due to membrane sphingolipids, which, in vitro, are sufficient for detectable Pak1 activation (146). Thus, membrane localization may activate Paks by multiple mechanisms. Another GTPase independent mode of Pak2 activation is via proteolytic cleavage (137). Caspase activation during apoptosis leads to Pak2 cleavage, releasing auto-inhibition of the kinase domain and thereby inducing activation (138). Interestingly, my analysis of the Pak2 sequence showed that Caspase cleavage reveals a putative N-terminal myristolation site

on the kinase domain containing fragment. The use of cryptic myristolation sites for membrane localization following proteolytic cleavage has been demonstrated for the Caspase target Bid (153). Although Caspase activated Pak2 has not been localized, it may utilize a mechanism similar to Bid and localize to membranes. Thus, membrane localization appears to be a highly significant aspect of Pak activation, suggesting active Paks encounter their targets at the membrane.

The work here showed that Nef also leads to activation of Pak2 on cellular membranes. This is consistent with the observation by others that myristolation targets Nef to membranes and is also important for Nak association (112). I showed here that virtually all of the active Pak2 is found in a complex containing Nef. Thus, Nef likely activates and retains Pak2 in a membrane complex. Moreover, most of the Pak2 in the cells, however, is inactive and not membrane associated. Nef may recruit Pak2 to membranes were it is then activated. Attempts so far, however, have not detected any Pak2 in the membrane fractions by western blot analysis, consistent with the low abundance of the Nef/Pak2 complex. Recruitment of Pak2 to cell membranes by Nef, therefore, cannot be formally concluded. However, as the kinase assay results demonstrate, there is indeed Pak2 on cell membranes. Future attempts using larger scale membrane preparations should demonstrate, by western blot, the presence of Pak2 on cell membranes. The effect of Nef on Pak2 membrane localization can then be assessed by comparing membrane associated Pak2 in control cells and cells expressing Nef. Such a demonstration would further substantiate a model in which Nef recruits Pak2 to the membrane, leading to Pak2 activation.

The role of endogenous GTPases in Nef mediated Pak2 activation was also addressed. Over-expression of RhoGDIα inhibited Nef mediated Pak2 activity. RhoGDIα over-expression has been shown to be an effective inhibitor of Rac and CDC42 binding to the Pak GTPase binding domain (143). The mechanism of action is uncertain. RhoGDIα has been suggested to inhibit GDP disassociation as well as to sequester GTPases in the cytosol (144;145). In either case the inhibition of RhoGTPase effector function inversely correlates with Nef association with active Pak2. Mutation of the Pak2 GTPase binding domain also ablates Nef induced activation of Pak2. While we cannot exclude the possibility that sphingolipids also play a subtle role in activation of Nef bound Pak2, we do not detect any effect of Nef on the activity of a Pak2 molecule mutated at the GTPase binding domain. GTPase binding domain mutant Paks are activated by sphingolipids (146). Thus, endogenous Rho family GTPases are likely the most important final effectors in Nef mediated Pak2 activation.

The specific Rho family GTPase(s) involved in Nef mediated Pak2 activation have not been identified. Rac1, Rac2, and CDC42 can all activate Pak2. It is possible that one, two, or all three of these play a role in Nef mediated Pak2 activation. My attempts to identify any of these GTPases in Nef immunoprecipitates by western blot have been unsuccessful (not shown). The use of specific dominant negative mutant GTPases has also been described (128). The most commonly used so-called ‘dominant negatives’ have an asparagine mutation at residue 17. This mutation is thought to either lock the GTPase in the GDP bound or nucleotide-free state. Like all GTPases, Rho family GTPases are activated by guanine nucleotide exchange factors (GEFs). The ‘dominant negative’ GTPase mutants cannot be activated, but bind GEFs and are thought

to sequester endogenous GEFs. This model is predicted mostly from work done on other similar 21 kDa GTPases. Although phenotypes have been reported with Rac and $CDC42_{N17}$ mutants, including reduced Nak association with Nef (128), the ability of these N17 mutants to confer a dominant negative phenotype toward their respective targets has not been directly shown. In fact, Lu et. al. showed that these mutants had little effect on Rac or CDC42 binding activity to the Pak GTPase binding domain over a broad range of expression (143). Moreover, $CDC42_{N17}$ at high expression levels was shown to have a slight positive effect on Pak activation (143). This is consistent with our PBD staining of 293T cells in which a very slight increase in staining was detected with over-expressed $CDC42_{N17}$. A similar slight activating phenotype of $CDC42_{N17}$ has also been observed when using the GTPase binding domain of the CDC42 effector WASP to detect activity in 3T3 cells (Abhi Seth, personal communication). When I attempted to use N17 mutants to inhibit Nef mediated Pak2 activation in 293T cells, I did not observe an inhibitory effect (not shown). It is possible that these mutants only confer a 'dominant negative' activity in limited cells types. Until their biochemical phenotypes are better characterized, these molecules cannot be used to identify the specific GTPases involved in Nef mediated Pak2 activation. Interestingly, a Cdc42 mutation D57Y was shown by Lu et. al. to inhibit endogenous CDC42 activity, but not Rac (143). The future use of specific, *de facto* dominant negative CDC42 and perhaps and analogous Rac may decipher if these GTPases are involved in Nef mediated Pak2 activation. It is also possible that yet undescribed endogenous RhoGTPases may play an important role.

The ability of Nef to induce Pak2 activation via Rho family GTPases is clear. Some have suggested that Nef may associate with Vav (in a complex with Pak1), a

guanine nucleotide exchange factor (GEF) that activates Rac and CDC42 (154). Others have not found Vav in the complex, but have suggested that Pix is also found in the Nef/Pak2 complex (155). As Pix has GEF activity toward Rac, these reports raised the possibility that Nef may actually mediate GTPase activation via regulation of endogenous GEFs. I took two approaches to test this hypothesis. I first developed a cell-based assay to detect total activity levels of Pak activating GTPases by FACS. This approach overcame two major limitations of an already existing method for detecting active GTPases using a GST-PBD 'pull-down' assay. The 'pull-down' assay can only assess activation levels of known GTPases with good antibodies for their detection. Furthermore, there is the potential for GTPase hydrolysis due to GTPase activating protein (GAP) activity in solution during the procedure. Our novel approach had the advantage of using fixed cells so that GAP activity would be minimized. Moreover, it should detect total cellular activity levels of all endogenous GTPases capable of binding the Pak2 GTPase binding domain. I showed that this method does indeed detect active GTPase. Moreover, it detected constitutive GTPase activity in control cells. Nef, however, did not induce a detectable alteration in GTPase activity levels. Consistent with this view, the conventional 'pull down' assay showed that stable Nef expression does not affect levels of GTP loaded Rac1 or Rac2 in T cells. Unfortunately, I was unable to detect any active CDC42 due to the low sensitivity of the antibody used; therefore, I cannot conclude that Nef does not affect CDC42 activation in T cells. Together, these findings, however, are consistent with a lack of a role for Nef in regulating GEF activity levels. Since we cannot rule out changes in GTPase activity levels falling below our limit of detection, it is possible that Nef mediates highly localized changes in GEF activity that

represent only a small fraction of total constitutive GEF activity. As the Nef/Pak2 complex is of low abundance, such localized regulation could be sufficient to mediate Pak2 activation. However, we have no data to validate such a model.

Instead this data suggests that Nef acts as an adaptor, recruiting Pak2 to cellular membranes were it encounters endogenous active GTPases. I detected constitutively active GTPases in 293T cells and CEM T cells. Moreover, I showed in a T cell line that Nef staining significantly overlaps with the staining pattern observed with the p21 binding domain of Pak. Thus, Nef may mediate Pak activation by spatial regulation, bringing Pak from the cytosol to sites of active GTPases. Nef and PBD staining showed a peri-nuclear distribution in CEM cells. Thus Pak activation may occur on intracellular membranes. Consistent with this view, others have localized Rho family GTPases that active Paks to endomembranes (144). Moreover, GTP bound CDC42 has also been shown to bind the gamma subunit of the coatamer complex that cycles between the ER and golgi (156). Interestingly, Nef has been shown to bind to the subunit of this complex, further attesting to the co-localization of Nef and active GTPases on intracellular membranes (67). Also supporting the possibility that Nef mediated Pak2 activation occurs on endomembranes, the GTPase Ras, has been recently shown to activate signaling cascades on Golgi and ER membranes (157).

The components of the Nef/Pak2 complex are not entirely known. Only a small fraction of expressed Nef and Pak2 in our experiments are found together in a tightly bound complex. This observation is consistent with a modified Nef sub-population specifically interacting with Pak2 or with another cellular factor limiting complex formation. This factor could simply be active GTPase. However, it is not possible to

rule out that other cellular factors may also be important. The SH3 ligand of Nef is critical for Nak activity (131), and presumably Pak2 activation. As neither Pak2 or Rho family GTPases contain SH3 domains, it is reasonable to speculate that another SH3 domain protein is found in the Nef/Pak2 complex. Vav was proposed to serve as this SH3 domain containing protein and was proposed to be activated by Nef, serving as a critical GEF for Pak activating GTPases (158). Others, however, have not found Vav in the Nef/Pak2 complex or have suggested that the Vav SH3 binding domain does not strongly interact with Nef in vitro (130;155). My data, moreover, is not consistent with activation of GEFs by Nef. Others have suggested that □Pix is found in the Nef/Pak2 complex (155). Pix binds with high affinity to Pak and, it is reasonable that Pix would co-precipitate with Pak (159). Pix has also been shown to regulate Pak activation both via Rac GEF activity as well as GTPase independent mechanisms (159). While we cannot rule out subtle contributions of Pix to Nef mediated Pak2 regulation, a Pix binding mutant of Pak2 was still activated by Nef (not shown). Thus, Pak2 bound Pix is not necessary for Nef activation of Pak2. It is also not known if Pak2 directly interacts with Nef. Fackler et. al. who identified Nak as Pak1 showed that in vitro Pak1 and Nef interact (127). This does not seem to the be the case in vivo. It is assumed that Pak2 and Nef directly interact in cells and that the highly homologous Pak1 may also complex with Nef in vitro. However, as we see no evidence for Pak1 activation either by in vitro kinase assay or by our in gel kinase assays (in which Pak1 activity can be detected (not shown)), it is unlikely that Nef and Pak1 from a tightly bound complex in cells. If they did, Pak1 would likely be activated as all the known GTPases that activate Pak2 at cellular membranes are known to also activate Pak1, which has an identical GTPase

binding domain. Thus the specificity of Nef for Pak2 likely comes at the level of complex formation. Future work, perhaps using purified components, will be needed to determine whether Nef and Pak2 directly interact, and what cellular factors, if any, are required to stabilize their interaction.

We were also able to demonstrate that Nef expression leads to phosphorylation of the Pak2 substrate Merlin in vivo. While we have not yet inhibited Pak2 activity to show that Merlin phosphorylation in Nef expressing cells is mediated by Pak2, my work strongly suggests that this is the case. First, Nef leads to phosphorylation of Merlin at position S518, a specific Pak2 substrate (149;150). Moreover, the primary isolate Nef_{D88-11} does not induce detectable Pak2 activation and also does not induce Merlin phosphorylation (Fig. 23). Thus, Nef activated Pak2 likely phosphorylates substrate in cells, which supports the conclusion that the Nef/Pak2 complex initiates Pak2 signaling cascades (Fig. 25).

The role of the Pak2 pathway in Nef function. No Nef mediated cellular phenotype is clearly dependent on Pak2. However, correlative studies provide some insight into possible roles for Pak2 in mediating Nef function. A high degree of genetic diversity is found amongst primary Nef isolates (160). This laboratory has characterized functional defects in eight primary HIV Nef isolates obtained from AIDS patients and a long term non-progressor. This work led by Dr. John Foster identified three isolates, 233, D90-7, and D88-11, that are defective for Pak2 activation as determined by in vitro kinase assay. These isolates have also been evaluated for CD4 down-modulation, MHC class I down-modulation, and infectivity enhancement. Nef 233 and D90-7 are functional in all of these functions. D88-11 is also functional for CD4 down-modulation and infectivity

enhancement, but partially defective for MHC class I down-modulation. The genetic basis for the Nak association defect in 233 was determined to be due to the 'mutation' F193I. At the same position, D90-7 also contains a substitution, F193Y, which is presumed to be the basis of the Nak association/activation defect in this isolate as well. The S189R 'mutation' found in D88-11 accounts for the Nak association/activation defect in that isolate. Introduction of F193I or S189R into a consensus Nef background, D.Con, was shown to be sufficient to ablate Pak activity demonstrated by our standard in vitro kinase assay procedure (68). We have shown that these point mutants also have CD4 and MHC class I down-modulation activities similar to the isolates from which the mutations were derived (i.e. D.ConF193I is fully functional for both, while D.ConS189R is completely functional for CD4 down-modulation, but partially defective for MHC class I down-modulation) (160). Thus, these studies reveal no obvious correlation between Pak2 activation and Nef induced CD4 down-modulation, infectivity enhancement or MHC class I down-modulation.

However, the S189R mutant is defective for both MHC class I down-modulation and Pak2 activation. The basis for the dual defect observed in $Nef_{D88\text{-}11}$ or $Nef_{D.ConS189R}$ is not clear. It could simply reflect the non-conservative mutation mediating global structural changes in Nef. Or perhaps residue S189 is in a region of the protein with overlapping function. Such a conclusion would suggest an even more complex view of Nef function in which overlapping domains have evolved distinct functions. A third possibility is that MHC class I and Pak2 activation are related phenomena. To this end, D.ConS189R and D.ConF193I mutants have been analyzed by John Blakemore and Rene Molina in our laboratory using the in gel assay. These data show defective, but detectable Pak2

activation by D.ConF193I. In contrast, D.conS189R shows no activity. Thus, D.ConF193I, and perhaps the isolates from which this mutant was derived, do in fact mediate Pak2 activation. Now that a Pak2 substrate, Merlin, has been identified, the ability of these Nefs to induce phosphorylation of Pak2 targets in vivo can be determined. If this residual activity proves sufficient to induce Merlin phosphorylation, a correlation between Pak2 and MHC class I down-modulation may exist. The first proline of the proline rich region of HIV Nef has also been shown by others to be important for Pak association. Rene Molina in this laboratory constructed this mutant, D.ConP71A, on the consensus Nef background and confirmed the importance of this residue for Pak2 activity by in vitro kinase assay. He also analyzed its ability to down-modulate MHC class I and found its phenotype to be similar to $Nef_{D88\text{-}11}$ and $Nef_{D.ConS189R}$, partially defective for this function. This could again be due to global structural changes associated with proline mutation, overlapping functional domains in Nef, or an association between Nef mediated Pak2 activation and Nef-induced MHC class I downmodulation. These data are summarized below in table 1. They suggest the exciting possibility that Pak2 activation by Nef is important for MHC class I down-modulation. However, such a correlation assumes that the minimal amount of Pak2 activation by D.ConF193I is sufficient to mediate Pak2 effector functions in cells. This possibility is worthy of future consideration.

Table 1: Summary of functional analysis of Nef primary isolates and mutants performed in our laboratory.

	D.Con	D90-77	233	$D.Con_{F193I}$	D88-11	$D.Con_{S189R}$	$D.Con_{P71A}$
CD4 ↓	+	+	+	+	+	+	+
Infectivity	+	+	+	+	+	+	N/A
MHC I↓	+	+	+	+	+/-	+/-	+/-
In vitro kinase activity	+	-	-	-	-	-	-
In gel kinase activity	+	N/D	N/D	+/-	-	-	N/A

N/A = Not Done

It is also tempting to implicate Pak2 in Nef mediated T cell activation. I have shown that stimulation of Jurkat cells with PHA leads to modest activation of an approximately 62 kDa and 68 kDa kinases using an in gel kinase assay with histone H4 substrate (not shown). These kinases could represent Pak1 and Pak2, suggesting their activation is associated with T cell activation. T cell activation is often considered in the Nef field as a specific event. Activation of the T cell receptor, however, is associated with a broad range of cellular responses including changes in morphology, gene transcription, and release of effector molecules (161). Moreover, the nature of a specific T cell response to activation varies based on its ontogeny and context of exposure to stimulus (161). Thus the aspects, if any, of T cell activation regulated by Nef need to be specifically defined. Gene array analysis and NFAT studies suggest that Nef plays a role in regulating gene transcription in an analogous manner to T cell receptor activation (32;93). Interestingly, Pak1 has been shown by others to be important for NFAT activity following T cell receptor stimulation (162). Some data shows a correlation between the modulation of

NFAT activity by Nef and Nef associated Pak2 activity (93). However, the direct Pak2 dependence of NFAT regulation by Nef has not been addressed. A Pak1 dominant negative mutant did not impair Nef regulation of NFAT (163). It is not known if this Pak1DN also inhibits Pak2.

Moreover, inconsistencies in the literature make it difficult to define a precise role of Nef in NFAT regulation. For example, in gene array studies Nef alone alters gene expression profiles (32). In NFAT studies Nef only has an effect when cells are also treated with PMA, a situation that is of questionable physiologic relevance (93). Thus, whether Nef regulates NFAT in vivo is not clear. Earlier studies have actually suggested a negative role for Nef in the regulation of IL-2 production following TCR stimulation (94). While the reason for these discrepancies is not known and may be in part due to differences in *nef* alleles used, it is also possible that Nef actually evolved to mediate a different phenomena associated with T cell activation. Perhaps due to the complex interplay of activation associated events, different effects on NFAT activity are observed in different settings, but none of these reflect a *de facto* Nef function. In any case, there is insufficient evidence to implicate Nef mediated Pak2 activation in NFAT activation and gene regulation in T cells.

It is also possible that Nef initiates multiple T cell activation associated pathways and that NFAT activation and Pak2 activation represent divergent pathways that share common upstream regulators. To this end, a recent report suggests that Nef induces sustained calcium elevation via association with an inositol phosphate receptor residing on the endoplasmic reticulum (164). Sustained calcium elevation is known to be critical for NFAT activity. In neutrophils, calcium has been shown to be important for Pak

activation in response to chemo-attractants (165). Thus Nef mediated Ca++ signaling could serve as a common upstream aspect of Nef mediated NFAT effects and Pak activation. However, the necessity of calcium signaling in Pak2 activation by Nef has not been tested.

The Pak2 pathway may regulate a cellular process distinct from gene transcription, but also associated with T cell activation. The known functions of the cellular factors identified in this pathway--Rho family GTPases, Pak2, and Merlin--are broad, making it difficult to speculate a specific role they might play in Nef function. Moreover, much of the work studying their cellular functions has been done in non-hematopoietic cells. However, a common aspect to RhoGTPase, Pak, and Merlin is their implication in the regulation of membrane and actin cytoskeleton dynamics (166-168). In T cells, Rac and CDC42 have been implicated in actin cytoskeleton rearrangements following T cell receptor stimulation (169). More recently, it has been shown that following engagement of the T cell receptor, a polarized axis of Golgi and lipid rafts forms along which cytokines are exocytosed toward the antigen presenting cell (170). Rho family GTPases have been implicated in such polarization of golgi during T cell activation (170). Although Merlin has not been specifically studied in hematopoietic cells, it is similar to the ERM (**E**zrin, **R**adixin, **M**oesin) proteins which play roles in linking the actin cytoskeleton to cellular membranes (168). Some models suggest Merlin negatively regulates ERM proteins (168). The role of phosphorylation by Pak2 in regulating Merlin function is not known, but is suggested to negatively regulate Merlin activity (168). Thus Merlin phosphorylation could lead to increased activity of ERM proteins. Interestingly, activity of Ezrin has been shown to be critical for T cell activation due to its role in the

generation of the immunological synapse (171). Via Merlin phosphorylation, Nef, furthermore, could regulate Ezrin activity. Thus Nef may play a role in regulating the membrane/cytoskeletal dynamics. Consistent with this view, Nef localizes to membrane and also appears to be integrally connected to the actin cytoskeleton network (114;115).

Use of systems described in the work presented here are useful for identifying and characterizing molecules regulated by Nef. These systems have shown that Nef modulates cellular proteins involved in the regulation of actin cytoskeleton. However, translating the effects of Nef activated Pak2 to cell phenotypes and eventually enhanced viral replication may require the development of new systems. Hematopoietic cells are faced with the unique challenge of having to migrate over large distances, recognize specific targets, and then alter their morphology as to execute effector functions in a directional manner. Thus, the cytoskeletal regulation in these cell types is undoubtedly complex and unique. Determining the precise morphological consequences of Nef expression at a minimum must be done in HIV target cells, specifically monocyte/macrophages and T cells. As cytoskeletal regulation is significantly altered by cellular transformation, the use of laboratory cell lines may be inappropriate for the study of Nef on cellular morphology (172). They may mask the importance of Pak2 activation for Nef associated cellular phenotypes. If analyzed in an appropriate setting, Nef expression may, for example, produce a clear morphological phenotype. However, development of such systems will be difficult, as high level transduction of primary hematopoietic cells is most easily accomplished with potent cellular activation with agents such as PHA. Such methods of stimulation are undoubtedly associated with their own morphological consequences. Our laboratory has been able to generate Nef

expressing lentivirus vectors. Lentiviruses can transduce non-dividing, primary human cells. Now that I have clearly shown that Nef activates Pak2 and regulates Merlin phosphorylation, development of systems that can assess the role of Nef on actin dynamics in a more physiological environment are justified.

The role of Pak2 in viral replication and disease: A possible masked phenotypic effect of Pak2 activation in laboratory cell systems suggests that appreciating a role for Pak2 in viral replication will also require the use of specialized systems. Indeed Nef does not enhance viral replication in many in vitro systems (8;9). Interestingly, in two in vitro systems where Nef is reported to have an effect on viral replication, there is correlative evidence implicating Pak2 activation in this process (173;174). Apparently stable SH3 ligand mutants of Nef are associated with attenuated viral replication in post-infection stimulated primary mononuclear cells and primary immature dendritic cell T cell co-cultures (173;174). Thus, a lack of Pak2 association correlates with impaired replication in these systems. The importance of Nef mediated Pak2 activation has also been studied in vivo (129;173;175;176). These studies utilize Nef mutants defective for Nak association. The first such study investigated the in vivo replication of an SIV_{mac239} encoding a Nef mutated at two arginine residues (RR-LL) (129). This mutant Nef was shown to be defective in its ability to associate with Nak (129). Following infection of macaques, the RR-LL mutant reverted before disease progression. The authors interpreted this as a demonstration that there is in vivo selection for Nef to activate Nak and that Nak activation is important for disease progression. However, the conclusions of this study were later challenged by observations made by our laboratory indicating that

the RR-LL mutant Nef is unstable and also defective for CD4 down-modulation (68). Thus the selective force driving reversion in these monkeys is not clear. Others have studied the importance of the SH3 binding domain in vivo. Infection of monkeys with a SIV_{mac239} mutated at the Nef SH3 binding domain has produced conflicting results. Three such studies have been performed and together suggest that there is significant (although not absolute) reversion of the PXXP mutant in vivo and that reversion does not always precede disease progression (173;175;176). Thus, Nak association is not likely necessary for lentivirus replication in all in vivo contexts. Nonetheless, there is in vivo selective pressure to maintain Nak activity and the PXXP motif across primate lentivirus Nefs in the midst of active viral replication, and the associated high rate of mutation.

Studies in transgenic mice suggest that in the process of optimizing the host environment for viral replication, Nef may directly mediate some of the pathogenic properties of lentiviruses (21). Interestingly, subsequent work using transgenic animals expressing equal or greater amount of a mutant PXXP mutant Nef showed no disease phenotype (177). Thus, events mediated by Nef's PXXP motif are critical for disease induction in this system. Moreover, disease induction was shown to not be a result of association of the Nef SH3 ligand with Hck as expressing wild-type Nef in a Hck null mouse still produces disease. The value of transgenic mouse models for the study of in vivo infection, however, is unclear and it remains to be demonstrated that specific mutants defective in Pak2 activation are nonpathogenic.

Implications for AIDS research: Elucidation of the interactions between virus and host is critical to our basic understanding of HIV biology. While the contribution of essential

viral genes to the viral life cycle are relatively well appreciated, current knowledge of how the virus utilizes as well as evades host biology is far from complete. Existing characterization of the Nef protein suggests that Nef plays an important role in regulating the host environment for efficient viral replication. The work presented here identifies and characterizes cellular factors regulated by Nef. Such work is essential to understanding the mechanisms by which Nef may modulate the host environment for more efficient, and more pathogenic, virus infection. As the role in the viral life cycle of the cellular factors described here are further elucidated, new therapeutics that inhibit viral replication by targeting host factors may be rationally approached. Moreover, the correlation between Pak2 activation and disease in the transgenic mouse model in which no viral replication occurs raises the possibility that the continued study of Nef mediated Pak2 activation and its downstream cellular consequences may lead to novel approaches to alleviate disease.

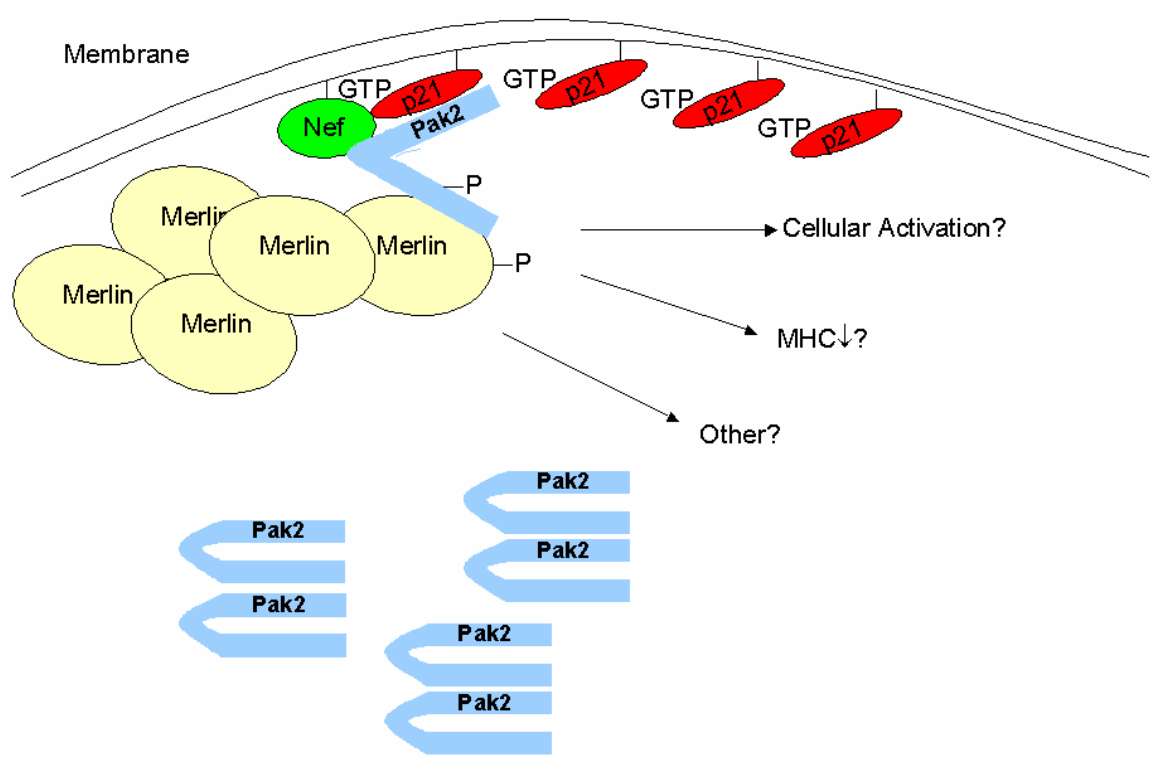


Figure 25: Model of Nef mediated Pak2 activation. Inactive Pak2 localizes to the cytosol. Nef leads to the recruitment of Pak2 to cell membranes where Pak2 encounters active p21 GTPases which, in turn, leads to autophosphoylation and activation of Pak2. Active Pak2 can then phosphorylate substrates such as Merlin depicted here. The activation of Pak2 may lead to cellular phenotypes such as MHC class I down-modulation or lymphocyte activation as discussed in text.

REFERENCE LIST

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

2. **Cullen, B. R.** 1994. The role of Nef in the replication cycle of the human and simian immunodeficiency viruses. Virology **205**:1-6.

3. **Klotman, M. E., S. Y. Kim, A. Buchbinder, A. DeRossi, D. Baltimore, and F. Wong-Staal**. 1992. Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proceedings of the National Academy of Sciences of the United States of America **89**:1148.

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

5. **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.

6. **Miller, M. D., M. B. Feinberg, and W. C. Greene**. 1994. The HIV-1 nef gene acts as a positive viral infectivity factor. Trends in Microbiology **2**:294-298.

7. **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. Journal of Virology **68**:2906-2914.

8. **Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene**. 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proceedings of the National Academy of Sciences of the United States of America **86**:9549-9553.

9. **Kim, S., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore**. 1989. Lack of a negative influence on viral growth by the nef gene of human immunodeficiency virus type 1. Proceedings of the National Academy of Sciences of the United States of America **86** :9544-9548.

10. **Arora, V. K., B. L. Fredericksen, and J. V. Garcia**. 2002. Nef: agent of cell subversion. Microbes & Infection **4**:189-199.

11. **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. New England Journal of Medicine **340**:1715-1722.

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

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

14. **Birch, M. R., J. C. Learmont, W. B. Dyer, N. J. Deacon, J. J. Zaunders, N. Saksena, A. L. Cunningham, J. Mills, and J. S. Sullivan**. 2001. An examination of signs of disease progression in survivors of the Sydney Blood Bank Cohort (SBBC). J Clin Virol **22**:263-270.

15. **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. New England Journal of Medicine **332**:228-232.

16. **Salvi, R., A. R. Garbuglia, A. Di Caro, S. Pulciani, F. Montella, and A. Benedetto**. 1998. Grossly defective nef gene sequences in a human immunodeficiency virus type 1-seropositive long-term nonprogressor. Journal of Virology **72**:3646-3657.

17. **Rhodes, D. I., L. Ashton, A. Solomon, A. Carr, D. Cooper, J. Kaldor, and N. Deacon**. 2000. Characterization of three nef-defective human immunodeficiency virus type 1 strains associated with long-term nonprogression. Australian Long-Term Nonprogressor Study Group. Journal of Virology **74**:10581-10588.

18. **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. Nature Medicine **5**:194-203.

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

20. **Duus, K. M., E. D. Miller, J. A. Smith, G. I. Kovalev, and L. Su**. 2001. Separation of human immunodeficiency virus type 1 replication from nef-mediated pathogenesis in the human thymus. Journal of Virology **75**:3916-3924.

21. **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.

22. **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. Journal of Virology **60**:754-760.

23. **Fackler, O. T., P. D'Aloja, A. S. Baur, M. Federico, and B. M. Peterlin**. 2001. Nef from human immunodeficiency virus type 1(f12) inhibits viral production and infectivity. Journal of Virology **75**:6601-6608.

24. **Jamieson, B. D., G. M. Aldrovandi, V. Planelles, J. B. Jowett, L. Gao, L. M. Bloch, I. S. Chen, and J. A. Zack**. 1994. Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity. Journal of Virology **68**:3478-3485.

25. **Alexander, L., Z. Du, M. Rosenzweig, J. U. Jung, and R. C. Desrosiers**. 1997. A role for natural simian immunodeficiency virus and human immunodeficiency virus type 1 nef alleles in lymphocyte activation. Journal of Virology **71**:6094-6099.

26. **Messmer, D., R. Ignatius, C. Santisteban, R. M. Steinman, and M. Pope**. 2000. The decreased replicative capacity of simian immunodeficiency virus SIVmac239Delta(nef) is manifest in cultures of immature dendritic cellsand T cells. Journal of Virology **74**:2406-2413.

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

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

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

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

31. **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. Nature Medicine **2**:338-342.

32. **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.

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

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

35. **Garcia, J. V., J. Alfano, and A. D. Miller**. 1993. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. Journal of Virology **67**:1511-1516.

36. **Mariani, R. and J. Skowronski**. 1993. CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proceedings of the National Academy of Sciences of the United States of America. **90**:5549-5553.

37. **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. Current Biology. **9**:622-631.

38. **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. Current Biology. **9**:613-621.

39. **Foti, M., A. Mangasarian, V. Piguet, D. P. Lew, K. H. Krause, D. Trono, and J. L. Carpentier**. 1997. Nef-mediated clathrin-coated pit formation. Journal of Cell Biology. **139**:37-47.

40. **Le Gall, S., F. Buseyne, A. Trocha, B. D. Walker, J. M. Heard, and O. Schwartz**. 2000. Distinct trafficking pathways mediate Nef-induced and clathrin-dependent major histocompatibility complex class I down-regulation. Journal of Virology **74**:9256-9266.

41. **Luo, T., B. L. Fredericksen, K. Hasumi, A. Endo, and J. V. Garcia**. 2001. Human immunodeficiency virus type 1 Nef-induced CD4 cell surface downregulation is inhibited by ikarugamycin. Journal of Virology **75**:2488-2492.

42. **Luo, T., S. J. Anderson, and J. V. Garcia**. 1996. Inhibition of Nef- and phorbol ester-induced CD4 degradation by macrolide antibiotics. Journal of Virology **70**:1527-1534.

43. **Sanfridson, A., B. R. Cullen, and C. Doyle**. 1994. The simian immunodeficiency virus Nef protein promotes degradation of CD4 in human T cells. Journal of Biological Chemistry. **269**:3917-3920.

44. **Rhee, S. S. and J. W. Marsh**. 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. Journal of Immunology **152**:5128-5134.

45. **Rhee, S. S. and J. W. Marsh**. 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. Journal of Virology **68**:5156-5163.

46. **Mangasarian, A., M. Foti, C. Aiken, D. Chin, J. L. Carpentier, and D. Trono**. 1997. The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. Immunity **6**:67-77.

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

48. **Harris, M. P. and J. C. Neil**. 1994. Myristoylation-dependent binding of HIV-1 Nef to CD4. Journal of Molecular Biology **241**:136-142.

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

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

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

52. **Anderson, S. J., M. Lenburg, N. R. Landau, and J. V. Garcia**. 1994. The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. Journal of Virology **68**:3092-3101.

53. **Hua, J. and B. R. Cullen**. 1997. Human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus Nef use distinct but overlapping target sites for downregulation of cell surface CD4. Journal of Virology **71**:6742-6748.

54. **Mangasarian, A., V. Piguet, J. K. Wang, Y. L. Chen, and D. Trono**. 1999. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. Journal of Virology **73**:1964-1973.

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

56. **Greenberg, M., L. DeTulleo, I. Rapoport, J. Skowronski, and T. Kirchhausen**. 1998. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. Current Biology. **8**:1239-1242.

57. **Bresnahan, P. A., W. Yonemoto, and W. C. Greene**. 1999. Cutting edge: SIV Nef protein utilizes both leucine- and tyrosine-based protein sorting pathways for down-regulation of CD4. Journal of Immunology **163**:2977-2981.

58. **Craig, H. M., M. W. Pandori, and J. C. Guatelli**. 1998. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. Proceedings of the National Academy of Sciences of the United States of America. **95**:11229-11234.

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

60. **Lock, M., M. E. Greenberg, A. J. Iafrate, T. Swigut, J. Muench, F. Kirchhoff, N. Shohdy, and J. Skowronski**. 1999. Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis. EMBO Journal **18**:2722-2733.

61. **Piguet, V., Y. L. Chen, A. Mangasarian, M. Foti, J. L. Carpentier, and D. Trono**. 1998. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. EMBO Journal **17**:2472-2481.

62. **Xu, T., E. Vasilyeva, and M. Forgac**. 1999. Subunit interactions in the clathrin-coated vesicle vacuolar (H(+))-ATPase complex. Journal of Biological Chemistry **274**:28909-28915.

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

64. **Mandic, R., O. T. Fackler, M. Geyer, T. Linnemann, Y. Zheng, and B. M. Peterlin**. 2001. Negative factor from siv binds to the catalytic subunit of the v-atpase to internalize cd4 and to increase viral infectivity. Molecular Biology of the Cell **12**:463-473.

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

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

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

68. **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. Journal of Virology **75**:1672-1680.

69. **Luo, T. and J. V. Garcia**. 1996. The association of Nef with a cellular serine/threonine kinase and its enhancement of infectivity are viral isolate dependent. Journal of Virology **70**:6493-6496.

70. **Janvier, K., H. Craig, S. Le Gall, R. Benarous, J. Guatelli, O. Schwartz, and S. Benichou**. 2001. Nef-induced CD4 downregulation: a diacidic sequence in human immunodeficiency virus type 1 Nef does not function as a protein sorting motif through direct binding to beta-COP. Journal of Virology **75**:3971-3976.

71. **Liu, L. X., F. Margottin, S. Le Gall, O. Schwartz, L. Selig, R. Benarous, and S. Benichou**. 1997. Binding of HIV-1 Nef to a novel thioesterase enzyme correlates with Nef-mediated CD4 down-regulation. Journal of Biological Chemistry. **272**:13779-13785.

72. **Cohen, G. B., V. S. Rangan, B. K. Chen, S. Smith, and D. Baltimore**. 2000. The human thioesterase II protein binds to a site on HIV-1 Nef critical for CD4 down-regulation. Journal of Biological Chemistry **275**:23097-23105.

73. **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.

74. **Le Gall, S., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J. M. Heard, and O. Schwartz**. 1998. Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. Immunity. **8**:483-495.

75. **Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger, and D. Baltimore**. 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity. **10**:661-671.

76. **Swigut, T., A. J. Iafrate, J. Muench, F. Kirchhoff, and J. Skowronski**. 2000. Simian and human immunodeficiency virus Nef proteins use different surfaces to downregulate class I major histocompatibility complex antigen expression. Journal of Virology **74**:5691-5701.

77. **Betts, M. R., J. P. Casazza, B. A. Patterson, S. Waldrop, W. Trigona, T. M. Fu, F. Kern, L. J. Picker, and R. A. Koup**. 2000. Putative immunodominant human immunodeficiency virus-specific CD8(+) T-cell responses cannot be predicted by major histocompatibility complex class I haplotype. Journal of Virology **74**:9144-9151.

78. **Riggs, N. L., H. M. Craig, M. W. Pandori, and J. C. Guatelli**. 1999. The dileucine-based sorting motif in HIV-1 Nef is not required for down-regulation of class I MHC. Virology **258**:203-207.

79. **Swann, S. A., M. Williams, C. M. Story, K. R. Bobbitt, R. Fleis, and K. L. Collins**. 2001. HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. Virology **282**:267-277.

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

81. **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. Nature Cell Biology **2**:163-167.

82. **Wan, L., S. S. Molloy, L. Thomas, G. Liu, Y. Xiang, S. L. Rybak, and G. Thomas**. - PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. Cell 1998 Jul 24;94(2):205-16205-166.

83. **Bell, I., C. Ashman, J. Maughan, E. Hooker, F. Cook, and T. A. Reinhart**. 1998. Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) zeta chain leads to TCR down-modulation. Journal of General Virology **79**:2717-2727.

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

85. **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.

86. **Sol-Foulon, N., A. Moris, C. Nobile, C. Boccaccio, A. Engering, J. P. Abastado, J. M. Heard, Y. van Kooyk, and O. Schwartz**. 2002. HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. Immunity. **16**:145-155.

87. **Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk**. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. [see comments]. Cell **100**:587-597.

88. **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. Nature Medicine **5**:997-103.

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

90. **Schrager, J. A. and J. W. Marsh**. 1999. HIV-1 Nef increases T cell activation in a stimulus-dependent manner. Proceedings of the National Academy of Sciences of the United States of America. **96**:8167-8172.

91. **Wang, J. K., E. Kiyokawa, E. Verdin, and D. Trono**. 2000. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. Proceedings of the National Academy of Sciences of the United States of America. **97**:394-399.

92. **Page, K. A., W. C. van Schooten, and M. B. Feinberg**. 1997. Human immunodeficiency virus type 1 Nef does not alter T-cell sensitivity to antigen-specific stimulation. Journal of Virology **71**:3776-3787.

93. **Manninen, A., P. Huotari, M. Hiipakka, G. H. Renkema, and K. Saksela**. 2001. Activation of NFAT-dependent gene expression by Nef: conservation among divergent Nef alleles, dependence on SH3 binding and membrane association, and cooperation with protein kinase C-theta. Journal of Virology **75**:3034-3037.

94. **Bandres, J. C. and L. Ratner**. 1994. Human immunodeficiency virus type 1 Nef protein down-regulates transcription factors NF-kappa B and AP-1 in human T cells in vitro after T-cell receptor stimulation. Journal of Virology **68**:3243-3249.

95. **Yoon, K., J. G. Jeong, and S. Kim** . 2001. Stable expression of human immunodeficiency virus type 1 Nef confers resistance against Fas-mediated apoptosis. AIDS Research & Human Retroviruses **17**:99-104.

96. **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.

97. **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 Ex Med **189** :1489-1496.

98. **Rasola, A., D. Gramaglia, C. Boccaccio, and P. M. Comoglio**. 2001. Apoptosis enhancement by the HIV-1 Nef protein. Journal of Immunology **166**:81-88.

99. **Ndolo, T., N. K. Dhillon, H. Nguyen, M. Guadalupe, M. Mudryj, and S. Dandekar**. 2002. Simian Immunodeficiency Virus Nef Protein Delays the Progression of CD4(+) T Cells through G(1)/S Phase of the Cell Cycle. Journal of Virology **76**:3587-3595.

100. **Miller, M. D., M. T. Warmerdam, K. A. Page, M. B. Feinberg, and W. C. Greene**. 1995. Expression of the human immunodeficiency virus type 1 (HIV-1) nef gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. Journal of Virology **69**:579-584.

101. **Goldsmith, M. A., M. T. Warmerdam, R. E. Atchison, M. D. Miller, and W. C. Greene**. 1995. Dissociation of the CD4 downregulation and viral infectivity enhancement functions of human immunodeficiency virus type 1 Nef. Journal of Virology **69**:4112-4121.

102. **Aiken, C. and D. Trono**. 1995. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. Journal of Virology **69**:5048-5056.

103. **Chowers, M. Y., M. W. Pandori, C. A. Spina, D. D. Richman, and J. C. Guatelli**. 1995. The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation. Virology **212**:451-457.

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

105. **Chazal, N., G. Singer, C. Aiken, M. L. Hammarskjold, and D. Rekosh**. 2001. Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity. Journal of Virology **75**:4014-4018.

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

107. **Luo, T., J. L. Douglas, R. L. Livingston, and J. V. Garcia**. 1998. Infectivity enhancement by HIV-1 Nef is dependent on the pathway of virus entry: implications for HIV-based gene transfer systems. Virology **241**:224-233.

108. **Schaeffer, E., R. Geleziunas, and W. C. Greene**. 2001. Human immunodeficiency virus type 1 Nef functions at the level of virus entry by enhancing cytoplasmic delivery of virions. Journal of Virology **75**:2993-3000.

109. **Zhou, J. and C. Aiken**. 2001. Nef enhances human immunodeficiency virus type 1 infectivity resulting from intervirion fusion: evidence supporting a role for nef at the virion envelope. Journal of Virology **75**:5851-5859.

110. **Zheng, Y. H., A. Plemenitas, T. Linnemann, O. T. Fackler, and B. M. Peterlin**. 2001. Nef increases infectivity of HIV via lipid rafts. Curr Biol **11**:875-879.

111. **Iafrate, A. J., S. Carl, S. Bronson, C. Stahl-Hennig, T. Swigut, J. Skowronski, and F. Kirchhoff**. 2000. Disrupting surfaces of nef required for downregulation of CD4 and for enhancement of virion infectivity attenuates simian immunodeficiency virus replication in vivo. Journal of Virology **74**:9836-9844.

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

113. **Kaminchik, J., R. Margalit, S. Yaish, H. Drummer, B. Amit, N. Sarver, M. Gorecki, and A. Panet**. 1994. Cellular distribution of HIV type 1 Nef protein: identification of domains in Nef required for association with membrane and detergent-insoluble cellular matrix. AIDS Research.& Human Retroviruses **10**:1003-1010.

114. **Niederman, T. M., W. R. Hastings, and L. Ratner**. 1993. Myristoylation-enhanced binding of the HIV-1 Nef protein to T cell skeletal matrix. Virology **197**:420-425.

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

116. **Luo, T., J. R. Downing, and J. V. Garcia**. 1997. Induction of phosphorylation of human immunodeficiency virus type 1 Nef and enhancement of CD4 downregulation by phorbol myristate acetate. Journal of Virology **71**:2535-2539.

117. **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 Journal **14**:484-491.

118. **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.

119. **Craig, H. M., M. W. Pandori, N. L. Riggs, D. D. Richman, and J. C. Guatelli**. 1999. Analysis of the SH3-binding region of HIV-1 nef: partial functional defects introduced by mutations in the polyproline helix and the hydrophobic pocket. Virology **262** :55-63.

120. **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. Journal of Biological Chemistry. **272**:17899-17902.

121. **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 [see comments]. Nature **385**:650-653.

122. **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. Journal of Biological Chemistry.

123. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer**. 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proceedings of the National Academy of Sciences of the United States of America. **91**:1539-1543.

124. **Luo, T., R. A. Livingston, and J. V. Garcia**. 1997. Infectivity enhancement by human immunodeficiency virus type 1 Nef is independent of its association with a cellular serine/threonine kinase. Journal of Virology **71**:9524-9530.

125. **Cullen, B. R.** 1996. HIV-1: is Nef a PAK animal?. Current Biology. **6**:1557-1559.

126. **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. Current Biology **9**:1407-1410.

127. **Fackler, O. T., X. Lu, J. A. Frost, M. Geyer, B. Jiang, W. Luo, A. Abo, A. S. Alberts, and B. M. Peterlin**. 2000. p21-activated kinase 1 plays a critical role in cellular activation by Nef. Molecular & Cellular Biology. **20**:2619-2627.

128. **Lu, X., X. Wu, A. Plemenitas, H. Yu, E. T. Sawai, A. Abo, and B. M. Peterlin**. 1996. CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1. Current Biology. **6**:1677-1684.

129. **Sawai, E. T., I. H. Khan, P. M. Montbriand, B. M. Peterlin, C. Cheng-Mayer, and P. A. Luciw**. 1996. Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques. Current Biology. **6**:1519-1527.

130. **Renkema, H. G. and K. Saksela**. 2000. Interactions of HIV-1 NEF with cellular signal transducing proteins. Frontiers in Bioscience **5**:D268-D283.

131. **Manninen, A., M. Hiipakka, M. Vihinen, W. Lu, B. J. Mayer, and K. Saksela**. 1998. SH3-Domain binding function of HIV-1 Nef is required for association with a PAK-related kinase. Virology **250**:273-282.

132. **Sells, M. A., U. G. Knaus, S. Bagrodia, D. M. Ambrose, G. M. Bokoch, and J. Chernoff**. 1997. Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Current Biology **7**:202-210.

133. **Huang, Y., L. Zhang, and D. D. Ho**. 1995. Biological characterization of nef in long-term survivors of human immunodeficiency virus type 1 infection. Journal of Virology **69**:8142-8146.

134. **Frost, J. A., S. Xu, M. R. Hutchison, S. Marcus, and M. H. Cobb**. 1996. Actions of Rho family small G proteins and p21-activated protein kinases on mitogen-activated protein kinase family members. Molecular & Cellular Biology **16**:3707-3713.

135. **Garcia, J. V. and A. D. Miller**. 1994. Retrovirus vector-mediated transfer of functional HIV-1 regulatory genes. AIDS Research.& Human Retroviruses **10**:47-52.

136. **Liu, X., C. N. Kim, J. Pohl, and X. Wang**. 1996. Purification and characterization of an interleukin-1beta-converting enzyme family protease that activates cysteine protease P32 (CPP32). Journal of Biological Chemistry **271**:13371-13376.

137. **Bokoch, G. M.** 1998. Caspase-mediated activation of PAK2 during apoptosis: proteolytic kinase activation as a general mechanism of apoptotic signal transduction?. Cell Death & Differentiation **5**:637-645.

138. **Walter, B. N., Z. Huang, R. Jakobi, P. T. Tuazon, E. S. Alnemri, G. Litwack, and J. A. Traugh**. 1998. Cleavage and activation of p21-activated protein kinase gamma-PAK by CPP32 (caspase 3). Effects of autophosphorylation on activity. Journal of Biological Chemistry **273**:28733-28739.

139. **Rudel, T. and G. M. Bokoch**. 1997. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science **276**:1571-1574.

140. **Fackler, O. T., X. Lu, J. A. Frost, M. Geyer, B. Jiang, W. Luo, A. Abo, A. S. Alberts, and B. M. Peterlin**. 2000. p21-activated kinase 1 plays a critical role in cellular activation by Nef. Molecular & Cellular Biology **20**:2619-2627.

141. **Kameshita I. and Fujisawa H.** 1989. A Sensitive Mehtod for Detection of Calmodulin-Dependent Protein Kinase II Activity in Sodium Dodecyl Sulfate-Polyacrylamide Gel. Analytical Biochemistry **183**:139-143.

142. **Sells, M. A. and J. Chernoff**. 2000. Emerging from the Pak: the p21-activated protein kinase family. Trends in Cell Biology **7**:162-167.

143. **Lu, W. and B. J. Mayer**. 1999. Mechanism of activation of Pak1 kinase by membrane localization. Oncogene **18**:797-806.

144. **Michaelson, D., J. Silletti, G. Murphy, P. D'Eustachio, M. Rush, and M. R. Philips**. 2001. Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. Journal of Cell Biology **152**:111-126.

145. **Scheffzek, K., I. Stephan, O. N. Jensen, D. Illenberger, and P. Gierschik**. 2000. The Rac-RhoGDI complex and the structural basis for the regulation of Rho proteins by RhoGDI. Nature Structural Biology **7**:122-126.

146. **Bokoch, G. M., A. M. Reilly, R. H. Daniels, C. C. King, A. Olivera, S. Spiegel, and U. G. Knaus**. 1998. A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. Journal of Biological Chemistry **273**:8137-8144.

147. **Benard, V., B. P. Bohl, and G. M. Bokoch**. 1999. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. Journal of Biological Chemistry **274**:13198-13204. – Replace with Bagrodia et al.

148. **Cannon, J. L., C. M. Labno, G. Bosco, A. Seth, M. H. McGavin, K. A. Siminovitch, M. K. Rosen, and J. K. Burkhardt**. 2001. Wasp recruitment to the T cell:APC contact site occurs independently of Cdc42 activation. Immunity **15**:249-259.

149. **Xiao, G. H., A. Beeser, J. Chernoff, and J. R. Testa**. 2002. p21-activated kinase links Rac/Cdc42 signaling to merlin 7. Journal of Biological Chemistry **277**:883-886.

150. **Kissil, J. L., K. C. Johnson, M. S. Eckman, and T. Jacks**. 2002. Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. Journal of Biological Chemistry **277**:10394-10399.

151. **Coffin, J. M.** 1996. HIV viral dynamics. AIDS **10 Suppl 3**:S75-S84.

152. **Lu, W., S. Katz, R. Gupta, and B. J. Mayer**. 1997. Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Current Biology **7**:85-94.

153. **Zha, J., S. Weiler, K. J. Oh, M. C. Wei, and S. J. Korsmeyer**. 2000. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science **290**:1761-1765.

154. **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. Molecular Cell **3**:729-739.

155. **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. Journal of Virology **73**:9899-9907.

156. **Wu, W. J., J. W. Erickson, R. Lin, and R. A. Cerione**. 2000. The gamma-subunit of the coatomer complex binds Cdc42 to mediate transformation. Nature **405**:800-804.

157. **Chiu, V. K., T. Bivona, A. Hach, J. B. Sajous, J. Silletti, H. Wiener, R. L. Johnson, A. D. Cox, and M. R. Philips**. 2002. Ras signalling on the endoplasmic reticulum and the Golgi 1. Nat Cell Biol **4**:343-350.

158. **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. Molecular Cell **3**:729-739.

159. **Manser, E., T. H. Loo, C. G. Koh, Z. S. Zhao, X. Q. Chen, L. Tan, I. Tan, T. Leung, and L. Lim**. 1998. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Molecular Cell **1**:183-192.

160. **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. Journal of Virology **67**:4639-4650.

161. **Kane, L. P., J. Lin, and A. Weiss**. 2000. Signal transduction by the TCR for antigen. Current Opinion in Immunology **12**:242-249.

162. **Yablonski, D., L. P. Kane, D. Qian, and A. Weiss**. 1998. A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK. EMBO Journal **17**:5647-5657.

163. **Manninen, A., R. G. Herma, and K. Saksela**. 2000. Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway. Journal of Biological Chemistry **275**:16513-16517.

164. **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.

165. **Lian, J. P., L. Crossley, Q. Zhan, R. Huang, P. Coffer, A. Toker, D. Robinson, and J. A. Badwey**. 2001. Antagonists of calcium fluxes and calmodulin block activation of the p21-activated protein kinases in neutrophils. Journal of Immunology **166**:2643-2650.

166. **Bokoch, G. M.** 2000. Regulation of cell function by Rho family GTPases. Immunologic Research **21**:139-148.

167. **Bagrodia, S. and R. A. Cerione**. 1999. Pak to the future. Trends in Cell Biology **9**:350-355.

168. **Gautreau, A., D. Louvard, and M. Arpin**. 2002. ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling. Current Opinion in Cell Biology **14**:104-109.

169. **Reif, K. and D. A. Cantrell**. 1998. Networking Rho family GTPases in lymphocytes. Immunity **8**:395-401.

170. **Serrador, J. M., M. Nieto, and F. Sanchez-Madrid**. 1999. Cytoskeletal rearrangement during migration and activation of T lymphocytes. Trends in Cell Biology **9**:228-233.

171. **Roumier, A., J. C. Olivo-Marin, M. Arpin, F. Michel, M. Martin, P. Mangeat, O. Acuto, A. Dautry-Varsat, and A. Alcover**. 2001. The membrane-microfilament linker ezrin is involved in the formation of the immunological synapse and in T cell activation. Immunity **15**:715-728.

172. **Pawlak, G. and D. M. Helfman**. 2001. Cytoskeletal changes in cell transformation and tumorigenesis. Current Opinion in Genetics & Development **11**:41-47.

173. **Carl, S., A. J. Iafrate, S. M. Lang, N. Stolte, C. Stahl-Hennig, K. Matz-Rensing, D. Fuchs, J. Skowronski, and F. Kirchhoff**. 2000. Simian immunodeficiency virus containing mutations in N-terminal tyrosine residues and in the PxxP motif in Nef replicates efficiently in rhesus macaques. Journal of Virology **74**:4155-4164.

174. **Fackler, O. T., D. Wolf, H. O. Weber, B. Laffert, P. D'Aloja, B. Schuler-Thurner, R. Geffin, K. Saksela, M. Geyer, B. M. Peterlin, G. Schuler, and A. S. Baur**. 2001. A natural variability in the proline-rich motif of Nef modulates HIV-1 replication in primary T cells. Current Biology **11**:1294-1299.

175. **Khan, I. H., E. T. Sawai, E. Antonio, C. J. Weber, C. P. Mandell, P. Montbriand, and P. A. Luciw**. 1998. Role of the SH3-ligand domain of simian immunodeficiency virus Nef in interaction with Nef-associated kinase and simian AIDS in rhesus macaques. Journal of Virology **72**:5820-5830.

176. **Lang, S. M., A. J. Iafrate, C. Stahl-Hennig, E. M. Kuhn, T. Nisslein, F. J. Kaup, M. Haupt, G. Hunsmann, J. Skowronski, and F. Kirchhoff**. 1997. Association of simian immunodeficiency virus Nef with cellular serine/threonine kinases is dispensable for the development of AIDS in rhesus macaques. Nature Medicine **3**:860-865.

177. **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. Journal of Virology **75**:9378-9392.

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