Nef-Mediated Lipid Raft Exclusion of UbcH7 Inhibits Cbl Activity in T Cells to Positively Regulate Signaling

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Summary

Lentiviral Nef increases T cell signaling activity, but the molecular nature of the stimulus involved is incompletely described. We explored CD4 T cell lipid raft composition in the presence and absence of Nef. Here, the E2 ubiquitin-conjugating enzyme UbcH7, which acts in conjunction with c-Cbl, is absent from lipid rafts. This Nef-mediated exclusion is associated with failure of ubiquitination of activated Vav. In the presence of Nef, lipid raft Cdc42 is activated and forms a ternary complex between the c-Cbl-interacting protein p85Cool-1/βPix and c-Cbl, displacing UbcH7 from rafts. Suppression of p85Cool-1/βPix expression restores UbcH7 raft localization and Vav ubiquitination and diminishes Cdc42 activity. Moreover, p85Cool-1/βPix knockdown attenuates HIV replication. Thresholds for activation of signaling involve the intricate balance of positive and negative regulators. Here we provide evidence for Nef disruption of a negative regulator of T cell signaling in promoting HIV replication.

Introduction

The Nef gene of HIV is important for pathogenicity, increasing viral infectivity and replicative capacity. Several functional attributes have been ascribed to Nef, including the downregulation of MHC class 1 A and B alleles to promote escape from the cellular immune response, downregulation of cell-surface CD4 to enhance virion release from the infected cell surface, and alteration of host cell death pathways to prevent apoptosis of infected cells while promoting death of activated bystander CD8 T cells (for reviews, see Doms and Trono, 2000; Fackler and Baur, 2002). In addition, Nef alters cell signaling to induce an activating stimulus in CD4 T cells (Fackler and Baur, 2002). The principal function and exact molecular mechanism involved in Nef alteration of signaling in the viral life cycle are not fully de-

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fined but are likely to contribute to pathogenicity. A key motif required for Nef signaling is the SH3 binding domain, essential for optimal spread of HIV in primary cells (Fackler et al., 2001; Manninen et al., 1998; Saksela et al., 1995). This domain also mediates association with a variety of signaling proteins including the Src family kinases, PAK2, Protein kinase C theta, Erk-1, Raf1, TCRζ, and Vav (for review, see Renkema and Saksela, 2000).

The molecular event facilitating Nef alteration of cell signaling has engendered much interest, and evidence has accumulated for a role at the plasma membrane. Nef is detectable in lipid rafts in T cells (Alexander et al., 2004; Wang et al., 2000) and is associated with the recruitment of activated p21-activated kinase (PAK) to these domains (Krautkramer et al., 2004). Nef activates the GTPases Cdc42 and Rac via the guanine nucleotide exchange factors (GEF) Vav and DOCK2/ELMO1 (Fackler et al., 1999; Janardhan et al., 2004; Lu et al., 1996). PAK is a downstream effector of Cdc42 and Rac; activation of these GTPases by Nef is probably responsible for the consistent observation of PAK activation in Nef-expressing cells. Despite evidence that Nef activates a Cdc42-dependent signaling path in lipid rafts, the initial trigger remains unclear.

To shed further light on the nature of Nef signaling in T cells, we undertook an analysis of differential protein expression in CD4 T cell lipid rafts in the presence and absence of Nef. We investigated proteins recruited or lost from rafts after Nef expression by 2D-PAGE analysis and mass spectrometry. Among proteins absent from Nef-expressing rafts was the E2 ubiquitin-conjugating enzyme UbcH7, which is implicated in the c-Cbl-mediated ubiquitination and negative regulation of T cell signaling molecules such as Lck, Vav, and TCRζ (Rao et al., 2002; Miura-Shimura et al., 2003; Wang et al., 2001). This raft loss of UbcH7 has functional consequences. In the presence of Nef, activated Vav fails to undergo ubiquitination, resulting in the accumulation of tyrosine-phosphorylated Vav in lipid rafts and increased Cdc42 activity. This effect of Nef was recapitulated by UbcH7 knockdown and did not occur after conventional T cell activation. Expression of Nef in signaling-defective Jurkat T cell lines revealed a requirement for Lck but not TCRζ in inhibiting c-Cbl function. We established that in the presence of Nef, a ternary complex forms between activated Cdc42, the PAK interactive exchange factor p85Cool-1/βPix (hereafter referred to as βPix), and c-Cbl that displaces UbcH7 from the c-Cbl RING finger and lipid rafts. This ternary complex has been shown previously to occur after expression of constitutively active Cdc42, resulting in failure of EGFR ubiquitination (Wu et al., 2003). The complex is likely to be responsible for UbcH7 raft displacement, as expression of a constitutively active Cdc42 mutant in Jurkat led to diminished expression of UbcH7 in lipid rafts. Destruction of the complex by knockdown of βPix by short hairpin-interfering RNAs (siRNA) resulted in UbcH7 relocation to lipid rafts in Nef-positive cells and diminished Nef-induced Cdc42 activity. We found that Cbl was

required for constitutive localization of UbcH7 in lipid rafts, and it is likely that the Cdc42-βPix-c-Cbl interaction displaces UbcH7 from the Cbl RING finger by steric hindrance. The relevance of these observations to the HIV life cycle was explored by βPix knockdown in infected CD4 T cells, which led to attenuation of HIV replication. These findings indicate that at least part of Nef's signaling activity in T cells derives from inhibition of a negative regulator of T cell signaling.

Results

Changes in the Protein Content of CD4 T Cell Lipid Rafts in the Presence of Nef

Plasma membrane lipid rafts are cholesterol- and sphingolipid-rich plasma membrane domains thought to contribute to compartmentalizing signal transduction events in different regions of the plasma membrane (Harder, 2004). Evidence exists for Nef commandeering T cell signaling machinery high in the T cell signaling pathway and probably within lipid rafts themselves. Nef targeting to lipid rafts in CD4 T cells is dependent on its myristoylated N terminus (Wang et al., 2000). Flag-tagged Nef expression vectors were used to demonstrate the presence of Nef in Jurkat CD4 T cell lipid rafts. Figure 1A compares the raft localization of wild-type SF2 Nef with a mutant (glycine to alanine) at position 2, the myristoylation site (Nef-G2A). Western blotting illustrates cofractionation of the lipid raft marker GM1 with Nef but not Nef-G2A. 2D-PAGE analysis and mass spectrometry were used to sequence proteins differentially expressed in lipid rafts of Jurkat CD4 T cells transfected with either Nef or Flag (pCMV-Tag4A) control vector (for complete gel images, see Figure S1 in the Supplemental Data available with this article online). Cells were harvested at 24 hr posttransfection for sucrose density gradient centrifugation and lipid raft isolation. Four replicate 2D-PAGE gels (with a linear pI range of 3–10) were obtained for both control and Nef-expressing lipid raft fractions to visualize changes in the lipid raft proteome in the presence of Nef. Each gel resolved around 1200 spots, and differentially expressed proteins were selected for protein identification after image analysis. In-gel digestion with trypsin and peptide extraction were carried out by an automated workstation. Tryptic peptides were desalted on an LC system and eluted into a tandem mass spectrometer. The database search was performed with a protein search tool by Swiss-Prot. In several examples, multiple spots represented the same protein, with variations due to posttranslational modification or alternative splicing (Figure S1). Names of the identified proteins and their Swiss-Prot accession numbers are listed in Table 1. Information about sequence coverage and peptide sequence is given in Table S1. By using this methodology, we identified ten proteins that showed increased expression or were exclusively present in Nef-expressing rafts and seven proteins that were decreased in expression or were absent from Nef-expressing rafts.

In the presence of Nef, the E2 ubiquitin-conjugating enzyme UbcH7 was absent from lipid rafts (Figure 1C). UbcH7 has been shown to act as the cognate E2-conjugating enzyme for Cbl family proteins, E3 ubiquitin ligases that mediate ubiquitination, and downregulation

of activated T cell signaling molecules (Yokouchi et al., 1999; Zheng et al., 2000). Among the identified proteins differentially expressed in lipid rafts in the presence of Nef, UbcH7 stands out as an attractive candidate for involvement in Nef signaling. This absence may interfere with c-Cbl-mediated negative regulation of T cell signaling proteins and thus provide a means by which Nef might promote T cell signaling.

In addition to UbcH7, several other notable changes in lipid raft protein composition were detected (Figure 1C; Table 1; Figure S1). Nef-expressing lipid rafts exhibited recruitment of components of the actin cytoskeleton and posttranslational modification of transgelin 2, consistent with previous observations of Nef-driven actin polymerization (Fackler et al., 1999). Nef-expressing rafts lack stathmin, which is phosphorylated and consequently inactivated by PAK. Stathmin negatively regulates microtubule dynamics (Andersen, 2000) by sequestering tubulin, by decreasing the concentration of free heterodimers available for polymerization (Belmont and Mitchison, 1996), or by inducing catastrophe at microtubule tips (Cassimeris, 2002). The raft analysis revealed that Nef is associated with recruitment of the RNA binding protein hnRNP E1 that localizes to spreading initiation centers present in the early stages of cell spreading prior to the formation of focal adhesions (de Hoog et al., 2004). Signaling proteins recruited to rafts in the presence of Nef include lactate dehydrogenase-A isoform that is preferentially expressed in activated lymphocytes (Wollberg and Nelson, 1992). Finally, Nef-positive rafts lack a known negative regulator of signaling, 14-3-3ε. Isoforms of 14-3-3 expressed in T cells bind the catalytic subunit of activated PI3K (p110), reducing its enzymatic activity (Bonnefoy-Berard et al., 1995), and the key T cell signaling attenuator c-Cbl after TCR stimulation (Liu et al., 1996).

UbcH7 Is Excluded from Nef-Expressing Lipid Rafts

To confirm the results obtained by 2D-PAGE, Jurkat CD4 T cells were transfected with Nef and Flag vectors and lipid raft fractions were obtained by sucrose density gradient centrifugation. Figure 2A demonstrates exclusion of UbcH7 from the lipid raft fractions of Nef-expressing cells. Comparison of UbcH7 raft localization after expression of Nef-G2A revealed that Nef must be physically present in lipid rafts for UbcH7 raft exclusion to occur (Figure 2A). The majority of signaling functions described for Nef require an intact polyproline motif. To establish whether this Nef domain is needed for UbcH7 lipid raft exclusion, a mutant Flag expression construct was created in which the FPVR motif of Nef (aa 72–75) was mutated to VRIT (Nef-Px-Mu). Expression of this mutant also abrogated the ability of Nef to exclude UbcH7 from lipid raft fractions (Figure 2A).

Confocal studies were then undertaken to further assess the redistribution of UbcH7 observed after 2D-PAGE analysis. Nef-EGFP fusion vectors were created expressing wild-type Nef, Nef-G2A, and Nef-Px-Mu and used to transfect Jurkat. 24 hr posttransfection, lipid rafts were patched by anti-cholera toxin antibody. As observed for Western blots of T cell lipid raft fractions, UbcH7 staining is downregulated in lipid rafts in Nef-EGFP-expressing cells but colocalized with GM1 after expression of the empty EGFP vector, Nef-G2A-EGFP
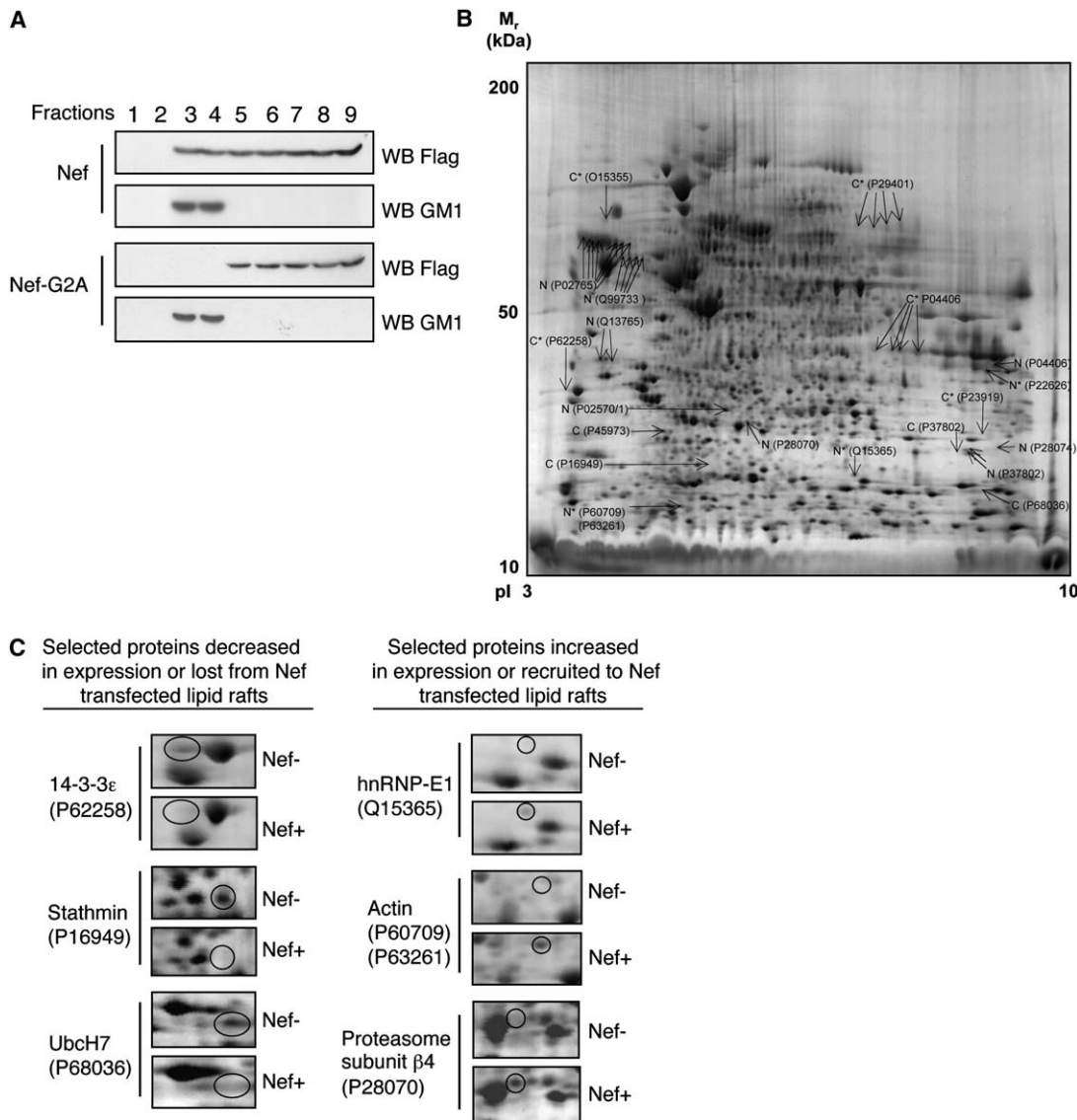
A

Fractions 1 2 3 4 5 6 7 8 9

Nef | WB Flag
     | WB GM1

Nef-G2A | WB Flag
        | WB GM1

B Mr (kDa)

200

C* (O15355)   C* (P29401)

N (P02765)
N (Q99733)   C* P04406
N (Q13765)
C* (P62258)
                              N (P04406)
                              N* (P22626)
N (P02570/1)   C* (P23919)
C (P45973)   C (P37802)   N (P28074)
                 N (P28070) N* (Q15365)   N (P37802)
C (P16949)   C (P68036)

N* (P60709)
(P63261)

10

pI 3   10

C Selected proteins decreased in expression or lost from Nef transfected lipid rafts

14-3-3ε (P62258)   Nef−
                    Nef+

Stathmin (P16949)   Nef−
                    Nef+

UbcH7 (P68036)   Nef−
                    Nef+

Selected proteins increased in expression or recruited to Nef transfected lipid rafts

hnRNP-E1 (Q15365)   Nef−
                    Nef+

Actin (P60709) (P63261)   Nef−
                          Nef+

Proteasome subunit β4 (P28070)   Nef−
                                 Nef+

Figure 1. Identification of Proteins Differentially Expressed in Jurkat Lipid Rafts in the Presence of Nef

(A) Western blot of Nef- or Nef-G2A-expressing Jurkat lysate fractions obtained after sucrose density gradient centrifugation. Fractions were immunoblotted to identify Nef with anti-Flag antibody and GM1 with cholera-toxin HRP.

(B) Synthetic 2D-PAGE image representing all protein spots present in the control versus Nef-transfected raft analysis highlighting the differentially expressed features along with their Swiss-Prot accession number. C, features present only in control rafts; N, features present only in Nef-transfected rafts; C*, features present in both control and Nef-transfected rafts but expressed to a higher extent in the control rafts; N*, features present in both control and Nef-transfected rafts but expressed to a higher extent in the Nef-transfected rafts. For complete gel figures and analysis, see Supplemental Data.

(C) Selected close-up examples of representative 2D-PAGE images showing changes in protein expression in Nef-transfected rafts.

and Nef-Px-Mu-EGFP (Figure 2B). Thus, Nef is associated with exit of UbcH7 from lipid rafts and this function requires both the Nef N-terminal myristoylation site and an intact Nef SH3 binding domain.

Failure of Vav Ubiquitination in Nef-Expressing CD4 T Cells

The functional relevance of Nef-mediated exclusion of UbcH7 from lipid rafts was investigated. Nef-interacting proteins known to be substrates for c-Cbl-mediated negative regulation after T cell activation stimuli include Lck, Vav, and TCRζ (Rao et al., 2002; Miura-Shimura et al., 2003; Wang et al., 2001). We focused on character-

izing Vav activity in the presence of Nef, as this has been directly linked to PAK activation, a highly conserved function of Nef. Vav GEF activity is important for HIV replication, as dominant-negative Vav suppresses HIV replication when expressed in infected cells (Fackler et al., 1999). In the normal immune response, Vav plays a crucial role in TCR signaling events, including Ca^{2+} flux and cytoskeletal reorganization (Cantrell, 2003). In turn, c-Cbl participates in turning off Vav activity through ubiquitination. After CD4 T cell activation, phosphorylated Vav undergoes c-Cbl association (Marengère et al., 1997), ubiquitination, and degradation (Miura-Shimura et al., 2003). We reasoned that if the exclusion

Table 1. Summary of Differentially Expressed Lipid Raft Proteins and Functional Roles

Functional Category	Increased in Expression or Exclusively Present in Nef-Expressing CD4 T Cell Lipid Rafts	Decreased in Expression or Absent from Nef-Expressing CD4 T Cell Lipid Rafts
Signal transduction	(P00338) L-lactate dehydrogenase A chain (EC 1.1.1.27) (LDH-A) (LDH muscle subunit) (LDH-M)	(O15355) protein phosphatase 2C γ isoform
	(P04406) glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (GAPDH)[a]	(P62258) 14-3-3 protein ε
		(P29401) transketolase
		(P23919) thymidylate kinase
Cytoskeletal and microtubule-organizing center regulation	(P37802) transgelin 2[a]	(P16949) stathmin (Oncoprotein 18)
	(P60709) (P63261) actin[a]	
Proteolysis	(P28070) proteasome subunit beta type 4 precursor	
	(P28074) proteasome subunit beta type 5 precursor	
Ubiquitination		(P68036) ubiquitin-conjugating enzyme E2-18 kDa UbcH7
mRNA processing and translation	(Q15365) poly(rC)-binding protein 1 (hnRNP-E1)	
	(Q13765) NASCENT polypeptide associated complex alpha subunit	
Nucleosome assembly	(Q99733) nucleosome assembly protein 1-like 4	
Ion channel		(P21796) voltage-dependent anion-selective channel protein 1 (VDAC-1)
Acute phase protein	(P02765) alpha-2-HS-glycoprotein precursor[a]	

Identified proteins are specified by Swiss-Prot accession number. Further information about sequence coverage and peptide sequences can be obtained from the Supplemental Data.
[a] Posttranslational modification detected by 2D-PAGE after Nef expression.

of UbcH7 from lipid rafts by Nef has functional relevance for Nef signaling, then it should be possible to demonstrate failure of ubiquitination of Nef-activated c-Cbl substrates.

Nef-mediated Vav activation was first confirmed after transfection of Jurkat with Flag or Nef vectors. Similar to the scenario occurring after anti-CD3/CD28 triggering, Nef expression resulted in Vav hyperphosphorylation in lipid raft fractions, as shown on Western blots from Nef-expressing versus control lysates (Figure 3A). The level of Vav GEF activity was then assessed with a Cdc42 activation assay. Here, active GTP bound Cdc42 binds to the p21 binding domains (PBD) of PAKs so GST-PBD can be used to pull down Cdc42-GTP. Levels of GTP bound Cdc42 in lipid rafts were assessed by glutathione agarose bound GST-PBD, and the fraction of PBD bound Cdc42 were assayed by immunoblotting (Figure 3B). This demonstrated that Nef increased the level of GTP-Cdc42 in lipid rafts, consistent with previous reports (Krautkramer et al., 2004). We next examined whether Vav becomes ubiquitinated in Nef-expressing CD4 T cell lipid rafts. c-Cbl has been implicated directly in the ubiquitination and negative regulation of Vav after T cell activation or conditions that promote both c-Cbl and Vav phosphorylation (Miura-Shimura et al., 2003). Figure 3C shows Western blots for ubiquitin after Vav immunoprecipitation from lipid rafts of Nef and control transfectants and T cells activated via ligation of CD3 and CD28. While Nef expression was associated with increased Vav GEF activity and phosphorylation, no ubiquitination of Vav could be detected in Nef-expressing CD4 T cell lipid rafts. In contrast, activation of Jurkat via plate bound anti-CD3/CD28 resulted in detectable Vav ubiquitination. In addition, after TCR stimulation of Jurkat, Vav could be coimmunoprecipitated with c-Cbl as would be expected for

a Cbl substrate (Marengère et al., 1997). However, in Nef-expressing cells, no such association could be demonstrated (Figure 3D).

To examine whether accessibility to UbcH7 might be responsible for failure of Vav ubiquitination in the presence of Nef, an siRNA targeting UbcH7 was used to diminish UbcH7 expression (Verma et al., 2004). Jurkat were transfected with this UbcH7 siRNA and a nonsilencing (NS) siRNA, whose sequence did not match any known human gene (Figure 3E). A comparison of the effect of UbcH7 knockdown on Vav activation with that occurring after antigen receptor triggering was undertaken. Similar to the results obtained after Nef expression, levels of GTP bound Cdc42 increased after UbcH7 knockdown (Figure 3F), and Vav tyrosine phosphorylation increases to levels comparable with conventional T cell activation (Figure 3G). In addition, we were unable to detect significant levels of Vav ubiquitination after UbcH7 knockdown (Figure 3G). These observations indicate that CD4 T cell c-Cbl-mediated negative regulatory activity is defective in Nef-expressing cells and suggest that the exclusion of UbcH7 away from raft-localized c-Cbl has functional consequences for Nef signaling.

Lck but Not TCRζ Is Required for Nef-Mediated UbcH7 Lipid Raft Exclusion

To establish whether UbcH7 lipid raft exclusion is idiosyncratic to the Nef T cell activation stimulus, the effect of antigen receptor triggering on UbcH7 raft location was studied. Jurkat were stimulated with plate bound anti-CD3/CD28 over a time course extending to 24 hr. It was not possible to demonstrate changes in expression of UbcH7 between raft and nonraft compartments either by Western blot (Figure 4A) or confocal microscopy (Figure 4B) throughout.
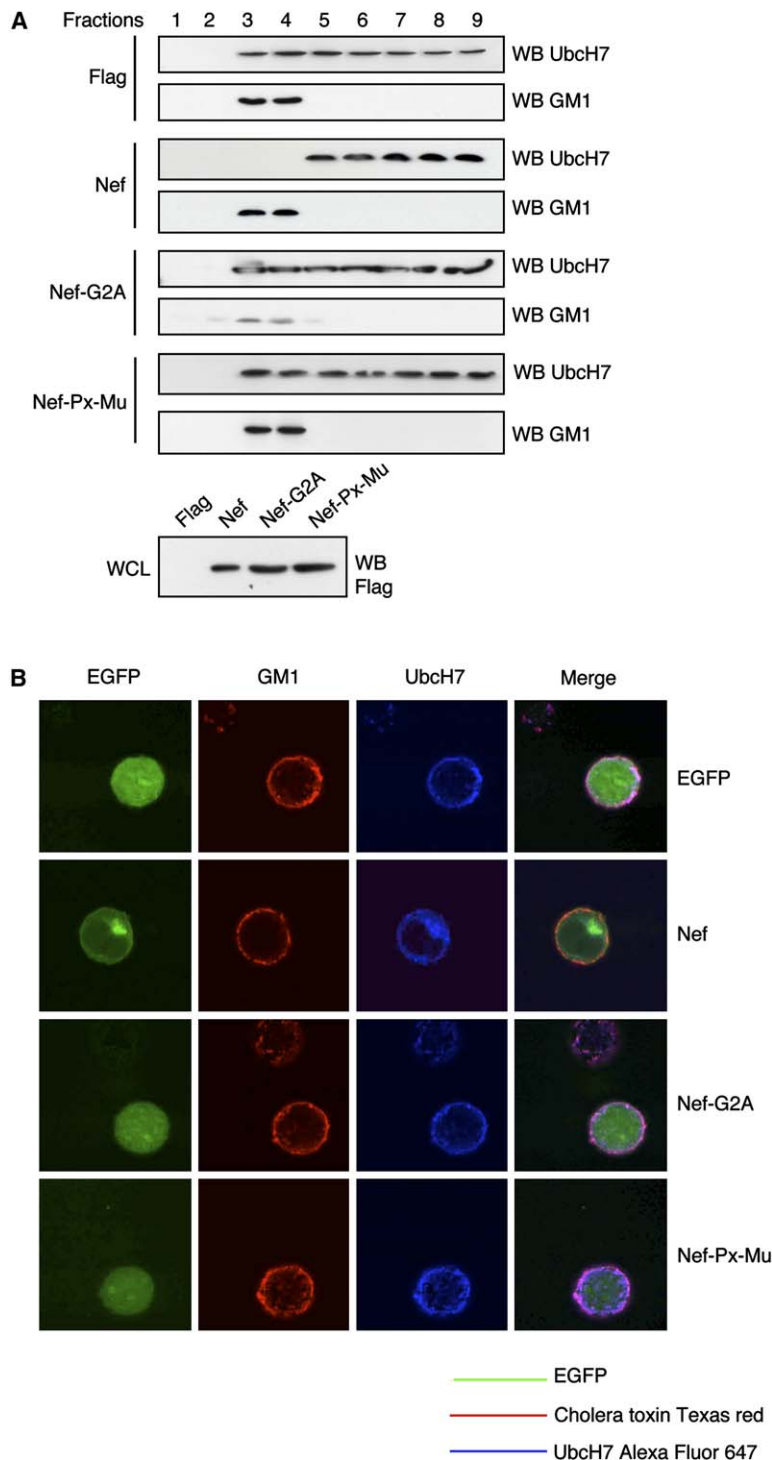
A

B

The requirement for upstream T cell signaling molecules in Nef-mediated downregulation of lipid raft UbcH7 was investigated with mutant Jurkat T cell lines defective in key T cell signaling molecules. JCaM1.6, genetically deficient in Lck (Straus and Weiss, 1992), and J.RT3-T3.5, functionally deficient in TCRζ (Ohashi et al., 1985), were transfected with Nef or Flag vectors and lipid raft fractions were prepared. Western blotting of GM1-positive fractions of Nef-transfected rafts revealed the absence of UbcH7 in J.RT3-T3.5 but not

JCaM1.6, thus indicating a requirement for Lck but not TCRζ in UbcH7 raft exclusion (Figure 4C). These results were confirmed by confocal analysis of J.RT3-T3.5 and JCaM1.6 transfected with EGFP and Nef-EGFP (Figure 4D). Next, Lck expression in JCaM1.6 was restored by either wild-type Lck or a kinase-dead mutant Lck-R273A (Rao et al., 2002), and localization of UbcH7 was examined. Interestingly, both Lck constructs restored the ability of Nef to downregulate UbcH7 expression in lipid rafts (Figures 4E and 4F), indicating that while Lck
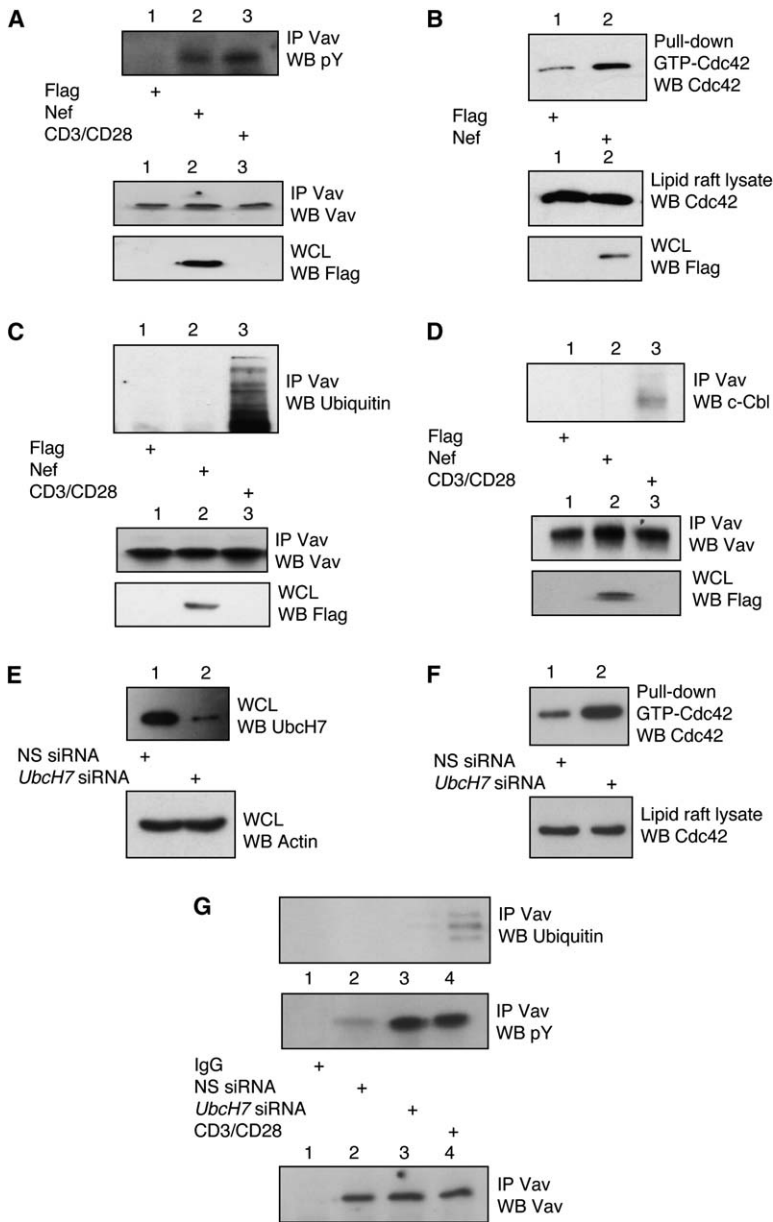
Figure 3. Failure of c-Cbl-Mediated Ubiquitination of Nef-Activated Vav

(A) Vav was immunoprecipitated from lipid rafts of Flag or Nef transfectants or Jurkat activated with anti-CD3/CD28, and anti-phosphotyrosine (pY) immunoblot was performed as shown in the top panel and Vav immunoblot below. Flag Western blot of WCL is in the lower panel.

(B) PBD-agarose was used to pull down GTP bound Cdc42 from lipid rafts of Flag- or Nef-transfected cells, and levels of GTP-Cdc42 were assessed by Western blot. The middle panel shows Cdc42 immunoblot from the same lipid raft samples. The bottom panel shows Flag immunoblot of WCL.

(C) Vav was immunoprecipitated from lipid rafts of Flag or Nef transfectants or Jurkat activated with anti-CD3/CD28. Immunoblot for ubiquitin is shown in the top panel and for Vav below. Flag immunoblot of WCL is in the lower panel.

(D) Jurkat were transfected with Flag or Nef vectors or activated with anti-CD3/CD28. Vav immunoprecipitate and c-Cbl Western blot is shown above and Vav Western blot from the corresponding immunoprecipitates below. The lower panel shows Flag immunoblot of the corresponding WCL.

(E) Jurkat were transfected with NS siRNA or *Ubch7* siRNA and UbcH7 protein level assessed at 48 hr by Western blot, with actin immunoblot below.

(F) Cdc42 immunoblot following pull-down of GTP-Cdc42 with PBD-agarose from cells treated as in (E) with Cdc42 immunoblot from lipid raft lysates shown below.

(G) Vav was immunoprecipitated from NS, or *Ubch7* siRNA transfected, and from CD3/CD28 activated Jurkat and Western blotted with anti-phosphotyrosine. Below is the corresponding anti-Vav immunoblot. The top panel illustrates ubiquitin immunoblot of the same Vav immunoprecipitates.

is required for this function of Nef, the kinase activity of Lck is dispensable.

Cdc42 Associates with βPix and c-Cbl in Nef-Expressing Cells

The mechanism by which Nef mediates exclusion of UbcH7 from lipid rafts and inhibition of Cbl-mediated ubiquitination was investigated. Crystallographic studies have established that UbcH7 interacts with Cbl via a weak association with the Cbl RING finger domain (Zheng et al., 2000). A known inhibitor of Cbl activity is Human Sprouty 2 (hSpry2). hSpry2 directly interacts with the c-Cbl RING finger domain, displacing UbcH7 from its binding site on the E3 ligase, thus abrogating epidermal growth factor receptor (EGFR) ubiquitination and endocytosis (Wong et al., 2002). We hypothesized that Nef might facilitate displacement of UbcH7 in such a manner by precipitating the interaction of an in-

hibitory protein with Cbl in an area at or in proximity to the RING finger. It has been demonstrated in conditions where Cdc42 activity is increased that a ternary complex can form between Cdc42 and c-Cbl, mediated by the PAK-interacting exchange factor βPix (Wu et al., 2003). Formation of this complex after expression of constitutively active Cdc42 mutants inhibits c-Cbl-mediated ubiquitination and degradation of EGFR. βPix proteins are binding partners of PAK (Bagrodia et al., 1999; Manser et al., 1998), Cbl-b (Flanders et al., 2003), and c-Cbl (Wu et al., 2003). The binding of c-Cbl to βPix is mediated via the βPix SH3 domain (Wu et al., 2003), and the Cbl family protein SH3 binding domain lies directly adjacent to the RING finger. We hypothesized that lipid raft Nef-activated Cdc42 might facilitate the formation of such a ternary complex of βPix, Cdc42, and c-Cbl that would displace UbcH7 from its binding site on the c-Cbl RING finger. To test this, we transfected Jurkat CD4 T cells

with either Flag or Nef vectors and immunoprecipitated Cdc42. It was possible to demonstrate coimmunoprecipitation of both βPix and c-Cbl with Cdc42 in the Nef-expressing T cell lysates (Figure 5A). In contrast, coimmunoprecipitation experiments carried out after T cell activation did not reveal formation of this ternary complex (Figure 5A). The requirement for Lck in inducing this ternary interaction complex in the presence of Nef was assessed by expressing Nef in JCaM1.6 alone, or together with Lck or Lck-R273A expression vectors. Figure 5B shows that the Nef-mediated interaction of Cdc42 with Cbl and βPix is not detectable in the absence of Lck but does not require the enzymatic activity of Lck to occur. These data indicate that Nef does indeed induce formation of a lipid raft Cdc42-βPix-Cbl complex in an Lck-dependent manner, which might be responsible for inhibition of c-Cbl activity by directly displacing the c-Cbl E2 UbcH7 away from its active site on the c-Cbl RING finger.

Expression of a Constitutively Active Cdc42 Mutant Is Associated with UbcH7 Raft Loss while βPix Knockdown Results in Relocation of UbcH7 to Lipid Rafts and Restores Ubiquitination of Nef-Activated Vav

To test this hypothesis further, the requirement for Cbl in facilitating UbcH7 entry to lipid rafts was examined after knockdown of c-Cbl and Cbl-b with siRNAs. Figure 6A illustrates the reduction in Cbl protein levels obtained after transfection of Cbl but not NS siRNA. Figure 6B demonstrates a reduction in level of UbcH7 detectable in lipid rafts after Cbl knockdown. We examined whether steric hindrance induced by association of activated Cdc42 and βPix with Cbl might explain the exit of UbcH7 from lipid rafts observed after Nef expression. A constitutively active Cdc42 mutant capable of constitutive GDP-GTP exchange, Cdc42(F28L), shown previously to induce Cdc42-βPix-Cbl formation (Wu et al., 2003), was transfected in Jurkat. UbcH7 lipid raft localization was assessed by Western blot (Figure 6C). Downregulation of UbcH7 raft expression was observed, suggesting formation of a Cdc42-βPix-Cbl complex that may prevent UbcH7 binding to the Cbl RING finger, probably via steric hindrance effects.

To investigate further whether the Nef-mediated Cdc42-βPix-Cbl complex formation was responsible for UbcH7 lipid raft displacement and failure of Vav homeostasis in Nef-expressing cells, we inhibited βPix expression. To do so, we chose several sequences in the human βPix gene and designed siRNA directed against βPix. These were tested for their ability to knock down βPix expression in Jurkat. Transfection of 20 μM βPix siRNA1 efficiently diminished expression of βPix when assessed by Western blot (Figure 6D, i) and confocal analysis (Figure 6D, ii) in comparison with NS siRNA. The effect of βPix knockdown on the subcellular distribution of UbcH7 was investigated in the presence of Nef. βPix siRNA1 and control NS siRNA were cotransfected with either control Flag or Nef expression vectors; lipid rafts were extracted and Western blotted for the presence of UbcH7. Figure 6E demonstrates that while UbcH7 remains excluded from raft fractions in Nef and NS siRNA transfectants, raft localization returns when βPix protein expression is diminished. We next

addressed whether βPix knockdown could restore Nef-activated Vav ubiquitination. Jurkat were transfected with Nef or Flag plasmids together with either βPix siRNA1 or NS siRNA. Vav was immunoprecipitated from lipid raft fractions obtained from these transfectants, and levels of ubiquitination were assessed by Western blotting. As predicted after βPix knockdown in Nef-expressing cells, Vav becomes detectably ubiquitinated in lipid rafts (Figure 6F). In addition, detection of Cdc42 activity in the same transfectants demonstrates that, after expression of βPix siRNA1, Nef-mediated Cdc42 activity is reversed (Figure 6G). Therefore, it appears that the formation of a ternary complex between Cdc42, βPix, and c-Cbl occurring in the presence of Nef results in lipid raft displacement of UbcH7 and failure of c-Cbl-mediated ubiquitination activity.

βPix Knockdown and HIV Replication

βPix siRNA1 was used to examine whether Nef interference with c-Cbl-mediated ubiquitination influences its function in enhancement of HIV replication. Figure 7A demonstrates the kinetics of βPix knockdown after transfection of βPix siRNA1 in Jurkat. Reporter EGFP-expressing HIV virus HIV NL-GI, where EGFP replaces Nef in HIV NL4-3 and Nef is replaced downstream of an IRES (Cohen et al., 1999), was then transfected with either βPix siRNA1 or NS siRNA. Viral production was compared for 48 hr posttransfection. Figure 7B shows βPix levels in the HIV-transfected cells. After introduction of NL-GI into βPix knockdown cells, HIV production was significantly diminished, as assessed by FACS analysis of EGFP expression (Figure 7C) and ELISA analysis of p24 production (Figure 7D). The data shown are representative of six replicate experiments. These results indicate that Nef-mediated Cdc42-βPix-Cbl association is important for HIV replication in CD4 T cells.

Discussion

The precise nature of the activation stimulus that Nef delivers to CD4 T cells has remained elusive. Signal transduction by T cells is through the translocation and activation of protein tyrosine kinases and the formation of a network of adaptor molecules (Kane et al., 2000). Nef has been proposed to act as an adaptor bringing signaling substrates into proximity to initiate signaling. While multiple interacting partners have been described, and several of these have been shown to change in activity in the presence of Nef, definition of a unifying single molecular unit involved has not been forthcoming.

In this study, we conducted a proteomic analysis of lipid rafts that revealed loss of UbcH7, hinting at a role for Nef in disruption of Cbl activity. We demonstrate that Cbl-mediated ubiquitination in lipid rafts of at least one of the Nef activated proteins, Vav, is diminished, leading to enhanced Cdc42 activity. This process requires an upstream Src kinase, Lck, but not TCRζ, and results in the formation of a ternary complex between Cdc42, βPix, and c-Cbl. The complex appears to be responsible for raft loss of UbcH7, as diminishing expression of βPix leads to relocation of UbcH7 to lipid rafts and ubiquitination of Nef-activated Vav. The Cdc42-βPix-Cbl-ternary complex formation has been described previously following expression of constitutively active
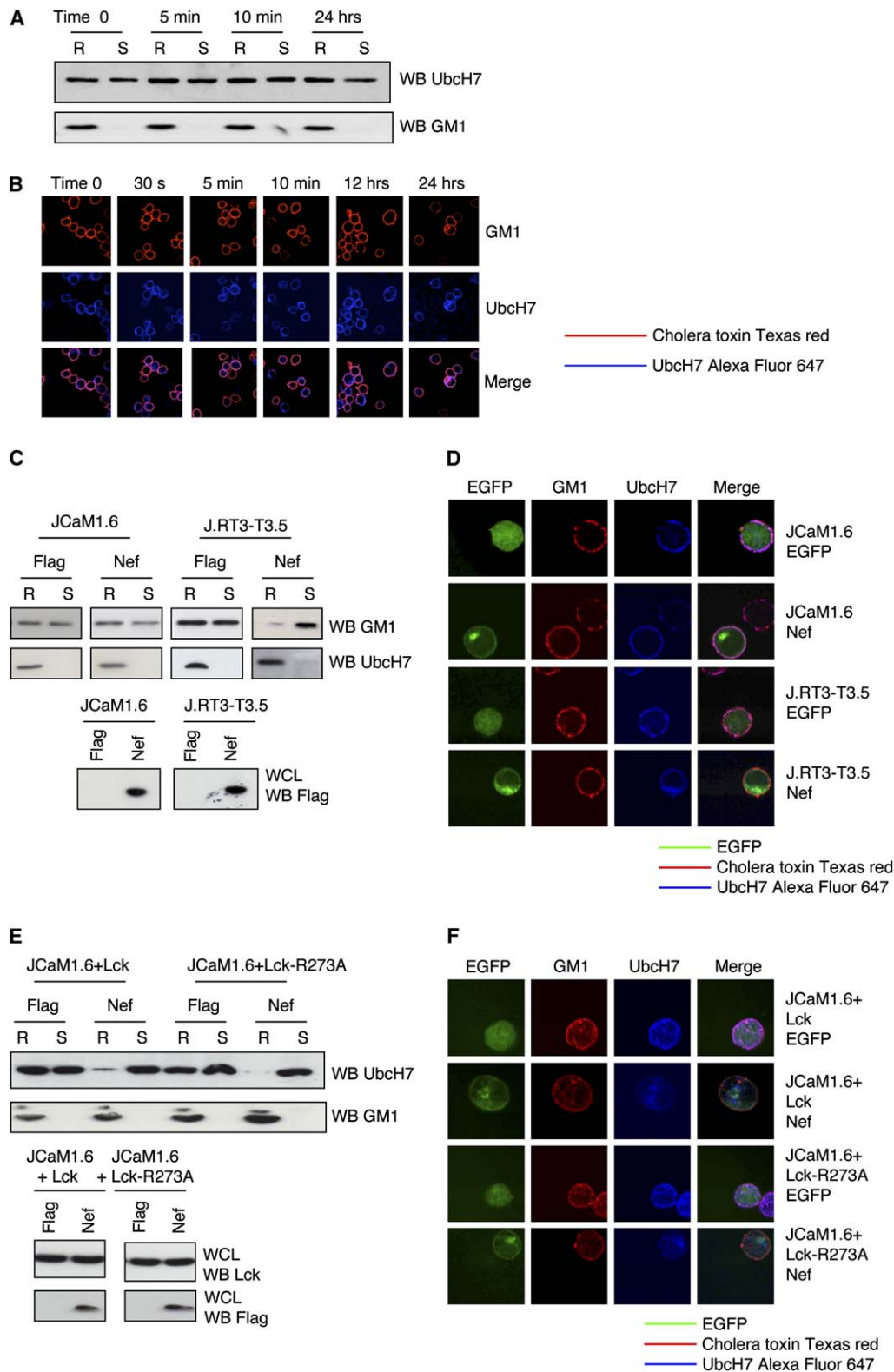
Figure 4. UbcH7 Raft Exclusion Is Distinct to the Nef Activation Stimulus and Is Reliant on Lck

(A) Western blot of UbcH7 in lipid raft (R) and nonraft (S) fractions of Jurkat stimulated with CD3/CD28 over a period of 24 hr. UbcH7 Western blot is in the top panel and GM1 below.

(B) Jurkat were stimulated with plate bound anti-CD3/CD28, harvested, and stained for GM1 with cholera toxin Texas red and UbcH7 Alexa Fluor 647 over a time course of 24 hr.

(C) JCaM1.6 and J.RT3-T3.5 cells were transfected with Flag or Nef vectors. Western blot for UbcH7 in lipid raft and nonraft lysates is shown in the top panel, with GM1 immunoblot below with cholera toxin. Flag Western blot of WCL is shown in the lower panel.
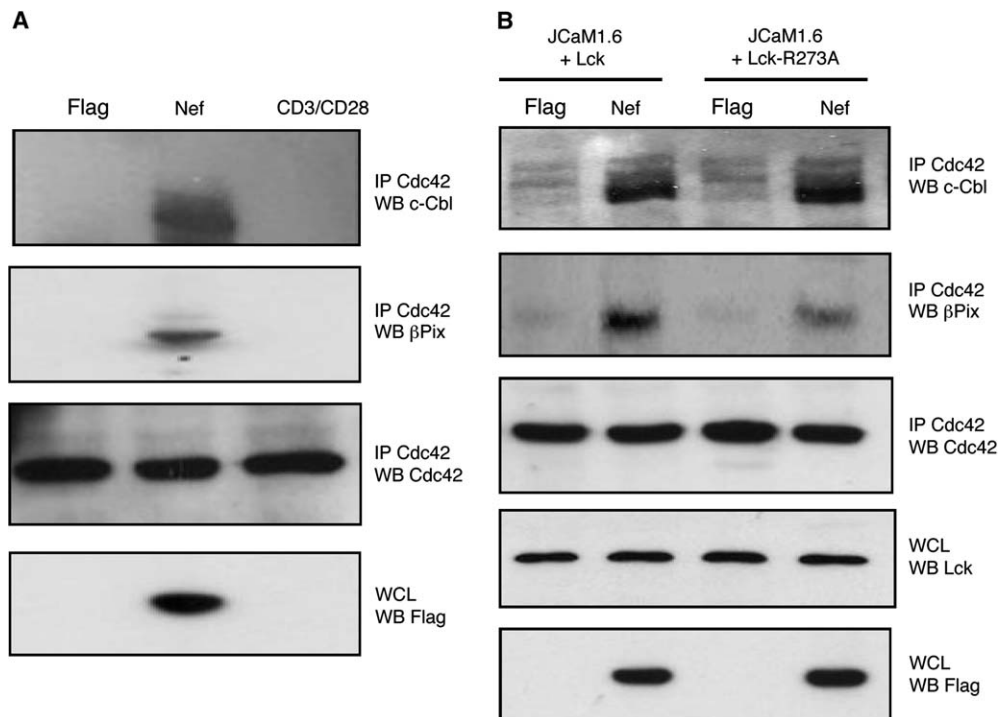
Figure 5. Cdc42 Associates with c-Cbl and βPix in the Presence of Nef

(A) Jurkat CD4 T cells were transfected with Flag or Nef or were activated with anti-CD3/CD28, and immunoprecipitates were performed with anti-Cdc42. The top panel shows Western blot for c-Cbl, the middle panel for βPix, and the lower panel for Cdc42. Flag expression in WCL is shown below.

(B) JCaM1.6 were transfected with Flag or Nef vectors and either Lck or Lck-R273A. Cdc42 was immunoprecipitated and Western blot for c-Cbl (top), βPix (middle), or Cdc42 (lower) was performed. Lck and Flag expression in WCL is shown below.

Cdc42 mutants, leading to failure of ubiquitination of EGFR, with resultant accumulation of surface EGFR and cellular transformation. The binding of activated Cdc42 to a βPix-Cbl complex here was thought to sterically interfere with the binding of Cbl to the EGF receptor, thus preventing E3 ligase activity. We found that expression of the mutant Cdc42, Cdc42(F28L), that is capable of constitutive GDP-GTP exchange, results in downregulation of lipid raft UbcH7 expression. It is likely that Cdc42-βPix-Cbl ternary complex formation also prevents the ubiquitin-charged E2 from gaining access to the Cbl RING finger by steric interference. Interestingly, diminishing Cbl expression with siRNAs resulted in loss of UbcH7 from lipid rafts, suggesting that interaction with Cbl is required for constitutive UbcH7 lipid raft localization in T cells.

Why Nef induces UbcH7 exit from lipid rafts but TCR stimulation fails to do so remains unclear. As a viral protein, Nef is not subject to the same negative regulatory constraints as endogenous T cell signaling mediators, which may lead to sustained imbalance in Cdc42 activity in lipid rafts and forward feed signaling. It might be expected that Nef should lead to transformation in cells

in which it is expressed. Nef has been shown to be transforming in certain cell types including fibroblasts (Briggs et al., 1997) and neurons in an SH3-dependent manner (Kramer-Hammerle et al., 2001). However, in the case of whole HIV infection, active viral replication leads to cellular cytopathicity, which likely overrides any transforming effect.

A paradigm exists for the control of ubiquitination by spatial localization of an E2 ubiquitin-conjugating enzyme, as UbcM2 is localized to the nucleus via importin-11 after ubiquitin charging (Plafker et al., 2004). It is possible that Nef parodies a yet undiscovered conventional physiological stimulus to interfere with c-Cbl function via control of the cellular localization of UbcH7. Nef has previously been shown to mimic integrin signaling by inducing the relocalization of the Eed transcriptional repressor to the plasma membrane in T cells (Witte et al., 2004). Lipid rafts have been implicated in the proper organization of integrin signaling (Palazzo et al., 2004; del Pozo et al., 2005). Nef-mediated recruitment of hnRNP E1 to lipid rafts is supportive of an integrin-type trigger. hnRNP E1 localizes to spreading initiation centers present in the early stages of cell spreading prior to the

(D) JCaM1.6 and J.RT3-T3.5 cells were transfected with EGFP or Nef-EGFP, stained for GM1 with cholera toxin Texas red and rafts patched with anti-cholera toxin antibody. UbcH7 was stained with UbcH7 Alexa Fluor 647.

(E) JCaM1.6 were transfected with Lck or Lck-R273A and Flag or Nef vectors before Western blot for UbcH7 and GM1 in raft and nonraft fractions. The lower panels show Western blot for Lck and Flag in WCL.

(F) JCaM1.6 were transfected with Lck or Lck-R273A and EGFP or Nef-EGFP before staining for GM1 with cholera toxin Texas red, raft patching, and subsequent UbcH7 Alexa Fluor 647 staining.
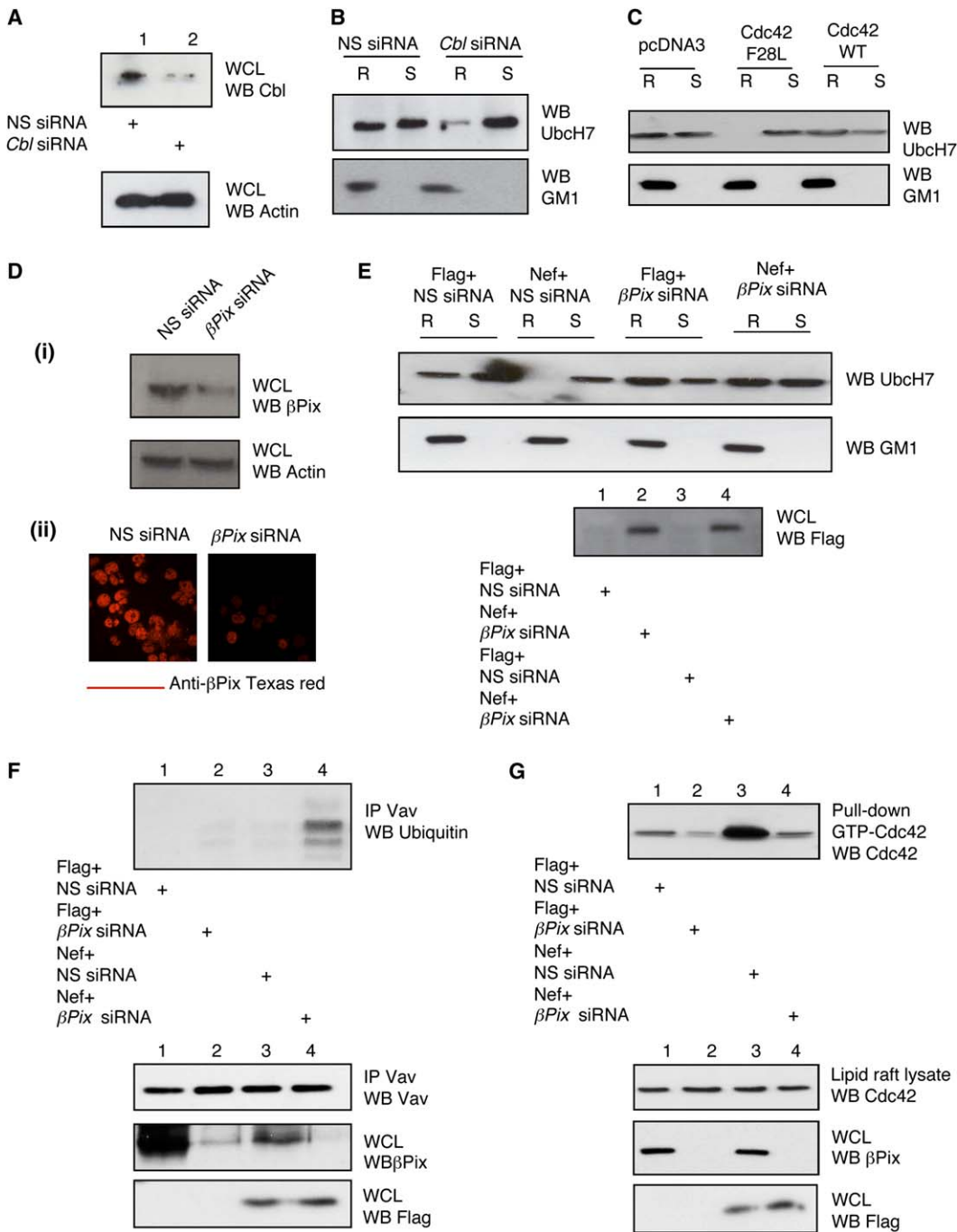
Figure 6. Cbl Is Required for UbcH7 Raft Localization, Expression of Cdc42(F28L) Displaces UbcH7 from Rafts, while βPix Knockdown Restores UbcH7 Raft Localization

(A) Jurkat were transfected with either NS siRNA or Cbl siRNA and Western blot for Cbl protein levels performed at 48 hr.

(B) The effect of Cbl knockdown on UbcH7 raft localization. The top panel shows UbcH7 protein levels in lipid raft fractions of Jurkat transfected with either NS siRNA or Cbl siRNA, and the lower panel shows GM1 Western blot.

(C) Jurkat were transfected with EGFP+empty vector (pcDNA3), Cdc42(F28L), or Cdc42WT, and Western blot for UbcH7 was performed on lipid raft (R) and soluble (S) fractions obtained after sucrose density gradient centrifugation. GM1 immunoblot is shown below. Transfection efficiency was comparable in all three transfections as assessed by EGFP expression on FACS analysis (data not shown).

(D) Western blot (i) and confocal (ii) images of βPix expression in the presence of NS siRNA and βPix siRNA1 at 48 hr post transfection.

(E) Lipid raft fractions obtained from cells transfected with Flag or Nef together with either NS siRNA or βPix siRNA. Fractions were Western blotted for UbcH7 and GM1 and WCL for Flag (bottom).

(F) Vav was immunoprecipitated from CD4 T cells transfected with Flag or Nef and either NS siRNA or βPix siRNA1. Western blot with anti-ubiquitin is shown in the top panel. The lower panel shows Vav Western blot from the same immunoprecipitates.

(G) Jurkat were transfected as in (C), lipid rafts were obtained, and Cdc42-GTP pull-down was conducted with PBD-agarose. Bound Cdc42 is shown in the top panel, lipid raft Cdc42 in the middle panel, and Flag immunoblot below.
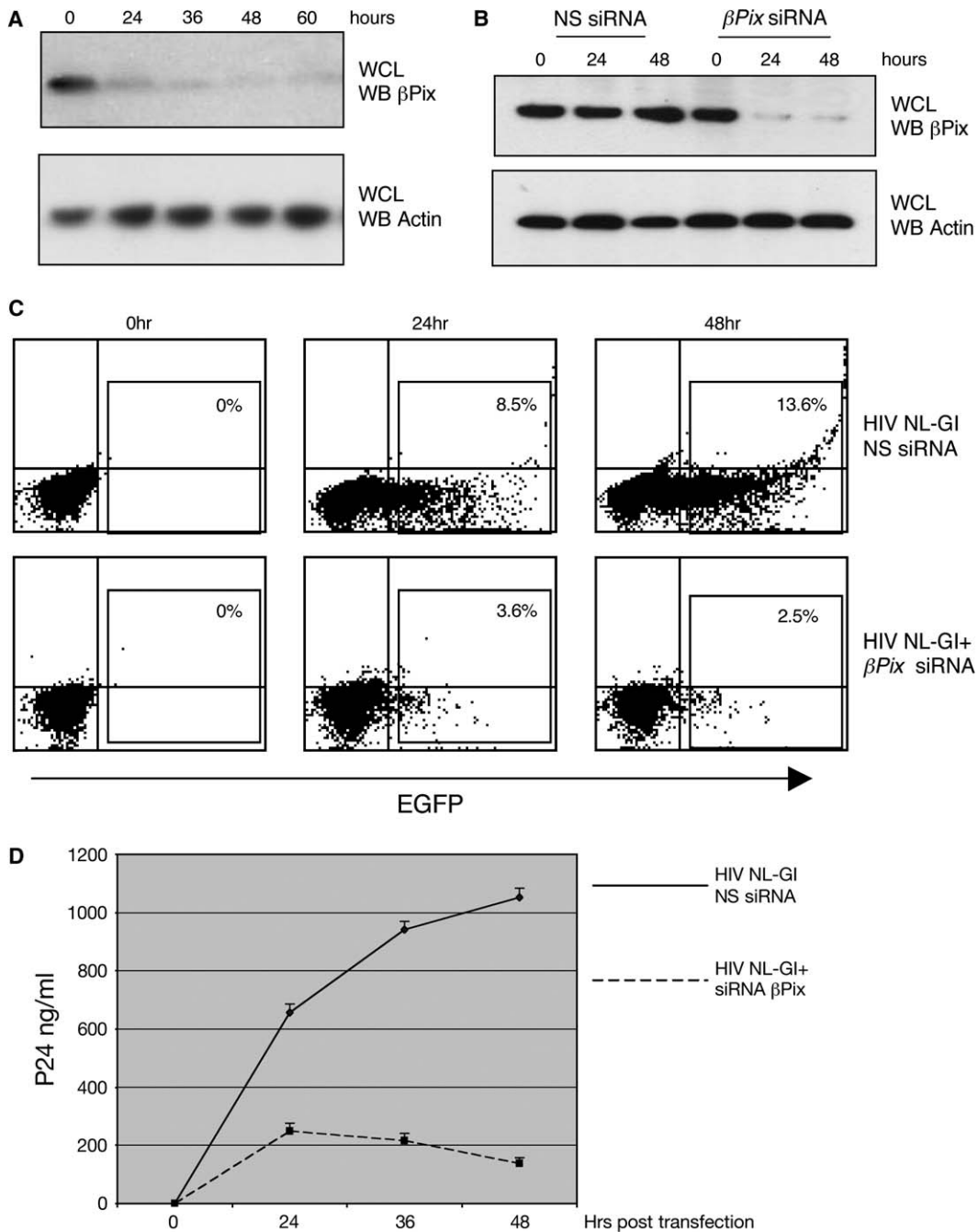
Figure 7. βPix Is Required for HIV Replication

(A) Time course demonstrating βPix knockdown after transfection of βPix siRNA1.

(B) Jurkat CD4 T cells were transfected with NL-GI HIV virus and either 20 μM *βPix* siRNA1 or NS siRNA, and the degree of βPix knockdown was assessed by Western blot.

(C and D) Levels of HIV production were documented by FACS analysis of EGFP expression (C) and by p24 ELISA, where values shown are averages ± SD from six separate experiments (D).

formation of focal adhesions that are induced by integrin triggering (de Hoog et al., 2004). Indeed, the requirement for Lck in inducing UbcH7 raft exit would also support such a stimulus, as Lck has been shown to be necessary for integrin activation in T cells (Fagerholm et al., 2002). It was interesting that the kinase domain of Lck was dispensable, hinting at an adaptor function for Lck in Nef-mediated UbcH7 raft downregulation. A previous study

has also implicated Lck in alteration of c-Cbl phenotype (in this case phosphorylation) in the presence of Nef and in the context of HIV infection of primary CD4 T cells (Yang and Henderson, 2005).

A consequence of Nef inhibition of c-Cbl-mediated ubiquitination is the maintenance of self-sustaining populations of GTP bound Cdc42 at the plasma membrane and in lipid rafts. How this dysregulation of signaling

functions to propagate virions is unclear. Cdc42 has been implicated in diverse cellular processes such as control of the actin cytoskeleton to help establish cell polarity, intracellular trafficking, and regulation of cell growth (reviewed by Cerione, 2004). It is possible that these roles may be usurped by Nef to govern aspects of the HIV life cycle. Localized plasma membrane Cdc42 activity may be required for virological synapse formation. Infected cells form a synapse with uninfected target cells that facilitates efficient cell-cell transfer of virions (Jolly et al., 2004; McDonald et al., 2003). It may be that Nef plays a role in the formation of this synapse by promoting local microtubule stabilization at the cell/cell interface via Cdc42 and PAK activation. The observed exit of the microtubule destabilizer stathmin from Nef-expressing lipid rafts is supportive of this hypothesis. Analogies have been made between the immunological and virological synapse, and interestingly in T lymphocytes, dominant-negative Cdc42 has been shown to block polarization toward antigen-presenting cells (Stowers et al., 1995). Ultimately, achieving heightened Cdc42 activity may be important in the HIV life cycle to direct HIV budding. HIV buds via the multivesicular body in a manner requiring TSG101 (tumor susceptibility gene 101 protein) and ESCRT-1 (endosomal sorting complex required for transport) (von Schwedler et al., 2003). The multivesicular body is specifically localized to sites of active exocytosis and enables secretory vesicle targeting and plasma membrane docking. Cdc42 has been shown to direct activity of the exocyst in yeast by binding Sec3p in its GTP bound form. Sec3p is an exocyst component that acts as a spatial landmark for polarized exocytosis. Cdc42 coordinates the vesicle docking machinery and the actin cytoskeleton for polarized secretion (Zhang et al., 2001). Most simply the inhibition of c-Cbl by Nef may serve to direct new gene expression akin to a weak form of T cell activation that has been proposed to provide a transcriptional environment favorable for completion of the HIV life cycle.

Ever since Nef was demonstrated to be essential for maintenance of high viral loads and progression to simian AIDS in adult rhesus macaques (Kestler et al., 1991), its primary function in achieving this effect has been under investigation. Identification of the principal host cell molecules involved is crucial to understanding how Nef exerts pathogenicity and in the rational design of intervention strategies. Our work demonstrates a role for Nef in interfering with an intrinsic negative feedback loop of T cell signaling. This inhibition of Cbl by Nef provides new candidate targets for inhibition of HIV replication.

Experimental Procedures

Cell Culture, Stimulation, Plasmids, Transfections, and p24 ELISA
Jurkat E6-1, JCaM1.6, and J.RT3-T3.5 cells were obtained from ATCC and were cultured in RPMI 1640 media (GIBCO-BRL) supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine (GIBCO-BRL). For stimulation of Jurkat with anti-CD3/CD28, 30 μl of 10 μg/ml OKT3 and anti-CD28 were added to 96-well plates and incubated at 37°C for 90 min. The wells were washed with PBS three times and 2×10^5 cells were added to each well in 0.2 ml media. Cells were incubated at 37°C and harvested at 24 hr. Anti-CD28 antibody (clone CD28.2) was obtained from Pharmingen. Flag-tagged Nef constructs were generated by cloning SF2 nef from pWT-IRES-EGFP (Simmons et al., 2001) into the pCMV-Tag-4A vec-

tor from Invitrogen. For the mutant G2A, the forward primer CGC GGA TCC GCG ACC ATG GCG GGC AAG TGG TCA AAA was used to amplify nef before insertion into pCMV-Tag-4A. The mutant Nef-PX-Mu was created by cloning PX-Mu-nef from the vector CN.94PXmu/Nef.Pxmu (Xu et al., 1999) before insertion to pCMV-Tag-4A. Nef-EGFP fusion expression vectors were created by cloning wild-type nef and mutants into the vector pEGFP-N3 from Clontech. Wild-type and kinase-dead Lck expression vectors PAlterMAX2-Lck and PAlterMAX2-R273A were obtained from Hamid Band, and the wild-type and constitutively active Cdc42 expression vectors, Cdc42(F28L), were from Richard Cerione. Transient transfection of Jurkat was carried out with AMAXA cell line nucleofector kit. Virus production was assayed by p24 ELISA; EGFP expression in HIV-infected cells was analyzed by FACSCalibur (Becton Dickinson).

Antibodies, Cdc42 Activation Assay, and siRNAs
The anti-Flag M2, anti-β Actin antibodies, and cholera-toxin HRP were obtained from Sigma. Antibodies to Cbl, βPix, and Cdc42 were obtained from Santa Cruz and Chemicon. Anti-cholera toxin antibodies were obtained from Sigma. Antibodies to UbcH7 were obtained from Upstate Biotechnology and Chemicon, and Vav and Lck antibodies were from Upstate Biotechnology and Santa Cruz. Anti-ubiquitin antibody was from Affiniti. Fluorescent conjugate secondary antibodies were obtained from Molecular Probes. Cdc42 activation assays were performed with the Cdc42 activation assay kit from Chemicon. Custom high performance purity (HPP) grade siRNAs were obtained from Qiagen. The target DNA sequence used to construct βPix siRNA1 was 5'-AAG AGCTCGAGAGACACATGG-3', for UbcH7 siRNA 5'- AAATGTGGGATGAAAAACTTC-3', and the nonsilencing target sequence 5'-AAT TCT CCG AAC GTG TCA CGT-3'. c-Cbl and Cbl-b siRNA sequences are available from Qiagen.

Lipid Raft Extraction, Immunoprecipitation, and Western Blots
Lipid rafts were isolated from cell lysates of 50×10^6 cells in Triton X-100 and floatation on sucrose density gradients as described previously (Cheng et al., 1999). Among 12 fractions collected from the top of the gradient, fractions 3 and 4 were confirmed as rafts by detection of GM1 by Western blot analysis. For immunoprecipitations, the lipid raft fraction 4 was mixed with 60 mM octyl β-D-glucopyranoside (Sigma), and solubilized fractions were incubated with the corresponding antibody and either Protein A or G-agarose. For Western blot analysis, immunoprecipitates or WCL were resolved on SDS-PAGE, transferred to PVDF membrane (Amersham Biosciences), and detected by the indicated antibodies by ECL system (Amersham Biosciences).

Confocal Analysis
For confocal analysis, cells were stained with cholera toxin to detect GM1, and lipid rafts were patched with anti-cholera toxin antibody. For intracellular staining with antibodies against UbcH7 or βPix, cells were fixed with 4% v/v paraformaldehyde and permeabilized with 1% v/v Triton X-100. Confocal microscopy was performed with a Bio-Rad Radiance 2000 laser scanning confocal and analyzed with LaserSharp 2000 software (Bio-Rad). All images were acquired in sequential scanning mode.

Two-Dimensional Gel Electrophoresis and Mass Spectrometric Analysis
See Supplemental Data.

Supplemental Data
Supplemental Data include eight figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/23/6/621/DC1/.

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