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Mutation of the ATP Cassette Binding Transporter A1 (ABCA1) C-Terminus Disrupts HIV-1 Nef Binding but Does Not Block the Nef Enhancement of ABCA1 Protein Degradation†

Zahedi Mujawar,‡ Norimasa Tamehiro,‡ Angela Grant,§ Dmitri Sviridov,∥ Michael Bukrinsky,§ and Michael L. Fitzgerald*,‡

‡*Lipid Metabolism Unit, Center for Computational and Integrative Biology, Richard B. Simches Research Center, Massachusetts General Hospital, 185 Cambridge Street, 7th Floor #7150, Boston, Massachusetts 02114,* §*Department of Microbiology, Immunology and Tropical Medicine, The George Washington University, Washington, D.C. 20037, and* ∥*Baker IDI Heart and Diabetes Institute, 75 Commercial Road, Melbourne, Victoria 3004, Australia*

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ABSTRACT: HIV-1 infection and antiretroviral therapy are associated with a dyslipidemia marked by low levels of high-density lipoprotein and increased cardiovascular disease, but it is unclear whether virion replication plays a causative role in these changes. The HIV-1 Nef protein can impair ATP cassette binding transporter A1 (ABCA1) cholesterol efflux from macrophages, a potentially pro-atherosclerotic effect. This viral inhibition of efflux was correlated with a direct interaction between ABCA1 and Nef. Here, we defined the ABCA1 domain required for the Nef−ABCA1 protein−protein interaction and determined whether this interaction mediates the ability of Nef to downregulate ABCA1. Nef expressed in HEK 293 cells strongly inhibited ABCA1 efflux and protein levels but did not alter levels of cMIR, another transmembrane protein. Analysis of a panel of ABCA1 C-terminal mutants showed Nef binding required the ABCA1 C-terminal amino acids between positions 2225 and 2231. However, the binding of Nef to ABCA1 was not required for inhibition because the C-terminal ABCA1 mutants that did not bind Nef were still downregulated by Nef. Given this discordance, the mechanism of downregulation was investigated and was found to involve the acceleration of ABCA1 protein degradation but did not to depend upon the ABCA1 PEST sequence, which mediates the calpain proteolysis of ABCA1. Furthermore, it did not associate with a Nef-dependent induction of signaling through the unfolded protein response but was significantly dependent upon proteasomal function and could act on an ABCA1 mutant that fails to exit the endoplasmic reticulum. In summary, we show that Nef downregulates ABCA1 function by a post-translational mechanism that stimulates ABCA1 degradation but does not require the ability of Nef to bind ABCA1.

HIV infection is associated with pro-atherosclerotic changes in lipid metabolism and an increase in the risk of cardiovascular disease (CVD) (*1*−*7*). Whereas numerous studies have investigated the role of antiretroviral therapy (ART) in driving this dyslipidemia (*8*), the effect of primary HIV infection on cellular cholesterol metabolism remains poorly characterized (*9*). Understanding the role of infection versus treatment is important because recent clinical trials have associated treatment interruption with a higher incidence of adverse cardiovascular events (*10*−*12*). These results suggest that early and persistent inhibition of viral replication may reduce the risk of CVD in infected individuals and indicates viral replication itself may induce certain aspects of HIV-associated dyslipidemia (*13*). In this regard, it is noteworthy that both treatment-naïve patients and HIV-infected individuals receiving ART have significantly lower levels of circulating high-density lipoprotein (HDL),[1] a change that is predicted to increase the incidence of atherosclerosis and CVD (*14*−*19*).

HIV replication can disrupt host lipid homeostasis (*20*), and this effect could be due to the critical role that cholesterol plays in the HIV life cycle (*21*). HIV assembly, budding, and infection of new target cells all depend on plasma membrane cholesterol (*22*−*27*). Depletion of virion-associated cholesterol attenuates fusion of HIV-1 to the host cell membrane, and cholesterol-sequestering compounds such as β-cyclodextrin permeabilize and inactivate virions, thus rendering them incompetent for cell entry (*28*−*32*). Additionally, monoclonal anticholesterol antibodies that remodel the plasma membrane of HIV-1 permissive human T-cells and macrophages result in inhibition of HIV-1 infection and production in vitro (*33*). Moreover, HIV-1 budding from the host cell is thought to occur at lipid rafts, and the cholesterol:phospholipid molar ratio of the viral envelope is

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*To whom correspondence should be addressed: Lipid Metabolism Unit, Center for Computational and Integrative Biology, Richard B. Simches Research Center, Massachusetts General Hospital, 185 Cambridge St., 7th Floor, Boston, MA 02114. Telephone: (617) 726-1465. Fax: (617) 643-3328. E-mail: mfitzgerald@ccib.mgh.harvard.edu.

[1]Abbreviations: ABCA1, ATP cassette binding transporter A1; apoA-I, apolipoprotein A-I; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FBS, fetal bovine serum; HDL, high-density lipoprotein; UPR, unfolded protein response.

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approximately 2.5 times that of the host cell surface membranes (*34*). Recently, mass spectrometry was used to quantitate the lipid constituents of the HIV envelope and compare this to the host cell membrane. The composition of the HIV-1 envelope lipid was similar to that of lipid rafts being enriched in cholesterol and sphingolipids, which further supports the hypothesis that HIV-1 buds from membrane microdomains (*35*). Thus, HIV may reprogram cellular lipid metabolism to maximize lipid rafts, the sites of virus assembly, budding, and release. Supporting this notion are studies that demonstrate HIV-1 Nef, a 27−29 kDa myristoylated viral protein, alters cellular cholesterol metabolism to maximize virion production. In particular, Nef expression increases the level of cholesterol biosynthesis by upregulating CYP51, which encodes a sterol 14α-demethylase that catalyzes the demethylation of lanosterol during cholesterol biosynthesis (*36*). Nef also upregulates other genes involved in de novo cholesterol biosynthesis, including HMG-CoA reductase, the rate-limiting enzyme for this process (*37*). Finally, Nef contains a C-terminal cholesterol recognition motif, preserved in all clades of HIV, which binds cholesterol both in vitro and in vivo (*36*). Because lipid raft-associated cholesterol is critical for HIV assembly and infectivity, it is thought that Nef serves as a carrier to enrich cholesterol at the sites of virion budding in the plasma membrane of infected cells (*26*, *38*). Thus, by upregulating its biosynthesis and binding cholesterol, Nef may ensure an adequate delivery of cholesterol to nascent virions that bud from infected cells (*36*).

To date, all of the pathways by which Nef is thought to maximize virion lipid content involve the synthesis of cholesterol. However, the fact that HIV infection lowers HDL levels suggests the virus may also disrupt pathways by which cells rid themselves of cholesterol, for example, via ABCA1-dependent cholesterol efflux (*39*). Indeed, we have found that Nef impairs ABCA1-dependent cholesterol efflux from human macrophages, a key step in the reverse cholesterol transport process by which the body rids itself of excess cholesterol. This viral-mediated inhibition of efflux was critical for the formation of infectious particles by HIV-1 and correlated with reduced ABCA1 protein levels and a direct interaction between ABCA1 and Nef (*40*).

Here we determined the domain on ABCA1 that allows it to physically interact with Nef and assessed whether this interaction mediates the ability of Nef to inhibit ABCA1 efflux function. Using a panel of ABCA1 C-terminal mutants (*41*), the binding of ABCA1 by Nef was found to depend upon a motif in the ABCA1 C-terminus between amino acids 2225 and 2231. Although binding of Nef to the ABCA1 C-terminal mutants was ablated, Nef was still able to significantly reduce the efflux activity and protein levels of the mutant ABCA1 transporters. Given this discordance, we further investigated the mechanism by which Nef was able to reduce ABCA1 protein levels. This Nef effect was associated with an acceleration of ABCA1 protein degradation but did not depend upon the ABCA1 PEST sequence-mediated calpain degradation of the transporter (*42*, *43*) and was not associated with activation of unfolded protein response (UPR) signaling pathways (*44*). In contrast, the Nef downregulation of ABCA1 was significantly dependent upon proteasomal function and repressed an ABCA1 mutant that fails to exit the endoplasmic reticulum. Thus, we conclude that Nef downregulates ABCA1 function by a novel post-translational mechanism that stimulates ABCA1 degradation but does not require the ability of Nef to bind the ABCA1 C-terminus.

MATERIALS AND METHODS

Reagents. The following reagents were purchased from the indicated suppliers: Metafectene (Biontex); Lipofectamine LTX (Invitrogen); LXR agonist TO-901317 (Calbiochem); [1,2-^{3}H]-cholesterol (PerkinElmer); human lipid free apolipoprotein A-I (Biodesign); calpeptin (BIOMOL); cycloheximide, lactacystin, epoxomicin, thapsigargin, tunicamycin, FLAG peptide, and anti-FLAG M2 affinity gel (Sigma); anti-ABCA1 rabbit polyclonal antibody (Abcam); anti-ABCA1 mouse monoclonal antibody (GenWay); anti-β-actin mouse monoclonal antibody (Millipore); anti-β-actin rabbit polyclonal antibody (Sigma); anti-CHOP mouse monoclonal antibody (ABR); anti-XBP-1 rabbit polyclonal antibody (Santa Cruz Biotechnology); and anti-HA mouse monoclonal antibody (Covance). The following reagents were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HIV-$1_{JR\text{-}CSF}$ Nef monoclonal antibody from K. Krohn and V. Ovod (*45*) and HIV-1 Nef polyclonal antibody from R. Swanstrom (*46*). We are grateful to M. Peterlin (University of California, San Francisco, CA) for pHSF2Nef, pHSF2NefΔXho, SF2-Nef_{WT}, and SF2-Nef_{G2A} constructs; O. Schwartz (Institut Pasteur) for AD8, AD8ΔNef viruses, and LAI-Nef_{WT} and LAI-Nef_{G2A} expression constructs; and G. Chimini (Centre d'Immunologie de Marseille-Luminy) for the YFP-ABCA1 construct. The FLAG-tagged ABCA1 ($ABCA1_{WT}$, $ABCA1_{\Delta 40}$, and $ABCA1_{\Delta 46}$) constructs and the anti-ABCA1 polyclonal antibody have been previously described (*41*, *47*). The $ABCA1_{DDDHLK\rightarrow AAAAAA}$ and $ABCA1_{\Delta PEST}$ mutants were generated and their sequences verified as previously described (*41*−*43*, *47*).

Cell Culture and Transfection Assays. HeLa, HEK 293, HEK 293T (ATCC), and HEK 293-EBNA-T (gift of B. Seed, Massachusetts General Hospital) cells were transfected using Metafectene (Biontex, Munich, Germany) following the manufacturer's protocol. In brief, the cells were seeded into 24-well poly-D-lysine-coated tissue culture plates at a density of 100000 cells/well and 24 h later were transfected with 0.5 μg of cDNA (in 30 μL of serum and antibiotic-free medium or PBS) with 2 μL of Metafectene (in 30 μL of serum and antibiotic-free medium or PBS). RAW 264.7 murine macrophages were transfected using Lipofectamine LTX reagent (Invitrogen) following the manufacturer's protocol. In brief, the cells were seeded into 12-well poly-D-lysine-coated tissue culture plates at a density of 100000 cells/well and 24 h later were treated with 1 μM TO-901317 (LXR agonist). After 24 h, the cells were transfected with 1 μg of cDNA (in 120 μL of Opti-MEM 1 reduced serum medium with 1 μL of PLUS Reagent and 3 μL of Lipofectamine LTX Reagent).

Immunoprecipitations. Immunoprecipitations were used to analyze the physical interaction of FLAG-tagged $ABCA1_{WT}$ and Nef_{WT} in lysates prepared from transfected HEK 293 EBNA-T cells seeded in six-well tissue culture plates. Twenty-four hours after cotransfection, the cells were washed with ice-cold PBS and lysed in 1 mL of TX-100 buffer [1% Triton X-100, 140 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 50 mM HEPES (pH 7.0), and a protease inhibitor cocktail]. The cell lysates were centrifuged (10 min at 2000*g* and 4 °C); the supernatants were transferred to fresh 1.5 mL Eppendorf tubes, and the ABCA1−Nef complexes were coprecipitated using the anti-FLAG M2 affinity gel (1 mg of total cell protein lysate determined by the Bradford assay incubated with 40 μL of anti-FLAG M2 affinity

gel overnight at 4 °C with rotation). After centrifugation, the anti-FLAG M2 affinity gel was washed four times with 1 mL of TX-100 buffer and transferred to fresh tubes. The FLAG−ABCA1 complexes were eluted from the affinity gel with 30 μL of FLAG peptide (0.5 mg/mL, 1 h, 4 °C), and the resulting supernatants were separated via 4 to 20% SDS−PAGE gradient gels and immunoblotted for the amount of precipitated Nef and ABCA1.

Cholesterol Efflux Assays. Cholesterol efflux assays were conducted as previously described (*41*, *47*). In brief, HEK 293-EBNA-T cells were seeded into 24-well poly-D-lysine-coated tissue culture plates at a density of 100000 cells/well and 72 h later were transfected in triplicate with the empty vector or the indicated cDNAs ($ABCA1_{WT}$, $ABCA1_{\Delta 40}$, $ABCA1_{\Delta 46}$, $ABCA1_{DDDHLK\rightarrow AAAAAA}$, or $ABCA1_{\Delta PEST}$ with Nef_{AS} or Nef_{WT}) using Metafectene (Biontex). In assays involving transfection of multiple cDNAs, an empty vector was used to maintain an equal amount of transfected DNA. Twenty-four hours post-transfection, the cells were incubated with 0.5 μCi/mL [^{3}H]cholesterol in complete medium (10% FBS/DMEM) for an additional 24 h. Non-cell-associated cholesterol was removed by two washes with 1× PBS followed by a 2 h incubation in 2 mg/mL fatty acid-free BSA/DMEM at 37 °C and two additional washes in 1× PBS. The cells were further incubated in medium alone (2 mg/mL fatty acid-free BSA/DMEM) or in this medium with 10 μg/mL lipid-free apoA-I for 20 h. Medium was collected from the cells and cleared of debris by a 5 min, 1000*g* spin. To measure total cholesterol uptake and efflux, cell layers were dissolved in 0.1 N NaOH for 1 h and the amount of radioactivity in the media and cell lysates was measured by scintillation counting. ApoA-I-dependent cholesterol efflux was expressed as the difference in the percentage of efflux [(medium counts per minute)/(medium + cell counts per minute) × 100] for the apoA-I-treated cells minus the percentage of efflux from the cells treated with medium alone.

Immunoblotting and Protein Degradation Assays. HEK 293-EBNA-T cells were seeded into 24-well poly-D-lysine-coated tissue culture plates at a density of 100000 cells/well and 24 h later were transfected in triplicate with the empty vector or the indicated cDNAs ($ABCA1_{WT}$ with Nef_{AS} or Nef_{WT}). Twenty-four hours after transfection and culture in complete medium (10% FBS/DMEM), the cells were treated with 100 μg/mL cycloheximide and cell lysates were subsequently collected 1, 4, 6, and 8 h after cycloheximide treatment using RIPA buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% sodium deoxycholate, 1% IGEPAL CA360, and protease inhibitor cocktail]. The amount of ABCA1, Nef, and β-actin in the lysates was assessed by immunoblotting and enhanced chemiluminescence using a Bio-Rad Molecular Imager ChemiDoc XRS+ system. Likewise, RAW 264.7 murine macrophages were seeded into 12-well poly-D-lysine-coated tissue culture plates at a density of 100000 cells/well and 24 h later were treated with 1 μM TO-901317 (LXR agonist). After 24 h, the cells were transfected with the indicated cDNAs (Nef_{AS} or Nef_{WT}). Twenty-four hours after transfection and culture in complete medium (10% FBS/DMEM), the cells were treated with 100 μg/mL cycloheximide and cell lysates were subsequently collected 1, 2, 4, 6, and 8 h after cycloheximide treatment using RIPA buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% sodium deoxycholate, 1% IGEPAL CA360, and protease inhibitor cocktail]. The amount of ABCA1, Nef, and β-actin in the lysates was assessed by immunoblotting and enhanced chemiluminescence using a Bio-Rad Molecular Imager ChemiDoc XRS+ system. The ABCA1 protein half-life was estimated by logarithmic graphs of the β-actin-normalized ABCA1 values versus time.

ABCA1 Localization by Confocal Microscopy and Ultracentrifugation. The cellular distribution of YFP- or GFP-tagged ABCA1 in 293 cells was determined using a Leica TCS SP confocal microscope and a 63× oil immersion lens. The effect of Nef expression on the cellular localization of ABCA1 was determined by cotransfection of GFP-Nef and YFP-ABCA1, or untagged Nef, GFP-ABCA1, and pDsRed2-ER. We further determined the cellular distribution of ABCA1 by ultracentrifugation of HEK 293 cell lysates expressing ABCA1 with or without Nef derived by douncing the cells in buffer [250 mM sucrose and 10 mM HEPES (pH 7.5)] and obtaining a 30000*g* membrane pellet (30P). The pellet was then suspended in 0.450 mL of 12.5% sucrose buffer and overlaid on a discontinuous sucrose gradient (0.319 mL of 20%, 0.319 mL of 26%, 0.638 mL of 32%, 1.275 mL of 36%, 0.85 mL of 40%, 0.638 mL of 46%, and 0.425 mL of 60% sucrose in buffer). The gradient was spun at 33000 rpm for 3 h with an SW55 Beckman rotor, and fractions were collected by needle puncture from the bottom of the tubes. Fractions were diluted with 3 volumes of HEPES buffer and centrifuged for 2.5 h at 100000*g*, and membrane pellets were suspended in a constant volume of SDS buffer for analysis by immunoblotting. Fractions enriched in endoplasmic reticulum membranes were identified by the presence of the ER resident protein SPTLC endogenously expressed in these cells.

Statistical Analysis. Data from the cholesterol efflux assays and cycloheximide assays were found to have equal variance and were further compared with two-tailed Student's *t* tests and ANOVA, respectively, using SigmaStat. A p value of < 0.05 was defined as statistically significant.

RESULTS

Previously, HIV-1 infection of macrophages was found to impair ABCA1 efflux activity through a process that depended upon Nef, a virus-encoded accessory protein. This effect lowered ABCA1 protein levels and was associated with a physical interaction between Nef and ABCA1 (*40*). Because we wanted to explore the structure−function relationship of the Nef−ABCA1 interaction and the ability of Nef to downregulate the transporter, we first tested whether this activity could be recapitulated in a nonmacrophage cell line that is readily transfected. To this end, we cotransfected cDNAs for Nef and ABCA1 into 293ET cells, which are a human embryonic kidney-derived 293 cell line that also expresses the SV40-T and Epstein-Barr viral nuclear antigens. We favor these cells because they support robust ABCA1 expression and efflux activity when transfected with pcDNA-based vectors expressing the ABCA1 cDNA driven by the cytomegalovirus (CMV) promoter. Next, protein lysates collected from the cells 24 h post-transfection were assessed for ABCA1 expression by immunoblotting. We found that co-expression of Nef strongly reduced protein levels of ABCA1 (Figure 1A). In contrast, cotransfection of the Nef cDNA with a pcDNA CMV-based vector encoding another transmembrane domain protein, cMIR (*48*), did not result in the downregulation of the cMIR protein (Figure 1B). This indicates that the effect of Nef on ABCA1 protein expression can be recapitulated in a nonmacrophage cell line and is not due to a nonspecific down-regulation of transmembrane proteins by Nef.

Because Nef can alter transcriptional pathways in infected cells, requiring an environment conducive to dynamic viral production,

we wanted to test if Nef expression results in the suppression of ABCA1 mRNA levels (*49*, *50*). To address this, 293ET cells were again cotransfected with ABCA1 and Nef (or a control vector containing the Nef sequence in an antisense orientation, Nef_{AS}) and quantitative RT-PCR was performed. This experiment showed that levels of ABCA1 mRNA were not being reduced by coexpression of Nef (Figure 1C). This result confirmed our previous findings that Nef also did not reduce the level of endogenous ABCA1 mRNA in macrophages (*40*). Additionally, we tested whether the ability of Nef to downregulate ABCA1 abundance was unique to the 293ET line because of SV40-T and EBNA viral antigen expression. This was not found to be the case as Nef was able to downregulate ABCA1 protein expression in HEK 293 cells, which do not express the SV40-T and EBNA antigens (Figure 1D). Moreover, when HeLa cells were cotransfected with the Nef and ABCA1 cDNAs, the level of ABCA1 expression was also reduced, indicating that this effect of Nef was not restricted to the HEK 293 line (Figure 1D). Finally, we tested whether the myristoylation of Nef was important for this ABCA1 downregulation. To address this question, we tested the ability of a Nef mutant (Nef_{G2A}), which blocks the myristoylation of Nef and prevents it from associating with lipid bilayers to reduce the level of ABCA1 expression (*51*). In contrast to Nef_{WT}, the Nef_{G2A} mutant showed little or no ability to downregulate ABCA1 despite being expressed at levels similar to that of Nef_{WT} (Figure 1E). In aggregate, these results indicate that Nef is able to downregulate ABCA1 expression in the 293ET cells, and mutation of the Nef myristoylation site blocks the ability of Nef to downregulate ABCA1.

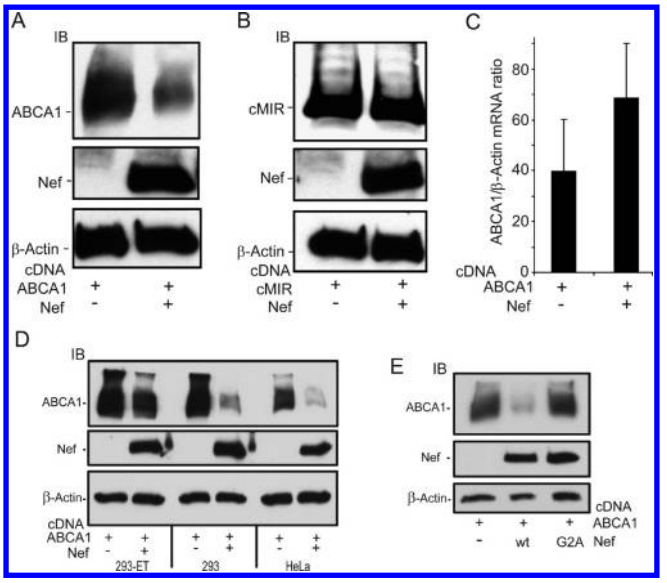

FIGURE 1: HIV-1 viral protein Nef downregulates ABCA1 protein expression by a post-transcriptional mechanism. (A) Human embryonic kidney cells (293ET) were transfected with an ABCA1 cDNA in the presence or absence of vectors expressing the HIV-1 Nef protein either in the antisense orientation (−) or in the sense orientation (+). Twenty-four hours post-transfection, protein lysates were prepared from the cells and the amount of ABCA1, Nef, and *β*-actin in the lysates was determined by immunoblotting (IB). (B) Coexpression of Nef with cMIR, another transmembrane protein, in 293ET cells does not effect cMIR protein expression. (C) Coexpression of Nef does not inhibit ABCA1 mRNA levels as determined by RT-QPCR assays indicating Nef is downregulating ABCA1 protein levels by a post-transcriptional mechanism ($n = 4$, ±standard error of the mean, $p = 0.37$). The Nef-mediated suppression of ABCA1 protein levels also occurs in HEK 293 and HeLa cells (D) and is abrogated by the Nef_{G2A} mutation, which blocks the myristoylation and membrane association of Nef (E).

Because the downregulation of CD4 and MHC I has been shown to depend on a physical interaction between Nef and these receptors (*52*−*54*), we next tested whether the Nef-mediated downregulation of ABCA1 was acting through the reported ability of Nef to bind ABCA1. To address this, 293ET cells were cotransfected with Nef or the Nef_{G2A} myristoylation mutant and FLAG-tagged ABCA1. Our previous study showed that insertion of the FLAG epitope into the first large N-terminal extracellular loop of ABCA1 preserves the ability of the transporter to bind and transfer cholesterol and phospholipid to apoA-I (*47*). FLAG-ABCA1 from the transfected cells was immunoprecipitated using anti-FLAG antibody beads, and after elution of FLAG-ABCA1 from the beads with the free FLAG peptide, the amount of Nef that coprecipitated with ABCA1 was assessed by immunoblotting. This analysis revealed that Nef_{WT} coprecipitated with ABCA1, whereas Nef_{G2A} did not (Figure 2, top left panel, IP), despite similar expression levels of the Nef_{G2A} mutant in the cell lysates used for the immunoprecipitations (Figure 2, middle right panel, input lysate). Thus, disrupting myristoylation

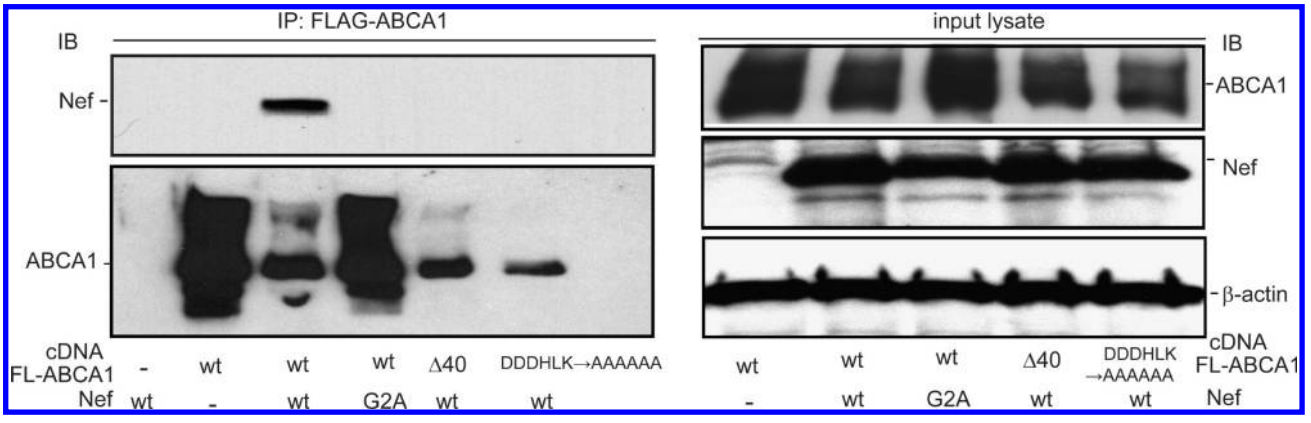

FIGURE 2: Physical interaction between Nef and ABCA1 depends upon Nef myristoylation and is disrupted by mutation of sequences between positions 2225 and 2231 of the ABCA1 C-terminus. 293ET cells were transfected with cDNAs that express wild-type FLAG-ABCA1 (FLAG tag located in the N-terminal first extracellular loop of ABCA1) or the indicated FLAG-ABCA1 C-terminal mutants in the presence of the indicated Nef constructs (ABCA1-Δ40, a 40-amino acid C-terminal deletion; ABCA1-DDDHLK-AAAAAA, sequences between positions −31 and −36 from the C-terminus selectively mutated to alanines; NefG2A, alanine mutation of the myristoylation motif). FLAG-ABCA1 was immunoprecipitated, and the amount of coprecipitated Nef and ABCA1 was determined by immunoblotting (left panels). The amount of ABCA1, Nef, and *β*-actin expressed in the transfected cells was further determined by immunoblotting the input protein lysates (right panels).

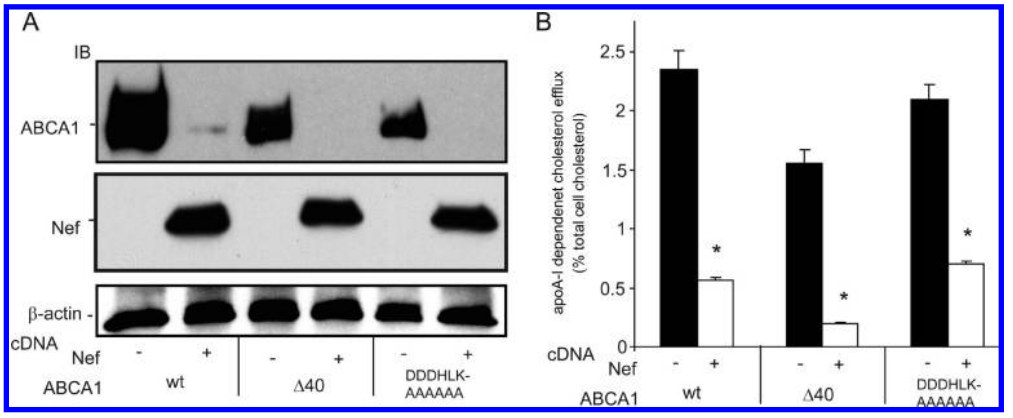

FIGURE 3: Disruption of the Nef−ABCA1 protein complex does not block the ability of Nef to downregulate ABCA1 expression. 293ET cells were transfected with cDNAs that express wild-type FLAG-ABCA1 or the indicated C-terminal FLAG−ABCA1 mutants as described in the legend of Figure 2. (A) Cell lysates were immunoblotted for the amount of ABCA1 and Nef, which shows Nef was still able to downregulate the expression of the ABCA1 C-terminal mutants that did not bind Nef. (B) Nef also maintains the ability to suppress the cholesterol efflux activity of the ABCA1 C-terminal mutants that did not bind Nef. Cells were transfected as described for panel A, loaded with [^{3}H]cholesterol for 24 h, washed, and incubated overnight with medium alone (DMEM, 1 mg/mL fatty acid-free BSA) or with medium and lipid-free apolipoprotein A-I (10 μg/mL). Scintillation counting was used to quantitate media and cell-associated [^{3}H]cholesterol, and the amount of apoA-I-dependent cholesterol efflux was graphed (percent of total cell-associated cholesterol) ($n = 3$, ±standard deviation, *$p < 0.05$, ABCA1 vs ABCA1 and Nef). Results are representative of two or more experiments.

of Nef blocked both its ability to physically interact with ABCA1 and its ability to downregulate protein levels of the transporter.

Previously, it has been shown that the Nef-mediated post-translational downregulation of the mannose receptor (MMR) required the presence of a motif (*S*DTKDLV) in the MMR cytoplasmic tail (*55*). Such motifs are present in other membrane proteins susceptible to Nef-mediated downregulation, including CD4, CCR5, and CXCR4. We found a similar sequence in the ABCA1 C-terminus (*S*DDDHLK) and tested whether Nef loses its ability to bind an $ABCA1_{\Delta 40}$ mutant, which lacks the last 40 amino acids of the ABCA1 C-terminus, including the *S*DDDHLK residues (*41*). Interestingly, the $ABCA1_{\Delta 40}$ mutant did not co-immunoprecipitate Nef (Figure 2, top left panel, IP), indicating that the last 40 amino acids of the ABCA1 C-terminus were required for Nef binding. To test whether mutation of just the ABCA1 residues between positions −30 and −40 that encompass the *S*DDDHLK motif disrupted the ability of ABCA1 to co-immunoprecipitate Nef, we generated an ABCA1 mutant that selectively changed these sequences to alanines ($ABCA1_{DDDHLK\rightarrow AAAAAA}$). Like the $ABCA1_{\Delta 40}$ mutant, $ABCA1_{DDDHLK\rightarrow AAAAAA}$ also failed to coprecipitate Nef, indicating that mutation of just the *S*DDDHLK motif in the C-terminus of ABCA1 could disrupt binding of Nef (Figure 2, top left panel, IP). Functional assessment of the $ABCA1_{\Delta 40}$ mutant showed that it possessed significantly less efflux activity than wild-type ABCA1, while the $ABCA1_{DDDHLK\rightarrow AAAAAA}$ mutant exhibited activity that did not differ significantly from that of ABCA1 (Figure 1 of the Supporting Information). Thus, the inability of these ABCA1 mutants to bind Nef could not be ascribed to a complete failure of their ability to efflux cholesterol to apoA-I. However, probing the anti-FLAG immunoprecipitates from cells cotransfected with the FLAG-ABCA1 mutants and Nef for the amount of the mutant ABCA1 transporters showed levels that were more comparable to the amount of $ABCA1_{WT}$ precipitated in the presence of Nef_{WT} than to the much greater amount of $ABCA1_{WT}$ that was precipitated in the presence of the Nef_{G2A} mutant that did not downregulate ABCA1 protein levels (Figure 2, bottom left panel, IP). This result raised the possibility that in spite of not physically interacting with Nef, the ABCA1 C-terminal mutants might still have been downregulated by Nef, which was further suggested by assessing the amount of total ABCA1 expressed in these cells (Figure 2, top right panel, input lysate). To investigate this possibility directly, additional 293ET cells were co-transfected with Nef or with a control vector (Nef_{AS}) along with $ABCA1_{WT}$, $ABCA1_{\Delta 40}$, or $ABCA1_{DDDHLK\rightarrow AAAAAA}$. Confirming the results of the co-immunoprecipitation experiment, we found the expression of $ABCA1_{\Delta 40}$ or $ABCA1_{DDDHLK\rightarrow AAAAAA}$ was still strongly inhibited by Nef coexpression (Figure 3A), which indicates that binding of Nef to ABCA1 was not a prerequisite for inhibiting protein expression of the ABCA1 mutants. Consistent with inhibition of mutant ABCA1 expression, efflux of cholesterol to apoA-I mediated by the $ABCA1_{\Delta 40}$ or $ABCA1_{DDDHLK\rightarrow AAAAAA}$ mutants was also significantly inhibited by Nef, as was the activity of $ABCA1_{WT}$ (Figure 3B). In summary, these results indicate that the Nef-mediated impairment of apoA-I-mediated cholesterol efflux is not critically dependent upon the ABCA1−Nef protein−protein interaction.

Given this discordance, we further explored the mechanism by which Nef was downregulating ABCA1 protein levels by testing whether it was caused by accelerated degradation of the transporter. To address this question, 293ET cells expressing Nef and ABCA1 were treated with cycloheximide to inhibit protein synthesis, and lysates of the treated cells were collected at a series of time points (from 2 to 8 h). ABCA1 protein expression was assessed in these lysates by immunoblotting (Figure 4A), and after the membranes had been probed for the level of β-actin protein, the amount of ABCA1 protein was quantified and expressed relative to the level in the vehicle-treated cells, thus providing an estimate of ABCA1 degradation (Figure 4B). Compared to that in cells expressing the control vector, degradation of ABCA1 was significantly enhanced in the cells expressing Nef, which reduced the half-life of the ABCA1 protein by more than 50% [ABCA1 half-life of 6.7 ± 1.5 h, ABCA1 and Nef half-life of 2.9 ± 0.4 h ($p < 0.05$)]. We further tested if Nef expression could accelerate the degradation of ABCA1 endogenously expressed in a macrophage environment. ABCA1 expression in RAW267 mouse macrophages was transcriptionally induced using the Liver-X-Receptor (LXR) agonist TO-901317 (10 μM, 24 h), and the cells were tranfected with the antisense vector or with the wild-type

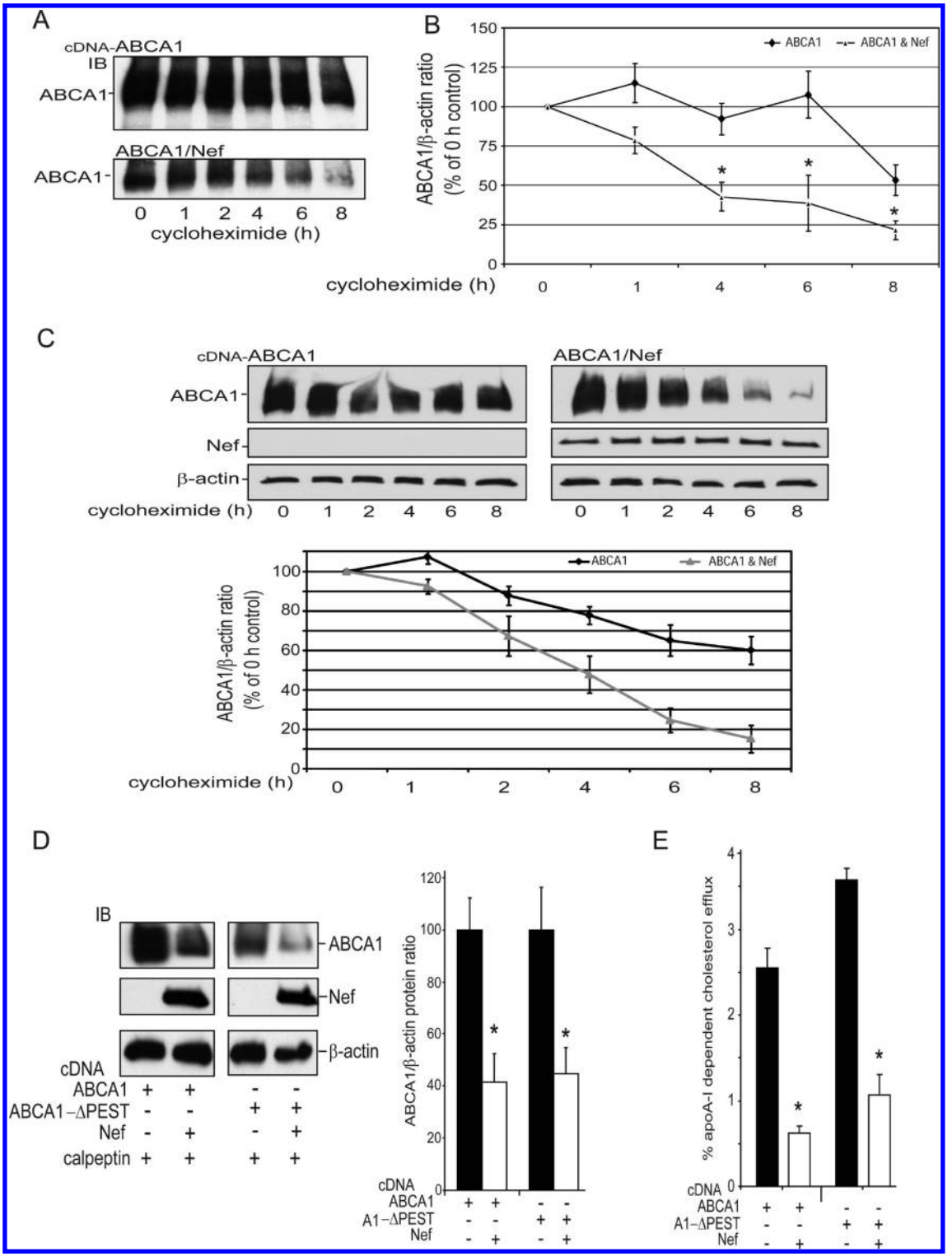

FIGURE 4: Nef downregulates ABCA1 protein expression by a mechanism that stimulates degradation of the transporter but does not depend upon the ABCA1 PEST motif. To determine the effect of Nef expression on the degradation of ABCA1, 293ET cells expressing ABCA1 alone or in the presence of Nef were treated with the protein synthesis inhibitor cycloheximide for the indicated time periods. (A) Immunoblots of ABCA1 expression in the presence and absence of Nef. (B) The ABCA1:β-actin protein ratio was quantitated using Bio-Rad ECL imaging and expressed as a percentage of the ABCA1 level in cells treated with vehicle only (0 h) [$n = 3$ (1 and 6 h), $n = 5$ (0, 4, and 8 h), ±standard error of the mean (*$p < 0.05$, ABCA1 vs ABCA1 and Nef)]. (C) Nef expression in RAW264.7 macrophages also significantly accelerates the degradation of endogenously expressed ABCA1 induced by treatment with an LXR agonist (TO-901217, 1 μM, 24 h). Representative immunoblots of ABCA1, Nef, and β-actin are shown in the top panels, and the ABCA1:β-actin protein ratio is graphed below ($n = 2$, ±standard error of the mean, $p < 0.01$, ANOVA) (D) Nef is able to significantly suppress the expression of ABCA1 and the ABCA1$_{\Delta PEST}$ mutant, and this effect is not blocked by calpain protease inhibitor calpeptin ($n = 3$, ±standard deviation, *$p < 0.05$, ABCA1 vs ABCA1 and Nef). (E) Nef is able to significantly suppress the cholesterol efflux activity of ABCA1 and the ABCA1$_{\Delta PEST}$ mutant ($n = 3$, ±standard deviation, *$p < 0.05$, ABCA1 vs ABCA1 and Nef). Results in panels A−E are representative of two or more experiments.

Nef vector and 24 h later were treated with cycloheximide. Again, expression of Nef was found to significantly reduce ABCA1 protein stability (Figure 4C) [ABCA1 half-life of 7.0 ± 1.0 h, ABCA1 and Nef half-life of 3.9 ± 0.4 h ($p < 0.01$)]. These results

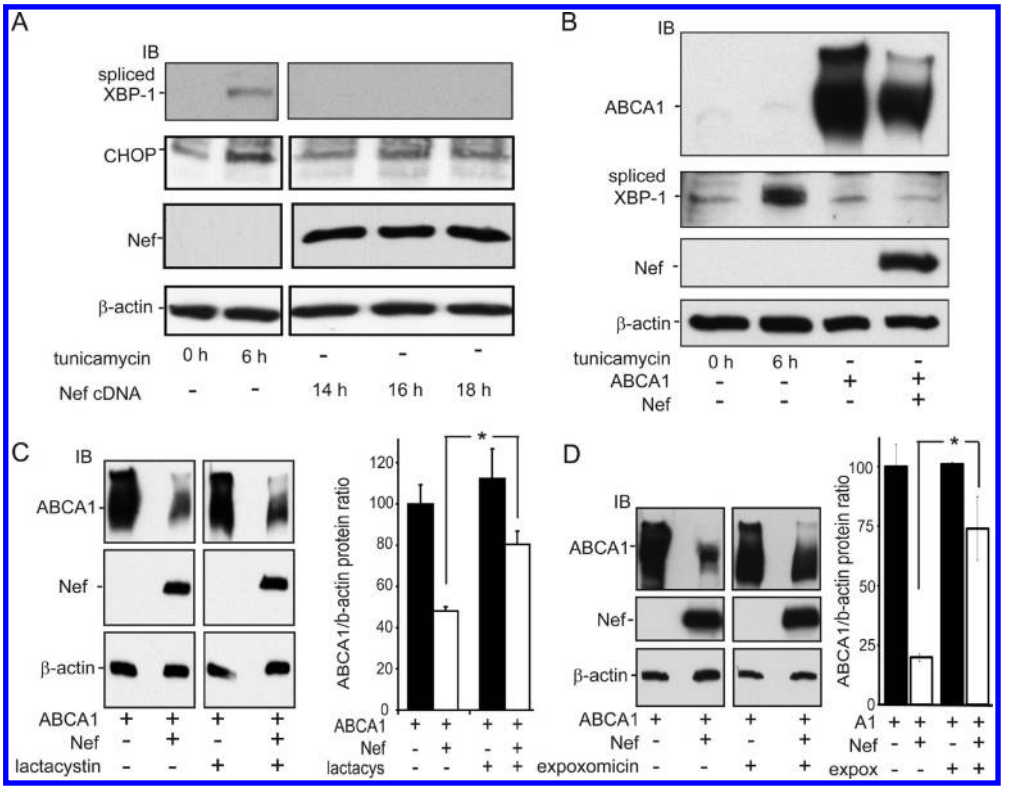

FIGURE 5: Nef inhibition of ABCA1 protein expression depends upon proteasomal activity but is not associated with signaling through the unfolded protein response pathway. (A) To test if expression of Nef alone stimulated UPR signaling in 293ET cells expressing Nef for the indicated time periods or treated with tunicamycin, an inhibitor of protein glycosylation that induces UPR signaling was analyzed for the expression of spliced XBP-1 and CHOP, two factors that mediate the UPR response and whose protein levels are induced upon UPR activation. In the tunicamycin-treated cells, levels of both spliced XBP-1 and CHOP increased, whereas in the cells expressing Nef, no such induction was apparent as determined by immunoblotting for the indicated proteins. (B) Likewise in cells expressing ABCA1 alone, or with Nef, levels of spliced XBP-1 were not induced. (C) Treatment of 293ET cells expressing ABCA1 and Nef with the proteasomal inhibitor lactacystin significantly blocks the ability of Nef to suppress ABCA1 protein levels ($n = 3$, ±standard deviation, $^*p < 0.05$, ABCA1 and Nef vs ABCA1, Nef, and lactacystin). (D) Treatment of 293ET cells expressing ABCA1 and Nef with the proteasomal inhibitor expoxomicin significantly blocks the ability of Nef to suppress ABCA1 protein levels ($n = 3$, ±standard deviation, $^*p < 0.05$, ABCA1 and Nef vs ABCA1, Nef, and expoxomicin). Results in panels A−D are representative of two or more experiments.

indicate that Nef was suppressing ABCA1 protein levels by a mechanism that stimulated the degradation rate of the transporter.

ABCA1 contains a PEST sequence (a motif rich in proline, glutamic acid, serine, and threonine residues) in its central intracellular cytoplasmic loop, and phosphorylation of this motif has been associated with enhancement of ABCA1 proteolytic degradation by calpain (*42*); on the other hand, apoA-I binding to ABCA1 inhibits this PEST-mediated degradation of the transporter (*43*). We thus tested whether the Nef-mediated ABCA1 downregulation required the PEST sequence of ABCA1 by generating and analyzing an $ABCA1_{\Delta PEST}$ mutant that has previously been shown to disrupt the calpain-mediated degradation of ABCA1. HEK 293ET cells were cotransfected with $ABCA1_{\Delta PEST}$ and Nef, or with the Nef_{AS} control vector, and protein levels (Figure 4C) and efflux activity (Figure 4D) of the $ABCA1_{\Delta PEST}$ mutant were assessed. As with ABCA1, Nef expression significantly repressed protein levels and the efflux activity of the $ABCA1_{\Delta PEST}$ mutant. Moreover, the ability of Nef to repress ABCA1 and the $ABCA1_{\Delta PEST}$ mutant was not blocked by calpeptin, an inhibitor of the calpain protease that induces the PEST-mediated degradation of ABCA1. Also, because Nef accelerated the degradation of ABCA1 in the cyclohexamide assays where saturating amounts of apoA-I were provided by the added fetal bovine serum, this indicates the binding of apoA-I to ABCA1 was not capable of blocking the Nef-mediated downregulation of ABCA1. In aggregate, these results indicate that Nef was increasing the rate of ABCA1 turnover by a mechanism that did not depend upon the PEST/calpain degradation pathway.

Because HIV-1 Tat, another viral accessory protein, has been reported to induce signaling through the unfolded protein response (UPR) (*56*), a pathway that can lead to the degradation of poorly folded proteins via endoplasmic reticulum-associated degradation (ERAD) (*57*), and another ATP-binding cassette transporter, ABCG2, is subject to ERAD (*58*−*60*), we questioned whether expression of Nef may also have the capacity to induce UPR signaling and thus explain its ability to enhance the degradation of ABCA1. To address this question, 293ET cells were transfected with Nef or with the Nef_{AS} control vector, and cell lysates were collected 14, 16, and 18 h post-transfection, a time frame in which Nef expression plateaus in this cell system. In parallel, additional cells were treated with tunicamycin, an inhibitor of protein glycosylation and a known inducer of UPR signaling. Lysates from these cells were then analyzed for activation of UPR signaling by immunoblotting for the level of spliced XBP-1 or CHOP/GADD153, two proteins whose upregulation during the UPR mediates transcriptional and degradative responses of this pathway (*44*). In cells treated with tunicamycin for 6 h, an increase in the levels of both spliced XBP-1 and CHOP was noted. However, in the cells transfected with Nef, no increase in the level of expression of these two UPR signaling components was detected (Figure 5A). These results indicated

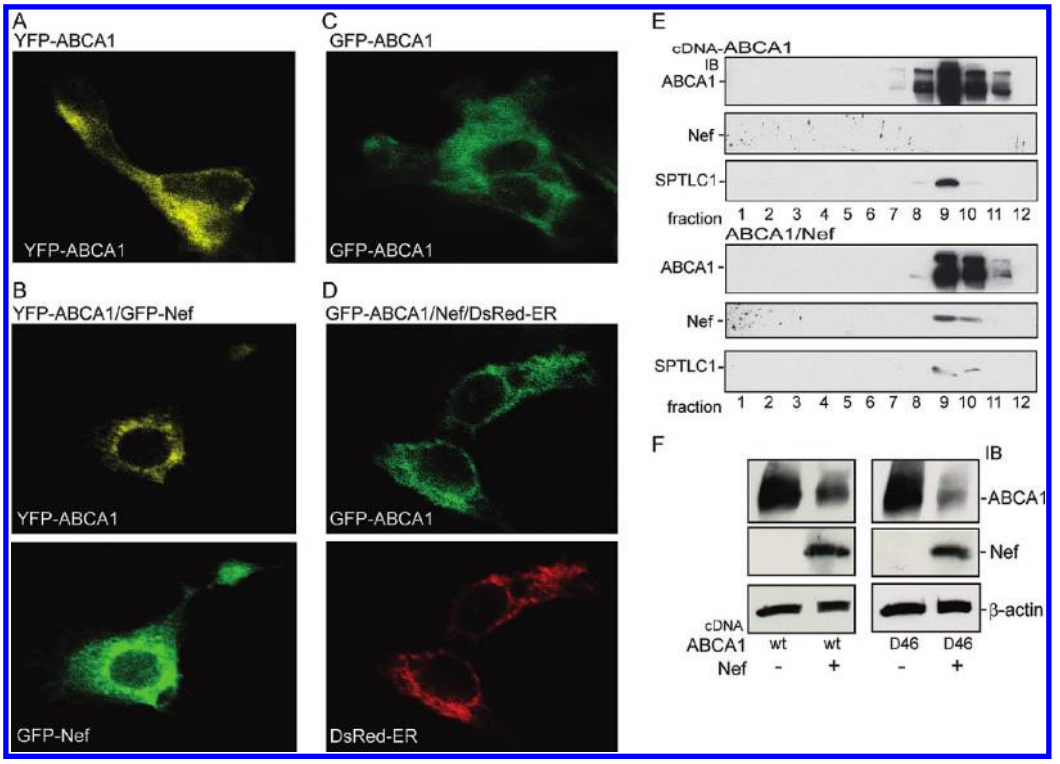

FIGURE 6: Nef causes a prominent trapping of ABCA1 in the ER. Confocal microscopy was used to image 293 cells expressing GFP-Nef and YFP-ABCA1. As opposed to the strong cell surface distribution of YFP-ABCA1 seen when not expressed with Nef (A), the transporter was predominantly localized to an internal organelle with a reticulate-like pattern reminiscent of the endoplasmic reticulum in cells expressing GFP-Nef (B). Likewise, GFP-ABCA1 expressed alone is prominently expressed at the cell surface (C), whereas when expressed with untagged Nef, the transporter is prominently relocalized to the endoplasmic reticulum as shown by the strong colocalization of GFP-ABCA1 and a DsRed-ER fusion protein that marks the ER (D). (E) The relocalization of ABCA1 by Nef is further demonstrated as analyzed by ultracentrifugation of 293 cell lysates using sucrose gradients. Immunoblots of the resulting fractions show that in cells expressing Nef, ABCA1 is more prominently localized to fractions that also contain the resident ER protein SPTLC1 endogenously expressed by these cells. (F) In 293ET cells, Nef is able to suppress the expression of an $ABCA1_{\Delta 46}$ mutant transporter that is trapped in the ER (shown are immunoblots of the indicated proteins).

that the 293ET cells were able to respond to signals that induce the UPR response but that expression of Nef alone does not induce this signaling as monitored by changes in the levels of spliced XBP-1 and CHOP.

Because ABCA1 is a large protein (2262 amino acids) with 12 transmembrane domains, it represents a complex target for chaperones of the endoplasmic reticulum to properly fold. Indeed, it has been reported that ER stress signals and UPR activation can lead to the degradation of ABCA1 (*61*, *62*). Thus, it was possible that the combined expression of ABCA1 and Nef could induce UPR signaling, whereas expression of Nef alone did not. To address this possibility, we first verified that induction of UPR signaling leads to suppression of endogenous full-length ABCA1 and the appearance of proteolytic fragments in THP-1 macrophages treated with tunicamycin (Figure 2A of the Supporting Information). Moreover, treatment of 293ET cells with tunicamycin also markedly suppressed the amount of ABCA1 protein after transfection of the ABCA1 cDNA (Figure 2B of the Supporting Information). These results indicated that 293ET cells were capable of mounting an unfolded protein response that suppresses ABCA1 expression. Thus, to test if the combined expression of ABCA1 and Nef induced UPR signaling, we coexpressed ABCA1 and Nef in 293ET cells and treated additional cells with tunicamycin in parallel, to serve as a positive control for induction of UPR signaling. Protein lysates prepared from these cells were immunoblotted for ABCA1 and Nef, which confirmed that coexpression of Nef reduced ABCA1 protein levels (Figure 5B). Additionally, when these membranes were stripped and probed for levels of spliced XBP-1, the cells treated with tunicamycin showed increased levels of spliced XBP-1, but in the cells expressing ABCA1 alone, or in the presence of Nef, little or no spliced XBP-1 was detected (Figure 5B). This result indicated that Nef stimulates the degradation of ABCA1 without inducing a detectable increase in the levels of spliced XBP-1, and further probing of these lysates also showed no induction of CHOP expression when Nef and ABCA1 were coexpressed.

The results described above indicate that the Nef-mediated degradation of ABCA1 was not associated with induction of ER stress as detected by an increased level of expression of CHOP or spliced XBP-1. However, these experiments did not rule out the possibility that Nef was triggering a degradative process that was dependent upon the activity of the cytoplasmic proteasome. To test this possibility, we treated cells expressing ABCA1 and Nef with the proteasomal inhibitor lactacystin (*63*). As expected, in vehicle-treated cells, Nef significantly reduced ABCA1 protein abundance by much more than 50%, whereas treatment of the cells with lactacystin significantly blocked the ability of Nef to downregulate ABCA1, resulting in an only 25% reduction in the level of ABCA1 expression (Figure 5C). Likewise, in the presence of the expoxomicin proteasomal inhibitor, the effect of Nef on ABCA1 protein expression was again significantly blocked by 75% (Figure 5D). Thus, the mechanism by which Nef was suppressing ABCA1 function depends, in part, on a proteasomal degradation process. Typically, the proteasomal turnover of integral membrane proteins occurs through ERAD, wherein the targeted protein is dislocated from the ER membrane and

ubiquitinated before being degraded by the proteasome (*57*). To begin to address whether Nef may be triggering the degradation of ABCA1 through this process, we first used confocal microscopy to colocalize GFP-Nef and YFP-ABCA1 in HEK 293 cells. In contrast to the normal prominent cell surface localization seen for YFP-ABCA1 in the absence of Nef (Figure 6A), the fluorescence of YPF-ABCA1 in cells also expressing Nef was largely confined to an intracellular location with a reticulate ER-like pattern (Figure 6B). This suggested that Nef might relocalize or trap ABCA1 in the ER. To test whether this was the case, we cotransfected HEK 293 cells with a nonfluorescent Nef construct, GFP-ABCA1, and a DsRed fusion protein that localizes to the ER by virtue of a KDEL ER targeting motif (*64*). In these cells, the GFP-ABCA1 construct expressed alone was again found to localize prominently to the cell surface (Figure 6C), whereas when expressed with Nef, GFP-ABCA1 was found to strongly colocalize with the DsRed-ER marker (Figure 6B). This result suggested that the mechanism by which Nef was triggering the degradation of ABCA1 was associated with a buildup of the transporter in the ER, a result that was further supported by sucrose gradient fractionation of the 293ET cells expressing ABCA1 alone or in the presence of Nef (Figure 6E). When expressed along with Nef, ABCA1 was found to more prominently localize to the fractions that also contained SPTLC1, an ER resident protein endogenously expressed by the 293ET cells.

Given these results, we questioned whether Nef would be able to downregulate an ABCA1 transporter that fails to exit the ER. To test this possibility, we coexpressed Nef with an ABCA1 mutant lacking the last 46 amino acids of the transporter ($ABCA1_{\Delta 46}$), which we have previously shown is prominently retained in the ER (*65*). As with ABCA1, coexpression of Nef markedly suppressed expression of the $ABCA1_{\Delta 46}$ mutant (Figure 6F). Together, these results suggest that the mechanism by which Nef suppresses the expression of ABCA1 involves retention of ABCA1 in the ER and a triggering of the ER-associated degradation of ABCA1.

DISCUSSION

We have investigated the mechanism by which Nef, a HIV-1 viral accessory protein, interacts with and suppresses the function of ABCA1, a cholesterol efflux transporter. HIV-1 infection of human macrophages has previously been shown to inhibit ABCA1-mediated cholesterol efflux, an effect that is important for HIV-1 viral infectivity and may contribute to the increased level of atherosclerosis seen in HIV-infected individuals (*66*). Here we defined what domain of ABCA1 allowed Nef to physically interact with the transporter and tested if this interaction was critical for the ability of Nef to suppress ABCA1 function. By analyzing a series of ABCA1 mutants, we found that the physical interaction between Nef and ABCA1 required a motif in the cytoplasmic C-terminal domain of ABCA1 between residues 2225 and 2231 (SDDDHLK). Deletion of the 40 C-terminal amino acids of ABCA1, including the SDDDHLK motif, or selective mutation of residues within this motif to alanines disrupted the physical interaction of Nef with ABCA1. However, these mutations did not block the ability of Nef to downregulate the efflux activity and protein expression of ABCA1. This indicates that a close physical association of Nef and ABCA1 is not essential for the Nef-mediated downregulation of ABCA1 efflux activity. In contrast, we did find that the Nef G2A mutation, which blocks the myristoylation of Nef and its association with membrane bilayers, did block the ability of Nef to both interact and downregulate ABCA1, a result that recapitulates what has been reported for the Nef inhibition of ABCA1 in macrophages (*40*). Finally, we show that Nef suppression of ABCA1 in both 293ET cells and RAW264.7 macrophages is acting at a post-translational level by a process that increases the level of degradation of ABCA1.

How may Nef be acting to increase the level of degradation of ABCA1? To date, a number of post-translational and proteolytic processes have been shown to regulate ABCA1 function. The most thoroughly described of these is the turnover of cell surface ABCA1 by a process that involves a calpain-mediated degradation of ABCA1 (*42*, *43*). The ability of calpain to degrade ABCA1 is regulated by a PEST sequence located in the central intracellular loop of ABCA1 near the first ATP binding cassette of the transporter. Given this degradative pathway can be modulated by the phosphorylation status of the ABCA1 PEST sequence, it was possible that Nef was stimulating the degradation of ABCA1 by modulating the phosphorylation of the ABCA1 PEST sequence and thus the calpain degradation of ABCA1. Our work indicates that this is not the case because Nef still downregulated the $ABCA1_{\Delta PEST}$ mutant, which is not subject to proteolysis mediated by calpain. Furthermore, treatment of cells expressing $ABCA1_{WT}$ and Nef with calpeptin, a specific inhibitor of the calpain-mediated degradation of ABCA1, did not block the ability of Nef to inhibit ABCA1 protein expression. Finally, because Nef accelerated the degradation of ABCA1 in the presence of saturating amounts of apoA-I, this indicates the ability of apoA-I to modulate the degradation of ABCA1 through the PEST motif was not capable of blocking the Nef-mediated downregulation of ABCA1 (*43*). In aggregate, these results indicate that the simulation of ABCA1 degradation mediated by Nef was not proceeding through the PEST/calpain pathway.

An alternative pathway described that stimulates degradation of ABCA1 is the induction of ER stress (*61*). Because Nef has been reported to induce cholesterol synthesis (*36*, *37*), this could induce ER stress and the unfolded protein response through the accumulation of excess cholesterol (*62*). We therefore tested whether Nef was triggering the degradation of ABCA1 through the UPR pathway. We first verified that tunicamycin, a known inducer of ER stress and UPR signaling, can lead to the induction of ABCA1 degradation in macrophages and that 293ET cells when treated with tunicamycin can initiate UPR signaling as detected by increased levels of spliced XBP-1 and CHOP. These results indicated that 293ET cells have the ability to mount a UPR that suppresses ABCA1 protein expression, yet the expression of Nef alone or coexpression of Nef and ABCA1 was not associated with an increased level of UPR signaling. This indicates that the Nef-mediated stimulation of the degradation of ABCA1 was likely not proceeding through a UPR mechanism.

However, the ER contains machinery that can dislocate large integral membrane proteins for degradation by the proteasome. Indeed, the cystic fibrosis ABC transporter can be degraded by such a mechanism (*67*), and our analysis demonstrated that Nef strengthens the association of ABCA1 with ER. We therefore tested whether the Nef-mediated suppression of ABCA1 was blocked by the proteasomal inhibitors lactacystin and expoxomicin (*63*). Both of these inhibitors significantly weakened the ability of Nef to downregulate ABCA1 expression. Moreover, Nef was able to suppress the expression of an ABCA1 mutant lacking the 46 C-terminal residues that we have previously shown to be largely trapped in the ER (*65*). This result suggests a two-tier

mechanism whereby Nef strengthens the association of ABCA1 with ER and also triggers the ER-associated degradation machinery to suppress ABCA1 efflux function. However, these inhibitors were only able to block approximately 50−75% of the Nef-mediated downregulation of ABCA1 expression. This suggests that Nef may also trigger the degradation of ABCA1 through alternative mechanisms that do not depend upon proteasomal activity. Because Nef has been reported to reroute cell surface CD4 and MHC1 molecules to the lysosome for degradation (*68*), and because ABCA1 can be routed to a late endosomal/lysosomal compartment (*69*), it is possible that Nef also stimulates ABCA1 degradation by rerouting the transporter from the cell surface to the lysosome. Indeed, our previous imaging experiments suggest that Nef may also be able to cause an at least transitory buildup of ABCA1 at the cell surface of macrophages (*40*). These divergent effects on ABCA1 localization indicate Nef may modulate multiple trafficking pathways to block ABCA1 function. Finally, it is noted that our estimate of the baseline ABCA1 protein half-life is greater by approximately 4 h compared to what has been previously published (*42*, *43*). Why this is the case remains to be investigated. However, it may be that in our assays where we have included fetal bovine sera and LXR agonist this reduces the level of ABCA1 protein turnover. The fact that Nef is able to accelerate ABCA1 degradation under these conditions suggests it is able to override the processes that sera and LXR agonists may trigger to stabilize ABCA1.

In summary, we have confirmed that the myristoylation status of Nef is critical for its ability to suppress the function of ABCA1 and physically interact with ABCA1. Although the ability of Nef to physically interact with the transporter may play additional regulatory roles not revealed by our assays, our results indicate that the interaction of Nef with the ABCA1 C-terminal domain is not required for Nef to suppress ABCA1 protein abundance. Although we cannot exclude the possibility that mutation of the ABCA1 C-terminus preserves a weak physical interaction between Nef and ABCA1 not detected in our assays, our work suggests that Nef is acting at a distance to stimulate ABCA1 degradation, at least in part through a process depending upon proteasomal activity that may act on ABCA1 located in the endoplasmic reticulum.

The critical question of whether the effect of Nef on ABCA1 is specific remains. Indeed, if Nef increases proteasomal activity in general, then it should increase the rate of general catabolism of proteins affected by this pathway. However, we previously demonstrated that ABCG1 and SR-BI are not affected by Nef (*40*), and results in this report show that another transmembrane protein, c-MIR, is not downregulated by Nef. One possible explanation is that these proteins are not metabolized through the mechanisms described here for ABCA1. This possibility appears unlikely given the high degree of similarity between ABCA1 and ABCG1 regulation (*70*). Another possibility is that Nef, by increasing the level of formation of lipid rafts, dislodges ABCA1 from the cell surface, making it susceptible to degradation. Yet another possibility is that weaker interactions of Nef with sites in other domains of ABCA1 may provide specificity for the effects of Nef. Clearly, additional studies are warranted to address these questions.

SUPPORTING INFORMATION AVAILABLE

Supplementary Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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