Tumor Suppressor Cylindromatosis (CYLD) Controls HIV Transcription in an NF-κB-Dependent Manner

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ABSTRACT
Characterizing the cellular factors that play a role in the HIV replication cycle is fundamental to fully understanding mechanisms of viral replication and pathogenesis. Whole-genome small interfering RNA (siRNA) screens have identified positive and negative regulators of HIV replication, providing starting points for investigating new cellular factors. We report here that silencing of the deubiquitinase cylindromatosis protein (CYLD), increases HIV infection by enhancing HIV long terminal repeat (LTR)-driven transcription via the NF-κB pathway. CYLD is highly expressed in CD4+ T lymphocytes, monocyte-derived macrophages, and dendritic cells. We found that CYLD silencing increases HIV replication in T cell lines. We confirmed the positive role of CYLD silencing in HIV infection in primary human CD4+ T cells, in which CYLD protein was partially processed upon activation. Lastly, Jurkat T cells latently infected with HIV (JLat cells) were more responsive to phorbol 12-myristate 13-acetate (PMA) reactivation in the absence of CYLD, indicating that CYLD activity could play a role in HIV reactivation from latency. In summary, we show that CYLD acts as a potent negative regulator of HIV mRNA expression by specifically inhibiting NF-κB-driven transcription. These findings suggest a function for this protein in modulating productive viral replication as well as in viral reactivation.

IMPORTANCE
HIV transcription is regulated by a number of host cell factors. Here we report that silencing of the lysine 63 deubiquitinase CYLD increases HIV transcription in an NF-κB-dependent manner. We show that CYLD is expressed in HIV target cells and that its silencing increases HIV infection in transformed T cell lines as well as primary CD4+ T cells. Similarly, reactivation of latent provirus was facilitated in the absence of CYLD. These data suggest that CYLD, which is highly expressed in CD4+ T cells, can control HIV transcription in productive infection as well as during reactivation from latency.

High-throughput RNA interference (RNAi) screening approaches are powerful tools to probe for HIV host interactions. Whole-genome small interfering RNA (siRNA) screens have identified a number of host factors essential for viral replication, but the relative importance of these factors in relevant cells remains to be verified (1–4). Similarly, these screens have the potential to identify cellular molecules which, when silenced, result in increased viral infection. The deubiquitinase cylindromatosis (CYLD) was identified as putative negative regulator of HIV infection in an siRNA screen (L. Pache, J. Marlett, A. Maestre, L. Manganaro, J. Murry, Y. Hwang, K. Olivieri, A. Diaz, P. D. De Jesus, R. König, F. D. Bushman, V. Simon, A. Fernandez-Sesma, J. A. Young, and S. K. Chanda, unpublished data).

CYLD, a multidomain protein, was first studied in association with skin malignancies (5, 6). The amino-terminal region of the 956-amino-acid-long CYLD protein is characterized by three cytoskeleton-associated protein (CAP) domains, two proline-rich (PR) motifs, a phosphorylation region (P), and a TRAF2 binding site (PVQES). The carboxy-terminal region is characterized by the ubiquitin-specific protease (USP) domain, which contains the zinc-binding B box that forms the catalytic domain responsible for CYLD deubiquitinase activity (Fig. 1A) (7). Mutations in this region abolish catalytic activity (8). Naturally occurring mutations are found in the USP and CAP regions (9). Several nonsynonymous mutations in the coding region of this gene have been linked to familial cylindromatosis (also termed “turban syndrome”) as well as to the sporadic Brooke-Spiegler syndrome (5, 10, 11). CYLD controls chronic inflammation during tumor progression, and its expression is downregulated in melanoma, hepatocellular carcinoma, and colon cancer (12, 13). Since CYLD is a lysine 63-linked deubiquitinase, it induces and suppresses different signaling pathways by specifically removing lysine 63-linked ubiquitin moieties (8, 14). Many of these pathways regulate the development (e.g., natural killer cells) and homeostasis (e.g., T cells and dendritic cells [DCs]) of the immune system as well as the

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quality of the immune responses mounted to different pathogens (15–19). For example, CYLD inhibits type I interferon signaling by deubiquitinating the pattern recognition receptor RIG-I and downregulating Toll-like receptor 2 (TLR2) signaling (20–22). The best-understood function of CYLD is the repression of the nuclear factor κB (NF-κB) pathway. Several molecules regulating this pathway (e.g., NEMO, TRAF2, TAK1, and TRAF6) are substrates of CYLD (7, 8, 14, 23, 24). Moreover, CYLD itself has been proposed to act as a negative regulator of the NFAT pathway (25, 26).

In the context of HIV infection, NF-κB is essential for efficient HIV transcription in transformed cell lines as well as in primary cells (27–30). NF-κB regulates HIV transcription by binding to two adjacent NF-κB/NFAT sites within the proviral long terminal repeat (LTR) promoter and enhancing the transcription of viral genes from the integrated provirus (31, 32). T lymphocytes are the main target cells for HIV, but only activated T cells support efficient HIV transcription and production of infectious particles (reviewed in reference 33). Resting CD4+ T lymphocytes, in contrast, fail to support productive viral transcription, which has been linked, at least in part, to low levels of active NF-κB (34–39).

Despite the reported importance of CYLD in T cell homeostasis and T cell activation, there are no reports on its effect on HIV replication. Here we describe that silencing of CYLD in a genome-wide siRNA screening resulted in an increase of viral infection. We hypothesized that CYLD modulates HIV infection by limiting viral transcription through negative regulation of the NF-κB pathway. Consistent with that hypothesis, we show here that HIV-1 LTR-driven transcription is increased in the absence of CYLD in an NF-κB-dependent manner. Moreover, we show that activation
of T cells leads to cleavage and, presumably, inactivation of CYLD. These findings are important because silencing of CYLD increases HIV infection of T cell lines as well as primary human T lymphocytes. In addition, CYLD was found to negatively regulate phorbol 12-myristate 13-acetate (PMA)-induced reactivation of latent HIV-1 gene expression. In conclusion, the deubiquitinase CYLD is a negative regulator of HIV-1 transcription.

MATERIALS AND METHODS

Cell isolation and cell culture. (i) Transformed cell lines. Human embryonic kidney 293T (HEK 293T) cells and TZM-bl cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. The A3R5 and Jurkat JLat (clone 8.4) T cell lines were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% FBS, 100 IU penicillin, 100 μg/ml streptomycin, 0.1 M HEPES, and 2 mM l-glutamine at 37°C. Jurkat JLat T cell lines were stimulated with 15 nM to 100 nM PMA (Sigma) for 1 h.

(ii) Primary cells. Human peripheral blood mononuclear cells (PBMCs) were obtained from Ficoll (Ficoll Histopaque; Sigma) density centrifugation from anonymous healthy blood donors (New York Blood Center). CD4+ T cells were negatively selected using magnetic beads (CD4+ T cell isolation kit II; Miltenyi Biotec) as per the manufacturer’s instructions. CD4+ T cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 IU penicillin, 100 μg/ml streptomycin, 0.1 M HEPES, 2 mM l-glutamine, and 20 units/ml interleukin-2 (IL-2) (NIH AIDS Reagent Program). Lymphocytes were activated with 1 μg/ml phytohemagglutinin-P (PHA) (Sigma) or with human T-activator CD3/CD28 at a 1:5 bead/cell ratio (Gibco by Life Technologies) for 48 h. CD4+ T cells were isolated from PBMCs using the MACS CD14 isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. CD14+ cells were differentiated into dendritic cells (DCs) or macrophages. DCs were obtained by adding 500 U/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech) and 1,000 U/ml human IL-4 (PeproTech) to the culture medium (RPMI, 10% FBS [HyClone] 100 U/ml l-glutamine, 100 μg/ml penicillin-streptomycin, 1 mM sodium pyruvate) and incubating for 5 to 6 days. Macrophages were obtained by incubating CD14+ monocytes with 2,000 U/ml human GM-CSF for 10 days. The GM-CSF containing medium was replenished at days 2, 5, and 8 as previously described (40).

Plasmids. pEAK Flag-CYLD was obtained by cloning CYLD cDNA into pEAK plasmid. We generated a pEAK Flag-CYLD (transcript variant 2) mutant that is resistant to CYLD short hairpin pGIPZ RHS430-99158676 by mutating nucleotides 2040, 2043, and 2046 in CYLD (NCBI accession number NM_001042355.1). Mutagenesis was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) and the primers CYLD shmut for (5’-GATTGAGCGCTGCAATCA=CATCCAGGGTCATTACAATTCTgcTTACTTAGACTCAACCT) and CYLD shmut rev (5’-CATCCAGGGTCATTACAATTCTgcTTACTTAGACTCAACCT) (39). Mutagenesis to obtain the CYLD C598A mutant was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) with primers CYLD C598A for (5’-CTCCAATATGCTAATG=CATCCAGGGTCATTACAATTCTgcTTACTTAGACTCAACCT) and CYLD C598A rev (5’-AGGTTGAGCTAAGTAAAgAAGTATTGTAATGACTTGTGC). The murine leukemia virus (MLV)-LTR-luciferase, HIV-LTR-luciferase, HIV-ΔNFκB-LTR-luciferase, and MLV-HIV-LTR-luciferase reporter plasmids were previously described (41). The LTR mutant viral clones were previously described (42). The c-fos-luciferase plasmid was previously described (44). The pGIPZ-RHS430 short hairpin RNA (CYLD shRNA) and pGIPZ-nontargeting (NT) shRNA vectors encoding CYLD-targeting short hairpins or nontargeting hairpins follow by puromycin selection (1 μg/ml).

Production of viral stocks. VSV-G-NL4.3 luciferase viral stocks were generated by transfection of HEK 293T cells with 20 μg of pNL4.3 luciferase and 3 μg VSV-G (pHCMV-G) per 75-cm² flask using 3 μg/ml polyethylenimine (PEI) (Polysciences) (44). Two days after transfection, supernatants were collected, clarified at 1500 rpm for 5 min, filtered (0.45 μm), aliquoted, and stored at −80°C. The molecular clone pNL4-3 Env-luciferase lacks a functional envelope and encodes luciferase in the position of Nef (pNL-Luc-E’; NIH AIDS Research Program [45]). Once pseudotyped with VSV-G (pHCMV-G), it was used for single-cycle infections (46).

VSV-G-pseudotyped viral stocks of wild-type HIV (HIV WT), HIV ΔUSF, HIV ΔNFL6L6 and HIV ΔNFκB/NFAT viruses were obtained by transfecting HEK 293T cells with the corresponding plasmids (42) together with VSV-G at a ratio of 1:5 using 3 μg/ml PEI. The replication-competent HIV molecular clones LAI and NL4.3 were obtained from the AIDS Research and Reference Reagent Program (47, 48). LAI and NL4.3 viral stocks were obtained by transfecting HEK 293T cells with the corresponding plasmids using PEI (3 μg/ml).

Infectivity titers of the viral stocks were determined by infecting TZM-bl reporter cells with serial dilutions in triplicate as previously described (44).

Lentiviral stocks used for the generation of stable HEK 293T, JLat 8.4, and A3R5 CYLD shRNAs and nontargeting shRNA were produced by transfecting pGIPZ-CYLDS-targeting short hairpin RNA or pGIPZ-nontargeting (NT) siRNAs (49) and VSV-G in HEK 293T cells using 3 μg/ml PEI at a ratio of 5:5:1.

Single-cycle infection experiments. HEK 293T cells were plated in 96-well plates and transfected with the indicated siRNA using RNAiMax reagent (Invitrogen). At 48 h posttransfection, cells were infected with VSV-G-pseudotyped NL4.3 Luc virus in the presence of Polybrene (5 μg/ml).

HEK 293T cells stably transduced with short hairpins directed against CYLD or nontargeting short hairpins were plated in 24-well plates and infected with VSV-G-NL4.3 Luc virus 24 h later. To determine firefly luciferase relative light values, cells were lysed in passive lysis buffer (PLB) at 24 to 48 h postinfection, incubated with luciferase substrate (Promega), and read immediately in a Victor3 multilabel counter (Perkin-Elmer).

Primary CD4+ T cells werePHA activated for 48 h before infection.
with VSV-G NL4.3 in the presence of Polybrene (2 μg/ml). RNA was collected form these cells at 24 h postinfection.

**Spreading-infection experiments.** A3R5 cells (0.5 × 10⁶), transfected with nontargeting shRNA or CYLD shRNA were infected (multiplicity of infection [MOI], 0.5) in a 48-well format (0.3 ml) and washed twice at 10 h postinfection. Every 2 days over a 10-day period, 100 μl of culture supernatant was collected, clarified, and stored at −80°C. Cultures were supplemented with 100 μl of fresh medium at each time point. At the end of the infection, the collected culture supernatants were used to infect TZM-bl cells in triplicate (15 μl, 96-well plates), and β-galactosidase activity was quantified 48 h after infection using Tropix (Perkin-Elmer) as previously described (50).

**Reactivation experiments.** JLat 8.4 cells (2 × 10⁵) transiently transfected with CYLD or nontargeting siRNAs were activated with PMA (200 ng/ml) at 24 h after AMAXA Nucleofection. Green fluorescent protein (GFP) expression was measured using a BD Biosciences FACSCalibur flow cytometer 48 h after PMA stimulation.

**Immunoblotting.** Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with complete protease inhibitor (Roche). Proteins were separated on 10% SDS-polyacrylamide gels (Invitrogen), transferred onto polyvinylidene difluoride membranes (Pierce), probed with antibodies, and visualized with SuperSignal West Pico or Femto (Pierce) on a Fluorochrom E System (Protein Simple) machine. The following antibodies were used: mouse M2 monoclonal antibody to FLAG (Sigma F1804), mouse monoclonal anti-CYLND N-terminal domain (Cell Signaling 4495), mouse monoclonal anti-CYLND N-terminal domain (Invitrogen), monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Santa Cruz 22323), monoclonal antitoxin (Sigma), monoclonal anti-IκBα (Cell Signaling), and monoclonal anti-

**Intracellular p24 staining.** JLat cells (3 × 10⁵) were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) as per the manufacturer’s instructions, followed by staining with anti-p24 antibody KC57 RD1 (1:100 dilution; Coulter Clone), HIV-1 p24 Gag-positive antigens were set by comparison with isotype-unrelated antibody control Msg1G1-RD1 (1:100 dilution; Coulter Clone). An average of 10⁵ cells were acquired per sample, and data were analyzed using Flowjo software.

**RNA isolation and quantitative real-time PCR (qRT-PCR).** Cellular RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions, followed by a treatment with the DNA-free DNase treatment and removal kit (AM1906; Ambion RNA by Life Technologies). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions.

The expression of host and viral transcripts was measured by quantitative real-time PCR using iQ SYBR green Supermix (Bio-Rad) according to the manufacturer’s instructions. The PCR temperature profile was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. Expression levels for individual mRNAs were calculated based on their threshold cycle (CT) (ΔΔCT values) using rps11 as a housekeeping gene to normalize the data. Primers for CYLD (CYLD for, 5′-GGTAATCCGTTGCAATCCTCAG; CYLD rev, 5′-AGTGGCTGCTGAGGTTCCATCC), rsp11 (rps11 for, 5′-GGGAGGAGCTAATGCAGCAG; and rps11 rev, 5′-ATGTCAGCTGCTGAGAACATC), and IκBα (IκBα for, 5′-GGCAAGGCTGTACGATTGA; IκBα rev, 5′-GAGGCGGTATCCACGAC) were designed using PrimerDesign software. Primers MH531, MH532, HIV LTP for, and HIV LTP rev were used to measure HIV mRNA expression (51, 52).

**Alu PCR.** At 1 day postinfection, cellular DNA was isolated using the DNeasy 96 Blood & Tissue kit (Qiagen) and quantitated using the Quant-iTTM PicoGreen R ds DNA assay kit (Invitrogen) on a Cytofluoro multichannel plate reader series 4000 (Applied Biosystems). In order to measure the amount of total viral cDNA, internal primers annealing between the U5 region and the R region were used. The proviral DNA content was measured using primers specific for the human Alu regions and primers annealing in the gap region together with an LTR-specific molecular beacon. PCRs were carried out using an Applied Biosystems 7500 Fast real-time PCR system. All steps were performed using an Epmotion 5075 robot (Eppendorf) (52).

**Luciferase reporter assay.** For MLV and HIV promoter-specific assays, CYLD shRNA and NT shRNA HEK 293T cells were transfected with 100 ng of MLV- and HIV-LTR-luciferase reporter plasmids and 100 ng of pRL-TK Renilla. Cells were lysed at 24 to 48 h posttransfection with passive lysis buffer (PLB). Firefly luciferase values were measured using the luciferase assay system (Promega), and Renilla luciferase values were measured using the Renilla assay system (Promega). Firefly luciferase data were normalized to Renilla luciferase values, and the data were represented as the fold change over NT shRNA HEK 293T cell values. All experiments were performed in duplicate or triplicate.

For the NF-κB promoter-specific assays, HEK 293T cells stably transfected with CYLD shRNA and NT shRNA were transfected with 50 ng of c-fos-luciferase reporter plasmids and 25 ng of p-RL-TK Renilla. Cells were lysed at 24 to 48 h posttransfection with PLB (Promega). Firefly luciferase values were measured using the luciferase assay system (Promega), and Renilla luciferase values were measured using the Renilla assay system (Promega). Firefly luciferase data were normalized to Renilla luciferase values, and the data were represented as the fold change over NT shRNA HEK 293T cell values. All experiments were performed in duplicate or triplicate.

Cell viability was determined using the CellTiter Glo assay system (Promega) as per the manufacturer’s instructions.

**Immunofluorescence.** HEK 293T cells were plated on coverslips, and 24 h later the cells were stimulated for 2 h with tumor necrosis factor alpha (TNF-α) (10 ng/ml). Cells were washed with PBS, fixed with 3% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and blocked in phosphate-buffered saline (PBS), 0.1% Triton X-100, and 1% BSA for 1 h.

Staining for p65 was performed overnight at 4°C using anti-p65 (Abcam ab32536). Coverslips were washed three times in PBS–0.1% Triton X-100 and incubated for 1 h with secondary antibody (Alexa Fluor 594; Molecular Probes). Nuclear staining was performed using Hoechst 33342 nucleic acid stain (Invitrogen). Coverslips were washed three times with washing solution and mounted on slides using Vectashield mounting medium (Vector Laboratories).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 software. P values are two sided, and values of <0.05 were considered to be significant.

**RESULTS**

**CYLD silencing increases HIV infection in single-cycle assays.** Following the identification of CYLD as a putative negative regulator of HIV replication through siRNA screening (Pache et al., unpublished data), we confirmed that transient silencing of CYLD increases HIV infection using two nonoverlapping siRNAs (CYLD_2 and CYLD_3). Figure 1B shows a 12- to 100-fold increase in HIV infection, as measured by luciferase expression, in HEK 293T cells silenced for CYLD (CYLD_3 siRNA or CYLD_2 siRNA) compared to cells transfected with nontargeting siRNAs (NT siRNA) (Fig. 1B, upper panel). Western blot analysis of siRNAs...
of HIV infection and that the catalytic activity of CYLD is necessary to decrease HIV infection. Of note, expression of the catalytically defective CYLD mutant (transcript variant 2) reduced HIV infectivity (Fig. 1C).

To investigate the molecular mechanism by which CYLD knockdown increased HIV infection, we complemented stable 293T CYLD shRNA and 293T NT shRNA (control) cell lines with a catalytically defective CYLD mutant that fails to remove lysine 63-linked ubiquitin moieties from substrates (CYLD C598A [8, 53]) and was rendered resistant to the CYLD shRNA by site-directed mutagenesis. As shown in Fig. 1C, the CYLD C598A mutant failed to reduce HIV infection in 293T cells containing the CYLD shRNA (Fig. 1C), suggesting that the catalytic activity of CYLD is necessary to decrease HIV infection. Of note, expression of the catalytically defective CYLD mutant C598A in 293T NT shRNA cells increases luciferase reporter expression, suggesting that this mutant might function as dominant negative. The efficiency of silencing was on average 80% by quantitative PCR (qPCR) analysis (data not shown) and Western blotting. The stable cell lines were probed with antibodies directed against the endogenous CYLD as well as with anti-FLAG antibodies to confirm expression of the shRNA-resistant FLAG-tagged CYLD variants (Fig. 1C, lower panel).

Taken together, these data suggest that CYLD acts as repressor of HIV infection and that the catalytic activity of CYLD is necessary for the repression.

Identification of the viral step affected by CYLD silencing. Next we determined the step in the viral replication cycle that is affected by the depletion of CYLD. We measured the production of total viral DNA and integrated provirus at 24 h after infection in HEK 293T cells transiently silenced for CYLD or controls. No differences were detected in cells with and without CYLD (Fig. 2A and B). In contrast, when we analyzed viral transcription upon CYLD silencing, we detected a 9-fold increase in HIV transcription in the CYLD silenced cells compared to the control cells (Fig. 2C). These data indicate that CYLD silencing enhances viral infection by increasing the levels of HIV mRNA transcription without affecting the levels of proviral DNA.

Increased HIV transcription in CYLD knockdown cells is NF-κB or NFAT dependent. To dissect the molecular mechanism by which CYLD controls HIV transcription, we took advantage of four HIV molecular clones that bear mutations in transcription factor binding sites in the LTR region: HIV WT, HIV ΔUSF, HIV ΔNFIL6, and HIV ΔNF-κB/NFAT (Fig. 3A) (42). We infected HEK 293T cells silenced for CYLD with VSV-G-pseudotyped viral stocks of these four viruses and analyzed the levels of HIV mRNA by qPCR at 1 day postinfection. All viruses except the one in which the NF-κB/NFAT sites were deleted displayed 6- to 9-fold-increased levels of HIV mRNA expression in the absence of CYLD (Fig. 3B). HIV ΔNF-κB/NFAT showed no increase in infection upon CYLD depletion, indicating that the effects of CYLD on HIV infection are dependent on the NF-κB/NFAT-binding site in the viral LTR.

To further confirm the involvement of the NF-κB pathway in CYLD-dependent HIV transcription, we next used a series of LTR reporter constructs (41). In contrast to that of HIV, MLV transcription is independent of NF-κB (54). Therefore, we compared luciferase reporter expression driven by an MLV LTR to that driven by an HIV LTR after transfection of 293T cells stably silenced for CYLD shRNA as well as control 293T NT shRNA cell lines (Fig. 3C). Additional constructs tested included an MLV with the HIV U3 region (containing the NF-κB/NFAT binding sites) and an HIV LTR lacking the NF-κB/NFAT sites (54). Figure 3D shows a 4-fold increase of luciferase expression driven by the
Several groups reported that CYLD inhibits NF-κB mechanism. Transcription was not affected. Activation by deubiquitinating different factors in this pathway (8, 14, 23). We wanted, therefore, to establish whether CYLD could act as a general inhibitor of NF-κB in our model systems. We first transfected 293T cells stably silenced for CYLD and the corresponding control cells (293T NT shRNA) with a reporter construct carrying the luciferase reporter gene under the control of the c-fos promoter, which contains binding sites for NF-κB. When we measured luciferase activity 24 h later, we found that the activity of the reporter gene was 6-fold increased in CYLD knockdown cells compared to control cells (Fig. 4A). The expression of the short hairpin-resistant form of CYLD decreased c-fos-dependent transcription to the level of the nontargeting control (Fig. 4A).

Collectively, these data suggest that CYLD silencing generally enhances NF-κB activity in HEK 293T cells.

HIV WT LTR and the MLV containing the HIV U3 region in the absence of CYLD. There was no difference in luciferase expression between CYLD knockdown cells and control cells with the MLV LTR and the HIV ΔNF-κB/NFAT LTR construct (Fig. 3D). Thus, CYLD silencing resulted in increased HIV LTR-dependent transcription, while the NF-κB/NFAT-independent MLV LTR-driven transcription was not affected.

**CYLD silencing increases NF-κB signaling as a general mechanism.** Several groups reported that CYLD inhibits NF-κB activation by deubiquitinating different factors in this pathway (8, 14, 23). We wanted, therefore, to establish whether CYLD could act as a general inhibitor of NF-κB in our model systems. We first transfected 293T cells stably silenced for CYLD and the corresponding control cells (293T NT shRNA) with a reporter construct carrying the luciferase reporter gene under the control of the c-fos promoter, which contains binding sites for NF-κB. When we measured luciferase activity 24 h later, we found that the activity of the reporter gene was 6-fold increased in CYLD knockdown cells compared to control cells (Fig. 4A). The expression of the short hairpin-resistant form of CYLD decreased c-fos-dependent transcription to the level of the nontargeting control (Fig. 4A).

We next analyzed the rate of degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IkBα) in response to tumor necrosis factor alpha (TNF-α) stimulation in the stable 293T CYLD shRNA cells compared to the stable 293T NT shRNA control cells. Figure 4B shows that IkBα was degraded more rapidly in the absence of CYLD. To confirm that the NF-κB pathway activation is enhanced in CYLD knockdown cells, we measured the expression level of IkBα, the transcription of which is induced by NF-κB, as part of a negative feedback loop (55, 56). We observed that the levels of IkBα mRNA were enhanced (10-fold) in CYLD knockdown cells compared to the control cells (Fig. 4C).

To further investigate the effect of CYLD silencing on the NF-κB pathway, we determined the subcellular localization of the NF-κB p65 subunit upon TNF-α stimulation in the presence or absence of CYLD by confocal microscopy. We observed a significant increase in NF-κB p65 nuclear translocation in CYLD knockdown cells (P < 0.001) (Fig. 4D and E).
CYLD is highly expressed in primary CD4+ T lymphocytes and is cleaved upon T cell receptor (TCR)-mediated activation. In order to determine the CYLD expression profiles in cells of the human immune system relevant to HIV replication, we purified and analyzed CD4+ and CD4− T cell populations from peripheral blood lymphocytes as well as CD14+ cells from peripheral blood lymphocytes obtained from anonymous blood donors. Monocytes were differentiated into immature dendritic cells (DCs) and...
Macrophages. CYLD protein levels in lysates of these three cell populations were analyzed by Western blotting. Figure 5A shows that CD4$^+$ T lymphocytes from both donors tested expressed more CYLD than the CD4$^-$ cell population. CYLD was detectable in monocyte-derived macrophages and monocytes derived immature DCs from both donors (Fig. 5B and C).

Previous studies using murine systems or immortalized human T cells suggested that CYLD might undergo proteolytic cleavage upon activation (25, 26, 57). Since productive HIV infection requires T cell activation, we tested whether CYLD was cleaved in primary human CD4$^+$ T cells. T cell activation was analyzed by immunoblotting. The CYLD antibody that was used for detection recognizes an N-terminal epitope and detects an N-terminal fragment of CYLD. *+, nonspecific band. (E) JLat 8.4 cells were stimulated for 24 h with PMA/ionomycin or PHA for 24 h. Primary CD4$^+$ T cells were stimulated for 5, 24, or 48 h with IL-2/PHA or IL-2 alone. Cell lysates were analyzed by immunoblotting for CYLD and actin expression. #, nonspecific band.

HIV transcription increases upon CYLD knockdown in immortalized and primary CD4$^+$ T lymphocytes. We next investigated the role of CYLD in spreading viral infection. The A3R5 T cell line was stably transduced with shRNA directed against CYLD or with nontargeting control shRNA. The efficiency of CYLD silencing was approximately 50% at the protein level as determined by Western blotting and densitometric analysis (Fig. 6A). Both cell lines were infected with replication-competent HIV NL4.3 and HIV LAI. Culture supernatants were collected every 2 days over a 10-day period and analyzed on TZM-bl reporter cells at the end of the infection. Both viruses replicated three times more efficiently in the cells expressing less CYLD (Fig. 6B).

In order to establish whether CYLD also plays a role in human primary CD4$^+$ T cells, we transiently silenced CYLD in purified CD4$^+$ T lymphocytes from two healthy donors. The efficiency of CYLD silencing was approximately 60% for both donors as determined by qPCR. We infected PHA-activated CD4$^+$ T cells silenced for CYLD or transfected with nontargeting siRNAs with a VSV-G-pseudotyped HIV NL4.3 lacking envelope and measured the levels of HIV mRNA at 24 h postinfection. We found that upon CYLD silencing, HIV mRNA expression was increased 2.8- to 5-fold in CYLD-silenced cells compared to control cells (Fig. 6C).

Overall, these data indicate that CYLD limits HIV transcription in transformed T cell lines as well as primary CD4$^+$ T cells.
Reactivation of latent HIV is enhanced in the absence of CYLD. The NF-κB pathway not only is important during the early stages of the HIV replication cycle to establish a productive infection but also plays a pivotal role in the establishment and maintenance of HIV latency (58). To determine whether CYLD influences reactivation of HIV from latency, we used the well-described Jurkat T cell lines (JLat) that harbor an integrated, latent HIV provirus encoding a GFP reporter (59). Expression of the latent provirus results in GFP expression and can be achieved by stimulation with cytokines (e.g., TNF-α) or mitogens (e.g., PMA) (51, 60, 61).

We first monitored the effect of CYLD silencing on HIV reactivation after PMA treatment of JLat cells (8.4 clone). Flow cytometry analysis showed a 2-fold increase of GFP expression in JLat cells silenced for CYLD compared to cells transfected with a nontargeting (NT) siRNA (Fig. 7A). Expression of the latent provirus results in GFP expression and can be achieved by stimulation with cytokines (e.g., TNF-α) or mitogens (e.g., PMA) or by silencing of cellular molecules (e.g., promyelocytic leukemia protein) (51, 60, 61).

We next stably silenced CYLD in JLat cells (clone 8.4) and analyzed Gag production upon PMA treatment. Both Western blot analysis (Fig. 7C) and intracellular p24 staining (Fig. 7D and E) showed a 4- to 6-fold increase in Gag p55 production. The intracellular Gag staining was required since the shRNA constructs used themselves expressed GFP, making it impossible to monitor HIV reactivation by direct GFP measurements as done in the transient-silencing experiments described above. We verified the levels of CYLD knockdown by immunoblot analysis (Fig. 7C).

We measured HIV mRNA expression by qPCR to ascertain that the enhanced level of viral protein production was caused by an increase in viral transcription. HIV transcription was determined at steady state and after PMA stimulation in JLat cells (clone 8.4) stably silenced for CYLD or transduced with a nontargeting control. We found that CYLD knockdown alone did not induce HIV reactivation, but upon PMA treatment, cells silenced for CYLD expressed 5- to 7-fold more HIV mRNA than the nontargeting control cells (Fig. 7A, upper panels). Thus, silencing of CYLD enhanced reactivation of latent provirus upon PMA reactivation in the JLat model system by increasing HIV transcription.

**DISCUSSION**

In this study, we dissect the molecular mechanism by which CYLD negatively affects HIV infection. We established that CYLD silencing enhances HIV transcription in different cell types, including primary CD4+ T cells (Fig. 1B and C, 2C, and 6C). The positive effect of CYLD knockdown on HIV infection was specifically linked to the presence of NF-κB/NFAT transcription factor-binding sites within the HIV LTR. Deletion of these sites abolished the effect of CYLD silencing on HIV transcription. Moreover, MLV transcription, which is known to be independent from the NF-κB/NFAT pathways, was not enhanced in cells lacking CYLD (Fig. 3A).
and B). Silencing of CYLD also improved viral replication in T cell lines (Fig. 6B) and facilitated viral reactivation in the JLat model (Fig. 7).

In agreement with previous reports, we found that in the absence of CYLD, NF-κB-dependent transcription was enhanced and IκBα degradation upon TNF-α stimulation was accelerated (Fig. 4). We observed, moreover, that the inhibitory effect of CYLD on HIV transcription was dependent on its catalytic activity (Fig. 1B). Overexpression of the short-hairpin-resistant form of CYLD in 293T CYLD shRNA cells decreased NF-κB-dependent transcription of a c-fos reporter to levels comparable to that in control cells (Fig. 4A), while it only partially decreased LTR reporter transcription. This residual activity may be due to the potency of Tat as a transactivator (reviewed in reference 62). In the absence of Tat, the majority of RNA polymerases are paused near the promoter, while in the presence of Tat, the elongation of the transcript is dramatically increased, resulting in more efficient HIV mRNA production (63). Of note, overexpression of the catalytically inactive mutant CYLD C598A in the control NT shRNA cells increased HIV infection (Fig. 1C). This effect could be due to

**FIG 7** Reactivation of latent HIV is enhanced in the absence of CYLD. (A) Flow cytometry profiles of JLat 8.4 cells transfected with CYLD siRNA or nontargeting siRNA (NT) in the absence of stimulation or treated with PMA. (B) Efficiency of silencing was determined by Western blot analysis (upper panel) and quantified by densitometric analysis (lower panel). (C) JLat 8.4 cells stably expressing shCYLD or nontargeting (NT) shRNA were stimulated with PMA for the indicated times. Efficiency of CYLD silencing (upper panel), HIV Gag production (middle panel), and actin expression (lower panel) were determined by Western blot analysis. *, nonspecific band. HIV Gag production was quantified by densitometric analysis. The level of Gag p55 expressed in NT shRNA JLat 8.4 cells after 24 h of PMA stimulation was set to 1. Values are means plus SEM from three independent experiments. (D) JLat 8.4 cells stably expressing shCYLD or NT shRNA were stimulated with PMA, and HIV Gag production was determined by flow cytometry using intracellular p24 staining. The plots shown are representative of four independent experiments. (E) Reactivation of HIV upon PMA stimulation of JLat 8.4 cells stably expressing shCYLD or NT shRNA as determined by p24 intracellular staining followed by flow cytometry. Values represent means plus SEM for four independent experiments. *, $P < 0.05$ (unpaired t tests, Prism software). (F) Quantitative PCR analysis of HIV-1 mRNA levels in JLat 8.4 cells stably expressing shCYLD or NT shRNA with and without PMA stimulation. HIV mRNA expression was analyzed using the $\Delta\Delta C_T$ method. Values represent means plus SEM for four independent experiments. HIV mRNA expression in the NT shRNA, PMA-treated group is set to 1, and values represent fold change over NT shRNA. Expression of HIV mRNA relative to the housekeeping gene rsp11 was 0.2. *, $P < 0.05$ (unpaired t tests, Prism software).
a dominant negative effect of the catalytically inactive version of CYLD. The C598A mutant is still able to bind to its targets but is unable to remove ubiquitin moieties, thus sequestering cellular targets from the endogenous wild-type CYLD. Catalytically inactive proteins that act in a dominant negative manner have been reported, including several deubiquitinas (64–66).

While we clearly show that CYLD acts in an NF-κB-dependent manner, our experimental methods did not distinguish between NF-κB and NFAT protein-specific contributions to HIV transcription. It is well established that CYLD negatively regulates NF-κB signaling (7, 8, 14, 23, 24), while the role of CYLD in the NFAT pathway is less well explored (25, 26). However, recently two groups reported that CYLD also inhibits NFAT signaling (25, 26). CYLD might, thus, act as an inhibitor of HIV transcription through distinct pathways.

Studies using knockout mice revealed a role for CYLD in T cell biology (67–70). In these studies, the absence of CYLD resulted in reduced accumulation of mature thymocytes and peripheral T lymphocytes. In addition, the differentiation of the CD4+ T lymphocytes into T regulatory cell subsets was halted (67–70). Cleavage of CYLD by the cellular paracaspase MALT1 was necessary for proper activation of CD4+ T cells in response to T cell receptor stimulation (57). Conversely, the loss of CYLD caused a hypersensitive T cell phenotype leading to autoinflammation (24). CYLD can also be cleaved by caspase 8, giving rise to an amino-terminal fragment of 25 kDa (71). Despite the importance of CYLD in CD4+ T cell homeostasis in mouse models, its role in human T cell biology is less well established. We observed that CYLD is highly expressed in the purified CD4+ fraction of human primary lymphocytes. Reminiscent of the findings of Staal et al. (57) and Thuille et al. (25) in mice, we observed CYLD processing in primary human CD4+ T cells upon activation. However, the CYLD fragment that we observed upon T cell activation migrated at a slightly higher size (45 kDa) than what was reported in the murine systems and in Jurkat T cells (40 kDa). Staal and colleagues observed CYLD cleavage within a few hours of stimulation, while in our T cell experiments CYLD cleavage became apparent only after 24 to 48 h of stimulation. Future studies will investigate whether a protease other than MALT1 or caspase 8 cleaves CYLD in CD4+ T cells in response to T cell receptor stimulation compared to that with PMA alone (24). CYLD could also be cleaved by caspase 8 as shown by the existence of an amino-terminal fragment of 25 kDa. Although the importance of this fragment is unclear, it may contribute to the phenotype of HIV-infected T cells suitable for supporting infectious HIV production (73–75).

The global activation status of the NF-κB pathway in the cell at the time of HIV-1 infection may be the key factor determining the outcome of infection (39). Establishment, maintenance, and reactivation of latency correlate with the activation levels of NF-κB (58, 76–78). The stimuli known to activate NF-κB (e.g., PHA and PMA) also induce HIV transcription from latent proviruses (61, 79). CYLD silencing failed to induce spontaneous reactivation of the silent provirus in the JLat model system, but HIV mRNA production was increased when CYLD knockdown was augmented with PMA activation compared to that with PMA activation alone (Fig. 7). These results indicate that for reactivation of the latent provirus, silencing of CYLD was insufficient, hinting at other restrictive cellular factors such as the chromatin environment or transcriptional interference (60). Of note, in a primary T cell model of latency, activation of NFAT instead of NF-κB has been proposed to be the main factor inducing reactivation of the latent provirus (42). Future studies investigating the role of CYLD in a primary model of latency are warranted to validate the observations.

Overall, our study shows that the deubiquitinase CYLD acts as a negative regulator of HIV transcription by inhibiting the NF-κB/NFAT pathway. Future studies will reveal whether modulation of CYLD plays a role in T cell activation and HIV infection in patients.

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