

A Genome-wide Short Hairpin RNA Screening of Jurkat T-cells for Human Proteins Contributing to Productive HIV-1 Replication*^[5]

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Short interfering RNAs (siRNAs) have been used to inhibit HIV-1 replication. The durable inhibition of HIV-1 replication by RNA interference has been impeded, however, by a high mutation rate when viral sequences are targeted and by cytotoxicity when cellular genes are knocked down. To identify cellular proteins that contribute to HIV-1 replication that can be chronically silenced without significant cytotoxicity, we employed a shRNA library that targets 54,509 human transcripts. We used this library to select a comprehensive population of Jurkat T-cell clones, each expressing a single discrete shRNA. The Jurkat clones were then infected with HIV-1. Clones that survived viral infection represent moieties silenced for a human mRNA needed for virus replication, but whose chronic knockdown did not cause cytotoxicity. Overall, 252 individual Jurkat mRNAs were identified. Twenty-two of these mRNAs were secondarily verified for their contributions to HIV-1 replication. Five mRNAs, *NRF1*, *STXBP2*, *NCOA3*, *PRDM2*, and *EXOSC5*, were studied for their effect on steps of the HIV-1 life cycle. We discuss the similarities and differences between our shRNA findings for HIV-1 using a spreading infection assay in human Jurkat T-cells and results from other investigators who used siRNA-based screenings in HeLa or 293T cells.

The use of RNA interference (RNAi)² to silence genes holds potential applications for antiviral therapy (1, 2). RNAi, directed to viral and/or host RNA sequences, has been employed to inhibit HIV-1 replication (3–6). These approaches are effective over the short term; however, over longer durations, they have elicited either a high rate of escape mutation when viral sequences are targeted or cytotoxicity when cellular genes are chronically suppressed (7–10).

If cellular genes important for HIV-1 replication (11, 12), but dispensable for host cell viability, could be identified, then silencing the RNAs from these genes may remedy the proclivity

for HIV-1 mutational escapes when viral sequences are targeted. Toward this objective, Brass *et al.* (13), König *et al.* (14), and Zhou *et al.* (15) have recently used libraries of presynthesized siRNA to knock down transiently HeLa/293T cell RNAs. They reported ~272, 278, and 304 annotated gene candidates that contribute to steps in the HIV-1 life cycle. Intriguingly, the identities of the gene candidates discovered by Brass *et al.* (13), König *et al.* (14), and Zhou *et al.* (15) are highly divergent with very little overlap when the three studies are compared with each other (16).

HeLa and 293T cells are highly efficient model cells for siRNA transfection. They are, however, poor representations of T-cells that are physiologically infected by HIV-1. In this respect, human T-cell lines, such as Jurkat, are better models, although the latter cells cannot be transfected efficiently with siRNAs. To perform a genome-wide RNAi screening in Jurkat T-cells, we decided to employ a shRNA library cloned in a retroviral vector. Our approach was to transduce Jurkat cells with the shRNA library and then select for cell clones that are individually integrated for single constitutively expressed shRNA. These shRNA clones are then infected with HIV-1. Because Jurkat T-cells are killed by HIV-1 infection, a cell would survive only if it harbored a shRNA that has knocked down sufficiently a gene required for lytic viral replication (Fig. 1A).

Execution of the above strategy identified 252 human genes whose chronic knockdown in Jurkat cells apparently interrupted productive HIV-1 infection. These 252 Jurkat genes are mostly distinct from the HeLa and 293T candidates described by Brass *et al.* (13), König *et al.* (14), and Zhou *et al.* (15). There were, however, many examples in our discovered genes that located to the same cellular signaling pathways as genes described by others.

EXPERIMENTAL PROCEDURES

Cell Lines—Jurkat and its derivative cell clones were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (HyClone), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Cells were selected and maintained in the same medium as described above with the addition of 0.125 μ g/ml puromycin.

Production of Virus-packaged shRNA Library and Stable Clones—Feline immunodeficiency virus vector-based shRNA library was produced as described in the manufacturer's protocol from SBI with the following modifications. 293T cells were propagated in Dulbecco's modified Eagle's medium (Invitro-

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² The abbreviations used are: RNAi, RNA interference; HIV-1, human immunodeficiency virus, type 1; siRNA, short interfering RNA; RT, reverse transcriptase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; VSV-G, vesicular stomatitis virus G.

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gen) with 10% fetal calf serum and transfected with 10 μg of the pPACK packaging plasmid mixture and 2 μg of the shRNA library lentivector constructs (SBI) using Lipofectamine and Plus reagents according to the manufacturer's protocol (Invitrogen). 24 and 72 h after transfection, conditioned culture media were collected to harvest the packaged viruses.

Viruses were quantified by reverse transcriptase (RT) (17). 10^6 – 10^7 cpm of RT units were used to infect 8×10^6 Jurkat cells. After 8 h of exposure to virus, cells were washed two times with PBS and resuspended in RPMI 1640 medium (Invitrogen). One week after transduction, the cells were selected with 0.125 $\mu\text{g}/\text{ml}$ puromycin containing growth medium for 3 weeks. For secondary validations, independent shRNA expressing vector constructs (Mission shRNA) were purchased from Sigma. These shRNA-packaged viruses were generated in 293T cells using ViraPowerTM Lentiviral Expression Systems (Invitrogen) according to the manufacturer's protocol.

shRNA-based Screening—Selected Jurkat clones were split into two flasks and subjected to HIV-1 infection or mock infection. Four weeks after infection, small RNAs were harvested using the mirVanaTM small RNA isolation kit (Ambion). cDNA synthesis and labeling were performed as described in the manufacturer's protocol. Reverse transcription was performed using 5 μg of total RNA in the presence of the 10 μM cDNA synthesis GNF primer (ATTTATTGTATCTGTGGGAGCCTC), 100 mM dithiothreitol, 10 mM of each dNTP, 1 \times reverse transcriptase buffer, and 200 units \times 4 of SuperScript[®] III Reverse Transcriptase (Invitrogen). The reaction mixtures were allowed to incubate at 42 °C for 1 h. The reaction was stopped by heat inactivation at 72 °C for 5 min. Half of the reaction mixture was then transferred to a tube containing 1 \times PCR buffer, 20 mM dNTP, 20 μM forward GNF primer (TGCATGTCGCTATGTGTTCTGGGA), and 20 μM reverse GNF primer (ACAAAGCACTGGAAGCTATCGAA) and Advantage[®] HF2 Taq polymerase (Clontech). PCR were performed under the following conditions: step 1, 94 °C for 4 min; step 2, 94 °C for 30 s and then 68 °C for 1 min; step 3, repeat step 2 for 20 cycles; and step 4, 68 °C for 3 min. An aliquot was made of 1 μl of the first round PCR into 4 new tubes, each containing 1 \times PCR buffer, 20 mM dNTP, 20 μM NRev GNF universal primer (AAAGAATGCTTATGGACGCTAGAA) and NFwd-Bio primer (Biotin-CTTCCTGTCAGA) and Advantage[®] HF2 Taq polymerase (Clontech). The PCR conditions were then as follows: step 1, 94 °C for 2 min, 50 °C for 2 min and then 68 °C for 1 min; step 2, 94 °C for 30 s and then 68 °C for 30 s; step 3, repeat step 2 for 18 cycles; and step 4, 68 °C for 3 min. The PCR products were then gel purified using the Qiagen QIAquick PCR purification kit as described in the manufacturer's protocol. After purification, the PCR product was treated with λ -exonuclease (New England Biolabs) at 37 °C for 2 h to remove the non-biotinylated strand. The digested products were purified as described above and then quantified. 10 μg of the purified products were hybridized on the GeneChip[®] Human Genome U133 +2 Array (Affymetrix) using the Affymetrix hybridization buffer and then stained with streptavidin-phycoerythrin (Molecular Probes). Following the standard Affymetrix protocol, the image of the processed chip was captured by a scanner controlled by Affymetrix GCOS software. Data were first nor-

malized by the quantile normalization method, and the variances were calculated based on analysis of variance analysis. The signals were summarized for replicates with two or more replicate values above 100. The fold changes were determined based on the ratio of the signals between the mock and infected samples. Pathway analysis was done by using the Ingenuity program.

Flow Cytometry— 1×10^6 cells were stained with anti-CXCR4-PE/anti-CD4-APC or their isotype controls (BD Biosciences) for 30 min at 4 °C. The stained samples were washed two times with PBS containing 1% fetal calf serum. After washing, the cells were resuspended with the wash buffer containing 0.5% of paraformaldehyde. Flow cytometry analyses were performed using a FACSCalibur (BD Biosciences) equipped with CellQuest software (BD Biosciences). Live cell gating was first done by measuring the forward (FSC) and sideward (SSC) light scatter of unstained Jurkat cells. Background level settings were determined based on the signals detected in Jurkat cells stained with isotype controls IgG_{1 κ} -APC and IgG_{2 $\alpha\kappa$} -PE. For monitoring of Jurkat cells killed by virus infection, Jurkat cells infected or mock-infected after 3 and 5 days with HIV-1 were harvested. Cells were washed twice with PBS, and the percentage of cell death was determined by the Annexin-V-FITC detection kit II (BD Biosciences) following the manufacturer's protocol.

Quantitative PCR—Total RNAs were isolated using the mirVana miRNA isolation kit (Ambion). The RNAs were first quantified by NanoDrop[®] Spectrophotometer (ND-1000; Grace Scientific, LLC). Real-time PCR was then performed using 1 μg of the total RNA and TaqMan[®] EZ RT-PCR Core Reagents (ABI Biosystems) and TaqMan Tamra Probes (TaqMan Gene Expression Assays, inventoried for the corresponding genes; ABI Biosystems) according to the manufacturer's protocol. Briefly, the RT step was done at 50 °C for 10 min, and the amplification was performed under the condition of 15 s at 95 °C and 1 min at 60 °C for 55 cycles in a 7300 Real Time PCR System (Applied Biosystems).

HIV-1 Reverse Transcription, p24 Antigen Enzyme-linked Immunosorbent Assay, and Transcription Analyses—Virus stock was prepared by transfecting 293T cells. Supernatant virus was quantified by RT (17) and/or the p24 Antigen enzyme-linked immunosorbent assay (RETRO-TEK; Zepeto-Matrix Corp.); typically, 10^6 – 10^7 cpm of RT units were used to infect 5×10^6 Jurkat cells. After 8 h of exposure to virus, cells were washed two times with PBS and resuspended into RPMI. After 24 h, cellular DNA and RNA were isolated using DNAzol[®] Reagent (Invitrogen) and TRI[®] Reagent (Sigma), respectively, and quantified by the Nanodrop Spectrophotometer. HIV proviral DNA was detected by quantitative PCR on 50 ng of genomic DNA using the primers TCAGCATTATCA-GAAGGAGCCACC (forward) TCATCCATCCTATTTGTTCCTGAAG (reverse). For HIV RNA quantification, reverse transcription was performed on 0.5 μg of cellular RNA with oligo(dT) as the RT primer, and quantitative PCR for HIV RNA was performed with 5% of the RT reaction using the same primers. A standard curve from 10^1 to 10^6 copies of pNL4.3 was used for quantification, and the glyceraldehyde-3-phosphate dehydrogenase gene and mRNA quantification was performed for normalization (forward, GCTCACTGGCA-

TGGCCTTCCGTGT and reverse, TGGAGGAGTGGGTGT-CGCTGTTGA). p24 Antigen enzyme-linked immunosorbent assay was carried out according to the manufacturer's protocol. Briefly, the conditioned supernatant was first diluted in PBS and then lysed in the lysis buffer. Samples together with the standards made of HIV-1 p24 (from concentrations of 0 to 125 pg/ml) were loaded on a pre-washed 96-well plate. The plate was then incubated overnight at 37 °C. The HIV-1 p24 Detector Antibody was then applied and incubated at 37 °C for 1 h after washing. Next, unhybridized antibody was washed away before the application of streptavidin-peroxidase working solution. The solution was incubated at 37 °C for 30 min. Color development was done by administration of the substrate working solution and incubation at 25 °C for 30 min. Stop solution was then applied to stop the reaction. The optical density (450 nm) was then acquired by a microplate reader (Mithras LB 940; Berthold Technologies).

Cell Proliferation Assay—Cell proliferation was determined using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay employing the Cell Counting kit 8 (CCK-8; Dojindo) according to the manufacturer's protocol. 10 μ l of CCK-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Dojindo) was added to 100 μ l of cells (around 5×10^3 cells/well) in a 96-well plate. The cell viability was determined by reading the optical density at 450 nm. The data were collected from three independent experiments. Results are presented as the mean \pm S.E. from at least three independent experiments.

Confocal Microscopy—Jurkat parental cells and clones were transfected with either GFP-Gag or GFP plasmid individually. Seventy-two hours after transfection, cells were allowed to settle on a chambered coverglass (Lab-Tek) for 3 h prior to imaging. Live images were captured by Leica TCS-NT (Leica Microsystem).

RESULTS

To identify genes in human T-cells important to HIV-1 replication that can be knocked down without cytotoxicity, we used a puromycin-marked feline immunodeficiency virus-based lentivector shRNA library that targets 54,509 human transcripts, including ESTs. This library has 3 to 5 shRNAs designed for each of the 54,509 human transcripts, and all the shRNAs are "bar-coded" for rapid identification using the GeneChip[®] Human Genome U133 +2 Array. Starting with the feline immunodeficiency virus-based shRNA lentivector library, we produced a corresponding VSV-G packaged shRNA virus library. We used the VSV-G shRNA virus to transduce Jurkat T-cells, a T-cell model commonly used in many HIV-1 studies due to its similar transcriptional profile when compared with that of CD4⁺ T cells (18). After transduction, puromycin was applied to select for cells that have integrated shRNAs. The selected cells were then challenged with a high titer stock of HIV-1 NL4-3 at a multiplicity of infection of 1.

In the above protocol, cells with a shRNA that knocks down an mRNA whose loss triggers cytotoxicity would be culled during puromycin selection. Because Jurkat cells die when infected by HIV-1 (under our infection conditions 92 and 96% of Jurkat

cells showed Annexin V death profile at days 3 and 5 after infection, data not shown), we anticipated that cells knocked down for an mRNA irrelevant to virus replication would succumb rapidly to virus infection. By contrast, a Jurkat shRNA clone silenced for a human mRNA needed for lytic HIV-1 replication would be expected to survive the infection. The shRNA in the survival cell can then be amplified by RT-PCR and identified via microarray bar-coding to reveal the targeted host mRNA (Fig. 1A).

To verify the proper execution of this strategy, we monitored each step for the anticipated outcome. We first optimized the experimental conditions for achieving a highly efficient (~72%) dose-dependent VSV-G virus transduction of Jurkat cells (supplemental Fig. S1). Next, Jurkat cells were transduced with the VSV-G-shRNA virus library, and the representativeness of the shRNA-transduced clones was verified. RNAs from two independently transduced cell populations were collected a few days after shRNA virus library transduction and were RT-PCR amplified and hybridized to the Human Genome U133 +2 Array (Fig. 1B, *Transductions 1 and 2*). Based on the array data, the concordance between two populations was determined to approximately 85.68–87.70%, supporting the reproducibility of the transductions. To determine the shRNA coverage within the shRNA-transduced cells, two separate hybridizations using the starting shRNA vector library (Fig. 1B, *Libraries 1 and 2*) were compared with the two shRNA transduction (*Transductions 1 and 2*). In this analysis, the complexity of the shRNA-transduced cells encompassed on average 84.34% of the total targeted mRNAs in the shRNA vector library. We note that the array patterns in the transduced cells (Fig. 1B, *Transductions 1 and 2*) are distinctly different from background hybridization visualized using RT-PCR of RNA from a mock-transduced Jurkat cell sample (Fig. 1B, *Bottom, Background neg Control*). Taken together, the results support that the shRNA virus transductions achieved robust (~85%), albeit not 100% representation, of the initial shRNA vector library.

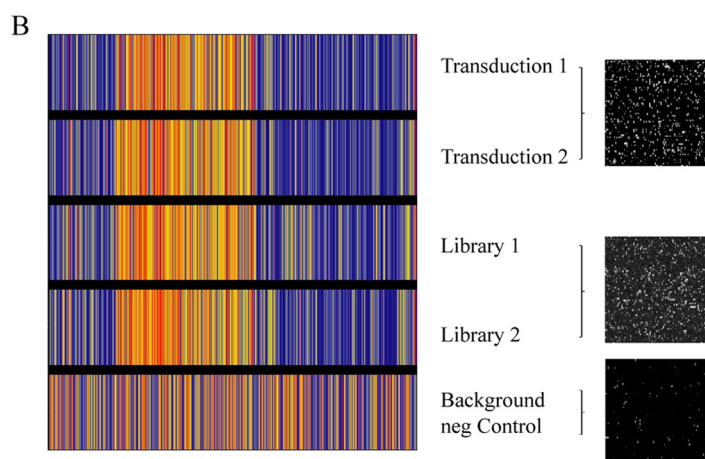
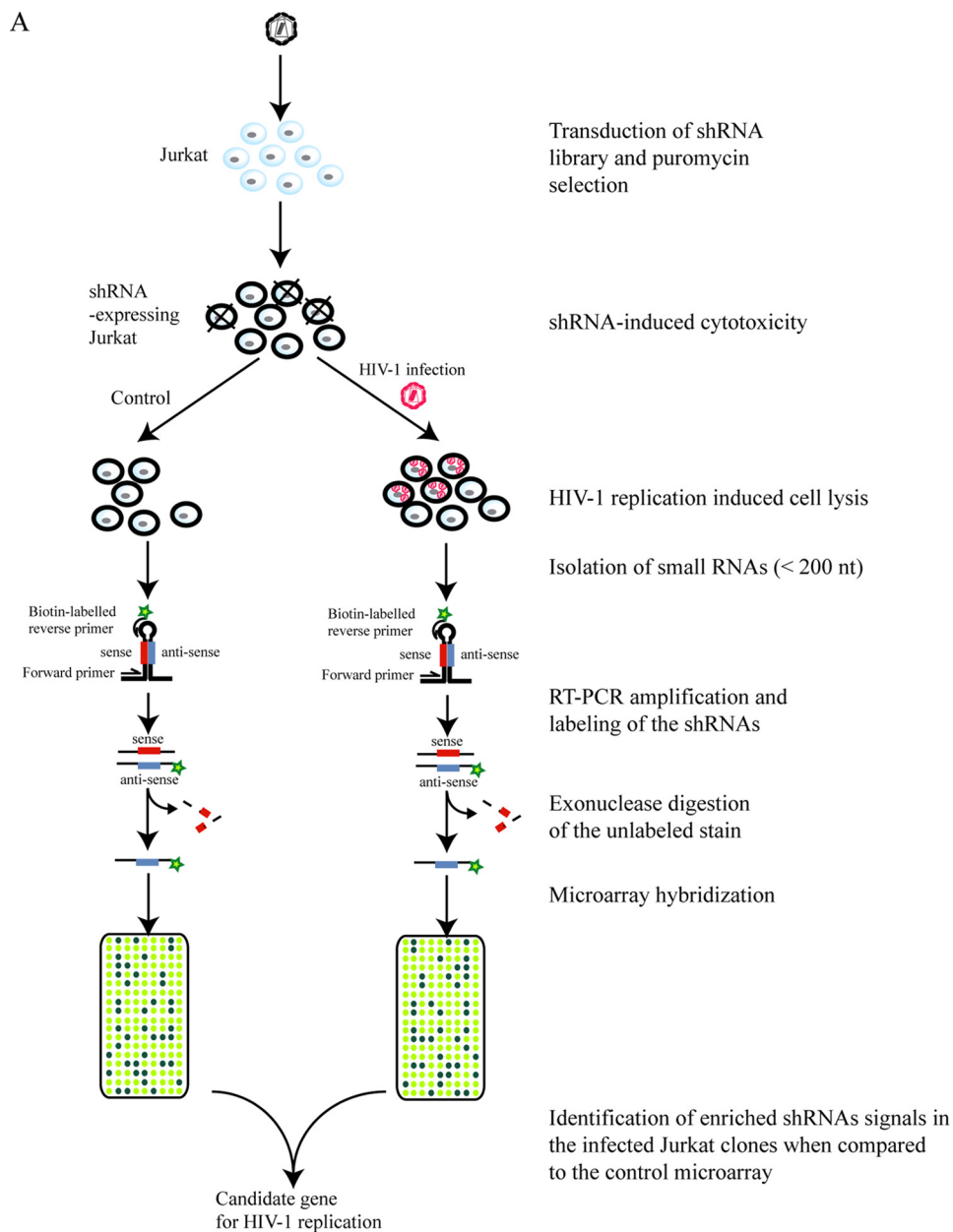
The transduced cells were selected next with puromycin. After 3 weeks of selection, 9,357 independent shRNA Jurkat clones remained, whereas 45,052 shRNA clones became undetectable. The shRNA clones that were no longer detected could arise in part from the following two reasons. First, a shRNA clone that has knocked down an mRNA whose loss is cytotoxic to the cell would be eliminated during puromycin selection. Second, some of the undetected 45,152 clones were relatively enfeebled, although not absolutely cytotoxic, and during the course of more than 3 weeks of tissue culture selection, these relatively enfeebled cells were outcompeted in proliferation by the more rapidly dividing cells that harbored the 9,357 detected shRNAs. Regardless of the two explanations, the results showed that 18.2% (or 9,357) of the starting 54,509 mRNAs could be tolerably knocked down without affecting Jurkat viability in tissue culture. In the 9,357 shRNA clones, we found 1,365 moieties that were targeted by more than one discrete shRNA. This number is higher than that expected by random (1309.02, $Z^* = -1.61$, standard error = 9.15, p value = 4.67×10^{-10}),

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suggesting that the knock down of some genes could confer a slight growth advantage to cells.

We next asked how many of the above 9,357 shRNA Jurkat clones could survive HIV-1 infection. The shRNA Jurkat clones were infected with HIV-1 NL4-3. After 4 weeks of infection, RNA from the survival cells was isolated, amplified by RT-PCR, and analyzed by microarray. The microarray hybridization revealed 252 distinct mRNAs that were enriched by at least 2–22-fold over control background (supplemental Fig. S2). Gene ontology analyses showed that these genes segregated into several functional classes (supplemental Fig. S3). These genes represented candidate factors that potentially contribute positively to HIV-1 replication in cultured Jurkat cells.

Next, we selected at random 22 of the 252 gene candidates for additional investigation. For each of the 22 selected genes, we employed 5 discrete shRNAs that targeted independent sequences within the mRNA. Transduction of 22×5 shRNAs resulted in the generation of 110 shRNA clones. These clones were confirmed for integration of the expected shRNAs (data not shown), and each was then challenged with infection by HIV-1. Based on the empirical experience in the literature, we expected that not all shRNAs designed to mRNAs will be successful in target knock down inside cells. In RT assays measuring supernatant virus production from these shRNA clones, consistent with the initial shRNA library screening results, 63 of 110 clones were able to restrict HIV-1 replication by slightly less than 50% to >90% when compared with HIV-1 infection of control Jurkat cells (Fig. 2A). To demonstrate that the effect of HIV-1 restriction is due to knock down of the intended mRNAs, we quantified in 25 selected cell clones without HIV-1 infection the shRNA-targeted mRNAs by RT-PCR (Fig. 2B). Fig. 2B shows that in these 25 shRNA clones, the ambient level of



the mRNA targeted by the respective shRNA was reduced by ~85–90% (the level of the corresponding mRNA in control cells was set as 1).

To verify further the above results, we performed two additional “control” experiments. First, a trivial explanation for reduced HIV-1 replication could be that the tested cells were nonspecifically reduced for cell surface CD4 and could no longer be infected by HIV-1. To exclude this possibility, we picked 9 cell clones and analyzed each by flow cytometry staining; none was found to be significantly different from parental Jurkat for CD4 (Fig. 3). Second, a reduction in HIV-1 replication could be a nonspecific result of shRNA-induced cytotoxicity. We deemed this explanation unlikely because all the cell clones were already selected for over 3 weeks in puromycin, prior to a second 4 weeks of HIV-1 infection. Nonetheless, 18 shRNA cell clones were chosen and compared head-to-head to parental Jurkat cells for relative proliferation rates in a CCK-8 metabolic assay. Cell growth rates, as reflected in the CCK-8 assay over a period of 4 days, were slightly variable as expected, but all were substantially robust and were little different from Jurkat control cells (Fig. 4).

All 252 candidate genes will need to be carefully studied to check if and how each might influence HIV-1 replication. Several HIV-1 relevant assays are available that can be used to address how candidate proteins affect cell surface co-receptor, reverse transcription, transcription, and Gag trafficking (Fig. 5). As a first step, we investigated 7 gene candidates. They were variously tested for their effects on cell surface CXCR4 expression (Fig. 5A), HIV-1 reverse transcription (Fig. 5B), HIV-1 transcription (Fig. 5C), and GFP-Gag trafficking (Fig. 5D). Detailed studies remain ongoing, but illustrative results are shown. Thus, knockdown of NRF1 affected cell surface CXCR4 expression, whereas knockdown of *EXOSC5*, *GALC*, *NCOA3*, *PRDM2*, *PRMT3*, and *STXBP2* did not (Fig. 5A). On the other hand, knockdown of *STXBP2* reduced the amount of reverse transcribed viral DNA (Fig. 5B); knockdown of *PRDM2* and *NCOA3* reduced HIV-1 transcription (Fig. 5C); and knockdown of *EXOSC5* affected GFP-Gag trafficking (Fig. 5D) in Jurkat cells. The knockdown of *GALC* showed no effect in these assays.

The published literature is in part consistent with the above findings. There is evidence for a role played by NRF1 in regulating the expression of CXCR4 (19). *NCOA3* and *PRDM2* have been reported to be transcriptional co-activators (20, 21), and Munier *et al.* (22) have suggested that *NCOA3* can interact with the HIV-1 LTR. *STXBP2* is an interactor of SYNTAXIN 3,

which acts on the membrane function and processes of the cell (23); however, it is unclear how knockdown of *STXBP2* (directly or indirectly) affects the amount of reverse transcribed HIV-1 proviral DNA. *EXOSC5* has been reported to be involved in RNA processing (24). Intriguingly, detailed proteomic data show that this protein interacts with nucleoporin (NUP210), a component of the nuclear pore complex (25), and a novel protein KIAA1217, which engages the TSG101-ESCRT and trans-Golgi pathways (supplemental Fig. S4). It remains to be established how knockdown of *EXOSC5* affects downstream GFP-Gag processing/trafficking.

Finally, we checked if the observed effects on HIV-1 replication in the shRNA cell clones could be confirmed to arise from the specific knockdown of the intended mRNA (Fig. 2B). A proof of causality can be established by reconstituting a shRNA knockdown cell with a vector that overexpresses the targeted mRNA. Due to limitations in obtaining full-length expression cDNAs, we currently could construct full-length expression plasmids for only three genes, *NCOA3*, *EXOSC5*, and *NRF1*. Consistent with the interpretation that suppression of HIV-1 replication was due to specific mRNA knockdown, when the *NCOA3*, *EXOSC5*, and *NRF1* shRNA cell clones were transfected with their cognate overexpression cDNAs, virus production, otherwise suppressed in the unreconstituted cells (Fig. 2A), was increased (Fig. 6).

DISCUSSION

Three recent studies have identified a large number of human genes that contribute to the HIV-1 life cycle using robotic approaches of transient siRNA library transfection of HeLa or 293T cells (13–15). Our current study has a similar goal of gene identification, but it is different in its use of durable transduction of a shRNA library into human Jurkat T cells. Neither the siRNA/HeLa/293T nor the shRNA/Jurkat strategy fully models HIV-1 replication in physiologically relevant primary cells. Nevertheless, because primary T-cells and macrophages are poorly transfectible with siRNAs, the successful execution of shRNA library transduction in Jurkat cells could pave the way for an approach that can be applied to primary cells.

The previous three siRNA screenings and the present shRNA analysis each reported more than 200 human genes that appear to be important for HIV-1 replication. It is noteworthy that the gene candidates in the previous three studies (13–15) have very little overlap with each other. In this regard, a direct comparison between our results and those reported earlier is difficult for several reasons. For instance, the strategy used by Brass *et al.*

FIGURE 1. A screening strategy using a shRNA library in a genome-wide identification of candidates important for HIV-1 replication in Jurkat cells. *A*, a VSV-G packaged shRNA virus (black) library was used to transduce Jurkat cells. Puromycin selection was employed to cull cells that did not integrate stably a shRNA. Clones that integrated a shRNA that targets a gene whose loss elicits cytotoxicity would similarly be eliminated. After selection and propagation, the shRNA Jurkat clones were divided into two pools, one infected with high titer HIV-1 NL4-3 (red) and the other carried as control. In the infected pool, only cells that have acquired a shRNA that knocked down a cellular mRNA important for HIV-1 replication would be protected from virus-induced lytic cell death. Small RNAs were isolated from survivor cells, and RT-PCR was performed using a specially designed biotin-labeled reverse primer. λ-Exonuclease was then applied to remove the non-biotin-labeled strand from the duplex. The remaining biotin-labeled strand was then hybridized to Affymetrix microarrays and then labeled with Cy3. By comparing the data extracted from microarrays of HIV-1-infected and uninfected samples, shRNAs enriched in the HIV-1-infected Jurkat cells identified virus-important candidate genes. *B*, confirmation of the representative complexity of shRNA virus library transduced Jurkat cells. To determine the shRNA complexity represented in the transduced cells (*Transduction 1* and *2*), RNAs were extracted and hybridized to microarrays as described in *A*. Results from the microarray hybridizations are represented as linear heat maps. The microarray results compared virus library-transduced cells (*Transduction 1* and *2*) with the starting vector library (*Library 1* and *2*). As a negative control (*Background neg Control*), Jurkat mock transduced cells were used in the same microarray analysis. Example raw microarrays images from each of the categories (*Transduction*, *Library*, and *Background neg Control*) are shown on the right. Spot density varied with the intensity threshold of detection.

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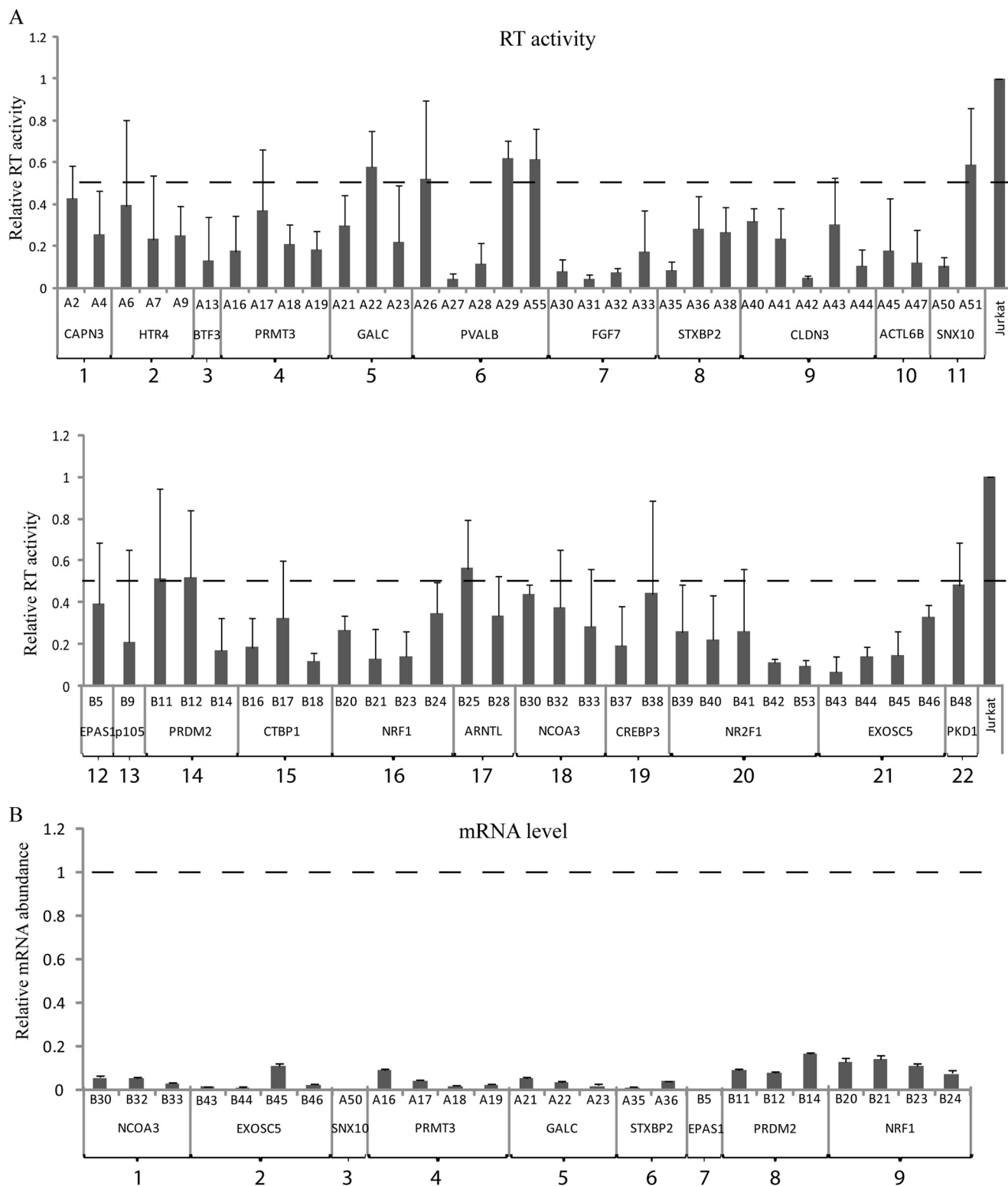


FIGURE 2. Verification of the effect from selected candidate genes on HIV-1 replication. *A*, Jurkat cells were stably transduced with the indicated individual shRNA, and the resulting cell clones were tested for their restriction of HIV-1 replication. Successful restriction of HIV-1 replication in the shRNA clones was shown by a reduction in RT activity in the cell culture supernatants compared with infection of control Jurkat cells (set as 1). The dotted line indicates a 50% reduction in RT activity achieved in the clonal cells. 63 selected clones (as discussed in the text) are shown. *B*, RT-PCR confirmation of the specific knockdown of the targeted mRNAs. The indicated mRNAs in selected clones were quantified by RT-PCR using gene-specific primers; the data were normalized to cellular 18 S RNA. The RT-PCR amount for each of the indicated mRNA in the parental Jurkat cell was set as 1.

(13) is quite distinct from ours. Hence, the cells used (HeLa versus Jurkat), the approaches (transient siRNA versus chronic shRNA), the assays (HeLa reporter versus lytic replication in

Jurkat), and the viruses (HXB2 versus NL4-3) are all different. We note that the Brass *et al.* (13) HXB2 virus is a mutated HIV-1 that does not express Nef or Vpu and is truncated for

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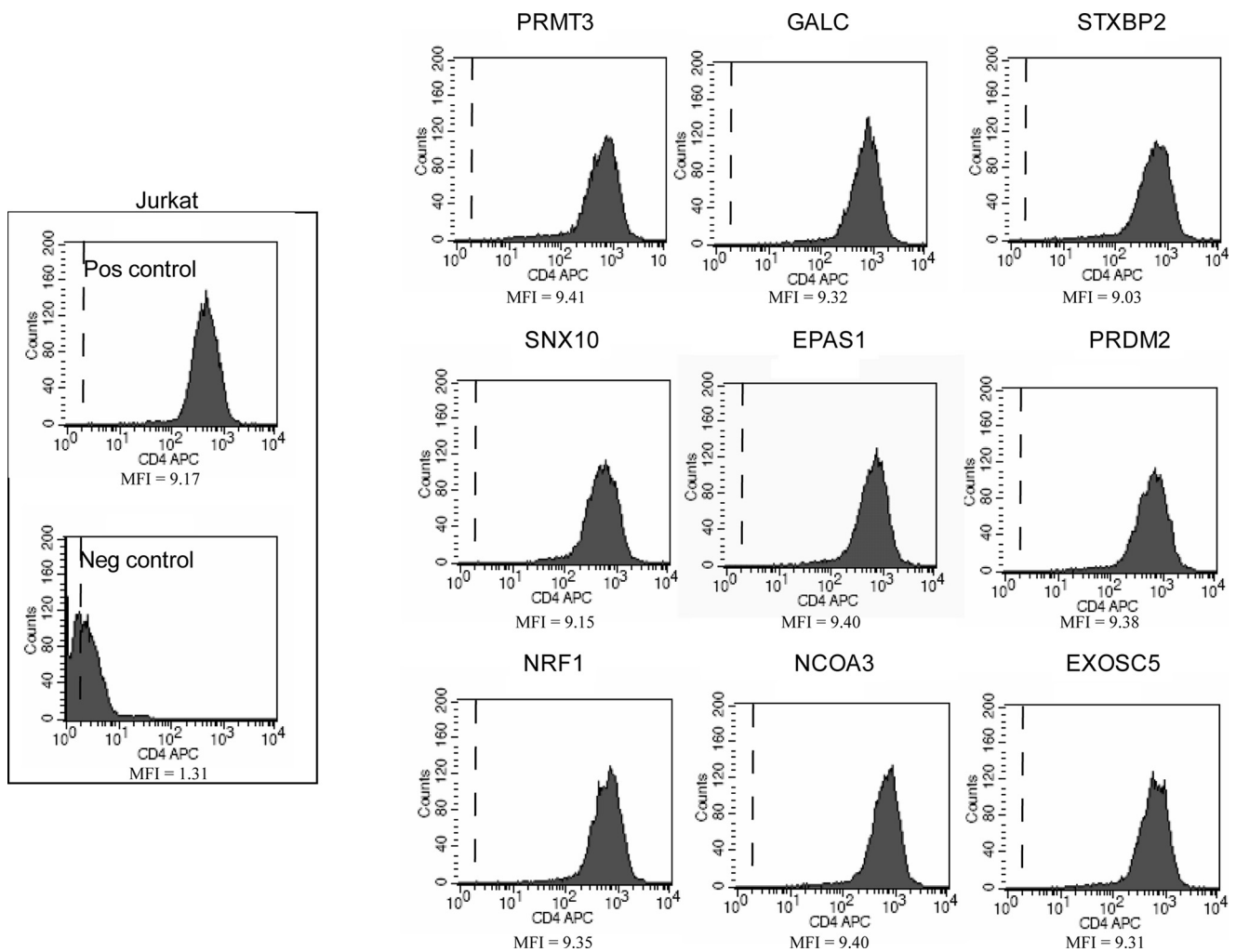


FIGURE 3. **Flow cytometric analysis of cell surface CD4 in shRNA Jurkat clones.** Expression of cell surface CD4 in selected shRNA Jurkat clones (PRMT3, GALC, STXBP2, SNX10, EPAS1, PRDM2, NRF1, NCOA3, and EXOSC5) was compared with that of normal Jurkat cells. An IgG_{k1} isotype control phycoerythrin staining of Jurkat cells was used as a negative control. Dotted lines are drawn to indicate the peak background intensity of the isotype control. MFI, mean fluorescent intensity.

Vpr, whereas our NL4-3 virus expresses full-length Nef, Vpu, and Vpr proteins (26–28).

We also recognize that there are several technical shortcomings to our shRNA screening. First, our shRNA-transduced cell clones covered only ~85% of the complexity of the shRNA vector library. Hence, in any single genome-wide screening, ~15% of the genome are not covered, and this gap can result in a significant number of “false negatives.” Statistically, an 85% coverage rate per screening suggests that nearly 100% coverage could be achieved by 4 or more independent repetitions of the same screening. Hence, the false negative omissions could be resolved with multiple shRNA library screenings. An additional source of false negatives could emerge because HIV-1 infection can result in the formation of syncytia. Syncytia formation between shRNA-transduced cells that resist HIV-1 infection with infected cells that are sensitive to HIV-1 infection could cause death of the former and result in the loss of signal from a shRNA cell that would otherwise be identified as a “hit” for a gene whose knockdown abrogates productive viral infection. The losses of such genes are false negatives. The extent of syncytia formation is, however, dependent on the experimental conditions and employed cells. In this regard, not all Jurkat cells

are the same for syncytia formation (supplemental Fig. S5). Indeed, in our experimental settings, syncytia formation was not prevalently seen. However, to the extent that some syncytia do occur, they could contribute to reducing the number of otherwise “positive” gene candidates. Second, our shRNA screening has some expected false positives. For example, we noted in our infection assays that on day 6 after infection 96% of Jurkat cells were killed by HIV-1. The possibility that HIV-1 infection, even when extended to 3 to 4 weeks, might not kill 100% of the Jurkat cells suggests that some of the survival cells “survive” independently of the ability of their resident shRNAs to knockdown mRNAs required for lytic HIV-1 replication. This limitation of quantitative HIV-1 killing of Jurkat cells predicts that a finite proportion of the identified candidates will be false positives. Thus candidate factors must remain “candidates” until they are secondarily proven for functional importance. Also, our shRNA library sequences are constrained by the need to fit the bar-coding in the GeneChip Human Genome U133 +2 Array. Alternate sequence designs for shRNA libraries could provide different stringencies and saturations in mRNA knockdown that might uncover other human genes.

shRNA Screening for Genes Contributing to HIV Replication

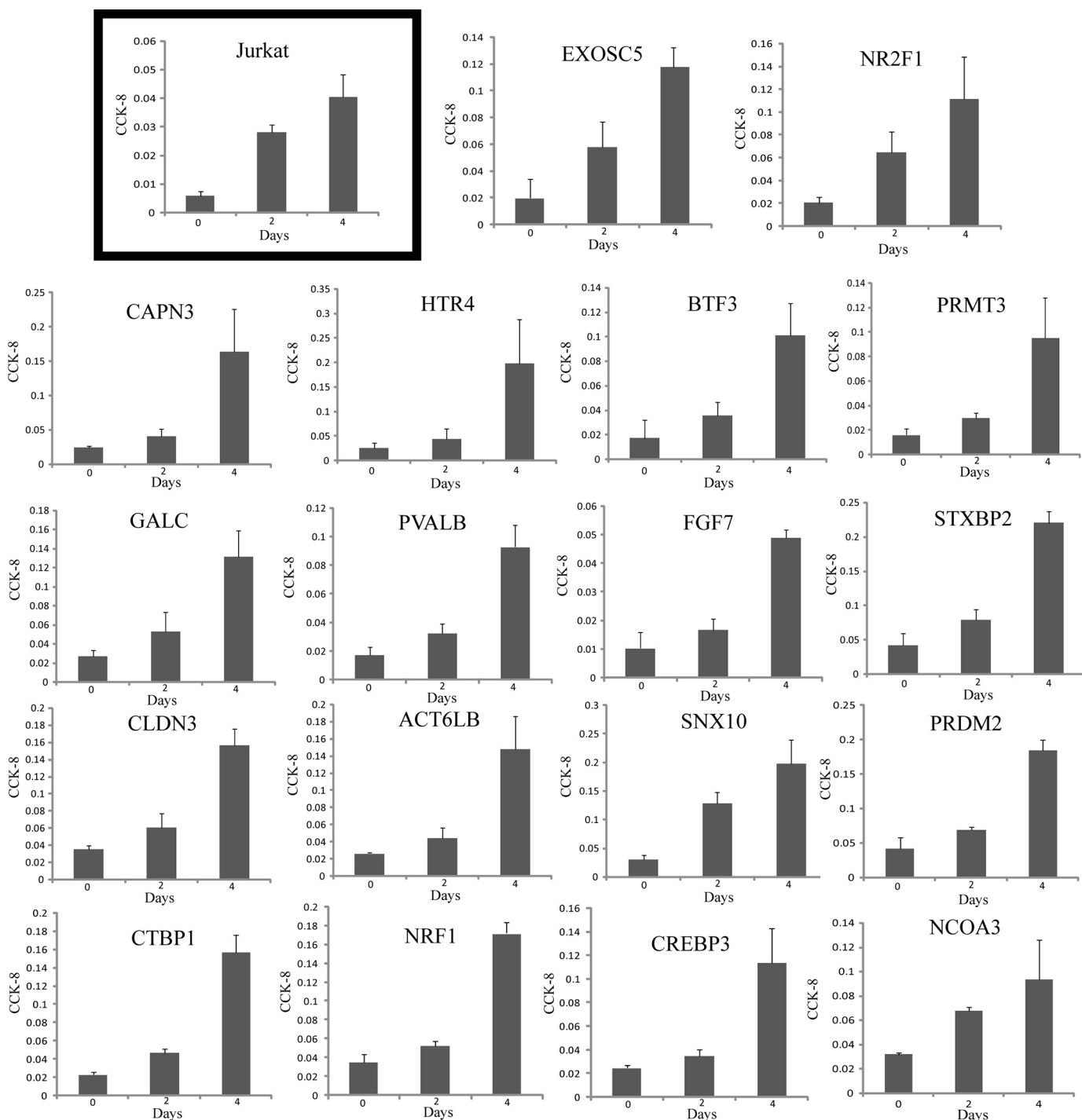


FIGURE 4. CCK-8 measurement of the proliferation of Jurkat and shRNA Jurkat clones. The CCK-8 assay measures the dehydrogenase metabolic activity of proliferating cells. The 450 nm absorbance increases proportionally with the number of dividing cells. The indicated shRNA Jurkat clones (EXOSC5, CAPN3, HTR4, BTF3, PRMT3, GALC, PVALB, FGF7, STXBP2, CLDN3, ACT6LB, SNX10, PRDM2, CTBP1, NRF1, CREBP3, and NR2F1) showed CCK-8 profiles similar to that from control Jurkat cells (boxed).

In considering the 252 gene candidates identified in this study, we realize that some of the gene candidates represent genes whose knockdown leads to latent HIV-1 infection, whereas others are genes whose knockdown results in abortive HIV-1 infection. These two possibilities underscore the need for all gene candidates to be further experimentally confirmed for their functional contributions to viral replication. Additional methodologies using modified vectors expressing red or

green fluorescent proteins coupled with flow cytometry sorting could be used to improve upon the discrimination between genes that contribute to latent viral infection *versus* those that confer abortive viral infection. Such approaches will be useful in further follow up analyses.

A careful parsing of our gene candidates revealed no identities between our genes and those reported by Brass *et al.* (13); there are three identities (*EPAS1*, *IBTK*, and *NUP98*) between

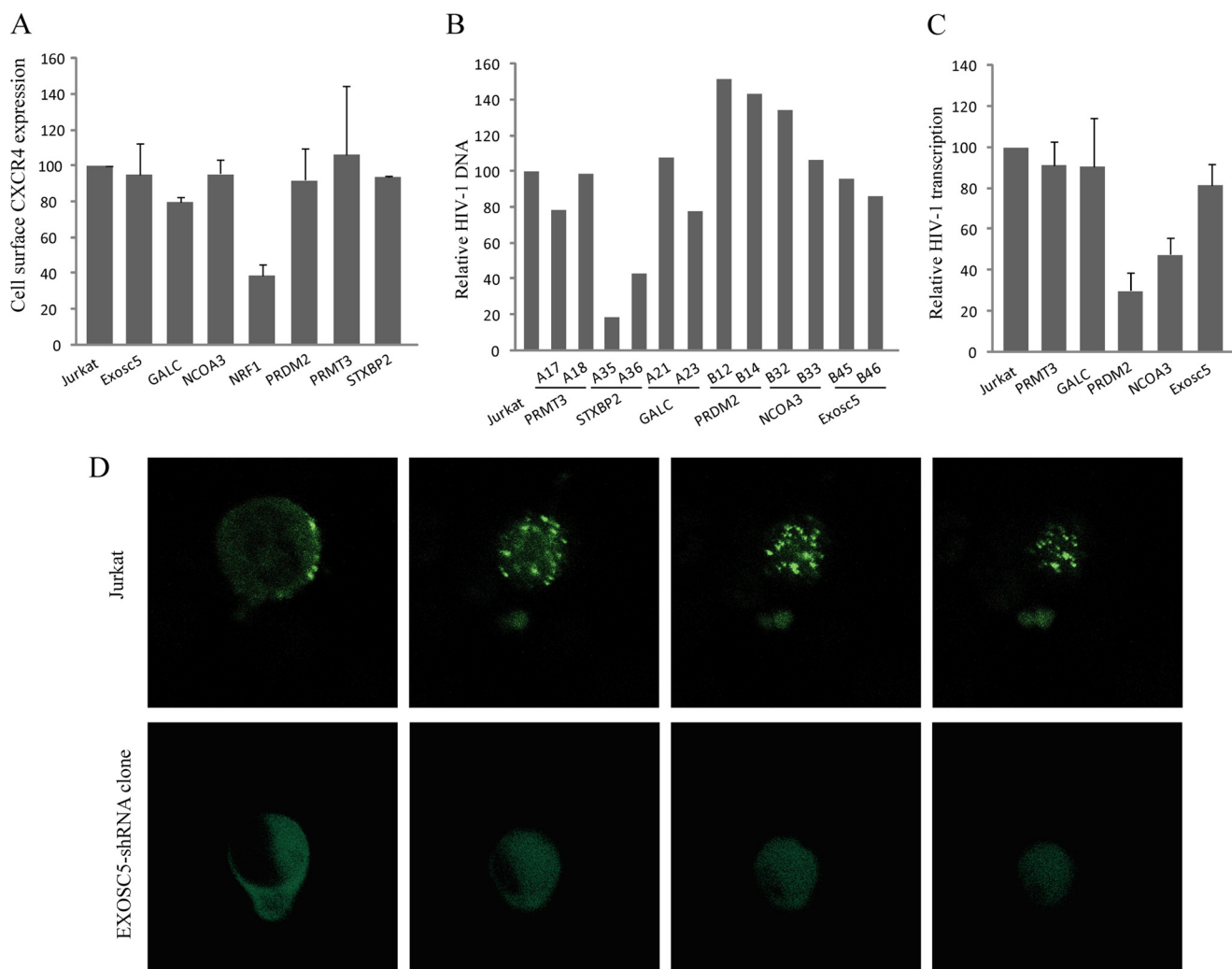


FIGURE 5. Analyses of the function contributed by candidate genes to the replicative life cycle of HIV-1. *A*, knockdown of NRF1 reduced the cell surface expression of HIV-1 co-receptor CXCR4. CXCR4 expression from the average values of three independent NRF1-shRNA clones was compared with parental Jurkat cells (set as 100) and with the average values from three of the other indicated shRNA clones. *B*, two different STXBP2-shRNA clones (A35 and A36) illustrate the reduced amount of reverse transcribed proviral DNA after HIV-1 infection. The proviral DNA level of infected control Jurkat cells was set as 100. Other shRNA clones, as indicated, did not show significant reduction. *C*, reduced HIV-1 transcription was seen in NCOA3 and PRDM2 shRNA clones compared with control Jurkat and the other indicated clones. Values are the averages from three independent clones. *D*, EXOSC5-shRNA cell clone showed a changed profile of transfected HIV-1 GFP-Gag. A “speckled” pattern for GFP-Gag was seen in the parental Jurkat cell (*upper panel*), whereas in an EXOSC5-shRNA clone a diffuse amorphous GFP-Gag pattern was visualized (*lower panel*). Images shown in the *upper* and *lower panels* represent “slices” from a single cell.

our study and König *et al.* (14), and three other identities (*NFKB1*, *RPL3*, and *RAB11A*) between our work and Zhou *et al.* (15). It has been noted elsewhere that the gene overlaps between Brass *et al.* (13), König *et al.* (14), and Zhou *et al.* (15) are remarkably scarce despite that the three studies utilized very similar siRNA transfections of HeLa/293T adherent cells (16). At first glance, the divergences in gene identities are puzzling. Further consideration, however, offers some possible clarity. For instance, we observed that many of our genes, although different in identity, mapped to the same cellular pathways populated by genes identified by Brass *et al.* (13), König *et al.* (14), and Zhou *et al.* (15). Thus, several candidates are located in the same NF- κ B signaling, estrogen-receptor signaling, peroxisome proliferator-activated receptor signaling, RAR activation, and caspase apoptosis routes (supplemental Fig. S6) with genes described by Brass *et al.* (13); other pathways are shared with König *et al.* (14) and Zhou *et al.* (15) (supplemental Fig. S6). Indeed, 42 distinct proteins in 5 pathways are

convergent with König *et al.* (14) (supplemental Fig. S6), and 41 distinct proteins in 7 pathways map with Zhou *et al.* (15). Several factors exhibited intriguing congruencies. Examples in (supplemental Fig. S5) show that whereas Brass *et al.* (13) identified AKT (29), our screening identified the AKT-immediate upstream factors, phosphatidylinositol 3-kinase (29), PTEN (30), and RAS (31). Additionally, whereas Brass *et al.* (13) identified NF- κ B p65 in HeLa, our work in Jurkat found the p65 partner, p50 (supplemental Fig. S7). Likewise, in the peroxisome proliferator-activated receptor γ pathway, Brass *et al.* (13) identified NCOR in HeLa, whereas we observed COUP in Jurkat (supplemental Fig. S8). Both NCOR and COUP act to negatively regulate peroxisome proliferator-activated receptor γ (32, 33). That the same pathways in Jurkat and HeLa cells are identified by different genes suggest that whereas a given pathway may be generally important for HIV-1 infection, different proteins in the pathway may be limiting for cellular proliferation *versus* HIV-1 replication depending on the cell type, the

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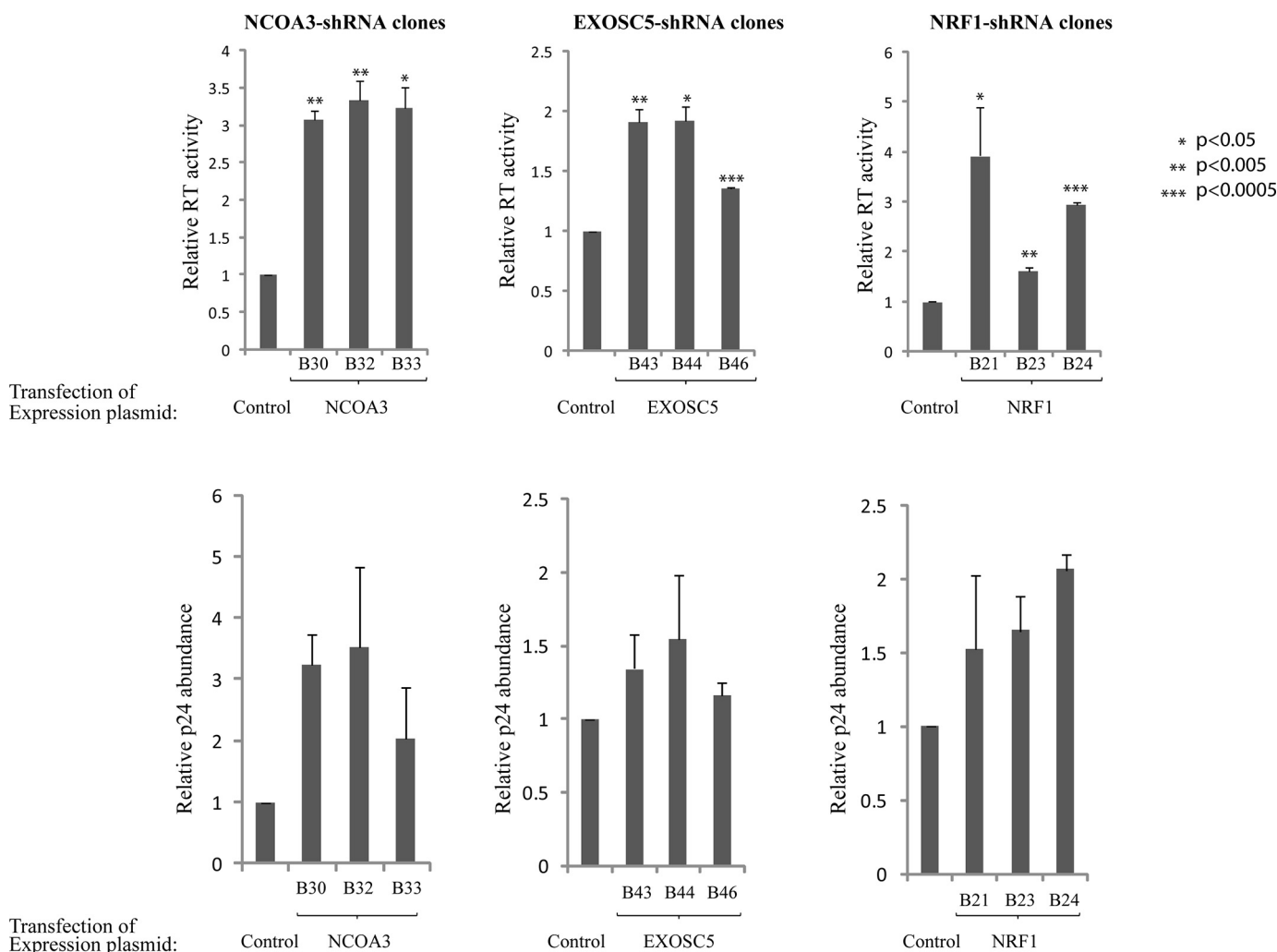


FIGURE 6. **Overexpression reconstitution of the cognate mRNAs targeted by shRNA knockdown rescued HIV-1 replication in shRNA Jurkat clones.** The overexpression of the cognate full-length cDNA, but not a control vector plasmid, increased HIV-1 replication as measured by supernatant RT production (*top*) and p24 quantification (*bottom*) in the indicated shRNA Jurkat clones.

functional assay (reporter *versus* virus replication), and the tissue culture conditions. The varying results underscore caution on interpreting findings from non-identical experimental systems. Conservatively, the four candidate sets should be viewed as starting points for winnowing down initial leads to the final proteins that must be proven by additional assays to be relevant for physiological HIV-1 replication in peripheral blood mononuclear cells and macrophages.

It is possible that the message from the different screening studies is that specific genes matter less than the cellular pathways that may be commonly critical to different settings of HIV-1 replication. If this interpretation is correct, then HIV-1 pathogenesis could be similar to tumor oncogenesis in which altered pathways and processes appear to hold generally greater importance than individual gene changes (34). Future knock-down studies in primary T-cells and macrophages will be required to confirm this notion for HIV-1.

Finally, recent progress in other screening approaches can serve to complement genome-wide RNAi findings. For example, advanced proteomic technologies have provided new insights on cellular proteins that may be important for HIV-1

replication in T-cells and macrophages (35, 36). If defined experimental conditions can be standardized, the analyses of complementary datasets accrued from RNAi screening, proteomic screening, and other high throughput methodologies such as pyrosequencing could contribute toward the elucidation of a coherent picture of the many cellular RNAs and proteins that can influence HIV-1 replication in human cells.

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