Report

Reactivation of Latent HIV-1 by Inhibition of BRD4

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SUMMARY

HIV-1 depends on many host factors for propagation. Other host factors, however, antagonize HIV-1 and may have profound effects on viral activation. Curing HIV-1 requires the reduction of latent viral reservoirs that remain in the face of antiretroviral therapy. Using orthologous genetic screens, we identified bromodomain containing 4 (BRD4) as a negative regulator of HIV-1 replication. Antagonism of BRD4, via RNA interference or with a small molecule inhibitor, JQ1, both increased proviral transcriptional elongation and alleviated HIV-1 latency in cell-line models. In multiple instances, JQ1, when used in combination with the NF-κB activators Prostratin or PHA, enhanced the in vitro reactivation of latent HIV-1 in primary T cells. These data are consistent with a model wherein BRD4 competes with the virus for HIV-1 dependency factors (HDFs) and suggests that combinatorial therapies that activate HDFs and antagonize HIV-1 competitive factors may be useful for curing HIV-1 infection.

INTRODUCTION

Elucidation of the host factors that modulate HIV-1 propagation has been a long-term goal of the HIV research community (Friedrich et al., 2011). In principle, this knowledge should allow us to develop therapies that seek to eradicate this disease (Choudhary and Margolis, 2011; Yang et al., 2009). Current antiretroviral therapy (ART) aimed at inhibiting viral enzymatic activities prevents HIV-1 replication and halts the virus's destruction of the host's immune system. However, patients develop viremia upon treatment interruption due to the reactivation of latent HIV-1 proviruses, and long-term ART has been associated with some adverse effects (Hakre et al., 2012; Margolis, 2010; Siliciano and Greene, 2011). To cure HIV-1, such latent reservoirs must be purged, the most promising strategy for this being viral activation performed in unison with ART to prevent de novo infection (Archin et al., 2012; Siliciano and Greene, 2011).

RESULTS

In an ongoing effort to saturate the HIV-1 dependency factory (HDF) siRNA screen (Brass et al., 2008), we found that depletion of the transcriptional regulator bromodomain containing 4 (BRD4) enhances HIV-1 infection with the use of either of two orthologous large-scale siRNA libraries (Figures 1A and 1B). BRD4 is a bromodomain protein that binds to acetylated histones and recruits transcription elongation factor b (PTEF-b), thereby increasing full-length mRNA expression (Jang et al., 2005; Yang et al., 2005). The establishment and maintenance of transcription of the HIV-1 genome is a critical step in the viral life cycle (Karn, 2011; Ott et al., 2011). After initiating transcription from the HIV-1 long terminal repeat (LTR), the host's RNA polymerase II complex pauses due to the influence of negative elongation factors (NELF and DSIF); this inhibition is overcome by the viral protein Tat, which binds to the viral mRNA's TAR element and recruits PTEF-b, leading to phosphorylation of both the polymerase and the negative elongation factors, resulting in transcriptional elongation (Karn, 2011; Ott et al., 2011; Zhang et al., 2007). Because the Tat/PTEF-b complex regulates viral transcription in a feed-forward manner, subtle alterations in the availability of either component strongly influence viral gene expression and activation (Karn, 2011; Karn and Stoltzfus, 2012; Ott et al., 2011). Of note, the majority of PTEF-b is associated with either the host cell's chromatin or its 7SK ribonucleoprotein (RNP) complex. The inaccessibility of PTEF-b presents a formidable barrier to viral activation that only Tat can overcome via its relocation of PTEF-b to the TAR element (Karn and Stoltzfus, 2012). BRD4 had also been previously implicated in HIV-1 biology. In different publications BRD4 has been suggested to be a positive or negative regulator of HIV-1 gene expression (Yang et al., 2005; Zhou et al., 2009). High level overproduction of a C-terminal portion of BRD4 has been observed to inhibit HIV-1 infection and compete with Tat for binding to PTEF-b in vitro (Bisgrove et al., 2007; Urano et al., 2008). The latter data suggest that BRD4 is necessary for basal viral transcription but might compete with Tat during active viral transcription (Bisgrove et al., 2007; Ott et al., 2011; Schröder et al., 2012). Together with our loss-of-function data, these studies prompted us to further investigate BRD4's role in the viral life cycle and, in particular, evaluate it as an antilatency target. To accomplish this, we individually transfected three unique siRNAs against BRD4 and monitored their effect on HIV-1 infection and the
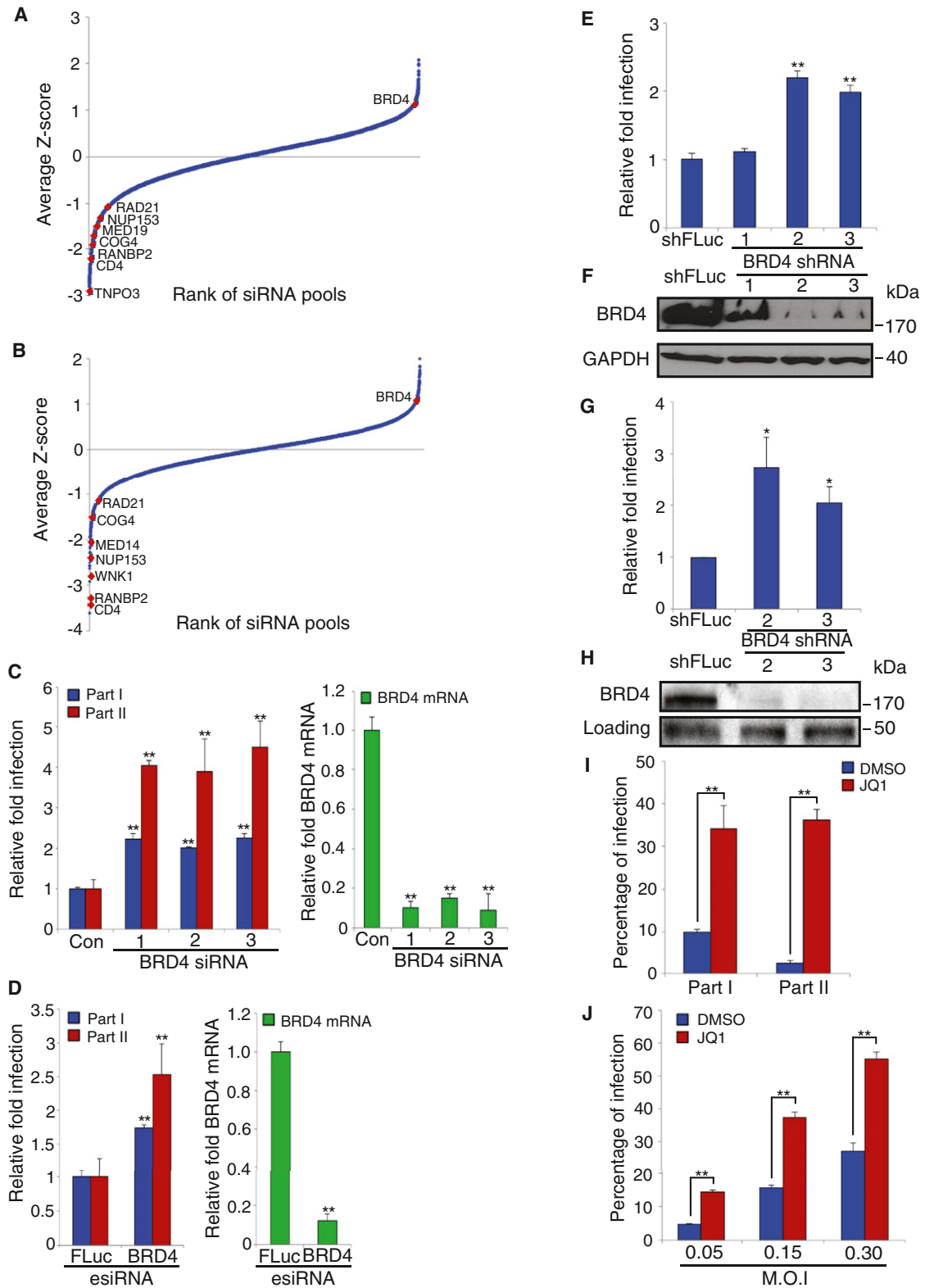
Figure 1. BRD4 Depletion or Inhibition Increases HIV-1 Replication

(A and B) The results of the indicated RNAi library screens with the siRNA pools ranked in order of average relative fold infection, from lowest to highest. The positions of known HDFs as well as the HIV-1-competitive factor (HCF), BRD4, from the screen are indicated. (A) Ambion Library. (B) esiRNA Library.

depletion of BRD4 mRNA levels (Figures 1C and 1D). Similar experiments were performed with the use of a diverse pool of siRNAs against BRD4, esiRNAs. In all cases the level of HIV-1 infection correlated with the levels of BRD4 depletion, suggesting that the effect on HIV-1 was attributable to the siRNA-mediated targeting of BRD4. We did routinely observe a greater difference in p24 staining in part II as compared to part I of these assays (Figure 1D), and we believe this may reflect the limitations of the image analysis for detecting absolute levels of p24, in addition to the potential of a nonlinear relationship existing between p24 levels detected in part I and percentage of infection scored in part II. Two out of three short hairpin RNAs (shRNAs) against BRD4 also increased HIV-1 infection in a T cell line in accordance with their depletion levels (Figures 1E and 1F). Importantly, we also detected higher levels of HIV-1 infection in primary human CD4$^+$ T cells after BRD4 depletion (Figures 1G and 1H). We then tested a small molecule, JQ1, which prevents BRD4-mediated transcriptional enhancement by interfering with its binding to acetylated histones (Delmore et al., 2011; Filippakopoulos et al., 2010). Consistent with BRD4's inhibiting HIV-1 infection and in keeping with a recent report (Banerjee et al., 2012), we found that JQ1 increased the replication of HIV-1 both in HeLa cells and in a T cell line (Figures 1I, 1J, and S1A) and that JQ1's proviral effect was dependent on the presence of BRD4 (Figure S1B).

We then investigated the mechanism of BRD4's inhibition of HIV-1. On the basis of previous studies and our observed effect of JQ1 on pseudotyped viruses (Figures 1E and 1G), we reasoned that inhibition of BRD4 may potentiate proviral transcription. In support of this notion, the depletion of BRD4 specifically increased the infection of HIV-1 proviruses in comparison to its effect on a γ-retrovirus (Moloney leukemia virus, MLV; Figure 2A). BRD4's loss also stimulated a stably integrated Tat-dependent reporter gene whose expression is driven by HIV-1's LTR, in contrast to the effect seen with CMV-promoter-dependent gene expression (Figure 2B). Similarly, the BRD4 inhibitor, JQ1, preferentially increased green fluorescent protein (GFP) expression from an HIV-1 LTR when compared to an MLV LTR (Figure 2C; Delmore et al., 2011; Filippakopoulos et al., 2010). Moreover, neither AP-1- nor NF-κB-dependent reporters were activated by JQ1 (Figure S2A). JQ1's enhancement of LTR activity was predominantly seen to be both Tat and TAR

dependent, suggesting that modulation of PTEF-b levels underlies the small molecule's effects (Figures 2D, 2E, and S2B). In studies using a T cell line stably expressing an HIV-1 LTR-GFP reporter gene we observed that the area under the GFP expression curve for cells stably expressing Tat and treated with JQ1 was 3.3-fold greater than that seen with Tat and DMSO, the percentage of GFP-positive cells increasing from 39% with Tat and DMSO to 54% with Tat and JQ1. Altogether, these data suggest that JQ1 in combination with Tat resulted in more cells reactivating latent viruses and doing so with greater magnitude. However, we did note a more modest stimulation of the HIV-LTR reporter in the absence of Tat (Figures 2D and 2E), and this was found to be TAR independent as well (Figure 2E). Consistent with the assumption that JQ1 enhanced the available levels of PTEF-b, we found that JQ1 treatment increased the association of Tat with PTEF-b (Figure 2F). Notably, JQ1 improved polymerase processivity along HIV-1 proviruses and increased all lengths of viral messenger RNAs (vmRNAs) tested (initial and elongated vmRNAs), the most substantial enhancement being seen for full-length vmRNAs (Figure 2G). Given the known interactions of both BRD4 and Tat with PTEF-b (Bisgrove et al., 2007), these data suggest a model wherein BRD4 competes with Tat for the localization of a limiting amount of PTEF-b and, therefore, upon BRD4's loss and/or chemical inhibition, more PTEF-b can bind to Tat and stimulate proviral transcriptional elongation.

Since the depletion or inhibition of BRD4 results in the production of 2- to 3-fold or greater viral gene expression, this suggests that a significant amount of proviruses, 50% to 75%, are not fully active. These quiescent viruses are reminiscent of latent proviruses present in HIV-1-infected individuals on long-term ART. To explore this similarity, we investigated BRD4's contribution to viral latency. Depletion of BRD4 in a promonocyte latency model, U1 cells, strongly increased both *gag* vmRNA expression and transcriptional processivity (Figures 3A–3C; Folks et al., 1987; Pomerantz et al., 1990). Chemical inhibition of BRD4 by JQ1 also enhanced transcriptional elongation in U1 cells (Figure 3D). U1 cells are impaired for vmRNA transcription because they express Tat containing a point mutation that reduces transcriptional activation (Cannon et al., 1994; Emiliani et al., 1998). In contrast, when we decreased BRD4 in another latency cell line, ACH2, which contains a provirus with a mutant TAR, we saw more moderate increases in vmRNA production as compared to that of the U1 cells (Figure S3A;

(C) HeLa MAGI cells were transfected with the indicated siRNAs for 72 hr, then infected with HIV-IIIB (part I). Forty-eight hours later, the viral supernatant was transferred to a new plate of HeLa MAGI cells for 48 hr (part II). Part I and II cells were stained for p24. Relative fold infection is normalized to the nontargeting control siRNA (Con). Identically transfected cells were assessed for BRD4 mRNA knockdown through quantitative PCR (qPCR) and normalized to the control siRNA (right).
(D) Cells were treated as in (C) but were transfected with esiRNA pools as indicated. Values were normalized to the nontargeting firefly luciferase (FLuc) esiRNA control pool. Identically transfected cells were assessed for BRD4 mRNA knockdown through quantitative PCR (qPCR) and normalized to the control FLuc esiRNA pool (right).
(E) Jurkat T cells were stably transduced with the indicated shRNAs, then infected with VSV-G NL4-3-GFP HIV-1. At 72 hr after infection, the percentage of GFP-positive cells was determined by flow cytometry and the relative fold infection normalized to the shFLuc-expressing control line.
(F) Western blot for cells in (E). kDa, kilodaltons.
(G) Primary human CD4$^+$ T cells were stably transduced with the indicated shRNAs, then infected with VSV-G NL4-3-GFP HIV-1. At 72 hr after infection, the percentage of GFP-positive cells was determined by flow cytometry and the relative fold infection normalized to the shFLuc-expressing control line.
(H) Western blot for cells in (G). The levels of a nonspecific band (loading) show relative protein loading.
(I) HeLa MAGI cells were treated with either DMSO or JQ1 (500 nM) for 1 hr, then infected with HIV-IIIB as in (C).
(J) Jurkat T cells were treated with either DMSO or JQ1 (500 nM) for 1 hr, then infected with VSV-G NL4-3-GFP HIV-1 using the indicated multiplicity of infections (M.O.I.). After 48 hr the percentage of GFP-positive cells was determined by flow cytometry.
Values represent the mean ± SEM; n > 3 throughout; *p < 0.05, **p < 0.01. Results were analyzed by unpaired t tests. See also Figure S1.
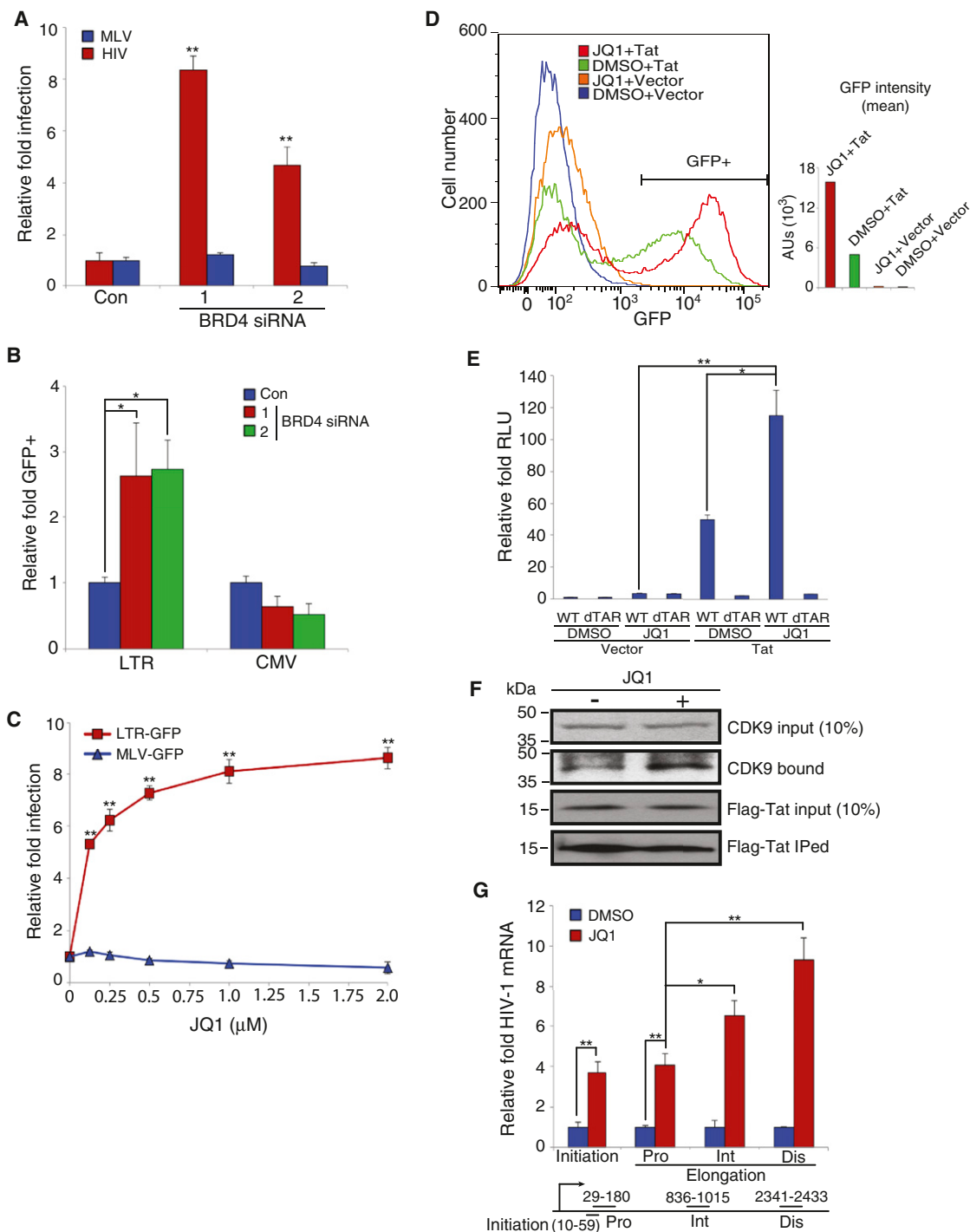
Figure 2. BRD4 Inhibition Stimulates HIV-1 Gene Expression and Increases Tat-Dependent Transcriptional Elongation and Tat-PTEF-b Association

(A) HeLa-T4 cells were transfected with the indicated siRNAs for 72 hr, then infected with either VSV-G HIV-YFP (HIV) or VSV-G MLV-GFP (MLV). After 48 hr the percentage of YFP of GFP positive cells was determined and normalized to the control siRNA.

(B) HeLa-Tat-III cells stably expressing either an LTR- or a CMV-driven d1-EGFP reporter gene were transfected with the indicated siRNAs. After 48 hr, GFP positive cells were measured and normalized to the control siRNA.

(C) HeLa-T4 cells stably transduced with a HIV-1 LTR-GFP reporter (LTR-GFP) or an MLV LTR-GFP reporter (MLV-GFP) were treated with the indicated concentrations of JQ1 for 48 hr, after which the relative fold infection was determined by the imaging of GFP-positive cells and cell nuclei (DNA stained).

(D) Jurkat T cells stably expressing an HIV-1 LTR-GFP reporter gene (LTRG cells) were transduced with gamma retroviruses expressing either Tat or the empty vector alone (vector). At 48 hr after transduction, cells were treated with DMSO or JQ1 (500 nM); 48 hr later the number of GFP expressing cells (GFP) was

Cannon et al., 1994; Emiliani et al., 1996). This is consistent with BRD4 antagonizing Tat's recruitment of PTEF-b.

Overcoming insufficient transcription is important for activating latent HIV-1 (Dahl and Palmer, 2009; Karn and Stoltzfus, 2012). Therefore, given JQ1's effect on transcriptional elongation, we evaluated its impact on latent HIV-1 when paired with compounds that boost transcriptional initiation by stimulating HDFs (HDF activators). Two such HDF activators are the natural product, Prostratin (12-deoxyphorbol 13-acetate; Gulakowski et al., 1997; Williams et al., 2004), and the cytokine, TNF-α (Duh et al., 1989; Folks et al., 1987), both of which stimulate the HDF, NF-κB, a transcription factor that is essential for HIV-1 LTR activity (Chan and Greene, 2012; Williams et al., 2004). Interestingly, when we tested JQ1 together with Prostratin in U1 cells, we discovered that this combined treatment synergistically increased latent viral gene expression >100-fold, most likely by enhancing both transcriptional initiation and elongation (Figure 3E; Barboric et al., 2001; Chan and Greene, 2012). Using several T cell J-Lat cell lines (Jordan et al., 2003), we observed increased HIV-1 LTR expression when using JQ1 and Prostratin in combination (Figures 3F and S3C). In addition, we found that stimulation of HIV-1 reporter gene expression in J-Lat T cell lines by either TNF-α or PMA was also enhanced by JQ1 (Figures 3G and 3H). It is unclear why these clonal lines do not achieve full activation under these conditions, but possible explanations include cell-to-cell variability and genetic drift. We note, however, that JQ1 alone had less effect in the J-Lat T cell lines as compared to the other cells tested, perhaps reflecting cell-line-specific activation states or requirements. In addition, when we evaluated JQ1 together with another latent HIV-1 activator, SAHA (suberoylanilide hydroxamic acid), a bifunctional cell-differentiating agent and nonspecific histone deacetylase inhibitor, we saw a modest improvement in HIV-1 gene expression in the J-Lat cell line A2 as compared to JQ1 treatment alone (Figure S3D; Archin et al., 2009; Richon et al., 1998).

On the basis of the enhanced viral activation produced by JQ1 alone or together with the HDF activators in the cell-line-latency models, we then tested these molecules in the setting of newly infected primary human T cells. Peripheral blood mononucleocytes (PBMCs) from uninfected volunteers were isolated, then either selected for CD4+ expression (donors 1–4) or depleted of CD8+ T cells (donors 5 and 6). The cells were stimulated with plate-bound CD3 and CD28 antibodies together with IL-2. Pretreatment of these activated T cells with JQ1 together with either PMA (donors 1 and 2) or Prostratin (donors 3–6) led to substantial increases in HIV-1-encoded reporter gene expression in all cases (Figure 4A). Interestingly, under these conditions, JQ1 alone had little to no effect on HIV-1 gene expression, similar to what was seen with the J-Lat T cell lines.

To explore the ability of JQ1 to both reactivate and stimulate the replication of HIV-1 within latently infected cells, we tested its effect on human CD4 T cells isolated from 19 ART-treated patients, all of whom had achieved durable viral suppression (plasma HIV RNA levels < 75 copies/ml) for a minimum of 52 weeks (Table S1). During analysis, patients had a median period of stable viral suppression of 226 weeks (interquartile range 149–390 weeks) and a median CD4+ T cell count of 698 cells/mm³ (interquartile range 595–1027). Cryopreserved PBMCs were rested for 3 days in media containing Nevaripine to prevent viral infection (600 nM), then washed and stimulated with Prostratin (250 nM), Phytohaemagglutinin (PHA, 2 μg/mL), or SAHA (500 nM) for 6–48 hr (see Experimental Procedures) in the presence or absence of JQ1 (250 nM).

Treatment with Prostratin, PHA, or SAHA resulted in detectable HIV replication in 12 of 19 patient samples (Table S2). Of these 12 samples, seven showed enhanced viral replication when JQ1 treatment was used in combination with either Prostratin or PHA (Figures 4B and 4E), with three of the seven patient samples having a negligible response to Prostratin or PHA treatment in the absence of JQ1 (donors 1, 3, and 11). Interestingly, JQ1 also inhibited the viral replication induced by either Prostratin or PHA in five patient samples in the absence of cytotoxicity (Figures 4C–4E, S4A, and S4B). Indeed, in two instances, treatment with JQ1 enhanced HIV-1 reactivation with one compound (either Prostratin or PHA) while inhibiting reactivation by the other (donors 3 and 9). A 6 hr treatment with SAHA reactivated latent HIV-1 in three samples (donors 2, 5, and 7; Table S2), but in each instance JQ1's addition suppressed SAHA's effect. In summary, we found that a heterogeneic response to latent HIV-1 reactivation occurs in T cells from different donors and that in multiple instances JQ1 could augment or synergize with Prostratin or PHA to reactivate latent viruses.

Tat-mediated transcription serves as a critical regulator of HIV-1 activation (Karn and Stoltzfus, 2012; Ott et al., 2011). Our data support the notion that PTEF-b levels are rate limiting for HIV-1's transcription in both transformed and primary cells (Isel and Karn, 1999; Kao et al., 1987). In this model, latent HIV-1 (Figure 4F, top panel) is initially reactivated by the binding

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assessed by flow cytometry. The percentage of GFP-positive cells was as follows: JQ1+Tat, 54.1%; DMSO+Tat, 39.0%; JQ1+vector, 0.335%; DMSO+vector, 0.0750%. The mean of GFP intensity was calculated for all sorted cells. Data are representative of three independent experiments.

(E) 293T cells were transfected with the indicated combinations of plasmids and treated with JQ1 (500 nM) or DMSO for 24 hr: wild-type (WT) or TAR-deleted (dTAR) HIV-1 LTR-luciferase reporter vectors, as well as a HIV-1 Tat expression vector (Tat) or the matching empty vector (vector). A dual luciferase assay was performed at 48 hr after transfection, and the relative light units (RLU) were normalized to the DMSO-treated cells transfected with the WT HIV-1 LTR-luciferase and empty vector plasmids.

(F) HeLa cells stably transduced with a FLAG-Tat were incubated with JQ1 (500 nM) or DMSO for 24 hr. Cells were then lysed and subjected to immunoprecipitation using anti-FLAG antibodies. An anti-CDK9 antibody was used to detect the endogenous CDK9 levels. An anti-FLAG antibody was used to detect the FLAG-Tat level.

(G) Jurkat T cells were treated with JQ1 (500 nM) or DMSO for 1 hr, then infected with VSV-G NL43-GFP HIV-1. After 48 hr cDNA was prepared and vmRNA length assessed with the use of the indicated primers. Initiation: 10–59 bp. Elongation: proximal (Pro, 29–180 bp), intermediate (Int, 836–1015 bp), and distal (Dis, 2341–2433 bp), numbers denoting the amplification region of the NL4-3 HIV-1 transcript as determined from the transcriptional start site. Data were normalized to the DMSO control.

Values represent the mean ± SEM; n > 3 throughout; *p < 0.05, **p < 0.01. Results were analyzed by unpaired t tests. See also Figures S1 and S2.
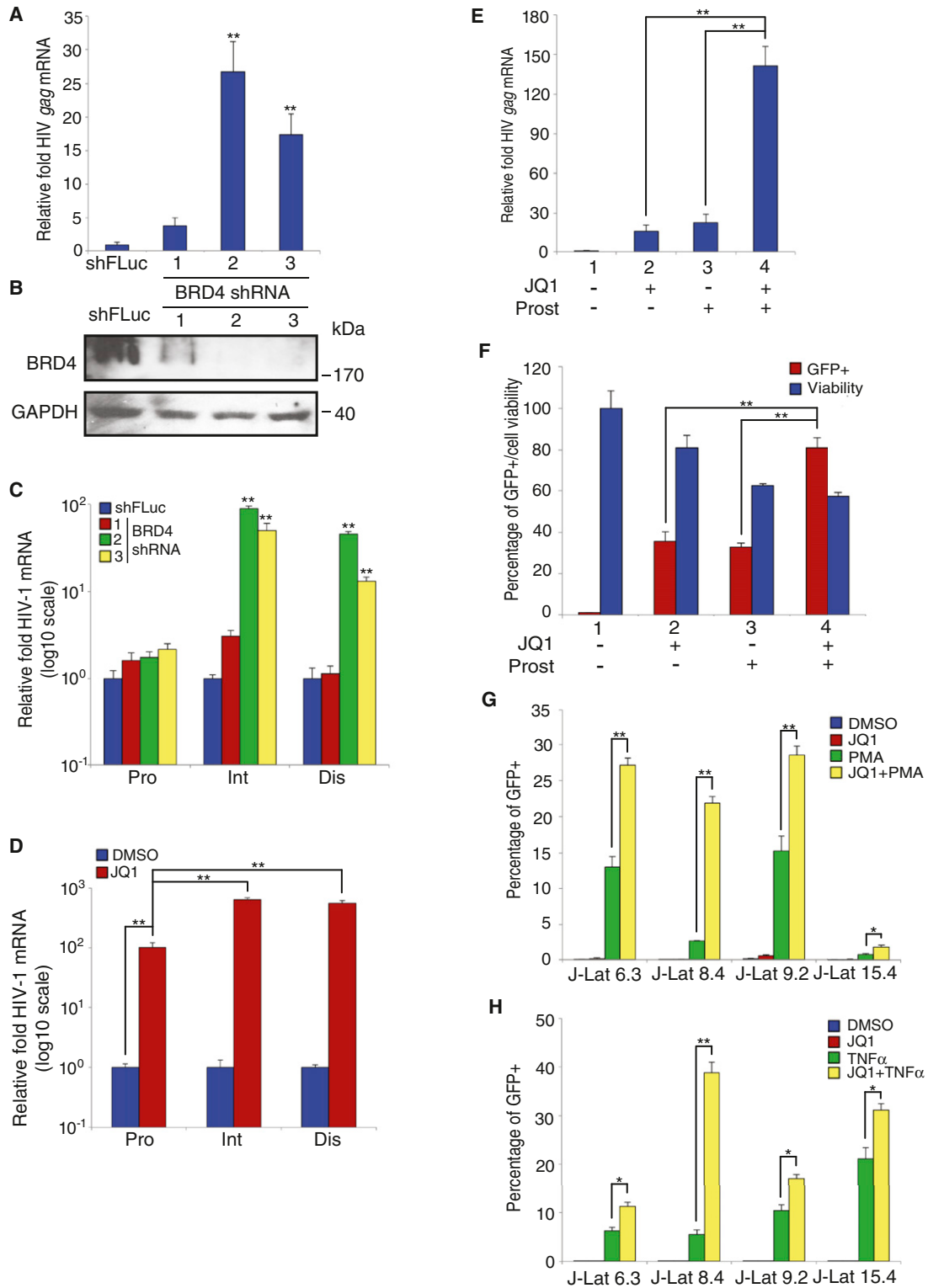
Figure 3. BRD4 Inhibition Alleviates HIV-1 Latency

(A) U1 cells were stably transduced with the indicated shRNAs, then HIV-1 gag mRNA levels were measured by qPCR and normalized to the shFLuc control.

(B) Western blot of cells in (A).

(C) A transcriptional elongation assay was performed as above using cells in (A). The level of transcripts was normalized to the shFLuc control. Note log scale on the y axis.

of transcription factors (i.e., NF-κB) to the LTR (middle panel). This early event produces sufficient Tat to recruit the limited amount of free PTEF-b, the majority of which is sequestered on chromatin by BRD4. Upon inhibition or depletion of BRD4, additional PTEF-b is released and can interact with Tat, thereby producing maximal transcription (stimulated reactivation, bottom panel). This PTEF-b-dependent stimulation is most significant when BRD4 is inhibited under conditions of high level LTR activity (Prostratin, PMA, PHA, or TNF-α), which increases transcriptional initiation and elevated Tat levels. On the basis of the increase in viral replication that occurs when NF-κB is activated and BRD4 is inhibited, our data demonstrate that in some instances the pairing of JQ1 with HDF-activators more effectively alleviates latency.

DISCUSSION

Beginning with orthologous large-scale genetic screening for host factors that modulate HIV-1 replication in vitro, we discovered that depletion of BRD4 increased viral replication in transformed cells and enhanced HIV infection in primary human CD4^{+} T cells. This work significantly extends elegant previous studies demonstrating that BRD4 modulates HIV-1 infection (Bisgrove et al., 2007; Urano et al., 2008). Indeed, we now show that BRD4's depletion or inhibition stimulated viral gene expression from both newly integrated and latent proviruses. Furthermore, we revealed that BRD4's loss enhanced HIV-1 gene expression by increasing Tat/PTEF-b association and host polymerase transcriptional elongation.

We found that in multiple instances a small molecule inhibitor of BRD4, JQ1, when used in combination with the transcriptional activators Prostratin or PHA, could trigger and/or markedly enhance the in vitro reactivation of latent HIV-1 in primary human T cells, thereby identifying a new first-in-class antilatency small molecule. While this manuscript was in preparation, Banerjee et al. also reported that JQ1 alone could stimulate HIV-1 replication in a T cell line and in the primary T cells from one of three patients receiving ART (Banerjee et al., 2012). Our study now significantly extends those observations by providing mechanistic insight into how BRD4, and thus JQ1, affect the HIV-1 life cycle. Importantly, we also describe how using JQ1 in combination with compounds known to alleviate viral latency, Prostratin or PHA, greatly increased reactivation of HIV-1 in latently infected primary T cells from 7 of 19 patients on long-term ART. Of note, our work shows that JQ1 alone does not reactivate HIV-1 in latently infected primary T cells, which differs from the single example of modest viral reactivation by JQ1

alone that was recently reported (Banerjee et al., 2012). Our data instead reveal that JQ1 potentiates the actions of several known transcriptional HIV-1 reactivating compounds, PMA, PHA, TNF-α, and Prostratin. Therefore, in comparison, our study demonstrates an enhanced potential for the use of agents that improve transcriptional elongation (e.g., JQ1) in reactivating latent HIV-1 reservoirs, via their pairing with small molecules that mobilize LTR-enhancing HDFs (e.g., NF-κB). Furthermore, by using such combinations to create synergies, it may be possible to improve the efficacy and minimize the toxicity of antilatency therapy (Choudhary and Margolis, 2011; Siliciano and Greene, 2011). A common mechanism of HIV-LTR activation shared by these compounds is the stimulation of NF-κB. In certain instances NF-κB's activity is dependent on interaction with BRD4 (Sharma et al., 2007). However Huang et al. determined that NF-κB transcriptionally activates a subset of genes in a BRD4-independent manner, and our data are consistent with the HIV-LTR being one such example (Huang et al., 2009).

In contrast to data generated through the use of several transformed cell lines or newly infected primary T cells, we observed a marked heterogeneity in the ability of the tested compounds to reactivate latent HIV-1 in primary T cells from patients on long-term ART. We also noted that the addition of JQ1 along with these HDF-activating compounds could either promote or inhibit viral reactivation. While the dichotomous effects of JQ1 are likely attributable to many factors, insight may come from earlier work showing that BRD4 is needed for basal HIV-1 LTR transcription, suggesting that interfering with this early action of BRD4 may prevent further increases in replication (Ott et al., 2011); additional possibilities include cell-lineage-dependent variations of BRD4 or PTEF-b expression or availability. Altogether, these results suggest that it will be useful to carry out personalized in vitro testing to identify efficacious combinations of HIV-1 reactivators, including JQ1.

Our work also demonstrates that in addition to HDFs that are needed by HIV-1 (Brass et al., 2008) and HIV-1 restriction factors (HRFs) that have evolved to specifically inhibit the virus (Yan and Chen, 2012), there exists a third functional class of host factors, HIV-1 competitive factors (HCFs); i.e., BRD4. Such factors have not been selected as being antiviral per se, but they can nonetheless blunt infections and promote latency by competing with HIV-1 for limiting cellular resources; e.g., PTEF-b. It follows, then, that antilatency strategies might be improved by combining HDF-activating therapies together with small molecules that transiently inactivate specific HRFs and/or HCFs. Because HIV-1 latency exists via multiple mechanisms, we deem it likely

(D) A transcriptional elongation assay was performed as above using U1 cells treated with either JQ1 (500 nM) or DMSO for 48 hr. The level of transcripts was normalized to the values of the DMSO-treated controls. Note log scale on the *y* axis.

(E) U1 cells were treated with either JQ1 (500 nM) or Prostratin (1 uM) alone, or the combination. After 72 hr cDNA was prepared and levels of gag mRNA determined.

(F) J-Lat A2 cells were treated with JQ1 (500 nM) or Prostratin (1 μM) for 72 hr. Cells were then assessed for GFP expression and viability. For the cell viability assays the relative luminescence units (RLU) of DMSO treated cells was set at 100%.

(G) The indicated J-Lat cell lines were treated with JQ1 (500 nM), PMA (200 nM), or the both for 72 hr. GFP positive cells were assessed using flow cytometry, and the percentage of positive cell was calculated.

(H) The indicated J-Lat cell lines were treated with the noted compounds (JQ1 (500 nM), TNF-α (10 ng/ml) alone or in combination. At 72 hr the percentage of GFP positive cells was determined by flow cytometry.

Values represent the mean +/− S.E.M., N > 3 throughout. *p < 0.05, **p < 0.01. Results were analyzed by unpaired t tests. See also Figure S3.
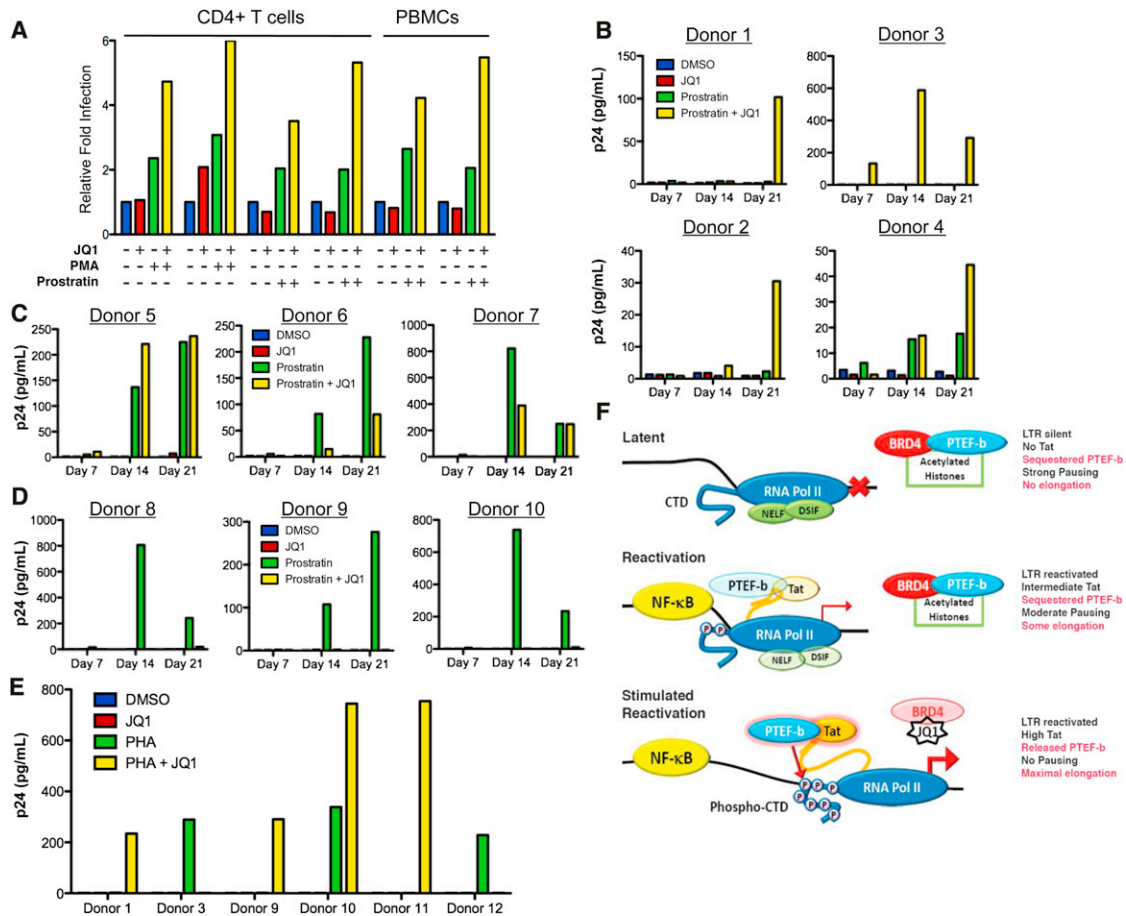
Figure 4. BRD4 Inhibition in Combination with HIV-1 HDF Activators Enhances HIV-1 Replication in Primary Human T Cells, as well as HIV-1 Reactivation in Latently Infected T Cells from Patients on Long-Term ART

(A) Primary CD4+ T cells or PBMCs were isolated from HIV-1-negative donors and treated with the indicated compounds (JQ1, 500 nM; PMA, 200 nM; or Prostratin, 1 μM) alone or with JQ1 and Prostratin combined for 1 hr, then infected with VSV-G NL4-3-GFP HIV-1. After 72 hr the number of GFP expressing cells was determined by flow cytometry and normalized to the DMSO control.

(B–D) CD8-depleted PBMCs isolated from ART-treated patients were rested for 3 days in the presence of Nevaripine (600 nM), followed by reactivation with DMSO or Prostratin (250 nM) for 48 hr in the presence or absence of JQ1 (250 nM). Viral replication was assessed by p24 ELISA on days 7, 14, and 21. Donors were numbered according to whether JQ1 had a beneficial effect on Prostratin reactivation (B) or a nonbeneficial or inhibitory effect (C and D).

(E) Viral replication was assessed as described above, but reactivation was assessed with DMSO or PHA (2 μg/mL) in the presence or absence of JQ1 (250 nM) for 48 hr.

(F) Model of BRD4 inhibition reactivating latent HIV-1 transcription. Latent HIV-1 (top panel) is initially reactivated by the binding of transcription factors (e.g., NF-κB) to the LTR, resulting in the production of sufficient Tat to modestly compete for some of the PTEF-b complexed with chromatin-associated BRD4 (acetylated histones) or 7SK-RNP (reactivation, middle panel). Upon antagonism of BRD4 by chemical inhibition with either JQ1 or RNAi, additional PTEF-b is made available from the chromatin-sequestered pool, thus lowering the barrier for interaction with Tat and producing maximal proviral transcription (stimulated reactivation, bottom panel). Enhanced viral transcription leads to the amplification of the feed-forward regulatory loop producing higher levels of Tat that in turn recruit the now more readily available PTEF-b (denoted as more intensely colored Tat and PTEF-b components). RNA pol II, RNA polymerase II; CTD = carboxy terminal domain of RNApol; AC, histone acetylation; NELF and DSIF, negative elongation factors removed after phosphorylation by PTEF-b.

See Figure S4 and Tables S1 and S2.

that continuing to improve and integrate our basic understanding of how host proteins modulate latency will provide additional insights and, in doing so, help guide the way to a cure.

EXPERIMENTAL PROCEDURES

Small Molecules

Prostratin and SAHA were from Santa Cruz (sc-203422, sc-220139). and PMA was from Sigma (P1585). Compounds were used at optimized concen-

trations: JQ1 (500 nM), Prostratin (1 μM), PMA (200 nM), SAHA (0.5 μM). See Extended Experimental Procedures for more details.

Genetic Screening for HIV-1 Modulators Using Orthologous RNAi Libraries

To identify host factors that modulate HIV-1 replication, we performed RNAi screens by using two genome-wide siRNA resources (Silencer Select, Ambion; Library 2, Mission esiRNAs, Sigma) and following a previously described protocol (Brass et al., 2008).

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EXTENDED EXPERIMENTAL PROCEDURES

Cell Lines, Viruses, Small Molecules, and antibodies

HeLa MAGI and HeLa-T4 cells were generously provided by the NIH AIDS reagent repository. The following cells were also generously provided by the NIH AIDS reagent program: HeLa-Tat-III/CMV/d1EGFP (Catalog # 11062; Parent et al., 2005), HeLa-Tat-III/LTR/d1EGFP (Catalog # 11063, (Parent et al., 2005)), Jurkat Clone E6-1 (Catalog # 177; Weiss et al., 1984), U1/HIV-1 (Catalog # 165; Folks et al., 1987), JLTRG reporter cells (Catalog # 11587, from Dr. Olaf Kutsch, University of Alabama, U.S.A.), J-Lat Tat-GFP Clone A2 (Catalog # 9854, (Jordan et al., 2003)), J-Lat full length clones 6.3, 8.4, 9.2 and 15.4 (Catalog # 9846, 9847, 9848, 9850, (Jordan et al., 2003)).

HIV-1 strains were generously provided by the NIH AIDS Repository: HIV-1 IIIB infectious strain (Brass et al., 2008) and NL4-3 (Pertel et al., 2011). VSV-G pseudotyped viruses were created by co-transfecting pCG-VSV-G and pCG-Gag-Pol vectors (Feeley et al., 2011) using the viral constructs (HIV-1-YFP, HIV-1 LTR-GFP [LTR-GFP], and MLV-GFP; Brass et al., 2008; Valle-Casuso et al., 2012).

Prostratin and Suberoylanilide Hydroxamic Acid (SAHA) were purchased from Santa Cruz (sc-203422, sc-220139). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (P1585). Compounds were used alone or paired using optimized concentration: JQ1 (500 nM), Prostratin (1 μM), PMA (200 nM), SAHA (0.5 μM). For HeLa and Jurkat cell lines, cells were pre-incubated with compounds for 1 hr before viral infection. Cells were cultured in the presence of compounds for another 48 hr before analysis. For J-Lat cell lines, cells were cultured for 72 hr in the presence of compounds before analysis. Recombinant Human Tumor Necrosis Factor α (TNFα) was purchased from Life Technologies (PHC3015).

Primary antibody, anti-HIV-1 p24 mouse monoclonal antibody mab-183 (NIH AIDS and Reagent Repository), and secondary antibody, goat anti-mouse Alex-Flour 488 (Invitrogen), for the HIV-1 modulator screens, were used for immunofluorescence detections of HIV-1 infection following a published protocol (Brass et al., 2008). A BRD4 polyclonal antibody was used for immunoblots (A301-985A, Bethyl Laboratories). GAPDH and CDK9 antibodies were purchased from Santa Cruz.

Plasmids

pQCXIP-Flag-Tat was constructed by subcloning C terminal Flag tag fused HIV-1 Tat into the pQCXIP retroviral vector (Clontech) using NotI and BamHI sites. Pseudoviruses were packed using pCG-VSV-G and pCMV-Gag-Pol vectors.

Genetic Screening for HIV-1 Modulators Using Orthologous RNAi Libraries

To identify host factors which modulate HIV-1 replication, we performed RNAi screens using two genome-wide siRNA resources (Silencer Select siRNAs, Ambion; Library 2, Mission esiRNAs, Sigma) following a previous established protocol (Brass et al., 2008). Briefly HeLa MAGI cells (part I) were reverse transfected with 50 nM final concentration siRNA using Oligofectamine (Invitrogen). Seventy two hours later the cells were infected with the infectious lab strain HIV-IIIB (NIH AIDS reagent repository). 48 hr later the cellular supernatant was removed and used to infect a fresh plate of HeLa MAGI cell (part II) of an additional 48 hr. In both parts I and II the cells were fixed (4% paraformaldehyde (Sigma) in D-PBS (Sigma), permeabilized (0.2% Triton X-100 in D-PBS) and stained for HIV-1 p24 expression using the anti-p24 antibody mab-183 (NIH AIDS and Reagent Repository, primary antibody) and goat anti-mouse Alexa-Flour 488 (Invitrogen, secondary antibody). Cellular DNA was stained using Hoechst 33342 (Invitrogen). Immunostained cells were imaged using an Image Xpress Micro microscope (Molecular Devices) and the images analyzed to determine percent infection and cell number (cell scoring module, Metamorph, Molecular Devices).

siRNAs and shRNAs

BRD4 siRNAs and shRNAs. All siRNAs were used at 50 nM final concentration using Oligofectamine transfection lipid (Invitrogen). Three unique BRD4 siRNAs (No. 1-3) were purchased from Ambion (s23901, s23902, s23903). The sequences targeted by these siRNAs are s23901: 5′- AGA TTG AAA TCG ACT TTG A −3′; s23902: 5′- CCT GAT TAC TAT AAG ATC A −3′; s23903: 5′- TGA GCA CAA TCA AGT CTA A −3′. The control siRNA is a pool of Silencer Negative siRNAs (No.1-4, AM4611, AM4613, Am4615 and AM4641, Ambion). FLuc and BRD4 esiRNAs were purchased from Sigma. Three unique BRD4 shRNAs (No. 1-3) were subcloned into the pAPM shRNA expression vector. pAPM is a microRNA (mir30)-based shRNA lentiviral vector (LV) where both the puromycin N-acetyltransferase cDNA and the shRNA are expressed via the spleen focus-forming virus (SFFV) promoter (Pertel et al., 2011). shRNAs targeting BRD4 were amplified by PCR from pGIPZ constructs (Open Biosystems) and cloned into the XhoI and EcoRI sites in pAPM as described previously (Pertel et al., 2011). Production of pAPM shRNA lentiviruses has been described previously (Bernasconi et al., 2010). The targeted sequences of BRD4 shRNAs: Sh1. 5′- ACA CTA TGT TTA CAA ATT GTT A −3′; Sh2. 5′- ACC CCG ACG AGA TTG AAA TCG A −3′, Sh3. 5′- CCA GGA CTT CAA CAC TAT GTT T −3′. FLuc. 5′- TAC AAA CGC TCT CAT CGA CAA G −3′. For experiments involving the transfer of viral supernatant (Figure 1c, d) the recipient cells used in "part two" were not treated with siRNAs.

Quantitative PCR

The Taqman primers and probe were designed and synthesized to measure BRD4 and GAPDH mRNA levels (IDT). BRD4 forward, 5′-AGG CAA AAG GAA GAG GAC G-3′; BRD4 reverse, 5′-CGA TGC TTG AGT TGT GTT TGG-3′; BRD4 probe, 5′-/56-FAM/ACA GCA AAA /ZEN/CCT GGC GTT CCC AC/3IABkFQ/-3′. GAPDH forward, 5′- ACA TCG CTC AGA CAC CAT G −3′; GAPDH reverse, 5′-TGT AGT TGA GGT CAATGA AGG G-3′; GAPDH probe, 5′-/56-FAM/AAG GTC GGA /ZEN/GTC AAC GGATTT GGT C/3IABkFQ/-3′. SYBR

Green Primers were designed to detect HIV gag and GAPDH mRNAs. Gag forward, 5′- GAC GCT CTC GCA CCC ATC TC −3′; Gag reverse, 5′- CTG AAG CGC GCA CGG CAA −3′. GAPDH forward, 5′- GCC TCT TGT CTC TTA GAT TTG GTC −3′; GAPDH reverse, 5′- TAG CAC TCA CCA TGT AGT TGA GGT −3′. qPCR for RNA processivity assay followed the previously published protocol (Chen et al., 2011). Proximal primers targeted base pairs (bp) 29-180 of the HIV-1 transcript. Forward, 5′- TGG GAG CTC TCT GGC TAA CT −3′; Reverse, 5′- TGC TAG AGA TTT TCC ACA CTG A −3′. Intermediate primers targeted bp 836-1015 of the HIV-1 transcript. Forward, 5′- GTA ATA CCC ATG TTT TCA GCA TTA TC −3′; Reverse, 5′- TCT GGC CTG GTG CAA TAG G −3′. Distal primers targeted bp 2341-2433 of HIV-1 transcript. Forward, 5′- GAG AAC TCA AGA TTT CTG GGA AG −3′; Reverse, 5′- AAA ATA TGC ATC GCC CAC AT −3′. Initial primers targeted bp 10-59 of the HIV-1 transcript. Forward, 5′- GTT AGA CCA GAT CTG AGC CT −3′; Reverse, 5′- GTG GGT TCC CTA GTT AGC CA −3′. Eluted mRNA samples (1 ug) were reverse transcribed using the random hexamers (0.1 uM) and SuperScript® III Reverse Transcriptase according to the manufacturer (Invitrogen). The primer pair aforementioned (0.1 uM each) was used to measure its mRNA level with either TaqMan® Fast Universal PCR Master Mix or Platinum® SYBR® Green qPCR SuperMix (Invitrogen) on a 7500 Fast PCR-System (Applied Biosystems). The relative input cDNA amount of GAPDH was used to normalize transcript levels. The following thermal cycles were used for qPCR: 95°C, 1 min/ 95°C, 5 s; 60°C, 30 s; 40 cycles.

Luciferase Assay
Luciferase assays were performed as described previously (Pertel et al., 2011). Briefly, 293T cells were transfected with the indicated firefly luciferase reporters, along with pRL-TK (Promega) as an internal transfection control. The cells were processed using the Dual-Glo Luciferase Assay System (Promega) and the plates were read using a Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific). The NF-κB and AP-1 luciferase reporters are described elsewhere (Pertel et al., 2011). The HIV-1 LTR luciferase reporter construct was obtained from the AIDS Research and Reference Reagent Program. The TAR deleted (dTAR) LTR luciferase reporter construct was a kind gift from Dr. Andrew Badley (Mayo Clinic). The HIV-1 Tat expression plasmid was a kind gift from Dr. Richard Mulligan (Harvard Medical School).

Cell-Viability Assay
Cell viability upon drug treatment was measured using CellTiter-Glo® Assay (Promega) based on quantitation of ATP level according to the manufacture's instruction.

Study Subjects
HIV-infected adults and HIV-seronegative controls were recruited from outpatient clinics at Massachusetts General Hospital and affiliated Boston area hospitals. The respective institutional review boards approved this study, and all subjects gave written consent.

Primary T Cell Experiments; Newly Infected Cells
PBMCs were isolated from buffy coats prepared from healthy donors using Ficoll-Paque Plus (GE Healthcare) following the protocol supplied by Miltenyi Biotec. CD4+ T cells were enriched from PBMC by positive selection using CD4 MicroBeads (Miltenyi Biotec) with purity routinely greater than 95%, as determined by flow cytometry after staining with allophycocyanin-conjugated anti-human CD4 (clone RPA-T4) (BD Biosciences). Primary human CD4+ T cells, or total PBMC, were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% FBS (Invitrogen), 4 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1 × MEM Non-Essential Amino Acids Solution (Invitrogen), 20 mM HEPES (Invitrogen), and 50 IU/mL recombinant, human IL-2 (Roche). Primary human CD4+ T cells or total PBMC were rested for 3d after purification then challenged with serial dilutions of VSV-G-pseudotyped pNL4-3 with an env-inactivating mutation and EGFP in place of nef (Pertel et al., 2011). Seventy two hours after challenge, the T cells were assayed for EGFP expression by flow cytometry. We routinely achieve greater than 10% infection with over 10,000 events captured. Infectivity data are represented as the average fold change in infection compared to the control from multiple M.O.I.s.

Primary T Cell Experiments Isolated from ART-Treated Patients; Latently Infected Cells
PBMCs were isolated from ART-treated patients, who had undetectable viremia for at least 52 weeks, as described above. The cells were rested in RPMI-1640 (Sigma-Aldrich) containing 10% FBS (Invitrogen), 4 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1 × MEM Non-Essential Amino Acids Solution (Invitrogen), 20 mM HEPES (Invitrogen), and 600 nM Nevaripine (Sigma-Aldrich) for 3 d. PBMCs were then washed once, and depleted of CD8+ T cells by negative selection using CD8 MicroBeads (Miltenyi Biotec). CD8-depleted PBMCs were plated at 2×10^6 cells/well in a 24-well plate (BD Falcon) and stimulated by Prostratin (250 nM), PHA (2 μg/mL) or SAHA (500 nM), in the presence or absence of JQ1 (250 nM). The T cells were split equivalently as needed to maintain appropriate cell numbers that would not lead to cell stress or death from overcrowding. Incubation with Prostratin or PHA was 48 hr, while incubation with SAHA was limited to 6 hr, prior to washing and resuspension in media containing 10 IU/mL recombinant, human IL-2 (Roche). Cells were cultured and viral replication was assessed by p24 ELISA (Perkin-Elmer) according to the manufacturer's instructions on day 7, 14, and 21.

Viability Staining of CD4+ T Cells
Primary CD4+ T cells from ART-treated patients were washed in 1mL sterile 1x PBS (Sigma) and incubated in 200 ul of 1x LIVE/DEAD violet viability dye for 15 min at 4°C. Cells were then washed and fixed in 4% paraformaldehyde and viability was assessed by flow cytometry using an LSR II (BD Biosciences). Data analysis was performed with the FlowJo software package (v.8.6, Treestar).

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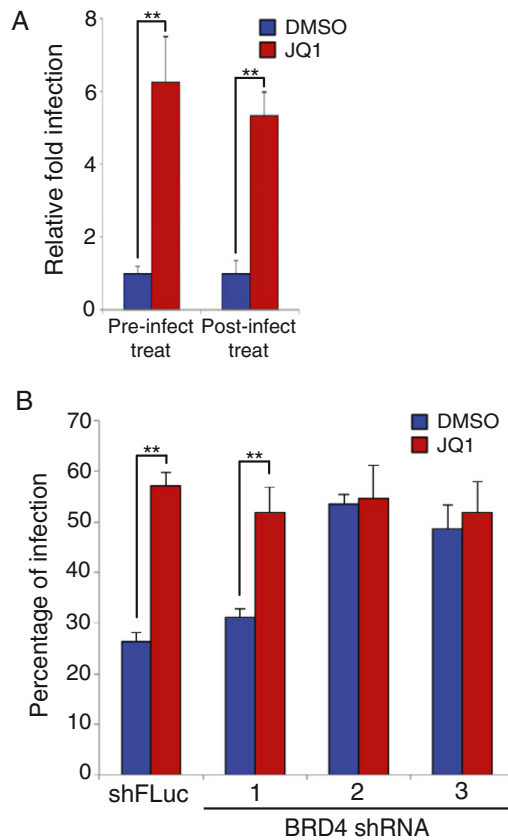
Figure S1. BRD4 Depletion Abolishes the JQ1 Effect on HIV Infection in Jurkat T Cells, Related to Figures 1 and 2

(A) HeLa-T4 cells were treated with JQ1 (500 nM) or DMSO either 24 hr before or 96 hr post infection of VSV-G NL4-3-GFP. After 48 hr of infection the percentage of GFP positive cells was determined and normalized to the DMSO group.

(B) Jurkat T cells stably transduced with the indicated shRNAs were incubated with JQ1 (500 nM) or DMSO for 24 hr and then infected with VSV-G NL4-3-GFP HIV-1. At 48 hr post infection the percentage of GFP positive cells was determined by flow cytometry.

Values represent the mean +/− S.E.M., N = 3. **p < 0.01. Results were analyzed by unpaired t tests.
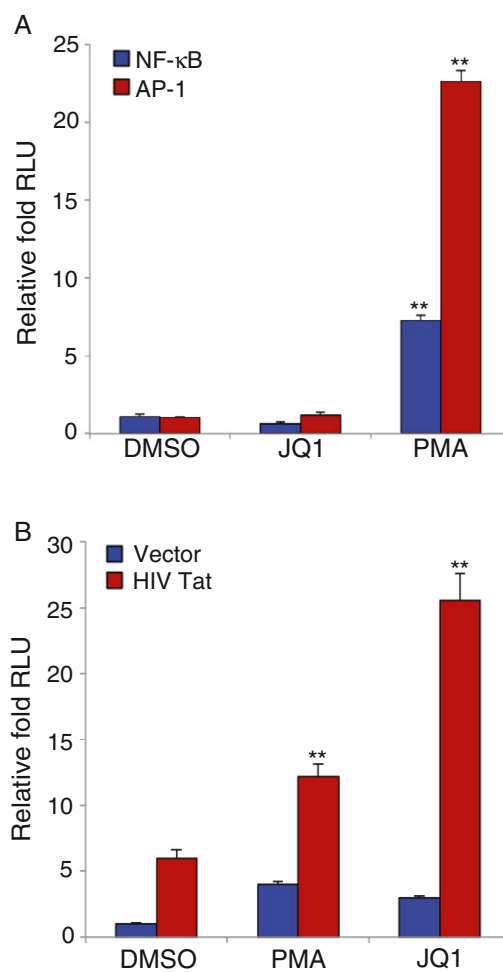
Figure S2. JQ1 Does Not Stimulate NF-κB or AP-1 Reporter Activity, Related to Figure 2

(A) 293T cells were transfected with either an NF-κB or AP-1 luciferase reporter. 24 hr after transfection the cells were treated with JQ1 (500 nM), PMA (100 nM), or DMSO for 20 hr and assayed for luciferase expression.

(B) 293T cells were transfected with an HIV-1 LTR luciferase reporter, along with either a Tat expression plasmid or an empty vector control. 24 hr after transfection, the cells were treated with JQ1 (500 nM), PMA (100 nM), or DMSO for 20 hr and assayed for luciferase expression.

Values represent the mean +/− S.E.M., N > 3 throughout. **p < 0.01. Results were analyzed by unpaired t tests.
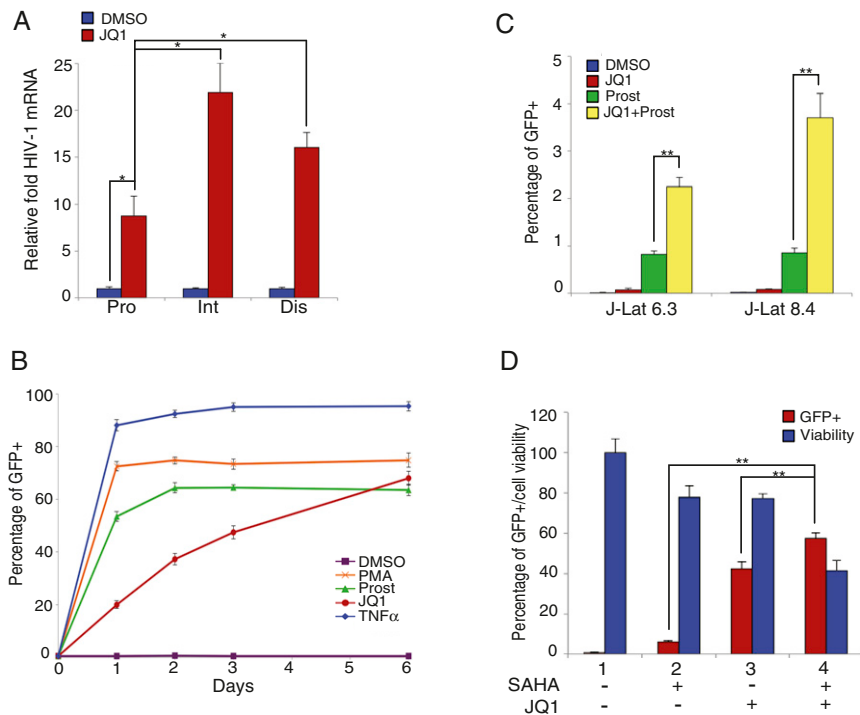
Figure S3. Effect of JQ1 Treatment on HIV-1 Reporter Gene Activity in Combination with Other HIV-1 Reactivating Compounds, Related to Figure 3

(A) Transcriptional elongation assays were performed using JQ1-treated ACH2 cells. mRNA was purified from these cells and subjected to reverse transcription. The primer sets targeting the proximal, intermediate and distal sites from the HIV-1 transcriptional initiation site were used to measure the relative level of local transcripts. The level of transcripts from DMSO-treated cells was set as 1.

(B) J-Lat A2 cells were incubated with the indicated compounds (PMA (200 nM), Prostratin (1 μM), JQ1 (500 nM) and TNF-α (10 ng/ml)) for 24 hr with the percentage of GFP positive cells determined by flow cytometry at the indicated days (0,1,2,3,6).

(C) The indicated J-Lat cell lines were treated with indicated compounds (JQ1 (500 nM), Prostratin (1 μM) alone or in combination. At 72 hr the percentage of GFP positive cells was determined by flow cytometry.

(D) J-Lat A2 cells were pre-treated with the indicated compounds (JQ1 (500 nM), 24 hr; SAHA (1.25 μM), 8 hr) alone or in combination. At 72 hr post-treatment the percentage of GFP positive cells was determined using flow cytometry. Cell viability was also measured with the relative luminescence units (RLU) of DMSO treated cells set at 100%.

Values represent the mean +/− S.E.M., N > 3 throughout. *p < 0.05, **p < 0.01. Results were analyzed by unpaired t tests.
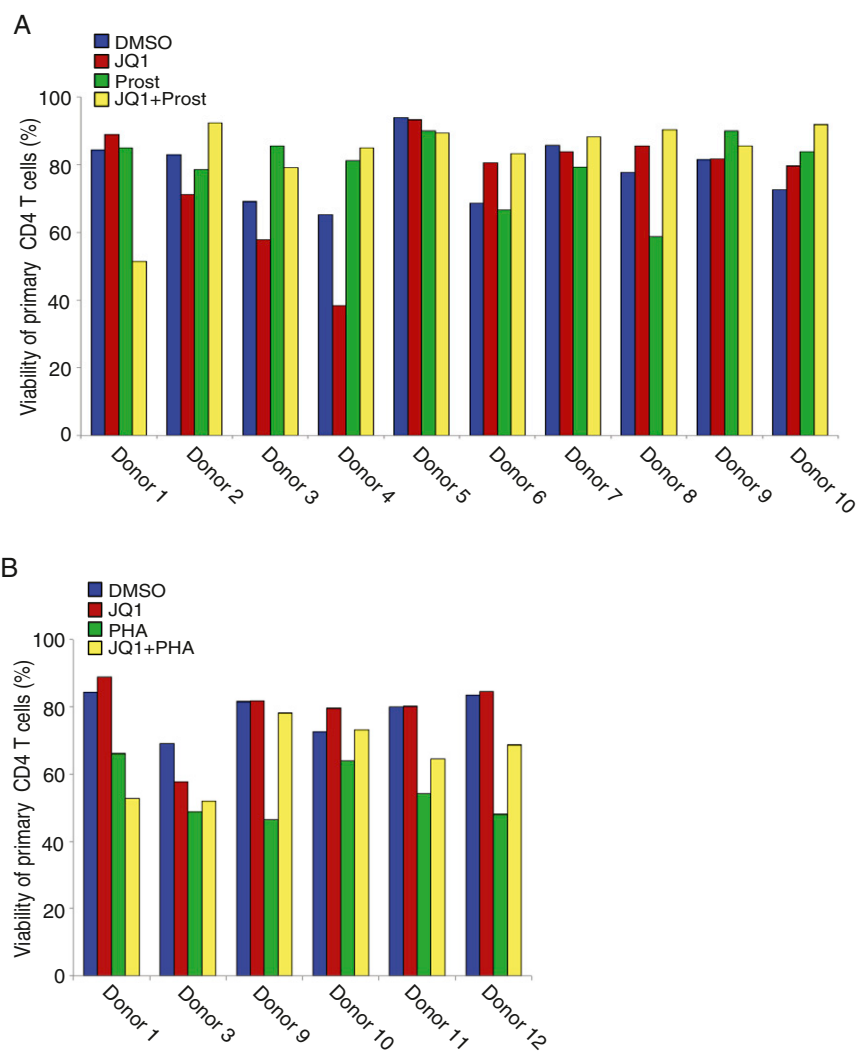
Figure S4. Treatment with JQ1, in Combination with Prostratin and PHA, Does Not Significantly Affect CD4$^+$ T Cell Viability, Related to Figure 4

Primary CD4$^+$ T cells from the 10 HAART-treated patients responsive to Prostratin (a) and the 6 patients responsive to PHA (b) were assessed for viability at Day 21 using the LIVE/DEAD Violet Viability/Vitality kit (Invitrogen). JQ1 treatment has no significantly adverse effect on the viability of primary CD4$^+$ T cells alone or in combination with activating agents.