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The BET bromodomain inhibitor JQ1 activates HIV latency through antagonizing Brd4 inhibition of Tat-transactivation

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ABSTRACT

Latent HIV reservoirs are the primary hurdle to eradication of infection. Identification of agents, pathways and molecular mechanisms that activate latent provirus may, in the presence of highly active antiretroviral therapy, permit clearance of infected cells by the immune system. Promoter-proximal pausing of RNA polymerase (Pol) II is a major rate-limiting step in HIV gene expression. The viral Tat protein recruits human Super Elongation Complex (SEC) to paused Pol II to overcome this limitation. Here, we identify the bromodomain protein Brd4 and its inhibition of Tat-transactivation as a major impediment to latency reactivation. Brd4 competitively blocks the Tat–SEC interaction on HIV promoter. The BET bromodomain inhibitor JQ1 dissociates Brd4 from the HIV promoter to allow Tat recruitment of SEC to stimulate HIV elongation. JQ1 synergizes with another latency activator prostratin, which promotes Pol II loading onto the viral promoter. Because JQ1 activates viral latency without inducing global T cell activation, this and other closely related compounds and their antagonization of Brd4 to promote Tat–SEC interaction merit further investigations as effective agents/strategies for eliminating latent HIV.

INTRODUCTION

Latent reservoirs of HIV are the primary hurdle to eradication of infection. The infected resting memory T cells, the best-characterized reservoir thus far, harbor integrated and transcriptionally silent proviruses that can evade host immune surveillance and resume active infection once the Highly Active Anti-Retroviral Therapy is interrupted. Currently, extensive efforts are being directed toward the development of effective strategies for eliminating these reservoirs. One strategy, nicknamed 'shock and kill', has recently gained much attention [reviewed in (1,2)]. It uses a 'shock' phase to reactivate latent proviruses and then the 'kill' phase to stop the spread of activated HIV by the Highly Active Anti-Retroviral Therapy and also eliminate the HIV-producing cells by immune responses and viral cytopathogenicity.

In devising the 'shock and kill' strategy, focus has been placed on finding ways to reactivate latent HIV without causing global T cell activation. To this end, a number of small molecule activators have been shown to stimulate HIV transcription in latently infected cells (1,2). However, these compounds are toxic, mutagenic (positive in Ames tests) or ineffective in trials involving enlarged sample size and prolonged treatment (3–5). Thus, better and more specific latency activators are urgently needed, which can only be achieved through the identification of the most relevant molecular mechanism and factors that contribute to viral latency.

Promoter-proximal pausing of initiated RNA polymerase II (Pol II) on integrated HIV proviral DNA has long been recognized as a major rate-limiting step in viral gene expression (6,7). To overcome this restriction, the HIV-encoded Tat protein recruits the human Super Elongation Complex (SEC) to paused Pol II through forming a multi-component complex that also contains the TAR RNA, a stem-loop structure located at the 5′ end of all nascent HIV transcripts (7–10). SEC contains two powerful transcription elongation factors P-TEFb and ELL2 that act by different mechanisms. Consisting CDK9 and cyclin T1, P-TEFb phosphorylates two negative elongation factors NELF and DSIF to antagonize their inhibitory actions and also Ser2 within the heptapeptide repeats that constitute the Pol II carboxy-terminal domain (CTD). ELL2 directly stimulates the processivity of Pol II through suppressing its transient pausing. Once recruited as part of

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SEC to the viral promoter by Tat and TAR, P-TEFb and ELL2 synergistically activate Pol II elongation to generate full-length HIV transcripts (7–10).

Besides HIV, many cellular genes especially those that control growth and development also use promoter-proximal pausing of Pol II and its release as a major mechanism regulating their expression [reviewed in (10)]. However, unlike HIV that relies on Tat to deliver P-TEFb/SEC to the paused Pol II, many cellular primary response genes use the bromodomain protein Brd4 to recruit P-TEFb to their promoters through the interactions of the Brd4 bromodomains with acetylated histones H3 and H4 (11–14). Interestingly, although the Brd4-P-TEFb interaction is required for expression of many cellular genes, it is inhibitory to Tat activation of HIV transcription, as Brd4 directly competes with Tat for binding to P-TEFb (11,15).

Recently, JQ1, a small-molecule inhibitor of BET bromodomains, has received much attention for its therapeutic potential against multiple myeloma and other cancer types that are addicted to the *c-Myc* oncogene (16–18). Displaying the highest affinity for the first bromodomain of Brd4 (19), JQ1 displaces Brd4 from acetylated chromatin thereby inhibiting transcription of *c-Myc* and other Brd4-dependent genes and inducing differentiation and growth arrest of cancer cells.

Given the intriguing role of the Brd4-P-TEFb interaction in affecting Tat-transactivation, we examined the impact of JQ1 on expression of integrated HIV provirus in well-characterized Jurkat T cell-based latency models. Our data indicate JQ1 as an effective latency activator that acts primarily by antagonizing Brd4's inhibition of Tat-transactivation. Brd4 is shown to competitively block the Tat–SEC interaction at the HIV promoter. JQ1 dissociates Brd4 to enable Tat to recruit SEC to stimulate Pol II elongation. In contrast, prostratin, an activator of protein kinase C (PKC) and NF-κB, stimulates mainly transcription initiation through promoting Pol II binding to the promoter and as such can synergize with JQ1 in activating HIV. Although affecting predominantly Tat-transactivation, JQ1 also weakly activates basal HIV transcription in Jurkat cells probably through partially releasing P-TEFb from the inactive 7SK snRNP. This secondary activity of JQ1 may help initiate latency activation. These data demonstrate that Brd4 is a previously unrecognized inhibitory factor that strongly suppresses Tat-transactivation in latently infected T cell lines, and that antagonizing this restriction by BET bromodomain inhibitors is a promising strategy for achieving targeted reactivation of latent HIV.

MATERIALS AND METHODS

Antibodies

The anti-AFF4 (A302-539A; Bethyl laboratories), -RNA Pol II (sc-899X; Santa Cruz Biotechnology) and -Pol II CTD-Ser2P (ab5095; Abcam) antibodies were purchased from commercial sources. The antibodies against CDK9, HEXIM1, Brd4, ELL2, CDK9-pT186 and LARP7 have been described previously (8,20).

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed essentially as described (21), except that no ultrasonic bath was used. Instead, overnight incubation with the various antibodies was performed. The sequences of the primers used in real-time PCR amplification of the HIV-1 promoter region are: 5′-LTR-f: 5′-GTTAGA CCAGATCTGAGCCT-3′ and 5′-LTR-r: 5′-GTGGGTT CCCTAGTTAGCCA-3′. Signals obtained by real-time PCR were normalized to input DNA, and signals generated by normal rabbit or mouse IgG (Santa Cruz Biotechnology) in control immunoprecipitations were subtracted from the signals obtained with specific antibodies.

Co-immunoprecipitation

The co-immunoprecipitation assay was performed essentially as described (8) with minor modifications. Briefly, for anti-Flag/HA IP, nuclear extracts (NEs) were incubated with anti-Flag or anti-HA agarose beads (Sigma) for 2 h; for anti-CDK9 IP, NEs were incubated with 5 μg anti-CDK9 antibody or normal rabbit IgG overnight and then incubated with protein-A beads (Invitrogen) for 1 h. The beads were washed extensively with buffer D0.3 M (20 mM HEPES-KOH [Ph7.9], 15% glycerol, 0.2 mM EDTA, 0.2% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 0.3 M KCl), eluted with 0.1 M glycine (pH 2.0) and analysed by western blotting with the indicated antibodies.

Luciferase assay

For luciferase assay conducted in HeLa cells, the HeLa-based NH1 cell line containing an integrated HIV-1 LTR-luciferase reporter construct but expressing no Tat (22), as well as its derivative NH2, which also harbors an integrated Tat-HA expression vector, was used. NH2 was generated by transfection of NH1 cells with pcDNA3-Tat-HA and then selection with G418 to obtain a stable clone. The production of Tat-HA in the G418-resistant clone NH2 was confirmed by western blotting. For luciferase assay in Jurkat cells, the Jurkat-based cell line 1G5 containing an integrated HIV-1 LTR-luciferase reporter gene (23) was nucleofected (Amaxa kit V) with 2 μg of an empty or a Tat-HA-expressing vector 24 h before the drug treatment. Cells were then treated with 5 μM JQ1 or 0.1% dimethyl sulfoxide (DMSO) for 24 h and subjected to luciferase assay using kit E1501 from Promega. Lysates were prepared from approximately equal number of cells on normalization among all the samples.

Brd4 knockdown

To reduce Brd4 expression in J-Lat A2 cells, 30 pmol of siBrd4 SMARTpool (L-004937-00, Dharmacon) or the non-target pool (D-001810-10, Dharmacon) were nucleofected (Amaxa kit V) into J-Lat A2 cells 24 h before drug treatment. Cells were then treated with 5 μM JQ1 or 0.1% DMSO for 24 h, and subjected to analysis by flow cytometry. To knock down Brd4 expression in HeLa cells, a HeLa-based cell line stably expressing a

Brd4-specific shRNA under the control of Cre recombinase, and a control HeLa cell line containing an integrated empty vector were used. The cell lines, shBrd4 sequence and the induction of Brd4 knockdown (KD) through infection with the adenovirus expressing Cre recombinase have been described previously (24).

RESULTS

JQ1 activates HIV transcription in latently infected Jurkat T cells

To determine the impact of JQ1 on HIV transcription, the J-Lat A2 cell line, a popular model created by the Verdin laboratory for studying HIV post-integrative latency, was used. This cell line was generated by transduction of human Jurkat T cells with an HIV vector expressing Tat-Flag and the green fluorescent protein (GFP) under the control of the viral 5′-LTR and an internal ribosome entry site (IRES) placed in between Tat and GFP [LTR-Tat-Flag-IRES-EGFP; (25)]. Surprisingly, unlike its suppression of *c-Myc* and Myc-dependent genes (16–18), JQ1 was found to stimulate the HIV LTR-driven GFP expression in J-Lat A2 cells in a dose- and time-dependent manner (Figure 1A and B). In contrast, the control enantiomer was completely inactive in these experiments.

In addition to J-Lat A2 cells, JQ1's stimulatory effect was also observed in 2D10 cells (Figure 1C and D); another Jurkat-based post-integrative latency model developed in the Karn laboratory (26). The HIV sequence contained in these cells encodes a partially attenuated Tat variant H13L and the short-lived d2EGFP protein in place of the *nef* gene. Moreover, like JQ1, another BET bromodomain inhibitor called iBet-151 (27) was also able to stimulate GFP expression in both J-Lat A2 and 2D10 cells in a dose- and time-dependent manner (Supplemental Figure S1). Finally, by performing quantitative RT-PCR with primers that hybridize to a distal portion of the GFP gene, JQ1 was shown to exert the stimulatory effect at the mRNA level (Figure 1E).

Brd4 KD further enhances JQ1 activation of HIV transcription

Among all the BET bromodomains, JQ1 is shown to target the first bromodomain of Brd4 with the highest affinity (19). Despite this strong selectivity, we would like to confirm that it is the inhibition of the function of Brd4, but not other BET proteins by JQ1 that resulted in the activation of HIV transcription. Toward this goal, we tested the effect of direct Brd4 KD by siRNA (siBrd4) on HIV LTR-driven GFP expression in J-Lat A2 cells. Brd4 is an abundant protein essential for cell growth and survival. The siBrd4-mediated removal of ~70% of cellular Brd4 did not cause obvious cell death but did activate the LTR-driven GFP expression from 1.05 to 4.79% GFP-positive cells (Figure 1F and Supplemental Figure S2 with representative FACS plots). The lack of a more robust activation was likely caused by the fact that there was still ~30% of total Brd4 in the KD cells. Nevertheless, the partially reduced Brd4 expression by

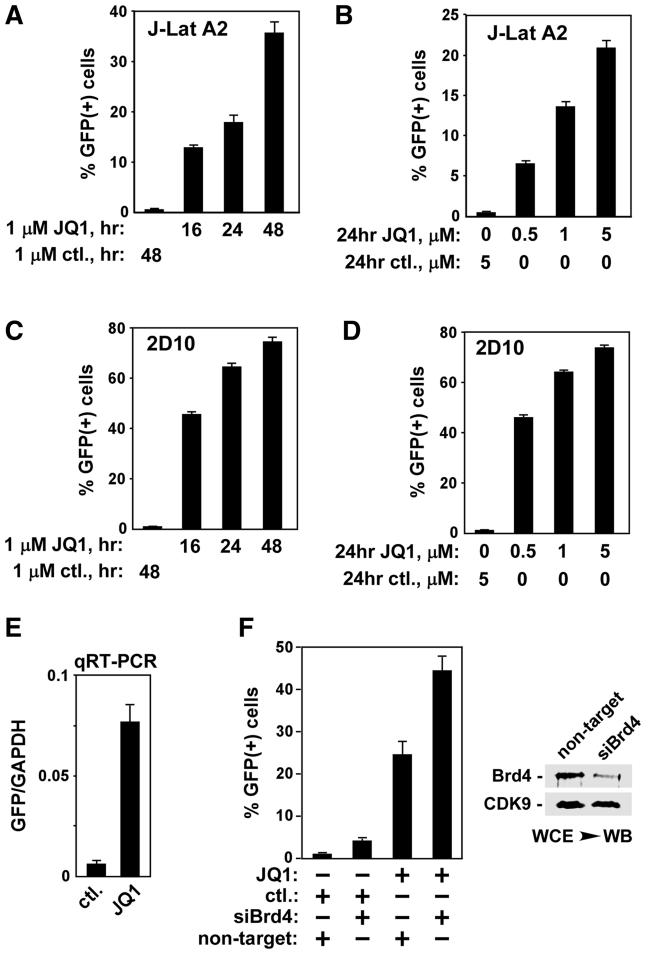

Figure 1. JQ1 activates HIV transcription in latently infected Jurkat T cells, and this effect is further enhanced by Brd4 KD. (**A** and **B**). J-Lat A2 cells were treated with 1 μM of JQ1 or the control enantiomer for the indicated periods of time (A) or for 24 h at the indicated concentrations (B). The induction of GFP expression was measured by flow cytometry (FACS) and expressed as percentages of GFP(+) cells of the entire population. (**C** and **D**). 2D10 cells were treated and analysed exactly as in A & B. (**E**) The ratios of the GFP/GAPDH mRNA levels in treated J-Lat A2 cells were determined by quantitative RT-PCR (qRT-PCR). (**F**) J-Lat A2 cells were nucleofected with either the Brd4-specific siRNA or a control non-target siRNA and then treated with JQ1 or the control enantiomer as indicated. The extent of Brd4 KD was determined by western blotting (WB) of whole cell extracts (WCE; right panel). The percentages of GFP(+) cells were measured by FACS (left panel). The error bars in all panels represent mean ± SD from three independent experiments.

siBrd4 further enhanced the stimulatory effect of JQ1 in A2 cells (an increase from 24.1 to 46.1% GFP-positive cells; Figure 1F and Supplementary Figure S2). Together, these data indicate an inhibitory role for Brd4 in suppressing HIV transcription in J-Lat A2 cells, and that antagonizing this inhibition by either JQ1 or Brd4 KD resulted in the activation of HIV latency.

Different from the aforementioned result in J-Lat cells, we previously showed that Brd4 siRNA reduced the HIV LTR-driven luciferase expression by 2- to 4-fold in HeLa cells (11). This cell line difference also caused the Tat-independent HIV transcription to respond differently to JQ1 (see later in the text). In addition, the discrepancy

between the two studies could be caused by the fact that J-Lat cells contain an integrated provirus, whereas the HIV LTR-luciferase construct was transiently transfected into HeLa cells in Yang *et al.*, 2005. The transiently transfected construct may be minimally assembled into chromatin or assembled differently from the integrated LTR, leading to different regulation by Brd4 that targets primarily acetylated chromatin.

JQ1 stimulates predominantly Tat-dependent HIV transcription

Given JQ1's stimulation of the production of GFP mRNA and protein in latently infected Jurkat cells, it is important to confirm that the stimulatory effect is due to the activation of the HIV 5′-LTR, but not any other unrelated viral or non-viral sequences in the integrated HIV vector. To this end, we examined the effect of JQ1 on expression of an integrated luciferase reporter gene that is driven solely by the HIV 5′-LTR in the Jurkat-based 1G5 cell line (23). To our surprise, JQ1 (5 μM for 24 h) only slightly increased the LTR-driven luciferase expression in the absence of Tat (Figure 2A). A broad titration of the drug (0.5–50 μM) at two different time points (6 and 24 h) also shows a small, but consistent, increase in luciferase expression (maximal 2.0-fold with 25 μM JQ1 for 6 h; Figure 2B and C). However, when Tat was expressed in 1G5 cells, JQ1 activated the HIV LTR much more potently (5.6-fold) without increasing the cellular Tat-HA level (Figure 2A).

Ruling out a cell type-specific phenomenon, the ability of JQ1 to significantly activate the Tat-dependent HIV transcription was also demonstrated in a pair of closely related HeLa-based cell lines NH1 and NH2 (Figure 2D). Both contain an integrated HIV LTR-luciferase reporter construct with Tat-HA stably expressed in NH2, but not NH1. However, different from the situation in the Jurkat-based 1G5 cell line, JQ1 caused a small reduction in basal LTR activity in NH1 cells (Figure 2D).

Ectopically expressed Tat is largely insufficient and relies on JQ1 to overcome Brd4 inhibition of Tat-transactivation

The data presented so far have revealed a critical role for JQ1 in overcoming Brd4's inhibition of Tat-transactivation. However, as in J-Lat A2 cells Tat is expressed under the control of HIV LTR from the same bicistronic mRNA as GFP, its level can be as low as that of GFP before the JQ1 treatment. In light of this consideration, we asked whether the constitutive Tat expression driven by the CMV promoter from a nucleofected plasmid would be sufficient to bypass the requirement for JQ1 to antagonize Brd4's inhibitory effect in J-Lat A2 cells. The data in Figure 2E and Supplementary Figure S3 indicate that ectopically expressed Tat only weakly increased the GFP protein and mRNA levels, in agreement with a previous observation obtained in another J-Lat cell line, 6.3 (28). However, when Tat was expressed in cells that were also treated with JQ1, the GFP expression was drastically enhanced to a level much higher than that produced by either Tat or JQ1 alone (Figure 2E and Supplementary Figure S3). This result indicates that the mere ectopic expression of Tat alone was largely insufficient to overcome the potent inhibition by Brd4, and that JQ1 inactivation of Brd4 was required to sensitize J-Lat A2 cells to Tat-transactivation.

JQ1 dissociates Brd4 and increases Tat binding to HIV promoter

JQ1 inhibits the binding of Brd4 to acetylated chromatin at the *c-Myc* gene promoter and *IgH* enhancers (16,17). We wanted to determine whether JQ1 also prevents Brd4 from binding to the HIV promoter, and if yes, how this would affect the occupancy of HIV Tat and the host cellular SEC and RNA Pol II at the promoter. To this end, quantitative ChIP analysis of the bindings of these factors to a small region overlapping with the HIV transcription start site was performed in J-Lat A2 cells before and after the JQ1 treatment. As a control, we also examined HIV promoter occupancy in cells treated with the well-known latency activator prostratin. For ease of comparison, 5 μM JQ1 and 1 μM prostratin were chosen, as they produced a similar degree of GFP expression in A2 cells (Figure 3A).

The first major noticeable effect of JQ1 is the ~8-fold reduction in the level of Brd4 bound to the HIV promoter compared with the vehicle DMSO (Figure 3B, panel 1). This result is consistent with the JQ1-induced reduction in Brd4 binding to the *c-Myc* gene promoter and *IgH* enhancers as reported previously (16,17), and suggests that the primary route by which Brd4 associates with the HIV promoter is through the interaction with acetylated histones. Notably, the prostratin-induced GFP expression was associated with only ~2-fold reduction of the promoter-bound Brd4 relative to DMSO (panel 1). This observation is again consistent with the idea that Brd4 is inhibitory to Tat-transactivation and thus must be removed from the HIV promoter to enable active transcription that is mostly driven by Tat. However, unlike JQ1, which directly and actively dissociated Brd4 from acetylated histones at the HIV promoter, the removal of Brd4 in prostratin-treated cells was likely the consequence of activated HIV transcription and thus indirect, which is why it was less robust than the direct Brd4 dissociation caused by JQ1.

Although JQ1 significantly reduced the HIV promoter occupancy by Brd4, it drastically increased the binding of Tat to the promoter compared with DMSO (Figure 3B, panel 2). Thus, the ability of JQ1 to antagonize Brd4's suppression of Tat-transactivation appears to be achieved through removing Brd4 from the HIV LTR so that Tat can subsequently bind. Although prostratin also increased the Tat association with the promoter, the effect was much less pronounced than that of JQ1 (panel 2), which correlates with the partial removal of Brd4 from the promoter in prostratin-treated cells (panel 1).

Although JQ1 increases SEC occupancy and phosphorylation of Pol II CTD, prostratin recruits more Pol II to HIV promoter

Consistent with the previous demonstrations that Tat recruits a SEC to the HIV LTR to activate transcription

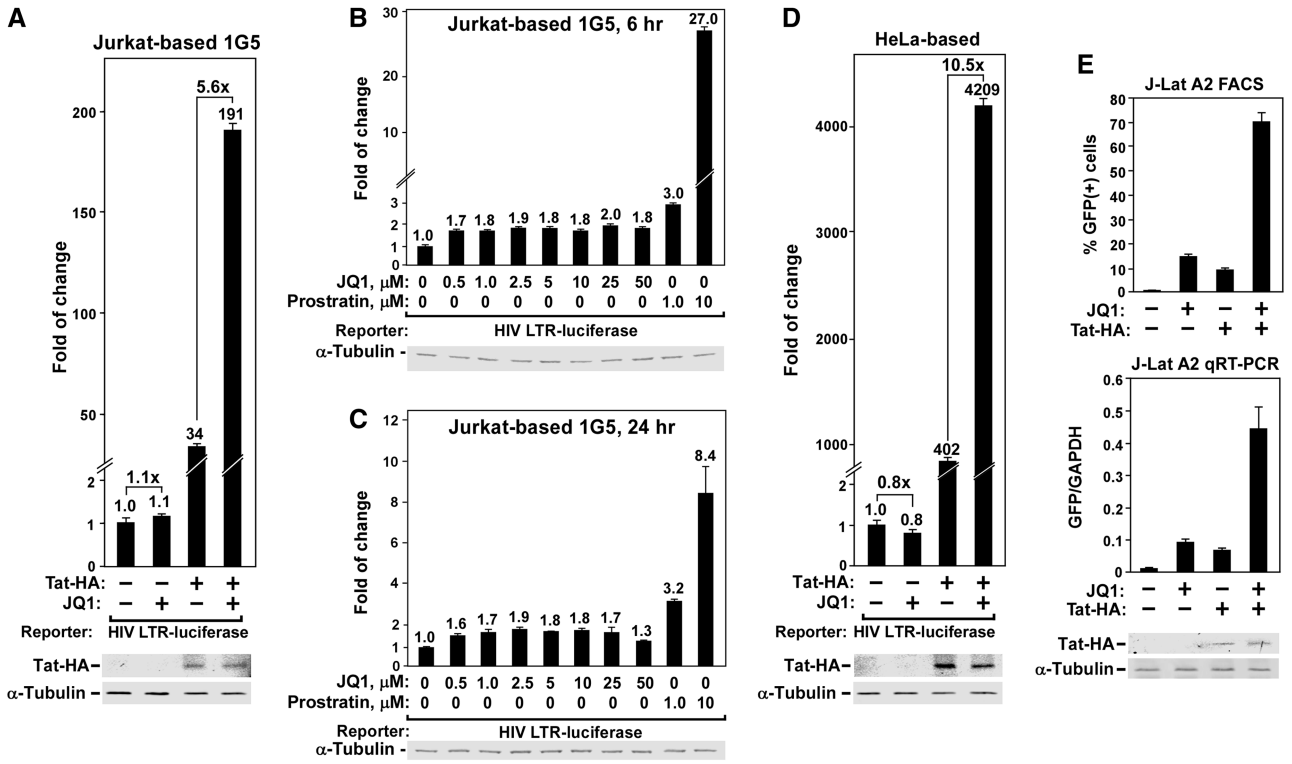

Figure 2. JQ1 stimulates predominantly Tat-dependent HIV transcription in both Jurkat and HeLa cells and ectopically expressed Tat is largely insufficient to overcome Brd4 inhibition of Tat-transactivation. (**A**) The Jurkat-based 1G5 cells containing an integrated HIV LTR-luciferase construct were nucleofected with the vector expressing Tat-HA (+) or nothing (−) and then treated with JQ1 or DMSO (−) as indicated. Whole cell extracts (WCE) were examined for the contained luciferase activities (top panel) and indicated proteins by western blotting (WB, bottom panel). (**B** and **C**) 1G5 cells were treated for 6 h (B) or 24 h (C) with the indicated concentrations of JQ1 or prostratin. WCE were examined for luciferase activities (top panels) and α-tubulin levels (bottom panels) as in A. (**D**) The HeLa-based NH1 cells expressing no Tat (left two columns) and NH2 cells stably expressing Tat-HA (right two columns), with both containing an integrated HIV LTR-luciferase construct, were treated with JQ1 or DMSO (−) and analysed as in A. (**E**) J-Lat A2 cells were treated as in A and analysed by FACS to determine the percentages of GFP(+) cells (top panel), by qRT-PCR for the GFP/GAPDH mRNA ratios (middle), and by WB for the indicated proteins in WCE (bottom). The error bars in all panels represent mean ± SD from three independent experiments.

(8,9), the JQ1-induced Brd4 dissociation and Tat binding to the HIV promoter were accompanied by significantly elevated promoter occupancy by AFF4, the scaffolding subunit of SEC, relative to the DMSO control (Figure 3B, panel 3). In addition, JQ1 also markedly increased the promoter binding by CDK9, another key SEC component (panel 4). These in turn resulted in enhanced phosphorylation of the Pol II CTD on Ser2 (CTD-Ser2P) as expected (Figure 3B, panel 5).

Interestingly, unlike JQ1, prostratin only slightly increased the levels of AFF4, CDK9 and CTD-Ser2P at the HIV promoter, compared with DMSO (Figure 3B, panels 3–5). However, it was able to significantly enhance the promoter occupancy by total Pol II, an effect that could not be achieved by JQ1 (panel 6). Furthermore, the induction of GFP expression by prostratin, but not JQ1, was completely inhibited by the pan-PKC inhibitor Gö6983 in both J-lat A2 and 2D10 cells (Supplementary Figure S4). These observations agree well with the demonstrated ability of prostratin to induce the PKC-dependent NF-κB activation, which in turn promotes polymerase recruitment to and transcription initiation from the HIV LTR (5).

Besides this ability, prostratin has recently been shown to also stimulate the expression of ELL2 (29), a dual-function transcription factor capable of promoting Pol II recruitment/initiation and entry into the pause site before its assembly into a SEC (30). Likely owing to their cooperative activation of different stages of the HIV transcription cycle—prostratin working on Pol II recruitment/transcription initiation, whereas JQ1 acting subsequently to enhance Pol II elongation—the two compounds synergistically activated the HIV LTR-driven GFP expression in A2 cells when used together (1 μM JQ1 and 0.5 μM prostratin alone produced 14.2% and 17.6% GFP+ cells, respectively, and their combination reached 78.6%; Figure 3C and Supplementary Figure S5).

JQ1-induced Brd4 dissociation is required for Tat to recruit SEC to HIV promoter

Is the increased promoter occupancy by the Tat–SEC complex in JQ1-treated A2 cells the consequence of JQ1-induced Brd4 dissociation or an independent event unrelated to Brd4 dissociation? To address this question, we performed ChIP analyses in HeLa-based NH1 cells that contain an integrated HIV LTR-luciferase gene, but express no Tat (22). The treatment with JQ1 also dissociated Brd4 (Figure 3D, panel 1) just as in J-Lat A2 cells. However, unlike in A2 cells where Tat was expressed from

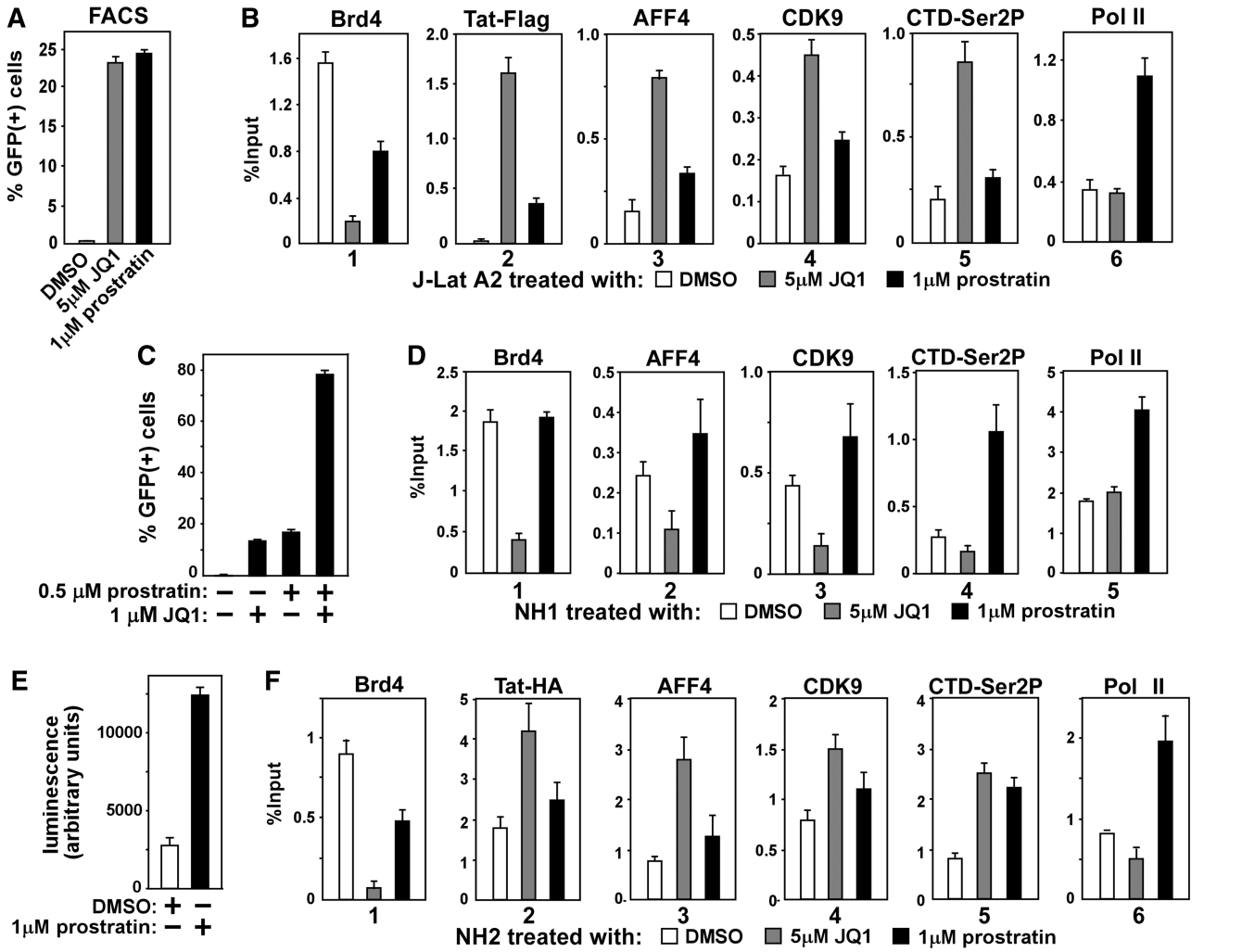

Figure 3. The JQ1-induced Brd4 dissociation enables Tat to recruit SEC to HIV promoter and induce Pol II CTD phosphorylation and viral transcription. (**A**) J-Lat A2 cells were treated with the indicated reagents and analysed by FACS for the percentages of GFP (+) cells in the population. (**B**) A2 cells were treated as in A and then subjected to ChIP analysis to determine the levels of the indicated factors bound to the HIV promoter. The signals were normalized to those of input. (**C**) A2 cells were treated with the indicated agents and analysed for GFP expression as in A. (**D**) The HeLa-based NH1 cells that contain an integrated HIV LTR-driven luciferase reporter gene, but express no Tat protein, were treated and analysed as in B. (**E**) Luciferase activities in lysates of NH1 cells treated with the indicated reagents were measured. (**F**) The HeLa-based NH2 cells stably expressing Tat-HA and containing the integrated HIV LTR-luciferase construct were treated and analysed by ChIP as in B. The error bars in all panels represent mean ± SD from three independent experiments.

the HIV vector shortly after JQ1 treatment, which in turn promoted SEC binding to the HIV promoter (Figure 3C), no Tat was produced in NH1 cells, and thus neither the Tat-dependent recruitment of SEC subunits AFF4 and CDK9 nor an increase in CTD-Ser2P was observed (Figure 3D, panels 2–4). On the contrary, JQ1 partially reduced the levels of AFF4, CDK9 and CTD-Ser2P at the promoter (panels 2–4), which is consistent with JQ1's partial suppression of Tat-independent HIV transcription in NH1 cells (Figure 2D). This result suggests that at least in HeLa cells, the Brd4 recruitment of P-TEFb may contribute in part to the subsequent SEC assembly on HIV promoter in the absence of Tat.

Just as in J-Lat A2 cells, prostratin significantly increased the HIV promoter occupancy by Pol II in NH1 cells (Figure 3D, panel 5). Compared with DMSO, it also enhanced the occupancy by AFF4, CDK9 and especially CTD-Ser2P (panels 2–4), suggesting that it can use a Tat-independent mechanism(s) to recruit SEC to the viral promoter to phosphorylate Pol II. Consistently, prostratin stimulated HIV transcription 4.7-fold in the absence of Tat (Figure 3E).

Finally, when Tat was stably expressed in HeLa-based NH2 cells, the JQ1-induced Brd4 dissociation from HIV promoter enabled Tat to recruit SEC and promote CTD-Ser2P (Figure 3F). The binding patterns of the various factors at the promoter bear striking similarity to those in JQ1-treated J-Lat A2 cells in which Tat expression is induced. Thus, by combining the data obtained in the engineered HeLa cells with (NH2) or without Tat (NH1) and comparing with those from J-Lat A2 cells, the notion that the JQ1-induced Brd4 dissociation is required for Tat to recruit SEC to HIV promoter and stimulate transcription can be firmly established.

ELL2 KD efficiently suppresses latency activation induced by JQ1

In light of the aforementioned ChIP data demonstrating JQ1's promotion of SEC formation on HIV promoter, we wanted to further confirm the functional significance of SEC in JQ1 activation of viral latency. To this goal, we knocked down the expression of ELL2, a key catalytic component of SEC besides P-TEFb, in Jurkat-based 2D10 cells and examined the impact on GFP production stimulated by either JQ1 or prostratin. qRT-PCR and Western analyses reveal that up to 74.2% of cellular ELL2 was removed by the ELL2 siRNA (Figure 4A). Under such conditions, JQ1's activation of LTR was reduced by 83.3%, whereas activation by prostratin was reduced by only 24.5% (Figure 4B). This result confirms the dependence on SEC function for JQ1's activation of HIV latency. It is also consistent with the aforementioned ChIP data showing that JQ1 and prostratin use largely different mechanisms to activate HIV LTR: Although JQ1 functions primarily at the elongation stage, where SEC is essential, prostratin stimulates mostly transcriptional initiation and promoter clearance.

Brd4 inhibits Tat-SEC interaction by competing with Tat for binding to P-TEFb on HIV promoter

To determine how Brd4 prevents Tat from recruiting a SEC to the HIV promoter, we tested whether Brd4 can directly inhibit the Tat-SEC interaction. Indeed, when wild-type Brd4 was overexpressed in cells, the levels of SEC components AFF4, ELL2, CycT1 and CDK9 bound to the immunoprecipitated Tat-HA were significantly decreased, even though they showed no change in NEs (Figure 4C). In contrast, overexpression of the Brd4 mutant ΔPID that lacks the C-terminal P-TEFb-interacting domain (15) failed to suppress the Tat-SEC interaction, suggesting that Brd4 and Tat compete for binding to the P-TEFb component of SEC.

Conversely, when the endogenous Brd4 expression was silenced by a specific shRNA, shBrd4, there was a marked increase in the interaction of Tat with the SEC subunits AFF4, ELL2, CycT1 and CDK9 (Figure 4D). Furthermore, confirming the ability of JQ1 to antagonize Brd4's inhibition of Tat-transactivation, the treatment of cells with JQ1 enhanced the Tat–SEC interaction (Figure 4E).

JQ1 increases CDK9 T-loop phosphorylation in Tat-dependent manner

The interaction of Tat with P-TEFb has been shown to increase CDK9's autophosphorylation and phosphorylation of the Pol II CTD (31,32). In light of the aforementioned observation that JQ1 stimulated the interaction of Tat with P-TEFb/SEC through eliminating the competition by Brd4, we asked whether an increased phosphorylation of Thr186, which is located at the tip of the CDK9 T-loop and required for CDK9's catalytic activity, could be accomplished by Tat with the help from JQ1. Indeed, in J-Lat A2 cells treated with JQ1, where Tat expression was induced by the drug, an increased CDK9 phosphorylation was detected with the antibody that specifically recognizes the phosphorylated form of Thr186 [Figure 5A; (20)]. In HeLa cells, JQ1 also increased Thr186 phosphorylation, and this effect was observed only under conditions of Tat expression (Figure 5B, lane 2).

JQ1 partially dissociates P-TEFb from 7SK snRNP in Jurkat T cells

The sequestration of P-TEFb in the inactive 7SK snRNP has been proposed as a key factor contributing to HIV latency (2). Latency activators such as prostratin and HMBA have been shown to release P-TEFb from this complex (29,33). To determine whether JQ1 could also target 7SK snRNP in addition to its suppression of Brd4's inhibition of Tat-transactivation, we analysed the associations of key 7SK snRNP components HEXIM1, an inhibitor of CDK9 kinase and LARP7, a 7SK RNA-binding protein, with the immunoprecipitated CDK9 in JQ1-treated Jurkat cells. Under the conditions that permitted efficient GFP induction, JQ1 partially dissociated HEXIM1, but not LARP7, from P-TEFb (Figure 5C). In comparison, the CDK kinase inhibitor flavopiridol efficiently dissociated both HEXIM1 and LARP7.

Thus, through directly targeting the 7SK snRNP, a portion of which has been detected at the HIV promoter before Tat activation (34), JQ1 could potentially increase the pool of active P-TEFb for subsequent SEC assembly at the promoter. This Tat-independent effect of JQ1, although relatively weak, could be responsible for JQ1's weak activation of basal HIV transcription (Figure 2B and C) and may also facilitate the initiation of latency activation at a time when the cellular Tat level is still below threshold (see Discussion later in the text). Finally, it is worth noting that unlike the situation in Jurkat cells, JQ1 was completely unable to disrupt 7SK snRNP in HeLa cells (data not shown), which may explain why it failed to activate basal HIV transcription in this cell line (Figure 2D).

DISCUSSION

A variety of restrictions on HIV gene expression (e.g. condensed chromatin structure, sequestration of host transcription factors in inactive complexes, transcriptional interference by adjacent promoters, block of nuclear RNA export, inhibition of HIV mRNA translation by miRNAs, etc.) have been proposed as important factors contributing to HIV proviral latency (2). The present study, however, indicates the Brd4 suppression of Tat-transactivation as another important and previously unrecognized inhibitory factor. Our data are consistent with a model (Figure 6) that Brd4 competitively blocks the interaction of Tat with P-TEFb at the HIV promoter, and that this inhibition is antagonized by JQ1, which dissociates Brd4 from the promoter to allow Tat to bind to P-TEFb and assemble a functional SEC on the TAR RNA for activation of HIV transcription and latency.

As Tat is produced from the same HIV mRNA whose production it stimulates, this viral protein fuels a powerful positive feedback circuit. In addition to its primary role as

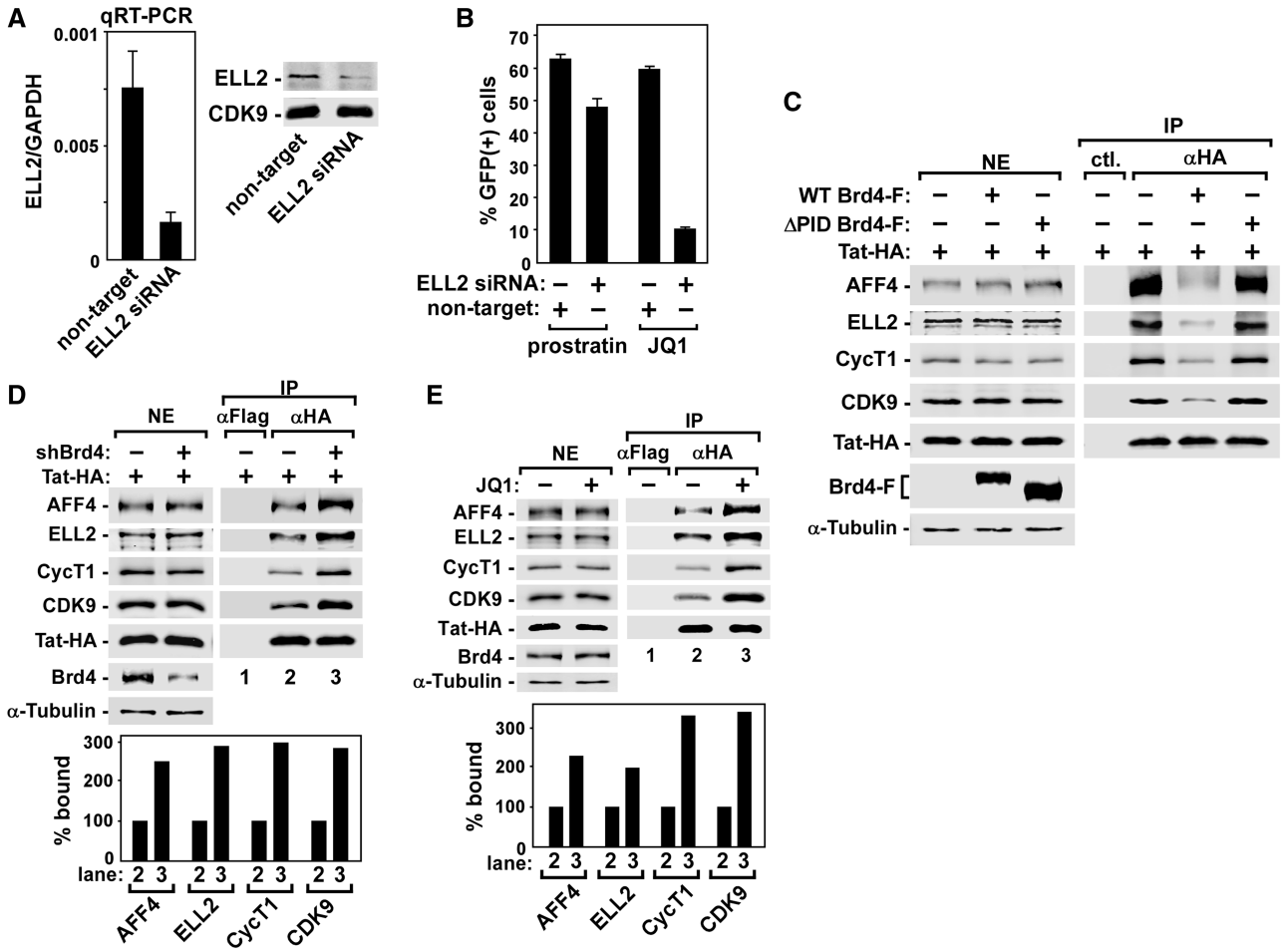

Figure 4. The SEC function is required for JQ1 activation of HIV transcription and Brd4 inhibits Tat-SEC interaction by competing with Tat for binding to P-TEFb on HIV promoter. (**A**) The ELL2-specific siRNA and a non-target control siRNA were transfected separately into 2D10 cells, and the ELL2 mRNA and protein levels were examined by qRT-PCR (left) and western blotting (right). (**B**) The same cells in A were examined for the impact of ELL2 KD on induction of GFP production by prostratin and JQ1. The percentages of GFP (+) cells were measured by FACS, and the error bars represent mean ± SD from three independent experiments. (**C** and **D**) HeLa cells were transfected with plasmids expressing the indicated proteins or shRNA. Nuclear extracts (NE) and immunoprecipitates (IP) obtained with either the specific (anti-HA) or control antibodies (ctl. IgG or anti-Flag) were examined by western blotting (WB) for the indicated proteins. The indicated SEC components bound to the immunoprecipitated Tat-HA in lanes 2 and 3 of D were quantified, normalized to the signals of Tat-HA and displayed in the bottom panel of D, with the signals in lane 2 artificially set to 100%. (**E**) HeLa cells transfected with the Tat-HA-expressing construct were treated with (+) or without (−) JQ1, analysed and quantified as in D.

an activator of Pol II elongation on integrated proviral DNA, Tat has also been shown to counteract several other mechanisms that block HIV transcription. For example, Tat can directly extract P-TEFb from the inactive 7SK snRNP [reviewed in (7)], enhance the formation of SEC through stabilizing the key SEC component ELL2 (8) and recruit HATs and chromatin remodeling complexes to the HIV LTR to create relaxed chromatin structure for activated transcription (35–38).

Owing to these multiple activities and also the feedback nature of signal amplification, the Tat level in an infected cell is recognized as one of the most important determinants in controlling the outcome of infection [reviewed in (1)]. In fact, small stochastic changes in Tat expression have been shown to determine whether a virus undergoes latency or productive infection (1,39). In latently infected cells, Tat exists at sub-threshold levels that are too low to allow productive HIV transcription and replication. Thus, through directly dissociating Brd4, an abundant nuclear protein and powerful inhibiter of Tat-transactivation, from the HIV LTR, JQ1 overcomes a prominent barrier to HIV latency activation.

Recently, JQ1 has been featured prominently as an anti-tumor drug owing to its potent anti-proliferative effect on cells of multiple myeloma, acute myeloid leukemia and other cancer types (16–18). This growth-inhibitory effect is mostly due to JQ1's suppression of expression of *c-Myc* and Myc-dependent genes that rely on Brd4 to recruit P-TEFb to their promoters (16,17). In this context, it is unsurprising and, at the same time, reassuring to learn that JQ1 does not cause global T cell activation (40), which will likely require the activation of many primary response genes that are dependent on Brd4 and Myc for expression (14,41). This important property of JQ1, coupled with its relatively low cytotoxicity (40) and largely Tat-specific effect in reversing HIV latency as

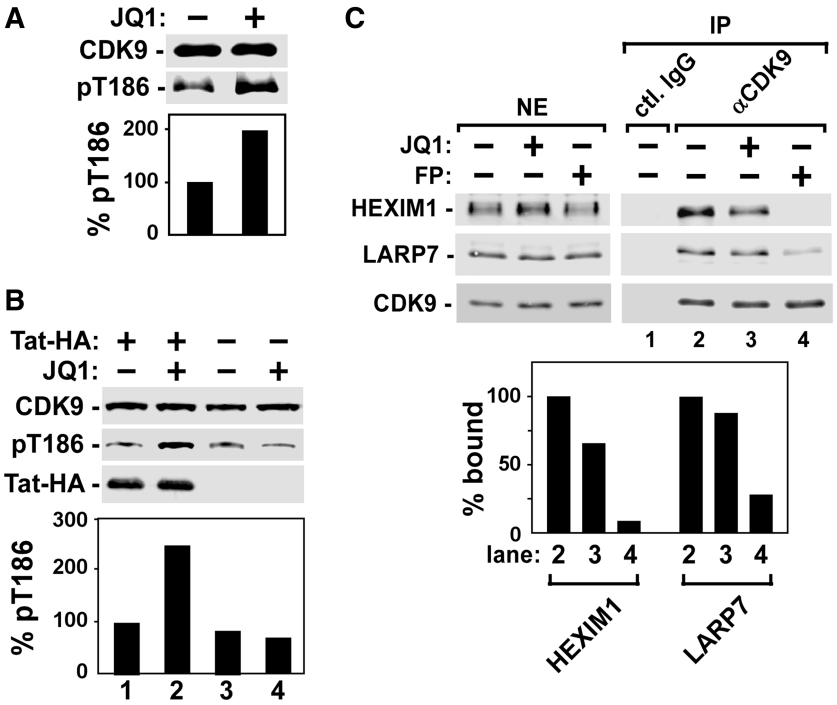

Figure 5. JQ1 enables Tat to increase CDK9 T-loop phosphorylation and partially dissociates P-TEFb from 7SK snRNP in Jurkat T cells. (**A**) The total CDK9 levels and the extent of CDK9 phosphorylation on Thr186 (pT186) were examined by Western analysis of affinity-purified CDK9 derived from J-Lat A2 cells treated with (+) or without (−) JQ1. The pT186 levels were quantified, normalized to those of total CDK9 and shown at the bottom, with the level in untreated cells set to 100%. (**B**) CDK9 affinity-purified from HeLa cells expressing Tat-HA or not and treated with or without JQ1 were analysed and quantified as in A for the phosphorylation on T186. The levels of Tat-HA in cell lysates were detected by anti-HA western blotting (WB). (**C**) Nuclear extracts (NE) of Jurkat cells untreated (−) or treated with JQ1 or flavopiridol (FP) were analysed by WB for the indicated proteins (left panel). Immunoprecipitates (IP) derived from NE with the indicated antibodies were examined by WB for the indicated proteins (right panel). The amounts of HEXIM1 and LARP7 bound to immunoprecipitated CDK9 were quantified, normalized to CDK9 and shown at the bottom, with the levels in untreated cells (lane 2) set to 100%.

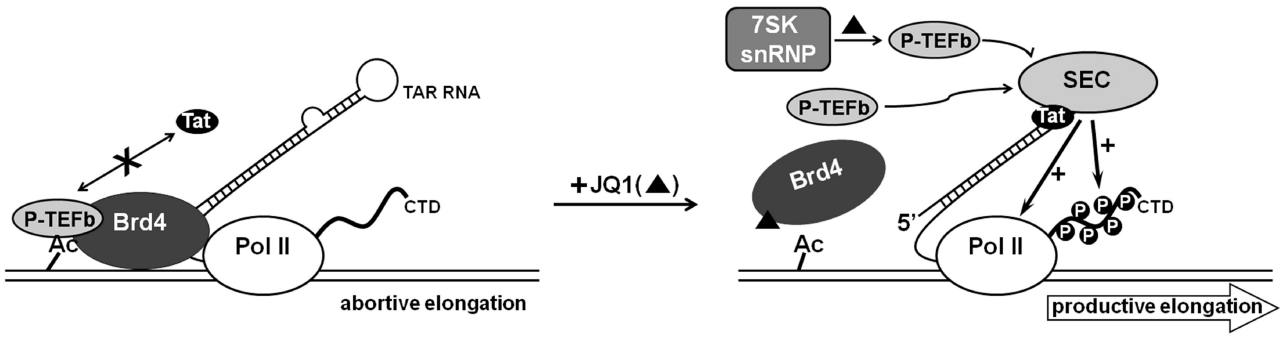

Figure 6. Diagram depicting Brd4 suppression of Tat-transactivation as a key inhibitory factor of HIV transcription and JQ1 as an effective antagonizer of this inhibition. Without JQ1, the HIV promoter-bound Brd4 (through interacting with acetylated histones, or Ac) competitively blocks the interaction of Tat with P-TEFb and prevents Tat from assembling a functional SEC. This leads to abortive transcriptional elongation. JQ1 dissociates Brd4 from the viral promoter to allow Tat to bind to P-TEFb and assemble a SEC on the TAR stem-loop structure for phosphorylation of the Pol II CTD and activation of productive elongation. Additionally, JQ1 also partially releases P-TEFb from the 7SK snRNP, providing another source of P-TEFb for SEC assembly at the promoter.

revealed in the current study, make this compound a very promising candidate in the design of targeted strategies to 'shock and kill' latent HIV reservoirs.

In addition to its stimulation of Tat-transactivation, JQ1 has also been shown to exert a small but highly reproducible positive effect on basal, Tat-independent HIV transcription in Jurkat T cells (Figure 2B and C). This secondary activity of JQ1 could be important, as it provides a potential means for JQ1 to kick-start the very first step of latency activation at a time when intracellular Tat level is still below a critical threshold. Mechanistically, the JQ1-induced partial disruption of 7SK snRNP (Figure 5C), which sequesters up to 90% of cellular P-TEFb in an inactive state (10), could be responsible for JQ1's weak stimulation of basal HIV transcription. This effect of JQ1 has also been noted by

Bartholomeeusen *et al.* (42) in a concurrent study. A small portion of 7SK snRNP has been reported to exist on the proviral promoter even before Tat-transactivation (34). The JQ1-induced disruption of the promoter-bound 7SK snRNP may provide an important source of P-TEFb for subsequent assembly of a functional SEC at the promoter (Figure 6). The direct transfer of P-TEFb from 7SK snRNP to SEC on HIV LTR is expected to bypass the requirement for Brd4 to recruit P-TEFb, which is blocked by JQ1 under such conditions.

During the course of submission and review of our manuscript, a concurrent study reports that JQ1 also mildly enables the recovery of virus from one of the three HIV-infected, ART-treated patients (40). The less-potent effect of JQ1 in resting primary T cells than in transformed latent cell lines is likely owing to the fact that the mechanism of JQ1 action is centered on the promotion of Tat–SEC interaction at the viral promoter (current study), and that resting CD4 T cells contain only very low levels of the key SEC component CycT1 (43). Therefore, more restrictive barriers will have to be removed before JQ1 can fully reverse latency in resting primary T cells.

Just as the establishment of viral latency in CD4 T cells is multifactorial (1,44), the reversion of HIV latency, especially in resting T cells, may benefit from a multi-pronged approach aimed at counteracting a multitude of restrictions. Given that JQ1 targets primarily the Tat-transactivation step and relies on the availability of key Tat cofactors *in vivo*, it is conceivable that JQ1 will benefit from cooperating with other latency activators that can create a more conducive environment for it to promote Tat function. Consistent with this idea, the current data indicate that JQ1 and prostratin synergistically activate HIV transcription and latency in J-Lat A2 cells (Figure 3C). Prostratin is known to activate NF-κB and ELL2, which in turn promote Pol II recruitment and entry into the pause site (5,30). These are all favorable conditions to allow JQ1 to exert its stimulatory effect.

Besides JQ1 and prostratin, several other small molecule activators (e.g. SAHA and 5HN) have also been reported to affect different cellular processes/pathways to reactivate latent HIV without significantly inducing global T cell activation (45,46). Full latency activation must rely on Tat and its interaction with SEC, which is strongly promoted by JQ1. Future studies will shed light on whether a cocktail of drugs that contains JQ1 as a key component plus other classes of latency activators can be used to efficiently activate latency through overcoming multiple restrictions in resting CD4 T cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5.

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