

Signal-induced Brd4 release from chromatin is essential for its role transition from chromatin targeting to transcriptional regulation

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ABSTRACT

Bromodomain-containing protein Brd4 is shown to persistently associate with chromosomes during mitosis for transmitting epigenetic memory across cell divisions. During interphase, Brd4 also plays a key role in regulating the transcription of signal-inducible genes by recruiting positive transcription elongation factor b (P-TEFb) to promoters. How the chromatin-bound Brd4 transits into a transcriptional regulation mode in response to stimulation, however, is largely unknown. Here, by analyzing the dynamics of Brd4 during ultraviolet or hexamethylene bisacetamide treatment, we show that the signal-induced release of chromatin-bound Brd4 is essential for its functional transition. In untreated cells, almost all Brd4 is observed in association with interphase chromatin. Upon treatment, Brd4 is released from chromatin, mostly due to signal-triggered deacetylation of nucleosomal histone H4 at acetylated-lysine 5/8 (H4K5ac/K8ac). Through selective association with the transcriptional active form of P-TEFb that has been liberated from the inactive multi-subunit complex in response to treatment, the released Brd4 mediates the recruitment of this active P-TEFb to promoter, which enhances transcription at the stage of elongation. Thus, through signal-induced release from chromatin and selective association with the active form of P-TEFb, the chromatin-bound Brd4 switches its role to mediate

the recruitment of P-TEFb for regulating the transcriptional elongation of signal-inducible genes.

INTRODUCTION

The regulation of the processivity of RNA polymerase (Pol) II is recognized as a key mechanism for controlling the expression of vast arrays of signal-inducible genes in metazoan (1,2). Shortly after transcriptional initiation, RNA Pol II pauses at the promoter-proximal region. The positive transcription elongation factor b (P-TEFb), a heterodimeric kinase predominantly composed of Cdk9 and Cyclin T1, promotes the transition of Pol II from pausing to processive mode by phosphorylating the C-terminal domain (CTD) of the largest subunit of Pol II, thereby leading to the synthesis of full-length transcripts (3,4). Hence, the availability of P-TEFb activity at promoter-proximal region is crucial for the expression of inducible genes. In cells, the activity of P-TEFb is tightly regulated (5,6). In actively growing cells, the majority of P-TEFb is sequestered in an inactive 7SK small nuclear ribonucleoprotein (snRNP) complex that also contains 7SK snRNA (7,8), HEXIM1/HEXIM2 (9–12), LARP7 (13,14) and MePCE/BCDIN3 (15–17). Upon stimulation by various signals, P-TEFb is liberated from the inactive complex, mostly due to the dephosphorylation at T-loop of Cdk9, the catalytic subunit of P-TEFb (5,18,19).

The efficient transcription of signal-inducible genes, however, relies not only on P-TEFb's liberation from the inactive complex, but also on its recruitment to promoters. Currently, bromodomain-containing protein Brd4, which belongs to the bromodomain and

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ET-domain (BET) protein family (20,21), is recognized as the most important general factor for P-TEFb recruitment (20–23). The two bromodomains of Brd4 are necessary and sufficient for its association with acetylated tails of histone H3 and H4 (24,25). In addition, a P-TEFb interacting domain (PID) located at the very C-terminus of Brd4 is essential for its binding to P-TEFb (26,27). The function of the ET domain is just being recognized as a region interacting with WHSC1L1/NSD3 for P-TEFb-independent regulation of H3K36 methylation (28). Originally termed mitotic chromosome associated protein (MCAP), Brd4 is found to be persistently associated with acetylated chromosomes during mitosis in a number of cell lines (24,25), which is critical for the recruitment of P-TEFb and the rapid expression of early G1 genes upon exiting mitosis (29–31). Thus, Brd4 is proposed to play an important role in transmitting epigenetic memory across cell divisions (29–31).

In addition to the relatively stable chromatin targeting of Brd4, its dynamic association with chromatin has been observed in multiple systems as well (32). For instance, signal-induced Brd4 occupancy at promoters has been shown to be crucial for the expression of a vast array of primary response genes both in lipopolysaccharide-stimulated macrophages (33) and in mitogen-activated Jurkat T cells (34). Moreover, a recent study revealed that subsequent to histone H3S10 phosphorylation and H4K16 acetylation, the binding of Brd4 to FOSL1 intronic enhancer is increased in serum-stimulated HEK293 cells (35). These observations indicate that Brd4 is dynamically redistributed to regulate gene expression under different circumstances.

How Brd4 transits from chromatin targeting to transcriptional regulation in response to stimulation, however, is not well understood (20). Here, by analyzing the chromatin-bound and -free fractions, we show that almost all Brd4 is associated with interphase chromatin in untreated cells. Upon ultraviolet (UV) or hexamethylene bisacetamide (HMBA) treatment, Brd4 is released from chromatin through signal-induced histone deacetylation, and this release is essential for P-TEFb recruitment to promoter and transcriptional elongation. Combined with previous studies (19,36), we propose a model in which the stimulation triggers the liberation of P-TEFb from the inactive 7SK snRNP complex, and the release of Brd4 from chromatin, thereby switching the role of Brd4 from chromatin targeting to P-TEFb recruitment for the efficient elongation of inducible genes.

MATERIALS AND METHODS

Chemicals

Hexamethylene bisacetamide (HMBA) (Cat#: 224235-50G, Batch#:01907JR) is from Sigma. Trichostatin A (TSA) and suberoyl bishydroxamic acid (SBHA) are from Calbiochem. Ethylenediaminetetraacetic acid (EDTA)-free complete protease inhibitor cocktail is from Roche. DNase I, SYBR Green Realtime PCR Master Mix Plus and the ReverTra Ace qPCR RT Kit

are from Toyobo Co. Japan. All other chemicals are from Amresco or Merck.

Antibodies

Rabbit anti-Sp1 (sc-5286), histone H3 (sc-59) and mouse anti- α -Tubulin (sc-10809) antibodies are from Santa Cruz. Mouse anti- β -Actin (A2228) antibody, mouse anti-HA agarose beads (A2095) and anti-Flag M2 affinity gel (A2220) are from Sigma. Rat anti-HA antibody is from Roche. Rabbit anti-histone H4 (05-858), H4K5ac (07-327), H4K8ac (07-328), H4K12ac (07-595), H4K16ac (06-762), H3K9/14ac (06-599), H3K4me3 (07-473) and H3K27me3 (07-449) are from Millipore. Rabbit anti-H3K36me3 (ab9050) is from Abcam. Rabbit anti-Cdk9, Cyclin T1 and HEXIM1 antibodies were raised in GeneScript (Nanjing, China) against the following peptides: RRRGSGITQQSTNQ (Cdk9, amino acids 343–356), SGNTDKPRPPPLPS (Cyclin T1, amino acids 702–715) and HRQERAPLSKFGD (HEXIM1, amino acids 346–359). Rabbit anti-Brd4 and Cdk9-pT186 antibodies were reported previously (19).

Plasmids

The constructs containing P-TEFb interaction domain (HA-PID, amino acids 1260–1362) (26) or N-terminal fragment (HA- Δ C, amino acids 1–721) of Brd4 were generated by inserting the respective polymerase chain reaction (PCR)-amplified complementary DNA (cDNA) fragments from pcDNA4c-huBrd4 (Addgene Plasmid 14441, with full-length human Brd4 cDNA) (37) into BamH I/Xba I sites of modified pcDNA3-HA or -Flag vector, and was confirmed by DNA sequencing. The plasmid with the short hairpin RNA (shRNA) targeting human Brd4 was reported previously (22).

Cell lines

HeLa cells, the HeLa cell with an integrated HIV-1 LTR-luciferase reporter gene (19) and the HeLa-based stable cell lines were maintained as reported previously (11,22). All other cell lines (Supplementary Figure S1) were maintained according to American Type Culture Collection (ATCC) instructions.

Treatment of cells with UV or various pharmacological compounds

Cells at 50–70% confluence were pre-incubated with histone deacetylase (HDAC) inhibitor TSA (400 nM) or SBHA (200 nM) for 1 h, followed by a treatment with 10 mM HMBA for 4 h, unless indicated otherwise. For UV irradiation, cells were exposed to 80 J/m² of UV in Spectrolinker XL-1000 (Spectronics) without culture medium, followed by an incubation in the original medium for 30 min (19). After treatments, the cells were subjected to nuclear fractionation immediately.

Modified nuclear fractionation

Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) and centrifuged at 1500g, 4°C for 5 min, and the packed cell volume (PCV)

was recorded. The cells were suspended in 5×PCV of ice-cold buffer A (10mM HEPES pH 7.9/1.5mM MgCl₂/10mM KCl/1mM DTT/0.5mM PMSF/1×protease inhibitor cocktail) for 10 min. The swollen cells were centrifuged, and the cell pellet was subjected to low-salt extraction by resuspending in 2×PCV of buffer A (with 1% NP-40) for 10 min to break the cell membrane. The nuclei were centrifuged at 5000g, 4°C for 5 min and the supernatant (~3.6×PCV) was saved. The nuclei were further extracted twice by resuspending in 1×PCV of buffer A (with 0.5% NP-40/75mM NaCl) for 5 min followed by centrifugation. The supernatants of the three extractions were combined, and saved as the low-salt fraction (LSF, ~5.6×PCV). Subsequently, the nuclei were subjected to high-salt extraction with 6×PCV of high-salt buffer (10 mM HEPES pH 7.9/20% glycerol/0.3 M NaCl/1.5 mM MgCl₂/0.4 mM EDTA/0.5% NP40/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride (PMSF)/1×protease inhibitor cocktail) on a rotator at 4°C for 30 min. The nuclei were centrifuged at 12000g at 4°C for 10 min and the supernatant was saved as the high-salt fraction (HSF, ~6.2×PCV). The volume and salt concentration of LSF were brought to the same as those of HSF. The remnant nuclear pellet was boiled in 6×PCV of sodium dodecyl sulfate (SDS)-loading buffer. The LSF and HSF were stored at -80°C, or used directly.

DNase I digestion of nuclei

Briefly, 1–2 × 10⁷ HeLa cells were subjected to three rounds of low-salt extraction as described above, and the nuclei were equilibrated with 500 μl of 1 × DNase I digestion buffer (40 mM Tris-HCl pH 7.5/8 mM MgCl₂/5 mM DTT/1 × protease inhibitor cocktail) and centrifuged at 5000g, 4°C for 5 min. The nuclei were incubated with 100 U of DNase I in 100 μl of 1 × DNase I digestion buffer at 30°C for 1.5 h, followed by centrifugation at 10000g, 4°C for 5 min. The supernatant was saved. A portion of digested nuclei was lysed with SDS-loading buffer and the rest was subjected to DNA isolation. The proteins in the supernatant and nuclear lysate were detected by western blotting and the isolated chromatin DNA was analyzed by ethidium bromide staining after agarose gel electrophoresis.

Salt-titrated extraction assay

The salt-titrated extraction was performed to test the strength of Brd4's interaction with chromatin. Cells were extracted with low-salt buffer for three times to remove chromatin-free P-TEFb and its related factors. The low-salt extracted nuclei (LSEs) were then extracted with 6 × PCV of high-salt buffer with indicated concentration of NaCl. After centrifugation at 12000g, 4°C for 10 min, the supernatants were saved as nuclear extracts (NEs) and the pellets were boiled in SDS-loading buffer to yield nuclear lysate fractions.

Immuno-affinity purification

Flag-tagged protein and its associated factors were isolated by anti-Flag Immuno-affinity purification (IP)

from NE, LSF or HSF of HeLa-based stable cells F1C2 (expressing Cdk9-Flag) (36), MCAP (HA-Brd4-Flag) (22) and HH8 (Flag-HEXIM1) (11) or transfected HeLa cells as described previously (38). The levels of proteins indicated in figures were determined by western blotting.

Luciferase assay

HeLa cells with an integrated HIV-LTR luciferase reporter gene (HIV-LTR-Luc) (19,39) were pre-incubated for 1 h with the indicated amount of HDAC inhibitors in six-well plates, followed by 6 h of incubation with 10 mM HMBA. Cells lysates were prepared to measure luciferase activity (19). The error bars were calculated based on three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out with HIV-LTR-Luc integrated HeLa cells as described previously (38). Immunoprecipitated DNA was analyzed by real-time PCR with SYBR Green Realtime PCR Master Mix Plus (Toyobo) and primers in HIV-LTR promoter region (-111 to -31, forward: 5'-GCT ACA AGG GAC TTT CCG CTG G, reverse: 5'-AGG ATC TGA GGG CTC GCC ACT). The PCR amplification was performed on Eppendorf Mastercycler ep realplex² with the following program: 95°C for 2 min followed by 35 cycles of 10 s at 95°C, 15 s at 60°C for annealing and 20 s at 70°C for extension. The results from two independent experiments were averaged and plotted as percentage of input.

Quantitative real-time-PCR

Total RNA was isolated from HeLa cells with an integrated HIV-LTR-Luc with Trizol (Invitrogen), and 2 μg of the RNA was digested with DNase I followed by phenol/chloroform extraction. The purified RNA was reverse-transcribed with ReverTra Ace qPCR RT Kit (Toyobo) in a total volume of 10 μl. For real-time PCR amplification, 2 μl of cDNA were used as template, with same conditions described in ChIP section. The HIV-LTR-Luciferase expression level was normalized to that of β-actin. Data were represented as fold enrichment compared to untreated sample. The error bars were calculated based on three independent experiments (38). The primers were as follows: HIV-LTR-Luciferase (+1 to +59, forward: 5'-GGG TCT CTC GAG TTA GAC CAG ATC TGA, reverse: 5'-GGG TTC CCT AGT TAG CCA GAG AGC; and +468 to +593, forward: 5'-CGC AGC CTA CCG TAG TGT TTG, reverse: 5'-ACT GAA ATC CCT GGT AAT CCG TT), and β-actin (forward: 5'-ATC GTC CAC CGC AAA TGC TTC TA, reverse: 5'-AGC CAT GCC AAT CTC ATC TTG TT).

RESULTS

Separation of chromatin-bound and -free P-TEFb and its related components

To study the chromatin recruitment of P-TEFb by Brd4, we developed a modified nuclear fractionation (MONF)

protocol based on Dignam's nuclear extraction method (40) and the concept of differential extraction (25,41) for fractionating the chromatin-bound and -free P-TEFb and its related components in HeLa cells (see 'Material and Methods' section, Figure 1A). After three rounds of extraction with a low-salt buffer containing 75mM NaCl, HEXIM1, a component of the inactive 7SK snRNP complex, was completely extracted into the LSF (Figure 1B, lane 1), indicating that the chromatin-free components related to P-TEFb can be completely removed from nuclei by this procedure. Remarkably, almost all Brd4 was extracted into the HSF that contained 0.3 M NaCl (Figure 1B, lane 2). P-TEFb showed a different distribution, for about 70–80% P-TEFb extracted into LSF, with the remaining Cyclin T1 and Cdk9 extracted into HSF (Figure 1B). Of note, the P-TEFb in LSF was fully sequestered in the inactive 7SK snRNP complex, as demonstrated by that Cyclin T1, which is the less abundant subunit of

P-TEFb, was completely removed by anti-HEXIM1 and anti-LARP7 depletion of LSF (Supplementary Figure S2A, left).

To test whether the nuclear P-TEFb and Brd4 observed in HSF were those associated with chromatin, we performed DNase I digestion of LSENS, with RNasin treatment as a control (see 'Material and Methods' section, Figure 1A). When chromatin DNA was fully degraded (Figure 1C, left panel, lane 2), Sp1, a sequence-specific DNA-binding protein, was released into the supernatant, whereas Lamin B, a nuclear matrix protein, remained in the nucleus (Figure 1C, right panel). Both Brd4 and P-TEFb in LSENS were completely released into supernatant upon DNase I digestion (Figure 1C, right panel, lane 2 and 4), indicating that similar to Sp1, their presence in the LSENS depends on their association with chromatin. As Brd4 and P-TEFb in LSENS could also be fully extracted into HSF with 0.3 M NaCl (Figure 1B, lane 2), therefore, the factors detected in HSF represent the

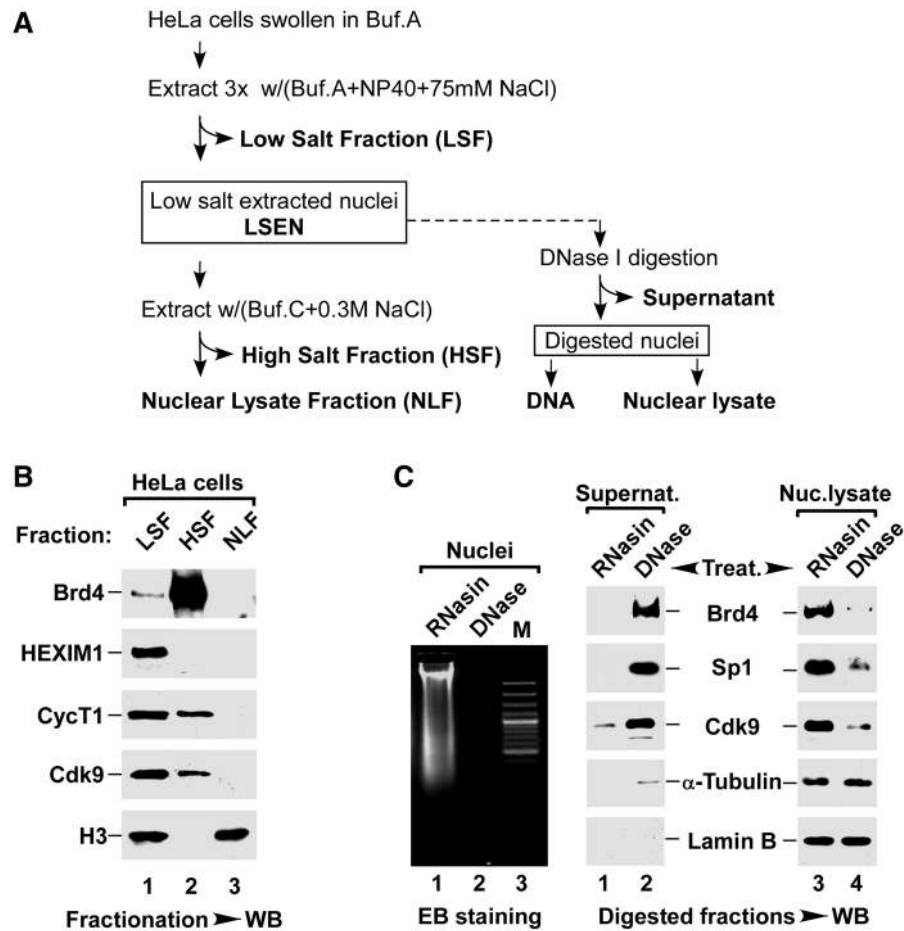


Figure 1. Almost all Brd4 is associated with chromatin in HeLa cells. (A) Schematics for modified nuclear fractionation (MONF) and DNase I digestion procedures. HeLa cells are suspended in buffer A, and the swollen cells are extracted three times with a low-salt buffer to yield low-salt fraction (LSF). The low-salt extracted nuclei (LSENS) are further extracted with a high-salt buffer to obtain high-salt fraction (HSF). Finally, the nuclear pellet is boiled in SDS-loading buffer, and saved as nuclear lysate fraction (NLF). Before further analysis, LSF, HSF and NLF are adjusted to the same volume and salt concentration. To verify that P-TEFb and its related factors in HSF reflected chromatin-bound components, LSENS are digested with DNase I and the supernatant is saved, whereas the nuclear pellet is subjected either to DNA isolation or to nuclear lysis. (B) The LSF, HSF and NLF fractions prepared from HeLa cells were analyzed by western blotting (WB) for the indicated proteins. (C) DNase I digestion assay of LSENS. Proteins in the supernatant and nuclear lysate after DNase I digestion of LSENS were analyzed by WB (right), and chromatin DNA was analyzed on ethidium bromide (EB) stained agarose gel (Left), with RNasin treatment as a control.

chromatin-bound population and those in LSF reflect the chromatin-free factors.

Almost all Brd4 targets to interphase chromatin

Brd4 has been shown targeting to chromosomes during mitosis (24). The detection of the vast majority of Brd4 in HSF indicated that almost all Brd4 also targets to interphase chromatin in HeLa cells (Figure 1B). To test whether it is a general phenomenon, we examined 10 randomly chosen human and mouse cell lines of different origins (Supplementary Figure S1). In all of them, it was consistently observed that almost all Brd4 is associated with chromatin, suggesting that chromatin targeting is a general property of Brd4 in mammalian cells.

UV or HMBA treatment induces the release of Brd4 from chromatin

Brd4 plays a key role in recruiting P-TEFb to promoters for genes' expression (22,23). Given that almost all Brd4 is already associated with chromatin in cells, we wondered how the chromatin-bound Brd4 mediates the recruitment of P-TEFb to gene promoters, especially those of the signal-inducible genes where increased P-TEFb occupancy is detected at the promoters upon stimulation (22,23). We therefore analyzed the distribution of Brd4 in chromatin-bound versus -free fractions of UV- or HMBA-treated cells. Interestingly, a significant portion of Brd4 appeared in LSF with a concomitant reduction of Brd4 in HSF of stimulated cells (Figure 2A), indicating that Brd4 was released from chromatin upon treatment. Although a large portion of Brd4 still remained on chromatin in the treated cells (HSF in Figure 2A and DNase I digestion assay in Supplementary Figure S2B), the salt-titrated extraction assay showed that 0.2 M NaCl was sufficient to remove all Brd4 from chromatin in treated cells instead of the 0.3 M salt required for the untreated cells (Figure 2B). These results suggested that the UV or HMBA treatment weakened the interaction of Brd4 with chromatin and thereby induced Brd4 release from chromatin.

Of note, after the removal of HMBA, the level of Brd4 in LSF was gradually reduced, and most Brd4 was gone from the chromatin-free fraction by 2 h (Figure 2C). Since total cellular levels of Brd4 did not change either during HMBA treatment or after HMBA removal (Figure 2C, bottom), the reduction of Brd4 in LSF indicated that the signal-released Brd4 re-associated with chromatin after HMBA withdraw. Thus, these data indicate that chromatin targeting is the default feature of Brd4 in untreated cells.

UV or HMBA treatment increases the levels of Brd4/P-TEFb complex in both chromatin-bound and -free fractions

Next, we asked whether the signal-released Brd4 plays a role in recruiting P-TEFb. It was noteworthy that upon UV or HMBA treatment, a portion of P-TEFb shifted from LSF to HSF (Figure 2A), suggesting an increased recruitment of P-TEFb to chromatin. Therefore, we analyzed the levels of Brd4 and its associated P-TEFb in

both the chromatin-bound and -free fractions in MCAP cells, a HeLa-based cell line stably expressing HA-Brd4-Flag, after anti-Flag affinity purification with excess amount of anti-Flag antibody (Figure 3A). In untreated cells, a trace amount of chromatin-free Brd4 and its associated P-TEFb was detected in LSF (Figure 3A, lane 1). Upon UV or HMBA treatment, the levels of Brd4/P-TEFb complex in LSF was dramatically elevated (Figure 3A, lanes 2 and 3), presumably due to the increased availabilities of both Brd4 that is released from chromatin (Figure 2A) and P-TEFb that is liberated from the inactive 7SK snRNP complex (Supplementary Figure S2A, right) (19). Concomitantly, more Brd4/P-TEFb complex was also detected in HSF upon UV and HMBA treatment (Figure 3A, compare lanes 5 and 6 with lane 4).

Both the chromatin-bound and signal-released Brd4 associates with P-TEFb that contains phosphorylated Cdk9-T186

The phosphorylation of T-loop of Cdk9 at Thr186 (Cdk9-pT186) is crucial for P-TEFb's kinase activity during transcription elongation (19,36). In UV- or HMBA-treated cells, however, the signal-induced liberation of P-TEFb from the inactive 7SK snRNP complex occurs after the dephosphorylation of Cdk9-pT186 (19). To examine the phosphorylation levels of the chromatin-free and -bound portions of P-TEFb, we fractionated F1C2 (stably expressing Cdk9-Flag) cells into LSF and HSF, and measured the extent of T186 phosphorylation by western blot analysis using normalized levels of total Cdk9 (Figure 3B). Consistent with the previous study (19), the extent of Cdk9-pT186 was dramatically decreased in LSF of UV- or HMBA-treated cells (Figure 3B, left). In contrast, high levels of Cdk9-T186 phosphorylation were detected in HSF from both untreated and treated cells (Figure 3B, right), indicating that the chromatin-bound P-TEFb is highly phosphorylated at Cdk9-T186.

Next, we analyzed the phosphorylation state of P-TEFb that is in association with Brd4, using the HEXIM1-bound P-TEFb isolated from the LSF of HH8 cells (a HeLa-based cell line stably expressing Flag-HEXIM1) as a control for the fully phosphorylated Cdk9-T186 (Figure 3C, lane 1) (19). Surprisingly, fully phosphorylated Cdk9-T186 was detected for the Brd4-associated P-TEFb purified from both chromatin-bound and -free fractions in UV-treated MCAP (expressing HA-Brd4-Flag) cells (Figure 3C, compare lanes 2 and 3 to lane 1). These data indicate that the signal-released Brd4 selectively associates with the transcriptionally active form of P-TEFb that has its Cdk9-T186 fully phosphorylated.

HDAC inhibitor blocks the UV- or HMBA-induced release of Brd4 from chromatin

Brd4 has been shown to associate with acetylated chromatin via its bromodomains (24,25). To test whether the UV- or HMBA-induced Brd4 release was achieved through a reduction in the acetylation levels of chromatin, we pre-treated cells with histone deacetylase (HDAC) inhibitors.

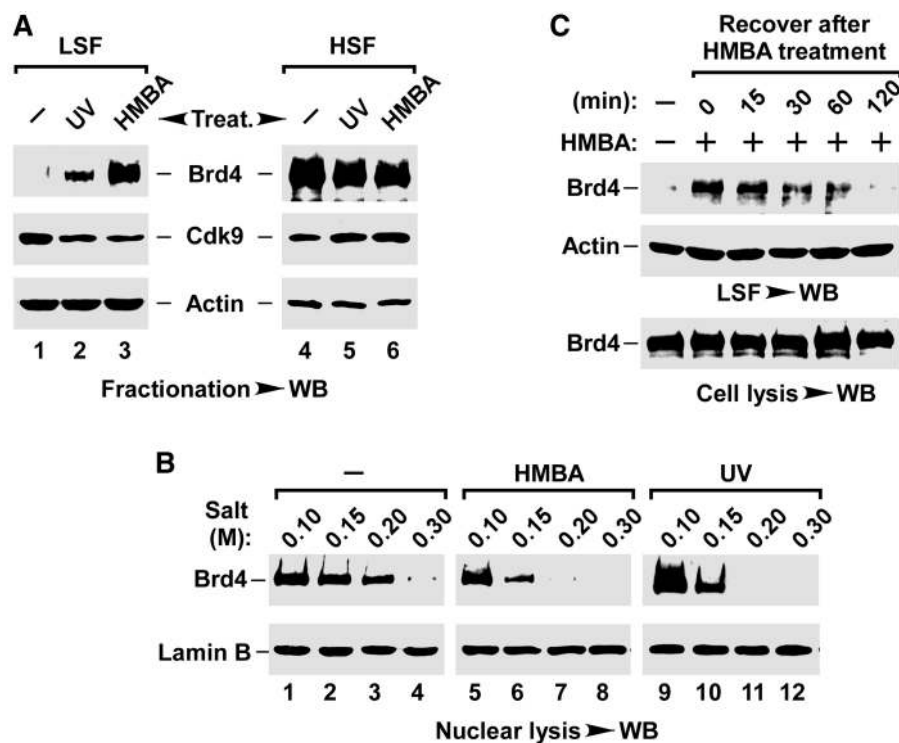


Figure 2. UV or HMBA treatment induces the release of Brd4 from chromatin. (A) LSF (left) and HSF (right) prepared from the UV- or HMBA-treated HeLa cells were subjected to western blotting (WB) for the indicated proteins. (B) Salt-titrated extraction assay of HeLa cells treated with HMBA or UV. The cells were extracted three times with low-salt buffer, and the nuclei were aliquoted and each was extracted with buffer containing indicated concentrations of NaCl. The nuclear pellets were lysed in SDS-loading buffer and analyzed by WB to determine the amount of Brd4 remained in chromatin. (C) The levels of released Brd4 in LSF (top) and the total amount of Brd4 in the cells (bottom) were determined by WB for HeLa cells that were treated with 10 mM HMBA for 4 h, and recovered in normal culture medium for the indicated period.

As expected, the Brd4 release induced by either UV or HMBA was markedly thwarted by HDAC inhibitor TSA (Figure 4A). Significantly, SBHA, a hybrid polar class HDAC inhibitor whose molecular structure is different from that of TSA (42), had a similar effect in blocking the UV- or HMBA-induced Brd4 release from chromatin (Supplementary Figure S3A). Moreover, the salt-titrated extraction assay showed that although the majority of Brd4 was extracted into buffer containing 0.15–0.2 M NaCl in HMBA-treated cells, TSA pretreatment prevented a significant portion of Brd4 from being extracted into these buffers (Figure 4B, compare lanes 7 and 8 with lanes 2 and 3). Collectively, these data indicate that the binding of Brd4 to chromatin is greatly stabilized when deacetylation is inhibited.

HDAC inhibitor blocks stimulation-induced deacetylation of nucleosomal histone H4K5ac/8ac

Brd4 has been shown to interact with both acetylated histone tails of H3 and H4 (25). Hence, the release of Brd4 from chromatin during HMBA or UV treatment is most likely caused by the deacetylation of nucleosomal histone H3 and H4. To test this idea, we examined the modification on nucleosomal histones H3 and H4 by western blot analysis of LSENs with modification-specific antibodies. Interestingly, after UV or HMBA treatment, the acetylation levels of nucleosomal histone H4 at lysine 5 (H4K5ac) and lysine 8 (H4K8ac) were reduced

(Figure 4C, compare lane 1 with lanes 3 and 5, with quantification based on three independent experiments in the bottom panel, which showed a consistent trend of reduction after treatment). In contrast, the acetylation levels of histone H4 at lysines 12 and 16 (H4K12ac, H4K16ac) (Figure 4C) and those of H3 at both lysine 9/14 (H3K9ac/K14ac), as well as the tri-methylation levels of histone H3 at lysines 4, 27 and 36 (H3K4me3, H3K27me3 and H3K36me3) (Supplementary Figure S3B), were not affected. As expected, the TSA pretreatment significantly blocked the signal-induced deacetylation of H4K5ac and H4K8ac (Figure 4C, lanes 4 and 6), suggesting the involvement of HDAC-dependent pathway(s) in HMBA- or UV-induced release of Brd4 from chromatin.

HDAC inhibitor blocks the Brd4-mediated recruitment of P-TEFb to chromatin

We reasoned that if the signal-induced Brd4 release from chromatin is essential for increased formation of Brd4/P-TEFb complex and its subsequent recruitment to chromatin, we should expect that when Brd4 release from chromatin is blocked, the signal-induced recruitment of Brd4/P-TEFb complex (Figure 3A) would also be deterred. To test this idea, we analyzed the levels of Brd4 and its associated P-TEFb in both the chromatin-bound and -free fractions in MCAP (stably expressing HA-Brd4-Flag) cells by immunoprecipitation. Indeed, as the TSA pretreatment blocked the release of

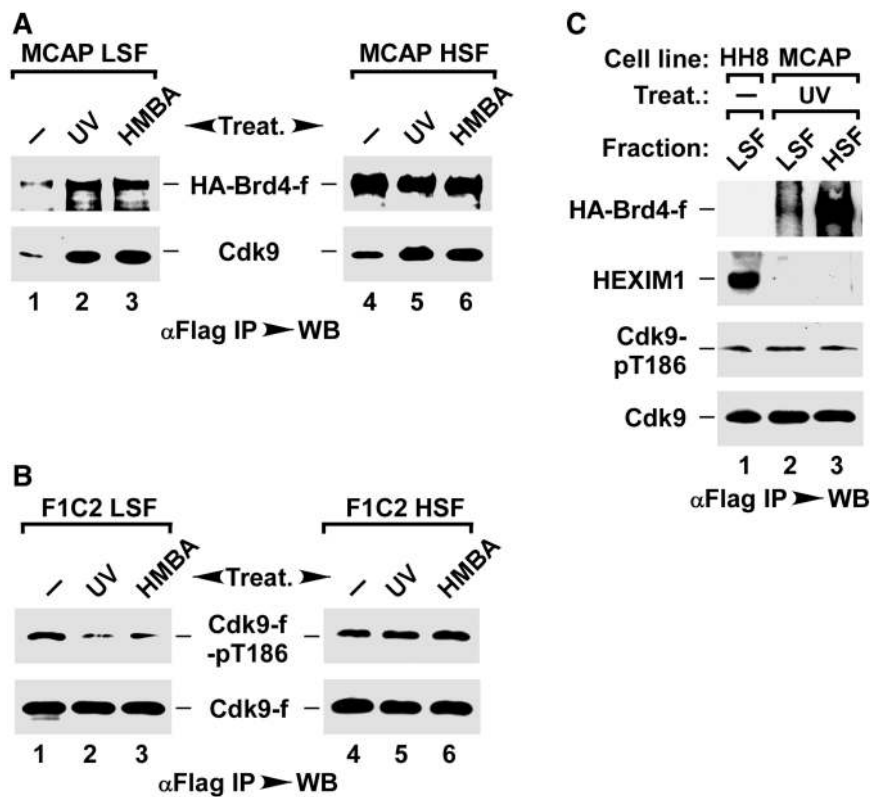


Figure 3. Both the chromatin-bound and signal-released Brd4 proteins associate with P-TEFb that contains phosphorylated CDK9 T-loop. (A) Levels of Brd4 and Cdk9 in anti-Flag immunoprecipitates from LSF (left) and HSF (right) of MCAP (HA-Brd4-f stable line) cells with indicated treatments were analyzed by western blotting (WB). (B) The levels of bulk and T186-phosphorylated (pT186) form of Cdk9 in anti-Flag immunoprecipitates derived from LSF (left) and HSF (right) of F1C2 (Cdk9-f stable line) cells treated with UV or HMBA were analyzed by WB. (C) Detection of Cdk9-T186 phosphorylation levels of Brd4-associated P-TEFb. Proteins were isolated from LSF and HSF of UV-treated MCAP cells by anti-Flag affinity purification. The levels of bulk and pT186 form of Cdk9 and indicated proteins were determined by WB, with HEXIM1-bound P-TEFb isolated from the LSF of HH8 (f-HEXIM1 stable line) cells as a control for the fully phosphorylated CDK9-T186. The sample volumes were adjusted to achieve equal loading of Cdk9 for the measurement of pT186 in (B, C).

chromatin-bound Brd4 (Figure 4A and D, LSF), it also diminished the UV- and HMBA-induced increase in the levels of Brd4/P-TEFb complex in chromatin-bound fractions (Figure 4D, HSF, compare lanes 10 and 12 with lanes 9 and 11).

Of note, neither the UV- or HMBA-induced disruption of 7SK snRNP (Supplementary Figure S3C) nor the association of P-TEFb with a Flag-tagged P-TEFb-interacting domain of Brd4 (amino acids 1260–1362) (Supplementary Figure S3D) was affected by TSA pretreatment. The expression levels of Brd4 and P-TEFb were also unaltered by these treatments (Supplementary Figure S3E). Therefore, the inhibitory effect of TSA on the signal-induced recruitment of Brd4/P-TEFb complex to chromatin is most likely achieved by impeding the Brd4 release from chromatin, which prevents the increased formation of Brd4/P-TEFb complex.

HDAC inhibitor blocks the stimulatory effect of HMBA on HIV-1 transcription

HMBA is a potent inducer for HIV-1 transcription (43), and HDAC inhibitors such as TSA can also activate HIV-1 expression (44). Thus, HMBA and HDAC inhibitors may be expected to have an additive effect in boosting

HIV-1 transcription. However, if the release of Brd4 from chromatin is essential for signal-induced P-TEFb recruitment to chromatin, HDAC inhibitors, which impede Brd4 release from chromatin (Figure 4A and Supplementary Figure S3A), would prevent HMBA from stimulating HIV-1 transcription. Therefore, we tested the combined effects of HDAC inhibitors and HMBA in HIV-LTR-Luc cells, a HeLa cell line with an integrated HIV-LTR luciferase reporter gene (19,39). Although TSA or HMBA individually stimulated HIV-1 promoter-driven luciferase activity, TSA attenuated the HMBA's stimulatory effect in a dose-dependent manner (Figure 5A). A similar effect was also observed with a different HDAC inhibitor, SBHA (Supplementary Figure S4).

Interestingly, quantitative RT-PCR analysis of the transcripts corresponding to the initiation and elongation regions (Figure 5B, top) (45) indicated that TSA augmented transcription mainly at the stage of initiation, whereas the stimulatory effect of HMBA occurred mostly at the stage of elongation (Figure 5B, bottom). When combined, TSA inhibited the HMBA-induced HIV-1 transcription specifically at the stage of elongation, rather than initiation (Figure 5B), similar to the effect of Brd4 knockdown by shRNA (Figure 5C).

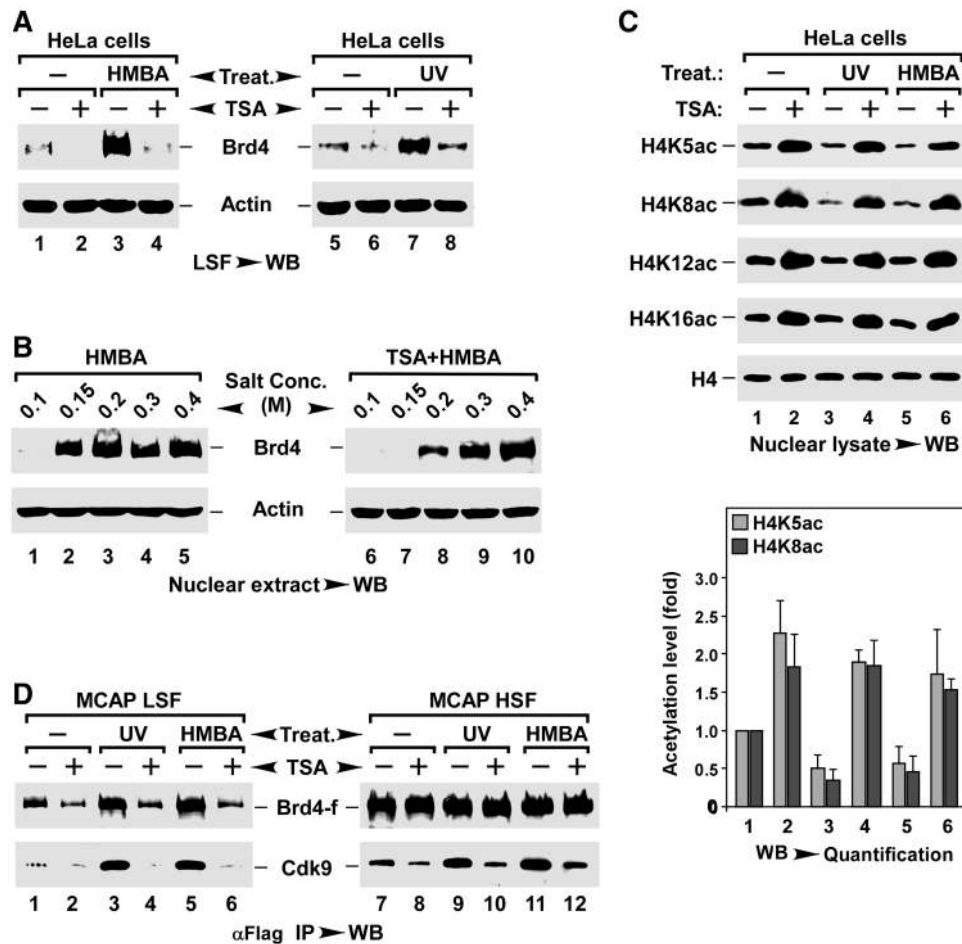


Figure 4. Impeding Brd4 release from chromatin abolishes Brd4-mediated P-TEFb recruitment. (A) The effect of TSA treatment on stimulation-induced Brd4 release. Brd4 in LSFs prepared from HeLa cells treated with UV (left) or HMBA (right) either with or without TSA pretreatment as indicated were analyzed by western blotting (WB). (B) Salt-titrated extraction of HeLa cells treated with TSA and/or HMBA. The cells were extracted with low-salt buffer to remove chromatin-free Brd4, and subsequently the nuclei were extracted with buffer containing indicated concentrations of NaCl. The levels of released Brd4 were analyzed by WB. (C) Effect of TSA treatment on stimulation-induced nucleosomal histone H4 deacetylation. WB analysis of nucleosomal histone H4 acetylation levels (top) with quantification (bottom, averaged from three independent experiments) for HeLa cells treated with UV or HMBA either with or without TSA pretreatment as indicated. (D) Effect of TSA pretreatment on the levels of UV- or HMBA-induced Brd4/P-TEFb complex. Anti-Flag immunoprecipitates from LSF (left) and HSF (right) of MCAP (HA-Brd4-f stable line) cells with indicated treatments were analyzed by WB.

To test whether the inhibitory effect of TSA on HMBA-stimulated HIV-1 transcriptional elongation was due to the inhibition of Brd4-mediated P-TEFb recruitment to HIV-1 promoter, we performed ChIP assay of HIV-LTR-Luc cells with anti-Cdk9 antibody. As expected, whereas HMBA enhanced the recruitment of P-TEFb onto HIV-1 promoter, this enhancement was markedly inhibited by TSA (Figure 5D).

Collectively, these data indicate that the signal-induced release of Brd4 from chromatin is essential for mediating P-TEFb recruitment to stimulate transcriptional elongation.

Ectopic expression of the ΔC -mutant of Brd4 induces the release of endogenous Brd4 from chromatin and augments HIV-1 transcription

Although the data presented so far strongly suggest that signal-triggered Brd4 release from chromatin is critical for

signal-induced elevation in HIV-1 transcription, we, however, cannot formally exclude the possibility that HDAC inhibitors and UV/HMBA treatments may influence transcription by other means. To avoid the side effects of these treatments, we tested whether the forced release of endogenous Brd4 from chromatin by the ectopic expression of HA- ΔC could enhance transcription. HA- ΔC , a HA-tagged Brd4 mutant (amino acids 1–721), which contains two bromodomains but lacks the P-TEFb interaction domain (PID), could associate with chromatin (Supplementary Figure S5A), but is unable to bind to P-TEFb (Figure S5B). HA-PID, a mutant that contains the P-TEFb interacting domain but not the bromodomains (amino acids 1260–1362), could associate with P-TEFb but not chromatin (Supplementary Figure S5A and B), and was used as a control.

While the ectopic expression of HA-PID did not alter the level of endogenous Brd4 in LSF (Figure 6A, compare

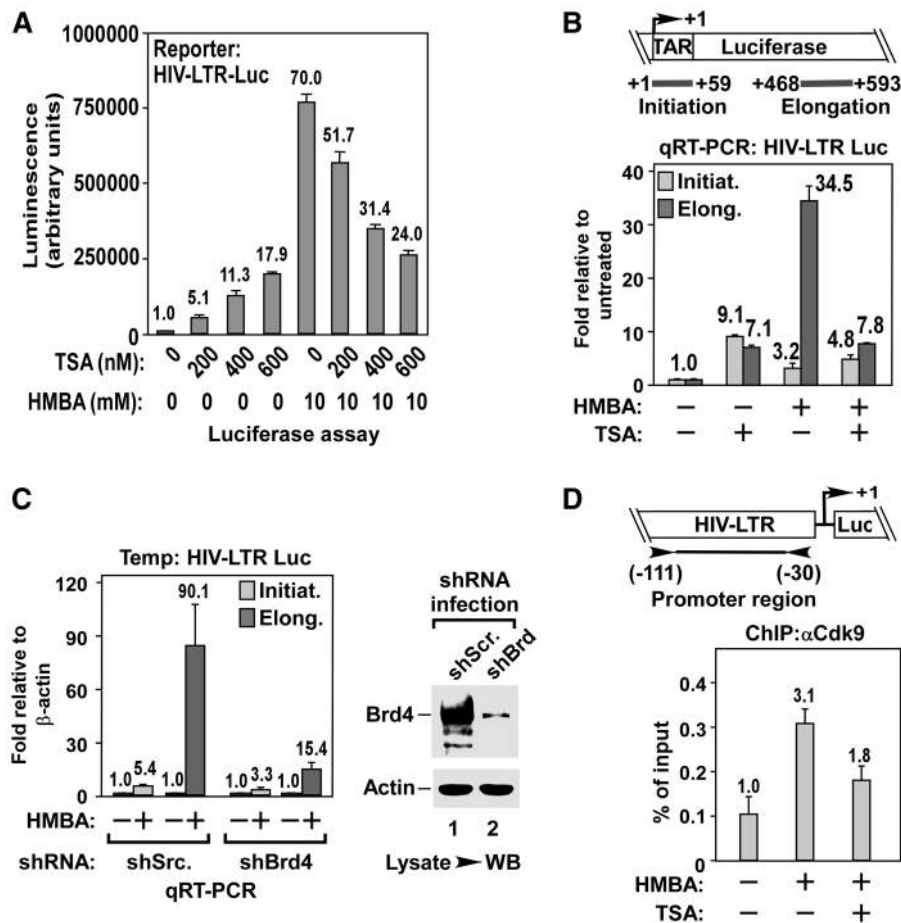


Figure 5. HDAC inhibitor blocks HMBA-stimulated HIV-1 transcriptional elongation. (A) Effect of HDAC inhibitor TSA on HMBA-stimulated HIV-1 expression. Luciferase activities of the cell lysates from HeLa cells with an integrated HIV-1 LTR-luciferase gene that were pretreated with the indicated amounts of TSA before incubated with HMBA were plotted based on three independent experiments. (B) The levels of transcripts corresponding to the regions of initiation (+1 to +59, TAR) or elongation (+496 to +593, luciferase) as illustrated on the top (45) were detected by qRT-PCR. HeLa cells with an integrated HIV-1 LTR-luciferase gene were pretreated with 400 nM TSA before incubated with 10 mM HMBA. The luciferase activities based on three independent experiments were plotted. (C) Effects of infection with lentivirus expressing scrambled shRNA or Brd4 shRNA on the levels of HMBA-induced HIV-1 transcription were measured by qRT-PCR (left) as in (B), and the results based on three independent experiments were plotted, with those untreated with HMBA setting to 1. The efficiency of Brd4 knockdown was analyzed by WB with β -actin as the loading control (right). (D) Chromatin immunoprecipitation (ChIP) assay with anti-Cdk9 antibody for HeLa cells with an integrated HIV-1 LTR-luciferase gene treated with TSA and/or HMBA. The precipitated DNA was analyzed by real-time PCR with the primers matching to the promoter region (top), and plotted as the percentage of input (bottom) based on two independent experiments.

lane 3 with lane 1), the ectopically expressed HA- Δ C increased the level of endogenous Brd4 in LSF (Figure 6A, compare lane 2 with lane 1), presumably by competitive binding of HA- Δ C to chromatin and thereby displacing a portion of endogenous Brd4 from chromatin. Accordingly, the ectopic expression of HA- Δ C, but not HA-PID, also enhanced HIV-1 transcription (Figure 6B), whereas both of them did not affect the levels of endogenous Brd4, P-TEFb and HEXIM1 (Supplementary Figure S5C). Taken together, these data support the notion that the availability of Brd4 in the chromatin-free state is limiting for gene transcription, and one mechanism for signal-induced transcriptional regulation is by modulating the amount of Brd4 mobilized from chromatin-bound into chromatin-free state for the subsequent P-TEFb recruitment and transcriptional elongation.

DISCUSSION

Combined with previous studies (19,36), the data presented above are consistent with a model (Figure 7) in which external signals trigger two parallel events: the liberation of P-TEFb from inactive 7SK snRNP and the release of Brd4 from chromatin. In untreated cells, most of the chromatin-free P-TEFb is sequestered in the inactive 7SK snRNP and almost all Brd4 is associated with acetylated chromatin. External stimuli, such as HMBA or UV, trigger the dephosphorylation of Cdk9 T-loop at T186 by the cooperative action of the activated phosphatases PP2B and PP1 α pathways, thereby liberating P-TEFb from the inactive 7SK snRNP (19). Meanwhile, the stimuli also induce the deacetylation of nucleosomal histone H4, and subsequently the release of Brd4 from chromatin by yet-to-be identified HDAC-dependent pathway. Through selective interaction with

the free P-TEFb that has its Cdk9 re-phosphorylated at T186 by an unknown kinase, the released Brd4 mediates the recruitment of the transcriptionally active form of P-TEFb to the promoter region via the interaction between Brd4/P-TEFb complex and specific adaptor

factors. Upon its arrival at the promoter region, P-TEFb catalyzes the phosphorylation of Ser2 at the CTD of Pol II, thereby leading to productive elongation. After the termination of the external signals, the released Brd4 re-associates with the acetyl-nucleosome until the next round of stimulation. This model incorporates two signal-induced processes, namely the liberation of P-TEFb from inactive 7SK snRNP and the release of Brd4 from chromatin for the subsequent recruitment of P-TEFb to chromatin to facilitate the expression of signal-inducible genes.

The observation that almost all Brd4 is associated with interphase chromatin in untreated cells is intriguing. Although Brd4 has been found to associate with chromosome during mitosis with little free Brd4 elsewhere as detected by immunofluorescence assays more than a decade ago (24), whether it has a similar distribution during interphase is unclear. Our data reveal, for the first time, that almost all Brd4 is associated with chromatin during interphase as well, and that it is a general phenomenon in mammalian cells (Figure 1B and Supplementary Figure S1). In addition, the observation that Brd4 re-associates with chromatin after the termination of stimulation (Figure 2C) indicates that chromatin targeting is the default feature of Brd4.

How the chromatin-associated Brd4 switches to a transcriptional regulation mode in response to stimulation has been enigmatic (20). The observation that Brd4 is released from chromatin upon UV or HMBA treatment (Figure 2A) provides a pivotal clue to this question. Our subsequent data demonstrated that the signal-released Brd4 selectively interacts with the transcriptionally active

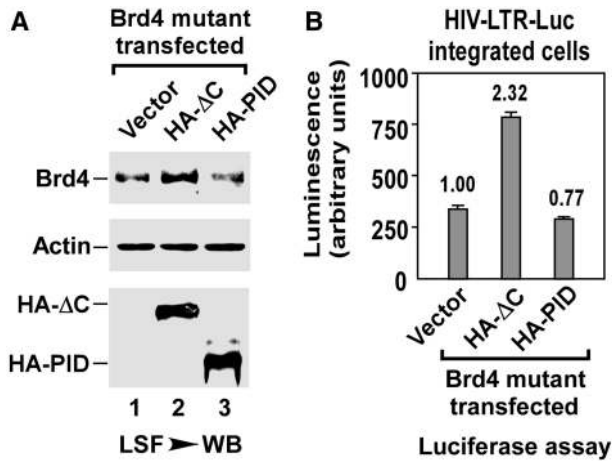


Figure 6. The forced release of endogenous Brd4 from chromatin by Δ C-mutant of Brd4 augments HIV-1 transcription. (A) The levels of endogenous Brd4 in LSFs from HeLa cells transfected with empty vector, HA-tagged Δ C-mutant of Brd4 (HA- Δ C) or HA-tagged PID (P-TEFb-interacting domain) of Brd4 (HA-PID) were analyzed by western blotting (WB). The levels of HA- Δ C and HA-PID in cell lysates were also detected by WB (bottom). (B) Luciferase activities of HIV-LTR-Luc integrated HeLa cells transfected with indicated Brd4 mutant constructs were plotted based on three independent experiments.

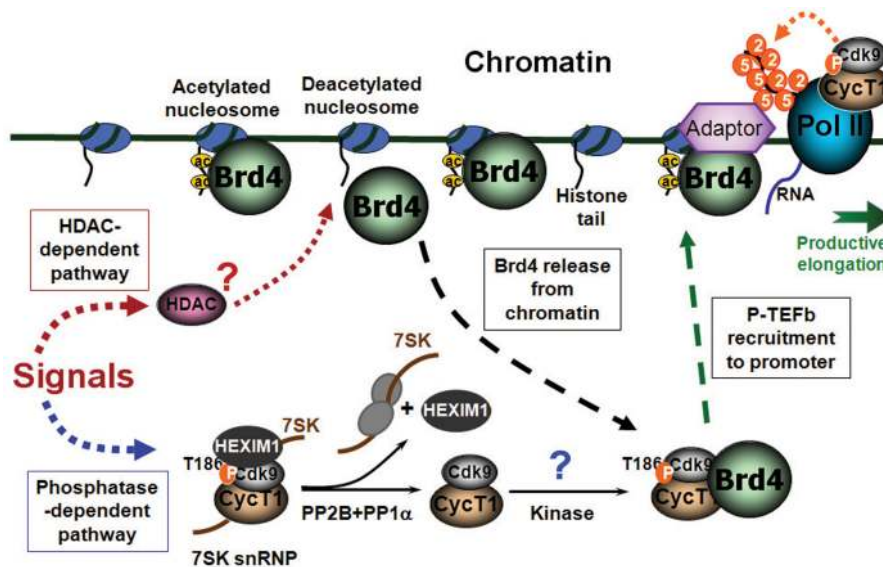


Figure 7. A model depicting the signal-induced functional transition of Brd4 from chromatin targeting to transcriptional regulation. In the absence of the external stimuli, almost all Brd4 is associated with acetylated chromatin (indicated as acetyl-nucleosome). The signals trigger histone H4 deacetylation (red dash line) by activating yet-to-be identified HDAC(s) (red question mark), which causes the release of Brd4 from chromatin (black dash line). Meanwhile, the signals also induce the dephosphorylation of Cdk9 at T186 (blue dash line) by the cooperative actions of the activated phosphatases (PP2B and PP1 α), thereby liberating P-TEFb from the inactive 7SK snRNP (19). Through selective association with the transcriptionally active form of P-TEFb that has its Cdk9T-loop re-phosphorylated by unknown kinase(s) (blue question mark), the released Brd4 mediates the recruitment of active P-TEFb to promoter-proximal region (green dash line), where P-TEFb modulates the processivity of Pol II, thereby leading to productive elongation. This recruitment is most likely through recognizing adaptor factors, such as Mediator or sequence-specific transcription factors.

form of P-TEFb (Figure 3) and mediates the recruitment of this active form of P-TEFb to chromatin (Figures 3 and 4) to enhance the transcription elongation of signal-inducible gene (Figure 5), thereby uncovering the transition process of Brd4 from chromatin-targeting state to transcriptional regulation mode. Of note, the Brd4 release alone, although essential, is insufficient for its functional transition, since almost all chromatin-free P-TEFb is sequestered in the inactive 7SK snRNP in untreated cells (Supplementary Figure S2A, left). The signal-induced liberation of P-TEFb from inactive complex during treatment (Supplementary Figures S2A, right and S3C) (19) is also necessary, as demonstrated by that the enhancement of transcription by the ectopic expression of HA- Δ C-Brd4 (Figure 6B) is much lower than that by HMBA treatment (Figure 5A), as HA- Δ C-Brd4 did not trigger the liberation of P-TEFb from inactive 7SK snRNP complex (Supplementary Figure S5D), whereas HMBA did (Figure S3C and refs. 19 and 36).

During UV or HMBA treatment, although the molecular details of the signal-induced release of Brd4 from chromatin are far from clear, the blocking effect of HDAC inhibitors on Brd4 release (Figure 4A and Supplementary Figure S3A) suggests that HDAC-dependent pathway(s) are involved, which is corroborated by the observation that UV or HMBA treatment triggered the deacetylation of nucleosomal histone H4K5ac and H4K8ac (Figure 4C). Whether the deacetylation at both H4K5 and K8 is necessary for Brd4 release from chromatin is currently unknown, but most likely the deacetylation at one position is sufficient to weaken the interaction between Brd4 and chromatin, as Brd4 mutants lacking either bromodomain associate more weakly with chromatin than the Brd4 with both bromodomains (25). In line with this feature, the salt-titrated extraction assay showed that the complete extraction of Brd4 from chromatin required 0.3 M salt in untreated cells, whereas 0.2 M salt is sufficient in cells treated with either UV or HMBA (Figure 2C). Hence, we may envision that most Brd4 binds to acetylated lysines via both bromodomains in untreated cells, and, upon treatment, it loses one or both contacts with chromatin. Remarkably, although Brd4 has been shown to bind di-acetylated histone H3 at K9/K14, di-acetylated H4 at K5/K12 and tetra-acetylated H4 at K5/8/12/16 (25), both UV and HMBA only caused the deacetylation of H4K5ac and H4K8ac but did not change the acetylation levels at other lysine residues on H3 and H4 tails (Figure 4C and Supplementary Figure S2B). Whether such 'selective deacetylation' is common for other types of stimuli and whether it implies a 'histone code' for signal-induced Brd4 release from chromatin are fascinating questions for future investigation.

The observation that Brd4 interacts with the transcriptionally active form of P-TEFb (Cdk9-T186 phosphorylated) in both chromatin-free and -bound fractions (Figure 3C) seemingly contradicts with our previous report of Brd4's association with the inactive form of P-TEFb (Cdk9-T186 dephosphorylated) (19). This is an important issue, as the phosphorylation of Cdk9-T186 is crucial for P-TEFb's kinase activity (19,36). A major

technical difference is that the dialyzed NE was used in the previous study (19). Therefore, we compared the phosphorylation state of Brd4-associated P-TEFb in dialyzed versus undialyzed NE, and found that Brd4 in undialyzed NE selectively binds to the P-TEFb with the phosphorylated Cdk9-T186, and that artificial dephosphorylation at Cdk9-T186 of Brd4-bound P-TEFb occurred during dialysis (see Supplementary Figure S6A). As the PID (amino acids 1260–1362) region of Brd4 is responsible for interacting with P-TEFb (26), we tested whether it possesses selectivity for T186-phosphorylated Cdk9. Similar to full-length Brd4, PID also selectively bound to the P-TEFb with fully phosphorylated Cdk9-T186 in HMBA-treated cells (Supplementary Figure S6B, lane 2). Collectively, these data demonstrated that Brd4 selectively interacts with the transcriptionally active (Cdk9-T186 phosphorylated) form of P-TEFb.

Since the dephosphorylation at Cdk9-pT186 is necessary for the liberation of P-TEFb from 7SK snRNP complex (Figure 3B and ref. 19), the association of Brd4 with phosphorylated P-TEFb in chromatin-free fraction indicates that re-phosphorylation of Cdk9-T186 of the liberated P-TEFb should have occurred before P-TEFb is recruited to chromatin. How the signal-liberated P-TEFb, which is dephosphorylated at Cdk9 T-loop by the concerted action of the activated PP2B and PP1 signal pathways (19), is converted to the phosphorylated form remains unclear. The observation that Brd4 could interact with dephosphorylated P-TEFb in the dialyzed NE implies that the dephosphorylated P-TEFb might bind Brd4 initially, but quickly becomes re-phosphorylated by an unknown kinase thereafter. A most recent study showed that the calcium/calmodulin-dependent kinase CaMK1D is involved in the regulation of the stability of Cdk9 as well as its phosphorylation levels of Cdk9-T186, but it is unlikely to be a direct kinase for Cdk9-T186 (46). So, for now, the identity of the kinase for Cdk9-T186 phosphorylation remains elusive.

To activate gene expression, Brd4/P-TEFb must be recruited to gene promoters. It has been assumed that the two bromodomains of Brd4 are responsible for the recruitment of Brd4 to gene promoters during transcriptional activation. However, if the deacetylation of nucleosomes leads to the release of Brd4 from chromatin, it is paradoxical that Brd4 is recruited to promoters for activating gene expression. Intuitively, the Brd4-mediated P-TEFb recruitment must be targeted to specific gene promoters. Therefore, it is likely that adaptor factors at gene promoters confers specificity for Brd4 recruitment. It has been reported that Brd4 co-purifies with Mediator components (23,47). Moreover, Brd4 was shown to bind to acetylated NF- κ B for transcriptional activation (48). Furthermore, Brd4 is able to recruit P-TEFb to naked DNA template in an *in vitro* study (22), indicating that factors other than acetylated nucleosome are at play. Based on these observations, it is very likely that in addition to acetylated nucleosomes, adaptors such as Mediator and/or sequence-specific transcription factors can draw Brd4/P-TEFb complex to specific promoters for transcriptional activation. During UV/HMBA-induced

global deacetylation, the adaptors may recruit Brd4/P-TEFb complex either independent of acetylated histones or together with locally acetylated histones.

In summary, this study reveals that most Brd4 targets to chromatin not only in mitosis but also during interphase. Evidently, not all chromatin-associated Brd4 functions in P-TEFb recruitment, but a portion of Brd4 on chromatin is stored in a passive reservoir at unstimulated state. Upon UV or HMBA treatment, signal-induced histone deacetylation leads to its release from chromatin, thereby switching Brd4's role from chromatin targeting to transcriptional regulation. The proposed missing links such as the unspecified HDAC(s) signal pathway for Brd4 release from chromatin, and the unknown kinase for the re-phosphorylation of Cdk9 T-loop (Figure 7), as well as the detailed molecular mechanism for recruitment of Brd4/P-TEFb complex to promoter-proximal region await future investigation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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