Molecular Cell, Vol. 19, 523–534, August 19, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.molcel.2005.06.027

The Bromodomain Protein Brd4 Is a Positive Regulatory Component of P-TEFb and Stimulates RNA Polymerase II-Dependent Transcription

Moon Kyoo Jang,[1] Kazuki Mochizuki,[1] Meisheng Zhou,[3] Ho-Sang Jeong,[2] John N. Brady,[3] and Keiko Ozato[1,*]

[1]Laboratory of Molecular Growth Regulation
[2]Laboratory of Molecular Genetics
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892
[3]Virus Tumor Biology Section
Basic Research Laboratory
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20892

Summary

Brd4 is a mammalian bromodomain protein that binds to acetylated chromatin. Proteomic analysis revealed that Brd4 interacts with cyclinT1 and Cdk9 that constitutes core positive transcription elongation factor b (P-TEFb). Brd4 interacted with P-TEFb in the living nucleus through its bromodomain. About half of P-TEFb was bound to the inhibitory subunit and functionally inactive. Brd4 interacted with P-TEFb that was free of the inhibitory subunit. An increase in Brd4 expression led to increased P-TEFb-dependent phosphorylation of RNA polymerase II (RNAPII) CTD and stimulation of transcription from promoters in vivo. Conversely, a reduction in Brd4 expression by siRNA reduced CTD phosphorylation and transcription, revealing that Brd4 is a positive regulatory component of P-TEFb. In chromatin immunoprecipitation (ChIP) assays, the recruitment of P-TEFb to a promoter was dependent on Brd4 and was enhanced by an increase in chromatin acetylation. Together, P-TEFb alternately interacts with Brd4 and the inhibitory subunit to maintain functional equilibrium in the cell.

Introduction

Many nuclear proteins that interact with chromatin contain bromodomains. Among them are proteins of the conserved BET family that carry two tandem bromodomains and an ET domain. Mammalian Brd4 studied here belongs to the BET family (Dey et al., 2000; Jeanmougin et al., 1997). Previous in vitro studies showed that bromodomains recognize acetylated histones and act as a module capable of deciphering acetylated histone codes (Dhalluin et al., 1999; Jacobson et al., 2000; Strahl and Allis, 2000). Studies of yeast and mammalian BET family proteins indicate that they recognize acetylated chromatin in vivo and regulate transcription and cell growth (Crowley et al., 2002; Denis et al., 2000; Dey et al., 2000; Houzelstein et al., 2002; Ladurner et al., 2003; Maruyama et al., 2002; Matangkasombut and Buratowski, 2003).

Brd4 (formerly MCAP) is a ubiquitously expressed nuclear protein of 200 kDa (Dey et al., 2000) that plays a role in the regulation of cell growth (Maruyama et al., 2002; Farina et al., 2004). Consistent with a role in cell growth, Brd4$^{-/-}$ mice are embryonic lethal (Houzelstein et al., 2002). By photobleaching assays, we showed that Brd4 and Brd2, another BET member, are mobile in the nucleus and transiently associate with acetylated chromatin, as has been reported for other nonhistone nuclear proteins (Dey et al., 2003). Interaction of Brd4 with acetylated chromatin is due to its affinity for acetylated histones H4 and H3, as has been shown for Brd2 (Kanno et al., 2004). Another salient feature of the BET proteins is that they remain bound to chromatin during mitosis, unlike other bromodomain proteins that are released from chromatin at that stage, suggesting their role in the transmission of memory across cell division (Dey et al., 2000, 2003; Kanno et al., 2004).

Based on the documented role for other BET proteins in transcription (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003; Denis et al., 2000; Kanno et al., 2004), Brd4 may also participate in transcription. Supporting this view, the human transcriptional mediator complex is reported to contain Brd4 or a Brd4-like protein (Jiang et al., 1998). However, the role of Brd4 in transcription has not been reported so far. Transcription initiation is a complex step that is coupled to elongation and mRNA processing (Proudfoot et al., 2002; Sims et al., 2004). Phosphorylation of serine 5 (Ser5) of the C-terminal domain (CTD) of the largest subunit of RNAPII by TFIIH signals the transition from initiation to elongation/promoter clearance. In addition, the P-TEFb is recruited to phosphorylate serine 2 (Ser2) of the CTD and releases promoter from negative elongation factors (Price, 2000; Zhou et al., 2000). P-TEFb is a heterodimer composed of cyclinT1, T2 or K, and cyclin-dependent kinase 9 (Cdk9) (Fu et al., 1999; Peng et al., 1998). Evidence based on inhibitor experiments indicates that P-TEFb is involved in most of RNAPII-dependent transcription (Chao and Price, 2001). Consistent with this view, cyclinT1/Cdk9 are shown to localize to transcriptionally active sites in the nucleus/chromosomes, although not all of them are associated with highly phosphorylated RNAPII (Herrmann and Mancini, 2001; Lis et al., 2000). P-TEFb binds to HIV Tat, a viral transactivator, and facilitates viral transcription and replication (Wei et al., 1998; Zhou et al., 2000). P-TEFb is also recruited to cellular promoters by interacting with a variety of transcription factors (Barboric et al., 2001; Kanazawa et al., 2003; Simone et al., 2002). Nevertheless, the mechanism regulating the recruitment of P-TEFb to a wide range of cellular promoter remains incompletely understood.

~50% of total P-TEFb in the cells is reversibly bound to the inhibitory subunit composed of 7SK small nuclear RNA (7SK snRNA) and MAQ1/HEXIM1 and ren-

*Correspondence: ozatok@nih.gov

dered kinase inactive (Nguyen et al., 2001; Yang et al., 2001; Chen et al., 2004; Michels et al., 2003; Yik et al., 2003). Upon dissociation of the inhibitory subunit, P-TEFb regains kinase activity and the ability to stimulate transcription. It is of importance to gain further understanding of the other pool of P-TEFb that is transcriptionally active and free of the inhibitory subunit.

We show that Brd4 is bound to P-TEFb without the inhibitory subunit, and it positively regulates the activity of P-TEFb in transcription. Both Brd4 and P-TEFb were recruited to the integrated HIV promoter in vivo, and their recruitment was enhanced by increased chromatin acetylation. Collectively, our data provide convincing evidence that Brd4 acts as a positive regulatory component of P-TEFb.

Results

Purification of the Brd4 Complex

In this study, the tandem affinity purification method (Ogryzko et al., 1998) was employed to identify proteins associated with Brd4. A murine Brd4 cDNA tagged with HA and FLAG at the N- and C terminus, respectively, (h-Brd4-f) was cloned in a retroviral vector and stably expressed in HeLaS3 cells. Nuclear extracts (NEs) from these cells were subjected to two-step immuno-affinity chromatography, and the immunopurified Brd4 complexes were separated on SDS gels. The identity of these bands was determined by MALDI-TOF and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Figure 1A). Two major bands of 85 kDa and 37 kDa copurified with h-Brd-f were identified as human cyclinT1 and Cdk9, respectively (Figure 1B and Table S1 available in the Supplemental Data with this article online). These bands were not found in control precipitates with FLAG-HA alone (control lanes in Figure 1B). CyclinT1 is a component of P-TEFb associated with Cdk9 (Peng et al., 1998; Price, 2000). Immunoblotting confirmed that Cdk9 and cyclinT1 were present in the Brd4 complex (Figure 1B, lanes 5–8).

Although less abundant than cyclinT1 and Cdk9, several components of the TRAP-Mediator complex, TRAP230, TRAP170, TRAP100, and TRAP80 (Fondell et al., 1996), were also found in h-Brd4-f precipitates, the results consistent with the previous report (Jiang et al., 1998) (Figure 1B). Immunoblot analysis confirmed the presence of TRAP230, TRAP100, and TRAP80 in the immunoprecipitates. Also revealed in immunoblotting was the presence of TRAP220 in the complexes, although this component was not conclusively verified by mass spectrometry (data not shown).

In Figure 1C, the h-Brd4-f complex prepared from NEs without dialysis also contained Cdk9 and cyclinT1, indicating that the interaction of Brd4 with P-TEFb is not due to an artificial dialysis product. We also examined whether Brd4-P-TEFb interaction is detectable in total cell extracts (TCEs), which were prepared as previously reported (Nguyen et al., 2001). Brd4 and cyclinT1/Cdk9 were coprecipitated from TCEs (Figure 1D). However, the cytoplasmic fraction contained little Brd4-P-TEFb complexes relative to the nuclear fraction (data not shown). These results strongly indicate that Brd4 associates with P-TEFb in the nucleus.

Endogenous Brd4 Interacts with P-TEFb

To further substantiate the specificity of Brd4-P-TEFb interaction, additional immunoblots were performed (Figure 2A). Besides cyclinT1, cyclinT2a/b, another Cdk9 partner, was present in the Brd4 complex, but other Cdks and cyclins were not. In Figure 2B, we tested whether other bromodomain proteins beside Brd4 interact with P-TEFb and found that among Brd4, Brd2, PCAF, and the N-terminal region of TAFII250 (TAFII250N; Kanno et al., 2004), only Brd4 coprecipitated cyclinT1 and Cdk9. To ascertain whether endogenous Brd4 interacts with P-TEFb, coimmunoprecipitation (CoIP) analysis was carried out with NEs from untransduced HeLa cells. In Figure 2C, anti-Brd4 antibody coprecipitated cyclinT1 and Cdk9, but preimmune sera did not. Reciprocally, anti-Cdk9 and anti-cyclinT1 antibodies both coprecipitated endogenous Brd4, whereas control IgG did not (Figure 2D).

To assess relative amounts of cyclinT1/Cdk9 bound to Brd4, immuno-depletion experiments were performed (Figure 2E). HeLa NEs were passed through anti-Brd4 antibody beads three times, which largely removed Brd4 from the extracts; in contrast, passage through preimmune IgG beads did not remove Brd4. Brd4 depletion led to partial removal of the heterodimer, in that ~50% of cyclinT1/Cdk9 remained in Brd4-depleted extracts, suggesting that about half of total cyclinT1/Cdk9 in the extracts interacted with Brd4.

Domain Analysis

To assess the domain in cyclinT1 required for interaction with Brd4, GST-cyclinT1 and deletion constructs were tested for their binding to ^{35}S-labeled Brd4 (Figures 3A and 3B). Full-length cyclinT1 and the deletion containing the central region (255–534) interacted with Brd4, whereas the N-terminal region (1–290) and C-terminal region (535–726) of cyclinT1 did not. GST-Cdk9 or GST itself did not interact with Brd4. Additional truncations found the central region (426–516) to be important for interaction with Brd4 (Figures 3A and 3B). Supporting these data, deletion of the central region abolished Brd4 binding. This region is similar in cyclinT1 and cyclinT2a/b (Peng et al., 1998) and is adjacent to the histidine-rich domain important for binding to RNAPII CTD (Taube et al., 2002). A Brd4 deletion, ΔC-term, that retained both bromodomains showed the same binding activity as wild-type (wt) Brd4.

To determine domains of Brd4 that interact with cyclinT1, wt Brd4 and its deletions were tested for binding with GST-cyclinT1 (Figures 3C and 3D). Whereas wt Brd4 and ΔC-term bound to cyclinT1, ΔBDI&II lacking both bromodomains did not. ΔBDI and ΔBDII, each lacking a single bromodomain, showed reduced binding, indicating that cyclinT1 interacts with Brd4 through either bromodomain. In Figures 3E and 3F, domain requirement was tested in vivo by CoIP. Brd4 and other deletions except for ΔBDI&II coprecipitated cyclinT1 and Cdk9, exhibiting the same domain requirement as that in vitro (Figure 3E). The binding of single bromodomain deletions to cyclinT1 to show a similar strength with that of wt Brd4 in vivo suggests a slight difference in binding affinity in vivo and in vitro. Consistent with data in Figure 3B, cyclinT1 lacking the central region failed to coprecipitate Brd4 (Figure 3F).

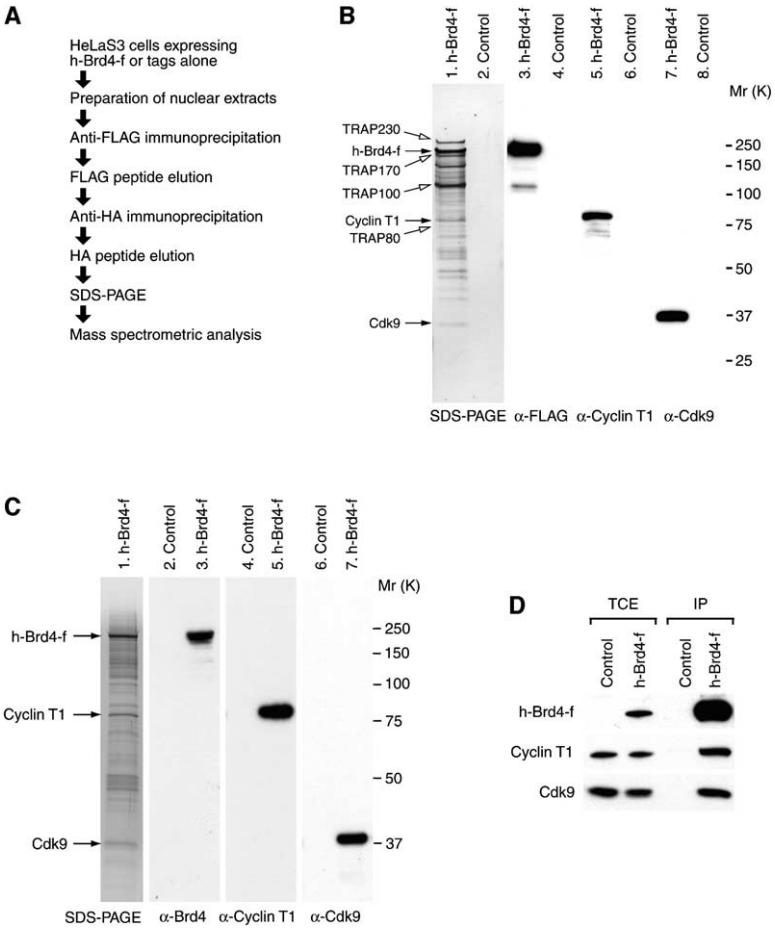

Figure 1. Brd4 Interacts with CyclinT1/Cdk9

(A) Two-step purification scheme to identify Brd4-associated proteins.

(B) Immunopurified h-Brd4-f complexes were fractionated on SDS-PAGE. Bands were analyzed by mass spectrometry and immunoblotting. Lanes 1 and 2, colloidal-blue staining. The positions of h-Brd4-f, P-TEFb subunits, and components of TRAP complex are marked by filled arrows and open arrows, respectively. Molecular size markers are shown on the right. Lanes 3–8, the complexes were immunoblotted with indicated antibodies.

(C) HeLaS3 NEs without dialysis were tested for coprecipitation of Brd4 and P-TEFb as in (B).

(D) TCEs with h-Brd4-f or control (FLAG-HA alone) were tested for coprecipitation. The precipitates were analyzed by immunoblotting with antibodies shown on the left. Abbreviations: TCE, input for total cell extracts and IP, FLAG immunoprecipitates.

Brd4 Interacts with CyclinT1/Cdk9 in Living Cells

CyclinT1 and Cdk9 distribute throughout the nonnucleolar space in the nucleus, forming speckle-like foci (Herrmann and Mancini, 2001). Brd4 also distributes as fine speckles in the nucleus except for the nucleoli (Dey et al., 2000). To examine colocalization of Brd4 and cyclinT1, immunostaining was performed for endogenous Brd4 in NIH3T3 cells expressing GFP-cyclinT1. In Figure 4A, both proteins distributed throughout the nucleus, but not in nucleoli. Similar to endogenous cyclinT1, GFP-cyclinT1 showed speckle-like structures and colocalized with Brd4 in various areas in the nucleus. CyclinT1 exhibits limited colocalization with RNAPII (Herrmann and Mancini, 2001; Lis et al., 2000). Staining of phosphorylated RNAPII revealed speckles of various sizes in the nucleus, but not the nucleoli. The superimposition of three images produced scattered merged signals in many areas, indicating colocalization of the three proteins.

To verify that Brd4 interacts with cyclinT1/Cdk9 in living cells, we utilized the bimolecular fluorescence complementation (BiFC) method (Hu et al., 2002), one of few methods that permit visualization of protein-protein interactions in living cells. This method also reveals the intracellular site of interaction of the proteins. CyclinT1 was fused to the N-terminal fragment of YFP (YN), whereas Brd4 was fused to the C-terminal fragment of YFP (YC). YN and YC alone, without fused proteins, were tested as controls. Cells were transfected with the different YN and YC pairs and pEBFP, the latter used to identify transfected cells (Figure 4B). No YFP complementation was seen upon cotransfection of YN plus Brd4-YC, or YN-cyclinT1 plus YC. However, upon cotransfection of YN-cyclinT1 plus Brd4-YC, intense YFP signals were detected that distributed throughout the nucleus (but not nucleoli). Some cells displayed relatively uniform YFP signals over the nucleus, whereas other cells showed speckle-like signals. To test domain requirements, BiFC experiments were performed with Brd4 deletions fused to YC (Figure 4B). Whereas the deletion lacking both bromodomains (ΔBDI&II) failed to complement YN-cyclinT1, other deletions (ΔBDI, ΔBDII, and ΔC-term) all showed efficient complementation, displaying the same domain requirements as in Figure 3. Immunoblot assay confirmed that Brd4 and the mutants were expressed at equivalent levels (Figure 4C). The efficiency of complementation was high in these experiments, because 65%–80% of BFP-positive cells showed YFP signals. These data demonstrate that Brd4 interacts with cyclinT1 in living nucleus through either bromodomain.

Brd4 Interacts with CyclinT1/Cdk9 without 7SK snRNA

About half of cyclinT1/Cdk9 in the cell is bound to the inhibitory subunit containing 7SK snRNA (Nguyen et al.,

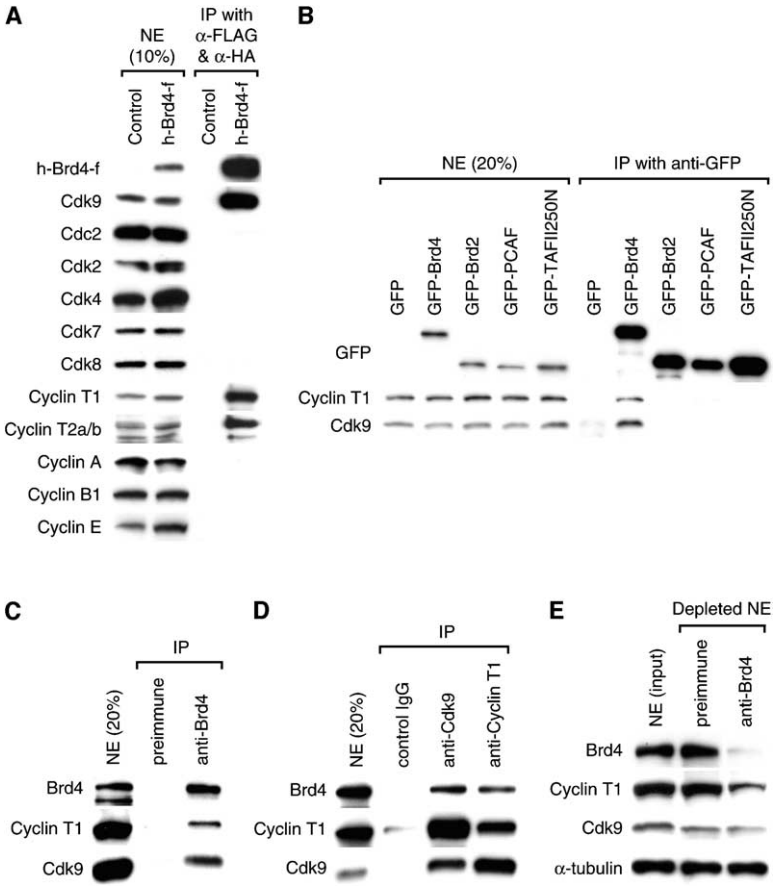

Figure 2. Endogenous Brd4 Interacts with CyclinT1 and Cdk9

(A) HeLaS3 NEs and immunoprecipitates purified as in Figure 1A were analyzed by immunoblotting with additional antibodies.

(B) NEs from HeLa cells transiently transfected with GFP constructs were precipitated by anti-GFP antibody, and input NEs (left, NE) or precipitates (right, IP) were analyzed by immunoblotting with antibodies shown on the left.

(C) HeLa NEs were precipitated with preimmune or anti-Brd4 antibody, shown on the top (IP), and precipitates were immunoblotted with antibodies, shown on the left.

(D) HeLa NEs were precipitated with the antibodies, shown at the top (IP), and precipitates were immunoblotted with antibodies, shown on the left.

(E) HeLa NEs were passed through preimmune or anti-Brd4 antibody beads three times, and supernatants were immunoblotted with antibodies, shown on the left.

2001; Yang et al., 2001). Data in Figure 2E suggested the possibility that Brd4 interacts with P-TEFb that is either with or without 7SK snRNA, but not both. To test this possibility, glycerol gradient centrifugation analysis was performed with NEs containing h-Brd4-f. In Figure 5A, cyclinT1/Cdk9 was partitioned into two major fractions. Complex I (fractions 9–13) contained cyclinT1/Cdk9 and Brd4, but not 7SK snRNA. Whereas, complex II (fractions 17–19) contained cyclinT1/Cdk9 and 7SK snRNA, but not Brd4, supporting the interaction of Brd4 with cyclinT1/Cdk9 without the inhibitory subunit. A modest amount of cdk9 was detected in fractions 5–7, consistent with the presence of free Cdk9 known to be present in the cells. The Brd4-P-TEFb complex sedimented between 460 kDa (TFIIH) and 120 kDa (CAK), whereas the P-TEFb containing 7SK snRNA sedimented along with Rpb1 at ~650 kDa. In keeping with these data, glycerol gradient centrifugation analysis performed with purified Brd4-immune complexes showed cosedimentation of cyclinT1/Cdk9 and Brd4 at around 340 kDa that did not have 7SK snRNA (Figure S1A). Additionally, Brd4 immuno-depleted materials when analyzed in glycerol centrifugation analysis showed little cyclinT1 and Cdk9 in complex I fractions in that they were found mostly in complex II (Figure S1B), further supporting the interaction of Brd4 with active P-TEFb.

These results were corroborated by CoIP experiments in Figure 5B, where Brd4-immune complexes precipitated by anti-FLAG antibody contained no detectable 7SK snRNA, whereas the cyclinT1-Cdk9 complexes precipitated by anti-Cdk9 or anti-cyclinT1 antibodies contained 7SK snRNA and Brd4 (see quantification in the bottom panel). Similarly, the Brd4-cyclinT1-Cdk9 complexes did not contain MAQ1/HEXIM1 (data not shown, see the accompanying paper by Yang et al., 2005). Essentially the same results were observed for endogenous Brd4 complexes (data not shown).

To study whether Brd4 regulates the amount of P-TEFb bound to the inhibitory subunit, we analyzed NIH3T3 cells expressing small interfering RNA (siRNA) for Brd4. Retroviral introduction of Brd4-siRNA reduced Brd4 protein levels to less than 10% without changing the expression of cyclinT1, Cdk9, and 7SK snRNA, indicating specific downregulation of Brd4 by siRNA (Figure 5C). As expected, control-siRNA did not alter expression of Brd4 or other proteins tested (Figure 5C). The levels of 7SK snRNA bound to P-TEFb in Brd4-siRNA cells were higher (~2-fold) than those in control-siRNA and parental NIH3T3 cells, indicating that a reduction in Brd4 expression increases binding of P-TEFb to the inhibitory subunit. We also examined whether Brd4 can interfere with the binding of 7SK snRNA to cyclinT1/Cdk9 in vitro. Immune complexes precipitated by anti-Cdk9 antibody from HeLa NEs (containing both Brd4 and 7SK snRNA) were incubated with recombinant wt or mutant Brd4, and the amount of free 7SK snRNA in supernatants was quantified (Figure 5D). Incubation with Brd4 and ΔC-term led to a 3-fold increase in free, unbound 7SK snRNA. In contrast, ΔBDI&II, the deletion that did not bind to P-TEFb, did not change the level of unbound

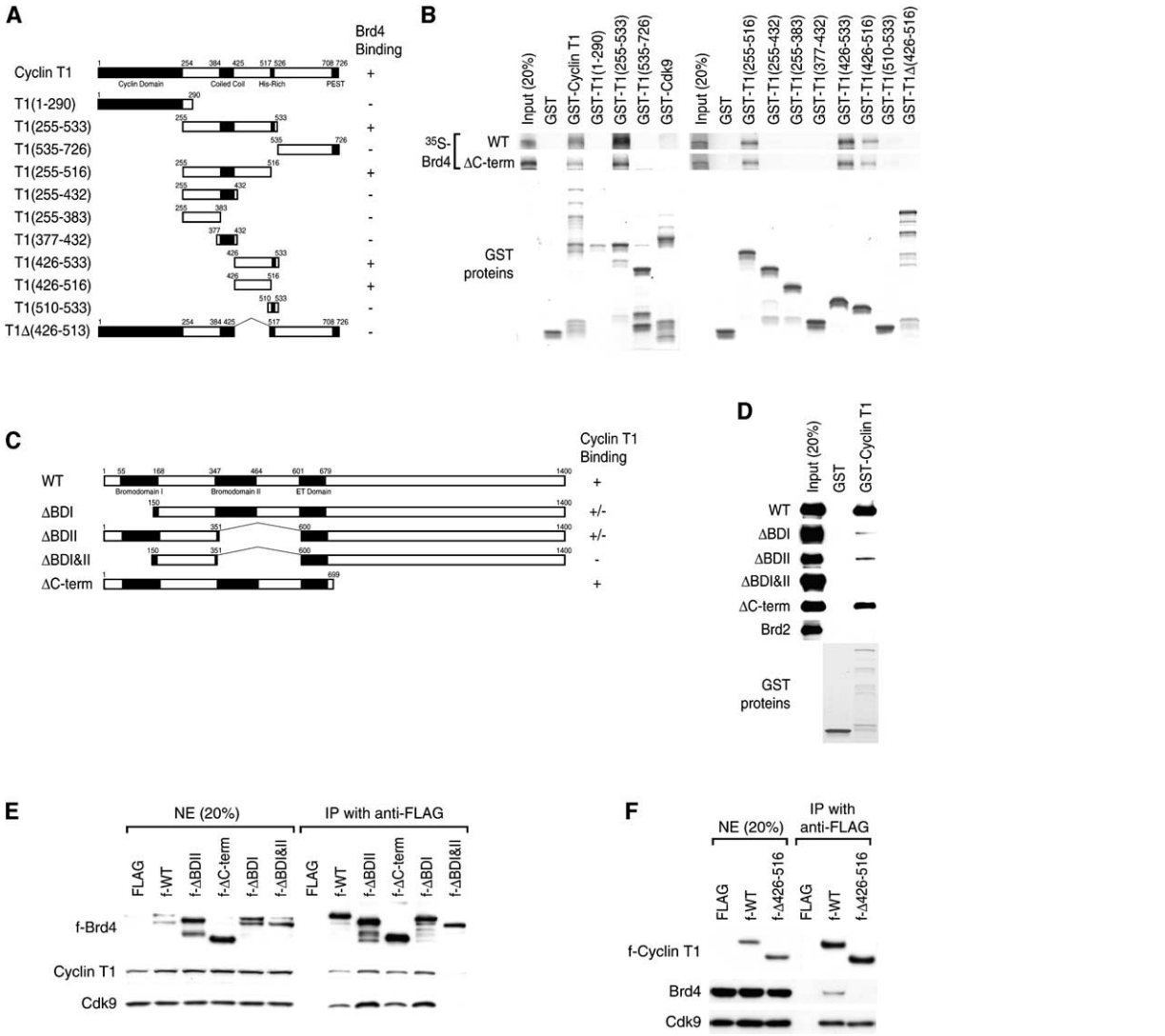

Figure 3. Domain Requirement

(A) Diagram of cyclinT1 deletions. Brd4 binding data are summarized on the right.

(B) GST-fusions were incubated with radiolabeled wt or ΔC-term Brd4, and bound materials were detected by autoradiography (top). Coomassie blue staining of GST-cyclinT1 proteins is shown at the bottom.

(C) Diagram of Brd4 deletions. CyclinT1 binding data are summarized on the right.

(D) Wt and Brd4 deletions tagged with 6xHis and FLAG and purified using baculoviral system were incubated with GST or GST-cyclinT1 beads, and bound materials were immunoblotted with anti-FLAG antibody (top). Coomassie blue staining of GST proteins is shown at the bottom.

(E) HeLa NEs containing FLAG, FLAG-tagged Brd4, or deletions were precipitated with anti-FLAG antibody and immunoblotted with indicated antibodies at left.

(F) HeLa NEs with FLAG alone, FLAG-tagged wt cyclinT1, or Δ(426–516) were precipitated by anti-FLAG antibody and immunoblotted with indicated antibodies at the left.

7SK snRNA. Further analysis by glycerol gradient centrifugation showed that the endogenous Brd4 comigrated with P-TEFb in complex I from control-siRNA cells (Figure 5E). Complex I was reduced in Brd4-siRNA cells, whereas P-TEFb levels in complex II were increased. These results indicate that P-TEFb binds alternately to Brd4 and the inhibitory subunit. The accompanying paper by Yang et al. (2005) presents additional evidence for mutually exclusive interaction of P-TEFb with Brd4 and the inhibitory subunit in vivo.

Brd4 Enhances RNAPII CTD Phosphorylation and Transcription by Interacting with P-TEFb

We tested whether Brd4 expression affects phosphorylation of Ser2 in RNAPII CTD, known to be mediated by P-TEFb. In Figure 6A, ectopic Brd4 expression increased Ser2 CTD phosphorylation without affecting Ser5 CTD phosphorylation. Expression of both Cdk9 and cyclinT1 also increased Ser2 phosphorylation without changing Ser5 phosphorylation. Ser2 phosphorylation was increased the most when Brd4, Cdk9, and

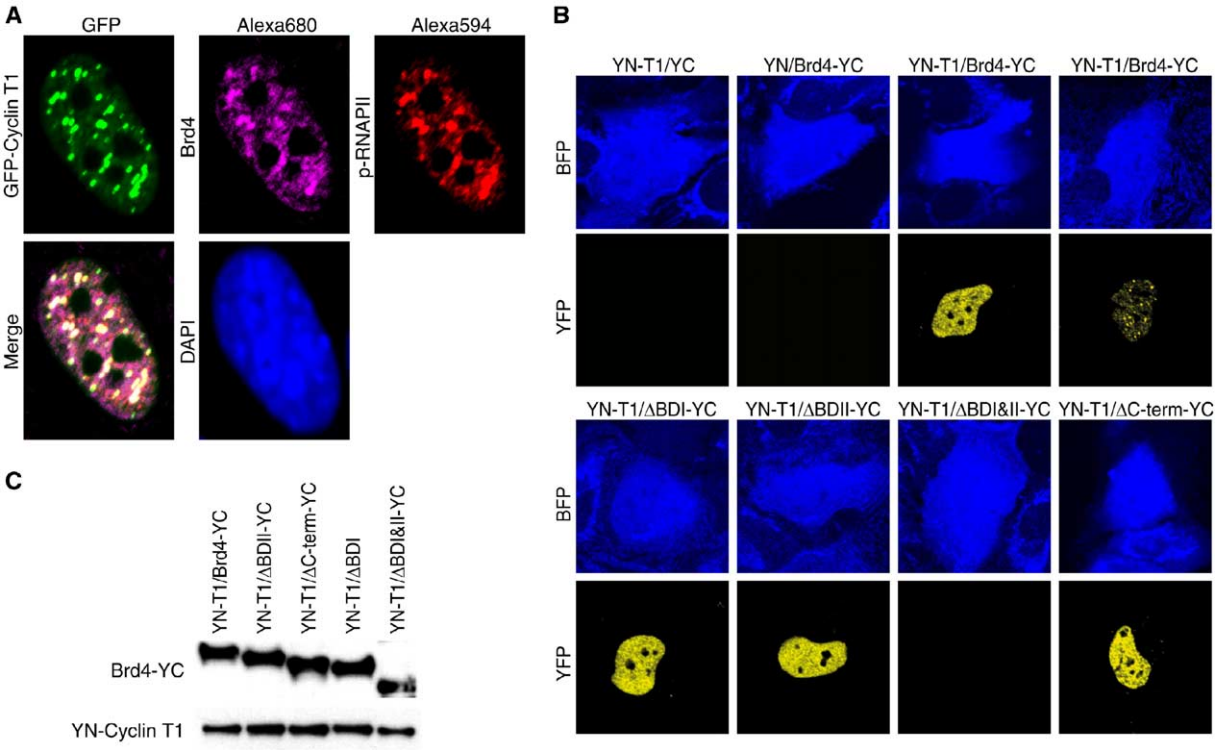

Figure 4. Interaction of Brd4 and CyclinT1 in Living Cells

(A) NIH3T3 cells transfected with GFP-cyclinT1 were stained with antibodies for Brd4 and phospho-RNAPII (Ser2) and viewed on a confocal microscope. All three proteins localized to nonnucleolar space in the nucleus with varied speckle-like structures. The triple merge image indicates patchy colocalization of the three proteins.

(B) HeLa cells were transfected with indicated combinations of YN and YC constructs along with pEBFP. BiFC signals were viewed as above. Blue images on upper panels indicate BFP run as transfection control. Intense YFP signals in the nucleus represent BiFC.

(C) Expression of Brd4-YC and YN-cyclinT1 was detected by immunoprecipitation and immunoblot with anti-GFP antibody.

cyclinT1 were ectopically expressed. The levels of RNAPII remained unaltered after transfection of these vectors. To further investigate the role of Brd4 in Ser2 CTD phosphorylation, we examined cells expressing Brd4-siRNA. In line with data in Figure 5C, Brd4-siRNA reduced Brd4 expression to ~10% without affecting RNAPII, cyclinT1, and Cdk9 expression (Figure 6B, lanes 1 and 4). In these cells, Ser2 phosphorylation was substantially reduced, whereas Ser5 phosphorylation was unaffected. We then transfected Brd4 into Brd4-siRNA cells and tested for Ser2 phosphorylation (Figure 6B, lanes 2 and 3 versus lanes 5 and 6). As expected, Brd4 transfection led to an increase in Brd4 levels in control-siRNA cells while leading to a meager increase in Brd4-siRNA cells, presumably due to siRNA inhibition of Brd4 expression. Transfection of Brd4 resulted in an increase in Ser2 phosphorylation in control-siRNA cells but only a small increase in Brd4-siRNA cells. Again, Brd4 transfection did not alter RNAPII, cyclinT1, and Cdk9 expression nor did it change Ser5 phosphorylation. These results indicate that Brd4 exerts a positive effect on Ser2 CTD phosphorylation by increasing functionality of P-TEFb.

P-TEFb stimulates the activity of cellular and HIV-1 promoters (Barboric et al., 2001; Kanazawa et al., 2003; Wei et al., 1998). To assess the role of Brd4 in transcription, we performed a reporter assay with the HIV-1 long terminal repeat (LTR) promoter and cellular promoters, including c-Myc and c-Jun (Figure 6C). Transfection of Brd4 increased activity of these reporters in a dose-dependent manner with the greatest increase observed with the HIV-1 LTR reporter, indicating that the Brd4 positively regulates transcription from these promoters. Supporting the specificity of Brd4 action, a mutant HIV-1 reporter with an altered TATA sequence gave a background luciferase activity, that was not increased by Brd4 expression (Figure 6C, Inset). Immunoblot analysis confirmed that Brd4 transfection increased Brd4 protein expression without altering cyclinT1/Cdk9 expression (Figure 6C). Promoter activity was also tested after co-transfection of both factors (Figure 6D); whereas transfection of Brd4 or cyclinT1 alone moderately increased promoter activity, transfection of both gave a greater increase. Reporter assays were next performed with Brd4 deletions. In Figure 6E, full-length Brd4, ΔBDI, and ΔBDII, all of which interact with P-TEFb, enhanced promoter activity, although the latter was less efficient than the former two. In contrast, ΔBDI&II, unable to interact with cyclinT1/Cdk9, failed to enhance promoter activity. These results support the view that Brd4 stimulates transcription by interacting with functionally active P-TEFb. It is of note that the ΔC-term deletion did not significantly enhance promoter activity, although it interacted with cyclinT1/Cdk9, suggesting a role for this

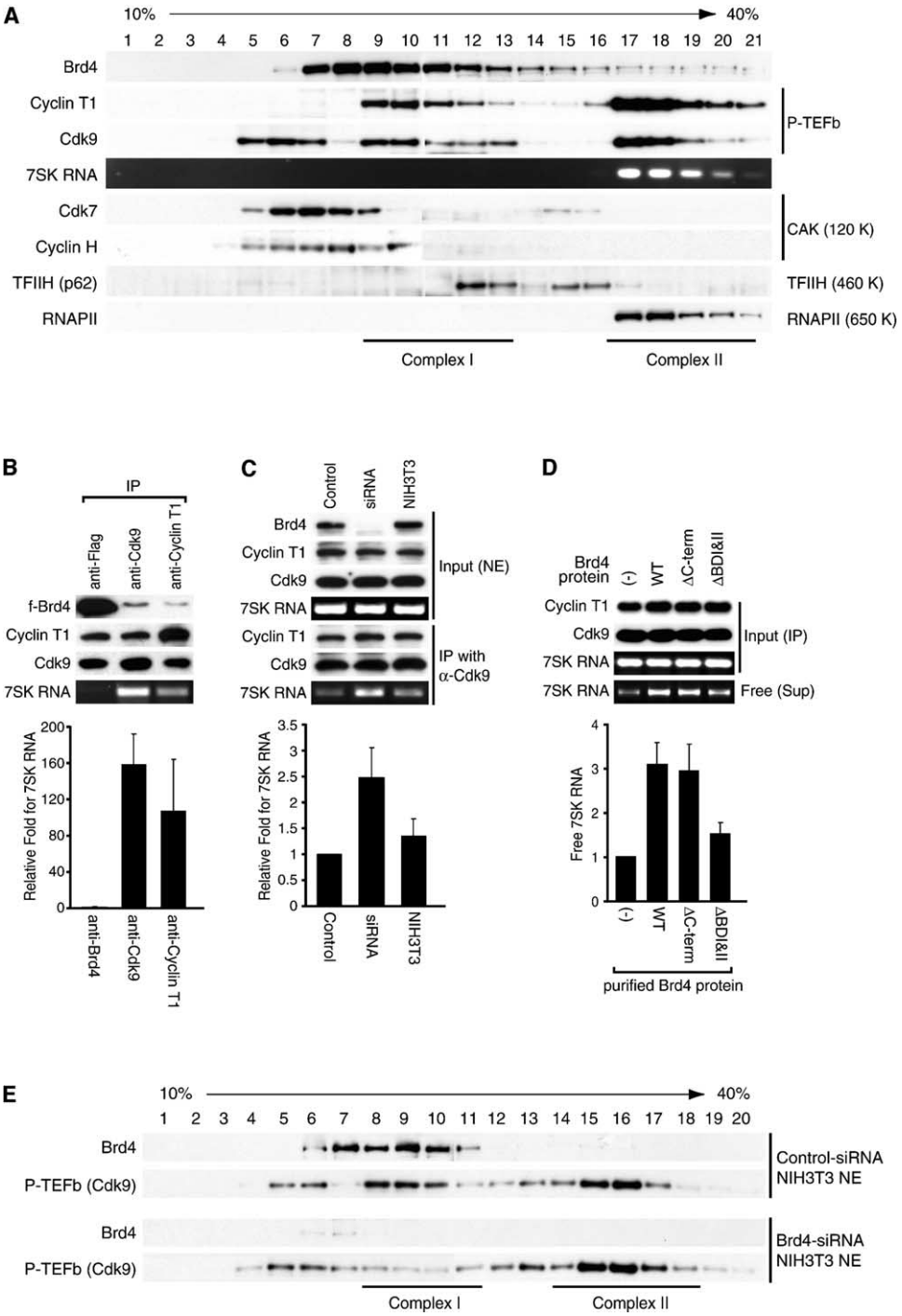

Figure 5. Brd4 Interacts with 7SK snRNA-Free CyclinT1/Cdk9

(A) NEs (0.5 mg) from HeLaS3 cells expressing h-Brd4-f were separated on a glycerol gradient centrifugation into 21 fractions and were analyzed by immunoblot or RT-PCR for indicated proteins/RNA, shown on the left. Estimated molecular weights of protein complexes were shown on the right.

(B) HeLa NEs containing h-Brd4-f were precipitated with antibodies, shown at the top, and analyzed by semiquantitative RT-PCR, real-time RT-PCR (bottom) and immunoblot, with antibodies shown on the left.

(C) Extracts from parental NIH3T3 or cells expressing control or Brd4-siRNA were precipitated with anti-Cdk9 antibody and analyzed as in (A) except by using primers for mouse 7SK snRNA.

(D) CyclinT1-Cdk9 complexes precipitated from HeLa NEs (400 μl) were incubated with ~1 μg of purified recombinant wt or mutant Brd4 or BSA (as control) for 1 hr at 4°C. 7SK snRNA in supernatants was detected by semiquantitative and real-time RT-PCR.

Results indicate the average of three independent experiments ± SD in (B)–(D).

(E) NIH3T3 NEs (0.3 mg) with control or Brd4-siRNA were analyzed by glycerol gradient centrifugation as in (A), and immunoblot for indicated proteins, shown on the left.

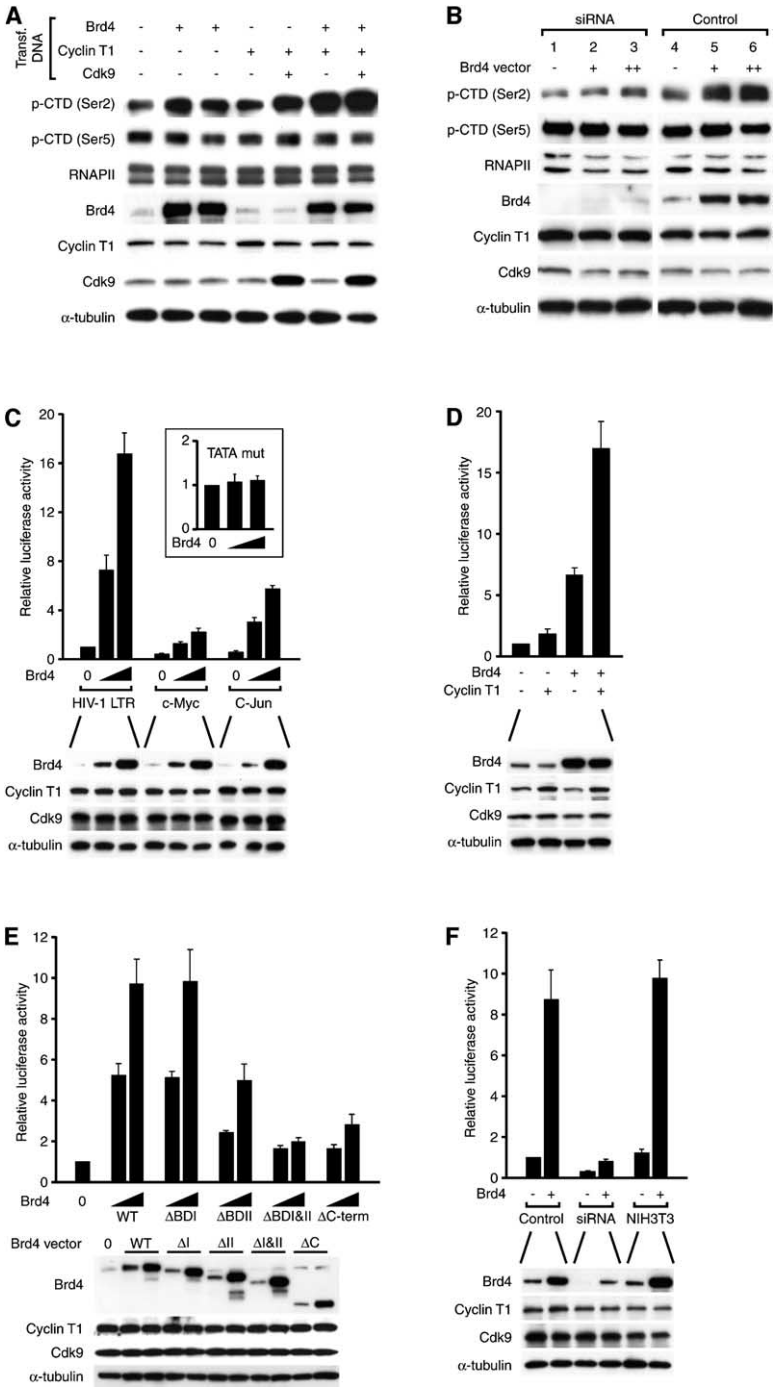

Figure 6. Brd4 Stimulates RNAPII CTD Phosphorylation and Enhances Promoter Activity

(A) Total extracts from HeLa cells transfected with indicated vectors (top) were immunoblotted with antibodies specific for phospho-Ser2 (H5) or phospho-Ser5 (H14) of RNAPII CTD. Immunoblots with additional antibodies were run as controls for transfection and loading.

(B) NIH3T3 cells expressing Brd4-siRNA or control-siRNA were transfected with increasing amounts of Brd4 vector, and total extracts were immunoblotted as in (A).

(C) HeLa cells were cotransfected with 20 ng of indicated luciferase reporter and 0.1 μg or 0.4 μg of pFLAG-CMV2-Brd4 vector for 24 hr. In all reporter assays, the amount of transfected DNA was kept constant by adding appropriate empty vectors. Shown at the bottom are immunoblot detection of Brd4, cyclinT1, Cdk9, and α-tubulin as controls for transfection and loading.

(D) HeLa cells were cotransfected with 20 ng of HIV-1 LTR reporter, 0.1 μg of pFLAG-CMV2-Brd4, and 0.4 μg of pcDNA3-cyclinT1 vectors. Luciferase assay and immunoblots were performed as in (C).

(E) HeLa cells were transfected with the HIV-1 LTR reporter and 0.1 or 0.25 ng of wt Brd4 or deletion vectors.

(F) Parental NIH3T3 cells or cells expressing control-siRNA or Brd4-siRNA were transfected with the HIV-1 LTR reporter and 0.5 μg of Brd4 vector.

Luciferase activities are expressed as relative units obtained from the average of three assays ± SD in (C)–(F).

domain in transcription (Figures 3D and 3E). To investigate whether a reduction in Brd4 negatively affects promoter activity, HIV reporter assays were performed with Brd4-siRNA cells (Figure 6F). Whereas promoter activity in control-siRNA cells was comparable to that of parental cells, reporter activity was significantly reduced in Brd4-siRNA cells. In control-siRNA and parental cells, Brd4 transfection increased promoter activity substantially, but it affected only modestly in Brd4-siRNA cells, in agreement with data in Figure 6B.

Brd4 Is Recruited to the HIV Promoter and Affects P-TEFb Recruitment In Vivo

To study whether Brd4 is recruited to a promoter in vivo and plays a role in P-TEFb recruitment, ChIP assays were performed for a stably integrated HIV-1 LTR reporter. NIH3T3 cells with an integrated HIV reporter were transduced with control-siRNA or Brd4-siRNA to produce cells with a normal or reduced Brd4 expression. Recruitment of Brd4 and Cdk9 was tested by real-time PCR with five primers corresponding to different re-

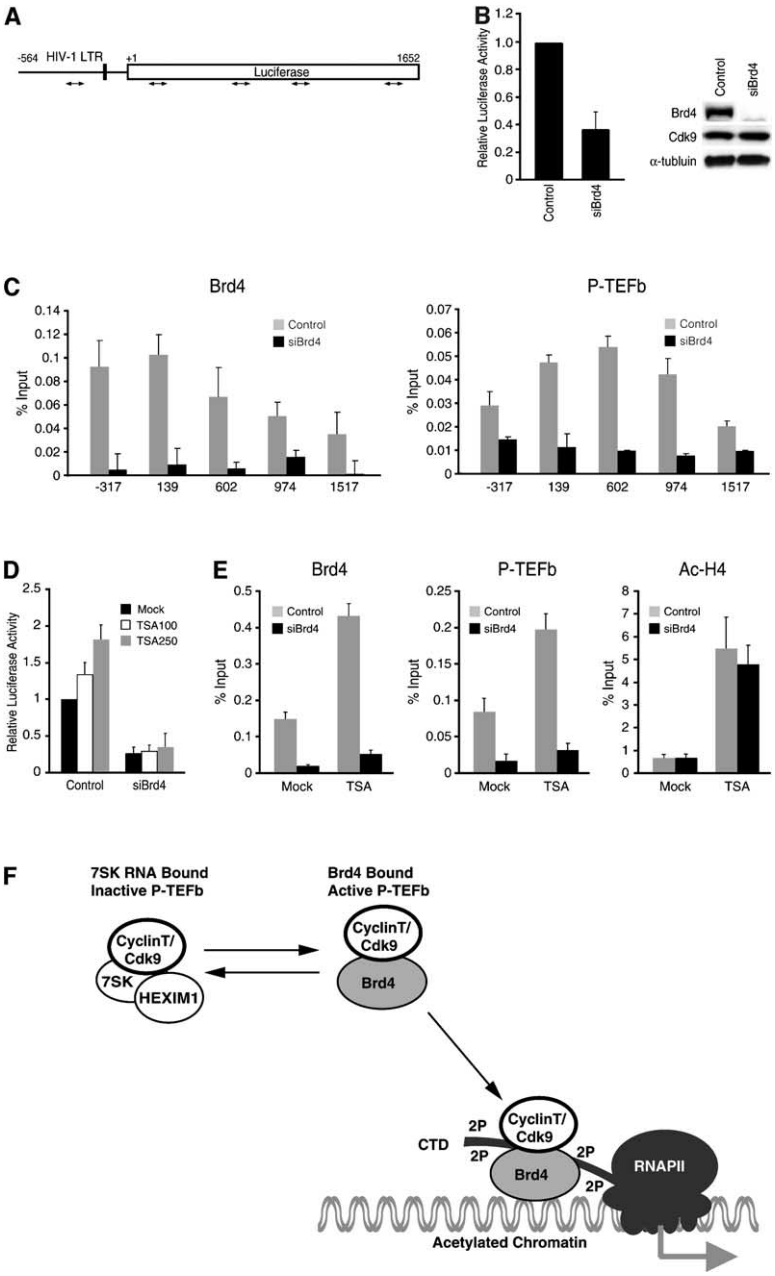

Figure 7. Brd4 Enhances the Recruitment of P-TEFb to the Integrated HIV-1 LTR Reporter

(A) Diagram of the HIV-1 LTR reporter stably integrated into NIH3T3 cells and the position of the primers tested for ChIP analysis.

(B) NIH3T3 cells with the integrated HIV-1 LTR reporter were transduced with control or Brd4-siRNA and selected by puromycin (2 μg/ml) for 4 days. HIV-1 LTR reporter activity and Brd4 expression were tested by luciferase assay and immunoblotting. Luciferase activities are expressed as relative units obtained from the average of three assays ± SD.

(C) Brd4 and Cdk9 bind to the integrated HIV-LTR sequence in vivo. NIH3T3 with control or Brd4-siRNA were subjected to ChIP assay by using anti-Cdk9 and anti-Brd4 antibodies shown on the top. Results indicate the average of two independent assays ± SD.

(D) NIH3T3 cells with the integrated HIV-1 LTR reporter were transduced as shown in (B). After treatment with TSA for 6 hr, HIV-1 LTR reporter activity was tested by luciferase assay. Luciferase activities are expressed as relative units obtained from the average of three assays ± SD.

(E) P-TEFb binding is regulated by Brd4 and acetylation of chromatin. NIH3T3 cells with control or Brd4-siRNA were treated with TSA (200 ng/ml) for 4 hr and subjected to ChIP assay by using anti-Cdk9, anti-Brd4, and anti-tetra acetyl-histone H4 antibodies shown on the top. Real-time RT-PCR was performed with a primer set (LTR-Luc139). Results indicate the average of two independent assays ± SD.

(F) A model for Brd4-P-TEFb interaction. Core P-TEFb (cyclinT1 and Cdk9) occurs in association with either Brd4 or the inhibitory subunit, representing the positive or negative regulatory component, respectively. P-TEFb activity is maintained at equilibrium by reversible exchange of the two subunits. By binding to acetylated chromatin, Brd4 facilitates the recruitment of P-TEFb to promoters and enhances their transcription. Interaction of Brd4 with the Mediator complex also increases P-TEFb recruitment.

gions of the reporter gene (Figure 7A). As expected, luciferase reporter activity was significantly lower in Brd4-siRNA cells than control-siRNA cells (Figure 7B). We first confirmed that two antibodies specific for Brd4 precipitated reporter fragments with all five primers, whereas preimmune sera did not, indicating that Brd4 is recruited to the integrated HIV-1 sequence (Figure S2B). As shown in Figure 7C, the amount of Brd4 bound to the promoter region (tested with the primer –317,139 and 602) was consistently greater than that bound to the downstream coding region (tested with primers 974 and 1517). In contrast, binding of Brd4 to the reporter was markedly diminished in Brd4-siRNA cells as detected by all five primers. To test the recruitment of P-TEFb to the HIV reporter, ChIP analysis was performed with Cdk9 antibody (Figure 7C). In control-siRNA cells, significant binding of Cdk9 was noted along the entire reporter gene, indicating that P-TEFb bound to both the promoter and coding regions of the reporter. Binding of Cdk9 was higher at the coding region than at the promoter (compare –317 and 602). Coinciding with reduced Brd4 binding, recruitment of Cdk9 was substantially lower in Brd4-siRNA cells than control-siRNA cells, seen with all five primers, indicating that Brd4 regulates recruitment of P-TEFb to the reporter. ChIP experiments were also performed with HeLa cells with a stably integrated HIV reporter, which also showed binding of Brd4 and Cdk9 to the reporter

gene (Figure S2). These results indicate that Brd4 binds to the HIV reporter and facilitates the recruitment of P-TEFb to the reporter.

Given that Brd4 is a chromatin binding protein preferentially interacting with acetylated histones, it was of importance to assess whether increased chromatin acetylation affects recruitment of Brd4 and of P-TEFb to the HIV reporter. To this end, we examined the effect of trichostatin A (TSA), a histone deacetylase inhibitor. In our previous study, TSA globally increased histone acetylation and binding of Brd4 to chromatin (Dey et al., 2003). In Figure 7D, TSA treatment for 6 hr enhanced the activity of integrated HIV reporter in control cells, but not in Brd4-siRNA cells. In accordance, ChIP assays in Figure 7E showed that TSA treatment enhanced binding of Brd4 to the HIV reporter in control-siRNA cells but only meagerly in Brd4-siRNA cells. The levels of Brd4 and P-TEFb were unaffected during 4 hr TSA treatment (data not shown, Dey et al., 2003). Coinciding with enhanced Brd4 recruitment, TSA significantly enhanced P-TEFb recruitment to the HIV reporter in control-siRNA cells but only modestly in Brd4-siRNA cells (middle). Moreover, TSA treatment enhanced histone H4 acetylation in the reporter region both in control- and Brd4-siRNA cells (right), indicating the importance of Brd4 binding for P-TEFb recruitment. Together, these results indicate that the recruitment of P-TEFb to the promoter is closely linked to binding of Brd4 to acetylated chromatin.

Discussion

Despite recent elucidation of the inhibitory subunit of P-TEFb, the property of the other half of P-TEFb, the functionally active counterpart, has remained elusive. We found that there are two separable cyclinT1-Cdk9 complexes in the cell, one bound to Brd4 and the other to the inhibitory subunit, indicating that Brd4 represents a component of the other P-TEFb complex. Brd4 interacted with P-TEFb through an internal region of cyclinT1. In our functional studies, an increase in Brd4 protein expression increased phosphorylation of RNAPII CTD by P-TEFb, stimulating transcription from multiple promoters. Conversely, an siRNA-based decrease in Brd4 expression decreased CTD phosphorylation and led to decreased transcription. The finding that a reduction in Brd4 expression increased P-TEFb binding to 7SK snRNA indicates that Brd4 and the inhibitory subunit bind to P-TEFb in a mutually exclusive manner. Our data suggest a model where P-TEFb reversibly switches its partner between Brd4 and the inhibitory subunit, thereby maintaining a dynamic equilibrium of its positive and negative activities (Figure 7F). In line with this model, Yang et al. (2005) show that several stress-inducing agents alter the equilibrium of the two complexes.

Given that Brd4 is expressed in most embryonic and adult tissues (Dey et al., 2000; Houzelstein et al., 2002) and Cdk9 and cyclinTs are likewise ubiquitous (Peng et al., 1998), the occurrence of the Brd4-P-TEFb complex is likely to be very broad, exerting a function of general importance. Interestingly, Brd2, another BET family protein with similar structural composition, did not interact with P-TEFb. Similarly, only cyclinT1/T2, but not other cyclins tested here, interacted with Brd4, indicating a highly selective interaction of the two proteins.

BiFC analysis demonstrated that the Brd4-P-TEFb complex resides in nonnucleolar regions of the nucleus in living cells. These results were in agreement with our CoIP data where Brd4-P-TEFb complexes were found mostly in the nuclear fraction, not in the cytoplasmic fraction. In line with these results, both cyclinT1/Cdk9 and Brd4 have been independently shown to localize to the nucleus except nucleoli (Herrmann and Mancini, 2001; Dey et al., 2000). We noted that Brd4 and cyclinT1/Cdk9 interact during the entire interphase. However, this interaction must be temporarily disrupted during mitosis, because Cdk9 and cyclinT1 are dispersed from mitotic chromosomes (Herrmann and Mancini, 2001), whereas Brd4 remains associated with them (Dey et al., 2003).

Activities of cellular and HIV promoters were elevated when Brd4 expression was elevated. Conversely, promoter activities were reduced when Brd4 expression was downregulated by Brd4-siRNA. Correlating with changes in promoter activity, Ser2 CTD phosphorylation was enhanced by Brd4 overexpression and reduced by Brd4 underexpression. This correlation indicates that Brd4 expression levels are an important determinant of the functionality of P-TEFb. In accordance, ChIP assays found that Brd4 and Cdk9 were both bound to the integrated HIV reporter and that Brd4 underexpression reduced Cdk9 recruitment. Based on these results, it seems reasonable to surmise that the recruitment of P-TEFb to a transcriptionally active gene is dependent on Brd4. Previous studies showed that P-TEFb is recruited to various promoters by specific activators (Barboric et al., 2001; Kanazawa et al., 2003). Thus, Brd4 may act in cooperation with other activators to recruit P-TEFb for some promoters. Nevertheless, the general notion that not all activators recruit P-TEFb suggests the broad importance of Brd4 in the recruitment of P-TEFb. It has been shown that P-TEFb binds to the CTD of RNAPII through a small histidine-rich tract in cyclinT1 (Taube et al., 2002). The interaction of P-TEFb with Brd4 would not prevent it binding to the CTD, because Brd4 binds to P-TEFb through the region outside of this tract. Because P-TEFb is thought to be broadly involved in transcription of many RNAPII-dependent genes (Chao and Price, 2001; Lis et al., 2000), Brd4 might affect transcription of many, if not most, P-TEFb-regulated genes.

Brd4 is highly mobile and transiently binds to acetylated core histones rich in transcriptionally active regions of chromatin (Dey et al., 2003). Through a dynamic mode of interaction, Brd4 may bring P-TEFb near transcriptionally active promoters in acetylated chromatin, thereby facilitating its interaction with RNAPII and the subsequent functions in elongation (Figure 7F). In support of this idea, an increase in chromatin acetylation led to an increase in P-TEFb recruitment to the HIV reporter, which was dependent on Brd4. It is of note that some Brd4 also interacts with components of the Mediator (Figure 1B). Thus, recruitment of P-TEFb to the promoter region and subsequent transcription may be further reinforced by additional interaction of Brd4 with the Mediator. In light of our ChIP results indicating

that Brd4 and P-TEFb apparently bind to the promoter and the coding region of the reporter, it is possible that P-TEFb interacts with Brd4 not only at the promoter region during preinitiation complex assembly but also along the coding region during the elongation step. Such extensive Brd4-P-TEFb interactions may help to stabilize the interaction of P-TEFb with RNAPII and facilitating their functions.

In summary, this paper identifies Brd4 as a positive regulatory component of P-TEFb. The accompanying paper by Yang et al. (2005) extends our findings by describing how the inhibitory subunit and Brd4 balance the positive and negative activities of P-TEFb.

Experimental Procedures

Purification of the Brd4 Complex

Full-length mouse Brd4 cDNA (Dey et al., 2000) tagged with HA and FLAG was cloned into a retroviral vector, pOZ, and transduced into HeLaS3 cells as described (Ogryzko et al., 1998). ~1 × 10^{10} cells resuspended in buffer A (10 mM HEPES, [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) were disrupted by using Dounce homogenizer and centrifuged to collect nuclear pellets. NE was extracted in buffer C (20 mM HEPES, [pH 7.9], 0.3 M KCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail) at 4°C for 30 min, followed by dialysis against buffer D (20 mM HEPES, [pH 7.9], 20% glycerol, 150 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF). NE was precipitated on anti-FLAG M2 agarose, and bound proteins were eluted with FLAG peptides (0.1 mg/ml; Sigma). The eluates were further precipitated on anti-HA agarose (Sigma). Bound proteins were eluted with HA peptides, separated on 10% SDS-PAGE, and stained with colloidal blue. Mass spectrometry was performed through the W.M. Keck Foundation Biotechnology Resource Laboratory. Data analysis was performed with the ProFound software.

Immunoprecipitation and Immunoblotting

Full-length or truncated Brd4 cDNAs (Maruyama et al., 2002) were cloned into pFLAG-CMV2 (Sigma), pEGFP-C1 (Clontech). Human cyclinT1 and Cdk9 cDNAs cloned by RT-PCR were inserted into pcDNA3, pFLAG-CMV2. NE (400 μg) from cells transfected with indicated vectors was incubated with indicated antibodies for 12 hr at 4°C and precipitated with protein G Sepharose beads (Amersham). Bound materials were eluted by FLAG peptides or in sample buffer, separated on 10% SDS-PAGE, and immunoblotted with the standard procedures. Antibodies to cyclinT1, cyclinT2a/b, or Cdk9 were from Santa Cruz. Antibodies to RNAPII and GFP were from Covance and Roche, respectively. Horseradish peroxidase-conjugated second antibodies were from Amersham and Jackson ImmunoResearch. Rabbit anti-Brd4 antibodies were described (Dey et al., 2000).

In Vitro Binding Assay

Full-length and truncated cyclinT1 and Cdk9 were cloned into pGEX4T and GST fusion proteins purified as described (Kanazawa et al., 2003). Fusion proteins (1 μg) bound onto glutathione beads were incubated with ^{35}S-labeled Brd4 for 4 hr at 4°C, and bound materials were detected by autoradiography. The purified recombinant Brd4 proteins from baculoviral system were incubated with GST-cyclinT1 bound to glutathione beads, and bound materials were immunoblotted with anti-Brd4 antibody.

Immunostaining and BiFC

Cells grown on coverslips were transfected with GFP-cyclin T1 for 24 hr and fixed in 4% paraformaldehyde in PBS and permeabilized in 0.1% Triton X-100. Cells were blocked and incubated with primary antibodies for 1 hr at room temperature, except for staining of phospho-RNAPII, for which cells were incubated for 16 hr at 4°C. Cells were then reacted with secondary antibodies (Alexa 680-conjugated donkey anti-rabbit IgG or Alexa594-conjugated goat anti-mouse IgM; Molecular Probes) for 1 hr. Cells were analyzed by an inverted Leica SP2 confocal microscope. BiFC analysis was performed as described (Hu et al., 2002; Kanno et al., 2004). Brd4 cDNA were cloned into pYC containing the C-terminal half of YFP (aa 155–238), and cyclinT1 was cloned into pYN containing the N-terminal half of YFP (aa 1–154). Cells were cotransfected with indicated plasmids along with pEBFP vector. After 20 hr incubation, cells were allowed to stand at 30°C for 1 hr, and YFP and BFP signals were viewed as above.

Glycerol Gradient Ultracentrifugation

NEs (0.5 mg) from HeLaS3 and NIH3T3 cells or the Brd4-P-TEFb complex immunopurified from h-Brd4-f NE (5 mg) were fractionated on a 4 ml 10%–40% glycerol gradient by centrifugation in an SW 60Ti rotor (Beckmann) at 45,000 rpm for 20 hr. 20 fractions (0.2 ml) were collected and analyzed by immunoblotting. For the analysis of the immunopurified Brd4-P-TEFb complex, Ctf18 immunoprecipitates were run in parallel to independently assess the size of the complex. Each fraction was tested for 7SK snRNA by semiquantitative RT-PCR.

Detection of 7SK snRNA

RNA purified from NEs, immunoprecipitates, or glycerol gradient fractions were reverse transcribed with Superscript II RTase (Stratagene). Semiquantitative PCR was performed by using the Taq DNA polymerase (Promega) with 25–30 cycles of amplification. For real-time PCR, the SYBR Green kit (Applied Biosystems) was used to monitor amplification of sample cDNA in the ABI Prism 7000 Sequence Detection System.

Reporter Assays and Brd4-siRNA Cells

Luciferase reporters were constructed in pGL3 (Promega). The HIV-1 LTR luciferase was a gift from A. DeVico. 1 × 10^5 cells seeded in 24-well plates were transfected with indicated expression vectors and luciferase reporters by using Fugene 6 (Roche). Total luciferase activities were measured by using a luciferase reporter assay system (Promega). Brd4-siRNA was expressed in NIH3T3 cells by using the pSUPER RNAi system (Brummelkamp et al., 2002). Brd4-siRNA oligomers that effectively reduced Brd4 expression were screened prior to experiments (siRNA sequences are in the Table S2). Cells were infected with viral supernatants from BOSC23 packaging cells transfected with pSUPERretro vector for Brd4- or control-siRNA for 2 days followed by puromycin selection (2 μg/ml) for 4 days. Brd4 expression levels were monitored by immunoblot prior to experiments.

ChIP Assays

NIH3T3 clones were isolated after stable transfection with pGL3/HIV-LTR-luciferase and two weeks of puromycin selection. Seven clones were pooled and transduced with control or Brd4-siRNA as above. ChIP assays were performed by using anti-Cdk9 antibody, anti-Brd4 antibodies, and anti-tetra acetyl H4 antibody (Upstate) with ChIP reagents provided by Upstate according to its recommended protocol. Briefly, 1 × 10^6 cells were crosslinked with 1% paraformaldehyde and sheared by sonication. 1 ml of 10-fold diluted reactions were incubated with antibodies (2 μl antiserum or 1 μg IgG) and immunoprecipitated by protein A agarose containing salmon sperm DNA. Precipitated materials were extensively washed with wash buffers, decrosslinked, and subjected to real-time PCR by using indicated primers.

Supplemental Data

Supplemental Data include two figures and two tables and are available with this article online at http://www.molecule.org/cgi/content/19/4/523/DC1/.

Acknowledgments

We thank E. Anderson for some mass-spectrometry analyses, T. Kerpola for advice on BiFC, and I. Dawid and C. Kayne for critically reading the manuscript.

Received: February 23, 2005
Revised: May 26, 2005
Accepted: June 27, 2005
Published: August 18, 2005

References

Barboric, M., Nissen, R.M., Kanazawa, S., Jabrane-Ferrat, N., and Peterlin, B.M. (2001). NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. Mol. Cell *8*, 327–337.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science *296*, 550–553.

Chao, S.H., and Price, D.H. (2001). Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. J. Biol. Chem. *276*, 31793–31799.

Chen, R., Yang, Z., and Zhou, Q. (2004). Phosphorylated positive transcription elongation factor b (P-TEFb) is tagged for inhibition through association with 7SK snRNA. J. Biol. Chem. *279*, 4153–4160.

Crowley, T.E., Kaine, E.M., Yoshida, M., Nandi, A., and Wolgemuth, D.J. (2002). Reproductive cycle regulation of nuclear import, euchromatic localization, and association with components of Pol II mediator of a mammalian double-bromodomain protein. Mol. Endocrinol. *16*, 1727–1737.

Denis, G.V., Vaziri, C., Guo, N., and Faller, D.V. (2000). RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. Cell Growth Differ. *11*, 417–424.

Dey, A., Ellenberg, J., Farina, A., Coleman, A.E., Maruyama, T., Sciortino, S., Lippincott-Schwartz, J., and Ozato, K. (2000). A bromodomain protein, MCAP, associates with mitotic chromosomes and affects G(2)-to-M transition. Mol. Cell. Biol. *20*, 6537–6549.

Dey, A., Chitsaz, F., Abbasi, A., Misteli, T., and Ozato, K. (2003). The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. Proc. Natl. Acad. Sci. USA *100*, 8758–8763.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., and Zhou, M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491–496.

Farina, A., Hattori, M., Qin, J., Nakatani, Y., Minato, N., and Ozato, K. (2004). Bromodomain protein Brd4 binds to GTPase-activating SPA-1, modulating its activity and subcellular localization. Mol. Cell. Biol. *24*, 9059–9069.

Fondell, J.D., Ge, H., and Roeder, R.G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc. Natl. Acad. Sci. USA *93*, 8329–8333.

Fu, T.J., Peng, J., Lee, G., Price, D.H., and Flores, O. (1999). Cyclin K functions as a CDK9 regulatory subunit and participates in RNA polymerase II transcription. J. Biol. Chem. *274*, 34527–34530.

Herrmann, C.H., and Mancini, M.A. (2001). The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. J. Cell Sci. *114*, 1491–1503.

Houzelstein, D., Bullock, S.L., Lynch, D.E., Grigorieva, E.F., Wilson, V.A., and Beddington, R.S. (2002). Growth and early postimplantation defects in mice deficient for the bromodomain-containing protein Brd4. Mol. Cell. Biol. *22*, 3794–3802.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell *9*, 789–798.

Jacobson, R.H., Ladurner, A.G., King, D.S., and Tjian, R. (2000). Structure and function of a human TAFII250 double bromodomain module. Science *288*, 1422–1425.

Jeanmougin, F., Wurtz, J.M., Le Douarin, B., Chambon, P., and Losson, R. (1997). The bromodomain revisited. Trends Biochem. Sci. *22*, 151–153.

Jiang, Y.W., Veschambre, P., Erdjument-Bromage, H., Tempst, P., Conaway, J.W., Conaway, R.C., and Kornbeg, R.D. (1998). Mammalian mediator of transcriptional regulation and its possible role as an end point of signal transduction pathways. Proc. Natl. Acad. Sci. USA *95*, 8538–8543.

Kanazawa, S., Soucek, L., Evan, G., Okamoto, T., and Peterlin, B.M. (2003). c-Myc recruits P-TEFb for transcription, cellular proliferation and apoptosis. Oncogene *22*, 5707–5711.

Kanno, T., Kano, Y., Siegel, R.M., Jang, J.M., Lenardo, M.J., and Ozato, K. (2004). Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. Mol. Cell *13*, 33–43.

Ladurner, A.G., Inouye, C., Jain, R., and Tjian, R. (2003). Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries. Mol. Cell *11*, 365–376.

Lis, J.T., Mason, P., Peng, J., Price, D.H., and Werner, J. (2000). P-TEFb kinase recruitment and function at heat shock loci. Genes Dev. *14*, 792–803.

Maruyama, T., Farina, A., Dey, A., Cheong, J., Bermudez, V.P., Tamura, T., Sciortino, S., Shuman, J., Hurwitz, J., and Ozato, K. (2002). A Mammalian bromodomain protein, brd4, interacts with replication factor C and inhibits progression to S phase. Mol. Cell. Biol. *22*, 6509–6520.

Matangkasombut, O., and Buratowski, S. (2003). Different sensitivities of bromodomain factors 1 and 2 to histone H4 acetylation. Mol. Cell *11*, 353–363.

Michels, A.A., Nguyen, V.T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and Bensaude, O. (2003). MAQ1 and 7SK RNA interact with CDK9/cyclin T complexes in a transcription-dependent manner. Mol. Cell. Biol. *23*, 4859–4869.

Nguyen, V.T., Kiss, T., Michels, A.A., and Bensaude, O. (2001). 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. Nature *414*, 322–325.

Ogryzko, V.V., Kotani, T., Zhang, X., Schiltz, R.L., Howard, T., Yang, X.J., Howard, B.H., Qin, J., and Nakatani, Y. (1998). Histone-like TAFs within the PCAF histone acetylase complex. Cell *94*, 35–44.

Peng, J., Zhu, Y., Milton, J.T., and Price, D.H. (1998). Identifcation of multiple subunits of human P-TEFb. Genes Dev. *12*, 755–762.

Price, D.H. (2000). P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol. Cell. Biol. *20*, 2629–2634.

Proudfoot, N.J., Furger, A., and Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501–512.

Simone, C., Bagella, L., Bellan, C., and Giordano, A. (2002). Physical interaction between pRb and cdk9/cyclinT2 complex. Oncogene *21*, 4158–4165.

Sims, R.J., 3rd, Belotserkovskaya, R., and Reinberg, D. (2004). Elongation by RNA polymerase II: the short and long of it. Genes Dev. *18*, 2437–2468.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Taube, R., Lin, X., Irwin, D., Fujinaga, K., and Peterlin, B.M. (2002). Interaction between P-TEFb and the C-terminal domain of RNA polymerase II activates transcriptional elongation from sites upstream or downstream of target genes. Mol. Cell. Biol. *22*, 321–331.

Wei, P., Garber, M.E., Fang, S.M., Fischer, W.H., and Jones, K.A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell *92*, 451–462.

Yang, Z., Zhu, Q., Luo, K., and Zhou, Q. (2001). The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature *414*, 317–322.

Yang, Z., Yik, J.H.N., Chen, R., He, N., Jang, M.K., Ozato, K., and Zhou, Q. (2005). Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol. Cell *19*, this issue, 535–545.

Yik, J.H., Chen, R., Nishimura, R., Jennings, J.L., Link, A.J., and Zhou, Q. (2003). Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. Mol. Cell *12*, 971–982.

Zhou, M., Halanski, M.A., Radonovich, M.F., Kashanchi, F., Peng, J., Price, D.H., and Brady, J.N. (2000). Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. Mol. Cell. Biol. *20*, 5077–5086.