Chromatin structure organization is crucial for regulating many fundamental cellular processes. However, the molecular mechanism that regulates the assembly of higher-order chromatin structure remains poorly understood. In this study, we demonstrate that Brd4 (bromodomain-containing protein 4) protein participates in the maintenance of the higher-order chromatin structure. Brd4, a member of the BET family of proteins, has been shown to play important roles in cellular growth control, cell cycle progression, and cancer development. We apply in situ single cell chromatin imaging and micrococcal nuclease (MNase) assay to show that Brd4 depletion leads to a large scale chromatin unfolding. A dominant-negative inhibitor encoding the double bromodomains (BDI/II) of Brd4 can competitively dissociate endogenous Brd4 from chromatin to trigger severely fragmented chromatin morphology. Mechanistic studies using Brd4 truncation mutants reveal that the Brd4 C-terminal domain is crucial for maintaining normal chromatin structure. Using bimolecular fluorescence complementation technology, we demonstrate that Brd4 molecules interact intermolecularly on chromatin and that replacing Brd4 molecules by BDI/II causes abnormal nucleosome aggregation and chromatin fragmentation. These studies establish a novel structural role of Brd4 in supporting the higher chromatin architecture.

Chromatin structure plays key roles in chromosomal functional events such as transcription, DNA replication, repair, and recombination. Mutations in genes encoding enzymes that modulate chromatin structure can result in perturbations of these important cellular processes and have been linked to many human genetic diseases (1). Aberrant chromatin structure is also manifested in cancer (2). Consequently, different processes involved in chromatin assembly and remodeling have become the potential drug targets for diseases such as cancer, neurodegenerative diseases, AIDS etc. A more complete understanding of the molecular mechanisms underlying the regulation of the chromatin structure is therefore vital for developing more rational drug therapies for these diseases.

Nucleosomes are the basic unit of chromatin, consisting of ~147 base pairs of DNA wrapped around a histone octamer containing two copies each of the core histones H2A, H2B, H3, and H4 (3). Neighboring nucleosomes are connected through a 10–50-base pair linker DNA to form a 10-nm “beads on a string” array. Nucleosome-nucleosome interaction drives the folding of a nucleosomal array into the 30-nm chromatin fiber and eventually into large scale structure (4). Besides compaction into chromosomes during mitosis, the chromatin fiber is organized, either in euchromatin that contains actively transcribed genes, or in heterochromatin that contains inactive genes (5). The folding of chromatin into a 30-nm fiber in vitro has been revealed by x-ray crystallography and electron microscopy studies (6–8). However, it is still unknown how the nucleosome fibers compact into condensed chromatin within a cell. Although a number of contributors to chromatin dynamics are known, their functional relationships have not been mechanistically defined. The mechanisms involved in higher-order chromatin architecture assembly and maintenance have remained elusive.

Reversible post-translational modifications of the N-terminal tails of histones are crucial for regulating the higher-order states of chromatin compaction (9). The bromodomain is an evolutionarily conserved motif present in many chromatin-associated proteins and histone acetyltransferases (10). It is the only known protein module capable of specifically binding to acetylated lysines at the histone tails (11). Structural studies have established the bromodomain/acetyl-lysine recognition as a pivotal mechanism for regulating protein-protein interactions in histone-directed chromatin remodeling (11). The crystal structure of the nucleosome core particle reveals that the
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...and basic N-terminal tail of histone H4 plays an important role in chromatin compaction (6), suggesting that bromodomain proteins binding to the acetylated H4 tail may be directly involved in this process.

Brd4 is a member of the BET family of proteins that harbor two bromodomains and an extra terminal domain (12). It is normally expressed as a long isoform (Brd4L, aa 1–1362) and a short isoform (Brd4S, aa 1–722). Brd4 binds to acetylated histones through its double bromodomains and becomes associated with both interphase chromatin and mitotic chromosomes (13). Brd4 knock-out in mice is embryonic lethal (14). This is consistent with its role in host cellular growth control and cell cycle progression (12, 15–18). Previous works from our group and others have identified Brd4 as an important target for a number of oncogenic viruses (19–23). The human Brd4 gene is also the target of translocation t(15;19) that defines a highly lethal carcinoma (24). Brd4 activation in human breast carcinomas induces a gene expression signature that efficiently predicts survival (25). It has also been identified as a novel target required for maintenance of acute myeloid leukemia (26). Most of the Brd4 functions have been linked to its role in transcription regulation (27–29). However, in our study, we observed that Brd4 depletion leads to enlarged nuclei indicating chromatin decondensation. Because the closely related Brd2 protein has been shown to possess a histone chaperone activity (30) and another bromodomain protein BrdT can bind hyperacetylated histone tail to induce a large scale chromatin reorganization (31), we decided to further examine how Brd4 binding to acetylated histones affects the chromatin structure.

In this study, we use micrococcal nuclease (MNase) assay and in situ chromatin imaging to show that Brd4 knockdown in human cells leads to highly decondensed chromatin structure. A mutant encoding the double bromodomains of Brd4 functions in a dominant-negative manner to dissociate Brd4L from chromatin and to cause a highly fragmented chromatin structure. Mechanistic studies demonstrate that the Brd4 CTD is crucial for maintaining normal chromatin structure. Bimolecular fluorescence complementation (BiFC) reveals that Brd4 molecules interact intermolecularly on chromatin through the N terminus and that replacing Brd4 molecules with the double-bromodomain mutant lacking the CTD causes abnormal chromatin fragmentation. Our study therefore provides mechanistic evidence to support a novel structural role of Brd4 in higher-order chromatin structure organization.

**Experimental Procedures**

**Recombinant Plasmid Construction**—Plasmids encoding the Xpress-tagged Brd4L (pcDNA4C-Brd4L), BDI/II (pcDNA4C-NLS-BDI/II), and CTD (pcDNA4C-NLS-CTD) have been described (19, 32). pCMV2-mBrd4 was kindly provided by Dr. Randall King (Harvard Medical School). Recombinant Plasmid Construction—Plasmids encoding the Xpress-tagged Brd4L (pcDNA4C-Brd4L), BDI/II (pcDNA4C-NLS-BDI/II), and CTD (pcDNA4C-NLS-CTD) have been described (19, 32). pCMV2-mBrd4 was kindly provided by Dr. Randall King (Harvard Medical School).

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**Immunofluorescent Staining**—Cells cultured on coverslips were fixed with 3% paraformaldehyde in PBS for 20 min. Immunofluorescent staining was performed as previously described (32, 34). The following primary antibodies were used: anti-Brd4N (recognizing Brd4 aa 156–284, 1:40,000), anti-Brd4C (recognizing Brd4 aa 1313–1362, 1:40,000), and anti-Brd2 (provided by Gerald Denis, 1:500), anti-Xpress (Invitrogen, 1:200), and anti-FLAG (Sigma, 1:20000). Secondary antibodies used were: Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, 1:1,000), Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, 1:1,000), and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, 1:500).

**Microscopy and Image Analysis**—All immunofluorescent images were collected using an inverted fluorescence microscope (Olympus, IX81) equipped with UPlanSApo ×40/0.95 NA lens (Olympus), UPlanSApo ×100/1.4 oil immersion lens (Olympus), and a high-resolution charge-coupled device camera (QImaging, FAST1394). Image data were analyzed and presented using SlideBook 5.0 software (Intelligent Imaging Innovations, Inc.). The scale bars were added using ImageJ software. Three-dimensional imaging of DAPI stained cells was performed using the “three-dimensional capture” mode of SlideBook 5.0 software with the step size set at 0.3 μm. The photos were assembled for presentation using the “make montage” function of ImageJ software. The immunofluorescent images of U2OS 2–6–5 cells were also analyzed using ImageJ software. The “adjust threshold” function of the software was used to select the YFP–LacI dots in the photos and the area data were acquired using “measure” function of the software.

**Micrococcal Nuclease Assay**—5 × 10^6 C33A cells were resuspended in 300 μl of HNB (15 mM Tris-HCl, pH 7.6, 0.5 mM sucrose, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 0.5% Triton X-100, 1 mM DTT, 0.5 mM PMSE). After 20 min incubation on ice, nuclei were isolated by centrifugation at 240 × g for 5 min. Nuclei were gently resuspended in 50 μl of nuclear buffer (20 mM Tris-HCl, pH 7.6, 70 mM NaCl, 20 mM KCl, 5 mM MgCl₂, and 3 mM CaCl₂). Nuclei suspension was incubated with 0.06 unit of micrococcal nuclease (Sigma) at 37 °C for 10 min. The digestion was terminated by addition of EDTA and EGTA to a 5 mM final concentration each. The nuclear pellets were collected by centrifugation at 6000 × g for 5 min, and resuspended in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM EDTA, 0.5% SDS) supplemented with RNase (Roche Applied Science, 100 μg/ml). After incubation at 37 °C for 20 min, Proteinase K (Roche Applied Science, 200 μg/ml) was added into the samples and incubated at 37 °C for 4 h. After phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in H₂O. Equal amounts of DNA samples were separated on a 1.2% agarose gel and detected with ethidium bromide. All MNase assay experiments were repeated more than three times.

**Immunoprecipitation and Western Blot Analysis**—At 48 h post-transfection, 293T cells were lysed in H buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 3 mM sodium butyrate, supplemented with protease inhibitor mixture and phosphatase inhibitor mixture, from Roche Applied Science) by passing through a 26-gauge needle 10 times. After a 20-min incubation on ice, the supernatant (S1) and the nuclear pellets were separated by centrifugation at 2,700 × g for 5 min. The pellets were resuspended in digestion buffer (H buffer with 20 mM MgCl₂) supplemented with 18 units of DNase I (Roche Applied Science) for every 10^7 cells. The mixture was incubated at 37 °C for 30 min, and centrifuged at 2,700 × g for 5 min. The supernatant (S2) was combined with S1 to make the whole cell lysates. Cell lysates for all of IPs except the one presented in Fig. 5C were prepared using this method. Nuclear extracts used in Fig. 5C were prepared as described previously (32, 34).

After preblocking in 1% BSA/PBS at 4 °C for 1 h, anti-FLAG M2-agarose beads (Sigma) were incubated with cell lysates at 4 °C for 4 h. For Brd4 IP, the lysates were immunoprecipitated using protein A beads (pre-blocked with 1% BSA in PBS at 4 °C for 1 h) mixed with 2 μg of either normal rabbit IgG or Brd4 antibody at 4 °C overnight. The beads were washed and eluted as described previously (32). Input and IP samples were resolved on a SDS-PAGE gel, transferred onto PVDF membrane, and immunoblotted with specific antibodies as indicated in the figure legends (ECL detection). Antibodies employed in the Western blot analysis include: Brd4C (1:80,000), Brd4N (1:80,000), AcH4 (Upstate, 1:2,000), Xpress (Invitrogen, 1:5,000), Actin (Chemicon, 1:150,000), HA-HRP (Roche, 1:1000), FLAG (Sigma, 1:1,000), Cyclin T1 (Santa Cruz, 1:200), and Cdk9 (Santa Cruz, 1:200). All of the IP experiments were repeated at least three times.

**RESULTS**

**Brd4 Knockdown Leads to Decondensed Chromatin Structure**—In our previous study, aberrant chromosome segregation and failures in cytokinesis were consistently observed when Brd4 was knocked down by RNA interference (33). We also noticed that, prior to the onset of the chromosome segregation defects, Brd4 knockdown cells first demonstrated enlarged nuclear shape that resembles chromatin decondensation. In this study, we further examined the chromatin structure induced by Brd4 knockdown in C33A cells. Efficient Brd4 knockdown was achieved using each of the four siRNAs (si1–4) targeting different regions of Brd4 gene (supplemental Fig. S1A). Brd4 knockdown frequently leads to increased cell size and enlarged/lobulated nuclei that are absent in the control siRNA-treated cells (Fig. 1A). Similar chromatin defects were observed in several other cell lines including 293T, U2OS, and primary human foreskin keratinocytes (data not shown).

Previous studies have demonstrated that Brd4 knockdown leads to G1 cell cycle arrest and does not increase DNA contents in cells (17, 18), the enlarged nuclei in Brd4 siRNA-treated cells therefore indicate chromatin decondensation (Fig. 1A). We then performed limited MNase digestion to further assess the chromatin structural defect induced by Brd4 knockdown. MNase digestion provides a useful tool for analyzing chromatin structure because it cleaves the linker DNA between adjacent nucleosomes, and depending on the extent of digestion, generates chromatin fragments with different sizes corresponding to nucleosome number, which could be detected as a specific ladder of DNA. The patterns of permeability to nuclease digestion reveal the chromatin organization of cells under different experimental conditions. As shown in Fig. 1B, compared with the control siRNA-treated cells, MNase digestion of chromatin...
from cells treated with Brd4 siRNA resulted in increased lower molecular weight DNA fragments especially the 1st, 2nd, and 3rd smallest bands and significantly reduced large DNA fragments migrating in the upper half of the gel. The changes in the DNA band pattern were confirmed by quantification using ImageJ (Fig. 1B, right panel). This increased chromatin accessibility to MNase reveals a decondensed chromatin structure associated with Brd4 knockdown, suggesting that the physiological level of Brd4 is essential for maintaining properly condensed chromatin architecture.

In a rescue experiment, co-expression of the full-length mouse Brd4 gene resistant to the human Brd4 siRNA could reverse the increased MNase cleavage in Brd4 knockdown cells, resulting in a digestion pattern similar to those observed in the control sample (supplemental Fig. S1B). This study demonstrates that mouse Brd4 protein is able to partially rescue the
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decondensed chromatin structure in Brd4 knockdown cells, providing further evidence to support the role of Brd4 in chromatin structure organization.

Because Brd4 knockdown leads to decondensed chromatin, we speculated that it might also induce chromatin unfolding. We then used a lac repressor-lac operator (lacR-lacO) array system in the U2OS 2-6-5 cell line to directly visualize the impact of Brd4 on the large scale chromatin structure in living cells. The U2OS 2-6-5 cell line contains a condensed heterochromatic region consisting of an array of 256 copies of the lacO repressor sequence (35). These cells were first transfected with either control or Brd4 siRNAs. Forty-eight hours later, the cells were re-transfected with a construct encoding a yellow fluorescent protein and lacR fusion protein (YFP-LacI) to directly visualize the lacO chromatin array by fluorescence microscopy. As shown in Fig. 1C, the lacO array was detected as a compact green nuclear dot in the control cells. However, in most of Brd4 knockdown cells, the sizes of these nuclear dots increased significantly (Fig. 1C). The areas of YFP-LacI dots were calculated using ImageJ to be 0.67 ± 0.27 μm² in the control cells and 1.45 ± 0.58 μm² in the Brd4 knockdown cells (Fig. 1D). The average area of the lacO region was 2.15 times larger in the Brd4 knockdown cells than in the control cells with a p value of 2.48E-07 (Student’s t test). This result reveals the unfolding of the subnuclear chromatin structure induced by Brd4 knockdown. Together, these studies demonstrate that the absence of Brd4 on chromatin leads to chromatin decondensation, supporting the Brd4 function in linking the nucleosome together and maintaining a properly condensed chromatin structure.

The Brd4 Double Bromodomain Mutant Functions as a Dominant-negative Inhibitor to Dissociate Brd4L from Chromatin and Cause Abnormally Fragmented Chromatin Structure—Brd4 associates with chromatin and mitotic chromosomes by binding to acetylated histone 3 (AcH3) and histone 4 (AcH4) through its double bromodomains (13). To further determine how this interaction contributes to chromatin structure organization, we developed a dominant-negative inhibitor encoding the double bromodomains of Brd4 (aa 1–470, a.k.a. BDI/II) to prevent Brd4L from localizing to cellular chromatin. In C33A cells, endogenous Brd4L normally localizes to punctate dots on chromatin as seen in the non-transfected cells (Fig. 2A). However, in cells expressing BDI/II, endogenous Brd4L was prevented from localizing to punctate chromatin dots (Fig. 2A). Co-immunoprecipitation (co-IP) experiments showed that the amount of Brd4 binding to AcH4 is dramatically reduced in cells expressing BDI/II (supplemental Fig. S2A). Notably, inhibition of Brd4 and AcH4 co-IP by BDI/II was not rescued by trichostatin A, a histone deacetylase inhibitor (supplemental Fig. S2A), suggesting that the decreased Brd4-AcH4 interaction is not due to any change in H4 acetylation. Western blot analysis also confirmed that the BDI/II expression does not affect H4 acetylation in cells (supplemental Fig. S2B). These studies demonstrate that the BDI/II molecule functions as a dominant-negative inhibitor to competitively bind to AcH4 and dissociate endogenous Brd4L from acetylated histones on chromatin. This effect is specific for Brd4, as BDI/II does not affect the nuclear localization pattern of another bromodomain protein, Brd2 (Fig. 2A).

Immunofluorescence microscopy demonstrated that most of the BDI/II expressing cells not only show dispersed Brd4 staining but also exhibit severely fragmented chromatin as indicated by 4,6-diamidino-2-phenylindole (DAPI) DNA staining (Fig. 2A). The BDI/II-positive cells are negatively stained for Annexin V, suggesting that the fragmented chromatin is not caused by cell death (data not shown). To confirm that the abnormal DAPI staining induced by BDI/II reflects a change in chromatin structure, we expressed this protein in C33A cells stably expressing GFP-H2B. GFP-H2B binds to chromatin efficiently and paints the nuclear chromatin with GFP fluorescent signal, allowing a clearer visualization of chromatin structure. The BDI/II expression in C33A/GFP-H2B stable cells also causes a highly fragmented chromatin structure as shown by the GFP-H2B fluorescent signal as well as DAPI staining (Fig. 2B). We therefore use the term “chromatin fragmentation” to describe this phenomenon of chromatin reorganization that appears to break chromatin into multiple fragments inside the nucleus (Fig. 2B, middle panel). In contrast to the BDI/II-positive cells, neighboring cells that do not express BDI/II display normal chromatin structure (Fig. 2B). To rule out an overexpression artifact, we also expressed Brd4L at a similar level in cells. Notably, exogenous expression of Brd4L does not induce visible changes in chromatin structure (Fig. 2B). This result indicates that association of Brd4 with chromatin is vital for maintaining the normal chromatin structure. The nuclear reorganization induced by BDI/II is consistent with a previous study showing that the shorter isoform of BrdT triggers a dramatic compaction of acetylated chromatin (31). Together, these studies demonstrate a structural role for double-bromodomain proteins in remodeling acetylated chromatin.

We then performed MNase analysis to examine the BDI/II-induced abnormal chromatin structural change at the molecular level. Compared with the control cells, MNase digestion of chromatin isolated from cells expressing BDI/II generates significantly more intense lower molecular weight DNA fragments (especially the 2nd, 3rd, and 4th smallest bands from the bottom of the gel) but less intense higher molecular weight DNA fragments (Fig. 3A). These increased lower molecular weight bands represent DNA fragments isolated from nucleosome dimers, trimers, and tetramers (Fig. 3A), indicating increased MNase cleavage between these nucleosome oligomers in the BDI/II expressing cells. Notably, the intensity for the smallest band from the bottom of the gel representing DNA fragments isolated from nucleosome monomers was not increased but slightly decreased in the BDI/II sample. This result suggests that BDI/II does not simply induce a global chromatin decondensation but rather an abnormally fragmented chromatin structure, which represents an uneven compaction of chromatin caused by aggregation of nucleosomes into different oligomers that could not be easily cut by MNase into monomers. Consistent with a normal nuclear morphology in cells expressing Brd4L (Fig. 2B), expression of full-length Brd4 does not cause a dramatic change in the MNase cleavage pattern compared with the control cells (Fig. 3A).
To further investigate how changing the Brd4 status modulates chromatin architecture at a higher resolution, we performed three-dimensional imaging of the chromatin structure in 293T cells expressing either BDI/II or Brd4L. As shown in Fig. 3B, Brd4L expression does not cause an obvious chromatin structural change when compared with the control cells. In contrast, in most of the BDI/II expressing cells, chromatin appears to aggregate into highly compact regions (Fig. 3B). This abnormal chromatin pattern is consistent with the result obtained in C33A cells and also supports the abnormal nucleosome oligomerization observed in Fig. 3A. In conclusion, our data show that dissociation of Brd4L from chromatin by the BDI/II dominant-negative inhibitor leads to abnormal chromatin reorganization. These observations support an important role of Brd4L in maintaining the properly folded chromatin structure.

The C-terminal Domain of Brd4 Is Important for Maintaining Normal Chromatin Structure—Because expression of the N-terminal double bromodomains of Brd4 can lead to abnormal chromatin reorganization that is absent in the Brd4L expressing cells, we decided to identify the molecular component(s) of Brd4L that allows it to maintain normal chromatin structure. We constructed a series of Xpress-tagged Brd4 C-terminal truncation mutants encoding aa 1–470 (BDI/II), 1–594, 1–730, and 1–1046 (Fig. 4A). All of these mutants can interact efficiently with AcH4 (supplemental Fig. S3), demon-
strating that these proteins are folded correctly. We then examined chromatin morphology in cells expressing these truncation mutants. Quantification of the percentage of transfected cells with the fragmented chromatin structure indicates that shorter fragments such as 1–730, 1–594, or 1–470 all cause chromatin fragmentation in 40–50% of the transfected cells, whereas expression of the 1–1046 molecule does not generate any obvious chromatin structure defect (Fig. 4B).

To test whether these Brd4 mutants could compete for AcH4 binding and dissociate endogenous Brd4L from chromatin, co-IP of Brd4 and AcH4 using an antibody that specifically recognizes the Brd4 C terminus was performed in cells transfected with either a vector control or a Brd4 mutant construct. Normal rabbit IgG was used as a negative control to show that there was not any nonspecific pulldown of AcH4 (Fig. 4C). In contrast, Brd4 co-IP pulled down significant amounts of AcH4 in the vector control sample (Fig. 4C). A similar amount of AcH4 was pulled down by the Brd4 antibody in the 1–1046-transfected cells (Fig. 4C), indicating that endogenous Brd4L molecules can still interact with AcH4 in the presence of 1–1046. However, the amount of AcH4 co-IP with Brd4 was dramatically reduced in cells expressing 1–470, 1–594, and 1–730 (Fig. 4C). Because the Brd4 antibody and the AcH4 antibody were both generated from rabbit, the co-IPed samples show some rabbit IgG reactive nonspecific bands in the AcH4 Western blot. However, it is clear that expression of the shorter Brd4 truncation mutants (1–470, 1–594, and 1–730) can efficiently inhibit the binding of Brd4 to AcH4, whereas the longest mutant (1–1046) cannot. The ability of these Brd4 mutants to cause competitive dissociation of Brd4L from chromatin clearly correlates with their efficiency in triggering the fragmented chromatin structure; further supporting that proper binding of Brd4L on chromatin is essential for maintaining normal chromatin structure (Fig. 4, B and C). This study also suggests that the shorter Brd4 mutants may associate with chromatin more tightly than Brd4L, providing
FIGURE 4. **The C-terminal domain of Brd4 is important for maintaining normal chromatin structure.** A, schematic diagrams of Brd4 molecules. Their abilities to cause fragmented chromatin structure are summarized on the right. Brd4 domains: BD, bromodomain; ET, extra-terminal domain; P, proline-rich region. B, 293T cells transfected with pcDNA4C vector or pcDNA4C encoding Xpress-tagged Brd4 truncation mutants were fixed 24 h post-transfection and stained with Xpress antibody and DAPI. The percentage of cells showing fragmented chromatin defects was quantified from ~200 positively transfected cells. Mean ± S.D. shown in the diagram were calculated from three independent experiments. C, cell lysates from 293T cells transfected with pcDNA4C vector or a pcDNA4C Brd4 truncation mutant were IPed with either normal rabbit IgG (Control IP) or Brd4C antibody (Brd4 IP). Co-IPed proteins were immunoblotted with specific antibodies as indicated. Arrow and asterisks mark the Xpress-tagged Brd4 mutants expressed in cells. D, 293T cells transfected with pcDNA4C vector, or pcDNA4C plasmid expressing Brd4 molecules were fixed 24 h post-transfection and stained with Xpress antibody and DAPI. The percentage of cells showing the fragmented chromatin defect was quantified from ~200 positively transfected cells. Mean ± S.D. shown in the diagram were calculated from three independent experiments. E, 293T cells were co-transfected with a FLAG-HA-Brd4L plasmid and either pcDNA4C vector or a pcDNA4C encoding BDI/II, BDI/II fusions, or Brd4L. Cell lysates were IPed with FLAG antibody. The precipitates were immunoblotted with specific antibodies as indicated. Asterisks mark the Xpress-tagged proteins expressed in the cells.
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a mechanistic basis for the dominant-negative effect of BDI/II in Brd4L chromatin association.

Expression of Brd4-(1–1046) and Brd4L generates barely any detectable chromatin structural defect (Figs. 2–4), whereas 1–470, 1–594, and 1–730 mutants can dissociate Brd4L from chromatin to induce dramatic chromatin fragmentation. The data indicate that the aa 731–1362 region of Brd4 may harbor important molecular component(s) for maintaining normal chromatin structure. To test this further, we generated constructs encoding the BDI/II domain fused directly to either aa 731–1046 or 1047–1362 (the CTD domain of Brd4) to determine whether these regions could rescue the BDI/II-induced abnormal chromatin structure (Fig. 4A). The LacI molecule was also fused to BDI/II to generate a fusion control with a similar molecular weight (Fig. 4A). Similar to the BDI/II molecule, BDI/II-(731–1046) and BDI/II-(1047–1362) fusions could efficiently interact with AcH4 (supplemental Fig. S4). Furthermore, just as for the Brd4 CTD, the BDI/II-(1047–1362) fusion protein could pull down the Cdk9 and Cyclin T1 subunits of P-TEFb (positive transcription elongation factor b), which is known to bind to the last 50 amino acids of Brd4 (32) (supplemental Fig. S4). These results confirm that the mutant proteins are folded correctly. They were then expressed in 293T cells for chromatin structure analysis. Similar to the BDI/II expressing cells, the BDI/II-LacI-positive cells displayed a fragmented chromatin pattern (supplemental Fig. S5). However, the BDI/II-(731–1046) and BDI/II-(1047–1362) positive cells showed normal chromatin texture (supplemental Fig. S5). As shown in Fig. 4D, the percentage of BDI/II-LacI-positive cells that displayed fragmented chromatin (56.1%) was similar to the BDI/II-positive cells (51.9%), whereas this percentage was much lower in cells expressing BDI/II-(731–1046) (1.8%), BDI/II-(1047–1362) (2.8%), or Brd4L (0.3%).

To test whether the BDI/II-(731–1046) and BDI/II-(1047–1362) fusions could compete for AcH4 binding and dissociate Brd4L from chromatin, lysates from cells co-transfected with a FLAG-HA-Brd4L construct and either one of the Xpress-tagged Brd4 fusion constructs were subjected to FLAG IP. As shown in Fig. 4E, FLAG antibody could efficiently IP FLAG-HA-Brd4L in all samples. In cells co-transfected with a vector control, a significant amount of AcH4 was co-IP with FLAG-HA-Brd4L construct and either one of the Xpress-Brd4L, whereas no major band was detected in the co-IP using cells transfected with a FLAG-HA vector (Fig. 6A). Using a similar approach, BDI/II–FLAG-HA was shown to effi-
ciently pulldown Xpress-BDI/II (Fig. 6A). Because the cell lysates used in the co-IP were pretreated with DNase I to digest the DNA linkers between nucleosomes (“Experimental Procedures”), it rules out the possibility that the protein-protein interaction detected by this co-IP was mediated by the chromatin. This biochemical analysis demonstrates that Brd4 can interact intermolecularly and this interaction is mostly mediated by the N terminus 1–470 aa domain of Brd4.

To ensure that the Brd4-Brd4 interaction detected by co-IP reflects true interaction in cells, we further examined the Brd4-Brd4 interaction using BiFC technology. For BiFC, two interacting proteins are fused individually to complementary fragments of a fluorescent reporter and expressed in live cells. Interaction of these proteins will bring the fluorescent fragments within proximity, allowing the reporter protein to reform its native three-dimensional structure and emit fluorescent signal. Therefore, protein interaction can be identified and located within the cell through visualization of the intensity and distribution of fluorescence in these cells (37). To test the BDI/II-BDI/II and Brd4L-Brd4L intermolecular interaction, these molecules were fused individually to either the N terminus (VN) or C terminus (VC) of Venus (38). Venus signal was detected only when both BDI/II-VN and BDI/II-VC constructs were co-expressed in cells (Fig. 6B). On the other hand, expression of either construct with the VN or VC control generated only very dim background signals (Fig. 6B). These observations support that BDI/II molecules can interact intermolecularly in cells. Similarly, co-expression of Brd4L-VN and Brd4L-VC fusion proteins in cells generated a strong BiFC positive signal, which is absent in cells transfected with either Brd4L-VN with the VC control or Brd4L-VC with the VN control (Fig. 6B). This result provides direct evidence to demonstrate that the Brd4 molecules can interact efficiently in cells through the BDI/II domain. FLAG staining was performed to detect the FLAG-tagged BiFC molecules. We noticed that most of the BiFC-positive cells show a dimmer FLAG signal in the center of each cell possibly because the BiFC interaction interferes with FLAG staining (Fig. 6B). Signals for both BDI/II BiFC and Brd4L BiFC are shown in a pattern of evenly spaced minute punctate dots on chromatin that closely resembles the staining pattern of...
endogenous Brd4 (Fig. 6B), suggesting that the interactions occur when either Brd4L or BDI/II binds to the native Brd4 binding sites on acetylated chromatin. These observations are significant as we speculate that this Brd4 interaction may contribute to its function in nucleosome binding and chromatin compaction.

Replacement of Brd4L by BDI/II on Chromatin Causes Abnormal Chromatin Fragmentation—Because the BiFC assay showed a clear pattern of Brd4 intermolecular interaction on chromatin, we tested BDI/II in this system to visualize how it affects the Brd4L-Brd4L interaction on chromatin to cause chromatin fragmentation. U2OS cells were transfected with Brd4L-VN, Brd4L-VC, and either a control vector or the Xpress-BDI/II construct. The vector control cells showed a normal Brd4L BiFC signal indicating a nicely spaced Brd4 interaction on chromatin (Fig. 7A). However, in most of the Xpress-BDI/II-positive cells, the BiFC signal was significantly reduced to a dimmer diffused pattern, indicating dissociation of Brd4L from chromatin (Fig. 7A). In addition, the Xpress-BDI/II expression led to significant chromatin fragmentation as observed in previous experiments (Fig. 7A, DAPI, middle and bottom panels). In many cases, the green BiFC signal is mainly detected in the nuclear space without DAPI staining, suggesting that the Brd4L BiFC molecules were excluded from the fragmented chromatin (Fig. 7A, bottom panel). The resulting chromatin fragmentation morphology suggests that although Brd4 molecules retain the ability to interact with each other when they are dissociated from chromatin by BDI/II, they can no longer support the normal chromatin structure. This result provides direct visualization of how BDI/II causes the fragmented chromatin phenomenon by disrupting the Brd4L-Brd4L interaction on chromatin, offering further evidence to support that normal localization of Brd4L on chromatin is important for maintaining the properly organized chromatin structure.

To further visualize how BDI/II causes chromatin fragmentation, U2OS cells were transfected with BDI/II-VN, BDI/II-VC, and either a control vector or the Xpress-BDI/II construct (Fig. 7B). In contrast to the normal BDI/II BiFC signal detected in the control cells, co-expression of Xpress-BDI/II causes a more severe chromatin fragmentation and redistribution of the BDI/II BiFC signal into large green fluorescent islands (Fig. 7B). Interestingly, this BDI/II BiFC signal precisely matches the DAPI staining pattern, suggesting that BDI/II BiFC occurs on fragmented chromatin in the presence of Xpress-BDI/II. This is significantly different from the dissociation of Brd4L BiFC from chromatin induced by Xpress-BDI/II (Fig. 7A, bottom panel). The phenomenon is consistent with the notion that Xpress-
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Brd4/II could bind to chromatin with similar affinity as the Brd4/II BiFC molecules and therefore could not compete away the Brd4/II BiFC from chromatin. Because the Brd4/II molecules could interact with each other, expression of Xpress-Brd4/II could potentially further promote the Brd4/II BiFC on chromatin. The Brd4/II BiFC present on the fragmented chromatin provides direct evidence to demonstrate that binding of Brd4/II to chromatin can replace/disrupt the Brd4L-Brd4L interaction on chromatin to introduce structural defects that lead to chromatin fragmentation.

DISCUSSION

Chromatin structure dynamics are crucial for regulating many fundamental cellular events. Aberrant chromatin structure that perturbs these important biological processes has been linked to many human genetic diseases and cancer. Elaborate mechanisms have been demonstrated for the assembly of primary and secondary chromatin structure. However, due to the technical difficulties of traditional approaches that prevented a clear visualization of large scale chromatin structure, the molecular mechanism(s) that regulate the assembly of the higher-order chromatin structure have yet to be fully elaborated.

In our study, we combined a set of complementary new techniques developed in recent years to examine the role of Brd4 in chromatin structure maintenance. We observed that Brd4 knockdown leads to enlarged nuclei. Using MNase assay and chromatin structure maintenance. We observed that Brd4 elaborated.

The collapse of chromatin structure upon Brd4 knockdown supports that Brd4 binding to acetylated histones may provide molecular forces to maintain local nucleosome-nucleosome interactions. Each Brd4 molecule carries two bromodomains, which may bind to adjacent acetyl-lysines either within a single histone or on different nucleosomes to promote the nucleosome array interaction, contributing to chromatin fiber-fiber oligomerization. The intermolecular interaction may allow Brd4 to act in multimeric complexes to bridge acetylated nucleosomes together and to support higher-order chromatin folding.

Knockdown of both Brd4L and Brd4S isoforms leads to a highly decondensed chromatin structure, whereas dissociation of Brd4L from chromatin by Brd4/II triggers a severely fragmented chromatin. These observations again suggest that binding of the Brd4L isoform to chromatin is essential for maintaining a properly organized chromatin conformation. Brd4/II binding causes a more dramatic change in chromatin structure than Brd4 knockdown. One possibility is that it could eliminate native Brd4 from chromatin more effectively or could displace other BET proteins besides Brd4. In addition, unlike Brd4 knockdown that removes Brd4 molecules from chromatin, binding of Brd4/II on chromatin may maintain the regional nucleosome organization and chromatin architecture through intermolecular interaction. However, the higher intrinsic affinity of Brd4/II for chromatin described above may cause these molecules to bind nucleosomes more tightly than Brd4L, leading to nucleosome aggregation and chromatin fragmentation. This augmented chromatin structural defect suggests that the Brd4 C-terminal domain, which is absent in Brd4/II, harbors important properties for maintaining the properly folded chromatin structure.

The Brd4 C-terminus has been implicated in recruiting P-TEFb complex for stimulating transcription elongation (27–29). Our studies show that the Brd4 C-terminus function in chromatin structure maintenance is independent of the P-TEFb-associated transcription regulation, providing a novel structural role of Brd4 in chromatin architecture maintenance. The Brd4 C-terminal domain may provide steric support for an optimal spacing between nucleosomes to prevent abnormal chromatin condensation. It could also harbor additional post-translational modifications and/or important protein-protein interactions (either between Brd4 molecules or for Brd4 and other cellular proteins) to promote the chromatin scaffold function. In addition, the Brd4 C-terminal domain could negatively regulate Brd4L interaction with chromatin to ensure an optimal chromatin compaction. This notion is supported by a previous study (39), and is also consistent with our data showing that the shorter Brd4 molecules bind more tightly to AcH4 to compete the Brd4L and AcH4 interaction (Fig. 4). Because native Brd4 short isoform exists in cells, our observation also points to a possibility that the long and short forms of Brd4 may bind to chromatin in a tightly regulated manner to provide a balanced distribution for proper chromatin condensation. The molecular basis for this differential chromatin affinity of Brd4 isoforms and how it pertains to the Brd4 physiological function in chromatin structure organization remain to be investigated.

In our study, we noticed that the severity of the chromatin defect caused by Brd4/II paralleled its expression level. This is probably because Brd4/II could interact with Brd4L and therefore higher concentrations of the dominant-negative inhibitor...
can better overcome this interaction to dissociate endogenous Brd4L from chromatin. However, expression of Brd4L, Brd4-(41–1046), BDBI/II-(731–1046), or BDBI/II-(1047–1362) at the similar level as BDBI/II does not cause chromatin fragmentation, suggesting that the BDBI/II phenotype is not likely to be an over-expression artifact.

The BDBI/II-induced chromatin reorganization is in line with previous studies reporting a histone chaperone activity of Brd2 (30). Furthermore, expression of the short form of BrdT has been shown to induce large scale chromatin condensation in a trichostatin A-dependent manner (31). In addition to studying the chromatin morphology, we further determined the protein–protein interaction between Brd4 molecules and Brd4 with acetylated histones. This study thus provides the first mechanistic insight into Brd4 function as a chromatin adaptor to support molecular interactions on nucleosome array for chromatin structure maintenance. Our studies also identified the Brd4 C terminus as a critical domain for maintaining a properly condensed higher-order chromatin structure. Interestingly, among all the known human BET family proteins, Brd4L is the only member that contains the unique C terminus, the rest of the Brd4 domains are highly conserved in all BET proteins. The biological significance of this unique Brd4 domain remains to be further investigated.

Brd4 is targeted by a number of oncogenic viruses, highlighting the significance of its biological function in ensuring normal cellular growth and cellular defense against viral infection (19–23). In addition, translocation t(15;19) deletes the C-terminal half of the Brd4 long isoform, resulting in a novel fusion oncogene Brd4-Nut in a highly aggressive carcinoma (24). It is tempting to speculate that viral protein interactions and the bromodomain protein interaction between Brd4 molecules and Brd4 with acetylated histones. This study thus provides the first mechanistic insight into Brd4 function as a chromatin adaptor to support molecular interactions on nucleosome array for chromatin structure maintenance. Dysregulation of Brd4-associated pathways has been shown to contribute to breast cancer progression (25). Brd4 has also been identified as an important therapeutic target for acute myeloid leukemia (26). The widespread presence of bromodomains in leukemogenic and cancer genes highlights the significance of studying bromodomains in malignant diseases. Comprehensive understanding of the Brd4 function will provide a paradigm for investigating the molecular mechanisms of other bromodomains. Important Brd4 activities uncovered in the context of higher-order chromatin organization and normal cellular growth may offer promising leads for developing efficient therapeutic strategies to cure Brd4-associated human cancers.

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