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Brd4 Is Required for Recovery from Antimicrotubule Drug-induced Mitotic Arrest: Preservation of Acetylated Chromatin[D]

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The mammalian bromodomain protein Brd4 interacts with mitotic chromosomes by binding to acetylated histone H3 and H4 and is thought to play a role in epigenetic memory. Mitotic cells are susceptible to antimicrotubule drugs. These drugs activate multiple response pathways and arrest cells at mitosis. We found that Brd4 was rapidly released from chromosomes upon treatment with antimicrotubule drugs, including the reversible agent nocodazole. Yet, when nocodazole was withdrawn, Brd4 was reloaded onto chromosomes, and cells proceeded to complete cell division. However, cells in which a Brd4 allele was disrupted (Brd4+/−), and expressing only half of the normal Brd4 levels, were defective in reloading Brd4 onto chromosomes. Consequently, Brd4+/− cells were impaired in their ability to recover from nocodazole-induced mitotic arrest: a large fraction of +/− cells failed to reach anaphase after drug withdrawal, and those that entered anaphase showed an increased frequency of abnormal chromosomal segregation. The reloading defect observed in Brd4+/− cells coincided with selective hypoacetylation of lysine residues on H3 and H4. The histone deacetylase inhibitor trichostatin A increased global histone acetylation and perturbed nocodazole-induced Brd4 unloading. Brd4 plays an integral part in a cellular response to drug-induced mitotic stress by preserving a properly acetylated chromatin status.

INTRODUCTION

Brd4 is a mammalian bromodomain protein that belongs to the conserved BET family (Dey *et al*., 2000). The 200-kDa nuclear protein contains two bromodomains and a long C-terminal domain. Previous analysis with fluorescence loss in photobleaching showed that Brd4 dynamically interacts with acetylated histones H3 and H4 in living interphase cells (Dey *et al*., 2003). Extending these observations, we found that Brd4 and the related Brd2 recognize acetylated histone codes through the bromodomains (Kanno *et al*., 2004) and that acetylated K14 on H3 and K5/12 on H4 are preferred binding sites of Brd4 (Dey *et al*., 2003). An additional feature of Brd4 is that it localizes to chromosomes during mitosis (Dey *et al*., 2000). In mitotic cells, Brd4 predominantly resides in the long axes of all chromosomes except centromeres where Brd4 is conspicuously absent. Similar to interphase cells, localization of Brd4 to mitotic chromosomes is attributed at least in part to its binding to acetylated histones. During interphase, Brd4 interacts with other proteins, including the cyclin T/Cdk9 complex, P-TEFb (Jang *et al*., 2005). Brd4 recruits P-TEFb to various promoters in acetylated chromatin and participates in RNA polymerase II-dependent transcription. Based on these properties, Brd4 is thought to play a role in the inheritance of transcriptional memory from one generation of cells to the next (Dey *et al*., 2003; Loyola and Almouzni, 2004).

Progress has been made in understanding molecular pathways that regulate the process of cell division (Mitchison and Salmon, 2001; Pines and Rieder, 2001). During mitosis, chromatin undergoes a dramatic change both in conformation and function. In higher eukaryotic cells, chromatin-binding proteins, including many bromodomain proteins (except for Brd4/2) as well as specific and general transcription factors, are released from chromatin during mitosis (Muchardt *et al*., 1996; Segil *et al*., 1996). Coinciding with extensive unloading of factors from chromatin, transcription becomes globally suspended, rendering the whole genome inert during mitosis (Muchardt *et al*., 1996; Segil *et al*., 1996; Gottesfeld and Forbes, 1997). Furthermore, core histones are generally underacetylated, and the activities of histone acetyltransferases and deaceylases decline during that time (Kruhlak *et al*., 2001). The general chromatin hypoacetylation correlates with the precipitous fall in transcriptional activities. However, some lysine residues on histones H3 and H4 remain acetylated on mitotic chromosomes, which may serve as a mark for transcriptional memory (Kruhlak *et al*., 2001). Transcription restarts at the end of telophase when chromatin-binding proteins and transcription factors are reloaded onto chromatin (Prasanth *et al*., 2003). Despite dramatic changes in the chromatin status revealed during mitosis, the contribution of chromatin to the progression of mitosis has remained poorly understood. Recent investigations, however, begin to shed light on this issue. For exam-

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Abbreviations used: APC, anaphase-promoting complex; JNK, c-Jun NH_2-terminal kinase; TSA, trichostatin A.

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ple, a short-term histone hyperacetylation brought by a histone deacetylase inhibitor, trichostatin A (TSA), is shown to hinder progression from prometaphase to metaphase (Cimini *et al*., 2003). The long-term treatment with the inhibitor causes chromosomal missegregation, because of alterations in pericentric heterochromatin (Taddei *et al*., 2001).

A number of structurally diverse agents, such as colchicine, *Vinca* alkaloids, and paclitaxel target microtubules and interfere with the formation of mitotic spindles. These drugs elicit potent antimitotic effects and arrest cells at around prometaphase (Bhalla, 2003; Jordan and Wilson, 2004). Nocodazole is one such drug whose effects are reversible: whereas the drug causes extensive depolymerization of microtubules and complete mitotic arrest, its withdrawal leads to rapid reformation of mitotic spindles and resumption of mitosis. For its reversible effect on mitosis, nocodazole has been extensively used in biochemical and cell biological studies.

Many microtubule-targeting agents, extracted from plant and animal products, are thought to have evolved as a strategy for self-protection. Alternatively, animal cells are also equipped with mechanisms to respond to antimitotic effects of these agents. Cells activate checkpoints, such as mitotic stress checkpoints and spindle assembly checkpoints (Rudner and Murray, 1996; Scolnick and Halazonetis, 2000; Nigg, 2001; Cleveland *et al*., 2003; Rieder and Maiato, 2004), to halt progression of mitosis. Activation of checkpoints presumably helps cells to prepare for future recovery from drug-induced damage (Sorger *et al*., 1997; Bhalla, 2003). Furthermore, several mitogen-activated protein kinases are activated in many cells in response to antimicrotubule drugs, including extracellular signal-regulated kinases 1/2, p38, and c-Jun NH_2-terminal kinases (JNKs) (Haynes *et al*., 1992; Takenaka *et al*., 1998; Wang *et al*., 1998). Activation of these kinases seems to protect the survival of some cells, whereas promoting apoptosis of other cells (Wang *et al*., 1998; Yamamoto *et al*., 1999).

Here, we show that addition and withdrawal of nocodazole causes a dramatic redistribution of Brd4 from and to mitotic chromosomes in mouse cells. This drug-induced Brd4 dynamics was impaired in Brd4+/− cells, in which specific lysine residues in histones H3 and H4 were underacetylated relative to those in Brd4+/+ cells. Our results indicate that drug-induced Brd4 dynamics is regulated by the patterns of acetylated histone codes that are recognized and maintained by Brd4. We show that Brd4 dynamics critically contributes to the recovery from nocodazole-induced mitotic arrest, facilitating resumption of mitosis after drug removal. This work supports the view that chromatin and Brd4 dynamics play an integral part in cellular responses to drug-induced mitotic stress.

MATERIALS AND METHODS

Cells and Drug Treatment

Murine embryonic stem (ES) cells (EB3) (Niwa *et al*., 2000) and Brd4+/− clones were maintained on gelatin-coated dishes in G-MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, 1000 U/ml leukemia inhibitory factor (Chemicon International, Temecula, CA), and 5 μg/ml blasticidin S. Cells (1.5×10^5/ml) were treated with 100 ng/ml nocodazole, colcemid, or paclitaxel (all purchased from Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO) at 37°C for indicated periods of time. For control experiments, cells were treated with equivalent concentration of DMSO. In some experiments, cells were treated with TSA (50 ng/ml) in the presence or absence of nocodazole (100 ng/ml) for 6 h.

Targeted Disruption of the Brd4 Gene

BAC clones containing the murine *Brd4* locus were isolated by the Do-It-Yourself PCR screening kits (Mouse ES BAC, Release I) (Incyte, Wilmington, DE). A targeting vector was constructed by placing the *Nco*I (7.5-kb) and *Hind*III-*Xho*I (3.4-kb) fragments upstream and downstream of the SA-IRES-linked selection cassette containing the puromycin resistant gene, respectively (Figure S1A) (Mountford *et al*., 1994; Niwa *et al*., 2000). Two $\times 10^7$ cells were electroporated with 100 μg of the linearized targeting vector at 800 V and 3 μF and selected in the presence of 0.3 μg/ml puromycin. Clones with a disrupted *Brd4* gene were identified by Southern blot analysis using probes external to the recombination construct (Figure S1B).

Transfection and Live Cell Imaging

Two $\times 10^4$ cells were seeded on a Lab-Tek chambered cover-glass (Nalge Nunc International, Naperville, IL) and after overnight incubation, cells were transfected with 0.2 μg of an expression vector for green fluorescent protein (GFP)-labeled wild-type Brd4 (Maruyama *et al*., 2002) using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions and incubated for 12 h before treatment. Cells were treated with indicated drugs for 4 h in phenol red-free media. For drug withdrawal experiments, mitotic cells were harvested after 8 h of nocodazole treatment by mitotic shake-off and reincubated in a Lab-Tek chamber for indicated times. Hoechst 33342 (1 μg/ml) was added to media immediately before microscopic observations. Cells were viewed with a Leica TCS-SP2 confocal laser scanning unit equipped with UV, Ar, and Kr laser beams and attached to a Leica DMIRE2 inverted microscope using a 63× oil immersion objective. Images were acquired by using the Leica confocal software version 2.5.

Immunofluorescence Staining

Nocodazole-treated cells were cytospun and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and permeabilized with methanol for 10 min. Antibodies against α-tubulin was purchased from Sigma-Aldrich. Rabbit antibodies to Brd4, indirect immunofluorescent staining and counterstaining with Hoechst 33342 were described previously (Dey *et al*., 2000). Immunostaining of untreated cells (control) was performed with cells cultured on poly-L-lysine-coated coverslips using the same procedures as described above. Staining with the human ANA-centromere autoantibody (CREST) (Cortex Biochem, San Leandro, CA) and Alexa Flour 568 goat anti-human IgG (Molecular Probes, Eugene, OR) was performed as described previously (Zhang *et al*., 2004). Stained cells were viewed as described above.

Flow Cytometry Analysis and Clonogenic Survival Assay

Cells (1.5×10^5/ml) treated with nocodazole for 8 h at 100 ng/ml were collected by mitotic shake-off and reincubated for indicated times. For detecting apoptosis, cells were stained with Annexin V-fluorescein isothiocyanate (BD Biosciences PharMingen, San Diego, CA) according to the manufacturer's instructions. Stained cells were analyzed on FACSCalibur with the CellQuest software (BD Biosciences, San Jose, CA). For assessing DNA contents, cells were stained with propidium iodide (25 μg/ml) and analyzed as described above. For clonogenic survival assay, mitotic cells harvested after 8 h of nocodazole treatment were plated in a well of six-well plates (200 cells/well) and incubated in fresh media for 7 d. Colonies were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and stained with crystal violet. Cell masses >0.5 mm in diameter were counted as colonies.

Cdk1 Kinase Assays

Whole cell lysates were prepared from 5×10^5 nocodazole-treated/released cells by incubating in 100 μl of NP-40 buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, the protease inhibitor cocktail (Roche Diagnostics), and phsophatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, and 0.1 mM ammonium molybdate) for 20 min on ice. After removing debris by centrifugation, 30 μg of clear lysates was immunoprecipitated with 4 μl of anti-cyclin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Precipitates were incubated with 10 μCi of [γ-^{32}P]ATP (MP Biomedicals, Irvine, CA) in the presence of 20 μg of histone H1 (Upstate Biotechnology, Lake Placid, NY) in a total reaction volume of 50 μl at 30°C for 10 min. Reaction mixtures were transferred to P81 phosphocellulose paper and counted in a scintillation counter.

Histone Purification and Immunoblot Analysis

Histones were isolated according to the method described previously (Braunstein *et al*., 1993) with modifications. One million cells were suspended in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$. 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 M sulfuric acid), incubated on ice for 30 min, and centrifuged. The supernatants were dialyzed against 0.1 M acetic acid twice and water three times. Immunoblot analysis was performed with 15 μg of extracts and antibodies for unacetylated histones H3 and H4 and those acetylated at indicated lysine residues (all from Upstate Biotechnology, except for H4AcK5, which was from Serotec, Oxford, United Kingdom). Reactions were visual-

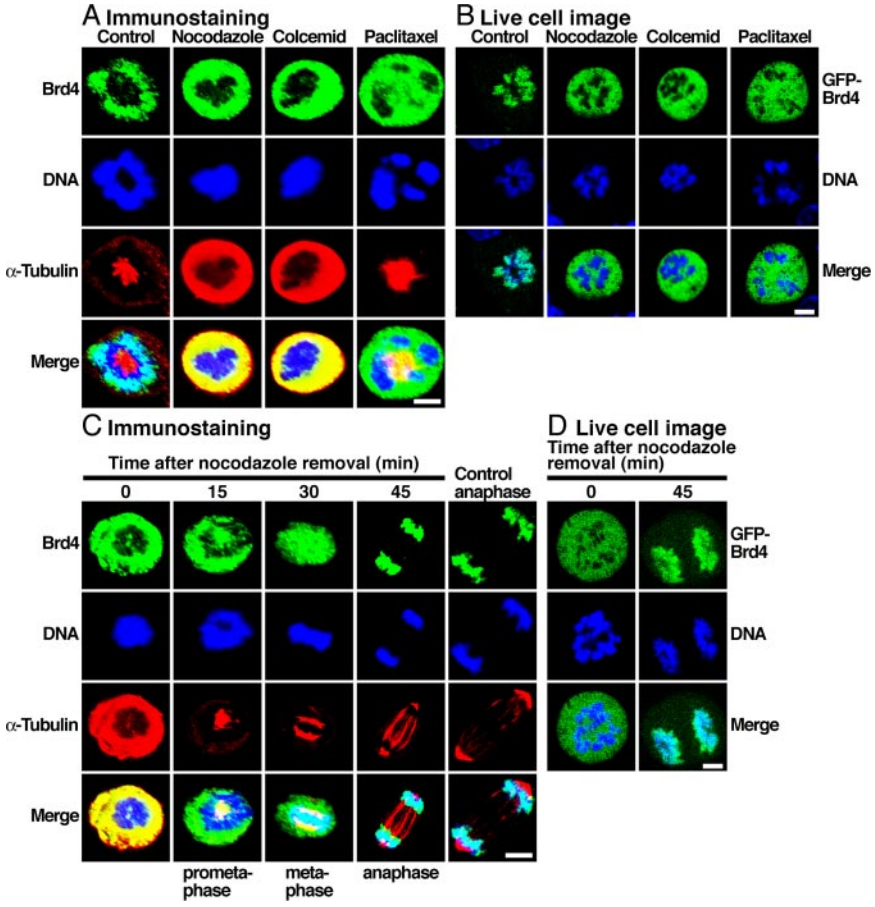

Figure 1. Redistribution of Brd4 after addition and removal of antimicrotubule drugs. (A) Murine ES cells (1.5×10^5/ml) were treated with indicated drugs (100 ng/ml) for 4 h. Cell were cytospun, fixed, and immunostained to detect Brd4 (green) and α-tubulin (red), and counterstained by Hoechst 33342 for DNA (blue). (B) Cells were transfected with GFP-Brd4 vector for 12 h and treated with indicated agents as described in A. GFP signals (green) were viewed along with Hoechst 33342 signal (blue) in live cells. (C) Cells that had been treated with nocodazole (100 ng/ml) for 8 h were washed and incubated in fresh media for indicated times and immunostained as described above. (D) GFP-Brd4-expressing ES cells were treated as described in C and viewed as described in B. Bar, 5 μm.

ized by enhanced chemiluminescence. To quantify immunoblot data, the band intensity was determined by the NIH Image (version 1.63) software.

RESULTS

Nocodazole Reversibly Releases Brd4 from Mitotic Chromosomes

During mitosis, Brd4 localizes to noncentromeric axes of chromosomes by binding mostly to acetylated histones H3 and H4 (Dey *et al*., 2000, 2003). We found that antimicrotubule drugs trigger dissociation of Brd4 from chromosomes in various cultured cells. Shown in Figure 1A is immunostaining of endogenous Brd4 in murine EB3 ES cells treated with nocodazole, colcemid, and paclitaxel. The former two drugs inhibit tubulin polymerization, whereas the latter promotes its polymerization (Jordan and Wilson, 2004). When treated with these drugs for 4 h at 100 ng/ml, Brd4 staining was detected entirely outside of condensing chromosomes stained for DNA: at this dose of the drugs, all mitotic cells in the fields showed complete dissociation of Brd4 from chromosomes. In contrast, Brd4 staining was restricted to chromosomes in mitotic cells treated with vehicle (DMSO) alone, as expected (see control in Figure 1A where Brd4 staining overlaps with DNA staining on a mitotic plate). Immunostaining of α-tubulin in Figure 1A verified that these drugs inhibited formation of proper mitotic spindles. To exclude the possibility that the observed dissociation of Brd4 from chromosomes is because of an artifact of fixation or other procedures used in this work, we visualized the movement of an exogenous Brd4 in live cells by transfecting a Brd4 vector fused to the GFP in ES cells. We have previously shown that GFP-Brd4 interacts with acetylated chromatin and behaves in a manner indistinguishable from that of the endogenous Brd4 (Dey *et al*., 2003). As shown in Figure 1B, GFP-Brd4 was again completely dispersed from mitotic chromosomes after drug treatment. These results indicate that Brd4 is unloaded from chromosomes in response to antimicrotubule agents.

Nocodazole is a reversible agent, and upon its removal, spindles reform and cells resume mitosis (Rieder and Maiato, 2004). We found that Brd4 reassociated with mitotic chromosomes after nocodazole was removed from the culture. In Figure 1C, cells that had been treated with nocodazole for 8 h were washed, mitotic cells were then incubated in fresh media for varying times up to 45 min, and the localization of Brd4 was examined by immunostaining. During the initial ~15 min after nocodazole withdrawal, Brd4 remained dissociated from chromosomes in most cells. Soon afterward, however, Brd4 began to relocalize to chromosomes. At ~30 min after drug withdrawal, when cells approached metaphase, Brd4 was seen reloaded onto chromosomes in the majority of cells. In these cells, reformation of mitotic spindles was evident (Figure 1C, see immunostaining of α-tubulin). Relocalization of Brd4 onto chromosomes was further increased in anaphase/telophase cells at ~45 min, when >75% of cells showed Brd4 on segregating chromosomes. In untreated control cells, Brd4 remained on mitotic chromosomes during the entire period of mitosis, including anaphase/telophase as noted previously (Figure 1C; Dey *et al*., 2000). Reassociation of GFP-Brd4 with mitotic chromosomes was also observed in live cells after nocoda-

zole removal (Figure 1D). Similar dissociation from and reassociation with mitotic chromosomes was observed for Brd4 in P19 and other cell types upon nocodazole addition and withdrawal (Nishiyama, Dey, Ito, and Ozato, unpublished data). Thus, Brd4 is unloaded from chromosomes after nocodazole treatment when mitosis is arrested and reloaded onto chromosomes upon drug withdrawal when mitosis restarts.

Brd4 Reloading Is Compromised in Brd4 Heterozygous (+/−) Cells

The dramatic redistribution of Brd4 after nocodazole addition and withdrawal suggested that Brd4 has a role in a cellular response to antimicrotubule drugs. We sought a genetic approach to address the significance of Brd4 dynamics in a mitotic stress caused by antimicrotubule drugs. With the aim of studying Brd4 null cells, we first constructed ES cells in which a *Brd4* allele was disrupted. Our targeting strategy (Figure S1) yielded many Brd4+/− clones. However, our attempts to disrupt the other *Brd4* allele were unsuccessful, suggesting that Brd4 is essential for cell growth. In accord with our results, Brd4−/− mice constructed by a gene trap strategy, are reported to be early embryonic lethal (Houzelstein *et al.*, 2002). To investigate a possible haploinsufficiency for *Brd4*, three Brd4+/− clones (C12, C16, and C27) were established. Brd4 protein levels in these clones and other Brd4+/− cells were consistently about one-half that of Brd4+/+ cells (Figure 2A). Brd4+/− cells from all three clones grew at a rate comparable with that of Brd4+/+ cells with no apparent growth disadvantage under normal culture conditions (Figure S2A). In addition, Brd4+/− clones and Brd4+/+ cells were equally capable of arresting mitosis after nocodazole addition, because they showed essentially the same mitotic indices observed at 2, 4, 6, and 8 h after drug addition (Figure S2B). This indicates that Brd4+/− cells did not prematurely enter mitosis upon nocodazole addition and mitotic checkpoints functioned in both cells (Scolnick and Halazonetis, 2000; Mikhailov and Rieder, 2002; Peters, 2002; Cleveland *et al.*, 2003). We noted, however, that mitotic indices were slightly reduced when tested at 12 h for both +/+ and +/− cells, indicating that a certain fraction of cells escape from mitotic arrest after extended nocodazole treatment (our unpublished data). The localization of Brd4 in +/− cells was identical to that in Brd4+/+ cells in the absence of nocodazole: it was restricted to mitotic chromosomes. Likewise, Brd4 was released from chromosomes both in +/− and +/+ cells after drug addition, indicating that Brd4 unloading occurred normally in +/− cells after nocodazole treatment (our unpublished data). However, Brd4+/− cells were clearly defective in Brd4 reloading after nocodazole withdrawal. Brd4 remained in the extrachromosomal space at 30 min after nocodazole removal when the majority of +/+ cells showed Brd4 reloading. Even at anaphase/telophase when chromosomes were segregating toward opposite poles, Brd4 resided in the space outside of chromosomes in many +/− cells (see an example in Figure 2B). In Figure 2C, we quantified the number of anaphase/telophase cells in which Brd4 was successfully reloaded onto chromosomes after nocodazole withdrawal. More than 70% of Brd4+/+ cells showed complete Brd4 reloading at 40 min. In contrast, only 20–30% of +/− cells had Brd4 on segregating chromosomes in all three Brd4+/− clones. There was a small proportion of Brd4+/+ cells in which Brd4 reloading seemed incomplete. This may be because of incomplete elimination of the drug from the cells and/or altered Brd4 expression levels. To exclude the possibility that the defect seen in +/− clones was because of transfection of targeting vector, we examined several ES clones obtained after transfection of a control vector. Brd4 in these clones was reloaded onto chromosomes after nocodazole removal in a manner identical to that of parental cells (our unpublished data). Once cells completed mitosis and entered G_1 phase, Brd4 associated with chromatin in Brd4+/− cells in a manner very similar to that in Brd4+/+ cells. This was evident by the identical salt solubility of Brd4 in +/+ and +/− cells in G_1 cells generated after nocodazole withdrawal (Figure S3). These results indicate that Brd4+/− cells are haploinsufficient for Brd4 reloading, suggesting the importance of Brd4 expression levels.

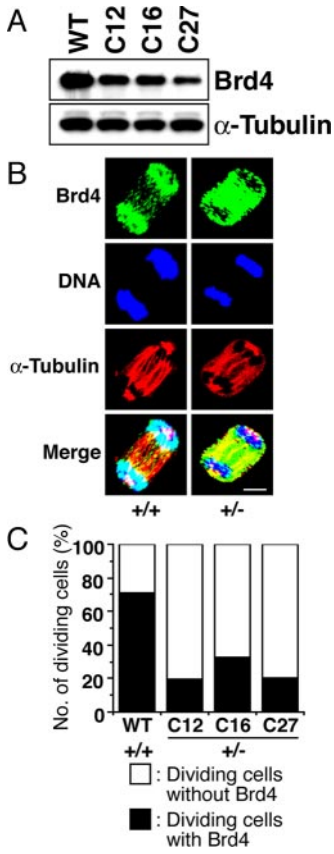

Figure 2. Defective Brd4 reloading in Brd4+/− cells. (A) Reduced Brd4 levels in Brd4+/− clones. Immunoblot analysis was performed with the whole cell lysates (30 μg) from parental ES cells (WT) and three representative Brd4+/− clones (C12, C16, and C27) for Brd4 and α-tubulin as a loading control. (B) Brd4+/+ and +/− cells were treated with nocodazole for 8 h; mitotic cells were incubated in fresh media for 40 min and immunostained for Brd4 localization. Left, an example of a Brd4+/+ cell in which Brd4 was fully reloaded on the segregating chromosomes. α-Tubulin was stained to visualize mitotic spindles. Right, an example of a Brd4+/− cell in which Brd4 remained outside of the segregating chromosomes. Bar, 5 μm. (C) Brd4 reloading was monitored by immunostaining of parental Brd4+/+ and three +/− clones that had been treated with nocodazole and released in fresh media for 40 min as described above. The black columns represent the percentage of cells in which Brd4 was reloaded on chromosomes, and the white columns represent those in which Brd4 was not reloaded on chromosomes. A representative of three independent tests is shown. More than 200 cells with segregating chromosomes (anaphase/telophase cells) were examined in each experiment.

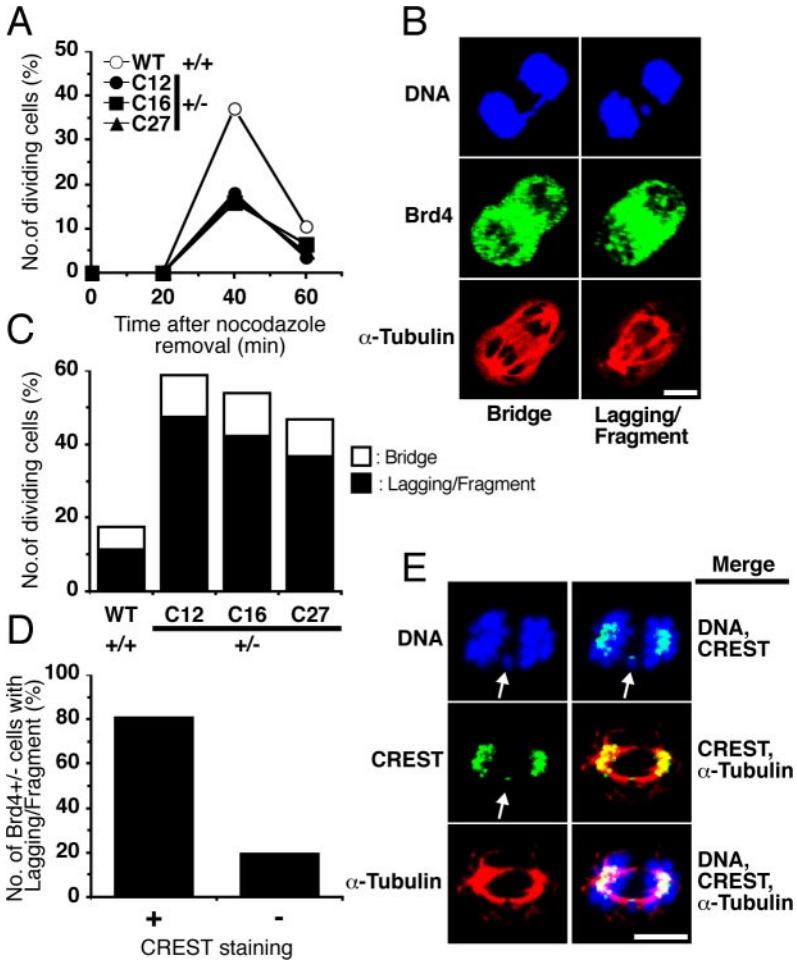

Figure 3. Brd4+/− cells are haploinsufficient in mitotic progression after nocodazole removal. (A) Impaired anaphase progression in Brd4+/− cells after nocodazole withdrawal. Brd4+/+ and +/− cells were treated with nocodazole (100 ng/ml) for 8 h. Mitotic cells were then allowed to proceed in fresh media for indicated times (minutes) and stained for Brd4 and DNA. Cells carrying segregating chromosomes were scored as those that progressed to anaphase/telophase. A representative of three independent tests is shown. At least 250 cells were examined in each experiment. (B) Abnormal chromosomal segregation in Brd4+/− cells. Segregating chromosomes were detected by visualizing stained DNA. An example of chromosomal bridge (Bridge) and misdistributed chromosome (Lagging/Fragment) is shown. Note that Brd4 remained unloaded in both cases. Bar, 5 μm. (C) The percentage of dividing cells carrying abnormal chromosomal segregation is shown. A representative of three independent tests is shown. More than 200 anaphase/telophase cells were inspected in each experiment. (D) The percentage of dividing Brd4 +/− cells showing CREST signals on misdistributed chromosomes is shown. More than 200 anaphase/telophase cells were inspected. (E) Brd4+/− cells incubated for 40 min after release from nocodazole treatment (100 ng/ml; 8 h) were fixed and immunostained with antibody for CREST (green) and α-tubulin (red) and counterstained for DNA (blue). CREST staining of a lagging chromosome is marked by arrow. Bar, 5 μm.

Brd4+/− Cells Are Haploinsufficient for the Ability to Recover from Nocodazole-induced Mitotic Arrest

If nocodazole-triggered Brd4 dynamics is a part of cellular responses to mitotic stress, then the capacity to reload Brd4 onto chromosomes may signify the cell's ability to recover from drug-induced mitotic arrest. To test this possibility, we next examined progression of +/+ and +/− cells to anaphase after nocodazole withdrawal. Cells that had been treated with nocodazole for 8 h were washed, mitotic cells were allowed to proceed in fresh media for varying times up to 60 min. Cells in anaphase/telophase carrying segregating chromosomes were detected by DNA staining. As shown in Figure 3A, there were few anaphase/telophase cells at 20 min, but their number was sharply increased at 40 min in +/+ cells, where ~40% of cells were in anaphase/telophase, in agreement with the timing of mitosis seen in Figure 1C. By 60 min most of +/+ cells completed mitosis and there were few remaining cells with segregating chromosomes. In contrast, +/− cells from all three clones showed much fewer anaphase/telophase cells at the peak time of 40 min: only ~15% of +/− cells carried segregating chromosomes at this stage. There were few anaphase/telophase cells before and after the peak time. These results indicate that a large fraction of Brd4+/− cells failed to resume mitosis and did not reach anaphase. That the percentage of anaphase/telophase cells in +/− clones was as low as +/+ cells at 20 min before the peak time indicates that +/− cells did not prematurely enter anaphase. Similarly, the results that the percentage of anaphase/telophase cells fell at 60 min in +/− clones in the same manner as in +/+ cells indicate that the reduction in anaphase/telophase cells in +/− clones was not because of a delayed progression to anaphase.

Increased Chromosomal Missegregation in Brd4+/− Cells

Although markedly reduced, a fraction of Brd4+/− cells reached anaphase after nocodazole removal. In many of these cells, segregating chromosomes did not carry Brd4, because it remained unloaded. It was of interest to assess whether progression to anaphase/telophase occurred normally in these Brd4+/− cells. To this end, we examined sister chromatid separation and chromosomal segregation in +/+ and +/− cells after nocodazole withdrawal. Cells that had been treated with nocodazole were allowed to proceed for 40 min in fresh media to reach anaphase/telophase, and the segregating chromosomes were morphologically inspected after staining of DNA and Brd4. We observed a significant increase in chromosomal missegregation in +/− cells, mostly the form of misdistributed chromosomes with a morphology resembling lagging chromosomes or chromosome fragments. In theses cells, some chromosomes/fragments seemed left behind near the mitotic plate, whereas other chromosomes moved toward spindle poles (Figure 3B). Chromosomal bridges were another type of abnormality observed after nocodazole treatment, in which sister chromatid arms remained incompletely separated (Figure

3B). Figure 3C compares the frequency of cells with abnormal chromosomal segregation in Brd4+/+ and +/− cells. In line with the reports that nocodazole treatment increases the frequency of chromosomal missegregation in human fibroblasts and PtK1 cells (Cimini *et al*., 1999, 2001), chromosomal missegragation was seen in ~17% of Brd4+/+ cells. However, the frequency of chromosomal missegregation was much higher in Brd4+/− cells: >50% of Brd4+/− cells carried abnormal chromosomes in all three clones. Kinetochore staining with CREST antibody revealed that most of misdistributed chromosomes in Brd4+/− cells were lagging chromosomes rather than chromosomal fragments (Figure 3E). Eighty percent of misdistributed chromosomes showed the kinetochore staining (Figure 3D). These results indicate that Brd4+/− cells are more prone to have chromosomal missegregation than Brd4+/+ cells after nocodazole treatment.

Nocodazole Treatment Increases Apoptosis as Well as Tetraploidy in Brd4+/− Cells, Leading to Reduced Proliferative Capacity

Given that Brd4+/− cells are defective in entering anaphase, it was of interest to assess whether some +/− cells succumb to programmed cells death after nocodazole withdrawal. Figure 4A examines the percentage of apoptotic cells after nocodazole removal by Annexin V staining. The percentage of Annexin V-positive apoptotic cells was ~30% in Brd4+/− cells, whereas that in Brd4+/+ cells was ~13%. Annexin V-positive cells were <10% at the time of release for both Brd4+/+ and +/− cells. These data indicate that Brd4+/− cells are susceptible to apoptotic cell death to a greater extent than Brd4+/+ cells after nocodazole treatment. It is possible that in addition to increased apoptosis, some Brd4+/− cells escaped from mitosis without completing cell division. If so, Brd4+/− daughter cells generated after nocodazole withdrawal may have tetraploid (or near teraploid) genomes. In Figure 4B, flow cytometry was carried out to assess DNA content in +/+ and +/− cells harvested after nocodazole release. The frequency of cells carrying 4N DNA was substantially higher in Brd4+/− cells than +/+ cells (55 vs. 35%). The above-mentioned data show that Brd4+/− cells are more susceptible to nocodazole-inflicted damage during mitosis than +/+ cells and raise the possibility that nocodazole treatment/withdrawal has a lasting effect on Brd4+/− cell growth. We thus performed clonogenic assays where cells were first treated with varying concentrations of nocodazole for 8 h, and mitotic cells were allowed to grow as colonies for subsequent 7 d in the absence of the drug. Of 200 cells seeded, ~95–175 colonies arose from untreated cells from both Brd4+/+ and +/− cells with some variability in experiments, indicating that the colony-forming ability was comparable in untreated Brd4+/+ and +/− cells. However, the colony-forming ability was markedly reduced in Brd4+/− cells that had been treated with nocodazole. Quantification of the number of colonies that arose after nocodazole treatment is presented in Figure 4C. In Brd4+/+ cells, nocodazole treatment reduced the number of colonies at most by 20%. In contrast, in all three Brd4+/− clones, nocodazole treatment at all does tested led to a more severe reduction in colony numbers. At the highest dose of the drug (500 ng/ml), the number of colonies fell to <50% of that of +/+ cells. These data indicate that the failure to reload Brd4 onto chromosomes after nocodazole removal has multiple functional consequences and results in growth inhibition in Brd4+/− cells.

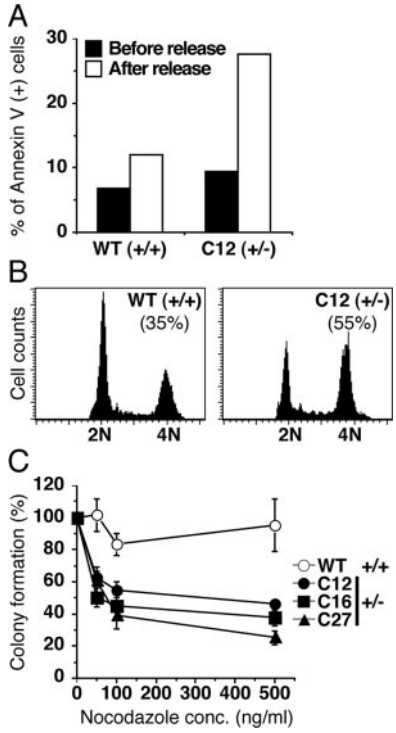

Figure 4. Impaired recovery from nocodazole-induced mitotic arrest in Brd4+/− cells. (A) The percentage of apoptotic cells after nocodazole removal. Cells released from nocodazole treatment (100 ng/ml for 8 h) were stained for Annexin V and analyzed by flow cytometry. The values represent the average of three experiments. (B) DNA contents after nocodazole withdrawal. Cells released from nocodazole as mentioned above were stained with propidium iodide and analyzed by flow cytometry. (C) Clonogenic survival after nocodazole treatment. Brd4+/+ and +/− cells were treated with indicated concentrations of nocodazole for 8 h. Two hundred mitotic cells were replated in a well and allowed to grow in fresh media, and colonies were counted 7 d later. Untreated Brd4+/+ and +/− cells yielded comparable numbers of colonies. Values represent the average of three independent assays.

Cyclin B1/Cdk1 Is Similarly Inactivated in Brd4+/+ and +/− Cells

Anaphase entry is triggered by the activation of the anaphase-promoting complex (APC), marked first by the inactivation of cyclin B1/Cdk1 (Clute and Pines, 1999; Nigg, 2001; Peters, 2002). Impaired anaphase entry observed with Brd4+/− cells after nocodazole removal suggested that these cells may be defective in activating the APC. To test this possibility, we examined Cdk1 kinase activity in Brd4+/+ and +/− cells. Cells were treated with nocodazole and released into fresh media for varying times. As presented in Figure 5A, Cdk1 kinase activity was the highest at the time of nocodazole removal (time 0). The kinase activity declined sharply thereafter and fell to background levels by 60 min both in Brd4+/+ and +/− cells. In accordance, cyclin B1 protein levels detected by immunoblot were the highest at time 0 and precipitously declined afterward both in Brd4+/+ and +/− cells at similar extents (Figure 5B). We also tested phosphorylation of Cdc27 and Cdk1 and found that it was also comparable in Brd4+/+ and +/− cells (our unpublished data). These results indicate that the APC was activated in Brd4+/− cells and that the processes that accompanied APC activation took place in Brd4+/− cells. Thus, the failure of Brd4+/− cells to enter anaphase

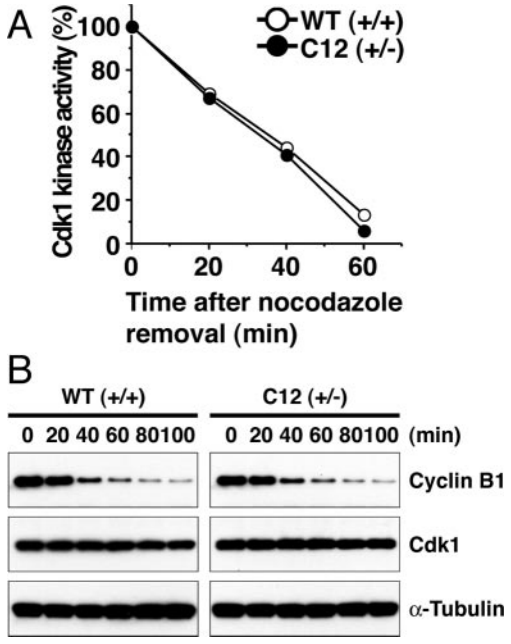

Figure 5. Comparable Cdk1 kinase activity and cyclin B degradation in Brd4+/+ and +/− cells. (A) Cdk1 activity was tested with histone H1 as a substrate using precipitates of lysates (30 μg) from Brd4+/+ and +/− cells that had been treated with nocodazole at 100 ng/ml for 8 h and incubated in fresh media for indicated times (minutes). Values represent the average of three samples. (B) Lysates in A were subjected to immunoblot analysis to detect cyclin B1 and Cdk1 expression. α-Tubulin was immunoblotted as a control.

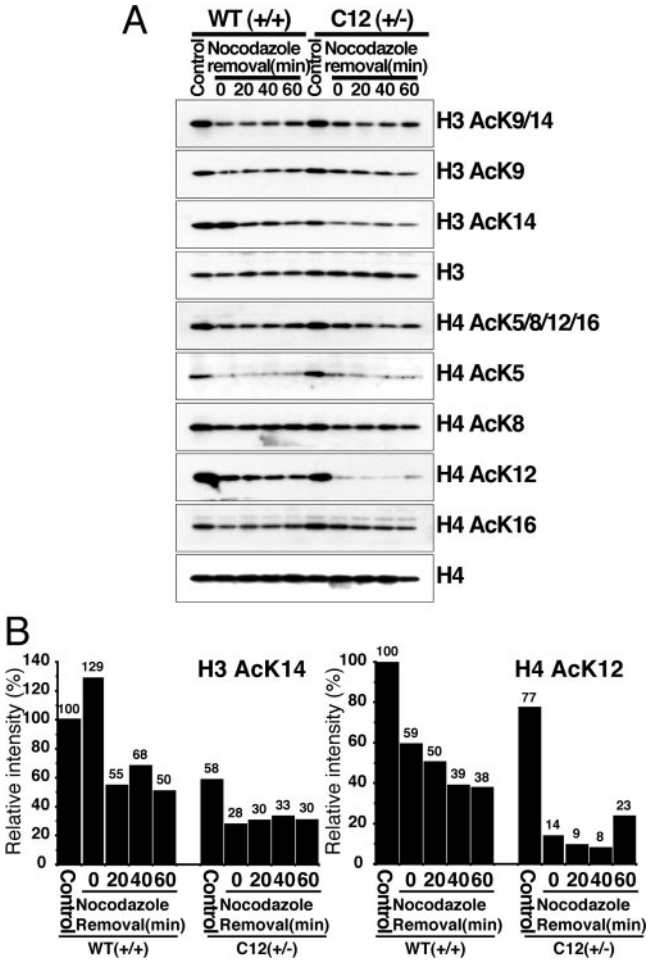

Figure 6. Histone hypoacetylation in Brd4+/− cells. (A) Acetylation of histones H3 and H4 was tested by immunoblot analysis with indicated antibodies using histones extracted from untreated (control) Brd4+/+ and +/− cells and treated with nocodazole followed by incubation in fresh media for indicated times (minutes). (B) Immunoblot data for acetylated K14 on H3 and acetylated K12 on H4 shown in A were quantified by densitometry analysis. The band intensity of histones acetylated at K14 and K12 was normalized with the intensity of unacetylated H3 and H4 on each time point and expressed as the percentage of intensity in which the value for untreated Brd4+/+ cells (control) was taken as 100%.

was apparently not because of the global lack of APC activation. However, it is still possible that Brd4+/− cells are impaired in some pathways downstream of APC activation, leading to the anaphase block observed after nocodazole withdrawal.

Underacetylation of the Brd4-binding Histone Residues in Brd4+/− Cells

The interaction of Brd4 with mitotic chromosomes depends largely on acetylated core histones, as is the case for binding of Brd4 to chromatin during interphase (Dey *et al*., 2003). We next sought to assess the status of chromatin acetylation in Brd4 +/− cells. If altered in +/− cells, we wished to address whether the alteration could account for the defects in Brd4+/− cells observed by nocodazole treatment. This question was of importance because it has not been clear whether Brd4 has a role in preserving chromatin acetylation, whereas it is clear that Brd4 binds to acetylated chromatin (Dey *et al*., 2003). Histones prepared from randomly growing cells (control), cells arrested at mitosis by nocodazole treatment, and cells released from nocodazole for varying times up to 60 min were tested for acetylation of H3 and H4 by immunoblot analysis. The use of specific antibodies allowed us to assess acetylation of individual lysine residues on the histones (Figure 6A). Antibodies for unacetylated H3 and H4 served as loading controls. As expected of histone hypoacetylation during mitosis, levels of acetylated H3 and H4 were lower in mitotically arrested and released cells than in randomly growing cells (0–60 min in Figure 6A) in both Brd4+/+ and +/− cells. All lysine residues tested here, K9 and K14 of H3 and K5, 8, 12, and K16 of H4 showed lower acetylation in mitotic (nocodazole treated/released) cells. Significantly, the levels of acetylated K14 on H3 and acetylated K12 on H4 were consistently lower in Brd4+/− cells than in +/+ cells in all stages of the culture examined (Figure 6A). This was confirmed by densitometry analysis of immunoblots in Figure 6B. The band intensity of these acetylated residues in Brd4+/− cells was ~50% of that +/+ cells. However, the level of acetylation in other residues was not altered in Brd4+/− cells. These results are interesting, because K14 of H3 and K12 of H4 are thought to be binding sites of Brd4 (Dey *et al*., 2003). We also noted that underacetylation of K12 of H4 in Brd4+/− cells relative to +/+ cells seemed more pronounced in nocodazole treated/released cells than in randomly growing cells. In contrast, K14 of H3 seemed underacetylated in +/− cells in all stages of cultures relative to +/+ cells. The reduction of acetyl H3 K14 and acetyl H4 K12 was observed with other +/− clones (our unpublished data). These results indicate that Brd4 has a role in the maintenance of acetylated K14 in H3 and K12 in

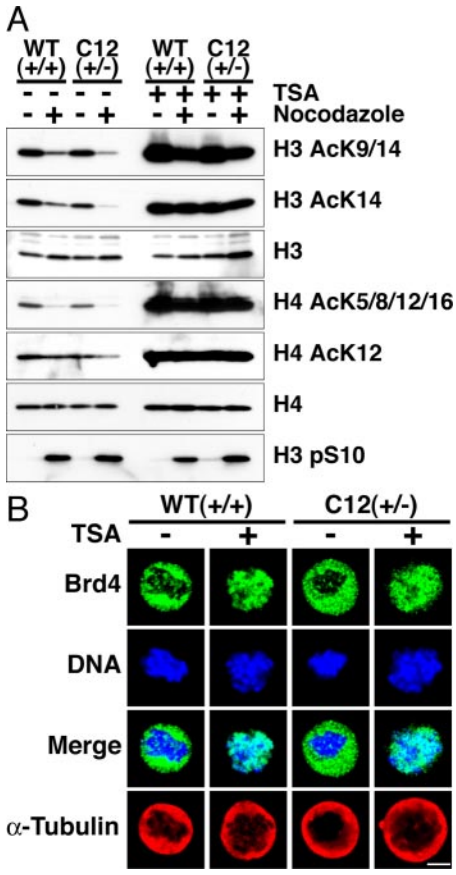

Figure 7. TSA inhibits nocodazole-induced Brd4 unloading. (A) Hyperacetylation of histones by TSA. Brd4+/+ and +/− cells were treated with nocodazole (100 ng/ml) or TSA (50 ng/ml) alone or both for 6 h, and histones H3 and H4 were analyzed for acetylation or phosphorylation of indicated residues. (B) Brd4+/+ and +/− cells were treated with nocodazole and TSA for 6 h as in A, and localization of endogenous Brd4 was visualized by immunostaining. Bar, 5 μm.

H4 in these cells. Because Brd4 itself does not catalyze histone acetylation/deacetylation, the maintenance of acetylated chromatin by Brd4 is likely to involve a nonenzymatic mechanism.

Enhanced Histone Acetylation Inhibits Nocodazole-induced Brd4 Unloading

The above-mentioned data suggested the possibility that histone acetylation is a key mechanism to regulate nocodazole induced redistribution of Brd4. To assess how an alteration of histone acetylation affects Brd4 dynamics, we next tested the effect of a histone deacetylase inhibitor, TSA (Yoshida *et al*., 1995), on nocodazole-induced Brd4 unloading. Brd4+/+ and +/− cells were treated with or without nocodazole for 6 h in the presence or absence of TSA (50 ng/ml). Figure 7A shows changes in the levels of H3 and H4 acetylation detected by immunoblot analysis. In the absence of TSA, both H3 and H4 were hypoacetylated in nocodazole-treated, mitotically arrested cells relative to untreated cells, as observed in Figure 6. K14 on H3 and K12 of H4 in Brd4+/− cells were less acetylated relative to those in Brd4+/+ cells, again noted in Figure 6. Importantly, TSA treatment greatly increased acetylation of H3 and H4 in both cells irrespective of nocodazole treatment: the levels of acetylation were comparable in Brd4+/+ and +/− cells, including those for K14 on H3 and K12 on H4. Furthermore, the increase seemed broad as seen in increases in diacetylated H3 and tetra-acetylated H4. In nocodazole-treated samples, Ser10 in H3 was phosphorylated at equivalent levels in Brd4+/+ and +/− cells in the presence or absence of TSA treatment, indicating that TSA did not inhibit nocodazole-induced mitotic arrest.

We next tested whether TSA treatment altered nocodazole-induced Brd4 unloading. Brd4+/+ and +/− cells were treated with TSA plus nocodazole under the same conditions as described above. Results of immunostaining are shown in Figure 7B. Brd4 was not released from chromosomes when cells were treated with TSA and nocodazole both in Brd4+/+ and +/− cells. In virtually all cells examined, Brd4 remained localized on mitotic chromosomes. When cells were treated with nocodazole alone, Brd4 was released from chromosomes tested in parallel as controls (Figure 7B). TSA treatment did not affect nocodazole-induced mitotic arrest as judged by DNA staining and cellular morphology. Similarly, TSA did not affect nocodazole-induced microtubule depolymerization, because α-tubulin was uniformly distributed in the presence and absence of TSA. These results indicate that histone acetylation has a major effect on nocodazole-induced Brd4 redistribution, and abnormal augmentation of histone acetylation inhibits nocodazole-induced Brd4 unloading. It is worth noting here that although TSA increased chromatin acetylation in a global manner, it did not restore the ability to enter anaphase, rather TSA treatment reduced the number of cells entering anaphase both in Brd4+/+ cells and +/− cells (our unpublished data). This is consistent with the previous reports showing that TSA treatment inhibits mitotic progression (Cimini *et al*., 2003; Mikhailov *et al*., 2004). These results indicate that Brd4 unloading is an event critical for recovery from nocodazole-induced mitotic arrest.

DISCUSSION

Here, we describe a striking dynamics that Brd4 displays in response to an antimitotic drug, nocodazole. Brd4 was released from prometaphase chromosomes after nocodazole addition. Brd4 was reloaded onto metaphase chromosomes within 30 min after nocodazole withdrawal and continued to associate with chromosomes for the remaining course of mitosis. Brd4+/− cells exhibited clear haploinsufficiency in reloading and manifested multiple deficiencies in the subsequent mitotic progression, illustrating that the ability to reload Brd4 onto chromosomes correlates with the capacity to recover from nocodazole-induced mitotic arrest and to complete cell division. We show that the observed Brd4 haploinsufficiency is linked to reduced acetylation of core histones in Brd4+/− cells. Together, our results indicate that acetylated histone codes on mitotic chromosomes influences an antimitotic stress response caused by nocodazole in cultured cells.

Unloading and Reloading of Brd4

Brd4 was released from chromosomes after addition of nocodazole and other microtubule-disrupting agents. Brd4 was released by these drugs not only in ES cells tested here but also in many other cell types, indicating that unloading of Brd4 is a widespread response of mammalian cells to antimitotic agents. Our evidence obtained from P19 cells indicates that Brd4 unloading is in part mediated by its C-terminal domain and is dependent on activation of a kinase

in the JNK pathway triggered by these drugs (Nishiyama, Dey, Ito, and Ozato, unpublished data).

Although the release of Brd4 from chromosomes upon addition of antimicrotubule drugs is a prominent event, it is not clear whether other chromosomal proteins are also released from chromosomes in response to these agents. Nevertheless, it is important to note here that antimicrotubule drugs have been widely used to identify proteins associated with mitotic chromosomes (Gasser and Laemmli, 1987; Saitoh *et al*., 1994; Uchiyama *et al*., 2005). These studies identified a number of nonhistone proteins, such as topoisomerase I and II, as constitutive chromosomal proteins. However, these studies would have excluded Brd4 and other proteins that might have been released from chromosomes after drug treatment from the list of "chromosomal proteins," because they used antimicrotubule drugs to prepare chromosomes. Our results may cast a cautionary note on the total reliance on these drugs for studying proteins associated with mitotic chromosomes.

Unloading of Brd4 was a temporary response that could be reversed after nocodazole removal: shortly after drug withdrawal, the bulk of Brd4 moved back to chromosomes. However, Brd4+/− cells were deficient in this process, because chromosomes remained without Brd4 in these cells. The failure to reload Brd4 onto chromosomes had a functional consequence, as observed by impaired entry into anaphase/telophase and an increased frequency of chromosomal missegregation. Some Brd4+/− cells apparently exited mitosis without cell division as evidenced by increased 4N cells, which may be consistent with comparable decrease in Cdk1 kinase activity in +/+ and +/− cells. Furthermore, Brd4+/− cells were impaired in the colony forming ability after nocodazole treatment, indicating that postnocodazole proliferation is more compromised in Brd4+/− cells than in +/+ cells. These data indicate that Brd4 reloading and associated events are an important part of cellular responses to drug-induced mitotic arrest. Paralleling these results, we found that defective Brd4 unloading is also detrimental to the recovery from drug-induced mitotic arrest (Nishiyama, Dey, Ito, and Ozato, unpublished data). Together, our work supports a view that Brd4 dynamics represents a cellular mechanism by which to minimize deleterious effects brought by theses drugs.

Mitotic deficiencies noted in nocodazole-treated Brd4+/− cells is not likely to be directly attributable to a defect in the classical mitotic stress checkpoint or spindle assembly checkpoints (Rudner and Murray, 1996; Scolnick and Halazonetis, 2000; Cleveland *et al*., 2003; Rieder and Maiato, 2004), because 1) nocodazole arrested Brd4+/+ and +/− cells similarly at prometaphase, both in terms of kinetics of arrest and dose of the drug; and 2) the timing of anaphase entry after nocodazole removal was the same in +/− and +/+ cells. In line with this, the APC was activated after nocodazole removal in Brd4+/+ and +/− cells in a similar manner, as supported by the comparable fall of Cdk1 kinase activity in +/+ and +/− cells after drug removal. However, it is possible that a pathway downstream from the APC activation may be compromised in +/− cells, contributing to the observed defects.

Brd4 Dynamics, the Histone Codes, and Mitotic Stress Responses

Detailed analysis of acetylated lysine residues in the core histones showed that Brd4+/− cells were hypoacetylated specifically at K14 of H3 and K12 of H4 in untreated as well as nocodazole-arrested and released cells. This indicates that Brd4 expression levels have a direct consequence on the pattern of histone acetylation. We previously observed that Brd4 binds to the acetylated K14 of H3 and acetylated K5/K12 of H4 but not other K residues (Dey *et al*., 2003). The present results indicate that Brd4 has an important role in preserving the acetylation of its binding site. These data provide the first evidence that Brd4 contributes to the maintenance of acetylated histone codes. Thus, inefficient Brd4 reloading observed with Brd4+/− cells may be accounted for by the insufficient acetylation of Brd4 target residues.

TSA markedly increased histone acetylation in Brd4+/+ and +/− cells, both during interphase and mitosis, and abolished histone hypoacetylation known for the mitotic stage. TSA also inhibited nocodazole-induced Brd4 unloading. This and the fact that Brd4 reloading was compromised in +/− cells where histones were hypoacetylated at specific residues indicate that Brd4 dynamics is dependent on proper histone acetylation patterns. This suggests that the recovery from mitotic arrest also depends on proper histone acetylation patterns. Supporting the critical role of appropriately acetylated histones in mitosis, a short-term TSA treatment is shown to delay the prometaphase/metaphase progression even in the absence of antimitotic drugs, causing inhibition of mitosis (Cimini *et al*., 2003). In addition, a histone deacetylase inhibitor is shown to prolong prophase, causing chromosomal decondensation (Mikhailov *et al*., 2004). Furthermore, TSA is shown to have a long-term negative effects on mitosis, causing defects in chromosomal segregation due to an alteration of pericentric heterochromatin (Taddei *et al*., 2001).

Nocodazole treatment triggers a dramatic movement of Brd4 from and to mitotic chromosomes. The redistribution of Brd4 was dependent on properly maintained histone acetylation, which was deficient in Brd4+/− cells. Our results indicate that the Brd4 dynamics is a mechanism ensuring the recovery from drug-induced mitotic arrest. The Brd4 dynamics described in this work may serve as a new paradigm to study the contribution of chromatin to mitotic stress responses.

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