Nucleophosmin/B23 Negatively Regulates GCN5-dependent Histone Acetylation and Transactivation*§

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Nucleophosmin/B23 is a multifunctional phosphoprotein that is overexpressed in cancer cells and has been shown to be involved in both positive and negative regulation of transcription. In this study, we first identified GCN5 acetyltransferase as a B23-interacting protein by mass spectrometry, which was then confirmed by in vivo co-immunoprecipitation. An in vitro assay demonstrated that B23 bound the PCAF-N domain of GCN5 and inhibited GCN5-mediated acetylation of both free and mononucleosomal histones, probably through interfering with GCN5 and masking histones from being acetylated. Mitotic B23 exhibited higher inhibitory activity on GCN5-mediated histone acetylation than interphase B23. Immunodepletion experiments of mitotic extracts revealed that phosphorylation of B23 at Thr199 enhanced the inhibition of GCN5-mediated histone acetylation. Moreover, luciferase reporter and microarray analyses suggested that B23 attenuated GCN5-mediated transcription in vivo. Taken together, our studies suggest a molecular mechanism of B23 in the mitotic inhibition of GCN5-mediated histone acetylation and transactivation.

The acetylation of nucleosomal histones by histone acetyltransferases (HATs)§ has been known for several decades. Histone-modifying enzymes, such as GCN5 (general control of amino acid synthesis 5) in the Spt-Ada-Gcn5 acetyltransferase (SAGA) and Esa1p in the NuA4 complex, can acetylate specific lysine residues in histone N-terminal tails (1). According to the histone code hypothesis (2), site-specific acetylation of histone tails may trigger chromatin remodeling that leads to retention of effector proteins to active promoters and formation of transcriptionally active chromatin regions.

GCN5 was originally identified as a transcriptional coactivator in yeast and was proposed to contribute to transcription by establishing interactions between certain activators and transcriptional complexes (3). GCN5 enhances transcription through its intrinsic acetyltransferase activity (4), which facilitates acetylation of histones and nonhistone substrates (3). Recruitment of the SAGA complex greatly increases transcriptional activation in vitro (5), and the requirement of GCN5 for chromatin remodeling has been demonstrated in vivo (6). However, the mechanism(s) that regulates GCN5 activity particularly in the context of histone acetylation has yet to be examined in detail. Potentially, GCN5 HAT activity can be regulated through posttranslational modifications, interacting with other proteins or at the level of substrate modifications. For example, phosphorylation by the Ku-DNA-dependent protein kinase or sumoylation may regulate GCN5-HAT activity (7, 8). Also, interaction with the SANT domain of the Ada2 affects GCN5-HAT activity in yeast (9). Likewise, modification of substrates, such as phosphorylation of H3 serine 10, increases GCN5 acetylation activity toward lysine 14 of H3 (10, 11).

Nucleophosmin/B23, also known as NPM1, NO38, or numatrin, has been identified as a phosphoprotein that possesses multiple cellular functions (12). It plays important roles in ribosome assembly, protein folding, and centrosome duplication (13). During interphase, the majority of B23 protein localizes to the nucleolus, whereas in mitosis it distributes throughout the nucleoplasm and associates with condensed chromosomes (14). B23 is phosphorylated by CDC2-cyclin B during mitosis (15), and inhibition of mitotic phosphorylation leads to dissociation of B23 from mitotic chromosomes concomitant with their decondensation (16). B23 is frequently targeted for genetic mutations by chromosomal translocations and point mutations in lymphomas and leukemias and is one of the most frequently mutated genes in acute myeloid leukemia (13). It has also been established that B23 can function as an acidic histone chaperone that helps assemble nucleosomes in vitro (17). B23 can either repress (18, 19) or stimulate transcription (20, 21), depending on the promoter context and/or inter-
acting transcription factors. There are controversial reports concerning the role of B23 in the regulation of tumor suppressor p53 stability and transcription. Depending on experimental settings, B23 can either increase or decrease p53 transcription (22–24). Recent knock-out studies suggest that deletion of B23 in mice leads to embryonic lethality, genomic instability, and activation of p53 and p21/Waf1 (25, 26).

In this study, we identified GCN5 as a B23-interacting protein. B23 inhibited GCN5-dependent acetylation of free and nucleosomal histones and blocked histones from being acetylated by GCN5. The inhibitory effect became more evident in the presence of mitotic B23. Mitotic extract depleted of threonine 199-phosphorylated B23 barely inhibited GCN5-dependent histone acetylation. B23 inhibited GCN5-dependent transactivation of the p21/Waf1 and Sp1 promoter. Microarray expression analysis revealed that a considerable number of genes were counterregulated by B23 and GCN5. We propose that B23 may negatively regulate GCN5-dependent transactivation through inhibition of GCN5-mediated nucleosomal acetylation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**U2OS (ATCC), 293T (ATCC), and NIH3T3 cells were cultivated in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Cells were transfected by FuGene 6 (Roche Applied Science) or Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. To establish stable inducible clones, pBI-tet-HA-Gcn5, pBI-tet-B23-FLAG, or pBI-tet-HA-Gcn5/B23-FLAG (see below) were co-transfected with pBabe-puro into U2OS Tet-on (Invitrogen) cells. Puromycin-resistant clones were selected and doxycycline-regulatable constructs were then verified by Western blot for inducible protein expression. Mitotic cells were collected by mitotic shake-off.

**Cloning and Site-directed Mutagenesis—**Human B23 open reading frame cDNA was amplified from the human testis Marathon-ready cDNA library (Clontech) using the following pair of primers: 5′-gca gtc gac acc aac ATG GAA GAT TCG and 5′-gcc gtt aac AAG AGA CTT CCT CCA CTG-3′. Sequencing analysis of the resulting plasmid confirmed that the B23 cDNA was identical to human B23 in GenBank™ (accession number BC006497). The plasmid pG5luc was obtained from Promega.

**Antibodies and Recombinant Protein Purification—**The following antibodies were used in this study: anti-B23 (B0556; Sigma), FLAG (M2; Sigma), phosphorylated B23 threonine 199 (phospho-B23-Thr199; Cell Signaling Technologies) and HA (HA.11; BabCO), anti-acetyl-H3K14 and acetyl-H4K8 (Upstate Biotechnology, Inc.), anti-His (H-15), GCN5 (H-75), histone H3 (FL-136), and β-actin (H300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). GST-cyclin E-CDK2 complex was purified with glutathione-agarose beads from 59 insect cells co-infected with baculoviruses expressing cyclin E and CDK2. GST-GCN5 and B23-His fusions were purified from Escherichia coli in essentially the same manner as described previously (28).

**Histone Acetyltransferase Assays—**Histones H3 and H4 were purchased from Upstate Biotechnology. HeLa cell short oligonucleosomes were the kind gifts from Dr. Jerry Workman. To prepare mononucleosomes, 5 μl of short oligonucleosomes (1 mg/ml) were mixed with 14 μl of Buffer R (10 mM Hepes-K+, pH 7.5, containing 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EGTA, 10% (v/v) glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride) and 0.6 μl of CaCl2 (100 μg/ml), followed by MNase (0.2 unit) digestion for 10 min at room temperature. To check the quality of the mononucleosomes, aliquots of the MNase-treated samples were treated with proteinase K at 37 °C for 30 min. DNA was isolated and analyzed on 1.1% agarose gel. More than 90% of the samples were mononucleosomes (data not shown). HAT assays were carried out in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, 0.25 μg/μl acetyl-CoA, 10% glycerol with protease inhibitor mixture (Roche Applied Science), and 0.05 μg/μl APHA Compound 8, a histone deacetylase (HDAC) inhibitor (Sigma). Each reaction contained 2 μg of H3, H4, GST-H3, or 0.5 μg of mononucleosomes as substrates and the indicated amount of GST-GCN5 and B23-His proteins. The reactions were carried out at 30 °C for 15 min and stopped by 2× SDS sample buffer followed by heating at 100 °C for 5 min. Acetylated histone H3 or H4 was identified by Western blot using anti-acetylated histone antibodies as described above.
Kinase Reaction with Interphase, Mitotic Lysates, or Cyclin E-CDK2—In total, 100 or 2 μg of purified His-tagged B23 (B23WT-His or B23T199A-His) bound to Ni²⁺-nitrilotriacetic acid beads was incubated with 250 μg of U2OS interphase or mitotic lysates or 1 μg of purified GST-cyclin E-CDK2 complex, respectively, for 90 min at 30 °C in a kinase reaction buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 50 μM ATP, and protease inhibitor mixture. B23-His proteins were then eluted from Ni²⁺-nitrilotriacetic acid beads by washing the beads with 10 mM Tris-HCl, pH 7.5, buffer containing 250 mM imidazole. The proteins were desalted by filtration through Zeba Desalt Spin Columns (Pierce) before use.

Luciferase Assays—To prepare interphase cell lysates for luciferase assay, 12 h after transfection, cells were first switched to serum-free medium for 12 h followed by cultivation in 10% FBS for 8 h and then harvested for luciferase assay. To prepare mitotic cell lysates for luciferase assay, 24 h after transfection, cells were treated with 0.1 μg/ml nocodazole for 12 h, released for 1 h, and then harvested by mitotic shake-off. The cells were lysed with 100 μl of passive lysis buffer (Promega) and incubated at room temperature for 30 min. The luciferase activities were measured using a TD20/20 Luminometer (Turner Designs) and normalized by β-galactosidase activities of the co-transfected pCMVβGal.

RT-PCR—Total RNA was purified from cells using Trizol reagent (Invitrogen). The cDNA was synthesized from 2 μg of total RNA with the use of Superscript II RNase H minus reverse transcriptase (Invitrogen) and oligo(dT) primer (Roche Applied Science) according to the manufacturer’s instructions. Human p21/Waf1-specific primers (5′-CCA GTG GAC AGC GAG CAG-3′; 5′-CCC TGC AGC AGA GCA GGA GGT-3′) were used to check p21 expression by RT-PCR. The proteins were then eluted from Ni²⁺-nitrilotriacetic acid beads by washing the beads with 10 mM Tris-HCl, pH 7.5, buffer containing 250 mM imidazole. The proteins were desalted by filtration through Zeba Desalt Spin Columns (Pierce) before use.

RESULTS

Microarray Analysis—U2OS-Tet-On inducible stable clones carrying pBI-tet vector, pBI-tet-HA-Gcn5, pBI-tet-B23-FLAG, and pBI-tet-HA-Gcn5/B23-FLAG were induced by the addition of 2 μg/ml doxycycline for 48 h. Total RNA was extracted from both induced and uninduced cells and subject to microarray hybridization using human 30k oligonucleotide chips according to the protocols of the Vanderbilt Microarray Shared Resources (available on VMSR website). Microarray expression data were analyzed with GenePix4.0 and Acuity4.0 software (Axon Instruments).

Immunoprecipitation, Mass Spectrometric, and Data Analyses—Immunoprecipitation of endogenous B23, GCN5, and FLAG-tagged GCN5 was performed in essentially the same manner as described in our previous studies (29). For mass spectrometric analysis, asynchronized U2OS cells were treated with or without 0.2 μg/ml nocodazole for 24 h and then lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25% Nonidet P-40 and protease inhibitor mixture (Roche Applied Science). Immunoprecipitation was conducted with 4 μg of anti-B23 antibody (Sigma) cross-linked to protein G-agarose beads. Beads were then washed eight times with lysis buffer. Bound proteins were eluted with 0.1 M glycine, pH 3.5, and precipitated in 10% trichloroacetic acid followed by an acetone wash. Samples were subjected to digestion for mass spectrometry as described in our previous studies (30). Briefly, the dry pellet was brought up in 8.0 M urea, reduced with Tris(2-carboxyethyl)phosphine (Bond-Breaker by Pierce), and treated with iodoacetamide to alkylate cysteines. The sample was then predigested with endoproteinase Lys-C, digested, and digested with trypsin. The resultant peptides were protonated with formic acid and loaded onto the back column of a three-phase MudPIT (multidimensional protein identification technology) setup (back column, Aqua C18 Reverse Phase-Luna Strong Cation Exchange; front column, Jupiter C18 Reverse Phase, all from Phenomenex) using a pressure cell (31). The sample peptides were then separated and analyzed by nano-LC-MS/MS using an UltiMate™ LC pump (LC Packings) in line with a linear ion trap mass spectrometer (LTQ; Thermo Finnigan) operating in data-dependent MS/MS mode. Five separate LC-MS/MS cycles were performed per sample, each varying by increased salt pulse concentration and followed by a linear organic gradient to resolve peptides. Spectra obtained from the LTQ were analyzed by DBDigger (32) using the human IPI (International Protein Index) data base, version 3.05. DTASelect was used to filter and organize the search results, whereas Contrast (33) was used to differentiate proteins that appeared in either synchronized or nocodazole-arrested cells.

Identification of GCN5 as a B23-interacting Protein in Nocodazole-arrested Cells by Mass Spectrometry—In order to explore the potential role of B23 in mitosis, we set out to identify B23 interacting proteins through a mass spectrometric approach. U2OS cells were first arrested by nocodazole. A majority of the U2OS cells were synchronized at G₂/M phase as shown by fluorescence-activated cell sorting analysis (Fig. S1). Protein complexes containing B23 were immunoprecipitated by anti-B23 antibody from both nocodazole-treated and -un-
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A.

B.

C.

FIGURE 1. Identification of GCN5 as a B23-interacting protein. A, MS/MS spectra of fragmented GCN5-specific peptide (+42) RQLLEKFRVEK (doubly charged), displayed as a series of a, b, and y ions according to Biemann nomenclature (52). Manual analysis of this spectrum corroborates DBDigger’s identification of putative B23-interacting partner GCN5. B, physical interaction between B23 and GCN5 in vivo. Left: co-immunoprecipitation of endogenous B23 and GCN5 from 293T cells. Cellular extract was immunoprecipitated (IP) with protein A/G beads conjugated with anti-B23, GCN5 antibodies, or IgG as a control. The immunoprecipitates were then separated by SDS-PAGE and probed with anti-B23 or GCN5 antibodies. Right: co-immunoprecipitation of endogenous B23 and GCN5 in pBl-HA-Gcn5/Flag-B23 U2OS-tet-On stable clone. U2OS-tet-On cells carrying pBl-HA-Gcn5/Flag-B23 were induced with 2 μg/ml doxycycline for 24 h, and immunoprecipitation was performed using anti-FLAG antibody or IgG as a control. The immunoprecipitates were separated by SDS-PAGE and probed with either anti-FLAG (for B23) or anti-HA (for GCN5) antibodies. C, GCN5 interacts with B23 in both nocodazole-treated (+NZ) and -untreated (−NZ) cells. Cells (293T) were arrested with 0.3 μg/ml nocodazole for 18 h, and an equal amount of protein lysates (200 μg) from nocodazole-treated and -untreated cells were used for immunoprecipitation as described in B.

B23 Interacts with GCN5 in Vitro and in Vivo—To confirm the mass spectrometric finding, we performed reciprocal immunoprecipitation experiments using antibodies against endogenous B23 and GCN5 in 293T cells, respectively. B23 and GCN5 were co-immunoprecipitated in each experiment (Fig. 2A, left), and the same results were obtained with their exogenous counterparts in U2OS-Tet-On cells stably expressing doxycycline-inducible HA-tagged GCN5 and FLAG-tagged B23 (Fig. 2A, right). Moreover, we showed that endogenous GCN5 associated with B23 in both nocodazole-treated and untreated 293T cells (Fig. 1C), indicating that the interaction may occur in both interphase and mitotic cells.

To verify the finding further, we performed a GST pull-down experiment using bacterially produced B23-His and GST-GCN5 fusion proteins. As shown in Fig. 2A, B23-His only precipitated protein A/G beads conjugated with anti-GCN5, whereas recombinant full-length mammalian GCN5 is competent for the acetylation of nucleosomal histones (27). In order to scrutinize the biological and biochemical significance of the
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**A.** GST pull-down

| GST pull-down | Input |
|---------------|-------|
| GST-GCN5 | B23-His |
| GST-GCN5 | + | - |
| GST-GCN5 | - | + |

**B.**

| GST-GCN5 derivatives | Input |
|----------------------|-------|
| GST-GCN5 | H3-AcK14 |
| GST-GCN5 | H3-AcK14 |
| GST-GCN5 | H3-AcK14 |
| GST-GCN5 | H3-AcK14 |

**C.**

| GST pull-down | Input |
|---------------|-------|
| GST-GCN5 | B23-His |
| GST-GCN5 | + | - |
| GST-GCN5 | - | + |

**FIGURE 2.** Deletion mapping of GCN5 interaction domains. **A,** physical interaction between B23 and GCN5 in vitro. Purified B23-His (lane 3) was incubated with GST-GCN5, and B23-GCN5 complexes were affinity-purified with glutathione beads. GCN5 and B23 were detected by Western blot using anti-GCN5 and anti-B23 antibodies, respectively. 

**B,** schematics of human GCN5 and its respective deletion derivatives. **C,** PCAF-N domain of GCN5 is involved in GCN5-B23 interaction. Purified B23-His was incubated with the indicated GST-GCN5 fusion proteins, and GCN5-B23 complexes were affinity-purified by glutathione-agarose beads (GST pull-down). B23-His and GST-GCN5 fusion proteins were detected by immunoblot using anti-B23 and GST antibodies, respectively.

**FIGURE 3.** B23 inhibits GCN5-mediated histone H3K14 and H4K8 acetylation in vitro. **A,** acetylation of purified histone H3 by GST-GCN5 in the absence (lane 2) or presence (lane 4) of B23-His. Acetylated H3K14 (H3-AcK14) was revealed by immunoblot using anti-acetyl-H3K14 antibody. Coomassie stain shows the amounts of histone H3, B23, and GCN5 loaded in each histone HAT reaction. **B,** B23 dose-dependent inhibition of H3K14 and H4K8 acetylation. An HAT assay was performed with increasing concentrations of B23, as indicated. H3-AcK14, acetylated H4K8 (H4-AcK8), B23-His, GST-GCN5, or GCN5-His was visualized by Western blot using antibodies against acetyl-H3K14, acetyl-H4K8, B23, and GCN5 antibodies or by Coomassie stain (marked with an asterisk). **C,** B23 blocks histones from being acetylated by GCN5 and does not function at a postacetylation stage. Scheme **A,** following HAT reaction in the absence (lane 1) or presence (lane 2) of GST-GCN5 for 15 min, purified B23-His was added (lanes 3 and 4), and the reaction continued for an additional 15 min. Scheme **B,** following HAT reaction without GST-GCN5 in the absence (lane 5) or presence (lane 6) of B23-His for 15 min, purified GST-GCN5 was added (lanes 7 and 8) and the reaction continued for an additional 15 min. Coomassie stain shows the amount of histone H3 loaded in each HAT reaction. Acetyl-H3K14, GST-GCN5, and B23-His were detected by Western blot as in **B.**

B23/GCN5 interaction, we performed an in vitro HAT assay using purified recombinant human GCN5. We first measured GCN5 HAT activity on lysine 14 of histone H3 (H3K14), since previous studies have demonstrated that H3K14 is the major histone acetylation site by GCN5 (36). As shown in Fig. 3A, in the presence of purified GST-GCN5, H3K14 was efficiently acetylated (lane 2), whereas the addition of B23 (B23-His) significantly reduced H3K14 acetylation (lane 4). Moreover, incubation with B23-His at increasing concentrations resulted in a progressive inhibition of both H3K14 and H4K8 acetylation (Fig. 3B), indicating a B23 dosage-dependent inhibition of GCN5 HAT activity. The PCAF-N domain has been shown to be required for PCAF autoacetylation in trans, which may in turn lead to enhancement of PCAF HAT activity (37). Whether GCN5 may use a similar mechanism to regulate its HAT activity or whether binding of B23 to the PCAF-N domain of GCN5 may impair this regulatory function remain to be determined.

All HAT assays throughout our experiments were performed in the presence of an HDAC inhibitor (APHA compound 8), which apparently did not perturb the B23-inhibitory function on GCN5-dependent HAT activity, excluding the possibility that B23 may serve as an HDAC. Since B23 is a core histone-binding protein (38), we wonder whether B23 may inhibit GCN5 activity through binding competition with GCN5 to histone substrates. As shown by the “order of addition experiment,” the addition of B23 subsequent to the GCN5-dependent HAT reaction failed to inhibit GCN5-mediated H3K14 acetylation (Fig. 3C, compare lanes 6 and 8, scheme B). This result indicates that B23 1) may not act at a postacetylation stage and 2) may as well block histones from being acetylated by GCN5 through direct binding competition with GCN5 to histone substrates, as shown previously for INHAT, a histone chaperone complex (39). The latter was supported by and in line with the fact that B23 is a histone chaperone (38) and can bind histone H3 independently of H3 acetylation (Fig. S2). This study, together with the interaction and deletion mapping data (Figs. 1 and 2), suggests that B23 may negatively regulate GCN5-dependent acetylation at both enzyme (GCN5) and substrate (histone) levels.
To evaluate the effect of B23 on GCN5 activity to acetylate chromatin histones, we tested whether the inhibitory activity of B23 may also occur with nucleosomes. According to a previous study, full-length mammalian GCN5, in the absence of any other SAGA component, can efficiently acetylate nucleosomal histone H3 (27). In line with this study, we showed that the recombinant full-length GCN5 (the long form GCN5L2) acetylated HeLa cell mononucleosomal H3K14 (Fig. 4, lane 2). In the presence of B23, the level of mononucleosomal H3K14 acetylation decreased (Fig. 4, lane 4). This inhibition may have significant impact on GCN5-dependent chromatin structure remodeling, as shown by an S7 nuclease sensitivity assay in GCN5- and B23-transfected cells (Fig. S3).

Phosphorylation of B23 at Threonine 199 Is Involved in B23-mediated Mitotic Inhibition of GCN5-dependent Histone Acetylation—To further explore the role of B23 in the regulation of GCN5-mediated histone acetylation in cells, we used anti-B23 monoclonal antibody to immunoprecipitate B23 from U2OS interphase and mitotic extracts. The immunopurified antibody-B23 complex was then applied to the GCN5-mediated histone H3 acetylation reactions. As demonstrated in Fig. 5A (top), although both interphase and mitotic B23 inhibited GCN5-mediated acetylation, mitotic B23 was more effective to inhibit GCN5-mediated histone acetylation activity. Work from others shows that B23 is phosphorylated by CDC2 kinase during mitosis, and accumulation of the highly phosphorylated mitosis-specific B23 correlates with mitotic chromosome condensation (15). Indirect evidence suggests a correlation between B23 dephosphorylation and mitotic chromosome decondensation (16). To evaluate potential contributions of mitotic B23 phosphorylation on the regulation of GCN5-mediated histone acetylation, we tested the effect of in vitro phosphorylated B23-His protein in the GCN5-mediated HAT assay. After incubation with mitotic or interphase extracts in a kinase reaction buffer containing ATP, mitotic phosphorylated B23 again showed higher inhibitory activity than interphase phosphorylated B23 (Fig. 5A, bottom, lanes 3 and 4). Together, both in vitro and in vivo purified B23 exhibited strong mitotic inhibitory activity on GCN5 (Fig. 5A), implying that mitotic phosphorylation of B23 may enhance its inhibitory activity toward GCN5-mediated histone acetylation.

A recent study suggests that threonine 199 of B23 (B23T199) is phosphorylated by CDK1 (CDC2) at the onset of mitosis, coinciding with chromosome condensation and nucleolar and nuclear envelope disassembly (40). The same phospho-

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**FIGURE 4.** B23 inhibits GCN5-mediated acetylation of mononucleosomal histones. HeLa cell mononucleosomes were used as substrates in GST-GCN5-mediated HAT reactions in the absence (lane 2) or presence (lane 4) of B23-His. Acetylated histone H3K14 (H3-AcK14), mononucleosomal H3, and GST-GCN5 were as revealed by Western blot using anti-acetyl-H3K14, H3, GCN5 antibodies, and B23-His by Coomassie, respectively. The indicated -fold change of H3-AcK14 was calculated based on the densitometric values of each lane normalized against the mononucleosome loading controls.

**FIGURE 5.** Phosphorylation of B23 threonine 199 in mitosis enhances B23-inhibitory activity on GCN5-mediated histone acetylation. A, top, interphase- and mitosis-derived B23 inhibit GCN5 HAT activity. B23 was immunoprecipitated by anti-B23 antibody from interphase (I) and mitotic (M) extracts. The HAT reaction was then carried out in the presence or absence of the immunopurified B23 (IPed-B23). Bottom, purified B23-His was incubated with the interphase or mitotic extracts in a kinase reaction buffer containing ATP and then added to the HAT reaction. Acetylated histone H3K14 (H3-AcK14), phosphorylated B23-Thr199 (B23-pT199), B23-His, and immunopurified B23 were revealed by Western blot using acetyl-H3K14, phospho-B23-Thr199, and B23 antibodies, respectively. Coomassie stain shows equal loading of GST-H3 in each HAT reaction. The indicated -fold change of H3-AcK14 was calculated based on the densitometric values of each lane normalized against the GST-H3 loading control. B, top, threonine 199 of B23 was preferentially phosphorylated in mitosis. Endogenous B23 or B23-pT199 was detected in interphase (I) and mitotic (M) extracts as above. Bottom, specificity of phospho-B23-Thr199 antibody. In vitro purified B23-His (WT or T199A) proteins were phosphorylated by baculovirus-produced cyclin E-CDK2 in the presence of ATP and visualized by Western blot using anti- phospho-B23-Thr199 antibody. C, B23-Thr199 phosphorylation is involved in mitotic inhibition of GCN5 HAT activity. Top, mitotic extracts were immunodepleted with anti phospho-B23-Thr199 antibody (depl) or control rabbit IgG (no depl) and then evaluated by an immunoblot using phospho-B23-Thr199, B23, and β-actin antibodies. Bottom, the resulting extracts were then added to the GCN5-mediated HAT reaction in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of B23-His (WT) or B23-T199A-His (T199A) that had been prephosphorylated by cyclin E-CDK2 as in B. GCN5 activity was evaluated in an immunoblot using anti-acetyl-H3K14 antibody. Coomassie stain shows equal loading of GST-H3 in each reaction.
B23 Modulates GCN5-dependent Transactivation—It is generally believed that GCN5-dependent transcription relies on its HAT activity (2, 3). The inhibitory effect of B23 on GCN5-dependent histone acetylation raised a question about whether B23 has any impact on GCN5-dependent transcriptional regulation. We thus evaluated the effect of B23 on GCN5-dependent transcription in the context of GCN5-regulated promoters. Our first experiment was designed to bring GCN5 to a reporter promoter followed by measuring the transactivation activity of GCN5 in the presence of B23. For this purpose, we fused GCN5 to GAL4 DNA-binding domain (pBIND-GCN5) and determined its activity on a pG5luc luciferase reporter that was under the control of five copies of GAL4 consensus binding sites. Previous studies demonstrated that fusion of GCN5 to the bacterial LexA DNA-binding domain activated transcription in yeast. It was suggested that this activity was attributable to the HAT and ADA2 binding domains of GCN5 (44). In line with the previous studies, transfection of pBIND-Gcn5 into 293T or U2OS cells activated the pG5luc luciferase reporter. Indeed, "add-back" of cyclin E-CDK2 phosphorylated wild-type B23-His but not B23T199A-His on GCN5-mediated histone acetylation. Despite the absence of cyclin E-CDK2 activity, we tested if "add-back" of cyclin E-CDK2-phosphorylated B23 may restore the inhibitory activity of phospho-B23-Thr\(^{199}\)-depleted mitotic extract. Indeed, "add-back" of cyclin E-CDK2-phosphorylated wild-type B23-His but not B23T199A-His mutant significantly restored the inhibitory activity of phospho-B23-Thr\(^{199}\)-depleted mitotic extract (Fig. 5C, bottom, lanes 3 and 4). Together, these studies suggest that phosphorylated B23-Thr\(^{199}\) contributes to the mitotic inhibitory activity of B23. However, our study does not exclude the possibility that mitotic phosphorylation of other B23 phosphorylation sites may also play an important role in the regulation of GCN5-mediated histone acetylation.

B23 in GCN5-dependent Histone Acetylation and Transactivation—It is generally believed that GCN5-dependent transcription relies on its HAT activity (2, 3). The inhibitory effect of B23 on GCN5-dependent histone acetylation raised a question about whether B23 has any impact on GCN5-dependent transcriptional regulation. We thus evaluated the effect of B23 on GCN5-dependent transcription in the context of GCN5-regulated promoters. Our first experiment was designed to bring GCN5 to a reporter promoter followed by measuring the transactivation activity of GCN5 in the presence of B23. For this purpose, we fused GCN5 to GAL4 DNA-binding domain (pBIND-GCN5) and determined its activity on a pG5luc luciferase reporter that was under the control of five copies of GAL4 consensus binding sites. Previous studies demonstrated that fusion of GCN5 to the bacterial LexA DNA-binding domain activated transcription in yeast. It was suggested that this activity was attributable to the HAT and ADA2 binding domains of GCN5 (44). In line with the previous studies, transfection of pBIND-Gcn5 into 293T or U2OS cells activated the pG5luc luciferase reporter. Indeed, "add-back" of cyclin E-CDK2 phosphorylated wild-type B23-His but not B23T199A-His on GCN5-mediated histone acetylation. Despite the absence of cyclin E-CDK2 activity, we tested if "add-back" of cyclin E-CDK2-phosphorylated B23 may restore the inhibitory activity of phospho-B23-Thr\(^{199}\)-depleted mitotic extract. Indeed, "add-back" of cyclin E-CDK2-phosphorylated wild-type B23-His but not B23T199A-His mutant significantly restored the inhibitory activity of phospho-B23-Thr\(^{199}\)-depleted mitotic extract (Fig. 5C, bottom, lanes 3 and 4). Together, these studies suggest that phosphorylated B23-Thr\(^{199}\) contributes to the mitotic inhibitory activity of B23. However, our study does not exclude the possibility that mitotic phosphorylation of other B23 phosphorylation sites may also play an important role in the regulation of GCN5-mediated histone acetylation.

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**FIGURE 6.** B23 inhibits GAL4-GCN5 transactivation activity. The reporter pG5luc and the normalization pCMV\(^\beta\)-Gal were transiently co-transfected into 293T and U2OS cells with the indicated plasmids. The values of the luciferase reporter activity were calculated after normalization with \(\beta\)-galactosidase activity. For 293T cells, the luciferase assay was performed 24 h after transfection. U2OS interphase and mitotic cells were prepared and measured as described under "Experimental Procedures." The results represent triplicate samples, and the data are shown in means ± S.D. The indicated \(p\) values (\(t\) test) of each experiment were obtained from calculations against the values of luciferase activity (293T) or \(\varepsilon\)-fold change (U2OS) of pBIND-Gcn5 (GAL4-GCN5) alone transfections (*) in 293T and U2OS cells, respectively. Note for comparison between interphase and mitotic experiments in U2OS cells, Fold change of relative luciferase activity was used.
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**A.** pBl-tet inducible clones

|        | Dox | Vector | Gcn5 | B23 | Gen5+B23 |
|--------|-----|--------|------|-----|----------|
| p21-actin fold change (p21/actin): | 2.09 | 1.0    | 1.0  | 1.0 | 0.94     |
| RT-PCR | | | |
| Western | | | |
| HA-GCN5-Flag-B23 | | | |
| Actin- | | | |

**B.**

| pCMVtag2C-Gcn5 | pCMVtag2C-B23 |
|----------------|--------------|
| Fold induced | > +9.92 |
| Fold repressed | < -9.92 |

**C.**

![Graph showing luciferase activity](image)

**FIGURE 7.** B23 inhibits GCN5-mediated transactivation in vivo. A, effect of GCN5 and B23 on p21/Waf1 expression. Total RNA was extracted from each doxycycline-inducible stable U2OS clone as indicated. Qualitative RT-PCR showed p21/Waf1 expression in each clone. The levels of B23 and GCN5 expression were shown by Western blot using anti-FLAG or -HA antibodies. β-Actin was used as loading controls for both RT-PCR and Western blot analyses. B, B23 blocks GCN5-mediated transactivation on the SP1 promoter. The reporter pSp1-luc and the normalization pCMV/β-galactosidase were transiently co-transfected into U2OS cells with the indicated plasmids. The values of the luciferase reporter activity were calculated after normalization with β-galactosidase activity. The data represent three independent experiments, and the results are shown as means ± S.D. C, effect of B23 on Gcn5-dependent global transcriptional activation. Microarray expression analyses were performed and analyzed in U2OS-Tet-On inducible cells carrying the pBI-tet vector (lane 1), pBI-tet-HA-Gcn5 (lane 2), pBI-tet-B23-FLAG (lane 3), or pBI-tet-HA-Gcn5/B23-FLAG (lane 4). The gene expression cluster map represents 147 genes whose expression showed significant counterregulation between GCN5 and B23. The scale value did not include the fold-change of genes (see genes marked by an asterisk in Table S1) whose expression were induced or repressed over 9.9-fold. Values of normalized -fold changes are shown in Table S1.

The data represent three independent experiments, and the results are shown as means ± S.D. C, effect of B23 on Gcn5-dependent global transcriptional activation. Microarray expression analyses were performed and analyzed in U2OS-Tet-On inducible cells carrying the pBI-tet vector (lane 1), pBI-tet-HA-Gcn5 (lane 2), pBI-tet-B23-FLAG (lane 3), or pBI-tet-HA-Gcn5/B23-FLAG (lane 4). The gene expression cluster map represents 147 genes whose expression showed significant counterregulation between GCN5 and B23. The scale value did not include the fold-change of genes (see genes marked by an asterisk in Table S1) whose expression were induced or repressed over 9.9-fold. Values of normalized -fold changes are shown in Table S1.

at the level of nucleosomal histone modification (47) and reinforce our finding that B23 may regulate GCN5-dependent transcription on certain promoters.

The p21/Waf1 promoter contains 6× Sp1 binding sites. Treatment of cells with sodium butyrate, an HDAC inhibitor, increases binding of GCN5 to the proximal region of p21/Waf1 promoter containing the Sp1 repeats concomitant with H3 hyperacetylation at the Sp1 sites (45). This suggests that GCN5 plays an important role in the regulation of Sp1 promoter activity. Histone H4 hyperacetylation has also been implicated in the regulation of TATA-less Sp1 promoter activity (46). We therefore utilized the Sp1 promoter activation as a model system to assess the roles of B23 and GCN5 in Sp1 promoter regulation. U2OS cells were transiently transfected with pSp1-luc reporter together with GCN5 and B23 alone or together (Fig. 7B). GCN5 activated the pSp1-luc reporter as exemplified by the 2-fold increase of luciferase activity (Fig. 7B, pCMVtag2C-Gcn5), whereas in the pCMVtag2C-B23 and pCMVtag2C-Gcn5 double-transfection experiment, the GCN5-dependent Sp1-luc activation was significantly inhibited (t test, p < 0.01) with the luciferase activity reducing to a background level (Fig. 7B, pCMVtag2C-Gcn5 + pCMVtag2C-B23). These results, in line with the inhibitory effect of B23 on GCN5-dependent histone acetylation, suggest that B23 may oppose GCN5-dependent activation of TATA-less Sp1 promoters.

To further evaluate the role of B23 in regulating GCN5-dependent transcriptional activation at a genome-wide scale, we carried out a microarray expression analysis in U2OS stable inducible clones carrying pBI-tet, pBI-tet-HA-GCN5, pBI-tet-B23-FLAG, or pBI-tet-HA-GCN5/B23-FLAG using Vanderbilt Microarray Shared Resources 30k human oligonucleotide chips. Gene expression profiles of GCN5- and B23-inducible clones were organized according to the dissimilarity of their hybridization signals meet all the following criteria: gene expression is 1) over 2.4-fold induced in GCN5-inducible cells, 2) over 2.4-fold repressed in B23-inducible cells, and 3) changed less than 1.1-fold (1.0 = no change) in GCN5/B23 double-inducible cells after normalization against the vector con-
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trols in at least two replicate experiments. Based on these criteria, we found about 147 genes that were counterregulated by GCN5 and B23 (Fig. 7C and Table S1). These results are consistent with a role of B23 in negatively modulating at least some of the GCN5-dependent transcription.

DISCUSSION

In this study, we demonstrate that B23, a multifunctional phosphoprotein, can inhibit GCN5-mediated free histone and nucleosomal acetylation as well as transactivation in vitro and in vivo. Our study supports the possibility that B23 may inhibit GCN5 catalytic activity via a dual mechanism involving both a direct interaction with GCN5 and a blockage of histone substrates from being acetylated by GCN5 (Figs. 1, 2, and 3C).

Our finding that B23 may negatively regulate GCN5-dependent transcription corroborates its inhibitory role on GCN5-mediated histone acetylation. Although B23 inhibitory activity is much less dependent on Thr199 phosphorylation in interphase (Figs. 5 and 6), phosphorylation of B23 Thr199 may play a role in regulating its inhibitory activity toward GCN5-dependent histone acetylation and transcription in mitosis (Figs. 5 and 6). According to the current hypothesis, the presence of active HATs and HDACs in mitotic cells may help to prime the cells for transcriptional reactivation immediately after chromosomes become decondensed in late mitosis (48), and Gcn5p is particularly critical for reactivation of transcription after mitotic silencing in yeast cells (49). Since B23 Thr199 becomes heavily phosphorylated at the onset of mitosis and dephosphorylated during anaphase (40), it is possible that this temporally regulated phosphorylation increases B23 inhibitory activity on GCN5 (Figs. 5 and 6), which may be a necessary step to prevent premature histone acetylation before the onset of mitotic transcriptional reactivation.

It has been demonstrated that B23 can either positively or negatively regulate transcription depending on the types of promoters and proteins it interacts with. For example, B23-RAR fusion proteins originated from acute promyelocytic leukemia cells can interact with co-repressors and co-activators, resulting in both transcriptional repression and activation (50). Similarly, B23 can both repress IRF1 (interferon regulatory factor 1)-mediated transcriptional activation (18, 19) and relieve YY1-induced transcriptional repression by direct interaction with YY1 transcription factor (20). Interestingly, a recent study shows that B23 works as an AP2α-binding transcriptional corepressor by remodeling local chromatin structure (51). We documented that B23 attenuated GCN5-dependent transcription at the Sp1 promoter in this study. Possible functional relevance to this stems from our observation that B23 hampers GCN5-dependent p21/Waf1 transcription, which is in agreement with the current model that nucleosomal histone deacetylation at p21/Waf1 promoter attributes to p53-independent inactivation of p21/Waf1 (47). A conceivable scenario is that inhibition of GCN5-mediated histone acetylation by B23 may prevail in a subset of genes depending on the contexts of their transcription activators and promoters. This idea is supported by our microarray data in which only a fraction of Gcn5-induced genes are suppressed by B23 (Fig. 7 and Table S1). Taken together, our observations demonstrate a novel function of B23 in the control of GCN5-dependent histone acetylation and transcription and may have significant impact on deciphering the mechanism(s) of GCN5- and B23-dependent transcriptional regulation during cell division.

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