Title: Regulation of nucleolar chromatin by B23/nucleophosmin jointly depends upon its RNA binding activity and transcription factor UBF

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Abstract

Histone chaperones regulate the density of incorporated histone proteins around DNA transcription sites and therefore constitute an important site-specific regulatory mechanism for the control of gene expression. At present the targeting mechanism conferring this site specificity is unknown. We previously reported that the histone chaperone B23/nucleophosmin associates with ribosomal RNA chromatin (r-chromatin) to stimulate rRNA transcription. Here, we report on the mechanism for site-specific targeting of B23 to the r-chromatin. We observed that, during mitosis, B23 was released from chromatin upon inactivation of its RNA binding activity by cdc2 kinase-mediated phosphorylation. The phosphorylation status of B23 was also shown to strongly affect its chromatin binding activity. We further found that r-chromatin binding of B23 was a necessary condition for B23 histone chaperone activity *in vivo*. In addition, we found that depletion of UBF (an rRNA transcription factor) decreased the chromatin binding affinity of B23, which in turn led to an increase in histone density at the r-chromatin. These two major strands of evidence suggest a novel cell cycle dependent mechanism for the site-specific regulation of histone density via joint RNA- and transcription factor-mediated recruitment of histone chaperones to specific chromosome loci.

Introduction

Chromatin, the constituent substance of all eukaryotic chromosomes, is a highly compacted structure consisting mainly of genomic DNA in association with the four-histone proteins H2A, H2B, H3 and H4. Due to chromatin's occluded nature significant chromatin remodeling is required to allow transcription factors to gain access to their DNA cognate sites. Thus chromatin remodeling is capable of acting as a specific controlling mechanism, playing a role in a number of important biological events including cell development, stress response and cell cycle progression. Several studies have established that transcription factors bound to specific DNA sequences are capable of attracting histone modifying and ATP-dependent chromatin remodeling enzymes, which in turn act to promote chromatins adoption of an open conformation (reviewed in (13)). In addition to the action of these recruited enzymes, histone chaperones have been suggested to play a role in forming open chromatin structures via their direct association with histones. During transcription elongation phases, histones in nucleosome are dynamically evicted from, and then deposited back onto DNA in concert with the progression of RNA polymerases. Several lines of evidence suggest that histone chaperones are involved in the regulation of histone density and thereby gene expression (29, 38). We also demonstrated that depletion of the histone chaperone TAF-I (16) changed the genetic expression profile of HeLa cells (10). Recent studies have suggested that a part of histone chaperones are recruited to the specific genes by interacting with certain DNA binding proteins (5, 7, 39). However, the molecular mechanism behind how histone chaperons achieve specific binding to particular genomic region is not well understood.

rRNA synthesis is closely tied to cell growth, and hence should, in theory, be tightly regulated in response to metabolic and environmental changes. Loading rate of RNA polymerase I (Pol I) onto the rRNA gene is a key regulatory step in controlling rRNA transcription levels (6). This step has been suggested to be regulated by the

transcription factor, UBF (Upstream Binding Factor) that acts to recruit the pol I complex (12). More recently, it was demonstrated that UBF plays roles in promoter escape (23) and transcription elongation (31) rather than pre-initiation complex assembly. Recent research has also demonstrated that UBF associates with the entirety of the rRNA genes including the intergenic region between rRNA coding regions (18). Therefore, it is likely that UBF plays a crucial role in defining rRNA gene loci. Another major factor determining rRNA expression levels is suggested to be the balance between 'active' and 'inactive' rRNA gene numbers. It was shown that only a half of the rRNA genes are actively transcribed in exponentially growing cells (4). Epigenetic mechanisms are suggested to play a key role in regulating this active/inactive rRNA gene balance. The NoRC complex (33) has a reported involvement with rRNA transcription regulation. NoRC binds to the promoter region of rRNA genes by interacting with TTF-I and recruits the Sin3 co-repressor complex (27, 42). It has also been reported that the SIRT1/Suv39h1/nucleomethylin complex mediates heterochromatin formation around rRNA genes in a manner sensitive to changing NAD+/NADH level (15). These chromatin modification enzymes create and maintain an inactive chromatin structure around rRNA genes.

Histone chaperones, nucleolin and the FACT complex, also have the important role in r-chromatin regulation. Nucleolin was reported to play a role in enhancing chromatin remodeling by SWI/SNF and ACF (1) and facilitate transcription by pol I (25) *in vitro*. Nucleolin and FACT have been shown to bind to r-chromatin and their knockdown reduces the pre-rRNA transcription level (3, 25). We previously identified a nucleolar protein, B23 as a component of Template Activating Factor-III, the factor responsible for mediating the structural changes of adenovirus chromatin to stimulate DNA replication *in vitro* (19). B23 exists as two isoforms, B23.1 and B23.2. The C-terminal RNA binding domain of B23.1 is lacking in B23.2. Following our initial discovery, B23 was subsequently shown to function as a histone chaperone *in vitro* (21). Recently, we have reported that B23 binds to r-chromatin and regulates the histone

density around rRNA genes (14). However, the mechanism by which B23 is recruited to, and subsequently associated with the r-chromatin remains unclear. Here, we clarify the molecular mechanism of how histone chaperone B23 is targeted to the r-chromatin. Our findings suggest a novel mechanism for histone chaperone targeting to the specific chromosome loci.

Materials and methods

Cell culture, transfection, and cell cycle synchronization

HeLa cells were maintained in MEM (Nissui) supplemented with 10% fetal bovine serum (FBS). 293T cells were maintained in Dulbecco's modified Eagle medium (Nissui) supplemented with 10% FBS.

Transient transfection of plasmid DNA was performed using GeneJuice (Novagen) according to the manufacturer's instruction. To establish stable cell lines, HeLa cells were transfected with pEGFP-Flag-B23.1, pEGFP-Flag-T4sA and pEGFP-Flag-T4sD (see below). Neomycin-resistant cells were selected by G418.

Mitotic cells were collected following synchronization by two cycles of thymidine blockage following nocodazole arrest. Briefly, cells were treated with 3 mM thymidine (Sigma) in the culture medium for 16 hrs, then released into a fresh culture medium without thymidine for 10 hrs and then finally subjected to a second block with 3 mM thymidine for 14 hrs. After the double block, cells were released into a fresh culture medium for 8 hrs, after which 50 ng/ml nocodazole (Sigma) was added and cells were incubated for 4 to 10 hrs. Mitotic cells were collected by gentle shaking of the incubation dishes. For the experiment shown in Fig. 6, mitotic cells were released into culture medium in the absence or presence of 50 ng/ml of actinomycin D (Act D) (Sigma). To inhibit the RNA polymerase II activity, cells were incubated in the culture medium containing 5 μg/ml of α-amanitin (Sigma) for 24 hrs. To examine the effect of α-amanitin, U1 snRNA was amplified by RT-PCR using primer set; 5'-cggggtaccatacttacctggcaggggaga-3', and 5'- cggggtaccgaattcaggggaaagcgcgaacg-3'.

siRNA transfection

siRNA was transfected using Lipofectamine RNAi MAX (Invitrogen). Stealth RNAs for negative controls (Stealth RNAi Negative Control Duplex, catalog No. 12935-300; Invitrogen), for B23.1 (NPM1-HSS143154), and for UBF

(UBTF-HSS111143, Invitrogen) were utilized.

Antibodies

The following antibodies were used in this study. Mouse monoclonal antibodies for B23.1 (Invitrogen), Flag-tag (M2; Sigma), UBF (F9, Santa Cruz Biotechnology), nucleolin (MS-2, Santa Cruz Biotechnology), and cyclin B (GNS1, Santa Cruz Biotechnology), and rabbit polyclonal antibodies for Histone H3 (Abcam), and phosphorylated Histone H3 (S10) (Millipore). Anti-H2A/H2B antibody was generated in rabbits by injecting recombinant His-tagged H2A/H2B (20). Specific antibodies against histones H2A and H2B were purified from rabbit serum by H2A/H2B immobilized HiTrap NHS-activated HP column (GE Healthcare).

Immunoprecipitation

Cells expressing Flag-tagged B23.1 or its mutants were lysed and sonicated in buffer A (50 mM Tris-HCl (pH 7.9), 0.1% Triton X-100) containing 100 mM NaCl. Anti-Flag-tag M2 Affinity Gel (Sigma) was added to the lysate, and the mixture was incubated at 4°C for 1 hr. The gels were washed extensively with buffer A containing 200 mM NaCl, and the proteins bound to the gels were eluted with the buffer containing 0.1 mg/ml of FLAG peptide (Sigma). Eluted proteins were separate on 13% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by silver staining and western blotting. For experiments involving immunoprecipitation for the subsequent detection of RNA, cells were incubated in a buffer (10 mM Hepes-NaOH (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl) containing 0.1% Triton X-100, and the NaCl concentration was adjusted to 0.42 M. The extracts were recovered after extensive centrifugation (15 krpm for 20 min), and the NaCl concentration was diluted to 0.2 M. Immunoprecipitation experiments were carried out as described above. The RNAs bound to precipitated proteins were purified with phenol:chloroform extraction and ethanol precipitation. RNAs were separated on 6% denaturing PAGE in 1xTBE, and

visualized with Gel Red (Biotium) staining.

Northern blotting

RNAs were prepared by immunorecipitation using anti-Flag antibody from nuclear extract, separated on 6% denaturing PAGE, and transferred to nitrocellulose membranes. Northern blotting assays were carried out using Alkphos Direct Labeling Reagents (GE Healthcare). The templates of probes were amplified by PCR using gene specific primer sets.

Chromatin immunoprecipitation (ChIP) assays

Chromatin extracts were prepared from cells fixed with formaldehyde by extensive sonication. In all ChIP experiments, the length of DNAs extracted from cell lysate was between 200 and 500 bp. ChIP assays were carried out according to the manual for the ChIP assay kit (Millipore). Cell lysates prepared from 2 x 10^6 cells were used for an immunoprecipitation assay, with 1% of the input cell lysate utilized as the input DNA sample. Precipitated DNAs were suspended in 50 μl of water and used as templates for PCR. The primer sets used to amplify A and B regions on the human rRNA gene were as follows. For Region A; 5'-TGTGAATTGGATGGTGGCGTTTTTGGGGA-3' and 5'-CAGGCGGCTCGAGCAGGAGC-3', for Region B; 5'-CGACTCTTAGCGGTGGATCACTC-3' and 5'-AAGCGACGCTCAGACAGGCGT-3'. PCR products were separated using a 6% PAGE and were visualized by Gel Red staining. Quantitative PCR (Q-PCR) was carried out using FastStart Universal Master (ROX) (Roche) and primer sets corresponding to the rRNA gene. For Fig. 7, HeLa cells were fixed and subjected to ChIP assays at 72 hrs after siRNA transfection. For Q-PCR reactions, the previously described primer sets (14) were used. For re-ChIP assays, precipitated protein-DNA complexes in the 1st ChIP assay were eluted in 20 μl of ChIP elution buffer (1% SDS, 10 mM Tris-HCl (pH 7.9), and 1 mM EDTA) containing 10 mM DTT, diluted with

dilution buffer (0.1% SDS, 1.1% Triton X-100, 16.7 mM Tris-HCl (pH 7.9), 1.2 mM EDTA, and 167 mM NaCl), and used for 2nd ChIP assays.

Indirect immunofluorescence

For immunofluorescence analyses, all procedures were carried out at room temperature. HeLa cells grown on coverslips were fixed with 1% or 3% paraformaldehyde for 10 min. Cells were then permeabilized for 5 to 10 min in a buffer (300 mM Sucrose, 3 mM $MgCl_2$ in PBS) containing 0.5% Triton X-100, and incubated in PBS containing 0.5% milk and 0.1% Triton X-100 for 30 min. The fixed and permeabilized cells were incubated with primary antibody for 1 hr. The cells on coverslips were washed with PBS containing 0.1% Triton X-100 (PBST), and incubated with secondary antibodies (anti-mouse IgG Alexa555, Invitrogen) for 30 min. Cells were washed extensively with PBST and the DNA was stained with TO-PRO-3 (Invitrogen) for 5 min. All fluorescence images were captured by a confocal microscope (LSM 5 Exciter, Carl Zeiss) with Plan-Apochromat 63x NA 1.4 oil immersion objective lens. Images were cropped, sized, and arranged into panels using Adobe photoshop CS3 (Adobe Systems).

Cell fractionation

Cells washed with PBS were incubated in a hypotonic buffer (10 mM Hepes-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM PMSF) on ice for 10 min, centrifuged at 2000 rpm for 3 min, and the supernatant removed. Cell pellets were suspended in hypotonic buffer containing 0.1% (v/v) Triton X-100 and incubated on ice for 2 min following which the soluble proteins were recovered. The cell pellets were suspended in an SDS-sample buffer as insoluble fractions. Equal amounts of total, soluble, and insoluble fractions were separated by SDS-PAGE and analyzed by western blotting.

Plasmid construction

Plasmids pET-14b-B23.1, -B23.2 and -T4sA have been described previously (19, 22). To construct pET-14b-T199D, the cDNA fragments containing a mutation at T199 were generated using a 2-step PCR reaction. In the first PCR reaction, two DNA fragments containing the mutation were amplified using T7 promoter primer and 5'-ATCTATACGAGATGATCCAGCCAAAAATG-3', and the T3 promoter primer and 5'-CATTTTTGGCTGGATCATCTCGTATAGAT-3', with pBS-Flag-B23.1 as a template. These DNA fragments were then separated and purified from an agarose gel and used in the second PCR reaction as templates. The second PCR reaction was performed with T7 and T3 promoter primers. Full length mutated cDNA was digested with *Bam*HI and cloned into a *Bam*HI-digested pBS-Flag vector. pBS-Flag-T199D was digested with *Nde*I and *Bam*HI and then subcloned into *Nde*I- and *Bam*HI-digested pET-14b. T219D and T234/237D expression vectors were constructed with the same procedure. Primer sets for T219D and T234/237D were 5'-AAAACCATCATCAGATCCAAGATCAAAAG-3' and 5'-CTTTTGATCTTGGATCTGATGATGGTTTT-3', and 5'-CAGGAAAAAGATCCTAAAGATCCAAAAGGA-3' and 5'-TCCTTTTGGATCTTTAGGATCTTTTTCCTG-3', respectively. T219/234/237D and T4sD expression vectors were prepared by the same method using the appropriate primers (described above) and plasmid vectors as templates. To construct pET-14b-T95D, the primer set, 5'-AAATAGATCCACCAGTGGTCTTAAGG-3' and 5'-GGTGGATCTATTTCAAAGCCCCCAAG-3' was used along with pET-14b-B23.1 as a template. To construct S125D, S125A, S70D and S254D expression vectors, primer sets, 5'-CAGAGGATGAAGATGAAGAGGAGGAG-3' and 5'-TCTTCATCCTCTGCATCTTCCTCCAC-3', 5'-CAGAGGCAGAAGATGAAGAGGAGGAG-3' and 5'-TCTTCTGCCTCTGCATCTTCCTCCAC-3', 5'-AAGGCGATCCAATTAAAGTAACA-3' and 5'-ATTGGATCGCCTTCGTAATTCAT-3', and

5’-AAGCAGATATAGAAAAAGGTGGT-3’ and 5’-TCTATATCTGCTTGCATTTTTGC-3’, were used respectively. The plasmids for expressing S70/125D and S70/125/254D (S3sD) were constructed using the identical protocol with appropriate primer sets (described above) and plasmid DNA templates. For expression of EGFP-Flag and Flag-tagged proteins in HeLa cells, each cDNA attached by Flag-tag at its 5’ terminus was cut out from a pBS-Flag vector by digestion with *Bam*HI, and then subcloned into a *Bam*HI-digested pEGFP-C1 vector (CLONTECH) or a *Bgl*II-digested pCAGGS vector. The sequences of all plasmids were confirmed using an ABI Prism BigDye(R) Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems) with the appropriate primers.

Purification of recombinant proteins and biochemical analyses

For expression and purification of recombinant proteins, BL21 (DE3) was transformed with pET-14b plasmids containing B23.1 mutant cDNAs. B23 proteins were expressed and purified as previously described (19). For histone transfer assays, core histones were preincubated with His-tagged proteins in a buffer (20 mM Hepes-NaOH (pH 7.9), 10% glycerol, 0.4 mg/ml BSA, and 50 mM NaCl) at room temperature for 10 min, and then 20 ng of 147 bp-long DNA fragment containing 5S rRNA gene was added. The mixture was incubated at 37°C for 1 hr, and analyzed by 6% PAGE in 0.5 x TBE buffer. The gel was run at 14.3 V/cm for 90 min and DNA was visualized with Gel Red staining. Filter binding assays and super coiling assays were carried out as described previously (20, 21).

Examination of rRNA transcription level in siRNA treated cells

rRNA transcription levels were examined as described previously (14). At 24 hrs after siRNA transfection, plasmid DNA for expression of Flag-tagged B23.1 proteins was transfected using GeneJuice (Novagen). Cells were collected 72 hrs after siRNA transfection and RNA was purified from transfected cells with an RNeasy kit

(QIAGEN). Reverse transcription was carried out with ReverTraAce (Toyobo). The synthesized cDNA was used as a template for quantitative PCR (Q-PCR) using FastStart Universal Master (ROX) (Roche). Q-PCR reactions were carried out using Applied Biosystems 7500 Fast Real-Time PCR System. Primer sequences used for RT-PCR were described previously (14).

Results

B23.1 is released from chromatin during mitosis.

We previously demonstrated that B23 is involved in the regulation of rRNA transcription through its histone chaperone activity (14) - however how B23 is targeted to r-chromatin was unknown. As shown in Fig. 1A, B23.1 is localized at the nucleoli in interphase cells, whereas it is randomly located during mitosis. We first examined whether B23.1 is associated with r-chromatin during mitosis. Asynchronous and mitotic HeLa cells were fixed and ChIP assays were performed (Fig. 1B). In agreement with previous findings (14) we found that in asynchronous cells, B23.1 and UBF were associated with both the promoter (region A) and coding regions (region B) of the rRNA gene. In mitotic cell extracts, rRNA gene DNA fragments precipitated with anti-UBF were clearly observable (lane 8), whereas those precipitated with anti-B23 were not (lane 7), indicating that B23 was released from r-chromatin during mitosis. Considering that B23 is phosphorylated during mitosis by the cdc2/cyclin B complex and that this phosphorylation inactivates the RNA binding activity (22), we tested whether the RNA binding activity of B23.1 was related to its chromatin binding activity. Extracts treated without or with increasing concentrations of RNase A were prepared from HeLa cells stably expressing GFP-Flag-B23.1 and subjected to immunoprecipitation with anti-Flag-tag antibody (Fig. 1C). GFP-Flag-B23.1 co-immunoprecipitated with chromosomal histone H3 (lane 6), however the level of co-precipitated histone H3 was significantly diminished when the extracts were treated with low concentrations of RNase A (lanes 7-10). These results suggest that the RNA binding activity of B23.1 is important for its association with chromatin.

The RNA binding activity of B23.1 is required for its association with r-chromatin.

To test the above notion, we next examined whether the B23.1 mutant T4sA associated with RNA and chromatin in mitotic cells. T4sA is incapable of being

phosphorylated due to the fact that its four cdc2-target sites, T199, T219, T234 and T237 (22), are replaced with alanine. We previously demonstrated that T4sA was associated with RNA regardless of whether or not it was pre-treated with cdc2/cyclin B kinase *in vitro* (22). If the RNA binding activity of B23.1 is required for its association with chromatin, we supposed that T4sA would remain associated with chromatin during mitosis. Cell extracts derived from asynchronous or mitotic cells stably expressing either Flag-B23.1 or -T4sA were subjected to immunoprecipitation (Figs. 2A and B). From asynchronous cell extracts, it can be seen that B23.1 and T4sA co-precipitated with a variety of proteins including nucleolin and histone H3 (Fig. 2A, lanes 3 and 5, Fig. 2B, lanes 9 and 11). Due to the fact that the mobility shift of endogenous and Flag-tagged B23.1 in mitotic extracts was significantly retarded due to hyper-phosphorylation and the amount of histone H3 phosphorylated at serine 10 was increased (Fig. 2B, lanes 2, 4, and 6), we took care to make sure that extracts were taken from cells properly synchronized at pro-metaphase. The mobility shift of mitotic Flag-T4sA was minimal (compare lanes 5 and 6, and 11 and 12), indicating that this mutant was not efficiently phosphorylated during mitosis. Interestingly, we found that nucleolin and histone H3 proteins efficiently co-precipitated with Flag-T4sA from mitotic extracts (lane 12). In parallel, we analyzed RNAs co-precipitated with Flag-tagged proteins from mitotic extracts (Figs. 2C and D). Flag-B23.1 co-precipitated with 5.8S and 5S rRNAs from asynchronous cell extracts (Fig. 2C) as previously reported (2, 41). However, Flag-B23.1 did not efficiently precipitate the RNAs from mitotic extracts (Fig. 2D, lane 5). In contrast, two small rRNAs were co-precipitated with Flag-T4sA even from mitotic extracts (lane 6). These results strongly support the idea that the RNA binding activity of B23.1 is regulated by cdc2-mediated phosphorylation *in vivo*.

We next examined whether Flag-T4sA remains associated with the r-chromatin during mitosis by ChIP assays (Fig. 2E). In asynchronous cells, Flag-B23.1 and Flag-T4sA bound to r-chromatin (white bars). During mitosis, the affinity of both

Flag-B23.1 and Flag-T4sA with the r-chromatin was markedly decreased as endogenous B23 did so (Fig. 1B), although the association level of T4sA was slightly higher than that of wild type B23.1.

The level of association between T4sA and r-chromatin detected in mitotic cells was decreased in comparison to that measured in asynchronous cells (Fig. 2E), whereas the degree of association between T4sA and chromosomal histone H3 in asynchronous and mitotic cells was not significantly altered (Fig. 2B, lanes 11 and 12). To explore this apparent inconsistency, we examined the localization of Flag-T4sA in mitotic cells (Fig. 2F). Under the assay conditions employed, soluble proteins were extracted and proteins associating with the nuclear structure were retained. As previously reported (28) in pro-metaphase cells, Flag-B23.1 was detected around the chromosome periphery (Fig. 2F). In comparison with wild type B23.1, T4sA was found to be more concentrated around the chromosome periphery. To address this point more quantitatively, cell fractionation experiments were carried out (Fig. 2G). Cells expressing either Flag-B23.1 or -T4sA were fractionated into soluble and insoluble fractions and the proteins existing in each fraction were analyzed by western blotting. Endogenous B23 and nucleolin appeared mainly in the insoluble fraction in asynchronous cells (lanes 3 and 9). In mitotic cells, however, these proteins were mainly in the soluble fraction (lanes 5 and 11). Throughout the cell cycle, histone H3 was observed only in the insoluble fraction. The fractionation pattern of Flag-B23.1 was similar to that of endogenous B23 (lanes 1-6), whereas Flag-T4sA was present in both the soluble and insoluble fractions in mitotic cells (lanes 11 and 12). The r-chromatin termed NOR in mitotic cells was visualized by UBF staining, however the co-localization of NOR with Flag-B23.1 or -T4sA was not clear (Fig. 2F). Thus, it is quite likely that the chromosomal histone H3 which co-precipitates with Flag-T4sA from mitotic extracts is itself derived from entire chromosome. These results suggest that the RNA binding activity of B23.1 is a necessary condition for its association with chromatin but is, by itself, not sufficient for maintaining an association with

r-chromatin.

B23.1 mutant mimicking mitotic phosphorylation does not efficiently associate with RNA and chromatin in asynchronous cells.

We next examined the chromatin binding activity of a phosphomimetic mutant, termed T4sD, in which 4 cdc2 target sites were replaced with aspartic acids in asynchronous cells (Fig. 3). Flag-tagged B23.1, T4sA and T4sD were transiently expressed in 293T cells and immunoprecipitation was carried out (Figs. 3A-C). We found that Flag-B23.1 and Flag-T4sA bound to a variety of proteins including nucleolin and histone H3 (Figs. 3A and B), and Flag-B23.1 also bound to 5.8S and 5S rRNA (Figs. 3C). Conversely, chromosomal histone H3 and nucleolin did not efficiently co-precipitate with T4sD (Fig. 3B, lane 8). Additionally, the amount of 5S and 5.8S rRNAs co-precipitated with Flag-T4sD was less than half of that co-precipitated with wild type B23.1 (Fig. 3C, lane 6). We noted that a low but distinct level of RNA was co-precipitated with T4sD from cell extracts, although T4sD does not associate with RNAs in vitro (Fig. 4). This may be due to the fact that T4sD forms an oligomer with endogenous B23 (Fig. 3B). Indeed, B23.1 and B23.2 were equally precipitated with Flag-tagged B23.1, T4sA, and T4sD (data not shown). To investigate the association of T4sD with the r-chromatin, ChIP assays were performed (Fig. 3D). The results clearly demonstrated that the association level of T4sD with r-chromatin was lower than that of B23.1. Taken in total, we concluded from these data that the RNA binding activity of B23.1 is crucial for its chromatin binding activity.

Cdc2-mediated phosphorylation at 4 consensus sites of B23 inactivates its RNA binding activity.

We next tried to identify critical phosphorylation site(s) affecting the RNA binding activity of B23.1. To this end, we constructed a series of phosphomimetic mutants of B23.1. Since 4 threonine residues were known to be phosphorylated by cdc2 kinase in

mitosis, 1 to 4 threonine residues were substituted by aspartic acid (designated as T199D, T219D, T234/237D, T3sD (T219/234/237D) and T4sD). The RNA binding activities of these mutant proteins were examined by filter binding assay (Fig. 4A). RNAs alone or RNAs mixed with B23.2, lacking the RNA binding domain of B23.1, were not retained on the membrane, whereas RNAs mixed with B23.1 were retained on the membrane in a B23.1 dose-dependent manner. The RNA binding activity of T199D was similar to that of wild type B23.1, whereas that of T219D was slightly lower. The RNA binding activity of B23.1 was gradually reduced in response to an increasing number of phosphomimetic mutations. When all 4 threonine residues were replaced with aspartic acids, the RNA binding activity was dramatically reduced. Since B23 is known to be phosphorylated at several different sites, we also examined the effect of other phosphorylation sites on the RNA binding activity of B23.1. One putative site, threonine 95 (T95) within the nuclear export signal sequence has been suggested as a likely phosphorylation site (40). Another potential phosphorylation regulatory site, serine 125 (S125), has been previously shown to be phosphorylated by CK2 kinase (37). Filter binding assays demonstrated that the RNA binding activities of T95D and S125D were slightly decreased (Fig. 4B). Since the number of phosphorylation site was likely to be important for affecting the RNA binding activity of B23.1, we constructed mutant proteins having two or three phosphomimetic mutations. Because B23 at the mitotic spindle pole was found to be phosphorylated at 3 serine residues (S70, S125, S254) (17), we constructed phosphomimetic mutants, S70D, S254D, S70/125D, and S3sD (S70/125/254D) and compared their RNA binding activities with that of T3sD containing three mutations at the cdc2 consensus sites (Fig. 4C). The RNA binding activity of the serine mutants was gradually decreased upon an increasing number of mutations as was seen in the case of cdc2 consensus sites (Figs. 4A and C). Importantly, S3sD showed about a 2-fold higher activity than T3sD (*P*-value=0.0097, *t*-test). From these data we concluded that the inactivation of the RNA binding activity depends on the number and position of phosphorylation sites.

Chromatin and RNA binding activities of B23.1 are required for its maximal stimulatory activity of rRNA transcription.

So far, we have demonstrated that the RNA binding activity of B23.1 was required for its association with chromatin. To examine the biological significance of this finding, we next examined whether the chromatin binding activity of B23.1 was necessary for its stimulatory effect upon rRNA transcription. To address this point, we first examined the histone binding activity of wild type and T4sD mutant B23.1 by immunoprecipitation (Fig. 5A). Both wild type and T4sD B23.1 proteins were bound to histones similarly even in the increasing concentrations of NaCl (lanes 6-8 and 10-12, respectively). We next examined the histone chaperone function of the T4sD mutant via histone transfer assay and supercoiling assay (Figs. 5B and C). His-tagged B23.1, B23.2 and T4sD similarly transfer histones to 147 bp-long DNA fragment, and the assembled nucleosome core particles were observed (Fig. 5B). In supercoiling assays, when nucleosome is assembled on relaxed circular DNA, supercoil is induced in the plasmid DNA. NAP1, a well-characterized histone chaperone, efficiently assembled nucleosome and supercoil was introduced into the plasmid DNA (Fig. 5C, lanes 11-13). Nucleosome was assembled on the plasmid DNA with increasing amounts of His-B23.1 and His-B23.2 (lanes 1-7) in agreement with previous studies (21), although the activity of B23 proteins was lower than that of NAP1. T4sD also showed a similar level of nucleosome assembly activity (lanes 8-10, and see the densitometric analysis). These results indicated that phosphorylation at the four cdc2-consensus sites did not affect the histone chaperone activity of B23.1. We next examined the histone chaperone activity of T4sD *in vivo*. HeLa cells were treated with control or B23.1 siRNA, and then Flag-tagged B23.1 and its mutants were produced by transient transfection. Under these conditions, the expression level of B23.1 in cells treated with B23.1 siRNA was decreased by ~ one third of that in control cells (cf. lanes 1 and 2 in Fig. 5D). Total RNA was extracted and the amount of 45S pre-rRNA was quantified by Q-PCR using

the primer set specific for 5'-ETS (external transcribed sequence) (Fig. 5E). Since the 5'-ETS region of pre-rRNA is quickly processed *in vivo*, the amount of 45S pre-rRNA should be taken as reflecting the ongoing transcription activity of rRNA. When endogenous B23.1 was depleted, the amount of 45S pre-rRNA was decreased to 70% of that in control cells. We also found that the amounts of UBF and the p194 subunit of RNA polymerase I were not significantly decreased by B23 depletion (data not shown). Decreased transcription level of pre-rRNA by B23 depletion is therefore likely to be the decreased RNA polymerase processivity by increased histone density along the r-chromatin (Fig.7 and (14)). However, pre-rRNA transcription level was recovered by the expression of Flag-B23.1 and -T4sA (Fig. 5E). In contrast, the expression of T4sD did not rescue the rRNA transcription, although T4sD showed the potential histone chaperone activity *in vitro* (Figs. 5A-C). Therefore, we concluded that the RNA binding activity of B23.1 is required for its correct targeting to the r-chromatin and that association of B23.1 with the r-chromatin plays a crucial role in achieving maximal stimulatory activity of rRNA transcription *in vivo*.

Nascent RNAs are dispensable for the recruitment of B23 on the r-chromatin.

We next explored which molecule(s) was involved in the recruitment of B23.1 to the r-chromatin. Because B23 associates with mature rRNAs (Fig. 2C), we hypothesized that B23 is recruited to r-chromatin via its association with nascent pre-rRNAs. In fact, we found that B23 also bound to pre-rRNA in cells (data not shown). Therefore, we examined whether the association of B23.1 with nascent pre-rRNA was required for its recruitment to r-chromatin. B23.1 is released from the r-chromatin during mitosis, with the association being restored 2 hrs after exit from mitosis (Fig. 6D, lanes 5 and 6). We examined the effect of pol I transcription initiation following mitosis on the recruitment of B23.1 to the r-chromatin. Mitotic HeLa cells were released for 2 hrs with or without low concentrations of Act D, and the extent of B23 association with the r-chromatin was examined using a ChIP assay. Low concentrations of Act D

specifically inhibits the pol I transcription (Fig. 6B). In addition, the nucleolar structure was not properly assembled and B23 was distributed throughout the nucleus with forming small foci (Fig. 6C). It should also be noted that distinct amounts of pre-rRNA remained during mitosis and the pre-rRNA level was decreased when cells underwent the entry into G1 phase, suggesting that pre-rRNA processing was not inhibited by Act D (Fig. 6B). ChIP assays demonstrated that the association of B23.1 with r-chromatin was recovered even in the presence of Act D (Fig. 6D, lane 9). Thus, we concluded that the newly synthesized pre-rRNA is not required for the recruitment of B23.1 to r-chromatin, although we cannot exclude the possibility that pre-rRNAs synthesized during the previous cell cycle and located at the r-chromatin may play a role in recruitment of B23.1 to the r-chromatin after re-entry into the new G1 phase. Next, we examined whether B23 is recruited to r-chromatin with RNAs transcribed by RNA polymerase II (Pol II). HeLa cells were cultured for 24 hrs in the absence or presence of α-amanitin, and ChIP assays were carried out. RT-PCR demonstrated that the amount of U1 snRNA transcribed by pol II was specifically reduced (Fig. 6E). Under this condition, ChIP assays demonstrated that both UBF and B23 remained associated with r-chromatin (Fig. 6F, lanes 3 and 4, and 7 and 8). These results suggest that the nascent RNAs are not required for the recruitment of B23 to r-chromatin.

UBF is involved in the recruitment of B23 on the r-chromatin.

Since UBF was found to be associated with the entire rRNA gene (18) and has previously been reported to be necessary for recruiting factors involved in linking rRNA transcription and processing (24), we next focused on the function of UBF in the recruitment of the B23-RNA complex to r-chromatin. To examine this point, ChIP assays were performed with control or UBF siRNA-treated HeLa cells (Fig. 7). The expression level of UBF protein in cells treated with UBF siRNA was reduced to less than 25% of the control siRNA treated cells, while those of B23 and histone H3 were

not changed (Fig. 7A). The enrichment of DNA fragments containing the rRNA genes were quantitatively analyzed by Q-PCR with several primer sets extending across the entire rRNA gene being employed (Figs. 7B-F). UBF was apparently enriched around the promoter and coding regions of the rRNA gene in control siRNA treated cells. UBF siRNA treatment significantly decreased the association level of UBF to the r-chromatin (Fig. 7B). B23 bound to the entire rRNA genes in control siRNA treated cells, whereas the B23 association was decreased by UBF siRNA treatment (Fig. 7C). This result indicated that UBF is involved in the recruitment of B23 to the r-chromatin. Additionally, we examined the effect of UBF siRNA treatment on the histone density around the r-chromatin that was shown to be regulated by B23 (14). Histone H2A/H2B and H3 were distributed evenly across the r-chromatin in control siRNA treated cells. Contrary to the B23 association level, histone density on r-chromatin was evenly increased by UBF siRNA treatment (Figs. 7D and E). The effect of UBF knockdown on the distribution pattern of B23 and histones around the r-chromatin was shown in Fig. 7F. These results suggest that UBF plays an important role in the recruitment of B23 to r-chromatin. In order to confirm this notion, we performed sequential ChIP assays (Fig. 7G). ChIP assays were first carried out with anti-UBF antibody, and the UBF containing complex was subsequently subjected to the second ChIP assays using anti-B23 and anti-UBF antibodies. Both on the regions A and B, we detected the co-localization of B23 and UBF (lane 5). The co-localization level of B23 and UBF on region A was higher than that of region B (lanes 5 and 6). These results strongly suggest that B23 is recruited to the r-chromatin through the joint actions of its own RNA binding activity and UBF's ability to regulate the histone density and thereby rRNA transcription level.

Discussion

Here we have shown that the r-chromatin binding of histone chaperone B23 is dependent on B23's RNA binding activity and is required for its stimulatory function of rRNA transcription. This conclusion was drawn from the following results; (i) the chromatin binding activity of B23.1 in cell extracts is sensitive to RNase treatment (Fig. 1C), (ii) T4sA, a mutant of B23.1 in which the RNA binding activity is not influenced by cdc2-mediated phosphorylation remains bound to chromatin during mitosis (Fig. 2), and (iii) T4sD, a mutant mimicking the mitotic phosphorylated state of B23.1, does not efficiently associate with RNA and r-chromatin (Fig. 3). The chromatin association of B23 was shown to be necessary for it to exert its histone chaperone activity in cells (Fig. 5). In addition, we demonstrated that recruitment of B23.1 to the r-chromatin depends not only upon its RNA binding activity but also on the presence of UBF. Our results suggest a novel regulatory mechanism for conferring target gene specificity to histone chaperones. The other important conclusion in this study is that the histone chaperone activity of B23.1 is indirectly regulated by cdc2-mediated phosphorylation during the cell cycle.

RNA molecules required for B23 recruitment to the r-chromatin.

We demonstrated that B23.1 is recruited to r-chromatin in an RNA binding activity-dependent manner. We found that nascent pre-rRNA was dispensable for the recruitment of B23 to r-chromatin (Fig. 6). In mitotic cells, since not only pol I transcription but also pre-rRNA processing is suggested to be inactivated, we cannot exclude the possibility that pre-rRNA synthesized during the previous cell cycle helps to recruit B23.1 to the r-chromatin. Another possibility is that mature rRNAs function as B23.1-recruiting RNAs. Several lines of evidence support this idea: (i) B23.1 associates with 5.8S and 28S rRNAs *in vitro* (22), (ii) Flag-tagged B23.1 was co-immunoprecipitated with mature rRNAs from cell extracts (Figs. 2C and 3C), and

(iii) endogenous B23 was co-precipitated with rRNAs from cell extracts (2, 35, 41). It is also possible that a minor population of non-ribosomal RNA is involved in the recruitment of B23.1. In this context, we examined the effect of pol II transcription inhibition on the association of B23 with r-chromatin (Fig. 6). However the association level of B23 with r-chromatin was not significantly reduced at least 24 hrs of pol II transcription inhibition. Clarification of the RNA binding specificity of B23.1 will provide a clue to determine which RNA molecule is required to recruit the B23 histone chaperone to the r-chromatin.

We have previously demonstrated that purified recombinant B23.1 did not show efficient nucleosome disassembly activity *in vitro* (20). However, the depletion of B23.1 increased the histone density around the rRNA gene in a histone chaperone activity-dependent manner (14). Thus, complex formation with RNA molecules may be required not only for the r-chromatin binding but also for stimulating the B23 histone chaperone function. In addition, given that acetylation of B23 enhances its histone chaperone activity (36), it would be interesting to examine the chromatin remodeling function of acetylated B23 in combination with its RNA binding activity.

Cell cycle-dependent regulation of histone chaperones.

In addition to the inactivation of the pre-rRNA processing pathway, an important consequence of mitotic phosphorylation of B23 may be the inactivation of rRNA transcription through r-chromatin regulation. During mitosis, most of genes are silenced and chromatin structure is highly condensed to ensure proper chromosome segregation. Silencing of rRNA transcription during mitosis by the phosphorylation-mediated inactivation of pol I machinery could be crucial for repetitive rRNA genes segregation (8). The inactivation of the histone chaperone function of B23 may be in part a mechanism for inactivation of rRNA transcription during mitosis. It was recently reported that rRNA gene clusters distributed throughout 10 chromosomes in diploid human cells are recombination hot spots for a variety of solid

tumors (34). Thus the rigorous regulation of B23-RNA complex formation in mitosis may be very important for maintaining the genome stability.

UBF function in recruiting the B23-RNA complex to the r-chromatin.

We demonstrated that the RNA binding activity of B23.1 is essential but not sufficient for its r-chromatin binding as shown in Fig. 2. UBF was found to be required for the recruitment of B23 to the r-chromatin to co-localize preferentially at the promoter region of rRNA gene (Fig. 7). However, the distribution patterns of UBF and B23 along the r-chromatin were different (Figs. 7B and C) and the direct interaction between UBF and B23 was not detected *in vitro* (data not shown). Thus, it is suggested that UBF recruits the B23 complex indirectly. Three possibilities exist for UBF's role in the recruitment of B23.1 to the r-chromatin. The first possibility is that the r-chromatin structure attracts the B23-RNA complex. It has been established that UBF binds to and then subsequently bends the r-chromatin via its HMG boxes to create a nucleosome-like structure (32). The r-chromatin structure associated with UBF may recruit the B23-RNA complex. The second possibility is that UBF removes the inhibitory factor for the B23-RNA complex assembly from r-chromatin. It was recently reported that UBF depletion increased the level of linker histone H1 on the r-chromatin (11, 26). Since HMG box proteins and histone H1 are suggested to associate with chromatin in a mutually exclusive manner (9), histone H1-bound chromatin may restrict the access of the B23-RNA complex. The third possibility is that UBF is needed for tethering the factor(s) that recruit B23 to the r-chromatin. Further analysis is required to distinguish these different possibilities.

In summary, here we have demonstrated that the histone chaperone B23.1 is recruited to the r-chromatin through its RNA binding activity and B23 recruitment is regulated by its cell cycle-dependent phosphorylation. This discovery represents a novel regulatory mechanism for targeting histone chaperones to specific genes. It was

recently reported that B23.1 is recruited to the specific gene transcribed by RNA polymerase II outside the nucleolus (30). These observations prompted us to speculate that B23.1 is differentially recruited to the different chromosome loci by changing the partner RNA molecules, implying that RNA molecules may specify the chromosome loci to be remodeled by B23.1. We feel that this hypothesis is noteworthy as it represents a new role for RNA participation in the chromatin regulation.

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Figure legends

Fig. 1. B23.1 is released from chromatin during mitosis

(A) Localization of GFP-Flag-B23.1. HeLa cells stably expressing GFP-Flag-B23.1 grown on coverslips were fixed with 3% paraformaldehyde and the localization of the protein was observed under confocal microscope. DNA was counterstained with TO-PRO-3. Bar under the panel indicates 10 μm. (B) ChIP assays of endogenous B23. Asynchronous and mitotic HeLa cells were subjected to ChIP assays using anti-Flag-tag as a control, anti-B23, and anti-UBF antibodies. The input (lanes 1 and 5) and co-immunoprecipitated (lanes 2-4 and 6-8) DNA were purified, and the DNA fragment harboring rRNA gene promoter (Region A; nucleotide positions between 42847-117 relative to transcription start site (+1)) and coding (Region B; 6623-6779) regions (top and middle panels, respectively) were amplified by PCR. PCR products were analyzed by 6% PAGE, and visualized with Gel Red staining. The amounts of PCR products amplified from input DNA were set as 1.0, and the enrichment was shown at the bottom of the panel. The positions of primers on rRNA gene are schematically represented at the bottom. (C) Immunoprecipitation of GFP-Flag-B23.1 with RNase treated cell extracts. HeLa cells expressing GFP-Flag-B23.1 were treated without (lanes 1 and 6) or with 1, 10, 100, 1000 μg/ml of RNase A (lanes 2-5 and 7-10). GFP-Flag-B23.1 was immunoprecipitated with anti-Flag-tag antibody, and separated by SDS-PAGE followed by western blotting with anti-B23.1 and -histone H3 antibodies.

Fig. 2. Mitotic phosphorylation of B23.1 is required for the inactivation of its chromatin and RNA binding activities

(A), (B) Immunoprecipitation of Flag-tagged proteins. Cell extracts were prepared from asynchronous (I) or mitotic (M) HeLa cells and HeLa cells expressing either Flag-B23.1 or -T4sA, and immunoprecipitation using anti-Flag-tag antibody was carried out. Immunoprecipitated proteins were separated by SDS-PAGE and detected

by silver staining (A) and western blotting (B). Input and immunoprecipitated proteins as in (A) (lanes 1-6 and 7-12, respectively) were analyzed by western blotting using anti-B23, -nucleolin, -histone H3 S10P (histone H3 phosphorylated at serine 10) and -histone H3 antibodies. Western blotting with anti-Cyclin B antibody was used as a mitotic marker (second panel). (C) RNAs co-precipitated with Flag-B23.1. Nuclear extracts were prepared from control and Flag-B23.1 expressing HeLa cells and immunoprecipitation with anti-Flag-tag antibody was carried out. The input and co-immunoprecipitated RNA was purified, separated on 6% denaturing PAGE, and analyzed by northern blotting with 5S and 5.8S rRNA gene probes (left and right panels, respectively). (D) RNA binding activity of B23.1 and T4sA during mitosis. Cell extracts were prepared from mitotic HeLa cells expressing either Flag-B23.1 or -T4sA as described in materials and methods, and immunoprecipitation with anti-Flag-tag antibody was carried out. Co-immunoprecipitated RNAs were purified, separated on 6% denaturing PAGE, and detected with Gel Red staining. Lane M indicates RNA molecular markers. (E) Cell cycle-dependent association of B23.1 and T4sA with the rRNA gene. Asynchronous and mitotic HeLa cells expressing either Flag-B23.1 or -T4sA were subjected to ChIP assays using control IgG and anti-Flag-tag antibody. Co-immunoprecipitated and input DNA was used as templates for Q-PCR using the primer sets for both regions A and B. The amount of amplified DNA precipitated with control IgG was set as 1.0, and the relative enrichment level of the DNA fragments by anti-Flag-tag immunoprecipitation are shown. White and black bars in the graph show the results of ChIP assays with asynchronous and mitotic cells, respectively. Q-PCR reactions were carried out with triplicate. (F) Localization of B23.1 and T4sA in mitotic cells. HeLa cells expressing either Flag-B23.1 or -T4sA grown on coverslips (left and right panels, respectively) were fixed in 1% paraformaldehyde and Flag-tagged proteins (green) and UBF (red) were detected by specific antibodies. DNA was counterstained with TO-PRO-3 (blue). Localization of proteins was observed under confocal microscope. Bar under the panels indicates 10 μm. (G) Extractability of

B23.1 and T4sA in asynchronous and mitotic cells. Asynchronous and mitotic (lanes 1-3 and 7-8, and 4-6 and 10-12, respectively) HeLa cells expressing either Flag-B23.1 or -T4sA were fractionated to soluble and insoluble fractions. Total cell extracts, soluble, and insoluble fractions (indicated by T, S, and I, respectively) were separated by SDS-PAGE and analyzed by western blotting using antibodies against nucleolin, Flag-tag, B23.1 and histone H3.

Fig. 3. Phosphomimetic B23.1 mutant does not efficiently associate with chromatin

(A), (B) Immunoprecipitation of Flag-tagged B23.1 proteins. 293T cells were transfected with empty vector or vectors for expression of Flag-B23.1, -T4sA, and -T4sD (lanes 1-4, respectively), and immunoprecipitation was carried out with anti-Flag-tag antibody. Precipitated proteins were separated by SDS-PAGE and detected with silver staining (A) or western blotting (B). Western blotting was carried out with anti-Flag-tag, -nucleolin, -B23, and -histone H3 antibodies. (C) RNA binding activity of T4sD in 293T cells. Cell extracts were prepared as described in Materials and methods. RNAs co-immunoprecipitated with Flag-tagged proteins were separated on 6% denaturing PAGE and visualized with staining by Gel Red. Lane M indicates RNA molecular markers. (D) r-chromatin association of B23.1 and T4sD. Flag-B23.1 or -T4sD was transiently expressed in 293T cells, and ChIP assays using anti-Flag-tag antibody (0.5 and 1.0 μg) were carried out. Anti-Myc-tag antibody (1 μg) was used as a control. Precipitated and input DNA was used as templates for PCR with specific primer sets (Region A and B). PCR product from control immunoprecipitation was set as 1.0, and the relative enrichment level of DNA fragment by anti-Flag-tag immunoprecipitation were indicated at the bottom and graphically represented. Gray and black bars in the graphs show the results of ChIP assays with Flag-B23.1 and -T4sD, respectively.

Fig. 4. Phosphorylation of 4 cdc2-consensus sites of B23 is crucial for efficient

suppression of its RNA binding activity

(A) RNA binding activity of B23 proteins containing mutations at cdc2 consensus sites. B23.1, B23.2, T199D, T219D, T234/237D, T3sD, and T4sD proteins (lanes 1-7, respectively) (200 ng) were separated by SDS-PAGE and visualized by CBB staining (left panel). T3sD and T4sD are B23.1 mutants in which T219, T234, and T237 and all 4 threonines (T), respectively, are replaced with aspartic acids (D). Purified proteins (50, 100, 200, and 400 ng) were mixed with ^{32}P-labeled HeLa cell total RNA (10 ng). The mixture was incubated and then filtered though nitrocellulose membranes. The membranes were extensively washed, and RNA retained on the membrane was detected with image analyzer (middle panels). The intensity of each spot was analyzed, and the RNA binding activity obtained with the same amount of B23 proteins were first calculated relative to that of B23.1 (1.0). Then the relative RNA binding activity at each protein amount was averaged. Means ± standard deviation (SD) obtained from twice of duplicate independent experiments are shown (center and right panels). (B) The effects of T95 and S125 phosphorylation on the RNA binding activity of B23.1. Purified proteins, B23.1, B23.2, T95D, S125A, and S125D (200 ng) were separated by SDS-PAGE (lanes 8-12, respectively) and visualized with CBB staining. The RNA binding activity of B23 mutant proteins was examined by filter binding assays as in (A). (C) The effects of the position and number of phosphorylation sites on the RNA binding activity of B23.1. Purified proteins, B23.1, B23.2, S70D, S125D, S254D, S70/125D, S3sD (S70/125/254D), and T3sD (T219/234/237D) (200 ng) were separated by SDS-PAGE (lanes 13-20, respectively) and visualized with CBB staining. The RNA binding activity of each protein was examined as in (A). For all experiments, statistical *P*-value was calculated by *t*-test and indicated with '*' for $P<0.05$ and '**' for $P<0.01$.

Fig. 5. RNA binding activity of B23.1 is required to facilitate rRNA transcription

(A) Histone binding activity of recombinant proteins. Recombinant B23.1 or T4sD (1

μg) was preincubated without (lanes 5 and 9) or with core histones (300 ng, lanes 6-8 and 10-12), and immunoprecipitation was carried out with anti-B23.1 antibody. Bound proteins were washed in a buffer containing 150 mM (lanes 4-6 and 9-10), 250 mM (lanes 7 and 11), or 400 mM (lanes 8 and 12) NaCl, and were separated by SDS-PAGE followed by silver staining. The asterisks indicate bands derived from the antibody. (B) The histone transfer activity of B23 proteins. Core histones (72 ng) were preincubated without (lane 1) or with 50 (lanes 2, 5, and 8), 150 (lanes 3, 6, and 9), or 450 (lanes 4, 7, and10) ng of recombinant B23.1, B23.2, or T4sD (lanes 2-4, 5-7, and 8-10, respectively), and then mixed with the 147 bp-long DNA fragment and further incubated. The mixtures were separated on 6% PAGE and DNA was visualized with Gel Red staining. (C) Supercoiling assays. B23.1, B23.2, T4sD, and NAP1 proteins were separated by SDS-PAGE and visualized by CBB staining (left panel). Core histones (1.8 pmol) were preincubated without (lane 1) or with 1.8 (lanes 2, 5, 8 and 11), 5.4 (lanes 3, 6, 9 and 12), or 16.2 (lanes 4, 7, 10 and13) pmol of recombinant B23.1, B23.2, T4sD, or NAP1 (lanes 2-4, 5-7, 8-10, or 11-13, respectively) and then mixed with plasmid DNA preincubated with topoisomerase I, and further incubated. DNA was purified and separated on a 1% agarose gel and visualized with Gel Red staining. Positions of relaxed (R) or supercoiled (S) circular plasmid DNA are indicated. The band intensity of lanes 4, 7, 10, and 13 were quantified and plotted (right panel). (D) Expression level of endogenous and exogenous B23 proteins. HeLa cells were transfected with control or B23.1 siRNAs. The cells were super-transfected 24 hrs after siRNA transfection with empty vector or vectors expressing either Flag-B23.1, -T4sA, or -T4sD. An equal number of cells was collected 72 hrs after siRNA transfection and subjected to western blotting with anti-Flag-tag, -B23.1, and -histone H3 antibodies. (E) rRNA transcription level of siRNA-treated cells. Total RNA was isolated from 7 x 10^5 cells prepared as in (D), and the rRNA transcription level was examined by quantitative RT-PCR using 5'-ETS-specific primer set. The amount of pre-rRNA was normalized by that of β-actin mRNA. Results are means with ± SD

obtained from three independent experiments. Statistical *P*-value is calculated by *t*-test and indicated with '*' for $P < 0.05$.

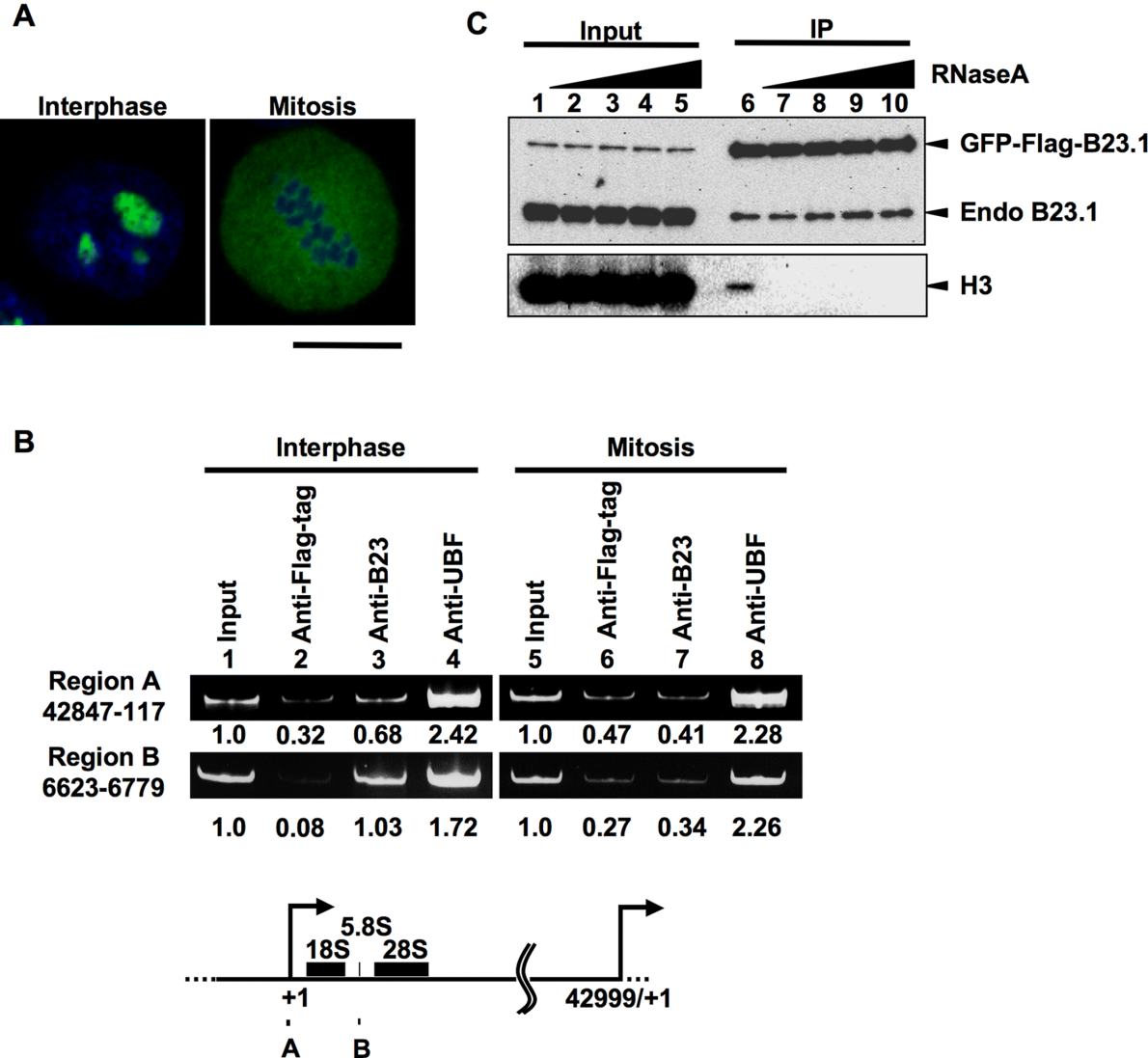
Fig. 6. Nascent rRNA is not crucial for the recruitment of B23-RNA complex to r-chromatin

(A)-(C) Effect of Act D on the rRNA transcription. HeLa cells were synchronized at mitosis and released for 2 hrs in growth medium in the absence or presence of 50 ng/ml Act D (see panel A). Total RNAs were purified and the rRNA transcription level was examined by quantitative RT-PCR using 5'-ETS-specific primer set (B). The amount of pre-rRNA was normalized by that of β-actin mRNA. Results are means with ± SD obtained from three independent experiments. Statistical *P*-value is indicated with '**' for $P < 0.01$. HeLa cells prepared as in (A) were fixed and subjected to immunofluorescence analysis with anti-B23.1 and anti-UBF antibodies (C). DNA was counterstained with TO-PRO-3. Bar at the bottom indicates 5 μm. (D) Effect of rRNA transcription initiation on the recruitment of B23 to r-chromatin. HeLa cells prepared as in (A) were subjected to ChIP assays using anti-Flag-tag and -B23 (lanes 4-6 and 7-9, respectively) antibodies. Precipitated (lanes 4-9) and input (lanes 1-3) DNA was used for PCR with region A and B primer sets. PCR products were analyzed on 6% PAGE and visualized with Gel Red staining. (E) Effect of α-amanitin on transcription. HeLa cells were cultured in the absence or presence of 5 μg/ml of α-amanitin for 24 hrs, and total RNA was isolated from 4.5×10^5cells. The amounts of pre-rRNA and U1 snRNA were examined by RT-PCR. PCR products were analyzed on 6% PAGE, visualized with Gel Red staining. (F) Effect of pol II inhibition on the association of B23 with r-chromatin. HeLa cells prepared as in (E) were subjected to ChIP assays using control IgG, anti-B23, and -UBF (lanes 2 and 6, 3 and7, and 4 and 8) antibodies. Precipitated and input DNA was amplified by PCR with the region A primer set.

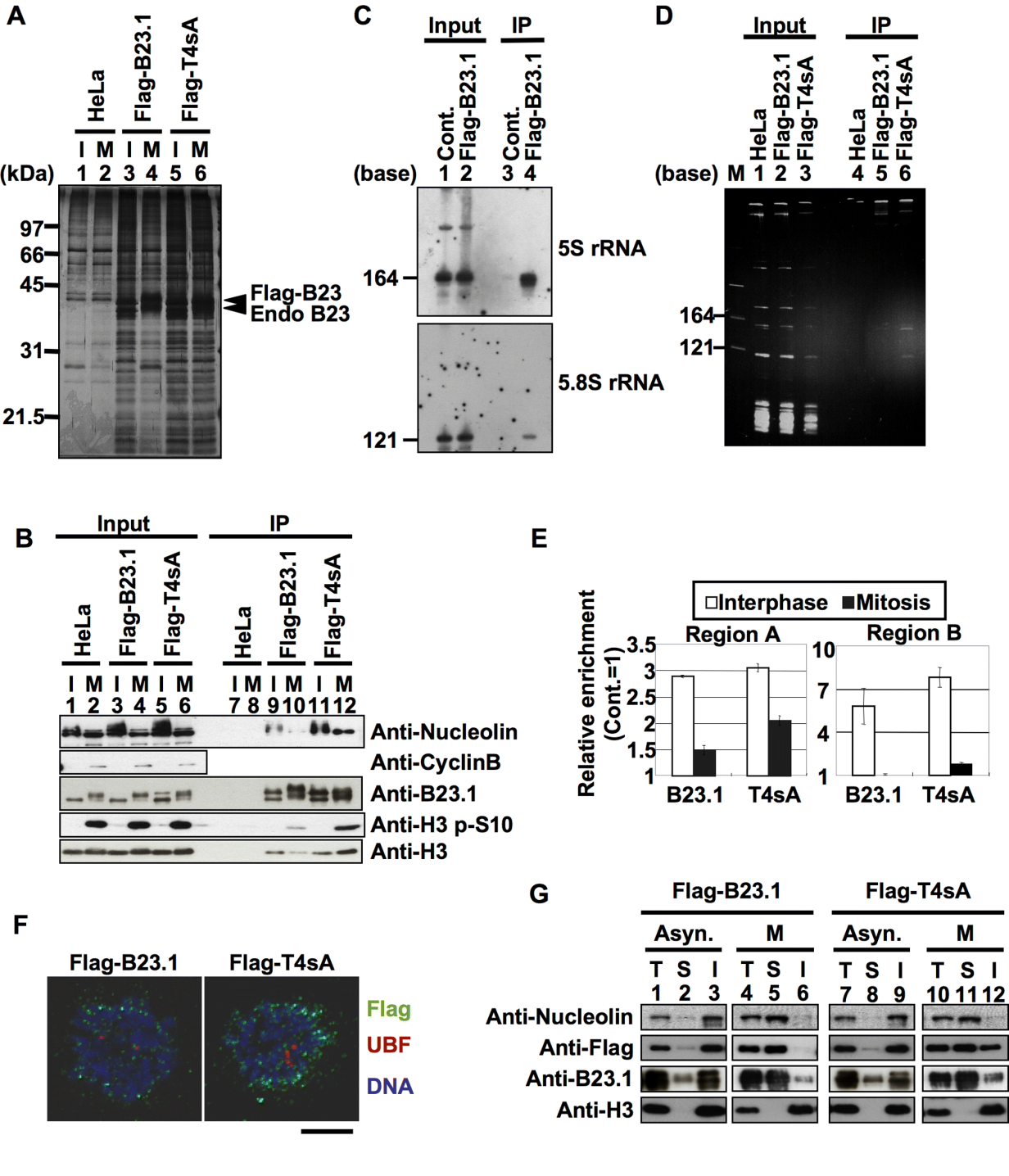
Fig. 7. UBF plays a crucial role in the recruitment of B23-RNA complex to the r-chromatin

(A) Expression level of UBF, B23.1, and histone H3 proteins in siRNA-treated HeLa cells. HeLa cells were transfected with control and UBF siRNAs (lanes 1-3 and 4-6, respectively). Cells were fixed 72 hrs after siRNA transfection and cell extracts were prepared. Increasing amounts of cell extracts were subjected to western blotting with anti-UBF, -B23, and -histone H3 antibodies. (B-F) Effect of UBF knockdown on the distributions of B23.1 and histones along the r-chromatin. Cell lysates prepared as in (A) were subjected to ChIP assays using anti-UBF (B), -B23 (C), -histone H2A/H2B (D), and -histone H3 (E) antibodies. Anti-Myc-tag antibody was used as a control. Precipitated DNA and input DNA were used as templates for Q-PCR using primer sets for the rRNA gene. The enrichment level of amplified DNA is shown as relative amount to input DNA (B-E). The X-axis of the graphs corresponds to the position along the 43 kbp-long rRNA genes shown schematically at the bottom. Blue and red lines in the graphs (B-E) show the results of ChIP assays with control and UBF siRNA-treated cells, respectively. Q-PCR reactions were carried out with triplicate. The amounts of B23.1, histone H2A/H2B, and histone H3 along the r-chromatin in UBF siRNA treated cells were estimated relative to those in control siRNA treated cells (F). Dotted lines show the average of change. (G) Co-localization of UBF and B23 on r-chromatin. Fixed HeLa cells were subjected to ChIP assays using control IgG and anti-UBF antibody (primary ChIP, lanes 2 and 3). The UBF containing complex was subjected to second immunoprecipitation with control IgG, anti-B23, and anti-UBF (lanes 4-6) antibodies. Precipitated (lanes 2-6) and input (lane 1) DNA was amplified by PCR. PCR products were separated by 6% PAGE and visualized with Gel Red staining.

Hisaoka, M. et al. Figure 1



Hisaoka, M. et al. Figure 2



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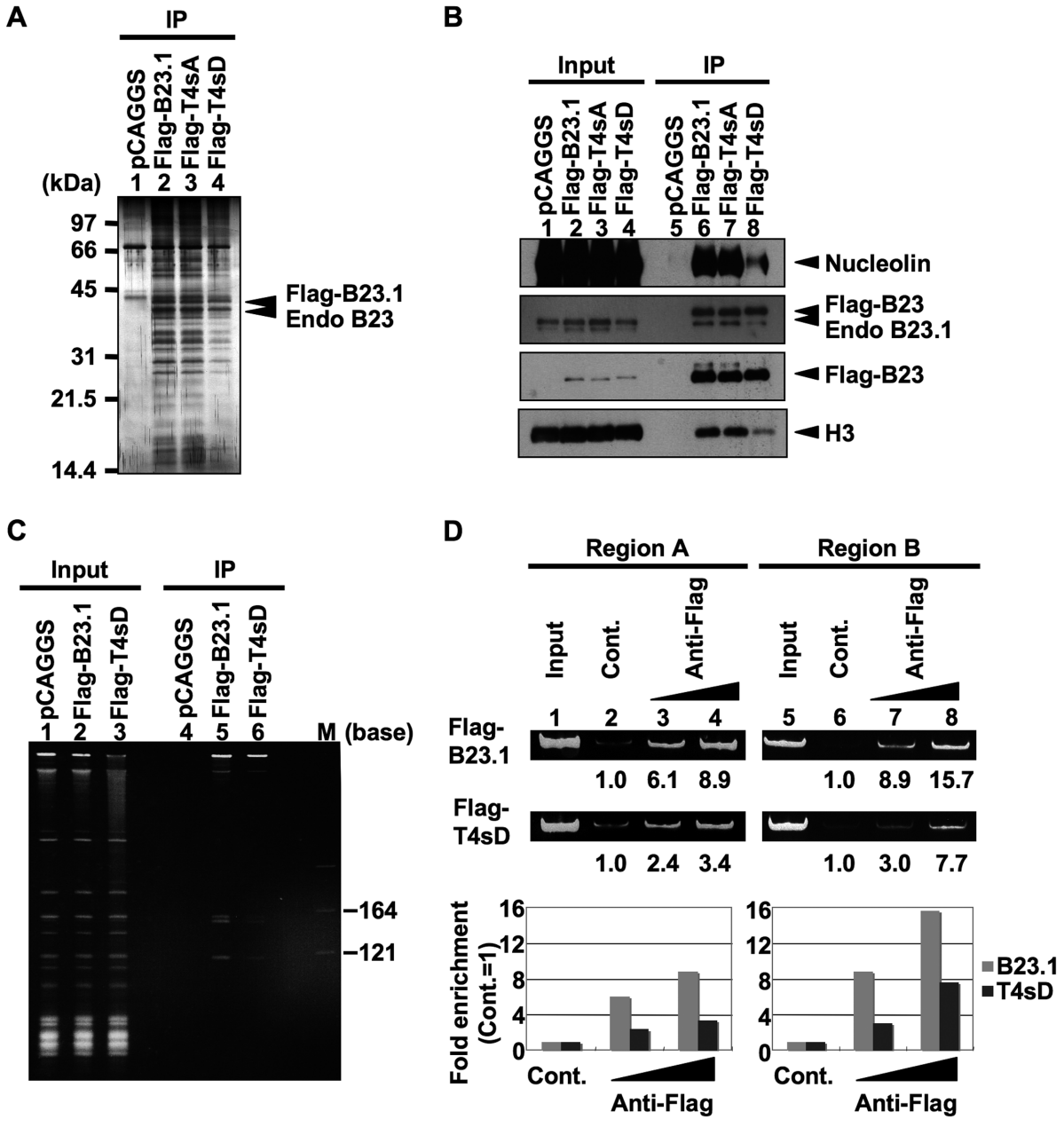
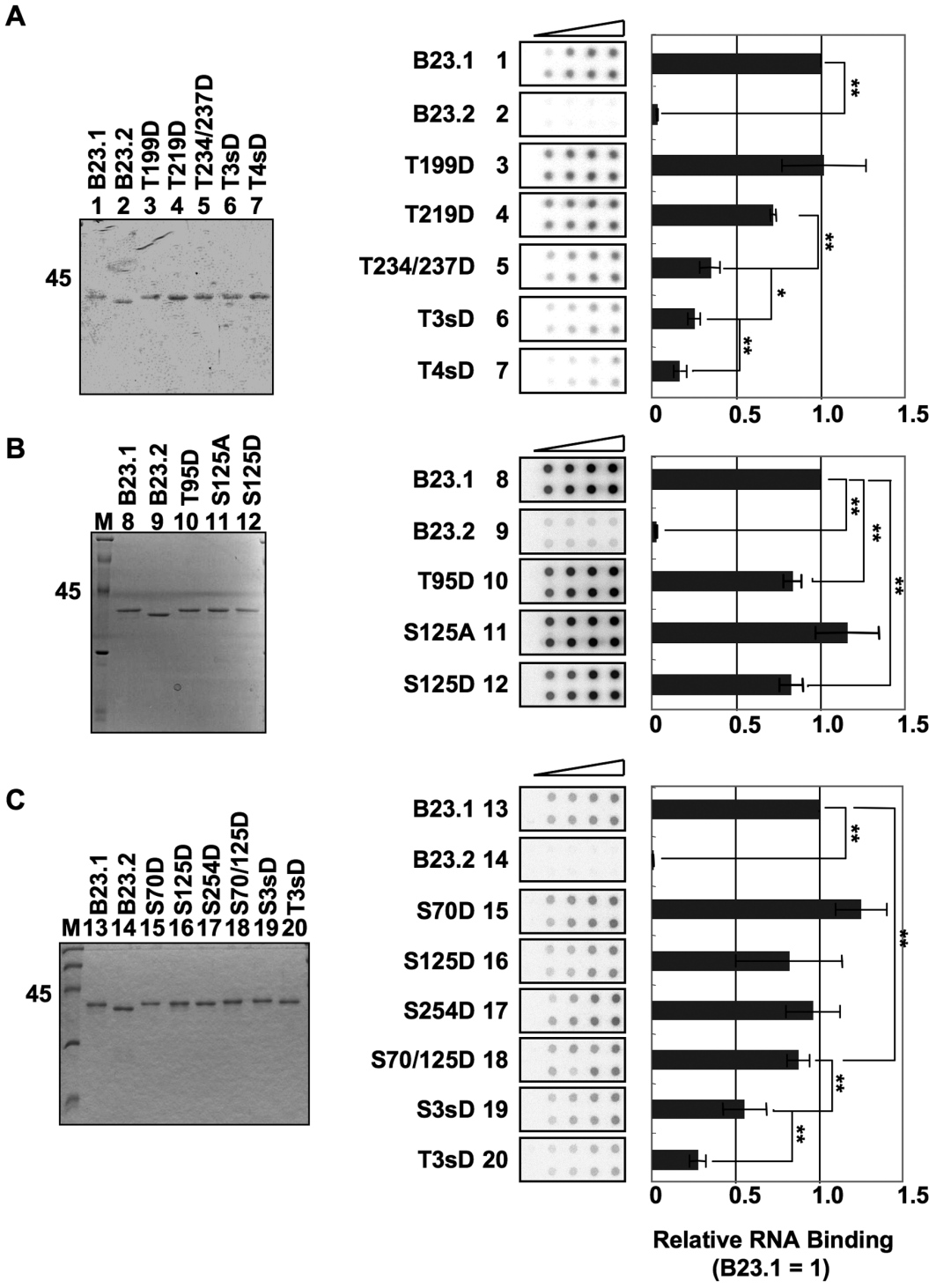


Hisaoka, M. et al. Figure 4



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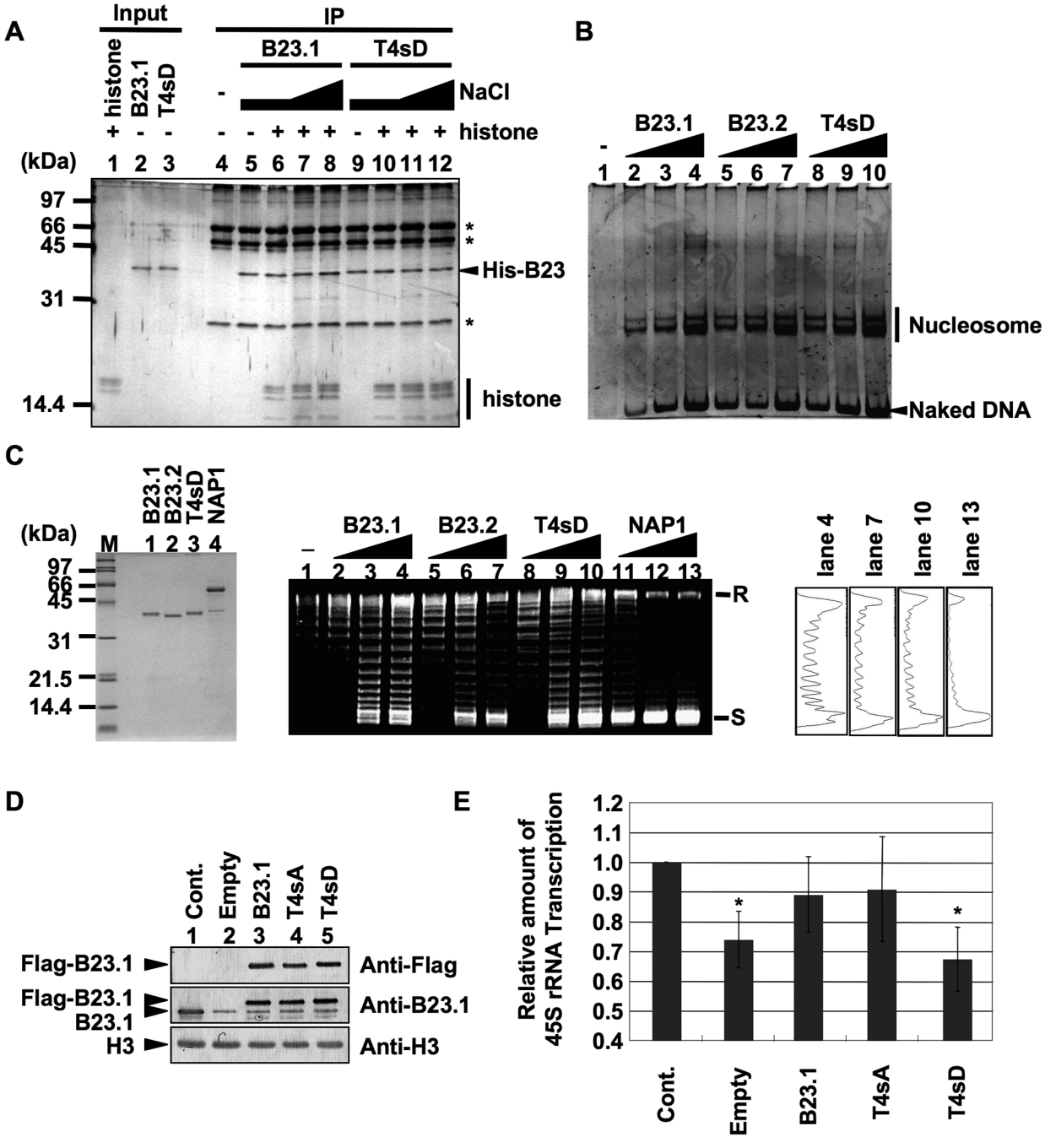
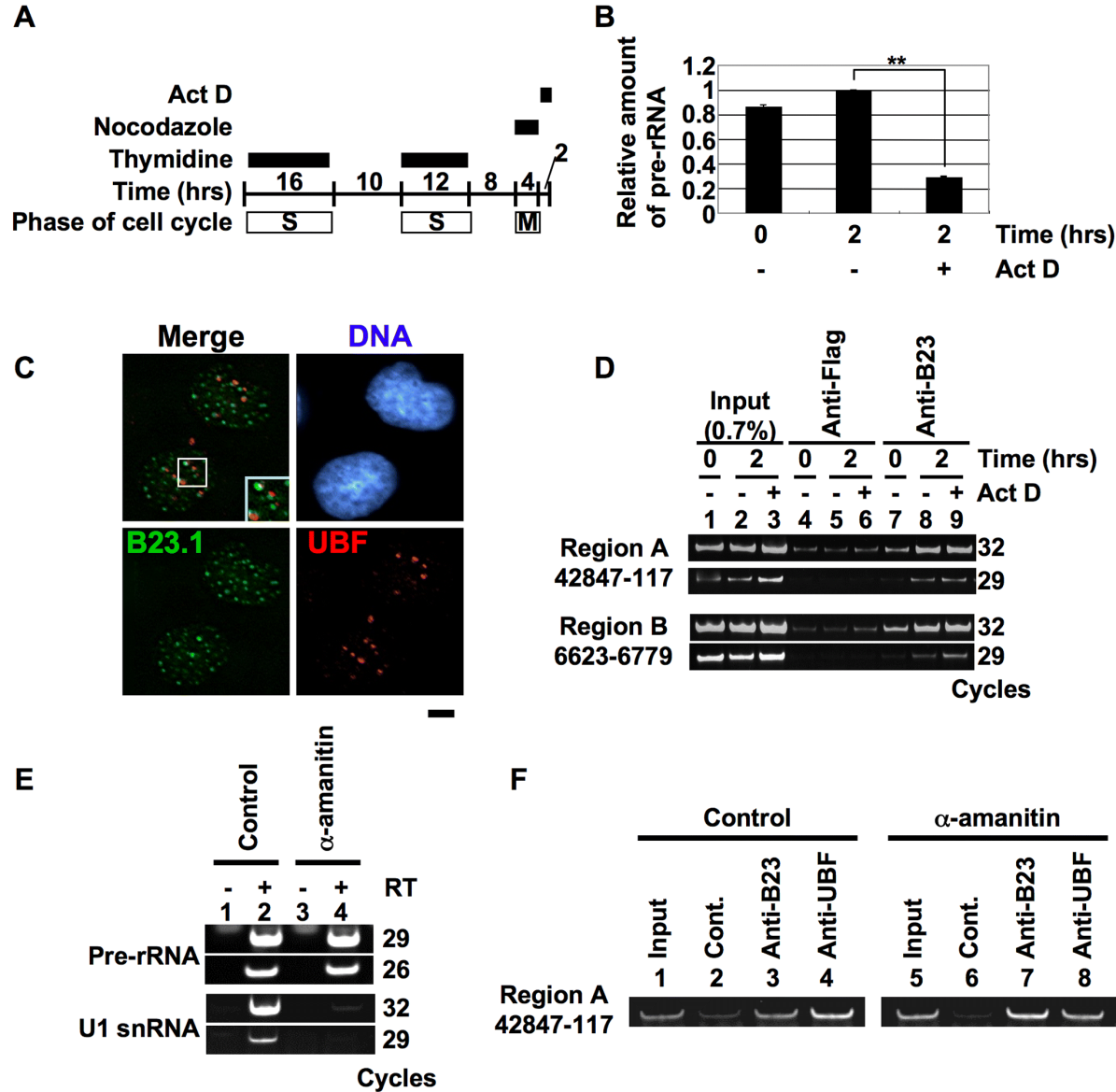


Hisaoka, M. et al. Figure 6



Hisaoka, M. et al. Figure 7