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Lysine 263 residue of NPM/B23 is essential for regulating ATP binding and B23 stability

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Abstract **Here, we show that Nucleophsomin/B23 provides lysine 263 as a critical binding site for ATP. Mutagenesis of lysine 263 to asparagine (K263N) disrupts B23 from ATP binding. While B23 WT exclusively localizes to the nucleolus, the B23-K263N is redistributed from the nucleolus to the nucleoplam. Notably, the K263N mutant is unstable, and displayed rapid degradation. Alteration of K263 induced B23 instability through increased ubiquitination and proteaosomal degradation. Moreover, mutation of K263 impedes the mitogenic effect of B23 in PC12 cells. Thus, K263 is a critical site for ATP binding and required for B23 stability, confining B23 in the nucleolus.**
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Keywords: Nucleolus/Nucleoplasm; Protein stability; Ubiquitination; Proliferation; Apoptosis

1. Introduction

B23/Nucleophosmin, a phosphonucleolar protein, has been implicated in several cellular processes, including cell proliferation [1], anti-apoptotic activity [2], cytoplasm/nuclear shuttling [3] and nucleolar/nucleoplasm transportation [4]. Downregulation of B23 mRNA delays the entry of cells into mitosis [5] and overexpression of B23 induces cell cycle arrest in normal fibroblasts, whereas it accelerates the entry into S phase in cells lacking p53, suggesting that B23 may have oncogenic potential [6–8]. In contrast, knocking down B23 prevents pre-ribosomal processing and causes cell death. Previous studies have suggested that B23 transport is critical for its function in the cell. Indeed, not only does B23 shuttle nucleus/cytoplasm [3], but also translocates from nucleolus to nucleoplasm during S phase [4]. The depletion of the GTP pool in culture condition caused B23 translocation to nucleoplasm [9]. B23 translocation can also be induced by certain anti-cancer drugs that cause growth inhibition [10]. Moreover, ATP promotes B23 translocation from nucleolus to nucleoplasm in isolated permeabilized cells [11], and positively charged lysine residues in the C-terminus of B23 have been implicated in binding ATP [12]. However, the ATP binding residue is unknown, and the physiological meaning of this event is not clear as well.

In this study, we identify lysine 263 in B23 as a specific binding site of ATP. Mutation of this site into asparagines (K263N) disrupts ATP binding and impedes B23 nucleolar residency. Moreover, K263N mutation increases the instability of B23 protein via ubiquitination and proteaosomal degradation, affecting cell proliferation. This demonstrates that K263 of B23 is critical for efficient nucleolar localization and protein stability as well as ATP binding.

2. Materials and methods

2.1. Cell cultures and reagent

PC12 cells were maintained in medium A (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 5% horse serum, and 100 units of penicillin–streptomycin). Stably-transfected PC12 cells with stably inducible B23 DNA were cultured in medium B (medium A with 100 μg/ml G418, 100 μg/ml hygromycin B, and 2 μg/ml tetracycline) and induced in medium B without 2 μg/ml tetracycline for 24 h. Anti-Akt, anti-GST, anti-NPM, anti-HA, anti-PARP and anti-Myc antibodies were obtained from Cell Signaling (Danvers, MA). Anti-α-Tubulin, anti-fibrillarin were acquired from Santa Cruz (Santa Cruz, CA). Adenosine triphosphate-agarose conjugated beads (ATP-beads) were from Fluka (Milwakuee, WI). All the chemicals not included above were from Sigma (St. Louis, MO).

2.2. ATP binding assay

ATP binding assays were performed as previously described [13]. Briefly, ATP-beads were incubated with 10 μg of purified GST-B23 protein in 400 μl binding buffer (40 mM HEPES, pH 7.5, 20 mM KCl, 5 mM $MgCl_2$, 5% glycerol) at 4 °C for 2 h. The beads were washed three times and associated protein was eluted in binding buffer containing 10 mM of soluble ATP.

2.3. Immunostaining and confocal analysis

PC12 cells grown on coverslips in 6-well plates were treated with antimycin A (5 μg/ml) for 1 h. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Immunolocalization was determined using anti-B23 and anti-fibrillarin antibodies with the Alexa Flour 488 goat anti-mouse and Alexa Flour 594 goat anti-rabbit fluorescent dye-conjugated antibodies. Cells were microscopically visualized fluorescent illumination. GFP-B23 WT and GFP-B23 K263N transfected PC12 cells were visualized using a Zeiss LSM confocal fluorescence microscope (Carl Zeiss, Jena, Germany).

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2.4. Pulse-chase determination of protein half-life

PC12 cells were transfected with myc-B23 WT and myc-B23 K263N and labeled with 50 μl/ml of [^{35}S]methionine for 2 h, and were chased by culturing in media for the indicated times. Total cell lysates were immunoprecipitated with anti-myc antibody and incubated with protein agarose A/G. The reaction mixtures were analyzed by autoradiography.

2.5. Measure of ATP concentration

PC12 cells were treated with or not antimycin A (5 μg/ml) or sodium azide(5 μM) for 12 h. Same amount of proteins were incubated with ATP bioluminescent assay mix (Sigma). Then reaction mixture was measured by luminotor.

2.6. Purification of nucleoli

Nucleoli were isolated from PC12 cells as previously described (18). In brief, purified nuclei were added to equal volumes of 0.88 M sucrose containing 0.05 mM $MgCl_2$. After centrifugation at $1200 \times g$ for 10 min at 4 °C, the nuclei were resuspended in 10 volumes of 0.34 M sucrose with 0.05 mM $MgCl_2$. Equal volumes of 0.88 M sucrose containing 0.05 mM $MgCl_2$ were centrifuged at $2000 \times g$ for 20 min at 4 °C. The supernatant containing the nucleoplasmic fraction was collected for further analysis. The nucleolar pellet was washed twice in 0.34 M sucrose containing 0.05 mM $MgCl_2$ and analyzed by Western blotting.

2.7. Ubiquitination assay

PC12 cells were co-transfected with myc-B23 and HA-ubiquitin. Transiently transfected PC12 cells obtained after 24 h were treated with or without 5 μg/ml antimycin A for 1 h and then harvested. Cells were lysed in TBS (20 mM Tris–Cl, pH 7.2, 137 mM NaCl) containing 2% sodium dodecyl sulfate (SDS) at 95 C, 10 min and then were added to four volumes of TBS containing 1% Triton X-100. These lysates were sonicated for 2 min and centrifuged at 4 C. Myc-B23 in the resulting lysate was immunoprecipitated with 2 μg anti-myc antibody and protein G/A agarose beads at 4 C for 4 h with rotation. The beads were washed three times with lysis buffer (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride and associated proteins were eluted in double-strength SDS sample buffer at 95 C for 7 min). Eluted proteins were subjected to immunoblotting against HA-tag to detect ubiquitinated B23. Immunoblotting against α-tubulin and myc-tag were a loading control.

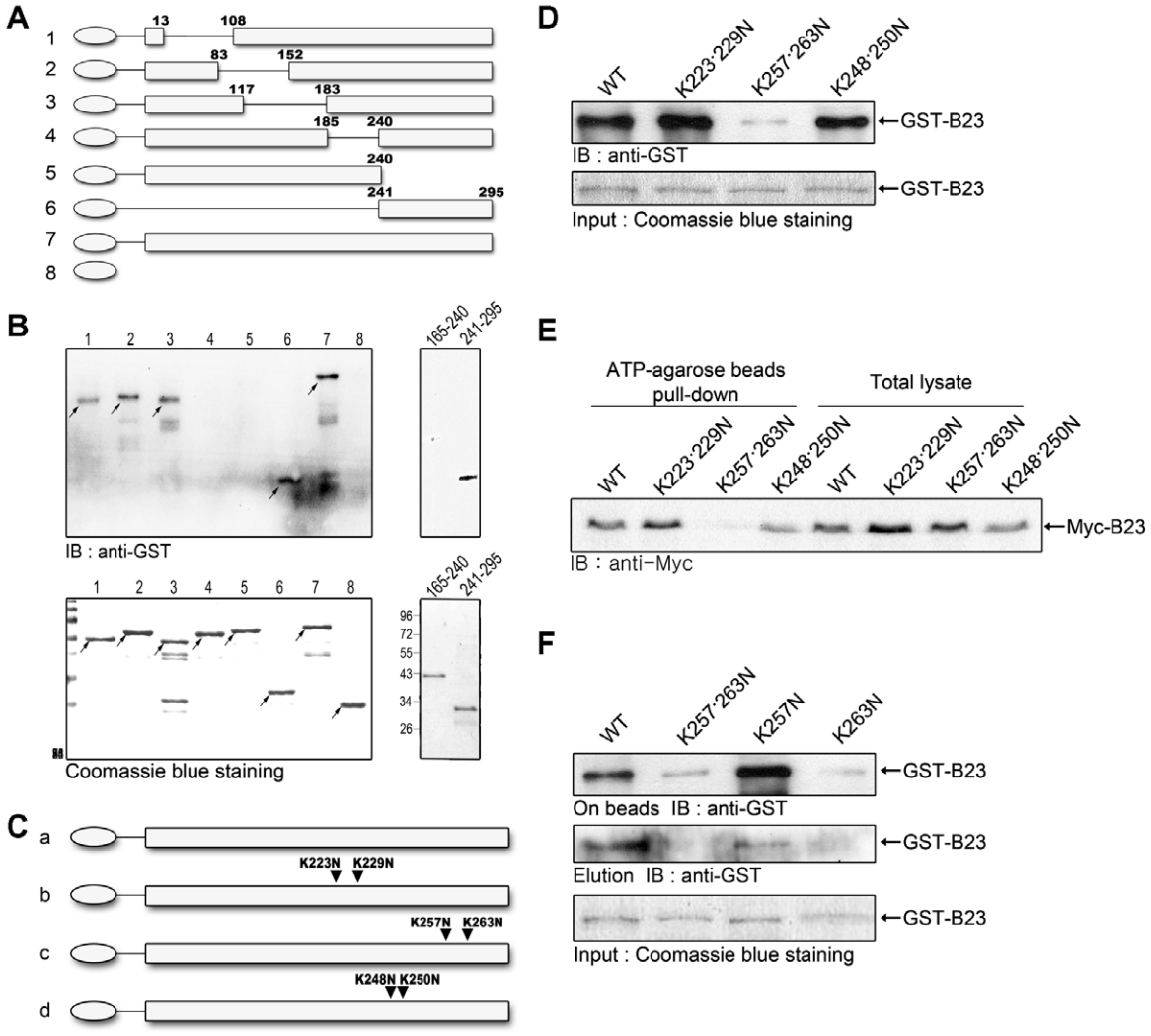

Fig. 1. Lysine 263 residue in B23 is critical docking site for ATP binding. (A) Diagram of GST-B23 deletion constructs. GST tag is depicted by the circles and B23 by squares. (B) GST-B23 deletion mutants were bound to ATP-beads (upper panel) and presented by Coomassie Brilliant Blue staining (bottom panel). B23 proteins are indicated by arrows. (C) Diagram of GST or Myc-B23 lysine mutant proteins. GST tag is depicted by the circles and Myc-tag by the squares. (D) In vitro binding assay. Purified GST-B23 mutant C fails to bind with ATP-beads. (E) ATP-pull-down assay using stably tranfected PC12 cells with inducible forms of Myc-B23 WT and mutants. (F) In vitro ATP binding assay using purified GST-B23 WT and mutants proteins. Associated GST-B23 proteins with ATP-beads were eluted in 10 mM soluble ATP.

2.8. Cell proliferation assay

Control vector, GFP-B23 WT and GFP-B23 K263N transfected cells (3×10^3 cells/well) were plated onto a 96-well plate and incubated in the medium containing 10% FBS and 5% HS without antibiotic for 6 h. The medium was then replaced 100 μl medium containing 10% FBS, 5% HS and 1% antibiotics. 10 μl of MTT (Roche, Germany) was added into each well, followed by incubation at 37 °C for 4 h. Finally, 100 μl of solubilization solution was added. The plate was incubated at 37 °C overnight, and the absorption was measured at 595 nm.

2.9. Cell growth assay

The cells were transfected with pGE(control vector) and Sh-B23 for 72 h and cell growth was measured by total cell count. For rescue B23 expression, after Sh B23 transfection for 36 h, rescue of B23 expression was performed by transfection of GFP-B23 WT and GFP-B23 K263N and GFP. Then cell growth was measured by absorbance measurement at 595 nm on total protein (Bradford assay).

3. Results

3.1. Lysine 263 residue of B23 is a critical docking site for ATP binding

Amino acid sequence analysis of B23 has revealed two putative ATP binding consensus sequences in the middle region 110–120 and C-terminal region 248–265 residues [12]. To map the ATP binding site of B23 in detail, we employed a series of recombinant GST-B23 deletion mutants to perform

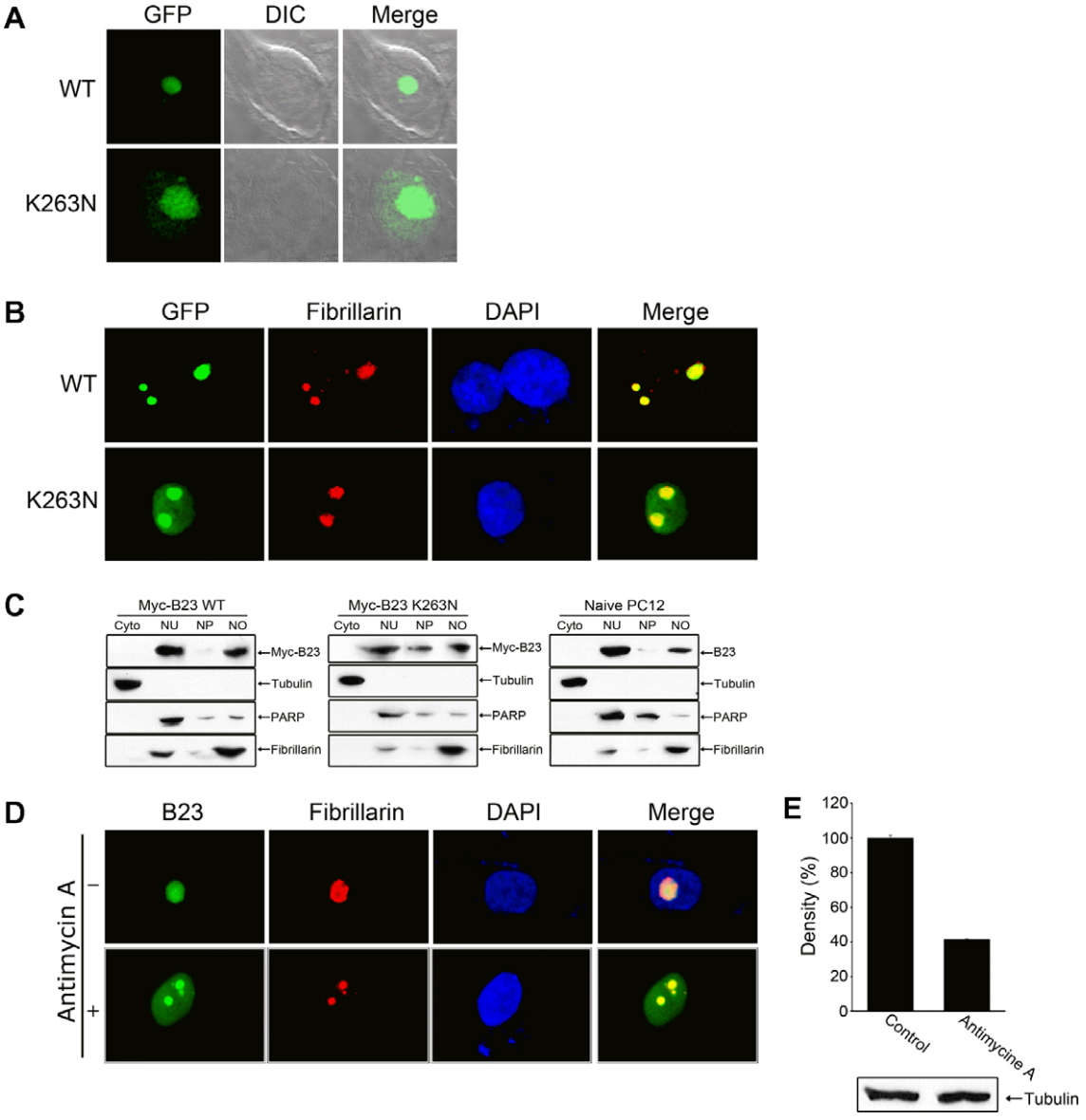

Fig. 2. ATP binding ability of K263 is essential for B23 nucleolar retention. (A) Cellular localization of GFP-B23 WT and GFP-B23 K263N were detected by differential interference contrast (DIC) microscope. Arrow head indicated the nucleolus. (B) B23 subcellular distribution. Immunofluorescent staining revealed GFP-B23 WT exclusively resided in the nucleolus, co-localized with fibrillarin but GFP- B23 K263N distributed entire nucleus. (C) Subcellular fractionation of Myc-B23 transfected PC12 cells and naïve PC12 cells by sucrose cushion. Cyto; cytoplasm, NU; nucleus, NP; nucleoplasm, NO; nucleolus. (D) Naïve PC12 cells were treated or not with 5 μg of antimycin A. After 12 h, immunofluorescence was performed by using α-B23 and α-fibrillarin antibody. Nuclei were stained with DAPI. Treatment with antimycin A resulted in B23 delocalization. (E) Naïve PC12 cells were treated or not with antimycin A for 12 h. Then same amount of cell lysates were incubated with ATP bioluminescent. Reaction mixture was measured by luminotor.

in vitro binding assay using ATP-agarose conjugated beads (Fig. 1A). Most of B23 deletion mutants retained the capacity to bind ATP. However, B23 deletion mutant that lacked the very C-terminal 241–295 residues, the mutant that lacked 185–240 and the fragment containing 165–240, lost the ability to bind ATP whereas the fragment containing 240–295 showed strong binding affinity with ATP, indicating that B23 likely possesses one ATP binding site and the very C-terminus of B23 is indispensable for ATP binding (Fig. 1B). To further assess the specific amino acid residue of B23 that directly binds to ATP, we used the selective GST-fused mutants that change lysines into asparagines in the regions of B23 and stably transfected PC12 cells with an inducible form of various Myc-B23 mutants [2] (Fig. 1C). Our in vitro binding assay revealed that only the B23 K257, 263N mutant failed to interact with ATP whereas B23 K223, 229N, and B23 K248, 250N mutants still bind to ATP, which is similar to B23 WT (Fig. 1D, top). The protein used for binding assay was verified (Fig. 1D, bottom). To ensure that B23 K257, 263N mutant is responsible for ATP binding, we performed an ATP binding assay using stably transfected PC12 cells with an inducible form of various Myc-B23 mutants. Compared with B23 WT, no ATP binding activity was observed in B23 K257, 263N mutant cells. Consistent with our in vitro binding assay, B23 K223, 229N, and B23 K248, 250N mutant cells showed robust interaction with ATP as B23 WT did (Fig. 1E). To define specific ATP binding site, we generated B23 K257N and B23 K263N mutants respectively. Our in vitro binding assay revealed that B23 K263N mutant completely lost its binding ability, whereas B23 K257N mutant still sustained the capability of ATP binding, strongly supporting lysine 263 as a critical docking site for ATP interaction in B23 (Fig. 1F).

3.2. K263 is essential for nucleolar localization of B23

Previous studies have shown that ATP depletion blocks B23 translocation from nucleolus to nucleoplasm [14]. To investigate the biological meaning of ATP binding to B23, the subcellular distribution of B23 was compared with that of K263N mutant, which lost its binding ability for ATP by confocal fluorescence laser microscopy using GFP-tagged protein. GFP alone diffused within the whole cell exclusive of the nucleoli (data not shown). Differential interference contrast (DIC) imagery revealed that GFP-B23 WT exhibited extensive accumulation into nucleoli, whereas GFP-B23 K263N was observed throughout the entire nucleus (Fig. 2A). Moreover, staining with anti-fibrillarin, a specific nucleolar marker protein, co-localized with GFP-B23 WT, providing evidence of B23 nucleolar localization. However, K263N mutant showed only partial co-localization with fibrillarin and complete overlap with 4′-6-diamidino-2-phenylindole (DAPI) staining, indicating that this mutant protein does not localize efficiently to nucleoli and is distributed the entire nucleus (Fig. 2B). To confirm the subnuclear distribution of B23 WT and B23 K263N, PC12 cells were subfractionated and underwent immunoblotting with anti-Myc and anti-B23 antibodies. Consistent with our microscopic observations, B23 WT exclusively localized to the nucleolar fraction, while the K263N mutant was evident in both the nucleoplasmic and cytoplasmic region, being slightly more abundant in the nucleolar region. Endogenous B23 in PC12 cells was detected in the nucleoli region whereas small amounts of B23 were present in the nucleoplasm (Fig. 2C). These results suggest that B23 WT is predominantly located in the nucleolus. However, K263N mutant failed to localize in the nucleolus as efficient as WT leading to its redistribution from the nucleolus. To further investigate the effect of ATP on nucleolar localization of B23, we evaluated that the deficiency of cellular ATP contents by antimycin A. Myc-B23 WT cells were treated with antimycin A in a several doses (0, 1, 2.5, and 5 μg/ml) for 1 h. Endogenous B23 was located mostly in the nucleolus, where fibrillarin specifically expressed (Fig. 2D, upper panels). Antimycin A elicited increased nucleoplasmic staining of B23 a dose dependent manner (data not shown). Notably, 2.5 μg/ml of antimycin A treatment lead to B23 delocalization from nucleolus is comparable to GFP-B23 K263N (Fig. 2D, bottom panels), indicating nucleolar localization of B23 requires ATP binding. 2.5 μg/ml of antimycin A treatment decreased more than 50% of ATP from in Myc-B23 WT cells. We observed similar result with another ATP synthesis inhibitor, sodium azaid (data not shown).

3.3. Alteration of K263 impedes B23 stability via ubiquitination and proteaosomal degradation

No protein degradation has been found in the nucleolus [15] and B23 is a stable protein with a half life greater than 24 h [16]. With the finding that ATP binding mutant, B23 K263N delocalized from the nucleolus, we wondered whether B23 subnuclear localization regulated B23 protein stability. To evaluate the effect of nucleolar localization of B23, we determined the half-life of B23-WT and the B23 K263N. A pulse-chase experiment revealed the stability of the B23-WT protein, whose half-life exceeded 24 h (Fig. 3). In contrast, the B23 K263N mutant that was incapable of binding ATP, exhibited

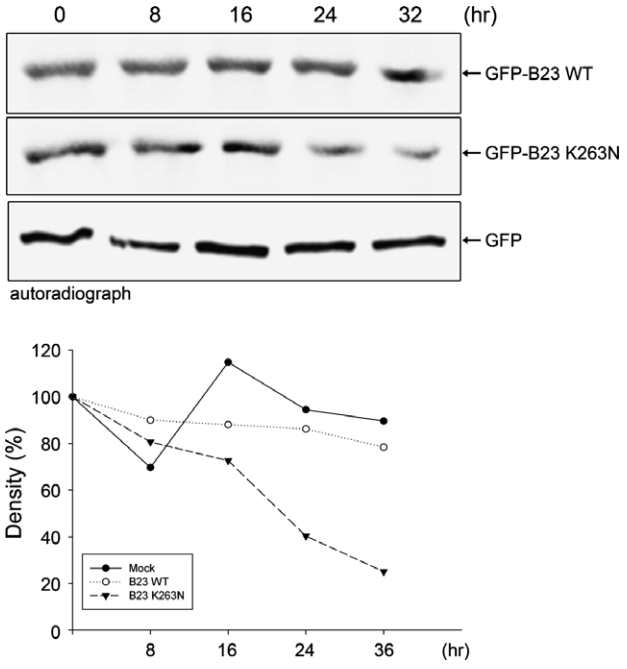

Fig. 3. B23 K263N is unstable than B23 WT. Half-life determination of B23 WT and K263N protein. PC12 cells were transfected with myc-B23 WT and myc-B23 K263N. After 24 h, cell were pulsed with [^{35}S] methionine for 2 h and then were chased for the indicated times. The amount of labeled B23 protein at each time point was quantified on a phosphorimager and normalized relative to the amount of radiolabeled B23 present in cells following the 0 h chase. The results are plotted.

a half-life of approximately 10 h, consistent with its more rapid degradation. Thus, one of probable reasons for the increased instability of B23 could be the alteration of K263 with its nucleoplasmic localization.

To search for the possible mechanism of B23 degradation via mutation of K263, we tested whether disruption of B23 K263 could induce B23 proteasomal degradation and ubiquitination. PC12 cells were transiently transfected with Myc-tagged B23 WT or K263N mutant and treated for 6 h with 20 μM MG-132, which is a 26 S proteasome inhibitor. The protein level of B23 WT remained intact and barely changed during the exposure to MG-132, whereas the protein level of K263N was protected in response to MG-132, suggesting that the instability of K263N at least partly mediated via proteasomal degradation (Fig. 4A). The dependency of proteasomal degradation suggested that increased instability of B23 might be due to ubiquitination. To test this possibility, PC12 cells were co-transfected with Myc-tagged B23 WT or K263N mutant was co-transfected with HA tagged ubiquitin plasmid. Under the normal condition, B23 WT revealed a low level of ubiquitin conjugation, while the B23 K263N mutant exhibited marked conjugation of ubiquitin. Furthermore, to validate ubiquitination of B23, the WT or mutant were untreated or were exposed to 10 μM of MG-132 for 4 h to allow accumulation and detection of B23 ubiquitination. Notable polyubiquitination of B23 K263N was observed even in the absence of MG-132, and markedly increased ubiquitination in the presence of MG-132, while B23 WT displayed a low level of ubiquitination that increased in the presence of MG-132 (Fig. 4B). These results support the idea that B23 K263N is more rapidly degraded and instable relative to B23 WT due to increased ubiquitination and proteasomal degradation.

We extend our study to test whether disruption of B23-ATP interaction induce B23 proteasomal degradation and ubiquitination. PC12 cells were treated with or without 20 μM MG-132 proteasome inhibitor for 2 hours prior antimycin A treatment. In cells pretreated with MG-132, B23 protein level appears slightly diminished, whereas in the absence of MG-132, B23 protein levels were obviously decreased in a dose response (Fig. 4C). In addition, PC12 cells were cotransfected with

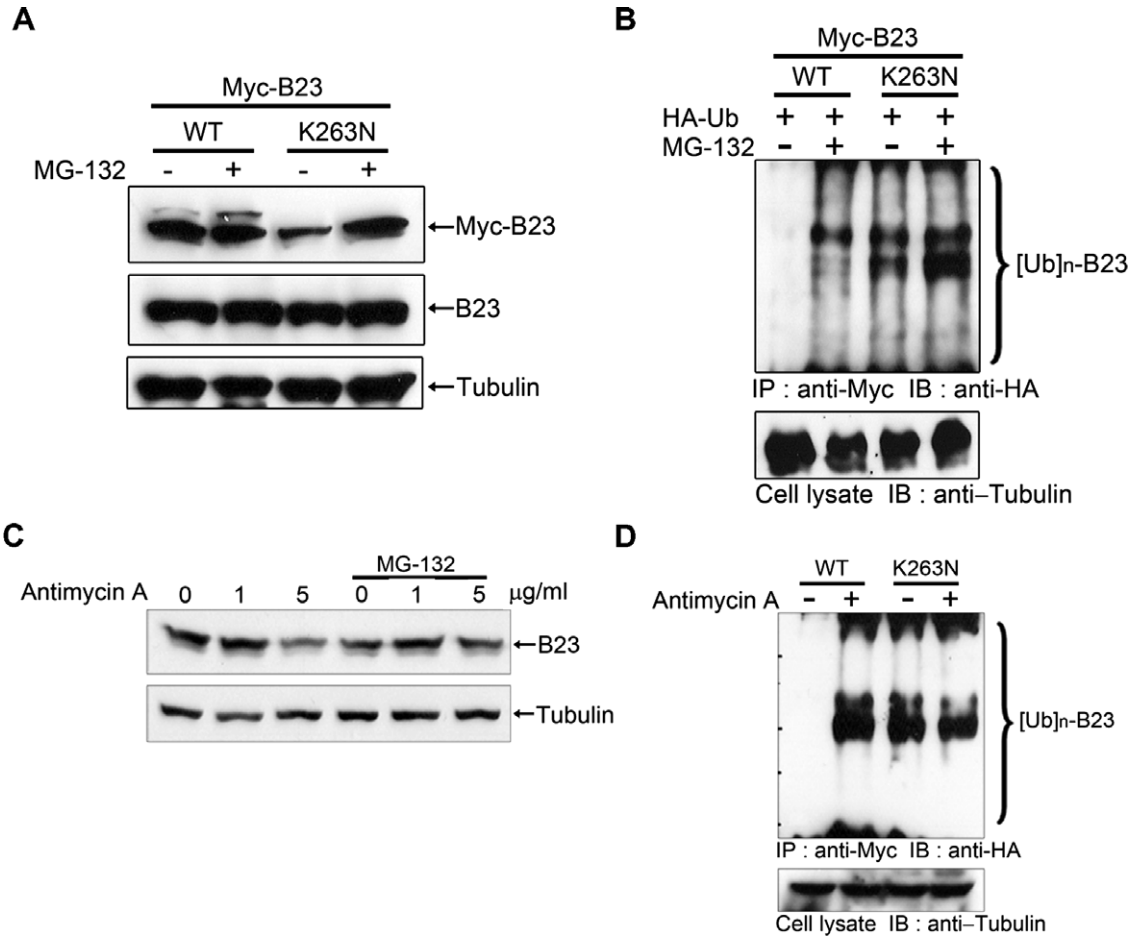

Fig. 4. Polyubiquitination and proteasomal degradation of B23 K263N. (A) Myc-B23 WT or Myc-B23 K263N cells were treated with or without 20 μM MG-132. B23 protein stability was observed by anti-B23 antibody. Immunoblotting assay with anti-Myc and anti-Tubulin antibodies revealed that a similar amount of Myc-B23 protein was expressed and a similar amount of protein was employed (middle and bottom panels). (B) Cells were co-transfected with Myc-B23 WT and HA-Ubiquitin, followed by no treatment or treatment with 20 μM MG-132 for 4 h before immunoprecipitation with anti-Myc antibody. (C) Naïve PC12 cells were pretreated with or without 20 μM MG-132 for 2 h. Then, cells were treated with antimycin A for 12 h with indicated concentrations. Cell lysates were probed with anti-NPM and anti-Tubulin antibodies. (D) In vitro ubiquitination assay. PC12 cells were cotransfected with HA-Ubiquitin and Myc-B23 WT or Myc-B23 K263N for 24 h. After treatment or non-treatment with 2.5 μg of antimycin A for 12 h, cell lysates were immunoprecipitated with anti-Myc antibody and performed immunoblotting with anti-HA antibody. Tubulin was used for verifying same amount of protein was employed.

Myc-tagged B23 WT or K263N mutant plasmid and HA tagged ubiquitin plasmid. Under normal condition, B23 WT revealed low level of ubiquitin conjugation, while B23 K263N mutant exhibited marked conjugation of ubiquitin. Moreover, depletion of ATP in Myc-B23 WT transfected cells displayed dramatically increased ubiquitination of B23. In contrast, antimycin A treatment has only negligible effect to ubiquitination of B23 K263N mutant cells (Fig. 4D). Presumably, it is due to saturated ubiquitination of B23 K263N. Hence we conclude that depletion of ATP from B23 binding is less stable relative to normal condition due to increased ubiquitination and proteasomal degradation.

3.4. B23K263N mutant is defective in restoring cell proliferation

Previous studies have shown that B23 protein level correlate with cell proliferation [17]. We undertook to examine the

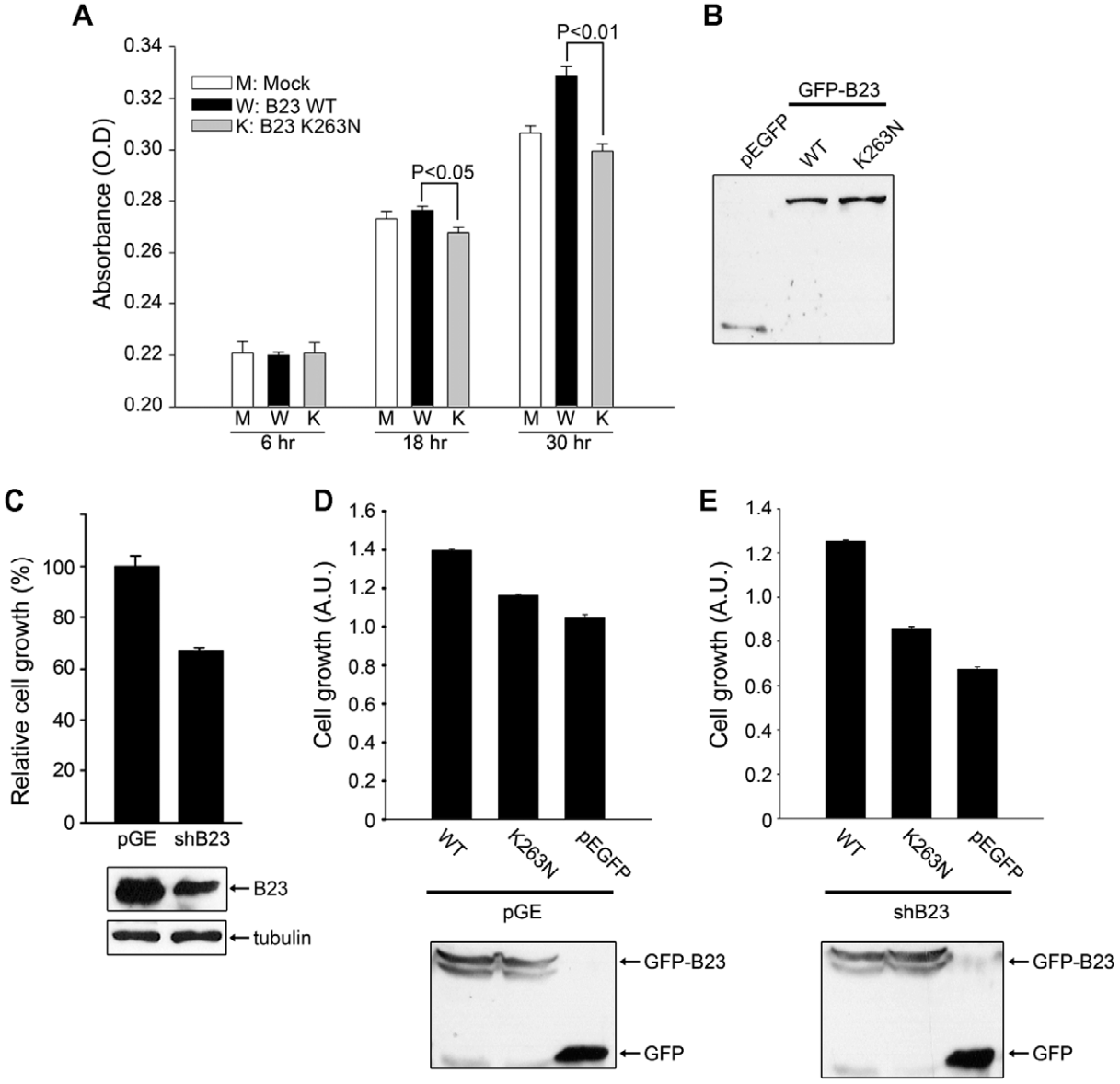

Fig. 5. K263N mutation is defective in restoring cell growth. (A) Effects of B23 WT and B23 K263N on PC12 cells proliferation were measured by MTT assay. Bars indicate mean of six replicates and each value represents the means ± S.E. of triplicate measurements. (B) Expression of GFP control, GFP-B23 WT and GFP-B23 K263N in PC12 transient transfectants by Western blot. (C) Knocking down of B23 with sh-B23 impairs cell growth. Data represent the relative cell number seen in sh-B23 transfected cells compared with control cells transfected with pGE-vector set to 100%, 72 h after sh-RNA transfection. An average of three independent experiments is shown. (D) Transient expression of B23 enhances cell growth but not for the K263N mutant. PC12 cells were transfected with indicated DNA for 24 h and analyzed using absorbance 595 nm measurements (Bradford assay) of total protein. The data are presented relative to control vector (pEGFP) transfected cells. GFP B23 expression was confirmed by immunoblotting with α-B23 antibody (bottom). *A.U.*, arbitrary units. (E) Depletion of endogenous by ShB23 for 36 h was followed by transfection with GFP-B23 WT, GFP-B23 K263N and pEGFP. GFP B23 expression was confirmed by immunoblotting with α-B23 antibody (bottom). The data are presented as in (D). At least 70% transfected cells were analyzed for each construct. These experiments were repeated three times $^{*}P < 0.01$ (Student's *t*-test).

growth and proliferation of B23 and B23 K263 mutant functions; Firstly, We determined the effect of increasing GFP-B23 WT and GFP-B23 K263N expression on PC12 cell growth rates in vitro via MTT assay Ectopic transient expression of GFP-B23 WT resulted in augmentation of proliferation rate, whereas GFP-B23 K263N decreased cell proliferation of PC12 cells (Fig. 5A). Western blot analysis showed expression of GFP-B23 WT and GFP-B23 K263N protein in transient transfected cells (Fig. 5B). Next, we depleted B23 by using B23 *s*hort *h*airpin RNA (shRNA) in PC12 cells followed by rescue with transient expression of GFP-B23 WT or GFP-B23 K263N. Depletion of B23 elicited considerable impairment of cell growth as measured by total cell count (Fig. 5A). Endogenous B23 levels were substantially diminished, whereas the tubulin was not affected (Fig. 5A, bottom). On the other hands, ectopic expression of B23-WT reproducibly enhanced cell growth (Fig. 5B). Notably, introduction of GFP-B23 WT into the cells depleted of endogenous B23 reconstituted cell growth, whereas GFP-B23 K263N or GFP alone had no effect (Fig. 5C), indicating that K263N mutant was defective in promoting cell growth. The expression of GFP-B23 and mutant were verified by fluorescence microscopy. The prominent effects in K263N transfected cells raise the possibility that the mutation might cause a structural alteration, and somehow is toxic to the cells, leading to an instable form, thereby failing to restore its mitogenic effect.

4. Discussion

The C-terminus of B23 is important for interaction with nucleic acids [18] and numerous lysine residues occur in the ATP binding motif [12]. In addition, we have recently shown that $PI(3,4,5)P_3$ directly binds to the C-terminus of B23 and inhibits DNA fragmentation in the nucleus. Having established the importance of the C-terminal region of B23, we presently demonstrate that the lysine 263 residue is a specific ATP binding residue of B23, and that the alteration of this residue in B23 results in the failure of B23 WT to either re-enter the nucleolus or to be retained in the nucleolus. Furthermore, we show that the K263N mutant is more unstable and susceptible for protein degradation, subsequentially contributing to decreased cell proliferation.

Independent of our study, it has been very recently demonstrated that the lysine 263 residue is a major sumolyation site in B23 and mutation of K263 into R abrogates its centrosomes localization, aggregating in outside of nucleolus [19]. Indeed, the K263N mutant of B23 was dispersed in both the nucleolus and nucleoplasm in our study, but no aggregation was detected (Fig. 2). B23 translocates from nucleolus to nucleoplasm during the stationary phase of growth [4] or during exposure to certain anti-tumor drugs [4,20]. Under conditions of lower ATP content, B23 delocalization from the nucleolus is blocked and newly synthesized rRNA is retained in the nuclei [14], suggesting that B23 translocation occurs in a regulated manner. However, it remains elusive how the K263 mutant of B23 (into R or N) delocalizes from the nucleolus. It can be argued that it is not possible to distinguish either the possibility that sumoylation of B23 regulates its nucleolar localization or that ATP binding ability of B23 is required for B23 nucleolar accumulation. In addition, we cannot rule out the simple but potent possibility that conformational change of B23 protein by mutation of K263 into R or N but not sumoylation (the other sumolyation site in the K230R mutant is mainly localized in the nucleolus). ATP binding abrogates nucleolar residency of B23, and consequently contributes to the destabilization of B23 protein either through the failure of protecting B23 from caspase-3 mediated cleavage [19] or due to increased proteasome dependent degradation and ubiquitination (Fig. 5). Nevertheless, K263 is a critical amino acid dictating B23 in the nucleolus and maintaining protein stability. K263 has been suggested to be a sumoylation site implicated in the protection of B23 from apoptotic degradation; presently, we introduce K263 as a critical ATP binding site that contributes to protein stability and cell proliferation.

B23 is a stable protein and its nucleolar localization is considered as a one of the possible reasons for its stability [16,21]. Our findings that B23 mutant, K263N substantially extrude its nucleolar localization (Fig. 3) but also decreases B23 protein level via proteasome dependent degradation and induces B23 ubiquitination (Figs. 4 and 5) argue that K263 is crucial for B23 protein stability. Presumably, B23 conformational change and delocalization increases B23 instability through its increased ubiquitination and proteasomal degradation. Although it remains to be determined how ATP binding ability maintains its nucleolar localization and protein stability on K263, our observations indicate that under certain circumstances B23 binds to ATP for protein stability, confining it in the nucleolus and ultimately resulting in cell proliferation.

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References

[1] Hingorani, K., Szebeni, A. and Olson, M.O. (2000) Mapping the functional domains of nucleolar protein B23. J. Biol. Chem. 275, 24451–24457.
[2] Ahn, J.Y., Liu, X., Cheng, D., Peng, J., Chan, P.K., Wade, P.A. and Ye, K. (2005) Nucleophosmin/B23, a nuclear PI(3,4,5)P(3) receptor, mediates the antiapoptotic actions of NGF by inhibiting CAD. Mol. Cell 18, 435–445.
[3] Borer, R.A., Lehner, C.F., Eppenberger, H.M. and Nigg, E.A. (1989) Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 56, 379–390.
[4] Yung, B.Y., Bor, A.M. and Yang, Y.H. (1990) Immunolocalization of phosphoprotein B23 in proliferating and non-proliferating HeLa cells. Int. J. Cancer 46, 272–275.
[5] Jiang, P.S., Chang, J.H. and Yung, B.Y. (2000) Different kinases phosphorylate nucleophosmin/B23 at different sites during G(2) and M phases of the cell cycle. Cancer Lett. 153, 151–160.
[6] Nozawa, Y., Van Belzen, N., Van der Made, A.C., Dinjens, W.N. and Bosman, F.T. (1996) Expression of nucleophosmin/B23 in normal and neoplastic colorectal mucosa. J. Pathol. 178, 48–52.
[7] Feuerstein, N., Chan, P.K. and Mond, J.J. (1988) Identification of numatrin, the nuclear matrix protein associated with induction of mitogenesis, as the nucleolar protein B23. Implication for the role of the nucleolus in early transduction of mitogenic signals. J. Biol. Chem. 263, 10608–10612.
[8] Feuerstein, N. and Mond, J.J. (1987) Identification of a prominent nuclear protein associated with proliferation of normal and malignant B cells. J. Immunol. 139, 1818–1822.
[9] Finch, R.A., Revankar, G.R. and Chan, P.K. (1993) Nucleolar localization of nucleophosmin/B23 requires GTP. J. Biol. Chem. 268, 5823–5827.

[10] Chou, Y.H. and Yung, B.Y. (1995) Cell cycle phase-dependent changes of localization and oligomerization states of nucleophosmin/B23. Biochem. Biophys. Res. Commun. 217, 313–325.
[11] Wu, M.H., Lam, C.Y. and Yung, B.Y. (1995) Translocation of nucleophosmin from nucleoli to nucleoplasm requires ATP. Biochem. J. 305 (Pt 3), 987–992.
[12] Chang, J.H., Lin, J.Y., Wu, M.H. and Yung, B.Y. (1998) Evidence for the ability of nucleophosmin/B23 to bind ATP. Biochem. J. 329 (Pt 3), 539–544.
[13] Chen, H.J. and Hwang, J. (1999) Binding of ATP to human DNA topoisomerase I resulting in an alteration of the conformation of the enzyme. Eur. J. Biochem. 265, 367–375.
[14] Finch, R.A. and Chan, P.K. (1996) ATP depletion affects NPM translocation and exportation of rRNA from nuclei. Biochem. Biophys. Res. Commun. 222, 553–558.
[15] Olson, M.O., Hingorani, K. and Szebeni, A. (2002) Conventional and nonconventional roles of the nucleolus. Int. Rev. Cytol. 219, 199–266.
[16] Itahana, K., Bhat, K.P., Jin, A., Itahana, Y., Hawke, D., Kobayashi, R. and Zhang, Y. (2003) Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. Mol. Cell 12, 1151–1164.
[17] Feuerstein, N., Spiegel, S. and Mond, J.J. (1988) The nuclear matrix protein, numatrin (B23), is associated with growth factor-induced mitogenesis in Swiss 3T3 fibroblasts and with T lymphocyte proliferation stimulated by lectins and anti-T cell antigen receptor antibody. J. Cell Biol. 107, 1629–1642.
[18] Wang, D., Baumann, A., Szebeni, A. and Olson, M.O. (1994) The nucleic acid binding activity of nucleolar protein B23.1 resides in its carboxyl-terminal end. J. Biol. Chem. 269, 30994–30998.
[19] Liu, X. et al. (2007) Sumoylation of nucleophosmin/B23 regulates its subcellular localization, mediating cell proliferation and survival. Proc. Natl. Acad. Sci. USA 104, 9679–9684.
[20] Yung, B.Y., Busch, H. and Chan, P.K. (1986) Effects of luzopeptins on protein B23 translocation and ribosomal RNA synthesis in HeLa cells. Cancer Res. 46, 922–925.
[21] Enomoto, T., Lindstrom, M.S., Jin, A., Ke, H. and Zhang, Y. (2006) Essential Role of the B23/NPM Core Domain in Regulating ARF Binding and B23 Stability. J. Biol. Chem. 281, 18463–18472.