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Nucleolar protein, Myb-binding protein 1A, specifically binds to nonacetylated p53 and efficiently promotes transcriptional activation

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ABSTRACT

Nucleolar dynamics are important for cellular stress response. We previously demonstrated that nucleolar stress induces nucleolar protein Myb-binding protein 1A (MYBBP1A) translocation from the nucleolus to the nucleoplasm and enhances p53 activity. However, the underlying molecular mechanism is understood to a lesser extent. Here we demonstrate that MYBBP1A interacts with lysine residues in the C-terminal regulatory domain region of p53. MYBBP1A specifically interacts with nonacetylated p53 and induces p53 acetylation. We propose that MYBBP1A dissociates from acetylated p53 because MYBBP1A did not interact with acetylated p53 and because MYBBP1A was not recruited to the p53 target promoter. Therefore, once p53 is acetylated, MYBBP1A dissociates from p53 and interacts with nonacetylated p53, which enables another cycle of p53 activation. Based on our observations, this MYBBP1A–p53 binding property can account for efficient p53-activation by MYBBP1A under nucleolar stress. Our results support the idea that MYBBP1A plays catalytic roles in p53 acetylation and activation.

1. Introduction

The tumor suppressor p53 is a critical mediator of cellular stress response, which maintains genomic integrity and prevents oncogenic transformation [1]. The protein p53 regulates many target genes that induces cell cycle arrest or apoptosis [2,3].

DNA damage is known to activate p53 as a transcription factor through post-translational modifications [4,5] such as phosphorylation, ubiquitination, and acetylation, which are critical in regulating p53 function [6–12]. The p300/CBP protein that possesses histone acetyltransferase activity and acetylates p53, acts as a coactivator of p53 and augments p53 transcriptional activity [13–15]. p53 acetylation occurs at multiple lysine residues in the C-terminal regulatory domain (CRD) of p53 (residues 370, 372, 373, 381, 382, and 386) in response to DNA-damaging agents [16–18]. p53 acetylation also correlates with its sequence-specific DNA binding [19], and augments recruitment of transcriptional activators to p53 [20]. Based on these observations, p53 acetylation is considered to play a vital role in p53 activation [21,22].

DNA damage induces repression of rRNA transcription by RNA polymerase I, resulting in disruption of nucleolar structure [23,24]. Low concentrations of actinomycin D (ActD) specifically inhibit RNA polymerase I-driven transcription, but do not affect RNA polymerase II-driven transcription [25,26]. Therefore, ActD treatment also induces nucleolar disruption [27].

We previously reported that nucleolar disruption induces acetylation and accumulation of p53 without phosphorylation. The nucleolar protein Myb-binding protein 1A (MYBBP1A) binds to p53 and facilitates p53 acetylation to enhance p53-mediated transcription by enhancing the p53–p300 interaction [28]. However, the mechanism by which MYBBP1A enhances p53 acetylation and induces p53 activity is still ambiguous.

Here we demonstrate that MYBBP1A specifically recognizes nonacetylated lysine residues of p53, promotes its acetylation, and dissociates from acetylated p53. The dissociated MYBBP1A may recognize nonacetylated p53 for another cycle of p53 activation. We predict that this MYBBP1A–p53 binding property may explain the effective p53-activating function of MYBBP1A.

2. Materials and methods

2.1. Cell culture and treatments

MCF-7 human breast cancer cells and H1299 p53-deficient human lung cancer cells were maintained in DMEM (Sigma). All

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media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Nacalai Tesque). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 and 100% humidity. To induce nucleolar stress, cells were exposed to ActD (5 nM).

2.2. siRNA and plasmid DNA transfection

For siRNA transfection, cells at 30–50% confluency were transfected using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All siRNAs were purchased from Invitrogen. The siRNA duplexes MYBBP1A, 5′-UCUUUCAGUCAGGUCGGCUGGUGAA-3′, p300 5′-AUUAUAGG AGAGUUCACCGGGCAGG-3′. Stealth RNAi negative control Medium or High GC was used as a negative control. Protein and RNA were extracted at 48 h after transfection of siRNA. For transfection of plasmid DNA, cells at 70–80% confluency were transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Protein was extracted 24 h after transfection of plasmid DNA.

2.3. Expression vectors, antibodies

cDNAs encoding full-length and indicated mutants of p53 and MYBBP1A were amplified by PCR and subcloned into the pcDNA3 plasmid (Invitrogen) containing sequences encoding FLAG, FLAG-HA, HA or myc sequences. β-Actin (Sigma) and anti-human-p53 (DO-1, Santa cruz) monoclonal antibodies and rabbit anti-p53-K382Ac (Cell Signaling Technology) polyclonal antibodies were used according to the manufacturers' instructions. Rabbit anti-human MYBBP1A antibody was raised against a synthetic peptide corresponding to 1265–1328 amino acids of human MYBBP1A.

2.4. GST pull-down assay

cDNAs encoding full-length human p53 or MYBBP1A and its deletion mutant derivatives were cloned into pGEX-4T-1 (Amersham Biosciences). GST-fusion proteins were expressed in BL-21 cells following induction with IPTG and purified with glutathione Sepharose 4B beads (Amersham). *In vitro* translated MYBBP1A was synthesized using an *in vitro* transcription/translation-coupled reticulocyte lysate system (Promega). Binding was performed in TNE buffer [150 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris–HCl (pH 8.0), 5 mM EDTA] for 30 min under rotation at 4 °C, and the beads were washed 5 times with TNE buffer. Beads were boiled in SDS sample buffer for 5 min, and the supernatants were loaded onto SDS–polyacrylamide gels followed by immunoblotting.

2.5. Coimmunoprecipitation and immunoblotting

Cells were lysed in TNE buffer. Extracted proteins were immunoprecipitated with antibody-coated protein G Sepharose (Amersham) beads. Bound proteins were separated by SDS–PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and detected with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Specific proteins were visualized using an enhanced chemiluminescence (ECL) immunoblot detection system (Amersham).

2.6. Chromatin immunoprecipitation (ChIP) and real-time PCR detection

ChIP assay was performed according to the published procedure [29]. The primers for real-time PCR were as follows:

p21-p53RE fw primer 5′-GTGGCTCTGATTGGCTTTCTG-3′
p21-p53RE rv primer 5′-CTGAAAACAGGCAGCCCAAG-3′

3. Results and discussion

3.1. Lysine residues in the CRD region of p53 play an important role in MYBBP1A–p53 interaction

We have previously demonstrated that cellular stresses induce nucleolar disruption, which leads to MYBBP1A translocation from the nucleolus to the nucleoplasm. Subsequently, MYBBP1A binds to p53 and promotes acetylation by enhancing p53–p300/CBP association, thereby activating the p53 function [28]. However, determination of the MYBBP1A–p53 binding properties remains. To investigate this, we attempted to dissect the mechanism of p53–MYBBP1A interaction. Co-immunoprecipitation experiments revealed the binding of endogenous MYBBP1A to p53 in MCF-7 cells under conditions of nucleolar stress (Fig. 1A), which is consistent with previous results.

Our previous results indicated that MYBBP1A binds to the CRD region in p53. Therefore, to further narrow the regions of p53 responsible for binding with MYBBP1A, we generated a series of truncation mutants of the p53 CRD region and tested the interaction with MYBBP1A by a GST pull-down assay (Fig. 1B). The GST pull-down assay determined that CRD-1 and CRD-3 interacted with MYBBP1A. In contrast, CRD-2 and CRD-4 did not bind to MYBBP1A. These data indicate that the 367–386 amino acid (aa) region of p53 is responsible for binding with MYBBP1A (Fig. 1C).

To identify the residues responsible for the binding, we subsequently performed alanine-scanning mutagenesis of the p53-CRD region construct and subjected it to GST pull-down assay (Fig. 1D). It was significant that substitution of lysine with alanine residues at K370, K372, K373, K381, K382, or K386, located in the 367–386 aa region specifically attenuated the MYBBP1A–p53 interaction (Fig. 1E). Moreover, MYBBP1A did not bind to the p53 6KA mutant, which bears simultaneous lysine substitutes 370, 372, 373, 381, 382, and 386 to alanines (Fig. 1F). These results (Fig. 1E and F) demonstrate that lysine residues in the CRD region of p53 play an important role in the p53–MYBBP1A interaction.

3.2. MYBBP1A binds specifically to nonacetylated p53

Because these lysine residues, responsible for the binding to MYBBP1A, are sites for p53 acetylation, we explored the effect of acetylation of these residues on binding to MYBBP1A. First, to test whether acetylation of lysine residues influences MYBBP1A–p53 binding, we generated a p53–6KQ mutant that mimics acetylated status (Fig. 2A). Our GST pull-down experiments demonstrated that the 6KQ mutant did not bind to MYBBP1A (Fig. 2B). Next we overexpressed p300 in H1299 cells to enhance p53 acetylation, and found that overexpression of p300 increased acetylation status of p53 and decreased MYBBP1A–p53 binding (Fig. 2C). Moreover, we generated a p53–6KR mutant (Fig. 2A), which acts as an acetylation-deficient missense mutant [21]. Co-immunoprecipitation experiments revealed that interaction between p53–6KR mutants and MYBBP1A is stronger than that between wild-type p53 and MYBBP1A (Fig. 2D, compare lanes 2, 3). We also examined the effect of decrease in p53 acetylation on p53–MYBBP1A interaction in MCF-7 cells by knockdown of p300. We found that p300 knockdown decreased endogenous p53 acetylation and increased the MYBBP1A–p53 interaction (Fig. 2E and F). Taken together, these data indicate that MYBBP1A specifically interacts with nonacetylated p53.

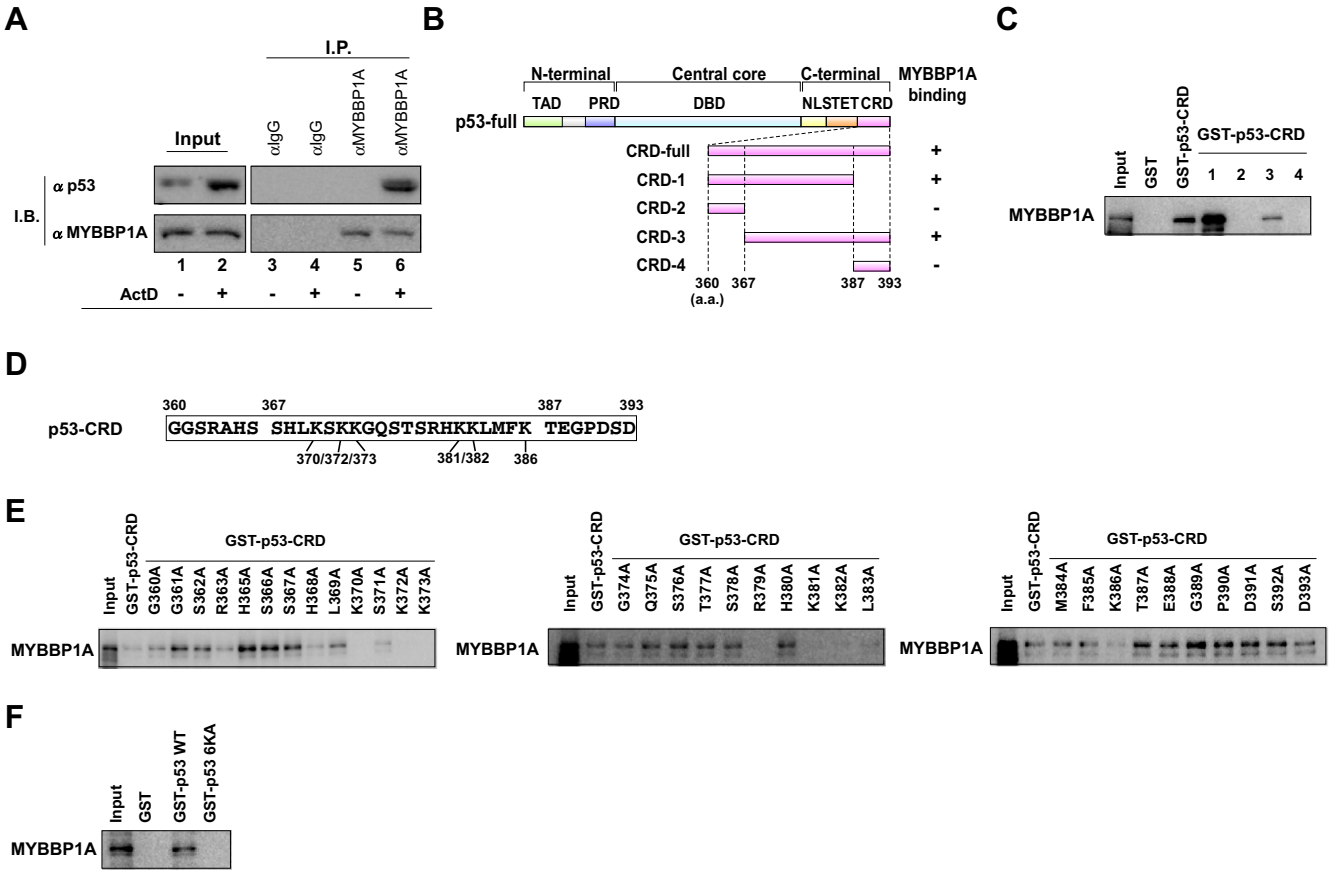

Fig. 1. Lysine residues in the CRD region of p53 play an important role in p53–MYBBP1A interaction. (A) Endogenous MYBBP1A associates with p53. MCF-7 cells were treated with a low dose of ActD (5 nM) for 6 h. The cell lysates were immunoprecipitated with normal rabbit IgG or anti-MYBBP1A antibodies and analyzed by immunoblotting using antibodies against MYBBP1A and p53. (B) Domain structure of the full-length p53 and various deletion mutants of CRD region. TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; NLS, nuclear localization signal; TET, tetramerization domain; CRD, C-terminal regulatory domain. (C) MYBBP1A directly binds to the CRD region in p53. *In vitro* translated MYBBP1A was incubated with GST-fused truncated p53 proteins. Bound proteins were analyzed by immunoblotting using an antibody against MYBBP1A. Input, 25% of the *in vitro* translated MYBBP1A used in the GST pull-down assay. (D) Amino acid sequence of human p53-CRD region. (E) Mutations in the lysine residues result in loss of the MYBBP1A interaction. *In vitro* translated MYBBP1A was incubated with p53-CRD region containing alanine-scanning mutations. Bound proteins were analyzed as in (C). (F) MYBBP1A did not bind to p53 6KA mutant. *In vitro* translated MYBBP1A was incubated with p53 6KA mutant carrying simultaneous substitutions of lysines 370, 372, 373, 381, 382, and 386 by alanines. Bound proteins were analyzed as in (C).

3.3. MYBBP1A does not interact with p53 on the target gene promoter

It is believed that acetylated p53 binds to target gene promoters to exert its transcriptional activity [19,21]. In this case, MYBBP1A dissociates from p53 before recruitment to target gene promoters because MYBBP1A does not bind to acetylated p53 (Fig. 2). To clarify that MYBBP1A does not interact with p53 on the target gene promoters, we performed a chromatin immunoprecipitation (ChIP) assay and examined p53 or MYBBP1A recruitments to the promoter region of the *p21* gene. As shown in Fig. 3A, p53 recruitment to the promoter was abrogated by knockdown of MYBBP1A, indicating that MYBBP1A is required for the recruitment of p53 to p21 gene promoter. In contrast, MYBBP1A itself was not detected on the promoter region (Fig. 3A and B). These results proved that MYBBP1A does not interact with p53 on the target gene promoter, although MYBBP1A is necessary for the p53 recruitment to the promoter.

3.4. MYBBP1A specifically binds to nonacetylated p53 and promotes transcriptional activation efficiently

Based on our results, we propose a model of p53 activation by MYBBP1A in response to nucleolar stress (MYBBP1A cycling model: Fig. 4). Under nucleolar stress, rRNA transcription is inhibited and the rRNA content in the nucleolus is decreased, resulting in nucleolar disruption and translocation of MYBBP1A to the nucleoplasm [28]. The nucleoplasm-located MYBBP1A binds to nonacetylated p53 to promote p53–p300 association, resulting in p53 acetylation and activation. Activated p53 binds to the target gene promoters and regulates gene expression, resulting in apoptosis or cell cycle arrest.

It should be noted that MYBBP1A is not recruited to the p53 target promoter nor does it bind to acetylated p53. Therefore, once p53 is acetylated, MYBBP1A dissociates from p53 and interacts with the nonacetylated form of p53, which leads to another cycle of p53 activation. These findings suggest that MYBBP1A plays "catalytic" roles in p53 acetylation and activation under nucleolar stress.

During nucleolar stress, the p53 protein level dramatically increases by escaping HDM2-mediated degradation, whereas the amount of MYBBP1A remains unchanged. Therefore, a limited amount of MYBBP1A is needed to activate the function of p53 that is accumulated because of nucleolar stress. Thus, our "MYBBP1A cycling model," in which MYBBP1A functions as a "catalyst," explains why MYBBP1A efficiently activates p53 in response to cellular stress sensed by the nucleolus.

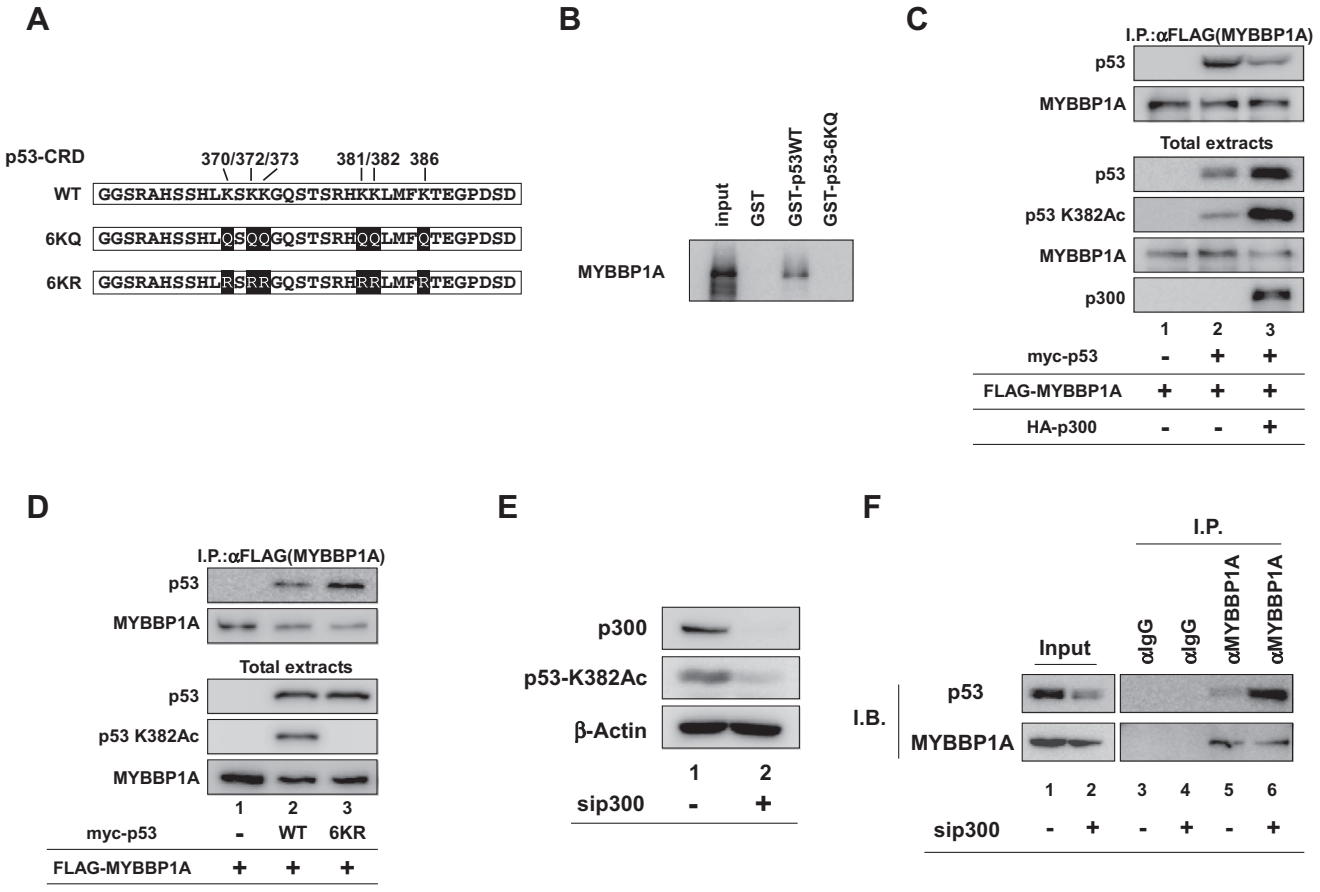

Fig. 2. MYBBP1A binds to non-acetylated p53 specifically. (A) Alignment of the sequence of the p53-CRD region and the 6KQ and 6KR mutants. Residues that have replaced the endogenous lysines are shown in black–white. (B) p53 6KQ mutant did not bind to MYBBP1A. *In vitro* translated MYBBP1A was incubated with the GST-fused full-length p53 proteins. Bound proteins were analyzed by immunoblotting. (C) Overexpression of p300 increased acetylation of p53 and decreased binding of p53 to MYBBP1A. H1299 cells were transfected with a combination of the expression vectors for FLAG-MYBBP1A, p53 and HA-p300 as indicated. Twenty-four hours after transfection, the cells were treated with ActD for 6 h. MYBBP1A was immunoprecipitated from the cell lysates using an anti-FLAG antibody and subjected to immunoblotting using an anti-p53 and -MYBBP1A antibody. (D) p53–6KR mutants bind to MYBBP1A stronger than WT-p53. H1299 cells were transfected with a combination of the expression vectors for FLAG-MYBBP1A, and wild type or mutated p53 as indicated. Twenty-four hours after transfection, the cells were treated with ActD for 6 h. MYBBP1A was immunoprecipitated from the cell lysates using an anti-FLAG antibody and subjected to immunoblotting using an anti-p53 antibody. (E and F) Interaction between p53 and MYBBP1A was increased by knockdown of p300. MCF-7 cells were treated with ActD for 6 h after transfection of control or p300 siRNA. The cell lysates were immunoprecipitated with normal rabbit IgG or anti-MYBBP1A antibodies and analyzed by immunoblotting using antibodies against MYBBP1A and p53.

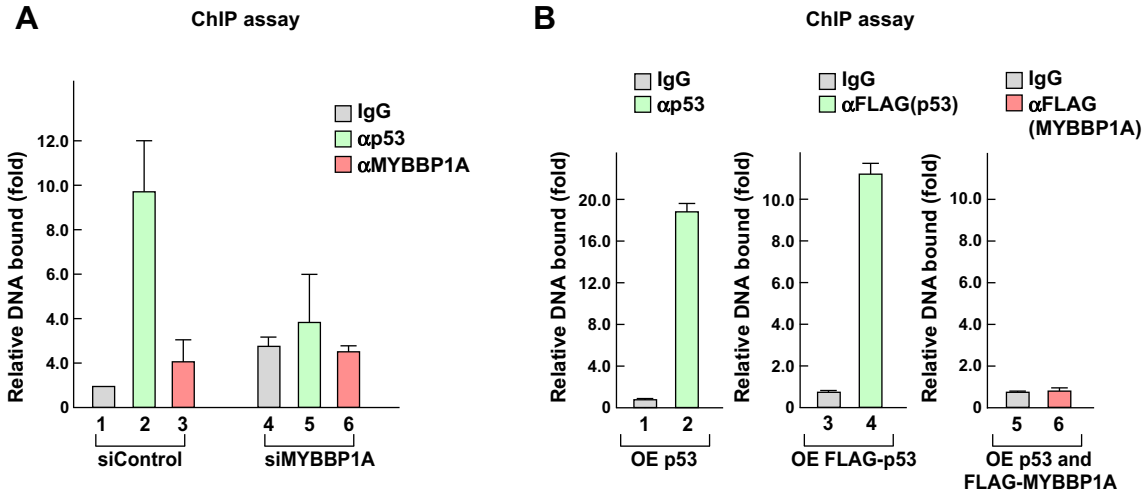

Fig. 3. MYBBP1A does not interact with p53 on the target gene promoter. (A) MYBBP1A is not recruited to p21 promoter in MCF-7 cells. MCF-7 cells were treated with indicated siRNA for 48 h before treatment with ActD for 6 h. A ChIP assay was performed using normal rabbit IgG, anti-p53, and anti-MYBBP1A antibodies. The p53-binding region of the p21 promoter was amplified and analyzed by qPCR. (B) MYBBP1A is not recruited to p21 promoter in H1299 cells. H1299 cells were transfected with indicated plasmids for 24 h before treatment with ActD for 6 h. A ChIP assay was performed using normal mouse IgG, anti-p53 or anti-FLAG antibodies. The p53-binding region of the p21 promoter was amplified and analyzed by qPCR. Bars represent mean + s.d. ($n = 3$).

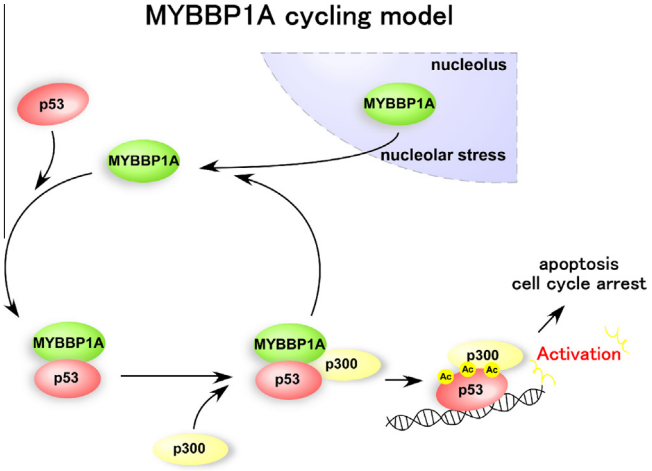

Fig. 4. Proposed model for the role of MYBBP1A on p53 activation following nucleolar stress. See the text.

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