Ebp1 Association with Nucleophosmin/B23 Is Essential for Regulating Cell Proliferation and Suppressing Apoptosis*

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Ebp1 and NPM/B23 are essential for cell proliferation and survival. Ebp1 possesses p42 and p48 isoforms. Whereas p42 exclusively resides in the cytoplasm, p48 localizes in both the cytoplasm and the nucleolus. Here, we show that Ebp1 forms a complex with B23, and this complex plays a critical role in cell proliferation and survival. p42 specifically associates with B23 upon epidermal growth factor stimulation, while p48 constantly binds B23. Moreover, Ser360 phosphorylation in p42, but not p48, is critical for the interaction. p48 constitutively binds B23 in the nucleolus, for which B23 Lys263 sumoylation is indispensable. By contrast, p42 selectively binds unsumoylated B23 mutants. Interestingly, B23 K263R, an unsumoylated mutant, triggers p42 nuclear translocation and interacts with it in the nucleus even in the absence of epidermal growth factor. In contrast, the nucleolar residency of p48 is abolished in B23 K263R cells. During the cell cycle, p42 selectively colocalizes with B23 in the mitotic cells, correlating with its phosphorylation status in mitosis. Knocking down of B23 or Ebp1 substantially decreases ribosome biogenesis and cell survival. Thus, B23 distinctly binds Ebp1 isoforms and regulates cell proliferation and survival through p42 and p48, respectively.

Ebp1, a member of the PA2G4 family of proliferation-regulated proteins, was initially identified as an ErbB3-binding protein in a yeast two-hybrid screen searching for the binding partners for the cytoplasmic region of ErbB3 (1). Overexpression of Ebp1 inhibits the growth of human breast and prostate cancer cells and induces cellular differentiation (2), probably through repressing transcription of both E2F1 and androgen receptor-regulated genes (3–5). Ebp1 participates in transcriptional regulation via its interaction with the Sin3-HDAC (6). Ebp1 also binds to the E2F1 promoter in a complex with the E2F1 transcription factor and Rb, which is regulated by the ErbB3 ligand heregulin (7). Recent evidence shows that EBP1 acts as a nucleolar growth-regulating protein linking between ribosome biosynthesis and cell proliferation. For instance, EB1 overexpression inhibits proliferation of human fibroblasts, and this effect is coupled to its nucleolar localization (8). Interestingly, Ebp1 is a part of ribonucleoprotein (RNP) complex and contains a dsRBD that mediates its interaction with dsRNA. Deletion of the Ebp1 dsRBD impairs its localization to the nucleolus and its ability to form RNP complexes (8). In the cytoplasm, Ebp1 is associated with mature ribosomes and that it is able to inhibit the phosphorylation of eukaryotic initiation factor 2 α (eIF2α). Ebp1 overexpression prevents eIF2α from phosphorylation, suggesting that it could be involved in protein translation control (9). Recently, we show that Ebp1 promotes cell survival by preventing apoptotic DNA fragmentation through a complex with active nuclear Akt, which is regulated by Ebp1 phosphorylation via PKCδ (10).

Ebp1 is ubiquitously expressed in all tissues and cells with two mRNA transcripts ~2.2 kb and 1.7 kb in size that encode two different alternatively spliced isoforms, p48 and p42, which differentially mediate PC12 cell survival and differentiation. p42 mRNA skips an exon consisting of 29 nucleotides, which contains 2nd ATG in p48 mRNA. Thus, p42 protein translation is initiated from the 3rd ATG. Although the two mRNA transcripts concentrations are comparable, in many mammalian cells, p48 is predominant form and p42 is barely detectable (1, 11). The molecular mechanism suppressing p42 protein level remains unknown. p48 that is 54 amino acids longer than p42 in the N terminus localizes in both the cytoplasm and the nucleus and suppresses apoptosis, whereas the shorter form p42 predominantly resides in the cytoplasm and promotes cell differentiation. EGF2 strongly stimulates p42 to bind ErbB3, and the association depends on PKC-mediated phosphorylation of Ebp1. By contrast, p48 does not bind to ErbB3 regardless of EGF treatment. Moreover, nerve growth factor (NGF) elicits extensive sprouting in p42 stably transfected PC12 cells, whereas p48 cells reveal modest neurite outgrowth (11). Therefore, p48 and p42 possess distinctive cellular functions.

Protein B23, also known as nucleophosmin (NPM) or NO38, is one of the most abundant proteins in the nucleolus (12, 13). B23 is implicated in ribosome biogenesis and cleaves the second internal transcribed spacer (ITS2) in 32S pre-rRNA, generating mature 28S rRNA (14, 15). B23 also resides in the cytoplasm and mediates centrosome duplication (16, 17) and senses cellular stress (18, 19). Like Ebp1, B23 is a component of pre-ribosomal ribonucleoprotein (RNP) complexes (20, 21). In addition to the above functions, B23 has a tumor suppression function through p53, because overexpression of B23 induces a p53-dependent cell cycle arrest in normal fibroblast cells (22). Never-
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in PBS at room temperature for 10 min. The cells were then permeabilized and blocked by 0.4% Triton X-100 and 2% FBS in PBS at room temperature for 15 min, washed with PBS three times and treated with several antibodies. Fluorescent images were taken by OLYMPUS IX71 fluorescence microscope.

Ribosome Biogenesis Assay—For monitoring ribosomal RNA processing, pulse-chase experiments using [methyl-3H]methionine were conducted based on the methylation of ribosomal RNA precursors and rapid turnover of the cellular pool of methionine, which provides [3H]methyl. Cells were infected with control adenovirus or virus expressing shRNA of Ebp1 or B23. In 48 h, the infected cells were starved of methionine for 30 min on 6-well plates with 1 ml of methionine-free medium and were labeled with 50 μCi of [methyl-3H]methionine (Amersham Biosciences) for 30 min. Cold methionine (15 μg/ml) was added to chase the label for 45 min. After washing with PBS, the total RNA was purified by an RNA isolation system (Promega) and the incorporated radioactivity was measured by a liquid scintillation counter. Equal amounts of radioactivity were loaded onto 1% agarose denaturing gel containing 0.55 M formaldehyde. The RNA was fractionated and transferred onto a Zeta-probe blotting membrane (Bio-Rad). The membrane was dried and sprayed by EN3HANCE (PerkinElmer Life Sciences) and exposed to Hyperfilm MP (Amersham Biosciences) at −80 °C with an intensifying screen for 7 days.

Genomic DNA Fragmentation—Oligonucleosomal fragmentation of genomic DNA was determined as described below. In brief, after transfection or infection, the cells were incubated with 50 μM VP16 or 1 μM staurosporine for 16 h. At the end of incubation, cells were pelleted, washed twice with ice-cold PBS, and lysed at 37 °C for 2 h in 500 μl of lysis buffer (100 mM Tris/HCl (pH 8.5), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K). Samples were treated with phenol/ chloroform and then centrifuged, the supernatants were treated isopropyl alcohol precipitation. This pellet was dissolved in TE buffer containing RNase A (10 μg/ml) at 37 °C for 2 h, then the same amount DNA (10 μg) was electrophoresed at 100 V for 1 h through a 2% agarose gel containing ethidium bromide in TAE buffer (0.04 M Tris acetate/0.001 M EDTA, pH 8.0). DNA bands were visualized under UV light.

Co-immunoprecipitation and in Vitro Binding Assays—A 10-cm plate of HEK293 cells were washed once in PBS, and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na2VO4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride), and centrifuged for 10 min at 16,000 × g at 4 °C. The supernatant was transferred to a fresh tube. The experimental procedures for co-immunoprecipitation and in vitro binding assays are as described (34). After SDS-PAGE, the samples were transferred to a nitrocellulose membrane. Western blotting analysis was performed with a variety of antibody.

Cell Synchronization—The cells were initially plated at a density of ~5 × 105 cells in a 10-cm dish. One day after seeding, the cells were incubated with 2 mM thymidine-containing medium, and 24 h later, the medium was removed, and the cells were washed twice with prewarmed PBS at 37 °C and incubated in fresh thymidine-free medium for 8 h.

Nevertheless, other studies demonstrate that high level expression of B23 correlates with uncontrolled cell growth, suggesting that B23 may have oncogenic potential (23–25). Overexpression of B23 in NIH-3T3 cells resulted in malignant transformation and tumorigenesis in nude mice (26). Such tumor cells also became resistant to UV-induced cell cycle arrest and apoptosis (27, 28). Conversely, mice lacking B23 die in early embryonic stages (29, 30), indicating an essential role for B23 in embryonic development. Employing PI(3,4,5)P3 column and NGF-treated PC12 nuclear extracts, we recently identified B23 as a nuclear PI(3,4,5)P3-binding protein. B23/PI(3,4,5)P3 complex mediates the anti-apoptotic effects of NGF by inhibiting DNA fragmentation activity of caspase-activated DNase CAD (31). ARF tumor suppressor binds B23 and enhances its sumoylation (32). However, the biological effects of this event remain unknown. Most recently, we show that B23 is sumoylated on both lysine 230 and 263 residues, but the latter is the major one. Mutation of Lys263 but not Lys230 into Arg abolishes its centrosomal and nucleolar residency (33).

In this report, we show that Ebp1 interacts with B23. p48 isoform constitutively associates with B23, which is mediated by B23 sumoylation; whereas p42 interacts with B23 in response to EGF stimulation, for which Ser360 phosphorylation by B23 sumoylation; whereas p42 interacts with B23 in

EXPERIMENTAL PROCEDURES

Cells and Reagents—PC12 cells were maintained in DMEM with 10% fetal bovine serum (FBS), 5% horse serum. Ebp1 p42 or p48 or B23 or control empty vector stably transfected PC12 cells (Tet-off cell lines) were maintained in DMEM containing 10% FBS, 5% horse serum, 100 μg/ml G418, 100 μg/ml hygromycin B, and 2 μg/ml tetracycline. The transfected genes were induced by culturing in medium without tetracycline for 24 h. HEK293 and HeLa cells were grown in DMEM containing 10% FBS. EGF was from Roche Applied Science. Anti-Ebp1 C antibody was from Upstate Biotechnology, Inc. Anti-Ebp1-N antibody was raised in rabbit against the N-terminal 22 residues in p48. All the chemicals not included above were purchased from Sigma. GFP-Ebp1 constructs are kindly supplied by Dr. Giulio Draetta (European Institute of Oncology, Milan, Italy).

Fluorescence Microscope Analysis—HEK293 cells were seeded on poly-L-lysine coated coverslips in 12-well dish. After overnight growth, cells were transfected using calcium phosphate method. Twenty-four hours after transfection, cells were incubated with DMEM without FBS for 24 h and then treated with 100 ng/ml EGF 30 min. For mitotic cell synchronisation, cells were treated with 100 ng/ml nocodazole for 12 h. PC12 or HeLa cells were treated with 50 μM VP16 or 1 μM staurosporine for 16 h. After apoptotic stimuli, cells were treated with 2 μM propidium iodide and 200 ng/ml Hoechst33342 for 10 min and then washed with PBS. Cells were fixed with 3% formaldehyde in PBS at room temperature for 10 min. The cells were then permeabilized and blocked by 0.4% Triton X-100 and 2% FBS in PBS at room temperature for 15 min, washed with PBS three times and treated with several antibodies. Fluorescent images were taken by OLYMPUS IX71 fluorescence microscope.

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The cells were then cultured in medium supplemented with 2 mM thymidine for an additional 16 h. After aspirating the medium, the cells were washed three times with PBS pre-warmed at 37 °C and then incubated in fresh medium. At various times after release from the second thymidine block, the cells were harvested and lysis. For mitotic arrest, we employed 100 ng/ml nocodazole for 12 h.

**Flow Cytometric Analysis of Cell Cycle Status**—The flow cytometric evaluation of the cell cycle status was performed by a modification of a published method (35). Briefly, 2 × 10^6 transfected or control or shRNA-treated HEK293 cells were centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol. Tubes containing the cell pellets were stored at −20 °C. After this, the cells were centrifuged at 1,000 × g for 10 min, and the supernatant was discarded. The pellets were resuspended in 30 μl of phosphate/citrate buffer (0.2 Na₂HPO₄, 0.1 citric acid, pH 7.5) at room temperature for 30 min. Cells were then washed with 5 ml of PBS and incubated with propidium iodide (20 g/ml)/RNase A (20 g/ml) in PBS for 30 min. The samples were analyzed on a Coulter Elite flow cytometer.

**RESULTS**

**B23 Binds to Ebp1**—Both Ebp1 and B23 localize in the nucleolus and prevent DNA fragmentation during apoptosis (10, 31). To explore whether they interact with each other, we conducted a binding assay with a variety of B23 fragments (Fig. 1A). GFP-p48 was transfected into HEK293 cells, and cell lysates were incubated with GST-B23 recombinant protein conjugated beads. Immunoblotting analysis reveals that GFP-p48 binds to full-length B23. Nonetheless, the strongest binding occurs to B23 truncates 14–107 and 84–151. Interestingly, B23 186–239 fails to interact with B23, suggesting that amino acids 186–239 is essential for B23 association with Ebp1. Immunoblotting with both anti-GFP and anti-Ebp1 reveals the same results (Fig. 1B, top and 2nd panels), indicating both the transfected and endogenous Ebp1 bind to B23. By contrast, GFP alone is unable to bind B23 (data not shown). To explore whether these 53 residues (amino acids 186–239) are sufficient for binding to Ebp1, we prepared the recombinant protein, and find that it binds to Ebp1 p48 much stronger than to p42, but it does not bind GFP control. GST control is unable to interact with any protein (Fig. 1C). To investigate which region of Ebp1 is implicated in binding to B23, we transfected
various GFP-tagged Ebp1 fragments into HEK293 cells, and their cell lysates were incubated with recombinant GST-B23 ΔA84–151. GST pull-down assay reveals that only full-length Ebp1 but not any other truncates associate with B23, indicating that the intact three-dimensional conformation is critical for Ebp1 p48 to interact with B23 in vitro (Fig. 1, D and E).

**EGF Regulates p42 Ebp1 Association with B23 in Mammalian Cells**—To examine whether the association between Ebp1 and B23 can be regulated by growth factors, we co-transfected Myc-B23 into HEK293 cells with various Ebp1 constructs, followed by EGF treatment for 30 min. Immunoprecipitation reveals that Ebp1 p42 displays even stronger affinity to B23 than p48. Truncation of the N-terminal 23 amino acids (p44 Ebp1) slightly increases the binding activity (Fig. 2A, upper left panel). To further explore the interaction between Ebp1 and B23, we serum-starved HEK293 cells and treated the cells with or without EGF. Coimmunoprecipitation studies demonstrate that both p48 and p42 are coprecipitated by anti-B23 antibody upon EGF stimulation. The specificity of the immunoprecipitated is verified with control IgG (Fig. 2B, upper left panel). Ebp1 p48 and p42 isoforms are verified in the whole cell lysates (upper right panel). The reciprocal immunoprecipitation was conducted with two different Ebp1 antibodies, which recognize both p42 and p48 (anti-Ebp1 C terminus) and p48 specific (anti-Ebp1 N terminus). The co-precipitated proteins were analyzed with anti-B23 antibody. Interestingly, B23 co-precipitates with p48 (anti-Ebp1 N) regardless of EGF stimulation, whereas B23 is selectively pulled down by anti-Ebp1 C antibody upon EGF stimulation (Fig. 2B, lower panel). The peptide corresponding to amino acids 186–239 of B23 strongly binds to both p42 and p48. To examine whether this short peptide overexpression inhibits B23/Ebp1 binding, we performed coimmunoprecipitation studies after transfection of B23 fragment. Compared with GST control, overexpression of GST-B23 (amino acids 186–239) completely disrupts the interaction between the association between endogenous B23 and Ebp1 isoforms (top and 2nd panels), indicating that residues 186–239 from B23 are implicated in binding to Ebp1. Neither anti-Ebp1 C nor anti-Ebp1 N antibody recognizes GST-B23 (amino acids 186–239) (data not shown). These findings suggest that B23 binding to p42 is up-regulated by EGF, whereas B23 constitutively associates with p48. Immunostaining with co-transfected GFP-p48 and Myc-B23 verifies that p48 always co-localizes with B23 in the nucleolus. By contrast, p42 predominantly resides in the cytoplasm, and EGF stimulation provokes its association with nucleolar B23 (Fig. 2D). Subcellular fractionation correlates with this observation. Myc-B23 mainly occurs in the nuclear fraction, and the cytosolic B23 might be centrosomal localized B23 (Fig. 2E).

**Ebp1 p42 Ser360 Phosphorylation Is Essential for Its Interaction with B23**—P42 binding to B23 requires EGF stimulation, indicating that EGF-mediated post-translational modification might regulate the complex formation. Our previous studies demonstrate that EGF or NGF triggers potent Ser360 phosphorylation in Ebp1. To assess whether this phosphorylation plays any role in regulating the interaction between Ebp1 and B23, we cotransfected Myc–B23 into HEK293 cells with various p42 constructs and the transfected cells were serum-starved overnight. GST pull-down assay shows that B23 strongly binds to p42 S360D, a phosphorylation mimic mutant, whereas wild-type p42 and S360A, a nonphosphorylated mutant, barely interact with B23. Neither wild-type p42 nor S360A was phosphorylated (data not shown). This finding underscores that p42 Ser360 phosphorylation is required for its association with B23 (Fig. 3A, right panels). In contrast, p48 Ebp1 wild-type and mutants reveal similar binding affinity to B23 (Fig. 3A, left panels). To further investigate the effect of Ser360 phosphorylation in p42 binding to B23, we co-transfected GFP-B23 into HEK293 cells with GST-p42 wt or S360A, respectively, and treated the co-transfected cells with EGF. As expected, wild-type p42 potently associates with B23 upon EGF stimulation, whereas S360A fails regardless of EGF treatment (Fig. 3B, top panel). Ebp1 S360 is potently phosphorylated in wild-type p42, which is completely abolished in S360A mutant (Fig. 3B, bottom panel). Hence, these data support that Ser360 phosphorylation is essential for the interaction between B23 and p42.

Murine p38-2G4 (grolification associate 2G4, PA2G4), a homolog of human Ebp1, was initially cloned as cell cycle-regulated protein (36). To examine whether human Ebp1 is also regulated by cell cycle, we monitored Ebp1 expression pattern and Ser360 phosphorylation. p48 remains unchanged in all phases, whereas p42 expression peaks in S phase and decreases in G2/M phase, and minimizes in G1 phase. Strikingly, p42 is selectively phosphorylated in G2/M phase, while p48 phosphorylation doublet remains constant in all phases (Fig. 3C). To explore whether the cell cycle-specific phosphorylation mediates p42 binding activity to B23, we co-transfected GST-B23 into HEK293 cells with GFP-p42 and p48, respectively. GST pull-down assay reveals that p48 interacts with B23 under both asynchronous and mitotic-arrested cells; by contrast, p42 selectively binds B23 in mitotic arrested cells but not in asynchronous cells (Fig. 3D, top panel). Interestingly, coprecipitated Ebp1 is specifically phosphorylated in mitotic cells (Fig. 3D, 2nd panel). GFP-p42 phosphorylation correlates with its binding activity (Fig. 3D, bottom panel). Immunostaining confirms that p42 colocalizes with B23 in mitotic cells but not in interphase cells (Fig. 3E). Taken together, these data demonstrate that Ser360 phosphorylation is essential for the association between p42 and B23.

**B23 Sumoylation Plays Distinctive Roles in Regulating Its Binding to p42 and p48 Ebp1**—Our previous study reveals that B23 can be sumoylated on both Lys230 and Lys265 residues with the latter the major site. To explore whether B23 sumoylation plays any role in the association between B23 and Ebp1, we co-transfected various B23 constructs into HEK 293 cells with Ebp1 p42 and p48, respectively. Co-immunoprecipitation studies demonstrate that unsumoylated B23 K230R and K263R mutants bind to p42, while wild-type B23 does not. Surprisingly, the robust interaction between p48 and B23 was completely blocked by K263R mutation, and it is also decreased by K230R mutation, demonstrating that Lys265 sumoylation is required for the association between B23 and p48. Interestingly, abolishing B23 sumoylation enhances its binding to p42 (Fig. 4A). To further examine the effect of sumoylation on the interaction between B23 and Ebp1, we conducted immunofluorescent staining studies. p48 co-localizes with wild-type B23 and K230R in the nucleus. However, B23 K263R does not reside in
EGF regulates the association between B23 and Ebp1. A, Ebp1 interacts with B23 in mammalian cells. Myc-B23 was co-transfected with various GFP-Ebp1 constructs into HEK293 cells, followed by EGF stimulation for 30 min. The cell lysates were incubated with anti-Myc agarose. Immunoblotting analysis of coprecipitated proteins reveals that both p42 and p48, as well as N-terminal 23 amino acids truncated p44, interact with B23 in vivo. As negative control, GFP does not (top panel). Verification of transfected and pulled down proteins are in second to bottom panels. B, EGF regulates endogenous B23 binding to Ebp1. HEK293 cells were serum-starved overnight, followed by 50 ng/ml EGF stimulation for 30 min. B23 antibody coprecipitates both p48 and p42 after EGF stimulation (upper left panel). Immunoblotting with anti-Ebp1 C antibody verifies both p42 and p48 expression in the cell lysates (upper right panel). P48-specific antibody (anti-Ebp1 N) precipitates B23, and EGF slightly enhances the interaction, whereas anti-Ebp1 C antibody, which recognizes both p42 and p48, weakly pulls down B23 in the absence of EGF. EGF substantially increases Ebp1 binding to B23 (lower left panel). C, B23 amino acids 186–239 overexpression disrupts its interaction with Ebp1. GST-B23 (amino acids 186–239) or GST was transfected into HEK293 cells. After 24 h, the cells were serum-starved overnight, followed by 50 ng/ml EGF treatment for 30 min. The interaction between endogenous B23 and Ebp1 is disrupted when B23 (amino acids 186–239) is overexpressed (top and second panels). D, immunofluorescent staining of B23 and Ebp1. GFP-p42 and Myc-B23 were cotransfected into HEK293 cells, followed by EGF stimulation for 30 min. B23 was stained with mouse monoclonal anti-Myc antibody, followed by Texas-Red conjugated anti-mouse secondary antibody. GFP-p42 co-localizes with B23 in the nucleolus irrespective of EGF stimulation (right panels). E, subcellular fractionation. GFP-p42 and Myc-B23 were cotransfected into HEK293 cells, followed by EGF stimulation for 30 min. The cytosolic and nuclear fractions from the transfected cells were prepared. GFP-p42 predominantly resides in the cytoplasm, and EGF treatment enhances its nuclear translocation, whereas GFP-p48 occurs in both the cytosolic and the nuclear fractions regardless of EGF treatment (top panel). Myc-B23 mainly distributes in the nuclear fraction. The cytosolic B23 might be centrosomally localized B23 (second panel). The identity of cytosolic and nuclear fractions is verified by the specific markers: tubulin and PARP.
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**FIGURE 3. Ebp1 p42 Ser360 phosphorylation is essential for its interaction with B23.** A, phosphorylation of p42 regulates its binding affinity to B23. Myc-B23 was cotransfected into HEK293 cells with GFP-Ebp1 wild-type and phosphorylation mutants. The transfected cells were serum-starved overnight. B23 was immunoprecipitated with anti-Myc-agarose, and the coprecipitated proteins were analyzed by Western blotting with anti-GFP antibody. Phosphorylation mimetic S360D, but not S360A or wild-type p42 binds to B23 in the absence of EGF (top right panel). p48 wild-type, S360A, and S360D mutants all interact with B23 (top left panel). Verification of transfected and pulled-down proteins (second to bottom panels). B, unphosphorylated p42 S360A does not bind to B23 in the presence of EGF. Wild-type or S360A p42 was co-transfected into HEK293 cells with GFP-B23. Twenty-four hours after serum starvation, the cells were treated with or without 50 ng/ml EGF for 30 min. p42 was pulled down with glutathione beads, and the associated proteins were analyzed with anti-GFP antibody (top panel). Verification of transfected and pulled-down proteins is shown in the second to fourth panels. C, p42 expression and phosphorylation is regulated by cell cycle. HEK293 cells were synchronized by double thymidine treatment. After release, different phase cells were collected and lysed and analyzed with anti-Ebp1 (C and N) and anti-phospho-Ser360 antibodies, respectively. D, p42 expression maximizes in the S phase, whereas p42 is selectively phosphorylated in G2/M phase. E, p42 binds B23 in mitotic cells. GFP-p42 or p48 was co-transfected into HEK293 cells with GST-B23. The transfected cells were treated with 100 ng/ml nocodazole for 12 h to arrest cells in mitosis. GST pull-down reveals that p42 strongly binds to B23 in mitotic but not asynchronous cells; in contrast, p48 associates with B23 in both cells (top panel). B23 coprecipitated Ebp1 is highly phosphorylated (second panel). Verification of the transfected proteins (third to fifth panels). Phosphorylation of p42 Ser360 is increased in mitotic cells, while p48 is phosphorylated in both mitotic and asynchronous cells (bottom panel). E, immunofluorescent staining of mitotic and interphase cells transfected with GFP-Ebp1 isoforms and B23. In mitotic cells, B23 and p42 co-localize, but p42 and p48 do not but p42 distribute in the nucleus in the interphase cells.

the nucleolus in the co-transfected cells, whereas p48 mostly localizes in the cytoplasm (Fig. 4B, arrows, bottom panel). Therefore, these results demonstrate that B23 nucleolar residency is necessary for p48 nucleolar localization (Fig. 4B, lower panels). p42 mainly distributes in the cytoplasm in both wild-type B23 and K230R-transfected cells. Surprisingly, p42 translocates into the nucleus, co-localizing with B23 K263R mutant, which distributes in the nucleoplasm (Fig. 4B, upper panels). Quantitative analysis reveals that p42 translocates into the nuclei of 30% B23 K263R transfected cells; by contrast, less than 10% B23 K263R transfected display p48 nucleolar residency (Fig. 4C). Subcellular fractionation demonstrates that p42 nuclear translocation increases in B23 K263R-transfected cells, whereas p48 nuclear distribution reduces in B23 K263R-transfected cells (Fig. 4D). Therefore, B23 sumoylation plays an essential role in dictating its association with Ebp1 isoforms.

**Ebpl and B23 Binding Is Essential for Cell Proliferation—NPM/B23 plays a critical role in rRNA processing. Its endoribonuclease activity is well documented (37, 38). B23 cleaves the second internal transcribed spacer (ITS2) in rRNA precursor, eliciting rRNA digestion by exonucleases and subsequent maturation into 28S rRNA, a major RNA component in the 60S ribosomal subunit (Fig. 5A) (14, 39). Ebp1 is part of ribonucleoprotein complexes and associates with different rRNA species (8). To determine the role of Ebp1 in dynamic processing of rRNA precursors and intermediates, we carried out pulse-chase labeling analysis of newly synthesized rRNA in PC12 cells, which were infected with adenovirus expressing shRNAs to deplete B23 or Ebp1, respectively. The expression level of endogenous B23 or Ebp1 is verified after shRNA knocking down (Fig. 5B). The pulse-labeled 47S rRNA precursor is readily detected in all treatments at the beginning of chasing (0 min), suggesting the transcription of the rRNA precursor is not inhibited by any of the treatments. At 45 min, the mature 28S rRNA is almost comparable to the 32S intermediate in cells infected with control adenovirus, indicating an active processing of the 32S precursor. By contrast, when cells...
were infected with B23 or Ebp1 shRNA, the level of 28 S rRNA is markedly decreased and the ratio of 28S/32S is reduced to 0.35 and 0.45, respectively (Fig. 5C), demonstrating that downregulation of B23 or Ebp1 p48 inhibits processing of the 32S intermediate for subsequent maturation of the 28S rRNA. Thus, like B23, Ebp1 also plays a critical role as an ITS2-specific endoribonuclease that cleaves 32S intermediate into 28S rRNA. Cell proliferation is actively coordinated with rRNA processing and ribosome assembly (8). To further explore the role of Ebp1 in cell proliferation, we conducted FACS analysis assay with HEK293 cells transfected with various Ebp1 constructs. The distribution of different cell phases in the transfected cells is summarized in the middle of Fig. 5D. Interestingly, compared with control and wild-type p42, its phosphorylation mimic, S360D, suppresses DNA synthesis, whereas the counterpart of p48 enhances DNA synthesis, fitting with previous findings that p42 and p48 possess opposite activities in cell growth (11). Wild-type p42 and its unphosphorylated mutant exhibit similar cell cycle profile as those of p48 (Fig. 5D, left panel), suggesting that phosphorylation plays a critical role in dictating Ebp1 cellular function in cell proliferation. On the other hand, knocking down of B23 with its shRNA evidently decreases cell distribution in S phase. In agreement with its anti-apoptotic role, ablation of B23 substantially increases cells in sub-G1 phase, indicating that depletion of B23 attenuates cell proliferation and triggers DNA fragmentation. Elimination of Ebp1 by its siRNA, which depletes both p42 and p48, also elicits S phase reduction. However, it does not significantly increase sub-G1 amount (Fig. 5E), suggesting that Ebp1 p48, which is the major isofrom, is necessary for cell proliferation but not as important as B23 for repressing apoptosis. Disrupting B23/Ebp1 complex by B23 (amino acids 186–239) prominently attenuates G2/M phase (Fig. 5F). Hence, both B23 and Ebp1 p48 are critical for ribosome biogenesis. Overexpression of Ser360 phosphorylated p48 enhances cell proliferation, whereas phosphorylated p42 inhibits cell growth.

Ebp1 and B23 Association Is Critical for Suppressing Apoptosis—Our previous studies demonstrate that B23 and Ebp1 play an important role in preventing apoptosis (10, 31). To explore whether B23/Ebp1 complex is critical for this event, we depleted Ebp1 from control and B23 stably transfected PC12 cells, followed by VP16 treatment. Quantitative apoptotic assay of propidium iodide uptake reveals that more than 50% cells are...
in apoptosis in the control cells after ablation of Ebp1. In B23-overexpressed cells, however, it was decreased to 35% (Fig. 6A, upper panel). The expression of Ebp1 and B23 is confirmed (Fig. 6A, lower panels). We conducted the similar experiments with Ebp1 p42 and p48 stably transfected PC12 cells. Ablation of B23 markedly elevates apoptosis in control cells and p42 stably transfected cells compared with p48 cells after VP16 treatment (Fig. 6B, upper panel). The expression of B23 and Ebp1 is confirmed (Fig. 6B, lower panels).

To further explore whether the association between B23 and Ebp1 is essential for preventing apoptosis, we transfected HeLa cells with GST-tagged B23 (amino acids 186–239) construct to disrupt the complex, and treated the cells with staurosporine for 12 h. Compared with B23 wild-type, overexpression of B23 (amino acids 186–239) fragment evidently increases apoptosis (Fig. 6C, left panel), underscoring that the association between B23 and Ebp1 is critical for preventing apoptosis. As predicted, (Δ186–293) truncation cripples the anti-apoptotic effect of B23. DNA fragmentation couples to the quantitative results (Fig. 6C, middle panel). The expression of GST-B23 constructs is confirmed (Fig. 6C, right panel). Collectively, these data support that the B23/Ebp1 complex is essential for blocking apoptosis.

DISCUSSION

In this report, we found that NPM/B23 potently associates with p48 Ebp1 in the nucleolus (Fig. 7). It also interacts with p42 Ebp1, nevertheless, which requires growth factor stimulation. We show that Ser360 phosphorylation in p42 is essential for its association with B23. In alignment with this observation, we found that p42 is selectively phosphorylated in mitosis. Accordingly, p42 robustly binds B23 in mitotic-arrested cells but not asynchronous cells. Although p48 constantly binds to B23, we discover that B23 sumoylation on Lys263...
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plays a critical role in the complex formation. On the contrary, unsumoylated B23 K263R mutant strongly associates with p42 Ebp1 even in the absence of growth factor, and provokes p42 nuclear translocation. Ablation of Ebp1 or B23 reduces ribosome biogenesis and decreases cell proliferation. Further, we show that abolishing the association between B23 and Ebp1 enhances DNA fragmentation and apoptosis. Thus, our findings demonstrate that B23/Ebp1 complex plays an important role in preventing DNA fragmentation, cell survival, and proliferation.

p38-2G4, a murine homolog of human Ebp1, was initially cloned as a cell cycle-specifically modified and proliferation-associated nuclear protein using a set of monoclonal antibodies prepared against DNA-binding proteins from Ehrlich ascites tumor cells (36). Consistent with this report, we found that p42 Ebp1 is expressed in a cell cycle-dependent manner. p42 is demonstrable in S and G2/M phases and negligible in G1 phase. By contrast, p48 is persistently expressed throughout the whole cell cycle. In alignment with p42 cell cycle specific expression profile, endogenous p42 protein level is much lower than p48 (Figs. 2B and 3C), although the two mRNA transcripts are comparable in all human tissue and cells (1). The molecular mechanism repressing p42 protein level remains unknown. Compared with the robust and constant Ser360 phosphorylation in p48, p42 phosphorylation selectively occurs in G2/M phase (Fig. 3C). FACS analysis reveals that p42 phosphorylation mimetic S360D mutant strongly decreases S phase (DNA synthesis) amount, whereas p48 counterpart increases S phase percentage, demonstrating that phosphorylation on serine 360 in p42 and p48 elicits opposite functions on cell proliferation (Fig. 5D).

At G1/S transition, the coordinated activation of E2F1 responsive promoters establishes a program of gene expression that is required for DNA replication. Hypophosphorylated retinoblastoma protein (Rb) binds E2F1 and blocks its transcriptional functions during G1 phase of the cell cycle, thereby preventing entry into S-phase. Mitogenic stimuli activate cyclin D/Cdk4 and Cdk6, which subsequently phosphorylate Rb and dissociate it from E2F1, resulting in cell entry into S phase. Interestingly, Ebp1 p42 associates with Rb and up-regulates its transcriptional suppressive activity on E2F1. Dephosphorylation of Ebp1 p42 enhances its binding to Rb (40). Conceivably, Ebp1 p42 phosphorylation decreases its

FIGURE 6. B23 and Ebp1 interaction enhances cell survival. A, depletion of Ebp1 sensitizes cells to apoptosis. Control and B23 stably transfected PC12 cells were induced in tet-off medium and infected with adenovirus expressing shRNA of Ebp1, followed by 50 μM VP16 treatment for 16 h. Apoptosis was analyzed by PI uptake assay. The upper panel shows quantification of apoptosis. Verification of Ebp1 and B23 expression level is shown (lower panels). B, ablation of B23 enhances cell death in Ebp1 stable cell lines. P42 and P48 stably expressed cell lines were induced in tet-off medium and infected with adenovirus expressing shRNA of B23, followed by VP16 treatment. Elimination of B23 substantially increases apoptosis in control and p42 cells, whereas apoptosis is significantly decreased in p48 compared with control (upper panel). Verification of Ebp1 and B23 expression levels is shown in the lower panels. C, disruption of B23/Ebp1 association elevates apoptosis. GST-tagged B23 wild-type, deletion mutant (Δ186–239), and fragment (amino acids 186–239) constructs were transfected into HeLa cells, followed by 1 μM staurosporine treatment. Compared with wild-type B23, both B23 (Δ186–239), unable to bind Ebp1, and B23 (amino acids 186–239), which disrupts B23/Ebp1 interaction, enhance DNA fragmentation (left panel). Quantitative analysis of propidium iodide uptake after staurosporine treatment is shown in the middle panel. Verification of B23 constructs expression is shown in the right panel. Significant differences (p values) were evaluated by counting five fields that contains at least 100 cells (Student’s t test).
binding to Rb, leading to E2F1 inactivation and DNA synthesis decrease.

p42 selectively binds B23 in response to EGF stimulation, for which Ser\textsuperscript{360} phosphorylation is critical. Immunoprecipitation with anti-Ebp1-C antibody, which recognizes both p42 and p48, pulls down B23 in an EGF-dependent manner. Nevertheless, immunoprecipitation with anti-Ebp1-N antibody, which selectively recognizes p48 but not p42, precipitates B23 regardless of EGF treatment. Surprisingly, B23 level in anti-Ebp1-C immunocomplex is much less than that in anti-Ebp1-N immunocomplex (Fig. 2B). Presumably, anti-Ebp1 C antibody possesses weaker immunoaffinity to both p42 and p48, leading to less amount of total Ebp1 precipitated than p48 pulled down by anti-Ebp1-N antibody. Interestingly, we observed that p42 specifically interacts with B23 in the mitotic arrest cells but not asynchronous cells, correlating with evident p42 phosphorylation on Ser\textsuperscript{360} (Fig. 3, C and D). On the other hand, different kinases phosphorylate nucleophosmin/B23 at different sites during G\textsubscript{2} and M phases of the cell cycle (41). NPM/B23 associates specifically with unduplicated centrosomes, and NPM/B23 dissociates from centrosomes by CDK2/cyclin E-mediated phosphorylation (16). Presumably, B23 phosphorylation in G\textsubscript{2}/M phase also contributes to its association with phosphorylated p42. We have previously shown that nuclear PI(3,4,5)P\textsubscript{3} lipid binds B23 in NGF-treated PC12 cells. B23/PIP3 complex directly interacts with caspase-activated Dnase (CAD) and prevents its endonuclease activity. Depletion of B23 enhances PC12 cells and hippocampal neuronal cell death (31). In addition, we found that Ebp1 p48 strongly binds nuclear active Akt, which is regulated by PKC. Ser\textsuperscript{360}-phosphorylated p48/p-Akt complex strongly blocks DNA fragmentation elicited by CAD or the cell-free apoptosomes (10). Here, we show that Ebp1 and B23 strongly bind to each other. Ablation of either one sensitizes apoptotic stimuli-triggered cell death and DNA fragmentation (Fig. 6, A and B). Moreover, overexpression of a fragment of B23 (amino acids 186–239), which implicates in binding to Ebp1, disrupts the complex and accelerates apoptosis (Fig. 6C), underscoring the interaction between B23/Ebp1 plays an important role in maintaining cell survival.

Recently, we report that B23 is sumoylated on both lysine 230 and 263 residues. Mutation of Lys\textsuperscript{263} but not Lys\textsuperscript{230} into Arg abolishes its centrosomal and nucleolar residency. Moreover, Rb binds to wild-type B23, but fails to interact with K263R. Sumoylation enhances B23 binding to Rb. Consequently, B23 potently stimulates E2F1-mediated transcriptional activity, of the N-terminal 54 residues, which forms one and half α-helix, exposes hydrophobic domain in p42 to hydrophilic solvent and elicits p42 refolding, resulting in recruitment of new binding partners to stabilize p42 (42, 43). Thus, p42 and p48 might exhibit different conformations, leading to divergent binding activities to various partners. It has been shown before that ARF tumor suppressor binds B23 and enhances its sumoylation (32). Conceivably, ARF might also regulate the association between B23 and Ebp1 and mediate their physiological functions. Hyperphosphorylated Rb is imported into the nucleolus in S or G\textsubscript{2} phase through binding to B23 (44). Ebp1 p42 also binds Rb, enhancing its transcriptional repressive activity (40). It remains unknown whether p48 also directly interacts with Rb. Given the cell proliferation stimulatory activity, imaginably, nucleolar p48 might sequester phosphorylated Rb into the nucleolus and release E2F1 from its inhibition. Based on our observation that S360D p48 enhances S phase (Fig. 5D), we predict that p48 Ser\textsuperscript{360} phosphorylation will augment this binding, facilitating cell proliferation. This putative oncogenic activity by p48 is consistent with most recent report that tamoxifen (TAM) treatment decreases p48 transcription and protein levels in the estrogen receptor (ER)-positive MCF-7 breast cancer cell line. In addition, the patients with high PA2G4-expressing tumors exhibit poorer clinical outcome. These results suggest that Ebp1 p48 may be a novel survival-associated breast cancer gene (45). The molecular mechanism underlying p42 and p48 contradictory tumor suppressive and oncogenic effects awaits further investigation. Clearly, the answers to this question will lead to better understanding about the physiological functions of Ebp1-B23 complex in cell proliferation and survival.

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