Novel nucleolar pathway connecting intracellular energy status with p53 activation

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Running title: The nucleolus and p53 activation
SUMMARY
In response to a shortage of intracellular energy, mammalian cells reduce energy consumption and induce cell cycle arrest, both of which contribute to cell survival. Here we report that a novel nucleolar pathway involving the energy-dependent nucleolar silencing complex (eNoSC) and Myb-binding protein 1a (MYBBP1A) is implicated in these processes. Namely, in response to glucose starvation, eNoSC suppresses rRNA transcription, which results in a reduction in nucleolar RNA content. As a consequence, MYBBP1A, which is anchored to the nucleolus via RNA, translocates from the nucleolus to the nucleoplasm. The translocated MYBBP1A induces acetylation and accumulation of p53 by enhancing the interaction between p300 and p53, which eventually leads to the cell cycle arrest (or apoptosis). Taken together, our results indicate that the nucleolus works as a sensor that transduces the intracellular energy status into the cell cycle machinery.

INTRODUCTION
Intracellular energy balance is important for cell survival. In response to nutrient or environmental stresses that cause reduction in the intracellular energy level, mammalian cells sense the intrinsic energy status and attempt to restore bioenergetic homeostasis through compensatory changes in the regulation of intermediate metabolic processes. One such mechanism is the reduction of ribosome biosynthesis, a major biosynthetic and energy-consuming process in mammalian cells; while the other mechanism is the arrest of cell proliferation. The LKB1-AMP-activated protein kinase (AMPK) pathway is reportedly involved in both regulatory processes (1-5). During energy starvation or glucose deprivation, when the cellular AMP/ATP ratio is increased, the LKB1-AMPK pathway is activated. This signaling in turn inhibits the mammalian target of rapamycin (mTOR)/p70 S6 kinase activity that is required for rapid and sustained serum-induced ribosomal biosynthesis (2). In addition to this regulation, AMPK, which is reportedly activated in response to reduced energy level, phosphorylates and activates p53, which leads to G1 cell cycle arrest (6). Inhibition of mTOR by AMPK and G1 cell cycle arrest by AMPK via p53 activation suppresses energy expenditure and protects cells from energy deprivation-induced apoptosis (4, 5).

On the other hand, a recent study in our laboratory revealed that glucose deprivation also reduces rRNA synthesis, which leads to downregulation of ribosome biosynthesis in HeLa cells lacking the LKB1-AMPK pathway (7). We found that a novel nucleolar protein, nucleomethylin (NML), forms an energy-dependent nucleolar silencing complex (eNoSC) with SIRT1 and SUV39H1 and functions in this process. Our results suggest that an energy-dependent change in the NAD+/NADH ratio regulates eNoSC, allowing the complex to couple the changing energy status with the level of rRNA transcription by...
regulating the epigenetic status of rRNA clusters. eNoSC promotes the restoration of energy balance and protects cells from energy deprivation-induced apoptosis (7).

With regard to p53 activation, several lines of evidence show that apart from ribosomal biogenesis the nucleolus has additional functions, including regulation of mitosis, cell cycle progression, and proliferation (8), and many forms of stress response such as p53 activation (9-11). Among them, several studies have shed light on the relationship between ribosomal biogenesis and p53 activation in response to nucleolar stress; p53 is reportedly activated by the reduction in ribosomal biogenesis through inhibition of rRNA transcription and processing (12-15). Inhibition of rRNA transcription by actinomycin D or TIF-IA siRNA treatment, which suppresses ribosomal biogenesis, causes disturbance of the nucleolus (9, 15-17). The ribosome-induced nucleolar disturbance is thought to activate p53 by blocking proteasomal degradation via MDM2. This can be achieved by negative regulation of MDM2 by the tumor-suppressor protein p19ARF or ribosomal proteins, both of which sequester MDM2, resulting in stabilization of p53 (18-20).

Very recently, we found that a nucleolar protein, Myb-binding protein 1a (MYBBP1A), is involved in p53 activation when ribosomal biogenesis is suppressed. MYBBP1A, which is anchored to the nucleolus through RNA, translocates from the nucleolus to the nucleoplasm when nucleolar RNA content is reduced by inhibition of rRNA transcription. The translocated MYBBP1A acetylates and accumulates p53 by facilitating the interaction between p53 and histone acetyltransferase p300 (Kuroda EMBO J. in press 2011). Given that nutrient shortage can reduce nucleolar RNA content by suppressing rRNA transcription, MYBBP1A may cause activation of p53 that is induced by glucose deprivation.

Here we report that NML senses the energy status and reduces nucleolar RNA content by suppressing rRNA transcription in response to glucose starvation, and that this leads to the translocation of MYBBP1A from the nucleolus to the nucleoplasm. Then, MYBBP1A activates p53 by enhancing its acetylation, which is a novel pathway, different from the already known LKB1-AMPK pathway. These findings indicate that NML and MYBBP1A may be the sensors in the nucleolus that connect intracellular energy status with the cell cycle machinery.

**EXPERIMENTAL PROCEDURES**

*Cell culture & Expression vector*

MCF-7 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium containing 1000 mg/l glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. WI-38 human normal fibroblast cells were cultured in Dulbecco’s modified Eagle’s medium containing 4500 mg/l glucose with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin.
MCF-7 cells were transfected with expression vectors encoding the respective proteins (pcDNA3-FLAG–mouse MYBBP1A, pcDNA3-FLAG-mouse NML).

**RNA interference**

For transfection of siRNAs, cells at 30 to 50% confluency were transfected with 20 nM of siRNA using LipofectAmine RNAi max (Invitrogen) according to the manufacturer’s protocol, and incubated for the indicated times. All siRNAs were purchased from Invitrogen. The sequence of siRNA duplexes is as follows:

- AMPKα1 #1 (5’-AAGAAUGGUACUCUUUCAAGAUGG-3’), AMPKα1 #2 (5’-CACAGAAGGAAUUAAGAUGAGG-3’),
- p53 (5’-UUCCGUCCCAGUAGAUUACCACUGG-3’), MYBBP1A #1 (5’-UUCACCAGCCGACCUGACUGAAAGA-3’), MYBBP1A #2 (GGUCCGAGAUAUUGCCUCGAA),
- NML #1 (5’-UAGGAUGCUGUGUGGUCUC-3’), NML #2 (5’-ACUGCUUGCGCUUAAUGUAUGAGG-3’). Stealth RNAi™ Luciferase reporter control duplex was used as a negative control.

**Cell cycle analysis**

Cells were harvested by trypsinization, fixed and stained with Guava® Cell Cycle Reagent (Millipore), and cell cycle populations were determined by using the Guava® EasyCyte system (Millipore) according to the manufacturer’s recommendations. The Guava® Luciferase reporter software (Millipore) was used to determine the cell populations in the different cell cycle phases.

**Trypan blue exclusion assay**

Cells were harvested by trypsinization, washed with PBS and mixed with 0.5% trypan blue (Nacalai Tesque). After 3 min of equilibration, the cells were counted under microscope.

**Immunofluorescence**

MCF-7 cells were reverse transfected with control or NML siRNA and incubated for 48 h, and then incubated in media containing various glucose concentrations (1000, 100, or 0 mg/l) for 24 h. Cells were fixed with 4% paraformaldehyde at room temperature for 15 min, and permeabilized with 0.5% Triton X-100 buffer (20 mM HEPES, 150 mM KCl, 0.5% TritonX-100) for 10 min at room temperature. Samples were blocked with 0.5% Goat serum for 1 h, washed with PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, and 8.1 mM Na$_2$HPO$_4$), and incubated with the indicated antibody for 2 h. Cells were washed with PBS and incubated with Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Invitrogen) or Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (Invitrogen) for 1 h. Cells were washed again with PBS, stained with 4, 6-diamidino-2-phenylindole (DAPI) (Dojindo), and mounted on slides with Vectashield (Vector Laboratory). Immunofluorescence was performed using
Biozero immunofluorescence microscopy (Keyence, Osaka, Japan).

**Immunoblotting**
For immunoblotting, cells were harvested by trypsinization, washed with PBS, and lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) plus protease inhibitor cocktail (Nacalai Tesque) for 30 min on ice. Lysates were cleared by centrifugation at 16100 × g for 30 min at 4°C. Protein concentrations were evaluated with a BCA kit (Thermo Scientific). Extracted proteins were separated by SDS-PAGE, transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk in TBS-T buffer (20 mM Tris at pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h, the membranes were incubated with the first antibody for 1 h. After washing with TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Bands were detected with Chemi-Lumi One (Nacalai tesque) or Immobilon Western blotting detection kit (Millipore).

**Immunoprecipitation**
For immunoprecipitation of the endogenous proteins, MCF-7 cells were lysed in TNE buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA and 0.5% NP-40) at 4°C for 30 min. The cleared lysate was incubated for 4 h with 2 μg of antibodies against the indicated proteins, 10 μl of protein G sepharose was added, and the sample was rotated for 2 h at 4°C. After washing four times with the same buffer, immunoprecipitates were separated by SDS-PAGE, and analyzed by immunoblotting with the indicated antibodies.

**Antibodies**
The antibodies used in these experiments were: β-Actin (Sigma), p53 (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG M2 antibody (Sigma), phospho-p53 (Ser15, Cell Signaling Technology, Beverly, MA), Acetyl-p53 (Lysine382, Cell Signaling Technology), p21 (F-5, Santa Cruz Biotechnology), Hdm2 (SMP14, Santa Cruz Biotechnology), PUMA (Cell Signaling Technology), AMPKα (Cell Signaling Technology), phospho-AMPKα (Thr172, Cell Signaling Technology), phospho-ACC (Cell Signaling Technology), p300 (N-15, Santa Cruz Biotechnology), UBF (Santa Cruz Biotechnology). Rabbit anti human-MYBBP1A antibody was raised against a synthetic peptide corresponding to 1265–1328 amino acids of human-MYBBP1A. Rabbit anti mouse-Mybbp1a antibody was raised against a synthetic peptide corresponding to 5-18 and 1321-1334 amino acids of mouse-Mybbp1a. NML antibody was prepared as described Murayama et al. (7).

**RNA purification and RT-qPCR**
Total RNA was isolated with FastPure™ RNA Kit (TAKARA) according to the manufacturer’s instruction. Total RNA (1μg) was reverse transcribed with PrimeScript™ 1st
strand cDNA Synthesis Kit (TAKARA). Real-time quantitative PCR analysis was performed using the Thermal Cycler Dice TP800 (Takara) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), as described in Murayama et al. (7). For PCR amplification, the specific primers, 5'-ATCGTCCACCGCAATGCTTCTA-3' and 5'-AGCCATGCAATCTCATCTTGTT-3' for β-Actin, 5'-GAACGGTGGTGTGTCGTTC-3' and 5'-GCGTCTCGTCTCGTCTCACT-3' for pre-rRNA were used.

**Nucleoli purification and quantitative determination of nucleolar RNA content**
Nucleoli were isolated from 1.2 × 10^7 MCF-7 cells in high purity by density gradient fractionation as previously described Andersen et al. (21). Total nucleolar RNA was prepared from the isolated nucleoli and quantified by spectrometry.

**RESULTS**

**Glucose limitation causes cell death in a p53-dependent manner**—p53 is reportedly activated in response to glucose limitation (6, 22, 23). To confirm this, we analyzed cell lysates from MCF-7 cells, which express wild type p53, cultured in various glucose concentrations by immunoblotting. We found that the protein levels of p53 and its downstream gene products, such as p21, HDM2, and PUMA, increased when glucose concentration was reduced (Fig. 1A). The induction of p53 downstream gene products was abrogated when p53 level was depleted by siRNA (Fig. 1A). Next, we tested whether the glucose limitation affected the viability of MCF-7 cells. A trypan blue exclusion assay indicated that glucose deprivation increased the percentage of dead cells. Furthermore, this was abrogated by p53 depletion (Fig. 1B). These results indicate that p53 is involved in cell death under nutrient stress. Then, we analyzed the modification of p53 and found that Ser15 phosphorylation and Lys382 acetylation were stimulated in cells deprived of glucose (Fig. 1C).

Next, we investigated the mechanism underlying the activation of p53 in response to glucose starvation in MCF-7 cells. AMPK is responsible for the phosphorylation of p53 under low glucose conditions (6, 22). Therefore, we tested the effect of glucose deprivation on the phosphorylation of AMPK itself and that of AMPK substrate acetyl-CoA carboxylase (ACC). Glucose limitation did not affect AMPK phosphorylation or ACC phosphorylation in MCF-7 cells (Fig. 1D). Moreover, depletion of AMPKα1 by siRNA did not affect the levels of p53, p53 phosphorylation, and p53 acetylation increased by glucose deprivation (Fig. 1E). These results suggest that AMPK is not involved in the activation of p53 in response to glucose deprivation in MCF-7 cells under our experimental conditions. Therefore, pathway(s) other than the LKB1-AMPK pathway may be involved in the upregulation
of p53 when glucose concentration is reduced in MCF-7 cells.

**MYBBP1A is implicated in the activation of p53 in response to glucose limitation**

Our recent work revealed that the nucleolar protein MYBBP1A activates p53 when ribosomal biogenesis was suppressed. MYBBP1A, which is anchored to the nucleolus by RNA, translocated from the nucleolus to the nucleoplasm when nucleolar RNA content was reduced by inhibition of rRNA transcription. The translocated MYBBP1A acetylates and accumulates p53 by facilitating the interaction between p53 and p300 (Kuroda EMBO J. in press 2011).

Glucose starvation reduces ribosomal biogenesis (1, 7). Therefore, the reduction in nucleolar RNA content in response to glucose starvation may cause the translocation of MYBBP1A, which eventually leads to the activation of p53. To test this possibility, we first examined whether glucose starvation reduced rRNA transcription in MCF-7 cells. As shown in Fig. 2A, rRNA transcription was reduced when glucose concentration was decreased. Nucleolar RNA content also reduced in parallel with the reduction in rRNA transcription (Fig. 2B). We next examined the effect of glucose starvation on the localization of MYBBP1A. MYBBP1A localized dominantly in the nucleolus when cells were cultured in medium containing normal glucose concentration (1000 mg/l) (Fig. 2C). However, MYBBP1A translocated from the nucleolus to the nucleoplasm in response to glucose starvation, whereas another nucleolar protein upstream binding factor (UBF) remained in the nucleolus (Fig. 2C left; 100 mg/l or 0 mg/l). Moreover, MYBBP1A and p53 localized diffusely in the nucleoplasm under glucose-starved condition (Fig. 2C right; 100 mg/l or 0 mg/l). The subcellular fractionation experiment confirmed the translocation of MYBBP1A (Fig. 2D). This quantitative assay showed that translocation of MYBBP1A to the nucleoplasm paralleled the reduction in nucleolar RNA content.

Then, we tested the interaction between p300 and p53 because we have already established that the translocated MYBBP1A strengthened their interactions (Kuroda EMBO J. in press 2011). We immunoprecipitated p300 from MCF-7 cell extracts cultured in medium with or without glucose, followed by immunoblotting. As shown in Fig. 2E, increasing amounts of p53 proteins were detected in the immunoprecipitants of p300. This result indicates that the interaction between p53, p300, and MYBBP1A is increased under the glucose-starved condition. Moreover, the GST pull-down experiment showed that p53 interacts with 929–1150 amino acid residues of MYBBP1A (Fig. 2F).

Next, we tested whether MYBBP1A was implicated in the activation of p53 in response to glucose deprivation by knockdown of MYBBP1A. siRNA mediated-MYBBP1A depletion almost abolished the enhancement of p53 acetylation induced by glucose starvation and partly suppressed the accumulation of p53 protein (Figs. 3A and S1A). Upregulation of
p53 downstream gene products, such as p21 and PUMA, under low glucose condition was also compromised by the depletion of MYBBP1A (Figs. 3A and S1A). In contrast, phosphorylation of p53 was barely influenced by this treatment (Figs. 3A and S1A). Moreover, re-expression of MYBBP1A in the MYBBP1A-depleted cells restored the acetylation of p53 under glucose-starved condition (Fig. 3B). Thus, MYBBP1A probably contributes to nutrient stress-induced p53 activation not by affecting its phosphorylation but by enhancing its acetylation. Consistent with this phenomenon, depletion of MYBBP1A abolished the enhanced interaction between p53 and p300 in the glucose-starved condition (Fig. 3C). Taken together, we consider that glucose starvation induces the translocation of MYBBP1A from the nucleolus to the nucleoplasm, where it stabilizes the interaction between p300 and p53, which causes acetylation and accumulation of p53. Then, we tested whether MYBBP1A was required to induce cell death in response to glucose starvation. As expected, depletion of MYBBP1A reduced the percentage of dead cells (Figs. 3D and S1B), indicating the involvement of p53 in cell death induced by low glucose concentration.

NML is implicated in the activation of p53 in response to glucose limitation—
We recently reported that the novel nucleolar protein NML formed a protein complex (eNoSC) with SIRT1 and SUV39H1 and suppressed rRNA transcription in response to glucose starvation (7). Thus, NML probably regulates p53 activation as follows: First, NML reduces rRNA transcription by glucose starvation, which leads to the reduction in nucleolar RNA content; next, as a consequence of the reduced nucleolar RNA content, MYBBP1A translocates from the nucleolus to the nucleoplasm; the translocated MYBBP1A strengthens the interaction between p53 and p300, resulting in the stimulation and activation of p53.

To address this possibility, we first examined whether NML was required for the reduction of both rRNA transcription and nucleolar RNA content under low glucose condition in MCF-7 cells. Our experimental results indicate that the reductions in rRNA transcription and nucleolar RNA content were alleviated by NML depletion (Figs. 4A, 4B and S1C, respectively). Next, we tested the effect of NML depletion on the localization of MYBBP1A (Figs. 4C, 4D and S1D). Immunofluorescence staining and immunoblotting experiments indicated that the translocation of MYBBP1A into the nucleoplasm in response to glucose limitation was weakened by the depletion of NML (Figs. 4C, 4D, and S1D). Consistent with this result, the enhancement of the interaction between p53 and p300 by glucose starvation was not observed when NML was depleted (Fig. 4E).

Finally, we tested the effect of NML on p53 activation. NML depletion efficiently abolished the enhancement of p53 acetylation in response to glucose starvation without
affecting the phosphorylation of p53; this was similar to the case of MYBBP1A depletion (Figs. 4F and S1E). Upregulation of p53 downstream gene products in glucose-starved condition was also weakened by the depletion of NML (Figs. 4F and S1E). Re-expression of NML in NML-depleted cells restored the acetylation of p53 under glucose-starved condition (Fig. 4G). MYBBP1A overexpression salvaged the NML-knockdown phenotype (Fig. 4H). This result supports the idea that MYBBP1A and NML function to activate p53 in the same pathway. Thus, NML contributes to p53 acetylation and activation by promoting MYBBP1A translocation into the nucleoplasm as a consequence of reduced nucleolar RNA content under the glucose-starved condition.

**NML-MYBBP1A pathway is involved in cell cycle arrest upon glucose deprivation**—

In this study, we found that the NML-MYBBP1A pathway contributes to p53 activation in response to glucose deprivation. To assess the physiological role of NML-MYBBP1A-mediated p53 activation in normal cells, we used a human normal fibroblast cell line, WI-38. As shown in Fig. 5A, glucose limitation-caused reductions in nucleolar RNA content were also alleviated by NML depletion in normal cells. Next, we tested the effect of NML depletion on the localization of MYBBP1A (Fig.5B). Immunoblotting experiments showed that the translocation of MYBBP1A into the nucleoplasm in response to glucose limitation was weakened by the depletion of NML (Fig.5B). Moreover, translocated MYBBP1A interacts with p53 and p300 (Fig. 5C). Next, we treated WI-38 cells with the control siRNA or siRNA for NML, MYBBP1A or p53 and then cultured them in media containing high (H; 4500 mg/l) or low (L; 300 mg/l) glucose, and tested the activation of p53 (Fig. 5D). Upon glucose starvation, p53 was acetylated, and the p53 downstream gene product p21 increased (Fig.5D). The upregulation of p53 acetylation and induction of p21 was reduced by siRNA treatment for NML or MYBBP1A (Fig. 5D). These results indicate that the NML-MYBBP1A pathway is involved in p53 activation in response to glucose starvation in normal as well as cancer cells. Next, we tested the effect of the NML-MYBBP1A pathway on cell proliferation under the glucose-starved condition (Fig. 5E). FACS analysis indicated that WI-38 cells were arrested in the G1 phase in medium containing low glucose (Fig. 5E). This enhancement of G1 cell cycle arrest was abolished when p53 was depleted (Fig. 5E). A similar phenotype was observed on depletion of NML or MYBBP1A (Fig. 5E). These results suggest that the NML-MYBBP1A pathway contributes to G1 cell cycle arrest by activating p53 in normal WI-38 cells when glucose concentration is reduced, which may contribute to limiting energy consumption under glucose starvation.

**DISCUSSION**

Here we have focused on the novel function of
the nucleolus that converts the signal of intracellular energy shortage into activation of p53. Two nucleolar factors are involved in this regulation. The first is the NML/SITR1/SUV39H1 protein complex eNoSC that detects intracellular energy status in terms of the NAD⁺/NADH ratio. Upon energy shortage, indicated by an increased NAD⁺/NADH ratio, eNoSC is activated via its SIRT1 subunit and suppresses rRNA transcription, which eventually leads to the reduction in nucleolar RNA content. The second is MYBBP1A that is tethered to the nucleolus by binding to nucleolar RNA. MYBBP1A translocates from the nucleolus to the nucleoplasm when nucleolar RNA content is reduced. The translocated MYBBP1A enhances the acetylation and accumulation of p53 by enhancing the interaction between p53 and p300 (Kuroda EMBO J. *in press* 2011). Thus, eNoSC functions as a detector and a signal transducer of the intracellular energy status; whereas MYBBP1A functions as an effector that activates p53 by enhancing its acetylation (Fig. 6).

In addition to the NML-MYBBP1A pathway, the LKB1-AMPK pathway is reportedly implicated in this regulation. Namely, in response to intracellular energy shortage, the increased AMP/ATP ratio activates AMPK, which leads to suppression of ribosomal biogenesis by inhibiting mTOR/p70 S6 kinase activity (3). In addition, activated AMPK enhances phosphorylation of p53 at its Ser15 and Ser46 residues, leading to p53 activation (6, 22). The biological significance and separate roles of the NML-MYBBP1A and LKB1-AMPK pathways remain to be determined. However, these two pathways may compensate for each other, thus improving their efficiency and reliability.

The suppression of ribosomal biogenesis or rRNA transcription by the LKB1-AMPK or NML-MYBBP1A pathways in response to energy deprivation is considered to be an adaptation mechanism that reduces energy expenditure. In addition, these pathways activate p53 under the energy-starved condition. However, the physiological relevance of p53 upregulation upon energy starvation is enigmatic. For example, our experimental results using WI-38, a human normal fibroblast cell line, indicate that upregulation of p53 in response to glucose starvation leads to G1 cell cycle arrest. Thus, the NML-MYBBP1A pathway reduces the energy consumption of WI-38 by repressing ribosomal biogenesis as well as arresting the cell cycle in the G1 phase. In contrast, our experimental results using MCF-7, a human breast tumor cell line, showed that glucose limitation-induced cell death in a p53-dependent manner. The molecular basis underlying this controversy on the role of p53 on cell fate remains to be determined. Notably, the acetylation of p53 reportedly enhances sequence-specific DNA binding of p53 and recruitment of a coactivator complex to promoter regions to activate p53-target gene expression (24,25). The functional consequences of p53 acetylation suggest that
the strength of acetylation may determine p53 regulation and cell fate. The activation of genes involved in cell cycle control requires partial acetylation, whereas the activation of proapoptotic genes requires full activation of p53 (26). Furthermore, we found that amount of MYBBP1A in the normal cell line is less than that in the cancer cell line (data not shown). The differences in cell fate due to activation of p53 between cell lines (e.g., normal cells vs. tumor cells) may be explained by the difference in the expression levels of MYBBP1A.

REFERENCES


**FOOT NOTES**

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The abbreviations used are: 1eNOSc, energy-dependent nucleolar silencing complex; 2MYBBP1A, Myb-binding protein la; 3AMPK, AMP-activated protein kinase; 4NML, nucleomethylin; 5mTOR, mammalian target of rapamycin, 6ACC, acetyl-CoA carboxylase; 7NPM, nucleophosmin; 8pre-rRNA, precursor rRNA

**FIGURE LEGENDS**

**Figure 1: Glucose limitation causes AMPK-independent p53 activation and induces cell death.**

A. MCF-7 cells were treated with control siRNA or siRNA for p53 for 48 h, and then incubated in media containing various glucose concentrations (1000, 100, or 0 mg/l) for 24 h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies.

B. MCF-7 cells were treated with control siRNA or siRNA for p53 for 48 h, and then incubated in media containing various glucose concentrations for the indicated times. The percentage of dead cells was analyzed by a trypan blue exclusion assay. Values are mean ± S.D., n = 3.

C. The cell lysates prepared from MCF-7 cells incubated in a various glucose concentrations for 24 h were analyzed by immunoblotting with the indicated antibodies.

D. MCF-7 cells were incubated in media containing various glucose concentrations for 24 h.
The cell lysates prepared from these cells were immunoblotted using the indicated antibodies.

E. MCF-7 cells were transfected with control siRNA or two independent siRNAs for AMPKα1 for 48 h, and then cultured in medium without glucose for 24 h. The cell lysates were analyzed by immunoblotting.

**Figure 2: Glucose limitation reduces nucleolar RNA content and causes MYBBP1A translocation into the nucleoplasm.**

A. MCF-7 cells were incubated in media containing the indicated glucose concentrations for 24 h, and pre-rRNA levels were determined by RT-qPCR, using primers that are specific for the 5' external transcribed spacer of pre-rRNA and normalizing for cell count. The level of the cells cultured in medium containing 1000 mg/l glucose was normalized to 100%. Values are mean ± S.D., n = 3.

B. Nucleolar RNA was isolated from the purified nucleoli of MCF-7 cells cultured for 24 h in media containing the indicated glucose concentrations. Total nucleolar RNA levels were quantified by spectrophotometry. The level of the cells cultured in medium containing 1000 mg/l glucose was normalized to 100%. Values are mean ± S.D., n = 3.

C. MCF-7 cells were cultured in media with the indicated glucose concentrations for 24 h, and the cells were stained using the indicated antibodies or DAPI (picture).

D. MCF-7 cells were cultured in media with the indicated glucose concentrations for 24 h. The cells were fractionated into nucleolar (NO) and nuclear (NE) fraction and proteins of each fraction were analyzed using indicated antibodies. UBF and histone H3 were shown as a loading control for nucleolar (NO) and nuclear (NE) fraction, respectively.

E. MCF-7 cells were cultured in media with a glucose concentration of 1000 mg/l or 0 mg/l for 24 h. The cell lysates were prepared, immunoprecipitated with normal rabbit IgG (IgG) or anti-p300 antibodies (p300), and analyzed by immunoblotting using the indicated antibodies.

F. In vitro translated FLAG-HA-MYBBP1A full-length and deletion mutants (Left) were incubated with GST-fused p53. After extensive washes, bound proteins were analyzed by immunoblot using HA antibodies (Right). Conserved motifs in MYBBP1A are shown in upper box.

**Figure 3: Glucose limitation enhances acetylation and accumulation of p53 in a MYBBP1A-dependent manner.**

A. MCF-7 cells were treated with control siRNA or siRNA for MYBBP1A#1 for 48 h, and then incubated in media containing various glucose concentrations (1000, 100, or 0 mg/l) for 24 h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies.

B. MCF-7 cells were treated with control siRNA or siRNA for MYBBP1A#1 and transfected
with mouse-MYBBP1A. Thirty six hours after transfection, cells were cultured in medium without glucose for 24h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies. Mouse-Mybbp1a (mMybbp1a) antibody cross-reacts with human-MYBBP1A.

C. MCF-7 cells were transfected with control siRNA or siRNA for MYBBP1A#1 for 48 h, and then cultured in medium without glucose for 24 h. The cell lysates were prepared, immunoprecipitated with normal rabbit IgG (IgG) or anti-p300 antibodies (p300), and analyzed by immunoblotting using the indicated antibodies.

D. MCF-7 cells were transfected with control siRNA or siRNA for MYBBP1A#1 for 48 h, and then cultured in media containing various glucose concentrations for the times indicated. The percentage of dead cells was analyzed using a trypan blue exclusion assay. Values are mean ± S.D., n = 3.

Figure 4: NML is involved in the activation of p53 in response to glucose limitation.

A. MCF-7 cells transfected with control siRNA or siRNA for NML#1 for 48 h were cultured in media containing the indicated glucose concentrations for 24 h. The pre-rRNA levels were determined by RT-qPCR and normalizing for cell count. The level of the cells transfected with control siRNA and cultured in medium containing 1000 mg/l glucose was normalized to 100%. Values are mean ± S.D., n = 3.

B. Nucleolar RNA was isolated from the purified nucleoli of the same set of MCF-7 cells as A. Total nucleolar RNA levels were quantified by spectrophotometry. The levels of the cells transfected with control siRNA and cultured in medium containing 1000 mg/l glucose was normalized to 100%. Values are mean ± S.D., n = 3.

C. MCF-7 cells transfected with control siRNA or siRNA for NML for 48 h were cultured in media with the indicated glucose concentrations for 24 h, and the cells were stained by the indicated antibodies or DAPI (picture).

D. MCF-7 cells were treated with control siRNA or siRNA for NML#1 for 48 h, and then incubated in media containing indicated glucose concentrations for 24 h. The cells were fractionated into nucleolar (NO) and nuclear (NE) fraction and proteins of each fraction were analyzed using indicated antibodies. UBF and histone H3 were shown as a loading control for nucleolar (NO) and nuclear (NE) fraction, respectively.

E. MCF-7 cells transfected with control siRNA or siRNA for NML#1 for 48 h, and then cultured in medium without glucose for 24 h. The cell lysates were prepared, immunoprecipitated with normal rabbit IgG (IgG) or anti-p300 antibodies (p300), and analyzed by immunoblotting using the indicated antibodies.

F. MCF-7 cells were treated with control or siRNA for NML#1 for 48 h, and then incubated in
media containing indicated glucose concentrations for 24 h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies.

G. MCF-7 cells were treated with control siRNA or siRNA for NML#2 and transfected with mouse-NML. Thirty six hours after transfection, cells were cultured in medium without glucose for 24 h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies. We used siRNA#2 in this experiment because siRNA#1 targeted mouse-NML as well as human-NML.

H. MCF-7 cells were treated with control siRNA or siRNA for NML#1 and transfected with mouse-MYBBP1A. Thirty six after transfection, cells were cultured in medium without glucose for 24 h. The cell lysates prepared from these cells were immunoblotted using the indicated antibodies. Mouse-Mybbp1a (mMybbp1a) antibody cross-reacts with human-MYBBP1A.

Figure 5: NML-MYBBP1A promotes G1 cell cycle arrest in response to glucose limitation.

A. WI-38 cells transfected with the control siRNA or siRNA for NML#1 for 36 h were cultured in media containing 4500 mg/l (H) or 300 mg/l (L) glucose for 60 h. Nucleolar RNA levels were quantified by spectrophotometry. The levels of the cells transfected with control siRNA and cultured in medium containing 4500 mg/l glucose was normalized to 100%. Values are mean ± S.D., n = 3.

B. WI-38 cells were treated with the indicated siRNAs for 36 h, and then incubated in media containing 4500 mg/l (H) or 300 mg/l (L) glucose for 60 h. The nucleolar and nuclear extract (NO and NE, respectively) were analyzed by immunoblotting using the indicated antibodies. UBF and histone H3 were shown as a loading control for nucleolar (NO) and nuclear (NE) fraction, respectively.

C. WI-38 cells were cultured in media containing 4500 mg/l (H) or 300 mg/l (L) glucose for 60 h. The cell lysates were prepared, immunoprecipitated with normal rabbit IgG (IgG) or anti-p300 antibodies (p300), and analyzed by immunoblotting using the indicated antibodies.

D. WI-38 cells transfected with the indicated siRNAs for 36 h were cultured in media containing 4500 mg/l (H) or 300 mg/l (L) glucose for 60 h. The cell lysates prepared from these cells were immunoblotted using the antibodies indicated. Adriamycin (ADR)-treated cells were used as a positive control for p53 activation.

E. The DNA contents of the same set of WI-38 cells as D were determined by FACS analysis. The percentages of the G1 phase cells were 48.00 ± 5.02% (siCont, H); 49.81 ± 1.33% (siNML, H); 50.49 ± 2.44% (siMYBBP1A, H); 52.97 ± 3.07% (sip53, H); 64.64 ± 2.77 % (siCont, L); 53.90 ± 4.17% (siNML, L); 56.43 ± 3.72% (siMYBBP1A, L); 50.39 ± 4.12 % (sip53, L). The DNA contents of adriamycin (ADR)-treated cells are shown in parallel; 45.97 ±2.30% (Cont.); 58.83 ±5.32% (ADR).
Figure 6: A model for p53 activation by NML-MYBBP1A in response to glucose limitation. See the discussion in detail.
Figure 2

A

Relative quantity of pre-rRNA (%)

100
80
60
40
20
0

1 2 3

pre-rRNA

B

Nucleolar RNA Content

Relative quantity of nucleolar RNA content (%)

100
80
60
40
20
0

1 2 3

Glucose Conc. (mg/l)

1000
100
0

C

MYBBP1A UBF DAPI

Glucose Conc. (mg/l)

1000
100
0

D

Glucose Conc. (mg/l)

No NE

MYBBP1A p53 UBF H3

1 2 3 4 5 6

E

Glucose Conc. (mg/l)

Input IgG p300

MYBBP1A p53 p300

1 2 3 4 5 6

F

GST-p53(Full) binding

+ + - + +

MYBBP1A

Input GST GST-p33

Full 1-1150 1-928 929-1328

LxxLL NES Leucine zipper-like motif Acidic region Basic repeat

1 200 400 600 800 1000 1200 1328 a.a.

1 1150

1 2784

929 1328
Figure 6

Low glucose

Apoptosis

Cell cycle arrest

Activation

p53

MYPB1A

300

Kinase

Nucleus

High glucose

Degradation

RNA transcription

Nucleus
Novel nucleolar pathway connecting intracellular energy status with p53 activation
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