Nucleophosmin (NPM1/B23) Interacts with Activating Transcription Factor 5 (ATF5) Protein and Promotes Proteasome- and Caspase-dependent ATF5 Degradation in Hepatocellular Carcinoma Cells*

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Background: NPM1 promotes whereas ATF5 inhibits HCC proliferation; NPM1 and ATF5 are regulated in an opposite manner in normal hepatocytes and HCC. Results: NPM1 competes against HSP70 for ATF5 binding and promotes proteasome- and caspase-dependent ATF5 protein degradation. Conclusion: NPM1 is a novel ATF5-interacting protein and abrogates ATF5 function in HCC. Significance: We reveal a mechanism by which NPM1 promotes HCC proliferation and survival via regulation of ATF5.

Nucleophosmin (NPM1/B23) and the activating transcription factor 5 (ATF5) are both known to subject to cell type-dependent regulation. NPM1 is expressed weakly in hepatocytes and highly expressed in hepatocellular carcinomas (HCC) with a clear correlation between enhanced NPM1 expression and increased tumor grading and poor prognosis, whereas in contrast, ATF5 is expressed abundantly in hepatocytes and down-regulated in HCC. Re-expression of ATF5 in HCC inhibits cell proliferation. We report here that using an unbiased approach, tandem affinity purification (TAP) followed with mass spectrometry (MS), we identified NPM1 as a novel ATF5-interacting protein. Unlike many other NPM1-interacting proteins that interact with the N-terminal oligomerization domain of NPM1, ATF5 binds via its basic leucine zipper to the C-terminal region of NPM1 where its nucleolar localization signal is located. NPM1 association with ATF5, whose staining patterns partially overlap in the nucleoli, promotes ATF5 protein degradation through proteasome-dependent and caspase-dependent pathways. NPM1-c, a mutant NPM1 that is defective in nucleolar localization, failed to stimulate ATF5 polyubiquitination and was unable to down-regulate ATF5. NPM1 interaction with ATF5 displaces HSP70, a known ATF5-interacting protein, from ATF5 protein complexes and antagonizes its role in stabilization of ATF5 protein. NPM1-promoted ATF5 down-regulation diminished ATF5-mediated repression of cAMP-responsive element-dependent gene transcription and abrogates ATF5-induced G2/M cell cycle blockade and inhibition of cell proliferation in HCC cells. Our study establishes a mechanistic link between elevated NPM1 expression and depressed ATF5 in HCC and suggests that regulation of ATF5 by NPM1 plays an important role in the proliferation and survival of HCC.

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide. The survival expectancy is usually 3 to 6 months for HCC patients due to late clinical presentation. The molecular pathways by which HCC develops and progresses remain largely unknown. The activating transcription factor 5 (ATF5; also known as ATFx) is a member of the ATF/CREB family of basic zipper (bZIP) proteins (1). It is highly expressed in the liver (2–4) and is down-regulated in HCC. Re-expression of ATF5 in HCC inhibits cell proliferation and induces G2/M arrest (5). ATF5 has been shown to be involved in other cellular processes such as differentiation of neural progenitor cells (6–8), repression of cAMP-induced transcription in JEG3 choriocarcinoma cells and PC12 pheochromocytoma cells (6, 9), regulation of apoptosis (10–17), and response to various types of cellular stresses (18–20).

ATF5 is known to subject to multilayered regulation that includes transcriptional regulation by EBF1 (21), translational regulation that is controlled by phosphorylated eIF2 (20, 23), and post-translational regulation that involves phosphorylation (2), acetylation (13), and Cdc34-dependent ubiquitin-methylation.

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This article contains supplemental Figs. S1–S3.

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3 The abbreviations used are: HCC, hepatocellular carcinoma; ATF5, activating transcription factor 5; TAP, tandem affinity purification; CRE, cAMP-responsive element; bZIP, basic zipper; PKA, protein kinase A; BAF, boc-asparty(OMe)-fluoromethylketone.
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diated proteolysis (9, 24, 25). We found recently that ATF5 interacts with chaperone protein HSP70, which protects ATF5 from otherwise extremely fast protein degradation in C6 glioma and MCF-7 breast cancer cells (12).

Using a tandem affinity purification (TAP) protocol (26, 27) followed by MS, we identified nucleophosmin (NPM1; also called B23 and NO38) as a major protein that interacts with ATF5. NPM1 is a nucleolar chaperone protein involved in diverse biological functions that include genomic stability and tumorigenesis (28, 29), ribosome biogenesis (30, 31), centrosome duplication (29, 32, 33), DNA repair (34), and response to cellular stress (28, 35). NPM1, which constantly shuttles between the nucleolus and cytoplasm (36), modulates the protein localization, aggregation, and stability of the tumor suppressors p53 (35, 37, 38) and p14ARF (39) and several other cellular factors, including the retinoblastoma susceptibility gene product pRB (40), the cell cycle G1-M checkpoint factor Gadd45α (41), and ribosomal protein S9 (42). In this report, we show that ATF5, via its bZIP region, interacts with the C-terminal region of NPM1 where its nucleolar localization signal resides. NPM1 competes with HSP70 for ATF5 binding and antagonizes the protective role of HSP70 to promote ATF5 protein degradation through proteasome-dependent and caspase-dependent pathways. Our results provide a mechanistic explanation for the observations that HCC express elevated NPM1 and depressed ATF5 and suggest that regulation of ATF5 by NPM1 plays an important role in the proliferation and survival of HCC.

MATERIALS AND METHODS

Cell Culture, Transfection, Stable Cell Lines, and DNA Constructs—HEK293, HepG2, and Hep3B cells were grown in DMEM (Invitrogen) with 10% FBS (Atlanta Biologicals), 100 μg/ml streptomycin, and 100 IU/ml penicillin. HEK293 cell lines stably expressing FLAG-HA-ATF5 or the control vector were selected and maintained in growth medium containing 800 μg/ml of G418 (Clontech) as described previously (26). Cell transfection was performed as described previously (13) using Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche). When used, MG132 (Calbiochem; 20 μM) and boc-aspartyl(OMe)-fluoromethylketone (BAF, Enzyme Systems Products; 50 μM) were added directly to cell culture (24 h after transfection if transfected cells were used) 4 h before harvesting cell lysates. Cycloheximide (Sigma; 10 μg/ml) was similarly added to cell culture for the time periods indicated.

The pCIN4 plasmids expressing FLAG-HA-ATF5, pLeGFP-FLAG-ATF5, and various truncation mutants, and HSP70-HA were described previously (12, 26). Mammalian expression vectors and retroviral vectors expressing Myc-NPM1 and various truncation mutants, FLAG-HA-NPM1, FLAG-HA-NPM1-c, and shRNA against NPM1 were kindly provided by Y. Zhang (University of North Carolina), K. Ye (Emory University), and W. Gu (Columbia University). pGEX2T-GST-ATF5 was kindly provided by O. J. Semmes (Eastern Virginia Medical School). pRSV-Cα (expressing the catalytic subunit of protein kinase A (PKA)) was obtained from A. Manni (Penn State College of Medicine).

Western Immunoblotting and Immunoprecipitation—Western immunoblotting and immunoprecipitation were performed as described previously (11–13). The antibodies used in the study were anti-NPM1 (Sigma), anti-FLAG (Sigma and Stratagene), anti-HA high affinity (3F10, Roche Applied Science), anti-ATF5 (Abcam), c-Myc (9E10, Santa Cruz Biotechnology), anti-actin (c1616, Santa Cruz Biotechnology), anti-α-HSP70 (Santa Cruz Biotechnology), and anti-ubiquitin (Cell Signaling). Secondary antibodies were from Jackson ImmunoResearch Laboratories, Sigma, and Santa Cruz Biotechnology.

Tandem Affinity Purification of ATF5-containing Protein Complexes and Mass Spectrometry Analyses—TAP of ATF5-containing protein complexes from HEK293-FLAG-HA-ATF5 cells were performed as described previously (26, 27). The purified proteins were separated on SDS-PAGE, and the protein bands were excised from the gel for MS analyses. An ABBScies 5800 Proteomics Analyzer (MALDI-TOF-TOF) and an MDS/Sciex 4000 QTrap (LC-MS/MS) were used for identification of ATF5-associated proteins at the Penn State College of Medicine Core Facility.

Reverse Transcription PCR and Quantitative Real-time PCR—RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s directions. To make cDNAs, RNA (2 μg) was primed with oligo(dT) and reverse-transcribed with SuperScript reverse transcriptase (Invitrogen) according to the manufacturer’s directions. Reverse transcription PCR (RT-PCR) and quantitative real-time PCR were carried out using the GoTaq polymerase or the GoTaq real-time PCR system (Promega) as described previously (13). The sequence of the primers used and the cycling parameters are available upon request.

Luciferase Reporter Assay—Luciferase assay was performed as described previously (26). Briefly, exponentially growing HEK293 cells were seeded at a density of 2 × 10^5 cells per well in a 96-well plate and allowed to grow for 24 h. Cells were treated with or without MG132 (20 μM) and BAF (50 μM) after cotransfection with pCRE-Luc or pGL3-Basic (control vector) reporter construct, pRL-Renilla (internal control), pRSV-Cα (expression vector for the catalytic subunit of PKA) (43), and a vector empty or expressing ATF5 and a vector empty or expressing NPM1 or NPM1-c. Cell lysates were prepared 24 h after cell transfection. Luciferase and Renilla activities were measured using the Dual-Luciferase reporter system (Promega) with a TD20/20 luminometer (Turner Designs). Relative luciferase activities were obtained by normalizing the luciferase activity against Renilla activity. Data are presented as mean ± S.E. (n = 3).

Protein Ubiquitination Assays—Cell lysates were prepared using cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 50 mM NaF, 1 mM NaVO_4, 1X complete protease inhibitor mixture (Roche Applied Science)) from HEK293 that were transfected with vectors expressing Myc-His-ubiquitin and other relevant genes and were treated with a proteasome inhibitor MG132 (20 μM) for 4 h. Polyubiquitinated species of tagged ATF5 were visualized by immunoblotting of ATF5 immunoprecipitates with antibodies that recognize ubiquitin and/or the tags.
In Vitro Caspase-3 Digestion Assay—Two μg of recombinant GST-ATF5 or GST-ATF5(D156A) was incubated with 50 ng of human recombinant activated caspase-3 (Sigma) in 20 μl of reaction buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM MgCl₂, 1 mM DTT) at 37 °C for 60 min. Reaction was stopped by adding an equal volume of electrophoresis sample buffer. Samples were separated by SDS-PAGE, and the input and cleaved products were visualized by Western immunoblotting or direct Coomassie Brilliant Blue staining.

Immunofluorescence Staining and Confocal Microscopy—Cells grown on polylysine-coated cover slips were fixed for 15 min in PBS containing 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 10 min, and incubated in blocking buffer (5% BSA in TBST) for 1 h. Cells were rinsed with PBS and incubated overnight at 4 °C in dilution buffer containing primary antibodies. The cells were washed three times with PBS before being incubated with an appropriate fluorochrome-conjugated secondary antibody for 1 h. Nuclear was stained by Hoechst 33342. Confocal images were taken using an Olympus IX81 motorized inverted microscope.

Flow Cytometry—Cell flow cytometry analysis was done as described previously (44). Briefly, HepG2 or Hep3B cells infected with retroviruses empty or expressing ATF5 and/or NPM1 were trypsinized, washed twice with 1× PBS, and pelleted by low speed centrifugation. Pellet was resuspended with 70% ethanol for 30 min at 4 °C. Cells were spun down and incubated with the DNA-binding dye propidium iodide solution (0.1% sodium citrate (w/v), 0.1% Triton X-100 (v/v), and 50 mg/liter propidium iodide in deionized water) for 1 h at room temperature prior to flow cytometric analysis.

Cell Viability and Colony Formation Assay—HepG2 and Hep3B cells were plated in a 96-well plate at 2 × 10³ cells per well and transfected with a vector empty (control) or expressing NPM1 and a vector empty or expressing ATF5. Cell viability was determined 5 days after transfection using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide kit (Invitrogen). Colony formation assay was performed as described previously (12). Colonies were visualized after staining with crystal violet. Only colonies containing >50 cells were scored. Data are presented as mean ± S.E. (n = 3) from three independent assays.

RESULTS

Identification of NPM1 as Novel ATF5-interacting Protein—To identify novel ATF5-interacting proteins, we established and characterized a HEK293 cell line that stably expressed a FLAG and hemagglutinin (HA) double-tagged ATF5 (HEK293-FLAG-HA-ATF5). The expression of the ectopic FLAG-HA-ATF5 with molecular mass of 35 kDa and of the endogenous ATF5 with molecular mass of 22 kDa in this cell line was determined by immunoblotting analyses of cell extracts with over-expressed FLAG-HA-ATF5 and after ATF5 immunodepletion as described previously (26). Immunoblotting analysis using cell extracts from this cell line and a control cell line that contained an empty vector indicated that the ectopically expressed FLAG-HA-ATF5 was expressed at near physiological level compared with that of the endogenous ATF5 (Fig. 1A). Thus, the composition and stoichiometry of the tagged ATF5 complexes in the HEK293-FLAG-HA-ATF5 clone likely will reflect those of native ATF5 complexes (26, 45). The FLAG-HA-ATF5-containing protein complexes were purified from the HEK293-FLAG-HA-ATF5 cells by TAP as described previously (26, 27). The purified proteins were separated on SDS-PAGE, and the protein bands were excised from the gel for MS analyses. A major protein band with an apparent molecular mass of 70 kDa was identified as HSP70 in the MS analysis (Fig. 1B), supporting our recent observation that HSP70 is an ATF5-interacting protein in C6 glioma and MCF-7 breast cancer cells (12). This finding also provided evidence that validated our TAP/MS approach. Our MS analysis also showed that the protein band that has an apparent molecular mass of 35 kDa contained both nucleophosmin (NPM1), a protein of 292 amino acids with a calculated molecular mass of 32 kDa, and ATF5, whose FLAG-HA-tagged form was the bait in the TAP and has 293 amino acids in length, also with a calculated molecular mass of 32 kDa. The two NPM1 peptides deduced from the MS (Fig. 1, C and D) called for >99% confidence in positive identification of NPM1.

To confirm ATF5-NPM1 interaction, we transfected HEK293 cells with FLAG-HA-ATF5 and Myc-NPM1 and performed coimmunoprecipitation analyses. Immunoblotting of the FLAG immunoprecipitate from the transfected cells with an anti-Myc antibody showed that FLAG-HA-ATF5 associated with Myc-NPM1 (Fig. 2A). Conversely, immunoblotting of the Myc-immunoprecipitated Myc-NPM1 from the transfected cells with an anti-HA antibody revealed the co-precipitated FLAG-HA-ATF5 (Fig. 2B). Immunoblotting of the immunoprecipitated NPM1 from either HEK293 (data not shown) or Hep3B (Fig. 2C) cells with an anti-ATF5 antibody showed that endogenous ATF5 was coimmunoprecipitated with NPM1, although a weaker association was detected in HEK293 cells likely due to relatively lower endogenous expression of NPM1 in that cell type. Additional immunoprecipitation immunoblotting analyses using cells transfected with various ATF5 and NPM1 truncations showed that the two molecules interact with each other with their C-terminal regions (supplemental Fig. S1). Interaction between endogenous ATF5 and NPM1 was further demonstrated in co-immunostaining analysis, which showed that the staining patterns of two proteins partially overlapped in the nucleus (Fig. 2D). Indicating the unusual strong interaction between the ATF5 and NPM1, interaction between the two proteins could be detected in 0.6 M NaCl (Fig. 1E), a salt concentration that interrupts most protein-protein interactions (31, 42). Furthermore, the strong interaction between NPM1 and ATF5 is RNA-independent, as pretreatment of cell extract with RNase A did not alter their association (data not shown). Together, these results indicated that NPM1 is a novel ATF5-interacting protein and they form stable complexes in vivo.

NPM1 Promotes ATF5 Degradation via Both Proteasome-dependent and Caspase-dependent Processes—Because ATF5 protein is normally unstable (12) and NPM1 is known to regulate protein stability of some of the key molecules in the cell, e.g. ARF (46–48), we examined whether NPM1 plays a role in the turnover of the ATF5 protein. FLAG-HA-ATF5 was cotransfected with a vector empty or expressing Myc-NPM1 or with vector expressing non-silencing shRNA or NPM1 shRNA into
HEK293 cells, and the protein levels of ATF5 and NPM1 were monitored by Western immunoblotting analysis. As shown in Fig. 3A, increased NPM1 expression led to dramatically decreased expression of ATF5. In contrast, down-regulation of NPM1 by shRNA resulted in up-regulation of ATF5. Showing endogenous ATF5 is subject to similar NPM1-dependent regulation, overexpression of NPM1 down-regulated, whereas depletion of NPM1 with an shRNA against NPM1 up-regulated the endogenous ATF5 in both HepG2 (Fig. 3B) and HEK293 (supplemental Fig. S2) cells. To determine the mechanism by which NPM1 regulates ATF5 expression, we first ruled out the possibility that NPM1 regulates ATF5 expression at the mRNA level or through translation. Total RNA was prepared from HepG2 cells transfected as in Fig. 3B, and the relative levels of ATF5 mRNA were determined by quantitative real-time PCR analysis. As shown in Fig. 3C, although ATF5 mRNA levels decreased in HepG2 cells transfected with an shRNA against ATF5, it remains constant when transfected with shRNA against NPM1 or when NPM1 was overexpressed, suggesting that NPM1 regulate ATF5 expression by post-transcriptional processes. To see whether NPM1 expression directly affected ATF5 protein stability, we transfected HEK293 cells with vector expressing FLAG-HA-ATF5 and vectors empty or expressing NPM1 or shRNA-NPM1 and assessed the decay of the ectopic FLAG-HA-ATF5 in the presence of cycloheximide (10 μg/ml), an inhibitor of protein translation. As shown in Fig. 3D, 3E, co-expression of NPM1 markedly enhanced, whereas co-expression of shRNA-NPM1 dramatically retarded ATF5 turnover. The half-life of the ectopically expressed FLAG-HA-ATF5 was ~3 h in vector-transfected HEK293 cells comparing with <1 h in cells transfected with NPM1 and >6 h in cells transfected with shRNA-NPM1 (Fig. 3F). To determine whether NPM1 regulates ATF5 protein stability via proteasome-dependent and caspase-dependent processes, we per-
formed immunoblotting analyses on HEK293 cells treated with an inhibitor for the 26 S proteasome (MG132; 20 μM) or a pan-caspase inhibitor (BAF; 50 μM) after transfection with various relevant constructs. As shown in Fig. 3G, presence of MG132 or BAF consistently up-regulated FLAG-HA-ATF5 protein levels (compare lane 1 with lanes 2 and 3), which was further increased by co-treatment with the two drugs (lane 4).

When NPM1 was overexpressed, expression of FLAG-HA-ATF5 was depleted as expected (compare lanes 1 and 5). Treatment of the cells with either MG132 or BAF dramatically reversed NPM1-promoted ATF5 degradation, whereas cotreatment with the two drugs completely blocked the effect of NPM1 on ATF5 degradation (lanes 6–8). These data indicated that NPM1 promotes ATF5 protein degradation via both proteasome-dependent and caspase-dependent processes.

**NPM1-promoted ATF5 Polyubiquitination Requires NPM1 to Shuttle between Nucleolus and Cytosol**—Mutations affecting the carboxyl-terminal domain of NPM1 occur in a significant percentage of adult patients with acute myeloid leukemia, and these alterations relocalize much of the NPM1 protein from its normal nucleolar stores to the cytoplasm (49, 50). To investigate whether nucleolar localization of NPM1 affects its ability to regulate ATF5 degradation via the proteasome-dependent pathway, we first demonstrated that overexpression of wild-type NPM1 enhanced the production of polyubiquitinated ATF5 in an in vivo ubiquitination assay. HEK293 cells were untreated or treated with MG132 after cotransfection with vectors expressing FLAG-HA-ATF5 and Myc-His-ubiquitin and a vector empty or expressing Myc-NPM1 (Fig. 4A) or a vector containing a non-silencing or an NPM1 shRNA (Fig. 4B).

Polyubiquitinated ATF5 species were visualized by immunoblotting FLAG immunoprecipitates with an anti-HA (Fig. 4A) or an anti-ubiquitin (Fig. 4B) antibody. These analyses provided strong evidence that NPM1 up-regulation stimulated whereas NPM1 down-regulation inhibited polyubiquitination of ATF5. We next compared wild type NPM1 and NPM1-c, a mutant NPM1 that has four nucleotides insertion at the C terminus that results into frameshift mutation and loss of certain NPM1 function together with changed subcellular localization (48), in their ability to interact with ATF5 and to enhance ATF5 polyubiquitination. Hep3B (Fig. 4C) or HEK293 (data not shown) were cotransfected with vectors expressing GFP-ATF5 and Myc-His-ubiquitin and a vector empty or expressing NPM1 or NPM1-c. Immunoblotting of HA immunoprecipitates with an anti-GFP antibody showed that although NPM1 expression increased the production of polyubiquitinated ATF5 as expected, the cytosol-localized NPM1-c not only failed completely to associate with ubiquitinated ATF5 (Fig. 4C, IP: HA; IB: GFP, compare lanes 1 and 3) but also inhibited ATF5 down-regulation (Fig. 4C, Lysate IB: GFP). These data indicate that NPM1 stimulates ATF5 polyubiquitination and that this activity is associated with the ability of NPM1 to maintain its nucleolar localization or its ability to shuttle between nucleolus and cytosol.

**NPM1 Stimulates Caspase-dependent ATF5 Cleavage at Site That Is Specifically Recognized by Caspase-3**—We next explored the mechanism by which NPM1 stimulated caspase-dependent ATF5 degradation. HEK293 cells were cotransfected with vectors expressing GFP-ATF5 and Myc-NPM1 or shRNA-NPM1 and were untreated or treated with BAF in the presence of MG132 to block the proteasome-dependent ATF5 degradation. Western immunoblotting analyses of the cell extracts using an antibody against GFP showed that beside the primary band of GFP-ATF5 located at the expected 58-kDa position, a smaller protein band (~45 kDa) reproducibly appeared in cells where GFP-ATF5 was down-regulated by...
NPM1 and disappeared in cells treated with BAF (Fig. 5A), suggesting caspase-dependent cleavage of GFP-ATF5. Significantly, a conserved DTLD sequence that matches the consensus caspase-3 recognition motif DxxD is located in the human (corresponding to amino acids 153–156), rat, and mouse ATF5 molecules. A cleavage at this site in the GFP-ATF5 fusion protein would produce a fragment with its size matching that of the smaller fragment recognized by the anti-GFP antibody (Fig. 5A). Consistent with a cleavage at the DTLD156 on ATF5, the smaller fragment was invisible when a similar blot was probed with an anti-ATF5 antibody that recognizes the C terminus of ATF5 (data not shown). To confirm that caspase-3 is responsible for ATF5 cleavage at the DTLD recognition site, we made a point mutation of ATF5 in which the Asp-156 was replaced by an Ala (ATF5D156A) and performed an in vitro caspase-3 digestion assay. As shown in Fig. 5B, although the GST-ATF5 generated the expected 45-kDa fragment after incubation of GST-ATF5 with caspase-3, the mutant GST-ATF5(D156A) failed to produce any cleaved product (Fig. 5B). These data support the conclusion that NPM1 plays a required role in the cleavage of ATF5 at the DTLD156 conserved site by caspase-3.

NPM1 Competes with HSP70 for ATF5 Binding and Antagonizes Protective Effect of HSP70 on ATF5—We have shown previously that chaperone protein HSP70 interacts with ATF5 and protects it from extremely fast degradation, extending its half-life of 0.1 h to >6 h in C6 glioma and MCF-7 cells (12). To further explore the mechanism by which NPM1 regulates ATF5 protein stability, we examined interaction between NPM1 and ATF5 in relation to HSP70. HEK293 cells were transiently transfected with indicated constructs and were treated with or without 26 S proteasome inhibitors MG132 (20 μM), pan-caspase inhibitor BAF (50 μM), or their combination. Immunoblotting analysis was performed as described in A. NPM1 and disappeared in cells treated with BAF (Fig. 5A), suggesting caspase-dependent cleavage of GFP-ATF5. Significantly, a conserved DTLD sequence that matches the consensus caspase-3 recognition motif DxxD is located in the human (corresponding to amino acids 153–156), rat, and mouse ATF5 molecules. A cleavage at this site in the GFP-ATF5 fusion protein would produce a fragment with its size matching that of the smaller fragment recognized by the anti-GFP antibody (Fig. 5A). Consistent with a cleavage at the DTLD156 on ATF5, the smaller fragment was invisible when a similar blot was probed with an anti-ATF5 antibody that recognizes the C terminus of ATF5 (data not shown). To confirm that caspase-3 is responsible for ATF5 cleavage at the DTLD156 recognition site, we made a point mutation of ATF5 in which the Asp-156 was replaced by an Ala (ATF5D156A) and performed an in vitro caspase-3 digestion assay. As shown in Fig. 5B, although the GST-ATF5 generated the expected 45-kDa fragment after incubation of GST-ATF5 with caspase-3, the mutant GST-ATF5(D156A) failed to produce any cleaved product (Fig. 5B). These data support the conclusion that NPM1 plays a required role in the cleavage of ATF5 at the DTLD156 conserved site by caspase-3.

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### FIGURE 3. NPM1 promotes proteasome- and caspase-dependent ATF5 degradation.

A. HEK293 cells were cotransfected with pCIN4-FLAG-HA-ATF5 and indicated constructs. Expression of FLAG-HA-ATF5, Myc-NPM1, and β-Actin (Actin) was monitored with immunoblotting analysis using anti-HA, anti-NPM1, and anti-actin antibodies, respectively. B. Expression of endogenous ATF5 was monitored in HepG2 cells transfected with indicated constructs. Immunoblotting (IB) analysis was performed as described in A. C. HepG2 cells were transfected with indicated constructs. Relative ATF5 mRNA abundance was determined by quantitative real-time PCR (QRT-PCR). ATF5 mRNA abundance in cells transfected with non-silencing shRNA (shRNA-NS) was used as control and was arbitrarily set at 100. D–F, HEK293 cells were transfected with indicated constructs and were treated with or without cycloheximide (CHX; 10 μg/ml) for indicated time periods. Antibodies against HA, Myc, and GFP (D) or against ACTIN, NPM1, and GFP (E) were used for the immunoblotting analyses. Both vectors empty (Vector) or containing shRNA-NPM1 expressed GFP, which is not labeled. In F, Western blots from three independent experiments as the ones shown in D and E were scanned, and the relative density of the ATF5 bands was calculated using GFP as a control (lower panels). Band density corresponding to cycloheximide (0) was set at 100%. The error bar depicts mean ± S.E. G, HEK293 cells were transiently transfected with indicated constructs and were treated with or without 26 S proteasome inhibitors MG132 (20 μM), pan-caspase inhibitor BAF (50 μM), or their combination. Immunoblotting analysis was performed as described in A.
bodies against HSP70 and Myc indicated that, although both HSP70 and NPM1 interacted with ATF5, they appeared in competition for ATF5 binding (Fig. 6A). Interestingly, overexpression of NPM1 led to increased NPM1 interaction with ATF5 and simultaneous loss of association between HSP70 and ATF5 (Fig. 6A). This is consistent with the interpretation that NPM1 binding to ATF5 is particularly strong (Fig. 2E) so that it displaces the protective HSP70 from the ATF5 protein complexes. To see whether overexpression of NPM1 would antagonize the protective effect of HSP70 on ATF5, we transfected vectors expressing GFP-ATF5, HSP70-HA, and Myc-NPM1 into HEK293 (data not shown) and Hep3B (Fig. 6B) cells and monitored the expression level of GFP-ATF5 with Western immunoblotting analyses. As shown in Fig. 6B, although GFP-ATF5 protein was stabilized significantly in Hep3B cells overexpressing HSP70, which is consistent with our previous findings (12), coexpression of Myc-NPM1 reversed the protective effect of HSP70 and depleted GFP-ATF5 in a dose-dependent manner. Together, these data show that NPM1 competes with HSP70 for ATF5 binding and antagonizes the protective effect of HSP70 on ATF5, accelerating the proteasome-dependent and caspase-dependent degradation of the ATF5 protein.

NPM1 Inhibits ATF5 Activity in CRE-dependent Gene Transcription—To explore the functional impact of NPM1 on ATF5 regulation, we examined whether NPM1 expression suppresses the inhibition of CRE-dependent transcription by ATF5 (6, 9). HEK293 cells were transfected with a reporter plasmid expressing luciferase under the control of the CRE element, Renilla control vector, RSV-Cα (expression vector for the catalytic subunit of PKA) (43), and vectors empty or expressing ATF5, NPM1 or NPM1-c. As shown in Fig. 7A, ATF5 repressed CRE-dependent gene expression promoted by PKA, which is consistent with previous observations (6, 9). In addition, we found that expression of NPM1 relieved ATF5-dependent CRE repression in a dose-dependent manner. In contrast, cells transfected with NPM1-c mutant or cells transfected with NPM1 but treated with MG132 and BAF showed no alleviation in ATF5-dependent CRE repression (Fig. 7A). To determine whether NPM1 regulation of ATF5 impacts the expression of endogenous CRE-dependent genes, we performed semiquantitative RT-PCR analysis on a number of genes, including PEPCK, c-IAP2, R-Ras, and YWHAH, which are known to be subject to CRE-dependent transcription regulation (51–55), using Hep3B cells transfected with a vector

FIGURE 4. NPM1 accelerates whereas down-regulation of NPM1 retards ATF5 polyubiquitination. A and B, HEK293 cells were transfected with indicated constructs and were treated with or without 26S proteasome inhibitors MG132 (20 μM). Anti-FLAG immunoprecipitates (IP) were immunoblotted (IB) with an HA (A) or anti-ubiquitin (Ub; B) antibody to show extensive polyubiquitination of ATF5. Lower panels were immunoblotting analyses using the same lysates to monitor the expression of Myc-NPM1, endogenous NPM1, and FLAG-HA-ATF5 in the cells. C, transfection, immunoprecipitation, and immunoblotting were performed as described in A except Hep3B cells and indicated antibodies were used. shRNA-NS, non-silencing shRNA.

FIGURE 5. NPM1 enhances caspase-dependent degradation of ATF5. A, HEK293 cells were transiently transfected with indicated constructs and were treated with or without pan-caspase inhibitor BAF (50 μM). Expression of full-length GFP-ATF5 and caspase-cleaved GFP-ATF5 fragment (GFP-ATF5[157–281]) was monitored by immunoblotting (IB) analysis using an anti-GFP antibody. β-Actin (Actin) was used as a loading control. B, in vitro digestion of GFP-ATF5 and GFP-ATF5(D156A) by caspase-3. In vitro cleavage of WT and mutant ATF5 proteins was monitored by Coomassie Brilliant Blue staining. Lower panels are immunoblots showing equal loading of GFP-ATF5 and GFP-ATF5(D156A), and caspase-3.
empty or expressing FLAG-HA-ATF5 and a vector empty or expressing Myc-NPM1. This analysis showed that expression of ATF5 suppressed the expression of all the four genes and that coexpression of NPM1 reversed the suppression on these genes by ATF5 (Fig. 7B). These data support the conclusion that NPM1-mediated ATF5 down-regulation results in activation of CRE-dependent gene transcription in Hep3B cells.

**NPM1 Promotes ATF5 Degradation**

![FIGURE 6. NPM1 competes with HSP70 for binding of ATF5 and reverses the protection of ATF5 by HSP70. A, HEK293 cells were transfected with indicated constructs. GFP immunoprecipitates (IP) were immunoblotted (IB) with antibodies against GFP (ATF5), HSP70, and Myc (NPM1). B, immunoblotting analysis monitoring expression of GFP-ATF5 and NPM1 in Hep3B cells transfected with indicated constructs. Different amount of Myc-NPM1 DNA construct was used in transfection: −, +, and ++ represent 0, 2, and 10 µg DNA.](image)

**FIGURE 7. NPM1 alleviates ATF5-dependent CRE repression, blocks ATF5-dependent inhibition of cell proliferation and colony formation potential, and removes ATF5-induced G2/M block in Hep3B cells.**

A, HEK293 cells were cotransfected with a reporter plasmid expressing luciferase under the control of the CRE sequence, Renilla control vector, RSV-β-Cα (expression vector for the catalytic subunit of PKA), and vectors empty (−) or expressing ATF5 (+), WT (NPM1) or C-terminal truncation of NPM1 (NPM1-c) at indicated DNA amount, and in the absence or presence of MG132 (20 µM) and BAF (50 µM) (MG + BAF). Luciferase activities were reported as relative units after correction with the Renilla activities. Expression of the transfected FLAG-ATF5, Myc-NPM1, and β-actin (loading control) was monitored by Western immunoblotting (IB) analysis (top panels). B, semi-quantitative RT-PCR analysis of PEPCK, c-IAP2, R-RAS, and YWHAH in response to ATF5 and NPM1 expression in Hep3B cells. β-Actin was used as control. C and D, expression of NPM1 reverses ATF5-induced inhibition of cell proliferation and survival in Hep3B cells. Hep3B cells were coinfected with a retrovirus empty (Control) or expressing NPM1 and a retrovirus empty (−) or expressing ATF5 (+). Cell viability and proliferation relative to vector control was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays 5 days after infection (C) and colony formation assay (D). *, p < 0.05; **, p < 0.01, two-tailed Student’s test. E, Hep3B cells infected with retrovirus as described in C were stained with propidium iodide, and cell cycle distribution (%) was analyzed by flow cytometry.

NPM1 Antagonizes Inhibitory Effect of ATF5 on HCC Cell Proliferation and Relieves ATF5-induced G2/M Blockade in HCC Cells—We next examined whether NPM1 antagonizes the inhibitory effect of ATF5 on the proliferation of HCC cells (5). Hep3B cells were co-infected with a control retrovirus or a retrovirus expressing NPM1 and a retrovirus empty or expressing ATF5. Cell viability was assayed 5 days later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. As shown in Fig. 7C, although Hep3B cells infected with the retrovirus expressing NPM1 grew similarly as cells infected with the control virus (compare bar 1 with bar 2), the cells infected with the retrovirus expressing ATF5 showed markedly reduced viability (compare bar 1 with bar 3). Hep3B cells infected with both retroviruses expressing NPM1 and ATF5 displayed a significant improvement in cell viability as com-
pared with cells expressing ATF5 alone (compare bar 3 with bar 4). Experiments with HepG2 cells produced similar results (supplemental Fig. S3A). Colony formation assays using the same Hep3B cells infected as in Fig. 7C demonstrated that infection of retroviruses expressing ATF5 reduced the colony forming potential of the host cells (Fig. 7D, compare bar 1 and bar 3), whereas co-infection with retroviruses expressing NPM1 reversed the inhibitory effect of ATF5 (compare bar 3 and bar 4). Our analysis further showed that ATF5 expression in Hep3B cells significantly increased the G$_{2}$/M cell population in the expanse of the G$_{1}$ cell population, which is consistent with a previous report (5), and co-expression of NPM1 relieved ATF5-induced G$_{2}$/M blockade in Hep3B cells (Fig. 7E). Similar results were obtained in parallel experiments using HepG2 cells (supplemental Fig. S3B). Together, these results show that NPM1 antagonizes the inhibitory effect of ATF5 on HCC cell proliferation and relieves ATF5-induced G$_{2}$/M blockade in HCC cells.

**DISCUSSION**

Both NPM1 and ATF5 have been implicated in the pathogenesis of several human malignancies. Although NPM1 has been described both as an oncogene and a tumor suppressor depending on the cell type and protein expression levels (37), and likewise, ATF5 is known to modulate cell proliferation and survival that is depended on cell types (56), whether they interact with each other physically and/or functionally was unknown. Using an unbiased approach, a combined TAP and MS analysis, we found that NPM1 is a major ATF5-interactor (Fig. 1). We show that in HCC cells, NPM1 competes against HSP70 in ATF5 binding and antagonizes the protective effect of HSP70 to promote ATF5 degradation via both proteasome-dependent and caspase-dependent processes (Figs. 3–6). Functionally, NPM1 alleviates ATF5-dependent CRE trans repression in HCC cells, antagonizes the inhibitory effect of ATF5 on HCC cell proliferation, and relieves ATF5-induced G$_{2}$/M blockade in HCC cells. Our findings revealed a mechanism that governs an inverse relationship for NPM1 and ATF5 in HCC and indicated that NPM1-mediated destruction of ATF5 contributes to HCC cell proliferation and survival.

NPM1 and related proteins (nucleophosmins/nucleoplasmins) all contain a well conserved N-terminal oligomerization domain that allows NPM1 to form a pentamer and then a decamer by packing two pentamers in a sandwich-like structure (57). These NPM1 oligomers are the structural units that engage in interaction with histones and several other basic proteins such as sperm nuclear basic proteins, including protamines and protamine-like type proteins (22, 57). Although ATF5 is a bZIP motif-containing protein, its interaction with NPM1, which is acidic with a pI of 5.1, does not seem to follow the same mechanism as histones and the like, however. ATF5 interacts with the C-terminal region of NPM1 (supplemental Fig. S1) where the nucleolar localization signal resides. In congruity, nucleolar localization signal-defective NPM1-c seemed to only weakly interact with ATF5 and did not promote ATF5 ubiquitination (Fig. 4C) and were ineffective in reversing ATF5-dependent CRE repression (Fig. 7A). These data are consistent with the possibility that NPM1-promoted ATF5 degradation requires NPM1 to stay in the nucleolus or be able to shuttle between the nucleoli and cytosol. A nucleolus-dependent ATF5-NPM1 interaction is also consistent with the partial colocalization of the two proteins in the nucleolus (Fig. 2D).

The competition between the two chaperone proteins, NPM1 and HSP70, for ATF5 binding (Fig. 6) seems to support the findings that NPM1 and HSP70 have opposite effects on ATF5 protein stability and functional potential (Figs. 3, 4, 5, and 7) (12). Noticeably, NPM1 expression was observed to dislodge HSP70 from ATF5 complexes (Fig. 6A) and out-competed HSP70 to drive down ATF5 protein expression and to alleviate ATF5-dependent CRE gene repression, inhibition of cell proliferation, and blockade of cell cycle at the G$_{2}$/M phases in HCC (Figs. 6 and 7 and supplemental Fig. S3).

Although we have determined that NPM1 promotes ATF5 polyubiquitination, we did not identify the specific ubiquitin E3 ligase(s) for this process. Among the possible candidates for this role include Rad6B and Fbw7, as the former was shown to be required for ubiquitin-dependent degradation of ATF5 in certain cell types (9), whereas the latter is localized in the nucleolus in an NPM1-dependent manner (47). In contrast, we have identified a conserved caspase-3 recognition site in ATF5 protein molecules and determined that NPM1 expression significantly elevated the sensitivity of this site to caspase-3 (Fig. 5A). Interestingly, the sequence of the conserved caspase-3 site in ATF5, DTLD, encompasses a threonine (Thr); phosphorylation at this site promises its conversion to a more fitting caspase-3 site, which is DEVD. Thus, two alternative mechanisms could be at work to facilitate NPM1-mediated ATF5 destruction by caspases. One is that NPM1 brings associated ATF5 closer to caspases directly and the other is that it entices an unknown protein kinase to phosphorylate the associated ATF5 at the DTLD site so that the ATF5 molecule is converted to a better matched target for caspases.

Taken together, our data support the conclusion that NPM1 is a major ATF5-interacting protein that antagonizes the protective role of HSP70 to promote ATF5 degradation via both proteasome-dependent and caspase-dependent processes. This work reveals a previously unknown mechanism that explains elevated expression of NPM1 and depressed expression of ATF5 in HCC cells. Our study shows that NPM1 is a critical modulator for ATF5 and that NPM1-promoted ATF5 degradation alleviates ATF5-dependent CRE repression, antagonizes the inhibitory effect of ATF5 on HCC cell proliferation, and relieves ATF5-induced G$_{2}$/M blockade in HCC cells.

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