Background: MEF/ELF4 can function as an oncogene. We demonstrated the role of MEF/ELF4 in acute myeloid leukemia.

Results: NPM1 inhibited the DNA-binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter whereas NPM1 mutant protein enhanced these activities of MEF/ELF4.

Conclusion: MEF/ELF4 activity may be activated by NPM1 mutant protein.

Significance: NPM1 mutant proteins have a role in MEF/ELF4-dependent leukemogenesis.

SUMMARY

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS transcription factors, can function as an oncogene in murine cancer models and is overexpressed in various human cancers. Here, we report a mechanism by which MEF/ELF4 may be activated by a common leukemia-associated mutation in the nucleophosmin gene. By using a tandem affinity purification assay, we found that MEF/ELF4 interacts with multifactorial protein nucleophosmin (NPM1). Coimmunoprecipitation and GST pull-down experiments demonstrated that MEF/ELF4 directly forms a complex with NPM1 and also identified the region of NPM1 that is responsible for this interaction. Functional analyses showed that wild-type NPM1 inhibited the DNA-binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter whereas NPM1 mutant protein (Mt-NPM1) enhanced these activities of MEF/ELF4. Induction of Mt-NPM1 into MEF/ELF4-overexpressing NIH3T3 cells facilitated malignant transformation. In addition, clinical leukemia samples with NPM1 mutations had higher human MDM2 (HDM2) mRNA expression. Our data suggest that enhanced HDM2 expression induced by mutant NPM1 may have a role in MEF/ELF4-dependent leukemogenesis.

Introduction

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS family of transcription factors, is characterized by an 85-amino acid ETS domain that recognizes a core sequence of GGAA or TTCC (1). MEF/ELF4 is

expressed in various normal and malignant hematopoietic cells and regulates the expression of various cytokines (interleukin-3 (1), granulocyte-macrophage colony stimulating factor (1), and interleukin-8 (2) as well as the cytolytic perforin molecule (3), antibacterial peptides lysozyome and human beta-defensin2 (4)), and matrix metalloproteinase (MMP)-9 expression (5). Furthermore, analyses of MEF/ELF4-deficient mice have revealed the essential role of MEF/ELF4 in the development and function of natural killer (NK) cells and NK-T cells (3). Recently, Smith *et al.* have shown that repression of Elf-4 by transcriptional repressor *Gfi1b* is important for the maturation of primary fetal liver erythroid cells (6). MEF/ELF4 also regulates the key aspects of hematopoietic stem cell behavior by controlling movement through the cell cycle from quiescence (G0) to G1 and G1 to S as well as resistance to myelosuppression (7, 8).

MEF/ELF4 is expressed in cancers such as leukemia (9), lymphoma, and ovarian cancer (10). Recently, Totoki *et al.* identified an intra-chromosomal inversion (Xq25) in hepatocellular carcinoma that generated a BCORL1-MEF/ELF4 fusion transcript (11). Experiments in several mouse models have suggested that MEF/ELF4 plays a role in tumorigenesis. For example, models of retrovirus-induced insertional mutagenesis have identified MEF/ELF4 as a gene that is involved in leukemic transformation (12). Sashida *et al.* have shown that overexpression of MEF/ELF4 enhances the expression of Mdm2, leading to decreased p53 expression (13) and enhanced transformation. In experiments with MEF/ELF4-overexpressing cells, they demonstrated that Ets1-induced p16 induction is suppressed, resulting in senescence suppression and tumor promotion.

Nucleophosmin (NPM1) is a nucleolar phosphoprotein (14) and a frequent target of genetic alterations in hematopoietic malignancies. NPM1 gene mutations have been found in approximately 60% of adult patients who have acute myeloid leukemia (AML) and a normal karyotype (15). These mutations lead to the aberrant cytoplasmic expression of NPM1 (NPMc+) due to nucleotide gain at the C-terminus (16, 17), which results in the loss of tryptophans residues essential for nucleolar localization and the gain of a new nuclear export signal (18). Increased NPM1 export into the cytoplasm probably perturbs multiple cellular pathways by delocalizing the proteins that interact with NPM1. By using a transgenic mouse model expressing the human NPMc+ mutation, it has been shown that NPMc+ confers a proliferative advantage in the myeloid lineage, suggesting that NPM1 mutations can participate in leukemia

development (19).

In the present study, we found that wild-type NPM1 (Wt-NPM1) downregulates, whereas mutated NPM1 (Mt-NPM1) upregulates, the transcriptional activity of MEF/ELF4 on the human MDM2 (HDM2) promoter. The expression of Mt-NPM1 in MEF/ELF4-overexpressing NIH3T3 cells resulted in enhanced malignant transformation. We also found that HDM2 mRNA expression in primary AML cells with *NPM1* mutations is significantly higher compared with AML cells without *NPM1* mutations. Taken together, our data suggest that NPM1 mutations may promote transformation by enhancing the oncogenic functions of MEF/ELF4.

EXPERIMENTAL PROCEDURES

Cell culture

293T cells (CRL-11268, ATCC, Manassas, VA, USA) were maintained at 37°C in DMEM (Invitrogen, Carlsbad, CA, USA) with bovine calf serum. U937 cells (CRL-1593.2; ATCC) were maintained with 10% (*v/v*) FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Fisher Scientific, Pittsburgh, PA, USA). NIH3T3 cells (CRL-1658; ATCC) were maintained under identical conditions with 10% (*v/v*) FBS and grown in RPMI 1640 (Fisher Scientific) with 10% FCS (HyClone, Logan, UT, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin. COS7 cells (CRL-1651; ATCC) were cultured in DMEM (Invitrogen) containing 10% FCS.

Tandem affinity purification assay

The cDNA of MEF/ELF4 was inserted into InterPlay N-terminal Mammalian TAP Vector (pTAP/MEF/ELF4; Stratagene, San Diego, CA, USA) comprising two affinity tags [immunoglobulin G (IgG)-binding domain and calmodulin-binding peptide] separated by the cleavage site of tobacco etch virus protease (20). 293T cells were transfected with pTAP or pTAP/MEF/ELF4 plasmids in a 10-cm dish. Transfected cells were collected and lysed in a solution containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% NP-40. The lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The resulting supernatant was incubated for 2 h at 4°C with IgG-Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK), after which the resin was washed and incubated with tobacco etch virus protease for 2 h at 16°C. Purification on calmodulin affinity resin (Stratagene) was performed according to the manufacturer's instructions. Purified proteins were precipitated with trichloroacetic acid, resolved with 1× sample buffer, and subjected to SDS-PAGE. Gels were stained with Coomassie blue, and protein bands were cut out. Proteins were

eluted with trypsin. The resulting peptides were analyzed with a Procise 49X cLC protein sequencer (Applied Biosystems, Foster City, CA, USA) (20).

In vitro translation

The cDNA molecules of Wt-NPM1 and Mt-NPM1 (21) were inserted into the pTnT vector (pTnT-NPM1; Promega, Madison, WI, USA) for *in vitro* translation. NPM1 protein (biotin-NPM1) was *in vitro*-translated with pTnT-NPM1 and labeled with biotinylated lysine (Transcend tRNA; Promega) by using a TnT Quick Coupled Transcription/Translation System (Promega). The cDNA of MEF/ELF4 was inserted into pET-3a (Novagen; VWR, Lisbon, Portugal), which allows the introduction of a His tag into the N-terminus of MEF/ELF4 (pET/MEF/ELF4). Overexpression of the recombinant protein (His-MEF/ELF4) was achieved in *Escherichia coli* BL21Gold (DE3) cells (Stratagene) transformed with the constructed plasmid pET/MEF/ELF4. His-MEF/ELF4 was isolated from cells broken in lysis buffer (STE buffer) with sonication and centrifuged at 15,000 × *g* for 10 min at 4°C (1).

Biotin-NPM1 was incubated with His-MEF/ELF4 or His (as a control) proteins at 4°C for 1 h. The mixture was loaded onto His spin traps (GE Healthcare) and eluted with 500 mM imidazole at pH 7.4. After SDS-PAGE and electroblotting, biotin-NPM1 in purified samples was detected by using a Transcend Non-radioactive Translation Detection System (Promega).

Immunoprecipitation and immunoblotting

MEF/ELF4 was cloned into p3xFLAG-CMV (Sigma, St. Louis, MO, USA) (FLAG-MEF/ELF4) from PCR products generated from pcDNA/MEF/ELF4 (1). Wt-NPM1 and Mt-A-NPM1 were cloned into pcDNA3.1/V5-His (pcDNA/V-Wt-NPM1 and Mt-A-NPM1, respectively) (Invitrogen) from PCR products generated from pcDNA/Wt-NPM1 and pcDNA/Mt-A (21). 293T cells were transfected with each plasmid by using Effectene Transfection Reagent (Qiagen, Berlin, Germany). After 48 h, cells were lysed by using a Universal Magnetic Co-IP Kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's instructions for nuclear extraction. Lysates were centrifuged at 15,000 rpm for 10 min at 4°C to remove the resin. The resulting supernatants were incubated for 4 h at 4°C with 5μg of antibodies against FLAG (Sigma), 5μg of antibodies against V5 (Invitrogen), or normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunoprecipitates were recovered, washed four times with ice-cold co-IP solution (Active Motif), and fractionated by SDS-PAGE. Separated

proteins were transferred to a membrane. After incubation in blocking buffer, membranes were probed with peroxidase-labeled antibodies against FLAG (Sigma), V5 (Invitrogen), or tag (Invitrogen). Detection was achieved with an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit, GE Healthcare). Quantification of Western blotting bands was performed by using AE-6982/C/FC and CS Analyzer ver. 3.0 software (ATTO, Tokyo, Japan).

GST and His pull-down assay

Fusion protein of GST and Wt-NPM1 (GST-NPM1) and GST-NPM1-deletion mutant constructs (Figure 1C) were generated by PCR with pcDNA/Wt-NPM1 as a template. PCR products were cloned in-frame into bacterial expression vector pGEX-T4. Plasmids that express GST fusion protein (GST-NPM1, GST-NPM1 deletion mutants) and His-MEF/ELF4 protein (pET/MEF/ELF4) or their controls were transfected into *E. coli*. Bacterial pellets were lysed in 1 mL of phosphate-buffered saline (PBS) with sonication. His-MEF/ELF4 or His alone was incubated with an equivalent amount of GST, GST-Wt-NPM1, or GST-Wt-NPM1 deletion mutants for 1 h at 4°C. Proteins were purified by using GST columns (MicroSpin GST Purification Module; GE Healthcare) or His columns. Bound proteins were analyzed by using SDS-PAGE/immunoblot.

EMSA

Recombinant proteins GST, GST-NPM1, His, and His-MEF/ELF4 were collected as described above. Nuclear protein from 293T cells transfected with pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 was extracted with an NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. EMSA was performed by using a LightShift Chemiluminescent EMSA Kit (Pierce). Recombinant protein or nuclear extracts were incubated with 20 fmol biotin 3' end-labeled oligonucleotides containing APET (an ETS binding site in the IL-3 promoter that was shown to bind to MEF/ELF4) (1). After electrophoresis, transfer, and crosslinking, the signal was detected by a peroxidase/luminol system (Chemiluminescent Nucleic Acid Detection Module; Pierce). To confirm specificity, a 200-fold excess amount of non-labeled oligonucleotides (APET-competitor) (1) was added. The DNA sequence of the APET oligonucleotide is 5'-CCTCAGTGAGCTGAGTCAGGCTTCCCC TTCCTGCCACAGGG-3'.

RNA interference

siRNA for NPM1 was transfected into 293T cells by using a GeneClip U1 Hairpin Cloning System (Promega) according to the manufacturer's instructions. The siRNA sequence-targeting NPM1 gene corresponded to nucleotides 103-125 of the coding region relative to the first nucleotide of the start codon, as described previously (22).

Luciferase assay

A 0.5μg aliquot of pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, pcDNA/Mt-A-NPM1, pcDNA/Mt-I-NPM1, or pcDNA/Mt-J-NPM1 was transfected into U937, 293T, and COS7 cells seeded in six-well dishes by using Nucleofecten (Qiagen) together with 0.1μg of pGL4 reporter plasmid (pGL4/APET (1), pGL4/ETSm-APET (1), pGL4/HDM2, or pGL4/HDM2mut) and 0.05μg of pLR-Bact vector. PGL4/ETSm-APET contains a mutation in the ETS binding site (ETSm-APET: 5'-CCTCAGTGAGCTGAGTCAGGCTgagCC TcgacGCCACAGGG-3'). pGL4/HDM2 contains a wild-type hdm2 (P2) promoter sequence from bp −82 to −122 (Wt-Ets: CAGGTTGACTCAGCTTTTCCTCTTGAG CTGGTCAAGTTCAG), and pGL4/HDM2mut contains a hdm2 (P2) promoter sequence with a mutated ETS site (Mt-Ets: CAGGTTGACTCAGCTTTTaCTCTTGAG CTGGTCAAGTTCAG) (23). Cell lysates were prepared 48 h after transfection, and luciferase activity was determined by using a Dual-Luciferase Reporter Assay System (Promega).

Anchorage-independent growth assay

NIH3T3 cells were plated on 24-well dishes in soft agar containing DMEM supplemented with 10% FCS after they were transfected with various combinations of empty vector, pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 and cultured for 2 weeks. Images were taken with a Leica DM IRBE Inverted Microscope (Leica Microsystems GmbH, Mannheim, Germany) with a 10× objective lens.

Immunochemistry

MEF/ELF4 was cloned into the pGFP-C3 vector (Clonetech, Mountain View, CA, USA) (pGFP-MEF/ELF4). 293T cells were transfected with the empty vector, pGFP-MEF/ELF4, pcDNA/V-Wt-NPM1, or pcDNA/V-Mt-A-NPM1. Cells were harvested 3 days after transfection. Cytospin samples were fixed for 15 min in PBS containing 4% paraformaldehyde. Fixed coverslips were washed twice in TBS, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in Image-iT FX Signal Enhancer (Invitrogen) for 30 min. Cells were incubated with primary antibody for 1 h and then washed extensively in TBS before

incubation with Alexa546-conjugated goat anti-mouse-IgG antibody (dilution, 1:2000; Invitrogen) for 1 h. Cells were covered with a drop of ProLong Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent images were obtained by using a confocal laser scanning microscope (LSM 5 Pascal V3.2; Carl Zeiss, Jena, Germany).

ChIP assay

293T cells were transfected with empty vector, pcDNA/MEF-FLAG, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 by using a nucleofection kit (Qiagen). After 48 h of culture at 26°C, cells were fixed by the addition of 1% formaldehyde in PBS for 10 min. Chromatin isolation and shearing were performed by using a OneDay Chip Kit (Diagenode, Liege, Belgium) and Shearing-Chip Kit (Diagenode) according to the manufacturer's instructions. Immunoprecipitation reactions were performed with anti-Flag monoclonal antibody (Sigma) or isotype control IgG (BD Biosciences, San Jose, CA, USA). Samples were analyzed by quantitative real-time quantitative reverse transcriptase-polymerase chain reaction (RQ-PCR) by using a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) as specified by the manufacturer. The primer sequences for the HDM2 promoter were 5'-GAACGCTGCGCGTAGTCTGG-3' (forward) and 5'-ACTGCAGTTTCGGAACGTGT-3' (reverse).

Clinical samples

Informed consent for sample collection was obtained according to protocols approved by the International Review Board of Nagasaki University, Nagasaki, Japan (approval number 33-3). Bone-marrow aspirates were collected from 22 AML patients before the initiation of chemotherapy. CD34-positive cells were isolated by using Ficoll density gradient centrifugation and magnetic beads (CD34 Isolation Kit; Miltenyi Biotec, Auburn, CA, USA) to minimize the confounding effect of MEF/ELF4 and NPM1 expression by mature myeloid cells. For the screening of NPM1 mutations, genomic DNA corresponding to exon 12 was amplified by using forward primer 5'-TTAACTCTCTGGTGGTAGAATGAA-3' and reverse primer 5'-CAAGACTATTTGCCATTCCTAAC-3', as reported previously. Amplified products were separated by agarose gel electrophoresis, purified by using a QIAquick gel extraction kit (Qiagen), and directly sequenced by using a DNA sequencer (3100; Applied Biosystems) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). When mutations were found by direct sequencing, the fragments were cloned into a pTOPO

vector (Invitrogen) and then transfected into the *E. coli* strain DH5A. At least four recombinant colonies were selected, and plasmid DNA samples were prepared by using a QIAprep Spin Miniprep Kit (Qiagen). Cloned fragments were sequenced to confirm the mutation of the *NPM1* gene.

Total RNA was harvested from purified CD34-positive cells by using an RNeasy Minikit (Qiagen). cDNA synthesis was undertaken by using an oligo (dT) primer with a PrimeScript II First-strand cDNA Synthesis Kit (Takara, Shiga, Japan). These cDNA molecules were measured by RQ-PCR with the primers listed in the Methods section of RQ-PCR.

RQ-PCR

RQ-PCR was performed by using a LightCycler TaqMan Master kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Twenty microliters of Universal ProbeLibrary probes (Exiqon, Vedbaek, Denmark) were added in the final reaction. Primers designed by using the Universal ProbeLibrary Assay Design Centre (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) were synthesized by Sigma. PCR amplification was performed by using a LightCycler 350S instrument (Roche). Thermal cycling conditions comprised 2 min at 40°C and 10 min at 95°C, followed by 45 amplification cycles at 95°C for 10 s, 60°C for 30 s, 72°C for 1 s, and then a 40°C cooling cycle for 30 s. Specific primers and probes were as follows: for HDM2, forward 5'-TCTGATAGTATTTCCCTTTCCTTTG-3', reverse 5'-TGTTCACTTACACCAGCATCAA-3', probe 5'-CGCCACTTTTTCTCTGCTGATCCAGG-3'; human MEF/ELF4, forward 5'-TGGAGACTCTCAGGGTCGAAA-3', reverse 5'-AAGCAACGGGATGGATGAT-3', probe 5'-TCACAGCTGGGAACACAGAG-3'; and human G6PDH, forward 5'-AAGCAACGGGATGGATGAT-3', reverse 5'-TCACAGCTGGGAACACAGAG-3', and probe 5'-CGCCACTTTTTCTCTGCTGATCCAGG-3'.

Statistical analyses

Comparisons of patient characteristics between two groups were performed with the Wilcoxon test. The results of *in vivo* experiments are presented as the mean ± SD of three independent experiments and compared by using one-way ANOVA followed by Scheffe's multiple comparison test. A P-value of 0.05 was considered statistically significant.

RESULTS

Identification of MEF/ELF4-binding protein

To identify the proteins that bind to MEF/ELF4, we performed the TAP procedure and analyzed the amino acid sequence of the protein complex, thereby identifying 25 proteins (including NPM1). NPM1 is essential for embryonic development and is frequently translocated or mutated in hematological malignancies (24). Therefore, we decided to focus on the interaction between NPM1 and MEF/ELF4.

Wt-NPM1 interacts with MEF/ELF4 in vivo and in vitro

To determine if Wt-NPM1 interacts with MEF/ELF4 in human cells, we transfected 293T cells with FLAG-MEF/ELF4 and V5-Wt-NPM1 expression plasmids and performed immunoprecipitations with mouse monoclonal anti-FLAG or anti-V5 antibody. As shown in Fig. 1A, FLAG-MEF/ELF4 protein co-precipitated with V5-Wt-NPM1 by the anti-V5 antibody (lane 1), but not by the isotype-matched control (lane 2). In reciprocal experiments, V5-Wt-NPM1 protein co-precipitated with Flag-MEF/ELF4 protein by the anti-Flag antibody (lane 3). These results showed the *in vivo* interaction between Wt-NPM1 and MEF/ELF4. To ascertain whether Wt-NPM1 protein interacted directly with MEF/ELF4, an *in vitro* association assay with biotin-labeled *in vitro*-translated Wt-NPM1 and bacterially recombinant His-MEF/ELF4 fusion protein was performed (Fig. 1B). Biotin-labeled Wt-NPM1 bound to His-MEF/ELF4 (lane 1), but not to His alone (lane 2). These results demonstrated that His-MEF/ELF4 bound directly to Wt-NPM1.

To characterize the region of Wt-NPM1 that binds MEF/ELF4, five distinct GST-NPM1 proteins were prepared (Fig. 1C). GST pull-down assays (Fig. 1D (a)) and His tag pull-down assays (Fig. 1D (b)) revealed that the N-terminal region of NPM1 (the F1, F2, and F3 fragments that contain the oligomerization domain) bound to His-MEF/ELF4, unlike the C-terminal region of NPM1 (F4 and F5).

Wt-NPM1 interferes with MEF/ELF4 binding to target DNA sequences

To assess the direct role of Wt-NPM1 in MEF/ELF4 action, we undertook EMSA. His-MEF/ELF4 bound to the APET probe (1), but no band was observed with His, GST, or GST-NPM1 (Fig. 2). The shifted band of MEF/ELF4 was diminished when the APET competitor was added to the reaction mixture. When Wt-NPM1 was added to the reaction mixture, the shifted band containing MEF/ELF4 was diminished. These results implied that Wt-NPM1 inhibits the DNA binding of MEF/ELF4 DNA through direct interactions.

Wt-NPM1 inhibits, whereas Mt-NPM1 enhances, MEF/ELF4-dependent

transcriptional activity

To study the functional relevance of the physical interaction between MEF/ELF4 and Wt-NPM1, we transfected pcDNA/MEF/ELF4 in combination with pcDNA/Wt-NPM1 and examined the activity of the APET promoter construct (1) in 293T cells (Fig. 3A). As reported previously, MEF/ELF4 activated the APET promoter by approximately 159-fold. Co-expression of Wt-NPM1 with MEF/ELF4 led to a significant decrease in luciferase activity. Similar data were obtained by using COS7 cells (Fig. 3B) and a human leukemia cell line, U937 (Fig. 3C).

Having shown that NPM1 expression attenuated the transcriptional activity of MEF/ELF4 in leukemia cells, we next assessed whether the inhibition of Wt-NPM1 expression *in vivo* enhanced MEF/ELF4-dependent transcriptional activity. The siRNA directed against Wt-NPM1 in 293T cells suppressed the expression of Wt-NPM1 protein by 60%-70% (Fig. 3D). Transient transfections were performed by using NPM1-knockdown 293T cells with pcDNA/MEF/ELF4 and PGL4/APET reporter plasmids. A luciferase assay revealed that MEF/ELF4-dependent transcriptional activity was significantly elevated in Wt-NPM1-knockdown cells by 1.8-fold (Fig. 3E). These results implied that Wt-NPM1 functioned as an inhibitor of MEF/ELF4.

Mutated nucleophosmin (Mt-NPM1) has been found in 50% of adult AML patients with normal karyotypes (15). It has been suggested that the mutation is a critical event for leukemogenesis. To determine the effect of Mt-NPM1 on the transcription-activating properties of MEF/ELF4, we transfected pcDNA/MEF/ELF4 in combination with pcDNA/Mt-A-NPM1, pcDNA/Mt-I-NPM1, or pcDNA/Mt-J-NPM1 and then examined the activity of the APET promoter construct in 293T cells (Fig. 3F). Co-expression of Mt-NPM1 with MEF/ELF4 led to a 315-fold increase in luciferase activity. Similar data were obtained with COS7 (Fig. 3G) and U937 (Fig. 3H) cells. To show the effect of the coexistence of both Wt- and Mt-NPM1, we transfected 293T cells with various amounts of plasmids that expressed Wt-NPM1 and Mt-A-NPM1. The expression of Mt-NPM1 enhanced MEF/ELF4-dependent APET promoter activation in a dose-dependent manner, even in the presence of Wt-NPM1 (Fig. 3I). Taken together, our results suggest that Wt-NPM1 has an inhibitory effect, whereas Mt-NPM1 has enhancing effect, on the function of MEF/ELF4.

Mt-NPM1 does not interact with MEF/ELF4 in vivo

Since the mutated region of Mt-NPM1 was

located outside the domain responsible for interaction with MEF/ELF4, we hypothesized that Mt-NPM1 might bind to MEF/ELF4. To test this hypothesis, we transfected 293T cells with FLAG-MEF/ELF4 and V5-Mt-A-NPM1 expression plasmids and performed immunoprecipitations with mouse monoclonal anti-FLAG or anti-V5 antibody. Contrary to our expectations, as shown in Fig. 4, FLAG-MEF/ELF4 protein and V5-Wt-A-NPM1 did not co-precipitate with each other (Fig. 4). These results showed that there is little *in vivo* interaction between Mt-A-NPM1 and MEF/ELF4.

Localization of MEF/ELF4 is unaffected by Mt-NPM1

Having shown that Mt-NPM1 enhances the transcriptional activity of MEF/ELF4, we next assessed whether Mt-NPM1 dislocates MEF/ELF4 into the cytoplasm. We transiently co-transfected a MEF/ELF4-GFP fusion protein vector together with the pcDNA/V-Wt-NPM1 or pcDNA/V-Mt-A-NPM1 expression vector into 293T cells. Wt-NPM1 protein and MEF/ELF4 localized to the nucleus (Fig. 5A (a)), whereas Mt-A-NPM1 protein localized to the cytoplasm (Fig. 5A(b)). Contrary to our expectations, the presence of Mt-A-NPM1 did not affect the subcellular distribution of MEF/ELF4. Western blot analysis of MEF/ELF4 and Wt- or Mt-NPM1 in nuclear and cytoplasmic proteins confirmed the nuclear localization of MEF/ELF4 even with Mt-NPM1 (Fig. 5B).

Wt-NPM1 inhibits, whereas Mt-NPM1 enhances, the oncogenic activity of MEF/ELF4

The overexpression of MEF/ELF4 in NIH3T3 cells increases the growth rate, enhances colony formation in soft agar, and promotes tumor formation in nude mice (10). To determine the effects of the interaction of NPM1 with MEF/ELF4 on cell behavior, we assessed the anchorage-independent growth of NIH3T3 cells after co-transfection of MEF/ELF4 with Wt-NPM1 or Mt-A-NPM1. Compared with NIH3T3 transfected with only MEF/ELF4, Wt-NPM1-coexpressing cells showed reduced anchorage-independent growth, whereas Mt-A-NPM1-coexpressing cells exhibited increased growth (Fig. 6).

MEF/ELF4 binds to the HDM2 promoter and activates its expression

In murine cells, MEF/ELF4 binds directly to the Mdm2 promoter, thereby promoting *Mdm2* expression (12). To ascertain whether MEF/ELF4 also directly regulates the promoter activity of *HDM2* (the human analog of Mdm2), we scrutinized the DNA sequence of the *HDM2* gene and found a conserved putative MEF/ELF4 binding site in the P2 promoter (Fig. 7B). To establish the association of MEF/ELF4 with the HDM2

promoter, we performed a ChIP assay with nuclear lysates from 293T cells expressing FLAG-MEF/ELF4. Immunoprecipitation with the FLAG antibody (but not with the control IgG) and subsequent PCRs revealed the recruitment of overexpressed MEF/ELF4 to the promoter region of the HDM2 gene (Fig. 7A). The luciferase assay revealed that MEF/ELF4 strongly transactivated the wild-type HDM2 promoter (Fig. 7B (a), Fig. 7C) and that the effect was abrogated by mutation of the ETS site (-122 to -82) (Fig. 7B (b), Fig. 7C). Compared with Wt-NPM1, the expression of Mt-A-NPM1 in 293T cells enhanced the association of MEF/ELF4 with the HDM2 promoter as detected by ChIP analysis (Fig. 7D). Taken together, these findings suggest that Mt-NPM1 upregulates HDM2 transcription by increasing the recruitment of MEF/ELF4 to the HDM2 promoter by dislocating Wt-NPM1 that interferes with its binding to the promoter.

Higher levels of HDM2 mRNA in clinical samples from AML patients with Mt-NPM1 and higher MEF/ELF4 expression

To determine the possible clinical relevance of MEF/ELF4, NPM1, and HDM2 in AML patients, we examined the mRNA levels of each in CD34-positive leukemic blasts from 22 AML patients with normal karyotypes. Fourteen patients had Wt-NPM1, and 8 patients had Mt-A-NPM1. There was no significant difference in the clinical characteristics of the Wt-NPM1 group and the Mt-NPM1 group (Table 1). Samples from the Mt-NPM1 group had significantly higher levels of HDM2 expression as compared to the Wt-NPM1 group ($p = 0.009$) (Fig. 8A). In addition, patients with high expression levels of MEF/ELF4 (the MEF/ELF4-H group) had significantly higher HDM2 expression than patients with low expression levels of MEF/ELF4 (the MEF/ELF4-L group) ($p = 0.03$) (Fig. 8B).

DISCUSSION

In the present study, we identified NPM1 to be a MEF/ELF4-binding protein. Wt-NPM1 inhibited the function of MEF/ELF4 (i.e., DNA-binding and transcriptional activities), whereas Mt-NPM1 augmented its function. Some of these effects of Wt-NPM1 and Mt-NPM1 on MEF/ELF4 were reproducible on the HDM2 promoter (one of the target genes of MEF/ELF4), suggesting that HDM2 expression is influenced by NPM1. Furthermore, we found that the expression of Mt-NPM1 in MEF/ELF4-overexpressing NIH3T3 cells resulted in enhanced malignant transformation. We also found that the mRNA level of HDM2 in primary leukemia cells was higher in patients with NPM1 mutations. Mef/Elf4 directly activates Mdm2 expression (13). Therefore, NPM1 mutation could enhance HDM2 expression through the

increased MEF/ELF4 activity, thereby promoting transformation by inhibiting the p53 pathway.

NPM1 is a multifunctional phosphoprotein that has been implicated in cell proliferation as well as regulation of transcription factors. It appears to repress or stimulate transcription. For example, Wt-NPM1 activates and inhibits p53 function through direct binding (22,25). Interferon regulatory factor-1 (IRF-1), a transcriptional activator, binds to Wt-NPM1, resulting in the inhibition of DNA binding and transcriptional activity (26). Our findings with Wt-NPM1 and MEF/ELF4 are consistent with these observations. Wt-NPM1 interacts directly with c-Myc and regulates the expression of endogenous c-Myc target genes at the promoter, which enhances c-Myc-induced proliferation and transformation (27). In contrast, the present study suggests that Wt-NPM1 inhibits (whereas Mt-NPM1 facilitates) the transformation induced by MEF/ELF4, suggesting that there is a contradiction in terms of NPM1 function. However, the overexpression of Wt-NPM1 without c-Myc activation has only a small effect on proliferation and has no effect on transformation, so Wt-NPM1 may mainly have a role in c-Myc-driven tumors. Interestingly, c-Myc, IRF-1, and MEF/ELF4 are all regulated during the cell cycle, and the levels of these transcription factors are highest in the G1 phase (28,29).

We found that Wt-NPM1 could interfere with the ability of MEF/ELF4 to bind to DNA, resulting in the inhibition of MEF/ELF4-dependent transcriptional activity. The mechanism by which Wt-NPM1 interferes with the DNA binding of MEF/ELF4 is unclear. We previously showed that the 120 amino acids of the N-terminal to the ETS domain in MEF/ELF4 (residues 87-206) are responsible for its binding to AML1 proteins (30); thus, MEF/ELF4 interacts with other proteins outside the DNA-binding domain. As mentioned above, the association of Wt-NPM1 and IRF-1 inhibits the DNA binding of IRF-1. Narayan *et al.* showed that IRF1 binds directly to Wt-NPM1 through a short linear motif in the nuclear localization sequence outside the DNA-binding domain (31). These results suggest that the inhibition of DNA binding by NPM1 may not be through a simple interference with the DNA-binding domain of MEF/ELF4. Determining the protein-binding interface of MEF/ELF4 may help to reveal the mechanism of NPM1-mediated transcriptional regulation.

The heterodimerization domain (residues 186-259) of NPM1 is essential for its interaction with p53 (22), and the c-Myc-binding region is within the NPM1 heterodimerization domain (27). In the case

of MEF/ELF4 and NPM1, the N-terminal regions of NPM1 (F1, F2, and F3) could bind to His-MEF/ELF4, implying that the oligomerization domain is important for the interaction.

Recently, it has been shown *in vivo* that NPM1 mutants actively contribute to leukemogenesis by conferring a proliferative advantage in the myeloid lineage. In zebrafish, forced expression of mutant NPM1 causes an increase in PU.1-positive primitive early myeloid cells (32). Furthermore, in a transgenic mouse expressing the human NPM1 mutant, although spontaneous AML was not found, myeloproliferation occurred in the bone marrow and spleen (33). Moreover, Vassiliou *et al.* showed that activation of a humanized mouse NPM1 mutant knock-in allele in mouse hematopoietic stem cells caused overexpression of the Hox gene, enhanced self-renewal, and expanded myelopoiesis, resulting in delayed-onset AML in one third of the mice (34). Taken together, these data suggest that NPM1 mutations initiate leukemia by activating a set of proliferative pathways. Mt-NPM1 enhances the transcriptional activity of MEF/ELF4, so the upregulation of HDM2 and subsequent downregulation of p53 may also have a role in leukemogenesis.

In vitro transfection studies and immunohistochemical observations in samples from AML patients have demonstrated that NPM1 mutants recruit Wt-NPM1 from the nucleolus and delocalize it to the nucleoplasm and cytoplasm (18) and that aberrant NPM1 accumulation in the cytoplasm may have a critical role in leukemogenesis. While Wt-NPM1 protein co-localizes with tumor suppressor p19ARF in the nucleolus, Mt-NPM1 delocalizes p19ARF from the nucleolus to the cytoplasm, which results in reduced p19ARF activities (e.g., Mdm2 and $p21^{cip1}$ induction, stimulation of NPM1) (35). Furthermore, by using OCI/AML3 human leukemia cells where mutant NPM1 is localized in the cytoplasm, Bhat *et al.* have recently shown that NPM1 co-localizing nuclear transcription factor, Forkhead box M1 (FOXM1), disappears from the cytoplasm following transient NPM1 knockdown (36). These data suggest that NPM1 may determine the intracellular localization of interacting transcription factors. However, in our experiments, Mt-NPM1 did not interact with MEF/ELF4 in vivo, and the subcellular distribution of MEF/ELF4 was not affected by the presence of Mt-NPM1. It seemed that Mt-NPM1 binds and dislocates Wt-NPM1 into the cytoplasm of leukemia cells, which eventually leads to uncontrolled transactivation of MEF/ELF4. Wt-NPM1 knockdown with siRNA against NPM1 also enhanced MEF/ELF4 activity (Fig. 3E),

suggesting that the depletion of an MEF/ELF4 inhibitor (i.e., Wt-NPM1) in the nucleus is responsible for the transactivation of MEF/ELF4. Taken together, it is likely that NPM1 mutants exert oncogenic functions at least in part through the upregulation of the activities of oncogenic transcription factors such as MEF/ELF4. The correlation between NPM1 mutations and the elevated expression of HDM2 in primary leukemia cells seems to support this theory.

In patients with AML, NPM1 mutations are mutually exclusive of recurrent genetic abnormalities. It can be speculated that the enhanced MEF/ELF4-HDM2-p53 pathway induced by NPM1 mutations may participate in leukemia development, especially in patients with a normal karyotype. The transactivation of MEF/ELF4 by E2F1 is inhibited by p53 (37), suggesting that p53 suppression induced by NPM1 mutation could lead to the activation of E2F1, resulting in the enhanced expression of MEF/ELF4. Our previous data showing the elevated expression of MEF/ELF4 in AML cells with a normal karyotype compared to that of AML cells carrying t(8;21) and t(15;17) seem to support this hypothesis. Our results suggest a new role for NPM1 and MEF/ELF4 in leukemia development.

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FIGURE LEGENDS

Figure 1

NPM1 interacts with MEF/ELF4. (A) 293T cells were transfected with the indicated expression plasmids. After 48 h, cell lysates were immunoprecipitated with anti-FLAG and anti-V5 antibodies. Immunoprecipitates were analyzed by 10% SDS-PAGE and subjected to immunoblotting with anti-V5 antibody (upper row) or anti-FLAG antibody (bottom row). (B) MEF/ELF4 interacts directly with NPM1 *in vitro*. *In vitro* association assays were undertaken by incubating His-MEF/ELF4 fusion protein immobilized by using a His-column with biotin-labeled MEF/ELF4 (lane 1). His alone was incubated with biotin-labeled NPM1 (lane 2) as a control. (C) NPM1 structure and the relative binding of MEF/ELF4 (schematic). HomoD, homodimerization domain, residues 1–117; AD/NLS, acidic domain/nuclear localization domain, residues 117–187; HeteroD, heterodimerization domain, residues 187–259; NBD, nucleic acid binding domain, residues 259–294. (D) The N-terminal portion of NPM1 is the MEF/ELF4-interacting domain. Bacterially expressed and purified GST, GST-NPM1, and GST-NPM1 mutants with deletions were mixed with bacterially expressed and purified His or His-MEF/ELF4 protein. Recombinant proteins were subjected to His or GST affinity columns, followed by immunoblotting with anti-GST or anti-His antibodies. (a): the reactive samples were subjected to analyses in His affinity column followed by immunoblotting with ant-His antibodies (left lower panel) or with anti-GST antibodies (left upper panel). (b): the reactive

samples were subjected to GST affinity columns, followed by immunoblotting with anti-GST antibodies (right upper panel) or with anti-His antibodies (right lower panel).

Figure 2

EMSA with recombinant His-MEF/ELF4, His, GST, and GST-Wt-NPM1. His-MEF/ELF4 was incubated with GST and GST-Wt-NPM1 at room temperature prior to EMSA by using a biotin-conjugated APET probe (lanes 1-4). An excess amount of unlabeled APET competitor was added to the reaction mixtures (lanes 5 and 6).

Figure 3

Wt-NPM1 inhibits, whereas Mt-NPM1 enhances, MEF/ELF4-dependent APET promoter transactivation. (A) 293T human kidney cell lines, (B) COS7 monkey kidney cell lines, and (C) U937 human hematological cell lines were co-transfected with the luciferase reporter gene of an artificial MEF/ELF4 target promoter (APET) and effecter genes. Target promoter and effecter genes were as follows: lane 1: PGL4/APET; lane 2: PGL4/ETSm-APET; lane 3: PGL4/APET and PcDNA/MEF/ELF4; lane 4: PGL4/APET, pcDNA/MEF/ELF4, and pcDNA/Wt-NPM1; lane 5: PGL4/ETSm-APET and pcDNA/MEF/ELF4; and lane 6: PGL4/APET and pcDNA/Wt-NPM1. Luciferase activity by PGL4/APET alone was assigned a value of 1.0. The analysis was performed in triplicate assays, and the results were reproducible. The results are shown as the mean ± SD (*$P < 0.05$). (D) 293T cells transduced with siRNA encoding vector (siWt-NPM1) were harvested 72 h after transduction for Western blotting. Hsp90 is shown as a control. sicNPM1, control siRNA non-relevant to the expression of NPM1; Wild, without transduction. (E) 293T cells were co-transfected with the luciferase reporter plasmid (PGL4/APET), expression plasmid (pcDNA MEF/ELF4), and siWt-NPM1 gene (pcDNA/siRNA-Wt-NPM1) or control. Luciferase activity by PGL4/APET alone was assigned a value of 1.0. The analysis was performed in triplicate assays, and the results were reproducible. The results are shown as the mean ± SD (*$P < 0.05$). (F) 293T cells were co-transfected with the luciferase reporter gene of an artificial MEF/ELF4 target promoter and effector genes. Target promoter and effector genes were as follows: lane 1: PGL4/APET; lane 2: PGL4/APET and PcDNA/MEF/ELF4; lane 3: PGL4/APET, pcDNA/MEF/ELF4, and Wt-NPM1; lanes 4, 5 and 6: PGL4/APET, pcDNA/MEF/ELF4, and Mt-A-NPM1, Mt-I-NPM1 or Mt-J-NPM1; and lanes 7, 8, 9 and 10: PGL4/APET and pcDNA/Wt-NPM1, Mt-A-NPM1, Mt-I-NPM1 or Mt-J-NPM1. Luciferase activity by PGL4/APET alone was assigned a value of 1.0. The analysis was performed in