

Structural Consequences of Nucleophosmin Mutations in Acute Myeloid Leukemia*

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Mutations affecting NPM1 (nucleophosmin) are the most common genetic lesions found in acute myeloid leukemia (AML). NPM1 is one of the most abundant proteins found in the nucleolus and has links to the MDM2/p53 tumor suppressor pathway. A distinctive feature of NPM1 mutants in AML is their aberrant localization to the cytoplasm of leukemic cells. This mutant phenotype is the result of the substitution of several C-terminal residues, including one or two conserved tryptophan residues, with a leucine-rich nuclear export signal. The exact molecular mechanism underlying the loss of nucleolar retention, and the role of the tryptophans, remains unknown. In this study we have determined the structure of an independently folded globular domain in the C terminus of NPM1 using NMR spectroscopy, and we report that the conserved tryptophans are critical for structure. This domain is necessary for the nucleolar targeting of NPM1 and is disrupted by mutations in AML with cytoplasmic NPM1. Furthermore, we identify conserved surface-exposed lysine residues that are functionally rather than structurally important for nucleolar localization. This study provides new focus for efforts to understand the pathogenesis of AML with cytoplasmic NPM1 and may be used to aid the design of small molecules that target the C-terminal domain of NPM1 to act as novel anti-proliferative and anti-leukemia therapeutics.

The gene encoding nucleophosmin (*NPM1*) is mutated in 50–60% of normal karyotype adult acute myeloid leukemia (AML),² making *NPM1* mutations the most common genetic lesions identified to date in *de novo* AML. NPM1 (nucleophosmin, also known as B23 or numatrin) is a ubiquitously expressed protein that belongs to the nucleoplasmin family of nuclear chaperones. Found predominantly in the nucleolus,

NPM1 is used as a marker for the granular component (1–3) (the site of pre-ribosomal subunit assembly (4)). NPM1 rapidly shuttles between the nucleoplasm and the cytoplasm (5–7) serving as a chaperone for both nucleic acids and proteins (8–11). NPM1 is essential for embryonic development (12) and appears to modulate diverse molecular functions, including ribosome biogenesis (13–16) and genomic stability (12). NPM1 is overexpressed in a number of solid tumors (17), is linked to the p53/MDM2 pathway via the tumor suppressor ARF (18–22), and is a transcriptional target of Myc (23, 24).

A distinctive feature of NPM1 mutants in AML is their aberrant localization to the cytoplasm of leukemic cells (NPMc+ AML) (25). Two consequences of mutation-related sequence changes at the C terminus of NPM1 promote aberrant cytoplasmic localization of mutant NPM1 proteins in NPMc+ AML. First, generation of an additional leucine-rich nuclear export motif reinforces Crm1 (XpoI)-dependent nuclear export of NPM1 leukemic mutants (26). Second, there is loss of tryptophan residues Trp²⁸⁸ and Trp²⁹⁰ (or Trp²⁹⁰ alone) that determine NPM1 nucleolar localization (25, 27, 28). However, the precise molecular mechanisms that regulate the steady state distribution of NPM1 are unknown. In this study, we tested the hypothesis that NPM1 tryptophans Trp²⁸⁸ and Trp²⁹⁰ are critical structural components of an independently folded domain that is essential for nucleolar targeting of NPM1. These studies provide a structural basis for understanding the mechanisms underlying NPMc+ AML.

EXPERIMENTAL PROCEDURES

Reagents—Mutants NPM1 W288A/W290A and NPM1 F268A/F276A were made by site-directed mutagenesis and mutants A and E by PCR using appropriate primers. NPM1 C-terminal domain variants, with the exception of mutant A, were cloned into a modified pRSETA vector (Invitrogen) expressing the protein fused to a tobacco etch virus protease-cleavable His₆-tagged lipoyl domain from *Bacillus stearothermophilus*. Proteins were expressed in *Escherichia coli* strain C41 and purified using Ni²⁺-chelating affinity chromatography. Fusion proteins were cleaved by tobacco etch virus protease digestion and removed in a second affinity step before concentration and final purification by size exclusion chromatography on a GF30 column (Amersham Biosciences). For isotopic labeling, K-MOPS minimal media containing ¹⁵NH₄Cl and/or [¹³C]glucose was used. Mutant A C-terminal domain was made using a Liberty CEM microwave peptide synthesizer (Matthews, NC) and standard Fmoc (*N*-(9-fluorenyl)methoxy-

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The atomic coordinates and structure factors (code 2vxd) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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² The abbreviations used are: AML, acute myeloid leukemia; NES, nuclear export signal; NOESY, nuclear Overhauser effect spectroscopy; eGFP, enhanced green fluorescent protein; MES, 4-morpholineethanesulfonic acid; PBS, phosphate-buffered saline; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; MOPS, 4-morpholinepropanesulfonic acid.

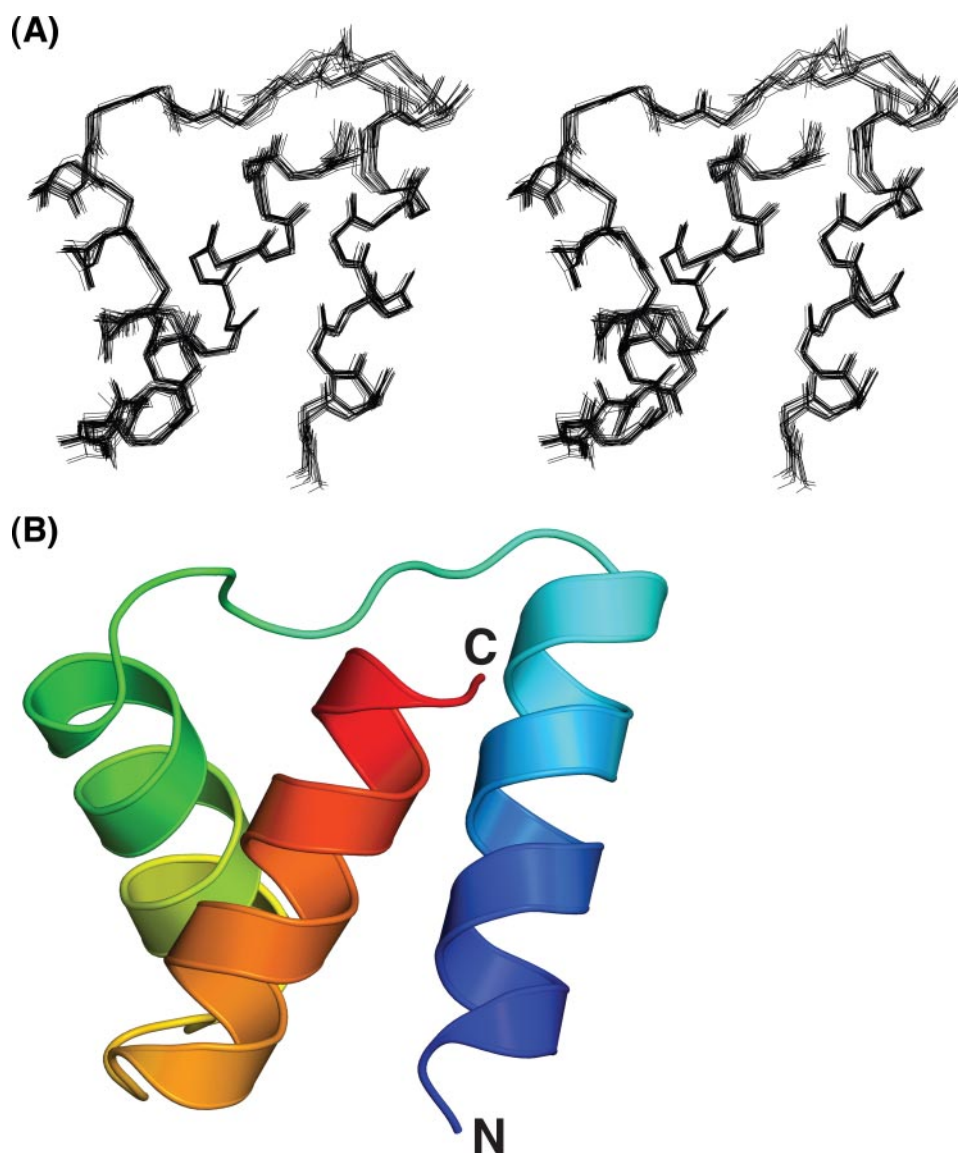


FIGURE 1. **Solution structure of the C-terminal domain of nucleophosmin.** *A*, overlay of the backbone atoms of the 20 lowest energy structures in stereo. *B*, schematic representation of the lowest energy structure colored *blue* to *red* from the N to C terminus (same orientation as in *A*). Prepared using the program PyMOL (53).

carbonyl)/*t*-butyl chemistry and purified by reverse-phase high pressure liquid chromatography. All C-terminal constructs have an additional two glycine residues at the N terminus as a result of the cloning and purification strategy; residue numbering is in context of the full-length protein and omits these glycines.

SDS-PAGE and electrospray mass spectrometry were used to confirm proteins were of the expected mass and to assess their purity. Protein concentration was determined spectroscopically using a Varian Cary 500 spectrometer (Varian, NC), and extinction coefficients were calculated for 280 nm (29, 30). All reagents were purchased from Sigma and were Anal-R grade or higher, except for isotopically enriched reagents, which were purchased from Cambridge Isotopes (Cambridge, UK).

Spectroscopic Measurements—The NMR spectra were recorded on Bruker Avance-800, Avance-II⁺ 700, and Avance-600 spectrometers. Two-dimensional NOESY, TOCSY, DQF-COSY, ¹⁵N-HSQC, constant time ¹³C-HSQC and ¹³C-TOCSY were recorded at 298 K. The mixing times

chosen were 80 ms for TOCSY and 120 ms for NOESY. Spectra were referenced relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate for signals of proton and carbon, or liquid ammonium for that of nitrogen. Approximately half the H β resonances were assigned stereospecifically using a combination of HNHB and DQF-COSY spectra. All the Val(H γ) and Leu(H δ) resonances were assigned stereospecifically using a 10% ¹³C-labeled sample of NPM1 C-terminal domain (31). All the NMR spectra were analyzed with Sparky (32).

All samples used for structure determination were 1.2 mM and prepared in 10 mM MES, pH 6.5, 150 mM NaCl, 5 mM dithiothreitol, 0.02% sodium azide, and 5% D₂O (homonuclear samples were in a deuterated version of this buffer). For hydrogen exchange experiments, the ¹⁵N-labeled NPM1 C-terminal domain was exchanged into NMR buffer containing 100% D₂O using a NAP-10 column (Amersham Biosciences), and a series of ¹H-¹⁵N-FHSQC spectra (33) were recorded over the course of 6 h. All one-dimensional ¹H NMR experiments were conducted in biophysics buffer (10 mM KP_i, pH 6.5, 150 mM NaCl, 5% D₂O).

Structure Determination—The distance constraints derived from the NOESY spectra were classified into four categories corresponding to inter-proton distance constraints of 1.8–2.8, 1.8–3.5, 1.8–4.75, and 1.8–6.0 Å, respectively. Hydrogen bond constraints of 1.8–2.1 Å were imposed on the distance between the hydrogen and the acceptor oxygen, whereas another constraint of 2.7–3.1 Å was imposed on the distance between the donor nitrogen and the acceptor oxygen. Torsion angle constraints were obtained from stereospecific assignment of residue side chains and incorporated in the structure calculation, along with the backbone ϕ and ψ angle constraints determined with the program TALOS (34). The structures were calculated using a standard torsion angle dynamics simulated annealing protocol with the program CNS (35). 150 structures were accepted out of 150, produced with no distance violations greater than 0.25 Å, and no angle violations greater than 5°. Of these the 20 lowest energy structures were selected for presentation and statistical representation.

Circular Dichroism—CD spectra of NPM1 were recorded between 260 and 190 nm at 30–60 μ M in the biophysics buffer

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using a Jasco-J815 spectropolarimeter (Jasco Inc., Easton, MD) and a 1-mm path length cuvette. The base line due to the buffer only was recorded and subtracted prior to normalizing the data for concentration. Thermal denaturation was followed at 222 nm with heating from 277 to 371 K at a rate of 1 K·min⁻¹. Data were fit to a standard two-state denaturation as described elsewhere (36).

Immunofluorescence—Full-length NPM1 variants were cloned into the mammalian expression vector pEGFP-C1 (BD Biosciences). NPM1 variants were thus expressed *in vivo* with an N-terminal enhanced green fluorescent protein (eGFP) tag.

NIH-3T3 murine fibroblasts were grown in Dulbecco's modified Eagle's medium GlutaMAXTM (Invitrogen) supplemented with 10% fetal calf serum. A day before transfection a confluent flask of cells was subcultured at a 1:8 dilution in 6-well plates containing glass coverslips. Transfection was performed using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. After 24 h coverslips were removed, washed with PBS, and fixed in 4% paraformaldehyde for 20 min. Cells were permeabilized with PBS + 0.1% Triton X-100 prior to blocking for 30 min with 20% fetal calf serum, 0.5% Tween 20 in PBS (FATS). Cells were stained with mouse anti-fibrillar (ab4566, AbCam, UK) at 1:500 in FATS for 1 h. After three washes with PBS + 0.1% Triton X-100, the cells were incubated in the dark for 1 h with Alexa FluorTM 594-conjugated goat anti-mouse antibody (Molecular Probes, Leiden, Netherlands) at 1:200 in FATS. The coverslips were dried in air and mounted using Fluoromount-GTM (Southern Biotech, Birmingham, AL). Images were collected using an MRC-2000 plus confocal apparatus (Bio-Rad) mounted on a Nikon eclipse E800 microscope using the ×60 oil objective lens.

RESULTS

The Structure of the NPM1 C-terminal Domain—Manual inspection of the sequence of NPM1, with assistance from the secondary structure prediction tool Jpred (37), indicated the potential for three short helices separated by short loops within the last 50 residues. This potential domain is separated from the N-terminal oligomerization domain by an ~120-residue acid-rich region that is predicted to be disordered. The DNA sequence corresponding to residues 243–294 of NPM1 was cloned and overexpressed in *E. coli*. Inspection by SDS-PAGE and electrospray-mass spectrometry following purification identified a pure protein of the expected mass. Both one-dimensional ¹H NMR and ¹⁵N-HSQC experiments produced well dispersed spectra that are characteristic of a folded protein (Fig. 3A).

The NMR spectra of residues Ser²⁴³ to Leu²⁹⁴ of NPM1 were assigned, and the solution structure was determined using standard techniques (38, 39). Residues 244–294 adopt a well defined tertiary structure with a root mean square deviation of 0.4 Å for backbone atoms (Fig. 1A). Experimental restraints and structural statistics for the 20 accepted lowest energy structures are summarized in Table 1. The coordinates for the structure are available from the Protein Data Bank (entry code 2vxd).

The C-terminal domain of NPM1 forms a well defined right-handed 3-helix bundle (Fig. 1B). Helix 1 is formed of residues

TABLE 1

Summary of conformational constraints and statistics for the 20 lowest energy accepted structures of C-terminal domain of nucleophosmin

| Structural constraints | |
|---|------------------------|
| Intra-residue | 823 |
| Sequential | 196 |
| Medium range ($2 \leq i - j \leq 4$) | 219 |
| Long range ($ i - j > 4$) | 118 |
| χ^1 angle constraints | 15 |
| ALOS constraints | 77 |
| Hydrogen bond constraints | 50 |
| Total | 1498 |
| Statistics for accepted structures | |
| Statistics parameter (\pm S.D.) | |
| r.m.s.d. ^a for distance constraints | 0.0084 \pm 0.0003 Å |
| r.m.s.d. for dihedral constraints | 0.345 \pm 0.029° |
| Mean CNS energy term (kcal·mol ⁻¹ \pm S.D.) | |
| <i>E</i> (overall) | 50.3 \pm 4.2 |
| <i>E</i> (van der Waals) | 15.8 \pm 2.9 |
| <i>E</i> (NOE and hydrogen bond constraints) | 3.1 \pm .2 |
| <i>E</i> (χ^1 dihedral and TALOS constraints) | 0.56 \pm 0.1 |
| r.m.s.d. from the ideal geometry (\pm S.D.) | |
| Bond lengths | 0.0015 \pm 0.00009 Å |
| Bond angles | 0.32 \pm 0.007° |
| Improper angles | 0.24 \pm 0.013° |
| Average atomic r.m.s.d. from the mean structure (\pm S.D.) | |
| Residues 3–54 (N, C- α , C atoms) | 0.40 \pm 0.07 Å |
| Residues 3–54 (all heavy atoms) | 0.94 \pm 0.08 Å |
| Structural quality | |
| Residues in most favored region of Ramachandran plot | 94.1% |
| Residues in additional allowed region of Ramachandran plot | 5.2% |
| Residues in disallowed region of Ramachandran plot | 0.3% |

^a r.m.s.d. means root mean square deviation; NOE means nuclear Overhauser effect.

Val²⁴⁴ to Glu²⁵⁶, with Lys²⁵⁷ and Gly²⁵⁸ then forming an H-bonded turn into the first loop Gly²⁵⁹–Pro²⁶⁴. Helix 2 runs from Glu²⁶⁵ to Phe²⁷⁶ until the second loop of Arg²⁷⁷–Asp²⁸⁰, and helix 3 is composed of residues Gln²⁸¹ to Lys²⁹². The core of the protein is formed largely by aromatic residues (Tyr²⁷¹, Phe²⁶⁸, Phe²⁷⁶, Trp²⁸⁸, and Trp²⁹⁰) with further contribution from residues Met²⁵¹, Ile²⁵⁵, Leu²⁶¹, Val²⁷², and Leu²⁸⁷. Sequence alignment of homologous proteins (Fig. 2D) reveals the hydrophobic core is largely conserved, with Trp²⁸⁸, Trp²⁹⁰, and Phe²⁶⁸ and Phe²⁷⁶ strictly conserved. Deletions, seen in the more distantly related proteins from *Lytechinus pictus* and *Asterina pectinifera*, are confined solely to loop 1, and it is highly likely that the fold is conserved throughout these species. Other residues strictly conserved are Pro²⁶², which ends loop 1, and three lysines as follows: Lys²⁶³ and Lys²⁶⁷ cluster on the surface of the protein, whereas Lys²⁴⁸ appears to form a salt bridge with Asp²⁸⁶ (Fig. 2C) and may be structurally important.

The NPM1 C-terminal domain has no significant sequence similarity to any protein or domain, but a search for structurally similar proteins using the program DALI (40) returned several matches, among which the most significant single domain protein was the recently published archaeal ribosomal protein S17e (41).

Effect of the Leukemia Mutations on the Structure of the NPM1 C-terminal Domain—NPMc+ mutations alter the sequence of the C terminus of the third helix in the structure. In the most common mutation, mutant A, the wild-type sequence WQWRKSL is replaced by the sequence CLAVEEVSLRK, whereas in the less common mutant E it is replaced with WQS-

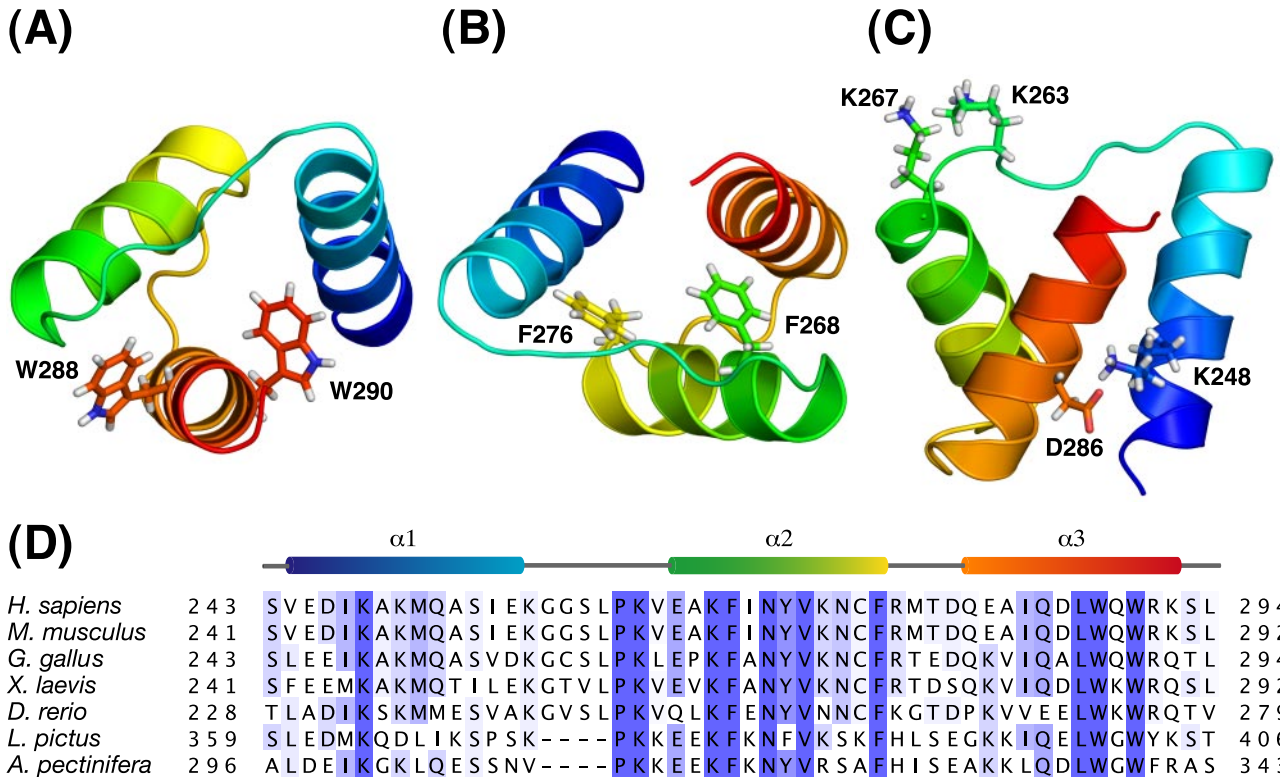


FIGURE 2. Sequence alignment and the positions of structurally and functionally important residues. A–C, schematic representations of the C-terminal domain of nucleophosmin colored blue to red from the N to C terminus with structurally and functionally important residues highlighted as sticks. Figure was prepared using the program PyMOL (53). A, Trp²⁸⁸ and Trp²⁹⁰ showing their positions in the core. B, Phe²⁶⁸ and Phe²⁷⁶, for comparison with A. C, residues Lys²⁴⁸ and Asp²⁸⁶ that form a salt bridge and Lys²⁶³ and Lys²⁶⁷, which cluster on the surface. D, structure-based sequence alignment of the C-terminal domain of nucleophosmin and related proteins. Sequences were aligned using the programs ClustalW (54) and Jalview (55) and shaded blue according to the degree of conservation. Proteins and their NCBI accession numbers are as follows: nucleophosmin 1 from *Homo sapiens* gi:10835063, *Mus musculus* gi:56206422, *Danio rerio* gi:31418910, *Gallus gallus* gi:45383996, NO38 from *Xenopus laevis* gi:64919, Mitotic Apparatus Protein p62 from *L. pictus* gi:1841528 and nucleolar protein from *A. pectinifera* gi:18146874.

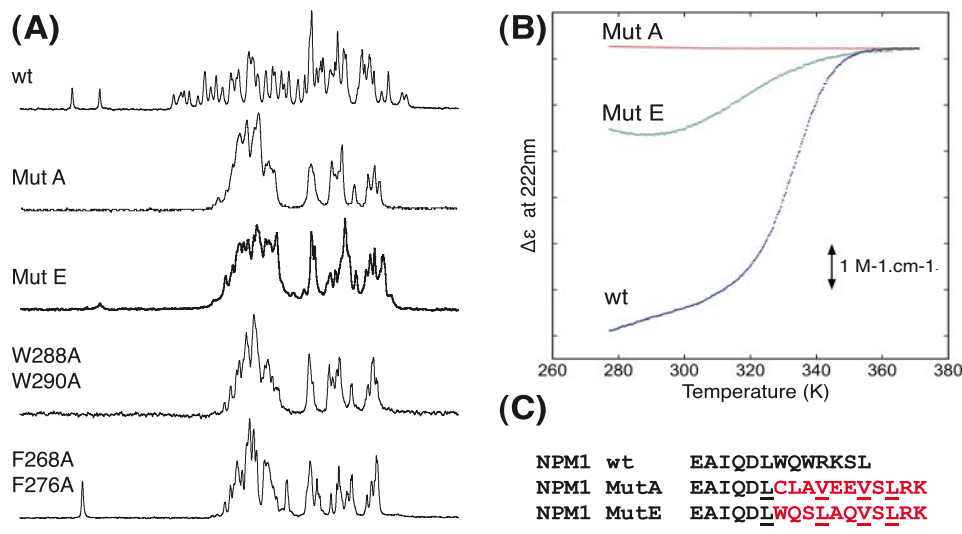


FIGURE 3. Biophysical analysis of nucleophosmin variants. A, amide region of one-dimensional ¹H NMR spectra of the C-terminal domains of the nucleophosmin variants: wild-type (wt); mutant A (Mut A); mutant E (Mut E); W288A/W290A (DW), and F268A/F276A. All mutants show a decrease in dispersion compared with wild type, indicating that all mutants are unfolded. B, traces from thermal melts of nucleophosmin C-terminal domain variants followed by differential absorbance at 222 nm by circular dichroism spectroscopy. The blue points correspond to wild type (wt), the green to Mutant E (Mut E), and the red to Mutant A (Mut A). Wild-type describes a typical two-state sigmoidal transition. Differential scanning calorimetry was used to corroborate this result and agreed within experimental error to a T_m of 62 °C and $\Delta H_{\text{unfolding}}$ of 40 kcal·mol⁻¹. Mutant A shows no sign of any transition, whereas mutant E exhibits some degree of transition although appears to never fully occupy the folded state of wild-type NPM1. C, table displaying the C-terminal sequences of wild-type NPM1 compared with mutants A and E. All naturally occurring mutations found in AML, including A and E, are because of a frameshift that results in the substitution of the extreme C terminus (including one or both of the tryptophans Trp²⁸⁸ and Trp²⁹⁰) with a leucine-rich NES. Substituted residues are colored red and those forming the NES motif are underlined.

LAQVSLRK (Fig. 3C). Several of the altered residues are at the interface between helix 3 and helices 2 and 1 and contribute to the hydrophobic core of the domain. In the NPMc+ mutants, these residues are replaced by smaller amino acids, and this would be predicted to greatly destabilize the domain. To test this hypothesis, we used NMR spectroscopy. The one-dimensional ¹H NMR spectra of both mutants A and E show a decrease in dispersion compared with NPM1wt (Fig. 3A), indicative of a loss of tertiary structure. Fig. 3B shows the CD spectra of NPM1wt and mutants A and E as a function of temperature. NPM1wt describes a sigmoidal unfolding transition typical of a folded protein, whereas mutant A shows no evidence of any such transition and yields a trace similar to that expected from an unstructured protein. Mutant E shows an intermediate response; there is evidence of a transition,

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but the protein never appears to fully occupy the folded state of the wild type.

These results suggest that the mutations found in AML lead to the destabilization of the C-terminal globular domain of NPM1. Mutant A, which lacks both Trp²⁸⁸ and Trp²⁹⁰, has no residual regular secondary structure. Interestingly, mutant E (in which Trp²⁸⁸ is retained), although largely destabilized, is clearly not completely unstructured. This mutant may retain some ability to localize NPM1 to the nucleolus, and this could explain why it has a stronger nuclear export signal than mutant A (42), which itself is completely unfolded.

A Folded C-terminal Domain Is Required for Nucleolar Targeting—Expressing NPM1 as an eGFP fusion in mammalian cells allows for the direct observation of its subcellular localization (43). In this manner the mutation of Trp²⁸⁸ and Trp²⁹⁰ to alanine in NPM1 has been shown to lead to loss of nucleolar localization and the accumulation of the protein in the nucleus (27, 28). To date it has not been clear how these residues contributed to the direction of NPM1 to the nucleolus. We have shown that Trp²⁸⁸ and Trp²⁹⁰ are part of the hydrophobic core of a folded domain (Fig. 2A), and it would be expected that their mutation would lead to a loss of structure. Indeed, the NPM1 C-terminal domain with Trp²⁸⁸ and Trp²⁹⁰ mutated to alanine lacks any distinct tertiary structure (as judged by one-dimensional ¹H NMR; see Fig. 3A) and does not undergo a cooperative unfolding transition when heated (Fig. 3B). This would suggest that mutation of these residues prevents nucleolar localization simply by preventing the correct folding of the C-terminal domain of NPM1. To test this hypothesis, we decided to make other mutations in the C-terminal domain that would be expected to disrupt its structure while retaining the tryptophans. To this end we chose to mutate phenylalanines 268 and 276 to alanine (F268A and F276A) as these residues are also strictly conserved, large hydrophobic residues that pack at the interfaces of the helices and make many contacts within the core (Fig. 2B, and compared with Fig. 2A). As expected the NPM1 domain containing the F268A/F276A mutations lacks any distinct tertiary structure (Fig. 3A). We next examined the effects of these mutations on subcellular localization by transfecting full-length green fluorescent protein-tagged NPM1 F268A/F276A into NIH-3T3 cells. The mutant eGFP-NPM1 F268A/F276A is nuclear and excluded from the nucleolus (Fig. 4), thus proving that nucleolar localization is a property of the C-terminal domain as a whole.

It has been reported by Falini *et al.* (27) that re-inserting Trp²⁸⁸ and Trp²⁹⁰ into mutant A leads to complete relocalization to the nucleolus. We investigated the C terminus of this mutant A C288W/A290W construct and found it to be folded, as judged by CD and NMR. Presumably, refolding the C terminus of mutant A allows it carry on its normal function within the nucleolus while making the nuclear export signal unavailable to the export machinery. Hence, to better distinguish the role of the NES from nucleolar targeting, we added the nuclear export signal from mutant A (LAVEEVSLRK) to the end of wild-type C-terminal domain with a GGS linker and found the full-length construct to be nucleolar. This indicates that a loss of structure and the addition of a nuclear export signal are nec-

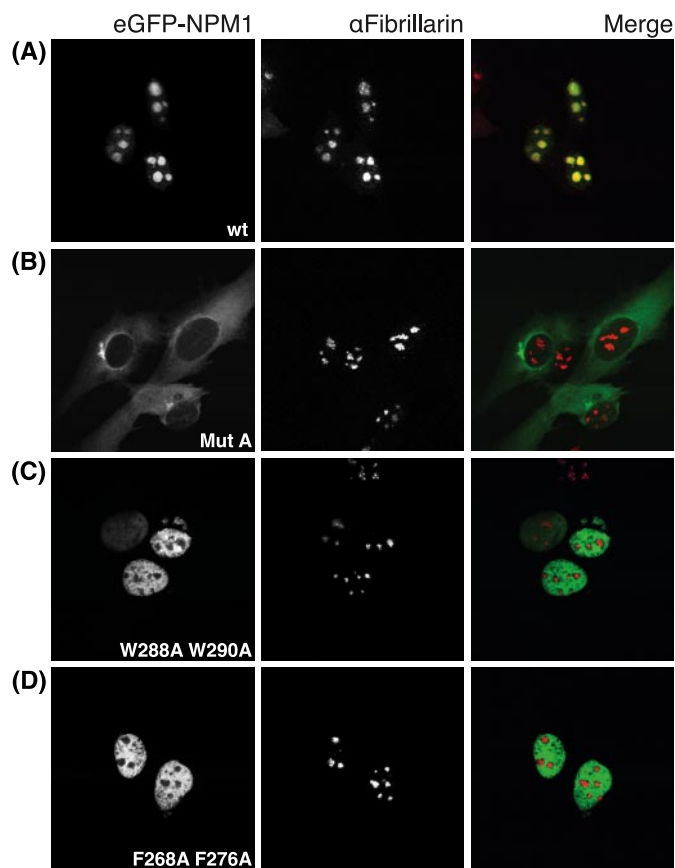


FIGURE 4. Cellular localization of C-terminal domain variants, structural mutants. NIH-3T3 cells were transfected with eGFP tagged full-length nucleophosmin variants, as indicated. A, wild type (wt); B, mutant A (Mut A); C, W288A/W290A; D, F268A/F276A (left panel). Cells were co-stained with anti-fibrillarlin antibody to highlight the nucleolus (middle panel). As expected, wild-type NPM1 is entirely nucleolar and mutant A is entirely cytoplasmic. Mutant eGFP-NPM1 W288A/W290A is nuclear and exclusive of the nucleolus, as too is mutant eGFP-NPM1 F268A/F276A.

essary to cause the aberrant cytoplasmic localization observed in NPMc+ AML.

Functionally Important Residues in the C-terminal Domain of NPM1—Within the C-terminal domain of NPM1 lysine residues 248, 263, and 267 are strictly conserved and surface-exposed (Fig. 5), thus indicating their potential as functionally important residues. To examine this, these residues were mutated to alanine, and the effects on domain stability and nucleolar localization were monitored.

Although Lys²⁴⁸ is surface-exposed, it appears to form a long range salt bridge with Asp²⁸⁶. Indeed the mutation K248A significantly destabilizes the domain and as such leads to Lys²⁴⁸ being classified as structurally important as opposed to being directly functional (data not shown).

Lysines 263 and 267 cluster and orientate freely on the surface of the domain (Fig. 5, and as judged by a lack of side chain nuclear Overhauser effects), and mutation of either Lys²⁶³ or Lys²⁶⁷, or both, increases the stability of the domain in line with the previously described effects of removing unpaired surface charges (44). Once established as not structurally detrimental, the nucleolar localization of the full-length NPM1 mutants K263A, K267A, and the double mutant K263A/K267A was studied *in vivo*. Mutant K263A was partially delocalized to the

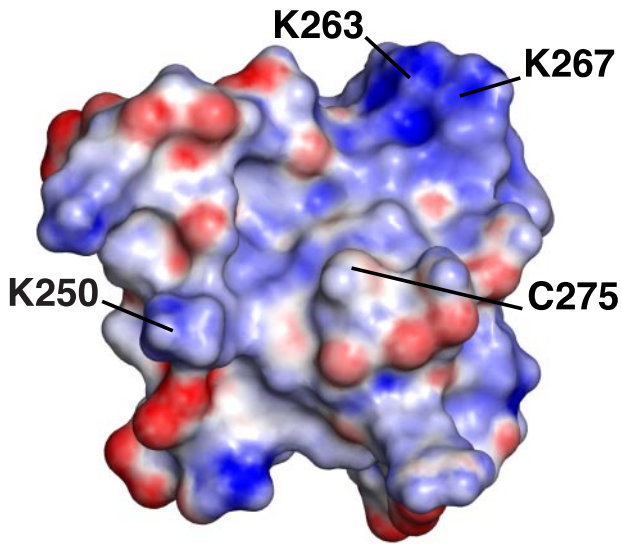


FIGURE 5. Electrostatic representation of the C-terminal domain showing functionally important residues. The electrostatic surface potential of the C-terminal domain of NPM1 as calculated by the program APBS (56) and colored using a linear color ramp from -20.0 kiloteslas (red) to $+20.0$ (blue). Lys²⁶³ and Lys²⁶⁷ are required for nucleolar localization, but Lys²⁵⁰ is not (see Fig. 6). The natural product avrainvillamide bound to Cys²⁷⁵ and displaced NPM1 from the nucleolus to the nucleoplasm (45).

nucleoplasm, and K267A and the double mutant were completely delocalized (Fig. 6).

As a control, the freely orienting surface lysine Lys²⁵⁰ was also mutated. The C-terminal mutant K250A was, predictably, more stable than the wild-type domain yet had no significant effect on nucleolar localization (Fig. 6). This important result emphasizes the specific functional requirement of residues Lys²⁶³ and Lys²⁶⁷ for nucleolar localization over any nonspecific charge effects.

DISCUSSION

Nucleophosmin is a member of the nucleoplasmin family of molecular chaperones and shares the N-terminal pentamerization domain and central acidic region characteristic of these proteins. In this study we have shown that NPM1 has an additional folded domain at its C terminus, which is unique to this subfamily. Although nucleoplasmin is found throughout the nucleus, NPM1 is one of the most abundant proteins in the nucleolus, and this C-terminal domain is necessary for its nucleolar localization. How it directs the protein to the nucleolus is not clear. It seems likely that it binds to a protein or nucleic acid ligand that is abundant in the nucleolus, and it is possible that the structural similarity to ribosomal protein S17e is functionally significant.

The natural product avrainvillamide has been shown to bind to the C-terminal domain of NPM1 via Cys²⁷⁵ (45) and sensitizes cells to apoptosis. It seems likely that avrainvillamide competes with the natural binding partner of this domain. Interestingly, Cys²⁷⁵ is on the same face as the two lysine residues that we have shown to be important for nucleolar localization (Fig. 5) suggesting that this region of the domain is involved in ligand binding.

Ribosome biogenesis is metabolically expensive and a rate-limiting step in cell division and growth. Consequently, ribosomal integrity is closely monitored by a variety of pathways (46).

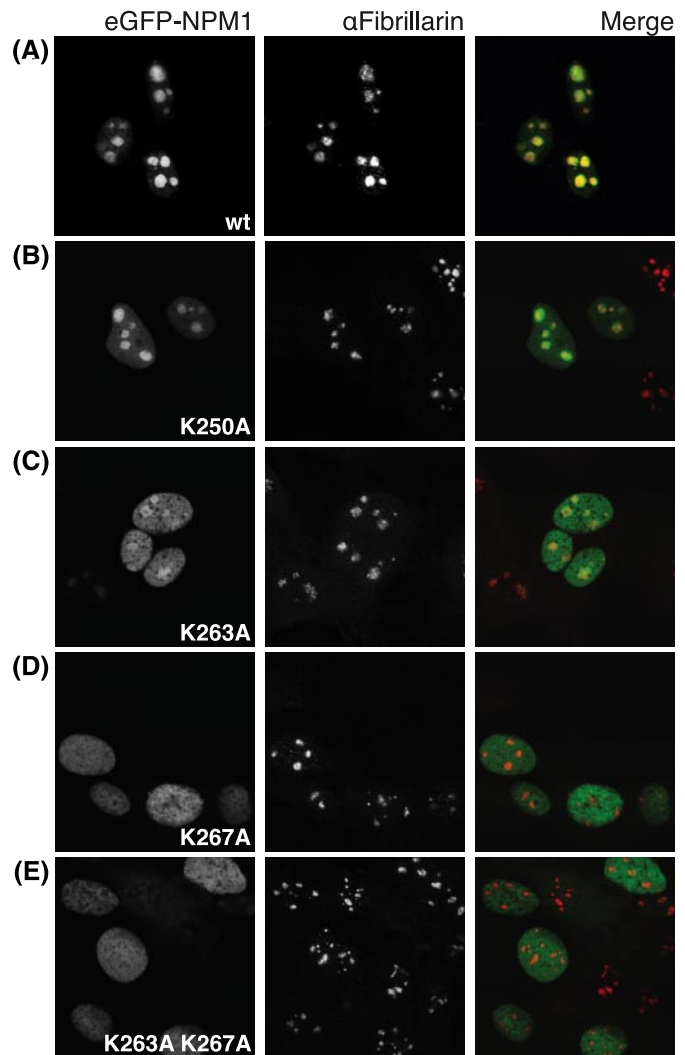


FIGURE 6. Cellular localization of C-terminal domain mutants, functional mutants. NIH-3T3 cells were transfected with eGFP tagged full-length nucleophosmin variants, as indicated. A, wild type (*wt*); B, K250A; C, K263A; D, K267A; E, K263A/K267A (*left panel*). Cells were co-stained with α -fibrillarlin antibody to highlight the nucleolus (*middle panel*). Wild-type NPM1 is entirely localized to the nucleolus, and mutant eGFP-NPM1 K250A is broadly similar. Mutant eGFP-NPM1 K263A is largely displaced into the nucleoplasm but displays some residual nucleolar retention. Mutant eGFP-NPM1 267A and the double mutant eGFP-NPM1 K263A/K267A show no residual nucleolar binding and are evenly distributed throughout the nucleoplasm.

Free ribosomal proteins, for example, activate the tumor suppressor p53 by inhibiting its negative regulator MDM2. There is growing evidence that defects in these pathways can lead to disease. Several blood disorders, for example, are caused by mutations in ribosomal proteins or in ribosome assembly factors (47–51), and mutations that prevent ribosomal protein binding to MDM2 have been reported in human cancers (52). These pathways also seem to be disrupted in NPMc+ AML. NPM1 binds to the p19/ARF tumor suppressor and is also regulated by other pathways that control cell growth (18–24). It seems likely that misdirection of p19^{ARF}, or another key regulatory molecule that binds NPM1, to the cytoplasm may play a key role in AML. The results of this study show that the aberrant cytoplasmic accumulation of NPM1 in NPMc+ AML results from both the gain of a nuclear export signal and the disruption of the structure of its C-terminal domain. Molecules

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that target the C-terminal domain of NPM1 interfere with this mislocalization could therefore have potential applications in leukemia therapy.

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