Nucleophosmin mutations in acute myeloid leukemia: A tale of protein unfolding and mislocalization

Luca Federici,^{1,2*} and Brunangelo Falini^{3*}

¹Ce.S.I. Center of Excellence on Aging, University of Chieti "G. D'Annunzio", 66013 Chieti, Italy

²Department of Experimental and Clinical Sciences, University of Chieti "G. D'Annunzio", 66013 Chieti, Italy

³Institute of Hematology, University of Perugia, 06100 Perugia, Italy

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Abstract: Nucleophosmin (NPM1) is an abundant, ubiquitously expressed protein mainly localized at nucleoli but continuously shuttling between nucleus and cytoplasm. NPM1 plays a role in several cellular functions, including ribosome biogenesis and export, centrosome duplication, chromatin remodeling, DNA repair, and response to stress stimuli. Much of the interest in this protein arises from its relevance in human malignancies. NPM1 is frequently overexpressed in solid tumors and is the target of several chromosomal translocations in hematologic neoplasms. Notably, *NPM1* has been characterized as the most frequently mutated gene in acute myeloid leukemia (AML). Mutations alter the C-terminal DNA-binding domain of the protein and result in its aberrant nuclear export and stable cytosolic localization. In this review, we focus on the leukemia-associated NPM1 C-terminal domain and describe its structure, function, and the effect exerted by leukemic mutations. Finally, we discuss the possibility to target NPM1 for the treatment of cancer and, in particular, of AML patients with mutated *NPM1* gene.

Keywords: NPM1; B23; acute myeloid leukemia; unfolding; G-quadruplex DNA; nucleolus

Introduction

Nucleophosmin domain organization, activities, and shuttling properties

Nucleophosmin (NPM1, also known as B23, No38, and numatrin) is an abundant, multifunctional

protein that was first identified as a phosphoprotein present in high quantities in the granular region of nucleoli (hence its name)^{1,2} but also capable of shuttling between nucleus and cytoplasm.³ Because of this feature, NPM1 activity has been associated to ribosomal RNA (rRNA) expression stimulation,⁴ preribosomal RNA assembly and processing,⁵ and ribosome export to the cytoplasm.⁶ In the last years, however, the number of cellular processes to which NPM1 participates, as well as the number of NPM1 cellular interactors, has grown exponentially and the protein is now thought to play a role also in chromatin remodeling,^{7,8} centrosome duplication,⁹ DNA replication, recombination, transcription, and repair as well as

Conflict of interest: B.F. applied for a patent on the clinical use of NPM1 mutants.

*Correspondence to: Luca Federici, Ce.S.I. Center of Excellence of Aging, University of Chieti, Via dei Vestini 31, 66013 Chieti, Italy. E-mail: lfederici@unich.it or Brunangelo Falini, Institute of Hematology, University of Perugia, Ospedale S. Maria della Misericordia, S. Andrea delle Fratte, 06132 Perugia, Italy. E-mail: faliniem@unipg.it.

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Figure 1. Schematic representation of NPM1 functional domains. Two leucine-rich nuclear export signals (NES), recognized by the exportin1-Crm1 transport system, are present in the N-terminal chaperone domain. A bipartite nuclear localization signal (NLS) is found at the central unstructured domain. Finally, a nucleolar localization signal (NoLS) is found at the C-terminal domain. The black bar indicates the region endowed with ribonuclease activity.

in the control of cell cycle progression and survival in response to a variety of stress stimuli.¹⁰

NPM1 belongs to the NucleoPhosMin/NucleoPlasMin family of proteins, whose members are found throughout the animal kingdom, but not in yeast and bacteria. They can be further classified into four groups, based on sequence similarities: NPM1 (nucleophosmin), NPM2 (nucleoplasmin), NPM3, and NPM-like invertebrate proteins.¹¹

The hallmark of all family members is the presence of a well-conserved N-terminal core domain that, following a short methionine-rich unstructured sequence of unknown function, extends for ~100 residues (Fig. 1) and folds as a eight-stranded beta-barrel, composed of two opposing antiparallel four-stranded sheets. Five single domains oligomerize to form a crown-shaped pentamer and, finally, two pentamers associate in a head-to-head fashion to generate a decameric structure that is thought to be the physiologically relevant assembly of all family members.^{12,13} The N-terminal domain functions as a classical histone chaperone: it is able to assemble histone octamers,¹² to transfer nucleosomes to naked DNA,⁷ to decondense *in vitro* sperm chromatin,⁸ and to assist the renaturation of chemically denatured proteins.¹⁴ The N-terminal domain has also been reported to mediate NPM1 interactions with a plethora of other proteins, including p53, p14arf, Fbw7γ, APE-1, ribosomal proteins RPL5, RPS9, RPL23, HIV proteins REV and TAT, and many others.^{10,15,16} Through these interactions, none of which has been

structurally characterized, NPM1 functions extend considerably beyond its role as a histone chaperone. Many of these proteins show nucleolar localization and it has been proposed that NPM1 favors their correct cellular localization, trafficking, stability, and activity, thus behaving as a nucleolar hub protein.¹⁷ The N-terminal domain is followed in NPM1 by a 120-residue long central domain, which is predicted to be natively unstructured throughout its length (Fig. 1). This domain contains two long acidic stretches that contribute to histone octamer assembly,¹² which are followed by a region that has been proposed to be responsible for a ribonuclease activity of the protein on pre-ribosomal particles.^{5,18} Finally, a short C-terminal domain is responsible for the nucleic acid-binding activity of the protein.¹⁸

Many of the functions played by NPM1 require its continuous shuttling between cytoplasm, nucleoplasm, and nucleoli. Accordingly, NPM1 sequence contains: (i) two leucine-rich nuclear export signals (NES) in the N-terminal core domain that serve for exportin1-Crm1-mediated export in the cytoplasm,^{9,19} (ii) a bipartite nuclear localization signal (NLS), located in between the two long acidic stretches of the central domain,¹⁸ and finally (iii) an aromatic-rich sequence at the C-terminal domain that functions as a nucleolar localization signal (NoLS)²⁰ (Fig. 1). As the C-terminal domain is typical of NPM1 and absent in NPM2 and NPM3 family members, only the former shows nucleolar

Figure 2. NPM1 C-terminal domain structure. (A) The terminal three-helix bundle is characterized by a small hydrophobic core mainly constituted by residues Phe268, Phe276, Trp288, and Trp290. The two tryptophan residues (or Trp290 alone) are mutated in the leukemia-associated variants. (B) The three-helix bundle here is shown in a different orientation to highlight the positive charges that are distributed throughout the surface of the domain. Residues Lys250, Lys257, Lys267, Asn270, Asn274, and Cys275 are located at the interface with G-quadruplex DNA. (C) Sequence alignment of the DNA-binding domain. Residues implicated in the formation of the hydrophobic core (in magenta) and in contacting G-quadruplex DNA (highlighted in blue, green, and yellow boxes for lysines, asparagines, and cysteine, respectively) are strictly conserved. An unstructured tail that precedes the three-helix bundle is necessary to confer high affinity for DNA and is also enriched in lysine residues (in cyan). Several residues in the tail, and at the interface with DNA, are subjected to posttranslational modifications: phosphorylation, acetylation, and sumoylation are highlighted with blue, red, and green stars, respectively.

localization, whereas the latter are predominantly nucleoplasmic.¹¹

Nucleophosmin and cancer

The interest in NPM1 has grown exponentially in the last few years, mainly because this protein is implicated in human malignancies. NPM1 has been found overexpressed in tumors of different histological origin, including gastric, ovarian, bladder, and prostate carcinomas.¹⁵ Furthermore, the NPM1 locus is frequently involved in chromosomal translocations or found deleted in various hematological malignancies.^{15,21} Notably, *NPM1* has been identified as the most frequently mutated gene in acute myeloid leukemia (AML) patients, accounting for ~30% of cases.²² Mutations map to the C-terminal domain of the protein and will be described in detail below.

The multifaceted activities of NPM1 and its relevance for histone chaperoning, ribogenesis, chromatin remodeling, centrosome duplication, and control of cell cycle progression and survival have been

the subject of a number of excellent reviews to which we refer the reader.^{10,15,16,23,24} Here, we focus on the C-terminal domain of the protein. We describe its structure, interactions, known physiological functions, and how they are modified in the NPM1 leukemic mutants. Finally, we discuss about the possibility to target the C-terminal domain of NPM1 for the specific treatment of AML patients carrying *NPM1* mutations.

The NPM1 C-Terminal Domain Structure

The terminal part of NPM1 sequence, following an ~120-residue long segment predicted to be natively unstructured, ends with a short segment that proved to be folded. This was shown by Grummit *et al.*, who determined the structure of this domain, encompassing residues 243–294 of the reference sequence.²⁵

The domain, shown in Figure 2(A,B), is composed of three helices that form a well-defined right-handed bundle. Helices H1 and H3 are almost coaxial but run with opposite polarities, whereas the connecting helix H2 is tilted, by ~45°, with respect

Mutant	Sequence	Protein
Wild-type	D L W Q W R K S L Stop GATCTCTG...GCAGT...GGAGGAAGTCTCTTTAAGAAAATAG	286 294 DLWQWRKSL
Mutant A (72–80.3%)	D L C L A V E E V S L R K Stop GATCTCTGTCTGGCAGT...GGAGGAAGTCTCTTTAAGAAAATAG	286 298 DLCLAVEEVSLRK
Mutant B (8–12%)	D L C H A V E E V S L R K Stop GATCTCTGCATGGCAGT...GGAGGAAGTCTCTTTAAGAAAATAG	286 298 DLCMAVEEVSLRK
Mutant C (<1%)	D L C V A V E E V S L R K Stop GATCTCTGCGTGGCAGT...GGAGGAAGTCTCTTTAAGAAAATAG	286 298 DLCVAVEEVSLRK
Mutant D (3.1–7%)	D L C L A V E E V S L R K Stop GATCTCTGCCTGGCAGT...GGAGGAAGTCTCTTTAAGAAAATAG	286 298 DLCLAVEEVSLRK
Mutant E (<1%)	D L W Q S L A Q V S L R K Stop GATCTCTG...GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG	286 298 DLWQSLAQVSLRK
Mutant F (<1%)	D L W Q S L E K V S L R K Stop GATCTCTG...GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG	286 298 DLWQSLEKVSRLK

Figure 3. Mutations at the C-terminal domain are associated with acute myeloid leukemia. Here, the most common mutations are shown. They consist in the insertion or duplication of small base stretches (underlined). For clarity, one every two codons in the terminal sequence of the gene is highlighted in bold and the corresponding amino acids are indicated in gray. The resulting amino acid sequences are also shown in the right column. The altered reading frame leads to the expression of mutated proteins that have a different C-terminal sequence and are longer by four residues (in italics). Both Trp288 and Trp290 (or Trp290 only in mutants E and F) are replaced and a new nuclear export signal (residues in gray boxes in the right column) appears in all cases.

to the other two. Each of the three helices interacts with the other two and the overall structure is stabilized by a small but well-defined hydrophobic core, mainly formed by four aromatic residues, namely Phe268, Phe276, Trp288, and Trp290 [Fig. 2(A)].

Sequence alignments indicate that the fold is likely to be very well conserved along the evolutionary tree, with no insertions or deletions in the helical regions and with a strict conservation of the hydrophobic core [Fig. 2(C)].

Several lysine and arginine residues are found in the three-helix bundle, distributed throughout its length, and contribute to the marked basicity of this domain [Fig. 2(B)]. Most of these residues are also well conserved and some of them are strictly conserved, suggesting a functional significance for nucleic acid binding [Fig. 2(C)]. Furthermore, the unstructured region immediately preceding the three-helix bundle (aa 225–242) is also enriched in basic residues, suggesting that a functional interplay with the three-helix bundle may take place [Fig. 2(C)].

A so-called nucleolar localization signal (NoLS) for NPM1 is present in the three-helix bundle. Importantly, although most of the identified NoLS are formed by sequence motives enriched in consecutive basic residues,¹⁷ which are thought to be recognized by specific nucleolar hub proteins (including NPM1), the NoLS of NPM1 is unique and constituted by the two tryptophan residues in helix H3. This was initially shown by observing that deletion of the terminal 288–294 segment in NPM1, which includes the two tryptophans [Fig. 2(C)], as well as

mutation of both tryptophans to alanine were sufficient to delocalize the protein in the nucleoplasm.²⁰

The role of these two residues in driving the cellular localization of NPM1 is further emphasized by the effect exerted by *NPM1* gene mutations in AML.

AML-Associated NPM1 Mutations: Implications for Protein Stability and Localization

NPM1 has been recently characterized as the most frequently mutated gene in AML patients, accounting for ~30% of all cases.^{22–24} *NPM1* mutations are always heterozygous^{22–24} and mutually exclusive with AML carrying recurrent genetic abnormalities.²⁶ Mutations cluster in the terminal exon (XII) of the *NPM1* gene and consist in the duplication or insertion of small nucleotide stretches (Fig. 3).^{22,23} Although more than 50 mutations have been reported so far, the consequences at the protein level are similar in all cases. The reading frame is altered and this leads to a variant NPM1 protein that has a different sequence in the last seven residues and is four residues longer.²² In the most frequent case, mutation A, which accounts for about 75% of cases, both Trp288 and Trp290 are lost, whereas in other mutants only Trp290 is replaced (Fig. 3). However, in all instances the NoLS is compromised. Furthermore, the new sequence in the variant protein matches the consensus for a leucine-rich NES that may be recognized by the exportin1-Crm1 export system (residues in gray boxes in Fig. 3). This new NES is added to and reinforces the two NES normally present in the N-terminal domain of the native protein.²⁷ Notably, when only Trp290 is replaced, a stronger NES motif is inserted to ensure maximal export in

the cytoplasm of the NPM1 mutant.²⁷ Very rarely, mutations may occur at exon XI of *NPM1* leading to a mutant protein that is truncated at its C-terminus and lacks both tryptophans.²⁸ The combined effect of the loss of either one or both tryptophan residues and the appearance of a new NES in the variant protein is mandatory to completely alter the shuttling properties of the protein.²⁹ In AML with *NPM1* mutations, the variant NPM1 protein is stably and aberrantly localized in the cytoplasm of leukemic cells (and therefore referred to as NPM1c+, where c+ stands for cytoplasmic positive).²² Furthermore, as the mutated protein is able to oligomerize with the NPM1 protein synthesized from the wild-type allele, through its unchanged N-terminal domain, the majority of wild-type NPM1 is also translocated in the cytosol and only a minimal portion of it is still retained in nucleoli.²⁹ This is the hallmark of this kind of leukemia that can be detected by immunohistochemistry.³⁰ Because of its distinctive features, AML with *NPM1* mutations has been included as a provisional distinct entity in the 2008 WHO classification of myeloid neoplasms.³¹

The observation that AML-associated *NPM1* mutations lead to the replacement of one or both of Trp288 and Trp290, and consequently alter the hydrophobic core of the C-terminal domain, led investigators to try to correlate the folding mechanism of the protein (wild-type versus the mutated one) with the altered cellular localization. NPM1 C-terminal domain behaves as a classical two-state folder that retains a fraction of residual structure at the denatured state.³² This residual structure could be mapped to helices H2 and H3, with both tryptophan residues belonging to the latter, whereas helix H1 is completely unstructured in the denatured state.³³ Therefore, AML-associated *NPM1* mutations by altering the protein sequence in the terminal part of helix H3 also alter the folding mechanism of the domain. Accordingly, it was shown that the isolated C-terminal domain from mutant A is completely unstructured, whereas the same domain from mutant F, which retains Trp288 (Fig. 3), is largely destabilized but retains some residual structure.²⁵

Intriguingly, the folding properties of the C-terminal domain have been unequivocally linked to the cellular localization of the whole protein. First, it was shown that by mutating Trp288 and Trp290 to alanine in the context of the native sequence, the C-terminal domain unfolds²⁵ and the protein loses its nucleolar localization.²⁹ This effect was also seen when perturbing the hydrophobic core of the domain by other means, such as by concomitantly mutating Phe268 and Phe276 to alanine.²⁵ Then, it was shown that a NPM1 variant protein, bearing the terminal sequence of mutant A (Fig. 3) but with the two tryptophan residues reinserted in their topological position, reacquires nucleolar localization.²⁹ Folding analysis of the C-terminal domain of such variant protein demonstrated that it folds similarly to the wild type.³⁴

Therefore, it appears that AML-associated *NPM1* mutations drive the cytoplasmic localization of the protein by combining two different effects.³⁵ First, rather than identifying a sequence-specific NoLS for NPM1, we should consider that a properly folded C-terminal domain as a whole is the NoLS. Mutations that unfold the domain lead to the loss of nucleolar localization and, conversely, mutations that refold the domain lead to a reacquisition of nucleolar localization, even in the context of the AML-variant protein sequence. When the leukemic NPM1 mutant protein loses nucleolar localization it does not remain in the nucleoplasm but goes to the cytoplasm. This is because of the second effect exerted by mutations, that is, the appearance of an additional NES that is recognized by the exportin1-Crm1 export system.^{29,35} This was shown by treating OCI-AML3 cells, which bear the *NPM1* mutation A (Fig. 3),³⁶ with a exportin1-Crm1 inhibitor and showing that, under this experimental setting, the protein loses nucleolar localization but remains blocked in the nucleoplasm.²⁹

To understand why a properly folded C-terminal domain is crucial for NPM1 nucleolar localization, we have to take into consideration the physiological functions played by this domain.

NPM1 C-Terminal Domain and Nucleic Acids Binding

NPM1 was initially suggested to be a putative nucleic acid-binding protein because of its close association to the granular region of nucleoli, which contains mature ribonucleoprotein particles on their way to becoming ribosomes.³⁷ Nucleic acid binding was later tested *in vitro* on both single and double stranded targets. It emerged that NPM1 binds nucleic acids independently from their sequence and with a preference for single-stranded DNA and RNA, over double-stranded DNA.³⁸ Later on, the nucleic acid-binding activity was mapped to the last 70 residues of the protein, through the use of a series of deletion mutants.¹⁸ As shown in Figure 2(C), this sequence includes the C-terminal three-helix bundle, which is preceded by a segment rich in lysine residues predicted to be natively unstructured.

NPM1 was also found to be endowed with ribonuclease activity³⁹ and, when tested on rRNA fragments synthesized from rDNA transcripts, showed preferential cleavage of a short region in the middle of the internal transcribed region 2 (ITS2) of rRNA, which is removed during ribosome maturation.⁵ Interestingly, the ribonuclease activity was later mapped to the central unstructured domain of the protein but with a twofold decrease in activity when the terminal three-helix bundle was deleted.¹⁸ These studies suggested a functional interplay between the central ribonuclease domain and the C-terminal domain, with the latter seemingly implicated in

substrate recognition and positioning. More recently, NPM1 was also found to be associated to mRNA during polyadenylation⁴⁰ and shown to bind the 3'-UTR region of the chicken connective tissue growth factor gene product promoting its degradation,⁴¹ suggesting that the NPM1 RNA-binding activity is not confined to pre-rRNA processing and ribosome assembly.

NPM1 has also been widely implicated in the control of gene transcription. In a number of cases this activity is exerted through the interaction with specific transcription factors and/or with HEXIM1, a negative regulator of PolII activity.⁴² However, at least in a few cases, direct interaction of NPM1 with promoter regions was observed. Recently, it was shown that NPM1 physically associates with a specific region in the promoter of the superoxide dismutase *SOD2* gene and that, in doing so, it participates to the transcriptional activation of the gene.^{43,44} Cancer-associated mutations at this region lead to loss of NPM1 binding and compromise both constitutive and inductive transcription of the *SOD2* gene.⁴⁴ The specific region recognized *in vivo* by NPM1 is predicted to fold as a hairpin by conventional Watson-Crick pairing, and contains a stretch of 10 consecutive guanines in the loop structure. Further studies on synthetic oligonucleotides, where both the structure and the sequence of the hairpin were challenged, showed that the C-terminal three-helix bundle alone displays low binding affinity against any oligonucleotide tested. A short lysine-rich unstructured region (aa 225–242) preceding the folded domain was always necessary to display full DNA-binding activity [see Fig. 2(C)].⁴⁵ Importantly, it was shown that the *SOD2* promoter sequence recognized by NPM1 also matches the sequence requirements for assuming a G-quadruplex fold and that this is the preferential structure, over the hairpin one, assumed by the oligonucleotide *in vitro* under physiological conditions.⁴⁵

NPM1 as a G-Quadruplex-Binding Protein

G-quadruplexes are noncanonical nucleic acid structures resulting from the formation of guanine tetrads, stabilized by Hoogsteen-type hydrogen bonds, that stack onto each other to form extremely stable assemblies.^{46,47} They can be formed both by DNA and RNA, and they are abundant at telomeric DNA, gene promoters, and mRNA 5'-UTRs.^{48,49} The importance of these structures in regulating several cellular processes at the translational and posttranslational levels is emerging clearly and a number of proteins have been shown to interact with and to stabilize or destabilize the G-quadruplex fold.⁵⁰

A well-characterized G-quadruplex sequence is present at the nuclease hypersensitive element III (NHE_{III}) of the *c-MYC* promoter, and regulates up to 90% of *c-MYC* gene transcription.^{51,52} The extended

Figure 4. A G-quadruplex sequence from the *c-MYC* promoter is shown in complex with the NPM1 DNA-binding domain (model derived from NMR data).⁵³ The G-quadruplex backbone is accommodated into a small positive groove formed by helices H1 and H2. Several interactions between lysine and asparagine residues and the DNA backbone phosphates stabilize the complex. The N-terminal tail is still unstructured in the complex even though it proved necessary for high-affinity binding. Lysine residues in the tail are highlighted. Mutation of Lys229 and Lys230 to alanine dramatically reduces the binding affinity.⁴⁵

C-terminal domain of NPM1 proved to bind this region with high affinity *in vitro*⁴⁵ and *in vivo* (unpublished results from Federici lab) and also to promote the G-quadruplex folding in the unstructured oligonucleotide.⁴⁵ These data suggest that NPM1 may be included in the constantly growing list of G-quadruplex-binding proteins.⁵⁰

The structure of the NPM1 C-terminal extended domain in complex with the G-quadruplex oligonucleotide from the *c-MYC* promoter was recently investigated.⁵³ It was shown that a region comprised between helices H1 and H2 of the terminal three-helix bundle forms a small groove where a stretch of consecutive backbone phosphates of the main G-quadruplex scaffold is accommodated (Fig. 4). The interacting groove is positively charged, with three lysines (K250, K257, and K267) and two asparagines (N270 and N274) at the interface that directly contact phosphate groups through salt bridges and hydrogen bonds [Figs. 2(C) and 4]. Conversely, base-specific interactions and interactions with nucleotides belonging to the G-quadruplex connecting loops appear marginal, explaining why NPM1 recognizes with similar affinities several G-quadruplexes with varying loop structures.^{34,45}

As mentioned above, a lysine-rich region that flanks the terminal three-helix bundle (residues 225–242) proved necessary for high-affinity recognition of all oligonucleotides tested, despite their structure.⁴⁵ The overall positive charge of this tail seems to be important for recognition, as shown by the loss of G-quadruplex affinity displayed by a double lysine to alanine mutant (K229A-K230A) in the tail.⁴⁵ Interestingly, this region was shown to be unstructured before and also after binding, and does not

participate to the final complex with the *c-MYC* G-quadruplex (Fig. 4).⁵³ A similar behavior was observed in a number of other interactions, both protein–DNA and protein–protein, where flanking unstructured regions that do not physically interact with the binding partner in the final complex nevertheless contribute to define the binding energy.^{54,55} Although the exact role played by this tail in nucleic acid recognition is currently under investigation, it has been recently shown that it significantly contributes to the thermal and chemical stability of the terminal three-helix bundle.⁵⁶ This stabilization is further increased by the additional presence of the N-terminal chaperone domain, suggesting an interplay between different functional domains in NPM1.⁵⁶

G-Quadruplex Binding and Nucleolar Localization

Nucleoli are highly dynamic structures that assemble around clusters of tandemly repeating rDNA genes. The rDNA gene is transcribed in a 47S pre-rRNA precursor that is then processed by removal of external and internal transcribed regions.⁵⁷ These regions, as well as the 28S region, were shown to be enriched in G-quadruplex sequences in the nontemplate strand.⁵⁸ Consistently with its role as a G-quadruplex-binding protein, NPM1 was later shown to bind these regions both *in vitro* and *in vivo*.³⁴ This activity was completely lost by the unstructured leukemic mutant A C-terminal domain. In addition, a refolding mutant A variant, where both tryptophans were reinserted in their topological position in the context of the mutant A sequence, reacquired (i) a folding similar to the wild-type domain, (ii) G-quadruplex binding at rDNA regions,³⁴ and (iii) nucleolar localization.²⁹ Consistently with this picture, it was also shown that treatment of OCI-AML2 cells, bearing wild-type *NPM1* at both alleles, with the G-quadruplex ligand TmPyP4 completely displaced NPM1 from nucleoli to the nucleoplasm.³⁴ Collectively, these data showed that a direct correlation exists between NPM1 C-terminal domain folding, G-quadruplex binding, and the nucleolar localization of the protein. Furthermore, as G-quadruplex regions are found at the nontemplate strand of the rDNA gene,⁵⁸ they will also be present in the pre-rRNA precursor, where they may be recognized by NPM1 C-terminal domain during pre-rRNA processing, even though this is still to be confirmed experimentally.

Posttranslational Modifications and Molecules That Bind NPM1 C-Terminal Domain

The C-terminal domain provides a strong nucleolar localization signal for NPM1 and, accordingly, the bulk of the protein is nucleolar in cells in G₀. However, in different cell cycle phases, in order to fulfil its many functions, NPM1 moves between nucleolus,

nucleoplasm, and cytoplasm. At least in part, this movement seems to be regulated by a variety of posttranslational modifications. NPM1 is phosphorylated at multiple sites by many kinases and NPM1 phosphorylation was shown to contribute to the regulation of cell cycle progression at different levels.⁵⁹ Interestingly, a number of phosphorylation sites are located at the positively charged unstructured arm preceding the C-terminal three-helix bundle [Figs. 2(C) and 4]. Among them, Ser227 is phosphorylated by PKC⁶⁰ and Thr234–Thr237 are phosphorylated by the Cdk1/cyclinB complex before mitosis.⁶¹ During mitosis, NPM1 is dispersed throughout the cell with a minor fraction localized at the spindle poles. Phosphorylation was shown to be necessary for this localization and, conversely, lack of phosphorylation was shown to trigger the spindle-checkpoint activation due to mitosis defects.^{62–64} NPM1 is also acetylated at multiple sites in its C-terminal end. It was shown that p300 acetylates Lys229, Lys230, Lys257, and Lys267, whereas SIRT1 promotes their deacetylation.⁶⁵ Lys229 and Lys230 are again located at the unstructured arm and their mutation to alanine was shown to severely reduce affinity for G-quadruplexes (Fig. 4).⁴⁵ Lys257 and Lys267 were instead shown to be located at the interface with the *c-MYC* G-quadruplex.⁵³ This would suggest that acetylation events may interfere with NPM1 binding at rDNA regions and, indeed, it was shown that acetylated NPM1 loses nucleolar localization and roams in the nucleoplasm where it binds and possibly activates RNAp-III.⁶⁵ The C-terminal domain is also subject of sumoylation at Lys230 and Lys263.⁶⁶ The effect exerted by sumoylation is still controversial, but it was proposed that it might interfere with pre-rRNA processing by NPM1 and play a role in controlling protein stability.⁶⁷ Collectively, it may be argued that the positively charged unstructured tail that precedes the three-helix bundle, which is necessary to confer high affinity for nucleic acids, is also the site of multiple regulatory events that affect NPM1 localization.

Recently it was shown that (+)-avrainvillamide, a naturally occurring alkaloid with antiproliferative effects, binds NPM1 and triggers an increase of p53 levels and apoptosis in LNCaP and T-47D cell lines.⁶⁸ (+)-Avrainvillamide functions as an electrophile, which can be subjected to covalent nucleophilic addition played by free thiols of protein targets to the unsaturated nitrone functional group of the drug. As to NPM1, mutagenesis and binding studies showed that the only cysteine that is alkylated by (+)-avrainvillamide is Cys275,⁶⁸ which is found at the interface with the *c-MYC* G-quadruplex [Figs. 2(C) and 4].⁵³ Even though this has to be experimentally proven, it may be hypothesized that Cys275 covalent linkage with this bulky alkaloid may interfere with G-quadruplex binding at

Figure 5. Proposed strategies for targeting NPM1 in AML. (A) As wild-type NPM1 oligomerizes with mutated NPM1 and is thus translocated to the cytosol, only a small fraction of the protein is retained in nucleoli. A drug able to dissociate residual wild-type NPM1 from nucleoli might cause nucleolar stress and disruption, ultimately leading to apoptosis in leukemic blasts. (B) An opposite strategy would be that of developing a drug acting as a pharmacological chaperone. Such drug should trigger mutated NPM1 refolding upon binding and shift, through mass effect, the folded–unfolded equilibrium toward the folded species. This would in turn facilitate the relocalization of both native and mutated NPM1 in nucleoli, thus counteracting the antiapoptotic effects played by NPM1c+.

nucleolar rDNA, thus releasing NPM1 from nucleoli. NPM1 dislocation would in turn lead to p53 stabilization through activation of the p14arf/HDM2 pathway.⁶⁹

Targeting NPM1 C-Terminal Domain for AML Treatment

NPM1 mutations are found in about 30% of AML patients^{22,24} and are highly stable during the course of the disease.⁷⁰ Moreover, *NPM1* mutations: (i) are specific for AML (usually *de novo* type with normal karyotype), (ii) are mutually exclusive of AML with recurrent genetic abnormalities, and (iii) show distinct gene and microRNA profiles.^{23,24,71,72} Notably, the small subset of leukemic stem cells isolated from these patients carry *NPM1* mutations and cytosolic NPM1, recapitulating the original disease when transplanted in immunocompromised mice.^{73,74}

All these evidences point to *NPM1* mutation as an early initiating event in AML²⁴ and make NPM1 an ideal target for therapy.⁷⁵ Thus, to develop molecular targeted therapies in this AML subtype is im-

portant. In fact, although patients carrying *NPM1* mutations without *FLT3* internal tandem duplication (ITD) are usually characterized by a relatively good outcome (overall survival 50–60%),^{76,77} a significant proportion of *NPM1*-mutated AML patients carrying an *FLT3*-ITD mutation still succumb of their disease.

NPM1 mutations have the double effect of unfolding the C-terminal domain and delocalizing the protein in the cytosol (NPM1c+). These events arguably lead to the loss of NPM1 nucleolar and nuclear functions but also, as it is emerging clearly by recent reports, to the gain of several unwanted cytosolic functions. For instance, it has been shown that mutated NPM1 carries to the cytoplasm and promotes the degradation of the tumor suppressors p14arf⁷⁸ and Fwb7γ,⁷⁹ which reflects in decreased p53 levels and increased c-MYC half-life. On the cytosolic side, it has been shown that NPM1c+ binds and inhibits caspases 6 and 8,⁸⁰ thus impacting on apoptosis and, more recently, that NPM1c+ binds and inhibits the PTEN deubiquitinating enzyme HAUSP, thus resulting in PTEN cytoplasmic polyubiquitinylation and degradation.⁸¹ These complex events, and others, show that NPM1 mislocalization heavily impacts on cell cycle regulation at multiple levels and have suggested a number of possible strategies to counteract *NPM1* mutations, which have been recently reviewed.⁷⁵ Here, we would like to emphasize two of these strategies that would pursue opposite finalities (Fig. 5).

A first strategy would be that of developing a drug that completely deprives nucleoli of their NPM1 pool [Fig. 5(A)]. In fact, it has been shown that in AML patients with *NPM1* mutations, which are always heterozygous, a small but detectable amount of wild-type NPM1 is always retained in nucleoli.²⁷ The rational for such approach is that, as NPM1 behaves as a hub protein in nucleoli, NPM1 nucleolar starvation would cause nucleolar stress and disruption of nucleolar structure [Fig. 5(A)]. We have recently shown that such approach is indeed worth investigation because a G-quadruplex ligand, like TmPyP4, completely displaces NPM1 from nucleoli and finally results in massive cell death.³⁴ However, and unexpectedly, OCI-AML3 cells, which bear heterozygous *NPM1* mutation A, proved more resistant to treatment than the control OCI-AML2 cells, bearing wild-type NPM1 at both alleles.³⁴ Even though the reasons for such increased resistance are still under investigation, it should be emphasized that G-quadruplex ligands may not be ideal for this purpose because, together with NPM1, they may also displace from nucleoli other proteins, including nucleolin,⁵⁸ thus proving toxic to normal cells too.³⁴ An ideal ligand, in order to be specific for NPM1, should target the surface of NPM1 that interacts with G-quadruplexes, rather than the G-

quadruplexes themselves. Current data suggest that (+)-avrainvillamide may act this way and might be used as a lead for developing new effective molecules.⁶⁸ However, more information is needed about its interaction with the NPM1 C-terminal domain as well as on the effect it exerts on relevant AML cell lines.

A second strategy would be that of developing a so-called pharmacological chaperone, that is, a molecule that, through binding and mass effect, would shift the equilibrium between the NPM1-mutated C-terminal domain unfolded and folded species toward the folded one [Fig. 5(B)]. This approach has already been used with success to rescue unstable mutants of the p53 tumor suppressors for cancer treatment⁸² and of phenylalanine hydroxylase for phenylketonuria treatment.⁸³ These studies showed that small molecules, selected either through drug libraries screening or rationally designed on the basis of crystal structures,⁸⁴ stabilize their targets *in vitro* and in cell lines, and may be used as effective leads for drug development. In our case, a pharmacological chaperone, by refolding its mutated C-terminal domain, should trigger NPM1c+ relocalization in the nucleoli of the leukemic blasts, thus counteracting the antiapoptotic effect exerted by NPM1c+ cytosolic activities [Fig. 5(B)]. As stated above, studies on refolding variants of the mutated protein³⁴, obtained through site-directed mutagenesis, indicated that upon refolding the protein reacquires both G-quadruplex-binding properties³⁴ and nucleolar localization,²⁹ suggesting that such approach may be worth further investigation.

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