Research Article

Born to Be Exported: COOH-Terminal Nuclear Export Signals of Different Strength Ensure Cytoplasmic Accumulation of Nucleophosmin Leukemic Mutants

Niccolò Bolli,[1] Ildo Nicoletti,[2] M. Felicetta De Marco,[1] Barbara Bigerna,[1] Alessandra Pucciarini,[1] Roberta Mannucci,[2] Maria Paola Martelli,[3] Arcangelo Liso,[4] Cristina Mecucci,[1] Francesco Fabbiano,[5] Massimo F. Martelli,[1] Beric R. Henderson,[6] and Brunangelo Falini[1]

[1]Section of Hematology and Immunology, University of Perugia, IBiT Foundation, Fondazione IRCCS Biotecnologie nel Trapianto; [2]Institute of Internal Medicine, University of Perugia, Perugia, Italy; [3]Institute of Hematology, University of Bari, Bari, Italy; [4]Institute of Hematology, University of Foggia, Foggia, Italy; [5]Institute of Hematology, Ospedale "Cervello," Palermo, Italy; and [6]Westmead Institute for Cancer Research, University of Sydney, Westmead Hospital, Sydney, Australia

Abstract

Creation of a nuclear export signal (NES) motif and loss of tryptophans (W) 288 and 290 (or 290 only) at the COOH terminus of nucleophosmin (NPM) are both crucial for NPM aberrant cytoplasmic accumulation in acute myelogenous leukemia (AML) carrying *NPM1* mutations. Hereby, we clarify how these COOH-terminal alterations functionally cooperate to delocalize NPM to the cytoplasm. Using a Rev(1.4)-based shuttling assay, we measured the nuclear export efficiency of six different COOH-terminal NES motifs identified in NPM mutants and found significant strength variability, the strongest NES motifs being associated with NPM mutants retaining W288. When artificially coupled with a weak NES, W288-retaining NPM mutants are not exported efficiently into cytoplasm because the force (W288) driving the mutants toward the nucleolus overwhelms the force (NES) exporting the mutants into cytoplasm. We then used this functional assay to study the physiologic NH_2-terminal NES motifs of wild-type NPM and found that they are weak, which explains the prominent nucleolar localization of wild-type NPM. Thus, the opposing balance of forces (tryptophans and NES) seems to determine the subcellular localization of NPM. The fact that W288-retaining mutants always combine with the strongest NES reveals mutational selective pressure toward efficient export into cytoplasm, pointing to this event as critical for leukemogenesis. [Cancer Res 2007;67(13):6230–7]

Introduction

Macromolecule transport into and out of the nucleus through the nuclear pore complex is essential for regulating cell functions (1) and, in fact, nucleocytoplasmic transport is altered in a variety of tumors (2). In acute leukemias, *NUP214* and *NUP98* gene translocations cause abnormal protein traffic through nuclear pores (3, 4). However, the most remarkable example of disrupted nucleocytoplasmic transport in leukemias involves nucleophosmin (NPM; ref. 5), a multifunctional shuttling phosphoprotein (6) residing mainly in the nucleolus (7). Indeed, ~30% of *de novo* acute myelogenous leukemia (AML) exhibiting distinctive clinicopathologic (5) and prognostic (8–11) features carry *NPM1* gene mutations that cause aberrant NPM accumulation in leukemic cell cytoplasm (5, 12). For this reason, we named this leukemia "cytoplasmic positive (NPMc+) AML" (5).

In NPMc+ AML, *NPM1* mutations alter tryptophans 288 and 290 (or 290 alone; ref. 5) and create a new nuclear export signal (NES) motif (13) at the NPM protein COOH terminus. Both abnormalities act in concert (5) to aberrantly localize NPM leukemic mutants in cytoplasm, as easily detected by immunohistochemistry (14, 15). Most of NPM leukemic mutants identified to date (16) lose both tryptophans 288 and 290, which are involved in NPM nucleolar localization (17); the others retain tryptophan 288. Six different mutation-related COOH-terminal NES sequences have been detected (16). Interestingly, COOH-terminal NES sequences and patterns of tryptophan loss are not randomly distributed (12). The L-xxx-V-xx-V-x-L NES motif, the most common in NPM mutants, is always associated with loss of both tryptophans (12). In contrast, NES motif variants (where valine at the NES second position is replaced by leucine, phenylalanine, cysteine, or methionine) are found only when tryptophan 288 is retained (12). Tryptophans 288 and 290 drive NPM into the nucleolus (17), but when tryptophan 288 is retained, immunohistochemistry shows that NPM mutants still localize in cytoplasm (14). Therefore, to get mutants out of the nucleus, the COOH-terminal NES sequence of NPM mutants retaining tryptophan 288 has to counterbalance the tryptophan 288 driving force to the nucleolus. This raises the question about whether variant COOH-terminal NES motifs have stronger nuclear export efficiency than the more common L-xxx-V-xx-V-x-L NES motif.

To assess whether the COOH-terminal NES motifs of NPM mutants differ in their nuclear export efficiency, we studied subcellular expression of different mutants at various concentrations of leptomycin B, a specific inhibitor of Exportin/Crm1 receptor (18, 19), and used a Rev(1.4)-eGFP fusion protein–based functional nuclear export assay (20) to measure the nuclear export activity of each COOH-terminal NES sequence after isolation from mutated NPM proteins (16). Seeking a functional explanation of nucleolus-restricted expression of wild-type NPM, we also used the same shuttling assay to assess the nuclear export strength of the two physiologic NH_2-terminal NES motifs (21, 22) of wild-type NPM.

We found that COOH-terminal NES motifs of NPM mutants vary greatly in their nuclear export strength, suggesting natural

Note: N. Bolli and I. Nicoletti contributed equally to this work.

Conflict of interest: B. Falini and C. Mecucci applied for a patent on clinical use of NPM mutants and have a financial interest in the patent.

Requests for reprints: Brunangelo Falini, Institute of Hematology, Policlinico, Monteluce, 06122 Perugia, Italy. Phone: 39-075-578-3190; Fax: 39-075-578-3834; E-mail: faliniem@unipg.it.

doi:10.1158/0008-5472.CAN-07-0273

selection of most efficient NES motifs in NPM mutants retaining tryptophan 288. This biological association favors maximum NPM mutant accumulation in cytoplasm and could be a critical event in leukemogenesis. Our results also account for the predominant nucleolar localization of wild-type NPM protein.

Materials and Methods

pEGFP-C1-NPM plasmid constructs. pEGFP-C1-NPMmA (5) and pEGFP-C1-NPMmE (12) were used as templates to generate the following derivative constructs: pEGFP-C1-NPMmA-C288W, in which tryptophan (W) 288, acting as a partial nucleolar localization signal (NoLS), was reinserted into NPM mutant A; pEGFP-C1-NPMmE-LxxxVxxVxL, in which the NPM mutant E COOH-terminal NES motif (LxxxLxxVxL) was replaced by the mutant A NES motif (LxxxVxxVxL); and pEGFP-C1-NPMmE-W288C, in which W288 in mutant E was replaced by a cysteine (C). Plasmids were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's instructions, with primers designed on the following sequences: pEGFP-C1-NPMmA-C288W, 5′-CTATTCAAGATCTCTGGCTGGCAGTGGAGGAAGTCTC; pEGFP-C1-NPMmE-LxxxVxxVxL, 5′-GATCTCTGGCAGTCTGTTGCCCAAGTCTCTTTAAG; and pEGFP-C1-NPMmE-W288C, 5′-GAGGCTATTCAAGATCTCTGTCAGTCTCTTGCCC.

Rev(1.4)-eGFP fusion plasmids containing NPM NES motifs. HIV-1 Rev protein shuttles between cytoplasm and nuclear compartments (23). Nuclear export efficiency of NES motifs from wild-type and mutated NPM proteins was measured using a Rev(1.4)-eGFP fusion protein–based functional nuclear export assay (20). The NES-mutated Rev(1.4)-eGFP and the positive control Rev(1.4)-NES3-eGFP (with the Rev NES reinserted) fusion plasmids were generated by one of the authors (B.H.) at the Westmead Institute for Cancer Research, Westmead Hospital (Sydney, Australia).

Double-strand DNA coding for several putative NES motifs from wild-type NPM (NES 42–49 and NES 94–102) and leukemic mutants at their COOH termini (LxxxVxxVxL, LxxxLxxVxL, LxxxCxxVxL, LxxxFxxVxL, and LxxxMxxVxL and mut6; refs. 16, 21, 22) was obtained by annealing partially complementary synthetic oligonucleotides bearing *Bam*HI-*Age*I compatible ends and ligating into *Bam*HI-*Age*I–digested Rev(1.4)-eGFP by standard procedures. Oligonucleotides are listed below: Rev(1.4)-NES-42–49-eGFP-F, 5′-GATCCATTATCTTTAAGAACGGTCAGTTTAGGA; Rev(1.4)-NES-42–49-eGFP-R, 5′-CCGGTCCTAAACTGACCGTTCTTAAAGATAATG; Rev(1.4)-NES-94–102-eGFP-F, 5′-GATCCAATAACACCACCAGTGGTCTTAAGGTTGAAA; Rev(1.4)-NES-94–102-eGFP-R, 5′-CCGGTTTCAACCTTAAGACCACTGGTGGTGTTATTG; Rev(1.4)-LxxxVxxVxL-eGFP-F, 5′-GATCCAGATCTCTGTCTGGCAGTGGAGGAAGTCTCTTTAAGAAAAAGA; Rev(1.4)-LxxxVxxVxL-eGFP-R, 5′-CCGGTCTTTTTCTTAAAGAGACTTCCTCCACTGCCAGACAGAGATCTG; Rev(1.4)-LxxxLxxVxL-eGFP-F, 5′-GATCCAGATCTCTGGCAGTCTCTTGCCCAAGTCTCTTTAAGAAAAAGA; Rev(1.4)-LxxxLxxVxL-eGFP-R, 5′-CCGGTCTTTTTCTTAAAGAGACTTGGGCAAGAGACTGCCAGAGATCTG; Rev(1.4)-LxxxCxxVxL-eGFP-F, 5′-GATCCAGATCTCTGGCAGTGCTGCTCCCAAGTCTCTTTAAGAAAAAGA; Rev(1.4)-LxxxCxxVxL-eGFP-R, 5′-CCGGTCTTTTTCTTAAAGAGACTTGGGAGCAGCACTGCCAGAGATCTG; Rev(1.4)-LxxxFxxVxL-eGFP-F, 5′-GATCCAGATCTCTGGCAGTGCTTCGCCCAAGTCTCTTTAAGAAAAAGA; Rev(1.4)-LxxxFxxVxL-eGFP-R, 5′-CCGGTCTTTTTCTTAAAGAGACTTGGGCGAAGCACTGCCAGAGATCTG; Rev(1.4)-LxxxMxxVxL-eGFP-F, 5′-GATCCAGATCTCTGGCAGTCCATGGAGGAAGTCTCTTTAAGAAAAAGA; Rev(1.4)-LxxxMxxVxL-eGFP-R, 5′-CCGGTCTTTTTCTTAAAGAGACTTCCTCCATGGACTGCCAGAGATCTG; Rev(1.4)-Mut6-eGFP-F, 5′-GATCCAGATCTCTGGCAAGATTTCTTAAATCGTCTCTTTAAGAAAATAGTTACA; Rev(1.4)-Mut6-eGFP-R, 5′-CCGGTGTAACTATTTTCTTAAAGAGACGATTTAAGAAATCTTGCCAGAGATCTG.

Cell cultures and transfection procedures. NIH-3T3 murine fibroblasts were cultured in DMEM supplemented with 10% bovine calf serum, 1% glutamine, and antibiotics. For transfection purposes, cells were seeded overnight on glass coverslips and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 24-h incubation, before any treatment, cells were incubated with 10 μg/mL cycloheximide (Merck Biosciences Ltd.) for 30 min. Leptomycin B (Merck Biosciences), a specific Crm1 inhibitor (19), was added at 20 ng/mL final concentration for standard assays and at doses ranging from 0.1 to 10 ng/mL for dose finding experiments (see below), always for 5 h; actinomycin D (Sigma-Aldrich) was added at 5 ng/mL final concentration for 3 h where indicated.

Immunofluorescence analysis. For immunofluorescence and confocal microscope studies, transfected and treated cells (see above) grown on glass coverslips were rinsed in PBS and fixed in 4% paraformaldehyde, pH 7.4 (10 min). Nuclei were counterstained with propidium iodide when necessary. Confocal analysis was done as previously described (24). Briefly, images were collected with a Zeiss LSM 510 confocal microscope (Carl Zeiss) using 488-nm (for eGFP) and 543-nm (for propidium iodide) laser lines for excitation. Acousto-optic tunable filter–controlled tuning of laser lines, pinhole diameters, and light collection configuration were optimized to obtain best signal-to-noise ratio and to avoid fluorescence crossover. LSM 510 software regulated the microscope; images were transferred to an SGI Octane workstation (Silicon Graphics) for further processing. Slices were reconstructed three-dimensionally using the shadow technique or isosurface analysis with Imaris software (Bitplane).

NES scoring system. A cell scoring method evaluated export activity of each NES inserted in pRev (1.4)-eGFP. Cells were observed with an Olympus AX70 epifluorescence microscope (Olympus) equipped with a 100×/1.4 numerical aperture oil immersion objective and a MNIBA optical filter cube (excitation, 470–495 nm; dichroic mirror, 505 nm; emission, 510–550 nm). A minimum of 200 cells for each slide were examined by two "blinded" independent observers. Cells with or without cytoplasmic fluorescence were counted and their percentage calculated using GraphPad Prism 4 software (GraphPad Software). Three experiments were done. Data showed good intertest reproducibility. The same slides were examined with a confocal microscope according to the above procedure. Confocal slices were projected on the *Z* axis using the maximum intensity method with the Imaris software (Bitplane); images were recorded and further processed with ImageJ (NIH).

Results

NPM leukemic mutants carrying W288 localize aberrantly in cytoplasm but still bind nucleoli. We reviewed the sequences of the 37 *NPM1* exon 12 mutations thus far identified (16) and the corresponding abnormalities at the COOH termini of the predicted NPM mutant proteins. Protein sequences carrying mutations of both tryptophans 288 and 290 were found in 23 of 37 (62.2%) of all NPM mutants and >95% of NPMc+ AML patients. This high frequency concurs with loss of both tryptophans in *NPM1* mutation A, which alone accounts for ~80% of NPMc+ AML cases. All NPM mutants carrying the most common COOH-terminal NES motif LxxxVxxVxL lack both tryptophans (Table 1; NES variant 1). W288 retention, although rarely observed in patients (<5%), occurred in 14 of 37 (37.8%) of NPM leukemic mutant sequences (Table 1). All NPM mutants retaining W288 displayed one of the five rare COOH-terminal NES motifs (Table 1; NES variants 2 to 6).

Because tryptophans 288 and 290 are crucial for targeting NPM to nucleoli (12, 17), we addressed the issue of whether NPM leukemic mutants carrying mutations of one (W290) or two (W288 and W290) tryptophans vary in their subnuclear expression pattern. Confocal microscopy compared the nucleolus-binding ability of eGFP-tagged NPM mutant A (lacking both W288 and W290) and eGFP-tagged NPM mutant E (retaining tryptophan 288) in NIH-3T3 transfected cells. NPM mutant A was detected in cytoplasm and was relocated in nucleoplasm but not in nucleoli after Exportin/Crm1 inhibition by leptomycin B (Fig. 1, *left*).

Table 1. NES from NPM leukemic mutants and correlation with tryptophan loss

NES variant	NES motif	Frequency of NES variant in NPM mutants [*n* (%)]	Frequency of NES variant in NPMc+ AML patients (%)	Mutation at both tryptophans associated with NES variant [*n* (%)]	Retention of tryptophan 288 associated with NES variant [*n* (%)]
1	*L*-xxx-*V*-xx-*V*-x-*L*	23 of 37 (62.2)	>95	23 of 23 (100)	0 of 23 (0)
2	*L*-xxx-*L*-xx-*V*-x-*L*	6 of 37 (16.2)	<1	1 of 6 (16.7)	5 of 6 (83.3)
3	*L*-xxx-*F*-xx-*V*-x-*L*	3 of 37 (8.1)	<1	0 of 3 (0)	3 of 3 (100)
4	*L*-xxx-*M*-xx-*V*-x-*L*	2 of 37 (5.4)	<1	0 of 2 (0)	2 of 2 (100)
5	*L*-xxx-*C*-xx-*V*-x-*L*	2 of 37 (5.4)	<1	0 of 2 (0)	2 of 2 (100)
6	*L*-WQD-*F*-LNR-*L*-*F*-KK-*IV*	1 of 37 (2.7)	<1	0 of 1 (0)	1 of 1 (100)

NOTE: Sequences were analyzed from seven studies, accounting for 2,879 adults and 107 children AML patients (8–11, 16, 37, 38).

Mutant E showed aberrant cytoplasmic expression but retained the ability to bind nucleoli (Fig. 1, *middle*). Removal of tryptophan 288 (mutant eGFP-NPMmE-W288C) abolished mutant E nucleolar binding in basal conditions and after leptomycin B incubation (Fig. 1, *right*). These findings show that tryptophan 288 drives some mutated NPM protein to nucleoli.

However, NPM leukemic mutants bearing tryptophan 288 accumulate aberrantly in leukemic cell cytoplasm, as documented by immunohistologic analysis of bone marrow biopsies from NPMc+ AML patients. Therefore, targeting of these mutants to nucleoli needs to be counterbalanced by a more efficient COOH-terminal NES-induced nuclear export. Consequently, the question arises about whether NES occurring with tryptophan 288 retention has to be stronger than the LxxxVxxVxL NES motif that is only found with loss of tryptophans 290 and 288.

Low doses of leptomycin B reveal different sensitivity of NPM leukemic mutants to Crm1 inhibition. We investigated whether the COOH-terminal NES motifs of NPM leukemic mutants carrying one (W290) or two (W288 and W290) mutated tryptophans vary in functional activity. NIH-3T3 cells were transfected with mutants carrying the NES of interest and incubated with low doses of leptomycin B to highlight differences in export efficiency of the different COOH-terminal NPM NES motifs. Leptomycin B incubation was done in NIH-3T3 cells transfected with NPM mutant A (containing the LxxxVxxVxL COOH-terminal NES motif and no tryptophans) or NPM mutant E (containing the LxxxLxxVxL COOH-terminal NES motif plus tryptophan 288). At conventional doses of leptomycin B (10–20 ng/mL), all NPM leukemic mutants completely relocated in the nucleus. We reasoned that a lower dose could reach a Crm1 inhibition threshold and, if different mutant NES sequences have different nuclear export efficiency, the NPM mutant nuclear relocation patterns would differ. Titration experiments showed that, at a dose of 0.5 ng/mL, NPM mutant A was almost completely nuclear (Fig. 2, *top*) whereas NPM mutant E still showed marked cytoplasmic positivity (Fig. 2, *middle*). As expected, similar numbers of NPM cytoplasmic positive cells were observed with mutant eGFP-NPMmE-W288C (Fig. 2, *bottom*).

These findings show that NPM mutant E is less sensitive to Crm1 inhibition than NPM mutant A (Fig. 2). They do not prove that the COOH-terminal NES motif causes this difference because there are two physiologic NES motifs at the NH_2 terminus of NPM

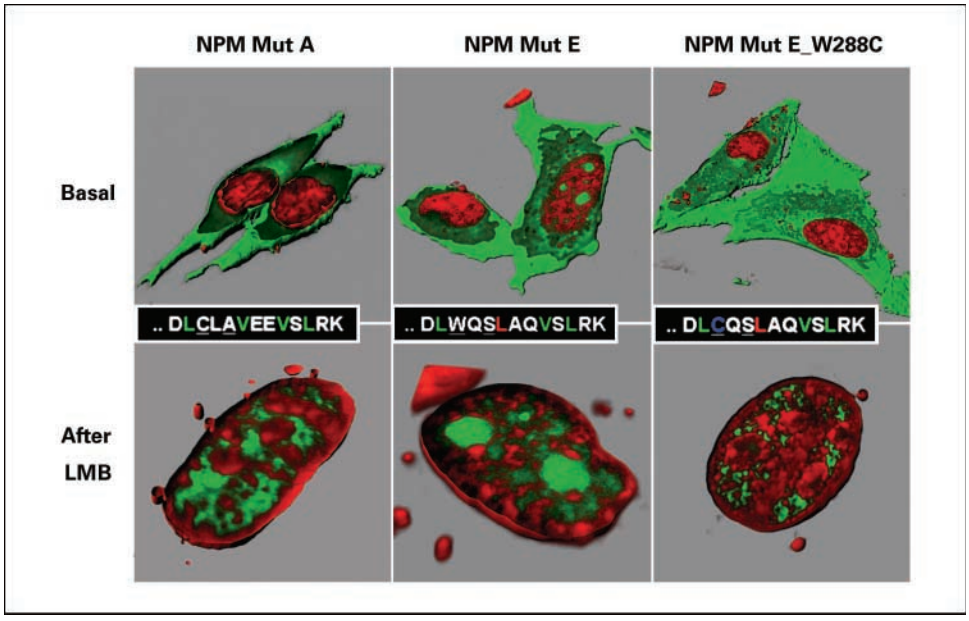

Figure 1. NPM leukemic mutants bearing W288 dislocate in the cytoplasm but retain ability to bind nucleoli. Subcellular localization of eGFP-NPM fusion protein in transfected NIH-3T3 cells. *Left,* NPM mutant A (*green*) is completely excluded from nucleus (*red*). *Middle,* NPM mutant E shows a partial nucleolar positivity due to presence of tryptophan 288; mutant E is no longer targeted to nucleoli (*right*) after substitution of W288 with cysteine. *LMB,* leptomycin B. Images are three-dimensional reconstructions of confocal sections (Zeiss LSM 510 and Imaris software).

and because NPM interacts with several nuclear and cytoplasmic proteins which may interfere with NPM mutant shuttling properties.

NPM leukemic mutants retaining W288 carry COOH-terminal NES motifs which are stronger than LxxxVxxVxL. To investigate the activity of both physiologic (NH_2-terminal) and mutation-induced (COOH-terminal) NES motifs of NPM systematically and without interfering factors, we conducted a nuclear export assay on isolated NPM NES sequences cloned into the Rev(1.4)-eGFP fusion protein (Fig. 3*A*). Because this construct contains a nuclear localization signal but lacks a NES motif, the recombinant Rev(1.4)-eGFP fusion protein produced in cells localizes in the nucleus (nucleoplasm and nucleoli). Inserting a functional NES between Rev(1.4) and eGFP protein restores shuttling activity, which is measured by immunofluorescence microscope analysis. This construct formed the basis of a functional shuttling assay designed to investigate the activity of each different NPM COOH-terminal NES motif. Actinomycin D, a RNA polymerase inhibitor, was used as an enhancer for detecting weak NES sequences because it increases nucleoplasmic availability of some nucleolar proteins (20, 25).

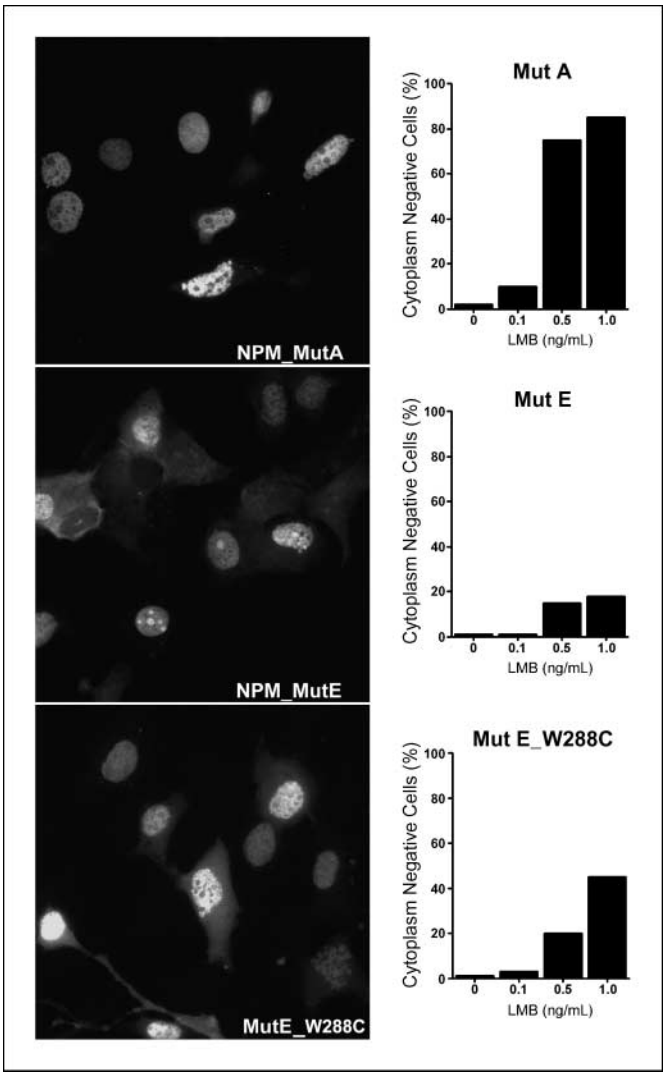

Figure 2. NPM leukemic mutant A is more sensitive to drug-dependent inhibition of Crm1 than mutant E. *Left column,* NPM leukemic mutant A (*top*) is relocated to the nucleoplasm by 0.5 ng/mL leptomycin B in nearly all transfected cells; NPM leukemic mutant E (*middle*) and artificial mutant E-W288C (*bottom*) are rather unaffected by the same leptomycin B dose. *Right column,* histograms show the percentage of transfected cells relocating the eGFP-NPM fusion proteins from cytoplasm to nucleus after incubation with increasing doses of leptomycin B.

Rev(1.4)-eGFP fusion protein showed nuclear localization in 100% of transfected cells, even in the presence of actinomycin D (Fig. 3*B*). Rev(1.4)-NES3-eGFP protein [with the physiologic NES motif of Rev reinserted between Rev(1.4) and eGFP] was cytoplasmic in most cells, even in basal conditions (Fig. 3*B* and *C*).

The NH_2-terminal NES motifs of NPM wild-type protein [i.e., amino acids 42–49 (21) and amino acids 94–102 (22)] displayed weak nuclear export efficiency. When transfected in NIH-3T3 cells, plasmids pRev(1.4)-NES-42–49 and pRev(1.4)-NES-94–102 encoded for fusion proteins which remained in the nucleus in most cells, both in basal conditions and after actinomycin D (Fig. 3*B and C*). The low nuclear export rate of these physiologic NES of NPM wild-type protein, in combination with signals driving NPM into the nucleoli (COOH-terminal tryptophans), provides a reasonable explanation for NPM nucleolus-restricted localization.

Significant functional export differences emerged in the six mutated NPM COOH-terminal NES motifs we investigated. Fusion proteins containing the most common NES motif LxxxVxxVxL (Rev-LxxxVxxVxL-eGFP) were nuclear in most transfected cells in basal conditions and produced cytoplasmic staining on actinomycin D incubation in ~40% of transfected cells (Fig. 3*B* and *C*). These findings indicate that NPM COOH-terminal LxxxVxxVxL is a functional NES motif with intrinsically weak activity. Therefore, NPM mutant A cytoplasmic localization depends on accumulated weak activity by the physiologic NES at the NH_2 terminus and the additional NES at the COOH terminus.

All NES motifs of NPM leukemic mutants retaining W288 produced a clear cytoplasmic staining in most transfected cells in basal conditions (Fig. 3*B* and *C*) and after actinomycin D incubation, indicating that these NES sequences exert stronger nuclear export efficiency than the LxxxVxxVxL NES. Leptomycin B incubation relocated all Rev(1.4)-NPM-NES-eGFP fusion proteins into the nucleus, confirming that protein export is NES dependent and Crm1 mediated (not shown).

These results show that NPM COOH-terminal LxxxVxxVxL is a functional NES motif with rather weak activity and that NPM leukemic mutants retaining W288 carry COOH-terminal NES motifs with stronger nuclear export efficiency.

A weak COOH-terminal NES motif and retention of tryptophan 288 is an unlikely combination. NPM leukemic mutants carrying a weak COOH-terminal NES motif and a tryptophan at position 288 have never been detected in primary AML samples (16). To investigate the consequences of a weak COOH-terminal NES motif combined with tryptophan 288 retention on NPM nucleocytoplasmic traffic, we used two artificial NPM leukemic mutants: (*a*) mutant A where tryptophan 288 was reintroduced (eGFP-NPMmA-C288W), and (*b*) mutant E (which normally retains W288) where the strong variant LxxxLxxVxL NES motif was replaced with the common weak LxxxVxxVxL NES (eGFP-NPMmE-LxxxVxxVxL). On transfection in NIH-3T3 cells, the bulk of both artificial proteins localized in the nucleoplasm and nucleoli and displayed greatly reduced cytoplasmic export as compared with the original NPM leukemic mutants A and E (Fig. 4).

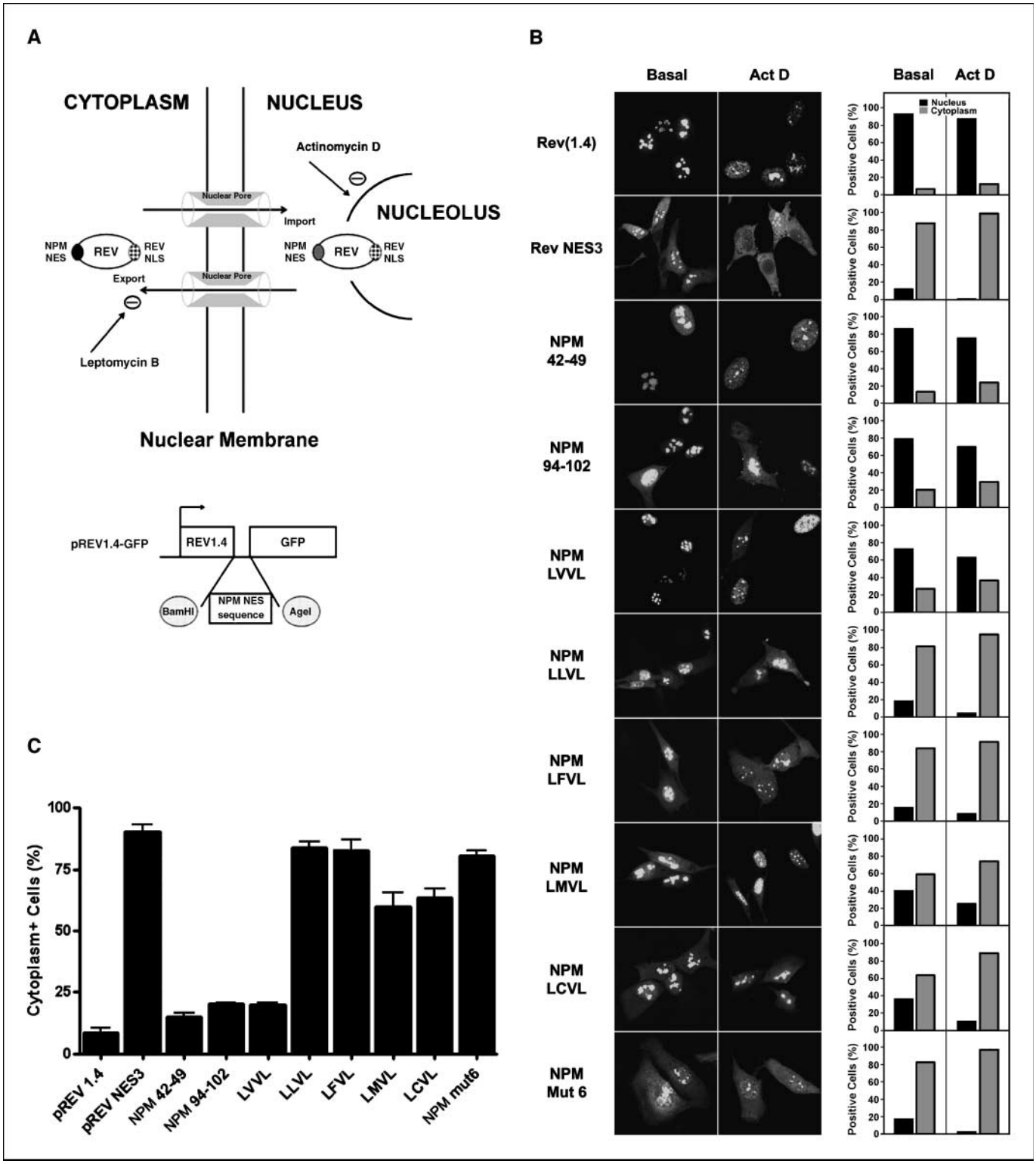

Figure 3. NPM COOH-terminal NES LxxxVxxVxL is weaker than all other NES motifs. *A,* schematic image of nucleocytoplasmic traffic in the Rev(1.4)-based NES efficiency assay, as previously described (20). Different NES test sequences from mutated NPM COOH terminus are challenged against Rev nuclear localization signal. NES activity is measured in basal state and after incubation with actinomycin D. *B,* subcellular localization in basal conditions and after actinomycin D (*Act D*) incubation is shown for each NPM COOH-terminal NES. The Rev(1.4) plasmid is nuclear by definition, having its own NES sequence inactivated by mutation. The positive control Rev(1.4)-NES3-eGFP peptide is cytoplasmic in the majority of transfected cells. The NH_2-terminal NES motifs of NPM wild-type protein (i.e., amino acids 42–49 and 94–102) and the NPM COOH-terminal LxxxVxxVxL NES motif drive cytoplasmic localization only in a minority of cells, whereas all other NPM NES motifs result in cytoplasmic positive staining in a greater proportion of cells. Cytoplasmic positivity of each protein was increased by actinomycin D. *Right, columns,* mean obtained from three independent experiments. *Nucleus,* nucleolar and/or nucleoplasmic eGFP positivity; *Cytoplasm,* partial or exclusive eGFP cytoplasmic staining. *C,* histogram showing different strength of various NPM NES motifs in basal conditions, measured as the percentage of cytoplasmic positive cells (partial or exclusive eGFP cytoplasmic staining).

These results indicate that the combination of a weak COOH-terminal NES motif with tryptophan 288 retention produces inefficient cytoplasmic accumulation of NPM leukemic mutants. Because NPM mutants are selected to lose two tryptophans and acquire a weak additional COOH-terminal NES, or to retain tryptophan 288 in association with a strong COOH-terminal NES, the main goal seems to be efficient cytoplasmic export of mutated NPM protein(s).

Discussion

In this article, we provide evidence for a functional variability among NES motifs of NPM wild-type protein and those created at the COOH termini of NPM leukemic mutants, and show that NES-mediated nuclear export efficiency plays a critical role in determining nucleolar localization of wild-type NPM and aberrant cytoplasmic expression of the mutant proteins. The close relationship between COOH-terminal NES motif strength and presence of one (W290) or two (W288 and W290) mutated tryptophans in NPM mutants suggests that COOH-terminal NES motifs are designed to counterbalance the tryptophan-mediated force driving NPM leukemic mutants to the nucleolus so as to achieve maximum cytoplasmic accumulation, which is probably a critical event in leukemogenesis.

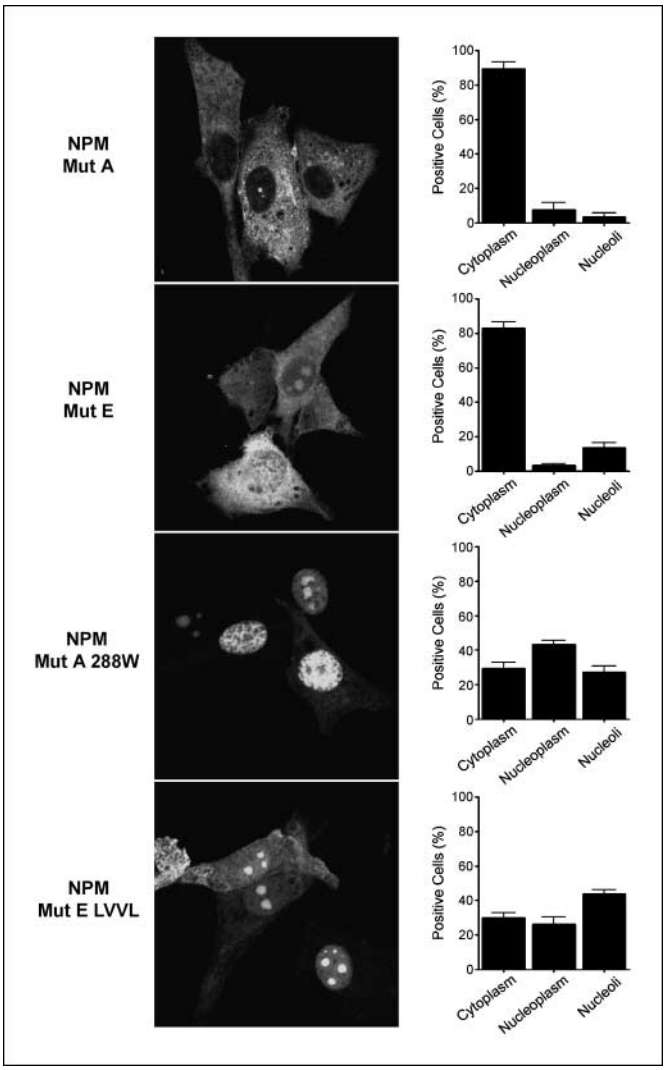

Figure 4. Artificial NPM leukemic mutants expressing both W288 and a weak NES localize mostly in the nucleus. *Top two rows,* NIH-3T3 cells transfected with NPM leukemic mutants pEGFP-C1-NPMmA and pEGFP-C1-NPMmE show a high proportion of cells expressing the fusion protein in the cytoplasm, as expected. *Bottom two rows,* NIH-3T3 cells transfected with artificial NPM leukemic mutants pEGFP-C1-NPMmA-C288W and pEGFP-C1-NPMmE-LxxxVxxVxL show a low proportion of cells expressing the fusion protein in the cytoplasm, as compared with the derivative proteins eGFP-NPMmA and eGFP-NPMmE.

Functional leucine-rich NES sequences commonly provide the signal for driving a protein out of the nucleus (26). NES motifs display a high natural variability in export activity (20, 27), which is probably related to differences in Crm1 affinity. The nuclear export efficiency of a NES motif represents a critical factor in intracellular localization of any shuttling protein. Others include the nuclear import rate, protein-protein (28, 29) or protein-nucleic acid interactions which lead to retention in cytoplasm or nucleus.

To assess how the balance between import and export signals dictates the restricted nucleolar distribution of the NPM wild-type protein (7), we used a Rev(1.4)-based shuttling assay (20) that gave us the possibility to quantify the efficiency of each of the two physiologic NES motifs of wild-type NPM [residues 94–102 (22) and residues 42–49 (21)] in a cellular system without confounding factors (e.g., other NES motifs in the same protein or changes in subcellular localization due to interactions with other proteins). Using this functional assay, we found that the two NES of NPM wild type exert weak nuclear export efficiency. This finding suggests that these NES cannot counterbalance the strength of the bipartite nuclear localization signal in the mid portion of NPM, which is responsible for nuclear import (30), and the force of the COOH-terminal tryptophans, which targets NPM to nucleolus (17, 30). Consequently, NPM wild type predominantly localizes in the nucleolus (Fig. 5, *left*). Although phosphorylation at threonine 95 (which physiologically regulates NPM NES 94–102 activity; ref. 22) probably does not occur in our artificial Rev(1.4)-based functional assay, our results support the theory of NES motif weakness (31) and fit with the observation that the least active NES motifs (20) belong to proteins that have specific nuclear functions, such as the transcriptional modulators p53 and activating protein-2. Our results also imply that the NES signal(s) needs to reach threshold activity for efficient protein export to cytoplasm.

In NPMc+ AML, an additional NES motif at the COOH terminus of NPM acting in concert with mutated tryptophans mediates aberrant cytoplasmic expression of NPM leukemic mutants (12). Here, we show that functional differences among the COOH-terminal NES motifs dictate NPM mutant nuclear export. NPM mutant COOH-terminal NES motifs retaining W288 exhibit stronger nuclear export activity than the NES motif L-xxx-V-xx-V-x-L, which is associated with mutations of both tryptophans 288 and 290. This finding strongly suggests that the COOH-terminal NES motifs of NPM mutants are naturally selected for efficiency in counteracting forces that drive NPM to nucleolus (i.e., tryptophans 288 and 290 flanking the core hydrophobic residues in the NES sequence) so as to achieve maximum mutant accumulation in cytoplasm (Fig. 5, *middle* and *right*). Under similar assay conditions, another cancer-associated protein (adenomatous polyposis coli tumor suppressor) was shown to contain both weak and strong NES, and cancer-linked mutations that truncate APC also underlie its export from nucleus to cytoplasm (32).

Interestingly, no NPM leukemic mutant combining a weak NES motif (L-xxx-V-xx-V-x-L) with W288 retention has ever been

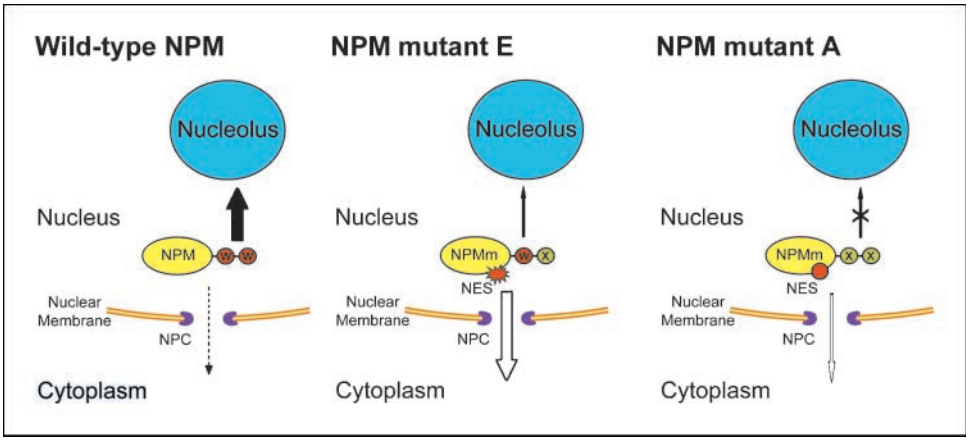

Figure 5. Opposing forces act to determine subcellular localization of wild-type NPM and NPM leukemic mutants. Wild-type NPM (*left*) is mainly localized in the nucleolus because the force (tryptophans 288 and 290; *dark arrow*) that drives the protein to the nucleolus overwhelms the scarce nuclear export activity (*dotted arrow*) of the two physiologic NES motifs (the mutation-related COOH-terminal NES is absent in NPM wild-type). NPM mutant E (*middle*) is delocalized in the cytoplasm because the residual force (tryptophan 288; *dark arrow*) that drives the mutant to the nucleolus is counterbalanced by the new COOH-terminal NES with strong nuclear export activity (*thick white arrow*). NPM mutant A (*right*) is delocalized in the cytoplasm because the lack of both tryptophans 288 and 290 abolishes the ability of the mutant to bind nucleolus (*dark arrow*) so that the new COOH-terminal NES motif, in spite of its weakness (*thin white arrow*), can cause export of the mutant to the cytoplasm. *Arrows,* forces dictating nucleocytoplasmic traffic of NPM. *NPM,* endogenous nucleophosmin; *NPMm,* leukemic NPM mutant; *W,* COOH-terminal tryptophan; *X,* mutated tryptophan; *NES,* COOH-terminal nuclear export signal motif; *NPC,* nuclear pore complex.

detected in NPMc+ AML patients (16). Artificial NPM mutants combining these two features (NPMmA-W288C and NPMmE-LVVL) are not efficiently exported into cytoplasm and accumulate in nucleoli and nucleoplasm. Added to different strength NES motifs, these observations point to an NPM mutant selection process. Mutant accumulation in cytoplasm seems to be a mandatory consequence of the mutation and possibly a critical event in leukemogenesis because only mutants that are efficiently exported into cytoplasm will probably give rise to a leukemic clone. Thus, NPM leukemic mutants seem to be "born to be exported." The finding that extremely rare variants of *NPM1* mutations occurring at exons other than exon 12 (33, 34) also result in additional NES and NPM mutant cytoplasmic dislocation further supports this view. NPM mutants have been shown to delocalize wild-type NPM and $p14^{ARF}$ into nucleoplasm and cytoplasm (12, 35), thus perhaps altering their functions (36). However, how altered NPM traffic contributes to leukemogenesis still remains an unsolved question.

Our results provide a rationale basis for immunohistochemical detection of cytoplasmic NPM being fully predictive of all types of *NPM1* mutations (14, 15). Finally, the findings presented in this article have a potential therapeutic effect because, if small molecules are to be designed to interfere with abnormal mutant traffic, all the complex structural and functional alterations that occur at the COOH termini of NPM mutants will need to be considered.

Acknowledgments

Received 12/1/2006; revised 4/4/2007; accepted 5/2/2007.

Grant support: Associazione Italiana per la Ricerca sul Cancro and the Fondazione Monte dei Paschi di Siena. N. Bolli is a recipient of a fellowship from Federazione Italiana per la Ricerca sul Cancro.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. G.A. Boyd for editorial assistance and Claudia Tibidò for secretarial assistance.

References

1. Macara IG. Transport into and out of the nucleus. Microbiol Mol Biol Rev 2001;65:570–94.
2. Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. Nat Rev Cancer 2004;4:106–17.
3. Fornerod M, Boer J, van Baal S, et al. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene 1995;10:1739–48.
4. Nakamura T. NUP98 fusion in human leukemia: dysregulation of the nuclear pore and homeodomain proteins. Int J Hematol 2005;82:21–7.
5. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 2005;352:254–66.
6. Borer RA, Lehner CF, Eppenberger HM, Nigg EA. Major nucleolar proteins shuttle between nucleus and cytoplasm. Cell 1989;56:379–90.
7. Cordell JL, Pulford KA, Bigerna B, et al. Detection of normal and chimeric nucleophosmin in human cells. Blood 1999;93:632–42.
8. Schnittger S, Schoch C, Kern W, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 2005;106:3733–9.
9. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 2005;106:3740–6.
10. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood 2005;106:3747–54.
11. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood 2006;107:4011–20.
12. Falini B, Bolli N, Shan J, et al. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. Blood 2006;107:4514–23.
13. Nakagawa M, Kameoka Y, Suzuki R. Nucleophosmin in acute myelogenous leukemia [letter]. N Engl J Med 2005;352:1819–20.
14. Falini B, Martelli MP, Bolli N, et al. Immunohistochemistry predicts nucleophosmin (NPM) mutations in acute myeloid leukemia. Blood 2006;108:1999–2005.
15. Pasqualucci L, Liso A, Martelli MP, et al. Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: impact on WHO classification. Blood 2006;108:4146–55.
16. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biological and clinical features. Blood 2007;109:874–85.
17. Nishimura Y, Ohkubo T, Furuichi Y, Umekawa H. Tryptophans 286 and 288 in the C-terminal region of protein B23.1 are important for its nucleolar localization. Biosci Biotechnol Biochem 2002;66:2239–42.
18. Fornerod M, Ohno M, Yoshida M, Mattaj IW. CRM1 is an export receptor for leucine-rich nuclear export signals. Cell 1997;90:1051–60.

19. Meissner T, Krause E, Vinkemeier U. Ratjadone and leptomycin B block CRM1-dependent nuclear export by identical mechanisms. FEBS Lett 2004;576:27–30.

20. Henderson BR, Eleftheriou A. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. Exp Cell Res 2000;256: 213–24.

21. Yu Y, Maggi LB, Jr., Brady SN, et al. Nucleophosmin is essential for ribosomal protein L5 nuclear export. Mol Cell Biol 2006;26:3798–809.

22. Wang W, Budhu A, Forgues M, Wang XW. Temporal and spatial control of nucleophosmin by the Ran-Crm1 complex in centrosome duplication. Nat Cell Biol 2005;7: 823–30.

23. Kalland KH, Szilvay AM, Brokstad KA, Saetrevik W, Haukenes G. The human immunodeficiency virus type 1 Rev protein shuttles between the cytoplasm and nuclear compartments. Mol Cell Biol 1994;14:7436–44.

24. Quentmeier H, Martelli MP, Dirks WG, et al. Cell line OCI/AML3 bears exon-12 NPM gene mutation-A and cytoplasmic expression of nucleophosmin. Leukemia 2005;19:1760–7.

25. Andersen JS, Lam YW, Leung AK, et al. Nucleolar proteome dynamics. Nature 2005;433:77–83.

26. la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S. NESbase version 1.0: a database of nuclear export signals. Nucleic Acids Res 2003;31:393–6.

27. Zhang MJ, Dayton AI. Tolerance of diverse amino acid substitutions at conserved positions in the nuclear export signal (NES) of HIV-1 Rev. Biochem Biophys Res Commun 1998;243:113–6.

28. Nikolaev AY, Li M, Puskas N, Qin J, Gu W. Parc: a cytoplasmic anchor for p53. Cell 2003;112: 29–40.

29. Bertwistle D, Sugimoto M, Sherr CJ. Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. Mol Cell Biol 2004;24: 985–96.

30. Hingorani K, Szebeni A, Olson MO. Mapping the functional domains of nucleolar protein B23. J Biol Chem 2000;275:24451–7.

31. Engelsma D, Bernad R, Calafat J, Fornerod M. Supraphysiological nuclear export signals bind CRM1 independently of RanGTP and arrest at Nup358. EMBO J 2004;23:3643–52.

32. Henderson BR, Fagotto F. The ins and outs of APC and β-catenin nuclear transport. EMBO Rep 2002;3:834–9.

33. Mariano AR, Colombo E, Luzi L, et al. Cytoplasmic localization of NPM in myeloid leukemias is dictated by gain-of-function mutations that create a functional nuclear export signal. Oncogene 2006;25:4376–80.

34. Albiero E, Madeo D, Bolli N, et al. Identification and functional characterization of a cytoplasmic nucleophosmin leukaemic mutant generated by a novel exon-11 NPM1 mutation. Leukemia. Epub 2007 Feb 15.

35. den Besten W, Kuo ML, Williams RT, Sherr CJ. Myeloid leukemia-associated nucleophosmin mutants perturb p53-dependent and independent activities of the Arf tumor suppressor protein. Cell Cycle 2005;4: 1593–8.

36. Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. Nat Rev Cancer 2006;6: 493–505.

37. Cazzaniga G, Dell'Oro MG, Mecucci C, et al. Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. Blood 2005;106: 1419–22.

38. Suzuki T, Kiyoi H, Ozeki K, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood 2005;106:2854–61.

Born to Be Exported: COOH-Terminal Nuclear Export Signals of Different Strength Ensure Cytoplasmic Accumulation of Nucleophosmin Leukemic Mutants

Niccolò Bolli, Ildo Nicoletti, M. Felicetta De Marco, et al.

Cancer Res 2007;67:6230-6237.