[Cell Cycle 4:11, 1593-1598, November 2005]; ©2005 Landes Bioscience

Report

Myeloid Leukemia-Associated Nucleophosmin Mutants Perturb p53-Dependent and Independent Activities of the Arf Tumor Suppressor Protein

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Received 09/12/05; Accepted 09/14/05

Previously published online as a Cell Cycle E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=2174

KEY WORDS

AUTHOR: PLEASE PROVIDE 5-10 KEY WORDS

ABBREVIATIONS

NPM	nucleophosmin
AML	acute myeloid leukemia
BSA	bovine serum albumin
MSCV	murine stem cell virus
IRES	internal ribosome entry site
GFP	green fluorescent protein
MEF	mouse embryo fibroblast
PFA	paraformaldehyde
Mab	monoclonal antibody
NES	nuclear export signal
Sh	short hairpin
His6	histidine hexamer

ACKNOWLEDGEMENTS

We thank all the other members of the Sherr/Roussel laboratory for suggestions and materials used in the course of these studies, and, in particular, Rose Mathew for providing primary MEFs and Richard A. Ashmun of the Flow Cytometry Laboratory for assistance with measurements of S-phase fractions. This work was supported by Cancer Center Core Grant (CA-21765), by NIH training Grant T32-CA-70089 (R.T.W.), and by ALSAC of St. Jude Children's Research Hospital. C.J.S. is an investigator of the Howard Hughes Medical Institute.

ABSTRACT

Nucleophosmin (NPM or B23) plays key roles in ribosome biogenesis, centrosome dupli-cation, and maintenance of genomic integrity. Mutations affecting the carboxylterminal domain of NPM occur in a significant percentage of adult patients with acute myeloid leukemia (AML), and these alterations create an additional nuclear export signal that relocalizes much of the protein from its normal nucleolar stores to the cytoplasm. When induced by oncogenic stress, the Arf tumor suppressor protein accumulates within the nucleolus, where it is physically associated with, and stabilized by, NPM. Ectopic overexpression of an NPM cytoplasmic mutant (NPMc) relocalized $p19^{Arf}$ and the endogenous NPM protein to the cytoplasm. NPMc-dependent export of $p19^{Arf}$ from the nucleus inhibited its functional interaction with the p53 negative regulator, Mdm2, and blunted Arf-induced activation of the p53 transcriptional program. Cytoplasmic NPM relocalization also attenuated Arf-induced sumoylation of Mdm2 and NPM and prevented wild type NPM from inhibiting $p19^{Arf}$ protein turnover. However, despite the ability of NPMc to interfere with these p53-dependent and independent activities of Arf, NPMc exhibited anti-proliferative activity in *Arf*-null NIH-3T3 cells. Overexpression of wild type NPM, but not NPMc, overcame premature senescence of *Atm*-null cells, a phenotype that can be rescued by inactivation of *Arf* or *p53*. Therefore, perturbation of Arf function appears to be insufficient to explain the oncogenic effects of the NPMc mutation. We favor the idea that NPMc also contributes to AML by dominantly perturbing other functions of the wild type NPM protein.

INTRODUCTION

The nucleolar phosphoprotein, nucleophosmin (NPM or B23) regulates several distinct biologic processes. NPM promotes ribosomal biogenesis by chaperoning pre-ribosomal particles from their site of synthesis in the nucleolus to the cytoplasm, thereby helping to govern their proper maturation.[1-4] NPM also binds to centrosomes throughout the G_1 phase of the cell cycle and negatively regulates their duplication prior to the G_1/S transition.[5,6] In the mouse, NPM is an essential gene whose inactivation in the germ line leads to a host of developmental defects that cause embryonic lethality at mid-gestation.[7] Even its partial loss-of-function leads to unrestricted centrosome duplication and genomic instability, which can contribute to myelodysplasia. Pertur-bations in NPM function also occur in various hematopoietic malignancies. Translocations in-volving the NPM gene that result in the formation of oncogenic fusion proteins occur in acute promyelocytic leukemia and in anaplastic large cell lymphomas.[8,9] Mutations affecting the carboxylterminus of the NPM protein (designated NPMc mutants) that relocalize the bulk of the protein from the nucleolus to the cytoplasm have recently been identified in ~35% of primary acute myeloid leukemias (AML) of adults 10 and in a smaller percentage of pediatric AML patients.[11] These mutations occur in leukemic cells that exhibit an otherwise normal karyotype and no previously described AML-associated genetic abnormalities, suggesting that they represent a novel class of tumor initiating lesions.

NPM binds stoichiometrically to the product of the *Arf* tumor suppressor gene (p19Arf in the mouse and $p14^{ARF}$ in humans) to affect Arf function and stability.[12-14] Like NPM, the bulk of Arf protein in cells is concentrated in the nucleolus, where $p19^{Arf}$ and NPM co-localize in high molecular weight complexes that also contain many other nucleolar proteins.[13] The *Arf* gene is not expressed in most normal tissues but is activated in response to tumor provoking stress signals induced by oncogenes such as mutant Ras or overexpressed Myc.[15] In turn, $p19^{Arf}$ binds to the p53 negative regulator, Mdm2, thereby

stabilizing p53 and inducing a p53-dependent tran-scriptional program that inhibits the proliferation of incipient tumor cells. Arf interacts with Mdm2 in the nucleoplasm, and it has therefore been suggested that nucleoplasmic sequestration of the two proteins, although it can occur, is not required for Arf to induce a p53 response.[16,17] Consistent with this idea, complexes between $p19^{Arf}$ and Mdm2 are distinct from those that con-tain p19Arf and NPM.[13,17] Although it is generally accepted that Arf exerts its effects in a p53-dependent manner, Arf also exhibits p53-independent tumor suppressive functions.18,19 For example, the Arf protein can interact with a number of transcription factors to negatively regulate their activities.[20-22] Arf can also inhibit ribosomal RNA processing[23] and induce the sumoylation of a wide variety of targets, including Mdm2 and NPM.[24-27] Given these functional and physical interactions between $p19^{Arf}$ and NPM, we initiated studies to determine if NPM mutants that relocalize to the cytoplasm might affect different Arf functions. We show that enforced ex-pression of NPMc acutely attenuates p53-dependent and -independent functions of Arf, and also affects endogenous NPM localization.

MATERIALS AND METHODS

Expression plasmids. Flag tagged human NPMc[10] was generated by PCR with a T7 sense primer and antisense primer (atgctcgagctattttcttaaagagacttcctccactgccagacagagatcttgaatag) using pcDNA3-FlagNPM as a template.[13] The reaction buffer included 50 ng of template, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4, 0.1% Triton X-100, 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 2.5 mM deoxynucleoside triphosphates, 25 pmol of each primer, and 2.5 U of Pfu DNA polymerase (Stratagene, La Jolla, CA). Each of 35 cycles consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72∞C for 2 minutes. The PCR product was purified (Qiagen, Valencia, CA), digested and inserted into the *Bam*HI and *Xho*I sites of the murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-green fluorescent protein (GFP) vector (MSCV-IRES-GFP). MSCV-IRES-GFP vectors encoding His-tagged Sumo-1[27] or Flag-tagged NPM,[13] and pSUPER.retro.neo-gfp (Oligoengine, Seattle, WA) expressing a short hairpin RNA (shRNA) targeting the 3′ untranslated region of mouse NPM[14] were described previously.

Cell culture. Human kidney 293T cells, mouse NIH-3T3 fibroblasts and Arf-inducible MT-Arf cells[28] were maintained in DMEM (Cambrex, East Rutherford, NJ) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), 4 mM glutamine (Invitrogen), and 100 units each of penicillin and streptomycin (Invitrogen) in an 8% CO_2-humidified incubator. Primary mouse embryo fibroblasts (MEFs; passage 3) derived from *Atm*-null mice were cultured as de-scribed.[29]

Retroviral vector production and infection. To produce ecotropic retroviruses, 3 x 10^6 293T cells in 100 mm diameter culture dishes were transfected with 0.5 μg of CAG4-Eco[30] and 1.5 μg of pMDgagpol[31] helper retroviral plasmids in combination with either 3 μg MSCV-IRES-GFP vectors encoding Flag-tagged wild type or mutant human NPM proteins, pSUPER.retro.neo-gfp (Oligoengine), or pSUPER.retro.neo-gfp expressing shRNA targeting mouse NPM mRNA.[14] Viruses harvested 24–60 hrs after transfection were pooled. Exponentially growing NIH-3T3 and MT-Arf cells (2 x 10^5 cells per 10 cm-diameter culture dish) were infected once with 2 ml ecotropic virus-containing supernatant supplemented with 6 μg of polybrene (Sigma, St. Louis, MO) per milliliter. Alternatively, cells were sequentially infected with two different viruses at 3 hr intervals for two rounds. MEFs were infected three times with 2 ml eco-tropic viruses at 3 hr intervals.

Immunofluorescence. Cells infected with the indicated viruses were trypsinized two days post-infection, re-seeded, and grown on coverslips for an additional 16–24 hrs. In experi-ments where *Arf* was induced with $ZnSO_4$, cells were re-seeded one-day post-infection and treated with 100 μM $ZnSO_4$ for 24 hours. To inhibit nuclear export, cells were exposed to 5 ng/ml leptomycin B (Biomol Research Laboratories Inc, Plymouth Meeting, PA) for 6 hours and either fixed with methanol-acetone [1:1 (vol/vol)] for 20 min at -20°C or with 4% para-formaldehyde (PFA) in PBS for 10 min at room temperature. Cells fixed in PFA were per-meabilized for 5 min with 0.3% Triton X-100 before staining. Cells were stained for 1 hr with the following primary antibodies: rat anti-$p19^{Arf}$ 5C3 monoclonal (MAb) hybridoma supernatant (1:500 dilution);[32] mouse anti-NPM MAb (1 μg/ml; Invitrogen); or mouse anti Flag-tag MAb (5 μg/ml; M2; Sigma). Coverslips were then incubated for 30 min with anti-rat or anti-mouse immunoglobulin (1:500 dilution; Invitrogen) conjugated to Alexa Fluor 594. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing DAPI to stain nuclei. Fluorescence signals were detected using an Axioskop 2 plus microscope fitted with an AxioCam HRc camera (Carl Zeiss Light Microscopy, Göttingen, Germany).

Immunoblotting. Procedures for immunoblotting were as described.[33] Briefly, cells were lysed in buffer containing Tween-20, and protein was quantified by BCA assay (Pierce Biotech-nology, Rockford, IL). Proteins separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen) were transferred to PVDF membranes (Millipore, Billerica, MA) and detected using antibodies to $p19^{Arf}$ (5C3), the Flag epitope (M2-HRP; Sigma), NPM (Invitrogen), Mdm2 (2A10; provided by A. Levine, Institute for Advanced Study, Princeton, NJ) and $p21^{Cip1}$ (F-5; Santa Cruz Biotech-nology, Santa Cruz, CA).

Cell cycle analysis and growth curves. NIH-3T3 and MT-Arf cells infected with retro-viruses encoding Flag-tagged wild type or mutant NPM proteins were treated with 100 mM ZnSO4 for 24 hours, harvested by trypsinization, and suspended in solution containing propidium iodide.[34] DNA content was determined by flow cytometric analysis, and MODFIT (Verity Software, Topsham, ME) was used to calculate the percentage of cells in S-phase. *Atm*-null MEFs infected with retroviruses encoding GFP, Flag-tagged wild type NPM, or mutant NPM proteins were seeded at low density (2 x 10^4 per 6 cm-diameter culture dish) two days post-infection. Cells from triplicate cultures were counted every day for six days.

In vivo sumoylation assay. MT-Arf cells were co-infected with MSCV-His tagged Sumo and either MSCV-IRES-GFP, MSCV-Flag-NPM or MSCV-Flag-NPMc. Two days after infection, cells were treated with 100 μM $ZnSO_4$ for 16 hours to induce $p19^{Arf}$ expression. Ten percent of the cells were lysed in RIPA buffer for direct immunoblotting, and the remaining cells were used to purify sumoylated proteins as previously described.[27] Briefly, cells were sonicated in lysis buffer containing 8 M urea and 10 mM imidazole. His-Sumo-conjugated proteins were pulled down with Ni-NTA resin (Qiagen), washed 5 times in urea lysis buffer containing 20 mM imidazole, and eluted with buffer containing 5% SDS, 0.72 M β-mercaptoethanol and 200 mM imidazole. Proteins electrophoretically separated on denaturing gels were transferred to PVDF membranes and visualized with antibodies to $p19^{Arf}$ (5C3), the Flag epitope (M2-HRP), NPM (Invitrogen) or Mdm2 (2A10).

RESULTS

NPMc delocalizes wild type NPM to the cytoplasm. We constructed a human NPM mutant containing a four base-pair insertion (TCTG) near the 3′ end of the cDNA coding se-quence (Fig. 1A). This mutated form (NPMc) encodes an altered protein identical to the most frequently observed class of NPM cytoplasmic mutants detected in AML patient samples (desig-nated mutant A by Falini and coworkers).[10] The resulting frame shift results in the substitution of 11 novel amino acids for the seven C-terminal residues of the wild type NPM protein and generates a consensus nuclear export signal (NES) (Fig. 1A) which is predicted to lead to the cytoplasmic relocalization of the NPMc protein.[35]

The NPM monomer homo-oligomerizes in cells to form high molecular mass complexes, but the domain required for multimerization is unaffected by alterations at the C-terminus.[3,13,36] Therefore, the enforced expression of NPMc is expected to act in a dominant manner to form hetero-oligomers with endogeneous NPM and relocalize both proteins to the cytoplasm.[37] We

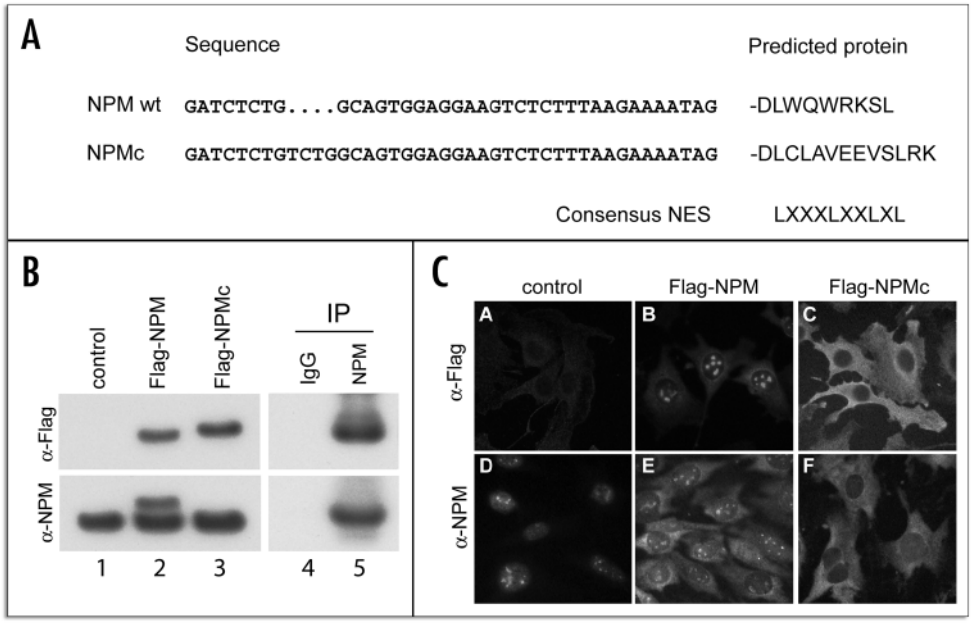

Figure 1. NPMc forms complexes with NPM and mobilizes it to the cytoplasm. (A) The sequence of NPMc alters its C-terminus and introduces a consensus NES. The NPMc mutant illustrated is the most common form detected in adult AML.[10] (B) Lysates of cells infected with a control ret-roviral vector or with those encoding Flag-tagged NPM or NPMc were blotted with antibodies to the Flag epitope (lanes 1–3, top) or with those directed to the C-terminus of wild-type NPM (bottom). The latter antibody does not detect the altered C-terminus of the mutant protein (lane 3, bottom). However, recovery of endogenous NPM with this antibody versus a control antibody (lane 4) co-precipitated Flag-tagged NPMc (lane 5). (C) Cells engineered to express Flag-tagged NPM or NPMc, or cells infected with the control vector, were analyzed by immunofluorecence with the same antibodies used for the analyses in panel B.

tagged the wild type and mutant forms of NPM with a Flag epitope and used retroviral vectors to introduce genes encoding either form of the protein into mouse NIH-3T3 fibroblasts (Fig. 1B). An antibody to the Flag tag detected the proteins, which were expressed at equivalent levels in infected cells (Fig. 1B, lanes 2, 3 top). In contrast, an antibody that specifically reacts with a C-terminal epitope of wild type NPM detected the faster migrating endogenous NPM protein as well as its more slowly migrating Flag-tagged counterpart (lanes 1, 2); important for the studies to follow, however, this antibody was unable to detect the altered C-terminus of the Flag-tagged NPMc protein (lane 3, bottom). Therefore, to study physical interactions between the mutant and wild type NPM proteins, lysates of cells engineered to express Flag-tagged NPMc were precipitated with antibody directed to the C-terminus of endogenous NPM, and immune complexes separated on denaturing gels were blotted with antibodies to the NPMc Flag epitope or to the endogenous NPM protein (Fig. 1B, lane 5). This confirmed that NPMc forms hetero-oligomers with the endogenous wild-type form.

NPM is a relatively abundant protein that primarily resides within the nucleolus, where it can be readily detected by immunofluorescence performed with the antibody directed to the C-terminus of wild type NPM (Fig. 1C, panel D). The exogenously expressed wild type protein also accumulated in the nucleolus (Fig. 1C, panel B), but its overexpression led to a net increase in cytoplasmic staining as well (panel E). In contrast, Flag-tagged NPMc not only predominately localized to the cytoplasm (panel C) but also delocalized endogenous NPM from nucleoli (panel F). Thus, when the level of expression of Flag-tagged NPMc approaches that of the endogenous NPM protein (Fig. 1B, lane 3), the latter can be delocalized to the cytoplasm.

NPMc delocalizes p19^{Arf} from nucleoli. The p19^{Arf} tumor suppressor is stoichiometri-cally bound to NPM and is stabilized by its association with NPM in high molecular mass com-plexes.[13,14] It has been argued that nucleolar sequestration of the Arf protein inhibits its p53-dependent activity, and that the ability of newly induced p19^{Arf} to antagonize Mdm2 and in-duce p53 instead depends upon a minor nucleoplasmic pool of Arf protein.[16,17] Using MT-Arf cells, an NIH-3T3 cell derivative engineered to express a zinc-inducible p19^{Arf} protein,[28] we studied the effect of Flag-tagged NPMc on p19Arf localization (Fig. 2A). Unlike parental NIH-3T3 cells which completely lack a functional Arf gene, MT-Arf cells express very low basal levels of p19^{Arf} in the absence of zinc, but synthesize increased levels of protein by two hours after induction and are maximally induced by 16 hours. Prior to zinc treatment, p19^{Arf} was not detected (Fig. 2A, panel A), but Flag-tagged NPM led to accumulation of p19^{Arf} in the nucleolus (panel B), due to the ability of NPM to inhibit regulated degradation of the Arf protein.[14] By contrast, NPMc rendered no such effect (panel C). Following zinc induction, p19^{Arf} accumulated in the nucleolus in cells infected with the control vector (panel D) or the vector expressing Flag-tagged NPM (panel E), but p19^{Arf} was delocalized to the cytoplasm by enforced expression of NPMc (panel F). When the p19^{Arf} protein was immunoprecipitated from lysates of cells ex-pressing Flag-tagged NPMc and proteins separated on denaturing gels were blotted with anti-bodies to the Flag tag or to NPM, both endogenous NPM and Flag-tagged NPMc were readily detected (data not shown). This confirmed that p19^{Arf} enters into complexes with both the wild type and mutant NPM proteins.

When introduced into MT-Arf cells, Flag-tagged NPMc accumulated in the cytoplasm whether or not zinc was added to the culture medium (Fig. 2B, panels A and B). Most of the Arf protein was delocalized to the cytoplasm when NPMc was coexpressed (panel F) as was much of the endogenous pool of NPM detected with antibodies selectively recognizing its C-terminus (panels I and J). NPM normally shuttles between the nucleus and cytoplasm in a manner that de-pends upon the Ran-Crm1 complex.[38,39] Addition of leptomycin B, a Crm1 inhibitor that inter-feres with NES-directed nuclear export, led to the reaccumulation of Flag-tagged NPMc (panel C) and endogenous NPM (panel K) within the nucleolus. Under these conditions, induced p19^{Arf} also reaccumulated in the nucleolus, even in the presence of NPMc (panel H). Thus, NPMc delo-calizes both wild type NPM and p19^{Arf} to the cytoplasm in a manner that depends upon its C-terminal NES.

NPMc attenuates Arf activity. We next infected MT-Arf cells with a control retroviral vector or one specifying a short hairpin (sh) RNA directed to noncoding sequences within en-dogenous NPM mRNA. As previously reported, the endogenous levels of NPM protein were re-duced by ~50% with NPM shRNA[14] (and data not shown). These same cells were transduced with vectors encoding either Flag-tagged wild type NPM or NPMc; p19^{Arf} was induced with zinc, and the expression of two canonical p53-responsive proteins, Mdm2 and p21^{Cip1}, was stud-ied. The NPM shRNA has no effect on the expression of the exogenously expressed Flag-tagged NPM proteins, which are encoded by cDNAs lacking the target sequence.

Antibodies directed to the C-terminus of wild type NPM detected the endogenous protein as well as the slower migrating Flag-tagged wild type species (Fig. 3, NPM blot, lanes 3 and 4). As already shown (Fig. 1B), this antibody did not detect NPMc (lanes 5 and 6). However, antibodies to the Flag epitope revealed expression of both the wild type and mutant Flag-tagged proteins (Fig. 3, Flag blot, lanes 3–6). Following zinc treatment, p19^{Arf} was induced (even numbered lanes). The higher levels of Arf protein observed in the presence of exogenous wild type NPM (lane 4 vs. 2) are

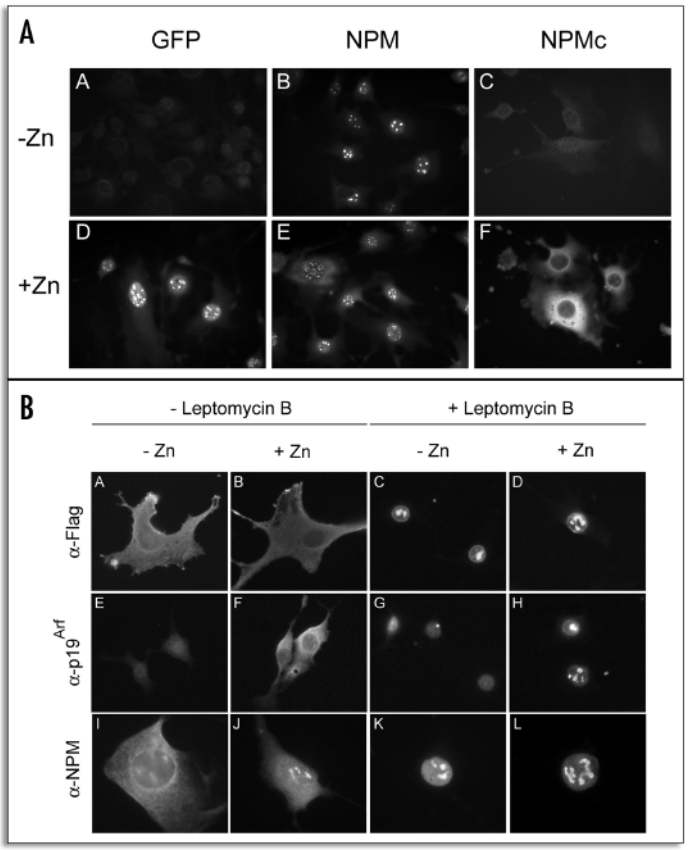

Figure 2. Inhibition of Crm1-mediated transport prevents NPMc-induced delocalization of NPM and $p19^{Arf}$. (A) MT-Arf cells infected with a control vector encoding GFP or those encoding Flag-tagged NPM proteins were left untreated (panels A–C) or were treated with zinc to induce the Arf protein (panels D–F). Cells were immunostained with the 5C3 monoclonal antibody di-rected to $p19^{Arf}$. (B) MT-Arf cells infected with a vector encoding Flag-tagged NPMc were in-duced with zinc and/or treated with leptomycin B as indicated at the top of the panels. Cells were immunostained with antibodies to the Flag-epitope, to $p19^{Arf}$, or to wild-type NPM as indicated at the left. All photographs were taken at a magnification of 630X.

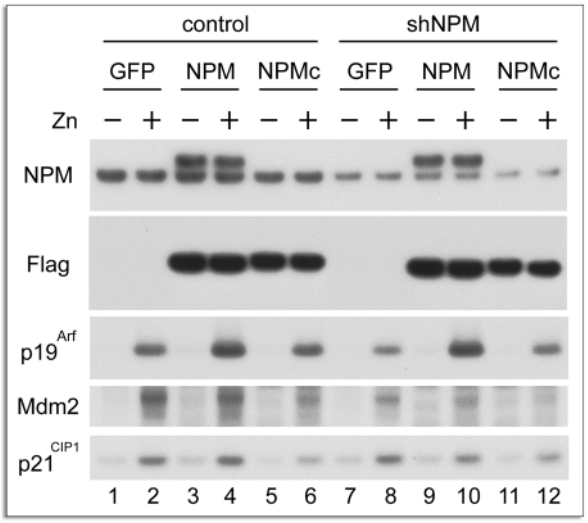

Figure 3. NPMc attenuates the $p19^{Arf}$-induced p53 response. Cells engineered to express Flag-tagged NPM (lanes 3, 4, 9, 10) or NPMc (lanes 5, 6, 11, 12) were treated with zinc (even numbered lanes) to induce the Arf protein. Some cells also expressed an shRNA directed to the endogenous NPM mRNA (lanes 7–12). Lysates prepared 24 hours after Arf induction were blot-ted with antibodies to the C-terminus of wild-type NPM, the Flag epitope, $p19^{Arf}$, or to the p53-responsive gene products, Mdm2 and $p21^{Cip1}$ as indicated at the left.

significant and reflect the ability of NPM to inhibit $p19^{Arf}$ turnover (see also Fig. 2A, panel B versus A).[14] Expression of Flag-tagged wild type NPM had no overt effects on the ability of $p19^{Arf}$ to trigger the p53-dependent induction of Mdm2 and $p21^{Cip1}$, but expression of NPMc attenuated this response (lane 6). Mdm2 induction was further compromised by the presence of NPM shRNA (lane 12).

Arf also exhibits p53-independent functions,[18,19] which include its ability to retard ribo-somal RNA processing[23] and to trigger the sumoylation of both NPM and Mdm2.[24,27] NPMc had no apparent ability to override Arf's effects on rRNA processing (data not shown), so we turned our attention to Arf-induced sumoylation. We again used MT-Arf cells engineered to express either Flag-NPM or Flag-NPMc and reinfected these cells with a vector encoding histidine-tagged (His6) Sumo-1. Arf was then induced, and the levels of $p19^{Arf}$ and p53-responsive Mdm2 were quantified by immunoblotting (Fig. 4A). Expression of Flag-NPM led to the accu-mulation of $p19^{Arf}$ even in uninduced cells (lanes 5 and 6), again reflecting the ability of NPM to retard the turnover of $p19^{Arf}$ expressed at very low basal levels.[28,32] However, Flag-tagged NPMc failed to render a similar effect (lanes 9 and 10), implying that cytoplasmic relocalization of $p19^{Arf}$ may prevent its stabilization. As before, Arf was induced to similar levels with zinc even in the presence of NPMc (lanes 11 and 12), but Mdm2 accumulation was compromised in its presence (Mdm2 blot, lanes 11, 12 vs. 3, 4).

His-tagged sumoylated proteins from these cell lysates were adsorbed to nickel-agarose resin in the presence of 8M urea and recovered by elution with imidazole. Immunoblotting of the sumoylated proteins with antibodies to NPM and to the Flag epitope revealed that the endogeneous and Flag-tagged forms of NPM were monosumoylated, even when $p19^{Arf}$ was not induced (Fig. 4B, lanes 2 and 6).[27] Induction of $p19^{Arf}$ with zinc increased the level of monosumoylated wild type Flag-tagged NPM and generated a more slowly migrating disumoylated species (lane 8), whereas NPMc sumoylation was unaffected by Arf induction (lanes 10 and 12). Expression of Flag-tagged NPMc abrogated monosumoylation of endogenous NPM (lanes 10 and 12) and also inhibited Arf-induced sumoylation of Mdm2 (lane 12 vs. 4). Taken together with the data in Figure 3, these findings indicate that NPMc can negatively regulate both p53-dependent and p53-independent effects of $p19^{Arf}$.

NPM has a growth-promoting activity not observed with NPMc. Because Arf induc-tion rapidly arrests the division cycle of MT-Arf cells,[28] we used flow cytometry to study the DNA content of Arf-induced cells engineered to co-express either of the two forms of Flag-tagged NPM. Our naïve expectation was that by attenuating Arf's effects, NPMc might prevent Arf-induced cell cycle arrest and provide cells with a proliferative advantage. Interestingly, in control NIH-3T3 cells that have sustained a deletion of the endogenous *Arf* gene, NPMc significantly reduced the fraction of cells in S phase as compared to cells transduced with the control vector or that over-expressing wild type NPM (Fig. 5A, left six bars). Hence, in the com-plete absence of *Arf*, NPMc has an anti-proliferative effect. In contrast, in MT-Arf cells that normally cycle more slowly, induction of $p19^{Arf}$ reduced the S phase fraction as expected, and exogenous NPM expression potentiated this effect, again most likely due to its ability to stabilize and increase the level of the Arf protein. However, the anti-proliferative effect of $p19^{Arf}$ was negated in cells expressing NPMc, consistent with its ability to acutely interfere with Arf function. Thus, NPMc mediates opposing effects depending on whether Arf activity is present or absent.

To look for longer term effects of NPM overexpression on cell proliferation, we tested the ability of exogenous wild type NPM and NPMc to affect the growth of primary mouse embryo fibroblasts (MEFs) lacking the *Atm* gene. Due to defective DNA damage sensing and repair, primary *Atm*-null MEFs undergo p53-dependent cell cycle arrest after only a few passages in culture.[40] Although the *Arf* gene is not induced by DNA damage signals, the absence of *Arf* limits the p53 response of Atm-null cells and thereby rescues their ability to proliferate.[29] NPMc was without effect in this assay, whereas the

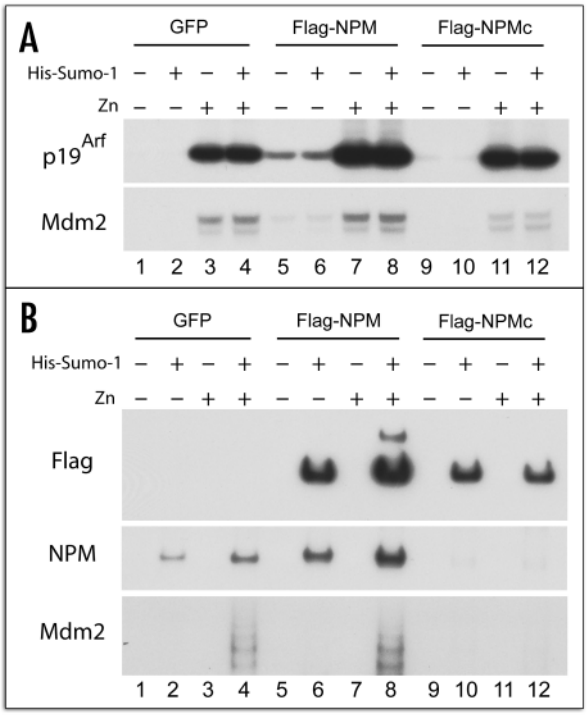

Figure 4. NPMc attenuates Arf-induced sumoylation of NPM and Mdm2. (A) Cells expressing GFP alone (control lanes 1–4), Flag-NPM (lanes 5–8), or Flag NPMc (lanes 9–12) were trans-duced with a vector encoding His6-Sumo-1 (even numbered lanes) and induced 48 hours later with zinc. Induction of $p19^{Arf}$ triggered Mdm2 expression (lanes 3, 4, 7, 8) which was attenuated by NPMc (lanes 11, 12). (B) The same lysates were affinity-purified on nickel-agarose in the presence of 8M urea, and sumoylated proteins recovered by elution with imidazole were sepa-rated on denaturing gels and blotted with antibodies to the Flag epitope, the wild-type NPM C-terminus, or Mdm2 as indicated at the left.

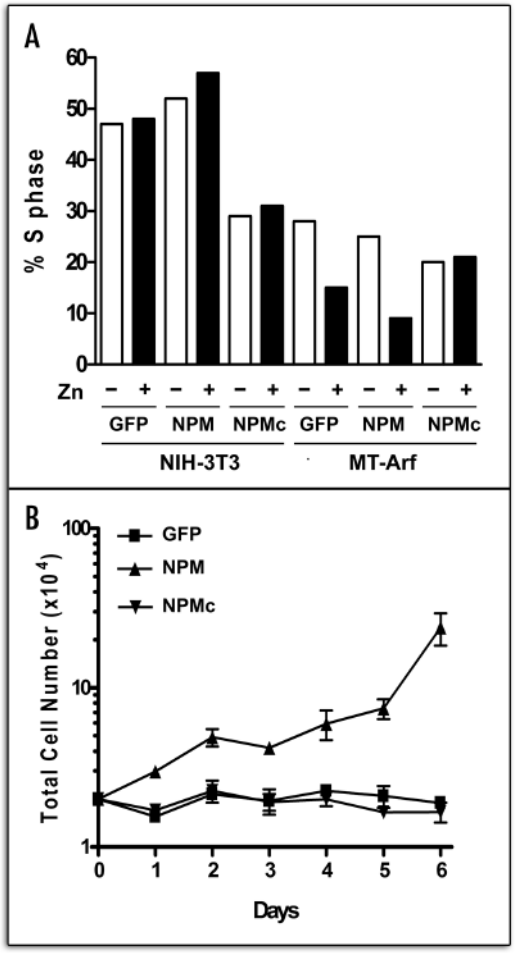

Figure 5. NPMc acutely blocks Arf's effects on S-phase entry but does not rescue premature se-nescence of Atm-null cells. (A) NIH-3T3 or MT-Arf cells engineered to express NPM or NPMc were treated with zinc, and their DNA content was measured by flow cytometry 24 hours after Arf induction. The percentage of the population in S-phase (DNA content between 2N and 4N) is indicated on the ordinate. (B) Early passage *Atm*-null MEFs were seeded at 2 x 10^4 in replica 60 mm-diameter culture dishes, and cells were enumerated daily. These cells have a very limited proliferative potential due to acquisition of DNA damage and activation of the p53 check-point.[29,40] Infection with a retrovirus encoding NPM, but not NPMc, induced proliferation.

wild type NPM protein provided *Atm*-null MEFs with a proliferative advantage (Fig. 5B). This experiment argues that, despite the ability of NPMc to inhibit $p19^{Arf}$ functions in short-term assays, the mutant protein lacks a proliferation-promoting function exhibited by its wild type counterpart.

DISCUSSION

AML-associated mutations that affect the NPM C-terminus create a novel NES that leads to increased Crm1-dependent export of the altered protein to the cytoplasm. NPM normally exists as a homo-oligomer, and its multimerization and interaction with p19Arf do not depend upon its C-terminal domain.[3,13] Indeed, a natural NPM isoform (B23.2) formed by alternative splicing of 3′ exons, has a substitution of two amino acids for the C-terminal[37] residues of NPM; this altered protein still predominately localizes to nucleoli, forms hetero-oligomers with full-length NPM, and continues to shuttle between the nucleus and cytoplasm. Therefore, the addition of the new NES in NPMc is responsible for its altered topology. Based on these considerations, it is not surprising that, when expressed at levels comparable to those of endogenous NPM, the NPMc protein behaves in a dominant manner to form complexes with and relocalize its endogenous counterpart from the nucleolus to the cytoplasm. Moreover, because the $p19^{Arf}$ protein is stoichiometrically bound to NPM, it too is carried into the cytoplasm in complexes with NPMc.

Relocalization of $p19^{Arf}$ attenuates its functions. When Arf was induced in the presence of NPMc, its ability to elicit a p53-dependent transcriptional response was diminished. Under these circumstances, the resulting levels of the canonical p53-responsive gene products, Mdm2 and $p21^{Cip1}$, were reduced, and the ability of $p19^{Arf}$ to limit the accumulation of cells in S-phase was compromised. We now appreciate that $p19^{Arf}$ interacts with at least two distinct E3 ligases, Mdm2 and Arf-BP1 (Mule/Hect9), to prevent their ubiquitination of p53.[41-43] As each of these ligases is normally found within the nucleus, relocalization of $p19^{Arf}$ to the cytoplasm by NPMc likely prevents these interactions and blunts Arf-mediated induction of the p53 response. Expression of NPMc also limited the ability of $p19^{Arf}$ to induce the sumoylation of Mdm2 and NPM. Because Arf-induced sumoylation appears to require the physical association of $p19^{Arf}$ with its targets, these findings provide some additional evidence that the interaction between Arf and Mdm2 was compromised. However, although p19Arf associates with NPMc and, in conjunction, with endogenous NPM in the cytoplasm, it did not stimulate their sumoylation under these circumstances. We therefore suspect that additional components required for the proper function of the sumoylation cascade were not mobilized with $p19^{Arf}$ to the cytoplasm.

Because Arf acts as a potent tumor suppressor, it is tempting to speculate that abrogation of its functions by NPMc contributes to the genesis of AML. However, the effects of NPMc in cells containing or lacking *Arf* activity were different. In immortalized *Arf*-null NIH-3T3 cells, NPMc inhibited S phase entry. If, as is likely, NPM plays an obligatory role in promoting cell proliferation by properly regulating centrosome duplication and thereby safeguarding genome integrity,[7] then its chronic inactivation by NPMc should induce checkpoint responses through Atm/Atr-based signaling pathways. To test the effects of NPM on the long term proliferation of primary cells, we introduced the wild type or mutant NPM genes into early passage *Atm*-null primary MEFs, whose premature senescence can be rescued either by *Arf* inactivation[29] or p53 loss.[40] In this setting, the enforced expression of wild type NPM endowed the cells with an increased proliferative potential, whereas NPMc had no effect. Since NPMc also interferes with the localization of endogenous NPM, we infer that its ability to attenuate Arf signaling is counterbalanced by inactivation of wild type NPM. Thus, NPMc may well contribute to myeloid malignancy by interfering with essential functions of wild type NPM.

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