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ORIGINAL ARTICLE

Nucleophosmin/B26 regulates PTEN through interaction with HAUSP in acute myeloid leukemia

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PTEN (phosphatase and tensin homolog deleted in chromosome 10) is a *bona fide* dual lipid and protein phosphatase with cytoplasmic (Cy) and nuclear localization. PTEN nuclear exclusion has been associated with tumorigenesis. Nucleophosmin (*NPM1*) is frequently mutated in acute myeloid leukemia (AML) and displays Cy localization in mutated nucleophosmin (NPMc+) AML. Here we show that NPM1 directly interacts with herpes virus-associated ubiquitin specific protease (HAUSP), which is known as a PTEN deubiquitinating enzyme. Strikingly, PTEN is aberrantly localized in AML carrying NPMc+. Mechanistically, NPM1 in the nucleus opposes HAUSP-mediated deubiquitination and this promotes the shuttle of PTEN to the cytoplasm. In the cytoplasm, NPMc+ prevents HAUSP from deubiquitinating PTEN, causing the latter to stay in the cytoplasm where it is polyubiquitinated and degraded. Our findings delineate a new NPM1–HAUSP molecular interaction controlling PTEN deubiquitination and trafficking.

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Keywords: PTEN; suppressor gene; Hausp; acute myeloid leukemia

INTRODUCTION

PTEN (phosphatase and tensin homolog deleted in chromosome 10) is a dual lipid and protein phosphatase with cytoplasmatic and nuclear localization. As an integrant of signal transduction networks operating in human cells, PTEN exerts diverse functions according to its conformational status and subcellular localization.[1–5] *PTEN*-inactivating somatic mutations have been described in several solid tumors[6–9] but they are not common in patients with acute myeloid leukemia (AML).[10–12] Alternative mechanisms leading to a modulation of anti-oncogenic control by PTEN at either the genetic or epigenetic level appear to be relevant; among them, the regulation of subcellular localization seems to have a major role.[1,3,4,13,14]

Nuclear PTEN is essential for tumor suppression and its nuclear import is mediated by linkage to a single molecule of ubiquitin (Ub).[15,16] This reaction, mediated by E3 Ub–protein ligase NEDD4-1 or other E3 ligases,[17–19] enables PTEN shuttling and leads either to PTEN nuclear import or, alternatively, to the addition of multiple Ub molecules, which can lead to proteasomal degradation.[16]

Approximately one third of cases of AML are characterized by an aberrant localization of the nucleophosmin (NPM1) protein due to different mutations in exon 12 of the gene, all invariably leading to the loss of a nucleolar localization signal and the acquisition of a novel cytoplasmic (Cy) export signal in the NPM1 protein.[20–23]

In this study, through assessing PTEN localization in a large number of primary AML samples we demonstrate a strong association between AML with mutated nucleophosmin (NPMc+) and PTEN nuclear depletion. We demonstrate that NPMc+ physically interacts with herpes virus-associated ubiquitin specific protease (HAUSP) and prevents it from deubiquitinating PTEN, causing the latter to shuttle to the cytoplasm where it is polyubiquitinated and degraded.

MATERIALS AND METHODS

Samples

Between July 2005 and June 2009, bone marrow samples from 102 consecutive patients with *de novo* AML, showing at least 70% leukemic infiltration, were collected when they presented at the Department of Hematology of the University of Rome Tor Vergata. A written informed consent was obtained from all patients.

Total RNA was extracted from Ficoll–Hypaque isolated mononuclear cells using the method of Chomczynsky and Sacchi. RNA was reverse-transcribed using random hexamer primers.

AML case tissues were characterized molecularly for *BCR–ABL*, *CBFB–MHY11*, *RUNX1/–RUNX1T1*, *DEK–CAN* fusion genes and for *NPM1* and *FLT3–ITD* (Fms-related tyrosine kinase 3 - Internal tandem duplication) mutations using methods reported elsewhere.[24–26]

Cell cultures

OCI–AML3 cells (carrying the exon-12 *NPM1* mutation A) and OCI-AML2, cells (kindly provided by Alicja Gruzcka, European Institute of Oncology, Milan) were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in α-minimal essential medium eagle (GIBCO-BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (GIBCO-BRL), 20 mM Hepes, 100 μg/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL).

The *PTEN*-deficient prostate cancer cell line (PC-3) was cultured as a monolayer in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mmol/l L-glutamine and 10 mmol/l HEPES.

The HEK293T human embryonal kidney cell line was cultured as a monolayer in Dulbecco's modified eagle medium containing 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin.

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Immunofluorescence assays

AML samples (cytocentrifuge preparations of Ficoll–Hypaque isolated cells, or directly diagnostic bone marrow smears) were fixed with methanol (5 min) and acetone (kept at −20 °C, 10 min) and washed in phosphate-bufferred saline (PBS). Slides were incubated overnight with the primary antibody, anti-PTEN (diluted 1:100 with PBS + 1% bovine serum albumin (BSA; clone 6H2.1, Cascade Bioscience Inc., Winchester, MA, USA) following two PBS washes and incubated for 2 h with the fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (diluted 1:20 with PBS + 1% BSA). After nuclear counterstaining with 4′,6-diamidino-2-phenylindole, slides were coverslipped with mounting medium and visualized using an Olympus BX61 fluorescent microscope equipped with a CoolSNAP EZ camera (Photometrics, Tucson, AZ, USA).

At least 100 cells were analyzed from each patient. The samples in which more than 70% cells had PTEN mainly in the cytoplasm were considered as representing patients with Cy–PTEN.

PC-3 cells were seeded on coverglass supports in complete medium; OCI–AML3 cell lines were prepared using a cytocentrifuge. Cells fixed with 4% paraformaldehyde were permeabilized in PBS-containing 0.1% Nonidet P-40 and blocked in 2% BSA. Slides were incubated overnight with the primary antibody following two PBS washes and incubated for 2 h with the secondary antibodies: Invitrogen Alexa Fluor 555-labeled goat anti-mouse and Invitrogen Alexa Fluor 488-labeled goat anti-rabbit (diluted 1:400 with PBS + 2% BSA) (Eugen, OR, USA).

PTEN was detected using an anti-PTEN antibody (clone 6H2.1, Cascade Bioscience Inc.) diluted 1:100 with PBS + 2% BSA. The nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole for 5 min in PBS. Finally, cells were rinsed and mounted in Fluoromount (Sigma-Aldrich, St Louis, MO USA). Images were acquired using a Zeiss LSM 700 (Jena, Germany) confocal laser scanning microscope.

Transfection

PC-3 cells were transfected with combinations of green fluorescent protein (GFP)–PTEN (0.1 μg), FLAG–NPM wild-type (NPMwt; 0.3 μg) and FLAG–NPMc + (0.5 μg). Immunofluorescence and western blot assays of subcellular fractions were performed after 48 h. Nuclear/Cy fractionation was carried out as described previously.[15]

For competition assays, HEK293T cells were transfected with increasing quantities of NPMc + (0.1, 0.3, 0.6, 0.9 μg). or combinations of FLAG–NPMc + (0.3 μg) and FLAG–NPMwt (0.1, 0.3, 0.6, 0.9 μg). After 48 h transfection, the lysates were subjected to co-immunoprecipitation (IP) and western blot analysis.

Immunoprecipitation

Total extracts were used for IP assays. Briefly, 1 mg of extract was incubated for 4 h at 4 °C with 4 μg of antibody (anti-mouse T26 specific to NPMc +) (Gruszka *et al.*[27]) and NPMwt (anti-mouse specific to NPMwt, Invitrogen ♯32–5200) and subsequently for 45 min at 4 °C with Dynabeads Protein G (Invitrogen, Dynal AS, Oslo, Norway). Samples were then blotted with anti-HAUSP polyclonal antibodies (MBL International, Woburn, MA, USA).

Western blot analysis

Cell pellets were resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 250 μM orthovanadate, 20 mM β-glycerophosphate and protease inhibitors (Sigma-Aldrich). Lysates were centrifuged at 10 000 *g* for 15 min at 4 °C and supernatants were stored at −80 °C. Twenty microgram aliquots of proteins were resuspended in a reducing Laemmli buffer (with β-mercaptoethanol) and loaded onto a 10% polyacrylamide gel, then transferred to nitrocellulose membrane. After blocking with 5% milk (Fluka, Sigma-Aldrich), the membranes were incubated with specific antibodies. Horseradish peroxidase-conjugated immunoglobulin G (IgG) preparations were used as secondary antibodies and the enhanced chemiluminescence procedure was employed for development (enhanced chemiluminescence kits, Amersham, Buckinghamshire, UK).

The autoradiograms obtained were scanned and exported for densitometry analysis. Protein signal intensities were measured using Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA). Signal quantity was normalized against the unrelated proteins β-actin (Cell Signaling Technology, Beverley, MA, USA), and α-tubulin (Abcam plc, Cambridge, UK).

Ubiquitination assay

HEK293T cells were co-transfected with GFP–PTEN, FLAG–NPMc + and HA–Ub or with GFP–PTEN, FLAG–NPMwt and HA–Ub, and 24 h later were treated with MG132 (Sigma-Aldrich, St Louis, MO, USA) for 4 h. HEK293T cells were IP with anti-GFP (Invitrogen, A11122) and analyzed for ubiquitination levels with anti-HA (Cell Signaling, C29F4) and anti-PTEN antibodies.

In vitro translation assay

A TNT T7 Quick PCR DNA transcription/translation kit (Promega, Madison, WI, USA) was used for HAUSP expression of PCR-generated DNA templates (primers for amplification of HAUSP full length: forward 5′–GGATCCTA ATACGACTCACTATAGGGAACAGCCACCATGAACCACCAGCAGCAGCAGCAGC AGCAGA–3′; reverse 5′–T(30)TCAGTTATGGATTTTAATGGCCTTTTC–3′). Precipitates were electrophoresed using SDS-polyacrylamide gel electrophoresis and blotted with antibodies against HAUSP and glutathione S-transferase (GST).

Förster resonance energy transfer assay

Förster resonance energy transfer (FRET) assay was performed by standard acceptor photobleaching confocal microscopy techniques using Cy3–Cy5 as a FRET pair and an indirect double immunofluorescence approach was used to identify physical interaction *in vivo* between NPM1wt and HAUSP proteins. HEK293T cells were seeded on coated glass coverslips and transfected with NPMc + . As controls, untransfected HEK293T cells were utilized. Cells were processed for double indirect immunofluorescence procedures, as described above. As fluorochromes for FRET detection, donkey anti-mouse IgG Cy3-conjugated and donkey anti-rabbit IgG Cy5-conjugated antibodies or donkey anti-mouse IgG Cy5-conjugated and donkey anti-rabbit IgG Cy3-conjugated antibodies were used (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). FRET studies were performed on a confocal microscope (TCS SP5, Leica Microsystems GmbH Wetzlar, Germany) using the implemented FRET acceptor photobleaching wizard. Acquisition settings were: objective Plan-Apochromat 63 × /1.4 NA oil immersion, pinhole 2 Airy units, format size 512 × 512. Pre- and postbleaching images were recorded serially by excitation of the Cy3-conjugated antibody at 543 nm (donor channel) with a helium–neon laser, and of the Cy5-conjugated antibody at 633 nm (acceptor channel) with a helium–neon laser. Low laser intensities were used to avoid bleaching effects during acquisition. Selections of cells were made by observations through the donor channel. The acceptor was bleached with high intensity (100%) power of the 633 nm laser line for 30 iterations. This iteration time was found to be effective for bleaching Cy5 fluorochrome in pilot experiments. The change in the fluorescence intensity between post- and prebleaching donor values, efficiency (E), was calculated using the formula E = (donor after bleaching − donor before bleaching) × 100/(donor after bleaching) and is shown as a percentage. Pseudocolored images showing FRET E values were also generated.

We analyzed 12 fields containing a variable number of cells from three independent experiments of NPM1–HAUSP double immunofluorescence. To confirm the validity of the FRET data, internal acquisition controls and external control experiments were performed. In the internal acquisition controls, region of interest were placed on donor regions that were unbleached. This was to verify that donor fluorescence values increased as a result of acceptor photobleaching, and that the increase was not caused by fluctuations in the imaging process (for example, by changes in the focal plane). In addition, in every acquisition field, a bleached region of interest was placed outside the cells. Field images that showed positive FRET values in the internal controls were discarded. As external FRET controls, additional groups containing cells immunoreacted with different combinations of primary and secondary antibodies were used as follows: 1 cells incubated with the NPMwt primary antibody alone (Invitrogen ♯32–5200) and subsequently incubated with both donkey anti-Cy5- and anti-Cy3-conjugated secondary antibodies (negative controls); 2 cells incubated with the HAUSP primary antibody alone and subsequently incubated with both Cy5- and Cy3-conjugated secondary antibodies (negative controls); and 3 cells incubated only with secondary antibodies (negative controls). The latter three control sets were used to identify eventual cross-reactions between secondary antibodies. All the controls were analyzed with the same setting as that used to analyze NPMwt–HAUSP immunostained cells. The cells were first studied visually to examine the localization and the co-expression of both NPM and HAUSP proteins. Only cells displaying a healthy appearance, a regular nuclear pattern (examined in the 4′,6-diamidino-2-phenylindole channel), and a

medium-high fluorescence intensity of both donor and acceptor were selected for FRET examination. For each cell, three compartments were analyzed: nucleus, nucleolus and cytoplasm.

Statistical analysis

Statistical analysis was done using Fisher's Exact Test using the 2×2 contingency.

RESULTS

To study PTEN localization in leukemia, we collected diagnostic bone marrow samples from 102 patients with AML. *NPM1* gene mutation analysis revealed that 34 cases were mutated and 68 were wild type. Among the 102 AML samples, 46 (45%) had cytoplasmic PTEN (Figures 1a and b). Based on *NPM1* status, cytoplasmic PTEN was detected in 21 of 68 (31%) cases with *NPMwt* and in 25 of 34 (74%) cases with NPMc+ ($P<0.0001$) (Figure 1c). Hence *NPM1* mutations strikingly correlated with PTEN nuclear depletion. Normal CD34+ cells show both nuclear and cytoplasmic localization of PTEN (Figure 1d).

No association was found between PTEN subcellular localization and other molecular AML subsets (Table 1) with the exception of cases of acute promyelocytic leukemia, bearing a PML–RAR-α translocation in which PTEN was predominantly detected in

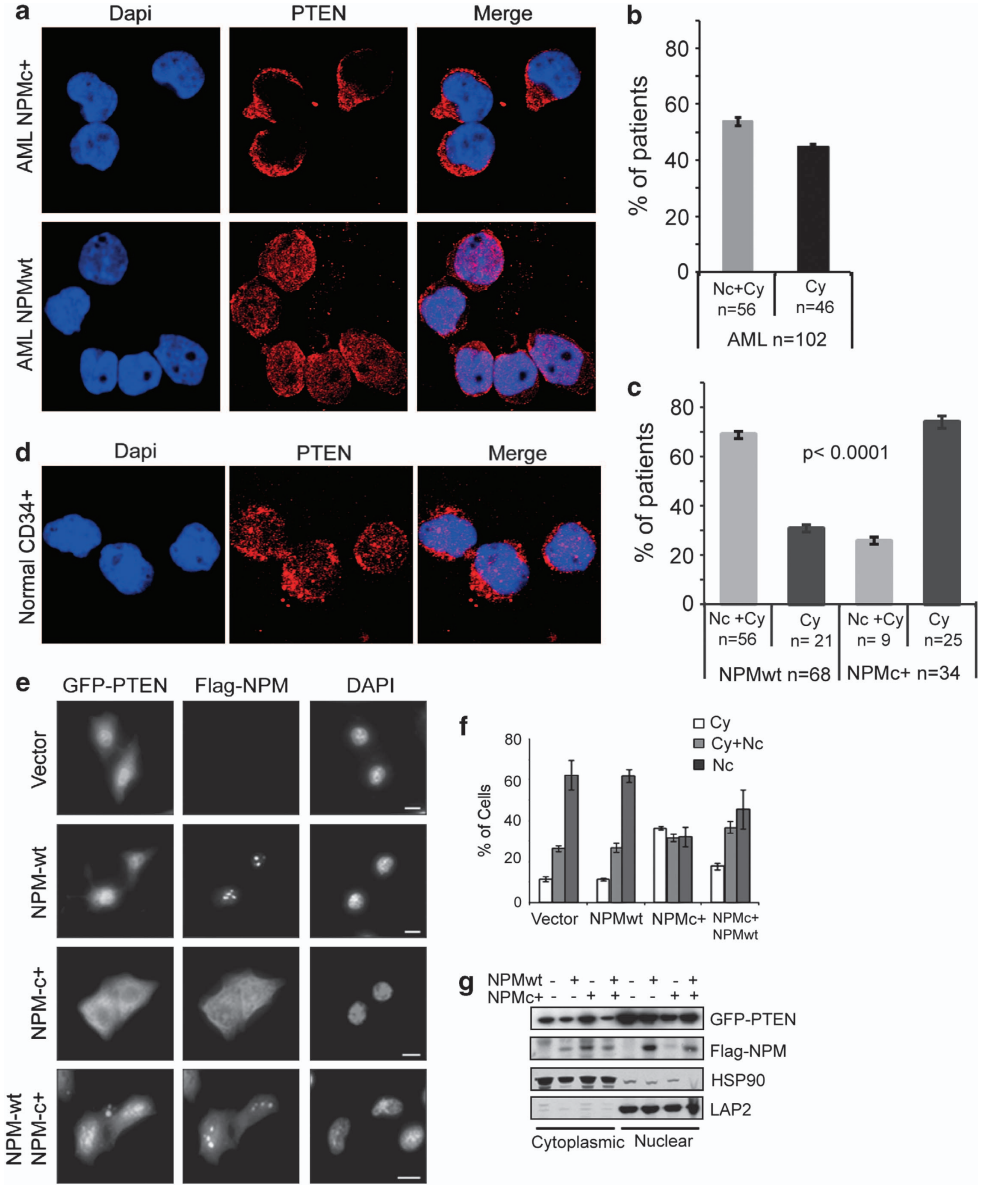

Figure 1. PTEN localization in NPMwt and NPMc+ AML patients and cell lines. (**a**) Representative images of AML bone marrow showing cytoplasmic (AML–NPMc+) or nuclear and cytoplasmic PTEN localization (AML–NPMwt). (**b**) Microscopy analysis of PTEN localization in 102 AML patients show 56 (55%) are nuclear and cytoplasmic (Cy) PTEN (Nc+Cy) and 46 (45%) are Cy–PTEN. In all patients, at least 100 cells were analyzed. (**c**) Distribution of PTEN localization in AML patients according to NPM1 gene status. (**d**) CD34+ stem cells show nuclear and cytoplasmic localization of PTEN. (**e**) Analysis of GFP–PTEN fusion protein, wild-type NPM–FLAG and NPMc+–FLAG fusion proteins transfected in PTEN-deficient prostate cancer cell line PC-3. GFP–PTEN is mainly in the nucleus when transfected alone or with wild-type NPM–FLAG. With NPMc+–FLAG fusion protein the GFP–PTEN green fluorescence is depleted from the nucleus and increased in the cytoplasm. (**f**) Cell count shows that the percentage of elements with predominantly Cy–PTEN is directly proportional to NPMc+ expression, and that there is a quantitative competition of the NPMwt versus NPMc+ protein. (**g**) Immunoblot analysis of subcellular fractions showing the effect of mutated FLAG–NPMc+ on GFP–PTEN localization.

Table 1. Molecular characterization of patients studied

	Nuclear exclusion of PTEN	*Nuclear and cytoplasmatic PTEN*
RUNX1/ − RUNX1T1	1	3
Inv 16	1	2
Dek/Can	1	1
Negative for the above alterations	41	47
Not valuable	2	3
FLT3/ITD +	12	9
FLT3/ITD −	30	44
Not valuable	4	3

Abbreviations: AML, acute myeloid leukemia; PTEN, phosphatase and tensin homolog deleted in chromosome 10; RUNX1/ − RUNX1T1, run related transcription factor 1/runt-related transcription factor 1; translocated to, 1.

the cytoplasm, as reported previously.[15] To determine whether mutant NPM1 affects PTEN localization *in vitro*, we transfected the *PTEN*-deficient prostate cancer cell line PC-3 with combinations of three different constructs expressing GFP–PTEN, FLAG–NPMwt and FLAG–NPMc + . After 48 h, GFP–PTEN was found mainly in the nucleus when transfected alone or with FLAG–NPMwt, whereas in the presence of mutated FLAG–NPMc + , nuclear GFP–PTEN was barely detectable and its localization was mainly cytoplasmic (Figures 1e and f).

Co-expression of both mutated and wild-type FLAG–NPM1 still led to PTEN delocalization but in a somewhat minor fashion, suggesting interference between the two molecules. Western blot analysis of subcellular fractions confirmed both the effect of mutated FLAG–NPMc + on GFP–PTEN localization and the competitive effect of FLAG–NPMwt (Figure 1g). We also would like to point out that Npmwt localizes mainly to the nucleus, whereas its mutated form has been found predominantly in the cytoplasmic fractionation. In addition, we would suggest that the

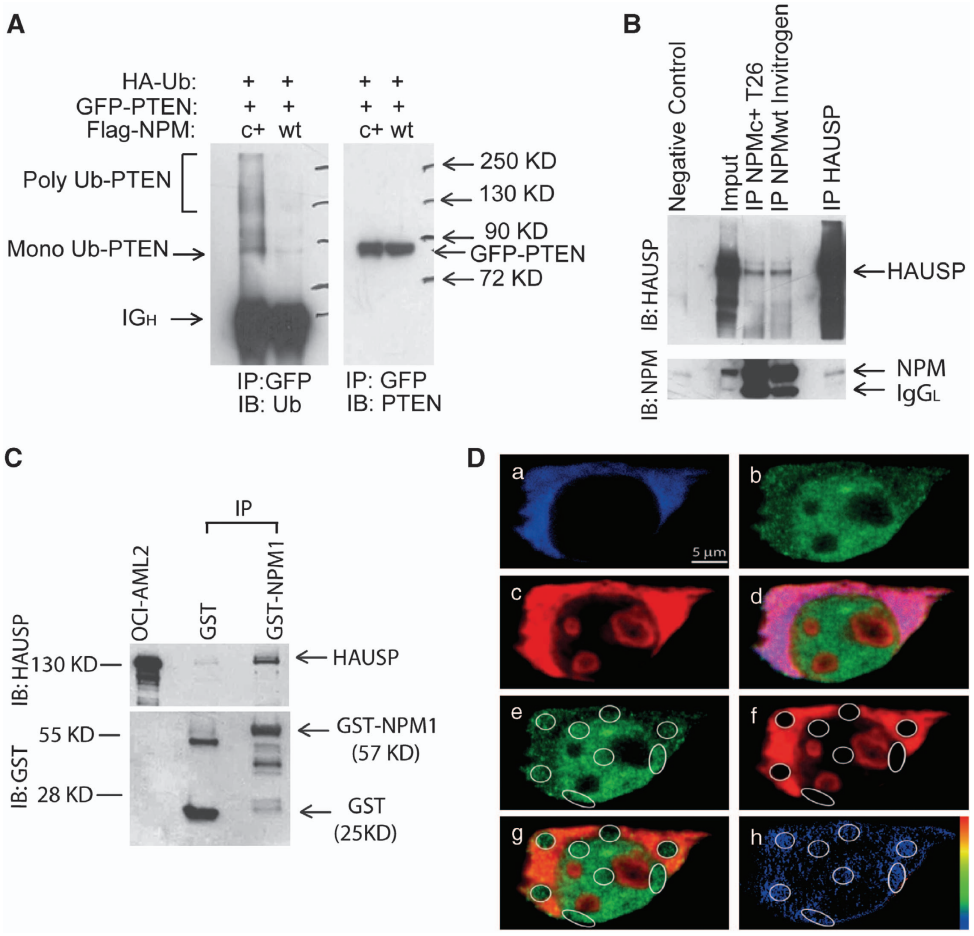

Figure 2. Interaction between NPM and HAUSP. (**A**) Lysates from HEK293T cells co-transfected with GFP–PTEN, FLAG–NPMc + and HA–Ub or with GFP–PTEN, FLAG–NPMwt and HA–Ub were IP with anti-GFP (lanes 1 and 2) and analyzed for ubiquitination levels. The lowest Ub–PTEN band is non-specific reactivity (represented as asterisks) and the upper one is monoubiquitinated PTEN. (**B**) Total lysates fractions of OCI–AML3 cells were IP with anti NPMc + T26, anti NPMwt or anti Hausp and analyzed with antibodies anti Hausp and anti NPM. (**C**) GST pulldown assay on *in vitro*-translated HAUSP incubated with equivalent amounts of GST and GST–NPM1 beads. A TNT T7 Quick PCR DNA transcription/translation kit (Promega) was used for HAUSP expression of PCR-generated DNA templates. Precipitated were electrophoresed by SDS-polyacrylamide gel electrophoresis and blotted with antibodies against Hausp and GST. (**D**) Confocal microscopy images of HEK293T cells immunolabeled for NPMwt (Cy5, red, acceptor channel) and HAUSP (Cy3, green, donor channel) for a FRET acceptor photobleaching assay. Bleached regions are indicated by the white ellipses. (a) NPMc + , trasfected cell, before bleaching. (b) HAUSP, before bleaching. (c) NPMwt, before bleaching. (d) Merge, NPMc + /HAUSP/NPMwt before bleaching. (e) Hausp, donor channel, after bleaching. (f) NPMwt, acceptor channel, after bleaching. (g) Merged HAUSP/NPMwt channels after bleaching. (h) Pseudocolored image showing a FRET E values map. Scale bars = 5 mm.

lower expression of Npmc + is likely due to the reduction of its protein stability as we transfected the respective plasmids with equal amounts.

To evaluate a possible physical interaction between NPM1 and PTEN, co-IP experiments were carried out. No interaction was detected between NPMwt or NPMc + and PTEN (data not shown).

Nuclear import of cytoplasmic PTEN is accelerated by mono-ubiquitination on lysine residue 13 or 289.[15] In acute promyelocytic leukemia cells, PTEN predominantly localizes in the cytoplasm due to its deubiquitination by HAUSP.[15] An *in vitro* ubiquitination assay revealed that the levels of mono- and polyubiquitinated PTEN were higher in FLAG–NPMc + -transfected HEK293T cells than in FLAG–NPMwt-transfected cells (Figure 2A). Co-IP analysis demonstrated a physical interaction between HAUSP and both NPMc + (anti-mouse T26 specific to NPMc +)[27] and NPMwt (anti-mouse specific to NPMwt, Invitrogen ♯32–5200) (Figure 2B). IP was also performed using IgG as a negative control, obtaining the same results (data not shown). In addition, molecular interaction analysis *in situ* by Förster resonance energy transfer (FRET) analysis demonstrated energy transfer in the FRET pair Cy5–Cy3, indicative of a physical direct association between HAUSP and NPM1 in OCI–AML3 cells. This interaction was detectable in both the nucleoplasm and the cytoplasm but not in the nucleolus where HAUSP is nearly absent (data not shown). Furthermore, *in vitro* binding experiments using a bacterially expressed GST-fused NPM1 protein and *in vitro*-translated HAUSP

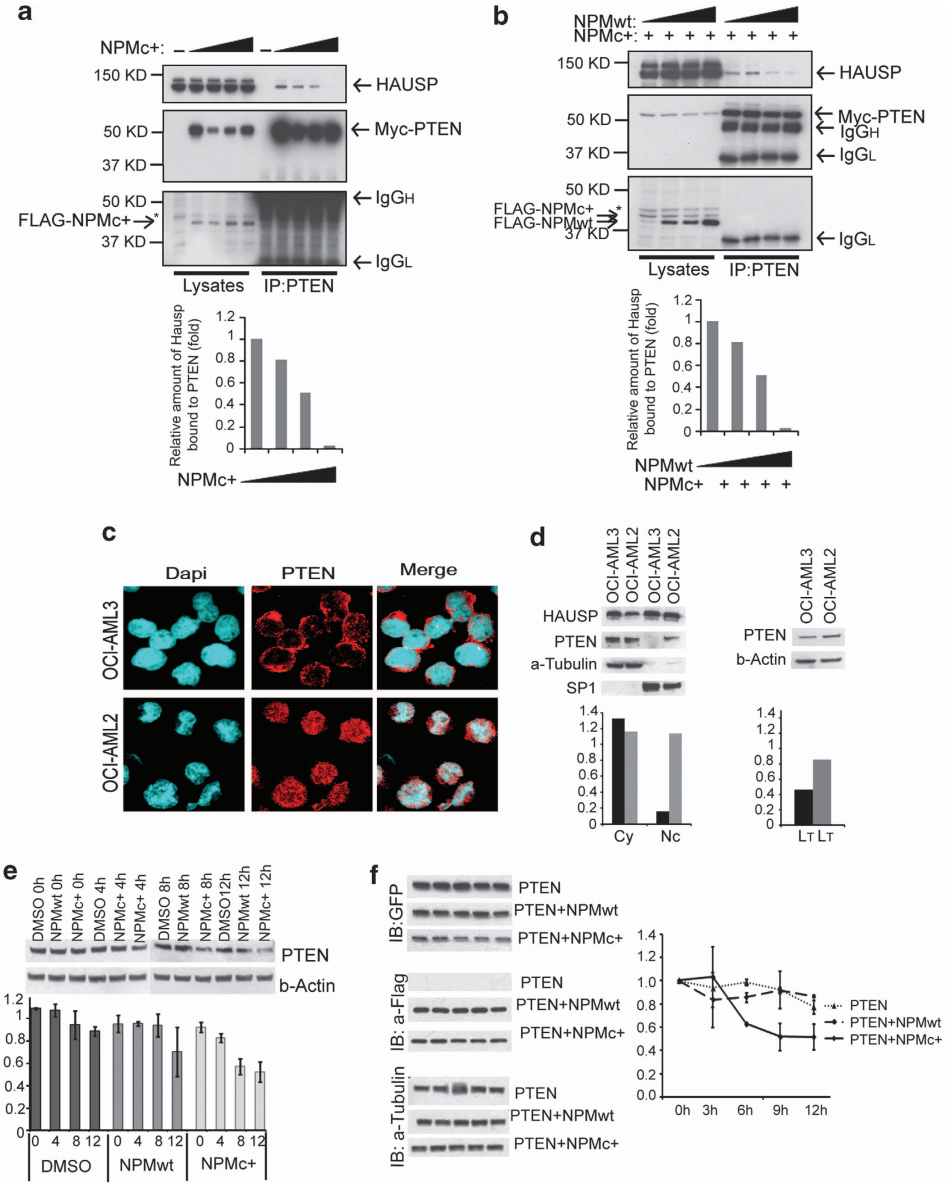

Figure 3. Regulation of PTEN localization and stability (polyubiquitination) by NPMc + . (**a**) HEK293T cells transfected with a vector control or increasing quantities of FLAG–NPMc + . As the NPMc + protein level increased, less HAUSP was pulled down together with PTEN. (**b**) HEK293T cells transfected with FLAG–NPMc + and increasing quantities of FLAG–NPMwt. NPMwt antagonized the PTEN–HAUSP molecular interaction in a quantitative fashion. The asterisks represent non-specific reactivity. (**c**) Immunofluorescence of PTEN in OCI–AML3 cells bearing NPMc + and in NPMwt-expressing OCI-AML2 cells. (**d**) Immunoblot of PTEN in OCI–AML3 cells bearing NPMc + and in NPMwt-expressing OCI-AML2 cells. We can observe that HAUSP is localized in both nucleus and cytoplasm. Cytoplasmic (Cy), Nuclear (Nc), Lysate total (L_T). (**e**) HEK293T cells transfected with NPMc + treated with CHX over 12 h. NPMc + enhanced PTEN degradation. Error bar correspond to analysis of two separated experiment. (**f**) HEK293T cells where co-transfected and treated with CHX using Gfp–PTEN alone and in combination with Flag–NPMwt or Flag–NPMc + . Error bar correspond to analysis of two separated experiment.

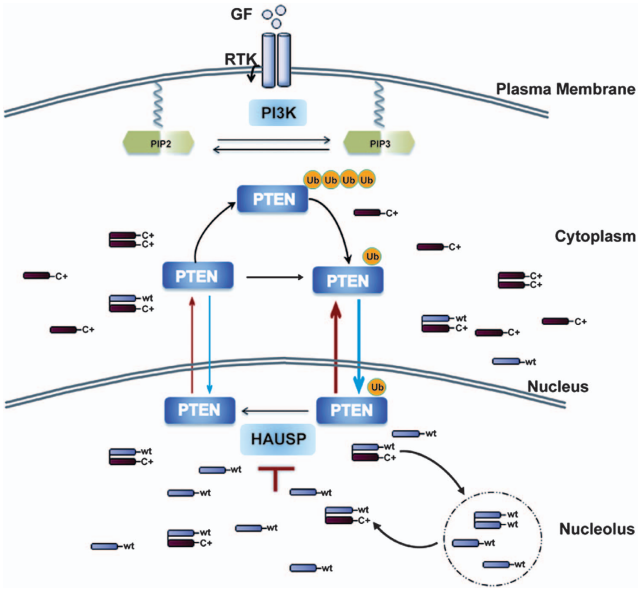

Figure 4. Proposed model of the effects of NPM1–HAUSP interaction on PTEN trafficking. NPMc+ displaces NPMwt from the nucleolus, and in the nucleoplasm NPMwt interacts directly with HAUSP, thereby preventing PTEN deubiquitination and its nuclear accumulation. In the cytoplasm, NPMc+ interacts with HAUSP and prevents PTEN deubiquitination and favors its polyubiquitination, which in turn results in cytoplasmic retention, loss of function and degradation.

revealed that NPM1 binds to HAUSP, corroborating the notion that the two proteins interact directly (Figure 2C).

The lack of an anchoring motif in NPMc+ drives most of the protein away from the nucleolus and, notably, the NPMwt counterpart follows.[21] In human embryonic kidney HEK293T cells transfected with a construct expressing NPMc+, we demonstrated an increase in energy transfer between NPMwt and HAUSP in both nucleus and cytoplasm (transfected $E = 8.38 \pm 1.17\%$; untransfected $E = 2.55 \pm 0.58\%$) (Figure 2D). Based on these findings, we hypothesize that both NPM1 and its mutant counterpart can bind HAUSP, thereby preventing PTEN deubiquitination in distinct compartments. Indeed, by HEK293T cells co-transfection with a construct expressing either NPMwt or NPMc+, we found a dose-dependent inhibition on the PTEN–HAUSP interaction, indicating molecular competition between HAUSP and both NPM species toward PTEN (Figures 3a and b). Finally, we found that OCI–AML3 NPMc+-expressing cells not only have less PTEN in the nucleus (Figures 3c and d) but also have a smaller overall quantity of the protein compared with OCI-AML2 NPMwt cells (Figure 3d). We therefore ascertained whether NPMc+ regulates PTEN stability. To this end, we treated NPMc+-transfected HEK293T cells with the inhibitor of protein translation, cycloheximide (CHX) over 12 h. We observed that PTEN stability decreases in cells transfected with NPMc+ (Figure 3e). We also co-transfected and treated with CHX the HEK293T cells using Gfp–PTEN alone and in combination with Flag–NPMwt or Flag–NPMc+. The data showed a clear decrease of PTEN life span in the presence of NPMc+ (Figure 3f).

DISCUSSION

Taken together our findings indicate that, when displaced outside its physiological localization in the nucleolus by NPMc+, a high molar ratio of nuclear NPMwt binds and inhibits HAUSP. This in turn leaves monoubiquitinated PTEN free to return to the cytoplasm. In the cytosol NPMc+ prevents HAUSP from deubiquitinating PTEN and as a consequence polyubiquitinated PTEN is degraded by proteasome activity. This could be explained by the fact that more PTEN ends up in the juxtamembrane zone, either directly linked in a low molecular weight fraction, or in a high molecular weight complex linked to the cellular membrane as a PTEN-associated complex (PAC).[28–30] It has been demonstrated that a location near the cell membrane favors ubiquitination and that PTEN–Ub has diminished phosphatase activity.[31] In keeping with this model, PTEN partitioning shifts decidedly toward the cytoplasm in NPMc+ AML cells, being both degraded and caught in the inactive Ub-linked configuration.

PTEN complete loss in normal hematopoietic stem cells leads to loss of stemness potential; in contrast, when PTEN is lost in a leukemic environment, the number of leukemia-initiating elements rises suggesting that failsafe mechanisms have to be evaded for PTEN loss to be tolerated by the leukemia-initiating cell.[32] PTEN complete inactivation in hematopoietic stem cells would lead to their depletion before they have an opportunity to progress to leukemia. This could explain why PTEN is seldom mutated or deleted in patients with AML.[10–12] On the other hand, a scenario wherein PTEN is depleted from the nucleus and polyubiquitinated in the cytoplasm in NPMc+ AML may be beneficial for maintaining blast cell survival and proliferation, while being much less detrimental to leukemia stem cell maintenance.

NPMc+ AML cells show a unique expression phenotype and usually have a normal karyotype. From a clinical point of view, the mutated protein has been shown to correlate with a less unfavorable prognosis in adult patients with AML.[33–35] In terms of pathogenesis, NPMc+ mutations are commonly regarded as fondant lesions of leukemia, as they confer distinctive biological and clinical features and show great stability during disease evolution and therapy-related clone selection. We propose that PTEN depletion from the nucleus of NPMc+-bearing cells deregulates growth and differentiation control in AML stem cells. Such deregulation differs from a condition of the complete loss of PTEN, in keeping with the notion that PTEN homeostatic control depends on the quantity and status of the protein in a dose-dependent fashion.[36]

The mechanisms regulating PTEN subcellular localization are not completely elucidated. Trotman *et al.*[16] showed that PTEN and Ub co-expression results in a significant acceleration of PTEN import and export rates, suggesting that PTEN ubiquitination may enhance trans-membrane shuttling. They also found that the nuclear resident fraction of PTEN is increased upon Ub overexpression. Notably, based on their findings nuclear PTEN was not quantitatively monoubiquitinated, suggesting that PTEN is deubiquitinated to remain nuclear or else re-exported.

Similar to PML–RAR-α displacing PML from nuclear bodies and affecting HAUSP function,[15] NPMc+ displaces NPMwt from the nucleolus and affects HAUSP function. In the nucleoplasm, NPMwt directly interacts with HAUSP, thereby preventing PTEN deubiquitination and its nuclear accumulation. In the cytoplasm, NPMc+ interacts with HAUSP, prevents PTEN deubiquitination and favors its polyubiquitination, which in turn results in its cytoplasmic retention, along with loss of function and degradation (Figure 4). However, we cannot exclude the possibility that in presense of NPMc+ a HAUSP-independent mechanism which is responsible for PTEN delocalization exists in addition to a HAUSP-dependent mechanism. In conclusion, the fact that aberrant PTEN post-translational modification and compartmentalization is so prevalent in AML suggests that PTEN-targeted therapies aimed at restoring normal PTEN trafficking, modification and abundance could be employed for treating this fatal disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

NIN designed, carried out and analyzed the experiments and co-wrote the manuscript. MSS carried out and analyzed the experiments and contributed in manuscript writing; MD and TO contributed with immunofluorescence analysis of patients samples; KLC, FG and IF carried out the experiments of western blot; FF performed the FRET analysis, analyzed the data and co-wrote the manuscript; GC contributed data analysis, interpretation of results and manuscript writing; LB, EC, and SA critically reviewed the manuscript, and amended the final report; FL-C. and PPP designed the study, supervised the research, co-wrote and edited the manuscript.

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