

Nuclear-Export of PU.1 with Mutated NPM1 Impedes Terminal Monocytic Differentiation of Acute Myeloid Leukemia Cells

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RESULTS

BACKGROUND

Nucleophosmin (NPM1) is the most recurrently mutated gene in de novo acute myeloid leukemia (AML) (~30% of AML)1. Despite being labeled a 'favorable risk' AML, only ~50% of patients with AML containing mutated-NPM1 (mNPM1) exhibit long-term survival 2, due to relapse that is typically resistant to current 2nd or 3rd-line treatments 3. Thus, new treatments are needed. This requires an understanding of how, at the molecular level, mutated-NPM1 confers clonal advantage. Previous discoveries provide important clues: wild-type NPM1 is a nucleolus-enriched phospho-protein that shuttles and chaperones several other proteins involved in chromatin remodeling and ribosome biogenesis 4,5,6. The recurrent mutations found in AML are insertions that produce a reading frame-shift the resulting mutated-NPM1 protein product gains a C-terminal nuclear export sequence (NES) and loses a nucleolar localization sequence 7 thus favoring nuclear-export over import mutated-NPM1 aberrantly accumulates in cytoplasm 1.7; this nuclear-export has been shown to be mediated by a nuclear export protein 'chromosome region maintenance (CRM1, also known as exportin 1 – XPO1)^{4,8}.

Another set of discoveries relates to the timing and positioning of NPM1-mutation in the multi-hit process of leukemogenesis – two different groups have shown that NPM1 mutations are culminating events that occur in committed progenitors. wherein they supplement earlier, founder mutations propagated from the germ line or hematopoietic stem cells (HSC) (e.g., DNMT3A-R882H) 9,10,11. That is, cells with an HSC phenotype isolated from the bone marrows of patients with NPM1-mutated AML, although harboring founder mutations, did not contain mutated NPM1, and did not initiate leukemia in immune-deficient mice, but instead produced multi-lineage engraftment. In contrast, cells with a progenitor phenotype from the bone marrows of the same patients contained both founder and NPM1 mutations and produced differentiation-arrested myeloblasts, that is, leukemic hematopoiesis 9,10,11. These observations created a strong circumstantial link between mutation of NPM1 and myeloid differentiation arrest of committed progenitors. Since progressive differentiation normally terminates exponential expansion of lineage-committed progenitors, failure of differentiation could be a key transforming event, causing persistent proliferation and clonal expansion. Supporting this notion, suppression of NPM1 expression using siRNA renewed differentiation and abrogated leukemia initiating capacity of NPM1-mutated AML cells 12. Since only a few of the hundreds of transcription factors expressed in hematopoietic cells are essential, master drivers of lineage-differentiation, this suggested to us that if mutated-NPM1 causes myeloid differentiation arrest, it may do so by interfering with the function of this master transcription factor circuit. This notion seemed particularly plausible because the master transcription factor drivers of terminal monocyte and granulocyte differentiation fates, PU.1 (SPI1) and CEBPA, are highly expressed in AML cells including leukemia stem cells (leukemia initiating cells), substantially exceeding expression levels in normal HSCs and often even normal committed progenitors 13,14,15,16,17,18. The protein-interactome of NPM1 in AML cells has not be comprehensively analyzed, therefore as an experimental first-step, we used mass-

spectrometry to comprehensively analyze the protein interactions of endogenous

NPM1 in AML cell nuclear and cytoplasmic fractions. The cell fate and potential

translational implications of observed interactions of NPM1/mutated-NPM1 were

protease inhibitors, 10% NP-40 was added to cell suspensions to break the cell membrane. Cell suspensions were centrifuged and supernatant was transferred to clean tubes and labeled as the cytoplasmic fraction. Nuclear pellets were treated with Benzonase and proteins were extracted with extraction buffer contained 5% NP-40_500mM_NaCl_5mM_dithiothreitol_and protease_inhibitor_cocktail_ The same extraction process was repeated two more time with 300 µL and 200 µL of extraction buffer with 150mM NaCl. The supernatant containing nuclear proteins was combined and transferred to clean tubes, and protein concentration was determined by BCA assay. Covalent bound antibody to protein G beads. Mouse anti-NPM1 (SCBT, SC-47725) and control IgG

were covalently coupled to Sepharose-protein A/G (SCBT, sc-2003) beads using dimethylpimelimidate (Sigma-Aldrich, D8388) Immunoprecipitation. Nuclear protein extracts were transferred to tubes with antibody bound protein

A/G beads and rocked gently at 4°C overnight. Nonspecific bound proteins were removed with 5 washes of 1X PBS containing 1% NP-40. Immunoprecipitation products were extracted from the protein A/G beads using Laemmli sample buffer Immunofluorescence analysis. Cells were cyto-spanned on to glass slides, and fixed in cold methanol

for 30min at -20°C. The fixed cells were blocked in 10% goat serum for 1 hour at room temperature, then incubated overnight at 4°C with the primary antibodies. Cells were washed in 1% goat serum with 0.1% Tween 20 and followed by incubation with secondary antibodies. Nuclei of cells were stained with fluorescence mounting medium (DAKO) containing DAPI (4',6-diamidino-2-phenylindole). Images were taken with Nikon Eclipse 400 microscope

Flow Cytometry Analysis. Cells were collected and washed with phosphate-buffered saline, and a total of 5×105 cells were stained with monoclonal mouse anti-human CD14-pycoerythrin- (clone: M5E2, cat. no. 301850; 1:100) and monoclonal rabbit anti-human CD11b-fluoroscein isothiocyanate-conjugated (clone: M1/70, cat. no. 101206: 1:100) antibodies (Biolegend, San Diago, CA.). The cells were incubated for 15 min at 4°C and then analyzed in a FC500 with data analysis using program CFX (Beckman Coulter Inc, Brea CA).

Protein identification by LC-MS/MS. Anti-NPM1 and isotype antibody immunoprecipitation products were subjected to SDS-polyacrylamide gel electrophoresis and stained with colloidal Coomassie Blue (Gel Code Blue, Pierce Chemical). Gel slices were excised from the top to the bottom of the lane: proteins were reduced with dithiothreital. (Sigma-Aldrich, D0632, 10mM), alkylated with iodoacetamide (Sigma-Aldrich, I1149, 55mM), and digested in situ with trypsin. Peptides were extracted from gel pieces 3 times using 60% acetonitrile and 0.1% formic acid/water. The dried tryptic peptide mixture was redissolved in 20 µL of 1% formic acid for mass spectrometric analysis. Tryptic peptide mixtures were analyzed by on-line LC-coupled tandem mass spectrometry (LC-MS/MS) on an Orbitrap mass

spectrometer (Theomo Fisher Scientific). Database Search and Data Validation. Mascot Daemon software (version 2.3.2; Matrix Science, London, UK) was used to perform database searches. To calculate the false discovery rate (FDR), the search was performed using the "decoy" option in Mascot. The spectral FDR and protein FDR are 0.35±0.17 % and 4.28±1.99 % respectively. A minimum Mascot ion score of 25 and peptide rank 1 was used for automatically accepting all peptide MS/MS spectra.

Label free relative protein quantitation (LFQ). Relative protein quantification was performed using spectral count-based LFQ. Statistical analysis was performed on all proteins identified with average spectral counts of ≥2 of at least one of the three experiments. The spectral count data was normalized by total spectral counts of the bait protein (NPM1) in each sample to adjust for differences in overall protein levels among samples. Proteins were considered to have a significant difference in abundance there was a difference of twofold or greater in normalized spectral counts between experiments and a p value ≤ 0.01 using a two-tailed t test.

Bioinformatic analysis. Proteins identified by label-free LC-MS/MS were analyzed by the Ingenuity Pathway Analysis Tool (IPA, Ingenuity Systems, Redwood City, CA). The "core analysis" function included in IPA (Ingenuity System Inc.) was used to interpret the data in the context of biological

RNA isolation, reverse transcription (RT) and real-time PCR. Total RNA from cultured cells was isolated. The cDNA was then synthesized from total RNA, Quantitative gene expression levels were detected using real-time PCR with the ABI PRISM 7500 Fast Sequence Detection System.

PROTEIN INTERACTIONS OF NPM1 AND MUTATED-NPM1 IN NUCLEUS AND CYTOPLASM

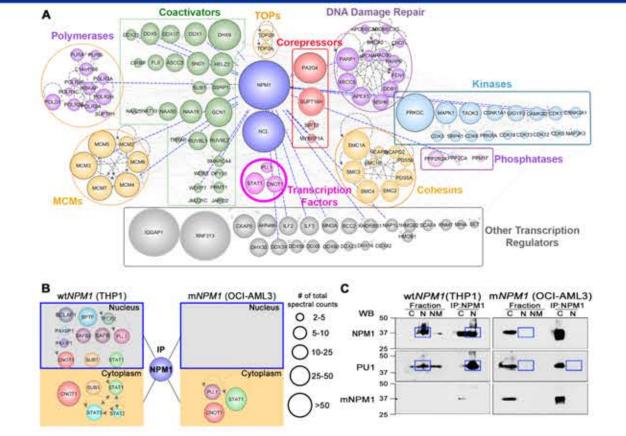
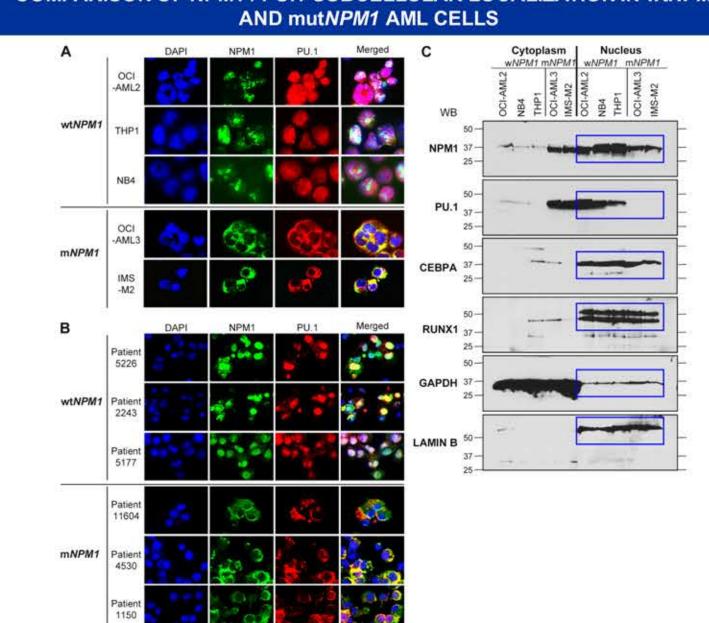


Figure 1: The NPM1 interactome includes key hematopoietic transcription factors and coregulators; these are dislocated to the cytoplasm with mutated-NPM1 (mNPM1) in mNPM1 AML cells. A) Transcription factors and coregulators pulled down with NPM1/mNPM1 from the cytoplasmic fraction of mNPM1 AML cells. Endogenous NPM1 and mNPM1 were immunoprecipitated from the cytoplasmic fractions of mNPM1 AML cells (OCI-AML3) and protein interactions analyzed by LCMS/MS. Coimmunoprecipitating proteins were organized into functional groups by Ingenuity Pathway Analysis. Individual protein enrichment is presented as total spectral counts, a semi-quantitative method for estimating the abundance of a specific protein in the co-immunoprecipitate. Larger circle size indicates higher number of total spectra counts for the protein. B) PU.1 was a major interaction partner of NPM1 in the nucleus of wtNPM1 AML cells, but in the cytoplasm of mNPM1 AML cells. Endogenous NPM1 and mNPM1 was immunoprecipitated from nuclear and cytoplasmic fractions of wtNPM1 (THP1) and mNPM1 AML cells. Only interactome transcription factors are shown (see supplementary material for additional data). Abundance of interacting proteins is indicated by the size of the circles. C) The interaction between NPM1 and PU.1 in the nucleus of wtNPM1 AML cells, and in the cytoplasm of mNPM1 AML cells, was also evident by immunoprecipitation-Western blot. The blue boxes indicate what would have been expected locations of NPM1 and PU.1 in nuclear fractions of mNPM1 AML cells.

COMPARISON OF NPM1 / PU.1 SUBCELLULAR LOCALIZATION IN wtNPM1 AND mutNPM1 AML CELLS



cells. A) KPT330 decreased cell counts of mNPM1 (OCI-AML3, IMS-M2) but not wtNPM1 (THP1) AML cells. Decitabine Figure 2. Immunofluorescence microscopy (IF) and Western blot (WB) to determine NPM1 and PU.1 localization in (DEC) was used to deplete DNMT1, and induced terminal differentiation of both mNPM1 and wtNPM1 cells. Cell counts by wtNPM1 and mNPM1 AML cell lines and primary cells. A) IF for NPM1 and PU.1 in wtNPM1 (OCI-AML2, THP1, NB4) and mNPM1 AML (OCI-AML3, IMS-M2) cell lines. DAPI stained for nuclei. Images were taken with Nikon Eclipse 400 microscope. automated counter. Mean±SD shown are of three independent replicates. B) KPT330 downregulated MYC and upregulated Magnification 630X. B) IF for NPM1 and PU.1 in wtNPM1 and mNPM1 AML primary cells from patients' bone marrow. p27/CDKN1B in mNPM1 but not wtNPM1 AML cells. Shown is a western blot. C) KPT330 upregulated the monocyte Images were taken with Nikon Eclipse 400 microscope. Magnification 630X. C) WB for PU.1 and NPM1 in cytoplasmic, differentiation marker CD14 and not the granulocyte differentiation marker CD11B in mNPM1 but not wtNPM1 AML nuclear and nuclear matrix fractions of wtNPM1 and mNPM1 AML cell lines. Blue boxes show what would have been cells. Flow cytometric analysis. D) KPT330 upregulated CSF1R (macrophage colony stimulating factor receptor) but not expected locations of NPM1 and PU.1 in nuclear fractions of mNPM1 AML cells. CEBPA and RUNX1 are other master differentiation-driving transcription factors that are highly expressed in AML cells - but in contrast to PU.1, were not major were used. Results are shown for three independent experiments. E) KPT330 induced monocytic morphology in mNPM1 interaction partners of NPM1 and were in the nucleus of both wtNPM1 and mNPM1 cells.

INHIBITING NUCLEAR-CYTOPLASMIC EXPORTATION RESTORED NUCELAR

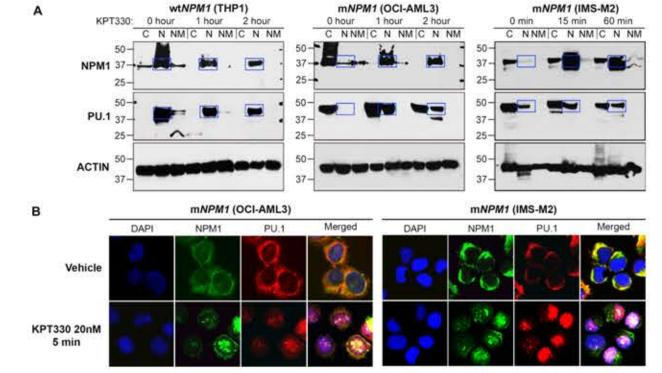
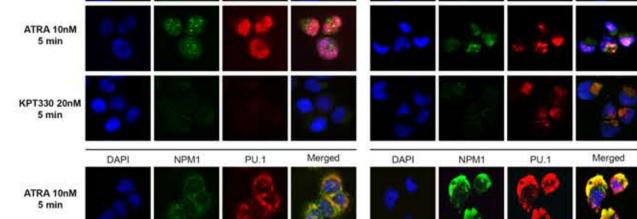


Figure 3. The nuclear export inhibitor selinexor (KPT330) sequestered both NPM1 and PU.1 into the nucleus of mNPM1 AML cells. A) Rapid relocalization of mNPM1 and much of PU.1 into the nucleus of mNPM1 AML cells by KPT330. wtNPM1 (THP1) and mNPM1 AML cells (OCI-AML3, IMS-M2) were treated with KPT330 20 nM and cell fractions (cytoplasm=C, nucleus=N, nuclear matrix=NM) were evaluated by Western blot for NPM1/mNPM1 and PU.1. Blue boxes show expected location of NPM1 and PU.1 in nuclear fractions. B) Immunofluorescence for NPM1 and PU.1 in vehicle versus KPT330 treated mNPM1 AML 23 cells. DAPI was used to stain for nuclei. Images were taken with Nikon Eclipse 400



ATRA TRANSFERRED STAT1 AND RARA TO THE NUCLEUS IN

mutNPM1 AML CELLS

Figure 5. ATRA 10 nM, but not KPT330, transferred STAT1 and RARA into the nuclei of mNPM1 AML cells. ATRA did not transfer mNPM1 or PU.1 into nuclei. DAPI stained nuclei. Immunofluoresœnœ microscopy images were taken with Nikon Eclipse 400 microscope. Magnification 630X.

ATRA TRIGGERED GRANULOCYTIC-DIFFERENTIATION OF

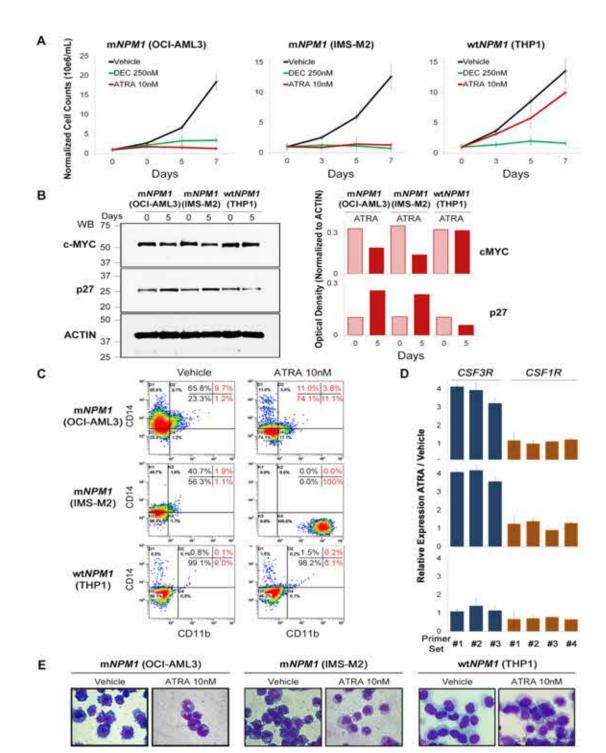


Figure 6. ATRA 10 nM triggered terminal granulocytic differentiation of mNPM1 but not wtNPM1 AML cells. A) ATRA decreased cell counts of mNPM1 (OCI-AML3, IMS-M2) but not wtNPM1 (THP1) AML cells. Decitabine (DEC) induced terminal differentiation of both mNPM1 and wtNPM1 AML cells. Cell counts were automated. Mean±SD shown are of three independent replicates. B) ATRA downregulated MYC and upregulated p27/CDKN1B in mNPM1 but not wtNPM1 AML cells. Western blot. C) ATRA upregulated CD11b (granulocyte marker) and not CD14 (monocyte marker) in mNPM1 but not wtNPM1 AML cells. Flow cytometry. D) ATRA upregulated CSF3R but not CSF1R expression in mNPM1 AML cells. QRT-PCR CSF3R (granulocyte colony stimulating factor receptor) expression in mNPM1 AML cells. QRT-PCR, multiple primer sets multiple primer sets were used in 3 independent experiments. E) ATRA induced granulocytic morphology changes in mNPM1 but not wtNPM1 AML cells. Giemsa-stained cytospin preparations of cells harvested on

ASSOCIATION OF OTHER TUMOR SUPPRESSORS WITH MUTATED-NPM1 MAY CONTRIBUTE TO DIFFERENTIATION ARREST

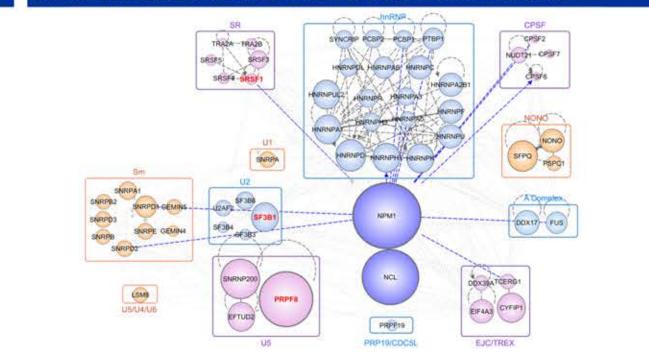


Figure 7. NPM1/mNPM1 in the cytoplasmic fraction of mNPM1 AML cells (OCI-AML3) interacts with splicing factors. Coimmunoprecipitating proteins were organized into functional groups by Ingenuity Pathway Analysis. Individual protein enrichment is presented as total spectral counts, a semi-quantitative method for estimating the abundance of a specific protein in the co-immunoprecipitate. Larger circle size indicates higher number of total spectra counts for the protein. Tumor suppressors identified by recurrent mutations and/or deletions in myeloid and other malignancies are highlighted in red.

HOX GENE UPREGULATION IN NPM1-MUTATED AML

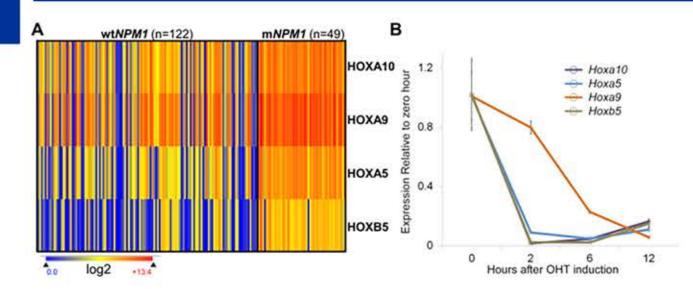
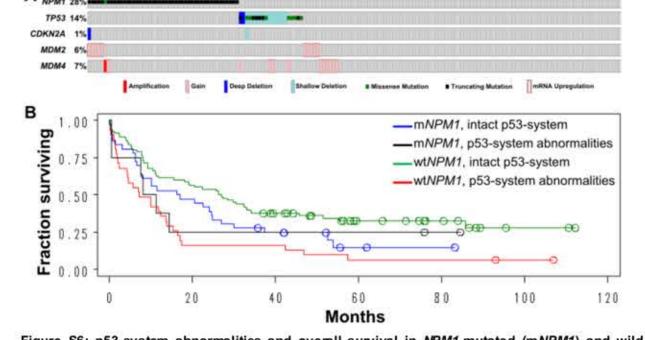


Figure 8. Relocation of Pu.1 into the nucleus suppressed Hox gene expression. A) High expression of HOX family genes in mNPM1 compared to wtNPM1 AML (gene expression by RNA-sequencing, primary AML bone marrow cells, TCGA). B) Pu.1 relocation into the nucleus suppressed Hox gene expression. Hematopoietic precursors knocked-out for Pu.1 were retrovirally transduced to express Pu.1 fused with the estrogen receptor (Pu.1-ER); Pu.1-ER is in the cytoplasm until the addition of the estrogen agonist tamoxifen (OHT) causes Pu.1-ER to translocate into the nucleus. Gene expression was measured by QRT-PCR.

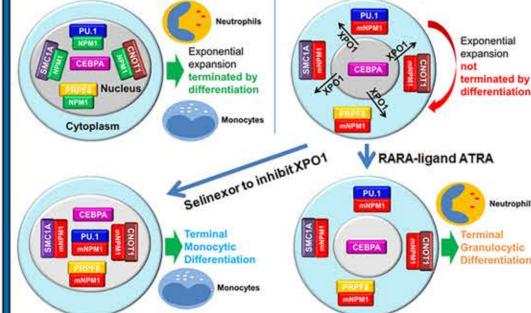
APOPTOSIS DEFECTS AND PROGNOSIS OF NPM1-MUTATED AND WTNPM1 AML



with p53-system abnormalities (n=31) data from TCGA (Cancer Genome Atlas Research)

Figure S6: p53-system abnormalities and overall survival in NPM1-mutated (mNPM1) and wild-type AML (wtNPM1). A) p53-system abnormalities in NPM1-mutated and wild-type AML (n=166, TCGA) (Cancer Genome Atlas Research). p53-system abnormalities were defined as TP53 mutation or deletion or expression or expression that was more than 1.5 standard deviations below the mean expression in 166 cases analyzed (heterozygous loss or homozygous deletion) and/or CDKN2A deletion (heterozygous loss or homozygous deletion) and/or MDM2/MDM4 copy number gain or expression level >1.5 standard deviations above the mean. Figure generated by CBioPortal. B) Overall survival in mNPM1 and wtNPM1 AML stratified by presence or absence of p53-system abnormalities (n=148, TCGA). mNPM1 with intact p53system (n=29); mNPM1 with p53-system abnormalities (n=8); wtNPM1 with intact p53-system (n=80); wtNPM1

GRAPHICAL CONCLUSION



PU.1 and with coregulators (e.g., SMC1A) for the master granulocyte differentiationdriving transcription factor CEBPA. Mutated-NPM1 protein acquires a nuclear-export signal, and PU.1/coregulators are inappropriately transported into the cytoplasm with it, thereby impeding the differentiation that usually terminates vigorous progenitor proliferation. Inhibiting nuclear export with selinexor retains PU.1 in the nucleus and triggers terminal monocytic differentiation of NPM1-mutated leukemia cells, while ATRA exploits the high amount of CEBPA relative to PU.1 in the nucleus of the leukemia cells to trigger terminal granulocytic differentiation.

SUMMARY

Failure to differentiate may propel clonal expansion of acute myeloid leukemia (AML) cells - self-renewal = proliferation without differentiation yet its underlying molecular mechanisms are mostly unknown. Nucleophosmin (NPM1) is the most frequently mutated gene in de novo AML. We analyzed the NPM1 protein-interactome comprehensively and discovered that it interacts with PU.1 (SPI1), the master driver of monocyte differentiation. Mutated-NPM1 protein acquires a nuclear export signal, and PU.1, like mutated-NPM1, was conspicuously located in the cytoplasminstead of nucleus of NPM1-mutated AML cells. NPM1 is exported by the nuclear exporter XPO1. A clinically available smallmolecule inhibitor of XPO1 binding to cargo (selinexor) retained both mutated-NPM1 and PU.1 in the nucleus and triggered terminal monocytic differentiation of AML cells. Thus, nuclear-export of PU.1 with mutated-NPM1 arrests differentiation and promotes proliferation.

SIGNIFICANCE

Relapsed NPM1-mutated AML is typically resistant to attempts at salvage, and long-term survival is expected for only ~50% of patients. One reason that first-line drugs somehow select for resistance to subsequent treatments is that seemingly different therapies have a common final intent of p53-dependent apoptosis (cytotoxicity). The inherently poor therapeutic index of cytotoxic therapy can be compromised further by alterations other than NPM1-mutation, e.g., TP53 mutation, MDM4 upregulation, that attenuate p53 function in AML cells. The molecular mechanisms of leukemogenesis by mutated-NPM1 shown here can be reversed by repositioning of clinically available compounds, to produce cell cycle exits by differentiation that do not require p53. Thus, the suggested approach appears rational also from higher level pathway and therapeutic index perspectives.

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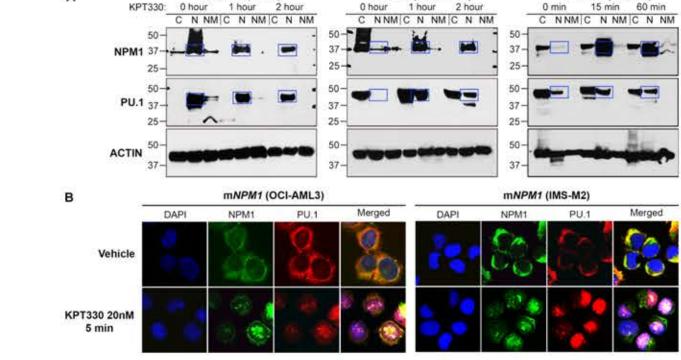
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NPM1/PU.1 IN mutNPM1 AML CELLS



INHIBITING NUCLEAR EXPORT OF NPM1/PU.1 INDUCES MONOCYTIC

DIFFERENTIATION OF mutNPM1 AML CELLS

KPT330 50 nM

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Figure 4. Nuclear localization of mNPM1/PU.1 by KPT330 triggered terminal monocytic differentiation of mNPM1 AML

10 00 0.1% 0.0% 10 0.0% 0.4%

but not wtNPM1 AML cells. Giemsa-stained cytospin preparations of cells harvested on day 5.

mNPM1 (OCI-AML3)

CSF3R

0 -----

Primer #1 #2 #3 #1 #2 #3 #4

CSF1R

mutNPM1 AML CELLS