Leukemogenic nucleophosmin mutation disrupts the transcription factor hub regulating granulo-monocytic fates

Xiaorong Gu, … , Babal K. Jha, Yogen Saunthararajah

*J Clin Invest.* 2018. [https://doi.org/10.1172/JCI97117](https://doi.org/10.1172/JCI97117).

**Graphical abstract**

---

**A) Normal myelopoiesis: Committed myeloid precursor**

1. Exponential proliferation
2. Terminated by differentiation

**B) AML: Mutant-NPM1 dislocates PU.1 into cytoplasm**

Exponential proliferation is decoupled from differentiation

**C) AML: Inhibiting nuclear export (XPO1) with selinexor**

1. Exponential proliferation
2. Terminated by differentiation

**D) AML: Inhibiting corepressors (DNMT1) with decitabine**

1. Exponential proliferation
2. Terminated by differentiation

---

**Find the latest version:**

[http://jci.me/97117/pdf](http://jci.me/97117/pdf)
Title: Leukemogenic nucleophosmin mutation disrupts the transcription factor hub regulating granulomonocytic fates

Authors: Xiaorong Gu¹, Quteba Ebrahem¹, Reda Z. Mahfouz¹, Metis Hasipek¹, Francis Enane¹, Tomas Radivoyevitch², Nicolas Rapin³,⁴,⁵, Bartlomiej Przychodzen¹, Zhenbo Hu⁶, Ramesh Balusu⁷, Claudiu V. Cotta⁸, David Wald⁹, Christian Argueta¹⁰, Yosef Landesman¹⁰, Maria Paola Martelli¹¹, Brunangelo Falini¹¹, Hetty Carraway¹², Bo T. Porse³,⁴,⁵, Jaroslaw Maciejewski¹¹,¹², Babal K. Jha¹, Yogen Saunthararajah¹,¹²

Affiliations:
¹ Department of Translational Hematology & Oncology Research, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio
² Department of Quantitative Health Sciences, Cleveland Clinic, Cleveland, Ohio
³ The Finsen Laboratory, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
⁴ Biotech Research and Innovation Center (BRIC), University of Copenhagen, Copenhagen, Denmark
⁵ Novo Nordisk Foundation Center for Stem Cell Biology, DanStem; Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark
⁶ Department of Hematology, Affiliated Hospital of Weifang Medical University, Weifang, China
⁷ Department of Internal Medicine, Division of Hematologic Malignancies and Cellular Therapeutics, University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City, Kansas
⁸ Department of Clinical Pathology, Tomsich Pathology Institute, Cleveland Clinic, Cleveland, Ohio
⁹ Department of Clinical Pathology, Case Western Reserve University, Cleveland, Ohio
¹⁰ Karyopharm Therapeutics, Newton, Massachusetts
¹¹ Institute of Hematology, CREO (Center for Research in Hematology-Oncology), University of Perugia, Perugia, Italy
¹² Department of Hematology and Oncology, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio

Key words: NPM1, leukemia, differentiation therapy, self-renewal, leukemia stem cells


Correspondence: Yogen Saunthararajah, MD, Taussig Cancer Institute, 9500 Euclid Avenue R40, Cleveland, OH 44195, tel: 216 444 8170, email: saunthy@ccf.org

Counts: Abstract: 196; Total Text – 11,828 ; Tables – 0; Figures – 12; Supplementary Material: Supplementary Methods, Supplementary tables (excel files) - 7, Supplementary figures - 19
**ABSTRACT**

*Nucleophosmin (NPM1)* is amongst the most frequently mutated genes in acute myeloid leukemia (AML). It is not known, however, how the resulting oncoprotein mutant-NPM1 is leukemogenic. To reveal the cellular machinery in which NPM1 participates in myeloid cells, we analyzed the endogenous NPM1 protein-interactome by mass-spectrometry, and discovered abundant amounts of the master transcription factor driver of monocyte lineage-differentiation PU.1 (SPI1). Mutant-NPM1, which aberrantly accumulates in cytoplasm, dislocated PU.1 into cytoplasm with it. CEBPA and RUNX1, the master transcription factors that collaborate with PU.1 to activate granulo-monocytic lineage-fates, remained nuclear, but without PU.1, their corepressor interactions were toggled from coactivators to corepressors, repressing instead of activating >500 granulocyte and monocyte terminal-differentiation genes. An inhibitor of nuclear export, selinexor, by locking mutant-NPM1/PU.1 in the nucleus, activated terminal monocytic fates. Direct depletion of the corepressor DNA methyltransferase 1 (DNMT1) from the CEBPA/RUNX1 protein interactome using the clinical drug decitabine activated terminal granulocytic fates. Together, these non-cytotoxic treatments extended survival by >160 days vs vehicle in a patient-derived xenotransplant model of *NPM1/FLT3*-mutated AML. In sum, mutant-NPM1 represses monocyte and granulocyte terminal-differentiation by disrupting PU.1/CEBPA/RUNX1 collaboration, a transforming action that can be reversed by pharmacodynamically-directed dosing of clinical small molecules.
INTRODUCTION

Nucleophosmin \((NPM1)\) is one of the most frequently mutated genes in de novo acute myeloid leukemia (AML) (~30% of cases) (1). Only ~50% of patients with \(NPM1\)-mutated AML can expect long-term survival with current anti-metabolite/cytotoxic treatments (2, 3), and there are no ‘precision’ molecular-targeted treatments for this disease, reflecting insufficient understanding of how mutant-NPM1 is leukemogenic. Previous discoveries have, however, provided important clues: NPM1 is a nucleus and nucleolus enriched phospho-protein that chaperones and shuttles several chromatin remodeling and ribosome biogenesis proteins between the nucleus and cytoplasm (4-6). The recurrent insertions into \(NPM1\) seen in AML, e.g., \(NPM1\)-W288Cfs*12, produce a reading frame-shift that eliminates a nucleolar localization sequence and adds a nuclear export sequence - mutant-NPM1 protein product thus accumulates aberrantly in cytoplasm (1, 7). This unbalanced export of mutant-NPM1 out of nuclei is mediated by a nuclear export protein ‘chromosome region maintenance 1’ (CRM1), also known as ‘exportin 1’ (XPO1) (5, 8). How such cytoplasmic dislocation of mutant-NPM1 confers a growth advantage to myeloid cells, however, is unknown.

These mutations, though highly recurrent in AML, are not seen in cancers of other tissues, suggesting a transforming effect specifically in a myeloid lineage, and other discoveries have clarified where in myeloid differentiation continua \(NPM1\) mutations originate: surface markers were used to sort bone marrow cells from patients with \(NPM1\)-mutated AML into hematopoietic stem cells (HSC) and lineage-committed myeloid progenitors, for subsequent DNA sequencing to document mutations (9-11). Some AML mutations, such as \(DNMT3A\)-R882H, were detected in both the HSCs and downstream progenitors (9-11). \(NPM1\) mutations were detected, however, only in the lineage-committed progenitors, wherein they supplemented mutations, e.g., \(DNMT3A\)-R882H, propagated from HSC (9-11). Upon xenotransplantation into immune-compromised mice, the HSC, lacking \(NPM1\) mutations, produced phenotypically normal multi-lineage hematopoiesis, but the lineage-committed progenitors containing mutated \(NPM1\) yielded leukemic hematopoiesis, wherein the cells did not mature and continued to replicate (9-11). Also indicating that \(NPM1\) mutations originate in and transform lineage-committed myeloid progenitors: (i) cytoplasmic NPM1 (mutant-NPM1 protein) is observed in myeloid but not in T or B cells from patients (12); (ii) \(NPM1\)-mutated AML cells that overcome inter-species barriers to initiate AML.
in immune-compromised mice (leukemia initiating cells or leukemia 'stem' cells) have surface phenotypes and transcriptomes of lineage-committed myeloid progenitors, e.g., granulocyte-monocyte progenitors (GMP) that normally differentiate into granulocyte- or monocyte-progenitors then terminally differentiated granulocytes or monocytes (9-11, 13, 14); (iii) bone marrow replacement (85-97% of cells) in patients with NPM1-mutated AML is by cells with surface phenotypes and transcriptomes of GMP (15); (iv) mutant-Npm1/Flt3-ITD knock-in into hematopoietic precursors in mice expands GMP in particular (16); and (v) AML mutations that precede NPM1 or FLT3 mutations and originate in germline or HSC (e.g., DNMT3A, RUNX1 mutations) expand lineage-committed daughter cells the most (17, 18), thereby increasing possibilities for mutations to NPM1 and/or FLT3 in these daughters (9-11).

Several lines of evidence have thus indicated that NPM1 mutations originate in and transform lineage-committed myeloid progenitors (e.g., GMP), a cellular context governed by a master transcription factor circuit containing PU.1 (SPI1), CEBPA and RUNX1 - a few of the ~100 transcription factors expressed in cells are masters, collaborating in couplets or triplets to powerfully determine cell fates and functions, illustrated by their remarkable capacity to convert cells of one lineage into another, even into embryonic stem cells (19). PU.1 cooperates with CEBPA and RUNX1 to command granulo-monocytic lineage-fates (20-27) – Pu.1 knock-out mice have reduced granulocytes and no monocytes (28, 29); Cebpa knock-out mice have no granulocytes (30); Runx1 knock-out mice abrogate definitive hematopoiesis (31); and ectopic expression of transcription factor ensembles containing PU.1 with CEBPA, or PU.1 with RUNX1, are sufficient to convert even fibroblasts into myeloid precursors/monocytes (29, 32-36). Functional compromise of this master circuit can hence be expected to impede myeloid differentiation, and accordingly, loss-of-function mutations/translocations of CEBPA and RUNX1 are highly recurrent in AML, a disease defined by myeloid differentiation arrest (37-39)(reviewed in(40)). Recurrent alterations to PU.1, however, have not been found (41). Here, upon the first mass-spectrometric analyses of protein-protein interactions of endogenous NPM1 affinity-purified from wildtype and NPM1-mutated AML cell nuclear and cytoplasmic fractions, we found that both wildtype and mutant-NPM1 interact with PU.1, and that mutant-NPM1 dislocates PU.1 into cytoplasm with it. Using several methods, we elucidated how this disruption of the PU.1/CEBPA/RUNX1 master transcription factor hub decouples proliferation from forward-
differentiation. Importantly, the transforming actions could be reversed by clinical small molecules, opening the door to non-cytotoxic differentiation-restoring treatments for patients with NPM1-mutated AML.

RESULTS

Protein interactions of endogenous NPM1 and mutated-NPM1 in nuclei and cytoplasm
Protein-protein interactions of endogenous NPM1 immuno-precipitated from nuclear and cytoplasmic fractions of NPM1-wildtype and mutated AML cells were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Separation of cells into fractions was essential, since mutant-NPM1 aberrantly accumulates in cytoplasm (1, 7). In nuclei of NPM1-wildtype AML cells, the NPM1 interactome was enriched for chromatin-remodelers, splicing factors, ribosomal proteins, transport proteins and hematopoietic transcription factors, most notable of which was PU.1 (Figure 1A, Table S1). NPM1 co-immunoprecipitated with PU.1 also in the reverse pull-down (Figure S1). PU.1 is essential for monocyte differentiation, contributes to granulocyte differentiation (23, 42-47), and is considered a master transcription factor, commanding other transcription factors and hundreds of genes to dictate cell fates (32). Critically, the transcription factors identified as interacting with NPM1 in nuclei of NPM1-wildtype AML cells were observed in cytoplasm of NPM1-mutated AML cells (Figure 1A).

We corroborated NPM1 interactions with PU.1, and dislocation of both into cytoplasm when NPM1 is mutated, using four additional methods: first, we immunoprecipitated endogenous NPM1 from cell fractions of wildtype (THP1) and NPM1-mutated AML cells (OCI-AML3) and then performed Western blots on the immunoprecipitates – these immunoprecipitation-Western blots again demonstrated abundant co-immunoprecipitation of PU.1 with wildtype-NPM1 from nuclei of NPM1-wildtype AML cells, and with mutant-NPM1 from cytoplasm of NPM1-mutated AML cells (Figure 1B); second, we used immunofluorescence microscopy (IF) to examine whole cell preparations of three NPM1-wildtype AML cell lines (OCI-AML2, THP1, NB4) and two NPM1-mutated AML cell lines (OCI-AML3, IMS-M2) – we found NPM1 and PU.1 in nuclei of the three NPM1-wildtype AML cell lines but both were in cytoplasm of the two NPM1-mutated AML cell lines (Figure 1C, S2); third, we used IF to visualize whole cell preparations of primary AML cells from three patients with
NPM1-wildtype AML and three patients with NPM1-mutated AML – again, both NPM1 and PU.1 were in nuclei of the three NPM1-wildtype AML primary cell populations but in the cytoplasm of the three NPM1-mutated AML primary cell populations (Figure 1D, S2); and fourth, we performed Western blots for NPM1, PU.1, and the master transcription factors RUNX1 and CEBPA that cooperate with PU.1 to drive granulo-monocytic differentiation (20-24) in cytoplasmic and nuclear fractions of three NPM1-wildtype AML cell lines (OCI-AML2, NB4, THP1) and two NPM1-mutated AML cell lines (OCI-AML3, IMS-M2): PU.1, CEBPA and RUNX1 were in nuclear fractions of the three NPM1-wildtype AML cell lines whereas PU.1, but not CEBPA or RUNX1, was instead in cytoplasmic fractions of the two NPM1-mutated AML cell lines (Figure 1E).

The master transcription factor expression pattern in AML cells
To interpret the pattern of master transcription factor expression in cytogenetically normal AML cells, we related this pattern to that seen at different stages of normal hematopoiesis, by using a gene expression resource that we generated and described previously (48, 49). Master transcription factors that produce HSC, HLF/PBX1/PRDM5, were expressed in the AML cells at <1/100th the levels seen in normal HSC but similar to that in normal GMP, granulocytes or monocytes (Figure 2A). Master transcription factors that produce granulocytes and monocytes, PU.1/RUNX1/CEBPA, were also expressed in the AML cells at levels similar to or exceeding (by up to 2-fold) that in normal GMP/granulocyte/monocytes, levels several-fold higher than in normal HSC (Figure 2A). The AML cells also clustered with normal GMP to terminally-differentiated granulocytes/monocytes and not HSC when expression levels of several known hematopoietic master transcription factors (PU.1, RUNX1, CEBPA, IRF8, GFI1, GATA1, GATA2, FLI1, TAL1, LMO2, EBF1, PAX5, HLF, PBX1, PRDM5, and ZFP37AML) were used for unbiased hierarchical clustering (Figure S3)(49).

PU.1 localizes at monocyte terminal-differentiation, but not myeloid-commitment, genes
PU.1/RUNX1/CEBPA, highly expressed in AML cells, would be expected to activate granulo-monocytic gene expression programs - we identified three such programs: (i) Proliferation program: ~300 genes identified in the literature as target genes of MYC, the master transcription factor regulator of cell growth and division (50)(Table S2, Figure 2B). We further validated that these genes are MYC-targets by analyzing separate public chromatin
immunoprecipitation-sequencing (MYC ChIP-seq) data from Encode (Figure S4A); (ii) Myeloid-commitment program: ~200 genes significantly upregulated in normal common myeloid progenitors (CMP) and GMP vs HSC/granulocytes/monocytes (Table S3, Figure 2B), identified by applying the Comparative Marker Selection algorithm to a public dataset of gene expression at different stages of myelopoiesis (51); and (iii) Terminal monocyte-differentiation program: >300 genes significantly upregulated in normal monocytes vs HSC/CMP/GMP (Table S4, Figure 2B), identified using the same method.

We then validated that the proliferation, commitment and monocyte-differentiation genes discriminated between HSC, committed myeloid progenitors and monocytes in our own separate database of gene expression in normal myelopoiesis (48, 49) (Figure S5). Then, using public data of Pu.1 binding to genomic sites in hematopoietic progenitors and monocytes (Pu.1 ChIP-seq)(52), we noted that Pu.1 localized at the monocyte-differentiation but not at the myeloid-commitment genes (Figure S4B). Accordingly, expression of monocyte-differentiation genes and Pu.1 positively correlated (correlation coefficients 0.01 to 0.66)(Figure 2B), but expression of commitment genes and Pu.1 negatively correlated (corr. coefficients -0.65 to -0.06)(Figure 2B). Proliferation and monocyte-differentiation gene expression negatively correlated (Figure S6).

The monocyte terminal-differentiation program is suppressed in NPM1-mutated AML cells
Consistent with the similar expression of PU.1/CEBPA/RUNX1 between AML cells and normal monocytes (Figure 2A), proliferation and myeloid commitment programs were also similarly expressed, both by pattern and magnitude (Figure 2C). Monocyte terminal-differentiation genes, however, were markedly suppressed in the AML cells, with levels ~3-fold lower than in normal monocytes (Figure 2A, C). Repression of the monocyte terminal-differentiation program despite PU.1/CEBPA/RUNX1 expression similar to or higher than in monocytes/granulocytes (Figure 2A) implies at least partial loss-of-function in this master transcription factor circuit – shown previously to occur by mutated or translocated RUNX1 and bi-allelically mutated CEBPA (37-39), and shown to occur here via the actions of mutant-NPM1. Notably, RUNX1, bi-allelic CEBPA, and NPM1 mutations, although very frequent in AML, are mutually exclusive (Figure 2D).
Mutant-NPM1 dislocates PU.1 into cytoplasm, and Pu.1 nuclear relocation transitions proliferative precursors to terminal monocytic fates

We then used two separate model systems to show that (i) mutant-NPM1 causes cytoplasmic dislocation of PU.1; and to reaffirm that (ii) Pu.1 nuclear relocation in a Pu.1-null myeloid context is sufficient to suppress key commitment/precursor genes, activate key monocyte terminal-differentiation genes, and trigger terminal monocytic fates.

The first model was HEK-293 cells co-transfected with expression vectors for NPM1 and PU.1, or mutant-NPM1 and PU.1. In the cells co-transfected to express NPM1 and PU.1, both NPM1 and PU.1 were almost exclusively nuclear localized as expected (Figure 3A). However, in the cells co-transfected to express mutant-NPM1 and PU.1, both mutant-NPM1 and PU.1 were mostly in the cytoplasm (Figure 3A). This data suggests mutant-NPM1 is responsible for the dislocation of PU.1 into cytoplasm.

The second model was Pu.1 knock-out (Pu.1−/−) murine hematopoietic precursors that were retrovirally transduced to express Pu.1 fused with the estrogen receptor (Pu.1-ER), generated and described previously (53): Pu.1-ER is in the cytoplasm unless estrogen is added, which causes its translocation into the nucleus. Reproducing previous data, Pu.1-ER translocation from cytoplasm into nuclei (Figure S7A) activated key monocyte differentiation genes (e.g., the macrophage colony stimulating factor receptor)(Figure S7B), induced morphologic changes of monocyte differentiation (Figure S7C) and terminated proliferation (Figure S7D). We showed previously that knock-down of Runx1 prevents the Pu.1-driven terminal monocytic differentiation (17, 20, 21). Thus, Pu.1 nuclear restoration, in a myeloid context containing Runx1, transitions indefinitely replicating precursors to terminally-differentiated monocytes.

The Pu.1−/− cells modeled another characteristic of NPM1-mutated AML cells: high expression of homeobox genes (e.g., Hoxa9, Hoxb5)(Figure 3B, C) – normally during myelopoiesis, there is a strong negative correlation between HOX gene and PU.1 expression (Pearson correlation coefficients -0.68 to -0.86, p<0.001)(Figure 3D), but incongruously, the AML cells express both simultaneously at high levels (Figure 3C). The high HOX gene expression in NPM1-mutated AMLs does not appear to be driven by the master transcription factors that produce HSC, since levels of these master transcription factors (HLF, PBX1, PRDM5) were ~1/1000th the levels of PU.1/RUNX1/CEBPA (Figure 3C). Instead, there was rapid suppression of Hoxa5, Hoxa9, Hoxa10
and Hoxb5 upon Pu.1-ER translocation into nuclei of Pu.1+ cells (Figure 3B), suggesting that PU.1 cytoplasmic dislocation can explain this feature of NPM1-mutated AMLs also.

Nuclear retention of mutant-NPM1 and PU.1 triggers monocytic differentiation

Protein macromolecules such as NPM1 require transport factors to enter (importins) and exit (exportins) nuclei. A specific exportin, XPO1 (CRM1), has been shown to mediate nuclear export of mutant-NPM1 (5) and consistent with this finding, XPO1 was the major exportin we found in the NPM1/mutant-NPM1 protein interactomes (Table S1). XPO1 interactions with transported cargo can be inhibited by the small molecule Selective Inhibitor of Nuclear Export (SINE) drug selinexor (KPT330)(54). Treatment of NPM1-mutated AML cells (OCI-AML3 and IMS-M2) with 10-20 nM of selinexor rapidly re-located both mutant-NPM1 and PU.1 to nuclei, seen both by Western blots of nuclear and cytoplasmic fractions (Figure 4A, B) and by IF (Figure 4C). The selinexor-induced nuclear retention of PU.1 terminated NPM1-mutated AML cell proliferation (Figure 5A) by monocytic differentiation, shown by downregulated protein levels of MYC (the master transcription factor regulator of cell growth and division)(Figure 5B), upregulated protein levels of p27/CDKN1B (a cyclin dependent kinase inhibitor that mediates terminal differentiation)(Figure 5B), upregulated expression of the monocyte lineage marker CD14 and the macrophage-colony stimulating factor receptor (MCSFR, CSF1R)(Figure 5C, D), but not expression of the granulocyte lineage marker CD11b nor the granulocyte-colony stimulating factor receptor (GCSFR, CSF3R)(Figure 5C, D), and morphologic changes of monocyte differentiation (bean-shaped nuclei, high nuclear/cytoplasmic ratio) (Figure 5E, S8) without inducing early apoptosis (no increase in Annexin-V-staining)(Figure S9). Thus, selinexor induced a constellation of events in NPM1-mutated AML cells that is consistent with terminal monocytic differentiation. The same treatment did not induce differentiation of NPM1-wildtype AML cells (THP1, OCI-AML2)(Figure 5A-E).

Low-dose selinexor in a patient-derived xenotransplant (PDX) model of dual NPM1/FLT3-mutated AML

Immune-deficient (NSG) mice were xenotransplanted with NPM1/FLT3-mutated primary AML cells (a sub-set of NPM1-mutated AMLs with poor prognoses)(55). After confirmation of bone marrow AML engraftment to ≥20% in 3 randomly selected mice, mice were randomized to treatment with vehicle or selinexor 2 mg/kg 4X/week
(Figure S10A). The 2 mg/kg dose was expected to produce plasma $C_{\text{max}}$ of ~200 nM based on previous pharmacokinetic studies, and was ~10-fold lower than the usual dose of >20 mg/kg used in mice for non-specific cytotoxic intent (54, 56, 57). At ~day 75, vehicle treated mice demonstrated signs of distress and the experiment was terminated for analysis: selinexor treated mice had no signs of distress and significantly lower bone marrow AML burden (~70% human CD45+ cells) than vehicle treated mice (>95% human CD45+ cells), with preservation of some murine hematopoiesis, shown by blood counts, bone marrow flow-cytometry for murine CD45+ cells, and morphology (Figure S10B-G). There was also a substantial AML burden in the spleens of vehicle-treated mice with >50-fold increase in splenic weight vs normal NSG spleen (~0.018 g)(extra-medullary AML features in monocytic AMLs); this burden was decreased ~3-fold reduction by selinexor (Figure S10D). IF confirmed restoration of some PU.1 into nuclei in AML cells from selinexor vs vehicle-treated mice (Figure S10E). Monocytic differentiation induction of the AML cells was evident by flow cytometry for human CD14 and by inspection of Giemsa-stained bone marrow cytospin preparations (Figure S10F, G).

Although there was substantial reduction in spleen AML burden, reduction in bone marrow AML burden was modest. We therefore examined if the disconnect between in vitro and in vivo potency reflected pharmacologic/pharmacodynamic factors that could be overcome with a higher, but still non-cytotoxic dose of selinexor, by comparing selinexor 2 mg/kg to 5 mg/kg (Figure 6A). Consistent with a molecular targeted effect that was saturated with a dose of 2 mg/kg, the higher dose of 5 mg/kg was not more efficacious: both doses produced similar restorations of PU.1 into nuclei in bone marrow AML cells (Figure 6B); selinexor 2 mg/kg reduced bone marrow AML burden from >95% to ~45% (Figure 6C), while 5 mg/kg reduced it to ~65% (the difference was not statistically significant); and both doses produced a >2-fold reduction in spleen AML burden vs vehicle (the difference was not statistically significant) (Figure 6D). A non-cytotoxic, differentiation-based mode of action for both doses was indicated by significant and similar ~2-fold increase in the CD14 monocyte lineage-differentiation marker (Figure 6E) and no increase in expression of the apoptosis/DNA-damage marker $\gamma$-H2AX in bone marrow AML cells (Figure 6F), and preservation of platelet and hemoglobin levels while receiving active therapy for ~50 days. There were eventual declines in platelets and hemoglobin, but driven by AML progression, demonstrated by concurrent increases in circulating myeloblasts (Figure 6G).
In short, a higher dose of the nuclear export inhibitor did not appear to be the solution to improving differentiation-restoring benefit in $NPM1/FLT3$-mutated AML in vivo, the genetic sub-set of $NPM1$-mutated AML that accounts for most clinical refractory/relapsed disease.

**Coregulator interactions of CEBPA and RUNX1 and repression of granulocyte differentiation genes**

We therefore queried if the master transcription factors CEBPA and RUNX1, that remained nuclear in $NPM1$-mutated AML cells, could be used for a complementary approach to differentiation-restoration: CEBPA and RUNX1 are expected to activate granulocytic fates (25, 27), however, the granulocyte differentiation program, like the monocyte differentiation program, is suppressed in AML vs normal granulocytes (**Figure S11, Table S5**). To investigate how, we examined the coregulator interactions of nuclear CEBPA and RUNX1 in $NPM1$-mutated AML cells using affinity-purification-LCMS/MS and Western blot: the CEBPA and RUNX1 protein interactomes were enriched for coregulators that repress transcription (corepressors, e.g., DNMT1, NURD, SIN3A complex, CBX) over coactivators that activate genes (e.g., SWI/SNF, NUA4, SETD1A)(**Figure 7, S12, Table S6, S7**). We showed previously a mechanism by which PU.1 synergizes with its master transcription factor partners: the transcription regulating domains of PU.1 and RUNX1 interacted to exclude corepressors and recruit coactivators (20, 21). In keeping with this motif, PU.1 introduction into the CEBPA/RUNX1 interactomes by selinexor switched CEBPA and RUNX1 interactions in $NPM1$-mutated AML cells from corepressors (e.g., DNMT1, NURD, SIN3A) to coactivators (e.g., SWI/SNF, NUA4, SETD1A), shown by immunoprecipitation-LCMS/MS and by immunoprecipitation-Western blot (**Figure 8, S12**).

Then, we evaluated use of the clinical small molecule decitabine to directly deplete the corepressor enzyme and scaffold protein DNA methyltransferase 1 (DNMT1) from the CEBPA/RUNX1 interactomes: DNMT1-depletion reconfigured CEBPA/RUNX1 interactions from corepressors to coactivators (**Figure 8, S12**). This activated terminal granulocytic fates: CD11b was upregulated (~20% of cells)(**Figure 9A**), granulocytic nuclear segmentation was induced in many cells (**Figure 9B, S8**), GCSFR was upregulated >6-fold (**Figure 8C**), MYC was downregulated (**Figure 5B**), p27/CDKN1B was upregulated (**Figure 5B**) and proliferation terminated (**Figure 5A**). Moreover, NPM1 expression decreases naturally during granulocyte-lineage differentiation (**Figure 9D**), and there was a progressive decrease in NPM1/mutant-NPM1 protein levels, progressive increase in PU.1
nuclear-retention (Figure 9E, S13), CD14 upregulation in ~60% of the cells (Figure 9A), monocytic morphology changes in many cells (Figure 9B, S8), and >6-fold upregulation of MCSFR (Figure 9C).

Combination differentiation-restoring therapy

Immune-deficient mice were xenotransplanted with NPM1/FLT3-mutated primary AML cells (55). After bone marrow AML engraftment to ≥20% was confirmed in 3 randomly selected mice (Figure 10A), mice were randomized to treatment with (i) vehicle as control; (ii) nuclear-export inhibition by selinexor 2 mg/kg oral gavage 4X/week; (iii) DNMT1-depletion by decitabine 0.1 mg/kg 3X/week subcutaneous alternating with 5-azacytidine 1 mg/kg 3X/week subcutaneous, with both drugs combined with tetrahydouridine 10 mg/kg intra-peritoneal to inhibit their otherwise rapid degradation in vivo by cytidine deaminase (a regimen optimized for non-cytotoxic DNMT1-depletion in vivo as previously described (58-63)); and (iv) combination nuclear export-inhibiting and DNMT1-depleting treatment (Figure 10A). Mice were closely followed and euthanized if there were signs of distress - blood counts at time-of-distress confirmed onset of anemia, thrombocytopenia and circulating AML (peripheral myeloblasts)(Figure 10B). Nuclear-export inhibition alone delayed distress onset by 10 days vs vehicle, a significant (p=0.01, Log Rank) but limited benefit (Figure 10C). DNMT1-depletion and combination nuclear-export inhibition/DNMT1-depletion, however, extended distress-free survival by >160 days vs vehicle in all the treated mice (Figure 10C). At euthanasia, there were similar >90% bone marrow AML burdens in all treatment groups (Figure S14), however, spleen AML burdens were decreased by a significantly greater extent by combination nuclear-export inhibition/DNMT1-depletion vs the other treatment groups (~2-fold vs DNMT1-depletion alone, p=0.02)(Figure 10D) (extra-medullary AML is a feature of monocytic AMLs, and spleen weights were >80-fold higher in vehicle-treated mice than normal NSG spleen). Growth fractions of bone marrow AML cells (SG2/M, cell cycle distribution measured by flow cytometry) were also smallest with combination treatment vs DNMT1-depletion (mean 29.4% vs 41.9%, p=0.002)(Figure 10E, S15). A non-cytotoxic differentiation-based mechanism-of-action was indicated in all treatment groups – with nuclear export inhibition by ~2-fold upregulation of monocyte lineage-marker CD14 (Figure 10F, S16) and monocytoid appearance of bone marrow AML cells (Figure 10G); with DNMT1-depletion by >2-fold upregulation of granulocyte lineage-marker CD11b or CD15 (Figure 10F, S17) and abnormal but recognizable nuclear segmentation (Figure 10G); with combination
treatment by even greater monocytic differentiation with >2-fold upregulation of CD14 (Figure 10F, S16) and monocytic morphology of bone marrow AML cells (Figure 10G); with all treatments by preservation of normal blood counts on active therapy for >8 months (Figure 10B), no increase in expression of the apoptosis/DNA damage marker γ-H2AX (Figure 10I, S18), and trivial sub-G1 fraction in bone marrow AML cells at time-of-distress (Figure 10E, S15).

**Resistance in vivo and in vitro was by avoidance of pharmacodynamic effect**

The NPM1/FLT3-mutated AML cells that resisted and progressed through several months of in vivo therapy to cause distress in the mice in the above experiment were analyzed for achievement of intended molecular pharmacodynamic effects – nuclear retention of mutant-NPM1/PU.1 by selinexor, and DNMT1-depletion by decitabine/5-azacytidine. In resistant cells, selinexor had failed to re-locate mutant-NPM1/PU.1 into nuclei (Figure S19A). This was also seen in vitro: NPM1-mutated AML cells (OCI-AML3) selected for resistance to selinexor over several months of in vitro culture (exponential proliferation in selinexor up to 50 nM added every 3 days) demonstrated persistent cytoplasmic dislocation of mutant-NPM1/PU.1, shown both by IF (Figure 11A) and by Western blot of cytoplasmic and nuclear fractions (Figure 11B). The selinexor-resistant cells were still sensitive to non-cytotoxic concentrations of decitabine (Figure 11C), as expected from the mechanism data shown earlier (Figure 7-9). Similarly, DNMT1 was not depleted from AML cells harvested from bone marrow at time-of-euthanasia (Figure S19B), consistent with previous documentation by us and others that in vitro resistance to decitabine and 5-azacytidine is by selection for malignant cells that avoid DNMT1-depletion (58-63).

Thus, resistance both in vitro and in vivo was by preventing intended molecular pharmacodynamic effects, underscoring the importance of the targeted pathways to the malignant phenotype. A figure summary of the overall data is provided (Figure 12).
DISCUSSION

Unbiased analyses of the NPM1 protein interactome revealed that it is a cofactor for PU.1, providing a mechanism explanation for why others have found that NPM1 is essential for monocyte/macrophage differentiation and function (64). Crucially, the NPM1/PU.1 interaction causes PU.1 functional deficiency when NPM1 is mutated, because mutant-NPM1 dislocates PU.1 into the cytoplasm with it.

PU.1, RUNX1 and CEBPA constitute a collaborating master transcription factor circuit that has as its purpose driving cells to terminal granulo-monocytic fates (20-27, 29, 32-36). This ensemble is similarly expressed in AML cells vs granulocytes/monocytes, but loss-of-function in the AML cells is evidenced by suppression of hundreds of monocyte and granulocyte terminal-differentiation genes vs monocytes/granulocytes, even as commitment and proliferation programs are similarly activated (21, 65-67). That PU.1 partial loss-of-function permits activation of myeloid-commitment genes but represses terminal-differentiation genes has been demonstrated in murine models: Pu.1-deficient HSCs could commit into the monocytic lineage, but subsequent activation of terminal-differentiation was suppressed, producing leukemic hematopoiesis – replicating lineage-committed cells impeded in their further maturation (42-47). This suspension of cells at an intermediate, inherently replicative stage of their advance along lineage-differentiation axes seems to hinge on partial but not complete loss-of-function in the PU.1/CEBPA/RUNX1 circuit, since experimentally, complete inactivation of PU.1, CEBPA, or RUNX1 kills AML cells, even as partial loss-of-function of any one of these is leukemogenic (17, 43, 68-72), and accordingly, NPM1, RUNX1 and bi-allelic CEBPA mutations, though highly recurrent in AML, are mutually exclusive (37-39).

PU.1/CEBPA/RUNX1 have been shown to promote exponential replication kinetics, by binding to MYC enhancers to produce high grade activation of MYC (the master transcription factor coordinator of cell proliferation), and by co-binding with MYC at its target genes (73-82) – this contrasts with the quiescence imposed by stem cell master transcription factors such as HLF in HSC (73-82). In computational analyses, such skewing of intrinsic replication rates logarithmically favors decoupling replication from forward-differentiation in lineage-progenitors as the most efficient strategy for malignant transformation (83). NPM1-mutations are such a
decoupling event, since suppression of mutant-NPM1 by siRNA unleashes forward-differentiation that terminates leukemia initiating capacity (84), and the connection with PU.1 shown here provides a mechanism.

Why does neoplastic evolution select to mutate \textit{NPM1} instead of \textit{PU.1} directly? We do not know for certain, but possibilities include that mutant-NPM1 creates the graded PU.1 loss-of-function that has been shown to be necessary for leukemogenesis (17, 43, 68-72), and/or that dislocation of proteins other than PU.1 contributes to transformation. Even though mutant-NPM1 dislocates several proteins, that PU.1 is key amongst these is suggested by: (i) Pu.1 partial loss-of-function is sufficient to transform normal into leukemic myelopoiesis (42-47, 72); (ii) Pu.1 nuclear re-introduction is sufficient to transition indefinitely replicating Pu.1-null myeloid precursors to terminal monocytic fates (17, 20, 21); (iii) PU.1 cytoplasmic dislocation can explain repression of >300 monocyte differentiation genes in AMLs since these genes are demonstrable PU.1 targets by ChIP-seq, and their expression positively correlates with PU.1 in normal myelopoiesis; (iv) PU.1 is absolutely required for the monocytic fates triggered by nuclear-export inhibition of NPM1-mutated AML cells (20-24, 29, 32-35); and (v) AML cells that avoid these nuclear-export inhibitor induced fates do so by avoiding PU.1 nuclear relocation. PU.1 cytoplasmic dislocation can moreover explain previously poorly understood features of \textit{NPM1}-mutated AMLs: (i) high HOX gene expression, since Pu.1-helmed differentiation advances suppress Hox genes; (ii) origin of \textit{NPM1} mutations in lineage-committed progenitors and not HSC (9-11), since Pu.1 elimination from HSC is anti-proliferative/anti-competitive (42), while its knock-down from lineage-committed myeloid progenitors is leukemogenic (42-47); and (iii) mutual exclusivity of \textit{NPM1} with \textit{RUNX1} or bi-allelic \textit{CEBPA} mutations (37-39), since some function of this master transcription factor hub is needed for existence as a lineage-progenitor (17, 43, 68-72).

Nuclear export of NPM1 is by the nuclear export protein XPO1. That inhibitors of XPO1 binding to cargo (e.g., KPT185, KPT8602) are 5-10 fold more potent in \textit{NPM1}-mutated than \textit{NPM1}-wildtype AML cells has already been reported (56, 85), however, the molecular mechanisms and pathways underlying this several-fold sensitivity were not known, and in clinical trials, selinexor (KPT330) has been evaluated in unselected AML genotypes and with traditional cytotoxic intent - dosages were escalated towards maximum tolerated levels of \textasciitilde 55-70 mg/m\textsuperscript{2} (93.5-119 mg fixed doses) and administered 1-2X/week in 3-4 week cycles, to achieve plasma C_{\text{max}} \textgreater 700 nM (54, 56, 57, 86), leading to a recommended phase 2 dose of 60 mg (~35 mg/m\textsuperscript{2}) 2X/week in 4 week cycles. The
molecular mechanism information in this report suggests an alternative application of selinexor or its more potent analogs that selects for patients with refractory/relapsed NPM1-mutated AML, and then uses significantly lower, better tolerated doses for a defined molecular pharmacodynamic objective of locking PU.1 in the nucleus, and for a downstream pathway objective of activating monocytic terminal-differentiation - cell cycle exits by terminal-differentiation can spare normal HSC (good therapeutic index) and do not require the master transcription factor regulator of apoptosis (cytotoxicity) p53 (40, 63, 65, 67, 87). Standard anti-metabolite/cytotoxic therapy on the other hand intends to upregulate p53, a goal undermined by genetic inactivation of p53 (e.g., by TP53 mutation/deletion, MDM2/4 gain) in many AMLs that confers resistance to clinical concentrations of cytotoxic drugs both in vitro and in vivo, even as the same concentrations destroy normal HSC (poor therapeutic index) (3, 88).

Having said this, the time-to-distress benefit of pharmacodynamically directed dosing of selinexor in a patient-derived xenotransplant model of NPM1/FLT3-mutated AML, a sub-set of NPM1-mutated AMLs with especially poor prognoses, was limited. This could reflect that the AML cells contained other genetic alterations, e.g., mutated FLT3, that arrest differentiation by other mechanisms - CEBPA is phosphorylated by the FLT3 pathway, modification that abrogates CEBP family interactions with coactivators (89), and impedes CEBPA-driven granulocytic differentiation (90). Underscoring this theme, corepressors (e.g., DNMT1), that oppose coactivators to repress rather than activate genes, were enriched in the protein interactomes of nuclear CEBPA and RUNX1 in NPM1-mutated AML cells, and altogether >500 granulocyte and monocyte terminal-differentiation genes were repressed. We showed previously that PU.1 and RUNX1 collaborate by excluding corepressors and recruiting coactivators (20, 21), a motif akin to corepressor/coactivator exchange at nuclear receptors upon binding of their cognate ligands. Accordingly, PU.1 introduction into the CEBPA/RUNX1 interactomes by selinexor toggled CEBPA/RUNX1 interactions from corepressors to coactivators. Directly depleting the corepressor DNMT1 from the CEBPA/RUNX1 interactome using non-cytotoxic concentrations of the clinical drug decitabine also rebalanced toward coactivators, and activated terminal granulocytic fates, as expected with a low nuclear PU.1/CEBPA ratio (23). Moreover, NPM1 expression naturally declines with granulocytic differentiation, possibly explaining why this treatment also decreased mutant-NPM1 and increased nuclear PU.1. Thus, leukemogenic alterations to RUNX1, FLT3, KMT2A and now NPM1 have been shown to converge onto
corepressor/coactivator imbalance in the PU.1/CEBPA/RUNX1 master transcription factor hub as a common final pathway by which terminal-differentiation is repressed in replicating progenitors (reviewed in (40)), explaining meaningful clinical activity of non-cytotoxic DNMT1-depletion by decitabine (or its pro-drug 5-azacytidine) in patients with myeloid malignancies containing sundry mutations and translocations (20, 21, 65-67, 91-94), and likely contributing to the greater efficacy of this approach than selinexor alone in the in vivo model containing both NPM1 and FLT3 mutations. Underscoring the importance of the targeted pathways to the malignant phenotype, resistance in vitro and in vivo was by AML cells that evaded nuclear export inhibition by selinexor and DNMT1-depletion by decitabine/5-azacytidine (58-63).

Altogether, knowledge that mutant-NPM1 dislocates PU.1 into cytoplasm can guide pharmacodynamically-directed dosing of clinical small molecules to reverse the dislocation and its effects on coactivator/corepressor content in the granulomonocyte master transcription factor hub, to thereby resume journeys of NPM1-mutated AML cells to terminal monocytic or granulocytic fates. This non-cytotoxic (p53-independent) pathway of action distinguishes such treatments from conventional cytotoxic induction/consolidation, suggesting evaluation would be appropriate in the ~50% of patients with NPM1-mutated AML and chemo-refractory disease.
METHODS

See also Supplementary Methods for methods we have previously described

Sources of cell lines and animals. OCI-AML2 and OCI-AML3 were purchased from DSMZ (Braunschweig, Germany), and THP1 cell lines were purchased from ATCC (Manassas, Virginia). IMS-M2 were a gift from Drs Maria Paola Martelli and Brunangelo Falini. The cell lines, including OCI-AML3 selected for resistance to selinexor, were additionally authenticated (Genetica cell line testing, Burlington, NC). NSG mice were purchased from Jackson Laboratories (Bar Harbor, Maine).

Immunofluorescence microscopy. Cells were cyto-spinned on to glass slides and fixed in cold methanol for 30 min 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 PU.1 (1:100, sc-352, Santa Cruz Biotechnology) and NPM1 (1:100, sc-47725, Santa Cruz Biotechnology). Cells were washed in 1% goat serum with 0.1% Tween 20 followed by incubation with secondary antibodies, Alexa Fluor 488 Goat Anti-Mouse (1:250, A1101, Invitrogen) or Alexa Fluor 568 Goat Anti-Rabbit IgG (1:500, A11036, Invitrogen), for one hour at room temperature in the dark. Cells were washed in 1% goat serum with 0.1% Tween 20 and 1X PBS. Nuclei of cells were stained with 0.25 μg/mL DAPI (4′,6-diamidino-2-phenylindole) for 3 minutes before mounting with fluorescence mounting medium (DAKO). Images were taken with Leica DM RBE microscope, connected to CRI Nuance multispectral imaging camera, running Nuance version 3.0.2 software (PerkinElmer). Confirmatory images were acquired using Leica SP8 inverted confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany) running Leica Application Suite X software. Of note, we found that paraformaldehyde fixation of cells (fixation with 4% paraformaldehyde for 10 min at room temperature, washed with 1X PBS and incubated with 0.2 % Tween-100 for another 10 min at room temperature) did not permit immunofluorescence detection of PU.1.

Treatment of a patient-derived xenotransplant model of dual NPM1/FLT3-mutated AML with nuclear-export inhibition and/or DNMT1-depletion. Patient-derived primary AML cells containing both NPM1 and
FLT3-ITD mutations were obtained from PROXE (catalogue#: DFAM-61786-V2)(55). AML cells were transplanted by tail-vein injection (3.0 x10⁶/mouse) into non-irradiated 6-8 week old NSG mice. Mice were anesthetized with isofluorane before transplantation. After confirmation of bone marrow AML engraftment to ≥20% in at least 3 randomly selected mice, remaining mice were randomized to treatment with vehicle (PBS), nuclear export inhibition by oral selinexor 2-5 mg/kg 4X/week (n=5 per group), DNMT1-inhibition (depletion) by tetrahydrouridine (THU) 10 mg/kg given intra-peritoneal followed by subcutaneous decitabine 0.1 mg/kg or subcutaneous 5-azacytidine 1 mg/kg 3X/week (the decitabine and 5-azacytidine were alternated each week; the subcutaneous route of administration of decitabine and 5-azacytidine was to produce low Cmax and long Tmax suited to non-cytotoxic DNMT1-depletion; tetrahydrouridine was used to inhibit cytidine deaminase that otherwise rapidly deaminates decitabine and 5-azacytidine in vivo (59-61, 67)). Tail-vein blood samples for blood count measurement by HemaVet were obtained prior to leukemia inoculation, and at intervals thereafter as indicated in the figures. Mice were observed daily for signs of pain or distress, e.g., weight loss that exceeded 20% of initial total body weight, lethargy, vocalization, loss of motor function to any of their limbs, and were euthanized by an IACUC approved protocol if such signs were noted.

**Bioinformatic and statistical analysis.** Protein interaction networks were constructed using Cytoscape 3.4. Briefly, identified proteins were represented as nodes in the network. The size of each node relates to the normalized relative quantification value as defined in ‘Label free relative protein quantitation (LFQ)’: protein node shape was set to ‘circle’, the length and width (diameter) of the circle were formatted by the continuous mapping function of the software to represent the normalized relative quantification value. Physical protein-protein interaction networks were predicted using STRING v 10.0 with high confidence (parameter value 0.70). Predicted protein-protein interactions were represented as Edges/Links connecting protein nodes, the thickness of each edge represented the statistical significance of the string prediction (Table S6, S7). Different colors were assigned to protein function complexes with blue for transcription factors, green for coactivators and red for corepressors.

Myeloid-commitment, monocyte and granulocyte terminal-differentiation genes were identified by applying Comparative Marker Selection (V10), Morpheus (https://software.broadinstitute.org/morpheus/)
Institute, MIT, USA)(95), an algorithm for identifying genes that discriminate between classes of samples, to a public database of gene expression at different stages of hematopoiesis (GSE24759(51)), to identify probes which significantly discriminated (500 probes in each direction) between CMP/GMP vs HSC/monocytes/granulocytes (commitment genes), monocytes vs HSC/CMP/GMP (monocyte terminal-differentiation genes) and granulocytes vs HSC/CMP/GMP (granulocyte terminal-differentiation genes). Statistical significance was determined by the 1000 permutations test and a p-value cutoff of ≤0.02. Expression of the commitment and monocyte differentiation genes was correlated with PU.1 expression in the same samples (Pearson correlation coefficient).

Expression of myeloid master transcription factors and myeloid differentiation programs in AML cells versus the normal hematopoietic hierarchy were compared using an integrated dataset, BloodPool, that we assembled and built as described previously (48, 49).

GEO Database numbers for analyzed ChIP-seq reads were (52, 96): GSM538017 (Pu.1 ChIP-seq in bone marrow macrophages), GSM537983 (Pu.1 ChIP-seq in peritoneal macrophages), and GSM1692857 (Pu.1 ChIP-seq in hematopoietic progenitors). Aligned ChIP-Seq reads were imported, analyzed, and visualized using Easeq (97). All values were normalized to reads per million per 1kbp.

Wilcoxon rank sum, Mann Whitney, and t tests were 2-sided and performed at the 0.05 significance level or lower (Bonferroni corrections were applied for instances of multiple parallel testing). Standard deviations (SD) and inter-quartile ranges (IQR) for each set of measurements were calculated and represented as y-axis error bars on each graph. Graph Prism (GraphPad, San Diego, CA) or SAS statistical software (SAS Institute Inc., Cary, NC) was used to perform statistical analysis including correlation analyses.

**Study approvals.** Bone marrow samples for research were obtained from patients with AML on a study protocol approved by the Cleveland Clinic Institutional Review Board (Cleveland, Ohio), with written informed consent prior to inclusion in the study. Experiments using patient-derived xenotransplant models of AML were approved by the Cleveland Clinic Institutional Animal Care and Use Committee (Cleveland, Ohio).

**SUPPLEMENTAL INFORMATION**

Supplementary Methods; Supplementary Excel Files (table S1-S7); Supplementary Figures 1-18.
AUTHOR CONTRIBUTIONS

XG and YS generated hypotheses, designed experiments and obtained funding. XG, QE, RM, MH, FE, TR, BP, NR, BP, BTP and CVC carried out experiments, generated and/or analyzed data. QE, RB, YL and BKJ also contributed to experimental design. ZH, DW, YL, MPM, BF, HC, JM and YS provided cells and reagents. XG and YS wrote the manuscript. All authors reviewed/commented on the work/manuscript.

ACKNOWLEDGMENTS

We thank the Harinder Singh laboratory for the gift of PUER cells. This work was supported by grants Velosano to X.G., P30 CA043703 from NIH/NCI and philanthropic funds from the James Oberle family, Robert and Jennifer McNeil, Leszek and Jolanta Czarnecki, and Dane and Louise Miller to Y.S., and Associazione Italiana Ricerca Cancro, AIRC IG 2016 n. 18568 and ERC Adv Grant 2016 n. 740230 to B.F.. Work in the B.P. Lab was supported through a centre grant from the NovoNordisk Foundation (Novo Nordisk Foundation Center for Stem Cell Biology, DanStem; Grant Number NNF15CC0027852). Karyopharm Therapeutics provided the selinexor compound.
REFERENCES


Figure 1: The NPM1 interactome includes the master transcription factor PU.1 which is cytoplasmically dislocated along with mutant-NPM1 in NPM1-mutated AML cells. A) Transcription factors pulled down with NPM1 and mutant-NPM1 from nuclear (N) and cytoplasmic (C) fractions of wildtype (wt) and NPM1-mutated (mut) AML cells. Endogenous NPM1 and mutant-NPM1 were immunoprecipitated from nuclear and cytoplasmic fractions.
cytoplasmic fractions of wildtype (THP1) and NPM1-mutated AML cells (OCI-AML3) and protein interactions were analyzed by LCMS/MS. Only interactome transcription factors are shown (additional data in Table S1). 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) NPM1 and PU.1 interaction in nuclei of wildtype AML cells, and in cytoplasm of NPM1-mutated AML cells, was also evident by immunoprecipitation-Western blot (WB). Blue boxes indicate expected locations of NPM1 and PU.1 if in nuclear fractions of NPM1-mutated AML cells. C) Immunofluorescence microscopy (IF) for NPM1 and PU.1 in wildtype (OCI-AML2, THP1, NB4) and NPM1-mutated (OCI-AML3, IMS-M2) AML cell lines. Nuclei were stained with DAPI. Images by Nikon Eclipse 400 microscope, magnification 630X. Secondary antibody alone controls are shown in Fig S1. D) IF for NPM1 and PU.1 in wildtype and NPM1-mutated AML primary cells from patients' bone marrow. Images by Nikon Eclipse 400 microscope, magnification 630X. Secondary antibody alone controls are shown in Fig S1. E) WB for PU.1, RUNX1, CEBPA and NPM1 in nuclear and cytoplasmic fractions of wildtype and NPM1-mutated AML cell lines. Blue boxes indicate expected locations of NPM1 and PU.1 in nuclear fractions of NPM1-mutated AML cells, red boxes highlight location in cytoplasm of these cells instead.
Figure 2. AML cells highly express the PU.1/RUNX1/CEBPA master transcription factor circuit that drives cells to terminal granulo-monocytic fates, but the monocyte-differentiation program is suppressed. A) Expression of granulo-monocytic (CEBPA, RUNX1, CEBPA) and HSC (HLF, PBX1, PRDM5) master transcription factors during normal myelopoiesis and in cytogenetically normal AML (CNAML). Gene expression data was integrated and normalized as previously described (48, 49). Bars = median±IQR, whiskers = range. HSC n=6, MPP n=2, CMP n=3, GMP n=7, neutrophils n=3, monocytes n=4, CNAML n=989. B) Negative correlation between myeloid-commitment and PU.1 gene expression, but positive correlation between monocyte-differentiation and PU.1 gene expression (Pearson correlation coefficients). Comparative Marker Selection (Morpheus, Broad) analysis of gene expression in HSC, CMP, GMP, colony
forming unit monocytes (CFUM) and monocytes from GSE24759 (51) identified ~200 myeloid commitment and ~300 terminal monocytic differentiation genes. MYC target genes identified by others using ChIP-Seq (98), validated by separate analyses (Figure S4). Also, Pu.1 localized at monocyte-differentiation but not commitment genes by ChIP-Seq (Figure S4). Gene sets were also validated in our separate gene expression database of normal hematopoiesis (Figure S5). Gene lists in Table S2-S4. C) CNAML expresses monocyte-differentiation genes at levels higher than in normal HSC, CMP or GMP, but ~3-fold lower than seen in normal monocytes. 100 CNAML shown (truncated from 989 analyzed)(49). p-values 2-sided Mann-Whitney test. D) NPM1, RUNX1 and bi-allelic CEBPA mutations in CNAML are highly recurrent but mutually exclusive. n=101, TCGA.
Figure 3. Two models were used to show that mutant-NPM1 dislocates PU.1 into cytoplasm, and that PU.1 nuclear relocation in Pu.1-null myeloid precursors represses key precursor genes (e.g., Hoxa9) and activates terminal monocytic fates. A) Mutant-NPM1, but not NPM1, translocates PU.1 into cytoplasm. HEK-293 cells were co-transfected with expression vectors for NPM1 and PU.1 or mutated-NPM1 (exon 12 TCTG insertion) and PU.1. After staining with anti-NPM1 (α-NPM1) and anti-PU1 (α-PU.1) antibodies, IF was used to evaluate cellular location of NPM1 and PU.1. Images by Nikon Eclipse 400 microscope, magnification 630X. B) Addition of estrogen (OHT) translocates Pu.1 into the nucleus in Pu.1-knockout (Pu.1−/) myeloid precursors retrovirally transduced to express Pu.1 fused with the estrogen receptor (Pu.1-ER) (53), activating terminal monocytic fates (Figure S7) and suppressing Hox gene expression. Hox gene expression measured by QRT-PCR, mean±SD three independent experiments. *p<0.01 (significant after Bonferroni correction) 2-sided t-test 12h vs 0h. C) Master transcription factor and HOX gene expression in NPM1-mutated and wildtype AML cells. Gene expression by RNA-sequencing, primary AML bone marrow cells, TCGA. D) Negative correlation between HOX and PU.1 gene expression in normal myelopoiesis. Gene expression in normal hematopoietic hierarchy from GSE24759 (HSC n=14, CMP n=4, GMP n=4, monocyte colony forming units n=4, monocytes n=5)(51). Pearson correlation coefficients.
Figure 4. The nuclear export inhibitor selinexor sequestered both mutant-NPM1 and PU.1 in nuclei of NPM1-mutated AML cells. A) Experiment schema. Cell fate outcomes are shown in Figure 5. B) Selinexor rapidly relocalized mutant-NPM1 and much of PU.1 into nuclei of NPM1-mutated AML cells. Wildtype (THP1) and NPM1-mutated AML cells (OCI-AML3, IMS-M2) were treated with selinexor 20 nM and cell fractions (cytoplasm=C, nucleus=N, nuclear matrix=NM) were evaluated by Western blot. Blue boxes show expected location of NPM1 and PU.1 in nuclear fractions. C) Immunofluorescence for NPM1 and PU.1 in vehicle vs selinexor treated NPM1-mutated AML cells. DAPI was used to stain for nuclei. Images by Nikon Eclipse 400 microscope, magnification 630X.
Figure 5. Nuclear retention of mutant-NPM1 and PU.1 by selinexor (Sel) triggered terminal monocytic differentiation of NPM1-mutated (mNPM1), but not wildtype (wNPM1), AML cells. A) Cell counts of mNPM1 (OCI-AML3, IMS-M2) and wNPM1 (OCI-AML2, THP1) AML cells. Decitabine (DEC) was used to deplete DNMT1. Cell counts by automated counter. Mean±SD of three independent experiments. *p<0.01 (significant after Bonferroni correction), t-test 2-sided Sel or Dec vs Veh at Day 5; n.s. p>0.025. B) Protein levels of MYC (master transcription factor driver of proliferation) and p27/CDKN1B (cyclin dependent kinase inhibitor mediating cell cycle exits by differentiation). Western blot. C) Monocyte lineage marker CD14 and granulocyte lineage marker CD11b expression. Flow cytometry. D) Cell morphology. Day 5. Giemsa-stain. Leica DMR microscope, 630X. Quantified in Figure S8. E) Macrophage colony stimulating factor receptor (MCSFR/CSF1R) or granulocyte colony stimulating factor receptor (GCSFR/CSF3R) expression. QRT-PCR, multiple primer sets were used for each gene (#1-3/4). Mean±SD three independent experiments. *p<0.01 (significant after Bonferroni correction), 2-sided t-test Sel vs Veh; n.s. p>0.0125.
Figure 6. The differentiation-restoring effect of selinexor in vivo was saturated at a dose of 2 mg/kg. Selinexor 2 mg/kg was compared to 5 mg/kg in a patient-derived xenotransplant model of dual NPM1/FLT3-mutated AML.  

A) Experiment schema. After confirmation of bone marrow AML engraftment to ≥20% in 3 randomly selected mice, remaining mice were randomized to vehicle, selinexor 2 mg/kg, or selinexor 5 mg/kg, by oral gavage 4X/week starting on day 21 (n=5/group). Treatment continued until signs of distress in vehicle treated mice (day 75) when the experiment was terminated for analyses.

B) Immunofluorescence microscopy for PU.1 and NPM1 location in bone marrow AML cells. DAPI was used to stain for nuclei. Images by Leica SP8 inverted confocal microscope, magnification 630X.  

C) Bone marrow AML burden. Flow cytometry for human CD45+ (AML) and murine CD45+ (normal) cells. Median±IQR. p-values Mann-Whitney test, 2-sided. Significance after Bonferroni correction is p<0.025.

D) Spleen AML burden. Median±IQR. p-values 2-sided Mann-Whitney test. Normal NSG spleen weight is ~0.018 g.

E) Serial blood counts. Increasing WBC were circulating myeloblasts. Tail-vein phlebotomy, blood counts by HemaVet. Mean±SD,*p<0.01 (significant after Bonferroni correction), Sel2 or Sel5 vs Veh at day 75, 2-sided t-test.

F) CD14 monocyte-lineage differentiation marker expression on bone marrow AML cells. Flow cytometry. Median±IQR. p-values Mann-Whitney test, 2-sided. Significance after Bonferroni correction is p<0.025.

G) γ-H2AX apoptosis/DNA-damage marker expression on bone marrow AML cells. Flow cytometry. Median±IQR. p-values 2-sided Mann-Whitney test (n.s. = p>0.025).
Figure 7. Coregulator interactions of nuclear CEBPA in NPM1-mutated AML cells. Endogenous CEBPA was affinity purified from nuclear fractions of OCI-AML3 cells, coregulator (coactivator and corepressor) interactions were analyzed by LCMS/MS and Western blot, and suggested CEBPA interactions in this context were biased toward corepressors. Quantification in Table S6.
Figure 8. Impact of PU.1 nuclear retention by selinexor, or DNMT1-depletion by decitabine, on coregulator interactions of nuclear CEBPA in NPM1-mutated AML cells. Selinexor 20 nM or decitabine 0.25 μM was added to OCI-AML3 cells at 0 and 24 hours, and cells were harvested at 48 hours. Endogenous CEBPA was affinity purified from nuclear fractions and coregulator interactions analyzed by LCMS/MS and Western blot. Quantification in Table S6. A) Depletion of the corepressor DNMT1 by decitabine, or nuclear retention of PU.1 by selinexor, rebalanced toward coactivators. B) Relative abundances of coregulator complexes with vehicle vs treatments. The individual proteins constituting the complexes are listed in panel A. Median±IQR. *p-value<0.0125 (significant after Bonferroni correction), Mann-Whitney test, 2-sided. C) CEBPA immunoprecipitation-Western blot to show co-immunoprecipitating master transcription factors (PU.1, RUNX1), a coactivator (PBRM1) and a corepressor (DNMT1). NPM1-mutated (OCI-AML3) and wildtype (THP1) AML cells.
Figure 9. Depletion of DNMT1 from the CEBPA/RUNX1 interactome by decitabine (0.25 µM/day x2) induced granulocyte/monocyte differentiation, while PU.1 nuclear retention by selinexor (20 nM/day x5) induced monocytic differentiation of NPM1-mutated AML cells (OCI-AML3). THP1 are NPM1-wildtype AML cells with high nuclear content of both PU.1 and CEBPA. 

A) Expression of the granulocyte lineage marker CD11b and the monocyte lineage marker CD14 in NPM1-mutated or wildtype AML cells treated with decitabine or selinexor. Flow cytometry on day 5. 

B) Cell morphology. Day 5. Giemsa-stain. Leica DMR microscope, 630X. Quantified in Figure S8. 

C) Expression of the granulocyte colony stimulating factor receptor (GCSFR, CSF3R) and macrophage colony stimulating factor receptor (MCSFR). QRT-PCR, day 5. Mean±SD, three independent experiments. Results with selinexor are in Figure 5. *p-value<0.01 (significant after Bonferroni correction), Dec vs Veh, 2-sided t-test. 

D) NPM1 mRNA expression during normal myelopoiesis. Gene expression data was integrated and normalized as previously described (48, 49). Mean±SD, p-value 2-sided t-test. 

E) NPM1 decreased, and nuclear PU.1 increased, after decitabine treatment. Serial Western blots nuclear and cytoplasmic fractions of NPM1-mutated AML cells OCI-AML3 after treatment with decitabine 0.25 µM on Day 0 and 1 (additional data Figure S13).
Figure 10. Combination differentiation-restoring treatment in vivo. A) Experiment schema. Immune-deficient mice were xenotransplanted with \( NPM1/FLT3 \)-mutated primary AML cells (55). After bone marrow engraftment to \( \geq 20\% \) AML was confirmed in 3 randomly selected mice, mice were randomly assigned to treatment with (i) vehicle; (ii) nuclear-export inhibition - selinexor 2 mg/kg 4X/week oral gavage; (iii) DNMT1-depletion - decitabine 0.1 mg/kg 3X/week alternating with 5-azacytidine 1 mg/kg 3X/week subcutaneous, combined with tetrahydrouridine 10 mg/kg intra-peritoneal (to inhibit in vivo degradation of decitabine/5-azacytidine by cytidine deaminase); or (iv) combination nuclear export-inhibitor/DNMT1-depletion. Mice were euthanized for signs of distress. B) Serial blood counts. Increasing WBC was by myeloblasts (inset). Tail-vein
phlebotomy, blood counts by HemaVet. Mean±SD. C) Survival (time-to-distress). p-values Log Rank test. D) Spleen AML burden at euthanasia. Median±IQR. *p<0.01, n.s. p>0.01 2-sided Mann-Whitney test. Photos show a spleen from a normal NSG mouse vs a vehicle-treated mouse for comparison, with H&E stained spleen sections showing AML infiltration (yellow arrows) and necrosis (white arrows) (magnification 400X). Normal NSG spleen weight is ~0.018 g. E) Cell cycle distribution of marrow AML cells at euthanasia. Mean±SD for percentage of cells in each cell cycle phase. p-value unpaired t-test 2-sided. Raw data Figure S14. F) Monocyte (CD14) and granulocyte (CD11b) lineage-differentiation marker expression in marrow AML cells at euthanasia. Flow cytometry. Median±IQR. *p<0.01 (significant after Bonferroni correction), n.s. p>0.01, Mann-Whitney test, 2-sided. Raw data Figure S15, S16. G) Morphology of marrow AML cells at euthanasia. Giemsa-stain. Leica DMR microscope, 630X. H) Apoptosis/DNA-damage marker γ-H2AX expression in marrow AML cells at euthanasia. Flow cytometry. Median±IQR. *p<0.01 (significant after Bonferroni correction), n.s. p>0.01, Mann-Whitney test, 2-sided. Raw data including positive control Figure S17.
Figure 11. Resistance is by avoidance of selinexor-induced nuclear relocation of mutant-NPM1/PU.1. OCI-AML3 NPM1-mutated cells were selected for resistance to selinexor by culture in selinexor up to 50 nM added every 3 days. A) NPM1 and PU.1 localization in the resistant cells by IF. DAPI was used to stain for nuclei. Images by Leica SP8 inverted confocal microscope, magnification 630X. B) NPM1 and PU.1 cytoplasmic vs nuclear localization in the resistant cells. Western blot of nuclear and cytoplasmic fractions. C) Parental and selinexor-resistant OCI-AML3 cells were sensitive to non-cytotoxic concentrations of decitabine. Cell counts by automated counter. Mean±SD for 3 independent experiments. *p<0.01 (significant after Bonferroni correction), Sel or Dec vs Veh at day 7, 2-sided t-tests.
<table>
<thead>
<tr>
<th>A) Normal myelopoiesis: Committed myeloid precursor</th>
<th>B) AML: Mutant-NPM1 dislocates PU.1 into cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Exponential proliferation</td>
<td>Exponential proliferation is decoupled from differentiation</td>
</tr>
<tr>
<td>2. Terminated by differentiation</td>
<td></td>
</tr>
<tr>
<td>[Diagram]</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 12. Summary.**

**A)** Master transcription factors PU.1/CEBPA/RUNX1 collaborate to recruit coactivators and activate granulo-monocytic differentiation genes. **B)** Cytoplasmic dislocation of PU.1 by mutant-NPM1 disrupts the collaboration, causing corepressor recruitment to nuclear CEBPA/RUNX1, and repression instead of activation of differentiation genes. **C)** Inhibiting nuclear export with selinexor retains mutant-NPM1/PU.1 in nuclei and activates monocyte differentiation genes, as expected with a high nuclear PU.1/CEBPA ratio. **D)** Inhibiting corepressors (e.g., DNMT1) recruited to nuclear CEBPA/RUNX1 activates granulocyte differentiation genes, as expected with a low nuclear PU.1/CEBPA ratio. The granulocytic direction of differentiation moreover naturally downregulates mutant-NPM1 to promote eventual PU.1 nuclear-retention.