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A Pin1/PML/P53 axis activated by retinoic acid in NPM-1c-acute myeloid leukemia

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Running heads: RA/arsenic activate a PML/P53 axis in NPM-1c-AML

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**Abstract**

Retinoic acid (RA) was proposed to increase survival of chemotherapy-treated Nucleophosmin-1 mutated Acute Myeloid Leukemia patients (NPM-1c AMLs). We reported that *ex vivo*, RA triggers NPM-1c degradation, P53 activation and growth arrest. PML organizes domains that control senescence or proteolysis. Here, we demonstrate that PML is required to initiate RA-driven NPM-1c degradation, P53 activation and cell death. Mechanistically, RA enhances PML basal expression through inhibition of activated Pin1, prior to NPM-1c degradation. Such PML induction drives P53 activation, favoring blasts response to chemotherapy or arsenic *in vivo*. This RA/PML/P53 cascade could mechanistically explain RA-facilitated chemotherapy response in NPM-1c AML patients.
**Introduction**

Nucleophosmin 1 (NPM-1) is a chaperone implicated in multiple processes, notably ribosomal biogenesis and growth control. *NPM-1* alterations were directly implicated in cancer development, through a variety of mechanisms, including chromosomal translocations or recurrent mutations [1]. In acute myeloid leukemia (AML), the most prevalent one is a short nucleotide insertion that induces a frame shift in the C-terminus of the protein, yielding NPM-1c [2]. Multiple properties were demonstrated for NPM-1c, including P53 inhibition or cytoplasmic sequestration of key regulatory proteins [3,4]. AML with NPM1-mutation is the most common subtype among AML (>1/3). More than half of the patients ultimately relapse when treated with conventional chemotherapy. NPM-1-associated AMLs in relapsed or aged patients unfit for chemotherapy represent a major unmet medical need.

Retinoic acid (RA) is a hormone with multiple effects on development and tissue homeostasis. RA has a dual effect on stem cell fate and differentiation following modulation of transcription. High doses of RA were also shown to inhibit Pin1, a protein-modifying enzyme involved in the activation of multiple growth suppressive pathways [5,6]. RA demonstrated unambiguous clinical efficacy in a variety of conditions including neuroblastoma and acute promyelocytic leukemia (APL) [7]. In APL, RA directly targets the driving PML/RARA oncoprotein for degradation and yields complete remissions (CR)[8] through activation of a PML/P53 senescence checkpoint [9,10]. In other AMLs, *in vitro* studies suggested some efficacy of RA [11,12]. However, contradictory results were reported in clinical trials that tested RA efficacy in non-APL AMLs [13]. Whereas the Austrian-German (AML HD98B) study reported that addition of RA to intensive
chemotherapy improved remission, event-free survival, and overall survival (OS) [13], the United Kingdom Medical Research Council (MRC) trial failed to demonstrate any overall advantage of adding RA to chemotherapy [14,15].

Intriguingly, the potential benefit of RA co-administration with chemotherapy seems restricted to patients bearing an NPM-1c mutation in the absence of fms-like tyrosine kinase 3 internal tandem duplication (FLT3/ITD) [16]. Whether this efficacy reflects RA-induced AML differentiation, as observed in several non-APL AML primary patient cells or models [11,12] remains to be elucidated. Interestingly, NPM-1c is degraded upon RA administration, suggesting that loss of NPM-1c expression may underlie, or at least contribute to, RA-driven differentiation and apoptosis ex vivo [17,18]. Ex vivo, NPM-1c degradation was accelerated by co-administration of arsenic trioxide (ATO), which similar to RA, may inhibit Pin1, a protein-modification enzyme implicated in growth control through multiple mechanisms [5,6,19]. Overall, the actual mechanism(s) of RA-enhancement of chemotherapy response in NPM-1c- AMLs remain to be elucidated.

PML (TRIM19) nucleates nuclear bodies (NBs) which are stress-responsive domains that exert growth suppressive properties [20]. In vivo, PML NBs are oxidative stress sensors controlling P53 activation [21]. PML plays a key role in the therapeutic response of APL [9, 22-25]. PML expression is altered in multiple tumor types, most often through PML protein loss upon activation of several degradation pathways, including Pin1 [26-30]. Interestingly, we previously reported impairment of PML NB formation in NPM-1c-driven AMLs [17,18].
Here, we unravel an unexpected role of PML in RA initiated responses of NPM-1c AML cells. PML is required to initiate RA-driven NPM-1c degradation, P53 activation and cell death. Mechanistically, RA stabilizes PML through inhibition of overexpressed and activated Pin1, enforcing growth arrest. Such RA-induced activation of the PML/P53 signaling cascade enhances chemotherapy or arsenic activity in vivo. Our studies identify PML as an unsuspected actor downstream of RA in NPM-1c AMLs.

Methods

Cell lines and treatments

OCI-AML3 or OCI-AML2 AML cells (harboring the NPM-1c mutation without FLT3-ITD or wild type (wt) NPM-1 respectively) were grown in minimum essential medium–α (MEM α) supplemented with 20% fetal bovine serum (FBS) and antibiotics. Cells were seeded at the density of 2 x 10⁵/ml.

RA (Sigma Aldrish) was used at 1 μM final concentration. The Pin1 inhibitor AG17724 (Sigma Aldrish) was used at 20 μM. Doxorubicin (Ebewe Pharma) or cytarabine (AraC) (Alexan, Ebewe Pharma) were used at 1 μM final concentration. Cell growth was assessed using the trypan blue dye assay.

Patient cells

Primary bone marrow (BM) blasts from AML patients were extracted following Ficoll separation and cultured in MEM-α supplemented with 20% FBS and antibiotics. Patients’ samples were collected following approval by the American University of Beirut.
Institutional Review Board and after patients provided written informed consent in accordance with the declaration of Helsinki. Patients characteristics are summarized in Supplementary table 1.

Two AML patients with NPM-1c who were judged unfit for conventional chemotherapy, received off-label compassionate RA (25 mg/m² daily) and ATO (0.15mg/kg daily) after informed consent.

**CRISPR OCI-AML3 cell lines**

PML expression was abrogated by CRISPR-mediated excision. A guide RNA targeting *PML* (Forward: 5’-GTCGGTGTACCGGCAGATTG; Reverse: 5’-AATCTGCGGTACACCGAC) was designed and cloned into pLAS5w.Ppuro-Cas9 plasmid for viral packaging. OCI-AML3 cells were infected with the corresponding viruses. Stable selection of knock-out cells was performed in the presence of 1μg/ml of puromycin, over a period of 2 weeks. Three OCI-AML3*^pml^- clones were generated and tested in this study. Similarly, P53 extinction was performed using a guide RNA targeting *P53* (Forward: 5’-CCATTGTTCAATATCGTCCG; Reverse: 5’-CGGACGATATTGAACAATGG). Recombinant Cas9 protein was synthesized from IDT to form Alt-R CRISPR/Cas9 RNP. OCI-AML3 cells were transiently transfected with Alt-R CRISPR/Cas9 RNP by using Nucleofector kit T (Amaxa) and applied program number X-01 in the nucleofector device (Lonza). The stable CRISPR knock-out clones were cloned by serial dilution to generate a single-cell separation. DNA from individual clones was extracted and the region surrounding the Cas9 cutting site was amplified by PCR and verified by sequencing to ensure the deletion of the target genes. Two OCI-AML3*^p53^- clones were generated and tested in this study.
Pin1 knock-down was performed using all in one plasmid pLAS5w.Ppuro-Cas9-gPin1. Lentiviruses were produced by transient transfection of HEK-293T cells. Infection of OCI-AML3 cells was performed by spinoculation for 90 minutes at 28000 rpm at 32°C. Stable selection of knock-out cells was performed in the presence of 1μg/ml of puromycin. Single cells were sorted in 96 well plate to have stable clones. Knock-out stable clones were verified by immunofluorescence and by western blot using the polyclonal anti-Pin1 antibody (cell signaling).

Statistical analysis

Data was reported as the average ± standard deviations. Statistical analysis was done using Student’s t test, p-value of less than 0.05 was considered as significant.

Immunoblotting, RNA Isolation and Quantitative RT-PCR, Microarray Analysis and Gene Set Enrichment analysis, Immunofluorescence and confocal microscopy, Pin1 activity assay, colony formation assay, Xenograft Animal Studies, Human CD45 staining and cell sorting are described in the Supplementary methods section.

Results

PML-dependent NPM-1c degradation activates P53

We and others demonstrated that RA triggers NPM-1c degradation, P53 activation, apoptosis and induce PML NBs formation in NPM-1c-expressing AML cell lines [17,18]. That PML NBs may be implicated in therapy-induced catabolism of other
oncoproteins [34] prompted us to investigate any role of PML in RA-triggered NPM-1c degradation. We thus generated CRISPR PML OCI-AML3 cell lines (Fig. 1A, Supplementary Fig. S1A). In these OCI-AML3<sup>pml/-</sup> cells, RA-mediated NPM-1c degradation was blocked (Fig. 1A, Supplementary Fig. S1A) and RA-induced cell death was abrogated (Fig. 1B, Supplementary Fig. S1B). When assessing the transcriptional effects of RA treatment in OCI-AML3 and control OCI-AML2 cells, a clear P53 signature was noted in OCI-AML3 cells (Supplementary Fig. S1C-E), in line with RA-induced P53 stabilization [17, 18]. To assess any role of P53 in cell death upon RA exposure, we also generated CRISPR P53 OCI-AML3 (OCI-AML3<sup>P53/-</sup>) cell lines (Fig. 1A, Supplementary Fig. S1F). In this model, again RA failed to initiate cell death (Fig. 1B, Supplementary Fig. S1G), although it efficiently degraded NPM-1c (Fig. 1A, Supplementary Fig. S1F). Collectively, RA-triggered, PML-facilitated, NPM-1c degradation likely explains P53 activation and growth arrest of AML cells.

**RA targets P53 prior to NPM-1c loss**

Further investigating the kinetics of response to RA, we unexpectedly obtained evidence for rapid P53 stabilization prior to any significant NPM-1c loss, but with a requirement for PML expression (Fig. 1C). Thus, NPM-1c loss is not the sole contributor to P53 activation. Remarkably, similar data were obtained upon ex vivo treatment of primary blasts derived from NPM-1c AML patients, where RA-triggered NPM-1c loss was only obtained after 48h, while P53 stabilization was generally observed as soon as 2h (Fig. 1D, E). Critically, such RA-triggered P53 activation was solely observed in NPM-1c AMLs patient samples (Fig. 1D, E). Thus, delayed NPM-1c degradation is most unlikely to explain early P53 activation upon RA-treatment.
RA stabilizes PML through Pin1 inactivation

RA also rapidly stabilized PML levels and induced PML NB-formation, solely in *NPM-1c*-positive patients' blasts or OCI-AML3 cells, with kinetics closely resembling those of P53 stabilization (Fig. 2A, Supplementary Fig. S2A-C). RA inconsistently enhanced *PML* gene expression, possibly through enhanced interferon production [35]. Previous studies have shown that RA inhibits the Pin1 enzyme and the latter regulates PML stability [5, 30, 36]. We thus compared the effects of RA and a Pin1 inhibitor (AG17724) on PML abundance, NB formation and P53 activation. Strikingly, RA or AG17724 similarly stabilized PML or P53 levels and promoted NB formation in *NPM-1c* AML patient blasts and OCI-AML3 cells (Fig. 2A-C, supplementary Fig. S2A-C). In contrast, *NPM-1-WT* AML cells were unresponsive to RA and AG17724 (Fig. 2A-C, Supplementary Fig. S2A). Pin1 inhibition did not initiate NPM-1c degradation (Supplementary Fig. S2D, S2E), implying that RA-induced PML stabilization is necessary, but not sufficient, to induce NPM-1c catabolism. Functionally, RA or AG17724 similarly lead to loss of clonogenic activity of OCI-AML3 cells in methylcellulose (Fig. 2D). To directly demonstrate Pin1 involvement in RA-response, we generated an OCI-AML3 cell-line with stable Pin1 down-regulation by ShRNA. Downregulation of Pin1 did not affect viability of *NPM-1c* AML cells (Supplementary Fig. 2F). Remarkably, PML and P53 or P21 activation by RA was abrogated following downregulation of Pin1 (Fig. 2E), and RA-induced cell death was lost (Supplementary Fig. 2F). Loss of clonogenic activity by RA or Pin1 inhibition were also abrogated (Fig. 2D). Thus, RA-induced activation of P53 and resulting growth arrest are initiated by Pin1 inhibition.
**PML is the primary target of RA and Pin1 inhibitors**

Pin1 directly controls both PML stability and P53 signaling [37]. PML and P53 are highly cross-regulated: PML controls P53 activation, but P53 transcriptionally induce PML expression [38, 39]. To decipher the respective roles of PML and P53 in response to Pin1, we compared RA and AG17724 response in OCI-AML3 and its pml-/- and P53-/- derivatives. Both drugs upregulated PML levels in P53-/- cells, and no (or minimal) induction of P53 was observed in OCI-AML3 pml-/- cells (Fig. 2F). These results establish that PML is the primary target of Pin1 inhibition in NPM-1c expressing cells. These results support a model wherein RA inactivates Pin1, to stabilize PML, induce NB formation, activate P53 and suppress growth. This does not exclude the possibility that P53 then constitutes a feed-forward amplification loop on PML expression.

**NPM-1c expressing cells exhibit high Pin1 basal level and activity**

The implication of Pin1 inhibition in RA-mediated effects in NPM-1c expressing cells prompted us to investigate Pin1 levels and activity. Strikingly, high Pin1 protein levels and activity were observed in NPM-1c expressing cell lines and primary blasts (Fig. 3A-C, Supplementary Fig3A). Treatment with RA significantly reduced Pin1 activity (Fig. 3C) without affecting Pin1 protein level (Supplementary Fig. S3B). In line with this increased Pin1 protein level and activity in NPM-1c expressing cells, both RA and AG17724 synergized with arsenic trioxide (ATO), and standard chemotherapy drugs in AML doxorubicine (Doxo) or cytarabine (AraC), to induce cell death in OCI-AML3 cells but not in OCI-AML2 cells (Fig. 3D).
RA and chemotherapy cooperate to clear NPM-1c-expressing cells *in vivo*

Clinical benefit of RA co-administration with chemotherapy seems restricted to AMLs bearing an *NPM-1c* mutation [16]. We thus examined the possibility of *in vivo* cooperation between RA and the standards of care of AML therapy: anthracyclins and cytarabine and examined any *PML*-dependency. We used xenografts from OCI-AML3 or OCI-AML3^{pml/-}, that were treated or not with RA for one week followed by either Doxo or AraC single agents, for one additional week. RA induced PML-dependent NPM-1c down-regulation and human P53 stabilization *in vivo* (Fig. 4A-D), while all three drugs ultimately induced human P53 phosphorylation (Fig. 4A, B). Importantly, RA synergized with Doxo or AraC to decrease abundance of human cells in treated mice, only in cells bearing intact PML (Fig. 4E-H). These observations suggest that, at least in this model, RA cooperates with chemotherapy to decrease AML burden, resembling the clinical observations made in AMLs.

**The RA/ATO combination has clinical activity in mice and AML patients**

RA and ATO trigger NPM-1c degradation, P53 activation and apoptosis in NPM-1c-expressing AML cell lines [17, 18]. RA upregulates PML, while ATO targets PML to enforce nuclear body formation and also inhibits Pin1 [6]. Thus, in principle, ATO could cooperate with RA through PML NB targeting [19]. To explore any *in vivo* relevance of these findings, we treated xenografts from OCI-AML3 or OCI-AML3^{pml/-} with RA and ATO for one week. RA/ATO induced PML-dependent NPM-1c degradation and human P53 stabilization *in vivo* (Fig. 5A) and decreased abundance of human cells in treated mice, again solely in cells bearing intact PML (Fig. 5B).
The combination of RA/ATO is a very well-tolerated therapeutic association in APL [40]. Two *NPM-1c* AML patients, unfit for conventional therapy, received this RA/ATO combination on an off-label compassionate basis. Blast clearance from peripheral blood and, to a lesser extent, from bone marrow were observed, although complete remission was not achieved (Fig. 5C). Longer follow-up after 2 months showed appearance of slowly growing AML cells. Thus, the RA/ATO combination may transiently target AML cells in some *NPM-1c* patients.

**Discussion**

The basis for RA-sensitivity of non-APL AMLs was initially believed to be RA-induced differentiation [11, 41]. Here, we report that PML constitutes an unsuspected actor downstream of RA and is required for its synergistic activity with other therapies in *NPM-1c* AMLs models. Previous *ex vivo* studies suggested that RA-driven NPM-1c degradation could be the molecular basis of its therapeutic activity through upregulation of ARF and resulting activation of P53. NPM-1c degradation should also correct multiple other phenotypes associated with NPM-1c, including sequestration of key regulators in the cytoplasm or transcriptional deregulation [4, 42-45]. Here, kinetic analysis of P53 activation upon RA treatment revealed that P53 upregulation actually preceded NPM-1c loss, suggestive for the existence of at least another P53 activation pathway. We indeed found that RA plays an essential role for growth arrest through Pin1 inhibition in both OCI-AML3 and primary patient blasts of *NPM-1c* AML cells only. Downstream of Pin1 inhibition, we identify the PML and P53 growth suppressors as its essential downstream
effectors [36, 46]. Analysis of *pml*<sup>−/−</sup> and *P53*<sup>−/−</sup> AML3 cells demonstrate that P53 is downstream of PML-triggered Pin1 or RA responses. Our discovery of the key role of PML downstream of RA-initiated Pin1 inhibition (Fig. 5D) suggests that RA-initiated Pin1 inhibition would upregulate PML and promote PML NB-formation, ultimately driving P53/senescence. These results suggest that in NPM-1c-driven AMLs, but not in NPM1-wild-type AMLs, impairment of PML NB formation is involved in the leukemic transformation and that RA-mediated restoration of PML NB contribute to the therapeutic effects.

In NPM-1c-positive AMLs, elucidation of the respective contributions of PML NB and NPM-1c degradation in the *in vivo* response, notably in combination with conventional chemotherapy, requires further investigations. Yet, the absence of RA effect on clonogenic activity of Pin1 down-regulated AML cells favors an important role of Pin1 inhibition in biological response and not only for early P53 activation (Fig. 2D). This model presents several feed-forward loops, all favoring anti-proliferative responses: RA-induced PML stabilization should facilitate PML-dependent NPM-1c degradation and P53 activation will enhance PML expression. Our results unravel a parallelism with the APL model: both involve oncoproteins that down-regulate basal P53 signaling. In both, therapy response involves degradation of the driving oncogene, PML NB reformation and P53 activation [8]. The co-existence of other major oncogenes (epigenetic regulators, FLT3) in *NPM-1c*-positive AMLs, most likely explains why RA/ATO is not curative on its own. Yet, RA could favor the action of chemotherapy, possibly by reverting basal Pin1 activation and P53 inhibition. Our observations could explain the clinical benefit of RA co-administration with conventional chemotherapy in
NPM-1c AMLs [13,16]. More broadly, the RA/ATO combination could target malignancies where Pin1 and/or PML are deregulated [26, 27]. In that respect, clinical responses to solid tumors were observed in some RA/ATO-treated APL patients who presented a synchronous another malignancy [47, 48], possibly reflecting activation of the RA/Pin1/PML/P53 axis unraveled by this study.
References

Figure legends

Figure 1. PML dependent NPM-1c degradation and P53 activation. **A.** Western blot analysis of PML, P53 and NPM-1c was performed on extracts of OCI-AML3, one clone of OCI-AML3\(^{pml-/-}\) (OCI-AML3\(^{pml-/-#1}\)) and one clone of OCI-AML3\(^{P53-/-}\) (OCI-AML3\(^{P53-/-#1}\)) cells after treatment with RA for 48 hours. Densitometry histograms represent an average of 5 independent experiments. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001. **B.** Cell growth (percent of control) was assessed using the trypan blue exclusion dye assay, in triplicate wells in OCI-AML3, one clone of OCI-AML3\(^{pml-/-}\) (OCI-AML3\(^{pml-/-#1}\)) and one clone of OCI-AML3\(^{P53-/-}\) (OCI-AML3\(^{P53-/-#1}\)) following treatment with RA for 48 hours (n=3). **C.** Western blot analysis of P53 and NPM-1c in OCI-AML3 and OCI-AML3\(^{pml-/-}\) after treatment with RA for 2, 12 and 24 hours as indicated. Densitometry histograms represent an average of 3 independent experiments. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001. **D.** Western blot analysis of NPM-1c and P53 in primary blasts derived from five NPM-1c AML patients and three NPM-1wt AML patients, after ex-vivo treatment with RA for 2h. Densitometry histograms represent an average of P53 and NPM-1c expression level in five NPM-1c AML patients and three NPM-1wt AML patients. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001. **E.** Western blot analysis of NPM-1c and P53 in primary blasts derived from five NPM-1c AML patients and three NPM-1wt AML patient, after ex-vivo
treatment with RA for 48h. Densitometry histograms represent an average of P53 and NPM-1c expression level in five NPM-1c AML patients and three NPM-1wt AML patients. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001.

**Figure 2. RA targets PML/P53 through Pin1 inactivation.**

**A.** Confocal microscopy of PML-NBs in primary blasts derived from one representative AML patient with NPM-1 wt and one representative AML patient with NPM-1c, after ex-vivo treatment with RA or AF-17724 for 2h as indicated. Histograms representate average of PML NB number per cell in 2 patients with NPM-1 wt and 2 patients with NPM-1c patients. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001.

**B.** Western blot analysis of PML, P53, and total NPM-1 (NPM1-wt) in primary blasts derived from three patients with NPM-1c AML and three patients with NPM-1 wt AML, after ex-vivo treatment with 20 µM of AG17724 or 1 µM of RA for 2h as indicated.

**C.** Western blot of PML and P53 in OCI-AML3 and OCI-AML2 cells following treatment with 20µM of AG17724 for 2h. Densitometry histograms represent an average of 3 independent experiments.

**D.** Colony formation assays in methylcellulose of OCI-AML3, and OCI-AML3 Pin1-KD cells, pre-treated with RA or AG17724 for 3 hours (n=3).

**E.** Western blot analysis of PML, P53, P21, or Pin1 in OCI-AML3 and OCI-AML3 Pin1-KD after treatment with 1 µM of RA for 2h. Densitometry histograms represent an average of 3 independent experiments. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**)
indicates p< 0.01; and (*** indicates p< 0.001. F. Western blot analysis of PML and P53 in OCI-AML3, OCI-AML3<sup>pml/-</sup> and OCI-AML3<sup>P53/-</sup> cells after treatment with 20 µM of AG17724 or 1 µM of RA for 2h as indicated. Densitometry histograms represent an average of 3 independent experiments. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001.

**Figure 3. NPM-1c expressing cells exhibit high Pin1 level and activity.** A. Western blot analysis of Pin1 in OCI-AML2, OCI-AML2-NPM-1wt, OCI-AML2-NPM-1c, OCI-AML3, OCI-AML3<sup>pml/-</sup> and OCI-AML3<sup>P53/-</sup>. B. Western blot analysis of NPM-1c and Pin1 in primary blasts derived from seven AML patients with *NPM-1c* (p4, p2, p5, p8, p7, p1 and p6) and six AML patients with *NPM-1wt* (p11, p13, p14, p10, p15 and p16). Densitometry histograms represent an average of Pin1 expression level in the seven tested NPM-1c AML patients and the six tested NPM-1wt AML patients. Densitometry was performed using ImageJ software. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001. C. Pin1 relative activity in OCI-AML2, OCI-AML3 and in primary blasts derived from three *NPM-1c* AML patients and two *NPM-1wt* AML patients after treatment with 1µM of RA as indicated. Statistical analysis was done using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001. D. Cell growth (percent of control) was assessed using the trypan blue exclusion dye assay in OCI-AML2 and OCI-AML3 cells following treatment with RA alone, AG-17724 alone or their combination with ATO, Doxorubicin and AraC for 72 hours as indicated (n=3). Statistical analysis was done
using Student’s t test, (*) indicates p< 0.05; (**) indicates p< 0.01; and (***) indicates p< 0.001.

**Figure 4. RA and chemotherapy cooperate to clear NPM-1c-expressing cells in vivo.** A-B. Eight-week-old NSG mice were injected with 2 million OCI-AML3 cells intravenously. At day 21 post-leukemic cells injection, RA was administered on a daily basis at the dose of 2.5 µg/g over a period of one week, followed by the administration of doxorubicin (2 µg/g) or cytarabine (60 µg/g) twice per week over a period of one week. Mice were sacrificed, BM was harvested from femurs and tibias of xenograft mice and then stained with the anti-human CD45 (hCD45) antibody. Western blot of NPM-1c, human P53, human P-P53 and PML in sorted hCD45 positive cells from BM harvested from untreated or treated NSG xenografted mice as indicated (2 mice per condition). C-D. Eight-week-old NSG mice were intravenously injected with 2 million OCI-AML3 pml-/- cells. Same treatment regimen was followed as indicated above. Western blot of NPM-1c, human P53 and P-P53 in sorted hCD45 positive cells from BM harvested from untreated or treated NSG xenografted mice as indicated. E-F. Graphs showing the percentage of hCD45 in OCI-AML3 xenografted NSG animals treated as described above (seven mice in the untreated group and in the groups treated with doxorubicin alone or RA in combination with cytarabine, nine mice for the group treated with RA alone or doxorubicin and RA, eight mice for the group treated with cytarabine alone). G-H. Graphs showing the percentage of hCD45 in OCI-AML3 pml-/- xenografted NSG animals treated as described above (three mice in each condition).
Figure 5. RA and Arsenic cooperate to clear NPM-1c-expressing cells. A. Eight-week-old NSG mice were injected with 3 million OCI-AML3 or OCI-AML3 pml-/ cells intravenously. At day 7 post-leukemic cells injection, ATO (5 µg/g/day) and RA (2.5 µg/g) were administered intraperitoneally every other day, over a period of 4 weeks. Western blot of human P53 and NPM-1c in sorted hCD45 positive bone marrow (BM) cells from NSG mice xenografted with OCI-AML3 or OCI-AML3 pml-/ cells, after in vivo treatment with ATO alone, RA alone or the combination of RA and ATO. B. Eight-week-old NSG mice were injected with 3 million OCI-AML3 or OCI-AML3 pml-/ cells intravenously. At day 7 post-leukemic cells injection, ATO and RA were administered every other day, over a period of 4 weeks intraperitoneally. At the end of treatment, bone marrow was harvested from femurs and tibias of xenografted mice and then stained with anti-hCD45 antibody. Graphs show the percentage of hCD45 in xenografted animals. C. Treatment schedule in two NPM-1c AML patients treated with RA and ATO as indicated. Percent of peripheral blood (PB) and bone marrow (BM) blasts are displayed. D. Proposed model on the molecular mechanisms of NPM-1c AML response to RA.
Supplementary materials and methods

Immunoblotting

One hundred µg of proteins were separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Blots were incubated with the following specific monoclonal antibodies, anti-NPM-1 recognizing both WT (abcam) and anti-P53 (Santa Cruz), or with the following polyclonal antibodies, anti-NPM-1 recognizing specifically the mutated NPM-1c (Invitrogen), a homemade chicken anti-PML, anti-PML (Novus) and an anti-Pin1 (Cell signaling). Proteins were then visualized using the enhanced chemiluminescence system (Bio-Rad).

RNA Isolation and Quantitative RT-PCR

RNA was isolated with RNeasy mini Kit (Qiagen). Reverse transcription was performed using iSCRIPT cDNA synthesis kit following the manufacturer’s instructions (Bio-Rad). Quantitative real-time PCR analysis was performed in triplicate with SYBR Green PCR Master kit (Roche) and GAPDH was used as an internal control. PCR primers sequences targeting P53 downstream genes were: P21 For, 5'-CGATGGAACCTCGACTTTGTCA; P21 Rev, 5'-GCACAAGGGTACAAGACAGTG; HDM2 For, 5'-CAGTAGCAGTGAATCTACAGGGA; HDM2 Rev, 5'-CTGATCCAACCAATCACCTGAAT; FAS For, 5'-AGATTGTGTGATGAAGGACATGG; FAS Rev, 5'-TGTTGCTGGTGAGTGTGCATT; GADD45 For, 5'-GAGAGCAGAAGACCGAAAGGA; GADD45 Rev, 5'-CAGTGATCGTGCGCTGACT; TP53INP1 For, 5'-TTCCTCCAACCAAGAACCAGA; TP53INP1 Rev, 5'-GCTCAGTAGGTGACTCTTCACT; GAPDH For, 5'-GACTTCAACAGCAAACCTCCCAC; GAPDH Rev, 5'-TCCACCACCCCTGTTGCTGTA. GAPDH was used as an internal control.
Microarray Analysis and Gene Set Enrichment analysis

cDNA microarray was conducted at the Curie Institute. The heatmap was composed of top 40 differentially expressed P53 target genes in NPM-1c mutant cells. Overall, 114 P53 target genes were analyzed based on reports from the literature [31]. Gene set enrichment analysis was performed using Hallmarks (h.all.v7.1) gene set database which contains 50 pathway gene sets. The number of permutations was 1000.

Immunofluorescence and confocal microscopy

OCI-AML3 or patients’ derived AML blasts were fixed with ice-cold methanol at -20°C for 20 minutes and cytospun onto glass slides. Immunostaining was performed with a rabbit polyclonal antibody against Pin1 (abcam), a mouse monoclonal antibody against human PML (Santa Cruz) or a homemade rabbit anti-PML antibody [32]. Primary antibodies were revealed by Alexa Fluor 594–labeled secondary antibody (Abcam). Staining of nuclei was performed with DAPI (4′, 6-diamidino-2-phenylindole) (Invitrogen). Images were acquired by confocal microscopy using a Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany) with a Plan Apochromat 63/1.4 numeric aperture oil-immersion objective, using Zen 2009 (Carl Zeiss).

Pin1 activity assay

Pin1 activity was measured using the SensoLyte® Green Pin1 Assay Kit that uses a fluorogenic substrate. Pin1 changes this substrate into the trans conformation that is readily cleaved to generate a fluorescent signal. Briefly, Cells were grown for 24 h at a density of 2×10^5/ml and then treated with 1 μM of RA for 2h. Cells were disrupted in lysis buffer (35 mM Hepes pH 7.4) and equivalent concentrations of cellular proteins were used in this assay. Proteins were incubated in the presence of Pin1 substrate for 2h in a 96-well
plate. Fluorescence was then monitored at Ex/Em=490/520nm. Increase in fluorescence intensity is directly proportional to the Pin1 activity.

**Colony Formation Assay**

AML cells were pretreated for 3hrs with 20 μM of AG17724 or 1μM RA. Cells were then embedded, at a density of 250 cells/well in 6-well plates, into methylcellulose (Stem Cell Technologies) supplemented with 20% FBS. After 10 days, colonies were counted using an inverted microscope device.

**Xenograft Animal Studies**

NOD/Shi-scid IL2rg−/− (NSG) mice were obtained from Jackson Laboratories (United States). All mouse protocols were approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut. Two or three million OCI-AML3 or OCI-AML3pml−/− cells were injected into the tail vein of 8-week-old mice. In long-term treatment, seven days post AML cells’ injection, mice were treated intraperitoneally with RA (2.5µg/g) and/or ATO (5µg/g/day) daily, over a period of 4 weeks. In short-term treatment, twenty-one days post AML cells’ injection, mice were treated intraperitoneally with RA (2.5µg/g) and/or ATO (5µg/g/day) daily for 7 consecutive days. Doxorubicine (Ebewe Pharma) (2 µg/g) or cytarabine (Alexan, Ebewe Pharma) (100 µg/g) were given intraperitoneally twice a week for one week in mice pretreated with RA daily for 7 days. RA was dissolved in dimethyl sulfoxide and diluted in 1x PBS supplemented with 5%Cremophor/5%Ethanol before its intraperitoneal administration to the mice.

**Human CD45 staining and cell sorting**

BM from the femurs and tibias of euthanized engrafted NSG animals with OCI-AML3 or OCI-AML3pml−/− cells, was flushed at the end of different treatments. To assess
the percentage of human engrafted cells following treatment with RA, ATO, doxorubicine, cytarabine or their combination, cell surface staining was performed using an anti-human CD45 Peridinin Chlorophyll Protein (PerCP) conjugated antibody (Becton Dickinson). Labeled samples were analyzed on a Guava flow cytometer. For cell sorting, BM cells were stained with anti-human CD45 PerCP or PE (BD biosciences) and sorted using a BD FACSaria cell sorter as described [33]. BM cells were also used to assess human P53, human p-P53, PML and NPM-1c protein levels by western blot, upon in vivo treatment using the monoclonal anti-human P53 (Abcam), the polyclonal anti-NPM-1c (Invitrogen), a homemade anti-PML and the monoclonal anti-p-P53 (S15) (Invitrogen) antibodies.
Supplementary Figure 1

A

B

C

D

E

F

G
Supplementary Figure 1. A. Western blot of PML, p53 and NPM-1c was performed on extracts of OCI-AML3 as a control, and three different clones of OCI-AML3_pml/- cells without or after treatment with RA for 2 and 24 hours as indicated. B. Cell growth (percent of control) was assessed using the trypan blue exclusion dye assay, in triplicate wells in OCI-AML3 as a control and in three different clones of OCI-AML3_pml/- cells following treatment with RA for 48 hours. C. Transcriptome microarray analysis of OCI-AML2 and OCI-AML3 upon treatment with RA for 6, 12 or 24h as indicated. D. GSEA analyses of P53 pathway in transcriptomic exploration of OCI-AML3 cells after RA treatment for 6, 12 or 24h as indicated. E. RT-qPCR for p21, HDM2, GAD45, FAS and P53INP1 transcript expression in OCI-AML2 and OCI-AML3 cells without or after RA treatment for 12h. F. Western blot analysis of p53 and NPM-1c was performed on extracts of OCI-AML3 as a control, and two different clones of OCI-AML3_p53/- cells without or after treatment with RA for 48 hours as indicated. G. Cell growth (percent of control) was assessed using the trypan blue exclusion dye assay, in triplicate wells in OCI-AML3 and in two different clones of OCI-AML3_p53/- cells without or after treatment with RA for 48 hours.
Supplementary Figure2. A. Confocal microscopy of PML-NBs in primary blasts derived from an *NPM-1-wt* AML patient (P10, left panel) or from an *NPM-1c* AML patient (P2, right panel), after *ex-vivo* treatment with RA or AG-17724 for 2h as indicated. B. Confocal microscopy of PML-NBs in OCI-AML3, after treatment with RA or AG17724 for 1 or 2h as indicated. C. Western blot of PML in OCI-AML3 following treatment with RA for 0.5, 1, 2, 6 or 24h as indicated (n=3). D. Western blot analysis of NPM-1c in primary blasts derived from three *NPM-1c* AML patients, after *ex-vivo* treatment with 20 µM of AG17724 for 48h. E. Western blot of NPM-1c in OCI-AML3 cells following treatment with 20 µM of AG17724 for 48h (n=3). F. Cell growth (percent of control) using the trypan blue exclusion dye assay, in triplicate wells in OCI-AML3 and OCI-AML3 Pin1-KD after treatment with 1 µM of RA for 48h.
**Supplementary Figure 3.**

A. Confocal microscopy analysis of Pin-1 in OCI-AML2 and OCI-AML3 cells (n=3). B. Western blot analysis of Pin-1 in OCI-AML3, without or after treatment with RA for 48h (n=2).
Supplementary Table 1: patients characteristics

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