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

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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 chemi-luminescence 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'-CGATGGAACTTCGACTTTGTCA; *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'-GACTTCAACAGCAACTCCCAC; *GAPDH* Rev, 5'-TCCACCACCCTGTTGCTGTA. 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

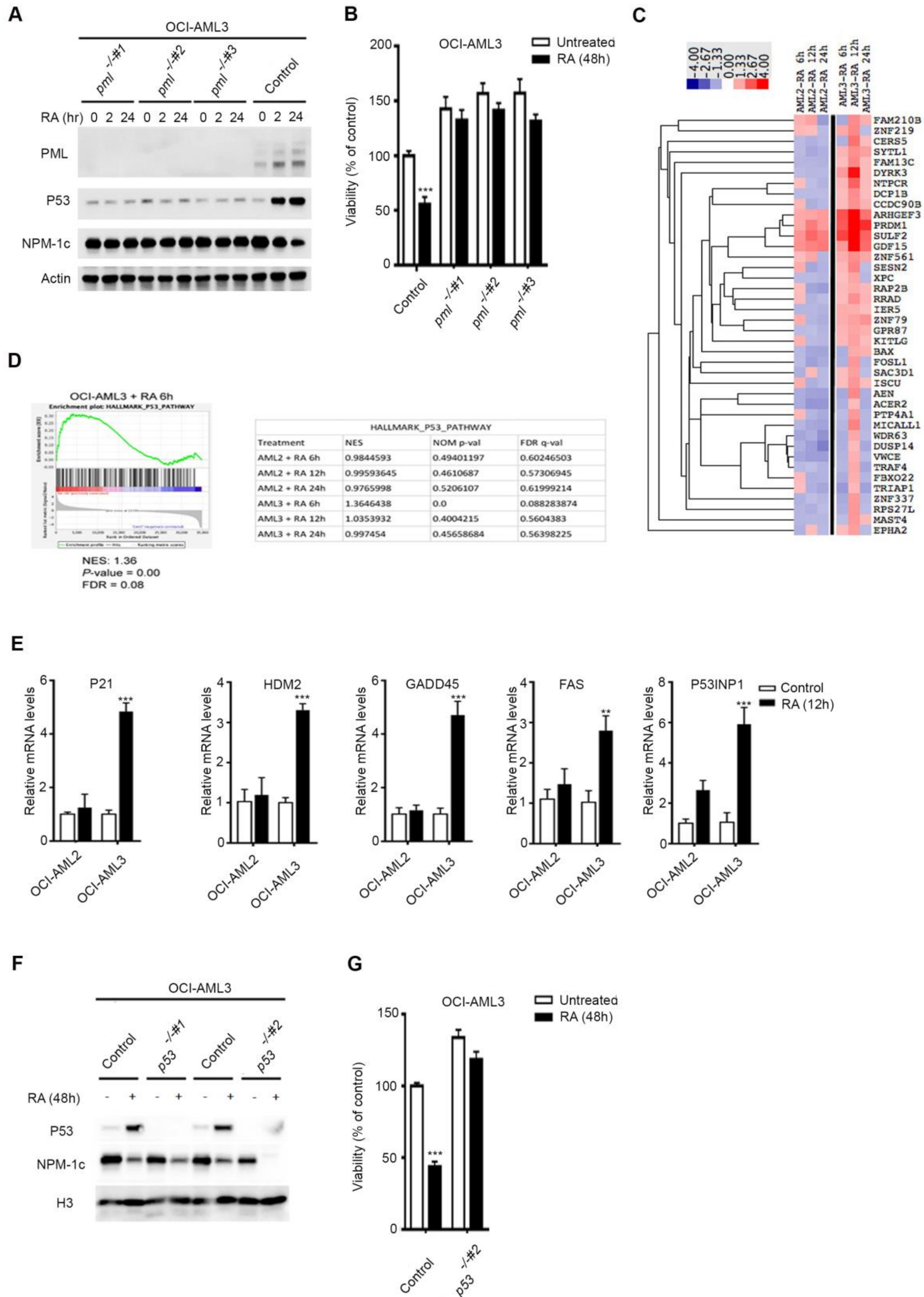
NOD/Shi-scid IL2rγ$^{-/-}$ (NSG) mice were obtained from Jackson Laboratories (United States). All mouse protocols were approved by the Institutional Animal Care and Utilization Committee of the American University of Beirut. Two or three million OCI-AML3 or OCI-AML3$^{pml-/-}$ cells were injected into 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-AML3$^{pml-/-}$ 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

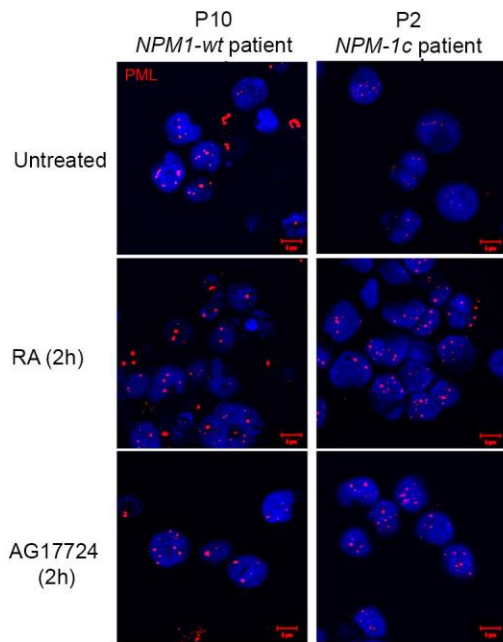


HALLMARK_P53_PATHWAY			
Treatment	NES	NOM p-val	FDR q-val
AML2 + RA 6h	0.9844593	0.49401197	0.60246503
AML2 + RA 12h	0.99593645	0.4610687	0.57306945
AML2 + RA 24h	0.9765998	0.5206107	0.61999214
AML3 + RA 6h	1.3646438	0.0	0.088283874
AML3 + RA 12h	1.0353932	0.4004215	0.5604383
AML3 + RA 24h	0.997454	0.45658684	0.56398225

Supplementary Figure1. 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 Figure 2

A



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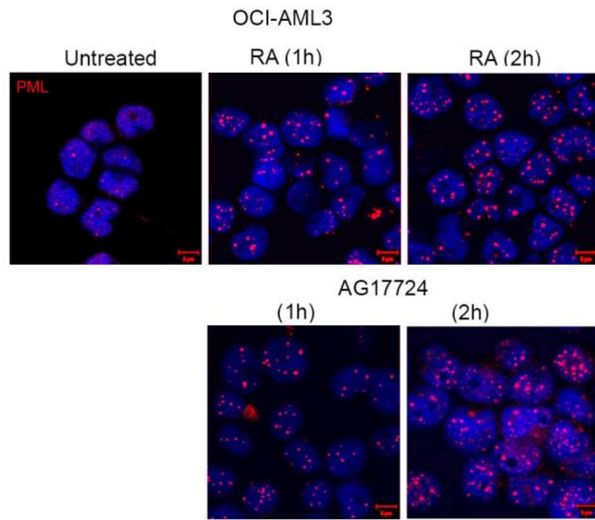

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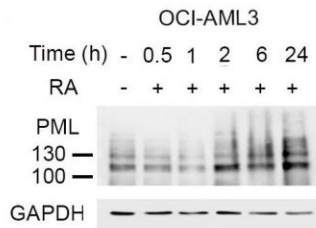

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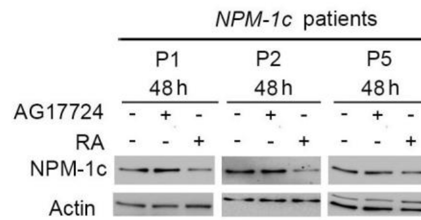

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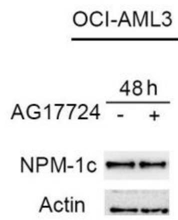

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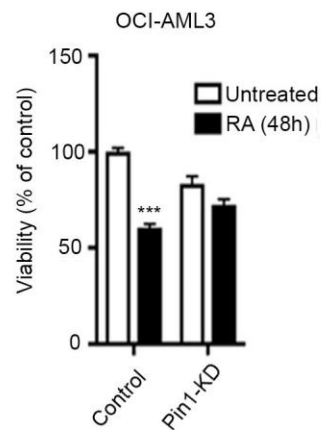
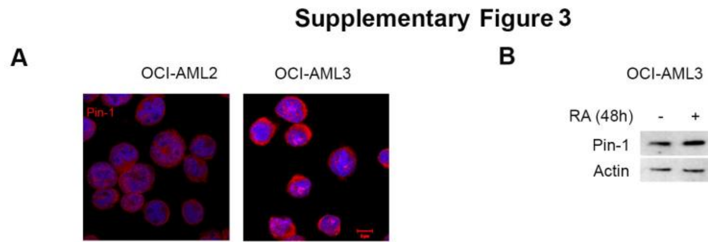


Supplementary Figure2. A. Confocal microscopy of PML-NBs in primary blasts derived from an *NPM-1-wt* AML patient (P10, left panel) or from an *NPM-1c* AML patient (P2, right panel), after *ex-vivo* treatment with RA or AG-17724 for 2h as indicated. **B.** Confocal microscopy of PML-NBs in OCI-AML3, after treatment with RA or AG17724 for 1 or 2h as indicated. **C.** Western blot of PML in OCI-AML3 follwoing 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 Figure3. 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

Patient number	Gender	Age	karyotype	Molecular profile	
				NPM1 status	FLT3 status
P1	Male	60	46,XY	Mutated	Wild type
P2	Male	65	46,XY	Mutated	Wild type
P3	Female	65	47,XX,+8	Mutated	Wild type
P4	Female	58	46,XX	Mutated	Wild type
P5	Male	65	46,XY	Mutated	FLT3-ITD
P6	Female	71	46,XY	Mutated	Wild type
P7	Male	45	46,XX	Mutated	Wild type
P8	Male	57	46,XY,t(15;17)(q24;q21)	Mutated	Wild type
P9	Male	27	46,XY,inv(16)(p13q22)	Wild type	Wild type
P10	Male	63	47,XY,+mar	Wild type	Wild type
P11	Female	54	46,XY	Wild type	FLT3-ITD
P12	Male	54	46,XY	Wild type	Wild type
P13	Male	68	46,XY	Wild type	FLT3-D835
P14	Male	50	46,XY,t(9;22)(q34;q11.2),add(14)(q32)	Wild type	Wild type
P15	Male	58	47,XY,+mar	Wild type	Wild type
P16	Male	22	46,XY	Wild type	Wild type