Clearance of PML/RARA-bound promoters suffice to initiate APL differentiation

Running title: APL differentiation by promoter clearance

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Key Points:

- PML/RARA loss or detachment from target promoters suffices to differentiate APL cells.
- PML/RARA degradation by arsenic thus explains arsenic-induced differentiation.

Abstract

PML/RARA, a potent transcriptional inhibitor of nuclear receptor signaling, represses myeloid differentiation genes and drives acute promyelocytic leukemia (APL). Association of the RXRA co-receptor to PML/RARA is required for transformation, RXRA promoting its efficient DNA-binding. APL is exquisitely sensitive to retinoic acid (RA) and arsenic trioxide (arsenic), which both trigger cell differentiation in vivo. While RA elicits transcriptional activation of PML/RARA targets, how arsenic triggers differentiation remains unclear. Here we demonstrate that extinction of PML/RARA triggers terminal differentiation in vivo. Similarly, ablation of RXRs loosens PML/RARA DNA-binding, inducing terminal differentiation of APL cells ex vivo or in vivo. RXRA sumoylation also directly contributes to PML/RARA-dependent transformation ex vivo, presumably by enhancing transcriptional repression. Thus, APL differentiation is a default program triggered by clearance of PML/RARA-bound promoters, rather than obligatory active transcriptional activation, explaining how arsenic elicits APL maturation through PML/RARA degradation.
Introduction

Acute promyelocytic leukemia (APL) is characterized by gene fusions involving Retinoic Acid Receptor-alpha (RARA) gene. The most common t(15;17) translocation fuses PML to RARA, yielding the PML/RARA fusion oncoprotein. PML, the key organizer of Nuclear Bodies (NB), is involved in redox sensing and hence confers sensitivity to arsenic trioxide.\(^1\)\(^-\)\(^3\) PML/RARA is a potent transcriptional repressor of retinoic acid (RA) signaling that interferes with gene expression programs involved in both hematopoietic progenitor self-renewal and terminal myeloid cell differentiation.\(^4\)\(^,\)\(^5\) Treatment of APL patients with RA induces terminal differentiation and transient remissions. Mechanistically, this is believed to reflect transcriptional reactivation of PML/RARA-silenced genes by RA, the ligand for RARA and PML/RARA,\(^6\) while RA-triggered PML/RARA degradation accounts for loss of self-renewal.\(^7\)\(^,\)\(^8\) Arsenic definitively cures a substantial proportion of patients as a single agent.\(^9\)\(^-\)\(^11\) Ex vivo studies demonstrated that arsenic primarily induces apoptosis of APL cells,\(^12\) although subsequent studies demonstrated partial or complete differentiation ex vivo and in vivo, respectively.\(^13\) Molecularly, arsenic degrades PML/RARA, but otherwise does not directly affect transcriptional regulation by RARA, raising the issue of the basis for differentiation.\(^14\)\(^,\)\(^15\) Arsenic also acts on normal PML to promote loss of self-renewal, likely explaining its clinical potency.\(^7\)\(^,\)\(^16\)

In normal cells, RARA is always associated to RXRs (Retinoid X Receptor) the universal partners of type II nuclear receptors to bind their responsive elements (RARE). RA binds to RARs inducing conformational changes within the RXRs/RARs complexes, thus resulting in enhancement of DNA binding, release of co-repressors, recruitment of co-activator complexes, and transcriptional activation of target genes. The RA pathway plays an important role in determining and regulating differentiation
pathways such as myelopoiesis. For example, RARA regulates the kinetics of granulocytic differentiation, while RXRA promotes monocytic differentiation. In contrast to RARA, PML/RARA homo-dimers bind DNA independently of RXRs ex \textit{celulo} suggesting than RXRs are not implicated in the transformation process. However, RXRs agonists may activate transcription from a PML/RARA-specific response element and may efficiently initiate APL cell differentiation. RXRA interacts with PML/RARA and CHIP-seq studies demonstrate that RXRA is always found at PML/RARA-bound promoters. The presence of RXRA in the PML/RARA complex greatly enhances its ability to bind DNA and to recognize highly degenerate sites. Additionally, RXRA may provide an independent repression signal through its sumoylation, the latter being sharply enhanced by PML/RARA. Accordingly, a PML/RARA mutant defective for RXR binding fails to initiate APL \textit{in vivo}, while silencing of RXRA induces apoptosis \textit{ex vivo}.

Here, we show that RXR excision from PML/RARA-driven APL relaxes association of the fusion and its target genes, inducing terminal differentiation of leukemic cells. Inactivation of PML/RARA by RNA interference also triggers differentiation. These unexpected observations provide a mechanistic basis for arsenic-induced APL differentiation.

\textbf{Materials and methods}

\textbf{Cell culture and Retroviral transductions}

cDNAs encoding PML/RARA and RARA were previously described. The Cre-ERT2 cDNA was inserted in MSCV-IRES-cRed (kind gift of R. Williams). The
murine RXRA WT and RXRA K113R cDNAs (kind gift of C. Egly) were inserted in MSCV-Babe-IRES-cRed. MSCV-FLT3ITD-IRES-GFP construct was previously described.\textsuperscript{32} ShRNA constructs were purchased from Sigma-Aldrich. The two shRNA constructs targeting human PML (5’-CACCCGCAAGACCAACAACAT-3’, 5’-GTGTACCGGCAGATTGTGGAT-3’), were inserted into pLKO.1-CMV-tGFP.

Bone marrow cells from 5-fluorouracil-treated mice (C57BL/6JRj) depleted in mature myeloid and lymphoid cells were cultured overnight with interleukin-3 (IL-3), IL-6 (10 ng/ml), and stem cell factor (100 ng/ml) (Eurobio Abcys). Bone marrow progenitors were infected twice by spinoculation with retroviral supernatant produced with Platinum-E cells. On the day following the second spinoculation, an equivalent 5 x 10\textsuperscript{3} cells was seeded per 1.1 ml of Methocult M3231 methylcellulose medium (Stem Cell Technologies) supplemented with 10 ng each of murine recombinant IL-3, IL-6, granulocyte-macrophage colony-stimulating factor and stem cell factor.\textsuperscript{25} One week after the initial infection, RXRA-expressing mCHERRY-positive cells were sorted and replated. One week later, secondary colonies were counted and cells analyzed by MGG staining and Western blot.

Cell cycle profiles were assessed using propidium iodide.

**Protein and cells analyses**

Cell lysates were resolved by SDS-PAGE and transferred on nitrocellulose membranes. We carried out detection with the chemi-luminescent substrate SuperSignal West Pico or Femto (Pierce Biotechnology). Antibodies : RP115 (RARA) was kindly provided by P. Chambon, anti-RXRα D-20 was purchased from Santa-
Cruz, anti-β-actin (20-33) and anti GAPDH (71.1) were purchased from Sigma-Aldrich.

H1299 cells stably expressing HIS-SUMO1 or HIS-SUMO2 were cotransfected (+) or not (-) with PML/RARA and RXR WT or K113R mutant. HIS-sumoylated proteins were purified by nickel pull-down and probed with the corresponding antibodies. Cos-7 cells were co-transfected with pSG5-RARA and pSG5-RXRA WT or pSG5-RXRA K113R constructs and treated with cycloheximide (0.1mg/ml, Calbiochem) and 1µM RA for 0, 1h, 3h and 6h. RARA and RXRA stability was then analyzed by Western Blot.

For flow cytometry analysis, cellular Fc receptors were blocked with rat IgG. We then carried out immuno-phenotypic analysis using fluorochrome-conjugated monoclonal antibodies to Mac1 and Gr-1 (clone RB6-8-C5, eBioscience). Staining was done at 4 °C for 20 min. We washed the cells twice and re-suspended them in HBSS with 2% FBS and 0.5 µg ml-1 propidium iodide. We gated dead cells out by high propidium iodide staining and forward light scatter. Immunofluorescences were performed using a homemade antibody against PML. For morphological analysis we performed a May-Grünwald-Giemsa staining (MGG).

**Transgenic mice and in vivo animal treatment**

PML/RARA transgenic mice were crossed with RXRAffe RXRBffe RXRGf/f mice. Bone marrow progenitors from RXRAffe RXRBffe RXRGf/f PML/RARA were infected with retroviruses encoding FLT3-ITD, yielding a transplantable APL (Figure 2A). The later was transduced by retroviruses encoding Cre-ERT2 and the leukemias were then serially transplanted in NMRI-Nude mice. Animal handling was done according to the guidelines of institutional animal care committees, using protocols.
approved by the “Comite Regional d’Ethique Experimentation Animale (CREEA) n°4”. Mice were treated with Tamoxifen (4-OHT, Sigma-Aldrich) by daily intra-peritoneal injections, 1mg/day during 1-5 days, and RA or arsenic as previously.37

RT-qPCR, CHIP and array experiments

Immortalized RARs−/− MEFs were retrovirally transduced with RXRA or RXRA K113R in presence or absence of PML/RARA and treated or not with RA (1µM) overnight. Total RNAs were isolated using the RNeasy kit (Qiagen) and first-strand cDNAs were synthetized using the SuperScript III reverse transcriptase (Invitrogen). Rarb and Act probes and primers for TaqMan assays were from Applied Biosystems. Quantification was performed by real-time quantitative PCR using the 7500 Fast Real-Time PCR system.

ChIP was performed using LowCell ChIP kit (Diagenode) according to manufacturer’s recommendations, except that chromatin was first incubated with antibodies overnight and then for 2 hours with beads. Antibodies used: anti GFP-FL and RXR ΔN 197 from Santa-Cruz, anti-H3, anti-H3 tri methyl K4, and Anti-PML+RARA fusion from Abcam. Real-time quantitative PCR was performed by real-time quantitative PCR using the Fast SYBR Green Master Mix (Applied Biosystems) and relative occupancy was calculated as fold enrichment over the control antibody anti-GFP. Amplicon for rab on chromosome 14: from 16 575 561 to 16 575 501, amplicon for hoxa1 on chromosome 6: from 52 153 671 to 52 153 580. RXRA and RXRB excised DNAs were quantified as above. Dpp9 gene was used as internal control.
Expression arrays and statistical analysis

RNA samples for array experiments were isolated as above and were hybridized on Affymetrix Human or Mouse Gene 1.1 ST Arrays. Log2 measures were obtained using RMA normalization. Threshold: 2 for human, 1.8 for mice, unless otherwise indicated. Expression profiles were normalized in batch using RMA method, independently in each series, yielding normalized log2 intensity measures. Log2 ratios were then obtained from these log2 intensities, using control samples as reference. Given two conditions A and B, in order to assess whether or not the genes up-regulated (respectively down-regulated) in these two conditions are significantly overlapping or not, we ranked the genes for both conditions and performed a chi2 test measuring the overlap between the top N genes in condition A and the top N genes in condition B (N ranging from 100 to 1200).

Results

RXRA sumoylation favors transformation of mouse primary hematopoietic progenitors.

PML/RARA enhances RXRA sumoylation.\textsuperscript{25} Purification of His-SUMO1 or His-SUMO2 conjugates demonstrated that the PML/RARA-enhanced RXRA sumoylation occurs on lysine K113 (Figure 1A, not shown). We observed higher RA-dependent transcriptional activation of \textit{Rarb}, a canonical RA primary target gene,\textsuperscript{38} in MEF transduced by RXRAK113R compared to RXRA (Figure 1B left panel) in line with studies showing that RXRA sumoylation contributes to transcriptional repression.\textsuperscript{30} We also detected a higher RA-dependent transcriptional activation in
the presence of PML/RARA (Figure 1B right panel) arguing that RXRA sumoylation is involved in PML/RARA-dependent transcriptional repression. Thus, PML/RARA-enhanced RXRA sumoylation contributes to PML/RARA-mediated repression.

We then tested the ability of the sumoylation-defective RXRAK113R mutant to modulate transformation of primary progenitors by PML/RARA, RARA or the MLL/ENL fusion, which does not interact with nuclear receptor signaling. RXRA slightly diminished RARA- or PML/RARA- triggered clonogenic activity (Figure 1C), most likely through RARA or PML/RARA destabilization. RXRK113R reduced clonogenic activity even further (Figure 1C-D), although it paradoxically stabilized the driving oncoproteins. Moreover, a significant induction of basal differentiation was observed (Figure 1E). In contrast, no effect of RXRA or RXRAK113R was observed on MLL/ENL-transformed cells. Directly measuring the half-life of RARA and RXRA upon RA exposure, we observed that RXRA sumoylation promotes their degradation (Figure 1F). Thus, RXRA not only contributes to DNA-binding of the complex, but its sumoylation also directly regulates PML/RARA-dependent differentiation block and clonogenic activity.

**RXRA loss induces differentiation and apoptosis.**

We next investigated the consequences of acute RXR ablation on survival and differentiation. We thus crossed PML/RARA transgenic mice driven by the MRP8 promoter with RXRA<sup>f</sup> f, RXRB<sup>f</sup> f, RXRG<sup>-/-</sup> mice. After backcrossing on the RXRA<sup>f</sup> f, RXRB<sup>f</sup> f, RXRG<sup>-/-</sup> mice, we obtained the PML/RARA transgene on a homozygous background for the RXRs floxed and null alleles. As those mice did not rapidly develop APL, we transduced their bone marrows with a retrovirus encoding FLT3-
ITD, a constitutively activated kinase that accelerates progression to APL.\textsuperscript{32,40} Transduced marrows were transplanted in irradiated Nude mice yielding aggressive APLs after 16 weeks which closely resemble previously studied ones (data not shown). These APLs were then transduced with a retrovirus co-expressing Cre-ERT2 and Cherry, yielding a transplantable APL where Tamoxifen (4-OHT) induces ablation of both RXRA and RXRB (Figure 2A).

We first assessed the effects of RA and 4-OHT in \textit{ex vivo} cultures of these primary blasts from murine APLs. APL spleen cells proliferated, even in presence of RA. Ablation of RXRs by 4-OHT induced cell death (data not shown), as previously shown in \textit{ex vivo} transformed cells.\textsuperscript{31} Yet, loss of cell viability was not observed for MLL/ENL-transformed cells (data not shown). Apoptosis induction by 4-OHT was confirmed by appearance of a sub-G1 peak (Figure 2B, arrow). While RA triggered PML/RARA degradation, RXRs ablation actually stabilized PML/RARA (Figure 2C-D, data not shown) in keeping with the fact that RXRA facilitates RARA degradation.\textsuperscript{41} Strikingly, 4-OHT treatment led to terminal differentiation of APL blasts, very similar to that triggered by RA. In contrast, the differentiation status of MLL/ENL-transformed cells was unaffected by ablation of RXRs (Figure 2E). Thus, loss of RXRs triggers apoptosis and differentiation of PML/RARA-transformed cells.

We then investigated the effect of RXRs ablation \textit{in vivo}. 4-OHT treatment a few days before the death of untreated animals inoculated with APL cells, significantly increased survival (Figure 3A, left) and led to rapid APL regression (Figure 3A, right). When 4-OHT-treated mice ultimately died of APL, blasts were primarily Cherry-negative, pointing to the selection of cells that had silenced CRE (data not shown). \textit{In vivo} ablation of RXRA and RXRB with 4-OHT treatment was complete, as demonstrated by Q-PCR. However, unexpectedly, a basal spontaneous
hemi-ablation of RXRB was constantly noted (Figure 3B). At the protein level, 4-OHT triggered the appearance of truncated RXRA-reactive protein species, but did not affect PML/RARA expression (Figure 3C). Critically, terminal blast differentiation was again observed 2 days post 4-OHT-exposure \textit{in vivo} (Figure 3D). Morphological differentiation was accompanied by loss of c-Kit expression and enhanced Mac1 and Gr1 expression (Figure 3E), very similar to RA treatment (Figure 3F). Acute ablation of RXRs in MLL/ENL-transformed cells \textit{in vivo} did not induce differentiation or tumor regression (data not shown). Thus, \textit{in vivo} loss of RXRs in APL blasts induces rapid terminal differentiation and APL regression.

**RXRA ablation detaches PML/RARA from its target sites.**

The presence of 4 DNA-binding domains in the PML/RARA//RXRA complex greatly facilitates its DNA binding, notably on non-canonical sites.\textsuperscript{22} We thus examined by CHIP whether 4-OHT would modify the occupancy or the chromatin environment of two primary targets, \textit{Rarb} and \textit{Hoxa1}.\textsuperscript{27} \textit{Ex vivo}, 4-OHT sharply decreased the amount of RXRA and PML/RARA associated with their binding sites. The remaining precipitated RXRA may be either residual full-length RXRA or PML/RARA-bound truncated RXRA. A small decrease in Histone H3 was reproducibly observed, with a significant increase in Histone H3 K4 trimethylation (Figure 4A). These observations are consistent with the proposal that, at least for a subset of targets, RXRA loss is accompanied by reduced DNA-binding of PML/RARA and transition of chromatin towards an active state.

To assess any global change in transcriptional regulation upon RXRs ablation, we performed transcriptomic arrays comparing \textit{ex vivo} 4-OHT and RA
treatments of primary APL cells at 4, 8 and 16 h. A set of genes was very reproducibly activated or repressed upon RA administration or ablation of RXRs in a time-dependent manner (Figure S1A,B). While genes induced or repressed by 4-OHT did not match those genes most potently modulated by RA (Figure S1A,B), a significant association was found between genes repressed over 2-fold by either treatment (Figure 4B), as well as between the 200 to 1000 top genes activated or repressed by RA and 4-OHT (data not shown). RXR-sensitive genes could not be identified as corresponding to a defined pathway. Finally, RXRs ablation blunted the basal expression levels of PML/RARA-activated targets such as \textit{Tgm2} (Figure 4C).\textsuperscript{42}

Collectively, this suggests that in APL, myeloid differentiation occurs through a series of subtle transcriptional changes, rather than massive activation of a specific master pathway.

**ShRNA inactivation of PML/RARA triggers differentiation \textit{in vivo}.

To directly investigate the effect of PML/RARA loss in established APL cells, we used lentiviral constructs expressing either a scrambled shRNA or a shRNA specifically targeting human \textit{PML} gene, thus destabilizing \textit{PML/RARA} expression, without affecting murine \textit{pml} (not shown). We transduced the vectors in murine APLs with identical efficiency and re-injected them in irradiated recipients. After 3 weeks, we observed a considerably lower proportion of GFP-expressing cells in the marrows of shRNA targeting PML/RARA compared to control shRNA mice (Figure 5A), formally demonstrating that sustained PML/RARA expression is required for APL growth. The majority of the remaining GFP-labeled cells had lost PML/RARA micro-speckled staining but, critically, now displayed terminal granulocytic differentiation
(Figure 5B-C), demonstrating that in vivo, down-regulation of PML/RARA expression promotes terminal myeloid differentiation.

**Reassessing the basis for arsenic-induced differentiation**

Similar to RXRA ablation or PML/RARA silencing, arsenic triggers differentiation and apoptosis whose respective extents, however, greatly vary with the experimental system used.\textsuperscript{12,13,15} To investigate the role of arsenic-initiated PML/RARA loss in transcriptional regulation, we first compared the mRNAs expression profiles of the NB4 APL cells treated with RA or arsenic. Up and down-regulated genes in response to these two unrelated agents were found to be extremely redundant (chi2 test p value < 1e-50), whatever the time considered (Figure 5D upper panel and 5E). Critically, a similar situation was observed in APL mice (Figure 5D lower panel, 5E, Figure S2).\textsuperscript{7} Thus, RA and arsenic regulate a common set of genes, most likely by a promoter clearance mechanism.

**Discussion**

Our results demonstrate that RXRA and its sumoylation play a key role in PML/RARA-initiated transformation. Unexpectedly, we observe that loss of RXRs leads to the terminal differentiation of APL blasts. Extinction of PML/RARA by RNA interference also induced differentiation of APL blasts. Our results therefore imply that promoter clearance is sufficient to differentiate APL cells, thus explaining how arsenic induces myeloid maturation in vivo.
PML/RARA interacts with the SUMO E2 enzyme UBC9 through its RING domain and enhances RXRA sumoylation. Here, we demonstrate that PML/RARA-enhanced RXRA modification occurs on lysine K113, blunts transcriptional activation and, critically, favors the ex-vivo transformation of primary mouse hematopoietic progenitors. Thus, PML/RARA recruits the sumoylation machinery onto RXRA to enhance repression by the PML/RARA-RXRA complex. Such recruitment of the sumoylation machinery onto chromatin may also be involved in the modification of other histone or non-histone proteins. Sumoylation of transcription factors and histones is primarily responsible for transcriptional repression, at least in part by antagonizing activating marks such as acetylation or ubiquitylation. RXRA sumoylation-dependent decrease in the transformation ability of PML/RARA is accompanied by the paradoxical stabilization of the fusion. Moreover, RXRA sumoylation is an important determinant of basal or RA-induced RARA degradation (Figure 1F). Retinoids or rexinoids induce the degradation of the RARA/RXRA complex and post-translational modifications have been implicated in activation-triggered degradation of many transcription factors. Thus, RXRA sumoylation could also be an important determinant of RXRA and RARA catabolism.

Previous studies have implicated RXRA in several aspects of APL pathogenesis. To examine the cellular effects of acute RXR loss, we designed a genetic system allowing excision of both RXRA and RXRB in murine APLs. Administration of 4-OHT induced terminal differentiation together with apoptosis, both ex vivo and in vivo. Loss of RXRs also initiated detachment of PML/RARA from some target genes, supporting the observation that the PML/RARA-RXRA complex, that contains at least four DNA-binding domains, has an enhanced affinity for DNA when compared to PML/RARA homodimers. Moreover, acute
RXRA excision enhances H3K4 tri-methylation, a modification associated with transcriptional activation. While RXRA down-regulation is required for granulocytic differentiation,\textsuperscript{17} we observed differentiation with some monocytic features upon RXRA ablation (Figure 2E). Recruitment of the RXRA/RARA complex onto the \textit{RARB} promoter after RA-induced PML/RARA degradation was proposed to be key for transcriptional re-activation and RA-response.\textsuperscript{50} Contrasting with this proposal, our observations of terminal differentiation upon RXR excision does not favor a model where transcriptional reactivation by RARA/RXRA is essential for differentiation (Figure 6).

PML/RARA degradation is essential to loss of self-renewal, while direct transcriptional activation is believed to trigger APL differentiation.\textsuperscript{6,7,51,52} Our observations of terminal differentiation upon PML/RARA silencing or its detachment from its target promoters suggest an alternative/complementary model wherein impeding PML/RARA binding to DNA suffices to initiate differentiation by a promoter clearing mechanism (Figure 6). This model explains the overlap between RA and arsenic targets and provides a molecular mechanism for arsenic-induced APL differentiation \textit{in vivo}.

PML/RARA has strong anti-apoptotic effects\textsuperscript{53} so that, in the absence of survival signals, loss of PML/RARA triggers apoptosis (Figure 2C, 6).\textsuperscript{24,54,55} RA activates potent anti-apoptotic genes such as \textit{MCL1},\textsuperscript{56} allowing the progression of an unabridged differentiation program \textit{ex vivo}. In RXRs-excised or arsenic-treated cells \textit{ex vivo}, the pro-apoptotic signals triggered by loss of PML/RARA DNA-binding are unopposed, precipitating cell death (Figure 6). Yet, growth factors enhance survival and promote terminal differentiation by arsenic \textit{ex vivo}\textsuperscript{57,58}. Our results could thus
explain the balance between treatment-induced differentiation and apoptosis in arsenic-treated APLs.\textsuperscript{14}

Collectively, our studies establish that PML/RARA clearance not only abrogate self-renewal,\textsuperscript{7,8} but also suffice to initiate APL differentiation. This demonstrates that APL maturation is a default program and further unifies the mode of action of retinoic acid and arsenic.

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**Authorship**

Contribution: J.H., A.V.P., J.A., A.d.R, performed experiments, analyzed data, and wrote the manuscript; L.P., M.L.B. performed experiments; H.d.T. designed experiments, analyzed data and wrote the manuscript. The authors have no conflicts of interest to disclose.
References


Figure legends

Figure 1. RXRA sumoylation enhances PML/RARA-mediated immortalization. (A) Sumoylation profile of transfected RXRA WT or K113R in H1299 cells stably expressing HIS-SUMO1 and expressing (+) or not (-) PML/RARA. (B) Analysis of rarb gene activation by RA (1µM) in MEFs expressing RXRA or RXRA K113R in presence (Rars-/MEFs, right panel) or in absence (MEFs, left panel) of PML/RARA. (C-D) Progenitors transduced with murine RXRA WT or K113R and PML/RARA, RARA or MLL-ENL were analyzed for: (C) clonogenicity, (D) morphology (bottom bar, 10 µm) (E) protein expression by Western blot (black dividing lines show grouping of images from different parts of the same exposed film). (F) Cos-7 cells co-expressing RARA and RXRA or RXRA K113R were treated with cycloheximide (0.1mg/ml) and RA (1µM) for the indicated time and half-life of RXRA and RARA proteins was analyzed by Western Blot. (B, C) Error bars represent standard deviations of three independent biological replicates. Significance of observed differences was evaluated using Student's t-test (∗ P 0.01–0.05; ∗∗ P 0.001–0.01; ∗∗∗ P < 0.001).

Figure 2. RXR loss induces growth differentiation and apoptosis of PML/RARA-transformed cells. (A) Generation of RXRAf/f RXRBf/f RXRG-/- PML/RARA FLT3-ITD Cre-ERT2 leukemia in mouse model. GFP-sorted spleen cells from leukemic mice were treated with 100nM 4-OHT or 1µM RA in culture and analyzed for: B cell cycle (C-D) PML/RARA expression (top bar, 10 µm) (E) differentiation. RXRAf/f RXRBf/f RXRG-/-, MLL/ENL Cre-ERT2 leukemia are shown as negative control. Bar, 10 µm.
Figure 3. RXR loss induces \textit{in vivo} differentiation. (A) Survival of mice inoculated with APL cells derived from RXRA\textsuperscript{f/f} RXRB\textsuperscript{f/f} RXRG\textsuperscript{-/-} PML/RARA FLT3-ITD Cre-ERT2 mice, untreated or treated with 4-OHT (left panel). Right panel: Cherry-positive cells after 5 days of treatment. (B-F) After 2 days treatment with 4-OHT or RA \textit{in vivo}, spleen cells were GFP sorted and analyzed as follows: (B) RXRA and RXRB ablation (C) proteins expression (black dividing lines show grouping of images from different parts of the same exposed film) (D) differentiation by MGG staining (top bar, 10 µm) and (E,F) flow cytometry.

Figure 4. RXR loss induces PML/RARA detachment from target genes. (A) APL mice were treated or not for 48h \textit{ex vivo} with 4-OHT. Immuno-precipitated DNA with the indicated antibodies was analyzed by qPCR as indicated. Error bars represent standard deviations of three independent biological replicates and significance was assessed as above. (B) Comparison of gene-expression activation (left panel, 1.7 fold up) or repression (right panel, 2 fold down) after 16h \textit{ex-vivo} treatment with 4-OHT (red) or RA (blue). See Figure S1A,B for primary data (C) \textit{Tgm2} gene expression after 16h \textit{ex-vivo} treatment with 4-OHT or RA (two biological replicates).

Figure 5. PML/RARA extinction triggers APL differentiation \textit{in vivo}. (A-C) Bone marrow APL blasts were infected with a GFP-expressing lentivirus encoding a scrambled shRNA sequence (Ctrl) or a shRNA sequence directed against human PML and injected in secondary recipients. Inoculated animals were analyzed 21 days after engraftment (6 mice, two independent experiments). (A) Proportion of GFP-
positive cells in the marrow. (B) PML/RARA or GFP immunofluorescence (bottom bar, 10 µm). Arrow points to differentiated GFP-positive cells with PML/RARA extinction. (C) FACS analysis of GFP-positive cells. (D) Gene expression analysis 6h or 12h after RA (blue) or As₂O₃ (red) treatment in human APL NB4 cell line (upper panels) or of APL mice (lower panel) See Figure S2 for representative data. (E) Chi2 test p values (median p-value for the different thresholds tested: N 100 to 1200) for association of the up and down-regulated genes. 935 refers to murine APLs, RA10 and RA100 indicate the doses of RA administered.

**Figure 6. Model.** Loss of PML/RARA DNA-binding through RXRA ablation, PML/RARA extinction or RA/arsenic-triggered catabolism clears target genes from the PML/RARA repressor, triggering differentiation. PML/RARA loss also induces apoptosis. The latter may be blocked by RA, growth factors or the micro-environment.
Figure 1.

A. For personal use only.

B. MEFs/ras-/- MEFs PML/RARA

C. Empty vector RXRA RXRA K113R

D. Empty vector RXRA RXRA K113R

E. COS-7 RXRA RXRA K113R

F. COS-7 RXRA RXRA K113R
Figure 4.

A

Fold enrichment

B

4OHT 14 genes
Overlapping 1 gene
Overlapping 10 genes

RA 390 genes
Activated genes

RA 83 genes
4OHT 117 genes
Repressed genes

C

Gene fold induction
Figure 5.

Panel A: Bar graph showing the percentage of GFP-positive cells in Ctrl and PML Sh RNA samples.

Panel B: Confocal images showing DAPI, PML/RARA, GFP, and Merge for Ctrl and PML Sh RNA conditions.

Panel C: Scatter plot showing the distribution of GFP positive cells in Ctrl and PML Sh RNA samples.

Panel D: Venn diagrams illustrating the overlap of genes from NB4 cell line 6h and 12h treated with RA and As2O3.

Panel E: Table summarizing the comparison of down and upregulated genes.

<table>
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<th>Sample</th>
<th>Comparison</th>
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<td>NB4</td>
<td>AR vs As: 3h</td>
<td>0</td>
<td>~1e-000</td>
</tr>
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<td>NB4</td>
<td>AR vs As: 12h</td>
<td>~1e-12</td>
<td>~1e-18</td>
</tr>
<tr>
<td>935</td>
<td>AR10 vs As: 6h</td>
<td>~1e-100</td>
<td>0</td>
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<tr>
<td>935</td>
<td>AR10 vs As: 12h</td>
<td>~1e-50</td>
<td>~1e-80</td>
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<tr>
<td>935</td>
<td>AR100 vs As: 12h</td>
<td>~1e-140</td>
<td>~1e-130</td>
</tr>
</tbody>
</table>

Chi2 test p values
(median p-value for the different thresholds N tested)
Clearance of PML/RARA-bound promoters suffice to initiate APL differentiation

Adeline Vitaliano-Prunier, Juliane Halftermeyer, Julien Ablain, Aurélien de Reynies, Laurent Peres, Morgane Le Bras, Daniel Metzger and Hugues de Thé