

RAR α 2 and PML-RAR similarities in the control of basal and retinoic acid induced myeloid maturation of acute myeloid leukemia cells

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Keywords: RAR α 2, retinoic acid, AML, silencing, PML-RAR

Received: May 16, 2016

Accepted: July 01, 2016

Published: July 13, 2016

ABSTRACT

Treatment of acute promyelocytic leukemia (APL) with *all-trans* retinoic acid (ATRA) is the first example of targeted therapy. In fact, the oncogenic fusion-protein (PML-RAR) typical of this leukemia contains the retinoid-nuclear-receptor RAR α . PML-RAR is responsible for the differentiation block of the leukemic blast. Besides PML-RAR, two endogenous RAR α proteins are present in APL blasts, i.e. RAR α 1 and RAR α 2. We developed different cell populations characterized by PML-RAR, RAR α 2 and RAR α 1 knock-down in the APL-derived NB4 cell-line. Unexpectedly, silencing of PML-RAR and RAR α 2 results in similar increases in the constitutive expression of several granulocytic differentiation markers. This is accompanied by enhanced expression of the same granulocytic markers upon exposure of the NB4 blasts to ATRA. Silencing of PML-RAR and RAR α 2 causes also similar perturbations in the whole genome gene-expression profiles of vehicle and ATRA treated NB4 cells. Unlike PML-RAR and RAR α 2, RAR α 1 knock-down blocks ATRA-dependent induction of several granulocytic differentiation markers. Many of the effects on myeloid differentiation are confirmed by over-expression of RAR α 2 in NB4 cells. RAR α 2 action on myeloid differentiation does not require the presence of PML-RAR, as it is recapitulated also upon knock-down in PML-RAR-negative HL-60 cells. Thus, relative to RAR α 1, PML-RAR and RAR α 2 exert opposite effects on APL-cell differentiation. These contrasting actions may be related to the fact that both PML-RAR and RAR α 2 interact with and inhibit the transcriptional activity of RAR α 1. The interaction surface is located in the carboxy-terminal domain containing the D/E/F regions and it is influenced by phosphorylation of Ser-369 of RAR α 1.

INTRODUCTION

Acute-promyelocytic-leukemia (APL) is characterized by a t(15:17) chromosomal translocation involving *PML* and *RARA*, which results in the expression of the oncogenic PML-RAR fusion protein [1–3] and a block in the myeloid maturation pathway [4]. The cyto-differentiating agent *all-trans* retinoic acid (ATRA) is used in the treatment of APL and it has changed the natural history of the disease [5–9].

The biological action of ATRA is mediated by RAR and RXR nuclear receptors (NRs). RAR α , RAR β , RAR γ , RXR α , RXR β and RXR γ are ligand-activated transcription-factors controlling the expression of target genes [10, 11]. The NR active forms consist of RAR/RXR heterodimers, in which the RAR moiety is responsible for ligand-binding [12–16]. ATRA binds/activates RAR α , RAR β and RAR γ with the same efficiency [17, 18]. The ligand-binding region of RARs is located in the

carboxy-terminal E-domain, which is maintained in PML-RAR (Supplementary Figure S1).

The molecular mechanisms underlying the differentiation block afforded by PML-RAR in APL blasts and those responsible for ATRA therapeutic activity are incompletely defined. PML-RAR may arrest the myeloid maturation of APL blasts exerting a dominant-negative effect on RAR α . Indeed, PML-RAR binds RAREs (Retinoic Acid Responsive Elements) of RAR α target-genes [19]. Part of PML-RAR action may also involve RAR α -independent mechanisms, as the fusion-protein binds to a larger set of DNA target-sequences than RAR α [19]. The relative contribution of PML-RAR and RAR α to the differentiation process ignited by ATRA in APL blasts is also largely unknown. ATRA-induced PML-RAR degradation may release RAR α from the dominant-negative effect exerted by the fusion-protein, permitting its ligand-dependent activation [2, 20, 21]. The situation is further complicated by the presence of three different RAR α isoforms (Supplementary Figure S1).

Using the NB4 model of APL and silencing/over-expression approaches, we provide evidence that PML-RAR and the RAR α splicing-variant, RAR α 2, inhibit basal and ATRA-dependent myeloid differentiation. In NB4 cells, knock-down of the major RAR α splicing variant, RAR α 1, exerts opposite effects relative to PML-RAR and RAR α 2. RAR α 2 action on myeloid differentiation is recapitulated in PML-RAR-negative and ATRA-sensitive HL-60 cells. PML-RAR and RAR α 2 directly bind/inhibit RAR α 1 transcriptional activity, indicating functional antagonism.

RESULTS

RAR α 2 is expressed, transcriptionally activated and degraded by ATRA in the APL-derived NB4 cell line

Four RAR α splicing-variant mRNAs, RAR α -v1, RAR α -v2, RAR α -v3 and RAR α -v4, are known (Supplementary Figure S1). RAR α -v1 and RAR α -v3 code for an identical protein (RAR α 1). RAR α -v4 is translated into RAR α 4 lacking the DNA-binding C-region. RAR α 2, the RAR α -v2 product, is devoid of the A-region [22]. We determined the levels of PML-RAR and RAR α splicing-variants in NB4 cells grown with and without ATRA (Figure 1A). In the absence of ATRA, large amounts of PML-RAR mRNA are measurable, while RAR α -v3 is the major endogenous RAR α transcript, followed by RAR α -v1, RAR α -v2 and RAR α -v4. PML-RAR and RAR α -v2 mRNAs are induced by ATRA.

High levels of PML-RAR and RAR α proteins are highlighted by an antibody [RP-alpha(F)] recognizing the F-region of the two receptors (Figure 1B). Although this antibody recognizes both RAR α 1 and RAR α 2, the RAR α bands determined upon Western blot analysis

(WB) are likely to correspond to RAR α 1. Indeed, when a selective anti-RAR α 2 antibody [Ab25alpha(A2)] is used, a specific RAR α 2 band is measurable only following immuno-precipitation (Figure 1B) or WB analysis with high amounts of extracts (see Figure 2C, upper). ATRA-treated NB4 cells show the expected degradation of PML-RAR and RAR α 1 proteins, which is associated with receptor activation [23, 24] and suppressed by the proteasome-inhibitor, MG132 (Figure 1B). Despite induction of the corresponding mRNA, the RAR α 2 protein band disappears upon ATRA challenge (Figure 1B). This effect is also consequent to proteasome-dependent RAR α 2 degradation, since MG132 blocks it (Figure 1B). ATRA causes a concentration-dependent stimulation of RAR α 1, RAR α 2 and PML-RAR transcriptional activity as well as a decrease in the relative protein levels in COS-7 cells co-transfected with the NRs and a retinoid luciferase-reporter (Figure 1C). The ATRA-dependent decrease in RAR α 1, RAR α 2, and PML-RAR proteins is suppressed by MG132 (Figure 1D). As observed for RAR α 1 (Figure 1D) and PML-RAR (data not shown) [24, 25], inhibition of RAR α 2 degradation by MG132 increases the induction of luciferase activity by ATRA.

Generation of NB4 derived cell populations stably and selectively silenced for PML-RAR, RAR α 1 and RAR α 2

We designed shRNAs targeting the fusion site of PML-RAR as well as the A/B-regions of RAR α 1 and RAR α 2 (Supplementary Figure S2). The RAR α 1-targeting shRNAs recognize also RAR α 4 and are expected to silence it too. However, RAR α 4 is undetectable in NB4 cells (data not shown) and it was not further considered. Following a preliminary screening based on transient transfection of COS-7 cells with PML-RAR, RAR α 1 or RAR α 2 cDNAs, we selected one shRNA for each receptor and one shRNA recognizing all the receptors (*ALLsh*). *ALLsh* efficiently down-regulates RAR α 1, RAR α 2 and PML-RAR in COS-7 cells (Figure 2A). The RAR α 1, RAR α 2 and PML-RAR shRNAs (*RA1sh*, *RA2sh* and *PMRsh*) reduce the levels of the targets specifically, while the negative-control shRNA (*SCRsh*) retroviral construct and the void vector (*VOID*) exert no effect.

We isolated NB4 cell populations with stable integration of the selected negative-control (*SCRsh-NB4*), the RAR α 1 (*RA1sh-NB4*), the RAR α 2 (*RA2sh-NB4*) and the PML-RAR (*PMRsh-NB4*) shRNAs. Specific knock-down of the targets is confirmed for *RA1sh-NB4* and *PMRsh-NB4* cells (Figure 2B), while the parental and *SCRsh-NB4* counterparts express similar levels of RAR α 1 and PML-RAR. The same amounts of PML proteins are evident in parental and *PMRsh-NB4* cells supporting the specificity of the PML-RAR targeting shRNA (Supplementary Figure S3). Following WB (Figure 2C-upper) or immuno-precipitation (Figure 2C-lower)

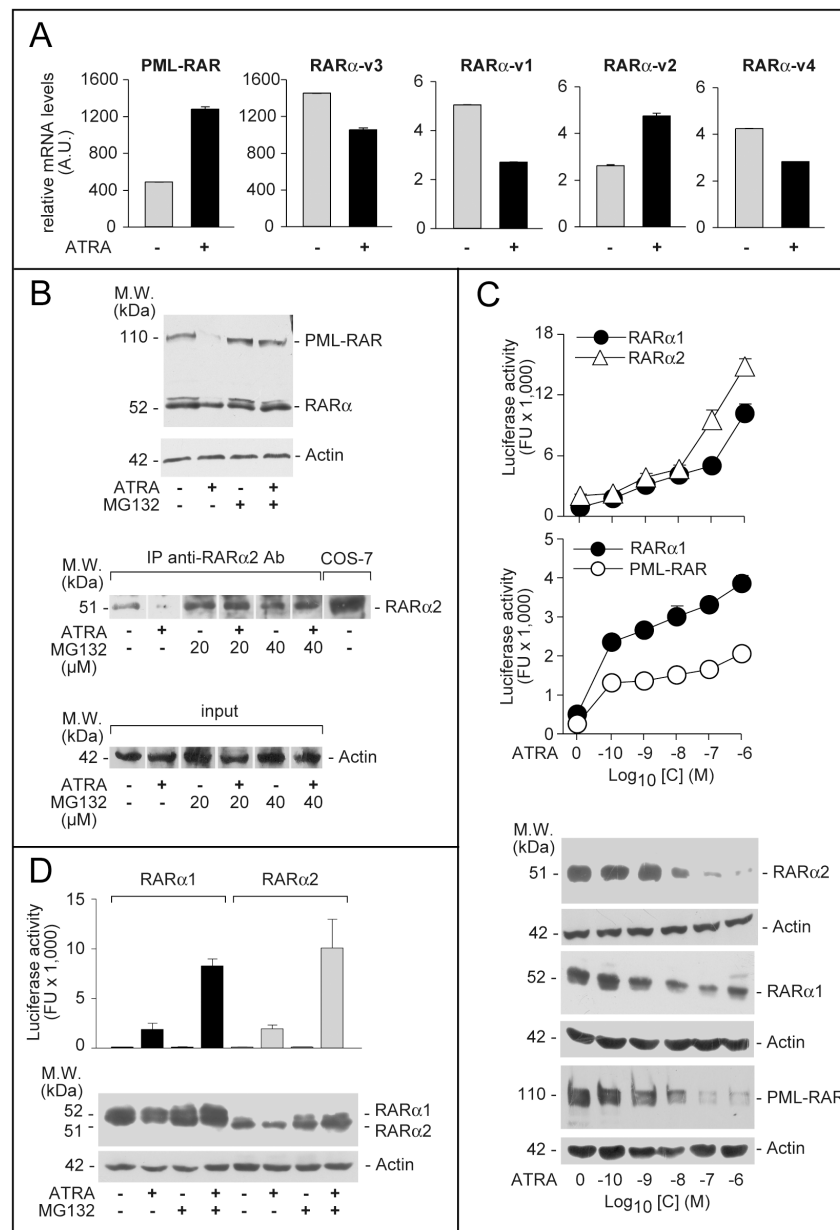


Figure 1: Expression, ATRA-dependent proteolytic degradation and transcriptional activity of PML-RAR, RAR α 2 and RAR α 1. **A.** NB4 cells were treated with vehicle (DMSO) or ATRA (0.1 μ M) for 48 hours. Total RNA was extracted and subjected to RT-PCR analysis using Taqman assays for the indicated mRNAs. The results are expressed as the mean \pm SD of 3 replicates. **B.** Upper: NB4 cells were treated with vehicle (DMSO) or ATRA (0.1 μ M) for 40 hours before addition of the proteasome inhibitor, MG132 (40 μ M) for 8 hours. Total protein extracts were subjected to Western blot analysis with an anti-RAR α antibody [RP alpha (F)]. Actin was used as a loading control. Lower: NB4 cells were treated as above with vehicle (DMSO), ATRA (0.1 μ M), the proteasome inhibitor, MG132 (20 and 40 μ M) or ATRA+MG132. Cell extracts were immuno-precipitated with an anti-RAR α 2 antibody [Ab25alpha2(A2)] coupled to protein G-sepharose beads (IP = immuno-precipitation) and the immuno-precipitates were subjected to Western blot analysis with the same anti-RAR α antibody used in the Upper panel. Equivalent amounts of protein extracts were used to immuno-precipitate RAR α 2, as indicated by the levels of actin in the extracts before addition of the anti-RAR α 2 antibody (input). COS-7 = Total extracts of COS-7 cells transfected with a *pcDNA3-RAR α 2* plasmid. The calculated molecular weight (M.W.) of the indicated proteins is shown on the left. **C.** COS-7 cells were transfected with *pcDNA3-RAR α 2*, *pcDNA3-RAR α 1* and *pSG5-PML-RAR* plasmids and the retinoid dependent Luciferase reporter, *β 2RARE-Luc*. Sixteen hours following transfection, cells were treated with DMSO or the indicated concentrations of ATRA for an extra 24 hours. Cell extracts were used for the measurement of luciferase activity and the indicated proteins by Western blot analysis. Luciferase activity data are expressed as the mean \pm SD of two replicates. **D.** COS-7 cells were transfected as in (C). Sixteen hours following transfection, cells were treated with vehicle (DMSO) or ATRA (1 μ M) for 16 hours and vehicle or MG132 (40 μ M) for an extra 8 hours. Cell extracts were used for the measurement of luciferase activity and the indicated proteins by Western blot analysis. Luciferase activity data are expressed as the mean \pm SD of two replicates.

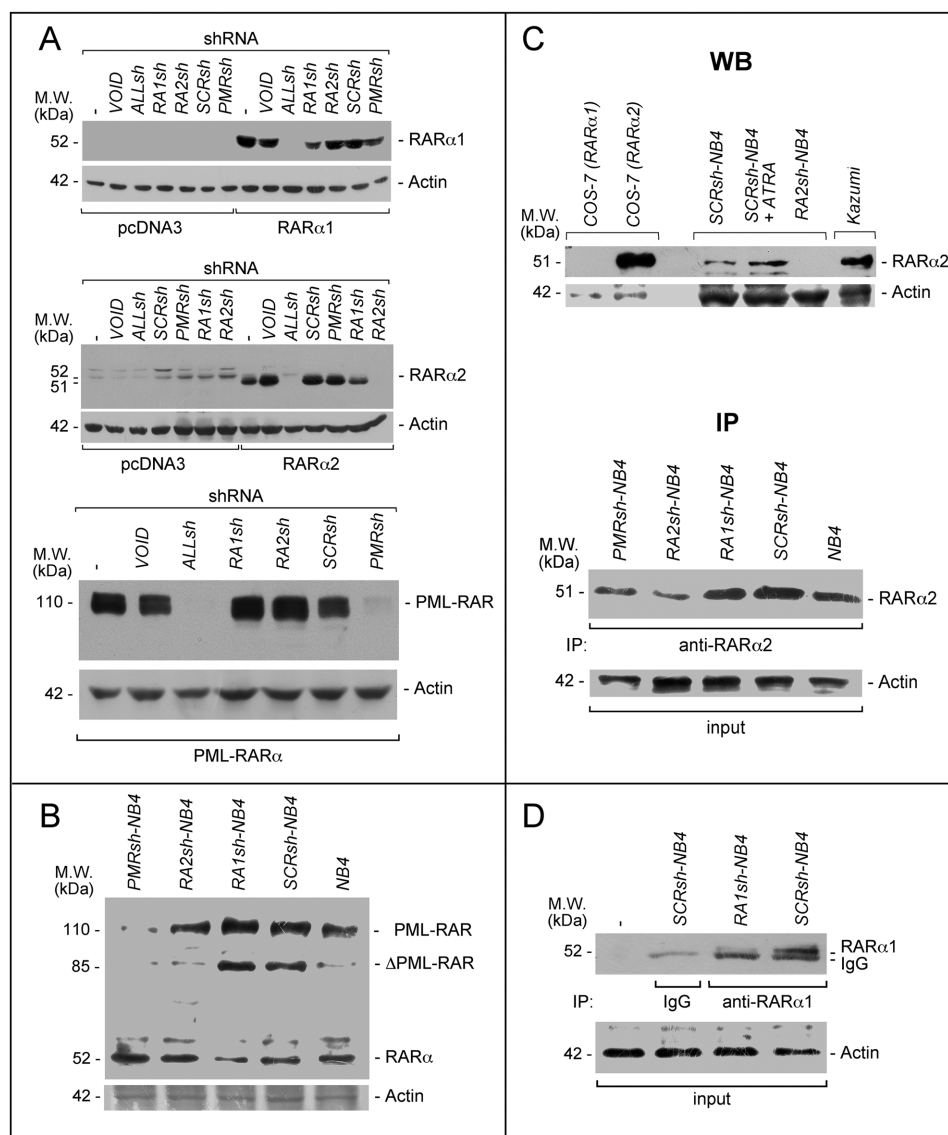


Figure 2: PML-RAR, RAR α 2 and RAR α 1 knock-down in COS-7 and NB4 cells. **A.** COS-7 cells were transiently transfected with the *pcDNA3*, *pcDNA3-RAR α 2*, *pcDNA3-RAR α 1* and *pSG5-PML-RAR* plasmids in the presence of the indicated shRNA-containing retroviral vectors and corresponding void vector (*VOID*). Sixteen hours following transfection, cell extracts were subjected to Western blot analysis using an anti-RAR α antibody [RP alpha (F)]. Actin is used as a loading control. *ALLsh* = shRNA targeting RAR α 1 (RAR α .v1 and RAR α .v3 mRNAs), RAR α 2 (RAR α .v2 mRNA) and RAR α 4 (RAR α .v4 mRNA); *RA1sh* = shRNA targeting RAR α 1; *RA2sh* = shRNA targeting RAR α 2; *PMRsh* = shRNA targeting PML-RAR; *SCRsh* = scramble shRNA (negative control). The (-) symbol represents extracts from COS-7 cell transfected in the absence of any shRNA. **B.** The indicated NB4 cell populations stably infected with shRNAs targeting PML-RAR (*PMRsh-NB4*), RAR α 1 (*RA1sh-NB4*), RAR α 2 (*RA2sh-NB4*) or scramble shRNA (*SCRsh-NB4*) as well parental NB4 cells (*NB4*) were grown under standard conditions for 48 hours. Cell extracts were subjected to Western blot analysis using the same anti-RAR α antibody as in (A). Actin is used as a loading control. **C.** Upper (WB = Western Blots): Extracts from the indicated COS-7 cells transfected with RAR α 1 and RAR α 2 expressing plasmids as well as the indicated NB4 cells [see (B)], were subjected to Western blot analysis with anti-RAR α 2 [Ab25alpha2(A2)] and β -actin (loading control) antibodies. *SCRsh-NB4* = cell treated with vehicle (DMSO) for 24 hours; *SCRsh-NB4+ATRA* = cell treated with ATRA (1 μ M) for 24 hours. *Kazumi* cells extracts are used as a control for RAR α 2 expression, as they contain high levels of the protein. Lower (IP = immunoprecipitations): Extracts from the indicated NB4 cell populations and parental NB4 cells [see (B)], were immuno-precipitated with an anti-RAR α 2 antibody [Ab25alpha2(A2)] coupled to Protein G sepharose beads. The immuno-precipitates were subjected to Western blot analysis with a different anti-RAR α antibody [RP alpha (F)]. Equivalent amounts of protein extracts were used to immuno-precipitate RAR α 2, as indicated by the levels of actin in the extracts before addition of the anti-RAR α 2 antibody (input). **D.** Extracts from the indicated NB4 cell populations [see (B)] were immuno-precipitated with an anti-RAR α 1 antibody [Ab10alpha1(A1)] or mouse immunoglobulin G (IgG) coupled to Protein G Sepharose beads or Protein G Sepharose beads alone (-). The immuno-precipitates were subjected to Western blot analysis with a different anti-RAR α antibody [RP alpha (F)]. The actin loading control of the immuno-precipitation experiment is shown (input). The calculated molecular weight (M.W.) of each protein is indicated on the left of each blot.

with the anti-RAR α 2 antibody [Ab25a(A2)], a remarkable and selective down-regulation of RAR α 2 is observed in *RA2sh-NB4* cells. If similar immuno-precipitation experiments are performed with an anti-RAR α 1 antibody, a decrease in the amounts of RAR α 1 is evident in *RA1sh-NB4* relative to *SCRsh-NB4* cells, confirming the WB results obtained on cellular extracts (Figure 2D).

PML-RAR and RAR α 2 silencing increases differentiation of NB4 cells in the absence or presence of ATRA, while RAR α 1 silencing exerts opposite effects in the presence of the retinoid

The consequences of RAR α 1, RAR α 2 and PML-RAR knock-down on *NB4* growth were evaluated in the absence/presence of ATRA (Figure 3A). In the absence of ATRA, *RA1sh-NB4* and *PMRsh-NB4* cells grow faster and more slowly, respectively, than *SCRsh-NB4* blasts. No difference is observed between *RA2sh-NB4* and *SCRsh-NB4* cells. Both PML-RAR and RAR α 2 silencing enhances ATRA growth-inhibitory action, while RAR α 1 knock-down exerts opposite effects.

Under basal conditions, a significant fraction of *PMRsh-NB4* and *RA2sh-NB4* cells show morphological signs of granulocytic maturation which are not observed in *RA1sh-NB4* cells, such as nuclear lobulation, increased cytoplasmic/nuclear ratio and appearance of cytoplasmic granules/vesicles (Supplementary Figure S4). As expected, ATRA induces morphological features of granulocytic differentiation in the majority of *SCRsh-NB4* cells. While similar features are observed in ATRA-treated *PRsh-NB4* and *RA2sh-NB4* cells, differentiation is much less evident in *RA1sh-NB4* cells. In the absence of ATRA, *SCRsh-NB4* blasts show the same low levels of CD11b and CD11c myeloid differentiation markers [26] (Figure 3B). Exposure to ATRA renders *SCRsh-NB4* cells highly positive for the two markers and it increases CD11b as well as CD11c mean-associated-fluorescence (MAF). In *RA1sh-NB4* cells, ATRA-dependent induction of these markers is suppressed, as indicated by CD11b-/CD11c-positivity and MAF. Relative to *SCRsh-NB4* cells, a substantial increase in CD11b-/CD11c-positivity is already observed in *PMRsh-NB4* and *RA2sh-NB4* blasts grown under basal conditions. The phenomenon is accompanied by enhanced MAF values after exposure to ATRA. Thus, *RA2sh-NB4* and *PMRsh-NB4* cells show similar patterns of basal and ATRA-dependent CD11b-/CD11c expression. We also defined the action of RARA α 1, RAR α 2 and PML-RAR shRNAs on 3 transcription factors controlling APL blast granulocytic maturation, *i.e.* PU.1 [27, 28], cEBP β [29, 30] and STAT1 α [31] (Figure 3C). RARA α 1 knock-down reduces the induction of PU.1, cEBP β and STAT1 α observed in ATRA-exposed parental and *SCRsh-NB4* blasts. In contrast, *PMRsh-NB4* and *RA2sh-NB4* cells show higher basal levels of STAT1 α and cEBP β as well as enhanced induction of PU.1, STAT1 α and cEBP β by ATRA.

To evaluate the effects of PML-RAR, RAR α 2 and RARA α 1 silencing on direct retinoid-targets, we focused on CD38 (Figure 3B) and paxillin (PXN) (Figure 3C) which are up-regulated by ATRA in *NB4* and other cell types [32–35]. Over 90% of all shRNA-infected cells are CD38⁺. ATRA increases CD38-MAF in *SCRsh-NB4* and *RA1sh-NB4* cells. The ATRA-dependent effect is considerably enhanced in *PMRsh-NB4* and *RA2sh-NB4* blasts. This enhancement is not evident in *RA1sh-NB4* cells, which show a slight reduction of the ATRA-dependent CD38-MAF increase in *SCRsh-NB4* blasts. Similar expression patterns are observed in the case of PXN. Thus, RAR α 2 and PML-RAR are negative determinants of myeloid differentiation and they down-regulate direct retinoid-responsive genes.

To evaluate whether the negative action of RAR α 2 on myeloid differentiation is specific to APL and dependent on PML-RAR expression, we performed studies in the *PML-RAR*⁺ and ATRA-sensitive *HL-60* model [45, 46]. In *HL-60* cells, RAR α -v3 is most abundant followed by RAR α -v1 and RAR α -v2 mRNAs, while negligible amounts of RAR α -v4 are measured (Supplementary Figure S5A). ATRA up-regulates RAR α -v2 and RAR α -v3 mRNAs. Relative to control *HL-60* populations (*SCRsh-HL60* and *Void-HL60* cells), RAR α 2 knock-down (*RA2sh-HL60*) (Supplementary Figure S5B) reduces basal cell-growth and enhances ATRA anti-proliferative action (Supplementary Figure S5C). In basal conditions, RAR α 2 knock-down increases the number of CD11b⁺ *RA2sh-HL60* cells (Supplementary Figure S5D). Following ATRA exposure (0.1 and 1.0 μ M), the number of CD11b⁺ cells and CD11b-MAF values are higher in *RA2sh-HL60* than *SCRsh-HL60* or *Void-HL60* cells. Both ATRA concentrations enhance CD11c-MAF induction in *RA2sh-HL60* relative to control cells. Finally, ATRA-dependent up-regulation of STAT1 α , cEBP β and PU.1 are enhanced by RAR α 2 knock-down (Supplementary Figure S5E). As for the consequences of RAR α 2 knock-down on the direct retinoid-responsive genes, all *RA2sh-HL60* cells are CD38⁺, while *SCRsh-HL60* and *Void-HL60* blasts are CD38⁻ (Supplementary Figure S5F). As expected, ATRA renders *SCRsh-HL60* and *Void-HL60* cells CD38⁺ and increases CD38-MAF in *SCRsh-HL60*, *Void-HL60* and *RA2sh-HL60* to the same extent. RAR α 2 knock-down stimulates ATRA-dependent induction of paxillin (Supplementary Figure S5E) as well as c-EBP ϵ and CYP-26A1 mRNAs (Supplementary Figure S5G). All this confirms and extends the *NB4* data, indicating that RAR α 2 exerts a negative action on myeloid maturation independently of PML-RAR expression.

RAR α 2 over-expression reduces ATRA-dependent differentiation of NB4 cells

To support the unexpected data obtained following RAR α 2 silencing in *NB4* cells, we took a specular approach and stably over-expressed the retinoid receptor

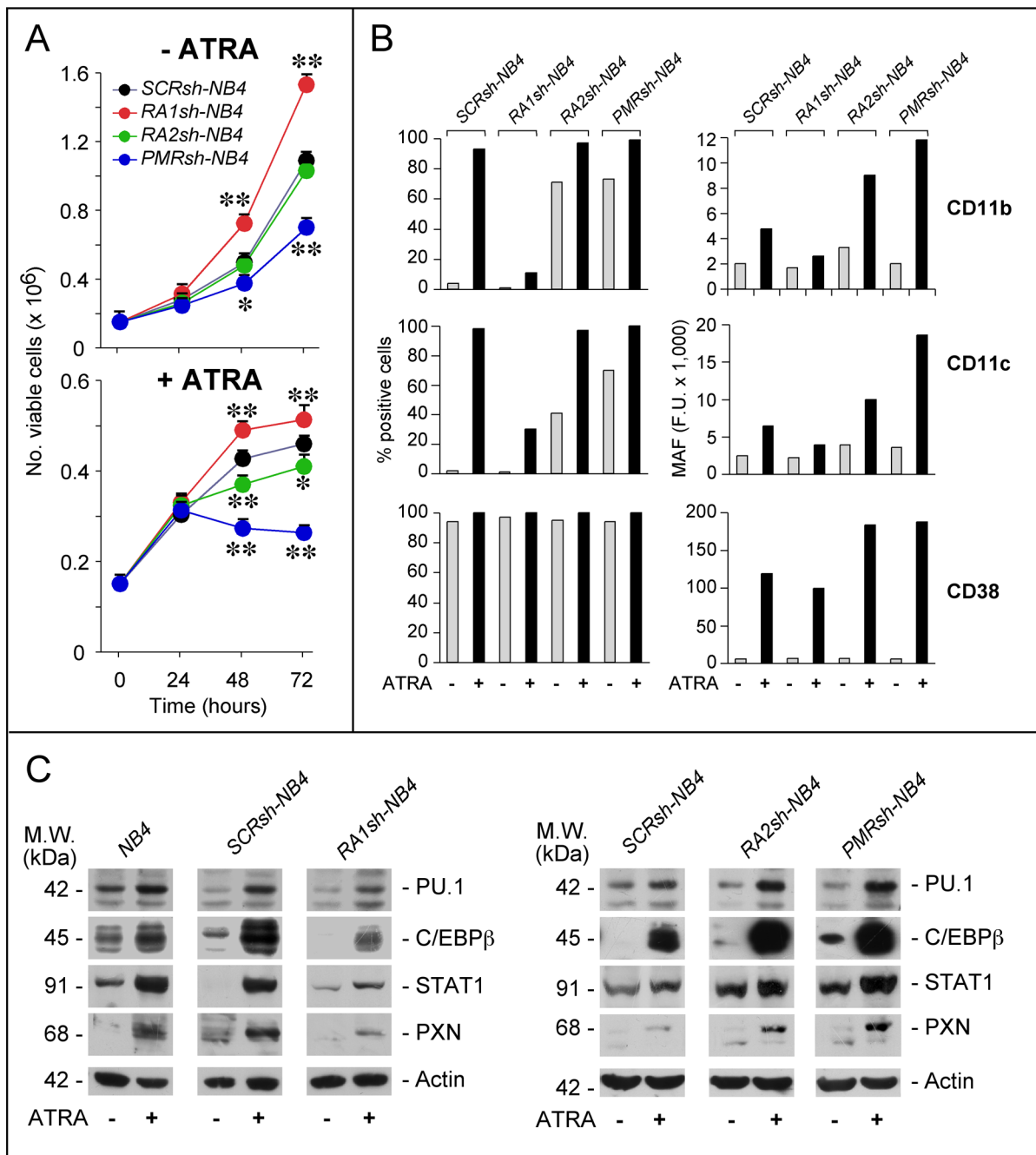


Figure 3: Effects of PML-RAR, RAR α 2 and RAR α 1 knock-down on the growth and differentiation of NB4 cells.

A. The indicated NB4 cell populations stably infected with shRNAs targeting PML-RAR (*PMRsh-NB4*), RAR α 1 (*RA1sh-NB4*), RAR α 2 (*RA2sh-NB4*) or the control scramble shRNA (*SCRsh-NB4*) were grown in the presence of vehicle (DMSO) or ATRA (1 μ M) for the indicated amount of time. The number of viable cells determined after staining with trypan blue is indicated. Each point is the mean \pm S.D. of three replicate cultures. ** = Significantly different relative to the corresponding *SCRsh-NB4* time point ($p < 0.01$ after Student's t-test); * = Significantly different relative to the corresponding *SCRsh-NB4* time point ($p < 0.05$ after Student's t-test). **B.** The indicated NB4 cell populations [see (A)] were grown in the presence of vehicle (DMSO) or ATRA (1 μ M) for 72 hours. Cells were subjected to FACS analysis for the indicated markers. The column graphs on the left indicate the percentage of CD11b-, CD11c- and CD38-positive cells. The graphs on the right indicate the MAF (mean-associated-fluorescence) values determined. The results are representative of two independent experiments. **C.** The indicated NB4 cell populations were treated as in (B) for 48 hours. Cell extracts were subjected to Western blot analysis for the indicated proteins. Actin is used as a loading control. The results shown in the upper and lower panels were obtained in separate experiments. Each line shows cropped lanes of the same gel, hence the results can be compared across the lanes, as they were obtained with the same exposure time. The calculated molecular weight (M.W.) of each protein is indicated on the left. The results are representative of at least two independent experiments.

in the same cellular context. To this purpose, we produced *NB4* cell populations stably transfected with a RAR α 2 plasmid (*pCDH-RAR2*) or the void vector (*pCDH*) (Figure 4A). Independent control (*pCDH-NB4a*; *pCDH-NB4b*) and RAR α 2 over-expressing (*pRAR2-NB4a*; *pRAR2-NB4b*) cell populations were compared for their growth in the absence/presence of ATRA. In *pCDH-NB4a*, *pRAR2-NB4a* and *pRAR2-NB4b*, the basal growth and the anti-proliferative action of ATRA are similar (Figure 4B). In untreated parental, *pCDH-NB4* and *pRAR2-NB4* cells, the same low levels of CD11b- and CD11c-MAF are observed (Figure 4C). However, the ATRA-dependent increase of CD11b-MAF in parental, *pCDH-NB4a* and *pCDH-NB4b* cells is suppressed in *pRAR2-NB4a* and *pRAR2-NB4b* cells. RAR α 2 over-expressing blasts show also a substantial inhibition of ATRA-dependent CD11c induction. In vehicle and ATRA-treated *pRAR2-NB4a*, *pRAR2-NB4b* and *pCDH-NB4a* cells, a similar trend is evident, if we compare the levels of PU.1, cEBP β and paxillin, (Figure 4D). Hence, RAR α 2 over-expression and RAR α 2 knock-down exert opposite effects on the myeloid-associated markers considered.

Silencing of PML-RAR and RAR α 2 exerts similar effects on the NB4 whole-genome gene-expression profiles

We compared the gene-expression profiles of *PMRsh-NB4*, *RA2sh-NB4*, *RA1sh-NB4* and *SCRsh-NB4* cells exposed to vehicle or ATRA for 48 hours [36, 37]. The time point was selected because it precedes terminal differentiation of *NB4* cells and it is characterized by large and ATRA-dependent variations of the whole-genome gene-expression profiles in parental *NB4* cells. We identified 5,567 genes whose expression is significantly modified in at least one of the comparisons considered ($p < 0.05$, BH, 0.6 fold-change threshold). Differentially expressed genes can be classified into 8 clusters according to their expression pattern by the K-means algorithm (Supplementary Table S1). *Cluster-1* and *Cluster-2* genes are up- or down-regulated in *PMRsh-NB4* cells under basal conditions and ATRA exerts no or very limited effects on their expression (Figure 5). In *PMRsh-NB4* cells, up-/down-regulation of the majority of genes reaches statistical significance. Although changes are smaller and often lack significance, the same regulation pattern of *Clusters-1/-2* genes is observed in *RA2sh-NB4* cells. *Cluster-3/-4* genes are up-regulated by ATRA in *SCRsh-NB4* and *RA1sh-NB4* cells to the same extent. ATRA-dependent up-regulation of *Cluster-3* genes is enhanced in *PMRsh-NB4* and *RA2sh-NB4* cells, while down-regulation of *Cluster-4* genes is repressed following PML-RAR and RAR α 2 knock-down (Figure 5). The differences reach statistical significance in a considerable fraction of genes. Once again, PML-RAR and RAR α 2 act on common gene-sets which are regulated by these *NRs*

in the same direction. *Clusters-5/-7* consist of numerous genes whose basal or ATRA-dependent expression is left generally unaffected by PML-RAR, RAR α 1 or RAR α 2 knock-down (Figure 6). *Cluster-5* genes are up-regulated, while *Cluster-7* genes are down-regulated by ATRA. Following ATRA treatment, *Clusters-6/-8* have similar profiles of expression relative to *Clusters-5/-7*. However, *Clusters-6/-8* genes are up-/down-regulated by PML-RAR and RAR α 2 knock-down also in basal conditions. Overall, the effects of RAR α 1 knock-down are small with few genes showing statistically significant alterations in their expression. However, a general trend towards inhibition of the ATRA-dependent effects, with particular reference to *Clusters-3/-5/-7*, is observed in *RA1sh-NB4* cells. This may be partially explained by incomplete silencing of RAR α 1 (see Figure 2A-2B).

We performed pathway enrichment analysis of the genes significantly regulated in *PMRsh-NB4* (comparisons c and g/d) and *RA2sh-NB4* cells (comparisons b and f/d) using annotated gene-collections (*Molecular Signatures* database) (Supplementary Table S2). In the 3 gene-collections considered, we found a significant overlap between the gene-sets enriched in *PMRsh-NB4* and *RA2sh-NB4* cells (Supplementary Figure S6A). Many of the genes regulated in *PMRsh-NB4* and *RA2sh-NB4* cells are direct PML-RAR targets [19] (Supplementary Figure S6B). The significant overlap between genes regulated by PML-RAR or RAR α 2 silencing and those regulated in NPM1-mutated blasts [38] (Supplementary Figure S7) may be of relevance for ATRA therapeutic action, since AMLs characterized by NPM1-mutations are deemed to be ATRA-sensitive [39–41]. Finally, enrichment in the genes of the GO “Immune-System-Process” (Supplementary Figure S8) may be linked to the neutrophil differentiation program triggered by PML-RAR and RAR α 2 knock-down. Indeed, emergency granulopoiesis is stimulated by inflammatory cytokines [42, 43], [44].

Finally, we evaluated the expression pattern of the genes contained in the “hematopoietic-cell-lineage” KEGG pathway (hsa04640) (Supplementary Figure S9), which is significantly enriched in genes regulated by PML-RAR ($p < 1E-6$) and RAR α 2 ($p < 3.38E-4$) silencing. As expected, the gene-regulation pattern observed following challenge with ATRA is consistent with myeloid differentiation. For instance, ATRA down-regulates CD135 (FLT3), a marker of hematopoietic stem-cells, while it up-regulates the neutrophil/monocyte marker, CD11b (ITGAM). Many ATRA-regulated mRNAs are also modulated by PML-RAR and RAR α 2 knock-down in the same direction.

RAR α 2 interferes with the transcriptional activity of RAR α 1 and PML-RAR

To evaluate whether *RAR α 2* inhibitory action on myeloid differentiation involves direct effects on RAR α 1

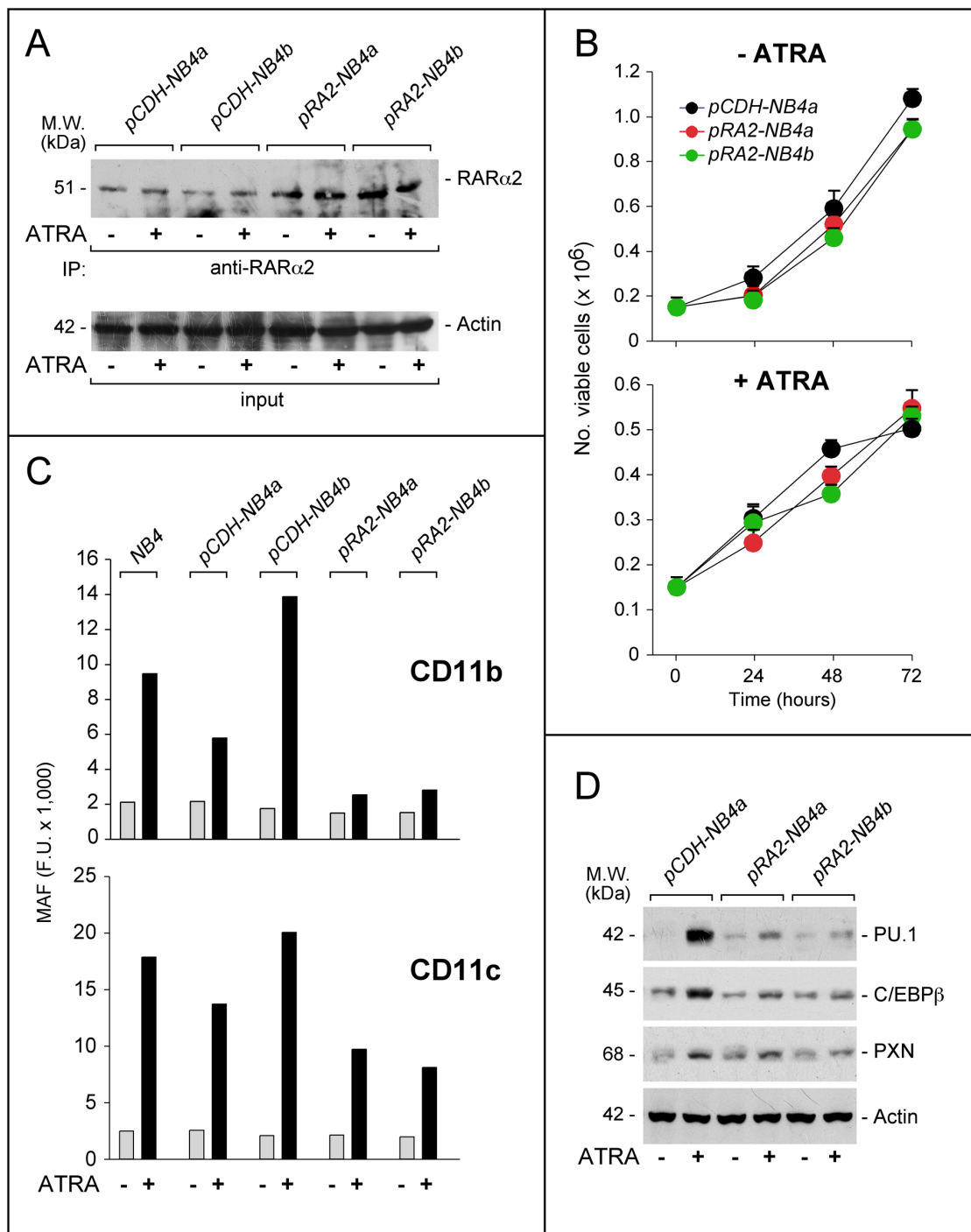


Figure 4: Effects of RARα2 over-expression on the growth and differentiation of NB4 cells. NB4 cells were transfected with the *pCDH-RA2* plasmid and the corresponding void vector, *pCDH*. Two distinct RARα2 expressing (*pRA2-NB4a* and *pRA2-NB4b*) and two (*pCDH-NB4a* and *pCDH-NB4b*) cell populations were isolated. **A**. Cells were treated with ATRA (1 μ M) for 24 hours. Cell extracts were immuno-precipitated with an anti-RARα2 antibody [Ab25alpha2(A2)] coupled to Protein G sepharose beads. The immuno-precipitates were subjected to Western blot analysis with a different anti-RARα antibody [RP alpha (F)]. Equivalent amounts of protein extracts were used to immuno-precipitate RARα2, as indicated by the levels of actin present in the extracts before addition of the anti-RARα2 antibody (input). **B**. Cells were treated with vehicle (DMSO) or ATRA (1 μ M) for the indicated amount of time. The number of viable cells determined after staining with trypan blue is indicated. Each point is the mean \pm S.D. of three replicate cultures. **C**. Cells were grown in the presence of vehicle (DMSO) or ATRA (1 μ M) for 72 hours and subjected to FACS analysis for the determination of CD11b and CD11c. The column graphs indicate the MAF (mean-associated-fluorescence) values determined. **D**. Cells were grown as in (C) and treated with vehicle (DMSO) or ATRA (1 μ M) for 48 hours. Cell extracts were subjected to Western blot analysis for the indicated proteins. Actin is used as a loading control. The calculated molecular weight (M.W.) of each protein is indicated on the left.

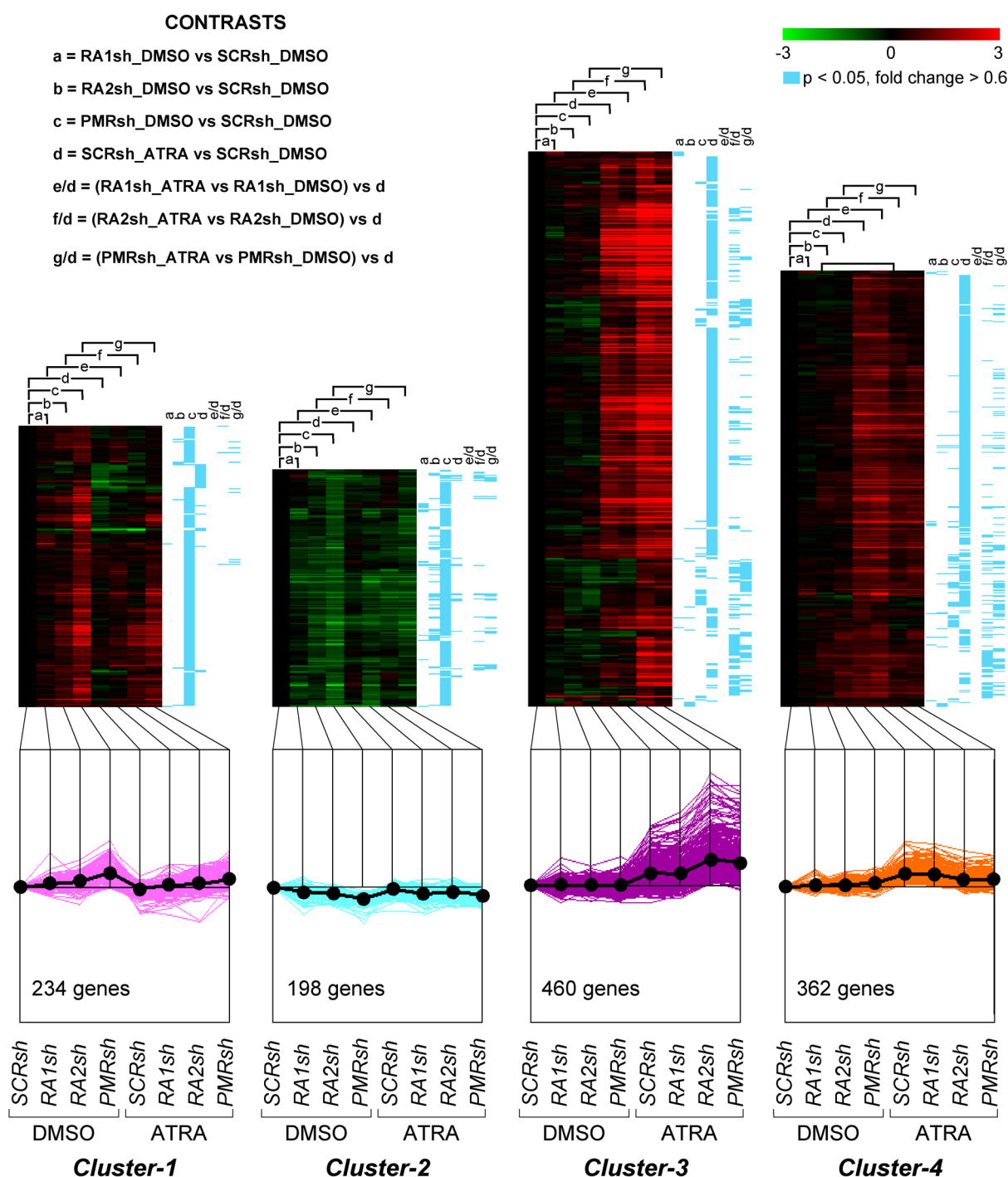


Figure 5: Perturbations of the basal and ATRA-dependent gene expression caused by PML-RAR, RAR α 2 and RAR α 1 knock-down in NB4 cells. Classification by K-means clustering: Cluster-1 through Cluster-4 genes. The indicated NB4 cell populations stably infected with PML-RAR (PMRsh-NB4), RAR α 1 (RA1sh-NB4), RAR α 2 (RA2sh-NB4) and scramble (SCRsh-NB4) shRNAs were grown in the presence of vehicle (DMSO) or ATRA (0.1 μ M) for 48 hours. Total RNA was used to perform whole-genome gene-expression microarray experiments. For each experimental group, data are reported as the \log_2 of the ratio vs basal expression in SCRsh-NB4 cells (SCRsh_DMSO). The genes regulated in at least one of the experimental conditions are grouped into eight Clusters and the figure illustrates Cluster-1 to Cluster-4. The upper graphs are heat-maps generated by hierarchical clustering using Pearson's distances. The statistical significance of the indicated comparisons (CONTRASTS, $p < 0.05$, \log_2 ratio > 0.6 or < -0.6) is shown on the right by the blue lines. The lower line graphs show the global expression profiles.

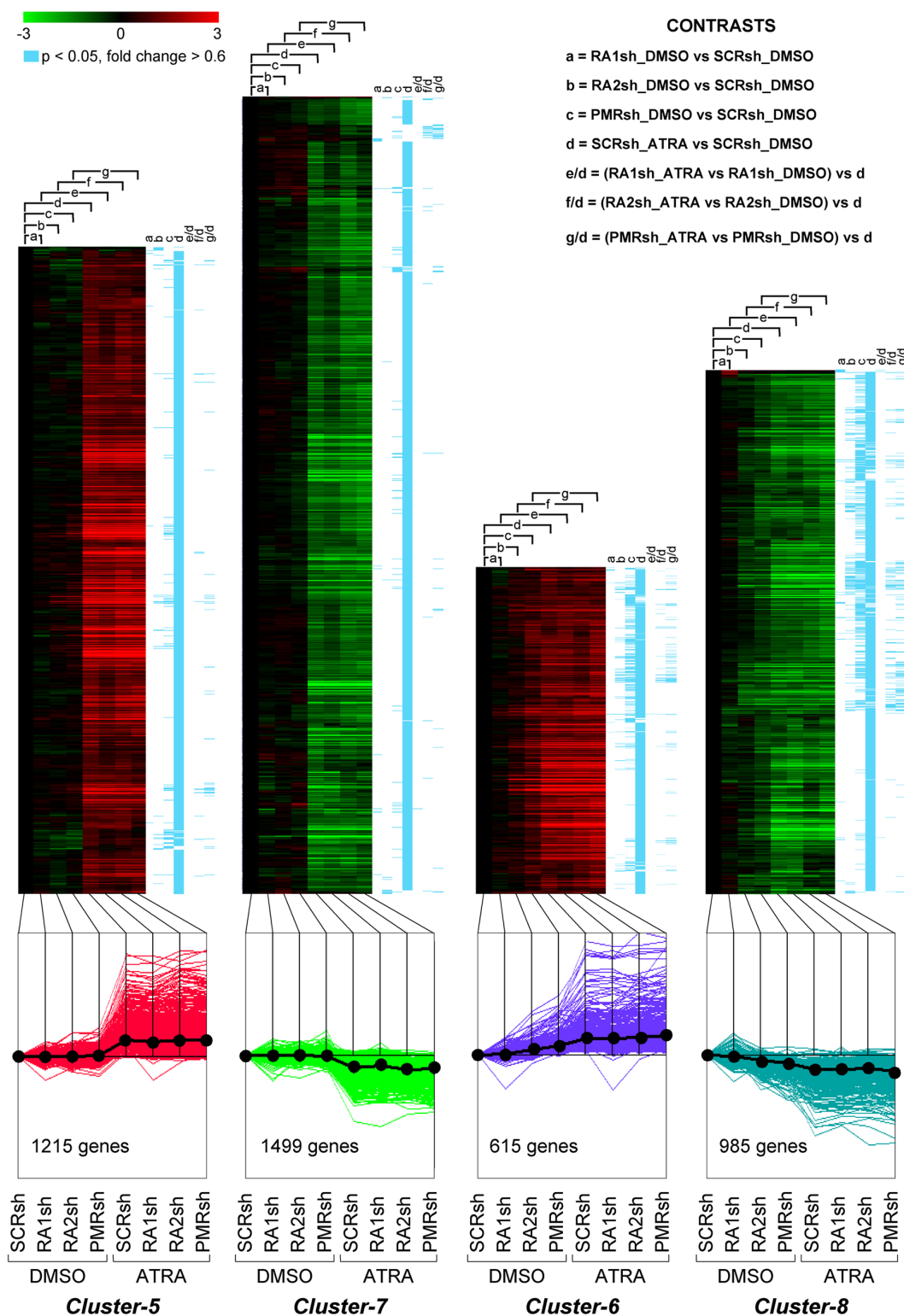


Figure 6: Perturbations of the basal and ATRA-dependent gene expression caused by PML-RAR, RAR α 2 and RAR α 1 knock-down in NB4 cells. Classification by K-means clustering: Cluster-5 through Cluster-8 genes. The indicated NB4 cell populations stably infected with PML-RAR (*PMRsh-NB4*), RAR α 1 (*RA1sh-NB4*), RAR α 2 (*RA2sh-NB4*) and scramble (*SCRsh-NB4*) shRNAs were grown in the presence of vehicle (DMSO) or ATRA (0.1 μ M) for 48 hours. Total RNA was used to perform whole-genome gene-expression microarray experiments. For each experimental group, data are reported as the log₂ of the ratio vs basal expression in *SCRsh-NB4* cells (*SCRsh_DMSO*). The genes regulated in at least one of the experimental conditions are grouped into eight Clusters and the figure illustrates *Cluster-5* to *Cluster-8*. The upper graphs are heat-maps generated by hierarchical clustering using Pearson's distances. The statistical significance of the indicated comparisons (CONTRASTS, $p < 0.05$, log₂ ratio > 0.6 or < -0.6) is shown on the right by the blue lines. The lower line graphs show the global expression profiles.

and PML-RAR transcriptional activity, we used a co-transfection approach in *COS-7* cells, a popular model characterized by no expression of PML-RAR or RAR α 2 and very low expression of RAR α 1 [17, 23, 26]. We performed co-transfection studies with RAR α 2 and RAR α 1 or PML-RAR in *COS-7* cells transiently expressing the β 2RARE-*tk-Luc* reporter. Separate transfection of RAR α 1, PML-RAR or RAR α 2 stimulates ATRA-dependent luciferase activity (Figure 7A). Simultaneous over-expression of RAR α 2 and RAR α 1 or PML-RAR reduces this stimulation, indicating cross-interference. Cross-interference is not due to effects on the amounts of RAR α 2, RAR α 1 or PML-RAR measured following separate and combined transfection in *COS-7* cells exposed to vehicle or ATRA (Figure 7B). Cross-interference is specific to RAR α 2 and RAR α 1 or PML-RAR, as indicated by the results obtained with RAR β 2 or RAR γ 2. Indeed, co-transfection with RAR α 2 increases or leaves unaffected luciferase activity as compared to separate RAR α 2, RAR β 2 or RAR γ 2 transfection (Figure 7C).

RAR α 2 binds to RAR α 1 and PML-RAR directly: insights into the structural determinants of these interactions

To establish whether cross-interference involve interactions between RAR α 2, RAR α 1 and/or PML-RAR, we performed pull-down experiments in *COS-7* cells transfected with RAR α 1. Vehicle and ATRA-treated *COS-7* extracts were incubated with glutathione-S-transferase(GST)-tagged RAR α 2 (GST-RAR α 2) or control GST (Figure 8A). Upon WB of the GST-RAR α 2 pull-down fraction with an anti-RAR α antibody, a band corresponding to transfected RAR α 1 is visible regardless of ATRA treatment. A similar band is not detected after GST pull-down. Superimposable results are observed if RAR α 1 is substituted by PML-RAR (data not show). Specular pull-down studies with *GST-RAR α 1* and *GST-RAR α 1DEF* (a RAR α 1 recombinant product consisting of the entire D/E/F) on extracts of *COS-7* cells transfected with *pHA-RAR α 2*, *pHA-SNAIL* and *pHA* confirm and extend the results (Figure 8B and Supplementary Figure S10A). In fact, the data obtained following WB with anti-HA antibodies indicate that RAR α 2 is pulled-down by both *GST-RAR α 1* and *GST-RAR α 1DEF*. ATRA does not affect this interaction. Similar experiments conducted with *GST-RAR α 1ABC* do not result in RAR α 2 pull-down (data not shown). The specificity of the interaction between RAR α 2 and the D/E/F regions of RAR α 1 is supported by the results obtained in *COS-7* cells transfected with the negative *pHA-SNAIL* and the positive *pSG5-RXR α* controls (Supplementary Figure S10B).

Far-Western experiments were performed (Figure 8C) on extracts of *COS-7* cells transfected with hemoagglutinin(HA)-tagged RAR α 2 and treated with vehicle or ATRA. Immobilized HA-RAR α 2 immuno-precipitates (anti-HA antibodies) were challenged

with GST-RAR α 1. Following incubation with GST-RAR α 1, but not GST, an anti-GST antibody highlights a band at the height of HA-RAR α 2. This demonstrates a direct interaction between HA-RAR α 2 and GST-RAR α 1, which is reproduced with the 2 GST-RAR α 1 derivatives consisting of the DEF regions containing (*GST-RAR α 1DEF*) or lacking the H12 helix [*GST-RAR α 1DEFD(408-416)*]. The GST-RAR α 1 protein consisting of the ABC regions (*GST-RAR α 1ABC*) does not interact with HA-RAR α 2. No quantitative difference in the RAR α 2/RAR α 1 interaction is observed in the absence/presence of ATRA.

To confirm the results obtained, we performed co-immuno-precipitation experiments in *COS-7* cells transfected with RAR α 2 alone or in combination with RAR α 1 using the specific anti-RAR α 2 antibodies. This was followed by WB with the distinct antibody [RPalpha(F)] detecting both RAR α 2 and RAR α 1. Co-precipitation of RAR α 1 (Figure 8D) is observed only in cells co-transfected with RAR α 2. The amounts of co-precipitated RAR α 1 are not influenced by ATRA treatment. RAR α 2 is immuno-precipitated only from cells transfected with the corresponding construct, confirming the specificity of the anti-RAR α 2 antibody. To get insights into the structural determinants of RAR α 2/RAR α 1 direct interaction, we performed similar co-immuno-precipitation experiments in *COS-7* cells co-transfected with RAR α 2 and selected RAR α 1 mutants of critical residues in the D/E/F regions (Figure 8E). Co-precipitation of RAR α 2 is observed in cells co-transfected with wild-type RAR α 1, the RAR α 1-S157A mutant (affecting a phosphorylation site at the C/D regions interface) and RAR α 1-I396E mutant (influencing the interactions with co-activators) [47]. In contrast, the RAR α 1-S369A mutant affecting the protein-kinase-A, mitogen-and-stress-activated-protein-kinase and p38-kinase phosphorylation site [23, 32, 48] does not interact with RAR α 2. The amounts of co-precipitated RAR α 1 and derived mutants are not significantly influenced by ATRA. Similar immuno-precipitation studies performed after co-transfection of RAR α 2, wild-type PML-RAR and the PML-RAR-S873A (corresponding to RAR α 1-S369A) confirm the above results. In fact, RAR α 2 co-immuno-precipitates only with wild-type PML-RAR (Figure 8F).

To evaluate whether interactions between RAR α 2 and RAR α 1 or PML-RAR are also observed in the native APL context, we performed further immuno-precipitation studies in our *NB4* models. RAR α 2 is immuno-precipitated in extracts from parental, *SCRsh-NB4*, *RA1sh-NB4* and *PMRsh-NB4* but not in *RA2sh-NB4* cells. An extra 52 kDa band is detectable in the immuno-precipitates of untreated *NB4*, *SCRsh-NB4* and *PMRsh-NB4* only upon long exposures and its intensity is dramatically increased by ATRA (Figure 9A). The band corresponds to RAR α 1, as it is absent in *RA1sh-NB4* cells. To evaluate whether RAR α 2 is capable of interacting with PML-RAR, WB experiments on the anti-RAR α 2 immuno-precipitates were

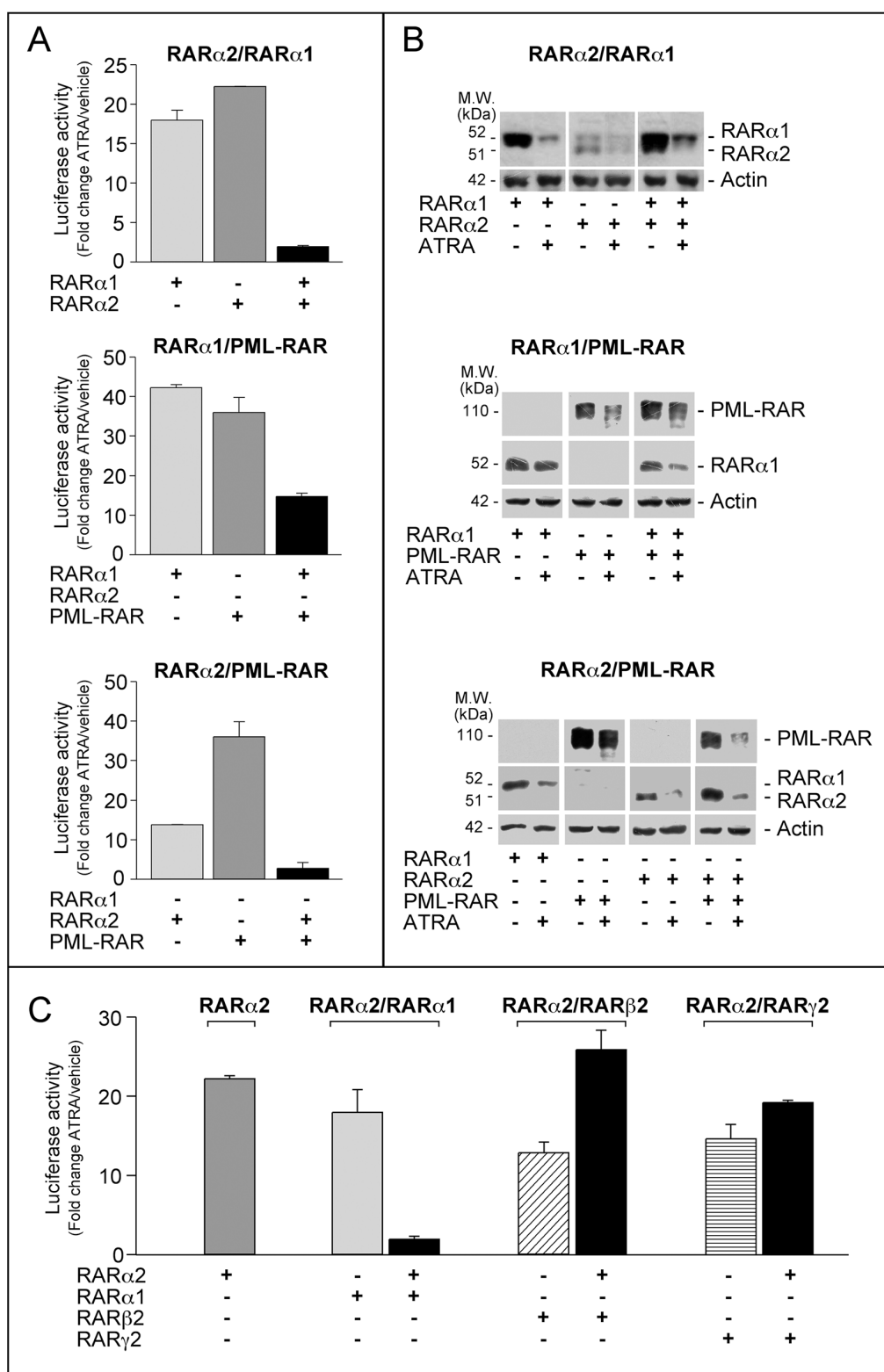


Figure 7: Interference between RAR α 2 and RAR α 1 or PML-RAR transcriptional activity. **A.** COS-7 cells were transfected with *pcDNA3-RAR α 2*, *pcDNA3-RAR α 1* and *pSG5-PML-RAR* plasmids alone or in combination and *β 2RARE-Luc*. Sixteen hours following transfection, cells were treated with DMSO or ATRA (1 μ M) for 24 hours. **B.** COS-7 cell extracts were subjected to Western blot analysis with an anti-RAR α antibody [RP alpha (F)]. The same amounts of extracts used for the determination of RAR α 1 and RAR α 2 were subjected to Western blot analysis to determine the loading control, β -actin. **C.** COS-7 cells were transfected with *pcDNA3-RAR α 2*, *pcDNA3-RAR α 1*, *pSG5-RAR β 2* and *pSG5-RAR γ 2* plasmids alone or in combination and *β 2RARE-Luc*. Sixteen hours following transfection, cells were treated with DMSO or ATRA (1 μ M) for a further 24 hours. Luciferase activity is expressed as the ratio of ATRA/DMSO luciferase activity (fold-change). Each value is the mean \pm SD of two replicates.

conducted with the antibody detecting PML-RAR, RAR α 2 and RAR α 1 (Figure 9B). In untreated *SCRsh-NB4* and *RA1sh-NB4* cells, PML-RAR and RAR α 2 co-immunoprecipitate. In the same cells treated with ATRA, the

amounts of co-immunoprecipitated PML-RAR increase. The co-immunoprecipitated PML-RAR band is observed in neither untreated nor ATRA treated *RA2sh-NB4* and *PMRsh-NB4* cells.

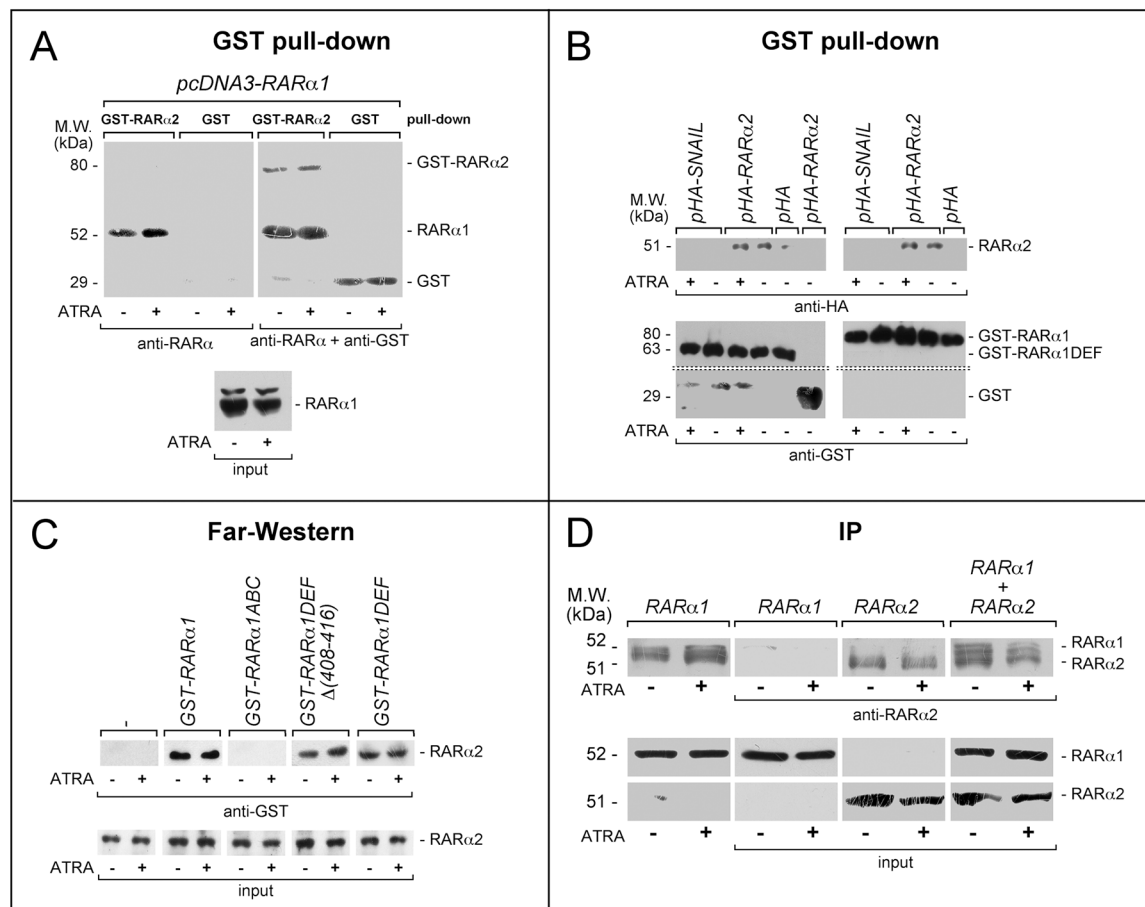


Figure 8: Functional and physical interactions between RAR α 2 and RAR α 1 or PML-RAR. **A.** GST pull-down: the GST-tagged recombinant protein, GST-RAR α 2 and GST were used. The two recombinant proteins conjugated to Glutathione-Sepharose beads were incubated with extracts of *COS-7* cells transfected with *pcDNA3-RAR α 1* and treated with vehicle or ATRA (1 μ M) for 4 hours. GST pull-down precipitates were blotted on nitro-cellulose filters, hybridized with an anti-RAR α [RP alpha (F)] (left panel) and subsequently with an anti-GST antibody (right panel). The blot was not stripped between the two hybridizations. Input: cell extracts (10 μ g of protein) representing 10% of the total amount of protein were subjected to Western blot analysis with the above anti-RAR α antibody. **B.** GST pull-down: the GST-tagged recombinant proteins, *GST-RAR α 1* and *GST-RAR α 1DEF* were used. The two recombinant proteins conjugated to Glutathione-Sepharose beads were incubated with extracts of *COS-7* cells transfected with *pHA-RAR α 2* as well as the negative controls, *pHA-SNAIL* plasmid and *pcDNA3* plasmid containing the HA tag (*pHA*). Transfected cells were treated with vehicle or ATRA as in (A). As an internal control of the experiment, we performed a pull-down assay with the GST protein coupled to Glutathione-Sepharose beads on extracts of cells transfected with *pHA-RAR α 2*. GST pull-down precipitates were subjected to Western blot analysis with anti-HA (upper panels) and anti-GST antibodies (lower panels). **C.** Far-Western: *COS-7* cells were transfected with a *pcDNA3* plasmid containing a haemoagglutinin (HA) tagged RAR α 2 cDNA (*pHA-RAR α 2*). Cell extracts were precipitated with agarose beads conjugated with an anti-HA monoclonal antibody. The immuno-precipitates were subjected to Far-Western analysis using the following GST-tagged RAR α 1 recombinant proteins: *GST-RAR α 1* = full-length RAR α 1; *GST-RAR α 1ABC* = RAR α 1 ABC regions; *GST-RAR α 1DEF* = RAR α 1 DEF regions; *GST-RAR α 1DEF Δ (408-416)* = RAR α 1 DEF regions lacking the H12 helix. Input: cell extracts (10 μ g of protein) representing 10% of the total amount of protein used for the immuno-precipitations were subjected to Western blot analysis with an anti-HA antibody. Each line shows cropped lanes of the same gel, hence the results can be compared across the lanes, as they were obtained with the same exposure time. **D.** Immunoprecipitations (IP): *COS-7* cells were transfected with *pcDNA3-RAR α 1* and *pcDNA3-RAR α 2* alone or in combination. Sixteen hours following transfection, cells were treated with vehicle or ATRA (1 μ M) for 4 hours. The indicated extracts were immuno-precipitated with anti-RAR α 2 antibodies and subjected to Western blot analysis with a different anti-RAR α antibody [RP alpha (F)]. The two leftmost lanes represent controls of RAR α 1 transfected cells directly submitted to Western blot analysis without immuno-precipitation. Equivalent amounts of protein extracts were used to immuno-precipitate RAR α 2, as indicated by the levels of RAR α 2 [Ab25alpha2(A2) antibody] and RAR α 1 [Ab10alpha1(A1)antibody]in the extracts (input). Each line shows cropped lanes of the same gel, hence the results can be compared across the lanes, as they were obtained with the same exposure time. (Continued)

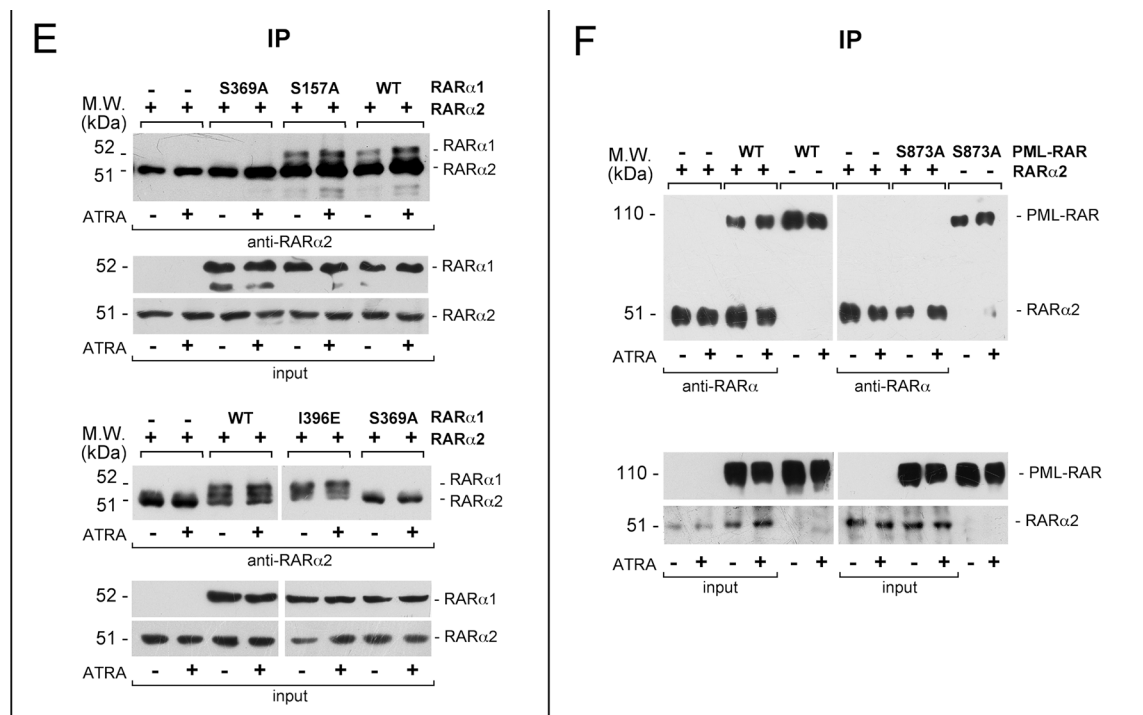


Figure 8: (Continued) Functional and physical interactions between RARα2 and RARα1 or PML-RAR. E. Immuno-precipitation (IP): COS-7 cells were co-transfected with wild-type (WT) RARα2 or WT RARα1 and RARα1 mutants and subjected to immune-precipitation and Western blot analysis as in (D). F. Immuno-precipitation (IP): COS-7 cells were co-transfected with wild-type (WT) RARα2 or WT PML-RAR and derived mutant. The extracts of transfected cells were treated and subjected to co-immuno-precipitation studies as in (D and E). Lanes 5, 6, 11 and 12 represent controls of PML-RAR and PML-RAR-S873A transfected cells directly submitted to Western blot analysis without immuno-precipitation.

In conclusion, our data demonstrate that RARα2 is capable of binding to RARα1 and PML-RAR and this binding may be at the basis of the observed functional interferences. The binding interface is located in the D/E/F region of RARα1 where the Ser-369 phosphorylation site plays a pivotal role in RARα2/RARα1 interaction. Binding of RARα2 to RARα1 and functional inhibition of the latter receptor may also partially explain the similarity in the effects afforded by RARα2 and PML-RAR silencing in NB4 cells.

DISCUSSION

It is believed that ATRA therapeutic action in APL involves degradation of PML-RAR [7, 49], which releases the suppressive effect exerted by the fusion protein on the product of the intact *RARA* allele, RARα [2, 3]. However, the existence of 3 RARα isoforms (RARα1, RARα2 and RARα4) adds complexity to the system. In the APL-derived NB4 cellular model, RARα2 and PML-RAR are negative regulators of the granulocytic differentiation program and act *via* common transcriptional mechanisms. The similarities between RARα2 and PML-RAR activities are observed not only in NB4 blasts under basal conditions, but

also upon exposure to ATRA. Indeed, RARα2 and PML-RAR knock-down enhances ATRA-dependent induction of myeloid markers and the expression of direct retinoid target-genes. By converse, RARα1 knock-down does not alter the basal differentiation state and gene-expression pattern of NB4 blasts, while it attenuates the ATRA-dependent induction of myeloid markers and the transcriptomic effects triggered by the retinoid. In NB4 cells, the negative effects of RARα2 on ATRA differentiating activity are confirmed by over-expression approaches. As RARα2 is a negative prognostic factor in multiple myeloma [50, 51], the oncogenic action of RARα2 may extend to other hematological malignancies and solid tumors. With respect to this, we have preliminary evidence that over-expression of RARα2 in a retinoid-responsive breast-cancer cell line inhibits ATRA-simulated activity of a RARE-containing reporter and suppresses ATRA-dependent induction of the retinoid targets, SMAD3 and β-catenin [52, 53].

Our gene-expression studies provide insights into the molecular mechanisms underlying RARα2 and PML-RAR involvement in the process of granulocytic maturation induced by ATRA in APL blasts. In physiological conditions, the process is constitutively

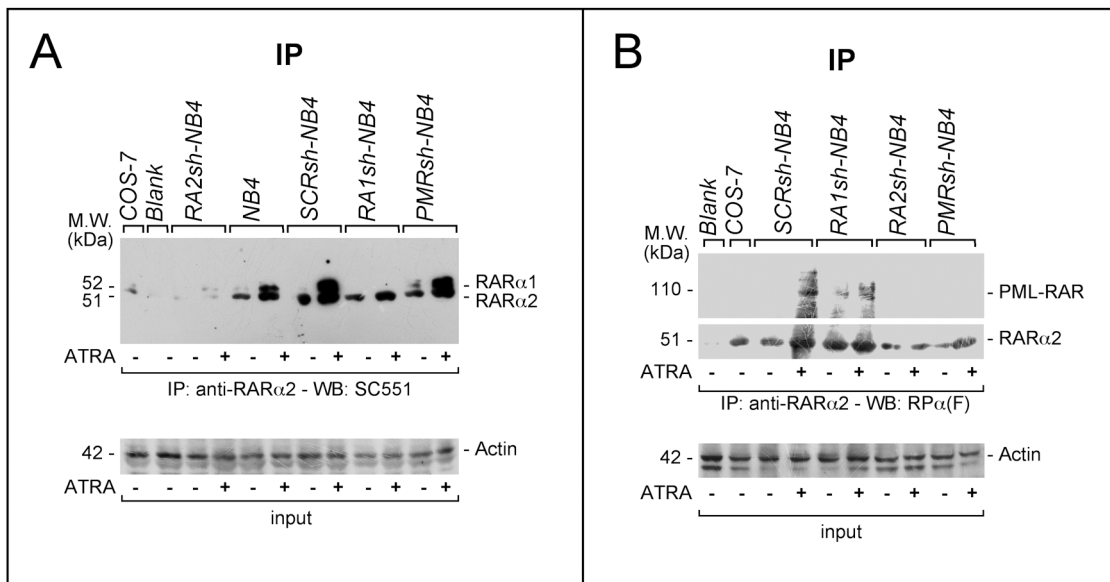


Figure 9: Physical interactions between RARα2 and RARα1 or PML-RAR in NB4 cells. A. and B. Immuno-precipitation (IP) experiments: *SCRsh-NB4*, *RA1sh-NB4*, *PMRsh-NB4*, *RA2sh-NB4* and *NB4* parental cells were treated with vehicle or ATRA (1 μM) for 4 hours. Cell extracts were immuno-precipitated using an anti-RARα2 antibody [Ab25α2(A2)]. This was followed by Western blot analysis of the immunoprecipitates with two distinct antibodies, which, in our experimental conditions, detect RARα2 and RARα1 (SC551) (A) or RARα2, RARα1 and PML-RAR [RP α(F)] (B), respectively. Input: cell extracts (10 μg of protein) representing 10% of the total amount of protein used for the immune-precipitations were subjected to Western blot analysis with an anti-actin antibody.

active during steady-state granulopoiesis, which is regulated by G-CSF, and GM-CSF [54, 55], and it is episodically stimulated during stress granulopoiesis, which is part of the innate immune response to infection/inflammation. Steady-state granulopoiesis requires the PU.1 and C-EBPα transcription factors [56–58], while stress granulopoiesis is stimulated by inflammatory cytokines like interferon [42, 43], IL-6, IL-3 and IL-1 [44]. ATRA seems to induce *NB4* cell differentiation *via* activation of both steady-state and stress granulopoiesis, since it induces PU.1, cEBPβ and IL-1. As for steady-state granulopoiesis, ATRA-dependent PU.1 induction is enhanced by PML-RAR and RARα2 knock-down, while it is blocked by RARα2 over-expression. By converse, cEBPα is down-regulated by ATRA and this is consistent with the absence of GM-CSF induction [59]. As for stress granulopoiesis, PML-RAR and RARα2 knock-down up-regulates the basal expression and enhances ATRA dependent induction of numerous inflammatory genes with particular reference to those involved in the interferon pathway, including STAT1 (Supplementary Table S1). Constitutive and ATRA-induced c/EBPβ protein as well as IL-1β mRNA levels are enhanced by PML-RAR and RARα2 knock-down. Consistent with this, over-expression of RARα2 down-regulates both basal and ATRA-dependent expression of c/EBPβ.

Given the similarity of the effects induced by RARα2 and PML-RAR knock-down, the two receptors

may act *via* common mediators or interact functionally. Functional studies confirm that RARα2, PML-RAR and RARα1 activate the same RARE-containing reporter in an ATRA-dependent fashion. Unexpectedly, however, RARα2 and PML-RAR interfere with RARα1 in terms of ligand-dependent transcriptional activity. This may explain the similarities between RARα2 and PML-RAR in terms of *NB4* differentiation and gene-expression profiles. The observed functional interferences support the concept that RARα1 is indeed a potential target of RARα2 and PML-RAR activity in APL cells. The significance of this is sustained by the data obtained on direct retinoid targets, like CD38 (Figure 3) CYP26A1 and RARβ (Supplementary Figure S11) in *NB4* cells. Indeed, time- and ATRA-dependent induction of CYP26A1 and RARβ mRNAs is blocked by RARα1 and enhanced by RARα2 or PML-RAR knock-down. Functional antagonism is at least partially explained by the ability of RARα2 and PML-RAR to physically interact with each other and RARα1. With the exception of the immuno-precipitation assays performed in *NB4* cells, our results demonstrate that direct binding is already observed in the absence of ATRA and it is not affected by the retinoid. However, in the *NB4* model, the levels of the RARα2/RARα1, PML-RAR/RARα1 and RARα2/PML-RAR complexes are increased by ATRA. The increase may be ascribed to RARα2 and PML-RAR induction by the retinoid (Figure 1). The interaction between RARα2 and RARα1 occurs also in PML-RAR-negative *HL-60* cells (Supplementary Figure S5B). Direct binding between the RARα2 and RARα1 or PML-RAR

involves the *D/E/F* regions. At present the binding interface is incompletely defined, although our data indicate that binding is influenced by phosphorylation of RAR α 1 Ser-369 and the corresponding Ser-873 residue of PML-RAR. The observation is of particular interest for the therapeutic use of ATRA as it may suggest rational combination strategies aimed at enhancing the anti-leukemic potential of the retinoid. In fact, RAR α 1 S369 is a target phosphorylation site of protein-kinase-A, mitogen-and-stress-activated-protein-kinase and p38-kinase [23, 32, 48]. From a therapeutic prospective, the action of combinations between ATRA and inhibitors of the three kinases should be specifically evaluated in appropriate pre-clinical models. With respect to this, it is noticeable that a p38-kinase inhibitor has been shown to boost the differentiating activity of ATRA in APL cells [32]. Thus, it would be tempting to speculate that at least part of the effect exerted by the p38-kinase inhibitor is related to suppression of the RAR α 2/RAR α 1 or RAR α 2/PML-RAR interactions. In conclusion, we propose that the RAR α 2/RAR α 1 and RAR α 2/PML-RAR heterodimers may be transcriptionally inactive and may explain at least part of the observed functional antagonisms.

MATERIALS AND METHODS

Reagents and constructs

MG132, and ATRA were from Calbiochem and Sigma. Custom-designed short hairpin RNAs (shRNAs) were purchased from Ambion (Supplementary Figure S2). The 5'- and 3'-end of each hairpin RNA contain an EcoRI and a BamHI site to allow oriented cloning into the (pGreenPuro, System Biosciences, Palo Alto, CA) retroviral vector. The cDNA of the RAR α 2 coding region, which was amplified from *NB4* cells, was cloned into the *pCDNA3* plasmid (Invitrogen) and transferred to the *pCDH-CMV* lentivirus vector (System Biosciences), utilizing the EcoRI-NotI sites.

Cells and infection/transfection procedures

NB4 [60], *HL-60* [61] and *COS-7* cells were cultured as described [36, 62, 63]. We generated *NB4* cell populations silenced for PML-RAR and the RAR α isoforms infecting cells with the *SCRsh*, *PMRsh*, *RA1sh* and *RA2sh* retroviral vectors according to standard protocols (System Biosciences). Following infection, *NB4* cells were selected in RPMI medium containing 10% bovine serum and puromycin (1.0 μ g/ml) for at least 15 days. Only *NB4* cell populations characterized by >95% positivity to GFP following FACS analysis were considered. Subsequent passages of the cell populations were performed in complete RPMI medium containing puromycin (0.5 μ g/ml). To isolate *NB4* populations over-expressing RAR α 2, cells were electroporated with *pCDH-CMV* lentiviral vectors (System Biosciences) containing

RAR α 2 (*pRA2*) using Neon Transfection (Invitrogen, Life Technologies). *HL-60* cells were infected as above with the *RA2sh*, *SCRsh* or the void (*Void*) retroviral vector.

Gene expression microarrays and real-time reverse-transcription-PCR

The *SCRsh-NB4*, *RA1sh-NB4*, *RA2sh-NB4* and *PMRsh-NB4* cell populations were treated with (DMSO) or ATRA (0.1 μ M) for 48 hours. Total RNA was reverse transcribed, labeled and hybridized to whole-genome gene expression microarrays (G4851B, Agilent, Santa Clara, CA) as already described [22]. Fluorescent signals were quantified with a laser scanner (Agilent). The microarray raw data were deposited in the Arrayexpress database (The accession No. E-MTAB-4713). Real-time reverse-transcription-PCR (RT-PCR) was performed with Taqman gene expression assays (C/EBP ϵ , Hs00152928_m1; CYP26A1, Hs00175627_m1; 18S endogenous control, 4333762F; RAR β 2, Hs00977143_m1; Applied Biosystems). The amplimers and Taqman probes used for the reverse-transcriptase RT-PCR assays of RAR α -v1 to RAR α -v4 were obtained from Life Technologies Italia (Monza, Italy) as detailed in Supplementary Methods.

RAR α 1, RAR α 2 and PML-RAR transactivation

COS-7 cells were transfected with RAR α 1, RAR α 2 and PML-RAR, alone or in combination in the presence of the RARE-containing β 2RARE-luciferase reporter [64]. The normalization plasmid is a renilla luciferase construct (Promega) [63].

FACS analysis, antibodies, immuno-precipitation and WB analyses

CD11b, CD11c and CD38 surface markers were determined with a Fluorescence Activated Cell Sorter (FACS, Becton and Dickinson) [26, 32]. Rabbit anti-RAR α polyclonal antibodies [RPalph(F)] and anti-RAR α 1 mouse monoclonal antibodies [Ab10a(A1)] were previously described [65]. Anti-RAR α 2 mouse monoclonal antibodies were raised against a synthetic peptide (amino-acids 1-29). Both Ab10a(A1) and Ab25a(A2) were purified on sulfoLink gel columns (Pierce Chemical) coupled to the immunizing peptide [66]. The other anti-RAR α (SC551), anti- β -actin, cEBP β , and anti-STAT-1 antibodies were from Santa-Cruz-Biotechnology. Anti-PU.1 and anti-paxillin antibodies were from Cell Signaling and Transduction Laboratories, respectively. WB analyses were performed as previously described [26] [32]. Immuno-precipitations were performed with antibodies immobilized on Protein-G-sepharose (Amersham). Agarose beads coupled to anti-HA antibodies were from Sigma (A2095).

Far-western and GST pull-down assays

COS-7 cells were transfected with a *pcDNA3* plasmid containing haemoagglutinin (HA)-tagged *RARα2* (*pHA-RARα2*). Extracts were precipitated with agarose-conjugated anti-HA monoclonal antibodies. The immunoprecipitates were subjected to Far-Western analysis using GST-tagged recombinant proteins [32]. For the pull-down experiments [32], we used the described *GST-RARα1*, *GST-RARα2* and derived recombinant proteins. *GST-RARα2* was obtained from *E. coli* cells transformed with an appropriate *RARα2* cDNA construct cloned in the *EcoRI*-*NotI* sites of *pGEX4T2*. The recombinant proteins conjugated to Glutathione-sepharose beads (Amersham) were incubated with extracts of *COS-7* cells transfected with *pHA-RARα2*, *pHA-SNAIL*, *pcDNA3-RARα1*, *pSG5-PML-RAR* or *pcDNA3* containing the HA-tag (*pHA*) and *pcDNA3* plasmids, for 4 hours. Pulled-down proteins were subjected to WB analysis using anti-HA, anti-*RARα* or anti-GST antibodies.

ACKNOWLEDGMENTS

We would like to acknowledge the help of Mr. Felice Deceglie and Mr. Alessandro Soave for the artwork.

CONFLICTS OF INTEREST

The authors declare no conflict of interest as to the data presented in this study.

GRANT SUPPORT

Grants from the Associazione Italiana per la Ricerca contro il Cancro (AIRC) and the Fondazione Italo Monzino to Enrico Garattini were fundamental for the completion of this work.

Author contributions

MG, MT, AZ, GP and MK performed various aspects of the experimental work; AR and GB performed and supervised the studies involving FACS and morphological analyses; MB and MF were involved in the computational analysis of the gene-expression studies; CR-E provided the tools and the expertise necessary to complete the molecular studies involving *RARα1* and *RARα2*; EG designed and supervised the entire study and wrote the manuscript.

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