S100A3 a partner protein regulating the stability/activity of RARα and PML-RARα in cellular models of breast/lung cancer and acute myeloid leukemia

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Abstract
All trans-retinoic acid (ATRA) is used in the treatment of acute promyelocytic leukemia (APL) and it is a promising agent also in solid tumors. The pharmacological activity of ATRA is mediated by the ligand-activated RAR and RXR transcription factors. In the present study, we define the basal and ATRA dependent RARα interactome in a RARα-overexpressing breast cancer cellular model, identifying 28 nuclear proteins. We focus our attention on the S100A3 calcium-binding protein, which interacts with RARα constitutively. In ATRA-sensitive breast cancer cells, S100A3 binds to RARα in basal conditions and binding is reduced by the retinoid. The interaction of S100A3 with RARα is direct and in lung cancer, APL and acute-myeloid-leukemia (AML) cells. In APL, S100A3 interacts not only with RARα, but also with PML-RARα. The interaction surface maps to the RARα ligand-binding domain, where the I396 residue plays a crucial role. Binding of S100A3 to RARα/PML-RARα controls the constitutive and ATRA-dependent degradation of these receptors. S100A3 knockdown decreases the amounts of RARα in breast- and lung cancer cells, inducing resistance to ATRA-dependent anti-proliferative/differentiating effects. Conversely, S100A3 knockdown in PML-RARα+ APL and PML-RARα− AML cells reduces the amounts of RARα/PML-RARα and increases basal and ATRA-induced differentiation. In this cellular context, opposite effects on RARα/PML-RARα levels and ATRA-induced differentiation are observed upon S100A3 over-expression. Our results provide new insights into the molecular mechanisms controlling RARα activity and have practical implications, as S100A3 represents a novel target for rational drug combinations aimed at potentiating the activity of ATRA.

Introduction
All-trans retinoic acid (ATRA) is used in the treatment of acute promyelocytic leukemia (APL) [1, 2], which is characterized by a chromosomal translocation involving the retinoid-receptor, RARα, resulting in the production of the PML-RARα oncogene [2–4]. ATRA is a promising agent in the treatment/chemoprevention of other neoplastic diseases, including breast cancer [3].

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ATRA activity is mediated by the ligand-activated transcription factors, RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ [4, 5]. These proteins consist of six regions A, B, C, D, E, and F from the N- to the C-terminus. The C-region is involved in DNA-binding, while the E-region contains the ligand-binding pocket [6–9]. Transcriptionally active retinoid-receptors consist of RAR/RXR heterodimers, in which RARs acts as the ligand-binding moiety. Unliganded RAR/RXR heterodimers bind to RAREs (retinoic-acid-responsive-elements) located in the regulatory regions of retinoid target-genes [6, 10]. Ligand-free RAR/RXR heterodimers are part of multi-protein complexes whose composition is modified upon ligand-binding. Specific RARs and RXRs interact with different proteins which modulate their transcriptional activity, phosphorylation/desphosphorylation [11], degradation [12] and subcellular localization [13].

Here, we use a proteomic approach to identify novel proteins interacting with RARα. Among the proteins showing a strong interaction with RARα, we focus on the calcium-binding protein, S100A3. The interaction of S100A3 with RARα is observed in breast cancer and lung cancer, acute-myeloid-leukemia (AML) as well as acute promyelocytic leukemia (APL) cells, in which S100A3 binds also to PML-RARα. S100A3 binding controls constitutive and ATRA-dependent degradation of RARα and PML-RARα. This interaction has opposite consequences on the anti-proliferative and differentiating activity of ATRA in breast cancer and lung cancer relative to AML and APL cells.

**Results**

**Identification of RARα interacting proteins**

RARα is the major determinant of ATRA anti-tumor activity in breast cancer [3, 5]. To identify novel RARα interacting proteins, we stably transfected a FLAG-tagged RARα (FLAG-RARα) plasmid and the corresponding void vector in ATRA-resistant MDA-MB-453 breast cancer cells [3, 14], generating RA-453 and FL-453 cells, respectively. In basal conditions, RA-453 express higher levels of RARs than parental MDA-MB-453 and FL-453 cells (Supplementary Fig. 1A). Unlike MDA-MB-453 and FL-453, RA-453 cells are responsive to the transcriptional and growth-inhibitory effects of ATRA. In fact, RA-453 cells transfected with a retinoid-dependent luciferase reporter (DR5-RARELuc) show stimulation of luciferase activity by ATRA. In addition, RA-453 growth is reduced by ATRA in a dose-dependent manner (Supplementary Fig. 1B).

To screen for RARα-binding proteins in RA-453 and FL-453 cells, we used a quantitative proteomic approach [15, 16] (Supplementary Fig. 2). Nuclear fractions enriched for DNA-binding (NABP, nucleic acid binding proteins) and histonic (INP, insoluble nuclear proteins) proteins were extracted from RA-453 and FL-453 cells exposed to vehicle or ATRA. Each nuclear fraction was immunoprecipitated with anti-FLAG antibodies and subjected to proteomic analysis. Twenty-eight of the proteins identified are present only in the RA-453 immunoprecipitates (Supplementary Table 1 and Supplementary Table 2). Ten proteins bind to unliganded RARα and binding is increased by at least 1.5-fold following treatment with ATRA (Supplementary Table 1). With the exception of CEP83 [17] and RL1D1 [18], all these interactors are histone proteins. Interestingly, CEP83 and RL1D1 are contained in the INP fraction. Thus, RARα-binding of these proteins may be indirect and mediated by one of the identified histones. The H2A/ core-histone protein shows maximal ATRA-dependent stimulation of RARα-binding.

Seventeen proteins, none of which is a histone, are identifiable in the NABP and INP fractions of vehicle-treated RA-453 cells (Supplementary Table 2). RARα-binding of all these proteins is diminished by ATRA.

S100A3, FABP5, and HSPB1 bind to unliganded RARα and the interaction is diminished by ATRA

We focused on the three RARα interactors, S100A3, FABP5, and HSPB1. S100A3 is a calcium-binding protein involved in transcription [19–21]. FABP5 protein binds and delivers ATRA to the PPARβ/δ nuclear-receptor [22–24]. HSPB1/HSP27 is a heat-shock protein whose expression is modulated by ATRA [25–27]. Detectable levels of FABP5, HSPB1/HSP27 (Supplementary Fig. 3A and Supplementary Fig. 3B), and S100A3 (Fig. 1a) are observed in vehicle and ATRA-treated FL-453 as well as RA-453 cells. ATRA treatment does not alter the basal expression of the three proteins.

We validated the observed interactions of S100A3, FABP5, and HSPB1/HSP27 with RARα using co-immunoprecipitation studies (Fig. 1a and Supplementary Fig. 3A, B). Anti-FLAG antibodies immunoprecipitate RARα only in RA-453 cells and the amount of immunoprecipitated RARα is similar in vehicle- and ATRA-treated RA-453 cells. In RA-453 cells, S100A3 (Fig. 1a), FABP5 (Supplementary Fig. 3A), and HSPB1/HSP27 (Supplementary Fig. 3B) co-immunoprecipitate with RARα and the levels of the three co-immunoprecipitated proteins are higher with vehicle than ATRA. This confirms the proteomic results and indicates that S100A3, FABP5, and HSPB1/HSP27 interact predominantly with unliganded RARα. The interaction of S100A3, FABP5, and HSPB1/HSP27 with RARα in RA-453 cells is also observed if the anti-FLAG immunoprecipitating antibodies are substituted.
by anti-RARα antibodies. Noticeably, anti-RARα antibodies immunoprecipitate not only FLAG-tagged RARα, but also endogenous RARα, which is expressed in both RA-453 and FL-453 cells, albeit at low levels. Further evidence supporting specific interactions between RARα and these proteins comes from the co-immunoprecipitation studies with anti-FABP5, anti-HSPB1 or anti-S100A3 antibodies.
RARα/S100A3 interaction is a general phenomenon and it occurs in the nucleus

We concentrated on S100A3, as the relevance of this protein for the biological activity of ATRA is unknown. To evaluate whether S100A3/RARα interaction is a general phenomenon, we performed co-immunoprecipitation studies in ATRA-sensitive tumor cell lines of different origin. We considered breast cancer SK-BR-3 and MCF-7 cells [3], lung cancer A-549 cells [28] as well as APL-derived PML-RARα+c NB4 blasts [29, 30] which synthesize measurable levels of S100A3 and RARα (Fig. 1b). RARα and S100A3 are detectable in the anti-S100A3 immunoprecipitates of vehicle-treated SK-BR-3, MCF-7, A-549, and NB4 cells (Fig. 1c, d). ATRA reduces the amounts of co-immunoprecipitated RARα, regardless of the cell line considered. In NB4 cells, S100A3 interacts also with PML-RAR and the interaction is reduced by ATRA (Fig. 1d).

We evaluated the subcellular distribution of the RARα/S100A3 interaction in SK-BR-3 cells, using quantitative immunoprecipitation, following separation of the nuclear and cytoplasmic fractions (Fig. 1e). As expected, RARα localizes predominantly to the nucleus [31–33], while S100A3 is evenly distributed in the cytosol and the nucleus (Fig. 1e-input). In basal conditions, the anti-S100A3 antibody co-immunoprecipitates significant amounts of RARα only from the nucleus and RARα nuclear protein levels are reduced by ATRA. Immunofluorescence experiments performed in MDA-MB-453 cells transiently transfected with RARα and S100A3 support these results (Fig. 1f), demonstrating punctate co-localization of RARα and S100A3 only inside the nucleus.

To validate the observation that S100A3 interacts with RARα/PML-RARα and to evaluate whether the calcium-binding protein recognizes other members of the RAR/RXR family, we used the COS-7 cellular model [31]. We performed co-immunoprecipitation experiments following overexpression of S100A3 with RARα, PML-RARα, RARβ, RARγ, or RXRα (Fig. 2a). RARα and PML-RARα are not the only S100A3-interacting retinoid-receptors, as anti-S100A3 antibodies co-immunoprecipitate also RARγ. As expected, binding of RARα, PML-RARα, and RARγ to S100A3 is reduced by ATRA. To confirm these results, we performed GST pull-down assays [32] in COS-7 cells over-expressing RARα, RARβ, RARγ, and RXRα. In basal conditions, only RARα and RARγ are specifically pulled down by GST-tagged S100A3 (GST-S100A3, Fig. 2b).

Definition of the RARα structural determinants of the interaction with S100A3

To get insights into the structural determinants of the RARα/S100A3 interaction, we performed GST pull-down assays using deletion/phosphorylation-mutants of the retinoid-receptor. We over-expressed the following RARα deletion-mutants in COS-7 cells: ΔAB, deleted for the A/B-regions; ΔH12(408–416), deleted for the H12-helix in the ligand-binding E-region; Δ403–462, deleted for a portion of the E- and the entire F-region; ΔF, deleted for the F-region.
ΔC, deleted for the DNA-binding C-region; ΔD, deleted for the D-hinge-region (Supplementary Fig. 4). In addition, we over-expressed PML-RARα and the RARα2 splicing-variant [32] and used PML as an internal control. Only ΔH12(408–416) and Δ403–462 lose the ability to interact with S100A3 (Fig. 2c). Deletion of the C- and D-regions causes a major reduction of the S100A3-interaction, although it does not abrogate binding (Supplementary Fig. 5). This last observation must be taken with caution as the absence of the C- or D-regions has major effects on the tridimensional structure of the RAR protein. Thus, the GST pull-down experiments confirm that PML-RARα interacts with S100A3. This interaction is due to the RARα moiety, as PML is not recognized by GST-S100A3.

RARα is phosphorylated at different residues in basal conditions or following exposure to ATRA [11]. Specific phosphorylation sites control the levels and functional activity of the retinoid-receptor. To evaluate whether some of the known phosphorylation sites play a role in the S100A3/RARα interaction, we performed GST pull-down studies with available Ser/Ala phosphorylation-mutants. We used the following phosphorylation-mutants: S74A-S77A, inactivating two p38 and CDK dependent phosphorylation sites of the B-domain [34, 35]; S154A and S157A,
inactivating the PKC-dependent phosphorylation sites of the C-domain [36]; S369A, inactivating a PKA/p38-dependent phosphorylation site in the E-domain [37] (Supplementary Fig. 4). None of the four phosphorylation mutant exerts a significant effect on the interaction with S100A3 (Fig. 2c). As ATRA activates the phosphorylation of all these mutated Ser-residues [11], the amino-acids are also unlikely to be involved in the diminution of RARα/S100A3 interaction caused by the retinoid (see also Fig. 3a, S74A-S77A).

To establish whether the interaction between S100A3 and RARα is direct, far-western experiments [32] were performed in COS-7 cells over-expressing S100A3 (Fig. 2d). The results demonstrate that GST-RARα interacts with S100A3. The interaction is reproduced with the GST-RARα derivative consisting of the DEF regions (GST-DEF) [32], but not with similar proteins consisting of the ABC-regions (GST-ABC) or the DEF regions lacking the H12-helix (GST-DEFΔH12). This demonstrates that S100A3 interacts with RARα in a direct manner and confirms the importance of the H12-helix in the interaction.

**S100A3 controls the transcriptional activity and the degradation of RARα and PML-RARα**

To evaluate whether the interaction with S100A3 exerts any effect on the transcriptional activity and the levels of the two retinoic-receptors, we co-transfected COS-7 cells with S100A3 and RARα or PML-RARα in the presence of a retinoid-dependent luciferase reporter. S100A3 reduces ATRA-dependent stimulation of RARα and PML-RARα, as assessed by the luciferase reporter (Fig. 3a). In vehicle-treated COS-7 cells, S100A3 upregulates both RARα and PML-RARα proteins. In the absence of S100A3, ATRA reduces the amounts of the two receptor proteins [12, 32], a phenomenon which is not observed in S100A3 over-expressing cells. The effects exerted by S100A3 on the levels of RARα and PML-RARα proteins are translational/posttranslational, as they are not accompanied by alterations in the amounts of the corresponding mRNAs (data not shown). The data suggest that S100A3 increases the levels of a transcriptionally-inactive form of RARα and PML-RARα. The specificity of the results is confirmed with the use of PML as a negative control.

To support the idea that the results obtained are due to the S100A3-interaction, we performed similar experiments with RARα2, RARβ, RARγ, and RARα deletion- or point-mutants maintaining (ΔAB and S74A-S77A) or losing (ΔH12 (408–416) and Δ403–462) the ability to bind S100A3 (Fig. 3a). S100A3 increases the protein levels and inhibits the ATRA-dependent luciferase activity of RARα2, RARγ, ΔAB, and S74A-S77A. In contrast, the amounts of RARβ, ΔH12(408–416), and Δ403–462 proteins are not modified by S100A3 overexpression. In the case of RARβ protein, S100A3 has also no influence on ATRA-dependent luciferase activity.

To determine the effects of S100A3 on the stability of the two proteins, COS-7 cells were transfected with S100A3 and RARα or PML-RARα before exposure to cycloheximide for different amounts of time in the absence (Fig. 3b) and presence of ATRA (Fig. 3c). In basal conditions, S100A3 increases the stability of RARα and PML-RARα (Fig. 3b, lower graphs). By the same token, S100A3 reduces ATRA-dependent degradation of the two retinoid-receptors, a phenomenon associated with ligand-dependent transcriptional activation [29]. As the proteasome is involved in the degradation of the two receptors [38], we
evaluated the effects of S100A3 on the ubiquitinylation of RARα. RARα, S100A3, and HA-tagged ubiquitin (pUb-HA) were over-expressed in COS-7 cells exposed to vehicle or ATRA. Cell extracts were immunoprecipitated with anti-RARα antibodies and blotted with antibodies recognizing poly-ubiquitylated proteins (Fig. 3d). Regardless of
The I396 residue of RARα is critical for the interaction with S100A3

Constitutive binding to RARα/PML-RARα, reduction of the binding by ATRA and inhibition of RARα/PML-RARα transcriptional activity suggest that S100A3 is a potential nuclear-receptor co-repressor. In addition, S100A3 contains a LKELLQKEL sequence (Supplementary Fig. 6), which is similar to the core alpha-helical-box (LXXI/HIXXXIL) of co-repressors [40].

The I396 residue is located in proximity to the RARα region involved in S100A3 binding (Supplementary Fig. 4) and the RARα I396E mutant (I396E) does not bind co-repressors [41]. Thus, we evaluated the capacity of I396E to bind S100A3, in the COS-7 model. Unlike RARα, I396E is not co-immunoprecipitated by anti-S100A3 antibodies (Fig. 4a). Consistent with the inability of S100A3 to interact with I396E, the calcium-binding protein has no effect on ATRA-dependent transcriptional activity or the steady-state levels of the RARα-mutant (Fig. 4b). As ATRA is a pan-RAR agonist, we supported the selectivity of these effects with the RARα agonist, AM580 [4]. We compared the dose-dependent effects of AM580 on RARα and I396E transcriptional activity (Fig. 4c, left). As for RARα, S100A3 reduces the luciferase activity of the retinoid-dependent transcriptional activity of I396E. Transcriptional inhibition by AM580 is accompanied by RARα-protein stabilization, while a similar effect is not observed with I396E (Fig. 4c, right).

We also evaluated whether S100A3 competes with the N-CoR co-repressor for RARα binding and we performed co-immunoprecipitation studies in COS-7 cells over-expressing combinations of the three proteins (Fig. 4d). For these studies, we used an N-CoR fragment (aa. 1629–2453) containing the RARα-binding domains [42]. Cell extracts were immunoprecipitated with anti-N-CoR antibodies and subjected to western blot analysis for RARα or S100A3. N-CoR antibodies co-immunoprecipitates RARα in N-COR and more so in N-COR + RARα transfected cells. Overexpression of S100A3 (N-COR + S100A3 and N-COR + RARα + S100A3 cells) competes for the binding of N-COR to RARα. This is consistent with S100A3 and N-COR binding to the same H12 region of RARα.

Effects of S100A3 on the cellular responses to ATRA in breast cancer cells

To evaluate whether modulation of S100A3 expression has any consequence on ATRA anti-tumor activity, we took a silencing approach. We designed three S100A3-targeting shRNAs (shS100A3-a, shS100A3-b, and shS100A3-c) and a scrambled control shRNA (shSCRAM). The specificity of our shRNAs is supported by the results obtained in COS-7 cells over-expressing S100A3 (Supplementary Fig. 7A). We infected SK-BR-3 cells with shS100A3-b and shS100A3-c lentiviruses alone or in combination, shSCRAM and the void lentivirus (VOVE) isolating the following cell populations: S100A3b/SK, S100A3c/SK, S100A3b+c/SK, SCRAM/CK, and VO/VE/5K. S100A3b/5K, S100A3c/5K, and S100A3b+c/5K express lower levels of S100A3 mRNA/protein than SCRAM/5K cells (Fig. 5a). As a consequence,
anti-RARα antibodies fail to co-immunoprecipitate S100A3 from S100A3 knockdown cells (Fig. 5b). Consistent with S100A3-dependent inhibition of RARα degradation, S100A3b/SCRAM, S100A3c/SCRAM, and S100A3b+c/SCRAM show lower levels of the retinoid-receptor than SCRAM/SCRAM cells (Fig. 5c). This effect is evident in basal conditions and following ATRA treatment.

We investigated the consequences of S100A3 silencing on the proliferation of SK-BR-3 grown under basal conditions. S100A3 knockdown exerts minor and divergent effects on the growth of SK-BR-3 cells, depending on the SCRAM/SCRAM or VOVE/SCRAM control line considered (Supplementary Fig. 7B). Given the key-role played by RARα in mediating ATRA anti-proliferative action in breast cancer cells, we also investigated the consequences of S100A3 silencing on ATRA-dependent growth inhibition of SK-BR-3 cells. Relative to SCRAM/SCRAM and VOVE/SCRAM, S100A3a/SCRAM, S100A3a+b/SCRAM, S100A3b/SCRAM, S100A3c/SCRAM,
were exposed to the indicated concentrations of the AM580 RAR agonist. Left: the bar graph illustrates the levels of luciferase activity. COS-7 cells were transiently co-transfected with an expression plasmid for the S100A3 cDNA (pCDNA3), wild-type RARα and the RARα I396E mutant (I396E) along with a luciferase reporter construct controlled by a retinoid responsive element. The negative control for the experiments is represented by cells co-transfected with the void expression plasmid (pSG5). Twenty-four hours following transfection, cells were treated with vehicle (DMSO) or ATRA (1 µM) for 1 h. At the end of the treatment, total cell extracts were immunoprecipitated with anti-S100A3 mouse monoclonal antibodies (IP: S100A3). A further negative control for the immunoprecipitations is represented by the extracts of COS-7 cells co-transfected with pSG5 and the S100A3 expression plasmid which were challenged with non-specific immunoglobulins G (IP: IgG). Following normalization for the content of S100A3 in the input, the various immunoprecipitates were subjected to western blot analysis with anti-RARα antibodies. All the blots were subjected to a western blot analysis with anti-S100A3, anti-N-COR antibodies, as indicated. Input = western blot analysis of the cell extracts before the indicated immunoprecipitation step. M.W. = molecular weights of the indicated proteins. Each immunoprecipitation is representative of at least two independent experiments providing the same type of results. b COS-7 cells were transiently co-transfected with an expression plasmid for the S100A3 cDNA (S100A3) or the corresponding void vector (pCDNA3), wild-type RARα and the RARα I396E mutant (I396E) along with a luciferase reporter construct controlled by a retinoid responsive element (g2RARE-Luc). Twenty-four hours following transfection, cells were treated with vehicle (DMSO) or ATRA (1 µM) for a further 24 h. Cell extracts were subjected to western blot analysis with anti-RARα (upper panels), anti-S100A3 (middle panels) and anti-actin (lower panels) antibodies. The same cell extracts were used for the measurement of luciferase activity, as illustrated by the bar graphs above the western blots. M.W. = molecular weights of the indicated proteins. c COS-7 cells were transiently transfected with the indicated expression plasmids as in (b). Twenty-four hours following transfection, cells were exposed to the indicated concentrations of the AM580 RARα agonist. Left: the bar graph illustrates the levels of luciferase activity. Each value is the mean ± SD of three replicate cultures. **Statistically significant comparison (p < 0.01, Student’s t-test). Right: the same extracts used for the determination of luciferase activity were subjected to western blot analysis with anti-RARα (upper panels), anti-S100A3 (middle panels) and anti-actin (lower panels) antibodies. d COS-7 cells were transiently co-transfected with the indicated combinations of S100A3, RARα, and a N-CoR fragment (aa. 1629–2453) containing the RARα-binding domains (NRI and NRII). Following normalization for the content of N-CoR in the input, cell extracts were immunoprecipitated with an anti-N-CoR antibody (IP: N-CoR) or with non-specific immunoglobulins G (IP: IgG) and subjected to western blot analysis with anti-RARα, anti-S100A3, and anti-N-CoR antibodies. Input = western blot analysis of the cell extracts before the indicated immunoprecipitation step. M.W. = molecular weights of the indicated proteins.

S100A3 interacts not only with RARα but also with the APL-specific PML-RARα oncogenic protein. Thus, we evaluated the functional consequences of S100A3 silencing in PML-RARα+ and APL-derived NB4 cells infected with shS100A3a (S100A3a/NB4), shS100A3c (S100A3c/NB4), shS100A3a + shS100A3c (S100A3a+c/NB4), shSCRAM (SCRAM/NB4), and VOVE (VOVE/NB4) (Fig. 6a). Unlike SCRAM/NB4, S100A3a/NB4, S100A3c/NB4, and S100A3a + c/NB4 cells do not express detectable amounts of S100A3 and show decreased levels of RARα and PML-RARα in basal conditions and more so after ATRA treatment. In addition, S100A3a/NB4, S100A3c/NB4, and S100A3a + c/NB4 grow more slowly than the VOVE/NB4 and SCRAM/NB4 controls (Fig. 6b). Finally, S100A3 knockdown enhances the anti-proliferative effect of ATRA, which is opposite to what is observed in SK-BR-3 and A549 cells.

The therapeutic action of ATRA in APL cells is consequent to a reversion of the differentiation block caused by PML-RARα. The phenomenon is recapitulated in NB4 cells which undergo granulocytic differentiation upon ATRA treatment [30, 31, 37, 44]. The basal levels of the granulocytic differentiation marker, NBT-reducing-activity [45], are higher in S100A3a/NB4, S100A3c/NB4, and S100A3a + c/NB4 cells than those of SCRAM/NB4 and VOVE/NB4 controls (Fig. 6c). In addition, ATRA causes a higher increase of NBT-reducing-activity in S100A3-silenced than control cells. S100A3 knockdown results in constitutive upregulation of the other differentiation markers, CD11c (Supplementary Fig. 8A, Fig. 6c) and CD38 (Fig. 6c). At the ATRA...
concentrations used, the expected induction of CD38 is already maximal in SCRAM/NB4, S100A3a/NB4, S100A3c/NB4, and S100A3a+c/NB4 cells. In contrast, silencing of S100A3 causes a reproducible enhancement of CD11c and CD11b (another differentiation marker) induction by ATRA (Fig. 6c). As expected, CD33 is constitutively expressed in NB4 cells and its expression is left unaffected by ATRA [4]. Consistent with all this, S100A3 knockdown is associated with morphological features of granulocytic differentiation (increase in the volume of the cytoplasm and the number of granulocytic vesicles), which are already visible under basal conditions and enhanced by ATRA (Supplementary Fig. 8B).

Among the genes induced by ATRA in APL cells [32], the PU-1, cEBPβ, cEBPε, and STAT1 transcription factors as well as the focal adhesion protein, paxillin, are involved in granulocytic differentiation [46, 47]. ATRA induces all these proteins in SCRAM/NB4 cells and this induction is magnified in S100A3a/NB4, S100A3c/NB4, and S100A3a+c/NB4 cells (Fig. 6d). Interestingly, S100A3 knockdown elevates the constitutive amounts of PU-1 and STAT1, which may explain the granulocytic-differentiation signs observed under basal conditions. As cEBPβ and paxillin are encoded by direct RAR target genes, we evaluated whether the increase in ATRA-dependent upregulation by S100A3 knockdown is consistent with a transcriptional effect.
Hence, we determined cEBPβ and paxillin mRNA levels in vehicle and ATRA-treated SCRAM/NB4, S100A3a/NB4, S100A3c/NB4 cells (Fig. 6e). The results obtained recapitulate what is observed at the protein level, demonstrating that S100A3 knockdown causes a substantial increase in ATRA-dependent induction of the two transcripts. The data support a transcriptional effect mediated by PML-RARα or RARα activation by the retinoid.

To confirm the results, we used a specular approach and we stably transfected S100A3 and a void vector in NB4 blasts obtaining oxS100A3/NB4 and pCDH/NB4 cells, respectively. S100A3 overexpression is associated with a consistent increase in the levels of both PML-RARα and RARα (Fig. 7a). This is accompanied by inhibition of the ATRA-dependent degradation of the two retinoid-receptors and a modest, though significant, reduction in the anti-proliferative effect of ATRA at 4 and 7 days (Fig. 7b). As for the differentiation state, the basal expression levels of NBT-reducing activity, CD11b, CD11c, cEBPβ, cEBPe, and PAX relative to the corresponding pCDH/NB4 control (Fig. 7d, e).

**S100A3 knockdown controls ATRA-dependent differentiation/proliferation of PML-RARα− HL-60 cells**

To establish the relevance of the cellular context and/or PML-RARα expression for the different effects afforded by S100A3 silencing in NB4 and SK-BR-3/A549, we considered the HL-60 AML cell line. HL-60 cells do not express PML-RARα, undergo granulocytic differentiation upon exposure to ATRA [48] and contain measurable levels of S100A3, which interacts with RARα (see VOE/HL and SCRAM/HL cells, Fig. 8a, b). We generated two cell populations of HL-60 cells stably silenced for S100A3 (S100A3b/HL; S100A3a+/c/HL) and two control populations (SCRAM/HL; VOE/HL) (Fig. 8a). As observed in NB4 cells, S100A3 silencing was associated with decreased steady-state levels of the RARα protein. Unlike the NB4 counterpart, S100A3 knockdown does not affect the basal activity (Fig. 7c), CD11b, CD11c, cEBPβ, cEBPe, and PAX relative to the corresponding pCDH/NB4 control (Fig. 7d, e).
growth (Fig. 8c) differentiation state (NBT assay, CD11b, CD11c, and CD38 expression) of HL-60 cells (Fig. 8d, e). In contrast, S100A3 silencing enhances ATRA growth-inhibitory and differentiating effects. As the results obtained in S100A3-silenced HL-60 and NB4 cells exposed to ATRA are substantially similar, our data indicate that the cellular context rather than PML-RARα expression is the major determinant of the different effects exerted by S100A3 on RARα functional activity in myeloid leukemia relative to breast cancer and lung cancer cells.

**Discussion**

The study reports on the identification of novel proteins interacting with RARα in breast cancer cells [3]. Among the
identified proteins, we focused our attention on S100A3, a member of the S100 family of calcium-binding proteins [49, 50]. There are no data regarding the potential involvement of S100A3 in the retinoid signal transduction pathway, although other members of the family, i.e. S100A9 and S100A10, have been implicated in ATRA-induced differentiation of APL cells [51–54]. Our data indicate that S100A3 binds to unliganded RARα directly and binding is reduced by ATRA. The H12-helix located in the ligand-binding E-region of RARα plays a crucial role in the interaction with S100A3. In particular, the I396 residue, laying in proximity to the H12-helix is of fundamental importance. The H12-helix and I396 are conserved in RARβ and RARγ, yet only RARγ interacts with S100A3. In S100A3, a sequence similar to the one involved in the binding of co-repressor molecules to RARs (Supplementary Fig. 4) is likely to underlay the ability to compete with the N-COR co-repressor for the binding to RARα. Our data are consistent with the idea that S100A3 may act as a RARα co-repressor and suggest that S100A3 and N-COR are unlikely to be part of the same co-repressor complex. The RARα/ S100A3 interaction is cell-context independent and it occurs predominantly in the cell-nucleus.

From a functional point of view, our results support the concept that the dynamic interaction with S100A3 is involved in the control of constitutive and ATRA-dependent RARα degradation in a cell-context independent manner. Similar effects are observed in the case of the APL-specific PML-RARα fusion protein. The studies performed in S100A3 over-expressing COS-7, SK-BR-3 (MG, unpublished results), and NB4 cells demonstrate an increase in the constitutive steady-state levels of both RARα and PML-RARα, due to a decrease in the proteasome-dependent degradation of the two proteins. The inhibitory effect on RARα and PML-RARα degradation by S100A3 is associated with a reduction in the ligand-dependent transcriptional activity of the two retinoid-receptors. This is the opposite of what is observed upon interaction of the p38 MAP-kinase with both receptors [37]. In fact, binding of p38 MAP-kinase to RARα and PML-RARα causes an increase in the degradation and a decrease in the transcriptional activity of the two receptors. The above observations indicate that proteasome-dependent degradation is a crucial determinant of RARα and PML-RARα function, although its inhibition is not necessarily associated with an increase in their transcriptional activity [37].

The control exerted by S100A3 on RARα modulates the anti-tumor activity of ATRA in breast cancer SK-BR-3 and lung cancer A549 cells. Here, S100A3 knockdown reduces the response to the anti-proliferative action of ATRA. In SK-BR-3 cells a similar effect is observed also as far as the retinoid differentiating activity. Induction of ATRA-resistance is consistent with the primary role exerted by RARα in mediating the anti-tumor action of the retinoid in breast cancer [3] and the decrease in the levels of RARα afforded by S100A3 knockdown. Interestingly, opposite effects are observed in PML-RARα+ NB4 and PML-RARα− HL-60 AML cells, where S100A3 knockdown increases the ATRA-dependent anti-proliferative and differentiating responses, despite down-regulation of RARα. In NB4 cells, S100A3 silencing induces not only ATRA-dependent but also basal differentiation. This is consistent with PML-RARα down-regulation by S100A3 silencing. Indeed, unliganded PML-RARα is believed to be responsible for the differentiation arrest of the APL blast and decreased expression of the translocation product by S100A3 knockdown may at least partially release the differentiation block.
Noticeably, this is the mechanism proposed for the potentiating action of arsenic trioxide on ATRA in APL [1, 2]. In NB4 cells, S100A3 knockdown seems to cause a larger reduction in the levels of PML-RAR\(\alpha\) than RAR\(\alpha\), altering the relative abundance of the two proteins in favor of the latter one. This may release residual RAR\(\alpha\) from the dominant-negative effect exerted by the oncogenic protein, potentiating the ATRA growth-inhibitory/differentiating...
effects. However, other myeloid-cell specific mechanisms must also be involved in the enhancement of the ATRA-dependent anti-proliferative effect afforded by S100A3 silencing, as indicated by the results obtained in PML-RARα− and AML-derived HL-60 cells.

In conclusion, the study demonstrates that S100A3 interacts with and is involved in the proteasomal-dependent degradation of RARα. Our results provide new insights on the molecular mechanisms underlying the control of RARα functional activity and may have practical implications. In fact, S100A3 represents a novel pharmacological target for the development of rational drug combinations aimed at potentiating the therapeutic activity of ATRA.
Materials and methods

Interactomic studies

The proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD00876 and further methodological details are available in Supplementary Methods.

Cells and infection procedures

The source of the cell lines and the infection procedures are described in Supplementary Methods. We generated SK-BR-3, A549, HL-60, and NB4 cell populations silenced for S100A3 with lentiviral vectors (pGREENpuro, System Biosciences) containing the shRNA. To isolate NB4 populations over-expressing S100A3, cells were infected with pCDH-CMV lentiviral vectors (System Biosciences) containing the human S100A3 cDNA. To this purpose, S100A3 was inserted in the XbaI and NotI sites of pCDH-CMV.

Transient overexpression and transactivation studies in COS-7 cells

COS-7 cells were transiently co-transfected with an expression plasmid for the S100A3 cDNA (S100A3) or the corresponding void vector (pcDNA3) in the presence of pSG5 plasmids containing RARα, PML-RARα, RARα2, PML or the RARα point as well as deletion-mutants described in Supplementary Figure 4. The methodologies used are as described [32].

Immunoprecipitation, far-western, GST pull-down assays, FACS analysis, immunoprecipitation, and western blot analyses

Immunoprecipitation, far-western, and GST pull-down assays were performed in COS-7 cells using already described approaches and methodologies [32]. Further details are available in Supplementary Methods. CD11b, CD11c, CD38, and CD33 markers were determined with a fluorescence activated cell sorter (FACS, Becton and Dickinson) [29, 37]. Western blot analyses were performed as previously described [29, 32, 37]. Agarose beads coupled to anti-HA antibodies were from Sigma (A2095).

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Author contributions

MG and MT supervised all the phases of the work and wrote the manuscript and contributed to the design and conduction of the study; GP and AZ performed the experimental work involving the use of cell lines; MK, AZ, GP, AA, and ML performed some of the experimental studies involving the use of cell lines. LB and RB conducted the interactomic studies involving the use of mass-spectrometry. MB and MF performed all the computational work; CRE participated in the design and conduction of some of the experimental work; EG designed and supervised the entire study and wrote the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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