Cancer Cell
Article

Recruitment of RXR by Homotetrameric RARα Fusion Proteins Is Essential for Transformation

Bernd B. Zeisig,[1,4] Colin Kwok,[1,4] Arthur Zelent,[1] Pattabhiraman Shankaranarayanan,[2] Hinrich Gronemeyer,[2] Shuo Dong,[3,*] and Chi Wai Eric So[1,*]
[1] Haemato-Oncology Section, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Greater London SM2 5NG, UK
[2] Department of Cancer Biology, Institut de Genetique et de Biologie Moleculaire et Cellulaire, IGBMC/CNRS/INSERM/ULP, 67404 Illkirch Cedex, Communauté Urbaine de Strasbourg, France
[3] Department of Medicine, Baylor College of Medicine, Houston, TX 77030, USA
[4] These authors contributed equally to this work.
*Correspondence: sdong@bcm.tmc.edu (S.D.), eric.so@icr.ac.uk (C.W.E.S.)
DOI 10.1016/j.ccr.2007.06.006

SUMMARY

While formation of higher-order oncogenic transcriptional complexes is critical for RARα fusion proteins in acute promyelocytic leukemia, the essential components and their roles in mediating transformation are still largely unknown. To this end, the present study demonstrates that homodimerization is not sufficient for RARα fusion-mediated transformation, which requires higher-order homotetramerization. Surprisingly, intrinsic homo-oligomeric DNA binding by the fusion proteins is also dispensable. Importantly, higher-order RXR/RARα fusion hetero-oligomeric complexes that aberrantly recruit transcriptional corepressors to downstream targets are essential for transformation. Intervention of RXR-dependent pathways by panRXR-agonists or RXRα shRNAs suppresses RARα fusion-mediated transformation. Taken together, these results define the oncogenic threshold for self-association and reveal the pathological significance of higher-order RARα fusion/RXR hetero-oligomeric complexes and their potential value as a therapeutic target.

INTRODUCTION

Acute leukemia is characterized by the presence of recurrent chromosomal abnormalities, which frequently results in formation of chimeric transcription factors (Look, 1997). A common theme shared by most of these leukemia-associated transcription factors (LATFs) is an acquisition of additional self-association domains (homodimerization and/or homo-oligomerization domains) from the fusion partners (So and Cleary, 2004). Emerging evidence indicates indispensable functions of these self-association domains in various LATFs, including MLL (Martin et al., 2003; So et al., 2003b), RARα (Kwok et al., 2006; Sternsdorf et al., 2006), and AML1 (Liu et al., 2006), fusions that account for the majority of the acute myeloid leukemia (AML). In all cases, disruption of self-association abrogates transformation. Moreover, forced self-association of truncated MLL (Martin et al., 2003; So et al., 2003b) or RARα (Lin and Evans, 2000; Minucci et al., 2000) via synthetic homodimerization/oligomerization domains can closely recapitulate many biochemical and transcriptional properties of bona fide fusion proteins and are capable of transforming primary hematopoietic cells (Kwok et al., 2006; Martin et al., 2003; So et al., 2003b; Sternsdorf et al., 2006). While these findings reveal a critical role of aberrant self-association in acute leukemogenesis, the underlying transformation mechanisms are still largely unknown.

SIGNIFICANCE

Emerging evidence indicates formation of higher-order transcriptional complexes as an essential step by various leukemia-associated transcription factors (LATFs), including MLL, RARα, and AML1, that account for the majority of acute myeloid leukemia (AML). To gain further insights into the underlying mechanisms, the present study functionally separates homodimerization and intrinsic homo-oligomeric DNA-binding properties of RARα fusions from transformation of primary hematopoietic cells. We reveal essential functions of homotetramerization and RXR recruitment, which results in the formation of higher-order hetero-oligomeric DNA-binding complexes that are required for RARα fusion-mediated transformation. These findings not only identify the key elements and potential avenues for therapeutic targeting of RARα-mediated leukemia, but also shed light on oligomeric transformation mechanisms reported with various LATFs.

Retinoic acid receptor α (RARα) fusion associates with almost 100% of acute promyelocytic leukemia (APL), which is uniquely sensitive to differentiation therapy with all-trans retinoic acid (ATRA) (Zelent et al., 2001). While wild-type RAR binds DNA very weakly, heterodimerization with retinoid X receptors (RXR) increases DNA-binding strength and defines the heterodimer-selective RA response element (RARE) repertoire (Chambon, 1996). In the absence of agonists or in the presence of antagonists/inverse agonists, the heterodimer represses the expression of certain target genes by recruiting corepressor complexes, containing histone deacetylases (HDAC) that silence gene expression by altering chromatin structure. ATRA binding results in allosteric conformational changes of the RARα/RXRα heterodimer resulting in dissociation of corepressor complexes and recruitment of histone acetyltransferase (HAT) containing coactivator complexes (Chen et al., 1997; Germain et al., 2002; Kurokawa et al., 1995; Onate et al., 1995). Conversely, oncogenic RARα fusions acquire high-affinity corepressor interaction ability, leading to constitutive transcription repression of their downstream target genes even in the presence of physiological levels of ATRA (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998). To date, in APL patients five different RARα fusions have been identified, all of which result in direct fusion of self-association domains from the partners to the truncated RARα protein (So and Cleary, 2004; Zelent et al., 2001). Biochemically, self-association of truncated RARα has been shown to confer (1) high-affinity binding with transcriptional corepressor complexes in an ATRA-dependent manner; (2) altered DNA-binding abilities as homo-oligomeric RARα fusions and/or higher-order hetero-oligomeric RARα fusions/RXRα complexes; and (3) potential dominant-negative effects on the pathways mediated by RARα partners (Dong et al., 2003; Dong and Tweardy, 2002; Dong et al., 1996; Lin and Evans, 2000; Minucci et al., 2000; Perez et al., 1993; Redner et al., 2000; So et al., 2000). Biologically, the self-association domains are also essential for RARα fusion mediated transformation of primary hematopoietic cells. Direct fusion of RARα with synthetic FKBP self-association domains is capable of transforming primary hematopoietic cells, and inducing leukemia in mice with long latency and reduced penetrance (Kwok et al., 2006; Sternsdorf et al., 2006). Conditional reversion of self-association can suppress transformation of primary hematopoietic cells by RARα fusion proteins (Kwok et al., 2006). While these findings reveal a crucial role of self-association and highlight it as a potential target for molecular therapy, we still have only very limited knowledge of the underlying transformation mechanisms, which are essential for rational drug targeting. For instance, it is not clear if there is an oncogenic threshold for self-association (e.g., homodimerization versus homotetramerization), what are the compositions of the oncogenic oligomeric effector complexes, and how do they affect gene expression and transformation?

To gain further mechanistic insights into these issues, the present study characterized the transformation mechanisms mediated by oligomeric Stat5b-RARα, which has two distinctive self-association domains, and exploited structural data that provide an opportunity to molecularly dissect the oncogenic threshold and functional requirements of self-association. We also investigated the importance of RXR in RARα fusion-mediated transformation.

RESULTS

Stat5b-RARα-Mediated Transformation of Primary Hematopoietic Cells Is Dependent on the Highly Conserved N-Terminal Domain but Not the Coiled-Coil Domain

While Stat5b-RARα (SR) was initially identified in an APL patient (Arnould et al., 1999), no transformation model is available for this fusion. To this end, the oncogenic property of SR in transformation of primary hematopoietic cells was assessed by a retroviral transduction/transformation assay (RTTA) that has successfully modeled the transformation properties of various LATFs including RARα fusion proteins (Du et al., 1999; Kwok et al., 2006; So et al., 2003a). As expected, primary bone marrow cells transduced with empty-vector viruses exhausted their proliferative capability in the second round of plating (Figure 1A). In contrast, SR-transduced cells acquired self-renewal property and could form compact GM-CFU-like colonies even in the third plating (Figures 1A and 1B). Immunophenotypic and histological analyses of the transduced cells were consistent with the phenotype of myeloid precursors (Figures 1B and 1C) reminiscent of PML-RARα- or PLZF-RARα-transformed cells (Kwok et al., 2006). Since Stat5b is activated in many forms of myeloid leukemia and constitutively active Stat5b alone could transform primary hematopoietic cells and induce leukemia in mice (Moriggl et al., 2005), we further showed that both the truncated Stat5b and RARα alone did not exhibit any detectable transformation property and failed to give colonies after second plating (Figure 1A). Thus, the enhanced self-renewal of primary hematopoietic cells mediated by SR is dependent on contributions from both the Stat5b and the RARα portions.

To gain further insights into the transformation mechanisms and identify critical domains required for SR-mediated transformation, we tested the myeloid transformation ability of various deletion mutants targeting specific domains in Stat5b (Dong and Tweardy, 2002). SR mutants with deletion of the Stat5b DNA-binding domain (ΔDBD) or SH2 domain (ΔSH) could still transform primary hematopoietic cells and gave compact third-round colonies, indicating that neither of these two domains is required for transformation (Figure 1A). Next, we tested the transformation ability of SR mutants targeting individual self-association domains. Unexpectedly, SR-ΔCC with deletion of the coiled-coil homodimerization domain could still efficiently transform myeloid progenitors and gave rise to significant numbers of compact third-round colonies (Figures 1A and 1B). In contrast, SR-ΔN with deletion of the N-terminal homotetramerization domain completely lost its transformation property and failed to produce third-round

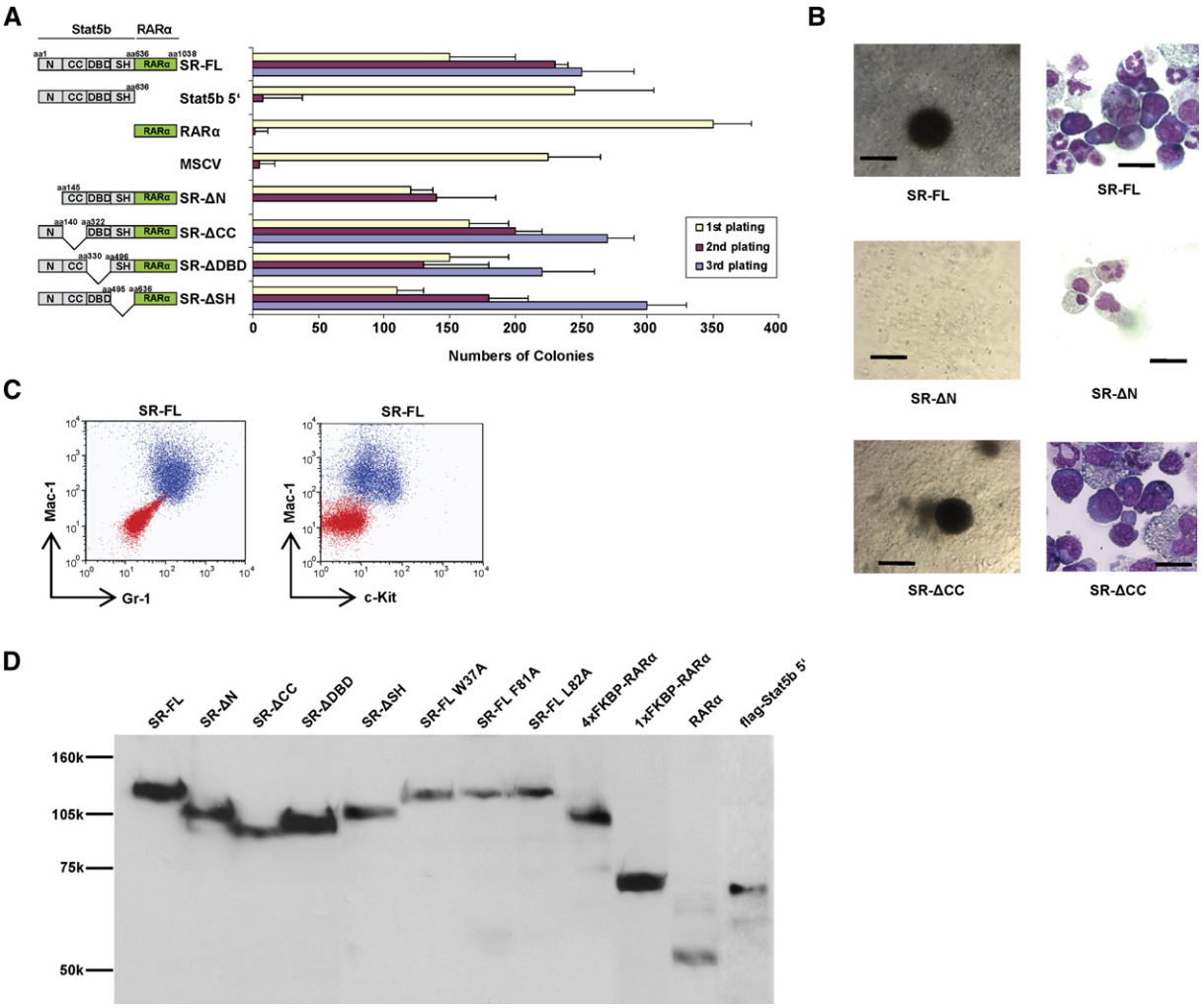

Figure 1. Enhanced Self-Renewal of Primary Hematopoietic Cells by SR Requires N-Terminal Tetramerization Domain but Not the Coiled-Coil Domain

(A) Schematic diagram of SR and mutants used in RTTA (left). The bar chart (right) represents the corresponding numbers of colonies after each plating in methylcellulose. Error bars indicate standard deviations (SD) of three independent experiments.

(B) Typical third-round colony (left) and cell morphology (right) of primary bone marrow cells transduced with indicated constructs. Scale bars in left and right panel are 100 μm and 10 μm, respectively.

(C) Phenotypical analysis of cells transformed by SR. Blue profiles represent stainings obtained with antibodies specific for the indicated surface markers. Red profiles show unstained controls.

(D) Western blot analysis with anti-RARα or anti-Flag antibodies shows expression of expected size proteins from all constructs used in RTTA.

colonies (Figures 1A and 1B), although comparable level of each mutant protein with expected size was detected by western blotting (Figure 1D). Taken together, these results suggest a specific requirement of homotetramerization for RARα fusion-mediated transformation.

Higher-Order Homotetramerization Rather Than Simple Dimerization Is Required for RARα Fusion-Mediated Transformation

To characterize the self-association property of these Stat5b domains in the context of RARα fusions, both coimmunoprecipitation and gel filtration assays were performed. Using an immunoprecipitation-western blot assay on 293 cells cotransfected with constructs expressing Flag- or Myc-tagged SR, self-association was observed in all the tested constructs but not in controls where the Flag-tagged constructs were replaced by empty vector (Figure 2A). These results confirm the presence of two distinctive self-association domains (N-terminal domain and coiled-coil domain) in the SR fusion. To further distinguish simple homodimerization from higher-order homo-oligomerization, gel filtration analysis was employed. In contrast to our previous results on PLZF-RARα, which almost exclusively eluted at a sharp peak corresponding to the estimated size of a homotetramer (Kwok et al., 2006), full-length SR was eluted in multiple fractions with a major

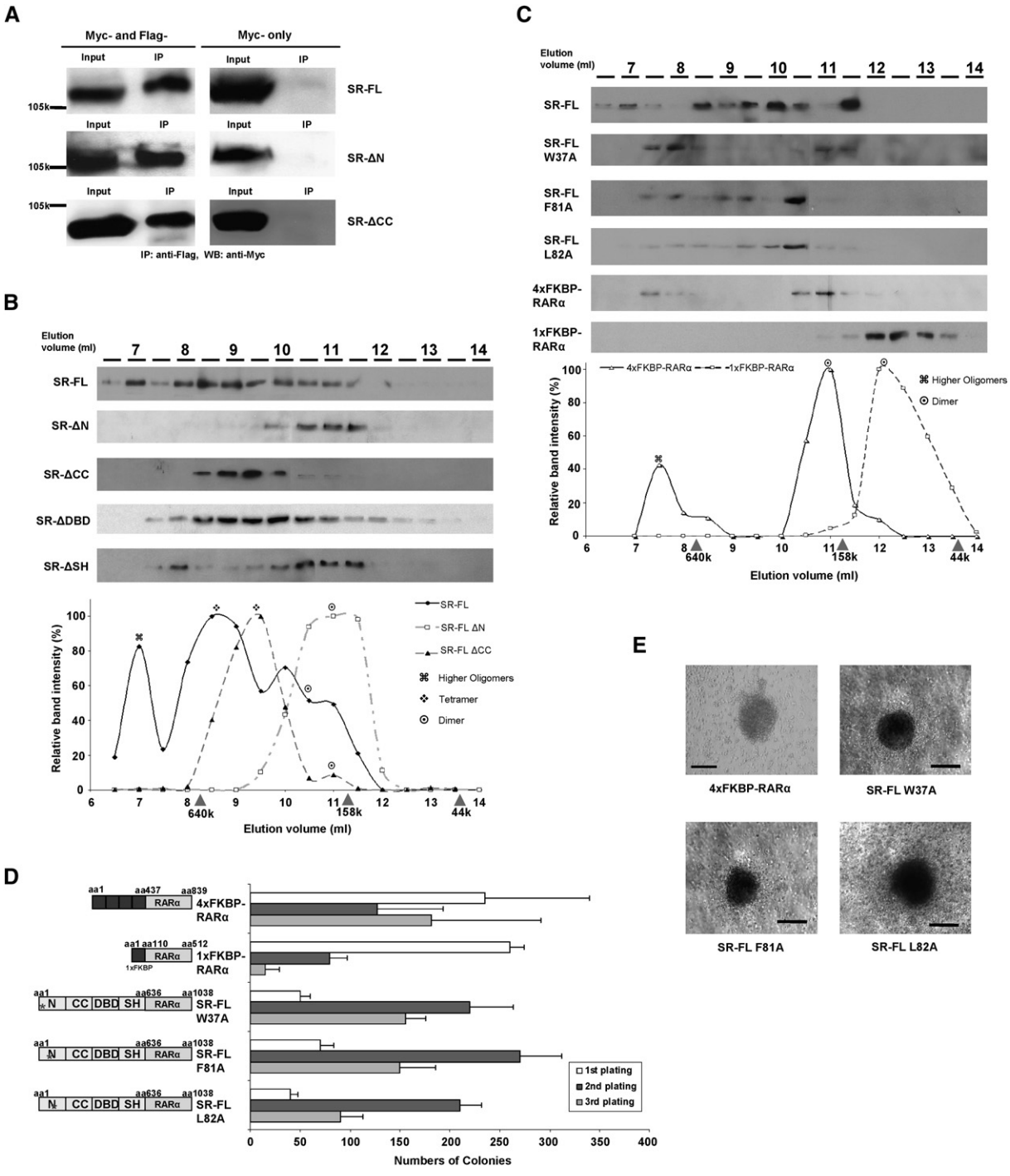

Figure 2. Homodimerization Is Not Sufficient for RARα Fusion-Mediated Transformation, which Requires Higher-Order Homo-tetramerization

(A) Cell lysates from cotransfected 293 cells were immunoprecipitated with anti-Flag antibody and western blotted with anti-Myc antibody.

(B and C) Gel filtration analysis using size exclusion column. Top panel shows the western blot using anti-RARα antibody on protein fractions collected from indicated elution volumes (ml). Constructs used in the experiments are shown in the left. Bottom panels are the gel filtration chromatograms plotting elution volume (ml) against intensity of the detected protein (%) with arrows indicating the elution volumes of corresponding size standard.

(D) Schematic diagram of SR point mutants and FKBP-RARα constructs (left) used in RTTA. The bar chart (right) represents the respective numbers of colonies after each plating. Error bars indicate SD of two independent experiments.

(E) Typical third-round colony morphology of cells transduced with indicated constructs. Scale bars, 100 μm.

peak of estimated molecular size about 500 kDa, consistent with a homotetramer. We also observed two minor peaks with molecular size about 200 kDa and >1 MDa, which could correspond to the sizes of a homodimer and a higher-order homo-oligomer, respectively (Figure 2B). Consistent with previous studies (Kwok et al., 2006; Minucci et al., 2000), endogenous RXR were dissociated from the RAR fusion complexes under stringent gel filtration conditions (Figure S1 in the Supplemental Data available with this article online). Analogous to the full-length SR, SR-ΔDBD and SR-ΔSH mutants eluted in multiple fractions covering homodimers, -tetramers, and –oligomers, although their relative distributions may not be the same (Figure 2B). Conversely, the SR mutants with deletion of a single self-association domain (ΔCC or ΔN) exhibited distinctive gel filtration elution profiles. The minor peak >1 MDa disappeared in both cases, suggesting that both self-association domains are required for the formation of higher-order homo-oligomeric complexes. The SR-ΔCC mutant was predominately eluted at a peak of about 400 kDa corresponding to a homotetrameric complex (Figure 2B). The SR-ΔN mutant was almost exclusively eluted at a single fraction of about 200 kDa corresponding to a homodimer (Figure 2B). These results reveal that the N-terminal domain is responsible for formation of homotetramers, while the coiled-coil domain mainly mediates homodimerization. Together with the transformation data, these results strongly suggest that homotetramerization rather than homodimerization is required for RARα-mediated transformation.

Since the self-association domains in Stat5b can have additional properties that may influence the transformation outcomes, we decided to validate this finding using a FKBP synthetic self-association domain that has been previously used for studying self-association property of various LATFs including RARα (Kwok et al., 2006; Martin et al., 2003; So et al., 2003b; Sternsdorf et al., 2006). To this end, we constructed a synthetic 1xFKBP-RARα carrying only one instead of four copies of the FKBP self-association domain and compared its self-association property with the previously described 4xFKBP-RARα (Kwok et al., 2006). In contrast to 4xFKBP-RARα, which displayed an elution profile with two major peaks corresponding to homo-oligomeric and -dimeric complexes, 1xFKBP-RARα was almost exclusively eluted at a fraction corresponding to a homodimer (Figure 2C), findings reminiscent of the full-length and ΔN mutant of SR, respectively. When these FKBP constructs were subjected to transformation assays, 4xFKBP-RARα, as expected, could efficiently enhance self-renewal of primary hematopoietic cells (Figure 2D&E). In contrast, cells transduced with 1xFKBP-RARα had gradually lost self-renewal capacity and displayed severely compromised ability to give rise to third-round colonies (Figure 2D). Western blotting confirmed similar expression levels of these constructs with the expected sizes (Figure 1D). Thus, these results together with the SR data strongly suggest that homodimerization is not sufficient for RARα fusion-mediated transformation of primary hematopoietic cells, which requires instead homotetramerization or higher-order homo-oligomerization.

Oncogenic SR Mutants and 4xFKBP-RARα Failed to Mediate Proficient Homo-Oligomeric DNA Binding

Aberrant transcriptional repression of RA-responsive genes is believed to be a major underlying transformation mechanism of RARα fusion and is relieved by retinoic acid therapy (Zelent et al., 2001). In contrast to the wild-type RARα, which binds DNA only in the presence of RXRα, we and others have shown that RARα fusion proteins via the self-association domains in the fusion partners could bind DNA either as homo-oligomers or hetero-oligomers with RXRα (Dong et al., 2003; Dong and Tweardy, 2002; Dong et al., 1996; Lin and Evans, 2000; Minucci et al., 2000; Perez et al., 1993; Redner et al., 2000; So et al., 2000). However, it is not clear if one or both of these DNA-binding properties are required for transformation, which may have critical implications for molecularly targeted therapies. To this end, we performed extensive gel shift analyses to assess the DNA-binding properties of various SR proteins. As expected, full-length SR could efficiently bind all tested RAREs as homo-oligomers (Figure 3A). Analogous to the full-length fusion, both SR-ΔDBD and the SR-ΔSH could also bind and exhibited similar RARE-binding patterns (Figure 3A). Conversely, SR-ΔN exhibited reduced DNA-binding abilities as compared with the full-length construct (Figure 3A). Surprisingly, the DNA-binding property of SR-ΔCC was significantly compromised even though it could efficiently transform primary hematopoietic cells. Proteins levels used in the assays were normalized by coupled in vitro transcription and translation assay using ^{35}S methionine (Figure 3B). The poor DNA-binding ability of transformation-competent SR-ΔCC suggests that homo-oligomeric DNA binding may not be essential for transformation.

Based on available crystal structural data and homology alignment among the Stat family proteins (Chen et al., 1998, 2003), we further developed and characterized several SR point mutants, three of which had maintained most properties of the full-length SR with the exception of their intrinsic homo-oligomeric DNA binding. Analogous to the SR-ΔCC mutant, all these mutants (SR-FL W37A; SR-FL F81A; SR-FL L82A) with a single point mutation at the N-terminal domain failed to bind DNA as homo-oligomeric complexes (Figure 3A). However, all of them maintained their ability to form homo-oligomeric, -tetrameric, and -dimeric complexes (Figure 2C). When these homo-oligomeric DNA-binding-deficient SR point mutants were assessed for their abilities to transform primary bone marrow cells, all maintained their transformation ability and gave rise to significant numbers of third-round compact colonies (Figures 2D and 2E). Consistently, both immunophenotype and morphology of the transformed cells were indistinguishable from those of full-length SR-transformed cells (Figure 2E and data not shown). The appropriate expression of each construct was confirmed by western blotting (Figure 1D). While these results strongly suggest that homo-oligomeric DNA binding is dispensable for

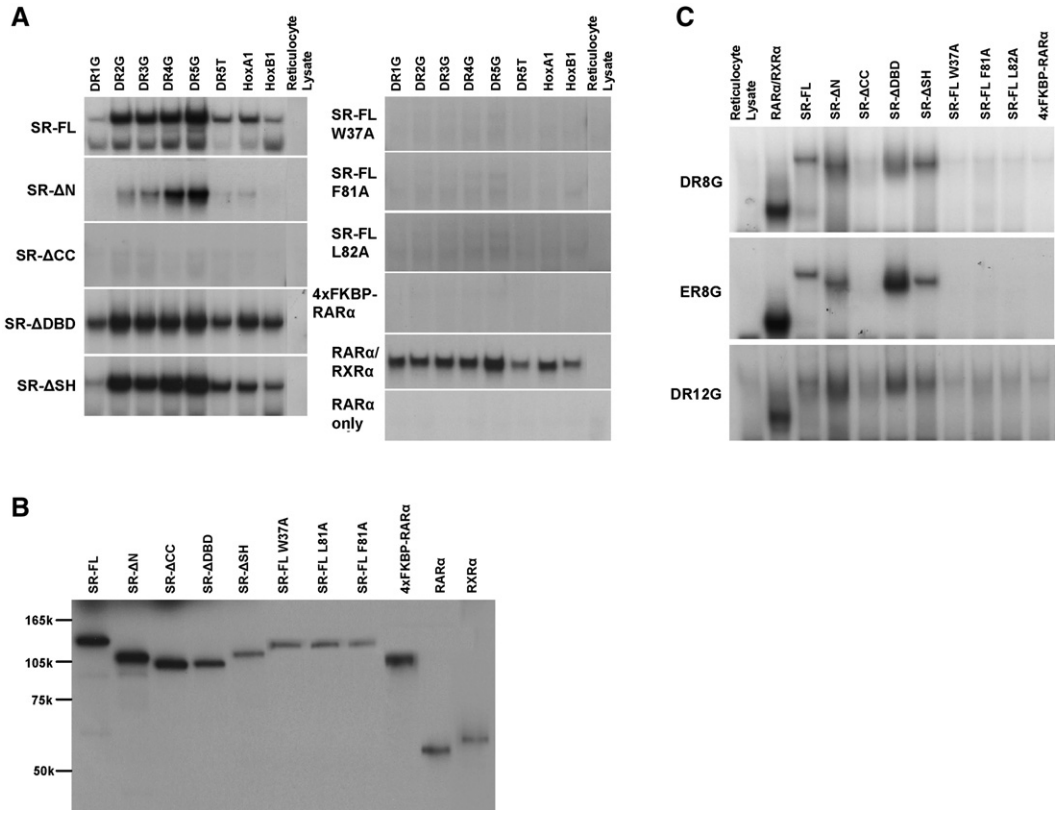

Figure 3. Intrinsic RARα Fusion Protein Homo-Oligomeric DNA Binding Is Not Required for Transformation

(A) Gel shift assays were performed with in vitro translated proteins (indicated in the left) using different RAREs (shown on top).

(B) Appropriate protein expression from constructs used for gel shift assays was confirmed by in vitro transcription/translation using ^{35}S-Met.

(C) Gel shift assays with "relaxed" forms of RAREs (left) using constructs indicated on top.

SR-mediated transformation, it is not clear if this is specific to SR or common for other RARα fusion proteins. We therefore assessed the DNA-binding properties of the synthetic 4xFKBP-RARα fusion, which can closely mimic the biochemical, transcriptional, and transformation properties of bona fide RARα fusions (Kwok et al., 2006). Analogous to the SR-ΔCC and the point mutants, 4xFKBP-RARα homo-oligomers failed to bind to any of the RAREs (Figure 3A).

Since homo-oligomeric RARα fusions have been speculated to bind to more relaxed RAREs such as DR8G, DR12G, and ER8G (Kamashev et al., 2004), we tested the ability of various RARα fusions to bind these RAREs. While full-length SR and SR-ΔN bound efficiently to these RAREs, all the SR point mutants and 4xFKBP-RARα failed to bind to DNA, although very weak binding was observed in SR-ΔCC (Figure 3C). In contrast to Kamashev et al., the wild-type RARα/RXRα heterodimer exhibited strong binding to all these relaxed forms of RAREs (Figure 3C), which was, however, not entirely unexpected in view of earlier reports (Kato et al., 1995; Tavera-Mendoza et al., 2006). The specificity of these bindings was confirmed by supershift assays using antibodies against RARα or RXRα (Figure S2). Taken together, the absence of DNA-binding ability in various transformation-competent RARα mutants indicates that homo-oligomeric DNA binding is not critically required for oncogenic transformation mediated by RARα fusions.

Higher-Order Hetero-Oligomeric SR/RXRα and 4xFKBP-RARα/RXRα Complexes Are the Dominant DNA-Binding Forms

Although our results uncoupled homo-oligomeric DNA binding from transformation, RARα fusions could potentially bind DNA as hetero-oligomers with RXR. To this end, we assessed their DNA-binding properties when complexed with RXRα. While the full-length SR efficiently bound RAREs as homo-oligomers, the presence of RXRα completely converted the SR homo-oligomers into SR/RXRα hetero-oligomers (Dong and Tweardy, 2002), which display a significantly relaxed DNA-binding selectivity compared to homo-oligomers (for example, bindings to DR1G, HoxB1, or p21 in Figure 4A). To further validate these results in vivo, we analyzed the DNA-binding complex in primary cells transformed by SR or MLL-ENL. Consistent with the in vitro data, SR binds DNA in vivo as hetero-oligomeric complexes with endogenous RXR (Figure 4B). More strikingly, RXRα could complex with

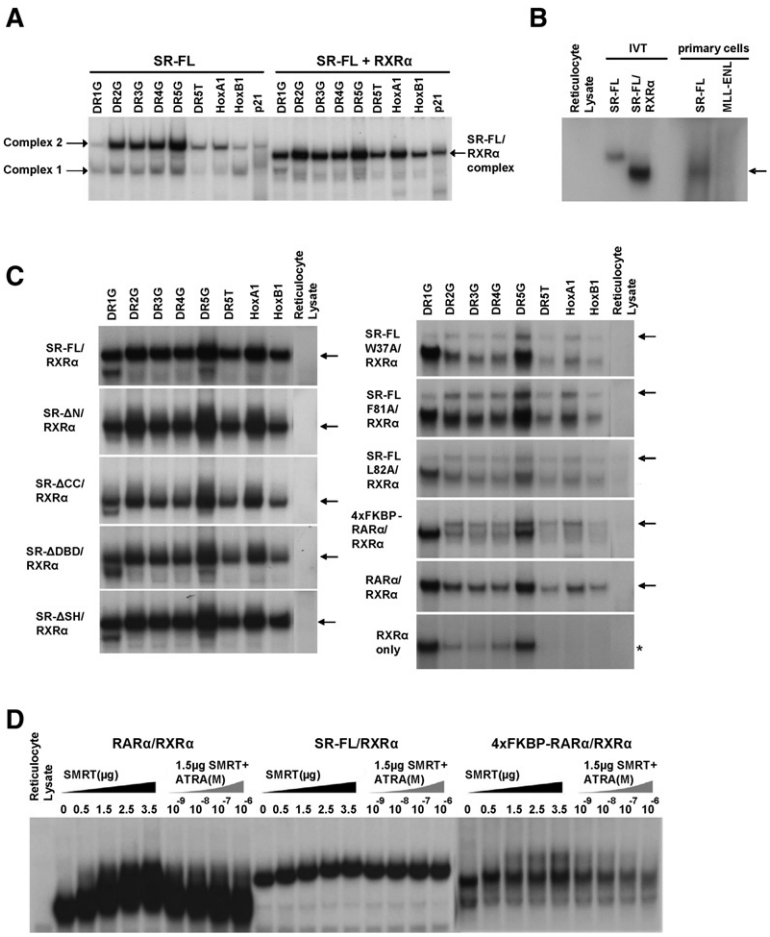

Figure 4. SR Forms Hetero-Oligomeric Complexes with RXRα and Corepressor SMRT to Bind RAREs

(A) Direct comparison between SR homo-oligomeric DNA binding and hetero-oligomeric DNA binding with RXR. Complex 1 and 2 refer to two different RARα fusion complexes with different apparent molecular sizes.

(B) Nuclear extracts from SR- or MLL-ENL-transformed primary cells were subjected to gel shift assay using DR5G. Retardation was compared to in vitro transcribed/translated (IVT) homo- and hetero-oligomeric complexes.

(C) Gel shift assays were performed with in vitro translated proteins (indicated in the left) together with RXRα using different RAREs (shown above). Arrows indicate RARα fusion/RXRα hetero-oligomeric complex. Asterisk shows homodimeric RXRα complex.

(D) IVT proteins (shown above) together with RXRα were used for gel shift assays using DR5G oligo with increasing amounts of SMRT in the absence or presence of indicated amounts of ATRA.

DNA-binding-deficient SR mutants to confer RARE binding (Figure 4C). These results strongly suggest that the higher-order SR/RXRα hetero-oligomer rather than the SR homo-oligomer is the dominant DNA-binding module. Consistently, similar data obtained with 4xFKBP-RARα further supported that higher-order RXRα hetero-oligomeric complexes correspond to the actual DNA-binding species derived from RARα fusion proteins (Figure 4C).

To further characterize the transcriptional properties of these higher-order hetero-oligomeric DNA-binding complexes, we assessed their ability to associate with the transcriptional corepressor SMRT and responses to ATRA treatment. RARα/RXRα heterodimers could bind to SMRT, but quickly dissociate at low concentration of ATRA (i.e., 10^{-8} M) (Figure 4D). In contrast, the binding of SMRT to SR/RXRα hetero-oligomers was refractory to ATRA treatment even at the concentration of 10^{-6} M (Figure 4D). Similarly, the association between 4xFKBP-RARα/RXRα hetero-oligomers and SMRT was more resistant to ATRA treatment (Figure 4D). Taken together, these results indicate that higher-order RARα fusion/RXRα hetero-oligomeric complexes can efficiently bind RAREs and associate with transcriptional corepressors that are refractory to physiological concentration of ATRA.

RXRα Is Required for Both Bona Fide and Synthetic RARα Fusion-Mediated Transformation

To further interrogate the roles of RXRα in APL, we employed a shRNA approach to evaluate its functional requirement for RARα fusion-mediated transformation of primary hematopoietic cells. Two independent shRNAs (sh563 and sh131) targeting murine RXRα were developed and validated for their ability to efficiently knock down the expression of murine RXRα (Figure 5A). Retroviral constructs carrying RARα oncoproteins were cotransduced with either RXRα shRNA or vector control. Although reduced numbers of colonies were obtained in the first plating due to lower transduction efficiency for simultaneous integration of two viruses into the genome, cells cotransduced with SR and vector control rapidly expanded and efficiently formed third-round compact colonies indistinguishable from the cells transduced with SR (Figures 5B and 5C and Figures S3A and S3B). Conversely, cells cotransduced with SR and either RXRα shRNA gradually lost self-renewal property and displayed significantly compromised ability to form third-round colonies in spite of a transient expansion in the second plating. These RXRα shRNAs also inhibited the ability of 4xFKBP-RARα to confer self-renewal, suggesting general and a critical

function of RXRα in RARα fusion-mediated transformation (Figures 5B and 5C and Figures S3A and S3B). To control for general toxicity and specificity of the shRNAs, they were independently cotransduced with retroviruses carrying a non-RARα oncoprotein, MLL-ENL. In contrast to RARα oncoproteins, neither of these RXRα shRNAs had any significant effect on MLL-ENL-mediated transformation (Figures 5B and 5C and Figures S3A and S3B).

To further extend the role of RXRα to the most common RARα fusion found in the human disease, we validated two independent shRNAs (sh616 and sh617) against human RXRα and tested their efficacy in inhibiting the growth of a PML-RARα leukemic cell line, NB4 (Figure 5D). Knockdown of hRXRα expression using sh616 or sh617 consistently resulted in (1) significant inhibition of proliferation (Figure 5E), (2) nearly complete ablation of clonogenic colony formation (Figure 5F and Figure S4), (3) loss of mitochondrial membrane potential (Figure 5G), and (4) induction of apoptosis (Figure 5G). In contrast, relatively mild impacts on differentiation were observed, as revealed by CD11c differentiation marker analysis (Figure S5). None of these effects were seen in cells transduced with the control shRNA construct, which did not affect the expression of endogenous hRXRα (Figures 5D–5G).

Clinically, it is known that a significant portion of APL patients develop ATRA resistance as a result of clonal evolution that selects for clones with mutations affecting the ATRA response such as ligand-binding domain in RARα (Lengfelder et al., 2005). Since our current approach directly targets RXR instead of RAR, we sought out to determine if it would also be effective for this subgroup, which is refractory to ATRA treatment and has a poor prognosis. We tested the efficacy of these two shRNAs in inhibiting the NB4-MR2 cells, an ATRA-resistant subclone derived from NB4 cells (Shao et al., 1997). In spite of their inert responsiveness to ATRA, each of these RXRα shRNAs could effectively (1) inhibit proliferation (Figure 5E), (2) ablate clonogenic colony formation (Figure 5F and Figure S4), (3) induce loss of the mitochondrial membrane potential, and (4) apoptosis (Figure 5G) but had apparently minimal, if any, effect on NB4-MR2 differentiation (Figure S5). Taken together, these results suggest that RXRα is a critical component for RARα fusion-mediated transformation and presents as a promising target for ATRA-resistant APL.

Inhibition of RARα Fusion-Mediated Transformation by RXR Agonist

To investigate the potential therapeutic value of targeting RXRα-mediated pathways in RARα fusion-mediated transformation, we used a well-characterized panRXR agonist, SR11237, which has been previously tested in combination with 8-CPT-cAMP, a protein kinase A (PKA) agonist, for the induction of differentiation and apoptosis of AML cell lines and patients' blasts (Altucci et al., 2005; Benoit et al., 1999). After the second plating, SR-transduced cells were split and plated into the third round in the absence or presence of SR11237. To demonstrate that the primary transformed cells from this assay are pathologically relevant to the human disease, the transduced cells were treated with ATRA. In contrast to untreated controls, where SR-transduced cells efficiently formed third-round colonies, cells treated with a pharmacological concentration (1 μM) of ATRA lost their self-renewal property and significantly compromised their ability to form third-round colonies, confirming the physiological relevance of these primary transformed cells (Figures 6A and 6B). When SR-transduced cells were treated with a combination of 100 μM 8-CPT-cAMP and 1 μM SR11237, a very significant growth inhibition (mean = 70%) was observed as compared with the mock control (Figures 6A and 6B). More interestingly, when cells were treated with 1 μM SR11237 alone, we also observed a significant inhibitory effect (mean = 50%) on colony formation (Figure 6A). To determine if this suppression is specific to SR or a more general phenomenon for RARα fusion-mediated transformation, we tested other RARα oncoproteins, including PML-RARα and 4xFKBP-RARα, and observed very similar inhibitory effects (Figure 6A). Notably, inhibition was not due to general toxicity of the drugs, as MLL-ENL-transformed cells were inert to these treatments (Figures 6A and 6B).

To further explore the mechanisms underlying the observed rexinoid effect, primary cells transformed by SR or MLL-ENL (control) were treated with SR11237 in combination with PKA or various retinoid antagonists. That 1 μM SR11237 still efficiently inhibits colony formation by SR-transformed cells even when coexposed to 100 μM RP-cAMP (a potent PKA inhibitor) suggested that this inhibitory effect was not related to cAMP-induced RXR desubordination (Figure 6C). To investigate if the observed rexinoid inhibition depended on transcriptionally active RARα/RXRα, which is required for ATRA-induced differentiation (Zelent et al., 2001), SR-transformed primary cells were treated with SR11237 and 1 μM of an RARα-specific antagonist (BMS614) or an inverse pan-RAR agonist (BMS493), which inhibits the transactivation by stabilizing corepressors binding to RAR (Altucci et al., 2005; Benoit et al., 1999; Germain et al., 2002). However, neither the RAR antagonist nor the inverse agonist had negative effect on SR11237-induced inhibition, strongly indicating a mechanism different from ATRA-induced differentiation (Figure 6C). Consistently, distinct results were obtained when the primary transformed cells were analyzed for differentiation markers after rexinoid or retinoid treatment. As expected, ATRA induced significant differentiation in primary transformed cells as reflected by its pronounced reduction in expression of c-kit progenitor marker and increased expression of myeloid differentiation markers Mac-1 and Gr-1 (Figure 6D). In stark contrast, SR11237-treated cells displayed only very mild effects on expression of these markers, whereas SR11237 together with cAMP analog displayed intermediate effects (Figure 6D). All this reveals that the observed rexinoid inhibition is distinct from ATRA-induced differentiation and cAMP/rexinoid-induced RXR desubordination. Consistently, SR11237 induced only weakly (<5-fold) the

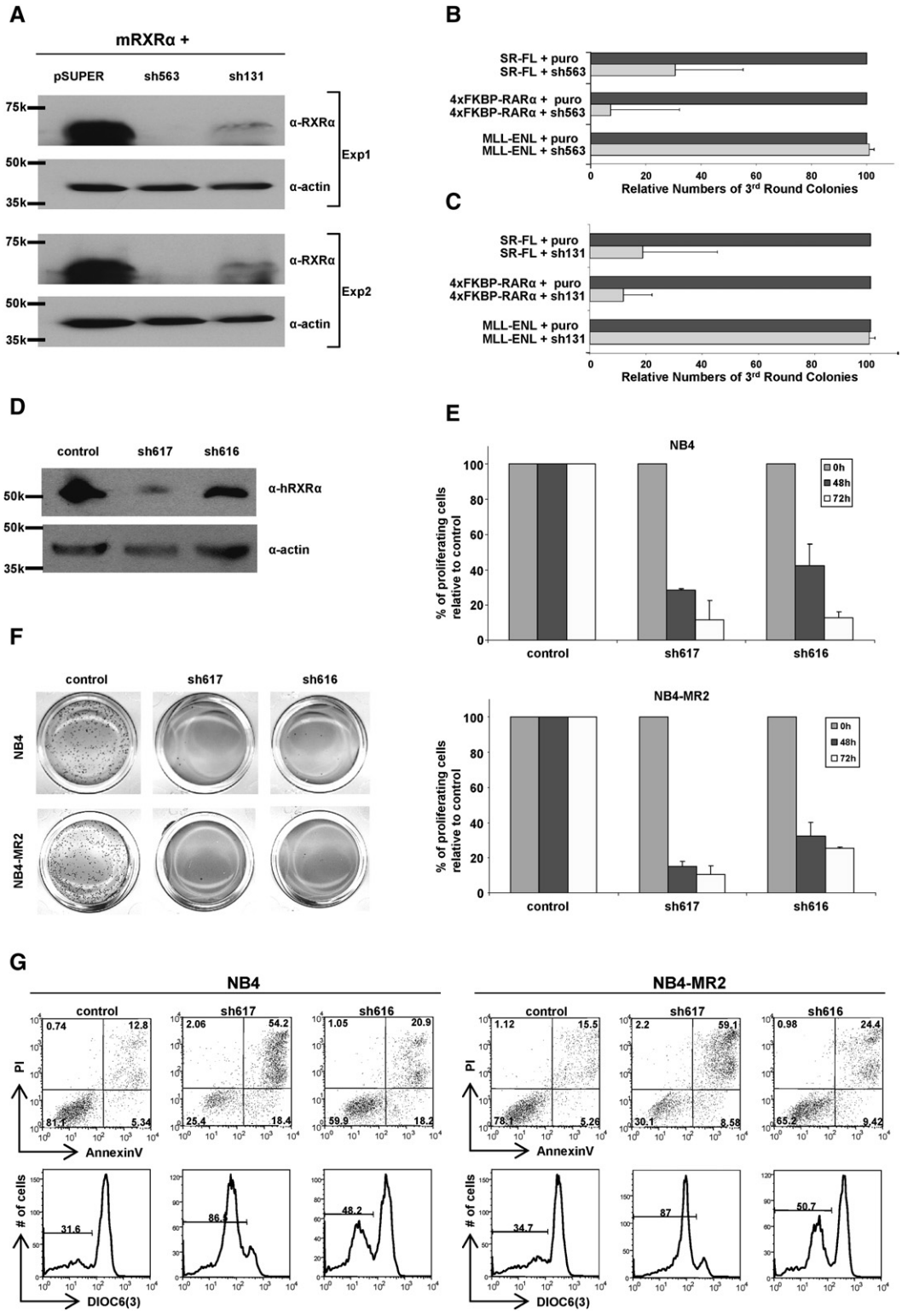

Figure 5. Expression of RXRα Is Required for RARα Fusion-Mediated Transformation

(A) Cell lysates from cotransfected 293 cells were western blotted with murine anti-RXRα (Santa Cruz) or anti-actin (Sigma) antibody as indicated. The results of two independent experiments are shown.

(B and C) The bar chart represents the relative numbers of third-round colonies of primary hematopoietic precursors cotransduced with retrovirus carrying fusion gene and empty vector or sh563 (B) or sh131 (C). Error bars indicate SD of two independent experiments.

(D) Lysates from transduced NB4 cells were blotted with anti-hRXRα antibody (provided by Hinrich Gronemeyer) and anti-actin antibody as indicated.

expression of an RARα-inducible gene, *RARβ*, which was, however, strongly upregulated (>150-fold) by ATRA in primary transformed cells (Figure 6E). Since pure rexinoids have been reported to induce apoptosis (Benoit et al., 2001; Aurélie Rossin, P.S., Annica Jacobson, Lucia Altucci, and H.G., unpublished data), which is consistent with our RXR shRNA data (Figure 5), and the observation of its modest impact on differentiation, we speculated that the effect of SR11237 might be a result of induction of apoptosis. Indeed, we could detect a significant loss of mitochondrial membrane potential and a dramatic induction of apoptosis (from 32% to 72%) by SR11237 in primary transformed cells (Figure 6F). These results support once more that SR11237-induced apoptosis is different from ATRA-induced differentiation or cAMP/rexinoid-induced RXR desubordination.

Previous studies have shown that rexinoids have the potential to induce apoptosis in various cell lines under low-serum conditions even in the absence of functional RARα (Benoit et al., 2001; Aurélie Rossin, P.S., Annica Jacobson, Lucia Altucci, and H.G., unpublished data). To further investigate the rexinoid-induced apoptosis in full-blown PML-RARα leukemic cells, NB4 cells were treated with SR11237 in the absence or presence of various apoptotic inhibitors under low-serum conditions (Figure 7). Analogous to the primary cell data, we detected significant induction of apoptosis (from 12% to 80%) (Figures 7A and 7B), activation of effector caspases (caspase 3/7) (Figure 7C), and loss of mitochondrial membrane potential (Figure 7D) in the presence of 1 μM SR11237. Addition of a pan-caspase inhibitor (Z-VAD-FMK) inhibited rexinoid-induced apoptosis, which was, however, inert to inhibitors of reactive oxygen species (ROS) such as L-NAC and GSH antioxidants (Figures 7A and 7B and data not shown). When cells treated with various rexinoids/retinoids were analyzed for apoptosis and expression of the CD11c myeloid differentiation marker, SR11237 alone induced significant apoptosis but not expression of CD11c, while ATRA or SR11237 in combination with cAMP analogs dramatically increased the CD11c expression without significant induction of apoptosis under identical experimental conditions (Figure 7D). Consistently, addition of Z-VAD-FMK caspase inhibitor suppressed SR11237-induced apoptosis without altering the differentiation status of the cells (Figure 7D). Taken together, these results reveal a critical cAMP-independent function of RXR agonists in inducing apoptosis without prior differentiation in RARα fusion-transformed cells.

DISCUSSION

Self-association, a widely used process for regulation of activity and specificity of proteins such as kinases and transcription factors involved in various biological processes, is frequently hijacked by oncoproteins to activate their oncogenic potential (So and Cleary, 2004). While the discovery of essential roles of self-association in mediating transformation of primary hematopoietic cells by various LATFs (Kwok et al., 2006; Liu et al., 2006; Martin et al., 2003; So et al., 2003b; Sternsdorf et al., 2006) has opened up an avenue for molecular targeting, it is not clear whether the targeting endpoint is homodimer or monomer, or if it is technically feasible to efficiently disintegrate oligomers into monomers. A recent study on AML1-ETO has indicated that a complete dissociation of tetrameric fusion into monomers can pose a serious technical challenge due to the relatively large hydrophobic interfaces that are frequently found in oligomers (Liu et al., 2006). Thus, defining the self-association threshold becomes a critical issue for not only understanding the underlying molecular mechanisms but also designing rational drug targeting. Using Stat5b-RARα with two distinctive self-association domains as a working model, we demonstrate that loss of homotetramerization, albeit with retention of homodimerization property, abolished its ability to transform primary hematopoietic cells. Similarly, simple homodimerization of truncated RARα by synthetic dimerizing modules (e.g., 1xFKBP) is not sufficient to mediate transformation, which requires higher-order homo-oligomerization (e.g., 4xFKBP). These results strongly suggest that disruption of homotetramers into homodimers instead of monomers may already be sufficient to abrogate the transformation ability of RARα oncoproteins. In contrast to the oligomerization domain of ETO in the AML1-ETO fusion protein, where the amphipathic monomer interacts with three other monomers through hydrophobic interactions that bury an extensive accessible surface area (ASA) of about 10K $Å^2$ (Liu et al., 2006), the N-terminal tetramerization domain of Stat proteins mediates the formation of homotetramers between two dimers with a total ASA of only about 2K $Å^2$ (Chen et al., 1998, 2003; Vinkemeier et al., 1998). More important, introduction of single point mutations in the N-terminal domain can abrogate homotetramerization of Stat proteins, suggesting relatively small or weak interaction interfaces, although any equivalent mutations will have to be empirically determined and validated in the context of RARα fusions. By defining the oncogenic threshold beyond homodimerization, the present study underscores the therapeutic potential of targeting the oligomerization properties in at least some RARα oncoproteins (Figure 8A).

While RAR alone is a poor DNA-binding protein unless heterodimerized with RXR (Chambon, 2005), we and others have previously shown that RARα fusion homo-oligomers acquire additional DNA-binding properties independent of RXR (Dong et al., 1996, 2003; Dong and

(E) Transduced NB4 (upper bar chart) and NB4-MR2 cells (lower bar chart) were subjected to MTS assay. Error bars indicate SD of two independent experiments.
(F) Typical INT-stained colony pictures of transduced NB4 (upper panel) and NB4-MR2 cells (lower panel) 6 days after plating in methylcellulose.
(G) Transduced NB4 (left side) and NB4-MR2 cells (right side) were analyzed for apoptosis using Annexin V/PI (upper panel) and mitochondrial membrane potential using DIOC6(3) (lower panel).

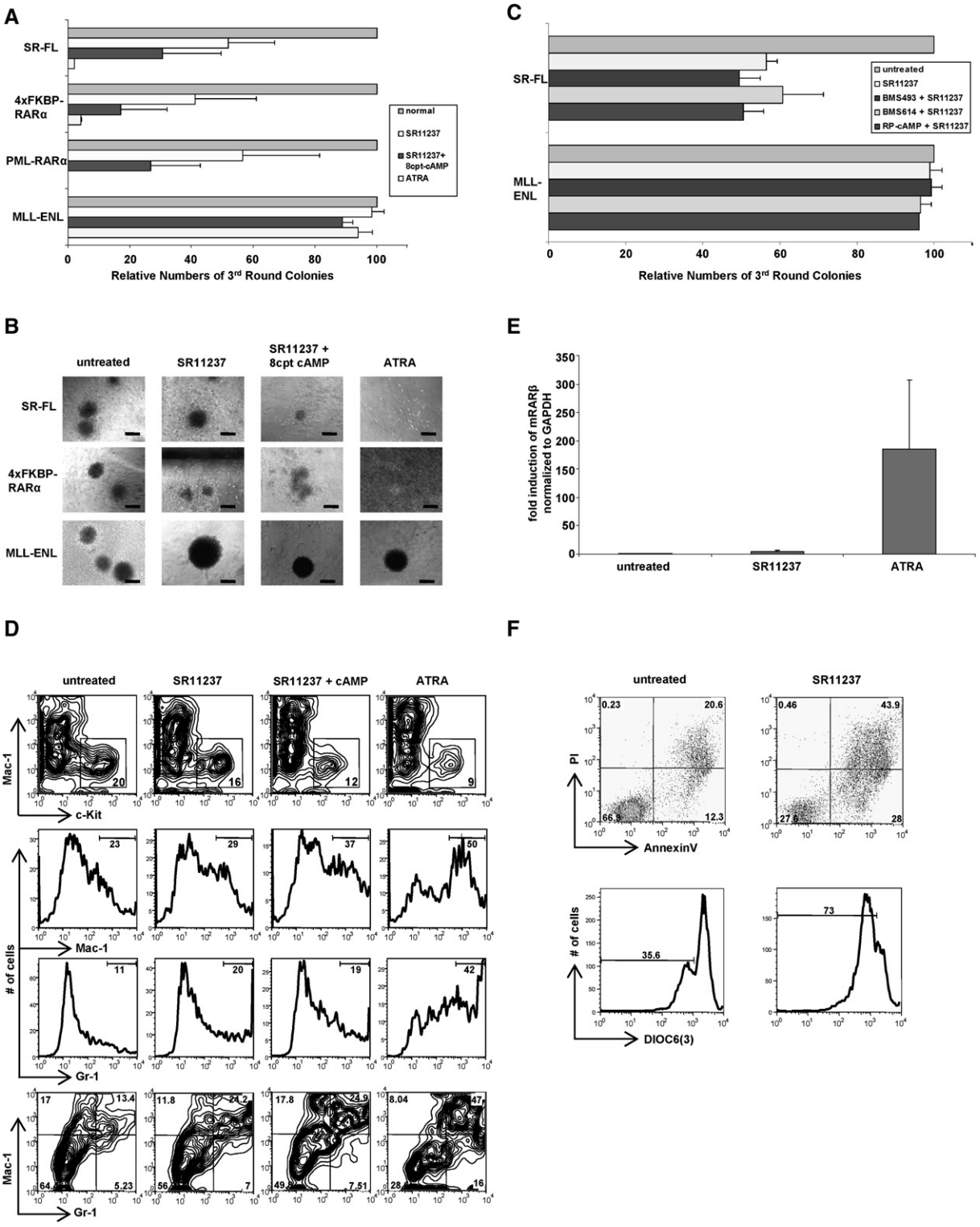

Figure 6. RXR Agonist Inhibits RARα Fusion-Mediated Transformation of Primary Cells by Inducing Apoptosis without Prior Differentiation

(A) The bar chart represents the relative numbers of third-round colonies of primary bone marrow cells transduced with indicated constructs (left). Error bars indicate SD of five independent experiments.

(B) Typical third-round colony morphology in the absence or presence of indicated drugs generated from transduced bone marrow cells as indicated. Scale bar, 100 μm.

Tweardy, 2002; Lin and Evans, 2000; Minucci et al., 2000; Perez et al., 1993; Redner et al., 2000; So et al., 2000). However, it is not clear which of these DNA-binding properties are critical for transformation or if both are required. In this study, we uncouple homo-oligomeric DNA binding from RARα fusion-mediated transformation by identifying several transformation-competent but homo-oligomeric DNA-binding-deficient mutants derived from both bona fide and synthetic RARα fusions. We also reveal higher-order hetero-oligomeric RXR/RARα oncoproteins as the dominant DNA-binding species to which RXRα confers specific DNA-binding properties to otherwise DNA-binding-deficient but transformation-competent RARα mutants, thereby revealing an important role of RXRα in modulating the transcription and transformation properties of oncogenic RARα fusion proteins (Figure 8B). While RXRα is a limiting cofactor for various nuclear receptors (Chambon, 2005), which may also be interfered and contribute to the repression phenotype observed upon shRNA and/or panRXR agonist treatment, these effects will be relatively minor, as primary hematopoietic cells transformed by MLL fusion were refractory to these treatments. Thus, intervention of RXRα-mediated pathways may represent a promising therapeutic avenue for effective targeting oncogenic RARα fusion complexes, particularly for ATRA-resistant APL cells (Figures 8B–8D).

Whereas ligand-dependent transactivation function of RXR is subordinated to some of its heterodimeric partners, such as wild-type RARs (Germain et al., 2002; Gronemeyer et al., 2004), crosstalk with other signaling pathways such as the one activated by elevated levels of intracellular cAMP can cause RXR desubordination due to corepressor release from the RAR partner (Altucci et al., 2005). PanRXR agonists had previously been used in conjunction with cAMP analogs or other RARα agonists to induce differentiation of both APL cells (Benoit et al., 1999; Kamashev et al., 2004) and RA-insensitive non-APL AML leukemic blasts (Altucci et al., 2005), suggesting that the molecularly targeted pathways by rexinoid can be context dependent, particularly in full-blown leukemic blasts or established cell lines. The present study reveals that combinatorial treatment with a pure rexinoid in the presence of elevated cAMP levels efficiently phenocopies the effect of ATRA with respect to the inhibition of growth/self-renewal of RARα fusion-transformed primary hematopoietic precursor cells (Figure 8C). Notably, we also observe a similar inhibition, albeit less pronounced, when using the rexinoid alone and demonstrate that SR11237 alone can activate caspase-mediated apoptosis, which is independent of cAMP and is different from ATRA-induced differentiation (Figure 8D). In established cell lines such as NB4, rexinoid-induced apoptosis is only observed in low-serum conditions when growth factors become limited. It is therefore an attractive hypothesis that the initial transformation event of hematopoietic precursors by RARα fusion oncoproteins may alter their growth factor requirements, possibly revealed by their altered self-renewal, but at the same time make them vulnerable against rexinoid-induced apoptosis. However, as transformed clones evolve, they acquire secondary mutations, such as activated Flt3 (Sohal et al., 2003), that reduce the growth factor dependence and may desensitize the response to rexinoid-induced apoptosis. Therefore, combinations of growth factor antagonists and rexinoids may represent alternative ways to target RARα fusion-transformed cells (Figure 8D).

Taken together, our results reveal formation of higher-order homotetrameric complexes and recruitment of RXRα as two essential components and potential therapeutic targets for RARα fusion-mediated transformation (Figure 8), thus further endorsing the therapeutic potentials of RXR agonists, which have already been used for treatment of cutaneous T cell lymphoma (Zhang and Duvic, 2003) and non-small-cell lung cancer (Rigas and Dragnev, 2005). Although the combination of ATRA and chemotherapy has greatly improved the overall survival of APL patients (Degos and Wang, 2001), there are still significant numbers of relapsed cases, which become ATRA resistant, and only half of them will respond to arsenical treatment (Lengfelder et al., 2005). Thus, future effort in identifying and validating additional molecular targets such as RXRα will provide not only important insights into APL pathogenesis but also effective therapeutic approaches, particularly for ATRA-resistant or relapsed APL patients.

EXPERIMENTAL PROCEDURES

Constructs

All the Stat5b-RARα (Dong and Tweardy, 2002), PML-RARα, FKBP-RARα (Kwok et al., 2006), GST-SMRT, and RXRα (Dong and Tweardy, 2002; So et al., 2000) constructs have been previously reported. The single point mutants of SR were made using the primer extension approach and have been fully sequenced across the amplified fragments. Details of shRNA constructs are described in the Supplemental Experimental Procedures.

RTTA

RTTA was performed as previously described (Kwok et al., 2006). For knockdown experiments, spinoculation was repeated three times before the plating. In vitro transformation was defined as enhanced self-renewal of primary hematopoietic cells beyond three rounds of plating in the serial replating assay.

Lentiviral Transduction, Clonogenicity, and Proliferation Assay

Lentiviral supernatant collected 48 hr after transfection of 293 cells was added to NB4 cells in the presence of 5 μg/ml polybrene for

(C) The bar chart shows the relative numbers of third-round colonies of SR- and MLL-ENL-transduced primary cells. Error bars indicate SD of two independent experiments.
(D) Primary cells transduced with SR were treated as indicated for 48 hr and analyzed for surface marker expression by FACS.
(E) RT-Q-PCR analysis for murine *RARβ* (*mRARβ*) on cDNA samples prepared from SR-transduced primary cells treated with indicated rexinoid/retinoid. Error bars indicate SD of two independent experiments.
(F) FACS plots show Annexin V/PI (upper panel) and DiOC6(3) (lower panel) stainings of SR-transduced primary cells.

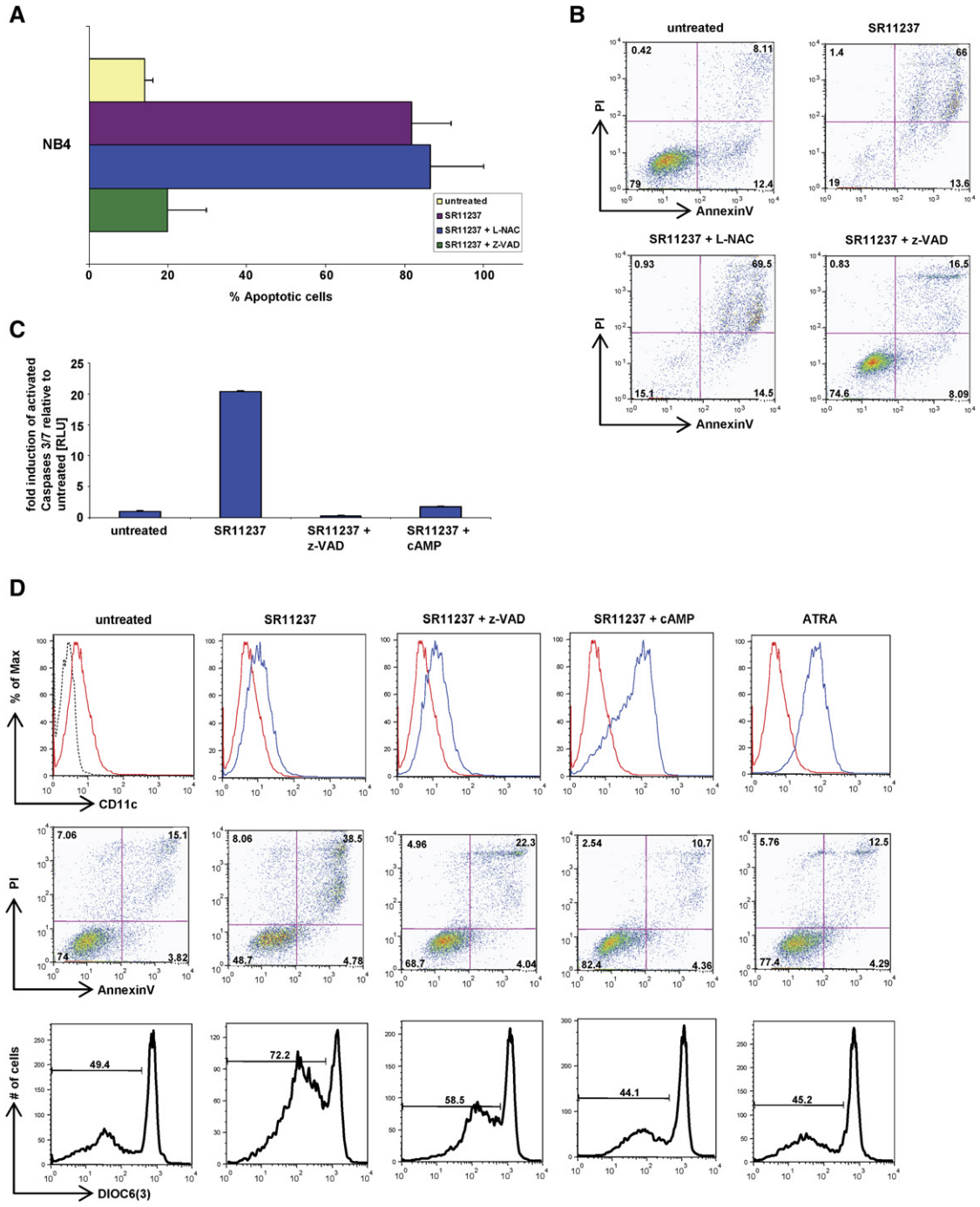

Figure 7. RXR Agonist Induces Caspase-Mediated Apoptosis in NB4 Cells without Prior Differentiation

(A) The bar chart represents the percentage of apoptotic NB4 cells after treatment (as indicated) for 72 hr. Error bars indicate SD of two independent experiments.

(B) FACS plots show Annexin V/PI staining of NB4 cells after treatment.

(C) The bar chart represents the fold induction of activated caspase 3 and 7 of treated NB4 cells relative to untreated control. The error bars indicate SD of measurements carried out in duplicates.

(D) NB4 cells treated for 60 hr as indicated were analyzed by FACS for expression of CD11c surface marker (upper panel: dotted, unstained; red, stained and untreated control; and blue, stained and treated samples), Annexin V/PI (middle panel), and DIOC6(3) (lower panel).

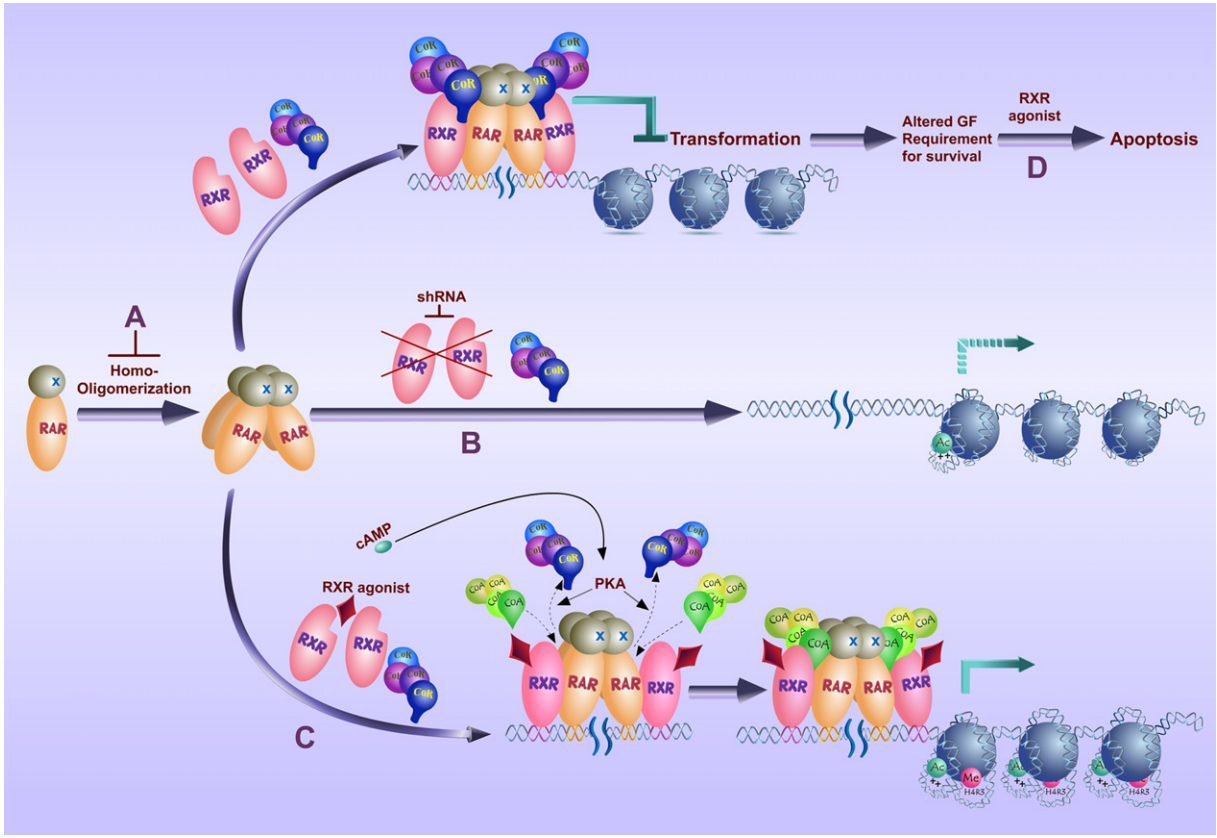

Figure 8. Potential Therapeutic Avenues by Targeting Homo-Oligomerization or RXR-Mediated Pathways in APL
Suppression of transformation by disruption of homo-oligomerization (A); knockdown of RXRα by shRNAs that inhibits the formation of effective DNA-binding complexes (B); pan-RXR agonist in combination of 8-CPT-cAMP by destabilizing the corepressor complexes and crosstalk with other cAMP-activated pathways (C); RXR agonists alone via induction of apoptosis, which can potentially further be augmented by growth factor inhibitors (D).

overnight transduction. Transduced cells were then selected for 3 days in the presence of 1 μg/ml Puromycin. For clonogenicity assay, 2000 transduced NB4 cells were plated in 1% methylcellulose (Stem Cell Technologies) and incubated for 6 days. Colonies were stained with INT (*p*-Iodonitrotetrazolium Violet, Sigma) at 100 μg/ml final concentration. For proliferation assay, 10,000 transduced NB4 cells were plated in triplicates into a 96-well microtiter plate in 100 μl volume. After 48 hr or 72 hr, 20 μl of MTS reagent (CellTiter96 Aqueous One Solution, Promega) was added and incubated for 1 hr in the dark at 37°C. Absorbance was recorded at 490 nm using a Photospectrometer (Dynatech Laboratories).

Phenotype Analysis

Immunophenotypic analysis was performed by FACS using fluorochrome-conjugated monoclonal antibodies to human CD11c (BU15 clone) (Caltaq) and murine c-Kit (2B8 clone), murine Mac-1 (M1/70 clone), murine Gr-1 (RB6-8C5 clone), murine B220 (RA3-6B2 clone), and murine CD3e (145-2C11 clone) (Pharmingen Inc. or eBiosciences Inc.), respectively. Staining was generally performed on ice for 15 min and washed twice before analysis using a BD LSR II system (Becton Dickinson Inc.).

Gel Filtration Study

Gel filtration assays were performed as previously described (Kwok et al., 2006). Collected fractions were analyzed by western blot using anti-RARα C-20 polyclonal antibody (Santa Cruz Biotech).

Coimmunoprecipitation Assay

The immunoprecipitation study was essentially performed as previously described (Kwok et al., 2006). The antibodies used in the immunoprecipitation assay were from Sigma-Aldrich (M2 anti-Flag antibody) and the Hybridoma Unit in the Institute of Cancer Research (9E10 anti-Myc antibody).

Gel Shift DNA-Binding Assay

The gel shift assay was carried out as previously described (So et al., 2000). The probes were made according to published sequences (Kamashev et al., 2004; So et al., 2000). Supershift was performed by incubating the indicated reaction in the presence of GST fusion proteins or specific antibody (anti-RARα C-20 polyclonal antibody or anti-RXRα D-20 polyclonal antibody from Santa Cruz Biotech).

Quantitative RT-PCR

RNA from primary cells was isolated using RNeasy mini (Qiagen) and reverse transcribed using SuperscriptII (Invitrogen). Quantitative RT-PCR (RT-Q-PCR) was carried out on a 7900HT Fast Real-Time PCR system (Applied Biosystems) using the Taqman Fast Reagent technology. Primer sequences are described in the Supplemental Experimental Procedures.

Assays for Apoptosis, Mitochondrial Membrane Potential, and Caspase Activity

Apoptosis was examined by staining with Annexin V/PI according to the manufacturer's protocol (Biovision) and analyzed by flow

cytometry. Mitochondrial membrane potential was assessed by incubating 2 × 10^5 cells with 50 nM DIOC6(3) (Invitrogen) for 30 min at 37°C and subsequently analyzed by flow cytometry. Selected viable NB4 and NB4-MR2 cells were prepared 48 hr prior to Annexin V/PI and DiOC6(3) stainings. Caspase 3/7 activity was measured using Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's instructions using MLX Microplate Luminometer (Dynex Technologies).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and five supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/1/36/DC1/.

ACKNOWLEDGMENTS

We thank Mel Greaves and Jon Wilson for useful discussion; Wyatt Yu, Amanda Wilson, Michael Cheung, and Jihui Qiu for excellent technical help; and Pui Yi Tse for professional artworks. This research is supported by project grants from Cancer Research UK (C.W.E.S.), the Leukaemia Research Fund (C.W.E.S.), European Union EPITRON LSHC-CT2005-518417 (H.G.), X-TRA-NET LSHG-CT2005-018882 (H.G.), and the Ligue Nationale contre le Cancer (H.G., équipe labelisée), and R21 grant CA119080 (S.D.) from the National Cancer Institute. C.W.E.S. is a fellow of the Association for International Cancer Research.

Received: November 20, 2006
Revised: April 10, 2007
Accepted: June 1, 2007
Published: July 9, 2007

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