Forced homo-oligomerization of RARα leads to transformation of primary hematopoietic cells

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Summary

Almost 100% of APL patients carry chimeric transcripts encoding truncated RARα fused to homo-oligomerization domains from partner proteins. To gain further insights into the cellular transformation mechanisms mediated by RARα fusion proteins, thorough structure/function analyses have been performed and identified the POZ homo-oligomerization domain as the minimal transformation domain that is necessary and sufficient for PLZF-RARα-mediated in vitro transformation of primary hematopoietic cells. A transformation-incompetent PLZF-RARα mutant defective in homo-oligomerization but not corepressor interaction could be rescued by synthetic FKBP-oligomerization domains. Furthermore, an artificial *FKBP-RARα* construct not only mimicked various biochemical properties of bona fide RARα fusion proteins but also mediated an ATRA-dependent transformation. Taken together, these findings endorse an oligomerization-dependent mechanism for RARα-mediated transformation and suggest a potential avenue for molecular therapy.

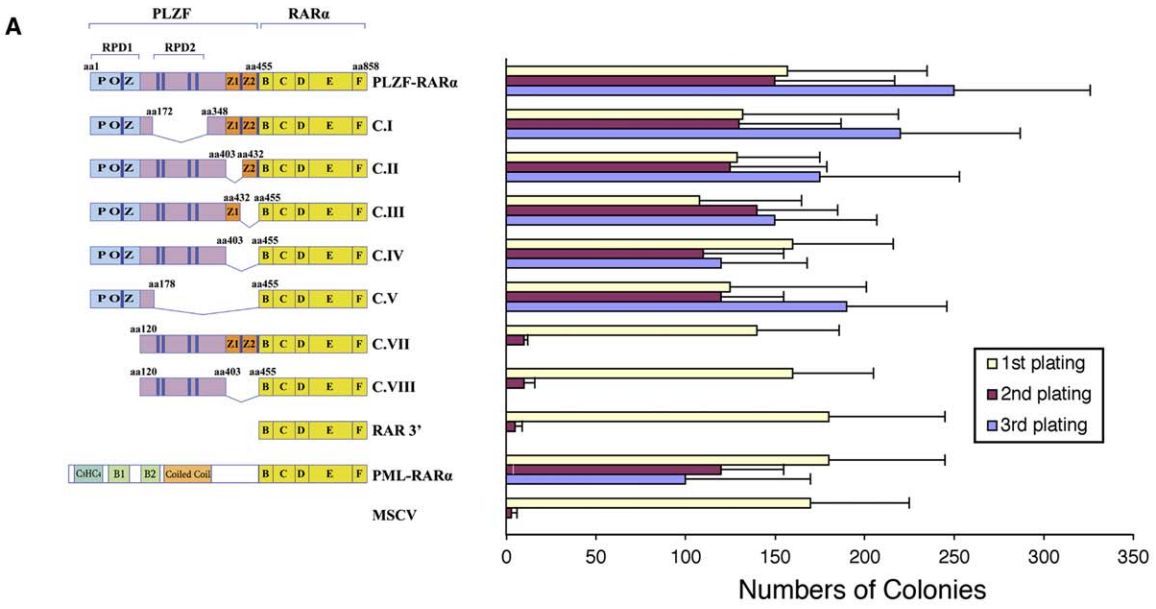
Introduction

Acute myeloid leukemias (AML) are characterized by selective expansion and differentiation block of immature myeloid precursors carrying specific chimeric transcription factors, which most commonly involve mutations of the retinoic acid receptor α (RARα), the core binding factor subunits (AML1 or CBFβ), or the mixed lineage leukemia (MLL) protein (So and Cleary, 2004). Chimeric RARα fusion can be found in almost 100% of acute promyelocytic leukemia (APL, AML-M3, a subtype of AML) that are remarkably sensitive to all-*trans*-retinoic acid (ATRA)-induced terminal differentiation (Zelent et al., 2001). The molecular mechanisms underlying the ATRA treatment of APL have been thoroughly studied in the last decade. It is now known that, in the presence of physiological doses (10^{-8} M) of ATRA, RARα/RXRα heterodimers will dissociate from transcriptional corepressor complexes composed of SMRT/NCoR, Sin3, and histone deacetylase (HDAC) and associate with transcriptional activation complexes consisting of CBP/p300, P/CAF, SRC-1, TIF2, and TRAM-1 (Chen et al., 1996, 1997; Kurokawa et al., 1995; Onate et al., 1995; Takeshita et al., 1997). This transcriptional switch from repression to activation will lead to expression of specific set of RA-inducible genes and normal differentiation of hematopoietic progenitors (Chambon, 1996). However, in the case of RARα fusion, the complete dissociation with transcriptional corepressor complexes will only occur at much higher pharmaceutical (10^{-6} M) or superpharmaceutical (for PLZF-RARα) concentrations, which may explain the effectiveness and the differential retinoic acid response of APL patients to ATRA treatment (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998). Further biochemical analyses reveal that the homo-oligomerization (formation of complexes with two or more identical subunits) domains present in all RARα partners including PML, PLZF, NPM, NuMA, and Stat5b are necessary and sufficient for the aberrant transcriptional repression properties, suggesting their critical roles in leukemic transformation (Dong et al., 1996, 2003; Dong and Tweardy, 2002; Lin and Evans, 2000; Minucci et al., 2000; Perez et al., 1993; Redner et al., 2000; So et al., 2000).

While significant progress has been made in understanding the biochemical and transcriptional properties of RARα fusions, the biological relevance of these findings in terms of transforming primary hematopoietic targets is still unclear. Since overexpression of RARα fusions are toxic to the majority of the hematopoietic cell lines (Ferrucci et al., 1997), very limited structure/function transformation studies have been performed, and

SIGNIFICANCE

While transcription factors are frequently targeted and converted into constitutive transcriptional activators or repressors in acute leukemia, acquisition of homo-oligomerization domains is a common feature associated with many oncogenic transcription factors, including RARα fusion proteins, which are present in almost all the APL patients. We have previously shown that forced oligomerization of truncated MLL can activate its transcriptional and oncogenic properties. Here, we further extend this finding to a different class of oncogenic transcriptional repressors by demonstrating that simple homo-oligomerization of RARα by either a bona fide fusion partner (PLZF) or synthetic oligomerization (FKBP) module can lead to oncogenic transformation of primary hematopoietic cells. These studies emphasize a prevailing and critical role for homo-oligomerization in oncogenic activation of chimeric transcription factors.


A
PLZF
RARα
RPD1
RPD2
PLZF-RARα
C.I
C.II
C.III
C.IV
C.V
C.VII
C.VIII
RAR 3'
PML-RARα
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0
50
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200
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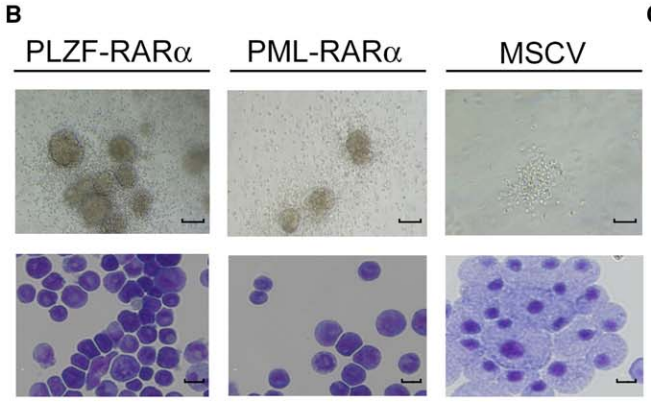
300
350
Numbers of Colonies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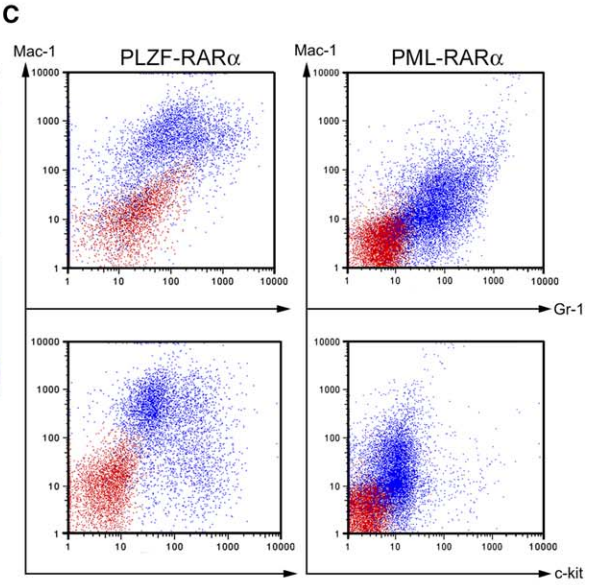
PML-RARα
MSCV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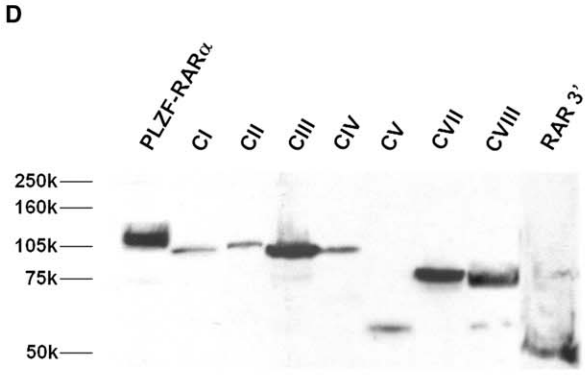
PLZF-RARα
PML-RARα
Gr-1
c-kit


D
PLZF-RARα
CI
CII
CIII
CIV
CV
CVII
CVIII
RAR 3'
250k
160k
105k
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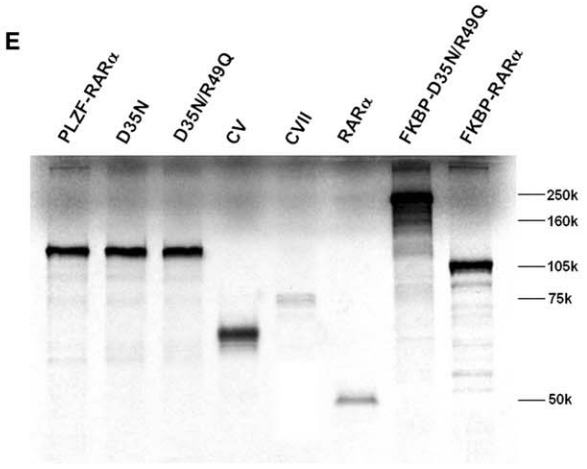
50k


E
PLZF-RARα
D35N
D35N/R49Q
CV
CVII
RARα
FKBP-D35N/R49Q
FKBP-RARα
250k
160k
105k
75k
50k

those have mainly exploited well-established cell lines such as U937 (Grignani et al., 1996, 1999; Lin and Evans, 2000; Ruthardt et al., 1997), which suffer from the pitfalls of carrying multiple nonrelevant genetic mutations that may not reflect the normal biology of the disease. To assess the impacts of oncoproteins on primary bone marrow cells, a retroviral transduction/transformation assay (RTTA), which has been widely used for various oncogenic transcription factors reported in human leukemia (Lavau et al., 1997; Slany et al., 1998; So et al., 2003a, 2003b), was employed to study RARα fusion proteins. Primary hematopoietic cells transduced with RARα fusions exhibited enhanced in vitro self-renewal and were capable of inducing APL-like diseases when transplanted into mice (Du et al., 1999; Minucci et al., 2002). These results not only reveal the primary impact of RARα fusion in promoting self-renewal but also provide a more physiologically relevant system to study the transformation property of RARα fusion.

To gain further mechanistic insights into the transformation mechanisms mediated by RARα fusion proteins, we performed detailed structure/function analysis using RTTA and identified the POZ homo-oligomerization domain as the minimal transformation domain for PLZF-RARα-mediated transformation of primary hematopoietic cells. We also provide experimental evidence showing that forced homo-oligomerization of RARα can mimic various biochemical properties of bona fide RARα fusion proteins and transform primary hematopoietic cells in an ATRA-dependent manner. Taken together, our results endorse the importance of a homo-oligomerization-dependent mechanism for RARα-mediated leukemogenic transformation.

Results

The POZ domain is the minimal transformation domain for PLZF-RARα-mediated transformation of primary hematopoietic cells

While we and others have shown previously that the POZ domain is critical for homo-oligomerization (formation of complexes with two or more identical subunits) and transcriptional activities of PLZF-RARα, its functional significance in transformation of primary hematopoietic cells has not been demonstrated (Dong et al., 1996; Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Hong et al., 1997; Lin et al., 1998). To this end, the ability of PLZF-RARα to transform primary bone marrow cells was assessed using RTTA as previously described (Du et al., 1999; Minucci et al., 2002; So et al., 2003a, 2003b). PML-RARα, which only has homo-oligomerization domains but not additional SMRT interaction domains was also used as a control in the experiment. In contrast to primary bone marrow cells transduced with MSCV vector alone that exhausted their self-renewal ability in the second round of plating, PLZF-RARα- or PML-RARα-transduced cells were able to form compact colonies in the third round of plating (Figure 1A). Morphological and immunophenotypic analysis of third round colonies from both PLZF-RARα and PML-RARα were consistent with early myeloid progenitors (c-kit^{lo}, Mac-1^{+}, Gr-1^{+}, B220^{-}, CD3^{-}), indicating that RARα oncoproteins could enhance self-renewal and partially arrest transduced cells at an early myeloid progenitor stage (Figures 1B and 1C and data not shown). It is interesting to note that, although they had similar cellular morphology and immunophenotype, the number of colonies from PLZF-RARα-transformed cells was significantly higher than the number of colonies from cells transformed with PML-RARα, suggesting that PLZF-RARα may be a more potent oncoprotein in conferring self-renewal ability to affected cells, which may correlate with its enhanced ability to associate with corepressor complexes (e.g., SMRT) (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Hong et al., 1997; Lin et al., 1998). These observed transformations were also dependent on the functional contributions from the PLZF or PML portions as cells transduced with a RARα 3′ construct encoding the truncated form of RARα failed to give raise third round colonies (Figure 1A). To identify the domain required for the enhanced in vitro self-renewal, the transformation ability of a series of deletion mutant constructs targeting different regions of PLZF (including the POZ domain that overlaps with transcriptional repression domain 1 [RPD1], proline-dependent phosphorylation sites, transcriptional repression domain 2 [RPD2], two individual Zn fingers) was assessed using RTTA (Figure 1A). Cells transduced with constructs (CI-CV) containing intact POZ/RPD1 domain consistently generated third round colonies, suggesting that the proline-dependent phosphorylation sites, PRD2, and both Zn fingers were dispensable for the in vitro transformation of primary hematopoietic cells. Conversely, constructs lacking the POZ domain (CVII, CVIII) failed to transform, indicating that an intact POZ domain is essential for the enhanced self-renewal (Figure 1A). The appropriate expression of each construct was confirmed by both Western blot and coupled in vitro transcription/translation assays (Figures 1D and 1E). The contrasting transformation results from CV (minimal POZ) and CVII (deletion of POZ) constructs suggested that the POZ domain is the minimal transformation domain required for enhanced in vitro self-renewal of primary hematopoietic cells.

The POZ domain is required for homo-oligomerization, high-affinity association with SMRT, and formation of high-molecular weight transcriptional complexes

To further characterize the biochemical properties of the *PLZF-RARα* constructs, their ability to form homo-oligomers and stable, high-molecular weight protein complexes and to interact with the nuclear receptor transcriptional corepressor (SMRT)

Figure 1. The POZ domain is the minimal transformation domain for PLZF-RARα-mediated transformation of primary hematopoietic cells

A: Schematic diagram of PLZF-RARα, PML-RARα, and the retroviral constructs used in RTTA (left). POZ, the POZ domain; RPD, repression domain; Z, zinc finger; C3HC4, RING finger motif; B1 and B2, Cys-rich B box; coiled coil, coiled coil dimerization domain. The bar chart (right) represents the corresponding numbers of colonies after each round of plating in methylcellulose. Error bars indicate standard deviations (SD).

B: Typical morphology (upper panel) and histological staining (lower panel) of third round colonies generated from bone marrow cells transduced with retroviruses expressing transforming construct (e.g., wild-type PLZF-RARα and PML-RARα) or nontransforming construct (e.g., MSCV alone). Scale bars in upper panel and lower panel are 100 μm and 10 μm, respectively.

C: Phenotypic analysis of cells transformed by PLZF-RARα or PML-RARα after the third plating. Blue profiles represent stainings obtained with antibodies specific for the indicated cell surface antigens. Red profiles represent unstained controls.

D: Western blot using anti-RARα antibody showed similar level of expression of expected size proteins from each construct.

E: Appropriate protein expression from constructs used for GST pull-down was confirmed by coupled in vitro transcription/translation assay.

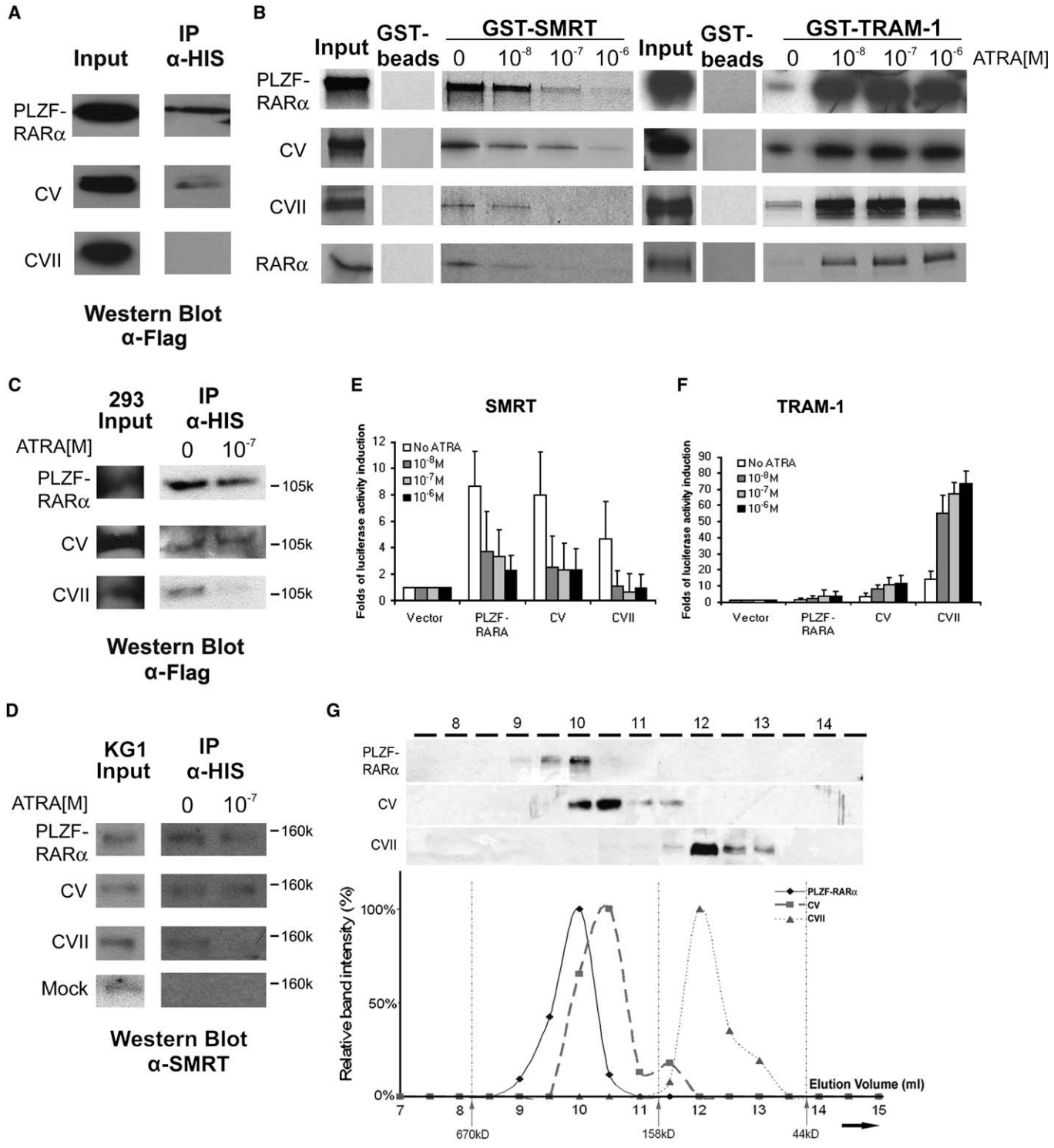


Figure 2. The POZ domain is required for homo-oligomerization, high-affinity association with SMRT, and formation of high-molecular weight complex by PLZF-RARα

A: Cell lysates were immunoprecipitated with anti-HIS antibody and Western blotted with anti-Flag antibody. Constructs used in cell transfection are listed in the left.

B: ^{35}S-labeled *PLZF-RARα* constructs indicated in the left were incubated with GST beads, GST-SMRT, or GST-TRAM-1 in the absence or presence of indicated amount of ATRA.

C: 293 cells cotransfected with Flag-tagged SMRT and HIS-tagged *PLZF-RARα* constructs were immunoprecipitated with anti-HIS antibody and Western blotted with anti-Flag antibody in the absence or presence of ATRA treatment. Constructs used for transfection are listed in the left.

D: KG1 cells transfected with Flag-tagged *PLZF-RARα* constructs were immunoprecipitated with anti-Flag antibody and Western blotted with anti-SMRT antibody in the absence or presence of ATRA treatment. Constructs used for transfection are listed in the left. Mock-transfected control was also indicated.

E and **F:** Mammalian two-hybrid assay using COS7 cells cotransfected with different pVP16(TA)-PLZF-RARα mutants in combination with GAL4-TK-luciferase reporter, Renilla luciferase internal control, and pGAL4-(DBD)-SMRT (**E**) or pGAL4-(DBD)-TRAM-1 (**F**). Transfected cells were cultured in DMEM supplement

and coactivator (TRAM-1) were studied. To assess their ability to form homo-oligomers, *PLZF-RARα* constructs were tagged with either Flag epitope or His epitope and cotransfected into 293 cells for immunoprecipitation assays. As a result, homo-oligomers were observed in both full-length PLZF-RARα and CV but not CVII mutant, confirming that the POZ domain is necessary and sufficient for mediating homo-oligomerization of PLZF-RARα oncoproteins (Figure 2A). Next, their ability to interact with transcriptional corepressor and coactivator was assessed using a GST pull-down assay with wild-type RARα as a control. In the absence of ATRA, PLZF-RARα was apparently most efficiently captured by the SMRT affinity beads, while CVII mutant and wild-type RARα lacking the POZ domain were least well bound, indicating that the POZ homo-oligomerization enhanced the binding to SMRT. When treated with increasing amounts of ATRA, CVII started to associate with TRAM-1 at 10^{-8} M ATRA and completely dissociated from SMRT at 10^{-7} M ATRA (Figure 2B). Conversely, although their association with TRAM-1 started at 10^{-8} M ATRA, the dissociation of SMRT from full-length PLZF-RARα and CV only occurred at 10^{-7} M and 10^{-6} M ATRA, respectively (Figure 2B). To confirm these interactions in vivo, both immunoprecipitation assays and a mammalian two-hybrid assay were employed. 293 cells cotransfected with Flag-tagged *SMRT* and different HIS-tagged *PLZF-RARα* constructs were immunoprecipitated with anti-HIS antibody and then subjected to Western blot using anti-Flag antibody. As expected, the specific band corresponding to SMRT protein was precipitated by anti-HIS antibody when cells were cotransfected with HIS-tagged *PLZF-RARα* constructs (Figure 2C). When treated with 10^{-7} M ATRA, SMRT dissociated from CVII but not from PLZF-RARα or CV, confirming the in vitro GST pull-down data. To demonstrate these interactions in leukemic cells, we transfected KG1 cells that express high level of SMRT with various Flag-tagged *PLZF-RARα* constructs for immunoprecipitation analysis using anti-Flag antibody. Endogenous SMRT protein was only precipitated in cells transfected with Flag-tagged *PLZF-RARα* construct but not in mock-transfected cells (Figure 2D). Consistently, endogenous SMRT completely dissociated from CVII but not from PLZF-RARα or CV in the presence of 10^{-7} M ATRA (Figure 2D), indicating strong corepressor binding associated with the POZ domain in leukemic cells context. To confirm these interactions in living cells, mammalian two-hybrid assays were performed using COS7 cells cotransfected with pGAL4(DBD)-SMRT or pGAL4(DBD)-TRAM-1 in combination with pVP16(TA)-PLZF-RARα mutants and pGAL4-TK-luciferase reporter. As a result, all PLZF-RARα mutants including CVII with weaker SMRT binding affinity can interact with SMRT and activate the reporter activity in vivo (Figure 2E). Both PLZF-RARα and CV resulted in around 8-fold activation, while CVII only had 5 folds as compared with vector control. When cells were treated with increasing amount ATRA, the SMRT interaction started to dissociate (Figure 2E). But both PLZF-RARα and CV could still maintain at least 2-fold activation even in the presence of 10^{-6} M ATRA. Conversely, the interaction between CVII and SMRT was very sensitive to ATRA treatment and was almost completely disrupted in the presence of 10^{-8} M ATRA (Figure 2E). On the other hand, the in vivo TRAM-1 association was far more prominent with CVII than with PLZF-RARα or CV (Figure 2F). CVII and TRAM-1 exhibited strong in vivo interaction and resulted in over 10-fold reporter activation, while PLZF-RARα and CV were only weakly associated with TRAM-1 and had activity just above (<2 folds) the basal level of the vector control (Figure 2F). In the presence of ATRA, CVII responded swiftly and resulted in almost 60-fold induction at 10^{-8} M ATRA and reached almost 80 folds in the presence of 10^{-6} M ATRA (Figure 2F). In contrast, PLZF-RARα was almost inert to the treatment and only had 3-fold activities in the presence of 10^{-6} M ATRA. In response to ATRA, CV had noticeable but mild induction and reached about 10-fold activation at 10^{-6} M ATRA (Figure 2F). Taken together, these results are consistent with the in vitro binding data indicating that the POZ oligomerization domain confers preferential binding ability to transcriptional corepressor (SMRT) over coactivator (TRAM-1) in an ATRA-dependent manner. Finally, we assessed the ability of these mutants to form stable high-molecular weight transcriptional complexes, which has been proposed as a critical feature for RARα oncoproteins (Lin and Evans, 2000; Minucci et al., 2000). Using gel filtration, we demonstrated that almost the entire population of full-length PLZF-RARα protein with apparent monomeric size of about 120 kDa was eluted at much higher-molecular weight fractions of about 400 kDa (Figure 2G). Similarly, CV with monomeric size of only about 65 kDa (Figure 1E) was capable of forming high-molecular weight complexes with slightly smaller estimated size, which probably reflects the reduced size of the mutant proteins. This suggest that both full-length PLZF-RARα and CV can form similar stable multiprotein transcriptional complexes, although a smaller subpeak with apparent molecular size similar to homodimers was only noticed in CV (Figure 2G). In marked contrast, almost the entire population of CVII protein lacking the POZ domain was eluted at fractions of apparent molecular weight less than 100 kDa (Figure 2G), which is consistent with the size of a monomer (Figure 1E). Taken together, these data indicated that the POZ domain is required for enhanced self-renewal, homo-oligomerization, efficient interaction with transcriptional coregulators, and formation of stable high-molecular weight complexes mediated by PLZF-RARα oncoprotein.

A POZ point mutant disrupting homo-oligomerization but not SMRT interaction results in loss of transformation potential

Complete removal of the POZ domain (CVII, CVIII) might affect the overall architecture and result in protein misfolding, which in turn could lead to the observed transformation and biochemical defects. Taking advantage of the X-ray crystallographic structure data available for the POZ domain of PLZF (Melnick et al., 2000, 2002), we made more sophisticated point mutants that maintain appropriate protein folding but selectively disrupt homo-oligomerization and/or repressor interaction. The integrity of the charged pocket in the POZ domain is critical for proper

with 10% charcoal/dextran-treated FCS in the absence or presence of indicated ATRA for 24 hr before cell lysis. Fruit fly luciferase activities normalized with Renilla luciferase activity were plotted as folds of induction compared with vector control-transfected cells. Error bars indicate standard deviations (SD).
G: Gel filtration analysis of PLZF-RARα mutants. Top panel indicates the Western blot results using anti-RARα antibody on protein fractions collected from indicated elution volumes (ml). Constructs used in the experiments are shown in the left. Bottom panel is the gel filtration chromatogram plotting elution volume (ml) against the relative band intensity of the detected proteins compared with the strongest protein band. Arrows indicate elution volumes of the corresponding size standard.

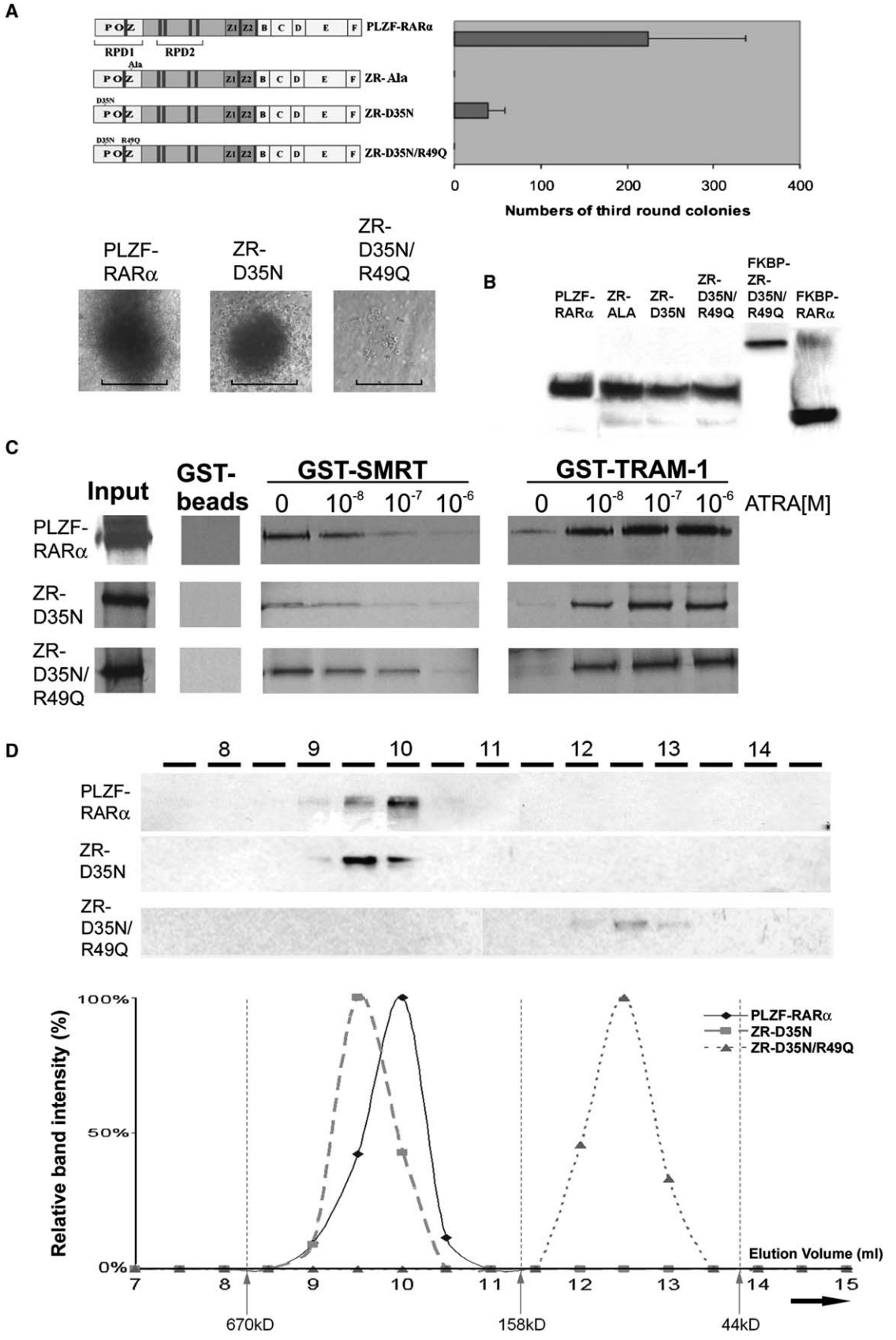


Figure 3. Self-association-defective point mutant can interact with corepressor but fails to enhance self-renewal of transduced primary hematopoietic bone marrow cells

folding and transcription repression (Melnick et al., 2000, 2002). In the context of PLZF, a double mutant carrying mutations of aspartic acid 35 to asparagine and arginine 49 to glutamine (D35N/R49Q) has proper folding but almost completely loses repression ability. Conversely, a D35N mutant carrying a single mutation of aspartic acid 35 to asparagine maintains most of the original properties of the wild type PLZF-RARα including transcription repression ability, albeit with an exception of slightly reduced ability to form homo-oligomer (Melnick et al., 2000, 2002). These mutants thus provided unique opportunities to study the functional requirement of homo-oligomerization in PLZF-RARα-mediated transformation. To this end, *PLZF-RARα* constructs carrying these mutations were made and assessed for their ability to transform primary hematopoietic progenitors, form homo-oligomers, high-molecular weight complexes, and interact with transcription corepressors and coactivators. In addition, an alanine PLZF-RARα mutant ($Ala^{48–52}$) that forms misfolded protein was also made and used as a negative control for the assay (Melnick et al., 2000, 2002). As expected, the misfolded Ala mutant was not able to transform primary cells (Figure 3A). Interestingly, the PLZF-RARα (D35N/R49Q) double mutant also lost its colony-forming ability in the third round of plating, although a similar number of colonies was obtained in the initial plating (Figure 3A and data not shown). Conversely, the PLZF-RARα (D35N) single mutant was still capable of forming compact third round colonies, although the numbers were significantly reduced (Figure 3A). The appropriate expression of each construct in packaging cells was confirmed by Western blot and coupled in vitro transcription/translation (Figure 3B and 1E).

To assess the biochemical properties of these mutants, GST pull-down revealed similar but distinctive in vitro binding properties of both D35N and D35N/R49Q mutants to SMRT and TRAM-1 (Figure 3C). While both mutants could associate with SMRT, the D35N/R49Q mutant had slightly higher binding affinity to SMRT than the D35N mutant that exhibited a weaker initial SMRT capture as compared with the wild-type PLZF-RARα, which may partially account for the weaker transformation ability exhibited by the D35N mutant (Figure 3C). In response to ATRA treatment, although both mutants started to associate with TRAM-1 at 10^{-8} M ATRA, significant dissociations of SMRT from D35N and D35N/R49Q mutants were only observed at 10^{-7} M and 10^{-6} M ATRA, respectively (Figure 3C). While the SMRT binding property of the D35N/R49Q mutant was different from the reported mutant in the context of PLZF, this finding is consistent with a recent report showing a strong corepressor binding ability by this D35N/R49Q mutant in the context of PLZF-RARα (Puccetti et al., 2005), suggesting that the corepressor binding is probably through the RARα portion or/and created as a result of novel protein folding when it is fused with RARα. When their ability to form high-molecular weight complexes was assessed, the D35N mutant displayed a very similar pattern as the wild-type PLZF-RARα with a single peak at about 400 kDa, while the D35N/R49Q mutant protein was almost exclusively eluted in the fractions with apparent molecular size smaller than 120 kDa, although a very small fraction of high-molecular weight complex was observed under long exposure (Figure 3D and data not shown). Taken together, these results further indicate that formation of stable high-molecular weight complexes via the homo-oligomerization domain is critical for cellular transformation mediated by PLZF-RARα.

Forced oligomerization of RARα rescues the transformation phenotype of the PLZF-RARα D35N/R49Q mutant

To ascertain the role of homo-oligomerization in PLZF-RARα-mediated transformation, we tested if resurrection of the oligomerization ability of the PLZF-RARα D35N/R49Q mutant (FKBP-D35N/R49Q) by a potent synthetic oligomerization module FKBP (M_{36}) could rescue the transformation phenotype. Both the biochemical and transformation properties of FKBP-D35N/R49Q mutants were assessed. Analogous to the original PLZF-RARα D35N/R49Q mutant, FKBP-D35N/R49Q associated with TRAM-1 at 10^{-8} M ATRA and dissociated from SMRT at 10^{-6} M ATRA (Figure 4A). Conversely, FKBP conferred strong homo-oligomerization ability to the otherwise self-association-deficient PLZF-RARα D35N/R49Q mutant. FKBP-D35N/R49Q could efficiently self-associate in the immunoprecipitation assay where PLZF-RARα D35N/R49Q was compromised in forming homo-oligomers (Figure 4B). Similarly, high-molecular weight complexes reappeared in gel filtration analysis when FKBP was fused to the otherwise monomeric FKBP-D35N/R49Q mutant (Figure 4C). Since the apparent molecular size of a monomeric FKBP-D35N/R49Q mutant is about 200 kDa (Figure 1E), the two major high-molecular weight peaks with apparent molecular sizes of about 550 kDa and 800 kDa are comparable to the expected size of homodimers and homotetramers, respectively, suggesting that FKBP-D35N/R49Q may associate with more heterogeneous homo-oligomeric high-molecular weight complexes, which has also been noticed in CV (Figure 2G). Taken together, these findings suggest that FKBP significantly enhanced the ability of D35N/R49Q to self-associate and form stable high-molecular weight complexes without dramatically affecting other biochemical properties. Before these constructs were tested in RTTA for their ability to transform primary hematopoietic cells, their expression was also confirmed by Western blotting and coupled in vitro transcription/translation assay (Figures 3B and 1E). In contrast to the PLZF-RARα D35N/R49Q mutant that failed to sustain replating after the second round of plating, cells transduced with FKBP-D35N/R49Q were capable of forming a significant number of third round compact colonies with morphology similar to that of wild-type

A: Schematic diagram of *PLZF-RARα* constructs used in RTTA (left). ZR, PLZF-RARα. The bar chart (right) represents the corresponding numbers of colonies in the third round plating. Error bars indicate standard deviations (SD). Pictures showed typical morphology of the third round colonies from bone marrow cells transduced with retroviruses expressing the indicated construct. Scale bars, 100 μm.

B: Western blot using anti-RARα antibody showed similar level of expression of expected size proteins from each construct in packaging cells.

C: ^{35}S-labeled *PLZF-RARα* constructs indicated in the left column were incubated with GST beads, GST-SMRT, or GST-TRAM-1 in the absence or presence of indicated amount of ATRA.

D: Gel filtration analysis. 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 panel is the gel filtration chromatogram plotting elution volume (ml) against the intensity of the detected proteins with arrows indicating the elution volumes of the corresponding size standard.

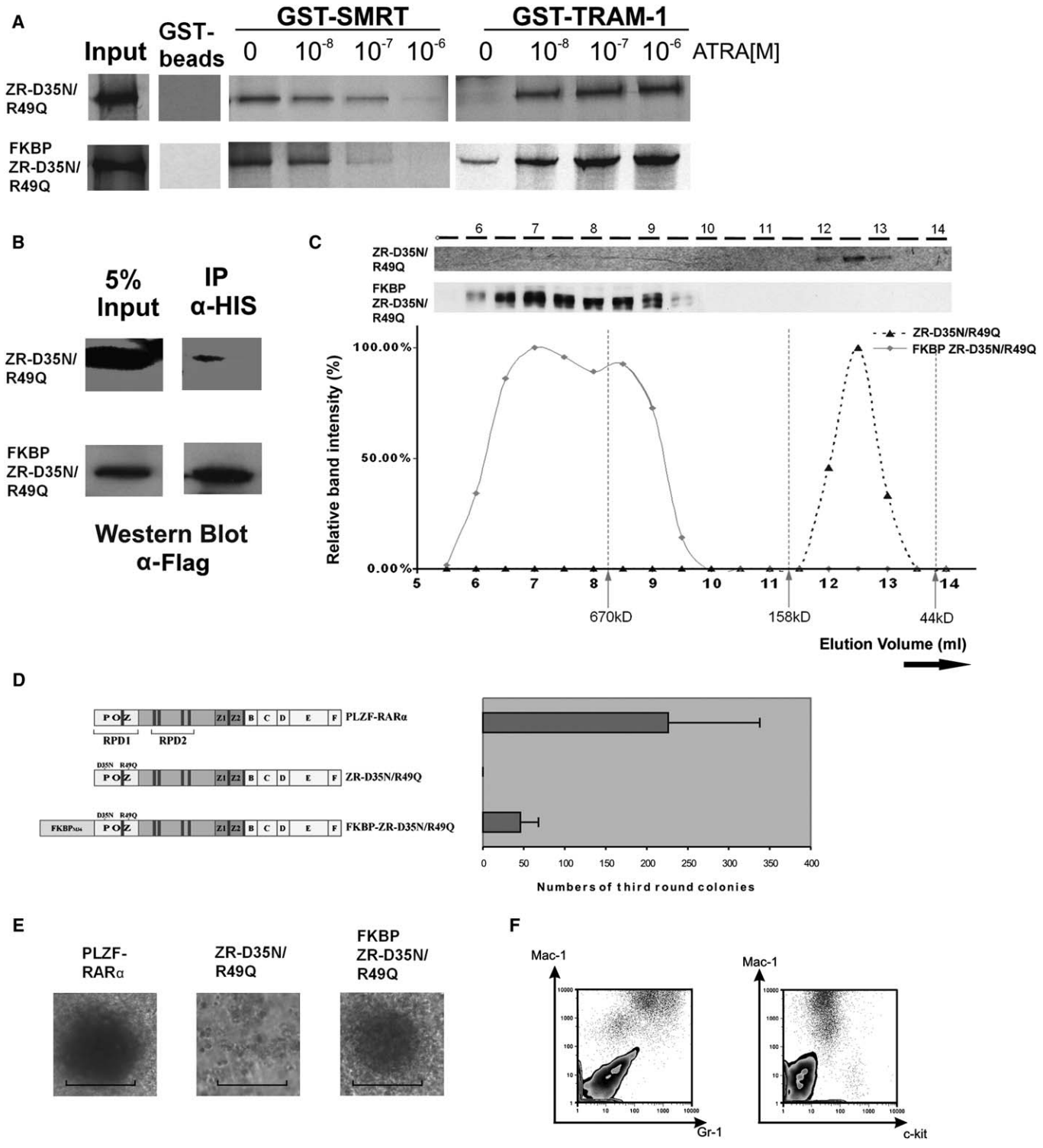


Figure 4. Rescue of transformation phenotype of self-association-defective point mutant by synthetic FKBP homo-oligomerization module

A: ^{35}S-labeled *PLZF-RARα* mutant constructs indicated in the left were incubated with GST beads, GST-SMRT, or GST-TRAM-1 in the absence or presence of indicated amount of ATRA. The same ZR-D35N/R49Q panel from Figure 3C was used in this figure for comparison, as these experiments were performed at the same time. ZR, PLZF-RARα.

B: Lysates extracted from 293 cells transfected with constructs shown in the left were immunoprecipitated with anti-HIS antibody and Western blotted with anti-Flag antibody.

C: Gel filtration analysis indicates reappearance of high-molecular weight complexes associated with FKBP-PLZF-RARα D35N/R49Q mutant. 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 panel is the gel filtration chromatogram plotting elution volume (ml) against the relative band intensity of the detected proteins with arrows indicating the elution volumes of the corresponding size standard.

PLZF-RARα-transformed cells in spite of being fewer in number (Figures 4D and 4E). To further characterize the FKBP-D35N/R49Q-transformed cells, FACS analysis revealed immunophenotypes of myeloid precursor (c-kit^{lo}, Mac-1^{+}, Gr-1^{+}) that were very similar to the wild-type PLZF-RARα-transformed cells (Figures 4F and 1C).

Forced oligomerization of RARα is sufficient to mimic bona fide chimeric RARα oncoproteins in transforming primary hematopoietic cells

The successful albeit partial rescue of the PLZF-RARα D35N/R49Q mutant by chimeric fusion with the FKBP oligomerization domain further endorses a key role of homo-oligomerization in RARα-mediated transformation. To this end, we tested if forced homo-oligomerization of RARα itself would be sufficient to transform hematopoietic progenitors in vitro. A *FKBP-RARα* synthetic construct was made by fusing FKBP directly to the 5′ truncated RARα, which was assessed for both its biochemical and transformation properties. FKBP-RARα was capable of forming strong homo-oligomers (Figure 5A) and stable high-molecular weight complexes with a major peak of apparent molecular weight of about 250 kDa (Figure 5B). FKBP-RARα also interacted with SMRT and TRAM-1 in an ATRA-dependent manner with similar or even slightly stronger binding affinities compared with wild-type PLZF-RARα (Figure 5C). These results indicate that FKBP-RARα closely mimics various biochemical properties of bona fide RARα oncoproteins.

When FKBP-RARα was expressed (Figures 1E and 3B) and tested in RTTA, it was capable of enhancing self-renewal of transduced primary hematopoietic cells and formed compact colonies in the third round of plating (Figure 5D). Consistently, the FKBP-RARα-transformed cells had very similar colony morphology and immunophenotypes to those of PLZF-RARα-transformed cells and were positive for Mac-1, Gr-1, and c-kit but negative for CD3 and B220 (Figures 5E and 5F and data not shown). To demonstrate the FKBP-RARα-mediated transformation acting on the same or similar pathways as the bona fide RARα fusion proteins, FKBP-RARα-transformed cells were treated with ATRA to see if the transformation could be reversed by intervening the retinoid pathways, which are major characteristics associated with RARα fusion-mediated transformation. As a control, cells transformed by a non-APL oncoprotein, E2A-PBX, were also included in the assay. In contrast to E2A-PBX-transformed cells that were refractory to ATRA treatment, the numbers of compact colonies after the third round of plating were significantly reduced when FKBP-RARα cells were treated with 10^{-8} M ATRA (Figure 5D). The size of the residual colonies after the ATRA treatment was also substantially smaller than the untreated counterpart (Figure 5F). The colony numbers further reduced with increasing dosage of ATRA and were almost completely eliminated when the concentration of ATRA reached the pharmaceutical dose of 10^{-6} M (Figure 5D). Taken together, these data indicate that forced homo-oligomerization of RARα can mimic various biochemical properties of bona fide RARα fusion proteins and transform primary hematopoietic cells in an ATRA-dependent manner.

Finally, we also investigated if homo-oligomerization is required for maintenance of transformation phenotype by FKBP-RARα. To this end, we tried to reverse the transformation by dissociating homo-oligomerization in cells transduced with FKBP-RARα using the FKBP homo-oligomer inhibitor drug AP21998. The effectiveness of AP21998 drug to dissociate FKBP-mediated homo-oligomerization was demonstrated by immunoprecipitation assay (Figure 5A) and further confirmed by gel filtration analysis where FKBP-RARα was eluted as mainly a monomeric fraction at around 100 kDa upon 1 μM AP21998 treatment (Figure 5B). To assess the effect of the drug on cell transformation, FKBP-RARα-transduced cells after the first plating were split into two, and half of them were treated with two consecutive rounds of AP21998. PLZF-RARα-transduced cells were used as a control for specificity and general toxicity of the drug in the assay. As a result, almost identical numbers of colonies were obtained in PLZF-RARα-transduced cells with or without the drug, indicating that the level of drug used was not generally toxic to cells (Figure 5D). Conversely, the numbers of third round colonies were significantly reduced when FKBP-RARα-transformed cells were treated with the drug (Figure 5D). Although complete inhibition had not be achieved, probably due to the poor accessibility of the drug in methylcellulose to the affected cells, the significant reduction of the transformed colony by AP21998 suggests a critical function of homo-oligomerization in maintaining the cellular transformation phenotypes.

Discussion

While forced homo-oligomerization is a well-known oncogenic mechanism to activate protein tyrosine kinases in chronic leukemias, myeloproliferative syndromes, and other malignances, its functional relevance in activating oncogenic transcription factors has not been clearly established (So and Cleary, 2004). We and others have recently shown that homo-oligomerization contributes to oncogenic activation of MLL in acute leukemias, where MLL chimeras are believed to act as constitutive transcriptional activators that aberrantly maintain expression of downstream target genes (Eguchi et al., 2004; Martin et al., 2003; So et al., 2003b). Here, we extend this finding to an opposite class of oncogenic transcription factors (e.g., RARα) with constitutive transcription repression activity. In contrast to MLL chimeras, RARα oncoproteins recruit transcriptional corepressor complexes that suppress expression of target genes, which are critical for self-renewal, cell differentiation, and/or apoptosis (Zelent et al., 2001). To understand the molecular mechanisms leading to oncogenic conversion of RARα by fusion proteins, we showed that the POZ homo-oligomerization domain of PLZF is necessary and sufficient when fused with RARα to enhance self-renewal and inhibit differentiation of primary

D: Schematic diagram of *PLZF-RARα* constructs used in RTTA (left). The bar chart (right) represents the corresponding numbers of colonies after the third round of plating in methylcellulose. Error bars indicate standard deviations (SD).

E: Typical morphology of the third round colonies generated from bone marrow cells transduced with retroviruses expressing indicated construct. Scale bars, 100 μm.

F: Phenotypic analysis of cells transformed by FKBP-PLZF-RARα D35N/R49Q after the third plating. Dotted profiles represent stainings obtained with antibodies specific for the indicated cell surface antigens. Zebra-plotted profiles represent unstained control.

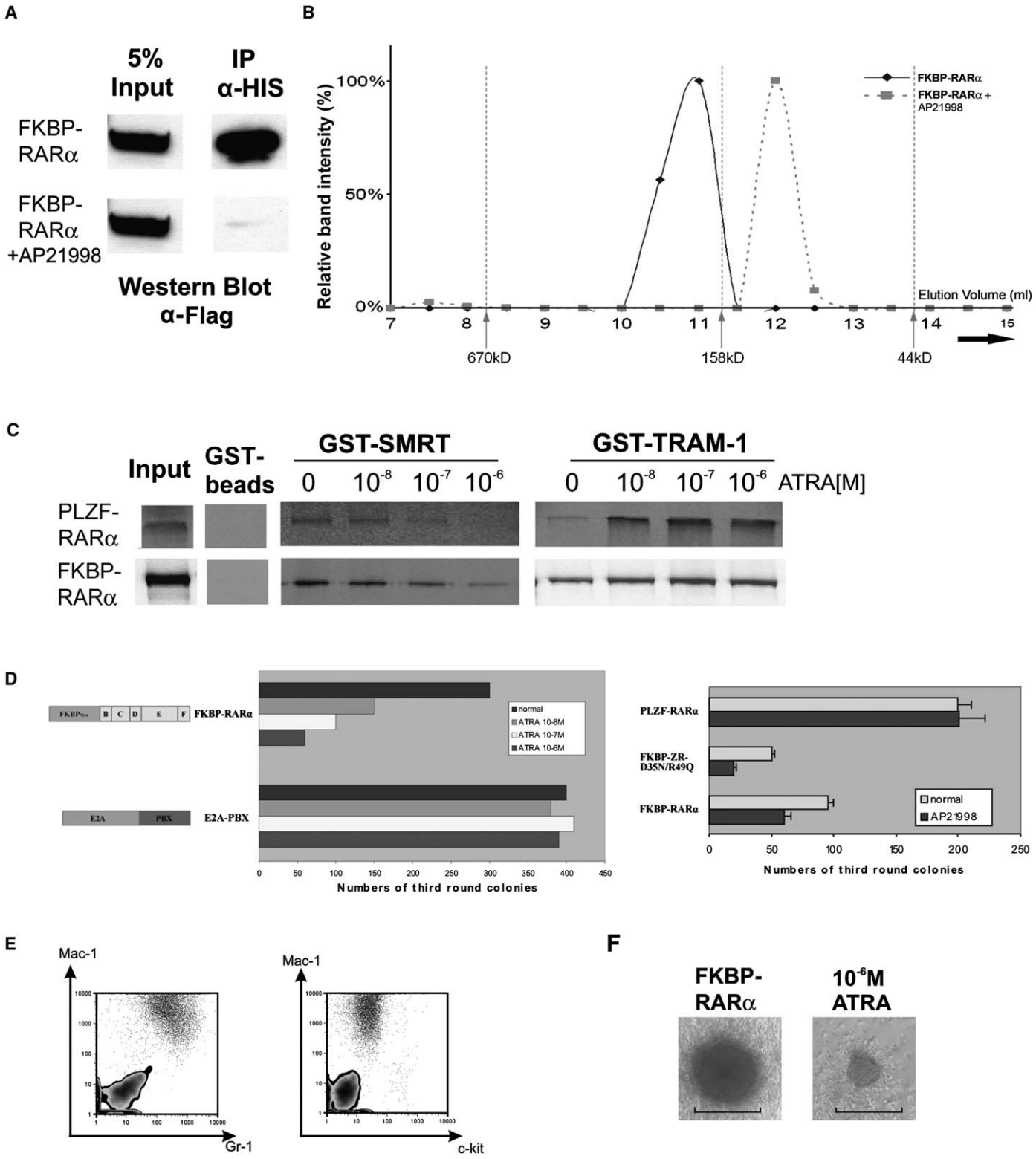


Figure 5. Forced homo-oligomerization of RARα results in transformation of primary myeloid progenitors in an ATRA-dependent manner

A: Lysates extracted from 293 cells transfected with constructs shown in the left were immunoprecipitated with anti-HIS antibody and Western blotted with anti-Flag antibody.

B: Gel filtration analysis of FKBP-RARα in the absence or presence of AP21998. Gel filtration chromatogram plotted elution volume (ml) against the intensity of the specific detected band with arrows indicating elution volumes of the corresponding size standard.

C: ^{35}S-labeled *RARα* mutant constructs indicated in the left were incubated with GST beads, GST-SMRT, or GST-TRAM-1 in the absence or presence of indicated amount of ATRA.

hematopoietic cells. Restoration of the homo-oligomerization capacity to the PLZF-RARα D35N/R49Q point mutant rescues the transformation phenotype, indicating that homo-oligomerization is an integral part of PLZF-RARα-mediated transformation. To this end, we further demonstrated that synthetic homo-oligomerization of truncated RARα (FKBP-RARα) mimics many biochemical properties of bona fide RARα oncoproteins and is sufficient to induce differentiation block on primary myeloid precursors. This transformation can also be partially reversed by either the specific homo-oligomerization inhibitor drug AP21998 or retinoic acid treatment. Collectively, these results emphasize a critical functional link between homo-oligomerization and transformation of primary hematopoietic cells by RARα oncoproteins.

Homo-oligomerization is a widely used and crucial mechanism for regulation of various normal biological processes from enzymatic activation and signal transduction to gene expression (Marianayagam et al., 2004); however, its precise contributions to the mode of action of chimeric transcription factors remain uncertain. Homo-oligomerization can not only enhance binding ability to existing interacting proteins/cofactors and downstream target genes, but also create novel binding interfaces to new target proteins and DNA sequences (Marianayagam et al., 2004). It is interesting to note that the transformation-defective PLZF-RARα D35N/R49Q point mutant exhibited in vitro binding properties similar to those of transcriptional corepressor SMRT and transcriptional coactivator TRAM-1 as compared with the rescued FKBP D35N/R49Q mutant, suggesting that recruitment of other complex components that were deficient in the PLZF-RARα D35N/R49Q mutant may be required for cellular transformation. In addition, the aberrant recruitment of critical components of transcriptional machinery by RARα oligomers may also have dominant-negative effects on other pathways that heavily rely on some of the limiting transcriptional cofactors (Dong et al., 1996, 2003; Dong and Tweardy, 2002; Lin and Evans, 2000; Minucci et al., 2000; Perez et al., 1993; Redner et al., 2000; So et al., 2000). Besides, the ability of oligomerization to increase the binding affinity of RARα to a broader range of DNA sequences may also result in deregulation of other downstream target genes. This is consistent with a recent study showing that PML-RARα oligomers not only have higher binding affinity to known retinoic acid response elements (RAREs) but also possess strong binding ability to a large number of other target sequences (Kamashev et al., 2004), suggesting that the gain of function via homo-oligomerization of RARα leading to deregulation of novel target genes may be critical for transformation. Thus, homo-oligomerization may act as a universal oncogenic amplifier that can not only enhance and/or alter the transcriptional properties of oncogenic transcription factors by both augmenting and broadening the binding affinity to interacting proteins and target DNA sequences, but also exert dominant-negative effects on other critical pathways. Future studies to identify transcriptional complexes and DNA binding selectivity associated with homo-oligomerized RARα will shed significant insights into these issues.

Previous studies showed that forced homo-oligomerization of truncated MLL by synthetic modules contributes but is not sufficient to induce rapid leukemic onset in vivo, which probably is due to the lack of additional functions contributed by the bona fide partners (Martin et al., 2003; So et al., 2003b). We also anticipate that synthetic homo-oligomerization of truncated RARα per se, although transforming in vitro, may not have all the oncogenic functions of bona fide RARα fusion proteins. (1) Most of the RARα partners, including PML, PLZF, and NPM, have been reported to have tumor suppressor or growth-suppressive activities (Falini et al., 2005; Felicetti et al., 2004; Grisendi et al., 2005; McConnell et al., 2003; Pandolfi, 2001). Acquisition of oligomerization domains from these bona fide partners will have dominant-negative effects on the tumor suppressor functions of these partner proteins by sequestering them into different subcellular compartments (Pandolfi, 2001). (2) The oligomerization domains of certain RARα partners, including PLZF and Stat5b, have been shown to possess independent repressor interaction domains that can significantly enhance corepressor binding, which may also be responsible for their refractory response to pharmaceutical level of ATRA treatment (Grignani et al., 1998; Guidez et al., 1998; He et al., 1998; Lin et al., 1998; Maurer et al., 2002; Nakajima et al., 2001). In addition, critical domains, such as the sumoylation site recently reported in PML, that are important for Daxx repressor recruitment and transformation will be absent in the synthetic homo-oligomeric RARα fusion (Zhu et al., 2005). (3) Hetero-oligomers (e.g., PLZF-RARα with PLZF) mediated by the bona fide oligomerization domain may also have different ability to interact with other components of the transcriptional complexes (e.g., RXR) that are critical for transformation (Kamashev et al., 2004). (4) Some of the reciprocal RARα-X proteins (e.g., RARα-PLZF, RARα-PML) have been demonstrated to be necessary to efficiently induce APL in vivo (He et al., 2000; Pollock et al., 1999). The lack of all these critical components in the synthetic FKBP-RARα fusion will predict a weak oncogenic property, which is also consistent with the recent observation that transgenic mice expressing synthetic RARα-dimers (using p50 or FKBP homo-oligomerization domains) developed leukemia with reduced penetrance and extended latency (Sternsdorf et al., 2006, this issue of *Cancer Cell*). Thus, homo-oligomerization is a critical but probably not the only component for RARα-mediated leukemogenesis.

Taken together, our results indicate that forced homo-oligomerization of truncated RARα can mimic many biochemical and transformation properties of bona fide RARα oncoproteins. The absolute requirement of homo-oligomerization domain for in vitro transformation of primary hematopoietic cells and its ability to rescue the transformation-defective PLZF-RARα mutant suggest its essential function for leukemogenic transformation, which may represent a promising avenue for therapeutic intervention.

D: Schematic diagram of retroviral constructs used in RTTA (left). Bar chart at the left represents the corresponding numbers of colonies after the third round of plating in the absence or presence of ATRA as indicated. Right bar chart represents the corresponding numbers of colonies after the third round of plating in the absence or presence of AP21998. Error bars indicate standard deviations (SD).

E: Phenotypic analysis of cells transformed by FKBP-RARα after the third plating. Dotted profiles represent stainings obtained with antibodies specific for the indicated cell surface antigens. Zebra-plotted profiles represent unstained control.

F: Typical morphology of the third round colonies generated from bone marrow cells transduced with retroviruses expressing FKBP-RARα in the absence or presence of ATRA. Scale bars, 100 μm.

Experimental procedures

Constructs

Mammalian and in vitro expression plasmids encoding full-length and various mutants of *PLZF-RARα*, *PML-RARα*, *RARα*, and *RXRα* constructed in pSG5 or pcDNA4 vectors have been previously described (Dong et al., 1996; Melnick et al., 2000, 2002; So et al., 2000). Inducible homo-oligomerization *FKBP-RARα* or *FKBP-PLZF (D35N)-RARα*, *FKBP-PLZF (D35N/R49Q)-RARα* synthetic constructs were made by fusing four to eight tandem repeats of self-oligomerizing FKBP mutant ($FKBP_{36M}$) carrying Phe to Met mutation (Ariad Pharmaceuticals, Inc.) (http://www.ariad.com/regulationkits/) to the RARα or PLZF-RARα mutant cDNAs, respectively. Retroviral constructs were made by transferring the inserts from the above expression plasmids into MSCV-hygro or MSCV-puro vectors (So et al., 2003b). GST-SMRT and GST-TRAM-1 have been previously reported (Dong and Tweardy, 2002)

RTTA

RTTA was performed as previously described (So et al., 2003b). Briefly, viral supernatants were collected 60 hr after transfection of Phoenix cells and used to infect hematopoietic progenitors and stem cells that were positively selected for c-Kit expression by magnetic activated cell sorting (MACS). Donor cells were harvested from the bone marrows of 4- to 10-week-old wild-type C57BL/6 mice. After spinoculation by centrifugation at 500 × g for 2 hr at 32°C, transduced cells were plated in 1% methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 20 ng/ml stem cell factor (SCF) and 10 ng/ml each of IL-3, IL-6, and GM-CSF (R&D Systems, Minneapolis, MN) in the presence or absence of appropriate drug selection (i.e., 1 mg/ml hygromycin or 1 μg/ml puromycin). Replating was repeated every 7 days. For drug studies, transduced cells were split into two wells after second plating. The homo-oligomerization inhibitor drug AP21998 was added to a final concentration of 1–5 μM in half of the wells to assess the effect of the drug on colony formation. Alternatively, appropriate amounts of ATRA were added to half of the wells to assess the effect on transformation. Transformation results were determined from at least two independent experiments.

Phenotype analysis

Immunophenotypic analysis was performed by FACS using fluorochrome-conjugated monoclonal antibodies to c-Kit (2B8 clone), Mac-1 (M1/70 clone), Gr-1 (RB6-8C5 clone), B220 (RA3-6B2 clone), and CD3e (145-2C11 clone) (Pharmingen Inc. or eBiosciences Inc., San Diego, CA). Staining was generally performed on ice for 15 min. Cells were washed twice in staining medium and resuspended in 1 μg/ml propidium iodine (PI) before analysis using a FACSCalibur machine (Becton Dickinson Inc.). Dead cells were gated out by high-PI staining and forward light scatter.

In vitro and in vivo interaction assays

GST pull-down and immunoprecipitation studies were essentially performed as previously described (Dong and Tweardy, 2002; He et al., 1998; So et al., 2000, 2003b). The antibodies used in the immunoprecipitation assay were from Sigma-Aldrich (M2 anti-Flag antibody) and Santa Cruz Biotech (anti-His antibody, anti-SMRT antibody). Mammalian two-hybrid analyses were performed as previously described (Guidez et al., 1998). Briefly, 0.4 μg GAL4(DBD)-SMRT or GAL4(DBD)-TRAM-1 expression vector was cotransfected with (1) 0.1 μg pGAL4-TK-Luc reporter containing five consensus GAL4 binding sites and thymidine kinase promoter upstream of the fruit fly luciferase gene and (2) 0.1 μg pRL-CMV internal control with Renilla luciferase driven by a CMV promoter, in combination with (3) 0.4 μg different pVP16 constructs encoding potent VP16 transactivation domain fused to PLZF-RARα mutants. Transfected cells were treated with different concentrations of ATRA for 24 hr before analysis. The relative fruit fly luciferase values were normalized with Renilla luciferase activity and presented as folds of induction compared with pVP16 empty vector controls. Standard deviations were calculated from three independent experiments.

Gel filtration study

293 cells were transfected in 10 cm plates with 20 μg of expression plasmid encoding the analyzed proteins. Forty-eight hours posttransfection, nuclear extracts were prepared in a high-salt elution buffer (500 mM NaCl, 20 mM HEPES [pH 7.5], 0.5 mM EDTA, 0.1% Triton X-100, 2 mM DTT, 0.2 mM PMSF, and protease inhibitor cocktail from Sigma). Gel filtration was carried out using an $ÄKTA_{FPLC}$ system with Superdex 200 gel filtration column (1.6 × 60 cm; Amersham Pharmacia) preequilibrated with running buffer (0.05 M TrisCl [pH 7.6], 200 mM NaCl, 3 mM EDTA, 0.2 mM PMSF) at a flow rate of 0.5 ml/min. Collected fractions were concentrated using the Howe Gyro-Vap evaporator before being analyzed on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes for Western blot detection using anti-RARα C-20 polyclonal antibody (Santa Cruz Biotech).

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