Cancer Cell
Article

PML/RARα Targets Promoter Regions Containing PU.1 Consensus and RARE Half Sites in Acute Promyelocytic Leukemia

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DOI 10.1016/j.ccr.2009.12.045

SUMMARY

PML/RARα is of crucial importance in acute promyelocytic leukemia (APL) both pathologically and therapeutically. Using a genome-wide approach, we identified in vivo PML/RARα binding sites in a PML/RARα-inducible cell model. Of the 2979 targeted regions, >62% contained canonical PU.1 motifs and >84% of these PU.1 motifs coexisted with one or more RARE half (RAREh) sites in nearby regions. Promoters with such PU.1-RAREh binding sites were transactivated by PU.1. PU.1-mediated transactivation was repressed by PML/RARα and restored by the addition of all-*trans* retinoic acid (ATRA). Genes containing such promoters were significantly represented by genes transcriptionally suppressed in APL and/or reactivated upon treatment with ATRA. Thus, selective targeting of PU.1-regulated genes by PML/RARα is a critical mechanism for the pathogenesis of APL.

INTRODUCTION

Acute promyelocytic leukemia (APL) is pathologically characterized by the accumulation of promyelocytic blast cells in the bone marrow and blood and cytogenetically defined by a typical t(15;17) (q22;q21) translocation occurring in most of the patients. This translocation results in a fusion protein known as the promyelocytic leukemia-retinoic acid receptor α (PML/RARα), which is capable of blocking cell differentiation at the promyelocytic stage. Although the pathogenetic importance of PML/RARα in APL has been recognized for over a decade, the mechanism by which PML/RARα drives the development of APL is far from clear. This is largely due to the limited number of PML/RARα target genes identified to date and to the structural and functional complexity of PML/RARα.

The fusion protein retains the oligomerization domain of PML and the DNA binding and ligand binding domains of RARα. It exists in multiple complexes including PML/RARα homodimers, PML/RARα oligomers (containing the homodimers plus one or two RXR), and PML/RARα-RXR heterodimers (Jansen et al., 1995). PML/RARα possesses a high affinity for corepressor molecules such as nuclear receptor corepressor, silencing mediator of retinoic and thyroid hormone receptors, and histone deacetylases. Thus, it acts as a strong transcriptional repressor for its target genes, which are thought to include genes indispensable for myeloid differentiation (Lin et al., 2001). In the

Significance

PML/RARα contributes to the development of APL through repression of genes important in myeloid development. Although previous reports have suggested substantial crosstalk between PML/RARα and PU.1, there is little understanding of how this occurs. Through a global approach, we demonstrate that PML/RARα predominantly targets PU.1-regulated promoters through both protein-protein interaction and DNA binding via RARE half sites forming complex binding motifs. Genes with such promoters are involved in hematopoiesis and primarily regulated by PU.1. In addition, these PML/RARα targets are significantly enriched in genes transcriptionally suppressed in APL and/or reactivated after ATRA treatment. Thus, selective targeting of PU.1-regulated genes by PML/RARα represents a major mechanism of transcriptional repression occurring in APL and is essential for the observed differentiation block.

presence of pharmacological concentrations of all-*trans* retinoic acid (ATRA), the ligand for both RARα and PML/RARα, the corepressor molecules disassociate from PML/RARα, restoring expression of genes essential for myeloid differentiation and so inducing the accumulated promyelocytes to differentiate into neutrophils.

Since PML/RARα retains the DNA binding domain of RARα, it has been a long-standing assumption that PML/RARα might primarily bind to RARα binding sites and thus suppress RARα-regulated genes. However, this assumption has become less likely after the observation that myeloid commitment is hardly affected in various RAR-deficient mice (Collins, 2002). This observation implies that genes indispensable for myeloid differentiation are probably regulated by transcription factors other than RARα or any other tested retinoid receptors. Identification of PML/RARα target genes has been lagging far behind, largely due to the complexity of DNA sequences recognized by PML/RARα and to the lack of means to distinguish target genes of PML/RARα from those of RARα. For instance, heterodimers of RARα and RXR bind typically to canonical retinoic acid responsive elements (RAREs) composed of direct repeats (DR) of two [A/G]G[G/T]TCA [RARE half (RAREh)] sites with five (DR5) or two (DR2) random nucleotides in between. However, it is important to note that most of the PML/RARα targets reported to date lack canonical RAREs in their promoter regions, e.g., *UBE1L*, *C/EBPβ*, and *BLR1*. Moreover, in vitro binding studies of PML/RARα using a selection/amplification technique have revealed that PML/RARα may bind to RAREh sites arranged in different orientations and with widely variable spacing in between (Kamashev et al., 2004), implying that the DNA binding spectrum of this fusion protein is more complex and versatile than previously thought. Furthermore, under certain circumstances, PML/RARα is able to interact with many other transcription factors, such as AP-1, GATA2, and Sp1, providing the potential for the fusion protein to target genes primarily regulated by other transcription factors and thus adding additional complexity to the binding spectrum of PML/RARα. For example, PML/RARα is reported to act as a repressor of AP-1-dependent transactivation, which can be reversed by the addition of ATRA (Doucas et al., 1993). Similarly, GATA2-dependent gene transcription appears to be influenced by PML/RARα (Tsuzuki et al., 2000).

Identification of bona fide and relevant in vivo binding sites of PML/RARα is obviously a multifaceted issue that requires a global approach. In the current study, we performed ChIP-on-chip studies on a PML/RARα-inducible cell model that mimics cells immediately after the t(15;17) translocation and the expression of PML/RARα.

RESULTS

Identification of In Vivo Binding Regions of PML/RARα

The PR9 cell line, constructed from U937 myeloid precursor cells, is a PML/RARα-inducible model that provides a system for studying early events associated with de novo expression of PML/RARα. To identify chromatin DNA targeted by PML/RARα, PR9 cells were treated with 100 μM of $ZnSO_4$ for 4 hr, a condition known to eventually induce many features similar to those of APL (Grignani et al., 1993). Also, under this condition, the amount of PML/RARα induced was more abundant than that of the endogenous RARα (Figure 1A and Figure S1A, available online), which might help to minimize the potential interference resulting from RARα, since a RARα-specific antibody was used to pull down DNA bound by PML/RARα. The precipitated DNA was then amplified, labeled, and hybridized to Affymetrix Human Promoter 1.0R arrays. Based on three biological replicates, a total of 2979 highly significant ChIP regions (Table S1) were identified through a model-based analysis of tiling-array algorithm (Johnson et al., 2006). Those ChIP regions, with an average size of 829 bps, represent 1981 unique RefSeq genes. To validate the ChIP-on-chip findings, we performed ChIP quantitative PCR (ChIP-qPCR) on DNA samples, precipitated using either an anti-PML antibody or an anti-RARα antibody, before and after PML/RARα induction. The PCR was performed with 25 primer pairs corresponding to six previously known targets of PML/RARα or RARα and 19 randomly chosen regions from the ChIP population. As illustrated in Figure 1B, enrichment of all the tested ChIP regions was significantly higher than that of non-relevant regions in DNA samples precipitated by either the anti-RARα or the anti-PML antibody after PML/RARα induction, although the degree of enrichment varied from one ChIP region to another (top). In contrast, most of the tested ChIP regions (17/25) were not enriched (<3 folds) in DNA samples precipitated by the two antibodies before PML/RARα induction (Figure 1B, bottom). These results indicate that the ChIP regions identified in our ChIP-on-chip experiments are specifically targeted by PML/RARα. Interestingly, of the eight ChIP regions whose enrichments were higher than three folds in DNA samples precipitated by the anti-RARα antibody before PML/RARα induction, five corresponded to known targets of RARα, i.e., *RARβ2*, *ITGB2*, *IL1B*, *CEBPE*, and *CDKN2D*, implying that a relatively small portion of the PML/RARα ChIP regions are targeted by RARα before expression of PML/RARα.

To provide additional controls, we assessed the expression level of PML/RARα protein in primary APL blasts, APL-derived NB4 cells (Lanotte et al., 1991), and a set of PR9 cell samples treated with varying concentrations of $ZnSO_4$, and we also evaluated ChIP enrichment in the cells with graded amounts of PML/RARα through ChIP-qPCR. As shown in Figure 1C, the amount of PML/RARα protein in primary APL samples fluctuated over a relatively wide range, as compared to NB4 cells (left panel). Meanwhile, the amount of PML/RARα protein in NB4 cells was approximately equivalent to that in PR9 cells treated with 50 μM to 75 μM of $ZnSO_4$ (Figure 1C, right panel). In ChIP-qPCR assays (Figure S1B), it was clearly shown that most of the tested ChIP regions were enriched even in PR9 cells treated with 50 μM of $ZnSO_4$, in which the amount of PML/RARα was obviously lower than that in NB4 cells. These results suggest that the amount of PML/RARα induced by 100 μM of $ZnSO_4$ in PR9 cells for 4 hr is biologically relevant, allowing the fusion protein to recognize bona fide binding sites upon its expression in hematopoietic cells.

Identification of Significantly Enriched Binding Sites in the PML/RARα ChIP Regions

We used three independent and complementary approaches to identify significantly enriched transcription factor binding

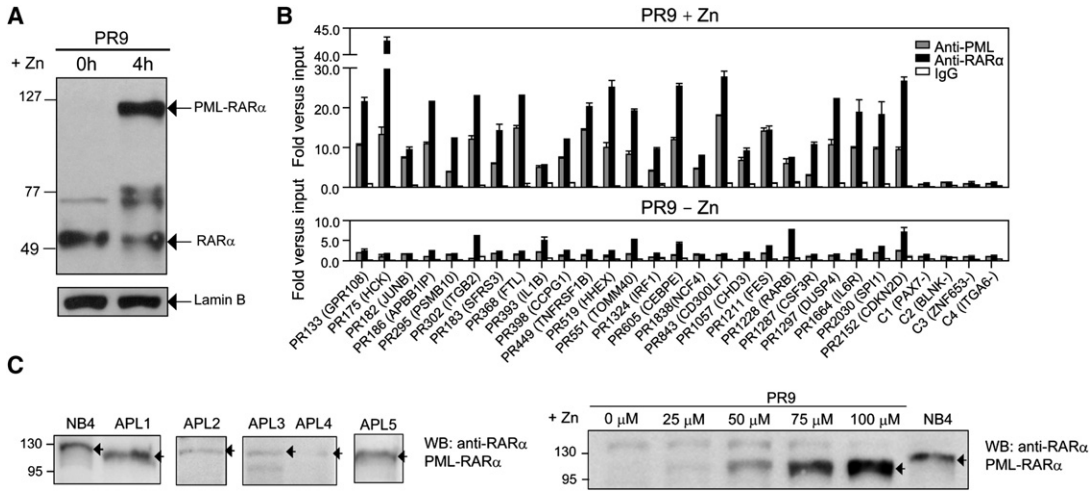

Figure 1. Expression of PML/RARα Protein in PR9, NB4, and Primary APL Cells, and Validation of the PML/RARα ChIP Regions

(A) Induction of the PML/RARα protein in $ZnSO_4$-treated PR9 cells. Western blot analysis was performed on PR9 cells treated with 100 μM $ZnSO_4$ for 4 hr and untreated PR9 cells. A time series induction of the PML/RARα protein by $ZnSO_4$ is shown in Figure S1A.

(B) Validation of the PML/RARα ChIP regions by ChIP-qPCR assays. ChIP-qPCR was performed on PR9 cells with (top panel) or without (bottom panel) $ZnSO_4$ treatment. Codes for individual ChIP regions are marked underneath and corresponding RefSeq genes are indicated inside the brackets. Negative controls are marked as C1 to C4. Data are shown as fold enrichment of ChIPed DNA versus input DNA. Error bars represent SD of triplicate measurements. See Table S1 for a full list of the PML/RARα ChIP regions.

(C, Left) Comparison of the amount of PML/RARα protein between NB4 cells and primary APL blasts (APL1 to APL5). (Right) Dose-dependent induction of PML/RARα protein in PR9 cells as compared to endogenous PML/RARα in NB4 cells. PR9 cells were treated with increasing concentrations of $ZnSO_4$ (25 μM, 50 μM, 75 μM, or 100 μM) for 4 hr. Western blots were performed using the anti-RARα antibody. The position of PML/RARα protein is indicated by arrowheads. ChIP-qPCR data obtained from PR9 cells treated with the same graded amounts of $ZnSO_4$ are shown in Figure S1B.

sites (TFBSs) within the PML/RARα ChIP regions. First, an enumeration-based de novo motif discovery approach (details in the Supplemental Experimental Procedures) was applied to uncover consensus motifs with statistical significance. Under a stringent cutoff (Z score > 10) (Xie et al., 2007), a series of subsequences were identified (Table S2). By comparing these subsequences with known TFBSs, we found that consensus binding sites for PU.1, ETS, and AP-1 were highly significant (Figure 2A, a). Second, 554 position weight matrices of known TFBSs from the TRANSFAC database were used to scan the ChIP regions. The matrices V$PU1_Q6, V$ETS_Q6, and V$AP1_C ranked at the top of the significant motifs (Figure 2A, b, and Table S3). Third, in view of the fact that position weight matrices of canonical RAREs (DR5 or DR2) were not included in the TRANSFAC database, we performed a direct scanning for such motifs in the ChIP regions. Canonical RAREs (DR5 plus DR2) even with one random mismatch, however, appeared to be at a relatively low frequency (13.26%) with a Z score of 9.28 (Figure 2A, c, and Table S4). In contrast, 81.97% of the ChIP regions contained at least one RAREh site (Z score of 18.23) (Figure 2A, c). Most of these RAREh-containing ChIP regions (64.21%) had two or more RAREh sites with different orientations and variable spacing in between ranging from a few nucleotides to many bases (Tables S1 and S4). These results appear to be consistent with previous findings that PML/RARα binds to RAREh sites in complex and versatile combinations (Kamashev et al., 2004; Meani et al., 2005).

Since PU.1 motifs represented the most significant and abundant binding sites next to RAREh sites in the ChIP regions, and since the vast majority of PU.1 motif-containing segments (84.24%) had one or more RAREh sites (Figure 2B), we assessed whether the coexistence of PU.1 consensus and RAREh sites (PU.1-RAREh) within the ChIP regions (an average size of 829 bp) was of statistical significance. As demonstrated in Figure 2C, the coexistence of PU.1 and RAREh sites, as identified by the ChIP-on-chip analysis of PML/RARα, was highly significant (Z score = 29.40). In contrast, a ChIP-on-chip analysis of PU.1 under the same condition revealed a moderate enrichment (Z score = 5.66) of RAREh sites in the PU.1 ChIP regions (Figure 2D). When the PU.1 ChIP regions were further divided into those shared with the PML/RARα ChIP regions and those specific to the PU.1 ChIP regions, the former, but not the latter, revealed a significant enrichment of RAREh sites (Z score: 11.34 versus −4.22; Figure 2D). These observations suggest that RAREh sites may provide a mechanism for PML/RARα to selectively target and modify PU.1-regulated genes.

Selective Recruitment of PML/RARα to PU.1-Bound Chromatin Regions after PML/RARα Induction

Since PU.1-RAREh sites were highly enriched in the PML/RARα ChIP regions, we questioned whether PU.1-regulated gene transcription could be influenced by PML/RARα on a large scale. To test this hypothesis, we first determined whether PU.1 and PML/RARα were co-associated with chromatin corresponding to the ChIP regions harboring PU.1-RAREh motifs. As demonstrated in ChIP-qPCR assays (Figure 3A), all of the tested PU.1-RAREh ChIP regions were significantly enriched in DNA samples, precipitated either by the anti-RARα or the anti-PU.1 antibody from $ZnSO_4$-treated PR9 cells, implicating the coexistence of

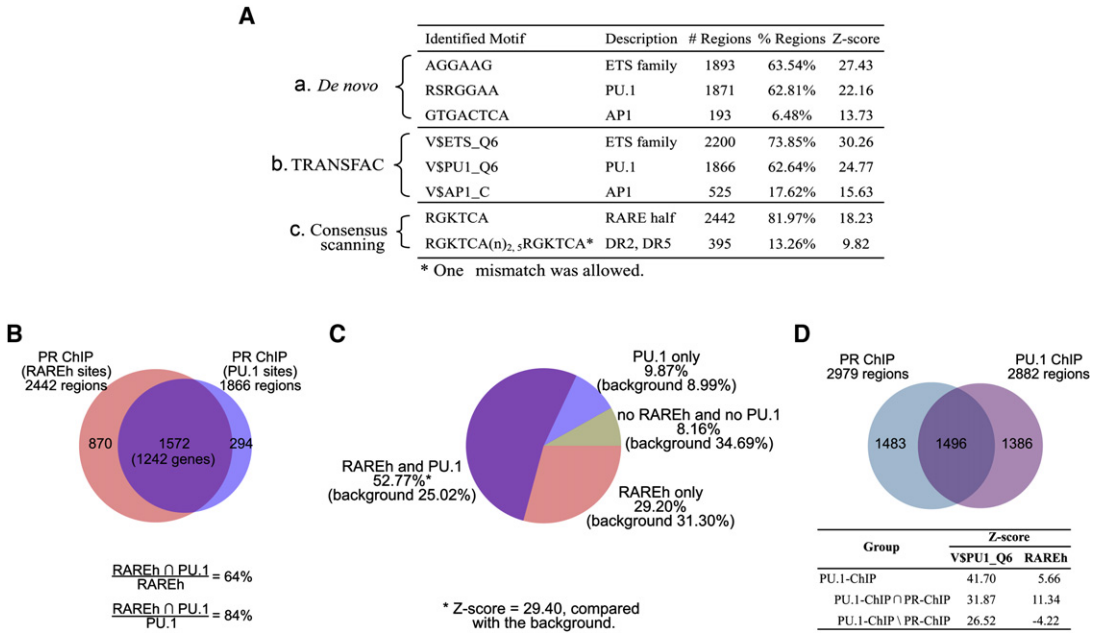

Figure 2. Identification of the Significant Motifs

(A) Motif discovery of the PML/RARα ChIP regions by three independent methods. The most significant motifs identified using de novo motif analysis and the TRANSFAC database are shown in (a) and (b), respectively. The lists of all significant motifs identified using the above two methods are provided in Tables S2 and S3. Results on scanning of RAREh and canonical RAREs are shown in (c). See Table S4 for a list of the RAREh combinations scanned.

(B and C) Schematic illustration of the PU.1-RAREh-containing PML/RARα ChIP regions. RAREh∩PU.1 is ChIP regions containing both RAREh and PU.1 sites.

(D) Schematic illustration of the comparison between PML/RARα ChIP regions and PU.1 ChIP regions. PU.1-ChIP∩PR-ChIP is the subpopulation of PU.1 ChIP regions that overlap with PML/RARα ChIP regions. PU.1-ChIP\PR-ChIP is the subpopulation of PU.1 ChIP regions that do not overlap with PML/RARα ChIP regions. PU.1 binding sites are defined by V$PU1_Q6 from TRANSFAC with the matrix similarity cutoff of 0.8, while RAREh sites are defined as perfect matches to RGKTCA consensus sequences.

PML/RARα and PU.1 on these ChIP regions. To further verify this observation, we performed sequential ChIP (re-ChIP) assays on $ZnSO_4$-treated PR9 cells. As demonstrated in both panels of Figure 3B, the ChIP regions immunoprecipitated with the first indicated antibody (anti-RARα/anti-PU.1) were re-immunoprecipitated with the second indicated antibody (anti-PU.1/anti-RARα), indicating the binding of PU.1 and PML/RARα to the same DNA.

To determine whether the binding of PU.1 to the ChIP regions was before the binding of PML/RARα, we conducted ChIP-qPCR on DNA samples prepared before and after PML/RARα induction using specific antibodies against PU.1 and RARα. As shown in Figure 3C, the difference of ChIP enrichment before and after the $ZnSO_4$ treatment was minimal in the PU.1 ChIP but dramatic in the PML/RARα ChIP, indicating the tethering of PML/RARα to chromatin regions pre-bound by PU.1.

Colocalization of PU.1 and PML/RARα on the same DNA fragments could be fortuitous or might indicate a physical interaction between the two proteins. To test this, coimmunoprecipitation (co-IP) assays were conducted using protein lysates from $ZnSO_4$-treated PR9 cells. As illustrated in Figure 3D, PU.1 was specifically detected in protein products immunoprecipitated by either the anti-RARα or the anti-PML antibody (left panel) and, likewise, PML/RARα was specifically detected in immunoprecipitates obtained with the anti-PU.1 antibody (right panel), suggesting that an interaction between PML/RARα and PU.1 occurred in vivo.

DNA Binding Assessment of PU.1 and PML/RARα to the PU.1-RAREh ChIP Regions

To further assess PU.1 and PML/RARα binding at the molecular level, we performed EMSA and DNase I footprinting assays on PU.1-RAREh-containing promoter sequences. As demonstrated in Figure 4A, when a 22 bp probe from the ChIP region of *HCK* covering the PU.1 motif was incubated with nuclear extracts from cells transfected with PU.1, specific protein-DNA complexes were observed (lane 2) and were supershifted by the anti-PU.1 antibody (lane 3), providing evidence that PU.1 binds to its motif identified in this setting. With increasing amounts of PML/RARα-containing nuclear extracts, the intensity of PU.1-DNA complexes decreased (Figure 4A, lanes 6–9), with concomitant accumulation of isotopic signals at the top of the gel; this was not seen with control extracts (Figure S2A). These results suggest that PML/RARα can interact with PU.1 in the presence of the probe DNA. Furthermore, DNase I footprinting assays on the ChIP region of *NCF4* provided a clear view of the binding of PU.1 and PML/RARα to a PU.1-RAREh-containing DNA fragment. As shown in Figure 4B and Figure S2B, in the presence of PU.1 alone, protection occurred only at the PU.1 motif, whereas in the presence of PML/RARα alone, no protection occurred at the RAREh sites, indicating that this fragment

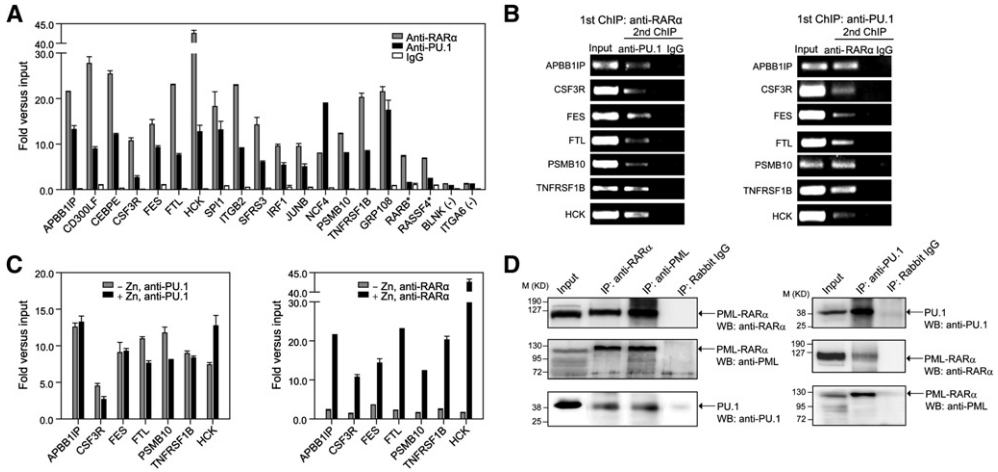

Figure 3. Molecular Evidence for the Interaction between PML/RARα and PU.1 on the PU.1-RAREh ChIP Regions

(A) Presence of both PU.1 and PML/RARα on the PU.1-RAREh ChIP regions after the induction of PML/RARα in PR9 cells as assayed by ChIP-qPCR. The canonical RARE-containing regions (asterisks) and the non-relevant regions (−) are included as controls. Data are shown as fold enrichment of ChIPed DNA versus input DNA. Error bars represent SD of triplicate measurements.

(B) Validation of the co-presence of PML/RARα and PU.1 on the same PU.1-RAREh ChIP regions through re-ChIP assays. ChIP products of the first indicated antibodies (1st ChIP) from $ZnSO_4$-treated PR9 cells were subjected to immunoprecipitation using the second indicated antibodies (2nd ChIP).

(C) Recruitment of PML/RARα to the PU.1-RAREh ChIP regions pre-bound by PU.1. ChIP-qPCR was performed using the anti-PU.1 and the anti-RARα antibodies. Data are shown as fold enrichment of ChIPed DNA versus input DNA. Error bars represent SD of triplicate measurements.

(D) Demonstration of the in vivo interaction between PML/RARα and PU.1 in PR9 cells after PML/RARα induction through co-IP assays. Western blots of input lysates or immunoprecipitates were analyzed using the indicated antibodies.

is primarily bound by PU.1 through its specific motif. In contrast, protection of the RAREh sites occurred only when both PU.1 and PML/RARα were present, demonstrating that PU.1 binding is a prerequisite for PML/RARα binding to its motifs.

Repression of PU.1-Dependent Transactivation by PML/RARα

Luciferase reporter assays were then conducted on 293T cells to evaluate the biological relevance of PU.1-RAREh-containing promoters on PU.1-mediated transactivation in the presence or absence of PML/RARα. Promoter regions that were tested included those for *SPI1/PU.1*, *SFRS3*, *HCK*, *PSMB10*, *IRF1*, and *NCF4*. In addition, a native *RARβ2* promoter with a canonical RARE and an artificial construct with three RARE copies (RARE3) were included as controls. As illustrated in Figure 5A, the PU.1-RAREh promoters shared several common features. First, promoter activities were significantly enhanced by cotransfection of PU.1 (Figure 5A, lane 7 versus 1), indicating PU.1-dependent transactivation of these promoters. Second, the PU.1-dependent transactivation was effectively suppressed by cotransfection of PML/RARα (Figure 5A, lane 8 versus 7). Third, the suppression of PU.1-dependent transactivation by PML/RARα was relieved by the addition of ATRA (Figure 5A, lane 9 versus 8), although the efficiency of the relief varied from one promoter to another. PML/RARα alone (Figure 5A, lane 3 versus 1) or with the addition of ATRA (lane 4 versus 3) caused minor/minimal changes in luciferase activity, providing additional evidence that transactivation of these promoters was primarily regulated by PU.1. Unlike PML/RARα, RARα had minimal impact on PU.1-mediated transactivation (Figure 5A, lane 10 versus 7), and the addition of ATRA resulted in minimal changes (lane 11 versus 10), consistent with the above finding that the PU.1-RAREh regions were targeted by PML/RARα rather than RARα.

In contrast to the PU.1-RAREh promoters, both the native *RARβ2* promoter and the artificial RARE3 segment revealed distinct features. For instance, PU.1 alone had minimal impact on luciferase activities of the *RARβ2* and RARE3 promoters (Figure 5A, lane 7 versus 1). However, the suppressive effect of PML/RARα alone was obvious (Figure 5A, lane 3 versus 1). Unlike that of the PU.1-RAREh promoters, ATRA-mediated transactivation of the canonical RARE promoters was significantly high in cells cotransfected with PML/RARα (Figure 5A, lane 4 versus 3), RARα (lane 6 versus 5), or empty vector (reflecting activity of endogenous RARα protein) (lane 2 versus 1), highlighting the regulatory nature of RAREs on these promoters. Interestingly, although ATRA-mediated transactivation was evident on the RAREs, this transactivation was even more enhanced by cotransfection of PU.1 (Figure 5A, lane 12 versus 2, lane 9 versus 4, and lane 11 versus 6). Although this interesting observation suggests that the ATRA-dependent transactivation can be potentiated by other factors, such as PU.1, and thus adds another feature to RARα-regulated promoters, it remains to be determined whether PU.1 can physically and functionally interact with RARα under physiological conditions.

It has been previously shown that PU.1 is a positive regulator of its own promoter (Chen et al., 1995a). Consistent with this finding, we also showed that PU.1 bound to its own promoter (Figure 5A). More importantly, we found that PML/RARα could bind to the *PU.1* promoter, resulting in repression of its transactivation (Figure 5A). Indeed, in line with the previous data (Mueller et al., 2006), we found that both the mRNA and protein levels of

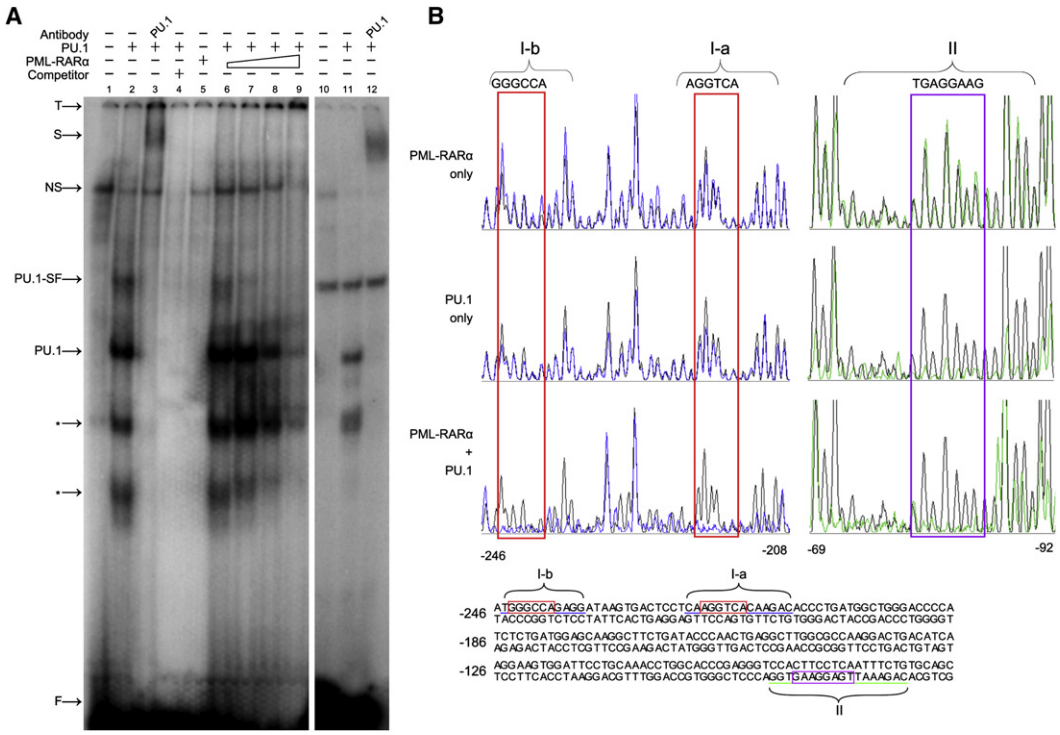

Figure 4. Molecular Evidence for the DNA Binding of PU.1 and PML/RARα to the PU.1-RAREh ChIP Regions

(A) Demonstration of the interaction between PML/RARα and PU.1 in the presence of DNA by EMSA assays. A PU.1 site-containing probe from the *HCK* promoter was used. See Figure S2A for a control experiment showing the PU.1-DNA complex with increasing amounts of control extracts. T, the top of the gel; S, supershifted PU.1-DNA complex with the anti-PU.1 antibody; NS, nonspecific complex; PU.1-SF, complex formed by PU.1 with other proteins in the nuclear extracts; PU.1, gel-shifted complex formed with PU.1 protein; *, complexes formed by proteolytic products of PU.1; F, unbound free probe.

(B) Binding site protection of the *NCF4* promoter by DNase I footprinting analysis. The blue traces (left) and green traces (right) represent, respectively, the fluorescence intensities of the RAREh sites containing *NCF4* coding strand (labeled with 5′-FAM) and the PU.1 site containing *NCF4* non-coding strand (labeled with 5′-HEX), both of which were incubated with the nuclear extracts from the 293T cells transfected with PML/RARα, PU.1, or PML/RARα-PU.1. The black traces represent the fluorescence intensity of the above regions incubated with control nuclear extracts from non-transfected 293T cells. Region I-a is a segment containing a perfect RAREh motif; region I-b is a segment containing a RAREh motif with one mismatch; region II is a segment containing a PU.1 consensus site. DNA sequences (bottom) showing three protected regions are underlined. Full-length electropherograms are included in Figure S2B.

PU.1 were reduced in $ZnSO_4$-treated PR9 cells and NB4 cells, as compared to untreated PR9 cells (Figure 5B). These observations suggest that downregulation of PU.1 in PML/RARα-positive cells is probably due to disruption of autoregulated PU.1 expression by PML/RARα.

Functional Assessment of PU.1 and RAREh Motifs

To test whether PU.1 and RAREh sites are responsible for the observed effects in Figure 5A, we mutated/truncated PU.1 and RAREh sites in *PSMB10* and *SFRS3* promoters and measured their luciferase activities. As shown in Figure 5C, mutation of the PU.1 sites was associated with significant reduction of PU.1-mediated transactivation (lane 7 versus 2) and the subsequent effects of PML/RARα (lane 9/7 versus lane 4/2), highlighting the biological significance of the PU.1 sites in these promoters. Although mutation of the PU.1 site in the *PSMB10* promoter correlated with significant reduction in PU.1-mediated transactivation, the mutated promoter appeared to retain some responsiveness; this is likely due to the presence of unidentified non-canonical PU.1 sites in the promoter. As expected, absence of the RAREh sites was strongly associated with loss of PML/RARα-mediated transrepression (Figure 5C, lane 4/2 versus lane 14/12) and loss of ATRA responsiveness (lane 5/4 versus lane 15/14). This further supports our contention that ATRA responsiveness of the PU.1-RAREh promoters is primarily via the formation of the PML/RARα-PU.1 complex on DNA.

Repression of PU.1-Dependent Transactivation by PML/RARα in Myeloid Cells

To test whether the results obtained in 293T cells could also be seen in hematopoietic cells, we performed transfection assays in myeloid precursor U937 cells, which expressed endogenous PU.1, using the same set of *PSMB10* promoters shown in Figure 5C. Similar to the result in 293T cells, transactivation of the wild-type promoter was significantly suppressed by cotransfection of PML/RARα in U937 cells (Figure 5D, lane 2 versus 1). Also consistent with the previous observations, mutation of the PU.1 motif (Figure 5D, PU.1-MT) was associated with significant reduction in induced activity (lane 3 versus 1) as well as significant loss of PML/RARα-mediated transrepression (lane 4/3 versus lane 2/1). When the cells were transfected with the

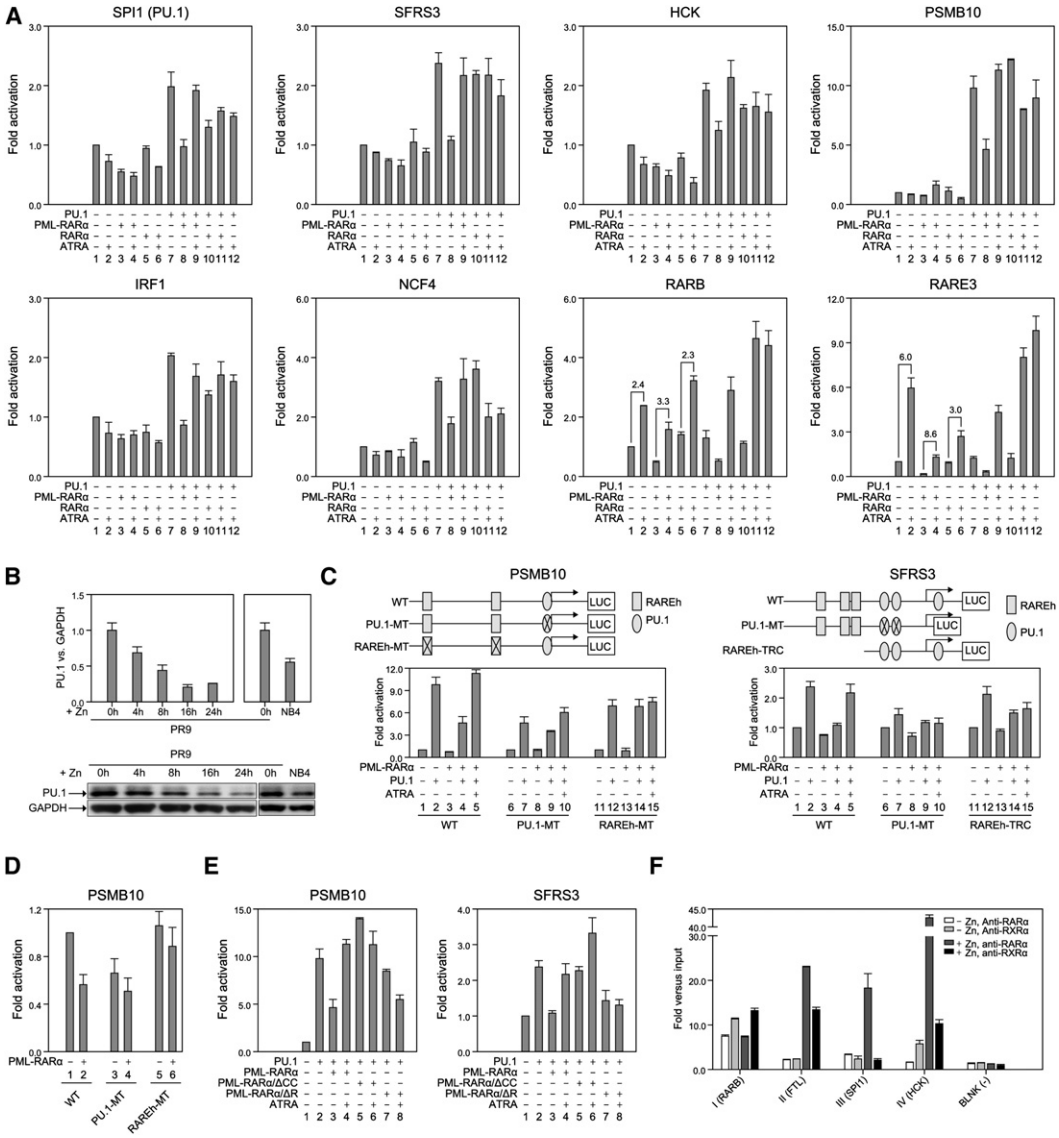

Figure 5. Evidence for Repression of PU.1-mediated Transactivation by PML/RARα

(A) Luciferase reporter assays on representatives of PU.1-RAREh-containing promoters (*SPI1*, *SFRS3*, *HCK*, *PSMB10*, *IRF1*, and *NCF4*) or canonical RARE-containing promoters (*RARB* and *RARE3*). Reporter plasmids and expression plasmids were cotransfected into 293T cells. SPI1, hematopoietic transcription factor PU.1; SFRS3, splicing factor, arginine/serine-rich 3; HCK, hematopoietic cell kinase; PSMB10, proteasome subunit, β type 10; IRF1, interferon regulatory factor 1; NCF4, neutrophil cytosolic factor 4; RARB, retinoic acid receptor β (RARβ2); RARE3, an artificial construct with three RARE copies.

(B) Expression of PU.1 in PR9 and NB4 cells. Real-time RT-PCR (top) and western blot analyses (bottom) were used to quantify PU.1 at both the mRNA and protein levels. mRNA levels were expressed relative to those in untreated PR9 cells. PR9 cells were treated with $ZnSO_4$ at the indicated time points.

(C) Mutation/truncation analysis of the promoter region of *PSMB10* and *SFRS3*. The PU.1 (gray ellipses) and RAREh sites (gray boxes) present in these constructs were mutated (X) or truncated, and the resultant constructs were assayed for the reporter gene activity in 293T cells.

(D) Repression of PU.1-mediated transactivation by PML/RARα in myeloid U937 cells. The same constructs of *PSMB10* used in (C) were tested.

(E) Requirement of both the DNA binding and protein-protein interaction domains for PML/RARα to function as an effective repressor. Expression plasmids encoding PML/RARα/ΔCC and PML/RARα/ΔR were used.

(F) Involvement of RXR in the PML/RARα ChIP regions. ChIP-qPCR was performed on chromatin prepared from $ZnSO_4$-treated PR9 cells using the antibodies specific to RXRα, RARα, and PU.1, respectively. Luciferase activities in (A) and (C)–(E) are normalized for transfection efficiency by co-transfection with a Renilla construct, and values are the mean ± SD obtained from at least three independent experiments. Results from real-time RT-PCR in (B) are normalized to GAPDH and show the mean of three replicates ± SD. Data in (F) are shown as fold enrichment of ChIPed DNA versus input DNA and error bars represent SD of triplicate measurements.

promoter devoid of RAREh sites (Figure 5D, RAREh-MT), the PML/RARα-mediated transrepression was essentially abrogated (lane 6/5 versus lane 2/1), indicating that the binding of PML/RARα to RAREh sites is required for the transrepression. In sum, the data obtained in hematopoietic cells confirm those obtained in 293T cells, supporting the notion that both PU.1

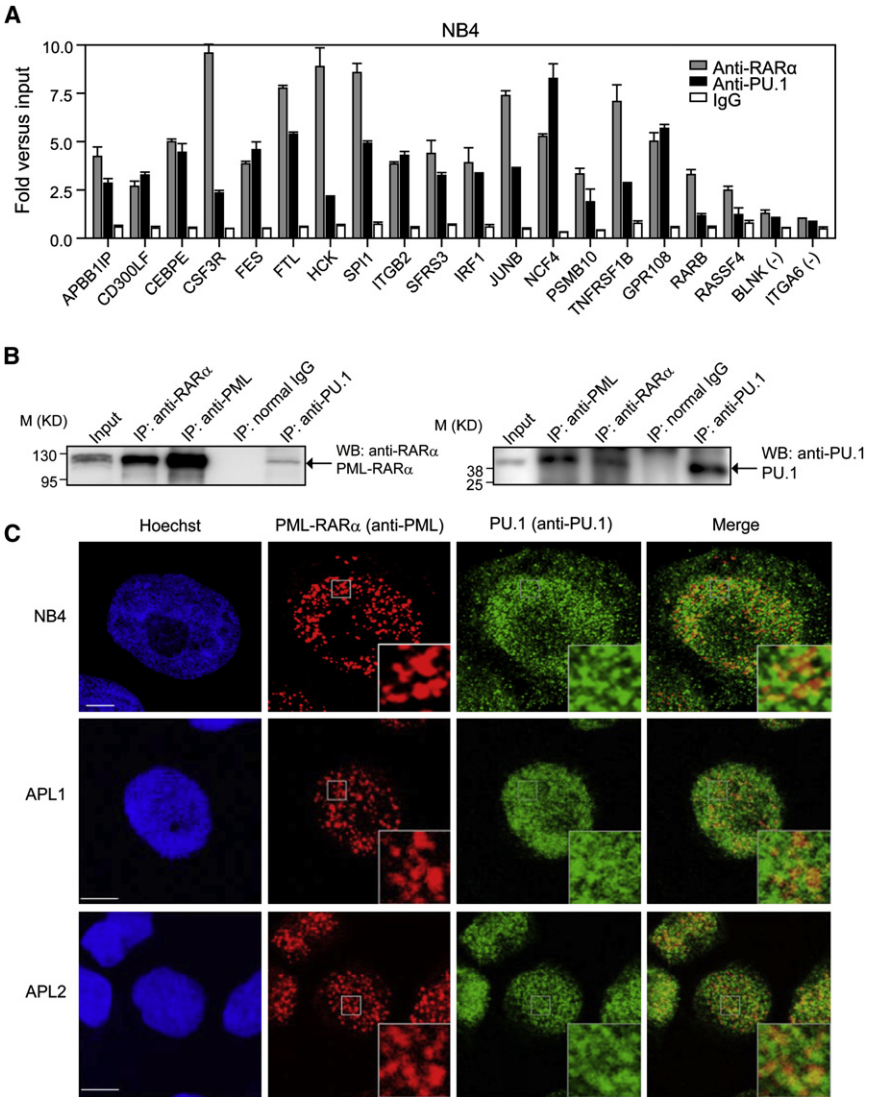

Figure 6. Selective Targeting of PU.1-regulated Genes by PML/RARα in NB4 Cells and Primary APL Blasts

(A) Recruitment of both PU.1 and PML/RARα to the PU.1-RAREh-containing PML/RARα ChIP regions in NB4 cells by ChIP-qPCR assays. Data are shown as fold enrichment of ChIPed DNA versus input DNA. Error bars represent SD of triplicate measurements.

(B) Demonstration of the in vivo interaction between PML/RARα and PU.1 in NB4 cells by co-IP assays. Western blots of input lysates or immunoprecipitates were analyzed using the indicated antibodies.

(C) Distribution of PML/RARα and PU.1 in primary APL blasts. Confocal immunofluorescence assays were performed using anti-PU.1 (green) and anti-PML (red) antibodies. Scale bars, 5 μm. Confocal microscopic images of blast cells from additional five primary APL patients are included in Figure S3.

and RAREh sites play important roles in PML/RARα-mediated transcriptional repression in APL.

Requirement of Both Protein-Protein Interaction and DNA Binding for PML/RARα to Act as an Effective Repressor on PU.1-RAREh Promoters

We have shown that PU.1 and PML/RARα interact to form a transcriptional complex. In an attempt to identify the regions of PML/RARα important in forming this complex, we tested two truncated forms of PML/RARα in luciferase reporter assays, i.e., PML/RARα/ΔCC, in which the coiled-coil domain is deleted (Jansen et al., 1995), and PML/RARα/ΔR, in which the DNA binding domain is deleted (Grignani et al., 1996). As demonstrated in Figure 5E, deletion of the coiled-coil domain was sufficient to abolish the repressive effects of PML/RARα on PU.1-dependent transactivation (lane 5 versus 3), while deletion of the DNA binding domain of PML/RARα had a moderate effect on mediating transrepression (lane 7 versus 3). However, loss of the DNA binding of PML/RARα was highly correlated with loss of the responsiveness of the promoters to ATRA (Figure 5E, lane 8 versus 7), consistent with the results shown in Figure 5C in which promoters without RAREh sites were used.

Involvement of RXR in the PML/RARα-PU.1-Bound ChIP Regions

Since RXR is known to be a crucial component of PML/RARα complexes (Zhu et al., 2007; Perez et al., 1993), we then assessed whether RXR was present in the PML/RARα-PU.1-bound ChIP regions. As evaluated by ChIP-qPCR using a RXRα-specific antibody, RXR was indeed present in some, but not all, of the tested ChIP regions (Figure 5F). This is consistent with the previous notion that PML/RARα exists in multiple forms of complexes, including PML/RARα homodimers devoid of RXR and oligomers and heterodimers containing RXR.

Molecular Evidence for Selective Targeting of PU.1-Regulated Genes by PML/RARα in APL-Derived NB4 Cells and Primary APL cells

With the model system of PR9 cells, we have identified PU.1-RAREh-containing promoters as important targets of PML/RARα. To test whether this finding is relevant to APL, we first conducted ChIP-qPCR and co-IP assays on the APL-derived cell line NB4. As demonstrated in the ChIP-qPCR data of NB4 cells (Figure 6A), most of the tested ChIP regions were significantly enriched by either the anti-RARα or the anti-PU.1 antibody, indicating the co-presence of PU.1 and PML/RARα on chromatin DNA corresponding to the ChIP regions. Moreover, co-IP assays on NB4 cells (Figure 6B) revealed similar phenomena as those shown in Figure 3D, i.e., PML/RARα was immunoprecipitated by the anti-PU.1 antibody and vice versa, demonstrating that PML/RARα interacts with PU.1 in NB4 cells as well. In addition, confocal microscopic images of PU.1 and

Table 1. Enrichment Analysis of Potential PML/RARα and PU.1 Targets with Gene Sets Associated with APL Models and Clinical Samples

			Total Potential Targets of PML/RARα				Potential Targets of PML/RARα-Containing PU.1-RAREh sites[b]				Potential Targets of PU.1 Identified by PU.1-ChIP			
Gene Set	Model	Number in Gene Set	Number in Gene Set	Fold Enrichment	Z Score	p Value	Number in Gene Set	Fold Enrichment	Z Score	p Value	Number in Gene Set	Fold Enrichment	Z Score	p Value
APL_UP	Primary APL	1514	226	1.2	3.1	5.17E − 04	139	1.2	2.2	1.17E − 02	205	1.1	1.1	1.12E −01
APL_DN	Primary APL	1482	355	1.9	12.7[a]	0.00E + 00	239	2.1	11.6[a]	0.00E + 00	343	1.8	11.2[a]	0.00E +00
PR9_Zn_UP	PR9 cells	1293	163	1.2	1.8	2.67E − 02	94	1.1	0.6	2.58E − 01	200	1.4	4.5	2.24E −06
PR9_Zn_DN	PR9 cells	1491	369	2.2	15.5[a]	0.00E + 00	250	2.4	14.1[a]	0.00E + 00	312	1.8	10.6[a]	0.00E +00
APL_RA_UP	Primary APL	1002	366	3.2	23.7[a]	0.00E + 00	247	3.5	20.9[a]	0.00E + 00	300	2.6	16.8[a]	0.00E +00
APL_RA_DN	Primary APL	1133	214	1.7	7.9	4.11E − 15	124	1.6	5.1	2.84E − 07	213	1.6	7.2	2.55E −13
NB4_RA_UP	NB4 cells	2275	586	2.4	22.3[a]	0.00E + 00	384	2.6	19.1[a]	0.00E + 00	567	2.3	20.1[a]	0.00E +00
NB4_RA_DN	NB4 cells	2833	309	1.0	0.8	1.78E − 01	179	1.0	−0.4	6.36E − 01	339	1.1	1.9	2.28E -02
PR_DN and RA_UP	all four models	714	284	3.5	22.4[a]	0.00E + 00	197	3.9	20.4[a]	0.00E + 00	248	2.9	17.8[a]	0.00E +00

UP, the upregulated gene set; DN, the downregulated gene set; RA, the gene set constructed from ATRA-treated samples; Zn, the gene set constructed from $ZnSO_4$-treated cells. PR_DN and RA-UP represents the gene set containing the genes downregulated in APL/$ZnSO_4$-treated PR9 cells and upregulated in ATRA-treated NB4/APL cells.

[a] The Z scores for the most significant gene sets.

[b] A full list of the PU.1-RAREh genes regulated in APL models and clinical samples can be found in Table S5, and the functional classification of these genes is provided in Table S6.

PML/RARα immunofluorescence, in NB4 and primary APL cells, allowed us to visualize in situ spatial relationships between the two proteins. As illustrated in Figure 6C and Figure S3, PU.1 and PML/RARα colocalized on microspeckles. It is important to note that the colocalization appears to occur on some rather than all of the microspeckles, further supporting the notion that PML/RARα selectively interacts with PU.1 in APL cells.

Evidence for Transcriptional Repression/ATRA Responsiveness of Genes Corresponding to the PU.1-RAREh ChIP Regions in APL

Still, an important question that remains to be addressed is whether genes corresponding to PML/RARα ChIP regions, particularly those corresponding to PU.1-RAREh ChIP regions, are transcriptionally suppressed in primary APL cells. To answer this question, we conducted a gene set-based analysis by comparing RefSeq genes corresponding to the total PML/RARα ChIP regions and the PU.1-RAREh ChIP regions with gene sets representing the differentially regulated genes from APL models/APL clinical samples. The gene sets were constructed from microarray data on PR9 cells after PML/RARα induction (Alcalay et al., 2003), clinical APL samples (Valk et al., 2004), ATRA-treated NB4 cells (this study, GEO accession number GSE19201), and ATRA-treated primary APL cells (Meani et al., 2005). We also included in the analysis a data set (PR_DN and RA_UP) created by selecting genes both downregulated in APL/$ZnSO_4$-treated PR9 cells and upregulated in ATRA-treated NB4/APL cells. There has been a long-standing assumption that downregulation of genes in APL cells is largely due to PML/RARα-mediated transcriptional repression and upregulation of genes in ATRA-treated APL cells is a consequence of the relief of that repression. As illustrated in Table 1, genes corresponding to both total and PU.1-RAREh ChIP regions were highly overrepresented (Z score > 10) in the gene sets relevant to PML/RARα-mediated transcriptional repression (including the gene sets downregulated in APL samples and $ZnSO_4$-treated PR9 cells, i.e., APL_DN and PR9_Zn_DN) and in those relevant to the responsiveness to ATRA (including those upregulated in ATRA-treated primary APL cells and ATRA-treated NB4 cells, i.e., APL_RA_UP and NB4_RA_UP). Through this analysis, we found that a significant portion of genes represented by the identified ChIP regions, especially those containing PU.1-RAREh sites, were transcriptionally suppressed in APL and/or reactivated upon ATRA treatment.

To test whether the genes corresponding to PU.1-RAREh ChIP regions and suppressed in APL and/or reactivated upon ATRA treatment (see Table S5 for the gene list) are functionally relevant to myeloid differentiation, we performed Gene Ontology (GO) enrichment analysis (http://www.pantherdb.org/). As illustrated in Table S6, significant enrichment was observed on GO terms involved in various aspects of myeloid differentiation, as highlighted by transcription factors, granulocyte-mediated immunity, and defense activities and some other myeloid-associated cascades. For example, transcription factors JUNB, IRF1, BHLHB2, ID2, and PU.1 are known to be early-lineage regulators of myelopoiesis and thus reduced levels of these genes/proteins are possibly consistent with the blocked differentiation characteristic of APL. Other significantly overrepresented GO categories included cell proliferation and differentiation, apoptosis,

cell cycle control, and signal transduction, all of which may collectively contribute to uncontrolled proliferation and decreased apoptosis in leukemic cells.

DISCUSSION

PML/RARα is the initiating factor in the development of APL and has a number of distinct features. In normal hematopoietic precursor $CD34^+$ Lin^- cells, the introduction of PML/RARα induces a differentiation block and promotes cell survival through a multistep process (Grignani et al., 2000). Similarly, the expression of this oncoprotein in hematopoietic PR9 cells induces many phenotypic changes like those in APL, such as inability to differentiate under certain stimuli, increased proliferation and sensitivity to ATRA (Grignani et al., 1993). Also, the expression of PML/RARα in murine bone marrow eventually causes disease with features characteristic of APL. Interestingly, a higher level of PML/RARα appears to be required during the initiation stage of oncogenic activities (Grignani et al., 2000; Nasr et al., 2008). Another interesting feature of this oncogenic protein is that its transcriptional repression effect can be relieved by ATRA, resulting in reactivation of genes essential for definitive myeloid differentiation. Exactly how PML/RARα exerts all of its effects on APL has been a continuing area of research, particularly as interference of RARα-mediated transcription alone does not recapitulate the disease phenotype. Based on this latter observation, it has been proposed that PML/RARα not only represses RAR signaling but also interferes with other myeloid essential transcription factors. In our work, we have investigated the early molecular effects of PML/RARα in hematopoietic progenitor cells and identified PU.1 as a factor that directs binding of PML/RARα to nearby RAREh sites, and in so doing creates a PML/RARα-PU.1 complex.

PU.1 has been identified as an important transcription factor in normal hematopoiesis and in generation of myeloid leukemias through disruption of its function (Tenen, 2003). Over a decade ago, PU.1 was reported to be expressed at high levels in granulocytic cells and was associated with the differentiation of myeloid cells from granulocytic precursors into mature neutrophils (Chen et al., 1995b). More recently, studies based on various in vitro and in vivo models have further demonstrated that PU.1 promotes the differentiation of committed myeloid progenitors (Rosenbauer and Tenen, 2007). Functional disruption of PU.1 or graded reduction in its expression may block myelomonocytic differentiation or maturation, resulting in accumulation of myeloid blasts and thus genesis of myelogenous leukemia in animal models (Dakic et al., 2005; Iwasaki et al., 2005). Overexpression of PU.1 has been shown to rescue myeloid differentiation in AML blasts that carry a mutant form of PU.1 (Rosenbauer et al., 2004).

In APL, expression of PU.1 is suppressed in leukemic cells. Treatment of these cells with ATRA restores PU.1 expression and induces neutrophil differentiation (Mueller et al., 2006). Importantly, conditional expression of PU.1 in PML/RARα leukemic cells is sufficient to induce neutrophil differentiation (Mueller et al., 2006), revealing biological effects similar to ATRA. With transgenic mice models, the penetrance rate of APL development is significantly increased in offspring once PML/RARα mice are crossed with $PU.1^{+/-}$ mice (Walter et al., 2005). To date, it is still unclear whether such crosstalk between PU.1 and PML/RARα was due to direct interaction of the two proteins or to indirect effects.

In the work presented here, we provide evidence that PU.1 and PML/RARα are in a transcriptional complex. The initiating factor for the complex is the recruitment of PML/RARα protein to chromatin DNA that contains both PU.1 and RAREh sites and has been bound by PU.1. The resulting DNA-bound PU.1-PML/RARα complex causes repression of PU.1-regulated genes. Through reporter assays using truncated proteins and promoters with mutant DNA binding sites, we have found that both protein-protein and DNA binding domains of PML/RARα are important for the effect, as are the PU.1 and RAREh sites.

The formation of PU.1-PML/RARα complexes on DNA and thus the selective targeting of PU.1-regulated promoters can be complex, requiring multiple essential elements. An obvious one is the presence of PU.1 motifs and thus the pre-bound PU.1 protein on the DNA region. The second one is the nearby presence of RAREh sites. These RAREh sites may form various DRs, everted repeats, and inverted repeats with widely variable spacing in between (see Table S4). However, due to the diversity and complexity of the RAREh-formed combinations, it is extremely challenging to extract some common features, except for RAREh, out of these DRs, everted repeats, and inverted repeats. Additional elements yet to be identified probably include histone modifications involved in the ChIP regions, since certain types of modifications appear to be a prerequisite for transcription factor binding (Guccione et al., 2006).

Our findings shed light on the functional correlation between PML/RARα and PU.1 in APL and raise some interesting issues. First, the promoter of PU.1 itself is targeted by PML/RARα, resulting in downregulation of PU.1. Reduced PU.1 in APL may fail to effectively activate its downstream target genes, suggesting that downregulated PU.1 targets in APL include not only those targeted by PML/RARα (i.e., containing PU.1-RAREh sites) but also those not targeted by PML/RARα (i.e., containing PU.1 motif only). Such an assumption is supported by the gene set analysis (Table 1), in which results revealed by the PU.1 ChIP regions are highly similar to those revealed by the PML/RARα ChIP regions. It may also explain why a dramatic effect of differentiation can be obtained in APL cells simply by restoring PU.1 expression (Mueller et al., 2006).

Another issue is whether PML/RARα exerts its oncogenic effect by targeting PU.1-regulated genes in the context of myeloid cells. Evidence that addresses this issue is that we have successfully detected the interaction between PML/RARα and PU.1 in hematopoietic PR9/NB4 cells through co-IP assays, but we have failed to do so in non-hematopoietic 293T cells co-expressing the two proteins (data not shown). The latter result is in accordance with previously published data (Yoshida et al., 2007). Accordingly, these data may also suggest that additional cofactors or modifications required for PML/RARα to interact with PU.1 are yet to be identified.

It has been previously shown that PU.1 is important in the pathogenesis of APL. However, the how and why were not known. Here we provide evidence for a direct interaction between PU.1 and PML/RARα in APL cells and the subsequent effect of this complex on PU.1-regulated genes. PU.1 is known to regulate genes by interacting, in either a synergistic or an

antagonistic manner, with other transcription factors such as C/EBPα, c-Jun, AML-1, IRF4, and GATA-1 (Tenen, 2003). Indeed, we found that the potential binding sites of many other transcription factors were enriched in PML/RARα ChIP regions, but at a moderate to low frequency (Table S3). While we have shown the importance of the interaction between PU.1 and PML/RARα in the pathogenesis of APL, it remains to be determined whether other yet unidentified transcription factors may also contribute to the differentiation block at the promyelocytic stage. However, data presented in this study provides a roadmap to future work and a more comprehensive understanding of APL.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents

PR9 is a PML/RARα-inducible model (Grignani et al., 1993) constructed from U937, a myeloid precursor cell line without the t(15;17) translocation but expressing many proteins important in myeloid development, including PU.1. To avoid the potential bias of clonal variations in culture, a single-cell subclone was selected.

NB4 is an APL-derived cell line, carrying the t(15;17) translocation and expressing the PML/RARα fusion protein (Lanotte et al., 1991).

The antibodies used are as follows and were purchased from Santa Cruz Biotechnology: PML (PG-M3), PML (H-238 x), RARα (C-20 x), and PU.1 (T-21 x). The rabbit IgG (ab46540) and anti-rabbit IgG HRP-linked antibodies (#7074) were obtained from Abcam and Cell Signaling, respectively.

Protein A Sepharose beads were purchased from GE Healthcare. ATRA (Sigma-Aldrich) was dissolved in ethanol as a stock solution at 1 mM and $ZnSO_4$ (Sigma-Aldrich) was dissolved in sterile water as a stock solution at 100 mM.

Patient Samples

APL blasts were obtained at disease onset from bone marrow samples of newly diagnosed patients with over 85% abnormal blasts and promyelocytic cells in their bone marrow. Informed consent was obtained from all patients according to procedures approved by the institutional review board from Ruijin Hospital, affiliated with Shanghai Jiao Tong University School of Medicine.

ChIP-on-Chip and Data Analysis

ChIP was performed according to the Affymetrix protocol as described previously (Carroll et al., 2005). The microarrays used were Affymetrix Human Promoter 1.0R Arrays, which cover 7.5–10 kb upstream through 2.45 kb downstream of transcriptional start sites for over 25,500 human genes. Three independent replicates were performed according to the instructions of the manufacturer.

ChIP-on-chip data were analyzed using a model-based analysis of tiling-array algorithm (Johnson et al., 2006) at the p value cutoff of 10^{-5} with input DNA as control. De novo motif analysis was performed by an enumeration-based method. In addition, TRANSFAC motifs, RAREh sites, and RAREh combinations were also evaluated. Details are available in Supplemental Experimental Procedures. Statistical significance of motifs was assessed by the binomial test and represented as Z scores.

Immunofluorescence Microscopy, Co-IP, and Western Blotting

Immunofluorescence microscopy, co-IP, and western blotting were performed as previously described with some modifications. Details are available in Supplemental Experimental Procedures.

EMSA and DNase I Footprinting Assay

EMSAs were conducted using a PU.1 site-containing *HCK* promoter in the presence of either nuclear extracts from transfected 293T cells or in vitro translation products. DNase I footprinting was performed using the Core Footprinting System (Promega) and analyzed with the 3730 DNA Analyzer (Applied Biosystems). Details are available in Supplemental Experimental Procedures.

Luciferase Reporter Assay

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen). U937 cells were transiently transfected using the Amaxa Nucleofector device (program V-01) with Nucleofector Kit V (Amaxa Biosystems). Luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega). Details are available in Supplemental Experimental Procedures.

Gene Set Analysis

A gene set was defined as a group of genes with same/similar biological features under a given condition. Statistical significance was assessed by the binomial test. Details are available in Supplemental Experimental Procedures.

Expression Microarrays

The expression microarrays used were Affymetrix hgu133Plus2. All experiments were performed according to the manufacturers' instructions. Details for microarray data analysis are available in Supplemental Experimental Procedures.

ACCESSION NUMBERS

Microarray gene expression data and ChIP-on-chip data are available at NCBI Gene Expression Omnibus under accession numbers GSE19201 and GSE19202, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2009.12.045.

ACKNOWLEDGMENTS

We thank anonymous reviewers for their constructive comments and suggestions. Also, we thank N. Kieffer and S. Khochbin for the helpful discussion and critical reading of the manuscript. We acknowledge Z. Qian, S. Dong and C. Lu for technical support. This work was supported in part by grants from the Ministry of Science and Technology of China (2006CB910405, 2006CB910700, 2007AA02Z335, 2006AA02Z302, and 2009CB825607), the National Natural Science Foundation (30730033, 30670436, and 30600260), the Chinese Academy of Sciences (KSCX2-YW-R-19 and GJHZ06), and the Shanghai Commission of Science and Technology (07SP07002). J.Z. is a member of the TB-VIR network (European Community Grants of FP7, 200973).

Received: August 22, 2008
Revised: September 30, 2009
Accepted: December 4, 2009
Published: February 16, 2010

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