Transcription and methylation analyses of preleukemic promyelocytes indicate a dual role for PML/RARA in leukemia initiation

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ABSTRACT

Acute promyelocytic leukemia is an aggressive malignancy characterized by the accumulation of promyelocytes in the bone marrow. PML/RARA is the primary abnormality implicated in this pathology, but the mechanisms by which this chimeric fusion protein initiates disease are incompletely understood. Identifying PML/RARA targets *in vivo* is critical for comprehending the road to pathogenesis. Utilizing a novel sorting strategy, we isolated highly purified promyelocyte populations from normal and young preleukemic animals, carried out microarray and methylation profiling analyses, and compared the results from the two groups of animals. Surprisingly, in the absence of secondary lesions, PML/RARA had an overall limited impact on both the transcriptome and methylome. Of interest, we did identify down-regulation of secondary and tertiary granule genes as the first step engaging the myeloid maturation block. Although initially not sufficient to arrest terminal granulopoiesis *in vivo*, such alterations set the stage for the later, complete differentiation block seen in leukemia. Further, gene set enrichment analysis revealed that PML/RARA promyelocytes exhibit a subtle increase in expression of cell cycle genes, and we show that this leads to both increased proliferation of these cells and expansion of the promyelocyte compartment. Importantly, this proliferation signature was absent from the poorly leukemogenic p50/RARA fusion model, implying a critical role for PML in the altered cell-cycle kinetics and ability to initiate leukemia. Thus, our findings challenge the predominant model in the field and we propose that PML/RARA initiates leukemia by subtly shifting cell fate decisions within the promyelocyte compartment.

Introduction

Acute promyelocytic leukemia (APL) is an aggressive malignancy characterized by the accumulation in the bone marrow of promyelocytes unable to undergo terminal differentiation. Approximately 97% of patients with APL express the PML/RARA fusion protein as a result of a t(15;17) chromosomal translocation and, remarkably, nearly all of them will respond to combination therapy with retinoic acid and arsenic trioxide.[1,2] Although treatable, the way in which PML/RARA initiates disease is still mysterious. In both humans and murine models, PML/RARA appears able to initiate leukemia[3-6] but several observations suggest that additional events are nevertheless needed to complete transformation: humans can be healthy carriers of PML/RARA[7] and, in animal models, a long latency is necessary for disease establishment, which still arises with incomplete penetrance.[3-5,8] In addition, concurrent events are invariably present in leukemic cells,[6,9-12] further supporting that PML/RARA-expressing cells need to undergo additional changes for complete transformation into leukemic clones.

At the molecular level, PML/RARA oncogenic activity is thought to result primarily from transcriptional repression. As the fusion protein homodimerizes, it can associate with an increased number of co-repressor molecules, such as histone de-acetylases and DNA methyltransferases.[13-15] Other epigenetic players including polycomb repressive complex 2 have also been suggested to participate in chromatin remodeling and transcription deregulation.[16] Further, the more relaxed DNA binding conformation of the homodimerized fusion protein allows targeting of an extended number of sites,[17] potentially contributing to gene deregulation at numerous sites in the genome. In leukemic cells, repression of two important regulators of myeloid cell development, C/EBPA and PU.1 has been described,[18-20] including repression of PU.1 targets,[21] effects which have been suggested to contribute to leukemogenesis. However, the transcriptional repression model of PML/RARA was built mostly on studies of fully transformed cells and cell lines, making it difficult to distinguish the contribution of PML/RARA from that of secondary events. In this study, we aimed to fill this gap by studying the effects of PML/RARA on preleukemic promyelocytes. As the preleukemic state cannot be studied in humans, we took advantage of the MRP8 murine model, which faithfully recapitulates features of human APL, and we assessed changes in gene expression, DNA methylation and proliferation caused by PML/RARA. Importantly, we carried out these studies in conditions representing the *in vivo* environment, in which

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PML/RARA preleukemic myeloid precursors eventually give rise to leukemic promyelocytes.

Methods

Murine model and bone marrow harvest

MRP8-PML/RARA transgenic mice have been described elsewhere.[3] Mice were bred and maintained at the University of California, San Francisco under standard conditions. For each individual sample, bones from one male and one female were pooled, thoroughly cleaned, ground and filtered. Cells were collected after centrifugation on Histopaque-1119 (Sigma #11191) and CKIT enrichment (Miltenyi Biotec #130-091-24).

Staining and flow cytometry

Cells were stained with a lymphocyte/erythrocyte/stem cell cocktail followed by secondary antibody. After blocking (Sigma #I4131), the suspension was stained with the specific cocktail. Live cells were double-sorted and purity after sorting was assessed. Compensation tubes were prepared by staining FVB/n spleen with fluorophore-conjugated CD45R/B220 antibodies.

RNA isolation and microarray analysis

Cells (25,000-50,000) were double-sorted into Trizol (Life Technologies #15596). Following phenol/chloroform extraction, RNA was purified on PicoPure columns (Life Technologies #KIT0204). Sample preparation, labeling, and array hybridizations were performed according to standard protocols from the University of California, San Francisco Shared Microarray Core Facilities (*http://www.arrays.ucsf.edu/protocols/*) and Agilent Technologies. The microarray was run on a murine Agilent platform comprising 41,174 probes, including 28,283 targeting protein-coding genes (27,285 known + 998 predicted), and 1,484 targeting non-coding RNA (950 known + 534 predicted). The dataset was quantile normalized[22] without background subtraction. Differential expression analysis using moderated t statistic with Benjamini–Hochberg false discovery rate (adjusted *P* value) correction was performed using the limma R/Bioconductor package.[23] Below, biological replicates refer to samples harvested from different litters, over the course of several months, while a technical replicate refers to the same biological sample run twice on the array. For the preleukemic *versus* normal study, data are representative of three or four biological replicates/population and are available on GEO (GSE54474). For the leukemic *versus* preleukemic study, data are representative of two biological replicates for the preleukemic group (plus 1 technical duplicate) and three biological replicates for the leukemic group (plus 1 biological and 1 technical duplicate) and are also available on GEO (GSE59431).

Genomic DNA isolation and genome-wide methylation analysis by enhanced reduced representation bisulfite sequencing

Cells (25,000-50,000) were double-sorted and genomic DNA was extracted using the PureGene kit (Qiagen). DNA (25 ng) was used to perform the enhanced reduced representation bisulfite sequencing (ERRBS) assay as previously described[24] and sequenced on a HiSeq2000 Illumina sequencer. Reads were aligned against a bisulfite-converted mm9 genome using Bowtie.[25] Downstream analysis was performed using R 2.15 and Bioconductor 2.12. Differential methylation analysis was performed using the MethylKit Bioconductor package,[26] and differentially methylated regions were annotated as follows: when overlapping with a gene body, the corresponding transcript is reported; when intergenic, all neighboring transcripts within a 50 kb window are reported; beyond 50 kb, the nearest transcriptional start site is reported. Data are representative of five biological replicates/population and are available on GEO (GSE54038).

Other information

Additional methods and details of the antibodies and reagents are described in the *Online Supplementary Document* and *Online Supplementary Table S13*.

Results

Refined characterization of leukemia-repopulating cells highlights the importance of CD34 as a marker of leukemogenic potential in MRP8 murine acute promyelocytic leukemia

As in humans, murine APL blasts display morphological characteristics of promyelocytes (Figure 1A). Although surface markers of promyelocytes differ in humans, several groups have demonstrated that in murine models, the

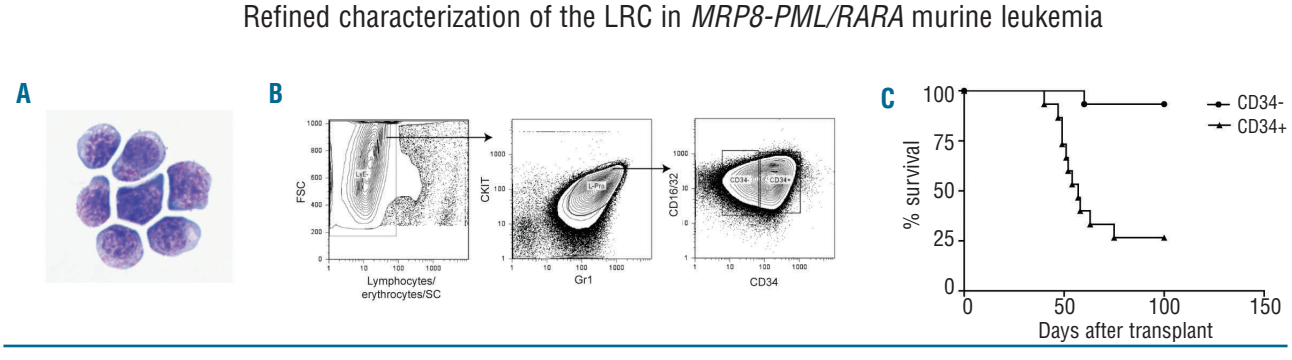

Figure 1. In murine APL, the leukemia-repopulating cell (LRC) is CKIT$^+$GR1^{int}CD34$^+$ and the ability to transplant the disease correlates with CD34 expression. (A) Representative morphology of murine APL cells. (B) Sorting strategy applied to assess the leukemogenic potential of the CD34$^+$ *vs.* CD34$^-$ fractions from the pool of CKIT$^+$GR1^{int} expressing cells in transplanted leukemia 1111. Cells expressing CD3, CD4, CD5, CD8a, TER119, B220 and SCA-1 were excluded with the lymphocyte/erythrocyte/stem cell-negative gate. (C) Survival curves of FVB/n mice transplanted with sorted CKIT$^+$GR1^{int}CD34$^+$ or CKIT$^+$GR1^{int}CD34$^-$ leukemic cells (pooled data for 100-1,000 cells).

leukemia-repopulating cell (LRC) is enriched in the CKIT^{+}CD34^{+}GR1^{int} population.[19,27] In this study, we provide additional data showing that the LRC loses its leukemia-repopulating ability concomitantly to down-regulating CD34 expression (Figure 1B,C).

By examining myeloid cell populations in wild-type animals, we found that normal promyelocytes have similar immunophenotypic characteristics to those seen in the leukemic mice, with loss of CD34 marking progression towards a more mature promyelocyte. It does, therefore, appear that the leukemogenic potential is acquired by cells expressing surface markers of promyelocytes, and we reasoned that studying these populations in wild-type *versus* PML/RARA bone marrow would help us identify key deregulations mediated by PML/RARA at this critical CD34^{+} to CD34^{-} transition.

Validation of a novel sorting strategy to isolate early and late promyelocyte populations

Importantly, to observe the impact of PML/RARA alone, bone marrow was harvested before 4 weeks of age from PML/RARA transgenic animals (designated as

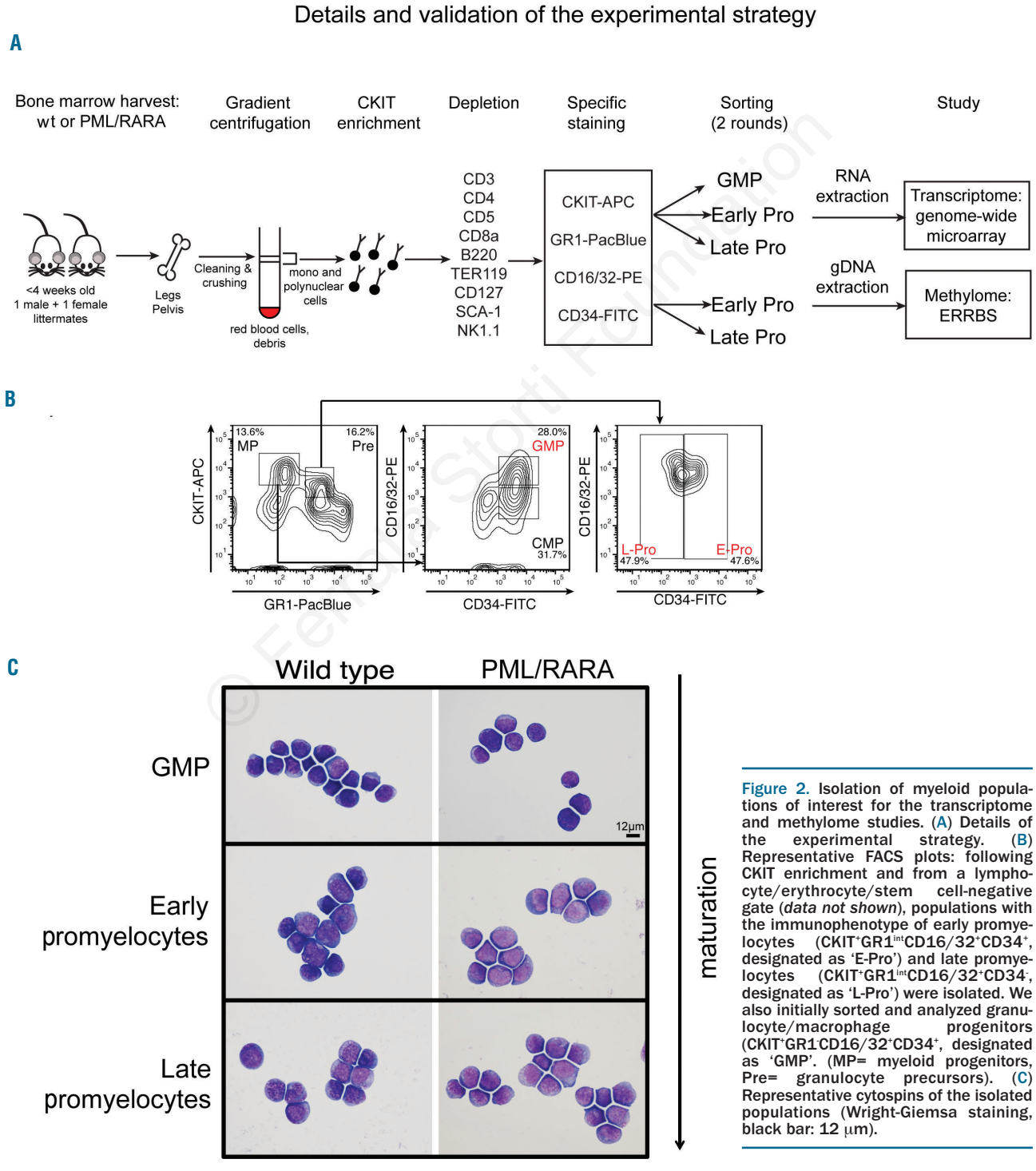

Figure 2. Isolation of myeloid populations of interest for the transcriptome and methylome studies. (A) Details of the experimental strategy. (B) Representative FACS plots: following CKIT enrichment and from a lymphocyte/erythrocyte/stem cell-negative gate (*data not shown*), populations with the immunophenotype of early promyelocytes (CKIT^{+}GR1^{int}CD16/32^{+}CD34^{+}, designated as 'E-Pro') and late promyelocytes (CKIT^{+}GR1^{int}CD16/32^{+}CD34^{-}, designated as 'L-Pro') were isolated. We also initially sorted and analyzed granulocyte/macrophage progenitors (CKIT^{+}GR1^{-}CD16/32^{+}CD34^{+}, designated as 'GMP'. (MP= myeloid progenitors, Pre= granulocyte precursors). (C) Representative cytospins of the isolated populations (Wright-Giemsa staining, black bar: 12 µm).

'preleukemic'/'PR') or their wild-type littermates, and processed for transcriptome and methylome profiling (Figure 2A,B).

Considering that the LRC in our cases of leukemia shows morphological and phenotypic characteristics of promyelocytes, we focused the majority of our analyses on the early promyelocyte ('E-Pro') and late promyelocyte ('L-Pro') populations, although the early granulocyte-macrophage progenitor (GMP) compartment was also isolated. Two rounds of sorting allowed us to reach near homogeneity (>95%) of the sorted cells, confirmed by post-sort flow cytometry (*data not shown*) and examination of cytospins (Figure 2C). As expected, accompanying maturation we observed a decrease in the nuclear:cytoplasmic ratio, lighter cytoplasm, and increasing primary granules, with no major morphological features distinguishing preleukemic from wild-type cells. Importantly, the expression pattern of cell surface antigens and myeloid markers

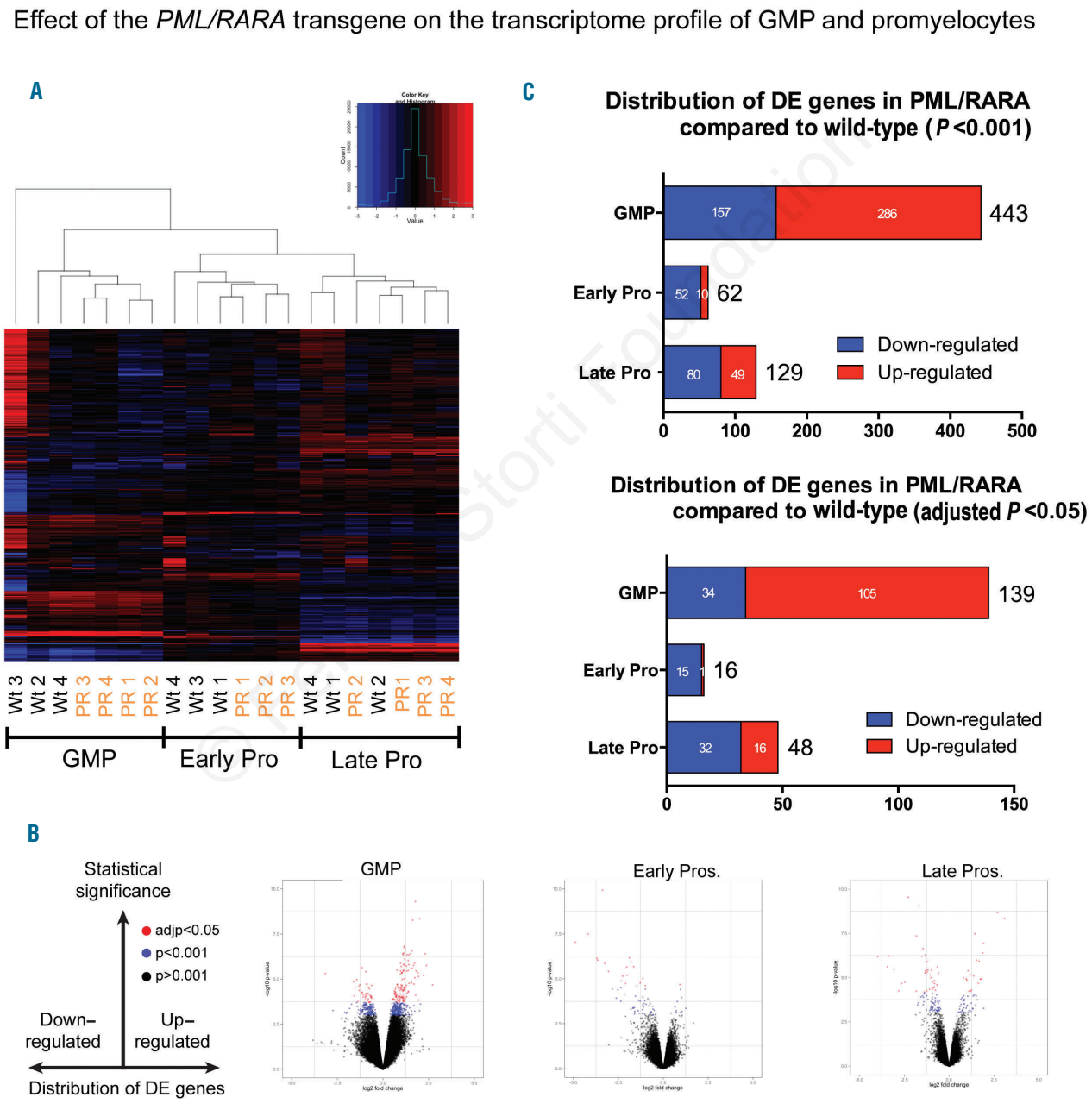

Figure 3. PML/RARA has a modest impact on the transcriptome. (A) Unsupervised clustering of samples showing segregation by maturation stages (median-centered heatmap, displaying the top 10% of probes by variance). (B) Volcano plots showing distribution of DE genes with PML/RARA expression in the different myeloid compartments, colored according to their statistical significance. (C) Distribution of DE genes between wild-type (wt) and PML/RARA GMP, early and late promyelocytes with a relaxed statistical cut-off of unadjusted *P*<0.001 (top) and with a more stringent statistical cut-off of adjusted *P*<0.05 (bottom).

validated our approach and we confirmed expression of the human transgene in all populations (*Online Supplementary Figure S1*).

PML/RARA alone has an unexpectedly limited impact on the transcriptome of preleukemic cells

RNA was prepared from these highly purified populations of GMP, E-Pro and L-Pro from wt and preleukemic mice and analyzed for differential gene expression utilizing genome-wide microarrays (Figure 2A). Unsupervised clustering of our samples showed segregation according to the stage of maturation (Figure 3A). However, samples did not all cluster by genotype, indicating a subtle effect of PML/RARA. Unexpectedly, volcano plots showed a predominance of gene up-regulation in the GMP, whereas in both the E-Pro and L-Pro populations the effect of the transgene was concordant with the described role of the fusion protein as a transcriptional repressor (Figure 3B).

Despite its ability to initiate APL and given the numerous previous reports describing PML/RARA as a strong transcriptional repressor, we found that the fusion protein surprisingly has only a modest effect on gene expression *in vivo*. Indeed, choosing a relaxed statistical cut-off of unadjusted $P<0.001$, we identified 443, 62 and 129 unique differentially expressed (DE) genes in the GMP, E-Pro and L-Pro populations, respectively (Figure 3C-top, *Online Supplementary Tables S1-S3* present the entire lists of DE genes). Using a more stringent statistical cut-off of adjusted $P<0.05$, we identified 139, 16 and 48 unique DE genes in the GMP, E-Pro and L-Pro populations, respectively (Figure 3C-bottom). To determine whether the deregulations observed are maintained along maturation, we assessed the overlap between our DE gene lists at the different stages. As seen in *Online Supplementary Table S4*, the strongest consistency was observed when comparing the E-Pro and L-Pro lists, indicating a continuing impact of the transgene in these populations. Importantly, we observed more DE genes in the L-Pro compartment, which correlated with stronger transgene expression (*Online Supplementary Figure S1B*), suggesting a dose-dependent effect.

Interestingly, although APL cells are characterized by a block of differentiation at the promyelocyte stage, PML/RARA-expressing preleukemic cells did not show altered expression of the master myeloid maturation regulators *Cebpa*, *Sfpi1* (PU.1), *Runx1* or *Gfi1* by microarray. However, we did observe differential expression of *Gata2* and *Irf8* (*Online Supplementary Figures S2* and *S5A*), two myeloid transcription factors which could potentially influence the course of myeloid maturation. We also observed, although to a lesser extent, some up-regulation in the presence of PML/RARA, with *Spp1* (osteopontin) being the most strongly up-regulated gene (8.5x, *Online Supplementary Figures S3* and *S5A*). Of note, we compared our lists of DE genes to those obtained in a prior study, which we present in *Online Supplementary Table S5* and discuss in the *Online Supplementary Document*.

PML/RARA alters the DNA methylation landscape of preleukemic promyelocytes in an unexpected direction

Whereas epigenetic alterations can be found in many acute myeloid leukemia samples, including fully transformed APL,[28,29] previous studies failed to identify any impact of PML/RARA on the DNA methylation landscape of preleukemic promyelocytes.[30] Applying the same protocol and stringent sorting strategy, E-Pro and L-Pro populations from wild-type or PML/RARA animals were isolated and analyzed by ERRBS to study their DNA methylation profiles. Mirroring the gene expression study, samples segregated by stage of maturation (Figure 4A). Methylation changes associated with PML/RARA expression were assessed revealing genome-wide changes at individual cytosines (differentially methylated cytosines, Figure 4B-top) and across 500 bp regions (differentially methylated regions, Figure 4B-bottom). Surprisingly, we identified a modest but clear pattern of altered DNA methylation with the fusion protein, but not in the expected direction. Indeed, hypomethylation was predominant in PML/RARA promyelocytes, contrary to our initial hypothesis that the fusion protein association with DNA methyltransferases would result in an increase in methylated DNA.

In the E-Pro and L-Pro populations, we identified differentially methylated regions associated with 193 and 449 unique genes, respectively (Figure 4C, full tables presented in *Online Supplementary Tables S6* and *S7*). As seen with the transcriptome dataset, the impact of PML/RARA seems to follow a dose-dependent pattern as the epigenetic profile was more strongly altered in the L-Pro compartment. However, we only found 11 loci similarly altered at the two promyelocyte stages, with a further seven undergoing a switch between the associated methylation change from the E-Pro to the L-Pro stage (*Online Supplementary Figure S4*). These results imply that DNA methylation *in vivo* is dynamic during maturation, and suggest that PML/RARA does not enforce a particular stable epigenetic landscape at most loci.

Oncogenic transcription factors are thought to alter chromatin conformation and transcriptional dynamics following their binding to DNA and recruitment of co-factors.[31] In light of previous studies showing co-recruitment of DNA and histone-modifying enzymes along with PML/RARA, we interrogated the interdependence between DE genes and differentially methylated regions. Using our lists of DE genes reaching $P<0.001$, we found three E-Pro and four L-Pro genes both associated with differential methylation and differential expression (Figure 4D). These data demonstrate that from a broad perspective, deregulation of gene expression by PML/RARA at the early steps of the leukemogenic process does not appear to result from altered methylation patterning on the DNA.

In PML/RARA preleukemic cells, expansion of the promyelocyte compartment and increased proliferation uniquely associate with the ability to initiate leukemia

To further characterize the impact of PML/RARA, we performed gene set enrichment analysis (GSEA) on our dataset using the C2 (curated) and C5 (gene ontology) gene set databases. Importantly, results highlighted enrichment for cell cycle and mitosis-related genes with PML/RARA in the E-Pro compartment, a novel finding not previously reported in an *in vivo* primary preleukemic context (*Online Supplementary Table S8*-green highlights and Figure 5A). Intrigued by these results, we further investigated the effect of PML/RARA on cell proliferation and, to delineate the association between phenotype and ability to initiate leukemia, we also investigated the p50/RARA transgenic line. This fusion, described by Lin and Evans,[32] has the benefit of recapitulating many features of the PML/RARA fusion protein (homodimerization, binding to retinoic acid-responsive elements, differentiation block *in*

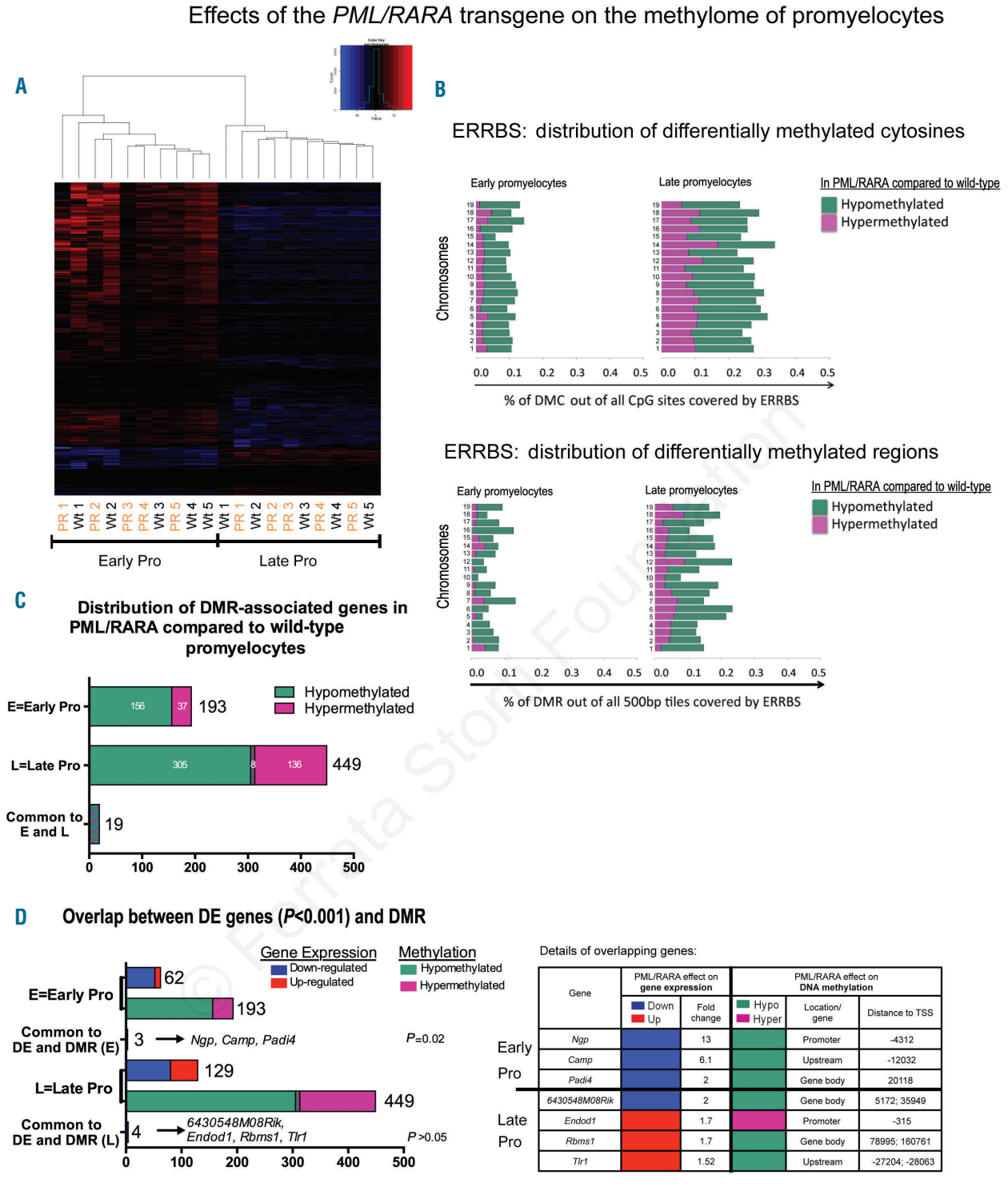

Figure 4. PML/RARA has a modest impact on the methylome. (A) Hierarchical clustering by methylation data, based on regions common to all samples, shows segregation by maturation stages. (B) Chromosomal distribution of differentially methylated cytosines (DMC-top) and differentially methylated regions (DMR-bottom) between PML/RARA and wild-type early and late promyelocytes. A total of 862 and 2,210 DMC were identified in the early and late promyelocyte comparisons, respectively (methylation difference >25% and q-value <0.01). A total of 136 and 347 DMR were identified in the early and late promyelocyte comparisons, respectively (methylation difference >25% and q-value <0.01). (C) Distribution of unique DMR-associated genes in PML/RARA promyelocytes compared to wild-type promyelocytes. A total of 193 and 449 genes were associated with the 136 and 347 DMR identified in the early and late promyelocyte comparisons, respectively (see ERRBS methods for annotation details). Eight genes associated with both hypomethylated and hypermethylated DMR in the late promyelocyte population. Of the 19 genes associated with DMR in both the early and late promyelocyte comparisons, the methylation changes were inconsistent (see *Online Supplementary Figure S4*). (D) Differentially expressed (DE) genes poorly correlate with DMR (left). The table (right) presents details on the transcript fold change and methylation changes and location (TSS= transcriptional start site). Refer to the *Online Supplementary Methods* for details on list generation, overlaps and statistics.

vitro). However, it is a poor initiator of leukemia *in vivo*,[33] making it a relevant tool to identify aspects of the preleukemic phenotype critical to the leukemogenic potential.

The blast/promyelocyte fraction was previously shown to be expanded in PML/RARA transgenic animals.[34] In this study, we provide additional data demonstrating that the expansion correlates with the acquisition of the promyelocyte immunophenotype, and is absent from the p50/RARA model (Figure 5B). To further delineate possible mechanisms behind this expansion, we investigated cell cycle kinetics by looking at the bromodeoxyuridine (BrdU) incorporation profile of sorted promyelocytes. Strikingly, we found that cells expressing PML/RARA have increased proliferation, as shown by enhanced BrdU incorporation, a phenotype that was confirmed both *in vitro* and *in vivo* (Figure 5C). Further establishing enhanced proliferation as a key feature for cells to be able to initiate a leukemogenic program, p50/RARA promyelocytes show no alteration of cell cycle kinetics compared to wild-type promyeloctes (Figure 5D).

PML/RARA alters part of the myeloid maturation program, in both murine preleukemic cells and human acute promyelocytic leukemia samples

Although PML/RARA promyelocytes are able to terminally differentiate *in vivo* at the preleukemic stage, we identified several aspects of the myeloid maturation program that are altered by the fusion protein, providing the first step towards a full differentiation block during leukemogenic progression.

Notably, within the list of DE genes reaching the statistical cutoff of P=0.001 or below, we identified a subset consistently dysregulated by PML/RARA at both the E-Pro and L-Pro stages. Among these 13 genes (represented by 15 probes), presented in *Online Supplementary Table S4*

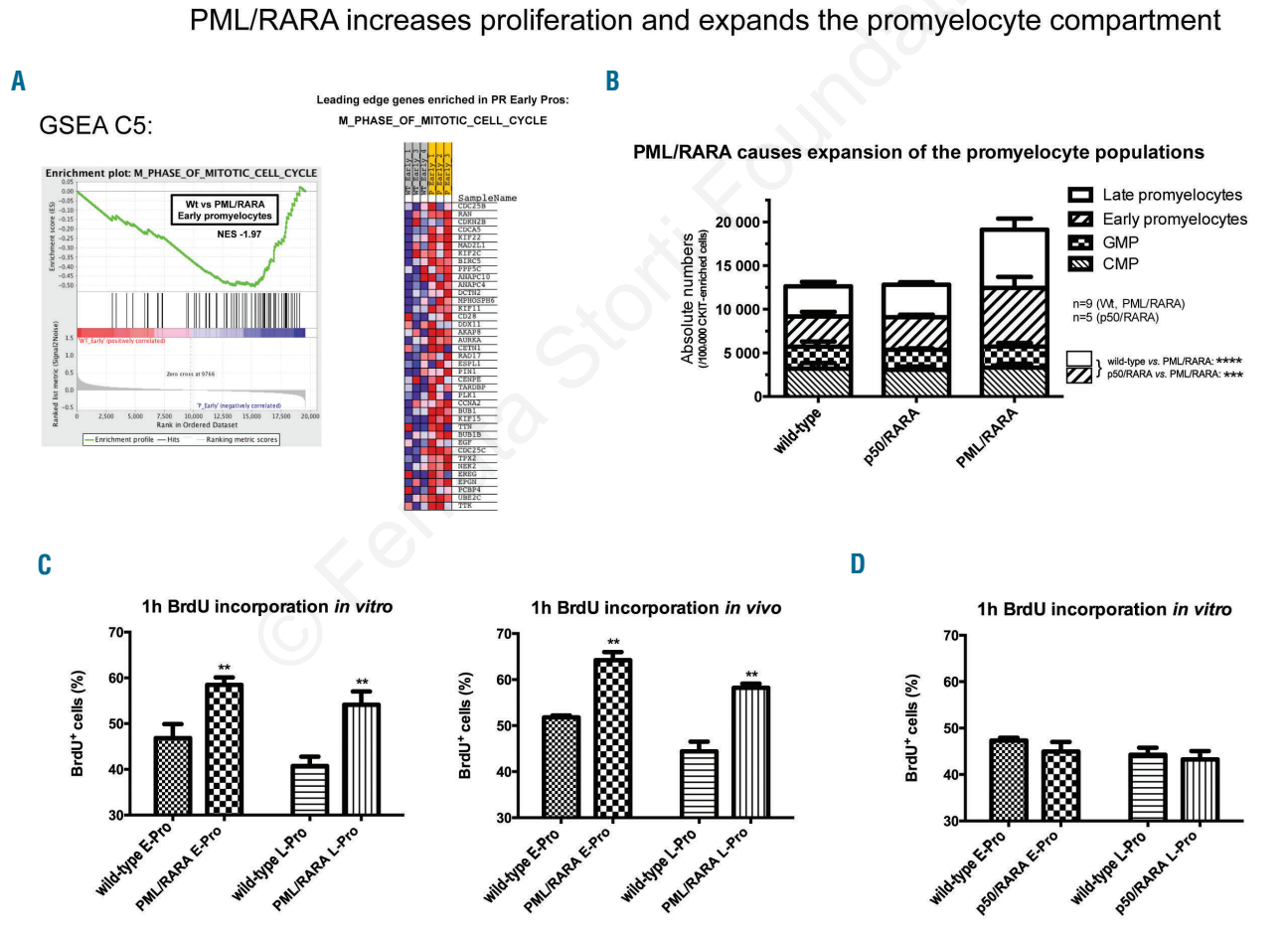

Figure 5. Increased proliferation in PML/RARA promyelocytes correlates with the ability to initiate leukemia and is dependent on the PML portion of the fusion, further implicating this moiety of PML/RARA as central to disease initiation. (**A**) Enrichment plot and heatmap of the leading edge genes for the GSEA C5 hit 'M_PHASE_OF_MITOTIC _CELL_CYCLE' in early promyelocytes. NES= normalized enrichment score. (**B**) Absolute numbers of cells in the common myeloid progenitor (CMP), GMP, early and late promyelocyte compartments (out of 100,000 CKIT-enriched cells) in wild-type, p50/RARA and PML/RARA bone marrow. A two-tailed t-test was performed to assess statistical significance of differences between groups (***P<0.001, ****P<0.0001). (**C**) Percentage BrdU$^+$ cells following 1 h incorporation *in vitro* and *in vivo* in wild-type *vs.* PML/RARA early and late promyelocytes. For all BrdU experiments, each bar represents the mean of technical triplicates with standard deviation, and the figure is representative of three independent experiments. A two-tailed t-test was performed to assess statistical significance of differences between groups (**P<0.01). An increase of approximately 24% and 32% of BrdU$^+$ cells was observed in the PML/RARA E-Pro and L-Pro, respectively. (**D**) Percentage BrdU$^+$ cells following 1 h incorporation *in vitro* in wild-type *vs.* p50/RARA promyelocytes.

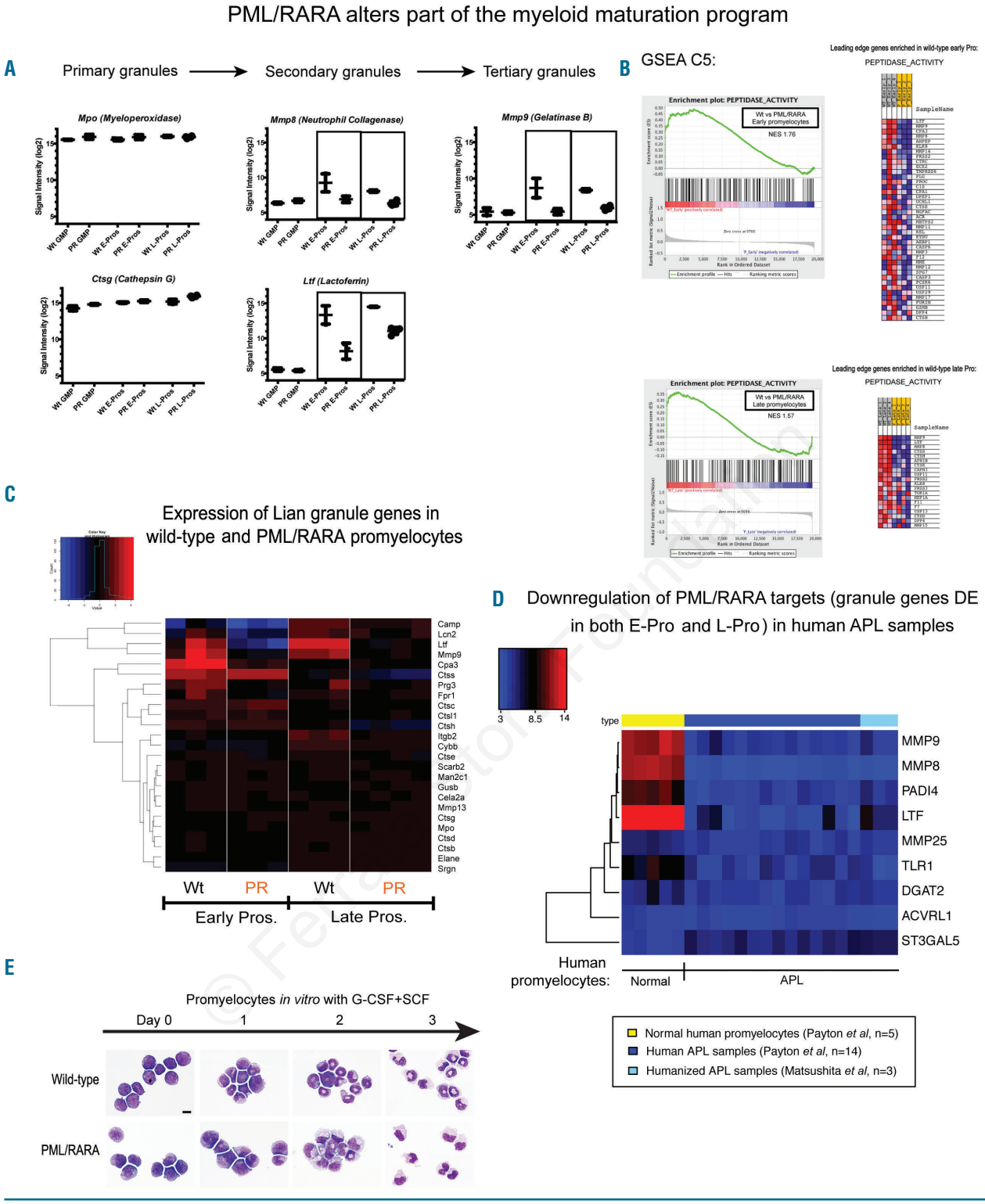

Figure 6. PML/RARA alters part of the myeloid maturation program, including by down-regulating a subset of secondary and tertiary granule genes normally induced during myeloid maturation. (A) Expression data from the microarray showing transcript levels of primary, secondary and tertiary granule genes. The Y axis displays the raw signal intensity with standard deviation off the array for each biological replicate (log_2 scale). The X axis displays the six groups interrogated, from the more immature to the more mature (from left to right: GMP, E-Pro, L-Pro) stage, in the FVB/n (Wt) or PML/RARA (PR) samples. (B) Enrichment plots and heatmaps of leading edge genes for the GSEA C5 hit 'PEPTIDASE_ACTIVITY' in the early (top) and late (bottom) promyelocyte populations showing enrichment in Wt relative to PR. NES= normalized enrichment score. (C) Supervised median-centered heatmap showing expression of genes included in the top GSEA C2 hit 'LIAN_NEUTROPHIL_GRANULE_CONSTITUENTS' (Lian *et al.*, Blood 2001;98(3):513-524) (Wt= FVB/n, PR= PML/RARA). (D) Unsupervised log_2-transformed heatmap shows gene expression in normal human promyelocytes (yellow, n= 5) as compared with both human PML/RARA APL (dark blue, n= 14) and humanized APL samples grown in murine recipients (light blue, n= 3). Nine of the 13 common genes highly dysregulated by PML/RARA at the early and late promyelocyte stages (*Online Supplementary Table S4*) could be assessed in the Payton and Matsushita datasets (Payton *et al.*, JCI 2009;119(6):1714-1726, Matsushita *et al.*, PLoS One 2014;9(11):e111082). (E) Sorted Wt and PML/RARA late promyelocytes were plated in the presence of G-CSF+SCF, and differentiation followed for 3 days. Wells were harvested every 24 h for cytospins. Unlike with PML/RARA, Wt promyeloyctes all matured into neutrophils by day 3 (Wright-Giemsa staining, black bar: 8 μm).

('CommonEarly-Late'), many belong to the family of neutrophilic granule genes, reaching a 35x down-regulation in the case of *Ltf* (lactoferrin, Figure 6A and *Online Supplementary Figure S5A*). Interestingly, this down-regulation affected only secondary and tertiary granule genes as primary granule expression levels remained unchanged (Figure 6A). Importantly, this observation was backed up by several GSEA results. When analyzing our expression data against the C5 database, results highlighted enrichment for peptidase activity in the wild-type E-Pro and L-Pro populations (Figure 6B and *Online Supplementary Table S8*-orange highlights). In addition, by GSEA C2 analysis (*Online Supplementary Table S9*-blue highlight), our dataset strongly associated with the results of Lian *et al.*,[35] who investigated the dynamics of neutrophilic granule expression. The heatmap generated with our promyelocyte expression data on this gene set illustrates that an important subset of granule genes is repressed by the presence of PML/RARA (Figure 6C). To investigate whether these findings were relevant to human leukemia, we explored data from Payton *et al.*[36] and Matsushita *et al.*[37] in which gene expression was compared in human APL samples and in humanized APL samples grown in murine recipients, respectively, *versus* normal human promyelocytes. As seen in Figure 6D, human APL samples show strong down-regulation of a subset of the genes most repressed in the mouse promyelocytes that express PML/RARA, mainly secondary granule genes. We thereby confirmed that target genes of PML/RARA in our murine model are also targets of PML/RARA in human APL samples derived from two different experimental models.

While the inability to express this subset of genes normally required for terminal differentiation does not appear sufficient to block maturation *in vivo*, we investigated the differentiation profile of sorted promyelocytes *in vitro* when cultured in the presence of pro-neutrophilic cytokines. As shown in Figure 6E, PML/RARA cells demonstrate impaired ability to complete neutrophilic differentiation. Hence, the fusion protein does alter the ability of cells to complete terminal differentiation, with such a phenotype more evident in an *in vitro* context.

Discussion

We aimed to understand mechanisms by which PML/RARA may initiate leukemia by examining how patterns of gene expression and methylation are altered in 4-

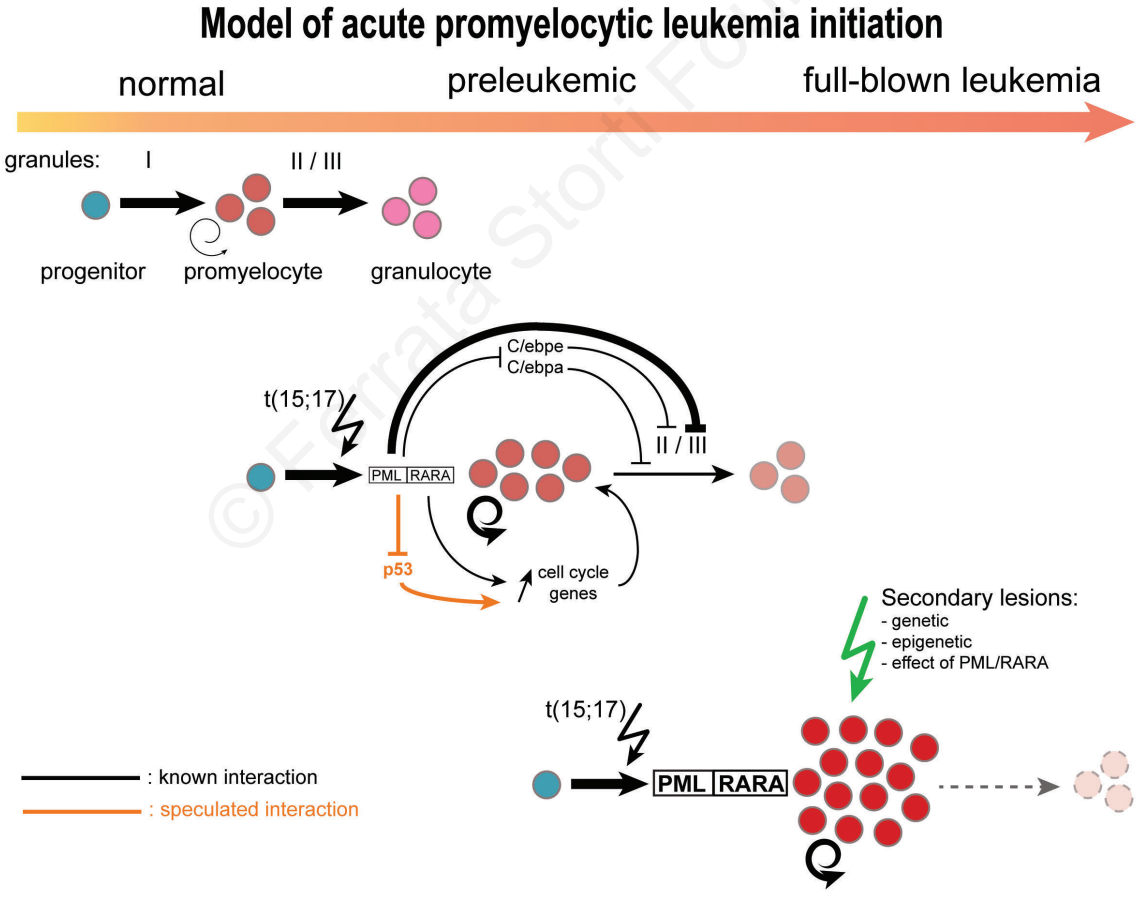

Figure 7. Model of APL initiation. As opposed to mediating broad cellular effects as commonly believed, PML/RARA initially exercises subtle changes within the promyelocyte compartment. *Cebpe* and *Cepba* appear modestly altered, which could nevertheless contribute to both the impaired ability to differentiate and the strong down-regulation of secondary and tertiary granule genes. As demonstrated for the *Ltf* gene, PML/RARA also directly represses transcription. The fusion protein also leads to a modest increase in cell cycle genes, which promotes proliferation and the expansion of the promyelocyte compartment, possibly through deregulation of a p53-mediated axis. Nonetheless secondary lesions need to accumulate in these cells in order to bypass the dominant maturation program to generate an acute phenotype, provided by additional mutations, epigenetic changes, and/or cumulative effects of the fusion protein itself.

week old mice expressing the *MRP8-PML/RARA* transgene, months prior to the acquisition of additional genetic lesions and the development of leukemia. As no preleukemic state can be studied in humans, this approach provides a unique tool to identify the effects mediated by PML/RARA, in the population most similar to the LRC in this model. It should be noted, however, that although our study focuses on the promyelocyte compartment, the PML/RARA translocation or the additional cooperative changes allowing the leukemic clone to arise in human APL might be acquired in earlier cellular compartments.

Briefly, our data demonstrate that PML/RARA markedly alters granule gene expression, while mediating an overall mild effect on the transcriptome and methylome. We also found that PML/RARA-expressing cells show an increased ability to proliferate, a phenotype which correlates with the ability to initiate leukemia. Collectively, our data support a model of leukemogenesis in which PML/RARA initiates leukemia by subtly shifting the balance within the promyelocyte compartment between continued proliferation and terminal maturation. Nevertheless, additional events are required to overcome the normally dominant program of terminal maturation and allow unchecked growth of transformed promyelocytes. Of note, we performed limited gene expression studies in fully transformed leukemic cells generated in our model (*Online Supplementary Figure S6* and *Online Supplementary Table S10*), which we discuss briefly in the *Online Supplementary Document*.

Deregulation of the epigenetic landscape was overall modest and hypomethylation was the predominant change in preleukemic cells expressing PML/RARA. Importantly however, we did identify consistent changes in all biological replicates at the preleukemic stage, contrasting with the findings of a previous study.[30] We did not observe a significant correlation between differential methylation and differential expression, but wondered whether genes associated with differentially methylated regions might develop differential expression at the later time-point of 12 weeks of age. Results presented in *Online Supplementary Figure S5B* do not indicate that this is the case. Of note, there has been a report of PML/RARA targeting epigenetic modifiers.[38] In the preleukemic context, although we found no differential expression of the DNA methylating enzymes nor of *Tet* genes involved in DNA hydroxymethylation, the chromatin modifiers *Cbx2* and *Padi4* were differentially expressed, possibly participating in alteration of chromatin conformation.

The strongest DE gene signature we observed was the down-regulation of secondary and tertiary granule genes. Interestingly, PML/RARA did not dampen expression of primary granule genes, providing a biological explanation for the excessive accumulation of these granules in APL cells. Of note, *Cebpe* has been described to control expression of such targets[39,40] and we did observe a slight down-regulation of *Cebpe* in our microarrays (decreased approximately 30%, but not reaching $P<0.001$). As noted in *Online Supplementary Figure S5B*, quantitative polymerase chain reaction data revealed modest down-regulation of *Cebpa*, which similarly did not reach $P<0.001$ in our microarrays, but when studied by quantitative polymerase chain reaction in similarly isolated cells was decreased 1.85x, with $P=0.01$. There is a possibility that this less than 2-fold down-regulation of *Cebpa* and *Cebpe* by PML/RARA plays a significant role in the observed large changes in granule gene expression. Interestingly, *Cebpa* was in a region of hypermethylation in PML/RARA E-Pro, indicating that although not a global mechanism in these cells, increased DNA methylation may be associated with subtle gene down-regulation at some loci.

How PML/RARA leads to the changes of gene expression and methylation we observed is an unanswered question. Within our top four most DE genes and a subset of differentially methylated regions selected from *Online Supplementary Figure S5B*, we found several RARA half sites within 20 bp of each other (*Online Supplementary Table S12*), a conformation representing a possible PML/RARA binding site.[17] To assess whether PML/RARA had been previously shown to directly target genes we identified as DE or as differentially methylated regions, we utilized a dataset of PML/RARA binding sites identified in human cells by Martens *et al.*[41] We found that the overlap between Martens' binding sites and our DE genes and differentially methylated regions was minimal (*Online Supplementary Figure S7A,B*). Finally, since we observed putative PML/RARA binding sites in our most down-regulated gene *Ltf*, we investigated binding of the fusion protein using chromatin immunoprecipitation. We confirmed binding and enrichment of PML/RARA at the *Ltf* locus (*Online Supplementary Figure S7C*), providing a mechanistic explanation for the strong deregulation observed.

Our data extend previous studies linking PML/RARA to altered proliferation,[42-44] providing the novel finding that altered cell-cycle kinetics is a feature of PML/RARA-expressing cells at very early stages of the leukemogenic process. We found that the enhanced capacity for promyelocytes to proliferate depends on the PML part of the fusion and correlates with the ability to initiate leukemia, in line with previous findings.[45,46] One outcome of *RARA* fusing to *PML* is the disruption of the *PML* nuclear bodies, known to control protein activity and stability through sequestration or post-translational modifications.[47] p53 activity, which plays a key role in ensuring appropriate responses to genotoxic stress, is modulated by normal PML, and this interplay is deregulated by PML/RARA (Ablain *et al.*[48] and references therein). Notably, several genes associated with the GSEA mitosis signature have been described to be direct targets of p53 for down-regulation (*Online Supplementary Table S8*). Up-regulation of those genes in PML/RARA promyelocytes might reflect deactivation of p53-mediated control as a consequence of nuclear body disruption.[48] Our data raise the hypothesis that efficient initiation of leukemia by PML/RARA may depend upon p53 deregulation rather than arising solely from the transcriptional properties of the fusion protein (Figure 7).

In this work we describe the impact of PML/RARA in carefully isolated myeloid cells *in vivo*, using a relevant murine model allowing for such studies. The data presented provide novel insights into the cellular effects of the fusion protein at early stages of leukemogenesis, but some caveats must be noted. The microarray and ERRBS analysis performed provide extended but not absolute coverage of the genome, and future experiments using RNA-seq and whole-genome coverage of methylation status might reveal additional effects of PML/RARA on regions not covered by our assays (additional non-coding genes, low-methylation regions as recently described[49]). Furthermore, although we provide evidence that gene down-regulation of *Ltf* occurs through direct targeting by the fusion protein, we were

unable to determine to what extent the changes identified were mediated by direct or indirect effects of PML/RARA. Nevertheless, our descriptive study provides the basis for interesting hypotheses that warrant testing. In particular, the role of p53 in the deregulated cell-cycle kinetics must be investigated. If alteration of p53 activity results from nuclear body disruption, it would also be interesting to study more broadly how post-transcriptional modifications are altered in PML/RARA-expressing cells, and to what extent the alterations contribute to leukemia initiation. We show that the predominant phenotype of PML/RARA-expressing cells (down-regulation of secondary and tertiary granule genes) is also a feature of human APL blasts. It should be noted however that leukemogenesis may differ between a mouse model and humans, and alternative APL models to our own (including those described by Westervelt *et al.*,[8] Matsushita *et al.*,[37] and Minucci *et al.*,[43] among others) also provide relevant settings to study PML/RARA-mediated transformation. Finally, further work to identify how cooperating lesions allow initiated cells to transition into full-blown leukemic blasts remains critical for understanding APL leukemogenesis.

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