Supporting Information

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SI Materials and Methods

Plasmids. The RARα-PLZF and PLZF-RARα fusion genes were cloned into the EcoRI site of two retroviral vectors that are based on Moloney murine leukemia virus, pBabe, as well as Moloney murine sarcoma virus, MSCV. These vectors contain a selectable marker, the green fluorescent protein (GFP) or the puromycin-N-acetyltransferase (puro or pac). The HA-hC/EBPα and hJUN-HA were kindly provided by D. G. Tenen (Harvard Medical School, Boston, MA) and by D. Bohmann (University of Rochester Medical Center, Rochester, NY), respectively. RARα-PLZF and PLZF-RARα were tagged at their N-terminal region in frame with a HA tag by PCR cloning, respectively, in pcDNA3.1 using the EcoRI and BamHI sites and then transferred in the HpaI and BglII sites of MSCV-neo. RARα-PLZF and hJUN were also tagged at their C-terminal region with the TAP tag, a gift from B. Coulombe (Clinical Research Institute of Montreal), within the retroviral vector MSCV-neo. A GFP-tagged protein RARα-PLZF was also obtained by subcloning the EcoRI/BamHI fragment containing the cDNA in the pEGFP-C2 expression vector (Clontech). These constructs were verified by sequencing. Both the HA and GFP tags did not alter the transcriptional properties of RARα-PLZF in transient transfection assays. The GST-C/EBPα and GST-HDAC1 in pGEX-ZT (Pharmacia), an expression vector for GST fusion proteins, were given by D. G. Tenen and C. Seiser (Medical University of Vienna, Austria), respectively. The wild-type and C/EBP binding site mutant h*CSF3R* proximal promoter (−1324 + 67) as well as a tetramer of this C/EBP binding site were kindly provided by D. G. Tenen and subcloned in the pXPII luciferase-reporter vector.

Chromatin Immunoprecipitation. The following oligonucleotides were used to amplify the mouse *Csf3r* promoter (ACCTGAAGTAAACGTTACCTG and TCCCGAAACTTCTCTTTCTAG) and the *Kit* promoter (GCGGGCAGTCGACCTTTAT and TCGACGTGTTAATGCCGGG).

Antibodies. Antibodies against C/EBPα (Santa Cruz Biotechnology, sc-61), HA (Covance, MMS-101R), HDAC1 (Santa Cruz Biotechnology, sc-8410 used in Western blot and IP), HDAC1 (gift from Alain Verrault, used for ChIP), c-Jun (Santa Cruz Biotechnology, sc-44), TAP (Open Bioscience CAB1001) as well as acetyl histone H3 (Upstate Biotechnologies, 06–599), RARα (Sigma-Aldrich, SAB1100358), G-CSFR (Santa Cruz Biotechnology, sc-9173), β-actin (Sigma-Aldrich, A5441), rabbit preimmune serum (Sigma-Aldrich), or rabbit IgG (Sigma-Aldrich, I-8140), goat anti-mouse and rabbit anti-goat peroxidase conjugated polyclonal antibodies (Calbiochem-Novabiochem International, 401215 and 401515, respectively), goat anti-rabbit conjugated HRP polyclonal antibody (Biorad, 172–1034), and goat anti-mouse conjugated HRP polyclonal antibody (Biorad, 170–6516) were used for immunoprecipitation, Western blotting, or chromatin immunoprecipitation.

Antibodies Used for Flow Cytometry Analysis. The Gr-1 and CD11b antibodies used were obtained from the hybridomas RB6-8C5 and M1/70.15.11.5HL (ATCC), respectively. Fluorescein isothiocyanate (FITC)-conjugated Annexin V (Biodesign) and fluorescinated goat anti-mouse (Caltech), fluorescein-conjugated anti-CD11b and biotinylated Gr-1 antibodies (eBiosciences, 11–0112 and 25–5931, respectively) were also used.

Electrophoretic Mobility Shift Assays. DNA binding assays were performed using nuclear extracts (10 μg) from 32D cells with the C/EBP site of the *CSF3R* (−57 to −37) promoter (1): wild type, AAG GTG TTG CAA TCC CCA GCC, and mutant AAG GTG TTC ACC AAC CCA GCC. The binding reaction was allowed to proceed for 30 min on ice in 50 mM Tris pH 7.8, 10 mM DTT, 250 mM KCl, 5% glycerol, 5 mg/mL of BSA and 300 ng of poly (dIdC) per reaction. Where indicated, proteins were preincubated for 30 min on ice with 2 μg of rabbit polyclonal antibodies against C/EBPα (Santa Cruz Biotechnology) or with unlabeled double-stranded oligonucleotide competitors at a molar excess of 100-fold. Protein–DNA complexes were resolved by electrophoresis on acrylamide gels. Gels were dried and exposed to a PhosphoImager screen (Molecular Dynamics).

GST–Pull-Down Assay. In vitro translated proteins were synthesized with the TNT-coupled reticulocyte lysate system (Promega) and labeled with [^{35}S]-Met (Amersham-Pharmacia) according to the manufacturer's protocol.

DNA Capture. DNA capture assay was performed as described previously (2). Biotinylated promoter fragments were amplified by PCR using 5′ biotinylated oligonucleotides from wild-type or C/EBPα mutated plasmids. Equal molar quantities of biotinylated DNA templates were then immobilized on magnetic resins conjugated with streptavidin according to the manufacturer's instructions. Nuclear extracts (NE) from BOSC cells overexpressing C/EBPα and 32D overexpressing TAP or RP-TAP were mixed and incubated with immobilized DNA templates in binding buffer (20 mM Tris [pH 8.0], 10% glycerol, 6.25 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.01% Nonidet P-40) in a final concentration of 100 mM NaCl and in the presence of an excess of 0.2μg/μL of Poly (dI-dC). After mixing, the samples were incubated by rotation for an hour at 30 °C. The resin coated with immobilized templates was then washed twice with binding buffer. The proteins bound to the immobilized templates were eluted from the templates by boiling of the beads for 5 min in SDS/PAGE sample buffer. Proteins in SDS/PAGE sample buffer were resolved on polyacrylamide gels and transferred to PVDF membranes for Western blot analysis.

1. Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG (1996) PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 88(4):1234–1247.

2. Grondin B, et al. (2007) c-Jun homodimers can function as a context-specific coactivator. *Mol Cell Biol* 27(8):2919–2933.

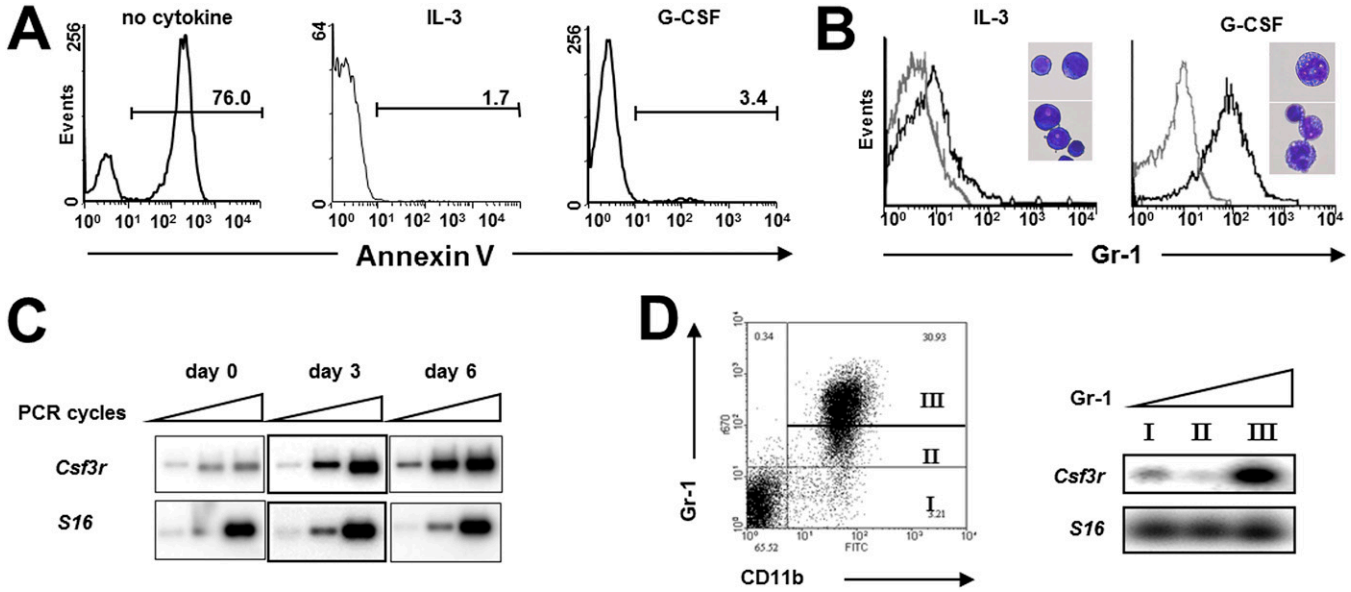

Fig. S1. G-CSF induces differentiation in myeloid 32D cells. (*A*) IL-3 or G-CSF suppresses apoptosis in 32D cells: The 32D immature myeloid cells were treated with IL-3 or G-CSF or deprived of cytokine for 24 h. Apoptosis was assessed by Annexin V labeling. (*B*) G-CSF treatment up-regulates Gr-1 expression. The 32D cells maintained in IL-3 or treated with G-CSF for 6 d. The 32D cells were labeled with Gr-1 and analyzed by flow cytometry. Black lines, Gr-1 staining; and gray lines, isotype-matched control antibody. *Insets*: cell morphology after 3 d of stimulation with IL-3 or G-CSF. (*C*) G-CSF treatment up-regulates *Csf3r* mRNA in 32D cells. Semiquantitative RT-PCR analysis of 32D cells undergoing granulocytic differentiation at the indicated times after G-CSF treatment. Amplification of ribosomal *S16* serves as a control for variations in input RNA. (*D*) Increased *Csf3r* expression in mature $Gr1^+$ granulocytes. $CD11b^+$ primary bone marrow cells were sorted into three fractions on the basis of Gr1 staining as indicated (*Left*). RT-PCR was performed as above on sorted cells (*Right*).

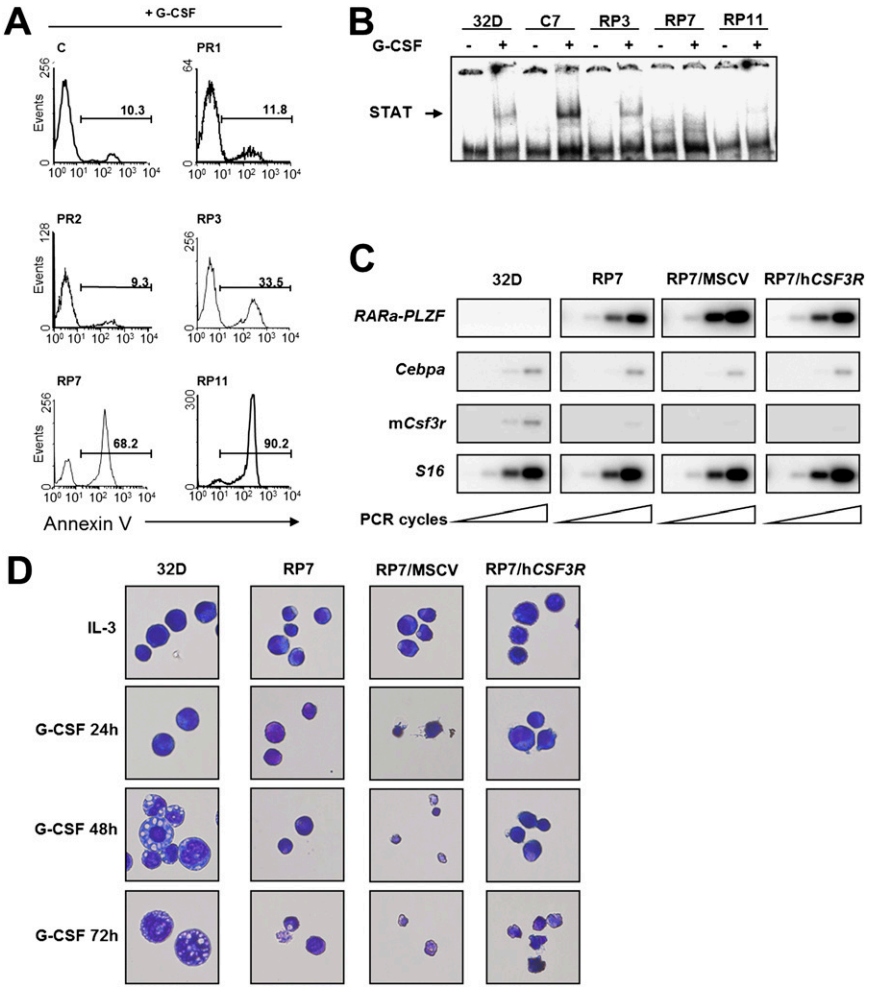

Fig. S2. The 32D cells expressing RAR-PLZF fail to respond to G-CSF treatment. (*A*) Flow cytometry analysis of 32D clones maintained in the presence of G-CSF. The 32D cells expressing the empty vector (C), PLZF-RARα (PR1, PR2), RARα-PLZF (RP3, RP7, RP11) were maintained with IL-3, washed, and then exposed to G-CSF for 24 h. Cells were labeled with Annexin V-FITC for apoptosis. Dead cells were excluded by propidium iodide staining. Data are representative of two to five independent experiments. (*B*) RARα-PLZF decreases STAT activation by G-CSF. Control cells (32D and C7) or RARα-PLZF (RP3, RP7, and RP11) expressing cells were exposed or not to G-CSF (30 min) after 2 h of growth factor deprivation. Gel retardation analysis of nuclear extracts was performed using a ^{32}P-labeled c-fos SIE probe. (*C*) Confirmation of *muCSF3R, C/EBPα and RARα-PLZF* expression in RP7 transfected cells. RP7 cells were either transduced with the hu*CSF3R* expression vector (RP7/hu*CSF3R*) or the empty MSCV vector (RP7/MSCV). Expression levels of *muCSF3R*, *C/EBPα*, and *RARα-PLZF* were measured by semi-quantitative RT-PCR. S16 is shown as a control for variations in input RNA. (*D*) Morphological analysis of 32D, RP7, RP7/MSCV, and RP7/huCSF3R upon G-CSF treatment. The 32D cells differentiated toward granulocytes, whereas RARα-PLZF expressing cells underwent apoptosis without differentiation. Ectopic expression of hCSF3R rescued apoptosis for 48 h but cells failed to survive or differentiate beyond that time point.

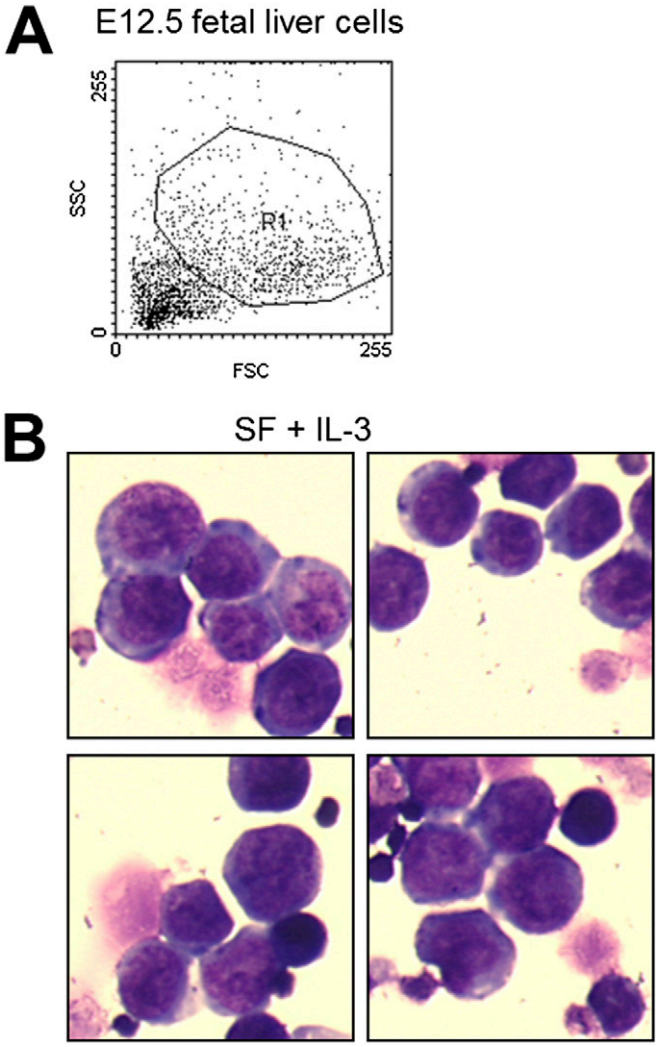

Fig. S3. Analysis of E12.5 fetal liver cells phenotype. (*A*) FACS analysis of E12.5 fetal liver cells. (*B*) Cytospin of E12.5 fetal liver cells. E12.5 fetal liver cells exposed to SF and IL-3 for 48 h contain mostly undifferentiated myeloid cells.

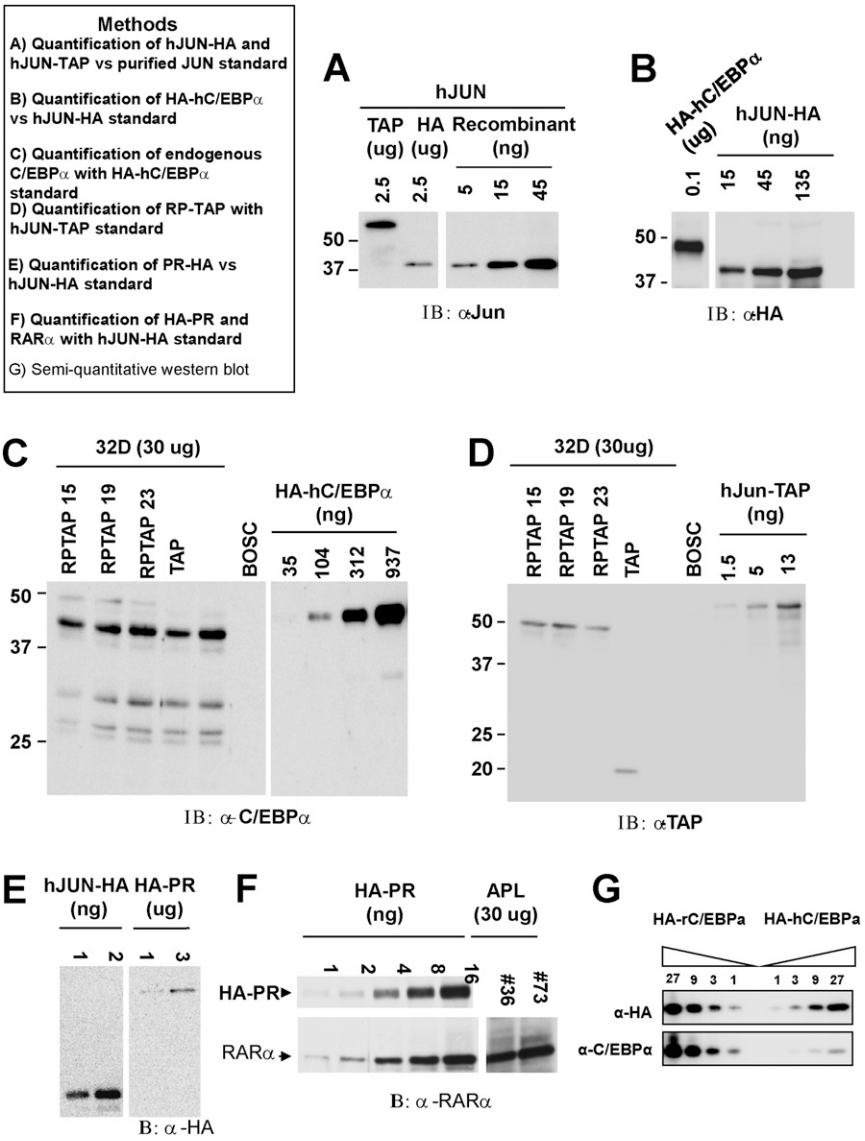

Fig. S4. Design of a semiquantitative Western blot to evaluate C/EBPα, RARα-PLZF, and RARα protein levels in 32D RP-TAP clones and in APL patients. (*A*) Quantification of hJUN-HA and hJUN-TAP using purified JUN protein. The concentrations of JUN protein in total protein extracts from BOSC cells expressing hJUN-HA and hJUN-TAP were estimated from a standard curve of purified recombinant JUN protein by semiquantitative Western blot analysis using antibodies against JUN. (*B*) Quantification of HA-hC/EBPα using hJUN-HA standard. A hJUN-HA standard curve (assessed in *A*) allows quantification of HA-hC/EBPα expressed in BOSC cells using an antibody against the HA tag. (*C*) Quantification of HA-PR using hJUN-HA standard. HA-PLZF-RARα concentrations were determined as in *B*. (*D*) Quantification of endogenous C/EBPα with the HA-hC/EBPα standard. C/EBPα protein levels in 32D cells and subclones were estimated against a standard curve of HA-hC/EBPα (assessed in *B*) using an antibody against C/EBPα. (*E*) Quantification of RP-TAP with hJUN-TAP standard. Semiquantitative Western blots of RARα-PLZF fusion protein were done as in *D* on 32D RPTAP clones using hJUN-TAP standard curve (assessed in *A*) and an antibody against the TAP tag. (*F*) Quantification of RARα using HA-PR standard. Antibody against RARα was used to quantify the protein amount of RARα in APL patients samples which were extrapolated from an HA-PR standard curve (assessed in *E*). The anti-RARα recognizes both wild-type RARα and PLZF-RARα proteins with the same efficiency. (*G*) Semiquantitative Western blot. Signals from tagged protein standards expressed in BOSC cells (HA-hC/EBPα or HA-rC/EBPα,) with antibodies against HA or C/EBPα.

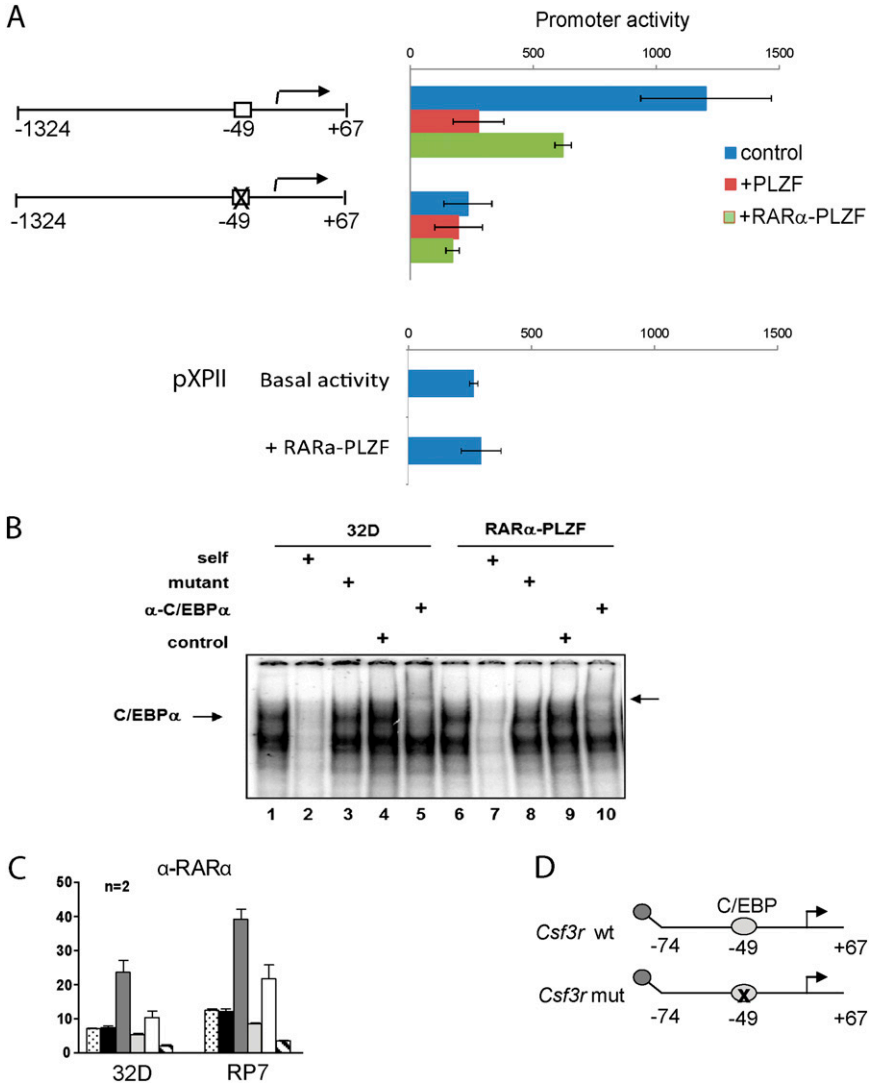

Fig. S5. C/EBPα DNA binding is not affected by RARα-PLZF but is required for transcription repression by PLZF and RARα-PLZF. (*A*) *CSF3R* promoter activity in 32D cells. The activity of the *CSF3R* promoter construct harboring or not a mutation at the C/EBP consensus sequence (as illustrated, *Upper*) and the empty luciferase reporter (pXPII, *Lower*) was tested by transient transfection in 32D in the presence of the empty MSCV vector (control), PLZF, or RARα-PLZF. Data represent the mean ± S.E.M of triplicate determinations and are representative of three independent experiments. (*B*) Gel retardation analysis of nuclear extracts from 32D cells or RARα-PLZF transfectants, using a ^{32}P-labeled C/EBPα probe derived from −70 to −40 of the *CSF3R* promoter. (*Left*) Arrow points to the band that was supershifted by an antibody to C/EBPα (*Right*, arrow). (*C*) Chromatin extracts from parental 32D cells or from RARα-PLZF expressing cells (RP7) were immunoprecipitated with an antibody against the RARα Nt domain (recognizing both wild-type RARα and RARα-PLZF) or a control rabbit IgG. Data are presented as fold enrichment over parental 32D cells. Because RARα-PLZF did not affect RARα protein levels in 32D cells (Fig. 2*C*), the twofold enrichment in myeloid promoter occupancy in RP7 cells suggests that RARα-PLZF occupies C/EBPα target promoters in 32D cells. (*D*) Illustration of the *Csf3r* promoter sequences that were immobilized on magnetic beads for DNA capture.

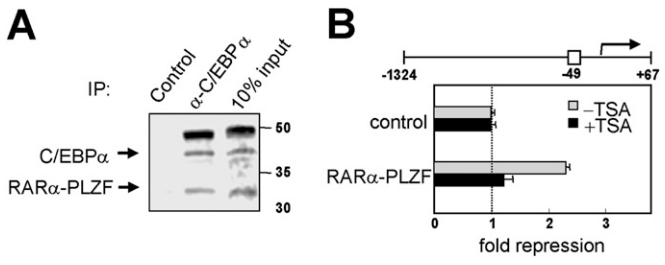

Fig. S6. RARα-PLZF binds to C/EBPα and HDAC1, and TSA treatment restores C/EBPα activity repressed by RARα-PLZF. (*A*) Coimmunoprecipitation of in vitro translated ^{35}S-RARα-PLZF and C/EBPα using anti-C/EBPα antibody or a rabbit preimmune serum (control) (n = 2). (*B*) TSA relieves the repression of *CSF3R* promoter activity by RARα-PLZF in transient transfections of 32D cells. After electroporation, performed as in Fig. S5*B*, the cells were cultured with or without TSA (10 ng/mL) (average of three independent experiments).

Table S1. List of primers for quantitative PCR analysis

Gene	5′→3′ Forward primer sequence	Reverse 5′→3′ primer sequence	Internal 5′→3′ primer sequence
		Semiquantitative RT-PCR	
Csf3r	ACATCTCCCTCCATGACTTT	GATGTTTTCATAAGATTTAGGG	TCCATCTGACTTAAACATTTTC
Cebpa	AAGAAGTCGGTGGACAAG	AAACCATCCTCTGGGTCTC	GAGTTGACCAGTGACAATGAC
S16	AGGAGCGATTTGCTGGTGTGA	GCTACCAGGGCCTTTGAGATG	CCAAATTTATGATCCGACAGT
RARa-PLZF	CTCTCCAGCACCAGCTTCCA	GACGGCCATGTCAGTGCCAG	AAAGCCTTTGTCTGTGATCAG
PLZF-RARa	AGCCTGAGGACTTGTCCTGA	GGGAAGCGGTTCCTGGATAG	AGAATGCACTTACTGGCTCATT
CSF3R	AGGAGCCCCCTTACCCACT	TGCTGTGAGCTGGGTCTGG	AGTCTGTATCACATCCACCT
		Quantitative PCR	
Csf3r	ACATCTCCCTCCATGACTTT	GATGTTTTCATAAGATTTAGGG	
Ltf	ACAGACAAGGTGGAAGTCCTT	TAGACTGGAACAGGCAAAACTC	
Ela2	ACAGTGGTGACTAACATGTG	CAAGGTTGTTACAGACCAAG	
Mpo	TCATTGGCACTCAGTTTAGGAA	TGTTCAGTAAACAGCAGAGACA	
Mir-223	CAGTGTCACGCTCCGTGTAT	AGCCACACTTGGGGTATTTG	
HPRT	GGCCAGACTTTGTTGGATTTG	CACAGGACTAGAACACCTGC	
		Chromatin immunoprecipitation	
Csf3r promoter	CTCTGGGAACAGGAGCTGAG	GCAGGGAGTAAGCCTGTCTG	
Ela2 promoter	AGCCAGGGCTACACAGAAAC	GAACTCAGGGCCTTATGCAG	
Mpo promoter	CCAGTGCTGGGATTAGAAGC	TGGGTTGTTTCCAGTTTTCA	
Ltf promoter	ACACCTGGTTGAGGGCAAT	TTATCTGTGCTGGGTGACCTC	
Mir-223 promoter	GGTTGAGAATGGGTGGAGAC	TGTGAGCAGGAAGGTCACAT	
Kit promoter	TGTGGGGGCTCCTGGTCTTA	TAGCGGCGCGCGACAG	
Hprt promoter	CTACCTCTGTAGTGCTGGGAT	CAGCATTTAGGAGGCAGAGG	
		DNA capture assay	
Csf3r promoter	GCAGCAGGGAAGCCAG	GCTTGTGCTCTCTCTTCTCTCTC	