Interplay between hypertriglyceridemia and acute promyelocytic leukemia mediated by the cooperation of peroxisome proliferator-activated receptor-α with the PML/RAR α fusion protein on super-enhancers

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Supplementary Data

Supplementary Methods

Supplementary Figures

Figure S1. Supporting data for the collaborative regulation of super-enhancers mediated by PPARα and PML/RARα.

Figure S2. Downregulation of target genes co-bound by PML/RARα and PPARα using the PPARα inhibitor GW6471.

Figure S3. Inhibition of cell growth in NB4 cells using the PPARα inhibitor GW6471.

Figure S4. Cell growth was increased upon the treatment of PPARα activator GW7647.

Supplementary Tables

Table S1. Primers used in this study

Table S2. Baseline characteristics of the APL patients having normal triglycerides and high triglycerides.

Table S3. RNA-seq patients' information.

Table S4. Super-enhancer associated genes co-bound by PPARα and PML/RARα.

Table S4 can be found in the accompanying Microsoft Excel file.

Table S5. OD values of NB4 cells treated with GW7647.

Supplementary References

Supplementary Methods

Cell lines and treatment

NB4 cells were routinely cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% of FBS (Gibco). All cell lines were maintained in a humidified atmosphere at 37°C with 5% CO_2.

NB4 cells were treated with PPARα antagonist GW6471 (Sigma, #G5045) at 5 μM or 10 μM. Five mg of GW6471 was dissolved in 806.9 μL of DMSO to prepare a 10 mM of the stock solution.

NB4 cells were treated with PPARα agonist GW7647 (Sigma, #G6793) at 0.5 μM. Five mg of GW7647 was dissolved in 994.5 μL of DMSO to prepare a 10 mM of the stock solution. Due to the high proliferation rate of cancer cells, NB4 cells were cultured under 5% of FBS upon the treatment of GW7647.

Treatment protocol for APL

According to Sanz risk, newly diagnosed APL patients were divided into low risk (a WBC count ≤ 10 × 10^9/L and a platelet count > 40 × 10^9/L), intermediate risk (a WBC count ≤ 10 × 10^9/L and a platelet count ≤ 40 × 10^9/L) and high risk (a WBC count > 10 × 10^9/L), respectively. They all received ATRA plus ATO–based induction therapy. ATRA (25 mg/m^2 per day) was given in an oral divided dose till CR and ATO was given intravenously at a dose of 0.16 mg/kg (10 mg maximum) per day till CR. Anthracycline (idarubicin or daunorubicin) was added to all high-risk patients and those intermediate risk patients experiencing leukocytosis during induction. Chemotherapy was not involved in low-risk patients, and hydroxyurea was applied to manage leukocytosis. After achieving complete remission with induction therapy, patients were randomized (1:1) into ATO and non-ATO groups for consolidation: ATRA-ATO versus ATRA–anthracycline for low-/intermediate-risk patients, or ATRA-ATO–anthracycline versus ATRA–anthracycline– cytarabine for high-risk patients. Patients with molecular complete response after consolidation therapy entered

maintenance therapy. Low- and intermediate-risk patients received three cycles of ATRA and ATO sequential treatment, while those of high-risk received five cycles of ATRA, ATO, and methotrexate treatment.

Antibodies

The antibodies to detect PPARα (Santa Cruz, #sc-398394), FLT3 (Cell Signaling, #3462S), phosphor-FLT3 (Tyr 589/591) (Cell Signaling, #3464S), STAT5 (Abcam, #ab32043), phospho-STAT5 (Y694) (Abcam, #ab32364,), and GAPDH (Cell Signaling, #D16H11,) were used in western blotting experiments. PPARα (Abcam, #ab227074) and IgG control antibody (Millipore, #12370 and #AP132) was used in CUT&Tag assays.

Inhibition of PPARα with siRNAs or chemical inhibitors

PPARα gene expression in NB4 cells was silenced by the small interfering RNA (siRNA) technique. The siRNA was synthesized by Genepharma (shanghai, China). The sequence is siPPARα-1: GUAGCGUAUGGAAAUGGGUUU; siPPARα-2: GAACAGAAACAAAUGCCAGUA. According to the protocol of Nucleofector Kit V (Lonza, Cologne, Germany), NB4 cells were electrotransferred with siRNA in the Amaxa Nucleofector II device (Lonza, program X-001).

The PPARα antagonist was GW6471, and the molecular weight was 619.67. A DMSO solvent with a final concentration of 5 - 10 μM was used as a control.

Cleavage Under Targets and Tagmentation (CUT&Tag) followed by sequencing

CUT&Tag assay was performed as described previously with modifications.[1] Briefly, 100000 cells were washed twice gently with wash buffer (20 mM HEPES pH 7.5; 150 mM NaCl; 0.5 mM Spermidine; 1× Protease inhibitor cocktail). 10 μL concanavalin A coated magnetic beads (Bangs Laboratories) were added per sample and incubated at RT for 10 min. Removed unbound supernatant and resuspended bead-bound cells with dig wash buffer (20 mM HEPES pH 7.5; 150 mM NaCl; 0.5 mM Spermidine; 1× Protease inhibitor cocktail; 0.05% Digitonin; 2 mM EDTA). A 1:50 dilution of primary antibody or IgG control antibody was incubated on a rotating platform overnight at 4°C. The primary antibody was

removed using magnet stand. Secondary antibody (anti-Rabbit IgG antibody) was diluted 1:100 in dig wash buffer and cells were incubated at RT for 60 min. Cells were washed using the magnet stand 2-3 times in dig wash buffer. A 1:100 dilution of pA-Tn5 adapter complex was prepared in dig-med buffer (0.01% Digitonin; 20 mM HEPES pH 7.5; 300 mM NaCl; 0.5 mM Spermidine; 1× Protease inhibitor cocktail) and incubated with cells at RT for 1h. Cells were washed 2-3 times for 5 min in 1 mL Dig-med buffer. Then cells were resuspended in the tagmentation buffer (10 mM $MgCl_2$ in Dig-med Buffer) and incubated at 37°C for 1 h. DNA was purified using phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. To amplify libraries, 21 μL of DNA was mixed with 2 μL of a universal i5 and auniquely barcoded i7 primer. A volume of 25 μL NEBNext HiFi 2× PCR Master mix was added and mixed. The sample was placed in a Thermocycler with a heated lid using the following cycling conditions: 72°C for 5 min (gap filling); 98 °C for 30 s; 14 cycles of 98 °C for 10 s and 63 °C for 30 s; final extension at 72 °C for 1 min and hold at 8 °C. The library clean-up was performed XP beads (Beckman Counter). The size distribution of libraries was determined by Agilent 4200 TapeStation analysis, and libraries were mixed to achieve equal representation as desired aiming for a final concentration as recommended by the manufacturer. Sequencing was performed in the Illumina Novaseq 6000 using 150bp paired-end following the manufacturer’s instructions.

Bioinformatic analysis for CUT&Tag-seq data and ChIP-seq data

PML/RARα ChIP-seq data for NB4 cells and H3K27ac ChIP-seq data for APL blasts were retrieved from our previous study, which are available at NCBI GEO under accession number GSE126720. All CUT&Tag-seq and ChIP-seq reads were aligned to the human genome (hg38) by Bowtie2 (version 2.2.9) under the following parameters -D 15 -R 2 -N 0 -L 22 -i S,1,1.15, and uniquely mapped reads were retained for further analyses. The sam files were converted to bam files and sorted with samtools. High-confident binding sites were called by MACS (version 1.4.2) with a *P*-value cutoff of 1e-10. The tdf file was generated by igvtools under the following parameters -z 5 -w 25 -e 250. The bedgraph files were generated by macs with the -B command. The overlap of peaks from different ChIP–

seq experiments was determined using the BEDTools2 suite.[2] For visualization, the binding of transcription factors and histone modifications were plotted by the UCSC Genome Browser (https://genome.ucsc.edu/) using the bedGraph files generated by MACS (version 1.4.2). Heat maps for PPARα and PML/RARα binding sites were generated by the bigWigSummary function from the UCSC database. Super-enhancers in APL blasts were identified using the ROSE algorithm.[3] In brief, constituent enhancers were calculated by merging individual enhancers according to H3K27ac ChIP-seq signals called by MACS (version 1.4.2) within 12.5 kb. The normalized ChIP-seq signals of H3K27ac in these constituent enhancers were ranked. To distinguish super-enhancers from typical enhancers, a cutoff was set as previously described.[3] ChIPseeker (v1.14.0) and R (3.4.1) were used for peak annotation to obtain target genes. The closest genes nearby PPARα bound super-enhancers were defined as the super-enhancer-associated PPARα-regulated targets, as previously described.[3] According to the KEGG database, pathway analysis was used to determine the genes' significant pathways near PPARα+PML/RARα+SEs co-bound regions. We used the Fisher's exact test to select the significant pathways, and the threshold of significance was defined by *P*-value and FDR.[4]

RNA extraction, reverse transcription, and RT-qPCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then 1 μg of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (RR047A, Takara). RT-qPCR was conducted using the TB Green® Premix Ex Taq™ (Tli RNaseH Plus) on the ABI ViiA 7 Real-Time PCR System. The relative expression level of each gene was calculated as 2-△△Ct, GAPDH was used as an internal control. Primers used in this study are listed in Table S4.

RNA-sequencing (RNA-seq) and data analysis

RNA-seq libraries were constructed according to the manufacture's instruction using the TruSeq RNA Sample Preparation Kit v2 (Illumina). About 1μg of total RNA was used and polyA-tailed mRNAs were purified from total RNA using oligo-dT attached magnetic beads.

The purified library was quantified using a Qubit 4 Fluorometer. High-throughput sequencing (150-bp paired-end reads) was done on the Illumina HiSeq 2500 or NovaSeq 6000 platform. Trim-galore was employed to remove the adapter sequences, and then the clean reads were aligned with STAR (v2.7.8) and quantified with HT-Seq (v0.13.5) using the GRCh38 human assembly. The differential expression assessment was conducted using the DESeq2 package. |log2(fold change) | >=1 and FDR < 0.05 were used for determining differentially expressed genes (DEGs). Gene expression levels were normalized to Transcripts Per Kilobase of exon model per Million mapped reads (TPM) using a customed script. To associate each binding peak (ChIP-seq) with the most likely regulated target gene (RNA-seq), we first extracted all differentially expressed genes (between APL with a high level of triglyceride and APL with a normal level of triglyceride) that located from 50 Kb upstream or downstream of the peak. The nearest gene to the peak was chosen as the potential target if multiple genes were found.

Gene set variation analysis (GSVA) and the gene set enrichment analysis (GSEA) analysis

GSVA, a popular framework for condensing information from gene expression profiles into a pathway or signature summary, was used to estimate the pathway activity of KEGG pathway signatures in each APL patient. The KEGG pathway gene sets were obtained from Molecular signatures database (MSigDB). A two-class comparison of GSVA scores of APL patients with high triglyceride levels (n=20) and the paired patients with normal triglyceride levels (n=20) was performed using the lmFit and eBayes functions implemented in the limma R package. Briefly, lmFit was used to fit a linear model to the GSVA scores divided in the high triglyceride- and normal triglyceride-group. Then, eBayes was used to compute t-statistics and log-odds ratios of differential pathway scores. Two-sided *P* values were corrected for multiple hypothesis testing using the Benjamini-Hochberg correction. Pathways with significantly normal/higher GSVA scores in APL patients with high triglyceride levels (q-value < 0.3) compared to those with normal triglyceride levels were identified as candidates for in-depth analysis. The DEGs between the two APL patient groups were used as input for GSEA implemented in the clusterProfiler

R package.

Western blotting

Cells were washed with PBS and lysed in RIPA lysis buffer (Beyotime Biotechnology, # P0013B) with 100 μM PMSF (Sigma) and 100 μM cocktail (Sigma) for 30 min; then lysates were obtained by sonication and centrifugation. Next, total protein was quantified using Bio-Rad *DC* Protein Assay (Bio-Rad, Hercules, CA, USA, #5000116) according to the manufacturer's instruction and diluted into 1 μg/μL using the RIPA lysis buffer and 5 × SDS-PAGE protein loading buffer (Beyotime Biotechnology, #P0015L). All the above steps were performed at 4 °C. Diluted protein samples were heated 100 °C for 10 min. Then 10 μg of total protein/sample was loaded and separated on 10% acrylamide tris-HCL-buffered SDS-PAGE gels (EpiZyme, Shanghai, China, #PG112), transferred to polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ, USA, #RPN303F), immunoblotted with indicated antibodies, and imaged using Immobilon Western Chemiluminescent HRP Substrate (Millipore, #WBKLS0500) on an image-quant LAS4000 (Fujifilm Life Science) or Amersham Imager 600 Luminescent Image analyzer (GE Healthcare Bio-Sciences).

Proliferation Assays

Cells were seeded at the concentration of 2000/well in a 96-wells plate. According to the manufacturer's instruction, cell proliferation was measured every 24 hours using Cell Counting Kit-8 (Dojindo, Tokyo, Japan, #CK04). All experiments were conducted in six-replicate.

Animal study

FVB/NJ mice were bred and housed in the animal facility of Ruijin hospital. The 6–8-week-old littermates had an initial body weight of about 22 ± 2 g. The mice were divided into two groups, 8-10 in each group. The mice were fed a high-fat diet (HFD, 60% fat) or a normal diet (ND). The mice in each group were fed and drank freely. Mice weights were recorded weekly. After six weeks, triglycerides (TG) were measured in mice to see if the high triglyceride model was successfully constructed. Lipid profile measurement was performed

according to the instructions of the lipid profile kit (KHB 146-V3.0-xx). The bone marrow cells were isolated from the femur and tibia of transplantable APL-onset mice carrying the PML/RARα transgene. Each mouse could obtain 1×10^5 of APL bone marrow cells. Cells were resuspended in 100-200 uL of DMEM and injected into mice by tail vein injection. Collected blood from the tail vein of each mouse on days 6, 13, and 17. Flow cytometry was used to detect the GFP positive rate (leukemia cells carried PML/RARα-GFP) to observe mice's pathogenesis dynamically.

Statistical analysis

Linear mixed-effect models were used to examine the relationship between triglycerides and white blood cell counts. The two-sided t-test was used to compare the measurements of the two groups. One-way ANOVA was used to compare multiple groups (simultaneous test for variance homogeneity). When comparing the cross-table data, the chi-square test was used to detect the statistical differences. When analyzing the differences in the data components of the 3x2 table, such as comparing the difference of risk level distribution in APL patients with normal or high triglyceride levels, the chi-square test with the Bonferroni correction was used. We used the Kaplan–Meier method to calculate whether the statistical difference between the two groups was significant for survival data. Binary logistic regression analysis was used to establish a model, and related variables were diagnosed by collinearity. The Delong test was used to compare the ROC curve areas and evaluate the models' performance. When the P value was less than 0.05, the difference was considered statistically significant. The Wilcoxon rank-sum test was used to assess the statistical significance of differences between two groups, and $p\text{-value} < 0.05$ indicated significant differences.

Supplementary Figures

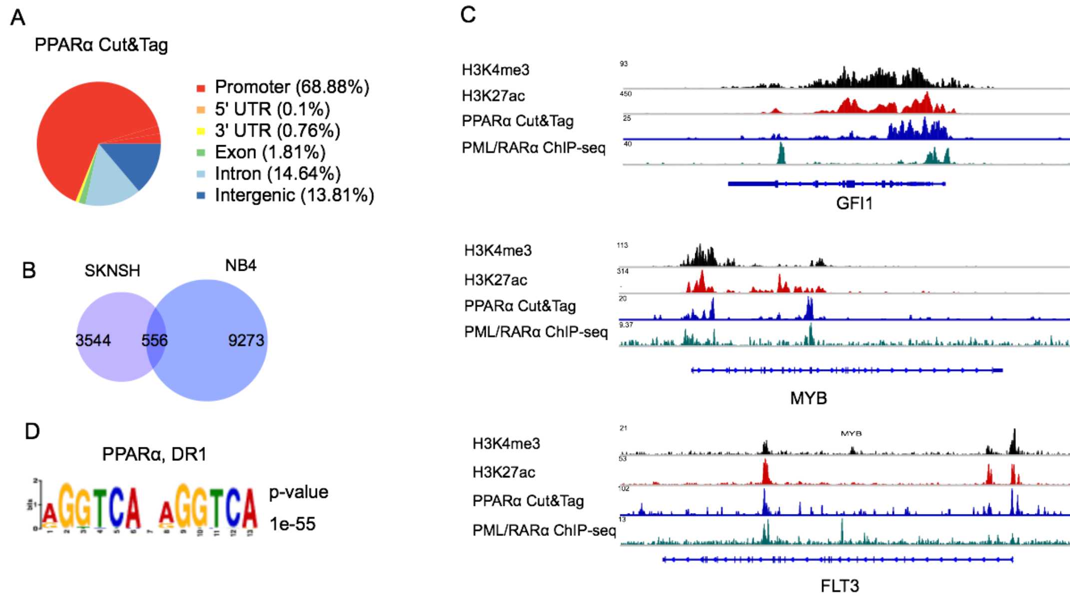

Figure S1. Supporting data for the collaborative regulation of super-enhancers mediated by PPARα and PML/RARα.

(A) Pie chart showing the distribution of 9,829 PPARα peaks in NB4 cells. UTR, untranslated region. (B) Schematic illustration of the comparison between PPARα binding sites in NB4 and SKNSH cells. (C) Genome browser view of H3K4me3, H3K27ac, PPARα, and PML/RARα co-binding at representative target genes in NB4 cells. (D) Motif discovery of PPARα Cut&Tag regions by directly scanning PPRE using AME algorithm.

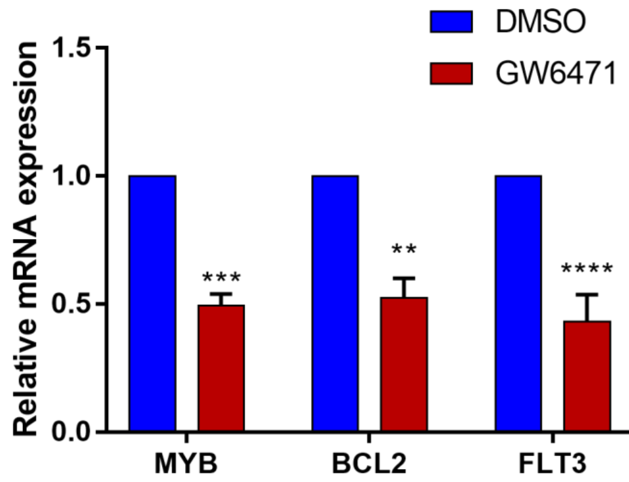

Figure S2. Downregulation of target genes co-bound by PML/RARα and PPARα using the PPARα inhibitor GW6471.

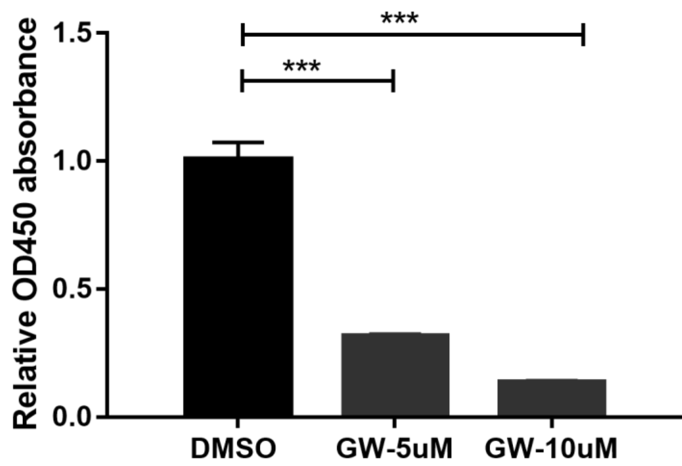

Figure S3. Inhibition of cell growth in NB4 cells using the PPARα inhibitor GW6471.

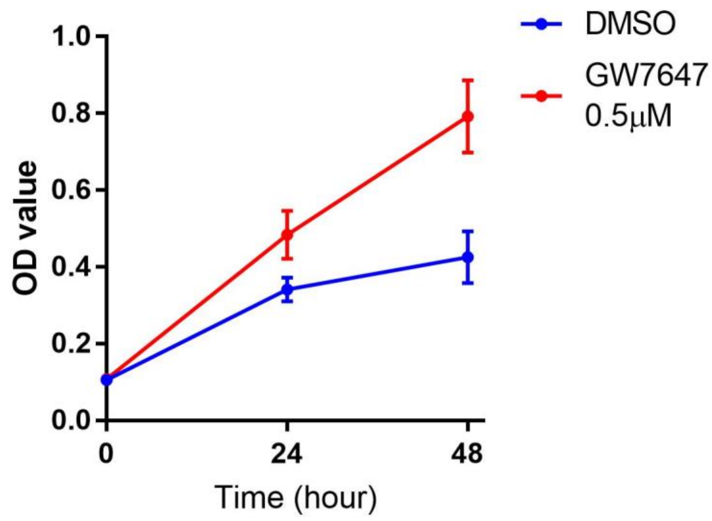

Figure S4. Cell growth was increased upon the treatment of PPARα activator GW7647. NB4 cells were cultured under 5% of FBS upon the treatment of GW7647.

Table S1. Primers used in this study

Name	Sequence
mGAPDH-F	GGCAAATTCAACGGCACAGT
mGAPDH-R	CGCTCCTGGAAGATGGTGAT
mFLT3-F	GAGCGACTCCAGCTACGTC
mFLT3-R	ACCCAGTGAAAATATCTCCCAGA
mPPARα-F	TGCAAACTTGGACTTGAACG
mPPARα-R	GATCAGCATCCCGTCTTTGT
hGAPDH-F	GGAGCGAGATCCCTCCAAAAT
hGAPDH-R	GGCTGTTGTCATACTTCTCATGG
hPPARα-F	CCAGTATTTAGGAAGCTGTCC
hPPARα-R	TGAAAGCGTGTCCGTGAT
hFLT3-F	AGGGACAGTGTACGAAGCTG
hFLT3-R	GCTGTGCTTAAAGACCCAGAG
hSTAT5A-F	CGAGTGCAGTGGTGAGATCC
hSTAT5A-R	TCCTCTGTCACGGACTCTGC
hMYB-F	AAGGTCGAACAGGAAGGTTATC
hMYB-R	ACTGTTCTTCTGGAAGCTTGT
hBCL2-F	TGTGGATGACTGAGTACCTGAACC
hBCL2-R	GGAGAAATCAAACAGAGGCCGCAT

Table S2. Baseline characteristics of the APL patients having normal triglycerides and high triglycerides.

	Normal TG		High TG		
	Median (IQR)	N (%)	Median (IQR)	N (%)	*P**
All patients		73 (100)		116 (100)	
Age at diagnosis, y	36.0 (26.5-47.5)		37 (30.8-48)		
≤ 40	-	40 (54.8)	-	64 (55.2)	0.182
> 40	-	33 (45.2)	-	52 (44.8)	
Sex					0.009
Male	-	26 (35.6)	-	64 (55.2)	
Female	-	47 (64.4)	-	52 (44.8)	
WBC	3.5 (1.5-7.5)		5.0 (2.0-17.2)		0.009**
≤ 10 $\times 10^9$/L	-	60 (82.2)	-	81 (69.8)	
> 10 $\times 10^9$/L	-	13 (17.8)	-	35 (30.2)	
Platelets	61.0 (25.8-130.5)		43.0 (21.0-92.3)		0.222
≤ 40$\times 10^9$/L	-	28 (38.4)	-	55 (47.4)	
> 40$\times 10^9$/L	-	45 (61.6)	-	61 (52.6)	
Sanz risk score					0.219
Low	-	25 (34.2)	-	27 (23.2)	
Intermediate	-	30 (41.4)	-	51 (44.0)	
High	-	18 (24.7)	-	38 (32.8)	
Differentiation syndrome	-	12 (16.4)	-	19 (16.3)	0.536
Early death	-	1 (1.4)	-	12 (10.3)	0.018

"-" indicates not applicable. *X^2 test compared distributions among TG groups. ** t-test was performed between the 2 groups. TG, triglycerides. There were no statistical differences between the two groups in treatment, supportive care, and time-to-treatment initiation.

Table S3. RNA-seq patients' information.

Patient ID	Group	Sex	Age	Risk level	WBC	PLT	PML/RARα transcript type
Pt01	Normal_TG	Female	36	High	19.1	59	L-type
Pt02	Normal_TG	Female	28	Low	5.4	228	S-type
Pt03	Normal_TG	Female	46	High	1.8	77	L-type
Pt04	Normal_TG	Male	21	Low	1.63	79	L-type
Pt05	Normal_TG	Female	49	High	16	22	L-type
Pt06	Normal_TG	Male	53	Low	1	158	S-type
Pt07	Normal_TG	Female	49	Intermediate	4.6	129	L-type
Pt08	Normal_TG	Male	21	Intermediate	6.7	170	L-type
Pt09	Normal_TG	Male	63	Intermediate	7.4	194	S-type
Pt10	Normal_TG	Female	21	Intermediate	6.7	159	L-type
Pt11	Normal_TG	Female	25	Intermediate	NA	NA	S-type
Pt12	Normal_TG	Female	31	Low	15.67	72	L-type
Pt13	Normal_TG	Male	26	Low	3.26	72	S-type
Pt14	Normal_TG	Female	21	Low	1.57	60	S-type
Pt15	Normal_TG	Male	44	Intermediate	1.89	29	L-type
Pt16	Normal_TG	Male	20	Intermediate	3.3	149	L-type
Pt17	Normal_TG	Female	42	Low	1.16	43	L-type
Pt18	Normal_TG	Female	54	Intermediate	0.75	32	L-type
Pt19	Normal_TG	Female	43	Intermediate	7.5	119	L-type
Pt20	Normal_TG	Male	27	Intermediate	0.83	3	L-type
Pt21	High_TG	Female	35	High	6.02	207	L-type
Pt22	High_TG	Male	52	High	5.1	153	S-type
Pt23	High_TG	Male	46	High	89	22	S-type
Pt24	High_TG	Male	31	High	133.8	10	S-type
Pt25	High_TG	Female	27	Intermediate	6.9	244	S-type
Pt26	High_TG	Female	32	Low	3	90	L-type
Pt27	High_TG	Male	55	Intermediate	3.6	183	L-type
Pt28	High_TG	Male	45	Intermediate	3.23	2	L-type
Pt29	High_TG	Female	64	Low	NA	NA	S-type
Pt30	High_TG	Female	39	Intermediate	1.26	9	L-type
Pt31	High_TG	Male	30	Intermediate	14.2	97	L-type
Pt32	High_TG	Male	37	Low	2.4	56	L-type
Pt33	High_TG	Female	34	Intermediate	4.18	4	L-type
Pt34	High_TG	Female	27	Intermediate	6.6	7	S-type
Pt35	High_TG	Male	54	Low	1.39	93	L-type
Pt36	High_TG	Female	48	Intermediate	0.84	77	L-type
Pt37	High_TG	Male	33	Intermediate	5.3	225	L-type
Pt38	High_TG	Male	29	Intermediate	3.6	7	S-type
Pt39	High_TG	Male	50	Low	15	134	L-type
Pt40	High_TG	Female	33	Low	0.5	43	V-type

TG, triglycerides

Table S4. Super-enhancer associated genes co-bound by PPARα and PML/RARα.

Table S4 can be found in the accompanying Microsoft Excel file.

Table S5. OD values of NB4 cells treated with GW7647

Batch Number	Time	DMSO			GW7647		
	(hour)	well 1	well 2	well 3	well 1	well 2	well 3
NO.1	0	0.106219	0.108182	0.104951	0.114309	0.105728	0.103331
	24	0.362222	0.350107	0.369361	0.422196	0.548603	0.531857
	48	0.449391	0.422263	0.451657	0.746224	0.863969	0.855373
NO. 2	0	0.104358	0.105255	0.104888	0.106487	0.104976	0.110888
	24	0.351326	0.330768	0.284784	0.514900	0.492744	0.392524
	48	0.518922	0.393908	0.315900	0.884464	0.766822	0.636208
NO.3	0	0.102851	0.102764	0.103489	0.103920	0.106660	0.103963
	24	0.378144	0.382101	0.409754	0.436997	0.549583	0.360702
	48	0.447968	0.408870	0.409754	0.710064	0.725402	0.786295

NB4 cells were cultured with 1640 containing 5% FBS. NB4 cells were treated with PPARα agonist GW7647 at 0.5 μM. DMSO served as control.

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