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Supplemental Information

The Histone Demethylase PHF8 Governs Retinoic Acid Response in Acute Promyelocytic Leukemia

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Inventory of Supplemental Information

SUPPLEMENTAL DATA

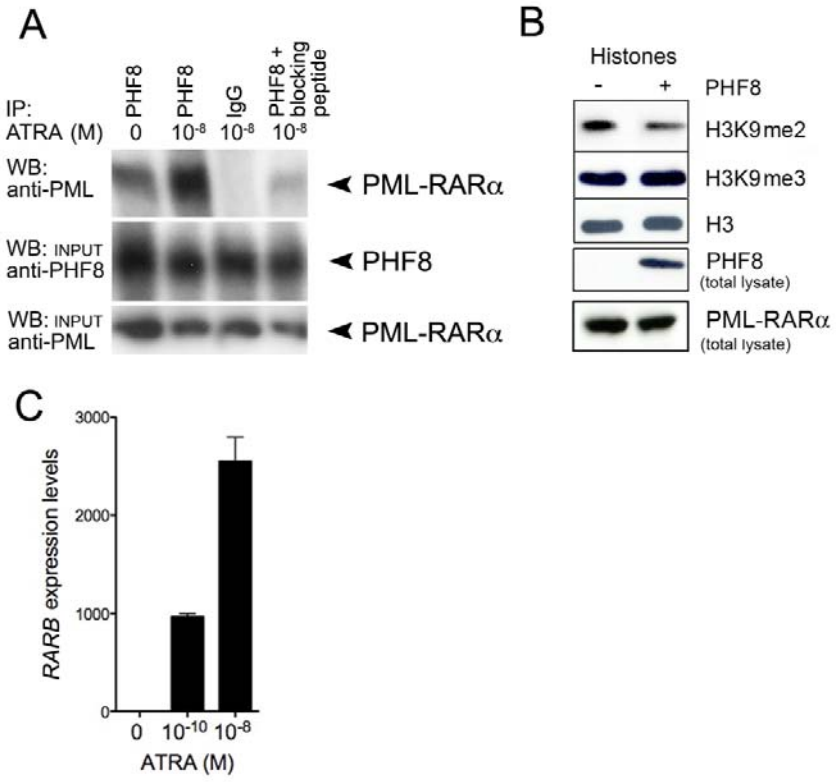
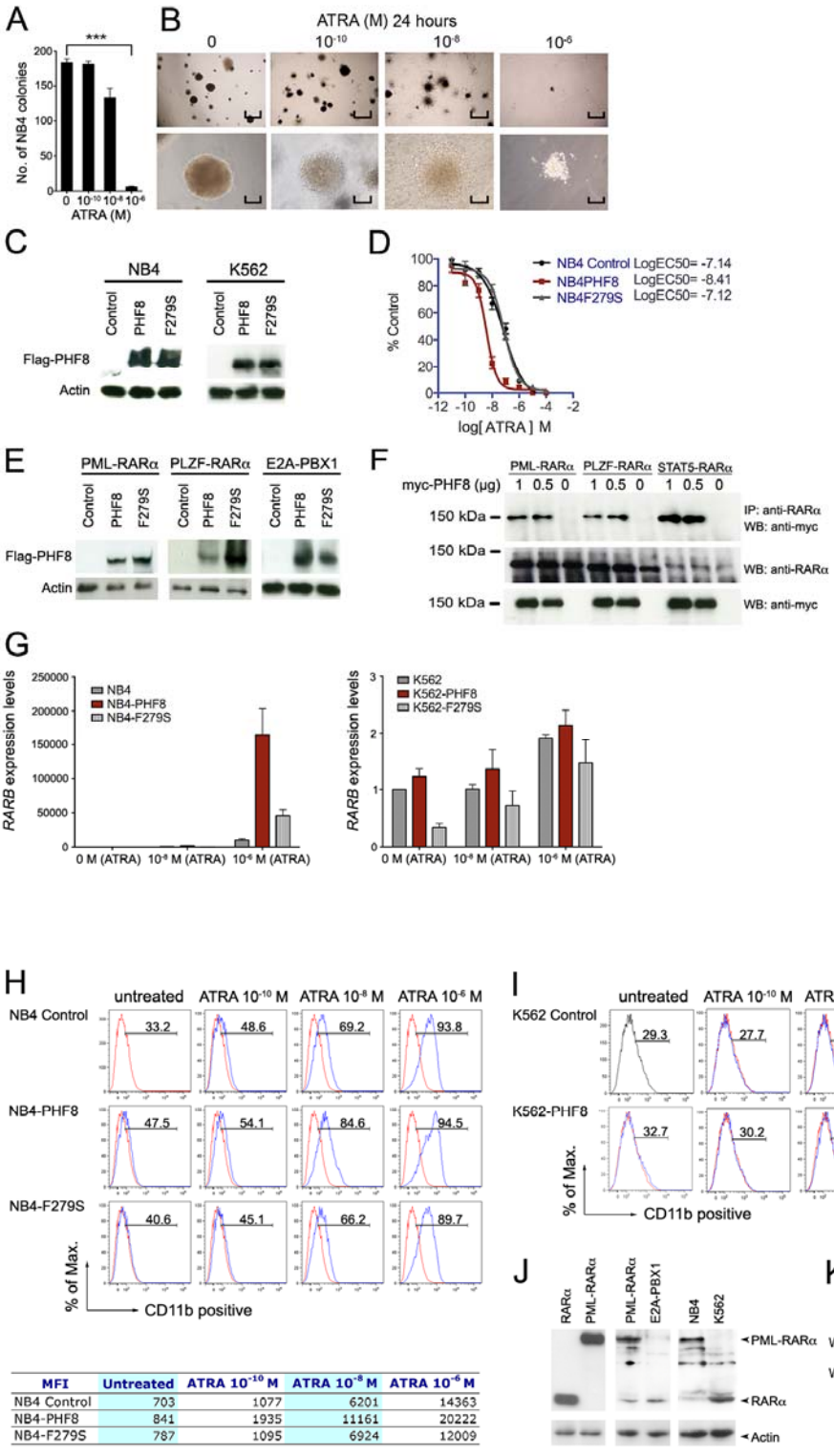


Figure S1, related to Figure 1. Increased Interaction between Endogenous PHF8 and PML-RARα upon ATRA Treatment. (A) CoIP analysis between endogenous PHF8 and PML-RARα in NB4 cells in the absence or presence of 24 hr ATRA treatment with or without PHF8 blocking peptides. (B) Histone demethylase activity of YFP-tagged PHF8 protein in 293T cells assessed by immunoblotting of purified histone extracts from sorted YFP positive (PHF8) or negative (Control) cells. (C) RT-qPCR detection of *RARB* expression levels from NB4 cells after treatment with ATRA at the indicated concentrations. Error bars indicate standard deviation of three independent experiments.



MFI	Untreated	ATRA 10^{-10} M	ATRA 10^{-8} M	ATRA 10^{-6} M
NB4 Control	703	1077	6201	14363
NB4-PHF8	841	1935	11161	20222
NB4-F279S	787	1095	6924	12009

Figure S2, related to Figure 2. PHF8 Enhances the Effect of Physiological Concentrations of ATRA in Human APL Cells. (A, B) NB4 cells were analyzed by colony formation assay (A, error bars represent s.e.m. of three independent experiments, ***$p<0.001$) and INT-stained after the ATRA treatment (B. Scale bar: top, 50 μm; bottom;, 200 μm). (C) Western Blot analysis of the ectopically expressed PHF8 in the indicated human cell lines expressing empty vector control, Flag-wild type PHF8 or catalytically inactive mutant Flag-PHF8-F279S. (D) Dose-response curve of colony formation in the presence of ATRA. The indicated cell lines were treated with increasing ATRA concentrations in a RTTA assay. After seven days, colonies were stained with INT and quantified. Non-treated cells were set to 100%, and drug response curves were analyzed with a nonlinear regression curve model. Error bars indicate standard deviation of three independent experiments. (E) Western Blot analysis of PHF8 in the indicated murine cell lines. (F) Representative co-immunoprecipitation (coIP) analysis of PHF8 in 293T cells expressing PML-RARα, PLZF-RARα, or STAT5-RARα together with indicated amount of myc-PHF8. Experiments were performed in the presence of ATRA. (G) RT-qPCR analysis of *RARB* expression in the indicated cells. Error bars indicate standard deviation of three independent experiments. (H-I) FACS analysis of CD11b expression in NB4 cell line derivatives (H) or K562 cell line derivatives (I). The bottom panel of (H) shows MFI (Median Fluorescence Intensity) of the FACS plots for NB4 cells. (J) Comparative analysis of endogenous RARα expression between the indicated cell lines by Western Blot. Panels represent 293T transfected cells as positive controls (left), c-Kit cells transformed with the indicated fusions (middle) and human cells lines (right). (K) Western blot analysis showing endogenous PHF8 expression levels in NB4 and NB4-MR2 cells lines.

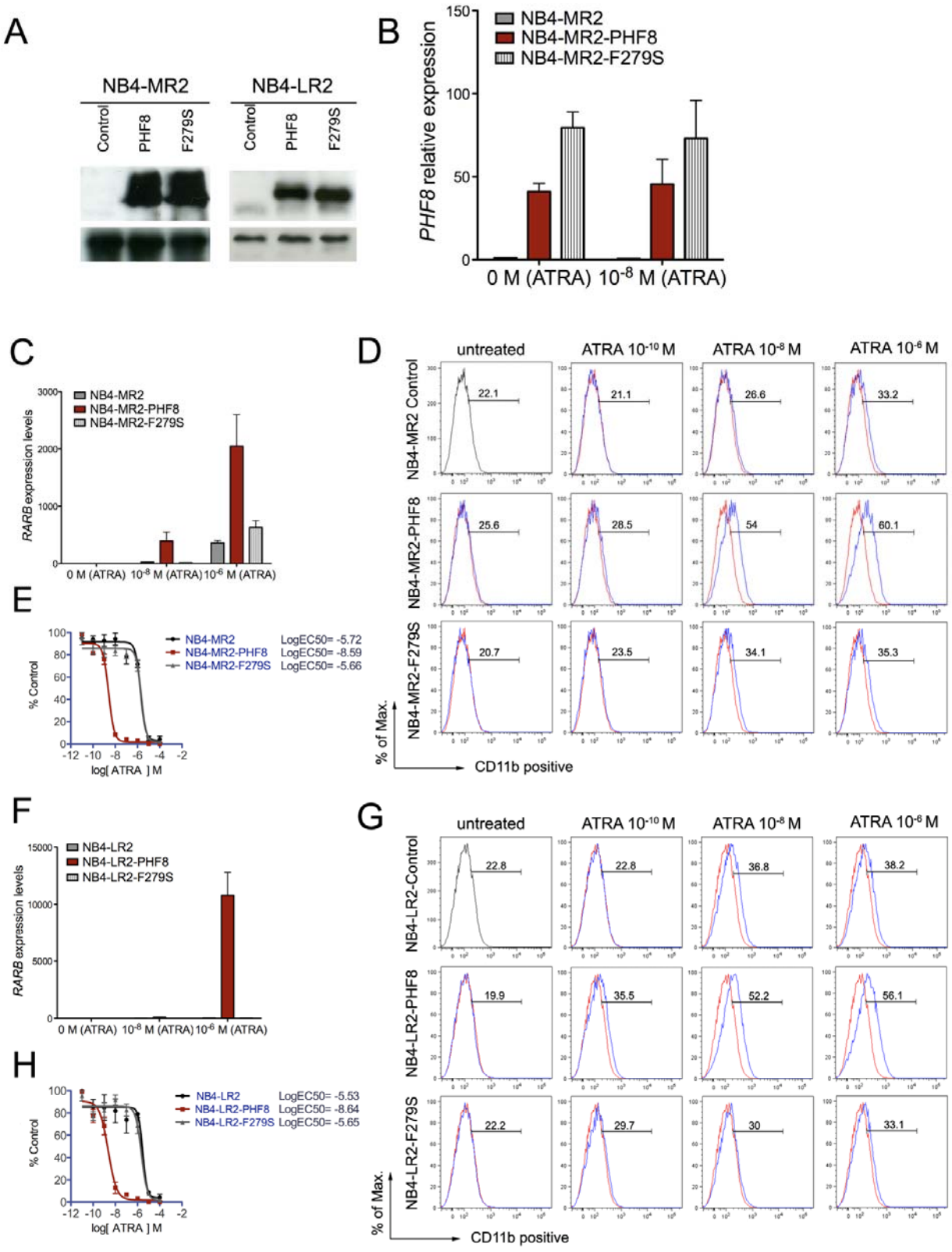

A
NB4-MR2
NB4-LR2
Control
PHF8
F279S
B
NB4-MR2
NB4-MR2-PHF8
NB4-MR2-F279S
PHF8 relative expression
0 M (ATRA)
10-8 M (ATRA)
C
RARB expression levels
D
untreated
ATRA 10-10 M
ATRA 10-8 M
ATRA 10-6 M
NB4-MR2 Control
NB4-MR2-PHF8
NB4-MR2-F279S
% of Max.
CD11b positive
E
% Control
log[ATRA] M
LogEC50= -5.72
LogEC50= -8.59
LogEC50= -5.66
F
NB4-LR2
NB4-LR2-PHF8
NB4-LR2-F279S
G
NB4-LR2-Control
H
LogEC50= -5.53
LogEC50= -8.64
LogEC50= -5.65

Figure S3, related to Figure 3. PHF8 Demethylase Activity is Essential for Sensitization of Human APL Cells to ATRA Treatment. (A) Western Blot analysis of the ectopically expressed PHF8 in control (NB4-MR2, NB4-LR2), wild type PHF8 (NB4-MR2-PHF8, NB4-LR2-PHF8) and catalytically inactive mutant PHF8-F279S (NB4-MR2-F279S, NB4-LR2-F279S) transduced cells. (B) RT-qPCR showing *PHF8* expression levels from the indicated NB4-MR2 cells. (C, F) RT-qPCR analysis for *RARB* expression levels in NB4-MR2 (C) or NB4-LR2 cells (F). (D, G) FACS analysis of NB4-MR2 cell lines (D) or NB4-LR2 cell lines (G) stained with CD11b. (E, H) Dose-response curve of colony formation in the presence of ATRA. The indicated cell lines NB4-MR2 (E) or NB4-LR2 (H) were treated with increasing ATRA concentrations in a RTTA assay. After seven days, colonies were stained with INT and quantified. Non-treated cells were set to 100%, and drug response curves were analyzed with a nonlinear regression curve model. Error bars (B, C, E, F, H) indicate standard deviation of three independent experiments.

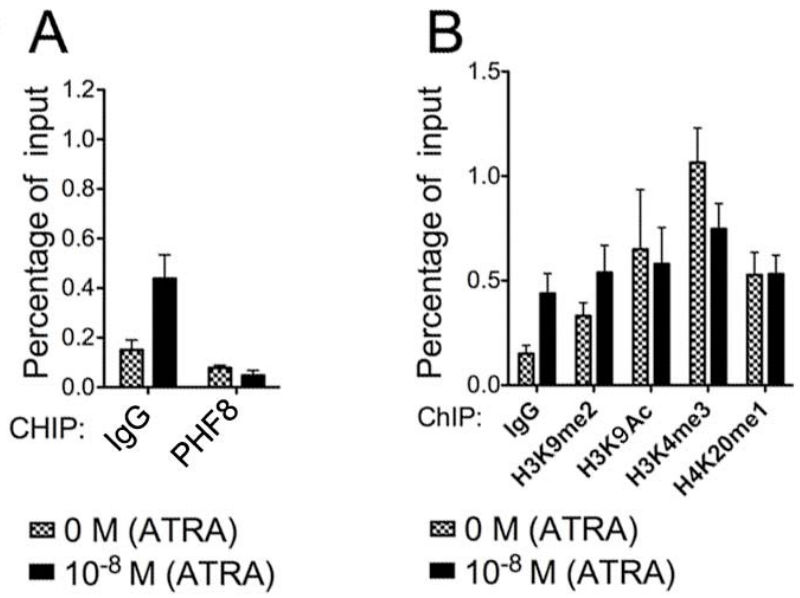


Figure S4, related to Figure 4. PHF8 Promoter Occupancy and Associated Histone Modifications after ATRA induction in K562 Cells. ChIP analysis of PHF8 (A) or various histone marks including H3K9me2, H3K4me3, H3K9Ac, and H4K20me1 (B) on RARE *RARB* in the K562 cells before and after 24 hr of ATRA treatment at 0 or 10^{-8} M. ChIP signals are presented as percentage of input where error bars indicate standard deviation of three independent experiments.

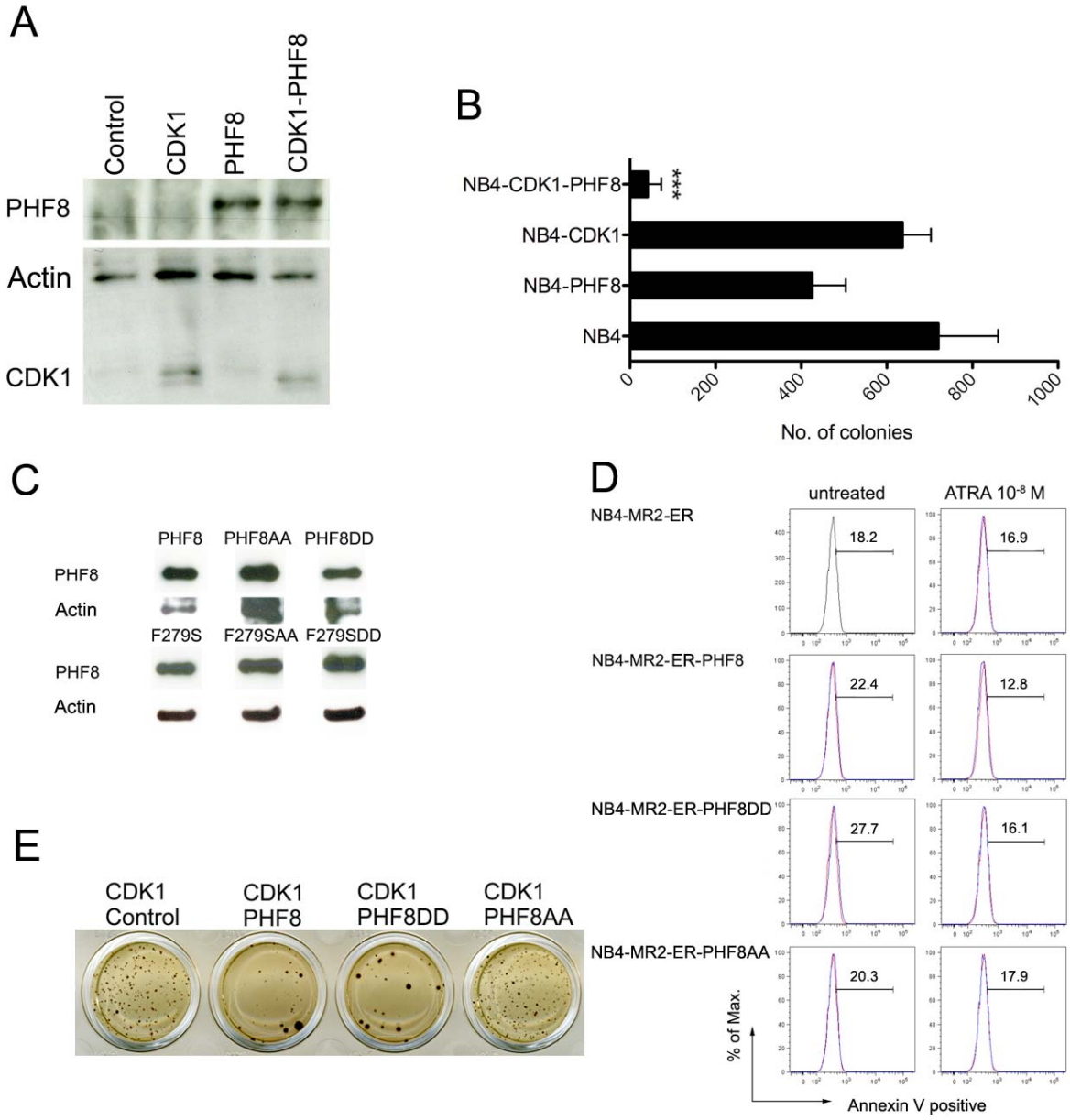

A
Control
CDK1
PHF8
CDK1-PHF8
PHF8
Actin
CDK1
B
NB4-CDK1-PHF8
NB4-CDK1
NB4-PHF8
NB4
***
0
200
400
600
800
1000
No. of colonies
C
PHF8
PHF8AA
PHF8DD
PHF8
Actin
F279S
F279SAA
F279SDD
PHF8
Actin
D
untreated
ATRA 10-8 M
NB4-MR2-ER
18.2
16.9
NB4-MR2-ER-PHF8
22.4
12.8
NB4-MR2-ER-PHF8DD
27.7
16.1
NB4-MR2-ER-PHF8AA
20.3
17.9
% of Max.
Annexin V positive
E
CDK1
Control
CDK1
PHF8
CDK1
PHF8DD
CDK1
PHF8AA

Figure S5, related to Figure 5. PHF8-mediated ATRA Response is Regulated by Serine-Phosphorylation. (A) Western blot analysis shows expression of Flag-PHF8 and myc-CDK1 proteins in NB4-MR2 cells transduced with empty vector control, wild type CDK1, wild type PHF8, or PHF8 and CDK1 together. (B) The bar chart represents the number of colonies of NB4 cells transduced with indicated constructs. Error bars represent s.e.m. of four independent experiments. ***$p<0.001$. (C) Western blot analysis shows expression of Flag-PHF8, Flag-PHF8AA and Flag-PHF8DD in NB4-MR2-ER cell lines. (D) FACS analysis of NB4-MR2-ER cells transduced with PHF8 variants and stained with AnnexinV apoptosis marker. (E) Typical INT-stained colony pictures of NB4-MR2 cells transduced with CDK1 and empty vector control or PHF8 wild type or PHF8AA or PHF8DD mutant.

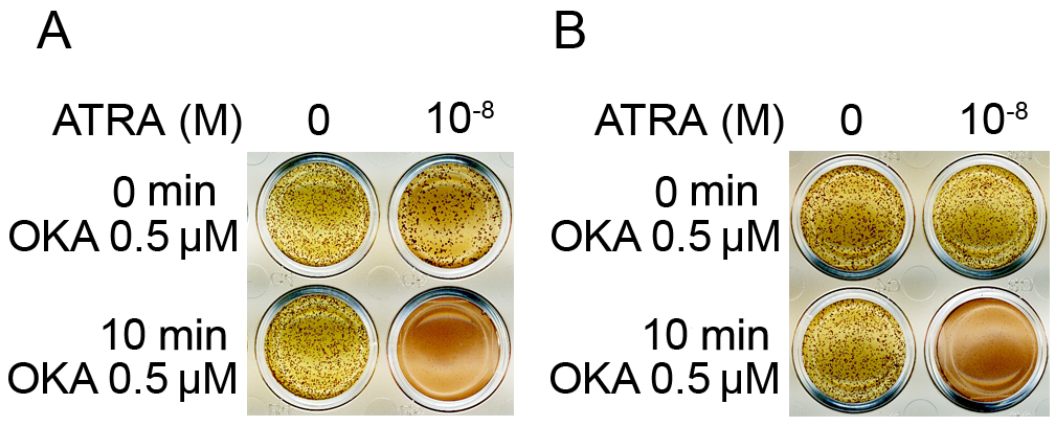


Figure S6, related to Figure 6. Combination Treatment with OKA and ATRA Induces Differentiation of Human APL Cells. Typical INT-stained colony pictures of human NB4 (A) and NB4-LR2 cells (B). Cells were treated with or without OKA/ATRA, at the indicated concentrations. Pictures represent typical results of three independent experiments.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

Plasmids encoding full-length of PML-RARα, PLZF-RARα in pSG5, pcDNA4 and MSCV vectors have been previously described (Kwok et al., 2006; Zeisig et al., 2007). pCMV-GFP-SMRT plasmid (a kind gift from Arthur Zelent). All PHF8 and CDK1 expression plasmids and ER inducible systems encoding full-length PHF8 and various mutants were generated by direct cloning or PCR based site-directed mutagenesis using PHF8 construct previously described (Qiu et al., 2010). All constructs were verified by DNA sequencing.

We used the following specific antibodies in this study: Flag M2 (Sigma-Aldrich, A8592), Anti-pSer (ab9332), RARα (C-20)(Santa-Cruz, sc-551), Actin (I-19)(Santa-Cruz, sc-1616). ChIP experiments were performed using PHF8 (Kleine-Kohlbrecher, D. et al., 2010) H3K4me3 (ab8580), H4K20me1 (ab9051), H4 (ab7311), H3 (ab1791), H3K9ac (ab10812), H3K4me3 (Active Motif 39159), H3K9/14ac (Diagenode, pAb-005-044), H3K9me2 (Millipore, 17-648).

Flow Cytometric Analysis of Mouse Cells

Immunophenotype analysis of murine bone marrow cells was performed using flow cytometry with fluorochrome-conjugated monoclonal antibodies against murine c-Kit (2B8 clone), murine Mac-1 (M1/70 clone) and murine Gr-1 (RB6-8C5 clone) (Biolegend). Staining was generally performed in SM [PBS; 0.2% FCS] and incubated on ice for 15 min, washed twice in SM and resuspended in SM containing 1 μg/mL of propidium iodide (PI) before analysis using a BD LSR II system (Becton Dickinson Inc.). Viable cells (PI-negative cells) were analyzed for cell surface marker expression.

Knockdown Experiments

For knockdown experiments, we followed the same RTTA procedure described above on primary murine or human haematopoietic cells by transducing them with pLKO.1 puro plasmids containing short hairpin RNAs (shRNAs) targeting PHF8 or scramble sequences

(Sigma-Aldrich SHCLNG).

Clone ID	Taxon	Symbol	Forward Oligo Sequence
TRC1 (SHC002)	human	shRNA control	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT
TRCN0000118318	human	shRNA 1 PHF8	CCGGCGACCCTGATAATAAGACCAACTCGAGTTGGTCTTATTATCAGGGTCGTTTTTG
TRCN0000118321	human	shRNA 3 PHF8	CCGGCCCAACTGTGAAGTCTTGCATCTCGAGATGCAAGACTTCACAGTTGGGTTTTTG
TRCN0000086825	mouse	shRNA 78 Phf8	CCGGGCAAGATGAAACTCGGTGATTCTCGAGAATCACCGAGTTTCATCTTGCTTTTTG
TRCN0000086826	mouse	shRNA 79 Phf8	CCGGGCGGACTGTACAGCTCATTAACTCGAGTTAATGAGCTGTACAGTCCGCTTTTTG

Histone Purification

Cells were grown and collected under the specific experimental conditions required. The cells were washed in PBS and lysed in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN3) at a cell density of 106 cells per ml. Lysed cells were incubated on ice for 10 minutes with gentle stirring and centrifuged at 6,500 x g for 10 minutes at 4°C to spin down the nuclei. Afterwards nuclei were washed in half the volume of TEB and centrifuged as before, the pellet was re-suspended in 0.2 N HCl at a density of $4x10^6$ nuclei per ml and incubated over night at 4°C. The following day, samples were centrifuged and supernatant containing the histone protein was used to determine protein content using the Bradford assay. The histone proteins were resolved by SDS–PAGE. Membranes were probed with described antibodies.

Chromatin Immunoprecipitation (ChIP)

Cells were cross-linked with 1% formaldehyde (Sigma) and chromatin DNA was sheared in 1% SDS lysis buffer to 300–500 bp average in size through sonication on ice in a Bioruptor (Diagenode) for 30 min (30 s on, 30 s off; high power load) before immunoprecipitation with control IgG or specific antibodies overnight at 4˚C and followed by incubation with protein A/G Dynal beads (Invitrogen) for an additional 1 hr. After washing and elution, incubation at 65˚C overnight reversed DNA-protein complex. Immunoprecipitated DNA was purified using QIAquick spin columns (Qiagen). For quantitative ChIP (qChIP),

purified immunoprecipitated DNA was used as a template for real-time PCR using primer/probe sets flanking specific gene regions. Primer sequences are derived from published literatures and are available upon request. Samples were run in triplicate, and the immunoprecipitated percentage of input was calculated using individual standard curves derived from serial dilution of input DNA. ChIPs were performed using specific antibodies to PHF8 (Kleine-Kohlbrecher et al., 2010), H3K9me2 (Millipore), H3K4me3, H3K9Ac, H4K20me1 (Abcam).

Annexin V staining

For apoptosis assays, cells from RTTA assays were washed in SM and resuspended in Annexin V binding buffer (25 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.2) and stained with Annexin V-FITC (Biolegend) and 1 mg/ml PI. Stained cells were then analyzed by flow cytometry.