Disruption of the PHRF1 Tumor Suppressor Network by PML-RARα Drives Acute Promyelocytic Leukemia Pathogenesis

Céline Prunier, Ming-Zhu Zhang, Santosh Kumar, Laurence Levy, Olivier Ferrigno, Guri Tzivion, and Azeddine Atfi

SUPPLEMENTAL DATA

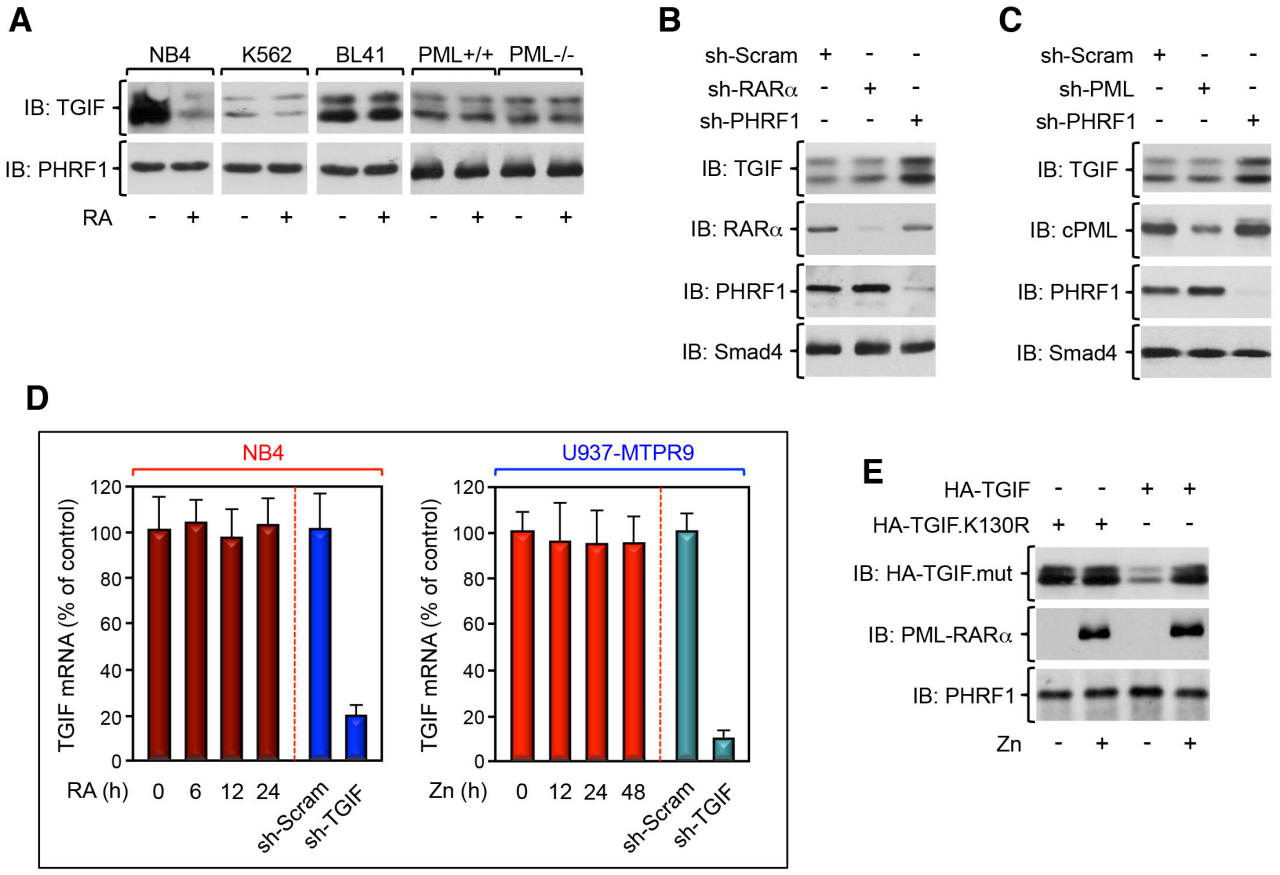

Figure S1: PML-RARα Promotes TGIF Stability. Related to Figure 1.

(**A**) RA stimulation had no effect on TGIF protein expression in the absence of PML-RARα. The indicated cell lines were treated with RA for 24 hr before being analyzed for TGIF or PHRF1 expression by immunoblotting using anti-TGIF or anti-PHRF1.

(**B**, **C**) PML or RARα deficiency had no effect on the abundance of the TGIF protein. U937 cells were transduced with lentiviruses encoding sh-Scram, sh-RARα, sh-PML, or sh-PHRF1 (used as a positive control). Cells lysates were analyzed by immunoblotting using anti-TGIF, anti-RARα, anti-PML, anti-PHRF1, or anti-Smad4 as a loading control.

(**D**) Ectopic expression of PML-RARα had no effect on TGIF mRNA expression. NB4 or U937-MTPR9 cells were treated with RA or Zn, respectively, for various time periods, and TGIF mRNA expression was determined by qRT-PCR and normalized on the basis of GAPDH expression. Measurement of TGIF mRNA expression in cells depleted for TGIF (sh-TGIF) was used as a positive control. Data are expressed as mean ± SD of triplicates from a representative experiment performed three times.

(**E**) PML-RARα stabilizes TGIF by a mechanism involving PHRF1. U937-MTPR9 cells were transfected with HA-TGIF or HA-TGIF.K130R and cultured in the absence or presence of Zn for 24 hr before being subjected to direct immunoblotting using anti-HA, anti-PML, or anti-PHRF1.

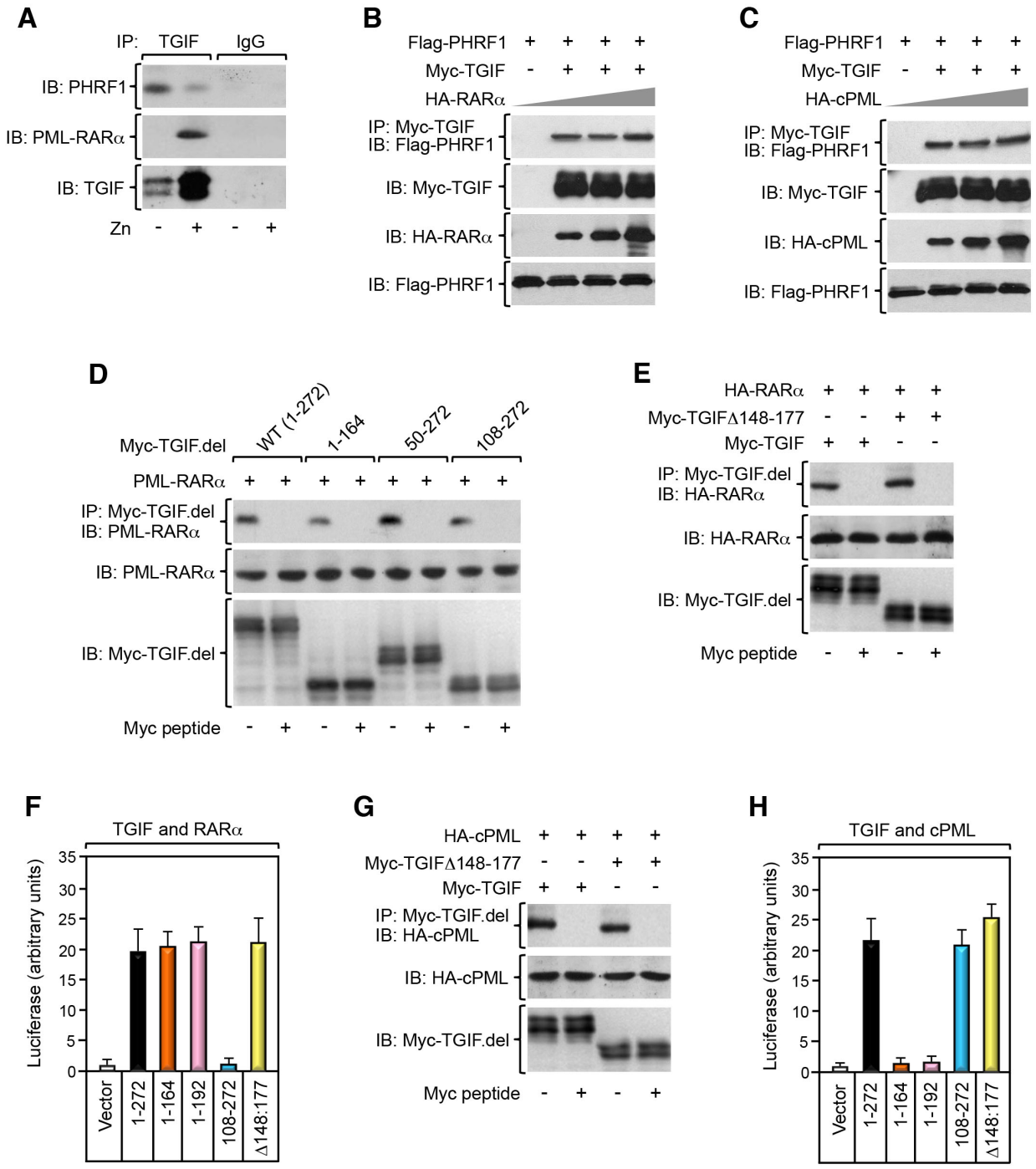

Figure S2: PHRF1 and PML-RARα Competitively Share TGIF. Related to Figure 2.
(A) TGIF associates with both PML-RARα and PHRF1. U937-MTPR9 cells were cultured in the absence or presence of Zn for 24 hr and cell lysates were immunoprecipitated with anti-TGIF or IgG. Interaction of PHRF1 or PML-RARα with TGIF was detected by immunoblotting using anti-PHRF1 or anti-RARα, respectively.

(**B, C**) Expression of RARα or cPML had no effect on the expression levels of TGIF or association of TGIF with PHRF1. U937 cells were transfected with the indicated combinations of HA-RARα, HA-cPML, Myc-TGIF, and Flag-PHRF1, and the association of Myc-TGIF with Flag-PHRF1 was assessed by coimmunoprecipitation. Expression of Myc-TGIF, HA-RARα, HA-cPML, and Flag-PHRF1 was determined by direct immunoblotting.
(D) Mapping of the domains in TGIF that mediate its interaction with PML-RARα. U937 cells were transfected with PML-RARα and the indicated TGIF deletion mutants fused to Myc. Cell extracts were immunoprecipitated with anti-Myc and immunoblotted with anti-RARα to detect PML-RARα. Expression of PML-RARα and TGIF deletion mutants was determined by direct immunoblotting. To attest to the validity of the interaction, anti-Myc was incubated with or without Myc peptide for 30 min prior to immunoprecipitation.
(**E, F**) Mapping of the domains in TGIF that mediate its interaction with RARα. U937 cells were transfected with HA-RARα and the indicated TGIF deletion mutants fused to Myc. Cell extracts were immunoprecipitated with anti-Myc and immunoblotted with anti-HA. Expression of HA-RARα and Myc-TGIF deletion mutants was determined by direct immunoblotting (E). U937 cells were transfected with pG5E1b-Luc together with Gal4-RARα and VP16-TGIF deletion mutants. Luciferase activity was measured and data are expressed as mean ± SD of triplicates (F).
(**G, H**) Mapping of the domains in TGIF that mediate its interaction with cPML. U937 cells were transfected with HA-cPML and the indicated TGIF deletion mutants fused to Myc. Cell extracts were immunoprecipitated with anti-Myc and immunoblotted with anti-HA. Expression of HA-cPML and Myc-TGIF deletion mutants was determined by direct immunoblotting (G). U937 cells were transfected with pG5E1b-Luc together with Gal4-cPML and VP16-TGIF deletion mutants. Luciferase activity was measured and data are expressed as mean ± SD of triplicates (H).

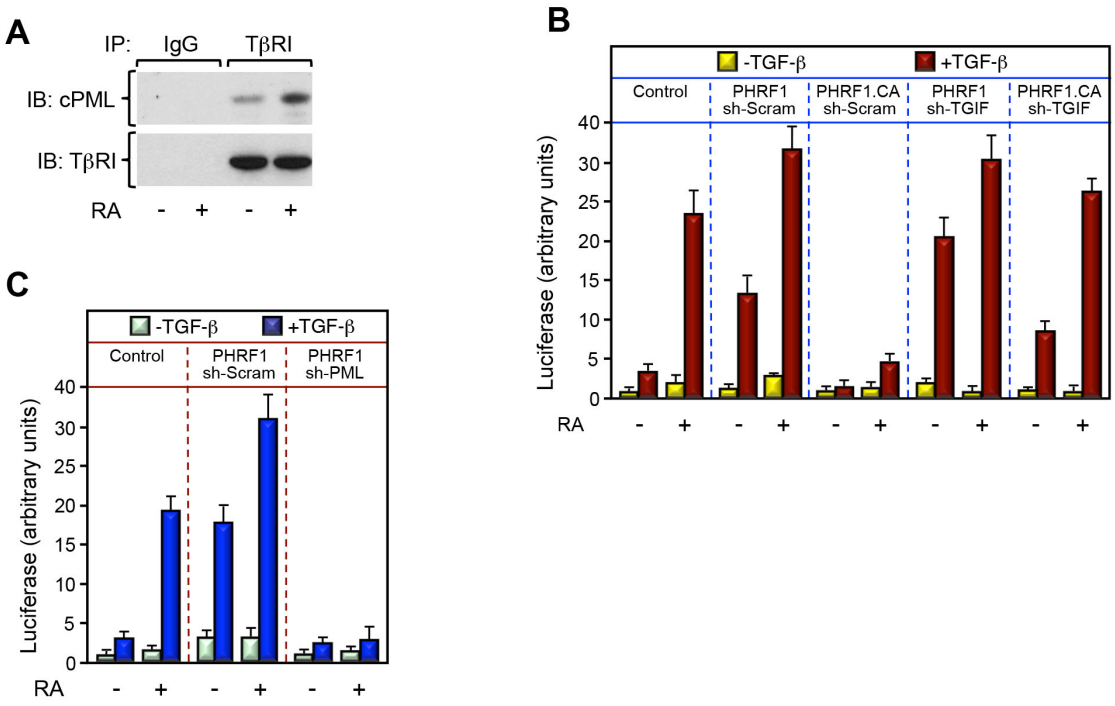

Figure S3: PML-RARα Disrupts TGF-β Signaling by Interfering with the Ability of PHRF1 to Trigger TGIF Degradation. Related to Figure 3.

(**A**) Degradation of PML-RARα restores the interaction between cPML and TβRI. NB4 blasts were treated with or without RA for 24 hr, and the association of endogenous cPML and TβRI was visualized by blotting anti-TβRI immunoprecipitates with anti-PML. Immunoprecipitates with IgG were used as negative controls.

(**B, C**) PML-RARα suppresses TGF-β-induced gene expression by antagonizing PHRF1. NB4 blasts were transfected with $CAGA_9$-Lux together with the indicated combinations of PHRF1, PHRF1.CA, sh-Scram, sh-TGIF, and sh-PML. Cells were then treated with RA for 24 hr prior to treatment with TGF-β for 16 hr. Luciferase activity was measured and data are expressed as mean ± SD (n = 3).

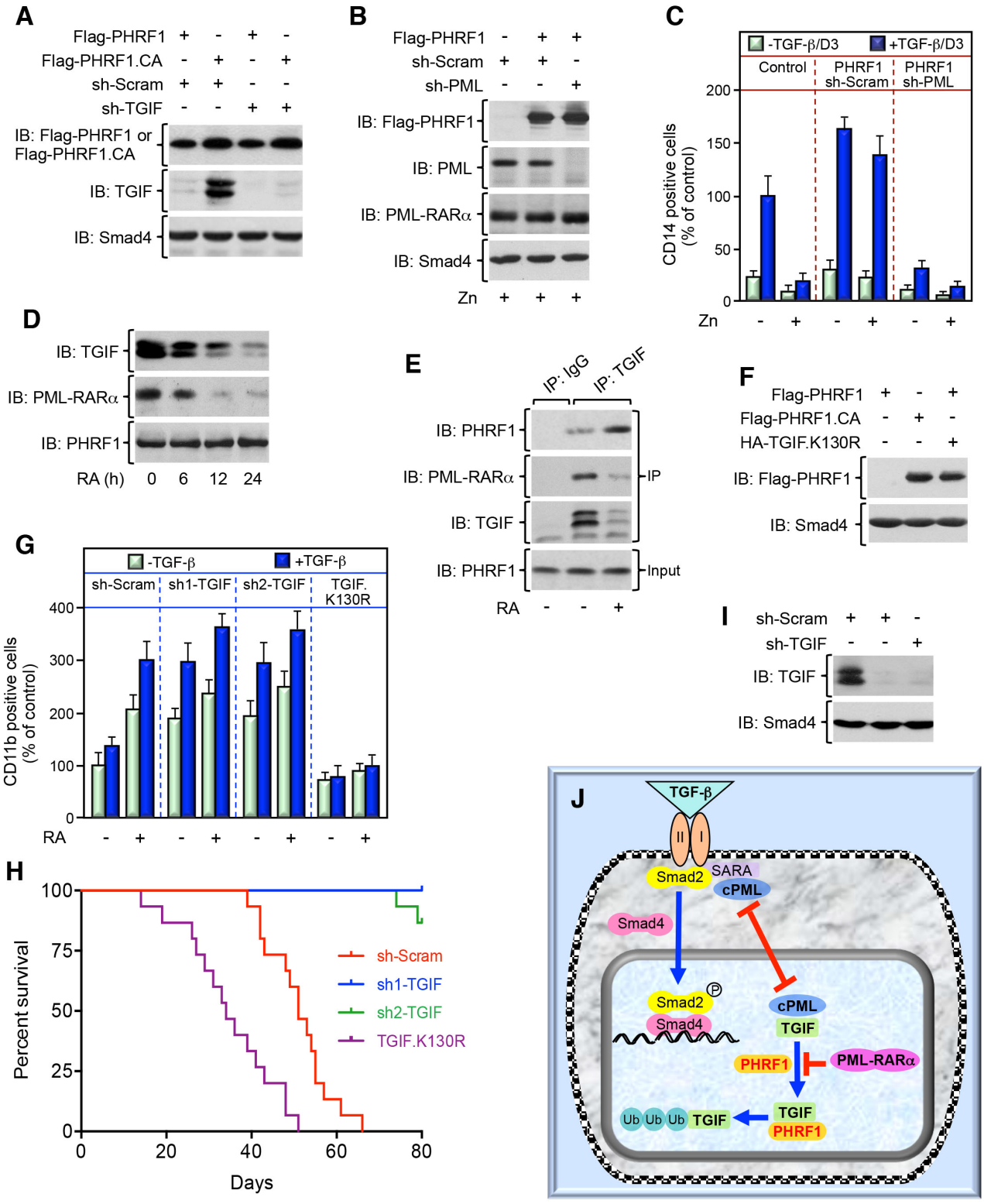
A
Flag-PHRF1
Flag-PHRF1.CA
sh-Scram
sh-TGIF
IB: Flag-PHRF1 or Flag-PHRF1.CA
IB: TGIF
IB: Smad4
B
Flag-PHRF1
sh-Scram
sh-PML
IB: Flag-PHRF1
IB: PML
IB: PML-RARα
IB: Smad4
Zn
C
-TGF-β/D3
+TGF-β/D3
Control
PHRF1 sh-Scram
PHRF1 sh-PML
CD14 positive cells (% of control)
Zn
D
IB: TGIF
IB: PML-RARα
IB: PHRF1
RA (h) 0 6 12 24
E
IP: IgG
IP: TGIF
IB: PHRF1
IB: PML-RARα
IB: TGIF
IP
IB: PHRF1
Input
RA
F
Flag-PHRF1
Flag-PHRF1.CA
HA-TGIF.K130R
IB: Flag-PHRF1
IB: Smad4
G
-TGF-β
+TGF-β
sh-Scram
sh1-TGIF
sh2-TGIF
TGIF. K130R
CD11b positive cells (% of control)
RA
H
Percent survival
Days
sh-Scram
sh1-TGIF
sh2-TGIF
TGIF.K130R
I
sh-Scram
sh-TGIF
IB: TGIF
IB: Smad4
J
TGF-β
II
I
Smad2
SARA
cPML
Smad4
Smad2
P
Smad4
cPML
TGIF
PHRF1
PML-RARα
Ub Ub Ub TGIF
TGIF
PHRF1

Figure S4: PML-RARα Drives APL Formation by Interfering with PHRF1-Driven TGIF Degradation. Related to Figure 4.

(**A**) Analysis of stable cell lines used in Figure 4A. Cell extracts from U937-MTPR9 cells stably expressing the indicated combinations of Flag-PHRF1, Flag-PHRF1.CA, sh-Scram, and sh-TGIF were analyzed by direct immunoblotting.

(**B, C**) Enforced expression of PHRF1 restored TGF-β-induced myeloid differentiation. U937-MTPR9 cells were transduced with the indicated combinations of retroviruses or lentiviruses encoding Flag-PHRF1, sh-Scram, and sh-PML. Extracts from cells cultured with Zn for 16 hr were subject to direct immunoblotting (B). Cells were cultured with or without Zn for 16 hr before being treated with or without TGF-β plus vitamin D3 (TGF-β/D3) for 5 days. Cell differentiation was evaluated by determining the number of positive CD14 cells (C).

(**D**) RA stimulation induced TGIF degradation in murine MRP8 blasts. MRP8 cells were treated with RA for various time periods, and TGIF, PML-RARα, or PHRF1 expression was determined by direct immunoblotting.

(**E**) RA stimulation promoted the assembly of the TGIF/PHRF1 complex at the expense of the TGIF/PML-RARα complex. MRP8 cells were treated with RA for 24 hr, and the association of TGIF with PHRF1 or PML-RARα was analyzed by immunoblotting anti-TGIF or IgG immunoprecipitates with anti-PHRF1 or anti-RARα, respectively.

(**F**) Expression of PHRF1 blocked APL development *in vivo*. Expression of Flag-PHRF1 was examined using spleen from mice transplanted with MRP8 cells stably expression Flag-PHRF1, Flag-PHRF1.CA, or Flag-PHRF1 plus HA-TGIF.K130R.

(**G, H, I**) TGIF deficiency suppressed APL formation. MRP8 cells were transduced with lentiviruses encoding control (sh-Scram) or two independent sh-RNAs targeting TGIF. Cells were treated with the indicated combinations of RA and TGF-β for 4 days and granulocytic differentiation was evaluated by determining the number of positive CD11b cells (G). Cells were transplanted to FVB mice, and the survival was recorded in a Kaplan-Meier graph (H). Expression of TGIF was determined by direct immunoblotting (I).

(**J**) A model depicting how PML-RARα disrupts the TGF-β tumor suppressor network.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Expression Vectors

The expression vectors pG5E1b-Luc, pCMV5-Myc-FAST1, pCMV5-HA-TGIF, pCMV5-HA-TGIF.K130R, pcDNA3-6xMyc-TGIF, pcDNA3-6xMyc-TGIFΔ148-177, pBICEP-CMV2-Flag-PHRF1, pBICEP-CMV2-Flag-PHRF1.CA, and pcDNA3-His-Ub were previously described (Demange et al., 2009; Ettahar et al., 2013; Faresse et al., 2008; Prunier et al., 2001; Seo et al., 2006; Seo et al., 2004). Dr. L. Di Croce kindly provided the expression vector encoding PML-PARα. pBabe-Zeo, pUMVC, and pCMV-VSV-G were obtained from Addgene. The gene reporter constructs pGL3-$CAGA_9$-Lux and pGL3-ARE_3-Lux were previously described (Dennler et al., 1998; Labbe et al., 1998). pEGFP-C2, pVP (VP16), pMT (Gal4), pLVX-Tet-Off, and pLVX-Tight-Puro plasmids were purchased from Clontech. The pGIPZ expression vectors encoding sh-Scram (scrambled), sh-PHRF1, sh-TGIF, sh-RARα, or sh-PML were purchased from Thermo-scientific.

To generate the Dox-repressible Flag-PHRF1 and Flag-PHRF1.CA expression vectors, the corresponding cDNAs were obtained by PCR and cloned into pLVX-Tight-Puro. A similar strategy was used to generate pVP-VP16-TGIF, pMT-Gal4-PML-RARα, pMT-Gal4-RARα, pMT-Gal4-cPML, pEGFP-TGIF, pCMV5-HA-cPML, pCMV5-HA-RARα, pBabe-Zeo-Flag-PHRF1, pBabe-Zeo-Flag-PHRF1.CA, or pBabe-Zeo-HA-TGIF.K130R.

Antibodies

Immunoprecipitation, immunoblotting, or FACS were performed using the following antibodies: anti-PHRF1 (Ettahar et al., 2013), anti-Flag M2, fluorescein-labeled sheep anti-mouse IgG (Sigma), Alexa-Fluor®568-labeled goat anti-mouse IgG (Invitrogen), anti-HA (Roche), anti-pSmad2 (UBI), anti-Smad2 (Zymed), anti-TβRI (R&D Systems), anti-CD14, anti-CD11b (BD Biosciences), anti-RARα, anti-c-Myc 9E10, anti-α-Tubulin, anti-SARA, anti-TGIF, anti-Smad4, and anti-PML (Santa Cruz Biotechnology).

To attest to the validity of the interaction in the experiments dedicated to mapping the domains in TGIF that mediate its interaction with cPML, RARα or PML-RARα, anti-Myc antibody was incubated with or without the Myc peptide for 30 min prior to immunoprecipitation.

Cell Treatments

For RA stimulation, cells were incubated in the absence or presence of 1 μM RA (Sigma) for 24 hr, unless the time is specified in figures. For TGF-β treatments, cells were cultured in the absence or presence of 200 pM TGF-β1 (Sigma) for the times indicated in figures. For Vitamin D3 treatments, cells were treated with 250 ng/ml Vitamin D3 (Sigma) for the times indicated in figures.

Transfection and Viral Transduction

Transient and stable transfections were performed using the Lipofectamine method according to the manufacturer's instructions (Invitrogen). To establish cells stably expressing HA-TGIF.K130R, Flag-PHRF1, or Flag-PHRF1.CA, cells were transduced with pBabe-Zeo-HA-TGIF.K130R, pBabe-Zeo-Flag-PHRF1, or pBabe-Zeo-Flag-PHRF1.CA and selected with Zeocin. At least five resistant colonies were pooled and expanded as a single cell line. To generate MRP8 cells harboring Dox-repressible Flag-PHRF1 or Flag-PHRF1.CA, cells were transduced with pLVX-Tet-Off together with pLVX-tight-Flag-PHRF1 or pLVX-tight-Flag-PHRF1.CA, selected with puromycin, pooled, and tested for the expression of Flag-PHRF1 or Flag-PHRF1.CA in the presence or absence of Dox by immunoblotting using anti-Flag M2 antibody. Of note, MRP8 blasts stably expressing Dox-repressible Flag-PHRF1 or Flag-PHRF1.CA were maintained in Dox-containing media to prevent myeloid differentiation. To generate cells depleted for PHRF1, TGIF, RARα, or cPML, cells were transduced with the pGIPZ lentiviruses expressing the corresponding shRNA or scrambled shRNA (sh-Sram), selected with puromycin, and pooled. To generate MRP8 cells depleted for TGIF, cells were transduced with pGIPZ-sh-TGIF for 48 hr and selected with puromycin for 24 hr before transplantation into recipient mice.

To generate lentiviruses producing sh-Scram, sh-PHRF1, sh-TGIF, sh-RARα, or sh-PML, the pGIPZ-shRNA plasmids were transfected into HEK293T cells along with the packaging mix and high-titter lentiviruses were purified by centrifugation following the manufacturer's guidelines (Thermo-scientific). A similar strategy was used to generate the lentiviruses, pLVX-Tet-Off**,** pLVX-tight-Flag-PHRF1, and pLVX-tight-Flag-PHRF1. To generate retroviruses encoding HA-TGIF.K130R, Flag-PHRF1, or Flag-PHRF1.CA, HEK293T cells were transfected with the corresponding pBabe-Zeo expression vector

together with the packaging expression vectors pUMVC and VSV-G. Conditioned medium containing retroviruses was used directly to infect cells.

Reporter Assays

Cells were transfected and treated with or without the indicated combinations of RA and TGF-β in medium containing 0.5% FCS. Then, cell extracts were assayed for luciferase activity with the dual luciferase assay system according to the manufacturer's instructions (Promega) and normalized.

For the mammalian two-hybrid system, cells were transfected with pG5E1b-Luc together with VP16-TGIF deletion mutants and Gal4-PML-RARα, Gal4-RARα, or Gal4-cPML. After 48 hr, cells were assessed for luciferase activity as described above. In all experiments, data are expressed as mean ± SD of triplicates from a representative experiment performed at least three times.

Histopathological Analyses

To examine infiltration of myeloid cells into the liver or spleen, tissues were fixed in formalin, paraffin embedded, and sections were stained with Harris Haematoxylin (H&E) solution (Sigma). Photographs were taken on a Nikon Eclipse 80i microscope with a Nikon Digital Sight camera (DS-Fi1) using NIS-Elements BR3.22.11 software. The weight of spleen is measured and the results were expressed as mean ± SD of all mice examined (n=15).

Real-Time PCR

Poly(A)+ RNA was isolated using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using total RNA and SuperScript 2 according to the manufacturer's instructions (Invitrogen). cDNA was mixed with iQ SYBR green supermix (BioRad) and sense and antisense primers, denatured at 95 °C for 3 minutes and amplified by 40 cycles of 95°C/57°C/72°C for 30 seconds each using an iCycler (BioRad). All samples were normalized to GAPDH and data are represented as percentage of control samples.

Primers used are:

-TGIF-Forward: 5'-GATCCAGAATGAAAGGCAAGA-3'

-TGIF-Reverse: 5'-GAAGAAAGGTCCAAGGGAATG-3'

-GAPDH-Forward: 5'-AGCCACATCGCTCAGACAC-3'

-GAPDH-Reverse: 5'-GCCCAATACGACCAAATCC-3'

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