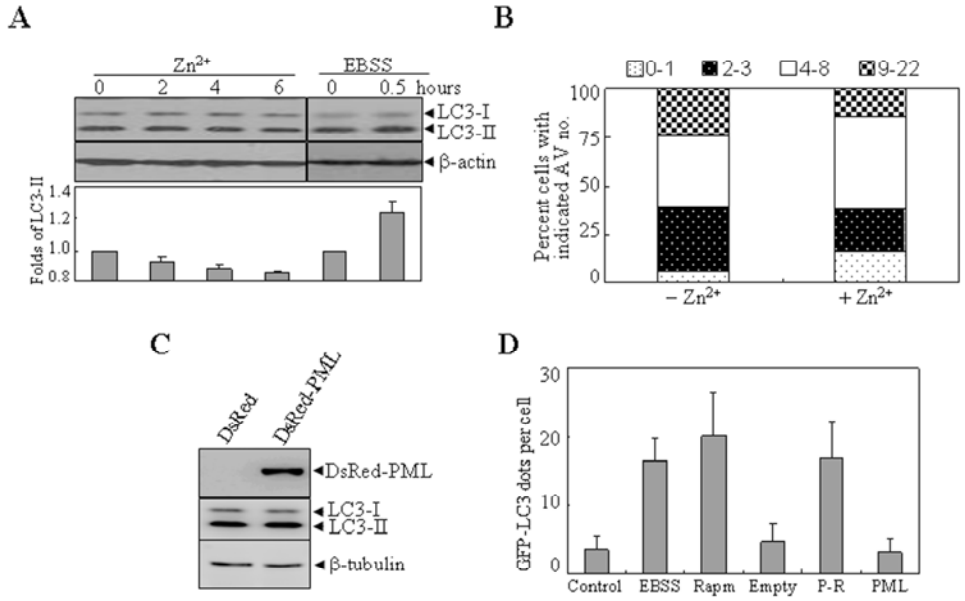
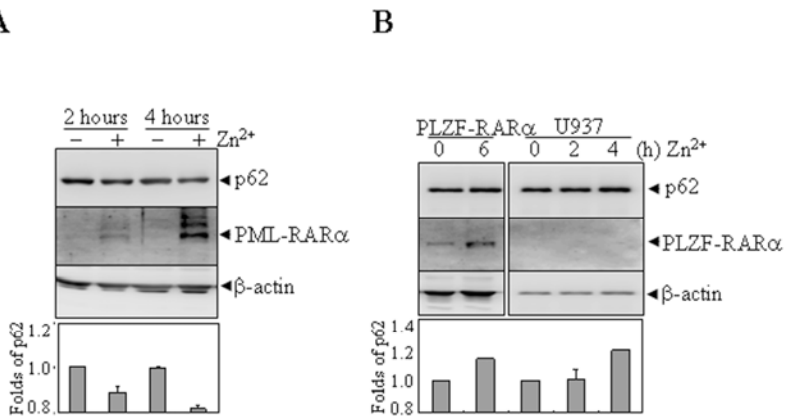
Supplemental materials for Huang Y et al:

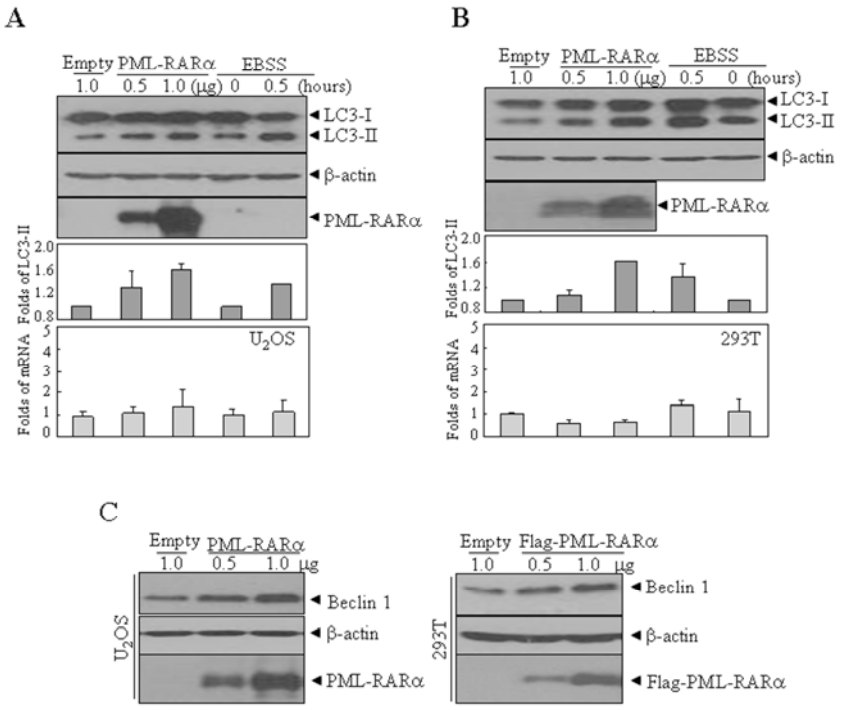
PML-RARα enhances constitutive autophagic activity through inhibiting Akt/mTOR pathway



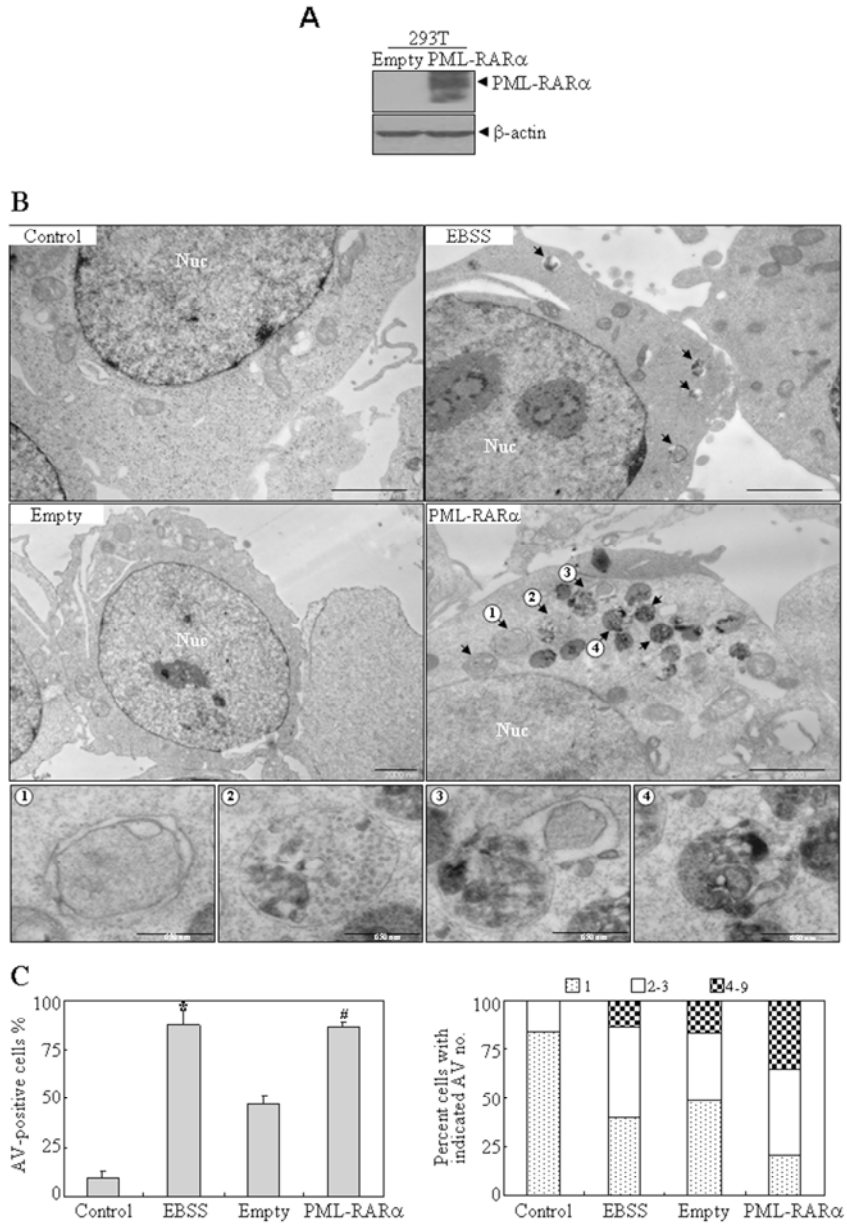
Supplemental Fig S1. The effects of PML expression and Zn^{2+} induction on autophagic activity in non-leukemic and leukemic U937 cells. (A) U937 cells were respectively treated with or without 100 μM $ZnSO_4$, or with EBSS for the indicated hours. Cell lysates were harvested for immunoblotting proteins as indicated. Relative LC3-II expression was determined by the ratio of densitometric value of LC3-II relative to the corresponding untreated controls. (B) U937 cells were treated with or without $ZnSO_4$ for 4 hours, and fixed and subjected to TEM analysis. The percent cells with the indicated AV numbers were summarized in the graph. (C) U_2OS cells were transiently transfected with 1.0 μg DsRed-PML or the empty vector. After transfection for 24 hours, the cell lysates were extracted for immunoblotting proteins as indicated. (D) Quantification of GFP-LC3 foci per cell was analyzed for the same experiment as performed in Figure 1E. "P-R" represents PML-RARα. All the experiments were repeated three times and similar results were obtained.



Supplemental Fig S2. The effect of PML-RARα expression on p62 protein. U937/PR9 (A), U937/PLZF-RARα and U937 (B) cells were respectively incubated with or without 100 μM $ZnSO_4$ for the indicated hours. The cell lysates were extracted and proteins as indicated were analyzed by western blot. The folds of p62 protein against the corresponding untreated cells were shown under the panel. These experiments were repeated three times and similar results were obtained.

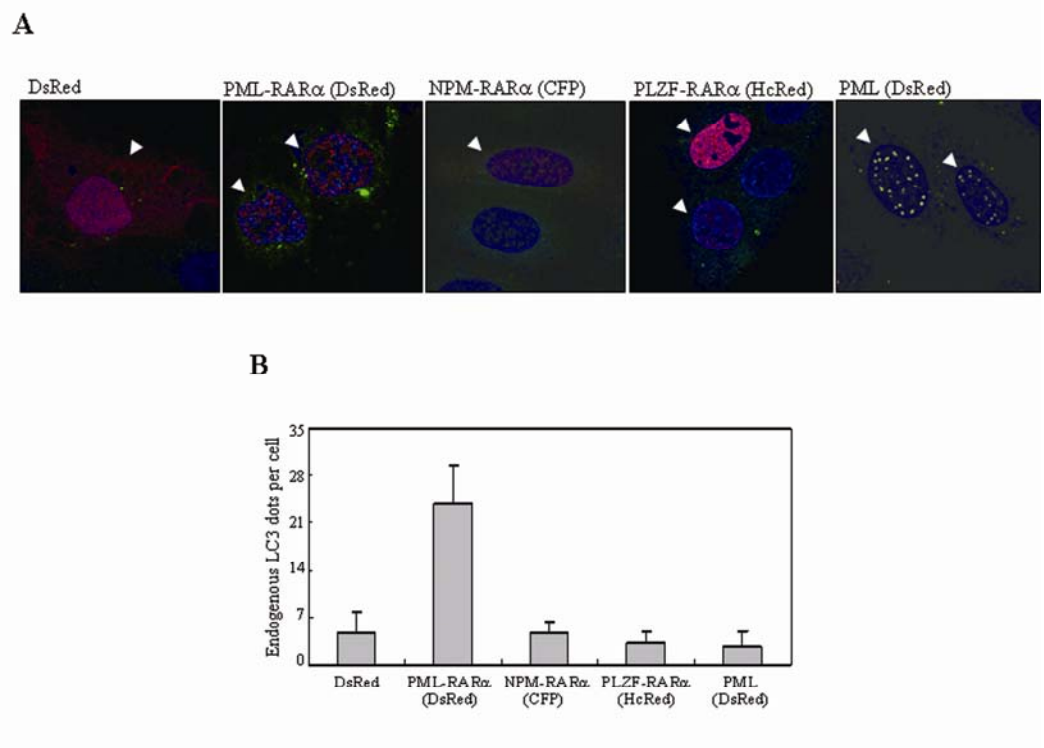


Supplemental Fig S3. Ectopic expression of PML-RARα enhanced LC3-II and Beclin 1 protein accumulation in non-leukemic cells. U_2OS (A, C) and HEK293T (B, C) cells were transfected with the indicated amounts of PML-RARα plasmid or the corresponding empty vector. EBSS treatment was used as a positive control. After 24 hours, the cellular proteins and mRNA were extracted. Western blot and real-time RT-PCR were performed to examine the indicated proteins and LC3 mRNA, respectively. Relative LC3-II expression levels was determined by the ratio of densitometric value of LC3-II relative to the untreated or vector controls. Independent experiments were repeated at least three times and similar results were obtained. All values are shown as means±S.D. of three independent experiments, each with triplicate samples.

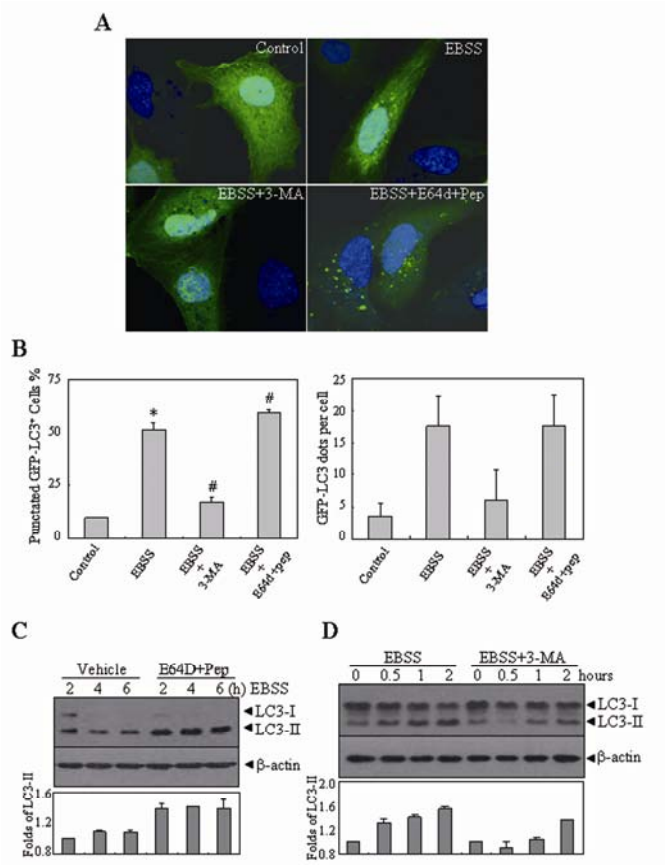


Supplemental Fig S4. Overexpression of PML-RARα enhances autophagosome formation in HEK293T cells. The cells were transfected with 1.0 μg pSG5 or 1.0 μg pSG5-PML-RARα expression plasmids. After 24 hours, the cells were fixed and subjected to TEM analysis. (A) The expression of PML-RARα after transfection was analyzed by western blot with RARα antibody and β-actin was used as a loading control. (B) The representative electron micrographs of HEK293T cells with the indicated treatment or indicated transfection were shown under TEM, with EBSS treatment for 1 hour as a positive control. AV structures were indicated with arrowheads. "Nuc" stands for nucleus. The indicated scale shown in images was 2000

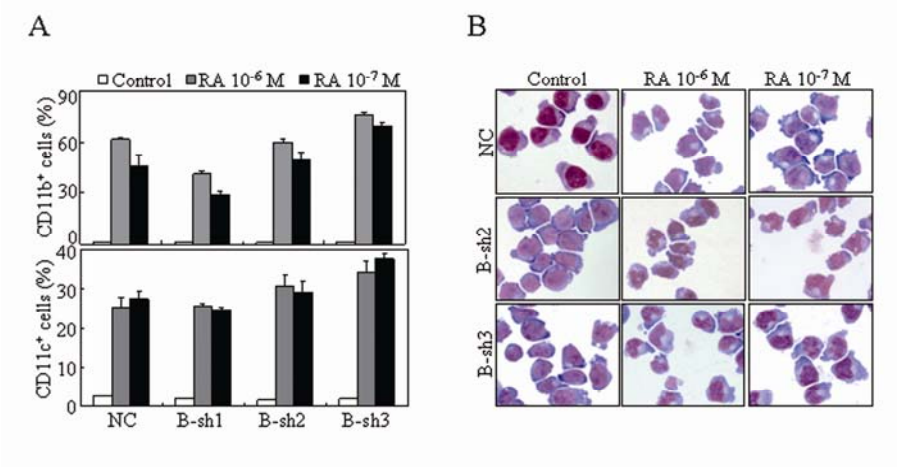
nm. Panels ①-④ were corresponding enlarged regions (scale bar = 650 nm) from the PML-RARα-expressing cell, showing typical AV structures with double- or mono-membranous vacuoles. (C) Quantification of the data in panel B was calculated and summarized. The percentages of AV^+ cells were shown in left panel and the percentages of cells with indicated AV numbers per AV^+ cell were shown in the right panel. The symbols * and # indicate *p* values of less than 0.05 compared with the control or cells transfected with empty vector, respectively. The experiments were repeated three times and similar results were obtained.



Supplemental Fig S5. The effects of APL-related fusion proteins and wild-type PML on the distribution of endogenous LC3. U_2OS cells were respectively transfected with the indicated plasmids. After transfection for 24 hours, endogenous LC3 protein was detected by indirect immunostaining with anti-LC3 antibody, followed by the observation with confocal microscopy. (A) Representative merged images of endogenous LC3 protein and the indicated transfected proteins were shown. Arrowheads point to cells with the expression of transfected protein. Of note, the upper cell expressed higher while the lower cell expressed a few of PLZF-RARα protein in PLZF-RARα (HcRed) plasmid-transfected cells. (B) The total numbers of endogenous LC3 dots per cell were calculated and analyzed from 30 cells with the indicated protein expression in an independent experiment. These experiments were repeated three times and similar results were obtained.



Supplemental Fig S6. Effects of lysosomal enzyme inhibitors and 3-MA on EBSS-induced GFP-LC3 aggregation and LC3-II enhancement. U_2OS cells, transfected with GFP-LC3 plasmid for 24 hours, were treated with or without EBSS in the absence or presence of Pepstatin A (10 μg/ml) and E64d (10 μg/ml) or 3-MA (10 mM) for 1 hour, respectively. The cells were then fixed and analyzed by confocal microscopy. (A) The representative images of the cells with the indicated treatment were shown. (B) Quantification of GFP-LC3-positive cells. The percentage of GFP-LC3-positive cells (left) and the total number of GFP-LC3 dots per cell (right) were calculated. The symbols * and # indicated *p* values of less than 0.001 and 0.05 compared with control or EBSS-treated cells, respectively. (C-D) U_2OS cells were treated with EBSS in the absence and presence of Pepstatin A (10 μg/ml) and E64d (10 μg/ml) (C) or 3-MA (10 mM) (D) for the indicated time. Relative LC3-II expression was determined by the ratio of densitometric value of LC3-II relative to the 2h EBSS plus vehicle-treated (C) or to the untreated controls (D). All experiments were repeated at least three times and similar results were obtained. The values are shown as means±S.D. from three independent experiments.



Supplemental Fig S7. The effect of Beclin 1 suppression on ATRA-induced leukemic cell differentiation. The indicated cells were treated with or without 10^{-6} M or 10^{-7} M ATRA for 24 hours. Then, CD11b- and CD11c-positive cells (A) as well as cell morphology (B) were examined by flow cytometry and microscopy, respectively. The y-axes in (A) represent mean±S.D. of triplicates from an independent experiment. These experiments were repeated three times and similar results were obtained.