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Supplemental Information

Promyelocytic Leukemia Protein Is an Essential Regulator of Stem Cell Pluripotency and Somatic Cell Reprogramming

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Supplemental Data

I. Supplemental Figures and Figures Legends

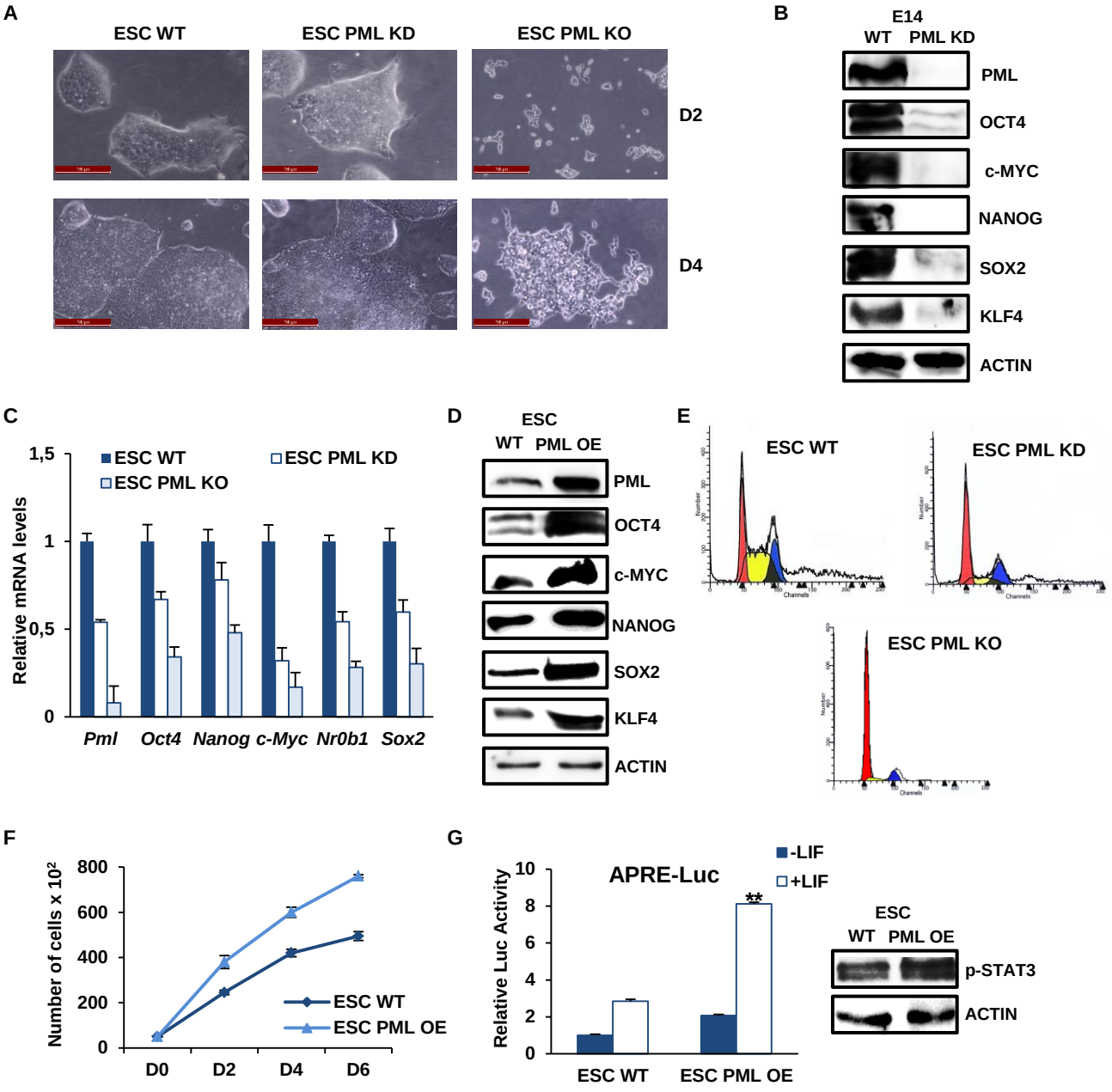

Figure S1 (related to Figure 1): PML is required for self-renewal and pluripotency of ESC. A. WT, PML KD and KO ESC colonies after 2 and 4 days in culture. Scale bar, 100µm. **B.** Pluripotency factors protein levels upon depletion of PML in E14 cell line. **C.** Relative mRNA levels of pluripotency factors in WT, PML KD and KO ESC. Error bars indicate +SD in three independent experiments (n=3). **E.** Cell cycle phase distribution of WT, PML KD and KO ESC. **F.** Growth curve of WT and PML OE ESC. Data represent the mean +/- SD of four independent experiments (n=4). **G.** The activity of APRE-Luc and p-STAT3 protein expression levels in ESC WT and PML OE ESC. Data represent the mean + SD of four independent experiments (n=4), * p<0,05.

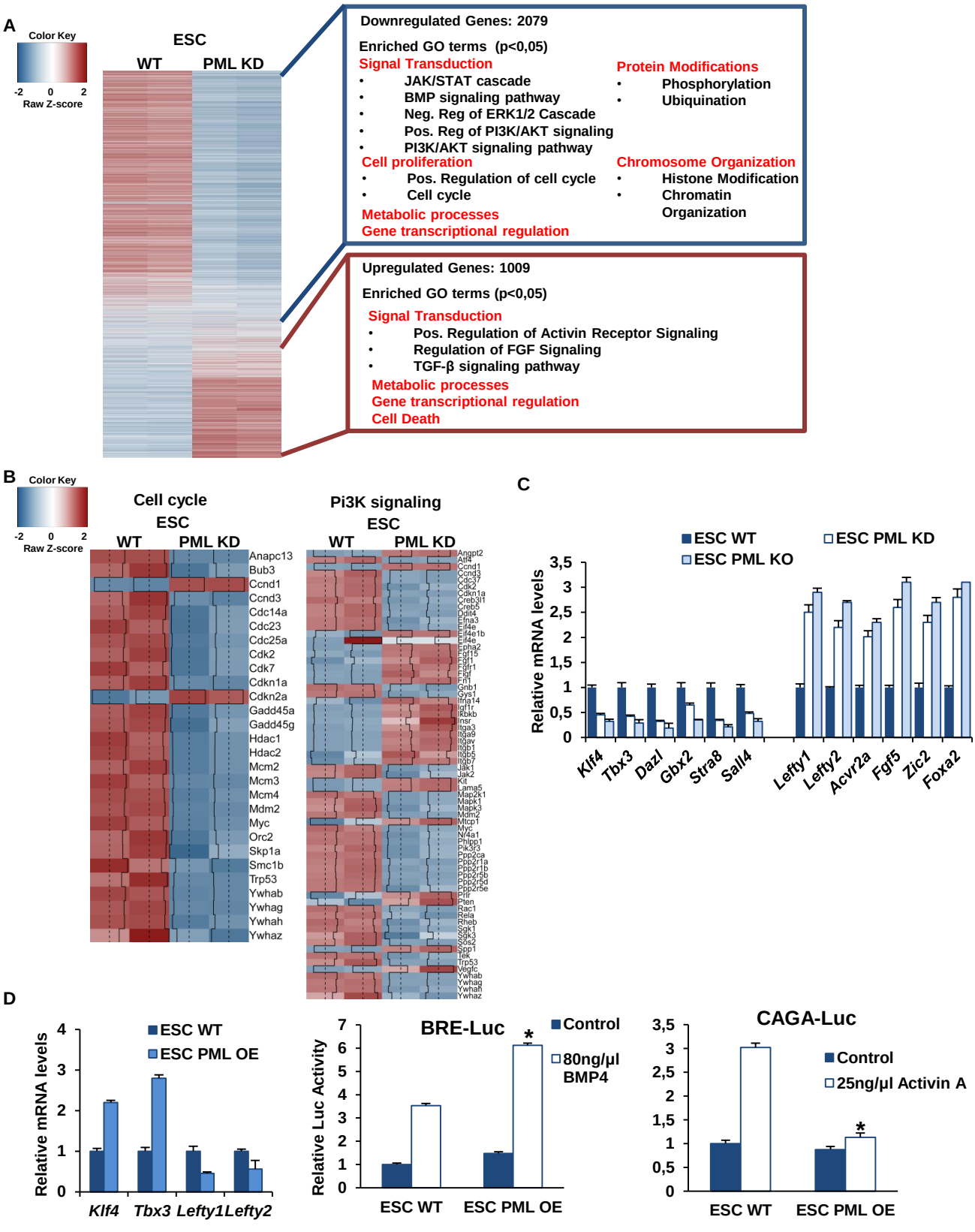

Figure S2 (related to Figure 2): Genome wide analysis from PML WT and PML KD ESC A. Heat map representing differential expression of genes in PML KD ESC compared to ESC WT. GO analysis was performed using RNEA software. Representative enriched terms were also shown. **B.** Heat map with the top representative cell-cycle (left panel) and pI3K signaling pathway (right panel) related genes. **C.** Relative mRNA levels of characteristic naïve and primed genes in PML KD, PML KO and control ESCs. Error bars indicate +SD of four independent experiments (n=4). **D.** Naïve and primed pluripotency factors expression levels in PML OE ESC cell line (left panel). BRE-Luc and CAGA-Luc reporters activity upon PML enforced expression (right panel). Data are shown as mean +SD of three independent experiments (n=3), p*<0,05.

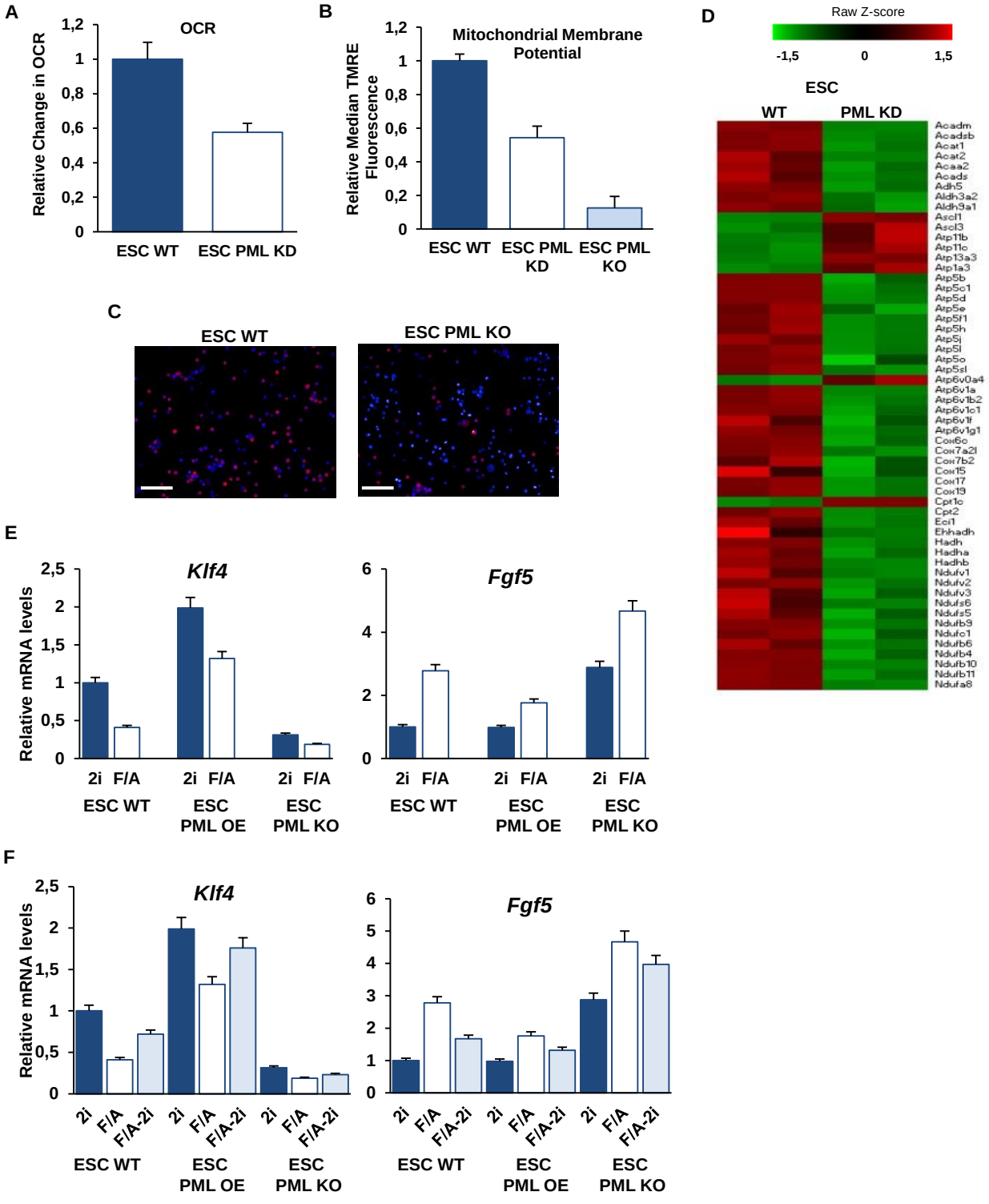

Figure S3 (related to Figures 2 & 3): PML is essential for naïve pluripotent state maintenance A. Oxygen Consumption Rate (OCR) measured in WT and PML KD ESC. **B.** Mitochondrial membrane potential of WT, PML KD and KO ESC measured by TMRE. Error bars indicate +SD of four independent experiments (n=4). **C.** Fluorescence images of TMRE incubated with WT and PML KO ESC. Scale bar, 50µm. **D.** Heat map showing OXPHOS and FAO related genes expression in WT and PML KD ESC. **E.** Relative mRNA levels of *Klf4* and *Fgf5* in WT, PML OE and PML KO ESC. Cells were cultured either in 2i/LIF or F/A for 6 days. **F.** RT-PCR analysis of *Klf4* and *Fgf5* in WT, PML OE and PML KO ESC. Cells were culture in: 2i/LIF (6 days), F/A (6 days) and F/A→ 2i/LIF (6 days F/A plus 6 days 2i/LIF).

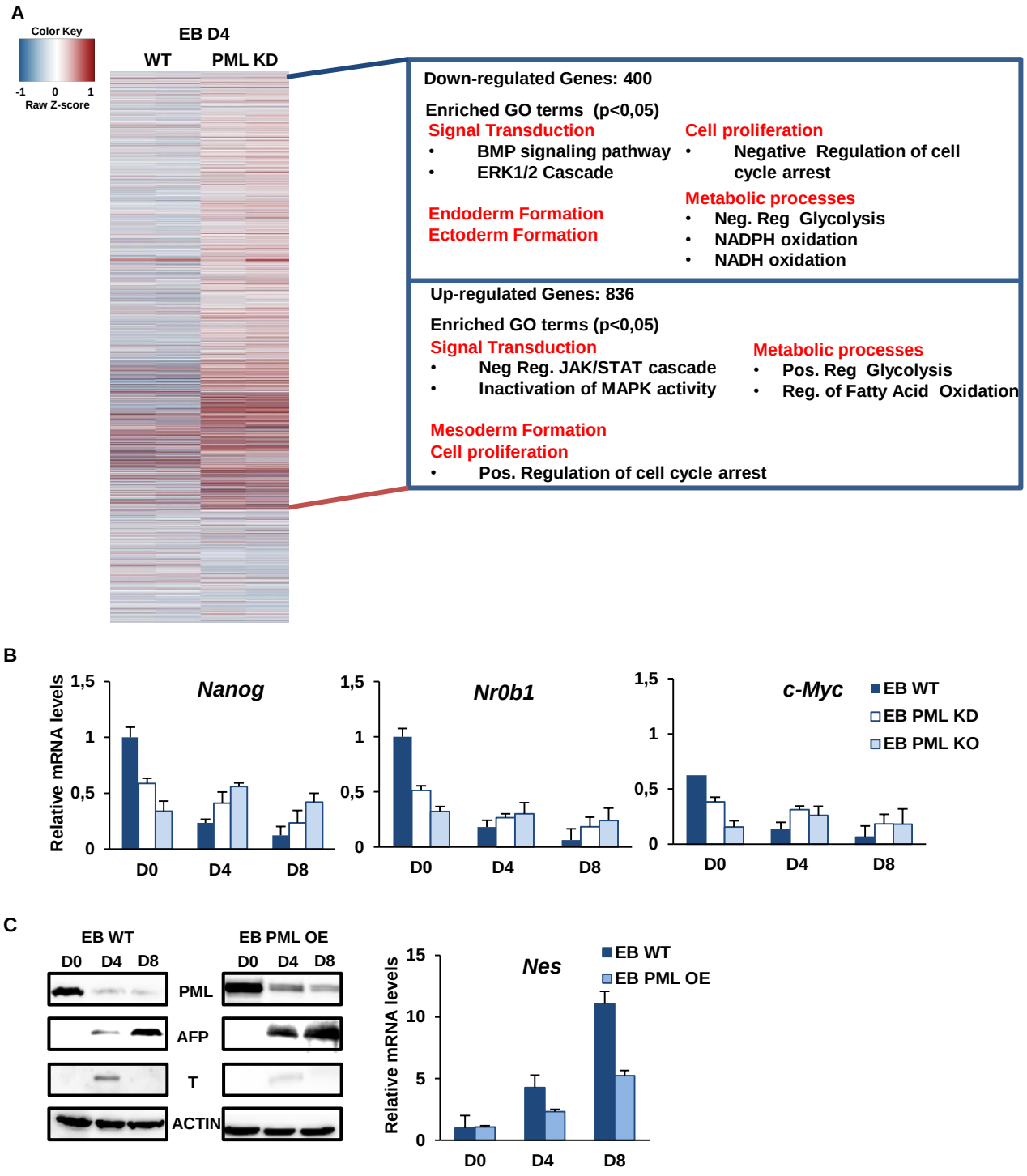

Figure S4 (related to Figure 4): Genome wide analysis from EB D4 PML WT and PML KD A. Heat map representing differential expression of genes in PML KD EB D4 compared to EB D4 WT. GO analysis was performed using RNEA software. Representative enriched terms were also shown. **B.** mRNA levels of *Nanog, c-myc, Nr0b1* in PML KD, KO and control EB D4. Error bars indicate +SD of four independent experiments (n=4). **C.** Protein levels of PML, AFP and T upon differentiation of WT and PML OE ESC (left panel). Characteristic ectodermal gene (*Nes*) mRNA levels (right panel). Data are shown as mean + SD of three independent experiments (n=3).

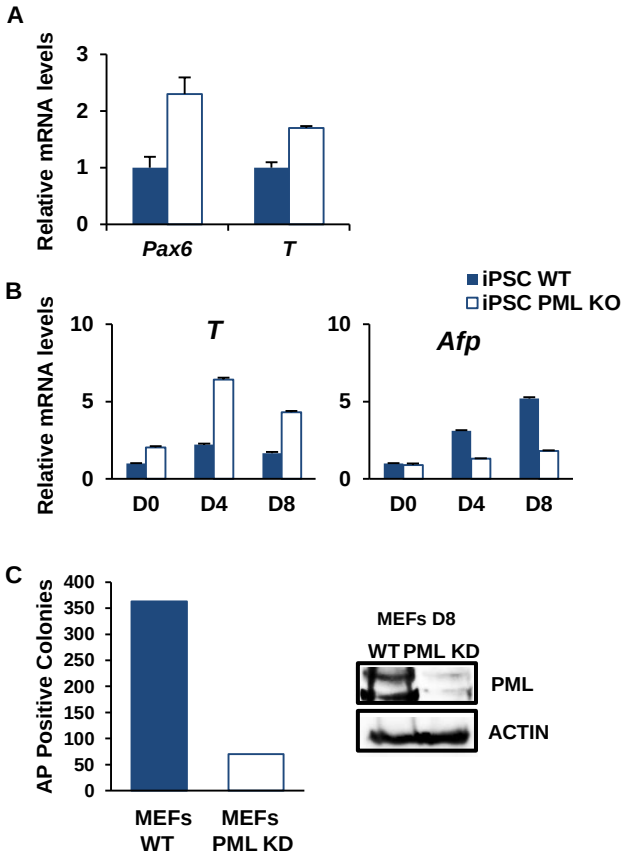

Figure S5 (related to Figure 5): Characterization of the derived *Pml*$^{-/-}$ and WT iPSC colonies. A. mRNA levels of developmental markers (*Pax6* and *T*) in *Pml*$^{-/-}$ and WT iPSC. **B.** *T* and *Afp* mRNA levels upon differentiation of *Pml*$^{-/-}$ or WT iPSC. **C.** AP-positive colony numbers 28 days after OSKM lentiviral transduction in combination with shRNA against *Pml.* PML protein expression levels at D8 of reprogramming process. Data are shown as mean + SD of four independent experiments (n=4).

Supplementary Experimental Procedures

Reverse Transcription and RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was subsequently reverse-transcribed from 2µg RNA by M-MLV Reverse Transcriptase (Takara) supplemented with RNase inhibitor (HT Biotechnology). Target genes expression was normalized against Actin. Primer sets used for real time PCR analysis are shown below. Relative abundance of each transcript was measured by quantitative real time PCR with SYBR Green I (Invitrogen).

1. ***Actin*** FOR: 5' GTGTGACGTTGACATCCGTA 3', REV: 5' GTAACAGTCCGCCTAGAAGC 3',
2. ***Afp*** FOR: 5' AAGCTGCGCTCTCTACCACCAGA 3', REV: 5' ACCACAGCCGGACCATT 3',
3. ***Eomes*** FOR: 5' GCTTCCGGGACAACTACGA 3', REV: 5' GAGAGGAGGCCGTTGGTCT 3',
4. ***Fgf5*** FOR: 5' GCAGAAGTAGCGCGACGTTT 3', REV: 5' TTGACTTTGCCATCCGGGTAG 3',
5. ***Fzd2*** FOR: 5'AGTTCCACGGGGAGAAGGG 3', REV: 5' AGCGGGTAGAACTGATGCAC 3',
6. ***Gata4*** FOR: 5' GCCAACTGCCAACTACCAC 3', REV: 5' GACCTGCTGGCGTCTTAGA 3',
7. ***Gata6*** FOR: 5' GCCACTGTGGAGACGAGA 3', REV: 5' CATATAGAGCCCCGAAGCA 3',
8. ***Hand1*** FOR: 5' ACCAGTTACATCGCCTACTTGA 3', REV: 5'CGCGACCACCATCCGTCTT 3',
9. ***Id1*** FOR: 5' GACTACATCAGGGACCTGCAGC 3', REV: 5' GGCCGCCAAGGCACTGATCTCG 3',
10. ***Id2*** FOR: 5' ATCCCCCAGAACAACAAGGT 3', REV: 5' ACCTTCTTGTTCTGGGGGAT 3',
11. ***Id3*** FOR: 5' CCAGGTGGAAATCCTGCACC 3', REV: 5' CTCTTGTCCTTGGAGATCACAA 3',
12. ***Jmjd3*** FOR: 5' GTACAGACCCCCGGAACC 3', REV: 5' TGGTGGAGAAAAGGCCTAAG 3'
13. ***Junb*** FOR: 5' ACCCCTACCGGAGTCTCAA 3', REV: 5' GGAGTAGCTGCTGCTGAGGTTG 3'
14. ***Lefty2*** FOR: 5' AGCACGCGACCGCTCCC 3', REV:5' CGATGCTCCATTCCGAACAC 3'
15. ***Nanog*** FOR: 5' CGCTGCTCCGCTCCATAACT 3', REV: 5' GCGCATGGCTTTCCCTAGTG 3',
16. ***Nr0b1*** FOR: 5' CTGGTGTGCAGCGTCTGA 3', REV: 5' GTGTTGGTCTCCGGATCTC 3',
17. ***Pax6*** FOR: 5' GGTGCTGGACAATGAAAACA 3', REV: 5' GGTACAGACCCCCTCGGATAA 3',
18. ***Pim-1*** FOR: 5' ATGCTCTTGTCCAAAATCAACTCGCTTGCC-3′, REV: 5′ TGATGAAGTCGAAGAGATCTTGCACCGGCT-3′
19. ***Oct4*** FOR: 5' CCCTGGGCGTTCTCTTTGGA 3', REV: 5' ACCAGGGTCTCCGATTTGCAT 3',
20. ***Otx2*** FOR: 5' CCGGAAACAGCGAAGGGA 3', REV: 5' GCTGTTGGCGGCACTTAG 3',
21. ***Sox17*** FOR: 5' CTCTGCCCTGCCGGGATGG 3', REV: 5' AATGTCGGGGTAGTTGCAATA 3',
22. ***T*** FOR: 5' GTTCCCGGTGCTGAAGGTAAAT 3', REV: 5' GCGAGTCTGGGTGGATGTAGA 3'
23. ***Wnt8a*** FOR: 5' GGGAACGGTGGAATTGTCCTG 3' REV: 5'GCAGAGCGGATGGCATGAA 3'

Co-Immunoprecipitation

IP of in vivo interacting protein complexes was performed using PML OE and PML KO stable ESC cell lines. Cells were lysed in EBC buffer (50mM Tris PH 8, 170mM NaCl, 0.5% NP40, 50mM NaF) containing 1 mM PMSF and protease inhibitors (Roche Applied Science). 200µg whole cell extracts were incubated with primary antibody overnight. The following day, 20µl of protein G sepharose beads were added to each sample and incubated at 4°C for 3hr. The beads were centrifuged and washed three times in 1 ml of NETN buffer (10Mm Tris PH 8, 250nM NaCl, 5mM EDTA, 0.5% NP40). SDS sample buffer was added and immunoprecipitated proteins were resolved by SDS-PAGE, followed by Western blotting as previously described (Niture and Jaiswal, 2009).

Antibodies

In this study, proteins were detected by primary antibodies against ACTIN (sc-47778, Santa Cruz), AFP (2137S, Cell Signaling), CCND1 (sc-8396, Santa Cruz), CDH1 (3195, Cell signaling), c-MYC (Sigma), NANOG (8600S, Cell Signaling), NROB1 (sc-13034X, Santa Cruz), OCT3/4 (sc-5279, Santa Cruz), PML (sc-18423, Santa Cruz), pRB (9309,Cell signaling), p-pRB(9308,Cell signaling), p-SMAD2/3 (3101, Cell Signaling), SNAI1 (3879, Cell Signaling), STAT3 (12640, Cell signaling), p-STAT3 (9131, Cell signaling), SOX2 (14962, Cell Signalling), T (sc-17745, Santa Cruz), VIM (5741, Cell Signaling) and ZEB1 (sc-25388, Santa Cruz).

Luciferase Assay

PML KD, PML OE and CGR8 ESC cell lines were transfected with APRE-Luc, CAGA-Luc or BRE-Luc reporter plasmids using Lipofectamine 2000 following the manufacture's protocol. Cells were stimulated with LIF (100ng/ml), Activin A (25ng/ml, R&D) and BMP4 (80ng/ml, R&D) respectively, 16h before harvesting for measurement of luciferase activity using the dual-luciferase reporter assay system (Promega).

Oxygen Consumption Rate (OCR)

Oxygen consumption rates were measured using a Clark-type electrode (Hansatech Instruments). Briefly, cells were washed and collected in 1 ml PBS buffer and subsequently transferred into a cylindrical chamber with a magnetic cylindrical stirring bar. The chamber was kept at 37^0C, and measurements were done for 10–15 min, depending on the oxygen consumption rate. The slope of the straight portion of the plot was used to derive the oxygen consumption rate. Cells were then collected for protein quantification. Rates were normalized to protein content.

Mitochondrial membrane potential measurement

Cells were washed with PBS, and 1 ml of DMEM with 100 nM TMRE (Invitrogen) was added to the culture plate for incubation at 37°C for 20 min. Cells were then resuspended in PBS and TMRE staining was analyzed at Ex/Em = 549/575 nm either by flow cytometry (BD FACS Canto II System) or by Operetta fluorescence microscope (PerkinElmer). Channel FL2 was used to detect the fluorescent signal as stained by TMRE.

Flow cytometry

For cell cycle analysis, 100.000 cells from each sample were trypsinized, washed with PBS, and fixed in 70% ethanol at 25°C for 10 min. Cells were suspended in 20 µl PBS and 10 µl RNase A (5 mg/ml).treated for 20 min at 37 ^{0}C and stained with propidium iodide (PI-Sigma) according to the manufacturer's protocol. Flow cytometry was performed on a BD FACS Canto II System and cell cycle distribution was analyzed using ModFIT LT Software.