Phosphatase PRL2 promotes AML1-ETO-induced acute myeloid leukemia

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Acute myeloid leukemia (AML) is a markedly heterogeneous hematological malignancy with poor prognosis. [1] Core-binding factor AML is cytogenetically defined by the presence of t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22), commonly abbreviated as t(8;21) and inv(16), respectively. [2-3] In both subtypes, the cytogenetic rearrangements disrupt genes that encode subunits of core-binding factor, a transcription factor that functions as an essential regulator of normal hematopoiesis. [2-3] The t(8;21) translocation, which generates the *AML1-ETO* fusion gene, is one of the most common chromosomal abnormalities detected in AML.[4] The AML1-ETO fusion transcription factor is present in approximately 4%-12% of adult and 12%-30% of pediatric acute myeloid leukemia (AML) patients.[4] AML1-ETO$^+$ AML remains a significant clinical problem, with 30% of patients relapsing and long-term survival rates ranging between 30 and 60%, indicating the need for improved therapeutic approaches.[2-4]

The **p**hosphatase of **r**egenerating **l**iver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins being validated as biomarkers and therapeutic targets in human cancer.[5] Notably, recent findings indicate that PRLs may play important roles in the pathogenesis of hematological malignancies.[6] Both PRL2 and PRL3 are highly expressed in some hematological malignancies, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma (MM) and acute lymphoblastic leukemia (ALL).[6] High PRL3 mRNA expression is associated with FLT3-ITD mutations and poor prognosis in AML patients with normal karyotype.[7] We have identified PRL2 to be important for the proliferation and self-renewal of hematopoietic stem cells (HSCs) through the regulation of KIT signaling.[8] Recently, we found that PRL2 mediates NOTCH and KIT signals in early T cell progenitors and that PRL2 is essential for oncogenic NOTCH1-induced T cell leukemia *in vivo*.[9-10] An improved understanding of how PRLs function and how they are regulated may establish PRLs as novel therapeutic targets in acute myeloid leukemia.

PRL2 is highly expressed in some subtypes of AML, including AML1-ETO$^+$ AML and AML with mixed lineage leukemia (MLL) translocations (Figure 1a). The PRL2 expression profiling data were obtained from the HemaExplorer, a Web server for easy and fast visualization of gene expression in

normal and malignant hematopoiesis.[11] We observed that the levels of PRL2 protein were elevated in several human AML cell lines compared to cord blood and peripheral blood mononuclear cells from healthy donors (Supplementary Figure S1a). However, the role of PRL2 in the proliferation and survival of human AML cells is largely unknown. Kasumi-1 is a human AML cell line with AML1-ETO and MV4-11 is a human B-myelomonocytic leukemia cell line with MLL translocation. We found that knockdown of PRL2 decreased the proliferation of both Kasumi-1 and MV4-11 cells (Figures 1b, 1c and S1b). We also found that ectopic expression of a PRL2 dominant-negative mutant PRL2/C101S-D69A (PRL2/CS-DA) reduced the proliferation of Kasumi-1 cells (Figure S1c).

Recently, we identified a small molecule PRL inhibitor (PRLi) using computer-based virtual screening. [12] Intraperitoneally injection of 15 mg/kg of PRLi into wild type mice daily for 3 weeks exhibited no toxicity and body weight and weights of major organs (liver, spleen, and kidney) were comparable to DMSO treated mice.[12] PRLi did not affect the viability of human cord blood mononuclear cells and $CD34^+$ cells.[10] Further, we found that pharmacological inhibition of PRL2 function decreased the proliferation and survival of human T-ALL cells in a dosage-dependent manner.[10] To determine the role of PRL2 in the proliferation and survival of human AML cells, we treated several human AML cell lines with PRL inhibitor (PRLi) and monitored cell proliferation and survival. We found that PRLi treatment of PRL2-expressing human AML cell lines resulted in decreased proliferation (Figure 1d). Furthermore, we found that primary human AML cells ($FLT3\text{-}ITD^+$ and $FLT3\text{-}ITD^-$) were sensitive to PRL2 inhibitor treatment in a dose-dependent manner (Figure 1d). Inhibition of PRL2 activity with PRLi resulted in apoptotic cell death of K562 cells that highly expresses PRL2 (Figure 1e). Importantly, we found that PRLi treatment significantly decreased ERK phosphorylation in human AML cells (Figure 1f), suggesting that PRL2 is a key mediator of signaling pathways in human leukemia cells.

Human leukemia-initiating cells (LICs) are enriched in the $CD34^+$ population of leukemia blasts. Human $CD34^+$ cells expressing AML1-ETO have nearly unlimited potential for multilineage cell generation, and represent a valuable tool for studying AML1-ETO-positive AML.[13] We found that

knockdown of PRL2 decreased the proliferation and colony formation of $CD34^+$ cells expressing AML1-ETO (Figures 1g and S1d). In addition, ectopic expression of a PRL2 dominant-negative mutant PRL2/CS-DA, but not the wild type PRL2, reduced colony formation of human $CD34^+$ cells expressing AML1-ETO (Figure S1e). While PRLi treatment does not affect the colony formation of human cord blood $CD34^+$ cells,[10] blocking PRL2 function with PRLi decreased the colony formation of human $CD34^+$ cells expressing AML1-ETO (Figure 1h). These data demonstrate that PRL2 is important for the proliferation and survival of human AML cells bearing AML1-ETO.

Both human and mouse models of AML have demonstrated that AML1-ETO is insufficient for leukemogenesis in the absence of secondary events. [13-14] However, a truncated form of the AML1-ETO fusion protein, AML1-ETO9a, is sufficient to cause leukemia in mice.[15] To determine the role of PRL2 in AML1-ETO-induced leukemia *in vivo*, we introduced AML1-ETO9a (AE9a) into Lin^- cells isolated from WT and *Prl2* null mice using a retrovirus carrying cDNA that encodes the leukemia associated oncogene AML1-ETO9a (MSCV-AML1-ETO9a-IRES-GFP). We found that loss of PRL2 decreased the proliferation of hematopoietic progenitor cells expressing AML1-ETO9a *in vitro* (Figures S2a). We also found that loss of PRL2 decreased the replating potential of hematopoietic progenitor cells expressing AML1-ETO9a *in vitro* (Figures 2a and S2b), suggesting that PRL2 may be essential for LIC maintenance.

We then transduced Lin^- cells isolated from WT and *Prl2* null mice with a retrovirus expressing AML1-ETO9a and transplanted transduced cells (GFP^+) into lethally irradiated recipient mice. *Prl2* null HSPCs expressing AML1-ETO9a show decreased repopulating potential in primary transplantation assays (Figure 2b). The frequency of GFP^+ cells in the bone marrow of recipient mice repopulated with *Prl2* null cells was significantly decreased compared to that of the WT cells 20 weeks following transplantation (Figure S2c), suggesting that PRL2 is important for the repopulating potential of HSPCs expressing AML1-ETO9a. At this point, none of the recipient mouse developed leukemia. To determine the role of PRL2 in leukemogenesis, we transplanted equal number of GFP^+ cells isolated from primary

recipient mice into lethally irradiated secondary recipient mice and monitored leukemia development. While the frequency of GFP^+ cells in the peripheral blood was comparable between wild type and *Prl2* null groups 40 days after transplantation (Figure S2d), the development of AML in host that received wild-type cells transduced with AML1-ETO9a retrovirus was rapid, with all control animals succumbing to disease within 15 weeks following transplantation (Figure 2c). In contrast, animals repopulated with *Prl2* null cells displayed significant protection from disease, with all animals remaining disease free 20 weeks following transplantation (Figure 2c), indicating that PRL2 is essential for the maintenance of leukemia-induced by AML1-ETO9a.

To determine the role of PRL2 in leukemia initiation and progression, we transduced wild type and *Prl2* null fetal liver cells, which are enriched for hematopoietic stem and progenitor cells, with a retrovirus expressing AML1-ETO9a and transplanted infected cells (GFP^+) into lethally irradiated recipient mice. Recipient mice repopulated with wild type fetal liver cells expressing AML1-ETO9a developed leukemia within 20 weeks following transplantation (Figure 2d). However, recipient mice repopulated with *Prl2* null fetal liver cells expressing AML1-ETO9a show delayed leukemia development and majority of *Prl2* null recipients are still alive 40 weeks following transplantation (Figure 2d). *Prl2* wild type recipients show splenomegaly and lethargic phenotype compared to *Prl2* null recipients (Figures S2e and S2f). These data demonstrate that PRL2 is important for leukemia progression.

PRL2 is highly expressed in AML-ETO positive AML cells (Figure S1a) and we found that ectopic AML1-ETO9a expression in mouse HSPCs increased the levels of both PRL2 mRNA and protein (Figures S3a and S3b). There is a putative AML1(RUNX1) binding site (TGTGGT) in the promoter region of *PRL2*, which is conserved between mouse and human. We examined the genome-wide occupancy of AML1-ETO in $AML1\text{-}ETO^+$ Kasumi-1 cells by performing the ChIP-seq assays using an anti-ETO antibody. [16] We found that AML1-ETO is associated with the *PRL2* promoter in Kasumi-1 cells (Figure 2e). We also found hematopoietic transcription cofactors, including E2A, LMO2, and p300, are associated with the *PRL2* promoter (Figure 2e), indicating that the AML1-ETO-

transcription factor complex (AETFC) binds the *PRL2* promoter.[16] We then examined the occupancy of AML1-ETO on *PRL2* promoter regions in Kasumi-1 cells by using an anti-RUNX1 (AML1) antibody in the ChIP assays. Using primer pairs that cover the putative AML1 binding site in the promoter region of *PRL2*, we found that AML1-ETO was associated with the promoter region of *PRL2* in Kasumi-1 cells (Figure 2f). These findings suggest that AML1-ETO may directly activate *PRL2* expression in hematopoietic cells.

To date, very little information exists in the literature on the role of PRL2 in human AML.[6-8] In this study, we have identified a critical role for PRL2 phosphatase in the proliferation and survival of human AML cells. Further, we demonstrated that PRL2 is essential for the leukemogenic potential of AML1-ETO9a *in vivo*. Given that some human AML cells are sensitive to PRLi treatment, PRL2 may play a general role in human AML. Our findings suggest that pharmacological inhibition of PRL2 holds potential as a novel therapy for acute myeloid leukemia, and might also be applicable to the treatment of other hematological malignancies.[10]

CONFLICT OF INTEREST

The authors declared that no conflict interest exists.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

MK, SC, YB, ZYZ, and YL. Designed the research. MK, SC, YB, CY, RG, XJS, CM, TAT, and ZHY. Performed the research; MK, and YL. Analyzed the data and performed the statistical analysis. HSB, MCY, RK, and JCM. Provided reagents to the study. ZYZ and YL. Wrote the manuscript. All authors read, comment on, and approved the manuscript.

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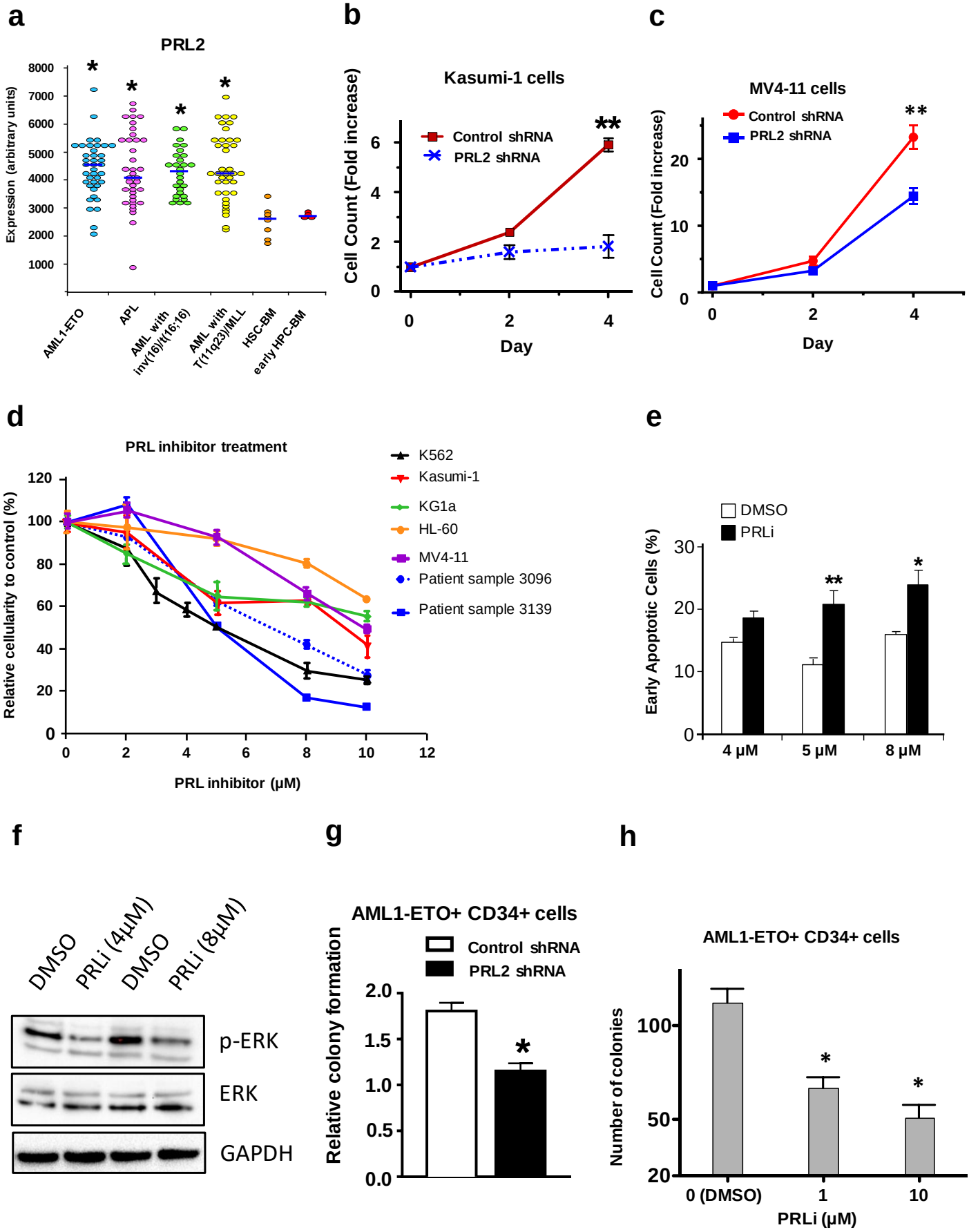
FIGURE LEGENDS

Figure 1. PRL2 promotes the proliferation and survival of human AML cells. (**a**) PRL2 is highly expressed in some subtypes of human AML compared to normal human bone marrow HSPCs (*P<0.05). (**b**) Kasumi-1 cells were transduced with lentiviruses expressing control or PRL2 shRNA. The proliferation of transduced cells (GFP^+) was measured over time (**p<0.01, n=3). (**c**) MV4-11 cells were transduced with lentiviruses expressing control or PRL2 shRNA. The proliferation of transduced cells (GFP^+) was measured over time (**p<0.01, n=3). (**d**) Inhibiting of PRL2 activity with a small molecule PRL inhibitor (PRLi) decreases the viability of human AML cell lines and primary human AML cells in a dosage-dependent manner. Patient sample 3096 is from an AML patient positive for FLT3ITD and FLT3TKD mutations. Patient sample 3139 is from an AML patients negative for FLT3ITD and NPM mutations. (**e**) Human K562 cells were treated with PRL inhibitor (PRLi) for 24 hours and apoptosis was determined by Annexin V and DAPI staining (*p<0.05, **p<0.01, n = 3). (**f**) Immunoblot analysis of ERK phosphorylation in human K562 cells following DMSO or a small molecule inhibitor (PRLi) treatment. Representative Western blot analysis of indicated proteins is shown. (**g**) Human cord blood $CD34^+$ cells expressing AML1-ETO were transduced with lentiviruses expressing control or PRL2 shRNA. Myeloid progenitors were quantified by using the methylcellulose culture (*p<0.05, n=3). (**h**) PRL inhibitor (PRLi) treatment decreases the colony formation of human cord blood $CD34^+$ cells expressing AML1-ETO in a dosage dependent manner (*p<0.05, n = 3).

Figure 2. PRL2 promotes AML1-ETO-induced leukemia *in vivo*. (**a**) Loss of PRL2 decreases the replating potential of AML1-ETO9a$^+$ progenitor cells. Myeloid progenitors were quantified by methylcellulose culture using wild type and PRL2 null Lin^- cells transduced with retroviruses expressing AML1-ETO9a. The methylcellulose cultures were serially replated, weekly, for 3 weeks. Data are means ± SD (*p<0.05, **p<0.01, n = 3 independent experiments). (**b**) *Prl2*$^{+/+}$ and *Prl2*$^{-/-}$ hematopoietic progenitor cells (Lin^-) were transduced with retroviruses expressing AML1-ETO9a and equivalent number of transduced cells (GFP^+) were injected into lethally irradiated recipient mice. The frequency

of donor-derived cells (GFP^+) in peripheral blood was determined every 4 weeks for 16 weeks by flow cytometry analysis. Data are means ± SD (*$p<0.05$, n = 5). (**c**) Two million GFP^+ cells isolated from the bone marrow of primary recipient mice were injected into lethally irradiated recipient mice. Kaplan-Meier curve shows the survival of mice transplanted with cells expressing AML1-ETO9a for the period of observation (***$p<0.001$, n=5). (**d**) GFP^+ fetal liver cells expressing AML1-ETO9a were injected into lethally irradiated recipient mice. Kaplan-Meier curve shows the survival of mice transplanted with cells expressing AML1-ETO9a for the period of observation (**$p<0.01$, n=6). (**e**) AML1-ETO directly binds to *PRL2* (*PTP4A2*) in Kasumi-1 cells revealed by genome-wide ChIP-seq analysis using an anti-ETO antibody.[16] (**f**) AML1-ETO was associated with the promoter of *PRL2* in human Kasumi-1 cells assayed by ChIP experiments using an anti-RUNX1 (AML1) antibody. RNA Pol-II antibody was used a positive control.

Figure 1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AML1-ETO
APL
AML with inv(16)/t(16;16)
AML with T(11q23)/MLL
HSC-BM
early HPC-BM
b
Kasumi-1 cells
Cell Count (Fold increase)
Control shRNA
PRL2 shRNA
Day
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PRL2 shRNA
Day
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Kasumi-1
KG1a
HL-60
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Patient sample 3096
Patient sample 3139
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PRLi
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DMSO
PRLi (4μM)
DMSO
PRLi (8μM)
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GAPDH
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AML1-ETO+ CD34+ cells
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PRL2 shRNA
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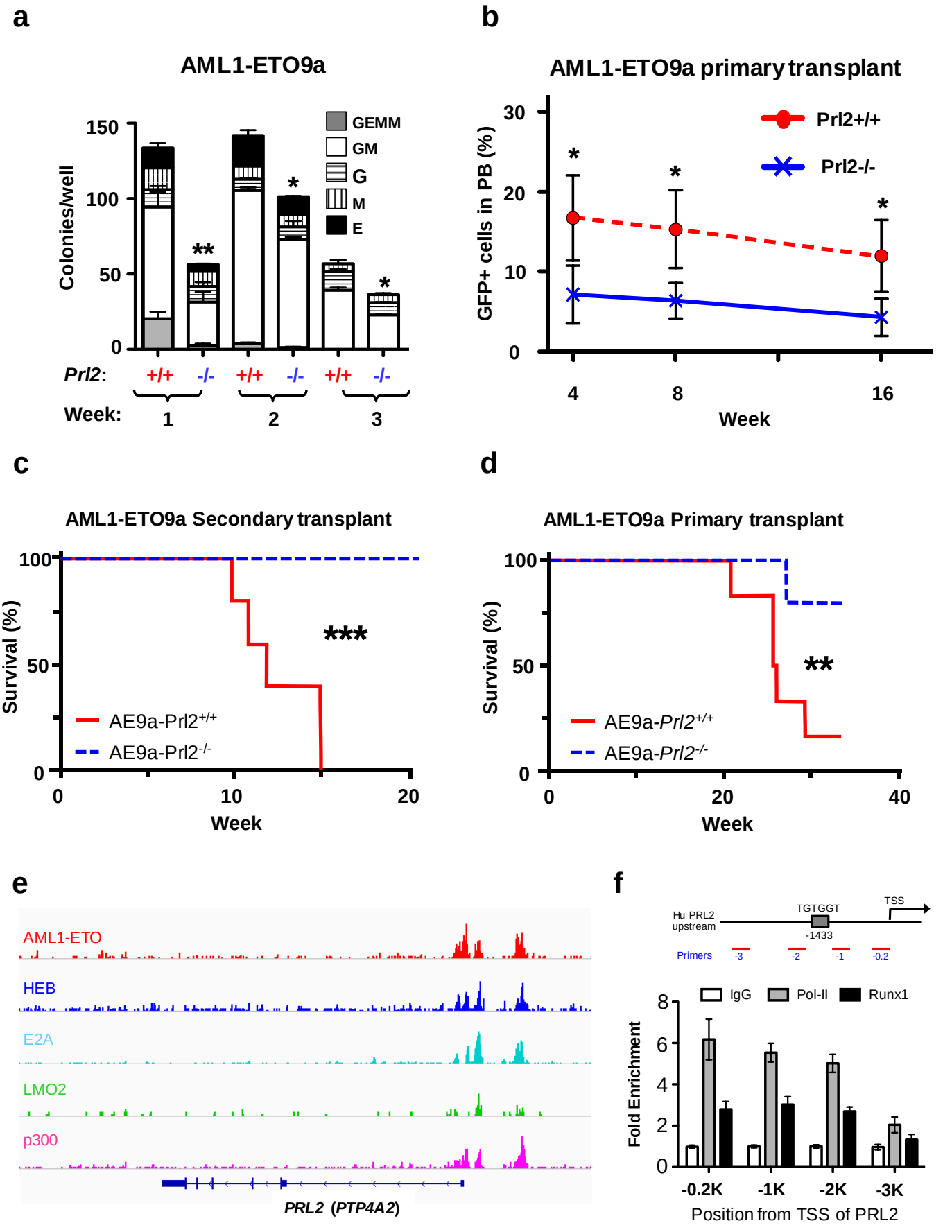
PRLi (μM)

Figure 2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Survival (%)
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Hu PRL2 upstream
TGTGGT
TSS
-1433
Primers
-3
-2
-1
-0.2
IgG
Pol-II
Runx1
Fold Enrichment
8
6
4
2
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-0.2K
-1K
-2K
-3K
Position from TSS of PRL2

Phosphatase PRL2 promotes AML1-ETO-induced acute myeloid leukemia

Supplemental Methods

Mice

Wild type C57BL/6 ($CD45.2^+$) and B6.SJL ($CD45.1^+$) mice were purchased from the Jackson Laboratories. $Prl2^{+/+}$ and $Prl2^{-/-}$ mice were maintained in the Indiana University Animal Facility and kept in Thorensten units with filtered germ-free air. All mice were 10-12 weeks of age at the time of analysis. Both male and female mice were utilized in the experiments. The Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine approved all experimental procedures.

Human AML cell lines, primary AML samples, and cord blood cells

Human AML cell lines, including K562, Kasumi-1, KG1a, HL-60, MV4-11, and MO7e were obtained from ATCC. All cell lines were authenticated by SRT profiling and tested for mycoplasma contamination. Primary AML samples and normal human cord blood samples were obtained after informed consent following the guidelines of the institutional review board of the Indiana University School of Medicine.

Production of Lentivirus

Lentiviral shRNA plasmid (pLB) was purchased from Addgene (11619). Oligonuculeotides targeting control (Luciferase) and human PRL2 cDNAs were cloned into the pLB plasmid. Oligonuculeotide sequences are available upon request. Lentiviral particles were generated by standard method using the third generation packaging system (pMDL, pMD2.G, and pRSV-Rev). Human AML cell lines were infected with high-titer lentiviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. The reduction of PRL2 proteins was determined by immunoblot analysis.

Immunoblotting analysis

Cells were washed with ice-cold PBS, and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) supplemented with a Complete Protease Inhibitor tablet (Roche Applied Science). Cell lysates were cleared by centrifugation at 15,000 rpm for 10 min. The lysate protein concentration was estimated using a BCA protein assay kit (Pierce). The protein samples were boiled with sample buffer, separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with appropriate antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique (ECL kit, GE Healthcare). Representative results from at least two independent experiments are shown. Antibodies were purchased from Cell Signaling (ERK1/2, pERK1/2, Actin and GAPDH). PRL2 antibody is a generous gift from Dr. Qi Zeng.

Apoptosis assays

Human AML cell lines were treated with DMSO or different concentration of PRL inhibitor (PRLi). 24 hours later, cell viability was evaluated by PI/Annexin V staining. Apoptotic cells were defined as PI^- Annexin V^+.

Production of Retrovirus

Retroviral particles were produced by transfection of Phoenix E cells with the MSCV-IRES-GFP or MSCV-AML1-ETO9a-IRES-GFP plasmids, according to standard protocols. Mouse hematopoietic progenitor cells were transduced on retronectin (Takara)-coated non-tissue culture plates with high-titer retroviral suspensions. Twenty-four hours after infection, GFP-positive cells were sorted by FACS. Transduced cells were then transplanted into lethally irradiated recipient mice. The presence of GFP^+ cells in the peripheral blood was measured by flow cytometry analysis.

Statistical Analysis

The animal sample size was based on previous studies evaluating the roles of AML1-ETP9a in leukemia and POWER analysis.[15] Using Chi-Square analysis, 5 mice per group will provide 80% POWER in detecting difference with 95% difference. Gehan-Breslow-Wilcoxon test was used for Kaplan-Meier survival curves. The other data were analyzed by paired or unpaired t test using GraphPad Prizm 5. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant. All experiments were repeated at least once.

Supplementary Figure Legends

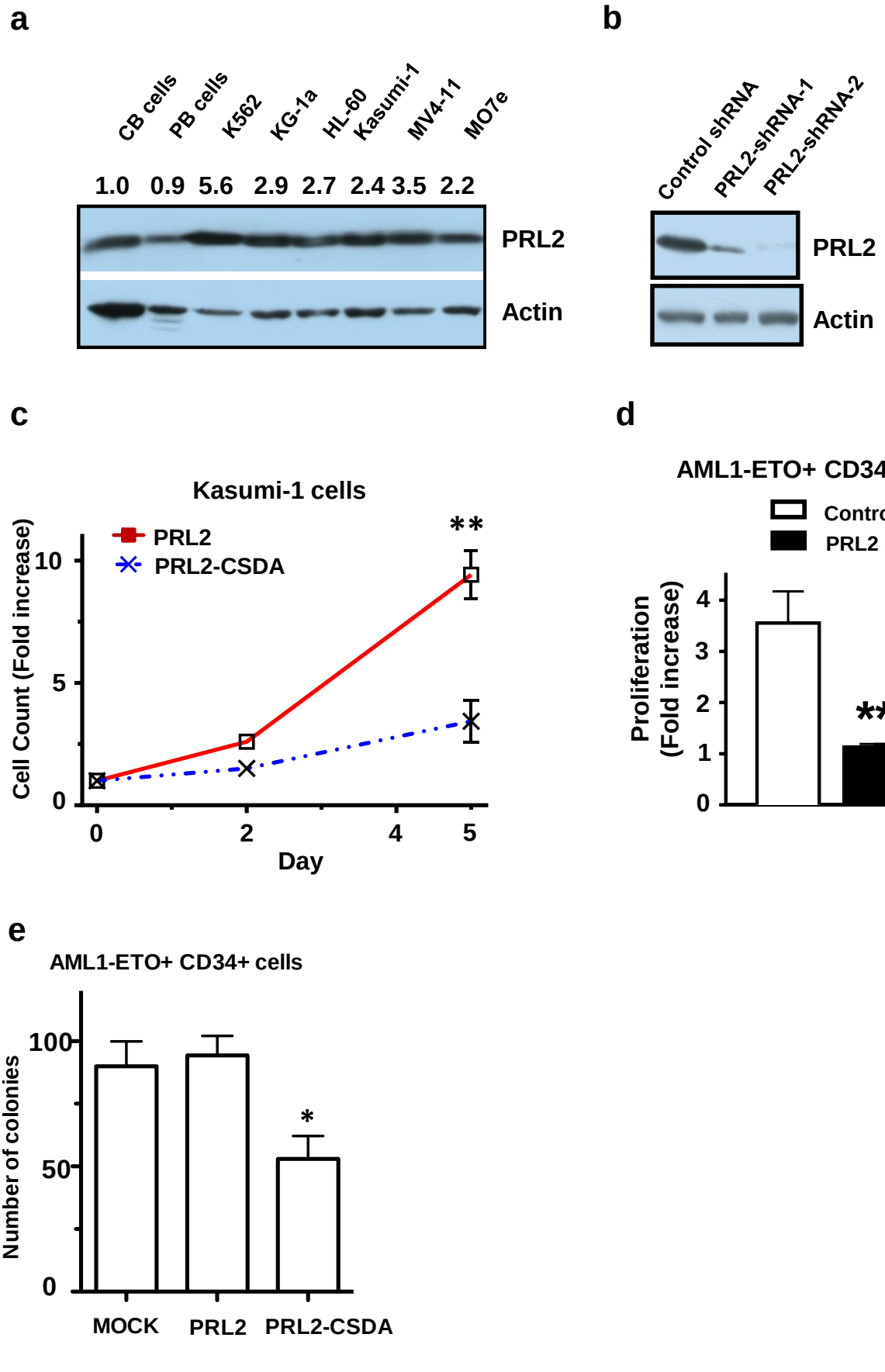
Figure S1. (**a**) PRL2 is highly expressed in several human AML cell lines compared to mononuclear cells from human peripheral blood and cord blood. (**b**) K562 cells were transduced with lentiviruses expressing control shRNA (Sh-Luc) or PRL2 shRNAs (PRL2-shRNA-1 and PRL2-shRNA-2). The level of PRL2 proteins was determined by western blot analysis. (**c**) Kasumi-1 cells were transduced with retroviruses expressing dominant-negative PRL2 (PRL2-CSDA) or GFP. The proliferation of transduced cells (GFP^+) was measured over time (**$p<0.01$, n=3). (**d**) Human cord blood $CD34^+$ cells expressing AML1-ETO were transduced with lentiviruses expressing control or PRL2 shRNA. The proliferation of transduced cells was measured over time (**$p<0.01$, n=3). (**e**) Human cord blood $CD34^+$ cells expressing AML1-ETO were transduced with retroviruses expressing WT or dominant-negative PRL2 (PRL2-CSDA). Myeloid progenitors were quantified by using methylcellulose culture (*$p<0.05$, n=3).

Figure S2. (**a**) Loss of PRL2 decreased the proliferation of AML1-ETO9a$^+$ progenitor cells (**$p<0.01$, n = 3). (**b**) Lin^- cells isolated from WT and *Prl2* null mice were transduced with retroviruses expressing AML1-ETO9a (AE9a). Equal number of transduced cells were seeded in methylcellulose culture. One week later, total cell number was measured from both groups (**$p<0.01$, n= 3). (**c**) Frequency of GFP^+ bone marrow cells in recipient mice repopulated with WT or *Prl2* null cells expressing AML1-ETO9a at 20 weeks post transplantation (**$p<0.01$, n= 5). (**d**) Frequency of GFP^+ cells in the peripheral blood of secondary recipient mice at 40 days after transplantation ($p>0.01$, n= 5). (**e**) Wild type recipient mice show

splenomegaly compared to *Prl2* null recipients. (**f**) Wild type recipient mice show thin and lethargic phenotype compared to *Prl2* null recipients.

Figure S3. (**a**) PRL2 mRNA was upregulated in mouse HSPCs expressing AML1-ETO9a (*p<0.05, n=3). (**b**) Ectopic expression of AML1-ETO9a increased the levels of PRL2 protein in mouse HSPCs.

Figure S1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PB cells
K562
KG-1a
HL-60
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PRL2
Actin
b
Control shRNA
PRL2-shRNA-1
PRL2-shRNA-2
PRL2
Actin
c
Kasumi-1 cells
PRL2
PRL2-CSDA
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Cell Count (Fold increase)
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AML1-ETO+ CD34+ cells
Control shRNA
PRL2 shRNA
Proliferation (Fold increase)
4
3
2
1
0
**
e
AML1-ETO+ CD34+ cells
Number of colonies
100
50
0
*
MOCK
PRL2
PRL2-CSDA

Figure S2

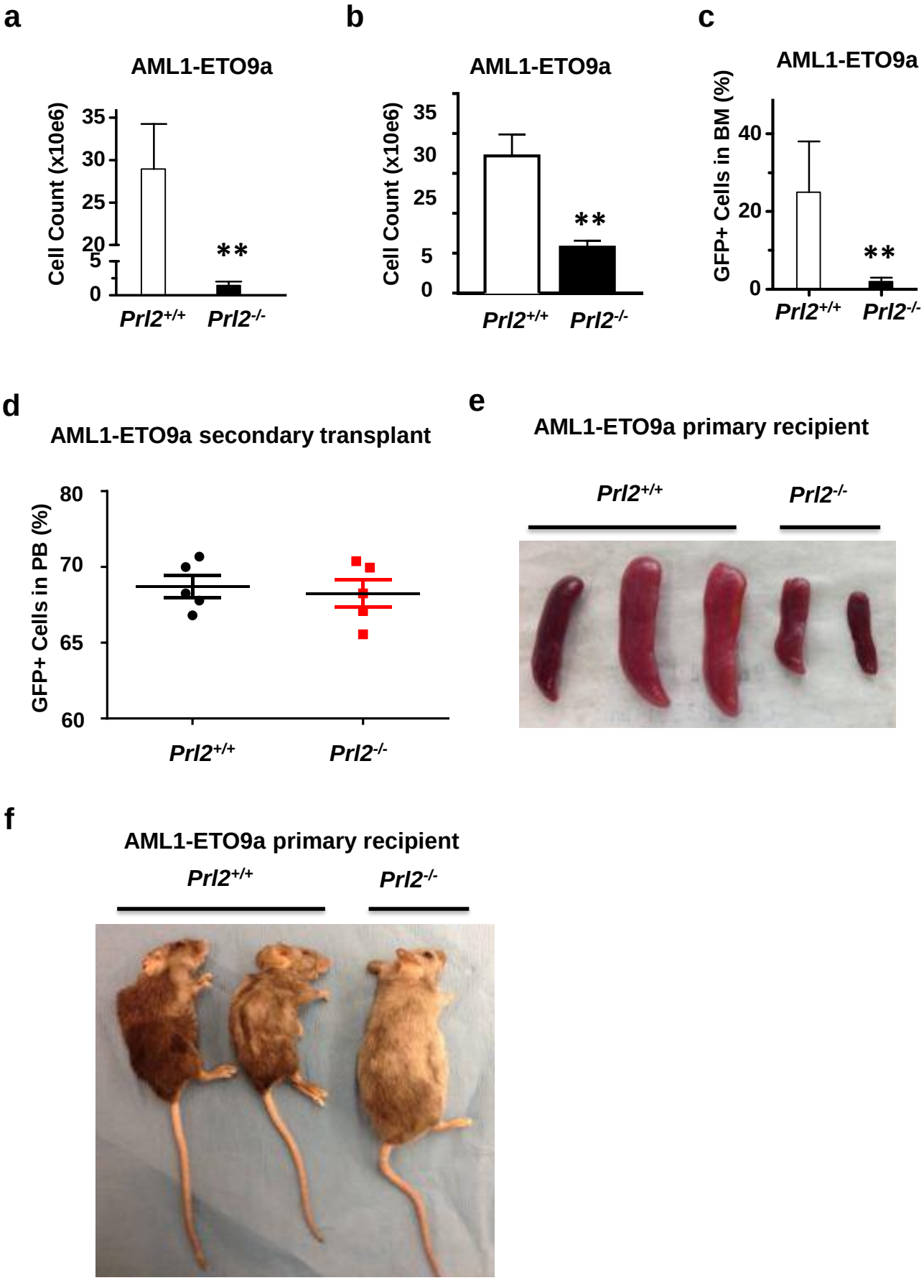
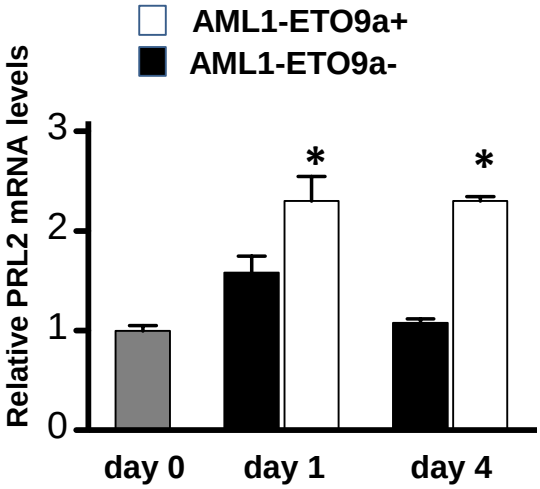
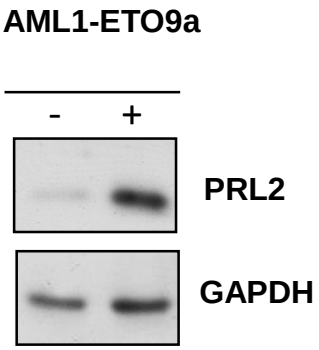

a
AML1-ETO9a
Cell Count (x10e6)
35
30
25
20
5
0
**
Prl2+/+
Prl2-/-
b
AML1-ETO9a
Cell Count (x10e6)
35
30
25
5
0
**
Prl2+/+
Prl2-/-
c
AML1-ETO9a
GFP+ Cells in BM (%)
40
20
0
**
Prl2+/+
Prl2-/-
d
AML1-ETO9a secondary transplant
GFP+ Cells in PB (%)
80
70
65
60
Prl2+/+
Prl2-/-
e
AML1-ETO9a primary recipient
Prl2+/+
Prl2-/-
f
AML1-ETO9a primary recipient
Prl2+/+
Prl2-/-

Figure S3

a


AML1-ETO9a+
AML1-ETO9a-
Relative PRL2 mRNA levels
3
2
1
0
*
*
day 0
day 1
day 4

b


AML1-ETO9a
-
+
PRL2
GAPDH