

Essential role of PU.1 in maintenance of mixed lineage leukemia-associated leukemic stem cells

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Key words

Acute myeloid leukemia, CSF-1R, mixed lineage leukemia, Spi-1, stem cells

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Funding Information

Ministry of Health, Labor and Welfare, Japan; Ministry of Education, Culture, Sports, Science and Technology, Japan; National Cancer Center Research and Development Fund, Japan; US National Institutes of Health (HL112719, CA32551, and 5P30-CA13330).

Received August 8, 2014; Revised December 5, 2014; Accepted December 14, 2014

Cancer Sci 106 (2015) 227–236

doi: 10.1111/cas.12593

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs).^(1,2) Leukemic stem cells (LSCs) are capable of the limitless self-renewal that is necessary for cancer initiation and maintenance. Conventional chemotherapies are often effective in reducing the total number of leukemia cells, but are not curative in many cases of AML. As LSCs are often resistant to conventional chemotherapies, residual LSCs are a potential cause of AML relapse. Thus, eradication of LSCs is critical to cure the disease.

Chromosome translocations that involve the mixed lineage leukemia gene (*MLL*) are frequently observed in human AML and often predict a poor prognosis.^(3–6) More than 60 genes have been identified as *MLL* fusion partners to date; chromosome rearrangements such as t(9;11), t(11;19), and t(10;11), which express *MLL*-AF9, *MLL*-ELL, and *MLL*-AF10, respectively, are commonly associated with AML.⁽⁵⁾ The *MLL* fusion proteins transform non-self-renewing myeloid progenitors into LSCs.^(7,8) Acute myeloid leukemia with *MLL* rearrangements consistently express *HOX* genes such as *HOXA7*, *HOXA9*, and *MEIS1*.^(9–11) The upregulation of *Hox* genes is critical for LSC induction and maintenance, but does not recapitulate the entire phenotype and biology of *MLL* leuke-

Acute myeloid leukemia is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). Rearrangements of the mixed lineage leukemia (*MLL*) gene are found in acute myeloid leukemia associated with poor prognosis. The upregulation of *Hox* genes is critical for LSC induction and maintenance, but is unlikely to support malignancy and the high LSC frequency observed in *MLL* leukemias. The present study shows that *MLL* fusion proteins interact with the transcription factor PU.1 to activate the transcription of *CSF-1R*, which is critical for LSC activity. Acute myeloid leukemia is cured by either deletion of *PU.1* or ablation of cells expressing *CSF-1R*. Kinase inhibitors specific for *CSF-1R* prolong survival time. These findings indicate that PU.1-mediated upregulation of *CSF-1R* is a critical effector of *MLL* leukemogenesis.

mias.^(12–15) Moreover, it is unlikely to support malignancy and the high LSC levels observed in *MLL* leukemias.⁽¹⁶⁾ These facts suggest that unknown critical mediators of leukemogenesis exist.

The present study shows that the upregulation of macrophage colony-stimulating factor (M-CSF) receptor (*CSF-1R*, also called *M-CSFR/c-FMS/CD115*) is critical for LSC activity in *MLL* leukemia. Acute myeloid leukemia was cured after eradication of cells expressing high levels of *Csf-1r* in mice. It was found that *MLL* fusions regulated *CSF-1R* transcription through a novel mechanism involving interaction with the transcription factor PU.1. These findings indicate that PU.1-mediated upregulation of *CSF-1R* is a novel therapeutic target for *MLL* leukemias.

Materials and Methods

Mice. C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). NGF-FKBP-Fas transgenic mice⁽¹⁷⁾ (Jackson Laboratory, Bar Harbor, ME, USA), *CSF-1R*-deficient mice⁽¹⁸⁾, *PU.1*-null/conditional deficient mice,⁽¹⁹⁾ and CreERT2 mice (TaconicArtemis (Germantown, NY, USA))⁽²⁰⁾ were maintained on a C57BL/6 genetic background. Mouse experiments were carried out in a specific pathogen-free environment at the

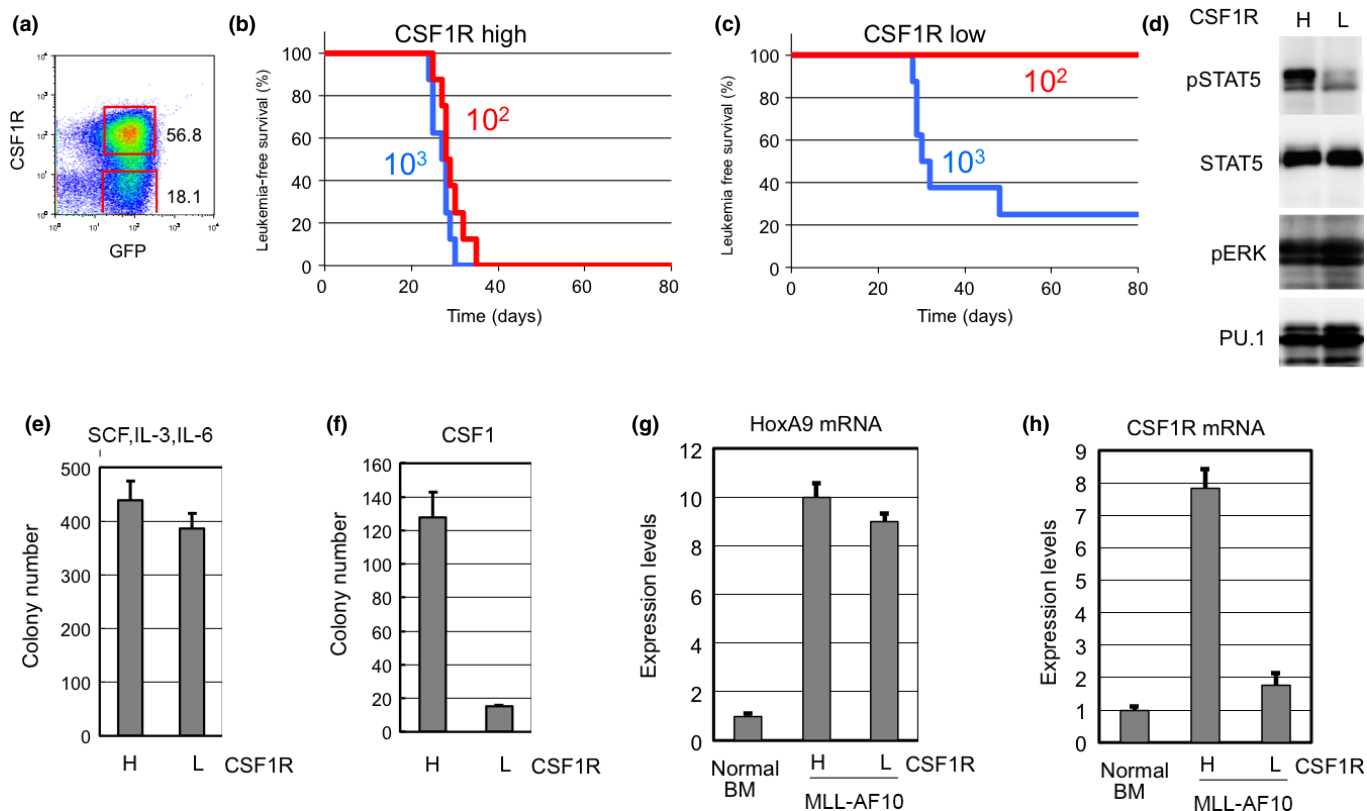


Fig. 1. Cells expressing high levels of macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R^{high}) show potent leukemia-initiating activity. (a) Bone marrow (BM) cells from mice with mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia were analyzed by flow cytometer for expression of GFP and Csf-1r. (b,c) Csf-1r^{high} and Csf-1r^{low} cells were sorted by flow cytometry. The indicated numbers of flow-sorted CSF-1R^{high} (b) and Csf-1r^{low} (c) cells were transplanted into sublethally irradiated mice, and leukemia-free survival was investigated. $n = 8$, $P < 0.001$. (d) Csf-1r^{high} (h) and Csf-1r^{low} (l) cells were analyzed for levels of total and phosphorylated signal transducer and activator of transcription 5 (STAT5 and pSTAT5), phosphorylated ERK p(ERK), and Pu.1 (e,f). Csf-1r^{high} and Csf-1r^{low} cells were analyzed for colony-forming activity in methylcellulose medium supplemented with interleukin (IL)-3, stem cell factor (SCF), and IL-6 (e) or with M-CSF (f). (g,h) Levels of HoxA9 (g) and Csf1r (h) mRNAs were measured in Csf-1r^{high} and Csf-1r^{low} cells prepared from BM of mice with acute myeloid leukemia.

National Cancer Center (Tokyo, Japan) animal facility according to institutional guidelines and with approval of the National Cancer Center Animal Ethics Committee.

Generation of AML mouse models. MSCV-MLL-AF10-ires-GFP was transfected with PLAT-E⁽²¹⁾ cells using the FuGENE 6 reagent (Roche Diagnostics Mannheim, Germany), and supernatants containing retrovirus were collected 48 h after transfection. The c-Kit⁺ cells (1×10^5 cells), which were selected from bone marrow (BM) or fetal liver cells using CD117 MicroBeads (Miltenyi Biotec Bergisch Gladbach, Germany), were incubated with the retrovirus using RetroNectin (Takara Bio Otsu, Japan) for 24 h in StemPro-34 serum-free medium (Invitrogen Waltham, MA, USA) containing cytokines (20 ng/mL stem cell factor [SCF], 10 ng/mL interleukin [IL]-6, and 10 ng/mL IL-3). The infectants were then transplanted together with BM cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by i.v. injection. Secondary transplants were carried out by i.v. injection of BM cells from the primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Treatment with AP20187, AraC, or Ki20227. AP20187 (10 mg/kg; gift from Ariad Pharmaceuticals Cambridge, MA, USA) was given daily by i.v. injection for 5 days, then 1 mg/kg AP20187 was given every 3 days thereafter as described previously.⁽¹⁷⁾ Ki20227⁽²²⁾ (20 mg/kg; gift from KIRIN Pharma) was

given orally daily from 7 days after transplantation. AraC (75 mg/kg) was given daily by i.v. injection for 5 days from 7 days after transplantation.

Immunofluorescent staining, flow cytometric analysis, and cell sorting. Bone marrow cells from AML mice were preincubated with rat IgG, and then incubated on ice with anti-CD115(CSF-1R)-PE (eBioscience San Diego, CA, USA) and anti-c-Kit-APC (2B8)-APC (BD Pharmingen San Jose, CA, USA). Flow cytometric analysis and cell sorting were carried out using the cell sorter JSAN (Baybioscience Kobe, Japan), and the results were analyzed using FlowJo software (Tree Star Ashland, OR, USA).

Reporter analysis. Csf1r-luciferase constructs were generated by ligation of WT and PU.1-lacking Csf1r promoter⁽²³⁾ with pGL4. For reporter analysis, SaOS2 cells were transfected with Csf1r-luc and pRL-CMV together with various expression constructs in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega Madison, WI, USA). Results of reporter assays represent the average values for relative luciferase activity generated from at least three independent experiments that were normalized using the activity of the enzyme from pRL-CMV as an internal control.

Immunoprecipitation and immunoblotting. For immunoprecipitation experiments, cells were lysed in a lysis buffer

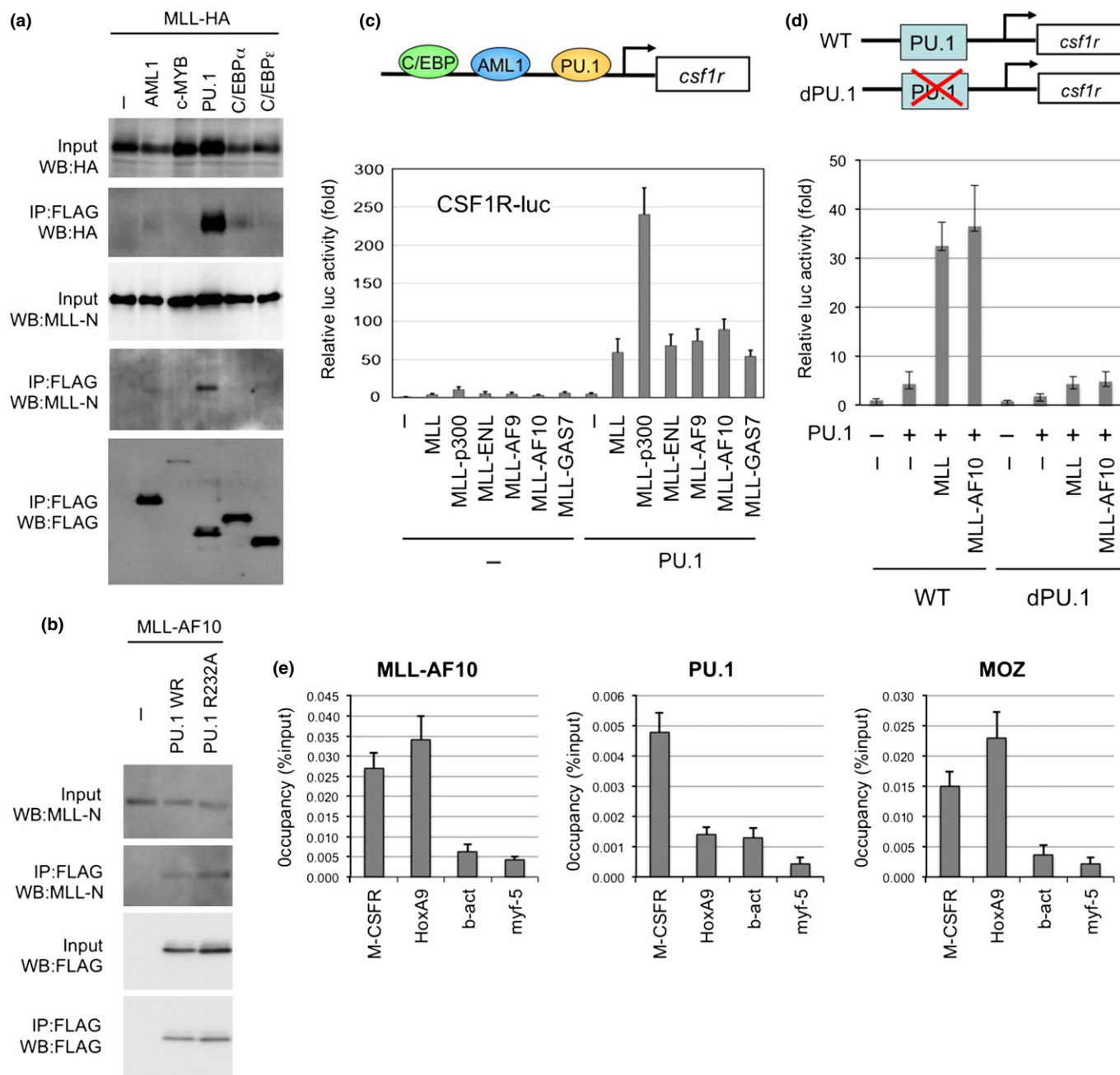


Fig. 2. PU.1-dependent upregulation of macrophage colony-stimulating factor receptor (CSF-1R) by mixed lineage leukemia (MLL) and MLL fusions. (a) Interaction of MLL with PU.1. 293T cells were co-transfected with MLL-HA and the indicated FLAG-tagged transcription factors, including FLAG-PU.1. Anti-FLAG antibody immunoprecipitates (IP:FLAG) or cell lysates (Input) were subjected to immunoblotting with anti-HA, anti-MLL-N, or anti-FLAG antibodies. (b) Interaction between MLL-AF10 and PU.1. 293T cells were co-transfected with MLL-AF10 and FLAG-tagged WT PU.1 or PU.1/R232A. Anti-FLAG antibody immunoprecipitates (IP:FLAG) or cell lysates (Input) were subjected to immunoblotting with anti-MLL-N or anti-PU.1 antibodies. (c) Effects of MLL, and MLL fusions on PU.1-mediated *Csf1r* promoter-driven transcription. SaOS2 cells were co-transfected with the *Csf1r*-luciferase construct and the indicated effectors. Luciferase activity was analyzed 24 h after transfection. Error bars represent SD ($n = 3$). (d) PU.1 binding site-dependence of MLL enhancement of *Csf1r* promoter-driven transcription. SaOS2 cells were transfected with the WT *Csf1r*-luciferase construct or its mutant lacking the PU.1-binding site, together with the indicated effectors. (e) ChIP of MLL-AF10 and PU.1. Bone marrow (BM) cells from acute myeloid leukemia mice (AML) induced by Flag-MLL-AF10, were subjected to ChIP analysis using anti-Flag (MLL-AF10), anti-PU.1, and anti-MOZ antibodies. Semiquantitative real-time PCR was carried out on the co-precipitated DNAs.

containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM DTT, 1 mM PMSF, and protease inhibitor. Cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads (Sigma) and gently rotated at 4°C overnight. The absorbed beads were washed six times with lysis buffer. Pre-

cipitated proteins were eluted from the beads by FLAG peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not carried out, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence using ECL plus Detection Reagents (Amersham Biosciences, Little Chalfont, UK).

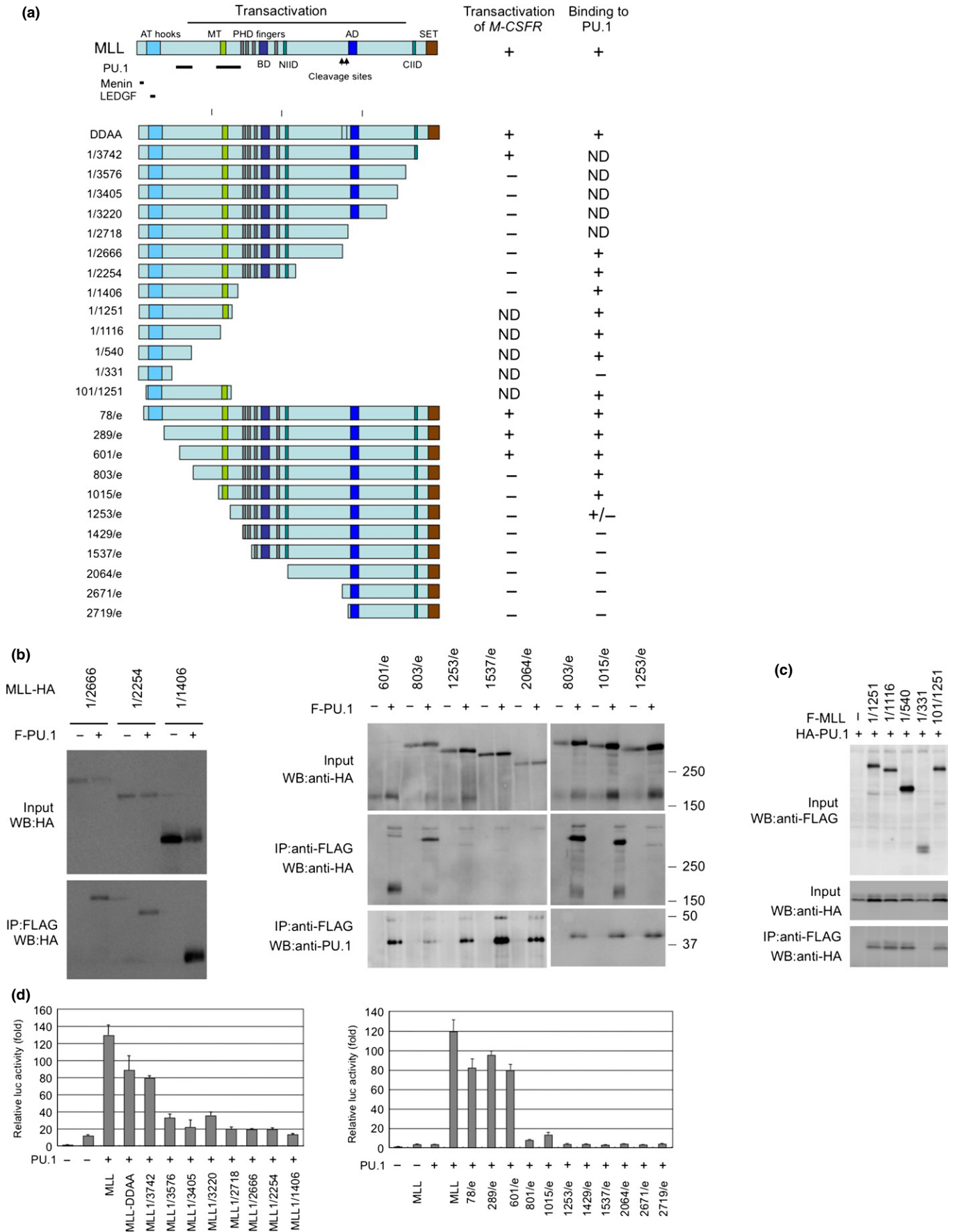


Fig. 3. Functional domains of mixed lineage leukemia (MLL) required for interaction with PU.1 and for PU.1-mediated activation of macrophage colony-stimulating factor receptor (*Csf1r*) promoter. (a) PU.1 binding and PU.1-mediated *Csf1r* promoter activity of MLL deletion mutants. The PU.1-, menin-, and LEDGF-interacting domains and the results for interaction with PU.1 and PU.1-mediated transactivation of *Csf1r*-luc are indicated. ND, not determined. (b,c) Pu.1 binding. 293T cells were co-transfected with WT or mutants of HA-tagged MLL and FLAG-tagged PU.1 (b), or with WT or mutants of FLAG-tagged MLL and HA-tagged PU.1 (c). Anti-FLAG antibody immunoprecipitates (IP:FLAG) or cell lysates (Input) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. (d) PU.1-mediated *Csf1r* promoter-driven transcription. SaOS2 cells were transfected with the *Csf1r*-luciferase construct and PU.1, together with deletion mutants of MLL. Luciferase activity was analyzed 24 h after transfection. Error bars represent SD ($n = 3$).

The primary antibodies used in this study were anti-FLAG (M2; Sigma-Aldrich (St. Louis, MO, USA)), anti-HA (3F10; Roche Diagnostics (Mannheim, Germany)), and anti-MLL-N⁽²⁴⁾ antibodies.

Statistical analyses. We used unpaired two-tailed Student's *t*-tests for comparisons and a log-rank test for survival data using JMP8 software (SAS Institute (Cary, CA, USA)).

Colony formation assays. Cells were cultured in 1% methylcellulose in Iscove's modified Dulbecco's medium containing 15% FBS, 1% BSA, 10 μ g/mL rh-Insulin, 200 μ g/mL human transferrin, 100 μ M 2-mercaptoethanol, 2 mM L-glutamine, and the following cytokines: 50 ng/mL rm SCF, 10 ng/mL rm IL-3, and 10 ng/mL rh IL-6; or 10 ng/mL mCSF-1. Cultures were maintained at 37°C under humidified conditions with 5% CO₂. Colonies containing >50 cells were counted on day 5.

Results

Upregulation of CSF-1R is critical for MLL-AF10-induced AML. Previous results indicated that the expression of CSF-1R was high in MOZ-TIF2-induced AML⁽²⁵⁾ and human AML.⁽²⁶⁾ Expression of *Csf-1r* was investigated in MLL-AF10-induced AML in mice. Results showed that *Csf-1r* expression was high in some AML cell populations (Fig. 1a). To assess LSC activity, cells expressing high (*Csf-1r*^{high}) and low (*Csf-1r*^{low/-}) levels of *Csf-1r* were purified and transplanted into irradiated mice. Transplantation of 10² flow-sorted *Csf-1r*^{high} cells was sufficient to induce AML in all mice transplanted (Fig. 1b). Conversely, no mice developed AML after transplantation of 10² *Csf-1r*^{low/-} cells (Fig. 1c). Thus, *Csf-1r*^{high} cells displayed stronger LIC activity compared to *Csf-1r*^{low/-} cells in MLL-AF10-induced AML.

Signal transducer and activator of transcription 5 (STAT5) and ERK, which are downstream effectors of CSF-1R, are activated in a variety of leukemias and myeloproliferative disorders. The phosphorylation status of these proteins was investigated in *Csf-1r*^{high} and *Csf-1r*^{low/-} cells from MLL-AF10-induced AML mice by immunoblot analysis with phospho-specific anti-STAT5 and anti-ERK antibodies. Stat5 was highly phosphorylated in *Csf-1r*^{high} cells but not in *Csf-1r*^{low/-} cells (Fig. 1d), whereas Erk1/2 were phosphorylated in both *Csf-1r*^{high} and *Csf-1r*^{low/-} cells. Further analyses are required to determine the role(s) of Stat5 during leukemogenesis.

As MLL-AF10-induced leukemia cells can form colonies in methylcellulose,⁽²⁷⁾ flow-sorted *Csf-1r*^{high} and *Csf-1r*^{low/-} cells were tested for colony formation in the presence of either M-CSF or multiple cytokines. *Csf-1r*^{high} cells and *Csf-1r*^{low/-} formed equivalent numbers of colonies when stimulated with multiple cytokines (Fig. 1e). However, *Csf-1r*^{low/-} cells showed reduced colony formation when stimulated with M-CSF alone (Fig. 1f). Quantitative RT-PCR analysis showed that HoxA9 was upregulated in both *Csf-1r*^{high} and *Csf-1r*^{low/-} cells (Fig. 1g) and that *Csf1r* mRNA was appropriately differ-

entially expressed (Fig. 1h). *Csf-1r*^{high} and *Csf-1r*^{low/-} cells were also observed in normal BM and fetal liver (Fig. S1). Populations of *Csf-1r*^{high} were reduced in *MLL*^{-/-} fetal liver cells, suggesting that *Csf-1r* expression is regulated by WT Mll as well as by Mll-fusions.

MLL fusions activate CSF-1R transcription through interaction with PU.1. Monocyte-specific expression of CSF-1R is reportedly regulated by transcription factors such as AML1, PU.1, and C/EBP.⁽²⁸⁾ To investigate MLL-mediated regulation of *CSF-1R* transcription, the interaction of MLL with several hematopoietic transcription factors was tested. Results showed that MLL strongly interacts with PU.1 (Fig. 2a). MLL-AF10 also interacted with PU.1 (Fig. 2b). Both MLL and MLL fusions very strongly stimulated PU.1-dependent activation of the *CSF-1R* promoter (Fig. 2c). Neither MLL nor MLLAF10 activated a *CSF-1R* promoter mutant lacking PU.1 binding sites (Fig. 2d). Interaction of MLL with AML1/RUNX1⁽²⁹⁾ and other factors was less strong, and MLL and MLL fusions did not activate the *CSF-1R* promoter in the presence of AML1 or C/EBP α (data not shown). Chromatin immunoprecipitation analysis indicated genomic localizations of MLL-AF10 and PU.1 on *Csf-1r* (Fig. 2e). These results suggest that MLL and MLL fusion proteins interact with PU.1 to activate *CSF-1R* transcription.

Immunoprecipitation analysis using MLL deletion mutants indicated that PU.1 interacts with at least two regions in the N-terminus of MLL (Figs 3a,S1). The menin and LEGDF-interacting domains⁽³⁰⁾ and the C-terminal SET domain, which is needed for histone methyltransferase activity,⁽³¹⁾ are not required for interaction with PU.1 (Fig. 3b,c) or the PU.1-dependent activation of *CSF-1R* by MLL (Fig. 3d), suggesting that interaction with menin and LEGDF and histone methyltransferase activity are not required for MLL-mediated transactivation of *CSF-1R*. PU.1 deletion analysis indicated that the ETS domain of PU.1 was required for the interaction of PU.1 with MLL (Fig. 4a,b). As the ETS domain is a DNA-binding domain, it is possible that the interaction between MLL/MLL fusions and PU.1 is DNA-dependent. However, this seems unlikely because MLL-AF10 also interacted with PU.1/R232A, which lacks DNA-binding capacity (Fig. 2b). Both the DEQ region and the ETS domain of PU.1 were required to activate PU.1-mediated transcription by MLL and MLL-AF10 (Fig. 4c).

To test whether MLL-AF10 stimulates PU.1-dependent induction of endogenous *Csf-1r*, *Pu.1*^{-/-} myeloid progenitors expressing the PU.1-estrogen receptor fusion protein (PUER) were used. These cells can differentiate into macrophages after restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT).⁽³²⁾ PUER cells were infected with MSCV-MLL-AF10-ires-GFP or control retroviruses. The GFP⁺ cells were sorted and cultured in the presence of 4-HT. Five days after the addition of 4-HT, flow cytometry analysis indicated a strong increase in *Csf-1r* expression by cells expressing MLL-AF10, but only a slight increase in cells infected with the control

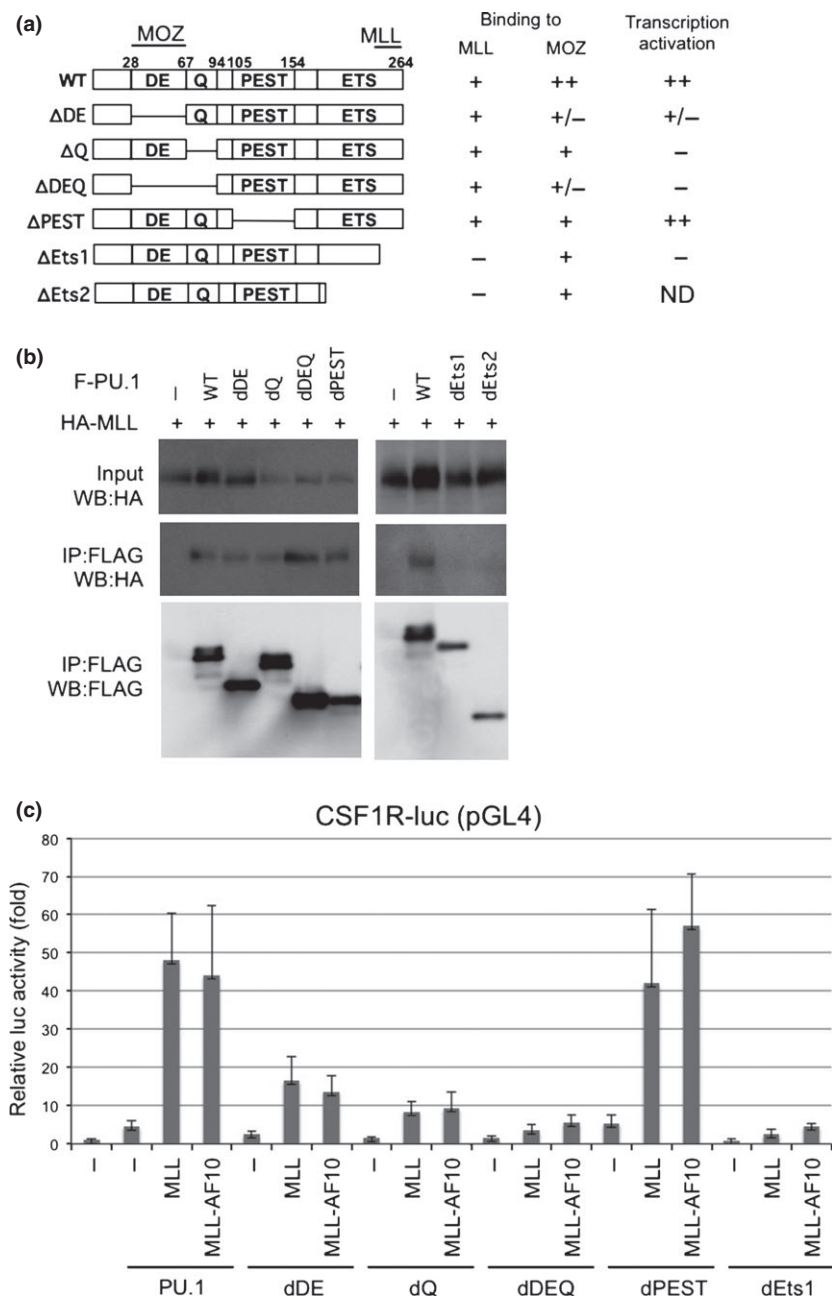


Fig. 4. Functional domains of PU.1 (a) Deletion mutants of PU.1. The MOZ- and mixed lineage leukemia (MLL)-interacting domains and the results for interaction with PU.1 and PU.1-mediated transactivation of macrophage colony-stimulating factor receptor (*Csf1r*)-luc are indicated. ND, not determined. (b) Interaction of PU.1 mutants with MLL. 293T cells were co-transfected with HA-tagged MLL and WT or mutants of FLAG-tagged PU.1. Anti-FLAG antibody immunoprecipitates (IP:FLAG) or cell lysates (Input) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. (c) SaOS2 cells were co-transfected with the *Csf1r*-luciferase construct and the indicated effectors. Luciferase activity was analyzed 24 h after transfection. Error bars represent SD ($n = 3$).

vector (Fig. 5a). Thus, MLL-AF10 induces expression of endogenous *Csf1r* in a PU.1-dependent manner.

To determine whether PU.1 is essential for initiation of MLL-AF10-induced AML, the WT and *Pu.1*^{-/-} fetal liver cells of E12.5 litter mates were infected with MLL-AF10 retrovirus and transplanted into irradiated mice. Although the mice with WT cells expressing MLL-AF10 developed AML 2–3 months after transplantation, mice with *Pu.1*^{-/-} cells were quite healthy for at least 6 months (Fig. 5b).

To determine whether PU.1 is required for maintenance of MLL-AF10-induced AML, AML mice were generated using fetal liver cells of *Pu.1* conditional KO mice (*Pu.1*^{fllox/fllox}ERT2-Cre). The BM cells of the AML mice were transplanted into secondary recipient mice and deletion of the *Pu.1* gene was induced 3 weeks after transplantation. All the control mice died within 1 month, whereas none of the mice with deletion

of *Pu.1* developed AML or died (Fig. 5c). The population of *Csf1r*^{high} cells in BM decreased within 4 days after deletion of *Pu.1* (Fig. 5d). By contrast, c-Kit-positive cells still remained. These results indicate that PU.1 is required for both development and maintenance of MLL-AF10-induced AML. The RT-PCR analysis indicated that levels of *Csf1r* mRNAs were decreased after *Pu.1* deletion but levels of *HoxA9*, *c-Kit*, and *Gapdh* mRNAs were stable at least 4 days after tamoxifen treatment (Fig. 5e). Chromatin immunoprecipitation analysis indicated that MLL-AF10 enrichment at the CSF-1R locus was reduced by deleting *Pu.1* (Fig. 5f).

CSF-1R is a promising target for AML therapy. To determine whether a high level of CSF-1R expression is an essential element of LICs, transgenic mice expressing drug-inducible FKBP-Fas suicide gene and EGFP under the control of the *Csf1r* promoter were used (Fig. 6a).⁽¹⁷⁾ In these mice, conditional ablation

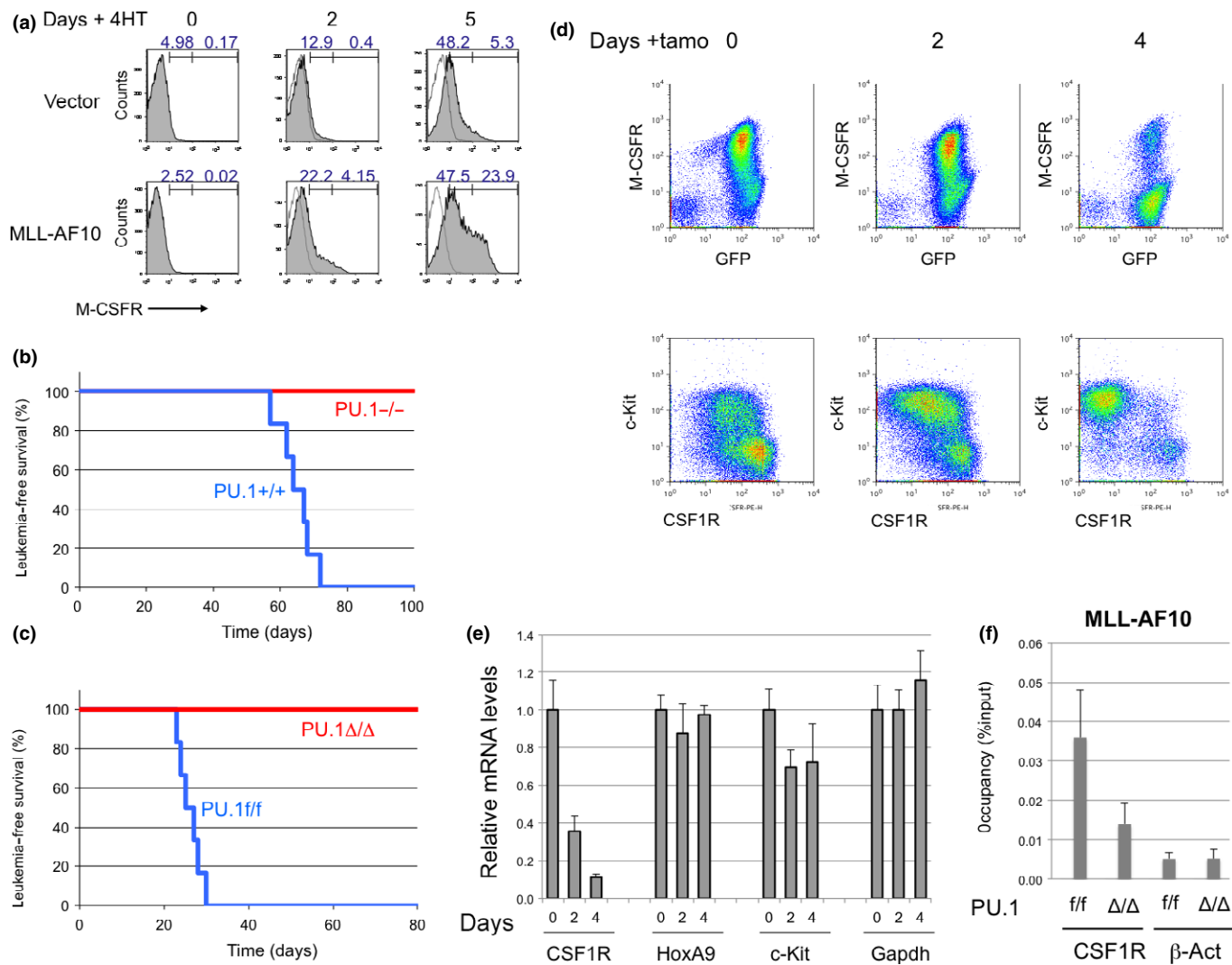


Fig. 5. PU.1 is critical for mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia (AML). (a) PUER cells infected with MSCV-GFP or MSCV-FLAG-MLL-AF10-ires-GFP retroviruses were exposed to 100 nM 4-hydroxytamoxifen (4-HT) for 0, 2, or 5 days and analyzed by FACS for macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R) expression. (b) Fetal liver cells of E12.5 PU.1^{+/+} and PU.1^{-/-} mouse embryo littermates were infected with either MLL-AF10, and transplanted into irradiated mice. Leukemia-free survivals of the mice were analyzed ($n = 6$, $P < 0.001$). (c) Fetal liver cells of E14.5 PU.1^{lox/lox} with ER-Cre were infected with MLL-AF10 and transplanted into irradiated mice. Bone marrow (BM) cells of the primary AML mice were transplanted into sublethally irradiated WT mice. Tamoxifen (tamo) (PU.1 Δ/Δ) or solvent (PU.1 f/f) was given to the secondary AML mice every 2 days by i.v. injection 17 days after transplantation, when GFP⁺ cells were detected in peripheral blood. Leukemia-free survivals of the secondary mice were investigated ($n = 6$, $P < 0.001$). (d,e) BM cells were prepared 0, 2, or 4 days after injection of tamoxifen and analyzed for expression of CSF-1R and c-Kit proteins (d) and for *Csf1r*, *HoxA9*, *c-Kit*, *Meis1*, and *Gapdh* mRNAs (e). (f) BM cells, untreated (f/f) or tamoxifen-treated for 4 days (Δ/Δ), were subjected to ChIP analysis using anti-Flag (MLL-AF10) antibodies. Semiquantitative real-time PCR was carried out on the co-precipitated DNAs.

of *Csf-1r*-expressing cells can be induced by injection of the AP20187 dimerizer.⁽¹⁷⁾ c-Kit⁺ BM cells of transgenic mice were infected with MLL-AF10 retrovirus and transplanted into lethally irradiated WT mice. These mice developed AML approximately 2 months after transplantation, and their BM cells were transplanted into secondary recipient mice. Seven days after transplantation, the mice were injected with AP20187 as described previously.⁽¹⁷⁾ All untreated mice, and none of the AP20187-treated mice, developed AML 4–6 weeks after transplantation (Fig. 6a), indicating that a high level of CSF-1R expression is a key LIC functional element in MLL-AF10-induced AML mice.

To determine if *Csf-1r* is essential for the development of MLL-AF10-induced AML, AML mice were generated using

E16.5 fetal liver cells from *Csf-1r*^{-/-}⁽¹⁸⁾ and *Csf-1r*^{+/+} littermate embryos. The mice transplanted with the WT cells developed AML 6–9 weeks after transplantation whereas those transplanted with *Csf-1r*^{-/-} cells developed AML 9–18 weeks after transplantation (Fig. 6b). Thus, the CSF-1R is required for efficient induction of AML by MLL-AF10.

The present results suggest that signaling through CSF-1R may be a suitable therapeutic target for kinase inhibitors in MLL fusion-induced leukemogenesis. The effect of the CSF-1R-specific inhibitor Ki20227 was tested with or without AraC in MLL-AF10-induced AML in mice. Ki20227 and AraC slowed the onset of AML (Fig. 6c) and inhibited the increase in GFP⁺ leukemic cells (Fig. 6d). The combination of Ki20227 plus AraC was more effective than either agent alone.

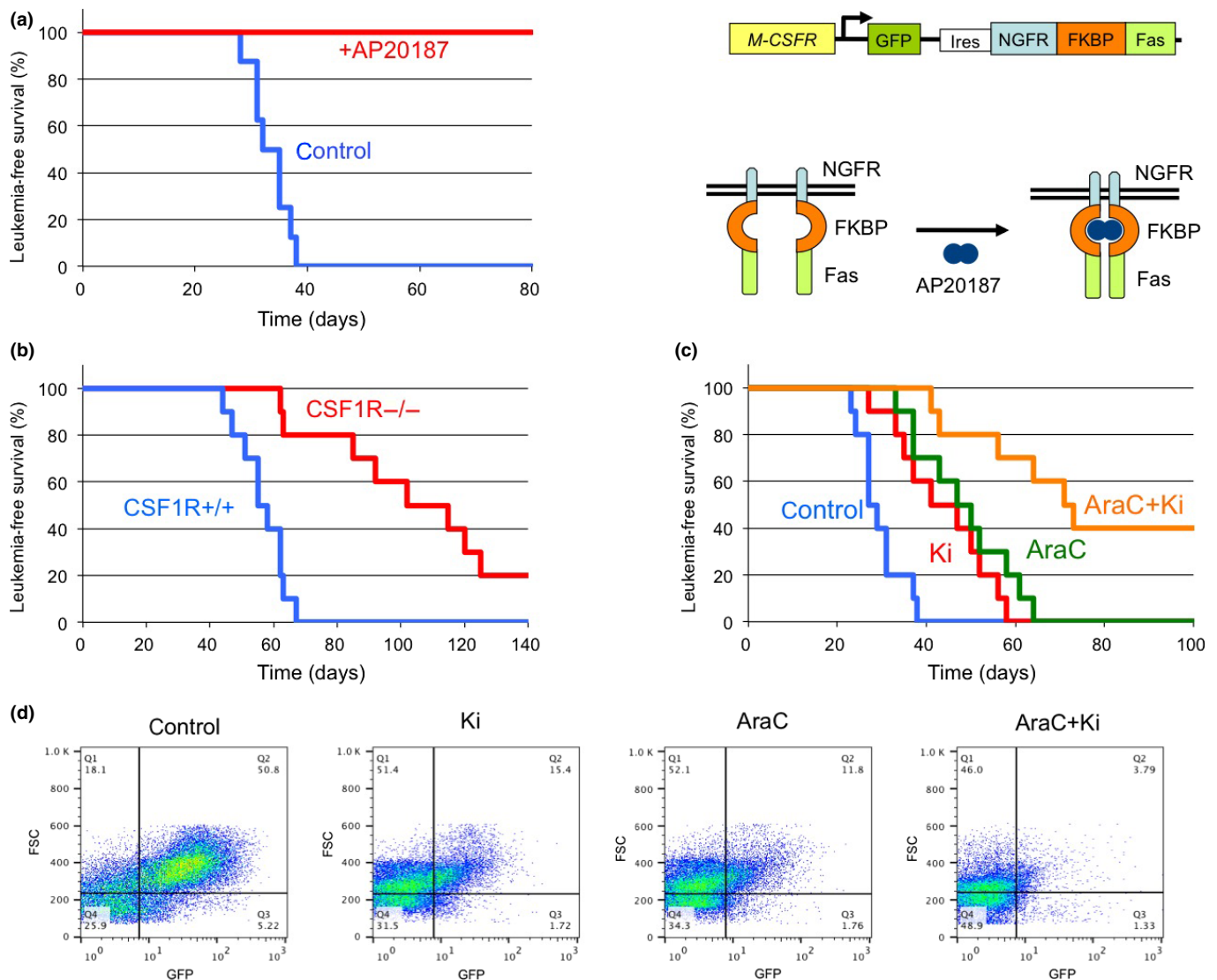


Fig. 6. Cure of mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia (AML) by ablation of cells expressing high levels of macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R^{high}). (a) Bone marrow (BM) cells from the transgenic (CSF-1R-EGFP-NGFR/FKBP1A/TNFRSF6) mice were infected with MSCV-MLL-AF10-ires-GFP and were transplanted into lethally irradiated C57BL/6 mice to induce AML. BM cells (1×10^4) of primary AML mice were transplanted into sublethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary AML mice was started by i.v. injection 3 weeks after transplantation. Leukemia-free survivals of the untreated ($n = 8$) and AP20187-treated ($n = 8$) secondary transplanted mice were investigated ($P < 0.001$). Right panel shows the structure of genes for the *Csf1r* promoter, EGFP, the NGFR-FKBP-Fas suicide construct, and activation of NGFR-FKBP-Fas. Note that in the transgenic mice, conditional ablation of cells expressing high levels of CSF-1R can be induced by exposure to the AP20187 dimerizer. (b) Fetal liver cells of E16.5 *Csf1r*^{+/+} and *Csf1r*^{-/-} mice littermate embryos were infected with MLL-AF10-ires-GFP and transplanted into irradiated mice. The leukemia-free survivals of the mice were analyzed ($n = 10$, $P < 0.001$). (c,d). BM cells (10^5) from AML mice with MLL-AF10 were transplanted into non-irradiated mice. Mice were treated with vehicle, Ki20227 (Ki), Ara-C, or Ki plus Ara-C (AraC+Ki). Leukemia-free survivals were analyzed (c) ($n = 10$, $P < 0.01$ [control vs Ki; AraC vs AraC+Ki]). Peripheral blood cells were prepared 21 days after transplantation and analyzed for expression of GFP (d).

Discussion

CSF-1R is a potential target for AML therapy. Acute myeloid leukemia is a highly malignant disease. Numerous genetic abnormalities are known in AML, among which chromosome translocations involving the *MLL* gene are associated with poor prognosis. Conventional chemotherapies are often effective in reducing the total number of leukemia cells, but are not curative in many cases of AML. Leukemic stem cells are capable of the limitless self-renewal necessary for cancer initiation and maintenance. As residual LSCs are a potential cause of AML relapse, eradication of LSCs is critical to cure the disease. The present results showed that LSCs are enriched in cells express-

ing high levels of CSF-1R. Relevant to our observations, a viral integration site of the Friend murine leukemia virus that is used in approximately 20% of virus-induced primary myeloid leukemias, was shown to be at the 5'-end of the *Csf1r* gene and to result in high expression of a normal-sized *Csf1r* mRNA.⁽³³⁾ Using a mouse model expressing a drug-inducible suicide gene controlled by the *Csf1r* promoter, ablation of *Csf1r*^{high} cells was shown to prevent AML mice from dying of the disease. Moreover, MLL-AF10-induced leukemia was suppressed by deletion of the *Csf1r* gene. These results clearly show that CSF-1R is a promising target for novel AML therapy. CSF-1R is a receptor tyrosine kinase that regulates the

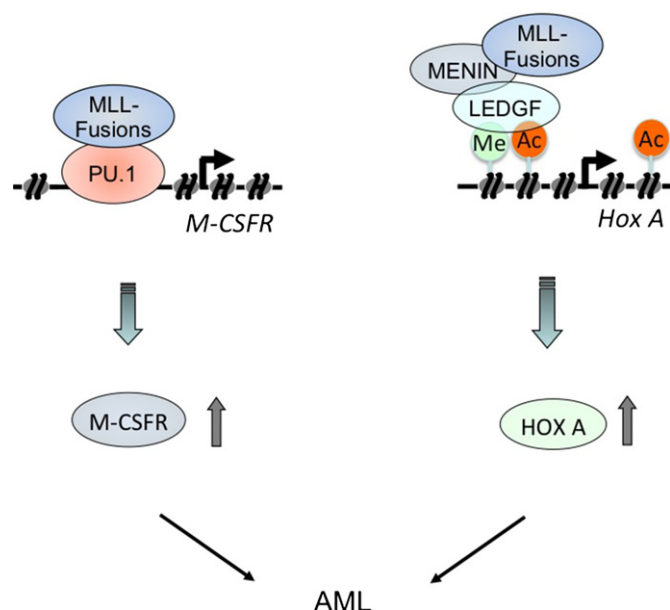


Fig. 7. Model for transcriptional regulation of macrophage colony-stimulating factor receptor (*Csf1r*) and *Hox* genes by mixed lineage leukemia (MLL) fusion proteins. MLL fusions induce the rapid onset of acute myeloid leukemia by activating two classes of pathways, the CSF-1R and *Hox* pathways. Left, MLL fusions stimulate constitutive CSF-1R expression by binding to PU.1 to induce leukemia. Right, MLL fusion proteins form a complex with menin and LEDGF to regulate the expression of *Hox* genes.

survival, proliferation, and/or differentiation of macrophages, osteoclasts, and Paneth cells.^(34–36) A tyrosine kinase inhibitor specific for CSF-1R slowed the progress of MLL-AF10-induced leukemia. CSF-1R upregulation has been detected in LSCs in MOZ-TIF2-induced AML⁽²⁵⁾ and human AML patients.⁽²⁶⁾

CSF-1R expression is critical for AML initiation but not for immortalization *in vitro*. MLL leukemias are invariably associated with the expression of *Hox* genes. Upregulation of *Hox* genes in MLL leukemias is critical for LSC maintenance; however, *Hox* upregulation alone does not recapitulate all the biological and clinical features of MLL leukemias and is unlikely to support malignancy and the high LSC frequency observed in MLL leukemias. Forced expression of the *HOXA9* gene can immortalize myeloid progenitors *in vitro*, but is not sufficient to initiate AML *in vivo*. By contrast, expression of MLL fusions such as MLL-AF10, MLL-AF9, and MLL-ENL is sufficient for both immortalization *in vitro* and initiation of AML *in vivo*. Our results indicate that cells expressing high levels of Csf-1r show strong AML initiation *in vivo* (Fig. 1b,c) whereas cells expressing high and low levels of Csf-1r showed equivalent colony formation *in vitro* (Fig. 1e). These findings suggest that CSF-1R expression is not important for immortalization *in vitro* but is critical for initiation of AML *in vivo*.

The LSC activity of MLL leukemia is known to be reduced after culture *in vitro*,⁽³⁷⁾ suggesting that a certain *in*

vivo microenvironment is required for LSC maintenance, as is also the case for hematopoietic stem cells. Our results show that the expression of Csf-1r is greatly reduced after culture *in vitro* (data not shown), suggesting that expression of CSF-1R is regulated by microenvironment-dependent epigenetics.

Differential regulation of CSF-1R and *Hox*. Both MLL and MLL fusion proteins form a complex with menin and LEDGF to regulate the expression of *Hox* genes.^(30,38) Our results show that PU.1 mediates the regulation of *Csf1r* transcription by MLL/MLL fusion proteins. The menin and LEDGF-interacting domains of MLL are not required for interaction with PU.1 or transactivation at the *Csf1r* promoter, suggesting that menin and LEDGF are unlikely to be involved in the regulation of CSF-1R expression. While expression of Csf-1r rapidly decreased after deletion of the *Pu.1* gene (Fig. 5d,e), *HoxA9* mRNA levels were stable at least 4 days after *Pu.1* deletion (Fig. 5e). Moreover, *HoxA9* mRNA levels were equivalent in AML cells expressing high and low levels of CSF-1R (Fig. 1h). These results suggest that expression of the *Csf1r* and *Hox* genes is independently regulated by MLL fusion proteins.

The MLL-AF10-induced AML was shown to be cured by deletion of *Pu.1* (Fig. 5c). However, deletion of Csf-1r prolonged survival time but did not cure the AML completely. These facts suggest that CSF-1R is not the sole critical PU.1-dependent mediator of leukemogenesis, and that there are other PU.1-target genes critical for maintenance of MLL fusion-induced AML. Such genes may be involved in the leukemogenesis in collaboration with *CSF-1R* and *Hox* genes.

It has been suggested that second mutations, such as activating point mutations, in receptor tyrosine kinases (e.g., FLT3 and c-KIT) are required for fusion genes such as AML1-ETO, PML-RAR α , or CBF β -MYH11 to induce acute leukemia.⁽³⁹⁾ In contrast, MLL fusions alone can induce the rapid onset of AML.⁽⁴⁰⁾ Our data suggest that MLL fusions induce the upregulation of the receptor tyrosine kinase *Csf-1r* and *Hox* genes, thereby inducing the rapid onset of AML by activating two classes of pathways (Fig. 7). These pathways provide potential molecular targets for new approaches in the treatment of these forms of leukemia.

Acknowledgments

We would like to thank Dr. D. E. Zhang for *Csf-1r* promoter mutant lacking PU.1-binding sites and Dr. Harinder Singh for PUER cells. This work was supported in part by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japanese National Cancer Center Research and Development Fund, and US National Institutes of Health grants HL112719, CA32551, and 5P30-CA13330.

Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. (A) Bone marrow (BM) cells from normal mice were analyzed by flow cytometry for expression of GFP and macrophage colony-stimulating factor receptor (Csf-1r). (B) Csf-1r^{high} and Csf-1r^{low/-} cells from normal mixed lineage leukemia (MLL)-AF10-induced AML mice were sorted by flow cytometry. Levels of *Csf1r* (H) mRNAs were measured in Csf-1r^{high} and Csf-1r^{low/-} cells prepared from normal and acute myeloid leukemia mouse BM. (C) *Mll*^{+/+} and *Mll*^{-/-} Fetal liver cells were analyzed by flow cytometer for expression of Csf-1r.