Discussion

Our recent discovery of the highly frequent G17V *RHOA* mutation in AITL and AITL-like PTCL-NOS led us to develop a novel method to detect this mutation [12]. The results of qAS-PCR analysis described here are correlated well with those derived from deep sequencing (Table 4), while qAS-PCR is superior to deep sequencing in terms of the cost and convenience. There is a pressing clinical need for a well-validated *RHOA* testing method with optimal analytical performance using the least amount of difficult-to-obtain patient specimens. We show here that even DNA samples subjected to whole-genome amplification or low quality/concentration DNA extracted from FFPE samples can serve as reliable material for our qAS-PCR method, if appropriate PCR procedure and primers are used. Allele-specific PCR for G17V *RHOA* mutation was mentioned in other report [13], although sensitivity and specificity of the methods were not described.

In a previous study, we defined the cut-off level of mutant allele frequencies determined by MiSeq as 0.02 [12]. In this study, we defined the cut-off level as 1.5×10^{-2} for qAS-PCR, but it remains to be determined whether these cut-off levels are sufficient to detect AITL. Given our finding that the mutated *RHOA* allele frequencies distributed below 0.05 in many AITL samples [12], the tumor cell content might be very low and could be detected in some cases only when the cut-off levels of qAS-PCR and deep sequencing are lowered. If we set the cut-off value lower, the sensitivity should be improved with the increase of false-positive results, raising a dilemma common to other clinical testings.

Several hotspot mutations that reveal distinct hematologic malignancies have been identified in conditions other than T-cell lymphomas. For example, detection of the V617F *JAK2* mutation is a part of the diagnostic criteria for myeloproliferative neoplasms in the latest version of WHO classification [1], although consensus is not reached about the detection methods and cut-off levels. Methods have been developed to detect this mutation including allele-specific PCR and a PCR-restriction fragment length polymorphism (RFLP) approach utilizing mutation sequence specificity for a restriction enzyme[15–18]. More recently, a V600E *BRAF* mutation in hairy cell leukemia [19], an L265P *MYD* mutation in Waldenström macrogloblinemia [20], and several mutations in *STAT3* in large granular lymphocytic leukemia [21] have been identified as diagnostics of these tumor types. In the future, it is likely that molecular alterations, including the G17V *RHOA* mutation, will be increasingly incorporated into the diagnostic criteria for hematologic malignancies. In summary, our novel method to detect the G17V *RHOA* mutation could provide an important clinical tool to diagnose AITL and AITL-like PTCL-NOS and in the future serve as a means to classify AITL and PTCL-NOS.

Supporting Information

File S1 Figures S1–S2 and Tables S1–S4. Figure S1. ROC curve for data of qAS-PCR and MiSeq. Horizontal axis shows 1-specificity and Vertical axis shows sensitivity of qAS-PCR method compared to the data of MiSeq. Figure S2. Effect of whole-genome amplification for qAS-PCR A, Comparison of [mut]/([wt]+[mut]) values by qAS-PCR and mutant allele frequencies as determined by MiSeq for 66 original samples (linear). B, Comparison of [mut]/([wt]+[mut]) values by qAS-PCR and mutant allele frequencies as determined by MiSeq for 66 original samples (log scale). C, Comparison of [mut]/([wt]+[mut]) values by qAS-PCR and mutant allele frequencies as determined by MiSeq for 29 whole-genome amplified samples (linear). D, Comparison of [mut]/([wt]+[mut]) values by qAS-PCR and mutant allele frequencies as determined by MiSeq for 29 whole-genome amplified samples (log scale). E, Comparison of [mut]/([wt]+[mut]) values by qAS-PCR for 15 pairs of original and whole-genome amplified samples in a log scale.
(PDF)

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Author Contributions

Conceived and designed the experiments: RN-M MS-Y SC. Performed the experiments: RN-M. Analyzed the data: RN-M MS-Y KY SY Y. Shiozawa TN KS MS SO KT NN. Contributed reagents/materials/analysis tools: TE HM NO T. Kato NK YY KI YO SS T. Komeno Y. Sato TI IK. Contributed to the writing of the manuscript: RN-M MS-Y SC.

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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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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.

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* leukemias.[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

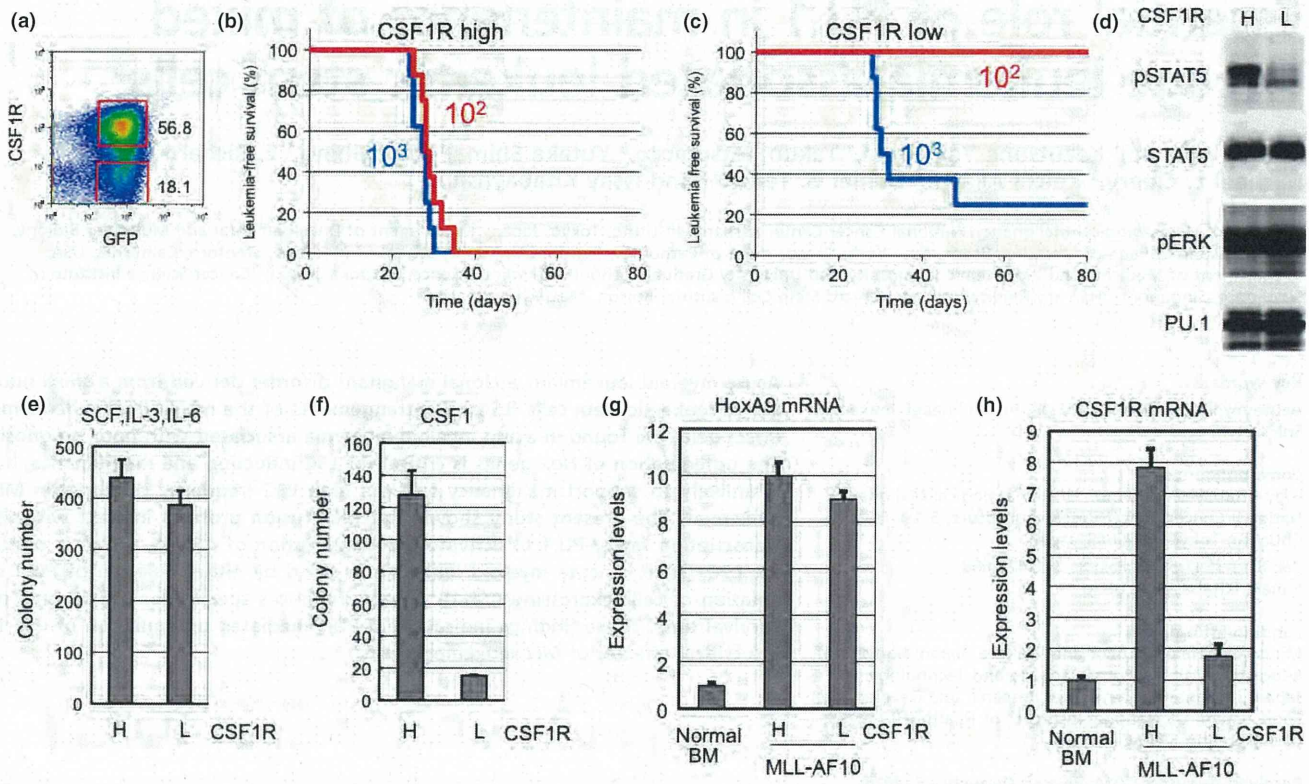

Fig. 1. Cells expressing high levels of macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R high) show potent leukemia-initiating activity. (a) Bone marrow (BM) cells from mice with mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia were analyzed by flow cytometer for expression 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 phRL-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 phRL-CMV as an internal control.

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

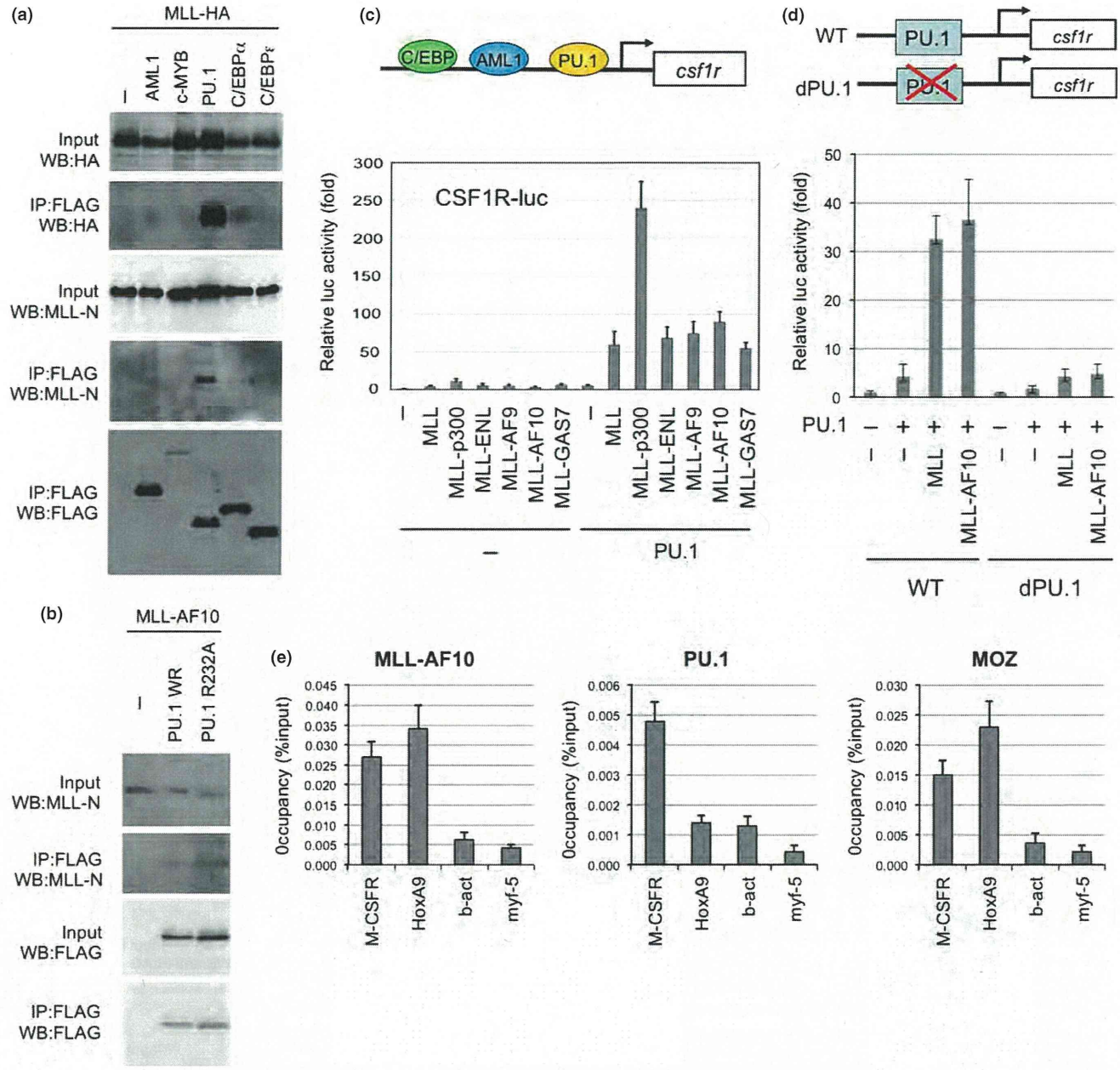

Fig. 2. PU.1-dependent upregulation of macrophage colony-stimulating factor receptor (CSF-1R) by mixed lineage leukemia (MLL) and MLL fusions. (a) Interaction of MLL with PU.1. 293T cells were co-transfected with MLL-HA and the indicated FLAG-tagged transcription factors, including FLAG-PU.1. Anti-FLAG 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/FR232A. 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. Precipitated 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).

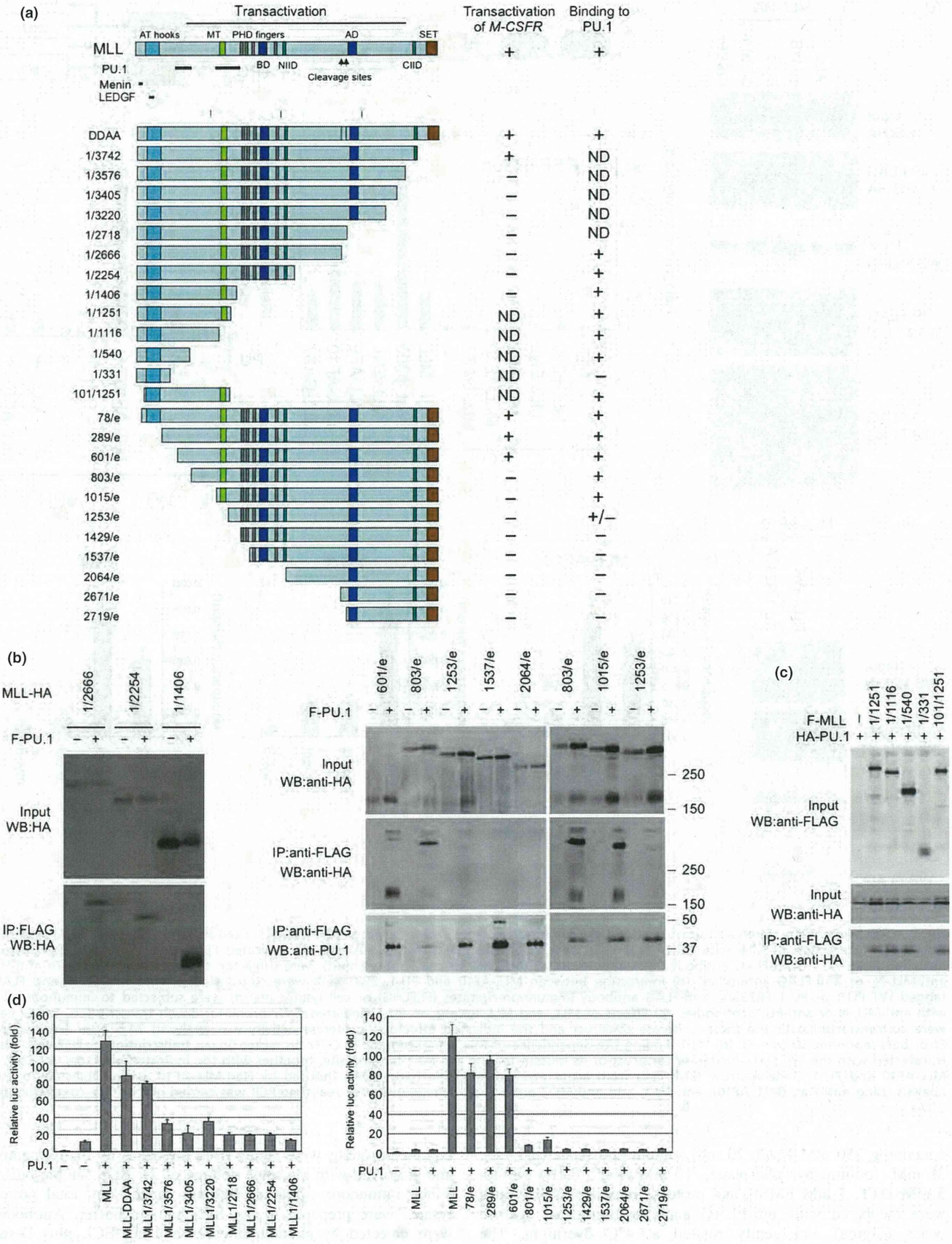

Fig. 3. Functional domains of mixed lineage leukemia (MLL) required for interaction with PU.1 and for PU.1-mediated activation of macrophage colony-stimulating factor receptor (*Csf1r*) promoter. (a) PU.1 binding and PU.1-mediated *Csf1r* promoter activity of MLL deletion mutants. The PU.1-, menin-, 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 μg/mL rh-Insulin, 200 μg/mL human transferrin, 100 μM 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 10^2 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 10^2 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 differentially 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/EBPα (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

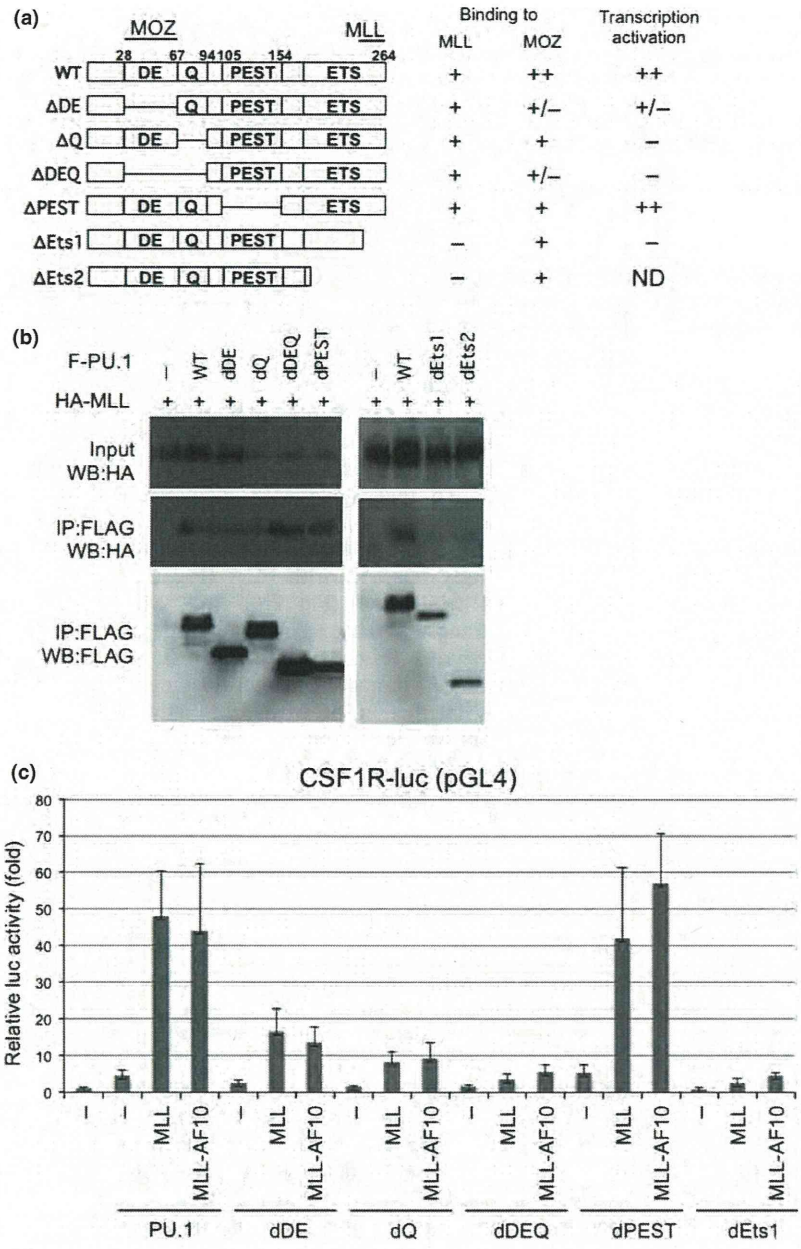

Fig. 4. Functional domains of PU.1 (a) Deletion mutants of PU.1. The MOZ- and mixed lineage leukemia (MLL)-interacting domains and the results for interaction with PU.1 and PU.1-mediated transactivation of macrophage colony-stimulating factor receptor (*Csf1r*)-luc are indicated. ND, not determined. (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 *Csf-1r* 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*$^{flox/flox}$ 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 Csf-1r^{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 *Csf-1r* 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 *Csf-1r* promoter were used (Fig. 6a).[17] In these mice, conditional ablation

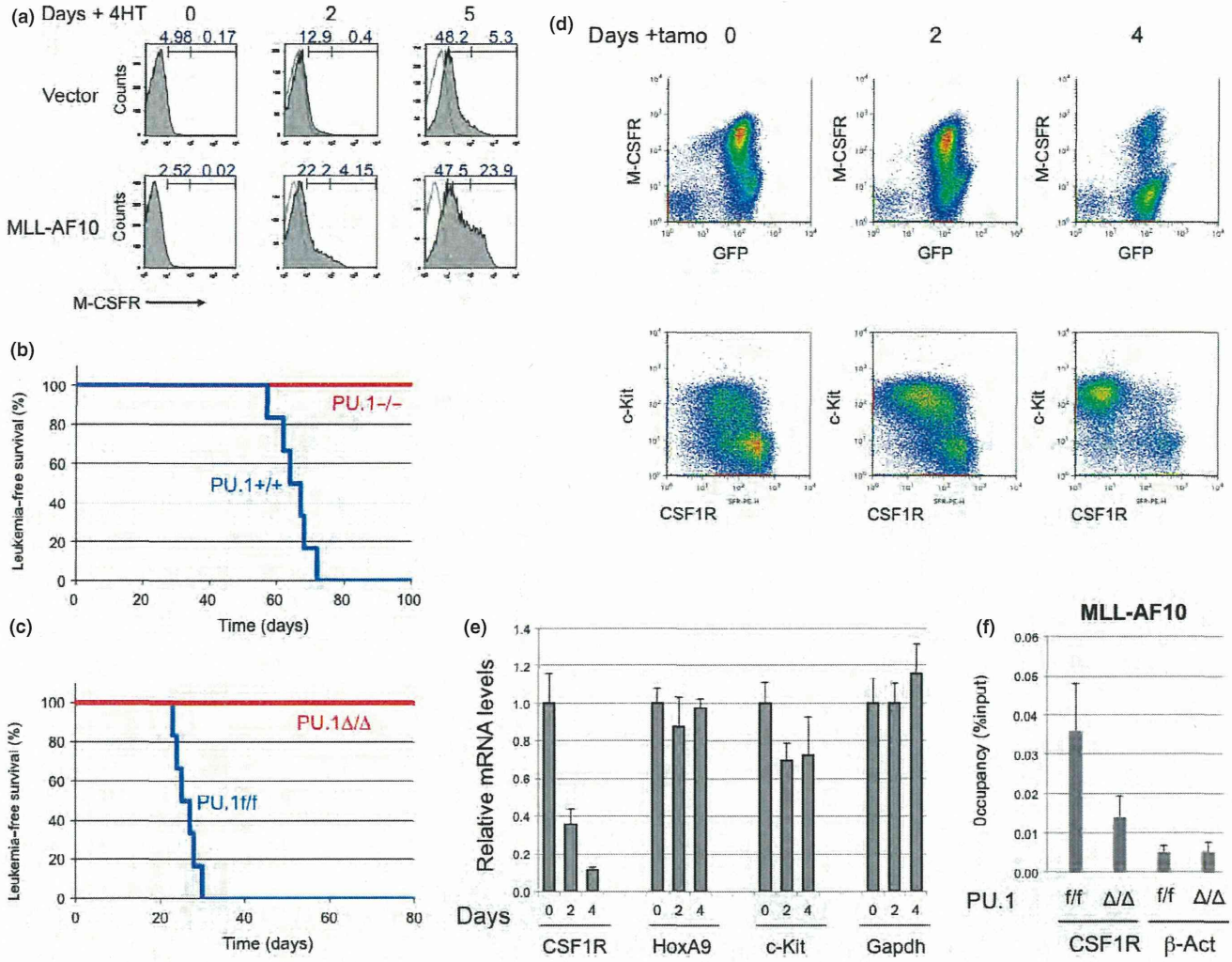

Fig. 5. PU.1 is critical for mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia (AML). (a) PUER cells infected with MSCV-GFP or MSCV-FLAG-MLL-AF10-ires-GFP retroviruses were exposed to 100 nM 4-hydroxytamoxifen (4-HT) for 0, 2, or 5 days and analyzed by FACS for macrophage colony-stimulating 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 $PU.1^{flox/flox}$ 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.1Δ/Δ) or solvent (PU.1f/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.

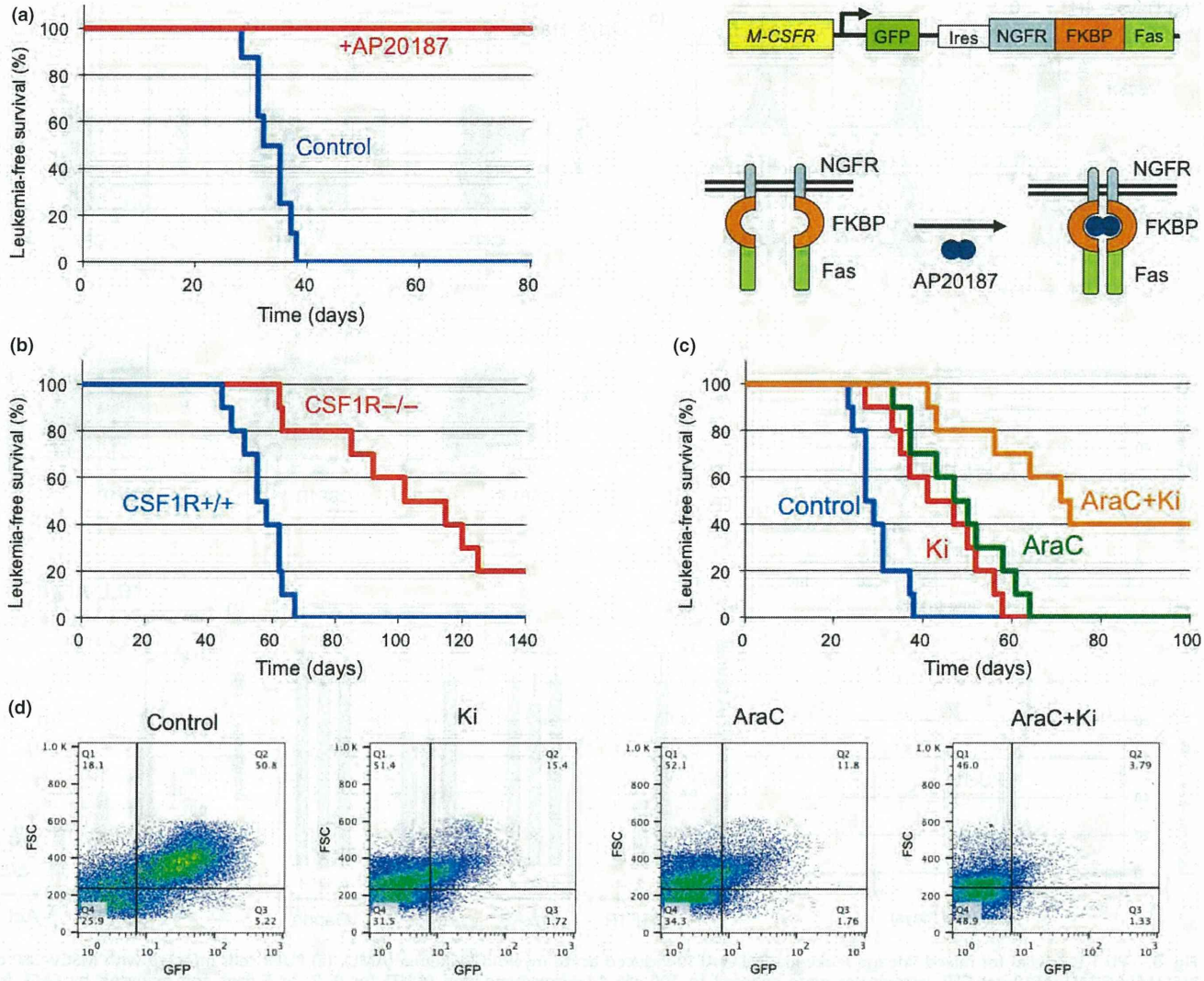

Fig. 6. Cure of mixed lineage leukemia (MLL)-AF10-induced acute myeloid leukemia (AML) by ablation of cells expressing high levels of macrophage colony-stimulating factor (M-CSF) receptor (CSF-1R^{high}). (a) Bone marrow (BM) cells from the transgenic (CSF-1R-EGFP-NGFR/FKBP1A/TNFRSF6) mice were infected 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 expressing 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 *Csf-1r* gene and to result in high expression of a normal-sized *Csf-1r* mRNA.[33] Using a mouse model expressing a drug-inducible suicide gene controlled by the *Csf-1r* promoter, ablation of Csf-1r^{high} cells was shown to prevent AML mice from dying of the disease. Moreover, MLL-AF10-induced leukemia was suppressed by deletion of the *Csf-1r* 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