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Essential role of PU.1 in maintenance of MLL-associated leukemia stem cells

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Abstract

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs). *MLL* gene rearrangements are found in AML 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. AML 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.

Keywords

Acute Myeloid Leukemia; CSF-1R; MLL; Spi-1; Stem Cells

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Author Contributions

YA, MS, KY, TK and YS performed research and analyzed data. ERS, MLK, KA, and DGT contributed vital new reagents. IK designed the research, analyzed data and wrote the paper.

Disclosure Statement

The authors have no conflicts of interest.

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Introduction

Acute myeloid leukemia (AML) is a clonal malignant disorder derived from a small number of leukemic stem cells (LSCs)[1, 2]. 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. Since 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]. Over 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]. MLL fusion proteins transform non-self-renewing myeloid progenitors into LSCs[7, 8]. AML 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 receptor (CSF-1R, also called M-CSFR/c-FMS/CD115) is critical for LSC activity in MLL leukemia. AML was cured upon eradication of cells expressing high levels of Csf-1r in mice. MLL fusions were found to regulate 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 CREA Japan (Tokyo). NGF-FKBP-Fas transgenic mice[17] (Jackson Lab.), *CSF-1R*-deficient mice[18], *PU.1*-null/conditional deficient mice[19], and CreERT2 mice (TaconicArtemis GmbH)[20] were maintained on a C57BL/6 genetic background. Mouse experiments were performed in a specific pathogen-free environment at the National Cancer Center 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), 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 (Militenyi Biotec), were incubated with the retrovirus using RetroNectin (Takara Bio) for 24 h in StemPro-34 SFM medium (Invitrogen) containing cytokines (20 ng/mL SCF, 10 ng/mL IL-6, 10 ng/mL IL-3). The infectants were then transplanted together with BM cells (2×10^5) into lethally irradiated (9

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Gy) 6- to 8-week-old C57BL/6 mice by intravenous (IV) injection. Secondary transplants were performed by intravenous injection of BM cells from the primary AML mice into sub-lethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, AraC, or Ki20227

AP20187 (gift from Ariad Pharmaceuticals; 10 mg/kg) was administered daily by IV injection for 5 d, and then 1 mg/kg AP20187 was administered every 3 d thereafter as described previously[17]. Ki20227[22] (gift from KIRIN Pharma; 20 mg/kg) were orally administrated daily from 7 days after transplantation. AraC (75 mg/kg) was administered daily by IV injection for 5 days from 7 days after transplantation.

Immunofluorescent staining, flow cytometric analysis, and cell sorting

BM cells from AML mice were preincubated with rat IgG, and then incubated on ice with anti-CD115(CSF-1R)-PE (eBioscience) and anti- c-Kit-APC (2B8)-APC (BD Pharmingen). Flow cytometric analysis and cell sorting were performed using the cell sorter JSAN (Baybioscience), and the results were analyzed using FlowJo software (Tree Star).

Reporter analysis

Csf1r-luciferase constructs were generated by ligation of wild-type 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). 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 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 6 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 performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence using ECL plus Detection Reagents (Amersham Biosciences, Buckinghamshire, United Kingdom). The primary antibodies used in this study were anti-FLAG (M2) (Sigma), anti-HA (3F10) (Roche), and anti-MLL-N[24] antibodies.

Statistical analyses

We performed unpaired two-tailed Student's *t*-tests for comparisons and a log-rank test for survival data using JMP8 software (SAS Institute).

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Colony formation assays

Cells were cultured in 1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM) containing 15% fetal bovine serum (FBS), 1% bovine serum albumin (BSA), 10 μg/mL rhInsulin, 200 μg/mL human transferrin, 100 μM 2-mercaptoethanol, 2mML-glutamine, and the following cytokines; 50 ng/mL rm SCF, 10 ng/mL rmIL-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]. Csf-1r expression was investigated in MLL-AF10-induced AML in mice. Results showed that Csf-1r expression was high in some AML cell populations (Figure 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 (Figure 1B). Conversely, no mice developed AML upon transplantation of 10^2 Csf-1r$^{low/-}$ cells (Figure 1C). Thus, Csf-1r^{high} cells displayed stronger LIC activity compared to Csf-1r$^{low/-}$ cells in MLL-AF10-induced AML.

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 (Figure 1D), while 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.

Since 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 (Figure 1E). However, Csf-1r$^{low/-}$ cells showed reduced colony formation when stimulated with M-CSF alone (Figure 1F). Quantitative RT-PCR analysis showed that HoxA9 was upregulated in both Csf-1r^{high} and Csf-1r$^{low/-}$ cells (Figure 1G) and that *Csf1r* mRNA was appropriately differentially expressed (Figure 1H). Csf-1r^{high} and Csf-1r$^{low/-}$ cells were also observed in normal BM and fetal lever (Supplemental Figure 1). Population of Csf-1r^{high} were reduced in *Mll*$^{-/-}$ Fetal liver cells, suggesting that Csf-1r expression is regulated by wild type 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

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tested. Results showed that MLL strongly interacts with PU.1 (Figure 2A). MLL-AF10 also interacted with PU.1 (Figure 2B). MLL and MLL fusions very strongly stimulated PU.1-dependent activation of the *CSF-1R* promoter (Figure 2C). Neither MLL nor MLLAF10 activated a *CSF-1R* promoter mutant lacking PU.1-binding sites (Figure 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 (ChIP) analysis indicated that genomic localizations of MLL-AF10 and PU.1 on *Csf-1r* (Figure 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 (Figures 3A and 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 (Figure3B and 3C) or the PU.1-dependent activation of *CSF-1R* by MLL (Figure 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 (Figures 4A and 4B). Since 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 (Figure 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 (Figure 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 upon 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. 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 vector (Figure 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 wild-type 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 wild-type cells expressing MLL-AF10 developed AML 2–3 months after transplantation, mice with *Pu.1*$^{-/-}$ cells were quite healthy for at least 6 months (Figure 5B).

To determine if 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 bone marrow (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, while none of the mice with deletion of *Pu.1* developed AML or died (Figure 5C). The population of Csf-1r^{high} cells in BM decreased within 4 days

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after deletion of *Pu.1* (Figure 5D). By contrast, c-Kit-positive cells were still remained. These results indicate that PU.1 is required for both development and maintenance of MLL-AF10-induced AML. 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 (Figure 5E). Chromatin immunoprecipitation (ChIP) analysis indicated that MLL-AF10 enrichment at the CSF-1R locus was reduced by deleting *Pu.1* (Figure 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[17] (Figure 6A). In these mice, conditional ablation 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 wild-type mice. These mice developed AML about 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 (Figure 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 wild-type cells developed AML 6–9 weeks after transplantation while those transplanted with *Csf-1r* $^{-/-}$ cells developed AML 9–18 weeks after transplantation (Figure 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. Ki20227 and AraC slowed the onset of AML (Figure 6C) and inhibited the increase in GFP+ leukemic cells (Figure 6D). The combination of Ki20227 plus AraC was more effective than either agent alone.

Discussion

CSF-1R is a potential target for AML therapy

AML 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. LSCs are capable of the limitless self-renewal necessary for cancer initiation and maintenance. Since 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.

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Relevant to our observations, a viral integration site of the Friend murine leukemia virus that is utilized 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 survival, proliferation and/or differentiation of macrophages, osteoclasts and Paneth cells[343536]. 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 demonstrate that cells expressing high levels of Csf-1r show strong AML initiation *in vivo* (Figures 1B and 1C) while cells expressing high and low levels of Csf-1r showed equivalent colony formation *in vitro* (Figure 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

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 LEGDF-interacting domains of MLL are not required for interaction with PU.1 or transactivation at the *Csf1r* promoter, suggesting that MENIN and LEGDF 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 (Figures 5D and 5E), HoxA9 mRNA levels were stable at least 4 days after *Pu.1* deletion (Figure 5E). Moreover, HoxA9 mRNA levels were equivalent in AML cells expressing high and low levels of CSF-1R (Figure 1H). These results suggest that expression of the *Csf1r* and *Hox* genes is independently regulated by MLL fusion proteins.

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The MLL-AF10-induced AML was demonstrated to be cured by deletion of *Pu.1* (Figure 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-RARa, or CBFb-MYH11 to induce acute leukemia[39]. By 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 (Figure 7). These pathways provide potential molecular targets for new approaches in the treatment of these forms of leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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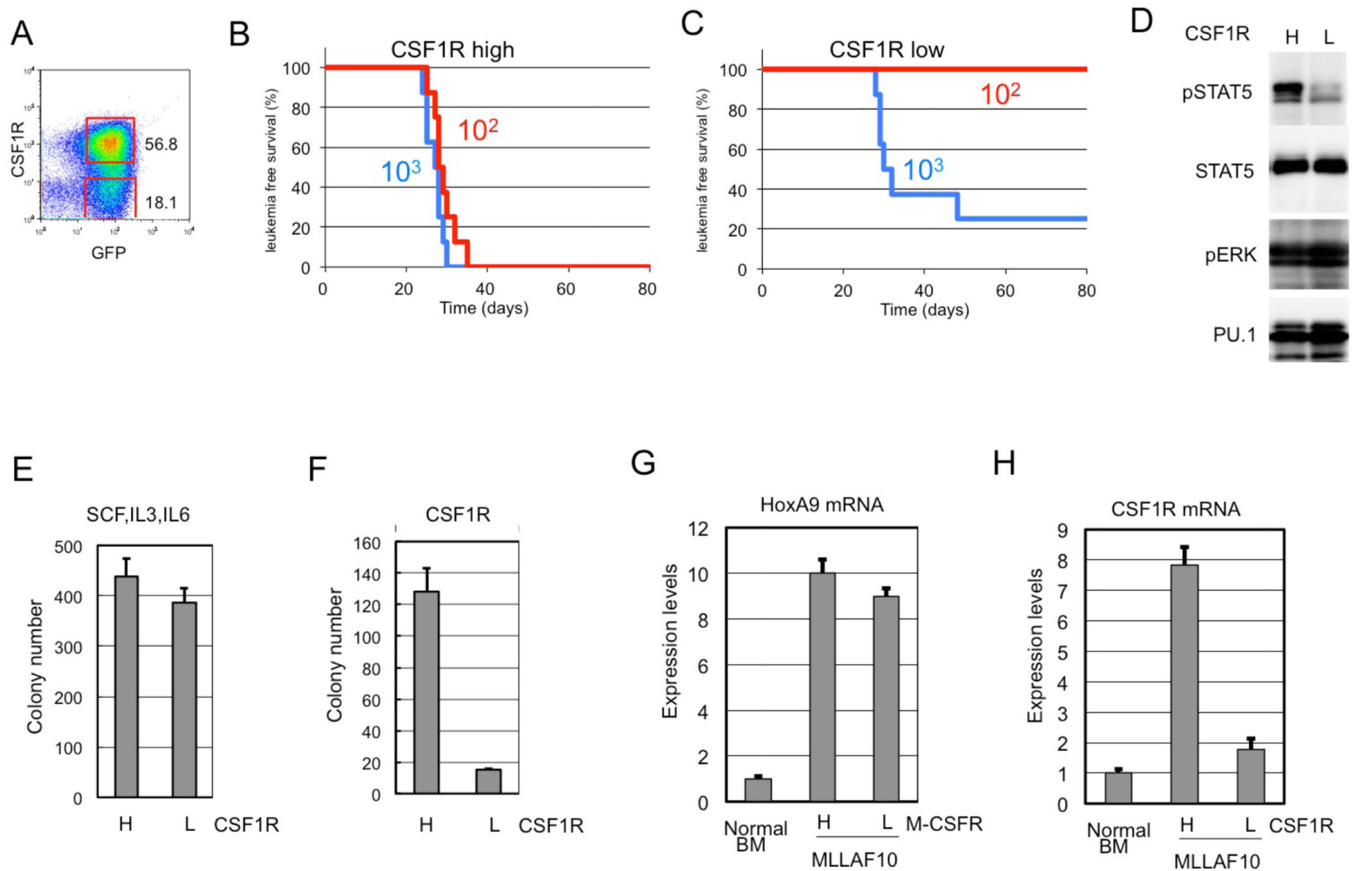

Figure 1. CSF-1R^{high} cells show potent leukemia-initiating activity

(A) The bone marrow (BM) cells from MLL-AF10–induced AML mice 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, and the indicated numbers of flow-sorted CSF-1R^{high} (B) and Csf-1r $^{low/-}$ (C) cells were transplanted into sub-lethally irradiated mice, and leukemia-free survival was investigated. $n = 8$, $P < 0.001$. (D) Csf-1r ^{high} and Csf-1r $^{low/-}$ cells were analyzed for levels of total and phosphorylated Stat5, phosphorylated 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 IL3, SCF and IL6 (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 AML mouse BM.

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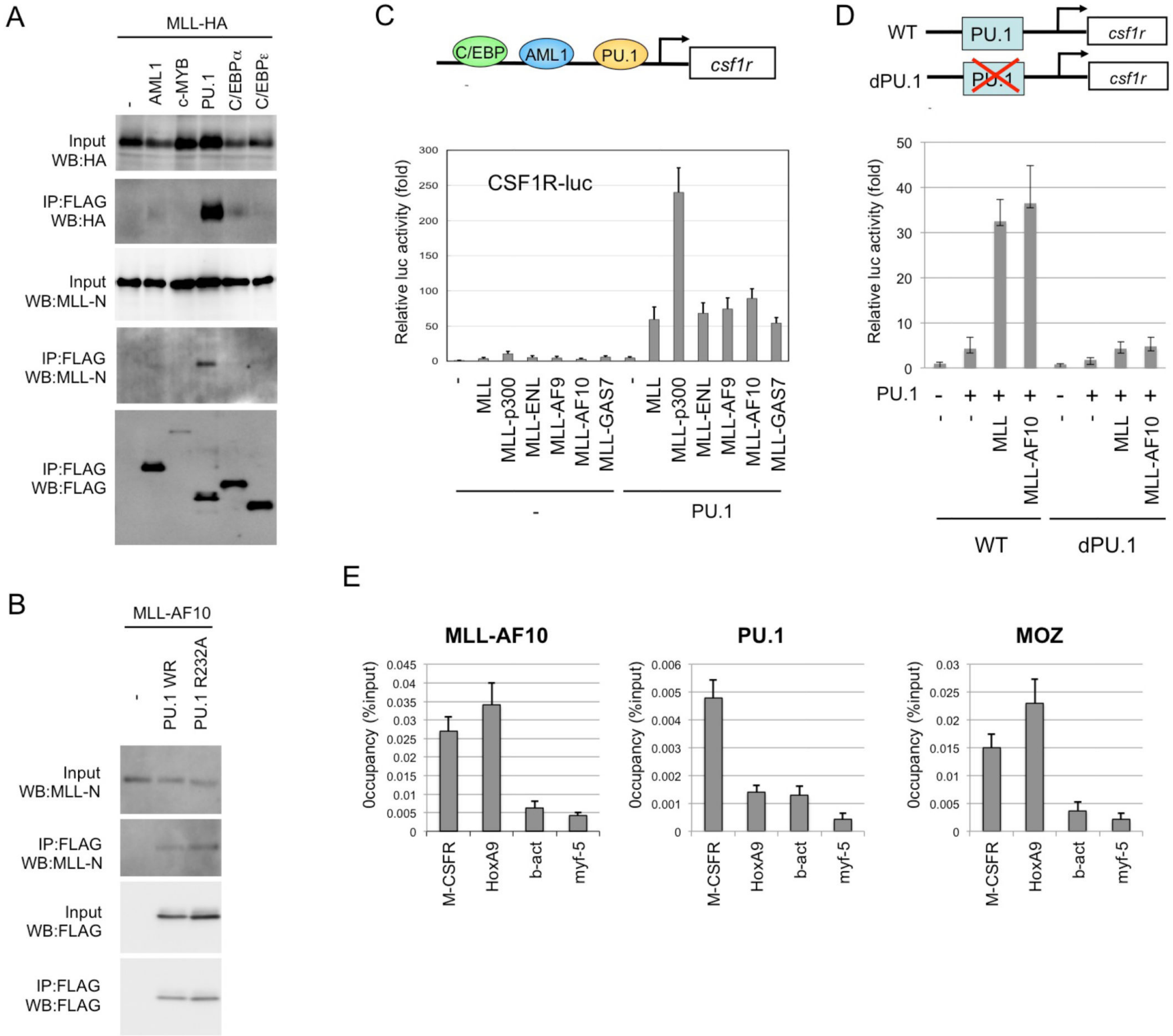

Figure 2. PU.1-dependent upregulation of CSF-1R by MLL and MLL fusions

(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 wild-type 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

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transcription. SaOS2 cells were transfected with the wild-type *Csf1r*–luciferase construct or its mutant lacking the PU.1-binding site, together with the indicated effectors. (E) Chromatin immunoprecipitation (ChIP) of MLL-AF10 and PU.1. The BM cells from AML mice 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 performed on the co-precipitated DNAs.

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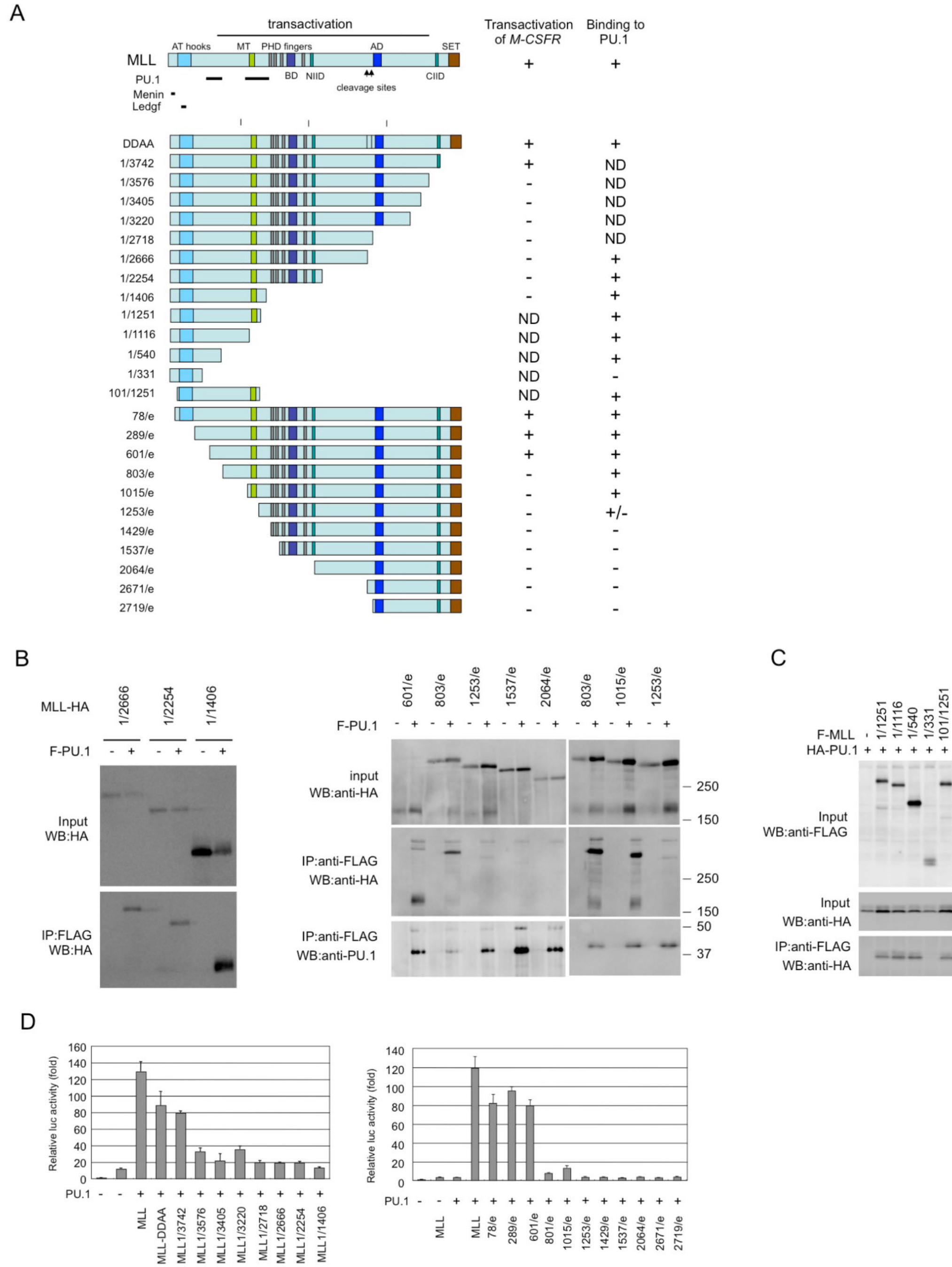

Figure 3. Functional domains of MLL required for interaction with PU.1 and for PU.1-mediated activation of *Csf1r* promoter

(**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 wild-type or mutants of HA-tagged MLL and FLAG-tagged PU.1 (**B**), or with wild-type or mutants of FLAG-tagged MLL and HA-tagged PU.1 (**C**). Anti-FLAG antibody immunoprecipitates (IP:FLAG) or cell lysates (Input) were

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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 ($n = 3$).

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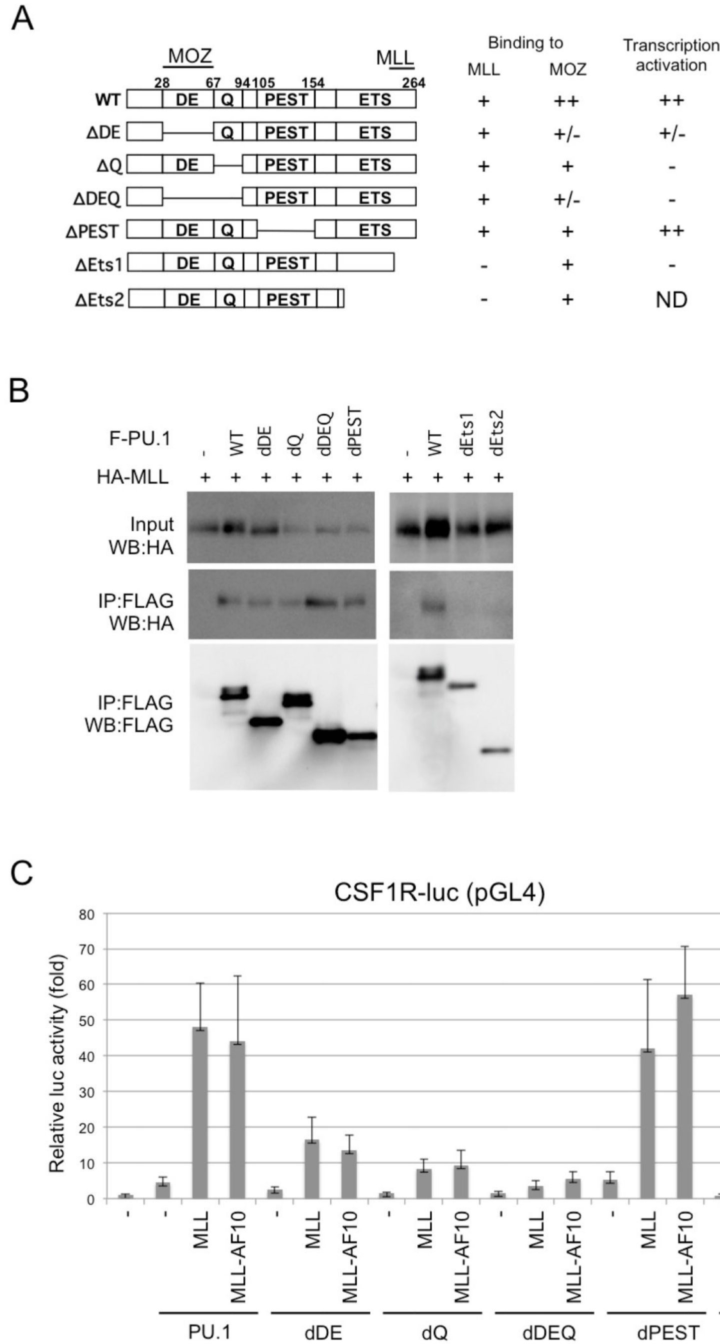

Figure 4. Functional domains of PU.1

(**A**) Deletion mutants of PU.1. The MOZ- and MLL-interacting domains and the results for interaction with PU.1 and PU.1-mediated transactivation of *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 wild-type 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 ($n = 3$).

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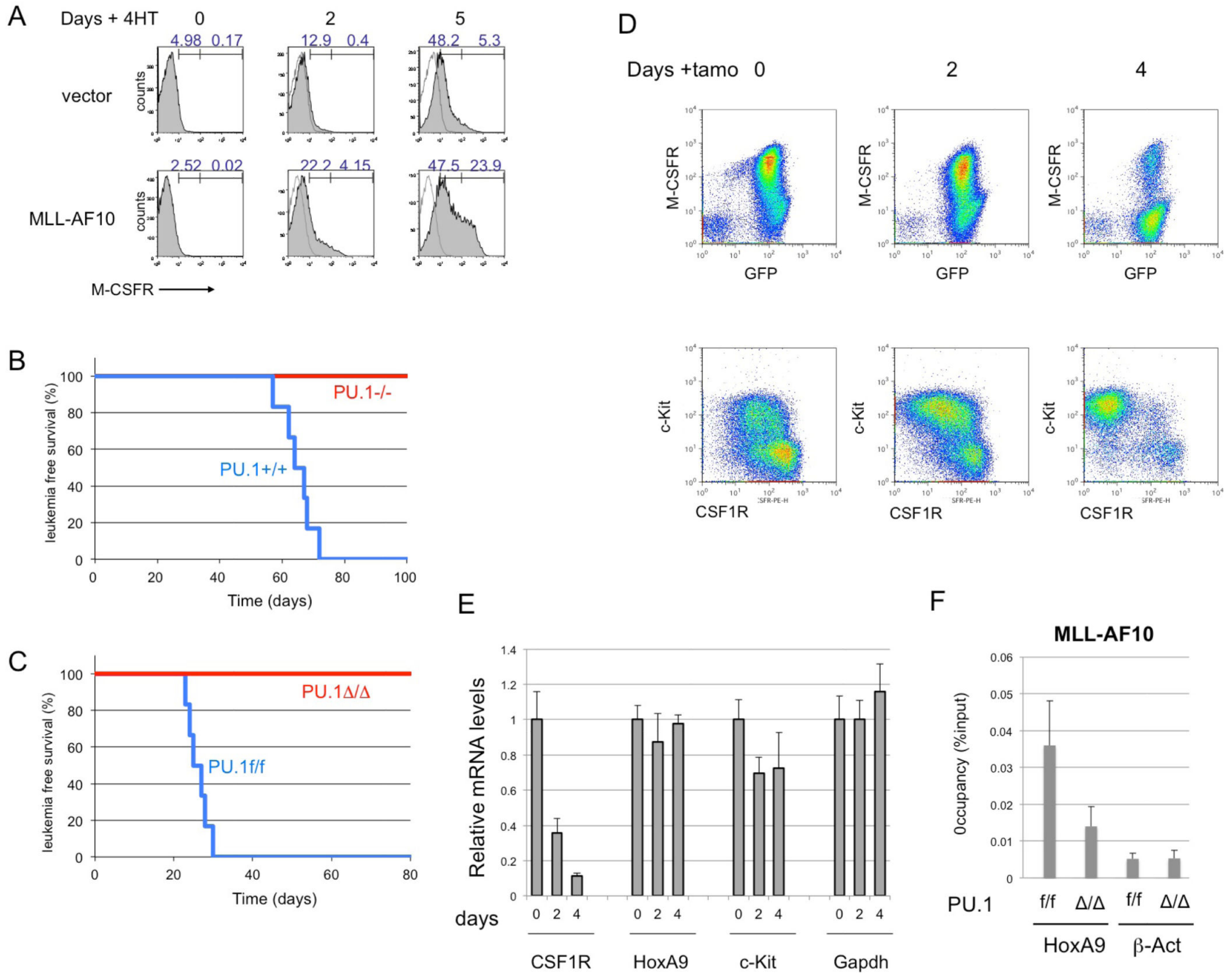

Figure 5. PU.1 is critical for MLL-AF10-induced AML

(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 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) The fetal liver cells of E14.5 PU.1$^{flox/flox}$ with ER-Cre were infected with MLL-AF10, and transplanted into irradiated mice. The BM cells of the primary AML mice were transplanted into sub-lethally irradiated wild-type mice. Tamoxifene (PU.1 / or solvent (PU.1f/f) was administered to the secondary AML mice every 2 d by intravenous injection 17 d 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) The BM cells were prepared 0, 2, or 4 days after injection of Tamoxifen and analysed for expression of CSF-1R and c-Kit proteins (D) and for *Csf1r*, HoxA9, c-Kit, Meis1 and Gapdh mRNAs (E). (F) The BM cells, untreated (f/f) or Tamoxifen-treated for 4 days (/), were subjected to

ChIP analysis using anti-Flag (MLL-AF10) antibodies. Semiquantitative real-time PCR was performed on the co-precipitated DNAs.

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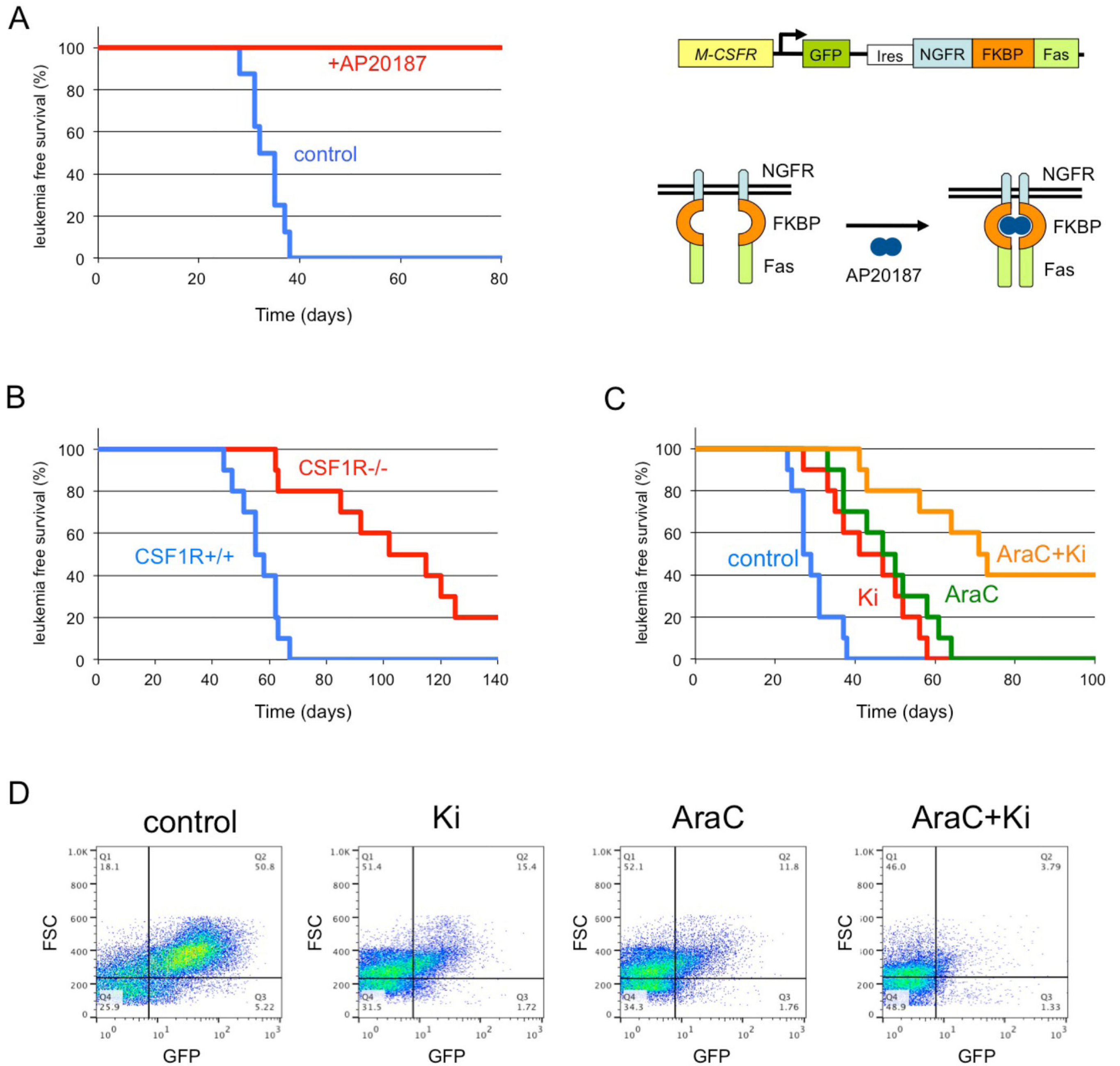

Figure 6. Cure of MLL-AF10-induced AML by ablation of CSF-1R^{high} cells

(A) The 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. Bone marrow cells (1×10^4) of primary AML mice were transplanted into sub-lethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary AML mice was started by IV injection three 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

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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. Vehicle, Ki20227, Ara-C, or Ki20227 plus Ara-C were administrated as described in EXPERIMENTAL PROCEDURES Leukemia-free survivals of the mice were analyzed (C). $n = 10$, $P < 0.01$ (control vs +Ki20227, AraC vs AraC+Ki) Peripheral blood cells were prepared 21 days after transplantation and analysed for expression of GFP (D).

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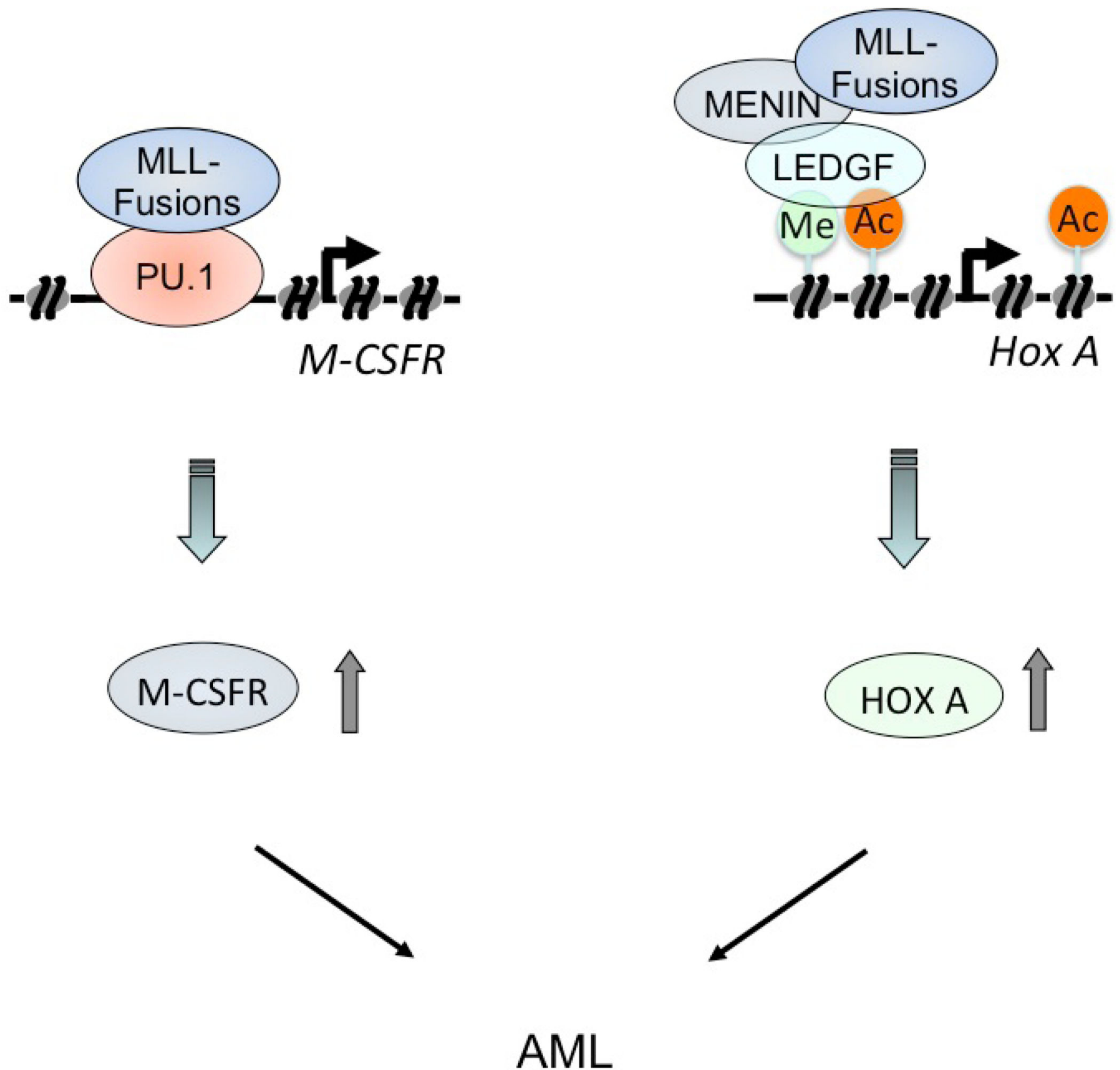

Figure 7.
Model for transcriptional regulation of *Csf1r* and *Hox* genes by MLL fusion proteins. MLL fusions induce the rapid onset of AML by activating two classes of pathways, CSF-1R pathway and Hox pathway.. MLL-fusions stimulated constitutive CSF-1R expression by binding to PU.1 to induce leukemia (left panel). MLL fusion proteins form a complex with menin and LEDGF to regulate the expression of Hox genes (right panel).