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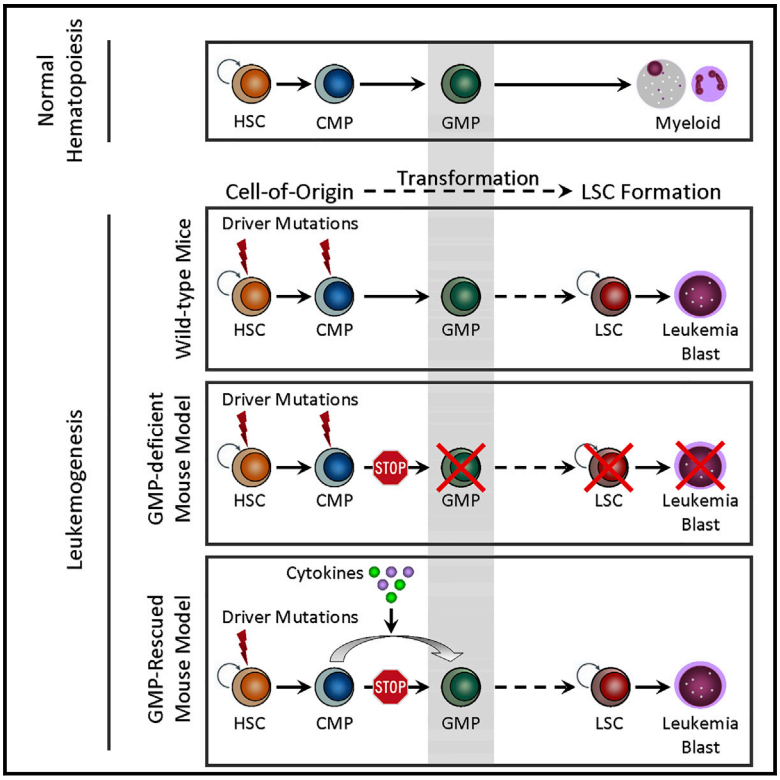
Cell Stem Cell

Hematopoietic Differentiation Is Required for Initiation of Acute Myeloid Leukemia

Graphical Abstract



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In Brief

Ye et al. show that myeloid differentiation is required for acquiring a leukemia stem cell (LSC) phenotype and AML initiation and that blocking GMP formation abrogates leukemic transformation. Cytokine-induced bypass of this block restores LSC and AML development, with GMPs providing a genomic environment permissive for activating LSC transcriptional programs.

Highlights

- Myeloid differentiation to GMPs is required for LSC formation and AML initiation
- Bypassing disrupted GMP differentiation restores AML LSC generation
- Normal GMPs and L-GMPs share a minimal transcriptional program
- GMPs provide a genomic environment permissive for L-GMP formation

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Article

Hematopoietic Differentiation Is Required for Initiation of Acute Myeloid Leukemia

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SUMMARY

Mutations in acute myeloid leukemia (AML)-associated oncogenes often arise in hematopoietic stem cells (HSCs) and promote acquisition of leukemia stem cell (LSC) phenotypes. However, as LSCs often share features of lineage-restricted progenitors, the relative contribution of differentiation status to LSC transformation is unclear. Using murine MLL-AF9 and MOZ-TIF2 AML models, we show that myeloid differentiation to granulocyte macrophage progenitors (GMPs) is critical for LSC generation. Disrupting GMP formation by deleting the lineage-restricted transcription factor C/EBPa blocked normal granulocyte formation and prevented initiation of AML. However, restoring myeloid differentiation in C/EBPa mutants with inflammatory cytokines reestablished AML transformation capacity. Genomic analyses of GMPs, including gene expression and H3K79me2 profiling in conjunction with ATAC-seq, revealed a permissive genomic environment for activation of a minimal transcription program shared by GMPs and LSCs. Together, these findings show that myeloid differentiation is a prerequisite for LSC formation and AML development, providing insights for therapeutic development.

INTRODUCTION

Leukemia stem cells (LSCs) are thought to be responsible for leukemia initiation, maintenance, and recurrence in acute myeloid leukemia (AML). Consequently, understanding the step-wise formation of LSCs might help overcome AML's resistance to current chemotherapy and disease relapse. Initial studies suggested that LSCs are restricted to a small sub-fraction of human AML cells phenotypically resembling normal hematopoietic stem cells (HSCs) (Bhatia et al., 1997). However, further characterization of LSCs using improved xenotransplantation models revealed the presence of functional LSCs sharing the surface phenotype of committed progenitors (McKenzie et al., 2005; Taussig et al., 2008). Recent studies of a large cohort of AML patients demonstrated enriched LSC activity within subsets phenotypically resembling normal lymphoid-primed multipotential progenitors (LMPPs) and granulocyte macrophage progenitors (GMPs) (Goardon et al., 2011). Leukemic LMPPs gave rise to leukemic GMPs (L-GMPs), but not vice versa, mirroring the hierarchy of normal hematopoiesis. Global gene expression profiles revealed that leukemic LMPPs and L-GMPs resembled their respective normal counterparts at the molecular level (Goardon et al., 2011). The similarities between LSCs and their normal counterparts both phenotypically and molecularly suggested that transformation to LSCs was completed at the progenitor stage. Therefore, LSCs may directly arise from progenitors that acquire aberrant self-renewal capacity. Alternatively, LSCs may originate from HSCs, yet full transformation occurs only upon progression to a more committed stage of differentiation.

A number of studies of human AML have suggested that HSCs are the likely cell of origin, but functional LSCs reside in more differentiated populations (Fialkow et al., 1989; Jan et al., 2012; Miyamoto et al., 1996; Shlush et al., 2014). Studies of murine leukemia models using retroviral expression of leukemia-associated fusion oncogenes MF9 and MOZ-TIF2 or a knockin mouse model carrying patient-derived CEBPA biallelic mutations demonstrated that both HSC and committed myeloid progenitor cells can be transformed and potentially serve as the cell-of-origin of LSCs (Huntly et al., 2004; Krivtsov et al., 2006; Bereshchenko et al., 2009). Regardless of cell-of-origin, LSCs from MF9 or MOZ-TIF2 mouse models phenotypically and molecularly resemble committed myeloid progenitor cells (Bereshchenko et al., 2009; Kirstetter et al., 2008; Kvinlaug et al., 2011; Somervaille and Cleary, 2006), consistent with features of LSCs in human AML patients. Therefore, the question remains to what extent differentiation impacts the complete transformation of LSCs from their cell-of-origin.

We and others have shown that C/EBPa plays a non-redundant role in the transition from common myeloid progenitors (CMPs) to GMPs (Zhang et al., 2004). Deletion of C/EBPa leads to a complete loss of GMPs and downstream progeny. Mice transplanted with C/EBPa knockout (KO) cells do not develop

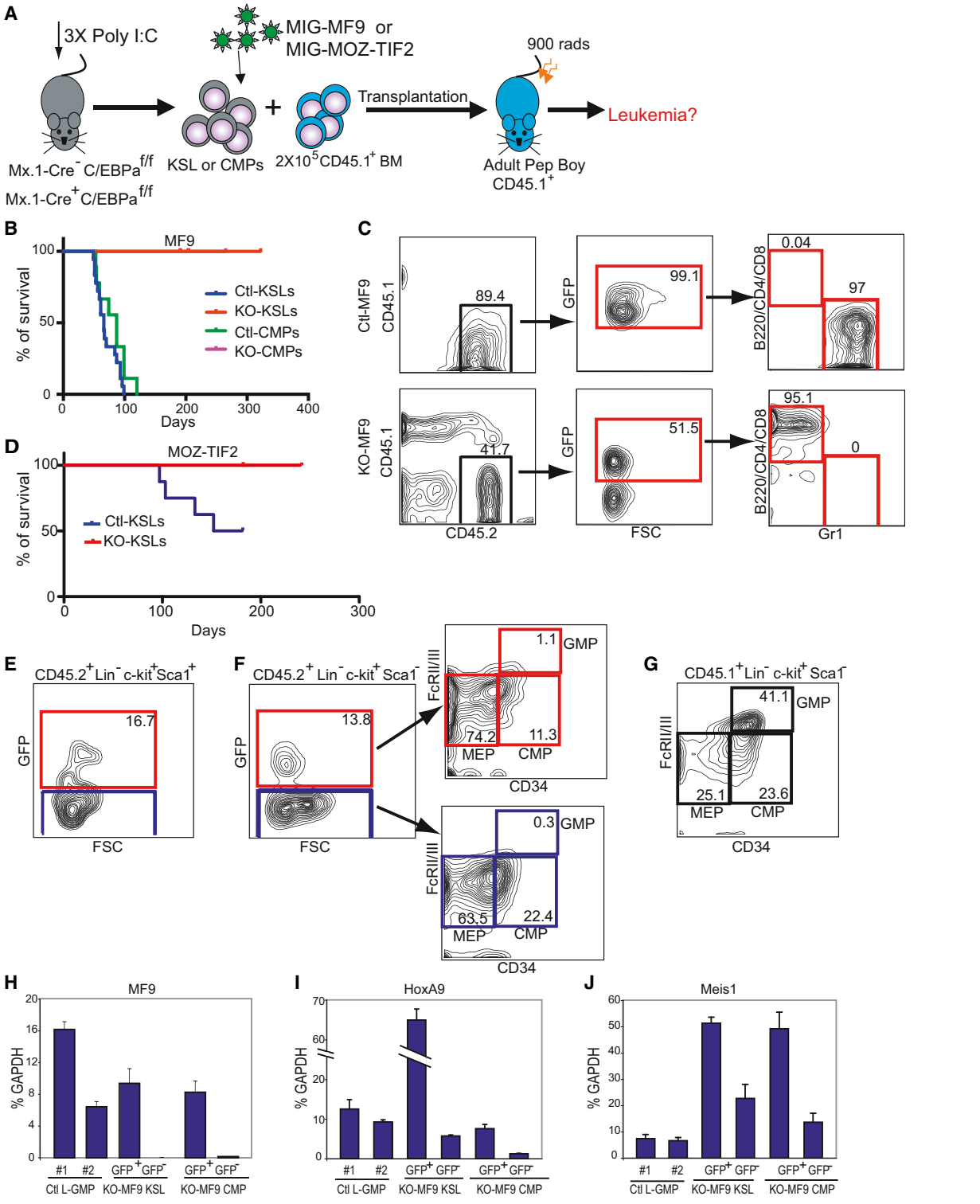

Figure 1. Loss of C/EBPa Abrogates AML Induced by MLL-AF9 or MOZ-TIF2-Transduced Hematopoietic Stem or Myeloid Progenitor Cells

(A) Outline of experiment strategy. Five days after poly(inosinic acid) poly(cytidylic acid) (Poly I:C) injections, $Lin^-Sca\text{-}1^+c\text{-}kit^+$ cells (KSLs) or their downstream common myeloid progenitors (CMPs) were isolated from control (Ctl) or C/EBPa conditional knockout (cKO) mouse bone marrow, transduced with either MIG-MF9 or MIG-MOZ-TIF2 retroviruses, and transplanted into lethally irradiated $CD45.1^+$ B6.SJL-Ptprca Pep3b/BoyJ (Pep Boy) congenic recipients along with 2×10^5 Pep Boy bone marrow cells for radioprotection.

(B) Kaplan-Meier survival analysis of mice receiving MF9-transduced Ctl or KO KSLs (Ctl: n = 18, blue; KO: n = 17, red) ($p < 0.01$) or CMPs (Ctl: n = 9, green; KO: n = 12, purple) ($p < 0.01$).

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AML, despite pre-leukemic features including HSC expansion, competitive repopulation advantage, and myeloid differentiation arrest (Bereshchenko et al., 2009; Ye et al., 2013; Zhang et al., 2004), consistent with failure to identify *CEBPA* null mutations in human AML patients so far, despite *CEBPA* mutations being present in 10% of AML patients (Nerlov, 2004). Therefore, we hypothesized that absence of leukemia in C/EBPa KO cells is due to lack of the critical myeloid target population. Here, we tested whether myelomonocytic commitment was required for LSC formation. Using a C/EBPa conditional KO (cKO) mouse model and MF9 and MOZ-TIF2 murine AML models, we demonstrate that regardless of cell-of-origin, myelomonocytic differentiation along the hierarchy of normal hematopoiesis is critical for LSC formation and leukemia development.

RESULTS

Loss of C/EBPa Abrogates MF9-Induced or MOZ-TIF2-Induced AML

To study the contribution of myelomonocytic differentiation to AML development, we used a well-characterized murine AML model induced by the MLL-AF9 fusion gene. $Lin^-Sca\text{-}1^+c\text{-}kit^+$ cells (KSLs) or CMPs isolated from poly(inosinic acid) poly(cytidylic acid) (Poly I:C)-treated Mx.1-Cre^- $C/EBPa^{f/f}$ control (Ctl) and Mx.1-Cre^+ $C/EBPa^{f/f}$ C/EBPa cKO mice were transduced with MSCV-MF9-IRES-GFP (MIG-MF9) retrovirus and transplanted into lethally irradiated congenic recipients along with a radioprotective dose of congenic bone marrow cells (Figure 1A). Development of AML was monitored by white blood cell (WBC) counts and flow cytometry of peripheral blood. All animals transplanted with MF9-transduced control (Ctl-MF9) KSLs (n = 18) or CMPs (n = 9) developed AML within 4 months. In contrast, none of the animals transplanted with MF9-transduced KO (KO-MF9) KSLs (n = 17) or CMPs (n = 12) developed AML up to 11 months on (Figures 1B, S1A, and S1B), consistent with a previous report using a different MLL-fusion gene, MLL-ENL (Ohlsson et al., 2014). Failure to induce AML in KO cells by MF9 was not due to homing or engraftment defects (Figure 1C). The lack of leukemia is not only limited to MLL-fusion AML, as MOZ-TIF2, which transforms cells through mechanisms different from MF9 (Aikawa et al., 2010; Kindle et al., 2005; Tam et al., 2013), gave a similar result, with no overt AML after 240 days post-transplantation (n = 12). Mice receiving MOZ-TIF2-transduced control cells developed AML with latencies comparable to those previously described (Huntly et al., 2004) (Figures 1D and S1C). These observations suggest that C/EBPa and/or C/EBPa-dependent myelomonocytic differentiation is required for AML development.

The L-GMP Population Is Absent in MLL-AF9 C/EBPa KO Recipients Despite High Expression of Meis1 and HoxA9

Next we examined the effects of MF9 on hematopoiesis in the absence of C/EBPa. GFP^+ KO KSLs persisted 11 months after transplantation (Figure 1E), generating B cells, CMPs, and megakaryocyte-erythroid progenitors (MEPs) (Figure 1F and data not shown). However, the GMP-like L-GMP compartment ($CD45.2^+Lin^-c\text{-}kit^+Sca\text{-}1^-CD34^+FcrRII/III^+$) was undetectable in KO-MF9 bone marrow (Figure 1F), like the failure of GMP formation in C/EBPa KO. In contrast, GMPs formed normally in the radioprotective $CD45.1^+$ WT bone marrow cells in the same chimeric recipient (Figure 1G), ruling out external effects on myeloid commitment. qRT-PCR analysis confirmed the expression of the MF9 in GFP^+ KO-derived KSLs and CMPs at levels comparable to those in Ctl-MF9 L-GMPs (Figure 1H). Hoxa9 and Meis1 are two key downstream targets of MLL-fusion oncogenes (Zeisig et al., 2004). Compared to untransduced GFP^- cells, *Hoxa9* and *Meis1* RNA in KO-MF9-derived KSLs or CMPs were both upregulated to levels that are either comparable to or higher than those in L-GMPs from Ctl-MF9 leukemic mice (Figures 1I and 1J). Therefore, key downstream targets of MF9 remain highly expressed in KO cells despite the absence of leukemia, in contrast to a report describing decreased expression in the absence of C/EBPa (Ohlsson et al., 2014). Our data are consistent with the report that overexpression of HoxA9 and Meis1 failed to rescue MLL-fusion leukemia in C/EBPa KO cells (Ohlsson et al., 2014). The absence of L-GMPs in MF9-transduced KO cells suggests that loss of C/EBPa itself, its associated GMP formation, or both abrogates leukemogenesis, affecting either LSC formation or maintenance.

C/EBPa Is Dispensable for the Maintenance of MF9-Induced AML

KSLs and CMPs from control and C/EBPa cKO were retrovirally transduced with MF9 before Poly I:C treatment and transplanted into lethally irradiated recipients (Figure 2A). Recipients with 5%–30% GFP^+ cells in blood were subjected to Poly I:C treatment to induce C/EBPa excision. No difference in survival was detected between the two groups (Figure 2B). Next we measured the frequency of C/EBPa KO LSCs by performing limiting dilution transplantation assays (Staber et al., 2014). The frequency of LSCs in

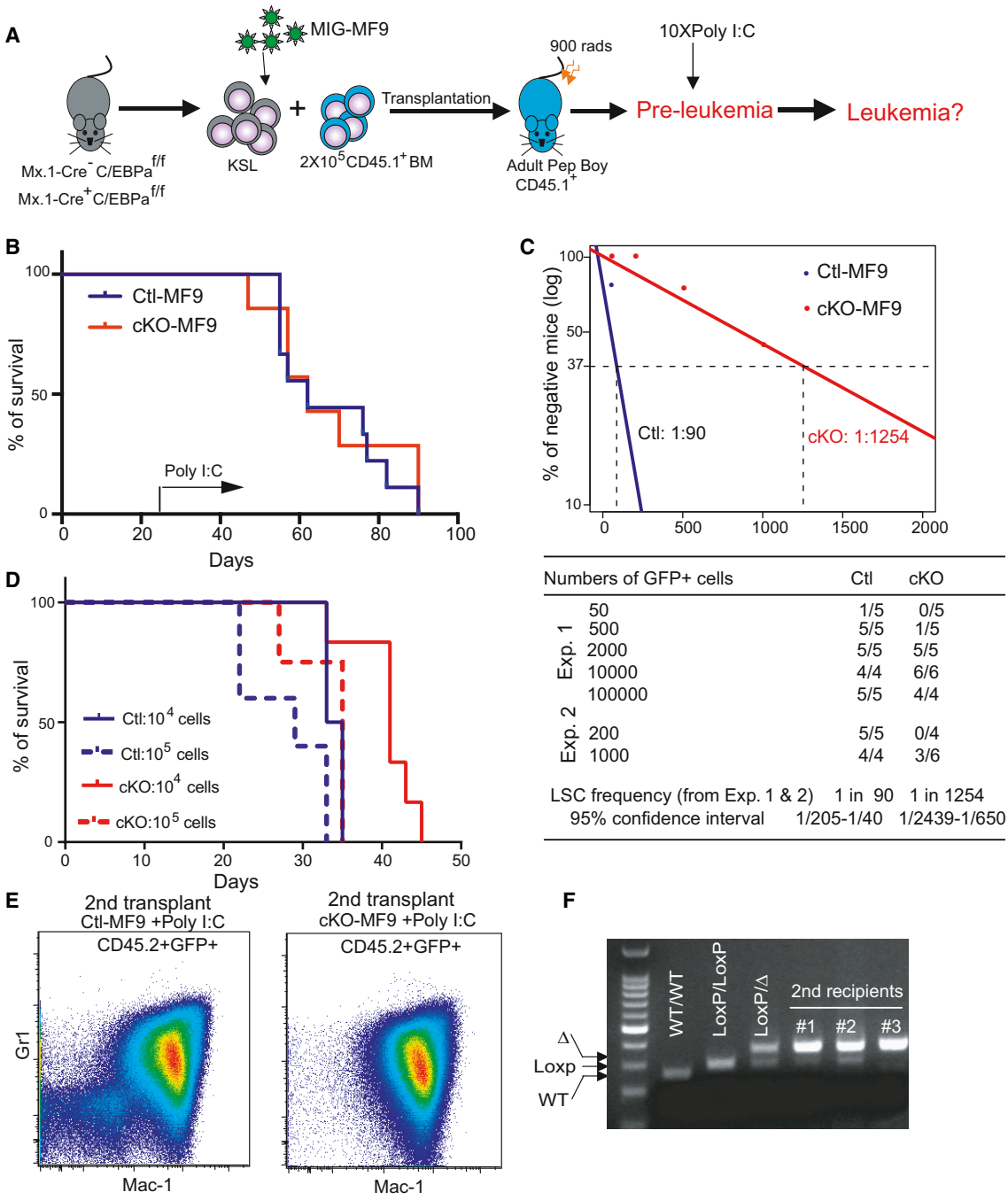
(C) Representative flow cytometry analysis of peripheral blood from mice transplanted with either MF9-transduced Ctl (Ctl-MF9) (upper) or KO (KO-MF9) (lower) KSLs 7 weeks after transplantation. Cells were analyzed for expression of GFP, CD45.2 (donor-derived), myeloid (Gr1), and lymphoid (B220, CD4, or CD8) markers and they demonstrated that GFP^+ cells in KO-MF9 recipients were predominantly lymphocytes, in contrast to predominantly myeloid cells in Ctl-MF9 recipients.

(D) Kaplan-Meier survival analysis of mice receiving either MOZ-TIF2-transduced Ctl (n = 8, blue) or KO (n = 12, red) KSLs ($p < 0.01$).

(E and F) Flow cytometry analysis of bone marrow from recipients transplanted with KO-MF9 KSLs 11 months post-transplantation. Representative plots of KO-MF9 recipient (Mouse #5) displaying the presence of MF9-transduced KO-donor-derived ($CD45.2^+GFP^+$) KSLs (E), CMPs, and megakaryocyte–erythroid progenitors (MEPs) (F) are shown, but note the absence of granulocyte macrophage progenitor (GMP)-like cells in both GFP^+ MF9-transduced (gated in red) and GFP^- untransduced (gated in blue) KO-derived cells (F).

(G) Flow cytometry plot showing the presence of CMPs, MEPs, and GMPs derived from $CD45.1^+$ congenic cells in bone marrow of a KO-MF9 recipient (Mouse #5).

(H–J) qRT-PCR showing levels of transcripts of the *MF9* fusion gene, *HoxA9*, and *Meis1* in MF9-transduced GFP^+ KSLs and CMPs isolated from KO-MF9 recipients (Mouse #3 and #4) 6 months post-transplantation as compared to levels in their untransduced GFP^- fractions in corresponding populations and levels in L-GMPs derived from Ctl-MF9 leukemic mice.



Numbers of GFP+ cells		Ctl	cKO
Exp. 1	50	1/5	0/5
	500	5/5	1/5
	2000	5/5	5/5
	10000	4/4	6/6
	100000	5/5	4/4
Exp. 2	200	5/5	0/4
	1000	4/4	3/6
LSC frequency (from Exp. 1 & 2)		1 in 90	1 in 1254
95% confidence interval		1/205-1/40	1/2439-1/650

Figure 2. Deletion of C/EBPa in Already Initiated MF9-Induced AML Does Not Eliminate Leukemia Development

(A) Schematic outline of experiment strategy. KSLs were isolated from Ctl or Mx.1-Cre^{+}C/EBPa$^{f/f}$ cKO mice prior to Poly I:C injections, transduced with MIG-MF9, and transplanted into lethally irradiated Pep Boy mice along with 2 × 10^{5} Pep Boy bone marrow cells. Poly I:C treatment was initiated 4 weeks post-transplantation in recipients carrying 5%–30% of GFP^{+} cells in peripheral blood. Leukemia development was monitored by survival and evaluated by the percentage of GFP^{+} cells in blood, spleen, and bone marrow.

(B) Kaplan-Meier survival analysis of recipients that received 10 Poly I:C injections following transplant receival of either Ctl-MF9 or cKO-MF9 KSLs (Ctl, n = 9, blue; cKO, n = 7, red). The Poly I:C treatment period is indicated by an arrow ($p > 0.05$).

(C) Limiting dilution assay measuring the frequency of leukemia stem cells (LSCs) after C/EBPa deletion in already initiated MF9-induced leukemia. Upper: logarithmic plot showing the percentage of negative recipients transplanted with different cell doses of GFP^{+} bone marrow cells isolated from either Ctl-MF9 or cKO-MF9 leukemic mice. Recipients surviving 4 months post-transplantation with no detectable GFP^{+} cells in blood, spleen, and bone marrow were considered non-responders. Lower: table showing the number of recipients that developed leukemia and the total number of recipients transplanted per cell dose. Frequencies of LSCs were calculated according to Poisson statistics using L-Calc software based on data from two independent experiments (Chi-square test; $p < 0.01$).

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Ctl-MF9 leukemic bone marrow (1:90) was reduced 14-fold in cKO-MF9 bone marrow (1:1,254) (Figure 2C). However, all recipients transplanted with 10^4 or more bone marrow cells from C/EBPa cKO mice succumbed to AML (Figures 2D and 2E). DNA genotyping confirmed C/EBPa deletion in those leukemic cells (Figure 2F). Thus, deletion of C/EBPa does not abolish AML development after leukemia has been initiated. We therefore conclude that the absence of leukemia in C/EBPa KO cells is due to the abrogation of formation of LSCs.

Transient Cytokine Stimulation Rescues Myeloid Differentiation in C/EBPa KO Cells

To elucidate whether impaired myelomonocytic differentiation rather than C/EBPa KO is responsible for failure of leukemia in KO-MF9 mice, we tested whether rescuing GMPs in C/EBPa KO cells would restore MF9-induced leukemia. C/EBPa is absolutely required for GMPs in steady state, but can be replaced by inflammatory cytokines during emergency granulopoiesis in infection; combined treatment with IL-3 and GM-CSF induced granulocyte differentiation in C/EBPa KO fetal liver cells in culture and in vivo (Hirai et al., 2006). 48 hr after we administered GM-CSF and IL-3 expression vectors by hydrodynamic injection, GMP-like cells (Figure S2A) and myeloid cells (Mac-1^+Gr1^+) (Figure S2B) were detected in the bone marrow of KO mice, but not in KO mice treated with empty vector. DNA genotyping confirmed excision of C/EBPa in rescued GMP-like and Mac-1^+Gr1^+ cells (data not shown).

We confirmed cytokine-induced rescue of GMP formation and myeloid differentiation in the C/EBPa KO transplantation setting (Figure S2C). Cytokine-induced myelopoiesis lasted 2–3 weeks, in accordance with transient elevation of GM-CSF and IL-3 levels (Hirai et al., 2006). Therefore, co-stimulation of GM-CSF and IL-3 at least partially rescued GMP formation and myelopoiesis in both primary and transplanted KO mice.

Rescue of Myeloid Differentiation Restores LSC Formation and AML Development in C/EBPa KO Recipients

MF9-transduced KO KSLs were transplanted into lethally irradiated congenic mice along with radioprotective CD45.1^+ bone marrow cells. After engraftment and reconstitution were confirmed 2–4 months after transplantation, mice were subjected to either GM-CSF + IL-3 vectors or empty vector injections (Figure 3A). Transient expansion of myeloid cells diminished 4 weeks after injection, an internal readout for effective cytokine stimulation (Figure S3A). At this time point, an L-GMP-like population appeared in cytokine-treated KO MF9 recipients, while no L-GMP-like cells were detected in MF9 untransduced KO cells in the same recipient or in KO-derived bone marrow with empty vector (Figures 3B and S3B). Cytokine-treated KO-MF9 recipients developed myeloblastic leukemia (cytokine rescued KO-MF9 AML) with extensive organ infiltration within an average of 60 days; no control animals succumbed to leukemia (Figures 3C and 3D). Genotyping of leukemic cells (CD45.2^+GFP$^+$Mac-1^+Gr1^+) from cytokine-rescued KO-MF9 leukemic mice confirmed derivation from KO donors (Figure 3E). Histological analysis demonstrated that leukemic cells from cytokine-rescued KO-MF9 AML were similar to those from Ctl-MF9 AML mice (Figure 3F). Phenotypic L-GMPs were fully formed in cytokine-rescued KO-MF9 AML (Figure 3G) (Krivtsov et al., 2006). Interestingly, bone marrow cells from cytokine-rescued KO-MF9 AML exhibited higher serial replating capacity than those from Ctl-MF9 AML (Figures S4A and S4B), perhaps due to the inherently increased self-renewal potential caused by C/EBPa deficiency (Ye et al., 2013; Zhang et al., 2013). Unlike Ctl-MF9 leukemic cells that grow well in liquid culture with SCF, TPO, IL-3, IL-6, and GM-CSF, cells from KO-MF9 AML bone marrow or spleen could not be maintained under these conditions, indicating that different conditions are required for MF9-KO AML cells to grow in culture, potentially explaining previous failed attempts to establish C/EBPa-independent MLL-fusion leukemia models using culture systems (Collins et al., 2014; Ohlsson et al., 2014). Cytokine-rescued KO-MF9 leukemic cells maintain long-term self-renewal in secondary transplantations, as all mice receiving cytokine-rescued KO-MF9 leukemia cells developed myeloid leukemia with latencies of about 40 days (Figures 4A and 4B) and harbored L-GMPs (Figure 4C). qRT-PCR confirmed complete loss of C/EBPa in those L-GMPs (Figure 4D). Reconstitution of leukemia and the presence of L-GMP in secondary recipients further indicate that C/EBPa is dispensable for leukemia maintenance in MF9-induced AML. Together, these data strongly suggest that myeloid differentiation, rather than the presence of C/EBPa, is required for MF9-induced LSC formation and subsequent AML development.

Cytokine-Rescued L-GMPs from C/EBPa KO Recipients Share Molecular Features with WT L-GMPs

Microarray analyses were performed on (1) L-GMPs from both primary and secondary Ctl-MF9 AML mice (Ctl L-GMP and 2^{nd} Ctl L-GMP); (2) cytokine-rescued KO-MF9 AML mice (KO L-GMP and 2^{nd} KO L-GMP); (3) GFP$^+$ untransformed KO KSLs; (4) GFP$^+$ CMPs from mice receiving KO-MF9 cells followed by empty vector injection (KO-MF9 KSL or CMP); and (5) CMPs and GMPs from WT mice (WT CMPs, WT GMPs) and CMPs from KO mice (KO CMPs). Unsupervised global clustering analysis demonstrated that KO L-GMPs clustered with Ctl L-GMPs, but did so separately from KO-MF9 KSLs or CMPs and WT or KO myeloid progenitors (Figure 5A). Consistently, gene set enrichment analysis (GSEA) analysis revealed the well-characterized MF9 self-renewal signature (Krivtsov et al., 2006) highly enriched in both Ctl L-GMPs and KO L-GMPs, but not in untransformed KO-MF9 CMPs (Figures 5B, 4C, and 4D). Thus our data support the notion that induction of myeloid differentiation beyond the

(D) Survival curves for mice transplanted with 10^4 or 10^5 GPF$^+$ bone marrow cells from primary mice receiving Poly I:C injections (Log-rank test: 10^4 groups: $p < 0.05$; for 10^5 groups: $p > 0.05$).

(E) Representative flow cytometry analysis of bone marrow from moribund secondary (2^{nd}) recipients showing similar surface expression of Mac-1 and Gr1 on GFP$^+$ cells.

(F) Genotyping of sorted GFP$^+$Gr1^+Mac-1^+ cells from bone marrow of 2^{nd} recipients showing deletion of C/EBPa alleles. WT (265 bp), LoxP (304 bp), and deleted alleles (Δ) (377 bp) were amplified by the one-PCR-reaction method.

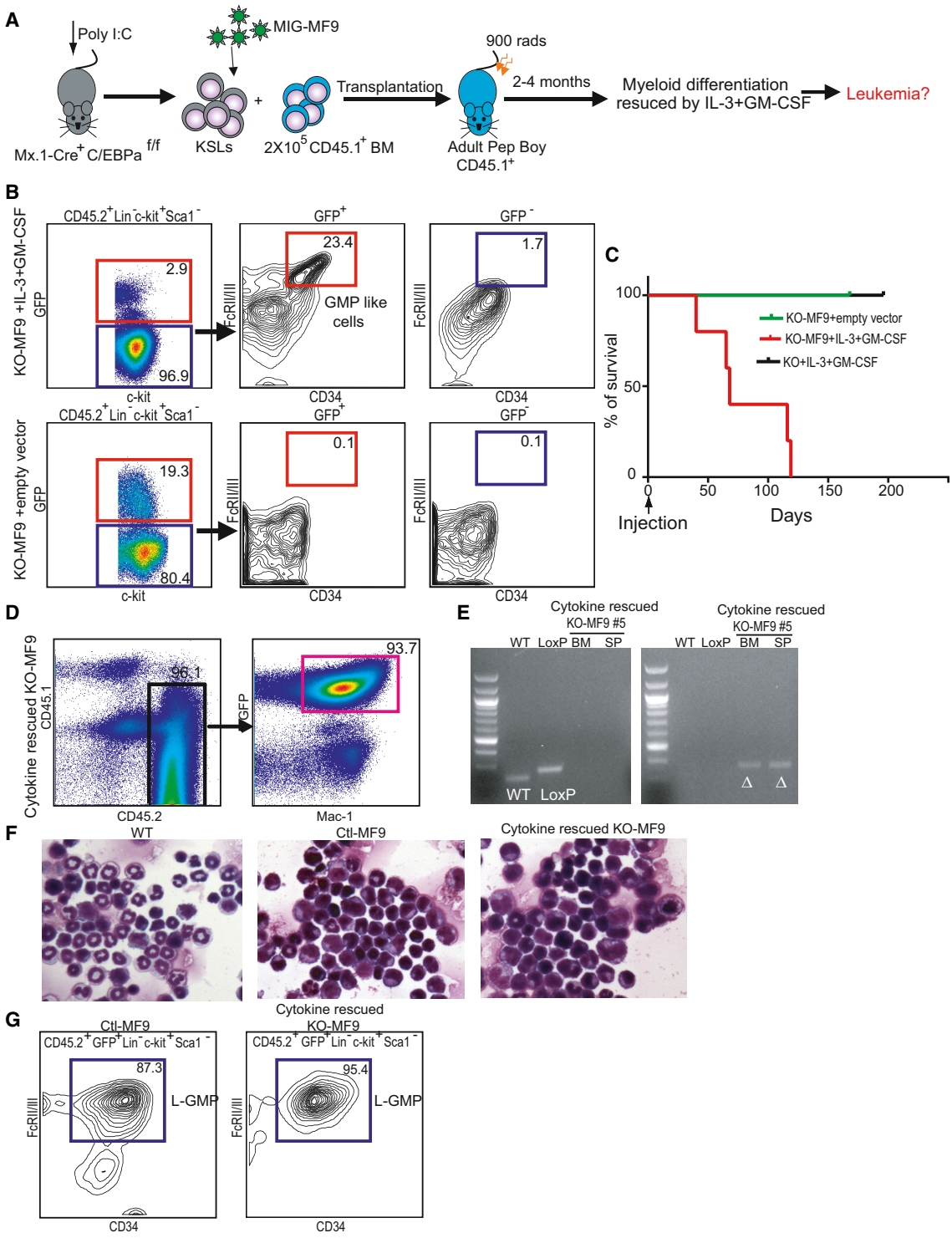

Figure 3. Rescue of Myeloid Differentiation Restores LSC Formation and AML Development

(A) Experimental outline. Mice were transplanted with MF9-transduced KO KSLs as described in Figure 1A. Myeloid differentiation was rescued by hydrodynamics-based injection of 1 μg IL-3 and GM-CSF-expressing vectors 2–4 months after transplantation.

(B) Representative flow cytometry plots of bone marrow (cytokine KO-MF9 Mouse #3) showing L-GMP-like cells ($Lin^-c\text{-}kit^+Sca\text{-}1^-CD34^+FcrRII/III^+$) in GFP^+ MF9-transduced KO-derived cells in recipients 4 weeks after injection with IL-3 and GM-CSF-expressing vector (upper), but not in the fraction of GFP^- MF9 untransduced KO cells or KO-derived cells from KO-MF9 recipients receiving empty vector injections (lower).

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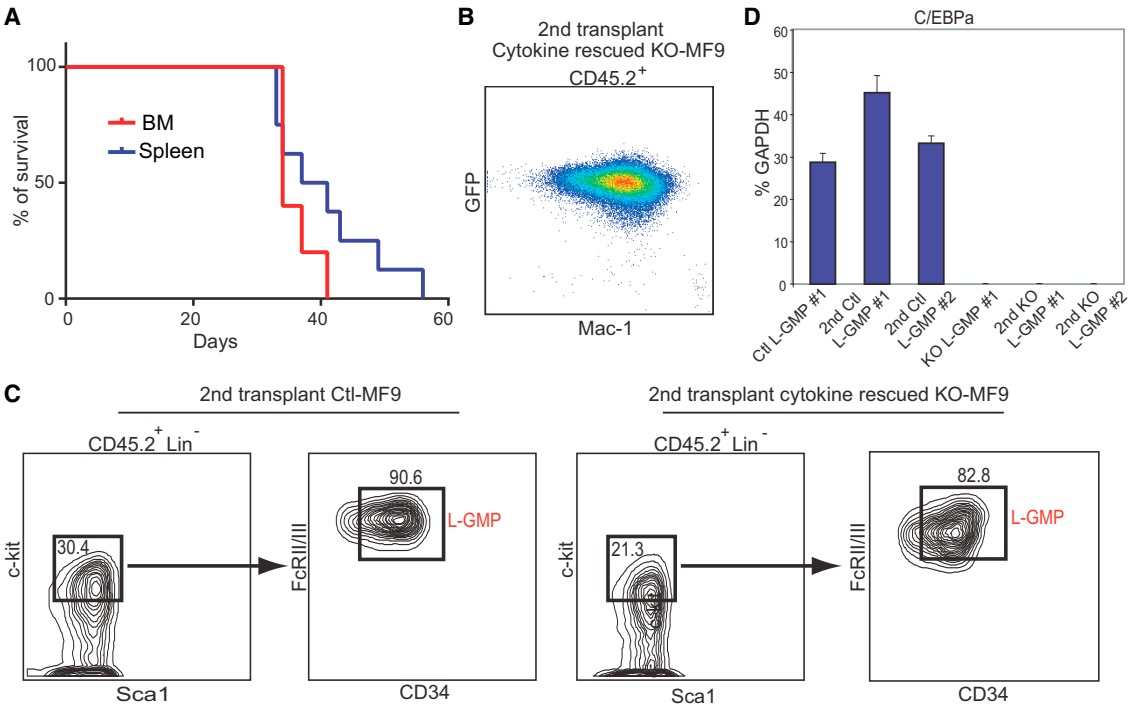

Figure 4. Myeloblastic Leukemia Develops in Secondary Recipients Transplanted with Cytokine-Rescued KO-MF9 Leukemic Bone Marrow

(A) Kaplan-Meier curve of survival for secondary recipients transplanted with 5×10^4 bone marrow (n = 5, red) and spleen (n = 8, blue) cells from cytokine-rescued KO-MF9 primary mice.

(B) Flow cytometry analysis of the spleen of secondary recipients transplanted with cytokine-rescued KO-MF9 leukemic mouse bone marrow showing abundant KO-derived Mac-1$^+$ myeloblasts.

(C) Representative plots of bone marrow from secondary recipients transplanted with either Ctl-MF9 (left) or cytokine-rescued KO-MF9 (right) bone marrow cells showing immunophenotypically indistinguishable leukemic GMP (L-GMP) populations.

(D) qRT-PCR showing levels of *C/EBPa* transcripts in L-GMPs from either Ctl-MF9 (Ctl L-GMP) or cytokine-rescued KO-MF9 (KO L-GMP) primary leukemic mice or their secondary recipients.

GMP stage restores the MF9-induced oncogenic program leading to AML.

We compared gene expression profiles of WT CMPs versus WT GMPs as well as untransformed KO CMPs versus control and KO L-GMPs and identified 902 (444 down- and 458 upregulated) differentially expressed genes (DEGs) in the transition from CMPs to GMPs (>1.5-fold-change, $p < 0.05$). Likewise, 1,262 (726 down- and 536 upregulated) DEGs were identified during MF9-induced leukemic transformation. We found that 45% of DEGs (412 genes, including 202 upregulated and 210 downregulated) in the transition from CMPs to GMPs overlapped with DEGs in L-GMP formation (Figures 6A and 6B and Table S1), supporting the hypothesis that the GMP stage is critical for MF9-induced AML. Among the 412 genes, 80% of upregulated and 60% of downregulated genes contained C/EBPa binding sites in promoter or enhancer regions based on C/EBPa ChIP-seq datasets (Ohlsson et al., 2014; Roe et al., 2015), consistent with the critical role of C/EBPa in GMP formation.

Next we determined biological pathways in myeloid differentiation and MF9-induced leukemogenesis by applying Ingenuity Pathway Analysis (IPA). Genes downregulated in the formation of both normal GMPs and L-GMPs were linked to lineage determination, self-renewal, cytokine interaction, and downstream signaling (Figure 6C). We observed significantly decreased expression of *MAML2* and *Gata3* in both GMPs and L-GMPs (Figure 6E), in line with the inhibitory role of the Delta-Notch pathway in myeloid differentiation and MF9-induced leukemogenesis (De Obaldia et al., 2013; Lobry et al., 2013). Analysis of upregulated

(C) Kaplan-Meier survival analysis of KO-MF9 recipients injected with either the combination of GM-CSF and IL-3 vectors (red) or empty vector (green) 2–4 months post-transplantation, or recipients transplanted with untransduced KO KSLs followed by GM-CSF and IL-3 vector injection (black). The day that mice received the hydrodynamics-based injection was set as Day 0 ($p < 0.01$).

(D) Representative flow cytometry analysis of moribund recipients of KO-MF9 2 months after the injection of IL-3 and GM-CSF vectors revealed abundant CD45.2$^+$GFP$^+$Gr1$^+$Mac-1$^+$ leukemic cells in bone marrow (cytokine-rescued KO-MF9 mice).

(E) Genotyping of sorted CD45.2$^+$Gr1$^+$Mac-1$^+$ cells from bone marrow and spleen of KO-MF9 AML mouse showing deletion of C/EBPa alleles. WT (236 bp), LoxP (275 bp), and Δ alleles (377 bp) were amplified by the two-PCR-reaction method.

(F) Wright-Giemsa staining of bone marrow cells derived from cytokine-rescued KO-MF9 mice showing immature myeloid blasts, morphologically indistinguishable from leukemia cells derived from MF9-transduced Ctl cells (Ctl-MF9).

(G) Flow cytometry showing the presence of an L-GMP population in moribund cytokine-rescued KO-MF9 leukemic recipients similar to that in Ctl-MF9 mice.

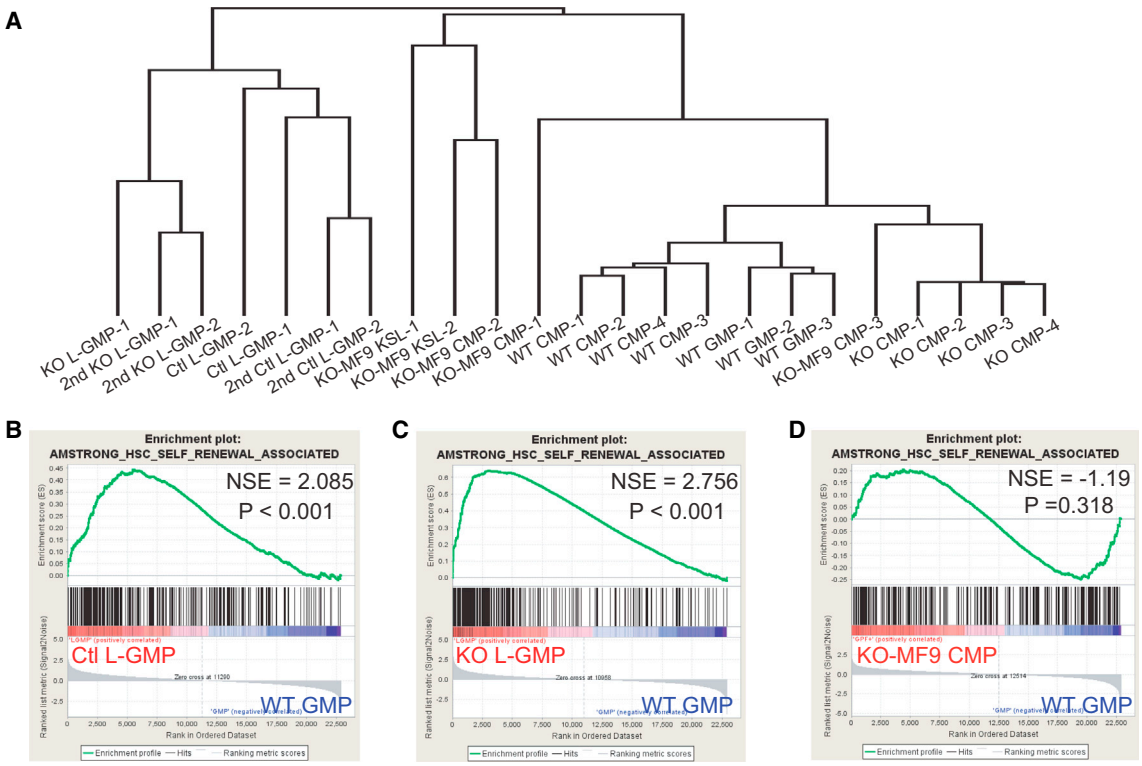

Figure 5. The MF9-Induced Oncogenic Program Is Restored in Cytokine-Rescued KO-MF9 L-GMPs in the Absence of C/EBPa

(A) Unsupervised clustering based on global gene expression of Ctl L-GMPs and KO L-GMPs from primary and secondary leukemic mouse bone marrow; GFP^+ MF9-transduced but untransformed KSLs and CMPs from KO-MF9 recipients injected with empty vector (KO-MF9 KSLs; KO-MF9 CMPs); and WT CMPs, WT GMPs, and KO CMPs.

(B–D) GSEA analysis of L-GMPs from Ctl-MF9 (B) and cytokine-rescued KO L-GMP (C) leukemia mice as well as MF9-transduced but untransformed KO CMPs (KO-MF9 CMP) (D) from KO-MF9 recipients, for enrichment of the MF9-induced leukemia self-renewal signature, as compared to WT GMPs. The normalized enrichment scores (NES) and p values are indicated in each plot.

genes showed association with myeloid cell function (Figure 6D). A significant proportion of genes, such as Idh1, was associated with metabolic pathways (Lu et al., 2012), consistent with the role of C/EBPa in metabolism (Darlington et al., 1995; McKnight et al., 1989; Wang et al., 1995). Altered Idh1 expression and metabolic pathways were reversed in cytokine-rescued KO L-GMPs (Figure 6F), indicating metabolic reprogramming with restoration of myeloid differentiation. The up- and downregulated genes included transcription factors well known for roles in myeloid progenitor differentiation (*Sox4*, *N-Myc*, and *Gfi-1*). Deletion of C/EBPa in normal CMPs led to dysregulation of these genes, which were restored in cytokine-rescued KO L-GMPs (Figures 6E and 6F). As C/EBPb plays a critical role in emergency granulopoiesis, it might rescue myeloid differentiation (Hirai et al., 2006). However, we observed only a slight increase in *C/EBPa* expression in KO-MF9 L-GMPs compared to WT L-GMPs or KO CMPs by both microarray and qRT-PCR (data not shown).

Alterations in H3K79me2 Modifications and DNA Accessibility during Myeloid Differentiation Correlates with Acquisition of the Transcriptional Program Permissive for Transformation of GMPs

To understand the uniqueness of GMPs in activating the 412 genes essential for MLL-fusion transformation (Figure 7A), we first re-analyzed the previous published histone H3 lysine K79 dimethyl (H3K79me2), an epigenetic mark associated with MF9 (Bernt et al., 2011). Approximately 80% of H3K79me2 marks showed overlap between GMPs and L-GMPs. We also observed increased H3K79me2 binding at ~60% of the upregulated genes during myeloid differentiation from KSLs to GMPs (Figure 7B). By contrast, analysis of previously identified MF9 direct downstream genes, a set of 139 genes including HoxA9 and Meis1 (Bernt et al., 2011), showed much weaker H3K79me2 binding in GMPs than in L-GMPs. In addition, there was no increased H3K79me2 modification at these loci during differentiation from KSLs to GMPs (Figure S5), consistent with reported decreased H3K79me2 at the HoxA gene cluster in GMPs compared to KSLs and L-GMPs (Bernt et al., 2011). These data suggest that GMPs are epigenetically ready for the activation of the transcriptional program permissive for MLL-fusion transformation, and high susceptibility of GMP to MF9 is unlikely to be caused by MF9 direct downstream targets.

As a second measure of changes in chromatin structure, we examined chromatin accessibility ±10 kb from the common 412 genes in WT KSLs, CMPs, and GMPs and in leukemic cells derived from WT and KO-MF9 mice using ATAC-seq (Buenrostro et al., 2013; Lara-Astiaso et al., 2014). Three types of clusters were identified, including a GMP-dominant cluster with

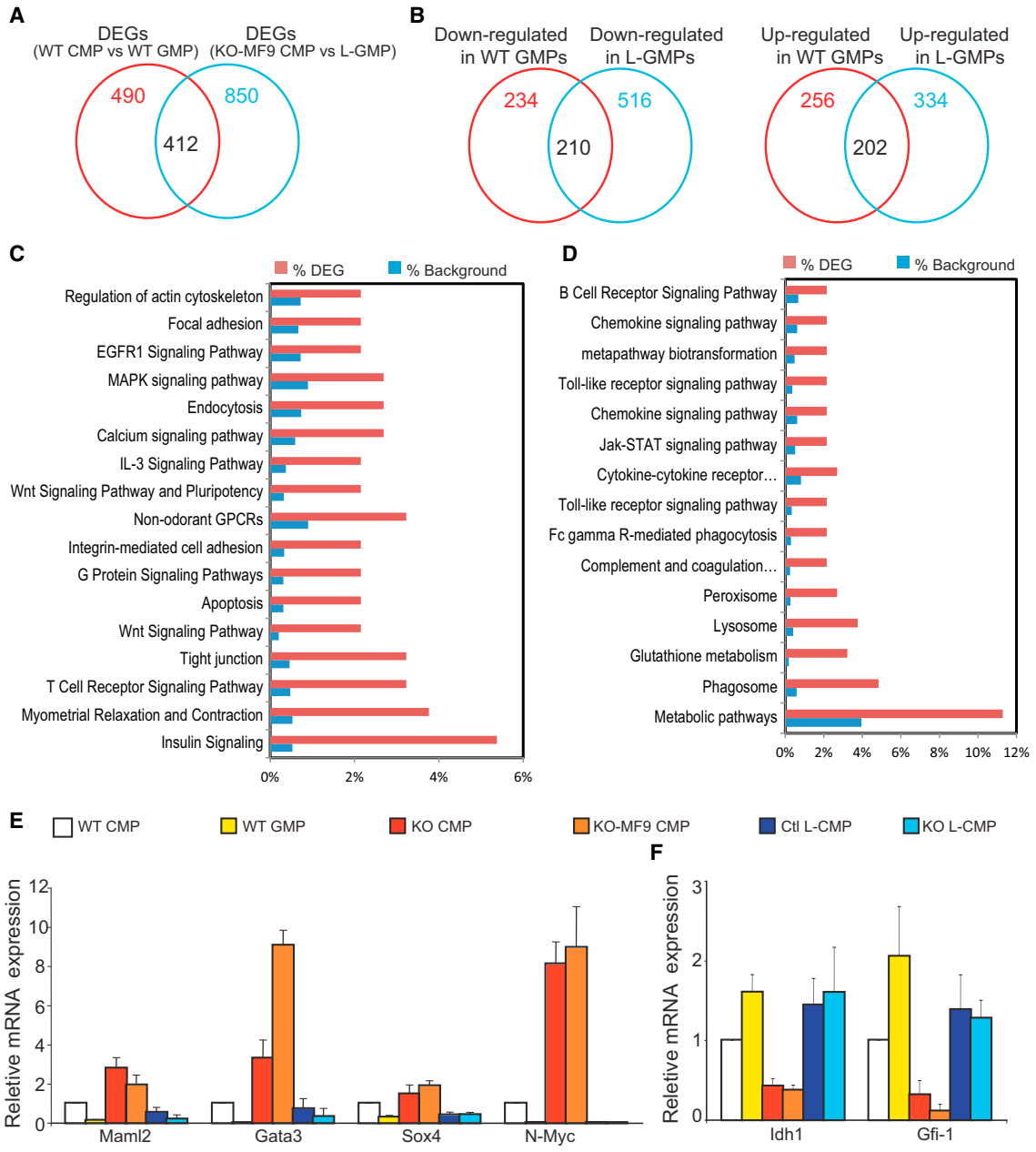

Figure 6. A Transcriptional Program Associated with Myeloid Lineage Commitment, Growth, and Metabolism Is Shared by GMPs and L-GMPs

(A and B) Venn diagram showing both the overall overlap (A) between genes differentially expressed during the transition from CMPs to GMPs and the formation of L-GMPs and the overlap in downregulated (left) and upregulated (right) genes individually (B) (Hypergeometric test; $p < 0.01$).

(C and D) Pathway analysis of genes downregulated (C) or upregulated (D) in the formation of GMPs and L-GMPs indicating enriched gene sets and pathways that are potentially involved in blocking or promoting myeloid differentiation and MF9-induced leukemogenesis. Blue bars represent the percentage of the number of genes in the pathway of interest relative to the total number of genomic genes (genomic background). Red bars represent the number of differentially expressed genes in the pathway of interest compared to all differentially expressed genes.

(E and F) qRT-PCR analysis of representative genes that were either downregulated (E) or upregulated (F) in the transition from CMPs to GMPs. However, these changes were reversed in C/EBPa KO CMPs. In contrast, in cytokine-rescued KO L-GMPs, levels of their expression were restored. The expression of each gene in WT CMPs was set as 1, and the relative fold changes of gene expression in other population were represented. Data represent mean value (±SD) of replicate arrays. (n = 3 different samples in each group.)

increasing accessibility in GMP and leukemic cells; the CMP-dominant cluster with decreasing accessibility from the CMP to GMP transition; and a KSL-dominant cluster with accessible sites enriched in KSLs (Figure 7C). Upregulated genes were relatively enriched in the GMP-dominant cluster, while downregulated genes were more enriched in the CMP-dominant one,

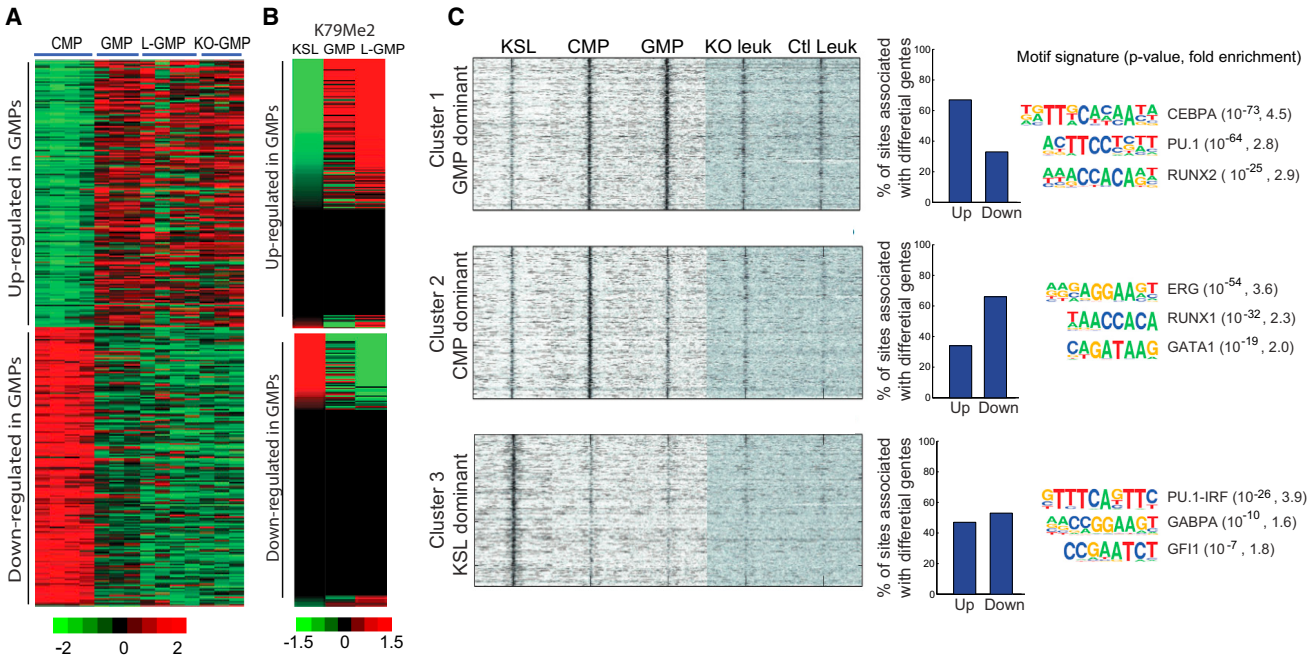

Figure 7. Changes in H3K79me2 Modification and DNA Accessibility during Myeloid Differentiation Correlate with the Acquisition of a Transcriptional Program Permissive for MLL-Fusion Transformation in GMPs

(A) Heatmap showing expression of the transcriptional program of 412 genes shared by GMPs and L-GMPs, including 210 genes (upper) that were down-regulated and 202 genes (lower) that were upregulated during the formation of GMPs and L-GMPs.

(B) Heatmap showing relative dimethylation of histone H3 lysine K79 (H3K79me2) binding strengths on the gene body (−2 kb upstream to transcription start site to the end of the untranslated region) of the 412 genes across WT KSLs, GMPs, and L-GMPs.

(C) Unbiased k-means clustering of ATAC-seq peaks ±10 kb to either side of the 412 common genes. k = 3 was chosen based on minimum average silhouette width. Motifs in ATAC-seq peaks were discovered de novo relative to a sequence composition-matched background set and cross-referenced to a known motif database to find matches to transcription factors (Homer2).

pointing to a correlation between the acquisition of the transcriptional program and alteration of local DNA accessibility during GMP and leukemic cell formation. Motif analysis of accessible regions demonstrated enrichment of C/EBPa and PU.1 binding sites in the GMP-dominant cluster and GATA motifs in the CMP-dominant cluster (Figure 7C), correlating with their function in the transition from CMPs to GMPs (Tenen et al., 1997). STAT3 and 5 are major downstream transducers of IL-3 and GM-CSF (de Groot et al., 1998), but we did not observe enrichment of STAT binding motifs in DNA-accessible regions over the 412 common genes, although the two cytokines rescued myeloid differentiation in KO cells (Hirai et al., 2006). Possibly, transient cytokine stimulation dramatically remodels chromatin configuration, leading to binding of STAT3/5 to regulatory elements inaccessible during normal myeloid differentiation, but critical for "emergency" granulopoiesis. DNA accessibility analysis performed on MF9 direct downstream targets demonstrated decreased genome accessibility during differentiation from KSLs and CMPs to GMPs at these loci (data not shown). Compact chromatin structure of MF9 direct targets in GMPs is in accordance with decreased H3K79me2, while increased H3K79me2 binding is more associated with increased genome accessibility. Approximately 90% of genes with increased H3K79me2 from KSLs to GMPs were also present in the GMP cluster. Together, these data suggest that GMPs provide an accessible genomic environment for establishment of a minimal transcriptional program essential for leukemic transformation that is separate from activation of MF9 direct targets.

DISCUSSION

One characteristic feature of AML is differentiation block. Here, we explored another aspect: whether differentiation is required for LSC formation. Using C/EBPa KO mice with the MF9 and MOZ-TIF2 leukemia models, we demonstrate that block of the CMP-GMP transition completely abrogates MF9-induced LSC formation and AML, regardless of the origin being from HSCs or myeloid progenitors. We also show that the CMP-GMP transition is dispensable for maintenance of MF9-induced AML. More importantly, we show that the absence of LSC formation and the failure of leukemia development are a result of the block in myeloid differentiation, rather than the absence of C/EBPa per se. Thus, we conclude that a defined differentiation stage, more specifically, the GMP stage, is required for MF9-induced AML (summarized in Figure S6). Similar results are observed with MOZ-TIF2. Therefore, the requirement of myelomonocytic differentiation is not restricted to the MF9 model but might play a more general role. As no *CEBPA* null mutations in AML patients have been described, our findings suggest that a residual function of CEBPA sustaining a certain extent of myeloid differentiation might be necessary for transformation in human AML.

The importance of C/EBPa in MLL-fusion-induced AML has recently been reported by other groups. Ohlsson et al. (2014) demonstrated that C/EBPa is dispensable in already established AML induced by MLL-ENL. Complete resistance of C/EBPa KO progenitors to MLL-ENL-induced leukemia led them to conclude that C/EBPa is required for MLL-fusion-related AML induction. Here, we extend their findings, demonstrating that the cellular state of GMPs, rather than C/EBPa, is essential. Furthermore, we generated a minimal transcriptional program necessary for GMP formation and subsequent LSC generation, and we demonstrated that H3K79me2 and alteration of chromatin accessibility during myeloid differentiation correlate with acquisition of an environment permissive for MLL-fusion transformation. Lastly, we demonstrated that other AML-associated oncogenes (i.e., MOZ-TIF2) require GMPs for AML initiation, suggesting that myeloid differentiation likely plays a broad role in AML development. In contrast to Ohlsson et al., we demonstrate that the absence of leukemia transformation in C/EBPa KO cells is not due to dysregulation of HoxA9 and Meis1, as HoxA9 and Meis1are highly expressed in C/EBPa KO cells with levels comparable to those of L-GMPs. This discrepancy may be due to different experimental approaches. Compared to using cells derived from culture, we prospectively isolated stem/progenitor populations from mice after stable reconstitution, minimizing changes during culture and providing more comparable populations.

Why is the cellular state of the GMP more susceptible to MLL-fusion-mediated transformation? The possibility that GMPs provide a transcriptional program permissive for the MLL-fusion oncogenic program is supported by the significant overlap in GMP and L-GMP transcriptomes. Changes in H3K79me2 modification and chromatin accessibility during myeloid differentiation correlate with acquisition of common molecular features shared by GMPs and L-GMPs, and alterations in H3K79me2 and genomic configuration do not find that MF9 direct targets are activated at the GMP stage. Therefore, susceptibility of GMPs to MF9 is unlikely due to a permissive environment required for activation of direct MF9 targets, and we hypothesize that the GMP transcriptional environment is a prerequisite for the MLL-fusion oncogenic program.

Total genomic sequencing of human AMLs has led to the identification of pre-leukemic HSCs (Jan et al., 2012; Shlush et al., 2014). These phenotypic HSCs carry leukemogenic mutations and show multilineage repopulating advantage over non-mutant normal counterparts, and yet they are incapable of generating overt malignancies. They are considered a reservoir for LSC generation and reformation during disease relapse. In addition to the complete block in GMP formation, C/EBPa KO HSCs exhibit enhanced self-renewal and repopulating ability but are unable to generate overt acute leukemia following transplantation (Ye et al., 2013; Zhang et al., 2004), fulfilling criteria for pre-leukemic HSCs. However, mice transplanted with MF9-transduced C/EBPa KO cells failed to develop leukemia. Indeed, MF9-expressing C/EBPa KO HSCs retain the capability to differentiate into multiple cellular lineages, yet they have completely lost their ability to generate L-GMPs and leukemic cells. Conversely, when the myeloid differentiation block was overcome by cytokine stimulation, MF9 recipients rapidly develop myeloblastic leukemia in the absence of C/EBPa. Our studies suggest that to develop frank AML, it is important for pre-leukemic HSCs to retain their ability to produce myeloid progeny, in line with recent findings in human AML patients that complementary mutations that lead to full-blown leukemia are only detected in differentiated populations (Jan et al., 2012; Shlush et al., 2014).

In conclusion, we have shown that myeloid differentiation plays a critical role in leukemia initiation. Our studies provide a mechanistic explanation for the frequent identification of LSCs as committed GMP-like cells in AML patients (Goardon et al., 2011). Furthermore, they support the concept that a critical step in LSC development is the progression from pre-leukemic stem cell to myeloid progenitor cells. Defining myelomonocytic differentiation as a key step in leukemic transformation, regardless of the initial cell-of-origin, will aide in the design of novel therapies targeting LSCs for clinical benefit.

EXPERIMENTAL PROCEDURES

Animals

All mice used in this study were housed in a sterile barrier facility approved by the IUCAC at the Beth Israel Deaconess Medical Center. Cre-mediated site-specific recombination of the loxP cassettes in C/EBPa cKO mice has been described previously (Ye et al., 2013). Excision of the C/EBPa loxP allele was analyzed on genomic DNA isolated from bone marrow and spleen cells by two methods. In a two-PCR-reaction assay, Primers 1 (P1) and P2 were used to detect the WT allele (236 bp) and the loxP allele (275 bp), while P3 and P4 were used to detect the excised allele (Δ) (377 bp). In a one-PCR-reaction method, P1, P3, and P4 primers were used to amplify WT (265 bp), lox (304 bp), and Δ (377 bp) alleles. Annealing temperature was 60°C. Primer sequences are in the Supplemental Experimental Procedures.

Flow Cytometry

Single-cell suspensions from various organs were analyzed by flow cytometry. Antibodies used are listed in the Supplemental Experimental Procedures. Stained cells were analyzed with an LSRII flow cytometer (BD Biosciences) or FACSAria (BD Biosciences) and sorted using a FACSAria. Diva software (BD) and FlowJo (Tree Star) was used for data acquisition and analysis, respectively.

Retroviral Transduction and Bone Marrow Transplant Assay

MLL-AF9 or MOZ-TIF2 retrovirus stocks were prepared as described in the Supplemental Experimental Procedures. 24 hr after transduction, KSLs (1–4 × 10^4 cells/mouse) or CMPs (5–10 × 10^4 /mouse) were injected into lethally irradiated B6.SJL-Ptprca Pepcb/BoyJ (Pep Boy) congenic recipients along with 2 × 10^5 Pep Boy mouse bone marrow cells for radioprotection.

qRT-PCR

RNA was extracted using RNeasy kit (QIAGEN), reverse transcribed using the Superscript First-Strand Synthesis System, and quantitatively assessed using a Rotor-Gene 6000 Real-time PCR machine (Corbett). For each sample, transcript levels of tested genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh). All experiments were performed with two to three technical replicates. Primers for qRT-PCR are listed in the Supplemental Experimental Procedures.

Hydrodynamics-Based Gene Transduction

Hydrodynamics-based gene transduction has been described previously (Hirai et al., 2006). 2 ml of PBS containing either 1 μg of pCAGGS expression vectors carrying cDNA encoding murine G-CSF and IL-3 or empty vector were injected into mice through the tail vein within 5–7 s using a 27G needle.

Limiting Dilution Assay to Measure Frequency of LSCs

$CD45.2^+$ GFP^+ bone marrow cells from MF9-transduced control or cKO leukemic mouse bone marrow after ten Poly I:C injections were injected into the retro-orbital venous sinus of lethally irradiated (900 rads) $CD45.1^+$ congenic recipients at 7 doses (10^5, 10^4, 2 × 10^3, 10^3, 500, 200, or 50 cells) along

with 2 × 10^5 CD45.1$^+$ congenic mouse bone marrow for radioprotection. Peripheral blood was obtained from each mouse every 2 weeks after transplantation for WBC counts and FACS analysis. Cells were stained with anti-CD45.2 and CD45.1 antibodies, to distinguish donor-derived cells from the host cells, and surface markers Mac-1 and Gr1, B220, and CD3, to identify myeloid and lymphoid cells.

Microarray Analysis

A total of 20 ng of purified total RNA was amplified using the Ovation Pico WTA System V2 (Nugen) and biotinylated with the FL-Ovation Biotin Module Version 2 (Nugen), according to the supplied protocol. cDNA was then hybridized to Affymetrix expression array Mouse Gene 1.0 ST chips. Expression values for all genes were calculated and normalized as previously described (Ye et al., 2013). Fold change cutoff ($\geq$1.5) and p value cutoff ($\leq$0.05) were used for differential expression. GSEA was used to test enrichment for the MF9 self-renewal signature in cytokine-rescued KO L-GMPs. Pathway analysis was carried out using IPA. Student t statistics was used for identification of differential expression, while p values for the enriched pathway analysis were calculated based on the Fisher exact test and further corrected using the Benjamini and Hochberg method. Results for pathway analysis were filtered for statistical significance using a nominal p value threshold of 0.05.

ATAC-Seq Analysis

ATAC-seq libraries from myoblasts from Ctl-MF9 and cytokine-rescued KO-MF9 leukemic mouse bone marrow were prepared as previously described (Buenrostro et al., 2013). Reads were trimmed for quality and adaptor sequences before aligning to mouse assembly mm9 using bowtie2. PCR and optical duplicates were removed using Picard. We discovered enriched regions by running MACS1.4 with the default parameters, and we normalized peak heights using sequencing depth. Previously published ATAC-seq libraries from KLS, CMP, and GMP populations were downloaded from GEO (GEO: GSE59992) (Lara-Astiaso et al., 2014) and processed as described above. In order to correlate chromatin accessibility with gene expression dynamics, all ATAC-seq promoter and enhancer peaks occurring within 20 kb of a differentially expressed gene were clustered using k-means (k = 3 based on the silhouette coefficient). Motif signatures were analyzed for each cluster using the 'findMotifsGenome' function with default parameters within Homer2.

Statistical Analysis

CRU frequencies were calculated with L-Calc software (StemCell Technologies) using Poisson statistics and the method of maximum likelihood to the proportion of negative recipients in a limiting dilution setting. Chi-square test was used on Kaplan-Meier survival analyses. Otherwise, statistical significance was assessed by the Student's unpaired t test.

ACCESSION NUMBERS

The accession number for array data reported in this paper is GEO:GSE61468.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes six figures, Supplemental Experimental Procedures, one table, and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.08.011.

AUTHOR CONTRIBUTIONS

Conceptualization, M.Y. and D.G.T.; Methodology, M.Y. and H.Z.; Investigation, M.Y., H.Z., M.C., E.L., R.S.W., C.S.B., A.K., S.A.A., and D.G.T.; Writing—Original Draft, M.Y. and H.Z.; Writing—Review and Editing, M.Y., H.Z., P.B.S., C.S.B., S.A.A., and D.G.T.; Funding Acquisition, D.G.T. and S.A.A.; Resources, H.Y., R.K., and J.Z.; Supervision, D.G.T.

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