ALOX5 exhibits anti-tumor and drug-sensitizing effects in MLL-rearranged leukemia

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MLL-rearranged acute myeloid leukemia (AML) remains a fatal disease with a high rate of relapse and therapeutic failure due to chemotherapy resistance. In analysis of our Affymetrix microarray profiling and chromatin immunoprecipitation (ChIP) assays, we found that ALOX5 is especially down-regulated in MLL-rearranged AML, via transcription repression mediated by Polycomb repressive complex 2 (PRC2). Colony forming/replating and bone marrow transplantation (BMT) assays showed that Alox5 exhibited a moderate anti-tumor effect both in vitro and in vivo. Strikingly, leukemic cells with Alox5 overexpression showed a significantly higher sensitivity to the standard chemotherapeutic agents, i.e., doxorubicin (DOX) and cytarabine (Ara-C). The drug-sensitizing role of Alox5 was further confirmed in human and murine MLL-rearranged AML cell models in vitro, as well as in the in vivo MLL-rearranged AML BMT model coupled with treatment of “5 + 3” (i.e. DOX plus Ara-C) regimen. Stat and K-Ras signaling pathways were negatively correlated with Alox5 overexpression in MLL-AF9-leukemic blast cells; inhibition of the above signaling pathways mimicked the drug-sensitizing effect of ALOX5 in AML cells. Collectively, our work shows that ALOX5 plays a moderate anti-tumor role and functions as a drug sensitizer, with a therapeutic potential, in MLL-rearranged AML.

Acute myeloid leukemia (AML), the most common type of acute leukemia attacking adults, is a heterogeneous disease characterized by the malignant expansion of dysfunctional myeloid progenitors and the suppression of normal hematopoiesis1. The mixed lineage leukemia (MLL) gene on chromosome 11q23 is the frequent target of chromosomal translocations and rearrangements in infant and adult leukemias. AML bearing MLL fusion often associates with poor prognosis and is resistant to most of the current clinical chemotherapies2–4. Though immense efforts have been exerted in developing novel therapeutic strategies in the past decades, the standard chemotherapy with intensive administration of chemotherapeutic drugs, e.g. the “7 + 3” regimen (i.e. combined treatment of anthracycline (daunorubicin or doxorubicin) and cytarabine (Ara-C)), remains the main therapeutic approach for treating AML, including MLL-rearranged AML1–4. Resistance to the standard chemotherapy remains the major cause of relapse and therapeutic failure7,9. Despite intensive chemotherapy, the overall survival of AML patients is still less than 30% for adults and 60% for children, and most of the patients with MLL-rearranged AML fail to survive longer than 5 years4,9–11. Therefore, it is crucial to understand the mechanisms underlying the pathogenesis and chemotherapy resistance of AML, such as MLL-rearranged AML, and identify potential new therapeutic targets that can suppress AML or improve the response to the currently existing chemotherapies.

The mechanism of AML drug response remains obscure. A number of key factors including transcriptional factors, oncogenes and tumor suppressors, as well as cell metabolism regulators, have been identified to play important roles in determining drug sensitivity or resistance1,9. Among these factors, the JAK/STAT signaling is known to be closely associated with myeloid malignancies. Activation of JAK/STAT pathway is a hallmark of the myeloproliferative neoplasms, and is found in many AML cases12,13. JAK/STAT pathway inhibitors, e.g. pacritinib and ruxolitinib, have already been studied in pre-clinical and clinical trials14,15. The proto-oncogene K-RAS is

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another factor involved in both leukemogenesis and therapeutic response. K-RAS mutations have been reported in many AML cases and are associated with prognosis and chemotherapy resistance. It holds great potential to cure AML by either targeting these factors directly or targeting a common upstream regulator that controls all these factors in AML.

The arachidonate 5-lipoxygenase gene (ALOX5) encodes a non-heme iron-containing enzyme of the lipoxygenase family. It catalyzes the production of leukotrienes (LTs) and reactive oxygen species (ROS) from arachidonic acid. ALOX5 is known to be involved in various physiological and pathological processes, including oxidative stress response, inflammation and cancer. It was reported previously that loss of Alox5 impairs the function of leukemic stem cells (LSCs) in BCR-ABL-induced chronic myelogenous leukemia (CML). To our surprise, our gene profiling reveals that ALOX5 expression is particularly down-regulated in MLL-rearranged AML. Both in vitro and in vivo studies were carried out to investigate the effects and underlying mechanism of ALOX5 in AML pathogenesis and drug response.

Results

**ALOX5 is especially down-regulated in MLL-rearranged AML.** In analysis of our Affymetrix microarray-based gene expression profiling of 100 human AML samples carrying MLL-rearrangements/t(11q23) (n = 12), inv(16) (n = 27), t(8;21) (n = 30) or t(15;17) (n = 31), along with 9 normal bone marrow (BM) control (3 each for CD34+ hematopoietic stem/progenitor cell, CD33+ myeloid progenitor cell, and mononuclear cell (MNC)) samples, we found that ALOX5 was expressed at a significantly lower level in MLL-rearranged AML, but not in other AML subtypes, relative to the normal controls (Fig. 1a). Notably, the expression level of ALOX5 in MLL-rearranged AML is also significantly lower than that in other AML subtypes (Fig. 1a); such pattern was also observed in an independent large-scale AML dataset (i.e., GSE14468) (Fig. 1b). The downregulation of ALOX5 in MLL-rearranged AML was verified through both qPCR (Fig. 1c) and Western blotting (Fig. 1d) in MLL-AF9 leukemic blast cells isolated from the relevant leukemic mice, as compared with normal controls.

To understand the mechanisms of the repression of ALOX5 in MLL-rearranged AML, we conducted chromatin immunoprecipitation (ChIP) assays. We found no significant enrichment of MLL fusion proteins at the promoter region of the ALOX5 locus (Fig. 1e–g). It is known that gene silencing mediated by the Polycomb repressive complex 2 (PRC2) and cofactors, e.g. EZH2 and SIN3A, is essential for MLL-rearranged AML. Here we show a significant enrichment of EZH2 and SIN3A at the ALOX5 promoter region. Histone H3 lysine 27 tri-methylation (i.e., H3K27me3), a marker for repressive transcription associated with EZH2, also exhibited high enrichment at these genomic loci (Fig. 1e–g). Therefore, it is highly likely that the PRC2 complex mediates the transcriptional repression of ALOX5 in MLL-rearranged leukemia.

**Anti-tumor effect of Alox5 in MLL-rearranged AML.** To assess the pathological role of Alox5 in MLL-rearranged AML, we cloned Alox5 CDS into MSCV-PIG retroviral vector, and then co-transduced MSCVneo-MLL-AF9 (MA9) with MSCV-PIG-Alox5 (Alox5) or MSCV-PIG (Ctrl) into mouse BM progenitor cells for in vitro colony-forming/replating assays. We showed that along with the increased number of passages, Alox5 overexpression showed a more significant degree of repression on MLL-AF9-induced colony forming (Fig. 2a). We also analyzed cell viability of those retrovirus transduced progenitor cells and showed that forced overexpression of Alox5 significantly suppressed cell viability (Fig. 2b). Knockdown of ALOX5 with siRNA did not show significant alterations in the viability of MONOMAC-6/t(9;11) cells (Supplementary Figs 1a–c and 3a,b).

In order to determine the in vivo effect of Alox5 in leukemogenesis, we performed primary BM transplantation (BMT) assay first and found that forced expression of Alox5 showed no significant influence on overall survival (medium overall survival: 108 days for the MA9 + Ctrl group vs. 96 days for MA9 + Alox5; P = 0.556, log-rank test) (Fig. 2c). We then isolated the leukemic blast cells from primary MLL-AF9-leukemic mice and transduced the cells with Alox5 or control retrovirus, and performed secondary BMT assays. Notably, Alox5 overexpression significantly delayed the progression of MLL-AF9 AML in secondary BMT recipients (median overall survival, 42.5 days (MA9_Alox5) vs. 37 days (MA9_Ctrl); P = 0.018, log-rank test) (Fig. 2d). While all the leukemic mice died from AML, overexpression of Alox5 resulted in a significant decrease in peripheral white blood cell count (Fig. 2e) and spleen size (Fig. 2f). Moreover, through flow cytometry analysis, we found that although the Alox5 overexpressing group and the control group have similar degrees of engraftment of MLL-AF9 donor cells in BM at their end points, the former has a significantly lower population of the Mac-1+/Gr-1+ leukemic blast cells than the latter (Fig. 2g,h). Tissue staining showed that, compared with the control group, the BM cells in the Alox5 overexpressing group were more differentiated, consistent with the flow cytometry results, and the infiltration of leukemic cells into spleen, liver, and peripheral blood was less severe (Fig. 2i). Thus, our data indicated that Alox5 exhibited a moderate anti-tumor effect in the maintenance of MLL-rearranged AML and restraints leukemic cell infiltration.

**Forced expression of Alox5 sensitizes MLL-rearranged AML cells to standard chemotherapy.** The anti-tumor role of Alox5 implies a therapeutic potential in treating AML. Since restoration of Alox5 expression/function alone showed only moderate inhibitory effect on AML progression, we sought to investigate whether Alox5 restoration could facilitate chemotherapeutic response and thus yield a more effective therapeutic effect. We first used lentivirus to overexpress human ALOX5 in MONOMAC-6/t(9;11) AML cells, and treated the transduced cells with doxorubicin (DOX) or cytarabine (Ara-C). Our results showed that overexpression of ALOX5 significantly sensitized MONOMAC-6 cells to DOX and especially Ara-C treatment (Fig. 3a–c; Supplementary Fig. 3c,d).

Similarly, in mouse BM progenitor cells transduced with MLL-AF9, co-expression of Alox5 remarkably enhanced the inhibitory effect of DOX or Ara-C on cell viability and colony forming capacity (Fig. 4a–c). We further tested the drug sensitizing effect of Alox5 in vivo. We transplanted MLL-AF9 AML cells with forced
expression of Alox5 (i.e., MA9_Alox5) or without (i.e., MA9) into secondary recipient mice, and after the onset of leukemia, the recipient mice were treated with or without DOX + Ara-C (i.e. “5 + 3” regimen). As shown in Fig. 4d, while DOX + Ara-C treatment alone only moderately (though statistically significantly) improved survival in mice carrying MLL-AF9 AML, forced expression of Alox5 dramatically improved the response of MLL-AF9 AML to the DOX + Ara-C treatment (Fig. 4d). As a result, amazingly over 70% of mice in the MA9_Alox5+ DOX/Ara-C group survived over 200 days, while all mice in the other groups died from AML within 70 days (Fig. 4d). The infiltration of leukemic cells into the spleen, liver, and peripheral blood was almost completely blocked in the MA9_Alox5+ DOX/Ara-C group of mice (Fig. 4e). Therefore, restoration of Alox5 holds great potential in improving chemotherapeutic response in MLL-rearranged AML patients.

Signaling pathways correlated with Alox5 overexpression in AML. To delineate the potential molecular mechanism underlying the anti-tumor and drug-sensitizing effects of Alox5, we performed RNA sequencing (RNA-seq) of two pairs of mouse BM leukemic blast cells collected from the MA9_Ctrl and MA9_Alox5 mice in secondary BMT assays shown in Fig. 2d. Through gene set enrichment analysis (GSEA) 36, we

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**Figure 1.** Expression of ALOX5 is significantly down-regulated in MLL-rearranged AML. (a) Comparison of ALOX5 expression between t(11q23)/MLL-rearranged AML and AML cases with inv(16), t(8;21) or t(15;17), or normal controls (NC) in our in-house AML dataset. The expression values were log2-transformed and mean centered. The P values were detected by t-test. (b) Comparison of ALOX5 expression between t(11q23) AML and other subtypes of AML cases in GSE14468. The expression values were log2-transformed and mean centered. The P values were detected by t-test. (c,d) Downregulation of Alox5 in MLL-AF9-AML mouse BM mononuclear cells, as compared with normal controls. Levels of Alox5 were detected through qPCR (c) and Western blotting (d). (e-g) EZH2 and SIN3A bind the promoter of ALOX5. Enrichment of MLL-N terminal (for both wild-type MLL and MLL fusions), MLL-C terminal (for wild-type MLL), EZH2, SIN3A, H3K27me3 or IgG at the ALOX5 promoter region was shown. Relative to transcription start site of ALOX5, locations of the three PCR amplicon sites are: ALOX5-A (e): −522~−234 bp; ALOX5-B (f): −259~−79 bp; ALOX5-C (g): +149~+596 bp. *P < 0.05; **P < 0.01, two-tailed t-test.
Figure 2. Anti-tumor effect of Alox5 in MLL-AF9-AML. (a) Inhibitory effect of Alox5 on MLL-AF9-induced colony forming/immortalization of normal mouse bone marrow progenitor cells. Normal mouse bone marrow progenitor cells were retrovirally transduced with MSCVneo-MLL-AF9 + MSCV-PIG (MA9 + Ctrl) or MSCVneo-MLL-AF9 + MSCV-PIG-Alox5 (MA9 + Alox5). Colony forming/replating assays (CFAs) were conducted for four passages. 20,000 cells were plated in each dish. (b) Alox5 overexpression represses cell viability of MLL-AF9-transduced mouse bone marrow progenitor cells. After the first passage, 10,000 of the above transduced cells were seeded in liquid culture medium. MTT assays were conducted 72 hours after seeding. (c) Effects of Alox5 on MLL-AF9-induced primary leukemia in vivo. Normal mouse bone marrow progenitor cells (CD45.1) were retrovirally co-transduced with MSCVneo-MLL-AF9 + MSCV-PIG (MA9 + Ctrl) or MSCVneo-MLL-AF9 + MSCV-PIG-Alox5 (MA9 + Alox5). 0.2 × 10^5 of the transduced cells were used as donor cells and transplanted into each primary recipient mouse (CD45.2). MA9 + Ctrl; n = 14; MA9 + Alox5: n = 16. (d) Inhibitory effects of Alox5 on the maintenance of MLL-AF9-induced AML in secondary BMT recipient mice. The secondary BMT recipients were transplanted with BM blast cells from the primary MLL-AF9 AML mice retrovirally transduced with MSCV-PIG (MA9-AML_Ctrl; n = 7) or MSCV-PIG-Alox5 (MA9-AML_Alox5; n = 8). Kaplan-Meier curves are shown. The P values were determined by log-rank test. (e) Total white blood cell (WBC) counts of the secondary BMT recipients were determined by Hemavet 950 at the mice’ end points. n = 5 for each group. (f) Weights of spleen and liver of each secondary BMT recipient at its end point are shown. n = 5 for each group. (g) Flow cytometry analyses of BM cells of secondary BMT recipients. The “blast” population was gated by FSC/SSC (framed out; upper panel) and the proportions of CD45.1+ (middle panel), Gr1+ and/or Mac1+ cells were analyzed (lower panel). (h) Statistical analysis of the Gr1+ Mac1+ population of secondary BMT recipients. n = 5 for each group. *P < 0.05; **P < 0.01, two-tailed t-test. (i) Wright-Giemsa staining of mouse peripheral blood (PB) and BM, or hematoxylin and eosin (H&E) staining of mouse spleen and liver of secondary BMT recipients.
found that IL-2/Stat5 signaling and K-Ras pathway were significantly suppressed in MA9_Alox5 AML cells as compared with MA9_Ctrl AML cells (Fig. 5a,b). Stat5 and K-Ras function as critical oncogenes in both solid tumors and leukemia, and both Stat5 and K-Ras signaling pathways were known to be closely associated with oncogenesis and drug sensitivity.9,15,16,38,39, c-Myc is a downstream target gene shared by Stat5 and K-Ras signaling pathways.18,19. We analyzed two independent AML patient databases and found a significant negative correlation between the expression levels of ALOX5 and c-Myc (Fig. 5c,d). In BM leukemic blast cells of MLL-AF9 leukemic mice, overexpression of Alox5 suppressed the expression of c-Myc and Flt3, an upstream key regulator of both Stat5 and K-Ras signaling.40 (Fig. 5e–f; Supplementary Fig. 3e–g). In order to determine whether repression of Stat5 and/or K-Ras pathways could mimic the drug sensitizing effect of ALOX5, we treated MONOMAC-6 cells with a combination of DOX or Ara-C, together with control, Stat5 inhibitor sc-355979, and/or K-Ras signaling inhibitor Tipifarnib.41 Results showed that a combined treatment of both sc-355979 and Tipifarnib significantly sensitized the cells to chemotherapy (Fig. 5g,h). In a data set composed of 82 AML samples (including 26 MLL-rearranged AMLs)43, those bearing K-Ras mutations (n = 5) are associated with a lower ALOX5 level (Supplementary Fig. 2a), indicating a potential positive feedback between K-Ras signaling and ALOX5. Noticeably, the only AML sample with mutated K-Ras that has relatively higher ALOX5 level also bears FLT3 mutation. The remarkable suppression of ALOX5 in MLL-rearranged AML bearing K-Ras mutation was further verified in human CD34+ derived MLL-AF9 cell lines (Supplementary Fig. 2b). Therefore, our results suggest that Alox5 exerts its anti-tumor and drug sensitizing effects in MLL-rearranged AML through suppressing the Stat and K-Ras signaling pathways.

Discussion

In contrast to the oncogenic role of ALOX5 reported previously in CML,26 here we show that ALOX5, suppressed by PRC2 at the transcription level, exhibits a moderate anti-tumor effect both in vitro and in vivo in MLL-rearranged AML, in which ALOX5 expression is especially repressed. More interestingly, we show that restoration of Alox5 expression can substantially increase the sensitivity of MLL-rearranged AML cells to standard chemotherapeutic agents such as DOX and Ara-C both in vitro and in vivo, as well as the underlying mechanism through suppressing the Stat and K-Ras oncogenic signaling pathways.

Interestingly, ALOX5 was largely known as an oncogene in solid tumors, e.g. prostate cancer and pancreas cancer.16,17. However, its role remains vague in leukemia. It was reported that Alox5 deficiency resulted in a significant reduction of LSCs in BM, and thus largely prolonged survival of BCR-ABL-induced CML mice.26 Nonetheless, there was no significant influence of Alox5 deficiency on normal hematopoietic stem cells (HSCs) or on the induction of lymphoid leukemia by BCR-ABL.26 Another study showed that Alox5 deficiency inhibited in vitro colony-forming capacity of BM progenitor cells induced by some AML oncogenic fusion genes such as AML1-ETO9a, a potent oncogenic isoform of AML1-ETO resulting from t(8;21)44, but had no significant effects on AML1-ETO9a-induced leukemogenesis in vivo45. Those findings together with our present results suggest that Alox5 likely plays distinct roles at different lineages (myeloid and lymphoid) or different stages (i.e. induction and maintenance) of various hematopoietic malignancies.

One of the major challenges in AML therapy is the high frequency of occurrence of therapeutic resistance and the subsequent relapse.7 Thus far, a variety of factors, such as JAK/STAT signaling and RAS pathway, have been identified to be closely related with chemotherapy response.8,13,14. Abnormal activation of JAK/STAT signaling is found in most de novo AML.13,15. K-Ras is a proto-oncogene essential for many solid tumors and hematopoietic malignancies.46 Although RAS (i.e. N-RAS or K-RAS) gene mutations were only found in no more than 10% of AML cases, activation of RAS pathway by mutations in upstream receptors, e.g. FLT3 and c-KIT, or downstream effectors, broadly exist in AML.47. A set of inhibitors have been developed to target JAK/STAT signaling (e.g.
OPB-31121 and pacritinib) or RAS pathway (e.g., Selumetinib), but their efficacy in clinical trials to treat hematopoietic malignancies was still limited. Our data suggest that restoration of ALOX5 expression/function could suppress both JAK/STAT and RAS signaling pathways simultaneously, and thus represents an alternative strategy, other than individual small-molecule inhibitors of JAK/STAT and RAS signaling pathways, to target these two critical oncogenic pathways in treating AML. Especially, restoration of ALOX5 expression/function in combination with the standard chemotherapy represents a potentially more effective therapeutic strategy for curing AML, at least MLL-rearranged AML. Thus, it would be interesting to identify small-molecule compound(s), such as natural product(s), that can specifically induce endogenous expression of ALOX5 expression in AML cells, and such compound(s) can be applied together with standard chemotherapy to treat MLL-rearranged AML.

Methods

Affymetrix exon array assays of human samples. As described previously, a total of 100 human AML (including 30 t(8;21), 27 inv(16), 31 t(15;17) and 12 MLL-rearranged) and 9 normal BM samples (including 3 each of CD34+ hematopoietic stem/progenitor, CD33+ myeloid, and mononuclear cell (MNC) samples) were analyzed by use of Affymetrix GeneChip Human Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA). Robust...
Multi-array Average (RMA) was used for the data normalization with Partek Genomics Suite (Partek Inc., St. Louis, MI). The complete microarray data set has been deposited into the GEO database under the accession code GSE34184 and GSE30285.

**Plasmid construction.** The CDS of ALOX5, encoding the human ALOX5 gene, was amplified through PCR using primers 5’-ATAACCGGTCCACCATGAGATGACGATGACAAGGCCTTCTCTCAGGTC-3’ and 5’-AGCGAATTCTCAGATGGGCACACTGTTCGGA-3’, and then cloned into pLJM1-EGFP lentiviral vector (Addgene, Cambridge, MA). The CDS of mouse Alox5 gene was amplified through PCR using primers 5’-AATCTCGAGCCACCATGAGATTAC AAGGATGACGATGACAAGGCCTTCTCTCAGGTC and 3’-CGAATTCTCAGATGGGCACACTGTTCGGA.
5'-ATTGAATTCTAGTGCTACGCTGGGAAT-3', and then ligated into a retroviral vector, namely MSCV-PIG (i.e., MSCV-puro-IRE6-GFP vector; bearing GFP gene), a kind gift from Drs. Gregory Hannon, Scott Hammond, and Lin He (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

**Cell culture.** MONOMAC-6 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% HEPES, 2 mM L-Glutamine, 100 × Non-Essential Amino Acid, 1 mM sodium pyruvate, 9 μg/ml insulin and 1% penicillin-streptomycin. Mouse leukemic cells were kept in RPMI 1640 supplemented with interleukin 3 (IL-3) and IL-6, 10 ng/ml; SCF 100 ng/ml; 10% FBS and 1% penicillin-streptomycin. siRNAs were transfected with Cell Line Nucleofector Kit V following program T-037, using the Amaxa Nucleofector Technology (Amaxa Biosystems, Berlin, Germany). Experiments were performed 48 h after transfection.

**Lentivirus production and infection.** pMD2.G, pMDLg/pRRE, pRSV-Rev and pLJM1-EGFP plasmids were purchased from Addgene (Cambridge, MA). 0.5 μg pMD2.G, 0.3 μg pMDLg/pRRE, 0.7 μg pRSV-Rev and 1.5 μg pLJM1-EGFP constructs, i.e. plJM1-Alox5 or pLJM1-EGFP control were co-transfected into HEK-293T cells in 60 mm cell culture dish with Effectene Transfection Reagent (QIAGEN, Valencia, CA). Lentiviral particles were harvested at 48 and 72 hours after transfection and concentrated with PEG-it® Virus Precipitation Solution (Biosystems, Berlin, Germany). Experiments were performed 48 h after transfection.

**Cell viability assays.** Cells were seeded into 96-well plates at the concentration of 10,000 cells/well in triplicates and MTT (Promega, Madison, WI) was used to assess cell proliferation and viability following the manufacturer's instructions.

**Chromatin immunoprecipitation (ChiP).** ChIP assay was conducted as described previously 50, with SABiosciences Corporation's ChampionChiP One-Day kit (Qiagen, Frederick, MD) following the manufacturer's protocol. Chromatin from THP-1 cells were cross-linked, sonicated into an average size of ~500bp, and then immunoprecipitated with antibodies against the N'-terminal of MLL (MLL-N), the C'-terminal of MLL (MLL-C), EZH2, SIN3A, H3K27Me3 or IgG (Abcam, Cambridge, MA). Purified DNA was amplified by real-time qPCR using primers targeting the promoter of Alox5 as described before 51.

**Western blotting.** Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and ruptured with RIPA buffer (Pierce, Rockford, IL) containing 5 mM EDTA, PMSF, cocktail inhibitor, and phosphatase inhibitor cocktail. Cell extracts were microcentrifuged for 20 min at 10,000 g and supernatants were collected. Cell lysates (20 μl) were resolved by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at 4 °C with anti-Alox5 antibody (Cell Signaling Technology Inc., Danvers, MA) or anti-ACTIN antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Membranes were washed 30 min with Tris-buffered saline containing 0.1% Tween-20, incubated for 1 hour with appropriate secondary antibodies conjugated to horseradish peroxidase, and developed using chemiluminescence substrates.

**In vitro colony forming and replating assays (CFAs).** CFAs were conducted as described previously with some modifications 52-56. Briefly, retrovirus vectors were co-transfected with pCL-Eco packaging vector (IMGENEX, San Diego, CA) into HEK293T cells using Effectene Transfection Reagent (Qiagen, Valencia, CA) to produce retrovirus. BM cells were harvested from a cohort of 4- to 6-week-old B6.SJL (CD45.1) donor mice after five days of 5-fluorouracil (5-FU) treatment, and primitive hematopoietic progenitor cells were enriched with Mouse Lineage Cell Depletion Kit (Millenyi Biotec Inc., Auburn, CA). An aliquot of enriched hematopoietic progenitor cells was added to retroviral supernatant together with polybrene in cell culture plates, which were centrifuged at 2,000 g for 2 hours at 32 °C (i.e., “spinoculation” 57-59) and then the medium was replaced with fresh media and incubated for 20 hours at 37 °C. Next day, the same procedure was repeated once.

Then, on the day following the second spinoculation, an equivalent of 2.0 × 10^4 cells were plated into a 35 mm Petri dish in 1.5 ml of Methocult M3230 methylcellulose medium (Stem Cell Technologies Inc, Vancouver, Canada) containing 10 ng/ml each of murine recombinant IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF), and 30 ng/ml of murine recombinant SCF (R&D Systems, Minneapolis, MN), along with 1.0 mg/ml of G418 and 2 μg/ml of puromycin. For each transduction, there were two duplicate dishes. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. The colonies were replated every 7 days under the same conditions. The colony-forming/replating assays were repeated 3 times.

**The maintenance, monitoring, and end-point treatment of mice.** C57BL/6 (CD45.2), B6.SJL (CD45.1) mice were purchased from the Jackson Lab (Bar Harbor, ME, USA) or Harlan Laboratories, Inc (Indianapolis, IN, USA) and maintained in house. Both male and female mice were used for the experiments. All laboratory mice were maintained in the animal facility at University of Cincinnati and the University of Chicago. All experiments on mice in our research protocol were approved by Institutional Animal Care and Use Committee (IACUC) of University of Cincinnati and the University of Chicago. All methods were performed in accordance with the relevant guidelines and regulations. The maintenance, monitoring, and end-point treatment of mice were conducted as described previously 57-59.

**Bone marrow transplantation assays.** For primary bone marrow transplantation (BMT) assays, normal BM cells of B6.SJL (CD45.1) mice were retrovirally co-transduced with MSCVneo-MLL-AF9 + MSCV-PIG (i.e., MA9 + Ctrl) or MSCVneo-MLL-AF9 + MSCV-PIG-Alox5 (i.e., MA9 + Alox5), through two rounds of spinoculation, as described previously 57-59. Then, the transduced donor cells were injected by tail vein into...
lethally irradiated (960 rads) 8- to 10-week-old C57BL/6 (CD45.2) recipient mice with 3 × 10^5 donor cells plus a radioprotective dose of whole BM cells (1 × 10^6; freshly harvested from a C57BL/6 mouse) per recipient mouse.

For secondary BMT assays, leukemic BM cells (with CD45.1 percentage >90%) were isolated from primary leukemic mice (CD45.2) that received BMT of wild-type BM progenitor donor cells (CD45.1) infected with MSCVneo-MLL-AF9 retrovirus alone. The leukemic cells from the primary recipients were retrovirally transduced with MSCV-PIG (MA9 Ctrl) or MSCV-PIG-Alox5 (MA9_Alox5), and then transplanted into sublethally irradiated (480 rads) 8- to 10-week-old C57BL/6 (CD45.2) secondary recipient mice via tail vein injection. 1.5 × 10^5 donor cells were transplanted into each secondary recipient mouse.

Peripheral blood (PB) cells were collected from the transplanted recipient mice monthly via tail bleeding and at any time that the mice showed signs of systemic illness to obtain the complete blood counts with white blood cell (WBC) differentials and the blood smears for the presence of immature or abnormal hematopoietic cells. The engraftment was assessed by flow cytometry analysis of CD45.1 in PB. Leukemic mice were euthanized by CO2 inhalation if they showed signs of systemic illness to collect specimens as controls for further analyses. The spleen, liver and thymus were weighed. Cells were obtained from PB, BM, spleen, and liver for flow cytometric analysis. Blood smear and BM cytospin slides were stained with Wright-Giemsa. For histological analysis, portions of the spleen and liver were fixed in formalin, sectioned, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

Gene set analyses. Gene Set Enrichment Analysis (GSEA) was used to analyze the signal pathway enrichment in different groups of samples. Hallmark gene sets obtained from MsigDB (The Molecular Signatures Database) were used as the “gene sets database” input. Vertebrate homology resource from the Mouse Genome Database (MGD) was extracted to convert between homologous human and mouse gene symbols.

Statistical software. The gene/exon array data analyses and qPCR data analyses were conducted by use of Partek Genomics Suite (Partek Inc, St. Louis, MI), TIGR Mutiple Array Viewer software package (TMeV version 4.6; TIGR, Rockville, MA), and/or Biocoordinator R packages. The t-test, Kaplan-Meier method, and log-rank test, etc. were performed with WinSTAT (R. Fitch Software), GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA), and/or Partek Genomics Suite (Partek Inc, St. Louis, MI). The P-values less than 0.05 were considered as statistically significant. Significance analysis of microarrays (SAM), embedded in the TMeV package (TIGR, Rockville, MA), was used to identify the genes that are significantly (p < 0.05; false discovery rate, FDR < 0.05) dysregulated in human AML samples relative to the normal controls.

Data availability. Data referenced in this study are available in The Gene Expression Omnibus. The Affymetrix exon array data and the microarray data are available under accession codes GSE34184 and GSE30285. The mouse RNA sequencing data is available under accession code GSE94840. The RNA sequencing results of the AML sample cohort analyzed for the correlation of ALOX5 levels and K-RAS mutations is under accession code GSE62190.

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Author Contributions
Y.W., J.C. and X.J. conceived the project and designed the research. Y.W., J.S., C.H., I.D., K.F., R.S., C.L., H.H., H.W., H.H., X.Q., J.J., J.C. and X.J. performed experiments and/or data analysis. Y.W., J.S., C.H., I.D., K.F., R.S., C.L., H.H., H.W., J.J., J.C. and X.J. contributed reagents/analytic tools and/or grant supports. J.C. and X.J. wrote the paper. All authors discussed the results and commented on the manuscript.

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