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Isocitrate dehydrogenase 1 mutations prime the all-trans retinoic acid myeloid differentiation pathway in acute myeloid leukemia

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Acute myeloid leukemia (AML) is characterized by the accumulation of malignant blasts with impaired differentiation programs caused by recurrent mutations, such as the isocitrate dehydrogenase (IDH) mutations found in 15% of AML patients. These mutations result in the production of the oncometabolite (R)-2-hydroxyglutarate (2-HG), leading to a hypermethylation phenotype that dysregulates hematopoietic differentiation. In this study, we identified mutant R132H IDH1-specific gene signatures regulated by key transcription factors, particularly CEBPα, involved in myeloid differentiation and retinoid responsiveness. We show that treatment with all-trans retinoic acid (ATRA) at clinically achievable doses markedly enhanced terminal granulocytic differentiation in AML cell lines, primary patient samples, and a xenograft mouse model carrying mutant IDH1. Moreover, treatment with a cell-permeable form of 2-HG sensitized wild-type IDH1 AML cells to ATRA-induced myeloid differentiation, whereas inhibition of 2-HG production significantly reduced ATRA effects in mutant IDH1 cells. ATRA treatment specifically decreased cell viability and induced apoptosis of mutant IDH1 blasts in vitro. ATRA also reduced tumor burden of mutant IDH1 AML cells xenografted in NOD–Scid–IL2rγ null mice and markedly increased overall survival, revealing a potent antileukemic effect of ATRA in the presence of IDH1 mutation. This therapeutic strategy holds promise for this AML patient subgroup in future clinical studies.

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Abbreviations used: 2-HG, 2-hydroxyglutarate; α-KG, α-ketoglutarate; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; BCR-ABL, BCR-ABL1; BCL, B cell lymphoma; ChIP, chromatin immunoprecipitation; GSEA, gene set enrichment analysis; IDH, isocitrate dehydrogenase; L-CFU, leukemic CFU; LC/MS, liquid chromatography–mass spectrometry; NBT, nitrotetrazolium blue; PML, promyelocytic leukemia; qChIP, quantitative ChIP; qPCR, quantitative PCR; RA, retinoic acid; TF, transcription factor.

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Despite progress in acute myeloid leukemia (AML) therapy, most patients relapse and die from the disease as a result of residual chemoresistant blasts. Thus, targeting this population is a mandatory requirement for effective AML therapy. In this context, mutations in isocitrate dehydrogenase 1 (IDH1) or IDH2 represent a promising therapeutic target. These mutations, originally identified in glioma (Dang et al., 2009), were then discovered in 15% of AML patients (Mardis et al., 2009) and have been found in an increasingly diverse set of other neoplasms, including hematological malignancies of both lymphoid and myeloid lineages (Lu et al., 2012; Fathi et al., 2014). Importantly, IDH mutations, which lead to the production of the oncometabolite (R)-2-hydroxylglutarate (2-HG), have been detected in preleukemic hematopoietic stem and multipotent progenitor cells as a persistent reservoir of surviving chemotherapeutic-resistant cells at remission (Shlush et al., 2014). Therefore, targeting IDH mutant cells might be essential to achieving long-term remission in the AML subgroup with IDH mutation.

An increasing number of studies have demonstrated that IDH1/2 mutations disrupt differentiation programs in nontransformed cells (Lu et al., 2012), as well as in hematopoietic and leukemic cells (Figueroa et al., 2010a; Sasaki et al., 2012; Losman et al., 2013). Moreover, tumors with IDH1/2 mutations exhibit a distinctive profile of 2-HG-dependent DNA and histone hypermethylation that alters gene expression (Figueroa et al., 2010a; Turcan et al., 2012), particularly for several genes involved in retinoic acid (RA) metabolism and signaling pathways (Chou et al., 2012; Guilhamon et al., 2013). Because RA is involved in granulocytic differentiation, we sought to specifically investigate the effects of all-trans-RA (ATRA) in the presence of the IDH1–R132H mutation in AML.

Our gene expression analyses demonstrate that IDH1–R132H mutation primes AML blasts to the granulomonocytic differentiation pathway compared with WT IDH1 AML cells and increases both H3K4me3 occupancy in the CEBPa gene promoter and expression of CEBPa, as well as downstream CEBPa target genes. Furthermore, we show a significant deregulation of RA-responsive target genes by IDH1–R132H mutation. Collectively, these observations suggest that 2-HG production resulting from IDH1 mutation biases hematopoietic differentiation toward the granulocytic lineage and further sensitizes mutant cells to ATRA-induced differentiation. Finally, we showed that the prodifferentiating effects of ATRA observed in the presence of IDH1 mutation at clinically achievable concentrations lead to a potent antileukemic response in AML cell lines and primary patient samples both in vitro and in vivo.

RESULTS AND DISCUSSION

As IDH mutations induce the production of neomorphic metabolite 2-HG, recent reports of 2-HG–dependent epigenetic programs (especially in genes involved in retinoid signaling pathways) are intriguing (Chou et al., 2012; Guilhamon et al., 2013). Seeking to determine whether AML cell lines engineered to express either the WT or mutant form of IDH1 (Fig. 1 A) and controlled for their production of 2-HG and α-ketoglutarate (α-KG; Fig. 1, B and C) have a specific gene signature, the gene expression profile of HL60 cells expressing the mutant IDH1–R132H was compared with WT cells by microarrays covering 23,924 human genes (Fig. 2). Computational analyses of these datasets identified two subsets of down- and up-regulated genes in mutant IDH1–R132H cells (Table S1) and their Gene Set Enrichment Analysis (GSEA) revealed significant enrichment of these signatures in published transcriptions of 417 AML patients (cohort data available under Gene Expression Omnibus (GEO) accession no. GSE6891; Verhaak et al., 2009; Fig. S1, A and B), which validated our gene profiles specific to AML cells expressing mutant IDH1. Classification of biological processes down- or up-regulated by IDH1–R132H mutation particularly highlighted its role in immune responses, signaling pathways, metabolic processes, and cell differentiation (Table S1 and Fig. 2 A).

Furthermore, functional annotation of IDH1–R132H target genes revealed an enrichment of genes previously described as responsive to or regulated by several key transcription factors (TFs) essential for myelopoiesis, such as PU.1, RUNX1, GATA1, MECOM, MNDA, CEBPB, and CEBPa (Table S2 and Fig. 2 B), and required for commitment to the monocytic and granulocytic lineages, such as CEBPa (Fig. 2 B). IDH1–R132H mutation also led to an increase in CEBPa expression at the RNA and protein level in both primary AML specimens (323 WT vs. 68 mutant IDH1 patients, respectively; GEO accession no. GSE6891; Verhaak et al., 2009; Fig. 2 C and D). As histone methylation is closely linked to mRNA expression and differentiation, especially in IDH mutant cells (Figueroa et al., 2010b; Kernytsky et al., 2015), we have shown that occupancy of the H3K4me3-activating locus in the CEBPa promoter was significantly increased in IDH1–R132H mutant HL60 and MOLM14 cells (Fig. 2 E). Because a peak of CEBPa expression occurred at the granulocyte-monocytic progenitor step of normal myelopoiesis (Radomska et al., 1998; Bagger et al., 2013; Hema-Explorer database; Fig. S1 C), it is likely that IDH1–R132H mutation can drive AML blasts to this differentiation pathway through increased CEBPa expression. RT-PCR and immunophenotypical flow analyses indicated a significant increase in CEBPa-target genes RXRα, EMILIN, and CD11b (Laiosa et al., 2006; Hasemann et al., 2014) in the presence of mutant IDH1–R132H compared with WT IDH1 (Fig. 2, F–I). Quantitative chromatin immunoprecipitation (qChIP) experiments showed that mutant IDH1–R132H influences the recruitment of endogenous CEBPa to several of these target genes (Fig. 2 H). Moreover, IDH1–R132H mutation also modulated other myeloid differentiation-associated TFs (CEBPβ, PU.1, and RUNX1) and genes involved in the differentiation-related MAPK signaling pathway and associated metabolic processes (Fig. 2, A and B; Table S1 and S2), thus inducing a myeloid differentiation bias in these cells. Our results are consistent with...
recently published data demonstrating that IDH mutations favor differentiation toward the granulomonocytic lineage (Sasaki et al., 2012; Kats et al., 2014), while blocking erythroid differentiation (Losman et al., 2013; Wang et al., 2013a). It is noteworthy that the reintroduction of the CEBPα TF in hematopoietic stem/progenitor cells has the same effects as IDH1–R132H mutation in biasing differentiation toward the granulomonocytic lineage, while blocking the erythroid differentiation (Schepers et al., 2007). Finally, the overexpression of CEBPα sensitizes cells to differentiation into the granulocytic lineage with various agents (Ferrari-Amorotti et al., 2006).

Interestingly, gene expression analyses also revealed that the IDH1–R132H mutation gene signature is highly enriched in genes responsive to treatment with small molecules such as retinoids (e.g., ATRA or RA; Fig. 2 J), especially in promyelocytic leukemia (PML)/RA receptor α–driven acute promyelocytic leukemia (APL) cell lines and patients (Fig. S1 D) or, in some cases, of non-APL primary cells (Altucci et al., 2005). These observations corroborate a recently published metaanalysis showing a dysregulation of several genes involved in RA-related pathways in other cancers with IDH1–R132 (Guilhamon et al., 2013). Collectively, our gene expression analyses indicate that IDH1–R132H mutation can prime AML blasts toward granulomonocytic differentiation through the activation of the CEBPα pathway and could sensitizes cells to ATRA-induced differentiation through the regulation of ATRA-responsive gene expression.

We tested this hypothesis using 25 primary AML patient specimens screened for their IDH1/2 mutational status in our cohort of 384 AML patients from Toulouse Hospital, which exhibit similar clinical features and distribution as those previously described in the literature (Abbas et al., 2010; Green et al., 2011; Table S3 and S4). We found that a clinically relevant concentration (1 µM) of ATRA induced the nuclear lobulation specifically associated with neutrophilic differentiation in eight primary AML patient samples harboring the IDH1–R132H mutation (Fig. 3 A) and producing 2-HG (Fig. 3 B). Moreover, treatment with ATRA was sufficient to increase the percentage of AML blasts expressing granulomonocytic differentiation markers (e.g., CD11b, CD15, and CD14) in 11 primary mutant IDH1–R132H AML sam ples harboring the IDH1–R132H mutation (Fig. 3 A) and producing 2-HG (Fig. 3 B). Moreover, treatment with ATRA was sufficient to increase the percentage of AML blasts expressing granulomonocytic differentiation markers (e.g., CD11b, CD15, and CD14) in 11 primary mutant IDH1–R132H AML samples (Fig. 3 C). Similar results were obtained for clinically ATRA-responsive APL samples used as a positive control (Fig. 3, A and C). In contrast, no increase in morphological changes and the CD14 marker, and only very modest decrease or increase in CD11b and CD15, respectively, were observed at any time point after ATRA treatment of primary AML blasts expressing WT IDH1 (Fig. 3, A and C). Consistent with data in primary
Figure 2. IDH1 mutation primes AML cells to the ATRA differentiation pathway through the activation of granulomonocytic differentiation-associated genes. (A) Gene ontology enrichment analysis of down- (122) and up-regulated (206) genes from RNA expression profile of the differentially expressed genes between WT IDH1 HL60 clones and mutant IDH1–R132H HL60 clones by 1.5-fold or more (n = 3 clones for each cell type). (B) Gene to TF associations within mutant IDH1–R132H target genes using data mining algorithm (Genomatix). (C) mRNA expression of C/EBPα in WT (gray; n = 323) or mutant (black; n = 68) primary AML patients from the AML patient cohort GSE6891 (Verhaak et al., 2009) and in WT (gray) or R132H (black) HL60 (dots) or MOLM14 (square). (D) Protein level of CEBPα in mutant IDH1 HL60 (R132H, clone 5 and 11) and MOLM14 (R132H) cells or of WT IDH1 HL60 (WT, clone 4 and 7) and MOLM14 (WT) cells by Western blot (n = 3). (E) ChIP experiments showing the relative enrichment of histone H3 trimethylation at lysine 4 (H3K4me3) on CEBPα promoter in mutant IDH1–R132H HL60 and MOLM14 cells versus WT IDH1–WT HL60 and MOLM14 cells, as indicated. The precipitated DNA fragments were subjected to real-time PCR analysis with two different pairs of primers amplifying the CEBPα promoter. Results were represented as
differentiation would preferentially lead to the reduction of R132H expressing cells to ATRA-induced differentiation. Least in part, responsible for the enhanced sensitivity of IDH1–R132H activity using a mutant IDH1–specific inhibitor AGI-5198 significantly reduced both the production of 2-HG and sensitized NB4, HL60, and MOLM14 cells to ATRA-induced differentiation (Fig. 4 F). In contrast, the inhibition of IDH1–R132H mutation (Fig. 4 E vs. Table S2), and IFN-stimulated genes are activated by RA (Chelbi-Alix and Pelicano, 1999; Shen et al., 2011), suggesting that cross talk may occur between these two distinct transcriptional programs.

Because ATRA-induced CD11b expression correlates with the expression of IDH1–R132H protein and with the level of 2-HG production (Fig. 4, A–C compared with Fig. 1, A and B), we asked whether exogenous 2-HG would sensitize AML cells to ATRA-induced differentiation. Pretreatment with a cell-permeable form of 2-HG (1-octyl-[R]-2-HG) increased the intracellular 2-HG concentration (Fig. 4 F) and sensitized NB4, HL60, and MOLM14 cells to ATRA-induced differentiation (Fig. 4 G). In contrast, the inhibition of IDH1–R132H activity using a mutant IDH1–specific inhibitor AGI-5198 significantly reduced both the production of 2-HG (Fig. 4 H) and the sensitivity of mutant IDH1–R132H cells to ATRA (Fig. 4 I). This confirms that 2-HG production is, at least in part, responsible for the enhanced sensitivity of IDH1–R132H expressing cells to ATRA-induced differentiation.

We also tested whether ATRA-induced terminal differentiation would preferentially lead to the reduction of cell proliferation and to cell death of mutant IDH1–R132H AML cells in vitro. As expected, 10-d treatment with ATRA induced a significant decrease in cell viability (Fig. 5 A) in mutant IDH1–R132H cells without affecting WT or control IDH1 cells. Moreover, ATRA treatment induced a mutant IDH1–specific apoptotic cell death, as shown by the increase of the Annexin V marker (Fig. 5, B and C) and cleavage of poly (ADP-ribose) polymerase and caspase-3, specifically in mutant IDH1–R132H AML cells (Fig. 5 D). This correlated with a marked decrease of antiprototic B cell lymphoma 2 (BCL2) protein (Fig. 5 D) in mutant IDH1–R132H cells. It is notable that it has been recently demonstrated that mutant IDH1/2 AML cells exhibited BCL2 dependency (Chan et al., 2015). Altogether, the reduction of cell viability and the induction of apoptosis led to a specific and marked reduction in the size and number of L-CFUs after ATRA treatment (Fig. 5, E and F).

To evaluate the ability of ATRA treatment to induce myeloid differentiation in vivo, we used a xenograft model based on immunodeficient NOD–scid IL2r<sup>α<sup>−</sup>-null (NSG) mice with primary AML samples or MOLM14 carrying the IDH1–R132H mutation (Fig. 6, E and F). Although the reduction of cell viability and the induction of apoptosis led to a specific and marked reduction in the size and number of L-CFUs after ATRA treatment when the disease was well established and showed that ATRA specifically differentiated mutant IDH1–R132H cells xenografted in NSG mice, as indicated by morphological changes (Fig. 6, B and C) and an increase of CD11b<sup>+</sup> myeloid cells in three primary AML patient samples (Fig. 6 D), bone marrow; Fig. 6 E, spleen; Fig. 6 F, peripheral blood) or MOLM14 cell line (Fig. 6 H). Furthermore, ATRA significantly reduced bone marrow tumor burden in a dose-dependent manner (Fig. 6 I) and led to a significant increase in overall survival of mice (Fig. 6 J). In contrast, we did not observe either prodifferentiative or antileukemic effects of ATRA in the WT IDH1 MOLM14 xenograft model (Fig. 6, K–M). Collectively, these data demonstrated that ATRA specifically induces differentiation and reduces tumor growth in the mutant IDH1–R132H subgroup of AML both in vitro and in vivo.

In summary, IDH1 mutation and its (R)-2-HG dysregulate downstream target pathways of myeloid-specific TFs, especially CEBPα, priming mutant IDH1–R132H AML blasts to the granulomonocytic lineage. Moreover, ATRA treatment down-regulates the expression of the protooncogene...
genic protein BCL2, followed by the reduction of cell viability through the induction of apoptotic cell death in vitro. ATRA also has an in vivo antileukemic effect, specifically in mutant IDH1–R132H AML cells. These data suggest that IDH1–R132H mutation could be a valuable biomarker to select AML patients for ATRA treatment. Results from clinical trials with ATRA have been conflicting (Schlenk et al., 2009; Burnett et al., 2010; Nazha et al., 2013), but because IDH1 mutational status of those patients was not monitored in these studies, it would also be enlightening to reanalyze results of clinical trials conducted in non-APL patients to address the impact of IDH mutations on ATRA therapy. Interestingly, high serum 2-HG levels represented a negative prognostic factor independent of other clinical and molecular features.

Figure 3. **IDH1–R132H mutation sensitizes to ATRA-induced differentiation of primary patient samples in vitro.** (A–C) ATRA treatment induces granulocytic differentiation of 11 primary patient samples carrying mutant IDH1–R132H (R132) compared with 10 WT IDH1 (WT) and 1 PML-RARα fusion protein (APL). (A) Primary WT IDH1, mutant IDH1–R132H AML, or APL patient samples were treated with ATRA at 1 µM for the indicated time, cytospun, and stained with May-Grünwald Giemsa. Cells were classified in five categories (from less to more differentiated; right). Bar, 10 µm. 100 cells per condition were counted three times for statistical analysis (Mann-Whitney test). **(B) Intracellular amounts of 2-HG and α-KG in 12 primary patient samples expressing WT IDH1 (WT) or mutant IDH1–R132H (R132H), determined by Liquid chromatography–mass spectrometry (LC/MS; method 2). The amounts were normalized using a labeled standard, [13C]-succinate, spiked with the same volume in each sample (y axis). A log_{10} scale was used. ***, P < 0.05; Mann-Whitney test. (C) Flow cytometry analysis of CD11b, CD15, and CD14 expression was performed in primary WT IDH1, mutant IDH1–R132H AML, or APL patient samples treated with 1 µM ATRA or vehicle for 12 d (mean ± SEM). *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001 indicate statistical significance of the observed differences (Wilcoxon matched pairs signed rank test).
Figure 4. IDH1–R132H mutation sensitizes to ATRA-induced differentiation of AML cell lines in vitro. (A–D) Analysis of ATRA-induced differentiation in HL60 and MOLM14 infected with retroviruses encoding GFP alone (CTL) or GFP and the indicated IDH1 variants. (A) Quantification of the CD11b expression measured by flow cytometry after 3-d treatment with 1 µM ATRA or solvent (ratio of mean fluorescence intensity in ATRA-treated to vehicle-treated cells ± SEM; n = 5). Mann-Whitney test. (B) Quantification of the CD11b expression measured by flow cytometry after 3-d treatment with 1 µM ATRA or solvent (ratio of mean fluorescence intensity in ATRA-treated to vehicle-treated cells ± SEM; n = 5) in different clones (CTL, clones 14–15; R132H Cl 5, R132H Cl 11). (C) Quantification of the CD11b expression measured by flow cytometry after 3-d treatment with 1 µM ATRA or solvent (ratio of mean fluorescence intensity in ATRA-treated to vehicle-treated cells ± SEM; n = 5) in different clones (HL60, WT Cl 14, WT Cl 2, R132H Cl 5, R132H Cl 11). (D) Quantification of the NBT reduction measured by flow cytometry after 3-d treatment with ATRA (ratio of absorbance in ATRA-treated to vehicle-treated cells ± SEM; n = 5) in different clones (HL60, MOLM14).
in AML patients (DiNardo et al., 2013; Wang et al., 2013b; Janin et al., 2014). Our observation that 2-HG levels correlate with the ability of ATRA to induce terminal differentiation further support the contention that this therapeutic strategy is likely to be most relevant for the category of AML patients producing highest levels of 2-HG and would be an alternative therapeutic strategy for AML patients developing resistance to mutant IDH inhibitors. It is noteworthy that this therapeutic approach may also be relevant and of benefit for AML patients harboring IDH2 mutations. Because IDH1 mutations are present in preleukemic hematopoietic stem cells and systemically conserved at relapse, this therapeutic strategy holds promise to specifically achieve a long-term remission for this AML patient subgroup.

**MATERIALS AND METHODS**

**AML patient samples.** AML and normal bone marrow samples were obtained from patients at the Department of Hematology (Centre Hospitalier Universitaire de Toulouse [CHU], Toulouse, France) after consent in accordance with the Declaration of Helsinki. Samples were stored at the HIMIP collection. In accordance with French law, HIMIP collection has been declared to the Ministry of Higher Education and Research (CD 2008–307 collection 1) and a transfer agreement was obtained (AC 2008–129) after approbation by ethical committees (Comité de Protection des Personnes Sud-ouest et Ouestremer II). Clinical and biological annotations of the samples have been declared to the Comité National Informatique et Libertés (i.e., Data Processing and Liberties National Committee). All patients were diagnosed at the Department of Hematology (CHU, Toulouse, France). Their characteristics are summarized in Table S3. Samples containing at least 80% blasts were grown in H4230 methylcellulose medium (STEMCELL Technologies) and ATRA or the control. Primary patient AML cells were plated in 35-mm Petri dishes in duplicate with or without ATRA at 1 µM. After 12-d incubation in a humidified CO2 incubator, cells were washed three times with PBS at 37°C. If the patient blasts were known to not grow in methylcellulose medium (assessed routinely at diagnosis in our center), they were co-cultured with human mesenchymal stem cells in IMDM (Life Technologies) supplemented with 15% bovine serum albumin, insulin, and transferrin (STEMCELL Technologies), 100 U/ml of penicillin and streptomycin (Invitrogen), 5 µM β-mercaptoethanol (Invitrogen), 1 mM pyruvate (Sigma-Aldrich), MEM 1X (Sigma-Aldrich), 100 ng/ml DNase (MP Biomedicals), 10 ng/ml hIL3, 100 ng/ml hSCF and 10 ng/ml human thrombopoietin (all from R&D Systems) at 10⁶ cells/ml. All the samples were then processed for treatment with different reagents.

**Targeted resequencing.** A panel of 31 genes frequently mutated in AML and myeloid malignancies, including TET2, CEBPA, DNMT3A, IDH1, and IDH2, was designed. Amplicon libraries were obtained from 112 ng DNA per sample, using HaloPlex Target Enrichment System (Agilent Technologies) according to the manufacturer’s protocol, with the exception that all volumes of reactions were halved. Sequencing was performed using a MiSeq sequencer (Illumina) following the manufacturer’s recommendations. Results were analyzed after alignment of the readings using SureCall Software version 3.0.1.4 (Agilent Technologies). BWA MEM algorithm was used for alignment and SNP PET SNP Caller (Illumina) algorithm was used to identify SNP and INDELs variants. Minimum allele frequency for variant calling was set at 5% with a minimum local depth of 40. All variants were manually checked using IGV version 2.3 software. Complementary detection of described polymorphisms was performed by an in-house software using Ensembl database.

| WT clones 4–7; R132H, clones 5–11; Mann-Whitney test. | (C) Dose-dependent increase of CD11b expression induced after 3-d treatment with 1 µM ATRA or solvent (ratio of mean fluorescence intensity in ATRA-treated to vehicle-treated cells ± SEM; n = 5) in HL60 cells parental, control (C14), WT (C12), or with IDH1–R132H mutations (clone 5 or 11). Two-way ANOVA. (D) Measurement of NBT reduction after 12-d treatment with 1 µM ATRA or control. The data correspond to the mean of three independent experiments (Mann-Whitney test). (E) Venn diagram of the overlapping genes enriched in mutant IDH1 HL60 cells (R132H) versus WT IDH1 HL60 cells both treated with 1 µM ATRA for 24 h with a FDR <0.05, considered as differentially expressed mRNA irrespective of the fold-change. Gene to TF associations specifically within 109 mutant IDH1–R132H target genes in response to ATRA using data mining algorithm (Genomatix). Similar screening and analysis was performed in WT IDH1 HL60 cells with exogenous 2-HG (WT+2-HG) versus WT IDH1 HL60 cells both treated with 1 µM ATRA for 24 h. (F and G) NB4, IDH1–WT HL60 clone, or MOLM14 were treated with or without 100 or 50 µM, respectively, 2-HG for 3 d with 1 µM ATRA or control. (F) Intracellular amounts of 2-HG in parental NB4 cell line or HL60 cell lines expressing WT IDH1 (WT) with or without treatment with 1-octyl-(R)-2-HG–2-HG determined by LC/MS. The amounts were normalized using a labeled standard, [13C]-succinate, spiked with the same volume in each sample (y axis). Each condition represents a triplicate set of measurements. **, P < 0.01; Mann-Whitney test. (G) Quantification of the CD11b expression measured by flow cytometry (ratio of mean fluorescence intensity in ATRA-treated to vehicle-treated cells ± SEM; n = 5; *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001, statistical significance of the observed differences (Mann-Whitney test).
Clonogenic assays. 1,000 AML cells/ml were plated in duplicate in 35-mm Petri dishes in H4230 Stem Cell Technologies methylcellulose medium (STEMCELL Technologies) supplemented with 10% of 5637 cell line–conditioned medium (Dos Santos et al., 2008) and the different reagents. After a 7-d incubation in a humidified CO2 incubator, 500 µl of MTT at 10 mg/ml was gently added to the methylcellulose and incubated for 2 additional hours at 37°C in a humidified CO2 incubator. Photographs of dishes were taken and dark purple (viable) colonies were counted.

Generation of mutant IDH1–R132H, WT IDH1–WT, and control (empty) AML cell lines. Human AML cell lines NB4 (DMSZ), HL60 (DMSZ), and MOLM14 cells (Matsumo et al., 1997), provided by M. Carroll (University of Pennsylvania, Philadelphia, PA), were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin and streptomycin (Invitrogen) in an incubator at 37°C and 5% CO2. HL60 and MOLM14 were transduced with IDH1–R132H, IDH1–WT, or control vector MIGR1 (CTL), as previously described by Figueroa et al. (2010a,b). For MOLM14, bulk preparations were used for all the experiments. For HL60, subclones of (IDH1–R132H, IDH1–WT, or control constructs were generated by sorting GFP-positive cells in each well of a 96-well plate with a FACS Sorter (FACSAria II SORP; BD). 10 subclones of each group (IDH1–R132H, IDH1–WT, or control) were established. Two clones of each category were then selected for further studies. They were grown...
Figure 6. **In vivo antileukemic activity of ATRA in cell line model and primary patient blasts carrying IDH1 R132H mutation.** (A) Schematic representation of the in vivo protocol used for B–F. Immunodeficient NOD-scid IL2rγnull (NSG) female mice were xenografted with IDH1 R132 or WT primary AML patient samples. When AML disease was established, mice were treated with subcutaneous (B–F) ATRA pellet at 2.5 mg (B and C) or 7.5 mg (D–F) pellet or control for 21 d. (B) Human cells were sorted from mice bone marrow, cytospun, and stained with May-Grünwald Giemsa. A representative picture is shown. (C) Cells were classified in 5 categories (from less to more differentiated in accordance of their morphological changes). 100 cells per condition were counted three times (Mann-Whitney test). Percentage of human (hCD45⁺·mCD45⁻·hCD33⁺·hCD44⁺) CD11b⁺ cells was measured by flow cytometry analysis in bone marrow (D), spleen (E), and peripheral blood (F) of primary patient samples-xenografted mice. (Patient 1, n = 5 untreated and 5 treated mice; patient 2, n = 6 untreated and 6 treated mice; patient 3, n = 9 untreated and 9 treated mice). (G) Schematic representation of the in vivo protocol used for H–M. (H–M) Immunodeficient NOD-scid IL2rγnull (NSG) female mice were xenografted with MOLM14 transduced with IDH1 R132H (H–J) or WT (K–M). When AML disease was established, mice were treated with subcutaneous ATRA pellet (five mice per group) or control (four mice per group). Blast differentiation and mice overall survival with or without ATRA (7.5 or 15 mg subcutaneous pellet) were monitored in vivo. After 6-d treatment, the percentage of human (hCD45⁺·mCD45⁻·hCD33⁺·hCD44⁺) CD11b⁺ cells was assessed by flow cytometry (H and K) and the total cell tumor burden in bone marrow (hCD45⁺·mCD45⁻·hCD33⁺·hCD44⁺·Annexin-V⁻) cells was analyzed by flow cytometry in mutant IDH1–R132H (I) or WT (J) MOLM14–xenografted mice. Mann-Whitney test. Survival of mutant IDH1–R132H (J) or WT (M) IDH1–WT MOLM14–xenografted mice treated with vehicle (CTL, n = 7) or ATRA (7.5 mg subcutaneous pellet, n = 7). *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001 indicate statistical significance of the observed differences (Log-rank [Mantel-Cox] test).
<10 passages after resuscitation. All clones were periodi-
cally authenticated by morphological inspection and pro-
tein expression and tested negative for mycoplasma.

**Reagents.** ATRA (Sigma-Aldrich) was dissolved in DMSO
(max. 1/1,000 final dilution). 1-Octyl ester of (R)-2-HG
was synthesized at the Organic Synthesis Core (Memorial
Sloane-Kettering Cancer Center, New York, NY) as previously
described in Losman et al. (2013) and dissolved in DMSO (max.
1/1,000 final dilution). Octylalcohol (Sigma-Aldrich) solution
in DMSO at the same concentration of (R)-2-HG was used as a
control solution to assure that only the effect of (R)-2-HG-
free acid on the cells was observed. AGI-5198 (EMD Millipore)
dissolved in dimethylfuran (max. 1/1,000 final dilution).

**Protein analysis.** Western blot analyses were performed as
previously described (Lu et al., 2012). Mutant IDH1-R132H
and total IDH1 were detected with rabbit monoclonal anti-
bodies to IDH1-R132H (clone H09; 1:1,000; Dianova) and
and with rabbit monoclonal antibody to IDH1 (clone 2DH1;
1:1,000; Cell Signaling Technology), caspase 3 by rabbit poly‑
clonal antibody (1:1,000; Cell Signaling Technology), Bcl‑2
1:1,000; Cell Signaling Technology), caspase 3 by rabbit poly‑
clonal antibody (1:1,000; Cell Signaling Technology), Bcl‑2
by rabbit polyclonal antibodies (1:1,000; Cell Signaling Tech‑
nology), and actin by mouse monoclonal antibodies (1:5,000;
Santa Cruz Biotechnology, Inc.). Standard secondary anti‑
bodies conjugated to HRP were used after incubation with
primary antibodies. Immunoreactive bands were visualized by
enhanced chemiluminescence (P32209; Thermo Fisher Sci‑
etific) with a Syngene camera.

**Extraction of metabolites.** Metabolite extraction was per‑
formed on frozen pellets (~80°C) of cells. Cell pellets were
lysed with a cold (~20°C) mixture of 70% methanol and
30% water, scraped, and vortexed 20 s for a maximal lysis.
The samples were centrifuged (18,000 g, 4°C, 30 min), and
the supernatants were collected. A calculated volume con‑
taining the equivalent of 0.5 million cells was dried under
nitrogen. Dried extracts were dissolved in 100:40 µl of mo‑
BILE phase A + 60 µl of mobile phase B

**Intracellular metabolite quantification.** Targeted
HPLC-MS/MS experiments were performed using an Ac‑
quity HPLC system coupled to a triple-quadrupole Xevo
TQ MS (Waters Corp.). The software interface was Mass‑
Lynx (version 4.1). Chromatographic separation was per‑
formed on Sequant ZICpHILIC 5 µm, 2.1 × 150 mm at
15°C (Merck). Mobile phase A consisted of an aqueous
buffer of 10 mM of ammonium carbonate in water with
ammonium hydroxide to adjust basicity to pH 10.5, and
mobile phase B of 100% AcN. Chromatographic elution
was achieved with a flow rate of 200 µl/min. After injection
of 10 µl sample, elution started with an isocratic step of 1 min
at 80% B, followed by a linear gradient from 80 to 40% of
phase B from 1 to 6 min. The chromatographic system was
then rinsed for 5 min at 0% phase B, and the run was ended
with an equilibration step of 9 min. The column effluent
was directly introduced into the electrospray source of the
mass spectrometer, and analyses were performed in the neg‑
ative ion mode. Source parameters were as follows: droplet
evaporation temperature, 350°C; desolvation and cone gas
flow rates, (L/h) 650 and 30; capillary voltage, −2.5 kV. Mass
resolution parameters LM 1 and HM 1 were fixed at 3 and
15, respectively. MRM transitions were optimized for 2‑HG
and α-KG detection. For 2-HG, the MRM transition m/z
147 to 129 was monitored with a collision energy of 12 eV
and a cone voltage of 15 V. For α-KG the MRM transition
m/z 144 to 101 was monitored with a collision energy of 10
eV and a cone voltage of 12 V. MassLynx software was used for
peak detection and integration.

**Quantitative PCR (qPCR).** cDNAs were synthesized from 500
ng total RNA using SuperScript III Reverse transcription
(Invitrogen). Real-time qPCRs were performed on a Light‑
Cycler 480 SW 1.5 apparatus (Roche) with Platinum Taq
DNA polymerase (Invitrogen) and a SYBR green mix con‑
taining the equivalent of 0.5 million cells was dried under
nitrogen. AGI-5198 (EMD Millipore) was dissolved in dimethylfuran (max. 1/1,000 final dilution).

**ChIP.** Cells (5,106 cells) were trypsinized and counted be‑
fore cross-link (5 min 1% formaldehyde/1% paraformalde‑
hyde added in the culture medium). Glycine was added at
125 mM final for 5 min to stop the cross-linking. Cells were
then rinsed with cold PBS. Cell nuclei were isolated after
incubation for 5 min on ice in buffer A (20 mM Heps, pH 7.8, 10 mM KCl, 0.25% Triton X-100, 1 mM EDTA,
and 0.5 mM EGTA, supplemented with protease inhibi‑
tors). After centrifugation, nuclei were resuspended in buf‑
er B (10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA,
0.5 mM EGTA, and protease inhibitors) for 10 min on ice.
Nuclei were lysed and chromatin extracted with lysis buffer
(10 mM Tris, pH 8.1, 40 mM NaCl, 0.1% SDS, 0.5% Triton
X-100, 0.05% NaDoc, 1 mM EDTA, 0.5 mM EGTA, and
protease inhibitors) at a final concentration corresponding
to 10 million cells/ml. Chromatin sonication was achieved
with a sonicator (epishear; Active Motif). MDW2 ChIP
was performed by incubating 500 µl (prepared from 5 million
cells) of chromatin prepared from H1299 cells with 20 µl
of protein G–Dynabeads (Life Technologies) and 5 mg of
CGBPα (C-18; Santa Cruz Biotechnology, Inc.) goat polyc‑
clonal antibody, or an irrelevant antibody. After overnight
incubation, immunoprecipitates were successively washed
with five buffers: W1 (10 mM Tris, pH 8.0, 150 mM KCl,
NP-40 0.5%, and 1 mM EDTA), W2 (10 mM Tris, pH 8.0,
100 mM NaCl, 0.1% NaDoc, and 0.5% Triton X-100), W3a
(10 mM Tris, pH 8.0, 400 mM NaCl, 0.1% NaDoc, and
0.5% Triton X-100), W3b (10 mM Tris, pH 8.0, 500 mM
NaCl, 0.1% NaDoc, and 0.5% Triton X-100), W4 (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NaDoc, 0.5% NP-40, and 1 mM EDTA), and W5 (10 mM Tris, pH 8.0, and 1 mM EDTA). Input and immunoprecipitated DNA were decross-linked overnight at 65°C, diluted in TE, and incubated first with RNaseA (37°C, 45 min), and then with proteinase K (55°C, 45 min). Proteins were removed with phenol-chloroform-isomyl-alcohol extraction and DNA was recovered by chromatography (nucleospin extract II columns; Macherey-Nagel). For ChIP-qPCR, 1/100 (0.5 µl) of input or immunoprecipitated DNA was analyzed by qPCR with SYBR green (Takara Bio Inc.) on a LightCycler 480 apparatus (Roche). Results are represented as the mean value of at least three independent experiments of immunoprecipitated chromatin (percentage of input) with the indicated antibodies after normalization using a control ChIP performed with an irrelevant antibody. Sequence of primers used for qPCR can be provided upon request.

**Gene expression analysis.** Total RNAs were extracted from AML clones expressing WT IDH1–WT or mutant IDH1–R132H gene using the QIAGEN kit according to the manufacturer’s instructions. RNA purity and integrity were monitored using NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer with RNA 6000 Nano assay kit. Only RNAs with no sign of contamination or marked degradation (RIN > 9) were considered good quality and used for further analysis. Transcriptome profiling assays were performed using the Affymetrix Human Gene GeneChip 2.0 ST arrays. In brief, 250 ng of total RNAs were reverse transcribed into cDNA, and then transcribed into cRNAs and labeled into biotinylated cRNA using the GeneChip WT PLUS Reagent kit (Affymetrix) according to the manufacturer’s standard protocols (P/N 4425209 Rev.B 05/2009 and P/N 702808 Rev.6). Labeled cRNA products were randomly fragmented and hybridized onto Affymetrix GeneChips. Upon hybridization, arrays were washed and stained using the Affymetrix GeneChip WT Terminal Labeling and Hybridization kit, before being scanned using a GeneChip Scanner 3000.

**Microarray data analysis.** CEL files generated after upon array scanning were imported into the Partek Genomics Suite (GS) 6.6 for further analysis. For this purpose, Partek options were set up for GC-content adjustment, robust multivariate background correction, quantile normalization, log-transformation, and mean summarization. Data were first preprocessed to estimate transcript cluster expression levels from raw probe signal intensities. Resulting expression data were then imported into R statistical environment for further processing. First, nonspecific filtering was applied to keep only transcript clusters, which belong to the main category. Next, quality of the dataset was assessed through box plots, relative log expressions, and pairwise sample correlations. Principal variance component analysis (PVCA) was also applied to the data. This method estimates the percentage of variability relative to each experimental parameter. As principal variance component analysis revealed a batch effect, the remove-batcheffect method of LIMMA package (R/Bioconductor) was used to correct for this effect. Finally, the Rank Product algorithm was used to estimate the statistical significance of the difference in gene expression between different conditions. Genes with pfp <0.05 and absolute fold change ≥1.5 were considered to be differentially expressed.

**Data exploration and mining.** Lists of DE mRNA (FC > ±1.5 and FDR < 0.05) obtained throughout the study were uploaded in the Genome Analyzer bioinformatics tool (Genomatix) for further functional analyses (GO term, TFs, and small molecules) based on the Genomatix literature mining. The significance of the association between each list and functions or canonical pathways was measured by Fisher’s exact test. As a result, a p-value was obtained, determining the probability that the association between the genes in our dataset and a function or canonical pathway can be explained by chance alone. Transcriptional gene regulatory networks were built based on the molecular relationship repertoire referenced in the Genomatix library. The Genomatix Upstream Regulators analytic was also used to identify the transcriptional regulators that could explain the experimental gene expression patterns, predict their activation state, and determine the biological functions affected by the regulatory cascade. We examined the enrichment of our IDH1 mutation gene signature with existing gene expression sets from AML patient specimens with clinicobiological data available under GEO accession no. GSE6891 using GSEA software.

**Analysis of granulomonocytic differentiation.** We treated cells with ATRA (Sigma-Aldrich) for the indicated time before analysis. We performed FACS analysis of CD11b, CD14, and CD15 expression on 5 × 10⁵ cells using the following anti-human monoclonal antibodies: CD11b-PE (2:100; Beckman Coulter), CD14-AF700 (2:100; BD) for GFP cell lines or CD14-FITC (2:100; BD) for patient samples, CD15-APC-H7 (2:100; BD), CD33 BV711 (2:100; BD), and CD44 PerCP Cy5.5 (2:100; BD) after washing with PBS. Antimurine antibody CD45-PerCP Cy5.5 was added for in vivo analysis. Flow cytometry analysis was performed using a Fortessa flow cytometer (BD), and data were analyzed with FlowJo software. A minimum of 10,000 events was collected. Apoptotic cells were stained with Annexin V-PE according to the manufacturer’s protocol (BD). We analyzed the level of the NBT reducing activity after differentiation using the method of Sakashita et al. (1991) with a slight modification. Treated or control cells were resuspended in phorbol-12-myristate-13-acetate (PMA) to a final concentration of 200 ng/ml. 20 µl of MTS was added in 200 µl of cell suspension control or treated. After a 2-h incubation at 37°C and 5% CO₂ in an incubator, the amount of formazan formed was assayed spectrophotometrically at 570 nm. For the morphological analysis of myeloid cell differentiation, we prepared cytocines with centrifugation in 150 µl PBS at a speed of 300 rpm for 5 min using Superfrost slides.
Plus positively charged glass slides. We stained cytospun slides at room temperature with May–Grünwald Giemsa (Sigma-Aldrich). 100 cells were counted in triplicate for each condition and examined for cellular morphology using an Axioskop microscope with Axicam camera (Carl Zeiss).

**In vivo treatment of AML in NSG mice.** Female NSG mice were produced at the UMS006 in Toulouse (France) using breeders obtained from Charles River. NSG mice were bred and housed at the Purpan Hospital. All animal experimental protocols were approved by the institutional Animal Care and Use Ethical Committee of the UMS006 and Région Midi-Pyrénées (approval #13-U1037-JES08). NSG mice were treated by an i.p. injection of busulfan (20 mg/kg) before i.v. xenotransplantation of AML. All the mice in the same experiment received equal numbers of cells (2 × 10⁶ cells for MOLM14-IDH1WT or IDH1R132H or 10⁵ primary patient specimens), and then a daily monitoring of mice for symptoms of disease (ruffled coat, hunched back, weakness, and reduced motility). At the disease establishment (after flow cytometric analysis of bone marrow aspirate or blood sample identifying human blasts), ATRA was administrated to xenografted mice by subcutaneous implantation of a 21-d release 2.5- or 7.5-mg ATRA pellet (Innovative Research of America). After 6 d (for xenografted mice with cell lines) or 21 d (for xenografted mice with primary AML patient sample) ATRA treatment, mice were humanely sacrificed to analyze bone marrow engraftment. For xenografted mice with cell lines, a second group of mice was used to follow overall mouse survival. Bone marrow (mixed from tibias and femurs) were crushed into HBSS with 1% SVF; washed in PBS, and dissociated into single-cell suspensions for analysis by flow cytometry of human leukemia cell engraftment and bone marrow cell tumor burden. CD33⁺CD45⁺hCD45⁺mCD45⁺AnnexinV⁻ cells were assessed by flow cytometry, and the differentiation of human cells was assessed with CD11b, CD15, and CD14 markers. For the morphological analysis of myeloid differentiation, 150,000 human blasts (CD33⁺CD45⁺hCD45⁺mCD45⁺AnnexinV⁻ cells) were sorted with a FACS Sorter (FACSARIA II SORT; BD), cytospun, and stained with May–Grünwald Giemsa staining.

**Statistical analyses.** The Kaplan–Meier method was used to estimate the leukemia-free survival in xenografted mice. Log-rank p-values were used for comparisons of the leukemia-free survival among three subgroups. The other statistical analyses were performed using the Mann–Whitney U-Test using the Prism software package (GraphPad Software). Results are expressed as mean ± SEM. Differences were considered as significant for p-values <0.05. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Online supplemental material.** Fig. S1 shows the validation of a specific gene signature in AML cells with IDH1–R132H mutation. Table S1 shows the list of up- and down-regulated genes in mutant IDH1–R132H AML cells compared with WT IDH1 cells. Table S2 shows the list of co-cited TFs mutant IDH1–R132H AML cells compared with WT IDH1 cells. Table S3 shows the clinical characteristics of 25 AML patients bearing or lacking an IDH1–R132 mutation. Table S4 shows the list of differentially expressed genes in mutant IDH1–R132H AML cells compared with WT IDH1 cells after ATRA treatment. Table S5 lists ATRA-responsive genes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150736/DC1.

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Author contributions: H. Boutzen, C. Récher, and J.E. Sarry designed the studies, interpreted the data, and wrote the manuscript; H. Boutzen performed the experiments; L. Gales and F.A. Castelli performed 2-HG and metabolomic experiments; G. Yang and O. Querfellini synthesized the cell permeable 2-HG; L. Vallar and T. Kaoma performed transcriptomic analyses; V. Mansat-de Mas, and E. Delabesse managed the tissue biobank and performed mutational analyses; P. Hirsch and F. Delhommeau contributed to NGS design and analysis; E. Saland, C. Larrue, M. David, and F. de Toni contributed to the in vivo experiments. F. Vergez, L. Le Cam, L.K. Linares, L. Stuani, J.C. Portias, and C. Junot assisted with data interpretation.

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