

Cancer-associated IDH2 mutants drive an acute myeloid leukemia that is susceptible to Brd4 inhibition

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Somatic mutations in the isocitrate dehydrogenase (IDH) genes *IDH1* and *IDH2* occur frequently in acute myeloid leukemia (AML) and other cancers. These genes encode neomorphic proteins that produce the presumed oncometabolite 2-hydroxyglutarate (2-HG). Despite the prospect of treating AML and other cancers by targeting IDH mutant proteins, it remains unclear how these mutants affect tumor development and maintenance in vivo, and no cancer models exist to study the action of IDH2 mutants in vivo. We show that IDH2 mutants can cooperate with oncogenic *Flt3* or *Nras* alleles to drive leukemia in mice by impairing the differentiation of cells of the myeloid lineage. Pharmacologic or genetic inhibition of IDH2 triggers the differentiation and death of AML cells, albeit only with prolonged IDH2 inhibition. In contrast, inhibition of the bromodomain-containing protein Brd4 triggers rapid differentiation and death of IDH2 mutant AML. Our results establish a critical role for mutant IDH2 in leukemogenesis and tumor maintenance and identify an IDH-independent strategy to target these cancers therapeutically.

[*Keywords:* AML; Brd4 inhibition; IDH mutants; targeted therapy; tumor maintenance]

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Acute myeloid leukemia (AML) is a heterogeneous cancer involving the accumulation of immature cells of the myeloid lineage (Shih et al. 2012; The Cancer Genome Atlas Research Network 2013). Genomic and functional studies have identified two broad classes of mutations that cooperate during AML development (Kelly and Gilliland 2002; Takahashi 2011). Class I mutations confer a proliferative and/or survival advantage of hematopoietic stem and progenitor cells (HSPCs) and include activating mutations in *NRAS* or *KRAS*, loss of the Ras-GAP *NF1*, or upstream activation of RAS signaling through mutations affecting the *FLT3* receptor tyrosine kinase. Class II mutations promote self-renewal and block the differentiation of HSPCs. Such mutations include translocations involving the *MLL1* gene or the t(8:21) fusion involving *AML1-ETO*. Alone, class I mutations tend to trigger chronic myeloid leukemia-like diseases, whereas class II mutations lead to a myelodysplastic syndrome (MDS)-

like state. Although an oversimplification, as a rule, class I and class II mutations cooperate to drive AML, leading to the aberrant proliferation and suppressed differentiation that are hallmarks of this disease.

Currently, most AML patients are treated with high-dose chemotherapy involving cytarabine (ara-C) and an anthracycline, a highly toxic drug combination to which >70% of patients develop resistance. However, the nature of certain genes mutated in AML suggests strategies for treating patients with molecularly targeted agents. For example, internal tandem duplication (ITD) mutations in *Flt3* lead to constitutive activation of its tyrosine kinase activity, and drugs targeting these mutations are in clinical trials (Stirewalt and Radich 2003; Leung et al. 2013). Similarly, MEK inhibitors, which interfere with RAS effector mechanisms, show efficacy in certain pre-clinical models (Lauchle et al. 2009). Finally, inhibitors of DOT1L, a histone methyltransferase needed for the oncogenic activity of *MLL* fusion oncoproteins, are entering clinical trials (Bernt et al. 2011; Daigle et al. 2011). Such therapies require that leukemia remains dependent on the targeted oncogene or pathway, which is often difficult

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to predict a priori. Still, not all novel AML targets directly interfere with oncogenic drivers or their downstream effectors. For example, the BET family member Brd4, which is not mutated in AML, has been identified as a target in AML owing to its ability to sustain a Myc-dependent self-renewal program activated by certain AML drivers (Dawson et al. 2011; Zuber et al. 2011b). Regardless of their mode of action, genetically and pathologically accurate mouse models of AML have been important in informing target development (Bernt et al. 2011; Zuber et al. 2011b).

Recently, somatic mutations in the isocitrate dehydrogenase (IDH) genes *IDH1* and *IDH2* have been identified at high frequency in AML and other tumor types (Parsons et al. 2008; Mardis et al. 2009; Yan et al. 2009; Amary et al. 2011; The Cancer Genome Atlas Research Network 2013). These genes encode key metabolic enzymes that convert isocitrate to α -ketoglutarate (α -KG). IDH mutations mainly impact certain active site residues (e.g., *IDH1*^{R132}, *IDH2*^{R140}, or *IDH2*^{R172}), resulting in loss of normal enzymatic function and the acquisition of a neomorphic activity that enables the mutant proteins to reduce α -KG to 2-hydroxyglutarate (2-HG) (Dang et al. 2009; Ward et al. 2010). The presumptive “oncometabolite” 2-HG can competitively inhibit multiple α -KG-dependent dioxygenases, including key epigenetic regulators such as histone demethylases and the DNA-demethylating TET proteins (Figuroa et al. 2010; Xu et al. 2011). Consequently, IDH mutants are associated with dramatic chromatin abnormalities, including globally altered histone and DNA methylation (Figuroa et al. 2010; Lu et al. 2012; Turcan et al. 2012). In the hematopoietic system and other cell types, these changes are associated with a differentiation block (Koivunen et al. 2012; Lu et al. 2012; Sasaki et al. 2012; Turcan et al. 2012).

The neomorphic action of IDH mutant proteins has created enthusiasm for targeting these enzymes with novel anti-cancer agents, and early studies using small molecules capable of inhibiting *IDH1*^{R132H} and *IDH2*^{R140Q} show some activity (Rohle et al. 2013; Wang et al. 2013). Still, there is a paucity of data documenting the oncogenic effects of IDH mutants on the development and maintenance of bona fide malignancies. On one hand, *IDH2* mutants block the differentiation of cultured HSPCs (Figuroa et al. 2010), which accumulate in the hematopoietic compartment of mice expressing *IDH1*^{R132H} (Sasaki et al. 2012). Also, enforced expression of this mutant can promote cytokine-independent growth and block the differentiation of an established erythroleukemic cell line in vitro (Losman et al. 2013). Still, in vivo models whereby IDH mutants drive a fully malignant disease have been lacking.

In this study, we describe a new mouse model in which *IDH2* mutants cooperate with other lesions to drive an aggressive AML that accurately recapitulates features of the human disease. We further use this system to study the basis of IDH-mediated oncogenesis and as a preclinical model for testing novel therapies.

Results

IDH2 mutants cooperate with *Flt3-ITD* or *Nras*^{G12D} to promote leukemia

Considering evidence that *IDH* mutations can block the differentiation of HSPCs (Figuroa et al. 2010; Sasaki et al. 2012), we hypothesized that they might act as canonical class II mutations and thus could cooperate with class I mutations to promote AML. We chose mouse models incorporating two common class I mutations observed in human AML: *FLT3-ITD* (Nakao et al. 1996) and *Nras*^{G12D} (Schubbert et al. 2007). *Flt3-ITD* knock-in mice develop a chronic myelomonocytic leukemia that never progresses to AML (Lee et al. 2007; Chu et al. 2012), whereas Mx-1-mediated activation of a latent “lox-stop-lox” *Nras*^{G12D} allele (Haigis et al. 2008) in hematopoietic cells results in a myeloproliferative disorder (Li et al. 2011; Wang et al. 2011).

We applied a mosaic mouse modeling approach in which HSPCs are isolated from 5-fluorouracil (5-FU)-treated *Flt3-ITD* mice, transduced with retroviral vectors expressing *IDH2* mutants or a vector control, and then assessed for tumorigenic potential following transplantation into sublethally irradiated syngeneic recipient mice (Schmitt et al. 2002). For experiments involving *Nras*^{G12D}, mice were pretreated with polyinosinic:polycytidylic acid (pIpC) to trigger cre-mediated oncogene activation (Supplemental Fig. 1A). Immunoblotting of protein extracts obtained from sorted GFP⁺ HSPCs confirmed expression of *IDH2* wild-type and mutant proteins (Supplemental Fig. 1B). Gas chromatography-mass spectrometry (GC-MS) analysis revealed that 2-HG levels were elevated in HSPCs expressing *IDH2*^{R140Q} and *IDH2*^{R172K} but not wild-type *IDH2*, confirming that the mutant alleles function as expected (Supplemental Fig. 1C).

Recipients of HSPCs expressing *Flt3-ITD* or *Nras*^{G12D} together with either *IDH2*^{R140Q} or *IDH2*^{R172K} displayed significantly reduced survival compared with recipients of HSPCs expressing *Flt3-ITD* or *Nras*^{G12D} transduced with empty vector or wild-type *IDH2* (Fig. 1A,B). Recipients of *Flt3-ITD*;*IDH2*^{R140Q} and *Flt3-ITD*;*IDH2*^{R172K}-expressing HSPCs died with a similarly short latency after transplantation (median leukemia-free survival = 97 d); similarly, both *Nras*^{G12D};*IDH2*^{R140Q} and *Nras*^{G12D};*IDH2*^{R172K}-expressing HSPC recipients also showed accelerated disease onset, although in this instance, the effect of *IDH2*^{R140Q} was less potent than *IDH2*^{R172K} (median leukemia-free survival = 178 d for *IDH2*^{R140Q} vs. 98 d for *IDH2*^{R172K}; $P < 0.0001$) (Fig. 1B). Complete blood counts (CBCs) showed that all *IDH2*^{R140Q} and *IDH2*^{R172K} recipients displayed leukocytosis (Fig. 1C,D), anemia (Fig. 1E,F), and gross splenomegaly (Fig. 1G). Importantly, disease could be transferred to secondary recipients by transplanting bone marrow (BM) cells derived from moribund mice, indicating that neoplastic cells arising in the presence of *IDH2*^{R140Q} or *IDH2*^{R172K} were fully malignant (Fig. 1H).

IDH2 mutations drive aggressive AML

To further characterize the hematopoietic malignancy induced by each *IDH2* mutant, we subjected moribund

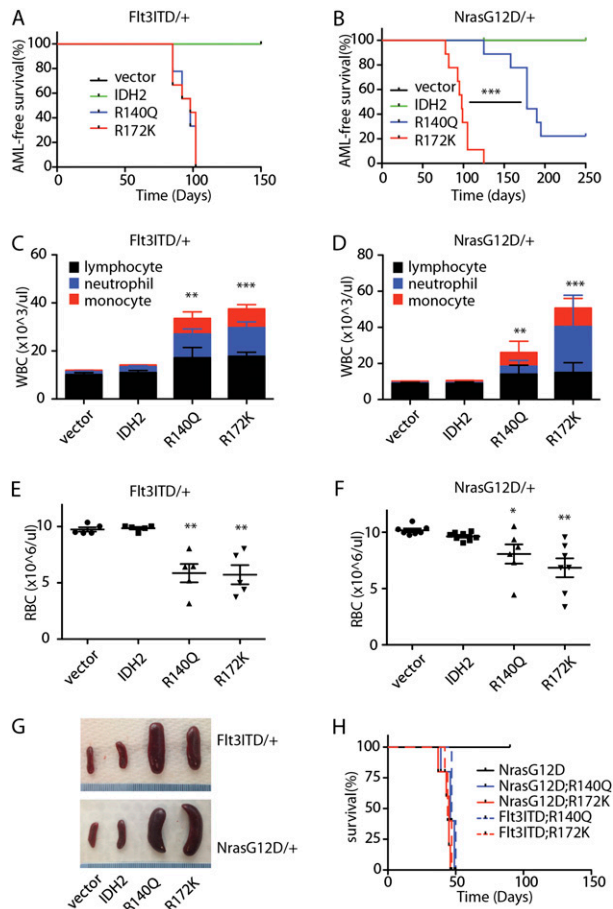


Figure 1. IDH2 mutants cooperate with class I mutations to promote leukemia. (A) Kaplan-Meier survival curve of mice transplanted with Flt3-ITD HSPCs transduced with empty MSCV-IRES-GFP vector (pMIG), IDH2 wild type (WT), or mutants (IDH2^{R140Q} and IDH2^{R172K}). *n* = 9. (B) Kaplan-Meier survival curve of mice transplanted with Nras^{G12D} HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). *n* = 9. (C) White blood cell (WBC) counts of mice transplanted with Flt3-ITD HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}) at 12 wk after transplantation. *n* = 5. (D) WBC counts of mice transplanted with Nras^{G12D} HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}) at 12 wk after transplantation. *n* = 5. (E) Red blood cell (RBC) counts of mice transplanted with Flt3-ITD HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}) at 12 wk after transplantation. *n* = 5. (F) RBC counts of mice transplanted with Nras^{G12D} HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}) at 12 wk after transplantation. *n* = 5. (G) Representative pictures of the spleens of recipient mice transplanted with Flt3-ITD or Nras^{G12D} HSPCs transduced with empty vector (pMIG), IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (H) Kaplan-Meier survival curve of the secondary recipient mice transplanted with Flt3-ITD or Nras^{G12D} HSPCs transduced with IDH2 wild type or mutants (IDH2^{R140Q} and IDH2^{R172K}). *n* = 5. (**P* < 0.05; (**)*P* < 0.01; (***)*P* < 0.001.

animals to histopathological analyses and immunophenotyping. At the time of sacrifice, all recipients of HSPCs harboring Flt3-ITD or Nras^{G12D} and an IDH2 mutant contained numerous circulating blasts in the peripheral blood (Fig. 2A). Leukemic cells replaced normal hematopoietic cells within the BM (Fig. 2B) and spleen and disseminated to normally nonhematologic organs such as the liver (Supplemental Fig. 2A,B). Flow cytometry revealed that all leukemic cells expressed mutant IDH2, indicated by GFP expression (Supplemental Fig. 3). Whether obtained from the spleen or BM, they expressed the myeloid marker Mac-1 and/or c-kit, a marker of more immature myeloid progenitors (MPs) (Fig. 2C,D,G).

Peripheral blood from recipient mice transplanted with HSPCs expressing the control vector or one encoding wild-type IDH2 contained mostly CD3⁺ or Thy1⁺ T cells and B220⁺ or CD19⁺ B cells, which is consistent with the cellular composition of blood from nonleukemic mice (Fig. 2E,H). In contrast, the peripheral blood from mice bearing leukemia driven by IDH2^{R140Q} or IDH2^{R172K} was overrun with CD3⁻ B220⁻ Mac1⁺ or Thy1⁻ CD19⁻ Mac1⁺ cells (Fig. 2F,I). Taken together, these features indicate that IDH2 mutants in combination with Flt3-ITD or NrasG12D drive disease resembling human AML.

In vitro studies indicate that the high 2-HG levels produced by IDH2 mutants can impair the functions of TET proteins that convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), resulting in a decrease of 5hmC levels (Figueroa et al. 2010). Accordingly, murine AMLs triggered by either IDH2^{R140Q} or IDH2^{R172K} showed a marked increase in 2-HG production relative to nonleukemic BM, the immortalized myeloid cell line 32D, and AML induced by other oncogenes (Fig. 3A). Interestingly, although in vitro studies indicate that IDH2^{R172K} produces more 2-HG than IDH2^{R140Q} (Ward et al. 2013), the 2-HG levels present in Flt3-ITD;IDH2^{R140Q} and Flt3-ITD;IDH2^{R172K} AMLs were comparable, perhaps owing to the lower expression of IDH2^{R172K} than that of IDH2^{R140Q} (data not shown). Analysis of DNA methylation by dot blotting of normal and leukemia DNA showed no significant difference in the global levels of 5mC in AMLs expressing IDH2 mutants. However, consistent with their ability to inhibit TET proteins, IDH2 mutant leukemia displayed a massive reduction in 5hmC levels (Fig. 3B).

IDH2^{R172} mutations are associated with poor prognosis in AML patients, while the clinical effects of IDH2^{R140} mutations are controversial (Marcucci et al. 2010; Paschka et al. 2010; Patel et al. 2012). As underlying differences in survival of patients with different AML genotypes can be linked to treatment response (Zuber et al. 2009; Patel et al. 2012), we asked whether IDH2 mutant AMLs were intrinsically sensitive or resistant to ara-C chemotherapy. Consistent with previous reports (Zuber et al. 2009), Nras^{G12D};AML1-ETO leukemic cells were much more sensitive to ara-C than Nras^{G12D};MLL-AF9 leukemic cells in a 3-d treatment assay (IC₅₀ = 39 vs. 63 nM; *P* = 0.03). In contrast, both Nras^{G12D};IDH2^{R140Q} and Nras^{G12D};IDH2^{R172K} AML cells were highly resistant to ara-C in vitro (IC₅₀ = 93 and 345 nM, respectively; *P* = 0.0001 for

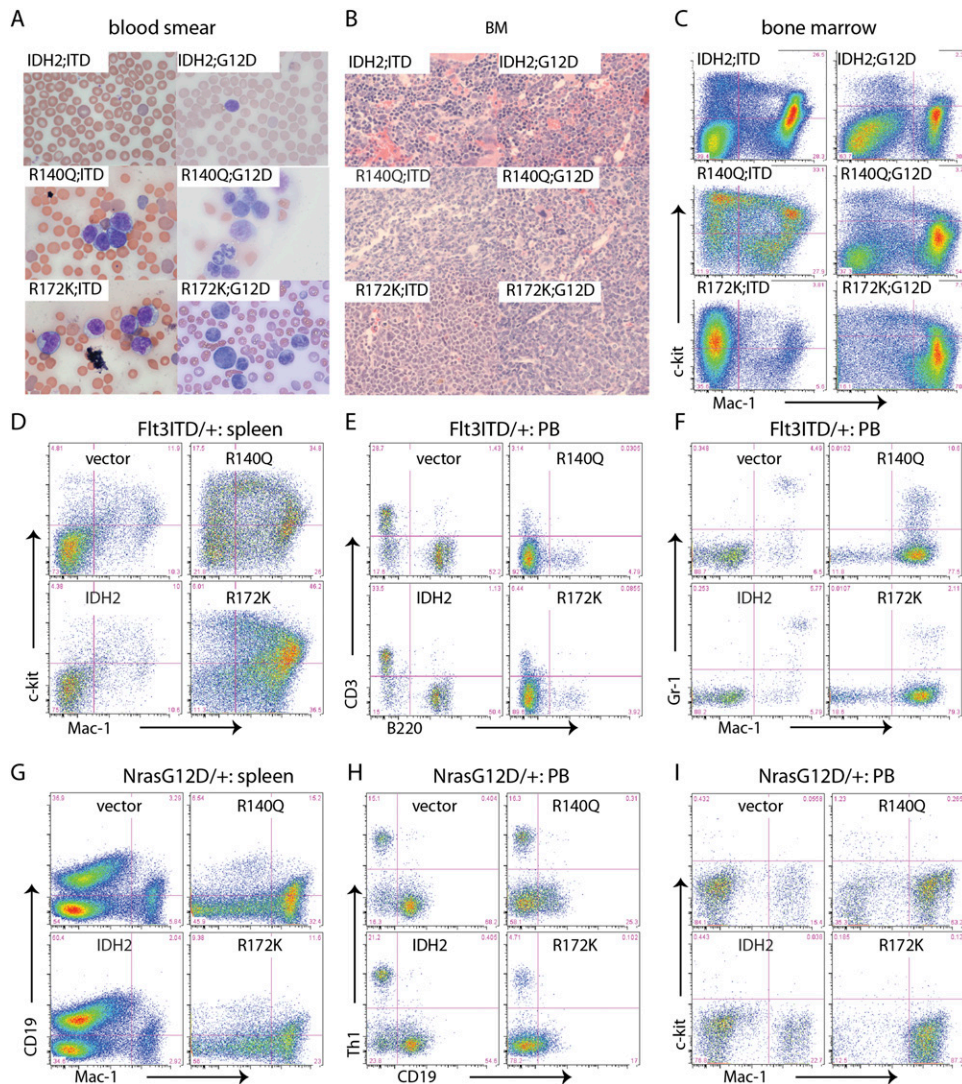


Figure 2. IDH2 mutations result in AML. (A) Blood smear of recipient mice transplanted with Flt3-ITD or Nras^{G12D} HSPCs transduced with IDH2 wild type (WT) or mutants (IDH2^{R140Q} and IDH2^{R172K}). (B) H&E staining of BM sections (400×) of recipient mice transplanted with IDH2 wild-type- or mutant-expressing cells. (C) Representative flow plots of BM cells from recipient mice transplanted with Flt3-ITD or Nras^{G12D} HSPCs transduced with IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (D) Representative flow plots of splenocytes stained with Mac-1 and c-kit of recipients transplanted with Flt3-ITD cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (E) Representative flow plots of peripheral blood stained with B220 and CD3 of recipients transplanted with Flt3-ITD cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (F) Representative flow plots of peripheral blood stained with Mac-1 and Gr-1 of recipients transplanted with Flt3-ITD cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (G) Representative flow plots of splenocytes stained with Mac-1 and CD19 of recipients transplanted with Nras^{G12D} cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (H) Representative flow plots of peripheral blood stained with CD19 and Thy1 of recipients transplanted with Nras^{G12D} cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}). (I) Representative flow plots of peripheral blood stained with Mac-1 and c-kit of recipients transplanted with Nras^{G12D} cells transduced with empty vector, IDH2 wild type, or mutants (IDH2^{R140Q} and IDH2^{R172K}).

Nras^{G12D};IDH2^{R140Q} vs. Nras^{G12D};AML1-ETO; $P < 0.0001$ for Nras^{G12D};IDH2^{R172K} vs. Nras^{G12D};AML1-ETO). Nras^{G12D};IDH2^{R172K} AML cells were even more resistant to ara-C than Nras^{G12D};IDH2^{R140Q} AML cells ($P = 0.0002$), an intriguing observation in light of the poorer prognosis of patients with IDH2^{R172} mutations (Fig. 3C). This resistance was more striking in vivo, where some ara-C-treated mice harboring IDH2 mutant AMLs died even earlier than vehicle-treated controls (Fig. 3D). Therefore, IDH2

mutants can cooperate with class I mutations to promote AML with pathological, biological, and molecular features of the human disease.

IDH2 mutants are sufficient to suppress hematopoietic differentiation and alter DNA methylation in vivo

To determine whether the tumorigenic phenotype driven by mutant IDH2 might derive from their ability to impair

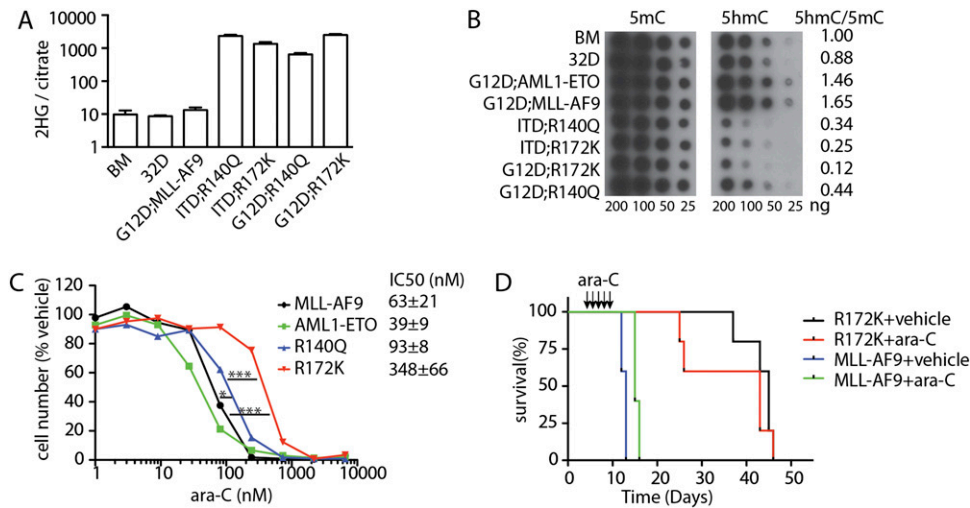


Figure 3. IDH2 mutant-induced AMLs display high 2-HG levels and altered DNA methylation and are chemoresistant. (A) 2-HG levels in BM, 32D, and MLL-AF9 or IDH2 mutant-induced AMLs. $n = 3-5$. (B) Representative dot blotting with antibodies against 5mC and 5hmC displaying DNA methylation of nonleukemic (BM and 32D) and leukemic cells. (C) Dose response of $Nras^{G12D};MLL-AF9$, $Nras^{G12D};AML1-ETO$, $Nras^{G12D};IDH2^{R140Q}$, and $Nras^{G12D};IDH2^{R172K}$ leukemic cells to ara-C. Leukemic cells were treated with vehicle or 1–6000 nM ara-C for 3 d. $n = 4$. (D) Kaplan-Meier survival curve of mice transplanted with $Nras^{G12D};MLL-AF9$ or $Nras^{G12D};IDH2^{R172K}$ leukemic cells treated with ara-C or vehicle. $n = 5$.

differentiation in vivo, we examined the impact of IDH2 mutants on hematopoiesis using a competitive reconstitution assay. Here, CD45.1 HSPCs were transduced with a control vector expressing GFP alone or together with GFP vectors encoding either wild-type IDH2, IDH2^{R140Q}, or IDH2^{R172K}; the resulting populations were then mixed with CD45.2 BM cells as a competitive reference and transplanted into lethally irradiated CD45.1 mice (Fig. 4A). Peripheral blood, spleen, and BM from recipient animals were analyzed for cellular composition, 2-HG production, and changes in DNA methylation.

As expected, white blood cell (WBC) counts in mice receiving HSPCs transduced with empty or wild-type IDH2-expressing vector increased with time after transplantation (Supplemental Fig. 4A). Under these conditions, transplanted cells were able to reconstitute a normal hematopoietic compartment, which displayed the expected distributions of Lin⁻ Sca⁺ c-Kit⁺ (LSK) HSPCs, Lin⁻ Sca⁻ c-Kit⁺ (MP), and other hematopoietic cells (Fig. 4B,D). If anything, empty vector and wild-type IDH2 appeared to confer a competitive disadvantage during hematopoietic reconstitution, as virtually no GFP⁺ cells remained after 12 wk, with most cells being derived from untransduced or competitor cells (Fig. 4E).

Mice reconstituted with HSPCs expressing IDH2^{R140Q} and IDH2^{R172K} showed a markedly different phenotype. Although at 4 wk post-transduction these mice retained WBC counts comparable with controls, by 12 wk, they showed significantly reduced WBC and had developed an anemia (Fig. 4F; Supplemental Fig. 4A) associated with significantly reduced BM cellularity (Fig. 4G). Nevertheless, IDH2 mutant cells (GFP⁺) predominated over controls (GFP⁻), suggesting that these cells outcompeted and excluded the nontransduced and cotransplanted CD45.2

cells (Fig. 4E). These changes were associated with an increased fraction of LSK⁺ and MPs in the BM (Fig. 4B–D). Although LSK cells expressing both IDH2 mutants showed slightly increased BrdU incorporation when compared with controls, the opposite occurred in the MP compartment, where IDH2 mutant MP cells displayed reduced BrdU incorporation (Fig. 4H,I). The reduced proliferation and increased percentage of MPs in recipients of IDH2 mutants were associated with a skewing of BM differentiation toward the myeloid lineage (Fig. 4D) comparable with phenotypes observed in *IDH1*^{R132H} knock-in mice (Sasaki et al. 2012). Thus, IDH2 mutant proteins block the differentiation of MP cells in vivo, leading to the accumulation of more immature cell populations.

As expected, BM cells derived from mice reconstituted with HSPCs expressing IDH2^{R140Q} or IDH2^{R172K} contained high levels of 2-HG (Fig. 5A). In contrast to what has been observed in other systems (Xu et al. 2011; Lu et al. 2012), no obvious changes in the global abundance of methylated histones (H3K4me3, H3K9me3, and H3K27me3) were observed in BM cells expressing IDH2 mutants (Supplemental Fig. 5) relative to vector-only or wild-type IDH2-expressing cells, raising the possibility that IDH2 mutants can have tissue-specific effects on chromatin biology and cell fate. Nevertheless, these BM cells showed significantly decreased 5hmC levels without overt changes in global 5mC levels (Fig. 5B), an effect that was also observed in c-kit-positive cells subjected to flow cytometry (Fig. 5C,D; Supplemental Fig. 5B). Overexpression of wild-type IDH2 did not have significant effects on 5hmC levels (Fig. 5B,D; Supplemental Fig. 5B). Hence, IDH2 mutants are sufficient to drive 2-HG production and alter DNA methylation during leukemogenesis.

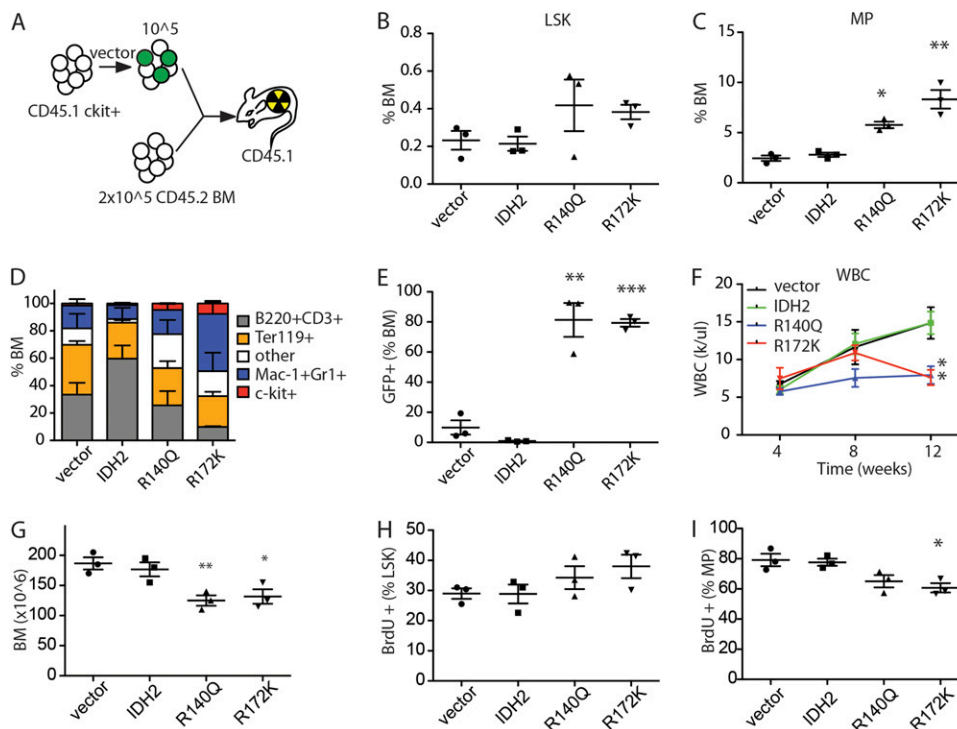


Figure 4. IDH2 mutations are sufficient to block differentiation of HSPCs and alter DNA methylation. (A) Schematic diagram of competitive transplant assay. Vector refers to transduction of cells with empty MSCV-IRES-GFP vector or one coexpressing wild-type IDH2 or an IDH2 mutant. CD45.1 c-kit⁺ HSPCs (10^5) transduced with vectors were mixed with 2×10^5 CD45.2 BM and transplanted into lethally irradiated CD45.1 recipient mice. (B) Percentage of lin⁻Sca-1⁺c-kit⁺ (LSK) cells in the whole BM of recipient mice 12 wk after transplantation. $n = 3$. (C) Percentage of lin⁻Sca-1⁻c-kit⁺ (MP) cells in the whole BM of recipient mice 12 wk after transplantation. $n = 3$. (D) Percentage of c-kit⁺, Mac-1⁺ or Gr-1⁺, B220⁺ or CD3⁺, and Ter119⁺ cells in the whole BM of recipient mice 12 wk after transplantation. $n = 3$. (E) Percentage of GFP⁺ cells in the whole BM of recipient mice 12 wk after transplantation. $n = 3$. (F) WBC counts of recipient mice with IDH2 wild-type- or mutant-expressing cells. $n = 4-5$. (G) BM cellularities of recipient mice with IDH2 wild-type- or mutant-expressing cells at 12 wk after transplantation. $n = 3$. (H) BrdU incorporation rate of LSK cells after 1 d of labeling. $n = 3$. (I) BrdU incorporation rate of MP cells after 1 d of labeling. $n = 3$. Of note, in B–G, vector, IDH2, or IDH2 mutants refers to mice transplanted with HSPCs transduced with the indicated vector but does not necessarily indicate that all of the cells analyzed expressed the constructs. Specifically, while BM from mice transplanted with IDH2 mutant cells almost completely consisted of GFP⁺ IDH2 mutant-expressing cells (shown in E), empty vector and wild-type IDH2 produced a competitive disadvantage, and BM consisted entirely of GFP⁻ normal competitors at this time point.

IDH2 mutants are required for leukemia maintenance

The availability of IDH mutant-driven leukemia models enabled us to assess whether ongoing expression of IDH2 mutants and their product, 2-HG, were required for tumor maintenance. AGI-6780 is a commercially available small-molecule inhibitor that targets IDH2^{R140Q} but not IDH2^{R172K} and has been shown to have anti-leukemic activity in human AML (Wang et al. 2013). To assess its activity against IDH2 mutant-driven murine AML, control Nras^{G12D};MLL-AF9 leukemia or Nras^{G12D} leukemias coexpressing IDH2^{R140Q} or IDH2^{R172K} were treated with AGI-6780 in vitro, and 2-HG production, cellular proliferation, and differentiation were monitored. Within 2 d, AGI-6780 inhibited 2-HG production in AML cells expressing IDH2^{R140Q} but not control or IDH2^{R172K}-expressing leukemia cells (Fig. 6A). Remarkably, this effect was associated with a 2-wk proliferative burst that was unique to IDH2^{R140Q}-expressing AML (Fig. 6B), after which these cells ceased to proliferate and underwent

differentiation (Fig. 6C,D). This proliferative burst and eventual differentiation have also been seen following AGI-6780 treatment of a human AML explant harboring the IDH2^{R140Q} allele (Wang et al. 2013).

Small-molecule inhibitors of the IDH2^{R172K} protein are not currently available. To test its role in leukemia maintenance, we designed shRNA specifically targeting human IDH2, thus inhibiting only the exogenously transduced IDH2 mutant used in this model (Fig. 6E,F). Control (shRen, a potent shRNA targeting Renilla luciferase) and IDH2-specific shRNAs were cloned downstream from a tetracycline-responsive element and dsRed and retrovirally transduced into IDH2 mutant leukemia cells also expressing a rtTA transgene, thereby making the shRNAs doxycycline-inducible (Zuber et al. 2011a). Cells were treated with doxycycline and analyzed for the prevalence of the dsRed marker (and thus the competitive fitness of shRNA-expressing cells) over time.

As expected, IDH2 shRNAs had no effect on MLL-AF9-driven AML. However, similar to the effects of AGI-6780,

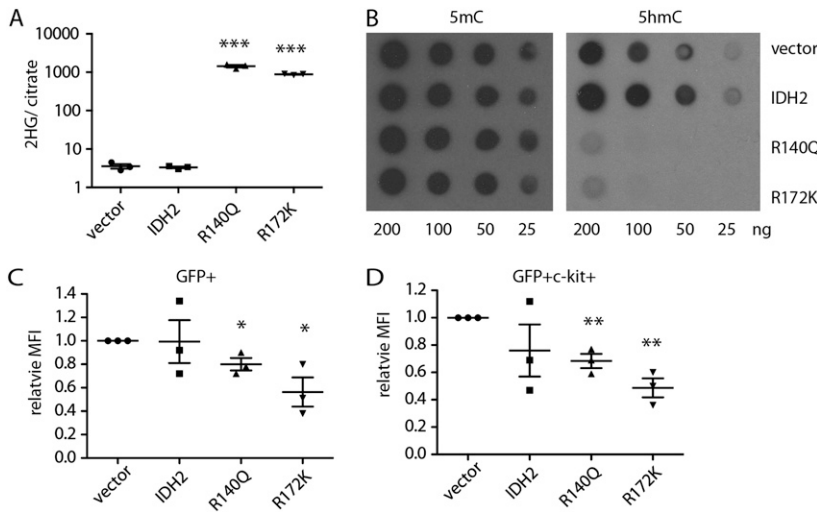


Figure 5. IDH2 mutants lead to abnormal DNA methylation in hematopoietic cells. (A) 2-HG levels in the whole BM cells of recipient mice. $n = 3$. (B) Representative dot blots showing 5mC and 5hmC levels of whole BM genomic DNA. (C) Relative mean fluorescence intensity (MFI) of 5hmC in GFP⁺ BM cells. $n = 3$. (D) Relative MFI of 5mC in GFP⁺ c-kit⁺ BM cells. $n = 3$.

inhibition of IDH2^{R172K} by RNAi led to an initial enhanced proliferation and subsequent depletion of shRNA-expressing cells from the population (Fig. 6H), an effect associated with myeloid differentiation (Fig. 6G). Thus, ongoing expression of IDH2 mutants is required to block differentiation, although long periods of IDH2 inhibition are required to reverse these effects. In principle, the delay in response may reflect the requirement for cell division to renormalize the epigenetic changes produced by mutant IDH2.

IDH mutant AMLs rapidly differentiate in response to Brd4 inhibition

The BET family protein BRD4 has been identified as a therapeutic target in AML produced by MLL fusion oncoproteins owing to its ability to sustain an aberrant self-renewal circuit controlled by Myc (Dawson et al. 2011; Zuber et al. 2011b). To test whether the aberrant self-renewal program produced by IDH2 mutants is also sensitive to Brd4 inhibition, we examined the impact of two independently validated Brd4 shRNAs (Zuber et al. 2011b) or the small-molecule Brd4 inhibitor JQ1 on leukemic cell proliferation, viability, differentiation, and Myc levels in IDH2 mutant AML. Cultured cells were infected with the doxycycline-inducible construct used above expressing either shRNAs targeting Brd4 or Renilla as a control. Brd4 suppression acutely reduced the competitive fitness of cultured Nras^{G12D};IDH2^{R172K} cells compared with controls, suggesting that Brd4 is essential to maintain IDH2 mutant leukemia (Fig. 7A).

In parallel, we tested response to the small-molecule JQ1 in vitro. As expected, Nras^{G12D};MLL-AF9 AML cells were highly sensitive to JQ1 (IC₅₀, 58 nM) (Zuber et al. 2011b), while Nras^{G12D};AML1-ETO AML cells were more resistant (IC₅₀, 1159 nM). IDH mutant AMLs were even more sensitive to JQ1 than Nras^{G12D};MLL-AF9-expressing cells (IC₅₀, 34 nM and 19 nM for Nras^{G12D};IDH2^{R140Q} and Nras^{G12D};IDH2^{R172K}, respectively; $P = 0.01$ for Nras^{G12D};IDH2^{R140Q} vs. Nras^{G12D};MLL-AF9;

$P < 0.0001$ for Nras^{G12D};IDH2^{R172K} vs. Nras^{G12D};MLL-AF9) (Fig. 7B).

The hypersensitivity of IDH mutant AMLs to Brd4 inhibition was associated with rapid differentiation after either shRNA Brd4 knockdown or JQ1 treatment (Fig. 7C–E). Nevertheless, JQ1 treatment did not affect 2-HG levels in either IDH2^{R140Q} or IDH2^{R172K} cells (Fig. 7F), perhaps indicating that Brd4 inhibition acts downstream from mutant IDH2 proteins and mediates its effects without directly affecting its neomorphic activity. However, as has been reported in MLL-AF9-driven AML, immunoblotting of IDH2 mutant AML lysates treated for 48 h with JQ1 revealed reduced levels of Myc (Fig. 7G). Therefore, IDH2 mutant AMLs are addicted to a Brd4-driven, Myc-dependent, self-renewal program that can be inhibited by JQ1 treatment.

Although the pharmacologic properties of JQ1 are not ideal for in vivo studies (Matzuk et al. 2012), we tested its activity against IDH2 mutant AML in vivo. Mice were transplanted with IDH2^{R172K} AML and, upon engraftment, treated (day 5) with vehicle or 50 mg/kg per day JQ1 by gavage for 2 wk (Filippakopoulos et al. 2010; Zuber et al. 2011b). Leukemic cells were apparent in the peripheral blood in vehicle-treated mice at day 19 but completely absent in JQ1-treated animals (Fig. 7H). JQ1-treated mice also displayed significantly improved erythropoiesis, leading to reduced anemia (Fig. 7I). While all vehicle-treated mice died within 3 d post-treatment, JQ1 treatment extended median survival to 18 d (Fig. 7J). Taken together, IDH mutant-driven AML is susceptible to Brd4 inhibition both in vitro and in vivo.

Discussion

A mouse AML model driven by IDH2 mutants suggests therapeutic approaches for AML treatment

In vivo cancer models are essential for understanding genetic alterations that contribute to oncogenesis and can provide preclinical systems for testing novel therapies.

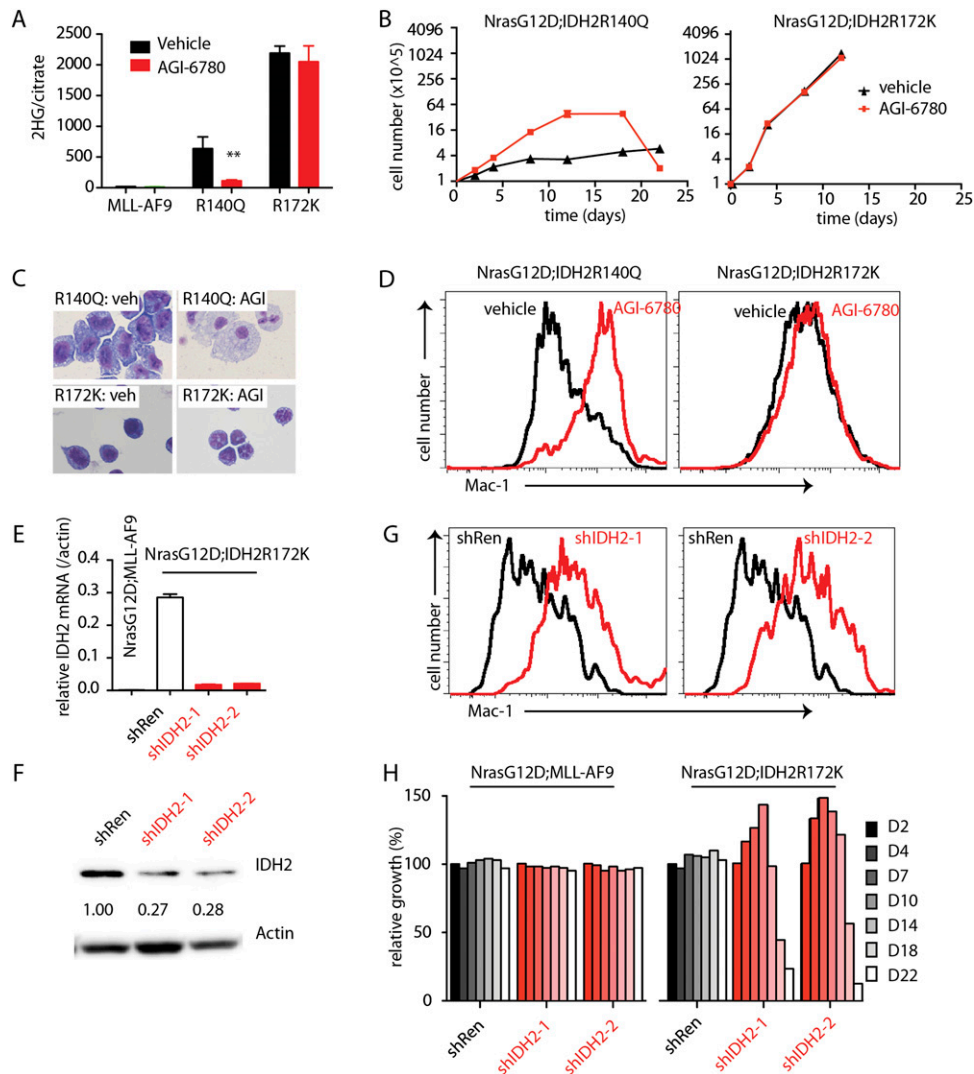


Figure 6. Inhibition of IDH2 mutants leads to loss of 2-HG and delayed differentiation. (A) 2-HG levels of leukemic cells treated with 5 μ M AGI-6780 for 2 d. $n = 3$. (B) Growth of leukemic cells treated with 5 μ M AGI-6780 or vehicle. $n = 4$. (C) Representative H&E staining of cytopsin of Nras^{G12D};IDH2^{R140Q} and Nras^{G12D};IDH2^{R172K} leukemic cells treated with vehicle or 5 μ M AGI-6780 at day 22. (D) Expression levels of Mac-1 by Nras^{G12D};IDH2^{R140Q} and Nras^{G12D};IDH2^{R172K} leukemic cells treated with vehicle or 5 μ M AGI-6780 at day 22. (E) Transcript levels of hIDH2 in Nras^{G12D};IDH2^{R172K} cells expressing shRen or shIDH2, normalized to actin mRNA. $n = 4$. (F) Western blot demonstrating hIDH2 knockdown in Nras^{G12D};IDH2^{R172K} leukemic cells by shRNAs against mutant IDH2. (G) Relative growth of Nras^{G12D};MLL-AF9 and Nras^{G12D};IDH2^{R172K} leukemic cells with shRen or shIDH2. $n = 3$. (H) Expression levels of Mac-1 by Nras^{G12D};IDH2^{R140Q} and Nras^{G12D};IDH2^{R172K} leukemic cells treated with shRen or shIDH2.

Here we demonstrate that the IDH2^{R140Q} and IDH2^{R172K} mutants observed in human cancers can be potent oncogenes in mice, acting as class II driver mutations that cooperate with the class I mutations Flt3-ITD and Nras^{G12D} to promote aggressive AML. Murine AMLs expressing IDH2 mutants display the histopathological and molecular features that are characteristic of the human disease and show a marked chemoresistance phenotype that may underlie the association between IDH2^{R172K} mutations and poor patient survival.

IDH2 mutant proteins promote AML by blocking the differentiation of HSPCs; in the absence of a cooperating event, this produces an MDS-like state, a disorder in

which IDH2 mutations are frequent in humans (Patnaik et al. 2012). While these effects correlate with the ability of IDH2 mutants to produce 2-HG and alter DNA methylation, our experiments do not prove a causal role for these downstream changes and disease etiology or maintenance. Still, in a parallel study (Lu et al. 2013), IDH2^{R172K} acts similarly to drive the malignant conversion of mesenchymal progenitor cells into sarcoma in vivo, which also correlates with its ability to produce 2-HG, block differentiation, and alter DNA methylation. Irrespective of the precise mechanism, these data imply a broad and potent action of IDH mutant oncogenes across diverse disease states.

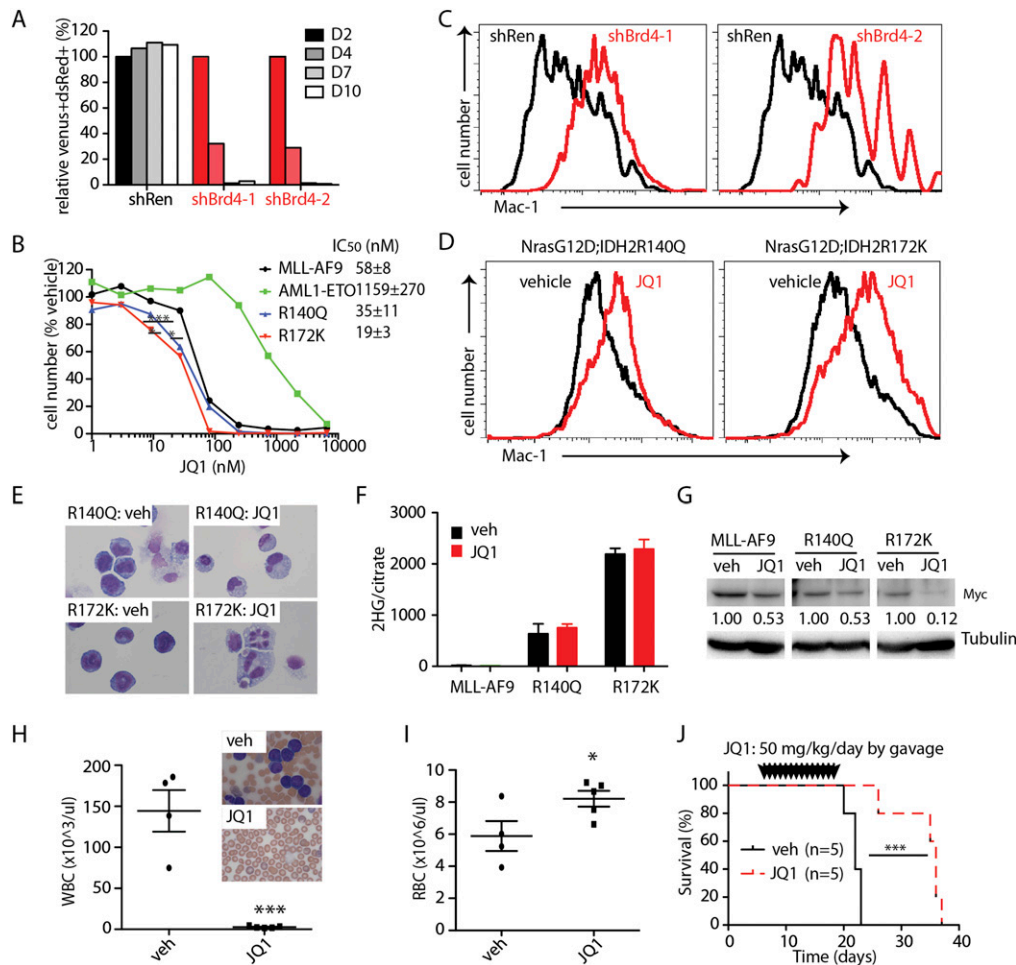


Figure 7. IDH mutant leukemias rapidly differentiate in response to Brd4 inhibition. (A) Relative growth of $Nras^{G12D};IDH2^{R172K}$ leukemic cells with Brd4 knockdown. $n = 3$. (B) Dose response of $Nras^{G12D};MLL-AF9$, $Nras^{G12D};AML1-ETO$, $Nras^{G12D};IDH2^{R140Q}$, and $Nras^{G12D};IDH2^{R172K}$ to JQ1. Leukemic cells were treated with vehicle or 1–6000 nM JQ1 for 3 d. $n = 4$. (C) Representative flow plots showing Mac-1 expression levels in $Nras^{G12D};IDH2^{R172K}$ leukemic cells with shRen or shBrd4 3 d after doxycycline induction. (D) Representative flow plots showing Mac-1 expression levels in $Nras^{G12D};IDH2^{R140Q}$ and $Nras^{G12D};IDH2^{R172K}$ leukemic cells treated with vehicle or 50 nM JQ1 for 2 d. (E) Representative cytospin staining of $Nras^{G12D};IDH2^{R140Q}$ and $Nras^{G12D};IDH2^{R172K}$ leukemic cells treated with vehicle or 50 nM JQ1 for 2 d. (F) 2-HG levels of $Nras^{G12D};MLL-AF9$, $Nras^{G12D};IDH2^{R140Q}$, and $Nras^{G12D};IDH2^{R172K}$ leukemic cells treated with vehicle or 50 nM JQ1 for 2 d. $n = 3$. (G) Western blotting of Myc levels in $Nras^{G12D};MLL-AF9$, $Nras^{G12D};IDH2^{R140Q}$, and $Nras^{G12D};IDH2^{R172K}$ leukemic cells treated with vehicle or 50 nM JQ1 for 2 d. The numbers indicate normalized Myc levels by densitometry. (H) WBC counts of $Nras^{G12D};IDH2^{R172K}$ recipient mice treated with vehicle or JQ1 at day 20 after transplant. Inserts show the representative blood smear of vehicle- or JQ1-treated mice. Vehicle, $n = 4$; JQ1, $n = 5$. (I) RBC counts of $Nras^{G12D};IDH2^{R172K}$ recipient mice treated with vehicle or JQ1 20 d after transplant. Vehicle, $n = 4$; JQ1, $n = 5$. (J) Kaplan-Meier survival curve of $Nras^{G12D};IDH2^{R172K}$ recipient mice treated with vehicle or JQ1. The mice were treated with vehicle or 50 mg/kg per day JQ1 by gavage from day 5 to day 18 after transplant. $n = 5$.

Owing to their similarity to the human disease, the AML models described herein may be useful for evaluating therapies to target IDH2 mutant leukemia. Using genetic and pharmacological tools to manipulate IDH2 activity, we show that IDH2 mutants are required for sustained 2-HG production and leukemia maintenance. Suppression of IDH2 mutant levels and its neomorphic activity triggered myeloid differentiation, albeit requiring prolonged IDH2 inhibition. This delayed response may reflect the requirement for multiple rounds of proliferation to restore normal epigenetic states but might also present a confounding factor when treating patients with

acute leukemia. In contrast, shRNA or small-molecule-mediated inhibition of Brd4 causes rapid terminal differentiation and elimination of IDH2 mutant leukemia cells even in the presence of sustained 2-HG levels. This effect is associated with loss of a Myc-dependent self-renewal circuit and triggers substantial anti-leukemic effects in vivo. Notably, $IDH2^{R172K}$ mutant AML, which is not inhibited by AGI-6780 (Wang et al. 2013) and is associated with poor prognosis (Marcucci et al. 2010; Paschka et al. 2010; Patel et al. 2012), displays the highest sensitivity to Brd4 inhibition.

While high-dose chemotherapy can be highly effective against AML, these agents produce substantial toxicity,

and patients frequently relapse with resistant disease. One notable exception involves the treatment of acute promyelocytic leukemia (APL), which can be cured without substantial toxicity by a combination of all *trans* retinoic acid (ATRA) together with arsenic or other molecularly targeted agents (Shen et al. 1997). In contrast to conventional chemotherapy, which triggers leukemia cell death, ATRA acts more directly by reversing the differentiation block produced by the driving PML-RAR α oncoprotein (Zhang et al. 2000). As shown here, similar effects can be achieved in murine IDH2 mutant AMLs following IDH2 or Brd4 inhibition, raising hope that improved agents will produce sustained anti-leukemic responses in patients. In any event, these observations validate IDH2 as a therapeutic target in AML and point to an alternative approach to IDH2 inhibition for treatment of IDH2 mutant cancers.

Materials and methods

Mice

All mouse experiments were conducted in accordance with institutional guidelines at Memorial Sloan-Kettering Cancer Center (MSKCC). Recipient C57Bl/6 mice (National Cancer Institute) were irradiated at a dose of 4.5 Gy (Cs137) for sublethal irradiation or two doses of 5.5 Gy for lethal irradiation before transplantation. Mice were transplanted with 1×10^6 or the indicated number of cells by tail vein injection. Mice were monitored for leukemogenesis by complete blood cell count (Hemavet, Drew Scientific), spleen palpation, and blood smear.

Plasmid construction

Human IDH2 wild type and mutants R140Q and R172K were cloned into a pMIG vector (Lu et al. 2012). shRNAs against Renilla (TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGATGTATAGATAAGCATTATAATTCCTATGCCTACTGCCTCGGA), IDH2 (shIDH-1, TGC TGTGACAGTGAGCGCCAAGCTGAAGAAGATGTGGAA TAGTGAAGCCACAGATGTATCCACATCTTCTTCAGCTT GATGCCTACTGCCTCGGA; shIDH2-2, TGCTGTTGACA GTGAGCGCTAAGACCGACTTCGACAAGAATAGTGAA GCCACAGATGTATTCTTGTGCGAAGTCGGTCTTATTGCCT ACTGCCTCGGA), and Brd4 (shBrd4-1, TGCTGTTGACAGTG AGCGCCCATGGATATGGGAACAATATAGTGAAGCCACA GATGTATATTGTTCCATATCCATGGGTTGCCTACTGCCT CGGA; shBrd4-2; TGCTGTTGACAGTGAGCGCACACAATCAA GTCTAAACTAGATAGTGAAGCCACAGATGTATCTAGTTTA GACTTGATTGTGCTGCCTACTGCCTCGGA) (Zuber et al. 2011b) were cloned into a TRIN (Tre-dsRed-mir30-PGK-Venus-IRES-Neo) vector (Zuber et al. 2011a).

Cell culture and transduction

BM cells were isolated from the indicated young adult donor mice, and c-kit-positive HSPCs were separated by autoMACS (Miltenyi Biotec, Inc.). HSPCs were cultured with stem cell medium as described (Schmitt et al. 2002; Zuber et al. 2009). IDH2 mutant AMLs were cultured with IMDM medium supplemented with 20% FBS and 0.2 ng/mL IL-3. Nras^{G12D};MLL-AF9 AML was cultured with RPMI-1640 plus 10% FBS. Retroviruses were made by calcium phosphate-mediated transfection of

Plat-E (Morita et al. 2000) packaging cells. HSPCs and AML cells were transfected by spinoculation.

Measurement of 2-HG

Frozen cell pellets were extracted with 1 mL of ice-cold 80% methanol containing 20 μ M deuterated 2-HG as an internal standard (D-hydroxyglutaric-2,3,3,4,4-d5). Methanol extracts were incubated at -80°C for 30 min and centrifuged at 21,000g for 20 min at 4°C to remove precipitated protein. Nine-hundred microliters was evaporated to dryness under a nitrogen gas stream. Dried organic acids were derivatized by the sequential addition of 50 μ L of 40 mg/mL methoxyamine hydrochloride in pyridine with incubation for 90 min at 30°C followed by 80 μ L of MSTFA + 1% TMCS (Thermo Scientific) and 70 μ L of ethyl acetate with incubation for 30 min at 37°C using an automated sample preparation platform (Gerstel). One microliter of the trimethylsilyl-derivatized organic acids was analyzed by GC-MS using an Agilent 7890A gas chromatograph with an HP-5MS capillary column connected to an Agilent 5975 C mass selective detector operating in splitless mode with electron impact ionization. Relative quantitation of 2-HG was determined from extracted ion chromatograms (EICs) for 2-HG (m/z : 349 or 247) normalized to the EICs of intracellular citrate (m/z : 465).

Histology and pathology assay

Bone, spleen, and liver were fixed in 10% formalin. Embedding, sectioning, and H&E staining were performed by IDEXX RADIL. Blood smears were stained with Hema-Quik II stain solution (Fisher Scientific). Cytospin was performed on Shandon Cytospin 4 and then stained with JorVet DipQuick stain.

Flow cytometry

Flow cytometry was performed on LSR II or Fortessa machines (BD Bioscience). All of the cell surface marker antibodies were from eBioscience. 5-hmC antibodies were from Active Motif (no. 39769). Cell surface marker staining was performed with Hank's balanced salt solution plus 2% FBS. For 5-hmC staining, cells were fixed and permeabilized with BD fix/perme buffer and then treated with 0.3 mg/mL DNase I for 30 min at 37°C . For the BrdU incorporation assay, mice were intraperitoneally (i.p.) injected with 100 mg/kg BrdU and then fed with 1 mg/mL BrdU in water for 24 h (Chen et al. 2008). BrdU staining was performed with a BD BrdU kit (BD Bioscience).

Dot blotting

Dot blotting was performed as described (Ko et al. 2010). Briefly, purified genomic DNA was quantified on NanoDrop and denatured by 0.1 M NaOH. Serial-diluted DNA was spotted on a nitrocellulose membrane. 5mC and 5hmC antibodies (Active Motif) were diluted at 1:1000 and 1:5000, respectively.

Western blotting

For IDH2 and Myc detection, cells were lysed in RIPA buffer, and lysates were separated with SDS-PAGE gel electrophoresis. For histone acid extraction, cells were lysed in hypotonic lysis buffer. The primary antibodies were anti-IDH2 (Abcam, ab55271), anti-c-Myc (Santa Cruz Biotechnology, sc-764), anti-actin (Sigma, T6074), anti-tubulin (Sigma, T9026), anti-H3K4me3 (Active Motif, 39916), anti-H3K9me3 (Active Motif, 39765), anti-H3K27me3 (Millipore, 07-449), and anti-H3 (Cell Signaling Tech, 4499).

Quantitative real-time PCR

mRNA was extracted from FACS-sorted cells with Trizol (Invitrogen), and then cDNA was made with SuperScript III (Invitrogen) according to the manufacturer's manual. Quantitative PCR was performed on a 7900HT Fast Real-Time system (Applied Biosystems). The sequences of PCR primers were mouse Actin forward (TGGTGATAAGTGGCCTTGAGTGT) and reverse (ATGCAAGGAGTGAAGAACACAGC) and hIDH2 forward (AGACCGACTTCCGACAAGAATAAG) and reverse (GACTGCACATCTCCGTCATAG).

Two-color shRNA competitive proliferation assay

Cells were transduced with shRNAs and then selected with G418 (US Biological). Infected cells were mixed with uninfected cells at a 1:1 ratio for the IDH2 knockdown experiment and 4:1 for the Brd4 knockdown experiment. Next, the shRNAs were induced by 1 mg/mL doxycycline (Sigma). The percentage of shRNA-expressing cells (Venus⁺dsRed⁺) was measured by a Guava easyCyte flow cytometer (Millipore) at the indicated time points.

Drug treatment

In vitro, cells were treated with vehicle, ara-C (Bedford Laboratories), AGI-6780 (Xcess Biosciences), or JQ1 for 3 d or the indicated times. Viable cells were counted by a Guava easyCyte flow cytometer. In vivo treatment, JQ1 was suspended in vehicle (0.5% [w/v] hydroxy-propyl-methylcellulose [Sigma], 0.2% [v/v] Tween 80 [Sigma]) at a concentration of 10 mg/mL. The suspension was sonicated before use. Mice with AMLs were treated with vehicle, 100 mg/kg per day ara-C by i.p. injection for 5 d, or 50 mg/kg per day JQ1 by gavage for 2 wk.

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