Supplementary Materials for

(*R*)-2-Hydroxyglutarate is Sufficient to Promote Leukemogenesis and its Effects are Reversible

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This PDF file includes:

Materials and Methods

Cell lines, Cell culture and Chemical treatments. 293TL cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. TF-1 cells (obtained from ATCC) were maintained in RPMI containing 10% FBS, 1% penicillin/streptomycin and 2 ng/mL recombinant human GM-CSF (R&D Systems). SCF ER-Hoxb8 cells (a kind gift from David Sykes, Massachusetts General Hospital and Mark Kamps, University of California-San Diego) were maintained in RPMI containing 10% FBS, 1% penicillin/streptomycin, 20 ng/mL recombinant mouse SCF (Peprotech) and 1 μM β-estradiol (Sigma). TF-1 cells infected with shRNA-expressing lentiviruses were selected with 1 μg/mL puromycin. Cells were treated with dimethyloxalylglycine (DMOG) (Frontier Scientific) for three hours. Cells were treated with 1 μM of the IDH1 inhibitor (a kind gift from Agios Pharmaceuticals, Inc.) prior to growth factor deprivation and EPO-stimulation or β-estradiol withdrawal.

Vectors. HA-tagged human IDH1 cDNAs (described previously (*8*)) were PCR-amplified and subcloned as StuI-NotI fragments into the LeGO-iG2 expression vector (*29*) (a kind gift from Kristoffer Weber, University Medical Center Hamburg-Eppendorf) and sequences were confirmed by DNA sequencing. Lentiviral pLKO.1 human TET1 shRNA vectors (TRCN0000075024 and TRCN0000075026), TET2 shRNA vectors (TRCN0000122172 and TRCN0000145351); and EglN1 shRNA vectors (TRCN0000001042; TRCN0000001044; TRCN0000001045 and TRCN00000010578) all carrying the puromycin resistance gene, were obtained from the Broad Institute TRC shRNA library. Lentiviral pLKO.1 human shRNA vectors used in the pooled shRNA screen were also all obtained from the Broad Institute TRC shRNA library.

Preparation of individual lentiviruses. Lentiviral particles were generated by calcium phosphate cotransfection of 293TL cells with LeGO-iG2 or pLKO expression vectors and the lentiviral packaging constructs psPAX2 (GAG-Pol) and pMD2.G (VSV-G) in a 2:2:1 ratio.

shRNA minipool creation. The shRNA minipool was generated as previously described (*30*). In brief, each of the ~800 shRNA plasmids was arrayed at a final concentration of 15 ng/μl. After pooling equal volumes of each shRNA, the pooled plasmids were used to transform ElectroMAX DH5α-E cells (Invitrogen) by electroporation and the cells were then plated onto two 24 × 24-cm^2 bioassay dishes (Nunc). DNA was purified from the plated transformants using a HiSpeed Plasmid Maxi Kit (Qiagen) and was used for virus production as previously described (*30*).

Lentiviral Infections. To perform lentiviral infections, TF-1 cells were spin-infected in 12-well plates as follows: 2 x 10^6 TF-1 cells were plated in growth media with lentiviral supernatant and 8 μg/mL polybrene, and the cells were centrifuged at 2000 rpm for 2 hours at RT. The infected cells were cultured overnight and then expanded the following morning. Puromycin selection of pLKO infections was started 24 hours after infection. Lentiviral expression was assessed: by flow cytometry for GFP and by western blotting

in the case of the LeGO-iG2 constructs; and by real-time qPCR and western blotting in the case of the individual pLKO constructs. The SCF ER-Hoxb8 cells were spin-infected as above, except that the cells and virus were centrifuged at 1000G for 90 minutes at RT. To perform large-scale infections for minipool screening, 3 x 10^7 TF-1 cells per replicate were resuspended in 24 ml of growth media containing 8 μg/ml polybrene. An appropriate volume of the pooled lentivirus was added to achieve an MOI of 0.3. This mixture was then split across a 12-well plate at 2 ml per well. A spin infection was performed by centrifugation at 2000 rpm for 2 hours at RT. After spin infection, cells were incubated for 4 hours and then supernatants were aspirated off and fresh media was added. 24 hours after infection the wells from each replicate were combined and cells were plated in growth media containing puromycin.

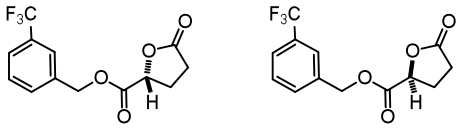
Immunoblot analysis. Whole cells extracts were prepared using RIPA lysis buffer (Boston Bioproducts) supplemented with a protease inhibitor cocktail (Roche), resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBST with 5% non-fat milk and probed with rabbit monoclonal anti-IDH1 antibody (Cell Signaling Technology), rabbit monoclonal anti-EglN1 antibody (Cell Signaling Technology), mouse monoclonal anti-HIF1α antibody (BD Transduction Laboratories), rabbit monoclonal anti-β-tubulin antibody (Cell Signaling), or mouse monoclonal anti-vinculin antibody (Sigma).

Real-time qPCR analysis. Total RNA was isolated using the RNeasy RNA isolation minikit with on-column DNase digestion (Qiagen). First strand cDNA was synthesized using the AffinityScript qPCR cDNA Synthesis kit (Stratagene). Real-time PCR was performed in duplicate using QuantiTect SYBR Green PCR master mix (QIAGEN) and the Mx3000P qPCR system (Stratagene). All values were normalized to β-actin levels. β-actin cDNA was amplified with sense primer (5'-ACCAACTGGGACGACATGGAGAAA-3') and antisense primer (5'-TAGCACAGCCTGGATAGCAACGTA-3'). TET1 cDNA was amplified with sense primer (5'-AGTAACACTGAGACCGTGCAACCT-3') and antisense primer (5'-AAGCCTGGAGATGCCTCTTTCACT-3'). TET2 cDNA was amplified with sense primer (5'-GGACATGATCCAGGAAGAGC-3') and antisense primer (5'-CCCTCAACATGGTTGGTTCT-3').

Esterification of (*R*)-2-HG and (*S*)-2-HG. To a solution of (*R*) or (*S*)-5-oxotetrahydrofuran-2-carboxylic acid (650 mg, 5.0 mmol) in acetonitrile (15 mL) was added *i*-Pr_2NEt (1.05 mL, 6.0 mmol, 1.2 equiv) and 3-(trifluoromethyl)benzyl bromide (0.92 mL, 6.0 mmol, 1.2 equiv). The mixture was heated to reflux for 10 minutes and allowed to stir at RT overnight. The mixture was concentrated under reduced pressure and the resulting residue taken up in ethyl acetate (50 mL). The organics were washed with 10% HCl (50 mL), 10% sat $NaHCO_3$ (50 mL) and brine (50 mL), and were dried over Na_2SO_4. Concentration gave a light yellow oil which was purified on silica gel, eluting with 1:1 hexanes:ethyl acetate to give the title compounds [(*R*)-enantiomer: 1.28 g, 89%; or (S)-enantiomer: 1.18 g, 82%] as a clear oil which later solidified upon standing in the freezer. R_f = 0.37 (1:1 hexanes:ethyl acetate). $[a]^{23}_D$ = + 2.1 (*c* 1.92, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): d 7.62 (d, *J* = 8.0 Hz, 1H); 7.61 (s, 1H); 7.56 (d,

J = 8.0 Hz, 1H); 7.52 (t, *J* = 7.52 Hz, 1H); 5.28 (s, 2H); 4.99 (dd, *J* = 4.5, 4.5 Hz, 1H); 2.63-2.51 (m, 3H); 2.35-2.29 (m, 1H). ^{13}C NMR (500 MHz, $CDCl_3$): d 176.0, 169.73, 136.0, 131.7 (q, *J* = 1.6 Hz), 131.1 (q, *J* = 32.5 Hz); 129.4, 125.5 (q, *J* = 3.8 Hz), 125.0 (q, *J* = 3.8 Hz), 123.9 (q, J = 271 Hz), 75.7, 66.6, 26.7, 25.8. IR (neat): 2962, 1786, 1750, 1329, 1202, 1115, 1070, 800, 701 cm^{-1}. HRMS (ESI+): calculated for $C_{13}H_{12}F_3O_4$ = 289.0688; found 289.0692.

(*R*)-2-HG: (*S*)-2-HG:



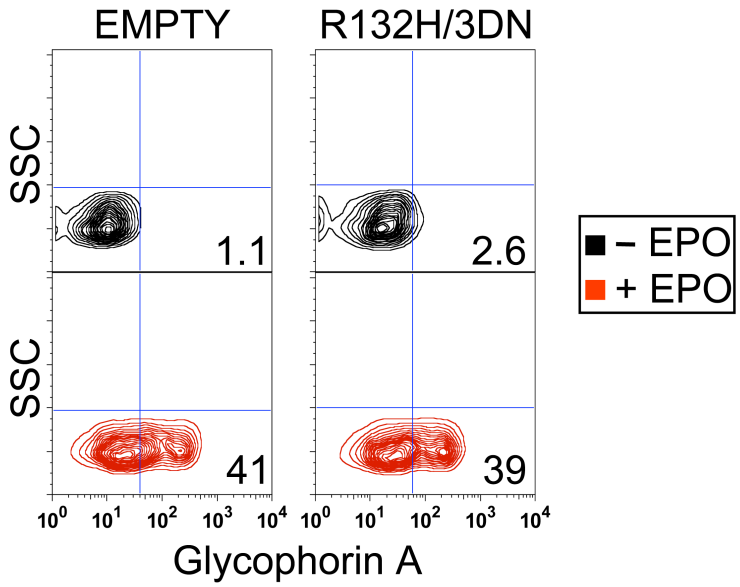
Liquid chromatography–electrospray ionization–mass spectrometry (LC–MS). Metabolites were extracted from exponentially growing TF-1 cells using 80% aqueous methanol (-80 °C) and were profiled by negative mode electrospray LC–MS. In experiments where relative 2-HG levels are reported, the methodology used was as previously described in (*8*). In experiments where absolute 2-HG levels are reported, the methodology used was as previously described in (*4*).

TF-1 Proliferation Assays. TF-1 stable cell lines were washed four times with plain RPMI. For growth factor-deprivation assays, cells were then counted and 1 x 10^6 cells were plated in duplicate or triplicate in 5 mL RPMI containing 10% FBS and 1% penicillin/streptomycin. Cell proliferation and viability were assessed by counting the number of viable cells/mL every 3 to 4 days using a Vi-Cell Cell Viability Analyzer (Beckman Coulter), and the cultures were periodically split and fed with fresh media lacking GM-CSF. For the shRNA minipool screen, after lentiviral infection each replicate was passaged 4 times in growth media containing puromycin. Cells were then washed four times with plain RPMI and plated at a density of 0.2 x 10^6 cells/mL in media lacking GM-CSF and were passaged for 10 days. Aliquots of 20 x 10^6 cells were collected on day 0 and day 10 of growth factor deprivation for DNA purification and Illumina sequencing, as previously described (*31*).

Differentiation Assays. TF-1 stable cell lines were washed four times in plain RPMI and then cultured overnight in media lacking GM-CSF. After 24 hours of GM-CSF starvation, recombinant human erythropoietin (Epoetin alfa, Amgen) was added to the cultures at a final concentration of 2 U/mL. 8 days later, erythroid differentiation was assessed by flow cytometry for Glycophorin A or fetal hemoglobin. Cell surface staining for Glycophorin A was performed as follows: cells were washed once in PBS supplemented with 2% FBS (PBS-FBS) and then resuspended in a 1:200 dilution of PE-Cy5-conjugated mouse anti-human CD235a antibody (BD Pharmingen) in PBS-FBS. The cells were incubated for 20 minutes at 4°C in the dark, then washed once with PBS-FBS. Intracellular staining for fetal hemoglobin was performed using a R-PE-conjugated Fetal Hemoglobin monoclonal antibody kit (Invitrogen) according to the manufacturer's directions. SCF ER-Hoxb8 stable cell lines were washed twice in sterile PBS and then cultured for 3 days in SCF media lacking β-estradiol. Monocytic differentiation was

assessed by flow cytometry using APC-conjugated mouse anti-human CD11b/Mac-1 (BD Pharmingen) and PerCP-Cy5.5-conjugated rat anti-mouse Ly-6G/Ly-6C (Gr1) (BD Pharmingen) as described above for Glycophorin A. The cells were analyzed on a four-color FACSCalibur cytometer (Becton Dickinson). All flow cytometry data was analyzed using FlowJo software (TreeStar, Inc.).

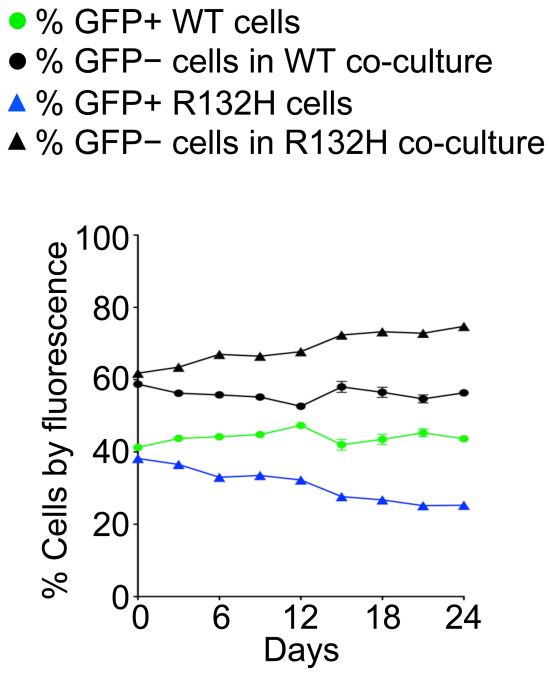
Fig. S1.



The differentiation block by IDH1 R132H requires its catalytic activity.

Differentiation of TF-1 cells infected with a lentivirus encoding catalytically-inactive mutant IDH1 (R132H/3DN), or empty vector, as determined by FACS for Glycophorin A after treatment with EPO for 8 days. Shown are representative results from three independent experiments.

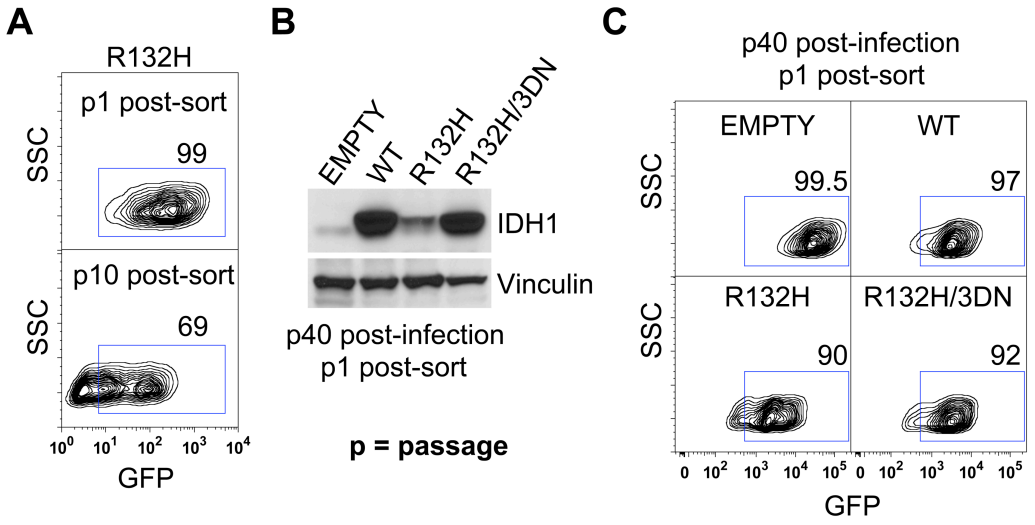
Fig. S2



Under growth factor-rich conditions mutant IDH expression confers a proliferative disadvantage to TF-1 cells.

TF-1 cells were infected with lentiviruses encoding IDH1 (wild-type or R132H) and GFP from a single IRES-containing mRNA. GFP positive cells (expressing wild-type IDH1 or IDH1 R132H) were mixed with uninfected (GFP negative) parental cells and were maintained in the presence of GM-CSF. Shown are percentage GFP-positive cells, as determined by FACS, as a function of time. Note that IDH1 R132H-expressing cells (blue) have a growth disadvantage when compared to GFP- parental cells (black) and GFP+ WT IDH1-expressing cells (green). Shown are mean values of duplicate experiments.

Fig. S3



Expression of mutant IDH is transcriptionally and post-transcriptionally downregulated in TF-1 cells upon prolonged passage in GM-CSF.

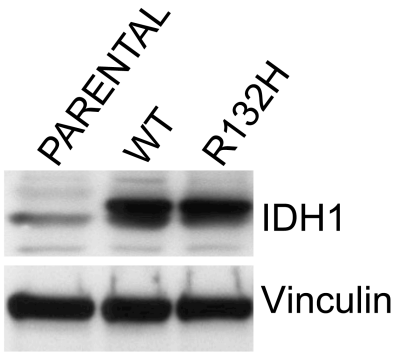
TF-1 cells were infected with lentiviruses encoding the indicated IDH1 variant (wild-type, R132H, or R132H/3DN) and GFP from a single IRES-containing mRNA. Empty = lentiviral vector encoding GFP alone.

(A) The percent GFP positive IDH1 R132H-expressing TF-1 cells as determined by flow cytometry immediately post-GFP sort and after 10 passages in GM-CSF.

(B and C) Immunoblot (B) and FACS (C) analysis of IDH1 expression in late-passage TF-1 cells immediately post-GFP sort. The cells in B and C had been passaged a total of 40 times and had been serially sorted every ten passages, for a total of 4 sorts.

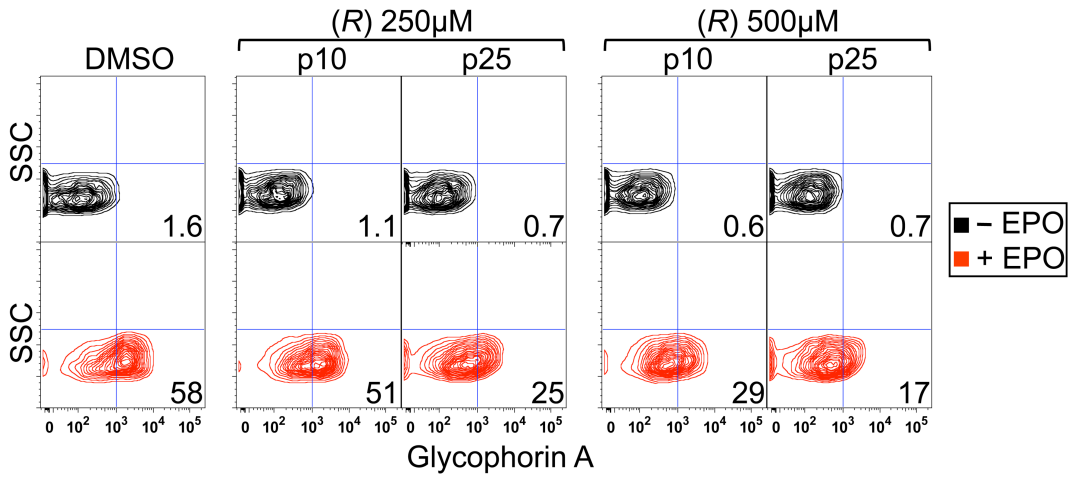
Shown are representative results from three independent experiments.

Fig. S4



Immunoblot analysis of IDH1 expression in parental ER-Hoxb8 cells and ER-Hoxb8 cells infected with lentiviruses encoding the indicated IDH1 variants. Shown are representative results from three independent experiments.

Fig. S5



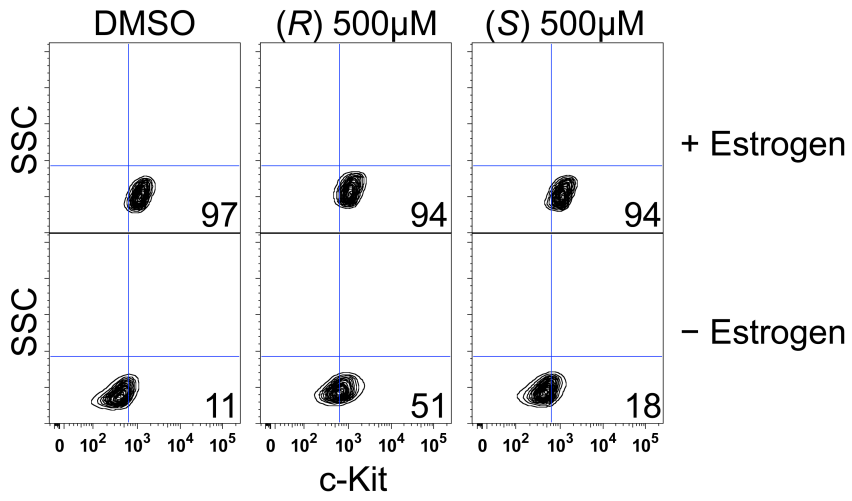
p = passage

TFMB-(*R*)-2-HG blocks EPO-induced differentiation of TF-1 cells in a dose-dependent and a passage-dependent manner.

Differentiation of TF-1 cells following pretreatment with DMSO, 250 μM or 500 μM TFMB-(*R*)-2HG for the indicated number of passages; as determined by FACS for Glycophorin A after treatment with EPO for 8 days.

Shown are representative results from five independent experiments.

Fig. S6

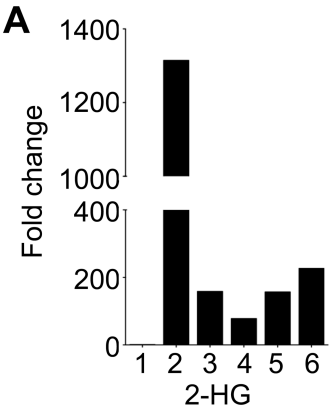


TFMB-(*R*)-2-HG blocks the downregulation of c-Kit cell surface expression upon estrogen withdrawal in ER-Hoxb8 cells.

c-Kit expression, as determined by FACS, in ER-HoxB8 cells following pretreatment of the cells with DMSO, 500 μM TFMB-(*R*)-2HG or 500 μM TFMB-(*S*)-2HG, 3 days after withdrawal of estrogen.

Shown are representative results from two independent experiments.

Fig. S7



B

		2-HG level	
		(ng/2x10^6 cells)	(mM)
1	Parental + DMSO	74 ± 2.2	0.12 ± 0.005
2	R132H (p3)	23,318*	> 37*
3	R132H (p10)	N.D.	N.D.
4	R132H (p30)	2,561 ± 49	4.04 ± 0.7
5	TFMB-(*R*)-2HG - 250μM	3,747 ± 68	5.90 ± 0.1
6	TFMB-(*R*)-2HG - 500μM	5,489 ± 594	8.65 ± 0.9

* values are above quantitative levels

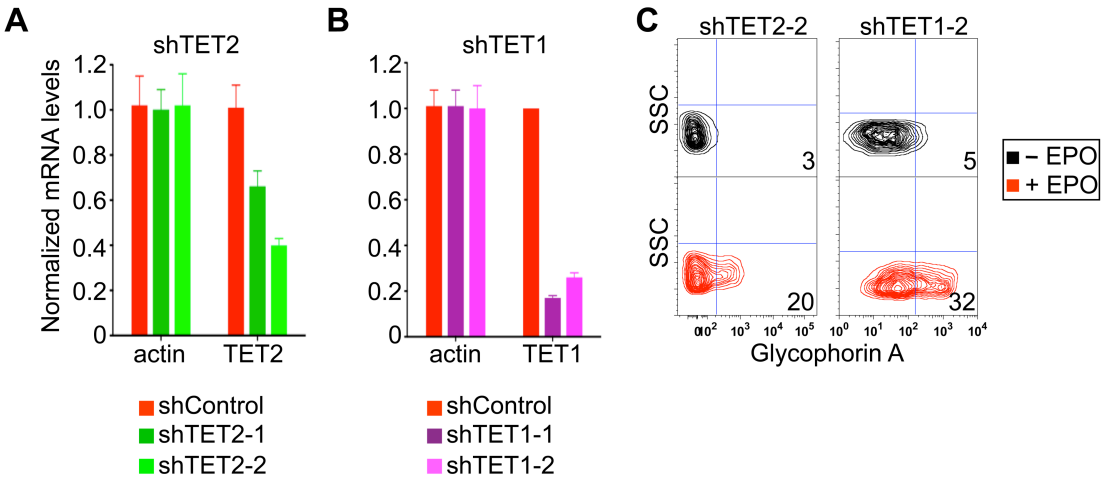
p = passage

Accumulation of 2-HG in TF-1 cells expressing IDH1 R132H or treated with TFMB-(*R*)-2-HG.

(A) LC-MS analysis of fold change (relative to parental TF-1 cells) in intracellular 2-HG levels under the following conditions: cultured in DMSO (1); expressing IDH1 R132H at passage 3 after infection (2), passage 10 after infection (3) and passage 30 after infection (4); and passaged in 250 μM (5) or 500 μM TFMB-(*R*)-2HG (6). Shown are mean values of triplicate experiments.

(B) Quantitative LC-MS analysis of intracellular concentrations of 2-HG under the indicated conditions. Shown are mean values of duplicate experiments ± SD.

Fig. S8

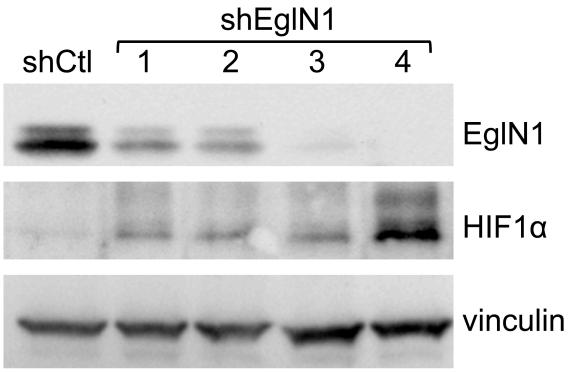


Knockdown of TET2 or TET1 in TF-1 cells using shRNAs.

(A and B) Quantitative real-time PCR analysis of TF-1 cells infected with lentiviruses encoding shRNAs targeting TET2 (A) and TET1 (B). Lentiviruses were obtained from the TRC collection of the Broad Institute, Cambridge, MA. mRNA abundance values were normalized to actin. Shown are mean values of triplicate experiments.

(C) Differentiation of TF-1 cells infected with lentiviruses encoding shRNAs targeting TET2 (left) and TET1 (right), as determined by FACS for Glycophorin A after treatment with EPO for 8 days. shTET2-1 (TRCN0000122172); shTET2-2 (TRCN0000145351); shTET1-1 (TRCN0000075024); shTET1-2 (TRCN0000075026). Shown are representative results from three independent experiments.

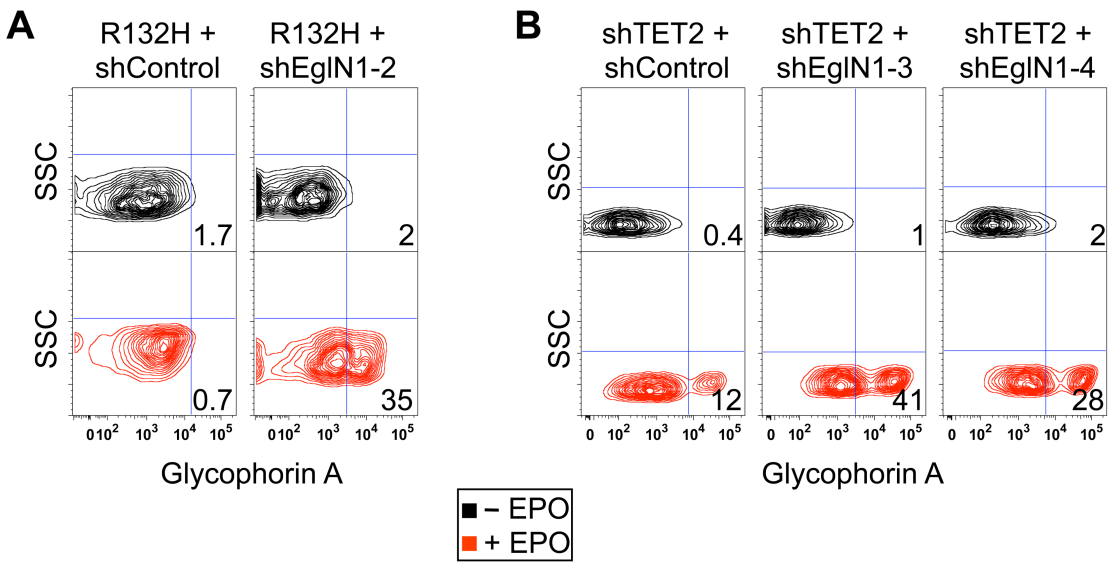
Fig. S9



Knockdown of EglN1 in TF-1 cells expressing IDH1 R132H and EglN1 shRNAs.

Immunoblot analysis of TF-1 cells expressing IDH1 R132H that were subsequently infected with lentiviruses encoding a non-targeting shRNA (shCtl) or shRNAs targeting EglN1 (shEglN1). Lentiviruses were obtained from the TRC collection of the Broad Institute, Cambridge, MA. shEglN1-1 (TRCN0000001042); shEglN1-2 (TRCN00000010578); shEglN1-3 (TRCN0000001044); shEglN1-4 (TRCN0000001045). Shown are representative results from four independent experiments.

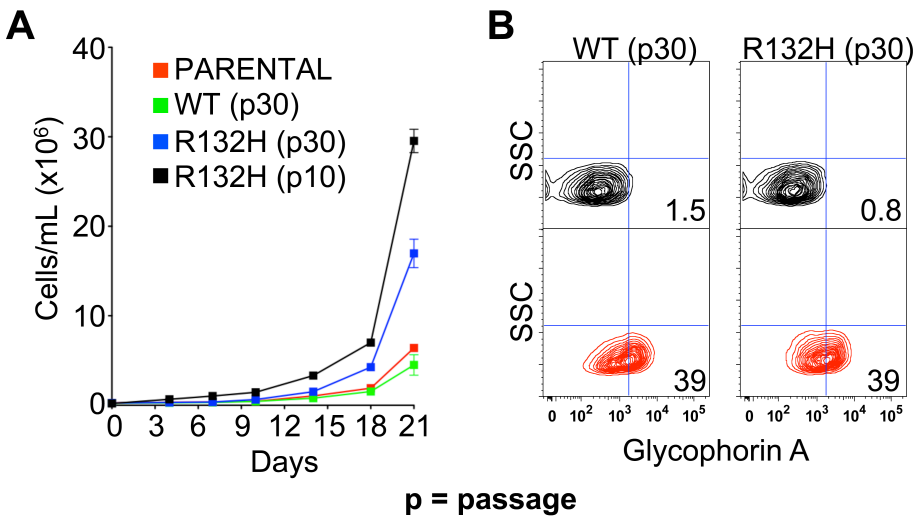
Fig. S10



TFMB-(*R*)-2-HG blocks EPO-induced differentiation of TF-1 cells in a dose-dependent and a passage-dependent manner.

Differentiation of TF-1 cells expressing IDH1 R132H (A) or expressing an shRNA targeting TET2 (B), infected with lentivirus encoding shRNAs targeting EglN1. Differentiation as determined by FACS for Glycophorin A after treatment with EPO for 8 days. Shown are representative results from four independent experiments.

Fig. S11



Late passage TF-1 cells that express low levels of mutant IDH1 remain growth factor-independent but recover the ability to differentiate in response to EPO.

(A) Proliferation of passage-10 and passage-30 TF-1 stable cell lines expressing the indicated variants of IDH1 under cytokine-poor conditions. Shown are mean values of duplicate experiments.

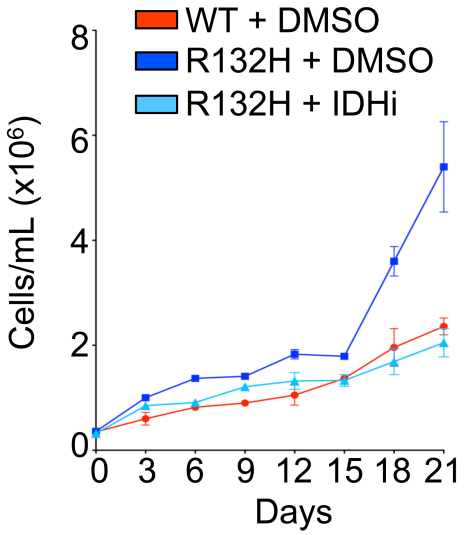
(B) Differentiation of passage-30 TF-1 cells, as determined by FACS for Glycophorin A after treatment with EPO for 8 days. Shown are representative results from three independent experiments.

Fig. S12

	2-HG level	
	(ng/2x10^6 cells)	(mM)
EP WT + DMSO	79 ± 0	0.15 ± 0
LP WT + DMSO	95 ± 0.7	0.15 ± 0
EP R132H + DMSO	8,075 ± 151	12.8 ± 0.21
EP R132H + 1 μM IDH1i	1,145 ± 70	1.80 ± 0.14
LP R132H + DMSO	373 ± 4.2	0.59 ± 0.007
LP R132H + 1 μM IDH1i	89 ± 0	0.14 ± 0

Quantitative LC-MS analysis of intracellular 2-HG levels in early-passage (EP = passage 15) and late-passage (LP = passage 40) TF-1 cells expressing the indicated IDH1 variants after treatment with DMSO or 1 μM of a small molecule inhibitor of IDH1 R132H (IDH1i) for 5 passages. Shown are mean values of duplicate experiments ± SD.

Fig. S13



Reversal of growth factor-independence in late-passage TF-1 cells treated with an IDH1 inhibitor.

Proliferation under cytokine-poor conditions of late-passage (p40) TF-1 cells expressing the indicated IDH1 variants after treatment with DMSO or 1 μM of a small molecule inhibitor of IDH1 (IDH1i) for 5 passages. Shown are mean values of duplicate experiments.

Table S1.

Genes targeted in shRNA minipool.								
ADI1	BCMO1	ETHE1	IDO1	KDM3B	PADI1	PRMT1	SETD6	TANC2
ADO	BCO2	EZH1	IDO2	KDM4A	PADI2	PRMT2	SETD7	TDO2
ALKBH1	C9orf64	EZH2	JARID2	MCEE	PADI3	PRMT3	SETD8	TET1
ALKBH2	C14orf169	FBXL19	JHDM1D	MEMO1	PADI4	PRMT5	SETDB1	TET2
ALKBH3	C17orf101	FTO	JMJD1C	METTL13	PHF1	PRMT6	SETDB2	TET3
ALKBH5	C20orf7	GLO1	JMJD4	MINA	PHF2	PRMT7	SHMT1	THUMPD2
ALKBH6	CARM1	GLOD4	JMJD5	MLL	PHF8	PRMT8	SMYD1	TMLHE
AS3MT	CDO1	GLOD5	JMJD6	MLL2	PHYH	PTGS1	SMYD2	UTY
ASH1L	COQ3	HAAO	JMJD7	MLL3	PHYHD1	PTGS2	SMYD3	
ASH2L	DOT1L	HGD	JMJD8	MLL5	PLA2G4B	RPE65	SMYD4	
ASPH	EHMT1	HIF1AN	KDM1A	NSD1	PLOD1	SETD1A	Suv39h1	
ASPHD1	EGLN1	HPD	KDM1B	P4HA2	PLOD2	SETD2	Suv39h2	
ASPHD2	EGLN2	HPDL	KDM2A	P4HA3	PLOD3	SETD3	Suv420h1	
ASXL1	EGLN3	HR	KDM2B	P4HB	PRDM2	SETD4	Suv420h2	
BBOX1	EHMT2	HSPBAP1	KDM3A	P4HTM	PRDM9	SETD5	TANC1	

List of genes targeted by shRNAs in custom minipool.

The 2-OG-dependent dioxygenases are indicated in grey. Also included in the minipool were other non-2-OG-dependent dioxygenases and histone methyltransferases. Lentiviruses were obtained from the TRC collection of the Broad Institute, Cambridge, MA.