Supporting Information

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SI Materials and Methods

AOM-DSS Administration and Sample Collection. CAC was induced using AOM-DSS. Eight-week-old male and female C57BL/6 wild-type mice were i.p. injected with 7.6 mg/kg AOM (Sigma-Aldrich) on day 0. Colitis was induced 7 d after AOM injection by oral administration of 3% (wt/vol) DSS (molecular weight, 50,000) (MP Biomedicals) in drinking water ad libitum for 7 d followed by 14 d of recovery with water alone. After recovery, a second cycle of DSS was repeated, and mice were killed after 3 wk of recovery (day 56). Body weight, stool consistency, and stool occult blood were monitored during the DSS treatment and recovery phases to generate a clinical activity score as described previously (1). Subsets of mice were killed on days 1, 3, 6, 7, or 8 of the first round of DSS administration or on day 4 of recovery from the first round of DSS administration. The colon was excised and processed for histology or for isolation of intestinal epithelial cells as previously described (2).

For metabolomics studies, urine was serially collected at baseline before AOM injection, after the first cycle of DSS (colitis stage), and the day before mice were killed (advanced neoplasia stage) by placing individual mice in a metabolic cage for 24 h. To determine whether elevated D2HG during colitis drives progression to colorectal cancer, mice were i.p. injected with 25 mg/kg D2HG (Sigma Aldrich) or vehicle once daily during DSS administration. For this study, a dose of 25 mg/kg was used, since a previous study reported that mice i.p. injected with 250 mg/kg/d D2HG exhibited circulating D2HG levels at the low range reported in acute myeloid leukemia patients (3); we wanted to achieve a smaller increase that is more reflective of D2HG levels during colitis (Table S2). When mice were killed, the colon was excised from the ileocecal junction to anus and was cut open longitudinally; polyp number and size were quantitated using a dissecting microscope and were prepared for histological evaluation. For biochemical assays, advanced neoplasias were excised using a dissecting microscope; colonic epithelial cells were isolated as previously described (2).

Metabolomic Analysis. Urinary organic acids were analyzed using GC-MS and were normalized to creatinine level (4). Derivatization to methyl ester lactones by methyl chloroformate, separation by 2D chiral column GC (GC × GC), and quantification by TOF MS (modified from ref. 5) were performed to differentiate D2HG and L2HG.

Human Tissue Samples. To measure D2HGDH, HOT, and Hif-1α mRNA expression, colonic mucosal biopsies were obtained from noninflamed, control patients ($n = 9$) and UC patients ($n = 21$). To visualize localization of D2HGDH in the colonic mucosa, one additional biopsy was obtained from noninflamed, normal patients and was formalin fixed and paraffin embedded for immunohistochemistry staining.

To measure D2HGDH expression in progressors and nonprogressors, we obtained retrospective colon mucosa samples of involved chronic UC (baseline). From the same patients, we obtained retrospective mucosa samples at follow-up, 1–8 y later, at which time 11 patients had progressed to high-grade dysplasia/adenocarcinoma (progressors), compared with 13 UC patients who remained dysplasia-free (nonprogressors). Progressor follow-up biopsies were of nondysplastic tissue taken 10–20 cm away from adenocarcinoma/high-grade dysplasia. Retrospective normal colon mucosa samples and inflamed samples from non-IBD patients were obtained as controls. Demographic and clinical information of patients is listed in Table S3. Parallel sections were viewed by a trained pathologist to verify the original diagnosis.

Histological Dysplasia/Adenoma Scoring. H&E-stained Swiss-rolled colon sections were scored histologically for adenocarcinoma (neoplasia). Scoring performed in a blind fashion by a qualified pathologist was based on these criteria: score 0, normal; 1, low-grade dysplasia; 2, high-grade dysplasia; 3, intramucosal adenocarcinoma; and 4, invasive adenocarcinoma.

Histological Inflammatory Scoring. Histological scoring of colitis was performed in a blind fashion on the basis of three parameters: the severity of inflammation, crypt damage, and ulceration, as previously described (6).

Enzymatic D2HG Assay. The D2HG concentration in mouse colon and cultured cells was measured using the enzymatic assay as previously described (7). Ten 10-μm paraffin-embedded sections of mouse colon were collected into a tube. The surface area of the tissue was estimated using a dissecting microscope; D2HG content was calculated as picomoles per cubic millimeter of tissue volume. D2HG concentration from cells was calculated as micromoles per milligram of protein.

IDH1 and IDH2 Sequencing. Tumor tissue, normal adjacent mucosa, and DSS-inflamed mucosa were microdissected from 7-μm paraffin-embedded sections of Swiss-rolled mouse colon. Genomic DNA was isolated from microdissected tissues using the QIAamp DNA FFPE Tissue Purification Kit (Qiagen). The PCR amplicons were generated and sequenced by Sanger sequencing using the primers listed in Table S4.

Immunohistochemistry. Five-micrometer paraffin-embedded sections of mouse colon or human colonic mucosal biopsies were incubated with D2HGDH antibody (ab136336; Abcam) as previously described (8). Elimination of primary antibody or IgG control antibody was used as a negative control. From each human immunostained slide five fields of mucosal area were digitally captured at 20× magnification microscopy (Zeiss Axioskop Plus). Using Image J software (NIH), the epithelium was defined as the region of interest using the brush tool in each digital image. DAB staining was quantified as previously described (9) and expressed as the percentage of the whole region of interest. Percentage values from the same section were then averaged.

Cell Lines. Polarized Caco2-BBE colonic epithelial cells and nontransformed IEC-6 rat small intestinal epithelial cells (American Type Culture Collection) were used to assess the effect of D2HG on cell migration, proliferation, and apoptosis. We also performed experiments using immune cell lines, which included a human T-cell line (Jurkat) and the Raw264.7 macrophage cell line (American Type Culture Collection). Cell lines were tested for mycoplasma contamination every 3 mo using a MycoScope PCR Mycoplasma Detection Kit (Genlantis). Cells were grown in DMEM supplemented with penicillin (40 mg/L), streptomycin (90 mg/L), and 10% FBS and were cultured under normoxia (5% CO_2, 95% humidified air) or, for some experiments, hypoxia (1% O_2, 5% CO_2, 94% N_2).

Caco2-BBE cells were transfected using Lonza electroporation with HIF-1α–ODD–pIRES containing a mutation to the ODD resulting in constitutive overexpression (a generous gift from Joseph Garcia, University of Texas Southwestern Medical Center, Dallas), 20 μM Stealth RNAi against Hif-1α (5′-CCAGCCG-CUGGAGACACAAUCAUAU-3′; Invitrogen), shRNA against

Hif-1α (a gift from Qiuyang Zhang, Baylor Scott & White Research Institute, Dallas), 20 μM siRNA against Hif-2 (SI00380212; Qiagen), 20 μM of three unique siRNAs pooled against Hif-2 (SR301415; OriGene), 20 μM Stealth RNAi against D2HGDH (RSS313522; Invitrogen), 20 μM of three unique siRNAs pooled against D2HGDH (SR318998; OriGene), or 20 μM Stealth RNAi siRNA Negative Control Med GC (siNC; Invitrogen).

Colonoid Culture. Colon enteroids (colonoids) were derived from isolated crypts collected from the length of the colon of wild-type mice and cultured in Matrigel Basement Membrane Matrix (Corning) as previously described (10). Epidermal growth factor, Noggin, and R-spondin-1 (ENR) culture medium contained Advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM Hepes, 1× L-glutamine, 1× N2, 1× B27 (all from Life Technologies), 50 ng/mL murine recombinant epidermal growth factor, 500 ng/mL R-spondin1, and 100 ng/mL Noggin (all from R&D Systems). Colonoids were grown in ENR medium for 9 d and treated with 100, 250, or 500 μM D2HG (Sigma-Aldrich) for 16 h. For collection of protein lysates for Western blot, colonoids were collected using Cultrex Organoid Harvesting Solution (Fisher Scientific) to remove Matrigel.

Cloning and Mutation of the 5′ Flanking Region of the Human *D2HGDH* Gene and Reporter Assays. The 5′ flanking region of the human *D2HGDH* gene was amplified by PCR using KOD Xtreme Hot Start DNA Polymerase (Millipore), human chromosome 2 genomic DNA (clone RP11-93H11; BacPac Resources, Children's Hospital Oakland Research Institute) as a template, and the following primers: 5′-GCTAAAGCTTGAGCTGAGATCAACCCCAGAA-3′ (underlined nucleotides indicate a HindIII site); 5′-ATCGCTCGAGGGAGACCTCGGGCTCCTCCTT-3′ (underlined nucleotides indicate a XhoI site). After sequence confirmation, the PCR product (GenBank accession no. KX227379; −1,286 to −17 from the transcriptional start site) was cloned into the pGL3 luciferase reporter vector (Promega) using XhoI and HindIII restriction sites. Putative transcription factor-binding sites within the full-length human D2HGDH promoter were identified using the web-based search programs Transcription Element Search System (TESS; www.cbil.upenn.edu/tess/), JASPAR (jaspar.genereg.net/), and UniPROBE (thebrain.bwh.harvard.edu/uniprobe/). To determine the importance in D2HGDH promoter activity of the putative consensus Hif-1α–binding site (5′-RCGTG-3′) located −127 to −131 from the transcription start site, site-specific mutation was introduced into the wild-type D2HGDH promoter in pGL3 by PCR amplification using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and the PAGE-purified primer 5′-GCAAGCGTGTTTCAATTTGTACAACAAGCATAAAACATGAAATTACCCTTG-3′. Nucleotide substitutions are indicated in bold. The PCR product was sequenced to ensure nucleotide substitution at the Hif-1α–binding site.

For reporter gene assays, Caco2-BBE cells were cotransfected with 1.6 μg of the pGL3 reporter construct and 20 ng of pRL-CMV (Renilla luciferase; Promega) as an internal control using Lonza electroporation and were harvested 72 h posttransfection. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer. Relative luciferase was calculated by normalizing firefly luciferase activity to Renilla luciferase activity of the pRL-CMV vector. Data were expressed as the normalized fold changes to controls.

ChIP. Caco2-BBE extracts were subjected to ChIP using Hif-1α antibody (14179; Cell Signaling) or rabbit IgG (2729; Cell Signaling) as a control and the ChIP assay kit materials (Active Motif). The immunoprecipitates were analyzed by real-time qPCR to detect coimmunoprecipitated DNA using a pair of D2HGDH promoter-specific primers targeting the putative Hif-1α–binding site (sense: 5′-CCTCCCTGCTTCTGCAAG-3′; antisense: 5′-AGTGGCCAAGGGTAATTTCA-3′) and normalized to input DNA.

LDH Release. The LDH detection kit (Takara Clontech) was used to measure cell cytotoxicity. An aliquot of 100 μL of culture medium was added to 100 μL of LDH reagent, and the percentage of viable cells was measured according to the manufacturer's protocol.

Epithelial Cell Migration. Cell migration was measured using the Oris Cell Migration Assay (CMA1.101; Fisher Scientific) according to the manufacturer's protocol. Oris Cell Migration 96-well plates have physical "stopper" barriers to create a central cell-free exclusion detection zone in the center of each well when plating. Caco2-BBE or IEC-6 cells were plated around the stoppers for 72 h. The stoppers were then removed, allowing cells to migrate into the exclusion zone, and cells were treated with D2HG or vehicle for 16 h. Cells were fixed with 4% paraformaldehyde, stained with Alexa Fluor 568 Phalloidin (Invitrogen) at 1:15 dilution for 1 h, and rinsed twice with 1× PBS. The Oris Detection Mask, which ensures that only fluorescence in the exclusion zone reaches the detector, was snapped onto the bottom of the plate, and fluorescence was measured using a plate reader.

TUNEL Staining. Immunofluorescent TUNEL staining was performed to measure apoptosis using the In Situ Cell Death Detection kit as described by the manufacturer (Roche). Nuclei were stained with DAPI.

Immune Cell Migration. To prepare conditioned medium, Caco2-BBE cells were grown to confluence on Transwells and treated apically with 250 μM D2HG or vehicle for 16 h. Medium in the basolateral chamber was then collected and used immediately as conditioned medium. Jurkat or Raw264.7 cells were plated in the upper chamber of a polycarbonate 24-well plate (3421; Costar) containing inserts with 5-μm pores. RANTES- (0.4 μg/mL), IP-10– (1.0 μg/mL), or IL-8– (0.4 μg/mL) neutralizing antibodies were added to aliquots of D2HG conditioned medium (R&D Systems). The conditioned medium from Caco2-BBE cells was added to the lower chamber as a chemotactic reagent. After 24-h incubation, the upper chamber was removed, and cells that had migrated to the lower chamber were collected and counted.

Luminex Multiplex Assays. To prepare conditioned medium, Caco2-BBE cells were grown to confluence on Transwells and treated apically with 250 μM D2HG or vehicle for 16 h. Medium in the basolateral chamber was then collected, and 42 cytokines/chemokines were measured using Luminex multiplex assays (HCYTOMAG-60K; EMD Millipore) according to the manufacturer's instructions with standards and samples in duplicate.

RNA Isolation and Real-Time qPCR Analysis. Total RNA was isolated from colon or cells using the RNeasy Kit (Qiagen). Real-time qPCR was performed as described previously (1). Final graphical data are $2^{-\Delta\Delta CT}$. Primers utilized for qRT-PCR are shown in Table S5.

Western Blot Analysis. Total protein was analyzed by Western blotting as described previously (1). Antibodies used were D2HGDH (TA331322; OriGene), HOT (SAB1410235; Sigma-Aldrich), Hif-1α (NB100-105SS; Novus), Hif-2 (20654; Abcam), PCNA (ab2426; Abcam), caspase 3 (9662S; Cell Signaling), and anti–β-actin (A1978; Sigma-Aldrich).

Statistics. The linear mixed model was used to determine significance across longitudinal measurements of the mouse urine metabolites during the CAC protocol. *P* values were obtained using an *F* test with the Kenward–Roger approximation (JMP

Genomics). A paired *t* test was used to determine significance of D2HGDH immunostaining between baseline and follow-up in progressors and nonprogressors (PRISM 6.0; GraphPad Software Inc.). The Kruskal–Wallis test, followed by Dunn's test, was used to analyze D2HGDH immunostaining across progressors, nonprogressors, normal, and non-IBD inflamed human colonic mucosal samples (PRISM). Other data are presented as mean ± SEM, for which an unpaired two-tailed Student's *t* test was used for single comparisons and one-way ANOVA with Bonferroni post hoc test for multiple comparisons (PRISM). Linear regression was used to analyze the correlation of urine D2HG level with polyp number, size, and dysplasia score and the correlation of D2HGDH mRNA expression with Hif-1α mRNA expression (PRISM). $P < 0.05$ was considered significant.

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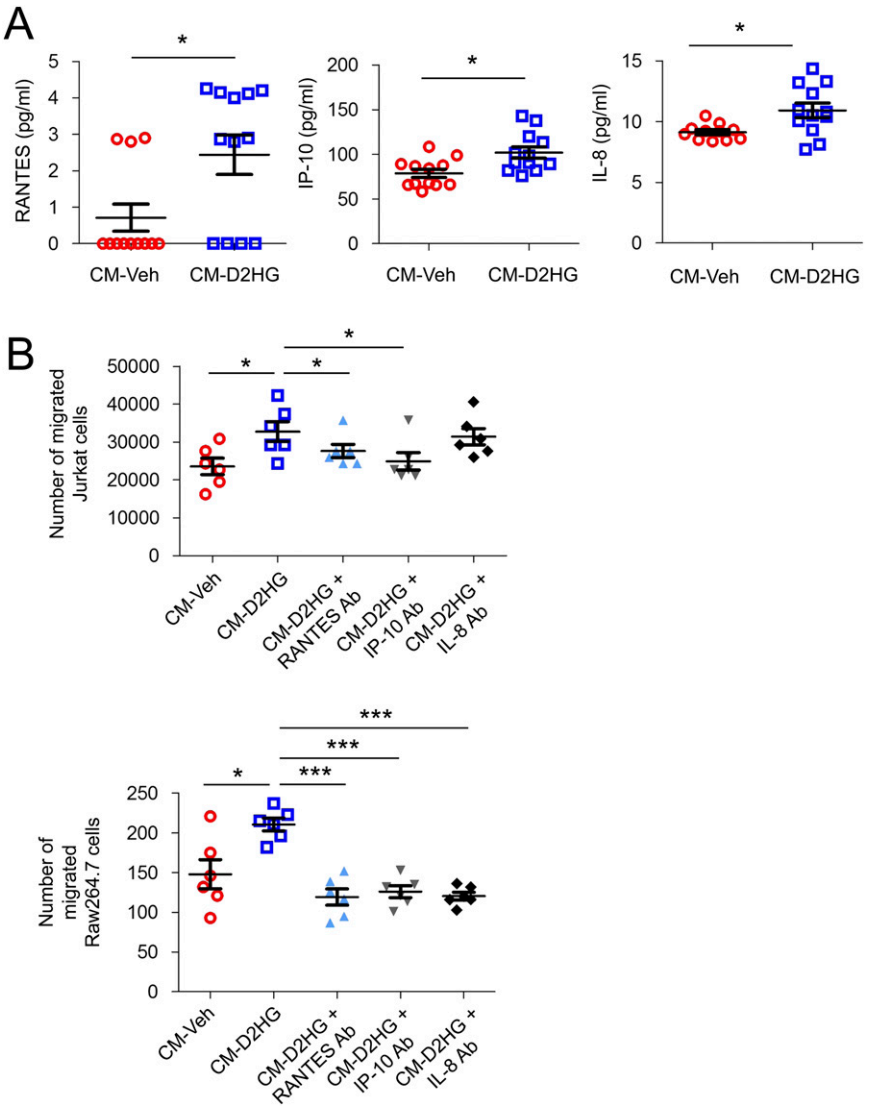

Fig. S1. D2HG enhances the secretion of RANTES, IP-10, and IL-8 from colon epithelial cells, which in turn recruit immune cells. (*A*) Caco2-BBE cells were grown to confluence on Transwells and treated apically with 250 μM D2HG or vehicle for 16 h. The medium in the basolateral chamber was then collected to be used as conditioned medium. Forty-two cytokines/chemokines were measured by Luminex multiplex assays. RANTES, IP-10, and IL-8 were significantly increased in CM-D2HG versus vehicle-conditioned medium (CM-veh). (*B*) The number of migrated Jurkat T cells and Raw264.7 macrophages after exposure to CM-veh or CM-D2HG for 24 h. Neutralizing antibodies to RANTES, IP-10, or IL-8 were added to the CM-D2HG before the 24-h incubation. Results are presented as individual data points ± SEM of 12 samples per group (*A*) or six samples per group (*B*). $*P < 0.05$, $***P < 0.005$ by unpaired, two-tailed Student's *t* test (*A*) or one-way ANOVA followed by Bonferroni's test (*B*).

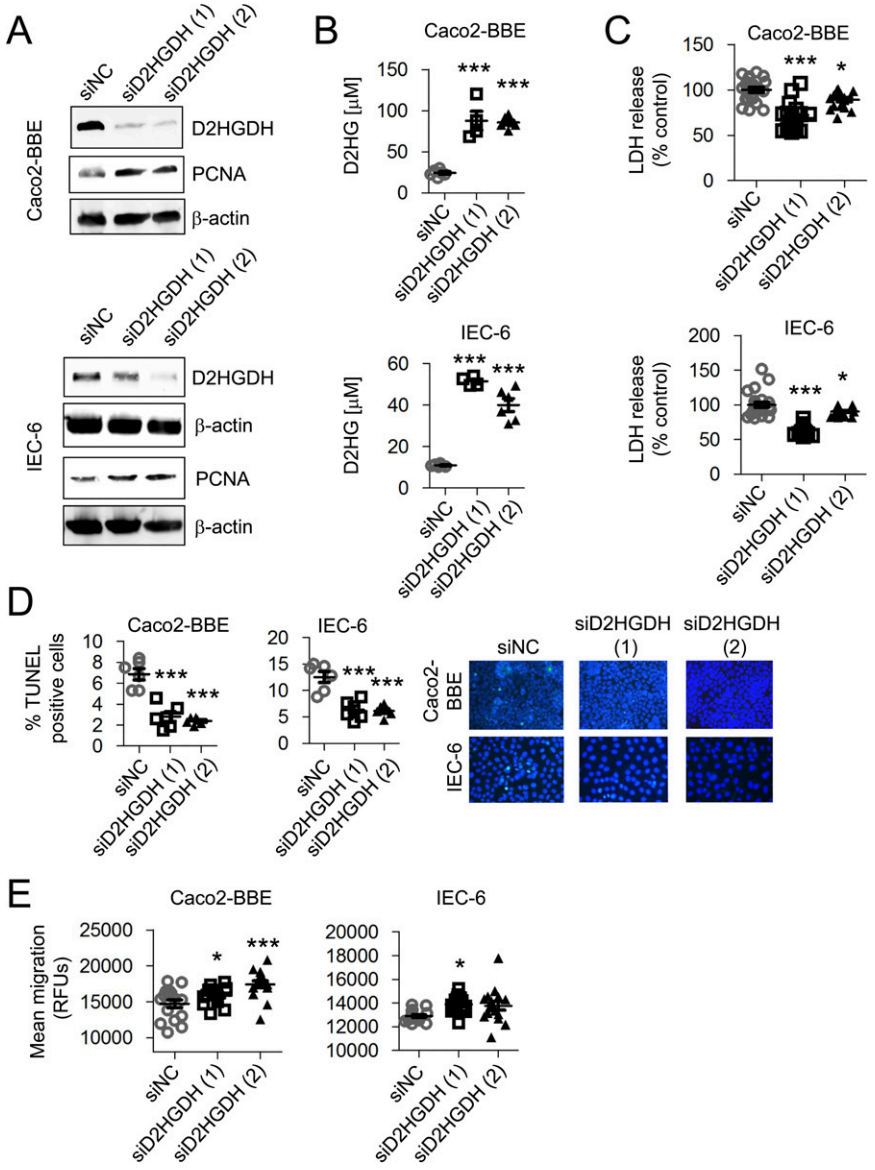

Fig. S2. Elevated D2HG via knockdown of D2HGDH enhances cell survival, proliferation, and migration in colonic epithelial cells. Caco2-BBE and IEC-6 cells were transfected with two independent RNAi constructs against D2HGDH (siD2HGDH) or RNAi negative control (siNC) for 48 h. (*A*) D2HGDH knockdown efficiency and PCNA protein expression by Western blot. (*B*) D2HG levels measured by enzymatic assay. (*C*) Cell cytotoxicity measured by LDH release. (*D*, *Left* and *Center*) The number of TUNEL$^+$ cells across six wells per group was counted to measure apoptosis. (*Right*) Photomicrographs of TUNEL (green) and DAPI (blue) staining. (*E*) Cell migration assay. Results are presented as individual data points ± SEM of four (*A* and *B*) or six (*D*) samples per group, 24 samples per group from three pooled, independent experiments (*C*), or 16 samples per group from two pooled, independent experiments (*E*). $*P < 0.05$, $***P < 0.01$ by unpaired, two-tailed Student's *t* test.

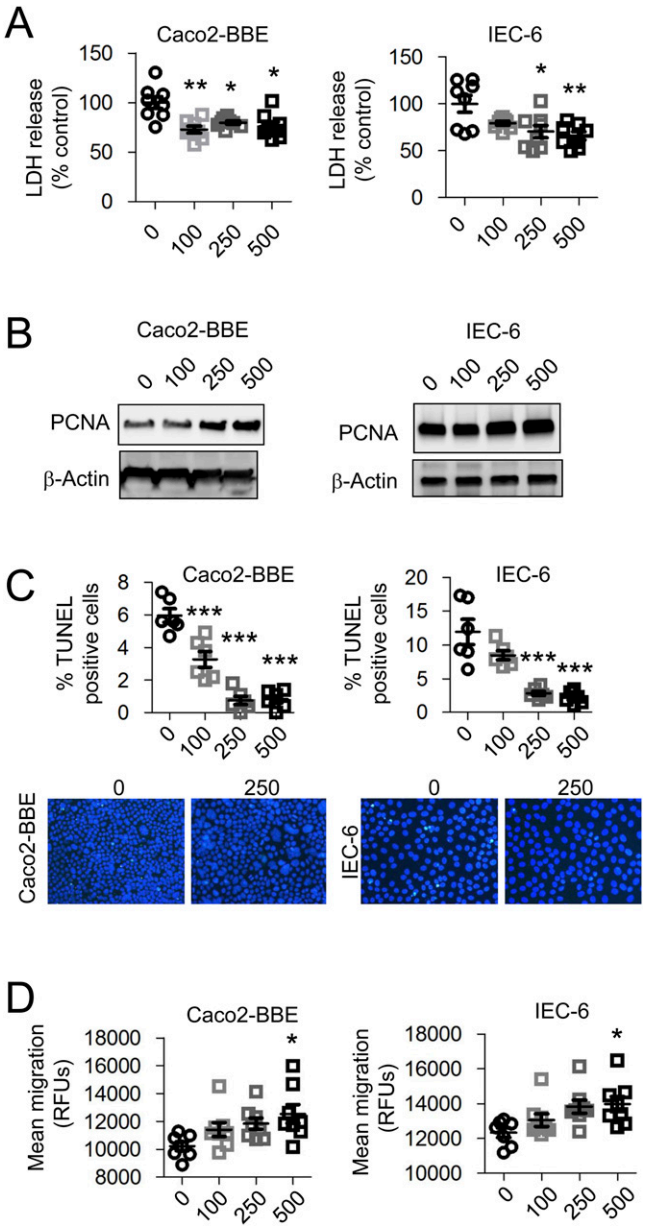

Fig. S3. D2HG enhances colonic epithelial cell survival, proliferation, and migration. Caco2-BBE and IEC-6 cells were treated with 100, 250, or 500 μM D2HG for 16 h. (*A*) Cell cytotoxicity measured by LDH release. (*B*) Representative Western blots of PCNA as a marker of proliferation. (*C*, *Upper*) The number of TUNEL$^+$ cells across six wells per group was counted to measure apoptosis. (*Lower*) Photomicrographs of TUNEL (green) and DAPI (blue) staining. (*D*) Cell migration assay. Results are presented as individual data points ± SEM of seven to eight samples per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ by one-way ANOVA followed by Bonferroni's test.

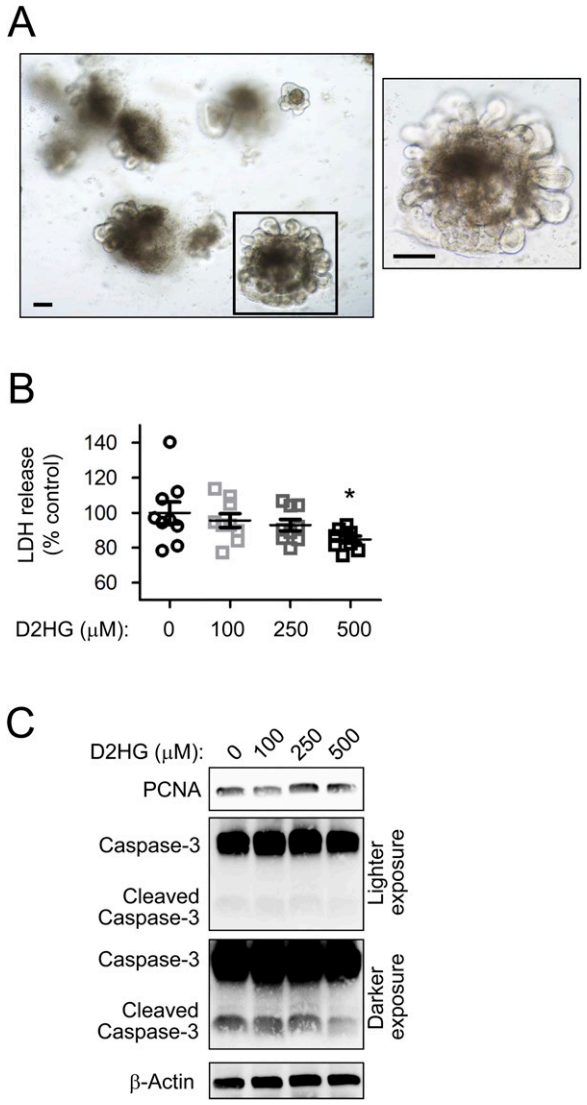

Fig. S4. D2HG enhances colonic enteroid (colonoid) survival and proliferation. (*A*) Representative images of colonoids derived from wild-type mouse colon. (Scale bars: 100 μm.) (*B*) Colonoids were grown in ENR medium for 9 d and treated with 100, 250, or 500 μM D2HG for 16 h. Cell cytotoxicity measured by LDH release. (*C*) Representative Western blots of PCNA as a marker of proliferation and cleaved caspase-3 as a marker of apoptosis. Results are presented as individual data points ± SEM of nine samples per group (*B*) or representative blots of three samples per group (*C*). *$P < 0.01$ by one-way ANOVA followed by Bonferroni's test.

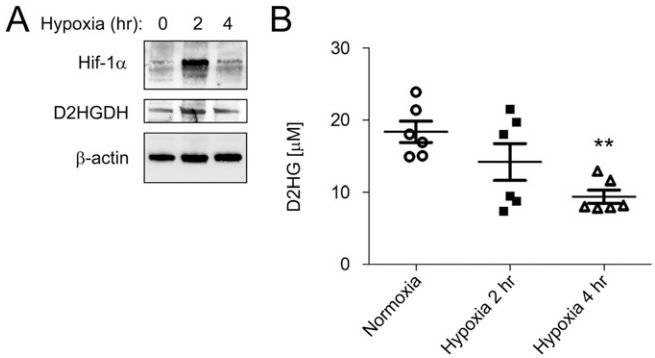

Fig. S5. Endogenous Hif-1α regulates D2HGDH and D2HG levels. Caco2-BBE cells were exposed to hypoxia to induce endogenous Hif-1α. (*A*) Representative Western blots of D2HGDH and Hif-1α protein expression. (*B*) D2HG levels measured by enzymatic assay. Results are presented as representative blots of three samples per group (*A*) or individual data points ± SEM of six samples per group (*B*). **$P < 0.01$ by one-way ANOVA followed by Bonferroni's test.

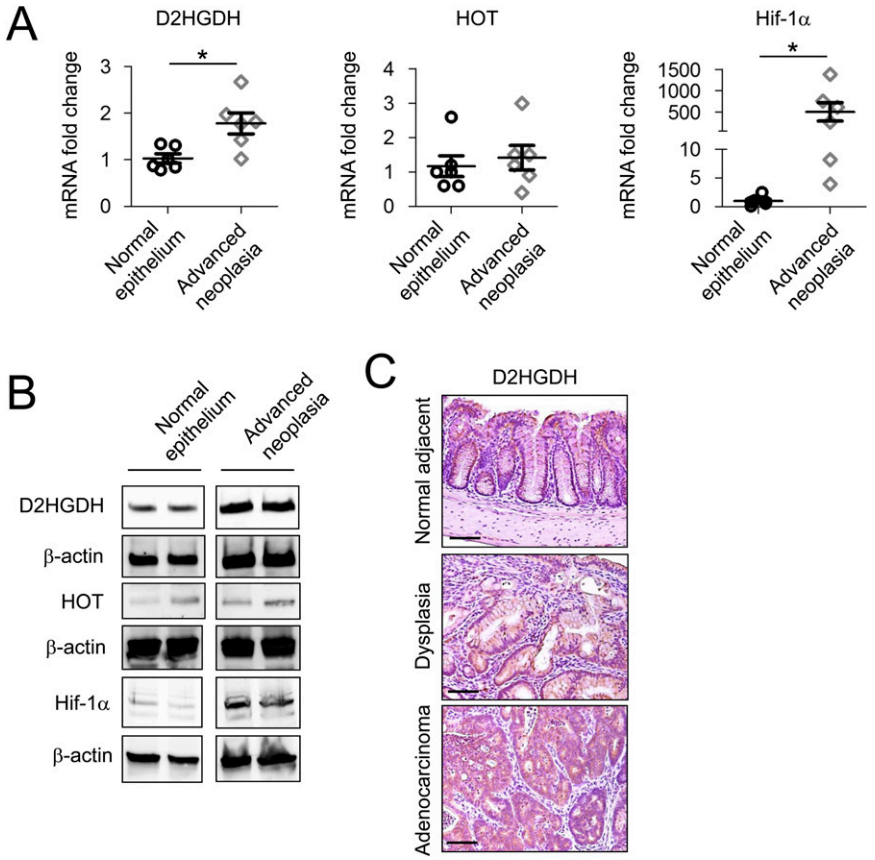

Fig. S6. D2HGDH expression is increased in advanced neoplasia. (*A* and *B*) D2HGDH, HOT, and Hif-1α mRNA (*A*) and protein (*B*) expression in mouse colon epithelial and advanced neoplasia measured by qPCR and Western blot, respectively. (*C*) Immunohistochemistry staining of D2HGDH (brown stain) in mouse colon. (Scale bars: 50 μm.) *$P < 0.05$ by unpaired, two-tailed Student's *t* test.

Table S1. Urine organic acids significantly altered during DSS colitis or advanced neoplasia stage of AOM-DSS–induced CAC compared with baseline

	Mean concentration in mmol/mol creatinine at each stage		
Organic acid	Baseline	Colitis	Advanced neoplasia
2-Oxoadipic	75	148	1
2-Hydroxyadipic	239	319	99
Glutaric	37	44	13
Glyceric	99	41	65
Citric	213	549	165
2-Hydroxyglutaric	49	70	11
Phenyllactic	10	6	2
4-Hydroxyphenyllactic	74	80	23
Thymine	39	66	33

Results are presented as means of 17 mice. $P < 10^{-6}$; FDR $<10^{-5}$.

Table S2. Urine D2HG and L2HG by GC × GC TOF

Metabolite	Stage		
	Baseline	Colitis	Advanced neoplasia
D2HG, mmol/mol creatinine			
Mean ± SEM	16.1 ± 4.6	24.5 ± 3.9	10.3 ± 1.8
P value vs. baseline		**0.04**	**0.01**
R^2, *P* value vs. polyp number		**0.71, 0.02**	0.06, 0.58
R^2, *P* value vs. polyp size		0.41, 0.12	0.04, 0.68
R^2, *P* value vs. dysplasia score		**0.59, 0.04**	0.01, 0.99
L2HG, mmol/mol creatinine			
Mean ± SEM	4.1 ± 1.1	3.5 ± 0.5	4.5 ± 0.8
P value vs. baseline		0.26	0.40
R^2, *P* value vs. polyp number		0.43, 0.11	0.15, 0.39
R^2, *P* value vs. polyp size		0.27, 0.23	0.18, 0.34
R^2, *P* value vs. dysplasia score		0.20, 0.30	0.01, 0.80

Results are presented as mean ± SEM of seven mice. Bold text indicates a statistically significant value.

Table S3. Demographic and clinical information of disease specimens

Variable	Progressor (*n* = 11)	Nonprogressor (*n* = 13)	Noninflamed control (*n* = 20)	Non-IBD inflamed (*n* = 20)
Gender	Males (9), females (2)	Males (9), females (4)	Males (16), females (4)	Males (9), females (11)
Age, y	Median, 59; range, 30–78	Median, 49; range, 19–87	Median, 49; range, 19–80	Median, 61; range, 22–83
Disease history	UC	UC	No history	Drug/toxic injury, infectious, or NOS active colitis
Disease location	Pancolitis (11), left-sided (0)	Pancolitis (7), left-sided (6)		
Region of specimens studied	Right colon (6), left colon (3), rectum (2)	Right colon (5), left colon (4), rectum (4)	Right colon (6), left colon (6), rectum (1), NOS (7)	Right colon (7), left colon (8), rectum (2), NOS (3)

Numbers in parentheses represent the number of patients. NOS, not otherwise specified.

Table S4. *IHD1* and *IDH2* PCR and sequencing primers

Primer name	Primer sequence
IDH1 sense	5′-GGAAATCCCCAAATGGCACCAT-3′
IDH1 antisense	5′-TTGGTCCCCATATGCATG-3′
IDH2 sense	5′-GAGCCCTAACGGAACGATC-3′
IDH2 antisense	5′-CTGGTCGCCATGGGCGTG-3′

Table S5. Real-time qPCR primer sequences

Primer name	Primer sequence
β-Actin sense	5′-TATGCCAACACAGTGCTGTCTGG-3′
β-Actin antisense	5′-TACTCCTGCTTGCTGATCCACAT-3′
D2HGDH sense	5′-CCCGTCTTTGACGAGATCAT-3′
D2HGDH antisense	5′-CATGATGAAGTCCCGTTCCT-3′
Human HOT sense	5′-TGCAGTGCCAACTACCTCAG-3′
Human HOT antisense	5′-TGATGGCTCTCGAAGTGATG-3′
Mouse HOT sense	5′-GGAAGCTTCATGGATGCAAT-3′
Mouse HOT antisense	5′-AACTCAGAGTGAGGGCTGGA-3′
Human Hif-1α sense	5′-CCACCTATGACCTGCTTGGT-3′
Human Hif-1α antisense	5′-TATCCAGGCTGTGTCGACTG-3′
Mouse Hif-1α sense	5′-GAAATGGCCCAGTGAGAAAA-3′
Mouse Hif-1α antisense	5′-CTTCCACGTTGCTGACTTGA-3′