Supporting Information

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SI Materials and Methods

Cell Lines. HCT116 and HT29 colorectal cancer cell lines were originally obtained from the American Type Culture Collection. The SW1222 cell line was a gift from Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The paired cell lines LS174T and LS180 were obtained from B. H. Tom (Northwestern University Medical Center, Chicago) (1). CCK81 was purchased from the Japan Health Foundation. All cell lines were cultured in complete DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). The stably transfected HCT116-CDX1 and LS174T-siCDX1 cell lines, together with their respective vector controls, were previously generated in our laboratory and maintained with geneticin selection pressure (2). Cells were incubated at 37 °C in a humidified environment at 10% CO_2 and either 21% or 1% O_2 and were grown to 50–80% confluence before the next passage or further experiments.

Hypoxic cells were grown in a humidified 1% oxygen and 10% CO_2 environment using a MiniGalaxy A incubator (RS Biotech Ltd). Medium was replaced two times per week after being prewarmed and equilibrated overnight in 1% oxygen. Cellular suspensions were obtained by trypsinization (0.5% trypsin for 2–5 min), and single cells were obtained by passing cells through a 20-μm filter (Celltrics; Partec). For differentiation and clonogenicity assays, single cells were plated in a 1:1 mix of DMEM/Matrigel in 96-, 48-, or 24-well plates, as previously described (3), and covered with DMEM. Colonies were grown for between 2 and 4 wk, depending on growth rates: HCT116 and HT29 (2 wk), LS180 (3 wk), and SW1222 and CCK81 (4 wk). For clonogenic assays, all colonies plated in 24-well plates were examined for morphology and size and were counted, with normoxic and hypoxic colonies with diameters greater than 400 and 200 μm, respectively, being classified as large colonies. For subcloning or replating experiments, colonies were recovered using Matrigel Recovery Solution as per the manufacturer's instructions.

DBZ is a γ-secretase inhibitor and blocks the Notch pathway. DBZ was a gift from Adrian Harris (CRUK, Oxford, United Kingdom) and originally purchased from Syncom. DBZ was added to medium to form the final concentrations as specified and was changed two times weekly.

Immunofluorescence. The mouse anti-human mucin antibody PR4D4 (4) was obtained from Cancer Research UK and is specific for goblet cells (3). The CDX1 purified mouse anti-human mAb was produced against a human CDX1 N-terminus peptide by our laboratory, as previously described (2). The mouse anti-human Ki67 mAb clone MIB-1 was obtained from DAKO. The activated Notch1 rabbit polyclonal antibody was obtained from Abcam (ab8925; 1:200), and the Bmi1 rabbit polyclonal antibody was obtained from Cell Signaling (2380S; 1:200). The mouse anti-human CA9 mAb clone M75 (1:100) was a gift from Adrian Harris (CRUK, Oxford, United Kingdom).

Immunofluorescent images were quantified using ImageJ (National Institutes of Health). Each colony was circumscribed, and mean tyramide 488 fluorescence was calculated minus the background. To normalize for the number of cells in each colony, the mean fluorescence was divided by the mean DAPI fluorescence minus the background. Thus, a fluorescence ratio (FR) was calculated (Eq. **S1**):

$$\mathrm{FR} = \frac{(\text{mean tyramide 488 fluorescence} - \text{background})}{(\text{mean DAPI fluorescence} - \text{background})}. \quad \textbf{[S1]}$$

Typical background values ranged from 1 to 2 arbitrary units. Typical tyramide 488 and DAPI fluorescence values ranged from 20 to 50 arbitrary units. The FRs of three to four colonies were calculated for each cell line and growth condition. Unpaired Student t test was used to compare the means of two populations. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

Western Blot. Western blots were performed using standard protocols. Antibodies used included Hif1α mAb (1:1,000; BD Biosciences), CA9 mAb (clone M75; 1:1,000), CDX1 mAb (1:4,000; in house), and β-tubulin (clone TUB 2.1; 1:5,000; Sigma Aldrich). HRP-conjugated secondary antibodies, swine anti-rabbit, goat anti-rabbit, or goat anti-mouse (DAKO), were used at a concentration of 1:1,000 to 1:5,000. Membranes were developed using the ECL Western Blotting Detection System (GE Healthcare).

Generation of *BMI1* and *CDX1* cDNA Transgene Plasmids. To assess the effects of *BMI1* and *CDX1* on the behavior of CRC cell lines, two plasmid vector constructs, pBI-CMV3-BMI1 (Fig. S7*A*) and pBI-CMV3-CDX1 (Fig. S7*B*), were synthesized that drove expression of an inserted cDNA sequence corresponding to *BMI1* and *CDX1*, respectively. The used vector pBI-CMV3 (Clontech) possessed a bidirectional CMV promoter with enhancer to drive expression of the transgene and the reporter ZsGreen1 GFP to allow for in situ tracking of plasmid transfection and expression. The ColE1 origin of replication within the plasmid allowed for propagation in prokaryotes, whereas the SV40 origin of replication within the plasmid allowed for propagation in eukaryotes.

A *CDX1* cDNA plasmid construct (pRc/CMV-CDX1) was a gift from Jaleh Malakooti (University of Illinois, Chicago, IL). A *BMI1* cDNA plasmid construct (pCMV-XL5-BMI1) was purchased from OriGene.

To clone the *CDX1* cDNA fragment into the target pBI-CMV3 vector, 1 μg pRc/CMV-CDX1 was digested with 2.0 units NotI restriction enzyme; terminal overhangs were filled in by the Klenow reaction. The linearized plasmid was further digested with 2.0 units HindIII restriction enzyme, gel-purified, and quantified to isolate the *CDX1* cDNA fragment. The target vector was prepared by digesting 1 μg pBI-CMV3 with 2.0 units each HindIII and *Eco*RV restriction enzymes followed by purification and quantitation. The cDNA fragment insert was ligated into the target vector, transformed into bacteria, extracted, and quantified. After sequencing the resulting plasmid, pBI-CMV3-CDX1, using the 5′-TGACGCAAATGGGCGGTAGG-3′ forward and 5′-ACCTCTAGAAATGTGGTATGGCTGA-3′ reverse primers to confirm identity and orientation of the *CDX1* cDNA insert, a plasmid DNA stock was synthesized.

To clone the *BMI1* cDNA fragment into the target pBI-CMV3 vector, 1 μg pCMV-XL5-BMI1 was digested with 2.0 units each SmaI and SacI restriction enzymes, purified, blunted with the Klenow fill-in reaction, gel-purified, and quantified to isolate the *BMI1* cDNA fragment. The target vector was prepared by digesting 1 μg pBI-CMV3 with 2.0 units *Eco*RV restriction enzyme followed by purification and quantitation. The cDNA fragment insert was ligated into the target vector, transformed into bacteria, extracted, and quantified. After sequencing the resulting plasmid, pBI-CMV3-BMI1, using the previously detailed se-

quencing primers to confirm identity and orientation of the *BMI1* cDNA insert, a plasmid DNA stock was synthesized.

Induction and Validation of *BMI1* and *CDX1* cDNA Transgene Expression in Cell Lines. The lumen cell lines LS180 and SW1222 as well as the dense cell lines DLD1 and HCT116 were selected for transfection experiments. Cells were harvested and separated into aliquots of 10^5 cells each for plating in 24-well plates. Cells were subsequently chemically transfected with 1 μg control or experimental vector DNA using the Lipofectamine LTX with PLUS lipofection system to assess the effects of forced expression of *BMI1* and *CDX1* on cell behavior, such as colony and lumen formation.

To evaluate transfection efficiency, cultures were photographed 24 h posttransfection with an inverted fluorescence microscope to assess the proportion of cells expressing the reporter ZsGreen1 GFP. All four cell lines exhibited high transfection efficiencies. To evaluate transfection persistence, cultures were subsequently photographed over 1 wk to assess the prevalence of cells expressing the fluorescent reporter. An example photographic time course for the HCT116 cell line is presented in Fig. S8.

To evaluate the effectiveness of the transfection procedure to induce expression of BMI1 or CDX1 protein, transfected cultures were sorted on the basis of ZsGreen1 expression through FACS (an example for the HCT116 cell line is presented in Fig. S9), and the ZsGreen1$^+$ cells (which successfully took up the transfected plasmid) were purified and lysed. Resulting cellular lysates were electrophoresed, transferred to a nitrocellulose membrane, and probed for expression of CDX1 and BMI1 with monoclonal antibodies by Western blotting (Fig. S10). A monoclonal antibody against β-tubulin was used as a loading control to establish that differences in protein expression were not attributable to variations in the amount of total protein loaded. Western blotting revealed that the cells transfected with vectors containing a cDNA insert expressed higher levels of BMI1 or CDX1 than did cells transfected with an empty control vector. Among the two dense lines DLD1 and HCT116, induction of BMI1 expression was not as strong as it was among the two lumen lines LS180 and SW1222, but an induction was observed nonetheless. CDX1 induction was extremely strong across all four cell lines. Thus, populations of cells expressing heightened levels of BMI1 and CDX1 proteins were successfully created and purified, allowing for downstream analyses of cell behavior as a function of these enforced higher protein expression levels.

To assess the clonogenic abilities of the transfected cell populations expressing higher levels of BMI1 or CDX1 than normal, transfected cells were sorted on the basis of ZsGreen1 expression, and the ZsGreen1+ population was purified. Transfected cells were grown in triplicate in 1,000-cell aliquots in Matrigel in 96-well plates for 2 wk to assess their clonogenic abilities in 3D. Although transgene expression was shown for 1 wk, the initiation of clones and/or differentiated luminal structures begins within hours or days of cell plating—the major events that influence clonogenicity would have occurred while the BMI1 or CDX1 cDNA was expressed highly.

After 2 wk of culture, wells were photographed, and the number and type of colonies within each well were assessed (Fig. 6). Examples of distinctive colonies identified within the pBI-CMV3-CDX1–transfected dense lines are presented in Fig. S11.

Transient Transfection with siRNA. Cells were seeded at 8×10^5 cells per 10-cm plate, transfected with siRNA at a final concentration of 20 nM, incubated overnight, and then placed in either normoxia or hypoxia for 72 h. Cells were then harvested for protein analysis or replated in Matrigel for further experiments. For CDX1 differentiation assay, cells were transfected, incubated overnight, plated in Matrigel, and grown in either hypoxia or normoxia for 72 h before being retrieved using BD Matrigel Recovery Solution and stained for CDX1.

The sequences of siRNA used are as follows:

HIF1α sense: 5′-UCAAGUUGCUGGUCAUCAGdTdT-3′,
HIF2α sense: 5′-ACUGCUAUCAAAGAUGCUGdTdT-3′, and
CA9 sense: 5′-GGAGGAUCUACCUGAAGUUdTdT-3′.

1. Tom BH, et al. (1976) Human colonic adenocarcinoma cells. I. Establishment and description of a new line. *In Vitro* 12:180–191.
2. Chan CW, et al. (2009) Gastrointestinal differentiation marker Cytokeratin 20 is regulated by homeobox gene CDX1. *Proc Natl Acad Sci USA* 106:1936–1941.
3. Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF (2010) Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci USA* 107:3722–3727.
4. Richman P, Bodmer W (1988) Control of differentiation in human colorectal carcinoma cell lines: epithelial-mesenchymal interactions. *J Pathol* 156:197–211.

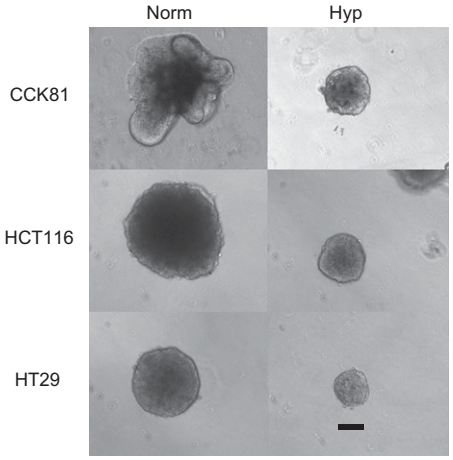

Fig. S1. Light microscopy of CCK81, HCT116, and HT29 colonies after 2–4 wk growth in Matrigel in normoxia or hypoxia. (Magnification: 20× objective; scale bar: 200 μm.)

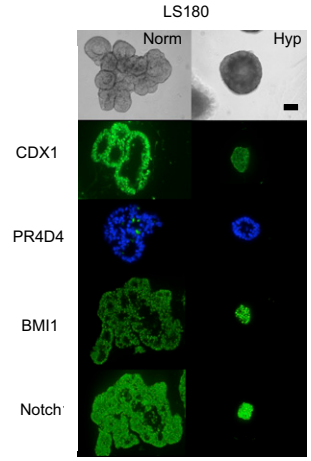

Fig. S2. Light microscopy and immunofluorescence of LS180 grown for 3 wk in 3D matrigel under normoxia and hypoxia (1% oxygen; 20× objective). (Scale bars, 200 μm.)

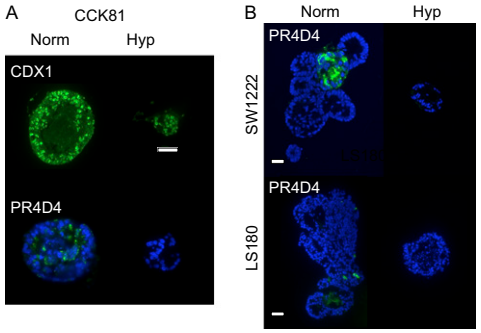

Fig. S3. (*A*) Immunofluorescence staining by monoclonal antibodies CDX1 and PR4D4 of CCK81 cell line colonies after 4 wk growth in Matrigel in normoxia or hypoxia. (Magnification: 20× objective; scale bar: 200 μm.) (*B*) Further examples of immunofluorescence staining by PR4D4 of SW1222 and LS180 colonies after 4 wk growth in Matrigel in normoxia or hypoxia. (Magnification: 20× objective; scale bar: 200 μm.)

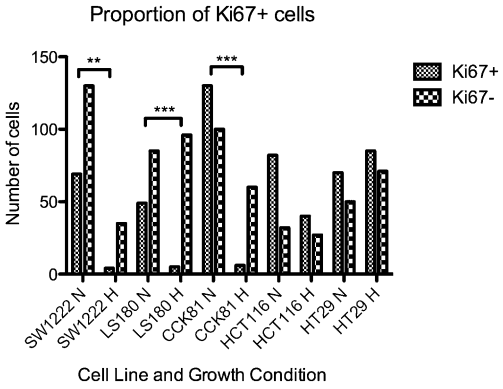

Fig. S4. Hypoxia reduces the proportion of Ki67+ve cells in cell lines that can differentiate (SW1222, LS180, and CCK81) compared with those that are mostly undifferentiated (HCT116 and HT29). Fisher exact test: $^{**}P < 0.01$ and $^{***}P < 0.001$.

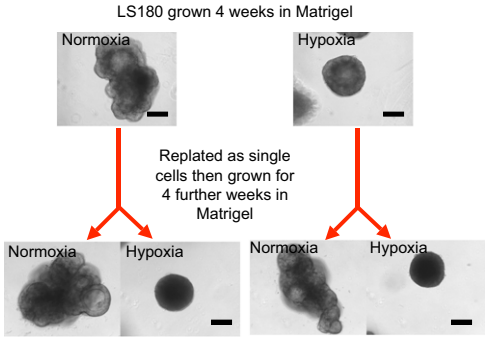

Fig. S5. LS180 colonies were grown in Matrigel in either normoxia or hypoxia for 4 wk, and then, they were retrieved and replated as single cells in Matrigel for another 4 wk in either normoxia or hypoxia. Cells from LS180 hypoxic dense colonies could still form differentiated colonies when regrown in normoxia. (Magnification: 20× objective; scale bar: 200 μm.)

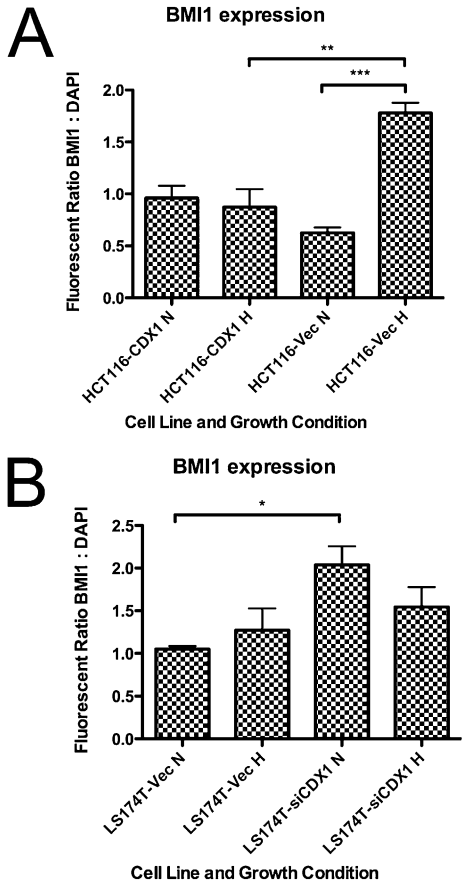

Fig. S6. Quantitation of BMI1 immunofluorescence for the paired cell lines (*A*) HCT116-CDX1; HCT116-vec and (*B*) LS174T-vec; LS174T-siCDX1 in normoxia and hypoxia (see Fig. 5).

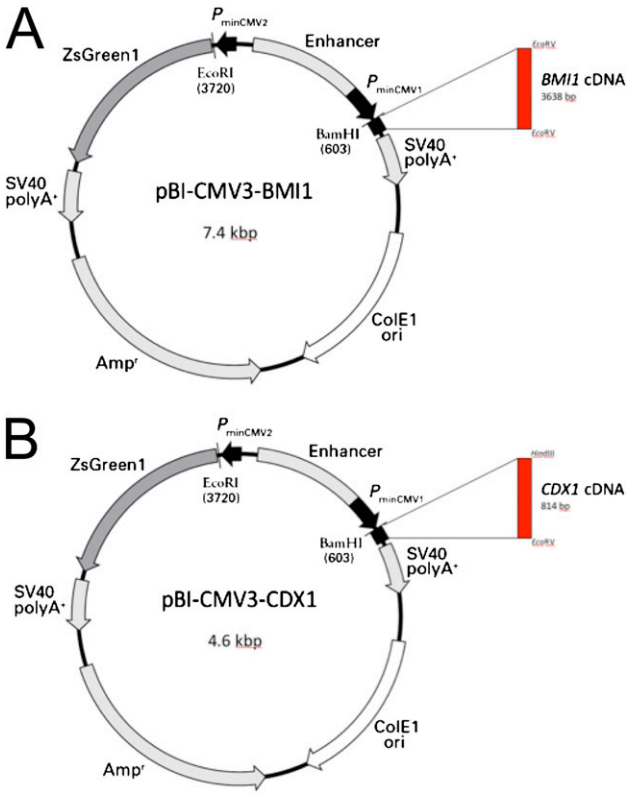

Fig. S7. Constructed plasmid vectors (Clontech) incorporating the *BMI1* (*A*) and *CDX1* (*B*) cDNAs that are constitutively expressed alongside a reporter GFP.

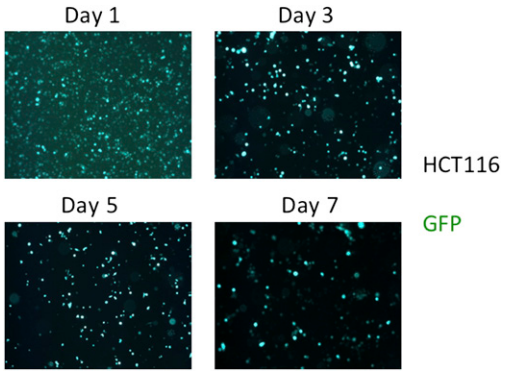

Fig. S8. Example photographs (10× magnification, 488-nm fluorescence excitation) of HCT116 cells transfected with the pBI-CMV3-CDX1 experimental vector. Even after 7 d, a significant minority of cells still expressed the vector.

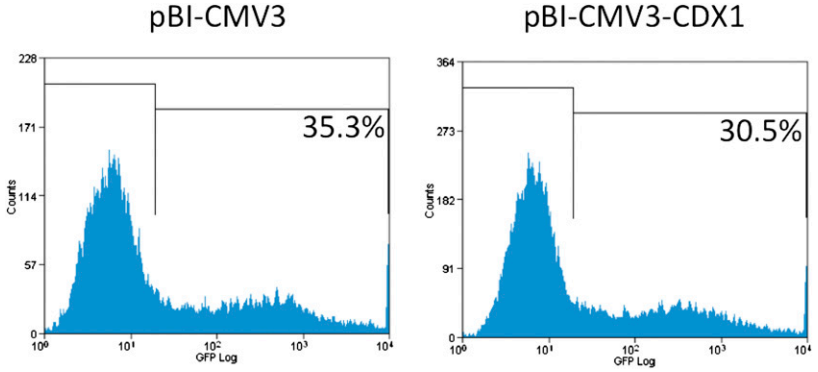

Fig. S9. Flow cytometry histograms of HCT116 cells transfected with pBI-CMV3 control or pBI-CMV3-CDX1 experimental vectors. The most fluorescent cells, corresponding to the right gates, were the cells that had successfully taken up the transfected plasmid and thus, were purified by FACS.

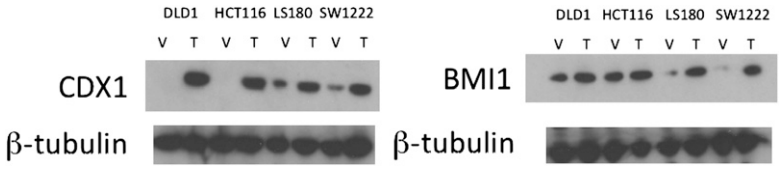

Fig. S10. Western blots showing induction of CDX1 (*Left*) and BMI1 expression (*Right*) within transfected cells. DLD1 and HCT116 expressed high levels of BMI1 endogenously, but transfection with the experimental pBI-CMV3-BMI1 did induce an increase in BMI1 expression.

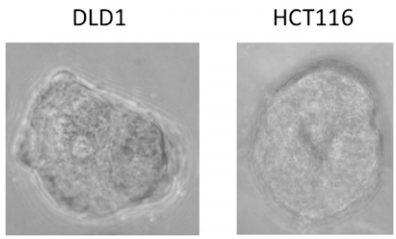

Fig. S11. Example photographs (10× magnification under phase contrast) of colonies from CDX1-transfected DLD1 and HCT116 cells. DLD1 and HCT116 were unable to differentiate in vitro to give rise to organized, polarized lumen-like structures, but the introduction of CDX1 seems to induce such ability.