Supporting Information

Sánchez-Tilló et al. 10.1073/pnas.1108977108

SI Discussion

In line with the literature, we found that nuclear translocation of β-catenin in familial adenomatous polyposis (FAP) adenomas is less evident than in FAP carcinomas. Nevertheless, it is worth noting that many cells in FAP adenomas start expressing ZEB1 despite the fact that only a moderate fraction of the total β-catenin has accumulated in the nucleus (Fig. S1*B*). This in vivo result correlates with the rapid induction of ZEB1 observed in HTC116 cells after only short incubation times with LMB, when levels of nuclear β-catenin are still moderate (Fig. 3*C* and Fig. S3*A*).

In addition, in some areas of FAP and $APC^{Min/+}$ tumors, ZEB1 was found not only in the nucleus but also atypically in the cytoplasm. There is already evidence of cytoplasmic expression of ZEB1 during development as well as in normal adult tissues and tumors (1–4). Because ZEB1 is only known as a transcription factor, any potential role of cytoplasmic ZEB1 remains to be determined. At the least, it suggests that ZEB1 function could be modulated by regulating not only its expression but also its intracellular localization.

As indicated above, secretion of hepatocyte growth factor (HGF) at the carcinoma invasive front contributes to the nuclear translocation of β-catenin in APC mutant colorectal carcinomas (CRCs) (5). HGF has been described to activate the LAMC2 promoter via JunD/Fra-2 (6), which in turn synergizes with β-catenin/TCF4 (7) and Smad3 (6) in the regulation of LAMC2. As ZEB1 is induced by β-catenin/TCF4 (this study), TGFβ (8) and Ras/Fra signaling (9), direct activation of LAMC2 by ZEB1 adds another level of complexity to the regulation of LAMC2 in CRC cells. In addition, and as indicated earlier, it could not be ruled out that ZEB1 could also activate LAMC2 indirectly via miR-200.

At this point, we could not exclude direct transcriptional regulation of MT1–MMP by ZEB1. Nevertheless, the E-box-like sequences we identified in its promoter deviate from the perfect consensus for ZEB1 binding (10). Its indirect regulation by ZEB1 is also uncertain because although MT1–MMP is a potential target for several microRNAs (e.g., miR-24, miR-26, and miR-181) (11), none of them have been linked to ZEB1.

SI Materials and Methods

Human and Mouse Tissues. Normal and tumor colorectal human samples were obtained from Institut d'Investigacions Biomèdiques August Pi i Sunyer's (IDIBAPS) Tumor Bank, and their use was approved by the local research ethics committee. The study analyzed 41 independent samples of human hereditary colorectal tumors: 12 FAP adenomas, 17 FAP carcinomas, and 12 Lynch carcinomas. Normal and adenomatous intestinal mice samples originated from 4-mo-old C57BL/6J wild-type APC and $APC^{Min/+}$ mice (The Jackson Laboratory) (12), respectively, and were kindly provided by L. Espinosa and V. Rodilla [Institut Municipal d'Investigació Mèdica (IMIM), Barcelona].

Antibodies. The commercial antibodies used in this article originated as follows: ZEB1 (H-102, E-20, and E-20-X from Santa Cruz Biotechnology), β-catenin (Ab6302 from Abcam; clone 14 from BD Biosciences; and 06–734 from Upstate-Millipore), E-cadherin (clone 36 from BD Biosciences) TCF4 (H-125 from Santa Cruz Biotechnology, and 6H5-3 from Upstate-Millipore), LAMC2 (D4B5, Upstate-Millipore), and α-tubulin (B5-1-2 from Sigma). MT1–MMP clone LEM2/15.8 was kindly supplied by A.G. Arroyo [Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid]. Secondary antibodies were obtained from Jackson ImmunoResearch: HRP-conjugated donkey antimouse IgG, HRP-conjugated goat-anti-rabbit IgG, Donkey Dylight™ 488-anti-rabbit IgG, Donkey Cy3™ antigoat IgG, Donkey Dylight™ 649 antimouse IgG, and Donkey Rho RedX™ antimouse IgG. For blocking in immunohistochemistry and immunofluorescence experiments and as control for ChIP assays, the following IgGs were used: normal goat IgG (Jackson ImmunoResearch, 5–8 mg/mL of IgG in normal serum), normal rabbit IgG (Santa Cruz Biotechnology), and normal mouse IgG (Epigentek, as included in the EpiQuik kit, see below).

Immunohistochemistry and Immunofluorescence Analyses. For tissue staining slides with 5-μm sections of formalin-fixed, paraffin embedded samples were deparaffinized and rehydrated before being subjected to antigen retrieval with 10 mM sodium citrate pH 6.0 for 20 min. All tissue samples were first screened by immunohistochemistry staining by the horseradish peroxidase and 3, 3′-diaminobenzidine (DAB) method before proceeding to mutiple immunoflourescence analysis. Slides for immunohistochemistry were first treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase, whereas slides for immunofluorescence staining were first incubated for 30 min with 0.1% sodium borohydride. In either case, slides were then incubated with a nonspecific binding blocking solution (5% goat or donkey normal serum plus 4% BSA in PBS, 0.5% Tween 20) followed by the corresponding primary (overnight at 4 °C) and HRP-conjugated or fluorochrome-conjugated secondary (1 h at 37 °C) antibodies. The immunohistochemistry reaction was developed with a DAB substrate Kit (Vector Labs) before slides were counterstained with hematoxilin and mounted in Di-N-butylPhthalate in Xylene solution (DPX, Sigma). Slides for immunofluorescence analysis were mounted in Vectashield-DAPI medium (Vector Labs). For immunofluorescence staining of established CRC cell lines, cells were grown in 8-well Permanox™ LabTek chambers (Nalgene-Nunc), fixed for 30 min with 4% paraformaldehyde solution (Electron Microscopy Sciences) and permeabilized with PBS-0.5% Triton X100. LabTek slides were then incubated with the blocking solution, primary, and fluorochrome-conjugated secondary antibodies and mounted with Vectashield-DAPI medium as described for tissue sections. Immunofluorescence was examined in a TCS SP5 Spectral confocal microscope (Leica) at the Microscopy Unit of the University of Barcelona, whose staff is here acknowledged for their technical advice. Images were analyzed using ImageJ software applying a median filter of 0.5–1 pixels.

Plasmids. The expression vector for ZEB1 was previously described (13). Other expression vectors were obtained from the following researchers: β-catenin and β-catenin^{SA} from J. Woodgett (Mount Sinai Hospital, Canada) (14), TCF4 and $TCF4_{d1-30}$ from M. Kato (University of Tsukuba, Japan) (15), and TLE-1 from S. Stifani (Montreal Institute, Canada) (16). Firefly luciferase reporters for the different promoters used in this article were obtained from the following researchers: −1,129/ + 55 of ZEB1 from M. Saito (University of Tokyo, Japan), −1,246 bp of human MT1–MMP from K. Lehti (Helsinki University, Finland), and 0.8 kb of human LAMC2 promoter from I. Schwarte-Waldhoff (Bochum University, Germany). TOPFLASH and FOPFLASH luciferase reporters (17) were obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD). pCMV-β-galactosidase was purchased from Clontech, and pBluescript SK vector from Stratagene-Agilent.

siRNAs and shRNAs. siRNA were ordered from Invitrogen or Integrated DNA Technologies using sequences described in the literature: ZEB1 (18), β-catenin (19) and TCF4 (20). As negative control, we used a scramble control siRNA (5′-GGUUACGAA-CUAAGCUAUA-3′) as well as (in Western blot/qRT-PCR studies) a siRNA against firefly luciferase as reported in ref. 21. Lentiviral particles encoding either a pool of three shRNAs against human ZEB1 (sc-38643-V)—whose sequences are different from those included in the siRNA oligonucleotide—or a control shRNA (sc-108080-V) were purchased from Santa Cruz Biotechnology.

Cell Lines and Cell Culture. SW480 and HCT116 cells (13) were cultured in Dulbecco's modified Eagle medium (Lonza) supplemented with 10% FCS (Sigma). In time-course studies with Leptomycin-B (LMB) (Sigma L2913, dissolved in 70% methanol as per manufacturer's instructions), cells were treated with 10 ng/mL of the drug for the period indicated in each individual experiment. In transcriptional experiments, cells were treated with LMB 48 h after transfection of the reporter vectors. After incubation with LMB, cells were washed twice with cold PBS, placed on ice, and processed immediately altogether for transcriptional, mRNA, or protein analyses.

Transient and Stable Cell Transfections. Cells were transiently transfected with expression or reporter vectors using Lipofectamine 2000 (Life Technologies-Invitrogen) and/or with siRNA oligonucleotides using Lipofectamine RNAiMAX (Life Technologies-Invitrogen). After 48 h, cells were processed for transcription, mRNA, or protein analyses. SW480 cells were stably knocked down for ZEB1 expression by infection with lentiviral particles encoding a pool of shRNAs along puromycin resistance (see above) followed by selection in puromycin-containing media.

Transcriptional Assays. In transcriptional experiments, cells were transfected with firefly luciferase reporter vectors and equal molar amounts of either cDNA-containing (denoted in figures as "+") or reference control empty ("−") expression vectors. As internal control for transfection efficiency, all points included cotransfection with 0.1 μg pCMV-β-gal. Total DNA was brought to the same amount by adding promoterless pBluescript SK vector. Firefly luciferase activity was assessed with a Luciferase Assay System kit (Promega Corporation), whereas β-galactosidase was determined with Luminiscent β-galactosidase Detection Kit II (Clontech). Relative luciferase activity (RLU) values throughout the article are expressed as the mean of duplicates and are representative of at least four independent experiments.

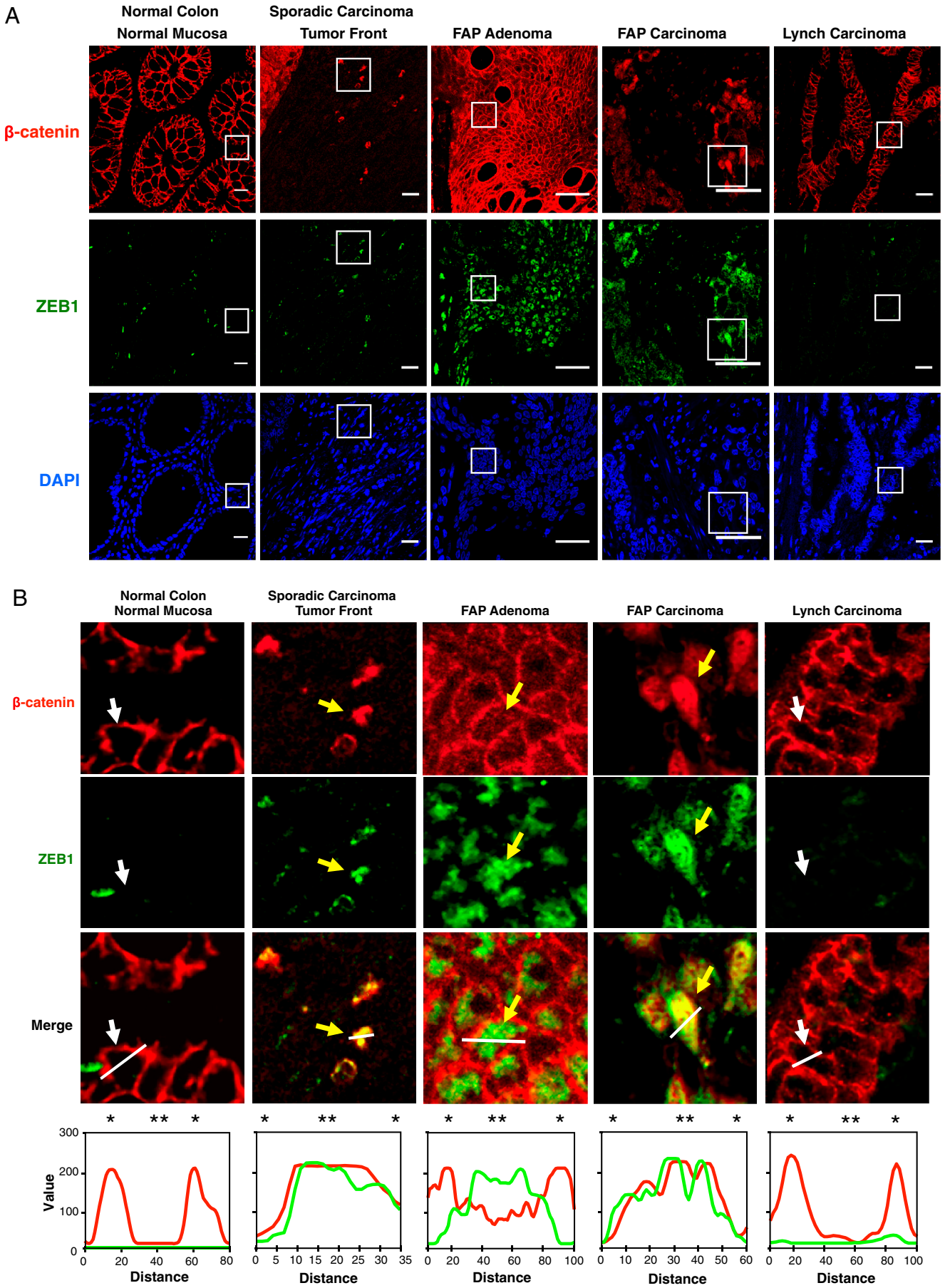
Site-Directed Mutagenesis. Identification of ZEB1 consensus binding sequences was conducted using MacVector software. ZEB1 binding sites at positions −213 and −96 in the human LAMC2 promoter were mutated using the QuickChange Lightning site-directed mutagenesis kit (Agilent) following manufacturer's instructions. Complementary mutant primers to opposite strands of the target DNA were designed using QuickChange Primer Design Program (www.agilent.com/genomics/qcpd) and purchased from Integrated DNA Technologies. Binding sites were mutated to sequences known to not bind ZEB1 (22). For the ZEB1 binding site at −213 of human LAMC2, the upper strand oligonucleotide sequence used is 5′-CCTTGGCCCGGGCCTATTGTGCGCCCTC-CTC-3′. For the ZEB1 binding site at −96 of the human LAMC2, the upper strand oligonucleotide sequence used is 5-GCTGGGT-CCTCCTTATTCACTATTGAGTCACACCCTGAAACA-3′. Mutations were confirmed by sequencing with BigDye® Direct Cycle Sequencing Kit (Life Technologies-Applied Biosystems) with primer 5′-CTGCCTTATTTCTGAGTTAGCG -3′.

RNA Extraction and Quantitative Real-Time PCR. Total RNA extracted with Trizol reagent (MRC Inc.) was used to synthesize cDNA with random hexamers and a commercial kit (Roche) according to manufacturer's instructions. mRNA was quantified by quantitative real-time PCR (qRT-PCR) at 60 °C using SYBR Green/ROX (GoTaq, Promega) and oligonucleotide primer sequences (Invitrogen or Integrated DNA Technologies) previously described in the literature: ZEB1 (23), β-catenin (24), TCF4 (25), E-cadherin (26), MT1–MMP (27), LAMC2 (6), cyclin D1 (28), and GAPDH (29). Relative mRNA levels were determined by Opticon Monitor 3.1.32 software (BioRad) by Ct method comparing values of genes to housekeeping GAPDH on corresponding cDNA. qRT-PCR data shown in the study are representative of at least three independent experiments.

Western Blot Assays. Western blot assays were performed as previously described (13). Briefly, SW480 and HCT116 cells were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% SDS, 50 mM Tris pH 8, 2 mM EDTA plus protease inhibitors) and loaded onto 8% poly acrylamide gels. Gels were then transferred to a PVDF membrane (Immobilon-P, Millipore). Following blocking for nonspecific antibody binding with 4% nonfat milk, membranes were incubated with the corresponding primary and HRP-conjugated secondary antibodies before the reaction was developed using Pierce's ECL Western Blotting Substrate or SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Western blots shown in the article are representative of at least four independent experiments.

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation (ChIP) assays were performed using the EpiQuick ChIP kit (Epigentek) as per manufacturer's instructions. Briefly, SW480 cells were incubated during 10 min with 1% formaldehyde solution (Electron Microscopy) at room temperature followed by incubation with 125 mM glycine. Lysates were sonicated in an Ultrasonic Liquid Processor (Misonix Inc.). Settings were as follows: 50 amplitude, 7 min (on for 10 s, off for 15 s on ice). Antibodies used for ChIP were as follows: mouse anti-TCF4 (6H5-3), rabbit anti-β-catenin (06–734), goat anti-ZEB1 (E-20-X), normal mouse IgG (as included in EpiQuick kit), normal rabbit IgG (Santa Cruz Biotechnology), and normal goat IgG (Jackson ImmunoResearch). DNA fragments were quantified by qRT-PCR as detailed above. Identification of DNA binding sequences for TCF and ZEB1 and design of primers for qRT-PCR was conducted using MacVector software. For the TCF binding site at position −578 of the human ZEB1 promoter, the primers amplifying the region between −674 and −477 were as follows: forward 5′- TGGAAGGGAAGGGAAGGGAGTC-3′ and reverse 5′-AGGCAGGGCTACCATCAGTC-3′. For the TCF binding site at position −161 of the human ZEB1 promoter, the primers amplifying the region between −325 and −101 were as follows: forward 5′-TTTACCTTTCCAACTCCGACAGC-3′ and reverse 5′-GGCTTTACGACATCACCTTCCTTAC-3′. For the ZEB1 binding site at position −213 of the human LAMC2 promoter, the primers amplifying the region between −256 and −126 were: forward 5′-CTCGCACATTCCAGGCAAAG-3′ and reverse 5′-CAGACAAACACACAGAGCACAAACC-3′. For the ZEB1 binding site at position −96 of the human LAMC2 promoter, the primers amplifying the region between −124 and −95 region were as follows: forward 5′-TGTGCTCTGTGTGTTTG-TCTGCC-3′ and reverse 5′-TGTGAATAAGGAGGACCCA-GCC-3′. The primers used for amplification of a fragment of the GAPDH promoter are included in the ChIP EpiQuik kit. In all qRT-PCRs of ChIP assays, values shown represent relative binding in relation to input and are the average of three independent ChIP experiments. Qualitative PCR of ChIP assays was performed using 1 μL of immunoprecipitated DNA. Following 28 cycles of amplification, PCR products were analyzed in a 1.5% agarose gel by Ethidium Bromide staining.

1. Spaderna S, et al. (2006) A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. *Gastroenterology* 131:830–840.
2. Franklin AJ, Jetton TL, Shelton KD, Magnuson MA (1994) BZP, a novel serum-responsive zinc finger protein that inhibits gene transcription. *Mol Cell Biol* 14:6773–6788.
3. Graham TR, et al. (2010) Reciprocal regulation of ZEB1 and AR in triple negative breast cancer cells. *Breast Cancer Res Treat* 123:139–147.
4. Gemmill RM et al. (2011) ZEB1-responsive genes in non-small cell lung cancer. *Cancer Lett* 300:66–78.
5. Vermeulen L, et al. (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 12:468–476.
6. Olsen J et al. (2003). Converging signals synergistically activate the LAMC2 promoter and lead to the accumulation of the laminin g2 chain in human colon carcinoma cells. *Biochem J* 371:211–221.
7. Hlubek F, et al. (2001) Expression of the invasion factor laminin γ2 in colorectal carcinomas is regulated by β-catenin. *Cancer Res* 61:8089–8093.
8. Shirakihara T, Saitoh M, Miyazono K (2007) Differential regulation of epithelial and mesenchymal markers by δEF1 proteins in epithelial mesenchymal transition induced by TGF-β. *Mol Biol Cell* 18:3533–3544.
9. Shin S, Dimitri CA, Yoon SO, Dowdle W, Blenis J (2010) ERK2 but not ERK1 induces epithelial-to-mesenchymal transformation via DEF motif-dependent signaling events. *Mol Cell* 38:114–127.
10. Remacle et al. (1999) New mode of DNA binding of multi-zinc finger transcription factors: δEF1 family members bind with two hands to two target sites. *EMBO J* 18:5073–5084.
11. Dalmay T, Edwards DR (2006). MicroRNAs and the hallmarks of cancer. *Oncogene* 25:6170–6175.
12. Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 247:322–324.
13. Sanchez-Tillo E, et al. (2010) ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene* 29:3490–3500.
14. Hino S, et al. (2005) Phosphorylation of β-catenin by cyclic AMP-dependent protein kinase stabilizes β-catenin through inhibition of its ubiquitination. *Mol Cell Biol* 25:9063–9072.
15. Sasaki T, et al. (2003) Lymphoid enhancer factor 1 makes cells resistant to transforming growth factor β-induced repression of c-myc. *Cancer Res* 63: 801–806.
16. Nuthall HN, Joachim K, Stifani S. (2004) Phosphorylation of serine 239 of Groucho/TLE1 by protein kinase CK2 is important for inhibition of neuronal differentiation. *Mol Cell Biol* 24:8395–8407.
17. Korinek V, et al (1997) Constitutive transcriptional activation by a β-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 275:1784–1787.
18. Eger A, et al. (2005) δEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* 24:2375–2385.
19. Li H, et al. (2009) Down-regulation of death-associated protein kinase-2 is required for β-catenin-induced anoikis resistance of malignant epithelial cells. *J Biol Chem* 284: 2012–2022.
20. Shulewitz M, et al. (2006) Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer. *Oncogene* 25:4361–4369.
21. Judge AD, Sood V, Shaw JR, et al. (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature Biotech* 23: 457–462.
22. Sekido R et al. (1994) The δ-crystallin enhancer-binding protein δEF1 is a repressor of E2-box-mediated gene activation. *Mol Cell Biol* 14:5692–5700.
23. Peña C, et al. (2005) E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: Clinicopathological correlations. *Hum Mol Genetics* 14:3361–3370.
24. Beiter K, et al. (2005) β-catenin regulates the expression of tenascin-c in human colorectal tumors. *Oncogene* 24:8200–8204.
25. Kalscheuer VM, et al. (2008) Disruption of the TCF4 gene in a girl with mental retardation but without the classical Pitt-Hopkins syndrome. *Am J Med Genet* 146A:2053–2059.
26. Yang S, et al. (2007) BMP-6 promotes E-cadherin expression through repressing δEF1 in breast cancer cells. *BMC Cancer* 7:211–224.
27. Miyoshi A, et al. (2005) Snail accelerates cancer invcasion by upregulating MMP expression and is associated with poor prognosis of hepatocellular carcinoma. *Br J Cancer* 92:252–258.
28. Khan NI, Bradstock KF, Bendall LJ (2007) Activation of Wnt/β-catenin pathway mediates growth and survival in B-cell progenitor acute lymphoblastic leukaemia. *Br J Haematol* 138:338–348.
29. Dohadwala M, et al. (2006) Cyclooxygenase-2-dependent regulation of E-cadherin: Prostaglandin E_2 induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. *Cancer Res* 66:5338–5345.


A
Normal Colon
Normal Mucosa
Sporadic Carcinoma
Tumor Front
FAP Adenoma
FAP Carcinoma
Lynch Carcinoma
β-catenin
ZEB1
DAPI
B
Normal Colon
Normal Mucosa
Sporadic Carcinoma
Tumor Front
FAP Adenoma
FAP Carcinoma
Lynch Carcinoma
β-catenin
ZEB1
Merge
Value
Distance

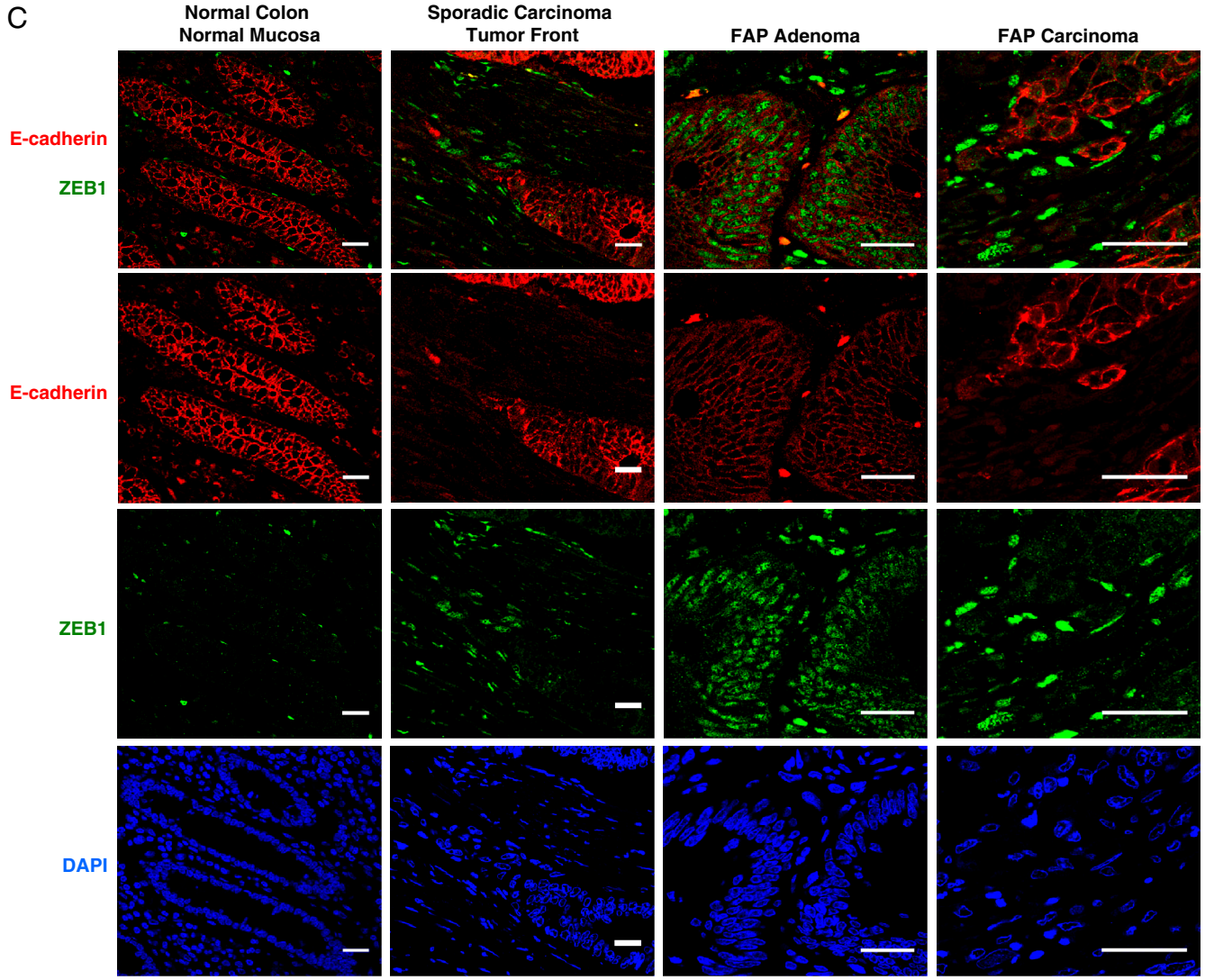
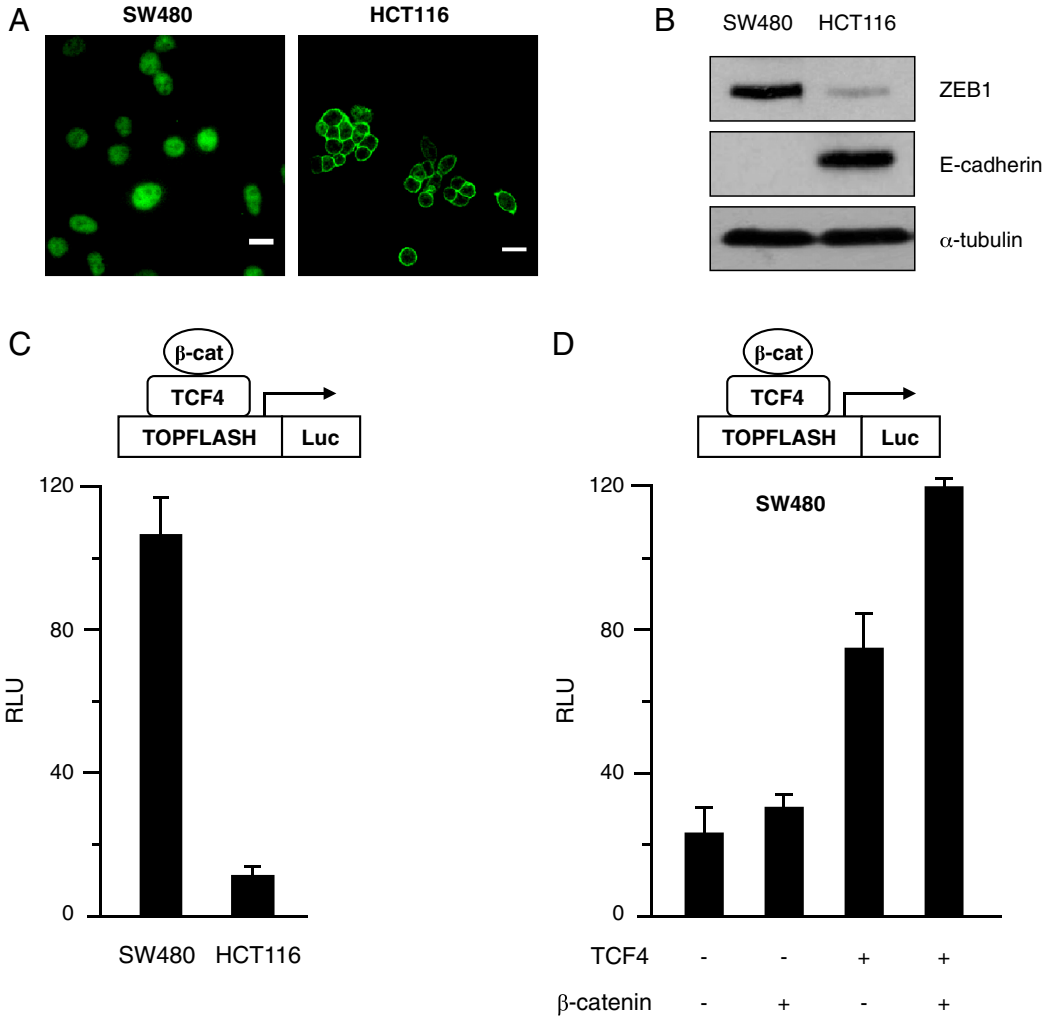


Fig. S1. (*A*), Single staining for β-catenin (red, clone 14 or Ab6302) and ZEB1 (green, H-102 or E-20) corresponding to Fig. 1*A*. Labeling with DAPI is also shown. In sporadic CRCs, dedifferentiated cancer cells at the tumor front were stained for ZEB1 (green) with E-20 and for β-catenin (red) with Ab6302, which was originally detected with Dylight™ 649 (invisible far red) but converted to red for representation. For the remaining samples (normal colon, FAP adenomas and carcinomas, and Lynch carcinomas), ZEB1 was stained with Ab H-102 and β-catenin with Ab clone 14. The area included within the white square is magnified in *B*. Scale bar, 25 μm. (*B*), Staining for β-catenin, ZEB1, and merge (β-catenin/ZEB1) in *A* is magnified to better appreciate intracellular localization of β-catenin and, where applicable, its colocalization with ZEB1. Absence of nuclear β-catenin and ZEB1 in normal colonic mucosa and Lynch CRCs are illustrated with a white arrow pointing toward a representative nucleus. Accumulation of nuclear β-catenin and ZEB1 expression in cells at the tumor front of sporadic CRCs and FAP colorectal adenomas and carcinomas are illustrated with a yellow arrow pointing toward a representative nucleus. (*Bottom*) An RGB profile shows relative levels of membrane (single asterisk, *) and cytoplasmic/nuclear (double asterisk, **) expression for β-catenin (red) and ZEB1 (green) across a cell section (white diagonal line in merged panel, relative distance values) for every sample. RGB profiles show no expression of either β-catenin or ZEB1 in the cytoplasmic/nuclear compartment of normal mucosa and Lynch carcinoma. Cytoplasmic/nuclear expression of β-catenin in the tumor front of sporadic CRCs and FAP adenomas and carcinomas correlates with ZEB1 expression. (*C*) Sections of normal colonic mucosa, tumor front of sporadic CRCs, and FAP adenomas and carcinomas were immunostained for E-cadherin (red, clone 36) and ZEB1 (green, H-102). A representative merge picture is shown along with single staining for either protein and DAPI. Scale bars, 25 μm.


A
SW480
HCT116
B
SW480
HCT116
ZEB1
E-cadherin
α-tubulin
C
β-cat
TCF4
TOPFLASH
Luc
120
80
40
0
RLU
SW480
HCT116
D
β-cat
TCF4
TOPFLASH
Luc
SW480
120
80
40
0
RLU
TCF4 - - + +
β-catenin - + - +

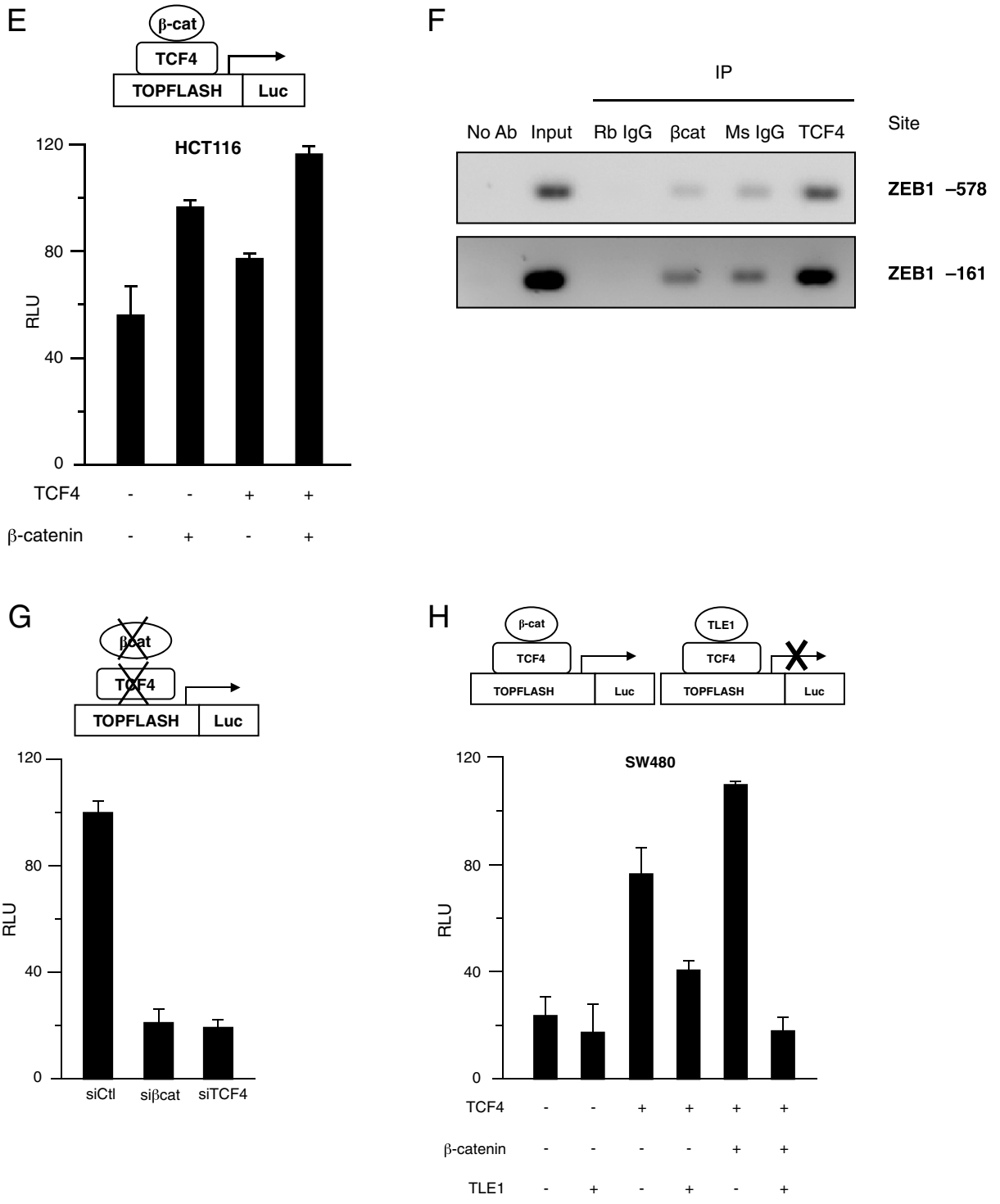


Fig. S2. (*A*) Intracellular localization of β-catenin (Ab6302) in SW480 and HCT116 cells. (*B*) Protein expression of ZEB1 (H-102) and E-cadherin (clone 36) in SW480 and HCT116 cells as assessed by Western blot. Expression of α-tubulin (B5-1-2) is included to control for loading. (*C*) β-catenin/TCF4-mediated transcription in SW480 and HCT116 cells. SW480 and HCT116 cells were transfected with 0.5 μg of the TOPFLASH luciferase reporter containing multimerized wild-type TCF binding elements. Only background RLU was detected in both cell types for the FOPFLASH reporter containing multimerized mutated TCF binding sites. (*D*) Same as in Fig. 2*B*, but for TOPFLASH reporter. SW480 cells were transfected with 0.5 μg of TOPFLASH along with 1 μg of empty vector or equal molar amounts of expression vectors for TCF4 and/or β-catenin. No RLU activity was detected for the FOPFLASH reporter. (*E*) Same as in *D* but in HCT116 cells. (*F*) TCF4 and β-catenin bind to the ZEB1 promoter. Qualitative PCR for fragments of the ZEB1 promoter precipitated in ChIP assays from SW480 cells with antibodies against TCF4, β-catenin, their respective control rabbit and mouse IgG, as well as with no antibody. The two amplified regions of the ZEB1 promoter contain potential TCF binding sites at positions −578 and −161. (*G*) As in *C*, SW480 cells were transfected with TOPFLASH along with 100 nM of siRNAs for β-catenin (siβ-cat), TCF4 (siTCF4), or a scrambled negative control siRNA (siCtl). No RLU activity was detected for the FOPFLASH reporter. (*H*) TLE1/TCF4 complexes inhibit transcriptional activation of TOPFLASH by β-catenin/TCF4. As in *D*, SW480 cells were transfected with TOPFLASH along combinations of 1 μg of empty vector or equal molar amounts of expression vectors for TCF4, β-catenin and/or TLE1. No transcriptional activity was detected for FOPFLASH reporter.

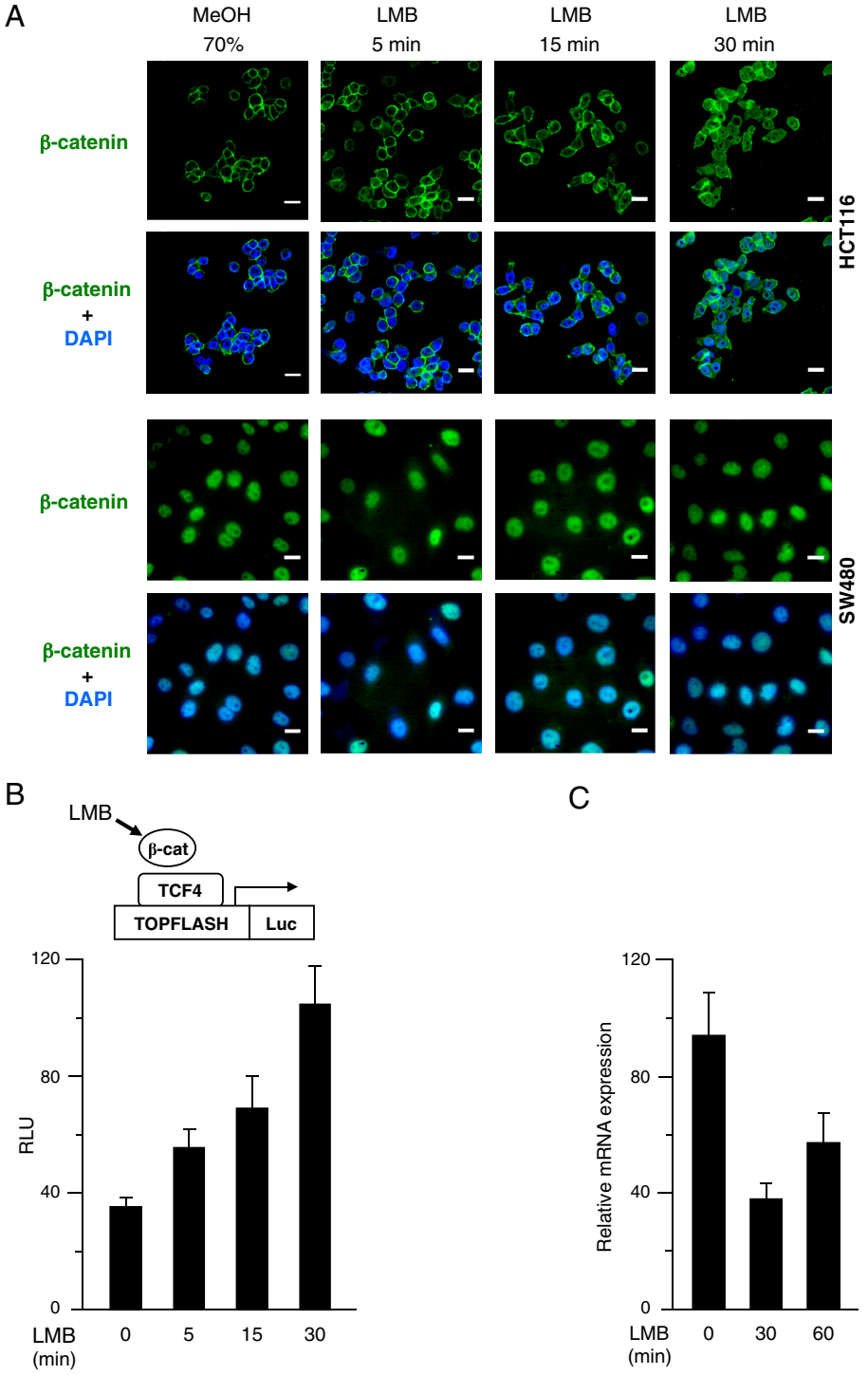


Fig. S3. (*A*) Intracellular localization of β-catenin in HCT116 and SW480 cells upon incubation with 10 ng/mL of LMB for different time periods. Cells were immunostained for β-catenin (Ab6302) and counterstained with DAPI. Cells were also subjected for 30 min to the same volume amount of the solvent in which LMB was resuspended (70% methanol). (*B*) HTC116 cells were transfected with 0.5 μg of TOPFLASH reporter and after 48 h treated with 10 ng/mL of LMB for the indicated periods before being processed for quantification of luciferase activity (see *SI Materials and Methods* above). No RLU activity was detected for the FOPFLASH reporter. (*C*) Downregulation of E-cadherin mRNA in HCT116 cells upon incubation with 10 ng/mL of LMB for the indicated time periods. Relative mRNA levels in respect to housekeeping GAPDH were determined by qRT-PCR.

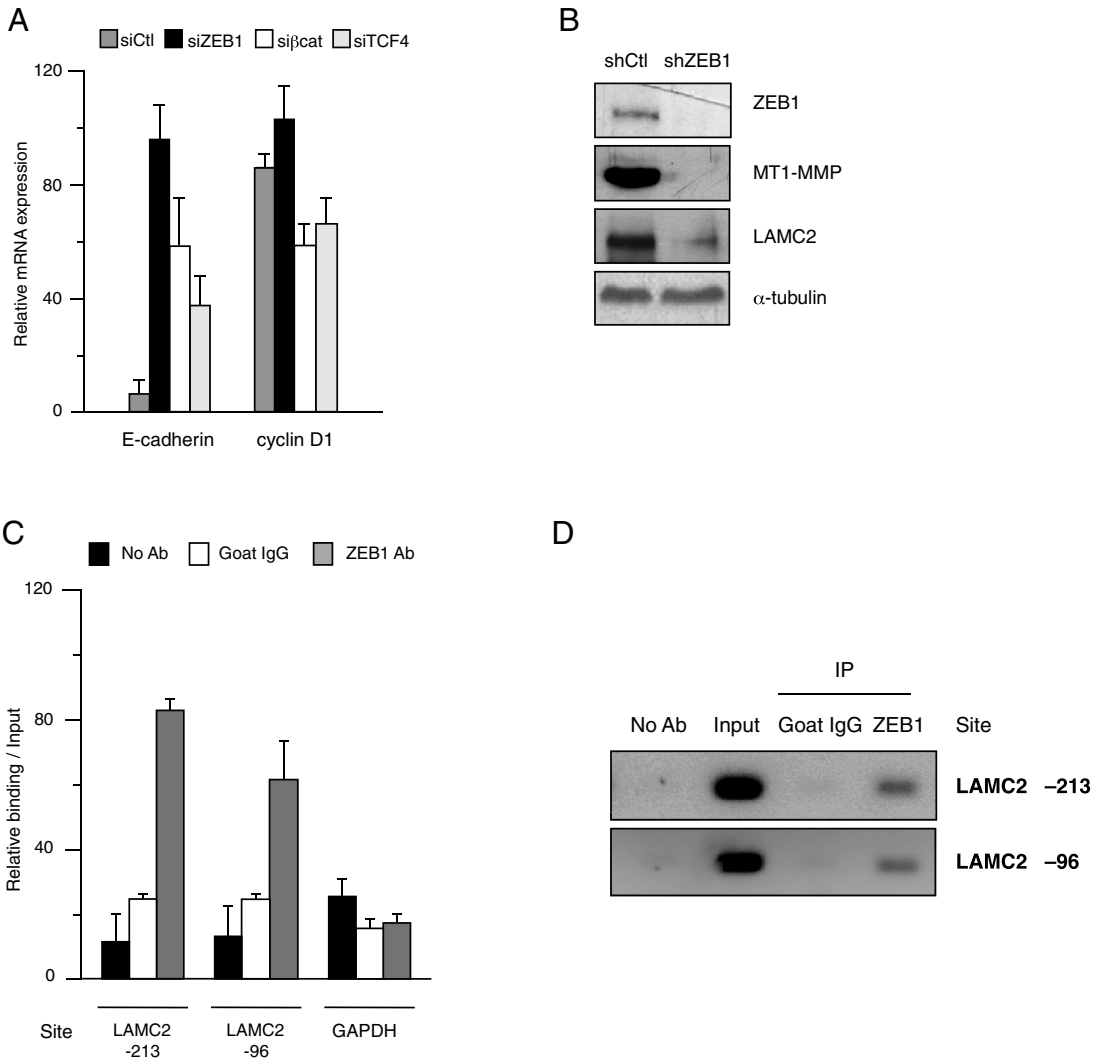
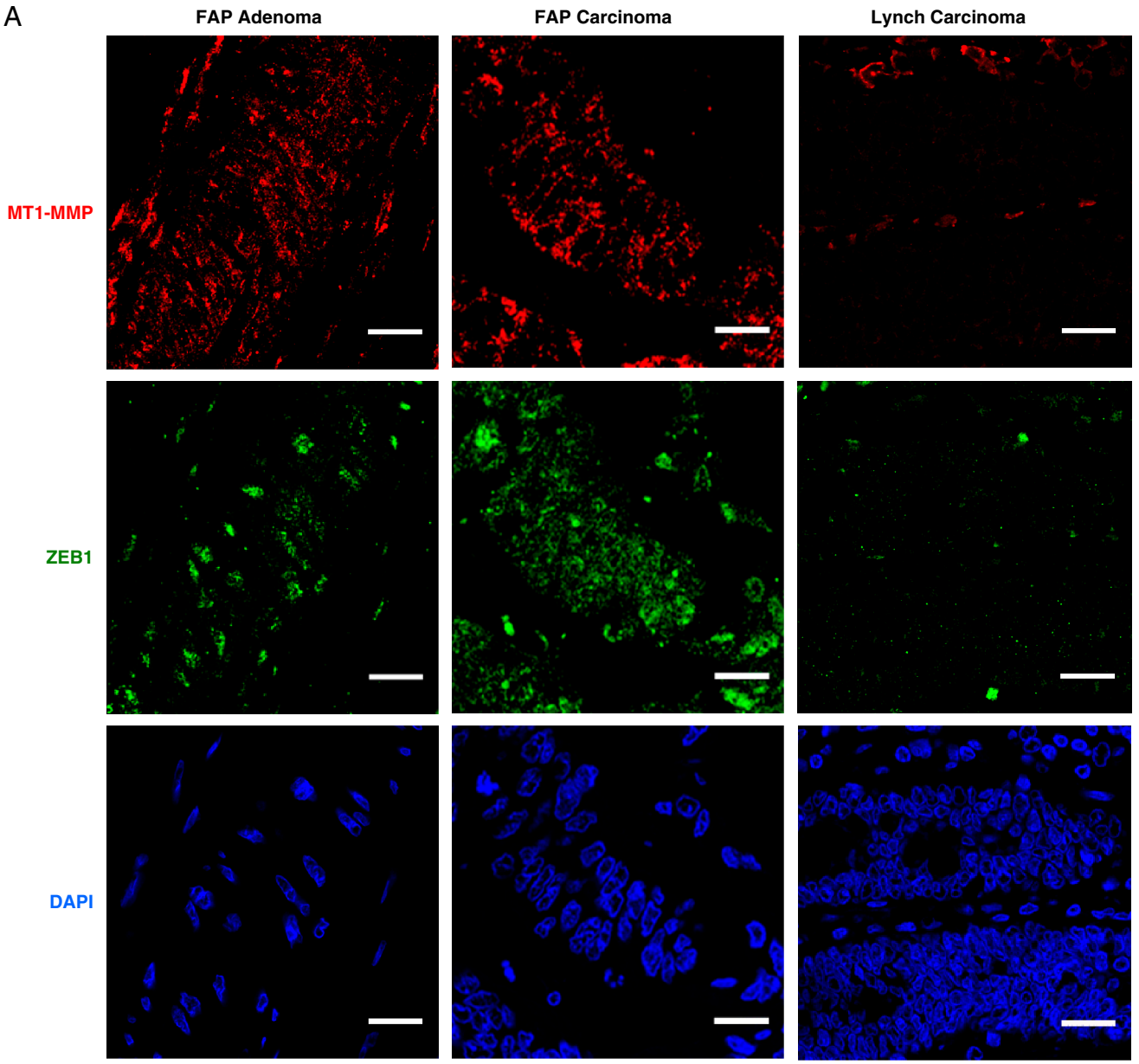
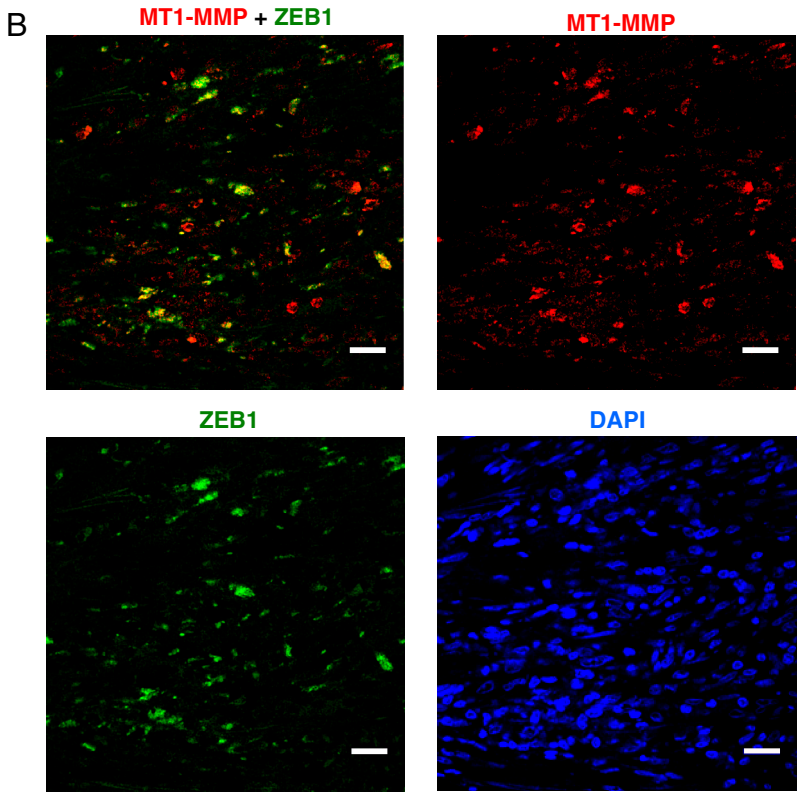


Fig. S4. (*A*) Relative mRNA expression of E-cadherin and cyclin D1 in SW480 cells transfected with a siRNA control, or specific siRNAs for ZEB1, β-catenin, and TCF4. Relative mRNA levels were determined by qRT-PCR and normalized respect to GAPDH. (*B*) ZEB1 regulates endogenous MT1–MMP and LAMC2 proteins. SW480 cells were stably knocked down for ZEB1 using specific lentiviral particles for shRNAs against ZEB1 (shZEB1) or control shRNA (shCtl). Upon selection with puromycin, cells were lysed and protein levels assessed by Western blot for ZEB1 (H-102), MT1–MMP (LEM2/15.8), LAMC2 (D4B5), and α-tubulin (B5-1-2). (*C*) ZEB1 binds to the human LAMC2 promoter in CRC cells with mutant APC. qRT-PCR of fragments of the LAMC2 and GAPDH promoters immunoprecipitated in ChIP assays from SW480 cells with ZEB1 Ab, control IgG or no Ab. The amplified LAMC2 promoter regions contain ZEB1 binding sites at positions −213 and −96. (*D*) Same as in *C*, but analysis of ChIP assays on the LAMC2 promoter was performed by qualitative PCR.


A
FAP Adenoma
FAP Carcinoma
Lynch Carcinoma
MT1-MMP
ZEB1
DAPI


B
MT1-MMP + ZEB1
MT1-MMP
ZEB1
DAPI

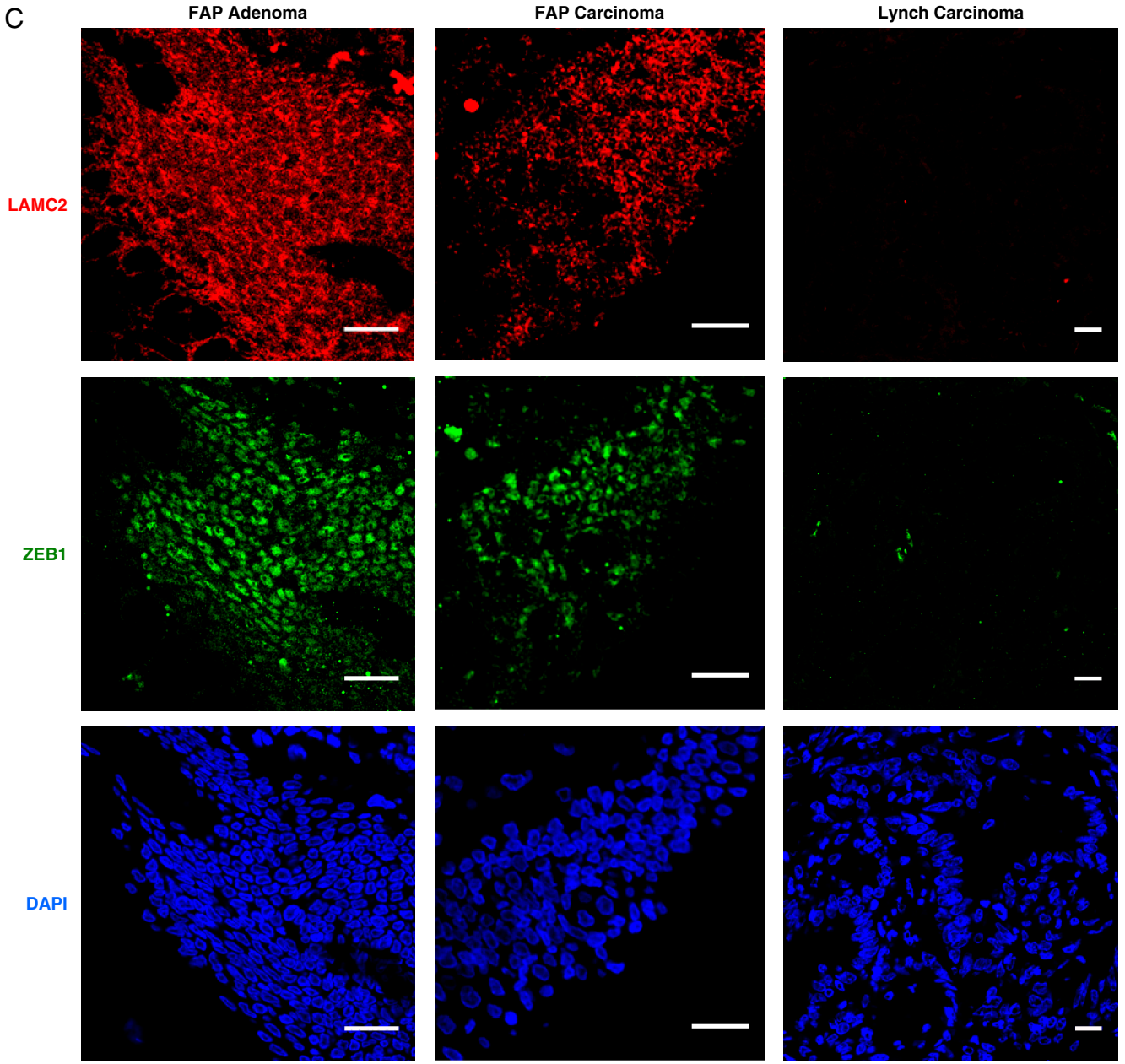


Fig. S5. (*A*) Single staining for MT1–MMP (red, LEM2/15.8) and ZEB1 (green, H-102) corresponding to Fig. 5*A*. Labeling for DAPI is also shown. Scale bar, 25 μm. (*B*) MT1–MMP colocalizes at the nucleus with ZEB1 in dedifferentiated cancer cells at the tumor front of sporadic CRCs. Sections were stained with MT1–MMP (red, LEM2/15.8) and ZEB1 (green, E-20). Dedifferentiated cancer cells at the tumor front were originally identified by their nuclear accumulation of β-catenin. MT1–MMP (red, LEM2/15.8) was first detected with Dylight™ 649 (invisible far red) but converted to red for representation. DAPI staining is also shown. Scale bar, 25 μm. (*C*) Single staining for LAMC2 (red, D4B5) and ZEB1 (green, H-102), corresponding to Fig. 5*B*. Labeling for DAPI is also shown. Over 90% of cells positive for ZEB1 in FAP adenomas and carcinomas coexpressed LAMC2. Scale bar, 25 μm.