Supplemental Material and Methods:

Western Blotting and Immunoprecipitation (IP)

For Western blotting of NF-κB pathway proteins and for immunoprecipation assays, cells were treated with experimental or control medium for 30 minutes. Total protein was immediately extracted using 200 μl of 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) according to the manufacturer's instructions. Protein concentrations were determined using the BCA-200 Protein Assay kit (Pierce, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes and incubated with primary antibodies overnight at 4^{o}C (Supplemental Table 1). Secondary antibody was either goat anti-rabbit or horse anti-mouse IgG (Cell Signaling Technology) conjugated with horseradish peroxidase (Cell Signaling Technology), and chemiluminescence was determined using the ECL detection system (Pierce, Rockford, IL) or the Super Signal West Dura detection system (Thermo Scientific, Waltham, MA). The membranes were stripped and re-probed with mouse anti-β-tubulin (Sigma, St. Louis, MO) as a loading control; lamin was used as a nuclear protein loading control. For immunoprecipitation assays, the experimental or control medium was removed, and the cells were washed in cold phosphate-buffered saline (PBS) and placed in lysis buffer containing 50 mM TrisHCL, 150 mM NaCl, 1% NP-40 with one tablet of Protease Inhibitor (Roche Life Sciences, Indianapolis, IN) per 50 ml of buffer. Equal amounts of protein were immunoprecipitated with saturating amounts of p65 overnight at 4°C, followed by a 1-hour incubation with 50 μl protein A-sepharose beads at 4°C. The beads were then washed, boiled, and the supernatants were used to immunoblot with antibodies against p50, IκBα, or PKAc. All blots were performed in two independent experiments.

Nuclear/Cytoplasmic Fractionation and Immunofluorescence (IF)

For nuclear/cytoplasmic fractionation and immunofluorescence, cells were treated with experimental or control medium for 30 minutes and the medium was then removed and replaced with neutral pH medium for 2 hours. Nuclear extracts were then isolated using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Rockford, IL) per manufacturer's instructions, and Western blotting was performed as above. Protein expression levels were quantified using NIH Image J 1.48 software, and the relative quantity of protein with respect to β-tubulin or lamin was calculated. All blots were performed in two independent experiments. For immunofluorescence, cells were fixed in 4% paraformaldehyde for 15 minutes. The sections were incubated for 10 minutes in phosphate-buffered saline, 2% bovine serum albumin and 0.2% Triton X-100, then washed and incubated for 1 hour at room temperature with 1:250 dilutions of polyclonal rabbit anti-human antibodies to p65 or p50 (Cell Signaling Technologies, Beverly, MA). The binding of primary antibodies was detected by a 1:250 dilution of goat anti-rabbit immunoglobulin (Ig)G fluorescein isothiocyanate–conjugated secondary antibody (Sigma-Aldrich). The cells then were stained with DAPI for 1 minute and washed with PBS three times. Cells were imaged with a Leica DM6000 B fluorescence microscope (Leica Microsystem, Buffalo Grove, IL).

Chromatin Immune-Precipitation (ChIP) Assay

ChIP assay was performed according to the protocol published by Nelson *et al.* with minor modifications. Cells were treated with experimental or control media for 24 hours. In brief, cells were cross-linked with 1.4% formaldehyde for 15 minutes at room temperature. The reaction was stopped by treatment with 125 mM glycine for 5 minutes at room temperature, and the cells were scraped and centrifuged to pellet. The cells were lysed in IP buffer containing 150 mM NaCl, 50 mM Tric-Hcl (pH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1.0% vol/vol), 0.1 M PMSF, and one Protease Inhibitor Cocktail Tablet per 50 ml of lysis buffer (Roche Applied

Science, Indianapolis, IN). Following lysis, cells were centrifuged, washed, and resuspended in the IP buffer. To shear the chromatin, the resuspended pellet was sonicated to generate DNA fragments of 0.5–1 kilobase. The sheared chromatin was cleared by centrifugation, and the supernatant (500 µg) was immunoprecipitated overnight at 4°C with 1µg of polyclonal rabbit anti-human p65 (Abcam) or anti-human p50 (Abcam); rabbit IgGs were used as isotype controls. Following immunoprecipitaton, the chromatin was again cleared by centrifugation, and 90% of the supernatant was transferred to prewashed Protein A-agarose beads (Upstate Biotechnologies, Billerica, MA) diluted 1:1 with IP buffer (20 µl beads:20 µl IP buffer), then rotated at 4°C for 45 min and again centrifuged to pellet. The beads were then washed 5–6 times with IP buffer without the protease inhibitors, and 100 µl of 10% Chelex 100 was added followed by boiling for 10 min. The DNA was then precipitated by centrifuging the sample at 12,000g at 4°C for 10 min, collecting the supernatant, washing the beads with 120 µl of H2O, followed by another centrifugation. The pooled supernatants served as the template for the PCR. For PCR, we used the CDX2 promoter forward primer 5′-GAGGGGTTGTGCGTAGAGTG-3′ and the CDX2 promoter reverse primer 5′-CCTTCCGTGATTAACGAGTGT-3′. 1% of reaction-sheared chromatin that did not undergo immunoprecipitation was used as an input control. PCR conditions consisted of 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. After amplification, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. All ChIP assays were performed in two independent experiments.

NF-κBp65 RNA Interference (siRNA)

NES-B10T cells were equally plated in 24-well tissue culture plates, and transfected using Lipofectamine RNAiMAX Regent (Invitrogen, Carlsbad, CA) and OptiMEM (Life Technologies) with 25 pmol/ml of the SMARTpool ON-TARGETplus NF-κBp65 siRNA (Thermo Scientific, Waltham, MA) for 72 hours per the manufacturer's instructions. As a control, cells were

transfected with ON-TARGETplus Non-targeting siRNA #1 (Thermo Scientific). After transfection, the medium was removed and replaced with growth medium. The efficiency of the siRNA for inhibiting p65 expression was determined by Western blotting at 72 hours. siRNA knockdown was performed in two independent experiments.

NF-κB Reporter Assay

Cells were equally seeded in 24-well plates one day before transfection. For transfection, we used an NF-κB reporter construct containing 4 DNA repeats of the NF-κB consensus binding site (generous gift of Zhiping Liu, University of Texas Southwestern); the renilla reporter pRL-TK (Promega, Madison, WI) plasmid was used to equalize for transfection efficiency. Cells were transfected for 5 hours with 500 ng/well of the NF-κB reporter construct along with 25 ng pRL-TK by using Lipofectamine LTX (Invitrogen, Grand Island, NY) per manufacturer's instructions. After transfection, the medium was removed and replaced with growth medium for 24 hours. Cells were treated with acidic bile salt medium for 1 hour, then changed to neutral pH medium for 6 hours. Luciferase activities were determined at 6 hours using the Dual Luciferase kit (Promega). Relative light units for firefly luciferase were normalized to renilla luciferase, and data were expressed relative to control cells. NF-κB reporter assays were performed in at least triplicate in two independent experiments.

Inhibition of Hydrogen Peroxide (H_2O_2) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase

Cells were pre-treated with 250 U/ml of polyethylene glycol (PEG)-catalase, a H_2O_2 scavenger, or with a 10 μM concentration of diphenylene iodonium (DPI), an NADPH oxidase inhibitor; these agents remained in the media for the duration of the experiment.

CDX2 Promoter Activity

Cells were equally plated in 24-well tissue culture plates. After 24 hours, the cells were co-transfected using Lipofectamine 2000 or 3000 (Invitrogen) with 0.4 µg of a plasmid containing the CDX2 promoter construct (pGL-3-CDX2 promoter, -562 bp) attached to a luciferase reporter and 25 ng of the renilla reporter pRL (Promega, Madison, WI) to control for transfection efficiency. After 24 hours, cells were treated with acidic bile salt medium or control medium for 24 hours. Cell extracts were then assayed for luciferase activities using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) per manufacturer's instructions. Data were expressed as relative light units for firefly luciferase normalized to renilla luciferase. All assays in cell lines were performed in triplicate in at least two independent experiments.

Qualitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In preliminary studies, we performed quantitative real-time PCR. However, in multiple experiments, threshold (Ct) values for expression of CDX2 mRNA in NES-B10T cells were high at baseline, which raised concerns regarding accurate quantification with this technique [1]. Therefore, we selected to use qualitative PCR (present vs absent) for all further analyses. Total RNAs were isolated by using the RNeasy Mini kit (Qiagen, Valencia, CA) per manufacturer's instructions, and quantitated by using the Nanophotometer (IMPLEN, Westlake Village, CA). Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) per manufacturer's instructions. The primer sequences were designed using Primer Express (Applied BioSystems, Foster City, CA) and manufactured by Integrated DNA Technologies (Coralville, IA). Each 50 µl PCR reaction contained 1 to 5 µl of cDNA (corresponding to 25 or 125 ng total RNA), 2.5 µM of each primer (12.5 pmol total), and 25 µl of 2X GoTaq® Green Master Mix (Promega, Madison, WI). For RT-PCR analysis of CDX2, the primer sequences were as follows: CDX2 forward: 5′- GAGCTGGAGAAGGAGTTT-3′ and CDX2

reverse: 5′-GGTGACGGTGGGGTTTAG-3′, 338 bp; GAPDH forward: 5′-TCCCACCTTTCTCATCCAAG-3′, and GAPDH reverse: 5′- GTCTGCAAAAGGAGTGAGGC-3′, 194 bp. PCR conditions for CDX2 consisted of 95°C for 5 minutes followed by 35 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; for GAPDH, PCR conditions consisted of 95°C for 5 minutes followed by 25 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Following amplification, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. GAPDH transcripts served as internal controls. The Barrett's epithelial cell line BAR-T served as a positive control for CDX2 mRNA expression [2]. All RT-PCR analyses were performed in two independent experiments.

Data Analyses

Quantitative data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired Student's t-test using the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). P values ≤ 0.05 were considered significant for all analyses.

References:

1. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004;15:155-66.

2. Matsuzaki J, Suzuki H, Tsugawa H, et al. Bile acids increase levels of microRNAs 221 and 222, leading to degradation of CDX2 during esophageal carcinogenesis. Gastroenterology 2013;145:1300-11.

Supplemental Table 1. Antibodies Used

Antibody	**Source information (cat#, vendor)**	**Dosage**	**Use**
p65	#4764, Cell Siganling	1:1000 dilution	WB
p105/p50	Ab7971, Abcam	1:1000 dilution	IP
p65	Ab7970, Abcam	1:1000 dilution	IP
phospho-IκBα(Ser32/26)	#9246, Cell Signaling	1:1000 dilution	WB
IκBα	#4814, Cell Signaling	1:2000 dilution	WB
phospho-p65(ser276)	101749, Santa Cruz	1:1000 dilution	WB
PKAc	610980, BD Biosciences	1:1000	WB
β-tubulin	T-5293, Sigma	1:3000 dilution	WB
Lamin	#2032, Cell Signaling	1:2000 dilution	WB
β-actin	A4700, Sigma	1:3000 diultion	WB

Supplemental Figures Legends:

Supplemental Figure 1. Immunofluorescence for cytoplasmic and nuclear p50 and p65 proteins in NES-G4T cells. After exposure to acid, bile salts, or acidic bile salts, there was strong nuclear localization of p50, but not p65. Immunofluorescence images shown are representative of the results from 2 independent experiments (scale bar = 50 μm).

Supplemental Figure 1