Supplemental Materials and Methods

Bioinformatics analysis of public databases

We analyzed public databases of The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). The data of RNA expression profiles and clinical information of EACs were downloaded from TCGA official website (https://portal.gdc.cancer.gov/repository), which contains 79 EAC and 9 normal esophagus samples. Three GEO datasets, including GSE13898[1], GSE92396 and GSE37203[2] , were downloaded from National Center for Biotechnology Information (NCBI) GEO database (https://www.ncbi.nlm.nih.gov/). In the GSE13898 dataset, genome-wide gene expression profiling was performed on biopsy samples of 75 primary EAC, 15 BE and 28 surrounding normal esophagus samples. The GSE92396 dataset is composed of surgical specimens of 12 EAC and 9 normal esophageal tissues. The GSE37203 datasets contains specimens of 37 EAC and 31 BE. The expression data were analyzed in R environment (version 4.0.3). Boxplots and violin plots were drawn using the R software. Hierarchical clustering analysis was performed by R package 'pheatmap'. Pearson correlation analysis was processed by R package 'Hmisc'. Limma package[3] (Version 3.26.9) was used to identify differentially expressed genes with the threshold of p value <0.05 and cut off of $\log2FC > \text{mean}(|\log2FC|)+2\times\text{sd}(|\log2FC|)$.

Human samples

The tissue microarray (TMA) included 67 de-identified archival cases of normal esophagus (n=5) and EACs (n=62). The TMA was constructed by Tissue Pathology Core at Vanderbilt University Medical Center, Nashville, TN. All tissue samples were verified by two pathologists and representative regions were selected and marked for construction of the TMA.

Animal experiments

The pL2-IL1β transgenic mice are a kind gift from Dr. Timothy Wang (Columbia University); a model of chronic esophageal inflammation that develops BE and EAC, as previously described[4]. The mice received drinking water containing 0.3% deoxycholic acid (DCA) at neutral pH at the age of three months. After 7 months of continuous DCA administration, the mice were sacrificed and subjected to histological analysis of the squama-columnar junctions at the gastro-esophageal junctions.

Cell culture

OE33 cell line was a generous gift from Dr. David Beer (University of Michigan, Ann Arbor, MI, USA) while OE19 cell line was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both cell lines were cultured in RPMI medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (GIBCO). Cell lines were guaranteed free of mycoplasma contamination by periodical tests using Mycoplasma Detection Kit (PCR) (SouthernBiotech, Birmingham, AL, USA). The authenticity of the cell lines was confirmed using short tandem repeat profiling (Genetica DNA Laboratories, Burlington, NC, USA).

Acidic bile salts (ABS) exposure

To mimic reflux condition, we and others have adopted exposure to ABS using in vitro models [5, 6, 7, 8, 9, 10, 11, 12]. ABS cocktail was prepared as an equimolar mixture of deoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and taurocholic acid dissolved in pH4 serum-free medium. Final concertation of 200 μM (40 μM of each bile salts mentioned above) was used to treat EAC cells for 20 min, followed by recovery times in regular media, unless otherwise specially stated.

Antibodies and reagents

Anti-Notch1/NICD antibody for Western blot (WB) or for immunofluorescence (IF), anti-p-NF-κB-p65 (S536), anti-NF-κB-p65 antibody, anti-β-Tubulin antibody, and HRP-linked anti-rabbit IgG, HRP-linked

anti-mouse IgG WB were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-NF-κB-p65 antibody for IF was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-DLL1 antibody targeting the C-terminal fragment used for WB and IF was ordered from Santa Cruz Biotechnology, while anti-DLL1 antibody specifically mapping the N-terminal region for WB was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-APE1 antibody for WB and IF, Goat anti-Rabbit IgG (H+L, Alexa Fluor 488) and Goat anti-Mouse IgG (H+L, Alexa Fluor 568) for IF were purchased from Thermo Fisher Scientific. Anti-β-Actin antibody was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Anti-P84 antibody was obtained from GeneTex (Irvine, CA, USA). E3330 (APE1 redox inhibitor) was obtained from Novus Biologicals (Littleton, CO, USA). APX2009, a novel derivative of E3330, was purchased from Sigma-Aldrich.Transfection reagents including Lipojet and Polyjet were purchased from SignaGen Laboratories (Rockville, MD, USA). Detailed information is listed in Supplemental Table 1.

Transfection and lentivirus infection

Scrambled siRNA (sc-29470) was purchased from Santa Cruz Biotechnology. APE1 siRNA (L-010237-00-0005) was obtained from Dharmacon (Lafayette, CO, USA). The flag-tagged coding sequence of APE1 was cloned in pcDNA3.1 mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA). The flag-tagged APE1 redox-deficient mutant (C65A) was developed by QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The coding sequence of NF-κB-p65 was cloned in pCMV mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA). For transient overexpression of APE1 and NF-κB-p65, mammalian expression plasmids or empty vector were transfected into OE33 cells and OE19 cells using PolyJet reagent (SignaGen Laboratories, Rockville, MD, USA). To establish stable cell lines, lentivirus particles expressing APE1 shRNA or control shRNA were constructed by VectorBuilder Inc (Santa Clara, CA, USA) and then transduced into

OE33 cells following standard procedures. The targeting sequence of APE1 shRNA is: 5'-GCCTGGACTCTCTCATCAATA-3'. Stable shAPE1 cell line was selected for at least 10 days with growth medium containing 10% FBS and puromycin (1 μg/ml).

RNA isolation and quantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Karlsruhe, Germany) and then cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), following the manufacturer's protocol. Quantitative RT-PCR was performed using a commercial product of SYBR® Select Master Mix purchased from Thermo Fisher Scientific and a CFX Connect real-time system (Bio-Rad). The threshold cycle number was determined by Bio-Rad CFX manager software version 3.0 and averaged on triplicated reactions. Melt curves were applied to evaluate the dissociation characteristics of the double-strained DNA templates during heating. Detailed sequences of PCR primers are listed in Supplemental Table 2. mRNA expressions of all samples were normalized to HPRT, and results were presented as relative fold change.

RNA-sequencing (RNA-seq) Data Analysis

OE33 cells with stable APE1-knockdown (shAPE1) or scrambled shRNA (shCtrl) were exposed to ABS for 20 min, followed by washing in PBS and recovery in regular medium for 6h. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). The experiments were performed in triplicate. The RNA samples were sent to GENEWIZ (South Plainfield, NJ) and subjected to high-throughput sequencing (Illumina HiSeq instrument, 4000 or equivalent). STAR aligner v.2.5.2b was used to align trimmed sequence reads and BAM files were generated. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. The raw counts were then normalized as transcripts per million (TPM), followed by further correction for batch effects using Combat. Limma package (Version 3.26.9) was applied for gene differential analysis and Gene Set

Enrichment Analysis (GSEA) was used to enrich potential pathways. P value <0.05 and cut off of log2FC > mean(|log2FC|)+2×sd(|log2FC|) were regarded as the thresholds.

Three-dimensional (3D) organotypic culture

3D organotypic cultures (OTC) of OE33 cells were performed as previously described before[13]. In brief, human esophageal fibroblasts (ScienCell, Carlsbad, CA, USA) were plated into 3D matrixes (75,000 cells/well) which contained collagen I (High concentration rat-tail collagen, Corning) and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and cultured for 7 days. After that, the OE33 cells were seeded on the top of the fibroblast matrix with the density of 500,000 cells/well and incubated for another 7 days. Before harvest, the OTC cells were treated with ABS cocktail (100 μM, 30min) from the top of the inserted chambers and allowed for 6h-recovery in complete medium. Subsequently, the samples were fixed in 70% ethanol and processed for paraffin embedding, H&E staining, and immunofluorescence.

Tumorosphere Formation Assay

Tumorosphere formation assay was conducted as previously described[14]. In short, single cell suspension was seeded into 6-well ultra-low attachment plates (Corning Incorporated, Corning, NY, USA) with low cell density ($5x10^3$/well) and cultured in serum-free DMEM/F-12 medium (GIBCO) supplemented with 2% B-27 (GIBCO), 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich) and 20 ng/ml basic fibroblastic growth factor (FGF, Sigma-Aldrich). The culture media was replaced with a fresh one every other day for 14 days to enrich cancer stem/progenitor cell populations. To investigate the potential effects of repeated ABS exposures (mimicking chronic GERD) on development and maintenance of cancer stemness, OE33 cells were exposed to ABS cocktail for 20 min every day for 14 days before tumorosphere culturing. To determine if APE1 redox function has a role on tumorosphere formation capacity, E3330, the APE1 redox inhibitor, was added into the tumorosphere medium on the

10th day of culturing. Figures of tumorospheres were captured by an Olympus CKX41SF Inverted Phase Contrast Microscope (Olympus, Tokyo, Japan) under white light. Tumorospheres with diameters greater than 20 μm were counted using ImageJ software (NIH, http://www.uhnresearch.ca/facilities/wcif/imagej /). The numbers and sizes were calculated and compared to the untreated group. To obtain paraffin-embedded sections, the medium with tumorospheres were transferred to 15ml tubes and the pallets were collected by centrifugation, followed by suspending in fresh 4% paraformaldehyde and incubating at RT for 40-50 min. After incubation, the mixtures were centrifuged again, and the supernatants were carefully removed. We then added 30-50ul of HistoGel (ThermoFisher Scientific) to each tube and mixed well with the pallets. Each histogel dome was put into a cassette and immersed in 70% ethanol, followed by standard procedures of paraffin embedding and slides cutting.

Cell nuclear/cytosol fractionation

EAC cell lines were treated with ABS cocktail, recovered in complete media and harvested at different time points. We used Cell Fractionation Kit (Cell Signaling Technology) to process the samples according to the supplier's protocol. In short, the cells were trypsinized to harvest, washed with PBS once and centrifuged at 500g for 5 min. Then, we discarded the supernatants and resuspended the pellets with Cytoplasmic Isolation Buffer (CIB). We vortexed the mixtures for 5 s and incubated on ice for 5 min. After incubation, we collected the supernatants as cytosolic fractions by centrifugation at 500g for 5 min. As for membrane fractions, the pallets were re-mixed with Membrane Isolation Buffer (MIB), vortexed for 15 s and incubated for 5 min. The supernatants (membrane extract) were collected again by centrifugation at 8000g 5 min. We resuspended the formed pellets with Cytoskeletal/Nuclear Isolation Buffer (CyNIB) and sonicated thoroughly at 20X speed. At last, the isolated cytosolic and nuclear

fractions were mixed with the 4X Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) and electrophoresed using SDS-PAGE.

Immunoprecipitation and Western blot analysis

Cells were lysed in RIPA buffer (Santa Cruz Biotechnology) supplemented with 1 × Halt protease inhibitor cocktail and 1 × Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific), followed by sufficient sonication and centrifugation of 12,000 rpm for 15 min at 4°C. Pierce BCA Protein Assay (ThermoFisher Scientific) was used to measure protein concentration. For immunoprecipitation, cell lysates were rotated together with protein A/G beads and 1ug of antibody or control IgG at 4°C overnight. After washed by PBST buffer (PBS (GIBCO) containing 0.1% Tween® 20 (Sigma-Aldrich)) for three times, the immunocomplexes were subjected to Western blot analysis. As for Western Blot analysis, samples were prepared by mixing 4X sample buffer and cell lysates and denatured at 85°C for 10 min. Next, proteins were separated by 10% SDS/PAGE gels and transferred to nitrocellulose membranes, sequentially being probed with indicated primary antibodies and HRP-conjugated secondary antibodies. β-actin was used as loading control. At last, protein bands were detected using commercial Immobilon Western Chemiluminescent HRP Substrate detection reagents (Thermo Fisher Scientific and MilliporeSigma).

Immunofluorescence staining

Immunofluorescence assays [15] were utilized to detect nuclear translocation of NOTCH1 and NF-κB-p65 proteins and determine location and expression of APE1 and DLL1 proteins in EAC cells. Immunofluorescence was applied on 3D OTC culture and tissues from the gastro-esophageal junction of the pL2-ILβ transgenic mice. Briefly, OE33 and OE19 cells were fixed with fresh 4% paraformaldehyde solution for 45 min at room temperature (RT) after recovery from ABS or control treatment. Cells were then permeabilized with PBS containing 0.25% Triton™ X-100 (Sigma-Aldrich). For paraffin

embedded slides, slides were oven heated at 65°C for 30 to 60 min, followed by standard procedures of deparaffinization. Next, the slides were subjected to antigen unmasking using Citrate Buffer, PH 6.0, 10X (Sigma-Aldrich) or Tris EDTA Buffer, pH 9.0, 1X (Genemed Biotechnologies Inc, South San Francisco, CA, USA). The slides were then incubated with 10% non-immune goat serum blocking solution (Thermo Fisher Scientific) for at least 30 min at RT. After that, primary antibodies against NOTCH1/NICD (1:200), NF-κB-p65 (1:200), APE1 (1:250), DLL1 (1:50) was added and incubated overnight at 4 °C in a humidified chamber. Secondary antibodies conjugated with Alexa Fluor 488 and/or 568 were used at the dilution of 1:400. The cells were then counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) and visualized by an Olympus BX51 fluorescence microscope (Olympus Co., Center Valley, PA).

Dual luciferase reporter assay

To evaluate the transcription activity of NOTCH1 and NF-κB in reflux conditions (ABS), two luciferase reporters were utilized. The NOTCH1 reporter (which includes 8xCSL) was a generous gift from Dr. Anthony Capobianco at University of Miami. For the NF-κB reporter, the pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega, Madison, WI) was used, containing five copies of an NF-κB response element (NF-κB-RE). OE33 and OE19 cells were co-transfected with the NOTCH1 reporter or the NF-κB reporter and Renilla plasmid using PolyJet transfection reagent. About 42-45h after transfection, the cells were treated with ABS cocktail or PBS, followed by PBS washing. Cells were allowed to recover for 3-6h in regular media before measuring the reporter luciferase activity. Dual luciferase assay (Promega, Madison, WI, USA) was applied to measure the reporter luciferase activity according to the manufacturer's protocol using Fluostar Optima microplate reader (BMG Labtech, Offenburg, Germany). The values of luciferase reporter activity of each group were normalized to the values of Renilla.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using ChIP-IT Express Enzymatic Kit (Active Motif, Carlsbad, CA, USA) referring to the manufacturer's instructions. Briefly, cells were plated in 100mm dishes until 60%-70% confluence was reached. After 6h recovery from ABS, cells were cross-linked with 1% formaldehyde (Sigma-Aldrich) and then washed with ice-cold PBS. The fixation reactions were stopped by adding Glycine Stop-Fix Solution, followed by washing with PBS again. Next, the cells were scraped using Cell Scraping Solution with PMSF and pelleted by centrifugation, followed by being re-suspended in lysis buffer supplemented with PIC and PMSF. Then the protein-chromatin complexes were further lysed by Dounce homogenizer and were sheared by Enzymatic Shearing Cocktail in order to get chromatin fragments with average length between 200bp and 1000bp.The supernatants were collected and incubated with the immunoprecipitation antibody (p-NF-κB-p65, S536, Cell Signaling Technology) and Protein G magnetic beads on an end-to-end rotator overnight at 4°C. Subsequently, the precipitated protein-DNA complexes were collected and further washed, eluted and de-crosslinked at 95°C for 15 min. The magnetic beads were isolated and discarded using a magnetic stand and the supernatants which contained purified DNA was analyzed by quantitative RT-PCR for NF-κB binding to DLL1 promoter. We designed six pairs of primers that cover the predicted NF-κB-p65 binding sites (Supplemental Table 3).

Immunohistochemistry (IHC)

The TMA slides were subjected to standard procedures of de-paraffinization and re-hydration, followed by antigen retrieval in sub-boiled Tris EDTA Buffer, pH 9.0 (Genemed Biotechnologies Inc) for 20 min. We then utilized IHC Select® Immunoperoxidase Secondary Detection system (MilliporeSigma) for staining according to the manufacture's protocol. The resulted slides were sequentially incubated with Blocking Reagent (at least 60 min at RT), primary antibodies against APE1 (1:300) and DLL1 (1:100) (1h at RT) or a negative control reagent, Secondary Antibody (30 min at RT), Streptavidin HRP (10 min

at RT). After rinse procedures, the slides were further counter-stained with Hematoxylin Counter Stain Solution for 10 min at RT, followed by dehydration using increasing concentrations of ethanol and xylene (Fisher Scientific). The intensity and frequency of staining were evaluated as scores based on the protocol described before[16]. For statistical analysis, we used index score as previously described [17] to compare protein expression of APE1 and DLL1 between EAC tissues and normal esophageal epithelium and to estimate their correlation with each other.

Statistics

All data were statistically analyzed by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The results were quantified and expressed as mean ± SEM. Differences between two independent variables were determined by Student's t-test while comparisons among multiple groups (≥3 groups) were estimated by one-way ANOVA, followed by the Bonferroni post-hoc test. Survival analysis was performed using Kaplan-Meier plots and Log-rank tests. $p<0.05$ was considered to be statistically significant. Each biochemical experiment was performed in triplicate unless otherwise specified.

References

1 Kim SM, Park Y-Y, Park ES, Cho JY, Izzo JG, Zhang D, *et al.* Prognostic biomarkers for esophageal adenocarcinoma identified by analysis of tumor transcriptome. PloS one 2010;**5**:e15074.

2 Silvers AL, Lin L, Bass AJ, Chen G, Wang Z, Thomas DG, *et al.* Decreased selenium-binding protein 1 in esophageal adenocarcinoma results from posttranscriptional and epigenetic regulation and affects chemosensitivity. Clinical Cancer Research 2010;**16**:2009-21.

3 Smyth GK. Limma: linear models for microarray data. Bioinformatics and computational biology solutions using R and Bioconductor: Springer, 2005:397-420.

4 Quante M, Bhagat G, Abrams JA, Marache F, Good P, Lee MD, *et al.* Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett-like metaplasia. Cancer cell 2012;**21**:36-51.

5 Nehra D, Howell P, Williams C, Pye J, Beynon J. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. Gut 1999;**44**:598-602.

6 Bernstein H, Payne CM, Bernstein C, Schneider J, Beard SE, Crowley CL. Activation of the promoters of genes associated with DNA damage, oxidative stress, ER stress and protein malfolding by the bile salt, deoxycholate. Toxicology letters 1999;**108**:37-46.

7 Jenkins G, Cronin J, Alhamdani A, Rawat N, D'souza F, Thomas T, *et al.* The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-κB activation in oesophageal cells, with a mechanism of action involving ROS. Mutagenesis 2008;**23**:399-405.

8 Peng D-F, Hu T-L, Soutto M, Belkhiri A, El-Rifai W. Glutathione peroxidase 7 suppresses bile salt-induced expression of pro-inflammatory cytokines in Barrett's carcinogenesis. Journal of Cancer 2014;**5**:510.

9 Nehra D, Howell P, Williams CP, Pye JK, Beynon J. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. Gut 1999;**44**:598-602.

10 Bernstein H, Payne CM, Bernstein C, Schneider J, Beard SE, Crowley CL. Activation of the promoters of genes associated with DNA damage, oxidative stress, ER stress and protein malfolding by the bile salt, deoxycholate. Toxicol Lett 1999;**108**:37-46.

11 Jenkins GJ, Cronin J, Alhamdani A, Rawat N, D'Souza F, Thomas T, *et al.* The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS. Mutagenesis 2008;**23**:399-405.

12 Peng DF, Hu TL, Soutto M, Belkhiri A, El-Rifai W. Glutathione Peroxidase 7 Suppresses Bile Salt-Induced Expression of Pro-Inflammatory Cytokines in Barrett's Carcinogenesis. J Cancer 2014;**5**:510-7.

13 Le Bras GF, Loomans HA, Taylor CJ, Revetta FL, Andl CD. Activin A balance regulates epithelial invasiveness and tumorigenesis. Laboratory investigation 2014;**94**:1134-46.

14 Fan X, Ouyang N, Teng H, Yao H. Isolation and characterization of spheroid cells from the HT29 colon cancer cell line. International journal of colorectal disease 2011;**26**:1279-85.

15 Peng DF, Hu TL, Soutto M, Belkhiri A, El-Rifai W. Loss of glutathione peroxidase 7 promotes TNF-alpha-induced NF-kappaB activation in Barrett's carcinogenesis. Carcinogenesis 2014;**35**:1620-8.

16 Hong J, Chen Z, Peng D, Zaika A, Revetta F, Washington MK, *et al.* APE1-mediated DNA damage repair provides survival advantage for esophageal adenocarcinoma cells in response to acidic bile salts. Oncotarget 2016;**7**:16688.

17 Peng D, Razvi M, Chen H, Washington K, Roessner A, Schneider-Stock R, *et al.* DNA hypermethylation regulates the expression of members of the Mu-class glutathione S-transferases and glutathione peroxidases in Barrett’s adenocarcinoma. Gut 2009;**58**:5-15.