Original research

Activation of NOTCH signaling via DLL1 is mediated by APE1-redox-dependent NF-κB activation in oesophageal adenocarcinoma

Lei Chen,[1,2] Heng Lu,[2] Dunfa Peng,[2,3] Long Long Cao,[2,4] Farah Ballout,[2] Kannappan Srirmajayam,[5] Zheng Chen,[2,3] Ajaz Bhat,[6] Timothy C Wang ,[7] Anthony Capobianco,[2,3] Jianwen Que,[7] Oliver Gene McDonald,[3,8] Alexander Zaika ,[2,3] Shutian Zhang,[1] Wael El-Rifai [2,3]

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2022-327076).

For numbered affiliations see end of article.

Correspondence to
Professor Wael El-Rifai, Surgery, University of Miami Miller School of Medicine, Miami, Florida, USA; wxe45@miami.edu
and Professor Shutian Zhang, Department of Gastroenterology, Capital Medical University Affiliated Beijing Friendship Hospital, Beijing, China; zhangshutian@ccmu.edu.cn

Received 28 January 2022
Accepted 3 June 2022

To cite: Chen L, Lu H, Peng D, *et al*. *Gut* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/gutjnl-2022-327076

ABSTRACT

Objective Oesophageal adenocarcinoma (EAC) arises in the setting of Barrett's oesophagus, an intestinal metaplastic precursor lesion that can develop in patients with chronic GERD. Here, we investigated the role of acidic bile salts, the mimicry of reflux, in activation of NOTCH signaling in EAC.

Design This study used public databases, EAC cell line models, L2-IL1β transgenic mouse model and human EAC tissue samples to identify mechanisms of NOTCH activation under reflux conditions.

Results Analysis of public databases demonstrated significant upregulation of NOTCH signaling components in EAC. In vitro studies demonstrated nuclear accumulation of active NOTCH1 cleaved fragment (NOTCH intracellular domain) and upregulation of NOTCH targets in EAC cells in response to reflux conditions. Additional investigations identified DLL1 as the predominant ligand contributing to NOTCH1 activation under reflux conditions. We discovered a novel crosstalk between APE1 redox function, reflux-induced inflammation and DLL1 upregulation where NF-κB can directly bind to and induce the expression of DLL1. The APE1 redox function was crucial for activation of the APE1-NF-κB-NOTCH axis and promoting cancer cell stem-like properties in response to reflux conditions. Overexpression of APE1 and DLL1 was detected in gastro-oesophageal junctions of the L2-IL1ß transgenic mouse model and human EAC tissue microarrays. DLL1 high levels were associated with poor overall survival in patients with EAC.

Conclusion These findings underscore a unique mechanism that links redox balance, inflammation and embryonic development (NOTCH) into a common pro-tumorigenic pathway that is intrinsic to EAC cells.

WHAT IS ALREADY KNOWN ON THIS SUBJECT?

⇒ Apurinic/apyrimidinic endonuclease (APE1) is aberrantly overexpressed in multiple cancer types, including oesophageal adenocarcinoma (EAC). Exposure to chronic gastro-oesophageal reflux, the main risk factor for Barrett's oesophagus (BE)-originated oesophageal tumourigenesis, induces APE1 dysregulation, activates intricate networks of redox-dependent transcription factors and promotes cancer cell survival.

⇒ Although activation of NOTCH signaling was reported in BE and EAC, the mechanisms underlying its activation in reflux conditions remain largely unknown.

WHAT ARE THE NEW FINDINGS?

⇒ Using 2D and 3D in vitro models as well as mouse and human data, we report a novel signaling axis where APE1 promotes activation of NOTCH through redox-dependent NF-κB activation and DLL1 induction. High levels of DLL1 are associated with poor overall survival in patients with EAC.

HOW MIGHT IT IMPACT ON CLINICAL PRACTICE IN THE FORESEEABLE FUTURE?

⇒ EAC is one of the leading causes of cancer death in the USA and Western countries, where standard chemotherapy has limited efficacy. Our findings provide a new perspective for the crosstalk between signaling networks, suggesting that targeting NOTCH or APE1-redox function can be a promising therapeutic strategy in patients with EAC.

INTRODUCTION

Oesophageal cancer is the sixth leading cause of cancer death worldwide.[1] Oesophageal adenocarcinoma (EAC), the predominant histopathological type of oesophageal cancer in the USA and Western countries, has been increasing rapidly over the past 40 years.[2] Patients with EAC have a 5-year survival rate below 20%.[3 4] Hence, there is an urgent need to identify novel molecular mechanisms and find new therapeutic targets for EAC. Chronic GERD is characterised by abnormal exposure of the lower oesophagus to a mixture of acidic gastric juice and bile salts. GERD is the main risk factor for metaplastic Barrett's oesophagus (BE) and its progression to EAC.[5 6] GERD conditions in patients with BE and EAC induce DNA damage along with aberrant activation of pro-inflammatory and pro-tumorigenic signaling pathways such as NF-κB and STAT3 pathways.[7 8]

Apurinic/apyrimidinic endonuclease (APE1), also known as APEX or redox factor 1 (REF1), is a dual-functional protein that plays important roles in both DNA base excision repair and reduction–oxidation (redox) transcriptional regulation.[9] APE1 interacts with a number of redox-dependent transcription factors (TFs) to induce transcription of target genes.[10 11] APE1 maintains the reduced status of cysteine residues on these TFs to enhance their DNA-binding affinity on target genes.[10 12] Aberrant overexpression of APE1 has been described in multiple cancers, including EAC.[8 13 14] Dysregulation of APE1 is closely related to cell proliferation, cell survival, angiogenesis, metastasis and drug resistance.[15 16] Exposure to acidic bile salts (ABS), the mimic of the reflux condition under GERD, induces oxidative stress and genotoxic DNA damage, with subsequent upregulation of APE1 to promote cell survival in EAC cells.[14 17]

The NOTCH pathway is a highly conserved cell signaling system, playing both oncogenic and tumour suppressive functions, depending on the cancer type and cellular context.[18 19] NOTCH signaling promotes cell differentiation, apoptosis, invasion, cancer stem-like properties, therapeutic resistance and tumour immune escape.[20–22] Mammals possess four different NOTCH receptors (NOTCH 1–4) and five Delta/Serrate/Lag2 ligands, referred to as JAG1, JAG2, DLL1, DLL3 and DLL4.[20] Once binding to a ligand from neighbouring cells, the NOTCH receptor undergoes consecutive proteolytic cleavages by ADAM17 and γ-secretase,[20] releasing the NOTCH intracellular domain (NICD) into the nucleus. NICD, the active form of receptor, forms a ternary complex with CBF1Su(H)–LAG1 (CSL) and protein mastermind-like 1 (MAML1) to transcriptionally regulate downstream targets such as HES and HEY gene families.[20 23]

Although NOTCH activation has been reported in BE and EAC,[24 25] the mechanisms underlying its activation in EAC and under reflux conditions remain largely unknown. This study demonstrates upregulation of NOTCH signaling in response to exposure to ABS, mimicking reflux conditions. Activation of NOTCH signaling occurred in an APE1-redox-dependent manner. Our findings uncover DLL1 as a critical ligand, activated by the APE1-NF-κB axis, to induce activation of NOTCH and promote stem-like properties in EAC tumourigenesis.

MATERIALS AND METHODS

For detailed resources, refer to online supplemental materials and methods and online supplemental tables 1-3.

RESULTS

NOTCH signaling is activated in patients with EAC and ABS-provoked cell lines

Gene set enrichment analysis (GSEA) of the TCGA and GEO datasets of the samples from patients with EAC predicted significant alterations of NOTCH signaling (online supplemental figure 1A–C). Using the TCGA-EAC dataset, we detected significant upregulation of mRNA levels of two receptors (NOTCH1 and NOTCH3), four ligands (DLL1, DLL4, JAG1 and JAG2) and several downstream targets (HES1, HEY1 and HEYL) (figure 1A). Analysis of the GSE13898 dataset (75 primary EAC and 15 BE) demonstrated similar results (figure 1B). Because chronic GERD is the primary risk factor for BE-derived EAC, we decided to validate the expression levels of NOTCH signaling components under reflux conditions (ABS exposure) using EAC cell lines. The results demonstrated consistent ABS-induced expression of several genes involved in the NOTCH signaling cascade, including NOTCH1, DLL1, DLL4, HES1 and HEY1 (figure 1C and online supplemental figure 1D). In addition, western blot analysis demonstrated a remarkable increase in the total DLL1 protein level, including the full length (75kDa) and cleaved c-terminal proteolytic fragment of DLL1 (30kDa), following exposure to ABS (figure 1D and online supplemental figure 1E). Consistent with activation of NOTCH, we observed an increase in the NICD (120kDa), the active form of full-length NOTCH1 (figure 1D and online supplemental figure 1E). These results suggest upregulation and activation of NOTCH signaling components in response to reflux conditions (ABS).

The hallmark of canonical activation of NOTCH signaling is nuclear localisation of NICD, which triggers transcription of downstream target genes. To confirm the nuclear translocation of NICD in response to ABS, we performed cytosol/nuclear fractionation in OE33 and OE19 cells. The results showed an accumulation of NICD in the nucleus in response to ABS exposure (figure 1E and online supplemental figure 1F). Immunofluorescence staining of NOTCH1/NICD demonstrated membranous immunostaining of NOTCH1 in the control (untreated) cells whereas cells exposed to ABS showed nuclear immunostaining indicative of cleaved NOTCH (NICD) (figure 1F,G, and online supplemental figure 1G,H). To confirm the transcriptional activity of ABS-induced nuclear NICD, we performed NOTCH/CSL luciferase reporter assays as a measure of NOTCH transcription activity. There was a significant increase in the luciferase activity ($p<0.001$) in response to ABS treatment compared with the control group (Ctrl) (figure 1H and online supplemental figure 1I).

As shown in figure 1D and online supplemental figure 1E, we detected ABS-induced increase and cleavage of DLL1, an important NOTCH ligand. To determine if DLL1 was indeed a crucial ligand for activation of NOTCH under ABS conditions, we performed a DLL1 antibody neutralisation experiment. DLL1 neutralisation not only reduced NICD at a base level (figure 1I, lane 3 vs lane 2) but also abrogated ABS-induced NOTCH1 cleavage compared with the IgG control (figure 1I, lane 6 vs lane 5). These results indicate that DLL1 is a critical ligand for ABS-induced NOTCH1 cleavage and activation.

NF-κB is an upstream TF for DLL1 that directly binds to the DLL1 promoter

As we detected changes in DLL1 at the mRNA level in response to ABS, our next step was to identify the upstream TFs that are induced with ABS and play a role in induction of DLL1 mRNA expression. First, we identified the differentially expressed genes (DEGs) defined by distinct DLL1 mRNA expression levels (DLL1-high vs DLL1-low) using tumor samples of the TCGA-EAC database. Based on the DEGs, we performed GSEA (http://software.broadinstitute.org/gsea/msigdb/collections.jsp#C3) and used a web-based tool (TRRUST, https://www.grnpedia.org/trrust/Network_search_form.php) for TF prediction. GSEA predicted 100 potential TFs critical for DLL1 transcription (online supplemental table 4, $p.adjust < 6e-34$), while 29 TFs were identified as candidates by TRRUST (online supplemental table 5). Overlapping the 100 enriched TFs of GESA and the TRRUST candidates highlighted 6 TFs, which included NF-κB (figure 2A). GSEA of the TCGA-EAC database confirmed enrichment of the NF-κB signature in DLL1-high compared with DLL1-low EAC samples (figure 2B). We further performed RNA sequencing using OE33 cells, in the presence or absence of exposure to ABS. RNA-seq analysis of OE33 cells demonstrated an increase in the expression levels of DLL1 and several NOTCH receptors (NOTCH1, NOTCH2 and NOTCH3), following exposure to

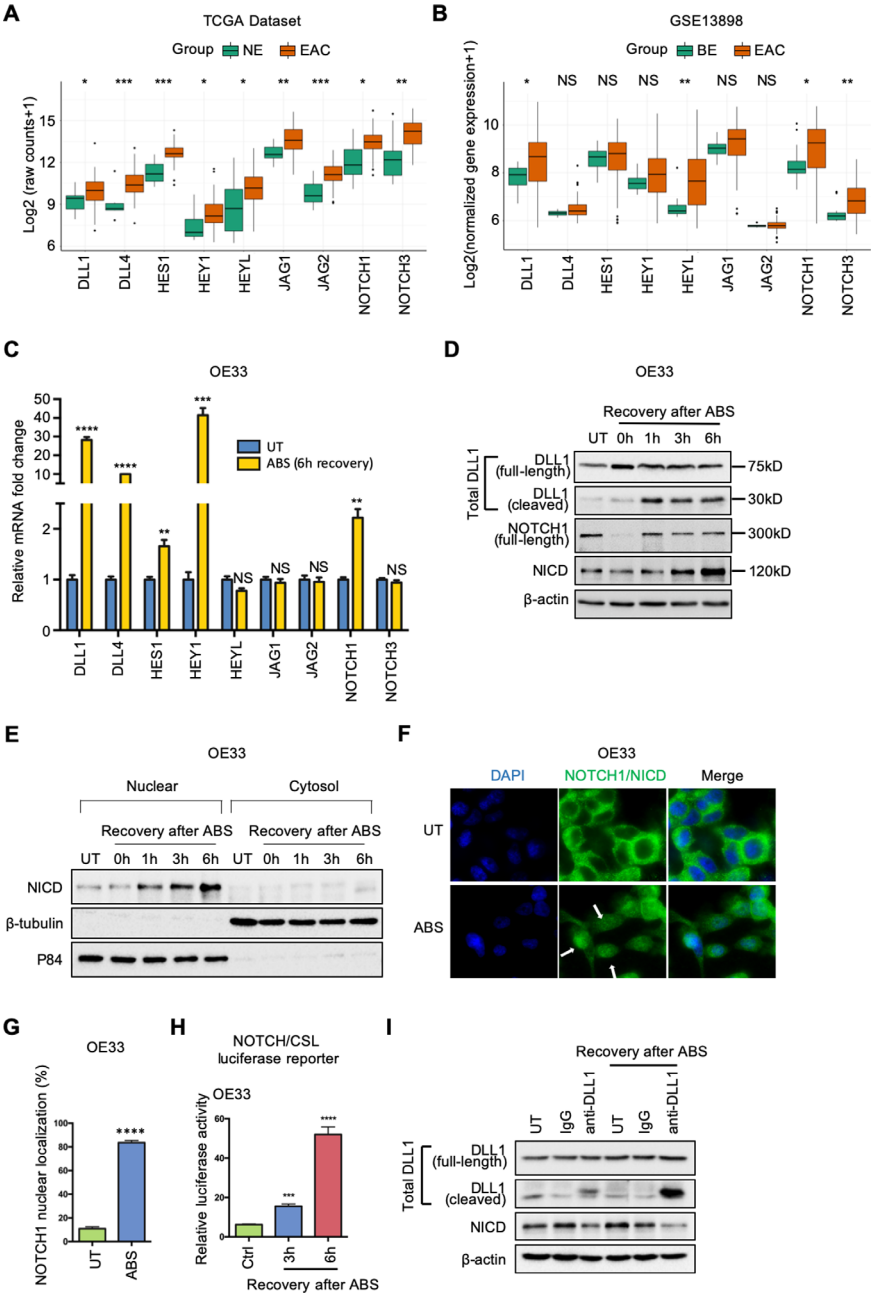

Figure 1 NOTCH signaling is activated in patients with oesophageal adenocarcinoma (EAC) and acidic bile salts (ABS)-provoked cell lines. (A) and (B) Grouped boxplot showing gene expression of core NOTCH signaling components in EAC/Barrett's oesophagus (BE) and normal oesophagus tissues in TCGA-EAC database (A) and GSE13898 dataset (B), respectively. (C) qRT-PCR showing mRNA expression levels of key components of NOTCH pathway in OE33 cells after 6h-recovery from ABS exposure. (D) Western blots of total DLL1 (including full-length DLL1 at 75 kDa and cleaved DLL1 at 30 kDa), full-length NOTCH1 (300 kDa), active NOTCH1 intracellular domain (NICD, 120 kDa) and β-actin in OE33 cells during indicated recovery time courses after ABS exposure. (E) OE33 cells were harvested for cytosol/nuclear fractionation after 6h-recovery from ABS exposure. Induction of NICD was examined by western blots. β-tubulin and p84 were used as loading control for cytosol fraction and nuclear fractions, respectively. (F) Representative immunofluorescent staining images of NOTCH1/NICD in OE33 cells after ABS exposure. DAPI was used for nuclear staining. (G) The percentage of NOTCH1 nuclear localisation was quantified using three independent fields. (H) NOTCH/CSL luciferase assays were performed in OE33 cells. (I) Anti-DLL1 antibody neutralisation blocked NOTCH1 cleavage and activation in OE33 cells. The cells were incubated with DLL1 antibody (10 µg/mL) or IgG control (10 µg/mL) in full medium before and during recovery of ABS exposure. All quantification analyses were shown as mean±SEM. Statistical significance was calculated using the Wilcoxon test in the public datasets for two group comparisons. t-Test was performed to analyse the experimental data for two group comparisons. *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001, NS, no significance.

ABS, as compared with untreated control (online supplemental figure 2A). GSEA analysis showed significant enrichment of the NF-κB signature (figure 2C and online supplemental figure 2B). Using western blot analysis, we confirmed the increase in NF-κB phosphorylation (S536) (figure 2D) and transcription activity (figure 2E) following exposure to ABS. Consistent with these data, immunofluorescence staining demonstrated an increase in nuclear NF-κB immunostaining following exposure to ABS (figure 2F,G). Of note, ectopic overexpression of p65 led to induction of DLL1 mRNA expression, suggesting a

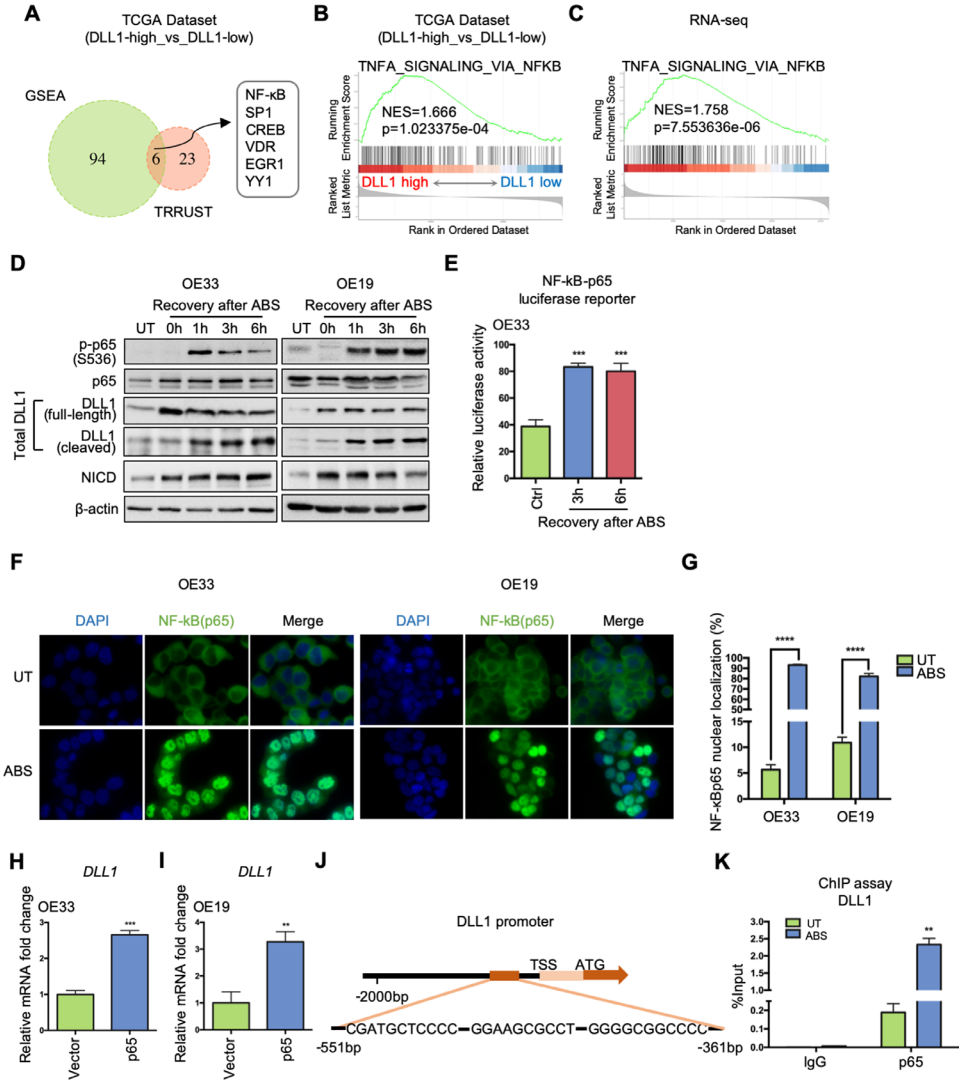

Figure 2 NF-κB is critical for acidic bile salts (ABS)-induced DLL1 transcription by directly binding to DLL1 promoter region. (A) DLL1-expression-based transcription factor enrichment analysis by gene set enrichment analysis (GSEA) and TRRUST website indicates NF-κB as one of the key transcriptional regulators in DLL1-high tumour samples in the TCGA-oesophageal adenocarcinoma (EAC) database. (B) GSEA of NF-κB signaling in the TCGA-EAC database, comparing DLL1-high EAC with DLL1-low EAC. (C) GSEA of NF-κB pathway in the RNA-seq datasets of OE33 cells, comparing control cells with ABS-treated cells (shCtrl vs shCtrl+ABS). (D) Western blots show protein level change of phosphor-p65, total p65, total DLL1 (including full-length ligand and its cleaved fragment), NICD and β-actin during 6h-recovery after ABS exposure in OE33 (left) and OE19 (right) cells. (E) NF-κB-p65 luciferase assay shows NF-κB transcriptional activity were elevated after ABS exposure in OE33 cells. Untreated cells worked as control (Ctrl). (F) Representative immunofluorescent staining images of NF-κB (p65) in OE33 (left) and OE19 cells (right) showing nuclear translocation after ABS exposure. DAPI was used for nuclear staining. (G) Percentage of NF-κB (p65) nuclear localisation was quantified using three independent fields. (H) and (I) DLL1 mRNA expression was detected after OE33 (H) and OE19 (I) cells were transfected with a p65 overexpression vector or control (Vector). (J) The ChIP primers amplified the DNA fragment containing three adjacent potential NF-κB binding sites in DLL1 promoter, −551 bp to −361 bp from transcription start site (TSS). (K) ChIP-qPCR was performed in OE33 cells by using phospho-NF-κB-p65 antibody to pull down chromatin after ABS exposure, compared with the untreated control. IgG works as control. Statistical data are shown as mean±SEM. **P<0.01, ***p<0.001 and ****p<0.0001 as calculated by t-test for two group comparisons.

possible direct role of NF-κB in transcription regulation of DLL1 (figure 2H,I).

Based on the above results, we analysed the DLL1 promoter to identify potential NF-κB binding sites using three online tools: JASPAR 2020 (http://jaspar.genereg.net/), Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and TRANSFAC-Gene Regulation (http://gene-regulation.com/pub/databases.html). This analysis identified twelve potential binding sites of NF-κB on the DLL1 promoter (online supplemental figure 2C). To confirm direct binding, we performed a ChIP assay using a phospho-NF-κB-p65 antibody, followed by qPCR using six primers' pairs (online supplemental table 3) that cover all potential binding sites. ChIP-qPCR results indicated that only one primer set covering three adjacent predicted binding sites (−551 bp to − 361 bp) (figure 2J) was amplified in the ChIP samples. This primer set showed a 12-fold increase of NF-κB binding on the DLL1 promoter after ABS exposure compared with the untreated group (UT) (figure 2K). There was no significant amplification detected in the IgG pull-down control sample (figure 2K). These novel findings confirmed the direct binding of NF-κB on the DLL1 promoter.

Exposure to ABS promotes NOTCH activation in an APE1-redox-dependent manner

Exposure of oesophageal cells to reflux conditions generates high levels of reactive oxygen species and oxidative stress necessitating activation of cellular redox mechanisms for the protection of transcription networks and maintaining cellular homeostasis. Our previous studies identified APE1 as an important signaling event in conditions of ABS exposure.[8 13 14] APE1 redox function is critical for protecting cellular signaling and activation of redox-dependent TFs such as STAT3 under reflux conditions.[8] We, therefore, hypothesised that APE1 played a critical role in promoting NF-κB transcription activity with subsequent transcriptional induction of DLL1 and NOTCH activation.

Indeed, ectopic expression of APE1 increased the levels of the three proteins (figure 3A). The NF-κB luciferase reporter assay consistently supported the role of APE1, showing

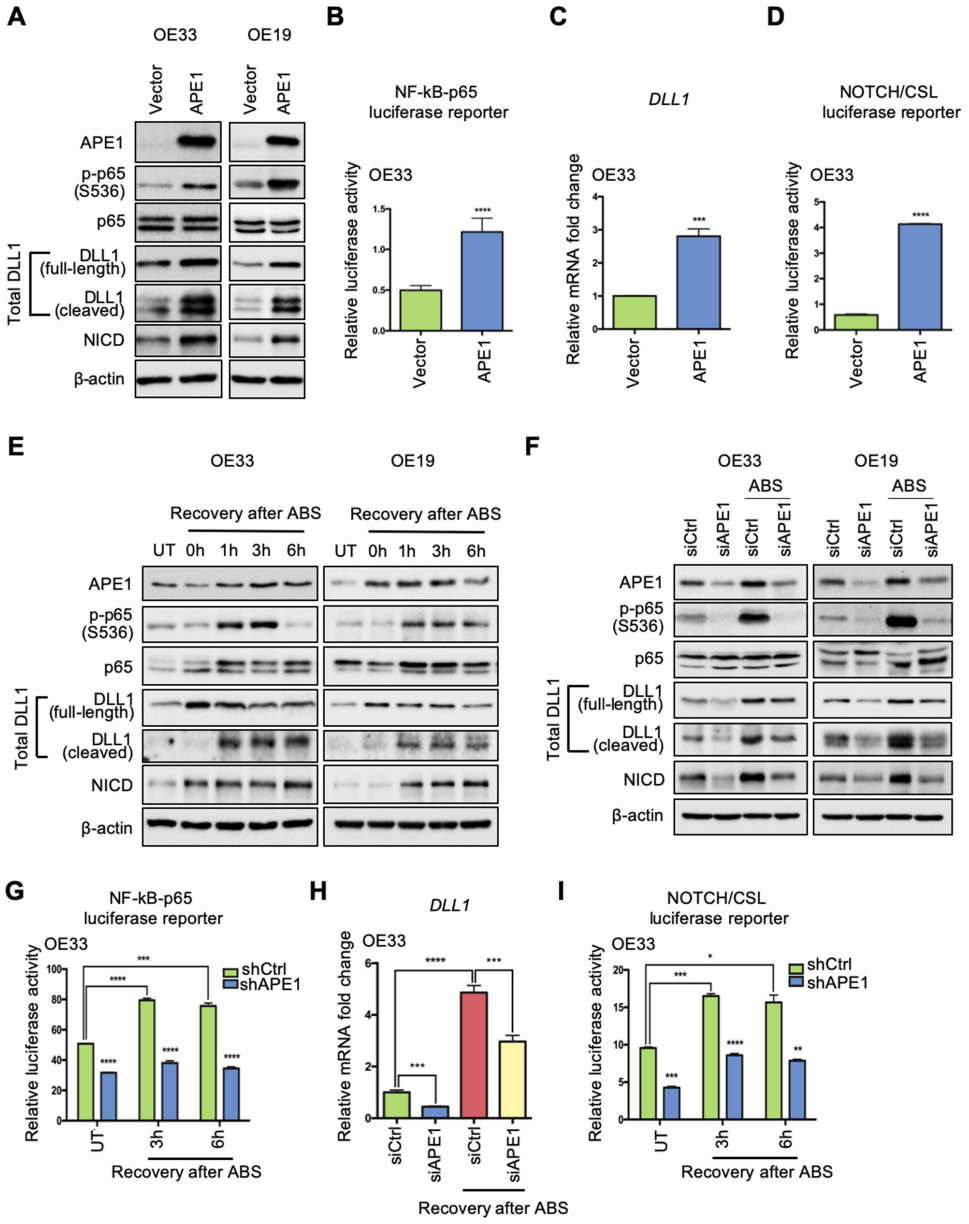

Figure 3 APE1 plays an essential role in acidic bile salts (ABS)-induced NF-κB-DLL1-NOTCH activation. (A) Western blots were used to detect protein levels of interested genes after ectopic APE1 overexpression in OE33 (left) and OE19 (right) cells. (B) NF-κB-p65 luciferase activity was examined after APE1 overexpression. (C) qRT-PCR for DLL1 mRNA expression was performed in the condition of APE1 overexpression. (D) NOTCH/CSL luciferase reporter assay was performed to confirm NOTCH transcriptional activity after APE1 overexpression. (E) Western blots were used for detecting protein expression of APE1, phosphor-p65, total p65, total DLL1, NOTCH intracellular domain (NICD) and β-actin at different time points of recovery from ABS exposure in OE33 (left) and OE19 (right) cells. (F) OE33 (left) and OE19 (right) cells were transfected with APE1 siRNA (siAPE1) or control siRNA (siCtrl) before ABS exposure; protein levels of interested genes were examined by western blot analysis. (G) OE33 cells with stable APE1 knockdown (shAPE1) or control (shCtrl) were exposed to ABS, NF-κB-p65 activity luciferase reporter assay was performed at 3h- and 6h- recovery time points after ABS exposure. (H) Transient knockdown of APE1 (siAPE1) blocked ABS-induced DLL1 upregulation in OE33 as comparing to control cells (siCtrl). (I) NOTCH/CSL luciferase activity was checked in APE1-knockdown (shAPE1) and scramble shRNA control (shCtrl) OE33 cells with or without ABS exposure. Statistical data are shown as mean±SEM. *P<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as calculated by t-test for two group comparisons.

induction of the luciferase activity with ectopic expression of APE1 (figure 3B). Using qRT-PCR, we found that APE1 overexpression upregulated DLL1 mRNA expression compared with the control (Vector) (figure 3C and online supplemental figure 3A). The NOTCH/CSL luciferase reporter assay confirmed that ectopic expression of APE1 significantly increased NOTCH luciferase activity, a measure of NOTCH transcription activity (figure 3D and online supplemental figure 3B). Based on these results, we next determined if changes in NF-κB-p65 phosphorylation, DLL1 induction and NOTCH activation are APE1-dependent under reflux conditions (ABS). Western blot analysis demonstrated an increase in APE1, phospho-p65, total DLL1, and active NICD following ABS treatment (figure 3E). APE1 silencing using siRNA abolished the ABS-induced increase in phospho-p65, DLL1 and NICD protein levels (figure 3F). We also used OE33 cells with stable APE1 knockdown (shAPE1) or control (shCtrl) for the NF-κB luciferase reporter assays. The results indicated that APE1 suppression abrogated ABS-enhanced NF-κB transcription activity (figure 3G). Transient (siAPE1) or stable (shAPE1) knockdown of APE1 abolished ABS-induced DLL1 transcripts (figure 3H and online supplemental figure 3C) and NOTCH/CSL luciferase activity (figure 3I and online supplemental figure 3D). Interestingly, DLL4 was not reduced on APE1 knockdown (online supplemental figure 3E,F), further confirming that DLL1 is the crucial NOTCH ligand in EAC. Collectively, these results indicate that changes in DLL1 and NOTCH activity are APE1-dependent under reflux conditions (ABS).

To determine the role of APE1 redox function in the ABS-induced NF-κB-DLL1-NOTCH activation, we used a stable shAPE1 cell line for reconstitution with wild-type APE1 (WT) or APE1-redox-deficient-mutant (C65A) under ABS conditions. The results indicated that the wild-type APE1, not the redox-deficient-mutant, restored protein levels of phospho-p65 (Ser536), DLL1 and NICD in response to ABS (figure 4A). In addition, the increase in phosphor-p65, DLL1 and NICD by ABS was abrogated by E3330, a pharmacologic small molecule inhibitor of APE1 redox activity (figure 4B). The use of E3330 also abrogated the ABS-induced NF-κB luciferase reporter activity (figure 4C), DLL1 expression (figure 4D,E), and NOTCH/CSL luciferase reporter activity (figure 4F,G). These results indicate that APE1 redox function was required for NF-κB activation, induction and cleavage of DLL1, and NOTCH1 cleavage and transcription activation under reflux conditions. To further validate the connection between APE1 and DLL1, we performed a Pearson correlation analysis of normalised gene expression in the GEO dataset of NE, BE and EAC (GSE13898). The results demonstrated a correlation between APE1 and NOTCH signaling

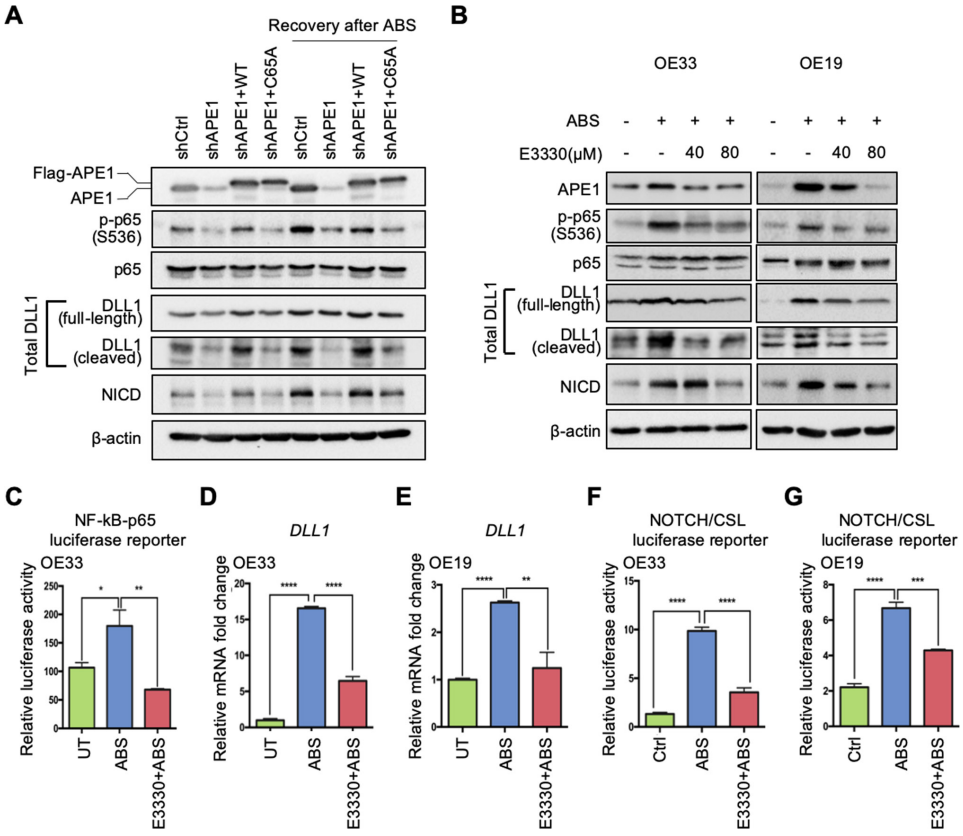

Figure 4 APE1 regulates NF-κB-DLL1-NOTCH axis through its redox function in response to acidic bile salts (ABS). (A) The APE1 stable silencing OE33 cells (shAPE1) were transfected with APE1-wild-type (WT) overexpression plasmid or APE1-redox-deficient-mutant (C65A) plasmid in the absence or presence of ABS exposure for western blots. (B) Western blots were used to detect protein levels of interested genes in the condition of ABS treatment with or without APE1 redox inhibitor, E3330, in OE33 (left) and OE19 (right) cells. (C) OE33 cells were maintained with 80 μM E3330 before and after ABS exposure, and then NF-κB-p65 activity luciferase reporter assay was performed. (D) and (E) qRT-PCR was used to detect DLL1 mRNA change in the condition of ABS exposure with or without E3330 application (80 μM) in OE33 (D) and OE19 (E) cells. (F) and (G) Luciferase reporter assays were performed to examine NOTCH transcriptional activity in the condition of ABS treatment with or without E3330 in OE33 (F) and OE19 (G) cells. Statistical data are shown as mean±SEM. *P<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as calculated by t-test for two group comparisons. NICD, NOTCH intracellular domain.

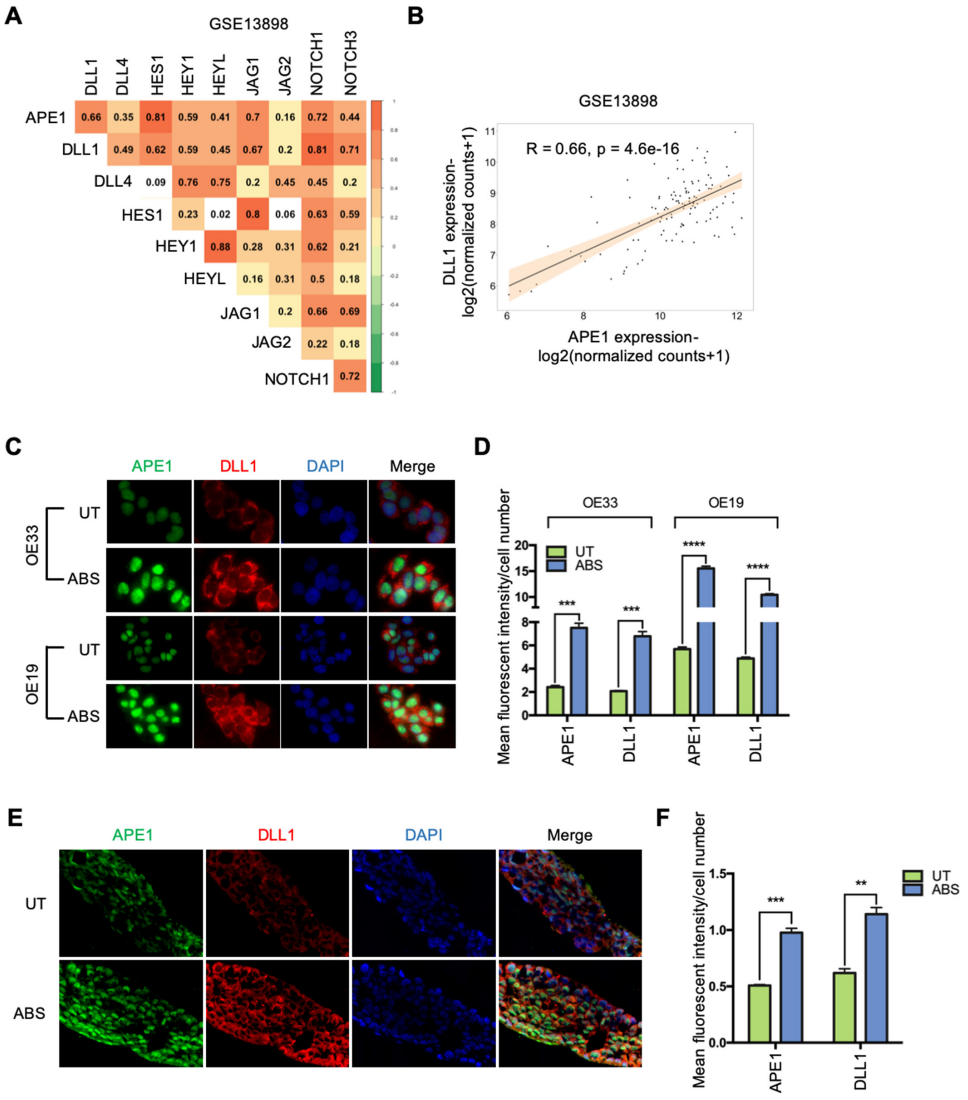

Figure 5 APE1 is correlated and co-overexpressed with DLL1 under acidic bile salts (ABS) exposure. (A) Pearson correlation analysis of APE1 and NOTCH signaling components in a GEO dataset of NE, Barrett's oesophagus and oesophageal adenocarcinoma (GSE13898). (B) APE1 has significant positive correlation with DLL1 at transcription level (R=0.66, p=4.6e−16) in the GSE13898 dataset. (C) Representative immunofluorescent staining images of APE1 (green) and DLL1 (red) in OE33 (upper) and OE19 (lower) cells with or without ABS treatment. (D) Mean fluorescent intensity of APE1 and DLL1 was quantified using three independent fields in OE33 (left) and OE19 (right) cells. (E) Representative immunofluorescent staining images of APE1 (green) and DLL1 (red) in OE33-derived 3D organotypic culture with or without ABS treatment. (F) Mean fluorescent intensity of APE1 and DLL1 was quantified using three independent fields. Statistical data are shown as mean±SEM. **P<0.01, ***p<0.001 and ****p<0.0001 as calculated by t-test for two group comparisons.

components (figure 5A). Of note, DLL1 was the only ligand that had a strong positive correlation with APE1 in patients with EAC and also significantly induced in our ABS-treated EAC cell models (figure 5B, R=0.66, p=4.6e−16). Immunofluorescence analysis demonstrated an increase in nuclear APE1 immunostaining levels together with upregulation of intracytoplasmic DLL1 after ABS treatment, as compared with untreated controls (UT) (figure 5C,D). Similar results were detected using 3D organotypic culture model of OE33 cells (figure 5E,F). Consistent with these findings, we detected an increase in the cleaved extracellular N-terminal fragment of DLL1 in the conditioned medium after 3h- and 6h-recovery from ABS exposure compared with UT controls (online supplemental figure 4). These results demonstrate an important role of APE1 redox function in the activation of NOTCH signaling via NF-kB-dependent transcription regulation of DLL1.

EAC tumorosphere formation is enhanced in response to ABS

NOTCH signaling plays a critical role in driving and maintaining stem cell properties.[24 26 27] We, therefore, investigated if the APE1-NOTCH axis promotes stem-like features in response to reflux conditions. To mimic the clinical conditions of GERD, we exposed OE33 cells to repeated episodes of ABS cocktail (200μM, pH=5.5) 20 min per day for 14 days, followed by a 3D tumorosphere formation assay.[28] OE33 cells with repeated ABS exposure displayed significant augmentation of tumorosphere number and growth compared with UT controls (figure 6A–C). Immunofluorescence staining demonstrated concomitant overexpression APE1 and DLL1 (online supplemental figure 5A,B). Western blots showed that cells exposed to repeated ABS treatment had higher levels of APE1, phospho-p65, DLL1 and NICD (figure 6D), confirming activation of this signaling cascade in

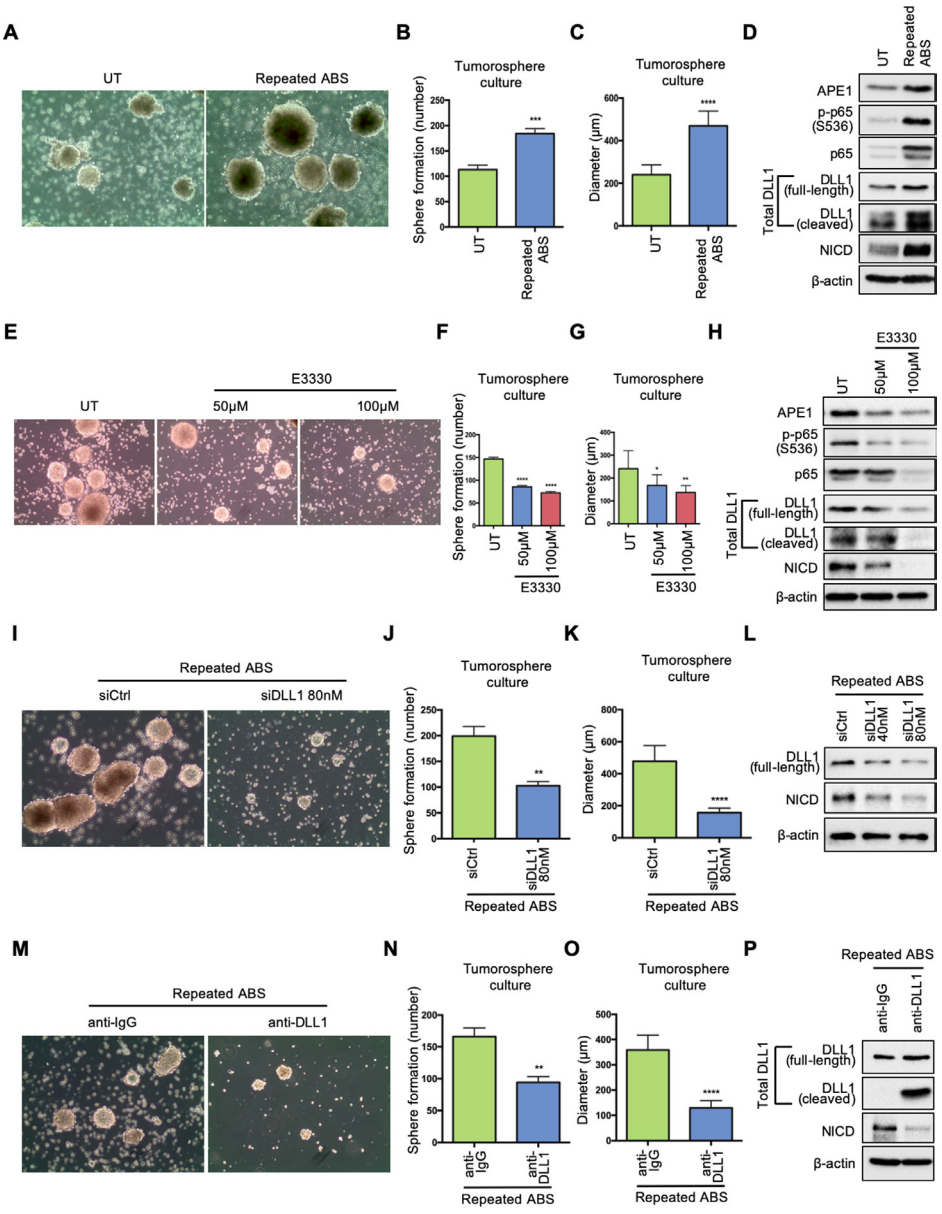

Figure 6 Oesophageal adenocarcinoma tumorosphere formation is enhanced in response to acidic bile salts (ABS). (A) OE33 cells were exposed to ABS (200 μM, pH=5.5) 20 min per day for 14 days; then, the cells were seeded for 3D tumorosphere culture; untreated OE33 cells (UT) were used as a control; representative images of the OE33-derived tumorospheres under white light. (B) and (C) Quantification of number (B) and size (C) of the tumorospheres in A. (D) Western blots of APE1, phosphor-p65, total p65, total DLL1 (full-length and cleaved form), NOTCH intracellular domain (NICD) and β-actin in the tumorospheres with and without repeated ABS exposure. (E) Tumorospheres derived from untreated OE33 cells were incubated with or without indicated doses of E3330 during culture; representative images of the OE33-derived tumorospheres with E3330 under white light. (F) and (G) Quantification of number (F) and size (G) of the tumorospheres in E. (H) Western blots of APE1, phosphor-p65, total p65, total DLL1 (full-length and cleaved form), NICD and β-actin in the tumorospheres with E3330. (I) Tumorospheres derived from repeated-ABS-treated OE33 cells were transfected with DLL1 siRNA (siDLL1) or scrambled control (siCtrl); representative images of the tumorospheres with or without transient knockdown of DLL1 under white light. (J) and (K) Quantification of number (J) and size (K) of the tumorospheres in I. (L) Western blots of full-length DLL1, NICD and β-actin in the tumorospheres with and without indicated concentration of DLL1 siRNA. (M) Tumorospheres derived from repeated-ABS-treated OE33 cells were incubated with DLL1 neutralising antibody or anti-IgG; representative images of the tumorospheres with DLL1 neutralisation under white light. (N) and (O) Quantification of number (N) and size (O) of the tumorospheres in M. (P) Western blots of total DLL1 (full-length and cleaved form), NICD and β-actin in the tumorospheres with DLL1 neutralising antibody or anti-IgG. Statistical data are shown as mean±SEM. *P<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 as calculated by t-test for two group comparisons.

response to reflux conditions. To determine if these phenotypic and molecular changes were dependent on APE1-redox function, we maintained OE33-derived tumorospheres with APE1-redox-specific inhibitor, E3330, for three consecutive days before imaging. E3330 (50 μM and 100 μM) gradually reduced the number and size of tumorospheres (figure 6E–G) compared with UT controls. The changes of immunofluorescence intensity (online supplemental figure 5C,D) and molecular signaling (figure 6H) were also reversed. We also used a recent derivative of E3330, APX2009, which is a potent inhibitor of APE1

redox function.[29 30] APX2009 decreased protein levels of phospho-p65, total DLL1 (both full-length and cleaved ligands) and NICD in the tumorospheres compared with the untreated group (online supplemental figure 5E).

To further confirm the vital role of DLL1 in NOTCH-mediated cancer cells' expansion in response to reflux, we transfected the tumorospheres derived from repeated-ABS-treated OE33 cells with DLL1 siRNA. Transient knockdown of DLL1 led to a remarkable decrease in tumorospheres' number and size (figure 6I–K) compared with control siRNA (siCtrl). In addition, western blots verified the DLL1 knockdown efficiency and demonstrated a reduced protein level of active NICD (figure 6L). Similarly, the use of DLL1 neutralising antibody abrogated tumorospheres' growth (figure 6M–O) and showed a decrease in NICD protein level (figure 6P) compared with the IgG control. These results demonstrate an important role of APE1-NOTCH signaling axis in promoting stem-like properties in EAC cells in response to reflux.

DLL1 is upregulated in neoplastic mouse and human oesophageal tissues and correlates with poor prognostic outcomes in human EAC

We validated our findings in vivo using L2-IL-1β transgenic mouse model of BE/EAC.[31] These mice develop neoplastic lesions at the gastro-oesophageal junctions that include high-grade dysplasia or EAC lesion following 6–8 months of exposure to deoxycholic acid in drinking water (figure 7A). Immunofluorescence staining demonstrated high levels of APE1 and DLL1 in the neoplastic glandular areas at the gastro-oesophageal junctions. APE1 immunostaining was predominantly nuclear, whereas DLL1 was detected on the cell surface (figure 7A). We also examined the protein expression levels of APE1 and DLL1 in human EAC tissues. We detected a co-overexpression pattern of APE1 and DLL1 by immunofluorescence (figure 7B) and IHC staining (figure 7C). To evaluate the correlation of APE1 and DLL1 in human EAC and normal oesophageal tissues, protein levels of APE1 and DLL1 were examined by IHC staining on tissue microarrays (figure 7D). We observed a coexpression pattern of APE1 and DLL1 in EAC tissues; IHC scores are summarised in online supplemental table 6). Statistical analysis of IHC index scores showed that protein levels of APE1 and DLL1 were significantly upregulated in EAC tissues compared with the normal oesophagus (figure 7E,F). Pearson correlation analysis revealed strong positive association between APE1 and DLL1 protein levels (figure 7G, R=0.73, p=1.7e−12). Survival analysis based on the TCGA-EAC database indicated poor prognostic outcome with a lower overall survival rate for patients with EAC with high levels of DLL1 (figure 7H) or HES5, a NOTCH downstream transcription target (figure 7I). A cartoon summarising our findings is shown in figure 8.

DISCUSSION

The incidence of BE and EAC has increased at alarming rates over the past four decades.[2 32] GERD, the main risk factor for EAC, is a clinical condition present throughout stages of BE-associated oesophageal tumourigenesis.[33] Exposure of oesophageal cells to reflux generates high levels of oxidative stress forcing cells to develop adaptive signaling mechanisms to maintain redox and cellular homeostasis. Although earlier studies have shown overexpression and activation of NOTCH signaling in EAC tumourigenesis,[24 34] the molecular mechanisms governing activation of NOTCH in EAC under reflux conditions remain unknown. In this study, our results uncover the underlying mechanisms of NOTCH activation in EAC. We report a novel signaling axis linking intracellular oxidative stress sensor (APE1), GERD-induced chronic inflammation (NF-κB) and NOTCH activation in EAC.

While an active scientific debate continues about origin of BE,[35–38] several recent studies support that BE originates from Lgr5+ progenitor cells[38] or Dclk1+ tuft cells[39] of gastric cardia. Aberrant NOTCH activation resulted in accelerated development of metaplastic BE-like lesions in the L2-IL1β mice.[31] Our analysis of public datasets demonstrated activation of NOTCH molecular signature in EAC. NOTCH is an evolutionary highly conserved cell-fate-control signaling pathway, which evolved with the initial appearance of multicellular organisms and conserved in all living organisms.[20] NOTCH signaling is essential for generating and maintaining cancer stem cell characteristics in EAC cells.[24] NOTCH activation can also induce transcriptomic alterations of multiple oncogenic pathways, such as c-MYC, KRAS, and Wnt/β-catenin,[39] creating a pro-tumorigenic environment for BE progression.

Although a recent study suggested that NOTCH signaling mediates differentiation in BE-like lesions with progression to adenocarcinoma in the L2-IL-1ß mouse model,[38] this study did not address the mechanisms of NOTCH activation. Using in vitro and in vivo models, we detected significant upregulation of NOTCH signaling components in response to reflux conditions. DLL1 was the most consistently up-regulated NOTCH ligand in the public datasets and in response to reflux conditions in our models. DLL1 is one of the three mammalian NOTCH ligands that belongs to Delta family. DLL1 overexpression promotes NOTCH signaling and cancer stem-like features in several tumour types.[40 41] We detected an increase in the full-length and cleaved intracellular form of DLL1. Interestingly, we found ABS-induced transcriptional upregulation of DLL1 with cleavage. DLL1 binds to NOTCH receptors to mediate its proteolytic cleavages that, in turn, release the NICD that enters the nucleus to bind to other transcription coactivators to induce expression of NOTCH targets.[20] We detected an increase in NICD with induction of expression of several NOTCH targets, such as HES1 and HEY1, following exposure to ABS. Of note, the use of neutralising DLL1 antibody abrogated ABS-mediated NOTCH activation, confirming its critical role in reflux conditions. These data indicate that reflux conditions play an important role in activation of NOTCH through DLL1.

Exposure to ABS during reflux conditions generates high levels of reactive oxygen species and oxidative stress with activation of pro-inflammatory signaling in EAC.[42–44] Through bioinformatics analysis of public datasets, NF-κB signature was prominent in EAC where GSEA demonstrated enrichment for NF-κB signature in tumours expressing high levels of DLL1. We identified and confirmed novel active NF-κB binding sites on the DLL1 promoter. Although the mechanisms by which NF-κB activation is induced and maintained under oxidative reflux conditions are not entirely understood, our data imply that APE1 redox function plays an important role in the process. APE1 has a unique redox function that protects and promotes TFs' activities and their binding affinity to target genes' promoters.[45] High levels of APE1, also known as REF1, have been described in EAC.[8 13 14] We found a positive correlation between APE1 and DLL1 in human EAC. These novel results uncover a novel link between inflammation and activation of NOTCH, showing crosstalk between APE1-redox, NF-κB and DLL1/NOTCH1 signaling in EAC. It is worth mentioning that the APE1 redox function can promote activation of the STAT3 TF in response to ABS in EAC.[8] We propose APE1 as an important redox protein that

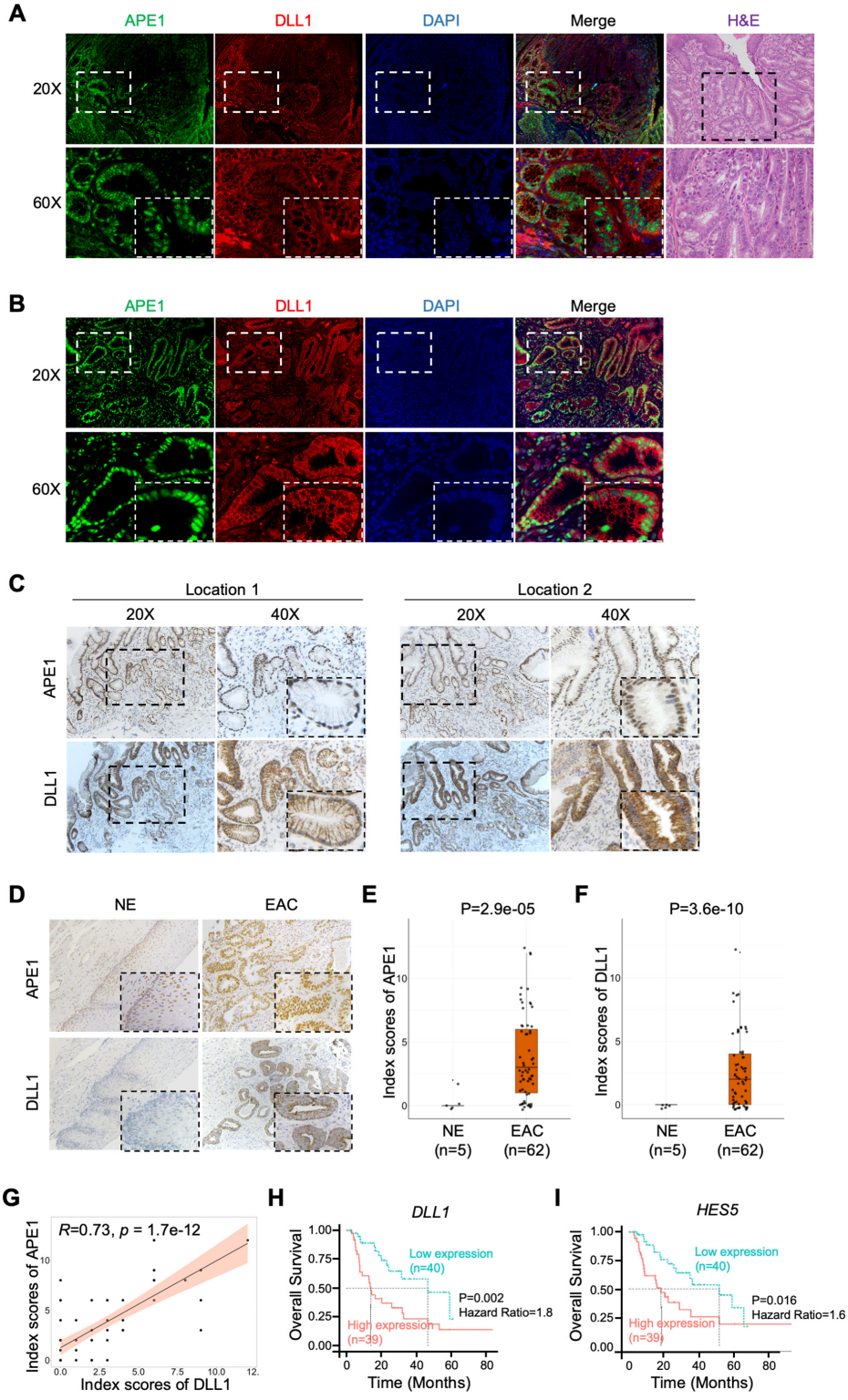

Figure 7 DLL1 is upregulated in neoplastic mouse and human oesophageal tissues and correlates with poor prognostic outcomes in human oesophageal adenocarcinoma (EAC). (A) and (B) Representative immunofluorescent staining images of APE1 (green) and DLL1 (red) in EAC tissue samples from pL2-IL1ß transgenic mouse (A) and human EAC tissue (B). DAPI was used for nuclear staining. (C) and (D) Representative IHC staining images of APE1 and DLL1 using the slides of the same human EAC tissue as B (C) and human EAC tissue microarrays (TMA) (D). (E) and (F) Comparisons of IHC index scores of APE1 (E) and DLL1 (F) between normal oesophageal epithelium (n=5) and EACs (n=62) in human EAC TMA were shown. (G) Pearson correlation analysis of the IHC index scores of APE1 and DLL1 protein from human EAC TMA. (H) and (I) Kaplan-Meier plots were used for the survival analysis in the TCGA-EAC database, comparing DLL1-high patients with DLL1-low patients (H), or comparing HES5-high patients with HES5-low patients (I).

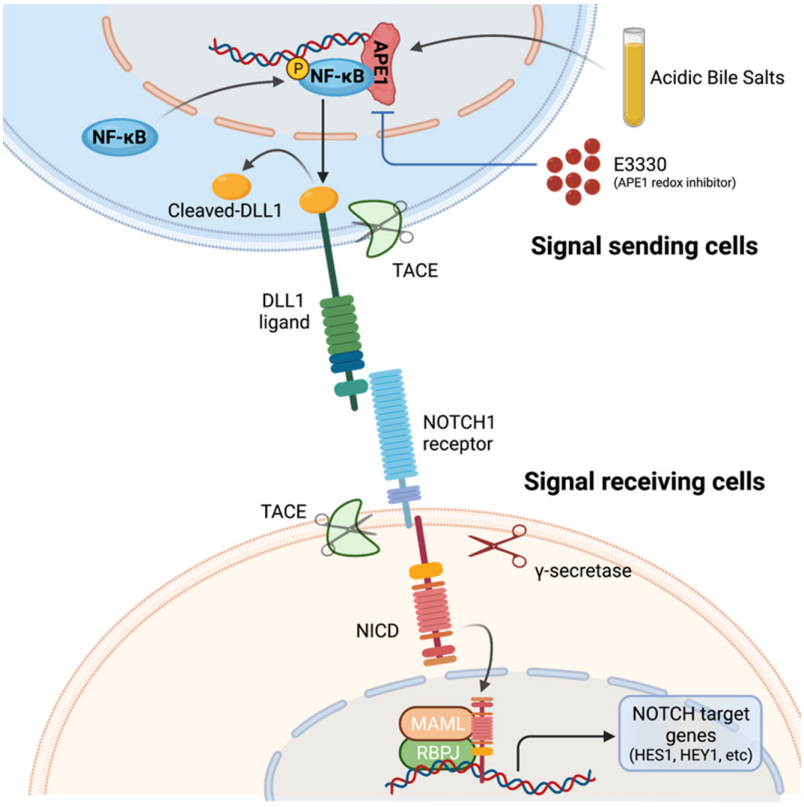

Figure 8 Schematic summary of APE1-redox-mediated NF-κB-DLL1-NOTCH activation in response to acidic bile salts (ABS) in oesophageal adenocarcinoma. Exposure to ABS, the in vitro mimic of GERD, increases APE1 protein level and activates APE1-redox-dependent transcription factor, NF-κB, which consequently upregulates DLL1 transcription by directly binding to DLL1 promoter region in the signal sending cells. Accumulated DLL1 protein on cell surface facilitates NOTCH1 cleavage in the signal receiving cells, releasing the active form of NOTCH1 receptor, NOTCH intracellular domain (NICD), which is translocated into cell nucleus, forms transcriptional complex with RBPJ and MAML, and activates transcription of downstream targets like HES1 and HEY1. APE1 redox-specific inhibitor, E3330, effectively blocks this ABS-activated NF-κB/DLL1/NICD signaling axis.

promotes pro-inflammatory oncogenic TFs in response to reflux conditions in EAC tumourigenesis.

Using repeated short exposures to ABS over 14 days to mimic chronic reflux conditions, we found that ABS not only activated APE1//NF-κB/DLL1/NOTCH signaling but also significantly enhanced tumorospheres formation capacity. APE1 small molecule redox inhibitors suppressed tumorospheres formation. We detected co-overexpression of APE1 and DLL1 in neoplastic lesions at the gastro-oesophageal junctions in the L2-IL1β mice. A similar expression pattern of APE1 and DLL1 was present in EAC tissue samples, where patients with EAC with DLL1-high expression levels had poorer clinical outcomes and shorter overall survival than those with DLL1-low levels.

In summary, our findings link inflammation (NF-κB), redox (APE1) and NOTCH signaling in EAC tumourigenesis under reflux conditions, providing a new perspective on the complex crosstalk of signaling networks. The results call for the development of novel therapeutic strategies that target APE1-redox or NOTCH signaling in patients with EAC.

Author affiliations
[1]Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, Beijing, China
[2]Department of Surgery, University of Miami Miller School of Medicine, Miami, Florida, USA
[3]Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, Florida, USA
[4]Department of Gastric Surgery, Fujian Medical University Union Hospital, Fujian, China
[5]Department of Molecular Pharmacology, University of Miami Miller School of Medicine, Miami, Florida, USA
[6]Sidra Medicine, Doha, Ad Dawhah, Qatar
[7]Department of Medicine, Columbia University Medical Center, New York, New York, USA
[8]Department of Pathology, University of Miami Miller School of Medicine, Miami, Florida, USA

Acknowledgements The research reported in this publication was supported by grants from the U.S. National Institutes of Health (NIH) (El-Rifai, W: R01CA206563, R01CA224366 and P01CA268991). LC received a scholarship from the China Scholarship Council. The Sylvester Comprehensive Cancer Centre (P30CA240139) shared resources were used in this study.

Contributors Conceptualisation and overall content: WE-R, SZ, LC. Methodology: LC, HL, AB, JQ and AZ. Validation: LC, HL and DLP. Formal analysis: LC and LLC. In vitro studies: LC, HL, KS, FB and ZC. In vivo studies: LC, HL and OGMD. Troubleshooting experiments: WE-R, TCW, AC and AZ. Data curation: LC, HL and WE-R. Supervision: WE-R, HL and DLP. Funding acquisition and resources: WE-R.

Funding This study was funded by the United States National Cancer Institute (Grant number: R01CA206563, R01CA224366, and P01CA268991).

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval The Institutional Research Ethics Committee approved the study design of deidentified human tissues or data. Written informed consents were received from all patients prior to participation. All animal studies were carried out following the protocols approved by the Institutional Animal Care and Use Committee of the University of Miami (UM-20-110).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

ORCID iDs
Timothy C Wang http://orcid.org/0000-0001-5730-3019
Alexander Zaika http://orcid.org/0000-0002-9993-238X
Wael El-Rifai http://orcid.org/0000-0002-5597-4588

REFERENCES

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020;70:7–30.
2 Edgren G, Adami H-O, Weiderpass E, *et al*. A global assessment of the oesophageal adenocarcinoma epidemic. *Gut* 2013;62:1406–14.
3 Gavin AT, Francisci S, Foschi R, *et al*. Oesophageal cancer survival in Europe: a EUROCARE-4 study. *Cancer Epidemiol* 2012;36:505–12.
4 Njei B, McCarty TR, Birk JW. Trends in esophageal cancer survival in United States adults from 1973 to 2009: a seer database analysis. *J Gastroenterol Hepatol* 2016;31:1141–6.
5 Pera M, Manterola C, Vidal O, *et al*. Epidemiology of esophageal adenocarcinoma. *J Surg Oncol* 2005;92:151–9.
6 Lagergren J. Adenocarcinoma of oesophagus: what exactly is the size of the problem and who is at risk? *Gut* 2005;54 Suppl 1:i1–5.
7 Peng D-F, Hu T-L, Soutto M, *et al*. Loss of glutathione peroxidase 7 promotes TNF-α-induced NF-κB activation in Barrett's carcinogenesis. *Carcinogenesis* 2014;35:1620–8.
8 Bhat AA, Lu H, Soutto M, *et al*. Exposure of Barrett's and esophageal adenocarcinoma cells to bile acids activates EGFR–STAT3 signaling axis via induction of APE1. *Oncogene* 2018;37:6011–24.
9 Vascotto C, Cesaratto L, Zeef LAH, *et al*. Genome-Wide analysis and proteomic studies reveal APE1/Ref-1 multifunctional role in mammalian cells. *Proteomics* 2009;9:1058–74.
10 Shah F, Logsdon D, Messmann RA, *et al*. Exploiting the Ref-1-APE1 node in cancer signaling and other diseases: from bench to clinic. *NPJ Precis Oncol* 2017;1:1–19.
11 Cardoso AA, Jiang Y, Luo M, *et al*. Ape1/Ref-1 regulates STAT3 transcriptional activity and APE1/Ref-1–STAT3 dual-targeting effectively inhibits pancreatic cancer cell survival. *PLoS One* 2012;7:e47462.
12 Zhang J, Luo M, Marasco D, *et al*. Inhibition of apurinic/apyrimidinic endonuclease I's redox activity revisited. *Biochemistry* 2013;52:2955–66.
13 Lu H, Bhat AA, Peng D, *et al*. Ape1 upregulates MMP-14 via redox-sensitive ARF6-mediated recycling to promote cell invasion of esophageal adenocarcinoma. *Cancer Res* 2019;79:4426–38.
14 Hong J, Chen Z, Peng D, *et al*. APE1-mediated DNA damage repair provides survival advantage for esophageal adenocarcinoma cells in response to acidic bile salts. *Oncotarget* 2016;7:16688–702.
15 Acharya A, Das I, Chandhok D, *et al*. Redox regulation in cancer: a double-edged sword with therapeutic potential. *Oxid Med Cell Longev* 2010;3:23–34.
16 Jorgenson TC, Zhong W, Oberley TD. Redox imbalance and biochemical changes in cancer. *Cancer Res* 2013;73:6118–23.
17 Sriramajayam K, Peng D, Lu H, *et al*. Activation of NRF2 by APE1/REF1 is redox-dependent in Barrett's related esophageal adenocarcinoma cells. *Redox Biol* 2021;43:101970.
18 Aster JC, Pear WS, Blacklow SC. The varied roles of Notch in cancer. *Annu Rev Pathol* 2017;12:245–75.
19 Ntziachristos P, Lim JS, Sage J, *et al*. From fly wings to targeted cancer therapies: a centennial for Notch signaling. *Cancer Cell* 2014;25:318–34.
20 Ranganathan P, Weaver KL, Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer* 2011;11:338–51.
21 Jin Y, Wang M, Hu H, *et al*. Overcoming stemness and chemoresistance in colorectal cancer through miR-195-5p-modulated inhibition of Notch signaling. *Int J Biol Macromol* 2018;117:445–53.
22 Radtke F, Fasnacht N, Macdonald HR. Notch signaling in the immune system. *Immunity* 2010;32:14–27.
23 Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci* 2013;132:2135–40.
24 Wang Z, Da Silva TG, Jin K, *et al*. Notch signaling drives stemness and tumorigenicity of esophageal adenocarcinoma. *Cancer Res* 2014;74:6364–74.
25 Song S, Maru DM, Ajani JA, *et al*. Loss of TGF-β adaptor β2SP activates Notch signaling and SOX9 expression in esophageal adenocarcinoma. *Cancer Res* 2013;73:2159–69.
26 Hovinga KE, Shimizu F, Wang R, *et al*. Inhibition of Notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate. *Stem Cells* 2010;28:1019–29.
27 Lu J, Ye X, Fan F, *et al*. Endothelial cells promote the colorectal cancer stem cell phenotype through a soluble form of Jagged-1. *Cancer Cell* 2013;23:171–85.
28 Johnson S, Chen H, Lo P-K. In vitro tumorsphere formation assays. *Bio Protoc* 2013;3:e325-e.
29 Sardar Pasha SPB, Sishtla K, Sulaiman RS, *et al*. Ref-1/APE1 inhibition with novel small molecules blocks ocular neovascularization. *J Pharmacol Exp Ther* 2018;367:108–18.
30 Logsdon DP, Shah F, Carta F, *et al*. Blocking HIF signaling via novel inhibitors of CA9 and APE1/Ref-1 dramatically affects pancreatic cancer cell survival. *Sci Rep* 2018;8:1–14.
31 Quante M, Bhagat G, Abrams JA, *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.
32 Lagergren J, Lagergren P. Recent developments in esophageal adenocarcinoma. *CA Cancer J Clin* 2013;63:232–48.
33 Coleman HG, Xie S-H, Lagergren J. The epidemiology of esophageal adenocarcinoma. *Gastroenterology* 2018;154:390–405.
34 Wang Z, Chen J, Capobianco AJ. The Notch signaling pathway in esophageal adenocarcinoma. *Cell Mol Biol* 2015;61:24–32.
35 Nicholson AM, Graham TA, Simpson A, *et al*. Barrett's metaplasia glands are clonal, contain multiple stem cells and share a common squamous progenitor. *Gut* 2012;61:1380–9.
36 Jiang M, Li H, Zhang Y, *et al*. Transitional basal cells at the squamous-columnar junction generate Barrett's oesophagus. *Nature* 2017;550:529–33.
37 Leedham SJ, Preston SL, McDonald SAC, *et al*. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. *Gut* 2008;57:1041–8.
38 Kunze B, Wein F, Fang H-Y, *et al*. Notch signaling mediates differentiation in Barrett's esophagus and promotes progression to adenocarcinoma. *Gastroenterology* 2020;159:575–90.
39 Kunze B, Middelhoff M, Maurer HC, *et al*. Notch signaling drives development of Barrett's metaplasia from Dclk1-positive epithelial tuft cells in the murine gastric mucosa. *Sci Rep* 2021;11:1–13.
40 Kumar S, Srivastav RK, Wilkes DW, *et al*. Estrogen-Dependent DLL1-mediated Notch signaling promotes luminal breast cancer. *Oncogene* 2019;38:2092–107.
41 Zhang C, Hai L, Zhu M, *et al*. Actin cytoskeleton regulator Arp2/3 complex is required for Dll1 activating Notch1 signaling to maintain the stem cell phenotype of glioma initiating cells. *Oncotarget* 2017;8:33353–64.
42 Dvorak K, Payne CM, Chavarria M, *et al*. Bile acids in combination with low pH induce oxidative stress and oxidative DNA damage: relevance to the pathogenesis of Barrett's oesophagus. *Gut* 2007;56:763–71.
43 Zhang HY, Hormi-Carver K, Zhang X, *et al*. In benign Barrett's epithelial cells, acid exposure generates reactive oxygen species that cause DNA double-strand breaks. *Cancer Res* 2009;69:9083–9.
44 Peng D-F, Hu T-L, Soutto M, *et al*. Glutathione peroxidase 7 suppresses bile salt-induced expression of pro-inflammatory cytokines in Barrett's carcinogenesis. *J Cancer* 2014;5:510–7.
45 Tell G, Quadrifoglio F, Tiribelli C, *et al*. The many functions of APE1/Ref-1: not only a DNA repair enzyme. *Antioxid Redox Signal* 2009;11:601–19.