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ORIGINAL PAPER

Alcohol drinking inhibits NOTCH–PAX9 signaling in esophageal squamous epithelial cells

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Abstract

Alcohol drinking has been established as a major risk factor for esophageal diseases. Our previous study showed that ethanol exposure inhibited PAX9 expression in human esophageal squamous epithelial cells *in vitro* and *in vivo*. In this study, we aimed to investigate the molecular pathways through which alcohol drinking suppresses PAX9 in esophageal squamous epithelial cells. We first demonstrated the inhibition of NOTCH by ethanol exposure *in vitro*. NOTCH regulated PAX9 expression in KYSE510 and KYSE410 cells *in vitro* and *in vivo*. RBPJ and NOTCH intracellular domain (NIC) D1 ChIP-PCR confirmed *Pax9* as a direct downstream target of NOTCH signaling in mouse esophagus. NOTCH inhibition by alcohol drinking was further validated in mouse esophagus and human tissue samples. In conclusion, ethanol exposure inhibited NOTCH signaling and thus suppressed PAX9 expression in esophageal squamous epithelial cells *in vitro* and *in vivo*. Our data support a novel mechanism of alcohol-induced esophageal injury through the inhibition of NOTCH–PAX9 signaling.

Keywords: ethanol; NOTCH; PAX9; RBPJ; NICD1; esophagus; esophageal squamous cell carcinoma

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No conflicts of interest were declared.

Introduction

Alcohol drinking is a known risk factor for esophageal diseases, e.g. gastroesophageal reflux disease (GERD) and esophageal squamous cell carcinoma (ESCC). As the most commonly seen gastrointestinal disorder, GERD causes heartburn and leads to substantial impairment of quality of life and work productivity. The prevalence of GERD is increasing worldwide, with the rate of weekly GERD symptoms affecting ~20% of people in the USA. Alcohol drinking worsens GERD symptoms [1]. Alcohol consumption reduces primary esophageal peristalsis and the resting pressure of the lower esophageal sphincter, and thus promotes reflux of gastric contents [2]. Alcohol also has direct noxious effects on the esophageal epithelium [3].

Esophageal cancer affected 17 650 adults and caused 16 080 deaths in the USA in 2019 [4]. In the world, it is the seventh most prevalent cancer and the sixth leading cause of cancer-related death, with more than 572 000 new cases and 508 000 deaths each year [5]. Oro-esophageal cancers have a stronger association with alcohol consumption than cancers of any other organ sites [6]. ESCC risk is associated with alcohol consumption in a dose-dependent manner. Alcohol drinking and smoking have additive or synergistic effects on carcinogenesis, and tobacco and alcohol use together explained more than 70% of cases of ESCC. A case-controlled study of the risk of ESCC showed a higher odds ratio (OR) for heavy drinkers (OR = 10) compared with tobacco smokers (OR = 5.8) [7]. Furthermore, heavy drinking led to an increased number of squamous cell carcinomas. Genetic polymorphisms of ethanol-metabolizing genes were reported to be associated with ESCC [8]. Consistent with human studies, animal studies have also shown that alcohol drinking or painting on the oro-esophageal epithelium inhibited squamous

differentiation and produced squamous hyperproliferation in rodents [9,10].

Certain mechanisms of alcohol-induced esophageal injury are supported by experimental data, whereas most are speculative or extrapolated from studies on cancers of other organ sites [11]. It is believed that ethanol enhances the penetration of carcinogens across the epithelium, stimulates cell proliferation, inhibits squamous differentiation, generates oxidative stress, interferes with DNA repair and synthesis, disturbs systemic metabolism of nutrients, impairs immune function, induces chronic inflammation, and promotes angiogenesis [12].

Our previous study showed that ethanol exposure inhibited PAX9 expression in esophageal squamous epithelial cells *in vitro* and *in vivo* [13]. PAX9 is a transcription factor of the PAX family characterized by a DNA-binding paired domain. Being expressed in somites, pharyngeal pouches, and mesenchyme, PAX9 is essential for the development of thymus, parathyroid, limb, palate, and teeth during mouse embryogenesis [14]. In mice, PAX9 regulates squamous differentiation and carcinogenesis in the oro-esophageal epithelium. In human ESCC, PAX9 expression in the esophagus of drinkers was significantly lower than that in the esophagus of non-drinkers. Mechanistically, we found that promoter hypermethylation was associated with PAX9 silencing in human tissues and that a demethylating agent reduced CpG methylation percentages and thus upregulated PAX9 expression in human ESCC cells. However, when cultured cells and mice were exposed to ethanol, the *PAX9* promoter did not undergo hypermethylation, even though PAX9 expression was downregulated [13]. These data suggested that alternative mechanisms were responsible for PAX9 downregulation by ethanol in our experimental settings.

Several signaling pathways have been suggested to regulate PAX9 expression [15,16]. Meanwhile, several signaling pathways have been identified as potential targets for ethanol's toxicological effects using a bioinformatics approach and microarray profiling, e.g. WNT, NOTCH, and SHH [17]. In this study, we aimed to investigate the molecular pathways through which alcohol drinking suppresses PAX9 in esophageal squamous epithelial cells.

Materials and methods

Ethics statement and human tissue samples

Formalin-fixed, paraffin-embedded sections of histologically normal human esophageal squamous epithelium were obtained from Ningxia Medical University General Hospital, with informed written consent and IRB approval. These 5-μm-thick sections were prepared from tissues originally harvested during esophagectomy from ESCC patients. All human samples were coded with patient identifiers removed. Clinical data, including alcohol drinking, were collected from the medical record. Patients with a self-reported history of heavy liquor drinking ($n = 5$) were regarded as 'heavy drinkers' (regular consumption of stronger than 50% ethanol v/v for more than 10 years) and those who reported never drinking alcohol ($n = 11$) were regarded as 'non-drinkers'. Occasional and light drinkers were excluded (supplementary material, Table S1). Positive immunostaining for cytokeratin 5 was used to validate the antigenicity of these tissue sections.

Cell culture and treatment

Human ESCC cells (KYSE510, KYSE410, KYSE450, and KYSE70) were obtained from the ATCC (Manassas, VA, USA) and the ECACC (Porton Down, Salisbury, UK) with proper authentication. These ESCC cell lines were selected for experiments based on their expression of NOTCH components and PAX9 (supplementary material, Figure S1). KYSE450 cells are known to carry a frame-shift *NOTCH1* mutation (https://portals.broadinstitute.org/ccle). KYSE510 and KYSE410 were exposed to ethanol, dibenzazepine (DBZ; a chemical NOTCH inhibitor) (Cayman, Ann Arbor, MI, USA), or recombinant human JAG1 (a NOTCH ligand; R&D Systems, Minneapolis, MN, USA). The human JAG1 recombinant protein containing the signal peptide and extracellular domain of JAG1 fused at the C-terminus to the Fc portion of human IgG (R&D Systems) was immobilized to the plastic surface of the culture plates by incubating plates with a solution of JAG1 (5 μg/ml) for 2 h at 37 °C. Cells were then seeded on JAG1-coated plates for 72 h. 3XFlag*NICD1* overexpression plasmid (Plasmid #20183; Addgene, Watertown, MA, USA) [18] or *RBPJ* siRNAs (Thermo Fisher, Waltham, MA, USA) were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All cell culture experiments were triplicated.

Analysis of signaling pathway activity

Cignal Finder 45-Pathway Reporter Array (Cat # CCA-901L; Qiagen, Valencia, CA, USA) was used to rapidly assess the activities of 45 signaling pathways in KYSE510 cells according to the manufacturer's protocol. In brief, reporter constructs resident in each well of the Cignal Finder array plate were resuspended with 50 μl of Opti-MEM medium (Thermo Fisher) and then mixed with 50 μl of diluted Attractene transfection reagent (Qiagen). KYSE510 cell suspension was diluted in Opti-MEM medium and then seeded in each well (50 000 cells per well). These cells were treated with either 0 or 100 mM ethanol for 24 h on the following day. Finally, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Animal experiment

Wild-type C57BL/6J mice, *Sox2CreER* mice, *Rosa*^{NICD1} mice, and conditional *Notch* deficiency mice (*K5CreER;* *N1*$^{fl/fl}$*;N2*$^{fl/fl}$) were obtained from the Jackson Laboratory

(Bar Harbor, ME, USA). *Sox2CreER* mice and *Rosa*^{NICD1} mice were crossed to generate tissue-specific NICD1-overexpressing mice (*Sox2CreER;Rosa*^{NICD1}). All animal experiments were approved by the IACUC at the North Carolina Central University (protocol number XC06142019). Mice were given tamoxifen (0.075 g/kg per day, i.p., for 5 days) to induce NICD1 overexpression or NOTCH1/NOTCH2 knockout in the esophageal squamous epithelial cells of *Sox2CreER; Rosa*^{NICD1} or *K5CreER;N1*$^{fl/fl}$*;N2*$^{fl/fl}$ mice, respectively. Mice were sacrificed at 2 weeks after tamoxifen induction to harvest the esophagus in formalin for histology, or the esophageal epithelium in liquid nitrogen for molecular analyses.

Western blotting

Total protein was extracted from the human ESCC cells and mouse tissues with a standard method. Proteins were detected with a rabbit monoclonal anti-cleaved NOTCH1 antibody (1:500, Cat # 4147; Cell Signaling Technology, Danvers, MA, USA), a rabbit monoclonal anti-PAX9 antibody (1:600, Cat # 12847; Cell Signaling Technology), a rabbit monoclonal anti-HES1 antibody (1:4000, Cat # 11988; Cell Signaling Technology), a rabbit monoclonal anti-RBPJ antibody (1:2000, Cat # 5313; Cell Signaling Technology), a rabbit polyclonal anti-c-MYC antibody (1:1000, Cat # 9402; Cell Signaling Technology), a mouse monoclonal anti-STAT3 antibody (1:1000, Cat # 9139; Cell Signaling Technology), a rabbit polyclonal anti-p-STAT3 antibody (1:1000, Cat # 9131; Cell Signaling Technology), a rabbit polyclonal anti-SOX2 antibody (1:2000, Cat # ab97959; Abcam, Cambridge, MA, USA), a rabbit polyclonal anti-ETV4 antibody (1:1000, Cat # ab135590; Abcam), and a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:40 000, Cat # ab8245; Abcam).

Immunohistochemical (IHC) staining

For IHC staining, deparaffinized sections were pretreated to retrieve antigens with a Tris-based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) before blocking with 10% normal serum and then applying either a rabbit monoclonal anti-NOTCH1 antibody (1:200, Cat # D1E11; Cell Signaling Technology), a rabbit polyclonal anti-NOTCH2 antibody (1:100, Cat # ab52302; Abcam), a rabbit polyclonal anti-activated NOTCH1 antibody (1:100, Cat # ab8925; Abcam), a rabbit monoclonal anti-PAX9 antibody (1:50; Cell Signaling Technology), or a rabbit monoclonal anti-HES1 antibody (1:50; Cell Signaling Technology) at 4 °C overnight. Tissue sections were then washed in PBS and incubated with biotinylated secondary antibodies for 30 min at room temperature. Detection of the antibody complex was carried out using the streptavidin–peroxidase reaction kit with DAB as a chromogen (ABC kit, Vector Laboratories).

NICD1 IHC staining intensity in histologically normal esophageal squamous epithelium was measured with ImageJ. The region of the esophageal epithelium was marked for analysis of the mean density of brown staining. The staining intensity was calculated by subtracting the mean intensity of the background.

RT-qPCR and ChIP-PCR

RT-qPCR was performed to quantify the expression levels of genes of interest with relevant primers and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a 96-well optical plate on an ABI 7900HT Fast Real-Time PCR system in triplicate (Applied Biosystems). RBPJ binding sites within the 5'-upstream DNA sequence of the mouse *Pax9* gene (ENSMUSG00000001497) were based on a previous RBPJ ChIP-seq study [19]. The ChIP-PCR analysis was performed using an EZ-ChIP kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions with antibodies to RBPJ and NICD1 that had been validated in previous ChIP-seq studies [19,20]. Immunoprecipitated DNA or input was PCR-amplified with the following primer pairs: Pax9 Primer1 (forward: 5'-CTT TCA AGG TGG CTC TAT GGT-3' and reverse: 5'-CAC AAA TTC TAT CTC CTT CCA GTT-3'; predicted size 366 bp); Pax9 Primer2 (forward: 5'-GGA AGA GGG GCA ACC AGA T-3' and reverse: 5';-TGA AGC TTT GGA GGT GGC GTC TAC-3'; predicted size 348 bp); positive control Hes1 (forward: 5'-TGT CTC TTC CTC CCA TTG G-3' and reverse: 5'-AAC TAC TGA GCA GTT GAA GG-3'; predicted size 244 bp).

Gene microarray analysis

Total RNA was extracted from control and ethanol-exposed (100 mM for 72 h) KYSE510 cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Microarray experiments were performed with Agilent SurePrint G3 Human GE v2 8x60K microarray (Agilent, Santa Clara, CA, USA). The raw data have been submitted to the NCBI GEO database (GSE96734), and data were analyzed as previously described [13]. Microarray data of squamous epithelium from control and ethanol-treated mice were obtained from our previous study for analysis of NOTCH pathway genes (GSE75373) [13]. Microarray data of two GEO datasets (GSE23400 and GSE20347) were downloaded and analyzed to compare *PAX9* and *HES1* mRNA expression in human ESCC versus matched normal tissues.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used for Student's *t*-test and Spearman's rank testing. Hierarchical clustering analysis was performed using the R package. $p < 0.05$ was considered statistically significant.

Results

Ethanol exposure modulates the activities of signaling pathways in KYSE510 and KYSE410 cells *in vitro*

Using the Cignal Finder 45-Pathway Reporter Array, we found that 20 signaling activities were significantly impacted in KYSE510 cells exposed to ethanol (100 mM for 24 h, $n = 4$ per group). Among these pathways, RBPJ, c-MYC, and STAT3 signaling activities were inhibited and SOX2 was activated (Figure 1A). Subsequent western blotting confirmed the downregulation of NICD1, c-MYC, and p-STAT3/STAT3 in KYSE510 cells. Since both *STAT3* and *c-MYC* are well-established NOTCH effectors [21,22], these data suggested the inhibition of NOTCH signaling by ethanol. Although SOX2 itself was not upregulated, its downstream transcriptional target, ETV4 [23], was significantly upregulated (Figure 1B).

Gene expression profiles in control and ethanol-exposed KYSE510 cells (100 mM for 72 h) were analyzed using gene microarrays ($n = 4$ per group; supplementary material, Table S2). Significance analysis of microarrays (SAM) revealed that 577 genes were upregulated and 952 genes downregulated in ethanol-exposed cells compared with control. A number of genes associated with squamous differentiation were among the downregulated genes including *AQP3*, *KRT1*, *KRT4*, *KRT13*, *KRT14*, *KRT23*, *KRT24*, *KRT31*, and *SPRR1B*. Four gene set analysis (GSA) approaches (GSA_GO, GSA_KB, GSA_CP, and GSA_TF, where GO = gene ontology; KB = knowledge-based; CP = canonical pathway; and TF = transcription factor) were used to identify differential enrichment of gene sets in the ethanol-exposed samples versus controls. GSA_GO showed downregulation of 'epidermis development' and 'ectoderm development'; GSA_KB showed downregulation of '*P63* target genes', 'basal layer genes', '*PAX9* target genes', and '*RBPJ* target genes'. GSA_CP showed upregulation of the *TGFβ* pathway, integrin pathway, and *NFκB* pathway, and GSA_TF upregulation of *NFκB* and *SMAD4*, in ethanol-exposed samples. These data suggested that ethanol exposure inhibited NOTCH signaling, promoted basal cell features, and suppressed squamous differentiation.

PAX9 is a downstream effector of the NOTCH signaling pathway in esophageal squamous epithelial cells *in vitro* and *in vivo*

Our previous study showed that ethanol exposure downregulated PAX9 expression in esophageal squamous epithelial cells *in vitro* and *in vivo*, and that PAX9 regulates squamous differentiation in the esophagus [13].

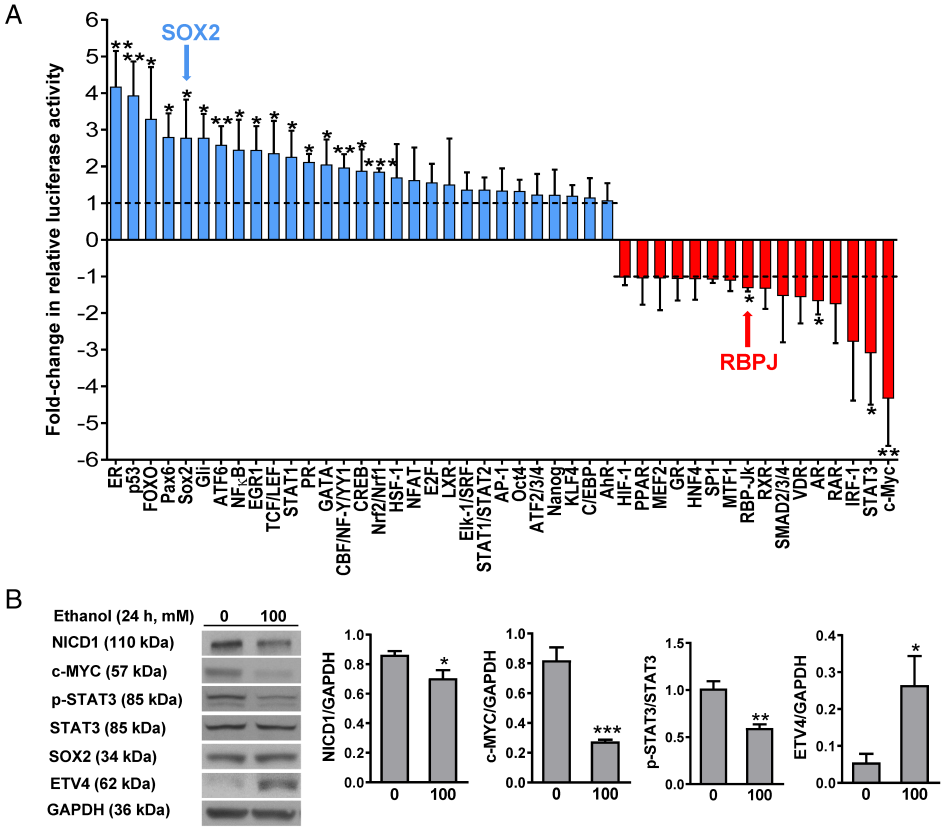

Figure 1. Multiple signaling activities in KYSE510 cells are modulated by ethanol. KYSE510 cells exposed to 100 mM ethanol for 24 h were analyzed with Cignal 45-Pathway Reporter Array (A, $n = 4$ per group). Data were normalized by signaling activities in control cells (black dotted lines). Expression of NICD1, NOTCH downstream genes (*c-MYC*, *p-STAT3*, *STAT3*), SOX2, and a SOX2 target gene (*ETV4*) were validated by western blotting (B). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Bar represents mean ± SD after triplicate experiments. *P* values were determined using Student's *t*-test.

RBPJ ChIP-seq also suggested *PAX9* as a potential downstream transcriptional target in neural stem cells [19]. Therefore, we hypothesized that PAX9 was a potential NOTCH effector in esophageal squamous epithelial cells as well. Western blotting showed that JAG1 (a NOTCH ligand) and NICD1 overexpression upregulated NICD1, HES1, and PAX9 expression in KYSE510 cells (Figure 2A,B).

When NOTCH is not activated, RBPJ is primarily a transcriptional repressor that exists in complexes with corepressors. The depletion of *RBPJ* resulted in the activation of canonical NOTCH target genes *in vitro* and *in vivo* [24]. Consistent with these observations, western blotting showed that *RBPJ* siRNA knockdown resulted in the upregulation of HES1 and PAX9 expression in KYSE510 cells (Figure 2C). A chemical inhibitor of NOTCH signaling, DBZ (25 or 50 μM for 24 h), caused dramatic downregulation of NICD1, HES1, and PAX9 expression in KYSE510 cells (Figure 2D). Similar results were found in KYSE410 cells (supplementary material, Figure S2).

To further determine whether NOTCH perturbation may affect PAX9 expression *in vivo*, *Sox2CreER;ROSA*^{NICD1} and *K5CreER;N1*$^{fl/fl}$*;N2*$^{fl/fl}$ mice were exposed to tamoxifen to overexpress NICD1 or knock out *Notch1/Notch2* in esophageal squamous epithelial cells. Western blotting and IHC demonstrated upregulation of NICD1 and PAX9 expression in mouse esophageal squamous epithelial cells of *Sox2CreER;ROSA*^{NICD1} mice (Figure 3A,B). On the contrary, NICD1 and PAX9 expression in mouse esophageal squamous epithelial cells of *K5CreER;N1*$^{fl/fl}$*;N2*$^{fl/fl}$ mice was significantly downregulated in comparison with the controls (Figure 3C,D). It was noteworthy that NOTCH deficiency in mouse esophagus inhibited squamous differentiation, as shown by thinning of the superficial layer (Figure 3D).

To determine whether *Pax9* is a direct transcriptional target of NOTCH signaling, we performed RBPJ and NICD1 ChIP-PCR. Two RBPJ binding sites were found within the 5'-upstream DNA sequence of the mouse *Pax9* gene based on a previous ChIP-seq study [19] (supplementary material, Figure S3). The ChIP-PCR

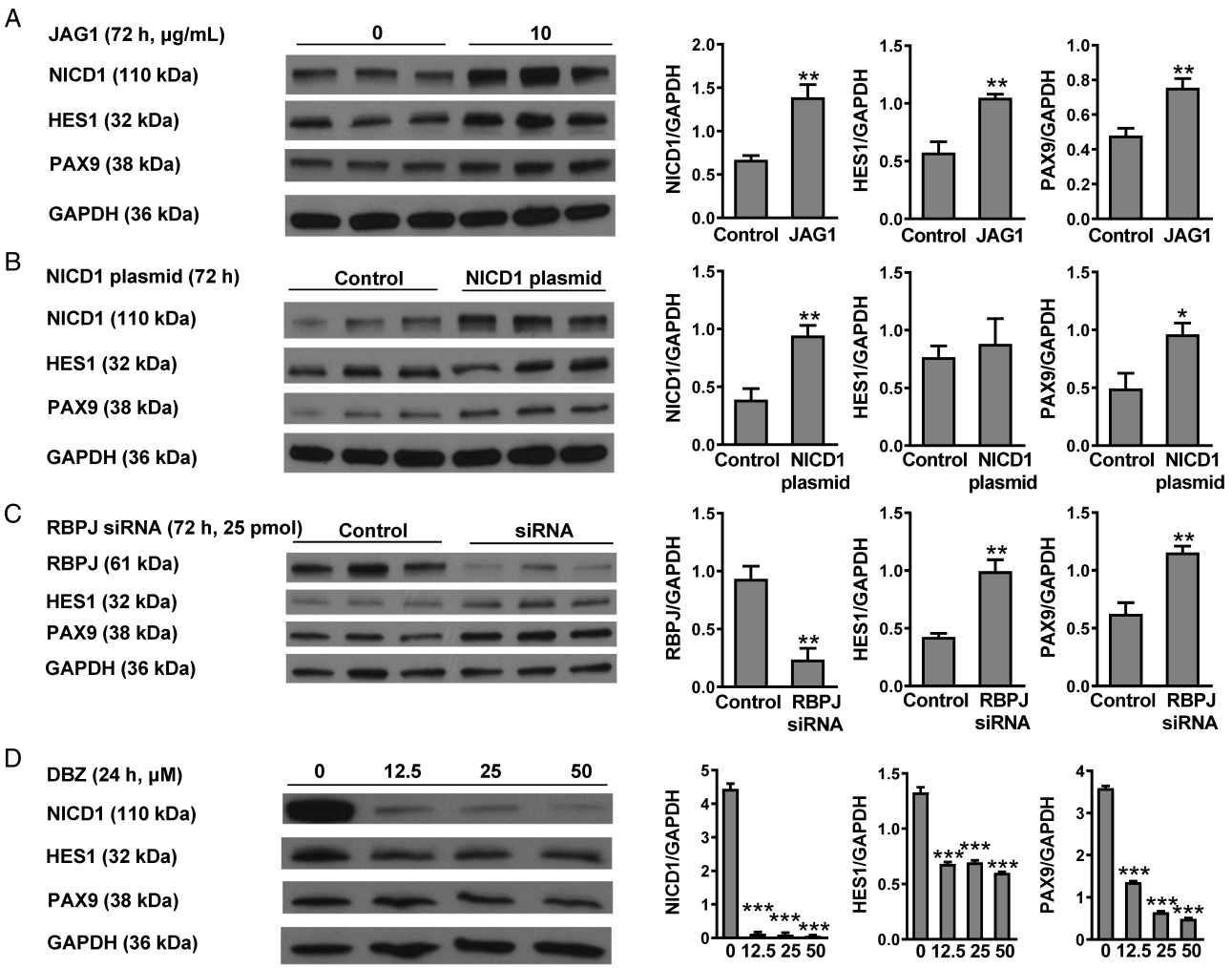

Figure 2. NOTCH perturbations modulate PAX9 expression in KYSE510 cells *in vitro*. Whole-cell lysates from KYSE510 cells exposed to JAG1 (A), NICD1 plasmid (B), *RBPJ* siRNA (C), or DBZ (D) were analyzed by western blotting for NICD1, HES1, and PAX9 expression. Experiments were performed in triplicate and protein expression was semi-quantitated for statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Bar represents mean ± SD. *P* values were determined using Student's *t*-test.

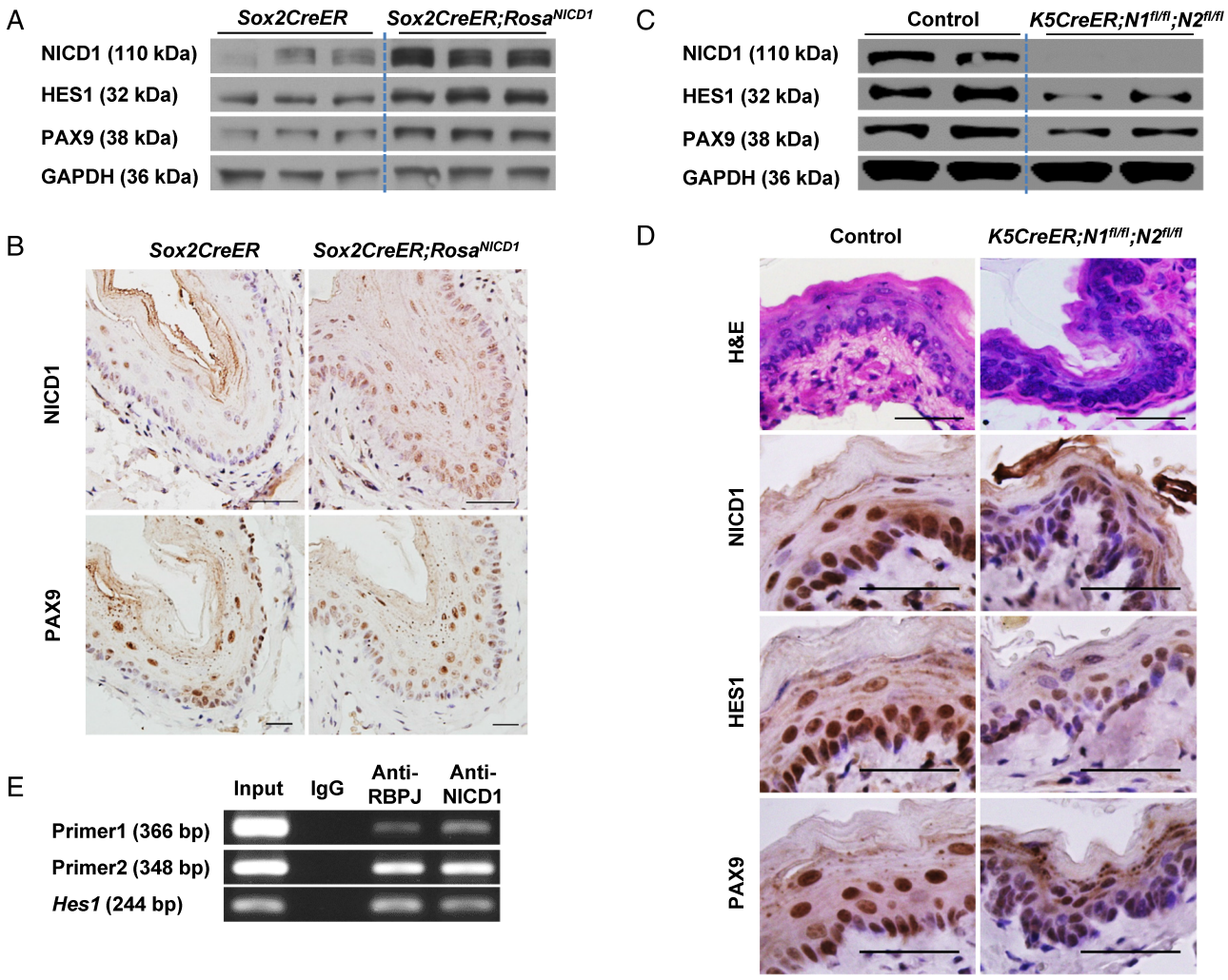

Figure 3. NICD1 perturbations modulate PAX9 expression in esophageal squamous epithelial cells *in vivo*. Western blotting (A, C) and IHC staining (B, D) showed overexpression of NICD1 and PAX9 in *Sox2CreER;Rosa*^{NICD1} esophagus (n = 3) and downregulation of NICD1 and PAX9 in *K5CreER;N1*$^{fl/fl}$*;N2*$^{fl/fl}$ esophagus (n = 3) compared with wild-type esophagus (n = 3). ChIP-PCR confirmed RBPJ or NICD1 binding to the 5′-upstream DNA sequence of the *Pax9* gene (E). Broken lines align samples of the groups. *$p < 0.05$, **$p < 0.01$. Scale bar = 50 μm.

result showed that RBPJ and NICD1 bound to the 5'-upstream DNA sequence of the *Pax9* gene in mouse esophagus (Figure 3E), suggesting that *Pax9* is a direct downstream target of NOTCH signaling in the esophageal squamous epithelial cells.

Ethanol suppresses PAX9 expression in KYSE510 and KYSE410 cells *in vitro* through inhibition of the NOTCH signaling pathway

To further understand the effect of ethanol on NOTCH–PAX9 signaling in esophageal squamous epithelial cells, KYSE510 and KYSE410 cells were exposed to ethanol. For the dose-dependent experiment, cells were exposed to ethanol at 25, 50, and 100 mM, which corresponded to physiologically relevant concentrations of 0.12, 0.23, and 0.46 g/dl. For the time-dependent experiment, cells were exposed to 100 mM ethanol for 24, 48, and 72 h. Western blotting showed downregulation of NOTCH–PAX9 signaling in KYSE510 cells (Figure 4A,B) and KYSE410 cells (supplementary material, Figure S4A,B) by ethanol in a dose- and time-dependent manner, suggesting that ethanol exposure inhibits NOTCH–PAX9 signaling in these cells *in vitro*.

Since ethanol has been reported to inhibit γ-secretase in vascular smooth muscle cells [25], we next determined whether NOTCH activation upstream and downstream of γ-secretase may have any effects on PAX9 expression in ethanol-exposed cells. KYSE510 cells were treated with JAG1 and/or ethanol. Western blotting showed that ethanol exposure counteracted the NOTCH activating effect of JAG1 on PAX9 expression in a dose-dependent manner (Figure 4C). Similar results were found in KYSE410 cells (supplementary material, Figure S4C). When KYSE510 cells were treated with ethanol and/or 3XFlag*NICD1* plasmid transfection, NICD1 overexpression counteracted the effect of ethanol exposure on PAX9 expression (Figure 4D).

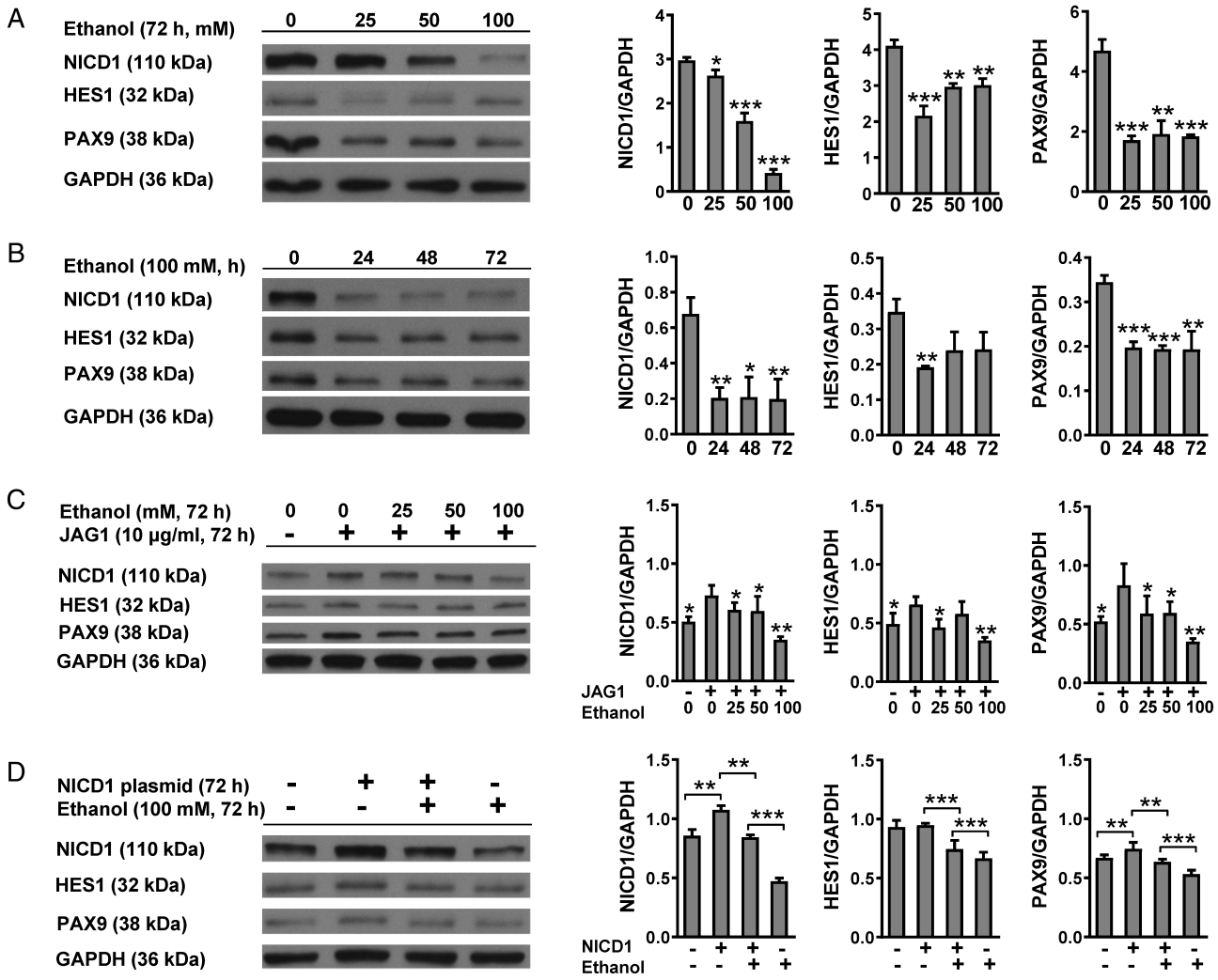

Figure 4. Ethanol exposure inhibits NOTCH signaling in KYSE510 cells *in vitro*. Whole-cell lysates from KYSE510 cells exposed to different concentrations of ethanol at various time points (A, B), JAG1 and/or ethanol (C), and NICD1 plasmid and/or ethanol (D) were analyzed by western blotting for NICD1, HES1, and PAX9 expression. Experiments were performed in triplicate and protein expression was semi-quantitated for statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Bar represents mean ± SD. *P* values were determined using Student's *t*-test.

Ethanol inhibits NOTCH signaling *in vivo*

To further elucidate the effect of ethanol on NOTCH signaling, we further analyzed mouse squamous epithelium obtained from our previous animal experiment in which wild-type mice were given ethanol in a sweetened drink *ad libitum*. A previous study showed that blood alcohol levels in the early morning after nocturnal feeding and drinking (20% w/v ethanol) were as high as 400 mg/dl (87 mM) [26], and this feeding protocol significantly downregulated PAX9 in our previous study [13]. We found inhibition of NOTCH signaling by both 20% ethanol for 4 weeks and 15% ethanol for 40 weeks. IHC staining showed that NOTCH receptors (NOTCH1, NOTCH2), NICD1, and HES1 expression were downregulated in the ethanol-exposed squamous epithelium compared with control, especially in mice exposed to 15% ethanol for 40 weeks (Figure 5A). Gene microarray data (GSE75373) were clustered with NOTCH pathway genes; samples exposed to 15% ethanol for 40 weeks were separated from the control and those exposed to 20% ethanol for 4 weeks (Figure 5B). RT-qPCR and western blotting confirmed NOTCH inhibition by alcohol drinking (Figure 5C,D).

Heavy liquor drinking is associated with NICD1 downregulation in human esophageal squamous epithelium

Previously we have shown that PAX9 expression in esophageal squamous epithelial cells is lower in the esophagus of drinkers than in the esophagus of non-drinkers [13]. Here we semi-quantitated NICD1 expression on formalin-fixed, paraffin-embedded tissue sections from heavy liquor drinkers and non-drinkers using IHC. As expected, NICD1 downregulation was observed in histologically normal esophageal squamous epithelium of heavy liquor drinkers compared with that from the non-drinkers (Figure 6 and supplementary material, Figure S5), suggesting that heavy liquor drinking is associated with NOTCH inhibition.

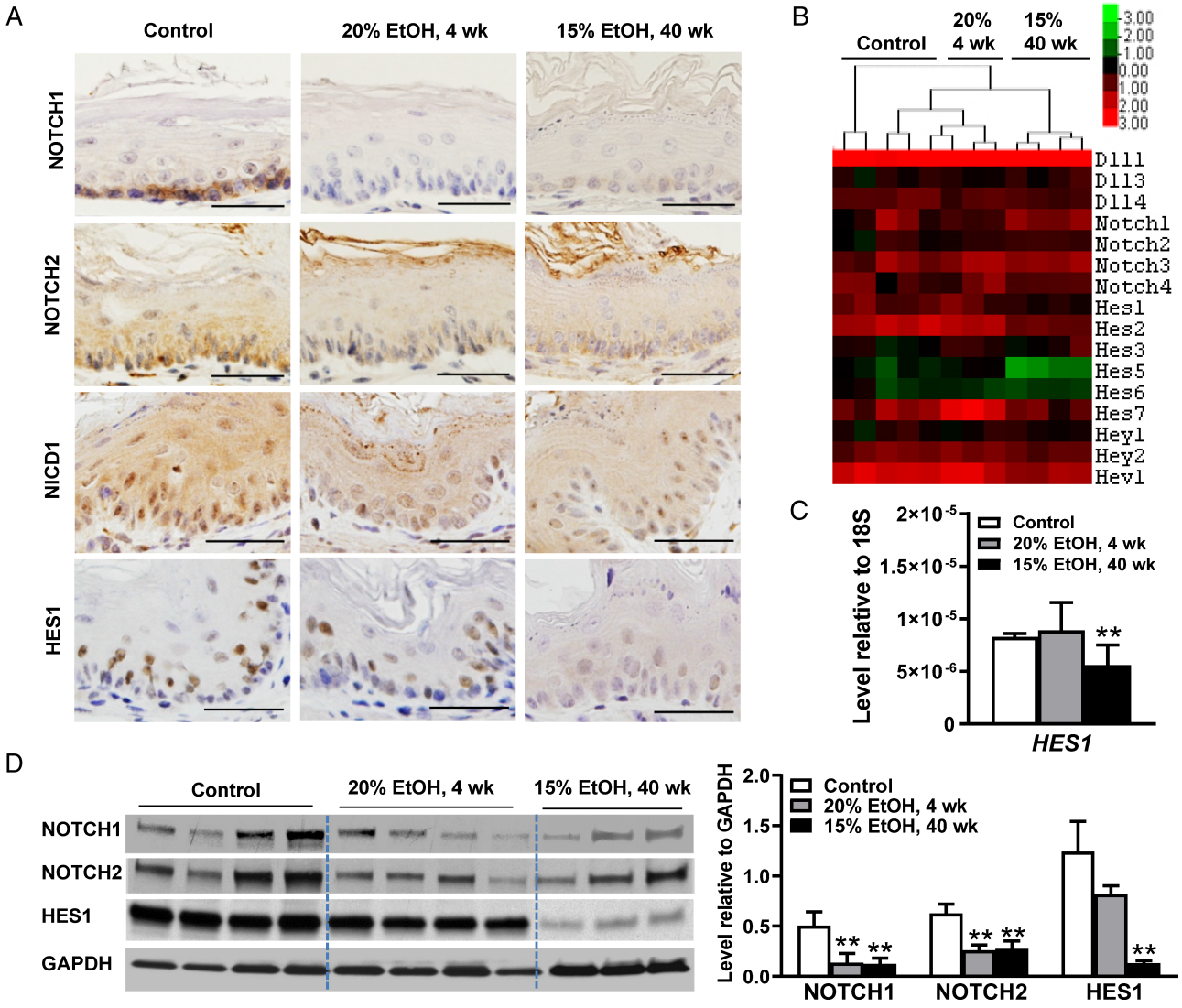

Figure 5. Ethanol exposure inhibits NOTCH signaling in the mouse squamous epithelium. IHC staining showed expression of NOTCH1, NOTCH2, NICD1, and HES1 in the ethanol-exposed mouse squamous epithelium compared with controls (A). Hierarchical clustering analysis of gene microarray data (GSE75373) was performed based on differential expression of *Notch* components (B). qRT-PCR and western blotting confirmed the downregulation of *Hes1* mRNA (C) and NOTCH components (D) in mouse squamous epithelium due to alcohol drinking (C–E). Broken lines indicate groups of samples and joining of panels. $^{*}p < 0.05$, $^{**}p < 0.01$. Bar represents mean ± SD ($n = 3$ per group). Scale bar = 50 μm. *P* values were determined using Student's *t*-test.

Moreover, analysis of the microarray data of two GEO datasets (GSE23400 and GSE20347) showed significant downregulation of *PAX9* and *HES1* mRNA expression in human ESCC (supplementary material, Figure S6A, B,D,E), compared with matched normal tissues. There was also a positive correlation between *PAX9* and *HES1* mRNA expression in these human esophageal squamous epithelium samples (supplementary material, Figure S6C,F), which is consistent with the regulation of PAX9 by NOTCH.

Discussion

Our previous study showed that ethanol exposure down-regulated PAX9 expression, suppressed squamous differentiation, and promoted carcinogenesis in the esophagus. In this study, we demonstrated that ethanol exposure inhibited NOTCH signaling and thus suppressed PAX9 expression in esophageal squamous epithelial cells *in vitro* and *in vivo*.

The NOTCH signaling pathway is mediated through ligands (e.g. JAG1) binding to NOTCH receptors (NOTCH1, 2, 3, 4). These receptors are then cleaved to allow its intracellular domain (e.g. NICD1) to be released from the membrane and enter the nucleus to form a transcriptional complex with RBPJ. NICD1 displaces the repressive cofactors bound to RBPJ and recruits a transcriptional activator complex, which initiates transcription of NOTCH downstream effectors such as HES1 [27]. In the normal esophageal epithelium of rodents and humans, NOTCH1, NOTCH2, and NOTCH3 are highly expressed, whereas NOTCH4 is expressed at a

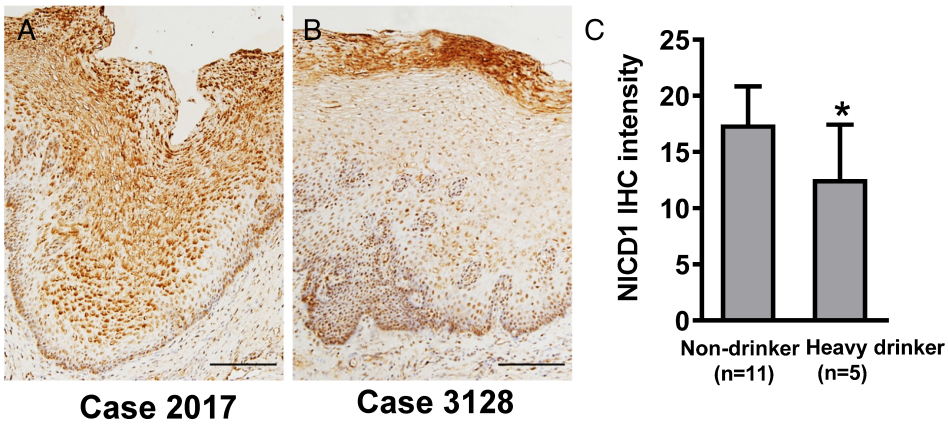

Figure 6. Downregulation of NICD1 in histologically normal esophageal squamous epithelium of heavy liquor drinkers and non-drinkers. NICD1 IHC is shown for a representative case of a non-drinker (A) and a representative case of a heavy liquor drinker (B). The staining intensity was measured with ImageJ for comparison between non-drinker's tissues (n = 11) and heavy liquor drinker's tissues (n = 5; C). *$p < 0.05$. Bar represents mean ± SD. Scale bar = 100 μm. P values were determined using Student's t-test.

minimal level [28,29]. NOTCH3 expression is subject to transcriptional regulation by NOTCH1, and loss of NOTCH signaling in mouse esophagus resulted in NOTCH3 loss [30]. NOTCH is known to regulate squamous differentiation in the esophagus [30,31], particularly in the commitment of keratinocytes to terminal differentiation by a HES1-dependent mechanism [32,33]. NOTCH also interacts with key regulators of squamous differentiation, such as P63 [34], IRF6 [35], NRF2 [36,37], and HPV8 E6 [38]. In this study, using a luciferase-based screening assay, we first demonstrated inhibition of NOTCH by ethanol exposure *in vitro* (Figure 1 and supplementary material, Table S2). We further showed that NOTCH regulated PAX9 expression *in vitro* and *in vivo* (Figures 2 and 3). These data explain our previous observation that PAX9 in esophageal squamous epithelial cells was downregulated by ethanol exposure and *Pax9* deficiency in mouse esophagus promoted cell proliferation and delayed cell differentiation [13]. NOTCH inhibition by alcohol drinking was further validated in mouse esophagus (Figure 5) and human tissue samples (Figure 6). Parallel to these observations, ethanol exposure significantly upregulated the expression of ETV4, a transcriptional target of SOX2 (Figure 1B). It has been well established that overexpression of SOX2 (a stem cell transcription factor) in the basal cells results in expansion of the basal compartment, inhibition of squamous differentiation, and histological changes leading to cancer in mice [39]. Consistent with our data, ethanol exposure was found to cause a massive, horizontal expansion of progenitor cell populations arising from single basal progenitor cells in the squamous epithelium of the tongue [40]. All these data support the notion that alcohol drinking results in expansion of the basal cell population and suppression of squamous differentiation in the esophagus.

PAX9 is a transcription factor of the PAX family characterized by a DNA-binding paired domain [14]. Several members of the *PAX* family are known to be regulated by NOTCH signaling, e.g. *PAX2* in the kidney [41], *PAX4* in blood [42], *PAX6* in the eye [43] and neuron [19], *PAX7* in muscle [44], and *PAX8* in the otic placode [45] and thyroid [46]. A genome-wide ChIP-seq analysis of NICD1/RBPJ targets identified *PAX9* as a potential downstream target in neural stem cells [19]. Consistent with this observation, RBPJ and NICD1 ChIP-PCR confirmed that *Pax9* is a direct downstream target of NOTCH signaling in mouse esophagus (Figure 3E). Modulation of PAX9 expression by NOTCH perturbations *in vitro* and *in vivo* further supported this claim (Figures 3 and 4).

It remains unclear how ethanol exposure inhibits NOTCH. Ethanol has been reported to suppress the NOTCH pathway through inhibition of γ-secretase proteolytic activity in vascular smooth muscle cells [25]. Consistent with this, our data showed that JAG1 was not able to rescue the NOTCH/PAX9-inhibitory effect of ethanol (Figure 4C), whereas NICD1 overexpression counteracted the effect of ethanol (Figure 4D). However, ethanol and in particular its metabolite (acetaldehyde) may attack amino acids [47], and thus inhibit NOTCH. It should be noted that the biological effects of ethanol are very complex and broad. Ethanol has been shown to enhance the expression and nuclear localization of 5-lipoxygenase and stimulate the biosynthesis of pro-inflammatory leukotriene B4 in oral cancer cells [48]. Ethanol-derived acetate may also feed into energy metabolism as a carbon source [49], or promote histone acetylation and thus regulate gene transcription [50]. Ethanol exposure increases the mutation rate through error-prone polymerases [51]. The concentration of ethanol may be an important factor in ethanol's biological effects. For example, liquor/spirit consumption was associated with higher risks of GERD and Barrett's esophagus, in which NOTCH was suppressed, whereas beer/wine consumption at low doses seemed to have the opposite effect [52]. Ethanol's effects on molecular pathways also depend on the context. For example, WNT signaling was suppressed by ethanol in human neural stem cells [53] but activated in fetal human and

mouse cerebral cortex [17]. The NOTCH pathway was inhibited by ethanol in smooth muscle cells [54] but activated in fetal human and mouse cerebral cortex [17] and human coronary artery endothelial cells [55]. In fetal alcohol syndrome models, SHH signaling in zebrafish was suppressed by ethanol [56]. However, in the liver, ethanol activated SHH signaling, and thus promoted carcinogenesis [57]. Therefore, further studies are needed to understand the molecular mechanisms of inhibition of NOTCH by ethanol exposure, which are relevant to alcohol-associated human esophageal diseases.

In summary, our data support a novel mechanism of alcohol-induced esophageal injury through inhibition of NOTCH–PAX9 signaling. As a result, chemical NOTCH activators may be used to prevent or treat alcohol-induced esophageal injury. Further characterization of the functional role of NOTCH–PAX9 signaling in esophageal squamous epithelial cells and its involvement in alcohol-induced esophageal injury is warranted and may reveal novel preventive and therapeutic opportunities for alcohol-associated esophageal diseases such as GERD and ESCC.

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Author contributions statement

MS, SR, HC, JL, CH, YL, YH, YL and ZX conducted the experiments and analyzed the data. MS, XC and ZX wrote and revised the manuscript. ZS, XC and ZX designed the experiments and supervised the whole process.

Data availability statement

Microarray data have been deposited in the NCBI GEO database under GEO Series accession number GSE96734 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE96734). The other microarray datasets used in this study are available in the NCBI GEO database under GEO Series accession numbers GSE75373 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75373), GSE23400 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23400), and GSE20347 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20347).

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. Expression of NOTCH components in human KYSE410, KYSE450, KYSE510, and KYSE70 cells assessed using western blotting

Figure S2. NOTCH perturbations modulate PAX9 expression in KYSE410 cells *in vitro*

Figure S3. RBPJ binding sites in the promoter region of the mouse *Pax9* gene (*Pax9*-201 transcript)

Figure S4. Ethanol inhibits NOTCH signaling in KYSE410 cells *in vitro*

Figure S5. Measurement of NICD1 IHC staining intensity in histologically normal esophageal squamous epithelium of a non-drinker (A) and a heavy liquor drinker (B) using ImageJ

Figure S6. Downregulation of *PAX9* and *HES1* in human ESCC compared with matched normal tissues (A, B, D, E) and correlation between *HES1* and *PAX9* mRNA expression (C, F)

Table S1. Clinical data and NICD1 staining intensity in the histological normal esophageal epithelium of humans

Table S2. Differentially expressed genes and enriched gene sets in KYSE510 cells exposed to ethanol (100 mM for 72 h)