HHS Public Access

Author manuscript

*J Pathol*. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

*J Pathol*. 2021 April ; 253(4): 384–395. doi:10.1002/path.5602.

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

**Menghan Shi**[a,b], **Shuang Ren**[a,b], **Hao Chen**[b], **Jing Li**[b,c], **Caizhi Huang**[b], **Yahui Li**[b], **Yuning Han**[c], **Yong Li**[d], **Zheng Sun**[a,*], **Xiaoxin Chen**[b,e,*], **Zhaohui Xiong**[b,*]

[a]Beijing Stomatological Hospital, Capital Medical University, 4 Tian-Tan-Xi-Li, Beijing 100050, China

[b]Cancer Research Program, Julius L. Chambers Biomedical Biotechnology Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707, USA

[c]Department of Thoracic Surgery, Ningxia Medical University General Hospital, Yinchuan, Ningxia 750004, China

[d]Department of Thoracic Surgery, National Cancer Center, Cancer Hospital of Chinese Academy of Medical Sciences, 17 Panjiayuan Nanli Road, Beijing 100021, China

[e]Center for Gastrointestinal Biology and Disease, Division of Gastroenterology and Hepatology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

## 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*.

[*]Co-corresponding authors: Z Sun, Beijing Stomatological Hospital, Capital Medical University, 4 Tian-Tan-Xi-Li, Beijing 100050, China. sunzheng12@vip.126.com; Xiaoxin Luke Chen, Cancer Research Program, Julius L. Chambers Biomedical Biotechnology Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707, USA. lchen@nccu.edu; Zhaohui Xiong, Cancer Research Program, Julius L. Chambers Biomedical Biotechnology Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707, USA. zxiong@nccu.edu;.

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 the 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 the GEO Series accession number 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).

No conflicts of interest were declared

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

## Introduction

Alcohol drinking is a known risk factor for esophageal diseases, e.g., gastroesophageal reflux disease (GERD), 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 >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 to 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 associate 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 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 drinkers' esophagus was significantly lower than that in non-drinkers' esophagus. Mechanistically, we found promoter hypermethylation was associated with PAX9 silencing in human tissues and 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, *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, 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 >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 System, 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 System) 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) 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) was used to rapidly assess activities of 45 signaling pathways in KYSE510 cells according to the manufacture's protocol. In brief, reporter constructs resident in each well of Cignal Finder array plate were resuspended with 50 μl Opti-MEM medium (Thermo Fisher) and then mixed with 50 μl diluted Attractene transfection reagent (Qiagen). KYSE510 cell suspension was diluted in Opti-MEM medium and then seeded in each well (50,000 cells/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*;*N*1$^{fl/fl}$;*N*2$^{fl/fl}$) 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/day, *i.p.* for 5 d) 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 (1:40000, 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 for overnight. Tissue sections were then washed in PBS and incubated with biotinylated secondary antibodies for 30 minutes at room temperature. Detection of the antibody complex was done 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 interest genes with relevant primers and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in an 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 were 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 a RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Microarray experiments were performed with Agilent SurePrint G3 Human GE v2 8×60K 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, 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 activities of signaling pathways in KYSE510 and KYSE410 cells *in vitro*

Using the Cignal Finder 45-Pathway Reporter Array, we found 20 signaling activities were significantly impacted in KYSE510 cells exposed to ethanol (100 mM for 24 h, n=4/group). Among these pathways, RBPJ, c-MYC, and STAT3 signaling activities were inhibited and SOX2 was activated (Figure 1A). Subsequent western blotting confirmed the down-regulation 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/group; supplementary material, Table S2). Significance Analysis of Microarrays (SAM) revealed that 577 genes were up-regulated and 952 genes down-regulated in ethanol-exposed cells as compared with control. A number of genes associated with squamous differentiation were among the down-regulated genes including *AQP3, KRT1, KRT4, KRT13, KRT14, KRT23, KRT24, KRT31, 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; 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, *NFκB* pathway, and GSA_TF upregulation of *NFκB* and *SMAD4*, in ethanol-exposed samples. These data suggested that ethanol exposure inhibited the 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 PAX9 regulates squamous differentiation in the esophagus [13]. RBPJ ChIP-seq also suggested *PAX9* as a potential downstream transcriptional target in neural stem cells [19]. Therefore, we hypothesized PAX9 as a potential NOTCH effector in esophageal squamous epithelial cells as well. Western blotting showed that JAG1 (a NOTCH ligand) and NICD1 overexpression up-regulated 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 up-regulation 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 down-regulation 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 were 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 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 down-regulation 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 to 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).

### 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 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 down-regulated in the ethanol-exposed squamous epithelium as compared with control, especially in those exposed to 15% ethanol for 40 weeks (Figure 5A). Gene microarray data (GSE75373) was 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 the esophageal squamous epithelial cells was lower in drinkers' esophagus than non-drinkers' esophagus [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 down-regulation was observed in histologically normal esophageal squamous epithelium of heavy liquor drinkers as compared to those from the non-drinkers (Figure 6; supplementary material, Figure S5), suggesting that heavy liquor drinking is associated with NOTCH inhibition.

Moreover, the analysis of microarray data of two GEO datasets (GSE23400, GSE20347) showed significant down-regulation of *PAX9* and *HES1* mRNA expression in human ESCC (supplementary material, Figure S6A,B,D,E), as 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*.

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 like HES1 [27]. In the normal esophageal epithelium of rodents and humans, NOTCH1, NOTCH2, and NOTCH3 are highly expressed whereas NOTCH4 is expressed at a 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], 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, 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 resulted in the 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 the 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], *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 *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 (Figure 3, 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 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 [53]. Ethanol's effects on molecular pathways also depend on the context. For example, WNT signaling was suppressed by ethanol in human neural stem cells [54] but activated in fetal human and mouse cerebral cortex [17]. NOTCH pathway was inhibited by ethanol in smooth muscle cells [55] but activated in fetal human and mouse cerebral cortex [17] and human coronary artery endothelial cells [52]. 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 NOTCH inhibition 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

WE thank Dr. Yan Shi and her staff, the Genomics Core Facility, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA for providing excellent microarray services. This work was supported by a Young Scientist Program from Beijing Stomatological Hospital, Capital Medical University (to MS, SR, YSP202006), a training grant from Beijing Stomatological Hospital, Capital Medical University (to MS, SR), and research grants from the National Institutes of Health (U54 AA019765, R21 AA028047, U54 CA156735, U54 MD012392).

## References

1. Richter JE, Rubenstein JH. Presentation and Epidemiology of Gastroesophageal Reflux Disease. Gastroenterology 2018; 154: 267–276. [PubMed: 28780072]
2. Ness-Jensen E, Lagergren J. Tobacco smoking, alcohol consumption and gastro-oesophageal reflux disease. Best Pract Res Clin Gastroenterol 2017; 31: 501–508. [PubMed: 29195669]
3. Bor S, Bor-Caymaz C, Tobey NA, et al. Esophageal exposure to ethanol increases risk of acid damage in rabbit esophagus. Dig Dis Sci 1999; 44: 290–300. [PubMed: 10063914]
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019; 69: 7–34. [PubMed: 30620402]

5. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394–424. [PubMed: 30207593]

6. Bagnardi V, Blangiardo M, La Vecchia C, et al. A meta-analysis of alcohol drinking and cancer risk. Br J Cancer 2001; 85: 1700–1705. [PubMed: 11742491]

7. Morita M, Kumashiro R, Kubo N, et al. Alcohol drinking, cigarette smoking, and the development of squamous cell carcinoma of the esophagus: epidemiology, clinical findings, and prevention. Int J Clin Oncol 2010; 15: 126–134. [PubMed: 20224884]

8. Wu C, Kraft P, Zhai K, et al. Genome-wide association analyses of esophageal squamous cell carcinoma in Chinese identify multiple susceptibility loci and gene-environment interactions. Nat Genet 2012; 44: 1090–1097. [PubMed: 22960999]

9. Maier H, Weidauer H, Zoller J, et al. Effect of chronic alcohol consumption on the morphology of the oral mucosa. Alcohol Clin Exp Res 1994; 18: 387–391. [PubMed: 8048743]

10. Simanowski UA, Suter P, Stickel F, et al. Esophageal epithelial hyperproliferation following long-term alcohol consumption in rats: effects of age and salivary gland function. J Natl Cancer Inst 1993; 85: 2030–2033. [PubMed: 8246289]

11. Poschl G, Seitz HK. Alcohol and cancer. Alcohol Alcohol 2004; 39: 155–165. [PubMed: 15082451]

12. Liu Y, Chen H, Sun Z, et al. Molecular mechanisms of ethanol-associated oro-esophageal squamous cell carcinoma. Cancer Lett 2015; 361: 164–173. [PubMed: 25766659]

13. Xiong Z, Ren S, Chen H, et al. PAX9 regulates squamous cell differentiation and carcinogenesis in the oro-oesophageal epithelium. J Pathol 2018; 244: 164–175. [PubMed: 29055049]

14. Peters H, Neubuser A, Kratochwil K, et al. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 1998; 12: 2735–2747. [PubMed: 9732271]

15. McGlinn E, van Bueren KL, Fiorenza S, et al. Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. Mech Dev 2005; 122: 1218–1233. [PubMed: 16169709]

16. Zhao H, Oka K, Bringas P, et al. TGF-beta type I receptor Alk5 regulates tooth initiation and mandible patterning in a type II receptor-independent manner. Dev Biol 2008; 320: 19–29. [PubMed: 18572160]

17. Hashimoto-Torii K, Kawasawa YI, Kuhn A, et al. Combined transcriptome analysis of fetal human and mouse cerebral cortex exposed to alcohol. Proc Natl Acad Sci U S A 2011; 108: 4212–4217. [PubMed: 21368140]

18. Ong CT, Cheng HT, Chang LW, et al. Target selectivity of vertebrate notch proteins. Collaboration between discrete domains and CSL-binding site architecture determines activation probability. J Biol Chem 2006; 281: 5106–5119. [PubMed: 16365048]

19. Li Y, Hibbs MA, Gard AL, et al. Genome-wide analysis of N1ICD/RBPJ targets in vivo reveals direct transcriptional regulation of Wnt, SHH, and hippo pathway effectors by Notch1. Stem Cells 2012; 30: 741–752. [PubMed: 22232070]

20. Xu J, Chi F, Guo T, et al. NOTCH reprograms mitochondrial metabolism for proinflammatory macrophage activation. J Clin Invest 2015; 125: 1579–1590. [PubMed: 25798621]

21. Kamakura S, Oishi K, Yoshimatsu T, et al. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat Cell Biol 2004; 6: 547–554. [PubMed: 15156153]

22. Palomero T, Lim WK, Odom DT, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A 2006; 103: 18261–18266. [PubMed: 17114293]

23. Watanabe H, Ma Q, Peng S, et al. SOX2 and p63 colocalize at genetic loci in squamous cell carcinomas. J Clin Invest 2014; 124: 1636–1645. [PubMed: 24590290]

24. Kulic I, Robertson G, Chang L, et al. Loss of the Notch effector RBPJ promotes tumorigenesis. J Exp Med 2015; 212: 37–52. [PubMed: 25512468]

25. Hatch E, Morrow D, Liu W, et al. Ethanol inhibits gamma-secretase proteolytic activity in vascular smooth muscle cells. Alcohol Clin Exp Res 2015; 39: 2115–2122. [PubMed: 26443551]

26. Song K, Coleman RA, Zhu X, et al. Chronic ethanol consumption by mice results in activated splenic T cells. J Leukoc Biol 2002; 72: 1109–1116. [PubMed: 12488491]

27. Borggrefe T, Oswald F. The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell Mol Life Sci 2009; 66: 1631–1646. [PubMed: 19165418]
28. Sander GR, Powell BC. Expression of notch receptors and ligands in the adult gut. J Histochem Cytochem 2004; 52: 509–516. [PubMed: 15034002]
29. Zhang Y, Yang Y, Jiang M, et al. 3D Modeling of Esophageal Development using Human PSC-Derived Basal Progenitors Reveals a Critical Role for Notch Signaling. Cell Stem Cell 2018; 23: 516–529 e515. [PubMed: 30244870]
30. Ohashi S, Natsuizaka M, Yashiro-Ohtani Y, et al. NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. Gastroenterology 2010; 139: 2113–2123. [PubMed: 20801121]
31. Ohashi S, Natsuizaka M, Naganuma S, et al. A NOTCH3-mediated squamous cell differentiation program limits expansion of EMT-competent cells that express the ZEB transcription factors. Cancer Res 2011; 71: 6836–6847. [PubMed: 21890822]
32. Blanpain C, Lowry WE, Pasolli HA, et al. Canonical notch signaling functions as a commitment switch in the epidermal lineage. Genes Dev 2006; 20: 3022–3035. [PubMed: 17079689]
33. Wang X, Pasolli HA, Williams T, et al. AP-2 factors act in concert with Notch to orchestrate terminal differentiation in skin epidermis. J Cell Biol 2008; 183: 37–48. [PubMed: 18824566]
34. Tadeu AM, Horsley V. Notch signaling represses p63 expression in the developing surface ectoderm. Development 2013; 140: 3777–3786. [PubMed: 23924630]
35. Restivo G, Nguyen BC, Dziunycz P, et al. IRF6 is a mediator of Notch pro-differentiation and tumour suppressive function in keratinocytes. EMBO J 2011; 30: 4571–4585. [PubMed: 21909072]
36. Wakabayashi N, Skoko JJ, Chartoumpekis DV, et al. Notch-nrf2 axis: regulation of nrf2 gene expression and cytoprotection by notch signaling. Mol Cell Biol 2013; 34: 653–663. [PubMed: 24298019]
37. Wakabayashi N, Shin S, Slocum SL, et al. Regulation of notch1 signaling by nrf2: implications for tissue regeneration. Sci Signal 2010; 3: ra52. [PubMed: 20628156]
38. Meyers JM, Spangle JM, Munger K. The human papillomavirus type 8 E6 protein interferes with NOTCH activation during keratinocyte differentiation. J Virol 2013; 87: 4762–4767. [PubMed: 23365452]
39. Liu K, Jiang M, Lu Y, et al. Sox2 cooperates with inflammation-mediated Stat3 activation in the malignant transformation of foregut basal progenitor cells. Cell Stem Cell 2013; 12: 304–315. [PubMed: 23472872]
40. Osei-Sarfo K, Tang XH, Urvalek AM, et al. The molecular features of tongue epithelium treated with the carcinogen 4-nitroquinoline-1-oxide and alcohol as a model for HNSCC. Carcinogenesis 2013; 34: 2673–2681. [PubMed: 23784083]
41. McLaughlin KA, Rones MS, Mercola M. Notch regulates cell fate in the developing pronephros. Dev Biol 2000; 227: 567–580. [PubMed: 11071775]
42. Hamidi H, Gustafason D, Pellegrini M, et al. Identification of novel targets of CSL-dependent Notch signaling in hematopoiesis. PLoS One 2011; 6: e20022. [PubMed: 21637838]
43. Onuma Y, Takahashi S, Asashima M, et al. Conservation of Pax 6 function and upstream activation by Notch signaling in eye development of frogs and flies. Proc Natl Acad Sci U S A 2002; 99: 2020–2025. [PubMed: 11842182]
44. Wen Y, Bi P, Liu W, et al. Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. Mol Cell Biol 2012; 32: 2300–2311. [PubMed: 22493066]
45. Jayasena CS, Ohyama T, Segil N, et al. Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. Development 2008; 135: 2251–2261. [PubMed: 18495817]
46. Yu XM, Jaskula-Sztul R, Ahmed K, et al. Resveratrol induces differentiation markers expression in anaplastic thyroid carcinoma via activation of Notch1 signaling and suppresses cell growth. Mol Cancer Ther 2013; 12: 1276–1287. [PubMed: 23594881]
47. Harris RA, Trudell JR, Mihic SJ. Ethanol's molecular targets. Sci Signal 2008; 1: re7. [PubMed: 18632551]

48. Guo Y, Wang X, Zhang X, et al. Ethanol promotes chemically induced oral cancer in mice through activation of the 5-lipoxygenase pathway of arachidonic acid metabolism. Cancer Prev Res (Phila) 2011; 4: 1863–1872. [PubMed: 21881027]

49. Odera JO, Xiong Z, Huang C, et al. NRF2/ACSS2 axis mediates the metabolic effect of alcohol drinking on esophageal squamous cell carcinoma. Biochem J 2020; 477: 3075–3089. [PubMed: 32776152]

50. Mews P, Egervari G, Nativio R, et al. Alcohol metabolism contributes to brain histone acetylation. Nature 2019; 574: 717–721. [PubMed: 31645761]

51. Voordeckers K, Colding C, Grasso L, et al. Ethanol exposure increases mutation rate through error-prone polymerases. Nat Commun 2020; 11: 3664. [PubMed: 32694532]

52. Morrow D, Hatch E, Hamm K, et al. Flk-1/KDR mediates ethanol-stimulated endothelial cell Notch signaling and angiogenic activity. J Vasc Res 2014; 51: 315–324. [PubMed: 25322777]

53. Filiberti RA, Fontana V, De Ceglie A, et al. Alcohol consumption pattern and risk of Barrett's oesophagus and erosive oesophagitis: an Italian case-control study. Br J Nutr 2017; 117: 1151–1161. [PubMed: 28478792]

54. Vangipuram SD, Lyman WD. Ethanol affects differentiation-related pathways and suppresses Wnt signaling protein expression in human neural stem cells. Alcohol Clin Exp Res 2012; 36: 788–797. [PubMed: 22150777]

55. Morrow D, Cullen JP, Liu W, et al. Alcohol inhibits smooth muscle cell proliferation via regulation of the Notch signaling pathway. Arterioscler Thromb Vasc Biol 2010; 30: 2597–2603. [PubMed: 20930168]

56. Ahlgren SC, Thakur V, Bronner-Fraser M. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. Proc Natl Acad Sci U S A 2002; 99: 10476–10481. [PubMed: 12140368]

57. Chan IS, Guy CD, Machado MV, et al. Alcohol activates the hedgehog pathway and induces related procarcinogenic processes in the alcohol-preferring rat model of hepatocarcinogenesis. Alcohol Clin Exp Res 2014; 38: 787–800. [PubMed: 24164383]

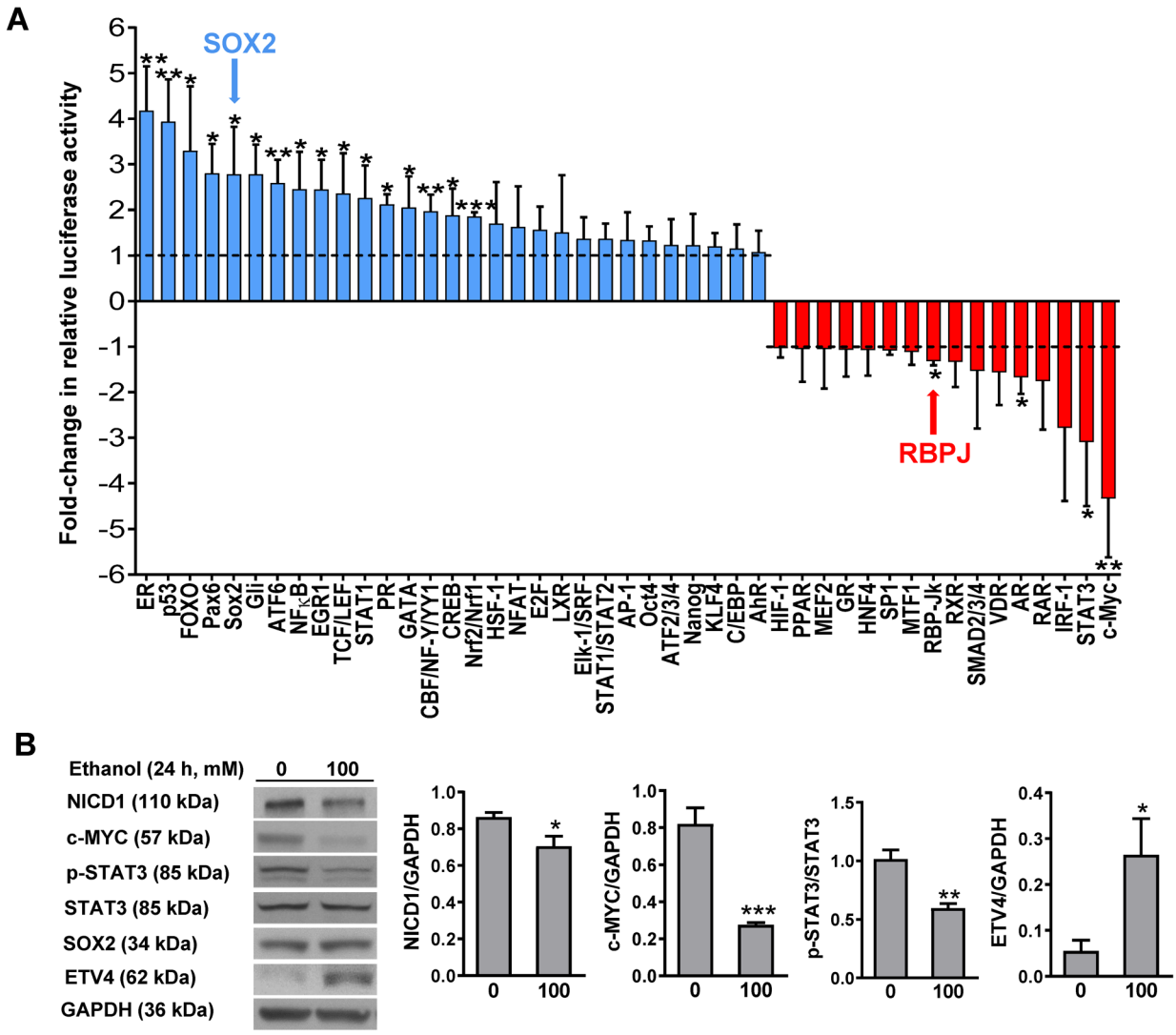


**Figure 1. Multiple signaling activities in KYSE510 cells are modulated by ethanol.**

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 represented mean ± SD after triplicate experiments. *P* values were determined using the Student's t-test.

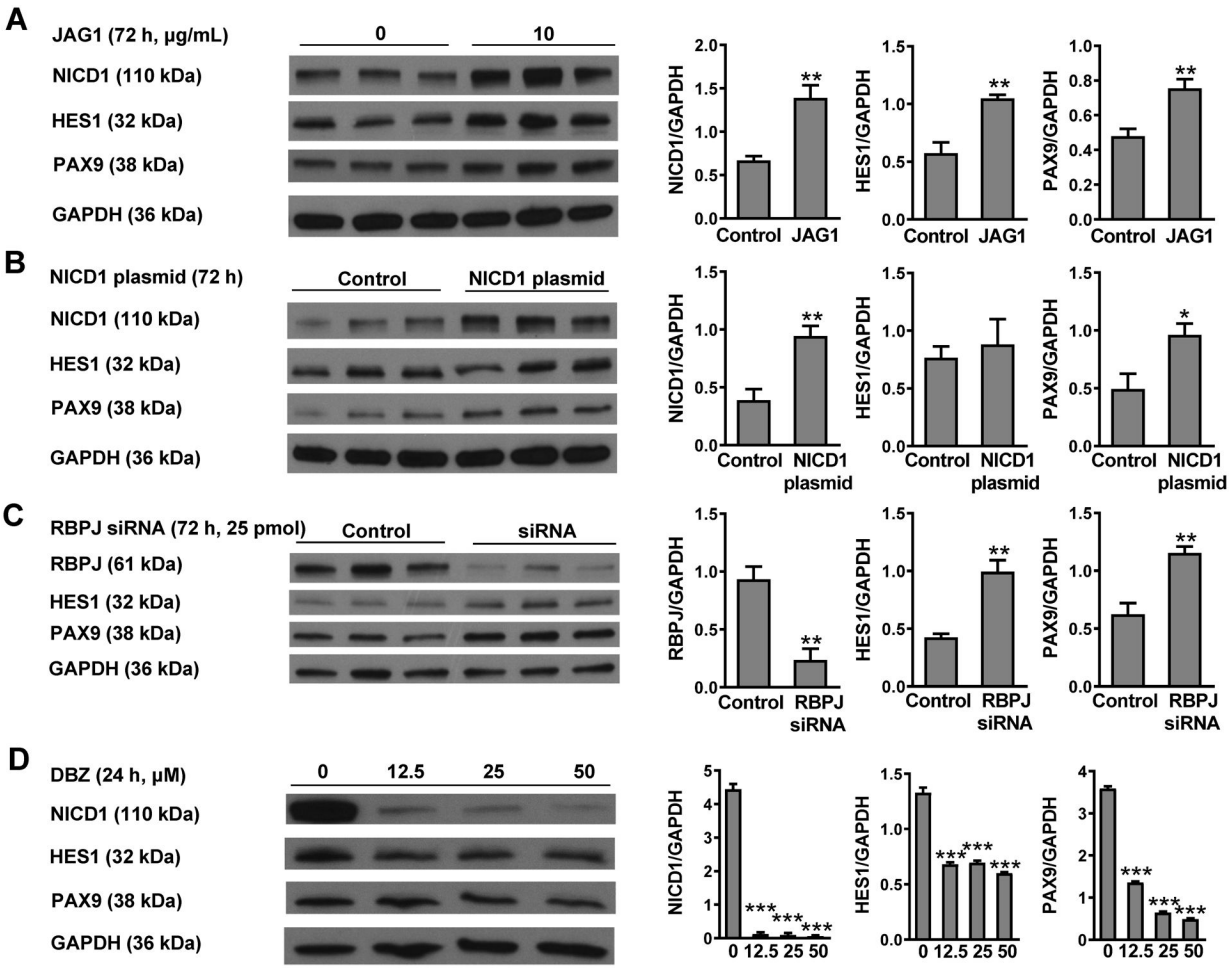


**Figure 2. NOTCH perturbations modulate PAX9 expression in KYSE510 cells *in vitro*.**

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 semi-quantitated for statistical analysis. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Bar represented mean ± SD. *P* values were determined using the Student's t-test.

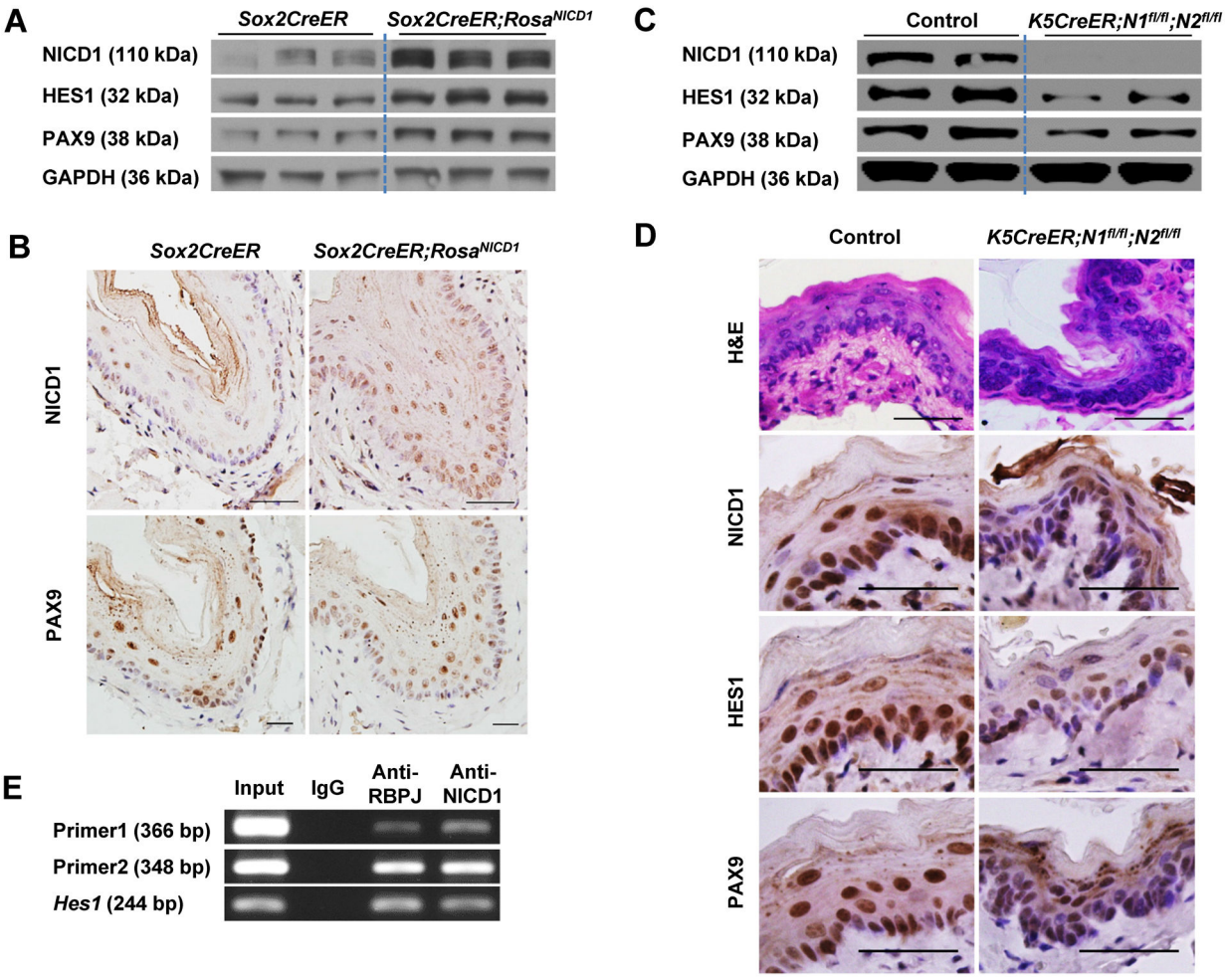


**Figure 3. NICD1 perturbations modulate PAX9 expression in esophageal squamous epithelial cells *in vivo*.**

Western blotting (A, C) and IHC staining (B, D) showed overexpression of NICD1 and PAX9 in *Sox2*CreER;*Rosa*$^{NICD1}$ esophagus (n=3) and down-regulation of NICD1 and PAX9 in *K5*CreER;*N1*$^{fl/fl}$;*N2*$^{fl/fl}$ esophagus (n=3) as 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 aligned samples of the groups. $^{*}p < 0.05$, $^{**}p < 0.01$. Scale bar=50 μm.

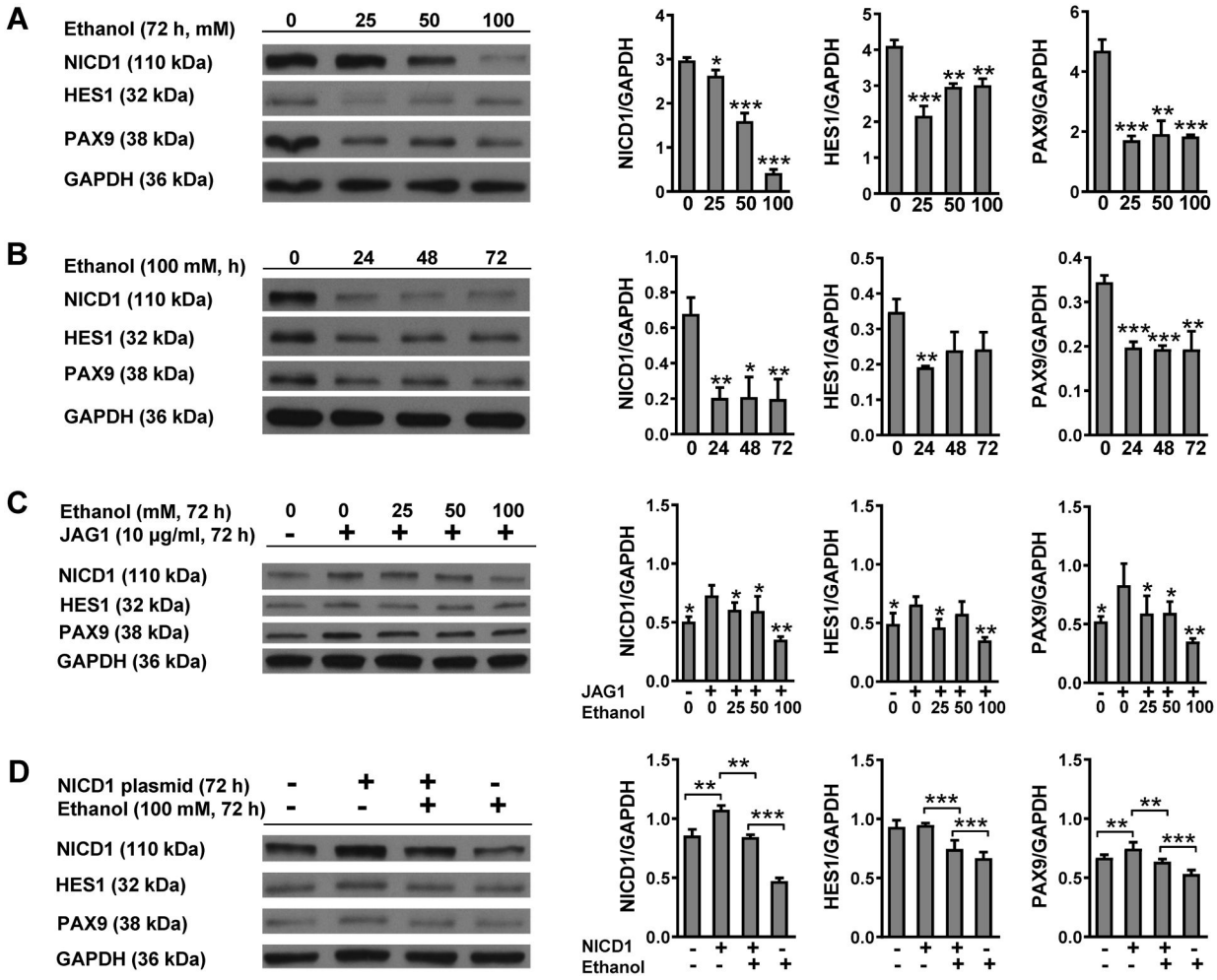


**Figure 4. Ethanol exposure inhibits NOTCH signaling in KYSE510 cells *in vitro*.**

Whole-cell lysates from KYSE510 cells exposed to different concentrations of ethanol at various time points (A, B), JAG1 and/or ethanol (C), 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 semi-quantitated for statistical analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bar represented mean ± SD. *P* values were determined using the Student's t-test.

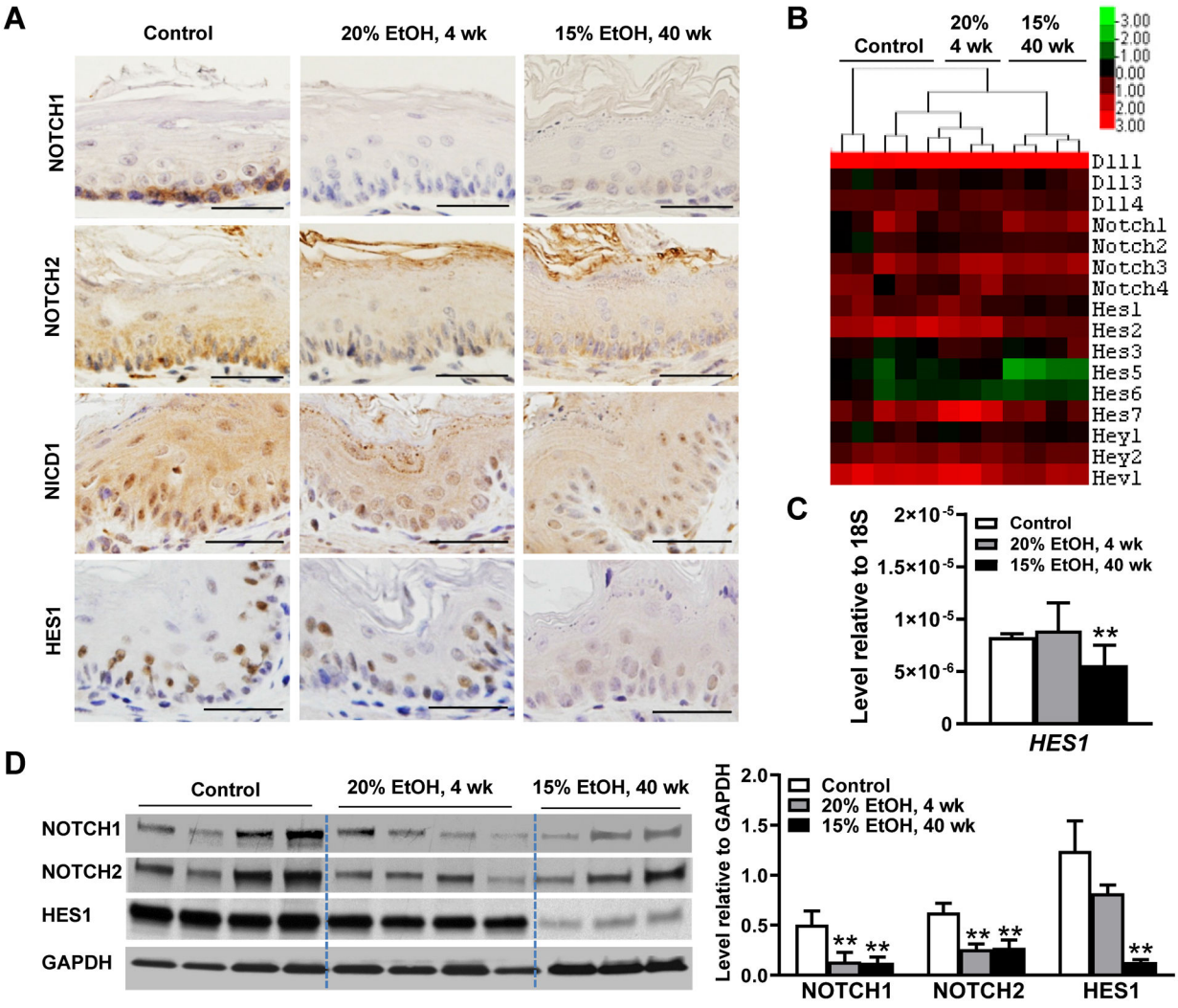


**Figure 5. Ethanol exposre inhibits NOTCH signaling in the mouse squamous epithelium.**

IHC staining showed expression of NOTCH1, NOTCH2, NICD1, and HES1 in the ethanol-exposed mouse squamous epithelium as 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 represented mean ± SD (n=3/group). Scale bar=50 μm. *P* values were determined using the Student's t-test.

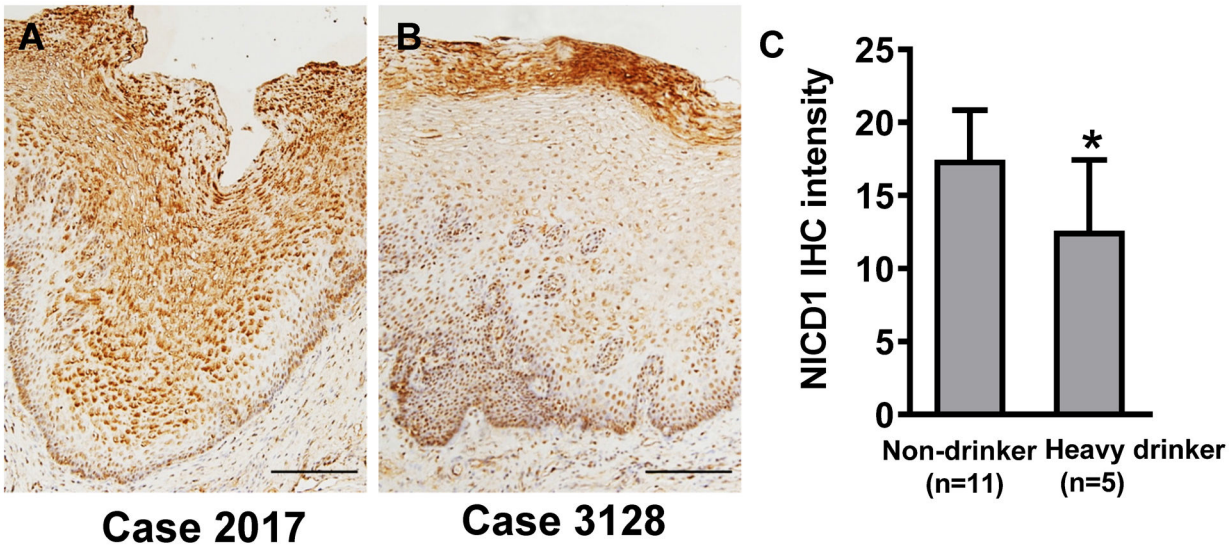


**Figure 6. Down-regulation of NICD1 in histologically normal esophageal squamous epithelium of heavy liquor drinkers and non-drinkers.**

NICD1 IHC is shown for a representative case of non-drinker (A) and a representative case of 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 represented mean ± SD. Scale bar=100 μm. *P* values were determined using the Student's t-test.