

## SUPPLEMENTARY

### Extended Materials and Methods

**Liquid Ethanol Diet.** Male Wild type (Wt) C57BL/6J mice (8-9-week-old) (n=20) were fed a control liquid diet (Lieber-DeCarli-shake and pour Bio-Serve, Inc. diet # F1259SP) for 5 days of acclimation. After 5 days, mice were randomly assigned to either remain on a liquid control diet (n=8) (pair-fed), or to an ethanol (EtOH) liquid diet (n=12) (Lieber-DeCarli-shake Bio-Serve, #F1258SP) for 5 days of a 1-2% v/v EtOH diet acclimation. After 5 days, EtOH-fed mice were switched to either 5% EtOH v/v plus vehicle [0.1% DMSO] (EtOH-treated, n=6), or 5% EtOH plus fenretinide (FEN (Apexbio, Inc) at 10 mg/kg/day (EtOH+Fen, n=6). A cohort of liquid control-fed mice were switched to Fen 10 mg/kg/day (pair-fed + Fen, n=4). All control liquid diet-fed mice were pair-fed to the mean 24-hour intake of 5% EtOH-treated mice. All groups remained on their diets for 25 days and had diets prepared fresh daily. After 25 days mice were anesthetized by 100 ul intraperitoneal injection with 100 mg/kg Ketamine/10 mg/kg Xylazine, and then sacrificed by cervical dislocation.

### **Intestinal Periodic Acid–Schiff, Alcian Blue Goblet Cell Staining and Mucosal Thickness Quantitation.**

Intestinal tissue from 2 cm portions of ileum and colon sections were fixed in buffered 4% paraformaldehyde (PFA, 1X phosphate buffered saline, pH 7.4) for 24 hours at 4 °C. Tissue were then paraffin embedded and sectioned (5 µM) and mounted on glass slides. Periodic acid–schiff (PAS) and alcian blue (AB, pH 2.5) goblet cell staining was performed on deparaffinized and rehydrated ileum and colon tissue sections using PAS or AB staining kit according to the manufacturer's protocol (Poly Scientific, Inc.). Approximately 10 PAS or AB positive regions of interest (ROI) fields per slide were captured using a Nikon Ts2-inverted microscope. With 1 slide per mouse, and 4-6 mice per dietary group, a total of 40-60 PAS and AB positive fields per experimental group were analyzed and quantified by color deconvolution densitometry analysis using Fiji ImageJ software [50]. *Mucosal thickness analysis:* Analysis of ileum and colon adherent mucosal layer thickness in PFA fixed intestinal tissue was

completed as previously described [19]. For each mouse, 10-15 random PAS positive mucosal fields were measured from outermost surface of ileum and colonic villi to luminal surface of the columnar epithelial cells using the distance measuring tool in Nikon NIS Elements image analysis software (Nikon, Inc).

**Immunohistochemistry and immunofluorescence microscopy.** Immunohistochemistry (IHC) was performed as previously described [16]. Deparaffinized and rehydrated intestinal ileum and colon sections were incubated with the following primary antibodies overnight at 4°C: MUCIN-2 (1:100, Rabbit polyclonal, #GTX100664, GenTex, Inc.), NICD (1:50, Rabbit mAb #4147, Cell Signaling, Inc.), SPDEF (1:50, Rabbit polyclonal, # 11467-1, Proteintech, Inc.), KLF4 (1:100, Goat polyclonal, #AF3158, R&D Inc), DLL1 (1:25, Sheep polyclonal, AF3970, R & D, Inc.), followed by incubations with either HRP- (SuperBoost Goat anti-Rabbit Poly HRP, Thermo, Inc), or fluorescent-conjugated secondary antibodies, (1:500, anti-goat, anti-rabbit or anti-sheep, IgG, Alexa Fluor 488 and 594, Thermo, Inc). Slide images were photographed using a Nikon Ts2-inverted fluorescent microscope. HRP-conjugated antibodies were visualized with 3,3'-diaminobenzidine (DAB). Approximately 10 FITC-488, TRITC-594, and DAB tissue positive regions of interest (ROI) fields per slide were captured using a Nikon Ts2-inverted fluorescent microscope. With 1 slide per mouse, and 4-6 mice per dietary group, a total of 40-60 FITC-488, TRITC-594, and DAB positive fields per experimental group were analyzed and quantified by fluorescence or color deconvolution densitometry analysis using Fiji ImageJ software [50].

**RNA Isolation and quantitative PCR.** Total RNA was extracted and purified from 2 cm portions of intestinal ileum and colon samples using the RNeasy Mini Kit (Qiagen, Inc). RNA quality and concentrations were determined using a NanoDrop One (Thermo, Inc) and cDNA synthesis libraries were constructed with 1 µg of total RNA using RevertAid First Strand cDNA Synthesis Kit. Quantitative PCR (qPCR) was performed as previously described [16] using SYBR Green PCR master mix on an Agilent

Mx3000P Real Time qPCR system (Agilent, Inc.). Gene specific primers (Table 1) were used to amplify target mRNAs that were normalized to the internal control mRNA, *36B4*.

**Statistical Analysis.** Group means differences are reported as means  $\pm$  SD and were analyzed using repeated measures ANOVA and Dunnett's multiple comparison test. Group means were treated as independent variables and computed using ANOVA and Dunnett's multiple comparison test when sample numbers were not equal. Significant differences were defined as p-values of less than 0.05, and all usage of the term "significant" throughout the text refers to means differences with a  $p < 0.05$ . All statistical analyses were performed using GraphPad Prism version 8.0 statistical software (GraphPad Software, Inc).

### Supplementary Figure Legends

#### Supplementary Figure 1.

**Alcohol and Fenretinide do not affect canonical Wnt or other intestinal cell types.** Quantitative PCR (qPCR) histograms of A-C) ileal, and G-I) colonic relative mRNA levels of canonical Wnt target genes *Axin2*, *Tcf4* *Lgr5*. qPCR histograms of D), E) ileal, and J), K) colonic relative mRNA levels of the enterocyte marker (Keratin 19, *Krt19*) and Paneth cell marker (Lysozyme, *Lyz1*). F) and L) qPCR histograms of ilea. and colonic relative mRNA levels of presenilin 1 (*Psen1*), a major subunit membrane gamma- ( $\gamma$ -) secretase. All histogram errors bars represent  $\pm$ SD, with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant. qPCR results represent gene fold changes relative to pair-fed mice normalized against the housekeeping gene *36B4*.

**Supplementary Figure 2. Fenretinide prevents alcohol mediated reductions to gut Notch ligands, DLL1 and DLL4.** Quantitative PCR (qPCR) histograms of A), B) ileal, and C), D) colonic relative mRNA levels of notch ligands Delta-like proteins 1 and 4 (DLL1) and (DLL4). Representative immunofluorescence images E), and F) and G) and H) quantification of ileal, and colonic DLL1 (yellow arrows). Magnification:  $\times 100$ ; scale bar = 100  $\mu\text{m}$ . All histogram errors bars represent  $\pm\text{SD}$ , with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant.

**Supplementary Figure 3. Fenretinide does not modulate gut retinoid signaling.** Quantitative PCR (qPCR) histograms of A) and C) ileal, and B) and D) colonic relative mRNA levels of canonical retinoic acid receptor (RAR) target genes RAR $\beta$ , and cytochrome P450 26a1 (*Cyp26a1*). All histogram errors bars represent  $\pm\text{SD}$ , with \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ns = not significant. qPCR results represent gene fold changes relative to pair-fed mice normalized against the housekeeping gene 36B4.

**Supplementary Figure 4.** Model of modulation of goblet cells and mucosal thickening by Alcohol and Alcohol plus Fenretinide. Fenretinide (Fen) treatments prevent alcohol mediated suppression of gut Notch ligands Delta-like proteins 1 and 4 (DLL1) and (DLL4). Maintenance of canonical Notch signaling by Fen, suppresses goblet cell specification genes such as *Spdef*, and goblet synthesis of mucin 2.