

## Supplementary Information

### Alcohol metabolism enriches squamous cell carcinoma cancer stem cells that survive oxidative stress via autophagy

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#### Supplementary Methods

##### *Genotyping for ALDH2 single nucleotide polymorphism*

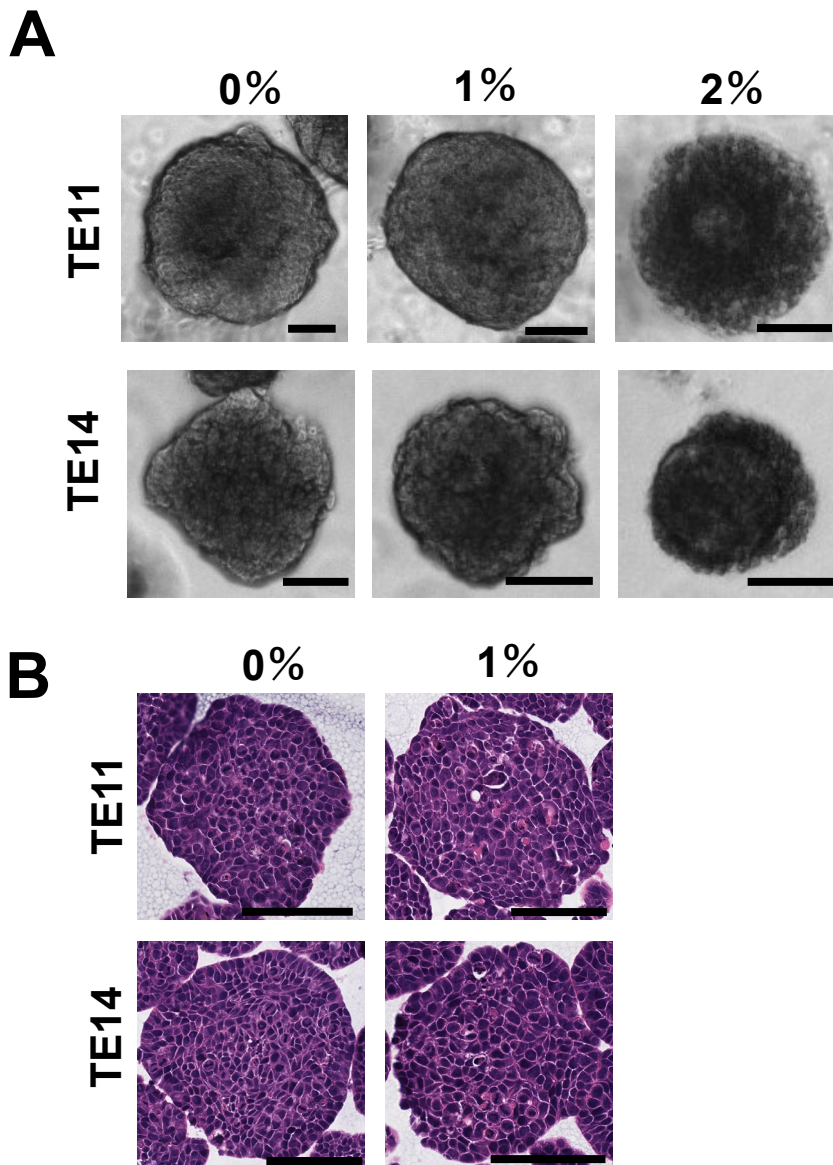
G Genomic DNA was isolated from TE11, TE14, ESC2-3, and HSC1-3 lines using the DNeasy Blood and Tissue Kit (Qiagen, #69504). Genotyping for the ALDH2\*2 single nucleotide polymorphism (rs671) was performed with the TaqMan drug metabolism genotyping assay (Thermo Fisher Scientific, #4351379) and TaqMan Genotyping Master Mix (Thermo Fisher Scientific, 4371353) using 10 ng of isolated genomic DNA per well. All reactions were carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems). Analysis was performed with the Applied Biosystems Genotyping Analysis Module using default settings on Thermo Fisher Cloud according to manufacturer's instructions.

##### *Immunoblot analysis*

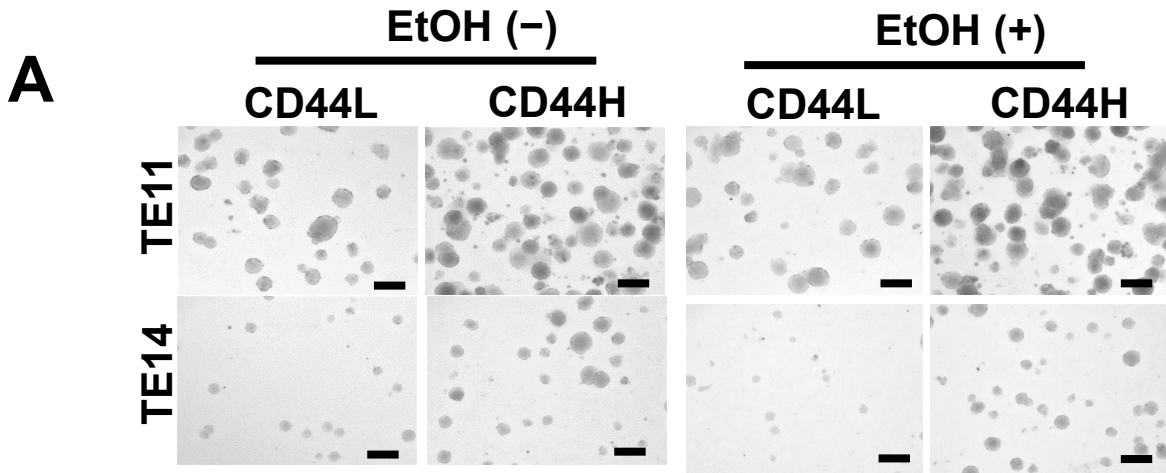
Immunoblot analysis of EtOH-exposed cells was performed as described previously (see reference [10] in the main text) utilizing anti-Phospho-p70 S6 Kinase (Thr389) (1:1000, #9205), anti-p70 S6 Kinase (1:1000, #9202), anti-Phospho-4E-BP1 (Ser65) (1:1000, #9451), anti-4E-BP1 (1:1000, #9452), and anti- $\beta$ -Actin (1:1000, #4967) as primary antibodies. Densitometry of resulting signals was performed with Image Lab software (Bio-Rad). The signal intensity for molecule of interest was calibrated by that of  $\beta$ -actin.

#### Supplementary Table S1 ALDH2 SNP genotypes in ESCC cell lines and patient-derived organoid lines

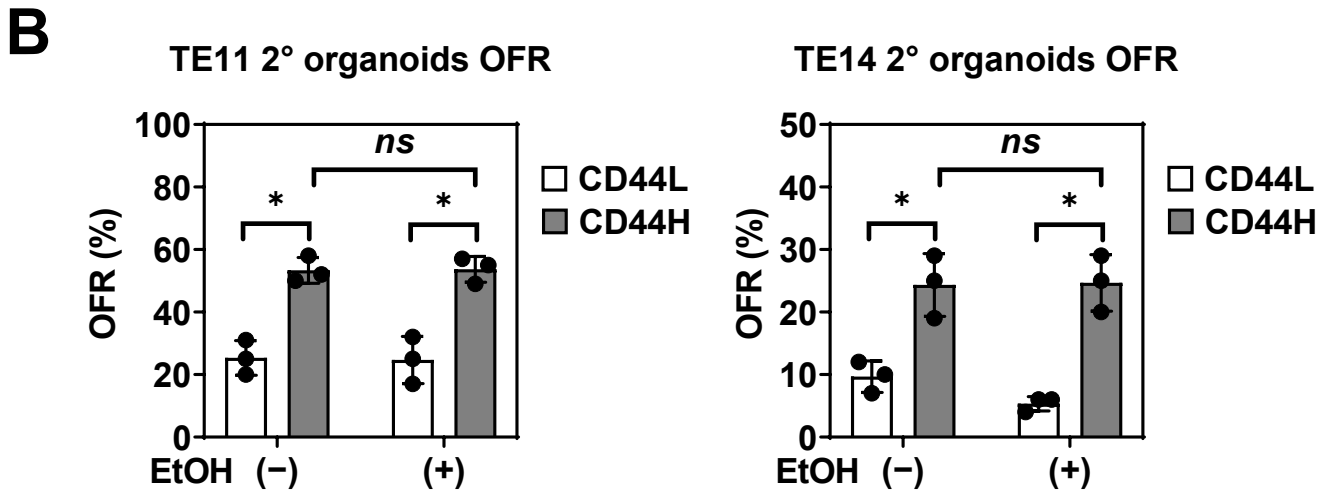
| Name | ALDH2 allele    | Characterization             |
|------|-----------------|------------------------------|
| ESC2 | ALDH2*1/ALDH2*1 | Homozygous (wildtype)        |
| ESC3 | ALDH2*1/ALDH2*1 | Homozygous (wildtype)        |
| HSC1 | ALDH2*1/ALDH2*1 | Homozygous (wildtype)        |
| HSC2 | ALDH2*1/ALDH2*1 | Homozygous (wildtype)        |
| HSC3 | ALDH2*1/ALDH2*1 | Homozygous (wildtype)        |
| TE11 | ALDH2*1/ALDH2*2 | Heterozygous (wildtype, SNP) |
| TE14 | ALDH2*1/ALDH2*2 | Heterozygous (wildtype, SNP) |



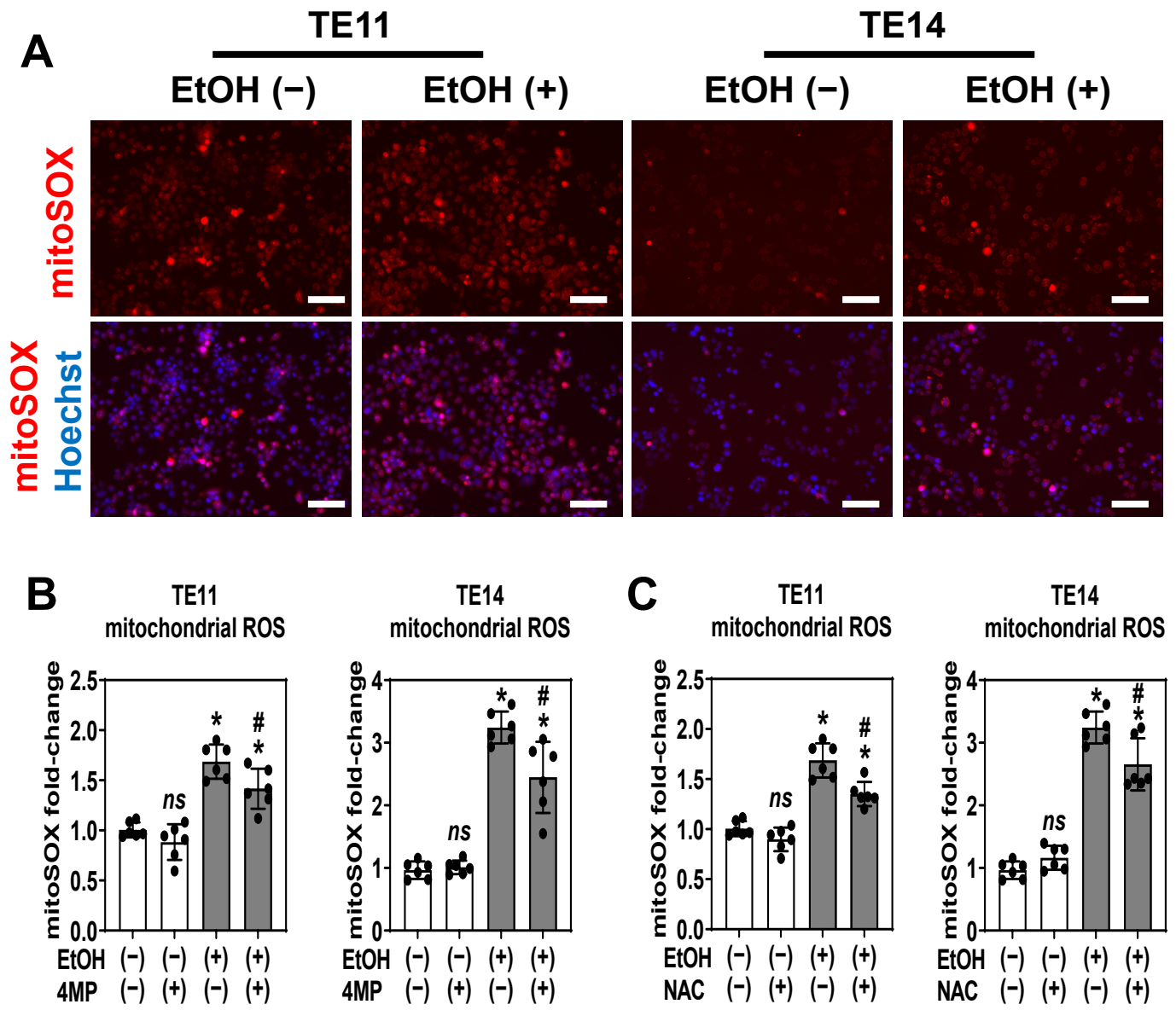
**Supplementary Figure S1      1% EtOH maintains viable 1° organoid structure.**  
 3D organoids generated with TE11 and TE14 cells were treated with or without EtOH at indicated concentrations for 4 days, starting from day 7. Phase-contrast images (A) and H&E staining images (B) of representative organoids are shown. Scale bar: 100  $\mu$ m. Note that viable structures were recovered for H&E staining when organoids were treated with 2% EtOH in (B). There are many organoids with no or few apoptotic cells in no-treatment control while most organoids have many apoptotic cells after EtOH treatment. Note that phase-contrast images were more obscure in organoids treated with 2% EtOH in (A). Viable structures were not recovered when organoids were treated with 2% EtOH in (B), suggesting that 2% EtOH impaired cell viability more severely than 1% EtOH.



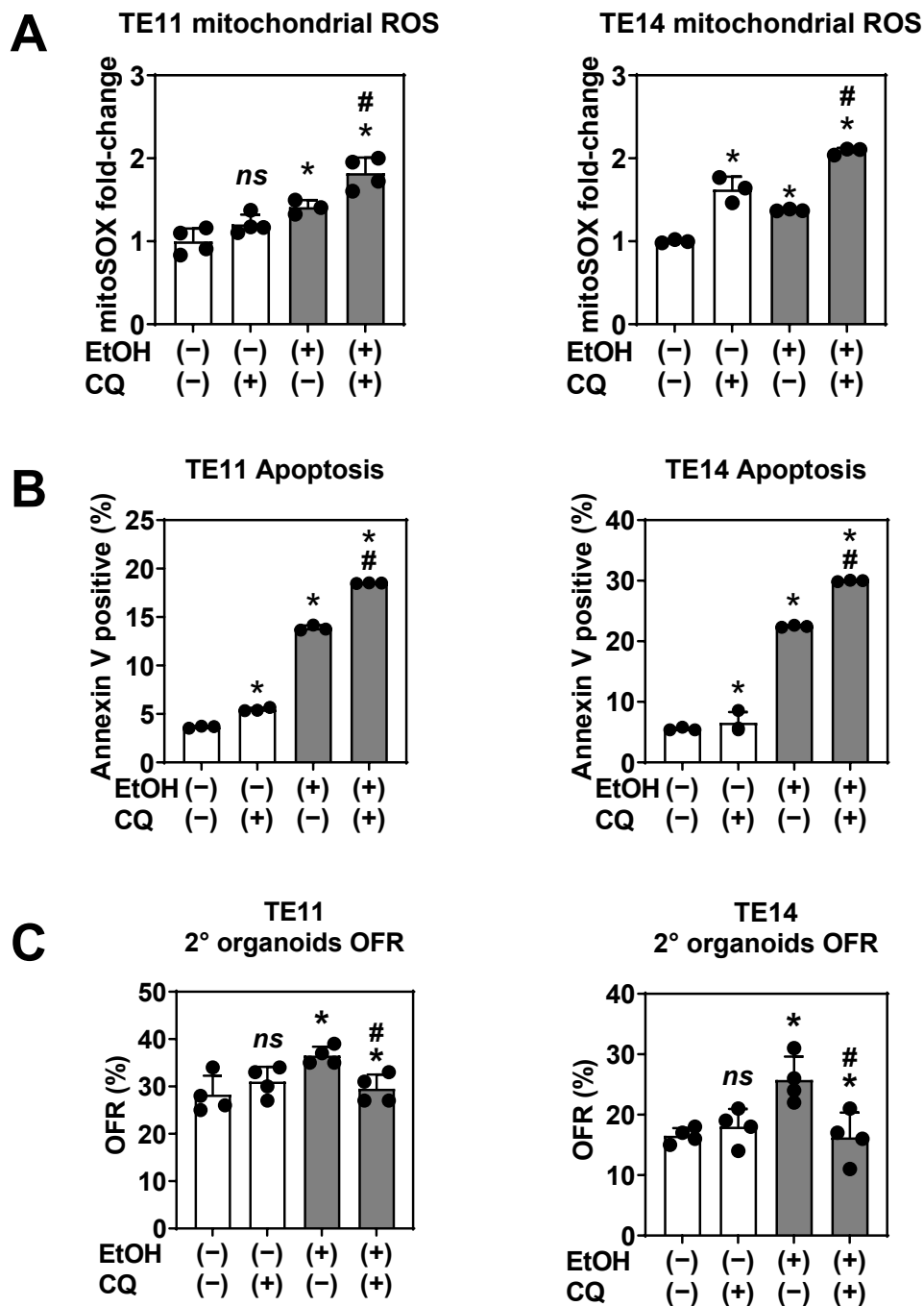
2° organoids grown in the absence of EtOH



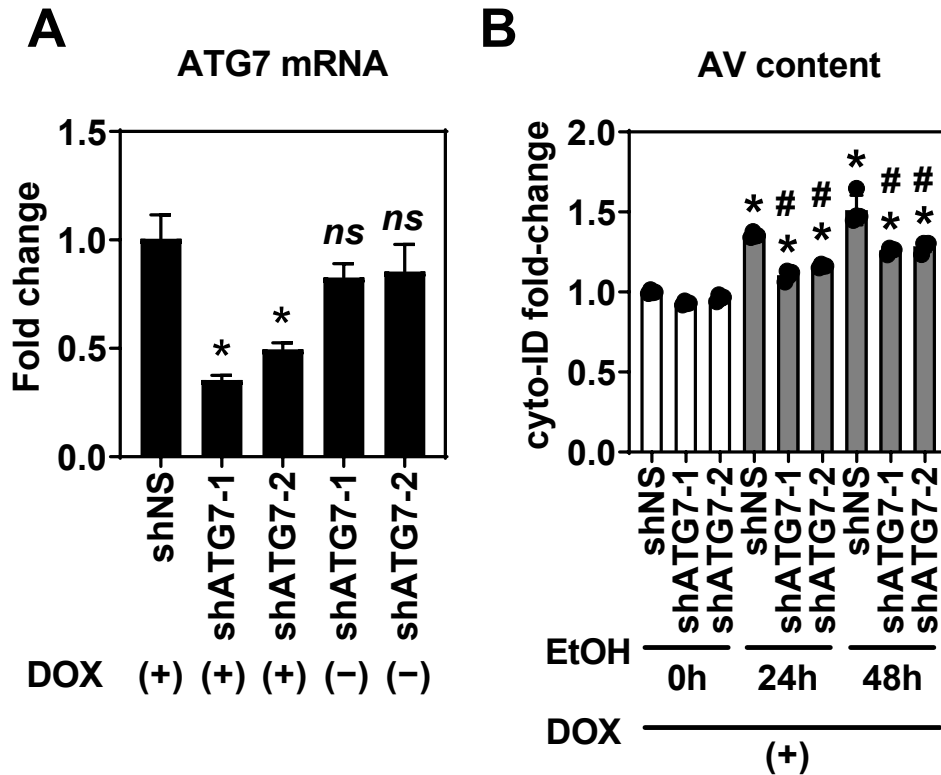
**Supplementary Figure S2 Characterization of FACS-purified CD44L and CD44H cells isolated from primary organoids treated with or without EtOH (A, B)** TE11 and TE14 organoids were treated with or without 1% EtOH for 4 days in 1° organoid. FACS-purified CD44L and CD44H cells of 1° organoids were passaged to grow 2° organoids in sub-culture (A). OFRs of 2° organoids were determined and plotted in bar graphs (B). Scale bar: 100  $\mu$ m. *ns*, not significant vs. CD44H in EtOH (-); \*,  $p < 0.05$  vs. CD44L.  $n=3$ .



**Supplementary Figure S3 ADH-mediated EtOH oxidation and oxidative stress are associated with increased mitochondrial superoxide production in EtOH-exposed SCC cells.** TE11 and TE14 cells were cultured in monolayer culture for 24 hours and treated with 1% EtOH for 6 hours with or without 2 mM of 4MP treatment (B) or 10 mM of NAC pretreatment (C). Relative mitochondrial ROS levels were measured by Keyence microscope. (A) Scale bar: 100  $\mu$ m. (B) *ns*, not significant vs. EtOH (-) and 4MP (-); \*,  $p < 0.05$  vs. EtOH (-) and 4MP (-); #,  $p < 0.05$  vs. EtOH (+) and 4MP (-).  $n=6$ . (C) *ns*: not significant vs. EtOH (-) and NAC (-); \*,  $p < 0.05$  vs. EtOH (-) and NAC (-); #,  $p < 0.05$  vs. EtOH (+) and NAC (-).  $n=6$ .

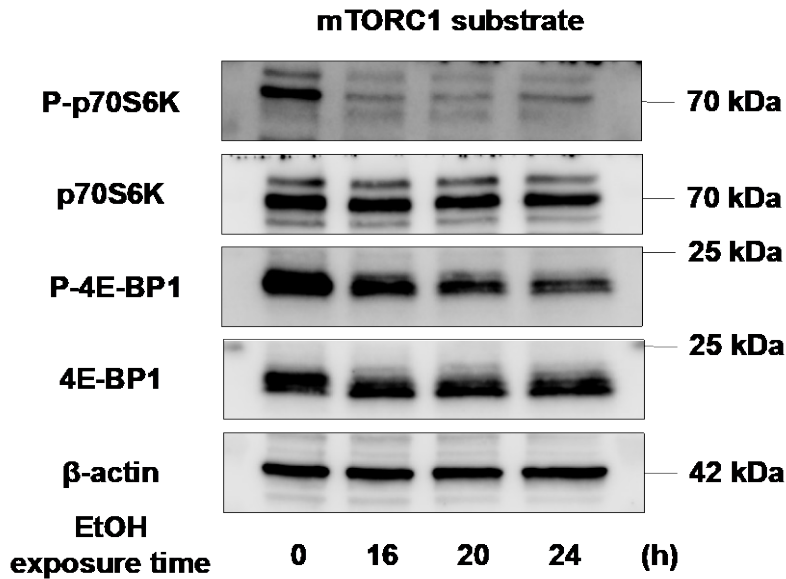
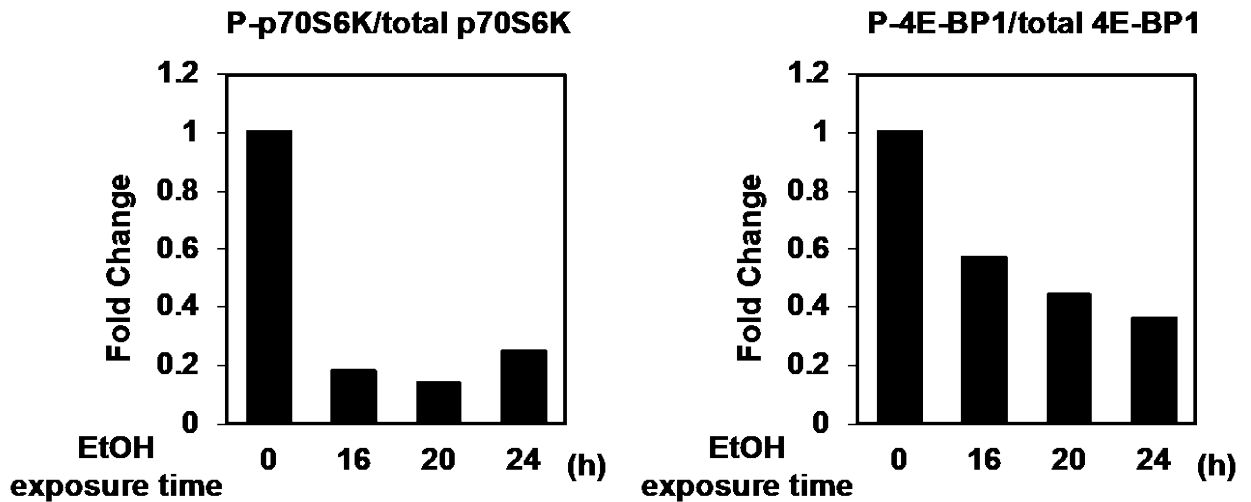


**Supplementary Figure S4 Autophagy limits EtOH-induced mitochondrial superoxide production and apoptosis to mediate CD44H cell enrichment.** (A) TE11 and TE14 cells were cultured in monolayer for 24 hours and treated with 1% EtOH for 6 h along with or without 2  $\mu$ M CQ. Relative mitochondrial ROS levels were evaluated by Keyence microscope. (B, C) TE11 and TE14 organoids were treated with 1% EtOH for 4 days along with or without 2  $\mu$ M CQ. Dissociated organoids were analyzed by Flow cytometry for Annexin V positive cells to determine apoptosis as shown in (B). 2° organoids were grown in the absence of EtOH and OFR was determined as plotted in graphs (C). *ns*, not significant vs. EtOH (-) and CQ (-); \*,  $p < 0.05$  vs. EtOH (-) and CQ (-); #,  $p < 0.05$  vs. EtOH (+) and CQ (-).  $n=4$  in (A) TE11 and (C),  $n=3$  in (A) TE14 and (B).

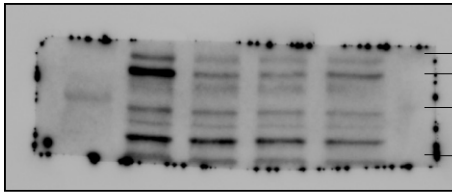


**Supplementary Figure S5 ATG7 knockdown by shRNA decreases EtOH-induced autophagy in SCC cells.** (A, B) TE11 cells with indicated genotypes were treated with or without DOX for 48 h to induce shRNA in monolayer culture. Without DOX treatment, shRNA did not affect ATG7 mRNA expression levels or ATG7 protein expression levels. (A) ATG7 mRNA expression levels were confirmed by qPCR. \*,  $p < 0.05$  vs. shNS.  $n=3$ . (B) TE11 cells with indicated genotypes were treated with 1% EtOH along with DOX in monolayer culture. Harvested cells were analyzed by flow cytometry to determine the AV content. \*,  $p < 0.05$  vs. shNS in 0 h EtOH; #,  $p < 0.05$  vs. shNS in 24 h or 48 h EtOH treatment.  $n=3$ .

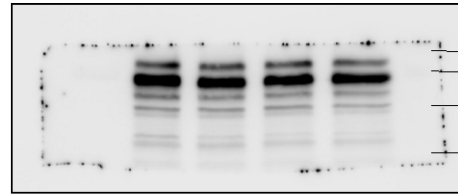


**A****B**

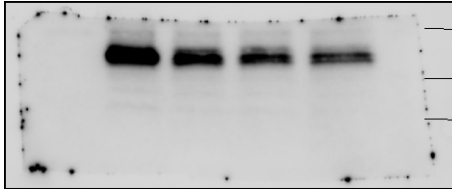
**Supplementary Figure S7 EtOH may suppress mTORC1 signaling in TE14 cells.** (A and B) TE14 cells were treated with 1% EtOH for indicated time periods and subjected to immunoblot analysis to determine phosphorylation of mTORC1 substrates p70 S6 kinase (p70 S6K) and 4E-BP1 proteins with  $\beta$ -actin serving as a loading control. Densitometry determined phosphorylation of p70 S6K (P-p70 S6K) and 4E-BP1 (P-4E-BP1) relative to total p70 S6K and 4E-BP1 proteins, respectively, in (B). Immunoblots represent one of two independent experiments with similar results. See **Supplementary Figure S8** for uncropped immunoblot images.



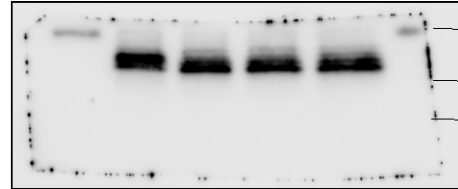
**P-p70S6K**



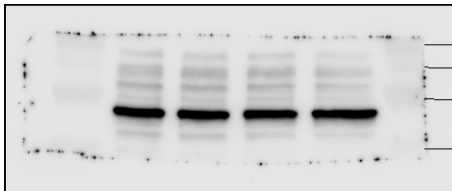
**p70S6K**



**P-4E-BP1**



**4E-BP1**



**$\beta$ -actin**

Supplementary Figure S8 Un cropped immune blot images corresponding to  
Supplementary Figure S7