## Materials and methods

**Materials:** The Taqman Gene expression master mix, Amplex UltraRed soluble fluorophore, Akt Pathway Total and Phospho 7-Plex panels, and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore were purchased from Invitrogen (Carlsbad, CA). ERK1/2 and $^{pT202/Y204}$-ERK single-plex bead-based assays were purchased from EMD Millipore (Billerica, MA). QIAzol Lysis Reagent for RNA extraction was obtained from Qiagen, Inc (Valencia, CA). MaxiSorp plates, and OptiPlates (96-well) used for enzyme-linked immunosorbent assays (ELISAs) were from Thermo Fisher Scientific (Rochester, NY). SuperBlock TBS, bicinchoninic assay reagents, and enhanced chemiluminescence (ECL) solutions were purchased from Pierce Chemical Company (Rockford, IL). Mouse monoclonal anti-ceramide and myriocin were purchased from Sigma-Aldrich Co (St. Louis, MO). Rabbit polyclonal antibody to large acidic ribosomal protein (RPLPO) was purchased from Proteintech Group, Inc (Chicago, IL). The A85E6 and A85G6 mouse monoclonal antibodies to AAH were generated, purified, and characterized as previously described [29, 30]. All other antibodies and immunodetection reagents for ELISAs were purchased from Abcam (Cambridge, MA), Upstate (Billerica, MA), Invitrogen (Carlsbad, CA), or Chemicon (Temecula, CA). Fine chemicals were purchased from CalBiochem (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO).

**In vivo experimental model:** Adult male Long Evans rats (Harlan Sprague Dawley, Inc., Indianapolis, Indiana) were pair-fed with isocaloric liquid diets (BioServ, Frenchtown, NJ) containing 0% or 37% (caloric content; 9.2% v/v) ethanol for 8 weeks [4]. Two weeks prior to initiating the study, rats were adapted to the liquid diets by incrementing ethanol from 0% to 11.8%, 23.6% and then 37% of the caloric content. Controls were adapted to ethanol-free liquid diets over the same period. After 3 weeks on the full liquid diets, rats in each group were treated on Mondays, Wednesdays, and

Fridays by intraperitoneal (i.p.) injection of myriocin (0.3 mg/kg) or saline. The myriocin and vehicle treatments were continued through the last 5 weeks of the study. Rats were monitored daily to ensure adequate nutritional intake and maintenance of body weight. Rats were housed under humane conditions and kept on a 12-hour light/dark cycle with free access to food. All experiments were performed in accordance with protocols approved by Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital, and they conform to guidelines established by the National Institutes of Health.

**Liver tissue processing:** At the end of the experiment, rats were fasted overnight, and then sacrificed by isoflurane inhalation. Livers were divided for freezing and fixation. Fresh tissue samples were snap-frozen in a dry ice/methanol bath and stored at -80°C for protein, ceramide, and RNA studies. Alternatively, tissue samples were immersion fixed in 4% buffered paraformaldehyde and embedded in paraffin. Histologic sections (5 μm thick) were stained with Hematoxylin and Eosin (H&E). Cryostat sections (10 μm thick) of paraformaldehyde fixed, non-embedded tissues were stained with Oil Red-O (ORO) to assess hepatic steatosis. Steatosis severity across the entire tissue sample (1 $cm^2$) was graded under code and at 100x magnification as follows: 0= not detectable staining; 1= staining confined to a 3-4 cell thick radius of hepatocytes in Zone 3; 2= hepatocyte ORO staining extends up to Zone 2; 3=ORO staining extends into and somewhat beyond Zone 2; 4= panlobular ORO staining. Inter-group comparisons were made by ANOVA tests with post hoc Tukey tests.

**Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays:** RNA was extracted from frozen liver using the RNeasy Mini Kit. Total RNA was reverse transcribed with random oligonucleotide primers and the AMV $1^{st}$ Strand cDNA Synthesis Kit. Gene expression was measured using a hydrolysis probe-based duplex qRT-PCR assay with β-actin as a reference gene as previously described [46]. Gene-specific primers and matched probes were determined with the ProbeFinder Software

(Roche, Indianapolis, IN). PCR amplifications were performed in a LightCycler 480 machine (Roche, Indianapolis, IN) and results were analyzed using the LightCycler® Software 4.0.

**Enzyme-linked immunosorbent assay (ELISA):** Immunoreactivity was measured by direct binding duplex ELISAs, and normalized to large acidic ribosomal protein (RPLPO) measured in the same wells [5]. Liver tissue homogenates were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, 0.1% Triton X-100, and protease and phosphatase inhibitors [5]. Protein concentration was measured with BCA reagents. Direct binding ELISAs were performed in 96-well MaxiSorp plates. Protein homogenates (100 ng/50 µl) were adsorbed to the well bottoms by overnight incubation at 4°C, and then blocked for 3 hours with 1% bovine serum albumin (BSA) in TRIS buffered saline (TBS). After washing, the samples were incubated with primary antibody (0.1-0.4 µg/ml) for 1 hour at 37°C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and Amplex UltraRed soluble fluorophore. Fluorescence intensity was measured (Ex 565 nm/Em 595 nm) in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Subsequently, the samples were incubated with biotin-conjugated antibodies to RPLPO, and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore. Fluorescence (Ex 360 nm/Em 450 nm) intensity was measured in a SpectraMax M5 microplate reader. Binding specificity was determined from parallel negative control incubations in which the primary or secondary antibody was omitted. The ratios of specific protein/RPLPO were calculated and used for inter-group statistical comparisons. Ceramide immunoreactivity was quantified by ELISA as previously described [5, 47].

**Bead-based multiplex ELISA:** Liver protein homogenates (100 µg samples) prepared as described earlier, were used to assess signaling through insulin and IGF-1 receptors, IRS-1, and Akt pathways with the Akt Total and Phospho 7-Plex Panels. The Akt Total 7-Plex panel measured: insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3β (GSK-3β). The Akt Phospho 7-Plex panel measured: $^{pYpY1162/1163}$-IR, $^{pYpY1135/1136}$-IGF-1R, $^{pS312}$-IRS-1, $^{pS473}$-Akt, $^{pT246}$-PRAS40, $^{pTpS421/424}$-p70S6K, and $^{pS9}$-GSK3β. Total ERK1/2 and $^{pT202/Y204}$-ERK1/2 were measured in single-plex assays. Immunoreactivity was measured in a BioPlex 200 (BioRad, Hercules, CA).

**Statistical analysis:** Data depicted in box plots reflect group medians (horizontal bar), 95% confidence interval limits (upper and lower box limits) and range (whiskers). Tabulated data reflect means ± SEMs for each group. Data (N=6/group) were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA), and inter-group comparisons were made using repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. Computer software generated P-values are shown within the graph panels or table.

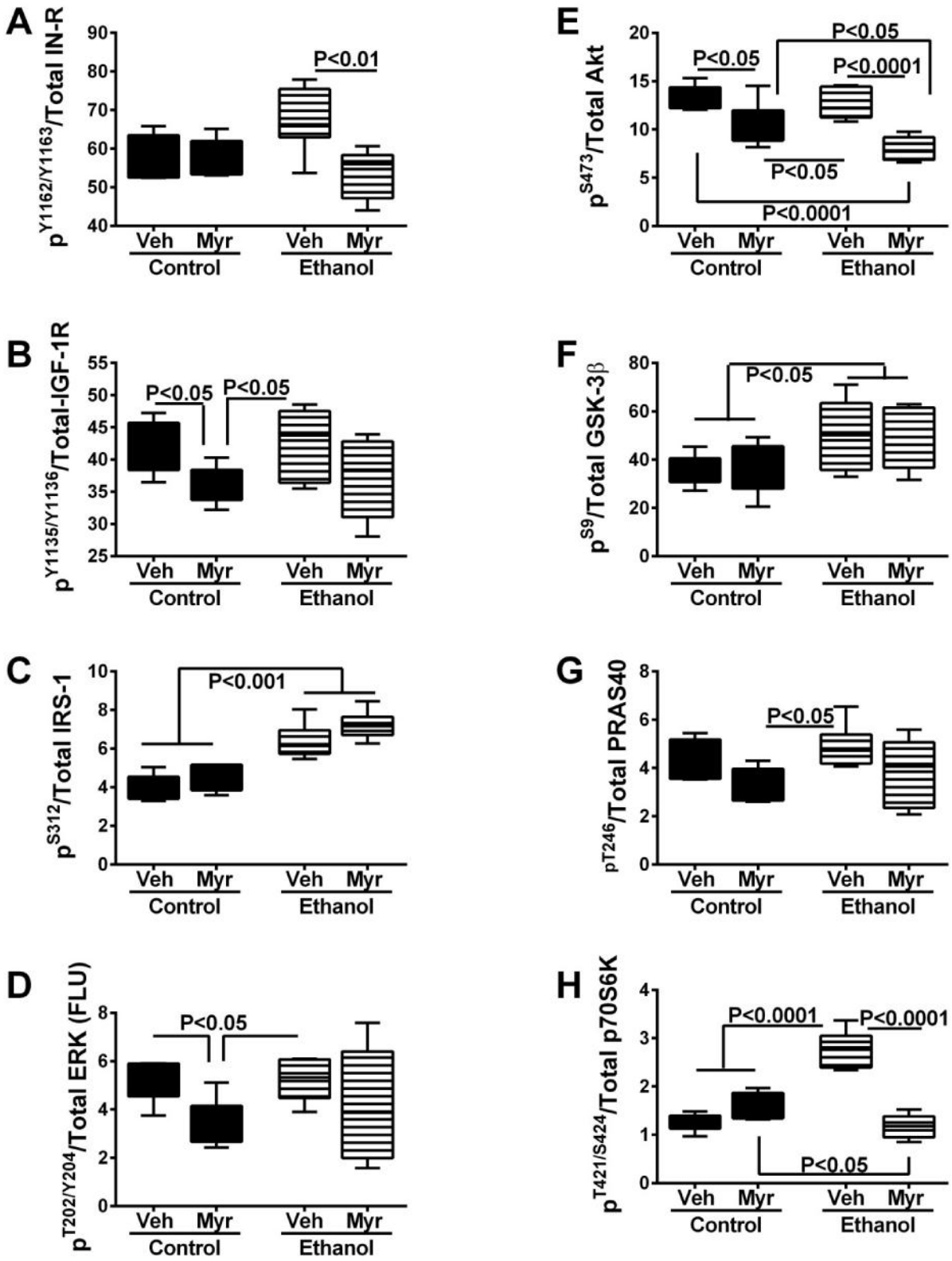
Supplementary Table 1: Effects of ethanol and myriocin on hepatic lipids*

| Lipid | C+V | C+M | Et+V | Et+M | F-Ratio | P-Value |
|---|---|---|---|---|---|---|
| ORO Grade | 2.0 ± 0.0 | 1.25 ± 0.16 | 3.75 ± 0.16 | 1.13 ± 0.29 | 53.01 | <0.0001 |
| Triglycerides | 8.4 ± 0.6 | 7.8 ± 0.5 | 7.3 ± 0.9 | 5.5 ± 0.7 | 2.76 | 0.066 |
| Nile Red | 1027 ± 53.4 | 1227 ± 120.4 | 1045 ± 53.1 | 997.7 ± 77.1 | 1.65 | 0.19 |
| Ceramide | 195651 ± 10301 | 198474 ± 14901 | 296502 ± 18904** | 176868 ± 11409 | 14.65 | <0.0001 |

Hepatic Oil red O (ORO) staining was graded on in histological sections using a semi-quantitative scale from 0 to 4 (see Supplementary Methods). Triglycerides and nile red fluorescence were measured in fresh frozen tissue using commercial assays. Ceramide immunoreactivity was measured by ELISA. Values corresponding to triglycerides, nile red, and ceramides reflect relative fluorescence units (arbitrary) normalized to protein content in the samples. *C+V = control + vehicle; Et + V = chronic ethanol fed + vehicle treatment. M= myriocin treatment (see Methods). **Post hoc Tukey's multiple comparison test demonstrated significantly higher ceramide levels in Et+V livers compared with all other groups (P<0.001).

**Supplementary Figure 1: Chronic ethanol feeding and myriocin treatment effects on relative levels of phosphorylated insulin, IGF and IRS signaling molecules in liver**. Liver tissue from control and chronic ethanol-fed rats that were treated with vehicle (Veh) or myriocin (Myr) was used to assess relative activation of insulin/IGF-1/IRS upstream and downstream signaling networks. Graphs depict the calculated ratios of (A) $^{pYpY1162/1163}$/total insulin receptor (R), (B) $^{pYpY1135/1136}$/total IGF-1R, (C) $^{pS312}$/total IRS-1, (D) $^{pT202/Y204}$/total ERK1/2, (E) $^{pS473}$/total Akt, (F) $^{pS9}$/total GSK3β, (G) $^{pT246}$/ total PRAS40, and (H) $^{pT246pS112}$ / total p70S6K measured by multiplex or single-plex ELISAs. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests of significance.

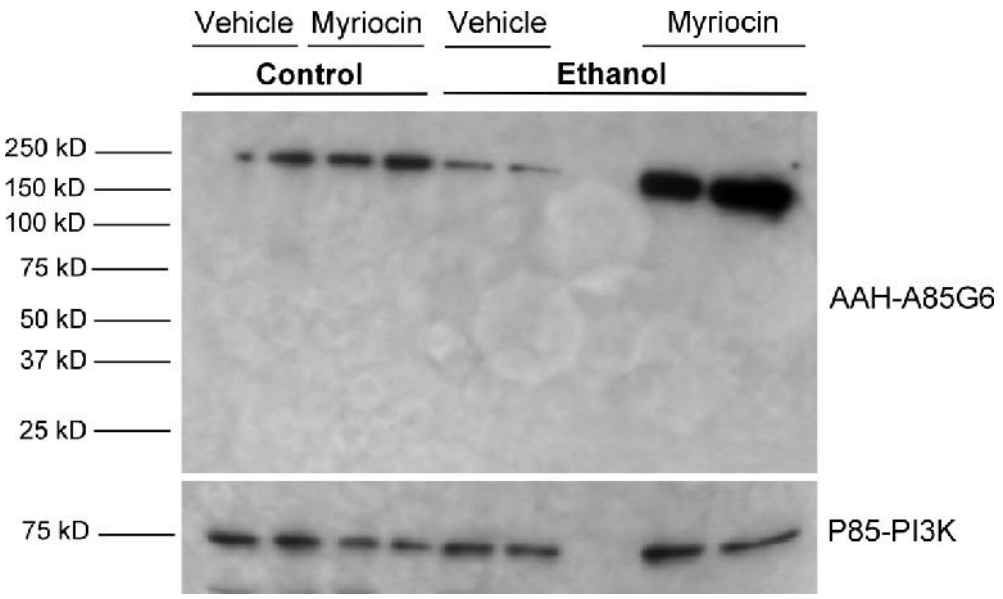
**Supplementary Figure 1**



**Supplementary Figure 2: Myriocin increases hepatic AAH expression in chronic ethanol-fed rats**. Adult male Long Evans rats were fed with isocaloric liquid diets containing 0% (control) or 37% ethanol (caloric content) for 8 weeks, and treated with vehicle or myriocin 3 times per week over the last 5 weeks of the experiment. Western blot analysis was performed with 30 µg liver protein homogenate per lane (measured using the BCA assay). Blots were probed with monoclonal antibodies to AAH (A85G6) or AAH/Humbug (A85E6). The latter recognizes the full-length AAH, Humbug, and N-terminal cleavage fragments of AAH. Immunoreactivity was revealed with HRP-conjugated secondary antibody, ECL reagents, and film autoradiography. Blots were re-probed with rabbit polyclonal antibodies to the p85 subunit of PI3 Kinase as a loading control. In A and B, the upper bands represent AAH, which migrate higher than the predicted ~86 kD due to post-translational modification by phosphorylation. The lower bands in B represent cleavage products of AAH or Humbug. Note sharply increased levels of AAH in myriocin-treated ethanol-exposed livers. Effects in control livers are subtle, corresponding to the already lower levels of ceramide in liver.

**Supplementary Figure 2**

A




B