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Hepatic Steatosis in Response to Acute Alcohol Exposure in Zebrafish requires Srebp Activation

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Abstract

Steatosis is the most common consequence of acute alcohol abuse and may predispose to more severe hepatic disease. Increased lipogenesis driven by the sterol response element binding protein (SREBP) transcription factors is essential for steatosis associated with chronic alcohol ingestion, but the mechanisms underlying steatosis following acute alcohol exposure are unknown. Zebrafish larvae represent an attractive vertebrate model for studying alcoholic liver disease (ALD), because they possess the pathways to metabolize alcohol, the liver is mature by 4 days post-fertilization (dpf), and alcohol can be simply added to their water. Exposing 4 dpf zebrafish larvae to 2% ethanol (EtOH) for 32 hours achieves ~80 mM intracellular EtOH and upregulation of hepatic *cyp2e1*, *sod* and *bip*, indicating that EtOH is metabolized and provokes oxidant stress. EtOH-treated larvae develop hepatomegaly and steatosis accompanied by changes in the expression of genes required for hepatic lipid metabolism. Based on the importance of SREPBs in chronic ALD, we explored the role of Srebps in this model of acute ALD. Srebp activation was prevented in *gonzo* larvae, which harbor a mutation in the membrane bound transcription factor protease 1 (*mbtps1*) gene, and in embryos injected with a morpholino to knock-down Srebp cleavage activating protein (*scap*). Both *gonzo* mutants and *scap* morphants were resistant to steatosis in response to 2% EtOH, and the expression of many Srebp target genes are down regulated in *gonzo* mutant livers.

Conclusion—Zebrafish larvae develop signs of acute ALD, including steatosis. Srebp activation is required for steatosis in this model. The tractability of zebrafish genetics provides a valuable tool for dissecting the molecular pathogenesis of acute ALD.

Keywords

liver; alcoholic liver disease; mbtps1; scap

Alcoholic Liver Disease (ALD) is among the most significant health problems associated with alcohol abuse: nearly 40% of alcohol related deaths in the U.S. are due to cirrhosis and liver failure (1). Chronic alcohol abuse can lead to severe liver disease whereas acute alcohol exposure causes transient steatosis (2). While steatosis is a generally benign and reversible outcome of binge drinking, it is also a precursor to developing steatohepatitis, fibrosis and cirrhosis associated with chronic ALD (3). Additionally, the consequences of transient, alcohol-induced steatosis may be amplified by genetic factors, as well as by other conditions associated with liver disease, such as viral infection or obesity (4,5).

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Chronic ethanol (EtOH) abuse leads to steatosis both due to decreased lipid oxidation resulting from impaired mitochondrial function (6,7) and to increased lipogenesis driven by the sterol regulatory element binding protein (SREBP) transcription factors (8). As outlined in Figure S1, SREBP activation occurs in response to low sterol concentrations, triggering the SREBP translocation from the endoplasmic reticulum (ER) to the Golgi via binding to SREBP cleavage activating protein (SCAP) (9,10). In the Golgi, SREBPs are cleaved by the membrane bound transcription factor proteases, MBTPS1 and MBTPS2 (also called site 1 and site 2 proteases) (11). This releases the active transcription factors to the nucleus where SREBP1 regulates genes essential for lipogenesis: acetyl-CoA carboxylase (*Acc1*) and fatty acid synthase (*Fasn*) and SREBP2 activates genes required for cholesterol biogenesis, including HMG CoA reductase (*Hmgcr*) and HMG CoA synthase (*Hmgcs1*). While SCAP is specific for the SREBPs, MBTPS1 and MBTPS2 also are required for activating the unfolded protein response and the hepatic acute phase response via cleavage of ATF6 (12) and CREBH (13) transcription factors, respectively (see Figure S1). This suggests a possible link between hepatic lipogenesis, ER stress and inflammation, each of which are central features of chronic ALD.

The pathophysiology of steatohepatitis caused by chronic alcoholism is well understood, whereas the mechanisms underlying steatosis following acute EtOH exposure are less clearly defined. The dearth of suitable animal models is a major obstacle to studying the genetic and cellular factors that contribute to hepatic pathology following acute EtOH exposure. Rodent models of ALD are informative for identifying key biochemical and cellular abnormalities after chronic EtOH. However, these models are time-consuming and costly (14) and, with the exception of chronic intragastric alcohol feeding (14), rodents are relatively resistant to ALD. This highlights the need for new approaches.

Among vertebrates, zebrafish have emerged as a powerful system because of its genetic tractability, optical clarity, and simple culture system (15). The large clutch size (number of offspring), rapid generation time and the ability to manipulate the externally developing zebrafish embryo allows for forward genetic screening, creation of transgenic fish, and transient knockdown of gene expression via morpholino injection (16). Zebrafish possess key enzymes that metabolize ethanol, including alcohol dehydrogenase (*adh3*) (17) aldehyde dehydrogenase (*aldh2*) (18) and cytochrome oxidase p450 2e1 (*cyp2e1*). Behavioral studies with adult and larval zebrafish exposed to alcohol (19-22) and the use of early zebrafish embryos to study fetal alcohol syndrome (23-28), demonstrate their utility for studying the effects of alcohol. None have yet reported using zebrafish to study ALD.

The *ex vivo* development of transparent zebrafish embryos allows detailed and dynamic analysis of embryonic lethal mutants. For example, homozygous deletion of *Mbtps1* in mice is embryonic lethal (29), requiring the creation of tissue-specific knock-out mice to study the role of this important gene in liver metabolism and steatosis (29). The zebrafish *mbtps1* mutant line, *gonzo* (*goz*), also do not survive to adulthood and die at 7 days post fertilization (dpf) (30,31). By 4 dpf, several measures of liver function are present, including the high expression of many hepatocyte-specific genes (32), bile secretion (33), glycogen storage and lipid metabolism (34,35). Thus, the role of *mbtps1* in hepatic physiology can be studied in *goz* mutants.

Here, we utilize zebrafish larvae to address the genetic basis of steatosis associated with acute ALD. Early zebrafish embryos are sustained by yolk, and although larvae are capable of feeding by 4 dpf (36), they can survive for several days without external nutrients. Thus 4-5 dpf zebrafish larvae present an ideal system to study the effects of alcohol on a mature liver without the metabolic changes introduced by nutrient metabolism. Exposing 4 dpf larvae to EtOH results in increased mortality, morphologic and behavioral abnormalities and

steatosis. Using *goz* mutants and morpholino knockdown of *scap*, we establish that the SREBP pathway is required for steatosis in this system. These findings provide mechanistic insight into the pathophysiology of acute ALD and present zebrafish as a new model for identifying genes that contribute to ALD.

EXPERIMENTAL PROCEEDURES

Animals

Adult wild type (WT) zebrafish and heterozygous carriers of the hi^{1487} allele (30,37)) of *mbtps1* (herein referred to as goz^{hi1487} were maintained on a 14:10 hour light:dark cycle at 28°C. Fertilized embryos collected following natural spawning were cultured at 28° C in fish water (0.6g/L Crystal Sea Marinemix-Marine Enterprises International: Baltimore, Maryland) containing methylene blue (0.002g/L). The Mount Sinai School of Medicine Institutional Animal Care and Use Committee approved all protocols.

Ethanol Treatment

At 4 dpf (between 96-98 hours post fertilization; hpf), 20-50 larvae were anesthetized with 2.5g/L tricane and transferred to 0, 0.5, 1, 2, 3, or 4% EtOH in fish water and cultured in sealed dishes for up to 32 hours. Each experiment was repeated on at least 3 clutches.

Tissue Alcohol Concentration Assay

Approximately 50 4 dpf larvae were treated with 0, 1 or 2% EtOH and collected at 8, 24, or 32 hours of EtOH exposure. Lavae were washed twice in phosphate buffered saline (PBS), homogenized on ice in 50 μl of PBS and pelleted by centrifugation. The supernatant was adjusted with PBS to equal 1 μl/larvae and EtOH concentration was quantified using the Ethanol Assay (Diagnostic Chemicals Limited: Oxford, CT). Briefly, the supernatant was incubated with alcohol dehydrogenase and nicotinamide adenine dinucleotide at room temperature. The UV absorbance at 340 nm was measured and the reading from untreated larvae at each time point was set as 0 mM. The concentration was calculated according to measurements of a standard containing 20 mM EtOH. Each sample was measured in triplicate and repeated on three clutches per time point.

Oil Red O Staining

Whole larvae were fixed with 4% paraformaldehyde, washed with PBS, infiltrated with a graded series of propylene glycol and stained with 0.5% Oil Red O in 100% propylene glycol overnight. Stained larvae were washed with decreasing concentrations of propylene glycol followed by several rinses with PBS and transferred to 80% glycerol. Steatosis was scored as 3 or more lipid droplets per liver. Images were captured on a Nikon SMZ1500 with a Nikon Digital Sight color camera.

Histologic Analysis

Larvae were fixed, embedded in HistoGel (Richard-Allan Scientific: Kalamazoo, MI) and paraffin according to standard procedures. 4 μm sections were stained with hematoxylin and eosin (H&E) and imaged on an Olympus BX41 microscope and a Nikon Digital Sight-Ri1 camera.

Reverse Transcription and Quantitative PCR

Poly dT primed cDNA was prepared from RNA isolated with the RNeasy kit (Quiagen) using Superscript II (Invitrogen: Carlsbad, CA). Real-time quantitative PCR (qPCR) was carried out on Light Cycler 480 (Roche: Basel, Switzerland) with one cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute using 1μmol/

L of gene specific primers (supplementary Table 1) and the iQ SYBR Green Supermix (Roche). *rpp0* was used as a reference, and expression was calculated using the cycle threshold (Ct) method ($2^{-Ct\ (target)}/\ 2^{-Ct\ (rppo)}$). Typically, 20 fish were pooled for each sample and 3-10 separate clutches were analyzed per experiment.

Morpholino injections

A splice blocking morpholino targeting *scap* (MO; 5′-cccgatactgcaagaagatt-3′ as described (31)) and a control morpholino (5′ aggttcctttggttttgcacagcgc-3′; Gene Tools, Philomath, OR) were diluted with water to 0.5 mM and approximately 4 nl was injected into >50 1-2 cell stage embryos using a Narishege IM-300 microinjector. Abnormal embryos were removed at 6 hpf.

Statistical Analysis

All evaluations were performed with MS Excel software. p values less than 0.05 were considered statistically significant.

RESULTS

Ethanol Induces Abnormalities in Zebrafish Larvae

Zebrafish hepatogenesis occurs between 1-3 dpf (38), and larval feeding begins on 4-5 dpf when the yolk has been completely utilized. Fasting induced steatosis occurs if larvae are not fed by 6 dpf (KCS and CG, unpublished). Thus, 4-5 dpf is the ideal time to assess the effects of alcohol without interference by the metabolic changes that accompany external nutrient metabolism or fasting.

To optimize a protocol for exposing zebrafish larvae to EtOH, we first assessed EtOH toxicity. 4 dpf larvae were exposed to EtOH concentrations ranging from 0.5 to 4.0% for 32 hours (i.e. until 128 hpf; Fig. 1A) and were visually scored for viability, morphology and behavioral changes. Exposure to 4% EtOH causes rapid mortality, while nearly all larvae exposed to 2% EtOH or less survived the entire exposure (Fig. 1B). Thus, 4 dpf larvae are affected by EtOH in a dose- and time-dependent manner, the LD_{50} is 2.6% at 32 hours of treatment and 2% EtOH is the maximal tolerable dose.

The cellular EtOH concentration was determined by an alcohol dehydrogenase assay using homogenate prepared from larvae exposed to 0, 171 and 343 mM EtOH (i.e. 0, 1 and 2% EtOH) for 8, 24, or 32 hours as a substrate. By 8 hours of exposure, tissue EtOH levels reached 40 mM in larvae treated with 1% EtOH and 63 mM in those treated with 2% (Fig. 1C). The level of internal alcohol remained constant at all time points, but at 24 hours of exposure, the internal EtOH concentration was higher in larvae treated with 2% compared 1% EtOH (Fig. 1C). The values measured in our experiments are comparable to those measured in other zebrafish studies (22,39) and are within an order of magnitude of the legal limit in humans.

If zebrafish metabolize EtOH, then the genes which metabolize EtOH, such as *cyp2e1*, *cyp3a4* and *aldh* (3) should be induced, and the generation of free oxygen radicals should cause oxidative stress and induce superoxide dismutase (*sod*) expression (40). While neither extended time (Fig. 1D) or increased concentration (Fig. 1E) of EtOH exposure caused changes in the expression these genes in extracts prepared from whole embryos, *cyp2e1* and *sod* expression increased significantly (p values <0.05), albeit modestly, in livers dissected from larvae treated with 2% EtOH for 32 hours. Strikingly, *bip* expression was induced more than 30 fold in the liver (Fig. 1F), indicating significant ER stress. Based on these data,

we conclude that zebrafish can ingest and metabolize EtOH, and this causes hepatic oxidative stress, signifying the suitability of this model for studying acute ALD.

Exposing early zebrafish embryos to EtOH disrupts patterning and causes morphologic abnormalities (25,28). To avoid these effects on early development, we began our treatment protocol at 4 dpf, yet we found EtOH concentrations of 2% or greater induced reproducible morphologic abnormalities (Fig. 2A). Fish with edema, hepatomegaly, body curvature were considered severely affected, whereas those which had only failed to inflate their swim bladder were scored as moderately affected (Fig. 2B). The number of affected fish increased as either time of exposure or EtOH concentration increased, with higher concentrations (3 and 4% EtOH) inducing more severe abnormalities (Fig. 2B). Alcohol induces profound behavioral effects in mammals as well as in adult (19,21,41) and larval (22) zebrafish. We also observed irregular and non-directional swimming, lethargy and immobility in zebrafish larvae treated with EtOH, and scored the number of animals exhibiting any of these behaviors as affected (Fig. 2C). These results demonstrate that EtOH ingestion by 4 dpf zebrafish larvae increases mortality and causes changes in morphology and behavior.

Acute Ethanol Exposure Induces Steatosis

Acute ethanol exposure in mammals causes steatosis (2). To determine whether this also occurs in zebrafish larvae, we treated 4 dpf larvae with 1% or 2% EtOH for 24 or 32 hours and visualized neutral lipids by whole mount oil red O staining (Fig. 3A). Untreated larvae and those treated with 1% EtOH (not shown) rarely developed steatosis by 5.5 dpf, whereas 2% EtOH treatment resulted in 53% and 64% steatosis at 24 and 32 hours of exposure, respectively (Fig. 3B). Histological analysis revealed macrovesicular lipid droplets in the hepatocytes of larvae treated for 32 hours with 2% EtOH (Fig. 3C). Despite this marked steatosis, there was no change in the expression of genes regulating several other hepatic functions (Fig. S2), suggesting that acute alcohol does not compromise liver function.

Steatosis in chronic ALD is due to both decreased lipid utilization due to mitochondrial dysfunction (6,7) and to lipogenesis driven by SREBP1-c (8). The molecular basis of steatosis associated with acute EtOH exposure is not known. To assess the effects of EtOH on hepatic lipid metabolism in zebrafish, qPCR was used to detect the expression of genes involved in lipid and cholesterol synthesis, lipid oxidation and transport. The expression of these genes was compared between the livers of 5.5 dpf fish treated with 2% EtOH to untreated fish. In Figure 3D, the fold change in expression is plotted as a single point for each clutch, with the mean fold change from all experiments indicated by an ‘X’. We found significant changes in the expression of genes in each category, including the putative Srebp target genes *fasn*, *hmgcs1* and the *hmgcra* and *hmgcrb* genes, which resulted from the genome duplication that occurred during teleost evolution (42).

Ethanol Mediated Steatosis Requires mbtps1 and scap: A Role for Srebps

To address whether acute alcohol-induced steatosis requires Srebps, we used *mbtps1* mutants and *scap* morpholino knock-down to prevent Srebp activation (Fig. S1). Both genes are expressed in the 5 dpf liver (Fig. 4A) suggesting they function at the time in which larvae develop steatosis in response to EtOH. The hi^{1487} allele of *goz* mutants (i.e. goz^{hi1487}) has a viral insertion in the intron preceding the initiator ATG of the *mbtps1* gene, resulting in a loss of function and embryonic lethality (30,37). However, unlike the *Mbtps1* knock-out mice which die *in utero* (43), precluding examination of hepatic function, goz^{hi1487} mutants survive until 7 dpf with fully mature livers. Additionally, injecting embryos with a morpholino to prevent *scap* splicing (31) should specifically prevent Srebp activation. We found no difference in the internal EtOH concentration of WT larvae and goz^{hi1487} mutants (Fig. 4B) or *scap* morphants (not shown) or the morphological changes induced by 32 hours

of treatment with 2% EtOH (Fig. 4C and not shown), indicating that these models do not alter the ability of zebrafish larvae to ingest and respond to alcohol. There was, however, a marked inhibition of steatosis in response to 32 hours exposure to 2% EtOH in *goz*^{hi1487} mutants (Figs. 4D and E) indicating a requirement for *mbtps1* in steatosis resulting from acute EtOH exposure.

The most straightforward interpretation of this data is that acute alcohol exposure causes hepatic Srebp activation, thereby inducing the expression of genes required for lipid and cholesterol synthesis (Fig. 3D), and that mutation of *mbtps1* blocks this pathway. Alternatively, as ATF6 is critical to the unfolded protein response (UPR), and this pathway has been implicated in ALD (44) the role of *mbtps1* in activating the ATF6 (12) may also be important (see Figure S1). However, with the exception of *bip* upregulation (Fig. 1F), we found that other Atf6 targets (Fig. S3A) and or *xbp1* splicing (Fig. S3B), markers of the UPR, were not induced the livers of EtOH treated larvae. Serum amyloid (*saa2*) is regulated by CREBH (13) and was substantially upregulated in response to EtOH (Fig. S3C), but the degree of upregulation varied from clutch to clutch.

These findings raised the possibility that failure to activate Atf6 and/or Crebh may contribute for the striking inhibition of steatosis in *goz*^{hi1487} mutants. Transient knock down of *scap* expression in WT fish by injecting embryos with a morpholino that blocks splicing of *scap* message is predicted to specifically prevent Srebp activation without affecting Atf6 or Crebh. *scap* morpholino injection did not adversely affect embryonic development or mortality and the same internal EtOH concentration was measured measured in *scap* morphants, larvae injected with a control morpholino and uninjected controls treated with 2% EtOH for 24 hours (not shown). In both sets of control larvae, i.e uninjected and those injected with a non-targeting morpholino, over 60% of the larvae developed steatosis in response to 2% EtOH in contrast, to only 25.7% steatosis in *scap* morphants (Fig. 4F).

If Srebp activation does not occur in *goz*^{hi1487} mutants in response to acute alcohol exposure, then Srebp target genes should not be upregulated in these fish. qPCR analysis of gene expression in the livers from 3 clutches of *goz*^{hi1487} larvae and their non-mutant siblings confirms this hypothesis. The expression of *acc1, fasn, hmgcra* and *hmgcs1* is significantly lower in untreated *goz*^{hi1487} livers compared to WTs, signifying that expression of these genes is regulated by Srebps during normal liver homeostasis (Fig. 5A). The hepatic expression of each of these genes is increased following 2% EtOH exposure in WT livers (Fig. 3D) whereas *acc1, fasn, hmgcra* and *hmgcs1* expression was markedly reduced in *goz*^{hi1487} (Fig. 5B). Interestingly, there is *hmgcrb* expression is the same in WT and *goz*^{hi1487} livers, regardless of EtOH exposure, suggesting that it is independent of Srebps. This is consistent with the finding that *hmgcrb* expression is not liver-specific and that it primarily functions in the zebrafish heart, not in the liver (45).

Importantly, changes in *echs1, cyp2e1* and *mtp* expression in response to EtOH are similar in WT and *goz*^{hi1487} livers, indicating the specificity of the effect on Srebp target genes. The Atf6 target gene, *bip*, is strongly induced in the liver following EtOH treatment, similar to what is observed in adult zebrafish brains following to chronic alcohol exposure (46). There is not a significant decrease in *bip* expression, however, in *goz*^{hi1487} livers (Fig 5C), suggesting that Atf6 may not be the primary regulator of *bip* in response to EtOH. We thus conclude that Srebp activation occurs in response to acute alcohol exposure, and is required for steatosis.

DISCUSSION

We demonstrate that 32 hours of continuous exposure to 2% EtOH causes zebrafish larvae to develop characteristic signs of acute ALD, including hepatomegaly, steatosis and changes in hepatic gene expression suggesting that alcohol is metabolized and causes oxidative stress (47). Symptomatic fish were generated rapidly and in large numbers via a relatively simple treatment protocol, providing distinct advantages over mammalian models. Genetic tractability is among the most useful attributes of zebrafish. We present two example, using *goz*^{hi1487} mutants and *scap* morphants, which demonstrate that zebrafish genetics can be used to quickly and efficiently identify genes required for alcohol-mediated steatosis.

Several studies established early zebrafish embryos as a model for fetal alcohol syndrome (23,25-27,48) and adult zebrafish are used to study the effects of alcohol on behavior (19-21,41,46). While we also find that EtOH induces morphological and behavioral abnormalities in zebrafish larvae, the effects we report are less likely to be attributable to disrupting developmental processes, since we expose larvae to alcohol once major developmental milestones are complete. Alcohol exposure of both early and late stage embryos does, however, appear to affect of cholesterol metabolism. Early embryos exposed to EtOH display growth retardation and cyclopia attributed to decreased cholesterol modification of sonic hedgehog (27) while we find that alcohol increases the expression of genes required for cholesterol biogenesis. It is possible that alcohol influences cholesterol metabolism by different mechanisms depending on the developmental stage.

The failure of *goz*^{hi1487} larvae to develop EtOH mediated steatosis demonstrates the role of *mbtps1* in acute ALD and implicates one of the Mbtps1 substrates - i.e. Srebps, Atf6 or Crebh - in this process. *scap* morphants are also resistant to EtOH mediated steatosis, but are not otherwise phenotypically affected. Moreover, as both *scap* morphants and *goz*^{hi1487} mutants internalize EtOH and display phenotypic and gene expression changes, indicating that these larvae are capable of metabolizing and responding to EtOH, but that they do not develop steatosis. This indicates that EtOH treatment activates Srebps, leading to target gene activation and subsequent increase in hepatic lipogenesis. Indeed, the hepatic expression of several Srebp target genes is dependent on *mbtps1*, even without alcohol, pointing to an essential and conserved function for Srebps hepatic lipid homeostasis. The Srebp1 and Srebp2 genes were recently identified in zebrafish through the ongoing zebrafish genome project, and it will be of interest to interrogate those transcripts to further define which Srebp is crucial for steatosis in this model.

While the most likely explanation of our data is that *de novo* lipogenesis driven by Srebps is required for steatosis, Atf6 may also contribute to steatosis in this setting, as a role for the UPR in ALD has been demonstrated in mammals (44). It is possible that the combined action of Srebps and Atf6 could explain the more substantial suppression of steatosis in *goz*^{hi1487} mutants compared to *scap* morphants. Arguing against this is the finding that several Srebp target genes are suppressed in *goz*^{hi1487} mutants, whereas there is no significant difference the induction of the Atf6 target gene, *bip*, in response to EtOH. Thus, the more likely explaination is that the differences in the efficacy of morpholinos, which act transiently so that there is only partial *scap* knockdown by 4 dpf, compared to the stable depletion achieved by homozygous mutation. In summary, we conclude that acute alcohol exposure causes Srebp activation, which drives the transcription of genes that are required for lipid and cholesterol synthesis, leading to de novo hepatic lipogenesis and steatosis.

The risk of ALD progression is dose- and pattern-dependent, but quantifying the level of alcohol consumption that provokes progression beyond past steatosis has not been possible. Moreover, variations in disease progression are thought to be influenced by as-yet-

unidentified genetic factors. While exciting progress has been made using mammalian models of ALD, uncovering the genes that contribute to this disease are a major hurdle. Knock-out mice are an unsurpassed means of testing genes suspected to play a role in ALD, however, these are costly and time consuming. The rapidly expanding library of publicly available zebrafish mutants (zirc.org) and the use of morpholinos to knockdown gene expression are a significant advantage of zebrafish. While the zebrafish larval model of ALD is not amenable to exploring the consequences of chronic alcohol abuse, such as fibrosis, this system offers several features that will complement mammalian studies of acute ALD. Additionally, as multiple organ systems are affected by alcohol abuse, we envision future investigations to incorporate studies on ALD with studies on the effects of alcohol on fields outside of hepatology.

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Abbreviations

acc1	acetyl-CoA carboxylase
ALD	Alcoholic Liver Disease
adh3	alcohol dehydrogenase
aldh	aldehyde dehydrogenase
ATF6	Activating Transcription Factor 6
bip	binding immunoglobulin protein
cpt1	carnitine palmitoyl transferase 1
chop	CCAAT/Enhancer-Binding Protein Homologous Protein
cyp2e1	cytochrome p450 2e1
cyp3a4	cytochrome p450 3a4
echs1	enoyl-CoA hydratase
edem1	ER degradation enhancer mannosidase alpha-like 1
EtOH	Ethanol
fabp10	fatty acid binding protein 10
fads2	fatty acid desaturase 2
fasn	fatty acid synthase
H&E	hematoxylin and eosin
hmgcr	HMG-CoA reductase
hmgcs1	HMG-CoA synthetase
hpf	hours post fertilization
mtp	microsomal triglyceride transfer protein

mbtps1	membrane bound transcription factor protease 1
qPCR	quantitative polymerase chain reaction
rpp0	ribosomal protein P0
saa2	serum amyloid
scap	SREBP cleavage-activating protein
sod	superoxide dismutase
SREBP	Sterol Regulatory Element Binding Protein

References

1. Kim WR, Brown RS Jr. Terrault NA, El-Serag H. Burden of liver disease in the United States: summary of a workshop. Hepatology. 2002; 36:227–242. [PubMed: 12085369]
2. Donohue TM Jr. Alcohol-induced steatosis in liver cells. World J Gastroenterol. 2007; 13:4974–4978. [PubMed: 17854140]
3. Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol. 2004; 34:9–19. [PubMed: 15670660]
4. Carmiel-Haggai M, Cederbaum AI, Nieto N. Binge ethanol exposure increases liver injury in obese rats. Gastroenterology. 2003; 125:1818–1833. [PubMed: 14724834]
5. Safdar K, Schiff ER. Alcohol and hepatitis C. Semin Liver Dis. 2004; 24:305–315. [PubMed: 15349807]
6. Cederbaum AI, Rubin E. Molecular injury to mitochondria produced by ethanol and acetaldehyde. Fed Proc. 1975; 34:2045–2051. [PubMed: 170139]
7. Hoek JB, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. Gastroenterology. 2002; 122:2049–2063. [PubMed: 12055609]
8. Ji C, Chan C, Kaplowitz N. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. J Hepatol. 2006; 45:717–724. [PubMed: 16879892]
9. Nohturfft A, DeBose-Boyd RA, Scheek S, Goldstein JL, Brown MS. Sterols regulate cycling of SREBP cleavage-activating protein (SCAP) between endoplasmic reticulum and Golgi. Proc Natl Acad Sci U S A. 1999; 96:11235–11240. [PubMed: 10500160]
10. Matsuda M, Korn BS, Hammer RE, Moon YA, Komuro R, Horton JD, Goldstein JL, et al. SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. Genes Dev. 2001; 15:1206–1216. [PubMed: 11358865]
11. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A. 1999; 96:11041–11048. [PubMed: 10500120]
12. Ye J, Rawson RB, Komuro R, Chen X, Dave UP, Prywes R, Brown MS, et al. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell. 2000; 6:1355–1364. [PubMed: 11163209]
13. Zhang K, Shen X, Wu J, Sakaki K, Saunders T, Rutkowski DT, Back SH, et al. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell. 2006; 124:587–599. [PubMed: 16469704]
14. Tsukamoto H, Mkrtchyan H, Dynnyk A. Intragastric ethanol infusion model in rodents. Methods Mol Biol. 2008; 447:33–48. [PubMed: 18369909]
15. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 2007; 8:353–367. [PubMed: 17440532]
16. Nasevicius A, Ekker SC. Effective targeted gene ‘knockdown’ in zebrafish. Nat Genet. 2000; 26:216–220. [PubMed: 11017081]

17. Reimers MJ, Hahn ME, Tanguay RL. Two zebrafish alcohol dehydrogenases share common ancestry with mammalian class I, II, IV, and V alcohol dehydrogenase genes but have distinct functional characteristics. J Biol Chem. 2004; 279:38303–38312. [PubMed: 15231826]
18. Lassen N, Estey T, Tanguay RL, Pappa A, Reimers MJ, Vasiliou V. Molecular cloning, baculovirus expression, and tissue distribution of the zebrafish aldehyde dehydrogenase 2. Drug Metab Dispos. 2005; 33:649–656. [PubMed: 15703303]
19. Dlugos CA, Rabin RA. Ethanol effects on three strains of zebrafish: model system for genetic investigations. Pharmacol Biochem Behav. 2003; 74:471–480. [PubMed: 12479969]
20. Gerlai R, Ahmad F, Prajapati S. Differences in Acute Alcohol-Induced Behavioral Responses Among Zebrafish Populations. Alcohol Clin Exp Res. 2008
21. Gerlai R, Lee V, Blaser R. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (Danio rerio). Pharmacol Biochem Behav. 2006; 85:752–761. [PubMed: 17196640]
22. Lockwood B, Bjerke S, Kobayashi K, Guo S. Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening. Pharmacol Biochem Behav. 2004; 77:647–654. [PubMed: 15006478]
23. Laale HW. Ethanol induced notochord and spinal cord duplications in the embryo of the zebrafish, Brachydanio rerio. J Exp Zool. 1971; 177:51–64. [PubMed: 4998733]
24. Blader P, Strahle U. Ethanol impairs migration of the prechordal plate in the zebrafish embryo. Dev Biol. 1998; 201:185–201. [PubMed: 9740658]
25. Bilotta J, Barnett JA, Hancock L, Saszik S. Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome. Neurotoxicol Teratol. 2004; 26:737–743. [PubMed: 15451038]
26. Reimers MJ, Flockton AR, Tanguay RL. Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish. Neurotoxicol Teratol. 2004; 26:769–781. [PubMed: 15451041]
27. Li YX, Yang HT, Zdanowicz M, Sicklick JK, Qi Y, Camp TJ, Diehl AM. Fetal alcohol exposure impairs Hedgehog cholesterol modification and signaling. Lab Invest. 2007; 87:231–240. [PubMed: 17237799]
28. Tanguay RL, Reimers MJ. Analysis of ethanol developmental toxicity in zebrafish. Methods Mol Biol. 2008; 447:63–74. [PubMed: 18369911]
29. Yang J, Goldstein JL, Hammer RE, Moon YA, Brown MS, Horton JD. Decreased lipid synthesis in livers of mice with disrupted Site-1 protease gene. Proc Natl Acad Sci U S A. 2001; 98:13607–13612. [PubMed: 11717426]
30. Nissen RM, Amsterdam A, Hopkins N. A zebrafish screen for craniofacial mutants identifies wdr68 as a highly conserved gene required for endothelin-1 expression. BMC Dev Biol. 2006; 6:28. [PubMed: 16759393]
31. Schlombs K, Wagner T, Scheel J. Site-1 protease is required for cartilage development in zebrafish. Proc Natl Acad Sci U S A. 2003; 100:14024–14029. [PubMed: 14612568]
32. Cheng W, Guo L, Zhang Z, Soo HM, Wen C, Wu W, Peng J. HNF factors form a network to regulate liver-enriched genes in zebrafish. Dev Biol. 2006
33. Farber SA, Pack M, Ho SY, Johnson ID, Wagner DS, Dosch R, Mullins MC, et al. Genetic analysis of digestive physiology using fluorescent phospholipid reporters. Science. 2001; 292:1385–1388. [PubMed: 11359013]
34. Sadler KC, Amsterdam A, Soroka C, Boyer J, Hopkins N. A genetic screen in zebrafish identifies the mutants vps18, nf2 and foie gras as models of liver disease. Development. 2005; 132:3561–3572. [PubMed: 16000385]
35. Schlegel A, Stainier DY. Microsomal Triglyceride Transfer Protein Is Required for Yolk Lipid Utilization and Absorption of Dietary Lipids in Zebrafish Larvae. Biochemistry. 2006; 45:15179–15187. [PubMed: 17176039]
36. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995; 203:253–310. [PubMed: 8589427]
37. Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N. Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci U S A. 2004; 101:12792–12797. [PubMed: 15256591]
38. Field HA, Ober EA, Roeser T, Stainier DY. Formation of the digestive system in zebrafish. I. Liver morphogenesis. Dev Biol. 2003; 253:279–290. [PubMed: 12645931]

39. Reimers MJ, La Du JK, Periera CB, Giovanini J, Tanguay RL. Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants. Neurotoxicol Teratol. 2006; 28:497–508. [PubMed: 16904866]
40. Kessova IG, Ho YS, Thung S, Cederbaum AI. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. Hepatology. 2003; 38:1136–1145. [PubMed: 14578852]
41. Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. Pharmacol Biochem Behav. 2000; 67:773–782. [PubMed: 11166068]
42. Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, et al. Genome duplication in the teleost fish Tetraodon nigroviridis reveals the early vertebrate proto-karyotype. Nature. 2004; 431:946–957. [PubMed: 15496914]
43. Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, et al. Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet. 2001; 28:241–249. [PubMed: 11431694]
44. Kaplowitz N, Ji C. Unfolding new mechanisms of alcoholic liver disease in the endoplasmic reticulum. J Gastroenterol Hepatol. 2006; 21:S7–S9. [PubMed: 16958678]
45. D'Amico L, Scott IC, Jungblut B, Stainier DY. A mutation in zebrafish hmgcr1b reveals a role for isoprenoids in vertebrate heart-tube formation. Curr Biol. 2007; 17:252–259. [PubMed: 17276918]
46. Damodaran S, Dlugos CA, Wood TD, Rabin RA. Effects of chronic ethanol administration on brain protein levels: a proteomic investigation using 2-D DIGE system. Eur J Pharmacol. 2006; 547:75–82. [PubMed: 16978605]
47. Yin HQ, Kim M, Kim JH, Kong G, Kang KS, Kim HL, Yoon BI, et al. Differential gene expression and lipid metabolism in fatty liver induced by acute ethanol treatment in mice. Toxicol Appl Pharmacol. 2007; 223:225–233. [PubMed: 17655900]
48. Arenzana FJ, Carvan MJ 3rd, Aijon J, Sanchez-Gonzalez R, Arevalo R, Porteros A. Teratogenic effects of ethanol exposure on zebrafish visual system development. Neurotoxicol Teratol. 2006; 28:342–348. [PubMed: 16574376]

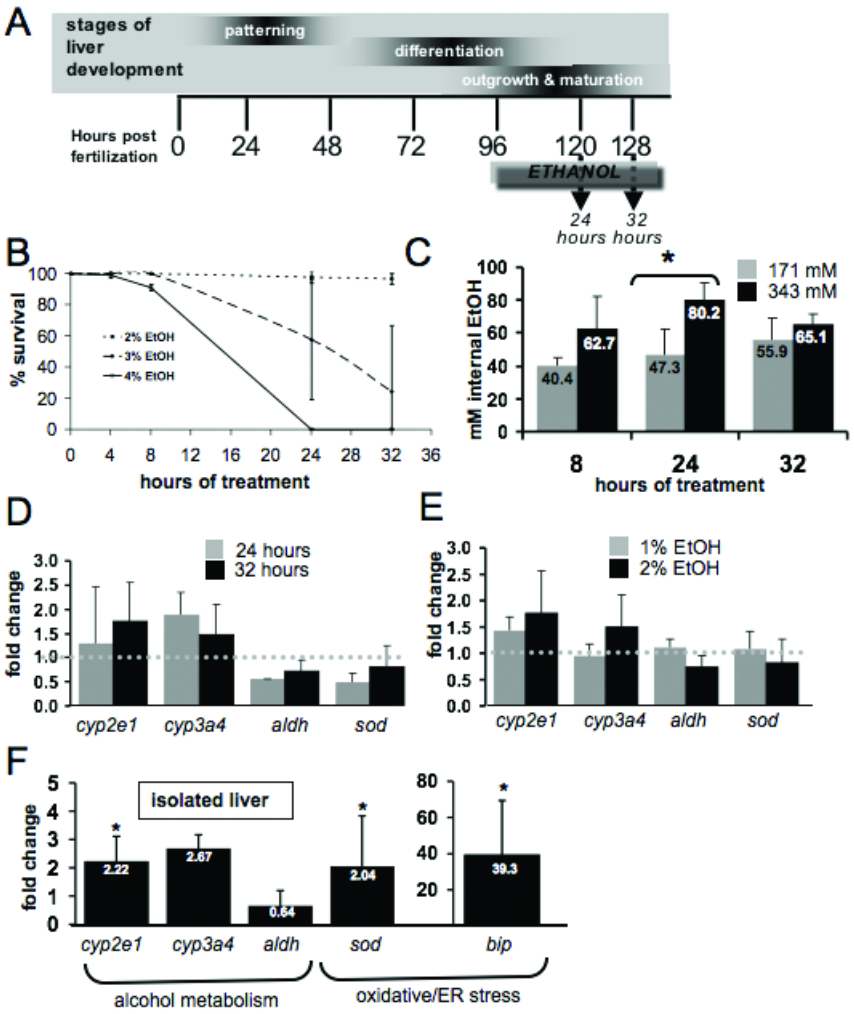

Figure 1. Acute EtOH exposure reduces viability of zebrafish larvae

(A) Outline of the EtOH treatment protocol. EtOH exposure began at 96 hpf and continued for up to 32 hours (128 hpf). (B) Larvae treated with 0-4% EtOH were scored for mortality and the average of 3 clutches is plotted during 32 hours of exposure; error bars show standard deviation. (C) Tissue EtOH concentration was determined using homogenates of whole larvae treated with 0, 171 and 343 mM EtOH (i.e. 0, 1 and 2%) as a substrate for an alcohol dehydrogenase activity using, and the measurements from untreated larvae was subtracted as background from measurements obtained from treated animals. Values indicate the average mM of internal EtOH in at least 3 separate clutches. * indicates p=0.046. (D-F) qPCR analysis of gene expression in whole larvae treated for 24 or 32 hours with 2% EtOH (D) or for 32 hours with either 1% or 2% EtOH (E). Values indicate the average fold change in gene expression compared to untreated controls in 3 clutches. (F) Livers were dissected from 4-8 clutches of larvae that were treated with 0% or 2% EtOH for 32 hours and qPCR analysis demonstrates a significant increase in the expression of *cyp2e1* and *sod* in response to EtOH (*indicates p value < 0.03).

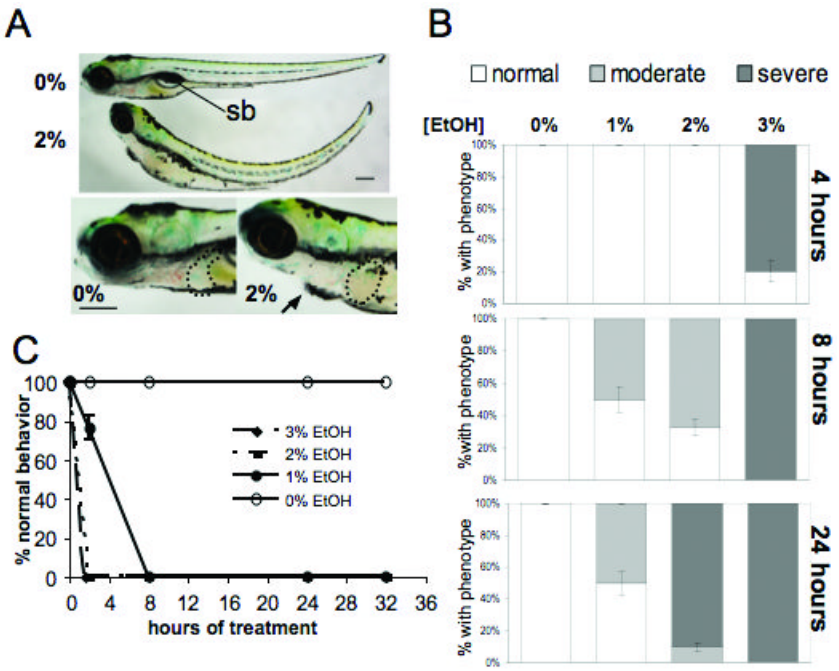

Figure 2. Zebrafish exposed to alcohol develop morphological and behavioral abnormalities

(A) 5.5 dpf larvae treated with 0% or 2% EtOH for 32 hours develop morphologic abnormalities including upward curvature of the trunk and tail, hepatomegaly (liver is outlined by a dotted line), pericardial edema (arrow), and failure to inflate the swim bladder (sb). Scale bar represents 250 μm. (B) Morphologic abnormalities induced by 0, 1, 2 and 3% EtOH become more severe with duration of exposure. Failure to inflate the sb was scored as a moderate abnormality and occurrence of one additional phenotype (i.e. edema, body curvature or hepatomegaly) was scored as severe. (C) Behavioral abnormalities induced by EtOH, including non-directional swimming and lethargy, was scored for animals treated with 0, 1, 2 and 3% EtOH. All experiments were repeated on 3 clutches and error bars indicate standard deviation.

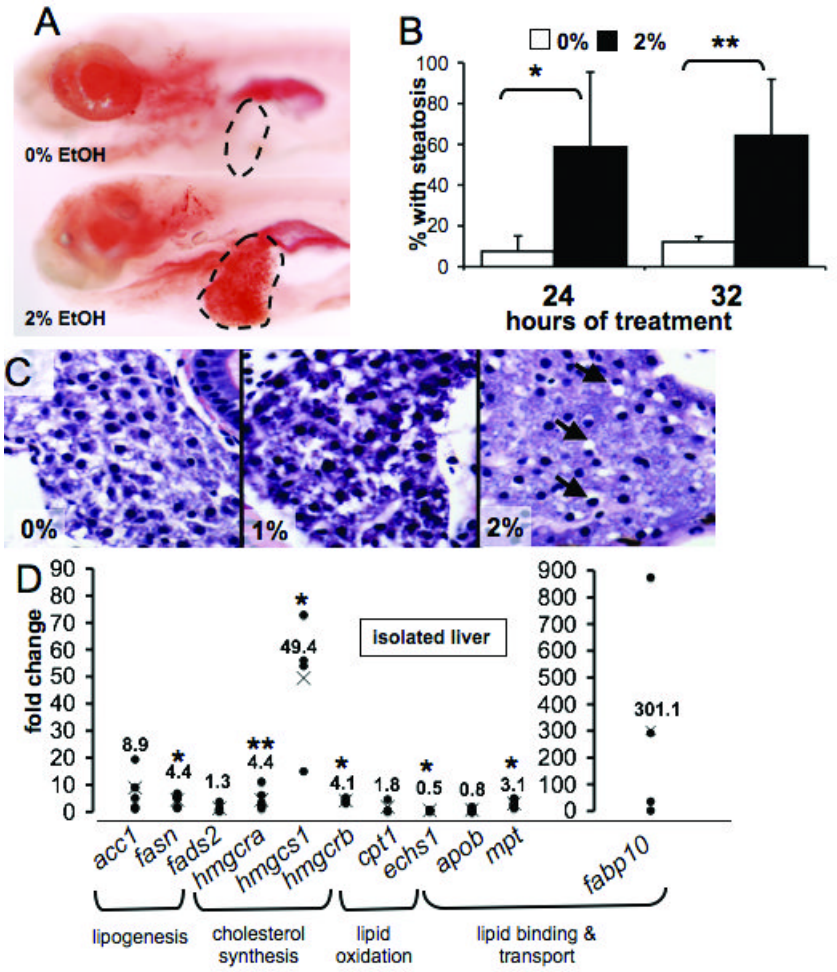

Figure 3. EtOH exposure results in steatosis and alteration of lipid metabolism

(A) Whole mount oil red O staining of 5.5 dpf larvae after exposure to 0% or 2% EtOH for 32 hours reveals steatosis. Dotted line outlines the liver. (B) 4 dpf larvae were treated with 0% or 2% EtOH, collected at 24 or 32 hours of exposure and stained with oil red O. The percent of larvae with steatosis was scored in at least 4 clutches with an average n of 22 fish per group; *p=0.07; **p=0.0002. (C) H&E staining of sections through the liver of 5.5 dpf larvae treated with 0, 1 or 2% EtOH for 32 hours. Large, clear cytoplasmic lipid droplets (arrows) are seen in livers from larvae treated with 2% EtOH. (D) Livers were dissected from 5.5 dpf larvae treated with 0% or 2% for 32 hours and the expression of genes involved in lipid metabolism was analyzed by qPCR. Each gene was analyzed in up to 8 clutches and each point represents the fold change of each gene in livers treated with 2% EtOH relative to their untreated siblings. The X indicates the mean fold change, which is indicated numerically. * indicates p <0.02 and ** indicates a p =0.07.

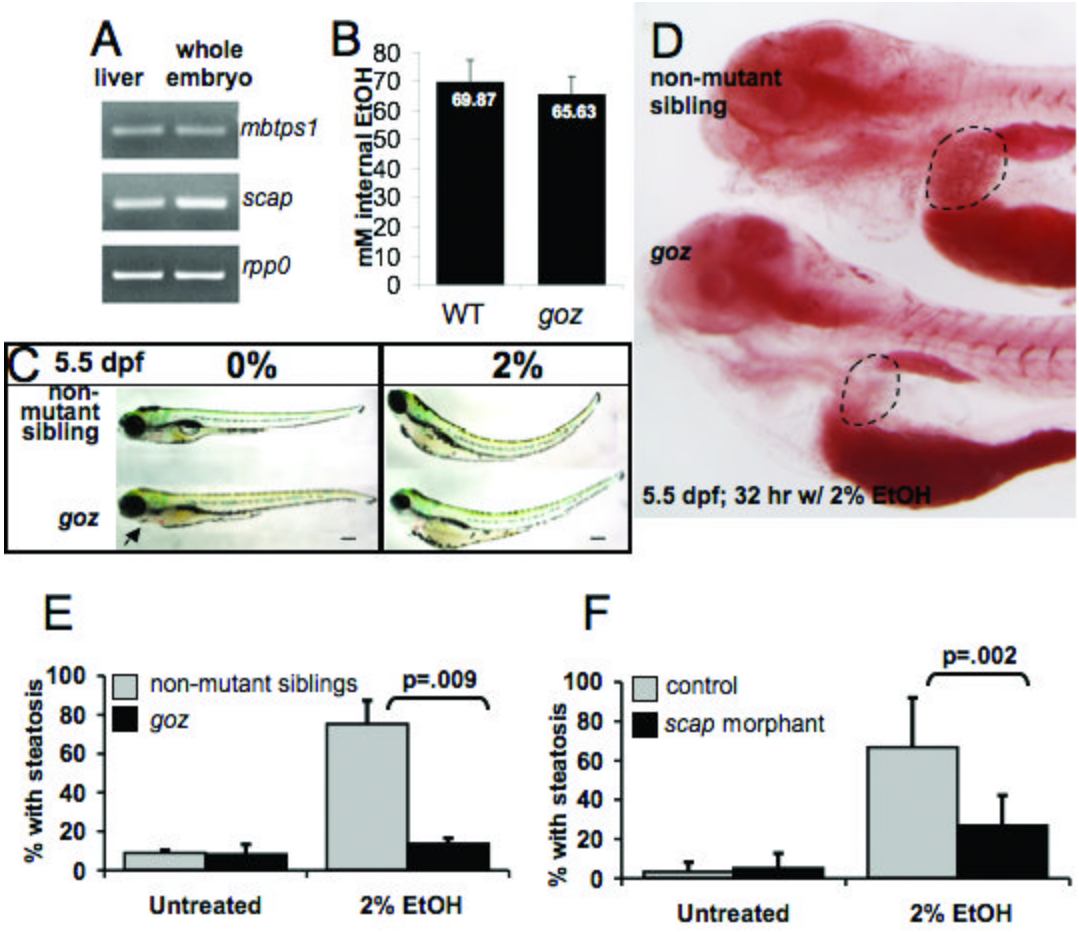

Figure 4. *goz*^{hi1487} mutants and *scap* morphants are resistant to EtOH induced steatosis

(A) PCR analysis of *mbtps1* and *scap* expression in 5 dpf liver and whole embryo extracts. *rpp0* is used as a control. (B) Tissue EtOH concentration was determined by spectrophotometric analysis of alcohol dehydrogenase activity using homogenates of whole 5.5 dpf larvae treated for 24 hours with 2% EtOH as a substrate. Values on each bar indicate the average mM of internal EtOH in 4 separate clutches of non-mutant siblings (WT) and *goz*^{hi1487} larvae. Error bars indicate the SEM. (C) Live images of 5.5 dpf larvae treated with 0% or 2% EtOH for 32 hours indicate that EtOH induces the same morphological changes in WT and *goz*^{hi1487} larvae. Additionally, *goz*^{hi1487} mutants have defective craniofacial development (30) illustrated by the small jaw (arrow). Scale bar = 250 μM. (D) *goz*^{hi1487} larvae and their non-mutant siblings were stained with oil red O on 5.5 dpf following 32 hours exposure to 2% EtOH. Neutral lipid accumulation is seen in the liver (dotted line) of WT fish (top) but not in *goz*^{hi1487} mutants (bottom). The percent of 5.5 dpf *goz*^{hi1487} mutants (E) or *scap* morphants (F) that develop steatosis with and without 32 hours of 2% EtOH exposure was scored in 4 *goz*^{hi148} clutches (n ≥ 14 fish per clutch; total n=230 larvae) and 5 clutches injected with *scap* or control morpholinos (MO; n≥ 8 fish per clutch, average clutch size of 21 and total n= 502 larvae) and averaged. Control larvae were non-mutant siblings in E and the sum of uninjected and control morpholino injected larvae in F. Error bars indicate SEM and p values are indicated.

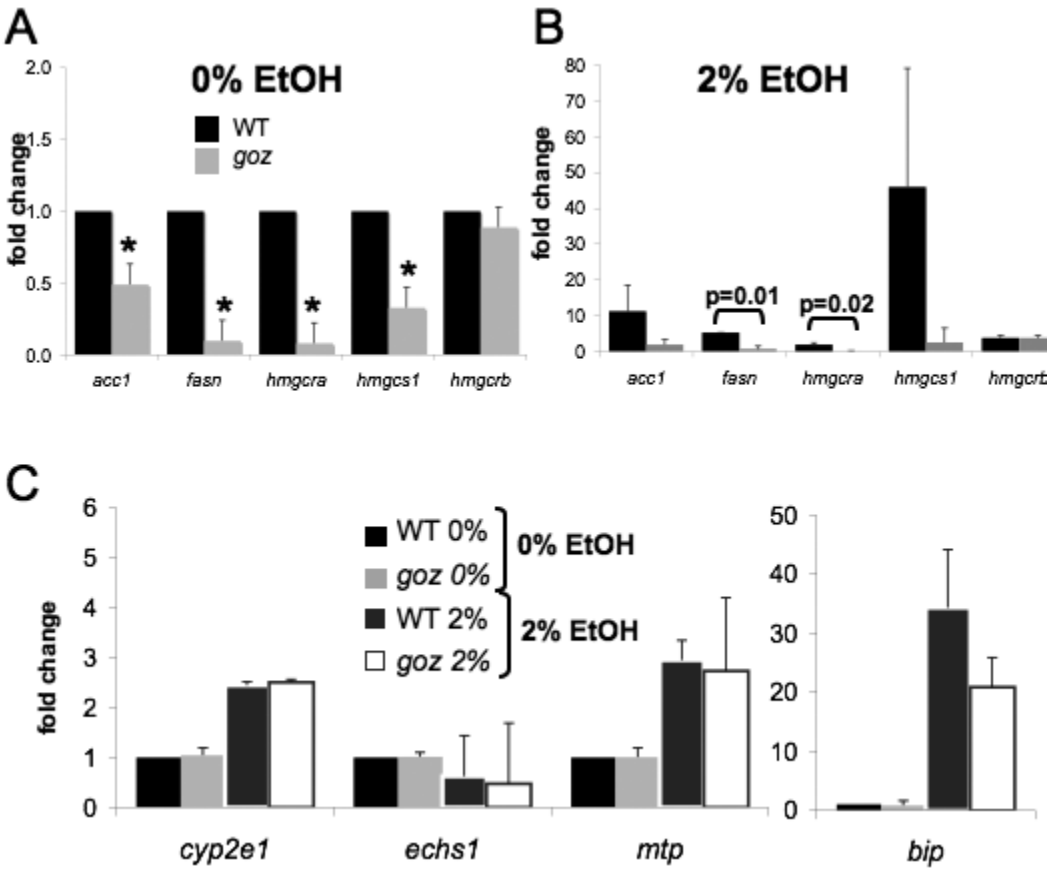

Figure 5. *goz*^{hi1487} mutants have decreased hepatic expression of Srebp target genes

(A-B) Livers were dissected from non-mutant siblings (WT) and *goz*^{hi1487} larvae on 5.5. dpf following 32 hours of exposure to 0% (A) and 2% EtOH (B) and qPCR analysis was used to detect gene expression relative to *rpp0*. The average fold change normalized to the expression in WT livers with no alcohol treatment is plotted. error bars indicate SEM. In (A) * indicates p<0.02. In (B), the p value corresponding to the different expression levels in WT and mutants is indicated. (C) qPCR was used to compare the expression of genes involved in EtOH metabolism (cyp2e1), lipid metabolism (*echs1* and *mtp*) and the Atf6 target, *bip* in the livers of WT and *goz*^{hi1487} larvae on 5.5. dpf following 32 hours of exposure to 0% and 2% EtOH.