TRANSCRIPTIONAL REGULATION OF THE HUMAN ALCOHOL DEHYDROGENASES AND ALCOHOLISM

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This work is dedicated to my parents and my brother for their unwavering support and unconditional love.

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ABSTRACT

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TRANSCRIPTIONAL REGULATION OF THE HUMAN ALCOHOL DEHYDROGENASES AND ALCOHOLISM

Alcohol dehydrogenase (*ADH*) genes encode proteins that metabolize ethanol to acetaldehyde. Humans have seven *ADH* genes in a cluster. The hypothesis of this study was that by controlling the levels of *ADH* enzymes, *cis*-regulatory regions could affect the risk for alcoholism. The goal was thus to identify distal regulatory regions of *ADH*s. To achieve this, sequence conservation across 220 kb of the *ADH* cluster was examined. An enhancer (4E) was identified upstream of *ADH4*. In HepG2 human hepatoma cells, 4E increased the activity of an *ADH4* basal promoter by 50-fold. 4E was cell specific, as no enhancer activity was detected in a human lung cell line, H1299. The enhancer activity was located in a 565 bp region (4E3). Four FOXA and one HNF-1A protein binding sites were shown to be functional in the 4E3 region. To test if this region could affect the risk for alcoholism, the effect of variations in 4E3 on enhancer activity was tested. Two variations had a significant effect on enhancer activity, decreasing the activity to 0.6-fold. A third variation had a small but significant effect. The effect of variations in the *ADH1B* proximal promoter was also tested. At SNP rs1229982, the C allele had 30% lower activity than the A allele.

In addition to studying the regulatory regions of *ADH* genes, the effects of alcohol on liver-derived cells (HepG2) were also explored. Liver is the primary site of alcohol metabolism, and is highly vulnerable to injuries due to chronic alcohol abuse. To identify the effects of long term ethanol exposure on global gene expression and alternative splicing, HepG2 cells were cultured in 75 mM ethanol for nine days. Global gene expression changes and alternative splicing were measured using Affymetrix GeneChip® Human Exon 1.0 ST Arrays. At the level of gene expression, genes involved in stress response pathways, metabolic pathways (including carbohydrate and lipid metabolism) and chromatin regulation were affected. Alcohol effects were also observed on alternative transcript isoforms of some genes.

Howard J. Edenberg, Ph.D.
Committee Chair.

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ABBREVIATIONS

μg	microgram
μl	microliter
μM	micromolar
^{0}C	degree centigrade
1Basal	*ADH1B* proximal promoter
4Basal	*ADH4* proximal promoter
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
AP-1	activator protein-1
Arg	arginine
bp	base pair
C/EBP	CCAAT/ enhancer binding protein
cDNA	complementary DNA
CDS	coding sequence
ChIP	chromatin immunoprecipitation
cm	centimeter
cRNA	complementary RNA
Ct	cycle threshold
CTF	CCAAT transcription factor
CYP2E1	cytochrome P450 2E1

Cys	cysteine
DBP	albumin D-site binding protein
DNA	deoxyribo nucleic acid
DNase	deoxyribonuclease
DSM	diagnostic and statistical manual of mental disorders
DTT	dithiothreitol
ECM	extra cellular matrix
EDTA	ethylene diamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
EST	express sequence tag
FB1	factor binds to the inducer of short transcript of Human Immunodeficiency virus-1
FBS	fetal bovine serum
FDR	false discovery rate
FoxA	forkhead box protein A
GI	gastrointestinal
Gln	glutamine
GRE	glucocorticoid response element
GSH	reduced glutathione
GSNO	*S*-nitrosoglutathione
h	hour(s)
Hap	haplotype
His	histidine

HMGSH	*S*-(hydroxymethyl) glutathione
HNF-1A	hepatocyte nuclear factor 1 alpha
ICD	international classification of diseases
IgG	immunoglobulin G
Ile	isoleucine
kb	kilo base pair
kDa	kilodalton
LCR	locus control region
M	molar
MEM	minimum essential medium
min	minute(s)
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
ng	nanogram
nm	nanometer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLIER	probe logarithmic intensity error
pmol	picomoles

qRT-PCR	quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
RMA	robust multi-array analysis
RNA	ribonucleic acid
s	second(s)
SNP	single nucleotide polymorphism
Sp1	specificity protein 1
SV40Basal	SV40 promoter
TBE	tris-borate EDTA buffer
TCA	tricarboxylic acid
TSS	transcription start site
UCSC	University of California, Santa Cruz
USF	upstream stimulatory factor
Val	valine

I. INTRODUCTION

1. Alcohol dehydrogenases

Medium-chain alcohol dehydrogenases (ADH) catalyze the reversible oxidation of ethanol and other alcohols to acetaldehyde (Edenberg and Bosron, 1997; Zakhari, 2006). ADHs are dimeric proteins that utilize NAD^+ as the coenzyme. Each ADH subunit is 40 kDa, binds two zinc ions and has catalytic and coenzyme binding domains (Hurley et al., 2002).

Figure 1. The primary pathway of alcohol metabolism. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase

Based on their sequence homology and kinetic properties, ADHs have been classified into different classes. In vertebrates, eight classes (I to VIII) have been identified, with no species encoding all eight classes (Duester et al., 1999; Peralba et al., 1999). Enzymes in classes I to V are present in multiple species including humans. Class VI is found only in rats and the deer mouse (Hoog and Brandt, 1995; Zheng et al., 1993). Classes VII and VIII are found in the chicken, and the amphibians, respectively (Kedishvili et al., 1997; Peralba et al., 1999). Less than 70% sequence homology has been observed between different classes, and only proteins within a class form dimers. The class III enzyme is the only ADH enzyme seen in invertebrates and thus is considered the ancestral

form that gave rise to other isozymes (Cederlund et al., 1991; Danielsson and Jornvall, 1992).

In humans there are seven ADH isozymes including three class I proteins. Class I proteins α, β and γ share greater than 90% similarity and can form homo- or heterodimers (Edenberg, 2000). The Class II ADH includes the π polypeptide; the class III includes the χ polypeptide; the Class IV, has the σ polypeptide isozyme, and no endogenous protein has been reported for class V.

Class	Gene	Protein	Tissue distribution	Common substrates
I	*ADH1A*	α	fetal and adult liver[1,2], adult kidney[3], adrenal gland[6]	ethanol[12], retinol[13]
I	*ADH1B*	β	fetal and adult liver[1,2], adult kidney[1,4], lung[1,4], blood vessels[5], adrenal gland[6]	ethanol[12], retinol[13]
I	*ADH1C*	γ	adult liver[2], fetal kidney[1], adrenal gland[6]	ethanol[12], retinol[13]
II	*ADH4*	π	fetal and adult liver[1,6], stomach[6], intestine[6], pancreas[6]	ethanol[12], retinol[13]
III	*ADH5*	χ	ubiquitous in adult[4,6] and fetus[6]	HMGSH[14,15], GSNO[16]
IV	*ADH7*	σ	adult stomach[7,8], upper GI tract[10,11], fetal liver[6]	retinol[13], ethanol[12]
V	*ADH6*	None	as mRNA in fetal and adult liver[6]	ethanol[12]

Table 1. Tissue distribution and substrate specificity of human ADH isozymes. HMGSH is *S*-(hydroxymethyl) glutathione and GSNO is *S*-nitrosoglutathione

[1](Smith et al., 1971)
[2](Smith et al., 1972)
[3](Smith, 1986)
[4](Duley et al., 1985)
[5](Allali-Hassani et al., 1997)
[6](Estonius et al., 1996)
[7](Yin et al., 1990)
[8](Yokoyama et al., 1995)
[9] (Zgombic-Knight et al., 1995)
[10](Dong et al., 1996)
[11](Yin et al., 1993)
[12](Edenberg and Bosron, 1997)
[13](Yang et al., 1994)
[14](Kaiser et al., 1991)
[15](Koivusalo and Uotila, 1991)
[16](Staab et al., 2008)

The seven *ADH* isozymes have overlapping substrate specificities (Table 1). All isozymes are active with ethanol, albeit with different Vmax and Km values (Edenberg and Bosron, 1997; Hurley et al., 2002). Class I enzymes have the lowest Km for ethanol and account for approximately 70% of alcohol metabolism in the liver (Hurley et al., 2002). Class II π- ADH, which has a Km of 34 mM for ethanol, contributes to most of the remaining 30% of alcohol metabolism in the liver (Hurley et al., 2002; Li et al., 1977). Class IV ADH has an intermediate Km value but the highest Vmax for ethanol (Kedishvili et al., 1995). It contributes mostly to alcohol metabolism in the stomach, where it is present at maximum concentration (Yin et al., 1990; Yokoyama et al., 1995). Class III ADH is a glutathione-dependent formaldehyde dehydrogenase that metabolizes glutathione adducts such as *S*-(hydroxymethyl) glutathione (HMGSH) and *S*-nitrosoglutathione (GSNO) more efficiently than primary alcohols and aldehydes (Kaiser et al., 1991; Koivusalo and Uotila, 1991; Staab et al., 2008).

In addition to dietary alcohol, other physiological substrates of ADH enzymes have been identified. One important substrate is retinol (vitamin A). Class I, II, and IV enzymes catalyze the oxidation of retinol to retinaldehyde, the first step in the synthesis of retinoic acid (Yang et al., 1994). Class IV ADH is the most active form of retinol dehydrogenase (Zgombic-Knight et al., 1995). Gene deletion studies in mice have shown that the Class IV ADH is protective against retinol deficiencies in the diet (Deltour et al., 1999; Molotkov et al., 2002). Other physiological substrates of ADHs include cytotoxic aldehydes generated during lipid peroxidation (Boleda et al., 1993), ω-hydroxy fatty acids (Boleda et al.,

1993), 3β-hydroxy-5β steroids (McEvily et al., 1988), 4-hydroxy-3methoxyphenyl ethanol (Mardh and Vallee, 1986) and 4-hydroxy-3methoxyphenyl glycol (Mardh et al., 1986; Mardh et al., 1985).

2. Human *ADH* cluster

In humans the seven ADH isozymes are encoded by seven genes *ADH1A* (encodes α), *ADH1B* (β), *ADH1C* (γ), *ADH4* (π), *ADH5* (χ), *ADH6* (no protein; only mRNA), *ADH7* (σ) (Table 1). The seven genes are present as a cluster spanning approximately 365 kb on chromosome 4q23 (Figure 2); a similar clustering of *ADH* genes is also observed in other mammals. In humans, all the seven genes have nine exons and eight introns (Edenberg, 2000). The direction of transcription is also the same and is from qter to pter (shown in the reverse orientation in Figure 2).

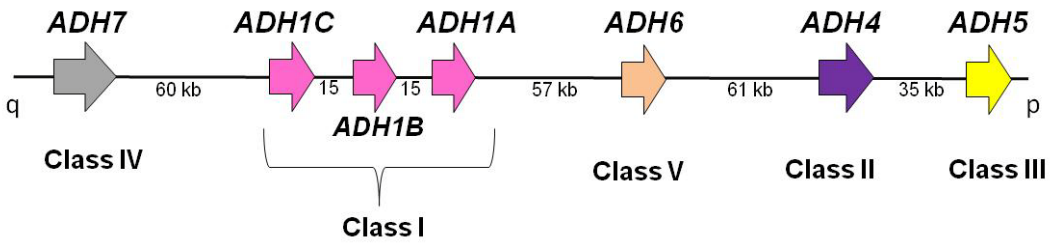

Figure 2. Diagram of the human *ADH* cluster. Seven alcohol dehydrogenase genes are shown in their transcriptional orientation (they are oriented on the chromosome 4q in the opposite direction). Arrows mark the genes and depict the direction of transcription. The genes range in size from 14.5 kb to 23 kb; intergenic distances are given in kb.

All *ADH* genes except *ADH7* are expressed at the highest levels in the liver; *ADH7* is highly expressed in the stomach and the upper gastrointestinal tract (Edenberg, 2000). In other tissues they are expressed to lower levels and each class has a distinct pattern of expression. *ADH5* is ubiquitously expressed and thus is the only *ADH* present in the brain. Tissue distribution of *ADH*s is summarized in Table 1.

With the exception of *ADH1C*, all *ADH*s are detected in fetal liver (Estonius et al., 1996). Class I *ADH*s exhibit temporal expression patterns during development. *ADH1A* and *ADH1B* are expressed in early (second trimester) and late (third trimester) fetal liver, respectively (Smith et al., 1971, 1972). Expression of *ADH1C* is observed only after birth (Smith et al., 1972). Once expressed, *ADH*s are expressed constitutively in adult organisms.

3. Additional pathways of alcohol metabolism

In humans, alcohol is metabolized predominantly in the liver by ADHs. Besides ADHs, oxidative metabolism of alcohol is also catalyzed by cytochrome P450 enzymes including (CYP2E1, CYP1A2 and CYP3A4) and hydrogen peroxide-dependent catalase (Handler et al., 1986; Handler and Thurman, 1988; Lieber, 2004; Lieber and DeCarli, 1968; Salmela et al., 1998; Zakhari, 2006). These three enzyme systems are localized to different sites within a cell; ADHs are present in the cytosol. CYP2E1 and catalase are present in microsomes and peroxisomes, respectively (Handler and Thurman, 1988; Lieber, 2004; Zakhari, 2006). The contribution of CYP2E1 to alcohol metabolism is minor because

CYP2E1 is induced only at elevated concentrations (Badger et al., 1993; Zakhari, 2006). Catalase also has a small role as it is limited by the availability of hydrogen peroxide (Lieber, 1984; Zakhari, 2006). Acetaldehyde generated from alcohol by any of these enzymes is further metabolized to acetate by aldehyde dehydrogenases (ALDH) (Hurley et al., 2002).

4. Alcoholism

Alcoholism is a complex disease affecting millions in the world, including 4 to 5% of the population in the United States at any given time (Li et al., 2007). Chronic alcohol abuse is associated with numerous health risks such as liver cirrhosis, cancer and cardiovascular disease (Cargiulo, 2007; Rehm et al., 2003). In addition, it has undesirable social consequences: traffic accidents, domestic violence, sexual assault and child malnutrition; it is the third leading cause of preventable deaths in the United States (Mokdad et al., 2004).

Diagnostic criteria for alcoholism have been defined in Diagnostic and Statistical Manual of Mental Disorders (DSM) and International Classification of diseases (ICD). According to the most recent DSM criteria (DSM-IV), a person is said to be alcohol dependent if he or she exhibits a maladaptive pattern of drinking with three or more of the following symptoms occurring at any time in a period of one year: tolerance, withdrawal, impaired control, neglect of activities, excessive time spent in alcohol-related activity and/or continued use despite knowledge of the problem (Grant, 1996; Hasin, 2003).

Alcoholism is influenced by both genetic and environmental factors. Evidence for genetic risk was obtained from family, twin and adoption studies (Birley et al., 2005; Goodwin et al., 1973; Goodwin et al., 1974; Kendler et al., 1997; Mayfield et al., 2008; McGue, 1997; McGue, 1999; Nurnberger et al., 2004; Prescott et al., 1999; Prescott and Kendler, 1999). Monozygotic twins of alcoholics exhibit greater risk for alcoholism whereas dizygotic twins of alcoholics are at approximately the same risk as full siblings (Kendler et al., 1997; Prescott et al., 1999). Children adopted away from alcoholic parents exhibit the same risk as the children brought up by their biological parents, further supporting the role of genetics in the risk for alcoholism (Goodwin et al., 1973; Goodwin et al., 1974). Together these studies suggest that greater than 50% of the risk for the disease is from genetic factors.

Several studies have been carried out to identify genes associated with the risk for alcoholism. *ADH* and *ALDH* were the first genes to be associated with alcoholism (Bosron and Li, 1986). Gamma-aminobutyric acid A receptor, alpha 2 (*GABRA2*) (Edenberg et al., 2004), cholinergic receptor, muscarinic 2 (*CHRM2*) (Luo et al., 2005; Wang et al., 2004), cholinergic receptor, nicotinic, alpha 5 (*CHRNA5*) (Wang et al., 2009), opioid receptor, kappa 1 (*OPRK1*) (Edenberg et al., 2008a; Xuei et al., 2007; Zhang et al., 2008a), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*) (Edenberg et al., 2008b) are some of the genes that have been reported recently in genome-wide association studies.

5. *ADH*s and alcoholism

The effects of ethanol on liver and other organs in the body are dependent on the concentrations of ethanol (Gronbaek, 2009). The rate at which ethanol is metabolized influences the concentrations of ethanol and acetaldehyde. Two important factors that could determine the rate of ethanol metabolism are (1). the kinetic properties of *ADH* enzymes, and (2). the levels of *ADH* enzymes. Several studies have reported association of variations in the coding and non-coding variations of *ADH*s with the risk for alcoholism (Birley et al., 2009; Edenberg and Foroud, 2006; Edenberg et al., 2006; Reich et al., 1998; Williams et al., 1999).

Functional variations in the class I *ADHs* have been studied extensively. There are three known alleles of *ADH1B* that vary at a single nucleotide position (Edenberg, 2007; Hurley et al., 2002). These single nucleotide polymorphisms (SNP) lead to non-synonymous changes in the amino acid sequence. The β1 subunit encoded by *ADH1B*1* has arginine (Arg) at positions 48 and 370. In the β2 subunit encoded by *ADH1B*2* subunit Arg at position 48 is changed to histidine (His) whereas in the β3 subunit encoded by *ADH1B*3*, Arg at position 370 is changed to cysteine (Cys). These substitutions result in enzymes with turnover rates 80- to 90-fold greater than *ADH1B*1*(Hurley et al., 2002). The protective effect of these variations was studied in the Asian populations where the *ADH1B*2* allele is most commonly seen. In Chinese men living in Taiwan, the frequency of the *ADH2*2* allele was 0.73 in the non-alcoholic population but reduced to 0.48 in alcoholics suggesting a protective effect (Thomasson et al., 1994; Thomasson et al., 1991).

Two alleles that alter the kinetic properties of the *ADH1C* enzyme have also been identified. The two alleles differ in two amino acid positions; the *ADH1C*1* allele has an Arg and isoleucine (Ile) at positions 272 and 350, respectively. The *ADH1C*2* allele instead has glutamine (Gln) and valine (Val) at the same positions. The protein encoded by *ADH1C*1* has 2.2-fold greater turnover rate than *ADH1C*2* and shown to be protective in Asian population (Hurley et al., 2002).

Besides *ADH* coding variations, variations in *cis*-regulatory elements that affect the levels of *ADH* enzymes have been associated with alcoholism. A SNP at position -136 (relative to the +1 translational start site) in the promoter of the *ADH4* gene affects the promoter activity in hepatoma cells, with the A allele having 2-fold higher activity than the C allele (Edenberg et al., 1999). This SNP has been associated with alcohol dependence in a Brazilian population (Guindalini et al., 2005). In the Japanese population, lower blood alcohol levels were observed in people with this regulatory variation in people with *ALDH2**487Glu/Glu genotype (Kimura et al., 2009).

Regulatory polymorphisms that affect the expression levels were also identified in a distal regulatory element 3 kb upstream of *ADH1C* promoter (Chen et al., 2005). The effect of various haplotypes of this region on basal promoter activity was studied. The haplotypes carried a combination of three SNPs and one 66 bp insertion / deletion. Insertion or deletion alone did not have any effect on the promoter function. However, a significant difference in activity was

observed in two haplotypes that differed at all four positions; one haplotype decreased the promoter activity by 57% whereas another had no effect

Because regulatory polymorphisms may play a critical role in affecting the genetic risks for alcoholism, a comprehensive knowledge of *ADH* transcriptional regulation is necessary.

6. Transcriptional regulation of *ADH*s

Regulation of transcription is accomplished through the complex interaction of *cis*-acting regulatory elements, proteins that bind these elements and the chromatin structure. *Cis*-elements that regulate gene expression include proximal promoters, enhancers, silencers, locus control regions (LCR), and insulators (Maston et al., 2006; West and Fraser, 2005). Enhancers, silencers and LCRs can control gene expression in an orientation-independent and position-independent way, and from locations as remote as 80 kb from the gene (Bondarenko et al., 2003; Maston et al., 2006). Enhancers bind activator proteins that activate transcription by recruiting general transcription factors and RNA polymerase II and/or by recruiting chromatin remodeling complexes that render the chromatin accessible to general transcription factors and RNA polymerase II. Silencers function by binding repressor proteins that inhibit assembly of general transcription factors and thereby repress expression. LCRs are complex regulatory modules with the ability to regulate transcription of multiple genes in the locus (Dean, 2006). Insulators are boundary elements that protect a gene

from the influence of neighboring *cis*-regulatory elements like enhancers or silencers (Bushey et al., 2008).

To understand the regulation of *ADH* expression the proximal promoter regions of *ADH*s have been studied extensively. In addition distant regulatory enhancer for class I ADH genes has been identified. However, distal regulatory mechanisms for the other classes of *ADH* genes have not been addressed yet.

Proximal promoters of the ADH genes have binding sites for multiple proteins (Figure 3). The transcription factors that are important for expression of *ADH*s include CCAAT/enhancer-binding protein (C/EBP) family, Specificity protein 1 (Sp1), CCAAT transcription factor (CTF), upstream stimulatory factor (USF), hepatocyte nuclear factor-1 (HNF-1) and Activator protein-1 (AP-1) (Edenberg, 2000).

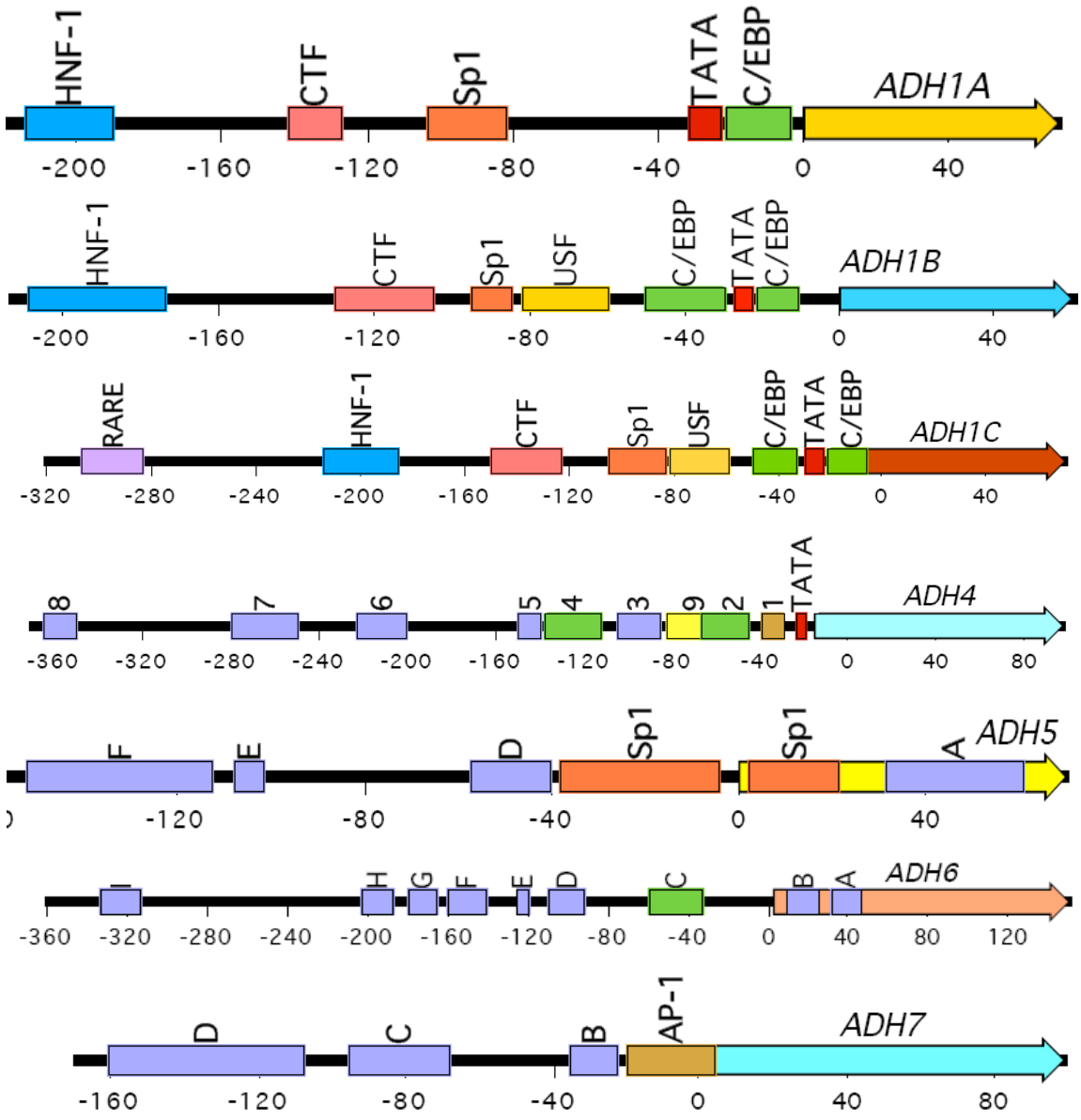

Figure 3. Schematic representation of *cis*-acting elements in the proximal promoters of *ADH* genes. Transcription factors known to bind a given site are shown above the site. Numbering is relative to the +1 transcription start site. Please refer to the text for references.

The human class I *ADH* genes share 80-90% identity in the region extending 270 bp upstream of the transcription start site (Brown et al., 1996). Two C/EBP sites flank the TATA box and both sites are bound by proteins (C/EBPα, C/EBPβ or Albumin D-site binding protein, DBP) in *ADH1B* and *ADH1C*. In *ADH1A* only the downstream site is bound by these proteins (Brown et al., 1994, 1996; Carr and Edenberg, 1990; Stewart et al., 1990a; Stewart et al., 1990b; van Ooij et al., 1992). Binding sites for USF, Sp1, HNF-1 and CTF are also present in the proximal promoters. Sp1, USF and HNF-1 enhance the expression, whereas CTF decreases the expression of *ADH1B* in transient transfection assays in hepatoma cells (Brown et al., 1996). In addition to these elements, *ADH1B* and *ADH1C* have a glucocorticoid response element (GRE) and a retinoic acid responsive element (RARE), respectively (Duester et al., 1991; Winter et al., 1990). The glucocorticoid response element (GRE) in *ADH1B* overlaps with the HNF-1 site and can bind purified glucocorticoid receptor (Winter et al., 1990). Dexamethasone, a synthetic glucocorticoid, can induce two- to four-fold expression from *ADH1B* promoters with GRE (Winter et al., 1990). A similar increase in endogenous expression of *ADH1* was observed in H4IIE-C3 rat hepatoma cells upon treatment with dexamethasone (Dong et al., 1988).

The retinoic acid responsive element (RARE) element in *ADH1C* is created by tandem duplication of 29 bp found in all class I *ADH* promoters. The duplicated downstream sequence can bind retinoic acid receptor and induce expression in the presence of retinoic acid (Duester et al., 1991; Harding and Duester, 1992).

ADH4 proximal promoter has nine protein binding sites, of which seven (sites 1 to 7) are bound by proteins present in liver extract (Li and Edenberg, 1998). Sites 8 and 9 are protected by extracts from kidney and spleen, respectively. C/EBP proteins bind to sites 2 and 4 and AP-1 binds to sites 1, 2 and also 4. Sites 2 to 7 act as positive regulators in rat hepatoma cells, but with different strengths. Site 8 acts as a negative element, decreasing the activity of the basal promoter by 21%.

ADH5 promoter is G-C rich and unlike other *ADH* genes, lacks a TATA box. There are ten (A to J) protein binding sites in the proximal 400 bp region (Hur and Edenberg, 1995). Minimal promoter with sites A through C is functional in H4IIE-C3 rat hepatoma cells, CV-1 African green monkey kidney cells, and HeLa cells. Sp1 binds to all three sites and activates expression. Binding of Sp1, however, is modulated by other members of Sp family and FB-1 transcription factor. Sp3, Sp4 and FB-1 compete with Sp1 to bind to site C and therefore decrease the activity of the promoter (Kwon et al., 1999; Lee et al., 2002). Sites E, G, H and I decrease activity in all cells studied. Sites D and F exhibit cell-specific activity; site D has a positive effect in H4IIE-C3 cells but no effect in the other cells. Conversely, site F acts a positive element in CV-1 and HeLa cells but as a weak negative element in H4IIE-C3 cells (Hur and Edenberg, 1995).

Post-transcriptional regulation, by two upstream AUG codons in the mRNA, of *ADH*5 has also been reported. Mutation at one or both of the upstream AUG codons increased gene expression by two-fold in examined cells (Kwon et al., 2001).

The *ADH6* promoter has nine (A to I) protein binding sites within 300 bp of the transcription start site (Zhi et al., 2000). All sites are bound by liver extract and act as positive elements in rat hepatoma cells. Sites C, D and E are recognized by C/EBPα. Two cell-specific elements are present further upstream, between -1.2 kb and -2.3 kb. Site 1 decreased the activity of the promoter in non-hepatoma cells while site 2 increased the activity in hepatoma cells.

The *ADH7* proximal promoter has four (A to D) protein binding sites, three of which are bound by proteins in the nuclear extract of different cells tested (Kotagiri and Edenberg, 1998). AP-1 binds strongly to site A and weakly to site C. Mutation in site A disrupts AP-1 binding and leads to a decrease in promoter activity, highlighting the importance of this site. C/EBP binds strongly to site B but decreases the activity of the promoter as observed in C/EBP overexpression studies. This effect could be one of the reasons why *ADH7* is not expressed in liver, where C/EBP proteins are present at high levels.

Known *cis*-regulatory elements in the proximal promoter regions do not entirely explain the tissue specific expression of *ADH*s in adults and the temporal expression of class I *ADH* genes in the fetus. In mice, 12 kb upstream and 23 kb downstream regions of *ADH1* were inadequate to induce *ADH1* expression in liver (Szalai et al., 2002). However, 110 kb upstream and 104 kb downstream regions were able to induce expression (Szalai et al., 2002). This indicates the presence of regulatory regions far from the promoter. In humans an HNF-1 binding site, 51 kb away from the class I *ADH* cluster, was identified (Su et al., 2006). This region was shown to regulate tissue specific expression of all the

class I genes and when deleted repressed the expression of each of the class I *ADH* genes in transgenic mice. The HNF-1 binding site was also shown to interact with the class I *ADH* promoters suggesting a DNA looping mechanism of activation.

7. Identification of *cis*-regulatory regions

In humans, 95% of the genome is non-coding sequence, and *cis*-regulatory regions are only a small part of this. Therefore, identifying *cis*-regulatory sequences like enhancers or silencers that can work from hundreds of kb away is a difficult task. Many approaches have been explored in the literature (Elnitski et al., 2006). The classical approach to search for regulatory regions of a gene of interest is to make deletion constructs of proximal regions and test these in reporter gene assays. However, this approach becomes cumbersome to identify distal regulatory regions. A more useful technique to identify distal regulatory regions has been the DNaseI hypersensitivity assay (Gazit and Cedar, 1980). It is based on the principle that the chromatin in the regulatory regions is more accessible to proteins and as a result, more sensitive to DNaseI, a non-specific endonuclease. Another technique that has been widely used in recent years for identifying or characterizing regulatory regions is chromatin immunoprecipitation (ChIP) (Dedon et al., 1991; Kuo and Allis, 1999). The function of regulatory regions is mediated via the binding of trans-acting transcription factors; thus studying DNA-protein interactions *in vivo* leads to identification of regulatory regions. Recently high-throughput versions of DNaseI

hypersensitive site assays and ChIP assays have been developed and used to identify regulatory regions on a genome-wide scale (Crawford et al., 2006a; Ren et al., 2000; Robertson et al., 2007; Sabo et al., 2006; Song and Crawford, 2010). However, these are still not cost-effective approaches for many research labs.

A computer based approach for identifying regulatory regions in the genome is comparative genomics (King et al., 2007; Miller et al., 2004). Comparative genomics involves cross-species sequence comparisons to identify evolutionarily conserved sequences. The underlying assumption for this strategy is that if a region is evolutionarily conserved, it implies a functional role (Hardison, 2000). Or if a region has a critical functional role, like gene regulation, then it is protected from mutations in the sequence. One of the first cellular enhancers discovered was identified through sequence conservation (Emorine et al., 1983). With the availability of genome sequences from increasing number of organisms, identifying regulatory regions through sequence conservation is a powerful tool.

8. Transcription factors

Transcriptional regulation is achieved through interaction of *cis*-regulatory regions with the trans-acting proteins. There are three kinds of transcription factors (Martinez, 2002; Tjian, 1996):

1. general transcription factors including RNA polymerase and transcription factor II family of proteins that are involved in initiation,

elongation and termination of transcription (Sikorski and Buratowski, 2009; Thomas and Chiang, 2006).

2. sequence-specific DNA binding proteins that bind *cis*-regulatory regions in the genome and control the expression of the corresponding genes; activator and repressor proteins fall under this group.

3. transcription cofactors mediate interactions between the basal transcription factors and sequence specific effectors. These include mediator complexes and chromatin remodeling complexes (Casamassimi and Napoli, 2007; Clapier and Cairns, 2009; Thomas and Chiang, 2006).

Activator proteins that are involved in regulatory mechanisms in this study are discussed below.

8.a. FoxA family

FoxA (previously known as Hepatocyte nuclear factor-3) transcription factors are a sub-family of forkhead box (Fox) proteins, which contain a 110 amino acid forkhead DNA binding domain (Weigel and Jackle, 1990). There are three FoxA proteins, FoxA1, FoxA2, and FoxA3, and they share 95% sequence identity in the forkhead domain. Forkhead domain has a ‘winged helix’ structure where three helices are arranged in a helix-turn-helix core, and flanked by loops (Clark et al., 1993). FoxA proteins also have trans-activation and histone interaction domains at the N and C-termini of the protein, respectively (Pani et al., 1992; Qian and Costa, 1995). FOXA proteins recognize VAWTRTTKRYTY

sequence, where V is A/C/G nucleotide, W is A/T, R is A/G, K is G/T and Y is C/T (Overdier et al., 1994)

FoxA proteins are highly expressed in the liver and regulate many liver-specific genes in adult organisms (Friedman and Kaestner, 2006; Schrem et al., 2002). Albumin (Herbst et al., 1991), aldoalse B (Gregori et al., 1994), transerythrin (Herbst et al., 1991) are some of the genes that are regulated by FOXA proteins. FOXA proteins play essential roles during development. They are expressed sequentially during development; FoxA2 appears by embryonic day 6.5 (E6.5), followed by FoxA1 and FoxA3 (Sasaki and Hogan, 1993). FoxA2 null mutations are embryonic lethal while FoxA1 and FoxA3 are postnatally lethal (Lee et al., 2005).

FoxA proteins belong to a class of transcription factors that function as pioneer factors, proteins that can bind highly compacted chromatin and alter the chromatin structure and enhance transcription (Zaret et al., 2008). During development, FoxA proteins have been shown to bind the enhancer of the albumin gene and open the chromatin (Chaya et al., 2001; Cirillo et al., 2002). FoxA1 has also been shown to act as pioneer factor in adult tissues (Carroll et al., 2005; Gao et al., 2003; Zhang et al., 2005).

8.b. HNF-1A

Hepatocyte nuclear factor -1α (HNF-1A) is a liver enriched transcription factor with POU and homeodomain DNA binding domains (Baumhueter et al., 1990). It also has three transactivation domains and a myosin-like dimerization

domain (Mendel et al., 1991a). It recognizes a consensus sequence GTTAATNATTAAC and binds to DNA as a dimer (Courtois et al., 1988; Frain et al., 1989). HNF-1A homodimers are stabilized by the protein dimerization cofactor of HNF-1 (DCoH). DCoH does not bind DNA nor does it interfere with the binding of HNF-1A to DNA (Mendel et al., 1991b). Like FoxA proteins, HNF-1A transcribes many liver specific genes like albumin (Lichtsteiner et al., 1987), α-antitrypsin (Courtois et al., 1987), α- and β-fibrinogen (Courtois et al., 1987), and others (Schrem et al., 2002).

9. Alcohol and the liver

In addition to understanding the genetic risk factors of alcoholism, it is also important to gain knowledge on the pathogenesis of the disease. Alcohol is chiefly metabolized in hepatocytes, parenchymal cells which form 85% of the total volume of a healthy liver (Tsukamoto and Lu, 2001). Liver is the most susceptible organ for alcohol induced injuries. Chronic alcohol abuse leads to alcoholic liver diseases, ALDs (Fleming and McGee, 1984; MacSween and Burt, 1986; Mann et al., 2003; McCullough and O' Connor, 1998). The most prevalent ALD is alcoholic steatosis or fatty liver, which is characterized by fat deposition in the liver and hepatomegaly (MacSween and Burt, 1986). Fatty liver, upon further exposure to alcohol, develops alcoholic hepatitis, where there is inflammation of the liver. The most severe form of ALD is cirrhosis in which fibrotic tissue replaces the normal liver tissues and leads to liver dysfunction. In a small

percentage (1- 2%) of people, cirrhosis leads to hepatocellular carcinoma (Seitz and Stickel, 2006).

Several molecular mechanisms have been implicated in the development and progression of ALD. Acetaldehyde, the break down product of alcohol, forms adducts with proteins, and disrupts their function (Niemela, 2001; Niemela et al., 1998; Worrall et al., 1990). Another key effect of alcohol metabolism is the altered energy state of the cell. In both ADH and ALDH catalyzed reactions, NAD^+ is reduced to NADH, increasing the NADH/NAD+ ratio in cells (Cunningham et al., 1986). This change in the redox state leads to inhibition of activity of many enzymes that are involved in metabolic pathways like carbohydrate metabolism (Badawy, 1977) . NADH also enters the electron transport chain and leads to the generation of reactive oxygen species (ROS) (Albano, 2006; Bailey et al., 1999; Wu and Cederbaum, 2009). ROS cause damage to mitochondrial membrane and also induce oxidative stress within the cell (Bailey and Cunningham, 2002; Bailey et al., 1999; Cunningham and Bailey, 2001). Upon chronic alcohol abuse, this oxidative stress overwhelms the cellular redox system and leads to the depletion of antioxidants such as reduced glutathione (Bai and Cederbaum, 2006; Garcia-Ruiz et al., 1994; Hirano et al., 1992). ROS also cause peroxidation of lipids which further increases the oxidative stress in the cell (Niemela, 2001; Niemela et al., 1998; Worrall et al., 1990).

Alcohol metabolism affects some of the key enzymes involved in lipid metabolism. Acetaldehyde decreases the DNA binding ability of the heterodimer

of proliferator-activated receptor-α (PPARα) and retinoid X receptor (RXR) (Galli et al., 2001). PPARα-RXR dimer is involved in the transcription of many fatty acid oxidation enzymes including carnitine palmitoyl transferase-1 (CPT1A), a rate limiting enzyme in the pathway (Aoyama et al., 1998; Zammit, 2008).

Another protein that is affected by ethanol is AMPK (AMP- activated protein kinase). Activation of AMPK leads to fatty acid oxidation and concurrent inhibition of fatty acid synthesis (Hardie et al., 1998). AMPK mediated regulation of the fatty acid oxidation is brought about by inhibition of acetyl-CoA carboxylase (ACC), and activation of malonyl Co-A decarboxylase (MCD). The activity of these two enzymes leads to a decrease in the concentration of malonyl Co-A and activation of CPT-1A. Ethanol decreases the activity of AMPK, thus inhibiting fatty acid oxidation and promoting fatty acid synthesis (You et al., 2004).

Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors involved in the transcription of many genes involved in fatty acid synthesis (Eberle et al., 2004). They play an important role in the development of alcohol induced fatty liver (You and Crabb, 2004a; You et al., 2002). Ethanol activates transcription from SREBP regulated promoters and leads to an increase in the expression of lipogenic enzymes (You et al., 2002).Thus, the combined effects of ethanol on PPARα and AMPK lead to the inhibition of fatty acid oxidation, increase in the fatty acid synthesis in the liver and development of fatty liver (Purohit et al., 2009; You and Crabb, 2004b).

Chronic alcohol abuse damages the lining of the intestine, ultimately exposing the liver to gut-derived bacterial endotoxins (Bode and Bode, 2005;

Keshavarzian et al., 1999). These endotoxins activate the liver macrophages, Kupffer cells, which release ROS and cause more damage to hepatocytes (Bode and Bode, 2005; Thurman, 1998). Kupffer cells also produce inflammatory cytokines such as TNF-α that contribute to liver inflammation. Acetaldehyde adducts, cell death seen in hepatocytes due to ROS also trigger an immune response against the alcohol-injured liver.

In addition, ROS and cytokines from hepatocyte and Kupffer cells, activate the hepatic stellate cells (HSC) (Nieto et al., 2002; Siegmund and Brenner, 2005; Wheeler et al., 2001). Upon activation HSC proliferate and increase the synthesis and secretion of extracellular matrix (ECM) proteins, particularly collagen (Cubero et al., 2009; Rojkind and Martinez-Palomo, 1976; Siegmund and Brenner, 2005). Accumulation of the ECM proteins alters the morphology of the liver, leading to the development of fibrotic liver.

10. Alternative transcript isoforms and diseases

One of the post-transcriptional processes that regulate gene function is splicing. In splicing, introns are removed and the remaining exons are spliced to form the mature protein coding or functional RNA (Wang et al., 2008). Exons or introns can be spliced in different arrangements and this process is termed alternative splicing. Diverse mRNA or proteins known as alternative isoforms can be generated from same gene by alternative splicing. Alternative splicing generates another layer of gene regulation and recent estimates suggest that 92-94% of the human genes are alternatively spliced (Wang et al., 2008). One-third

of alternative splicing events are cassette exons (exons that can be included or excluded in the transcript). Another type of alternative splicing event is where an alternative donor or acceptor splice site is used, generating a different 5’ and 3’ end of the exon. An intron can also be included during splicing to synthesize an alternatively spliced form. Alternative isoforms can also be generated by using an alternative transcription start site, alternative poly (A) site (alternate poly adenylation site that signals transcription termination) (Chen and Manley, 2009). Different ways in which alternative isoforms could be generated are shown in Figure 4. In this dissertation, all events mentioned above, that generate alternative isoforms will be referred to as alternative splicing events.

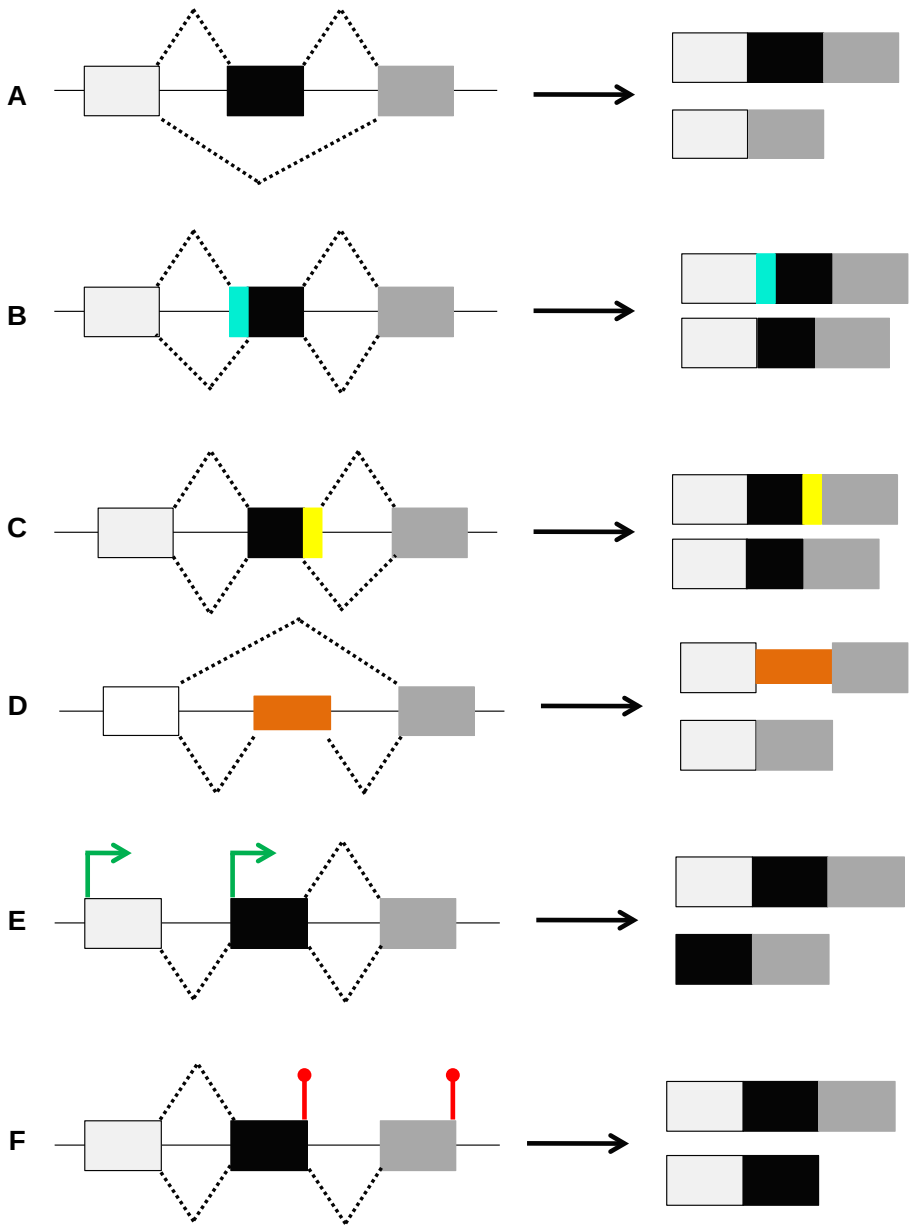

Figure 4. Generation of alternative transcript isoforms. Multiple ways in which alternative isoforms of a transcript can be synthesized are shown. (A) Inclusion or exclusion of a cassette exon. (B) Use of alternate acceptor site during splicing. (C) Use of alternate donor site during splicing. (D) Retention of an intron. (E) Use of alternate start site (indicated by an arrow) and (F) Use of alternate poly(A) site (indicated by a red line ending with a circle). Exons are shown as boxes. Dashed lines indicate splicing events and dark lines indicate introns.

Splicing defects can be the primary cause of a disease, as seen in spinal muscular atrophy (Zhang et al., 2008c), tauopathies (Gallo et al., 2007), atypical Cystic fibrosis (Buratti et al., 2001), Hutchinson-Gilford progeria syndrome (De Sandre-Giovannoli and Levy, 2006) and others (Garcia-Blanco et al., 2004; Orengo and Cooper, 2007). Alternative splicing has also been implicated in cancer, where key cancer genes like BRCA-1 have been shown to be alternatively spliced (Hoffman et al., 1998). One of the aims of this study was to examine the effects of alcohol on alternative splicing to gain insight into the molecular mechanisms of alcohol induced liver injuries.

11. Global transcriptional profiling

One of the aims of this study was to examine global effects of alcohol on gene expression and alternative transcript isoforms. The most popular approach to study these genome-wide changes is to use microarrays. Microarray technology allows the hybridization, in parallel, of thousands of labeled molecules in a sample to surface bound nucleic acid probes and the subsequent quantitation of bound molecules.

Recently Affymetrix GeneChip® Human Exon 1.0 ST Arrays (for simplicity these will henceforth be referred to as Exon arrays) have been developed. Unlike the traditional 3’ arrays that have probes targeting only the 3’ end of a transcript (Affymetrix, 2003), exon arrays have probes against the entire length of the transcript (Affymetrix, 2005a). Therefore, exon arrays can be used to not only quantitate gene expression, but also identify alternative isoforms. On average,

each exon in a transcript has one probe set and each probe set has four perfect match probes. In genes with only one exon, such as histones or CEBP family of transcription factors, more than one probe set is present per exon. There are a total of 1.4 million probe sets. From highest to lowest levels of confidence of annotation, probe sets are classified into core, extended or full probe sets (Affymetrix, 2005a). Core probe sets are supported by the RefSeq and full length mRNA GenBank records with full coding sequence (CDS) information. Extended probe sets, on the other hand, come from other cDNA evidence, including other human mRNA, EST sequences, ENSEMBL gene collections, syntenically mapped mRNA from mouse, rat and human, mitoMap mitochondrial genes, microRNA registry genes, vegaGene , and vegaPseudoGene records (Affymetrix, 2005a). Full probe sets are based on computational gene predictions like Genescan (Affymetrix, 2005a).

General work flow for exon array analysis includes the following steps: data normalization, summarization for each probe set, present/absent filtering, statistical analysis of differential gene expression and alternative splicing and finally biological significance of the affected genes in the experiment. Quantile normalization of the data is carried out because signals from different hybridizations may be at different scales. Data summarization generates a summary measure for a probe set from the signals of all of the (usually 4) probes in the set. Robust multi-array analysis (RMA) and the probe logarithmic intensity error (PLIER) are two of the commonly used approaches for data summary (Affymetrix, 2005c; Irizarry et al., 2003). Both algorithms use an error model-

based approach to summarize signals. RMA assumes that the error is proportional to the normalized and background-adjusted probe intensity. On the other hand, PLIER assumes that the error is proportional to the probe intensity without background correction.

After the probe set summary signals are obtained, it is necessary to remove signals that are not significantly different from the background. Pre-analysis filtration of absent probe sets decreases the noise and enhances the ability to detect real differences (McClintick and Edenberg, 2006). A detection above background correction was proposed for exon arrays to remove probe sets with signals near background (Clark et al., 2007). In addition to removing probe sets based on signal, other filtering steps have also been shown to decrease the noise and improve detection of alternative splicing events. Filtering probe sets based on hybridization specificity (bind to unique or more regions), or by fraction present (a probe set should be present in a pre-defined fraction of arrays used in the experiment) has been shown to improve the ability to detect real differences (McClintick and Edenberg, 2006; Mieczkowski et al., 2010; Xing et al., 2008).

Once probe set signals are obtained, signals from all the present probe sets in the gene are summarized to estimate the expression level of the gene of which they are part. Many approaches have been proposed for estimating gene expression. PLIER can be used to generate gene level estimates in a manner similar to probe set summarization (Affymetrix, 2005b). A simpler approach is to use median intensity of all the probe sets in a transcript (Clark et al., 2007). Clark

et al. reported that, in their data, median intensity of all core probe sets was a good estimate of gene expression. Once the probe set and gene signals are estimated, standard statistical tests like Student t-test can be used to look for genes or probe sets that have significantly different expression values between the test conditions. When the p-value is below the pre-defined threshold, for example, 0.05, the difference is said to be significant. Because of the large number of tests carried out in microarrays, multiple testing corrections are applied to the analyses to control the false positive rate. A false discovery rate (FDR) method of correction for multiple testing was proposed by Benjamini and Hochberg (1995). FDR estimates the number of false positives that can be expected at a particular p-value, although it does not identify which results are false positives. Similar to p-value, a threshold for FDR can be used to identify significantly affected genes

Analysis of the Exon array data to identify differentially expressed genes or alternatively spliced exons is challenging because exons and genes are not independent elements and cannot be treated so for statistical analysis. Therefore it is difficult to differentiate gene expression and alternative splicing. The estimation of overall expression of a gene is affected by the number of alternatively spliced probe sets or exons in the gene. For example, in the absence of any alternative splicing, comparison of gene expression values in two conditions should detect differentially expressed genes. However, if an exon is alternatively spliced, this also influences gene expression estimates and the gene could be falsely detected as differentially expressed. Equally, the detection

of alternative splicing in exons is dependent on the gene expression levels. It is not possible to use probe set expression values in two conditions to compare and detect alternative spliced exons, because if a gene is differentially expressed, then levels of all probes are affected and simply comparing the probe set expression values could lead to all probe sets detected as differentially spliced.

The goal is to detect differential gene expression as well as alternatively spliced exons. Two common approaches are used in the literature to detect alternative splicing. In the splicing index approach, probe set values are normalized to the gene signals (Clark et al., 2007; Srinivasan et al., 2005). Dividing probe set values by gene values should account for differential gene expression. It is, however, dependent on how the gene expression values are estimated. The second approach to analyze exon array data is two-way ANOVA. The two-way ANOVA model for alternative splicing includes experimental conditions and probe sets within the transcript as factors. The interaction between the probe set and experimental factors (like treatment condition) is used to identify probe sets that are alternatively spliced in the experimental conditions. However, both splicing index and two-way ANOVA do not overcome the effect of alternative splicing on differential gene expression. Also, both assume that all probe sets across a gene will have a similar response to changes in gene expression. This has been shown not to be true: one common observation is that the ends of the transcripts respond differently than the probe sets away from the ends (Bemmo et al., 2008; Whistler et al., 2010). This leads to an edge bias

effect in which many probe sets towards 5’ and 3’ ends are detected falsely as alternatively spliced (Bemmo et al., 2008; Whistler et al., 2010).

In this dissertation, exon arrays were used to study the effects of alcohol on gene expression and alternative splicing.

12. Research objectives

There are three main objectives of this research. The first objective was to investigate the transcriptional regulation of the *ADH* genes. Understanding the regulation of *ADH* genes is important because of the key role they play in alcohol metabolism and risk for alcoholism. A sequence conservation approach was taken to identify putative distal regulatory elements in the *ADH* cluster. These putative regions were tested for activity *in vitro* and a strong enhancer was identified and characterized.

The second objective was to study the effects of variations on the *cis*-regulatory regions in the *ADH* cluster. Many variations in the *ADH* cluster have been associated with the risk for alcoholism. Since most do not affect the structure of the encoded proteins; they could either affect gene regulation or merely be associated (in linkage disequilibrium) with variants that affect function in some way. The effect of variations on the activity of two *cis*-regulatory regions of *ADH* cluster was examined in this study. The first region that was examined was the enhancer identified in this study. Two variations greatly affect activity and we hypothesize that these could affect the risk for the disease. The second region that was examined was the *ADH1B* proximal promoter. There was already

evidence of association of two SNPs in this region with alcoholism (Edenberg et al., 2006). We examined the functional effects of variations in this region and observed that one variation decreases the activity of the promoter.

The third objective was to understand the effects of chronic alcohol exposure on global gene expression and alternative splicing in HepG2 human hepatoma cells. Microarray technology allowed us to explore the effects of ethanol at a global level and to gain more insight into the alcohol-induced liver injuries observed in chronic alcoholics. This was the first study to explore the effects of ethanol on alternative splicing.

II. MATERIALS AND METHODS

1. Identification of putative distal regulatory elements

To identify putative distal regulatory regions in the *ADH* cluster, comparative genomics approach was taken. University of California, Santa Cruz (UCSC) genome browser (March 2006, NCBI36/hg18 assembly) 28-Way Cons Track was used to identify evolutionarily conserved regions (Blanchette et al., 2004; Chiaromonte et al., 2002; Kent et al., 2003; Siepel et al., 2005). As sequence conservation was absent or not reported in the non-placental vertebrates at the time, only the placental mammals for which multiple alignments were available were used for this study: Rhesus, bushbaby, tree shrew, rat, mouse, guinea pig, rabbit, shrew, hedgehog, dog, cat, horse, and cow. If at least 50% sequence conservation was observed, the region was considered a putative regulatory region.

2. Cloning of test fragments

For testing putative distal regulatory elements, proximal promoters of *ADH4* (4Basal; −41 to −299 bp relative to *ADH4* +1 CDS) and *ADH1B* (1Basal; −10 to −169 bp relative to *ADH1B* +1 CDS) were amplified by PCR from human DNA using R-Taq polymerase (Midsci, St. Louis, MO). SV40 promoter was amplified from the pGL3 control vector (Promega, Madison, WI). Oligonucleotides used in PCR are listed in Table 2. All promoters were subcloned into KpnI and XhoI sites in the pXP2 luciferase reporter plasmid (Nordeen, 1988). Putative regulatory elements were cloned upstream of the

4Basal promoter; the position of each fragment relative to the nearest CDS and restriction sites are given in Table 3. Subfragments of 4E were cloned into BamHI and SalI sites of pXP2, upstream of 4Basal.

To test *ADH1B* proximal promoter haplotypes, the region extending to 1484 bp upstream of *ADH1B* +1 CDS was PCR amplified using high fidelity platinum Pfx polymerase (Invitrogen, Carlsbad, CA). Primers used are listed in Table 2. PCR cycle conditions were as follows: 94 ^{0}C/ 5 min, (94 ^{0}C/15 s, 62 ^{0}C/20 s, 68 ^{0}C/ 90 s)x 10 cycles, (94 ^{0}C/15 s, 60 ^{0}C/20 s, 68 ^{0}C/ 90 s)x 30 cycles, 68 ^{0}C/ 7 min. DNA from five different individuals was used as a template. All test fragments were cloned into KpnI and BglII sites in pXP2 luciferase reporter vector (Nordeen, 1988). Clones were sequenced by ABI BigDye terminator v3.1 cycle sequencing kit and five different haplotypes were obtained (Table 10).

Primer	Sequence	Description
HE3475	GTGGTACCGGGCTTTTCTCTATTATTTTA	4Basal _F
HE2492	CCCTCGAGAAGCTTCAAACTCCTACCCA	4Basal _R
HE3639	GTGGTACCAATCCAGTGGGTGTGGC	1Basal _F
HE3640	CCAAGCTTGTCTTCTCTGCCCACCAG	1Basal _R
HE3641	GTGGTACCCTGCGATCTGCATCTCAATTA	SV40 Basal _F
HE3642	CCAAGCTTAGTACCGGAATGCCAAGC	SV40 Basal _R
HE3481	CGGGATCCCAAGCCAGAATGAAAAGGTAGAC	4E _F
HE3482	CCAAGCTTAGCCAGAGCACAAATAATGGAG	4E _R
HE3623	CGGGATCCCCAAGCCAGAATGAAAAGGTA	4E1 _F
HE3633	GCGTCGACTTGCGATTTCTCTGGGATG	4E1 _R
HE3627	CGGGATCCTCAGGTCCATTCTGTGAACG	4E2 _F
HE3635	GCGTCGACTGTAGTCTCCCCTCTCTTGCTG	4E2 _R
HE3629	CGGGATCCCAGATAACAGCAAGAGAGGGG	4E3 _F
HE3636	GCGTCGACCAGCCAGAGCACAAATAATGG	4E3 _R
HE3530	CGGGATCCGCAGTCTCTATGTATTCTCTTGCC	4-5-a_F
HE3531	CCCCGGGGCTCAGTGGGCTTGTAAACG	4-5-a_R
HE3477	CGGGATCCTGAGGTGATAGATACCCTATTTA	6-4-a_F
HE3478	CCAAGCTTTTTGAGAACTGGGTTAGGTT	6-4-a_R
HE3526	CCCAAGCTTTTACAGAAAAGCCAACGCTG	1A-6-a_F
HE3527	CCCGGGGTCACCAGAGGGATGTGTTTG	1A-6-a_R
HE3524	CGGGATCCCTGTGATTGATTGGGTGTCG	1A-6-b_F
HE3525	CCCCGGGGGGGGAGGATTTAGCACCTATT	1A-6-b_R
HE3528	CGGGATCCAATAGGTGCTAAATCCTCCCC	1A-6-c_F
HE3529	CCCGGGGTCAAGAGATGTCTGGCTGTGAC	1A-6-c_R
HE3483	CGGGATCCAACCAATCTGCCCTGTG	1A-6-d_F
HE3484	CCAAGCTTGGAAGGAGGGGGTGAGATAG	1A-6-d_R
HE3485	CGGGATCCGTTTTCTGAGGCTTCCC	1A-6-e_F
HE3486	CCAAGCTTCCCTGATGTGATTATTGTGC	1A-6-e_R
HE3116	GTGGTACCCTGGGGCTATCTTCTTTCCG	*ADH1B* proximal promoter_F
HE3003	CGAGATCTGTCTTCTCTGCCCACCAGC	*ADH1B* proximal promoter_R

Table 2. Primers used to clone test fragments. Forward (F) and reverse (R) primers used for amplification of promoters and test regions are given.

Fragment	Nearest + 1 CDS	Location	Restriction sites
4-5-a	ADH5	-24,880 to -23,299	BamHI & SmaI
4E	ADH4	-14,506 to -13,003	BamHI & HindIII
6-4-a	ADH4	-20,549 to -19,471	BamHI & HindIII
1A-6-e	ADH6	-32,319 to -31752	BamHI & HindIII
1A-6-d	ADH6	-34,944 to -33,238	BamHI & HindIII
1A-6-c	ADH6	-46,988 to -45,523	BamHI & SmaI
1A-6-b	ADH6	-48,607 to -46,968	BamHI & SmaI
1A-6-a	ADH6	-49,682 to -48,534	HindIII & SmaI
4E1	ADH4	-14,506 to -13,973	BamHI & SalI
4E2	ADH4	-14,057 to -13,539	BamHI & SalI
4E3	ADH4	-13,567 to -13,003	BamHI & SalI

Table 3. Putative distal regulatory elements. The putative distal regulatory fragments that were tested in this study are listed along with the nearest (with respect to the direction of transcription in the cluster) +1 CDS. Location is relative to the nearest +1 CDS based on the human genome NCBI build 36.

3. Transient transfections and reporter gene assays

HepG2 human hepatoma cells (HB-8065; ATCC, Manassas, VA) were cultured in MEM (ATCC) with 10% FBS (Invitrogen, Carlsbad, CA), 4 mM glutamine (Thermo Scientific Hyclone, Waltham, MA) and 1X Penicillin and Streptomycin (Thermo Scientific Hyclone) on cell bind surface plates (Corning Inc, Corning, NY) at 37 ^{0}C. For transient transfection assays of putative distal regulatory elements, 3 x 10^5 cells were seeded per well in 12-well plates. 24 h after seeding, cells were transfected in complete media with 500 ng of test DNA, along with 15 ng of pCMV β-galactosidase plasmid (Clontech, Mountain View, CA) and 485 ng of pUC19 DNA, using 2 μl of Fugene HD (Roche, Indianapolis, IN) per 1 μg of DNA. Cells were harvested 30 h after addition of DNA by scraping into ice-cold 1X PBS, pelleted by centrifugation and suspended in 100 μl of 1X Reporter lysis buffer (Promega, Madison, WI). Cell extracts were prepared by repeated cycles of freeze-thawing; 5 μl of the extract was used for each assay. Luciferase assays were carried out using the Luciferase assay system (Promega, Madison, WI), with activity measured on a Spectromax LS (Molecular devices, Sunnyvale, CA). β-galactosidase assays were carried out using the Galacto-Light System (Tropix, Benford, MA).

For transfection assays with *ADH1B* proximal promoter haplotypes, 8 x 10^5 cells were seeded per well in 6-well cell binding surface plates. 24 h after seeding, cells were transfected in serum free media with 2 μg of test DNA, along with 140 ng of CMV-galactosidase plasmid (Clontech, Mountain View, CA) and 1.2 μg of pUC19 DNA using 3 μl Fugene HD. Complete medium was added 5 h

after addition of DNA and cells were cultured for another 24 h. Cells were harvested 30 h after addition of DNA, and processed and assayed as described above. 20 μl and 5 μl of the extract were used for Luciferase and β-galactosidase assays, respectively; activity was measured on a Lmax Plate Luminometer (Molecular Devices).

H1299 human lung carcinoma cells (ATCC CRL-5803) were cultured in high glucose DMEM (Sigma- Aldrich, St. Louis, MO) with 10% FBS, 2 mM glutamine and 1X Penicillin and Streptomycin on plastic plates (BD Biosciences, San Jose, CA) at 37 ^{0}C. Cells were seeded at 7 x 10^{5} per well in 6-well plates (BD biosciences, San Jose, CA). 24 h after seeding, cells were transfected in complete media with 2 μg of test DNA, along with 135 ng of β-galactosidase plasmid and 1.1 μg of pUC19 DNA using 3 μl of Fugene HD per 1 μg of DNA. Cells were harvested 30 h after addition of DNA, and processed and assayed as described above. 15 μl and 2.5 μl of the extract were used for Luciferase and β-galactosidase assays, respectively; activity was measured on a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Sparks, MD).

All test constructs were transfected at least in triplicate in each individual experiment, with experiments repeated at least three times. Promoter activity was defined as luciferase activity normalized to β-galactosidase activity, to correct for the transfection efficiency. A t-test assuming unequal variances was carried out in Microsoft Excel, considering each individual transfection as an independent data point.

4. Electrophoretic mobility shift assays (EMSA)

EMSAs (Sambrook et al., 1989) were carried out with double-strand oligonucleotides designed to span the putative transcription factor binding sites (Table 4). Oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA) with a 5′ 6-FAM label on one of the strands, which was annealed to the complementary unlabeled oligonucleotide. For annealing, the two single-stranded oligonucleotides were diluted to 5 μM in annealing buffer (10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0) and 50 mM NaCl) and mixed in a 1:1 ratio. Oligonucleotides were heated to 95 ^{0}C for 3 min, cooled to 5 ^{0}C above their melting temperature over a period of 5 min, cooled further to 5 ^{0}C below melting temperature over a 1 h period. They were finally allowed to cool to 20 ^{0}C over a period of 3 h.

Nuclear extracts were prepared from HepG2 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermoscientific Pierce, Waltham, MA), following the manufacturer's protocol. Protein concentrations were measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein binding reactions were carried out with 0.2 or 0. 4 pmol of the annealed oligonucleotides and 10 μg of the nuclear extracts in 10 mM Tris-HCl (pH 7.5), 60 mM potassium chloride, 2.5 mM magnesium chloride, 1 mM EDTA, 1 mM DTT, 750 ng of poly (dIdC) and 7% glycerol. Oligonucleotides were incubated with the nuclear extract for 30 min at 25 ^{0}C. In competitor assays, unlabeled competitor oligonucleotides in 50-fold molar excess to the labeled oligonucleotides were added to the reaction before addition of the probe. For supershift assays, 2 μg of the antibody was added to

each reaction. FOXA1 (sc-9186x), FOXA2 (sc-9187x) and IgG (sc-2028) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-protein complexes were electrophoresed on 6% polyacrylamide Novex DNA Retardation Gels (Invitrogen) in 0.5 x TBE running buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.3) and scanned with fluorescent image analyzer FLA-5100 (Fujifilm, Valhalla, NY) at 473 nm with the LPB filter.

Oligonucleotide	**Sequence**	**Description**
HE3781	5′6-FAM-TTTCAAGATCAGCAATTTGACAGCAAACATGAACTTTGTA	EMSA Oligo 1
HE3782	5′6-FAM-CTGCTTCCCTAACAAACACTGAAAAGATCAA	EMSA Oligo 2
HE3941	5′6-FAM-TAAGCATGTTGTCTTATTTGTTAATATGTTACATAATAC	EMSA Oligo 3
HE3784	5′6-FAM-TTAGTTTCTTCCCACTAAATAAAAACAAACAGAAGTTTTC	EMSA Oligo 4
HE3918	GCCCATTGTTTGTTTTAAGCC	FOXA consensus
HE3829	TCCCATACCCCCATTTAAGCC	FOXA consensus mutated
HE4017	AAGTTAATGATTAAC	HNF-1A consensus

Table 4. Oligonucleotides used in EMSA. Only the sense strand is shown; complementary oligonucleotides were annealed to these and double stranded oligonucleotides were used in EMSA.

5. Site directed mutagenesis

Mutants of the potential transcription factor binding sites in fragment 4E3 were generated by overlap extension PCR (Sambrook et al., 1989). Oligonucleotides in which the potential transcription factor binding sites were mutated (Table 5) were synthesized by Integrated DNA Technologies (Coralville, IA). In the first step of the PCR, two fragments were generated such that they overlap at the mutated sequence. Products were gel extracted and 75 ng of each product was used as template in the extension step. Products with overlapping ends were mixed with R-Taq polymerase and 10 PCR cycles were run without primers. HE3629 and HE3636 were then added and PCR was continued for another 25 cycles to amplify the full-length fragment. Products of the extension step were column purified and cloned into BamHI and SalI sites in the pXP2 vector, upstream of the *ADH4* promoter.

6. Generation of the 4E haplotypes

Different haplotypes of the 4E3 region were generated by site-directed mutagenesis with the Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed with the SNP at the center (Table 5). The amplified products were digested with DpnI to remove the template plasmid, and then transformed into DH5α competent cells (Invitrogen). Transformants were sequenced to confirm the presence of the SNP at the desired site.

Oligonucleotide	Sequence	Description
HE3825	TTCAAGATCAGCAATTTGACA**CACCCGT**TGAACTTTGTAATCAAACAGAC	Site 1 mutant
HE3843	AATCAAACCTCTGCTTCCCTA**CACCCGT**CTGAAAAGATCAAACGGG	Site 2 mutant
HE3876	GCA**CAGCCC**CTAATTTGTTAATATGTTACATAATACTTACCTCACAGGGTT	Site 3 mutant
HE3872	GCATGTTGTCT**ACCCCCA**TAATATGTTACATAATACTTACCTCACAGGGTT	Site 4 mutant
HE3874	GTAAGCATGTTGTCTTATT**CACCCG**TATGTTACATAATACTTACCTCAC	Site 5 mutant
HE3823	AGTTTCTTCCCACTAAATAAAA**CACCCGT**GAAGTTTTCTCTTAGCTAACA	Site 6 mutant
HE3881	CA**CAGCCC**CTAA**CCCCCA**TAATATGtTACATAATACTTACCTCACAGGGTT	Sites 3 and 4 mutant
HE3817	CCCAAATTTCATCGAACA**T**CCTAAAACTTTCAAGATC	rs7678936 T allele
HE3819	GTTGTCTTATTTGTTAATATG**G**TACATAATACTTACCTCACAG	rs7678890 G allele
HE3821	GTCTCTTTTCTGG**A**AAATCAGAGATCTGTCATTG	rs11401494 T allele

Table 5. Primers used in site-directed mutagenesis. Only the forward primer is listed, with the mutated site bold and underlined. Complementary oligonucleotides were used as reverse primers.

7. Long-term treatment of HepG2 cells with ethanol

Two million HepG2 cells were seeded per 75 cm^2 flask (Corning Inc., Corning, NY). Six hours after seeding, ethanol was added to a final concentration of 75 mM to one set of flasks. Every 24 h, media was replaced in both control and ethanol treatment flasks with fresh media, without or with ethanol, respectively. Four days after seeding, cells were trypsinized and seeded into new flasks at two million viable cells per flask, with media changes continuing daily as before. Five days after the split (a total of 9 days without or with ethanol), cells were collected by trypsinization. Four independent experiments were carried out.

The numbers of viable cells in the control and ethanol treatment flasks were measured by trypan blue exclusion. Briefly, 0.1 ml of 0.4% Trypan blue stain (Sigma T8154) was added to 0.5 ml of trypsinized cells, and allowed to stand at room temperature for 5 min. Cells that were not stained by the dye were counted as viable.

8. RNA extraction, labeling and hybridization

Trypsinized cells were pelleted by centrifugation at 2500 rpm for 3 min. The cell pellet was suspended in 5 ml of Trizol (Invitrogen, Carlsbad, CA) and total RNA was extracted following the manufacturer's protocol. RNA was further purified on RNeasy Mini kit columns (Qiagen, Valencia, CA). The quality of the RNA was tested on a Bioanalyzer (Agilent Technologies, Palo Alto, CA), with RNA integration numbers (RIN) from 9.6 to 10. Starting with 1 μg of total RNA, ribosomal RNA (rRNA) was reduced from the total RNA using RiboMinus

Human/Mouse Transcriptome Isolation Kit (Invitrogen, Carlsbad, CA). cDNA was synthesized with random primers tagged with the T7 promoter. This cDNA was used as template for cRNA synthesis by T7 RNA polymerase, using the GeneChip Whole Transcript Sense target labeling assay kit (Affymetrix, Santa Clara, CA). The sense strand of cDNA was then synthesized from cRNA, fragmented and terminally labeled with biotin. Fragmented and labeled cDNAs were subsequently hybridized to GeneChip Human Exon 1.0 ST Arrays (Affymetrix). Chips were stained, washed and scanned following the standard Affymetrix protocols. Two technical replicates (processed in separate batches) of each of the 8 independent samples (4 control, 4 ethanol treated) were used.

9. Exon array data analysis

Affymetrix CEL files were imported into Expression console software (Affymetrix) and analyzed at the core exon level (HuEx-1_0-st-v2.na28.hg18 annotation) using the PLIER algorithm (Affymetrix, 2005c). For further steps, control probe sets (e.g. those against intron regions) were excluded. Because cross-hybridizing probe sets were shown to be a major cause of false predictions of differential alternative splicing (Xing et al., 2008), only unique probe sets (a probe set in which all probes perfectly match only one sequence) were included. Thus 228,871 PLIER probe set signal estimates representing 17,881 genes were imported into Partek® Genomics Suite software version 6.4 (Partek, St. Charles, MO), quantile normalized, and log2 transformed. Technical replicates were normalized and transformed independently.

To remove signals that were not significantly different from the background, “detection above background” (DABG) p-value was calculated. Exon arrays have 25,000 background probe sets that do not match any sequence in the genome. Detection above background p-value was calculated by comparing the signal from each probe to the median intensity of background probes with the same GC content (Affymetrix, 2005b; Clark et al., 2007). Probe sets were also filtered based on fraction present (McClintick and Edenberg, 2006). This approach was shown to reduce false positives (McClintick and Edenberg, 2006) and to improve correlation between fold changes obtained in array data and quantitative reverse transcription PCR (qRT-PCR) data (Mieczkowski et al., 2010). In fraction present filtering, a probe set was considered to be present if it had a detection above background p-value ≤ 0.05 in all the arrays in at least one of the treatment conditions (ethanol or control, in this case).

A group of probe sets covering all the known isoforms for a given gene are referred to as a Transcript cluster; however, for simplicity these will be called genes. In addition to probe sets, genes were also subjected to present/absent filtering (McClintick and Edenberg, 2006). A gene was considered to be present if at least 50% of its probe sets were called present in all the samples in at least one treatment group (Clark et al., 2007). In the genes that were called present, only probe sets that were present were analyzed. After filtering steps, 127,805 representing 10,738 genes were included for further analysis.

To determine if a gene is differentially expressed or alternatively spliced or both, two-way analysis of variance (ANOVA) was carried out in Partek ® Genomics Suite (St. Louis, MO). The ANOVA model included experimental factors [treatment condition (ethanol *vs*. control) and batch ID (technical replicate)] along with the probe sets in each gene. With this model, p-values for differential expression and alternative splicing were obtained and based on these p-values, false discovery rate (FDR) (Benjamini and Hochberg, 1995) was calculated for differential gene expression and alternative splicing.

In this study, a gene was detected as differentially alternatively spliced if the alternative splicing FDR was ≤ 3%. If a gene meets this cutoff for differential alternative splicing, it means that one or more of the probe sets are affected by ethanol differently from other probe sets in the gene. However, this does not indicate which probe set was affected. Thus, differentially alternatively spliced genes were visually inspected to determine which probe set was differentially alternatively spliced. To assist in this closer examination, the effects of ethanol on each probe set in the gene were studied. One-way ANOVA was carried out to estimate the fold change and significance of this change (p-value) for each probe set. Fold change of each probe set was compared to the other probe sets in the gene to determine if the probe set was responding to alcohol differently from other probe sets.

Based on visual inspection, genes that were detected as differentially alternatively spliced (FDR ≤ 3%) were classified into three groups- probably differentially alternatively spliced, probably not differentially alternatively spliced

and uncertain. A gene was called probably differentially alternatively spliced if a probe set in the gene displayed a fold change significantly different in magnitude or direction from other probe sets in the gene. For example, *C1R* gene had a FDR of 0.3% for differential alternative splicing and thus was inspected for further analysis. In this gene four probe sets had a fold change in the range of 1.4 to 1.6 while another probe set had a 2.5-fold change. Because of this difference, the probe set and the gene were considered probably differentially alternative spliced.

To qualify as differentially alternatively spliced in the presence of ethanol, at least one exon had to be significantly different from the others (p-value > 0.05 in one-way ANOVA). For example, *ACAD11* was identified as differentially alternatively spliced with FDR of 0.001%. It had 18 probe sets that were included in the analysis. Most of these probe sets were affected by only a small magnitude by ethanol. One probe set however, was apparently increased in expression by 3-fold but the change was not significant (p-value for that probe set was 0.1). Therefore this gene was grouped under probably not differentially alternatively spliced. A gene was also considered probably not differentially alternatively spliced if the probe sets towards the 5' or 3' end were exhibiting probable edge effects (refer to Figure 19); these could represent real biological effects but because they are also artifactually produced (Bemmo et al., 2008; Whistler et al., 2010) we chose not to further examine them. A gene was classified as uncertain, if it was difficult to say if the gene had an alternative isoform with ethanol or which probe set in the gene was alternatively spliced. An example is shown in

Figure 20. A gene was also grouped as uncertain if the adjacent probe set in the same exon did not behave the same way. In the absence of known alternative events, it was not possible to say if these are alternate donor and acceptor sites or technical artifacts.

To identify biological pathways or processes affected by ethanol treatment, differentially expressed genes were analyzed by Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com). Steps in the exon data analysis are shown in Figure 5.

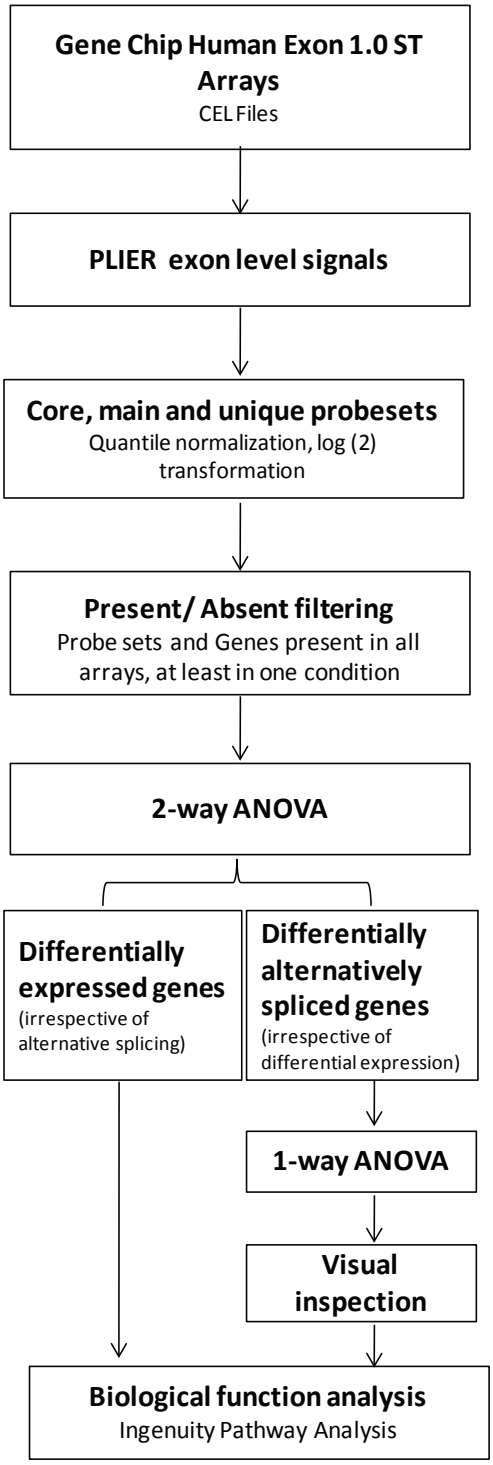

Figure 5. Exon array data analysis. The workflow of exon array data analysis is shown.

10. Validation of differential gene expression by qRT-PCR

Quantitative real time RT-PCR assays (Taqman gene expression assays) were carried out to confirm differential gene expression changes that were observed in microarray data. The synthesis of the first strand of cDNA was carried out using Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) or High-capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA) following manufacturer's directions. For seven genes (*FGG*, *GCLC*, *IDH2*, *TGFBR3*, *HK2* and *CPT1A*) 2 μg of total RNA was used as a template and the first strand of cDNA was synthesized with Superscript First-Strand Synthesis System. For the remaining genes (*HTR3B*, *SREBF1*, *BHMT*, *CHRNA5*, *PCK1*, *TGFB1*), the High-capacity cDNA Reverse Transcription kit was used with 1 μg or 1.5 μg of total RNA. Two independent cDNA synthesis reactions were carried out on each sample from the four independent experiments. Aliquots of the cDNA were used in real time PCR reactions with Taqman Gene expression assays (Applied Biosystems). Real time PCR reactions were carried out in triplicate on each of the two independently synthesized cDNAs from each sample. Mitochondrial ribosomal protein L3 (*MRPL3*) was used as an endogenous control, because our microarray data demonstrated that *MRPL3* had very low variation among samples and between control and ethanol conditions. Fold change for ethanol over control was calculated by delta delta Ct method (Livak and Schmittgen, 2001).

11. Validation of alternative splicing by qRT-PCR

To identify if there are alternative isoforms of the gene with respect to the test exon, PCR was carried out with primers flanking the test exon (Table 6). The High-capacity cDNA Reverse Transcription kit was used with 1.5 μg of total RNA and random primers (Invitrogen, Carlsbad, CA). For each condition (control or ethanol) cDNA from each of the four independent experiments was pooled and PCR was carried out using R-Taq polymerase (Midsci). PCR products were visualized on a 1.5% agarose gel to determine if the exon was alternatively spliced.

Quantitative real time RT-PCR assays (SYBR green assays) were carried out to confirm differential alternative splicing changes that were observed in microarray data. For *BPGM* and *CD55* 1.5 μg of total RNA from each of the four independent experiments was used as a template and the first strand of cDNA was synthesized with Superscript First-Strand Synthesis System and oligo d(T) primers (Promega, Madison, WI). For *MBD5* the High-capacity cDNA Reverse Transcription kit was used with 1.5 μg of total RNA and random primers (Invitrogen, Carlsbad, CA). Primers used to amplify each gene are listed in Table 6. Real time PCR reactions were carried out in duplicate on cDNA from each of the four independent experiments. For each experiment sample, the mean of Ct for control was subtracted from the mean of the Ct for ethanol and fold change was calculated by $2^{-(\text{delta Ct})}$.

Primer	Primer sequence	Gene
HE3679	ATGGAGTGCTTTATGTCAGTCCCAGTG	*MBD5*
HE3680	ACATCTTCTGCGGTTCTCTGTTTCACA	*MBD5*
HE3687	CCGAGGAAGGGAAGGTGGAGC	*MBD5*
HE3689	CACCCTGTGGGATTTGGTGTACAGTC	*MBD5*
HE3695	GGTATGCGGTGGTGTGATCGTA	*CD55*
HE3697	AACGTGAAACACGTGTGCCCTG	*CD55*
HE3768	TTGACAGGTTTGCTTGGGACGC	*CD55*
HE3769	GACTGCCTTTTTTCTCCTTGCTCTG	*CD55*
HE3694	CACTTCAGGTACTACCCGTCTTCTATCTGG	*CD55*
HE3711	CCAGAGGTGTGGGGTGGAAGT	*ACSL1*
HE3712	GGTGAGTGACCATTGCTCCTTTGG	*ACSL1*
HE3690	TGCTGCTGCTGCTGGTGGC	*BPGM*
HE3693	TTCAAATGGGCTAATATTCAAGGACAGC	*BPGM*
HE3691	ACTTTCTGTTTACACAAGAGTTGCCTCC	*BPGM*
HE3704	GATGGGAATGTGATGCTGCCTTCTAGTA	*NR2C1*
HE3705	CCCAGTGCTGGCAAGTGATGAA	*NR2C1*

Table 6. Primers used for validation of alternative splicing.

III. RESULTS

1. Identification of an enhancer in the *ADH* cluster

A comparative genomics approach was used to identify distal regulatory elements that affect expression of the *ADH* genes. Using the UCSC Conservation track, conservation in the intergenic regions between *ADH4* and *ADH5*, *ADH4* and *ADH6*, and *ADH6* and *ADH1A* was explored. Conservation in placental mammals, Rhesus, bushbaby, tree shrew, rat, mouse, guinea pig, rabbit, shrew, hedgehog, dog, cat, horse, and cow, was studied and regions with at least 50% conservation were identified as putative *cis*-regulatory regions. Eight putative regions were identified and their effect on the activity of the *ADH4* Basal promoter (4Basal) was tested in transient transfection assays in the HepG2 human hepatoma cells. The position of tested fragments in the *ADH* cluster is shown in Figure 6.

Six of the eight regions tested decreased the activity of the promoter (Figure 7), while another had no effect. Among these six negative regulatory regions, the greatest effect was observed at site 4-5-a which is 23 kb upstream of *ADH5*. It decreased the promoter activity to 0.26-fold. The region 6-4-a, 19.5 kb upstream of the *ADH4* also decreased the promoter activity by 70%. A similar effect was observed with 1A-6-d but other regions (1A-6-a, 1A-6-c , 1A-6-e) between *ADH1A* and *ADH6* had modest effects.

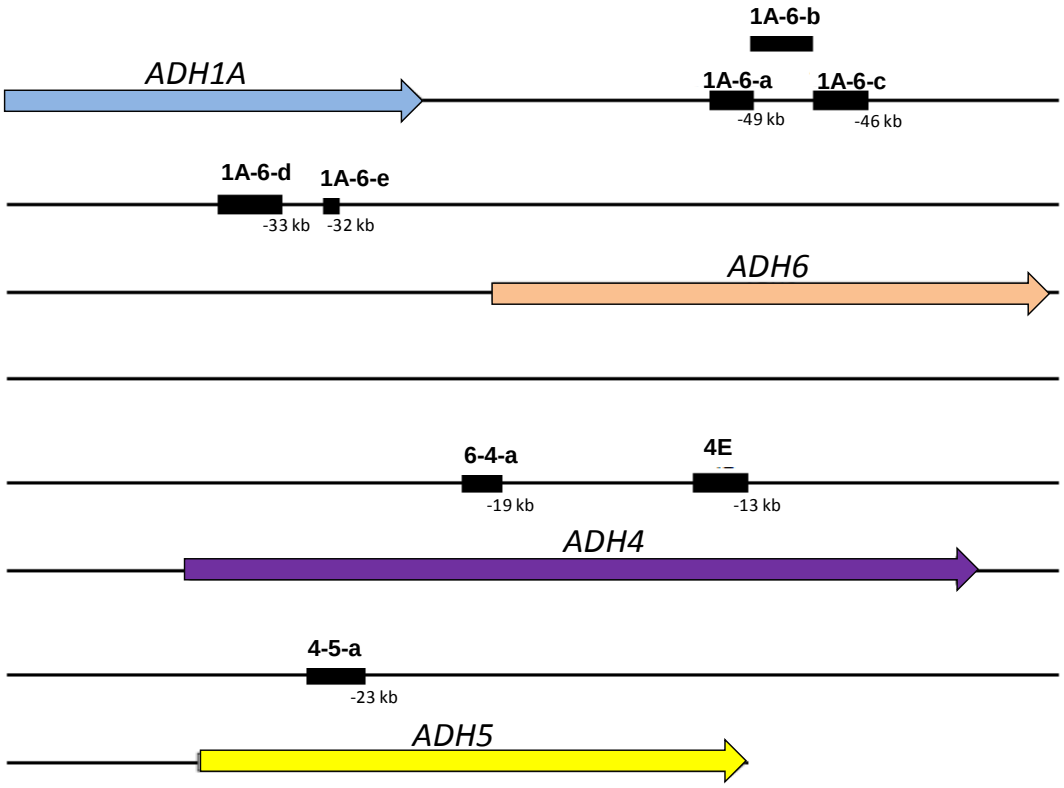

Figure 6. Location of the tested putative regulatory regions. Eight regions that were conserved in the 220 kb of the *ADH* cluster (Figure 2) studied are shown. The direction of the arrows represents the direction of transcription. Location relative to the nearest +1 CDS (in the direction of transcription) based on the human genome NCBI build 36 is also indicated.

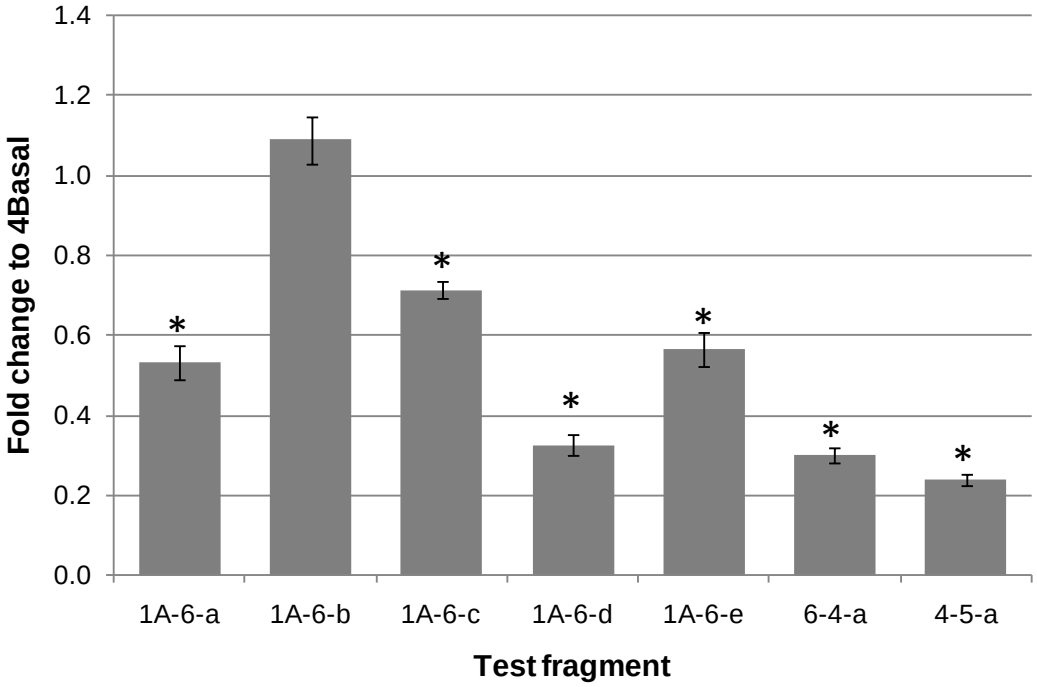

Figure 7. Six putative regulatory regions decrease transcription. Conserved regions were subcloned into pXP2 reporter vector upstream of 4Basal, and transiently transfected into HepG2 cells (n = 12). Transcription was determined as luciferase activity normalized to the internal control β-galactosidase. Fold change was calculated as ratio of the activity of each construct to the activity of 4Basal (set to one and therefore not shown); bars indicate the standard errors of the mean. *p-value ≤ 1×10^{-5}.

In contrast to these negative elements, a 1504 bp conserved region 13 kb upstream of the *ADH4* translation start site, (4E), caused a 50-fold increase in activity (p-value = 6×10^{-15}; Figure 8). In the absence of a promoter, the 4E fragment had weak activity, only 80% of the activity of the 4Basal promoter (p-value = 9×10^{-4}; Figure 8). Because of its strong enhancer effect, further studies were carried out with 4E.

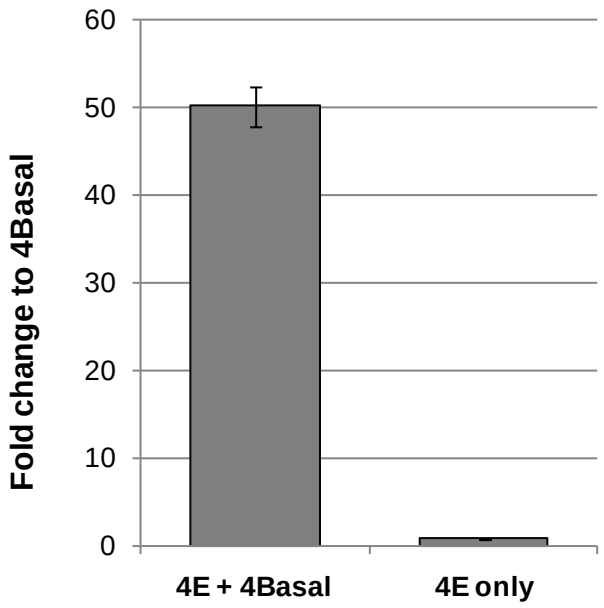

Figure 8. 4E enhances the activity of the *ADH4* promoter. Test plasmids with only the promoter (4Basal), 4E upstream of the promoter (4E+4Basal) and 4E in the absence of any promoter (4E only) were transiently transfected into HepG2 cells (n = 16). Promoter activity was determined as luciferase activity normalized to the internal control β-galactosidase. Fold change was calculated as ratio of the promoter activity of each construct to the promoter activity of 4Basal (set to one and therefore not shown); bars indicate the standard errors of the mean.

2. Characterization of the enhancer element 4E

2.a. Effect of 4E on heterologous promoters

To determine if the enhancer acts specifically on the *ADH4* promoter, it was tested in combination with two heterologous promoters. 4E increased the activity of another *ADH* promoter, the *ADH1B* basal promoter (1Basal), by 180-fold (p-value = 2×10^{-12}) in HepG2 cells. It increased the activity of the SV40 promoter (SV40Basal) by 56-fold (p-value = 8×10^{-10}).

2.b. Function of 4E in non-hepatoma cells

Cell specificity of 4E was tested in H1299 cells, a lung carcinoma cell line. Of the seven *ADH*s, only *ADH1B* is known to be expressed in lungs. However, the transfected minimal promoters of both *ADH4* and *ADH1B* did show activity above the vector-only background. Therefore, the enhancer activity of 4E was tested upstream of *ADH4*, *ADH1B* and SV40 promoters. No enhancer activity was detected with any of the three promoters (Table 7). These results suggest that 4E shows cellular specificity.

Promoter	Fold change in hepatoma cells (mean ± Std error)	Fold change in lung cells (mean ± Std error)
4Basal	50.2 ± 2.2 *	0.64 ± 0.1 *
1Basal	180 ± 5.3 *	0.97 ± 0.2
SV40Basal	56 ± 2.9 *	0.80 ± 0.1 *

Table 7. Cell specific activity of 4E. 4E was subcloned upstream of *ADH4*, *ADH1B*, and SV40 promoters and activity of each construct was normalized to the activity of the respective promoter to obtain the fold change (mean ± standard error). *p-value ≤ 0.05.

2.c. Localization of sequences required for 4E enhancer activity

To localize functional elements within the 1504 bp 4E region, the region was subdivided into three fragments, 4E1, 4E2 and 4E3 (Figure 9). The enhancer effect of 4E was contained within the 4E3 fragment, which enhanced the activity of 4Basal by 46-fold (p-value = 5×10^{-15}) (Figure 9). 4E1 repressed the activity of 4Basal by 50% (p-value < 0.001), whereas 4E2 increased the activity by 1.2-fold (p = 0.05). As the activity of 4E3 was not different from the parent fragment 4E (p-value = 0.13), further characterization was focused on the 4E3 fragment.

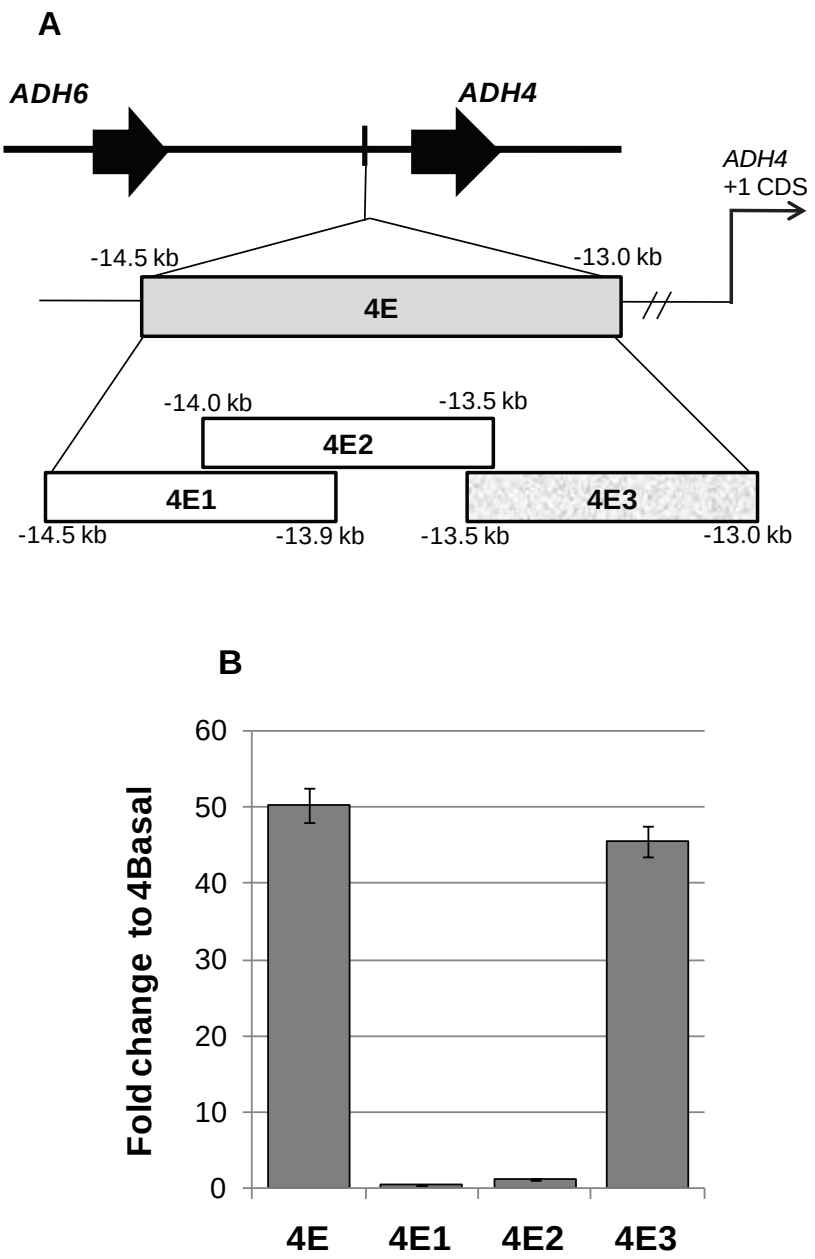

Figure 9. The enhancer function of 4E is located in a 565 bp region. (A) Position of 4E relative to flanking genes; the direction of the arrows represents the direction of transcription. This region is enlarged below, along with sub-fragments tested for activity. (B) The effects of subfragments of 4E upon the *ADH4* basal promoter were tested in transient transfections in HepG2 cells (n = 16). Fold change was calculated as ratio of the normalized luciferase activity of each construct to that of 4Basal; bars indicate the standard errors of the mean.

2.d. Identification of potential protein binding sites in 4E

Five potential Forkhead box protein A (FOXA, previously known as hepatocyte nuclear factor 3 binding sites and one hepatocyte nuclear factor 1-α binding site (HNF-1A; Figure 10) were identified using transcription factor prediction software Promo (Courtois et al., 1988; Farre et al., 2003; Messeguer et al., 2002; Overdier et al., 1994). Oligonucleotides were synthesized to cover these potential FOXA binding sites (Table 4, Materials and Methods) and tested in electrophoretic mobility shift assays (EMSA) with HepG2 nuclear extract. With all four oligonucleotides, at least two high-molecular weight DNA-protein complexes were observed (Figure 11). Competitor assays were carried out with unlabeled FOXA specific and non-specific oligonucleotides to determine if it is a FOXA specific complex. Unlabeled FOXA consensus oligonucleotide (Verschuur et al., 2005) disrupted the strong, high molecular weight complex. On the other hand, the complex was intact when a mutated FOXA consensus oligonucleotide was added, confirming that it is a FOXA-specific complex. A non-specific competitor oligonucleotide also had no effect on these complexes (Figure 11). The FOXA-specific complex was also perturbed in supershift assays with FOXA1 and FOXA2 antibodies, while it was unaffected with control IgG antibody (Figure 11).

rs7678936 G/T

CAGATAACAGCAAGAGAGGGGAGACTACAAAGCCCAAATTTCATCGAACA**G**CCTAAAACT

Oligo 1 **Site 1**

TTCAAGATCAGCAATTTGAC**AGCAAACA**TGAACTTTGTAATCAAACAGACTTCAAATCAA

Oligo 2 **Site 2** Oligo 3 **Site 3** **Site 4**

ACCTCTGCTTCCCTA**ACAAACA**CTGAAAAGATCAAACGGGGTAAGCA**TGTTGTC**T**TATTT**

Site 5 rs7678890 G/T

***GT*TAATAT**G**T**TACATAATACTTACCTCACAGGGTTGTGGTCAAGATTCAATTGATTATGT

Oligo 4

GTACAAAGTGCCTGATGTTAACTAGGCACTCAATGAGGGTTAGTTTCTTCCCACTAAATA

Site 6

AA**AACAAACA**GAAGTTTTCTCTTAGCTAACATTTTAAATATCAAACAAGATTTATAACAG

AGACAAACCCTATTATAAAAATCAAACTATTAAACAGAATAAGACTTCTTTCCCTTCCCC

AAGATGAAGACTACAGGAGCAAAGGTCATTTAGCTGGAAATTTAATTGCATATTTACTAG

rs11401494 del/A

AATTGGGTATTTCAAAGTCTCTTTTCTGGAAATCAGAGATCTGTCATTGAAGAGTCAC

ATTGCTCCATTATTTGTGCTCTGGCTG

Figure 10. Annotated genomic sequence of the 4E3 region. Six potential transcription factor binding sites, identified using Promo (Farre et al., 2003; Messeguer et al., 2002), are shown in bold along with the site name above the sequence. Sites 1 to 4 and site 6 were identified as putative FOXA sites where as site 5 was identified as putative HNF-1A site. Two nucleotides shared by sites 4 and 5 are shown in italics. Oligonucleotides used in EMSA are underlined. Known variations within the sequence are represented with an arrow.

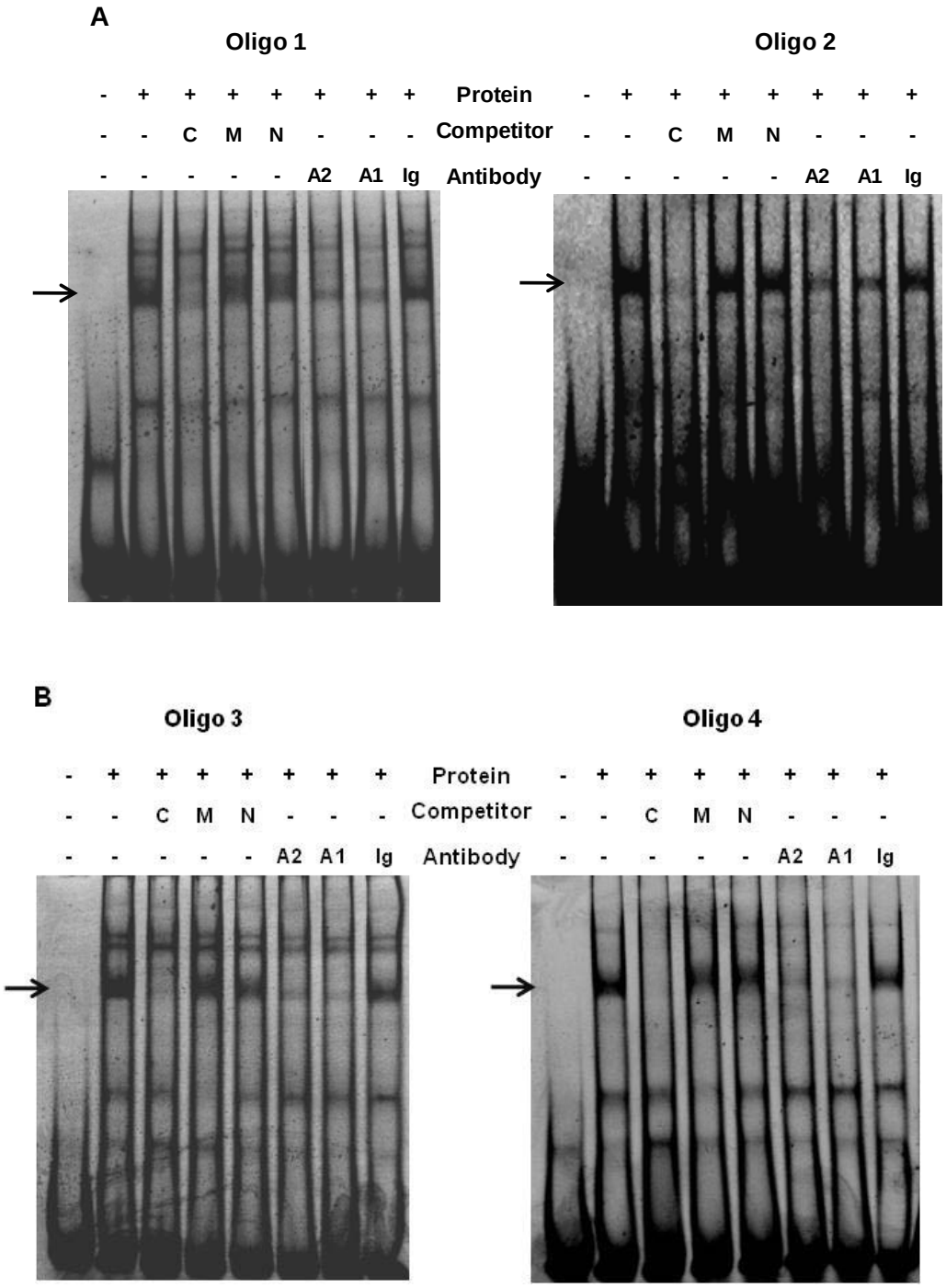

Figure 11. FOXA proteins bind to putative sites in 4E3. Electrophoretic mobility shift assays were carried out with 5′FAM labeled oligonucleotides of interest and 10 μg of HepG2 nuclear extract. Competition experiments were carried out in the presence of 50-fold molar excess of either a FOXA consensus (C), FOXA consensus mutant (M), or a non-specific (N) oligonucleotide. In

supershift assays, 2 μg of either FOXA2 antibody (A2), FOXA1 antibody (A1) or goat IgG (Ig) antibody was added. (A) Gel shifts with Oligos 1 and 2 that span sites 1 and 2, respectively, are shown. (B) Gel shifts in which Oligo 3 (sites 3, 4, and 5) and Oligo 4 (site 6) were used as probes are shown. FOXA specific bands are indicated by arrows, other bands were non-specific.

In Oligonucleotide 3, Site 5 was predicted to be a HNF-1α site; it overlaps with site 4 by two nucleotides. In EMSA assays with Oligonucleotide 3, multiple high molecular weight bands were observed, but the FOXA specific complex was the most prominent (indicated by an arrow on Figure 11). While none of the bands were disrupted by HNF-1A competitor (Su et al., 2006), increasing concentrations of the HNF-1A competitor led to a stronger FOXA specific complex (Figure 12).

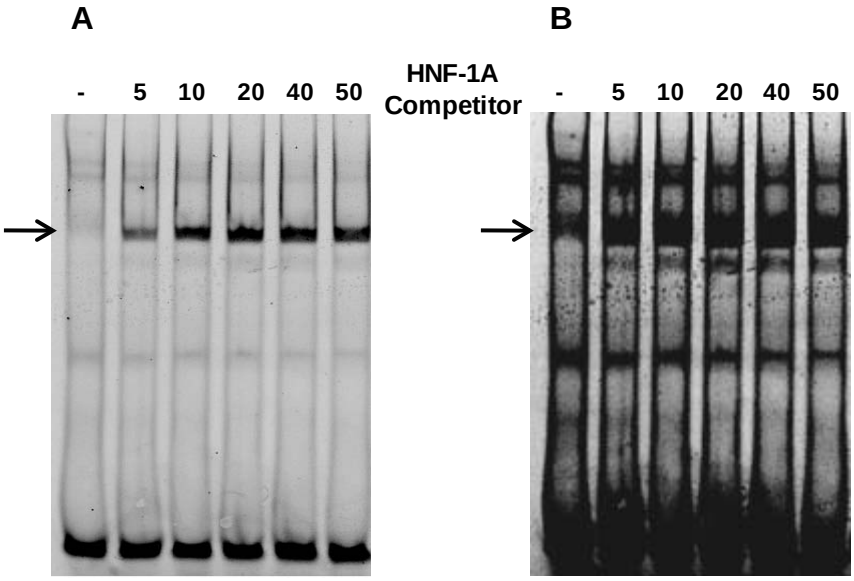

Figure 12. HNF-1A competitor increases FOXA binding. EMSA assays with Oligonucleotide 3 were carried out in the presence of increasing concentrations (5 to 50-fold molar excess) of HNF-1A consensus oligonucleotide. (A) Short exposure of the gel. (B) Longer exposure of the gel. FOXA specific bands are indicated by arrows, other bands were non-specific.

2.e. Effect of mutations on enhancer activity

To test the functional role of the putative FOXA sites in the 4E3 region, each of these sites was mutated and tested in transient transfections of HepG2 cells. Mutation of sites 1 and 4 decreased the activity by 60% and 65%, respectively (Figure 13), whereas mutations at sites 2, 3, and 5 reduced the activity by at least 40%. Disruption of site 6 did not have a significant effect.

To test the function of these sites in a combinatorial fashion, multiple sites were mutated. A double mutant of sites 1 and 4 lost most of the activity, exhibiting only 0.2-fold of the wild type enhancer. The activity was further decreased to 0.1-fold when multiple sites were mutated in conjunction with site 1 (Figure 13).

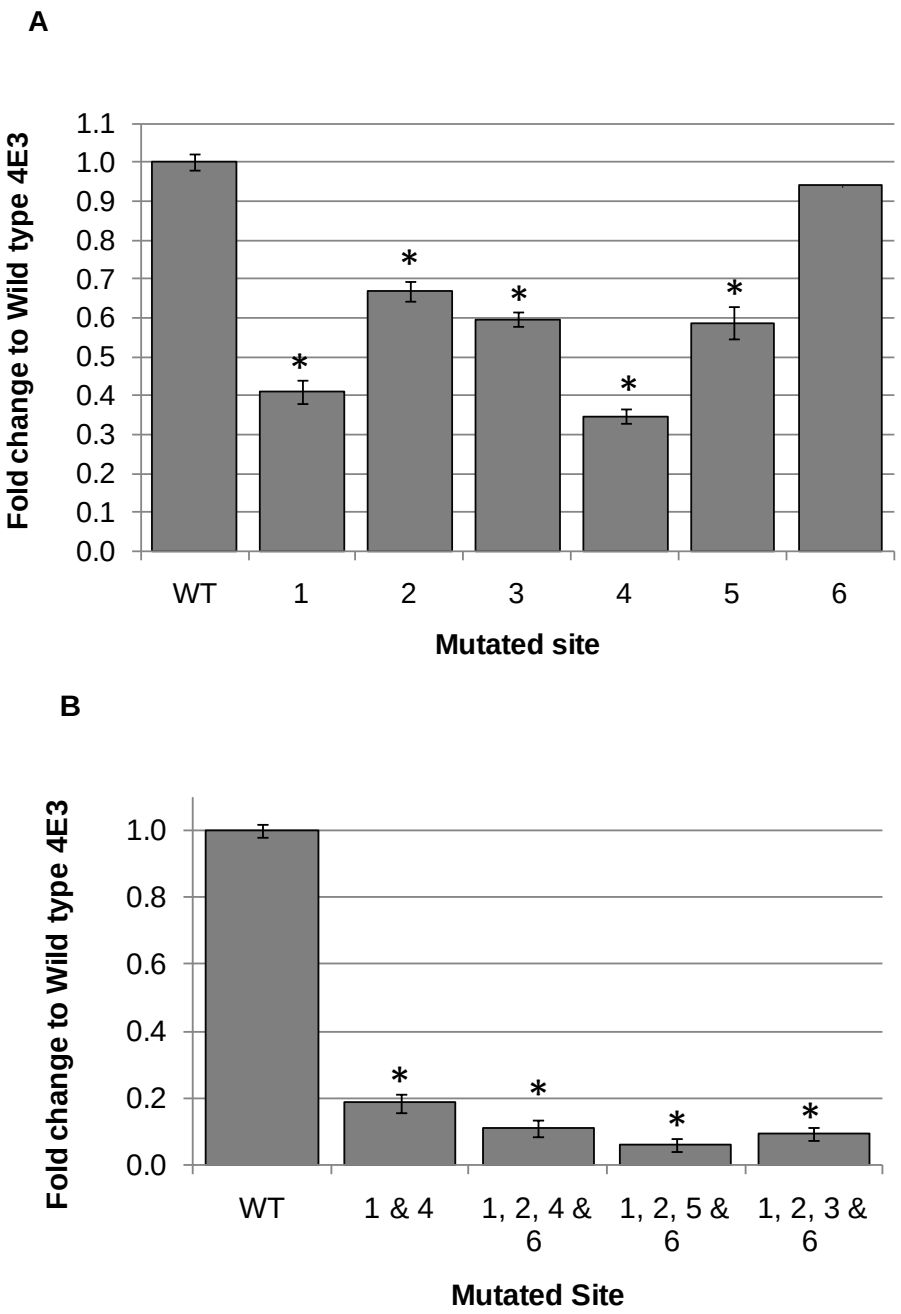

Figure 13. Effects of site-directed mutations on enhancer function. Potential FOXA and HNF-1α sites were mutated in 4E3 and tested in transient transfections of HepG2 cells (n = 16). Fold change was calculated as ratio of the enhancer activity of each mutated construct to the wild type; bars indicate the standard errors of the mean. (A) Activity of single site mutants. (B) Activity of double or multiple site mutants. *p-value $\leq 1.5 \times 10^{-7}$.

3. Effects of regulatory variations on gene expression

One of the hypotheses of this study is that variations in the regulatory regions of the *ADH* genes could lead to different levels of expression, thereby affecting the risk for alcoholism. Therefore, the effects of variations in two key regulatory regions, enhancer 4E3 and *ADH1B* proximal promoter, in the *ADH* cluster were studied.

3.a. Effects of natural variations on 4E3 enhancer activity

There are three known SNPs in the 4E3 enhancer region (NCBI dbSNP Build 130). The position of each SNP is shown in Figure 10. The genotype and allele frequencies for rs7678936 and for rs7678890 are shown in Table 8. Data for rs11401494 are unavailable.

In transient transfections, four different haplotypes of 4E3, cloned upstream of the *ADH4* promoter, were tested. The activities of all haplotypes were normalized to the activity of the most common haplotype in most populations: rs7678936 G, rs7678890 T, and rs11401494 del, which is denoted as haplotype 1. The activity of haplotype 2 (T, T, del) was only 60% that of haplotype 1. Haplotype 3 (G, G, del) displayed a similar decrease in activity. Insertion of A at position -13,058 (Haplotype 4: G, T, A) had a very small but significant effect (0.9-fold change relative to the haplotype 1, p-value = 0.02; Figure 14).

A

	rs7678936				
	Allele		Genotype		
Hapmap Population	**T**	**G**	**T/T**	**T/G**	**G/G**
CEU	0.1	1		0.1	0.9
CHB		1			1
JPT		1			1
YRI	0.3	0.7	0.1	0.4	0.5
ASW	0.3	0.7	0.1	0.4	0.5
CHD	0	1	0	0	1
GIH	0	1	0	0	1
LWK	0.3	0.7	0.1	0.4	0.5
MEX	0	1	0	0.1	0.9
MKK	0.3	0.7	0.1	0.4	0.5
TSI	0.1	0.9	0	0.1	0.9

B

	rs7678890				
	Allele		Genotype		
Hapmap Population	**T**	**G**	**T/T**	**T/G**	**G/G**
CEU	1	0.1	0.9	0.1	
CHB	1		1		
JPT	1		1		
YRI	0.7	0.3	0.5	0.4	0.1

Table 8. Allele and genotype frequencies of SNPs in the 4E3 region. Allele and genotype frequencies in different populations for the two SNPs in the enhancer region 4E3 were obtained from HapMap (release 27, Phase II and III; hapmap.ncbi.nlm.nih.gov). Data for rs11401494 are not available. (A) Data for rs7678936. (B) Data for rs7678890. Populations are Utah residents with ancestry from northern and western Europe (CEU), Han Chinese in Beijing, China (CHB), Japanese in Tokyo, Japan (JPT), Yoruba in Ibadan, Nigeria (YRI), African ancestry in Southwest USA (ASW), Chinese in Metropolitan Denver, Colorado (CHD), Gujarati Indians in Houston, Texas (GIH), Luhya in Webuye, Kenya (LWK), Mexican ancestry in Los Angeles, California (MEX), Maasai in Kinyawa, Kenya (MIKK), and Toscans in Italy (TSI).

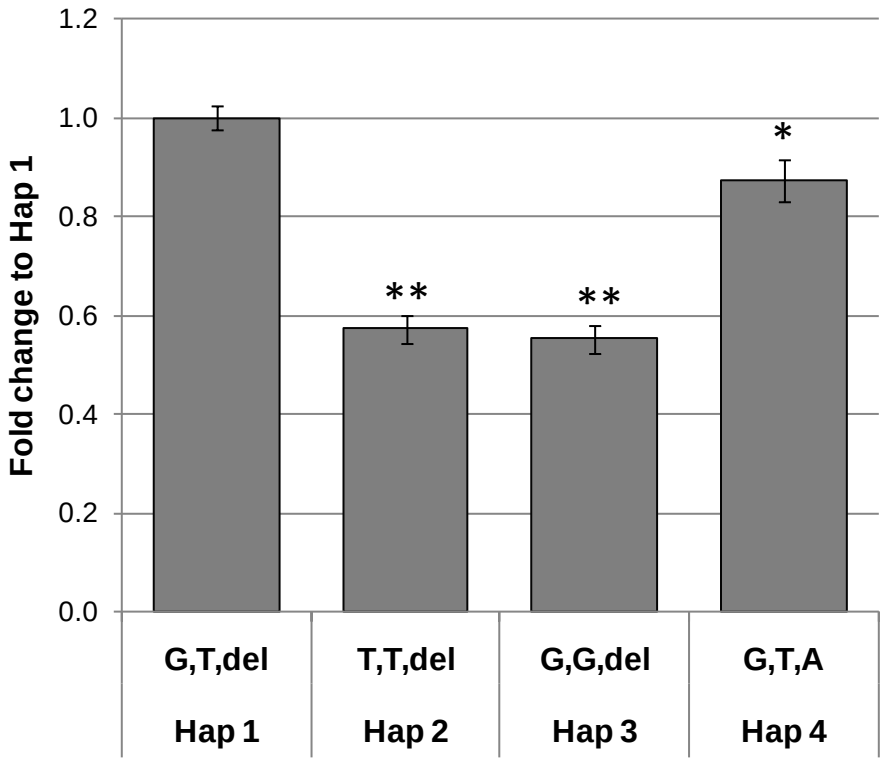

Figure 14. Effects of polymorphisms on enhancer function. Four different haplotypes of the 4E3 region were tested in transient transfections in HepG2 cells (n = 20). Alleles of rs7678936, rs7678890 and rs11401494, respectively are shown on the x-axis. The error bars indicate standard errors of the mean. The promoter activity of each haplotype was normalized to the promoter activity of Haplotype 1 (G,T, del). *p-value = 0.02; **p-value ≤ 8 x 10^{-13}.

3.b. Effects of polymorphisms on *ADH1B* promoter activity

In association studies of alcohol dependence (defined as meeting alcohol dependence criteria by DSM-IIIR (American Psychiatric Association, 1987) plus Feighner definite alcoholism (Feighner et al., 1972), three SNPs in the *ADH1B* region were significantly associated (Edenberg et al., 2006). Two of these variations (rs1229982 and rs1159918) are located in the proximal promoter region, while rs1353621 is in the first intron. Allele and genotype frequencies of rs1229982 and rs1159918 are shown in Table 9.

	rs1229982				
	Allele		Genotype		
Hapmap Population	A	C	A/A	C/A	C/C
CEU	0.2	0.8	0.0	0.3	0.7
CHB	0.0	1.0	0.0	0.1	0.9
JPT	0.0	1.0	0.0	0.1	0.9
YRI	0.6	0.4	0.3	0.5	0.2
ASW	0.5	0.5	0.2	0.5	0.2
CHD	0.1	0.9	0.0	0.1	0.9
GIH	0.1	0.9	0.0	0.2	0.8
LWK	0.5	0.6	0.2	0.6	0.3
MEX	0.2	0.8	0.0	0.3	0.7
MKK	0.4	0.6	0.2	0.5	0.3
TSI	0.2	0.8	0.1	0.3	0.6

	rs1159918				
	Allele		Genotype		
Hapmap Population	T	G	T/T	T/G	G/G
CEU	0.3	0.7	0.1	0.4	0.5
CHB	0.2	0.8	0.0	0.3	0.6
JPT	0.2	0.8	0.0	0.3	0.7
YRI	0.9	0.1	0.8	0.2	0.0
ASW	0.7	0.3	0.5	0.4	0.1
CHD	0.1	0.9	0.0	0.3	0.7
GIH	0.3	0.7	0.1	0.5	0.4
LWK	0.7	0.3	0.5	0.4	0.2
MEX	0.7	0.3	0.5	0.4	0.1
MKK	0.7	0.3	0.5	0.4	0.1
TSI	0.3	0.7	0.1	0.4	0.6

Table 9. Allele and genotype frequencies for two SNPs in the *ADH1B* proximal promoter region. Allele and genotype frequencies in different populations for the two SNPs that were associated with alcoholism were obtained from HapMap (release 27, Phase II and III; hapmap.ncbi.nlm.nih.gov). Populations are as in Table 8.

The effects of rs1229982 and rs1159918 SNPs, along with the other natural variations in the region of -1484 bp to -10 bp (with respect to the coding sequence start site were tested). The Single Nucleotide Polymorphism database (dbSNP; www.ncbi.nlm.nih.gov/projects/SNP/*)* reports 12 SNPs in this region (Figure 15). The region was PCR amplified from DNA of various individuals to generate the natural haplotypes, and cloned into the pXP2 reporter plasmid. Five different haplotypes, haplotype 1 to haplotype 5 (Table 10), were obtained. The five haplotypes had the same allele for five SNPs (rs28913901, rs28913903, rs28913904, rs3076071 and rs28913905) but differed in the remaining seven positions.

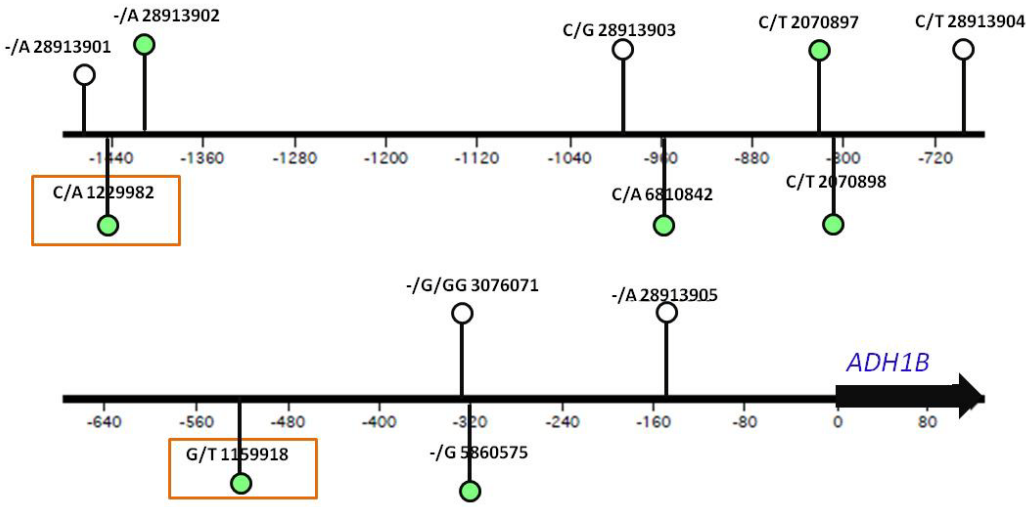

Figure 15. Variations in the *ADH1B* proximal promoter region. Twelve variations in the proximal promoter are shown as vertical lines with the two alleles and the SNP ID (rs number) at the end. Filled circles represent those variations that were tested in the transfection assays. For some variations (shown as open circles), the same allele was present in all the haplotypes tested. Rectangular boxes represent SNPs that were associated with alcoholism in European Americans. Position is relative to the ADH1B + 1 translational start site.

Haplotype	1229982	28913902	6810842	2070897	2070898	1159918	5860575
1	**A**	-	A	C	C	**T**	-
3	**C**	T	C	T	T	**G**	-
4	**C**	T	C	C	C	**T**	-
2	**A**	T	C	T	C	**G**	-
5	**C**	T	A	C	C	**T**	G

Table 10. Tested haplotypes of the *ADH1B* proximal promoter. Five haplotypes with seven SNPs that were obtained from cloning the proximal promoter region are shown; they were identical at all other positions. Alleles of the two SNPs that were associated with alcoholism are shown in bold.

Five haplotypes were tested in HepG2 cells by transient transfections. The activity of each was normalized to that of haplotype 1, which had the sequence closest to the reference sequence in Genbank (accession number NT_016354.17). A significant (p-value $\leq 5.4 \times 10^{-7}$) decrease in the promoter activity relative to haplotype 1 was observed in haplotypes 3, 4 and 5, whereas no difference in activity was seen with haplotype 2 (p-value = 0.39; Figure 16). Analysis of the combination of SNPs in the five haplotypes suggested that SNP rs1229982 at -1444 position might be responsible for the decrease in the activity. The promoter with the rs1229982 C allele at this position leads to a 30% decrease in the promoter activity.

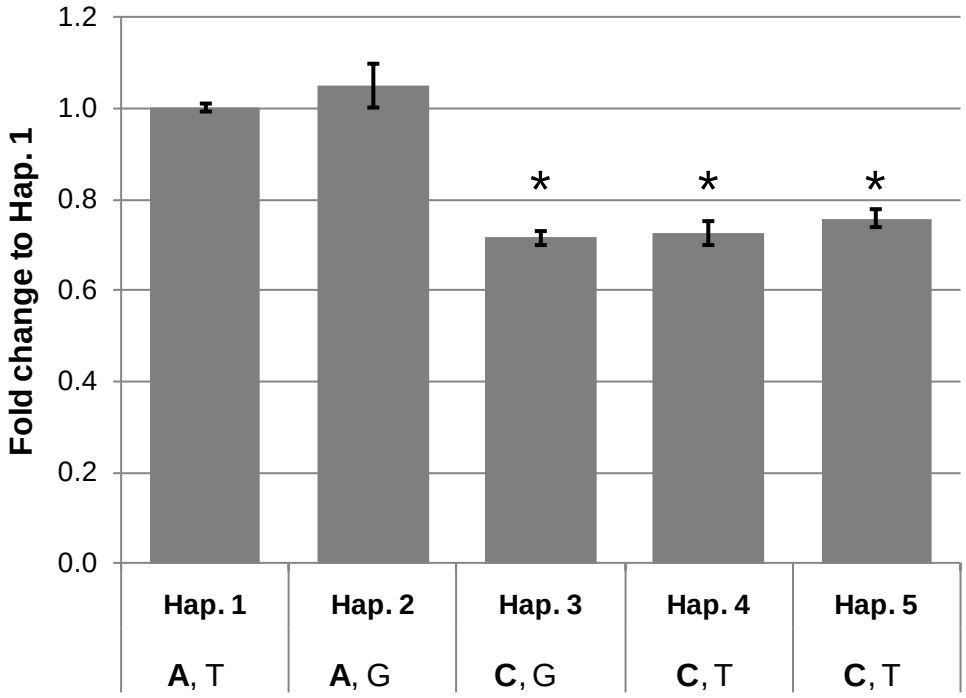

Figure 16. Variations in the *ADH1B* promoter affect activity. Five different haplotypes of the *ADH1B* proximal promoter region were tested in transient transfections in HepG2 cells (n = 12). The promoter activity of each haplotype was normalized to the promoter activity of Haplotype 1. Alleles of rs1229984, and rs1159918, respectively are shown on the x-axis. The error bars indicate standard errors of the mean. * p-value ≤ 6.6×10^{-4}.

4. Effects of alcohol on gene expression

To determine the effects of long term ethanol exposure on global gene expression, human hepatoma cells (HepG2) were exposed to 75 mM (0.34%) ethanol for nine days, with fresh media daily. To investigate the effect of ethanol on cell growth, the number of viable cells was counted by trypan blue exclusion. After the initial four day exposure to 75 mM ethanol, there were only 48% as many viable cells as in the control cultures. Two million viable cells were again seeded in new flasks and cultured in ethanol for the next five days. There were again 48% as many viable cells in the ethanol-treated cultures as in the control cultures. The decrease in viable cell number could be due to a longer lag phase, or a reduced rate of cell growth, or cell death. However, before trypsinization, cells appeared to be similar in morphology in both ethanol and control conditions. We did not observe many floating or dead cells in the presence of ethanol.

Effects of ethanol on gene expression were studied using Affymetrix GeneChip® Human Exon 1.0 ST Arrays. Among the 17,881 genes represented by main and unique probe sets, 10,738 genes (60%) were reliably detected in at least one of the treatment conditions, and only those were used for further analysis. Genes with just one probe set reliably detected were also excluded (516 genes). Two-way ANOVA was applied to identify genes that were differentially expressed. Steps in the data analysis are shown in Materials and Methods (Figure 5).

Of the 10,222 genes that were analyzed many were differentially expressed (Table 11). Most of the expression differences were modest, with absolute fold change between 1.1 and 1.3 (Figure 17).

Number of genes affected by ethanol		
FDR 15%	**FDR 10%**	**FDR 3%**
3242	2423	1093

Table 11. Effects of ethanol on gene expression at different false discovery rates. Number of genes significantly differentially expressed by ethanol at three levels of False Discovery Rate are shown. FDR was calculated by Benjamini and Hochberg method (1995).

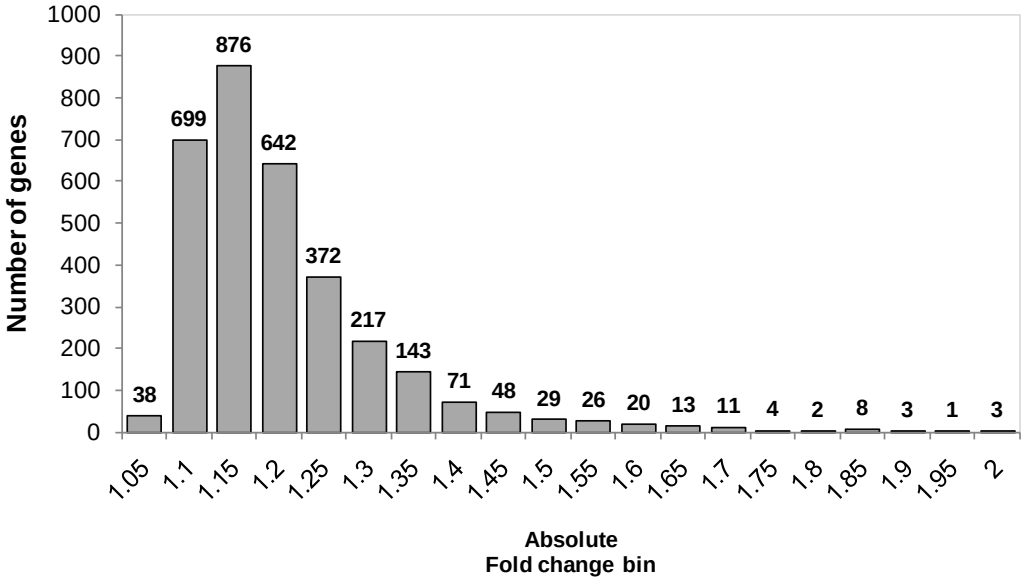

Figure 17. Distribution of fold changes of differentially expressed genes. Distribution of fold changes of differentially expressed genes (FDR ≤ 15%). Each bin represents number of genes that have absolute fold change equal to or less than the given value, but greater than the previous bin value (e.g. bin 1.1 shows the number of genes with absolute fold change between 1.0501 and 1.1000).

At a stringent false discovery rate (FDR) of 3% a total of 1,093 genes were differentially expressed due to chronic exposure to ethanol. To assess biological pathways affected by chronic ethanol treatment, differentially expressed genes (FDR ≤ 3%) that had a minimum of 10% change in expression level (991 genes) were analyzed. Once the pathways were identified using this stringent FDR, genes associated with these pathways were added that were at a relaxed FDR (≤ 15%) and with at least 10% fold change. This provided more insight into the effects of the alcohol on these pathways. Canonical pathways that were significantly affected by chronic ethanol treatment ($p \leq 0.05$) are listed in Table 12.

Ingenuity Canonical Pathways	**p-value**	**Molecules**
Arginine and Proline Metabolism	0.0001	*ALDH1B1, SAT2, PRODH2, ODC1, BCKDHB, CKB, DAO, C22ORF30, ALDH3A2, AGMAT, ALDH3B1, GATM, ASL, P4HA2*
Acute Phase Response Signaling	0.0001	*ECSIT, ITIH3, HAMP, SERPING1, AMBP, SERPINA3, SERPIND1, C1R, SHC1, HMOX1, JUN, MRAS, CFB, SERPINE1, AGT, HPX, TTR, C1S, IL6R, STAT3, CEBPB, PLG, HP, RBP5, FGA*
Pyruvate Metabolism	0.0001	*NKD1, ALDH1B1, PC, ACSS1, PCK1, BCKDHB, C22ORF30, PCK2, ALDH3A2, ACSS2, ALDH3B1, RWDD2B, UEVLD, ACSL1*
Fructose and Mannose Metabolism	0.0002	*GMPPB, PFKFB3, HK2, PFKFB1, PFKFB4, DUSP18, PFKP, PFKL, ALDOC, MPI*
Glycolysis/Gluconeogenesis	0.0003	*ALDH1B1, ENO2, ACSS1, PFKP, PFKL, HK2, ALDH3A2, ACSS2, ALDH3B1, RWDD2B, UEVLD, PTGR1, ACSL1, ALDOC*
Glycine, Serine and Threonine Metabolism	0.0006	*GNMT, BHMT, GLYCTK, AGXT, SARDH, DAO, C22ORF30, CBS, GCAT, SARS2, GATM, SHMT2, ETNK2*
Ascorbate and Aldarate Metabolism	0.0019	*ALDH1B1, C22ORF30, CYP24A1, ALDH3A2, ALDH3B1, BCKDHB*
Estrogen-Dependent Breast Cancer Signaling	0.0019	*JUN, TERT, IGF1R, MRAS, CREB3L4, HSD17B1, HSD17B2, HSD17B8, EGFR*
Renin-Angiotensin Signaling	0.0024	*PTK2B, GNAQ, SHC3, STAT3, SHC1, PAK1, JUN, ADCY1, ITPR3, MRAS, PRKCE, PRKD1, AGT*

Ingenuity Canonical Pathways	**p-value**	**Molecules**
Propanoate Metabolism	0.0025	*ALDH1B1, ALDH3A2, ACSS2, ACSS1, ALDH3B1, UEVLD, ALDH6A1, CCBL1, ACSL1, GCDH, ACADS*
IL-3 Signaling	0.0045	*SHC1, STAT6, PAK1, JUN, FOXO1, MRAS, PRKCE, STAT3, PRKD1*
Glycerolipid Metabolism	0.0060	*ALDH1B1, GPAM, LIPC, PNPLA3, GLYCTK, ALDH3A2, DGKG, ALDH3B1, LIPG, LPIN2, AGPAT3, PTGR1*
Prolactin Signaling	0.0065	*SHC1, JUN, MRAS, PRKCE, NMI, STAT3, CEBPB, PDK1, PRKD1*
Thrombopoietin Signaling	0.0089	*SHC1, THPO, JUN, MRAS, PRKCE, STAT3, PRKD1*
Urea Cycle and Metabolism of Amino Groups	0.0093	*CKB, SARDH, AGMAT, GATM, ASL, ODC1*
T Helper Cell Differentiation	0.0117	*TGFBR2, STAT6, IL4R, TGFB1, IL6R, IL10RB, RORC, STAT3*
Nitrogen Metabolism	0.0117	*SARDH, CA9, PTPRG, HAL, TGM4, GLS2, CCBL1*
Oncostatin M Signaling	0.0126	*SHC1, TIMP3, EPAS1, MRAS, STAT3*
FXR/RXR Activation	0.0138	*LIPC, UGT2B4, PCK2, FOXO1, NR0B2, SREBF1, FOXA1, FASN, HNF4A, VLDLR, MTTP*
Citrate Cycle	0.0151	*PC, PCK2, IDH2, PCK1, OGDH*
PPARα/RXRα Activation	0.0166	*ACOX1, TGFBR3, GNAQ, NR2C2, MAP4K4, NCOA3, ABCA1, TGFBR2, SHC1, JUN, NR0B2, TGFB1, FASN, ADCY1, MRAS*

Ingenuity Canonical Pathways	**p-value**	**Molecules**
Glycerophospholipid Metabolism	0.0166	*NAPEPLD, PNPLA3, PLA2G15, HMOX1, GPAM, GDE1, LPCAT2, C22ORF30, DGKG, LIPG, LPIN2, PLA2G12B, AGPAT3, ETNK2*
Bile Acid Biosynthesis	0.0191	*ALDH1B1, SOAT2, ALDH3A2, ALDH3B1, PTGR1, HSD3B7*
D-glutamine and D-glutamate Metabolism	0.0200	*TGM4, GLS2*
PXR/RXR Activation	0.0209	*CPT1A, PCK2, FOXO1, NR0B2, CES3, ALDH3A2, HNF4A*
Cholecystokinin/Gastrin-mediated Signaling	0.0263	*SHC1, JUN, PTK2B, ITPR3, MRAS, GNAQ, PRKCE, FNBP1, PRKD1, EGFR*
GNRH Signaling	0.0269	*MAP3K15, EGR1, GNAQ, CREB3L4, PAK1, JUN, ADCY1, ITPR3, MRAS, PRKCE, PRKD1, DNM2, EGFR*
Cell Cycle: G1/S Checkpoint Regulation	0.0288	*HDAC6, CCNE1, TGFB1, HDAC11, CDK4, ABL1, BTRC, HDAC5*
TR/RXR Activation	0.0347	*F10, SLC16A3, HP, UCP2, LDLR, SREBF1, FASN, PFKP, PCK1, FGA, AKR1C2, NCOA3*
NRF2-mediated Oxidative Stress Response	0.0355	*DNAJC3, GCLC, HERPUD1, TXNRD1, HMOX1, JUN, GPX2, MRAS, PRKCE, DNAJA3, JUND, PRKD1, ACTA1, EPHX1*
Glycosaminoglycan Degradation	0.0355	*MGEA5, KLB, NAGLU, GALNS, SULF2*
Coagulation System	0.0355	*PLG, F10, SERPINA5, F7, SERPINE1, FGA, SERPIND1*

Ingenuity Canonical Pathways	p-value	Molecules
Androgen and Estrogen Metabolism	0.0380	*UGT2B4, STS, SULF2, METTL7B, HSD3B7, HSD17B1, HSD17B2, HSD17B8*
Fatty Acid Metabolism	0.0407	*ALDH1B1, CPT1A, ALDH3A2, ACOX1, ALDH3B1, CYP4F11, PTGR1, ACSL1, GCDH, ACADS*
Glutathione Metabolism	0.0417	*ACSS2, GPX2, GCLC, IDH2, RAB15, ANPEP*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0447	*IL4R, CTGF, LEPR, FGFR1, IL6R, FGFR2, TGFBR2, TGFB1, TIMP1, IGF1R, PDGFRB, AGT, EGFR*
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.0468	*MYO5A, HMOX1, NAPEPLD, PAK1, PTK2B, LYN, PRKCE, ACTA1, PRKD1*
G Beta Gamma Signaling	0.0468	*SHC1, PAK1, ADCY1, MRAS, GNAQ, PRKCE, PRKD1, DNM2, EGFR*
Phospholipase C Signaling	0.0468	*NAPEPLD, GNAQ, CREB3L4, HDAC5, HDAC6, SHC1, HMOX1, HDAC11, ITPR3, ADCY1, LYN, MRAS, PRKCE, ARHGEF3, PLA2G12B, FNBP1, PRKD1*
C21-Steroid Hormone Metabolism	0.0479	*CYP21A2, HSD3B7, HSD17B2*

Table 12. Pathways affected by chronic ethanol exposure. Genes that were differentially expressed (FDR ≤ 3%; difference ≥ 10%) with chronic exposure to ethanol were analyzed by Ingenuity Pathway Analysis. Pathways (and genes in them) that were significantly affected ($p \leq 0.05$) are shown.

An overlap between significant pathways was observed. For example, most of the genes in the Pyruvate metabolism pathways were also present in Glycolysis/ Gluconeogenesis pathway. Similarly, many signaling molecules like *SHC1*, *GNAQ*, *ADCY1* were present in multiple pathways. Due of this overlap between pathways, we studied pathways with similar functions together. Therefore, pathways like glycolysis/ gluconeogenesis, citrate cycle, fructose and mannose metabolism were considered under effects of alcohol on carbohydrate metabolism. We also focused our analysis on liver associated functions like acute phase response pathways, or on pathways that are known to play a role in alcohol induced liver injury like the oxidative stress response.

Alcohol affected many genes in stress response pathways. Acute phase proteins like fibrinogen (*FGA*) and hemeoxygenase1 (*HMOX1*), were changed by 1.2 and 1.3-fold, respectively. Multiple genes associated with the Nrf2-mediated oxidative stress response were also affected by chronic ethanol treatment. Nrf2-regulated proteins including glutamate-cysteine ligase, catalytic subunit (*GCLC*), glutathione peroxidase 2 (*GPX2*), thioredoxin reductase (*TXNRD1*) were upregulated by at least 20%. Superoxide dismutases were also upregulated, but modestly (1.13-fold; Table 13).

Several genes involved in hepatic fibrosis were affected by ethanol. Fibroblast growth factor receptor family members *FGFR1* and *FGFR2* were upregulated by 1.49- and 1.53-fold, respectively. Transforming growth factor B (*TGFB1*) and its receptor *TGFBR2* were increased by 23% and 12% respectively. Other receptors that were affected include insulin-like growth factor

receptor (*IGFR1*, 1.29-fold), epidermal growth factor receptor (*EGFR*, 1.12-fold), platelet derived growth factor beta polypeptide (*PDGFRB,*-1.3-fold) and Interleukin-4 receptor (*IL-4R*, -1.19-fold). Alcohol also had an effect on cell cycle genes. Cyclin D1 (*CCND1*), Cyclin E1 (*CCNE1*) increased in expression. Ethanol had opposing effects on cyclin dependent kinase 4 and 6 (*CDK4* and *CDK6*); *CDK4* decreased in expression while *CDK6* increased in expression.

Chronic ethanol treatment affected genes encoding proteins involved in cellular metabolism (Table 13). Several genes involved in the metabolism of amino acids were affected by ethanol. Genes encoding urea cycle enzymes arginase (*ARG2*) and arginosuccinate lyase (*ASL*) were changed by -1.17 and -1.15-fold. Prolyl 4-hydroxylase, alpha polypeptide II (*P4HA2)*, an enzyme involved in the formation of 4-hydroxyproline increased in expression whereas pyrroline-5-carboxylate reductase (*PRODH2*) decreased in expression. Multiple genes in the metabolism small amino acids (glycine, serine, threonine) were also affected. Mitochondrial serine and threonine t-RNA synthetases (*SARS2*, *TARS2)* decreased in expression with ethanol.

The expression of many genes related to carbohydrate metabolism decreased upon ethanol treatment. Transcript level of phosphofructokinase (*PFKL)*, which catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1, 6-bisphosphate in the glycolytic pathway, was decreased by 1.25 fold. Pyruvate carboxylase (*PC*) enzyme that plays a crucial role in gluconeogenesis and lipogenesis was also decreased by 1.22-fold. Enzymes involved in fructose metabolism were also affected by ethanol. Liver isozyme 6-phosphofructo-2-

kinase/fructose-2,6-biphosphatase 1 (*PFKFB1*) which catalyzes both the synthesis and degradation of fructose-2,6-biphosphate increased by 1.41-fold with ethanol.

Glycerolipid metabolism, glycerophospholipid metabolism and fatty acid metabolism pathways were considered as effects of alcohol on lipid metabolism. Key fatty acid oxidation enzymes carnitine palmitoyltransferase 1A (*CPT1A*) and acyl-Coenzyme A dehydrogenase (*ACADS*) were decreased by 50% and 24%, respectively. Most of the enzymes involved in glycerolipid metabolism were upregulated by ethanol. Mitochondrial glycerol-3-phosphate acyltransferase (*GPAM*) and 1-acylglycerol-3-phosphate O-acyltransferase 3 (*AGPAT3*) increased in expression by 1.3- and 1.5-fold, respectively. Choline kinase alpha (*CHKA*) and ethanolamine kinases (*ETNK1*, *ETNK2*) were also upregulated.

Genes regulated by nuclear receptors, including farnesoid X receptor (FXR) and retinoid X receptor (RXR), were affected by chronic ethanol treatment. Nuclear receptor subfamily 0, group B, member 2 (*NROB2*) was downregulated by 1.3-fold, whereas very low density lipoprotein receptor (*VLDLR*), and ATP binding cassette members (*ABCB1* and *ABCB4*) were upregulated by 1.4 and 1.16-fold respectively.

In addition to identifying pathways that were affected by ethanol, changes in the expression of genes of interest, including those involved in alcohol metabolism, transcription factors, chromatin regulation or those that have been associated with the risk for alcoholism, were also examined. Of genes involved in alcohol metabolism, three aldehyde dehydrogenase genes were affected by

ethanol. The expression of *ALDH3B1* and *ALDH2* was decreased by 1.32-fold and 1.12-fold, respectively, whereas *ALDH1B1* increased by 1.13-fold. Histone acetyltransferase and deacetylases, *MYST4* and *HDAC11* were also affected by ethanol.

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
Amino acid metabolism					
2623441	*ACY1*	aminoacylase 1	-1.19	0.03	0.07
2397732	*AGMAT*	agmatine ureohydrolase (agmatinase)	-1.15	0.00	0.65
2535976	*AGXT*	alanine-glyoxylate aminotransferase	-1.19	0.00	0.84
3638607	*ANPEP*	alanyl (membrane) aminopeptidase	1.17	0.01	0.04
3722182	*AOC2*	amine oxidase, copper containing 2 (retina-specific)	1.27	0.09	0.76
3541383	*ARG2*	arginase, type II	-1.17	0.06	0.87
3005363	*ASL*	argininosuccinate lyase	-1.15	0.00	0.38
2914820	*BCKDHB*	branched chain keto acid dehydrogenase E1, beta polypeptide	-1.20	0.00	1.00
2817251	*BHMT*	betaine-homocysteine methyltransferase	1.46	0.00	0.98
2817212	*BHMT2*	betaine-homocysteine methyltransferase 2	1.23	0.00	0.16
3958005	*C22orf30*	chromosome 22 open reading frame 30	1.19	0.03	0.60
3933923	*CBS*	cystathionine-beta-synthase	-1.12	0.00	0.83
3476330	*CCDC92*	coiled-coil domain containing 92	1.80	0.06	0.72
3580769	*CKB*	creatine kinase, brain	-1.42	0.00	0.75
3430868	*DAO*	D-amino-acid oxidase	1.20	0.00	0.81
2864118	*DMGDH*	dimethylglycine dehydrogenase	1.14	0.11	1.00
3845175	*GAMT*	guanidinoacetate N-methyltransferase	-1.11	0.06	0.63
3622386	*GATM*	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	-1.40	0.00	0.64
3945014	*GCAT*	glycine C-acetyltransferase (2-amino-3-ketobutyrate coenzyme A ligase)	-1.40	0.00	0.93
3955185	*GGT5*	gamma-glutamyltransferase 5	-1.28	0.12	0.92

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3903598	*GGT7*	gamma-glutamyltransferase 7	1.11	0.04	0.22
3457891	*GLS2*	glutaminase 2 (liver, mitochondrial)	-1.14	0.00	0.93
2623611	*GLYCTK*	glycerate kinase	-1.23	0.00	0.95
2907513	*GNMT*	glycine N-methyltransferase	-1.56	0.00	0.64
3466687	*HAL*	histidine ammonia-lyase	-1.57	0.00	0.99
3638760	*IDH2*	isocitrate dehydrogenase 2 (NADP+), mitochondrial	-1.27	0.00	0.68
2720145	*LAP3*	leucine aminopeptidase 3	1.12	0.13	0.14
3621140	*LCMT2*	leucine carboxyl methyltransferase 2	1.11	0.11	0.65
2956438	*MUT*	methylmalonyl Coenzyme A mutase	-1.22	0.06	0.96
3031711	*NOS3*	nitric oxide synthase 3 (endothelial cell)	-1.17	0.06	0.87
2540157	*ODC1*	ornithine decarboxylase 1	1.15	0.00	0.63
3158060	*OPLAH*	5-oxoprolinase (ATP-hydrolysing)	-1.14	0.09	0.91
2875193	*P4HA2*	prolyl 4-hydroxylase, alpha polypeptide II	1.17	0.00	0.25
3860003	*PRODH2*	proline dehydrogenase (oxidase) 2	-1.23	0.00	0.38
3175971	*PSAT1*	phosphoserine aminotransferase 1	1.22	0.06	0.27
2626802	*PTPRG*	protein tyrosine phosphatase, receptor type, G	1.20	0.01	0.11
2458607	*PYCR2*	pyrroline-5-carboxylate reductase family, member 2	-1.15	0.06	0.85
3157647	*PYCRL*	pyrroline-5-carboxylate reductase-like	-1.22	0.06	0.74
3228813	*SARDH*	sarcosine dehydrogenase	-1.15	0.00	0.82
3861738	*SARS2*	seryl-tRNA synthetase 2, mitochondrial	-1.16	0.00	0.95
3743883	*SAT2*	spermidine/spermine N1-acetyltransferase family member 2	-1.19	0.01	0.51
3418007	*SHMT2*	serine hydroxymethyltransferase 2 (mitochondrial)	-1.12	0.00	0.95

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
2786322	*SLC7A11*	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	1.54	0.00	0.12
2358320	*TARS2*	threonyl-tRNA synthetase 2, mitochondrial (putative)	-1.12	0.06	0.94
2620348	*TGM4*	transglutaminase 4 (prostate)	1.47	0.01	0.00
Carbohydrate metabolism					
3218077	*ALDOB*	aldolase B, fructose-bisphosphate	-1.24	0.04	0.99
3750767	*ALDOC*	aldolase C, fructose-bisphosphate	-1.29	0.00	0.91
3458700	*B4GALNT1*	beta-1,4-N-acetyl-galactosaminyl transferase 1	1.36	0.01	0.11
3957486	*DUSP18*	dual specificity phosphatase 18	1.33	0.02	0.42
3403015	*ENO2*	enolase 2 (gamma, neuronal)	-1.30	0.00	0.92
2342176	*FPGT*	fucose-1-phosphate guanylyltransferase	1.18	0.13	0.15
3667241	*FUK*	fucokinase	-1.19	0.04	0.84
3540552	*FUT8*	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	1.43	0.00	0.27
3770923	*GALK1*	galactokinase 1	-1.16	0.06	0.87
3704513	*GALNS*	galactosamine (N-acetyl)-6-sulfate sulfatase	1.21	0.01	0.00
3784602	*GALNT1*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	1.31	0.02	0.02
2684187	*GBE1*	glucan (1,4-alpha-), branching enzyme 1	-1.21	0.02	0.97
3596147	*GCNT3*	glucosaminyl (N-acetyl) transferase 3, mucin type	1.39	0.00	0.92
3972929	*GK*	glycerol kinase	-1.31	0.04	0.35
2674653	*GMPPB*	GDP-mannose pyrophosphorylase B	-1.25	0.02	0.98
2691014	*GSK3B*	glycogen synthase kinase 3 beta	1.14	0.13	0.98
3867538	*GYS1*	glycogen synthase 1 (muscle)	-1.16	0.00	0.00
2489545	*HK2*	hexokinase 2	-1.33	0.00	0.82

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3601955	*MPI*	mannose phosphate isomerase	-1.26	0.00	0.64
2999948	*OGDH*	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	-1.24	0.00	0.67
3378541	*PC*	pyruvate carboxylase	-1.22	0.00	0.80
3890640	*PCK1*	phosphoenolpyruvate carboxykinase 1 (soluble)	-1.35	0.00	0.94
3529508	*PCK2*	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	-1.13	0.01	0.19
2515707	*PDK1*	pyruvate dehydrogenase kinase, isozyme 1	-1.19	0.02	0.10
3667093	*PDPR*	pyruvate dehydrogenase phosphatase regulatory subunit	1.19	0.00	0.75
4009811	*PFKFB1*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	1.41	0.01	0.57
2377094	*PFKFB2*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	-1.15	0.05	0.56
3233605	*PFKFB3*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	-1.29	0.00	0.86
2673312	*PFKFB4*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	-1.18	0.00	0.94
3923632	*PFKL*	phosphofructokinase, liver	-1.25	0.01	0.27
3232349	*PFKP*	phosphofructokinase, platelet	-1.16	0.01	0.88
3928040	*RWDD2B*	RWD domain containing 2B	-1.32	0.00	0.99
2798538	*SDHA*	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	-1.10	0.14	0.53
3365487	*UEVLD*	UEV and lactate/malate dehyrogenase domains	1.13	0.02	0.52
Lipid metabolism					
3434594	*ACADS*	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	-1.24	0.01	0.15
3543673	*ACOT2*	acyl-CoA thioesterase 2	-1.21	0.04	0.13
3771215	*ACOX1*	acyl-Coenzyme A oxidase 1, palmitoyl	-1.25	0.00	1.00
2796553	*ACSL1*	acyl-CoA synthetase long-chain family member 1	1.42	0.00	0.00

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3901696	*ACSS1*	acyl-CoA synthetase short-chain family member 1	1.17	0.03	0.00
3883064	*ACSS2*	acyl-CoA synthetase short-chain family member 2	-1.18	0.00	0.92
3923426	*AGPAT3*	1-acylglycerol-3-phosphate O-acyltransferase 3	1.49	0.00	0.52
2734047	*AGPAT9*	1-acylglycerol-3-phosphate O-acyltransferase 9	1.28	0.06	0.94
3274758	*AKR1C2*	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, ty	1.25	0.00	0.51
3714068	*ALDH3A2*	aldehyde dehydrogenase 3 family, member A2	1.19	0.01	0.29
2898499	*ALDH5A1*	aldehyde dehydrogenase 5 family, member A1	1.14	0.06	0.61
3457794	*APOF*	apolipoprotein F	-1.26	0.03	0.66
3379326	*CHKA*	choline kinase alpha	1.13	0.06	0.98
3379644	*CPT1A*	carnitine palmitoyltransferase 1A (liver)	-1.51	0.00	0.67
3833992	*CYP2S1*	cytochrome P450, family 2, subfamily S, polypeptide 1	-1.15	0.11	0.22
3015040	*CYP3A43*	cytochrome P450, family 3, subfamily A, polypeptide 43	-1.18	0.13	0.95
3853658	*CYP4F11*	cytochrome P450, family 4, subfamily F, polypeptide 11	1.31	0.00	0.16
3823340	*CYP4F12*	cytochrome P450, family 4, subfamily F, polypeptide 12	1.18	0.10	0.04
2709235	*DGKG*	diacylglycerol kinase, gamma 90kDa	1.23	0.00	0.33
2708720	*EHHADH*	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	-1.22	0.03	0.97
2781813	*ELOVL6*	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	1.12	0.09	0.71
3408018	*ETNK1*	ethanolamine kinase 1	1.11	0.11	0.38
2451870	*ETNK2*	ethanolamine kinase 2	1.31	0.00	0.00
2334740	*FAAH*	fatty acid amide hydrolase	-1.17	0.06	0.86

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3774635	*FASN*	fatty acid synthase	-1.10	0.03	0.01
3821995	*GCDH*	glutaryl-Coenzyme A dehydrogenase	-1.23	0.01	0.65
3683276	*GDE1*	glycerophosphodiester phosphodiesterase 1	1.34	0.00	0.56
3729014	*GDPD1*	glycerophosphodiester phosphodiesterase domain containing 1	1.40	0.08	0.99
3306984	*GPAM*	glycerol-3-phosphate acyltransferase, mitochondrial	1.31	0.01	0.05
2945518	*GPLD1*	glycosylphosphatidylinositol specific phospholipase D1	1.19	0.03	0.01
3821015	*LDLR*	low density lipoprotein receptor	-1.20	0.00	0.57
3299585	*LIPA*	lipase A, lysosomal acid, cholesterol esterase	1.23	0.04	0.87
3595691	*LIPC*	lipase, hepatic	1.16	0.03	0.67
3787855	*LIPG*	lipase, endothelial	1.53	0.00	0.01
2708855	*LIPH*	lipase, member H	2.26	0.00	0.09
3661718	*LPCAT2*	lysophosphatidylcholine acyltransferase 2	1.64	0.02	0.77
2469910	*LPIN1*	lipin 1	1.13	0.03	0.35
3796335	*LPIN2*	lipin 2	-1.13	0.01	0.85
2737257	*MTTP*	microsomal triglyceride transfer protein	1.23	0.02	0.78
3962219	*NAGA*	N-acetylgalactosaminidase, alpha-	1.12	0.06	0.66
3065480	*NAPEPLD*	N-acyl phosphatidylethanolamine phospholipase D	1.32	0.02	0.33
3294142	*PLA2G12B*	phospholipase A2, group XIIB	1.28	0.00	0.71
3666124	*PLA2G15*	phospholipase A2, group XV	1.11	0.02	0.83
3947952	*PNPLA3*	patatin-like phospholipase domain containing 3	1.18	0.02	0.10
3434142	*PRKAB1*	protein kinase, AMP-activated, beta 1 non-catalytic subunit	1.12	0.07	0.90

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3220673	*PTGR1*	prostaglandin reductase 1	1.43	0.00	0.80
3352904	*SC5DL*	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like	1.31	0.09	0.24
3289235	*SGMS1*	sphingomyelin synthase 1	1.26	0.06	0.18
2738664	*SGMS2*	sphingomyelin synthase 2	1.67	0.01	0.00
2359885	*SLC27A3*	solute carrier family 27 (fatty acid transporter), member 3	-1.17	0.04	0.50
3415763	*SOAT2*	sterol O-acyltransferase 2	-1.20	0.01	0.64
3747966	*SREBF1*	sterol regulatory element binding transcription factor 1	-1.15	0.03	0.78
3967689	*STS*	steroid sulfatase (microsomal), isozyme S	1.52	0.00	0.04
3137901	*TTPA*	tocopherol (alpha) transfer protein	1.96	0.00	0.08
Acute phase response					
2460296	*AGT*	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1.14	0.02	0.01
3221800	*AMBP*	alpha-1-microglobulin/bikunin precursor	1.12	0.00	0.36
3442475	*C1R*	complement component 1, r subcomponent	1.48	0.00	0.00
3403168	*C1S*	complement component 1, s subcomponent	1.39	0.00	0.17
2902958	*C4B*	complement component 4B (Chido blood group)	-1.14	0.04	0.69
2377165	*C4BPA*	complement component 4 binding protein, alpha	-1.19	0.12	0.54
3223776	*C5*	complement component 5	-1.20	0.08	0.74
2902844	*CFB*	complement factor B	-1.23	0.01	0.72
3851020	*ECSIT*	ECSIT homolog (Drosophila)	-1.24	0.01	0.64
2790626	*FGA*	fibrinogen alpha chain	1.24	0.01	0.04
2790652	*FGG*	fibrinogen gamma chain	1.40	0.06	0.40

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3838094	*FTL*	ferritin, light polypeptide	1.29	0.11	0.63
3830306	*HAMP*	hepcidin antimicrobial peptide	-1.41	0.01	0.99
3667858	*HP*	haptoglobin	-1.28	0.02	0.82
3360874	*HPX*	hemopexin	-1.35	0.01	0.92
3000503	*IGFBP1*	insulin-like growth factor binding protein 1	-1.19	0.03	0.25
3996551	*IKBKG*	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	1.22	0.04	0.10
2496962	*IL1R1*	interleukin 1 receptor, type I	1.19	0.12	0.82
2657831	*IL1RAP*	interleukin 1 receptor accessory protein	-1.14	0.08	0.94
2360257	*IL6R*	interleukin 6 receptor	-1.19	0.01	0.86
2857416	*IL6ST*	interleukin 6 signal transducer (gp130, oncostatin M receptor)	1.20	0.13	0.25
4027009	*IRAK1*	interleukin-1 receptor-associated kinase 1	-1.12	0.04	0.71
2624178	*ITIH3*	inter-alpha (globulin) inhibitor H3	-1.42	0.00	0.84
3160895	*JAK2*	Janus kinase 2 (a protein tyrosine kinase)	1.43	0.04	0.86
3733065	*MAP2K6*	mitogen-activated protein kinase kinase 6	1.29	0.08	0.57
2904946	*MAPK13*	mitogen-activated protein kinase 13	-1.15	0.08	0.92
3832760	*NFKBIB*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	-1.11	0.10	0.80
2934682	*PLG*	plasminogen	1.46	0.01	0.11
3442579	*RBP5*	retinol binding protein 5, cellular	-1.24	0.02	0.64
2892341	*RIPK1*	receptor (TNFRSF)-interacting serine-threonine kinase 1	1.18	0.13	0.51
3549757	*SERPINA3*	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1.23	0.00	0.41

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2444529	*SERPINC1*	serpin peptidase inhibitor, clade C (antithrombin), member 1	1.23	0.06	0.91
3937743	*SERPIND1*	serpin peptidase inhibitor, clade D (heparin cofactor), member 1	1.10	0.01	0.66
3016148	*SERPINE1*	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-1.30	0.00	0.23
3331355	*SERPING1*	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	1.41	0.01	0.03
3757840	*STAT3*	signal transducer and activator of transcription 3 (acute-phase response factor)	1.10	0.01	0.32
3783565	*TTR*	transthyretin	1.12	0.01	0.82
Oxidative stress response					
3646164	*DNAJA3*	DnaJ (Hsp40) homolog, subfamily A, member 3	-1.11	0.01	0.62
2656569	*DNAJB11*	DnaJ (Hsp40) homolog, subfamily B, member 11	1.18	0.12	0.94
2343289	*DNAJB4*	DnaJ (Hsp40) homolog, subfamily B, member 4	1.25	0.13	0.66
3018866	*DNAJB9*	DnaJ (Hsp40) homolog, subfamily B, member 9	1.27	0.06	0.25
3487432	*DNAJC15*	DnaJ (Hsp40) homolog, subfamily C, member 15	1.17	0.12	0.11
3497270	*DNAJC3*	DnaJ (Hsp40) homolog, subfamily C, member 3	1.38	0.01	0.95
2340350	*DNAJC6*	DnaJ (Hsp40) homolog, subfamily C, member 6	1.14	0.06	0.87
2382970	*EPHX1*	epoxide hydrolase 1, microsomal (xenobiotic)	1.29	0.00	0.33
2957700	*GCLC*	glutamate-cysteine ligase, catalytic subunit	1.21	0.03	0.70
3568603	*GPX2*	glutathione peroxidase 2 (gastrointestinal)	1.24	0.00	0.26
2957462	*GSTA4*	glutathione S-transferase alpha 4	1.19	0.11	0.60
3662387	*HERPUD1*	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	1.14	0.02	0.03
3944129	*HMOX1*	heme oxygenase (decycling) 1	1.34	0.00	0.83

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3696666	*NQO1*	NAD(P)H dehydrogenase, quinone 1	1.10	0.07	0.17
3917851	*SOD1*	superoxide dismutase 1, soluble	1.13	0.13	0.27
3429460	*TXNRD1*	thioredoxin reductase 1	1.22	0.01	0.59
3952880	*TXNRD2*	thioredoxin reductase 2	-1.19	0.00	0.77
Hepatic fibrosis or hepatic stellate activation					
2863964	*ARSB*	arylsulfatase B	1.14	0.09	0.44
2351063	*CSF1*	colony stimulating factor 1 (macrophage)	1.10	0.11	0.26
2974330	*CTGF*	connective tissue growth factor	-1.22	0.02	0.15
3002640	*EGFR*	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.12	0.02	0.17
3229338	*FCN1*	ficolin (collagen/fibrinogen domain containing) 1	-1.20	0.04	0.79
3132016	*FGFR1*	fibroblast growth factor receptor 1	1.49	0.00	0.05
3310041	*FGFR2*	fibroblast growth factor receptor 2	1.53	0.00	0.00
3704513	*GALNS*	galactosamine (N-acetyl)-6-sulfate sulfatase	1.21	0.01	0.00
2835792	*GM2A*	GM2 ganglioside activator	1.10	0.09	0.10
2675120	*HYAL3*	hyaluronoglucosaminidase 3	1.13	0.08	0.12
4025339	*IDS*	iduronate 2-sulfatase	1.26	0.03	0.01
3918574	*IFNAR1*	interferon (alpha, beta and omega) receptor 1	1.25	0.08	0.02
3918447	*IFNAR2*	interferon (alpha, beta and omega) receptor 2	1.32	0.05	0.35
3610804	*IGF1R*	insulin-like growth factor 1 receptor	1.13	0.01	0.89
3654175	*IL4R*	interleukin 4 receptor	-1.19	0.00	0.99
2724308	*KLB*	klotho beta	1.20	0.01	0.50
2340433	*LEPR*	leptin receptor	1.40	0.03	0.00
3304012	*MGEA5*	meningioma expressed antigen 5 (hyaluronidase)	1.23	0.02	0.21

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3388830	*MMP3*	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	-1.30	0.11	0.96
3962219	*NAGA*	N-acetylgalactosaminidase, alpha-	1.12	0.06	0.66
3721795	*NAGLU*	N-acetylglucosaminidase, alpha-	-1.12	0.01	0.65
2791197	*PDGFC*	platelet derived growth factor C	1.18	0.11	0.63
2881239	*PDGFRB*	platelet-derived growth factor receptor, beta polypeptide	-1.31	0.00	0.99
3087703	*PDGFRL*	platelet-derived growth factor receptor-like	1.15	0.11	0.62
3773340	*SGSH*	N-sulfoglucosamine sulfohydrolase	-1.10	0.05	0.73
3908358	*SULF2*	sulfatase 2	1.51	0.00	0.01
3863021	*TGFB1*	transforming growth factor, beta 1	1.23	0.00	0.28
2615360	*TGFBR2*	transforming growth factor, beta receptor II (70/80kDa)	1.12	0.02	0.97
2422722	*TGFBR3*	transforming growth factor, beta receptor III	1.31	0.00	0.80
3976341	*TIMP1*	TIMP metallopeptidase inhibitor 1	1.24	0.01	0.18
3772661	*TIMP2*	TIMP metallopeptidase inhibitor 2	1.28	0.05	0.30
3943504	*TIMP3*	TIMP metallopeptidase inhibitor 3	1.25	0.00	0.14
Cell cycle or Apoptosis					
3191724	*ABL1*	c-abl oncogene 1, receptor tyrosine kinase	1.12	0.00	0.59
3838067	*BAX*	BCL2-associated X protein	-1.13	0.15	0.17
3261165	*BTRC*	beta-transducin repeat containing	1.12	0.01	0.44
3338192	*CCND1*	cyclin D1	1.12	0.06	0.21
3828112	*CCNE1*	cyclin E1	1.31	0.02	0.06
3145107	*CCNE2*	cyclin E2	1.28	0.08	0.50
3551303	*CCNK*	cyclin K	1.24	0.07	0.68
3458783	*CDK4*	cyclin-dependent kinase 4	-1.14	0.03	0.49

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3061319	*CDK6*	cyclin-dependent kinase 6	1.15	0.14	0.13
2905169	*CDKN1A*	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.10	0.07	0.37
3042001	*CYCS*	cytochrome c, somatic	-1.26	0.09	1.00
2897576	*E2F3*	E2F transcription factor 3	1.19	0.07	0.23
3257098	*FAS*	Fas (TNF receptor superfamily, member 6)	1.36	0.03	0.19
3421300	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	1.19	0.06	0.09
Nuclear receptor transcribed genes					
3218528	*ABCA1*	ATP-binding cassette, sub-family A (ABC1), member 1	1.21	0.00	0.04
3060182	*ABCB1*	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1.17	0.14	1.00
3060117	*ABCB4*	ATP-binding cassette, sub-family B (MDR/TAP), member 4	1.17	0.08	0.06
3475879	*ABCB9*	ATP-binding cassette, sub-family B (MDR/TAP), member 9	1.16	0.11	0.08
3665029	*CES3*	carboxylesterase 3	-1.17	0.02	0.56
3910429	*CYP24A1*	cytochrome P450, family 24, subfamily A, polypeptide 1	-1.30	0.00	0.60
3502437	*F10*	coagulation factor X	-1.20	0.02	0.64
2473149	*NCOA1*	nuclear receptor coactivator 1	1.12	0.11	0.72
3887635	*NCOA3*	nuclear receptor coactivator 3	1.24	0.00	0.06
2402883	*NR0B2*	nuclear receptor subfamily 0, group B, member 2	-1.29	0.01	0.89
2659393	*OSTalpha*	organic solute transporter alpha	-1.32	0.00	0.45
3738629	*SLC16A3*	solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	-1.30	0.00	0.22
2907887	*SLC22A7*	solute carrier family 22 (organic anion transporter), member 7	1.14	0.10	0.38

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3866785	*SULT2A1*	sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	-1.17	0.10	0.87
3381817	*UCP2*	uncoupling protein 2 (mitochondrial, proton carrier)	-1.34	0.00	0.97
2772341	*UGT2B4*	UDP glucuronosyltransferase 2 family, polypeptide B4	2.17	0.00	0.84
3160175	*VLDLR*	very low density lipoprotein receptor	1.41	0.01	0.28
Signaling molecules					
3000342	*ADCY1*	adenylate cyclase 1 (brain)	1.27	0.00	0.00
2677723	*ARHGEF3*	Rho guanine nucleotide exchange factor (GEF) 3	1.25	0.00	0.01
2359993	*CREB3L4*	cAMP responsive element binding protein 3-like 4	-1.16	0.00	0.79
2480383	*EPAS1*	endothelial PAS domain protein 1	-1.13	0.01	0.59
3210808	*GNAQ*	guanine nucleotide binding protein (G protein), q polypeptide	1.17	0.03	0.59
2903782	*ITPR3*	inositol 1,4,5-triphosphate receptor, type 3	-1.18	0.01	0.89
4001785	*MAP3K15*	mitogen-activated protein kinase kinase kinase 15	1.21	0.02	0.16
2496727	*MAP4K4*	mitogen-activated protein kinase kinase kinase kinase 4	1.14	0.03	0.55
2644565	*MRAS*	muscle RAS oncogene homolog	1.20	0.02	0.46
2580955	*NMI*	N-myc (and STAT) interactor	1.63	0.00	0.02
3382861	*PAK1*	p21 protein (Cdc42/Rac)-activated kinase 1	1.63	0.00	0.00
3559192	*PRKD1*	protein kinase D1	1.46	0.00	0.00
3091301	*PTK2B*	PTK2B protein tyrosine kinase 2 beta	-1.13	0.03	0.29
3568616	*RAB15*	RAB15, member RAS onocogene family	1.47	0.00	0.17
2436985	*SHC1*	SHC (Src homology 2 domain containing) transforming protein 1	1.15	0.02	0.01

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
Alcohol metabolism					
3169331	*ALDH1B1*	aldehyde dehydrogenase 1 family, member B1	1.13	0.01	0.11
3432090	*ALDH2*	aldehyde dehydrogenase 2 family (mitochondrial)	-1.12	0.06	0.71
3337329	*ALDH3B1*	aldehyde dehydrogenase 3 family, member B1	-1.32	0.00	0.80
3571727	*ALDH6A1*	aldehyde dehydrogenase 6 family, member A1	-1.60	0.00	0.65
2975257	*ALDH8A1*	aldehyde dehydrogenase 8 family, member A1	-1.22	0.09	0.69
Transcription factors					
3945914	*ATF4*	activating transcription factor 4 (tax-responsive enhancer element B67)	-1.11	0.06	0.21
3858993	*CEBPA*	CCAAT/enhancer binding protein (C/EBP), alpha	-1.14	0.02	0.94
3888613	*CEBPB*	CCAAT/enhancer binding protein (C/EBP), beta	1.12	0.02	0.89
3134013	*CEBPD*	CCAAT/enhancer binding protein (C/EBP), delta	-1.16	0.10	0.99
3836266	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	-1.26	0.14	0.85
3561703	*FOXA1*	forkhead box A1	-1.15	0.01	0.16
3510858	*FOXO1*	forkhead box O1	-1.12	0.01	0.63
2891556	*FOXQ1*	forkhead box Q1	-1.20	0.05	0.57
3539070	*HIF1A*	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	1.13	0.13	0.72
3754797	*HNF1B*	HNF1 homeobox B	-1.14	0.04	1.00
3886453	*HNF4A*	hepatocyte nuclear factor 4, alpha	1.12	0.01	0.01
2516967	*HOXD1*	homeobox D1	-1.33	0.00	0.41
2907730	*SRF*	serum response factor (c-fos serum response element-binding transcription factor)	-1.11	0.04	0.76
3458337	*STAT6*	signal transducer and activator of transcription 6, interleukin-4 induced	-1.20	0.00	0.68

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
Chromatin regulation					
2544662	*DNMT3A*	DNA (cytosine-5-)-methyltransferase 3 alpha	-1.11	0.03	0.01
3882012	*DNMT3B*	DNA (cytosine-5-)-methyltransferase 3 beta	1.25	0.00	0.01
3945006	*H1F0*	H1 histone family, member 0	-1.15	0.05	0.82
3048869	*H2AFV*	H2A histone family, member V	-1.13	0.11	0.50
2611504	*HDAC11*	histone deacetylase 11	-1.32	0.01	0.66
3758845	*HDAC5*	histone deacetylase 5	-1.12	0.02	0.89
3976848	*HDAC6*	histone deacetylase 6	-1.16	0.01	0.94
2947073	*HIST1H1B*	histone cluster 1, H1b	1.18	0.00	0.76
2946353	*HIST1H1D*	histone cluster 1, H1d	1.31	0.05	0.78
2900074	*HIST1H2BN*	histone cluster 1, H2bn	1.13	0.11	0.04
2946324	*HIST1H3D*	histone cluster 1, H3d	1.11	0.07	0.06
2899233	*HIST1H3E*	histone cluster 1, H3e	1.14	0.12	0.12
2946383	*HIST1H4H*	histone cluster 1, H4h	1.31	0.01	0.01
2509740	*MBD5*	methyl-CpG binding domain protein 5	1.43	0.00	0.00
3656855	*MYST1*	MYST histone acetyltransferase 1	-1.15	0.02	0.02
3252382	*MYST4*	MYST histone acetyltransferase (monocytic leukemia) 4	1.19	0.11	0.68
Genes associated with alcoholism					
3521174	*ABCC4*	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1.14	0.01	0.75
3606304	*AKAP13*	A kinase (PRKA) anchor protein 13	1.22	0.01	0.47
3927226	*APP*	amyloid beta (A4) precursor protein	1.13	0.00	0.15

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3673684	*CDT1*	chromatin licensing and DNA replication factor 1	-1.22	0.00	0.05
3603436	*CHRNA5*	cholinergic receptor, nicotinic, alpha 5	1.36	0.02	0.06
2772968	*COX18*	COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)	-1.23	0.01	0.77
3720228	*CRKRS*	Cdc2-related kinase, arginine/serine-rich	1.19	0.02	0.29
3652424	*EEF2K*	eukaryotic elongation factor-2 kinase	1.11	0.01	0.90
3260383	*ENTPD7*	ectonucleoside triphosphate diphosphohydrolase 7	1.12	0.01	0.04
2948587	*FLOT1*	flotillin 1	-1.15	0.01	1.00
3199511	*FREM1*	FRAS1 related extracellular matrix 1	1.82	0.00	0.07
2469825	*GREB1*	GREB1 protein	1.24	0.01	0.00
3636391	*HOMER2*	homer homolog 2 (Drosophila)	1.27	0.01	0.27
3349660	*HTR3B*	5-hydroxytryptamine (serotonin) receptor 3B	2.13	0.00	0.27
3779362	*IMPA2*	inositol(myo)-1(or 4)-monophosphatase 2	-1.24	0.01	0.68
3817733	*JMJD2B*	jumonji domain containing 2B	-1.17	0.00	0.77
3517793	*KLF12*	Kruppel-like factor 12	1.30	0.00	0.18
2667809	*OSBPL10*	oxysterol binding protein-like 10	1.17	0.01	0.31
2698996	*PCOLCE2*	procollagen C-endopeptidase enhancer 2	1.88	0.00	0.01
3358361	*PDDC1*	Parkinson disease 7 domain containing 1	-1.16	0.02	0.41
2361401	*PMF1*	polyamine-modulated factor 1	-1.19	0.02	0.75
2480168	*PRKCE*	protein kinase C, epsilon	1.20	0.00	0.00
3213847	*SHC3*	SHC (Src homology 2 domain containing) transforming protein 3	1.31	0.02	0.64
2902884	*SKIV2L*	superkiller viralicidic activity 2-like (S. cerevisiae)	-1.16	0.02	0.88

Transcript cluster ID	Gene	Gene Description	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3369366	*SLC1A2*	solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.17	0.01	0.53
2610544	*SLC6A11*	solute carrier family 6 (neurotransmitter transporter, GABA), member 11	1.23	0.01	0.79
3751794	*SLC6A4*	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	1.20	0.00	0.01
3318666	*SMPD1*	sphingomyelin phosphodiesterase 1, acid lysosomal	1.18	0.03	0.90
2378662	*TRAF5*	TNF receptor-associated factor 5	1.84	0.00	0.04
3316375	*TSPAN4*	tetraspanin 4	-1.20	0.00	0.92
3896976	*TXNDC13*	thioredoxin domain containing 13	1.23	0.00	0.00
3815328	*WDR18*	WD repeat domain 18	-1.19	0.01	0.87
3989089	*ZBTB33*	zinc finger and BTB domain containing 33	1.27	0.01	0.15
3971923	*ZFX*	zinc finger protein, X-linked	1.16	0.00	0.80
3078478	*ZNF786*	zinc finger protein 786	1.20	0.03	0.67

Table 13. Differentially expressed genes within pathways that were significantly affected by chronic alcohol exposure. Pathways significantly affected by ethanol were identified by Ingenuity Pathway analysis (Table 12). Pathways (and genes in them) with related functions were grouped into broader categories. For example, Glycolysis/ Gluconeogenesis and Citrate cycle are grouped under carbohydrate metabolism. Fold change (E/C) is ethanol/ control. Differentially expressed genes (FDR ≤ 15% and difference ≥ 10%) within the key pathways discussed in the text are shown. Other genes are listed in Appendix.

4.a. Validation of differential gene expression results by qRT-PCR

Quantitative real time RT-PCR assays (Taqman gene expression assays) were carried out to confirm differential gene expression changes that were observed in microarray data. Genes were selected from the key pathways that were affected by chronic ethanol treatment. Even small expression changes in the array data were confirmed by these assays. For example *SREBF1* decreased in expression by 15% in the array data and was confirmed to be decreased by 19% in qRT-PCR (Figure 18). A total of thirteen genes out of fourteen genes testes were confirmed with fold changes very similar to that observed in the array data (Figure 18).

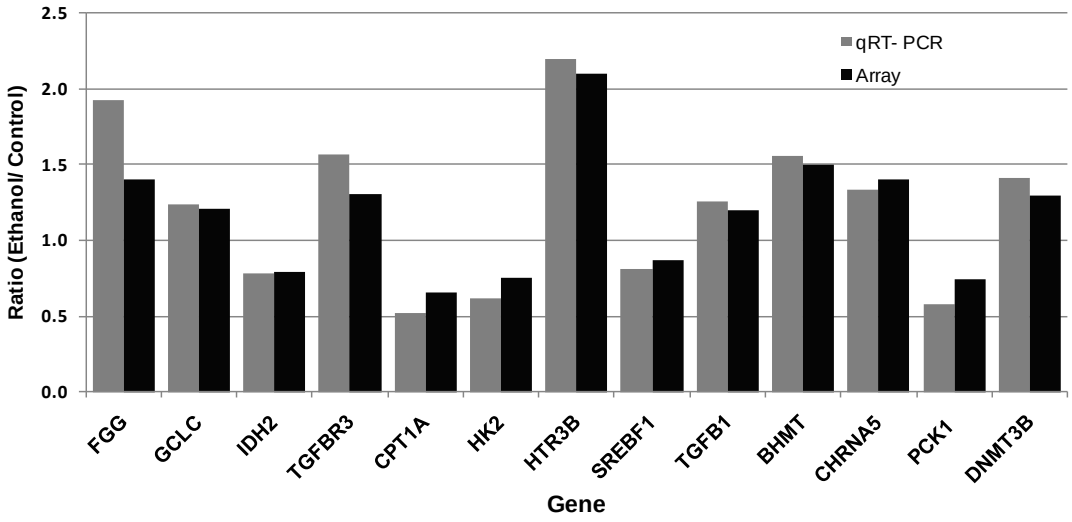


Figure 18. qRT-PCR validation of differential gene expression. Genes from key pathways affected by chronic alcohol exposure were validated by qRT-PCR. Fold change is the ratio of expression under ethanol treatment over control treatment. For all genes p-value ≤ 5 x 10^{-6}. For comparison, fold changes observed in array data are shown.

5. Effects of chronic alcohol exposure on RNA splicing

Effects of ethanol on splicing were explored using Affymetrix GeneChip® Human Exon 1.0 ST Arrays. Two-way ANOVA generated p-values (and FDRs calculated using this p-value) were used to identify genes that were differentially alternatively spliced due to chronic ethanol exposure. Some of these genes were also differentially expressed. Of the 10,222 genes that were analyzed many genes were differentially alternatively spliced after treatment with ethanol (Table 14).

	Number of genes		
	FDR 15%	**FDR 10%**	**FDR 3%**
Total Alternatively spliced[1]	**1323**	**948**	**458**
Alternatively spliced only[2]	687	559	323
Differentially expressed and Alternatively spliced[3]	**636**	**389**	**135**

Table 14. Effects of chronic ethanol exposure on splicing at different false discovery rates. [1]Irrespective of the differential expression FDR. [2]Alternative splicing without differential expression at that FDR. [3]Both alternative splicing and differential expression. FDR was calculated by the method of Benjamini and Hochberg (1995).

Due to the complexity of identifying true alterative splicing events (discussed in Introduction), FDR ≤ 3% was used for detecting genes that were differentially alternatively spliced upon chronic ethanol treatment. A total of 458 (4.5%) of the detected genes were differentially alternatively spliced at an FDR ≤ 3%. To detect true events, the effects of alcohol on each probe set was visualized in the context of the gene using a custom track in the UCSC genome browser (Kent et al., 2002). Multiple factors were considered during this inspection. How is the expression of each probe set affected by ethanol? If a probe set changes in expression with ethanol, is this effect different from how the other probe sets in the gene are affected? To aid in this analysis, a one-way ANOVA was carried out on probe sets. The fold change obtained for each probe set along with the p-value (which indicates if the effect is significant) from one-way ANOVA were used to identify probe sets that were probably alternatively spliced in a gene. The analysis was restricted to genes with a minimum of three present probe sets.

In many genes, probe sets at the 5’ end of the transcript were either called absent or exhibited a decrease in expression with ethanol treatment (Figure 19). A pattern was also noticed at the 3’ end of the transcripts, with an increase in the expression in the presence of ethanol. Examples of these edge effects are shown in Figure 19. In most cases it was difficult to distinguish between edge effects and true alternate promoter and alternate poly(A) events and we therefore focused on other alternate events such as putative cassette exons. However, in some cases multiple probe sets indicated the presence of an alternate promoter

(for example, in *MBD5*, *KANK1*). We considered these alternate promoters to be probably differential alternative events.

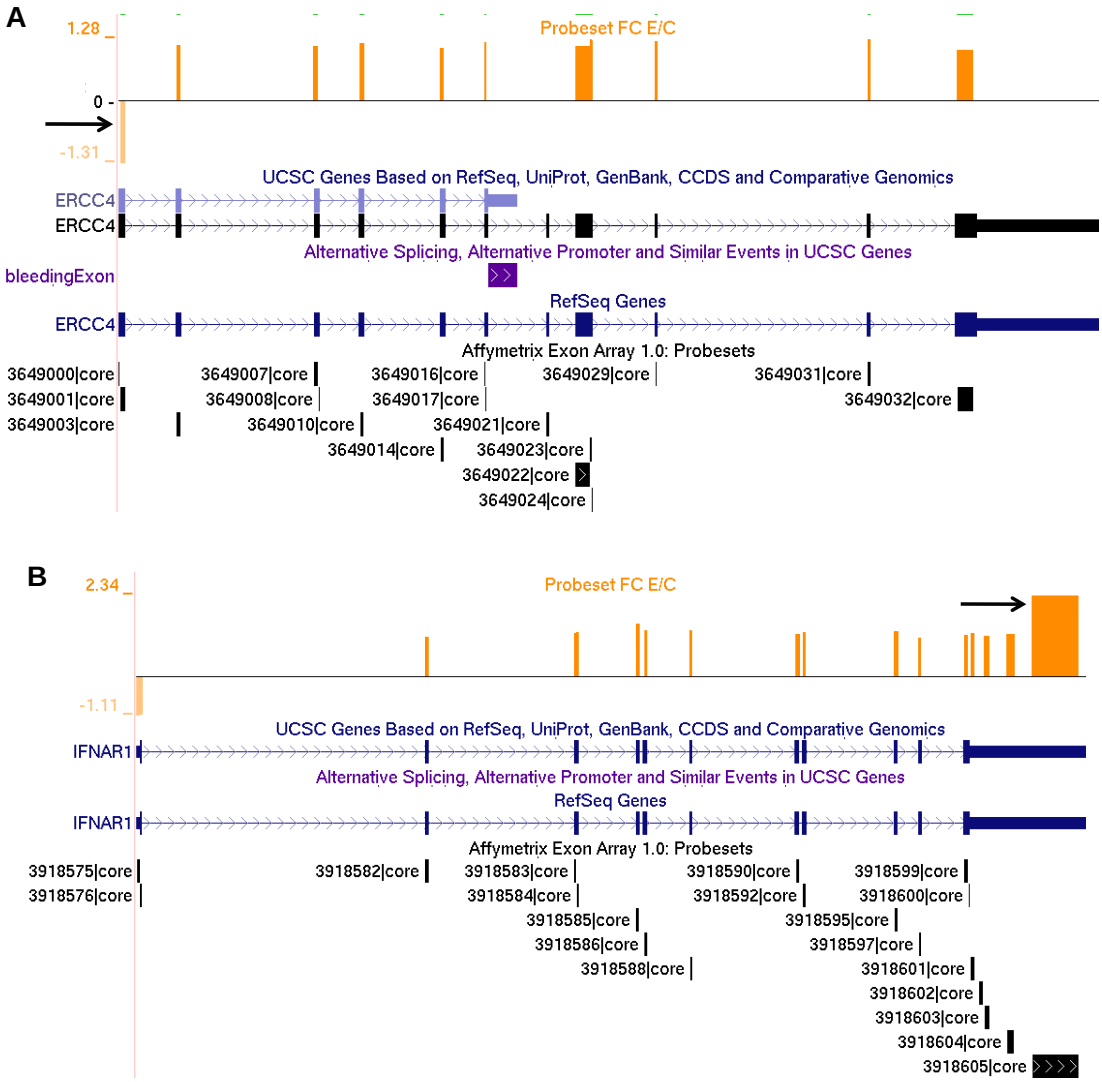
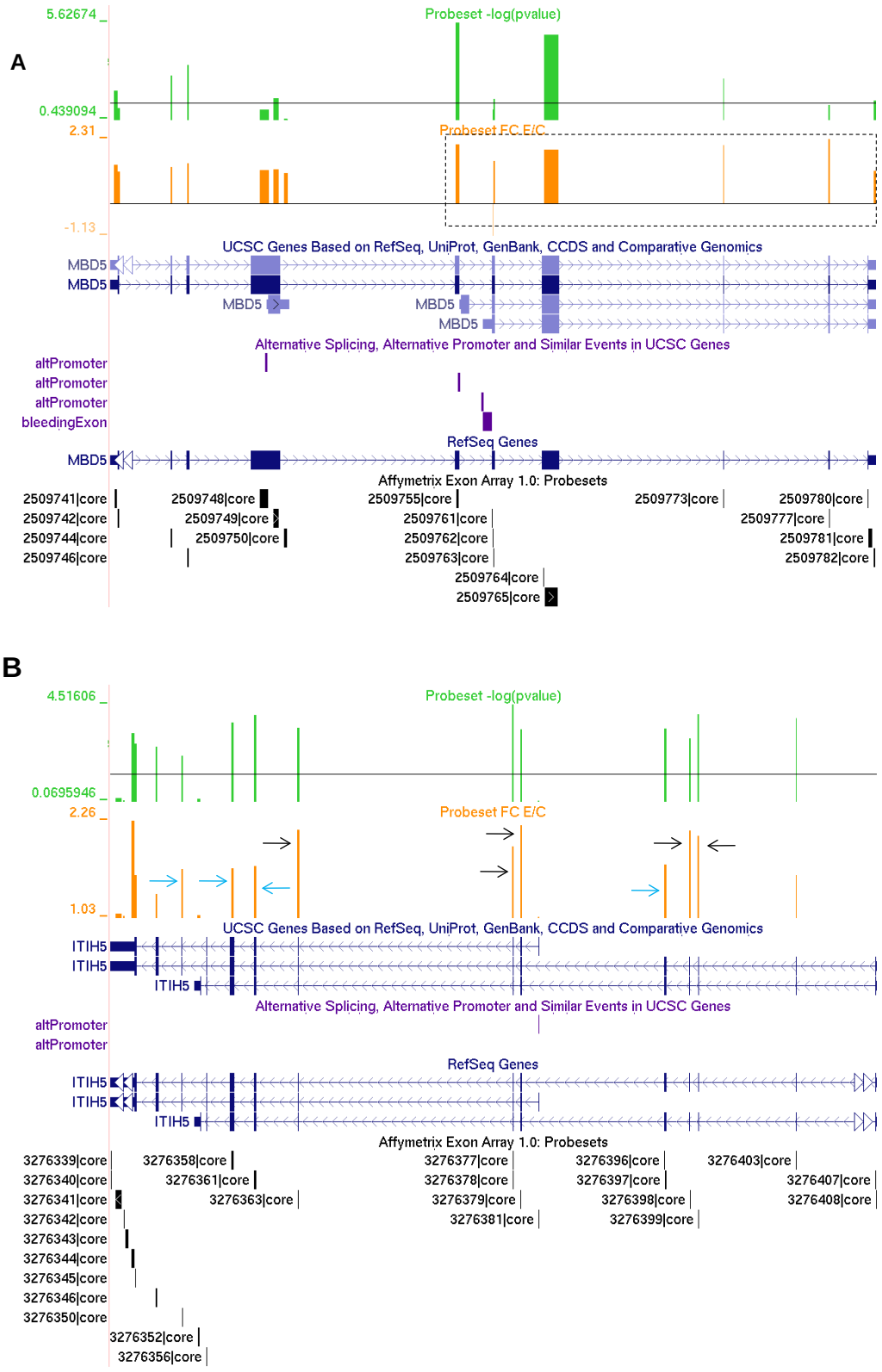


Figure 19. 5' and 3' edge effects in exon array data. Both UCSC genes and Refseq tracks are shown. In these tracks exons are represented as boxes, and intervening introns as lines. Arrows on introns show the direction of transcription. The alternative splicing events track, which displays all known alternative splicing events, is also shown. Probe set FC E/C is a custom track that displays fold change, ethanol/ control, for each probe set from the data in this study. (A) An example of 5' edge effect; affected probe set indicated by an arrow (B) An example of 3' effect, indicated by an arrow. 5' edge effect is also observed in this gene.

A total of 458 genes that were alternatively spliced were visually inspected and grouped into three categories (described in detail in Materials and Methods, section 9) 1. Probably differentially alternatively spliced, if a probe set has a significant fold change between ethanol and control, and behaves differently from the remaining probe sets in the gene; 2. Probably not differentially alternatively spliced if there was no indication of differential alternative splicing or if only edge effects were observed; 3. Uncertain if the changes appear to be probably differentially alternatively spliced but it is difficult to conclude if the alternative event is real. An example of each category is shown in Figure 20. 142 genes (31%) appeared to be probably alternatively spliced while 199 (43%) genes were probably not alternatively spliced. The remaining genes were grouped into the uncertain category. In genes that were detected as probably alternatively spliced probe sets that are probably differentially spliced are listed in Table 15.


A
5.62674
Probeset -log(pvalue)
0.439094
2.31
Probeset FC E/C
-1.13
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
MBD5
Alternative Splicing, Alternative Promoter and Similar Events in UCSC Genes
altPromoter
bleedingExon
RefSeq Genes
Affymetrix Exon Array 1.0: Probesets
2509741|core
2509742|core
2509744|core
2509746|core
2509748|core
2509749|core
2509750|core
2509755|core
2509761|core
2509762|core
2509763|core
2509764|core
2509765|core
2509773|core
2509777|core
2509780|core
2509781|core
2509782|core
B
4.51606
Probeset -log(pvalue)
0.0695946
2.26
Probeset FC E/C
1.03
UCSC Genes Based on RefSeq, UniProt, GenBank, CCDS and Comparative Genomics
ITIH5
Alternative Splicing, Alternative Promoter and Similar Events in UCSC Genes
altPromoter
RefSeq Genes
Affymetrix Exon Array 1.0: Probesets
3276339|core
3276340|core
3276341|core
3276342|core
3276343|core
3276344|core
3276345|core
3276346|core
3276350|core
3276352|core
3276356|core
3276358|core
3276361|core
3276363|core
3276377|core
3276378|core
3276379|core
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3276407|core
3276408|core

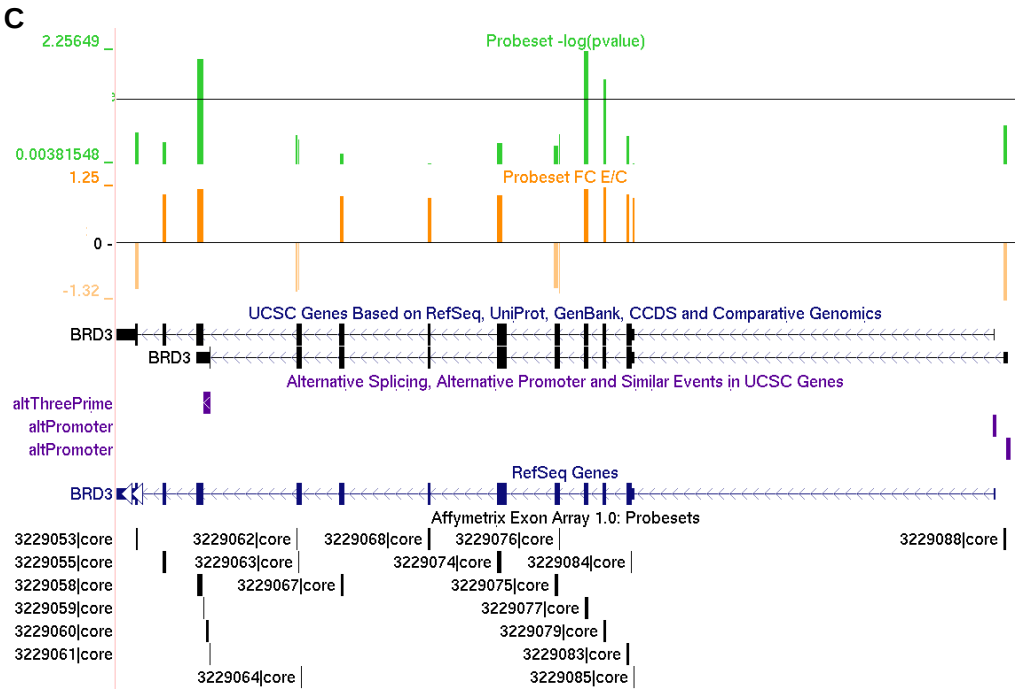

Figure 20. Examples of different groups of alternatively spliced genes. USCS genome browser images with exon array custom track are shown. (A) An example of a gene that was grouped as probably differentially alternatively spliced is shown. One half of the probe sets have lower fold change than the other half, suggesting expression of a shorter form of the transcript with ethanol. (B) Gene classified as Uncertain. Among the probe sets that were significantly affected, some (indicated by blue arrows) had a smaller fold change while others (indicated by black arrows) had a much higher fold change; some remained unaffected. Thus it was difficult to say if the gene was alternatively spliced or not. (C) Gene that was classified as probably not differentially alternatively spliced by looking at the expression of probe sets in the gene. Only three probe sets were significantly affected by ethanol but were similar in expression to each other.

From the 142 genes that appeared probably differentially alternative spliced, 317 probe sets were matched to known alternative splicing or promoter or termination events from the data available in the UCSC genome browser. Of these only 39 probe sets appeared to be affected by ethanol. 26 of these probe sets were cassette exons.

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3308034	3307939	ABLIM1	actin binding LIM protein 1	0.00	0.04	2.10	1.13	
2796582	2796553	ACSL1	acyl-CoA synthetase long-chain family member 1	0.00	0.00	2.66	1.42	
3716956	3716950	ADAP2	ArfGAP with dual PH domains 2	0.00	0.02	1.56	1.21	Cassette Exon
3716961	3716950	ADAP2	ArfGAP with dual PH domains 2	0.00	0.02	1.85	1.21	
3263610	3263555	ADD3	adducin 3 (gamma)	0.02	0.13	1.53	1.15	
3940676	3940631	ADRBK2	adrenergic, beta, receptor kinase 2	0.00	0.02	2.35	1.25	
3509892	3509885	ALG5	asparagine-linked glycosylation 5, dolichyl-phosphate beta-glucosyltransferase homolog (S. cerevisiae)	0.00	0.06	2.05	1.12	
3704741	3704717	ANKRD11	ankyrin repeat domain 11	0.01	0.02	2.03	1.17	
3282143	3282117	ANKRD26	ankyrin repeat domain 26	0.01	0.19	1.84	1.24	
3282161	3282117	ANKRD26	ankyrin repeat domain 26	0.01	0.19	1.66	1.24	
3282188	3282117	ANKRD26	ankyrin repeat domain 26	0.01	0.19	2.23	1.24	
3985233	3985218	ARMCX5	armadillo repeat containing, X-linked 5	0.02	0.07	1.44	1.18	
3261950	3261923	AS3MT	arsenic (+3 oxidation state) methyltransferase	0.00	0.15	-1.83	-1.06	
3137642	3137530	ASPH	aspartate beta-hydroxylase	0.01	0.02	1.50	1.16	Cassette Exon
2950757	2950753	BAK1	BCL2-antagonist/killer 1	0.01	0.55	1.37	-1.04	
2950763	2950753	BAK1	BCL2-antagonist/killer 1	0.01	0.55	-1.66	-1.04	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
2996330	2996321	BBS9	Bardet-Biedl syndrome 9	0.00	0.05	2.09	1.39	
2996339	2996321	BBS9	Bardet-Biedl syndrome 9	0.00	0.05	2.26	1.39	
3025504	3025500	BPGM	2,3-bisphosphoglycerate mutase	0.00	0.53	-1.69	1.06	Cassette Exon
3732463	3732448	BPTF	bromodomain PHD finger transcription factor	0.00	0.11	2.33	1.21	Cassette Exon
3393845	3393834	C11orf60	chromosome 11 open reading frame 60	0.02	0.01	1.93	1.42	
3393857	3393834	C11orf60	chromosome 11 open reading frame 60	0.02	0.01	2.16	1.42	
3471832	3471819	C12orf30	chromosome 12 open reading frame 30	0.00	0.47	2.00	1.05	
3588702	3588658	C15orf41	chromosome 15 open reading frame 41	0.00	0.01	1.79	1.15	
3442492	3442475	C1R	complement component 1, r subcomponent	0.00	0.00	1.91	1.48	
3442494	3442475	C1R	complement component 1, r subcomponent	0.00	0.00	2.53	1.48	
2377258	2377229	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	0.02	0.07	-1.38	1.17	Cassette Exon
3964164	3964154	CERK	ceramide kinase	0.01	0.05	1.50	1.09	
2685924	2685908	CLDND1	claudin domain containing 1	0.00	0.10	2.04	1.15	Cassette Exon
3261992	3261971	CNNM2	cyclin M2	0.00	0.06	-1.08	1.13	
3452866	3452865	COL2A1	collagen, type II, alpha 1	0.00	0.02	1.83	1.25	
3452867	3452865	COL2A1	collagen, type II, alpha 1	0.00	0.02	2.52	1.25	
3924402	3924372	COL6A1	collagen, type VI, alpha 1	0.00	0.23	1.55	1.12	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3855119	3855104	CRLF1	cytokine receptor-like factor 1	0.00	0.02	-1.26	1.14	
3284910	3284882	CUL2	cullin 2	0.02	0.14	2.74	1.20	
2837366	2837266	CYFIP2	cytoplasmic FMR1 interacting protein 2	0.00	0.04	1.73	1.13	
2903043	2903034	CYP21A2	cytochrome P450, family 21, subfamily A, polypeptide 2	0.00	0.02	2.35	1.19	
3526553	3526544	DCUN1D2	DCN1, defective in cullin neddylation 1, domain containing 2 (S. cerevisiae)	0.00	0.01	2.00	1.20	Cassette Exon
2429199	2429147	DENND2C	DENN/MADD domain containing 2C	0.02	0.25	2.06	1.21	Alt Three Prime
3449809	3449760	DENND5B	DENN/MADD domain containing 5B	0.01	0.15	1.71	1.11	Alt Five Prime
3438704	3438617	EP400	E1A binding protein p400	0.00	0.14	2.72	1.07	
3883731	3883690	EPB41L1	erythrocyte membrane protein band 4.1-like 1	0.00	0.01	-1.15	1.20	Cassette Exon
3260855	3260829	FAM178A	family with sequence similarity 178, member A	0.00	0.08	1.77	1.24	
3260884	3260829	FAM178A	family with sequence similarity 178, member A	0.00	0.08	1.71	1.24	
3260885	3260829	FAM178A	family with sequence similarity 178, member A	0.00	0.08	1.75	1.24	
3884894	3884892	FAM83D	family with sequence similarity 83, member D	0.00	0.45	-1.34	-1.02	
3536809	3536786	FBXO34	F-box protein 34	0.01	0.13	1.26	1.12	
3310204	3310041	FGFR2	fibroblast growth factor receptor 2	0.00	0.00	2.75	1.53	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
2678828	2678714	FHIT	fragile histidine triad gene	0.01	0.07	-1.37	1.12	
3355064	3355056	FOXRED1	FAD-dependent oxidoreductase domain containing 1	0.00	0.05	1.47	-1.11	Bleeding Exon
3183263	3183238	FSD1L	fibronectin type III and SPRY domain containing 1-like	0.01	0.18	2.13	1.15	
2836609	2836518	GALNT10	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (GalNAc-T10)	0.00	0.05	1.72	1.12	
3468153	3468103	GNPTAB	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	0.00	0.01	2.10	1.29	
3438161	3438061	GPR133	G protein-coupled receptor 133	0.01	0.34	-1.44	1.04	Cassette Exon
3334428	3334415	GPR137	G protein-coupled receptor 137	0.01	0.19	1.52	1.10	
3050476	3050462	GRB10	growth factor receptor-bound protein 10	0.03	0.19	1.38	1.05	
2469862	2469825	GREB1	GREB1 protein	0.00	0.01	1.90	1.24	
3267040	3267036	GRK5	G protein-coupled receptor kinase 5	0.01	0.05	2.07	1.17	
2927734	2927722	HEBP2	heme binding protein 2	0.03	0.83	-1.28	-1.01	
3258934	3258910	HELLS	helicase, lymphoid-specific	0.03	0.31	1.67	1.12	Cassette Exon
2610128	2610094	IL17RC	interleukin 17 receptor C	0.02	0.63	-1.42	-1.03	
2610086	2610056	IL17RE	interleukin 17 receptor E	0.00	0.60	-1.74	1.04	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3272344	3272205	INPP5A	inositol polyphosphate-5-phosphatase, 40kDa	0.02	0.40	-1.51	-1.03	Bleeding Exon
3339471	3339423	INPPL1	inositol polyphosphate phosphatase-like 1	0.00	0.07	1.36	1.05	
3159484	3159483	KANK1	KN motif and ankyrin repeat domains 1	0.00	0.02	-1.19	1.08	Alt Promoter
3159513	3159483	KANK1	KN motif and ankyrin repeat domains 1	0.00	0.02	3.21	1.08	Cassette Exon
3159526	3159483	KANK1	KN motif and ankyrin repeat domains 1	0.00	0.02	1.91	1.08	
3687311	3687308	KCTD13	potassium channel tetramerisation domain containing 13	0.01	0.09	1.69	1.09	
3117386	3117384	KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3	0.00	0.05	-1.44	1.18	
2764216	2764192	KIAA0746	KIAA0746 protein	0.01	0.00	2.45	1.56	
3239168	3238962	KIAA1217	KIAA1217	0.00	0.01	1.77	1.25	Cassette Exon
3239170	3238962	KIAA1217	KIAA1217	0.00	0.01	1.71	1.25	Cassette Exon
3737294	3737274	KIAA1618	KIAA1618	0.00	0.03	2.47	1.07	
3886104	3886072	L3MBTL	l(3)mbt-like (Drosophila)	0.01	0.04	2.11	1.18	
2674065	2674047	LAMB2	laminin, beta 2 (laminin S)	0.01	0.15	-1.51	-1.09	
3402854	3402836	LEPREL2	leprecan-like 2	0.00	0.27	1.75	1.06	
2352526	2352501	LRIG2	leucine-rich repeats and immunoglobulin-like domains 2	0.01	0.55	1.69	1.04	
3600255	3600212	LRRC49	leucine rich repeat containing 49	0.01	0.21	-1.75	1.28	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
2814812	2814756	MAP1B	microtubule-associated protein 1B	0.00	0.01	1.58	1.24	
3723725	3723687	MAPT	microtubule-associated protein tau	0.01	0.00	2.39	1.47	
3807768	3807753	MBD1	methyl-CpG binding domain protein 1	0.01	0.14	1.42	1.06	Cassette Exon
3807800	3807753	MBD1	methyl-CpG binding domain protein 1	0.01	0.14	1.41	1.06	
2509748	2509740	MBD5	methyl-CpG binding domain protein 5	0.00	0.00	1.20	1.43	Alt Promoter
2509755	2509740	MBD5	methyl-CpG binding domain protein 5	0.00	0.00	2.12	1.43	Alt Promoter
3611221	3611126	MEF2A	myocyte enhancer factor 2A	0.02	0.11	2.06	1.15	Cassette Exon
3834786	3834778	MEGF8	multiple EGF-like-domains 8	0.00	0.12	2.21	1.09	
3945116	3945084	MICALL1	MICAL-like 1	0.01	0.47	2.54	1.05	
3453679	3453592	MLL2	myeloid/lymphoid or mixed-lineage leukemia 2	0.03	0.04	1.46	1.09	
3939483	3939470	MMP11	matrix metallopeptidase 11 (stromelysin 3)	0.02	0.61	1.48	1.05	
4027609	4027585	MPP1	membrane protein, palmitoylated 1, 55kDa	0.02	0.01	1.51	1.15	Cassette Exon
2692888	2692883	MUC13	mucin 13, cell surface associated	0.00	0.00	1.03	2.99	
3431233	3431220	MVK	mevalonate kinase	0.02	0.35	1.68	1.04	
3341235	3341221	MYO7A	myosin VIIA	0.00	0.25	1.48	1.04	
3722722	3722700	NAGS	N-acetylglutamate synthase	0.01	0.16	-1.65	-1.15	
3485325	3485292	NBEA	neurobeachin	0.00	0.01	2.46	1.47	
3485354	3485292	NBEA	neurobeachin	0.00	0.01	3.20	1.47	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3776154	3776139	NDC80	NDC80 homolog, kinetochore complex component (S. cerevisiae)	0.02	0.11	2.07	1.20	
3468771	3468743	NT5DC3	5'-nucleotidase domain containing 3	0.00	0.01	1.94	1.25	
3382895	3382861	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	0.00	0.00	1.18	1.63	
3175284	3175274	PCSK5	proprotein convertase subtilisin/kexin type 5	0.00	0.00	2.01	1.35	
3407496	3407453	PDE3A	phosphodiesterase 3A, cGMP-inhibited	0.02	0.32	2.12	1.10	
3755379	3755359	PIP4K2B	phosphatidylinositol-5-phosphate 4-kinase, type II, beta	0.03	0.16	1.44	1.06	
2977650	2977621	PLAGL1	pleiomorphic adenoma gene-like 1	0.01	0.00	2.33	1.44	Cassette Exon
2640646	2640579	PLXNA1	plexin A1	0.00	0.40	-1.30	1.04	
3426598	3426502	PLXNC1	plexin C1	0.00	0.00	1.92	1.34	
2694842	2694817	PLXND1	plexin D1	0.03	0.73	1.31	-1.01	
3878439	3878429	POLR3F	polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa	0.00	0.12	2.03	1.17	
3976654	3976639	PORCN	porcupine homolog (Drosophila)	0.03	0.78	1.64	-1.02	
2948431	2948425	PPP1R10	protein phosphatase 1, regulatory (inhibitor) subunit 10	0.02	0.00	-1.00	1.24	
2948433	2948425	PPP1R10	protein phosphatase 1, regulatory (inhibitor) subunit 10	0.02	0.00	-1.00	1.24	
3134053	3134034	PRKDC	protein kinase, DNA-activated, catalytic polypeptide	0.01	0.57	1.21	1.04	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3185569	3185558	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)	0.01	0.50	1.23	1.04	
3771474	3771464	QRICH2	glutamine rich 2	0.00	0.02	2.20	1.17	
2369994	2369950	QSOX1	quiescin Q6 sulfhydryl oxidase 1	0.01	0.08	1.63	1.12	
3580293	3580234	RAGE	renal tumor antigen	0.01	0.05	2.75	1.23	
2843588	2843579	RMND5B	required for meiotic nuclear division 5 homolog B (S. cerevisiae)	0.00	0.59	1.54	-1.05	Bleeding Exon
3221027	3220977	ROD1	ROD1 regulator of differentiation 1 (S. pombe)	0.00	0.52	1.99	1.04	Cassette Exon
3847294	3847252	SAFB2	scaffold attachment factor B2	0.01	0.59	1.47	1.04	
3832949	3832918	SAMD4B	sterile alpha motif domain containing 4B	0.01	0.22	1.50	1.07	
2829436	2829416	SEC24A	SEC24 family, member A (S. cerevisiae)	0.00	0.97	1.85	-1.01	
2622567	2622547	SEMA3F	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	0.01	0.02	1.50	1.13	
3764552	3764527	SEPT4	septin 4	0.00	0.05	2.00	1.20	
2738684	2738664	SGMS2	sphingomyelin synthase 2	0.00	0.01	2.42	1.67	
3557360	3557350	SLC22A17	solute carrier family 22, member 17	0.01	0.02	1.68	1.16	Cassette Exon
3472326	3472312	SLC24A6	solute carrier family 24 (sodium/potassium/calcium exchanger), member 6	0.02	0.96	-1.35	-1.00	
2645287	2645275	SLC25A36	solute carrier family 25, member 36	0.03	0.38	1.45	1.12	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
2584925	2584904	SLC38A11	solute carrier family 38, member 11	0.02	0.02	2.67	1.80	
2545588	2545549	SLC5A6	solute carrier family 5 (sodium-dependent vitamin transporter), member 6	0.01	0.53	1.21	-1.03	
3751824	3751794	SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	0.01	0.00	1.76	1.20	
3262461	3262433	SLK	STE20-like kinase (yeast)	0.00	0.46	-1.55	1.05	Cassette Exon
3592394	3592366	SPATA5L1	spermatogenesis associated 5-like 1	0.02	0.64	1.47	-1.04	
3140741	3140640	STAU2	staufen, RNA binding protein, homolog 2 (Drosophila)	0.01	0.05	2.39	1.28	Cassette Exon
3766288	3766284	STRADA	STE20-related kinase adaptor alpha	0.00	0.07	1.42	1.08	Retained Intron
3908375	3908358	SULF2	sulfatase 2	0.01	0.00	1.07	1.51	
2660827	2660800	SUMF1	sulfatase modifying factor 1	0.00	0.21	-1.53	1.07	
3283067	3282974	SVIL	supervillin	0.01	0.00	1.70	1.17	Cassette Exon
3283123	3282974	SVIL	supervillin	0.01	0.00	1.92	1.17	Alt Promoter
3968223	3968122	TBL1X	transducin (beta)-like 1X-linked	0.00	0.00	2.16	1.13	
4007736	4007734	TFE3	transcription factor binding to IGHM enhancer 3	0.01	0.04	1.29	1.17	Cassette Exon
3631259	3631214	TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	0.03	0.01	1.94	1.23	Cassette Exon
3301869	3301857	TM9SF3	transmembrane 9 superfamily member 3	0.00	0.12	1.88	1.13	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3502641	3502632	TMCO3	transmembrane and coiled-coil domains 3	0.00	0.02	1.62	1.13	
3909102	3909064	TMEM189-UBE2V1	TMEM189-UBE2V1 readthrough transcript	0.00	0.07	2.77	1.21	Cassette Exon
3107258	3107242	TMEM67	transmembrane protein 67	0.02	0.23	2.12	1.20	Cassette Exon
3449083	3449068	TMTC1	transmembrane and tetratricopeptide repeat containing 1	0.01	0.02	2.13	1.27	
2317444	2317434	TPRG1L	tumor protein p63 regulated 1-like	0.01	0.40	1.50	1.06	
3634080	3634071	TSPAN3	tetraspanin 3	0.01	0.00	1.09	1.11	
3896997	3896976	TXNDC13	thioredoxin domain containing 13	0.00	0.00	1.07	1.23	
2962727	2962683	UBE2CBP	ubiquitin-conjugating enzyme E2C binding protein	0.00	0.68	1.37	1.04	
3311757	3311715	UROS	uroporphyrinogen III synthase	0.02	0.21	1.74	1.16	Alt Promoter
2542699	2542651	WDR35	WD repeat domain 35	0.00	0.98	-1.25	-1.00	
3699223	3699178	WDR59	WD repeat domain 59	0.00	0.00	1.55	1.12	Alt Promoter
3846617	3846594	ZBTB7A	zinc finger and BTB domain containing 7A	0.02	0.22	-1.32	1.04	
3571260	3571248	ZFYVE1	zinc finger, FYVE domain containing 1	0.01	0.02	1.79	1.17	
3907571	3907561	ZNF335	zinc finger protein 335	0.00	0.73	1.21	1.01	
3078504	3078493	ZNF425	zinc finger protein 425	0.01	0.21	2.35	1.24	
2564648	2564634	ZNF514	zinc finger protein 514	0.02	0.15	2.15	1.24	
3843919	3843906	ZNF8	zinc finger protein 8	0.00	0.03	1.75	1.15	

Probe set ID	Transcript cluster ID	Gene	Gene Description	Differential Alternative splicing FDR	Differential gene expression FDR	Probe set Fold Change (E/C)	Gene Fold-Change (E/C)	Known Alternative event
3621172	3621160	ZSCAN29	zinc finger and SCAN domain containing 29	0.02	0.09	-1.27	1.13	Alt Five Prime

Table 15. Probe sets probably differentially alternatively spliced in response to chronic ethanol treatment. Probe sets that are probably alternatively spliced in response to chronic ethanol treatment are shown. Fold change (E/C) is ethanol/ control. Probe set fold change refers to the change in the expression of the indicated (Probe set ID) probe set with ethanol. Gene fold change refers to the difference in the expression of the gene, taking into consideration all the probe sets in the given transcript cluster (Transcript cluster ID).

5.a. Validation of differential alternative splicing

Four genes, *MBD5* (methyl CpG binding protein), *CD55* (CD55 molecule, decay accelerating factor for complement), *BPGM* (2,3-bisphosphoglycerate mutase), and *ACSL1*(acyl-CoA synthetase long-chain family member 1) that were detected as differentially alternatively spliced were tested in validation studies. These genes were selected because they are associated with important functions like chromatin regulation and fatty acid metabolism. *MBD5*, *CD55* and *ACSL1* were also detected as differentially expressed.

MBD5 was considered as probably alternatively spliced because of the presence of a possible alternative promoter, that leads to a shorter transcript (2.3 kb) that shares five exons with the longer transcript (5.3 kb; Figure 20). While probe sets corresponding to only the long transcript had a mean fold change of 1.3, the probe sets corresponding to both isoforms had an average 2.1-fold change with ethanol. To quantitate the fold change for each test exon, qRT-PCR reactions were carried out. For *MBD5* primer pairs were designed to amplify regions with 1.3- and 2.1-fold change. A similar change in expression was also observed in qRT-PCR assays; 1.4-fold (± 0.12 standard error of the mean) and 2.5 (± 0.4 standard error of the mean) increase in expression was observed for the corresponding regions.

CD55, *BPGM*, and *ACSL1* had what appeared to be differentially alternatively spliced cassette exon. In *CD55* there was a -1.4-fold change in expression of probe set 2377258 with ethanol while other probe sets increased in expression. To test whether this exon is alternatively spliced, primers were

designed to flank the exon and two products of 383 bp or 266 bp were expected. Two transcript isoforms were detected for *CD55* (Figure 21). In qRT-PCR assays, a 0.7 $\pm$ 0.1, fold decrease in the expression of test exon with ethanol was observed. To determine if this decrease in expression is specific to this exon, another exon which did not appear to be alternatively spliced was also tested. A similar change in expression (0.7 $\pm$ 0.2) was observed for this exon also, indicating that *CD55* does not undergo a true differential alternative splicing event.

In *BPGM* one probe set (3025504) displayed a significant -1.7-fold decrease in expression while others did not have any significant change. The test exon was 320 bp long and thus products of 384 bp or 67 bp were expected if the exon was included or excluded from the transcript. In PCR multiple bands were seen, including bands that appeared to be of the expected size (Figure 21).

In *ACSL1* one probe set (2796582) increased in expression by 2.6-fold while others were increased on average 1.4 fold. To detect if isoforms of the gene with and without the cassette exon exist, primers were designed to amplify across the putative cassette exon, yielding either 232 bp or 181 bp products. Only one isoform was detected for *ACSL1* and therefore it was not considered further (Figure 21).

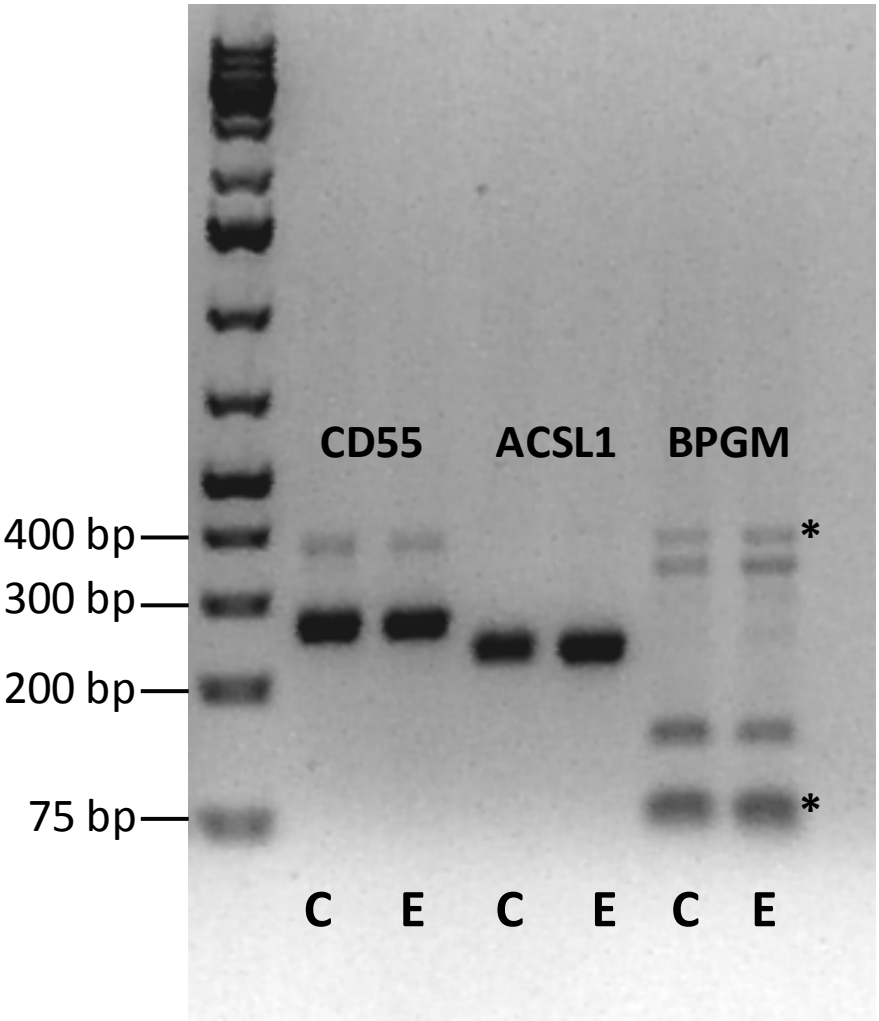

Figure 21. Detection of alternative isoforms for validation. PCR was carried out with primers flanking the test exon to determine if the exon is alternatively spliced. cDNA from control (C) and ethanol (E) samples was used as the template. Size of the DNA ladder is indicated on the left. For BPGM, two bands that appeared to be of the expected size are indicated by an asterix.

IV.DISCUSSION

1. Regulation of *ADH*s by distal *cis*-regulatory regions

Cis-regulatory regions play a critical role in the regulation of the gene expression. Because polymorphisms in regulatory sequences may affect the genetic risks for alcoholism, understanding the regulation of *ADH* expression is important. The known *cis*-regulatory elements in the *ADH* cluster do not completely account for expression levels of all *ADH* genes in the liver. We sought to identify distal regulatory regions in the *ADH* cluster. Sequence conservation implies an important functional role. Therefore, to discover putative distal regulatory regions, sequence conservation in the non-coding regions of the *ADH* cluster extending from the 3' end of *ADH1A* to the transcriptional start site of *ADH5* was studied. We chose to restrict our search to the intergenic regions, although regulatory regions could also be present in the intron sequences.

Eight conserved regions were identified and tested in the context of the *ADH4* basal promoter in human hepatoma cells (HepG2). Most were negative regulatory regions that decreased the activity of the promoter by at least 40% (Figure 7). The greatest effect was observed with 4E, a 1504 bp region 13 kb upstream of the *ADH4* translational start site. 4E increased the activity of its cognate *ADH4* promoter 50-fold in HepG2 cells (Figure 8). In preliminary studies, this enhancer activity was observed to be distance and orientation dependent (data not shown). 4E also enhanced the activity of the *ADH1B* promoter by 180-fold. In HepG2 cells the *ADH1B* proximal promoter has weaker activity than *ADH4* promoter. This probably explains the larger increase in activity observed

with the *ADH1B* promoter. 4E also enhanced the activity of another strong promoter, SV40 early promoter, 56-fold.

The 4E enhancer was not functional in a non-hepatic cell line, H1299 lung carcinoma cells, suggesting that it has cell-type specificity (Table 7). By deletion analysis, it was observed that the enhancer activity was present in a 565 bp region, 4E3 (Figure 9). Six putative transcription factor binding sites were identified in 4E3 by sequence homology, and their roles were tested by electrophoretic mobility shift assays (EMSA) and site directed mutagenesis. Sites 1, 2, 3, 4, and 6 were confirmed to be FOXA binding sites by EMSA with competitor oligonucleotides and by supershift assays (Figure 11). Oligo 3, which spans sites 3, 4, and 5, produced multiple high molecular weight bands, but the FOXA specific complex was the most prominent (Figure 12). Site 5, which overlaps with the site 4 by two nucleotides, was predicted to be a HNF-1A site. While none of the bands were disrupted by HNF-1A competitor, a stronger FOXA specific complex was detected. It is possible that HNF-1A binds weakly to site 5 and this binding interferes with the binding of the FOXA to the overlapping site 4.

Site-directed mutagenesis studies showed the greatest effect on enhancer function when either site 1 or site 4 was mutated, with activity decreasing to 40% of the wild type (Figure 13). Mutating multiple sites affected activity more dramatically. All combinations of multiple mutants tested displayed approximately a multiplicative reduction in activity compared to the respective single mutants, suggesting that each site is acting independently (Figure 13). When sites 1, 2, 4 and 6 were mutated, activity decreased to 10% of the wild type. In a genome-

wide mapping of DNase I hypersensitive sites in HepG2 cells (Crawford et al., 2006b) , a hypersensitive peak was detected across the region of transcription factor binding sites that we characterized in 4E3 (UCSC Open chromatin track, March 2006, NCBI36/hg 18 assembly). This indicates that the site identified here is accessible to transcription factors *in vivo*.

FOXA and HNF-1A proteins are liver-enriched factors that regulate many liver-specific genes. Thus, it is not surprising that they are utilized to enhance the expression of *ADH* genes that are expressed at highest levels in the liver. FOXA proteins can function as pioneer factors and bind to highly compacted chromatin, alter the chromatin structure and enhance transcription. Evidence of such regulation was observed in the expression of the albumin gene during development (Cirillo and Zaret, 1999). This could be a possible mechanism by which FOXA activates *ADH4* expression from the 4E3 enhancer.

Among the other seven putative distal regulatory regions that were tested, six regions repressed the activity of the 4Basal promoter. Highest repression was observed with the region between *ADH4* and *ADH5*, with the promoter activity decreasing by 76% (Figure 7). A similar decrease in activity was observed with fragment 6-4-a, the region immediately upstream (relative to the translational start site of *ADH4*) of 4E. Fragments 1A-6-c and 1A-6-a also decreased the activity while1A-6-b did not have any significant effect. 1A-6-a, 1A-6-b and 1A-6-c were overlapping fragments that span 4.1 kb on genomic sequence. It is possible that fragmenting this 4.1 kb region decreased the functional effect of the region.

A more comprehensive analysis with more overlap between fragments is necessary to understand the role of this region.

It is noteworthy that a strong enhancer is flanked by negative regulatory regions. Because 4E3 functions on heterologous promoters *in vitro,* including the *ADH1B* and SV40 promoters, it is conceivable that in the absence of intervening boundary elements this enhancer could act on the *ADH6* promoter 60 kb away or other *ADH* promoters in the cluster. One possible function of the negative regulatory regions maybe to modulate the function of the 4E3 enhancer. Another possibility is that these regions play an important role in other tissues. A more detailed study is necessary to understand the role of these regions in the regulation of *ADH* expression. In summary, multiple distal *cis*-regulatory regions in the *ADH* cluster that could regulate the expression of *ADH* genes were identified.

2. Regulatory variations and effects on function

Several variations in genes encoding alcohol dehydrogenases have been associated with the risk for alcoholism (Birley et al., 2009; Edenberg and Foroud, 2006; Edenberg et al., 2006; Reich et al., 1998; Williams et al., 1999). Although the most widely-studied *ADH* variations affect the sequence of the encoded enzymes, one (rs1800759) is known to affect regulation of gene expression (Edenberg et al., 1999). The expression level of ADH enzymes could affect the flux through the alcohol metabolic pathway and thereby influence the effects of

alcohol and the risk for alcoholism. As part of this dissertation, haplotypes of two *cis*-regulatory regions in *ADH* cluster were tested.

Because of its enhancer function, variations in the 4E3 region could greatly affect the expression levels of *ADH4*. There are three SNPs in this region, rs7678936 (G/T), rs7678890 (T/G) and rs11401494 (del/T). Three haplotypes were tested. Haplotype 2 (T, T, del) and haplotype 3 (G, G, del) were less active (0.6-fold) than Haplotype1 (G, T, del), so it was not possible to attribute the effect to a single SNP. rs11401494 had a small effect on the function of the enhancer. The three SNPs are not present in any of the transcription factor binding sites identified in this study, however, rs7678936 is adjacent to a putative FOXA site and rs7678890 is adjacent to site 5 (HNF-1A).

It would be of interest to determine whether these variations will contribute to differential susceptibility to alcoholism. From allele frequencies, rs7678936 (G/T) and rs7678890 (T /G) appear to be in linkage disequilibrium, meaning the rs7678936 G allele travels with the rs7678890 T allele. The GT haplotype is at a fifty percent frequency in African populations (YRI, LWK, MIKK) whereas it appears to have reached higher frequencies in European (CEU) and have become fixed in Asian populations (CHB, JPT) or populations with Asian ancestry (CHD, GIH). Therefore, the role of these variations in the risk for alcoholism will be more important in populations of African descent.

The effect of variations was also investigated in the *ADH1B* proximal promoter region because there is evidence of association of two SNPs (rs1229982 and rs1159918) in this region with the risk for alcoholism in a

European-American population (Edenberg et al., 2006). We tested five haplotypes and observed that the presence of the C allele of rs1229982 reduced the activity of the promoter by 30%. Although the magnitude of the effect is small, it could be of significance considering alcoholism is a complex disease in which multiple genes contribute to the disease. This functional effect supports our hypothesis that variations in the regulatory polymorphisms could be associated with the disease and vice versa. The frequency of the rs1229982 C/C genotype is high in some populations such as CHB, JPT. It would be of interest to study the effect of this SNP on the risk for alcoholism in these populations.

In this study, we did not detect a functional effect for rs1159918 and other SNPs that were tested in this region. One possible explanation for the absence of functional effect even though it was associated with alcoholism is that it is in linkage disequilibrium (LD) with another SNP that is associated with the risk for alcoholism. From the available HapMap LD data in the *ADH* cluster, only rs6810842 ($r^2 = 0.949$) is in LD with rs1159918 in the CEU population. This SNP was, however, tested in this study and was not observed to have any functional effect, hinting at a possibility of another SNP associated with alcoholism.

In view of the effect of variations on the enhancer activity and also on the promoter activity that was observed in this study, it is important to consider the presence of variations in a test region when studying *cis*-regulatory regions. Testing a haplotype which has much lower activity could mislead one to ignore the function of the region. The small magnitude of effect that was observed with the negative regulatory regions in this study could be one such example. Thus, it

is necessary to study other haplotypes in test regions to completely appreciate the function of these regions.

In summary, results from this study support our hypothesis that variations could have a functional effect on the expression levels of *ADH*, and that these could be associated with alcoholism.

3. Effects of alcohol on gene expression

The effects of extended alcohol exposure on global gene expression in a human hepatoma cell line, HepG2 were studied. HepG2 cells do not express class I *ADH* genes but express *ADH4* at moderate levels (Tian and Edenberg, 2005). They also do not express cytochrome P450 2E1 (*CYP2E1*). Thus, this model system helped to identify the direct effects of alcohol and also effects due to limited metabolism of alcohol. An advantage was to identify effects of alcohol directly on human hepatocyte-like cells in the absence of potential signaling from neighboring cells, such as hepatic stellate cells and Kupffer cells which are known to play a role in alcohol induced liver injury (Siegmund and Brenner, 2005; Thurman, 1998; Wheeler et al., 2001).

HepG2 cells were cultured in the presence of 75 mM ethanol for 9 days. The legal limit of 0.08% blood alcohol level corresponds to 17.4 mM ethanol. Alcoholic beverages range from approximately 4.5% ethanol (900 mM) for beer to 12% ethanol (2 M) for wine to 41.1% ethanol in spirits (7M). Because 80% of alcohol is absorbed in the small intestine and most of alcohol is carried to the liver via portal vein, liver is exposed to high concentrations of alcohol (Swift,

2003). Prolonged exposure of HepG2 cells to ethanol led to a decrease in the number of viable cells relative to untreated cells. This decrease in cell number could be due to a prolonged lag phase, or slow cell growth or due to cell death. Ethanol has been shown to have apoptotic effects in HepG2 cells and hepatocytes (Higuchi et al., 1996; Neuman et al., 1993; Neuman et al., 1999). However, we did not observe visible differences in morphology between cells in ethanol and control conditions, and also did not notice non-adherent (dead) cells in the presence of ethanol.

Significant differences in gene expression between control and ethanol treated cells were observed. Many of these were small changes (10 to 30%). Statistical power obtained from eight arrays used for each condition along with multiple probe sets that target each gene, allowed us to detect these small changes. We were able to validate changes as small as 15% in qRT-PCR studies

Chronic ethanol treatment of HepG2 cells had an effect on the expression of genes in key metabolic pathways and stress response pathways. Some of the pathways that may be key to understanding different aspects of alcohol induced liver disease are discussed below. Effects of alcohol on genes involved in chromatin regulation and genes associated with alcoholism are also discussed.

3.a. Acute phase response

Liver responds to infection, inflammation, injury to tissue, or malignancy, with the acute phase response. Acute phase response is characterized by

increase or decrease in the levels of multiple proteins (Ceciliani et al., 2002; Gabay and Kushner, 1999). Several genes involved in the acute phase response were affected by ethanol (Table 13). Interleukin 6 (IL-6) and IL-1 are the primary mediators of acute phase response in the liver; although the genes encoding these mediators were not detected in our data, the genes encoding their receptors and signal transducers were affected by ethanol. *IL6R* decreased in expression whereas *IL1R1* increased. The signal transducer membrane proteins (*IL6ST* and *IL1RAP*) associated with these receptors were also affected. The intracellular signal transducer of IL-6, Janus kinase 2 (*JAK2*) increased in expression by 43% while transcription factor Signal transducer and activator of transcription 3 (*STAT3*) increased modestly. Positive acute phase proteins (protein that increase in expression in acute phase response) like fibrinogens (*FGG*, *FGA*), angiotensiongen (*AGT*), *SERPIN*s (A3, D1, G1) increased in expression by ethanol (Table 13). Increase in the expression of *FGG* was confirmed by qRT-PCR validation. We observed a 1.9-fold change in *FGG* expression, larger than that observed using arrays (1.4- fold; Figure 18).

Chronic ethanol exposure had an opposite effect on some positive acute phase proteins: heptoglobin (*HP*), hemopexin (*HPX*), hepcidin antimicrobial peptide (*HAMP*), complement C4 binding protein (*C4BPA*) decreased in expression. Decrease in the expression of *C4BPA* and *HAMP* proteins with ethanol was observed in other studies (Bykov et al., 2007; Ohtake et al., 2007). Recent evidence suggests that the decrease in the expression of hepcidin antimicrobial peptide along with the increase in the expression of ferritin probably

leads to the iron overload (Chick et al., 1987; Ohtake et al., 2007). In our chronic alcohol exposure model *HAMP* and ferritin light chain (*FTL*) were changed by -1.41- and 1.29-fold, respectively (Table 13). Similar observations were made in the serum levels of ferritin and hepcidin antimicrobial peptide in alcoholics (Chick et al., 1987; Ohtake et al., 2007). Reactive oxygen species, possibly from parenchymal and non-parenchymal cells, have been implicated in the downregulation of hepcidin expression (Harrison-Findik et al., 2006). In a mouse model this effect was shown to be independent of Kupffer cells (Harrison-Findik et al., 2009). Similar findings in our model system indicate that oxidative stress generated in hepatocytes could be sufficient to change the expression of hepcidin. Therefore, these results indicate that chronic alcohol exposure modulates the receptivity of hepatocytes to the acute phase response, and also the expression of acute phase reactant proteins.

3.b. Nrf2 oxidative stress response pathway

Alcohol metabolism generates reactive oxygen species and creates a state of oxidative stress in hepatocytes (Albano, 2006; Bailey et al., 1999; Wu and Cederbaum, 2009). In response to oxidative stress, the transcription factor Nuclear factor-eythroid 2-related factor 2 (Nrf2) regulates expression of multiple genes involved in antioxidant defenses (Itoh et al., 1997; McMahon et al., 2001). Increase in the levels of Nrf2 transcript and protein were observed in animal livers and HepG2 cells expressing *CYP2E1* and exposed to alcohol (Gong and Cederbaum, 2006). Deletion of Nrf2 in mice was shown to aggravate alcohol

induced liver damage (Lamle et al., 2008). In our data we did not observe an increase in the expression of Nrf2. However, many Nrf2 transcribed genes were differentially expressed. This could be due to the activation of Nrf2 instead of an increase in the levels of expression. Under non stress conditions, kelchlike ECH-associated protein 1 (Keap1) sequesters Nrf2 and targets it for ubiquitination. When there is oxidative stress, Keap1 dissociates from Nf2, leading to a functional Nrf2 (Itoh et al., 1999; Itoh et al., 2003).

One of the Nrf2 transcribed genes that was affected by chronic alcohol exposure is the catalytic subunit (*GCLC*) of the glutamate cysteine ligase (GCL). This enzyme carries out the rate-limiting step of glutathione (GSH) synthesis (Lu, 1999). With chronic ethanol exposure there was a 20% increase in the expression of the enzyme. This increase in expression was also confirmed by qRT-PCR (Figure 18). In addition to GCL activity, the synthesis of GSH is also regulated by the availability of cysteine (Lu, 1999). *SLC7A11*, one of the subunits of amino acid transport system χc^- involved in the uptake of cystine (Bannai, 1986), was differentially expressed (1.54-fold). Although it is not an Nrf2 transcribed gene, the increase in *SLC7A11* could be an additional response of the cell to maintain the cellular GSH levels.

Ethanol also affected other Nrf2 transcribed antioxidant enzymes: glutathione s-transferase alpha 4 (*GSTA4)*, glutathione peroxidase 2 (*GPX2*), superoxide dismutase 1 (*SOD1*), thioredoxin reductase 2 (*TXNRD2*) increased in expression. Multiple genes belonging to the DNAJ/Hsp40 family of chaperone proteins were upregulated with ethanol. DNAJ members stimulate the ATP

hydrolysis of Hsp70 proteins, and are thus essential for the activity of Hsp70 proteins (Qiu et al., 2006). In summary, limited metabolism of alcohol and direct alcohol exposure generates an oxidative stress response in HepG2 cells.

3.c. Amino acid metabolism

Several genes involved in the metabolism of amino acids were downregulated by ethanol. Genes encoding urea cycle enzymes arginase (*ARG2*) and arginosuccinate lyase (*ASL*) decreased in expression. Multiple enzymes in argenine proline metabolism were affected by ethanol. Glycine amidinotransferase (*GATM*) and guanidinoacetate N-methyltransferase (*GAMT*) mediate the conversion of argenine to creatine and were downregulated with ethanol. Two key enzymes involved in the synthesis of polyamine putrescine were affected by ethanol. Ornithine decarboxylase (*ODC*) that converts ornithine to putrescine (Russell and Snyder, 1968) increased in expression by 15%. In contrast agmatinase (*AGMAT*) which also leads to the synthesis of putrescine from argmatine (Russell and Snyder, 1968), decreased in expression by the same magnitude. Opposing effects of alcohol on ODC activity during liver regeneration have been reported earlier. After partial hepatectomy, acute exposure to alcohol inhibited ODC activity where as chronic exposure to alcohol increased activity (Diehl et al., 1988; Diehl et al., 1990; Poso and Poso, 1980). Our data suggest that the increase in the expression of ODC could be one possible factor in the increase in the activity of ODC after chronic ethanol treatment.

Two enzymes involved in methionine metabolism were also affected by ethanol. An increase in expression was observed for Betaine-homocysteine methyltransferases (*BHMT*, *BHMT2*) which carry out the folate independent conversion of homocysteine to methionine (Finkelstein and Martin, 1984). For BHMT the array data was validated by qRT-PCR. Another pathway of homocysteine catabolism is transsulfuration of homocysteine to cystathione and is catalyzed by cystathionine β-synthase (CBS) (Finkelstein and Mudd, 1967). In our data *CBS* was downregulated by a modest 12%. This increase in the expression of *BHMT*s and decrease in *CBS* expression could be an adaptive response to maintain the levels of essential amino acid methionine in the cells. In addition to being a part of proteins, methionine also acts as a precursor to methyl group donor, S-adenosyl methionine (SAM). SAM acts as a methyl-group donor for methyltransferase-catalyzed reactions.

3.d. Carbohydrate metabolism

Effects of alcohol on the activity of enzymes involved in carbohydrate metabolism have been recognized for many years. Alcohol alters $NADH/NAD^+$ ratio and this affects the activity of many enzymes in glycolysis, citrate cycle. In our data we saw a decrease in the expression of many genes involved in carbohydrate metabolism (Badawy, 1977). Two key regulatory proteins in the glycolytic pathway, hexokinase (*HK2*) and phosphofructokinase (*PFKL*) were decreased in expression due to ethanol treatment. The ethanol induced change in *HK2* expression was validated by qRT-PCR (Figure 18). TCA cycle enzyme,

oxoglutarate dehydrogenase (*OGDH*) was decreased by 1.24-fold while isocitrate dehydrogenase (*IDH3B*) decreased modestly. Both these enzymes are inhibited by NADH, and carry out key regulatory steps in TCA cycle. Phosphoenol pyruvate carboxykinase (*PCK*) catalyzes the formation of phosphoenolpyruvate from oxaloacetate and is an important control point in gluconeogenesis. The cytosolic form, *PCK1*, decreased by 1.35-fold; we further confirmed this by qRT-PCR (Figure 18). Pyruvate carboxylase (*PC*) that catalyzes the carboxylation of pyruvate to oxaloacetate, decreased. In addition to pyruvate, various precursors like lactate, amino acids and glycerol, enter the gluconeogenic pathway. To enter into the gluconeogenic pathway, glycerol has to be phosphorylated by glycerol kinase (*GK*) (Walker et al., 1993). There was a -1.33-fold change in the expression of glycerol kinase, indicating an additional mechanism by which alcohol inhibits hepatic gluconeogenesis. These results indicate that alcohol not only modulates the activity of some enzymes involved in the carbohydrate metabolism, it also affects their expression at the level of transcription.

3.e. Lipid metabolism

Key genes involved in the fatty acid beta-oxidation pathway were affected by ethanol. Acyl-CoA synthetases (ACS) are involved in the activation of fatty acids (Soupene and Kuypers, 2008). With chronic ethanol treatment, acyl-CoA synthetases *ACSL1*, *ACSS2* were differentially expressed by 1.42 and -1.18-fold, respectively. CPT1A transports fatty acyl-coA esters across the mitochondrial inner membrane and its deficiency results in a decreased rate of fatty acid beta-

oxidation (Aoyama et al., 1998; Zammit, 2008). *CPT1A* decreased by 50% in HepG2 cells exposed to ethanol and this decrease was confirmed by qRT-PCR (Figure 18). Acyl-CoA dehydrogenase, short chain (*ACADS*) is one of enzymes which carry out the first step in the beta-oxidation of fatty acids (Kelly et al., 1993). It was downregulated by 24%. In peroxisomal oxidation of fatty acids, this reaction is catalyzed by Acyl-CoA oxidase, palmitoyl, *ACOX1*(Reddy and Hashimoto, 2001). *ACOX1* also decreased in expression. In summary, many of the enzymes involved in the oxidation of fatty acids were expressed at lower levels when HepG2 cells were exposed to ethanol.

Ethanol also had a modest (15% decrease) effect on the expression of serum response regulatory binding factor 1 (*SREBF1*). We confirmed array results with qRT-PCR (Figure 18). *SREBF1* transcribes many genes that participate in fatty acid synthesis, including fatty acid synthase. Our data contradicts previous findings where an increase in protein and mRNA expression and the activity of *SREBF1* was observed (You et al., 2002). Acetaldehyde was shown to be responsible for the increase in the transcription from SREBF1 promoters. It is possible the decrease in expression in our data is because limited metabolism of alcohol in HepG2 cells does not generate enough acetaldehyde to trigger an increase in the expression of SREBF1.

Exposure of cells to ethanol for 9-days also affected many genes in the glycerolipid or glycerophospholipid metabolism. Glycerol-3-phosphate acyltransferase, mitochondrial (*GPAM*) and 1-acylglycerol-3-phosphate O-acyltransferase 3 (*AGPAT*) were increased by 1.31- and 1.48-fold. GPAM is

involved in the first step of de novo synthesis of triglycerides and glycerophospholipids (Dircks and Sul, 1997). Overexpression of this protein in mice was shown to increase serum and liver triglycerides, while decreasing fatty acid β-oxidation and steatosis (Linden et al., 2006). AGPAT3 belongs to the family of lysophosphotaide acyl transferases that are involved in acylation of glycerol-3-phosphate to 1-acyl glycerol-3-phsphate. AGPAT3 is localized to the golgi membrane and over expression of this protein inhibits the formation of Golgi membrane tubules and protein trafficking (Schmidt and Brown, 2009).

Lipins are 1, 2-diacylglycerol-3-phosphate (phosphatidate) phosphatases. Lipin 1 (*LPIN1*) increased in expression whereas *LIPN2* decreased with ethanol. Both are expressed in the liver (Donkor et al., 2007). In addition to phosphatase activity, LIPN1 also has transcriptional coactivator activity (Finck et al., 2006). It associates with PPARα and PPARγ coactivator-1α and modulates the expression of many enzymes involved in fatty acid oxidation and synthesis.

In summary, genes encoding multiple enzymes involved in fatty acid metabolism and triglyceride synthesis were affected upon chronic alcohol exposure. These effects could partly explain the development of alcohol induced fatty liver. Action of ethanol on *LIPN1* could be a new mechanism by which alcohol affects fatty acid metabolism in the liver. By affecting proteins like *AGPAT3* which regulates golgi membrane, alcohol could lead to more cellular damage.

3.f. Genes involved in chromatin regulation

Recent studies indicate that ethanol affects epigenetic modifications of histones and DNA (Bonsch et al., 2006; Bonsch et al., 2004; Lee and Shukla, 2007; Park et al., 2005; Shukla and Aroor, 2006). In HepG2 cells cultured in ethanol for 9 days, a 1.25-fold increase in the expression of *DNMT3B*, one of the *de novo* DNA methyl transferases, was observed. A similar increase (1.31-fold) of *DNMT3B* expression was observed in validation assays. In addition to *DNMT3*, methyl-CpG binding domain protein 5 (*MBD5*) expression was also increased by 43%. The MBD family of proteins binds to methylated CpG regions in the DNA leading to transcriptional repression.

Effects of alcohol on DNA methylation are controversial. A 40% decrease in global DNA methylation was reported in the livers of rats fed ethanol for 9-weeks (Lu et al., 2000). In chronic alcoholics, however, a hypermethylation of DNA in peripheral blood cells with a decrease in the expression of *DNMT3B* was reported (Bonsch et al., 2006; Bonsch et al., 2004). In our experiment with HepG2 cells, global DNA methylation levels were not measured.

Genes encoding histone deacetylases and histone acetylases were affected by ethanol exposure. *HDAC5, HDAC6* and *HDAC11* decreased in expression. Histone acetylase *MYST4* increased in expression by 20% where as *MYST1* decreased modestly (11%). HDAC5 (Zhang et al., 2002) and HDAC11 (Villagra et al., 2009) proteins are involved in the regulation of transcription, while HDAC6 is a cytoplasmic protein associated with microtubules (Zhang et al., 2008b).

In a study that examined the acute effects of alcohol on histone modifications in isolated rat hepatocytes and rat liver, ethanol was shown to increase the acetylation of histone 3 (H3), lysine 9 (K9) acetylation (Park et al., 2005). As acetylation of histones is regulated by histone acetylases and deacetylases, it is possible that this effect is brought about by modulating the expression of histone deacetylases and acetylases as was observed in our data.

3.g. Genes associated with alcoholism

Alcoholism is a genetic disorder and multiple genes have been associated with this disease. Forty four genes that were differentially expressed in our data have been previously associated with alcoholism in humans (Bierut et al., 2010; Edenberg and Foroud, 2006; Hill et al., 2004; Kalsi et al., 2009). Some of these genes, including Insulin-like growth factor 1 receptor (*IGF1R*), Cholinergic receptor, nicotinic, alpha 5 (*CHRNA5*), 5-hydroxytryptamine (serotonin) receptor 3B (*HTR3B*) play a role in alcohol induced liver injury in the liver, or act as receptors in the brain. We confirmed the effect on expression of *HTR3B* by qRT-PCR (Figure 18). Knowledge that ethanol affects the transcription of the genes associated with the disease provides mechanistic insight into why these genes are associated with the disease.

4. Effects of alcohol on alternative splicing

Recently it has been predicted that 92 to 94% of genes in the human genome encode alternative isoforms (Wang et al., 2008) and there is increasing

evidence that alternative isoforms could lead to disease (Garcia-Blanco et al., 2004; Orengo and Cooper, 2007). For the first time, the effects of chronic ethanol exposure on alternative isoforms were explored in this study using Affymetrix human Exon arrays. Due to the inherent difficulties associated with exon array data analysis, a stringent FDR was used. Resulting genes were also visually inspected to identify true alternative splicing events. Only 31% of the genes that passed a 3% FDR filter were probably alternatively spliced. One of the main causes for many probably not alternatively spliced are the 5' and 3' edge effects that were observed in most of the genes. There was a decrease in the expression of 5' probe set with ethanol whereas there was an increase in the expression of 3' probe set. Bemmo et al. (2008) reported that probe sets at the 5' and 3' ends of the transcript behave differently and termed this "edge bias." They attributed the 5' effects to high GC content at the 5' end of the transcript and the 3' effects could be due to the random amplification protocol. Whistler et al. (2010) reported that approximately 75% of false positives are due to this edge bias effect (Whistler et al., 2010). Other reasons for false positives include saturated or non-responsive probe sets (Bemmo et al., 2008; Whistler et al., 2010).

In validation studies, four genes were tested. Three of the tested events appeared to be cassette exons, whereas one was an alternative promoter. Of the three cassette exons, only two (*BPGM* and *CD55*) were confirmed to be alternatively spliced. For *BPGM* the decrease in the expression of the test exon was confirmed by qRT-PCR assays. For *CD55*, although a decrease in the expression of the test exon was observed, a similar decrease in the remaining

part of the transcript was also observed. This observation suggests that this is not a true ethanol affected differential alternative splicing. However, the fraction of the transcript with the test exon in the RNA was very small (as indicated by the density of the band in Figure 21). It is possible that the assays carried out are not sensitive to detect a 40% fold change at low concentrations of the transcript.

Another gene that was validated for differential alternative splicing was *MBD5*. In *MBD5*, probe sets in one half of the transcripts were increased in expression by 1.3-fold and probe sets in the second half of the transcript were increased approximately by 2-fold. Although no alternative isoforms are reported in the Refseq database, a shorter isoform is reported in the UCSC gene track. The expression of shorter isoform (2.5 kb) was increased by 2.1-fold upon ethanol treatment. This was validated by qRT-PCR assay. Not much is known about the function of this protein. A haploinsufficiency in this gene was reported to be the cause of a 2q23.1 microdeletion syndrome that leads to intellectual disabilities, seizures and speech impairment (Williams et al., 2010). Interestingly, the short isoform lacks the 70 amino acid methyl binding domain that is present in all MBD proteins. So, further studies are required to understand the function of short form of the transcript.

BPGM encoded 2,3-Bisphophoglycero mutase enzyme catalyzes both the synthesis and degradation of 2,3-diphosphoglycerate (2,3-DPG), which modulates the affinity of hemoglobin to oxygen (Joulin et al., 1988). The function of the alternative forms of this protein is not known. *BPGM* is mainly expressed in

erythrocytes. It will be interesting to study if chronic alcohol exposure has the same effect on BPGM alternative splicing in erythrocytes.

In summary, identification of differential alternative splicing due to alcohol exposure is very complicated. It requires a great deal of manual analysis of the data and validation studies to identify true differential alternative isoforms.

5. Future directions

As part of this dissertation multiple *cis*-regulatory regions in the 360 kb *ADH* cluster have been identified. In this study 4E3, a strong distal enhancer of *ADH4* was identified. All experiments were carried out by transient transfections in human hepatocyte-like cells. Transient transfection assays cannot duplicate the full regulatory complexity present *in vivo*, including additional *cis*-regulatory regions and the complexity of chromatin structure. Future studies could study this region in an animal model. A transgenic mouse model of human *ADH4* can be generated by integrating a bacterial artificial chromosome (BAC) carrying human *ADH4* along with its neighboring non-coding sequences into mouse genome. The animal model can be used to answer many questions regarding the function of 4E3. The magnitude of enhancer activity of 4E3 along with its contribution to tissue specificity in all tissues can be determined. The mechanism of activation can also be examined. If FOXA mediated physical interaction of the promoter and enhancer and DNA looping occurs, it can be identified using chromosome conformation capture technique (3C) (Dekker et al., 2002). 3C technique involves formaldehyde crosslinking of interacting chromatin regions, restriction digestion,

intermolecular ligation and subsequent PCR. If FOXA proteins function as pioneer factors at 4E3 enhancer in the embryonic stage, chromatin immunoprecipitation technique can be used to investigate chromatin profile before and after activation.

The most interesting question that arises with genes that are present in a cluster in genome is whether they are regulated by a locus control region (LCR). By definition, LCRs are regulatory regions that regulate the expression of multiple genes in a cluster. It will be interesting to study the role of 4E3 on regulation of other *ADH* genes in the cluster. This also can be studied in an animal model if other *ADH* genes are included in the BAC.

The function of negative regulatory regions identified in this study in cells other than hepatic cells has not been explored. It is possible that these could play a greater functional role in non-liver tissues. Tissue specificity of these regions can be studied in non-hepatic cells or in an animal model.

Variations play an important role in the risk for alcoholism. Two variations in the 4E3 enhancer were observed to affect the enhancer activity. These could play a significant role in alcoholism. Therefore, association studies have to be carried out to determine their affect on the risk for alcoholism.

In our study of the effects of chronic ethanol treatment of HepG2 cells, we observed many hallmark effects of alcohol on gene expression along with some new effects. Because these cells are acting as a good model system for alcohol effects, it is possible to further explore other effects of alcohol. Effects of alcohol on global methylation can be studied using chromatin immunoprecipitation with

anti-methyl cytosine antibody combined with high throughput techniques like sequencing or microarrays. Effects of alcohol on histone modifications can also be studied in a similar approach.

To identify effects of alcohol on alternative isoforms an alternative approach like RNA-sequencing (RNA-seq) can be employed. RNA-seq data allow detection of alternative isoforms using the reads at the splicing junctions.

APPENDIX

Genes differentially expressed with chronic ethanol exposure.

All differentially expressed genes (FDR ≤ 3, fold change ≥ 10%), excluding genes listed in Table 13.

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3230610	ABCA2	-1.11	0.02	0.29
2708287	ABCC5	1.33	0.01	0.05
3682182	ABCC6	-1.14	0.03	0.33
2777276	ABCG2	1.38	0.02	0.45
3500772	ABHD13	1.17	0.02	0.98
3528759	ABHD4	1.27	0.00	0.76
3282268	ACBD5	-1.10	0.00	0.64
2459837	ACTA1	-1.23	0.01	0.25
2566021	ACTR1B	-1.24	0.01	0.98
3907011	ADA	1.13	0.02	0.48
3011492	ADAM22	1.31	0.01	0.45
2358393	ADAMTSL4	-1.42	0.00	0.87
3716950	ADAP2	1.21	0.02	0.00
3320123	ADM	-1.18	0.00	0.82
3336801	ADRBK1	-1.15	0.01	0.14
3940631	ADRBK2	1.25	0.02	0.00
3554360	ADSSL1	-1.30	0.02	0.97
3039791	AGR2	2.36	0.01	0.52
3903361	AHCY	-1.12	0.00	0.86
3293187	AIFM2	-1.18	0.01	0.47
3868160	AKT1S1	1.22	0.00	0.84
3838522	ALDH16A1	-1.26	0.01	0.87
3646542	ALG1	1.17	0.00	0.66
3391093	ALG9	1.13	0.01	0.83
3427032	AMDHD1	1.20	0.00	0.85
2696309	AMOTL2	-1.27	0.00	0.92

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
2445982	ANGPTL1	1.58	0.02	0.93
3299970	ANKRD1	-1.58	0.00	0.41
3704717	ANKRD11	1.17	0.02	0.01
2754673	ANKRD37	-1.27	0.01	0.98
3651672	ANKS4B	-1.22	0.01	0.92
2487082	ANTXR1	1.35	0.00	0.00
2487412	ANXA4	-1.35	0.01	0.39
3295032	AP3M1	1.12	0.01	0.09
3015241	AP4M1	-1.24	0.01	0.44
2766893	APBB2	1.15	0.01	0.33
2622196	APEH	-1.20	0.03	0.62
3356115	APLP2	1.10	0.02	0.07
3945572	APOBEC3C	-1.31	0.01	0.85
3945614	APOBEC3F	-1.32	0.00	0.85
3469319	APPL2	1.15	0.01	0.10
3203569	AQP3	-1.30	0.02	0.56
3022409	ARF5	-1.15	0.03	0.96
3371928	ARFGAP2	-1.11	0.02	0.21
2746693	ARHGAP10	1.50	0.01	0.13
3289189	ASAH2	1.68	0.00	0.04
3743371	ASGR1	-1.17	0.01	0.13
3137530	ASPH	1.16	0.02	0.01
2315951	ATAD3A	-1.20	0.02	0.91
2726072	ATP10D	1.54	0.01	0.34
3464983	ATP2B1	1.16	0.02	0.07
3863087	ATP5SL	-1.12	0.02	0.71
3974556	ATP6AP2	1.16	0.02	0.25
3514736	ATP7B	1.12	0.01	0.06
3834046	AXL	-1.18	0.00	0.13
2669930	AXUD1	-1.16	0.02	0.75
3908963	B4GALT5	1.11	0.02	0.07
3803120	B4GALT6	1.42	0.01	0.11
3240452	BAMBI	1.28	0.01	0.02
3910360	BCAS1	1.29	0.00	0.34
3447694	BCAT1	1.13	0.03	0.11

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3656829	BCKDK	-1.23	0.01	0.88
2713382	BDH1	-1.25	0.02	0.93
2608725	BHLHE40	-1.37	0.00	0.99
3346548	BIRC3	1.67	0.03	0.43
3882190	BPIL1	1.14	0.01	0.94
2903343	BRD2	1.10	0.01	0.22
3436544	BRI3BP	1.16	0.02	0.59
2375664	BTG2	1.18	0.02	0.04
2899340	BTN2A2	1.26	0.02	0.97
2899413	BTN3A3	1.25	0.01	0.85
3286776	C10orf10	-1.30	0.00	0.47
3252690	C10orf11	1.25	0.02	0.19
3308378	C10orf82	1.20	0.01	0.45
3334847	C11orf2	-1.27	0.01	0.99
3393834	C11orf60	1.42	0.01	0.02
3396144	C11orf61	1.23	0.02	0.13
3332938	C11orf79	1.13	0.02	0.42
3474787	C12orf27	1.27	0.02	0.53
3410384	C12orf35	1.39	0.02	0.71
3472000	C12orf51	1.19	0.03	0.39
3572461	C14orf1	1.16	0.01	0.31
3578069	C14orf139	1.40	0.01	0.21
3573933	C14orf145	1.53	0.03	0.07
3573994	C14orf145	1.28	0.01	0.66
3578089	C14orf49	1.17	0.00	0.46
3588658	C15orf41	1.15	0.01	0.00
3652271	C16orf52	1.18	0.01	0.19
3688424	C16orf58	-1.17	0.00	0.60
3678542	C16orf89	1.33	0.02	0.26
3744324	C17orf68	-1.15	0.02	0.47
3864597	C19orf61	1.21	0.01	0.99
3820310	C19orf66	-1.17	0.01	0.47
2437273	C1orf2	1.15	0.02	0.01
2361697	C1orf66	-1.22	0.01	0.38
2359736	C1orf77	-1.11	0.02	0.99

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3960005	C1QTNF6	1.51	0.01	0.10
3442514	C1RL	1.14	0.01	0.09
3890109	C20orf108	-1.10	0.02	0.12
3904594	C20orf117	1.12	0.01	0.42
3901665	C20orf3	1.11	0.02	0.90
2621949	C3orf60	-1.31	0.01	0.53
2882834	C5orf4	-1.11	0.03	0.86
2986146	C6orf122	1.73	0.01	0.00
2950798	C6orf125	-1.24	0.00	0.97
2902736	C6orf48	-1.22	0.01	0.43
2931700	C6orf97	-1.51	0.00	0.52
2337786	C8A	-1.22	0.02	0.98
3130823	C8orf41	1.13	0.02	0.79
3168210	C9orf127	1.22	0.03	0.83
3186207	C9orf91	1.18	0.01	0.31
3168066	CA9	-1.74	0.00	0.13
3392332	CADM1	1.26	0.00	0.20
3456353	CALCOCO1	1.23	0.01	0.17
3772775	CANT1	1.19	0.01	0.35
3449368	CAPRIN2	1.37	0.02	0.44
3591838	CASC4	1.15	0.01	0.62
4005859	CASK	1.30	0.02	0.20
3456630	CBX5	-1.57	0.00	0.69
3226737	CCBL1	-1.18	0.02	0.98
3767531	CCDC46	1.64	0.02	0.20
2881860	CCDC69	1.20	0.02	0.07
2674138	CCDC71	-1.16	0.01	0.85
2948821	CCHCR1	-1.16	0.01	0.98
3753956	CCL16	-1.17	0.01	0.98
3625326	CCPG1	1.41	0.03	0.42
2936657	CCR6	-1.24	0.01	0.90
3601229	CD276	1.12	0.01	0.42
3708858	CD68	-1.16	0.01	0.74
2881370	CD74	1.19	0.00	0.45
2895841	CD83	1.24	0.02	0.66

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3402315	CD9	1.20	0.00	0.19
4025771	CD99L2	1.16	0.00	0.58
2324084	CDA	1.37	0.01	0.48
3666409	CDH1	-1.19	0.00	0.67
3970642	CDKL5	1.37	0.02	0.30
3396770	CDON	1.22	0.00	0.00
3863669	CEACAM1	1.14	0.01	0.80
3350908	CEP164	1.13	0.02	0.26
3190420	CERCAM	1.27	0.01	0.78
3906160	CHD6	1.14	0.02	0.02
4014251	CHM	1.15	0.03	0.39
2662435	CIDEC	-1.29	0.01	0.88
3815649	CIRBP	-1.14	0.02	0.83
3469687	CKAP4	1.13	0.01	0.00
3007960	CLDN4	1.21	0.02	0.87
2949330	CLIC1	1.18	0.03	0.00
3577940	CLMN	1.20	0.02	0.75
3381063	CLPB	1.15	0.01	0.60
2395890	CLSTN1	1.15	0.02	0.94
3129065	CLU	1.27	0.00	0.33
2836856	CNOT8	-1.21	0.02	0.61
3452865	COL2A1	1.25	0.02	0.00
3892974	COL9A3	1.14	0.03	0.00
3543935	COQ6	-1.23	0.00	0.13
3662723	COQ9	-1.13	0.01	0.86
2768197	CORIN	1.38	0.02	0.24
3378830	CORO1B	-1.16	0.01	0.48
3512843	CPB2	1.33	0.00	0.22
2842255	CPLX2	1.24	0.01	0.99
2711604	CPN2	1.16	0.02	0.43
3421446	CPSF6	1.17	0.00	0.37
2477073	CRIM1	-1.11	0.01	0.53
3855104	CRLF1	1.14	0.02	0.00
3934245	CSTB	-1.16	0.02	0.60
2527786	CTDSP1	-1.28	0.01	0.57

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2954355	CUL7	-1.12	0.01	0.72
3986230	CXorf57	1.32	0.02	0.15
2515240	CYBRD1	1.74	0.00	0.01
3120051	CYC1	-1.23	0.01	0.92
3771642	CYGB	1.19	0.01	0.08
4031068	CYorf15B	1.39	0.03	0.96
2903034	CYP21A2	1.19	0.02	0.00
2344888	CYR61	-1.50	0.00	0.48
3940001	CYTSA	1.14	0.02	0.40
2854445	DAB2	1.28	0.03	0.94
2673830	DALRD3	-1.25	0.00	0.95
3723071	DBF4B	1.16	0.01	0.10
2999710	DBNL	-1.23	0.01	0.05
3526544	DCUN1D2	1.20	0.01	0.00
3113280	DEPDC6	1.36	0.00	0.20
3937183	DGCR8	1.22	0.00	0.43
3567187	DHRS7	1.25	0.01	0.82
3235373	DHTKD1	-1.13	0.00	0.85
3598613	DIS3L	1.17	0.01	0.60
3133325	DKK4	2.03	0.00	0.93
3296386	DLG5	1.24	0.00	0.32
3552083	DLK1	1.13	0.01	0.75
2515471	DLX1	1.83	0.00	0.05
3624145	DMXL2	1.29	0.01	0.64
3820758	DNM2	-1.23	0.00	0.84
3303165	DNMBP	1.16	0.00	0.95
3068097	DOCK4	1.24	0.01	0.77
3850725	DOCK6	-1.14	0.00	0.00
3522398	DOCK9	1.43	0.01	0.00
2424524	DPYD	1.36	0.02	0.11
3802924	DSC3	2.17	0.00	0.11
3783529	DSG2	1.13	0.03	0.45
3552847	DYNC1H1	1.12	0.02	0.13
3695199	DYNC1LI2	1.14	0.01	0.11
3521372	DZIP1	1.31	0.02	0.22

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3463112	E2F7	1.27	0.01	0.09
2612371	EAF1	-1.11	0.02	0.42
3980380	EDA	1.24	0.02	0.52
2830861	EGR1	-1.20	0.02	0.09
4021433	ELF4	1.17	0.02	0.07
3679959	EMP2	1.17	0.01	0.79
3345427	ENDOD1	1.32	0.02	0.21
3150579	ENPP2	2.41	0.00	0.01
3883690	EPB41L1	1.20	0.01	0.00
3713794	EPN2	1.11	0.03	0.14
3015778	EPO	-1.33	0.01	0.31
3400413	ERC1	1.20	0.00	0.98
3648995	ERCC4	1.13	0.01	0.01
2462329	ERO1LB	1.49	0.01	0.00
3445768	ERP27	1.55	0.01	0.42
2395177	ERRFI1	-1.18	0.00	0.90
3921068	ETS2	-1.15	0.00	0.53
3541937	EXDL2	1.18	0.02	0.84
3566304	EXOC5	1.14	0.00	0.86
3328389	EXT2	1.24	0.00	0.00
3502411	F7	-1.17	0.02	0.68
3979101	FAAH2	1.27	0.02	0.14
3226253	FAM102A	1.30	0.00	0.06
3278813	FAM107B	-1.18	0.02	0.89
3471538	FAM109A	-1.16	0.01	0.30
2523354	FAM117B	1.26	0.02	0.01
3355021	FAM118B	1.20	0.01	0.44
3225952	FAM129B	-1.14	0.01	1.00
2777487	FAM13A1	-1.52	0.00	0.96
2867145	FAM172A	1.30	0.01	0.36
3798778	FAM38B	1.31	0.03	0.54
3798829	FAM38B2	1.17	0.01	0.20
2658785	FAM43A	-1.20	0.03	0.79
2634058	FAM55C	1.32	0.01	0.17
3803290	FAM59A	1.24	0.01	0.43

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3665550	FAM65A	-1.13	0.01	0.71
3152558	FAM84B	1.28	0.02	0.02
2904663	FANCE	-1.22	0.01	0.62
2742581	FAT4	1.26	0.02	0.04
3576749	FBLN5	1.61	0.00	0.00
3623031	FBN1	1.52	0.00	0.00
3230530	FBXW5	-1.16	0.01	0.15
3809621	FECH	1.14	0.03	0.37
2338487	FGGY	-1.19	0.01	1.00
3125993	FGL1	1.33	0.02	0.72
2568687	FHL2	-1.25	0.01	0.84
3401099	FKBP4	1.14	0.03	0.01
2360468	FLAD1	-1.11	0.03	0.58
3527864	FLJ10357	-1.12	0.00	0.85
3898355	FLRT3	1.34	0.01	0.88
3544625	FLVCR2	1.15	0.01	0.85
3227159	FNBP1	1.16	0.00	0.42
3190061	FPGS	-1.20	0.01	0.08
2732655	FRAS1	1.23	0.01	0.67
3421579	FRS2	1.17	0.02	0.75
3661152	FTO	1.11	0.01	0.94
3608398	FURIN	1.15	0.01	0.56
3130757	FUT10	1.16	0.01	0.80
3847462	FUT6	1.13	0.02	0.00
3830189	FXYD1	-1.65	0.00	0.39
3110272	FZD6	1.39	0.01	0.97
2745547	GAB1	1.18	0.03	0.03
3623683	GABPB1	1.16	0.01	0.24
2401581	GALE	-1.18	0.01	0.99
2511045	GALNT13	1.41	0.03	0.64
3454892	GALNT6	1.24	0.00	0.36
3706439	GARNL4	-1.19	0.00	0.29
2474594	GCKR	1.30	0.01	0.52
3663228	GINS3	1.19	0.01	0.76
3471005	GIT2	1.17	0.02	0.10

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3759335	GJC1	1.59	0.00	0.12
2989537	GLCCI1	1.58	0.02	0.83
3422855	GLIPR1	1.81	0.00	0.14
3451246	GLT8D3	1.24	0.02	0.32
3468103	GNPTAB	1.29	0.01	0.00
3191338	GPR107	1.23	0.00	0.22
3475782	GPR109A	-1.60	0.02	0.99
3475794	GPR109B	-1.56	0.00	0.94
2386747	GPR137B	1.35	0.03	0.04
3662851	GPR97	-1.22	0.01	0.33
3683377	GPRC5B	1.61	0.00	0.00
3830002	GRAMD1A	-1.17	0.02	0.95
2584712	GRB14	1.48	0.01	0.87
2469157	GRHL1	-1.18	0.03	0.92
3755903	GSDMB	1.32	0.03	0.19
3574074	GTF2A1	1.15	0.03	0.13
3192525	GTF3C4	1.11	0.03	0.37
2954771	GTPBP2	-1.12	0.02	0.53
3257750	HECTD2	1.43	0.02	0.13
3396249	HEPACAM	1.44	0.00	0.43
3354380	HEPN1	1.41	0.02	0.74
2735459	HERC3	-1.24	0.01	0.96
3096575	HGSNAT	1.31	0.03	0.19
3260666	HIF1AN	1.15	0.02	0.46
3325907	HIPK3	1.16	0.03	0.99
2948926	HLA-B	1.30	0.01	0.09
3881282	HM13	1.16	0.01	0.00
3351841	HMBS	-1.12	0.03	0.20
3815493	HMHA1	-1.38	0.01	0.16
3557268	HOMEZ	1.18	0.00	0.48
3851720	HOOK2	-1.15	0.02	0.15
3667890	HPR	-1.33	0.00	0.48
3711262	HS3ST3B1	1.11	0.02	0.06
3721815	HSD17B1	-1.15	0.02	0.07
3671076	HSD17B2	-1.22	0.01	1.00

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2903488	HSD17B8	-1.21	0.02	0.58
3656737	HSD3B7	-1.18	0.03	0.84
3934187	HSF2BP	1.34	0.00	0.06
3308397	HSPA12A	1.30	0.00	0.00
3925439	HSPA13	1.22	0.03	0.09
3394123	HYOU1	1.11	0.00	0.64
3766621	ICAM2	1.18	0.03	0.04
2957499	ICK	1.21	0.01	0.68
2948630	IER3	-1.31	0.01	0.90
2403261	IFI6	1.35	0.01	0.00
3257246	IFIT1	1.80	0.02	0.11
3629610	IGDCC3	1.24	0.00	0.40
3359134	IGF2	1.14	0.02	0.49
3918535	IL10RB	1.26	0.02	0.42
3339261	IL18BP	1.18	0.02	0.13
2359817	INTS3	1.21	0.01	0.19
3430776	ISCU	1.15	0.01	0.41
2438482	ISG20L2	1.21	0.01	0.41
3573123	ISM2	-1.23	0.00	0.55
3656223	ITGAL	-1.33	0.00	0.79
3276337	ITIH5	1.59	0.00	0.00
3488985	ITM2B	1.21	0.01	0.66
3577160	ITPK1	-1.20	0.01	0.75
3897505	JAG1	-1.27	0.00	0.99
3357237	JAM3	1.22	0.02	0.13
2333429	JMJD2A	1.13	0.02	0.43
2459296	JMJD4	-1.18	0.01	0.97
2415084	JUN	-1.14	0.01	0.11
3854877	JUND	-1.24	0.01	0.87
3470793	KCTD10	1.20	0.03	0.72
3867092	KDELR1	1.10	0.02	0.97
3572041	KIAA0317	1.19	0.02	0.13
3905073	KIAA0406	1.14	0.01	0.36
2764192	KIAA0746	1.56	0.00	0.01
3203990	KIAA1161	1.23	0.02	0.87

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3604147	KIAA1199	1.14	0.00	0.15
3238962	KIAA1217	1.25	0.01	0.00
3655628	KIF22	-1.12	0.03	0.39
3464747	KITLG	1.66	0.01	0.96
2724308	KLB	1.20	0.01	0.50
3147508	KLF10	1.35	0.00	0.04
2469213	KLF11	1.25	0.01	0.55
3803418	KLHL14	1.35	0.01	0.03
3757108	KRT19	-1.18	0.01	0.45
2925510	L3MBTL3	1.66	0.00	0.05
2371065	LAMC1	1.22	0.00	0.20
3631498	LARP6	1.33	0.01	0.43
2828796	LEAP2	-1.29	0.03	0.67
3841474	LENG8	1.14	0.00	0.49
3577078	LGMN	1.16	0.01	0.99
3866898	LIG1	-1.13	0.02	0.90
3463727	LIN7A	-1.34	0.01	0.59
3965936	LMF2	-1.14	0.03	0.19
3416996	LOC440104	1.20	0.02	0.06
3651509	LOC81691	-1.40	0.01	0.51
3847356	LONP1	-1.20	0.00	0.72
2393711	LRRC47	-1.13	0.02	0.16
3016791	LRWD1	-1.13	0.03	0.60
3529877	LTB4R2	1.33	0.01	0.01
3402444	LTBR	1.14	0.01	0.28
3098977	LYN	1.25	0.03	0.38
2866590	LYSMD3	1.21	0.01	0.86
3362826	LYVE1	1.60	0.02	0.78
4013359	MAGT1	1.14	0.01	0.85
3633347	MAN2C1	-1.18	0.01	0.42
3444906	MANSC1	1.57	0.00	0.56
2814756	MAP1B	1.24	0.01	0.00
4002081	MAP7D2	1.23	0.00	0.48
3723687	MAPT	1.47	0.00	0.01
3286921	MARCH8	1.16	0.01	0.60

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3702293	MBTPS1	1.12	0.00	0.05
3394264	MCAM	1.27	0.01	0.13
3502259	MCF2L	1.11	0.01	0.00
3329343	MDK	1.23	0.01	0.90
3844822	MED16	-1.21	0.01	0.48
3223551	MEGF9	1.20	0.02	0.14
3618333	MEIS2	1.25	0.00	0.29
2909263	MEP1A	2.07	0.00	0.21
3604236	MESDC1	1.14	0.01	0.97
3796244	METTL4	1.25	0.01	0.09
3416895	METTL7B	1.21	0.00	0.16
3638204	MFGE8	1.60	0.00	0.00
3304012	MGEA5	1.23	0.02	0.21
3320717	MICAL2	1.14	0.01	0.44
3256560	MINPP1	1.18	0.01	0.88
3845647	MKNK2	-1.14	0.00	0.86
3434525	MLEC	1.19	0.02	0.01
3699080	MLKL	1.25	0.01	0.89
3528864	MMP14	1.19	0.02	0.34
3845681	MOBKL2A	-1.24	0.01	0.18
3064501	MOGAT3	-1.42	0.00	0.79
3708874	MPDU1	-1.23	0.00	1.00
4027585	MPP1	1.15	0.01	0.02
2365958	MPZL1	1.18	0.00	0.71
3738205	MRPL12	-1.14	0.02	0.82
3675101	MRPL28	-1.14	0.01	0.99
3771120	MRPL38	-1.12	0.02	0.99
2902633	MSH5	-1.24	0.02	0.65
3942179	MTMR3	1.16	0.00	0.20
3697183	MTSS1L	1.11	0.03	0.13
2692883	MUC13	2.99	0.00	0.00
2896484	MYLIP	-1.23	0.01	0.84
2692447	MYLK	1.11	0.01	0.91
3458248	MYO1A	1.19	0.01	0.20
3752709	MYO1D	1.18	0.02	0.58

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3624607	MYO5A	1.69	0.00	0.00
3417809	NAB2	-1.22	0.01	0.93
3229628	NACC2	-1.13	0.02	0.54
3721795	NAGLU	-1.12	0.01	0.65
3181460	NANS	1.17	0.02	0.65
3675369	NARFL	-1.13	0.02	0.80
2559619	NAT8	1.48	0.02	0.65
3770390	NAT9	-1.26	0.00	0.75
3485292	NBEA	1.47	0.01	0.00
3649811	NDE1	-1.15	0.01	0.95
3154317	NDRG1	-1.15	0.01	0.50
3555736	NDRG2	-1.14	0.01	0.26
2379068	NENF	1.19	0.02	0.06
3601051	NEO1	1.36	0.00	0.33
3690154	NETO2	1.33	0.00	0.97
3340410	NEU3	1.12	0.02	0.34
3942062	NF2	1.17	0.00	0.56
2603987	NGEF	-1.18	0.03	0.98
3564071	NIN	1.37	0.01	0.04
3956909	NIPSNAP1	-1.24	0.00	0.63
3182984	NIPSNAP3B	1.32	0.02	0.66
3660075	NKD1	1.13	0.01	0.67
3708553	NLGN2	1.21	0.01	0.31
4030371	NLGN4Y	1.46	0.02	0.67
3203582	NOL6	-1.11	0.03	0.61
2431112	NOTCH2	1.22	0.01	0.56
3146012	NPAL2	1.54	0.02	0.48
3891048	NPEPL1	1.13	0.01	0.04
3774029	NPLOC4	1.12	0.01	0.61
2738378	NPNT	1.37	0.00	0.65
3632492	NPTN	1.23	0.02	0.99
2612175	NR2C2	1.13	0.02	0.44
3225096	NR6A1	1.15	0.00	0.57
3770588	NT5C	-1.21	0.01	0.79
3304624	NT5C2	1.25	0.01	0.84

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3468743	NT5DC3	1.25	0.01	0.00
2915828	NT5E	1.53	0.01	0.80
3681674	NTAN1	1.10	0.02	0.41
3322251	NUCB2	1.37	0.02	0.58
3751463	NUFIP2	1.15	0.02	1.00
3376155	NXF1	1.19	0.00	0.21
3432438	OAS1	1.19	0.01	0.66
2912980	OGFRL1	1.81	0.02	0.31
2609904	OGG1	-1.24	0.01	0.68
3981120	OGT	1.15	0.03	0.61
3319119	OLFML1	2.14	0.00	0.36
3942531	OSBP2	1.37	0.02	0.44
3549517	OTUB2	1.28	0.01	0.33
3335952	PACS1	1.15	0.00	0.65
3815116	PALM	1.21	0.02	0.08
3457696	PAN2	-1.12	0.03	0.16
2392528	PANK4	-1.11	0.03	0.77
2910218	PAQR8	-1.12	0.03	0.14
2699059	PAQR9	1.33	0.02	0.79
2708229	PARL	-1.19	0.01	0.68
2639054	PARP14	1.26	0.03	0.84
3629567	PARP16	1.24	0.03	0.32
3368054	PAX6	1.20	0.03	0.40
3887165	PCIF1	-1.13	0.02	0.74
3175274	PCSK5	1.35	0.00	0.00
3727712	PCTP	1.26	0.01	0.82
2487696	PCYOX1	1.24	0.01	0.66
3262198	PDCD11	1.10	0.02	0.69
2589017	PDE11A	1.41	0.01	0.68
3089535	PDLIM2	-1.20	0.01	0.17
3766796	PECAM1	1.47	0.01	0.00
2318656	PER3	-1.12	0.01	0.10
3957445	PES1	-1.12	0.01	0.64
3753690	PEX12	1.21	0.02	0.98
2390518	PGBD2	1.17	0.01	0.66

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
2953751	PGC	-1.43	0.00	0.63
4001556	PHKA2	-1.23	0.00	0.38
3259978	PI4K2A	1.14	0.03	0.05
3301914	PIK3AP1	1.24	0.00	0.18
2905404	PIM1	-1.32	0.01	0.97
3173673	PIP5K1B	1.23	0.02	0.15
3732230	PITPNC1	1.44	0.01	0.10
3822723	PKN1	-1.13	0.01	0.98
3190683	PKN3	-1.16	0.01	0.90
3922975	PKNOX1	1.19	0.01	0.04
2977621	PLAGL1	1.44	0.00	0.01
3828067	PLEKHF1	1.17	0.01	0.73
2479433	PLEKHH2	1.46	0.02	0.01
3845782	PLEKHJ1	-1.27	0.01	0.52
3759849	PLEKHM1	1.21	0.00	0.50
2453370	PLXNA2	1.23	0.00	0.00
3426502	PLXNC1	1.34	0.00	0.00
3746574	PMP22	1.51	0.00	0.61
3073013	PODXL	1.16	0.01	0.02
3881786	POFUT1	1.20	0.02	0.91
3572982	POMT2	1.17	0.01	0.32
3820342	PPAN	-1.14	0.01	0.55
2948425	PPP1R10	1.24	0.00	0.02
3838004	PPP1R15A	-1.31	0.00	0.98
2518488	PPP1R1C	1.20	0.02	0.01
3272761	PRAP1	1.24	0.02	0.89
3838809	PRMT1	-1.13	0.02	0.26
3874751	PRNP	1.19	0.00	0.93
3973692	PRRG1	1.21	0.03	0.11
3717737	PSMD11	1.11	0.02	0.85
2353717	PTGFRN	1.12	0.02	0.00
3402736	PTMS	1.32	0.01	0.50
3871192	PTPRH	-1.15	0.00	0.99
3329983	PTPRJ	1.25	0.01	0.04
3081862	PTPRN2	1.23	0.00	0.00

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3757917	PTRF	1.18	0.01	0.68
3157817	PUF60	-1.17	0.02	0.84
2315739	PUSL1	-1.20	0.01	0.41
3923498	PWP2	-1.13	0.00	1.00
3625440	PYGO1	1.74	0.02	0.53
3771464	QRICH2	1.17	0.02	0.00
3820727	QTRT1	-1.19	0.01	0.65
2888648	RAB24	-1.15	0.03	0.88
3384321	RAB30	1.26	0.02	0.57
3189617	RALGPS1	1.45	0.01	0.09
3497659	RAP2A	1.35	0.00	0.50
2675208	RASSF1	-1.19	0.01	0.65
3315952	RASSF7	-1.31	0.02	0.33
2546054	RBKS	-1.29	0.00	0.70
3472468	RBM19	1.11	0.00	0.03
3976519	RBM3	-1.16	0.03	0.50
3696226	RBM35B	-1.47	0.00	0.52
2766788	RBM47	1.46	0.00	0.00
3378411	RBM4B	1.21	0.01	1.00
3390542	RDX	1.29	0.02	0.81
3168309	RECK	1.55	0.00	0.14
2765865	RELL1	1.11	0.00	0.51
2407786	RHBDL2	1.48	0.03	0.49
3710870	RICH2	1.42	0.01	0.00
3397877	RICS	1.14	0.02	0.77
3878836	RIN2	1.33	0.03	0.05
3861658	RINL	-1.15	0.01	0.75
3933205	RIPK4	-1.13	0.02	0.72
3821908	RNASEH2A	-1.11	0.02	0.51
2562867	RNF103	1.36	0.01	0.54
3844704	RNF126	-1.21	0.02	0.89
2647458	RNF13	1.13	0.00	0.90
2897172	RNF144B	-1.20	0.02	0.72
3704352	RNF166	-1.11	0.02	0.50
2924253	RNF217	1.20	0.03	0.26

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
2333907	RNF220	-1.15	0.01	1.00
2339872	ROR1	1.55	0.00	0.10
2435261	RORC	-1.17	0.01	0.53
3230440	RP11-216L13.5	-1.57	0.00	0.58
2321466	RP1-21O18.1	-1.13	0.01	0.88
3978819	RRAGB	1.37	0.02	0.78
2964231	RRAGD	2.15	0.00	0.01
2675763	RRP9	-1.20	0.01	0.41
3691326	SALL1	1.34	0.00	0.02
3556454	SALL2	-1.19	0.02	0.49
3909777	SALL4	1.62	0.01	0.07
3252534	SAMD8	1.14	0.01	0.52
3904691	SAMHD1	1.40	0.01	0.48
2594089	SATB2	1.27	0.00	0.33
3844978	SBNO2	-1.19	0.02	0.94
3602004	SCAMP5	1.16	0.01	0.36
3605780	SCAND2	1.27	0.01	0.12
3091475	SCARA3	1.41	0.00	0.28
2898934	SCGN	1.38	0.01	0.98
3414969	SCN8A	1.31	0.01	0.29
3441885	SCNN1A	1.19	0.02	0.91
3728037	SCPEP1	1.29	0.00	0.27
2592532	SDPR	-1.40	0.00	0.72
3790479	SEC11C	1.30	0.03	0.18
3942350	SEC14L2	1.32	0.00	0.05
3574207	SEL1L	1.16	0.02	0.82
2622547	SEMA3F	1.13	0.02	0.01
2872047	SEMA6A	1.52	0.00	0.45
2328273	SERINC2	-1.33	0.00	0.93
3549708	SERPINA4	1.18	0.01	0.83
3549740	SERPINA5	1.30	0.00	0.46
3387259	SESN3	3.26	0.00	0.62
3687277	SEZ6L2	1.13	0.03	0.19

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3250863	SGPL1	1.17	0.01	0.07
3982811	SH3BGRL	1.56	0.01	0.12
4001850	SH3KBP1	1.15	0.02	0.95
3205659	SHB	-1.26	0.00	0.68
3708919	SHBG	-1.24	0.02	0.77
2875491	SHROOM1	-1.16	0.00	0.47
3920171	SIM2	1.19	0.02	0.01
3542847	SIPA1L1	1.14	0.00	0.89
3873629	SIRPA	1.94	0.00	0.01
3494706	SLAIN1	1.62	0.03	0.32
3617312	SLC12A6	1.12	0.02	0.42
3907987	SLC13A3	1.15	0.02	0.18
3522327	SLC15A1	1.47	0.02	0.02
2921402	SLC16A10	1.15	0.01	0.13
2427469	SLC16A4	1.28	0.03	0.75
3768412	SLC16A6	-1.53	0.00	0.13
2485636	SLC1A4	1.21	0.01	0.22
2353337	SLC22A15	-1.23	0.02	0.30
3557350	SLC22A17	1.16	0.02	0.01
2877861	SLC23A1	-1.20	0.02	0.77
3598430	SLC24A1	1.10	0.02	0.16
3738224	SLC25A10	-1.23	0.01	0.99
3742384	SLC25A11	-1.17	0.03	0.58
3990762	SLC25A14	1.21	0.01	0.75
3561532	SLC25A21	1.59	0.01	0.34
3251023	SLC29A3	1.14	0.02	0.98
3887452	SLC2A10	1.14	0.02	0.72
2701927	SLC33A1	1.18	0.02	0.50
3354443	SLC37A2	-1.20	0.01	0.25
3394092	SLC37A4	-1.34	0.01	0.21
2584904	SLC38A11	1.80	0.02	0.02
3542063	SLC39A9	1.11	0.03	0.72
3469180	SLC41A2	1.38	0.02	0.13
3183111	SLC44A1	1.15	0.03	0.26
3820612	SLC44A2	1.60	0.00	0.01

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3895330	SLC4A11	-1.14	0.01	0.97
3988165	SLC6A14	2.07	0.01	0.69
3087659	SLC7A2	-1.19	0.02	0.39
3684100	SLC7A5P1	-1.27	0.01	0.40
3340449	SLCO2B1	-1.18	0.02	0.94
3892812	SLCO4A1	-1.12	0.00	0.08
3945877	SMCR7L	1.14	0.02	0.04
3713195	SMCR8	1.22	0.02	0.17
3740838	SMG6	1.11	0.02	0.65
3134511	SNAI2	1.59	0.00	0.14
2824089	SNORA13	-1.19	0.01	0.98
3833757	SNRPA	-1.11	0.02	0.98
3899346	SNX5	-1.11	0.01	0.41
3035702	SNX8	-1.15	0.01	0.59
2920085	SOBP	1.31	0.00	0.08
3352948	SORL1	-1.16	0.00	0.98
3733590	SOX9	1.16	0.00	0.85
3883441	SPAG4	-1.32	0.00	0.92
2882098	SPARC	1.84	0.00	0.02
2411575	SPATA6	1.33	0.03	0.64
3413950	SPATS2	1.22	0.01	0.83
3293840	SPOCK2	1.51	0.00	0.00
3455973	SPRYD3	-1.10	0.02	0.78
3876990	SPTLC3	1.29	0.01	0.01
3984468	SRPX2	1.22	0.01	0.04
3894322	SRXN1	1.15	0.02	0.73
3470597	SSH1	1.20	0.02	0.20
3154398	ST3GAL1	-1.13	0.02	0.82
3846709	STAP2	-1.16	0.01	0.43
3381317	STARD10	1.19	0.01	0.60
3980078	STARD8	1.39	0.00	0.00
2999485	STK17A	-1.24	0.01	0.75
2951916	STK38	1.26	0.02	0.87
3189932	STXBP1	1.34	0.02	0.03
3558418	STXBP6	1.18	0.01	0.05

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3261765	SUFU	1.14	0.03	0.20
3908358	SULF2	1.51	0.00	0.01
3417184	SUOX	1.16	0.01	0.94
3282974	SVIL	1.17	0.00	0.01
3375396	SYT7	1.26	0.02	0.14
3094778	TACC1	1.20	0.02	0.14
3361021	TAF10	-1.15	0.02	0.15
2950214	TAP1	1.14	0.02	0.68
3773241	TBC1D16	1.20	0.01	0.59
3634458	TBC1D2B	1.28	0.01	0.16
3968122	TBL1X	1.13	0.00	0.00
3644249	TBL3	-1.13	0.03	0.19
3472755	TBX3	1.15	0.03	0.42
3063856	tcag7.1177	1.17	0.02	0.00
3893910	TCEA2	1.16	0.01	0.02
2622095	TCTA	-1.26	0.00	0.89
3264299	TECTB	1.36	0.00	0.33
2845829	TERT	-1.19	0.02	0.97
2731757	THAP6	1.15	0.03	0.37
3589458	THBS1	-1.19	0.00	0.79
3956854	THOC5	1.19	0.00	0.35
3816611	THOP1	-1.22	0.01	0.60
2708498	THPO	-1.15	0.00	0.50
3558012	TINF2	-1.13	0.01	0.42
3631214	TLE3	1.23	0.01	0.03
3176209	TLE4	1.29	0.02	0.23
3448481	TM7SF3	1.16	0.01	0.66
3502632	TMCO3	1.13	0.02	0.00
3182229	TMEFF1	1.65	0.01	0.18
3412345	TMEM117	1.18	0.01	0.38
3194613	TMEM141	-1.38	0.00	0.68
3987029	TMEM164	1.36	0.00	0.12
2526971	TMEM169	1.32	0.02	0.57
4025500	TMEM185A	1.19	0.00	0.42
3745525	TMEM220	-1.28	0.02	0.41

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3675116	TMEM8	-1.16	0.02	0.81
3449068	TMTC1	1.27	0.02	0.01
3425134	TMTC3	1.29	0.02	0.04
3645555	TNFRSF12A	-1.28	0.02	0.80
3481410	TNFRSF19	1.13	0.02	0.13
2956052	TNFRSF21	1.15	0.01	0.03
2444283	TNFSF4	-1.36	0.03	0.77
2599153	TNS1	-1.35	0.00	1.00
3748026	TOM1L2	1.15	0.02	0.07
3954331	TOP3B	1.14	0.01	0.76
3191147	TOR1B	1.12	0.01	0.27
3145149	TP53INP1	1.32	0.00	0.95
3597338	TPM1	1.12	0.02	0.04
3644541	TRAF7	-1.14	0.02	0.42
3677752	TRAP1	-1.13	0.02	0.90
3819104	TRAPPC5	1.18	0.01	0.64
2748061	TRIM2	1.56	0.01	0.57
3763656	TRIM25	1.14	0.00	0.66
3978579	TRO	1.28	0.01	0.21
2991150	TSPAN13	1.18	0.01	0.01
3634071	TSPAN3	1.11	0.00	0.01
3418492	TSPAN31	-1.11	0.02	0.94
3978169	TSPYL2	1.25	0.03	0.21
3891342	TUBB1	-1.27	0.00	0.65
3959593	TXN2	1.17	0.02	0.10
3217736	TXNDC4	1.21	0.01	0.51
2356115	TXNIP	1.26	0.01	0.95
3195139	UAP1L1	-1.30	0.01	0.93
2947877	UBD	1.50	0.01	0.24
3431143	UBE3B	1.13	0.00	0.38
2319560	UBE4B	1.14	0.00	0.08
3758967	UBTF	1.11	0.02	0.46
3486025	UFM1	1.14	0.00	0.19
3438527	ULK1	1.13	0.02	0.01
3770979	UNC13D	-1.17	0.02	0.99

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
2936731	UNC93A	-1.99	0.00	0.94
3379269	UNC93B1	-1.17	0.01	1.00
3597603	USP3	1.21	0.01	0.03
3685051	USP31	-1.10	0.02	0.78
3990566	UTP14A	1.18	0.03	0.21
2949380	VARS	-1.14	0.02	0.35
2818517	VCAN	1.38	0.02	0.05
3351806	VPS11	-1.14	0.01	0.10
3569374	VTI1B	1.28	0.02	0.84
2760371	WDR1	-1.10	0.03	0.77
3976716	WDR13	-1.24	0.01	0.61
3569926	WDR22	1.18	0.02	0.44
3933817	WDR4	-1.12	0.02	0.72
3699178	WDR59	1.12	0.00	0.00
3591704	WDR76	1.17	0.03	0.53
3722084	WNK4	1.11	0.03	0.84
3715109	WSB1	-1.25	0.00	0.00
3848437	XAB2	-1.13	0.02	0.84
3956589	XBP1	1.12	0.01	0.00
3687452	YPEL3	1.13	0.02	0.44
3075566	ZC3HAV1	1.29	0.01	0.06
3703665	ZCCHC14	1.18	0.02	0.30
3307120	ZDHHC6	1.25	0.01	0.12
3668898	ZFP1	1.33	0.01	0.72
3620590	ZFP106	1.15	0.02	0.18
3860410	ZFP82	1.35	0.03	0.51
3571248	ZFYVE1	1.17	0.02	0.01
3151473	ZHX1	1.24	0.01	0.11
3906062	ZHX3	1.17	0.01	0.68
3645881	ZNF174	1.25	0.03	0.98
3359751	ZNF195	1.31	0.03	0.33
3835544	ZNF227	1.24	0.02	0.10
3835467	ZNF234	1.23	0.00	0.76
3954525	ZNF280B	1.19	0.02	0.06
3902081	ZNF337	1.23	0.02	0.52

Transcript cluster ID	Gene	Gene Fold-Change (E/C)	Differential gene expression FDR	Differential Alternative splicing FDR
3129304	ZNF395	-1.13	0.01	0.39
3831698	ZNF420	1.23	0.01	0.01
3821410	ZNF440	1.38	0.02	0.32
3184896	ZNF483	1.89	0.02	0.40
3826601	ZNF493	1.31	0.00	0.17
3819968	ZNF559	1.16	0.00	0.33
3842301	ZNF581	-1.18	0.01	0.43
3856075	ZNF682	1.41	0.02	0.90
3840372	ZNF701	1.24	0.02	0.73
3851374	ZNF709	1.44	0.02	0.46
4023006	ZNF75D	1.40	0.02	0.46
2904528	ZNF76	-1.23	0.02	0.77
3908831	ZNFX1	1.17	0.02	0.68
3029129	ZYX	-1.21	0.00	0.62
3741875	ZZEF1	1.14	0.01	0.02

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Zhi, X., Chan, E.M., and Edenberg, H.J. (2000). Tissue-specific regulatory elements in the human alcohol dehydrogenase 6 gene. DNA and cell biology *19*, 487-497.

CURRICULUM VITAE

EDUCATION

Ph.D. in Biochemistry and Molecular Biology

2005 - 2010

Indiana University, Indianapolis, USA

Master of Professional Studies in Biochemistry and Molecular Biology

2001 - 2005

University of Maine, USA

Master of Science in Microbial Gene Technology

1999 - 2001

Madurai Kamaraj University, India

Bachelor of Science in Microbiology

1996 - 1999

Madras University, India

EXPERIENCE

Research, Indianapolis

- Identified and characterized an enhancer element upstream of alcohol dehydrogenase 4 (*ADH4*) gene.
- Studied the effects of single nucleotide polymorphisms in the regulatory regions of alcohol dehydrogenase genes, *ADH4* and *ADH1B*.
- Analyzed the effects of long term ethanol treatment on global gene expression and alternative splicing in human hepatoma cell line by microarray analysis.

Research, Maine

- Identified two partial Phosphoribulokinase gene fragments in the sea slug DNA, providing evidence of horizontal gene transfer from the alga, *Vaucheria litorea* to the mollusk, *Elysia chlorotica.*
- Studied the stability of plastid proteins in the alga and the sea slug.
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Teaching, Maine

- Taught undergraduate lab courses in Introductory Biochemistry, Fundamental Chemistry and Microbiology.
- Set up labs for multiple lab courses including Advanced Biochemistry, Introductory Biochemistry, Fundamental Chemistry, and Introductory Microbiology.
- Evaluated lab reports and examinations for each course.

Research, Madurai

- Cloned *yieL* gene from *Escherichia coli* K-12 MG1655 and characterized the function of its gene product as endo-b-1,4-xylanase.

PUBLICATIONS

- Pochareddy, S. and Edenberg, H.J. Identification of FOXA dependent enhancer of human Alcohol dehydrogenase 4 (*ADH4)*. *Gene (2010) 460: 1-7*
- Pochareddy, S. and Edenberg, H.J. Effects of long-term ethanol treatment on global gene expression in HepG2 cells (in preparation).
- Rumpho, M.E., Pochareddy, S., Worful, J.M., Summer, E.J., Bhattacharya, D., Pelletreau, K.N., Tyler, M.S., Lee, J., Manhart, J.R., and Soule, K.M. Molecular characterization of the calvincycle enzyme phosphoribulokinase in the Stramenopile alga *Vaucheria litorea* and the plastid hosting. *Molecular Plant (2009) 2 : 1-13*
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CONFERENCE ABSTRACTS

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- Pochareddy, S. and Edenberg, H.J. Identification and characterization of an enhancer in the *ADH* cluster. Mechanisms of Eukaryotic transcription, Cold Spring Harbor, 2009.
- Dastoor, F., Pochareddy, S., and Rumpho, M.E. Phosphoribulokinase in *Elysia chlorotica*. 4th International Symbiosis Congress, Halifax, 2003.