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# TGF-beta Receptors and Alcohol Sensitivity in Drosophila

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TGF-BETA RECEPTORS AND ALCOHOL SENSITIVITY IN *DROSOPHILA*


A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Human and Molecular Genetics at Virginia Commonwealth University


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# LIST OF ABBREVIATIONS

*baboon*..................................................................................................*babo*

*Chloride Intracellular Channel*..................................................................*Clic*

Loss of Function...........................................................................LOF

Neuromuscular Junction.............................................................NMJ

*punt*.............................................................................................*put*

*saxophone*...................................................................................*sax*

*thickveins*....................................................................................*tkv*

Transforming Growth Factor-Beta.............................................TGF-β

*wishful-thinking*.............................................................................*wit*

ABSTRACT


TGF-B RECEPTORS AND ALCOHOL SENSITIVITY AS MODELED IN *DROSOPHILA*

Kristyn N. Sennett, B.S.


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Clic proteins influence ethanol-related behavior in flies and other species and also

mediate TGF-β signaling. These findings suggest that Clics and the TGF-β signaling

pathway might work together to modulate behavioral responses to ethanol. I used the

*Drosophila* model to address the hypothesis that TGF-β signaling is important for

ethanol sensitivity. Ethanol sensitivity was blunted by multiple transposon insertions in

the TGF-β receptor gene *thickveins*. Collectively, however, I found no consistent

correlation between expression of *thickveins* and altered ethanol sensitivity in flies

harboring transposons. I therefore also assessed ethanol sensitivity in flies with loss of

function point mutations in *thickveins*. Ethanol sensitivity was not altered in these

additional *thickveins* genotypes, contrary to my major hypothesis. My analysis of

*thickveins* suggests that TGF-β signaling might influence ethanol sensitivity, but if so there must be a complex relationship between the function of this pathway and sensitivity to alcohol.

INTRODUCTION

*Alcohol Abuse*

Alcohol is a legal drug that has a depressant effect on the central nervous system. Consumption of alcohol results in an initial elated feeling, but drinkers might also experience reasoning impairment, sedation and slowed motor functions as well as slurred speech as they become increasingly intoxicated. It is estimated that over 90% of adults in America have had at least one encounter with alcohol consumption and that 44% of adults in America are drinkers (consume more than 12 drinks per year) (DHHS, 2000). Beyond the general social acceptance, people likely consume alcohol for other perceived benefits such as stress relief. However, the negative impact alcohol has on a person's health far outweigh the benefits. On a physiological level, it is well documented that moderate to heavy drinking can lead to liver cirrhosis, cancer, and high blood pressure leading to stroke and heart attack (DHHS, 2000). Furthermore, heavy drinking can lead to alcohol abuse, dependence and other mental health disorders associated with increased consumption of alcohol.

A person is considered an alcohol abuser when they drink excessively despite role impairment and other social issues (APA, 2000). If not treated, alcohol abuse can morph into alcohol dependence marked by the development of tolerance to alcohol and withdrawal symptoms when trying to quit (APA, 2000). Approximately 7.4% of adults

who consume alcohol in the United States are alcohol abusers or alcoholics (APA, 2000). The costs of alcohol abuse are staggering with hundreds of billions of dollars spent annually in the U.S. on effects of alcohol abuse (DHHS, 2000). However there are few effective treatments to combat alcohol abuse and none that address the underlying causes of the disease.

Alcohol abuse is considered a complex disease with both environmental and genetic components. While there is an environmental aspect, 50-60% of risk variance is thought to come from genetics (APA, 2000). The genetic component of this disease is also complex with what is thought to be a polygenic mode of inheritance with many genes of small effect size. In a longitudinal genetic study done by Schuckit (1994) evidence was found that men who were at risk of developing alcohol abuse due to a family history of alcohol abuse or dependence had a lower level of response to alcohol. That is to say that these men did not become intoxicated at the same doses as men without family histories for alcohol abuse. This has led to the development of the theory that if an individual is initially less sensitive to alcohol (i.e. has decreased level of response to alcohol) then that individual is at greater risk for developing alcohol abuse tendencies (Schuckit, 1994). The identification of this inverse relationship between sensitivity to alcohol and alcohol abuse has led to further genetic studies such as this one and different preventative techniques.

Ethanol has a wide variety of targets throughout the human body resulting in the biological and behavioral effects seen upon consumption of alcohol. The nervous system is the main target of these effects with GABA-A receptors, NMDA receptors, and nicotinic acetylcholin receptors (nAChR) being some of the more well characterized

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targets of ethanol (Spanagel, 2009). Recent studies have implicated cAMP (Moore et al., 1998), as well as GABA receptor genes (Dzitoyeva et al., 2003; Paul, 2006) and other genes such as *Chloride Intracellular Channel* (*Clic*) (Bhandari et al., 2012) in alcohol sensitivity. While there is strong evidence to support these genes being involved in the genetic pathways that impinge on alcohol sensitivity, these studies by no means have identified all the genes that are involved. The limited data on the biological pathways involved as well as the lack of effective treatment available provide the rationale for additional studies to uncover the genetic pathways involved in the behavioral responses to alcohol.

*Drosophila as a Model*

*Drosophila melanogaster* is a well-established genetic invertebrate model. It has been utilized in many studies on abused substances including alcohol. While it is an invertebrate species, it exhibits many of the same behavioral responses to alcohol as do mammals. An initial exposure to low levels of ethanol results in a hyperactive behavior in the fly, which is also observed in other species (Guarnieri and Heberlein, 2003). Higher concentrations of ethanol and longer exposures to ethanol result in the loss of postural control and sedation (Guarnieri and Heberlein, 2003). Repeated exposures to ethanol can lead to the development of tolerance similar to that observed in vertebrates (Scholz et al., 2000; Guarnieri and Heberlein, 2003; Mackay and Anholt, 2006). This behavioral conservation makes *Drosophila* an excellent tool for modeling responses to alcohol.

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Many assays have been developed to measure acute ethanol sensitivity in *Drosophila.* The Grotewiel lab developed an assay called ethanol Rapid Iterative Negative Geotaxis (eRING) that combined startle-induced climbing and ethanol exposure to measure the sedative effects of ethanol on negative geotaxis (Bhandari et al., 2009). I used this assay extensively to examine acute ethanol sensitivity. More details on eRING can be found in Chapter 1.

In addition to behavioral conservation, there is also significant genetic conservation between *Drosophila* and other species. Approximately 2/3 of all human disease genes are represented by obvious orthologs in *Drosophila* (Mackay and Anholt, 2006). Because of the genetic conservation observed, there is also conservation of biological pathways controlled by those genetics. The conservation observed on a molecular level in *Drosophila* allows for the powerful genetic analysis of pathways important in biology and disease. This conservation in *Drosophila* applies well to genes involved in ethanol sensitivity. For example, several genetic pathways such as those involving neuropeptide F, cAMP, and GABA receptors have been identified and well characterized as being involved in ethanol sensitivity in *Drosophila*  and other species (Rodan and Rothenfluh, 2010).

*Drosophila* are an advantageous model in other regards as well. Primarily, they are a cost effective model compared to other models such as mice. Additionally, their quick generation time (about 2 weeks) allows for quick propagation of offspring for experimentation. Their strongest advantage is the availability of many genetic reagents. Finally, as mentioned above, their behaviors are easily observed and assessed which is beneficial for this study.

Chloride intracellular channel and ethanol behavior

Chloride intracellular channels (CLICs) constitute a family of proteins with possible functions as chloride channels, regulators of TGF-β signaling and other biochemical processes. In mammals there are 6 CLIC (CLIC1-6) proteins, 2 in worms, and one in flies (Bhandari et al., 2012). The fly Clic protein has significant homology to Clic4 and other Clic proteins protein in mammals.

While the predicted function of CLIC as a channel protein is somewhat controversial, other studies have suggested additional functions of CLICs in other biological processes. These additional functions of CLIC include interactions with ryanodine receptors (Jalilian et al., 2008), 14-3-3 proteins (Suginta et al., 2001), binding to A-kinase anchoring proteins (AKAP) (Shanks et al., 2002) and involvement in the TGF-β signaling pathway (Shukla et al., 2009). Most recently a study across species identified CLIC as having a role in sensitivity to alcohol (Bhandari et al., 2012). Bhandari *et al* (2012) found that ethanol sensitivity was decreased in flies with attenuated *Clic* function and  that worms with loss of the two *Clic* genes had blunted sensitivity to ethanol as well as enhanced acute functional tolerance. Further they found that ectopic expression of *Clic4* had blunted ethanol induced ataxia (Bhandari et al., 2012).  These studies strongly suggest that the *Clic* family of genes plays a conserved role in ethanol sensitivity, although they do not address potential molecular mechanisms of Clic action. Interestingly, Shukla *et al.* (2009) showed that vertebrate CLIC4 functions in the Transforming Growth Factor Beta (TGF-β) signaling pathway by assisting in SMAD transcription factor function. Thus, it is possible that fly Clic also functions in this

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pathway and therefore that TGF-β signaling might be an important regulator of ethanol sensitivity.

*TGF-β Signaling*

TGF-β signaling has been implicated in a wide range of biological processes including growth, cell differentiation, adhesion and cell death (Massague, 1998). In *Drosophila* most studies on TGF-β signaling have focused on the larval neuromuscular junction (NMJ) during development. Its role in the NMJ has been found to assist in synapse development and function (Keshishian and Kim, 2004).

TGF-β signal transduction occurs in response to binding of a TGF-β ligand. In vertebrates as well as in invertebrates there are several different classes of ligands that have different functions depending on developmental timing and tissue (Massague, 1998). In *Drosophila*, there are two primary classes of ligands, Bone Morphogenetic Protein (BMP) and Activin. In the BMP class are the specific ligands decapentaplegic, screw and glass bottom boat which all participate in retrograde signaling at the NMJ (from muscle to presynaptic receptor) (McCabe et al., 2003; Keshishian and Kim, 2004; Gesualdi and Haerry, 2007; Bayat et al., 2011). The Activin class of TGF-β ligands includes *Drosophila* Activin, maverick, dawdle, and myoglianin which participate in anterograde signaling (neuron to post synaptic muscle receptor) (Gesualdi and Haerry, 2007; Bayat et al., 2011).

Ligands present in high enough quantities will dimerize and bind to their appropriate receptor complexes. Receptor complexes are the result of association of two Type I and two Type II transmembrane serine/threonine kinase receptors. In

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*Drosophila*, the Type I receptors are thickveins (TKV), baboon (BABO), and Saxophone (SAX) and the Type II receptors are wishful-thinking (WIT) and punt (PUT). Of these receptors, there is evidence that BMP ligands bind to the TKV, SAX, PUT and WIT receptor complexes on the neuron (Marques et al., 2002; McCabe et al., 2003; Keshishian and Kim, 2004) and the Activin ligands bind to BABO and PUT receptor complexes on the muscle (Figure 1) (Marques et al., 2002; Lee-Hoeflich et al., 2005).

Upon ligand binding (specifically the ligand binds to the type I receptors), the kinase function becomes activated by the cross phosphorylation of the cytoplasmic domains of the receptors (Type II receptors phosphorylate the Type I receptors which in turn phosphorylate the Type II receptors) (Massague, 1998). Once activated the Type I receptors are able to phosphorylate a series of downstream SMAD proteins in the cytoplasm. The SMAD phosphorylated by TKV and SAX in *Drosophila* is Mothers against decapentaplegic which is an R-SMAD (aka receptor-regulated SMAD) (Keshishian and Kim, 2004) (Figure 1). The SMADs phosphorylated by BABO is simply called dSMAD (aka *Drosophila* SMAD) (Keshishian and Kim, 2004) (Figure 1). Once SMADs are phosphorylated they participate in signal transduction by binding with a Co-SMAD and translocating into the nucleus where they are then able to bind to DNA and act as transcription factors. Vertebrate CLIC4 functions in the TGF-β signaling pathway by binding to a molecule called Schnurri-2 once the pathway has been activated (Shukla et al., 2009). Together, CLIC4 and Schnurri-2 translocate to the nucleus and once there dissociate. CLIC4 is then able to bind with SMADs in the nucleus and act with them as a transcription factor complex (Shukla et al., 2009).

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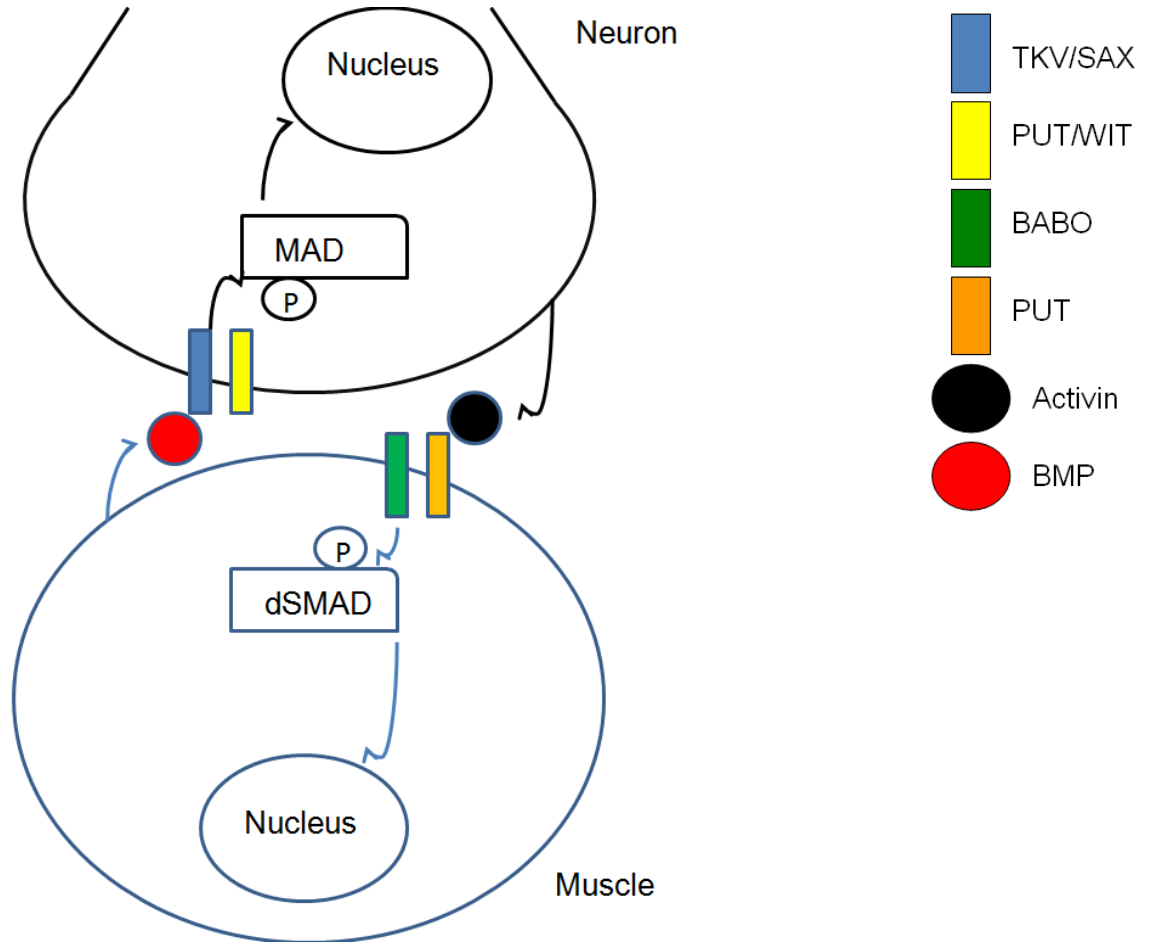
**Figure 1. TGF-β Signaling in the *Drosophila* Neuromuscular Junction. Adapted from Bayat et. al, 2012.**

*Aim of this Thesis*

The global hypothesis for this study is that TGF-β receptors influence acute sensitivity to ethanol. Therefore the primary aim of this thesis is to characterize the TGF-β receptor gene *tkv* in *Drosophila* ethanol sensitivity. Additionally, I performed a preliminary genetic analysis of two additional TGF-β receptor genes, *babo* and *wit*, in ethanol sensitivity. To address these aims, I molecularly characterized several transposon insertions in the TGF-β receptor genes and then assessed the effects of the transposon insertions on gene expression. Additionally, I assessed ethanol sensitivity in *tkv*, *wit* and *babo* transposon insertion lines and explored whether previously characterized point mutations in *tkv* or RNAi-mediated knock-down of this gene altered ethanol sensitivity. Finally, I also determined whether internal alcohol concentrations were altered in *tkv* transposon strains. The global prediction from my major hypothesis is that mutations that disrupt the normal transcription and function of these TGF-β receptor genes will blunt acute ethanol sensitivity.

CHAPTER 1

**Materials and Methods**

*Detailed protocols can be found in the Appendix.

*1.1 Fly Husbandry, Fly Strains, and Genetics*

Flies were reared on standard *Drosophila* medium (10% sucrose, 2% yeast, 3.3%

cornmeal, 1% agar, 0.2% Tegosept) supplemented with active yeast at 60% relative

humidity and 25°C under a 12-hour light/dark cycle. All flies carrying *thickveins* (*tkv*),

*wishful-thinking* (*wit*) and *baboon* (*babo*) transposons (Table 1) were backcrossed for

seven generations to *w[A]* (the laboratory's standard stock containing the $w^{1118}$ allele)

by selecting for the w+ eye color marker in the transposons (Thomas and Grotewiel,

unpublished). Homozygous lethal *tkv* mutant chromosomes were maintained *in trans* to

the CyO balancer chromosome. $tkv^7$ and $tkv^8$ (Table 1), previously characterized point

mutations at two different locations in *tkv*, were moved into the *w[A]* genetic background

by backcrossing for 7 generations to d07811 homozygous transposon insertion flies that

had been previously backcrossed to *w[A]* as described above. *tkv7* and *tkv8* were

tracked through the backcross by selecting for $tkv^7$/d07811 or $tkv^8$/d07811 based on eye

color.

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**Table 1. TGF-β Receptor Genetic Manipulations.** Each allele was backcrossed to $w^{1118}$ for seven generations.

| Gene | Allele | Class | Location | Confirmed? | Source | Viability |
|------|--------|-------|----------|------------|--------|-----------|
| *thickveins* | d07811 | P-element transposon | 2L:5237460 | yes | Harvard/L. Thomas | homozygous viable |
| | f02766 | PBac transposon | 2L:5221749 | yes | Harvard/L. Thomas | homozygous viable |
| | KG05071 | P-element transposon | 2L:5259281 | no | Bloomington/L. Thomas | homozygous lethal |
| | c06013 | PBac transposon | 2L:5237496 | yes | Harvard/L. Thomas | homozygous lethal |
| | f03305 | PBac transposon | 2L:5234131 | yes | Harvard/L.Thomas | homozygous lethal |
| | 8 | LOF point mutation | 2L:5221353 (C>A) | yes | Bloomington | homozygous lethal |
| | 7 | LOF point mutation | 2L: 5220203 (G>A) | yes | Bloomington | homozygous lethal |
| *wit* | d02492 | P-element transposon | 3L:4071839 | yes | Harvard/L. Thomas | homozygous viable |
| | e01243 | PBac transposon | 3L:4071615 | yes | Harvard/L. Thomas | homozygous viable |
| | e00566 | PBac transposon | 3L:4064700 | yes | Harvard/L. Thomas | homozygous viable |
| *baboon* | k16912 | P-element | 2R:4840513 | yes | Bloomington/L. Thomas | homozygous lethal |
| | c050710 | PBac transposon | 2R:4841108 | yes | Harvard/L. Thomas | homozygous lethal |
| | c04236 | PBac transposon | 2R:4846414 | yes | Harvard/L. Thomas | homozygous lethal |

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All UAS lines used in RNAi experiments were in the Vienna *Drosophila* Research Center (VDRC) standard background containing a $w^{1118}$ mutation (Table 2). Gal4 lines, *Mef2 Gal4* and *Actin/CyO-* were backcrossed to *w[A]* for seven generations as above (Thomas and Grotewiel, unpublished). To control for genetic background, progeny from Gal4 flies crossed to w[VDRC] and UAS flies crossed to *w[A]* were used for eRING experiments.

**Table 2. UAS-RNAi and GAL4 Lines.** UAS lines were in the w[VDRC] background and GAL4 lines were backcrossed to $w^{1118}$.

| Allele | IR Target Site | | Viability |
|--------|----------------|---|-----------|
| 3059 | 5221k-5221.2k | ::actin-Gal4 | viable |
| | | ::mef2-Gal4 | viable |
| 105834 | 5219.8k-5220.3k | ::actin-Gal4 | viable |
| | | ::mef2-Gal4 | adult lethal |

## 1.2 Transposon Insertion Confirmation

All transposon insertions were confirmed prior to and after backcrossing. Flies were collected under anesthesia ($CO_2$) and then killed by freezing at -20°C. Genomic DNA was isolated using a revised version of the Qiagen[TM] DNAeasy Blood and Tissue (250) kit. Confirmation of transposon insertions was carried out via standard PCR with 200ng of genomic DNA and primers specific to each transposon insertion location (Table 3). PCR reactions were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining.

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**Table 3. PCR primers for Allele Confirmation.** Primers used to confirm location of transposon insertions and point mutations.

| Gene | Allele | Primer ID | Primer Sequence |
|---|---|---|---|
| *Thickveins* | d07811 | MSG86 | 5'- CGACGGGACCACCTTATGTTATTTCATCATG -3' |
| | | VCU233 | 5'- CGTATCGACGAATGTGCAAC -3' |
| | f02766 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU236 | 5'- GTCTTTGGGAAGCGTAAAG -3' |
| | KG05071 | MSG86 | 5'- CGACGGGACCACCTTATGTTATTTCATCATG -3' |
| | | VCU239 | 5'- CCGGAGGTGTGAAGAAAAG -3' |
| | c06013 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU233 | 5'- CGTATCGACGAATGTGCAAC -3' |
| | f03305 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU237 | 5'- GTAGCGCCGTGCCATATAG -3' |
| | 8 | VCU355 | 5'- CAGCATAAACACGGACAGGG -3' |
| | | VCU357 | 5'- GTGAGTCCCCTATTAATCCATAC -3' |
| | 7 | VCU350 | 5'- GGTCGGAACTGCGTATTCAAC -3' |
| | | VCU352 | 5'- CCGATACCATCACGCTGGC -3' |
| *wishful-thinking* | e00566 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU246 | 5'- CTGTCACAGAAATCACACATC- 3' |
| | e01243 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU248 | 5'- GCTACGATGCCTTGCTCTC -3' |
| | d02492 | MSG86 | 5'- CGACGGGACCACCTTATGTTATTTCATCATG -3' |
| | | VCU243 | 5'- GTTTTGCCGTTTGACTTTTC- 3' |
| *Baboon* | k16912 | MSG86 | 5'- CGACGGGACCACCTTATGTTATTTCATCATG -3' |
| | | VCU210 | 5'- GCAGAGCAGTTTTTCCAACC -3' |
| | c05710 | PBac3' | 5'- CCTCGATATACAGACCGATAAAACACAT -3' |
| | | VCU212 | 5'- GGACATGCTTATCAGTCGCC -3' |
| | c04236 | PBac5' | 5'- CAGTGACACTTACCGCATTGACAAGCAC -3' |
| | | VCU214 | 5'- TTTGGATTTGGCTTGTTTCC -3' |

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## 1.3 tkv$^7$ and tkv$^8$ Mutation Confirmation

The presence of the point mutations in tkv$^7$ and tkv$^8$ were confirmed prior to and after backcrossing into the w[A] background. Genomic DNA was isolated and standard PCR was performed as above. Primers were designed to flank the point mutations by at least 50 base pairs (Table 3). Amplification was confirmed by agarose gel electrophoresis. The PCR samples were treated with ExoSap-IT to eliminate remaining primers and dNTPs from the PCR reaction. Treated PCR samples were sequenced at ACGT Inc. (Chicago, IL), and analyzed using Applied Biosystems Sequence Scanner v1.0 (© 2005).

## 1.4 mRNA Isolation and cDNA Synthesis

Flies were collected under anesthesia, frozen at -20$^{\circ}$C, and then homogenized in Trizol$^{®}$ (Invitrogen #15596-018). An mRNA pellet was isolated, washed with 75% EtOH, dried at room temperature, reconstituted with DEPC water, placed at 4$^{\circ}$C overnight and stored at -20$^{\circ}$C until needed. The mRNA concentration was measured using spectrophotometry (Ultraspec 200 Pharmcia Biotech).

Reverse transcription was performed by adding 6ug (total volume of 17uL) of mRNA to a DNAse enzyme (Invitrogen$^{TM}$ #AM1906). After treatment with DNAse, the DNAse enzyme was inactivated by a DNAase inactivation agent (Invitrogen$^{TM}$ #AM1906). cDNA synthesis was then carried out by Oligo(dT) (Invitrogen$^{TM}$ #58862), DTT (Invitrogen$^{TM}$ #Y0147),1$^{st}$ Strand Buffer (Invitrogen$^{TM}$ #Y02321), dNTPs, and Superscript II$^{®}$ enzyme (Invitrogen$^{TM}$ #91681). All cDNA samples underwent a quality control check to confirm that there was minimal genomic DNA present by standard PCR

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with primers VCU86 (5'- CACGGGAAAACACTGGCAATC -3') and VCU87 (5'-
CTGGCCCAGATCAGAGGTT -3') to detect *Doc3* .


*1.5 Quantitative Real Time PCR*

Quantitative real time PCR (qRT-PCR) was performed on an Applied

Biosystems[TM] 7500 Fast Real-Time PCR System to determine expression of the TGF-β

receptor gene of interest. Prior to examining expression levels, all primer sets (Table 4)

were tested to ensure (1) proper amplification of cDNA serial dilutions and (2)

appropriate dissociation characteristics. Primers found to have slopes in the cDNA

dilutions of ~-3.5 and single dissociation peaks were used to measure expression levels

of the TGF-β receptor genes (Figure 2) in cDNA diluted 1:4. Diluted cDNA was added to

ddH$_2$O, SYBER green (Quanta Biosciences), and primers from a 3pmol/μL working

concentration in triplicate. Data were analyzed with 7500 Fast System software v1.3.1

(Applied Biosystems © 2001-2004).  Relative expression of the TGF-β receptor genes

was determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

**Table 4. qRT-PCR Primers**

| Gene | Primer ID | Primer Sequence |
|------|-----------|-----------------|
| *thickveins* | VCU310 | 5'- CGAGGAGGAGCGTACATATG -3' |
| | VCU311 | 5'- GAAGACAACGGTGGTTTTCTC -3' |
| | VCU312 | 5'- TCGCAGCAGACAATGTTCTTG -3' |
| | VCU313 | 5'- CAGGTCACGGTTGCAGAA G -3' |
| *wishful-thinking* | VCU204 | 5'- GCCCAGATGGCTACACTTTC -3' |
| | VCU205 | 5'- GACTGGCTGCAAATGGAAG -3' |
| | VCU206 | 5'- GCGCTGTTGAGCTATTTCG -3' |
| | VCU207 | 5'- GCATTCGCTGAAAGTTAGGG -3' |
| *baboon* | VCU258 | 5'- AACCTGATCCCAGCATTGAG -3' |
| | VCU259 | 5'- GGGATACCAGCACTCCTTCA -3' |
| | VCU260 | 5'- GGTGGTTTGCATCGAGAAGT -3' |
| | VCU261 | 5'- TGGGATACCAGCACTCCTTC -3' |
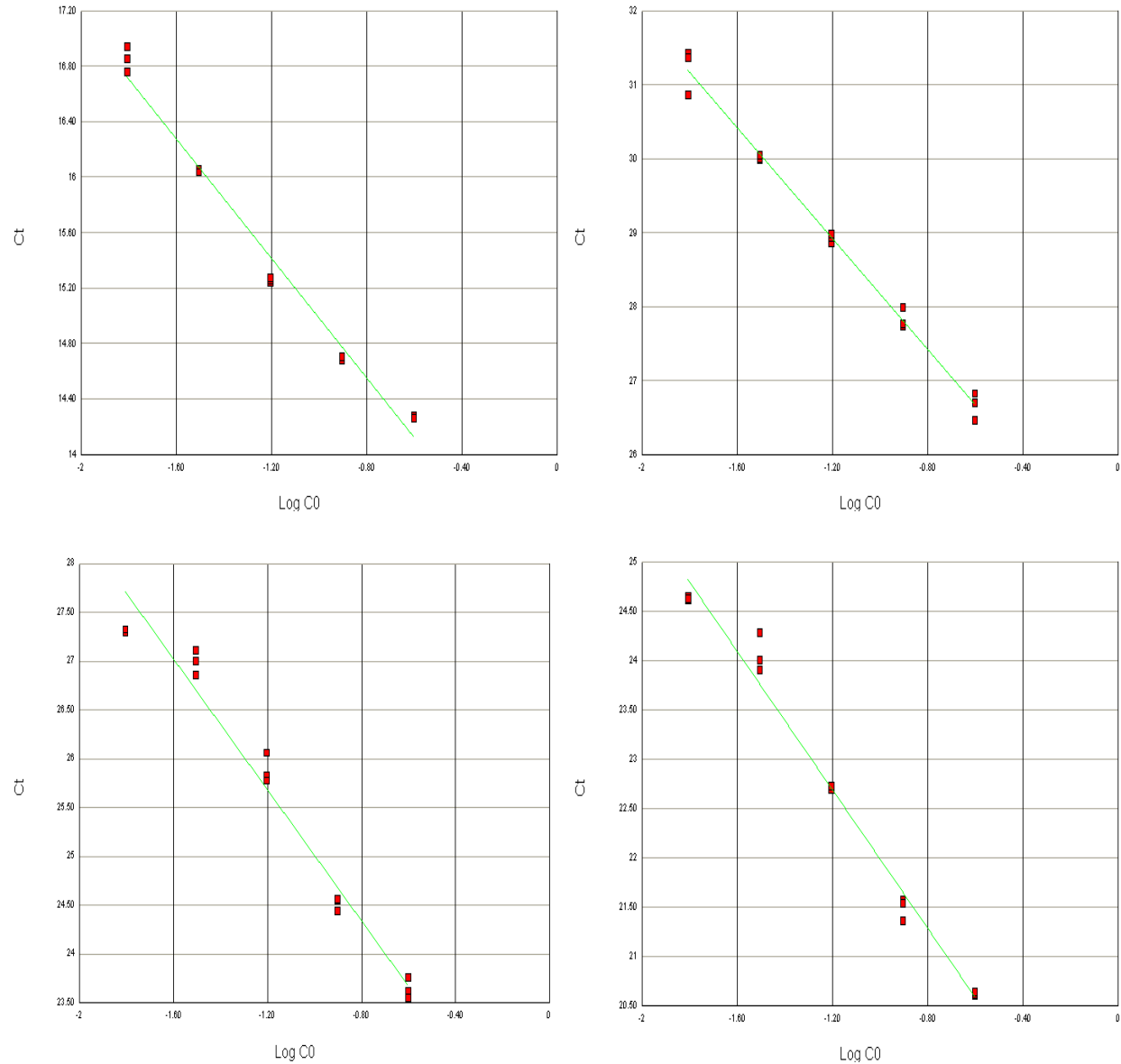
**Figure 2. Standard Curves of Primers Used in qRT-PCR for Each Gene and Control.** (A.) *Actin* primers VCU45 and VCU46, slope= -3.16, r2= 0.98. (B.) *tkv* primers VCU310 and VCU312, slope= -3.75, r2= 0.99. (C.) *wit* primers VCU205 and VCU207, slope= -3.35, r2= 0.97. (D.) *babo* primers VCU258 and VCU259, slope= -3.5, r2= 0.98

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## 1.6 Ethanol Rapid Iterative Negative Geotaxis

To test sedation in response to acute exposure to ethanol, flies were tested using the ethanol Rapid Iterative Negative Geotaxis assay (Bhandari et al., 2009). In this assay, groups of flies are stimulated to climb (startle-induced negative geotaxis) in the presence of ethanol. Ethanol time-dependently and dose-dependently inhibits climbing behavior. Groups of 25 female flies, approximately 3-5 days old, of a single genotype were collected under brief $CO_2$ anesthesia and placed in individual non-yeasted food vials. Flies were allowed to recover from anesthesia at 25°C and 60% humidity overnight prior to behavioral testing.

Base line negative geotaxis was determined by a water test in which flies were placed in vials containing a cotton plug with 500μL of dd$H_2O$. Each vial was placed in a rig in between a camera and a light source (Figure 3 modified from Bhandari, Bettinger et al. 2009). The rig containing the vials of flies was rapidly banged against the table 3 times to induce negative geotaxis behavior. A picture was taken (Canon Power Shot G3 Digital Camera) after 4 seconds to allow time for the flies to climb up the sides of the vials. This was repeated after a one minute rest period twice. Ethanol exposure occurred the same way as above except that 20% EtOH was added to the cotton flug. Ethanol tests were run under continuous exposure to EtOH for 20 minutes.
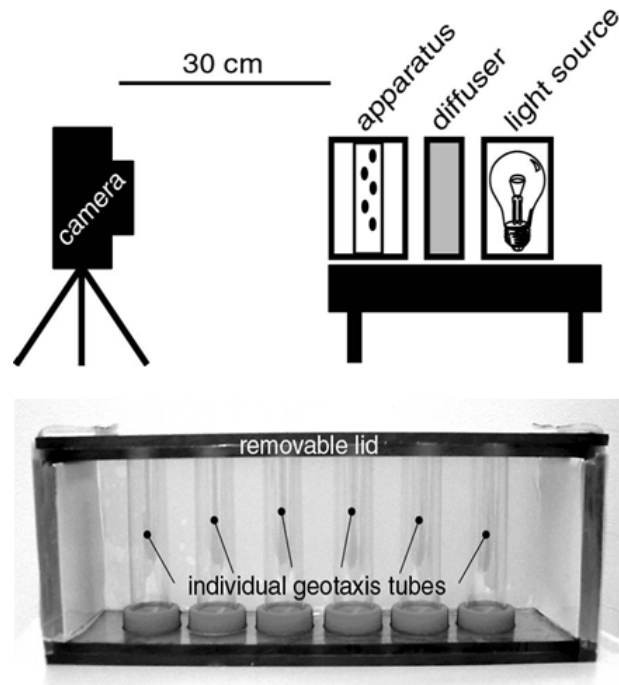
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**Figure 3. ethanol Rapid Iterative Negative Geotaxis.** (Above) Depiction of eRING

set-up with camera in front of an apparatus holding vials that contain flies, a cotton plug,

and water or ethanol vapor. (Below) Apparatus containing vials that during testing hold

flies with either water or ethanol vapor.

Pictures of flies from eRING studies were prepared for analysis using Adobe ® Photoshop ® 5.0 (© 1989-1998). Pictures were then analyzed using Scion Image (B 4.0.2 © 2000). For each time point of each individual vial tested, negative geotaxis (cm) values were calculated in Microsoft Excel from the data points extracted using Scion Image. T50 values (the time required for ethanol to inhibit negative geotaxis by 50%) were interpolated using a 3$^{rd}$ order polynomial calculation in GraphPad Prism v4.02 (© 1992-2004).

*1.7 Internal Ethanol Content*

To test any pharmacokinetic differences in the various genotypes tested, internal ethanol content was examined at 0 (water), 5 and 10 minutes of ethanol (20%) exposures in eRING assays using female flies collected and recovered as described above. After exposure to ethanol, each set of flies was frozen at -20°C until homogenized in ice-cold ddH$_2$O and centrifuged at maximum speed for 20 minutes. An aliquot of the resulting supernatant was added to alcohol reagent (Pointe Scientific Inc, #A7504-150) and incubated at 30°C for 5 minutes. Absorbance at 340 nm was taken for each sample in triplicate and preliminary internal ethanol concentrations were determined from linear regression/interpolation from a standard curve. Fly volumes were determined by weighing 25 female flies before and after drying at 55°C for up to 24 hours. Final ethanol concentrations were calculated from the volume of water in flies as follows: mM interpolation x (3/10) x (200uL / [# of flies x uL/fly]).

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CHAPTER 2

**Molecular and Behavioral Characterization of the TGF-β Receptor Gene, *tkv*, in Ethanol Sensitivity.**

*Introduction*

Vertebrate Clic proteins are required for TGF-β signaling in cardiomyocytes (Shukla et al., 2009) and preliminary studies in our laboratory suggested that RNAi-mediated knockdown of the Type I TGF-β receptor gene *thickveins* (*tkv*) blunts ethanol sensitivity in flies (data not shown, Bhandari and Grotewiel, unpublished). Thus, I hypothesized that *Clic* might influence ethanol sensitivity via its participation in TGF-β signaling. I chose to investigate this potential mechanism by exploring the role of TGF-β receptors in ethanol sensitivity. The simplest prediction from my hypothesis is that decreased *tkv* expression will blunt acute sensitivity to ethanol. Such a change in ethanol sensitivity in response to altered TGF-β signaling would be consistent with our proposed mechanism of CLIC action in ethanol sensitivity (Bhandari et al., 2012).

*Transposon Insertion Mutants in tkv*

Prior to genetic analysis of the TGF-β receptor *tkv* in acute ethanol sensitivity, I obtained several transposon insertions in *tkv* (Table 1, Figure 4). To confirm the positions of the transposon insertions, I performed standard PCR on genomic DNA

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isolated from each strain using primers that flanked the reported positions of the transposons in combination with a primer that annealed to the terminal repeats with each transposon (Table 1, Figure 4). I confirmed the positions of four transposon insertions (d07811, f02766, f03305, and c06013) (Figure 4). Despite vigorous attempts with multiple primer pairs, I was unable to confirm the location of KG05071 and it formally remains unmapped. I am confident, however, that this transposon insertion disrupts *tkv* function based on my assessment of complementation for viability and *tkv* expression (see below). All transposon insertions were backcrossed for seven generations to *w[A]*, our standard laboratory reference stock, to control for genetic background effects in our experiments (Thomas and Grotewiel, unpublished).
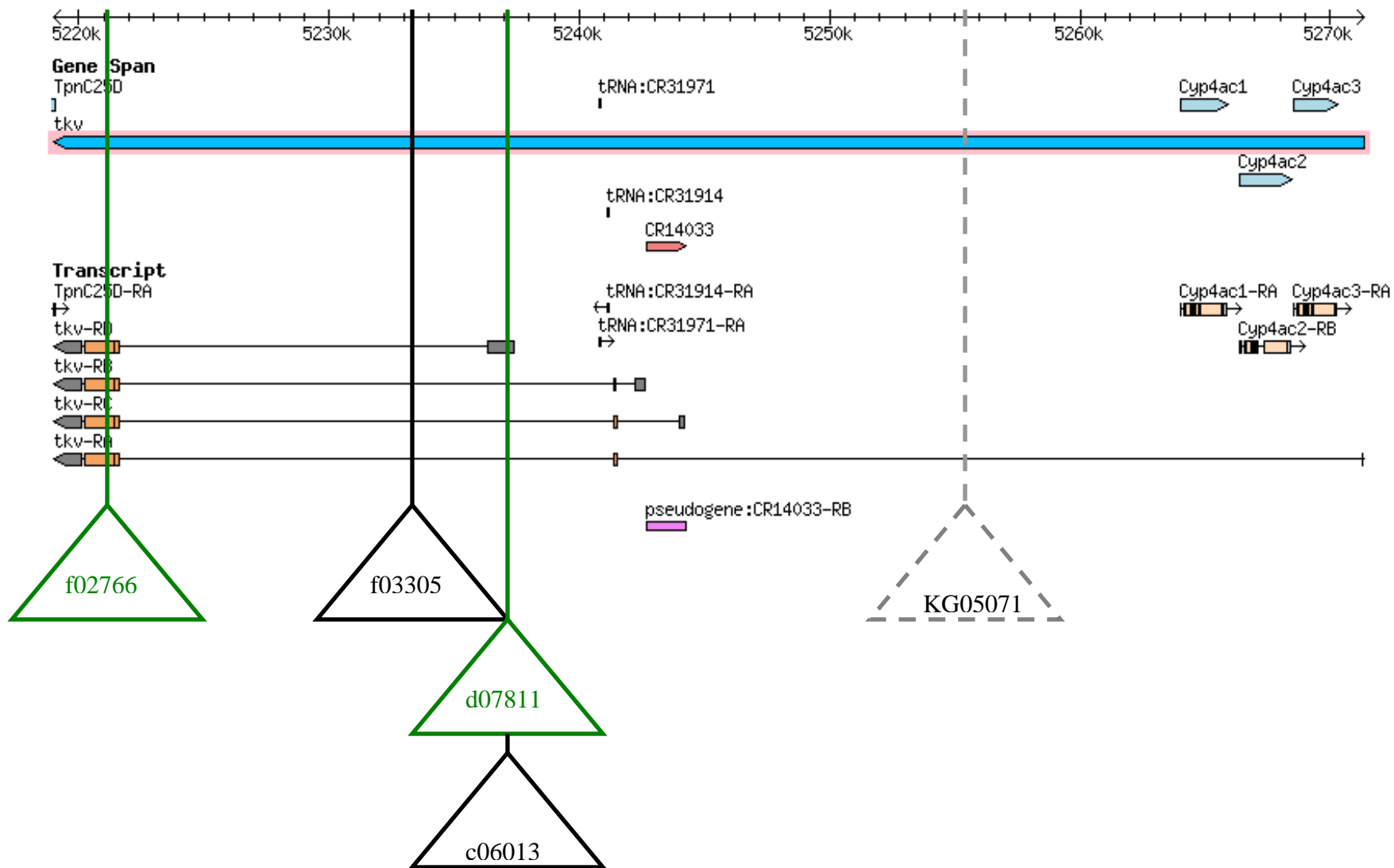
**Figure 4. Mapped Transposons in the *thickveins* Gene.** All transposons confirmed at the predicted positions except KG05071. Green indicates homozygous viable, black indicates homozygous lethal.

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To assess my major hypothesis, I analyzed *tkv* expression and acute ethanol sensitivity in parallel in all *tkv* transposon mutants. I determined *tkv* expression by quantitative Real-Time PCR (qRT-PCR) for each transposon insertion line. Total expression of *tkv* was significantly decreased in c06013/+ and KG05071/+ heterozygotes (23.33% and 61.2% respectively), but not in the f03305/+ heterozygote or in d07811/d07811 and f02766/f02766 homozygotes compared to *w[A]* control flies (defined as 100%) (Figure 5a, Table 7).

I assessed acute ethanol sensitivity in ethanol Rapid Iterative Negative Geotaxis (eRING) assays (Bhandari et al., 2009) using 20% ethanol. eRING assays measure bang-induced climbing in the continuous presence of ethanol vapor. By determining T50 values (the time required for climbing to be inhibited 50% by ethanol) from these studies, I can determine their acute sensitivity to ethanol. Typically, *w[A]* control flies exhibit T50 values of 5-8 minutes under these conditions. Flies homozygous for the transposon insertions d07811 and f02766 (Figure 6a and b) or heterozygous for KG05071 (Figure 6c) had a significant increase in T50 values, indicating that these transposon lines are less sensitive to ethanol than control animals. T50 values were unchanged, however, in other flies heterozygous for the transposons c06013 and f03305 compared to controls (Figure 6d and e). Thus, ethanol sensitivity is blunted in flies harboring some, but not all, transposon insertions evaluated.

24
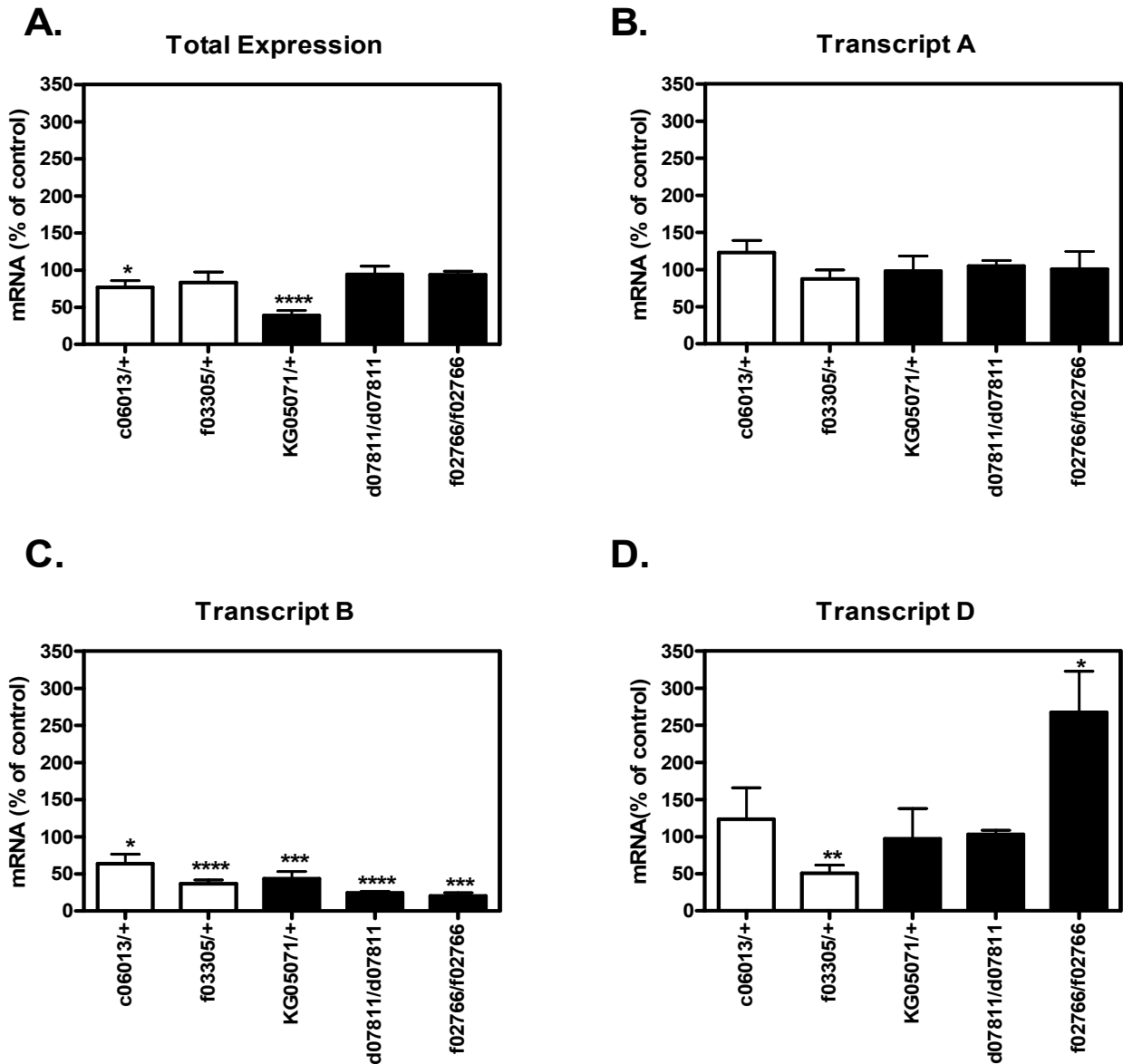
**Figure 5. qRT-PCR Analysis of *tkv* Expression in Simple Heterozygous and Homozygous Transposon Insertion Strains.** (A.) Total expression of all transcripts of *tkv* . (B.) Expression of *tkv* transcript A. (C.) Expression of *tkv* transcript B. (D.) Expression of *tkv* transcript D. One sample t-test, * p = 0.05-0.01, ** p-value = 0.009-0.005, *** p-value= 0.004-0.001,**** p-value= 0.0009- <0.0001. Filled bars indicate genotypes with blunted ethanol sensitivity.

25
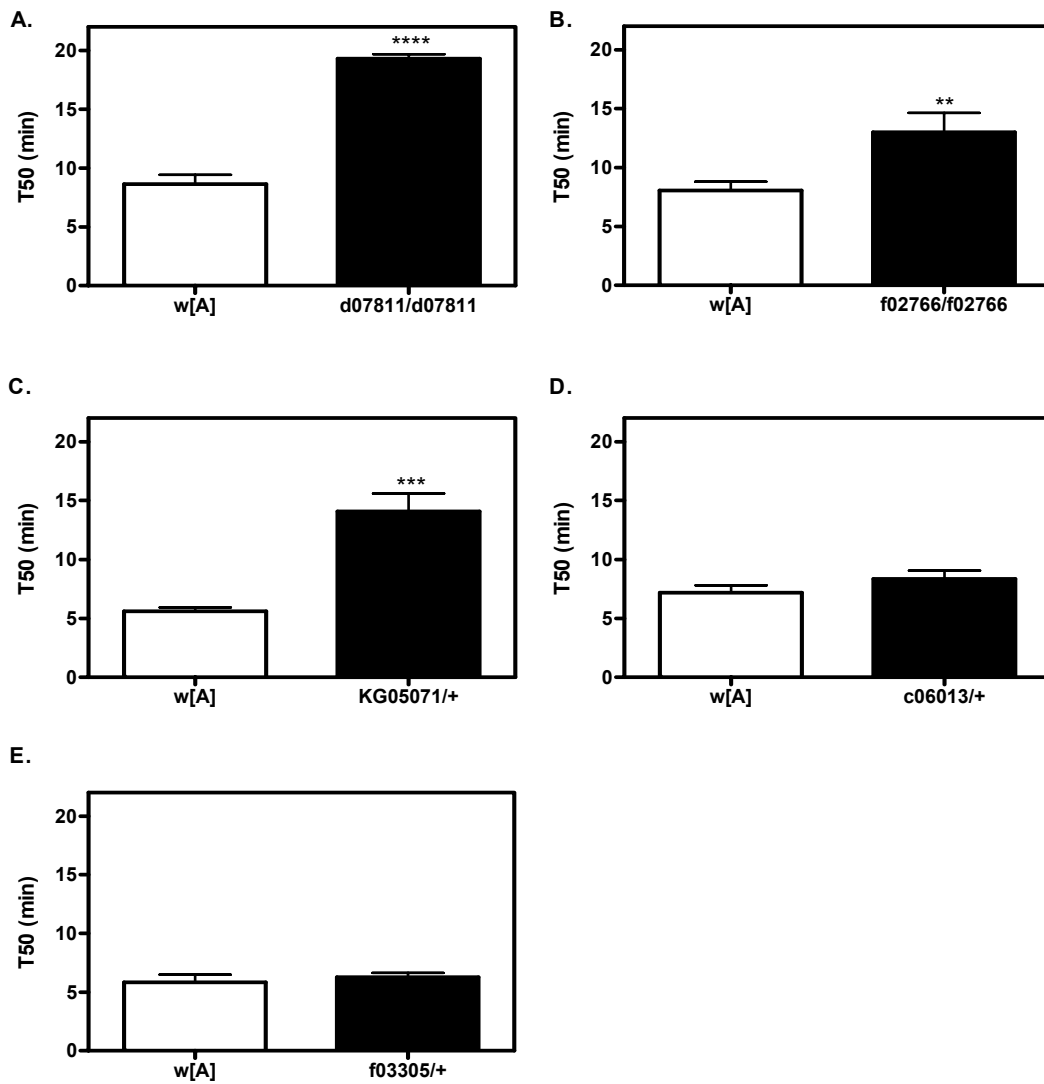
**Figure 6. Ethanol Sensitivity in Simple Heterozygous and Homozygous *tkv***

**Transposon Insertion Strains.** Ethanol sensitivity (T50) values in control *w[A]* (open bars) and *tkv* transposon lines (closed bars). T50 values are increased in (A) d07811 homozygotes (t test, p<0.0001), (B) f02766 homozygotes (p=0.0054) and (C) KG05071/+ heterozygotes (p= 0.001) compared to *w[A]* control flies. T50 values in (D) c06013 and (E) f03305 heterozygotes were unchanged relative to *w[A]* controls. n= 8-15.

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The increased T50 values in 3 independent strains with transposon insertions in *tkv* suggest that this gene might influence acute ethanol sensitivity. I note, however, that total expression of *tkv* does not appear to correlate with ethanol sensitivity. Specifically, KG05071/+ heterozygotes and d07811 and f02766 homozygous flies have blunted ethanol sensitivity, but only KG05071/+ flies have decreased total expression of *tkv*. Further, although c06013/+ had a significant decrease in total *tkv* expression, ethanol sensitivity was not substantially altered in this genotype. If *tkv* influences ethanol sensitivity, therefore, this affect cannot be explained by changes in total *tkv* expression measured by qRT-PCR.

There are four (A-D) predicted transcripts from the *tkv* locus. I therefore hypothesized that there might be a pattern of changes in expression of the individual *tkv* transcripts that correlates with the altered ethanol sensitivity in transposon insertion genotypes. To measure expression of each transcript individually, we developed qRT-PCR assays using isoform-specific primers for transcript A, B and D (data not shown, Chan and Grotewiel, unpublished). Transcript C was not consistently detected in multiple experiments using several different primer pairs, indicating that *tkv*-C is not highly expressed in adults or that none of the primers used to detect this transcript work well. Given the difficulty of detecting transcript C, expression of this isoform was not considered further.

Expression of transcript A was not altered in any of the transposon lines (Figure 5b), while transcript B was significantly decreased in all transposon lines tested (Figure 5c). Transcript D was decreased in f03305/+ flies only (Figure 5d). Taken together the qRT-PCR results indicate that there is differential expression among the isoforms but

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not necessarily among the mutants. More importantly, I found no clear pattern of change in expression of *tkv* isoforms that explained the blunted sensitivity to acute ethanol.

It seemed possible that ethanol uptake or metabolism might be altered in *tkv* transposon insertion lines and that the resulting changes in internal ethanol concentrations might explain the T50 values found in these animals. To measure internal ethanol content, flies were exposed to either water (0 min time point) or 20% ethanol for 5 or 10 minutes. As expected, internal ethanol concentrations increased with time of ethanol exposure (Figure 7). I found no differences in internal ethanol concentration in control *w[A]* and *tkv* transposon insertion strains (Figure 7). Altered internal ethanol content, therefore, cannot explain the presence or absence of altered sensitivity to acute ethanol in *tkv* transposon strains.
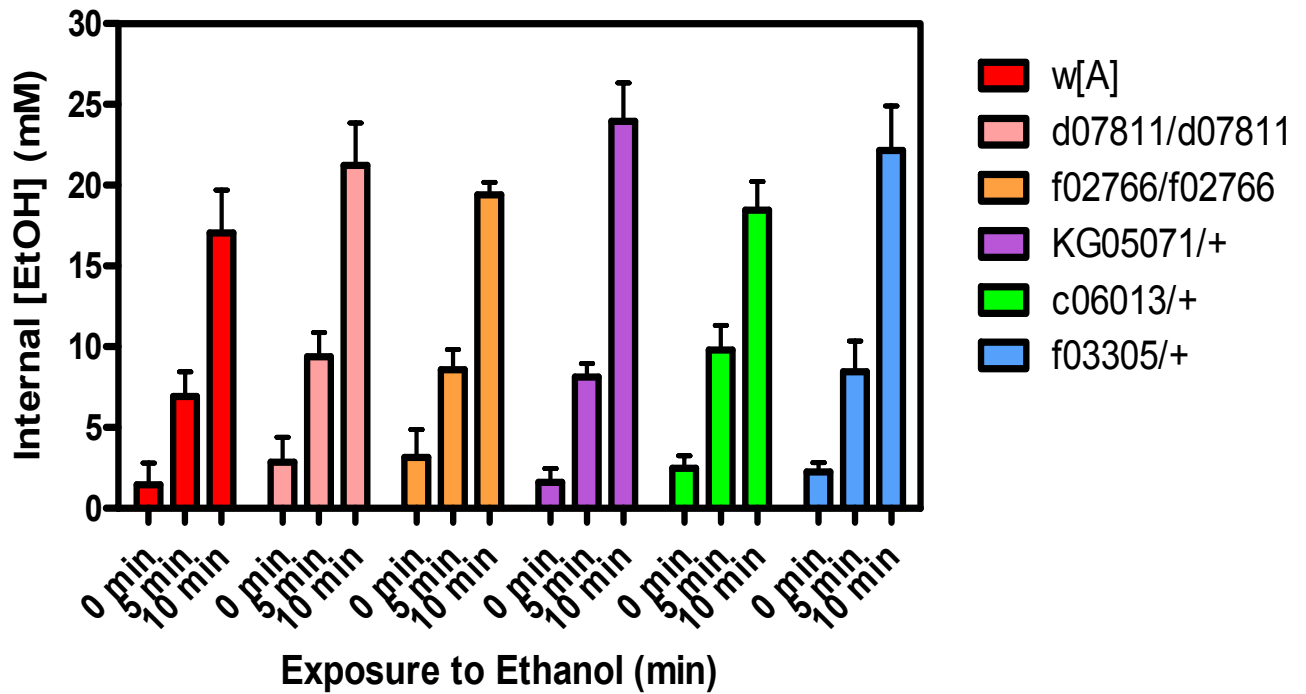
**Figure 7. Internal Ethanol Content of Simple Heterozygous and Homozygous Transposon Strains.** Time (p<0.0001), but not genotype (n.s.), influenced internal ethanol content (two-way ANOVA).

*Complementation for Ethanol Sensitivity*

Genetic complementation is a powerful approach to determine whether a locus plays a role in a particular process or phenotype. I exploited this approach to further probe the possible connection between *tkv* and sensitivity to acute ethanol. Complementation works best with recessive alleles and I consequently excluded KG05071 from the behavioral portion of these studies because it had a dominant effect on ethanol sensitivity (Fig 5c).

I first determined all combinations of the transposon insertions that complemented for viability.  Many combinations of the *tkv* alleles *in trans* were not viable (i.e. noncomplementation, Table 5). The combination of molecular mapping (Table 1, Figure 4), gene expression changes (Figure 5) and these complementation studies strongly indicate that transposons KG05071, c06013 and f03305 are all loss of function alleles of *tkv*.  Importantly, f03305 and c06013 were viable *in trans* to d07811, raising the possibility that I can use these allelic combinations in complementation analyses to further probe the role of *tkv* in ethanol sensitivity.

Table 5. Complementation for Viability in *tkv* Transposon Insertions

|  | c06013 | f03305 | d07811 | 7 | 8 |
|---|---|---|---|---|---|
| KG05071 | failed | failed | full | failed | failed |
| c06013 |  | failed | full | failed | failed |
| f03305 |  |  | full | failed | failed |
| d07811 |  |  |  | full | full |

I next hypothesized that f03305 and c06013 *in trans* to d07811 would not complement for ethanol sensitivity and *tkv* expression. c06013/d07811 and f03305/d07811 both exhibited a significant decrease in total *tkv* expression compared to *w[A]* controls (Figure 8a). I also examined the expression of individual *tkv* isoforms as part of this series of complementation experiments. Expression of *tkv*-A was not significantly changed in f03305/d07811 or c06013/d07811, while *tkv*-B was decreased in both transheterozygotes (Figure 8b and c). *tkv*-D was not changed in c06013/d07811, but this isoform was decreased in f03305/d07811 (Figure 8d). These qRT-PCR studies confirm that c06013/d07811 and f03305/d07811 are partial loss of function genotypes.

To determine whether f03305 and c06013 complemented d07811 for acute ethanol sensitivity, I compared T50 values from eRING studies using 20% ethanol in *w[A]* controls, flies heterozygous for f03305, c06013 and d07811, and in flies with f03305 and c06013 *in trans* to d07811. T50 values were significantly greater in f03305/d07811 flies compared to *w[A]* controls and f03305 and d07811 heterozygotes (Figure 9a). Similarly, c06013/d07811 also exhibited a blunted sensitivity to ethanol when compared to *w[A]* control flies, but T50 values in this transheterozygous combination were not significantly different than c06013 and d07811 heterozygotes (Figure 9b). The simplest interpretation of these data is that f03305 and c06013 do not complement d07811 for sensitivity to acute ethanol and therefore that *tkv* is involved in this behavioral response to ethanol.
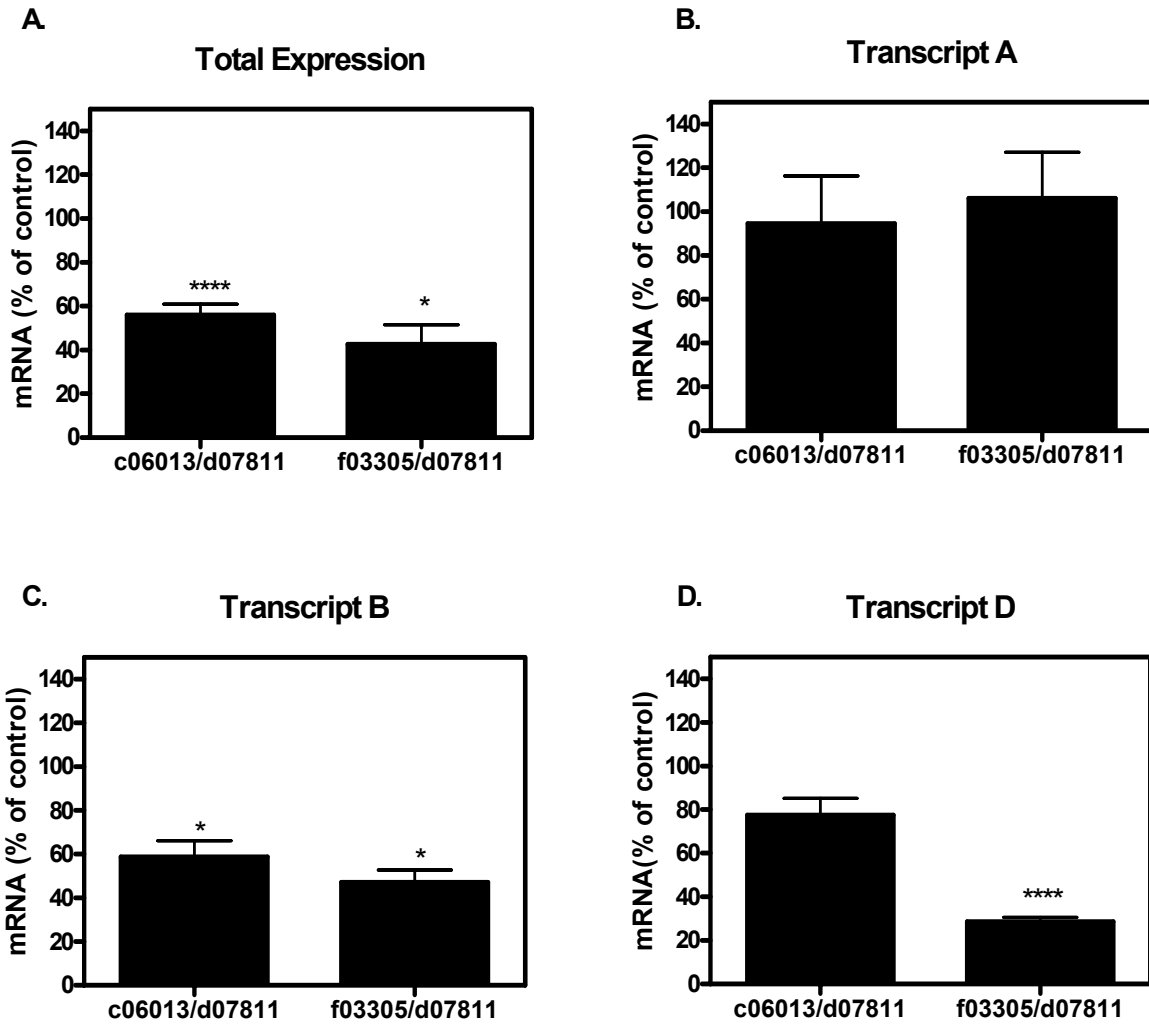
**Figure 8. qRT-PCR Analysis of *tkv* Expression in Transheterozygotes.** (A.) Total expression of all transcripts of *tkv*. (B.) Expression of *tkv* transcript A. (C.) Expression of *tkv* transcript B. (D.) Expression of *tkv* transcript D. * p-value = 0.05-0.01, ** p-value = 0.009-0.005, *** p-value= 0.004-0.001,**** p-value= 0.0009- <0.0001.
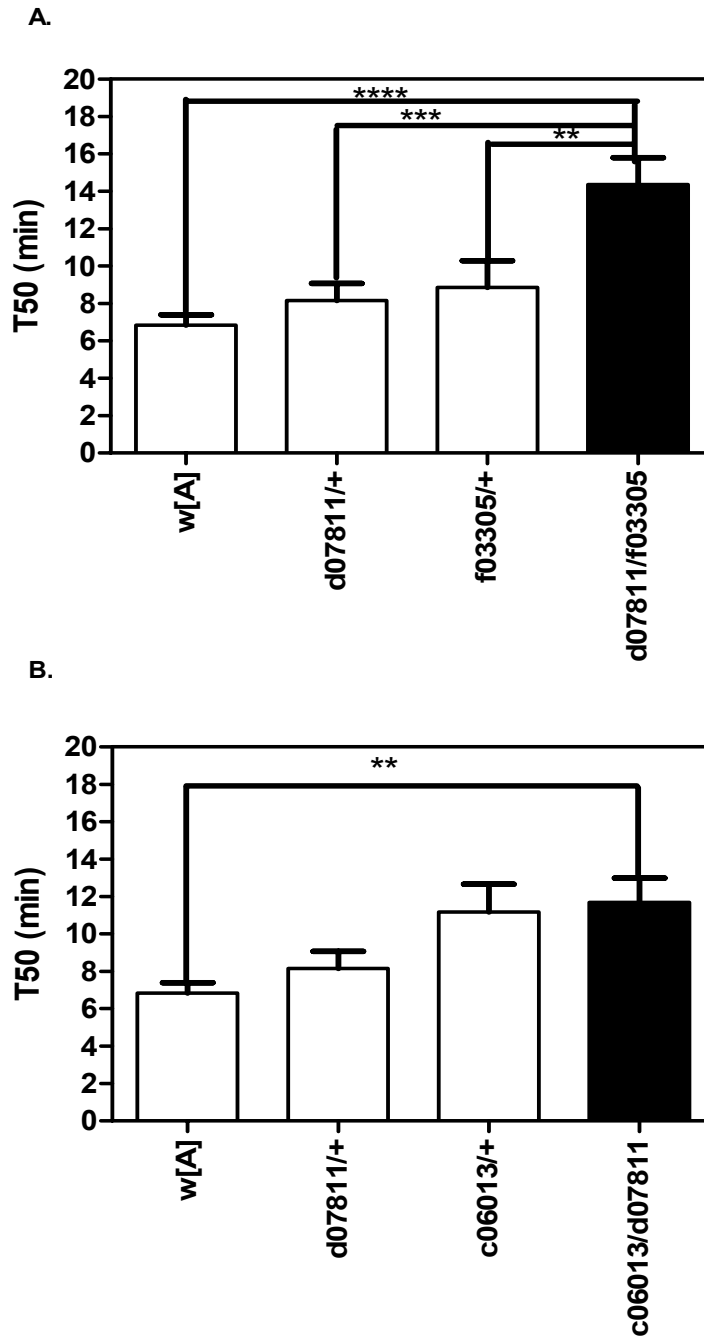
**Figure 9. Ethanol Sensitivity in Transheterozygote Insertion Strains** * p-value =
0.05-0.01, ** p-value = 0.009-0.005, *** p-value= 0.004-0.001,**** p-value= 0.0009-
<0.0001. Filled bars indicate ethanol phenotype. n= 17-19.

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I also assessed internal ethanol concentrations to determine whether the blunted ethanol sensitivity in f03305 and c06013 *in trans* to d07811 was associated with altered ethanol uptake or metabolism. As in the simple transposon strains there was a significant effect of time but there was not a significant effect of genotype and no significant interaction between time and genotype (Figure 10). The blunted ethanol sensitivity in f03305/d07811 and c06013/d07811 flies is therefore likely due to an altered pharmacodynamic effect of ethanol as opposed to an altered pharmacokinetic effect.

Taken together, the expression analysis of *tkv* and ethanol sensitivity in flies with independent alleles of *tkv* in transheterozygotes is consistent with a decrease in total expression of *tkv* or possibly the *tkv*-B isoform altering the sensitivity to ethanol (Table 6). Additionally, my analysis of internal ethanol content in these complementation studies indicates that the altered ethanol sensitivity in *tkv* mutants is related to a change in the pharmacodynamic properties of ethanol in these animals.  Although the data from my studies on transposon mutants are somewhat complex, multiple experiments support the hypothesis that the TGF-β receptor *tkv* influences ethanol sensitivity in flies.

Table 6. Complementation for Ethanol Sensitivity in *tkv*

|  | d07811 |
| --- | --- |
| c06013 | does not complement |
| f03305 | does not complement |
| tkv7 | does complement |
| tkv8 | does complement |

34
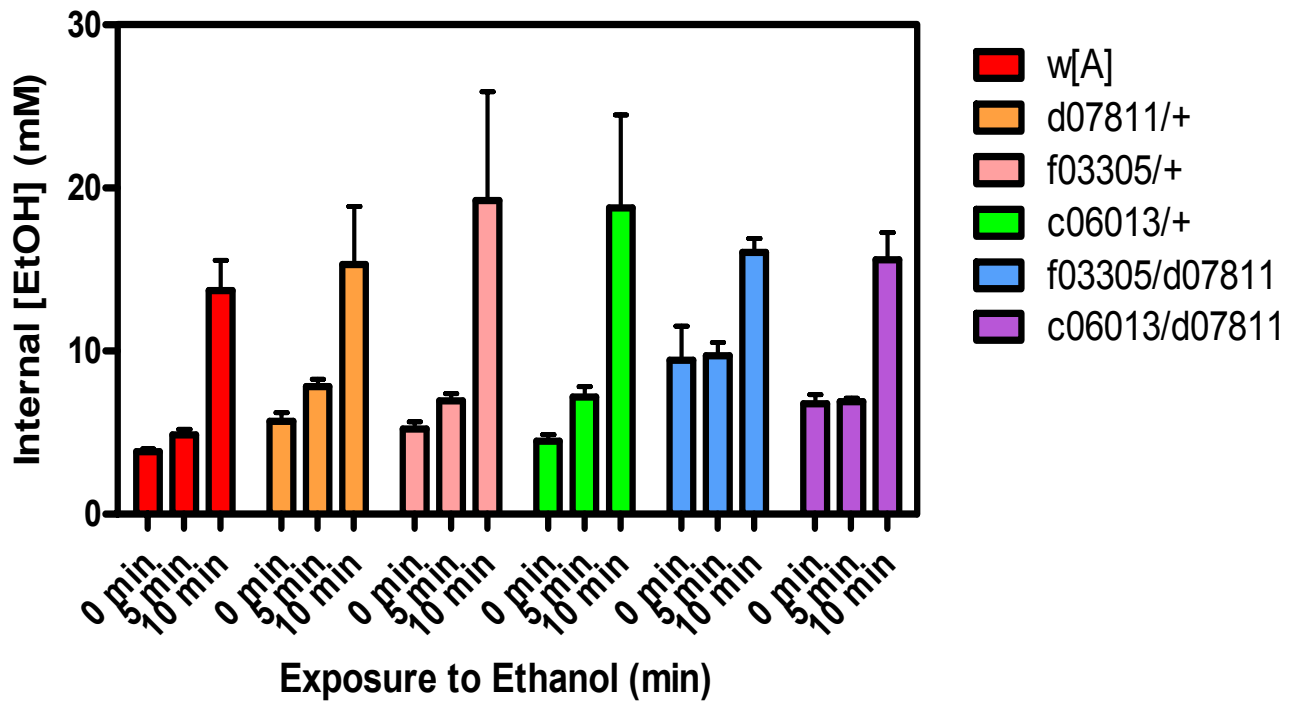
**Figure 10. Internal Ethanol Content of *tkv* Transheterozygote Insertion Strains.**

There was no effect of genotype on internal ethanol (two-way ANOVA, n.s.), but there

was a significant effect of time (p<0.0001).

*Point Mutant Loss of Function tkv Alleles*

Given the complexities in my data from studies using transposon insertion mutants, I decided to further test the global hypothesis that *tkv* influences ethanol sensitivity by using previously characterized point mutant loss of function alleles in *tkv*. I predicted that flies harboring *tkv* point mutations would exhibit blunted sensitivity to ethanol or that the *tkv* point mutations would not complement *tkv* transposon insertions for ethanol sensitivity.

Two homozygous lethal point mutant alleles (*tkv$^7$* and *tkv$^8$*) were obtained from the *Drosophila* Stock Center (Bloomington, Indiana). *tkv$^7$* contains a single point mutation that changes G>A and consequently a glutamic acid to a lysine (E528K) at a conserved residue in the C-terminus of the kinase domain (Nellen et al., 1994). *tkv$^8$* contains an independent point mutation that changes C>A and introduces a predicted termination in the extracellular domain of TKV at C156 (Nellen et al., 1994). Both point mutants were backcrossed to a d07811 stock that had been previously backcrossed to *w[A]*, thereby moving the *tkv* point mutants into the *w[A]* background. Each point mutation (heterozygous) was confirmed by DNA sequencing before and after backcrossing (Figure 11 and 12).
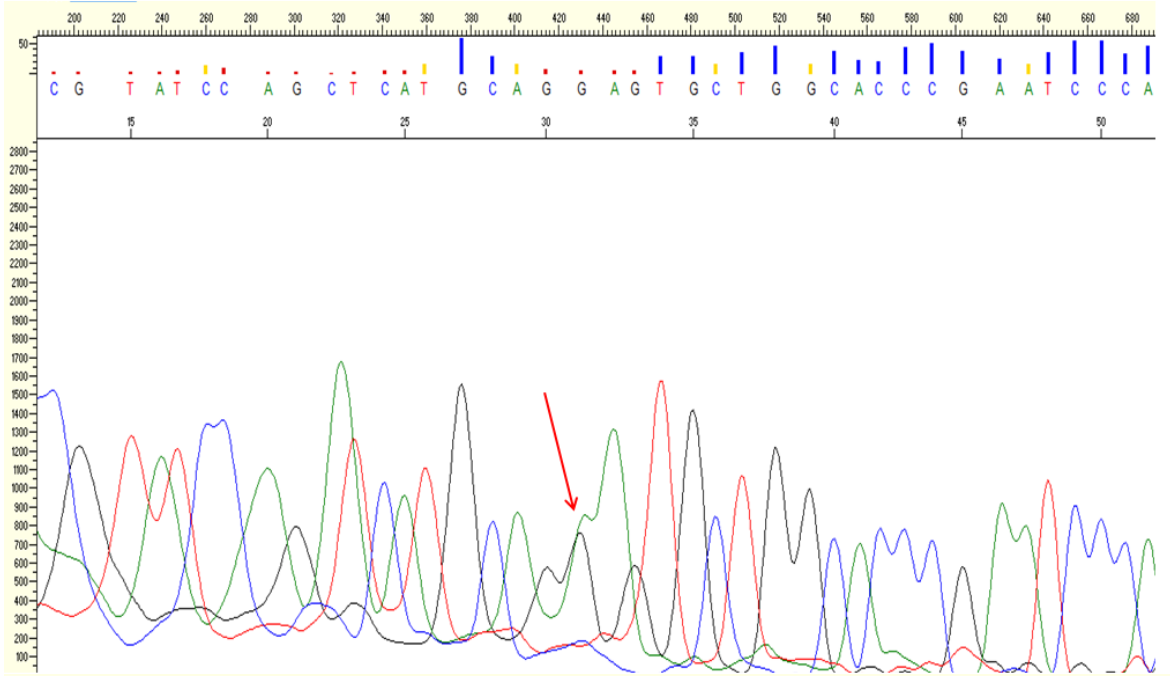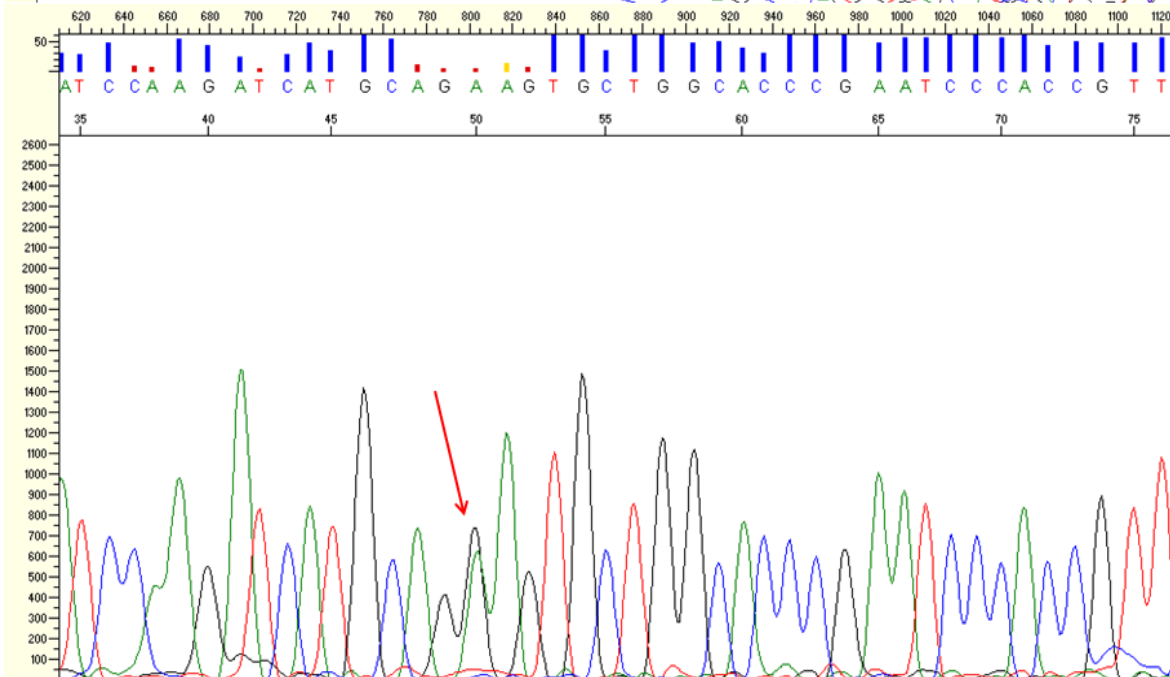
**Figure 11.Sequence Analysis of *tkv*[7] Point Mutation. (**A.) Sequence of *tkv*[7] point mutation prior to backcross. (B.) Sequence of *tkv*[7] point mutation after backcross. Arrow indicates heterozygous locus containing the point mutation.

37
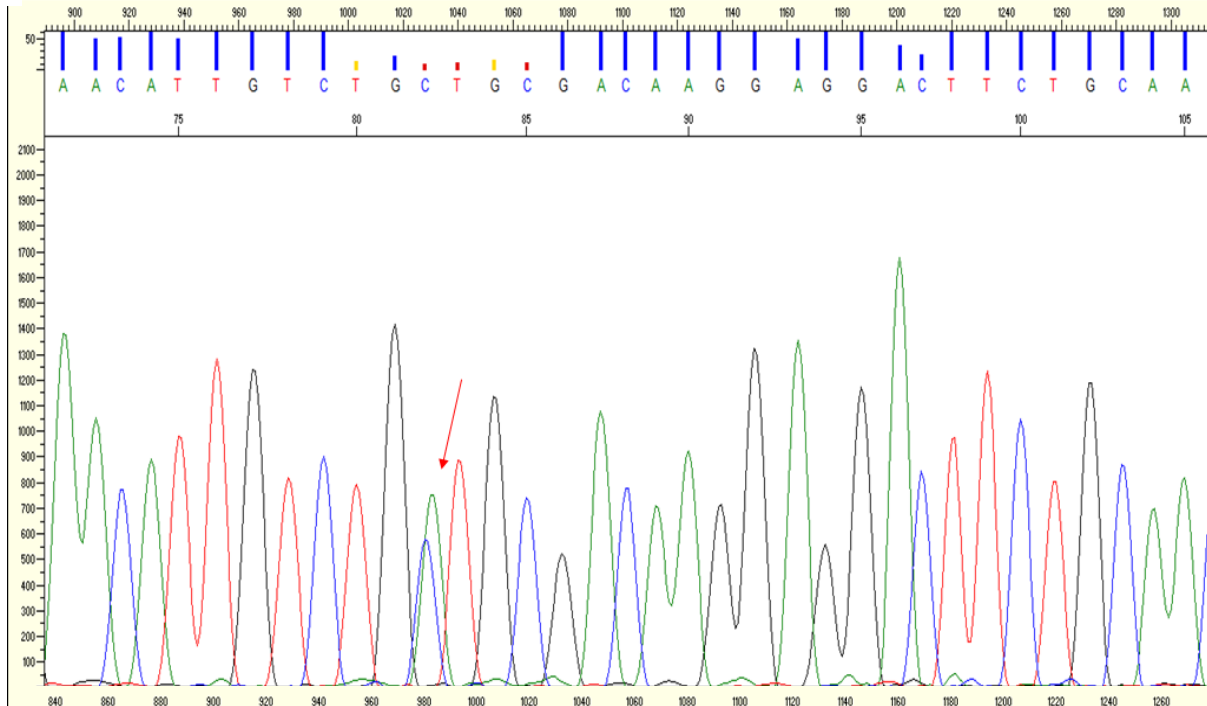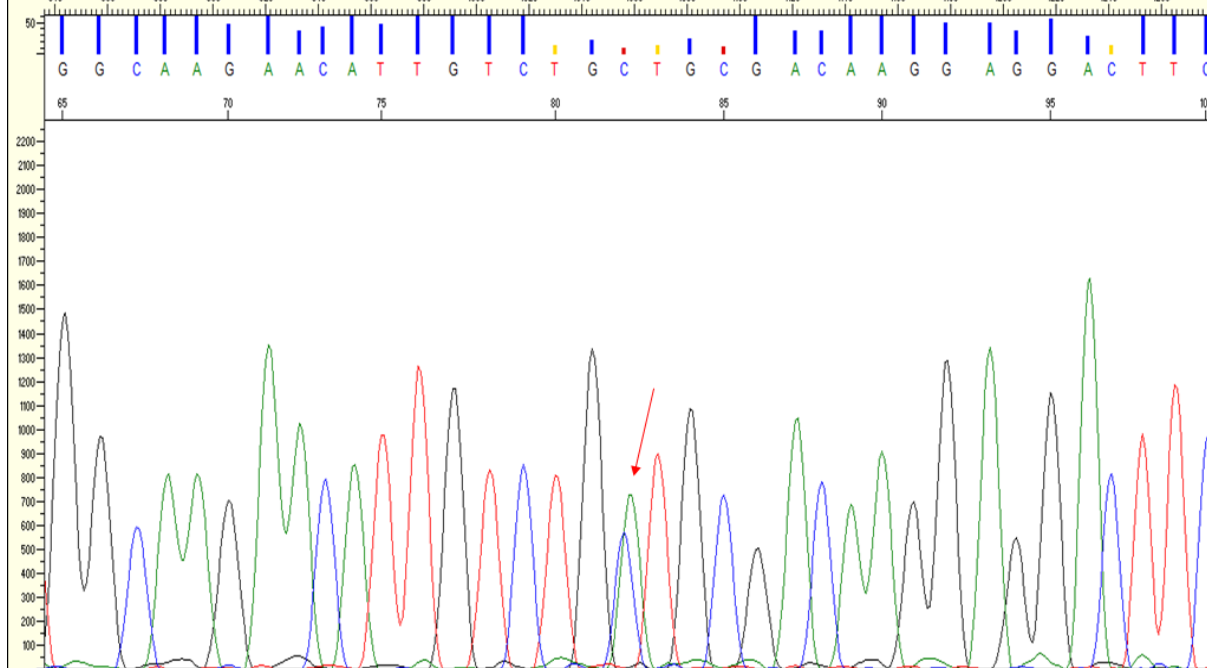
**Figure 12. Sequence Analysis of *tkv*<sup>8</sup> Point Mutation.** (A.) Sequence of *tkv*<sup>8</sup> point mutation prior to backcross. (B.) Sequence of *tkv*<sup>8</sup> point mutation after backcross. Arrow indicates heterozygous locus containing the point mutation.

I evaluated ethanol sensitivity in $tkv^7$ and $tkv^8$ heterozygotes as well as these two $tkv$ alleles *in trans* to the d07811 transposon. In contrast to my results with transposon insertions, I found no significant change in T50 values in $tkv^7$/+ or $tkv^7$/d07811 compared to *w[A]* controls or d07811/+ heterozygotes (Figure 13a). Similarly, I found no effect of the $tkv^8$ allele when tested as a heterozygote or *in trans* to d07811 (Figure 13B). These results are not consistent with my data derived from studies with transposon insertions and therefore do not support the hypothesis *tkv* is important for ethanol sensitivity in flies.

**A.**

**B.**
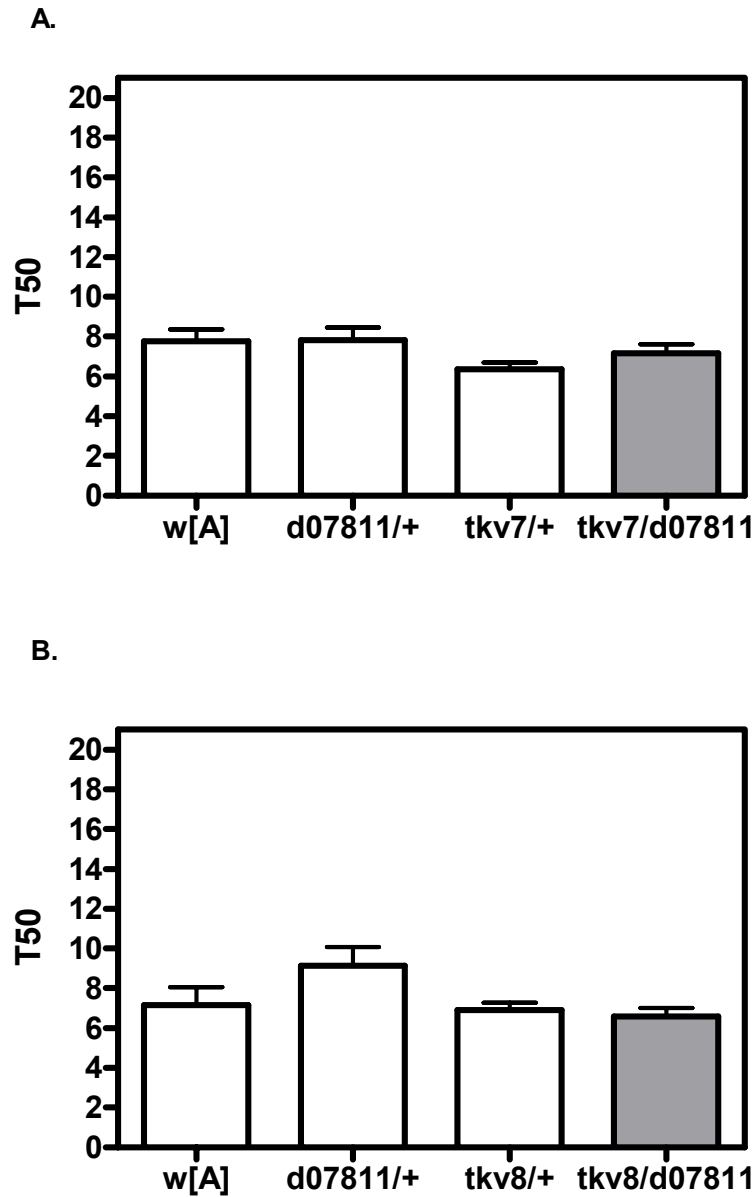
**Figure 13. Ethanol Sensitivity in *tkv*[7] and *tkv*[8] Heterozygous and Transheterozygous Mutants.** (A.) There is no significant difference in T50 values between control and *tkv*7/+ or control *tkv*[7]/d07811. (B.) There is no significant difference in T50 values between control and *tkv*[8]/+ or control *tkv*[8]/d07811. n= 7-10

*Global and Tissue-Specific Knockdown of tkv by RNAi*

Preliminary studies of RNAi-mediated knockdown of *tkv* ubiquitously or in the muscle indicated that *tkv* influences acute sensitivity to ethanol (data not shown, Bhandari and Grotewiel, unpublished). I attempted to confirm these preliminary studies by determining whether ubiquitous or muscle-specific expression of two independent UAS-*tkv*-inverted repeat (IR) lines (3059 and 105834, Table 2) altered ethanol sensitivity. Based on the preliminary data, I hypothesized that the two independent UAS *tkv* IR transgenes would exhibit a significant blunting of ethanol sensitivity upon global and muscle-specific knockdown.

In initial studies using 20% ethanol, I found that the flies for this experiment did not become sufficiently sedated and therefore that acute ethanol sensitivity could not be determined. I reasoned that increasing the ethanol concentration might help resolve differences between genotypes and therefore used 30% ethanol for subsequent RNAi studies. In each RNAi experiment, I compared T50 values for flies harboring a single copy of (ubiquitous) Actin-Gal4 or (muscle) mef2-Gal4, flies with a single copy of UAS-*tkv*-IR 3059 or 105834, and flies with Actin-Gal4 or mef2-Gal4 driving UAS-*tkv*-IR 3059 or 105384. All flies used in these RNAi studies were generated in a F1 hybrid *w[A]*/w[VDRC] genetic background. In contrast to the preliminary studies, I found no substantive difference between flies with a single copy of the Gal4 driver and presumed *tkv* knockdown flies (Figure 14). These data do not support the hypothesis that *tkv* influences ethanol sensitivity, although the low relative sensitivity of Actin-Gal4/+ flies could be masking any significant change in ethanol sensitivity in 3059/Actin-Gal4 or 105834/Actin-Gal4 flies.

41
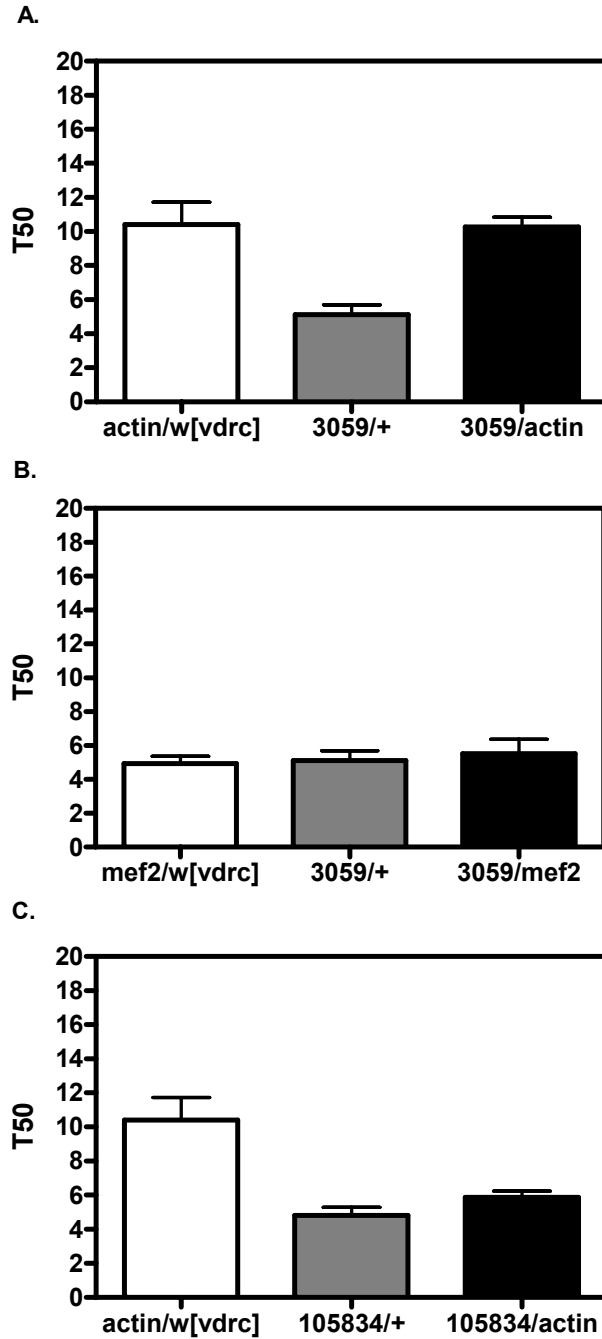
**Figure 14. Ethanol Sensitivity of *tkv* RNAi Knockdown Flies.** (A.) Global knockdown of the UAS 3059 *tkv* allele with actin-Gal4. (B.) Muscle-specific knockdown of the UAS 3059 *tkv* allele with Mef2-Gal4. (C.) Global knockdown of the UAS 3059 *tkv* allele with actin-Gal4. n= 10-12.

*Conclusions*

Initial complementation for viability strongly indicates that the transposons used in this study were in the *tkv* gene and caused loss of function of TKV. Analysis of acute ethanol sensitivity in these transposon insertions revealed a significantly blunted sensitivity in some but not all of these *tkv* mutated flies (Table 7). Expression analysis indicated that total *tkv* expression is not correlated to sensitivity (Table 7). However since the expression of *tkv-B* is decreased across all transposon insertions this might indicate that this is the transcript being acted on by the transposon insertions. These results support the hypothesis that *tkv* does influence acute ethanol sensitivity.

However, RNAi knockdown of *tkv* and LOF alleles for *tkv* that were tested as part of this study do not support this hypothesis. This is in part due to inconclusive results of the RNAi studies where the controls with significant resistance to ethanol might be masking any blunted ethanol sensitivity in the knockdown flies. Further studies should be done on *tkv* to determine how *tkv* is influencing acute sensitivity to ethanol and its role in the mechanism for Clic involvement in acute ethanol sensitivity.

**Table 7. *tkv* Transposon Expression and EtOH Sensitivity Summary**

| genotype | total tkv expression | tkv-A expression | tkv-B expression | tkv-D expression | EtOH Sensitivity |
|---|---|---|---|---|---|
| d07811/d07811 | 94% | 105% | 24%* | 103% | 223%* |
| f02766/f02766 | 94% | 100% | 20%* | 268%* | 161%* |
| | | | | | |
| KG05071/+ | 39%* | 98% | 44%* | 97% | 252%* |
| c06013/+ | 77%* | 123% | 64%* | 124% | 116% |
| f03305/+ | 83% | 87% | 37%* | 51%* | 107% |
| | | | | | |
| c06013/d07811 | 56%* | 95% | 59%* | 77% | 175% |
| f03305/d07811 | 43%* | 106% | 47%* | 29%* | 215%* |

all measures as percent of w[A] control, * indicates significance p<0.05

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CHAPTER 3

**Preliminary Molecular and Behavioral Characterization of the TGF-β Receptor**

**Genes *wishful-thinking* and *baboon* in *Drosophila* Ethanol Sensitivity**

*Introduction*

For a more complete view of TGF-β receptors and their roles in acute ethanol

sensitivity, I examined the TGF-β receptor genes *wishful-thinking* (*wit*) and *baboon*

(*babo*). *babo* encodes a Type I receptor and *wit* encodes a Type II receptor. As with *tkv*,

I hypothesized that decreased expression of *wit* or *babo* would blunt sensitivity to

ethanol. Below is preliminary data for both *wit* and *babo* expression and acute ethanol

sensitivity in a series of flies with transposon insertions in these two genes.

*A. wishful-thinking*

To study the influence of *wit* on acute ethanol sensitivity, I obtained three

transposon insertions in the gene. These transposon insertions are near or in the 5' and

3' UTRs, locations that I confirmed by standard PCR with primers that annealed to the

transposon and near the reported insertion sites (Table 1, Figure 15).
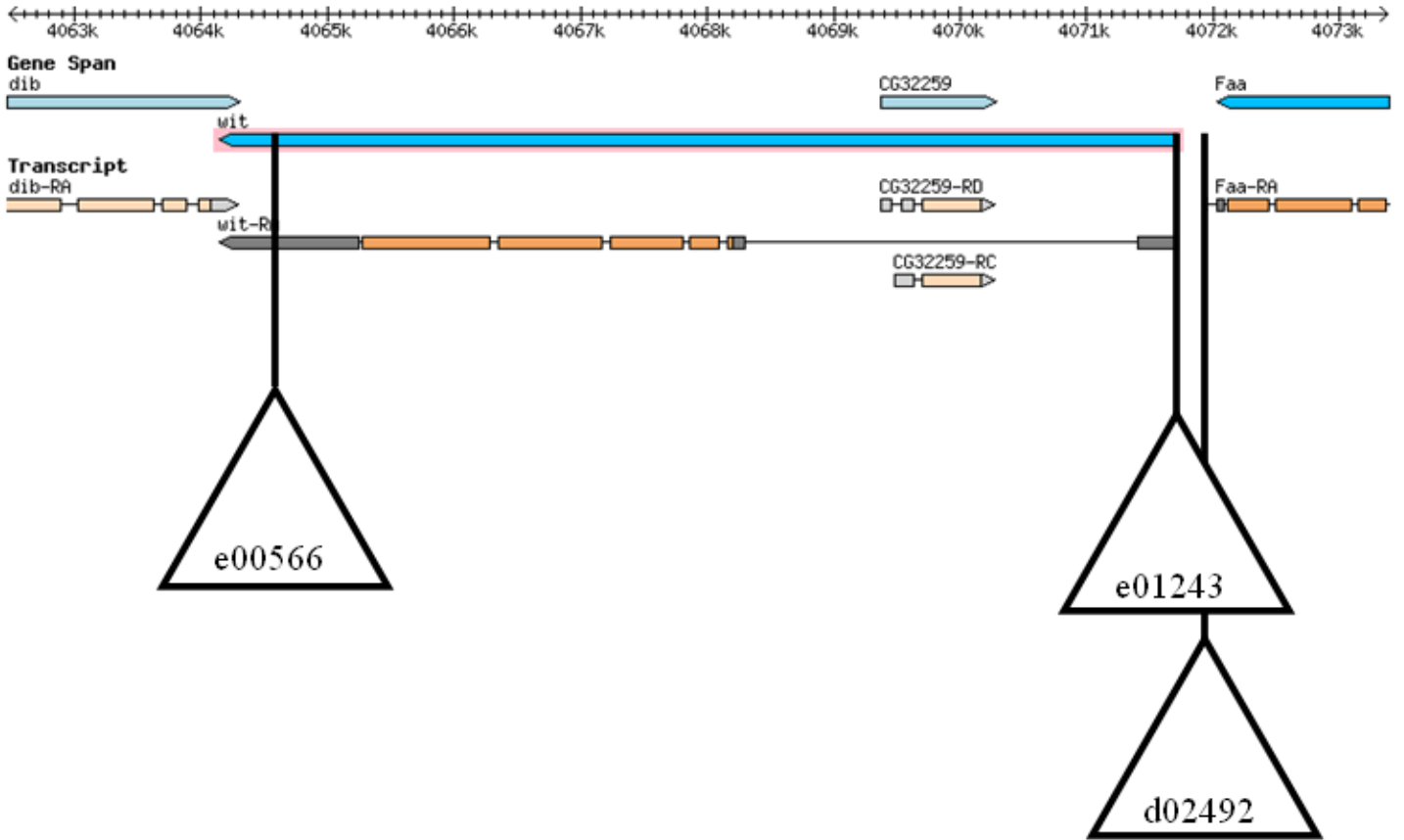
**Figure 15. Mapped Transposon Insertions in the *wishful-thinking* Gene.**

I evaluated total *wit* expression by qRT-PCR for the three homozygous viable transposon insertions, e00566, e01243, and d02492. This analysis revealed that in the [e00566] flies there was a significant upregulation of *wit* expression by over 4-fold (Fig. 16). There was a significant decrease in expression in the d02492 transposon carrying flies, but there was no significant change in expression in the e01243 transposon line (Figure 16).

To assess acute ethanol sensitivity in these transposon lines, I used the eRING assay with 20% ethanol. The T50 values from all three wit transposon strains were significantly different from *w[A]* control animals (Figure 17), suggesting that that *wit* might influence acute ethanol sensitivity. Although complex, these results could lead to follow-up studies that more formally address the role of *wit* in acute ethanol analysis using RNAi knockdown and point mutant LOF alleles.
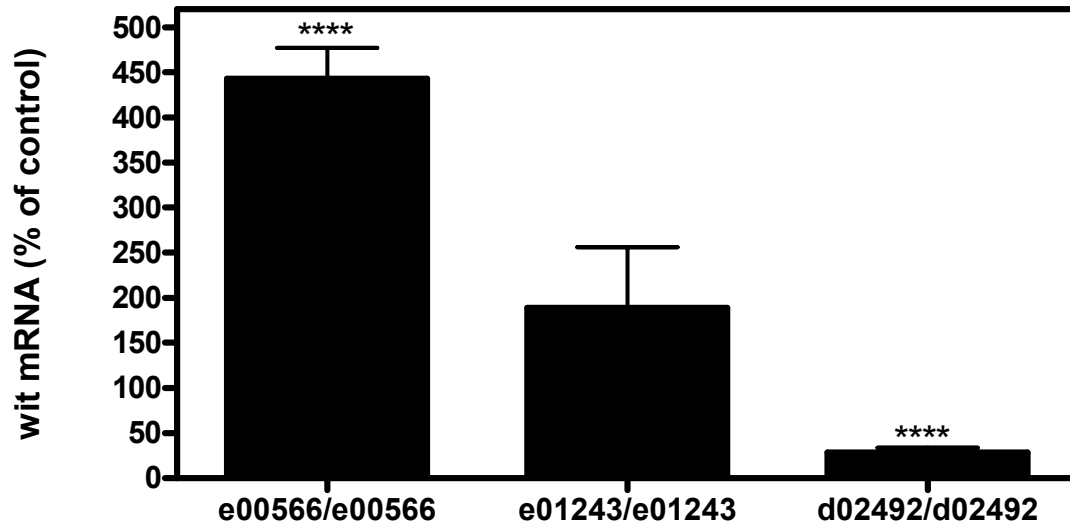
**Figure 16. qRT-PCR Analysis of *wit* Expression in Transposon Strains.**

 **** p-value 0.0009- <0.0001.
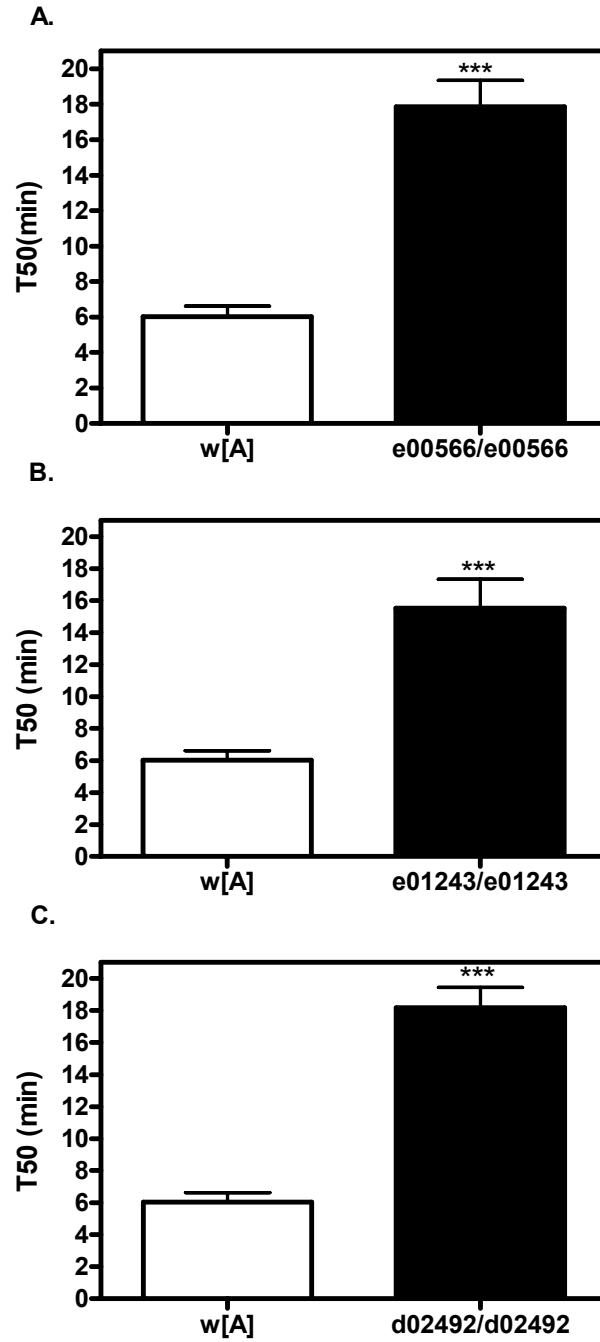
**Figure 17. Ethanol Sensitivity in *wit* Transposon Insertion Strains.**

(A.) e00566/e00566. (B.) e01243]/[e01243. (C.) d02492/d02492.

***p-value 0.004-0.001. Open bars indicate control, filled bars indicate transposon

insertion. n= 9-10.

*B. baboon*

As with *wit*, I used transposon insertions in *babo* to do a preliminary assessment of the influence of this gene on acute ethanol sensitivity. These transposons were located in the non-coding 5' UTR and introns of the *babo* gene (Table 1, Figure 18). I used standard PCR to confirm the three transposon insertions (k16912, c05710, and c04236) used in this study (Table 1).

I evaluated total expression of *babo* by qRT-PCR and observed a significant decrease in the expression of *babo* in the c05710 and k16912 heterozygous transposon lines (Figure 19). There was no change in expression of c04236 heterozgyotes. I also performed an eRING assay on these transposon lines to assess acute ethanol sensitivity. There was no significant difference between these transposon lines and the *w[A]* control in T50 values when exposed to 20% ethanol (Figure20).

The lack of a blunted acute ethanol phenotype observed in this preliminary study does not support the hypothesis that *babo* influences acute ethanol sensitivity. Based on these results, further studies of *babo* will likely not be fruitful in studying acute ethanol sensitivity.
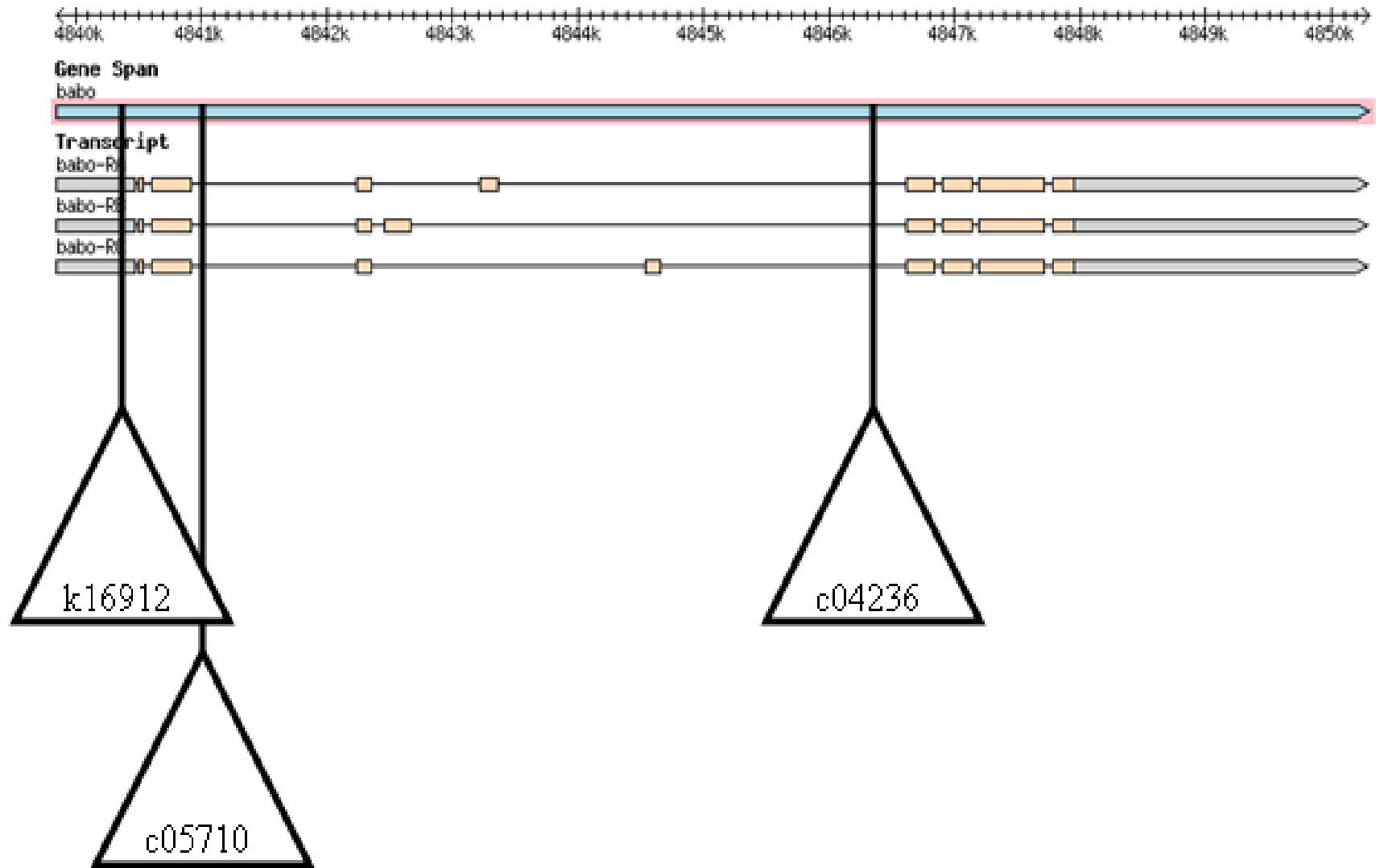
**Figure 18. Mapped Transposon Insetions in the *baboon* Gene.**
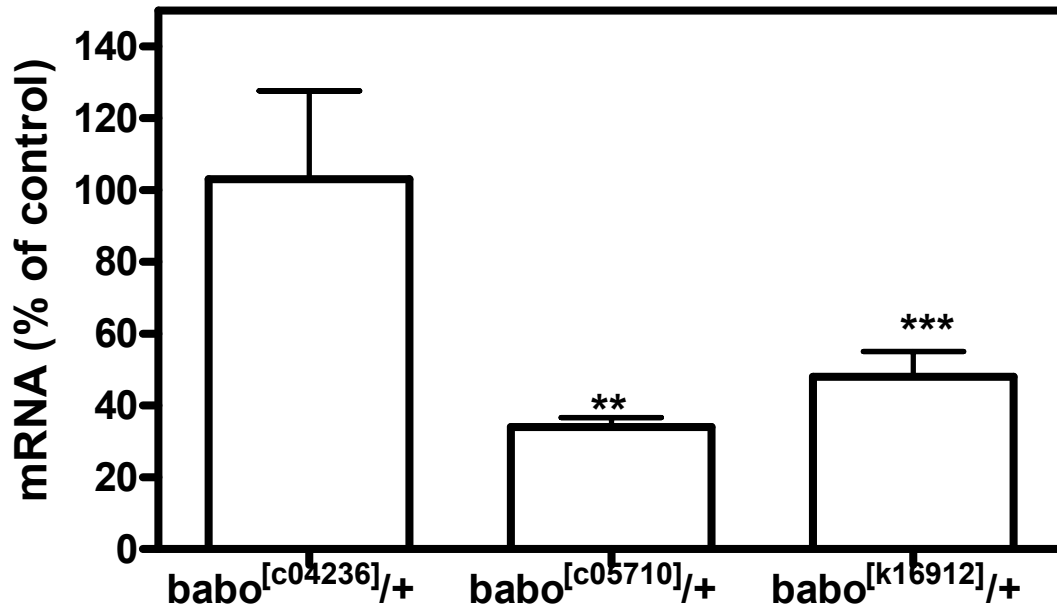
**Figure 19. qRT-PCR Analysis of *babo* Expression in Transposon Strains.**

 **p-value 0.009-0.005, ***p-value 0.004-0.001.
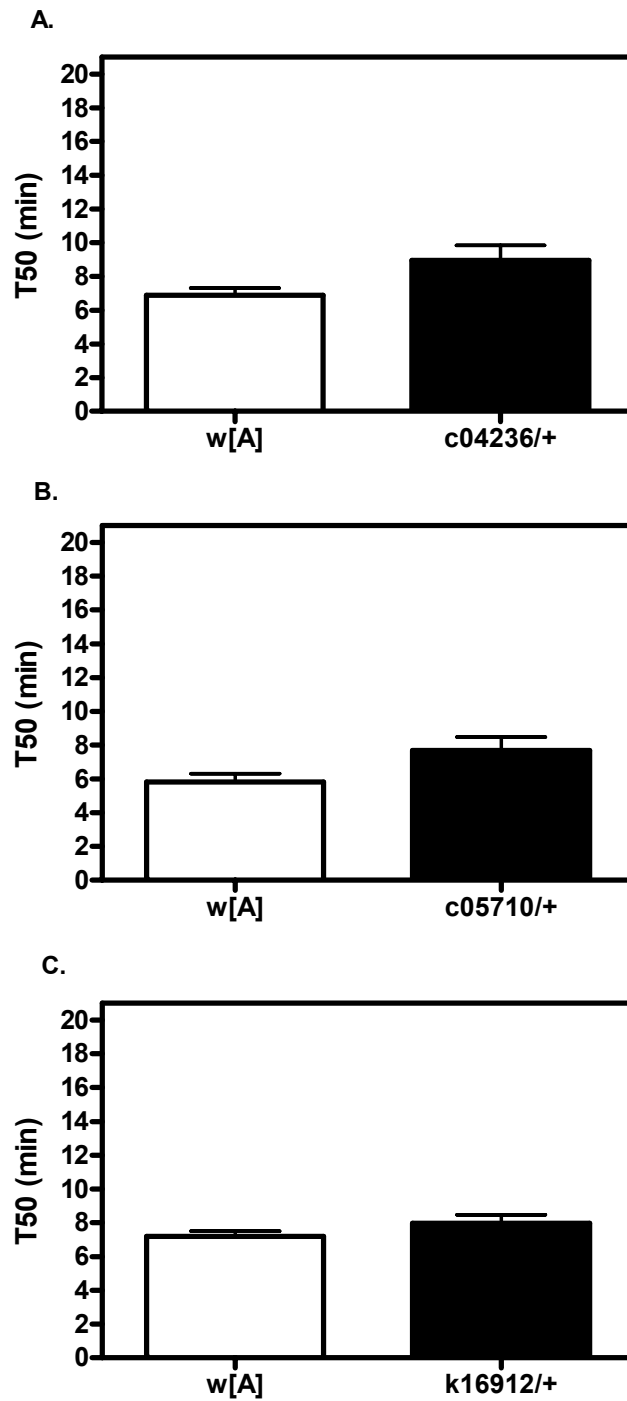
**Figure 20. Ethanol Sensitivity in *babo* Transposon Insertion Strains.**

(A.) c04236/+. (B.) c05710/+. (C.) k16912/+. There is no significant difference in T50

values in *babo* mutants compared to controls. Open bars indicate controls, closed bars

indicate transposon insertions. n=9-10.

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*Conclusions*

These preliminary data suggest there might be very different roles of *wit* and *babo* in acute ethanol sensitivity. I observed significant increases in T50 values of homozygous wit transposon insertions by eRING analysis. This may indicate there might be a potential influence of *wit* on acute ethanol sensitivity. I suggest that further studies be done to determine this potential role of *wit* on ethanol sensitivity. However, due to the evidence from these eRING studies of transposon insertions in *babo* that *babo* might not influence acute ethanol sensitivity, I do not believe further studies on *babo* would be beneficial.

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CHAPTER 4

**Summary**

Alcohol abuse and dependence are diseases with far reaching consequences. It is estimated that nearly 50-60% of the risk variance for developing alcohol abuse or dependence is genetic (APA, 2000). The genetic component of alcohol abuse is considered polygenic with many genes of presumed small effects influencing the risk variance. In an effort to further understand the genetic components of alcohol abuse and dependence, large scale meta-analyses have been done to populate a list of potential influencing genes.

From this analysis the *Clic* gene was identified. *Clic* is known to be involved a wide range of biochemical processes beyond the chloride intracellular channels as the name suggests. A recent study done by Bhandari *et al.* (2012) shows a cross species detailed analysis of the influence of *Clic* on acute ethanol sensitivity. Specifically in *Drosophila*, the decreased expression of *Clic* blunts acute ethanol sensitivity (Bhandari et al., 2012). While this study provides strong evidence for the influence of *Clic* on acute ethanol sensitivity, it does not provide a mechanism for why this change of sensitivity occurs when *Clic* expression is decreased. As mentioned above, *Clic* is involved in many different biochemical pathways including binding to AKAP proteins, interactions

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with ryanodine receptors and 14-3-3, as well as involvement in the TGF-β signaling pathway (Suginta et al., 2001; Shanks et al., 2002; Jalilian et al., 2008) (Shukla et al., 2009). The later pathway was the focus of this thesis research project.

Shukla *et al*. (2009) showed that vertebrate *Clic4* functions within the TGF-β signaling pathway by associating with Schnurri-2 upon activation of the pathway, translocating from the cytoplasm to the nucleus where it dissociates from Schnurri-2 and then binding of SMADs to regulate transcription of downstream genes. This direct involvement in signal transduction in the TGF-β signaling pathway suggested that this might be the mechanism by which *Clic* influences acute ethanol sensitivity. To further determine if this potential mechanism for the change in ethanol sensitivity observed when *Clic* is mutated in flies, I examined TGF-β receptor genes.

In both mammals and invertebrates, such as *Drosophila*, there are two TGF-β receptor classes, type I and type II. These receptors associate to form a heterotetramer when the TGF-β ligand binds. In *Drosophila* there are three Type I receptor genes (*thickveins*, *baboon*, and *saxophone*) and two Type II receptor genes (*wishful-thinking* and *punt*).

The global hypothesis for this study was that TGF-β receptors, specifically *thickveins*, influence acute sensitivity to ethanol in *Drosophila*. To first examine this genetic influence on acute ethanol sensitivity, I obtained transposon insertions in the *tkv* gene and confirmed the presence of each transposon. Once confirmed, I examined the expression of *tkv*, acute ethanol sensitivity and internal ethanol concentrations in these transposon insertion lines.

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These experiments show that five independent transposon insertions in *tkv* exhibit a broad range of *tkv* expression as determined by qRT-PCR. These mutants further exhibited a broad range of T50 values when tested in the eRING assay. No direct correlation was found between the expression of *tkv* and the sensitivity to ethanol in these mutants. I hypothesized that since there are four different isoforms of *tkv* that there might be a differential expression among these isoforms in these transposon mutants. There was a significant decrease in the *tkv*-B transcript in all the transposon strains. This decreased expression may indicate that the B transcript of *tkv* might be the specific isoform that is being affected by the transposon insertions. Otherwise, no simple correlation was found in the expression of these simple isoforms and ethanol sensitivity. Further, to test what was being observed in these mutants was not a result of a pharmacokinetic effect, I examined the internal ethanol content in these mutants. No difference was seen among the genotypes indicating that the sensitivity observed was not a pharmacokinetic effect but a pharmacodynamic effect.

To follow up on these transposon experiments, I performed a series of supporting experiments, including complementation experiments, RNAi knockdown and LOF point mutations. When *in trans* to each other, selected transposons did not complement for acute ethanol sensitivity. This further supported my hypothesis that *tkv* influences acute ethanol sensitivity. The presumed knockdown of *tkv* by RNAi did not exhibit blunted acute ethanol sensitivity. This lack of expected blunting of acute ethanol sensitivity was also found in studies with the two LOF alleles tested as well as the LOF allele *in trans* to the d07811 transposon insertion. Since there was a blunting of ethanol sensitivity observed in some of the transposon lines and in the complementation experiment, *tkv*

might influence sensitivity to ethanol, however this was not supported by follow-up experiments addressed above.

In summary, some of my data strongly implicate *tkv* in ethanol sensitivity and some of my data do not. The role of *tkv* in ethanol sensitivity, therefore, remains unclear.  If *tkv* is important for ethanol sensitivity as predicted by my major hypothesis, there must be a fairly complex relationship between *tkv* expression and the behavior. For example, it is possible that the qRT-PCR data do not reflect protein expression levels, that *tkv* expression during development is important for the behavior, that the behavior is sensitive to altered expression of *tkv* in select tissues only, or some combination of these or other complexities. To address this I propose using western blot analysis of *tkv* in different tissues and at different ages of the fly. Alternatively, it is possible that *tkv* simply is not important for ethanol sensitivity in flies and that the altered T50 values in *tkv* transposon lines are due to changes in expression of other nearby or possibly distant genes. For example, several cytochrome P450 genes reside in the 5' region of the *tkv* transcription unit and it could be possible that disruption of these embedded genes influences ethanol sensitivity. The genes local to *tkv* could be examined by a combination of qRT-PCR analysis and western blot analysis. These additional studies that address *tkv* protein expression levels, *tkv* expression during development and expression of genes near *tkv* could help resolve the relationship between *tkv* and ethanol sensitivity.

In addition to *tkv*, I obtained preliminary data from the Type I receptor gene *babo* and the Type II receptor gene *wit*. Expression analysis and eRING analysis of acute ethanol sensitivity of transposon lines in *babo* exhibit no changes that would indicate

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that *babo* is influencing ethanol sensitivity. On the other hand, follow-up studies should be done on *wit* because eRING analysis of acute ethanol sensitivity potentially indicates that *wit* might be influencing acute ethanol sensitivity.

Overall, the evidence for TGF-β receptors role in influencing acute sensitivity to ethanol is unfortunately inconclusive. However, it is important to note that there is some evidence for *tkv* and *wit* on the influence of ethanol sensitivity that should be followed-up on in further studies. Finally, there should be follow-up studies that confirm that the mechanism by which *Clic* influences acute ethanol sensitivity in *Drosophila* is through the TGF-β signaling pathway.

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APPENDIX

Protocols

*A. gDNA Isolation*

**DNeasy Genomic DNA Isolation**

1. Place up to 50 adult flies in a labeled 1.5 mL snap-cap tube. Add 180 µL sterile

    PBS and 10 µL RNaseA (10mg/mL). Smash and grind with drill/pestle for 15

    seconds. Incubate at room temperature for 2 minutes.

2. Add 20 µL proteinase K solution (kit) and 200 µL Buffer AL (kit) to each tube.

    Vortex and then incubate at 56 C for 10 minutes.

3. Add 200 µL 100% ethanol to each tube and vortex to mix.

4. Pipette mixture from step 3 to a labeled DNeasy column and place in a 2 mL

    collection tube (kit). Spin at 9000 rpm (6600 x g) for 1 minute. Discard flow-

    through and collection tube.

5. Place the DNeasy column in a fresh 2 mL collection tube (kit) and add 500 µL of Buffer AW1 (kit). Spin at 9000 rpm (6600 x g) for 1 minute. Discard flow-through and collection tube.

6. Place the DNeasy column in a fresh 2 mL collection tube (kit) and add 500 µL of Buffer AW2 (kit). Spin at maximum rpm for 3 minutes. Discard flow-through and collection tube.

7. Place the DNeasy column in a new, labeled 1.5 mL snap-cap tube. Add 200 µL distilled H2O to membrane in column and incubate 2 minutes at room temperature. Spin at 9000 rpm (6600 x g) for 1 minute to elute genomic DNA.

8. Dilute 10 µl of each sample 1:10 in a separate 0.5 mL tube with distilled sterile H2O and determine $A_{260}/A_{280}$ value.

Genomic DNA concentration (µg/mL) = $A_{260}$ Value x (50mg/mL) x 10

Reagents and Supplies:
1.5mL snap-cap tubes
0.5 ml snap-cap tubes (for determining concentration)
Sterile PBS (pH 7.4)
100% ethanol
RNase A solution (10 mg/ mL in TE)
56 C water bath or heat block
Distilled sterile H2O

Contributed by Mike Grotewiel as modified version of Quiagen protocol

(12/2003).Updated: 4/2008

*B. mRNA Isolation, cDNA Synthesis, Absolute qRT-PCR Quantification and Relative qRT-PCR Quantification*

**RNA Preparation (25 fly prep)**

A. <u>Fly collection and initial preparation:</u>

1. Wear gloves and collect 25 flies in a 1.5 ml snap cap tube. Freeze at -80$^{\text{o}}$C (long-term storage) or at -20$^{\text{o}}$C (short-term storage).

2. Wipe down bench and all pipettes, pipette boxes, etc. with 100% ethanol. Place clean plastic pestles in 50 ml conical tube, cover pestles with chloroform, soak for 20 minutes. Transfer pestles to new clean empty 50 ml conical tube and allow to air dry. All water used is DEPC water.

B. <u>RNA extraction:</u>

1. Add 250 µl Trizol to each tube of flies. Homogenize for 1 minute with drill and pestle.

2. Add an additional 250µl Trizol to each tube and vortex for 15 seconds. Incubate for 5 minutes at room temperature.

3. Add 100µl chloroform to each tube. Vortex for 15 seconds. Incubate for 3 minutes at room temperature.

4. Centrifuge samples at maximum speed (14,000 x g) for 15 minutes at $4^{o}C$ (in cold room).

5. Label new 1.5 ml tubes.  Use pipette to remove upper aqueous phase and put in new tube (200 µl max).  If you accidentally pipette any fly parts or pink liquid, centrifuge samples again and then remove upper layer.  Discard tubes with fly parts and pink liquid.

6. Add 250µl isopropanol to each tube containing the upper aqueous phase.  Invert tube 5 times.  Incubate 10 minutes at room temperature.

7. Centrifuge samples at maximum speed (14,000 x g) for 10 minutes $4°C$.

8. A white pellet on the bottom of each tube should be visible.  Remove liquid from tube with pipette.  Add 500µl 75% ethanol (made with DEPC water) to each tube, invert 5 times to wash.

9. Centrifuge samples at maximum speed (14,000 x g) for 5 minutes $4^{o}C$.

10. Use a 200 and then a 20 µl pipette to remove all liquid by pellet.  Clean a piece of glass plate with ethanol and then chloroform.  Invert tubes on the cleaned glass plate.  Air dry until no liquid droplets remain and white pellet becomes clear (30-60 min).

11. Add 50µl DEPC water to each pellet, allow to sit at room temperature for ~60 minutes, and resuspend by pipetting up and down.  Be patient, this can take awhile.

12. Measure RNA concentration:  Blank is 100µl DEPC water.  Samples are 98µl DEPC water and 2 µl of RNA sample.  $ABS_{260}$ x 40µg/ml x 50 = concentration RNA. Store RNA samples at

$-80^{o}C$ until needed.

**DNAse treatment and reverse transcription**

**\*\*The following two steps need to be done on same day\*\***

A.  DNAse treatment

1.  Set heat blocks to 37$^o$C and 65$^o$C.

2.  Place 6μg RNA in 0.5 ml snap cap tube.  Bring total volume to 17 μl with DEPC water.

3.  Add 2 μl DNAse buffer and 1 μl DNAse enzyme.  Incubate at 37°c for 25 minutes.

4.  Pulse centrifuge samples and then add 2 μl DNAse inactivation reagent.  Incubate 2 minutes at room temperature.  While incubating flick tube gently to resuspend the inactivation reagent throughout the sample.  Sample should be cloudy white.

5.  Spin down 10,000 x g for 1.5 minutes.

6.  Use pipette to transfer 11 μl of clear upper phase to new 1.5 ml tubes.


B.  Reverse transcription of RNA (~3 μg)

1.  Add 1 μl of oligo-dT to each sample of DNAse treated RNA.

2.  Incubate at 65$^o$C for 15 minutes.  Thaw 1$^{st}$ strand buffer, DTT, and 10mM dNTP on ice.

3.  After 65$^o$C incubation, put tubes on ice for 1 minute then pulse spin in centrifuge.  Keep all samples on ice throughout.

4. Add to each tube:
4 µl of 5x 1$^{st}$ strand buffer
2 µl of 0.1M DTT
1 µl 10mM dNTPs
1 µl Superscript 2 enzyme (keep in the cold block)
5. Incubate at 42°C for 50 minutes.

6. Store samples at -20°C.

7. Run standard PCR on 1-2µl of cDNA with RT-PCR primers as quality control

check. Use VCU86/VCU87 (detects Doc3 cDNA and gDNA) unless you are working

with Doc3 as a gene of interest (see M.S.G. for alternative primer set).


Notes:

1. Use RNAse-free reagents only.

2. DNAse treatment reagents from Applied Biosystems (AM1906).

3. Reverse transcriptase protocol adapted from company protocol (Gibco/BRL).

   4. Reverse transcriptase and buffers are from Invitrogen (18064014)

5. Oligo-dT from Invitrogen (18418-012).

**Real-Time PCR: cDNA titration and dissociation curve for new primers**

A. Reaction Set-Up

1. Dilute primers from 10 pmol/µl to 3 pmol/µl (e.g. 35 µl $H_2O$ + 15 µl 10 pmol/µl primer).

2. Dilute control cDNA per table below. Scale as appropriate for the number of primer pairs.

| cDNA dilution | Example: 1 primer pair | Example: 2 primer pairs |
|---|---|---|
| 1:4 | 6 µl water + 2 µl cDNA | 12 µl water + 4 µl cDNA |
| 1:8 | 4 µl water + 4 µl 1:4 cDNA | 8 µl water + 8 µl 1:4 cDNA |
| 1:16 | 4 µl water + 4 µl 1:8 cDNA | 8 µl water + 8 µl 1:8 cDNA |
| 1:32 | 4 µl water + 4 µl 1:16 cDNA | 8 µl water + 8 µl 1:16 cDNA |
| 1:64 | 4 µl water + 4 µl 1:32 cDNA | 8 µl water + 8 µl 1:32 cDNA |

Note: RT-PCR is highly sensitive. Knobs on pipettes MUST be taped at all possible steps when pipetting replicate samples. Change tips after each volume is dispensed.

3. Make master mix for each cDNA dilution according to the table below for experiments in triplicate. Pulse vortex (and pulse centrifuge if necessary).

|  | each well in plate final (n=1) | Master for 1 primer pair (n=4) | Master for 2 primer pairs (n=8) | Master for 3 primer pairs (n=12) | Master for 4 primer pairs (n=16) |
|---|---|---|---|---|---|
| water | 4.5 µl | 18 µl | 36 µl | 54 µl | 72 µl |
| SYBR | 7.5 µl | 30 µl | 60 µl | 90 µl | 120 µl |
| cDNA | 1 µl | 4 µl | 8 µl | 12 µl | 16 µl |
| F primer | 1 µl | XX | XX | XX | XX |
| R primer | 1 µl | XX | XX | XX | XX |

4.  Label one tube for each primer pair for each cDNA dilution (e.g., for 2 primer pairs and 5 cDNA dilutions, label 2 x 5 = 10 tubes total).

5.  Aliquot master mix @ 49.4 µl /tube from step 4, one tube for each primer pair for each cDNA dilution.  Some master mix will remain unused.

6.  Make a master mix of primers for each primer pair by combining/mixing 25 µl of the forward and 25 µl of the reverse primers.  Add 7.6 µl of the primer master mix to the appropriate tubes from step 5.  Pulse vortex (and pulse centrifuge if necessary).

7.  Aliquot in triplicate 15µl of each reaction into a 96 well plate.  Some reaction mix will remain unused.  Spin plate at 1000 rpm for 2 minutes using the centrifuge in Rita Shiang's lab.

B.  Amplification and Data Acquisition

1.  Turn on 7500 Fast System machine.  Launch 7500 Fast System software via desktop icon.  Place plate in machine.

2.  Click File -> New.  Set 'Assay' to 'Absolute Quantification (Standard Curve)'.  Set 'Run Mode' to 'Standard 7500'.  Click 'Next'.

3.  If using new primers, click on 'New Detector', add name, leave description blank, select SYBR as the reporter and choose a color.  When finished adding new primers, click on appropriate detectors, click 'Add', click 'Next'.

4.  A 'New Documents Wizard' interface should be visible.  Assign detectors (primers) and cDNA dilutions to wells by highlight wells and then checking appropriate 'Use' box next to the detector.  With wells highlighted, also assign roles

for detectors as 'Standard' by using drop down menu under 'Task' for each detector and assign dilution information in the 'Quantity' box in decimal format:

1/4 = 0.25
1/8 = 0.125
1/16 = 0.0625
1/32 = 0.03125
1/64 = 0.015625
Click 'Finish".


5.  An interface with 3 tabs should be visible.  In the 'Set-Up' tab, information on primer pair and cDNA dilution should be visible.

6.  Using the 'Instrument' tab, set 'Sample Volume' to 15 $\mu$l, set 'Run Mode' to Standard 7500, and 'Data Collection' as Stage 3, Step 2 (60.0 @ 1.00).

7.  Click 'Add Dissociation Stage'.  **VERY IMPORTANT**

8.  Name your data file via File -> Save As ->SDS Documents -> Grotewiel Lab -> Your Name -> year.mo.da description (e.g. 2011.02.05 daGal4 Akt RNAi).

9.  Click 'Start', wait a couple of minutes to make sure the lid on the machine is heating up.  Run will take ~2.5 hours.  Return to remove plate and retrieve data file.


**Real-Time PCR:  Relative Quantification**

<u>Reaction Set-Up</u>

1.  Dilute primers from 10 pmol/µl to 3 pmol/µl (e.g. 35µl $H_2O$ + 15µl 10pmol/µl primer).

2.  Dilute cDNA samples as determined in cDNA titration/dissociation curve above.

Note:  RT-PCR is highly sensitive.  Knobs on pipettes MUST be taped at all possible steps when pipetting replicate samples.  Change tips after each volume is dispensed.

3.  Make master mix for each diluted cDNA sample according to table below for experiments in triplicate.  A typical experiment would use only 2 primer pairs.  Pulse vortex (and pulse centrifuge if necessary).

|  | each well in plate final (n=1) | Master for 2 primer pairs (n=8) | Master for 3 primer pairs (n=12) | Master for 4 primer pairs (n=16) |
|---|---|---|---|---|
| water | 4.5 µl | 36 µl | 54 µl | 72 µl |
| SYBR | 7.5 µl | 60 µl | 90 µl | 120 µl |
| cDNA | 1 µl | 8 µl | 12 µl | 16 µl |
| F primer | 1 µl | XX | XX | XX |
| R primer | 1 µl | XX | XX | XX |

4.  Label one tube for each primer pair for each diluted cDNA sample (e.g., for 2 primer pairs and 2 cDNA samples, label 2 x 2 = 4 tubes total).

5.  Aliquot master mix @ 49.4 µl /tube from step 4, one tube for each primer pair for each cDNA sample.  Some master mix will remain unused.

6.  Make a master mix of primers for each primer pair by combining/mixing equal volumes of the forward and the reverse primers.  Add 7.6 µl of the primer master mix to the appropriate tubes from step 5.  Pulse vortex (and pulse centrifuge if necessary).

7.  Aliquot in triplicate 15μl of each reaction into a 96 well plate.  Some reaction mix will remain unused.  Spin plate at 1000rpm for 2 minutes using the centrifuge in Rita's lab.

<u>Amplification and Data Acquisition</u>

1.  Turn on 7500 Fast System machine.  Launch 7500 Fast System software via desktop icon.  Place plate in machine.

2.  Click File -> New.  Set 'Assay' to 'Relative Quantification Plate'.  Set 'Run Mode' to 'Standard 7500'.  Click 'Next'.

3.  Click on appropriate detectors, click 'Add', click 'Next'.

4.  A 'New Documents Wizard' interface should be visible.  Assign detectors (primers) to wells by highlight wells and then checking appropriate 'Use' box next to the detector.  With wells highlighted, also assign roles for detectors as 'Endo' (endogenous control) or 'Target' (gene of interest) by using drop down menu under 'Task' for each detector.  Check that wells are labeled with E (endogenous control) or T (target).  Click 'Finish'.

5.  An interface with 3 tabs should be visible.  Using the 'Set-Up' tab, assign genotypes or treatment groups by highlighting groups of wells and labeling them by typing in the 'Well Inspector'.  Genotypes can also be assigned without 'Well Inspector' by highlighting and simply typing.

6.  Using the 'Instrument' tab, set 'Sample Volume' to 15 μl, set 'Run Mode' to Standard 7500, and 'Data Collection' as Stage 3, Step 2 (60.0 @ 1.00).

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7. Name your data file via File -> Save As ->SDS Documents -> Grotewiel Lab ->

Your Name -> year.mo.da description (e.g. 2011.02.05 daGal4 Akt RNAi).

8. Click 'Start', wait a couple of minutes to make sure the lid on the machine is

heating up.  Run will take ~1.5 hours.  Return to remove plate and retrieve data file.

*C.  Standard PCR*

1X Reaction Master Mix

| | |
|---|---|
| $H_2O$ | 25.7uL (or volume to achieve total reaction volume of 50uL) |
| 10X buffer | 5uL |
| 2.5mM dNTPs | 5uL |
| 50mM MgCL | 2uL |
| Primer 1 | 5uL |
| Primer 2 | 5uL |
| Taq | 0.3uL |
| cDNA | 2uL (or volume to achieve 200ng) |
| Total Reaction Volume: 50uL | |

Master Mix should be put in a 0.5mL PCR tube. Reactions run on MSG1 Thermo Cycler

program in Grotewiel Lab.

*D. Agarose Gel Electrophoresis with Ethidium in the Gel*

## A. Pouring the gel

1. Select the comb and gel based on the number of samples.

2. Determine the gel volume using the attached chart.

3. Pour appropriate amount of 1XTAE into dedicated agarose container.

4. Add 1% w/v agarose.  For 100 ml gel, use 100 ml 1XTAE plus 1 g agarose.

5. Microwave gel mixture 1 minute.  Swirl, and continue microwaving until agarose is melted. Typically this requires less than 1 additional minute.

6. Insert casting tray so that gaskets seal on sides.

7. Pipet 2-6 $\mu$l ethidium bromide into bottom of casting tray.  See attached chart.

8. Pour hot gel mixture into casting tray.  Stir gel-ethidium bromide mixture with comb.

9. Insert comb and let gel cool until solid.  If the gel will not be used within 4 hours it must be covered in 1XTAE buffer once it solidifies.

10. Once the gel is solid, gently remove combs and cover the gel in 1XTAE buffer.

## B. Sample preparation and loading

1. Pipet 10X glycerol dyes (1/10 sample volume) into tube caps.  For a 20 $\mu$l reaction, use 2$\mu$l 10X glycerol dyes.

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2. Cap tubes and centrifuge briefly to collect all solutions in the bottom of the tubes.

3. Load 15 μL of 1 kb DNA ladder (with the dye already included) into the molecular weight standards lane.

4. Load appropriate volume of samples, one sample per lane.

5. Load the gel so that all samples, including the molecular weight standards, are centered.

## C. Running the gel

1. Add 2-6 μl ethidium bromide to bottom buffer tank and disperse with pipet tip.

2. Place gel cover with electrodes on gel apparatus.

3. Connect electrodes to power supply, red to red and black to black.

4. Ensure that the red electrode is at the bottom of the gel. *RUN TO RED!*

5. Run gel at 50-100 V until lower dye band has migrated approximately 75% of the distance to the bottom. Check every 20 minutes initially, then more often.

## D. Imaging the gel

1. Remove gel from apparatus using the tray and place on transilluminator.

2. Turn on epi-white light. Center gel, zoom and focus camera.

3. Close door, turn off epi-white light, and turn on UV light.

4. Adjust exposure time until good image appears.

5. Adjust aperture and focus until very good image appears.

6. Freeze image. Annotate as appropriate.

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7. Save file in "TIF" format to your folder on "Grotewiel-1" and print.

8. Image can be manipulated in Photoshop on "Grotewiel-1".

### E. Table

| gel unit | gel size | # of samples | buffer | agarose | ethidium |
|---|---|---|---|---|---|
| B1A | 7 X 8 cm | 6, 10 | 300 ml | 35 ml | 2 μl X 2 |
| B1 | 9 X 11 cm | 10, 14 | 500 ml | 60 ml | 4 μl X 2 |
| B2 | 12 X 14 cm | 12, 20 | 800 ml | 100 ml | 6 μl X 2 |
| A2 | 20 X 25 cm | 16, 24, 36 | 3000 ml | 300 ml | 15 μl X2 |

*E. ethanol Rapid Iterative Negative Geotaxis*

**Fly Preparation and Collection**

*Beginning 21/2 Weeks Prior to Experimentation*

- About 2-4 days prior to seeding bottles the bottles should be taken from the cold room and put into the environmental chamber to dry.

- For each genotype being tested seed 5 to 8 bottles (do not over seed, use only 10 females).

- After seeding bottles should be put back in the environmental chamber.

*1-11/2 Weeks Prior to Experimentation*

- After 1 week in the environmental chamber the parents should be removed from the bottles (as long as there are a significant number of larvae and pupae present on the sides of the bottle). These parents can be used to seed a new set of bottles for a second brood or dumped depending on the needs of the experiment.

- Bottles should be placed back into the environmental chamber. If a second brood has been set up repeat the above steps of waiting a week then this time dumping the parents.

*Week of Experimentation*

- After an additional 5-10 days there should be enough flies to collect from the first brood for experimentation. About 1 day prior to collecting set out 3 vials of food for each genotype and control being tested in the environmental chamber from the cold room.  These vials should be **non-yeasted**.

- Collection of these flies should be done with minimal use of anesthesia.

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- Flies of a single sex should be collected.  Typically only females are collected but this can differ depending on the gene and the restraints of the experiment.

- 25 flies of a single sex are collected then placed into a 1.5mL tube to restrict the amount of anesthesia getting into the vial. The flies from the 1.5mL tube are dumped into the vial.  Label the vial accordingly and place the vial on its side until the flies have woken up.

- Once 3 vials for each genotype and control are collected, place the vials back into the environmental chamber overnight. Testing should begin the day after collection.

**Experimental Preparation**

*Day of/Day Prior to Experimentation*

- Flugs need to be prepared the day of or the day before experiment and not before to prevent problems with the flugs.

- Flugs are found on the top shelf in the fly room of the middle bench on the side facing the dissecting microscopes.

- Flugs come larger than necessary for the experiment. Cut flugs with a blade width-wise so that they are roughly the same size. You can cut a single flug into about 3-4 pieces while maintaining the same general size.

- Place glue on one side of the flug without getting too much on the flug. Glue used for this procedure is found in the top drawer in the middle of the bench with the dissecting scopes.

- Place the flug with the glue into a clean vial (i.e. without food) with forceps. Gently guide the flug to the bottom of the vial so that glue does not get on the sides of the vial. At this point the flug is pointing vertically. When you do get it so that it is touching the bottom of the vial, gently push it so that the side with the glue is facing the bottom of the vial. Smash the flug into the bottom of the vial so that it fits snuggly and will not come loose.

- Repeat this for as many vials needed for the experiment. Once they are all completed place them upside down in a vial box and place them either on the bench in the fly room or in the RING testing room.

*Day of Experiment*

- Prior to experiment and bringing flies into the room the humidity in the RING testing room should be set so that the humidity is about 55-60%. This is done by turning on the humidifier on the right wall and adjusting accordingly. Make sure there is water supply in the humidifier reservoirs.

- Dilute ethanol to the concentration that was found to be optimal in dosage testing (make enough for the total experiment). Water used for this dilution should be ddH20 which is from the purifier in the back of the lab.

- Get a bottle/tube of ddH2O as well from the same source

- Turn the light source on under the light diffuser on the table

- Place memory card in camera and align the camera and tripod so that it frames the table and produces a straight picture. You may wish to take a test picture before the test to ensure correct alignment.

- Bring the flies into the test room to acclimate at least a few minutes before the test begins.

**Experimental Procedure**

*Water Test*

1. Secure flugs to the vial by pressing down on the flugs with the end of forceps
2. Pipette 500uL of ddH$_2$0 onto the flug being careful not to drip water onto the edges of the vial.
3. Flip flies from each vial into individual vials with flugs for one set of flies. Cover each vial quickly with an orange cap so that no flies escape.
4. Place the capped vials into the rig with the orange cap on the bottom. Carefully place the top of the rig so that the vials fit securely in the grooves.
5. Place a label on the right panel of the rig describing the genotype, the test (i.e. water), and date.
6. Place the rig in front of the light diffuser and make sure the camera is turned on.
7. Rap the rig three times on the table and start the timer after 3$^{rd}$ rap.
8. When the timer reaches 3 seconds, press the shutter button on the camera to take the picture
9. Wait until the timer reaches 1 minute and rap the rig against the table 3 times.
10. When the timer reaches 1 minute 6 seconds, press the shutter button on the camera to take the picture
11. Repeat steps 9 and 10 for minute 2.

12. Remove the top of the rig and flip flies back into their original vials until ready to do EtOH test.

13. Repeat all steps for each set of flies that are being tested.

NOTE: You may wish to record the pictures being taken in a notebook for reference when doing data analysis.

*EtOH Test*

1. Secure flugs to the vial by pressing down on the flugs with the end of forceps

2. Pipette 500uL of diluted EtOH onto the flug being careful not to drip EtOH onto the edges of the vial.

3. Flip flies from each vial into individual vials with flugs for one set of flies. Cover each vial quickly with an orange cap so that no flies escape.

4. Place the capped vials into the rig with the orange cap on the bottom. Carefully place the top of the rig so that the vials fit securely in the grooves.

5. Place a label on the right panel of the rig describing the genotype, the test (i.e. EtOH), and date.

6. Rap the rig three times on the table and start the timer after 3$^{rd}$ rap.

7. When the timer reaches 3 seconds, press the shutter button on the camera to take the picture

8. Wait until the timer reaches 1 minute and rap the rig against the table 3 times.

9. When the timer reaches 1 minute 6 seconds, press the shutter button on the camera to take the picture

10. Repeat steps 9 and 10 for each minute thereafter. Continue until over ½ of the flies climb only ½ way up the vial in each vial or until the 20th repetition.

11. Flip flies back into their vials. At this point the testing is done for this set of flies. You may either throw out these flies or keep them for other types of testing.

12. Repeat the above steps for each set of flies being tested.

**Clean-Up**

1. Remove flugs from vials and throw out the flugs. Place vials and orange caps into a gray bin beside the sink in the fly room to be cleaned.

2. Turn off light source, camera and humidifier

3. Make sure rig is put away and everything is in order

4. Turn off light and close and lock door to testing room when done.

**Data Analysis and Processing**

*Photo Processing*

1. Put memory card into card reader and plug card reader into the computer. Copy pictures from card into a folder on your desktop.

2. Open Adobe Photoshop 5.0

3. In Photoshop open pictures you wish to edit. (NOTE: Photoshop has an upper limit of the number of pictures that can be opened at a time. 18 pictures is usually a good number to work with at a time)

4. On the right hand side of the screen a menu should be present in the menu press the "Create New Action" button.  This will cause a screen to appear asking you to

name and change certain settings. You can name the action if you wish otherwise press "Record" to continue.

5. Use the marquee tool to draw an outline around the vials and the panel with the label. In the Image menu in the toolbar select crop.

6. In the Image menu in the toolbar select Mode, then select grayscale. It will ask if you wish to discard color information, press ok to continue.

7. In the Image menu in the toolbar select Image Size. This will come up with a screen where you can adjust the picture. Change image height to 3 inches. This will automatically adjust the width. Press ok to continue.

8. In the File menu in the toolbar select Save As. Save a copy of the picture in TIFF format to the folder you created on your desktop.

9. Close the picture that was being edited.

10. Press the stop button on the right hand menu to stop the recording

11. Press play for each picture, this will do all of the above steps in editing the picture.

12. Continue for all the pictures for the experiment.

13. Close Photoshop and open Scion image.

14. In Scion Image open options in the Analyze menu in the toolbar. Make sure that only X&Y Center is checked, the max measurement is set to 8000, the field width is 9 and the digits to the right of decimal point are 5. In the Options menu select preferences and make sure the ClipBoard Buffer Size is set 99999 and the boxes for Invert Y-axis and Desktop Friendly are checked. If these are not the defaults

then change them, and in the file menu select record preferences to make these the default choices.

15. In the toolbar open the Special Menu.  In the Special menu select Load Macros.

16. In the Macros folder select the ScreenMacros

17. Open TIFF formatted pictures in the file menu (NOTE: It might be easier to start off with only 3 or 4 pictures)

18. Once the pictures are open, select Subtract Background from the Special Menu

19. Select Threshold in the Special Menu. All pictures that are opened in Scion Image will be edited when Subtract Background and Threshold are executed. It is therefore unnecessary to repeat this process for each picture open.

20. Choose the eraser tool and erase anything in the picture that is not a fly.

21. Choose the pencil tool and make the "ink" white.  Draw lines to separate any flies that look to be joined in the picture.

22. In the Analyze menu select Analyze Particles. In the screen that pops up make sure minimum particle size is set to 3 and max is set to 50.  Make sure the options for Label Particles and Reset Measurement Counter are selected only.

23. The picture will now have numbers where the flies are. Click on the picture and press Ctrl-C to copy these values.

24. Paste the values into a blank excel sheet labeling each set of values for reference.

25. Repeat this for each picture.

*Analysis*

Analysis needs to be done in sets based on genotype and EtOH concentration.

1. Open the Excel file named "eRING Reduction MASTER 2007".  In this file change the date to correspond with the date tested.  For each set you will also need to change the genotype, EtOH concentration and Time depending on the picture.

2. Open the Excel file named "eRING Compiled". Nothing needs to be changed in this file.

3. In the "eRING Reduction MASTER 2007" paste the values for the first picture of the set. Change any information regarding the genotype, EtOH and time as necessary.  For the first picture of the set the time should be set to 0. For each picture after this in the set increment time by one (i.e. Time 0 would be the first picture, Time 1 would be the second picture and so on.)

4. In the "eRING Reduction MASTER 2007" once the values are pasted in the left hand side of the sheet, press Ctrl-Shift-E. This will run the Macros for the two Excel sheets.

5. Save the "eRING Reduction MASTER 2007" by selecting Save As from the File menu and save it accordingly. Close the "eRING Reduction MASTER 2007" file. DO NOT save the "eRING Compiled" file at this time.

6. Open a new "eRING Reduction MASTER 2007" file and change the date, genotype, EtOH concentration and time to correspond to the second picture of the set.

7. Repeat steps 3-5 for each picture in the set.  For water there will only be 3 value sets to analyze for each genotype. For EtOH there will be more than three value sets. Once the mean reaches below 0.5 in the "eRING Compiled" file you do not need to analyze any further pictures for that set.

8. Once the set is complete save the "eRING Compiled" file by selecting Save As from the File menu and save it accordingly.

9. Repeat this process for each set.


*Data Processing and Clean-Up*

1. In the water "eRING Compiled" files, copy the individual vial values from the tab labeled "Individual Vial T50s". Paste these values into a new excel sheet and average the values for each vial.

2. In the "eRING Compiled" files for EtOH, create a copy of the "Individual Vial T50s" sheet by right clicking the tab and selecting Create Copy and Move to End from the window that pops up.

3. In the copied sheet on the right hand side, copy the values for each vial and paste them at one time point lower. At time point 0 paste the average water values for that genotype by copying those values and right clicking and selecting Paste Special. A window will pop-up, select values and press OK.

4. Looking at the graph, delete values for each vial where the values seem to level off.

5. Copy these values and paste them into a PRISM file to create a line graph.

6. Copy the T50 values from the copied and now modified "Individual Vial T50s" into PRISM to make a bar graph. Each graph (line and bar) should contain the mutant values and the control *w[A]* values.

*F.  Internal Ethanol Content*

**Ethanol Content Protocol**

**Use pre-chilled solutions throughout assay for consistency**

1.  Grow/collect flies and prepare Flugged vials as you normally would for eRING assays.  Use 25 flies/vial and typically 1 vial/genotype with 3-5 vials total per test. Expose one group to $ddH_2O$ (0 minutes) and other groups to ethanol (0.5 ml in Flug) during eRING tests for 5 and 10 minutes or other times as appropriate.

2.  After each water or ethanol exposure, transfer flies to labeled 1.5 ml snap-cap tubes and store at $-20°C$.  Continue water and ethanol exposures until you have a complete set of frozen flies from each genotype at each time-point.

3.  Homogenize frozen flies with drill/pestle in 200 μl ice-cold $ddH_2O$ for 30 seconds. Keep flies on ice before and after homogenization.

4.  Centrifuge homogenized flies at maximum speed at $4°C$ for 20 minutes.  Prepare 25 mM standard by adding 4.37 μl of 100% ethanol to 2996 μl ice-cold $ddH_2O$.  Prepare remaining standards using the table below.  Store standards on ice.

5.  Transfer 100 μl of clear supernatant to new labeled 1.5 ml snap-cap tube.  Lipid or other crud will stick to the outside of the pipette tip.  DO NOT TOUCH PIPPETTE TIP TO NEW TUBE!  Store 100 μl supernatants on ice.

6.  Add 300 μl of cold ethanol reagent to 1.5 ml snap-cap tubes for each sample (in triplicate, 3 tubes/sample) and standard (in duplicate, 2 tubes/standard).

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7. Add 10 µl of each sample supernatant and standard to the corresponding tube from step 6.  Mix by single pulse vortexing.

8. Incubate at 30°C in heat block for 5 minutes.

9. Read absorbance of 100 µl of each reaction at 340 nm and interpolate values for samples in Prism.

10. Final ethanol concentration in samples determined as:  mM interpolation x(3/10)x (200 µl/[# flies x µl/fly]).  Fly volume in µl is determined as indicated on next page.  Each vial of flies is an N of one.

Notes:
1.  Standards

| Standard | Volume of ddH$_2$O | Volume of 25 mM |
|---|---|---|
| 0 mM | 1000 µl | 0 |
| 5 mM | 800 µl | 200 µl |
| 10 mM | 600 µl | 400 µl |
| 15 mM | 400 µl | 600 µl |
| 20 mM | 200 µl | 800 µl |
|  |  |  |
| 25 mM | 2996 µl | 4.37 µl (100% ethanol, 17.16 M) |

2. Alcohol Reagent:  Dilute per manufacturer's instructions.  Good for at least 2 weeks at 4°C.
3.  Reaction is maximal at ~2 minutes and has a stable product (i.e. A340) out to at least 12 minutes.
4.  Use all cold reagents for consistency.
5.  A 10 minute exposure to vapor from 50% ethanol in an eRING assay should lead to a final internal ethanol concentration of ~150 mM in control flies.  Each vial of flies is an N of one.

**Fly Volume Protocol**

1. Grow/collect flies as you normally would for eRING assays. Use 25 flies/vial and typically 3 vials/genotype. Each vial of flies is an N of one.

2. Drill 2-3 holes in the lids of 1.5 ml snap-cap tubes (one tube for each vial of flies in step 1) using a flame-heated needle (~20 gauge, large enough for $CO_2$ to enter but small enough to retain flies). Be careful!

3. Weigh each 1.5 ml snap-cap tube from step 2 using the Mettler PB153S balance and record the tube weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube weight*.

4. Anesthetize flies in vials and transfer to weighed tubes from step 3. Place tubes upside down on $CO_2$ plate in quarantine area to keep the flies anesthetized.

5. Weigh each tube containing flies from step 4 using the same balance and record the weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube wet weight*.

6. Place the tubes with flies in a 55°C dry incubator for 18-24 hours to desiccate.

7. Weigh each tube containing desiccated flies from step 6 using the same balance and record the weight out to 3 decimal places on the side of each tube or elsewhere. This is the *tube dry weight.*

8. Determine the <u>total weight</u> of each fly: ([tube wet weight] – [tube weight])/# flies. This should be ~1.5 mg/fly. Females will be bigger than males.

9. Determine the <u>water weight</u> of each fly: ([tube wet weight] – [tube dry weight])/# flies. This should be ~0.8 mg/female and 0.6 mg/male.

10. One mg of water weight = one µl of water volume.  The water volume is used to calculate the internal ethanol concentration (see previous page).

**Citations:**

Moore, M. S., J. DeZazzo, A. Y. Luk, T. Tully, C. M. Singh and U. Heberlein (1998). "Ethanol intoxication in Drosophila: Genetic and pharmacological evidence for regulation by the cAMP signaling pathway." Cell **93**(6): 997-1007.

Bhandari, P., J. C. Bettinger, A. G. Davies, K. Kendler and M. Grotewiel (2009). "An assay for evoked locomotor behavior in Drosophila reveals a role for integrins in ethanol sensitivity and rapid tolerance." Alcohol Clin Exp Res **33**(10): 1794-805.

*G. Sequencing Sample Preparation*

PCR samples with sequencing primers using 200ng of gDNA.

Take 5ul of each sample and add it to 2ul of ExoSap-IT

Run samples on the following Thermal Cycler Protocol:

    37C for 30min

    80C for 15min

    4C for ever


Add 2ul of forward primer, and 1ul of water to ExoSap cleaned PCR products.

Use the Low Cost method of primer extension sequencing at ACGT Inc.

VITA


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