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Genetics and Genomics of Alcohol Responses in Drosophila

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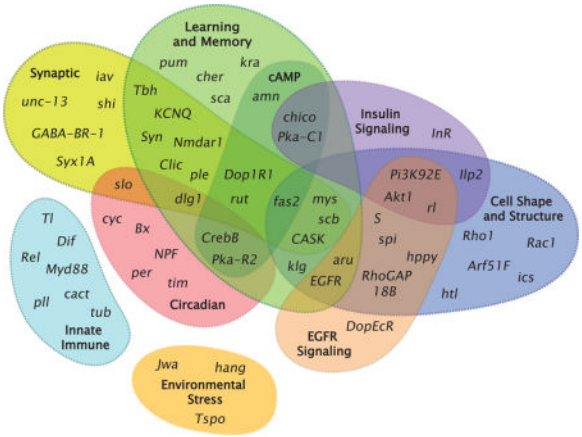
Abstract

Drosophila melanogaster has become a significant model organism for alcohol research. In flies, a rich variety of behaviors can be leveraged for identifying genes affecting alcohol responses and adaptations. Furthermore, almost all genes can be easily genetically manipulated. Despite the great evolutionary distance between flies and mammals, many of the same genes have been implicated in strikingly similar alcohol-induced behaviors. A major problem in medical research today is that it is difficult to extrapolate from any single model system to humans. Strong evolutionary conservation of a mechanistic response between distantly related organisms, such as flies and mammals, is a powerful predictor that conservation will continue all the way to humans. This review describes the state of the Drosophila alcohol research field. It describes common alcohol behavioral assays, the independent origins of resistance and tolerance, the results of classical genetic screens and candidate gene analysis, and the outcomes of recent genomics studies employing GWAS, transcriptome, miRNA, and genome-wide histone acetylation surveys.

Graphical abstract



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Classical genetics and genomics share a common thread of unbiased investigation that is not limited by one's preconceptions. Classical genetics of the kind usually practiced in Drosophila begins with single-gene mutations that disrupt a phenotype of interest. This works as a perfect filter, and only mutant alleles with large effect size are recovered. The one-gene-at-a-time approach must eventually assemble collections of these functionally validated genes into a sensible network. In a sense, genomics operates in the opposite direction. At the start, networks of genic differences are correlatively associated with a phenotype, and it is at the end that functional validation of network members occurs.

Because classical Drosophila genetics already facilitated gene-phenotype discovery, Drosophila labs were slow to embrace modern GWAS, RNA-seq, and ChIP-seq analyses. For the most part, technological advances in Drosophila focused on better tools for mutating genes that simplified the molecular identification of the gene- and tissue-specific analysis of gene function and expression—two of the most difficult aspects of genetic studies in Drosophila (Bellen *et al*, 2011). This review explores the recent genetic and genomic advances in the study of alcoholism-related phenotypes in *Drosophila melanogaster*. Throughout this article, the terms *Drosophila* or *flies* will be used to refer exclusively to *D. melanogaster*.

Model organism research has been essential for determining how the nervous system adapts to and is altered by alcohol, and each model organism affords distinct advantages and limitations. As a model system, Drosophila represents an appealing compromise. While the fly genome has about half the number of genes as the human genome, most of the genes found in mammals also exist in flies (Rubin *et al*, 2000). Furthermore, Drosophila show many of the same ethanol responses as humans do. Low doses of ethanol produce hyperactivity, moderate doses incoordination, and high doses sedation (Wolf *et al*, 2002). Flies also acquire ethanol tolerance and exhibit functional dependence, symptoms of withdrawal, disinhibition, and ethanol preference in drinking assays and will undergo unpleasant experiences to acquire ethanol.

Despite the great evolutionary distance between flies and mammals, many of the same genes have been implicated in strikingly similar alcohol-induced behaviors. A major problem in medical research today is that it is difficult to extrapolate from any single model system to humans. Strong evolutionary conservation of a mechanistic response between distantly related organisms, such as flies and mammals, is a powerful predictor that conservation will continue all the way to humans. An advantage of Drosophila as an experimental animal—which one cannot overemphasize—is the value of the Drosophila community, whose culture of sharing and open access to newly developed genetic tools dates back more than 100 years. Flies are one of the few animals in which community resources provide access to mutations and RNAi and overexpression transgenes for almost all genes. Low cost makes practical the screening of large numbers of genes, and the rapid life cycle means that new combinations of genetic tools can be quickly created.

With respect to alcoholism research, Drosophila is a unique model organism in that wild Drosophila, like humans, have a close relationship with ethanol (Nunney, 1996). *D. melanogaster* females find food attractive for egg laying once a fermentation is well

underway, and both the adult and offspring are more resistant to the fermentation products ethanol, acetic acid, and CO_2 than some of their close drosophilid competitors are (McKenzie and Parsons, 1972; McKenzie and Parsons, 1974; McKenzie and McKechnie, 1979; Parsons and Spence, 1981; Capy and Gibert, 2004). Furthermore, a low concentration of ethanol in the food increases fitness—perhaps by sanitizing the food and increasing its caloric density (Parsons and Spence, 1981). These attributes provide *D. melanogaster* with a transient advantage for capitalizing on this niche.

The collection of complex behaviors displayed by flies—walking, climbing, flying, sleeping, learning, courting, mating, fighting, and communicating—offer a rich variety of ways to probe ethanol effects. However, the most common approach for the initial phase of testing has been to use the simplest behavior to measure—sedation. Alcohol resistance is the innate sensitivity to alcohol and is quantified during the first alcohol exposure. Alcohol tolerance is defined as alcohol-induced alcohol resistance. Often alcohol resistance and the capacity to acquire alcohol tolerance are measured in the same experiment. Both rapid and chronic tolerance have been studied in flies (Berger *et al*, 2004). Rapid tolerance is tolerance produced by a single exposure that is present after alcohol clearance, while chronic tolerance is the product of repeated alcohol exposure. Acute tolerance (manifest while the alcohol exposure is occurring) has not been studied in flies. Both rate of sedation and rate of recovery from sedation have been used as metrics. Adult flies are ideal for studying the mechanism of functional tolerance because the adult does not acquire metabolic tolerance (Scholz *et al*, 2000). Thus, neural alcohol adaptation can be studied in the absence of confounding changes in the rate of alcohol metabolism.

Two very common assays

The most common methods for measuring ethanol resistance and tolerance in flies are the inebriometer and visual observation. The inebriometer (graphically described in Fig.1A) is a large vertical tube through which an ethanol/air mixture continuously flows (Cohan and Graf, 1985; Weber, 1988). The tube contains a radial spiral of scoop-like ledges. Flies are placed in the top, where they grasp and stand on the scoops at the top. But as they succumb to ethanol intoxication they lose the ability to hang on to a scoop tumbling between scoops and eventually out the bottom, where they are machine counted. This device separates flies based on their rate of intoxication or their degree of incoordination to a given ethanol dose. Differences in the mean elution time (MET) are a measure of the relative resistance of flies to ethanol intoxication. The inebriometer can be used to quantify innate resistance and—when applied to flies that have previously been exposed to ethanol—tolerance. A caveat concerning the inebriometer is that it is events other than sedation or simple incoordination can influence the elution time, for instance flies undergoing severe seizures should elute extremely rapidly because they cannot hang on. In practice, this has not been a problem.

Quantifying ethanol resistance visually is more often used in fly labs because it requires no special equipment. In its simplest incarnation, flies are placed in vials whose top is sealed with a cotton plug to which an ethanol solution is added (e.g., 1 ml of 35% ethanol; Fig.1B). In these evaporative vial exposures, ethanol vapor concentration climbs over time, and time is used as a proxy for ethanol concentration. At early time points, low-dose ethanol produces

hyperactivity. As the ethanol concentration climbs, flies become uncoordinated and fall from the wall (flies are naturally negatively geotactic). At later time points, incoordination becomes so severe that flies are unable to climb and remain on the bottom of the vial. This is followed by a loss of postural control followed by sedation. Usually, the time of occurrence of one or more of these endpoints is recorded. This assay has many variations. Sometimes the source of ethanol is from the bottom, sometimes it is pumped into the vial at fixed concentration, sometimes the vials are agitated to better evaluate when the capacity to initiate climbing is lost, and sometimes automation is used to quantify the behaviors (Cowmeadow *et al*, 2005; Wen *et al*, 2005; Ramazani *et al*, 2007; Bhandari *et al*, 2009; Pohl *et al*, 2013). Crabbe *et al*, (2005) have shown, in mice, that different alcohol-induced ataxia assays have distinct genetic dependencies; that is, no single behavioral assay appears able to capture all genes that affect alcohol induced behavior. Whether this is true in the fly is not yet known because a thorough comparison of the genetic sensitivity of the different Drosophila sedation (or hyperactivity or ataxia or tolerance) assays has not yet been conducted. However, there are some examples that show that different dependences exist—for example, different RhoGap variants underlying ethanol stimulation and intoxication (Rothenfluh *et al*, 2006).

In the evaporative assay, there is daily variation in the rate of sedation, probably due to changes in the ethanol evaporation rate caused by variation in humidity or barometric pressure. As a result, all stocks to be directly compared should be tested at the same time. This variability has sometimes led non-Drosophila researchers to doubt whether using such behavioral endpoints is meaningful. If the behavioral endpoint is meaningful, it should consistently occur at the same internal ethanol concentration. Confirmation that a biological endpoint reflected a response to a specific dose of ethanol was most thoroughly demonstrated by Pohl *et al*, (2013), who repeated an evaporative sedation assay over many days. When 50% of the population was sedated (determined by visual inspection, referred to as K50), the internal ethanol and water content of the flies was determined. Despite the fact that the K50 varied from 29 to 71 minutes, the internal ethanol concentration at K50 showed little variation. It was determined to be 111 mM +/− 2.3 mM ethanol (n=39, Fig.2). This was not a ceiling effect since heavily sedated flies achieve more than twice this hemolymph concentration (Cowmeadow *et al*, 2005). Thus, using behavioral endpoints and time as a proxy for ethanol concentration is reasonable as long as the experiments to be directly compared are performed at the same time.

Ethanol preference

Quantifying alcohol consumption in flies has been challenging due to the small meal size. The most commonly used method is the CAFE assay, which emulates two-bottle choice and compares consumption from microcapillary tubes of ethanol-laced and ethanol-free food (Ja *et al*, 2007). In the CAFE assay flies prefer ethanol food and following starvation can be made to drink to pharmacologically relevant levels (45 mM equivalent to ~0.21 %BAC; Devineni and Heberlein, 2009) although Pohl *et al*, (2012) showed that, without forced starvation, the consumption rate and sip size produced a peak ethanol concentration of about 4 mM (equivalent to ~0.013 %BAC). Flies also will overcome aversive stimuli to consume ethanol (Devineni and Heberlein, 2009; Kaun *et al*, 2011). Furthermore, Devineni and

colleagues measured ethanol preference in a series of mutants sensitive or resistant to ethanol and found a strong correlation between increased ethanol preference and the ability to acquire tolerance. The capacity for tolerance was suggested as a reason for increased consumption over time (Devineni *et al*, 2011).

In non-consumptive assays, preference can be quantified by pairing olfactory cues to an ethanol stimulus. The flies are later tested with the olfactory cues to infer their preference for ethanol. This is usually done through use of the Y-maze (Fig.3; Kaun *et al*, 2011). Kaun *et al*, (2011) used the Y-maze to probe the rewarding aspects of ethanol. Male flies were trained to associate the intoxicating effect of ethanol with an odorant. Thirty minutes after training, the males show aversion to the ethanol paired odorant but 24 h later they show increased attraction to the odorant. Importantly, this associative learning is dependent on dopaminergic signaling. In adult Drosophila, multiple intoxicating ethanol exposures will increase the attraction to an odor paired with the ethanol treatment. In addition, Shohat-Ophir *et al*, (2012) show that sexual rejection in male flies increases ethanol intake, indicating a compensation between natural and drug rewards. Another non-consumptive method to quantify preference is the proboscis extension response (PER). In this assay, an immobilized fly is allowed to smell or taste a substance. Extension of the proboscis indicates that the substance is favorable (Shiraiwa and Carlson, 2007). Finally, preference has also been measured by recording the differential oviposition onto a particular food substrate. Foods containing the greatest number of eggs are interpreted as preferred (Richmond and Gerking, 1979).

Classical genetic screens and candidate gene analysis

Genetic analysis of alcohol responses in flies are initiated from two fundamentally distinct origins, these are unbiased 1) classical genetic screens and candidate gene surveys and 2) genomic surveys. Classical genetic screens identify important genes but do not provide a context for their understanding. Genes have memberships in many pathways, and not knowing which membership is relevant for a phenotype can be limiting. However, as more and more genes are identified and epistatic interactions between genes are evaluated, pathways and gene ontology relationships start to become clear. Ideally, mutant analysis should be done to saturation. This was key to the Drosophila developmental genetics breakthroughs made in the 1980's (Nusslein-Volhard and Wieschaus, 1980). This has not yet been achieved for alcohol responses because of the small number of fly alcohol research labs. Over time, mutant analysis is starting to implicate specific pathways over and over. The Venn Diagram in Fig.4 groups alcohol-resistance and tolerance genes identified by classical genetic approaches into the gene ontology-type sets. Throughout this document, gene names are in italics and protein names are capitalized in roman type.

Learning and Memory genes

Alcoholism research in flies began in the laboratory of Ulrike Heberlein. Using the inebriometer, they identified an allele of *amnesiac* (*amn*) that altered ethanol sensitivity (Moore *et al*, 1998). A known memory gene, *amn* was thought to modulate adenylate cyclase. Follow-up work showed that other genetic manipulations of cAMP that affected

learning also altered ethanol sensitivity (Fig.4 and reviewed in Heberlein *et al*, 2004). Because cAMP signaling was already linked to mammalian alcohol responses (reviewed in Diamond and Gordon, 1997), an important aspect of this work was the demonstrated conservation of alcohol responses between flies and mammals. The learning gene category quickly became the most highly represented category connected to alcohol responses. Berger *et al*, (2008) showed that from a collection of 52 learning mutants, 21 had altered ethanol responses and that because resistance and tolerance could be independently affected, resistance and tolerance must have distinct genetic origins.

Three genes known to function in learning or memory—*kra, rut*, and *sca*—were later shown to play a role in naive ethanol preference. Mutations in *kra* that affect long-term memory, ethanol sensitivity, and tolerance to ethanol were tested for ethanol preference using the CAFE assay. Although *kra* mutants showed decreased preference relative to controls, this decreased preference is not an intrinsic property of memory mutants since not all memory mutants affect preference (Devineni and Heberlein, 2009). Xu *et al*, (2012) also measured drinking preference for the learning mutants *rutabaga* (*rut*) and *dunce* (*dnc*). The *rut* gene encodes adenylate cyclase, and *dnc* encodes cyclic nucleotide phosphodiesterase; mutations in the former decrease cAMP while mutations in the latter increase cAMP. Mutants for *rut* showed reduced preference, whereas a mutation in *dnc* did not affect preference. Furthermore, *rut* animals are hypersensitive to ethanol while *dnc* animals show normal ethanol LOR (Moore *et al*, 1998; Devineni and Heberlein, 2009). The involvement of *scabrous* (*sca*; a learning gene) was shown in conditioned ethanol preference assay (Kaun *et al*, 2011). The Scabrous protein can act as a ligand of the Notch cell membrane receptor (Lee *et al*, 2000). Notch signaling pathways have been identified in genomic surveys of alcohol-responsive genes in both flies and mammals (Melendez *et al*, 2012; Morozova *et al*, 2015). The study of *Notch* with regards to ethanol responses in flies should proceed rapidly since the fly community has been accumulating *Notch*-specific tools since the 1917 discovery of *Notch* in Drosophila (Morgan, 1917).

BK-type Ca^{2+}-activated K^+ channels—the *slo* gene

The role of the Drosophila *slo* gene in alcohol tolerance was first seen in studies examining homeostatic regulation of *slo* in response to organic solvent sedation (reviewed in Atkinson, 2016). Ghezzi *et al*, (2004) showed that flies acquire benzyl alcohol (BA) tolerance, BA sedation induces *slo* expression, *slo* mutants block BA tolerance, and transgenic *slo* induction in the nervous system can phenocopy functional tolerance. Cowmeadow *et al*, (2005, (2006) made the same observation about the relationship between *slo* and functional ethanol tolerance and showed that ethanol and BA produced mutual cross tolerance. This indicated that these drugs produced tolerance by overlapping mechanisms, a fact that would prove useful in other analyses (below). The role of BK channels in tolerance is conserved in mammals. Mammalian BK channels, encoded by *slo*, are a well-known target of ethanol (Knott *et al*, 2002). In the rat supraoptic nucleus, ethanol tolerance is acquired by miRNA regulation that suppresses the translation of ethanol-sensitive mRNA isoforms while permitting the translation of isoforms that encode ethanol-resistant BK channels (Pietrzykowski *et al*, 2008).

Tolerance and withdrawal have long been hypothesized to be mechanistically related. The underlying idea is that drugs, such as alcohol, trigger adaptive changes that homeostatically counter an effect of the drug, resulting in functional tolerance (Martin, 1968; Solomon and Corbit, 1974). However, after drug clearance, the adaptation persists and becomes counter-adaptive—generating a symptom of withdrawal that is opposite to the effect of the drug (Solomon, 1980; Koob, 1996; Littleton, 1998; Koob and Le Moal, 2006).

Working with *slo*, we showed that the response of *slo* to alcohol sedation satisfied the criteria of the homeostatic theory of tolerance and withdrawal (Ghezzi *et al*, 2010). It has been shown that organic solvent exposure depresses excitability by increasing the neural refractory period (Lin and Nash, 1996; Ghezzi *et al*, 2010). Our work showed that alcohol-induced expression of *slo* decreases the refractory period, thus increasing the capacity of the nervous system for repetitive firing. After alcohol clearance, *slo*-mediated increased excitability persists, reducing the seizure threshold of the flies and producing a withdrawal symptom. All of these changes can be blocked by a mutation in *slo* and phenocopied by transgenic *slo* expression (Ghezzi *et al*, 2010; Ghezzi *et al*, 2014). Alcohol withdrawal hyperexcitability is also observed in humans (Finn and Crabbe, 1997).

To understand how *slo* "senses" sedation, the transcriptional response of *slo* to alcohol was investigated. Histone acetylation changes are symptomatic of transcription factor action as the transcription factors manipulate changes in gene expression. Therefore, we mapped alcohol-induced histone H4 acetylation changes across the *slo* transcriptional control region to identify sites of ethanol-induced transcription factor activity. BA-induced acetylation spikes mapped to three CREB binding-site motifs (Wang *et al*, 2007). These were later shown to be sites of BA-induced phosphoCREB binding. Dominant negative mutations in CREB were shown to block BA-induced acetylation changes, induction of *slo*, and behavioral tolerance (Wang *et al*, 2009). This demonstrated that *slo* "senses" alcohol-sedation in part through the action of the CREB transcription factor.

Another DNA element to be identified was the 60 n 6b element, which was proposed to be a negative regulator of *slo* that limited alcohol induction of the gene. This was confirmed by precisely deleting the element from the endogenous *slo* promoter region. Without this element, alcohol induced *slo* to a greater extent in the CNS, and tolerance persisted for an abnormally long period of time (normal BA tolerance = ~10 days, mutant = >28 days; normal ethanol tolerance = ~10 days, mutant = >21 days). We also observed an increase in the intensity of the withdrawal response. No other behavioral changes were detected in the deletion mutant (Li *et al*, 2013; Krishnan *et al*, 2016). The transcription factor(s) that bind 6b have not yet been identified. These experiments convincingly demonstrated the utility of using changes in histone acetylation to map alcohol-responsive DNA elements. In light of this, we used genome-wide histone acetylation assays to identify other alcohol-responsive genes (below).

Other Synaptic Genes

Drosophila and mammals share many of the same neurotransmitters (NTs), including norepinephrine, epinephrine, serotonin, dopamine, acetylcholine, glutamate, histamine, gamma-aminobutyric acid (GABA), and glycine (Jackson *et al*, 1990; Monastirioti, 1999). In

mammals, ethanol has been shown to affect a number of these neurotransmitter signaling pathways (Lovinger *et al*, 1989).

Dopamine was shown to be necessary in Drosophila for normal ethanol-induced hyperexcitability and preference—a well-known association in mammals. However, in flies dopaminergic signaling does not directly modulate the sensitivity to ethanol sedation (Shen *et al*, 1995; Boileau *et al*, 2003; Kong *et al*, 2010b). Dopamine signaling in flies is also crucial for retrieval of intoxication-dependent odor-associated ethanol preference when tested in the Y-maze (Kaun *et al*, 2011). Female flies are choosy about the ethanol concentration of food for oviposition. In an oviposition assay, Azanchi *et al*, (2013) demonstrated that ethanol aversion and preference were modulated by distinct competing dopaminergic neuropil. Other NT systems implicated in ethanol behaviors include the $GABA_B$ receptor encoded by *GABA-B-R1*—shown to be important for normal ethanol sensitivity (Dzitoyeva *et al*, 2003)—and the NMDA receptor, (Nmdar1 subunit)— shown to be important in flies to fully express ethanol tolerance (Maiya *et al*, 2012). Finally, knockdown of a Ca^{2+}-sensitive PKC (*Pkc53E*) in serotonergic neurons resulted in ethanol resistance, a phenotype that could be reversed by pharmacological inhibition of serotonin reuptake (Chen *et al*, 2010).

Additionally, Drosophila and mammals share the NPF/NPY neuropeptides that are relatively similar in sequence and function in hunger and stress signaling (Nässel and Wegener, 2011). Reduced NPF expression in flies and reduced NPY expression in mammals promote increased ethanol consumption and resistance (Thiele *et al*, 1998). Reducing the expression of NPF or its receptor, NPFR, were both shown to cause ethanol resistance, while overexpression of either one produced sensitivity. However, manipulating NPF/NPFR1 did not change sensitivity to diethyl ether, suggesting this neuropeptide does not modulate general sedation but modulates sedation specific to ethanol (Wen *et al*, 2005). Shohat-Ophir and Heberlein (2012) showed that NPF signaling reflects the reward state in flies, that ethanol is perceived as a reward that is registered in the NPF-NPFR signaling axis, and that modifying NPF levels can mask or enhance the rewarding effects of ethanol.

Two NTs, less common in mammals but used in flies, are octopamine and tyramine (Evans and Gee, 1980; Lewin, 2008). Tyramine and octopamine have been suggested to serve as the functional equivalents of epinephrine and norepinephrine in flies (Roeder *et al*, 2003). This biogenic amine is synthesized from tyrosine (tyrosine → tyramine → octopamine) and both tyramine and octopamine receptors are represented in the fly. The tyramine β-hydroxylase gene, *Tbh*, is involved in the rate-limiting step of octopamine synthesis and is transcriptionally repressed by *Bacchus* (Monastirioti *et al*, 1996; Scholz, 2005; Chen *et al*, 2013). Both genes were shown to be important for normal ethanol sensitivity (Chen *et al*, 2013). An increase in octopamine through knockdown of *Tbh* causes ethanol sensitivity, and mutations in *Bacc* were shown to attenuate sensitivity. Expression of *Tbh* was shown to be both necessary and sufficient in a small set of neurons for expressing innate olfactory ethanol preference in flies. Flies deficient in octopamine synthesis had less olfactory preference for ethanol (Schneider *et al*, 2012).

Other synaptic proteins that have been linked to ethanol responses include the *Syntaxin A1* gene (*Syx1A*; Krishnan *et al*, 2012), *synapsin* (*syn*; Godenschwege *et al*, 2004), and *Shibire* (*Shi*, encodes Drosophila dynamin; Krishnan *et al*, 2012). Recently, Munc13-1, a presynaptic protein linked to Ca^{2+}-triggered vesicle exocytosis in mammals (Rizo and Südhof, 2002), was also shown to affect ethanol behaviors. Das *et al*, (2013) mapped the ethanol binding site in the fly protein and showed that reducing its expression increases ethanol preference in the CAFE assay. The synaptic scaffolding gene, *dlg1*, encodes both DlgA and DlgS97 (homologs of the mammalian PSD-95 and SAP97, respectively). Mutations affecting DlgS97 but not DlgA play a role in ethanol tolerance—a role conserved in mammals. Dlg97 interacts with N-methyl-D-aspartate (NMDA) receptors and the calcium/calmodulin-dependent protein kinase CASK. Hypomorphic mutants of both the NMDA receptor 1 gene (*Nmdar1*), or *CASK*, also reduce ethanol tolerance (Maiya *et al*, 2012). Moreover, *dlg1* interacts with *fas2*, a cell-adhesion molecule linked to ethanol sensitivity (Cheng *et al*, 2001).

One-gene-at-a-time mutant analysis has also identified a number of channel genes whose activity is important for alcohol responses. For instance, the Drosophila *KCNQ* gene encodes a K^+ channel inhibited by ethanol and linked to ethanol resistance and tolerance. Cavaliere *et al*, (2012) showed that transgenic overexpression of *KCNQ* produces ethanol resistance while suppression of KCNQ expression increases sensitivity. In mammals, KCNQ2/3 forms the M-current channel (Wang *et al*, 1998), which is inhibited by ethanol in dopaminergic VTA neurons (Koyama *et al*, 2007). Another ion channel gene associated with ethanol responses across species is *Clic*. The *Clic* gene was identified as the highest-ranked alcohol-responsive gene in mouse and human transcriptome studies. Mutations in the *C. elegans* orthologues produced ethanol resistance (Bhandari *et al*, 2012) as did genetic knockdown of *Clic* in flies (Chan *et al*, 2014). *Clic* encodes a mysterious protein that functions as an anion-specific ion channel (Littler *et al*, 2008).

Circadian rhythm genes

Ethanol can disrupt circadian rhythms, and some ethanol responses cycle in a circadian manner (Spanagel *et al*, 2005). Van der Linde and Lyons (2011) showed that while ethanol resistance shows circadian rhythmicity, ethanol tolerance does not. Pohl *et al*, (2013) then genetically tested four genes that encode core components of the intracellular clock for an effect on resistance and tolerance. They observed that mutations in *per*, *tim*, and *cyc* but not in *Clk* prevented tolerance without any effect on resistance, reiterating the idea that resistance and tolerance have independent origins. Further, because not all of the clock mutants disrupted ethanol tolerance, these data were interpreted to mean that the role of circadian genes in tolerance is independent of their role in generating circadian rhythms. This outcome is reminiscent of the report by Andretic *et al*, (1999) in which mutations in *per*, *cyc* and *Clk* prevented sensitization to cocaine while mutations in *tim* did not. Some of the other mutants to be discussed below and listed in Fig.4 also disrupt circadian rhythmicity. In flies, an allele of *slo* was actually the first circadian rhythm mutant shown to alter an ethanol response (described above). Ceriani *et al*, (2002) showed that loss-of-function mutations in *slo* produced circadian arrhythmia, a result later extended to mammals

(Meredith *et al*, 2006; Montgomery *et al*, 2013). BK channels appear to act as an output of the central pacemaker cells.

A genetic screen for mutations affecting cocaine sensitivity identified an allele of *Beadex* (*Bx*), also known as *dLmo*, that encodes a transcriptional repressor involved in producing circadian rhythms (Tsai *et al*, 2004). Lasek *et al*, (2011a) showed that reduced *Bx* expression reduced ethanol resistance and increased *Bx* expression increased ethanol resistance. Additionally, Lasek *et al*, (2011b) performed a microarray analysis to identify genes whose expression was inversely related to the expression of the *Bx* repressor. *Bx* was shown to be a negative regulator of *Alk* (Drosophila homolog of anaplastic lymphoma kinase gene). *Alk* regulates *Erk* activity in the fly brain (Gouzi *et al*, 2011), and it is likely that it affects ethanol behaviors through *Erk*. In mice, *Bx* (*Lmo4*) and *Alk* orthologs also influence ethanol sensitivity and have been shown to modulate ethanol drinking (Lasek *et al*, 2011a; Lasek *et al*, 2011b; Dutton *et al*, 2016). *Alk* is induced by ethanol and may be involved in ethanol dependence (Dutton *et al*, 2016; Schweitzer *et al*, 2016). Finally, *Alk* was strongly associated with ethanol dependence in a human GWAS meta-analysis (Wang *et al*, 2011).

EGFR/ERK, PI3K/Akt Signaling

Work from two different laboratories demonstrated that the EGFR/ERK signaling pathway modulates baseline ethanol resistance. Screening for ethanol-resistant mutants, Corl *et al*, (2009) identified an allele of the *happyhour* (*hppy*) gene, an ortholog of a vertebrate ste20 kinase gene, and showed that it negatively regulates EGFR/ERK pathway signaling. Ethanol resistance in *hppy* loss-of-function mutations was shown to be a product of increased EGFR/ERK signaling. Evolutionary conservation of the ethanol link to this pathway was then demonstrated by showing that administration of erlotinib, an EGFR/Erb1 tyrosine kinase inhibitor, produced ethanol sensitivity in flies and mice and reduced ethanol preference in rats in a two-bottle choice paradigm.

Other experiments continued to connect the dots upstream and downstream from EGFR and ERK using unbiased genetic screens and epistatic interactions. The depicted pathway in Fig. 5. represents the order of steps largely determined by epistatic interactions. Not all genes in a process have been mutationally identified. Therefore arrows and inverted T’s do not necessarily mean that the proteins physically interact but only mean that signaling relevant to ethanol sensitivity occurs between the two proteins. The connection between steps of the pathway were confirmed by genetically activating and inactivating the pathway at each step and measuring ethanol resistance. Epistatic interactions between genes described in the pathway (Fig.5.) were consistent (Corl *et al*, 2009; Eddison *et al*, 2011). This generally holds true more often in flies than mammals due to the smaller gene families.

The EGFR/ERK pathway was implicated in modulating the ethanol level of response a second time in experiments examining steroid hormone effects on ethanol sensitivity. The steroid hormone effects are usually attributed to transcriptional changes produced by steroid receptor transcription factors. Petruccelli *et al*, (2016) recently provided the first evidence that a non-nuclear G-protein-coupled steroid receptor has an impressively important role in modulating ethanol resistance. The DopEcR (Dopamine Ecdysone Receptor) is functionally similar to the mammalian G-protein-coupled estrogen receptor but has the highest sequence

similarity to mammalian β2-adrenergic receptor. DopEcR responds to dopamine and ecdysone but activates different downstream cascades (Srivastava *et al*, 2005); however, only ecdysone signaling through the receptor affects ethanol LOR. In the model proposed by Petruccelli *et al*, decreased DopEcR signaling allows EGFR/Erk to be more active, promoting resistance (Fig.5).

The role of the *arouser* gene (*aru* in the Fig.5) is also worth special notice. This *Eps8* (Epidermal growth factor receptor kinase substrate-8) homolog is an adapter signaling protein that works in two pathways, in different cells, and with distinct mechanisms to establish normal ethanol sensitivity. Eps8 proteins are known regulators of actin dynamics that alter both synaptic architecture, NMDAR activity, and affect ethanol sensitivity in mice (Offenhauser *et al*, 2006).

PI3K/Akt signaling negatively regulates *aru*, and *aru* negatively modulates synapse number in the CNS and at the NMJ. Loss of *aru* expression specifically in the LNv circadian pacemaker neurons (identified as PDF+ cells) causes ethanol sensitivity. In a different set of neurons, the EGFR/Erk signaling pathway also modulates *aru* to affect ethanol sensitivity (Eddison *et al*, 2011). Receptors other than EGFR can also activate ERK signaling. Drosophila has two FGFR receptor orthologs, called *heartless* (*htl*) and *breathless* (*btl*), that might also activate Erk to alter ethanol responses. Mutations in *htl* but not *btl* specifically altered behavioral hyperactivity observed in response to low doses of ethanol (King *et al*, 2014).

Insulin signaling

The insulin signaling pathway became a focus in alcohol research in flies because ethanol level of response could be modulated by cAMP signaling within the insulin-producing cells (Corl *et al*, 2005). Inhibition of cAMP-dependent PKA in cells expressing the insulin-like peptide gene *Ilp2* reduced ethanol resistance. Reduced resistance was also produced in *InR* mutants and in *chico* (encodes the insulin-receptor substrate) mutants (Rodan *et al*, 2002; Corl *et al*, 2005). Insulin signaling is known to regulate many physiological processes and signaling pathways such as the Pi3K/Akt and MAPK/ERK pathways (reviewed in Claeys *et al*, 2002). Both of these pathways have been shown to be involved in ethanol responses in flies (above).

The Rothenfluh lab (Peru Y Colon de Portugal *et al*, 2012; Acevedo *et al*, 2015) demonstrated that InR, Arf6 (fly gene name *Arf51F*), and S6 Kinase (*S6K*) form a signaling pathway that modulates ethanol sedation (but not the hyperlocomotion effects of low-dose ethanol). The described pathway InR—>Arf6—>S6K represents the flow of information only, and intermediate steps remain to be added. Genetics and biochemical experiments showed that stimulation of InR activated both the Arf6 small GTPase and S6K and made flies ethanol resistant. Suppressing expression (mutation or RNAi) of any one of the three genes made flies more ethanol sensitive. The activating phosphorylation of S6K and ethanol resistance were epistatically blocked by inhibition of Arf6, while the changes in ethanol resistance produced by manipulating either InR or Arf6 could be epistatically blocked by altering S6K activity. The described pathway provides points of integration with other signaling molecules—mTOR was shown to regulate S6K activity and ethanol responses as

in mammals, and Rac1 was shown to regulate Arf6 activity, providing integration into the RhoGap pathway described below. As in mammals, S6 ribosomal subunit phosphorylation is a marker of neural activity in flies. In Acevedo *et al*, this phosphorylation is hypothesized to be causally related to sedation. This group proposes that strong ethanol exposure, which suppresses the InR/Arf6/S6K pathway, could mechanistically underlie differences in ethanol sedation. This would most likely be through its known effects on the actin cytoskeleton and dendritic conformation (Dimitroff *et al*, 2012).

Rho GTPases and Actin cytoskeleton dynamics

Rothenfluh and colleagues provide biochemical, cytological, and genetic evidence that the three RhoGAP isoforms encoded by *RhoGAP18B* (a.k.a. *whir*) modulate the actin cytoskeleton, cell morphology, and distinct aspects of the ethanol sedation response (Rothenfluh *et al*, 2006; Ojelade *et al*, 2015a). As shown in Fig.6, each RhoGAP18B isoform negatively regulates one of the rho-family GTPases—Cdc42, Rho1, and Rac1—that have complementary or opposing effects on cell morphology. Changes in cell morphology have been associated with changes in ethanol responses (Ojelade *et al*, 2015a). Activation of Rho1 or Rac1 produces behavioral resistance to ethanol sedation.

The *Rsu1* gene (Ras suppressor1; fly gene name is *ics*), a regulator of Rac1, was identified in an unbiased screen for mutations that produce ethanol resistance. Rsu1 protein was shown to physically associate with Rac1 but not CDC42 or Rho1. An experimentally supported model was produced in which Rsu1-regulation by integrins represses Rac1 activity (but not CDC42 or Rho1; see Fig.6), which could change ethanol responsivity through the action of Rac1 on actin polymerization and cell shape (Bhandari *et al*, 2009; Ojelade *et al*, 2015b). A mutation in *Rsu1* was also shown to affect the preference of male flies for drinking ethanol food, and in a human GWAS, *Rsu1* was associated with alcohol dependence. Furthermore, in a separate GWAS, there was shown to be an association between an *Rsu1* SNP and ventral striatum activity (shown with fMRI) in a reward-anticipation paradigm (Ojelade *et al*, 2015b).

Stress genes

In flies, stress pathways were implicated in ethanol tolerance because specific patterns of heat shock mimic ethanol tolerance (Scholz *et al*, 2005). The *hangover* gene encodes a Zn-finger nuclear protein and was identified in an unbiased screen for rapid-tolerance mutants. The mutant allele may affect ethanol responsivity because of its role in responding to environmental stressors (Scholz, 2005); albeit it might also be because of its effect on expression of the FasII cell-adhesion molecule (Schwenkert *et al*, 2008). A subsequent human sibling pair study identified SNPs in the human homolog, *ZNF699*, that were associated with human alcohol dependence (Riley *et al*, 2006). Finally, *ARL6IP5* (alias *Jwa*, *addicsin*), has been called a stress gene in mammals that increases expression in response to oxidative stress and heat shock (Chen *et al*, 2007). The Drosophila homolog of *Jwa* is required for ethanol tolerance (Li *et al*, 2008). TSPO (also called the peripheral benzodiazepine receptor) is a mitochondrial outer-membrane protein proposed to be involved in cellular stress regulation (Batoko *et al*, 2015). TSPO interacts with the diazepam binding inhibitor (DBI), which has been proposed to contribute to drug dependence. DBI

can reduce $GABA_A$ receptor activity (Bormann, 1991; Ostuni *et al*, 2008). Mutations in the *TSPO* gene affect ethanol resistance in a sex-specific manner that appears to be mediated in part through ROS signaling and Caspase activity (Lin *et al*, 2015).

Innate immune system

In mammals, the innate immune TLR signaling pathways are activated by ethanol and have been linked to ethanol level of response, preference, and alcoholic liver disease (reviewed in Warden and Mayfield (this volume) and Cui *et al*, 2014; Crews *et al*, 2015; Gao *et al*, 2011). In flies, the response of the homologous Toll pathway is well-conserved. However the polarity of the effect on sensitivity is reversed. Troutwine *et al*, (2016) manipulated each step in the Toll pathway and showed that pathway activation at any step produced ethanol resistance while pathway suppression produced sensitivity without affecting the capacity for tolerance, once again indicating that resistance and tolerance have distinct mechanistic origins (as did Kong *et al*, 2010a). Previously, Morozova *et al*, (2007) and Kong *et al*, (2010a) showed that innate immune pathway genes were induced 90 to 120 min after ethanol vapor, while Troutwine *et al*, (2016) showed that the pathway itself is activated 30 minutes after ethanol treatment.

Blood-Brain Barrier

The *moody* gene, identified in an unbiased genetic screen, encodes glial expressed orphan GPCRs. A mutation in *moody* compromises blood-brain barrier (BBB) integrity and causes ethanol resistance (Bainton *et al*, 2005; Schwabe *et al*, 2005), probably by altering many aspects of brain physiology. In mammals, changes in BBB integrity have also been proposed to be produced by chronic ethanol consumption (reviewed in Szabo and Lippai, 2014).

Genomic analyses of alcohol responses in flies

Transcriptome studies

Morozova *et al*, (2006, 2007, 2009, 2011) conducted a variety of creative transcriptome analysis studies. Each publication probes alcohol responsivity in a different way. This body of work describes the behavioral and transcriptional changes after a single ethanol exposure and 2 hours later, after a second exposure. Gene candidates and gene networks were validated using mutants or RNAi knockdown. This group began looking at transcriptome changes following each ethanol treatment (Morozova *et al*, 2006) and then at gene expression differences between fly lines bred for ethanol resistance or sensitivity for 35 generations (Morozova *et al*, 2007). In 2009, this was followed up by examining the basal (ethanol-naive) gene expression profile of 40 wild-derived inbred lines (Morozova *et al*, 2009). In Morozova *et al*, (2011), the opposite tack was taken and a transposon-mutagenesis screen was used to identify genes affecting ethanol sensitivity and tolerance. Transcriptome data was used to generate transcription correlation networks around 9 genes and 12 genes that when mutated produced sensitivity and resistance, respectively.

The *Men* gene was identified in all four studies as important in determining level of response. *Pdk* was also identified in two of the studies. *Men* encodes malic enzyme and *Pdk* encodes pyruvate dehydrogenase kinase; both are genes involved in energy metabolism that

might also influence fatty-acid synthesis (Morozova *et al*, 2006; Morozova *et al*, 2007; Morozova *et al*, 2009; Morozova *et al*, 2011). The human homolog of *Men* was shown to be associated with increased cocktail drinking in a GWAS (Morozova *et al*, 2009). In the network analysis, the *Men* gene and *psq* gene (a transcription factor important in neural development) were also major hub genes. The *nuf* and the *Bx* (*dLmo*) genes were identified in two of the described studies. Nuf is thought to be a cytoskeleton binding protein that influences dendrite morphology. Interestingly it has been shown to genetically interact with *Bx* (*dLmo*) that when mutated affects fly cocaine and alcohol responses. Mutations in the mammalian homolog called *Lmo4* produce similar phenotypes in mice (discussed above; Lasek *et al*, 2010; Lasek *et al*, 2011a). Furthermore, some of the identified genes (*Toll*, *Imd*, *Thor*) implicated the innate immune system in setting the level of response. A previous microarray study by Kong *et al*, (2010a) had also identified innate immune genes (e.g. *Tl*, *cact*, *MyD88*, *imd*, *rel*, and *Spn27a*) as ethanol responsive, and recently Troutwine *et al*, (2016) tested each step in the Toll pathway and showed an effect on sensitivity but not tolerance. Similarly, *Sirt1*, shown to be important for tolerance, was also flagged by Morozova *et al*, (2006), functionally tested by Kong *et al*, (2010a), and later extensively studied by Engel *et al*, (2016). An important general observation was that gene modules associated with innate ethanol sensitivity and tolerance were distinct (Morozova *et al*, 2009; Morozova *et al*, 2011; Morozova *et al*, 2014). However, while Kong *et al*, (2010) reported a correlation between sedation sensitivity and tolerance, they also note that the two show substantial genetic separation. The independence of sensitivity and tolerance has also been reported in single-gene studies (e.g. Troutwine *et al*, 2016; Berger *et al*, 2008).

GWA and QTL Studies

Morozova *et al*, (2015) presents an analysis that merits a separate discussion. This work makes use of the *Drosophila melanogaster* Genetics Reference Panel (DGRP)—a community resource of 205 lines derived from wild-caught female Drosophila (Mackay *et al*, 2012; Huang *et al*, 2014). These lines were sister-brother inbred for 20 generations, and then the genome was sequenced. Transcriptome data were also determined for 40 lines. The entire collection is available from a community stock center, and a website provides access to the data and software tools needed for GWAS (http://dgrp2.gnets.ncsu.edu/). The stocks and data have been used by different labs to perform GWA and QTL analysis on a wide range of traits with the understanding that the data will be shared on the website. Morozova *et al*, (2015) used these stocks for a GWA study and an extreme QTL study. Innate resistance and tolerance were measured for all 205 lines. The lack of correlation between the degree of ethanol sensitivity and tolerance further illuminated the genetic independence of these ethanol responses (Fig.1C in Morozova *et al*, 2015).

The GWA yielded a single candidate, the *cut* gene, which encodes a homeobox transcription factor that is a target for Notch signaling and whose homolog has been implicated in mammalian ethanol responses (Melendez *et al*, 2012). For extreme QTL mapping, an advanced intercross population was made by crossing the three most sensitive and most resistant DGRP lines. The resultant population was maintained by random mating of large numbers of animals for 25 generations to break up linkage groups. Then ethanol sensitivity and tolerance responses were measured for 2000 males and 2000 females, and the genomes

of the top 10% of each category were sequenced. A significant association with ethanol sensitivity was found in a gene cluster associated with catecholamine metabolism. By comparing the rank-ordered DGRP and extreme QTL analysis gene lists, an additional 62 genes crossed the significance threshold, 42 of which had already been associated with an ethanol response in an animal. Gene networks identified include genes involved in cAMP, Notch, Wingless, Hedgehog and EGF signaling and genes involved in dopamine synthesis.

A number of the studies by Morozova *et al*, note that there is a substantial lack of overlap between GWAS and single-gene studies. They propose that this might be because large effect genes are under strong selection pressure and so vary little in natural populations. As a result they cannot be identified in natural population GWAS. It should be noted that such genes are amenable to study by the single-gene approaches described earlier.

For more than 50 years investigators have worried whether alcohol drinking in their model system is motivated by the pharmacological effects of alcohol or its caloric content (Rodgers *et al*, 1963). This problem appears to have been largely resolved in at least some modern rodent drinking paradigms (Ford *et al*, 2007, Lyons *et al*, 2008; reviewed in Brabant *et al*, 2014). The assay most often used to measure Drosophila alcohol drinking is the CAFE assay —a two-bottle choice analog in which upside-down flies drink ethanol-laced and ethanol-free liquid food from two sets of 5 ul pipettes (Ja *et al*, 2007). This is a difficult way for flies to feed and may represent a calorically restricted state. Pohl *et al*, (2012) performed calorie-balancing experiments indicating that flies consumed the ethanol food primarily for its caloric value. However, other work indicates that it is the pharmacological effects that motivate ethanol drinking (Devineni and Heberlein, 2009; Xu *et al*, 2012; Peru Y Colon de Portugal *et al*, 2014). Sekhon *et al*, (below) has done much to resolve this disagreement.

In Sekhon *et al*, (2016), the Kliethermes laboratory used DGRP lines to quantitatively evaluate the genetic overlap between food and ethanol consumption in male flies. They observed a strong correlation (r=0.68) between the amount of food and the amount of ethanol solution consumed. Gene-behavior correlations showed that ~30% of the genes associated with increased ethanol consumption overlapped with those genes associated with increased food consumption. This provides a quantitative measure of how much of ethanol preference in flies is driven by the food value of ethanol.

Among the 384 genes associated with both food and ethanol consumption were *fruitless* (*fru*), *D2-like Dopamine Receptor* (*Dop2R*), *Vesicular Monoamine Transporter* (*Vmat*), *Casein kinase II* (*CkIIβ*), and *DOPA decarboxylase* (*Ddc*). Among the 507 genes associated only with ethanol preference were a number of the validated genes that have been described in previous sections. These include *NPF*, *NPFR*, *amn*, *Bx* (*dLmo*), and *Men*. An examination of eleven species of Drosophila also showed a strong correlation between food and ethanol consumption (r=0.71). Surprisingly, at least in male flies, olfactory preference and consumption preference were largely not genetically related. The approach by Sekhon *et al*, provides a metric for determining the degree to which any given drinking assay responds to ethanol as a food or as a pharmacologically active compound.

miRNA expression

miRNAs have emerged as powerful regulators of global gene expression that can coordinately regulate the translational efficiency of multiple genes at time. This may be important in multigenic adaptive processes such as those implicated in alcohol addiction. In a survey of early ethanol-induced changes in miRNA expression, Ghezzi and colleagues (2016) identified a small set of 14 miRNAs whose expression changed within 30 minutes of ethanol sedation. Of these, two (out of seven tested) were functionally validated to exert an effect on ethanol sensitivity: miR-6 and miR-310. These miRNAs have been associated with synaptic transmission and development. Expression of miR-6 in the CNS has been associated with suppression of proapoptotic genes (Ge *et al*, 2012). Loss of the miR-310–313 cluster showed a significant increase in expression of the active zone protein Bruchpilot (gene is *brp*) and enhancement of neurotransmitter release (Tsurudome *et al*, 2010). Previously, *brp* was linked to ethanol tolerance (Ghezzi *et al*, 2013). Interestingly, many of their other putative targets are well-known alcohol-related genes. These include *Arfip* (Peru Y Colon de Portugal *et al*, 2012), *CASK* (Maiya *et al*, 2012), *Tl* (Troutwine *et al*, 2016), *kn* (Ghezzi *et al*, 2013), *tao* (King and Heberlein, 2011), *AcCoAS* (Kong *et al*, 2010a), *rho* (Berger *et al*, 2008), and *Teh2* (Ghezzi *et al*, 2013).

It is important to note that all mammalian miRNA families are represented in Drosophila. Thus, analysis of miRNA regulation in flies can be highly informative and applicable to the biology of higher organisms, including humans (Ibanez-Ventoso *et al*, 2008). A miR-310 homolog has been previously associated with alcohol disorders in humans. The human miR-92 is the sequence-related homolog of the Drosophila miR-310, and it was found to be upregulated in the PFC of human alcoholics and has been associated with the regulation of immune TLR signaling, synaptic signaling and angiogenesis and is a biomarker for traumatic brain injury (Nunez and Mayfield, 2012).

Histone acetylation

In our efforts to understand the contribution of changes in gene transcription to the production of functional ethanol tolerance, we chose to monitor alcohol-induced histone H4 acetylation as a proxy for increases in gene transcription. The rationale for this was that enhanced histone acetylation is a feature of gene activation and that H4 acetylation is functionally involved in producing the chromatin conformation needed for gene expression (Shogren-Knaak *et al*, 2006). However, mRNA abundance is heavily influenced by the regulation of mRNA stability (e.g. by miRNAs, see above and; Gurtan and Sharp, 2013; Bethune *et al*, 2012). Thus, histone acetylation is a generic probe that is a better benchmark of the rate of transcription than the measurement of mRNA abundance is. The chromatin-immunoprecipitation (ChIP) was used to genomically map H4 acetylation spikes induced in fly heads 24 h after ethanol sedation. Unfortunately, ~1,100 genes responded, and it is unlikely that all of these contribute to tolerance. To winnow the wheat from the chaff, we intersected this gene set with a gene set in which benzyl alcohol had been used to induce sedation. Ethanol and benzyl alcohol produce functional tolerance by similar or highly overlapping mechanisms (discussed above). Benzyl alcohol treatment produced a similar number of altered genes, but the intersection contained only 146 genes. We hypothesized that these 146 genes were important for tolerance and were coregulated. Evidence for

coregulation was found using a modENCODE Drosophila community resource that provides RNA-seq data from different developmental stages and from animals that had been treated with a variety of environmental stressors (Celniker and Graveley, 2012). These were used in organizing the 146 genes into coregulated cohorts. Using this dataset, Ghezzi *et al*, compared the histone H4-identified genes to the modENCODE dataset and found that the 146 subset could be organized into four highly correlated gene clusters. Gene ontology revealed a cluster enriched in transcription and chromatin regulation genes, a cluster enriched for genes associated with adenyl nucleotide binding and the cytoskeleton, and a cluster that contained synaptic proteins. The fourth cluster was not a good match for any single gene ontology term. Eighteen candidates were tested by mutation and/or RNAi analysis and eleven were observed to affect the acquisition of tolerance. A number of these were encoded proteins that were already known to interact. Among the identified genes, *pumilio*—a known translation regulator that modulates neural excitability (Schweers *et al*, 2002)—has shown up in many independent studies.

Sir2

Histone acetylation is determined by the antagonistic activity of histone acetylases (HATs) and histone deacetylases (HDACs). Two transcriptome studies have identified the *Sir2* (called *Sirt1* in flybase) HDAC gene as ethanol responsive and by mutant analysis to influence ethanol level of response (Morozova *et al*, 2006; Kong *et al*, 2010a). The mammalian homolog of *Sir2* is called *SIRT1*. Engel *et al*, (2016) described *Sir2*'s effect on alcohol responses in detail. *Sir2* mutations perturb normal ethanol resistance, tolerance, and preference. Knockdown of *Sir2* in the adult nervous system but not other tissues causes increased resistance and reduced tolerance. Wild-type flies sedated with ethanol vapor increase their preference for ethanol food (CAFE assay), however in *Sir2* mutants this phenomenon is reversed—animals show high naive preference that reduces with prior vapor sedation. The *Sir2* HDAC is known to affect transcription and is induced by ethanol sedation. Transcriptome analysis indicated that expression of many genes were changed (≥2X) in the mutant. In *Sir2* mutants, *Synapsin* (*Syn*) mRNA and protein were reduced after ethanol exposure. *Syn* mutants phenocopy *Sir2* mutants with regards to resistance and tolerance.

The mammalian *SIRT1* HDAC has long been linked to the pathological effects of ethanol in the liver, where it has been shown to activate NF-κB by deacetylation. Ethanol metabolism depresses [NAD+], which suppresses *SIRT1* and thereby activates NF-κB (reviewed in You *et al*, 2015; and Cui *et al*, 2014). Troutwine *et al*, (2016) has shown that increasing the activity of the Drosophila NF-κB transcription factors in the nervous system promotes ethanol resistance. Thus, *Sir2* inhibition or mutation may also promote resistance through NF-κB activation.

Closing Remarks

Classical genetic approaches and modern genomic studies are unbiased gene-discovery methods that together are well suited for identifying alcohol-response gene networks. In recent years, these approaches have been converging on overlapping sets of genes. An

understanding of the molecular nature of alcohol responses and alcohol neuroadaptations in the fly model system can be invaluable to those whose only interests are the responses of mammalian model systems because the fly model system provides points of comparison for identifying the conserved molecular effects of ethanol. Responses conserved over such large evolutionary distances are the ones that are most likely to also be conserved in humans and could be a useful key for identifying which druggable targets are likely to work in humans.

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- The genetics of alcohol behaviors have been extensively studied in the Drosophila model system.
- Single gene genetic analysis and genomic studies have identified pathways affecting alcohol responses.
- Alcohol resistance and tolerance have distinct genetic origins.

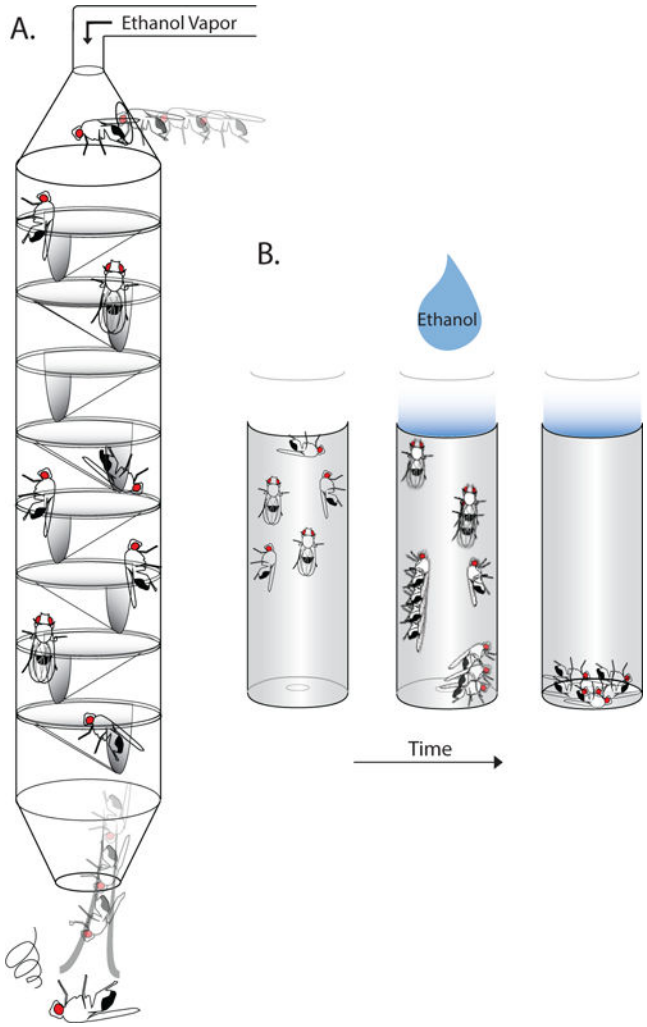

Figure 1. Two common assays

A) The Inebriometer. The illustration depicts the inebriometer: a large vertical glass tube with scoop-like ledges on which flies can walk or stand. While ethanol vapor is pumped through the apparatus, flies are introduced through a small opening at the top and allowed to breath the vapor. As flies succumb to the ethanol effects and lose postural control, they elute from the bottom of the column. B) Visually monitoring ethanol sedation in vials is an extremely common assay. Figure not drawn to scale.

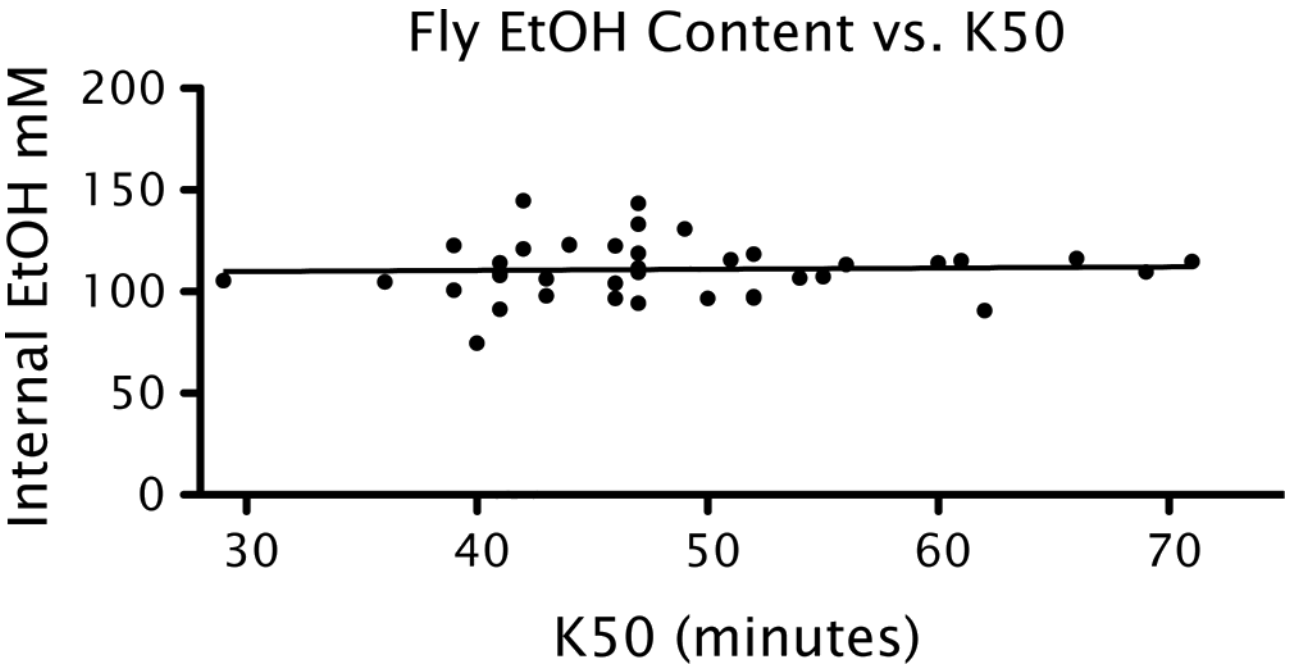

Figure 2. The 50% knockdown time (K50) is a behavioral endpoint that occurs at a fixed ethanol concentration even when environmental conditions produce extreme differences in K50 time

The internal ethanol concentration of 39 groups of 10 flies quantified at the time when half of the flies in each group succumbed to ethanol sedation. While the K50 time differed greatly, the internal ethanol remains fixed within a narrow range around 111 mM +/− 2.3 (Pohl *et al*, 2013 used with permission).

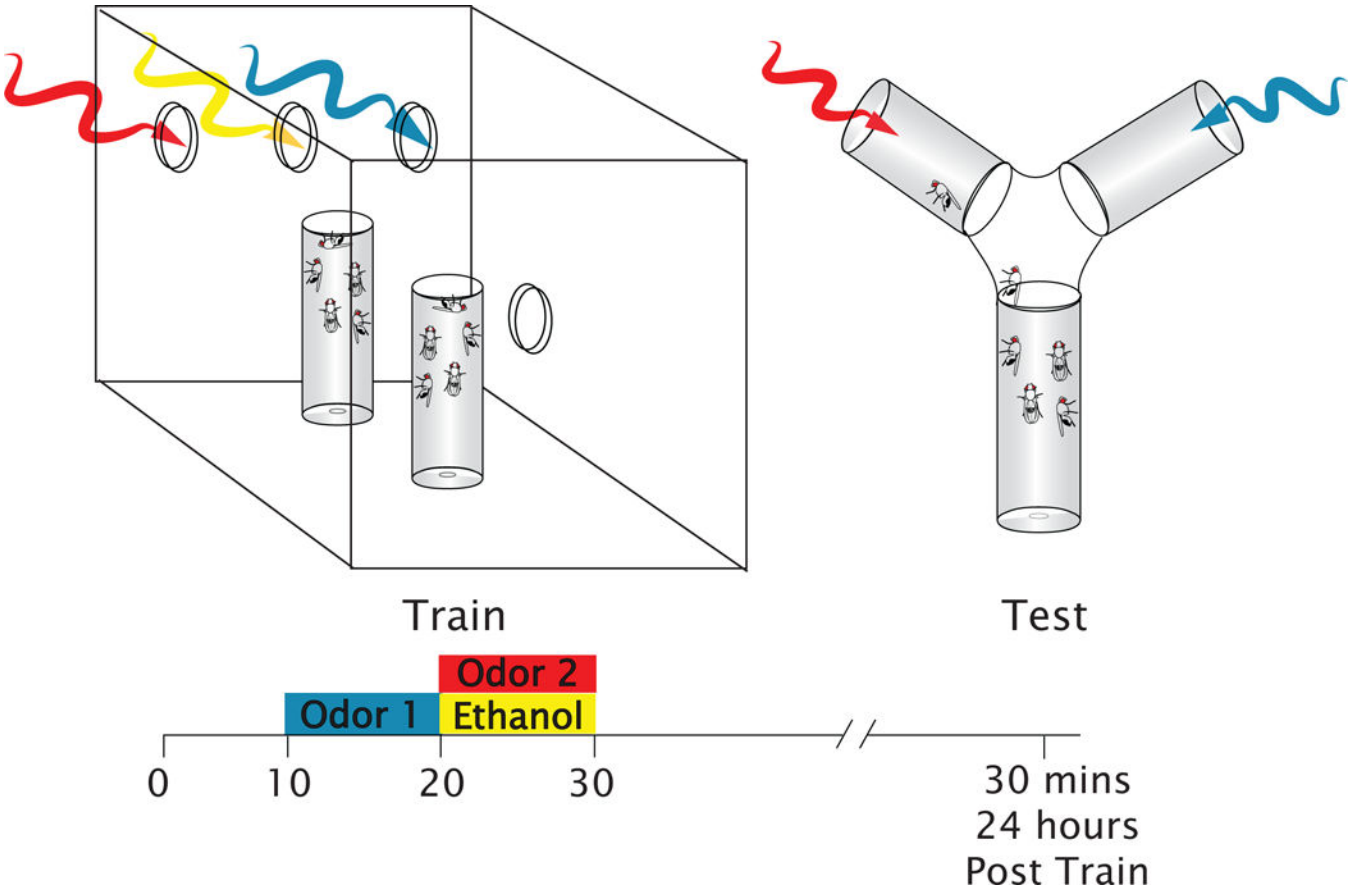

Figure 3. The ethanol-conditioned odor preference assay

The illustration depicts the training (left) and the test (right) stages of the assay. During training, groups of flies are exposed to two odorants separately. One of the odors is paired with an intoxicating ethanol exposure, while the other odor is unpaired (as shown in the timeline). Flies are later tested (30 mins or 24 hours post training) in a Y-maze where the odors alone are pumped in through either arm of the Y. Flies choose which arm to approach. Figure not to scale.

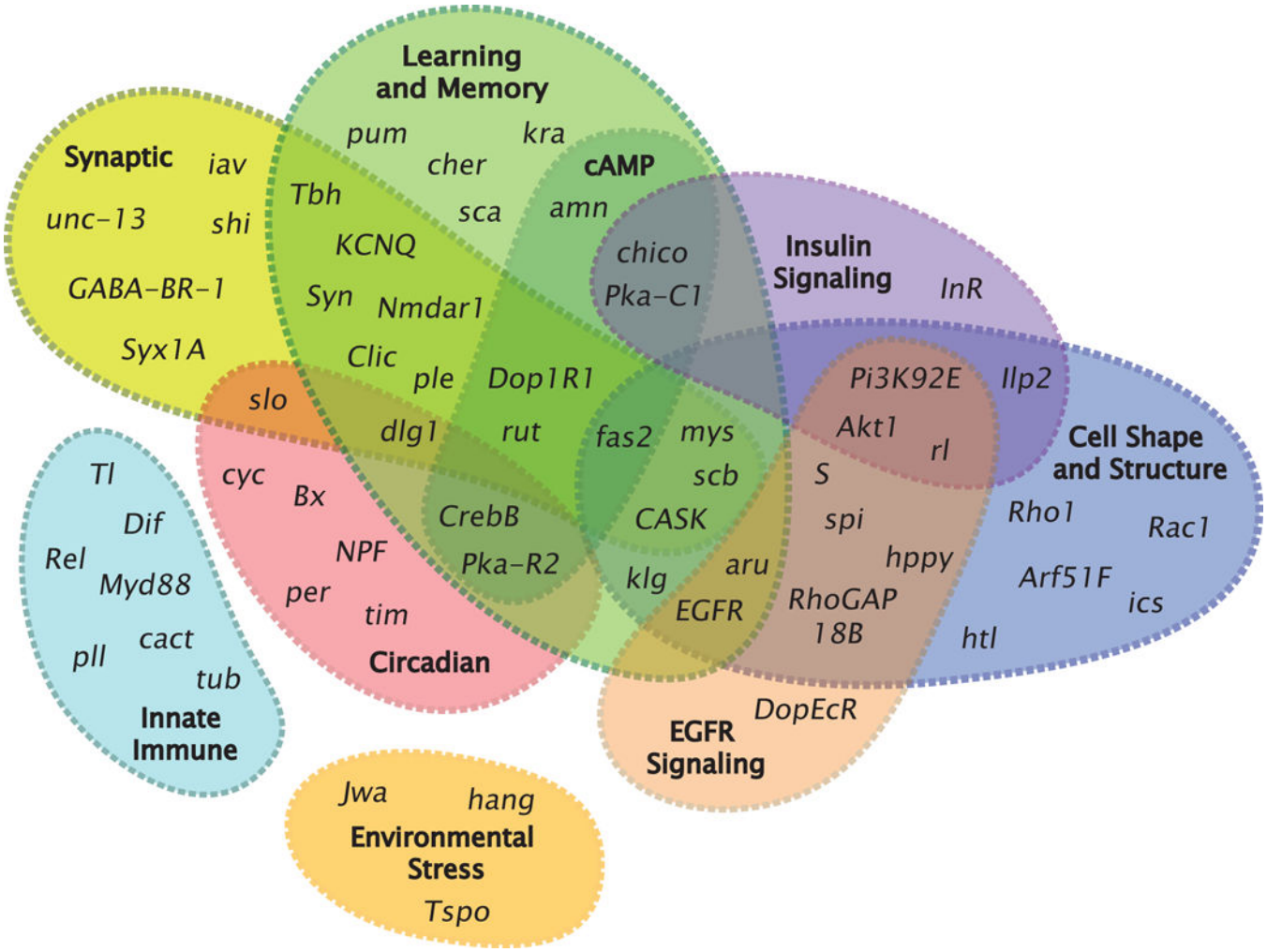

Figure 4. Ethanol resistance, tolerance, and preference genes identified by one-gene-at-a-time genetics organize into GO-like gene categories

The Venn diagram shows how overrepresented gene categories/functions become apparent once sufficient numbers of genes are identified. The evidence for each gene is as follows: *ple* (Bainton *et al*, 2005), *cher*, *klg, kra, pum* (Berger *et al*, 2008), *mys, scb* (Bhandari *et al*, 2009), *Clic* (Bhandari *et al*, 2012), *KCNQ* (Cavaliere *et al*, 2012), *Fas2* (Cheng *et al*, 2001), *chico*, *Ilp2*, *InR* (Corl *et al*, 2005), *Egfr*, *hppy*, *rl* (*Erk*), *S, spi* (Corl *et al*, 2009), *slo* (Cowmeadow *et al*, 2005), *unc-13* (Das *et al*, 2013), *GABA-B-R1* (Dzitoyeva *et al*, 2003), *Akt1*, *aru*, *Pi3K92E* (Eddison *et al*, 2011), *Syn* (Godenschwege *et al*, 2004), *Nmdar1*, *sca* (Kaun *et al*, 2011), *htl* (King *et al*, 2014), *Dop1R1* (Kong *et al*, 2010b), *shi*, *Syx1A* (Krishnan *et al*, 2012), *Bx* (*dLmo*) (Lasek *et al*, 2011a), *Jwa* (Li *et al*, 2008), *CASK*, *dlg1* (Maiya *et al*, 2012), *amn*, *Pka-C1*, *rut* (Moore *et al*, 1998), *ics* (*Rsu1*) (Ojelade *et al*, 2013), *Pka-R2* (Park *et al*, 2000), *Arf51F* (Peru Y Colon de Portugal *et al*, 2012), *DopEcR* (Petruccelli *et al*, 2016), *cyc*, *per*, *tim* (Pohl *et al*, 2013), *Rac1*, *Rho1*, *RhoGAP18B* (Rothenfluh *et al*, 2006), *hang* (Scholz *et al*, 2005), *iav*, *Tbh* (Scholz, 2005), *cact*, *Dif*, *Myd88*, *pll*, *Rel*, *Tl*, *tub* (Troutwine *et al*, 2016), *CrebB* (Wang *et al*, 2007), *NPF* (Wen *et al*, 2005). The learning and memory category contains many more genes that have been only cursorily characterized (see text).

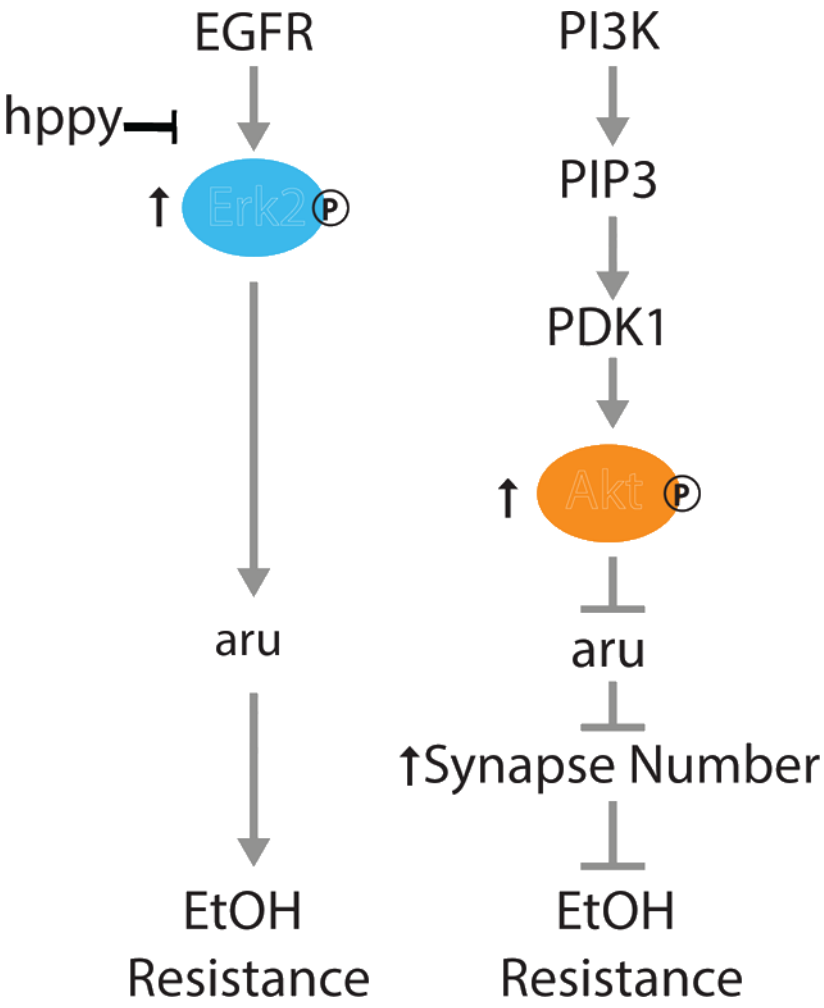

Figure 5. The Aru protein is regulated by both EGFR and PI3K signaling pathways to alter ethanol level of response by different mechanisms

The ethanol-relevant activity of these pathways appears to occur in different developmental stages. The genetic evidence does not necessarily indicate that all steps occur in the same cell (Eddison *et al*, 2011). Gray arrows indicate activation, and inverted T indicates inhibition.

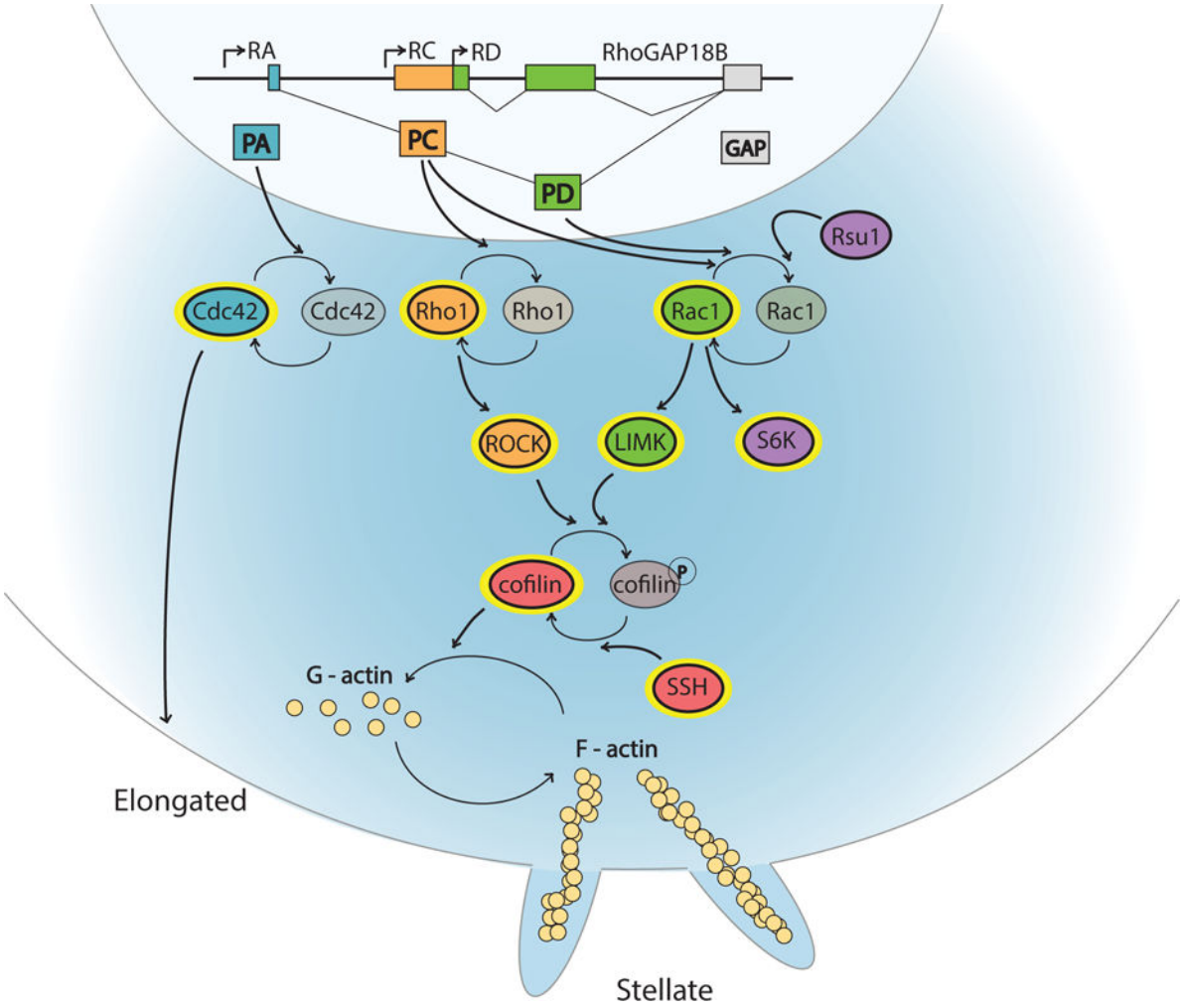

Figure 6. RhoGAP18B regulates Rho GTPases, cell shape, and ethanol resistance
Adapted from Ojelade ***et al***, (2015a).