Natural variation in protein kinase D modifies alcohol sensitivity in *Caenorhabditis elegans*

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Abstract

Differences in naïve alcohol sensitivity between individuals are a strong predictor of later life alcohol use disorders (AUD). However, the genetic bases for alcohol sensitivity (beyond ethanol metabolism) and pharmacological approaches to modulate alcohol sensitivity remain poorly understood. We used a high-throughput behavioral screen to measure acute behavioral sensitivity to alcohol, a model of intoxication, in a genetically diverse set of over 150 wild strains of the nematode *Caenorhabditis elegans*. We performed a genome-wide association study to identify loci that underlie natural variation in alcohol sensitivity. We identified five quantitative trait loci (QTL) and further show that variants in the *C. elegans* ortholog of protein kinase D, *dkf-2*, likely underlie the chromosome V QTL. We found that resistance to intoxication was conferred by *dkf-2* loss-of-function mutations as well as partly by a PKD inhibitor in a *dkf-2*-dependent manner. Protein kinase D might represent a conserved, druggable target to modify alcohol sensitivity with application towards AUD.

Significance statement

Here, we identify a novel role for protein kinase D (*dkf-2*) in *C. elegans* alcohol sensitivity. PKD, like protein kinase C, might represent a newly discovered druggable target to modify alcohol response. Identifying causal variants in genes of the remaining loci will likewise provide new insights into the genetic basis of variation in naïve alcohol sensitivity in *C. elegans* and other organisms.

Classification: Research Report

Keywords: GWAS, alcohol sensitivity, protein kinase D

Introduction

Alcohol Use Disorder (AUD) is one of the most common psychiatric diseases in the US now affecting more than one in 10 American adults (Grant et al., 2015; 2017). Many more bear the burden of alcohol misuse through indirect effects, such as drunk driving, lowered productivity, and a stressed medical system, which together cost the US about a quarter of a trillion dollars annually (Sacks et al., 2015). Despite the devastating effects of this disease, relatively few treatment options are available (Franck & Jayaram-Lindstrom, 2013). It is thus critical that new and effective ways to reduce AUD by treatment and prevention are developed.

Although research into the molecular mechanisms of alcohol has produced many impactful discoveries, a complementary approach has been to identify the specific genetic variants that contribute to population-wide differences in AUD risk (Schuckit, 2018). Identification of the genetic factors that predict AUD could help to target at-risk individuals with early interventions that curtail the development of alcohol-dependence, and also provide actionable insights into the biological foundations of AUD.

One of the best predictors of future drinking problems is alcohol sensitivity (Schuckit, 1994; 1996; 1998). People with low naïve alcohol sensitivity are up to four times as likely to become alcoholics compared to people with normal or high alcohol sensitivity. Differences in alcohol sensitivity exist before AUDs develop and are highly heritable (Verhulst et al., 2015). Although genome-wide association studies (GWAS) for alcohol use disorder in human populations have accelerated greatly in recent years, it remains difficult to identify causal variants from these studies. Identifying causal genetic variants that underlie differences in alcohol sensitivity, then, would make ideal biomarkers for early intervention and AUD risk management. Further, although the complex phenotype of AUD is difficult to model, the relatively simple but highly predictive trait of sensitivity to alcohol intoxication can be easily modeled in the nematode *C. elegans*.

C. elegans has played a key role in identifying molecules with conserved effects in mammalian alcohol responses. Genetic screens identified the BK-potassium channel (SLO-1) as the major target for ethanol in nematodes (Davies et al., 2003), and subsequent work used *C. elegans* to identify the role of the NPY (Davies et al., 2004) receptor and SWI/SNF chromatin remodelers in acute alcohol response (Mathies et al., 2015). Mutations in these genes, which were first identified using *C. elegans*, predictably alter alcohol responses in mice and humans (Mathies et al., 2015; Du et al., 2005; Robinson & Thiele, 2017). Nearly all studies on alcohol response in *C. elegans* have relied on a single genetic background, the laboratory wild-type strain N2, save for one recent study that analyzed the alcohol responses of a recombinant inbred line panel derived from crosses of six wild strains (van Wijk et al., 2023). By leveraging the largely unexplored genetic diversity of

hundreds of recently gathered *C. elegans* wild strains from around the world, additional genes with important orthologous roles in human alcohol sensitivity and addiction can be identified (Crombie et al., 2023).

We performed a GWAS with a collection of 152 *C. elegans* wild strains to identify five quantitative trait loci (QTL). We next show that the ChrV QTL is likely explained by variation in *dkf-2*, a highly conserved ortholog of human Protein Kinase D (PKD). Although PKD's upstream activator, protein kinase C (PKC), has an established role in cross-species alcohol response (Lee & Messing, 2008), our results are, to our knowledge, the first evidence that PKD similarly affects alcohol sensitivity. We next show that a selective PKD inhibitor trends towards differentially conferring intoxication resistance in a *dkf-2*-dependent manner. These findings highlight the power of harnessing natural genetic diversity in a powerful model organism to identify novel genetic effectors of medically important traits such as alcohol sensitivity.

Results

A genome-wide association study of alcohol sensitivity identifies five loci

To calculate a heritability index for alcohol sensitivity, we first assayed a subset of 12 highly genetically diverse *C. elegans* wild strains for acute behavioral sensitivity to alcohol using egg-laying (Fig. 1A). We found that sensitivity ranged widely in the population and was about 42% heritable, mirroring the heritability of alcohol sensitivity observed in human populations ($H^2 \approx 0.3$-0.5; Verhulst et al., 2015). To confirm that egg-laying serves as a general readout for alcohol-induced depression in neuromuscular function as in previous studies (Davies et al., 2003), we likewise measured depression in locomotion for six strains and found a strong correlation between the measures (R^2=0.87; Supplemental Fig. 1). After establishing significant heritable trait variation in the diversity panel, we expanded our assays and measured alcohol intoxication sensitivity of 152 wild strains from around the world (Fig. 1a; right). We conducted a GWAS and identified five QTL significantly associated with differences in alcohol sensitivity (Fig. 1B). At all five of these loci, alternative (ALT) single nucleotide variants (SNVs) were associated with lower alcohol sensitivity. Although the most significant marker was ChrII:2,464,417 ($-\log_{10}(p)$= 5.768), we chose not to pursue this QTL for fine mapping, because it was within a hyper-variable region of low sequence confidence (Lee et al., 2021). The next-most significant SNV, at ChrV:16,792,754 ($-\log_{10}(p)$=5.492), marked a QTL spanning ~2 Mb from V:16,638,047-18,330,166, where the ALT allele predicted significantly lower alcohol sensitivity (Fig. 1B).

Fine mapping

To identify potential causal variants in the chromosome V QTL, all rare and common SNVs in the interval were tested for association with alcohol intoxication sensitivity differences, with priority given to alleles with high predicted effects on gene function (*e.g.*, nonsynonymous substitutions, early stop codons, splice-site variants, etc.)(Fig. 2B). Fine mapping identified several high-impact variants in the two nearest genes to peak QTL marker, *dkf-2* (V:16,764,489 C->T; V:16,758,168 A->G) and *immt-2* (V:16,789,765 C->T; V:16,790,476 G->A), that segregated with differences in sensitivity to ethanol intoxication (Fig. 2A; second and third panels). Within each gene, candidate causal variants were almost completely linked; however, in a few strains, candidate causal variants between *dkf-2* and *immt-2* were not in strong LD. This difference allowed us to identify which variants in these two genes segregated best with reduced alcohol sensitivity. We found that strains with ALT alleles in *immt-2*, but not *dkf-2*, were more sensitive to alcohol than strains where ALT alleles were present in both (Fig. 2C). Interestingly, PKDs are downstream of PKC, a known effector of alcohol response (Wallace et al., 2007). Further, *dkf-2* deletion mutants were shown to restore movement in a TDP-43 model of neurodegeneration (Liachko et al., 2014), suggesting an ameliorative effect on neuromuscular depression. These data together suggest that ALT variants in *dkf-2* might underlie the QTL.

Probing the role of Protein kinase D in alcohol sensitivity

To better understand the effect of the candidate variants on gene function, we asked whether *dkf-2* loss-of-function mutants showed altered alcohol sensitivity. We obtained two *dkf-2* deletion alleles in the N2 laboratory wild-type background: *ok1704*, a 716 bp deletion in exons 11 and 12, and *tm4076*, a 1,646 bp deletion that interrupts an obligate exon of all six known isoforms (Fig. 3A). We found that strains harboring either of the two deletion alleles were resistant to intoxication relative to the N2 strain (Fig. 3C). Because the loss-of-function mutants displayed lower alcohol sensitivity relative to *dkf-2* REF strains, we hypothesized that the candidate causal variant(s) in *dkf-2* in wild strains are hypomorphic, because the ALT allele at the marker likewise covaried with lower alcohol sensitivity.

To causally test the role of candidate alleles in *C. elegans* alcohol sensitivity, we attempted to knock-in the top candidate variants in *dkf-2* (V:16,764,489 or V:16,758,168) into the laboratory strain N2. Although we failed to generate the desired conversion mutants, we were able to obtain a novel deletion allele, *vx40*, in *dkf-2* (Fig. 3A). In *vx40* two exonic bases near the PAM site were deleted, causing a putative frame-shift loss-of-function allele. We likewise found that this novel deletion conferred decreased alcohol sensitivity. Interestingly, *vx40* affects only four of the six predicted *dkf-2* isoforms (a, d, e, and f), one

fewer than *ok1704* deletion (a, c, d, e, and f) and two fewer isoforms than the *tm4076* deletion (Hillier et al., 2009)(Fig. S4).

To test whether *dkf-2* plays a role in the alcohol sensitivity of the wild strains and to probe the nature of the natural candidate causal variants, we generated two independent *dkf-2* deletion alleles in each of two representative wild strain backgrounds: PS2025, which harbors the *dkf-2* REF alleles, and NIC1, which harbors the *dkf-2* ALT alleles. We found that novel deletions in the ALT background did not change alcohol sensitivity, but deletions in the REF background significantly reduced alcohol sensitivity (Fig. 3D).

It is worth noting that although NIC1 has the same phenotype in the hemizygosity tests (mean = 0.504) as in GWAS assays (mean sensitivity = 0.463), it was more sensitive than previous experiments in the assays with deletion of *dkf-2* (mean sensitivity = 0.623). This difference was likely caused by aberrantly high untreated egg-laying measures for this set of assays (mean eggs/hour = 84.2) relative to earlier tests (mean eggs/hour = 37) and relative to PS2025 in the same tests (mean eggs/hour = 67.5). Thus, higher pre-measurements could have inflated the sensitivity of NIC1 in these assays relative to other experiments reported here. We are agnostic as to the reasons for this notable difference. Still, the results from this experiment demonstrate that deletion of the *dkf-2* gene affects alcohol sensitivity in a background dependent manner, suggesting natural ALT alleles in *dkf-2* are driving differences in alcohol sensitivity.

We next performed a reciprocal hemizygosity test. We crossed WT PS2025 and NIC1 strains with an N2 strain carrying the *dkf-2* deletion allele *tm4076*. Interestingly, we found that F_1 *dkf-2* hemizygotes resembled their respective REF or ALT allele parental phenotypes, *i.e.*, the addition of the REF *dkf-2* allele recovered sensitivity but addition of the ALT allele did not. Together, these results provide evidence that the candidate ALT alleles represent causal hypomorphic natural variants (Fig. 3F).

To determine whether *dkf-2* alleles exert their effects on alcohol sensitivity by gross differences in ethanol metabolism, we measured internal alcohol concentrations for the laboratory strain (N2), a *dkf-2* REF wild isolate (PS2025), a *dkf-2* ALT wild isolate (NIC1), as well as two *dkf-2* knockout mutants in each of the REF and ALT backgrounds. We found no differences in internal alcohol concentration after one hour of ethanol exposure between any of the strains (Supplemental Fig. 4). Moreover, alcohol concentrations were consistent with established measures (Alaimo et al., 2012). These results suggest that phenotypic differences in alcohol sensitivity were not caused by differences in ethanol metabolism or uptake.

Because protein kinases represent druggable targets, we next asked whether *dkf-2* affects alcohol sensitivity using a selective protein kinase D inhibitor (CID 755673 – Tocris Bioscience). We chose to assay the laboratory strain N2 and an N2 deletion mutant *dkf-2(ok1704)* to avoid potential confounding effects of wild strain variation in drug permeability and metabolism. CID 755673 has been shown to disrupt multiple cellular responses of all three human PKDs *in vitro* (Sharlow et al., 2008). Although CID 755673 has not been used in *C. elegans* before, previous studies have found remarkably conserved function, inhibition, and activation dynamics for *C. elegans* DKF-2 and human PKDs. For example, DKF-2A exogenously expressed in human HEK293 cells is predictably activated by pharmacological activation of endogenous upstream human PKCs (Feng et al., 2007). Likewise, DKF-2A expressed in human cell culture shows robust phosphorylation activity towards human PI4KIIIβ when treated with a PKC activator – a known native target of human PRKD2 (Aicart-Ramos et al., 2016). We found that the *dkf-2(ok1704)* deletion mutant showed no significant difference in alcohol response when treated with the inhibitor, and strains that carry a wild-type copy of *dkf-2* trended towards a decrease in alcohol sensitivity (Fig. 3E; two-sided t-test p=0.07, Cohen's d: 0.584 medium effect).

Discussion

By harnessing natural variation in wild strains of *C. elegans*, we identified five QTL associated with variation in alcohol sensitivity. Further, we demonstrated a new role for protein kinase D (*dkf-2*) in alcohol sensitivity. To our knowledge, protein kinase D has eluded identification using classical forward-genetic screens for ethanol sensitivity in the *C. elegans* laboratory strain N2 – nor has PKD attracted attention in the wider field of alcohol research to date. Only a single alcohol study has considered PKD, not in a role for sensitivity, but as a potential therapeutic target in experimental alcoholic pancreatitis (Yuan et al., 2021).

Protein kinases and alcohol response

Although other protein kinases are known effectors of alcohol response (*e.g.*, PKCs (Harris et al., 1995; Wallace et al., 2007), ERKs (Lee & Messing, 2008), PKAs (Pandey et al., 2005), and CAM kinases (Zhang et al., 2011)), this study is the first to implicate protein kinase D (PKD). PKDs are serine/threonine protein kinases that affect a wide range of cellular processes (Fu & Rubin, 2011; Ellwanger & Hauser, 2013). Most often, PKDs are downstream effectors of protein kinase C (PKC) and diacylglycerol (DAG) via phospholipase C (PLC) β and γ, which are in turn activated by a variety of neurotransmitters, hormones, and other stressors (Fu & Rubin, 2011; Waldron & Rozengurt,

2003) (Fig. 3B). Recent studies, however, show that PKDs are sometimes activated via PKC-independent autophosphorylation, but the precise mechanism and functional consequences of this pathway remain obscure (Elsner et al., 2019; Reinhardt et al., 2020).

As integrators of signal inputs from PKC and DAG, PKDs affect a wide array of downstream cellular processes, like class II HDAC activity (Parra et al., 2005; Dequiedt et al., 2005), neuronal function and development (Fu et al., 2009; Bisbal et al., 2008; Czondor et al., 2009), and Golgi and membrane dynamics (Rozengurt, 2011), as well as a conserved role in activation of immune and stress tolerance pathways (Kim et al., 2010; Feng et al., 2007; Ren et al., 2009) (Fig. 12b). PKD could plausibly modify alcohol sensitivity through one or more of these pathways, but immune and stress tolerance pathways represent especially promising candidate mechanisms. In *C. elegans, dkf-2* plays a role in innate immune response, where it promotes PMK-1 (ortholog of p38) activity, and downregulates *daf-16*, a transcription factor that promotes stress tolerance and lifespan through the Insulin/IGF-1-like signaling (IIS) pathway (Lin et al., 1997; Lin et al., 2001). Thus, PKD sometimes interacts with the p38 MAPK cascade, a pathway containing *nsy-1* (ortholog of human MAP3K), mutants of which are resistant to the toxic effects of ethanol and other drugs of abuse (*e.g.*, methamphetamine, MDMA)(Gomez, 2013; Schreiber & McIntire, 2011).

Given the size of the genomic locus and coding sequence of *dkf-2*, it is puzzling that this gene was not identified in earlier forward genetic screens. In some of the *dkf-2* deletion mutants, particularly the *tm4076* mutant where all isoforms of the gene were predicted to be affected, we found a low penetrance sterility phenotype as well as poor gut health. Spontaneous mutagenesis and natural selection in wild populations, on the other hand, could have produced variants subtle enough to affect essential gene function without causing lethality or sterility (Ossowski et al., 2010; Schroeder et al., 2017).

Candidate genes in the remaining QTL

Although we dissected only one of the five QTL identified in the GWAS, statistical fine mapping of the remaining QTL suggests several promising candidate genes underlying these loci (Fig. 1B). For Chromosome III, top candidates include *cdh-1*(cadherin), *cosa-1*(human CNTD1 (cyclin N-terminal domain containing 1)), and the potassium channel *twk-45*, a particularly notable candidate, as the largest hit from earlier alcohol sensitivity screens is the BK-potassium channel *slo-1*. Further, a GWAS in human populations for interactive effects of alcohol drinking and blood pressure identified multiple significant associations in a human orthologue of *twk-45*, *KCNK3* (Feitosa et al., 2018). The QTL on Chromosome X is more difficult to parse, because the low recombination rate of the X chromosome leads to much larger candidate regions containing a larger number of linked genes. The most significant SNVs from fine mapping of the X chromosome peak include *let-*

4 and *slo-2*. Given the known roles of *slo-1* and *slo-2* in *C. elegans* alcohol response, *slo-2* represents a promising causal candidate gene (Bettinger & Davies, 2014; Dopico et al., 2014; Scott et al., 2017; 2018). Other promising candidate genes in the X chromosome peak include *atrn-1*, a mutant with known locomotion phenotypes and levamisole hypersensitivity from previous *C. elegans* studies, and *slt-1* (human orthologue *SLT-1*, *SLT-2*, *SLT-3*), a gene with important roles in neuronal development via SLT-ROBO signaling. Interestingly, a recent human GWAS on externalizing behaviors, including substance abuse, identified associations with variants in human *SLT2* (Linner et al. 2021). Due to local genomic hyper-variability (Lee et al., 2021), the QTL on ChrII, ChrIII, and ChrIV were difficult to dissect. In *C. elegans*, certain regions of the genome are highly variable from the laboratory strain N2 as well as other related haplotypes (Lee et al., 2021). In effect, hyper-variability leads to difficulty in assembling genome sequences against a reference genome, causing areas of low coverage. Although hyper-variable regions are difficult to dissect, they still could represent true effectors of *C. elegans* alcohol sensitivity. We attempted CRISPR knock-ins of other variants in the ChrII and ChrIII QTL but failed to generate even deletion mutants.

Conclusions

Here, we used a high-throughput behavioral screen to measure acute behavioral sensitivity to ethanol intoxication in a diverse set of over 150 wild strains of the nematode *Caenorhabditis elegans*. Using GWAS, we identified five loci associated with alcohol sensitivity. Next, we causally validated the role of a highly conserved orthologue of PKD in *C. elegans* alcohol response. We further find that a PKD inhibitor trends towards affecting alcohol intoxication sensitivity in a *dkf-2*-dependent manner. Together, these data reveal a new role for PKD in natural variation to alcohol intoxication, a trait that significantly predicts later life alcohol abuse in human populations. If future studies demonstrate a conserved role for PKD in mammalian alcohol response, these findings represent an exciting potential avenue for novel AUD treatments. Already, work in mammals has shown that PKCs are druggable targets with substantial effects on AUD traits (Lee & Messing, 2008). However, relative to PKCs, PKDs were only recently discovered (Fu & Rubin, 2011), thus, far fewer studies and available reagents are available for researchers to exploit. Future work should aim to tease apart the mechanisms by which PKD affects sensitivity to ethanol intoxication.

Methods

Animals

Animals were grown at room temperature (20°C) using nematode growth medium agar plates seeded with OP50 *E. coli* bacterial lawns as previously described (Brenner, 1974). Strains were propagated for at least three generations before behavioral testing, to minimize epigenetic effects of starvation on assays. To prevent mutational drift in our test populations, strains were re-thawed from the original stock every 1-2 months (Cook et al., 2016). Animals were age synchronized to day-one adulthood using timed-egg lays. When needed, nematode growth plates were cleaned using serial passage rather than bleaching to prevent inadvertent selection effects.

Assay for alcohol intoxication sensitivity

Nematode growth medium (NGM) agar (12 mL) was dispensed into a 6-cm diameter Petri dish and allowed to set for 48 hrs. Next, agar plates were seeded with ~0.75 mL of OP50 *E. coli* 48 hours prior to testing. At the start of each assay, 10 clonal, day-one adult worms were picked to an untreated control plate, where they were allowed to lay eggs for one hour. Animals were then transferred to an ethanol-treated plate, where they were again allowed to lay eggs for one hour before removal. For each assay, alcohol intoxication sensitivity was calculated by dividing the number or eggs laid on the ethanol plate by the number of eggs laid on the untreated control plate subtracted from one (alcohol sensitivity = 1 - (# eggs laid on ethanol / # eggs laid on control)). To minimize the influence of nuisance variables, strains were tested in blocks of 18-22 variably interleaved across replicates. Genotype averages were calculated using alcohol sensitivity scores from about 10 individual assays. Ethanol plates were prepared by depositing 280 µL of 200 proof ethanol (Sigma) beneath the agar pad 25 minutes before animals are transferred for behavioral testing (Davis et al., 2014). After one hour, this exogenous dose results in an internal ethanol concentration of ~80 mM in day-one adult nematodes (Alaimo et al., 2012; Scott et al., 2017). To minimize between assay variation, all ethanol plates were massed to 17.5 -18g before application of ethanol.

Heritability calculation

Broad sense heritability (H^2) was calculated using $H^2 = V_G/(V_G+V_E)$, where V_G is between strain variance and V_E is residual variance. H^2 was estimated using a linear mixed-effect model (*lme4* (R 4.4.0)) where genotype was a random effect (Shaver et al., 2023)

PKD inhibitor assays

Alcohol sensitivity assays were conducted as described above. To ensure drug effects, animals were treated with an exogenous concentration of CID 75563 (2.0 µM) that is high relative to its IC50 for human PKDs (0.18-0.28 µM), but still far below the IC50 for non-target protein kinases (*e.g.*, PKC = 10.0 µM; PLK1 = 20.3 µM) (Sharlow et al., 2008).

Exogenous treatment of *C. elegans* to drugs often requires orders of magnitude higher concentrations than effective internal concentrations (Rand and Johnson, 1995; Burns et al., 2010). Animals were pre-treated with CID 755673 (DMSO) for one hour prior to alcohol sensitivity assays. During behavioral assays, both the baseline and alcohol egg-laying plates contained CID 75563 (2.0 µM) and 0.01% DMSO. Treated animals were tested alongside yoked controls whose plates were treated with DMSO to account for non-specific solvent effects.

Determining internal ethanol concentrations

Internal ethanol concentrations were measured as described previously (Alaimo et al., 2012). In brief, we used a colorimetric assay that uses alcohol metabolic enzymes (ADH & ALDH) to generate quantitatively sensitive shifts in absorbance at 340 nm (Megazyme). Day-one adult animals from each strain were subjected to the same alcohol sensitivity assay as described above. After one hour on ethanol treatment plates, 200 animals of each strain were picked into 120 uL of DI H_20. The resulting dilution factor was calculated using estimates of day-one adult worm volumes by Andrews (2019). To prevent evaporation of ethanol from samples, worms were homogenized on ice using a Pyrex grinder for about two minutes per sample. Samples were centrifuged, and supernatant was extracted using a micropipette. Average ethanol concentrations for each strain were determined against a standard colorimetric absorbance curve.

GWAS

GWAS was performed using alcohol sensitivity phenotypes of 152 *C. elegans* wild strains and SNV markers from the publicly available CeNDR VCF release 20231213. Association mapping used a linear mixed model optimized for *C. elegans* (rrBLUP) implemented in cegwas2 (https://github.com/AndersenLab/cegwas2-nf) (Endelman et al., 2011; Yu et al., 2006; Zdraljevic et al., 2017). SNVs with minor allele frequency less than 5% were filtered out using BCFtools (Li, 2011). Missing genotypes were imputed using PLINK 1.9 (Purcell et al., 2007; Chang et al., 2015). Strain relatedness was accounted for using a kinship matrix generated with the A.mat function in rrBLUP (Endelman, 2011). Because many markers were in high linkage, significance thresholds were adjusted by using number of independent tests in the marker set (Noble, 2009; Johnson et al., 2010). Fine mapping was performed by analyzing genotype-phenotype relationships for all SNVs in the QTL interval identified by GWAS, rather than only common SNVs (MAF greater than or equal to 5%). Pairwise linkage disequilibrium (r^2) was calculated between each candidate SNV in the interval and the genome-wide significant SNV from GWAS (Mueller, 2004).

Fine mapping

NIC1 and PS2025 strains were chosen based first on the fact that they did not share many variants in common in the defined QTL, and second that their alcohol sensitivity phenotypes differed by about one SD. NIC1 (mean=0.463) was a little less than one SD below the average phenotype for the ALT group (0.568; SD=0.176) and PS2025 (mean=0.570) was likewise about one SD below the average phenotype for the REF group (mean=0.692; SD=0.093).

CRISPR-Cas9 genome editing

Ribonucleoprotein (RNP) complexes for co-CRISPR were used to generate mutants (Farboud & Meyer, 2015; Prior et al., 2017; Woo et al., 2013). Cas9 protein and sgRNA were incubated together to form an RNP complex *in vitro*. The assembled RNP complex and repair template were then directly injected into the gonad of a *C. elegans* hermaphrodite, generating edits in the germline. For ease of screening for desired transformants, a gRNA for a mutation into the locus *dpy-10* was co-injected that causes a dominant Roller phenotype (Arribere et al., 2014; Prior et al., 2017). After confirmation of desired edits by Sanger sequencing, the *dpy-10* marker allele was crossed out by selecting F_2's lacking the Roller phenotype.

Target and repair sequences for vx40 mutant:

dkf-2 crRNA: 5' UGAGGCUCUACAGUUUAUCA 3'

dkf-2 ssDNA repair oligo: 5'
GGCCATAAGTGTTTTTTAGTTAACGACAGCTTCCTTGACTTTAACGCGAAGCTCTG

GAAGCTTTACTCCGAGATGTCAAATAGTTCCTCCAGCATTCAAAAATAAGGTTTTTTAAGCTTTTTAA
ACTATCTTTAAACTTT 3'

dpy-10 crRNA: 5' GCUACCAUAGGCACCACGAG 3'

dpy-10 ssDNA repair oligo: 5'
CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCAT

GCGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT 3'

Strains

CK600 – N2 strain *dkf-2* loss-of-function (*tm4076*)

RB14668 – N2 strain *dkf-2* loss-of-function (*tm1704*)

JPS1349 – N2 strain *dkf-2* loss-of-function (*vx40*)

ECA3728 – NIC1(ALT) strain *dkf-2* loss-of-function

ECA3729 – PS2025(REF) strain *dkf-2* loss-of-function

ECA3730 – PS2025(REF) strain *dkf-2* loss-of-function

ECA3731 – NIC1(ALT) strain *dkf-2* loss-of-function

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Competing interests: The authors declare no competing interest.

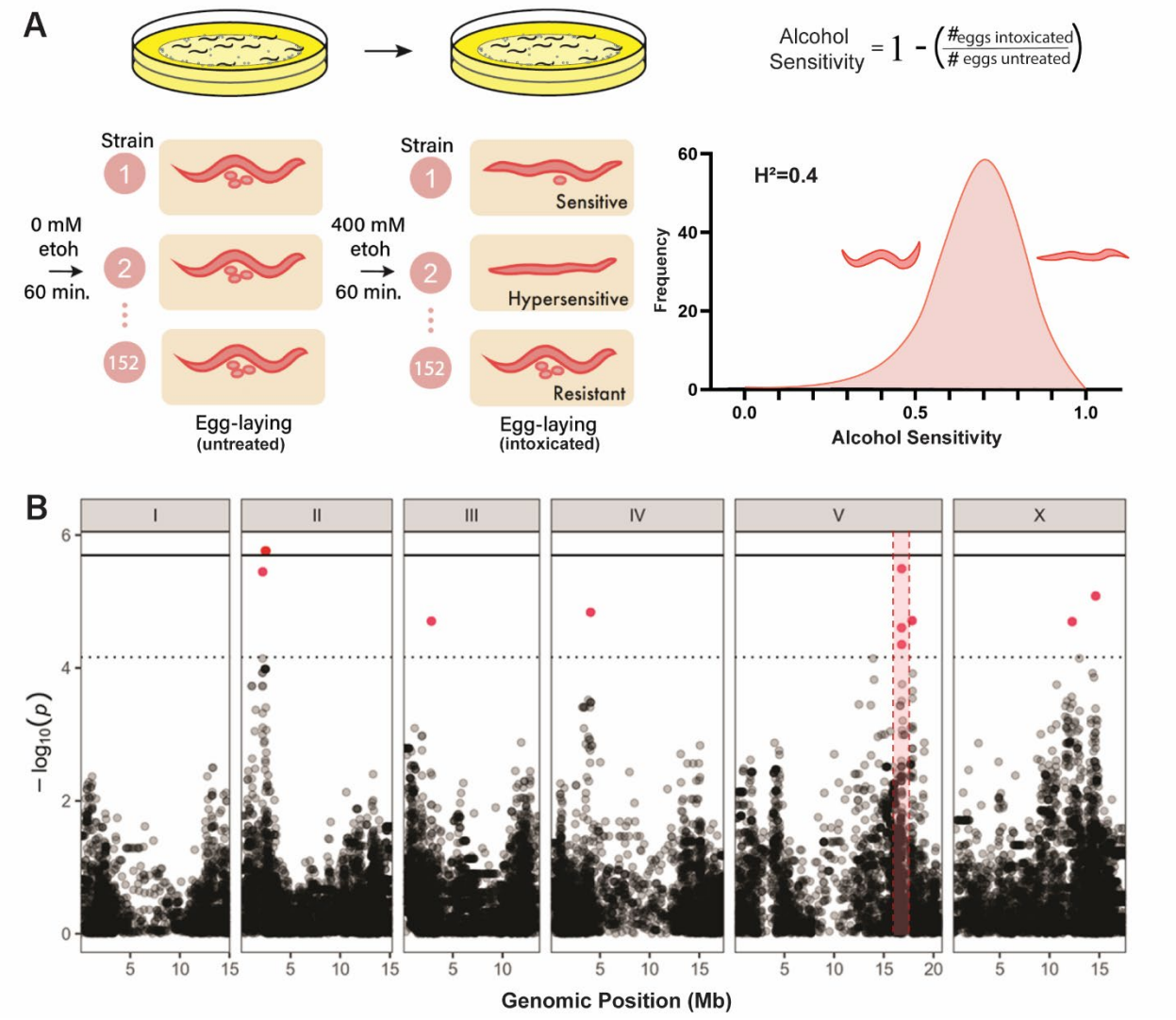

Figure 1: GWAS of natural variation in alcohol sensitivity of 152 *C. elegans* wild strains.

(A) Overview of high-throughput behavioral screen for naïve alcohol sensitivity in 152 *C. elegans* wild isolates. In each assay, basal egg-laying rates were measured using ten clonal adults, and strain average phenotypes were calculated as the average phenotype score across ~10 replicates for each genotype. Distribution of alcohol intoxication sensitivity in the population is depicted in top right corner. **(B)** Manhattan plot of GWAS for alcohol sensitivity. Each panel represents a chromosome (I-V & X); each point represents an SNV marker. X-axis represents genomic position (Mb), and y-axis denotes significance ($-\log_{10}(p)$). The black dotted line denotes genome-wide significance cutoff after correcting for the number of independent association tests. Highlighted with a vertical red box is the ChrV QTL that was pursued for fine mapping.

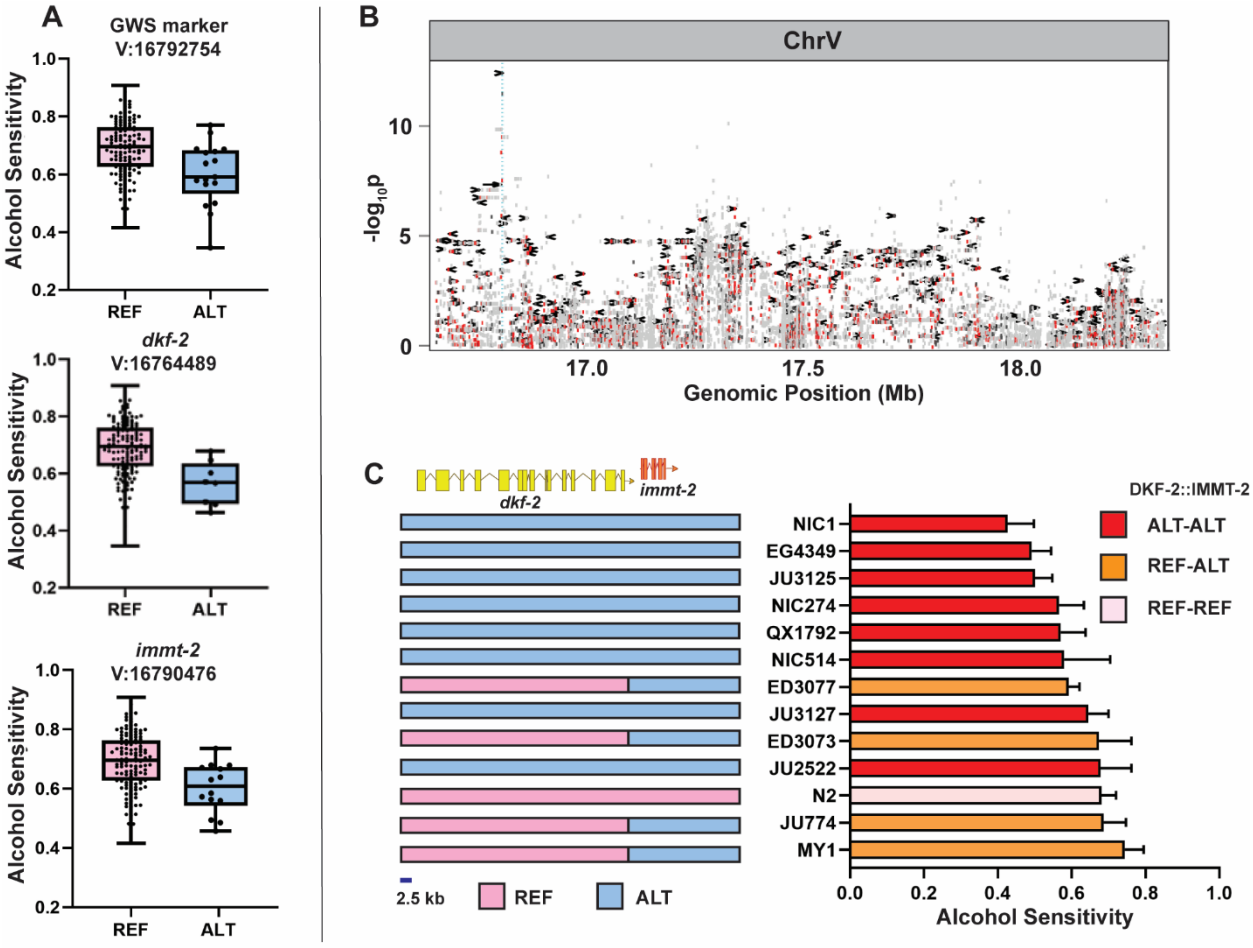

Figure 2: Fine mapping chromosome V QTL associated with decreased sensitivity.

(A) Each point in boxplots represents the average alcohol sensitivity of a single strain. Y-axis denotes alcohol intoxication sensitivity, and genotype at SNV is shown along the X-axis (REF or ALT). Average phenotypes of all strains were segregated by their genotype at the denoted allele. Top graph shows strains segregated by genotype at the genome-wide significant SNV identified by GWAS. Middle and bottom graphs show phenotypes segregated by genotype at the two high impact mutation candidate variants identified by fine mapping. **(B)** Results from fine mapping. Each line represents an SNV in the significant interval (V:16,638,047-18,330,166) from GWAS. Y-axis denotes significance. Color fill of each SNV represents predicted variant effect, where red denotes high-impact variants, gray represents low-impact variants, and light gray represents intergenic/linker variants. **(C)** Left shows the genotype of six strains at the two high-impact variants predicted in each candidate gene nearest the GWAS peak marker, *dkf-2*, and *immt-2*. In a small subset of strains (left; pink and blue multicolored bars), candidate variants in *dkf-2* were not in strong LD with candidate variants in *immt-2*. (Right) Average intoxication sensitivity of each genotype arranged from least to most sensitive (left) (mean & SEM; n=10-15). Color

433 denotes genotype shown in left panel, where red denotes ALT-ALT, orange denotes ALT-REF,
434 and pale pink denotes REF-REF N2 lab strain.

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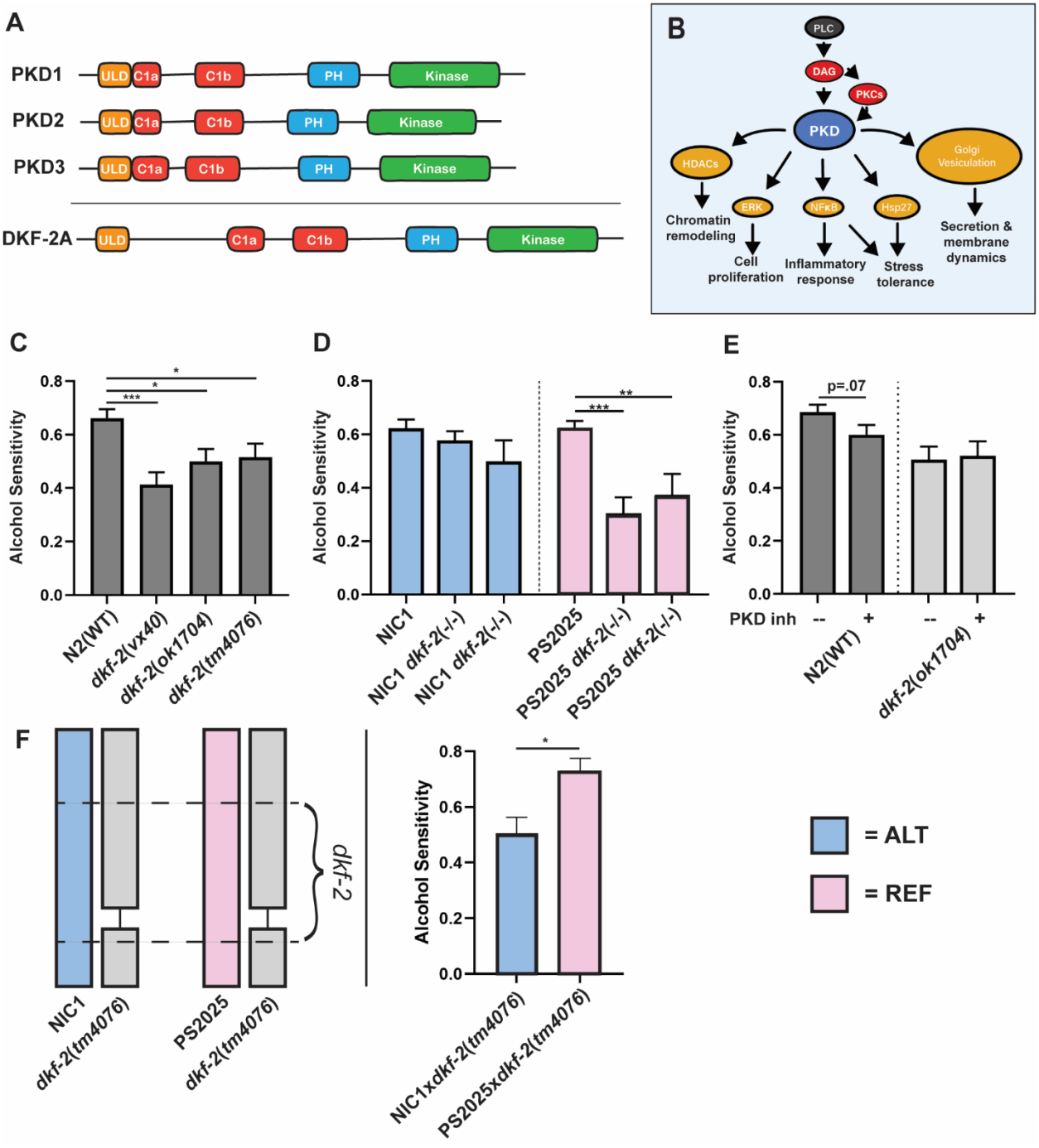

Figure 3: Independent deletion alleles in the highly conserved *C. elegans* orthologue of PKD (*dkf-2*) predictably reduce alcohol sensitivity.

(A) Protein kinase D is functionally conserved from humans to *C. elegans*. The human gene products (PKD1, PKD2, PKD3) are compared to one isoform of the *C. elegans* ortholog (DKF-2-A). Orthologous functional domains are labeled. **(B)** Known interactions of PKD. PKDs are most often downstream effectors of DAG-PKC signaling cascades. Activated

PKDs mediate a wide array of cellular processes *e.g.*, chromatin dynamics, cell growth and proliferation, innate immunity and inflammatory response, general stress tolerance and Golgi vesiculation. Recent studies show that PKD can activate via PKC-independent autophosphorylation of the conserved ULD domain **(C)** Mean alcohol sensitivities (SEM) of three *dkf-2* deletion alleles (*vx40*, *ok1704*, and *tm4076*) were significantly lower than intoxication sensitivity of *dkf-2* lab wild-type (N2) animals (mean & SEM; n=15; *p*=0.0001; p=0.0104; p=0.0167). **(D)** Deletion mutants in a wild isolate (NIC1) harboring candidate hypomorph allele(s) in *dkf-2* do not cause a reduction in alcohol sensitivity (mean & SEM; n=18; p=0.35; p=0.1573), whereas deletions in a strain without the candidate hypomorph alleles cause significant reductions in alcohol sensitivity (mean & SEM, n=18; p=0.0001; p=0.0052). **(E)** Treatment with a PKD inhibitor (CID 755673) trends towards lower alcohol sensitivity in the N2 lab strain (mean & SEM, n= 18-20, two-sided t-test p=0.07, Cohen's d: 0.584 -medium effect) but shows no effect on alcohol sensitivity in a *dkf-2* deletion mutant (RB1468(*ok1704*)). **(F)** Strains that are hemizygous for ALT genotype at *dkf-2* are resistant to alcohol, while strains that are hemizygous for the REF genotype at *dkf-2* are sensitive to alcohol (mean & SEM; n=6; p=0.01). NIC1 (ALT) and PS2025 (REF) were mated to a *dkf-2* deletion mutant (*tm4076*). F_1 progeny from these crosses were assayed for their alcohol sensitivity. Alcohol sensitivity was higher in PS2025 (REF)x*dkf-2*(-/-) F_1's relative to NIC1(ALT)x*dkf-2*(-/-) F_1's.

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