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

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

121 **Probing the role of Protein kinase D in alcohol sensitivity**

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

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

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

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

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

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

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

173 Because protein kinases represent druggable targets, we next asked whether *dkf-2* affects
174 alcohol sensitivity using a selective protein kinase D inhibitor (CID 755673 – Tocris
175 Bioscience). We chose to assay the laboratory strain N2 and an N2 deletion mutant *dkf-*
176 *2(ok1704)* to avoid potential confounding effects of wild strain variation in drug
177 permeability and metabolism. CID 755673 has been shown to disrupt multiple cellular
178 responses of all three human PKDs *in vitro* (Sharlow et al., 2008). Although CID 755673 has
179 not been used in *C. elegans* before, previous studies have found remarkably conserved
180 function, inhibition, and activation dynamics for *C. elegans* DKF-2 and human PKDs. For
181 example, DKF-2A exogenously expressed in human HEK293 cells is predictably activated
182 by pharmacological activation of endogenous upstream human PKCs (Feng et al., 2007).
183 Likewise, DKF-2A expressed in human cell culture shows robust phosphorylation activity
184 towards human PI4KIIIβ when treated with a PKC activator – a known native target of
185 human PRKD2 (Aicart-Ramos et al., 2016). We found that the *dkf-2(ok1704)* deletion
186 mutant showed no significant difference in alcohol response when treated with the
187 inhibitor, and strains that carry a wild-type copy of *dkf-2* trended towards a decrease in
188 alcohol sensitivity (Fig. 3E; two-sided t-test p=0.07, Cohen's d: 0.584 medium effect).

189

190 **Discussion**

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

199 **Protein kinases and alcohol response**

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

208 2003) (Fig. 3B). Recent studies, however, show that PKDs are sometimes activated via
209 PKC-independent autophosphorylation, but the precise mechanism and functional
210 consequences of this pathway remain obscure (Elsner et al., 2019; Reinhardt et al., 2020).

211 As integrators of signal inputs from PKC and DAG, PKDs affect a wide array of downstream
212 cellular processes, like class II HDAC activity (Parra et al., 2005; Dequiedt et al., 2005),
213 neuronal function and development (Fu et al., 2009; Bisbal et al., 2008; Czondor et al.,
214 2009), and Golgi and membrane dynamics (Rozengurt, 2011), as well as a conserved role in
215 activation of immune and stress tolerance pathways (Kim et al., 2010; Feng et al., 2007;
216 Ren et al., 2009) (Fig. 12b). PKD could plausibly modify alcohol sensitivity through one or
217 more of these pathways, but immune and stress tolerance pathways represent especially
218 promising candidate mechanisms. In *C. elegans, dkf-2* plays a role in innate immune
219 response, where it promotes PMK-1 (ortholog of p38) activity, and downregulates *daf-16*, a
220 transcription factor that promotes stress tolerance and lifespan through the Insulin/IGF-1-
221 like signaling (IIS) pathway (Lin et al., 1997; Lin et al., 2001). Thus, PKD sometimes
222 interacts with the p38 MAPK cascade, a pathway containing *nsy-1* (ortholog of human
223 MAP3K), mutants of which are resistant to the toxic effects of ethanol and other drugs of
224 abuse (*e.g.*, methamphetamine, MDMA)(Gomez, 2013; Schreiber & McIntire, 2011).

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

232 **Candidate genes in the remaining QTL**

233 Although we dissected only one of the five QTL identified in the GWAS, statistical fine
234 mapping of the remaining QTL suggests several promising candidate genes underlying
235 these loci (Fig. 1B). For Chromosome III, top candidates include *cdh-1*(cadherin), *cosa-*
236 *1*(human CNTD1 (cyclin N-terminal domain containing 1)), and the potassium channel *twk-*
237 *45*, a particularly notable candidate, as the largest hit from earlier alcohol sensitivity
238 screens is the BK-potassium channel *slo-1*. Further, a GWAS in human populations for
239 interactive effects of alcohol drinking and blood pressure identified multiple significant
240 associations in a human orthologue of *twk-45*, *KCNK3* (Feitosa et al., 2018). The QTL on
241 Chromosome X is more difficult to parse, because the low recombination rate of the X
242 chromosome leads to much larger candidate regions containing a larger number of linked
243 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

278 Animals were grown at room temperature (20°C) using nematode growth medium agar
279 plates seeded with OP50 *E. coli* bacterial lawns as previously described (Brenner, 1974).
280 Strains were propagated for at least three generations before behavioral testing, to
281 minimize epigenetic effects of starvation on assays. To prevent mutational drift in our test
282 populations, strains were re-thawed from the original stock every 1-2 months (Cook et al.,
283 2016). Animals were age synchronized to day-one adulthood using timed-egg lays. When
284 needed, nematode growth plates were cleaned using serial passage rather than bleaching
285 to prevent inadvertent selection effects.

286 **Assay for alcohol intoxication sensitivity**

287 Nematode growth medium (NGM) agar (12 mL) was dispensed into a 6-cm diameter Petri
288 dish and allowed to set for 48 hrs. Next, agar plates were seeded with ~0.75 mL of OP50 *E.*
289 *coli* 48 hours prior to testing. At the start of each assay, 10 clonal, day-one adult worms
290 were picked to an untreated control plate, where they were allowed to lay eggs for one hour.
291 Animals were then transferred to an ethanol-treated plate, where they were again allowed
292 to lay eggs for one hour before removal. For each assay, alcohol intoxication sensitivity was
293 calculated by dividing the number or eggs laid on the ethanol plate by the number of eggs
294 laid on the untreated control plate subtracted from one (alcohol sensitivity = 1 - (# eggs laid
295 on ethanol / # eggs laid on control)). To minimize the influence of nuisance variables,
296 strains were tested in blocks of 18-22 variably interleaved across replicates. Genotype
297 averages were calculated using alcohol sensitivity scores from about 10 individual assays.
298 Ethanol plates were prepared by depositing 280 µL of 200 proof ethanol (Sigma) beneath
299 the agar pad 25 minutes before animals are transferred for behavioral testing (Davis et al.,
300 2014). After one hour, this exogenous dose results in an internal ethanol concentration of
301 ~80 mM in day-one adult nematodes (Alaimo et al., 2012; Scott et al., 2017). To minimize
302 between assay variation, all ethanol plates were massed to 17.5 -18g before application of
303 ethanol.

304 **Heritability calculation**

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

308 **PKD inhibitor assays**

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

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

320 **Determining internal ethanol concentrations**

321 Internal ethanol concentrations were measured as described previously (Alaimo et al.,
322 2012). In brief, we used a colorimetric assay that uses alcohol metabolic enzymes (ADH &
323 ALDH) to generate quantitatively sensitive shifts in absorbance at 340 nm (Megazyme).
324 Day-one adult animals from each strain were subjected to the same alcohol sensitivity
325 assay as described above. After one hour on ethanol treatment plates, 200 animals of each
326 strain were picked into 120 uL of DI H_20. The resulting dilution factor was calculated using
327 estimates of day-one adult worm volumes by Andrews (2019). To prevent evaporation of
328 ethanol from samples, worms were homogenized on ice using a Pyrex grinder for about two
329 minutes per sample. Samples were centrifuged, and supernatant was extracted using a
330 micropipette. Average ethanol concentrations for each strain were determined against a
331 standard colorimetric absorbance curve.

332 **GWAS**

333 GWAS was performed using alcohol sensitivity phenotypes of 152 *C. elegans* wild strains
334 and SNV markers from the publicly available CeNDR VCF release 20231213. Association
335 mapping used a linear mixed model optimized for *C. elegans* (rrBLUP) implemented in
336 cegwas2 (https://github.com/AndersenLab/cegwas2-nf) (Endelman et al., 2011; Yu et al.,
337 2006; Zdraljevic et al., 2017). SNVs with minor allele frequency less than 5% were filtered
338 out using BCFtools (Li, 2011). Missing genotypes were imputed using PLINK 1.9 (Purcell et
339 al., 2007; Chang et al., 2015). Strain relatedness was accounted for using a kinship matrix
340 generated with the A.mat function in rrBLUP (Endelman, 2011). Because many markers
341 were in high linkage, significance thresholds were adjusted by using number of
342 independent tests in the marker set (Noble, 2009; Johnson et al., 2010). Fine mapping was
343 performed by analyzing genotype-phenotype relationships for all SNVs in the QTL interval
344 identified by GWAS, rather than only common SNVs (MAF greater than or equal to 5%).
345 Pairwise linkage disequilibrium (r^2) was calculated between each candidate SNV in the
346 interval and the genome-wide significant SNV from GWAS (Mueller, 2004).

347 **Fine mapping**

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

354 **CRISPR-Cas9 genome editing**

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

364 Target and repair sequences for vx40 mutant:

365 *dkf-2* crRNA: 5' UGAGGCUCUACAGUUUAUCA 3'

366 *dkf-2* ssDNA repair oligo: 5'
367 GGCCATAAGTGTTTTTTAGTTAACGACAGCTTCCTTGACTTTAACGCGAAGCTCTG

368 GAAGCTTTACTCCGAGATGTCAAATAGTTCCTCCAGCATTCAAAAATAAGGTTTTTTAAGCTTTTTAA
369 ACTATCTTTAAACTTT 3'

370 *dpy-10* crRNA: 5' GCUACCAUAGGCACCACGAG 3'

371 *dpy-10* ssDNA repair oligo: 5'
372 CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCAT

373 GCGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT 3'

374

375 **Strains**

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

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

378 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.

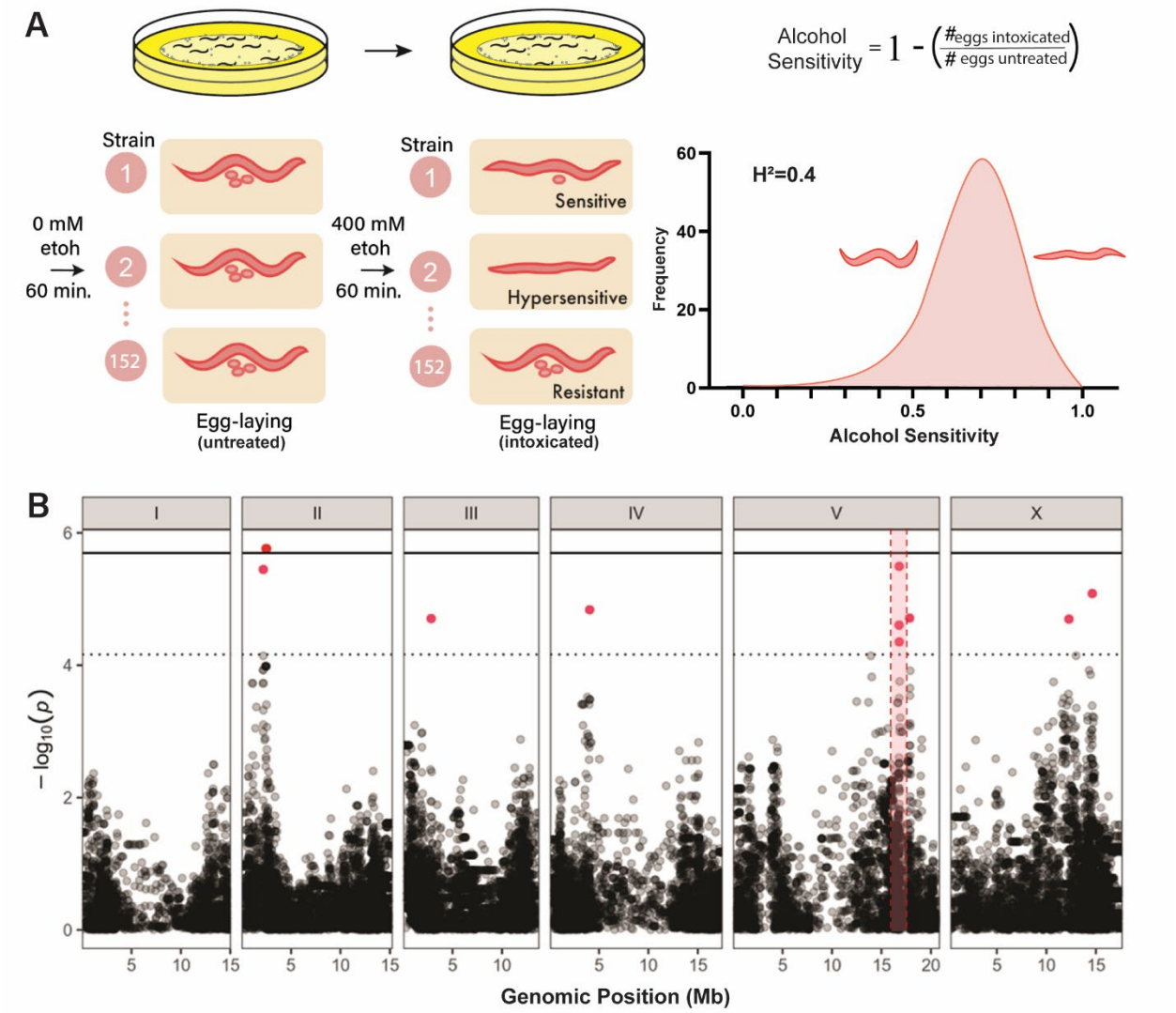

Figure 1: GWAS of natural variation in alcohol sensitivity of 152 *C. elegans* wild strains.

(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.

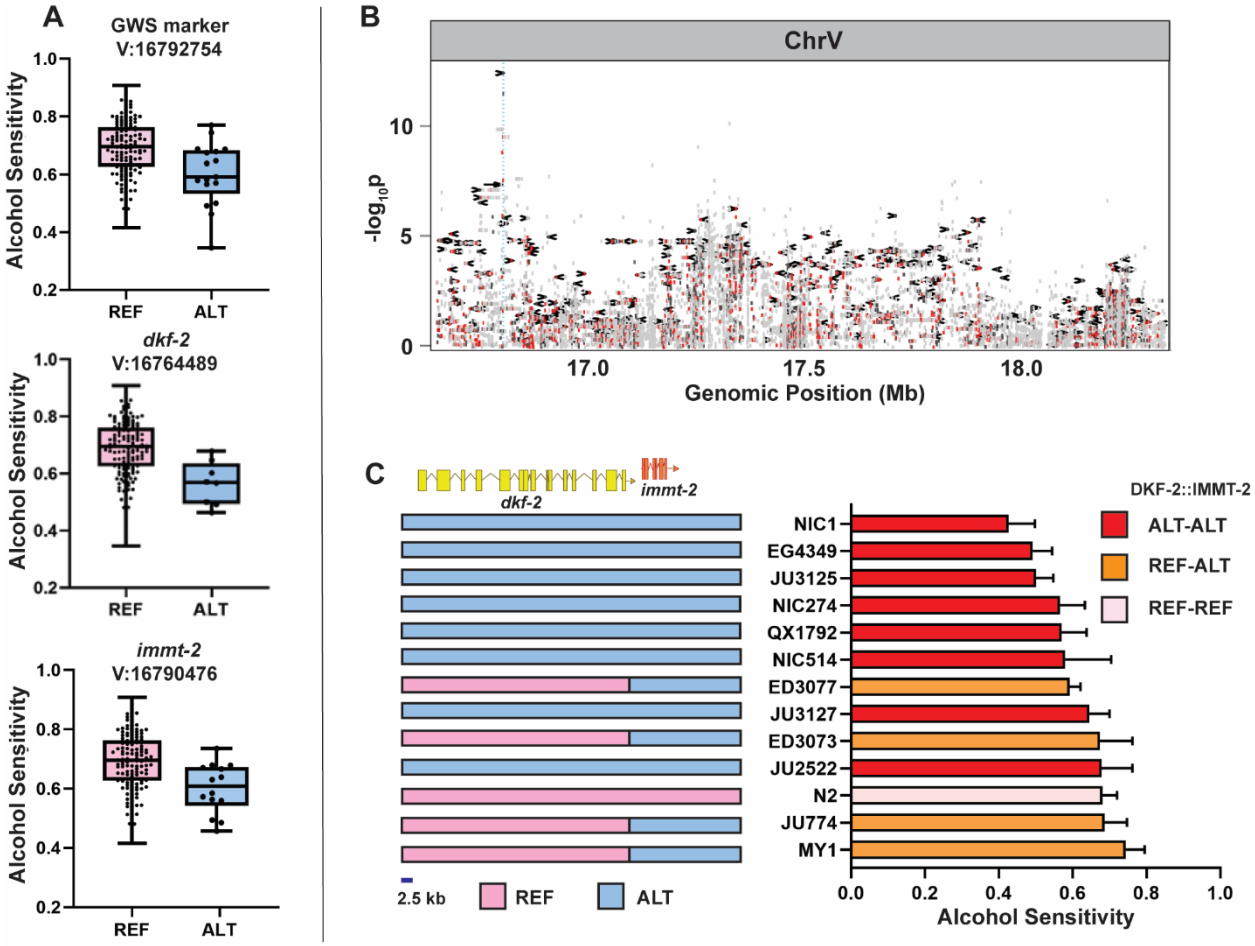

Figure 2: Fine mapping chromosome V QTL associated with decreased sensitivity.

(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

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

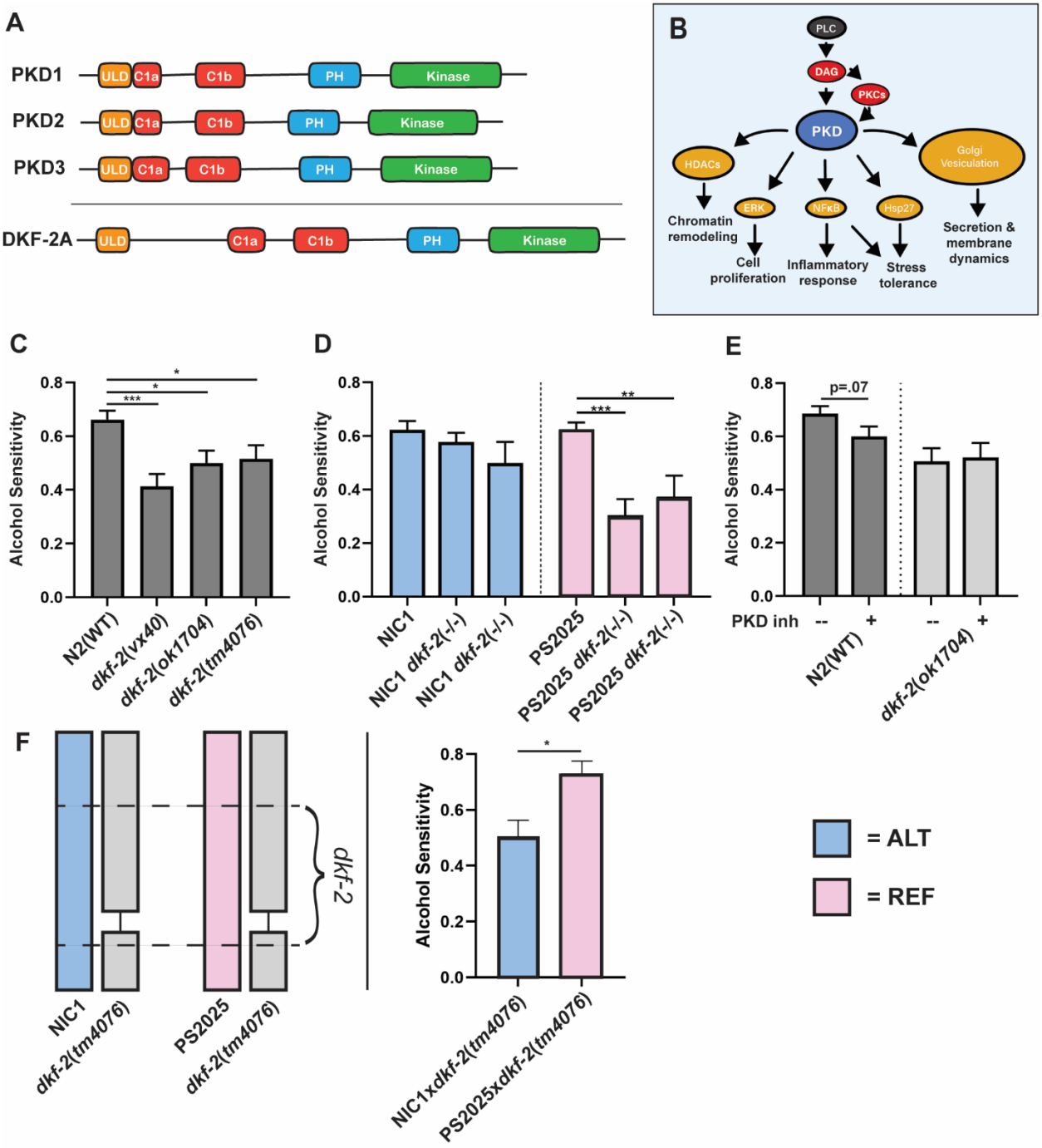

Figure 3: Independent deletion alleles in the highly conserved *C. elegans* orthologue of PKD (*dkf-2*) predictably reduce alcohol sensitivity.

(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

454 PKDs mediate a wide array of cellular processes *e.g.*, chromatin dynamics, cell growth and
455 proliferation, innate immunity and inflammatory response, general stress tolerance and
456 Golgi vesiculation. Recent studies show that PKD can activate via PKC-independent
457 autophosphorylation of the conserved ULD domain **(C)** Mean alcohol sensitivities (SEM) of
458 three *dkf-2* deletion alleles (*vx40*, *ok1704*, and *tm4076*) were significantly lower than
459 intoxication sensitivity of *dkf-2* lab wild-type (N2) animals (mean & SEM; n=15; *p*=0.0001;
460 p=0.0104; p=0.0167). **(D)** Deletion mutants in a wild isolate (NIC1) harboring candidate
461 hypomorph allele(s) in *dkf-2* do not cause a reduction in alcohol sensitivity (mean & SEM;
462 n=18; p=0.35; p=0.1573), whereas deletions in a strain without the candidate hypomorph
463 alleles cause significant reductions in alcohol sensitivity (mean & SEM, n=18; p=0.0001;
464 p=0.0052). **(E)** Treatment with a PKD inhibitor (CID 755673) trends towards lower alcohol
465 sensitivity in the N2 lab strain (mean & SEM, n= 18-20, two-sided t-test p=0.07, Cohen's d:
466 0.584 -medium effect) but shows no effect on alcohol sensitivity in a *dkf-2* deletion mutant
467 (RB1468(*ok1704*)). **(F)** Strains that are hemizygous for ALT genotype at *dkf-2* are resistant to
468 alcohol, while strains that are hemizygous for the REF genotype at *dkf-2* are sensitive to
469 alcohol (mean & SEM; n=6; p=0.01). NIC1 (ALT) and PS2025 (REF) were mated to a *dkf-2*
470 deletion mutant (*tm4076*). F_1 progeny from these crosses were assayed for their alcohol
471 sensitivity. Alcohol sensitivity was higher in PS2025 (REF)x*dkf-2*(-/-) F_1's relative to
472 NIC1(ALT)x*dkf-2*(-/-) F_1's.

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