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

Benjamin L. Clites[1], Brooke Frohock[1], Emily J. Koury[2], Oluwatoyin C. Ogunbi[1], Maya K. Mastronardo[3], Bhuvan V. Kanna[1], Erik C. Andersen[2], Jonathan T. Pierce[1]

[1] Waggoner Center for Alcohol & Addiction Research, Center for Learning and Memory, Department of Neuroscience, The University of Texas at Austin, Austin TX 78712, USA

[2] Department of Biology, Johns Hopkins University, Baltimore MD 21218, USA

[3] Cell, Molecular, Developmental Biology and Biophysics Graduate Program, Johns Hopkins University, Baltimore MD 21218, USA

Corresponding author: jonps@utexas.edu

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## Abstract

Differences in naïve alcohol sensitivity among individuals are a strong predictor of later-life alcohol use disorders (AUD). However, the genetic bases of 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 and identified five quantitative trait loci (QTL) that underlie natural variation in alcohol sensitivity. We validated that a variant in the conserved ubiquitin-like domain of a *C. elegans* ortholog of protein kinase D, *dkf-2*, likely underlies the chromosome V QTL. Furthermore, lower alcohol sensitivity, *i.e.* resistance to intoxication, was conferred by *dkf-2* loss-of-function mutations. Protein kinase D might represent a conserved, druggable target to modify alcohol sensitivity with application towards AUD.


## Article summary

We investigated the genetic basis of differences in alcohol sensitivity, a key predictor of alcohol use disorder. We measured alcohol-induced behavioral changes in over 150 genetically diverse nematode strains. Using a genome-wide association study, which links genetic differences to traits, we identified five genomic regions associated with alcohol sensitivity. We then showed that variation in a gene encoding protein kinase D influences resistance to intoxication. These findings identify a conserved molecular pathway that affects alcohol sensitivity and highlight a potential target for intervention. They show how natural genetic variation can reveal mechanisms underlying complex, human-relevant traits.

## Introduction

Alcohol Use Disorder (AUD) is one of the most common psychiatric diseases in the USA, affecting more than one in 10 American adults (Grant et al., 2015; 2017). Many more people bear the burden of alcohol misuse through indirect effects, such as drunk driving, lowered productivity, and a stressed medical system, which together cost the USA 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 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. Major progress towards identifying over 100 genetic variants that are associated with problematic patterns of alcohol consumption has been achieved in recent years (*e.g.*, Walters et al., (2018), Linner et al., (2021), Saunders et al., (2022)). However, candidate variants and their associated genes need to be functionally validated.

One of the best predictors of future drinking problems is alcohol sensitivity (Schuckit, 1994; 1996; 1998). People with low naïve alcohol sensitivity need to drink more to feel intoxicated and 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). Genome-wide association studies (GWAS) for traits correlated with alcohol use disorder in human populations, including alcohol sensitivity, have advanced rapidly in recent years. Any validated causal variants 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 alcohol sensitivity 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 alcohol sensitivity (Mathies et al., 2015). Mutations in these genes, which were first identified using *C. elegans*, predictably alter alcohol sensitivity 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 reference strain N2, except 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 *C. elegans* wild strains from around the world, additional genes with important orthologous roles in human alcohol sensitivity with relation to AUD can be identified (Crombie et al., 2023).

86 We performed a GWAS with a collection of 152 *C. elegans* wild strains to identify five
87 quantitative trait loci (QTL) that underlie differences in alcohol sensitivity for egg laying
88 behavior. We next showed that the chromosome V QTL is likely explained by variation in
89 *dkf-2*, a highly conserved ortholog of human Protein Kinase D (PKD). Although PKD's
90 upstream activator, protein kinase C (PKC), has an established role in cross-species
91 alcohol response (Lee & Messing, 2008), our results are likely the first evidence that PKD
92 similarly affects alcohol sensitivity. These findings highlight the power of harnessing natural
93 genetic diversity in a tractable model organism to identify novel genetic effectors of
94 medically important traits such as alcohol sensitivity.

95

96 **Materials and Methods**

97 **Animals**

98 Animals were grown at room temperature (20°C) using nematode growth medium agar
99 plates seeded with OP50 *E. coli* bacterial lawns as previously described (Brenner, 1974).
100 Strains were propagated for at least three generations before behavioral testing, to
101 minimize effects of starvation on development and physiology of the animal or its recent
102 lineage. To prevent mutational drift in our test populations, strains were re-thawed from the
103 original stock every one to two months. Animals were synchronized to day-one adulthood
104 using timed-egg lays.

105 **Assay for alcohol intoxication sensitivity**

106 Nematode growth medium (NGM) agar (12 mL) was dispensed into a 6-cm diameter Petri
107 dish and allowed to set for 48 hrs. Next, plates were seeded with ~0.6 mL of OP50 *E. coli* 48
108 hours prior to testing. At the start of each assay, 10 clonal, day-one adult worms were
109 picked to an untreated control plate, where they were allowed to lay eggs for one hour.
110 Animals were then transferred to an ethanol-treated plate, where they were again allowed
111 to lay eggs for one hour before removal. For each assay, alcohol intoxication sensitivity was
112 calculated by dividing the number or eggs laid on the ethanol plate by the number of eggs
113 laid on the untreated control plate subtracted from one (alcohol sensitivity = 1 - (# eggs laid
114 on ethanol / # eggs laid on control)). To minimize the influence of nuisance variables,
115 strains were tested in blocks of 18-22 variably interleaved across replicates. Genotype
116 averages were calculated using alcohol sensitivity scores from about 10 individual assays.
117 Ethanol plates were prepared by depositing 280 µL of 200 proof ethanol (Sigma) beneath
118 the agar pad 25 minutes before animals are transferred for behavioral testing (Davis et al.,
119 2014). After one hour, this exogenous dose results in an internal ethanol concentration of
120 ~80 mM in day-one adult nematodes (Alaimo et al., 2012; Scott et al., 2017). To minimize

121 between-assay variation, all ethanol plates were massed to 17.5-18 g before application of
122 ethanol.

123 **Heritability calculation**

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

127 **Determining internal ethanol concentrations**

128 Internal ethanol concentrations were measured as described previously (Alaimo et al.,
129 2012). In brief, we used a colorimetric assay that uses alcohol metabolic enzymes (ADH &
130 ALDH) to generate quantitatively sensitive shifts in absorbance at 340 nm (Megazyme).
131 Day-one adult animals from each strain were subjected to the same alcohol sensitivity
132 assay as described above. After one hour on ethanol treatment plates, 200 animals of each
133 strain were picked into 120 µL of distilled water. The resulting dilution factor was calculated
134 using estimates of day-one adult worm volumes by Andrews (2019). To prevent evaporation
135 of ethanol from samples, worms were homogenized on ice using a Pyrex grinder for about
136 two minutes per sample. Samples were centrifuged, and supernatant was extracted using
137 a micropipette. Average ethanol concentrations for each strain were determined against a
138 standard colorimetric absorbance curve.

139 **GWAS**

140 GWAS was performed using egg-laying behavioral sensitivity to alcohol phenotypes of 152
141 *C. elegans* wild strains and SNV markers from the publicly available CaeNDR *C. elegans*
142 release 20250625 and NemaScan (Widmayer et al., 2022). Fine mapping was performed by
143 analyzing genotype-phenotype relationships for all SNVs in the QTL identified by GWAS,
144 rather than only common SNVs (MAF greater than or equal to 5%).

145 **Strain selection for QTL validation**

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

152 **CRISPR-Cas9 genome editing**

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

162 Target and repair sequences for *vx40* mutant:

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

164 *dkf-2* ssDNA repair oligo: 5'
165 GGCCATAAGTGTTTTTTAGTTAACGACAGCTTCCTTGACTTTAACGCGAAGCTCTG

166 GAAGCTTTACTCCGAGATGTCAAATAGTTCCTCCAGCATTCAAAAATAAGGTTTTTTAAGCTTTTTAA
167 ACTATCTTTAAACTTT 3'

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

169 *dpy-10* ssDNA repair oligo: 5'
170 CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCAT

171 GCGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT 3'

172 **Strains**

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

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

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

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

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

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

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

180 **PKD inhibitor assays**

181 Alcohol sensitivity assays were conducted as described above. To ensure drug effects,
182 animals were treated with an exogenous concentration of CID 75563 (2.0 μM) that is high

183 relative to its IC50 for human PKDs (0.18-0.28 μM), but still far below the IC50 for non-
184 target protein kinases (*e.g.*, PKC = 10.0 μM; PLK1 = 20.3 μM) (Sharlow et al., 2008).
185 Exogenous treatment of *C. elegans* to drugs often requires orders of magnitude higher
186 concentrations than effective internal concentrations (Rand and Johnson, 1995; Burns et
187 al., 2010). Animals were pre-treated with CID 755673 (DMSO) for one hour prior to alcohol
188 sensitivity assays. During behavioral assays, both the baseline and alcohol egg-laying
189 plates contained CID 75563 (2.0 μM) and 0.01% DMSO. Treated animals were tested
190 alongside same-day controls whose plates were treated with DMSO to account for non-
191 specific solvent effects.

192 **Statistical comparisons**

193 Data were analyzed using SPSS (v29.0.2.0, IBM) or Excel 2022 (365 MSO, Version 2510
194 Build 16.0.19328.20190, 64-bit) with standard planned two-sided t- or ANOVA tests and the
195 using the Holm-Bonferroni method to correct for a priori planned multiple comparisons
196 method and Bonferroni method for post hoc multiple comparisons test.

197

198 **Results**

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

200 To calculate heritability for *C. elegans* alcohol sensitivity, we assayed a subset of 12
201 genetically diverse wild strains for acute behavioral sensitivity to alcohol using egg-laying
202 behavior (Fig. 1a). Egg laying, which relies on the coordinated action of neurons in a defined
203 circuit, has been exploited to discover and characterize the function of dozens of
204 conserved molecules (*e.g.*, Desai et al., 1988; Schafer, 2006; Medrano and Collins, 2023).
205 Alcohol-induced depression of egg-laying behavior has served as a model of intoxication in
206 *C. elegans* (Davies et al., 2003; Davis et al., 2014). We found that alcohol sensitivity
207 differed in the population and was about 42% heritable, mirroring the heritability of alcohol
208 sensitivity observed in human populations ($H^2 \approx 0.3$-$0.5$; Verhulst et al., 2015). To confirm
209 that egg-laying behavior serves as a general readout for alcohol-induced depression in
210 neuromuscular function as in previous studies (Davies et al., 2003; Davis et al., 2014), we
211 measured alcohol-induced depression in locomotion for six strains and found a strong
212 correlation between the measures ($R^2 = 0.87$; Supplementary Fig. 1).

213 After establishing significant heritable trait variation in the diversity panel, we expanded our
214 assays and measured alcohol sensitivity of 152 wild strains from around the world (Fig. 1a;
215 right). We focused on behavioral sensitivity to alcohol using egg-laying because this trait is
216 easily quantified (number of eggs laid during one hour). We conducted a GWAS and

217 identified five QTL significantly associated with differences in alcohol sensitivity (Fig. 1b;
218 Supplementary Table 1; Supplementary Fig. 2).

219 We chose to pursue the QTL on chromosome V with a peak marker at 16,792,754. This QTL
220 encompassed an approximately 1 Mb interval, where the ALT allele predicted significantly
221 lower alcohol sensitivity, *i.e.*, resistance to intoxication (Fig. 1b; Fig. 2a). To identify
222 potential causal variants in the chromosome V QTL, all rare and common SNVs in the
223 interval were tested for association with alcohol sensitivity differences, with priority given
224 to alleles with high predicted effects on gene function (*e.g.*, nonsynonymous substitutions,
225 early stop codons, splice-site variants, etc.) (Fig. 2b). Fine mapping identified several high-
226 impact variants in the two nearest genes to the peak QTL marker, *dkf-2* (V:16,764,489 C->T)
227 and *immt-2* (V:16,790,476 G->A), that segregated with differences in sensitivity to ethanol
228 intoxication (Supplementary Fig. 3). The gene *dkf-2* encodes a *C. elegans* ortholog of
229 protein kinase D (PKD) (Feng et al., 2007). Interestingly, PKDs function downstream of PKC,
230 a known effector of alcohol response (Wallace et al., 2007), so we focused our efforts on
231 this candidate gene. Further, deletion of *dkf-2* was shown to restore movement in a TDP-43
232 model of neurodegeneration (Liachko et al., 2014), suggesting an ameliorative effect on
233 neuromuscular depression. These data together suggest that ALT variants in *dkf-2* might
234 underlie the QTL for alcohol sensitivity.

235 **Validating the role of Protein kinase D in alcohol sensitivity**

236 To test whether *dkf-2* represented a causal gene for alcohol sensitivity differences, we
237 assayed *dkf-2* loss-of-function mutants. We obtained two *dkf-2* deletion alleles in the N2
238 laboratory wild-type background: *ok1704*, a 716 bp deletion in exons 11 and 12, and
239 *tm4076*, a 1,646 bp deletion that interrupts an obligate exon of all six known isoforms (Fig.
240 3a; Supplementary Fig. 4). We found that strains harboring either of the two deletion alleles
241 had lower alcohol sensitivity relative to the N2 strain (Fig. 3b). We also generated a novel
242 deletion allele, *vx40*, that deletes two exonic bases causing a putative frame-shift loss-of-
243 function allele. This novel deletion also conferred decreased alcohol sensitivity.
244 Interestingly, *vx40* affects only four of the six predicted *dkf-2* isoforms (a, d, e, and f), one
245 fewer than the *ok1704* deletion (a, c, d, e, and f) and two fewer isoforms than the *tm4076*
246 deletion (Hillier et al., 2009) (Supplementary Fig. 4). These results provide evidence that
247 loss of *dkf-2* confers low alcohol sensitivity.

248 Because the loss-of-function mutants displayed lower alcohol sensitivity relative to strains
249 that harbor the *dkf-2* REF allele, we hypothesized that the candidate causal natural
250 variant(s) in *dkf-2* are hypomorphic, because the ALT allele at the marker likewise covaried
251 with lower alcohol sensitivity. To test whether *dkf-2* plays a role in the alcohol sensitivity of
252 the wild strains and to probe the nature of the natural candidate causal variants, we

253 generated two independent *dkf-2* deletion alleles in each of two representative wild strain
254 backgrounds: NIC1, which harbors the *dkf-2* ALT alleles, and PS2025, which harbors the
255 *dkf-2* REF alleles. We found that novel deletions in the ALT background did not change
256 alcohol sensitivity, but deletions in the REF background significantly reduced alcohol
257 sensitivity (Fig. 3c).

258 We next performed a hemizygosity test. We crossed PS2025 and NIC1 wild strains with an
259 N2 background strain carrying the *dkf-2* deletion allele *tm4076*. Interestingly, we found that
260 $F_1$ *dkf-2* hemizygotes resembled their respective REF or ALT allele parental phenotypes (Fig.
261 3d). These results provide evidence that the candidate ALT alleles represent likely causal
262 hypomorphic *dkf-2* natural variants. It is worth noting that, although NIC1 has the same
263 phenotype in the hemizygosity tests (mean = 0.504, Fig. 3d) as in GWAS (mean sensitivity =
264 0.463), it was more sensitive than previous experiments in the assays with deletion of *dkf-2*
265 (mean sensitivity = 0.623, Fig. 3b). This difference was likely caused by aberrantly high
266 untreated egg-laying measures for this set of assays (mean eggs/hour = 84.2) relative to
267 earlier tests (mean eggs/hour = 37) and relative to PS2025 in the same tests (mean
268 eggs/hour = 67.5). Thus, higher basal untreated-measurements of egg laying before
269 ethanol-treated measurements could have inflated the sensitivity of NIC1 in these assays
270 relative to other experiments reported here. We do not know the reasons for this notable
271 difference, but the results from this experiment demonstrate that deletion of the *dkf-2* gene
272 affects alcohol sensitivity in a background-dependent manner, suggesting that natural ALT
273 alleles in *dkf-2* drive differences in alcohol sensitivity.

274 To determine whether *dkf-2* alleles exert their effects on alcohol sensitivity by gross
275 differences in ethanol metabolism, we measured internal alcohol concentrations for the
276 lab strain (N2), a *dkf-2* wild strain with REF alleles (PS2025), a *dkf-2* wild strain with ALT
277 alleles (NIC1), as well as two *dkf-2* deletion mutants in each of the REF and ALT
278 backgrounds. We found no differences in internal alcohol concentrations after one hour of
279 ethanol exposure between any of the strains (Supplementary Fig. 5). Moreover, alcohol
280 concentrations were consistent with established measures (Alaimo et al., 2012). These
281 results suggest that phenotypic differences in alcohol sensitivity were not caused by
282 differences in ethanol metabolism or uptake. Taken together, we conclude that *dkf-2* is a
283 causal gene that contributes to alcohol sensitivity in *C. elegans*.

284 **An isoleucine-to-valine variant in DKF-2 contributes to differences in alcohol**
285 **sensitivity**

286 To investigate specific variants in *dkf-2* that could causally account for natural variation in
287 alcohol sensitivity, we considered two that are predicted to have impacts on gene function
288 Fig. 4a). First, we considered V:16,758,168 that is predicted to cause an ALT missense

289 variant (I263V) in DKF-2 isoforms a, e, and f, as well as a missense variant (I41V) in isoform
290 d. The variant is located three nucleotides from the start of a splice site for these isoforms.
291 Second, we considered V:16,764,489 that is predicted to cause a missense variant L78F in
292 isoform c of DKF-2.

293 To test if either of the natural ALT variants conferred lower alcohol sensitivity, we generated
294 knock-in alleles for I236V or L78F in N2 and PS2025 background strains. These strains
295 harbor REF variants at both loci and are more sensitive to alcohol compared to our
296 comparison strain NIC1 that has both ALT variants. To control for off-target genome-editing
297 effects, we generated two independent strains for each knock-in variant replacement,
298 resulting in eight CRISPR-engineered strains to compare with three wild strain controls (Fig.
299 4b). We plotted data for each unique strain and statistically compared each CRISPR-
300 engineered strain with its wild background correcting for multiple comparisons (Fig. 4b).
301 We found that for three out of four of the engineered strains the I263V ALT variant conferred
302 significantly decreased alcohol sensitivity compared to their corresponding genetic
303 background (two-sided t-tests: N2 vs. N2 I263V #1, $p = 0.0247$; N2 vs. N2 I263V #2, $p =$
304 $0.0850$; PS2025 vs. PS2025 I263V #1, $p = 0.0306$; PS2025 vs. PS2025 I263V #2, $p = 0.0497$;
305 Fig. 4b). By contrast, we found no significant change in alcohol sensitivity with allele
306 replacement of the L78F variant. These results functionally validate that the DKF-2 I263V
307 natural variant underlies the differences in alcohol sensitivity.

308 To infer how the I263V ALT variant could alter function of DKF-2 in alcohol sensitivity, we
309 considered conservation of this residue at primary and tertiary protein structure levels. At
310 the primary sequence level, I263 in DKF-2 is poorly conserved with human and mouse
311 protein kinase D2 and D3 (PRKD2 and 3) (Supplementary Fig. 6). However, predicted 3D
312 structures show that I263 is likely located at an analogous position across nematode,
313 mouse, and human orthologs. Specifically, I263 and its human equivalent, K96, both
314 represent the terminal residue in an alpha helix within the ubiquitin-like (ULD) domain (Fig.
315 4c). This predicted alpha helix structure is supported by the crystal structure (2.2 Å
316 resolution) of the ULD domain from the *C. elegans* PKD paralog DKF-1 (Fig. 4c, Reinhardt et
317 al., BioEssays 2020). The ULD helps PKD dimerize, which shifts it to a more active state.
318 Thus, the I263V natural variant could decrease DKF-2 activity by interfering with
319 dimerization.

320 **Pharmacological probing of *dkf-2* and alcohol sensitivity**

321 Because protein kinases represent druggable targets, we next asked whether DKF-2 affects
322 alcohol sensitivity using a selective protein kinase D inhibitor (CID 755673 – Tocris
323 Bioscience). We chose to assay the laboratory-adapted strain N2 and a deletion mutant
324 *dkf-2(ok1704)* in that genetic background to avoid the potential confounding effects of

variation in metabolism of wild strains. 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*, previous studies found remarkably conserved functions 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 (two-sided t-test, $p = 0.84$), whereas the N2 strain displayed a non-significant trend towards a decrease in alcohol sensitivity (Supplementary Fig. 7; two-sided t-test $p = 0.07$, Cohen's d: 0.584 suggesting a medium effect). Given the non-significant change in alcohol sensitivity, future *C. elegans* studies might consider optimizing treatment or using a different PKD inhibitor.

## 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 lab has considered PKD, not in a role for behavioral sensitivity to alcohol, but as a potential therapeutic target in experimental alcoholic pancreatitis (Yuan et al., 2021; Yuan et al., 2022).

### Protein kinases and alcohol response

Although other protein kinases are known effectors of behavioral responses to alcohol (*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) (Supplementary Fig. 8). Recent studies, however, show that PKDs are sometimes activated via PKC-independent autophosphorylation, but the precise mechanism and

360 functional consequences of this pathway remain obscure (Elsner et al., 2019; Reinhardt et
361 al., 2020).

362 Using CRISPR-engineered variant swapping, we found evidence that the I263V natural
363 variant contributed to differential alcohol sensitivity. Inspection of this residue on
364 predicted tertiary structure suggests that it resides in the alpha helix within the ULD. The
365 ULD domain mediates dimerization of PKD upon membrane recruitment (via
366 diacylglycerol), which in turn enables trans-autophosphorylation of the activation loop
367 thus enabling kinase activation (Reinhardt et al., 2020). This mechanism makes PKD
368 activation concentration- and localization-dependent, offering a regulatory checkpoint.
369 The ULD therefore is a key regulatory domain to switch PKD from an inactive (or low
370 activity) state to an active signaling state. We hypothesize that the I263V ALT variant
371 reduces the function of DKF-2 by interfering with this conserved domain and its associated
372 functions.

373 As integrators of signal inputs from PKC and DAG, PKDs affect a wide array of downstream
374 cellular processes. These include class II HDAC activity (Parra et al., 2005; Dequiedt et al.,
375 2005), neuronal function and development (Fu et al., 2009; Bisbal et al., 2008; Czondor et
376 al., 2009), and Golgi and membrane dynamics (Rozengurt, 2011), as well as a conserved
377 role in activation of immune and stress tolerance pathways (Kim et al., 2010; Feng et al.,
378 2007; Ren et al., 2009) (Supplementary Fig.8). PKD could plausibly modify alcohol
379 sensitivity through one or more of these pathways, but immune and stress tolerance
380 pathways represent especially promising candidate mechanisms. In *C. elegans, dkf-2* plays
381 a role in innate immune response, where it promotes PMK-1 (ortholog of p38 MAP kinase)
382 activity, and downregulates *daf-16*, a transcription factor that promotes stress tolerance
383 and lifespan through the Insulin/IGF-1-like signaling (IIS) pathway (Lin et al., 1997; Lin et al.,
384 2001). Thus, PKD sometimes interacts with the p38 MAPK cascade, a pathway containing
385 *nsy-1* (ortholog of human MAP3K), mutants of which are resistant to the toxic effects of
386 ethanol and other drugs of abuse (*e.g.*, methamphetamine, MDMA) (Gomez, 2013;
387 Schreiber & McIntire, 2011).

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

395 **Candidate genes in the remaining QTL**

396 Although we dissected only one of the five QTL identified in the GWAS, statistical fine
397 mapping of the remaining QTL suggests several promising candidate genes underlying
398 these loci (Fig. 1B). We considered genes with natural variants predicted to alter function
399 and *C. elegans* orthologs of human genes that have been associated with alcohol
400 sensitivity and alcohol use disorder across 20 GWA studies (Table 3). For chromosome II,
401 the most significant variant predicted to affect genes expressed in adult stage *C. elegans*
402 was one in the bidirectional promoter for *K02F6.5* and *K02F6.8* genes that encode
403 potassium channel tetramerization domain (KCTD) proteins. This result is intriguing given
404 that the most significant single gene of large effect conveying low alcohol sensitivity is the
405 BK potassium channel SLO-1 (Davies et al., 2003), and two KCTD proteins (6 and 16) have
406 been found by independent human GWAS to be associated with the following alcohol
407 traits: drinks per week, Aspartate aminotransferase platelet ratio index in high alcohol
408 intake, Alcohol use disorder (consumption score), and Fibrosis-4 index in high alcohol
409 intake (Saunders et al., 2022; Linnér et al., 2019; Kranzler et al;., 2019; Innes et al., 2020).
410 Although the BK channel does not appear to rely on KCTD proteins to tetramerize, KCTD
411 affect other potassium channels and have additional roles such as interacting with Gβγ to
412 alter cAMP signaling (Sloan et al., 2023). The chromosome II region also included *sup-9*,
413 which is orthologous to human *KCNK3* potassium channel gene that has also been
414 associated with an alcohol trait (Diastolic blood pressure x alcohol consumption (light vs
415 heavy) interaction (2df test)) (Feitosa et al., 2018). Because alcohol sensitivity in *C. elegans*
416 correlates with anesthetic sensitivity, the potassium channel *sup-9* is a strong candidate
417 (Morgan et al., 2007). Loss of function confers resistance to halothane-induced paralysis,
418 whereas gain of function in its channel component, *sup-10*, causes hypersensitivity
419 (Singaram et al., 2011). For chromosome III, the QTL peak was in *pll-1,* which is an ortholog
420 of human PLCL1 that was associated with alcohol consumption in a meta-analysis of
421 human GWAS (Kapoor et al., 2013). The edge of this QTL contains the acetylcholine
422 transporter *snf-6*, which was isolated in the original forward-genetic screen for mutants
423 with low alcohol sensitivity (Davies et al., 2003; Kim et al., 2004).

424 The QTL on chromosomes IV and X are more difficult to parse. This result might be
425 explained in part by the low recombination rate of the X chromosome leading to QTL with a
426 larger number of linked genes. Due to local genomic hyper-divergence (Lee et al., 2021),
427 the QTL on chromosomes II, III, and IV were difficult to dissect. In *C. elegans*, certain
428 regions of the genome contain high levels of genetic variation from the laboratory strain N2
429 as well as other related haplotypes (Lee et al., 2021; Moya et al., 2025). In effect, hyper-
430 divergence leads to difficulty in comparing genome sequences against a reference
431 genome. Although hyper-divergent regions are difficult to dissect, they still could represent
432 true effectors of *C. elegans* alcohol sensitivity.

## 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.

## Data availability

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Strains and plasmids are available upon request.

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**Author contributions:** B.C., E.C.A., and J.T.P. designed research; B.C., T.C.O., B.V.K., and J.T.P. performed research; M.K.M. performed mapping; S.J.K. contributed reagents; B.C., E.C.A., and J.T.P. analyzed data; J.T.P. and B.C. acquired funding; and B.C. wrote first draft, B.C., E.C.A., and J.T.P. worked on final paper.

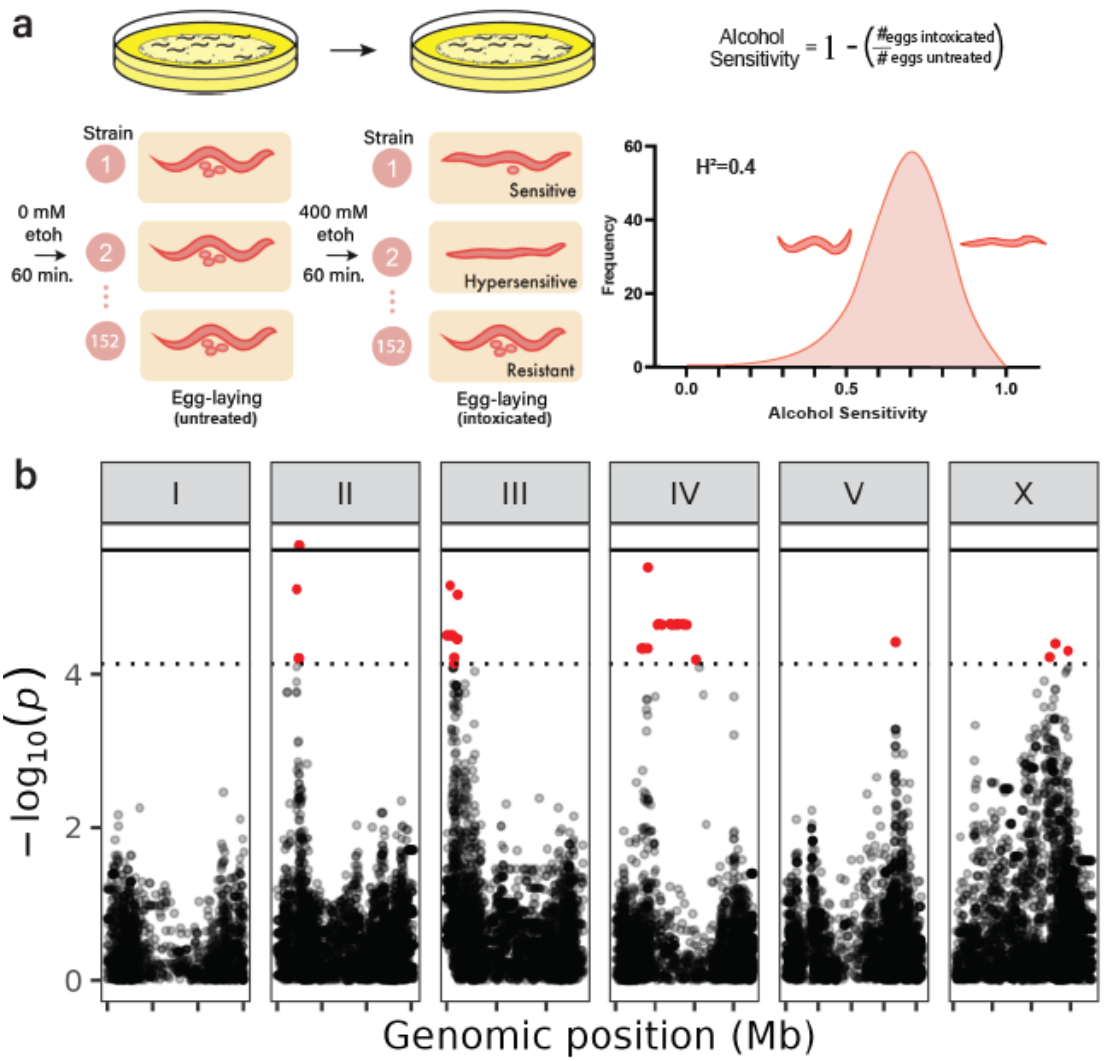


**Figure 1: GWAS of natural variation in alcohol sensitivity of 152 *C. elegans* wild strains.**

**(a)** Overview of high-throughput behavioral screen for naïve alcohol sensitivity in 152 *C. elegans* wild strains. 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 approximately 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 ($-\log_{10}(p)$). Horizontal black lines denote genome-wide significance cutoff after correcting for the number of independent association tests: Eigen (dotted) and Bonferroni (solid). Highlighted with a vertical red box is the ChrV QTL that was pursued for fine mapping.

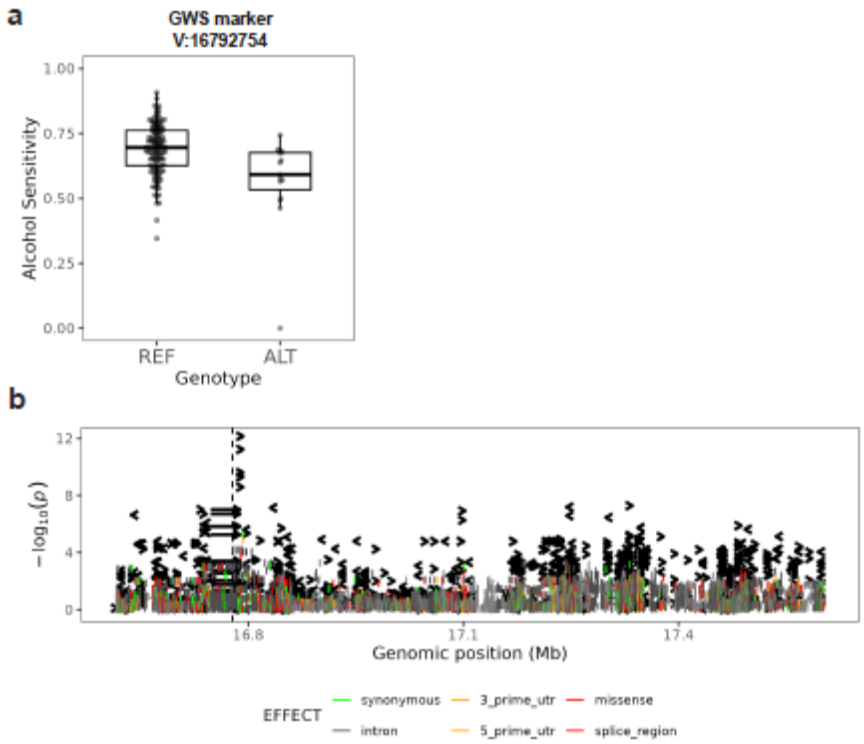


**Figure 2: Fine mapping the chromosome V QTL associated with decreased sensitivity.**

**(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.

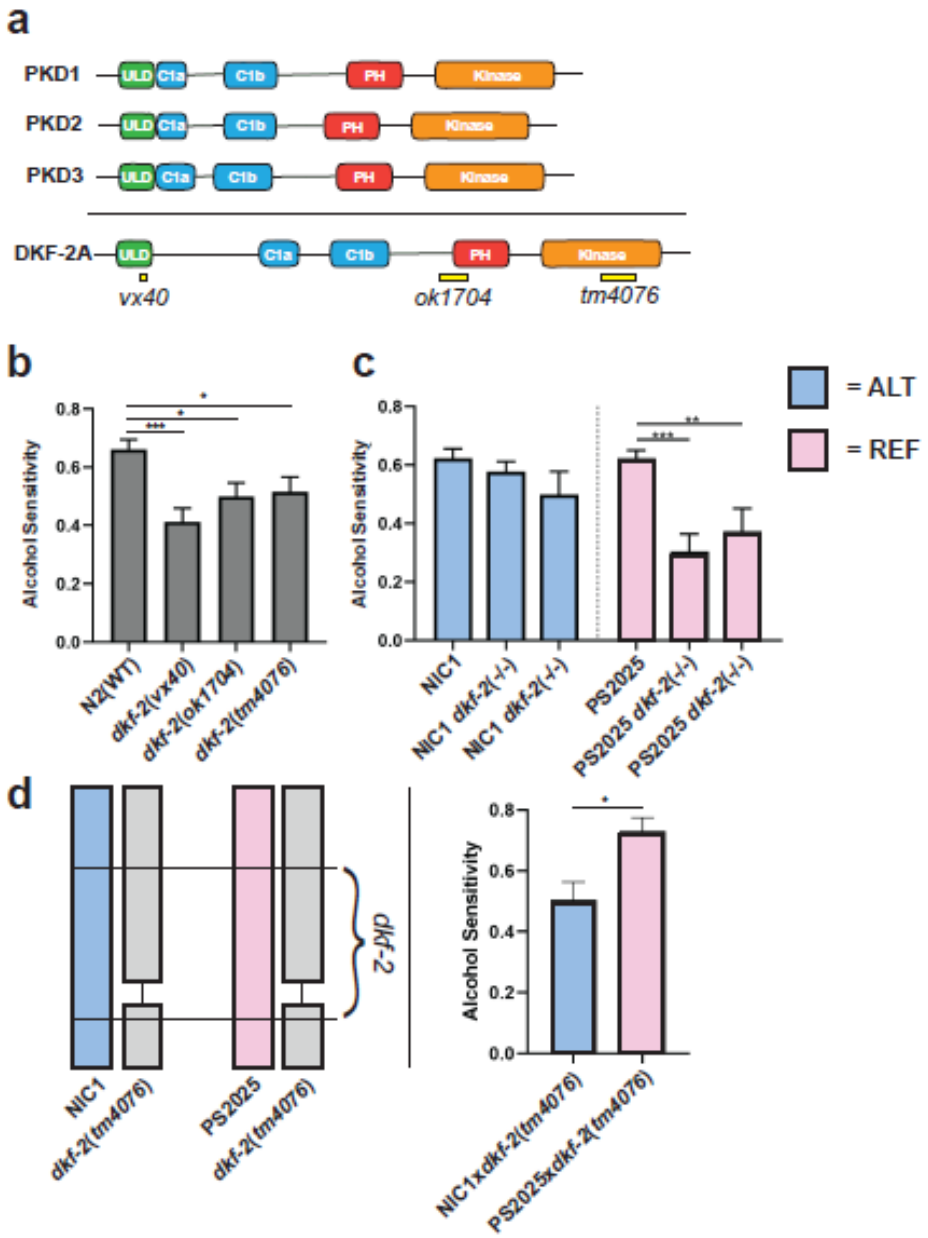


**Figure 3: Independent deletion alleles in the highly conserved *C. elegans* ortholog of PKD (*dkf-2*) predictably reduce alcohol sensitivity.**

**(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)** 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). **(c)** Deletion mutations in a wild strain (NIC1) harboring candidate hypomorphic allele(s) in *dkf-2* does not cause a reduction in alcohol sensitivity

513 (mean & SEM; n=18; p=0.35; p=0.1573), whereas deletions in a strain without the
514 candidate hypomorphic alleles cause significant reductions in alcohol sensitivity (mean &
515 SEM, n=18; p=0.0001; p=0.0052). **(d)** Strains that are hemizygous for ALT genotype at *dkf-2*
516 are resistant to alcohol, although strains that are hemizygous for the REF genotype at *dkf-2*
517 are sensitive to alcohol (mean & SEM; n=6; p=0.01). NIC1 (ALT) and PS2025 (REF) were
518 mated to a *dkf-2* deletion mutant (*tm4076*). $F_1$ progeny from these crosses were assayed for
519 their alcohol sensitivity. Alcohol sensitivity was higher in PS2025 (REF)x*dkf-2*(-/-) $F_1$'s
520 relative to NIC1(ALT)x*dkf-2*(-/-) $F_1$'s. Planned two-sided t-tests were used for comparisons.

521

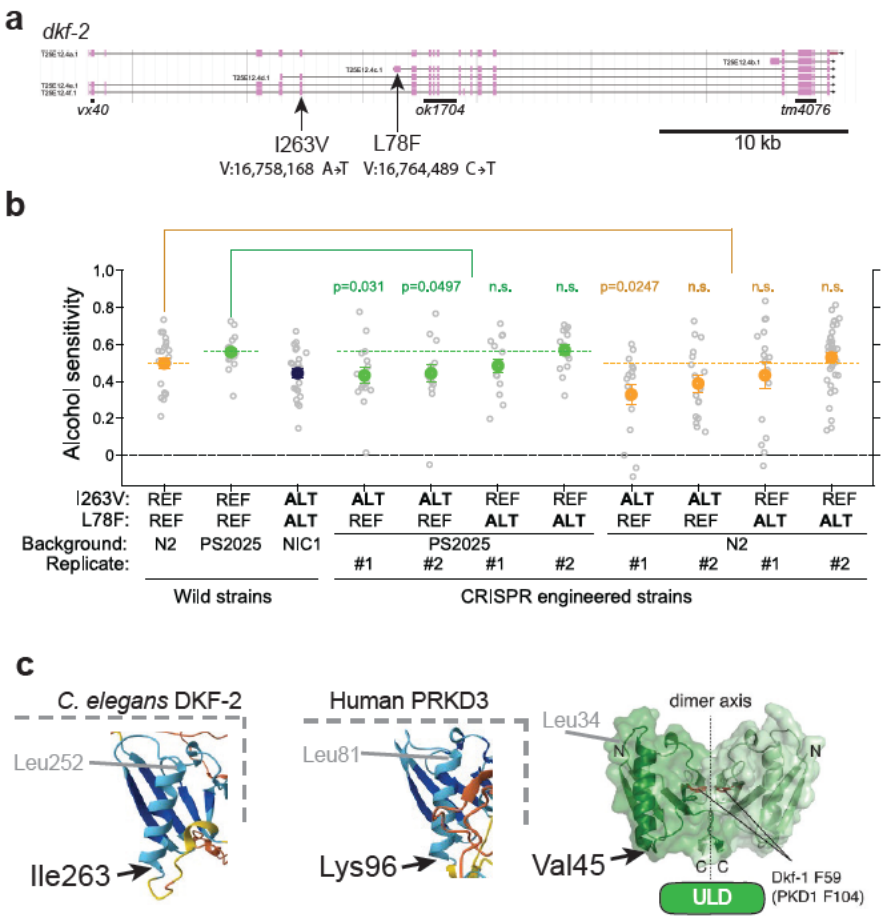


**Figure 4: The DKF-2(I263V) variant contributes to alcohol sensitivity. (a)** Schematic diagram of the *dkf-2* gene locus and location of ALT variants I263V and L78F. **(b)** Alcohol sensitivity plotted for 11 strains that comprise three wild strains and four CRISPR/Cas12 engineered strains made in duplicate. Replacing the ALT variant I263V into PS2025 or N2 backgrounds conferred significant decrease in alcohol sensitivity for three out of four strains, *i.e.,* resistance to intoxication. Swapping the ALT variant L78F had no significant effect in either background. Horizontal colored dashed lines added to help compare to mean of corresponding REF-REF background strains. Planned two-sided t-tests were corrected for multiple comparisons using the Holm–Bonferroni method. Corrected p-values are shown color coded to compare with the corresponding background strain. **(c)** Predicted structure of *C. elegans* DKF-2 and human PRKD3 share a single alpha helix and beta sheets in the ubiquitin-like domain (ULD). The I263 residue is in an analogous position at one side of the alpha helix. Crystal structure of *C. elegans* protein kinase D paralog DKF-1 shown for comparison (modified from Reinhardt et al., 2020).

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