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1 # CRISPR Turbo Accelerated Knock Out (CRISPy TAKO) for rapid *in vivo* screening of gene
2 # function

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20 **Abstract:**

21 The development of CRISPR/Cas9 technology has vastly sped up the process of genome editing
22 by introducing a bacterial system that can be exploited for reverse genetics-based research.
23 However, generating homozygous knockout (KO) animals using traditional CRISPR/Cas9-
24 mediated techniques requires three generations of animals. A founder animal with a desired
25 mutation is crossed to produce heterozygous F1 offspring which are subsequently interbred to
26 generate homozygous F2 KO animals. This study describes a novel adaptation of the
27 CRISPR/Cas9-mediated method to develop a homozygous gene-targeted KO animal cohort in
28 one generation. A well-characterized ethanol-responsive gene, *MyD88*, was chosen as a
29 candidate gene for generation of *MyD88*$^{-/-}$ mice as proof of concept. Previous studies have
30 reported changes in ethanol-related behavioral outcomes in MyD88 KO mice. Therefore, it was
31 hypothesized that a successful one-generation KO of MyD88 should reproduce decreased
32 responses to ethanols sedative effects, as well as increased ethanol consumption in males that
33 were observed in previous studies. One-cell mouse embryos were simultaneously electroporated
34 with four gRNAs targeting a critical Exon of MyD88 along with Cas9. DNA and RNA analysis of
35 founder mice revealed a complex mix of genetic alterations, all of which were predicted to ablate
36 MyD88 gene function. This study additionally compared responses of Mock treatment control
37 mice generated through electroporation to controls purchased from a vendor. No substantial
38 behavioral changes were noted between control cohorts. Overall, the CRISPR/Cas9 KO protocol
39 reported here, which we call CRISPR Turbo Accelerated KnockOut (CRISPy TAKO), will be useful
40 for reverse genetic *in vivo* screens of gene function in whole animals.

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41 **Introduction:**

42 Clustered regulatory interspaced short palindromic repeats (CRISPR) paired with CRISPR
43 associated protein 9 (Cas9) is currently the dominant and preferred gene editing tool in scientific
44 research. CRISPR based screens of gene function *ex vivo* have been tremendously useful for
45 identifying genes involved in tumor suppression[1], mitochondrial function[2], and dendritic
46 development[3]. High throughput CRISPR loss-of-function reverse genetic screens allow for the
47 rapid identification of genes involved in phenotypes of interest. However, *in vitro* screens are
48 limited by phenotypes that can be readily assayed in cell culture, e.g., cellular proliferation, drug
49 sensitivity, and cell survival. Further, the acquisition of transcriptome data has greatly outpaced
50 our capacity to functionally study genes of interest. For many biological questions, particularly
51 those that pertain to dysfunction of the central nervous system where behavioral abnormalities
52 are the primary phenotype of interest, *in vivo* tests of behavior must be employed. Because
53 behavior is the phenotype of interest, *in vitro* screens are unsatisfactory. In this study, we sought
54 to develop a method with moderately high throughput that could be used *in vivo* to screen genes
55 for effects on behavior.

56 Global gene knockout (KO) animal models are a gold standard approach that have been
57 widely used to study and delineate the effects of individual molecules in whole organisms. The
58 recent application and widespread adoption of CRISPR/Cas9 technology dramatically facilitated
59 KO animal generation. However, the standard method of creating CRISPR KO animals, a.k.a.,
60 CRISPy Critters[4], typically requires three generations to produce experimental animals that can
61 be phenotypically evaluated and therefore is unsuitable for moderate-high throughput *in vivo*
62 screens (Fig. 1A). Briefly, in a typical CRISPR KO animal study, CRISPR reagents are introduced
63 to one-cell embryos that develop into founder (F0) animals that are screened for the desired
64 mutation. F0 animals are typically an eclectic mix of wild-type and mutant animals. The mutants
65 may be heterozygotes, homozygotes, or compound heterozygotes, and most mutant alleles differ
66 in the individual mutations they harbor in the target gene of interest. A founder animal that harbors

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67 a desirable mutation (typically a frameshift or a large deletion) is then mated to wild-type (WT)
68 mice to produce heterozygous F1 offspring. Subsequently, heterozygotes are interbred to
69 produce homozygous F2 mutant KO offspring. These F2 mutant animals have both alleles of the
70 gene of interest inactivated, they all harbor the same mutation in the gene of interest, and they
71 can be compared to WT littermate controls for relevant phenotypic changes. Although this
72 CRISPR approach to creating gene KO animals represents a dramatic savings in time, effort, and
73 expense compared to traditional embryonic stem cell based gene targeting approaches, the
74 CRISPR approach still requires considerable time and expense because three generations of
75 animal production is time consuming and results in substantial animal care and housing
76 expenses. This process also requires a considerable amount of personnel time for colony
77 maintainance and genotyping.

78 We endeavored to establish a one generation CRISPR KO approach in which F0 animals
79 could be directly used to test for the behavioral consequences of gene inactivation. We reasoned
80 that a very high efficiency CRISPR mutagenesis approach could be used to efficiently create F0
81 animals in which both alleles of the gene of interest are mutated and are functionally inactivated
82 (i.e., gene KOs) (Fig. 1B). Although each F0 animal may have different mutations, they would all
83 be functionally and phenotypically equivalent if a critical part of the gene were sufficiently mutated.

84 Our long-term goal is to employ this accelerated technique to vastly speed up the
85 screening process of testing novel ethanol-responsive genes for involvement in ethanol-related
86 behavioral phenotypes, including ethanol consumption. Therefore, we initially piloted this
87 approach *in vitro* on two novel ethanol-responsive long noncoding RNA (lncRNA) genes. We
88 subsequently sought to validate this method *in vivo* by mutating a gene previously shown to alter
89 behavioral responses to ethanol when inactivated using traditional global KO technology. *MyD88*
90 was chosen as a well-characterized ethanol-responsive gene for proof-of-concept as prior studies
91 have evaluated the effects of MyD88 global KO on ethanol-related behaviors, including ethanol
92 drinking[5] and response to ethanol's acute sedative/hypnotic and motor ataxic effects[6,7]. Single

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93 generation F0 MyD88 KO animals were hypothesized to exhibit decreased ethanol-induced
94 sedative/hypnotic effects, decreased sensitivity to ethanol-induced motor ataxia, and a male-
95 specific increase in ethanol consumption relative to controls.

96 To further streamline this accelerated KO mouse protocol, we reasoned that for first pass
97 screening of genes for behavioral phenotypes, isogenic animals purchased directly from a vendor
98 could be used as a control group for comparison to KOs. However, one concern is that the
99 CRISPR procedure itself, irrespective of the gene being mutated, could exert deleterious effects
100 that could lead to false positive or negative results. Therefore, we also created in-house Mock
101 treatment controls that were produced under an identical protocol to the KOs except that the
102 Mock-treated animals were created with procedures that lacked crRNAs. This Mock-treated
103 control group was directly compared to isogenic C57BL/6J WT mice (Jax controls) purchased
104 from the Jackson Laboratory (JAX). We hypothesized that these two control groups would not
105 differ on behavioral endpoints of interest.

106 In this report, we describe implementation and validation of a novel technique for the
107 accelerated production of CRISPR KO mice in one generation. Animals produced via this protocol
108 are herein affectionately referred to as CRISPR Turbo Accelerated KnockOuts (i.e., CRISPy
109 TAKOs). We report that our CRISPR protocol can reliably produce a large number of F0 KO
110 animals and that the ethanol phenotype of MyD88 CRISPy TAKOs largely recapitulates results
111 previously reported for traditional MyD88 global KOs. Furthermore, for the behaviors tested in this
112 study, vendor purchased mice and Mock treatment controls did not differ substantially. Together,
113 these results establish the CRISPy TAKO method for screening gene function *in vivo*. This
114 method has moderately high throughput and will be especially useful for phenotypes, such as
115 behavioral responses, that cannot be assayed *in vitro*.

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Materials and Methods:

116 Animals:

117 All experiments were approved by the Institutional Animal Care and Use Committee of the
118 University of Pittsburgh and conducted in accordance with the National Institutes of Health
119 Guidelines for the Care and Use of Laboratory Animals. C57BL/6J male and female mice used to
120 generate embryos for electroporation and the purchased control group were procured from The
121 Jackson Laboratory (Bar Harbor, ME). CD-1 recipient females and vasectomized males were
122 procured from Charles River Laboratories, Inc. (Wilmington, MA). Mice were housed under 12-
123 hr light/dark cycles, with lights on at 7 AM and had *ad libitum* access to food (irradiated 5P76
124 ProLab IsoProRMH3000; LabDiet, St. Louis, MO) and water.

125 gRNA Design:

126 Guide RNAs (gRNAs) were generated using a commercially available two-piece system
127 termed ALT-R™ CRISPR/Cas9 Genome Editing System (IDT DNA, Coralville, IA). This system
128 combines a custom CRISPR RNA (crRNA) for genomic specificity with an invariant trans-
129 activating crRNA (tracrRNA) to produce gRNAs[4]. crRNAs were designed using the computational
130 program CCTop/CRISPRator[8,9], which gauges candidate sgRNAs for efficiency and specificity.

131 Four crRNAs were used to target the ethanol-responsive lncRNA gene *4930425L21Rik*
132 (see Table 1 for gRNA target sequences). These four crRNAs bind within a 366bp region that
133 includes the putative promoter and first Exon (see Fig. 2A). Similarly, four crRNAs were used to
134 target the lncRNA gene *Gm41261* (see Table 1 for gRNA target sequences). These four crRNAs
135 bind with a 316bp region that includes the putative promoter and first Exon (see Fig. 2C). Four
136 crRNAs were also selected for *MyD88* (see Table 1 for gRNA target sequences) that bind within
137 a 209bp region that includes *MyD88* Exon 3 and flanking DNA (see Fig. 3A). For each project,
138 the four crRNAs were annealed separately with tracrRNA in a 1:2 molar ratio then combined into
139 a single solution.

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140 CRISPR/Cas9 Mutagenesis:

141 Female C57BL/6J mice were superovulated with 0.1mL of CARD HyperOva (CosmoBio,
142 #KYD-010) between 10 AM and 11 AM, followed by 100 IU of human chorionic gonadotropin
143 (Sigma, #CG10) 46-48hrs later. Donor females were caged overnight with C57BL/6J males
144 starting 4-6hrs post-gonadotropin injection and allowed to mate. Embryos were harvested from
145 oviducts between 9 AM and 10 AM the following morning, cumulus cells were removed using
146 hyaluronidase, and embryos were cultured under 5% CO_2 in KSOM medium (Cytospring, #K0101)
147 for 1-2hrs. Embryos were electroporated in 5µL total volume of Opti-MEM medium
148 (ThermoFisher, #31985088) containing 100ng/µL of each sgRNA and 100 or 200ng/µL Alt-R®
149 S.p. Cas9 Nuclease V3 protein (IDT, #1081058) with a Bio-Rad Gene-Pulser Xcell in a 1mm-gap
150 slide electrode (Protech International, #501P1-10) using square-wave pulses (five repeats of
151 3msec 25V pulses with 100msec interpulse intervals). Two different concentrations of Cas9
152 protein were used to assess which produced greater mutagenesis in embryos targeting genes
153 *4930425L21Rik* and *Gm41261*; only the 200ng/µL concentration was used for MyD88 embryos.
154 Electroporated embryos were placed back into culture under 5% CO_2 in KSOM. For
155 *4930425L21Rik* and *Gm41261*, embryos were cultured for 3 days until the morulea/blastocyst
156 stage and subsequently analyzed for mutations. For MyD88, one- or two-cell embryos were
157 implanted into the oviducts of plug-positive CD-1 recipient (20-40 embryos per recipient) that had
158 been mated to a vasectomized male the previous night. Mock-treated controls were manipulated
159 in parallel as described above, except that the electroporation mix lacked the *MyD88*-specific
160 crRNAs (i.e., only tracrRNA and Cas9 protein were used).

161 Genotyping:

162 For *4930425L21Rik* and *Gm41261*, DNA was amplified from individual embryos using a
163 Qiagen Repli-G kit (Qiagen, #150025) and subject to PCR genotyping under the following

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164 settings: 95°C for 5min (1x); 95°C for 30sec, 60°C for 30sec, 72°C for 1min (40x); 72°C for 10min
165 (1x). Primers for PCR amplification of *4930425L21Rik* and *Gm41261* are listed in Table 1. PCR
166 amplicons (WT = 613 anf 506bp, respectively) were analyzed by agarose gel electrophoresis and
167 Sanger sequencing.
168 For *MyD88*, DNA was isolated from tail snips of MyD88 CRISPy TAKO and Mock-treated
169 control offspring using Quick Extract (Lucigen, #QE09050). Primers for *MyD88* genotyping are
170 listed in Table 1. PCR amplicons (WT = 494bp) were analyzed by agarose gel electrophoresis
171 and Sanger sequencing.

172 Subcloning:
173 Samples that did not produce clear chromatograms were subcloned to identify allelic
174 variants. The TOPO™ TA cloning kit (ThermoFisher Scientific, #K457501) was used according
175 to manufacturers instructions, with slight modifications. Briefly, sample PCR product was
176 incubated at room temperature for 15mins with TOPO reagents, then the TOPO vector mixture
177 was incubated with chemically competent DH5α (ThermoFisher Scientific, #18265017) cells on
178 ice for 30min. Cells were then heat-shocked for 45sec in a 42°C water bath then immediately
179 placed back on ice for 2min. S.O.C. medium (ThermoFisher Scientific, #15544034) was added
180 and cells were incubated in a bacterial shaker at 37°C for 90min at 225rpm. Cells were plated on
181 kanamycin-resistent LB plates and incubated at 37°C for 16-18hrs. Single colonies (n = 10 per
182 sample) were collected and their DNA was used for PCR. Colonies that produced a single PCR
183 band were then Sanger sequenced.

184 RNA Preparation:
185 Brain cerebellar tissue from one Mock treatment control (n = 1 male), one Jax control (n
186 = 1 male), and 6 MyD88 mutants (n = 3 male, n = 3 female) were used for RT-PCR analysis. All

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187 mice were 11-12 weeks of age at time of sacrifice. Total RNA was isolated using TRIzol
188 (Invitrogen, #15596018) according to the manufacturer's protocol, and purified with a TURBO
189 DNA-*free*™ Kit (Invitrogen, #AM1907). Total RNA was analyzed for purity and concentration using
190 a Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). One microgram of purified
191 RNA was converted into cDNA using Superscript™ III First-Strand Synthesis System (Invitrogen,
192 #18080051) with random hexamer primers. PCR primers were used that span from Exon 2 to
193 Exon 4 (see Supplementary Table 1) of MyD88. A reaction that lacked reverse transcriptase was
194 used as a negative control for each sample tested. RT-PCR amplicon size is 280bp for WT, and
195 99bp when Exon 3 is lacking.

196 Behavioral Testing:

197 All mice were moved into a reverse light-cycle housing/testing room (lights off at 10 AM)
198 at 5 weeks and allowed to acclimate for 2-3 weeks before the start of experiments. Experiments
199 were performed in the housing room (ethanol drinking) or an adjoining room [loss of the righting
200 response (LORR), rotarod]. Mice were group-housed 4 to 5 per cage based on genotype and
201 sex. The same mice were sequentially tested on the rotarod, LORR, and drinking assays, with
202 4-7 days between assays.

203 Drugs:

204 Injectable ethanol solutions were prepared fresh daily in 0.9% saline (20%, v/v). Ethanol
205 (Decon Laboratories, Inc.) was injected intraperitoneally (i.p.) at 0.02mL/g of body weight.

206 Rotarod:

207 In order to assess ethanol-induced motor ataxia, mice were trained on a fixed speed
208 rotarod (Ugo Basile, Gemonio, Province of Varese, Italy) at 11rpm. Training was considered
209 complete when mice were able to remain on the rotarod for 60sec. Following training, mice were

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210 injected with ethanol (2g/kg, i.p.) and every 15min mice were placed back on the rotarod and
211 latency to fall was measured until mice were able to remain on the rotarod for 60sec.

212 Loss of the Righting Response (LORR):

213 Sensitivity to the sedative/hypnotic effects of ethanol was determined using the LORR
214 assay. Mice were injected with ethanol (3.5g/kg, i.p.) and when mice became ataxic, they were
215 placed in the supine position in V-shaped plastic troughs until they were able to right themselves
216 3 times within 30sec. LORR was defined as the time from being placed in the supine position until
217 they regained their righting reflex. Body temperatures were maintained using a heat lamp
218 throughout the assay.

219 Two-Bottle-Choice Every-Other-Day (2BC-EOD) Drinking:

220 Mice were given access to ethanol (15%, v/v) and water for 24hr sessions every other day
221 for 12 days starting at 12 PM. Water alone was offered on off days. Purchased drinking bottles
222 were 15mL with 3.5-inch sipper tubes (Amuza, San Diego). The side placement of the ethanol
223 bottles was switched with each drinking session to avoid side preference. Ethanol solutions were
224 prepared fresh daily. Bottles were weighed before placement and after removal from the
225 experimental cages. Empty cages with sipper bottles were used to control for leakage, and
226 leakage amount was subtracted from amount consumed by the mice. The quantity of ethanol
227 consumed, and total fluid intake, was calculated as g/kg body weight per 24hr. Preference was
228 calculated as amount ethanol consumed divided by total fluid consumed per 24hr.

229 Statistical Analysis:

230 Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., La
231 Jolla, CA) for two-tailed Mann-Whitney test and two-way ANOVA (with mixed-effects analysis (i.e.
232 when technical failures are present), multiple comparisons, and repeated measures when

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233 appropriate). Significant main effects were subsequently analyzed with Benjamini, Krieger, and
234 Yekutieli two-stage linear step up procedure post-hoc analysis[10]. Technical failures were
235 appropriately removed from analysis.

236 The two control groups were first compared to one another; if no difference was found
237 between control groups, these groups were pooled and tested against the MyD88 KO group.
238 Graphs show control groups plotted separately even though they were analyzed together, unless
239 noted otherwise. Because of well-known sex differences on the behaviors of interest, and
240 because male and female mice were tested on separate days, each sex was analyzed separately.
241 Statistical significance was defined as $p \leq 0.05$ and $q \leq 0.05$. All data are presented as mean ±
242 S.E.M.

243 **Results:**

244 CRISPR/Cas9-mediated Mutagenesis:

245 Preliminary testing of the CRISPy TAKO method occurred *in vitro* using embryos
246 electroporated at the one-cell stage, cultured until the blastocyst stage, then genotyped (Fig. 2).
247 To enhance CRISPR mutagenesis frequency, each gene was targeted simultaneously with four
248 gRNAs that were tiled across a small section of the gene. In addition, we tested two
249 concentrations of Cas9 protein (100 and 200ng/µL) that were higher than the minimum amount
250 we typically use (i.e., 50ng/µL).

251 The first gene targeted was an unannotated ethanol-responsive gene, *4930425L21Rik*,
252 using 4 gRNAs that span ~400bp of the putative promoter and first Exon (Fig. 2A). Agarose gel
253 electrophoresis of PCR amplicons that span the targeted locus indicated that 3 of 5 embryos
254 tested at 100ng/µL Cas9 had obvious indels whereas 2 embryos (#'s 1.2 and 1.3; Fig. 2A, B) had
255 amplicons that were grossly indistinguishable from the 613bp WT control amplicon (Fig. 2B).
256 Sanger sequencing revealed #1.2 as heterozygous for WT and a 21bp deletion (Fig. 2A). At
257 200ng/µL Cas9 protein, all seven embryos assessed were found to harbor deletions of varying

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258 sizes (Fig. 2A, B). Thus, 0% of the embryos electroporated with 200ng/µL Cas9 harbored WT
259 amplicons that were visible on the agarose gel or detectable by amplicon bulk sequencing.
260 The second gene targeted was another unannotated ethanol-responsive gene, *Gm41261*.
261 Four gRNAs spanning ~350bp within the putative first exon were used (Fig. 2C). Agarose gel
262 electrophoresis of PCR amplicons that span the targeted locus indicated that 1 of 5 embryos
263 tested at 100ng/µL Cas9 had an obvious indel (#5.6; Fig. 2C, D), whereas the other 4 of 5 embryos
264 had amplicons that were indistinguishable from the 506bp WT control amplicon (Fig. 2D). Sanger
265 sequencing revealed one embryo (#5.3; Fig. 2C, D) was homozygous WT, whereas the other four
266 embryos harbored various small deletions (Fig. 2C). At 200ng/µL Cas9, all six embryos assessed
267 were found to harbor deletions of varying sizes (Fig. 2C, D). Although one embryo (#7.5) had a
268 PCR product approximately the size of the WT amplicon (506bp), Sanger sequencing revealed a
269 14bp deletion. Sanger sequencing also revealed a sequence inversion in #8.1, along with a 16bp
270 insertion directly following the inverted sequence (Fig. 2C). Thus, 5 of 6 embryos electroporated
271 at 200ng/µL Cas9 protein did not harbor detectable WT amplicons by agarose gel or amplicon
272 bulk sequencing. Because the higher 200ng/µL Cas9 concentration showed greater mutagenic
273 activity in both *4930425L21Rik* and *Gm41261*, this concentration was utilized in targeting MyD88.
274 As proof-of-concept and to validate our method *in vivo*, we created MyD88 CRISPy TAKO
275 mice. The four gRNAs were tiled across a 209bp region of *MyD88* that included Exon 3 (Fig. 3A).
276 Exon 3 was targeted because prior traditional global MyD88 KO studies demonstrated that
277 deletion of this Exon inactivates MyD88[11] and imparts an alcohol behavioral phenotype[5-7].
278 Implantation of embryos electroporated with *MyD88* gRNAs yielded 54 offspring (n = 26
279 females, n = 28 males). Thirty-one offspring (n = 16 females, n = 15 males) were derived from
280 electroporation of Mock-treated control embryos that were handled identically except that the
281 crRNAs were omitted from the electroporation solution. All mice born from electroporated
282 embryos were genotyped for gross indels at *MyD88* Exon 3 using endpoint PCR. The 494bp WT
283 PCR amplicon was invariant and readily detectable in Jax and Mock-treated control samples as

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284 expected (Fig. 3B and data not shown). In stark contrast, 52 of 54 MyD88 KO mice displayed
285 gross indels that were readily apparent following gel electrophoresis of PCR products. PCR
286 results are shown in Figure 3B for the subset (n = 15/sex) of the MyD88 mutant mice created that
287 were selected for behavioral phenotyping. The indels varied from animal to animal and were
288 approximately 50-300bp smaller than the 494bp WT amplicon. To accurately characterize the
289 mutations present, we sequenced the PCR products of the mutated mice selected for behavior
290 As illustrated in Figure 3A, deletions removed Exon 3, deleted spice junctions, and/or are
291 predicted to create frameshifts.

292 Cerebellar tissue from a random subset of MyD88 CRISPy TAKOs and controls were used
293 for RT-PCR analysis using PCR primers that bind to Exons 2 and 4 to examine MyD88 mRNA.
294 This analysis revealed the expected 280bp fragment in Jax and Mock WT control samples (Fig.
295 3C). In contrast, none of the six MyD88 CRISPy TAKO mice examined peoduced a fragment of
296 this size. Five of the six samples produced a predominant band of ~99bp was would be expected
297 for MyD88 mRNA that lacked Exon 3. One sample produced a major band of ~210bp and may
298 represent a splicing defect. Thus, MyD88 CRISPy TAKO mice are likely to be functional KOs.

299 <u>Ethanol-Induced Loss of Righting Response (LORR)</u>:

300 No difference in ethanol LORR (3.5g/kg, i.p.) was found between the Mock and Jax control
301 groups for males or females (p = 0.9671 and p = 0.7345, respectfully; Fig. 4). Therefore, Mock
302 controls and Jax controls were combined and compared to MyD88 KOs (for completeness, control
303 results are plotted separately). Male MyD88 KOs exhibited a significant reduction in ethanol-
304 induced LORR duration when compared to controls (p = 0.0068; Fig. 4A). No difference was
305 observed in females for ethanol-induced LORR (Fig. 4B).

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306 Ethanol-Induced Motor Incoordination:

307 The ataxic effects of an acute ethanol injection (2g/kg, i.p.) were measured using a
308 constant speed (11rpm) rotarod test. For male mice, comparison of Mock and Jax controls
309 showed a significant effect of time [$F (2.281, 41.05) = 36.41$, $p < 0.0001$], and time x genotype [F
310 $(9, 162) = 3.209$, $p = 0.0013$], but no effect of genotype (Fig. 5A). Because of the time x genotype
311 interaction, control groups were not combined for this analysis. Repeated measures two-way
312 ANOVA of all three groups revealed a significant effect of time [$F (2.474, 71.73) = 59.01$, $p <$
313 0.0001], and an effect of time x genotype [$F (18, 261) = 1.964$, $p = 0.0120$] but no effect of
314 genotype (Fig. 5A). Posthoc comparisons revealed that male Mock control mice recovered more
315 quickly than Jax controls at the 15min timpoint ($q = 0.0042$).

316 For females, comparison of Mock and Jax controls revealed a significant effect of time [F
317 $(2.775, 55.51) = 89.05$, $p < 0.0001$], but no effect of genotype or time x genotype (Fig. 5B).
318 Therefore, the two control groups were combined (data plotted separately for completeness) and
319 compared to Myd88 KOs. There was a significant effect of time [$F (2.664, 87.90) = 148.3$, $p <$
320 0.0001], and genotype [$F (1, 33) = 4.721$, $p = 0.0371$], but no effect of the time x genotype
321 interaction (Fig. 5B). Post-hoc analysis did not reveal any significant differences.

322 Two-Bottle Choice Every-Other-Day (2BC-EOD) Drinking:

323 Mice were tested for ethanol drinking using an intermittent every-other-day, two bottle free choice
324 consumption assay over a period of 12 days. Mock and Jax control groups were first compared
325 against each other; two-way ANOVA mixed-effects analysis was used for all 2BC-EOD statistical
326 analyses. For males, there was a significant effect of time for ethanol intake [$F (3.333, 62.00) =$
327 5.740, $p = 0.0011$], ethanol preference $(2.702, 50.27) = 10.85$, $p < 0.0001$], and total fluid intake
328 [$F (3.392, 63.09) = 17.98$, $p < 0.0001$], but there was no effect of genotype or time x genotype
329 interaction for any of these parameters (Fig. 6A-C). Therefore, both Mock and Jax control groups
330 were combined (data plotted separately for completeness) and compared to MyD88 KOs.

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331 Analysis of ethanol intake in males between the combined control group and MyD88 KO revealed
332 a main effect of time [F (4.123, 129.5) = 10.67, p < 0.0001] and genotype [F (1, 32) = 4.850; p =
333 0.0350], but no interaction between the two (Fig. 6A). Post-hoc analysis revealed that MyD88 KO
334 males had significantly greater intake on day 10 (q = 0.0285) compared to controls. For
335 preference in males, there was an effect of time [F (3.365, 105.7) = 24.02, p < 0.0001] but no
336 effect of genotype or time x genotype (Fig. 6B). For total fluid intake in males, there was a main
337 effect of time [F (3.915, 122.9) = 36.79, p < 0.0001] and genotype [F (1, 32) = 8.897, p = 0.0054],
338 but no time x genotype interaction between MyD88 KO and controls (Fig. 6C). Post-hoc analysis
339 revealed significantly increased total fluid consumption in MyD88 KOs vs controls on days 4, 6, 8
340 (q = 0.0118), and day 10 (q = 0.0002) (Fig. 6C).

341 In females, Mock and Jax control groups were first compared. There was a significant
342 effect of time on ethanol intake [F (2.412, 47.27) = 8.979, p = 0.0002] and ethanol preference [F
343 (2.626, 51.47) = 22.58, p < 0.0001], but no effect of genotype or time x genotype interaction on
344 either parameter (Fig. 6D, E). Therefore, both control groups were combined (data plotted
345 separately for completeness) and compared to MyD88 KOs. For ethanol intake, females showed
346 a main effect of time [F (2.632, 86.85) = 12.50, p < 0.0001] but not genotype or time x genotype
347 interaction (Fig. 6D). Similarly, for ethanol preference a significant effect of time [F (3.317, 109.5)
348 = 29.10, p <0.0001] but not genotype and time x genotype interaction was observed (Fig. 6E).
349 Thus, ethanol intake and preference did not differ between female MyD88 KOs and the combined
350 control group.

351 Comparing total fluid intake in female Mock and Jax controls revealed significant main
352 effects of time [F (2.818, 55.22) = 9.800, p < 0.0001] and genotype [F (1, 20) = 10.41, p = 0.0042],
353 but no time x genotype interaction (Fig. 6F). Therefore, for female total fluid intake, control groups
354 were not combined and each genotype was considered separately. This analysis revealed a
355 significant effect of time [F (2.650, 84.79) = 11.20, p < 0.0001] and genotype [F (2, 33) = 4.221, p

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356 = 0.0223] (Fig. 6F), but no time x genotype interaction. Post-hoc analysis did not reveal any
357 significant differences.

358 **Discussion:**

359 The current study reports on a CRISPR/Cas9-mediated mutagenesis protocol that is
360 suitable for rapid screening for the phenotypic effects of gene KO *in vivo*. Traditional CRISPR
361 mouse KO procedures require three generations of animal breeding and genotyping, which is
362 time consuming and expensive. In contrast, with the CRISPy TAKO protocol described here, first
363 generation gene-targeted F0 mice can be rapidly produced and screened for phenotypic effects.
364 Although individual F0 mice harbor a variety of mutant alleles for the gene of interest, careful
365 project design ensures that each F0 animal is functionally equivalent, i.e., a gene KO. F0 animals
366 can be directly screened for phenotypes of interest. If no phenotype is detected, the gene is
367 rapidly eliminated from further consideration. If an interesting phenotype is observed, a F0 animal
368 can be bred as in traditional approaches to establish a true breeding line that can first be tested
369 to confirm the phenotype. This will ensure rigor and reproducibility in the experimental pipeline.
370 Subsequently, the line can be maintained long-term and more detailed, rigorous mechanistic
371 studies can be conducted. The CRISPy TAKO approach can save valuable time and minimize
372 animal numbers and financial resources.

373 There are several keys to the success of this approach. First, we use embryo
374 electroporation to facilitate genetic modification of a large number of animals with minimal effort.
375 Large numbers of embryos (n = 30-50) can be simultaneously transfected with CRISPR reagents
376 at a very high efficiency[12-16]. This avoids the limiting bottleneck of directly injecting each individual
377 embryo. Second, achieving a very high level of indel formation that ablates function of the gene
378 of interest in each animal is critical. We observed that 52 of 54 of animals harbored inactivating
379 indels, while the other two harbored small mutations that were not charecterized. To achieve this
380 high KO efficiency, we simultaneously utilized four gRNAs that targeted a small, functionally

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381 important portion of the gene of interest. In other experiments, we have observed that the
382 mutagenesis efficiency of a single gRNA is highly variable. Simultaneous use of two gRNAs tends
383 to increase mutagenesis efficiency. We reasoned that an even higher number of gRNAs tiled
384 across a small but functionally important part of a gene would result in even higher efficiency. We
385 are unsure if four is the optimal number of gRNAs, but this should be rigorously explored in future
386 studies. For *in vivo* proof-of-concept, we focused on a small, single Exon of the *MyD88* gene that
387 results in a null allele when disrupted[5-7,11]. This approach should also work by targeting the
388 promoter or any region that is critical for function of the gene and/or gene product. It should also
389 be noted that we observed that 200ng/µL Cas9 protein in the electroporation mix produced a
390 much higher rate of indel formation compared to 100ng/µL. While 200 ng/µL is 4x the minimum
391 amount we typically use in our lab for most CRISPR embryo electroporation experiments, this
392 amount is less than that reported in the literature[15,16].

393 The CRISPy TAKO approach could be further streamlined and throughput increased if
394 control animals for comparison could be procured directly from a vendor. However, it is
395 conceivable that the *in vitro* embryo manipulation / CRISPR electroporation procedure could
396 introduce some unknown variable that could impact the phenotype of interest regardless of the
397 gene targeted for modification. Therefore, we compared phenotypes of control animals procured
398 directly from JAX with isogenic controls that were produced in-house, in parallel to the MyD88
399 TAKOs. This in-house control group was created using procedures that were identical to those
400 used to create MyD88 TAKOs except that crRNAs were omitted from the electroporation
401 reactions. We observed near complete concordance between these Mock controls and Jax
402 control animals for the behavioral phenotypes of interest. We only observed a subtle female-
403 specific difference in total fluid consumption in the 2BC-EOD assay (Fig. 6G) and a male-specific
404 genotype x time interaction on the rotarod (Fig 5A). We conclude that mice purchased directly
405 from a vendor can be used as a control group for screening CRISPy TAKO mice for behavioral
406 alterations provided the controls are the same genetic background as those animals that served

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407 as embryo donors. Using a single vendor-derived control group will substantially increase
408 throughput and reduce expenses.

409 As proof-of-concept of the CRISPy TAKO approach, we focused on *MyD88* as a candidate
410 gene. We sought to functionally validate our approach by comparing behavioral phenotypes
411 observed with those previously reported for global MyD88 KO mice produced using traditional
412 gene targeting technology, which displayed robust alterations in ethanol-induced behavioral
413 responses and ethanol drinking behavior[5-7]. Overall, similar behavioral results were observed
414 between traditional MyD88 KOs and MyD88 CRISPy TAKOs (Table 1). Consistent with previous
415 findings, the MyD88 KO females show faster recovery time from ethanol's incoordination effects
416 (Fig. 5B), but contrary to those studies, no difference between male MyD88 KOs and controls is
417 reported here (Fig. 5A). Also consistent with previous reports, albeit with a milder effect size[5],
418 MyD88 KO males had greater consumption of ethanol than controls (Fig. 6A). However, KO males
419 in the present study did not have a difference in preference when compared to controls (Fig. 6B),
420 but had significantly increased total fluid intake compared to controls (Fig. 6C), suggesting these
421 male mice drink more fluid in general, and it is not specific to ethanol. Altered total fluid intake in
422 MyD88 KO females compared to controls (Fig. 6F) is consistent with the published literature[5].

423 It is unclear if the results presented here show a milder phenotype than those previously
424 reported[6,7], or if these differences are simply due to experimental variation that is common in
425 behavioral studies between labs, facilities, and universities[17,18]. Although all studies were
426 conducted using C57BL/6J mice, the current study utilized mice sourced directly from JAX,
427 whereas Blednov et al., 2017a/b used mice sourced from JAX that were bred in-house for an
428 unspecified number of generations. It is also possible that the CRISPy TAKO approach is slightly
429 less sensitive than the traditional KO approach for detecting phenotypic changes. The most likely
430 explanation for such a possibility is that F0 CRISPR mice may be mosaic and it is conceivable
431 that tail DNA genotyping is not reflective of the genetic changes that occur in the brain of the
432 mutant animals. It is possible that some WT MyD88 is expressed in the brain of some F0 animals,

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433 however this is unlikely because RT-PCR analysis of a subset of TAKO MyD88 mice did not
434 reveal WT bands (Fig. 3C). Nonetheless, the approach described here should be very useful for
435 a first pass screening method to identify genes with a large effect on a phenotype of interest. The
436 usefulness of this approach for detecting subtle genotypic differences requires further evaluation.

437 One limitation of the approach as outlined is the potential for off-target effects of CRISPR
438 mutagenesis. This approach uses multiple gRNAs simultaneously along with a relatively high
439 concentration of Cas9, both of which could lead to off-target effects. Although off-target effects
440 were not examined in this study, they are unlikely to explain the phenotypic changes we observed.
441 The main behavioral phenotypes observed in the MyD88 CRISPy TAKO mice are the same as
442 those observed in MyD88 global KOs that were produced using traditional, non-CRISPR gene
443 targeting techniques. Furthermore, several studies have reported that off-target effects in
444 CRISPR/Cas9 animals is minimal with careful selection of gRNAs as done in the present study[19-
445 21].

446 In summary, we propose using the CRISPy TAKO approach for rapidly screening large
447 numbers of genes *in vivo* to identify those that have large effects on a phenotype of interest. Once
448 such a gene is identified, an individual animal that harbors a confirmed KO allele should be mated
449 to establish a true breeding mutant KO line. A true breeding line will be useful for future studies
450 to (a) confirm the phenotype of interest, (b) to test for and rule out the potential impact of off-target
451 mutations, (c) to enable the rigorous testing of control and KO littermates derived from
452 heterozygous matings, and (d) to provide an unlimited source of uniform animals for further, in-
453 depth analyses and long term line maintenance.

454 We conclude that the CRISPy TAKO method can be used for efficient, moderate
455 throughput, *in vivo* screens to identify genes that impact whole animal responses when ablated.
456 This method avoids the extensive animal breeding, time, and resources required with traditional
457 CRISPR animal KO approaches. This method should find widespread use in studies where

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458 moderate to large numbers of genes must be rapidly screened for effects that cannot be
459 interrogated *in vitro*, such as whole animal behavioral responses.

460 Conflict of Interest:
461 The authors declare that the research was conducted in the absence of any commercial or
462 financial relationships that could be construed as a potential conflict of interest.

463 Author Contributions:
464 Project conception and gRNA design divised by GEH. *In vitro* analysis conducted by AS and GEH.
465 *In vivo* project design, organization, and analysis conducted by SLP. SLP and AS managed the
466 behavioral experimentation together. All authors contributed to writing and editing of the
467 manuscript.

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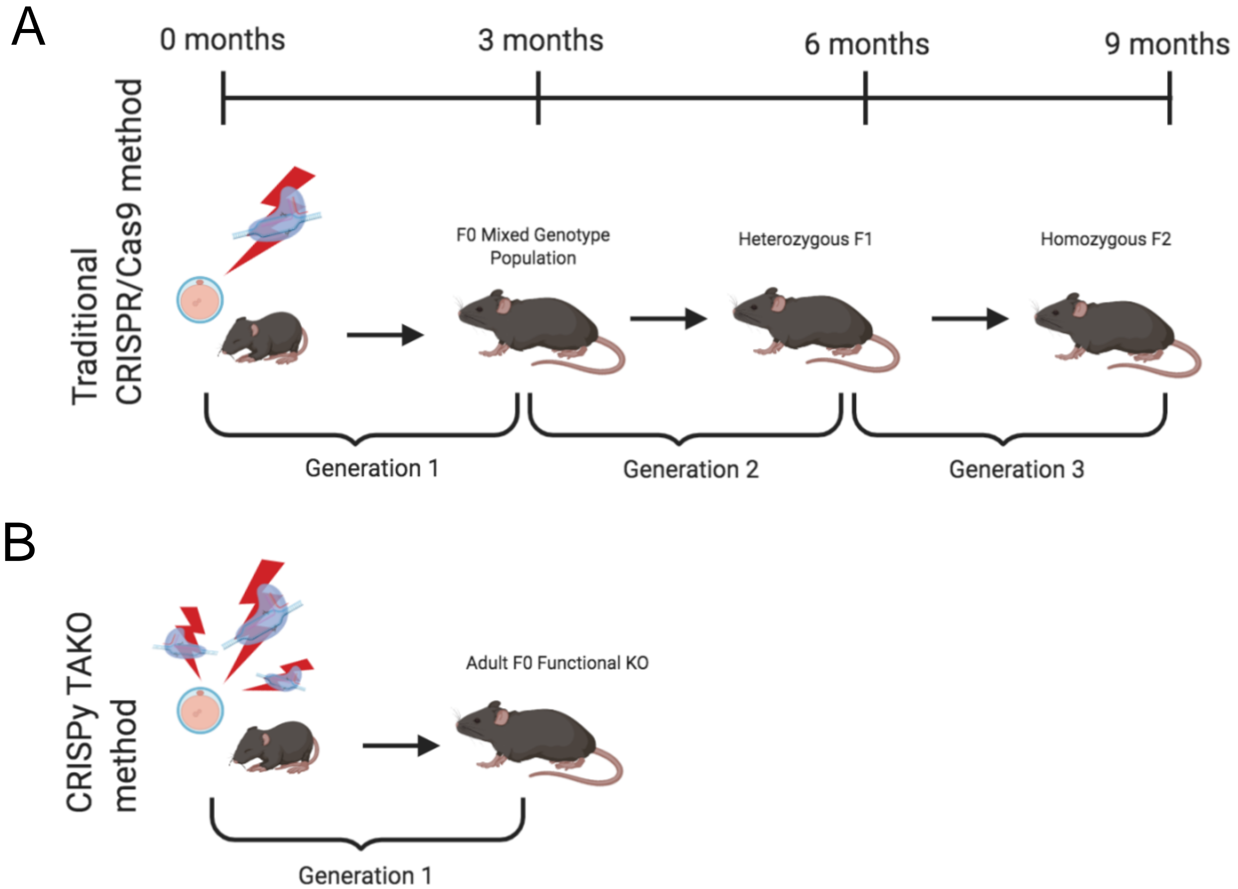

Figure 1: Comparison of the timeline required to produce knockout (KO) mice for behavioral testing using traditional and the CRISPy TAKO approaches. **(A)** Traditional CRISPR/Cas9-mediated method to create a stable KO line. Founder (F0) animals are an eclectic mix of wild-type, heterozygous, and homozygous KOs. A founder with an inactivating mutation is selected for breeding to establish a KO line of mice. First generation offspring (F1) are heterozygous and must be interbred to produce an F2 generation. A subset (~25%) of the F2 generation are homozygous KO mice and can be compared for behavioral phenotypes with WT littermates. **(B)** CRISPy TAKO method for creating functional KO mice. By using multiple gRNAs that target a small but functionally critical part of the gene of interest, most F0 mice harbor biallelic mutations that functionally inactivate the gene of interest and are suitable for behavioral phenotyping.

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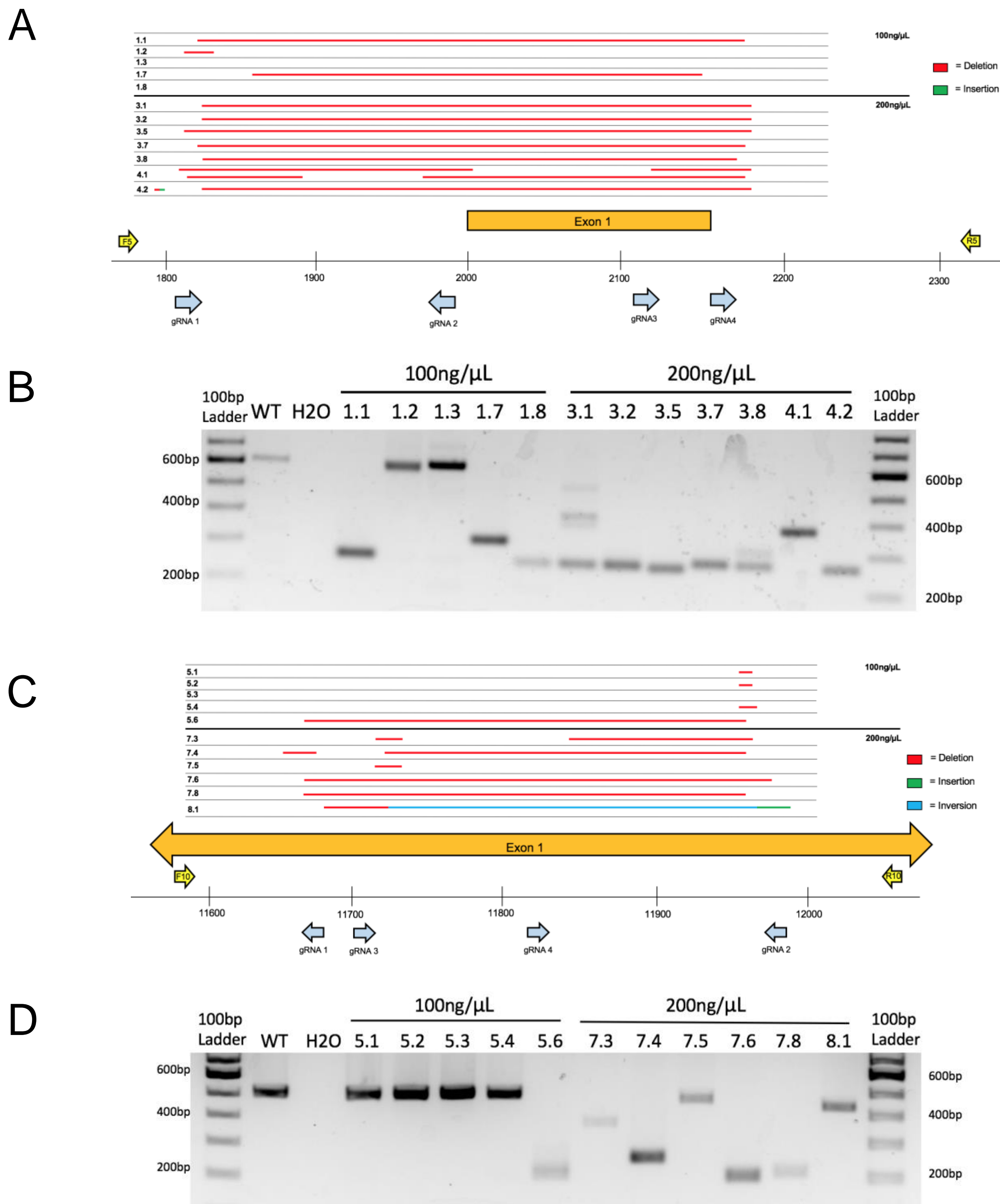

Figure 2. Embryo CRISPy TAKO genotypes for *4930425L21Rik* and *Gm41261*. (A) Sequence results for the major product(s) of TAKO embryos targeting gene *4930425L21Rik* electroporated with 100ng/µL and 200ng/µL Cas9 protein. Full deletions are shown in red. Sequence insertions are shown in green. Individual animal tag numbers are presented on the left. The gRNAs and PCR primers used are shown as blue and yellow arrows, respectively. (B) Agarose gel electrophoresis of PCR amplicons for *4930425L21Rik* in embryos. Samples 1.1 through 1.8 were electroporated with 100ng/µL Cas9 protein while samples 3.1 through 4.2 were electroporated with 200ng/µL Cas9 protein. (C) Sequence results from TAKO embryos targeting gene *Gm41261* with 100ng/µL and 200ng/µL Cas9. Sequence inversions are shown in blue. (D) Agarose gel electrophoresis of PCR amplicons for *Gm41261* in embryos. Samples 5.1 through 5.6 were embryos electroporated with 100ng/µL Cas9 while samples 7.3 through 8.1 were electroporated with 200ng/µL Cas9 protein.

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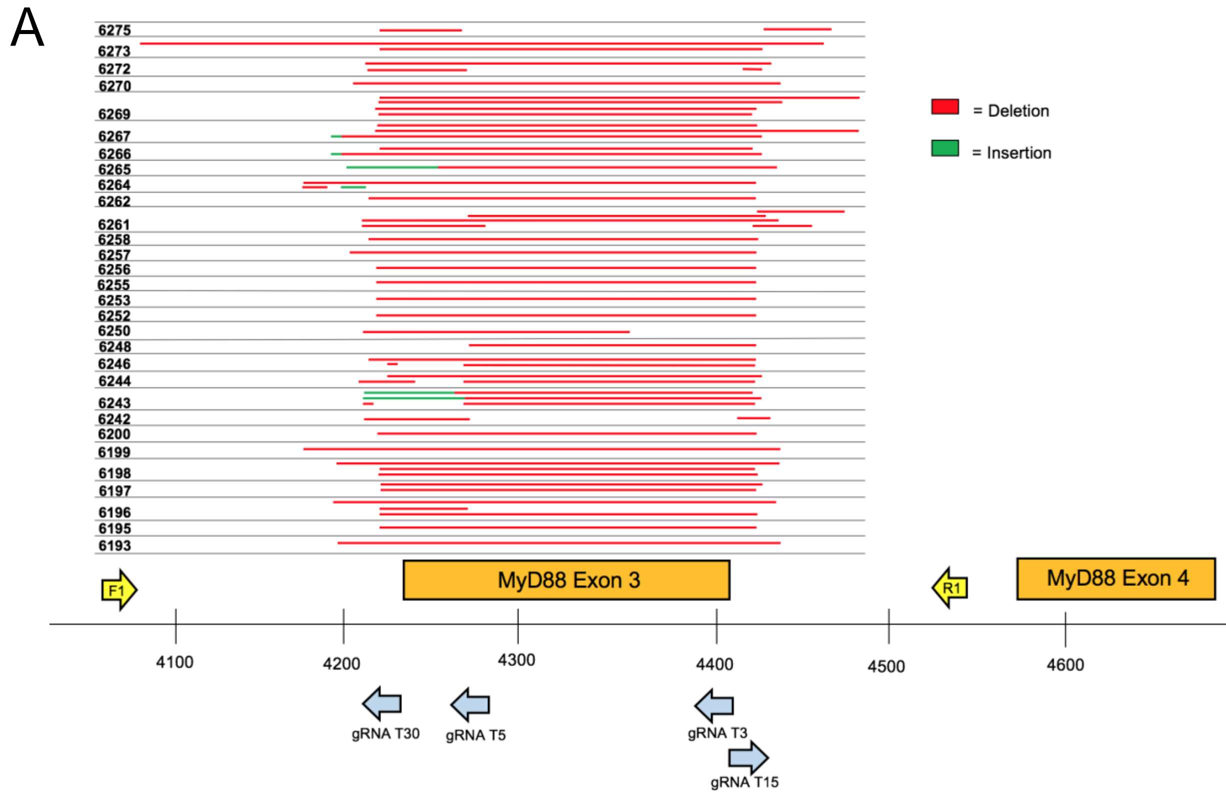

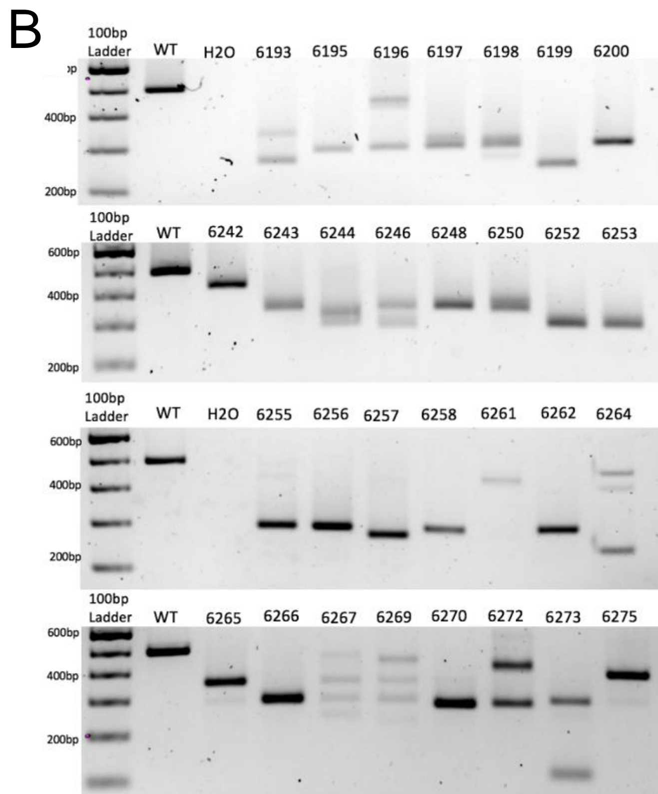

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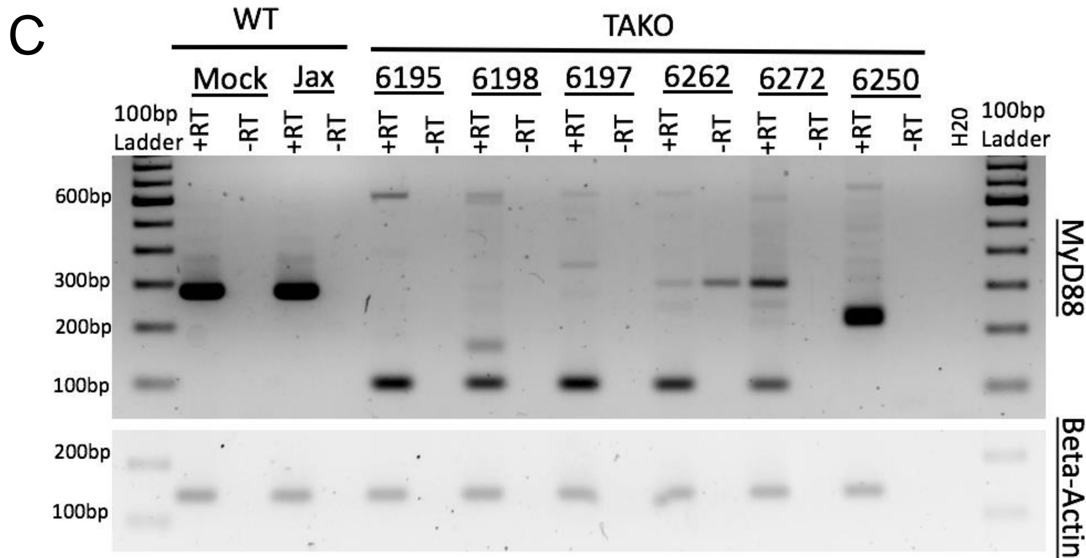

Figure 3: *In vivo* MyD88 CRISPy TAKO genotypes. (A) Sequencing results from mice selected for behavioral experimentation. CRISPR/Cas9 mutagenesis of MyD88 exon 3 was performed and animals used for experimentation were sequenced to confirm successful deletion. Full deletions are shown in red. Sequence insertions are shown in green. Individual animal tag numbers are presented on the left. The gRNAs and PCR primers used are shown as blue and yellow arrows, respectively. (B) PCR results from DNA of MyD88 CRISPy TAKO mice used for experimentation. Jax used as WT control. (C) RT-PCR results from cerebellar brain tissue from a random subset of Mock, Jax, and MyD88 CRISPy TAKO mice showing abnormal *MyD88* RNA transcripts in TAKO mice. RT-PCR using Beta-actin was used as a control.

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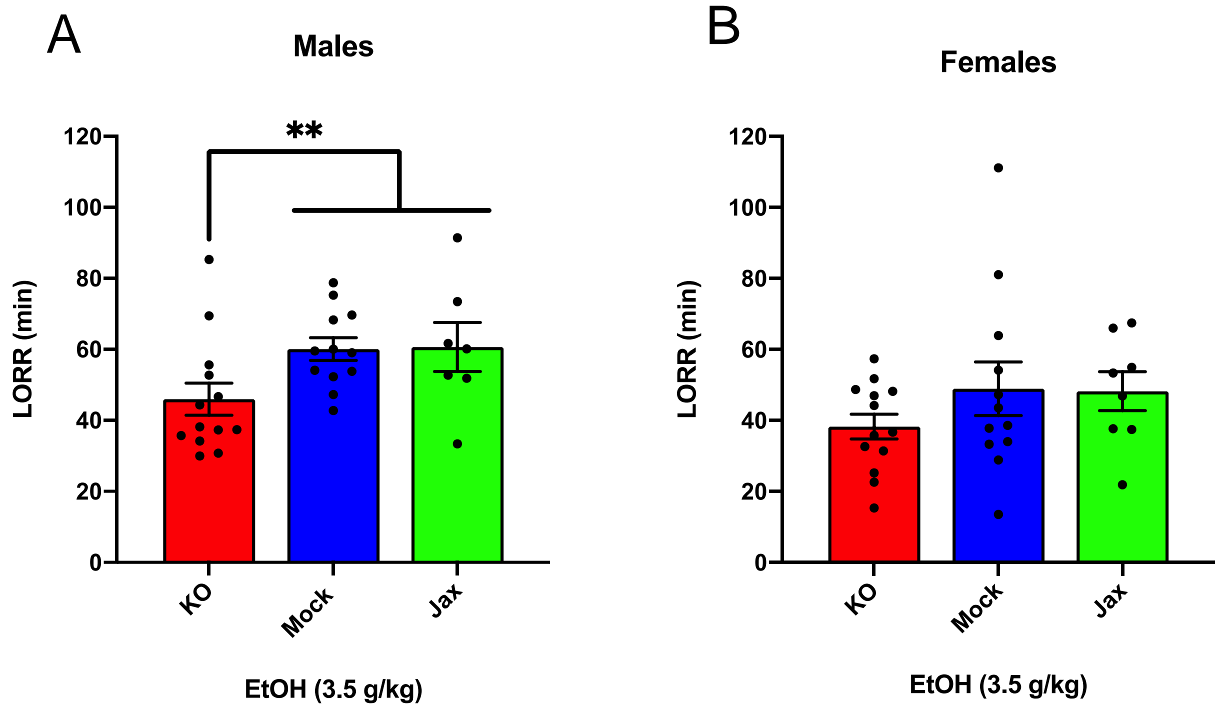

Figure 4: Duration of LORR induced by ethanol (3.5 g/kg, i.p.) injection in male **(A)** and female **(B)** MyD88 KOs, Mock controls and Jax controls. For both males and females, mock controls and Jax controls did not differ and therefore were pooled and compared to same sex MyD88 KOs (data are plotted separately for completeness). KO males had reduced duration of LORR compared to the combined control group (**$p < 0.01$). For females, no difference was observed between MyD88 KOs and the combined control group. Values represent mean ± SEM. Data were analyzed using a two-tailed Mann-Whitney test.

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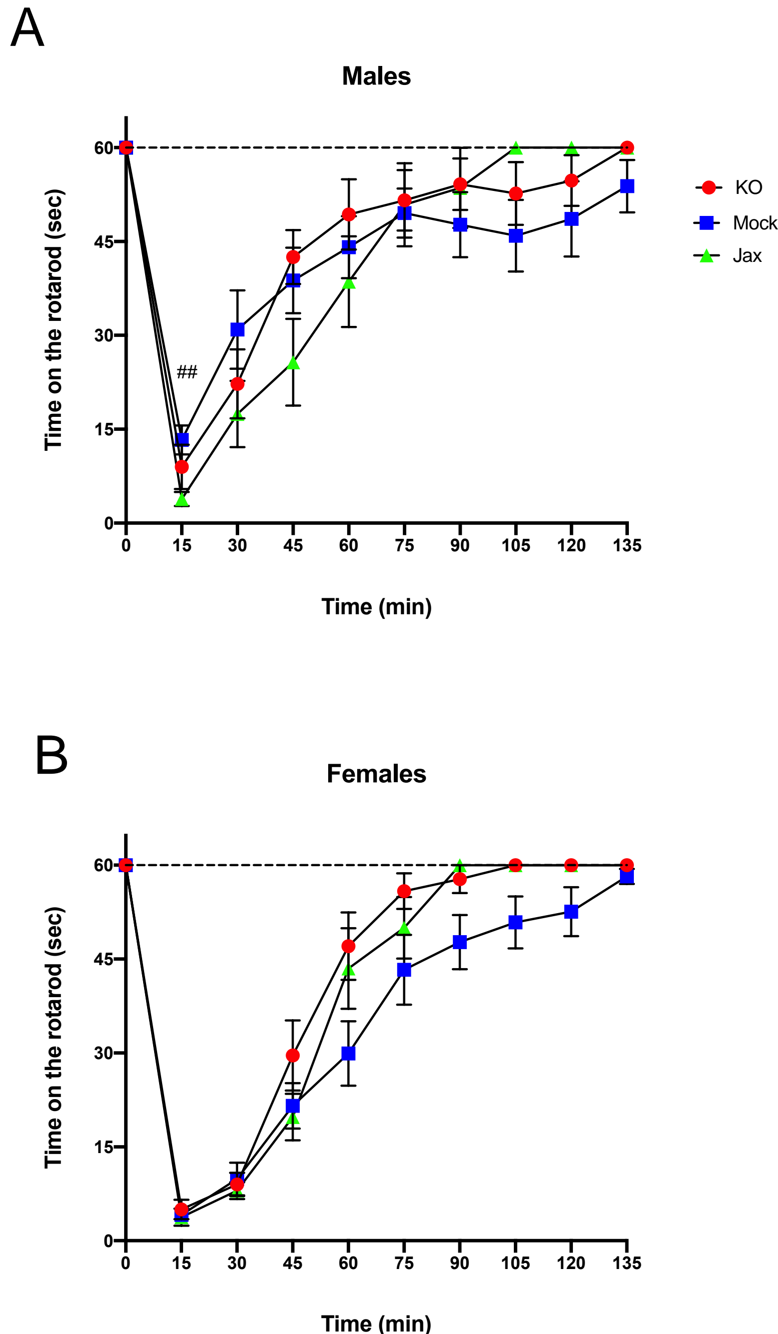

Figure 5: Recovery from ethanol (EtOH)-induced motor incoordination in MyD88 KO and control mice. **(A)** A significant time x genotype interaction was observed for male mock controls and Jax controls. Therefore, control groups were not combined. Jax controls recovered more slowly compared to Mock controls at the 30 min timepoint (##q < 0.01). **(B)** Female mock controls and Jax controls did not differ and were combined for comparison to MyD88 KOs (data are plotted separately for completeness). Post-hoc analysis did not reveal any significant differences between groups at any time points. Data represent time in seconds on the rotarod after injection of EtOH (2 g/kg, i.p.). Values represent mean ± SEM. Data were analyzed by repeated-measures 2-way ANOVA with multiple comparisons followed by Benjamini, Krieger, and Yekutieli post hoc-test.

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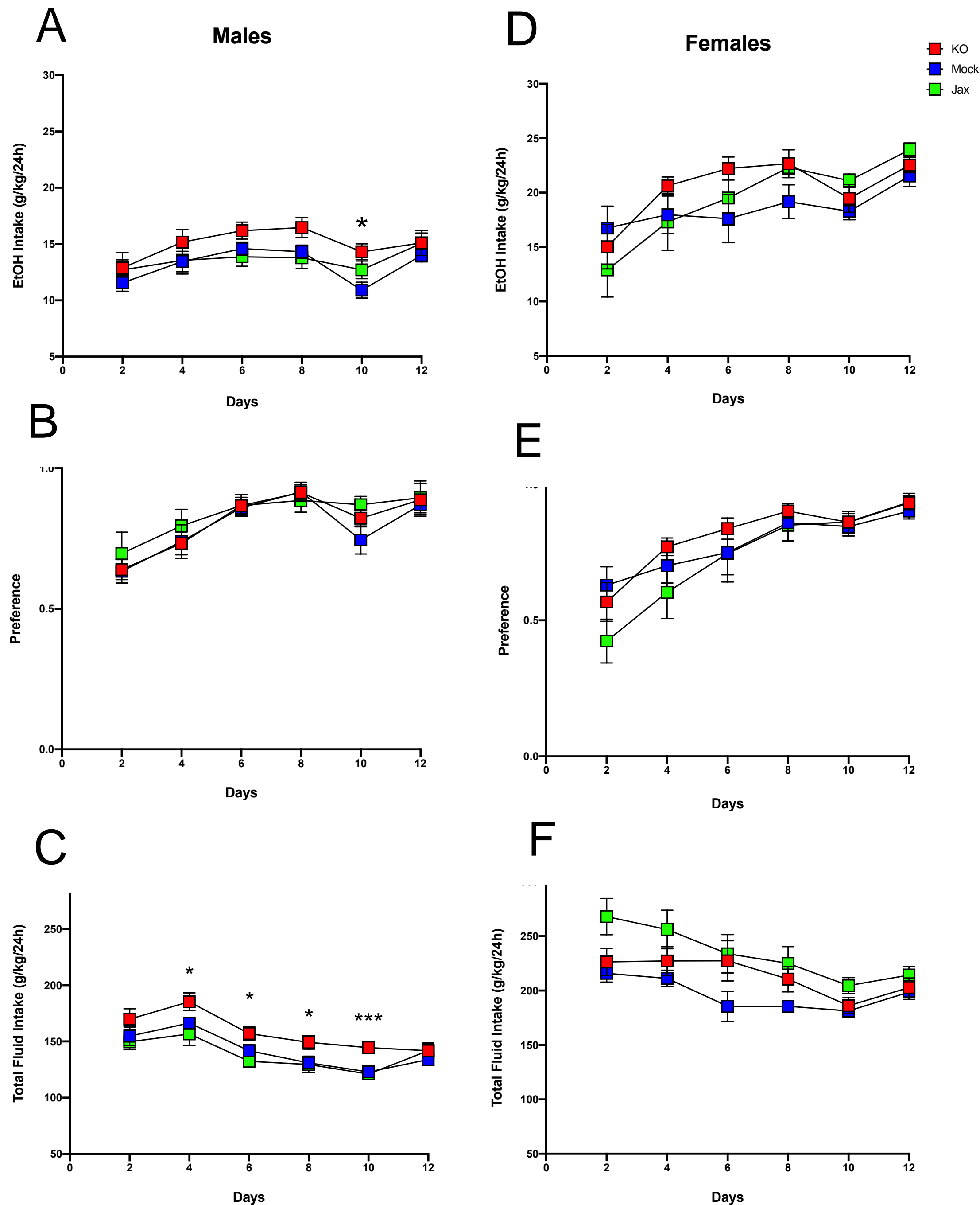

Figure 6: Two-bottle choice, every-other-day drinking in MyD88 KO, Mock control and Jax control mice. Left, males; right, females. **A and D.** Ethanol (EtOH) intake (g/kg/24 h), **B and E.** EtOH preference. **C and F.** Total fluid intake (g/kg/24 h) in control (Jax C57BL/6J, n = 8; Mock-treatment control, n = 13-14) versus mutant mice (n = 13-14 MyD88 KO). Values represent mean ± SEM. Data were analyzed by repeated-measures 2-way ANOVA (mixed-effects analysis where appropriate) with multiple comparisons followed by Benjamini, Krieger, and Yekutieli post-hoc tests (*p < 0.05 and ***p < 0.001 between MyD88 KO and combined controls.

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	CRISPy TAKO		Previous Reports	
	Males	Females	Males	Females
Rotarod	No change	Faster recovery	Faster recovery[6,22]	Faster recovery[6]
EtOH Loss of Righting Reflex	Reduced duration	No change	Reduced duration[6,22]	Reduced duration[6]
2BC-EOD (15% v/v)	Increased ethanol drinking	Reduced total fluid intake	Increased ethanol drinking and preference. Reduced total fluid intake[5]	Reduced total fluid intake[5]

Table 1: Comparison of TAKO cohort results with previous findings.