1 **Title:**
2
3 Genetic characterization of ABC pesticide transporters establishes *Drosophila* as an *in vivo* model for
4 toxicogenetics
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29 **Abstract:**
30

31 Pesticides remain one of the most effective ways of controlling agricultural and public health insects, but much
32 is still unknown regarding how these compounds reach their targets. Specifically, the role of ABC transporters in
33 pesticide absorption and excretion is poorly understood, especially compared to the detailed knowledge in
34 mammalian systems. Here, we present a comprehensive characterization of pesticide transporters in the model
35 insect *D. melanogaster*. An RNAi screen was performed, which knocked down individual ABCs in targeted
36 epithelial tissue, examining the subsequent changes in sensitivity to the pesticides spinosad and fipronil. This
37 simultaneously implicated a novel ABC drug transporter *CG4562* but also highlighted a predominant role for the
38 P-glycoprotein orthologue *Mdr65*. Further characterization of the P-glycoprotein family was performed via
39 transgenic overexpression and immunolocalization, finding that *Mdr49* and *Mdr50* play enigmatic roles in
40 pesticide toxicology perhaps determined by their different subcellular localizations within the midgut. Lastly,
41 heterologous expression of the *Mdr65* orthologue from the major malaria vector *Anopheles gambiae* was used to
42 establish an *in vivo* characterization system for the characterization of P-glycoproteins from non-model insects in
43 *D. melanogaster*. This study provides the basis for establishing *Drosophila* as a model for toxicology research
44 regarding drug transporters.
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Introduction:

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51 Small molecule pesticides have been the most versatile method of control for both agricultural and public health
52 insect pests. However, there is an urgent need for new compounds due to the emergence of pesticide resistance
53 and unacceptably high deleterious effects on off-target species. One obstacle in the design of new pesticides is
54 bioavailability, or the proportion of a drug that can cross epithelial barriers and reach its target within the body.
55 Although a variety of different factors determine a drug's ability to cross these epithelia [1], transporters are
56 particularly interesting from a genetic perspective. By translocating molecules across the lipid bilayer, they can
57 either decrease or increase bioavailability depending on their localization and direction of transport. Such drug
58 transporters have been extensively studied in the context of pharmaceutical development; regulatory agencies
59 have specified guidelines for transporter conscious drug design [2], and companies have been founded which
60 focus exclusively on drug transporter services (https://www.solvobiotech.com/). However, the role of these
61 transporters in pesticide toxicology has so far been understudied.

62

63 Of particular interest is the ATP-binding cassette (ABC) superfamily. Each functioning ABC transporter is
64 composed of two conserved nucleotide binding domains (NBDs) and two transmembrane domains (TMDs)
65 involved in substrate recognition [3]. Individual transporters are classified into families (A-H) based on
66 homology, which can suggest function but does not allow prediction of which specific substrates are transported
67 by members of a given family. Selected members of the A, B , C , and G families are known to transport
68 xenobiotics in mammalian systems [4]. Perhaps the best studied transporter is P-glycoprotein (aka ABCB1, P-
69 gp), a from the B family. This protein was first identified in a cancer resistant mammalian cell line [5] , and has
70 been found to act as a polyspecific drug transporter across a range of different taxa.

71

72 Much work on ABCs in insects has been focused on their role in detoxifying pesticides and plant secondary
73 metabolites [6]. Most of this work has been indirect, implicating pesticide transporters via their upregulation in
74 resistant populations or following pesticide exposure. While some transporters have been functionally
75 characterized [7–9], the diversity of transporters that act on pesticides is poorly understood. Like other taxa, P-
76 glycoprotein has been the most studied drug transporter in insects. A family wide expansion of P-gp was
77 previously found in Lepidoptera (moths and butterflies), which may underpin their extreme polyphagy [10].
78 CRISPR-Cas9 removal and *in vitro* studies have also highlighted their conserved role as drug transporters in this
79 order [11–13]. However, a more detailed characterization of P-gp *in vivo* is lacking.

80

81 The study of ABC transporters like P-gp is often difficult *in vitro* despite a wide variety of assays being available
82 [14]. More direct methods of transport such as monolayer cell assays and *in vivo* mice models are more accurate
83 but are relatively expensive and thus not available to all laboratories. The fruit fly *Drosophila* represents a
84 potentially powerful system to study ABC transporters *in vivo* at least with respect to toxins such as pesticides
85 [15]. Apart from its advantages as a genetically tractable model organism, it also has physiologically well
86 characterized epithelial tissues such as the midgut [16], Malpighian tubules [17] and blood-brain-barrier (BBB;
87 [17]). Recent papers have also considered the four P-glycoprotein paralogues in *Drosophila. Mdr65* was
88 localized to the BBB and shown to efflux fluorescent dyes [19] . Deletion of this gene along with the tubule
89 predominant *Mdr49* and midgut predominant *Mdr50* significantly increased sensitivity to a variety of pesticides
90 [20–23]. Strikingly, removal of *Mdr49* and *Mdr50* displayed opposite pesticide responses (more tolerant or more
91 sensitive) depending on which pesticide was used. Similar results were found when RNAi was used to target

92 these two genes [23]. This apparent paradox does not have a proven explanation but may be rooted in the
93 localization of these transporters within tissues and cells. Such differences in localization among the well-studied
94 ABCC (MRP) transporters in mammals have explained such opposing effects [24]. However, these questions
95 have so far not been investigated in insects.
96
97 Here we present a series of experiments which sought to use *D. melanogaster* to i) identify novel pesticide
98 transporters ii) characterize their effect on pesticide toxicology *in vivo* and iii) establish *Drosophila* as a
99 heterologous pesticide transporter model.
100
101 **Results:**
102
103 *An in vivo RNAi screen identifies putative pesticide transporting ABCs*
104
105 Of the 56 total ABC transporters in *D. melanogaster*, 37 belong to the B, C, or G subfamilies that have been
106 previously implicated as drug transporters in mammalian systems. For our study we further only considered
107 transporters they had an expression level of >5 RPKM in a given tissue on the FlyAtlas expression database or
108 were present above that threshold in a separate midgut transcriptome [25,26]. This narrowed the list to 33
109 transporter genes and 59 combinations of transporter tissue combinations tested in this study (Table S1).
110
111 An RNAi screen was performed whereby individual transporters knocked down in a single tissue were assessed
112 for their insecticide tolerance. Knock down of several transporters in a single epithelium yielded differences in
113 sensitivity compared to its respective control genotypes, while the majority showed either no difference or were
114 inconsistent (Figure 1; Table S2). Most notably, knock down of *Mdr65* substantially increased the sensitivity of
115 both spinosad and fipronil (Figure 1). To a far lesser extent knockdown (KD) of CG32091, CG3164, and
116 *CG4562* in the midgut tissue increased tolerance to spinosad. Furthermore, KD of CG8799 and *CG9664* in the
117 tubules increased tolerance to spinosad. However, the variation inherent in bioassays and RNAi preclude firm
118 conclusions being drawn from only the RNAi data alone.
119
120 *CRISPR-Cas9 of uncharacterized ABC transporter increases spinosad tolerance*
121
122 *CG4562*, *CG3164*, and *CG32091* were selected for knockout (KO) using CRISPR-Cas9 as they showed the most
123 consistent differences in the RNAi toxicology screen. A KO line for *CG3164* was unable to be established as it
124 did not homozygose from the balancer stock, suggesting the gene plays an essential role. KO of *CG32091* and
125 *CG4562* were successful. Of these, only *CG4562* showed a decrease in spinosad sensitivity which mimicked the
126 RNAi phenotype (Figure 2; Table S3). This suggests that *CG4562* plays a role in spinosad transport in *D.*
127 *melanogaster*, but the magnitude of this change was far less than that observed for *Mdr65* knockdown.
128
129 *Overexpression of P-glycoprotein in Drosophila*
130
131 In the RNAi screen *Mdr65* knockdown provided the most consistent impact on the response to spinosad and
132 fipronil across the three tissues tested (Figure 1). Further, CRISPR-Cas9 based KO of *Mdr65* and its paralogues
133 *Mdr49* and *Mdr50* indicates that each of the genes has a role in insecticide response [20]. We thus sought to
134 expand upon these findings using transgenic UAS responder lines containing the ORFs of *Mdr49*, *Mdr50* and
135 Mdr65. Overexpression of *Mdr65* at the BBB increased tolerance to spinosad and showed no difference against
136 fipronil in contrast to the striking increase in susceptibility observed with RNAi in this tissue (Figure 3A,B).

137 Very minor differences in response were observed when *Mdr49* or *Mdr50* were driven in their respective tissues
138 of the tubules or the midgut respectively (Figure 3 C-F; Table S4).
139
140 Transgenic expression of these P-gp genes with a strong ubiquitous driver yielded a larger effect. Both *Mdr49A*
141 and *Mdr65* increased sensitivity to spinosad while *Mdr50* overexpression lines were slightly more resistant
142 (Figure 4B,D; Table S5). With the pesticide nitenpyram ubiquitous expression of both *Mdr65* and *Mdr50* caused
143 resistance while overexpression of *Mdr49* caused increased sensitivity (Figure 4 A,C). Overexpression of
144 *Mdr49B* had limited effect. These results agree with previous findings [20,23], demonstrating that global
145 deletion or expression of genes can have variable effects on the direction of toxicological change (more
146 susceptible or resistant) depending on the gene and pesticide under investigation.
147
148 Rescue experiments whereby we replaced an endogenous copy of P-gp with itself in a specific tissue. The
149 expression of *Mdr49A* or *Mdr49B* in the Malpighian tubules of the Mdr49KO background did not have any
150 measurable effect on the toxicology of spinosad or fipronil (Figure 5 A,B; Table S6). Likewise, the
151 reintroduction of *Mdr50* into the midgut increased survival but only very subtly; the effect was observed only at
152 one dose (Figure 5 C,D). A much clearer effect was seen with the rescue of *Mdr65*. Significantly higher survival
153 was observed in both pesticides when a functional copy of *Mdr65* was added into the Mdr65KO background
154 specifically at the BBB (Figure 5 E,F). These results highlight the subtlety of the effect seen with *Mdr49* and
155 *Mdr50*, but also suggest the *Mdr65* rescue system as a viable way of detecting transgene function *in vivo*.
156
157
158 *Immunolocalization of endogenous P-gp*
159
160 Given that overexpression of P-glycoprotein orthologues in *Drosophila* yielded qualitatively different
161 phenotypes depending on the insecticide and gene in question, we sought to localize these proteins with
162 immunofluorescence. The C219 antibody was used, which targets an epitope common to all P-glycoprotein
163 paralogues found in *Drosophila*, and a genotype with a wild type P-gp gene (Myo-GAL4) was used in
164 comparison with all three P-gp KOs. No staining was found in the fat body in any genotype under investigation,
165 suggesting that this tissue does not contain notable P-gp expression (Figure S1). The dissected central nervous
166 system showed strong staining in all genotypes except Mdr65KO, confirming that the MDR65 protein is the
167 predominant P-gp orthologue at the BBB (Figure S2). Removal of *Mdr49* had a drastic effect and abolished
168 almost all tubule expression especially that concentrated in the lumen (Figure S3 C'). The remaining non-
169 luminal P-gp is presumably due to expression of *Mdr50* or *Mdr65* present in small quantities in the tubule.
170
171 Lastly, the midgut was examined which provided the most complicated yet interesting tissue under study. In wild
172 type and Mdr65KO flies, P-glycoprotein was found localized primarily to a region of the posterior midgut
173 (defined as R5 by [27]) , which matches transcriptomic data for these genes (Figure 6 A-B). There were also
174 lower amounts of expression detectable in the anterior midgut near the gastric caeca. However, Mdr50KO and
175 Mdr49KO genotypes appeared to show opposing subcellular localizations. While Mdr50KO showed an apical
176 specific phenotype, Mdr49KO localized only to the basolateral side (Figure 6 C-D). This suggests that different
177 paralogues differ by their subcellular localization despite residing in the same tissue.
178
179 *Transgenic Expression of P-glycoprotein from a malaria vector*
180
181 We lastly sought to extend our findings to P-glycoproteins from pest species such as the malarial vector
182 *Anopheles gambiae*. Strong, ubiquitous expression of *AgP-gp* (AGAP005639) in *Drosophila* resulted in

183 increases in sensitivity to both nitenpyram and spinosad with effect sizes on par with or greater than the
184 endogenous *Drosophila* proteins (Figure 7; Table S5). Given the larger effect sizes observed when rescuing the
185 Mdr65KO at the blood brain barrier, *AgP-gp* was also expressed specifically at the blood brain barrier in an
186 Mdr65KO background this suggested a higher tolerance to the pesticide spinosad, but not fipronil (Figure 8;
187 Table S7). The expression of *Mdr49A* or *Mdr49B* did not yield any effect in this system suggesting that different
188 paralogues may not be active in this tissue.
189
190 **Discussion**
191
192 The emergence of resistance and unacceptably high externalities posed by the current generation of pesticides
193 necessitates novel, safe, and effective compounds for insect control. Bioavailability presents an obstacle to
194 rational pesticide design, and this is worsened by a poor understanding of insect drug transporters *in vivo*. Here,
195 we develop the *Drosophila* model system for transporter mediated toxicology by i) characterizing the diversity
196 of pesticide transporters in *Drosophila* ii) providing a more in-depth characterization of P-glycoprotein and iii)
197 heterologously expressing an orthologous mosquito transporter in *D. melanogaster*.
198
199 *The diversity of ABCs involved in pesticide transport*
200
201 One of the goals of this study was to identify which ABCs were involved in pesticide transport, and a toxicology
202 screen of RNAi lines was performed to address this. Identification of the previously known *Mdr65* gene
203 suggested that the assay could detect true positives and the screen further suggested a previously uncharacterized
204 gene (*CG4562*) which transports spinosad in the midgut (Figure 1). This was confirmed by CRISPR-Cas9 KO,
205 which phenocopied the RNAi knockdown (Figure 2). *CG4562* belongs to the ABCC family, several members of
206 which have been implicated in drug transport in humans [28] and other insects such as the human body louse
207 [29], red flour beetle [8] and even in *D. melanogaster* [30] . Unlike several other ABCC drug transporters
208 identified in insects, removal of *CG4562* increases tolerance to spinosad rather than increasing sensitivity. This
209 runs counter to the narrative of ABCs as "resistance" genes and highlights a more complex role for transporters
210 in influencing the distribution of compounds around the body. Similar findings in mammalian ABCC genes have
211 shown that transporters localizing to the basolateral side of tissues like the intestine have the effect of pumping
212 compound into the body [24]. While *CG4562* was not localized in this study, we hypothesize it to be present at
213 the basolateral membrane and thus pump toxin out of the midgut cell into the hemolymph.
214
215 However, several limitations of the assay may have skewed a bias of the RNAi screen towards false negatives.
216 For example, *Mdr49* and *Mdr50* were not detected in the RNAi screen despite CRISPR-Cas9 KO yielding clear
217 effects [20]. This may stem from considering each tissue in isolation or may also represent a limitation of the
218 strength of the knockdown achieved in this study, either of which could mask subtle effect sizes. Future studies
219 could make use of the next generation of genetic tools in *Drosophila* such as somatic CRISPR for tissue specific
220 removal or simply making heritable mutations in a larger range of transporters [31] which would likely elicit
221 larger effects. Combinatorial knockouts or knockdowns may also be an option as synergistic action has been
222 previously associated with toxicology in both mammals [32] and insects [33].
223
224 *P-glycoprotein plays a predominant role in insecticide toxicology*
225

226 Knockdown of the previously known *Mdr65* gene at the blood brain barrier created an effect size that was an
227 order of magnitude higher than for any other transporter, suggesting a predominant role for this protein in
228 pesticide toxicology. Such a role would match the situation in mammals where P-glycoprotein has by far the
229 largest effect in most tissues [28]. Interestingly, P-glycoprotein has only been rarely associated with insecticide
230 *resistance,* defined as the abnormally high levels of tolerance to pesticides causing control failures. Selected
231 studies have shown P-gp's role in resistance [34], but this mechanism does not appear to be widespread among
232 insects compared to other methods like target site mutations or upregulation of drug metabolizing enzymes [35].
233 Indeed, in the current study only strong ubiquitous expression was able to generate changes in toxicology and
234 these were modest compared to the effect of removing the gene (Figure 1, Figure 3). P-glycoprotein thus appears
235 to play a strong role in the toxicology of pesticides, but increased expression may have only a limited effect on
236 pesticide resistance. Interestingly, an opposite role is played by some cytochrome P450s; overexpression of
237 *Cyp6g1* confers insecticide resistance, while removal has a limited effect [36].
238
239 Strikingly, the ubiquitous expression of *Mdr49* or *Mdr50* caused opposite changes in toxicology (increased
240 tolerance or increased sensitivity) depending on both the pesticide and transgene, agreeing with previous
241 CRISPR-Cas9 data [20,23]. These differences may be due to changes in localization, and immunostaining at the
242 cellular level indicated a change in subcellular localization of *Mdr49* and *Mdr50* genotypes (Figure 6). However,
243 it is currently unclear how any potential localization changes cause the observed changes in toxicology among
244 pesticides. Another hypothesis is that a compensatory mechanism exists whereby the removal of one transporter
245 impacts another as was seen for organic anion transport in the tubule [37]. Lastly, there may be restrictions as to
246 which tissue a given paralogue can act. Ubiquitous expression of different paralogues yielded different effects
247 (Figure 4), and only certain paralogues *(Mdr65* and *AgP-gp*) were shown to be functional in the BBB rescue
248 system (Figure 8). Future work will be needed to address this paradox, which promises to have important
249 implications for toxicokinetics and for basic research on how transporter gene's function.
250
251 *Drosophila as a model system for ABC mediated toxicology*
252
253 The characterization of the P-gp orthologue from *A. gambiae* showed that it was able to influence the
254 toxicological profile of the pesticide spinosad when expressed both ubiquitously and in a tissue specific *Mdr65*
255 rescue system at the BBB (Figure 7,8). Although this system no doubt requires optimization, the successful
256 heterologous expression of a pest transporter *in vivo* opens the door for the study and comparison of these genes
257 in the future. So far it has been challenging to find suitable expression systems for drug transporters and
258 *Drosophila* represents an unexplored alternative. Side by side expression of transporters from pest species (e.g.,
259 mosquitoes, agricultural pests) with non-target organisms (e.g., humans, bees), in a *Drosophila* like system could
260 provide the basis for pest selective insecticides in the future. Such heterologous expression in *Drosophila* has
261 been used to previously characterize pesticide target proteins [38], and given the challenging interpretation of *in*
262 *vitro* transporter data [14], *Drosophila* may provide a welcome alternative.
263
264 **Materials and Methods:**
265
266 *Previously generated Drosophila genotypes*
267
268 Several driver genotypes were used in this study which expressed GAL4 in defined epithelial tissues (Luong et.
269 al under review). The Mex-Gal4 line was previously reported (Phillips and Thomas, 2006) and drove expression
270 specifically in the midgut. Similarly, the Myo1A GAL4 driver (Myo-GAL4; 67057) was used as an alternative to

271   Mex-G4 in the P-gp rescue experiments as it also drives expression in the midgut enterocytes. The
272   Urate_Oxidase-GAL4 (UO-GAL4) localized expression specifically in the Malpighian tubules [39]. Lastly, a
273   previously published driver which localized GAL4 to the subperineural glial cells of the BBB was used (SPG-
274   GAL4; [40]. Strong ubiquitous expression was also achieved with Actin-GAL4 (Bloomington #3953).
275
276   Publicly available UAS-RNAi lines were obtained from the transgenic RNAi project (Trip) or the Vienna
277   *Drosophila* RNAi Centre (VDRC) and contained RNA interference sequences targeting an individual ABC
278   transporter under the control of a UAS promoter (Table S1; [41,42]. Crossing of any one of these flies to a
279   GAL4 would express the RNAi construct and knock down the specified ABC in the specified tissue.
280
281   *Generation of CRISPR-Cas9 knockout genotypes*
282
283   Selected genes identified in the RNAi screen were confirmed by removing the majority of the gene from the
284   genome using CRISPR-Cas9. Pairs of sgRNAs for each were first designed via the CRISPR optimal target finder
285   [43]. These were selected so that one sgRNA targeted a sequence at the 5' and 3' end of the gene. Each sgRNA
286   was cloned into the Bsbi site in the pU6 vector by annealing overlapping custom primers (Table S2). Pairs of
287   pU6-sgRNA plasmids were then injected into fly lines expressing Cas9 under the control of the Nanos promoter
288   either on the 2nd or 3rd chromosome (Bloomington # 78781 & 78782). In either case the chromosome carrying
289   Cas9 was crossed out so that all deletions were eventually in identical backgrounds apart from the ABC
290   transporter being removed.
291
292   *Transgenic expression genotypes*
293
294   Transgenic lines containing an open reading frame (ORF) of a given transporter regulated by a GAL4 Inducible
295   UAS promoter were created. ORFs were first amplified with gene specific primers and cloned into the pGEM-T-
296   easy shuttle vector. The fragments were then digested with flanking NotI sites, subcloned into the NotI site of the
297   pUASt-AttB vector [44], and injected into the AttP40 (Bloomington #25709) or the VK13 (Bloomington
298   #24864) *D. melanogaster* lines harboring AttP sites and an endogenous source of φC31 integrase. This strategy
299   was used to introduce *Mdr49A, Mdr49B, Mdr50, Mdr65,* and the P-gp derived from the VK7 stain of Anopheles
300   gambiae (*AgP-gp*).
301
302   In addition to expression in "wild type" backgrounds, transgenic expression of these P-gp genes was performed
303   in P-gp KO backgrounds [20] in order to observe any complementation (rescue) of the initial toxicology
304   phenotype. In order to match known endogenous expression patterns, *Mdr49* was expressed specifically in the
305   Malpighian tubules, *Mdr50* specifically in the midgut, and *Mdr65* specifically in the BBB. For *Mdr49* and
306   *Mdr50,* stable lines were made carrying i) the deletion ii) the UAS-transgene and iii) the GAL4. As the KO and
307   transgenic GAL4 cassette and KO was on the same chromosome recombination was used to generate flies
308   carrying both alleles. Each of the two previously characterized *Mdr49* isoforms (A and B; [21] were considered
309   separately. For *Mdr65,* the transgene and the BBB-GAL4 cassette (on the 2nd chromosome) were both
310   independently crossed into the Mdr65KO background separately and finally crossed together to make the rescue
311   line. For the heterologous BBB rescue system, the UAS-Pgp construct was inserted into the VK13 landing site
312   (3rd chromosome) and was recombined with the Mdr65KO allele (also 3rd chromosome) and crossed into a
313   background with BBB-GAL4 on the 2nd. Due to genetic incompatibilities irrespective of the genes under
314   investigation, BBB-GAL4 was unable to be homozygosed with the Mdr65KO 3rd chromosome, so all rescue
315   lines were maintained and assayed over a 2nd chromosome CyO balancer.

316

Insecticide bioassays

318

Fipronil (CAS# 120068-37-3) and Spinosad (CAS# 168316-95-8) were both obtained as analytical standards from Sigma. Insecticide bioassays were performed as described previously [20]. First instar larvae were obtained by collecting between 150-200 females and 100 males of defined genotypes and allowing them to lay eggs on cherry juice agar plates for 24 hours. After this period, the plates were changed, and the eggs washed onto a fine mesh. Eggs were then transferred onto fresh cherry juice agar plates and left undisturbed for an additional 24 hours which generated a population of 1st instar larvae. 50 larvae were transferred to standard media dosed with a defined concentration of insecticide. Survival was scored after 16 days by counting the number of total pupae in the vial. For genotypes carrying a CyO balancer, 100 larvae were picked/vial and total numbers of straight wing flies were counted as there were no significant differences among the number of CyO emerging individuals (data not shown). Raw mortality data was then analyzed in the R statistical environment.

329

Immunolocalization of P-glycoprotein

331

In order to analyze protein localization of C219 P-gp, immunostaining was performed as described previously [19] , with some modifications. In brief, third instar larvae were dissected in SF-900 II insect medium (SFM, Cat. No 10902-096, Gibco) enriched with penicillin-streptomycin mix (50x, Cat. No P4458, Sigma-Aldrich) and placed on ice. Following dissections, the tissues were incubated in 4% formaldehyde solution (Methanol free, Thermo Scientific, 28906) for 60 min and then in 3% Blocking Solution (BS, 3% BSA, 0.1% Triton in PBS1X) overnight at 4°C. Tissues were incubated with mouse anti-P-glycoprotein C219 antibody at 3.6µg/mL (Glycoprotein Monoclonal Antibody C219, Cat. No MA1-26528, Invitrogen) overnight at 4°C. On the third day the tissues were incubated with Alexa Fluor 555-conjugated anti-mouse IgG secondary antibody (Invitrogen) (1:1,000 in BS) for 1.5 hours at RT. After BS washes, actin filaments were stained with Alexa Fluor 488-conjugated phalloidin (1:50 in BS for 30 min at RT, Invitrogen Thermo Scientific, A12379) and nuclei were stained with DAPI (1:100 in BS for 20 min at RT, AppliChem, Cat. No A1001). Tissues were then gently rinsed twice with PBS1X, prior to mounting in Vectashield (Vector Laboratories, H-1000-10) on SuperFrost+ slides. Imaging was conducted using a Leica TCS SP8 confocal laser scanning microscope unit, equipped with an inverted microscope (DMI6000 CS), a high-end Scientific fluorescence CCD camera (Leica DFC365FX) and a set of the appropriate high quality lasers, housed in the Institute of Molecular Biology and Biotechnology (IMBB) Microscope Facility of Foundation for Research & Technology-Hellas (FORTH).

348

Acknowledgments

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495
496

497   **Figure/Table Legends**

498

499   Figure 1: ABC Transporter RNAi screen
500   A knockdown of ABC transporters was performed in the BBB (left), Midgut (middle), and Malpighian tubules
501   (right), followed by a bioassay on either spinosad (bottom) or fipronil (top). Vertical dashed lines distinguish the
502   different ABC transporters targeted by RNAi. Horizontal solid lines signify how the genetically matched control
503   line behaved, while boxes reflect the degree of difference between the knockdown and control. In other words,
504   the further from the vertical line a box is the more susceptible (lower) or resistant (higher) the knockdown was.

505   Boxes are color coded based on their genetic background. From Bloomington TRIP stocks, AttP2 was grey,
506   AttP40 was blue. From the VDRC, GD was orange and KK was red.
507
508   Figure 2: CRISPR-KO of candidate drug transporters
509   Bioassay data from the KO of CG32091 and *CG4562* is shown. The genetic control background (Nanos-Cas9)
510   and the CG32091 showed a strong response, indicated by a low survival proportion on the y-axis. Removal of
511   *CG4562* showed a higher proportion of survival across all doses of spinosad (x-axis). Error bars represent 95%
512   confidence intervals generated by Schneider-Orelli'scorrection.
513
514   Figure 3: Tissue specific overexpression of P-gp paralogues
515   Bioassay data is shown for both spinosad and fipronil against genotypes selectively overexpressing a single P-
516   glycoprotein orthologue (*Mdr49*; C-D, Mdr50; E-F, or Mdr65; A-B) in the specific tissue corresponding to their
517   proposed localization. Grey bars indicate genetically matched controls in all samples while colors correspond to
518   specified overexpression genotypes.
519
520   Figure 4: Global overexpression of P-gp paralogues
521   Bioassay data is shown for the pesticides spinosad (B,D) and nitenpyram (A,C), the latter chosen due to the
522   difficulty of scoring fipronil toxicology in this genetic background. As the *Mdr65* overexpression genotype was
523   generated in a distinct background it was considered separately (A-B), while all other overexpression constructs
524   were analyzed jointly (C-D). In each panel the y-axis represents survival while the x-axis signifies the dose of
525   the pesticide under investigation.
526
527   Figure 5: Genetic rescue of *Drosophila* Knockouts
528   Genetic rescue of the three knockouts Mdr49KO, Mdr50KO, and Mdr65KO was tested for their pesticide
529   toxicological response. Spinosad (B,D,F), Fipronil (A,C), and Nitenpyram (E) were used against different
530   genotypes, with the latter being chosen due to the difficulty of scoring fipronil toxicology in this genetic
531   background. In each panel the x-axis represents pesticide dose and the y-axis represents survival corrected with
532   abbots correction. Error bars represent 95% confidence intervals generated by Schneider-Orelli's correction.
533
534   Figure 6: Immunolocalization of P-glycoprotein paralogues
535   Immunostaining is shown for the control genotype (Myo; A) and Mdr65KO (B), Mdr49KO (C), and Mdr50KO
536   (D). For each row, zoomed out images of the whole midgut are shown along with zoomed images based
537   depending on where fluorescence was detected. Brightfields are shown along with confocal images and merges
538   for each column. In each image, DAPI stained nuclei are in blue, phalloidin stained actin is in green, and the
539   C219 antibody localization (P-gp) is in red.
540
541   Figure 7: Global overexpression of P-glycoprotein in *A. gambaie*
542   The single P-glycoprotein orthologue from Anopheles gambiae was transgenically expressed in *D.*
543   *melanogaster* under the control of a strong ubiquitous promoter (Actin-GAL4). These transgenic flies (dark
544   grey) were then compared with their matched controls (light grey) using a larval toxicology assay against
545   Nitenpyram (left) or Spinosad (right). In each panel the x-axis represents pesticide dose and the y-axis represents
546   survival corrected with abbots correction. Error bars represent 95% confidence intervals generated by Schneider-
547   Orelli's correction.
548
549   Figure 8: Genetic rescue of *Drosophila* Mdr65KO with *AgP-gp*
550   Three different UAS-P-gp constructs expressing *Mdr49A* (Pink), *Mdr49B* (Purple) and *AgP-gp* (dark grey) were
551   expressed in a Mdr65KO background and tested on the pesticides nitenpyram (left) and spinosad (right). In each

552   panel the x-axis represents pesticide dose and the y-axis represents survival corrected with abbots correction.

553   Error bars represent 95% confidence intervals generated by Schneider-Orelli's correction. Although there is no

554   established statistical test for Schneider-Orelli's correction non-overlapping 95% confidence intervals are

555   considered a very conservative estimate and significance stars were included in this graph reflecting this.

556

557

558   Figure S1: P-gp staining of the *Drosophila* brain

559

560   The central nervous system was stained was stained for the wild-type control (Myo-GAL4; Top row) along with

561   Mdr65KO (2nd row down), Mdr49KO (3rd row down), Mdr50KO (bottom). Blue indicates nuclei while red

562   indicates the C219 P-gp signal.

563

564   Figure S2: P-gp staining of the *Drosophila* fat body

565

566   The fat body was stained was stained for the wild-type control (Myo-GAL4; Top row) along with Mdr65KO (2nd

567   row down), Mdr49KO (3rd row down), Mdr50KO (bottom). Blue indicates nuclei while red indicates the C219

568   P-gp signal.

569

570

571   Figure S3: P-gp staining of the *Drosophila* Malpighian tubule

572

573   The Malpighian tubule was stained was stained for the wild-type control (Myo-GAL4; Top row) along with

574   Mdr65KO (2nd row down), Mdr49KO (3rd row down), Mdr50KO (bottom). Blue indicates nuclei while red

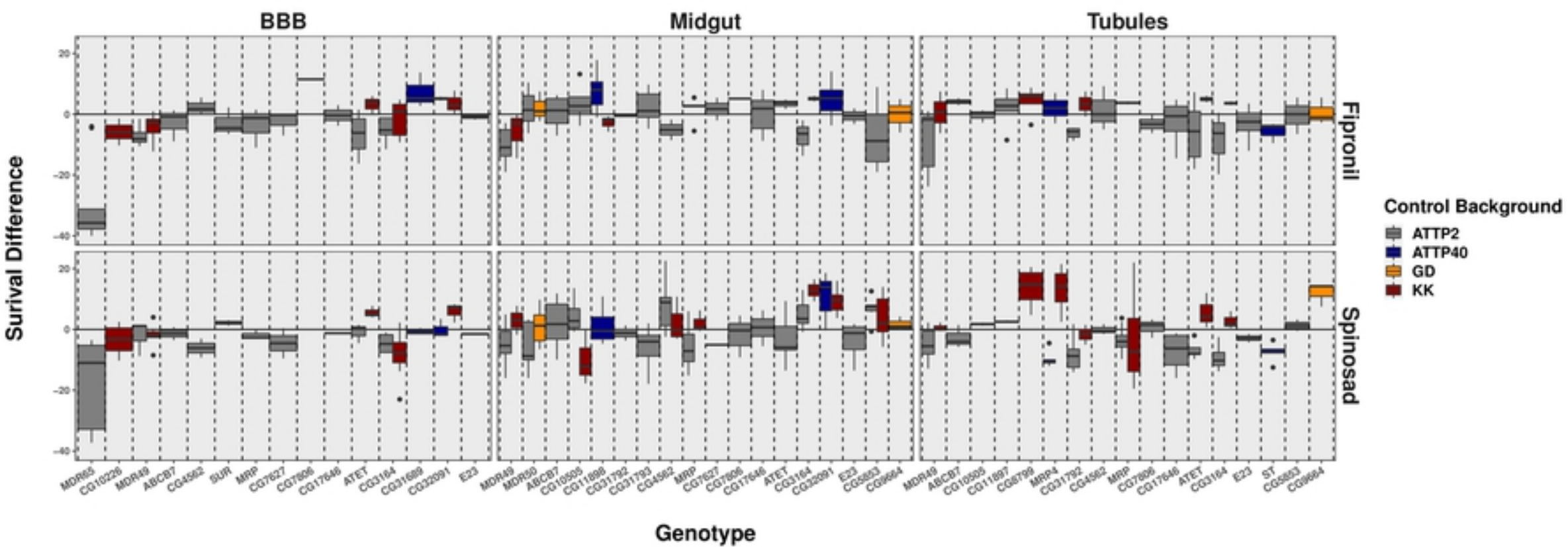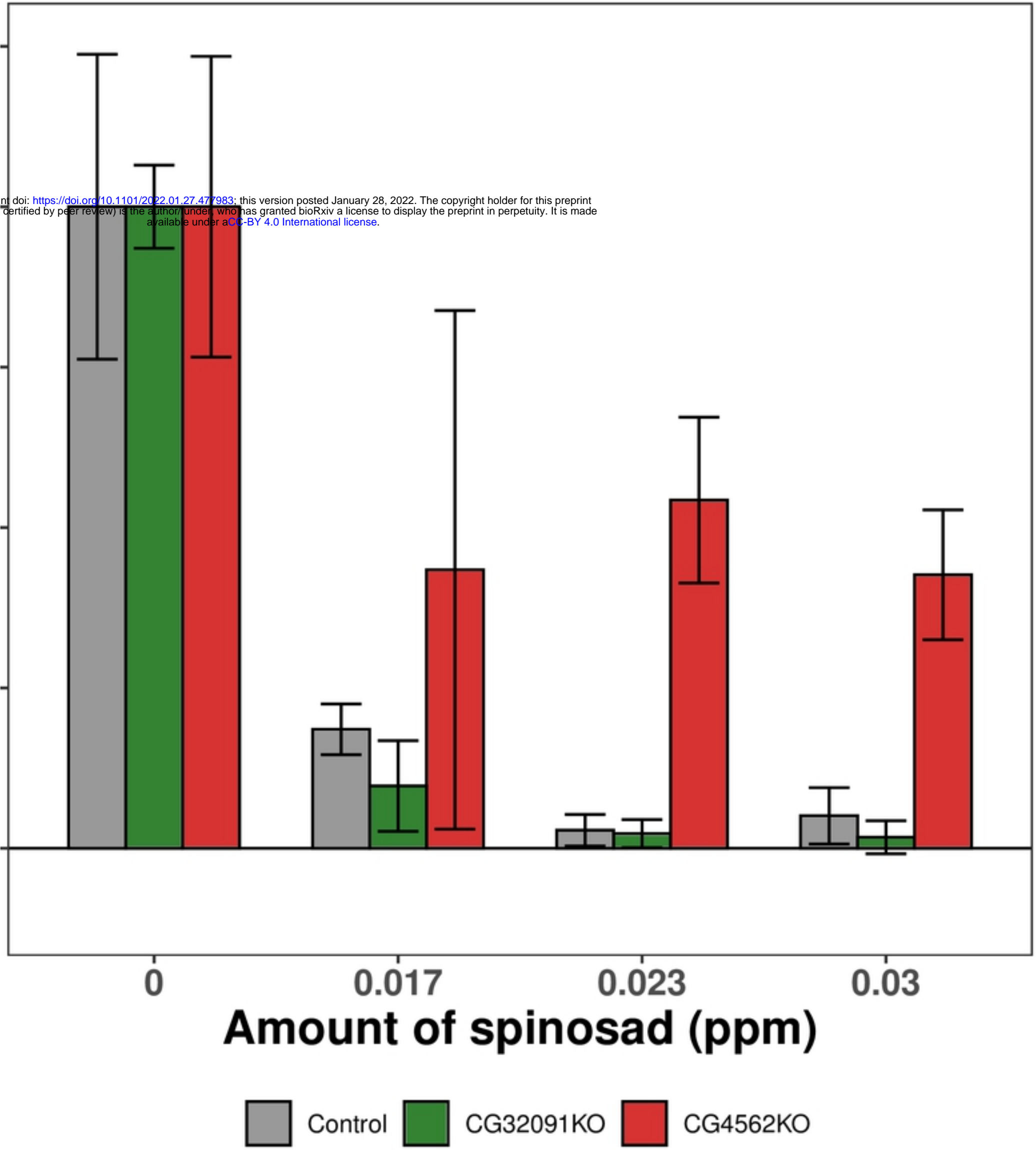575   indicates the C219 P-gp signal. Green indicates actin.
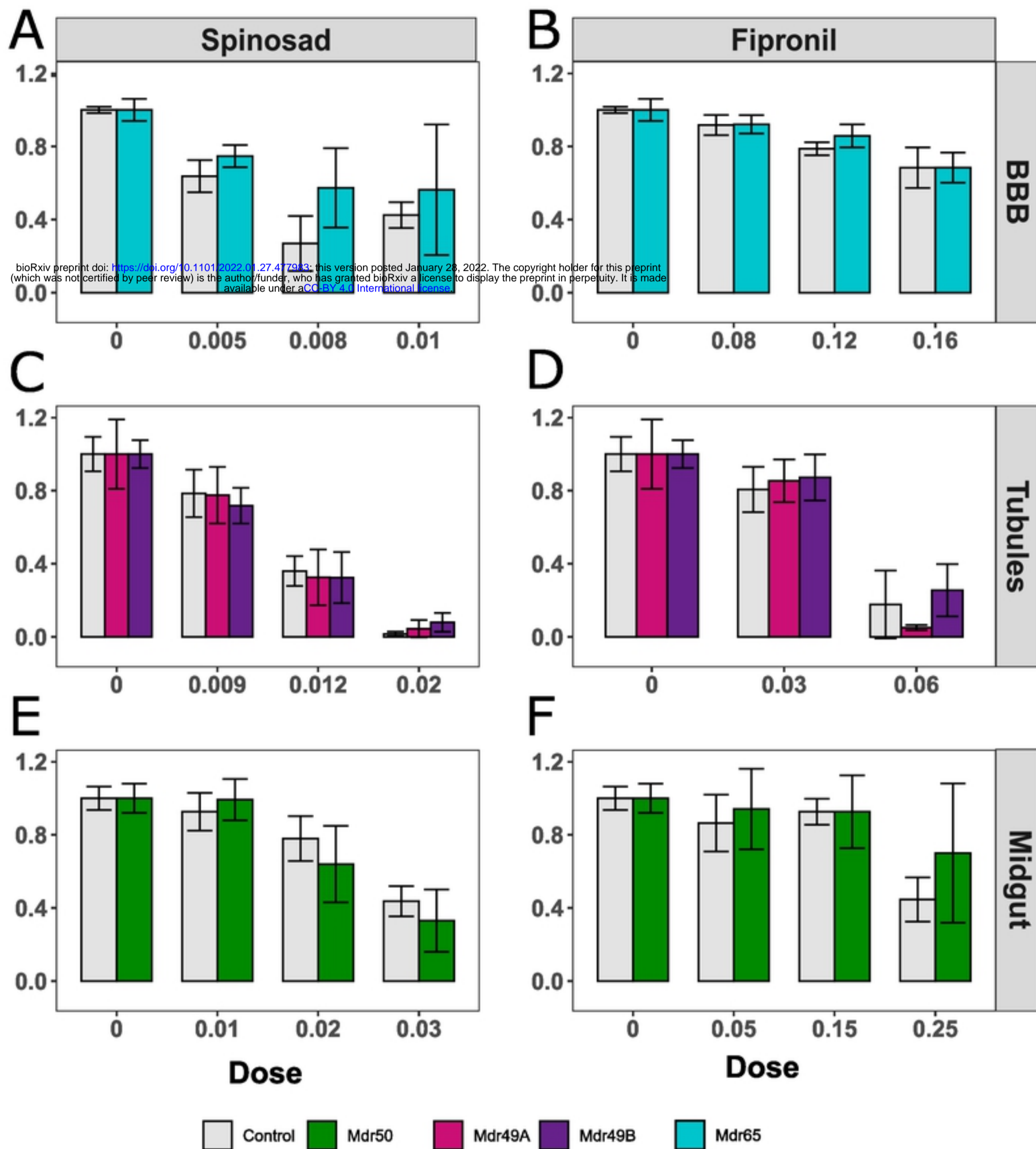
576

Figure 1

Figure 2

Figure 3

Figure 4

NUCLEI ACTIN C219

A Myo – GAL4

GC
HG MG
MT
100 µm
100 µm
MERGE 100 µm
100 µm
100 µm

B Mdr65KO

100 µm
MG
HG
MT
GC
100 µm
100 µm

C Mdr49KO

HG
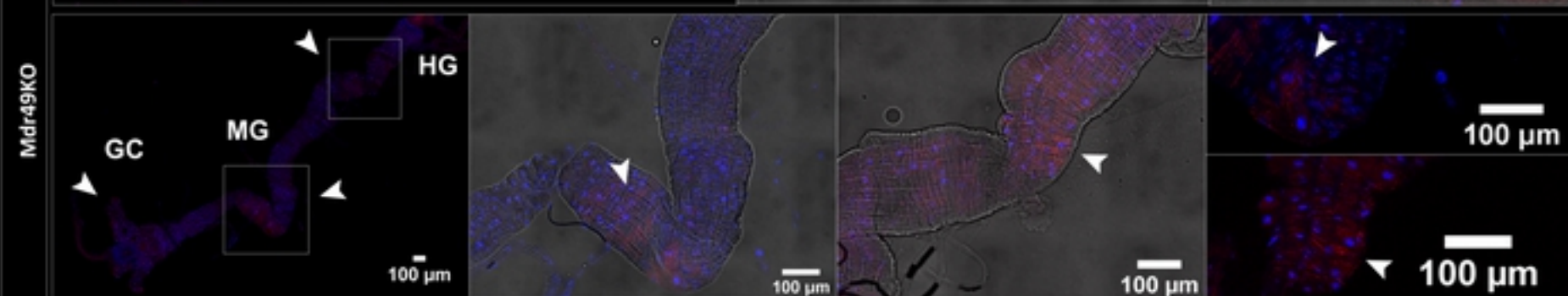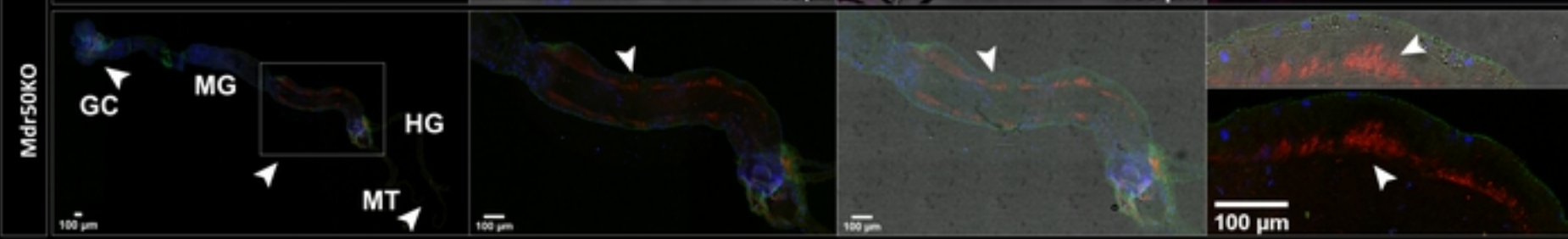GC MG
100 µm
100 µm
100 µm
100 µm
100 µm

D Mdr50KO

GC MG
HG
MT
100 µm
100 µm
100 µm
100 µm

Figure

Figure

Figure