1 **Quantitative tests of albendazole resistance in beta-tubulin mutants**

2 J.B. Collins[1a], Skyler A. Stone[2a], Emily J. Koury[2], Anna G. Paredes[2], Fiona Shao[2], Crystal Lovato[2], Michael
3 Chen[2], Richelle Shi[2], Anwyn Y. Li[2], Isa Candal[2], Khadija Al Moutaa[2], Nicolas Moya[1] and Erik C. Andersen[1,‡]
4
5 **Affiliations:**
6 1. Department of Biology, Johns Hopkins University, Baltimore, MD, 21218, USA
7 2. Department of Molecular Biosciences, Northwestern University, Evanston, IL 60208, USA
8
9 a. Equal contribution
10
11 **‡Corresponding author:**
12 Erik C. Andersen
13 Department of Biology
14 Johns Hopkins University
15 Bascom UTL 383
16 3400 North Charles St.
17 Baltimore, MD 21218
18 412-516-1282
19 erik.andersen@gmail.com
20

21 **Short Title: Albendazole resistance in beta-tubulin mutants**
22

23 **Conflicts of interest:** none
24

25

26 **Abstract:**

27   Benzimidazole (BZ) anthelmintics are among the most important treatments for parasitic nematode

28 infections in the developing world. Widespread BZ resistance in veterinary parasites and emerging resistance in

29 human parasites raise major concerns for the continued use of BZs. Knowledge of the mechanisms of resistance

30 is necessary to make informed treatment decisions and circumvent resistance. Benzimidazole resistance has

31 traditionally been associated with mutations and natural variants in the *C. elegans* beta-tubulin gene *ben-1* and

32 orthologs in parasitic species. However, variants in *ben-1* alone do not explain the differences in BZ responses

33 across parasite populations. Here, we examine the roles of five *C. elegans* beta-tubulin genes (*tbb-1*, *mec-7*,

34 *tbb-4*, *ben-1*, and *tbb-6*) to identify the role each gene plays in BZ response. We generated *C. elegans* strains

35 with a loss of each beta-tubulin gene, as well as strains with a loss of *tbb-1*, *mec-7*, *tbb-4*, or *tbb-6* in a genetic

36 background that also lacks *ben-1* to test beta-tubulin redundancy in BZ response. We found that only the

37 individual loss of *ben-1* conferred a substantial level of BZ resistance, although the loss of *tbb-1* was found to

38 confer a small benefit in the presence of albendazole (ABZ). The loss of *ben-1* was found to confer an almost

39 complete rescue of animal development in the presence of 30 µM ABZ, likely explaining why no additive effects

40 caused by the loss of a second beta-tubulin were observed. We demonstrate that *ben-1* is the only beta-tubulin

41 gene in *C. elegans* where loss confers substantial BZ resistance.

42

43 **Keywords:** beta-tubulin, benzimidazole, anthelmintic resistance, *C. elegans*

44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62

Highlights:

- Loss of *ben-1* provides almost complete rescue of development in albendazole (ABZ)

- Loss of different beta-tubulin genes does not confer ABZ resistance

- Loss of *ben-1* and a second beta-tubulin does not enhance the *ben-1* level of ABZ resistance

1. Introduction

Parasitic nematode infections are among the most common infectious diseases of humans and pose significant health and socioeconomic risks for endemic regions. Upwards of 1.5 billion individuals are estimated to be infected with at least one parasitic nematode species globally, with infections causing anemia, impaired cognitive development, reduced growth, diarrheal disease, intestinal obstructions, and lymph edema (Salikin et al., 2020). Anti-helminth drugs, or anthelmintics, are used in endemic areas to control infections and limit adverse health effects caused by parasitic nematodes. Anthelmintics are often delivered through mass drug administration (MDA) programs designed to deliver essential medicines to regions with infected populations.

One of the most common anthelmintics delivered in MDA programs is albendazole (ABZ), a drug belonging to the benzimidazole (BZ) class of anthelmintics. The BZ drug class is included in many MDA programs because of its broad-spectrum activity, capable of treating a wide variety of intestinal helminths, as well as being safe and affordable to easily deliver to large populations (Banerjee et al., 2023). Studies of the mode of BZ action have found that they inhibit the polymerization of microtubules by targeting beta-tubulin (Hastie and Georgopoulos, 1971; Sheir-Neiss et al., 1978). A study of BZ response in the free-living model nematode *Caenorhabditis elegans* found that larvae exposed to BZs were developmentally impaired and uncoordinated in locomotion (Chalfie and Thomson, 1982). Subsequent experiments showed that animals with loss-of-function mutations in the beta-tubulin gene *ben-1* were found to exhibit wild-type growth and movement in the presence of BZs (Driscoll et al., 1989). Wild-type growth, despite the loss of *ben-1*, is thought to be possible because another beta-tubulin gene acts redundantly and compensates for the loss of *ben-1*. The *C. elegans* genome contains five additional beta-tubulin genes (*tbb-1, tbb-2, mec-7, tbb-4,* and *tbb-6)* that are differentially expressed in various tissues and are thought to supply beta-tubulin function when *ben-1* is lost (Hurd, 2018).

Orthologs of *ben-1* were found to be the target of BZs in parasitic nematodes. A beta-tubulin gene (*tbb-isotype-1*) from *Haemonchus contortus*, a small-ruminant parasite, was found to rescue BZ susceptibility when expressed in a *C. elegans* strain that lacked *ben-1* (Kwa et al., 1995, 1994, 1993). Unlike *C. elegans,* the *H. contortus* genome contains only four genes encoding beta-tubulins (*tbb-isotype-1*, *tbb-isotype-2*, *tbb-isotype-3*, and *tbb-isotype-4*). A smaller complement of beta-tubulin genes, combined with expression differences between each of the four genes has led to the conclusion that loss of *tbb-isotype-1* likely causes lethality, indicating that BZ resistance in parasites is probably dependent on altered function variants in beta-tubulin. However, parasitic

118   nematodes currently lack the genetic tools, such as genome editing, to validate resistance genes using targeted

119   mutations. Exploration of anthelmintic resistance is dependent on *C. elegans* as a complement to research in

120   parasites, and a cycle of discovery has been proposed to explore and validate the mechanisms of BZ resistance

121   using both free-living and parasitic nematodes (Wit et al., 2021).

122   Anthelmintic resistance is a major concern in the control of parasites. Resistance to the BZ drug class

123   has become nearly ubiquitous in many nematode species of veterinary importance and is now an emerging

124   problem in nematode infections of humans (Howell et al., 2008; Kaplan, 2004; Krücken et al., 2017). The

125   development of resistance to BZs makes the control of infections difficult and costly. To address the emergence

126   of BZ resistance, it is necessary to understand the underlying genetics contributing to resistance. After suspected

127   resistance-associated variants are identified in parasites, they can be validated in *C. elegans* using CRISPR-

128   Cas9 genome editing. Studies of BZ resistance have identified non-synonymous variants at codons 134, 167,

129   198, and 200 of *ben-1* orthologs in parasites (Avramenko et al., 2019; Kwa et al., 1994; Mohammedsalih et al.,

130   2020; Venkatesan et al., 2023). Every known beta-tubulin variant associated with BZ resistance in parasitic

131   nematodes has been shown to cause resistance in *C. elegans* by the introduction of the variant into the *ben-1*

132   gene (Dilks et al., 2021, 2020; Kitchen et al., 2019; Kwa et al., 1994; Venkatesan et al., 2023). These variants in

133   parasite beta-tubulin genes are thought to alter a putative BZ binding site, preventing BZs from inhibiting beta-

134   tubulin, preserving the normal formation of microtubules, and allowing nematodes to survive and develop

135   normally in the presence of BZ treatment.

136   Despite the validation of variants in *ben-1* orthologs as a mechanism of resistance to BZs, *ben-1* is not

137   the only gene involved in BZ resistance. Genome-wide association studies in wild populations of *C. elegans*

138   have identified multiple genomic loci independent of *ben-1* that are associated with BZ resistance (Hahnel et al.,

139   2018; Zamanian et al., 2018). Fully understanding the genetics of resistance is necessary to inform strategic

140   decisions that improve the efficacy of existing treatments, as well as lead to the development of new treatments

141   and control strategies. Thus, it is imperative to identify all genes associated with BZ resistance. Here, we explore

142   the effects that loss of each beta-tubulin gene has on BZ resistance in *C. elegans*. The gene *ben-1* has been

143   extensively studied and confers the greatest level of BZ resistance. However, the roles of the other *C. elegans*

144   beta-tubulin genes (*tbb-1*, *tbb-2*, *mec-7*, *tbb-4*, and *tbb-6*) in BZ resistance are not well understood. We have

145   compared the effects of single gene deletions of each beta-tubulin gene on nematode development when

146   exposed to a single concentration of ABZ that previously has been found to confer a significant impact on the

147   development of the wild-type N2 strain of *C. elegans* (Dilks et al., 2021, 2020). We find that the loss of *ben-1*

148   conferred the highest level of resistance and the loss of *tbb-1* conferred moderate resistance. To test for genetic

149   redundancy among beta-tubulin genes, we used CRISPR-Cas9 genome editing to delete each beta-tubulin gene

150   in a genetic background that already has lost *ben-1* function. The loss of each beta-tubulin gene in the *ben-1*

151   deletion background did not confer a detectable change in ABZ resistance compared to the loss of *ben-1* alone.

152   Overall, we find that the loss of *ben-1* alone is sufficient to confer the maximum level of *C. elegans* ABZ resistance

153   at the concentration tested.

154

155   **2. Materials and Methods**

156   ***2.1 Generation of phylogeny of selected nematode beta-tubulins***

157   Five nematode species were selected to make a phylogenetic tree of beta-tubulins to observe levels of

158   conservation. All nematode species selected are Clade V nematodes as the association of *ben-1* orthologs with

159   BZ resistance has most often been validated in this clade. *C. elegans* and *Caenorhabditis briggsae* were selected

160   as two closely related free-living nematode species. *Pristionchus pacificus*, another free-living nematode, was

161   selected because of its high-quality genome and evolutionary divergence from *C. elegans*. Many parasite

162   genomes are relatively poor quality and lack detailed gene annotations, so we chose two parasite species with

163   well annotated genomes, the hookworm *Necator americanus* and *H. contortus*, to include in the phylogenetic

164   tree.

165   *Orthofinder* (Emms and Kelly, 2019) was used to identify beta-tubulin sequences (Supplementary Table

166   1) from each species. Data were obtained from the following sources: WormBase Parasite (WBPS18) (*H.

167   contortus*, *N. americanus*, *P. pacificus*), WormBase (WS279) (*C. elegans*), and from a previous publication (*C.

168   briggsae*) (Moya et al., 2023). Ortholog sequences were aligned using *Mafft,* and the phylogenetic tree was

169   generated and annotated using *IQTREE* (Katoh et al., 2002; Minh et al., 2020). *IQTREE* performs automatic

170   model selection. The selected model was LG+G4, which uses the LG model (Le and Gascuel, 2008) to examine

171   amino-acid exchange rates and a discrete gamma model with four categories (G4) (Yang, 1994) to examine

172   heterogeneity across amino acid sites. Branch support was estimated with 1000 iterations of ultrafast bootstrap

173   approximation (Minh et al., 2013). Putative clades were identified in the generated tree and colored by clade.

174

2.2 C. elegans strains and maintenance

Nematodes were grown on plates of modified nematode growth media (NGMA) containing 1% agar and 0.7% agarose and seeded with the *Escherichia coli* strain OP50 (Andersen et al., 2014). Plates were maintained at 20°C for the duration of all experiments. Before each assay, animals were grown for three generations to reduce the multigenerational effects of starvation.

CRISPR-Cas9-edited strains were generated as previously described (Dilks et al., 2020; Hahnel et al., 2018) (Supplementary File 2), except for VC364 *tbb-1(gk207)*, which was acquired from the *Caenorhabditis* Genetics Center (Minneapolis, MN). All single deletions were generated in the reference N2 genetic background. All double deletions were generated in the ECA882 *ben-1(ean64)* genetic background (Dilks et al., 2021, 2020). Progeny from injected animals (F1) were individually placed onto NGMA plates to reproduce and then sequenced using Sanger sequencing to confirm the presence of the desired edit. At least two generations of animals after single-animal passage were Sanger sequenced to confirm successful genome edits. Two independent edits of each strain were generated to control for any potential off-target effects caused by CRISPR-Cas9.

188

2.3 Nematode food preparation

The OP50 strain of *E. coli* was used as a nematode food source on NGMA plates. Bacterial food for the liquid-based high-throughput assay was prepared as previously described (Widmayer et al., 2022). Briefly, a frozen stock of the HB101 strain of *E. coli* was used to inoculate and grow a one liter culture at an OD_{600} value of 0.001. Six cultures containing one liter of pre-warmed 1x Horvitz Super Broth (HSB) and an OD_{600} inoculum grew for 15 hours at 37°C until cultures were in the late log growth phase. After 15 hours, flasks were removed from the incubator and transferred to 4°C to halt bacterial growth. Cultures were pelleted using centrifugation, the supernatant removed, and washed with K medium. Bacteria were resuspended in K medium, and the OD_{600} value was determined. The bacterial suspension was diluted to a final concentration of OD_{600}100 before being aliquoted to 30 mL and frozen at -80°C.

199

2.4 Albendazole stock preparation

201 A 100 µM stock solution of albendazole (Fluka, Catalog #: A4673-10G) was prepared in dimethyl

202 sulfoxide (DMSO), aliquoted, and stored at -20°C. A frozen ABZ aliquot was defrosted shortly before adding the

203 drug to the assay plates.

204

205 **2.5 High-throughput phenotyping assay (HTA)**

206 A previously described HTA was used for all ABZ response phenotyping assays (Shaver et al., 2023).

207 Two independent assays made up of three bleaches each were performed. Strains underwent three generations

208 of growth to control for any starvation effects and were then bleach synchronized in triplicate to control for

209 variation caused by bleach effects. Embryos were concentrated at 0.6 embryos/µL in 50 µL of K medium (Boyd

210 et al., 2012). A volume of 50 µL of the embryo solution was dispensed into each well of a 96-well plate. Both

211 DMSO and ABZ conditions contained 48 wells of N2 and ECA882, and 24 wells of each of the other tested

212 strains for each replicate bleach. Embryos were allowed to hatch overnight at 20°C with constant shaking at 180

213 rpm. The following morning, HB101 aliquots were thawed at room temperature, combined, and diluted to $OD_{600}30$

214 with K medium, and kanamycin was added at a concentration of 150 µM to inhibit further bacterial growth and

215 prevent contamination. The final well concentration of HB101 was $OD_{600}10$ and the final concentration of

216 kanamycin was 50 µM, and each well was treated with either 1% DMSO or 30 µM ABZ in 1% DMSO. Animals

217 were grown for 48 hours with constant shaking at 180 rpm, after which, animals were treated with 50 mM sodium

218 azide in M9 buffer to straighten and paralyze the animals for imaging. Following 10 minutes of exposure to

219 sodium azide, each plate was imaged using a Molecular Devices ImageXpress Nano microscope (Molecular

220 Devices, San Jose, CA) with a 2X objective (Shaver et al., 2023).

221 Independent assays included identical strain sets except as follows: Strains with a deletion of *tbb-2* were

222 found to be too developmentally delayed to use in these assays. The ECA3746 *ben-1(ean64)*; *mec-7(ean257)*

223 strain was removed from assay one because of an insufficient quantity of embryos after bleach synchronization.

224 Smaller significant effects on animal development were observed for some single deletions in control conditions

225 of assay one but not in assay two, indicating that significance assigned to the observed small effects could be

226 the result of high levels of replication, making even small differences significant.

227

228 **2.6 Data cleaning and analysis**

229   High-throughput   assay   images   were   processed   using   CellProfiler

230   (https://github.com/AndersenLab/CellProfiler). Processed image data were cleaned and processed using the

231   *easyXpress* (Nyaanga et al., 2021) R package as previously described (Shaver et al., 2024). The two assays

232   were cleaned and processed independently. All statistical comparisons and figure generation were performed in

233   R(4.1.2) (R Core Team, 2020). We used the *Rstatix* package *tukeyHSD* function on an ANOVA model generated

234   with the formula *phenotype ~ strain* to calculate differences in the responses of the strains. Figure 3 was

235   generated using data from assay one because of the large amount of variation shown in animal response for the

236   VC364 *tbb-1(gk207)* strain in assay two, thought to be caused by human error. Figure 4 was generated using

237   data from assay two, because of the loss of the ECA3746 strain in assay one. All data are presented in

238   supplemental figures.

239

240   **3. Results**

241   ***3.1 Beta-tubulins are well conserved among Clade V nematode species***

242   We wanted to determine how each of the six beta-tubulin genes from *C. elegans* were related to each

243   other, as well as to orthologs from other nematode species (Hurd, 2018). Phylogenetic analysis found five

244   putative clades of beta-tubulin proteins (Figure 1). *Caenorhabditis elegans tbb-1* and *tbb-2* share a common

245   clade with the *tbb-isotype-1* beta-tubulins from *H. contortus* and *N. americanus*. *Caenorhabditis elegans mec-7*

246   and *tbb-4* are in separate clades with *tbb-isotype-3* and *tbb-isotype-4* clustering with each gene, respectively.

247   The genes *ben-1* and *tbb-isotype-2* each cluster into separate clades. The gene *tbb-6*, a beta-tubulin unique to

248   *C. elegans,* could not be placed into the tree because of a high level of divergence. The high levels of

249   conservation of beta-tubulins among Clade V species highlight the ability to use *C. elegans* as a model system

250   to investigate the broad roles of beta-tubulins in BZ resistance across diverse nematode species.

251

252   ***3.2 The loss of ben-1 is the only beta-tubulin gene to confer high levels of ABZ resistance***

253   CRISPR-Cas9 genome editing was used to generate deletions of each beta-tubulin gene in the N2

254   laboratory strain genetic background (Figure 2). Edited strains with single deletions of each beta-tubulin gene

255   were phenotyped in DMSO and ABZ using a previously described high-throughput assay (HTA) that

256   quantitatively measures nematode development (Shaver et al., 2023; Widmayer et al., 2022). Briefly, strains

257   were bleach synchronized and embryos were titered into 96-well plates. The following day, arrested L1 larvae

258   were given OP50 *E. coli* with either 1% DMSO or 30 µM ABZ and 1% DMSO. Plates were incubated for 48 hours

259   at 20°C with constant shaking at 180 rpm. Animals were then treated with sodium azide and imaged to quantify

260   the lengths of each animal in each well of a 96-well plate. Median animal lengths were calculated from each well

261   of an assay plate and normalized across independent growths, plates, and bleaches. Deletion of each beta-

262   tubulin gene in the same genetic background enables the determination of the quantitative effects that each

263   gene has on BZ response, as well as to determine if the loss of each beta-tubulin gene impacts development in

264   control conditions. Median animal length after 48 hours of exposure was normalized to control conditions, and

265   then statistical comparisons were made between N2 and each strain. The loss of *tbb-1* had the most significant

266   impact on development in control conditions, indicating that the loss of *tbb-1* is detrimental (S Figs. 4,6). The

267   loss of *ben-1* was the only strain to confer high levels of resistance to ABZ, almost fully rescuing development

268   compared to control conditions (Figure 3, S Figs. 3,5). The loss of *tbb-1* was found to confer a moderate level of

269   resistance, with animal development significantly less affected than the wild-type strain but still heavily affected

270   by ABZ as compared to control conditions.

271

272   ***3.3 The loss of ben-1 confers the highest level of ABZ resistance compared to other beta-tubulin mutants***

273   To determine if other beta-tubulin genes play a redundant role in ABZ resistance with *ben-1*, we

274   generated individual deletions of *tbb-1*, *mec-7*, *tbb-4*, and *tbb-6* in the *ben-1(ean64)* genetic background. We

275   exposed these double beta-tubulin mutants to DMSO and ABZ in the same high-throughput development assay

276   described above to determine if the loss of a second beta-tubulin alters the levels of BZ resistance observed in

277   the single *ben-1* mutant. Similarly to the single deletion assay, small significant differences were observed for

278   multiple strains compared to the wild-type strain in control conditions, except for the strain ECA3628 *ben-*

279   *1(ean64)*; *tbb-4(ean282)* (S. Figs 8,10), which likely has off-target effects of gene editing that impacted growth

280   compared to the independently edited second strain. Small differences in the summarized median length reflect

281   differences in the developmental rate that could be caused by the combined effects of the loss of multiple beta-

282   tubulins. Strains with the loss of a second beta-tubulin were found to be equally resistant when compared to the

283   loss of *ben-1* alone (Figure 4, S Figs. 7,9). As previously noted, the loss of *ben-1* almost fully rescued

284 development at 30 μM ABZ compared to the control strain, possibly preventing any small effects conferred by

285 the loss of a second beta-tubulin from being observed.

286

287 **4. Discussion**

288   Despite the role beta-tubulin variants have in BZ resistance, the collective understanding of BZ resistance

289 comes from studies of *C. elegans ben-1* and orthologs in parasites. Fully understanding the mechanisms

290 underlying BZ resistance is imperative to the future of BZs as anthelmintic treatments. Here, we take an important

291 first step to test additional beta-tubulin genes in BZ resistance.

292

293 *4.1 ben-1 plays the largest role in ABZ resistance in C. elegans*

294   We examined the role that five of the six *C. elegans* beta-tubulin genes play in ABZ resistance by

295 generating strains with a loss of each gene, as well as strains with a loss of an additional beta-tubulin in a *ben-*

296 *1* mutant background. Because of detrimental effects on development, strains with a loss of *tbb-2* could not be

297 measured for responses to ABZ. Consistent with previous studies, the loss of *ben-1* was sufficient to confer the

298 maximum level of ABZ resistance, though it is important to note that the loss of *tbb-1* conferred a moderate level

299 of resistance. Loss of a second beta-tubulin in a strain with a loss of *ben-1* did not confer a detectable

300 enhancement of resistance. However, we can not definitively conclude if any other beta-tubulin gene acts

301 redundantly with *ben-1* in ABZ resistance. The assay that we used to measure ABZ resistance uses one

302 concentration that previously was found to differentiate susceptible strains from *ben-1* mutant strains (Dilks et

303 al., 2021, 2020). It remains possible that enhancement of ABZ resistance could be detected at higher ABZ

304 concentrations where the single contribution of *ben-1* might not be sufficient to cause resistance alone. Another

305 caveat is that only a single trait, development, was measured. ABZ affects multiple traits, including fecundity and

306 competitive fitness over multiple generations (Shaver et al., 2024). Future studies should investigate multiple

307 traits at different ABZ concentrations to fully understand the role of all beta-tubulin genes in the ABZ response.

308

309 *4.2 BZ resistance is complicated by differences in beta-tubulin copy number, levels of expression, and*

310 *resistance alleles*

311   We tested the role of each beta-tubulin gene in ABZ response by deleting much of the coding sequence.

312   Therefore, these results are binary for the presence or absence of each beta-tubulin gene. Amino-acid altering

313   variants from parasites have been validated in ABZ resistance using *C. elegans* and shown to cause ABZ

314   resistance equivalent to a strain with a loss of *ben-1* (Dilks et al., 2021, 2020; Venkatesan et al., 2023). However,

315   these variants likely do not cause loss of *tbb-isotype-1* function in parasites (Saunders et al., 2013). What could

316   be causing this discrepancy between loss-of-function variants in *C. elegans* and potential altered function

317   variants in parasitic nematodes? In species with highly expressed beta-tubulin genes that have BZ-sensitive

318   alleles, loss-of-function alleles would cause fitness defects, similar to what we see with *tbb-1* and *tbb-2* (Figure

319   4). In these species, benzimidazole resistance must be mediated by altered function variants. In species with

320   less highly expressed (or tissue-specific) beta-tubulin genes that have BZ-sensitive alleles, loss-of-function

321   alleles could cause BZ resistance because other beta-tubulin genes can substitute for essential functions, similar

322   to what we see with *ben-1* (Hurd, 2018). Interestingly, the *H. contortus* beta-tubulin gene *tbb-isotype-2* is shown

323   to be equally related to *tbb-isotype-1* and *ben–1*, and loss-of-function alleles of this gene have been documented

324   in some highly resistant *H. contortus* populations (Saunders et al., 2013). Additionally, the phenotypic

325   classification of BZ-resistance phenotypes differs between these two species and can be explained by

326   differences in loss-of-function vs. altered function mutations. In *C. elegans* where *ben-1* variants or mutations

327   can cause loss of function, the BZ-resistance phenotype is recessive (Dilks et al., 2021). By contrast, putative

328   BZ-resistance alleles in *H. contortus* are hypothesized to cause dominant BZ resistance (Silvestre et al., 2001).

329

330   Beyond coding variants or mutations in beta-tubulin genes, changes in the levels and tissue-specific

331   expression can alter BZ resistance. Previously, we found that some *C. elegans* wild strains with clear ABZ

332   resistance do not have variants that alter the coding sequence of *ben-1* but instead have much lower expression

333   levels of *ben-1* as compared to the rest of the population (Zhang et al., 2022). These strains are resistant because

334   the susceptible beta-tubulin protein is not expressed. Additionally, we found that the expression of *ben-1* in

335   cholinergic neurons alone is sufficient to confer susceptibility to ABZ (Gibson et al., 2022), highlighting that

336   variants modifying expression in specific tissues could confer resistance in a unique way independent of the

337   beta-tubulin coding sequence. These observations from both *C. elegans* and *H. contortus* demonstrate that more

338   attention should be paid to the number of beta-tubulin genes, their levels of expression, the sites of expression,

339 and the putative BZ-resistance alleles found in each beta-tubulin gene. To definitively understand BZ resistance

340 mediated by beta-tubulin genes, we must also drastically improve parasitic nematode genomes and gene models

341 because most species lack full descriptions of their beta-tubulin complement.

342

343 **5. Future directions**

344 The role of *ben-1* and *tbb-isotype-1* beta-tubulins in BZ resistance has been thought to be similar and

345 has established *C. elegans* as an essential model for parasite BZ resistance research. However, BZ treatment

346 is typically fatal in susceptible parasites (Prichard, 1988), as well as documented ovicidal effects of BZs against

347 parasite embryos (Boes et al., 1998). Conversely, the same effects are not typically seen in *C. elegans* where

348 the most significant impact is often on the developmental rate (Shaver et al., 2022). The loss of *tbb-1* or *tbb-2*

349 was deleterious and loss-of-function mutations in either gene would likely be rapidly selected against in the wild

350 (*i.e.*, no variants are observed in natural *C. elegans* strains) (Crombie et al., 2024), similarly to the predicted loss

351 of *tbb-isotype-1*. It is important to note that *tbb-1* and *tbb-2* have known resistance alleles at amino acid position

352 200, and future studies should edit both genes to make them harbor BZ-sensitive alleles to more closely

353 approximate the beta-tubulin complement and alleles in *H. contortus.* Such studies could offer an improved

354 model system for investigating BZ resistance. However, studies of BZ resistance need to investigate variants

355 beyond single amino-acid alterations. Our results demonstrate that a variety of factors such as copy number,

356 expression, and tissue-specific function can all affect BZ resistance. To continue to broaden our understanding

357 of BZ resistance, we must expand to a whole-genome approach that investigates variants across every single

358 beta-tubulin gene and beyond that single class of genes.

359

360

Data Availability

All code and data are openly available at https://github.com/AndersenLab/2024_beta_tubulin_manuscript

References

Andersen, E.C., Bloom, J.S., Gerke, J.P., Kruglyak, L., 2014. A variant in the neuropeptide receptor npr-1 is a major determinant of Caenorhabditis elegans growth and physiology. PLoS Genet. 10, e1004156.

Avramenko, R.W., Redman, E.M., Melville, L., Bartley, Y., Wit, J., Queiroz, C., Bartley, D.J., Gilleard, J.S., 2019. Deep amplicon sequencing as a powerful new tool to screen for sequence polymorphisms associated with anthelmintic resistance in parasitic nematode populations. Int. J. Parasitol. 49, 13–26.

Banerjee, S., Mukherjee, S., Nath, P., Mukherjee, A., Mukherjee, S., Ashok Kumar, S.K., De, S., Banerjee, S., 2023. A critical review of benzimidazole: Sky-high objectives towards the lead molecule to predict the future in medicinal chemistry. Results in Chemistry 6, 101013.

Boes, J., Eriksen, L., Nansen, P., 1998. Embryonation and infectivity of Ascaris suum eggs isolated from worms expelled by pigs treated with albendazole , pyrantel pamoate, ivermectin or piperazine dihydrochloride. Vet. Parasitol. 75, 181–190.

Boyd, W.A., Smith, M.V., Freedman, J.H., 2012. Caenorhabditis elegans as a Model in Developmental Toxicology, in: Harris, C., Hansen, J.M. (Eds.), Developmental Toxicology: Methods and Protocols. Humana Press, Totowa, NJ, pp. 15–24.

Chalfie, M., Thomson, J.N., 1982. Structural and functional diversity in the neuronal microtubules of Caenorhabditis elegans. J. Cell Biol. 93, 15–23.

Crombie, T.A., McKeown, R., Moya, N.D., Evans, K.S., Widmayer, S.J., LaGrassa, V., Roman, N., Tursunova, O., Zhang, G., Gibson, S.B., Buchanan, C.M., Roberto, N.M., Vieira, R., Tanny, R.E., Andersen, E.C., 2024. CaeNDR, the Caenorhabditis Natural Diversity Resource. Nucleic Acids Res. 52, D850–D858.

Dilks, C.M., Hahnel, S.R., Sheng, Q., Long, L., McGrath, P.T., Andersen, E.C., 2020. Quantitative benzimidazole resistance and fitness effects of parasitic nematode beta-tubulin alleles. Int. J. Parasitol. Drugs Drug Resist. 14, 28–36.

Dilks, C.M., Koury, E.J., Buchanan, C.M., Andersen, E.C., 2021. Newly identified parasitic nematode beta-tubulin alleles confer resistance to benzimidazoles. Int. J. Parasitol. Drugs Drug Resist. 17, 168–175.

401 Driscoll, M., Dean, E., Reilly, E., Bergholz, E., Chalfie, M., 1989. Genetic and molecular analysis of a
402   Caenorhabditis elegans beta-tubulin that conveys benzimidazole sensitivity. J. Cell Biol. 109, 2993–3003.
403 Emms, D.M., Kelly, S., 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics.
404   Genome Biol. 20, 238.
405 Gibson, S.B., Ness-Cohn, E., Andersen, E.C., 2022. Benzimidazoles cause lethality by inhibiting the function of
406   Caenorhabditis elegans neuronal beta-tubulin. Int. J. Parasitol. Drugs Drug Resist. 20, 89–96.
407 Hahnel, S.R., Zdraljevic, S., Rodriguez, B.C., Zhao, Y., McGrath, P.T., Andersen, E.C., 2018. Extreme allelic
408   heterogeneity at a Caenorhabditis elegans beta-tubulin locus explains natural resistance to
409   benzimidazoles. PLoS Pathog. 14, e1007226.
410 Hastie, A.C., Georgopoulos, S.G., 1971. Mutational resistance to fungitoxic benzimidazole derivatives in
411   Aspergillus nidulans. J. Gen. Microbiol. 67, 371–373.
412 Howell, S.B., Burke, J.M., Miller, J.E., Terrill, T.H., Valencia, E., Williams, M.J., Williamson, L.H., Zajac, A.M.,
413   Kaplan, R.M., 2008. Prevalence of anthelmintic resistance on sheep and goat farms in the southeastern
414   United States. J. Am. Vet. Med. Assoc. 233, 1913–1919.
415 Hurd, D.D., 2018. Tubulins in C. elegans. WormBook.
416 Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol.
417   20, 477–481.
418 Katoh, K., Misawa, K., Kuma, K.-I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence
419   alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066.
420 Kitchen, S., Ratnappan, R., Han, S., Leasure, C., Grill, E., Iqbal, Z., Granger, O., O'Halloran, D.M., Hawdon,
421   J.M., 2019. Isolation and characterization of a naturally occurring multidrug-resistant strain of the canine
422   hookworm, Ancylostoma caninum. Int. J. Parasitol. 49, 397–406.
423 Krücken, J., Fraundorfer, K., Mugisha, J.C., Ramünke, S., Sifft, K.C., Geus, D., Habarugira, F., Ndoli, J.,
424   Sendegeya, A., Mukampunga, C., Bayingana, C., Aebischer, T., Demeler, J., Gahutu, J.B., Mockenhaupt,
425   F.P., von Samson-Himmelstjerna, G., 2017. Reduced efficacy of albendazole against Ascaris lumbricoides
426   in Rwandan schoolchildren. Int. J. Parasitol. Drugs Drug Resist. 7, 262–271.
427 Kwa, M.S., Kooyman, F.N., Boersema, J.H., Roos, M.H., 1993. Effect of selection for benzimidazole resistance
428   in Haemonchus contortus on beta-tubulin isotype 1 and isotype 2 genes. Biochem. Biophys. Res.
429   Commun. 191, 413–419.
430 Kwa, M.S., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated
431   with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol. Biochem. Parasitol. 63, 299–
432   303.
433 Kwa, M.S., Veenstra, J.G., Van Dijk, M., Roos, M.H., 1995. Beta-tubulin genes from the parasitic nematode
434   Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. J. Mol. Biol. 246, 500–510.
435 Le, S.Q., Gascuel, O., 2008. An improved general amino acid replacement matrix. Mol. Biol. Evol. 25, 1307–
436   1320.
437 Minh, B.Q., Nguyen, M.A.T., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol.
438   Biol. Evol. 30, 1188–1195.
439 Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., Lanfear, R.,
440   2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol.
441   Biol. Evol. 37, 1530–1534.
442 Mohammedsalih, K.M., Krücken, J., Khalafalla, A., Bashar, A., Juma, F.-R., Abakar, A., Abdalmalaik, A.A.H.,
443   Coles, G., von Samson-Himmelstjerna, G., 2020. New codon 198 β-tubulin polymorphisms in highly
444   benzimidazole resistant Haemonchus contortus from goats in three different states in Sudan. Parasit.
445   Vectors 13, 114.
446 Moya, N.D., Stevens, L., Miller, I.R., Sokol, C.E., Galindo, J.L., Bardas, A.D., Koh, E.S.H., Rozenich, J., Yeo,
447   C., Xu, M., Andersen, E.C., 2023. Novel and improved Caenorhabditis briggsae gene models generated
448   by community curation. bioRxiv. https://doi.org/10.1101/2023.05.16.541014
449 Nyaanga, J., Crombie, T.A., Widmayer, S.J., Andersen, E.C., 2021. easyXpress: An R package to analyze and
450   visualize high-throughput C. elegans microscopy data generated using CellProfiler. PLoS One 16,
451   e0252000.
452 Prichard, R.K., 1988. Anthelmintics and control. Vet. Parasitol. 27, 97–109.
453 R Core Team, 2020. R: A Language and Environment for Statistical Computing.
454 Salikin, N.H., Nappi, J., Majzoub, M.E., Egan, S., 2020. Combating Parasitic Nematode Infections, Newly
455   Discovered Antinematode Compounds from Marine Epiphytic Bacteria. Microorganisms 8.
456   https://doi.org/10.3390/microorganisms8121963

457 Saunders, G.I., Wasmuth, J.D., Beech, R., Laing, R., Hunt, M., Naghra, H., Cotton, J.A., Berriman, M., Britton,
458   C., Gilleard, J.S., 2013. Characterization and comparative analysis of the complete Haemonchus
459   contortus β-tubulin gene family and implications for benzimidazole resistance in strongylid nematodes. Int.
460   J. Parasitol. 43, 465–475.
461 Shaver, A.O., Miller, I.R., Schaye, E.S., Moya, N.D., Collins, J.B., Wit, J., Blanco, A.H., Shao, F.M., Andersen,
462   E.J., Khan, S.A., Paredes, G., Andersen, E.C., 2024. Quantifying the fitness effects of resistance alleles
463   with and without anthelmintic selection pressure using Caenorhabditis elegans. bioRxiv.
464   https://doi.org/10.1101/2024.02.01.578300
465 Shaver, A.O., Wit, J., Dilks, C.M., Crombie, T.A., Li, H., Aroian, R.V., Andersen, E.C., 2023. Variation in
466   anthelmintic responses are driven by genetic differences among diverse C. elegans wild strains. PLoS
467   Pathog. 19, e1011285.
468 Shaver, A.O., Wit, J., Dilks, C.M., Crombie, T.A., Li, H., Aroian, R.V., Andersen, E.C., 2022. Variation in
469   anthelmintic responses are driven by genetic differences among diverse C. elegans wild strains. bioRxiv.
470   https://doi.org/10.1101/2022.11.26.518036
471 Sheir-Neiss, G., Lai, M.H., Morris, N.R., 1978. Identification of a gene for beta-tubulin in Aspergillus nidulans.
472   Cell 15, 639–647.
473 Silvestre, A., Cabaret, J., Humbert, J.F., 2001. Effect of benzimidazole under-dosing on the resistant allele
474   frequency in Teladorsagia circumcincta (Nematoda). Parasitology 123, 103–111.
475 Venkatesan, A., Jimenez Castro, P.D., Morosetti, A., Horvath, H., Chen, R., Redman, E., Dunn, K., Collins,
476   J.B., Fraser, J.S., Andersen, E.C., Kaplan, R.M., Gilleard, J.S., 2023. Molecular evidence of widespread
477   benzimidazole drug resistance in Ancylostoma caninum from domestic dogs throughout the USA and
478   discovery of a novel β-tubulin benzimidazole resistance mutation. PLoS Pathog. 19, e1011146.
479 Widmayer, S.J., Crombie, T.A., Nyaanga, J.N., Evans, K.S., Andersen, E.C., 2022. C. elegans toxicant
480   responses vary among genetically diverse individuals. Toxicology 479, 153292.
481 Wit, J., Dilks, C.M., Andersen, E.C., 2021. Complementary Approaches with Free-living and Parasitic
482   Nematodes to Understanding Anthelmintic Resistance. Trends Parasitol. 37, 240–250.
483 Yang, Z., 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over
484   sites: approximate methods. J. Mol. Evol. 39, 306–314.
485 Zamanian, M., Cook, D.E., Zdraljevic, S., Brady, S.C., Lee, D., Lee, J., Andersen, E.C., 2018. Discovery of
486   genomic intervals that underlie nematode responses to benzimidazoles. PLoS Negl. Trop. Dis. 12,
487   e0006368.
488 Zhang, G., Roberto, N.M., Lee, D., Hahnel, S.R., Andersen, E.C., 2022. The impact of species-wide gene
489   expression variation on Caenorhabditis elegans complex traits. Nat. Commun. 13, 3462.

490

491 **Legends to Figures**
492 **Figure 1. Phylogenetic relationship of nematode beta-tubulins.** Beta-tubulin gene models from three free-
493 living nematodes, *Caenorhabditis elegans* (*C.e.*), *C. briggsae* (*C.b.*), and *Pristionchus pacificus* (*P.p.*), and two
494 parasitic nematodes, *Haemonchus contortus* (*H.c.*) and *Necator americanus* (*N.a.*), were used to generate a
495 tree showing the relationship between beta-tubulin genes. Branches are colored by putatively assigned clades.
496 Sequence data were obtained from the following sources: WormBase Parasite (WBPS18) (*H. contortus*, *N.
497 americanus*, *P. pacificus*), WormBase (WS279) (*C. elegans*), and from a previous publication (*C. briggsae*)
498 (Moya et al. 2023).

499

500 **Figure 2. Gene models and locations of deletion alleles generated in *C. elegans* beta-tubulin genes.** Gene
501 models of the longest isoforms are presented for each *C. elegans* beta-tubulin gene, with exons (orange), introns
502 (gray lines), and untranscribed regions (gray boxes) shown. Regions that were deleted using CRISPR-Cas9
503 genome editing are shown as black lines under each model. Deleted regions of *tbb-1* are shown as two black
504 lines because strains with two independent deletion alleles were used. Gene model data were obtained from
505 WormBase (WS279).

506

507 **Figure 3. Only loss of *ben-1* causes resistance to ABZ.** Median animal lengths of strains grown in 30 µM ABZ
508 that have been regressed for bleach effects and then normalized to the mean of all median animal lengths from
509 the control condition are shown. Each point represents the summarized measurements of an individual well
510 containing five to 30 animals. Data are shown as box plots with the median as a solid horizontal line and the
511 75th and 25th quartiles on the top and bottom of the box, respectively. The top and bottom whiskers extend to

512 the maximum point within the 1.5 interquartile range from the 75th and 25th quartiles, respectively. Statistical
513 significance compared to the wild-type strain is shown above each strain ($p < 0.05$ = *, $p < 0.0001$ = ****, ANOVA
514 with Tukey HSD).

516 **Figure 4. None of the other beta-tubulin genes act redundantly with *ben-1* in ABZ response.** Median animal
517 lengths of strains grown in 30 μM ABZ that have been regressed for bleach effects and then normalized to the
518 mean of all median animal lengths from the control condition are shown. Each point represents the summarized
519 measurements of an individual well containing five to 30 animals. Data are shown as box plots with the median
520 as a solid horizontal line and the 75th and 25th quartiles on the top and bottom of the box, respectively. The top
521 and bottom whiskers extend to the maximum point within the 1.5 interquartile range from the 75th and 25th
522 quartiles, respectively. Statistical significance compared to the Δ*ben-1* strain is shown above each strain ($p <$
523 0.05 = *, $p < 0.0001$ = ****, ANOVA with Tukey HSD).

525 **Supplemental Figure 1. Distribution of raw animal lengths for each strain in assay two after exposure to**
526 **ABZ.** Raw median animal lengths, summarized by well, for each strain are shown for DMSO (0 μM) and ABZ
527 (30 μM) conditions. Wells are colored by the corresponding replicate bleach synchronization (red=1, green=2,
528 blue=3).

530 **Supplemental Figure 2. Distribution of raw animal lengths for each strain in assay one after exposure to**
531 **ABZ.** Raw median animal lengths, summarized by well, for each strain are shown for DMSO (0 μM) and ABZ
532 (30 μM) conditions. Wells are colored by the corresponding replicate bleach synchronization (red=1, green=2,
533 blue=3).

535 **Supplemental Figure 3. Only loss of *ben-1* causes ABZ resistance.** Median animal lengths of strains grown
536 in 30 μM ABZ that have been regressed for bleach effects and then normalized to the mean of all median animal
537 lengths from the control condition are shown. Each point represents the summarized measurements of an
538 individual well containing five to 30 animals. Data are shown as box plots with the median as a solid horizontal
539 line and the 75th and 25th quartiles on the top and bottom of the box, respectively. The top and bottom whiskers
540 extend to the maximum point within the 1.5 interquartile range from the 75th and 25th quartiles, respectively..
541 Statistical significance compared to the wild-type strain is shown above each strain ($p < 0.05$ = *, $p < 0.0001$ =
542 ****, ANOVA with Tukey HSD).

544 **Supplemental Figure 4. Loss of beta-tubulin genes affects animal lengths in control conditions.** Median
545 animal lengths of strains grown in 1% DMSO are shown. Each point represents the summarized measurements
546 of an individual well containing five to 30 animals. Data are shown as box plots with the median as a solid
547 horizontal line and the 75th and 25th quartiles on the top and bottom of the box, respectively. The top and bottom
548 whiskers extend to the maximum point within the 1.5 interquartile range from the 75th and 25th quartiles,
549 respectively. Statistical significance compared to the wild-type strain is shown above each strain ($p < 0.05$ = *, p
550 < 0.0001 = ****, ANOVA with Tukey HSD).

552 **Supplemental Figure 6. Loss of beta-tubulin genes affects animal lengths in control conditions.** Median
553 animal lengths of strains grown in 1% DMSO are shown. Each point represents the summarized measurements
554 of an individual well containing five to 30 animals. Data are shown as box plots with the median as a solid
555 horizontal line and the 75th and 25th quartiles on the top and bottom of the box, respectively. The top and bottom
556 whiskers extend to the maximum point within the 1.5 interquartile range from the 75th and 25th quartiles,
557 respectively. Statistical significance compared to the wild-type strain is shown above each strain ($p < 0.05$ = *, p
558 < 0.0001 = ****, ANOVA with Tukey HSD).

560 **Supplemental Figure 7. Additional loss of beta-tubulin genes in a Δ*ben-1* background did not confer a**
561 **detectable level of increased ABZ resistance.**Median animal lengths of strains grown in 30 μM ABZ that have
562 been regressed for bleach effects and then normalized to the mean of all median animal lengths from the control
563 condition are shown. Each point represents the summarized measurements of an individual well containing five
564 to 30 animals. Data are shown as box plots with the median as a solid horizontal line and the 75th and 25th
565 quartiles on the top and bottom of the box, respectively. The top and bottom whiskers extend to the maximum
566 point within the 1.5 interquartile range from the 75th and 25th quartiles, respectively. Statistical significance

567 compared to the Δ*ben-1* strain is shown above each strain ($p < 0.05 = *$, $p < 0.0001 = ****$, ANOVA with Tukey
568 HSD).
569

570 **Supplemental Figure 8. Loss of multiple beta-tubulins affects animal lengths in control conditions.**
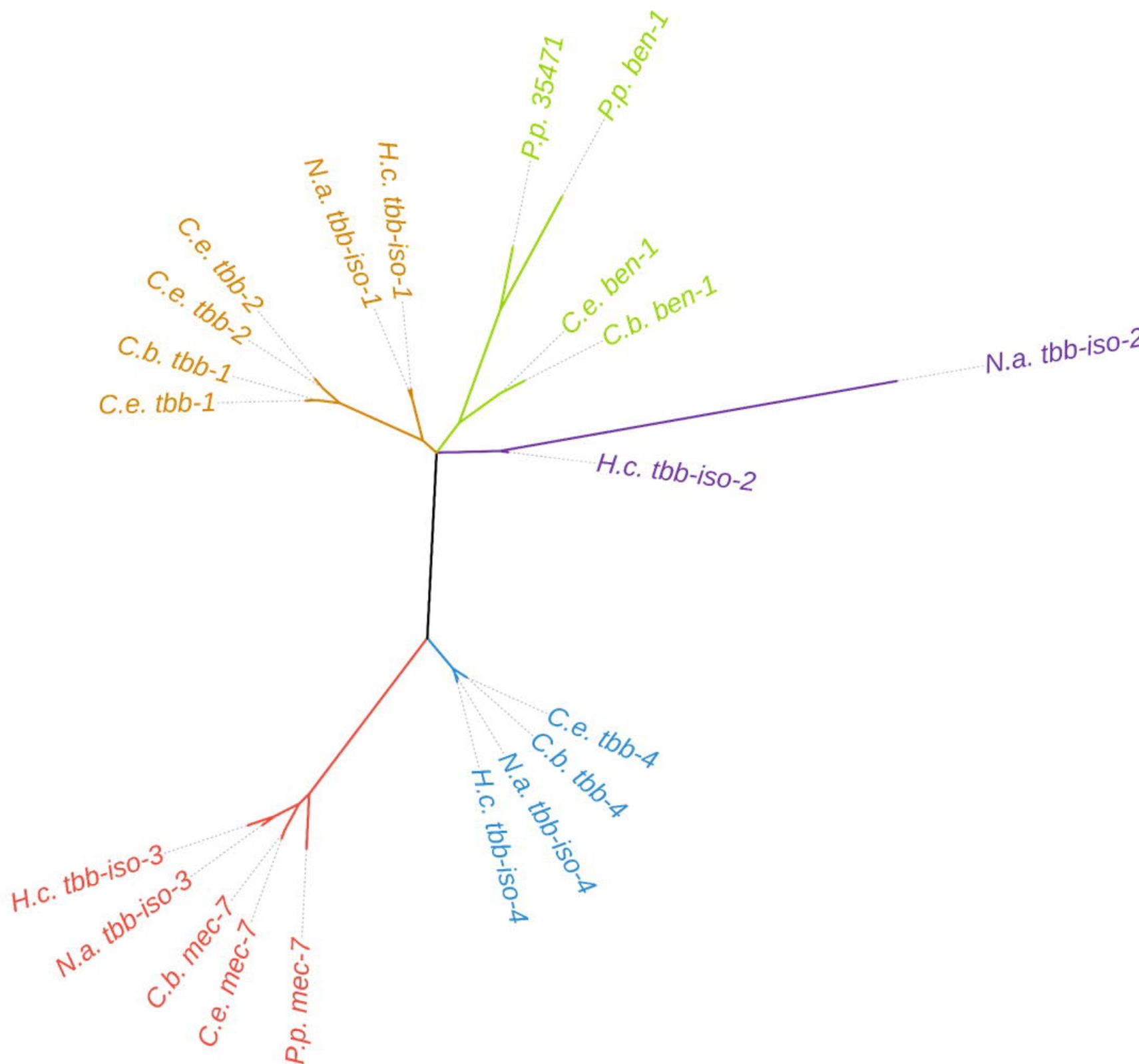571 Median animal lengths of strains grown in 1% DMSO are shown. Each point represents the summarized
572 measurements of an individual well containing five to 30 animals. Where applicable, data from both independent
573 edits in Assay 2 are shown. Data are shown as box plots with the median as a solid horizontal line, with the 75th
574 and 25th quartiles on the top and bottom of the box, respectively. The top and bottom whiskers extend to the
575 maximum point within the 1.5 interquartile range from the 75th and 25th quartiles, respectively. Statistical
576 significance compared to the wild-type strain is shown above each strain ($p < 0.05 = *$, $p < 0.001 = ***$, $p < 0.0001$
577 $= ****$, ANOVA with Tukey HSD).
578

579 **Supplemental Figure 9. Additional loss of beta-tubulin genes in a Δ*ben-1* background did not confer a**
580 **detectable level of increased ABZ resistance.** Median animal lengths of strains grown in 30 μM ABZ that have
581 been regressed for bleach effects and then normalized to the mean of all median animal lengths from the control
582 condition are shown. Each point represents the summarized measurements of an individual well containing five
583 to 30 animals. Data from both independent edits in Assay 1 are shown. Data are shown as box plots with the
584 median as a solid horizontal line, with the 75th and 25th quartiles on the top and bottom of the box, respectively.
585 The top and bottom whiskers extend to the maximum point within 1.5 interquartile range from the 75th and 25th
586 quartiles, respectively. Statistical significance compared to the Δ*ben-1* strain is shown above each strain ($p <$
587 $0.05 = *$, $p < 0.001 = ***$, $p < 0.0001 = ****$, ANOVA with Tukey HSD).
588

589 **Supplemental Figure 10. Loss of multiple beta-tubulin genes affects animal lengths in control conditions.**
590 Median animal lengths of strains grown in 1% DMSO are shown. Each point represents the summarized
591 measurements of an individual well containing five to 30 animals. Data are shown as box plots with the median
592 as a solid horizontal line and the 75th and 25th quartiles on the top and bottom of the box, respectively. The top
593 and bottom whiskers extend to the maximum point within the 1.5 interquartile range from the 75th and 25th
594 quartiles, respectively. Statistical significance compared to the wild-type strain is shown above each strain ($p <$
595 $0.05 = *$, $p < 0.001 = ***$, $p < 0.0001 = ****$, ANOVA with Tukey HSD).
596

Tree scale: 1

tbb-1

tbb-2

mec-7

tbb-4

ben-1

tbb-6

Transcript length (kb)

Median animal length