

Interactions of *Caenorhabditis elegans* β -tubulins with the microtubule inhibitor and anthelmintic drug albendazole

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Abstract

Parasitic nematodes are major human and agricultural pests, and benzimidazoles are amongst the most important broad-spectrum anthelmintic drug class used for their control. Benzimidazole resistance is now widespread in many species of parasitic nematodes in livestock globally and an emerging concern for the sustainable control of human soil-transmitted helminths. β -tubulin is the major benzimidazole target, although other genes may influence resistance. Among the 6 *Caenorhabditis elegans* β -tubulin genes, loss of *ben-1* causes resistance without other apparent defects. Here, we explored the genetics of *C. elegans* β -tubulin genes in relation to the response to the benzimidazole derivative albendazole. The most highly expressed β -tubulin isotypes, encoded by *tbb-1* and *tbb-2*, were known to be redundant with each other for viability, and their products are predicted not to bind benzimidazoles. We found that *tbb-2* mutants, and to a lesser extent *tbb-1* mutants, were hypersensitive to albendazole. The double mutant *tbb-2 ben-1* is uncoordinated and short, resembling the wild type exposed to albendazole, but the *tbb-1 ben-1* double mutant did not show the same phenotypes. These results suggest that *tbb-2* is a modifier of albendazole sensitivity. To better understand how BEN-1 mutates to cause benzimidazole resistance, we isolated mutants resistant to albendazole and found that 15 of 16 mutations occurred in the *ben-1* coding region. Mutations ranged from likely nulls to hypomorphs, and several corresponded to residues that cause resistance in other organisms. Null alleles of *ben-1* are albendazole-resistant and BEN-1 shows high sequence identity with tubulins from other organisms, suggesting that many amino acid changes could cause resistance. However, our results suggest that missense mutations conferring resistance are not evenly distributed across all possible conserved sites. Independent of their roles in benzimidazole resistance, *tbb-1* and *tbb-2* may have specialized functions as null mutants of *tbb-1* or *tbb-2* were cold or heat sensitive, respectively.

Keywords: benzimidazole; albendazole; *Caenorhabditis elegans*; drug resistance; tubulin; microtubules

Introduction

Parasitic nematodes are among the most common human pathogens and infect almost 2 billion people (World Health Organization 2015). Mass drug administration programs primarily use the benzimidazole (BZ) anthelmintic drug class to control infections and billions of doses have been dispensed, mainly to children (Becker et al. 2018). Unfortunately, previous use in livestock led to the evolution of resistance, which is now globally widespread for multiple parasitic nematode species of domestic animals (Kotze and Prichard 2016; Rose Vineer et al. 2020). Thus, resistance in human parasitic nematodes seems inevitable and is already emerging (Krücken et al. 2017; Schulz et al. 2018; Furtado, Medeiros, et al. 2019; Orr et al. 2019).

The complex life cycle of parasitic nematodes, including the requirement for obligate hosts, makes parasites difficult to study. The free-living nematode *Caenorhabditis elegans* has been used to

study the BZ mode of action (Spence et al. 1982; Stasiuk et al. 2019; Dilks et al. 2020, 2021; Hahnel et al. 2020; Wit et al. 2020). In classic work, Driscoll et al. (1989) screened *C. elegans* for mutants resistant to the BZ derivative benomyl and found that all 28 alleles occurred in the same gene, the β -tubulin *ben-1*. This result is consistent with the observation that BZs bind the β -tubulin subunit of microtubules and block polymerization (Friedman and Platzer 1980; Lacey and Prichard 1986; Lacey and Gill 1994; Aguayo-Ortiz et al. 2013). Several residues are thought to be involved in BZ binding, and these residues are mutated in β -tubulins of drug-resistant parasitic nematodes (Kwa et al. 1994, 1995; Redman et al. 2015; Kotze and Prichard 2016; Avramenko et al. 2019) and confer strong resistance when introduced into *C. elegans ben-1* using genome editing (Kitchen et al. 2019; Dilks et al. 2020, 2021).

β -tubulins are highly conserved among eukaryotes (Luduena 1998), and *ben-1* is one of 6 *C. elegans* β -tubulins (Hurd et al. 2010).

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The most highly and widely expressed β -tubulins in *C. elegans*, *tbb-1* and *tbb-2*, have the Y200 residue that is correlated with BZ resistance, but *ben-1* has the sensitive F200 amino acid. Although they differ in microtubule dynamics and their susceptibility to microtubule-severing enzymes, *tbb-1* and *tbb-2* act redundantly with each other for embryonic viability (Wright and Hunter 2003; Ellis et al. 2004; Lu et al. 2004; Honda et al. 2017). Other *C. elegans* β -tubulins, including *ben-1*, function primarily in the nervous system (Hurd 2018; Nishida et al. 2021).

Complete loss of *ben-1* confers BZ resistance has no detectable growth disadvantages on or off drug under laboratory conditions (Driscoll et al. 1989; Hahnel et al. 2018; Dilks et al. 2020, 2021). Indeed, many *C. elegans* wild isolates carry unique *ben-1* variants with no appreciable effects on fitness (Hahnel et al. 2018). In contrast, only a few point mutations appear to occur in resistant parasitic nematodes (primarily F167Y, E198A, and F200Y in the isotype-1 β -tubulin gene) although deletion of the isotype-2 β -tubulin in the small ruminant parasite *Haemonchus contortus* has also been observed in resistant isolates (Kotze and Prichard 2016; Avramenko et al. 2019). The limited number of mutations and the absence of clear loss-of-function mutations in the major resistance gene in parasitic nematodes, which is expected to be more frequent, imply that loss-of-function mutations would cause considerable fitness costs in the absence of drug (Wit et al. 2020). The reported resistant alleles likely retain function but no longer bind the BZ drugs. Although *C. elegans ben-1* is clearly the major target of BZ, other currently unknown genes can modify resistance in the field both in parasites and *C. elegans* (Zamanian et al. 2018; Furtado, de Aguiar, et al. 2019). Mutations in the *C. elegans* stress response, BZ uptake or metabolism modify BZ sensitivity (Jones et al. 2015; Fontaine and Choe 2018; Matoušková et al. 2018; Stasiuk et al. 2019).

Here, we explore the role of *C. elegans ben-1* and resistance to the BZ derivative albendazole (ABZ), particularly with respect to the major β -tubulin isotypes. We found that *ben-1* is redundant with *tbb-2* as double mutants are uncoordinated (Unc) and short in the absence of drug, phenotypes resembling wild-type animals exposed to ABZ. *tbb-2* mutants are more sensitive to ABZ than the wild type. By contrast, *tbb-1 ben-1* double mutants showed no obvious defects. These data indicate that *ben-1* and *tbb-2* are major mediators of ABZ sensitivity. As only one of the previously reported *ben-1* alleles (Driscoll et al. 1989) was available from the *Caenorhabditis* Genetics Center, we conducted a screen for ABZ-resistant mutants and found that 15 out of 16 mutations occurred in the *ben-1* coding region, consistent with *ben-1* being the major target of BZs in *C. elegans*. Surprisingly, although the BEN-1 sequence is highly conserved and protein nulls are fully resistant and viable, we found that ABZ missense mutations resistant to ABZ seemed to be biased toward a limited number of residues.

Materials and methods

Strains, growth conditions, and ABZ treatment

Strains were maintained at 15°C on NGM (nematode growth media) spread with the OP50 strain of *Escherichia coli* as the food source (Brenner 1974). Strains are listed in Supplementary Table 1 and information about genes can be found at WormBase. Hatch rates were determined for complete broods of 6 hermaphrodites as previously described (Mains et al. 1990). Double mutants were made using standard genetic procedures, often aided by linked morphological markers, which were removed before analysis. All tubulin genotypes were confirmed by PCR or sequencing.

ABZ (Sigma #A4673) was diluted to the appropriate concentration in dimethyl sulfoxide (DMSO) so that 20–50 μ l could be added to 60-cm Petri dishes containing 10 ml of NGM. This solution was quickly spread over the entire surface, and concentrations were calculated assuming uniform diffusion throughout the agar. After 1 day, plates were spread with OP50 bacteria, which was allowed to grow for 2 days at room temperature before storage of the plates at 4°C. In other reports, ABZ in DMSO is often added to cooled molten agar, but we found that this procedure often forms a precipitate. Although our effective concentration may not be comparable to plates made by adding ABZ to molten agar, or to liquid culture, our results were dose-dependent and reproducible even after many months of plate storage.

For measurements of larval growth, L4 hermaphrodites were transferred to the assay temperature and the next day, 15–50 gravid worms were moved to fresh NGM plates without drug (Supplementary Fig. 1). The plates were incubated for approximately 2 h at 25°C, approximately 3 h at 20°C, or approximately 7 h at 11°C to produce semisynchronous broods [for the temperature-sensitive mutations *tbb-2(qt1)*, animals laid embryos at the permissive temperature of 20°C to bypass the temperature-sensitive period, after which they were transferred to the restrictive temperatures of 25°C]. Approximately 30–70 eggs were then transferred with a platinum wire worm pick to plates with or without ABZ. Hatching rates were near 100% in the presence or absence of drug. Plates were incubated for the specified times, during which drug-free control animals grew to the L4 or young adult stage without hatching of the next generation, which would have made it difficult to identify arrested animals. Animal lengths were measured from photographs using ImageJ software (Schneider et al. 2012) using the segmented line tool to trace the center of the animal along the anterior/posterior axis. As we found that the effects of DMSO added to NGM had no detectable effects on growth in these assays (Supplementary Fig. 2a), we did not include DMSO in controls in these plate assays.

In addition to solid plate-based assays, we also performed a high-throughput phenotyping assay in response to ABZ (Supplementary Fig. 3; Dilks et al. 2020, 2021). In short, a small piece of NGM agar (NGMA) with a starved population of individuals was placed on a new 6-cm NGMA plate at 20°C (Andersen et al. 2014). After 2 days, gravid adults from these plates were spot bleached to remove contamination, and the next morning, L1 larvae were placed on new 6-cm NGMA plates. These individuals were then grown for 5 days when a large population of L4 larval individuals was present on the plates. Five L4s were then placed on new 6-cm NGMA plates with multiple replicate populations per strain. After 4 days of growth, plates were bleach synchronized and the embryos were diluted to approximately 1 embryo per microliter. Fifty microliters of this diluted embryo suspension was placed into each well of a 96-well plate. After these embryos hatched, these populations were then fed bacterial lysate (García-González et al. 2017) mixed with either ABZ in 1% DMSO or 1% DMSO alone. After 48 h of growth, images of each well of animals were taken using an ImageXpress Nano (Molecular Devices, San Jose, CA). Images were analyzed using the easyXpress package (Nyaanga et al. 2021), which facilitates the measurement of individual nematode sizes from images and calculates summary statistics for sizes of populations of nematodes. In these highly sensitive assays, the solvent DMSO has detectable effects *C. elegans* development, reproduction, and lifespan. Therefore, differences among strains within the control condition (DMSO alone) were controlled by subtracting the mean

control condition value from each drug condition replicate for each strain using a linear regression model ($drug_phenotype \sim mean_control_phenotype$). In this way, we addressed the differences among strains that were caused by the drug condition and the variation in the control condition does not affect the variation in the drug condition (Evans et al. 2021).

Screen for ABZ resistance

Ethyl methanesulfonate (EMS, Sigma) mutagenesis of the wild-type reference strain N2 (HR1988) was conducted as per Brenner (1974) using 40 μ M EMS for 4 h at room temperature. A number of approaches and ABZ concentrations were used and screens are summarized in Supplementary Table 2. Mutagenized animals were placed on plates without drug and groups of 20–30 F1 gravid adults, which contain putative homozygous resistant F2 embryos, were picked onto plates that ranged from 1.5 to 50 μ M ABZ at 20°C. In some screens, mutagenized animals were picked directly to ABZ plates and gravid F1 progeny were counted after a week. Wild-type *E. coli* (AMA1004, which grows into thicker lawns than OP50) was added to all plates after 1 week. The extra food allowed mutations with weaker ABZ resistance more time to outgrow their nonmutant siblings. Plates were screened for movement, or increased growth if we saw no movement defects (the latter yielded *sb156*). Resistant worms were transferred to fresh ABZ plates and only 1 strain, derived from a single animal, was retained per selection plate. As some screens were nonclonal, strains harboring identical *ben-1* DNA changes were deemed duplicates (2 pairs were found, *sb146* and *sb147*; *sb143* and *sb159*). Thirteen unique *ben-1* mutations and 1 mutation (*sb156*) with no changes in the *ben-1* coding sequence were found among 9,500 haploid genomes (Supplementary Tables 1 and 2). *sb144* was outcrossed 6 times, and *sb151* 5 times, *sb163*, 6 times and *sb164*, 1 time to remove background mutations.

Additional screens took advantage of the *tbb-2 ben-1* Unc phenotype that we report here, so screens for movement on ABZ should be biased against new *ben-1* alleles. HR2038 *tbb-2(gk129)* was mutagenized as described above (Supplementary Tables 1 and 2). This screen yielded 2 mutants among 10,100 haploid genomes in 1 screen and 5 mutants out of 51,560 haploid genomes in another screen (Supplementary Tables 1 and 2).

ben-1 DNA sequencing

ben-1 was amplified using Phusion Taq DNA polymerase (Thermo Scientific) and sequenced in 4 segments (Supplementary Fig. 4). After amplification, DNA was extracted for Sanger sequencing from the gel using Bioneer AccuPrep PCR/Gel Purification Kit. All sequencing were carried out at the University of Calgary Core DNA services.

Statistical analysis

Statistical analyses of agar plate-based studies were calculated in Prism software. The worm length data were compared to controls run on the same day using a 2-tailed Mann–Whitney Rank-Sum test because most data sets failed normality tests. High-throughput assay data were analyzed using the R statistical environment and comparisons were made using an ANOVA with a post hoc Tukey HSD test. All data and scripts are available on Github (https://github.com/AndersenLab/2021_Pallotto).

Results

To measure drug sensitivity, we scored larval lengths from semi-synchronized broods of embryos laid over a several-hour period,

followed by 3 days of growth on plates at 20°C (unless otherwise stated). Control animals were L4/young adults by this time, before any hatching of the F1 generation. We found that 7.5 μ M ABZ caused partial growth inhibition of the wild type so most of our experiments used this dose to detect either weak resistance or increased sensitivity (Supplementary Fig. S2b). We also found maximal differences between control and 7.5 μ M ABZ treatment of the wild type after 3 days postembryo laying (Supplementary Fig. S2c). We present much of our data in 2 parts. First, we normalize each strain to the average length of the wild-type run in parallel to see if mutations affect normal growth. Second, to compare relative drug sensitivities among strains, we normalize each drug-treated strain to that strain's average length on parallel, nondrug plates. This comparison controls for any growth impairments off drug. A value of 1.0 signifies complete resistance. This approach should be sufficient to qualitatively divide strains into 4 categories: resistant, partially resistant, sensitive, or hypersensitive. Unless otherwise stated, 2-tailed Mann–Whitney Rank-Sum tests are used to compare data. Data that are not normalized, showing absolute length in microns, are included in Supplementary Figs. for all experiments.

ben-1 is redundant with *tbb-2* but not *tbb-1*

ben-1 null alleles display no mutant phenotype other than complete resistance to ABZ (Driscoll et al. 1989; Hahnel et al. 2018; Dilks et al. 2020, 2021), indicating likely redundancy with other *C. elegans* β -tubulin genes. Previous work has shown that the maternal contributions of the major tubulin isotypes *tbb-1* and *tbb-2* are redundant with each other for embryonic viability (Wright and Hunter 2003; Ellis et al. 2004; Lu 2004). Therefore, we made double mutants with the canonical *ben-1(e1880)* mutation and null alleles of either *tbb-1* or *tbb-2*. Both tubulin mutations are deletions that remove the start codons. Notably, *tbb-2 ben-1* double mutants showed a short and Unc phenotype in the absence of ABZ, but each single mutant was wild type. Indeed, the double mutant resembled the wild-type strain when it is exposed to BZ drugs (Fig. 1a). This result was also recapitulated in length measurements after 3 days of growth (Fig. 1b, Supplementary Fig. 5a). The *tbb-2* and *ben-1* single mutants were, respectively, 0.91 and 0.93 the length of the wild-type controls run in parallel (Fig. 1b). If the mutations were additive, we expect the double mutant to be $0.91 \times 0.93 = 0.85$ of the wild type. The observed average length was 0.67, indicating a mutual enhancement ($P < 0.0001$). A similar effect was not seen for *tbb-1 ben-1* double mutants where the observed value was 0.80, which was higher than the predicted expected value of 0.72 and could indicate a weak suppression. The *tbb-2 ben-1* mutant phenotype and the phenocopy in the wild type after ABZ exposure suggest that TBB-2 and BEN-1 have redundant functions in the cells that are affected by ABZ.

Previous reports of redundancy between *tbb-1* and *tbb-2* were based on embryonic viability after depleting maternal stores (Wright and Hunter 2003; Ellis et al. 2004; Lu 2004). To determine if *ben-1* plays a role in early development, we compared the *tbb-1 tbb-2* double mutant to the *tbb-1 tbb-2 ben-1* triple mutant segregating from heterozygous mothers. Both the double and the triple mutant strains showed ~25% arrested growth after hatching. We also found little to no increase in embryonic lethality in the triple mutant with *ben-1*: 3.1% of the embryos of *tbb-1 tbb-2 ben-1/+* selfed mothers failed to hatch ($N = 1,564$) compared to 2.1% of unhatched embryos for *tbb-1 tbb-2/+* ($N = 1,356$). Therefore, *ben-1* has no essential zygotic role in the embryo.

We next asked how ABZ would affect β -tubulin mutants. The *ben-1(e1880)* (amino acid change G104D) and *ben-1(ean65)*

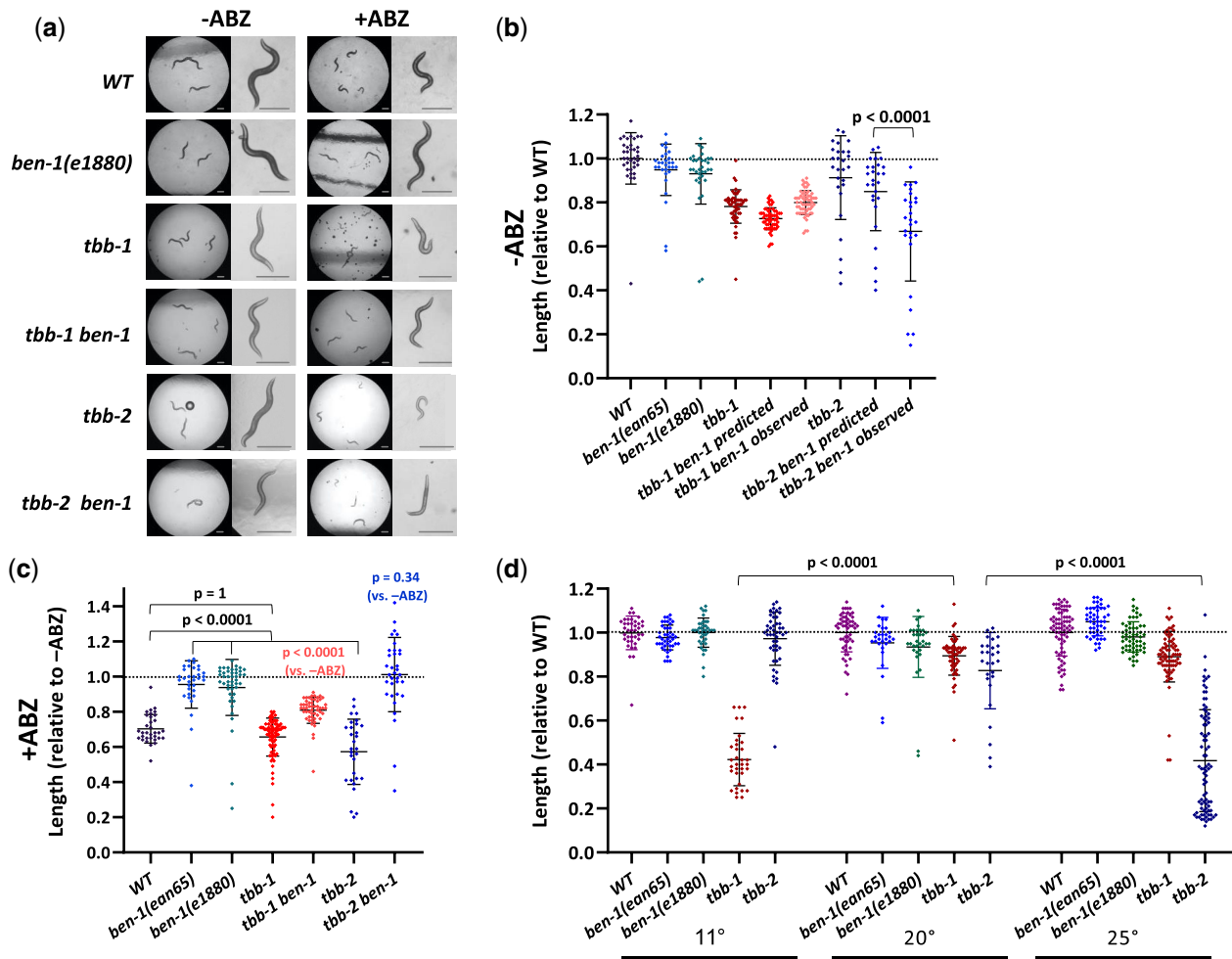


Fig. 1. Genetic interactions of β -tubulin genes. Unless otherwise stated, experiments represent 3 days of growth at 20°C, during which time controls grew to L4/Adulthood. a) Images of the effects of ABZ on strains at low and high magnification. *tbb-2* mutants may be hypersensitive to ABZ and *tbb-2 ben-1* double mutants grown in the absence of ABZ resemble the wild type exposed to drug. Scale bars = 250 μ m. The spots in some images are crystals that sometimes form on NGM. b) Worm lengths, in the absence of drug were normalized to the average length of the wild type. A representative wild-type sample is shown, but values of each strain were normalized to the wild type grown in parallel. The distribution of expected lengths of the double mutants of *tbb-1* and *tbb-2* with *ben-1(e1880)* was determined by multiplying each single mutant value by the average of *ben-1(e1880)*. The observed value *tbb-2 ben-1* double mutant was lower than expected. c) Effects of ABZ on tubulin mutants normalized to the average length of the same strains grown in parallel off drug. The *tbb-2* mutant appears more sensitive but was completely rescued (average near 1.0) by a mutation in *ben-1*. The *tbb-1* mutant showed normal sensitivity (but see Fig. 2) and only showed partial rescue of the *ben-1* defect. d) Although both *tbb-1* and *tbb-2* single mutants grew well at 20°C, the *tbb-1* mutant was cold sensitive and the *tbb-2* mutant was heat sensitive. Growth was measured after 8 days at 11°C, 3 days at 20°C, and 2 days at 25°C, during which times controls grew to L4/Adult. Note that the *tbb-1* and *tbb-2* 20°C data are that same as that used in (b). Mean and standard deviations are indicated. Two-tailed Mann-Whitney Rank-Sum test were used to calculate P-values. [Supplementary Fig. 5](#) contains raw data in microns.

(deletion of exons 2–4) mutant strains showed near-complete resistance compared to growth of the mutant off drug (Fig. 1c, [Supplementary Fig. 5a](#)). The *tbb-2* mutant strain was more sensitive to ABZ than the wild type ($P < 0.0001$), consistent with the observation that the *tbb-2 ben-1* double mutant showed mutant phenotypes off drug. Notably, *tbb-2 ben-1* growth was not affected by the addition of ABZ ($P = 0.34$) indicating that *ben-1* ABZ resistance is epistatic to *tbb-2* hypersensitivity. By contrast, the *tbb-1* mutant showed the same sensitivity to drug as the wild type ($P = 1$, but see below). The *tbb-1 ben-1* double mutant was still partially sensitive to ABZ ($P < 0.0001$).

High-throughput measurement of the ABZ response

We performed a high-throughput assay that is more sensitive than plate-based assays, to test how different combinations of tubulin mutations affect responses to ABZ. Populations of

nematodes were grown from the L1 larval stage for 48 h in both ABZ and DMSO (control) conditions to measure both the effects on ABZ responses and potential changes in normal growth conditions. In control conditions, we found that *ben-1(e1880)* mutant, the *tbb-1* deletion allele, and the double mutant grew more slowly than the wild type (Fig. 2a). There was no synergistic interaction of *tbb-1* with *ben-1(e1880)* as the double mutant grew as well as the *tbb-1* single mutant ($P = 0.54$, we were unable to perform this assay with the *tbb-2* deletion allele or this mutation in combination with *ben-1(e1880)* because any strains harboring the *tbb-2* were too sick and slow-growing for this assay). The differences between strains in control conditions, especially the strains containing *ben-1(e1880)*, were surprising because previous studies reported that the *ben-1(e1880)* allele did not cause noticeable growth defects on plates ([Driscoll et al. 1989](#)). However, the highly sensitive assays used here detected a significant difference ($P < 0.0001$, Tukey's HSD). Conversely, the *ben-1(ean65)* deletion

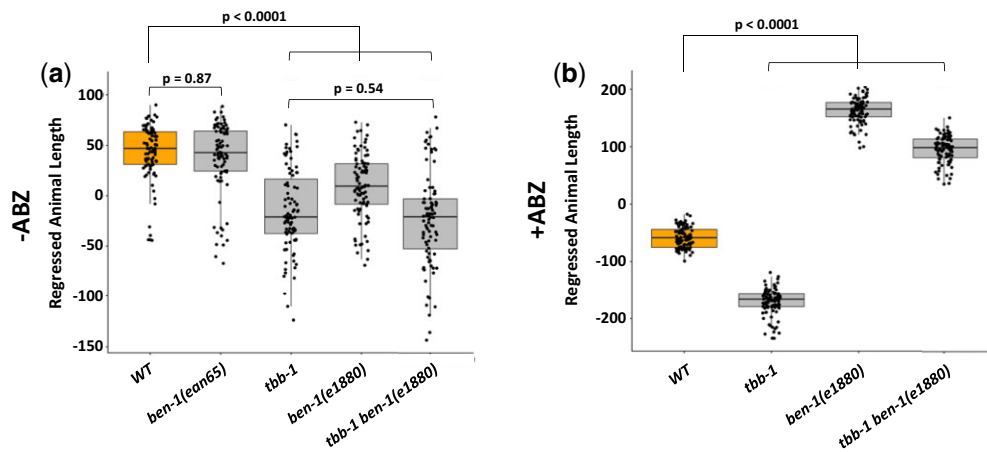


Fig. 2. High-throughput analysis of β -tubulin mutant allele combinations. Animals were grown in the absence (a) or presence (b) of ABZ. Strain names are shown on the x-axis with the allele shown under the box plot. Regressed median animal length of a population of animals is shown on the y-axis (see Materials and Methods). Each point represents the median animal length calculated from a well containing approximately 50 animals. Data are shown as Tukey's box plots with the median displayed as a horizontal line and the edges of the box representing the 25th and 75th quartiles. Whiskers are the extended 1.5 interquartile range. Tukey's HSD is used to calculate significance.

strain, which removes exons 2–4 created using targeted genome editing (Hahnel et al. 2018; Dilks et al. 2020), showed no changes in growth in this assay. This result suggests that the *ben-1(e1880)* strain likely has other mutations that affect fitness or the mutation has some neomorphic growth defects that can be revealed in liquid culture. In response to ABZ treatment, the *ben-1(e1880)* strain was the most resistant (Fig. 2b). The combination of *ben-1(e1880)* and the deletion of *tbb-1* was also highly resistant compared to the wild type ($P < 0.0001$, Tukey's HSD). The strain with the deletion of *tbb-1* alone was the most significantly ABZ-sensitive strain. We did not find increased *tbb-1* ABZ sensitivity relative to the wild type in our plate-based assay (Fig. 1c), indicating that the high-throughput method is likely more sensitive. Alternatively, the different environment of the liquid-based assays could create physiological conditions that sensitize the *tbb-1* mutant to the effects of the drug.

The *tbb-1* mutant is cold-sensitive and the *tbb-2* mutant is heat sensitive

Although *tbb-1* and *tbb-2* are redundant for viability, single mutants have different effects on tubulin dynamics in the early embryo and *tbb-2* also shows reduced hatching at 25°C compared to *tbb-1* (Wright and Hunter 2003; Ellis et al. 2004; Lu et al. 2004; Honda et al. 2017). *tbb-1* length measurements were less variable than those of *tbb-2* at 20°C, perhaps reflecting another difference (Fig. 1d, Supplementary Fig. 5). We examined temperature-dependent growth between the extremes of efficient *C. elegans* laboratory growth, 11°C and 25°C. We found that *tbb-1* was severely compromised at 11°C ($P < 0.0001$ compared to 20°C), and *tbb-2* showed the opposite pattern at 25°C ($P < 0.0001$ vs 20°C, Fig. 1d, Supplementary Fig. 5b). The greater variability of *tbb-2* than *tbb-1* at 20°C may reflect being near its restrictive temperature.

Screens for ABZ resistance

Null alleles of *ben-1*, including large deletions, lead to BZ resistance (Driscoll et al. 1989; Chen et al. 2013; Katic and Großhans 2013; Hahnel et al. 2018; Dilks et al. 2020). To understand the range of *ben-1* mutations that can cause ABZ resistance and to possibly uncover genes other than *ben-1* that contribute to resistance, we selected for ABZ-resistant mutants after mutagenesis

under a variety of conditions (Materials and Methods, Supplementary Table 2). In the first screen of 9,500 haploid genomes carried out with 7.5–50 μ M ABZ, we identified 13 independent mutants based on either movement or improved growth on drug. As discussed below, 12 had sequence changes in the coding region of *ben-1*. This result yields an aggregate forward mutation rate of 1/730 *ben-1* mutations/gamete, the same frequency as found by Driscoll et al. (1989). This rate is higher than the average mutation rate for *C. elegans* genes of 1/2,000 following standard EMS mutagenesis (Brenner 1974; Park and Horvitz 1986).

Only 1 mutation in the initial screen, *sb156*, lacked changes in the *ben-1* coding region (see below) and could represent an alternative target or modifier of ABZ resistance. To bias against additional mutations in *ben-1*, we carried out screens in the *tbb-2* mutant background. We reasoned that a new allele of *ben-1*, although able to grow on ABZ, would be Unc as found for the *ben-1 tbb-2* double mutant strain (Fig. 1, a and b). Therefore, we screened for movement, rather than either movement or growth as we did in the first screen (Driscoll et al. (1989) screened only for movement). Some of the animals were screened at a lower dose 1.5 μ M ABZ (the rest were screened at 7.5 μ M) because *tbb-2* mutants are more sensitive to the drug than the wild type (Fig. 1, a and c). These screens yielded 2 mutations, but at a much lower an aggregate rate of 1/5,050 per haploid genome, indicating the screen was indeed biased against frequent *ben-1* alleles. However, sequencing showed that both of these strains had *ben-1* mutations (see below). In the absence of drug, *ben-1(sb163) tbb-2* showed normal movement, while movement of *ben-1(sb164) tbb-2*, like the *tbb-2* double with the canonical allele *ben-1(e1880)*, was impaired. This demonstrates allele specificity of the *ben-1* alleles *sb163* and *sb164* with *tbb-2*. A third larger screen at 1.5–50 μ M ABZ yielded 5 mutations at the low rate of 1/10,310 per haploid genome, but again yielded no good candidates for non-*ben-1* mutations. All double mutant strains grew better than the parent *tbb-2* strain on 7.5 μ M ABZ over several generations but had only marginally better movement than the parent on ABZ, especially as young larvae. These mutants have not been sequenced but are likely *ben-1* alleles for several reasons. Like *ben-1 tbb-2* double mutants, they are Unc and short off the drug. After backcrossing, crossovers separating resistance from the Unc and short phenotypes were relatively rare, indicating linkage consistent with the

2 cM map distance between *ben-1* and *tbb-2*. Therefore, although the 2 screens in the *tbb-2* background were likely biased against *ben-1* mutations, our criterion for choosing mutants with improved movement may not have been sufficiently rigorous to exclude *ben-1* alleles. Alternatively, non-*ben-1* mutations leading to resistance in the *tbb-2* background may be rare.

Several of our *ben-1* mutants had slow growth in plate assays off drug relative to the wild-type parent strain run in parallel (Fig. 3a, strains are arranged in rank order of resistance found in Fig. 3b, Supplementary Fig. 6). As most strains were not outcrossed after mutagenesis (Materials and Methods, Supplementary Table 2), differences in un-outcrossed strains must be treated with caution. When growth of each strain on ABZ was normalized to its growth in the absence of drug to account for possible background effects, mutations varied from fully to partially resistant (Fig. 3b, note that *sb151* and *sb163*, which showed the lowest resistance, were backcrossed least 5 times). Driscoll et al. (1989) also found mutations with different levels of resistance when scored for paralysis. Although *sb156* (which lacks mutations in the *ben-1* coding sequence) outgrows the wild type after several generations, resistance is not seen after 3 days of growth at 7.5 μ M ABZ and animals are Unc on drug. Resistance in terms of

growth was seen in the 3-day growth assay at the lower dose of 1.5 μ M ($P = 0.005$, Fig. 3, c and d).

Sequence changes in ABZ resistance mutations

All mutant strains from the first 2 screens, except *sb156*, had sequence changes in the *ben-1* coding region (Fig. 4, Supplementary Table 1). Among these mutants, several of the *ben-1* mutations are likely nulls, including nonsense alleles (*sb152*, Q31Stop; *sb158* W344Stop; *sb154* Q375Stop) as well as a splice donor mutation (*sb153*, a stop occurs after 54 intron-encoded amino acids following amino acid 157). All other mutations are in codons that encode amino acids conserved between BEN-1, TBB-2, *H. contortus* ISO-1 (the gene mutated in BZ-resistant isolates of this ruminant parasite), and β -tubulins from *Drosophila*, human, and *Saccharomyces cerevisiae* (Fig. 4, Supplementary Fig. S7 shows mutations relative to the 6 *C. elegans* β -tubulins). We sequenced the canonical e1880 (G104D) allele (Driscoll et al. 1989) and found that it occurred at a different nucleotide but in the same codon as *sb145* (G104S). Meanwhile, another pair of mutations also had different changes at a shared codon, *sb144* (G142E) and *sb164* (G142R). Two mutations have changes reported in other organisms: *sb159* E198K is found in BZ-resistant fungi and parasitic

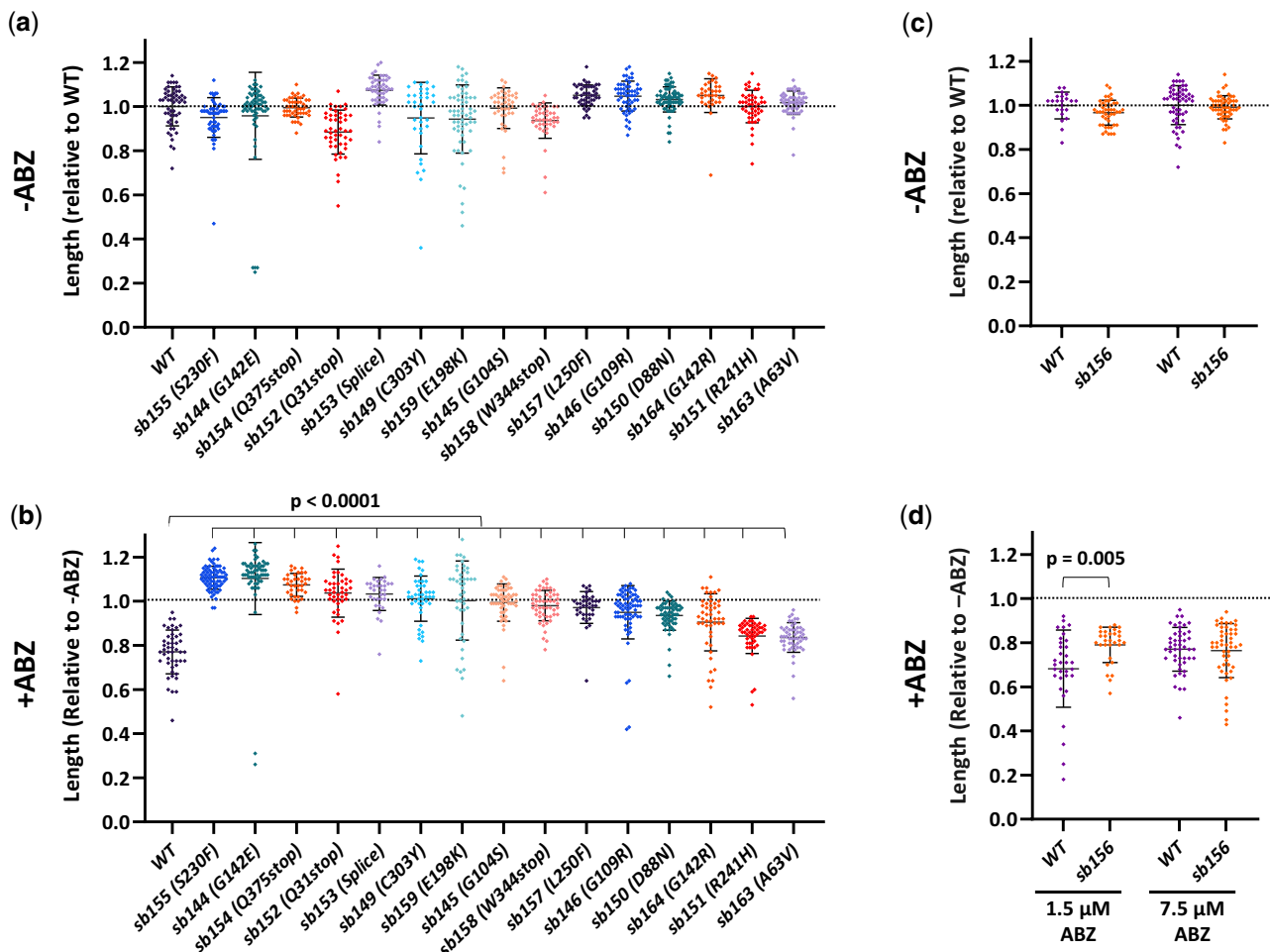


Fig. 3. Sensitivity of mutant strains to ABZ. Strains are presented in rank order of ABZ resistance after 3 days of growth at 20°C, during which time controls grew to L4/Adulthood. a) Growth of *ben-1* mutations relative to wild type cultured in parallel (a representative wild type is shown). b) Growth on 7.5 μ M ABZ was normalized to the average of the same strain grown in parallel in the absence of drug shown in (a). *sb151* and *sb163* showed the least resistance. c) Growth of the wild type and *sb156*, which does not have a lesion in the *ben-1* coding region, relative to the wild type. d) Assays run in parallel to (c) at the indicated levels of ABZ. *sb156* shows partial resistance at the lower dose. Two-tailed Mann-Whitney Rank-Sum test were used to calculate P-values. Mean and standard deviations are indicated. Supplementary Fig. 6 contains raw data in microns.

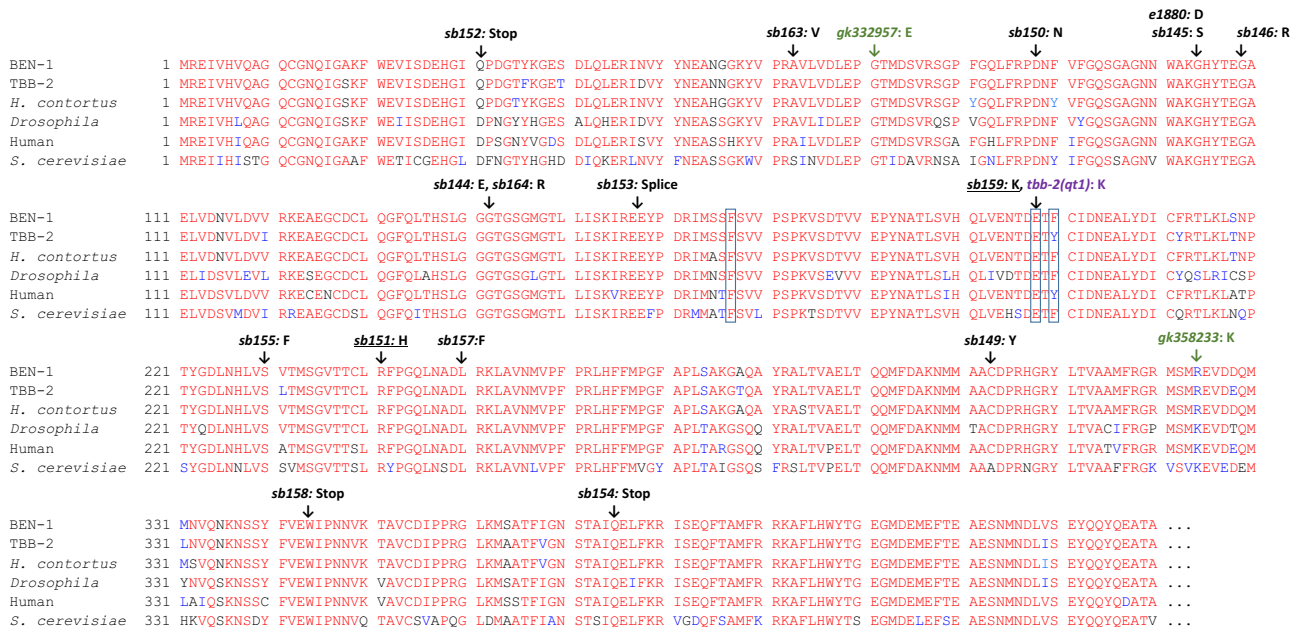


Fig. 4. Multiple sequence alignments showing location of ABZ-resistant mutants. BEN-1 is compared to *C. elegans* TBB-2 and β -tubulins from the ruminant parasite *H. contortus*, *Drosophila*, human and *S. cerevisiae*. Locations of mutations found in our screen are indicated in black along with the canonical allele *e1880*. Boxed residues indicate the positions most frequently mutated in parasites. Underlined alleles correspond to BZ-resistant mutants found in other organisms. Green represents alleles from the Million Mutant Project and purple denotes the change in *tbb-2(qt1)*. Sequences are truncated to exclude the nonconserved C-terminal regions. *H. contortus* iso-1 ACS29564.1, *Drosophila* NP_651606.2, human BAD96759.1, *S. cerevisiae* NP_116616.1. For alignments to the other *C. elegans* β -tubulins see [Supplementary Fig. 7](#).

nematodes and was recently found to be resistant when edited into *C. elegans ben-1* (Jung et al. 1992; Liu et al. 2014; Mohammedsalih et al. 2020; Dilks et al. 2021). The *sb151* R241H is found in benomyl-resistant *S. cerevisiae* mutants (Thomas et al. 1985). EMS induces GC to AT transitions and would have not induced *ben-1* mutations corresponding to the common parasite mutations F167Y, E198A, and F200Y (see Discussion).

Although our data are not precise enough to correlate small changes in resistance with particular structural changes, it is notable that *sb151* (R241H) and *sb163* (A63V) had the lowest levels of resistance (Fig. 3b), implying they retain some wild-type *ben-1* function. Each strain was outcrossed 5–6 times and grew well in the absence of drug, so the lack of full resistance is unlikely to come from background mutations induced by mutagen or any dominant-negative effects of the *ben-1* mutations. The alanine-to-valine of *sb163* is the most conservative change in our collection. Because *sb163* was non-Unc in combination with *tbb-2*, it likely retains some wild-type *ben-1* function. As mentioned above, the same R241H lesion seen in *sb151* is benomyl-resistant and cold-sensitive for growth in yeast. As *S. cerevisiae* has only a single β -tubulin gene, this mutation must retain wild-type function in yeast (Thomas et al. 1985). In analogy with the yeast R241H mutations, we tested *sb151* for cold sensitivity. Like *ben-1* null alleles, *sb151* had little effect on growth at 11°C, 20°C, or 25°C in the absence of drug (Fig. 5a, Supplementary Fig. 8a). If *sb151* compromises normal *ben-1* function more at lower temperatures, it should be more resistant and we found a slight increase of resistance at 11°C vs 25°C ($P < 0.0001$, Fig. 5b).

ABZ resistance in other β -tubulin mutant genes

The *C. elegans* genome encodes 3 other β -tubulin genes in addition to *ben-1*, *tbb-1*, and *tbb-2*. We tested 2 *ben-1* mutations generated by the Million Mutant Project (Thompson et al. 2013) that have amino acid changes found in other *C. elegans* wild-type β -tubulin genes

and so might be considered conservative substitutions that retain function (Fig. 4, Supplementary Fig. 7). These mutants were identified after random mutagenesis without subsequent selection for ABZ resistance. The *ben-1(gk332957)* G71E change is shared with the divergent β -tubulin TBB-6. This mutation nevertheless compromises but does not eliminate wild-type *ben-1* function as it shows partial resistance ($P < 0.0001$ vs the wild type, Fig. 5, c and d, Supplementary Fig. 8b). The *gk358233* R324K allele is likely a permissible change as it is found in fly, human, and yeast β -tubulins as well as TBB-4, TBB-6, and MEC-7 (Fig. 4, Supplementary Fig. 7). As expected for a functional protein, this allele was still sensitive to ABZ (Fig. 5, c and d, Supplementary Fig. 8b). Both of the Million Mutant Project alleles showed compromised growth off drug, but the strains were not outcrossed.

The *tbb-4*, *tbb-6*, and *mec-7* genes each encode the F200 residue that correlates with BZ sensitivity, but mutations in these genes are not found in wild ABZ-resistant strains (Hahnel et al. 2018). As *tbb-4* is expressed in some of the same neurons as *ben-1* (Hao et al. 2011; Nishida et al. 2021), we asked if loss of both *tbb-4* and *ben-1* would alter movement, similar to the *tbb-2 ben-1* double mutant. However, the double mutant was not Unc off of drug. The single *tbb-4* mutant was not hypersensitive to ABZ (Fig. 5e, f, $P = 0.44$ compared to the wild type exposed to ABZ, Supplementary Fig. 8c).

Although TBB-2 is predicted not to bind ABZ as it is Y200, the *qt1* allele has been implicated in benomyl resistance (Wright and Hunter 2003). This *tbb-2* mutation encodes the E198K change that is ABZ resistant when edited into *ben-1* (Dilks et al. 2021) and is present in the *ben-1(sb159)* allele from our screen. It is also found in BZ-resistant parasitic nematodes and fungi (Jung et al. 1992; Liu et al. 2014; Mohammedsalih et al. 2020). Wright and Hunter (2003) found that *tbb-2(qt1)* prevented embryonic spindle orientation defects caused by benomyl microtubule depolymerization at the first embryonic cleavage, particularly at higher temperatures. We found a small increase in resistance in *tbb-2(qt1)* in our

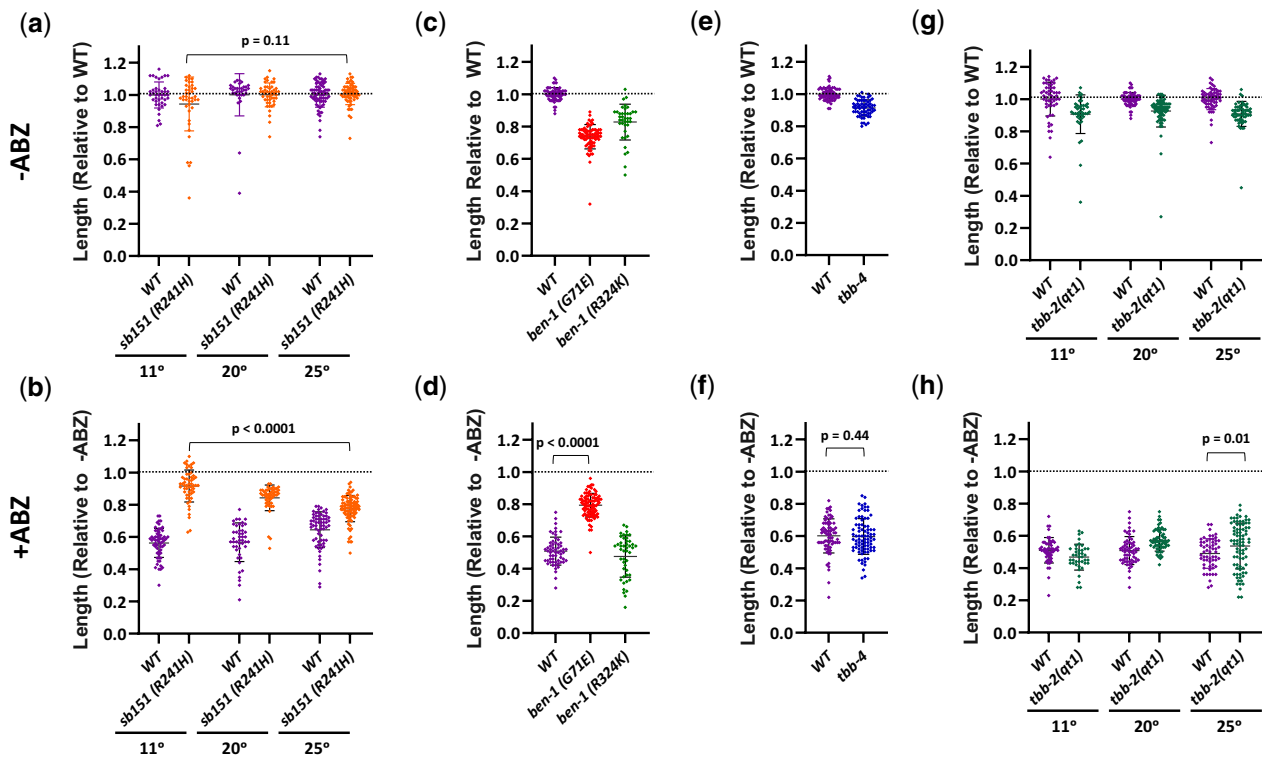


Fig. 5. Phenotypes of selected mutations in *ben-1* and other tubulin genes. Animals were measured after 8 days at 11°C, 3 days at 20°C, and 2 days at 25°C, during which time controls grew to L4/Adulthood. Unless otherwise indicated, experiments were performed at 20°C. Upper panels (a, c, e, and g) were grown in the absence of ABZ and were normalized to the wild-type run in parallel. Lower panels (b, d, f, and h) are the corresponding experiments grown on 7.5 μ M ABZ and are normalized to the strain grown in parallel in the absence of drug. a, b) Although the corresponding *S. cerevisiae* is cold-sensitive for growth, *sb151* was not. *sb151* did cause a slight reduction in *ben-1* function at 11°C as indicated by better growth on ABZ at the lower temperature. c, d) The *ben-1* G71E mutation (*gk332957*) from the Million Mutant Project showed partial resistance to ABZ but R324K (*gk358233*) did not, indicating that the latter mutation does not compromise function. e, f) Loss of *tbb-4*, which includes the sensitive F200 residue did not alter drug sensitivity. g, h) The temperature-sensitive mutation *tbb-2(qt1)* showed slight resistance at its restrictive temperature of 25°C. Two-tailed Mann-Whitney Rank-Sum test were used to calculate P-values. Mean and standard deviations are indicated. [Supplementary Fig. 8](#) contains raw data in microns.

growth assays at the restrictive temperature of 25°C ($P = 0.01$, [Fig. 5, e and f, Supplementary Fig. 8d](#)).

Discussion

The [World Health Organization \(2017\)](#) includes ABZ on its list of 100 Essential Medicines. Billions of ABZ doses have been administered for treatment of parasitic nematodes, mainly to children. Previous widespread use of BZ in agriculture caused the evolution of resistance, often rendering BZ drugs ineffective for a number of livestock parasitic nematode species. Resistance amongst human helminths seems highly likely and concerns are growing about its emergence ([Moser et al. 2017](#)). β -tubulins are the major target of BZ drugs in both fungi ([Thomas et al. 1985](#); [Jung et al. 1992](#); [Liu et al. 2014](#)) and nematodes ([Driscoll et al. 1989](#); [Kwa et al. 1995](#); [Wit et al. 2020](#)). To better understand the genetics of BZ resistance, we used *C. elegans* as a model. The β -tubulin *ben-1* gene was known to be the major target of the BZ class of drugs ([Driscoll et al. 1989](#); [Hahnel et al. 2018](#)). We explored genetic interactions of *ben-1* and ABZ with the major β -tubulin isotypes, *tbb-1* and *tbb-2*, and conducted forward genetic screens to examine the types of mutations that lead to ABZ resistance.

Interaction of *ben-1* with other β -tubulin genes

Assigning paralogous functions among β -tubulins within an organism, or inferring homology by descent of β -tubulins between

organisms, is problematic because of their slow rate of evolution. The exception to tubulin conservation occurs in the C-terminus, which shows little similarity between tubulin paralogs within a species or between tubulins from different species. A few specializations have been ascribed to these regions ([Hurd 2018](#)). Although mutations in *C. elegans ben-1* and *iso-1* of the ruminant parasite *H. contortus* both confer BZ resistance, which might imply homology, levels and cellular patterns of expression may be more critical to define shared functions than primary sequence ([Saunders et al. 2013](#)).

To better understand BZ resistance, we sought to clarify the functional relationships between *ben-1* and the major β -tubulin isotypes *tbb-1* and *tbb-2*. Unlike BEN-1, neither TBB-1 nor TBB-2 is predicted to bind BZ as they encode Y200 rather than the sensitive F200 residue. The *tbb-1* and *tbb-2* genes act redundantly with each other for viability ([Wright and Hunter 2003](#); [Ellis et al. 2004](#); [Lu et al. 2004](#)). Of these 2 genes, we found that *tbb-2* shows greater functional overlap with *ben-1*. In the absence of ABZ, *tbb-2* and *ben-1* are redundant for movement, body morphology, and growth ([Fig. 1](#)). For these phenotypes, the *tbb-2 ben-1* double mutant resembles the wild type exposed to ABZ. A simple model is that TBB-2 and BEN-1 are expressed in the cells responsible for the ABZ-induced phenotypes. Consistent with this observation, *tbb-2* mutants had a greater increase in ABZ sensitivity relative to the wild type than did loss of *tbb-1*: *tbb-2* sensitivity was apparent in the plate-based assay but *tbb-1* sensitivity was only seen in the

high-throughput assay, which was more sensitive and where physiological conditions might enhance ABZ sensitivity (Figs. 1 and 2). The phenotypic similarities between *tbb-2* mutants and *ben-1* mutants could be caused by shared isotype-specific functions. Another possibility is that the overall higher levels of *tbb-2* expression relative to *tbb-1* (Nishida et al. 2021) could be important. If the stronger interactions that we observed in *tbb-2* double mutants are simply a matter of higher overall β -tubulin levels, it might be possible to increase BZ toxicity in parasites with sub-clinical doses of microtubule inhibitors that target all microtubules, rather than only those microtubules that include β -tubulin isoforms with F200. Another possibility for differences between *tbb-1* and *tbb-2* may stem from cross-regulation between tubulins. TBB-1 is upregulated in the *tbb-2* mutant used in our study (Ellis et al. 2004). Although not predicted to bind ABZ, TBB-1 might increase incorporation of BEN-1 into microtubules compared to TBB-2. If *tbb-2* (but not *tbb-1*) mutants increase BEN-1 expression, this could sensitize microtubules to ABZ.

***tbb-1* and *tbb-2* have nonoverlapping functions**

tbb-1 and *tbb-2* are redundant for viability although single mutants of *tbb-1* and *tbb-2* have only subtle effects on tubulin dynamics and modest effects on hatching rates (Wright and Hunter 2003; Ellis et al. 2004; Lu et al. 2004; Honda et al. 2017). If each member of a redundant gene pair efficiently provides the same functions, selection might not act to preserve both members of the pair (Nowak et al. 1997). However, if the members of the gene pair also have nonoverlapping essential functions, selection will retain both copies. Indeed, *tbb-1* and *tbb-2* may be specialized for growth at different temperatures, as *tbb-1* mutants grew poorly at 11°C and *tbb-2* mutants had compromised growth at 25°C (Fig. 1). This range matches the substrate temperatures of *C. elegans* collected from the Hawaiian Islands (4–23°C) (Crombie et al. 2019, 2022).

Only certain BEN-1 residues might mutate to cause ABZ resistance

We conducted forward genetic screens to explore the types of mutations that can confer ABZ resistance. Consistent with the idea that *ben-1* loss leads to ABZ resistance (Driscoll et al. 1989; Hahnel et al. 2018; Dilks et al. 2020, 2021), we found a number of nonsense alleles (*sb152*, *sb158*, and *sb154*) and a splice donor mutation (*sb153*) among the 16 alleles we sequenced. These mutations are likely protein nulls and are distributed throughout the gene (Fig. 4). One might suspect that the high conservation of β -tubulins would imply that most *ben-1* residues are critical for function a priori and so our screen could have identified missense mutations in a large proportion of the conserved sites. Outside the nonconserved C-terminus, *ben-1* shows 77% and 97% identity with β -tubulins from the yeast *S. cerevisiae* and the parasitic nematode *H. contortus*, respectively. Comparisons to other *Caenorhabditis* species also indicate that *ben-1* evolution is highly constrained (Hahnel et al. 2018). However, of the 12 missense mutations (we include the canonical allele *e1880* in this total), 7 have what might be considered unusual properties.

Several lines of evidence indicate that relatively few *ben-1* missense mutations can lead to sufficient loss of activity to confer resistance. For example, we found 2 mutations that correspond to BZ-resistant mutations in other parasites and fungi. In those organisms, the genes are essential, so mutations likely represent specific changes that block drug binding and retain sufficient function to compete in the wild in the absence of drug. Such mutations in the nonessential *C. elegans* gene *ben-1* should be rare

compared to those mutations that cause loss of function, if most *ben-1* missense mutations were to confer resistance. Fifteen mutations leading to resistance have been reported in parasitic nematodes and fungi. However, only 3 can be created in one step in *ben-1* as GC to AT transitions, which accounts for 90% of EMS-induced *C. elegans* mutations (Thompson et al. 2013; Supplementary Table 1) and these mutations do not include the F167Y, E198A, and F200Y commonly found in resistant parasites. EMS can induce H6Y found in *Aspergillus nidulans*, E198K found in *A. nidulans*, *Gibberella zeae*, and *H. contortus*, and R241H found in *S. cerevisiae* (Thomas et al. 1985; Jung et al. 1992; Liu et al. 2014; Hahnel et al. 2018; Mohammedsalih et al. 2020). We found 2 of these 3 mutations, *sb159* (E198K) and *sb151* (R241H). *sb163* also appears to be another mutation that retains function as it was selected to retain movement in the *tbb-2* background. Another 3 ABZ-resistant missense mutations have been reported in wild *C. elegans* isolates (Hahnel et al. 2018), and of these S145F and M257I can be induced by EMS. These alleles may differ from the BZ-resistant mutations in the species described above in that they need not retain wild-type functions.

Another indication that a limited number of missense changes in *ben-1* can mutate to cause ABZ resistance is that we found 2 pairs of mutations that occur at different nucleotides within the same codon and cause different amino acid changes. *e1880* (G104D) and *sb145* (G104S) both alter amino acid 104 while *sb144* (G142E) and *sb164* (G142R) are both at position 142. This overlap could indicate that these positions are uniquely critical for protein function.

Our screen appeared to more efficiently identify nonsense and splicing mutations than missense alleles, again implying an unexpected rarity of amino acid changes that can confer resistance. We isolated 4 of 35 possible EMS-induced *ben-1* nonsense and splicing mutations. By contrast, we found only 12 of the 389 possible *ben-1* missense mutations that can be induced by EMS (excluding the nonconserved C-terminus, Tahsin Hassan Rahit KM and Tarailo-Graovac M, personal communication). Perhaps a more compelling example of the paucity of missense alleles that can confer ABZ resistance is that in wild *C. elegans* populations, where *ben-1* mutations may arise after environmental BZ exposure, Hahnel et al. (2018) found that only 3 of 25 resistance mutations were missense.

Thus, the assumption that high conservation of β -tubulin indicates that most missense alleles would result in severe loss of function may not be valid. This hypothesis is consistent with a systematic survey of the *S. cerevisiae* β -tubulin gene. Reijo et al. (1994) changed clusters of charged amino acids to alanine and found that only 11 of 55 alleles were lethal (although many viable alleles would cause fitness costs in nature). Only 5 were strongly resistant to benomyl. This result suggests that in *C. elegans* most *ben-1* missense mutations would not confer ABZ resistance as they could retain sufficient function to deliver the BZ poison to the microtubule. Another explanation is that many missense changes in tubulins, with their many protein-protein contact sites, may have a propensity to act in a dominant-negative or neomorphic fashion, resulting in an Unc, slow growing or a lethal phenotype that might have been missed in our screens (Phillips et al. 2004; Lu and Mains 2005; Lee et al. 2021).

ben-1* is the major ABZ target in *C. elegans

With the widespread use of ABZ in human populations, it is critical to understand the genetics of nematode drug resistance. *ben-1* is clearly the major target in *C. elegans* under laboratory conditions. The resistant allele *sb163* (A63V) that retains at least partial

ben-1(+) function could represent a new mutation that may arise in parasites. If most *ben-1* missense alleles do not confer resistance, parasitic nematode species with a redundant Y200 containing paralogs might be more likely to acquire resistance through nonsense and deletion mutations than the F167Y, E198A, and F200Y commonly found in BZ-resistant parasites. Additional genes also influence BZ resistance in both wild *C. elegans* and in parasitic nematodes (Hahnel et al. 2018; Zamanian et al. 2018; Furtado, de Aguiar, et al. 2019). We did find 1 mutation (*sb156*) with no lesions in the *ben-1* coding region and this mutant had the weakest resistance in our study. Further optimization of our screens to recover weak resistance may uncover additional genes. A better understanding of the interplay of BZ drugs and tubulin genetics can also inform treatments of other types of human diseases that are treated with BZ drugs, including cancer (Prasher and Sharma 2022).

Data availability

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

[Supplemental material](#) is available at GENETICS online.

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Conflicts of interest

None declared.

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