Genomic landscape of drug response reveals novel mediators of anthelmintic resistance

Authors: Stephen R. Doyle[1,†,*], Roz Laing[2,†,*], David Bartley[3], Alison Morrison[3], Nancy Holroyd[1], Kirsty Maitland[2], Alistair Antonopoulos[2], Umer Chaudhry[4§], Ilona Flis[2], Sue Howell[5], Jennifer McIntyre[2], John S. Gilleard[6], Andy Tait[2], Barbara Mable[2], Ray Kaplan[5+], Neil Sargison[4], Collette Britton[2], Matthew Berriman[1], Eileen Devaney[2,‡], James A. Cotton[1,‡]

† These authors contributed equally to this work

‡ These authors contributed equally to this work

§ Current address: School of Veterinary Medicine, University of Surrey; Surrey, United Kingdom

+ Current address: Department of Pathobiology, School of Veterinary Medicine, St. George's University; St. George's, Grenada

*Corresponding authors: email: stephen.doyle@sanger.ac.uk; email: Rosalind.Laing@glasgow.ac.uk

Affiliations:

1. Wellcome Sanger Institute; Hinxton, United Kingdom
2. Institute of Biodiversity Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow; Glasgow, United Kingdom
3. Moredun Research Institute; Penicuik, United Kingdom
4. Royal (Dick) School of Veterinary Studies, University of Edinburgh; Edinburgh, United Kingdom
5. Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia; Athens, United States
6. Department of Comparative Biology and Experimental Medicine, Host-Parasite Interactions Program, Faculty of Veterinary Medicine, University of Calgary; Calgary, Canada

Abstract:

Understanding the genetic basis of anthelmintic drug resistance in parasitic nematodes is key to improving the efficacy and sustainability of parasite control. Here, we use a genetic cross in a natural host-parasite system to simultaneously map resistance loci for the three major classes of anthelmintics. This approach identifies novel alleles for resistance to benzimidazoles and levamisole and implicates the transcription factor, *cky-1*, in ivermectin resistance. This gene is within a locus under selection in ivermectin resistant populations worldwide; functional validation using knockout and gene expression experiments supports a role for *cky-1* overexpression in ivermectin resistance. Our work demonstrates the feasibility of high-resolution forward genetics in a parasitic nematode, and identifies variants for the development of molecular diagnostics to combat drug resistance in the field.

One-Sentence Summary:

Genetic mapping of known and novel anthelmintic resistance-associated alleles in a multi-drug resistant parasitic nematode

Main Text: Over a billion people and countless livestock and companion animals require at least annual treatment with anthelmintic drugs to control parasitic worm (helminth) infections. The rapid and widespread evolution of resistance to these drugs is a significant health concern in livestock (*1*) and places an economic burden on food production. Resistance is present on every continent where anthelmintics are used; in many places, individual drug classes are now ineffective, and some farms have resistance to every major class of drug (*2*), threatening the economic viability of livestock farming. In Europe, gastrointestinal helminths of livestock are responsible for annual production losses of €686 million, of which €38 million is associated with anthelmintic resistance (*3*). Drug resistance is also now a major concern in the treatment of helminths infecting dogs (*4*, *5*), with multiple drug resistance to all major anthelmintic classes in the dog hookworm now common in the USA (*6*). The same classes of drugs to which veterinary parasites have rapidly evolved resistance are also used to control related human-infective helminths, which are targeted at scale by some of the largest preventative chemotherapy programmes in the world. Although less established in human-infective helminths, the emergence of widespread anthelmintic resistance – echoing the current global emergency around antimicrobial resistance – will have serious socio-economic and welfare impacts on people infected with parasitic worms and derail hard-won progress towards the proposed eradication and elimination of helminths over the next decade (*7*, *8*).

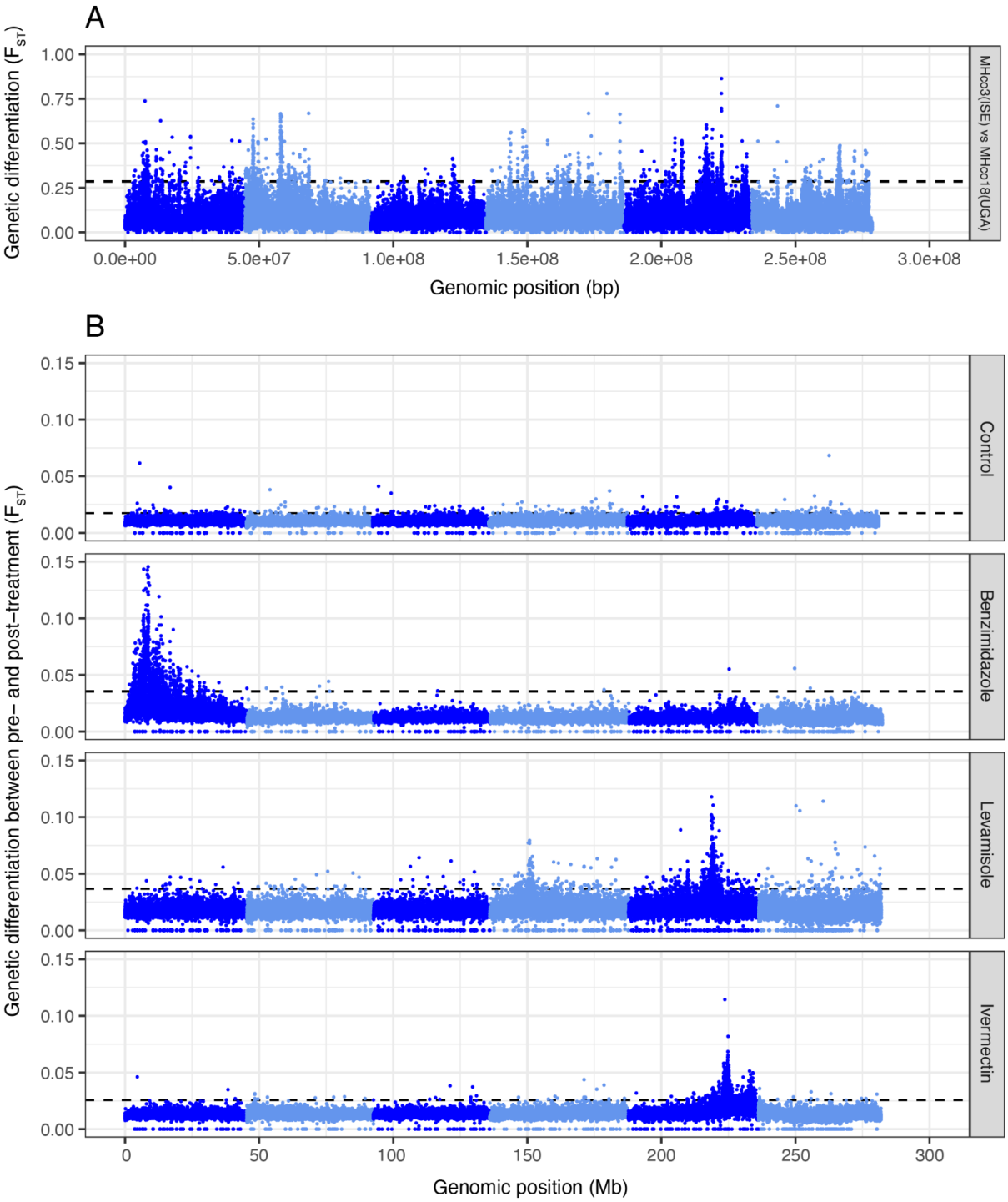
Despite extensive efforts, the causal mutations and mechanisms of resistance in parasitic helminths remain largely unresolved. Many candidate "resistance genes" have been proposed for most drug classes; these candidates are primarily homologues of genes that confer resistance in the free-living model nematode *Caenorhabditis elegans,* and are subsequently assayed for differences in genetic variation and/or gene expression in parasite isolates that vary in their response to treatment (*9–11*). A successful example of this approach is the identification of variants of β-tubulin that inhibit tubulin-depolymerisation by benzimidazole-class anthelmintics (*12*, *13*). These variants, particularly at amino acid positions 167, 198 and 200 of β-tubulin isotype 1 (*14–16*), have subsequently been shown to be

associated with resistance in many parasitic species for which benzimidazoles have been extensively used, and a number of these parasite-specific mutations have been functionally validated in *C. elegans* (*17*, *18*). However, these three variants are unlikely to explain all phenotypic variation associated with resistance (*19*, *20*), and it is unknown to what degree other variants contribute to benzimidazole resistance in parasitic species. For other drug classes, few candidate genes have been functionally validated or shown to be important in natural parasite populations. For example, concurrent mutation of three glutamate-gated chloride channels (*glc-1, avr-14, avr-15*) enables resistance to high levels of ivermectin by *C. elegans (21)*, yet no strong evidence of selection on these channels in any parasitic species has been demonstrated to date. On the one hand, the many genes proposed may reflect that resistance is a complex, quantitative trait where similar resistance phenotypes can be derived from variation in multiple loci. Alternatively, resistance may be species and/or population-specific, and evolve independently under subtly different selection pressures (*22*). However, some candidates are likely to have been falsely associated with resistance, as most studies present relatively weak genetic evidence from the analysis of single or few candidate loci in small numbers of helminth populations that often differ in both drug susceptibility and geographic origin. Many helminth species are exceptionally genetically diverse (*23–26*), and consequently, candidate gene approaches have limited power to disentangle causal variation from linked but unrelated background genetic variation, a situation that is exacerbated by the experimental intractability and inadequate genomic resources available for many parasitic helminths (*9*).

Here we describe a genome-wide forward genetics approach using the parasitic nematode *Haemonchus contortus* as a model to identify genetic variation associated with resistance to three of the most important broad-spectrum anthelmintic drugs globally: ivermectin, levamisole, and benzimidazole. *H. contortus* is an economically important gastrointestinal parasite of livestock and one of only a few genetically tractable parasites used for drug discovery (*27*, *28*), vaccine development (*29*, *30*), and anthelmintic resistance research (*22*). Our approach has exploited a genetic cross between the susceptible MHco3(ISE) and multi-drug

115 resistant MHco18(UGA) strains of *H. contortus* (**fig. S1 A**), allowing us to investigate
116 resistance in a natural host-parasite system while controlling for confounding
117 genetic diversity that differentiates parasite strains (see **Supplementary materials**
118 regarding the establishment and validation of the cross). Using an eXtreme
119 Quantitative Trait Locus (X-QTL) (*31*, *32*) analysis framework, whereby pools of F3-
120 generation progeny from F2 adults treated *in vivo* were sampled pre- and post-
121 treatment for each drug (**fig. S1 B**; n = 3 parasite populations per drug class
122 maintained in independent sheep; **fig. S2**) and analysed by whole-genome
123 sequencing (**table S1**), we aimed to identify drug-specific quantitative trait loci
124 (QTLs) associated with resistance throughout the genome. These QTLs and specific
125 variants were independently validated using genome-wide variation from
126 populations of *H. contortus* obtained from ten US farms of known resistance
127 phenotype (see **Supplementary materials** for a description of the US farms and
128 quantitative phenotyping**; table S2, fig. S3, fig. S4**), and from more than 350
129 individual parasites sampled throughout the world where *H. contortus* is endemic
130 (*25*, *33*).
131
132 A key feature and thus advantage of using a genetic cross to map anthelmintic
133 resistance loci is that the high degree of within-strain diversity and genome-wide
134 genetic divergence is controlled by admixture in the F1 generation of the cross. The
135 susceptible and resistant parental *H. contortus* strains of the cross are highly
136 genetically differentiated throughout their genomes (**Fig. 1A;** mean F_{ST} = 0.089 ±
137 0.066 SD; n = 16,794,366 single nucleotide variant sites), typical of two parasite
138 strains sampled from different continents (*25*, *34*). In subsequent generations, both
139 susceptible and resistant alleles segregate at moderate frequencies in the absence
140 of selection, and genetic recombination breaks down the linked genetic variation
141 that defines and differentiates the parental strains. This was evident by a
142 significantly lower genome-wide genetic differentiation in the F3-generation control
143 population (genome-wide mean F_{ST} = 0.012 ± 0.004) and absence of discrete peaks
144 of high genetic differentiation (**Fig. 1B: Control**). In contrast, after each drug
145 treatment, discrete QTLs that differ between each drug class were revealed: after
146 benzimidazole treatment, we identified a major QTL on chromosome 1 (**Fig. 1B:**

147 **Benzimidazole**); after levamisole, two QTLs on chromosome 4 and 5 (**Fig. 1B:**
148 **Levamisole**); and after ivermectin, a major QTL on chromosome 5 and minor QTLs
149 on chromosomes 2 and 5 (**Fig. 1B: Ivermectin**).
150



151

152

153 **Fig. 1. A genetic cross followed by drug selection reveals discrete QTLs associated**

154 **with each anthelmintic drug class.**

155 (**A**) Genome-wide comparison of susceptible MHco3(ISE) and multidrug-resistant

156 MHco18(UGA) parental strains revealed broad-scale genetic differentiation on all

157 chromosomes. (**B**) Comparison of genome-wide differentiation between F3 generation

158 pooled infective-stage larvae (L_3, n = 200) sampled pre- and post-treatment revealed

159 distinct genomic regions or QTLs associated with benzimidazole, levamisole, and
160 ivermectin drug treatment. An untreated control where sampling was time-matched to the
161 treated groups is shown for comparison. In all plots, each point represents the mean
162 genetic differentiation (F_{ST}) from three biological replicates in five kb sliding windows, and
163 the dashed line represents the genome-wide mean F_{ST} + 3 SD for each comparison (See
164 **fig. S2** for genome-wide replicate data). Individual chromosomes are indicated by
165 alternating dark and light blue shading.
166
167
168
169 ***Variation at β-tubulin isotype 1 and a novel β-tubulin isotype 2 variant is***
170 ***associated with high levels of benzimidazole resistance***
171 The β-tubulin isotype 1 (HCON_00005260) gene and, in particular, nonsynonymous
172 changes at coding positions 167, 198, and 200 have been widely associated with
173 benzimidazole resistance in *H. contortus* (*13*, *15*, *16*, *35*) and other nematodes
174 frequently exposed to benzimidazole treatment (*17*, *36*). After benzimidazole
175 selection, a single broad QTL was found on chromosome 1 (**Fig. 2A;** see
176 **Supplementary materials** for further discussion of the genetic structure of the QTL)
177 containing the β-tubulin isotype 1 locus. Within this gene, we identified a significant
178 increase in the frequency of a Phe200Tyr variant (a phenylalanine [reference
179 susceptible variant] to tyrosine [resistant variant] substitution at position 200) from
180 pre- to post-treatment and relative to untreated controls (**Fig. 2B;** P = 1.7e-26,
181 genome-wide Cochran–Mantel–Haenszel (CMH) test between replicates). We also
182 identified a small increase in frequency of the Phe167Tyr variant (mean $freq_{\text{pre-treatment}}$
183 = 0.14 to $freq_{\text{post-treatment}}$ = 0.20), however, no variation was found at the Glu198
184 position. Considering the previous association between these variants and
185 benzimidazole resistance, we conclude that the Phe200Tyr variant is the primary
186 driver of phenotypic resistance in the X-QTL population.
187
188 *Haemonchus contortus* has multiple β-tubulin genes (*37*), and deletion of the β-
189 tubulin isotype 2 gene (HCON_00043670) on chromosome 2 has been associated
190 with increased levels of resistance beyond that of mutations in the isotype 1 gene
191 alone (*14*). Here, we found no evidence of deletions in isotype 2. However, a minor

192 but not significant increase in genetic differentiation between pre- and post-
193 treatment populations was found at this locus, and a Glu198Val variant at a
194 homologous site to a known resistance variant in isotype 1 was present at a low
195 frequency in the genetic cross ($freq_{\text{pre-treatment}}$ = 0.260 to $freq_{\text{post-treatment}}$ = 0.323; not
196 significant genome-wide CMH). However, on the US farms, the Glu198Val variant
197 did vary in frequency between farms and was significantly correlated (r = 0.978, *P* =
198 1.02e-6; Pearson's correlation) with EC_{50} values for benzimidazole resistance (**Fig.**
199 **2C**). The variance observed in EC_{50} among resistant farm populations was not
200 caused by variation in the frequency of the Phe200Tyr mutation of the isotype 1
201 gene, as this variant was already at high frequency in all populations, except for the
202 farm that was susceptible to benzimidazoles (Farm 1; **fig. S5**). These data suggest
203 that once the isotype 1 Phe200Tyr variant has reached near fixation in the
204 population, the Glu198Val variant of isotype 2 mediates higher levels of
205 benzimidazole resistance than conferred by the Phe200Tyr variant alone. As such,
206 this novel allele present in β-tubulin isotype 2 should be considered, in addition to
207 the well-characterised isotype 1 variants, as a genetic marker for benzimidazole
208 resistance.
209
210 In addition to the association with benzimidazole resistance, it has been suggested
211 that the β-tubulin isotype 1 Phe200Tyr variant in *H. contortus* (*38–40*) and also at an
212 equivalent variant site in a β-tubulin gene in the human-infective filarial nematode
213 *Onchocerca volvulus* (*41*) is associated with ivermectin resistance. Here we found
214 no evidence of selection on either the Phe167Tyr or Phe200Tyr variants (or any
215 variant found in the region) in X-QTL analyses of ivermectin treatment (**fig. S6A**), nor
216 any correlation with ivermectin EC_{50} on the US farms (**fig. S6B**). These data reaffirm
217 that mutations in β-tubulin isotype 1 are specific to benzimidazole resistance.

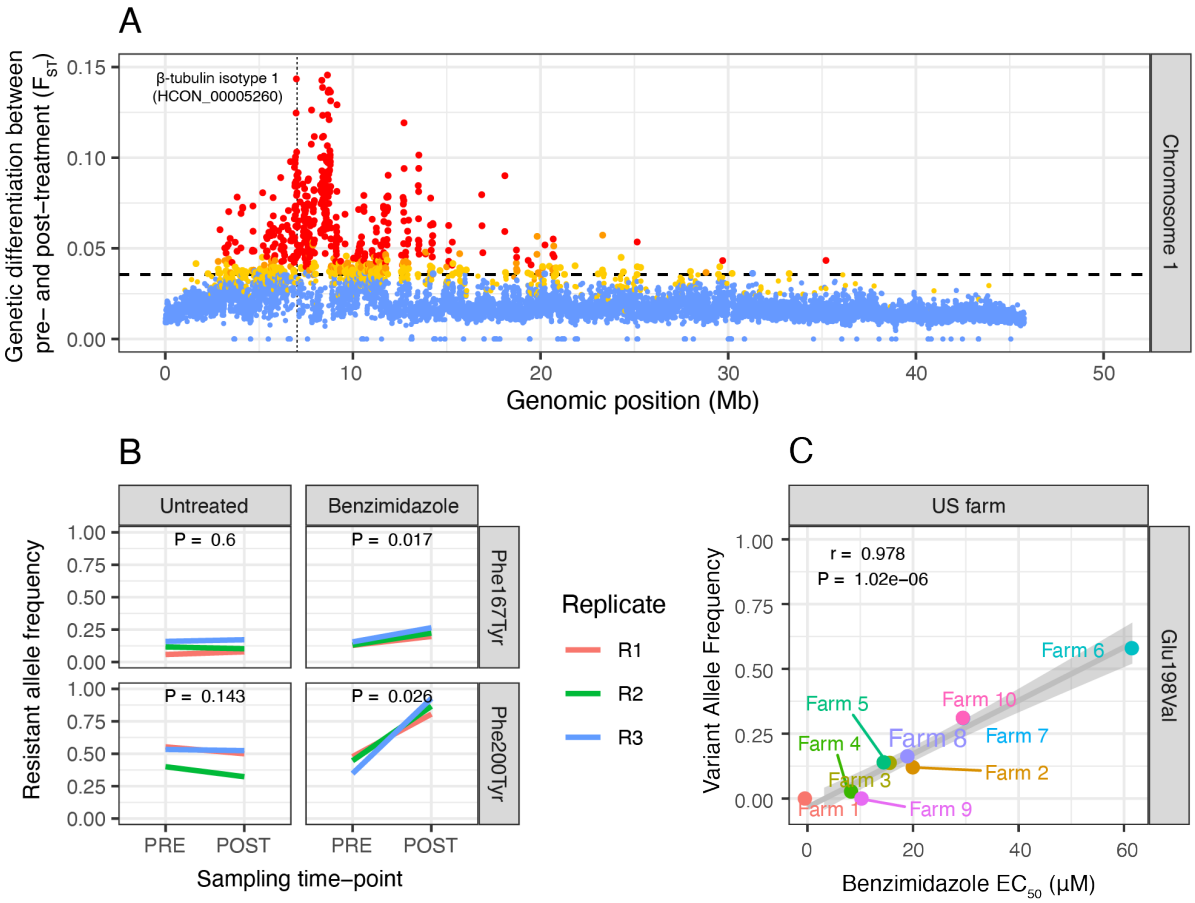


Fig. 2. Characterisation of QTL associated with benzimidazole resistance. **(A)** Chromosome-wide genetic differentiation between pre- and post-benzimidazole treatment on chromosome 1. Each point represents the mean F_{ST} in a five kb window; points are coloured based on the concordance of individual replicates indicated by none (blue), 1 of 3 (yellow), 2 of 3 (orange), or all 3 (red) above the genome-wide threshold. The genome-wide threshold is defined as the mean + 3 SD of the chromosome-wide F_{ST} indicated by the horizontal dashed line, whereas the vertical dashed line highlights the position of the β-tubulin isotype 1 (HCON_00005260) gene. **(B)** Allele frequency change at Phe167Tyr and Phe200Tyr variant positions of β-tubulin isotype 1 pre- and post-treatment, including untreated time-matched control. Coloured lines show biological replicates. *P*-values are calculated using pairwise t-tests of allele frequency by sampling time point (i.e., pre- and post-treatment). **(C)** Correlation between benzimidazole EC_{50} concentration (μM) observed at particular farms and Glu198Val variant frequency of β-tubulin isotype 2 (HCON_00043670) on US farms. Pearson's correlation (r) and associated *P*-value together with the trendline and standard error of the linear regression are shown.

236

237 ***Levamisole selection implicates acetylcholine receptors, including a novel acr-***
238 ***8 variant, with resistance***

239 The anthelmintic activity of levamisole is due to its antagonistic effect on nematode
240 nicotinic acetylcholine receptors (*42*), and resistance in *C. elegans* is typically
241 associated with variation in subunits of these receptors or other accessory proteins
242 that contribute to acetylcholine-mediated signalling (*43*). Here we identified two
243 major QTLs on chromosomes 4 and 5 that contain a tandem duplication of the
244 acetylcholine receptor subunit β-type *lev-1* (HCON_00107690 & HCON_00107700)
245 and acetylcholine receptor subunit *acr-8* (HCON_00151270), respectively (**Fig. 3A**).
246

247 The *H. contortus acr-8* gene (**Fig. 3B**) has long been implicated in levamisole
248 resistance; a truncated isoform of *acr-8* containing the two first exons and a part of
249 intron 2 (previously called *Hco-acr-8b*) (*44*), and subsequently, a 63 bp indel
250 between exons 2 and 3 have been associated with resistance based on their
251 presence in several resistant isolates (*45*). However, the functional consequence of
252 these variants in mediating levamisole resistance *in vivo* is not yet clear. Here, we
253 identified two larger deletion variants spanning 31,527,022 to 31,527,119 (97 bp) or
254 31,527,121 (99 bp) on chromosome 5 that increased in frequency from 73.47% in
255 the pre-treatment population to 86.58% after levamisole treatment (**Fig. 3C;** paired
256 t-test across replicates, $P = 0.1$). However, the *acr-8* indel was present in the
257 levamisole susceptible parental MHco3(ISE) strain (59.05%) and was present at only
258 a slightly higher frequency in the resistant MHco18(UGA) strain (63.55%). Thus,
259 these data argue that the *acr-8* indel is a poor marker of levamisole resistance.
260

261 We did, however, identify a nonsynonymous variant (Ser168Thr) in *acr-8* that was
262 strongly correlated with resistance across multiple datasets. In the X-QTL analyses,
263 Ser168Thr increased to a high frequency after drug selection in the F2 generation
264 (**Fig. 3D**; position 31,521,884; genome-wide CMH: $P = 1.6e-15$; allele frequency
265 change pre- vs post-treatment: $P = 1.0e-4$; in time-matched no-treatment control: P
266 $= 0.4$). It was also found at a high frequency in the USA field population with the
267 highest levamisole drug resistance phenotype (Farm 7; $freq_{Ser168Thr} = 0.64$). This

268 association was supported in global diversity data of *H. contortus* (*25*), where we
269 found the Ser168Thr variant fixed in parasites from the Kokstad (KOK; South Africa)
270 population ($freq_{Ser168Thr}$ = 1.0; n = 4), the only population with confirmed levamisole
271 resistance in that study, whereas the variant was absent in all other populations
272 analysed. The identification of Ser168Thr prompted us to look beyond *H. contortus*;
273 a reanalysis of levamisole resistance in resequencing data from the closely related
274 clade V parasitic nematode *Teladorsagia circumcincta (46)* revealed a homologous
275 non-synonymous variant at high frequency in resistant parasites (Ser140Thr in
276 Cont419:G75849C ; $freq_{Ser140Thr}$ = 0.972), which was absent in the susceptible
277 population to which it was compared. Although a serine to threonine substitution is
278 a relatively conservative change, we found the serine residue to be highly conserved
279 among clade V nematodes (**fig. S7**), particularly among the parasite species,
280 whereas in the free-living *Caenorhabditis spp.*, threonine is encoded at this position.
281 In *C. elegans, acr-8* is genetically and functionally distinct relative to *acr-8* of
282 parasitic nematodes and is not a component of the native levamisole receptor (*47*);
283 the *C. elegans* functional homolog *lev-8*, which can be transgenically substituted by
284 *H. contortus acr-8* to produce a functional receptor (*48*), does encode a serine at
285 this homologous position. The *H. contortus* ACR-8 Ser168Thr variant lies
286 immediately downstream of the cys-loop domain within the ligand-binding pocket
287 and is immediately adjacent to a highly conserved tryptophan residue essential for
288 ligand binding (*49*, *50*) (**Fig. 3E**). Importantly, key residues downstream of the
289 conserved tryptophan have previously been shown to influence levamisole
290 sensitivity of closely related receptor subunits (*51*). Thus, we hypothesise that the
291 Ser168Thr variant facilitates a change in the molecular interactions within the
292 binding pocket of ACR-8, resulting in a decreased sensitivity to levamisole.
293
294 The identification of *lev-1* genes within the chromosome 4 QTL is compelling, with
295 three intronic variants of *lev-1* (top variant position 14,995,062 in HCON_00107700;
296 *P* = 1.7e-20; CMH test) among of the top ten most differentiated SNPs on this
297 chromosome. However, it remains unclear what effect the overall observed variation
298 in the *lev-1* genes has on levamisole resistance. Although multiple non-synonymous
299 variants were also identified (seven and three variants for HCON_00107690 and

300 HCON_00107700, respectively), none were predicted to cause high-effect changes
301 in the protein sequence and exhibited only relatively minor shifts in allele frequency
302 upon levamisole treatment. In *C. elegans*, several dominant resistant variants of *lev-*
303 *1* have been described (not found in the data described here); however, *lev-1* can
304 be lost without affecting the function of the receptor (*43*). Examination of variation in
305 *lev-1* expression in addition to genetic variation may be required to elucidate the
306 role of *H. contortus lev-1* subunits in levamisole resistance. Close to the *lev-1* genes
307 and toward the centre of the QTL, four of the top ten variants in chromosome 4
308 were found in HCON_00107560 (top non-synonymous variant: Arg934His at
309 position 14,781,344; P = 1.0e-21; CMH test), an ortholog of *C. elegans kdin-1*.
310 Highly conserved with mammalian orthologs (*52*), *kdin-1* has been shown to co-
311 localise with acetylcholine receptors at rat neuromuscular junctions during
312 development (*53*) where, via a PDZ domain, it participates in the coordination of
313 signalling components including ion channels and neurotransmitters. The precise
314 role of HCON_00107560 or *kdin-1* in *H. contortus* or *C. elegans*, respectively,
315 remains unknown; however, its putative association with levamisole response here
316 warrants further investigation.
317
318 Signals of selection on two components of the pentameric acetylcholine receptor
319 prompted us to look for selection on the remaining subunits. Although the
320 expression of *unc-63* (HCON_00024380) and *unc-29.3* (HCON_00003520) mRNAs
321 were significantly reduced in the larvae of resistant MHco18(UGA) strain (*54*), we
322 found no evidence of selection on the region of the genome containing these genes.

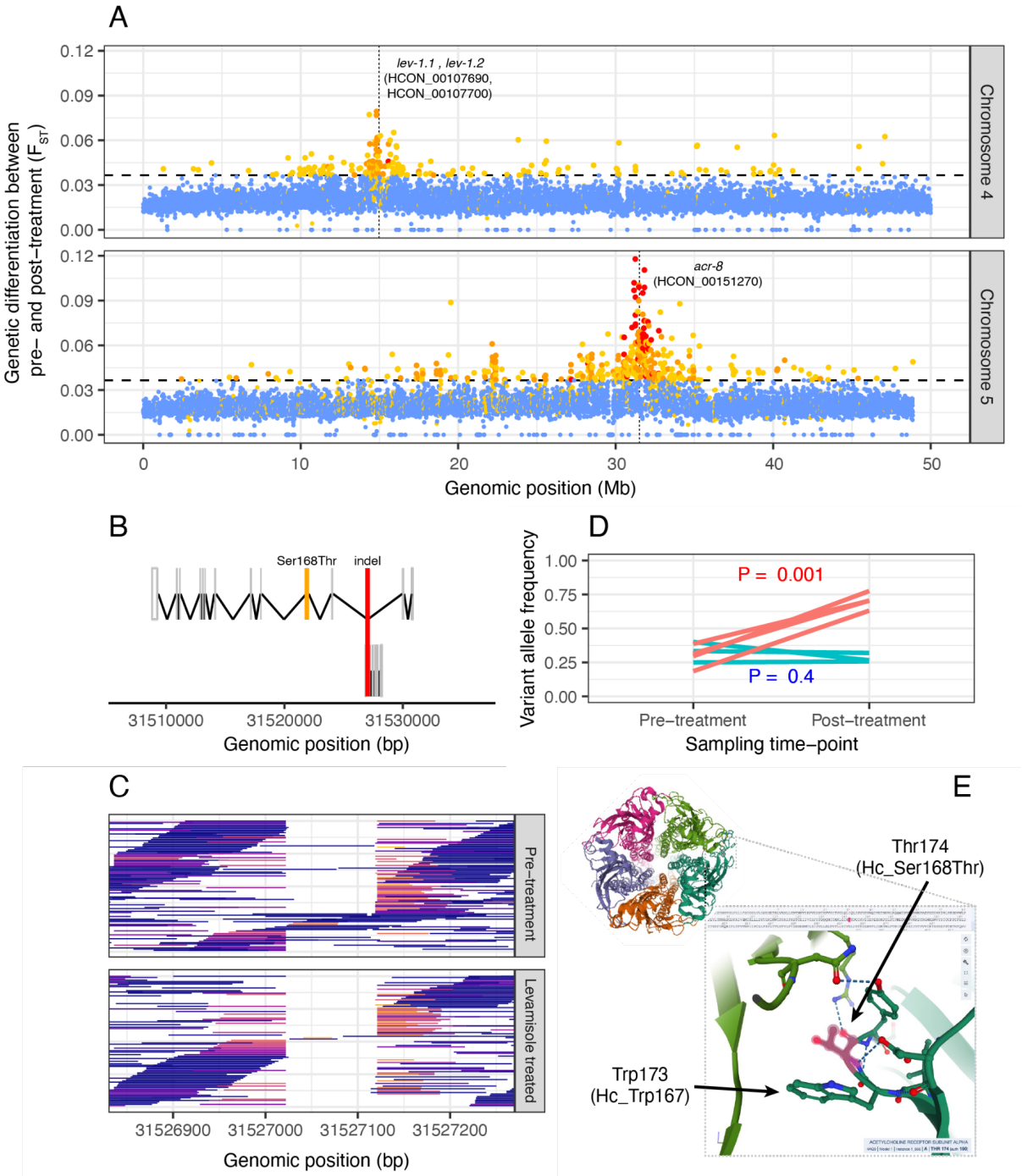


Fig. 3. Characterisation of QTL associated with levamisole resistance.

(**A**) QTL between pre-treatment and levamisole-treated parasites on chromosome 4 (top) and chromosome 5 (bottom). Each point represents the mean F_{ST} in a five kb window; points are coloured based on the concordance of individual replicates indicated by none (blue), 1 of 3 (yellow), 2 of 3 (orange), or all 3 (red) above the genome-wide threshold (horizontal dashed line; mean + 3 SD of the chromosome-wide F_{ST}). (**B**) Gene model for *acr-8* (top; HCON_00151270) and a cuticle collagen (bottom; HCON_00151260), highlighting

332 the position of the overlapping *acr-8*/levamisole-associated indel and the Ser168Thr variant
333 of *acr-8*. (**C**) Visualisation of sequencing reads supporting the *acr-8* intronic indel. Mapped
334 reads are coloured to reflect the degree to which they have been clipped to allow correct
335 mapping in the presence of the deletion, i.e. reads that have not been clipped are blue,
336 whereas reads that are moderate to highly clipped are coloured red to yellow, respectively.
337 (**D**) Comparison of Ser168Thr variant frequency between pre- and post-levamisole
338 treatment (red) and time-matched untreated controls (green). (**E**) Structure of the
339 pentameric cys-loop acetylcholine receptor of *Torpedo marmorata* (Protein Data Base ID:
340 4AQ9), one of the few species from which the receptor's structure has been resolved (*55*).
341 The Trp[Ser/Thr]Tyr motif is highly conserved among the clade V nematodes (**fig. S7**) and
342 the distantly related alpha subunit of *T. marmorata*; Thr174, the homologous position of the
343 *H. contortus* Hc_Ser168Thr variant of *acr-8,* lies within the acetylcholine binding pocket at
344 the interface of the alpha and gamma subunits and adjacent to Trp173 (*H. contortus*
345 Hc_Trp167), a residue essential for ligand binding.
346
347
348
349 ***A resolved ivermectin QTL implicates* cky-1 *as a novel mediator of resistance***
350 Ivermectin is a critically important broad-spectrum drug used to control several
351 human- and veterinary-infective helminths worldwide and is also widely used as an
352 acaricide targeting ticks and mites. We recently identified a ~5 Mb QTL associated
353 with ivermectin resistance from 37 to 42 Mb on chromosome 5 from the analysis of
354 a backcross experiment (*34*, *56*), and subsequently, we identified evidence of
355 selection in the same chromosomal region in ivermectin-resistant field populations
356 from Africa and Australia (*25*). Although the introgression region from the backcross
357 was broad (*57*), the genetic architecture of the QTL was consistent with a single
358 dominant variant driving resistance, and we were able to demonstrate that most
359 candidate genes previously proposed to be associated with resistance were not
360 under direct ivermectin selection. However, we were unable to confidently identify
361 any novel candidate driving mutation among the ~360 genes lying within the region
362 (*34*).
363

364 Here, we confirm the QTL within the previously implicated chromosome 5 region at
365 ~37.5 Mb (*34*, *56*) but with significantly increased resolution (**Fig. 4A**). We have
366 narrowed the genetic association to approximately 300 kb wide (region:
367 ~37,250,000-37,550,000), based on the region of highest differentiation between
368 independently replicated pre- and post-treatment X-QTL samples (**Fig. 4B**). This
369 region was also highly differentiated between pre-treatment larvae and adult male
370 worms that survived ivermectin treatment *in vivo* **(fig. S8 A,B),** and between larvae
371 that survived treatment with an EC_{75} dose of ivermectin and those sensitive to an
372 EC_{50} dose *in vitro* **(fig. S8 C,D)***.* Together, these results confirm that this locus is
373 under direct selection and mediates resistance in both the parasitic stages *in vivo*
374 and in the free-living stages *in vitro* (see **Supplementary materials** for further
375 discussion). Finally, this was the only region in the genome where increased levels
376 of ivermectin resistance (i.e., EC_{50}) was associated with a loss of genetic diversity in
377 moderately or highly resistant field populations relative to susceptible populations
378 (**Fig 4C**), consistent with a selective sweep in response to ivermectin-mediated
379 selection.
380
381 The main chromosome 5 QTL contained 25 genes and included an expansion of
382 protein kinases (8/21 genes present in the genome with the InterPro identifier
383 IPR015897), some of which had the highest statistical association with resistance;
384 for example, HCON_00155240 (intronic position 37,336,132, P = 3.3e-13; position
385 37,235,944, P = 1.2e-12) and HCON_00155270 (intronic position 37,343,439, P =
386 1.0e-10). These protein kinases are, however, novel leads with no previous
387 association to drug resistance, and a lack of functional orthologs and observed
388 gene expansion made it difficult to further infer and test a role for these genes in
389 ivermectin resistance.
390
391 Towards the middle of the QTL, we identified *cky-1* (HCON_00155390; positions
392 37,487,982 - 37,497,398) as a new mediator of resistance, based on several lines of
393 evidence. In the X-QTL data, *cky-1* contained eight moderately to highly
394 differentiated significant non-synonymous variants (top variant: position 37,497,061
395 [Ser583Pro], P = 9.6e-09; CMH test). In a complementary study, we showed *cky-1*

396 was the only gene in the region significantly upregulated in both males and females
397 of the resistant MHco18(UGA) isolate relative to MHco3(ISE) and was one of the
398 most upregulated genes genome-wide (RNA-Seq paper). In this study, RT-qPCR of
399 *cky-1* from the parental isolates of the cross and two unrelated ivermectin-resistant
400 *H. contortus* strains revealed significant overexpression in ivermectin-resistant
401 relative to sensitive strains (**Fig. 4E**), an observation that was replicated between
402 sensitive and ivermectin-resistant strains of the related parasite, *T. circumcincta*
403 (**Fig. 4E**). To explore *cky-1* further, we assayed *C. elegans* developmental and
404 pumping behavioural phenotypes, both known to be perturbed by ivermectin
405 exposure (*58*), to test the role of differential expression of *cky-1* on the resistant
406 phenotype in the presence of ivermectin. While complete knockout of *cky-1* was
407 non-viable, both a balanced deletion (VC2274) (**Fig. 4E**) and RNAi knockdown (**Fig.**
408 **4F**) of *cky-1* increased the sensitivity of *C. elegans* to ivermectin relative to the
409 ivermectin-susceptible N2 strain. Finally, in two independent transgenic *C. elegans*
410 strains, we found that overexpression of *cky-1* partially improved fitness in the
411 presence of ivermectin relative to the N2 control (**Fig. 4G**). The level of *cky-1*
412 expression is, therefore, associated with and contributes to the ivermectin
413 resistance phenotype in nematodes.
414
415 Two additional, less prominent QTLs on chromosome 5 at ~46 Mb and on
416 chromosome 2 at ~3 Mb were also identified after ivermectin treatment (**Fig. 4A;**
417 see **Supplementary materials** for a description of the two QTLs). The second
418 chromosome 5 QTL was identified as a candidate region associated with resistance
419 in the backcross (*34*); however, we did not have the statistical power to differentiate
420 it from the main QTL in that experiment. Here, the QTL appeared to segregate
421 independently of the prominent 37.5 Mb peak, providing more robust evidence of a
422 second resistance-conferring variant on chromosome 5. Although the main
423 chromosome 5 QTL at 37.5 Mb was present in all selection experiments with
424 ivermectin, the secondary QTLs were variable between replicates and experiments.
425 To further refine the association, we exposed the F5 generation of the cross to a
426 half standard dose of ivermectin, followed by a double dose thereafter. The
427 rationale was first to identify low-effect variants (responding to the half dose

treatment), then select a subset of variants that conferred resistance at high doses (see **Supplementary materials** for additional background). In these experiments, we consistently detected the main chromosome 5 QTL but not the less prominent chromosome 2 QTL. Additionally, we detected the presence of at least three new minor QTLs (**fig. S10**, supplementary text). Of practical significance, the identification of novel replicate-specific variants in addition to the main chromosome 5 QTL highlights the consequence of under-dosing in selecting novel variants, and emphasises the importance of correct dosing in the field.

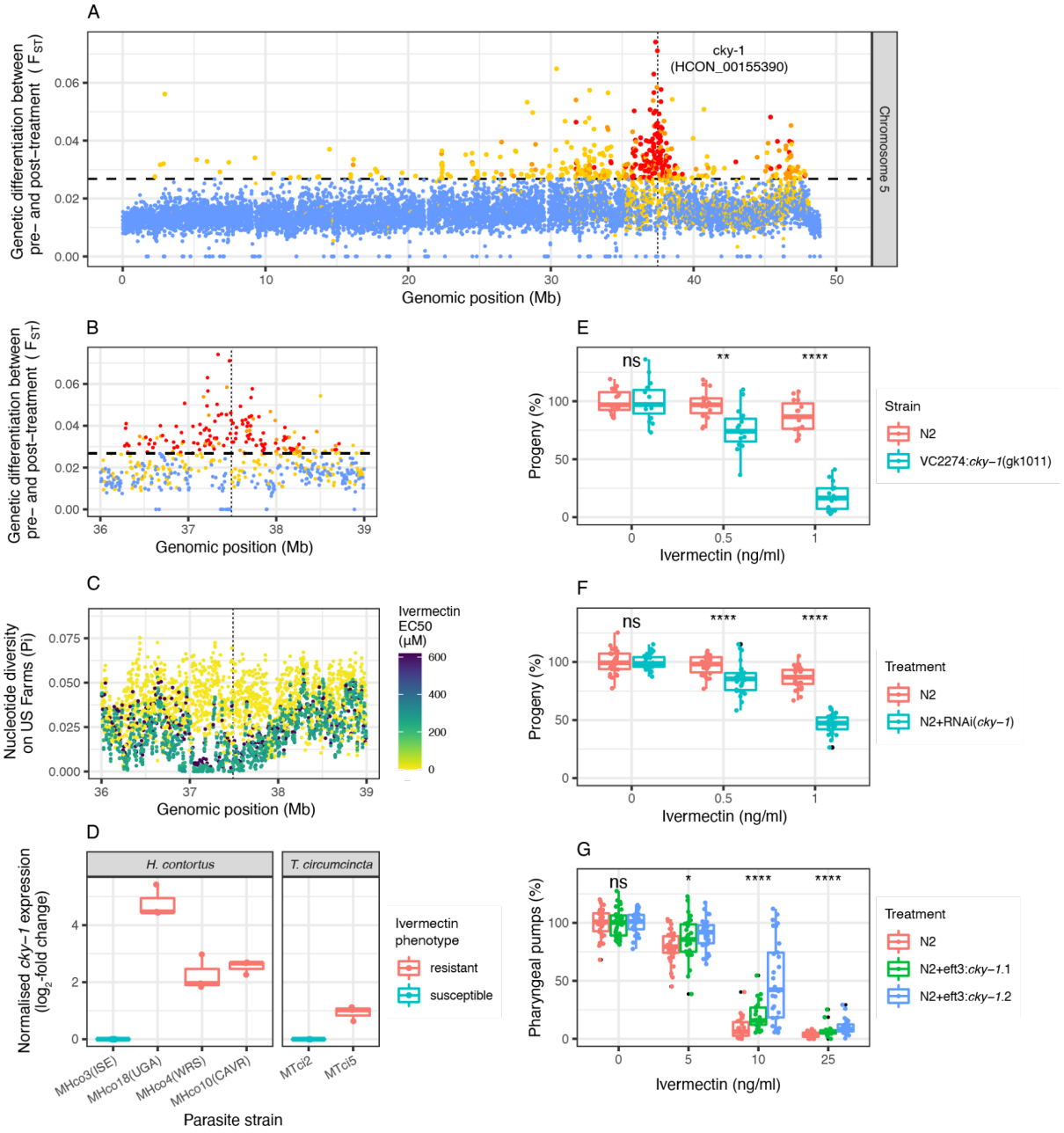


Fig. 4. Characterisation of QTL associated with ivermectin resistance.

(**A**) QTL between pre- and post-ivermectin treatment on chromosome 5. Each point represents the mean F_{ST} in a five kb window; points are coloured based on the concordance of individual replicates indicated by none (blue), 1 of 3 (yellow), 2 of 3 (orange), or all 3 (red) above the genome-wide threshold (horizontal dashed line; mean + 3 SD of the chromosome-wide F_{ST}). A magnified aspect of the main chromosome 5 QTL, highlighting (**B**) genetic differentiation (F_{ST}) in the X-QTL cross, and (**C**) nucleotide diversity (Pi) on US farms, where each farm is coloured by the degree of ivermectin resistance (EC_{50}) measured by larval development assays. In A, B and C, the position of *cky-1* is indicated by the

447 vertical dashed line. (**D**) RT-qPCR analysis of *cky-1* expression in both *H. contortus* and *T.*
448 *circumcincta* strains that differ in their ivermectin resistance phenotype. Data represents
449 log_2-transformed expression normalised to actin or GAPDH control genes for *H. contortus*
450 and *T. circumcincta,* respectively. Downregulation of *cky-1* expression in *C. elegans* by
451 either (**E**) a balanced deletion or (**F**) RNAi-knockdown increases ivermectin sensitivity
452 relative to the control N2 strain, based on developmental assays measuring the percentage
453 of progeny surviving to adulthood relative to DMSO controls. (**G**) Overexpression of *cky-1*
454 increases resistance to ivermectin relative to the control *C. elegans* N2 strain based on a
455 pharyngeal pumping assay. In **E, F,** and **G,** each point represents an independent treatment
456 condition, which is normalised to a DMSO control without ivermectin. A Kruskal-Wallis test
457 was used to determine whether treatment condition differed from untreated control, where
458 ns = not significant, * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

459 **Discussion**

460 Anthelmintics are currently the most important tool for controlling parasitic worm
461 infections in humans and animals worldwide, and this is likely to remain true for the
462 foreseeable future. However, this paradigm of control is threatened by the
463 emergence and spread of anthelmintic-resistant parasites. Despite the large health
464 and economic impacts resulting from increasing levels of anthelmintic resistance,
465 multiple complicating factors have hindered the ability to determine the genetic loci
466 responsible for resistance. Here we demonstrate an efficient approach to map
467 multiple drug resistance-conferring loci for three of the most important anthelmintic
468 drugs in the globally distributed and genetically tractable parasitic nematode, *H.*
469 *contortus*. We have identified novel variants and loci likely involved in resistance to
470 each of these drug classes; these include the β-tubulin isotype 2 Glu198Val variant
471 correlated with benzimidazole resistance in field populations, the *acr-8* Ser168Thr
472 variant associated with levamisole resistance in both the cross and field populations
473 of *H. contortus*, and *cky-1* as a novel candidate gene that mediates ivermectin
474 response. Our approach was validated by identifying QTLs and variants previously
475 associated with drug resistance, for example, the β-tubulin isotype 1 Phe200Tyr
476 variant associated with benzimidazole resistance and the *acr-8* indel variant
477 associated with levamisole resistance. However, for the latter, we provide evidence
478 against the indel being a reliable marker of resistance. Finally, we note an absence
479 of many previously proposed ivermectin-associated candidate genes in the QTL
480 described, highlighting both the limitation of candidate gene approaches and the
481 power of genome-wide forward-genetic strategies to robustly identify regions of the
482 genome containing known and novel mediators of resistance (*9*).
483

484 We have refined a previously identified QTL for ivermectin resistance on
485 chromosome 5 (*34*) to ~300 kb, and together with functional genetic evidence from
486 expression and knockout experiments, we have explicitly tested the role of our
487 proposed candidate in the main ivermectin QTL on chromosome 5, the NPAS4
488 ortholog *cky-1*. This gene encodes an activity-dependent basic Helix-Loop-Helix
489 (bHLH)-PAS family transcription factor shown in mammals to regulate the
490 excitation/inhibition balance upon neuronal activation to limit excitotoxicity (*59*) and

491 during the development of inhibitory synapses to control the expression of activity-
492 dependent genes (*60*). It is yet to be determined if this is a conserved molecular
493 function in nematodes; however, it is tempting to speculate that the
494 hyperexcitability as a result of induced activation of ion channels by ivermectin at
495 the neuromuscular junction is, at least in part, controlled by a "retuning" of the
496 excitation/inhibition balance to limit toxicity. The role of *cky-1* in ivermectin
497 resistance is supported by: (i) genetic differentiation between susceptible and
498 resistant strains around this locus relative to genome-wide variation that is
499 replicated in geographically and genetically diverse strains here and elsewhere (*25*,
500 *34*, *61*), (ii) the presence of non-synonymous variants that are highly differentiated
501 before and after treatment, (iii) increased gene expression of *cky-1* in resistant
502 strains relative to susceptible strains (supported by genome-wide RNA-seq (RNA-
503 seq paper)), (iv) knockdown of the *C. elegans* ortholog leading to hypersensitivity to
504 ivermectin, and (v) partial induction of resistance in *C. elegans* by overexpression of
505 *cky-1*. We acknowledge that overexpression of *cky-1* in *C. elegans* does not
506 recapitulate the high levels of ivermectin resistance seen in *H. contortus* or, for
507 example, by concurrent mutation of glutamate-gated chloride channels in *C.*
508 *elegans* (*21*); while this may argue against *cky-1* as a universal mediator of
509 resistance, it likely reflects the challenge of using a heterologous expression system
510 in which there is an assumption that the biology (and, therefore, response to
511 treatment) is concordant between the free-living and parasitic species, and/or may
512 reflect the multigenic nature of ivermectin resistance in different species (*62–64*).
513 Given the lack of an obvious causal non-synonymous variant, we hypothesise that a
514 non-coding variant that influences the expression of *cky-1* is under selection in
515 resistant strains of *H. contortus*; however, such variants are difficult to validate
516 without genotype and transcriptional phenotype data from a large number of
517 individual worms.
518
519 It is broadly accepted that the mode of action of ivermectin is on ligand-gated ion
520 channels, and ivermectin resistance has been associated with variants in glutamate-
521 gated channels (*65*). Concurrent mutation of a number of these channels (*glc-1, avr-*
522 *14* and *avr-15*) confers high-level resistance in *C. elegans* (*21*) and selection on at

523 least one of these channels (*glc-1*) in wild strains (*66*) has been demonstrated. We
524 find no evidence to suggest that genetic variation in these channels confers
525 ivermectin resistance in *H. contortus*. Transcriptional changes in these channels in
526 resistant, relative to drug-susceptible, parasite strains have been demonstrated
527 previously; for example, the glutamate-gated chloride channel subunits (*glc-3, glc-*
528 *5*), as well as p-glycoprotein ABC transporters (*pgp-1, pgp-2, pgp-9*) (*54*) in the
529 MHco18(UGA) strain. Similarly, a *pgp-9* copy number variant was associated with
530 ivermectin resistance in a genetic cross and bulk segregant experiment in the
531 related nematode *T. circumcincta (46)*, while transgenic overexpression of the
532 equine parasitic nematode *Parascaris univalens pgp-9* modulated ivermectin
533 sensitivity in *C. elegans* (*67*). However, none of these genes were identified in
534 regions of differentiation after treatment in this study, suggesting these genes are
535 not the direct target of selection. However, we cannot exclude that variation in
536 expression of these genes may be a downstream response to selection on a
537 transcriptional regulator such as *cky-1*.

538 The use of genetic crosses, in which the genetics of the parasites can be controlled,
539 is the ideal way to generate populations of individuals in which the relationship
540 between genotype and phenotype can be assayed. Our approach here relies on
541 selecting populations of parasites using drug treatment, however, advances are still
542 required to improve phenotyping of resistance in individual parasites. The ability to
543 do so would improve our understanding of the molecular basis of drug resistance
544 phenotypes and enable more sophisticated genetic approaches to unravel the role
545 of the minor signatures of selection we observe in this experiment. Recent
546 advances in single larvae whole-genome sequencing (*68*) and low-input RNA
547 sequencing (*69*), even at single-cell resolution (*70*), now provide the tools to allow a
548 more precise understanding of molecular and cellular phenotypes for drug response
549 and may help to fully understand the role of *cky-1*. The identification of *cky-1* as a
550 putative candidate offers new plausible hypotheses relevant to a resistant
551 phenotype, whereby *cky-1* may act: (i) during development to establish a neuronal
552 architecture that is more tolerant to hyperexcitability such as that caused by
553 ivermectin, and/or (ii) in response to ivermectin exposure by initiating transcription
554 of downstream genes to modulate the excessive excitation/inhibition imbalance,

555 thereby mitigating the lethal effect. These hypotheses will require further validation,
556 aided in the first instance by identifying the downstream targets of *cky-1*. However,
557 it is clear that the molecular mechanisms by which parasites develop ivermectin
558 resistance are more complex than previously appreciated. Broader, systems biology
559 approaches are likely needed to understand the relationship between direct
560 evidence of selection in the genome and the downstream transcriptional responses
561 that enable parasite survival when challenged with ivermectin. By defining the
562 genomic landscape of anthelmintic resistance even in a single resistant strain, our
563 results refocus effort away from candidate genes with limited support and redefine
564 our understanding of the evolution of anthelmintic resistance in helminths of
565 veterinary and medical importance.

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Author contributions:

Conceptualisation: RL, AT, ED, JAC

Investigation: SRD, RL, DB, AM, KM, AA, CB, UC, IF, JM

Formal analysis: SRD, RL

Software: SRD

Resources: DB, AM, RK, NS

Supervision: CB, JSG, NS, MB, ED, JAC

Project administration: NH, ED

Writing - Original Draft: SRD, JAC, RL, ED

Writing - Review & Editing: All authors

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852

853 **Competing interests:**

854 Authors declare that they have no competing interests.

855

856 **Data and materials availability:**

857 Raw sequencing data for this study are outlined in **table S1** and are archived under
858 the ENA study accession PRJEB4207. The *H. contortus* genome assembly and
859 manually curated annotation resources are publicly available at
860 https://parasite.wormbase.org/Haemonchus_contortus_prjeb506/Info/Index/. The
861 code used to generate and analyse data and to plot figures can be found at
862 https://github.com/stephenrdoyle/hcontortus_X-QTL.

863

864 **Supplementary Materials**

865 Materials and Methods

866 Supplementary Text

867 Figs. S1 to S10

868 Tables S1 to S2