The gene *scb-1* underlies variation in *Caenorhabditis elegans* chemotherapeutic responses

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ABSTRACT

35

36   Pleiotropy, the concept that a single gene controls multiple distinct traits, is

37   prevalent in most organisms and has broad implications for medicine and agriculture.

38   The identification of the molecular mechanisms underlying pleiotropy has the power to

39   reveal previously unknown biological connections between seemingly unrelated traits.

40   Additionally, the discovery of pleiotropic genes increases our understanding of both

41   genetic and phenotypic complexity by characterizing novel gene functions. Quantitative

42   trait locus (QTL) mapping has been used to identify several pleiotropic regions in many

43   organisms. However, gene knockout studies are needed to eliminate the possibility of

44   tightly linked, non-pleiotropic loci. Here, we use a panel of 296 recombinant inbred

45   advanced intercross lines of *Caenorhabditis elegans* and a high-throughput fitness

46   assay to identify a single large-effect QTL on the center of chromosome V associated

47   with variation in responses to eight chemotherapeutics. We validate this QTL with near-

48   isogenic lines and pair genome-wide gene expression data with drug response traits to

49   perform mediation analysis, leading to the identification of a pleiotropic candidate gene,

50   *scb-1* for some of the eight chemotherapeutics. Using deletion strains created by

51   genome editing, we show that *scb-1*, which was previously implicated in response to

52   bleomycin, also underlies responses to other double-strand DNA break-inducing

53   chemotherapeutics. This finding provides new evidence for the role of *scb-1* in the

54   nematode drug response and highlights the power of mediation analysis to identify

55   causal genes.

56

INTRODUCTION

57

58   Pleiotropy refers to the well established notion that a single gene or genetic

59 variant affects multiple distinct traits (Paaby and Rockman 2013), and the discovery of

60 pleiotropic genes can provide meaningful insights into the molecular mechanisms of

61 these traits (Tyler *et al.* 2016). It has become easier to identify pleiotropic genes with the

62 advent of reverse-genetic screens and quantitative trait locus (QTL) mapping (Paaby

63 and Rockman 2013). For example, pleiotropic QTL for diverse growth and fitness traits

64 have been identified in organisms such as yeast (Cubillos *et al.* 2011; Jerison *et al.*

65 2017; Peltier *et al.* 2019), *Arabidopsis* (McKay *et al.* 2003; El- Assal *et al.* 2004; Fusari

66 *et al.* 2017), *Drosophila* (Brown *et al.* 2013; McGuigan *et al.* 2014), and mice (White *et*

67 *al.* 2013; Leamy *et al.* 2014; Lin *et al.* 2014). These studies have led to important

68 questions in the field of evolutionary genetics regarding the 'cost of complexity' (Fisher;

69 Orr 2000), as a single mutation might be beneficial for one trait and harmful for another

70 (Wagner and Zhang 2011). Furthermore, human association studies have identified

71 pleiotropic variants associated with different diseases (Sivakumaran *et al.* 2011;

72 Pavlides *et al.* 2016; Chesmore *et al.* 2018), highlighting both the ubiquity and

73 importance of certain immune-related genes and oncogenes across unrelated diseases

74 (Borrello *et al.* 2008; Gratten and Visscher 2016). Perhaps the strongest evidence of

75 pleiotropy exists for molecular phenotypes. Large-scale expression QTL (eQTL)

76 mapping studies have identified single regulatory variants that control expression and

77 likely the functions of hundreds of genes at once, opening a window into the

78 mechanisms for how traits are controlled (Keurentjes *et al.* 2007; Breitling *et al.* 2008;

79   Rockman *et al.* 2010; Albert and Kruglyak 2015; Hasin-Brumshtein *et al.* 2016; Albert *et*

80   *al.* 2018).

81   The nematode *Caenorhabditis elegans* provides a tractable metazoan model to

82   identify and study pleiotropic QTL (Paaby and Rockman 2013). A large panel of

83   recombinant inbred advanced intercross lines (RIAILs) derived from two divergent

84   strains, N2 and CB4856 (Rockman and Kruglyak 2009; Andersen *et al.* 2015), has been

85   leveraged in several linkage mapping analyses (Li *et al.* 2006; Gutteling *et al.* 2007b,

86   2007a; Kammenga *et al.* 2007; Seidel *et al.* 2008, 2011; Reddy *et al.* 2009; McGrath *et*

87   *al.* 2009; Doroszuk *et al.* 2009; Viñuela *et al.* 2010; Rockman *et al.* 2010; Bendesky *et*

88   *al.* 2011, 2012; Bendesky and Bargmann 2011; Rodriguez *et al.* 2012; Andersen *et al.*

89   2014; Glater *et al.* 2014; Snoek *et al.* 2014; Balla *et al.* 2015; Schmid *et al.* 2015; Singh

90   *et al.* 2016; Zdraljevic *et al.* 2017, 2019; Lee *et al.* 2017; Zamanian *et al.* 2018a; Evans

91   *et al.* 2018; Brady *et al.* 2019). Quantitative genetic analysis using these panels and a

92   high-throughput phenotyping assay (Andersen *et al.* 2015) has facilitated the discovery

93   of numerous QTL (Zamanian *et al.* 2018b), several quantitative trait genes (QTG)

94   (Brady *et al.* 2019) and quantitative trait nucleotides (QTN) (Zdraljevic *et al.* 2017, 2019)

95   underlying fitness-related traits in the nematode. Additionally, three pleiotropic genomic

96   regions were recently found to influence responses to a diverse group of toxins (Evans

97   *et al.* 2018). However, overlapping genomic regions might not represent true pleiotropy

98   but could demonstrate the co-existence of tightly linked loci (Paaby and Rockman

99   2013).

100   Here, we use linkage mapping to identify a single overlapping QTL on

101   chromosome V that influences the responses to eight chemotherapeutic compounds.

102   We show that these drug-response QTL also overlap with an expression QTL hotspot

103   that contains the gene *scb-1*, previously implicated in bleomycin response (Brady *et al.*

104   2019). Although the exact mechanism of *scb-1* is yet unknown, it is hypothesized to act

105   in response to stress (Riedel *et al.* 2013) and has weak homology to a viral hydrolase

106   (Kelley *et al.* 2015; Zhang *et al.* 2018). Together, these data suggest that the

107   importance of *scb-1* expression might extend beyond bleomycin response. We validated

108   the QTL using near-isogenic lines (NILs) and performed mediation analysis to predict

109   that *scb-1* expression explains the observed QTL for four of the eight drugs. Finally, we

110   directly tested the effect of *scb-1* loss of function on chemotherapeutic responses. We

111   discovered that expression of *scb-1* underlies differential responses to several

112   chemotherapeutics that cause double-strand DNA breaks, not just bleomycin. This

113   discovery of pleiotropy helps to further define the role of *scb-1* by expanding its known

114   functions and provides insights into the molecular mechanisms underlying the

115   nematode drug response.

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118

119   **MATERIALS AND METHODS**

120   **Strains**

121   Animals were grown at 20°C on modified nematode growth media (NGMA)

122   containing 1% agar and 0.7% agarose to prevent burrowing and fed OP50 (Ghosh *et al.*

123   2012). The two parental strains, the canonical laboratory strain, N2, and the wild isolate

124   from Hawaii, CB4856, were used to generate all recombinant lines. 208 recombinant

125   inbred advanced intercross lines (RIAILs) generated previously by Rockman *et al.*

126   (Rockman and Kruglyak 2009) (set 1 RIAILs) were phenotyped for expression QTL

127   mapping (detailed below). A second set of 296 RIAILs generated previously by

128   Andersen *et al.* (Andersen *et al.* 2015) (set 2 RIAILs) was used more extensively for

129   drug phenotyping and linkage mapping. The set 2 RIAILs were used for linkage

130   mapping because they addressed the three main disadvantages of the set 1 RIAILs

131   detailed previously (Andersen *et al.* 2015), namely a structured population, the

132   laboratory-derived variant in *npr-1 (Sterken et al. 2015)*, and the *peel-1 zeel-1*

133   incompatibility (Seidel *et al.* 2008, 2011). Because of these limitations, the set 2 RIAILs

134   were generated using QX1430 and CB4856. QX1430 is from the N2 strain background

135   but contains a transposon insertion in *peel-1* and the CB4856 *npr-1* allele introgressed

136   on chromosome X (Andersen *et al.* 2015). Near-isogenic lines (NILs) were generated by

137   backcrossing a selected RIAIL for several generations to the parent strain (N2 or

138   CB4856) (Brady *et al.* 2019) using PCR amplicons for insertion-deletion (indels) variants

139   to track the introgressed region. NILs were whole-genome sequenced to verify

140   introgressions were only in the targeted genomic intervals. CRISPR-Cas9-mediated

141   deletions of *scb-1* were described previously (Brady *et al.* 2019). All strains are

142   available upon request or from the *C. elegans* Natural Diversity Resource (Cook *et al.*

143   2016). Primers used to generate ECA1114 can be found in the **Supplemental**

144   **Information**.

145

High-throughput fitness assays for linkage mapping

146
147   For dose responses and RIAIL phenotyping, we used a high-throughput fitness

148   assay described previously (Andersen *et al.* 2015). In summary, populations of each

149   strain were passaged and amplified on NGMA plates for four generations. In the fifth

150   generation, gravid adults were bleach-synchronized and 25-50 embryos from each

151   strain were aliquoted into 96-well microtiter plates at a final volume of 50 µL K medium

152   (Boyd *et al.* 2012). The following day, arrested L1s were fed HB101 bacterial lysate

153   (Pennsylvania State University Shared Fermentation Facility, State College, PA;

154   (García-González *et al.* 2017)) at a final concentration of 5 mg/mL in K medium and

155   were grown to the L4 larval stage for 48 hours at 20°C with constant shaking. Three L4

156   larvae were sorted into new 96-well microtiter plates containing 10 mg/mL HB101

157   bacterial lysate, 50 µM kanamycin, and either diluent (1% water or 1% DMSO) or drug

158   dissolved in the diluent using a large-particle flow cytometer (COPAS BIOSORT, Union

159   Biometrica; Holliston, MA). Sorted animals were grown for 96 hours at 20°C with

160   constant shaking. The next generation of animals and the parents were treated with

161   sodium azide (50 mM in 1X M9) to straighten their bodies for more accurate length

162   measurements. Animal length (median.TOF), optical density integrated over animal

163   length (median.EXT), and brood size (norm.n) were quantified for each well using the

164   COPAS BIOSORT. Nematodes get longer (animal length) and become thicker and

165   more complex (optical density) over developmental time. Because animal length and

166   optical density are highly correlated, we calculated a fourth trait (median.norm.EXT) that

167   normalizes optical density by animal length (median.EXT / median.TOF). Phenotypic

168   measurements collected by the BIOSORT were processed and analyzed using the R

169   package *easysorter* (Shimko and Andersen 2014) as described previously (Brady *et al.*

170   2019). Differences among strains within the control conditions were controlled by

171   subtracting the mean control-condition value from each drug-condition replicate for each

172   strain using a linear model (*drug_phenotype ~ mean_control_phenotype*). In this way,

173   we are addressing only the differences among strains that were caused by the drug

174   condition and the variance in the control condition does not affect the variance in the

175   drug condition. For plotting purposes, these residual values (negative and positive

176   residuals) were normalized from 0 to 1 where 0 refers to the smallest residual

177   phenotypic value in that condition and 1 refers to the largest.

178

179   **Dose-response assays**

180   Four genetically divergent strains (N2, CB4856, JU258, and DL238) were treated

181   with increasing concentrations of each of the eight drugs using the high-throughput

182   fitness assay described above. The dose of each drug that provided a reproducible

183   drug-specific effect that maximizes between-strain variation while minimizing within-

184   strain variation across the four traits was selected for the linkage mapping experiments.

185   The chosen concentrations are as follows: 100 μM amsacrine hydrochloride (Fisher

186   Scientific, #A277720MG) in DMSO, 50 μM bleomycin sulfate (Fisher, #50-148-546) in

187   water, 2 μM bortezomib (VWR, #AAJ60378-MA) in DMSO, 250 μM carmustine (Sigma,

188   #1096724-75MG) in DMSO, 500 μM cisplatin (Sigma, #479306-1G) in K media, 500 μM

189   etoposide (Sigma, #E1383) in DMSO, 500 μM puromycin dihydrochloride (VWR,

190   #62111-170) in water, and 150 μM silver nitrate (Sigma-Aldrich, #S6506-5G) in water.

191

192   **Linkage mapping**

193   Set 1 and set 2 RIAILs were phenotyped in each of the eight drugs and controls

194   using the high-throughput fitness assay described above. Linkage mapping was

195   performed on each of the drug and gene expression traits using the R package

196   *linkagemapping* (https://github.com/AndersenLab/linkagemapping) as described

197   previously (Brady *et al.* 2019). The cross object derived from the whole-genome

198   sequencing of the RIAILs containing 13,003 SNPs was loaded using the function

199   *load_cross_obj("N2xCB4856cross_full")*. The RIAIL phenotypes were merged into the

200   cross object using the *merge_pheno* function with the argument *set = 1* for expression

201   QTL mapping and *set = 2* for drug phenotype mapping. A forward search (*fsearch*

202   function) adapted from the *R/qtl* package (Broman *et al.* 2003) was used to calculate the

203   logarithm of the odds (LOD) scores for each genetic marker and each trait as $-n(ln(1-$

204     $R^2)/2ln(10))$ where R is the Pearson correlation coefficient between the RIAIL genotypes

205   at the marker and trait phenotypes (Bloom *et al.* 2013). A 5% genome-wide error rate

206   was calculated by permuting the RIAIL phenotypes 1000 times. The marker with the

207   highest LOD score above the significance threshold was selected as the QTL then

208   integrated into the model as a cofactor and mapping was repeated iteratively until no

209   further significant QTL were identified. Finally, the *annotate_lods* function was used to

210   calculate the effect size of each QTL and determine 95% confidence intervals defined

211   by a 1.5 LOD drop from the peak marker using the argument *cutoff = proximal*.

212

213   **Modified high-throughput fitness assay for NIL validation**

214   NILs and *scb-1* deletion strains were tested using a modified version of the high-

215   throughput fitness assay detailed above. Strains were propagated for two generations,

216   bleach-synchronized in three independent replicates, and titered at a concentration of

217   25-50 embryos per well of a 96-well microtiter plate. The following day, arrested L1s

218   were fed HB101 bacterial lysate at a final concentration of 5 mg/mL with either diluent or

219   drug. After 48 hours of growth at 20°C with constant shaking, nematodes were treated

220   with sodium azide (5 mM in water) prior to analysis of animal length and optical density

221   using the COPAS BIOSORT. As only one generation of growth is observed, brood size

222   was not calculated. A single trait (median.EXT) was chosen to represent animal growth

223   generally, as the trait is defined by integrating optical density over length. Because of

224   the modified timing of the drug delivery, lower drug concentrations were needed to

225   recapitulate the previously observed phenotypic effect. The selected doses are as

226   follows: 12.5 µM amsacrine in DMSO, 12.5 µM bleomycin in water, 2 µM bortezomib in

227   DMSO, 250 µM carmustine in DMSO, 125 µM cisplatin in K media, 62.5 µM etoposide

228   in DMSO, 300 µM puromycin in water, and 100 µM silver in water.

229

230   **Expression QTL analysis**

231   Microarray data for gene expression using 15,888 probes were previously

232   collected from synchronized young adult populations of 208 set 1 RIAILs (Rockman *et*

233   *al.* 2010). Expression data were corrected for dye effects and probes with variants were

234   removed (Andersen *et al.* 2014). Linkage mapping was performed as described above

235   for the remaining 14,107 probes, and a significance threshold was determined using a

236   permutation-based False Discovery Rate (FDR). FDR was calculated as the ratio of the

237   average number of genes across 10 permutations expected by chance to show a

238   maximum LOD score greater than a particular threshold vs. the number of genes

239   observed in the real data with a maximum LOD score greater than that threshold. We

240   calculated the FDR for a range of thresholds from 2 to 10, with increasing steps of 0.01,

241   and set the threshold so that the calculated FDR was less than 5%.

242   Local eQTL were defined as linkages whose peak LOD scores were within 1 Mb

243   of the starting position of the probe (Rockman *et al.* 2010). eQTL hotspots were

244   identified by dividing the genome into 5 cM bins and counting the number of distant

245   eQTL that mapped to each bin. Significance was determined as bins with more eQTL

246   than the Bonferroni-corrected 99th percentile of a Poisson distribution with a mean of

247   3.91 QTL (total QTL / total bins) (Brem *et al.* 2002; Rockman *et al.* 2010; Evans *et al.*

248   2018). We identified nine eQTL hotspots (II, IVL, IVC, IVR, VL, VC, VR, XL, and XC). To

249   avoid false positives, we increased the LOD threshold for QTL to be counted in the

250   hotspot analysis to a LOD > 5 or LOD > 6. At a LOD > 5, six of the nine eQTL hotspots

251   persist (IVL, IVR, VC, VR, XL, and XC), and at a LOD > 6, three persist (IVL, IVR, and

252   XL). We further looked for spurious eQTL hotspots in ten permuted datasets. At a LOD

253   > 5, we identified four hotspots, and at a LOD > 6, we identified one hotspot.

254

255   **Mediation analysis**

256   A total of 159 set 1 RIAILs were phenotyped in each of the eight drugs and

257   controls using the standard high-throughput fitness assay described above. Mediation

258   scores were calculated with bootstrapping using the *mediate* function from the

259   *mediation* R package (version 4.4.7) (Tingley *et al.* 2014) for each QTL identified from

260   the set 1 RIAILs and all 49 probes (including *scb-1,* A_12_P104350) that mapped to the

261   chromosome V eQTL hotspot using the following models:

262

263   (1) Mediator model: *lm(expression ~ genotype)*

264   (2) Outcome model: *lm(phenotype ~ expression + genotype)*

265

266   The output of the *mediate* function can be summarized as follows: the total effect

267   of genotype on phenotype, ignoring expression (*tau.coef*); the direct effect of genotype

268   on phenotype, while holding expression constant (*z0*); the estimated effect of

269   expression on phenotype (*d0*); the proportion of the total effect that can be explained by

270   expression data (*n0*). This mediation proportion (*n0*) can be a useful way to identify the

271   impact of gene expression on the overall phenotype. However, cases of inconsistent

272   mediation (where the direct effect is either smaller than or in the opposite direction of

273   the indirect mediation effect) render this measurement uninterpretable with values

274   greater than one or less than zero (MacKinnon *et al.* 2007). We used the estimated

275   effect of expression on phenotype (*z0*) as the final mediation score for this reason.

276   Because the effect size can be positive or negative, mediation scores range from -1 to

277   1, and we evaluated the absolute value of mediation estimates to compare across traits.

278   Each mediation estimate generated a p-value, indicating confidence in the estimate,

279   derived from bootstrapping with 1000 simulations. The likelihood of *scb-1* mediating a

280   given QTL effect was calculated relative to the other 48 probes with an eQTL in the

281   region (**Table S1**). Traits in which *scb-1* was at or above the 90th percentile of this

282   distribution were prioritized over other traits.

283

Statistical analysis

285   Broad-sense heritability was calculated from the dose response phenotypes

286   using the *lmer* function in the *lme4* R package (Bates *et al.* 2014) with the formula

287   *phenotype ~ 1 + (1|strain)* for each dose. For the NIL and *scb-1* deletion high-

288   throughput assays, statistical significance of phenotypic differences between each strain

289   pair was tested using the *TukeyHSD* function (R Core Team 2017) on an ANOVA model

290   with the formula *phenotype ~ strain* to assess differences between strains in the control-

291   regressed phenotype data.

292

Data Availability

294   **File S1** contains the results of the original dose response high-throughput fitness

295   assay. **File S2** contains the residual phenotypic values for all 159 set 1 RIAILs, 296 set

296   2 RIAILs, and parent strains (N2 and CB4856) in response to all eight

297   chemotherapeutics. **File S3** contains the linkage mapping results for the set 2 RIAILs for

298   all 32 drug-response traits tested in the high-throughput fitness assay. **File S4** is a VCF

299   that reports the genotype of ECA1114. **File S5** contains the simplified genotype of all

300   the NILs in the study. **File S6** contains the raw pruned phenotypes for the NIL dose

301   response with the modified high-throughput fitness assay. **File S7** contains the pairwise

302   statistical significance for all strains and high-throughput assays. **File S8** contains the

303   microarray expression data for 14,107 probes from Rockman *et al.* 2010. **File S9**

304 contains the linkage mapping results for the expression data obtained with the set 1

305 RIAILs. **File S10** contains the location of each eQTL hotspot and a list of genes with an

306 eQTL in each hotspot. **File S11** contains the linkage mapping results from the set 1

307 RIAILs for all 32 drug-response traits tested in the high-throughput fitness assay. **File**

308 **S12** contains the pairwise mediation estimates for all 32 drug-response traits and all 49

309 probes. **File S13** contains the raw pruned phenotypes for the *scb-1* deletion modified

310 high-throughput fitness assay. The datasets and code for generating figures can be

311 found at https://github.com/AndersenLab/scb1_mediation_manuscript. Supplemental

312 materials are available at Figshare.

313

314 **RESULTS**

315 **Natural variation on chromosome V underlies differences in responses to several**

316 **chemotherapeutics**

317   We measured *C. elegans* development and chemotherapeutic sensitivity as a

318 function of animal length (median.TOF), optical density (median.EXT), and brood size

319 (norm.n) with a high-throughput assay developed using the COPAS BIOSORT (see

320 Methods) (Andersen *et al.* 2015; Zdraljevic *et al.* 2017, 2019; Evans *et al.* 2018; Brady

321 *et al.* 2019). Animal length and optical density (animal thickness and composition) are

322 both measures of nematode development, and brood size is a measure of nematode

323 reproduction (Andersen *et al.* 2015). Because optical density is calculated as a function

324 of length and these traits are related, a fourth trait that captures the optical density

325 normalized by length (median.norm.EXT) was also included. We exposed four

326 genetically divergent strains (N2, CB4856, JU258, and DL238) to increasing doses of

327 eight chemotherapeutic compounds. Five of these compounds (bleomycin, carmustine,

328 etoposide, amsacrine, and cisplatin) are known to cause double-strand DNA breaks

329 and/or inhibit DNA synthesis (Dorr 1992; Ketron *et al.* 2012; Dasari and Tchounwou

330 2014; Montecucco *et al.* 2015; Nikolova *et al.* 2017). The remaining three compounds

331 either inhibit protein synthesis (puromycin) (Azzam and Algranati 1973), inhibit the

332 proteosome and subsequent protein degradation (bortezomib) (Piperdi *et al.* 2011), or

333 cause cellular toxicity in a poorly defined way (silver nitrate) (Kaplan *et al.* 2016) (**Table

334 1**). In the presence of each drug, nematodes were generally shorter, less optically

335 dense, and produced smaller broods compared to non-treated nematodes (**Figure S1,

336 File S1**). We observed significant phenotypic variation among strains and identified a

337 substantial heritable genetic component for most traits (average H^2 = 0.52 +/- 0.53).

338 We exposed a panel of 296 RIAILs (set 2 RIAILs, see Methods) to all eight

339 chemotherapeutics at a selected concentration that both maximizes among-strain and

340 minimizes within-strain phenotypic variation (**File S2**). Linkage mapping for all four traits

341 for each of the eight drugs (total of 32 traits) identified 79 QTL from 31 traits (one trait

342 had no significant QTL), several of which have been identified previously (Zdraljevic *et

343 al.* 2017; Evans *et al.* 2018; Brady *et al.* 2019) (**File S3, Figure S2**). Strikingly, a QTL on

344 the center of chromosome V was linked to variation in responses to all eight compounds

345 (**Figure 1**). In all cases, the CB4856 allele on chromosome V is associated with greater

346 resistance to the drug than the N2 allele (**Figure S2, File S2, File S3**). We previously

347 identified this genomic interval as a QTL hotspot, defined as a region heavily enriched

348 for toxin-response QTL (Evans *et al.* 2018). Because several of the chemotherapeutics

349 share a similar mechanism of action, a single pleiotropic gene might underlie the

350 observed QTL for multiple drugs.

351   In order to isolate and validate the effect of this QTL, we constructed reciprocal

352 near-isogenic lines (NILs) by introgressing a genomic region on chromosome V from the

353 resistant CB4856 strain into the sensitive N2 background and vice versa (**File S4**, **File**

354 **S5**). We used a modified high-throughput assay (see Methods) to measure length and

355 optical density of a population of animals grown in the presence of the drug for 48 hours

356 (from larval stages L1 to L4). In this modified assay, less drug was required to observe

357 the same phenotypic effect as before (**Figure S3, File S6**). Statistical significance was

358 calculated in a pairwise manner for each strain (see Methods; **File S7**). For all eight

359 chemotherapeutics tested, the strain with the N2 introgression was significantly more

360 sensitive than its CB4856 parent and/or the strain with the CB4856 introgression was

361 significantly more resistant than its N2 parent (**Figure 2, File S6, File S7**). These data

362 confirm that one or more genetic variant(s) within this region on chromosome V cause

363 increased drug sensitivities in N2.

364

365 **Expression QTL mapping identifies a hotspot on the center of chromosome V**

366   Genetic variation can affect a phenotype most commonly through either

367 modifications of the amino acid sequence that lead to altered protein function (or even

368 loss of function) or changes in the expression level of the protein. In the latter case,

369 measuring the intermediate phenotype (gene expression) can be useful in elucidating

370 the mechanism by which genetic variation causes phenotypic variation. More

371 specifically, cases with overlap between expression QTL (eQTL) and drug-response

372 QTL suggest that a common variant could underlie both traits and provide evidence in

373 support of causality for the candidate gene in question (Huang *et al.* 2015; Sasaki *et al.*

374 2018).

375   To identify such cases of overlap between expression QTL and the drug-

376 response QTL on chromosome V, we need genome-wide expression data for the

377 RIAILs. In a previous study, expression of 15,888 probes were measured using

378 microarrays for a panel of 208 RIAILs (set 1 RIAILs, see Methods) between N2 and

379 CB4856 (Rockman and Kruglyak 2009) (**File S8**). This study used the variation in gene

380 expression as a phenotypic trait to identify eQTL using linkage mapping with 1,455

381 variants (Rockman *et al.* 2010). They identified 2,309 eQTL and three regions with

382 significantly clustered distant eQTL (eQTL hotspots), suggesting that these regions are

383 pleiotropic, wherein one or more variant(s) are affecting expression of multiple genes.

384 We recently performed whole-genome sequencing for these strains and identified

385 13,003 informative variants (Brady *et al.* 2019). Using this new set of variants, we re-

386 analyzed the eQTL mapping by performing linkage mapping analysis for a selected

387 14,107 of the 15,888 probes without genetic variation in CB4856 (Andersen *et al.* 2014).

388 We identified 2,540 eQTL associated with variation in expression of 2,196 genes

389 (**Figure 3A, File S9**). These eQTL have relatively large effect sizes compared to the

390 drug-response QTL. On average, each eQTL explains 23% of the phenotypic variance

391 in gene expression among the RIAIL population. Half of the eQTL (50.2%; 1,276)

392 mapped within 1 Mb of the gene whose expression was measured and were classified

393 as local (see Methods) (Albert and Kruglyak 2015). The other half (49.7%; 1,264) were

394 found distant from their respective gene, and over a third (37%; 940) were found on

395   different chromosomes entirely. In general, eQTL effect sizes increased, max LOD

396   scores decreased, and confidence intervals became smaller compared to the original

397   mapping results (**File S9**). These differences and the additional eQTL observed

398   between this analysis and the original are possibly caused by the integration of new

399   genetic markers. Additionally, we found several differences in methodology between the

400   current approach and the previous one. These differences include ignoring the

401   population structure of the set 1 RIAILs, adding the forward-search marker-regression

402   linkage mapping, and altering the linkage mapping method itself (see Methods,

403   (Rockman *et al.* 2010)).

404   We noticed regions of the genome that appeared to be enriched for distant

405   eQTL. We identified eQTL hotspots in a similar manner to the previous study (see

406   Methods) and found a total of nine eQTL hotspots (**Figure 3B, File S10**). Six of the nine

407   eQTL hotspots withstood more stringent filtering methods (see Methods), and three (left

408   of chromosome IV, right of chromosome IV, and left of chromosome X) were the most

409   significant. These three hotspots also overlap with the most significant eQTL hotspots in

410   the previous study (Rockman *et al.* 2010). Notably, three of the eQTL hotspots (center

411   of chromosome IV, right of chromosome IV, and center of chromosome V) overlap with

412   the previously identified drug-response QTL hotspots on chromosomes IV and V

413   (**Figure 3B**) (Evans *et al.* 2018). The overlap of these eQTL and drug-response QTL

414   hotspots could provide strong candidate genes whose expression underlies the

415   differences in nematode drug responses generally. Expression of one gene of interest,

416   *scb-1*, has been previously implicated in response to bleomycin (Brady *et al.* 2019) and

417   resides within the eQTL hotspot region on the center of chromosome V (**File S10, Table**

418   **S1**). Although the exact mechanism of how *scb-1* responds to bleomycin is unknown, its

419   putative hydrolase activity (Kelley *et al.* 2015; Zhang *et al.* 2018; Brady *et al.* 2019)

420   suggests that it might act to break down chemotherapeutic compounds. These data

421   suggest that variation in expression of *scb-1* and responses to these eight

422   chemotherapeutics (including bleomycin) could be mechanistically linked through the

423   metabolic breakdown of chemotherapeutic drugs.

424

425   **Mediation analysis suggests that *scb-1* expression plays a role in responses to**

426   **several chemotherapeutics**

427   Mediation analysis seeks to explain the relationship between an independent and

428   a dependent variable by including a third intermediary variable. We can use mediation

429   analysis to understand how certain genetic variants on chromosome V (independent

430   variable) affect drug responses (dependent variable) through differential gene

431   expression of genes within the eQTL hotspot (mediator variable) (**Figure S4**). We

432   measured brood size, animal length, and optical density in response to all eight

433   chemotherapeutics in the set 1 RIAILs and performed linkage mapping for these traits

434   (**File S2, File S11, Figure S5**). Although the power to detect QTL with these strains is

435   lower than in our original mapping set (set 2 RIAILs; see Methods) (Andersen *et al.*

436   2015), we still identified overlapping QTL on chromosome V for half of the drugs tested

437   (bleomycin, cisplatin, silver, and amsacrine) (**Figure S5, File S11**).

438   We calculated the effect that variation in expression of *scb-1* had on drug-

439   response traits compared to the other 48 genes with an eQTL in the chromosome V

440   eQTL hotspot using mediation analysis (see Methods). We estimated that the effect of

441   expression variation of *scb-1* on bleomycin response is 0.65 (set 1 RIAILs, **Figure 4,**

442   **Figure S6, File S12**). Moreover, out of all 49 genes with an eQTL in the region (**Table**

443   **S1**), *scb-1* was a clear mediation score outlier. All of the remaining three

444   chemotherapeutics with a QTL on the center of chromosome V in the set 1 RIAIL

445   mapping showed moderate evidence of *scb-1* mediation, with *scb-1* falling well above

446   the 90th percentile of mediation estimates for all genes with an eQTL in this region

447   (**Figure 4, Figure S6, File S12**). We further performed this mediation analysis on all 32

448   drug-response traits, regardless of the presence of a QTL in the set 1 RIAIL panel

449   (**Figure S6**, **File S12**). Etoposide and puromycin also showed evidence of *scb-1*

450   mediation. This *in silico* approach indicated that expression of *scb-1* might be an

451   intermediate link between genetic variation on chromosome V and responses to several

452   of the chemotherapeutic drugs tested.

453

454   **Expression of *scb-1* affects responses to several chemotherapeutics that cause**

455   **double-strand DNA breaks**

456   To empirically test whether *scb-1* expression modulates the chromosome V QTL

457   effect for each drug, we used the modified high-throughput assay (see Methods) to

458   expose two independently derived strains with *scb-1* deletions (Brady *et al.* 2019) to

459   each chemotherapeutic (**Figure 5, Figure S7, File S13**). Statistical significance was

460   calculated in a pairwise manner for each strain (see Methods; **File S7**). Because RIAILs

461   with the CB4856 allele on chromosome V express higher levels of *scb-1* than RIAILs

462   with the N2 allele (**File S8, File S9**), we expect that loss of *scb-1* will cause increased

463   drug sensitivity in the CB4856 background but might not have an effect in the N2

464   background. We validated the results of Brady *et al.* and confirmed that ablated *scb-1*

465   expression causes hyper-sensitivity to bleomycin in both N2 and CB4856 (**Figure 5,**

466   **Figure S7, Figure S8 File S7, File S13**). We also observed similarly increased

467   sensitivity to cisplatin with *scb-1* deletions in both backgrounds. Furthermore, removing

468   *scb-1* shows moderately increased sensitivity in the CB4856 background for amsacrine

469   and in the N2 background for carmustine. The remaining four drugs did not show a

470   significantly different phenotype between the parental N2 and CB4856 strains,

471   suggesting these traits might be less reproducible or that *scb-1* variation does not

472   underlie these drug differences. Overall, these results provide evidence for the

473   pleiotropic effect of *scb-1*, which appears to mediate responses to at least four of the

474   eight chemotherapeutic drugs.

475

476   **DISCUSSION**

477   In this study, we identified overlapping QTL on the center of chromosome V that

478   influence sensitivities to eight chemotherapeutic drugs. Because five of these drugs are

479   known to cause double-strand DNA breaks, we hypothesized that this genomic region

480   might be pleiotropic – a single shared genetic variant affects the responses to each

481   drug. Because this variant might affect drug responses by regulating gene expression

482   levels, we looked for the co-existence of drug-response QTL and expression QTL on

483   chromosome V. We identified 2,540 eQTL and nine eQTL hotspots, including a region

484   on the center of chromosome V. We calculated the mediation effect of all 49 genes with

485   an eQTL that maps to this hotspot region and identified *scb-1* as a candidate gene

486   whose expression influences the responses to several chemotherapeutics. We used

487   CRISPR-Cas9-mediated *scb-1* deletion strains to empirically validate the role of *scb-1* in

488   the chemotherapeutic response. In addition to bleomycin (Brady *et al.* 2019), we

489   discovered that responses to cisplatin, amsacrine, and carmustine are affected by *scb-1*

490   expression. In this study, we found evidence that several overlapping QTL are

491   representative of pleiotropy at the gene level and further elucidated the function of *scb-1*

492   as a potential response to double-strand DNA break stress.

493

494   **Mediation of drug-response QTL using gene expression to identify causal genes**

495   Mediation analysis often suggests potential candidate genes that underlie

496   different traits (Huang *et al.* 2015; Sasaki *et al.* 2018) and could be applied to drug

497   responses. Using *C. elegans* strains and high-throughput assays, we can rapidly

498   validate hypotheses generated by mediation analysis. Three of the eight

499   chemotherapeutics that map to an overlapping drug-response QTL and were potentially

500   mediated by *scb-1* were validated using targeted deletion strains.

501   Although mediation analysis provided moderate evidence that expression of *scb-*

502   *1* could also play a role in sensitivity to etoposide and puromycin, we observed no

503   experimental evidence of this relationship. Additionally, we have evidence that

504   expression of *scb-1* might mediate response to carmustine. However, mediation

505   analysis disagrees. The discrepancy between the mediation analysis and validation of

506   causality using targeted deletion strains could be partially explained by one of several

507   possibilities. First, different traits were measured in each experiment. The mediation

508   analysis used traits measured over 96 hours of growth in drug conditions spanning two

509   generations, but the causality test used traits measured over 48 hours of growth in drug

510   conditions within one generation. Second, the precision of our mediation estimates was

511   likely reduced by the poor quality drug traits for the set 1 RIAIL panel (Andersen *et al.*

512   2015). Indeed, bortezomib, carmustine, etoposide, and puromycin did not map to the

513   center of chromosome V using the set 1 RIAILs (**Figure S5**). Expression data for the set

514   2 RIAIL panel would likely generate more accurate mediation estimates, especially if

515   data were collected using RNA sequencing to avoid the inherent reference bias of

516   microarray data (Zhao *et al.* 2014). Third, our mediation analysis was performed using

517   expression data collected in control conditions and phenotype data collected in drug

518   conditions. This analysis will only provide evidence of mediation if the baseline

519   expression differences affect an individual's response to the drug. Collecting expression

520   data from drug-treated nematodes could help us learn more about how gene expression

521   varies in response to treatment with the chemotherapeutic. Finally, as we only directly

522   assessed the complete loss of *scb-1* in drug sensitivity, it is still possible that reduction

523   of function (or change in function) caused by a single nucleotide variant or other

524   structural variation in CB4856 could validate the role of *scb-1* in responses to these

525   drugs.

526   This study demonstrates the power of pairing genome-wide linkage mapping of

527   gene expression and drug response data using simple colocalization as well as more

528   complex mediation analysis techniques. In addition to providing a resource for

529   candidate gene prioritization within a QTL interval, mediation analysis can help to

530   identify the mechanism by which genetic variation causes phenotypic differences. This

531   type of approach could be even more powerful using genome-wide association (GWA)

532   where the lower linkage disequilibrium between variants also has smaller confidence

533 intervals in some genomic regions. Smaller intervals have fewer spurious overlapping

534 eQTL, which could help to narrow the list of candidate genes. Although mediation

535 analysis is only effective if a change in expression is observed and might not be useful

536 for identifying effects from protein-coding variation, many current studies show that the

537 majority of genetic variants associated with complex traits lie in regulatory regions

538 (Hindorff *et al.* 2009). Whole-genome expression analysis could provide the missing link

539 to the identification of causal genes underlying complex traits.

540

541 **New evidence for the pleiotropic function of *scb-1***

542 We identified eight chemotherapeutics with a QTL that mapped to a genomic

543 region defined as a QTL hotspot on the center of chromosome V (Evans *et al.* 2018).

544 Multiple genes in close proximity, each regulating an aspect of cellular growth and

545 fitness, might underlie each QTL independently. Alternatively, genetic variation within a

546 single gene might regulate responses to multiple (or all) of the eight drugs tested,

547 particularly if the gene is involved in drug transport or metabolism or if the drug

548 mechanisms of action were shared (*e.g.* repair of double-strand DNA breaks).

549 Expression of *scb-1*, a gene previously implicated in modulating responses to

550 bleomycin, was found to reduce sensitivity to half of the drugs tested. This pleiotropic

551 effect of *scb-1* provides new evidence for the function of the gene and possible

552 molecular mechanisms underlying nematode drug responses. It is hypothesized that

553 SCB-1 might function as a hydrolase that metabolizes compounds like bleomycin

554 (Brady *et al.* 2019) or somehow plays a role in the nematode stress response (Riedel *et*

555 *al.* 2013). Both hypotheses are consistent with our data, explaining why nematodes with

556   low expression of *scb-1* are highly sensitive to the compound. Furthermore, all four of

557   these chemotherapeutics, whose responses are mediated by expression of *scb-1*, are

558   known to cause double-strand DNA breaks (Dorr 1992; Ketron *et al.* 2012; Dasari and

559   Tchounwou 2014; Nikolova *et al.* 2017). Although the results for bortezomib, puromycin,

560   and silver were inconclusive, we found no clear evidence that expression of *scb-1*

561   dictates their responses. Together, these data suggest a potential role for *scb-1*

562   specifically in response to stress induced by double-strand DNA breaks. However, the

563   lack of sensitivity in etoposide, which also causes double-strand DNA breaks

564   (Montecucco *et al.* 2015), indicates that this response might be more complex.

565   The exact variant that causes the differential expression of *scb-1* is still unknown.

566   Importantly, *scb-1* lies within an eQTL hotspot region where it is hypothesized that

567   genetic variation at a single locus might regulate expression of the 49 genes with an

568   eQTL in this region. It is possible that the same causal variant that regulates expression

569   of *scb-1* could also underlie the QTL for the remaining four chemotherapeutics through

570   differential expression of other genes. For example, mediation analysis for both

571   bortezomib and etoposide indicated that expression variation of a dehydrogenase

572   (*D1054.8*) may underlie their differential responses (**File S12**). Alternatively, the causal

573   variants underlying these drug-response QTL might be distinct but physically linked in

574   the genome. This result would suggest a cluster of genes essential for the nematode

575   drug response. Overall, our study highlights the power of using mediation analysis to

576   connect gene expression to organismal traits and describes a novel function for the

577   pleiotropic gene *scb-1*.

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Acknowledgments

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FIGURES LEGENDS & TABLES

592 **Table 1 : Main mechanism of action for eight chemotherapeutic drugs**

Drug	Drug class	Mechanism of action
Amsacrine	Topoisomerase inhibitors	DNA intercalation and inhibition of topoisomerase II, causing DNA double-strand breaks, cell cycle arrest, and cell death
Bleomycin	Antitumor antibiotic	Forms complexes with iron that reduce molecular oxygen to form free radicals which in turn cause DNA single- and double-strand breaks
Bortezomib	Proteosome inhibitors	Reversibly inhibits the 26S proteosome and inhibits nuclear factor (NF)-kappaB causing disruption of various cell signaling pathways, cell cycle arrest, and cell death.
Carmustine	Alkylating agents	Alkylates and cross-links DNA causing cell cycle arrest and cell death
Cisplatin	Alkylating agents	Alkylates and cross-links DNA causing cell cycle arrest and cell death
Etoposide	Topoisomerase inhibitors	Binds to and inhibits topoisomerase II causing an increase of DNA single- and double-strand breaks, cell cycle arrest, and cell death
Puromycin	Aminonucleoside antibiotic	Acts as analog of 3' terminal end of aminoacyl-tRNA and incorporates itself into growing polypeptide chain causing premature termination and inhibition of protein synthesis
Silver	NA	Multi-faceted induction of apoptosis

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598   **Figure 1.** A large-effect QTL on the center of chromosome V underlies responses to

599   several chemotherapeutics. Linkage mapping results with the set 2 RIAILs for a

600   representative trait for each drug are shown (amascrine: median.norm.EXT, bleomycin

601   median.TOF:, bortezomib: median.TOF, carmustine: norm.n, cisplatin: median.TOF,

602   etoposide: norm.n, puromycin: median.TOF, silver: median.TOF). Genomic position (x-

603   axis) is plotted against the logarithm of the odds (LOD) score (y-axis) for 13,003

604   genomic markers. Each significant QTL is indicated by a red triangle at the peak

605   marker, and a blue rectangle shows the 95% confidence interval around the peak

606   marker. The percentage of the total variance in the RIAIL population that can be

607   explained by each QTL is shown above the QTL. The dotted vertical line represents the

608   genomic position of *scb-1*.

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617   **Figure 2.** Near-isogenic lines validate the chromosome V QTL. (A) NIL genotypes on

618   chromosome V are shown, colored orange (N2) and blue (CB4856). From top to

619   bottom, strains are N2, ECA232, ECA1114, and CB4856. The dotted vertical line

620   represents the location of *scb-1*. (B) NIL phenotypes in eight chemotherapeutics (12.5

621   µM amsacrine, 12.5 µM bleomycin, 2 µM bortezomib, 250 µM carmustine, 125 µM

622   cisplatin, 62.5 µM etoposide, 300 µM puromycin, and 100 µM silver) are plotted as

623   Tukey box plots with strain (y-axis) by relative median optical density (median.EXT, x-

624   axis). Statistical significance was calculated for each strain pair (**File S7**). Significance

625   of each strain compared to its parental strain (ECA232 to N2 and ECA1114 to CB4856)

626   is shown above each strain pair and colored by the parent strain against which it was

627   tested (ns = non-significant (p-value > 0.05); *, **, ***, and **** = significant (p-value <

628   0.05, 0.01, 0.001, or 0.0001, respectively).

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635   **Figure 3.** Expression QTL mapping identifies several hotspots. (A) The genomic

636   locations of the eQTL peaks derived from linkage mapping using the set 1 RIAILs (x-

637   axis) are plotted against the genomic locations of the probe (y-axis). The size of the

638   point corresponds to the effect size of the QTL. eQTL are colored by the LOD score,

639   increasing from purple to pink to yellow. The diagonal band represents local eQTL, and

640   vertical bands represent eQTL hotspots. (B) Quantification of eQTL hotspots identified

641   by overlapping distant eQTL. The number of distant eQTL (y-axis) in each 5 cM bin

642   across the genome (x-axis) is shown. Bins above the red line are significant and marked

643   with an asterisk. The bins with the blue asterisks are most significant and have been

644   identified in a previous analysis. The dotted vertical line represents the genomic position

645   of *scb-1*. Grey rectangles below the plot represent locations of the drug-response QTL

646   hotspots previously identified.

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655 **Figure 4.** Mediation analysis for the eQTL hotspot on the center of chromosome V.

656 Mediation estimates calculated as the indirect effect that differences in expression of

657 each gene plays in the overall phenotype (y-axis) are plotted against the genomic

658 position of the eQTL (x-axis) on chromosome V for 49 probes (including *scb-1* (red

659 diamond)) that map to the chromosome V eQTL hotspot (set 1 RIAILs). A

660 representative trait for each drug from the set 1 linkage mapping analysis are shown:

661 amascrine (median.EXT), bleomycin (median.EXT), cisplatin (median.TOF), and silver

662 (median.norm.EXT). The 90th percentile of the distribution of mediation estimates for

663 each trait are represented by the horizontal grey lines. The confidence intervals for the

664 QTL (set 1 RIAILs) are shown with the vertical blue dotted lines. The confidence of the

665 estimate increases (p-value decreases) as points become less transparent.

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670 **Figure 5.** Testing the role of *scb-1* in drug responses. (A) Strain genotypes on

671 chromosome V are shown, colored orange (N2) and blue (CB4856). From top to

672 bottom, strains are N2, ECA1132, ECA1134, and CB4856. Deletion of *scb-1* is indicated

673 by a grey triangle. The dotted vertical line represents the location of *scb-1*. (B)

674 Phenotypes of strains in eight chemotherapeutics (12.5 µM amsacrine, 12.5 µM

675 bleomycin, 2 µM bortezomib, 250 µM carmustine, 125 µM cisplatin, 62.5 µM etoposide,

676 300 µM puromycin, and 100 µM silver) are plotted as Tukey box plots with strain (y-

677 axis) by relative median optical density (median.EXT, x-axis). Statistical significance

678 was calculated for each strain pair (**File S7**). Significance of each deletion strain

679 compared to its parental strain (ECA1132 to N2 and ECA1134 to CB4856) is shown

680 above each strain pair and colored by the parent strain against which it was tested (ns =

681 non-significant (p-value > 0.05); *, **, ***, and **** = significant (p-value < 0.05, 0.01,

682 0.001, or 0.0001, respectively).

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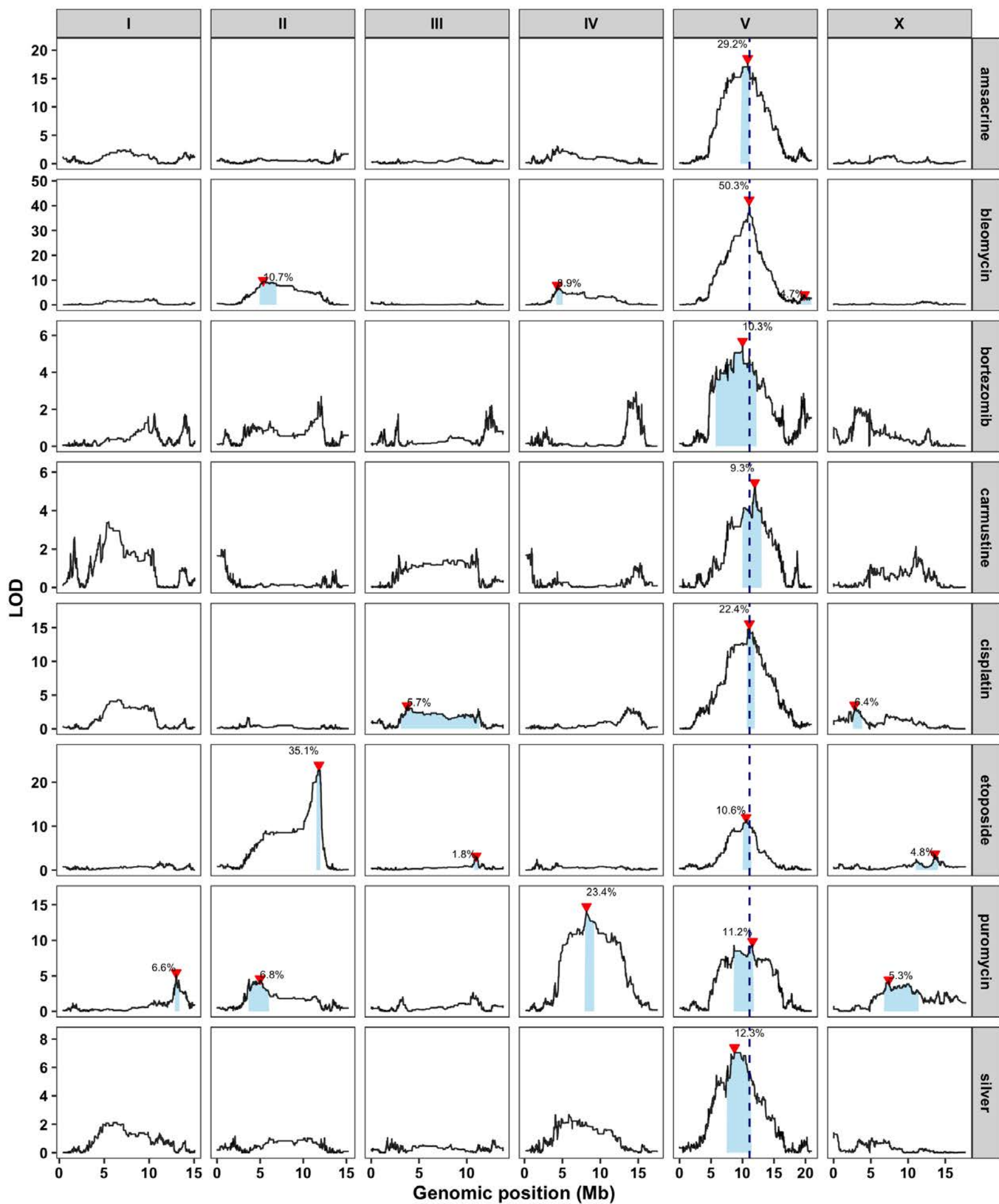902   Zdraljevic, S., B. W. Fox, C. Strand, O. Panda, F. J. Tenjo *et al.*, 2019 Natural variation

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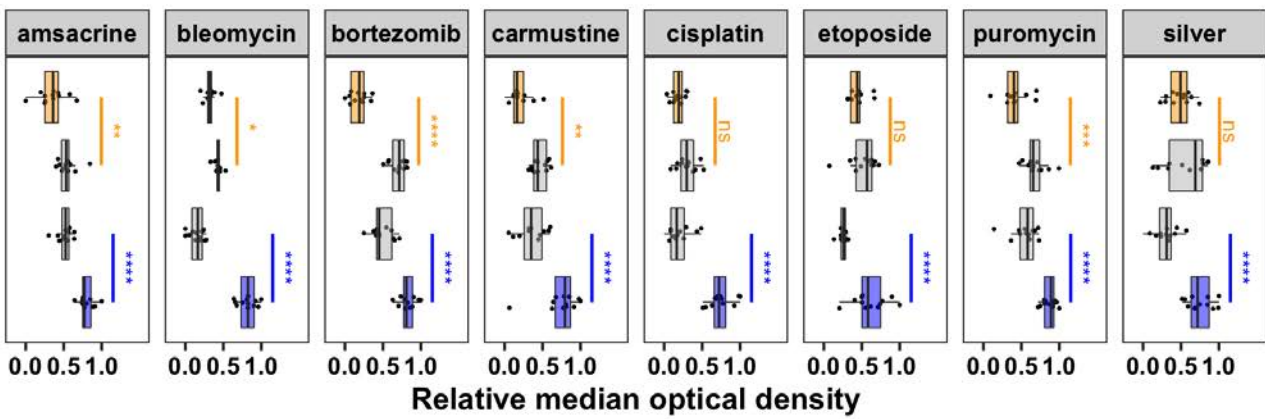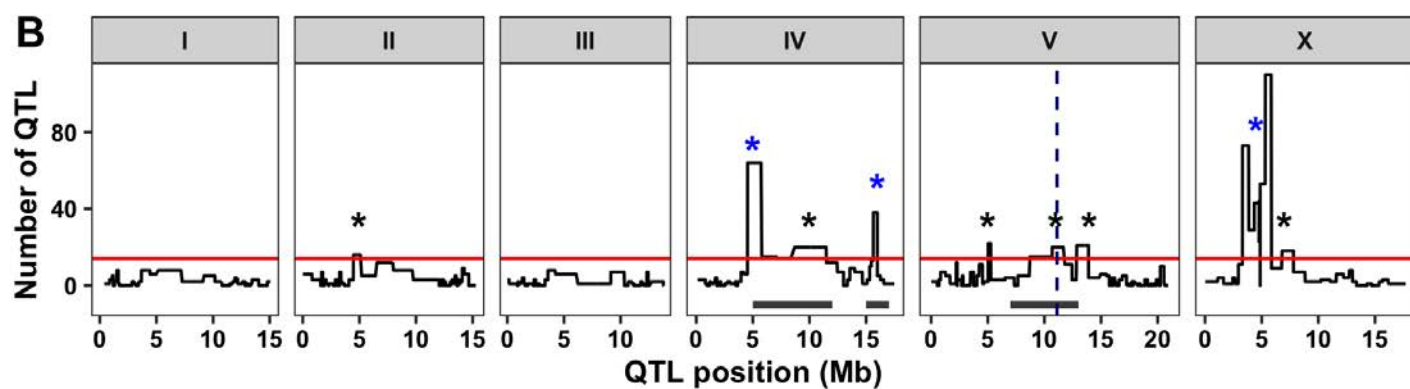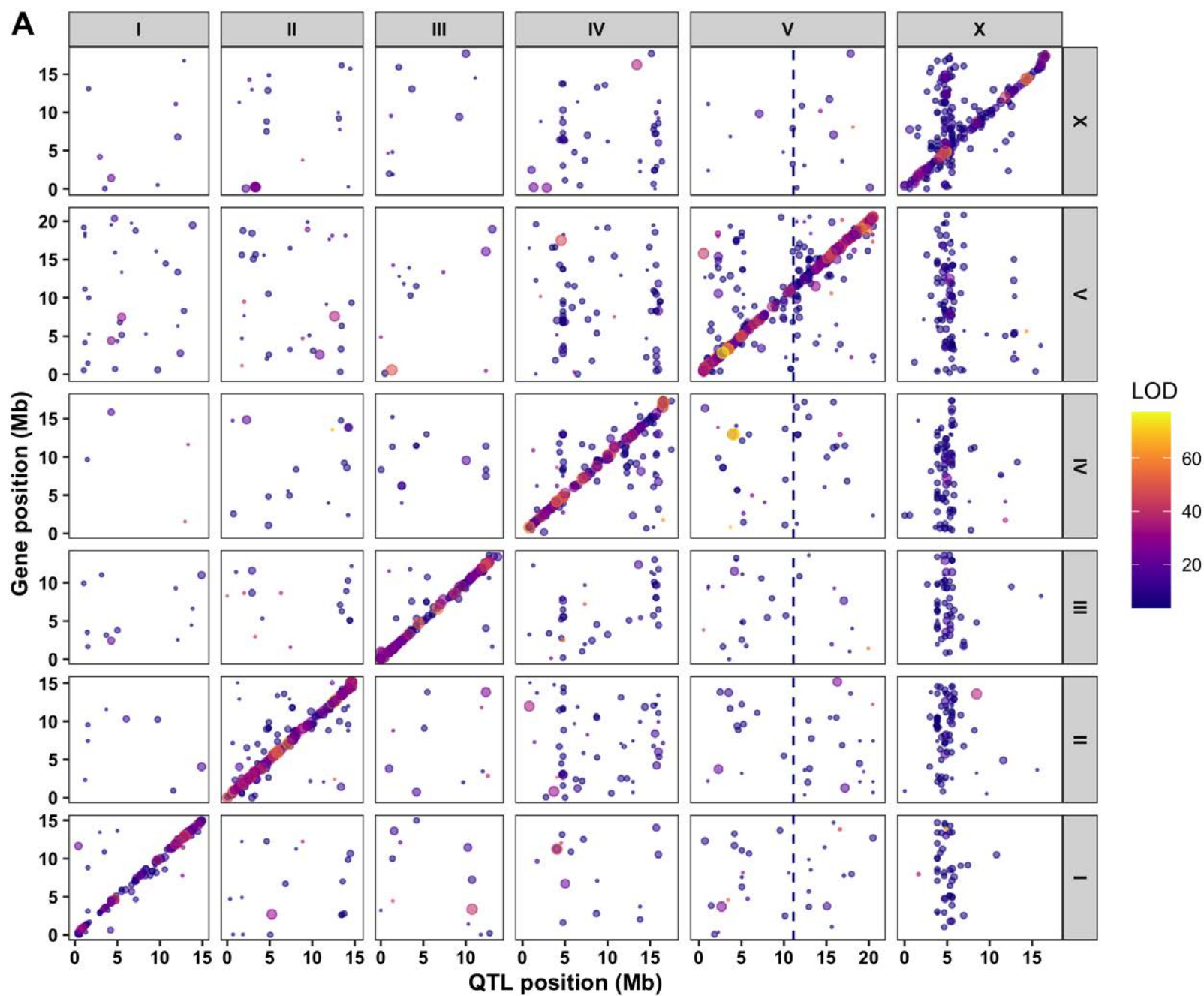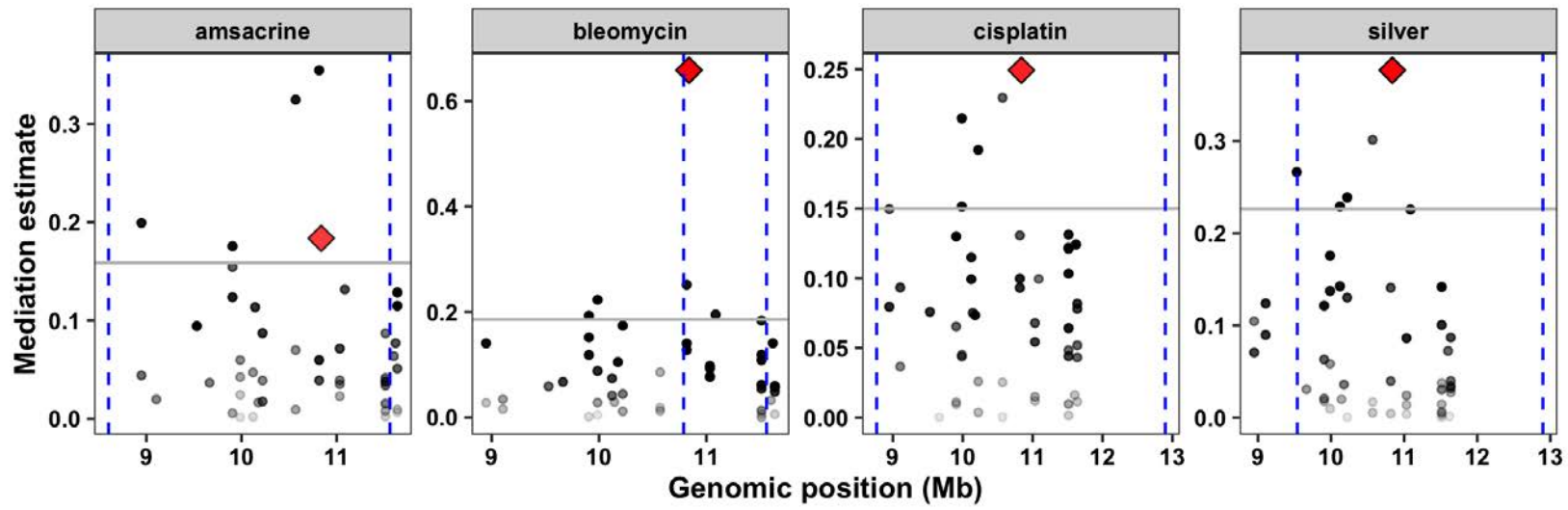
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