A role for worm *cutl-24* in background- and parent-of-origin-

dependent ER stress resistance

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Abstract

Organisms in the wild can acquire disease and stress resistance traits that far outstrip the programs endogenous to humans. Finding the molecular basis of such natural resistance characters is a key goal of evolutionary genetics. Standard statistical-genetic methods toward this end can perform poorly in organismal systems that lack high rates of meiotic recombination, like *Caenorhabditis* worms. Here we discovered unique ER stress resistance in a Kenyan *C. elegans* isolate, which in inter-strain crosses was passed by hermaphrodite mothers to hybrid offspring. We developed an unbiased version of the reciprocal hemizygosity test, RH-seq, to explore the genetics of this parent-of-origin-dependent phenotype. Among top-scoring gene candidates from a partial-coverage RH-seq screen, we focused on the neuronally-expressed, cuticlin-like gene *cutl-24* for validation. In gene disruption and controlled crossing experiments, we found that *cutl-24* was required in Kenyan hermaphrodite mothers for ER stress tolerance in their inter-strain hybrid offspring, and was a contributor to the trait in their purebred progeny. These data establish the Kenyan strain allele of *cutl-24* as a determinant of a natural stress-resistant state, and they set a precedent for the dissection of natural trait diversity in invertebrate animals without the need for a panel of meiotic recombinants.

Introduction

Understanding mechanisms of trait diversity in organisms from the wild is a central goal of modern genetics. Classical methods toward this end, namely association and linkage mapping, have enabled landmark successes in the field (Flint and Mott 2001). These tools are well-suited to organismal systems in which highly polymorphic, well-mixed recombinant populations exist in the wild or can be generated in the lab. Such features are lacking in the nematode worm *Caenorhabditis.* The laboratory strain of *C. elegans* has been a fixture of the biology literature for decades (Nigon and Félix 2017), but dissecting natural variation in nematodes has proven to be a challenge owing to low rates of polymorphism and meiotic recombination (Hillers and Villeneuve 2003). In a few model *C. elegans* systems, landmark resources have been established to overcome these limitations (Li *et al.* 2006; Reddy *et al.* 2009; Rockman and Kruglyak 2009; McGrath *et al.* 2009; Doroszuk *et al.* 2009; Seidel *et al.* 2011; Bendesky *et al.* 2012; Ghosh *et al.* 2012, 2015; Balla *et al.* 2015; Andersen *et al.* 2015; Large *et al.* 2016; Ben-David *et al.* 2017), including recent progress with genome-wide association scans (Webster *et al.* 2019a; Na *et al.* 2020; Evansid *et al.* 2021). Despite these advances, the power of natural variation genetics remains out of reach for many nematode strains and species.

The reciprocal hemizygosity test (Stern 2014) is a strategy to map genotype to phenotype that complements linkage and association approaches. This approach starts with an F1 hybrid formed from the mating of two genetically distinct lines of interest. An induced mutation in the hybrid at a given gene disrupts the allele from each parent in turn, uncovering the other allele in a hemizygous state. The set of hemizygote mutants allow a comparison of phenotypes conferred by the two parents' alleles, controlled for background and ploidy. Strengths of the method include the ability for trait dissection without a panel of meiotic recombinants, and the potential for genetic insights into traits unique to hybrid backgrounds (heterosis, for example, and imprinting and other parent-of-origin effects; Comings and MacMurray 2000; Reinhold 2002;

Wolf and Wade 2009; Timberlake 2013; Monk *et al.* 2019)). Our group previously established

genome-wide reciprocal hemizygosity mapping in yeast (RH-seq; (Weiss *et al.* 2018)), and we

reasoned that nematodes could serve as a useful testbed for an extension of the method to

multicellular animals.

For a case study using RH-seq, we set out to identify natural stress resistance states in wild

worms, and to probe their genetics. We chose to focus on ER protein folding quality control,

which is essential in eukaryotes for development and stress response, and is a linchpin of

diabetes, neurodegenerative disease, and other human disorders (Hebert and Molinari 2007;

Roth *et al.* 2010). Surveying *C. elegans* isolates, we characterized a Kenyan strain with a ER

stress resistance phenotype, and we discovered a robust parent-of-origin effect in hybrids

derived from this strain. To enable the search for determinants of this trait variation, we

developed an implementation of RH-seq for the worm which achieved unbiased, partial

coverage of the genome. From the results we focused on one gene hit, *cutl-24*, for validation of

its impact on ER stress response.

Materials and Methods

Worm strains

Wild-type strains of *C. elegans* AB1 (Adelaide, Australia), CB4856 (Hawaii, USA), CB4932

(Taunton, Great Britain), ED3052 (Ceres, South Africa), ED3077 (Nairobi, Kenya), GXW1

(Wuhan, China), JU1088 (Kakegawa, Japan), JU1172 (Concepcion, Chile), JU1652

(Montevideo, Uruguay), JU262 (Indre, France), JU393 (Hermanville, France), JU779 (Lisbon,

Portugal), MY16 (Munster, Northwest Germany), N2 (Bristol, Great Britain) and PX179 (Eugene,

USA) were used in this study, all from the *Caenorhabditis* Genetics Center.

Tunicamycin resistance assays

Tunicamycin resistance assays were performed as described (Henis-Korenblit *et al.* 2010) with

modifications as follows. For Figure 1, 2-3 day 1 gravid adults of different strains were placed

onto plates containing 5 µg/mL tunicamycin (InSolution, Sigma) which was added to NGM

media before pouring and allowed to lay eggs for 12 hours. The number of eggs was counted

(typically 50-200) and compared with the number of animals that reached the adult stage within

96 hours at 20°C. In controls on untreated (DMSO) plates, ~100% of eggs hatched for a given

strain. For each bar in Figure 2, 5-10 L4 hermaphrodites of the indicated strain were incubated

with 10-20 males of the indicated strain for 24 hours to allow mating. The hermaphrodites were

then transferred onto plates containing 5 µg/mL tunicamycin (InSolution, Sigma) as above and

allowed to lay eggs for 12 hours. The proportion of eggs from the indicated cross that developed

to adulthood in the presence of tunicamycin was calculated the same as in Figure 1 and was

normalized to the analogous quantity from wild-type ED3077. For Figure 5, the experimental

condition was the same as above except 10 µg/mL tunicamycin (InSolution, Sigma) was used

and the number of animals that reached the adult stage was counted after 66-68 hours at 25°C.

RH-seq in *C. elegans*

RH-seq parental strain construction.

To enable the heat-induced Mos1 transposon system (Bessereau *et al.* 2001; Williams *et al.*

2005; Duverger *et al.* 2007) in the ED3077 and N2 backgrounds, we proceeded as follows.

For mutagenesis in N2, we used IG358 (oxEx229 [Mos1 Substrate + *myo-2p*::GFP]), an N2

transgenic which carries multiple copies of the Mos1 transposon and pharynx-specific green

fluorescent protein (GFP) expression, and IG444 (frEx113 [(pJL44) transposase + *col-*

12p::DsRed]), an N2 transgenic which carries the coding sequence of the Mos1 transposase

under the control of a heat-shock promoter and a coelomocyte-specific DsRed reporter.

For the corresponding strains in the ED3077 background, we injected the pSM-Mos1

transposon plasmid at 25 ng/µl, pCFJ421 (P*eft-3::gfp::h2b*) at 10 ng/µl, and pSM at 65 ng/µl to

yield ED3077-gG. We injected pJL44 (P*hsp16.48::MosTase::glh-2utr*) at 50 ng/µl, pCFJ90

(P*myo-2::mCherry::unc-54utr*) at 2 ng/µl, and pSM at 48ng/µl to create ED3077-3R.

After mutagenesis, to enable the collection of progeny from mutant hermaphrodites in one

background mated to wild-type males from the other (see below), we used labeled strains for

the latter. To make these, wild-type ED3077 and wild-type N2 were injected with pCFJ104

(P*myo-3::mCherry::unc-54utr*) and pCFJ90 (P*myo-2::mCherry::unc-54utr*) respectively at 5 ng/µl,

after which the respective transgene was integrated into the worm genome by UV/TMP

treatment. The worms were outcrossed 3 time.

RH-seq hemizygote construction and phenotyping

Our RH-seq workflow proceeded in 13 rounds in N2. For each round of hemizygotes bearing

mutations in the N2 alleles of genes, we first made Mos1-ready N2 animals by picking 100-200

L4 hermaphrodites of IG358 and 200 L4 males of IG444 onto one NGM plate. After 12 hrs at

25°C, the hermaphrodites and males were transferred to a new NGM plate for egg-lay. 100-200

F1 progeny exhibiting GFP and mCherry marker expression (*i.e.*, containing both transposase

and transposon arrays) were hand-picked at the L4 stage.

In these Mos1-ready N2 animals, we induced Mos1 transposition at the young adult stage by

heat-shock treatment as described (34 °C for 1 h, 20 °C for 1 h and 34 °C for 1 h) (Boulin and

Bessereau 2007). We collected the putatively mutant eggs and selfed them for two generations

to ensure appreciable numbers of clones of a given mutant genotype. We collected 20,000-

50,000 F3 progeny and sorted in a Union Biometrica COPAS Biosort for those that had lost the

substrate array and transposase array (*i.e.,* they lacked GFP and mCherry expression) to

prevent continued transposition for the rest of the experiment.

We transferred 10,000-20,000 of these putatively mutant N2 hermaphrodites to NGM plates with

5,000-10,000 labeled wild-type ED3077 males (see above) and allowed mating to occur for one

and half day at 15°C. The mated hermaphrodites were lysed, and ~30,000 eggs were

transferred to one plate (5-10 plates per round) containing 10 µg/mL tunicamycin, followed by

incubation for development for 66-68 hrs at 25°C.

Afterward, 3,000-12,000 surviving worms were collected and sorted, with only hermaphrodite

adults (assessed based on size) and hybrid progeny of the marked ED3077 parent (assessed

based on mCherry marker expression) retained for DNA isolation and sequencing (see below).

11 rounds of hemizygotes bearing mutations in the ED3077 alleles of genes proceeded as

above, except that we mated ED3077-gG with ED3077-3R to make Mos1-ready ED3077, and

after mutagenesis, we mated the resulting putatively mutant hermaphrodites in the ED3077

background to mCherry-marked wild-type N2 males to yield hemizygotes.

Genomic DNA extraction from *C. elegans*

Animals from hemizygote pools that had been collected after control or tunicamycin treatment

from a given RH-seq round were collected and snap-frozen in liquid nitrogen. Genomic DNA

(gDNA) from the worm pellets were extracted using the DNeasy Blood & Tissue Kit (Qiagen)

and a *C. elegans* gDNA extraction protocol from the Kaganovich lab (University Medical Center

Göttingen, http://www.kaganovichlab.com/celegans.html). The purified gDNA was quantified

using a Nanodrop.

Transposon insertion sequencing library construction

Illumina transposon sequencing libraries were constructed using the FS DNA Library Kit (NEB)

as follows. By following the protocol, gDNA was enzymatically digested and ligated with E-

adapters (a duplex whose upper strand is longer than the lower). The E-adapter upper arm was

GGGCGTAGATTACCGTCCGCGACTCGTACTGTGGCGCGCC*T (*T indicates T overhang),

and the E-adapter lower arm was /Phos/GGCGCGCCACAGTACTTGACTGAGCTTTA/3ddC/.

Sequences containing the 3' junction of each inserted Mos1 transposon to the genome were

amplified using a Mos1 transposon primer (5'-

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNN

NNNXXXXXXGATTTAAAAAAAACGACATTTCATAC-3' N, random sequence; XXXXXX,

barcode indices; GATTTAAAAAAAACGACATTTCATAC, Mos1 transposon sequences) and a

primer homologous to a region of the adapter

(CAAGCAGAAGACGGCATACGAGATACATCGGGGGCGTAGATTACCGTCCGCGACTC).

The thermocycler protocol was as follows: 98°C for 30 seconds, (98°C for 10 seconds, 65°C for

1 minute and 15 seconds) x 24, 65°C for 10 minutes, 4°C hold. Single-end sequencing of 100

bp was then done on a NovaSeq machine at the Vincent J. Coates Genomics Sequencing Lab

at the University of California, Berkeley. In total, we sequenced 22 libraries from pools of

hemizygotes bearing mutations in the ED alleles of genes (representing one tunicamycin-

treated and one untreated pool from each of 11 rounds), and 26 libraries from pools bearing

mutations in the N2 alleles of genes (representing one tunicamycin-treated and one untreated

pool from each of 13 rounds), generating 3-20 million reads per library (Table S3).

RH-seq read-mapping

For a given library of reads reporting transposon insertion sequencing from hemizygotes after

tunicamycin or control treatment for RH-seq, we first removed adapter sequences from the end

of the reads using fastx_clipper from the fastx toolkit. Then, using fastx_trimmer from the fastx

toolkit, we removed the first 6 bp of every read, so every read would now begin with the barcode

index sequence. We then demultiplexed the barcode indexes using the fastx_barcode_splitter

from the fastx toolkit. Using an adapted custom python script, we retained for analysis only

reads containing the last 28 bp of the Mos1 transposon, allowing for no mismatches. For this

subset of reads containing the transposon, we excised the sequence immediately flanking the

end of the transposon sequence, and mapped the remainder of the read (containing the stretch

of genome adjoining the transposon insertion) to the respective genome using BLAT (minimum

sequence identity = 100; tile size= 12).

For pools of hemizygotes bearing transposon insertions in the N2 alleles of genes, we mapped

to the N2 genome from BioProject PRJNA13758, release WS271. For hemizygotes with

insertions in the ED3077 alleles, we mapped to a ED3077 reference genome made as follows.

We downloaded raw genome sequencing reads for ED3077 from

https://www.elegansvariation.org/ (Cook *et al.* 2017). These reads were aligned to genome

assembly WS271 of the reference sequence of the *C. elegans* N2 strain using bowtie2 with

default parameters. Samtools, bcftools, and bgzip were used to call SNPs, retaining those with

a quality score of >20 and combined depth of >5 and <71. We then generated a pseudogenome

by replacing the reference N2 allele with that of ED3077 at each SNP using bcftools.

We eliminated reads whose genomic sequence portion was shorter than 30 bp and/or mapped

to more than one location in the genome. For analysis of a given sequencing library, we inferred

that reads that mapped to genomic positions within 100 bp of each other originated from the

same transposon mutant clone and assigned the sum of their counts to the position which had

the most mapped reads. Then, for a given insertion in a given library, we considered the number

of reads mapped to that position, n_{insert}, as the relative proportional abundance of the mutant in

the worm pellet whose gDNA was sequenced. In order to compare abundances across samples

and libraries, we divided n_{insert} by the total number of reads in the sample to get the normalized

abundance.

RH-seq data analysis

We used N2 genome annotations from BioProject PRJNA13758, release WS271, to determine

whether an inferred transposon insertion position was genic or non-coding, and we retained only

those insertions which were genic. For a given insertion observed in sequencing from a given

RH-seq round (comprising a pool of hemizygotes split into tunicamycin- and control-treated

subpools), we tabulated the $\log_2$ ratio of the normalized abundance in the control sample

divided by the normalized abundance in the tunicamycin-treatment sample, which we call the

tunicamycin effect on the respective transposon mutant as detected by RH-seq. In formulating

this ratio, we assigned a pseudocount of 1 for each case of an insertion with zero reads in either

the tunicamycin-treated or control sample of its respective round. For insertions detected in

multiple rounds, we averaged the tunicamycin effect across all rounds before proceeding. For a

given gene, we compiled all transposon insertion mutants in the gene as observed across all

RH-seq rounds, and we used a two-sample Wilcoxon test to compare the tunicamycin effects

between two sets of mutants: those in the N2 allele of the respective gene and those in the

ED3077 allele. We corrected for multiple testing using the Benjamini-Hochberg method.

Generating Cas9 mutants of *cutl-24*

CRISPR-mediated genome editing was performed as described (Friedland *et al.* 2013) with

modifications as follows. Purebred N2 and, separately, ED3077 as day 1 adult animals were

injected with pDD162 (P*eft-3::Cas9::tbb-2utr*), pCFJ90 (P*myo-2::mCherry*) and two different

pU6::sgRNAs targeting *cutl-24*. Surviving worms were separated, and F1 mCherry-positive

animals were collected; their progeny, representing the F2 generation, were genotyped for the

cutl-24 gene. Mutant genotypes are reported in Supplementary Table 4.

***cutl-24* expression analysis**

For Figure 6, the *cutl-24* expression pattern was obtained from (Dillman *et al.* 2015;

Hashimshony *et al.* 2015). For Figure 7, the *cutl-24* expression pattern in single cell resolution

was obtained from (Packer *et al.* 2019). Cell type labels follow the format

CellType:StartTime_EndTime, where times represent development following fertilization: ILso,

Inner labial socket; AMso, Amphid socket; CEPsh, Cephalic sheath; CEPso, Cephalic socket;

ADEsh, Anterior dereid sheath; PHB_and_possibly_PHA, PHB/PHA neuron;

ILsh_OLLsh_OLQsh, Inner/outer labial sheath; XXX, Glia and excretory cells; ASG, ADL, AWA,

ASH and ADE, neurons of other types; AMso_PHso, Amphid and phasmid socket;

ADE_CEP_PDE, neurons.

Code availability

Custom Python and R scripts used for RH-seq data analysis are available at

https://github.com/annagflury/RHseq-scripts.

Data availability

RH-seq data have been deposited in the Sequence Read Archive (SRA), submission ID

SUB9564728.

Results

Tunicamycin resistance in wild *C. elegans* and its parent-of-origin dependence

Tunicamycin, a N-glycosylation inhibitor and ER stress inducer, causes developmental delay in

nematodes (Richardson *et al.* 2011). To identify *C. elegans* strains resistant to this defect, we

collected eggs from each of a panel of wild isolates and the laboratory strain N2, exposed them

to a toxic concentration of tunicamycin, and tabulated the number of successfully developed

adults after 96 hours at 20°C. Among these strains, the predominant phenotype was of marked

sensitivity, in which most eggs exposed to tunicamycin failed to reach adulthood, and indeed

never reached the larval stage of development (Figure 1). All isolates we assayed fit this

description except one: ED3077, originally isolated from a park in Nairobi, Kenya, had a rate of

development in the presence of the drug outstripping that of the rest of the panel by >2-fold

(Figure 1). We targeted this tunicamycin resistance phenotype in ED3077 as a compelling target

for genetic dissection, and we chose to use N2 as a representative of the tunicamycin-sensitive

state.

To begin to investigate the genetics of tunicamycin resistance in ED3077, we mated this strain

to N2, and subjected the resulting F1 hybrid eggs to our tunicamycin development assay.

Results made clear that the hybrid phenotype depended on the direction of the cross (Figure 2).

ED3077 hermaphrodites, mated to N2 males, yielded eggs that developed in tunicamycin

conditions at a rate approaching that of the ED3077 purebred. By contrast, hybrids with N2 as

the hermaphrodite parent had almost no ability to develop in the presence of tunicamycin

(Figure 2). Thus, the ED3077 phenotype was partially dominant over that of N2, but only when

ED3077 was the hermaphrodite in the cross. We hypothesized that the mechanism of this

maternal effect could bear on the genetics of the trait difference between purebred ED3077 and

N2. In what follows, we describe our experiments to probe both facets of the system.

RH-seq reveals allelic effects of *cutl-24* in an inter-strain hybrid

We sought to identify the genetic basis of the parent-of-origin-dependent tunicamycin resistance

trait in the ED3077 x N2 system using RH-seq. The method requires large cohorts of hybrids

harboring disrupting mutations in one of the two copies of each gene in turn, and we chose the

Mos1 transposon system (Bessereau *et al.* 2001; Williams *et al.* 2005) for this purpose. We set

up a workflow to generate transposon mutants in ED3077 or N2 and mate each to the wild-type

of the respective other parent, yielding hemizygote eggs (Figure 3). In each case we used

hermaphrodites as the transposon mutant parent and males as the wild-type, in light of the

importance of the hermaphrodite genotype in hybrids (Figure 2). We incubated hemizygote eggs

in tunicamycin or untreated control conditions, and then collected animals that reached

adulthood for DNA isolation and sequencing.

We reasoned that deploying this RH-seq setup at moderate scale could reveal candidate

determinants of tunicamycin resistance in the ED3077 x N2 hybrid. To this end, we generated

and detected a total of 74,893 hemizygote genotypes from DNAs of animals reaching adulthood

in tunicamycin and in control conditions, after quality-control filtering of sequencing data

(Supplementary Tables 1 and 2). Focusing on the 2,722 highest-coverage genes, we tested for

a difference in normalized sequencing representation among tunicamycin-treated adults

between two cohorts of hemizygotes—those with the ED3077 allele uncovered and those with

the N2 allele uncovered. In this test, several genes exhibited suggestive evidence for allelic

effects unique to tunicamycin resistance, though none reached significance after correction for

multiple testing (Supplementary Table 3). We considered each such gene to represent a

potential candidate determinant of tunicamycin response, whose *bona fide* function could be

validated in single-gene experimental follow-up.

Manual inspection pointed to *cutl-24*, encoding a largely uncharacterized protein containing a

cuticlin-like domain, as a candidate of particular interest on the basis of the robust potential

impact of allelic variation (Figure 4). In RH-seq data, genotypes with the ED3077 allele

disrupted in the hybrid, leaving the N2 uncovered and functional, were at low abundance in

tunicamycin-treated adults relative to controls; genotypes with the ED3077 allele intact and the

N2 allele disrupted were abundant in these samples (Figure 4). On this basis we hypothesized

that the ED3077 allele of *cutl-24* had pro-resistance activity in the hybrid, to an extent greater

than the effects of the N2 allele.

***cutl-24* as a determinant of tunicamycin resistance in hybrids and purebreds**

To validate the role of *cutl-24* in trait variation in the ED3077 x N2 system, we first focused on

the hybrid background, where this gene had been a top scorer in our RH-seq screen. We used

Cas9 to generate stable lines of purebred N2 and ED3077 harboring a nonsense mutation in

cutl-24 (Supplementary Table 4). We mated each mutant as the hermaphrodite to the respective

wild-type male of the other strain, as in our RH-seq setup (Figures 3-4), and we subjected the

progeny to development assays in the presence of tunicamycin. The results showed that

disrupting *cutl-24* in ED3077 hermaphrodites, followed by crossing to wild-type male N2,

compromised tunicamycin resistance of the hybrid by >2-fold (Figure 5a), consistent with the

trend we had observed in RH-seq (Figure 4). Mutating *cutl-24* in N2 hermaphrodites, followed

by mating with wild-type ED3077 males, had no such effect and instead appeared to confer a

benefit in the hybrid (though this trend was not significant; Figure 5a). These data establish *cutl-*

24 as a driver of the unique tunicamycin resistance phenotype of the hybrid progeny of ED3077

hermaphrodites, and serve as a validation of the RH-seq approach.

We also examined the importance of *cutl-24* in the male parent of inter-strain hybrids. *cutl-24*

mutation in ED3077 males conferred only a slight, non-significant drop in tunicamycin resistance

of their hybrid progeny, in matings with N2 hermaphrodites; the same was true when N2

mutants were the male parent of a cross with ED3077 hermaphrodites (Figure 5b). That is, *cutl-*

24 mutation in males had little if any impact on hybrid tunicamycin response, regardless of

background. This result echoed our finding from wild-type crosses that hermaphrodite genotype

matters most for tunicamycin resistance in hybrids (Figure 2). Together, our genetic analyses

establish *cutl-24* as a determinant of the parent-of-origin-dependent tunicamycin resistance in

ED3077 x N2 hybrids, with the ED3077 allele in the hermaphrodite sufficient to explain the

hybrid resistance phenotype entirely.

We hypothesized that *cutl-24* could also contribute to tunicamycin resistance in a purebred

context. As expected, the N2 purebred performed so poorly in tunicamycin resistance assays

that *cutl-24* mutation effects could not be resolved (Figure 5c). Such a detection floor in the

assay signal was not an issue for purebred ED3077, in which we observed a 20% drop in

tunicamycin resistance in the *cutl-24* mutant relative to wild-type (Figure 5c). This modest but

significant phenotype was much less dramatic than the impact of *cutl-24* mutation we had

detected in hybrids (Figure 5a). Such a contrast points up the behavior of the hybrid as a

sensitized background, in which *cutl-24* dependence is amplified and drives striking parent-of-

origin effects (Figure 5a-b). In purebred ED3077, we infer that *cutl-24* acts instead as a modifier

of tunicamycin resistance, likely one of many determinants of a complex genetic architecture.

Evidence for *cutl-24* as a neuronal factor

To help formulate models for *cutl-24* activity and function, we characterized the localization of its

gene product with an expression analysis approach. In whole-worm developmental timecourses

(Dillman *et al.* 2015; Hashimshony *et al.* 2015), *cutl-24* expression was high in embryos and

lower but appreciable in larvae and adults (Figure 6). Lineage-traced single-cell transcriptomes

through development (Packer *et al.* 2019) found *cutl-24* in glia of the inner labial sensilla and, at

lower levels, in many other neurons (Figure 7). In adult hermaphrodites, RNA tagging for a

marker of outer labial sensilla neurons and PVD nociceptors revealed significant *cutl-24*

expression (Smith *et al.* 2010). These results establish the presence of the *cutl-24* gene product

in a range of neuronal cells, and raise the possibility that *cutl-24* may exert its effect on

development and stress resistance in this context.

Discussion

The search for genes underlying natural trait variation has been a mandate for geneticists since

Mendel. Wild nematodes have great potential as a study system for ecological genetics (Frézal

and Félix 2015; Cook *et al.* 2017). In these animals, landmark studies have identified variants

with Mendelian (de Bono and Bargmann 1998; Reddy *et al.* 2009; McGrath *et al.* 2009;

Bendesky *et al.* 2012; Ghosh *et al.* 2012, 2015; Balla *et al.* 2015; Large *et al.* 2016) and

maternal-effect (Ben-David *et al.* 2017; Ewe *et al.* 2020) modes of action that underlie

phenotypes of interest. Many of these successes have derived from model advanced intercross

mapping populations in *C. elegans* (Li *et al.* 2006; Rockman and Kruglyak 2009; Doroszuk *et al.*

2009; Andersen *et al.* 2015). In non-model nematode strains and species, statistical genetics

continues to pose challenges, owing to population structure among wild isolates (Webster *et al.*

2019b) and low meiotic recombination rates in lab crosses (Hillers and Villeneuve 2003).

In this work, we have established strain differences and parent-of-origin effects in worm ER

stress resistance. And we have used a new tool, RH-seq, alongside classical genetics to identify

cutl-24 as a determinant of the variation. By discovering maternal-effect and development-

related functions of this uncharacterized gene, our study underscores the utility of the natural

variation approach, and RH-seq in particular, in nematodes.

The RH-seq approach in *C. elegans*

RH-seq shares with several other worm screening methods (Frézal *et al.* 2018; Burga *et al.*

2019; Webster *et al.* 2019b) the ability to streamline phenotyping assays by bulk sequencing of

naturally varying strains. The chief distinction of RH-seq is its ability to map genotype to

phenotype without recombinant progeny from crosses, in the lab or in the wild (Weiss *et al.*

2018; Weiss and Brem 2019). As such, here the technique has enabled genetic dissection in

non-model *C. elegans* ED3077 in the absence of a panel of recombinant inbred lines. More

broadly, the utility of RH-seq for any worm strains or species will be a function of mutagenesis

throughput and phenotyping signal-to-noise, which will govern coverage and statistical power in

a given implementation. Our mapping of *cutl-24* in a screen of limited depth and power serves

as a compelling demonstration of the method.

Maternal effects of strain variation and *cutl-24* function

Starting from the discovery of a unique tunicamycin resistance phenotype in the Kenyan *C.*

elegans isolate ED3077, we found that ED3077 x N2 hybrid embryos develop best in

tunicamycin when they arise from ED3077 hermaphrodite parents rather than ED3077 males.

Furthermore, these hybrid embryos rely on parent *cutl-24* far more than do ED3077 purebreds,

under tunicamycin treatment. It is tempting to speculate that hybrids act as a sensitized

background for *cutl-24* mutation owing to stresses from genome incompatibility, as has been

documented in other *C. elegans* crosses (Dolgin *et al.* 2007; Snoek *et al.* 2014). If so,

hermaphrodite *cutl-24* could help maintain homeostasis under this burden, when compounded

by tunicamycin.

Our work leaves open the mechanism by which *cutl-24* in hermaphrodites affects developing

progeny. Given its expression in multiple neuronal types, *cutl-24* could participate in the newly

described pathway linking neuronal serotonin in mothers with stress-protective transcription in

their progeny (Das *et al.* 2020). Alternatively, acting in labial sensilla in particular, *cutl-24* could

follow the precedent from other maternal-effect genes that directly modulate egg-laying and egg

structure in the worm (Daniele *et al.* 2020; Min *et al.* 2020; Baugh and Hu 2020). Or *cutl-24*

transcripts could be expressed in the germline and maternally deposited in the embryo for

activity early in development, as is the case for other well-characterized maternal-effect factors

(Robertson and Lin 2015).

In any such case, ED3077 hermaphrodites, reared under standard conditions in our assay

design, would lay eggs better equipped to handle environmental and/or genetic challenges, in

part mediated by *cutl-24*. Consistent with this notion, in a previous study of wild *C. elegans*, the

ED3077 strain was distinguished by a marked ability for L1 larvae to develop successfully after

starvation (Webster *et al.* 2019b). The emerging picture is one of a unique, broad stress-

resistant phenotype in this isolate. Further work will establish its complete genetic architecture

and the evolutionary forces that drove its appearance in the wild.

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Figure captions

Figure 1. Tunicamycin resistance phenotypes of wild *C. elegans* isolates. The *y*-axis

reports the proportion of eggs from the indicated wild isolate that developed to adulthood in the

presence of tunicamycin. For a given column, each dot represents results from one replicate

population, and the bar height reports the mean. ***, $p < 0.001$.

Figure 2. Tunicamycin resistance of inter-strain hybrids depends on the parent of origin.

The *y*-axis reports the proportion of eggs from the indicated cross that developed to adulthood

in the presence of tunicamycin, normalized to the analogous quantity from wild-type ED3077.

For a given column, each dot represents results from one replicate population, and the bar

height reports the mean. ED, ED3077; herm, hermaphrodite. **, $p < 0.01$.

Figure 3. Making hemizygote mutants for RH-seq. RH-seq requires hemizygote hybrids

(purple) from matings between mutants of one background (red) and wild-types of another

(blue). Top: arrays (circles) harboring the Mos1 transposon substrate (green) and heat-shock-

inducible transposase enzyme gene (yellow) in the red background come together into one

strain. Center: after heat shock, a transposon copy integrates into the genome (straight black

line) of an egg of the red background, which is fertilized by a wild-type male of the blue

background. Bottom: the resulting F1 hybrids are hemizygous throughout the soma and are

used as input into a sequencing-based tunicamycin resistance assay.

Figure 4. RH-seq reveals *cutl-24* as a candidate gene at which allelic variation contributes

to tunicamycin resistance. Each row reports results from a hemizygote mutant in which the

Mos1 transposon has inserted into the N2 (a) or ED3077 (b) allele of *cutl-24* in the inter-strain

F1 hybrid background. For a given row, the red triangle indicates the Mos1 insertion position in

the *cutl-24* locus; the heat map reports the relative abundance of the hemizygote, as measured

by RH-seq, after tunicamycin selection as a difference from the analogous quantity from a

control culture.

Figure 5. *cutl-24* in ED3077 mothers is required for tunicamycin resistance in their inter-

strain hybrid progeny. Shown are tunicamycin resistance measurements in (a) F1 hybrids

whose hermaphrodite parent of the indicated background harbored a mutation in *cutl-24,* and

wild-type controls*;* (b) F1 hybrids whose male parent of the indicated background harbored a

mutation in *cutl-24,* and wild-type controls*;* and (c) purebred *cutl-24* mutants and wild-type

controls. In each panel, phenotype measurements were normalized to the analogous quantity

from a wild-type ED3077 included in the experiment as a standard. For a given column, each

dot represents results from one replicate population, and the bar height reports the mean. ED,

ED3077; herm, hermaphrodite. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

Figure 6. *cutl-24* expression across *C. elegans* developmental stages. In a given panel,

each bar reports average expression (FPKM, Fragments Per Kilobase of transcript per Million

mapped reads) of *cutl-24* in wild-type animals of the N2 background from (Dillman *et al.* 2015;

Hashimshony *et al.* 2015). in the indicated developmental stage (a) or sex of adult animals (b).

Error bars report standard deviation ($n = 2$).

Figure 7. *cutl-24* is expressed in neurons. In a given panel, each bar reports *cutl-24*

expression (TPM, Transcripts Per Million kilobases) in the indicated cell type from single cell

transcriptomes (Packer *et al.* 2019), in embryos at the indicated developmental time in minutes

(a) or in L2 larvae (b). The highest expression was seen in inner labial socket (ILso), cephalic

socket (CEPso), and phasmid socket (PHso) cells; for other abbreviations in labels, see

Methods.

Supplementary table captions

Supplementary Table 1. RH-seq library sizes. Each row reports sequencing results from hemizygotes in the N2 x ED3077 diploid hybrid background, made from transposon mutants of the indicated strain mated to the wild-type of the other.

Supplementary Table 2. Abundance of hemizygote mutants in tunicamycin RH-seq. Abundances of transposon-mutant Mos1 insertion site in the F1 hybrid of N2 or ED3077 allele from RH-seq. Each row reports results of sequencing one transposon insertion in the N2 x ED3077b diploid hybrid after selection of the transposon mutant pool, reflecting the abundance in the pool of the respective hemizygote clone harboring the insertion. Gene, chromosome, and position report the fine-scale position of the insertion. Allele, the strain parent's homolog in which the transposon insertion lay. Control_count and treatment_count report read counts of the transposon insertion sequenced after selection of the mutant pool in the indicated condition, normalized for library size. Transposon insertions not detected in any replicate of the indicated selection were assigned an abundance of 1.

Supplementary Table 3. Effects of allelic variation in tunicamycin RH-seq. Each row reports the results of reciprocal hemizygote tests of tunicamycin resistance of hemizygote transposon mutants at the indicated gene in the N2 x ED3077 diploid hybrid. N2_control_count, N2_treatment_count, ED3077_control_count, and ED3077_treatment_count report normalized abundances of a hemizygote harboring a transposon insertion in the indicated parent's homolog after culture in the indicated condition, as a mean across transposon mutants, from all biological replicates. The last two columns report results of a two-tailed Mann-Whitney statistical test for a difference in the abundance after tunicamycin selection, relative to the abundance in an untreated control, of hemizygotes harboring transposon insertions in the two parents' homologs. The Benjamini-Hochberg method was used to correct for multiple testing.

626 **Supplementary Table 4. *cutl-24* mutants.** Each pair of rows reports the context of a Cas9-

627 induced mutation (red) in the *cutl-24* gene of the indicated *C. elegans* strain. Uppercase, exonic

628 sequence; lowercase, intronic sequence.

Figure 1

Figure 2

Figure 3

4a

ED3077 male X N2 mutant   log2(Treatment/Control)

4b

N2 male X ED3077 mutant   log2(Treatment/Control)

Figure 4

Figure 5

6a

6b

Figure 6

7a

7b

Figure 7