Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system

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We report the use of clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated endonuclease Cas9 to target genomic sequences in the *Caenorhabditis elegans* germ line using single-guide RNAs that are expressed from a *U6* small nuclear RNA promoter. Our results demonstrate that targeted, heritable genetic alterations can be achieved in *C. elegans*, providing a convenient and effective approach for generating loss-of-function mutants.

CRISPR and CRISPR-associated (Cas) systems are adaptive mechanisms evolved by bacteria and archaea to repel invading viruses and plasmids[1,2]. CRISPR-Cas systems incorporate foreign DNA sequences into host CRISPR loci to generate short CRISPR RNAs (crRNAs) that direct sequence-specific cleavage of homologous target double-stranded DNA by Cas endonucleases[3,4]. Recent work with the *Streptococcus pyogenes* type II CRISPR system, which requires the nuclease Cas9, a targeting crRNA and an additional trans-activating crRNA, has shown that fusing crRNA and trans-activating crRNA to form a single guide RNA (sgRNA) is sufficient to direct Cas9-mediated target cleavage[4]. This system has been used for genome engineering in yeast[5], *Drosophila melanogaster*[6], human and mouse cell lines[7–10], and in zebrafish and mouse[11,12]. Here we configured Cas9 and sgRNAs for targeted gene disruption in the nematode *C. elegans*.

We first generated vectors to express Cas9 and sgRNAs in the germ line (**Fig. 1a**). We added an SV40 nuclear localization signal (NLS) to the 3′ end of the open reading frame encoding Cas9 to ensure the enzyme would be properly localized to the nucleus[8,10]. To drive expression of transcripts encoding this Cas9–SV40 NLS fusion protein, we used the promoter sequence from the gene *eft-3*, selected for its effectiveness in driving expression in the germ line[13]. Although in previous studies vectors containing RNA polymerase III (pol III) promoters have been used to transcribe small RNAs[14] or sgRNAs in mammalian systems[8–10], no equivalent vector has been described in *C. elegans*. Studying conserved upstream and downstream regulatory sequences flanking a *U6* small nuclear RNA (snRNA) gene in *C. elegans*, we derived a putative pol III promoter for expression of sgRNA (**Fig. 1a** and **Supplementary Fig. 1**). It has been suggested that optimal expression from pol III promoters occurs when the first base transcribed is a purine[15,16]. Combining this finding with the known sequence requirements of cleavage guided by CRISPR-Cas, our sgRNA expression system enables the selection of target sequences of the form G/A(N)$_{19}$NGG, where the G/A(N)$_{19}$ is a 20-nucleotide sequence that will recognize a homologous stretch of double-stranded DNA in the genome, and the 3′ NGG sequence represents the essential protospacer-associated motif (PAM)[1] (**Fig. 1b**).

We designed sgRNAs complementary to coding sequences in the *unc-119* and *dpy-13* genes. We selected these genes for targeting because loss-of-function alleles have been isolated at these loci that cause easily identifiable uncoordinated (Unc) or dumpy (Dpy) phenotypes, respectively[17,18]. Studies have indicated that CRISPR-Cas–guided double-strand breaks can be repaired through the process of nonhomologous end joining (NHEJ), which generates insertions and deletions (indels) in the vicinity of the cleavage site[7–9,11]. We reasoned that indels disrupting the coding sequences of *unc-119* and *dpy-13* would mimic previously identified alleles that cause Unc and Dpy phenotypes.

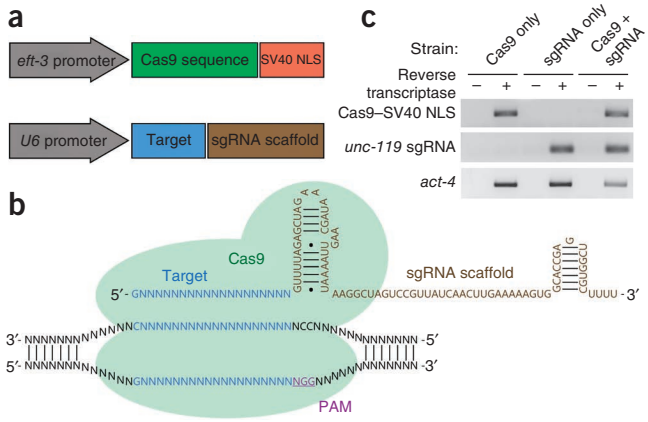

Figure 1 | Vectors that drive expression of Cas9 and sgRNAs in *C. elegans*. (**a**) Schematics of the cassette encoding Cas9–SV40 NLS driven by the *C. elegans eft-3* promoter and the target and scaffold sequence of the sgRNA driven by a pol III promoter. (**b**) Schematic of Cas9 interacting with sgRNA and its genomic target. (**c**) RT-PCR analysis of total RNA from strains expressing the indicated vectors with primers specific for Cas9 sequence or *unc-119* sgRNA. Transcripts from the *act-4* gene were monitored as a loading control.

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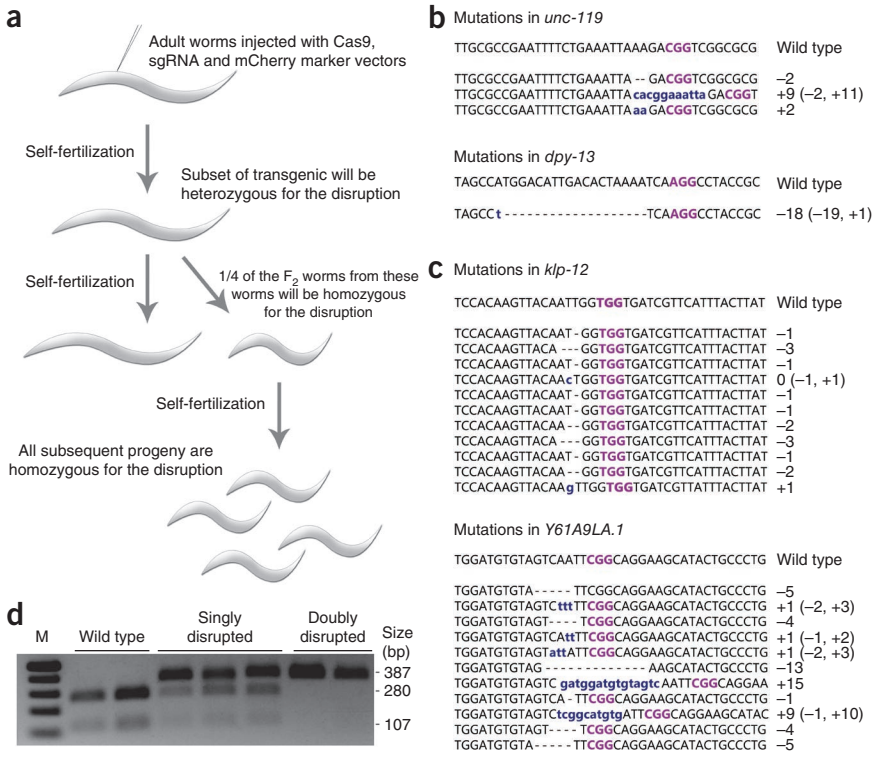

Figure 2 | Heritable, targeted gene disruptions in the germ line using CRISPR-Cas systems. (**a**) Experimental design to screen for mutants with phenotypes derived from worms injected with CRISPR-Cas expression vectors. (**b**,**c**) Sequences of the indel mutations found in our *unc-119* and *dpy-13* mutant lines (**b**) and *klp-12* and *Y61A9LA.1* mutant lines (**c**). Insertions are marked in blue, deletions are marked by dashes, and the PAMs are marked in purple. Numbers to the right of the sequences indicate the net loss or gain of bases for each allele, with the number of bases inserted (+) or deleted (–) indicated in parentheses. (**d**) Restriction digest analysis of PCR amplicons spanning the *klp-12* cleavage site from seven F_1 worms (1% agarose gel). M, 1 kb plus DNA ladder (Thermo Scientific).

To verify expression of both Cas9-SV40 NLS and sgRNAs, we microinjected the gonads of wild-type adult worms, generating transgenic progeny that carry each expression vector alone or both in stable extrachromosomal arrays[19]. We isolated total RNA from these transgenic lines and performed reverse transcription (RT)-PCR assays to detect transcripts. These assays confirmed that Cas9–SV40 NLS and sgRNAs were transcribed in transgenic worms (**Fig. 1c**), indicating that the *eft-3* and pol III promoters in our vectors were active.

We next investigated whether our Cas9-sgRNA expression system could direct targeted cleavage and disruption of *unc-119* and *dpy-13* in the germ line. We microinjected worms with vectors encoding Cas9, one of the two sgRNAs and a vector driving expression of mCherry in body-wall muscles to label transformed F_1 progeny. No mCherry fluorescent F_1 worms exhibited Unc or Dpy phenotypes. We isolated these mCherry-fluorescent worms and screened their F_2 progeny for Unc or Dpy phenotypes (**Fig. 2a**). In two replicate experiments in which we expressed Cas9 and the *unc-119*–specific sgRNA in worms, we recovered Unc F_2 progeny from 1/27 and 1/105 isolated F_1 worms (**Table 1**). In a third experiment in which we targeted the *unc-119* locus using higher concentrations of our expression vectors (Online Methods), we recovered Unc F_2 progeny from 1/60 F_1 worms. When targeting the *dpy-13* locus, we recovered Dpy F_2 progeny from 1/210 individual F_1 worms (**Table 1**). In all four experiments, when we identified Unc and Dpy F_2 progeny, we recovered them at a frequency of 25% from individual F_1 worms. All of the F_3 progeny from Unc and Dpy F_2 mutant worms exhibited Unc and Dpy phenotypes, respectively (**Supplementary Fig. 2** and **Supplementary Videos 1**–**3**). These observed patterns of inheritance are consistent with recessive loss-of-function mutations originating in the germ line of injected worms. We did not recover mutant worms from progeny of F_1 worms that did not express our mCherry marker or from worms injected with Cas9 vector or sgRNA vector alone (**Supplementary Table 1**), suggesting that both components are required for cleavage. To verify that disruptions targeted *unc-119* and *dpy-13*, we isolated DNA from mutant worms and sequenced regions spanning the predicted sites of cleavage. The genomes of all Unc mutants and the Dpy mutant had unique indels located in the expected target sequences, occurring 3–4 bases upstream of the PAM sequence (**Fig. 2b**). All of the identified indels are predicted to alter the coding sequence of each gene and would lead to the production of truncated proteins. These molecular changes are consistent with the phenotypes we observed, resembling previously characterized loss-of-function mutants. These results indicate that our vector system enables the expression of Cas9 and sgRNAs in the germ line to achieve targeted, heritable gene disruptions.

To extend our initial results and test whether we could also recover worms carrying disruptions that do not lead to visible phenotypes, we selected two additional loci (*klp-12* and *Y61A9LA.1*) with no known loss-of-function phenotypes and generated sgRNAs to target them. We microinjected these worms with Cas9, sgRNA and mCherry expression vectors as above, and isolated mCherry fluorescent F_1 progeny. We then genotyped these F_1 worms by sequencing regions of genomic DNA spanning expected cleavage

Table 1 | Summary of experiments targeting four loci

Experiment	Gene	Injected worms	F_1 worms	Disruptions	Frequency %
1	*unc-119*	ND	27	1	1/27 (3.7)
2	*unc-119*	ND	105	1	1/105 (0.9)
3	*unc-119*[a]	ND	60	1	1/60 (1.7)
4	*dpy-13*	ND	210	1	1/210 (0.5)
5	*klp-12*[a]	12	66	53	53/66 (80.3)
6	*klp-12*[a]	14	35	27	27/35 (77.1)
7	*Y61A9LA.1*[a]	11	72	13	13/72 (18.1)

ND, not determined.
[a]Fivefold higher concentrations of expression vectors were used (Online Methods).

sites. In two replicate experiments targeting *klp-12* and one targeting *Y61A9LA.1*, we generated disruptions in 53/66 (80.3%), 27/35 (77. 1%) and 13/72 (18.1%) of the F_1 worms screened, respectively (**Table 1** and **Fig. 2c**). At the *klp-12* locus, 27 out of 80 F_1 worms carrying a disruption were homozygous for a single disruption, and the remaining worms were heterozygous for a single disruption or carried two unique disruption alleles (**Supplementary Fig. 3**). We speculate that doubly targeted mutant F_1 worms are generated through two sequential break and repair events. The first event may occur in the haploid oocyte, where NHEJ-mediated repair introduces an indel. The second event likely occurs later in the sperm-contributed chromosome, where either a second, unique indel is introduced by NHEJ, or the already disrupted chromosome is used as a template in homologous recombination and the error is copied, yielding a homozygous mutant. We followed the inheritance of four *klp-12* alleles identified in F_1 worms by genotyping single F_2 worms not expressing mCherry and confirmed the heritability of all of these disruptions (**Supplementary Fig. 3**).

To demonstrate an additional screening strategy to identify disruptions that do not cause obvious phenotypes, we designed our sequence targeting the *klp-12* sgRNA to overlap with the recognition sequence of the restriction enzyme MfeI. When CRISPR-Cas–mediated cleavage occurs at this site, any indels spanning the restriction enzyme recognition sequence would lead to a restriction fragment length polymorphism in PCR amplicons generated from mutant genomic DNA. Using this approach, we identified wild-type worms, singly disrupted worms and doubly disrupted worms (**Fig. 2d**) that we confirmed by our sequencing analysis described above. One caveat to this restriction enzyme–based analysis is that worms carrying non-heritable mutations in a large fraction of somatic cells may exhibit digestion patterns that appear similar to patterns from worms with heritable disruptions. Sequencing of DNA from single F_1 and F_2 worms not expressing mCherry as described above (**Supplementary Fig. 3**) would be required to subsequently confirm the heritability of mutations. However, these results indicate that when possible, this method can provide a convenient way to prescreen a large number of candidate F_1 progeny for gene disruptions and decrease the number of worms requiring validation by sequencing.

To assess the possibility of CRISPR-Cas cleavage at off-target loci in our mutant strains, we searched for other sites in the genome that could potentially be targeted by our sgRNAs. Evidence suggests that the 12 nucleotides in the target sequence proximal to the PAM are the most critical determinants of cleavage specificity and may constitute a 'seed' region[20]. We scanned the genome for sequences of the form $(N)_{12}NGG$, and selected candidate off-target sites for each sgRNA that contained the minimum number of mismatches in these sequences. We sequenced the genomic regions spanning these potential cleavage sites in several of our mutant strains and found no evidence of cleavage nor indels at these loci (**Supplementary Fig. 4**). However, this was not a systematic assessment of the specificity of CRISPR-Cas–guided cleavage in *C. elegans*, and future work will be required to investigate the potential for off-target cleavage.

The discovery that RNA-guided endonucleases can cleave target sequences in the nuclei of eukaryotic cells has enabled genome editing in cultured cells, yeast, vertebrates and *Drosophila*. Here, through the use of a *U6* snRNA pol III promoter to drive expression of sgRNA, we demonstrated that CRISPR-Cas–guided cleavage can be used to introduce heritable mutations in *C. elegans*. In principle, the methodology described here could be applied to other model organisms in which efficient delivery of DNA to the germ line is feasible. Our results suggest that CRISPR-Cas–based systems have great potential for heritable genome editing in a wide variety of multicellular eukaryotes.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.E.F., K.M.E. and J.A.C. conceived of and designed experiments, with help from Y.B.T.; A.E.F. and J.A.C. assembled vectors; A.E.F. and J.A.C. performed microinjections and screened mutants; A.E.F., J.A.C. and Y.B.T. performed off-target genotyping analysis; A.E.F., K.M.E. and J.A.C. wrote the manuscript with input from Y.B.T., M.P.C. and G.M.C.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Strains and maintenance. The Bristol N2 strain (provided by members of the *Caenorhabditis* Genetics Center, University of Minnesota) was used in all experiments described. All worms were grown on nematode growth medium (NGM) agar plates seeded with the *Escherichia coli* bacterial strain OP50 and maintained using standard procedures[21].

Identification of a conserved *U6* snRNA pol III promoter. To develop a pol III promoter expression vector, we identified a conserved *U6* snRNA locus by performing BLAST-like Alignment Tool (BLAT) searches using the consensus *U6* snRNA sequence[22]. One locus on chromosome IV was selected for further analysis, and alignment and conservation tracks were extracted from the UCSC genome browser[23]. We identified ~80 base pairs of upstream sequence and 10 base pairs of downstream sequence conserved among several nematode species (see **Supplementary Fig. 1** for alignment). We therefore conservatively chose to include 500 bases of upstream sequence and 237 bases of downstream sequence flanking the snRNA sequence.

sgRNA targeting sequence identification and selection. Using the known sequence requirements of CRISPR-Cas–guided cleavage, we searched for target sequences in the *C. elegans* genome on the basis of the following criteria: (i) sequences had to be of the form G/A(N)$_{19}$NGG; and (ii) if a knockout of a protein-coding gene is desired, sequences contained in known open reading frames should be targeted. Although this is not a strict requirement, it likely ensures that a disruption will create an allele that shifts the canonical reading frame, often producing premature termination codons. (iii) Where possible, it is also desirable to look for target sequences that have a restriction enzyme recognition sequence a few bases upstream of the PAM (this will facilitate prescreening F_1 progeny by restriction digests). To actually select these sgRNA target sequences, we copied the genomic sequence spanning all of the coding exons and intervening intronic sequences of a gene of interest from Wormbase into Microsoft Word and, using the asterisk character as a wildcard, searched for strings that met the above criteria.

Plasmid construction. To create the Cas9–SV40 NLS expression vector, a worm codon-optimized open reading frame encoding Cas9 with an internal intron sequence and a 3′-end–fused SV40 nuclear localization signal sequence (see **Supplementary Table 2** for a full sequence) was synthetically produced (Genscript Inc.) and inserted into the vector pUC57. This intron containing open reading frame was PCR-amplified using the oligonucleotide primers cas9 start F/cas9 tbb-2 UTR R (see **Supplementary Table 2** for a full list of primers used in this study). The promoter region from the *eft-3* gene and 3′ UTR from the gene *tbb-2* were PCR-amplified from plasmid pCFJ601 (obtained from Addgene through the gift of E. Jorgensen and C. Frokjaer-Jensen) using the primers pUC57 EcoRI Peft-3 F and Peft-3 cas9 start R or tbb-2 UTR F and tbb-2 UTR pUC57 R, respectively. These three PCR products (promoter, Cas9–SV40 NLS + intron and 3′ UTR) were then inserted into a pUC57 plasmid digested EcoRI and HindIII using the Gibson assembly method as previously described[24]. To create the pol III promoter expression vector, we ordered two overlapping gBlocks gene fragments (IDT) collectively containing the 500 upstream nucleotides flanking a conserved *U6* snRNA locus, a target sequence with homology to a portion of the coding sequence of the *unc-119* gene, remaining sequence corresponding to the sgRNA and 237 nucleotides downstream of the *U6* snRNA locus (see **Supplementary Table 2** for full sequences). The two gBlocks were stitched together by PCR using the primers U6prom EcoRI F and U6prom HindIII R. This PCR product was then digested with EcoRI and HindIII and ligated into a pUC57 plasmid that had been digested with EcoRI and HindIII, creating vector pU6::unc-119 sgRNA. To generate the *dpy-13* sgRNA expression vector, we used the pU6::unc-119 sgRNA vector above as a template and amplified two overlapping PCR fragments using the primers U6prom EcoRI F and dpy-13 gRNA R or dpy-13 gRNA F and U6prom HindIII R. These PCR products were gel-purified and then mixed together in a second PCR with primers U6prom EcoRI F and U6prom HindIII R. This final PCR product was digested with EcoRI and HindIII and ligated into a pUC57 plasmid that had been digested with EcoRI and HindIII, creating the vector pU6::dpy-13 sgRNA.

To generate the *klp-12* sgRNA expression vector, we used the pU6::unc-119 sgRNA vector above as a template and amplified two overlapping PCR fragments using the primers U6prom EcoRI F and klp-12 gRNA R and klp-12 gRNA F and U6prom HindIII R. These PCR products were gel-purified and then mixed together in a second PCR with primers U6prom EcoRI F and U6prom HindIII R. This final PCR product was digested with EcoRI and HindIII and ligated into a pUC57 plasmid that had been digested with EcoRI and HindIII, creating the vector pU6::klp-12 sgRNA.

To generate the *Y61A9LA.1* sgRNA expression vector, we used the pU6::unc-119 sgRNA vector above as a template and amplified two overlapping PCR fragments using the primers U6prom EcoRI F and Y61A9LA.1 gRNA R or Y61A9LA.1 gRNA F and U6prom HindIII R. These PCR products were gel-purified, and then mixed together in a second PCR with primers U6prom EcoRI F and U6prom HindIII R. This final PCR product was digested with EcoRI and HindIII and ligated into a pUC57 plasmid that had been digested with EcoRI and HindIII, creating the vector pU6::Y61A9LA.1 sgRNA.

DNA microinjection. Plasmid DNA was microinjected into the germ line of adult hermaphrodite worms using standard methods as described previously[25]. Injection solutions were prepared to contain a final concentration of 100 ng/µl for two replicate *unc-119* experiments and the *dpy-13* experiment, and 500 ng/µl for a third *unc-119* experiment and all *klp-12* and *Y61A9LA.1* experiments. In all injections, we used the vector pCFJ104 (Pmyo-3::mCherry) as a co-injection marker. The vectors used in this study were present at the following final concentrations in injection mixes: 100 ng/µl total concentration-Peft-3::Cas9-SV40 NLS::tbb-2 3′UTR at 50 ng/µl, pU6::unc-119 or dpy-13 sgRNA at 45 ng/µl and pCFJ104 at 5 ng/µl; 500 ng/µl total concentration-Peft-3::Cas9-SV40 NLS::tbb-2 3′UTR at 250 ng/µl, pU6::unc-119 or klp-12 or Y61A9LA.1 sgRNA at 225 ng/µl, and pCFJ104 at 25 ng/µl.

When vectors were injected separately and the total DNA concentration in our injections mixes was lower than 100 ng/µl, the final concentration of DNA was adjusted to 100 ng/µl by adding DNA ladder.

We have noticed that injections with a total DNA concentration of 500 ng/µl can lead to sterility (up to 25%) of F_1 adult progeny.

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At present it is difficult to conclude whether the cause of this sterility is due to an overall increase in plasmid DNA delivered in injections or due to an increased concentration of a particular plasmid in our injection mix. This increase in sterility did not drastically affect our ability to recover fertile worms carrying disruptions at the *unc-119*, *klp-12* and *Y61A9LA.1* loci. If sterility does become an issue, we suggest testing several concentrations of each plasmid when trying to generate targeted disruptions in genes of interest.

RNA isolation and RT-PCR assays. Total RNA was isolated from lines stably carrying plasmids as extrachomosomal arrays using Tri reagent (Sigma) as recommended by the manufacturer. RT-PCR assays were performed using the OneStep RT-PCR kit (Qiagen) according to the protocol described by the manufacturer. Thirty nanograms of total RNA was used as input for each reaction. The sequences of primers used are provided in **Supplementary Table 2**.

Screening for disruptions in worms with no obvious phenotypes and genotyping. To screen for disruptions in the *klp-12* gene, we placed F_1 worms in 5 µl of single worm lysis buffer (10 mM Tris pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20 and 100 µg/ml proteinase K) and lysed the worms for 1 h at 60 °C, followed by incubation at 95 °C to inactivate the proteinase K. We then amplified a region of genomic DNA spanning the predicted disruption site by PCR using Phusion high fidelity polymerase (Thermo Scientific) as recommended by the manufacturer, using all 5 µl of worm lysate as a template (see **Supplementary Table 2** for a list of all primers used for PCR amplification and genotyping). PCR amplicons were then cleaned using the Genejet PCR purification kit (Thermo Scientific) as recommended by the manufacturer. The PCR product (5 µl) was then digested with the restriction enzyme MfeI (NEB) per manufacturer recommendations, and digestion products were resolved on a 1% agarose gel, stained with 100 µg/ml ethidium bromide and detected using a UV transilluminator.

To genotype all other worms and loci of interest, single worms were lysed, relevant regions were amplified by PCR and PCR products were cleaned as described above. Cleaned PCR products were then sequenced by Sanger sequencing methods (Genewiz).

To monitor inheritance of targeted disruptions at the *klp-12* locus, we followed the F_2 progeny of three F_1 worms carrying four alleles with disrupted sequences (two worms carrying a homozygous mutation and one worm carrying two independent disruptions). We sequenced single F_2 progeny from these worms (five F_2 worms from each of the homozygous mutants and 18 F_2 worms from the worm carrying two independent disruptions). For all four alleles, the allele found in the F_1 generation was passed on faithfully to the F_2 generation. In the case of the F_1 worm carrying two independent disruptions, we isolated homozygous F_2 mutant worms carrying each independent mutant allele at the expected Mendelian frequencies of 25%.

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