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

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

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Abstract

CRISPR-Cas systems have been used with single-guide RNAs for accurate gene disruption and conversion in multiple biological systems. Here we report the use of the endonuclease Cas9 to target genomic sequences in the *C. elegans* germline, utilizing 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.

Clustered, regularly interspaced, short palindromic repeats (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 *S. pyogenes* type II CRISPR system, which requires the nuclease Cas9, a targeting crRNA, and an additional trans-activating crRNA (tracrRNA), has shown that fusing the crRNA to the tracrRNA 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[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 germline (Fig. 1a). An SV40 nuclear localization signal (NLS) was added to the 3′ end of the Cas9 open reading frame 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 utilized the promoter sequence from the gene *eft-3*, selected for its effectiveness in driving expression in the germline[13]. While previous studies have utilized vectors containing RNA polymerase III (pol III) promoters to transcribe small RNAs[14] or sgRNAs in mammalian systems, no equivalent vector has been described in *C. elegans*. Studying conserved upstream and downstream regulatory sequences flanking a U6 snRNA gene in *C. elegans*, we derived a putative pol III promoter for sgRNA expression (Fig. 1a; Supplementary Fig. 1). It has been suggested that optimal expression from pol III promoters occurs when the first base transcribed is a

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Author Contributions

A.E.F., K.M.E., 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., J.A.C wrote the manuscript, with input from Y.B.T., M.P.C, and G.M.C.

purine[15, 16]. Combining this finding with the known sequence requirements of CRISPR-Cas guided cleavage, our sgRNA expression system enables the selection of target sequences of the form $G/A(N)_{19}NGG$, where the $G/A(N)_{19}$ represents 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. These genes were selected 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 non-homologous end joining (NHEJ), generating 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 causing Unc and Dpy phenotypes.

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

We next investigated whether our Cas9/sgRNA expression system could direct targeted cleavage and disruption of *unc-119* and *dpy-13* in the germline. We microinjected animals with vectors expressing 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-positive F_1 animals displayed Unc or Dpy phenotypes. We isolated these mCherry-positive animals and screened their F_2 progeny for Unc or Dpy phenotypes (Fig. 2a). In two replicate experiments expressing Cas9 and the *unc-119*-specific sgRNA, we recovered Unc F_2 progeny from 1/27 and 1/105 isolated F_1 animals (Fig. 2b). In a third experiment targeting the *unc-119* locus using higher concentrations of our expression vectors (see Supplementary Methods), Unc F_2 progeny were recovered from 1/60 F_1 animals. When targeting the *dpy-13* locus, we recovered Dpy F_2 progeny from 1/210 individual F_1 animals (Fig. 2b). In all four experiments, when Unc and Dpy F_2 progeny were identified, they were recovered at a frequency of 25% from singled F_1 animals. All of the F_3 progeny from Unc and Dpy F_2 mutant animals displayed Unc and Dpy phenotypes, respectively (Fig. 2c and Supplementary Movies 1–3). These observed patterns of inheritance are consistent with recessive loss-of-function mutations originating in the germline of injected animals. We were unable to recover mutant animals from progeny of F_1 animals not expressing our mCherry marker, or from animals injected with Cas9 or sgRNA 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 animals and sequenced regions spanning the predicted sites of cleavage. The genomes of all Unc mutants and the Dpy mutant possessed a unique indel located within the expected target sequences, occurring three to four bases upstream of the PAM sequence (Fig. 2d). 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 observe, resembling previously characterized loss-of-function mutants. These results indicate that our vector system enables the expression of Cas9 and sgRNAs in the germline to achieve targeted, heritable gene disruptions.

To extend our initial results and test whether we could also recover animals 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 animals with Cas9, sgRNA, and mCherry expression vectors as above, and isolated mCherry-positive F_1 progeny. We allowed these F_1 animals to lay eggs and then genotyped these animals by sequencing regions of genomic DNA spanning expected cleavage sites. In two replicate experiments targeting *klp-12* and one targeting *Y61A9LA.1*, we generated disruptions in 80.3%, 77. 1%, and 18.1% of the F_1s screened, respectively (Fig. 3a, 3b). Interestingly, at the *klp-12* locus, 27 out of 80 F_1 animals carrying a disruption were homozygous for a single disruption while other animals carried two unique disruption alleles. We speculate that these doubly-targeted mutant F_1s 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 NHEJ introduces a second, unique indel, or through homologous recombination uses the already-disrupted chromosome as a template and copies the error, yielding a homozygous mutant. We followed the inheritance of four *klp-12* alleles identified in F_1 animals by genotyping single mCherry-negative F_2 animals and confirmed the heritability of all of these disruptions (Supplementary Fig. 3).

To demonstrate an additional screening strategy capable of identifying disruptions that do not cause obvious phenotypes, we designed our *klp-12* sgRNA targeting sequence to overlap with that of a restriction enzyme, *Mfe*I. 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 (Figure 3C). Using this approach, we were able to distinguish between wild type animals, singly-disrupted animals, and doubly-disrupted animals (Figure 3C) that were confirmed by our sequencing analysis described above. These results indicate that when possible, this method can provide a convenient way to pre-screen a large number of candidate F_1 progeny for sequence disruptions and reduce the number of animals 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 within these sequences. We sequenced the genomic regions spanning these potential cleavage sites in several of our mutant strains and found no evidence of cleavage or indels at these loci (Supplementary Fig. 2). However, these results do not systematically assess the specificity of CRISPR-Cas guided cleavage in *C. elegans* and future work will be required to further 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 sgRNA expression, we demonstrate that CRISPR-Cas guided cleavage can 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 germline is feasible. Our results suggest that CRISPR-Cas based systems possess great potential for heritable genome editing in a wide variety of multicellular eukaryotes.

Methods

Strains and maintenance

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

Identification of a conserved U6 snRNA pol III promoter

In order to develop a pol III promoter expression vector, we identified a conserved U6 snRNA locus by performing 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 approximately 80 base pairs of upstream sequence and 10 base pairs of downstream sequence conserved among several nematode species (see Supplementary Figure 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:

1. Sequences had to be of the form $G/A(N)_{19}NGG$, where the $G/A(N)_{19}$ represents 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).
2. If a protein-coding gene knockout is desired, sequences contained within 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.
3. Where possible, it is also desirable to look for target sequences that possess a restriction enzyme recognition sequence a few bases upstream of the PAM. This will facilitate pre-screening 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 Cas9 open reading frame 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 kind gift of E. Jorgensen and C. Frokjaer-Jensen) using the primers *pUC57 EcoRI Peft-3 F*/*Peft-3 cas9 start R* and *tbb-2 UTR F*/*tbb-2 UTR pUC57 R*, respectively. These three PCR products (promoter, Cas9-SV40 NLS +

intron, and 3′ UTR) were then inserted into an EcoRI/HindIII digested pUC57 plasmid 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/U6prom HindIII R*. This PCR product was then digested with EcoRI and HindIII and ligated into an EcoRI/HindIII digested pUC57 plasmid, 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*|*dpy-13 gRNA R* and *dpy-13 gRNA F*|*U6prom HindIII R.* These PCR products were gel purified, and then mixed together in a second PCR reaction with primers *U6prom EcoRI F*|*U6prom HindIII R.* This final PCR product was digested with EcoRI and HindIII and ligated into an EcoRI/HindIII digested pUC57 plasmid, 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*|*klp-12 gRNA R* and *klp-12 gRNA F*|*U6prom HindIII R.* These PCR products were gel purified, and then mixed together in a second PCR reaction with primers *U6prom EcoRI F*|*U6prom HindIII R.* This final PCR product was digested with EcoRI and HindIII and ligated into an EcoRI/HindIII digested pUC57 plasmid, 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*|*Y61A9LA.1 gRNA R* and *Y61A9LA.1 gRNA F*|*U6prom HindIII R.* These PCR products were gel purified, and then mixed together in a second PCR reaction with primers *U6prom EcoRI F*|*U6prom HindIII R.* This final PCR product was digested with EcoRI and HindIII and ligated into an EcoRI/HindIII digested pUC57 plasmid, creating the vector pU6::*Y61A9LA.1* sgRNA.

DNA microinjection

Plasmid DNA was microinjected into the germline of adult hermaphrodite animals using standard methods as described previously[25]. Injection solutions were prepared to contain a final concentration of 100ng/uL for two replicate *unc-119* experiments and the *dpy-13* experiment, and 500ng/uL for a third *unc-119* experiment and all *klp-12* and *Y61A9LA.1* experiments. In all injections, we used the vector pCFJ104 (P*myo-3*::mCherry) as a co-injection marker. The vectors used in this study were present at the following final concentrations in injection mixes:

(P*eft-3*::Cas9-SV40 NLS::*tbb-2* 3′UTR) : 50 ng/uL

(pU6::*unc-119* or *dpy-13* sgRNA) : 45 ng/uL

pCFJ104: 5 ng/uL

and

(P*eft-3*::Cas9-SV40 NLS::*tbb-2* 3′UTR) : 250 ng/uL

(pU6::*unc-119* or *klp-12* or *Y61A9LA.1* sgRNA) : 225 ng/uL

pCFJ104: 25 ng/uL

When vectors were injected separately for Figure 1C, the final concentration of DNA was adjusted to 100 ng/uL by adding DNA ladder.

We have noticed that injections with a total DNA concentration of 500 ng/uL can lead to sterility (up to 25%) of F_1 adult progeny. At present it is difficult to conclude if 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 significantly affect our ability to recover fertile animals 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 animals with no obvious phenotypes and genotyping

To screen for disruptions in the *klp-12* gene, we placed F_1 animals in 5 uL 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, 100 ug/mL proteinase K) and lysed the animals for one hour 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 uL 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. 5 uL of PCR product were then digested with the restriction enzyme MfeI (NEB) per manufacturer recommendations, and digestion products were resolved on a 1% agarose gel, stained with 100 ug/mL ethidium bromide, and detected using a UV transilluminator.

To genotype all other animals and loci of interest, single animals 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 animals carrying four alleles with disrupted sequences (two animals carrying a homozygous mutation and one animal carrying two independent disruptions). We sequenced single F_2 progeny from these animals (Five F_2s from each of the homozygous mutants and 18 F_2s from the animal carrying two independent disruptions). We demonstrated that 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 carrying two independent disruptions, we were able to isolate homozygous F_2 mutant animals carrying each independent mutant allele at the expected Mendelian frequencies of 25%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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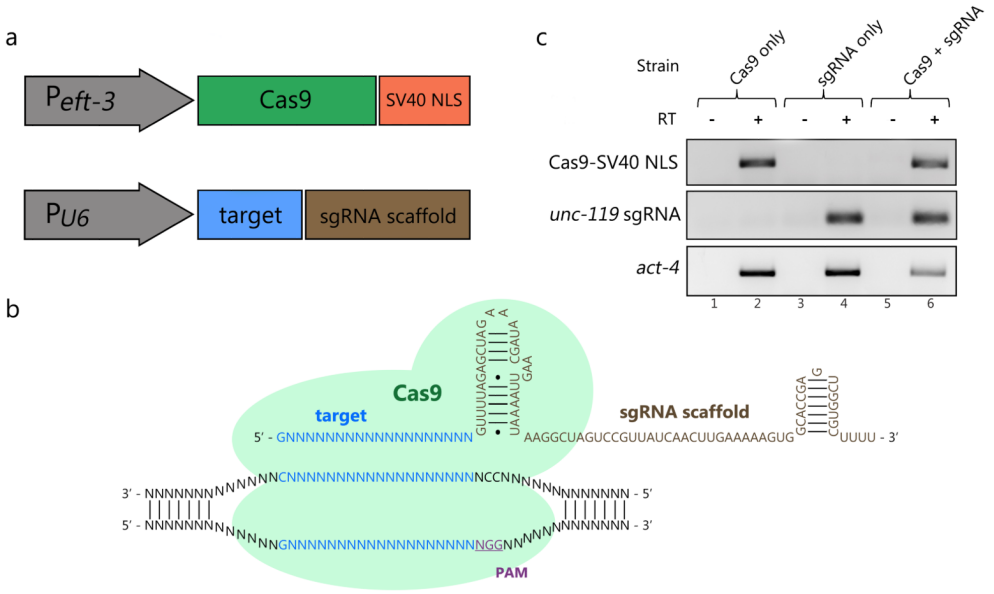

Figure 1.

A set of vectors that drive expression of Cas9 and sgRNAs in *C. elegans*. (A) The *C. elegans eft-3* promoter drives transcription of Cas9 with a 3′ SV40 nuclear localization sequence. A pol III promoter (derived from a U6 snRNA locus) drives transcription of the sgRNA, which contains a target sequence and a scaffold sequence. (B) A schematic illustration of Cas9 interacting with sgRNA and its genomic target. (C) RT-PCR results demonstrating expression of Cas9 and sgRNA transcripts. Total RNA was tested from strains carrying Cas9 vector alone (lanes 1 and 2), *unc-119* sgRNA vector alone (lanes 3 and 4), and both vectors (lanes 5 and 6) with primers specific for Cas9 (top panel) or *unc-119* sgRNA (bottom panel). For all samples, control reactions were run in the absence of Reverse Transcriptase (-RT; lanes 1, 3, and 5).

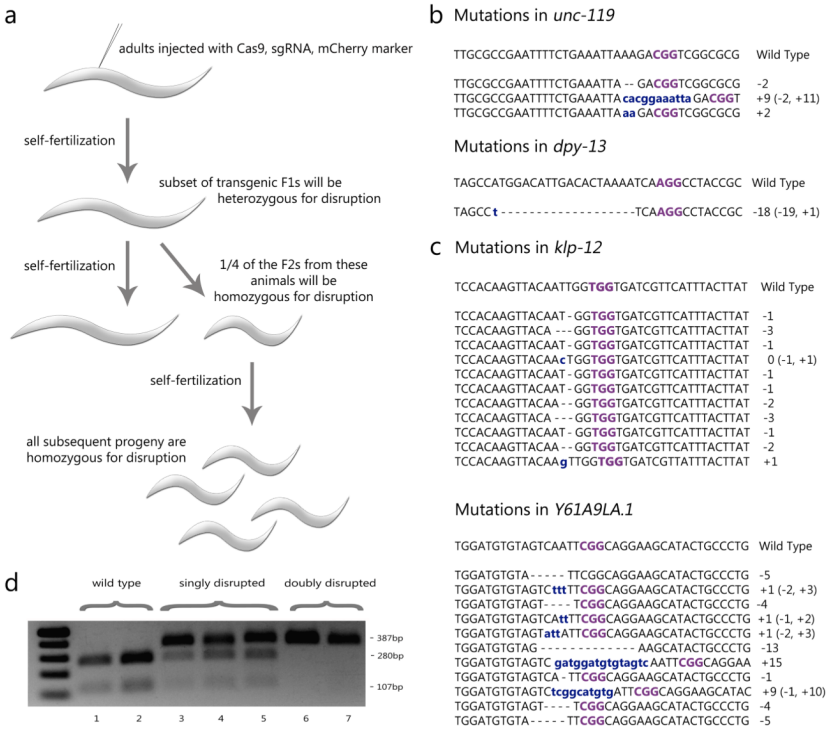

Figure 2.

Heritable, targeted gene disruptions in the germline using CRISPR-Cas systems. (A) Wild type (Bristol N2) adults were injected with vectors expressing Cas9, sgRNA, and a body wall muscle-specific mCherry marker. mCherry-positive F_1 animals were isolated, a small fraction of which were heterozygous for the disruption. Next, the F_2 animals were screened for mutant phenotypes, reflecting homozygous disruption. All further progeny of these F_2 mutants were homozygous for the disruption. (B) A table summarizing the results of the four experiments, in which 4 disruptions were found out of 402 mCherry-positive F_1 animals. (C) Images of worms from our wild type background line, a disrupted *unc-119* line, and a disrupted *dpy-13* line. (D) Sequences of the indel mutations found in our mutant lines. Insertions are marked in blue, deletions are marked by dashes, and the PAM is marked in purple. *The third experiment targeting the *unc-119* locus utilized five-fold higher concentrations of expression vectors (see Supplementary Methods for details).

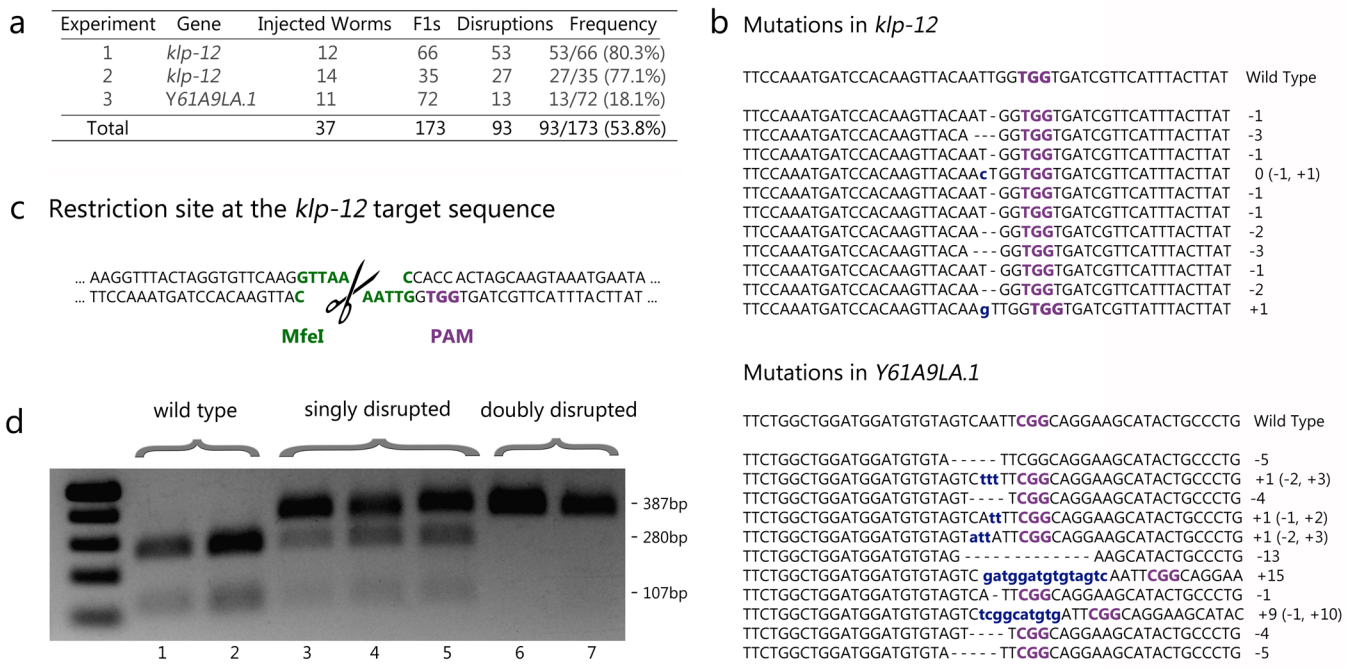

Figure 3.

Heritable, targeted gene disruptions in genes that lead to no obvious phenotypes. (A) A table summarizing the results of the three experiments, in which 93 disruptions were found out of 173 mCherry-positive F_1 animals. (B) Sequences of the indel mutations found in several of our mutant lines. Insertions are marked in blue, deletions are marked by dashes, and the PAM is marked in purple. (C) Sequence at the *klp-12* locus showing the target PAM site in purple and the *Mfe*I restriction site in green. (D) An image of a 1% agarose gel showing a restriction digest of PCR amplicons spanning the *klp-12* cleavage site from seven F_1 animals. Wild type sequences in lanes 1 and 2 are cut into bands of 280bp and 107bp, while doubly disrupted sequences remain full length at 387bp in lanes 6 and 7. Lanes 3, 4, and 5 show all three bands, indicating worms that are singly disrupted.

Table 1

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