Heat induced Cas9 visible (not very Strng)
Heat Chock promoter :: Cas9::gfp

CRISPR/Cas9-Targeted Mutagenesis in *Caenorhabditis elegans*

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Cas9::GFP expression is mild and not visible very much.

ABSTRACT The generation of genetic mutants in *Caenorhabditis elegans* has long relied on the selection of mutations in large-scale screens. Directed mutagenesis of specific loci in the genome would greatly speed up analysis of gene function. Here, we adapt the CRISPR/Cas9 system to generate mutations at specific sites in the *C. elegans* genome.

CURRENT methods to generate mutations in the genome of *Caenorhabditis elegans*, including chemical mutagenesis and imprecise excision of transposons, all rely on recovering mutations in large-scale mutagenesis screens. Recently, several groups reported the use of the *Streptococcus pyogenes* CRISPR/Cas9 system to generate double-strand break (DSB)-induced mutations in specific genomic loci in model systems including yeast (Dicarlo *et al.* 2013), flies (Bassett *et al.* 2013; Gratz *et al.* 2013; Yu *et al.* 2013), mammalian cells (Cho *et al.* 2013a; Mali *et al.* 2013), and zebrafish (Hwang *et al.* 2013). Because of the enormous potential for targeted genome engineering, we here investigate the suitability of the CRISPR/Cas9 system for use in *C. elegans*. This article is one of six companion articles in this issue (Chiu *et al.* 2013; Cho *et al.* 2013b; Katic and Grosshans 2013; Lo *et al.* 2013; Tzur *et al.* 2013) that present different approaches to and features of Cas9-CRISPR genome editing in *C. elegans*.

The *S. pyogenes* CRISPR/Cas system effects site-specific cleavage of double-stranded DNA through a complex containing the Cas9 endonuclease and two noncoding RNAs (CRISPR RNA or crRNA, and trans-activating crRNA or tracrRNA) (Gasiunas *et al.* 2012; Jinek *et al.* 2012). Target site specificity is mediated by a 20-nt spacer region in the crRNA that is complementary to the target DNA and a 3-nt motif (NGG) following the target site in the DNA [termed protospacer adjacent motif (PAM)] (Gasiunas *et al.* 2012; Jinek *et al.* 2012). Thus a wide range of target sites can be chosen. Conveniently, a single synthetic guide RNA (sgRNA) that fuses the 3′ end of crRNA to the 5′end of tracrRNA is sufficient to target Cas9 to a specific site and generate DSBs (Jinek *et al.* 2012) (Figure 1A).

To promote expression of Cas9, we codon optimized the *S. pyogenes* Cas9 coding sequence for *C. elegans*, introduced artificial introns, and attached SV40 and *egl-13* nuclear localization signals to the N and C termini, respectively, of the encoded Cas9 protein (Figure 1B). To express Cas9 in the germline, we placed the Cas9 coding sequence under control of the *eft-3* or *hsp-16.48* promoters and the *tbb-2* 3′-UTR, each of which has been shown to be compatible with germline expression (Bessereau *et al.* 2001; Merritt *et al.* 2008; Frøkjær-Jensen *et al.* 2012). To visualize expression of Cas9, we also generated Cas9::EGFP fusion vectors. We did not detect EGFP expression after injection of *Peft-3::Cas9::EGFP* (>20 animals examined). Injection of *Phsp-16.48::Cas9::EGFP* did result in visible EGFP expression, 5 hr after heat-shock induction for 1 hr at 34°. Expression did vary between experiments: one series of injections resulted in high expression in 5/5 animals examined (Figure 1C), while a second series of injections showed only weak expression in 1/12 animals examined. Because even low expression levels may provide sufficient enzymatic activity, in further experiments we tested both *Peft-3-* and *Phsp-16.48*-containing constructs for activity.
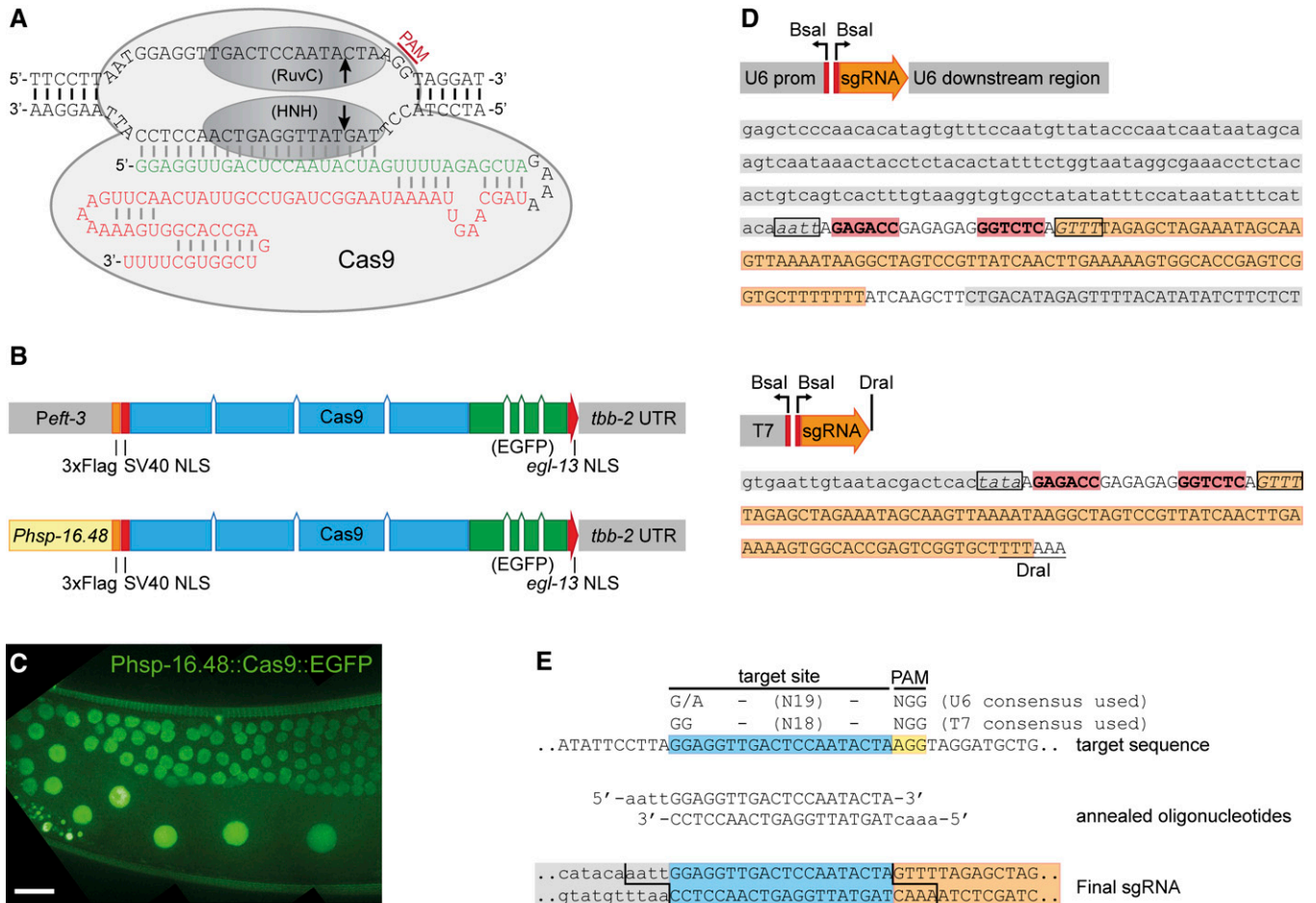
Figure 1 Experimental design and germline Cas9 expression. (A) Cas9/sgRNA in complex with a target site. RuvC and HNH endonuclease domains together generate a double-strand break. In the sgRNA sequence, green bases are crRNA derived and red bases tracrRNA derived. (B) Schematic of the Cas9 expression vectors used in this study, placing Cas9 or Cas9::EGFP under control of the *eft-3* promoter or the *hsp-16.48* heat-shock promoter. Versions lacking EGFP are not shown. (C) Germline expression and nuclear localization of Cas9::EGFP expressed from the *hsp-16.48* heat-shock promoter. Shown is a maximum-intensity projection of a Z-stack. Bar, 10 μm. (D) Diagrams and sequences of the U6::sgRNA and T7::sgRNA vectors. Gray background, promoter or downstream regions; orange background, sgRNA sequence downstream of the target recognition sequence; red background, *Bsa*I recognition sites; boxed nucleotides, sequences left as 5' overhang after *Bsa*I digestion. (E) Example of cloning a target sequence into the U6::sgRNA vector. The 20-bp target site is outlined in blue and the PAM in yellow. The consensus sequences we used for target site selection are also indicated. Detailed materials and methods are available in File S1.

To provide the sgRNA, we tested two different approaches. First, we generated a vector containing a T7 promoter upstream of the sgRNA sequence for *in vitro* transcription of the sgRNA. Second, we generated a vector expressing the sgRNA under control of the regulatory sequences of an RNA polymerase III transcribed U6 snRNA on chromosome III, to enable *in vivo* transcription (Thomas *et al.* 1990). Both vectors contain *Bsa*I restriction sites for simple insertion of the target recognition sequence as an oligomer linker (Figure 1, D and E).

As a first test of functional activity, we generated a reporter construct carrying an out-of-frame copy of EGFP and *lacZ* downstream of the *myo-2* promoter. Imprecise repair of a DSB in a linker region between the first ATG and EGFP can result in a frameshift, leading to EGFP expression. We co-injected the reporter (15 ng/μl) with *Peft-3*::Cas9 or *Phsp-*

16.48::*Cas9* (50 ng/μl), a U6-driven sgRNA targeting the linker region (50 ng/μl), and a *Pmyo-3*::*mCherry* co-injection marker (5 ng/μl). We also tested injection of lower Cas9/ sgRNA concentrations (20 ng/μl both) together with *Pst*I-digested λ DNA (20 ng/μl), to promote generation of more complex extrachromosomal arrays. Per condition we injected 10 animals, and *Phsp* expression was induced by a 1-hr heat shock at 34° after the injection. None of the injections with *Peft-3*::Cas9 yielded viable transgenic F$_1$'s. Instead, we observed mCherry-expressing dead embryos, indicating a deleterious effect of this construct. A series of test injections showed that the embryonic lethality is concentration dependent, ranging from 30% at 1 ng/μl to 100% at 20 ng/μl (see Supporting Information, Table S1). In contrast, 89% of the transgenic lines obtained from the injections with *Phsp-16.48*::*Cas9* expressed EGFP in the pharynx, indicating the

Table 1 Number of transgenic and EGFP-expressing F$_1$ animals obtained using Cas9/sgRNA directed against an EGFP frameshift reporter

			Results	
sgRNA concentration[a]	Phsp-16.48::Cas9 concentration[a]	No. P0 injected	Transgenic F1	F1 expressing EGFP
20	20	10	126	114 (90%)
50	50	10	32	27 (84%)

[a] All concentrations are in nanograms per microliter. Injections with 20 ng/μl Cas9/sgRNA are supplemented with 20 ng/μl of PstI-digested λ DNA. All injections include 5 ng/μl of the Pmyo-3::mCherry marker to identify transgenic animals and 15 ng/μl of the out-of-frame EGFP reporter.

presence of an extrachromosomal array with at least one frame-shifted copy of the reporter (Table 1). The injection of a lower concentration Phsp::Cas9 diluted with λ DNA resulted in a higher number of transgenic offspring, although the fraction expressing EGFP was similar (90% and 84%, respectively, Table 1). Control injections lacking the sgRNA did not show EGFP expression, demonstrating specific Cas9/sgRNA activity (50 transgenic F$_1$'s examined).

We also examined 18 stable transgenic lines obtained from EGFP-expressing F$_1$ animals. Of these, 15 expressed EGFP in most (>90%) of the F$_2$ transgenic animals. The small fraction of EGFP-negative transgenics could be due to mosaic inheritance of the extrachromosomal reporter array. Since Cas9 expression is induced by heat shock only in the injected P$_0$ animals, these findings may indicate that DSBs were generated in the germline of the P$_0$. Taken together, Cas9/sgRNA appears to efficiently generate DSBs in our plasmid-based reporter.

We next wanted to determine whether Cas9/sgRNA can be used to generate heritable mutations at a specific genomic locus in C. elegans. For this purpose, we generated sgRNA constructs targeting the lin-5 coding sequence near the known ev571 mutation. We injected Phsp::Cas9 together with either in vitro transcribed sgRNA or the U6::sgRNA plasmid, as well as the Pmyo-3::mCherry co-injection marker (Table 2). For each combination we injected 20 P$_0$ animals, selected individual F$_1$ animals expressing mCherry, and examined their F$_2$ progeny for the presence of Lin-5 offspring. Animals injected with in vitro-produced sgRNA failed to produce lin-5 mutants (Table 2). In contrast, injections with U6::sgRNA yielded a total of 10 F$_1$ animals that produced approximately one-quarter Lin-5 offspring (Table 2). We confirmed the presence of mutations at the lin-5 locus by

sequence analysis, identifying several deletions and a 7-bp insertion (Figure 2). For each F$_1$ line we sequenced two mutant F$_2$ animals independently, and in each case both animals harbored exactly the same mutation, strongly suggesting that the mutations were inherited from the parent and were not generated de novo by somatic events. Two mutations could not be resolved: Sanger sequencing traces from both sides degrade into double peaks at the sgRNA target site. This can result from the presence of a repeated sequence, and we speculate that DSB repair resulted in the duplication of a short DNA sequence. Injections with the lower concentration of Cas9 and sgRNA expression plasmids coupled with λ DNA yielded higher numbers of transgenic F$_1$ animals, but ultimately produced the same number of lin-5 mutants (Table 2).

Finally, we targeted three additional loci—dpy-11, rol-1, and unc-119—using Phsp-16.48::Cas9 and U6::sgRNA (Table 2). As for lin-5, we selected transgenic F$_1$ animals and looked for the presence of visible mutants in the F$_2$ generation. For dpy-11 and unc-119, we identified two transgenic F$_1$'s each that segregated approximately one-quarter mutant progeny, from a total of 20 and 41 transgenic F$_1$ animals selected, respectively (Table 2). Homozygous mutations in dpy-11 or unc-119 were readily identified in all cases (Figure 2). For rol-1, from 284 transgenic F$_1$'s, we observed three plates with only a single Rol F$_2$ animal. Sequencing of these mutants did confirm the presence of mutations at the target site (Figure 2). It appears therefore that the rol-1 phenotypes generated by our sgRNA are only partially penetrant. Together, these results confirm the ability of our approach to generate mutations at specific loci in the genome.

Here, we adapted the CRISPR/Cas9 system for use in C. elegans and demonstrate its ability to efficiently generate

Table 2 Number of transgenic F$_1$ and mutant F$_2$ progeny produced using Cas9/sgRNA directed against genomic loci

	sgRNA			Transgenic F$_1$	
Method/target	Concentration[a]	Phsp-16.48::Cas9 concentration[a]	No. P$_0$ injected	No. selected	With mutant progeny
U6 × lin-5	20	20	20	92	5
U6 × lin-5	50	50	20	24	5
T7 × lin-5	10	50	20	29	0
T7 × lin-5	150	50	20	124	0
U6 × rol-1	20	20	40	144	1
U6 × rol-1	50	50	20	140	2
U6 × dpy-11	50	50	20	20	2
U6 × unc-119	50	50	20	41	2

[a] All concentrations are in nanograms per microliter. Injections with 20 ng/μl Cas9/sgRNA are supplemented with 35 ng/μl of PstI-digested λ DNA. All injections include 5 ng/μl of the Pmyo-3::mCherry marker to identify transgenic animals.

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lin-5:    GAACAGGAGCTTACTGAGACTCTTCGGGCGACG  wild type
          GAACAGGAGCTTACTGAGACTCT-CGGGCGACG  -1
          GAACAGGAGCTTACTGAGACT--TCGGGCGACG  -2
          GAACAGGAGCTTACTGAGACTC--GGGCGACG   -3
          GAACAGGAGCTTACTGAGACTC---GGGCGACG  -3
          GAACAGGAGCTTACTGAGACT----GGGCGACG  -4
          GAACAGGAGCTTACTGAGA------GGCGACG   -7
          GAACAGGAGCTT----------TCGGGCGACG   -11
          GAACAGGAGCTTACTGAGACGGACGACTCTTCGGGCGACG  +7
          GAACAGGAGCTTACTGAGACT (+n)  CTTCGGGCGACG  +n
          GAACAGGAGCTTACTGAGACTCT (+n)  TCGGGCGACG  +n

dpy-11:   AGCTTGCAAGGATCTTCAAAA----------------------------------AGCATGGAACGC  wild type
          AGCTTGCAAGGATCTTCAAAACATGGAACATATACAATTGGAACATGGAACATATATTGTTCGAGCATGGAACGC  +42
          AGCTTGCAAGGATCTTCA---         (+n)         ---ATGGAACGC  -6/+n

unc-119:  CGGTGGTTATAGCCTGTTCGGTTACCGGTGGGG  wild type
          CGGTGGTTATA--------GGTTACCGGTGGGG  -8
          CGGTGGTTATAGCCTGTTCGGTGTACCGGTGTACCGATGTACCGGTGGGGTACCGGTGGGG  +28

rol-1:    TTAATGGAGGTTGACTCCAATACTAAGGTAGGA  wild type
          TTAATGGAGGTTGACTCCAA--CTAAGGTAGGA  -2
          TTAATGGAGG------------------A      -22
          TTAATGGAGGTTGACTCCAA (+n) AAGGTAGGA  -4/+n
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Figure 2 Genomic mutations generated by Cas9/sgRNA. Mutations are shown relative to the wild-type sequences. Three mutations could not be resolved by sequencing and may correspond to insertion of a repeated sequence. Blue indicates sgRNA target site, and yellow is the PAM motif.

genomic mutations. For *dpy-11*, *lin-5*, and *unc-119*, we obtained on average one mutant from every 5 or 6 P_0 animals injected. For *rol-1*, the frequency was much lower (three mutants of 60 P_0 injections), but the partial penetrance of the phenotype likely caused us to miss several mutations. The approach is not only efficient but also fast: cloning, mutant isolation, and sequencing of mutations can be completed in 10 days.

A recently published CRISPR/Cas9 method for *C. elegans* uses *Peft-3* to drive Cas9 expression (Friedland *et al.* 2013). However, we found that expression of Cas9 from the *eft-3* promoter causes embryonic lethality. This contrasting result may be due to differences in the exact Cas9 protein produced. While the reason for the observed lethality is unclear, use of the heat-shock promoter to provide a pulse of expression only in the injected animal circumvents this problem.

Five companion articles (Chiu *et al.* 2013; Cho *et al.* 2013b; Katic and Grosshans 2013; Lo *et al.* 2013; Tzur *et al.* 2013) also report the successful application of CRISPR/Cas9 in *C. elegans*. These groups use various approaches to provide Cas9 and sgRNA, including injection of Cas9 RNA or protein and *in vitro*-produced sgRNA. Thus, although in our case heat-shock-induced Cas9 coupled with U6-driven sgRNA proved most efficient, it appears that the methodology to provide these two components can be highly flexible.

In addition to generating mutants, the DSBs produced by Cas9/sgRNA enable several other applications of genome engineering, including insertion of exogenous DNA through homologous recombination (Katic and Grosshans 2013; Tzur *et al.* 2013), and are likely to become an important tool for *C. elegans* researchers.

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GENETICS

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File S1

Materials and Methods

Culture conditions and strains: The wild-type *C. elegans* strain N2 was maintained under standard culture conditions as previously described (BRENNER 1974). All experiments were performed at 25 °C, unless otherwise noted.

Plasmid construction: We generated four Cas9 expression constructs: pMB62 and pMB63 drive expression from the *eft-3* promoter, and are identical except for the presence of EGFP in pMB62. pMB66 and pMB67 drive expression from a heat shock promoter, and are again identical except for the EGFP fusion in pMB66. To generate these expression constructs, we first amplified the *tbb-2* 3' UTR from N2 genomic DNA by PCR, using a forward primer (5'-AAGAATTCATGCAAGATCCTTTCAAGCA), and reverse primer (5'-AAGAGCTCTGATCCACGATCTGGAAGATT) with EcoRI and SacI restriction sites, respectively. The resulting PCR product was cloned into pBluescript SK(+) digested with EcoRI and SacI. Next, we PCR amplified the *eft-3* promoter from pCFJ601 (FRØKJÆR-JENSEN *et al.* 2012) using a forward primer containing SalI (5'- AAGTCGACGCACCTTTGGTCTTTTATTGTCA), and a reverse primer containing XbaI and EcoRI sites (5'-AAGAATTCCCCGGGTCTAGATGAGCAAAGTGTTTCCCAACTG). The resulting PCR product was cloned into the *tbb-2* construct digested with SalI and EcoRI. To add Cas9, we ordered a synthetic plasmid containing the 3xFlag tag, the SV40 NLS, the Cas9 coding sequences with artificial introns, and the *egl-13* NLS (Genscript). All sequences were codon optimized for *C. elegans* using the *C. elegans* Codon Adapter (REDEMANN *et al.* 2011). These sequences were cloned between the *eft-3* promoter and *tbb-2* 3' UTR using XbaI and EcoRI sites also present in the synthetic plasmid. A unique SpeI site was added following the *tbb-2* UTR to facilitate future cloning efforts, by ligating a short oligonucleotide linker into an existing NdeI site, resulting in vector pMB63. To generate pMB62, *C. elegans* optimized EGFP coding sequences were PCR amplified from pMA-mEGFP (a kind gift from Tony Hyman) using primers containing PstI sites (5'-AACTGCAGATGTCCAAGGGAGAGGAGCTC and 5'-AACTGCAGCTTGTAGAGCTCGTCCATTCCGTG), and cloned downstream of Cas9 using PstI. To generate the heat-shock expression constructs pMB66 and pMB67, the heat shock promoter *Phsp-16.48* was amplified from vector pJL44 (BESSEREAU *et al.* 2001) using a forward primer containing a KpnI site (5'-AAGGTACCGCTGGACGGAAATAGTGGTAAAG) and a reverse primer containing an SpeI site (5'-AAACTAGTTCTTGAAGTTTAGAGAATGAACAGTAA), and inserted into pMB62 and pMB63 from which the *eft-3* promoter was removed using KpnI and XbaI (SpeI and XbaI digestions result in compatible overhangs).

To generate the T7 sgRNA vector pMB60, the T7 promoter sequence followed by the BsaI cloning sites and the chimeric crRNA-tracrRNA sequences was ordered as a gBlocks Gene Fragment (IDT), and cloned blunt into cloning vector pMK digested with PvuII. To generate the U6 sgRNA vector pMB70, the U6 promoter sequence (THOMAS *et al.* 1990) followed by the BsaI cloning

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sites and the chimeric crRNA-tracrRNA sequences was ordered as a gBlocks Gene Fragment (IDT), and cloned blunt into cloning vector pBluescript SK+ digested with EcoRV. The sgRNA sequences were then transferred from pBluescript to pMK using PvuII sites present in both vectors. Finally, to add potential 3' regulatory sequences, we PCR amplified and inserted an 888 bp region downstream of the U6 snRNA using primers containing HindIII (5'- AAGCTTCTGACATAGAGTTTTACATATATCTTCTCTG) and SalI(5'- GTCGACCGAAGAGCACAGAAAAATTGG).

The Cas9 activity reporter plasmid pLM47 (*Pmyo-2::ATG::sgRNA target::EGFP::lacZ::unc-54UTR*) was constructed by replacing the C23 microsatellite of a previously generated microsatellite instability reporter (pLM3, sequence available upon request) with an oligonucleotide linker containing a suitable sgRNA target sequence (GGATAACAGGGTAATTCTACCGG). The EGFP and LacZ coding sequences are out of frame with the first ATG, and require Cas9/sgRNA induced mutagenesis to be expressed.

sgRNA target site selection and cloning: The selection of a suitable sgRNA target site is limited by two requirements. First, the three nucleotides immediately following the target site have to correspond to the PAM consensus sequence of NGG (note that these three nucleotides are not actually incorporated in the sgRNA). Second, the promoters used may impose restrictions on the 5' nucleotides. In our case, efficient transcription from the T7 promoter is promoted by the incorporation of GG as the first two nucleotides of the RNA produced (IMBURGIO *et al.* 2000), while optimal transcription from a polymerase III promoter appears to require a purine as the first nucleotide of the RNA (FRUSCOLONI *et al.* 1995; ZECHERLE *et al.* 1996). We therefore used the following sgRNA consensus sites: G/A-(N19)-NGG for the U6 vector, and GG-(N18)-NGG for the T7 vector. Though we chose to use these conservative consensus sites, it may be possible to ease the restrictions on the 5' nucleotides by using different promoters (especially for *in vivo* production of the sgRNA), or by extending the sgRNA sequence on the 5'-end with one or two nucleotides that do not participate in target recognition. To find suitable sites in the *lin-5* and *rol-1* genomic sequences, we searched for these consensus sequences using ApE – A plasmid Editor (http://biologylabs.utah.edu/jorgensen/wayned/ape/).

To facilitate cloning of different target sites into our vectors, we designed these to be digested with BsaI, a restriction enzyme that cuts outside of the recognition sequence. Two BsaI sites are juxtaposed such that upon digestion, the recognition sites themselves are eliminated, and two overhangs are created that exactly match the last four nucleotides of the U6 or T7 promoter, and the first four nucleotides of the sgRNA sequence. To insert the target sites, we ordered phosphorylated forward and reverse oligonucleotides that can be annealed to generate linkers compatible with BsaI digested T7 or U6 vector. For *lin-5:* lin-5_T7_Fwd: 5'-tataGGAGCTTACTGAGACTCTTC, lin-5_U6_Fwd: 5'-aattGGAGCTTACTGAGACTCTTC, and lin-5_Rev: 5'-aaacGAAGAGTCTCAGTAAGCTCC. For *rol-1*: rol-1_U6_Fwd: 5'- aattGGAGGTTGACTCCAATACTA and rol-1_Rev: 5'-aaacTAGTATTGGAGTCAACCTCC. For *dpy-11*: dpy-11_U6_Fwd: 5'-aattGCAAGGATCTTCAAAAAGCA and dpy-11_Rev: 5'-

aaacTGCTTTTTGAAGATCCTTGC. For *unc-119*: unc-119_U6_Fwd: 5'-aattGTTATAGCCTGTTCGGTTAC and unc-119_Rev: 5'-aaacGTAACCGAACAGGCTATAAC. Oligonucleotides were annealed by heating 0.5 µmol of each oligonucleotide in annealing buffer (25 mM Tris-HCl, 10 mM MgCl$_2$, pH 7.5) and slowly cooling to room temperature. Annealed oligonucleotides were ligated in vectors digested with BsaI, and inserts were verified by sequencing.

In vitro transcription: *In vitro* transcribed sgRNA was generated with the life technologies MEGAscript T7 kit, using 1 µg of DraI digested plasmid as a template. After a 4 hour incubation, the sgRNA was purified by Ammonium Acetate precipitation per manufacturer's instructions, and resuspended in RNAse free water.

Imaging: Imaging of *Pmyo-2::EGFP* and *Phsp-16.48::Cas9::EGFP* expressing animals was performed on an Andor Revolution spinning disc confocal microscope. Z-stacks with 1 µm slice distance were taken at several locations along the length of the worm. Stacks were then stitched together using the ImageJ pairwise stitching plugin. Finally, a maximum intensity projection of 9 slices was generated.

Injections and Heat shock induction: Plasmids and RNA were injected using standard *C. elegans* microinjection procedures. To induce expression from the *hsp-16.48* promoter, injected animals were heat shocked for 1 h at 34 °C on agar plates floating in a water bath, 30 min to 1 h after injection.

Reagent availability: The sgRNA and Cas9 expression plasmids will be made available through Addgene (http://www.addgene.org).

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Table S1 Concentration dependency of the embryonic lethality caused by *Peft-3::Cas9*

Peft-3::Cas9 concentration	Transgenic F1		
	Embryonic lethal	Viable	% Emb
0 ng/µl	3	28	10
1 ng/µl	6	14	30
2 ng/µl	12	15	44
5 ng/µl	6	7	46
10 ng/µl	20	4	83
20 ng/µl	17	0	100

Injections consisted of 50 ng/µl of sgRNA, 5 ng/µl of the *Pmyo-3::mCherry* marker to identify transgenic animals, and the indicated amounts of *Peft-3::Cas9*. To inject a constant amount of DNA (75 ng/µl), injections with less than 20 ng/µl of *Peft-3::Cas9* were supplemented with empty pBluescript vector. Results represent the transgenic progeny derived from 6 injected animals over a 28 hour period.

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