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’-AAGAATTCAATGCAAGATCCTTTCAAGCA), and reverse primer (5’-AAGAGCTCTGATCCACGATCTGGAAATT) 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’-AAGAATTCCCCGGTCTAGATGAGCAAAGTGTTTCCCAAACGTG). 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 Ndel 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’-AACTGCAGATGCTTGAAGTAGAGCTCGTCCTCCTCGT), 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 Kpnl site (5’-AAGGTACCGCTGCCAGAAATAGTGTAAG), and a reverse primer containing an SpeI site (5’-AAACTAGTTCTGAGTTAGAGCTGCAGTAA), and inserted into pMB62 and pMB63 from which the *eft-3* promoter was removed using Kpnl and XbaI (SpeI and XbaI digestions result in compatible overhangs).

To generate the T7 sgRNA vector pMB60, the T7 promoter sequence followed by the Bsal cloning sites and the chimeric crRNA-tracrRNA sequences was ordered as a gBlocks Gene Fragment (IDT), and cloned bluntly 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 Bsal cloning
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’-AAGCTTCATAGAGTTTACATATATCTTCTCTG) and SalI(5’-GTCGACGAGAGCAGACAGAAAAATTGG).

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’-aaacTAGTATTGGAGTCACCTCC. For dpy-11: dpy-11_U6_Fwd: 5’-aattGCAAGGATCTTTCAAAAGCA and dpy-11_Rev: 5’-
aaacTGCTTTTGAAGAA
TCCTTG
For unc-119: unc-119_U6_Fwd: 5’-aattGTTATAGCCTGTTCGT
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 MgCl₂, 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 Dral 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).

Literature Cited


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

<table>
<thead>
<tr>
<th>Peft-3::Cas9 concentration (ng/µl)</th>
<th>Transgenic F1</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Embryonic lethal</td>
<td>Viable</td>
<td>% Emb</td>
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<tr>
<td>0 ng/µl</td>
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<tr>
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<td>0</td>
<td>100</td>
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</table>

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.