Heritable custom genomic modifications in *C. elegans* via a CRISPR-Cas9 system

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Short Running Title: CRISPR-Cas9 in *C. elegans* genome editing

**Key words:** CRISPR-Cas9; germline; *C. elegans*; homologous recombination; genome editing

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Abstract

We adapted the CRISPR-Cas9 system for template-mediated repair of targeted double-strand breaks via homologous recombination in *Caenorhabditis elegans*, enabling customized and efficient genome editing. This system can be used to create specific insertions, deletions and base pair changes in the germline of *C. elegans*. 
Genome engineering has proven to be a useful tool in biological research. Specific and accurate insertions, deletions and replacements allow for numerous invaluable ways to examine gene function. Yet, the accurate and efficient insertion or deletion of large, specific sequences has been challenging in most metazoans (Gaj et al. 2013). In the nematode Caenorhabditis elegans, creating heritable mutations had until recently involved random mutagenesis, and the insertion of genes into the genome had been limited to random loci, often resulting in the silencing of the inserted transgene in the germline due to repetitive insertions (Jackstadt et al. 1999; Kelly et al. 1997; Wilm et al. 1999). In the last few years new methods for genome editing have become available for this model system, all of which involve the creation of a specific double strand break in the genome. The most widely applied method uses the Mos1 transposon and transposase (Frokjaer-Jensen et al. 2012; Frokjaer-Jensen et al. 2010; Frokjaer-Jensen et al. 2008; Robert and Bessereau 2007). Although this method enables precise genome editing, it is limited by the availability of a transposon at the desired locus.

In bacteria and archaea, CRISPRs (clustered regularly interspaced short palindromic repeats) and Cas (CRISPR-associated) proteins are used to protect the cell against invading viruses and exogenous DNA (Sorek et al. 2013; Terns and Terns 2011; Wiedenheft et al. 2012). In the type II CRISPR systems, the Cas9 endonuclease specifically cleaves the exogenous DNA by interacting with two types of RNAs: tracrRNA (trans-activating CRISPR RNA), and crRNA (CRISPR RNA), that contain sequences complementary to the invading element and are generated from the CRISPR loci. Given the potential for RNA-mediated programmable DNA cleavage, this system was pared down to two components by using Cas9 from Streptococcus pyogenes and an engineered single guide RNA (sgRNA) (Jinek et al. 2012). Recently, this simplified system was transferred to several eukaryotic cell cultures and was shown to actively create specific double strand breaks, enabling changes in the genomes of those cells (Cho et al. 2013; Cong et al. 2013; DiCarlo et al. 2013; Jinek et al. 2013; Mali et al. 2013). Moreover, this system can now be used for genome editing in metazoan organisms such as mice, flies and fish (Gratz et al. 2013; Hwang et al. 2013; Shen et al. 2013; Yu et al. 2013).

We have recently reported the successful use of the CRISPR-Cas9 system in the nematode Caenorhabditis elegans, establishing a robust strategy for creating random insertion and deletion (indel) mutations in the germline of the worm by expressing Cas9 and sgRNA targeted to the desired site (Friedland et al. 2013). Although this method provides an efficient and facile strategy to create loss-of-function gene mutations, it involves nonhomologous end joining and therefore does not allow for user-specified changes, the complete removal of genes, or the introduction of tags. Here we report the use of this method for template-mediated repair of targeted double-strand breaks via
homologous recombination, enabling customized and efficient genome editing. This article is one of six companion articles (Chiu et al. 2013; Cho et al. 2013; Katic et al. 2013; Lo et al. 2013; Waaijers et al. 2013), that present different approaches to and features of CRISPR-Cas9 genome editing in *C. elegans*.

Results

Site-directed gene insertion using the CRISPR-Cas9 system

We hypothesized that targeted DNA double-strand breaks created by the CRISPR-Cas9 system can also be repaired by the homologous recombination pathway, and that specific insertions and/or deletions can be engineered into the germline using a donor vector (Figure 1A). To test this hypothesis, we injected young adult worms with a mixture of four plasmids (see Supporting Information) containing our codon optimized Cas9 driven by the *eft*-3 promoter, a *klp*-12 targeting sgRNA driven by a U6 snRNA promoter (Friedland et al. 2013), an mCherry reporter driven by a body wall muscle promoter to label F1 progeny and serve as a marker for extrachromosomal array formation, and a donor vector containing an *eft*-3 promoter-driven GFP transgene flanked on either side by 1.5kb of sequence homologous to regions upstream and downstream of the Cas9-induced cleavage site at the *klp*-12 locus (Figure 1B and Figure 2A). We isolated F1 progeny expressing GFP and mCherry, and then screened for F2 animals maintaining broad GFP but not mCherry expression, because these animals may have lost the extrachromosomal array and integrated the GFP transgene into the *klp*-12 locus. We additionally surveyed these GFP-expressing F2 animals by amplifying regions of genomic DNA specific for the recombinant product (Figure 2B). Using this combined screening approach, 1/72 (1.3%) isolated F1 animals generated progeny producing the expected PCR amplicon, indicating that homologous recombination occurred (Table 1). Consistent with the heritable nature of this recombination event, we obtained homozygous lines by isolating F2 worms producing 100% GFP-expressing F3 and subsequent progeny (Figure 2C). We further verified that the transgene was inserted seamlessly by sequencing our recombinant-specific PCR amplicon (Supplemental Figure 1).

A one-step CRISPR-Cas9-mediated gene replacement strategy

Next, we tested whether we could use our expression vector system to knockout a complete gene and replace it with a GFP transgene. We generated an sgRNA
targeting the lab-1 gene, and verified its effectiveness in generating indel mutations at the appropriate locus by direct sequencing when injected with Cas9 (data not shown)(Friedland et al. 2013). We then injected this sgRNA expression vector, our Cas9 expression vector, the mCherry reporter vector, and a donor vector containing a GFP transgene driven by the baf-1 promoter, flanked by 1020 and 1029 base pairs upstream and downstream of the lab-1 coding region, respectively (Supporting Information and Figure 3A). This donor vector was designed to completely remove the 652 bp of lab-1 and replace it with Pbaf-1::GFP. We screened for successfully engineered genomes in two independent experiments by using a similar PCR assay as described above, and we isolated F1 worms that had recombinant progeny in 1/40 (2.5%) and 4/24 (16.7%) of the worms (Table 1 and Figure 3B). Again, isolated from the original insertion strain were homozygous recombinant progeny that express GFP through multiple generations, both in somatic and germline tissues (Figure 3C and Supplemental Figure 2). Immunostaining using LAB-1-specific antibodies confirmed that the protein was absent in our recombinant line (Figure 3D). Sequencing of a PCR product spanning the recombinant locus confirmed the excision of the lab-1 gene and its replacement with the GFP transgene (Supplemental Figure 3). Consistent with the proposed functions for lab-1 in promoting sister chromatid cohesion and accurate meiotic chromosome segregation (de Carvalho et al. 2008), we observed increased embryonic lethality (45%, n=651; Emb) and a high incidence of males (11%, n=361; Him) in our lab-1 knock-out GFP knock-in line. These levels are similar to those reported for the lab-1 RNAi depleted worms (57% Emb and 6% Him), and higher than those observed for the lab-1(tm1791) hypomorph mutant (22% Emb and 4% Him) (de Carvalho et al. 2008), underscoring the importance of generating full knock-outs for assaying the null phenotypes of genes. We did not observe any new or unexpected phenotypes for either of the strains reported here.

Finally, to test whether the inserted promoter might regionally perturb gene expression, we assessed by RT-qPCR the expression of asfl-1 and T05F1.11, which flank lab-1 (Supplemental Figure 4). We found no significant change in the expression of T05F1.11 (P=0.153 by the two-tailed Mann-Whitney test, 95% C.I.), but the expression of asfl-1, which lies upstream of lab-1, exhibited a small yet significant change (P=0.046 by the two-tailed Mann-Whitney test, 95% C.I.). Further experiments will be required to determine whether this increase is due to a polar position effect exerted by the inserted promoter-gfp fusion, or a secondary effect of the lab-1 knock-out. Altogether, these results demonstrate that CRISPR-Cas9 is a useful system for the seamless replacement of genes in the C. elegans genome.

Discussion
The results presented here show the powerful use of the CRISPR-Cas9 system to accurately engineer the *C. elegans* genome, thereby enabling the creation of almost any custom mutation desired. We were able to insert and delete genes, as well as do both in one step. The transgenes were seamlessly inserted and were expressed both in the soma and the germline, indicating the functionality and specificity of this method. Although we did not observe any off-target effects, it would be a good practice to outcross any mutation created using the CRISPR-Cas9 system. This method (also see accompanying *C. elegans* studies by Chiu et al., Cho et al., Katic et al., Lo et al. and Waaijers et al., in this issue of GENETICS) now paves the way to various in vivo applications including the replication of mutations identified in other organisms and the assessment of protein-protein binding sites as well as the amino acids predicted to undergo post-translational modifications. Tagging of genes in their own natural sites will become easier, thus enabling scientists to monitor their expression more accurately and facilitating immunoprecipitation experiments. Different gene mutation projects have provided the worm community with a growing collection of invaluable base change and deletion mutants. The CRISPR-Cas9 system now provides the community the ability to expand this collection and obtain precise gene deletions or targeted changes in a simple and rapid manner.

Other methods have been previously implemented in *C. elegans* to create similar engineering capabilities like ZFNs (zinc-finger-nucleases), TALENs (transcription activator-like effector nucleases), MosSCI (Mos1-mediated single copy insertion) and MosDel (Mos1-mediated deletion) (Frokjaer-Jensen et al. 2012; Frokjaer-Jensen et al. 2010; Frokjaer-Jensen et al. 2008; Wood et al. 2011). The CRISPR-Cas9 method offers comparable and sometimes superior efficiency, along with three additional advantages: 1) It does not require the complex engineering of a special nuclease for every project, 2) it does not require any positive genetic selection marker, and 3) it can be applied to practically any locus given that the only requirement for the targeting site is the presence of G/A(N)\(_{19}\)NGG. These advantages, combined with the fact that this method calls for no specific genetic background, should now make it possible to sequentially create lines harboring multiple mutations in tightly linked genes that would otherwise require difficult genetic crosses. Finally, our results not only more generally demonstrate the versatility of the CRISPR-Cas9 system for customized engineering, now reported for several metazoan species, but also more specifically represent a significant step forward in facilitating a wide-range of gene function and regulatory studies in *C. elegans*.
Acknowledgments

We thank Yoav Mayshar, John Aach and Elisabeth Altendorfer for valuable suggestions. pAD010 was a kind gift from Yosef Gruenbaum. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This work was supported by US National Institutes of Health (NIH) grant R01GM072551 to M.P.C., by an NIH Early Independence Award (1DP5OD009153) and additional support from Harvard University to J.A.C., and by an NIH National Human Genome Research Institute Center of Excellence in Genome Sciences grant (P50 HG005550) to G.M.C. A.E.F. is supported by a Ralph Ellison/American Federation for Aging Research postdoctoral fellowship.
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Figure legends

**Figure 1**: Rationale and design applied in adapting the CRISPR-Cas9 system for homologous recombination-mediated genome engineering in *C. elegans*.

A) Schematic representation of the elements generating the engineered product. Cas9 (yellow) interacts with the sgRNA carrying a G/A(N)_{19}NGG sequence, where G/A(N)_{19} corresponds to 20 nucleotides complementary to the homologous genomic target, cleaving the target double-stranded DNA (bolts) at the 3’ NGG sequence that corresponds to the essential protospacer-associated motif (PAM). A donor vector that shares homology with the genomic locus, provides a template for repairing the break, thus inserting custom mutations (red), deletions and insertions (green) to the recombinant outcome.

B) Experimental design to generate and screen for worms carrying engineered genomes. Adult worms were injected with CRISPR-Cas9 expression vectors containing Cas9, a mCherry marker, donor template sequence and the targeting sgRNA.

**Figure 2**: Insertion of the Peft-3::GFP::tbb-2 3’UTR transgene into the klp-12 locus by CRISPR-Cas9 mediated homologous recombination.

A) Design of donor repair template and diagram of expected recombinant product. The donor template (top image) contains 1.5 kb of homology (solid black lines) flanking the Cas9 cleavage site at the klp-12 locus (middle image, red triangle). The donor also carries a GFP transgene (green box) flanked by the eft-3 promoter (yellow box) and the tbb-2 3’UTR (blue box), and mutations to introduce a HindIII recognition sequence (M1) and to destroy the protospacer-associated motif (PAM) of the sgRNA target sequence (M2). Any recombination events (bottom image) can be screened using PCR primers complementary to the transgene (arrows P2 or P3) and complementary to genomic regions outside the homology arms of the donor (arrows P1 or P4; dashed lines mark outer genomic regions).

B) PCR confirmation of recombinant animals. Left panel, top image; PCR assay using primers specific for a recombination event (P3 and P4 from A) sampling lysates of pooled F2 animals from two different lines carrying the transgene in an extrachromosomal array along with our body wall muscle mCherry marker (lanes 1 and 2) and a candidate recombinant line (lane 3) identified by fluorescence microscopy. Left panel, bottom image; PCR assay using primers complementary to the tbb-2 3’UTR to serve as a loading control. Right panel; PCR assays testing the recombinant line from the left panel, amplifying recombinant-specific PCR products spanning upstream (lane 1, primers P1 and P2) and downstream (lane 2, primers P3 and P4) of the cleavage site.
C) GFP expression in a strain in which a GFP transgene was inserted into the *klp-12* locus. Bar, 100 μm.

**Figure 3:** Insertion of the *Pbaf-1::GFP* transgene into the *lab-1* locus and excision of the *lab-1* gene by CRISPR-Cas9 mediated homologous recombination.

A) Design of donor repair template and diagram of expected recombinant product. The donor template (middle image) contains 1020 bases upstream to the *lab-1* ATG and 1029 downstream of the *lab-1* STOP codon regions of homology (solid black lines). The Cas9 cleavage site at the *lab-1* locus (652 bp) is located 310 bases downstream of the ATG (arrow, upper illustration). The donor also possesses a GFP transgene (884 bp, green box) flanked by the *baf-1* promoter (286 bp, green arrow).

B) PCR detection of recombinant specific species. PCR amplicon generated by using primers annealing 1167 base pairs upstream of the *lab-1* ATG and to the *baf-1* promoter. A-D, F-H negative clones. E, a positive clone. Arrowhead, ~1200 bp fragment.

C) GFP expression in a strain in which a GFP transgene was inserted and *lab-1* was deleted. Bars, 100 μm. Insets depict GFP expression in the head (i), embryo (ii) and oocyte (iii). Bars, 10 μm.

D) Anti-LAB-1 (red) and DAPI (blue) co-staining of late pachytene nuclei in the germlines of wild type (top) and *lab-1* deleted and *gfp* inserted (bottom) worms. Bars, 10 μm.

**Table 1:** Summary of experiments creating homologous recombination-mediated mutations

**Supporting Information Figure legends**

**Supplemental Figure 1:** Confirmation of seamless recombination at the *klp-12* locus.

Upper panel shows a sequence trace of the upstream recombinant junction. Endogenous upstream *klp-12* sequence is flanked by the M1 and M2 mutations described in Figure 2A, and the beginning of the *eft-3* promoter sequence. Bottom panel shows a sequence trace of the downstream recombinant junction, marking the end of the *tbb-2* 3’UTR followed again by endogenous *klp-12* sequence. All introduced sequences are labeled in red lower case letters.
Supplemental Figure 2: Immunostaining of germline nuclei with an anti-GFP antibody to examine the expression of the Pbaf-1::GFP transgene inserted into the lab-1 locus.

Shown are meiotic nuclei in early prophase I (transition zone and early pachytene) (A), and diakinesis (B), from whole mounted gonads of wild type and lab-1 knockout GFP knock-in worms, co-stained with DAPI and anti-GFP. Bars, 10µm.

Supplemental Figure 3: Confirmation of seamless recombination at the lab-1 locus.

Sequence resulting from the PCR product generated by using primers 1100 base pairs upstream and downstream of lab-1. Sequences from the lab-1 upstream region (violet), baf-1 promoter (yellow), plasmid backbone (light blue), and gfp (green) are marked. The site by which the upstream fragment was cloned is marked with brackets.

Supplemental Figure 4: lab-1 knock-out and GFP knock-in effect on the expression of flanking genes. RT-qPCR expression analysis of lab-1 (A), asfl-1 (B), and T05F1.11 (C). Relative ± SEM values are presented. Sample values were normalized to gpd-1 (GAPDH).
A

- **Cas9** target
- **sgRNA scaffold**
- **Donor Template**
- **Recombinant outcome**

B

1. **Cas9** marker Donor sgRNA
2. **Self fertilization**
3. **Isolation of marker carriers**
4. **Assay recombination events by PCR**
5. 1/4 of the F2 will be homozygous for the recombination
Table 1: Summary of experiments creating homologous recombination-mediated mutations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Gene</th>
<th>Injected worms</th>
<th>F1 Worms</th>
<th>Recombinant worms</th>
<th>Frequency (%)</th>
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<tr>
<td>A</td>
<td><em>klp-12</em></td>
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<tr>
<td>B</td>
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<td>40</td>
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<tr>
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<td><em>lab-1</em></td>
<td>7</td>
<td>24</td>
<td>4</td>
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