Heritable Gene Knockout in *Caenorhabditis elegans* by Direct Injection of Cas9–sgRNA Ribonucleoproteins

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**ABSTRACT** We present a novel method of targeted gene disruption that involves direct injection of recombinant Cas9 protein complexed with guide RNA into the gonad of the nematode *Caenorhabditis elegans*. Biallelic mutants were recovered among the F1 progeny, demonstrating the high efficiency of this method.

Clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated, Cas9-derived RNA-guided endonucleases (RGENs) enable targeted mutagenesis in cells and organisms (Cho et al. 2013a; Cong et al. 2013; DiCarlo et al. 2013; Gratz et al. 2013; Hwang et al. 2013; Jinek et al. 2012; Mali et al. 2013; Wang et al. 2012). In addition to other nuclease-mediated gene targeting methods (Morton et al. 2006; Wood et al. 2011), heritable genome editing was recently achieved in *Caenorhabditis elegans* using transgenes, driving the expression of Cas9 and a single guide RNA in *C. elegans* (sgRNA) (Friedland et al. 2013). Here we report that Cas9 protein, used as an alternative to a Cas9-encoding plasmid or mRNA, which can be silenced in nematodes, can induce efficient genome editing in *C. elegans*. This article is one of six companion articles (Chiu et al. 2013; Cho et al. 2013b; Katic and Grosshans 2013; Lo et al. 2013; Tzur et al. 2013; Waaijers et al. 2013) that present different approaches to and features of Cas9–CRISPR genome editing in *C. elegans*.

We first chose to target *dpy-3*, a gene on the X chromosome, because both homozygous and hemizygous mutations in this gene cause visible phenotypes (Blaxter 1993). We designed two sgRNAs complementary to the coding sequence of *dpy-3* (Figure 1, A and B). These sites are unique within the genome, and sequence alignment analysis showed that there were no possible off-target sequences in the entire genome, with fewer than four base mismatches to the target sequences (Supporting Information, File S1, Figure S1, and Table S1). We briefly incubated purified Cas9 protein with the two in vitro transcribed sgRNAs and injected the ribonucleoprotein (RNP) complexes into the gonads of adult *C. elegans* worms (Figure 1A). Among many injected P0 animals, five exhibited bloated gonad after microinjection, which is indicative of successful injection into the gonad. The F1 progeny of these five P0 animals were further examined for mutations.

The F1 animals were subjected to the T7 endonuclease I (T7E1) assay (Kim et al. 2009) to detect small insertions or deletions (indels) generated via the error-prone non-homologous end-joining (NHEJ) pathway used to repair double-strand DNA breaks (DSBs) induced at the target site. Mutations were detected in the F1 progeny of two P0 animals at frequencies of 1/24 (labeled as A-1 F1) and 3/33 (labeled as D-1 F1, D-2 F1, and D-3 F1). Sequence analysis of PCR products derived from the mutant F1 animals showed small deletions at one site or deletions that spanned both sites, suggesting that the RGENs may have acted on both targeted sequences (Figure 1B). We observed more than two mutations in the A-1 F1 and D-1 F1 animals, suggesting multiple mutational events in these animals, most likely in both the germ cells and somatic cells. Thus it appears that the nuclease maintained its activity in the embryos even after fertilization of the eggs.
Notably, we isolated two F1 animals from the same P0 animal (labeled D) that exhibited the dumpy (Dpy) phenotype, which is the expected phenotype of homozygous dpy-3 mutants (Figure 1, B and E). One Dpy F1 animal, D-2 F1, was examined along with other F1 animals using the T7E1 assay without collecting additional progeny. Sequence analysis of the PCR products derived from this animal showed both a small deletion and the wild-type sequence, suggesting that a mosaic mutation had occurred, probably in the hypodermis, which conferred the Dpy phenotype. The other animal, labeled D-3 F1, escaped from the plate after laying eggs, making it only possible to examine its F2 progeny. Sequence analysis showed that the F2 animals, all of which exhibited the Dpy phenotype, contained two independent mutations.

Figure 1 Heritable mutagenesis induced by the Cas9–sgRNA RNP complex in C. elegans. (A) Schematic representation of the Cas9 protein–sgRNA complex injection. Purified Cas9 protein and in vitro transcribed sgRNA were mixed and injected into the gonads of P0 animals. F1 and/or F2 animals were examined for mutations using the T7E1 assay and sequencing. The progeny were also examined for the visible Dpy and Unc phenotypes when appropriate. (B–D) Sequence analyses of the F1 mutant progeny (B) and F2 progeny (C) from the dpy-3 targeting experiments, and sequence analysis of the F1 progeny from the unc-1 targeting experiments (D). Target sequences of the sgRNAs are underlined within the genomic sequences of the corresponding genes. The nature of the mutations is indicated at the end of each sequence. —, deletion; +, insertion. Red characters represent nucleotides that do not match the genomic sequence. The blue sequences are the protospacer-adjacent motif (PAM) sequences. (E) Images of wild-type (N2 strain) and mutant worms (dpy-3 and unc-1) created by Cas9/sgRNA RNP-mediated gene knockout. Bar, 400 μm.
follow-up analysis of the progeny of F1 animals after tor-like effector nucleases (TALENs) (Kim Lee et al. observed, reminiscent of chromosomal deletions induced deletions that spanned the two RGEN sites were frequently

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1D). Similar to the deletions observed at the sequences, suggesting that this animal was a mosaic (Figure

4/24, 24/24 (100)).

animals showed that two (B-1 F1 and B-2 F1) contained

mutations in the

dpy-3 gene. In total, we observed 4/121 F1 animals with mutations and at least one case of biallelic heritable mutations in the dpy-3 targeting experiment. The results of the dpy-3 targeting experiment are shown in Table 1.

We next chose another X-linked gene, unc-1 (Rajaram et al. 1998; Chen et al. 2007), for targeting with the Cas9–sgRNA RNP complexes. We examined the F1 progeny of four P0 animals injected with the Cas9–sgRNA RNP complexes and found that two P0 animals produced mutant F1 progeny at frequencies of 5/32 (labeled as A-1 ~5 F1) and 4/24 (labeled as B-1 ~4 F1). Sequence analysis of PCR products derived from four F1 animals out of the nine mutant F1 animals showed that two (B-1 F1 and B-2 F1) contained single mutations, and a third animal (A-1 F1) contained two mutations (Figure 1D). The fourth animal, B-3 F1, contained two different mutations as well as the wild-type sequences, suggesting that this animal was a mosaic (Figure 1D). Similar to the deletions observed at the dpy-3 locus, deletions that spanned the two RGEN sites were frequently observed, reminiscent of chromosomal deletions induced using zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) (Kim et al. 2009, 2013; Lee et al. 2010, 2012). Although we did not perform direct follow-up analysis of the progeny of F1 animals after unc-1 targeting, we did find progeny that showed the expected uncoordinated (Unc) phenotype on the P0 plates from which mutant F1’s were picked after a few generations had passed (Figure 1E). This observation suggests that some of the mutations identified in the F1 animals were indeed transmitted through the germline. The results of the unc-1 targeting experiment are shown in Table 1.

In summary, we were able to disrupt two endogenous genes in C. elegans using Cas9 protein complexed with in vitro transcribed sgRNAs. It is worth noting that no subcloning steps are needed to generate new RGENs. In con-
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Communicating editor: O. Hobert

Note added in proof: See Genetics 195: Katic and Groshans 2013 (pp. 1173–1176) and Chiu et al. 2013 (pp. 1167–1171) for related works.

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