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 F_1 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 F_1 progeny of these five P0 animals were further examined for mutations.

The F_1 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 nonhomologous end-joining (NHEJ) pathway used to repair double-strand DNA breaks (DSBs) induced at the target site. Mutations were detected in the F_1 progeny of two P0 animals at frequencies of 1/24 (labeled as A-1 F_1) and 3/33 (labeled as D-1 F_1, D-2 F_1, and D-3 F_1). Sequence analysis of PCR products derived from the mutant F_1 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 F_1 and D-1 F_1 worms, 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.

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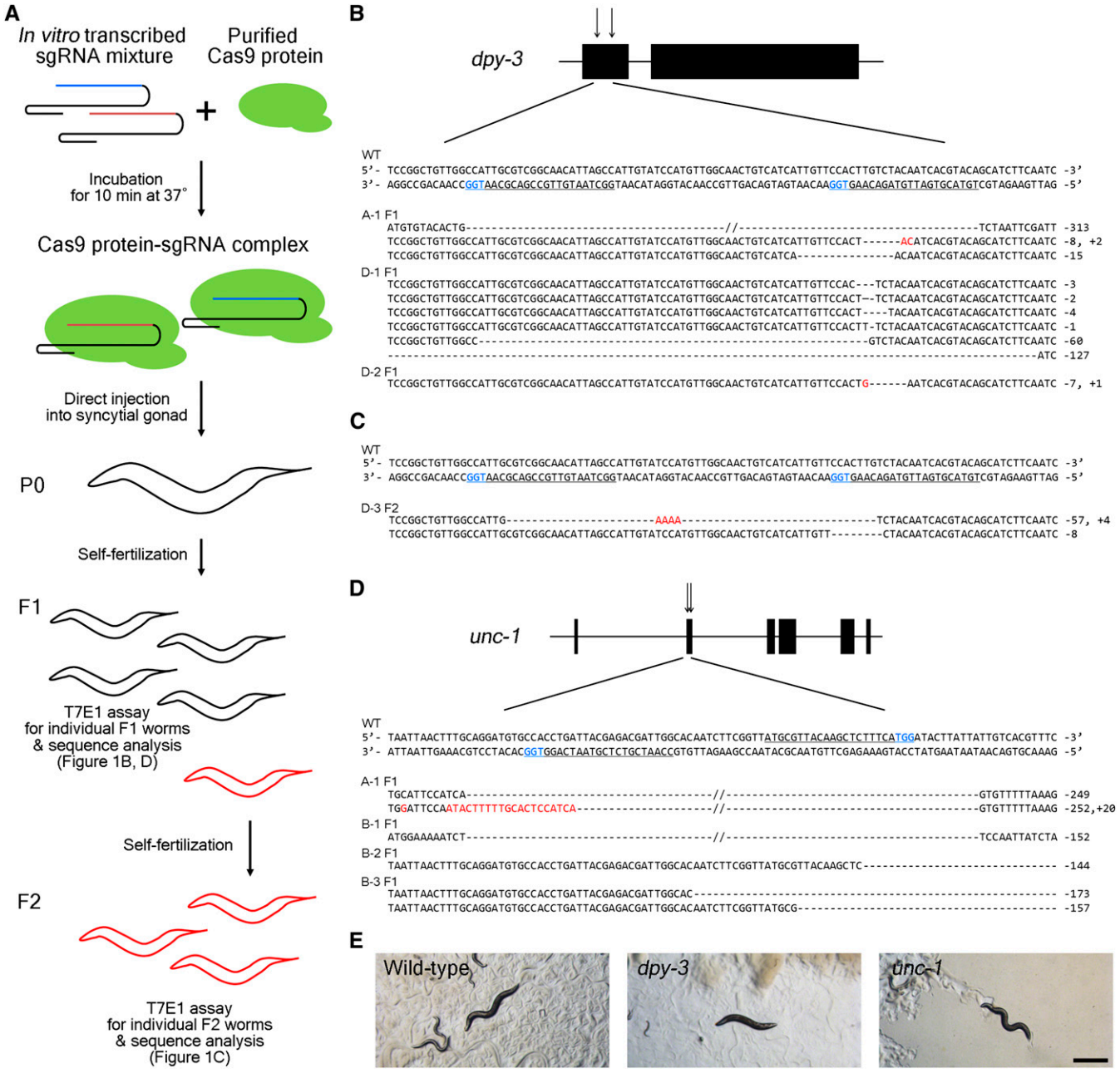

Figure 1 Heritable mutagenesis induced by the Cas9–sgRNA RNP complex in *C. elegans*. (A) Schematic representation of the Cas9 protein–sgRNA complex injection. Purified Cas9 protein and *in vitro* transcribed sgRNA were mixed and injected into the gonads of P0 animals. F_1 and/or F_2 animals were examined 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 F_1 mutant progeny (B) and F_2 progeny (C) from the *dpy-3* targeting experiments, and sequence analysis of the F_1 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.

Notably, we isolated two F_1 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 F_1 animal, D-2 F_1, was examined along with other F_1 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 F_1, escaped from the plate after laying eggs, making it only possible to examine its F_2 progeny. Sequence analysis showed that the F_2 animals, all of which exhibited the Dpy phenotype, contained two independent mutations

Table 1 Summary of RGEN-mediated mutagenesis performed in this study

Target gene	Injected P0	No. of analyzed F_1	No. of F_1 with mutations	Mutation frequency (%)	No. of analyzed F_2	No. of F_2 with mutations	Mutation frequency (%)
dpy-3	A	24	1	1/24 (4.2)	—	—	—
	B	24	0	0	—	—	—
	C	24	0	0	—	—	—
	D	33	3	3/33 (9.1)[a]	24	24	24/24 (100)
	E	16	0	0	—	—	—
	Total	121	4	4/121 (3.3)			
unc-1	A	32	5	5/32 (16)	—	—	—
	B	24	4	4/24 (17)	—	—	—
	C	18	0	0	—	—	—
	D	22	0	0	—	—	—
	Total	96	9	9/96 (9.4)			

[a] F_1 mutant animals derived from the D P0 animal were labeled as D-1, D-2, and D-3. Although D-3 F_1 itself was not examined because of its loss during the experiment, the F_2 animals from D-3 F_1 were subjected to sequence analysis. See the main text for details.

(Figure 1C, Table 1). This result suggests that the D-3 F_1 animal had two independent mutations that most likely occurred in both the oocyte- and the sperm-derived chromosomes. Based on the phenotype and the sequence analysis, we propose that the D-3 F_1 animal contained the biallelic mutations in the *dpy*-3 gene. In total, we observed 4/121 F_1 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 F_1 progeny of four P0 animals injected with the Cas9–sgRNA RNP complexes and found that two P0 animals produced mutant F_1 progeny at frequencies of 5/32 (labeled as A-1 ~5 F_1) and 4/24 (labeled as B-1 ~4 F_1). Sequence analysis of PCR products derived from four F_1 animals out of the nine mutant F_1 animals showed that two (B-1 F_1 and B-2 F_1) contained single mutations, and a third animal (A-1 F_1) contained two mutations (Figure 1D). The fourth animal, B-3 F_1, 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 F_1 animals after *unc-1* targeting, we did find progeny that showed the expected uncoordinated (Unc) phenotype on the P0 plates from which mutant F_1's were picked after a few generations had passed (Figure 1E). This observation suggests that some of the mutations identified in the F_1 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 contrast, the construction of new ZFNs or TALENs involves recombinant DNA technology. Limited analysis showed that no mutations were observed at the most likely off-target sequences of the *dpy-3* gene in the mutant F_2 animals (Figure S1), suggesting that this method can induce specifically targeted mutations. However, newly formed mutants should still be outcrossed with the wild-type strain to remove any off-target mutations that may have occurred. From our results described above, we propose the following simple and general procedure for generating gene-specific mutations using RGENs: (1) microinject the Cas9–sgRNA complex into ~10 P0 animals, (2) After the P0 animals have laid F_1 eggs, clone 10–20 individual F_1 animals from each P0 plate and allow them to grow into adults and lay eggs, (3) examine the individual F_1 animals using the T7E1 assay, and (4) from the plates with F_1 animals with mutations, pick and examine individual F_2 animals for germline-transmitted mutations after they have laid eggs.

Here, we combine two RGENs to target a single gene and find that RGENs often cleave two sites simultaneously, which gives rise to deletions that span the two sites. Despite the lack of a direct proof of simultaneous hits by the two sgRNAs, this result suggests that it will be worth trying targeting multiple sites simultaneously in different genes in nematodes. The injection of the Cas9 protein into nematodes has certain critical advantages over the use of a Cas9-encoding plasmid or mRNA. First, the ribonucleoprotein complexes act on targets immediately after injection. In contrast, mRNA or plasmids must be transcribed and/or translated after injection. Second, unlike exogenous mRNA that can be silenced in *C. elegans*, protein RGEN complexes remain active *in vivo*. Third, this procedure can be used in other species, such as parasitic nematodes, because there is no need to change the promoters or to optimize codon usage to express Cas9 in different organisms.

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

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