Targeted Heritable Mutation and Gene Conversion by Cas9-CRISPR in *Caenorhabditis elegans*

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ABSTRACT We have achieved targeted heritable genome modification in *Caenorhabditis elegans* by injecting mRNA of the nuclease Cas9 and Cas9 guide RNAs. This system rapidly creates precise genomic changes, including knockouts and transgene-instructed gene conversion.

THE ability to heritably delete and modify DNA greatly facilitates the study of gene function. In *Caenorhabditis elegans*, random genome modification through chemical means has long been a mainstay of geneticists. *Mos1* transposon-based insertional mutagenesis was developed in the last decade (Bessereau *et al.* 2001; Williams *et al.* 2005) and provided the basis for more targeted approaches of DNA modifications and deletions such as MosTIC (Robert and Bessereau 2007), MosSCI (Frøkjaer-Jensen *et al.* 2008, 2012), and MosDEL (Frøkjær-Jensen *et al.* 2010). These techniques all rely on the induction of DNA double-strand breaks through reactivation of an inserted *Mos1* transposon, followed by homologous recombination with an exogenously provided template. Although exceptionally useful, they require a *Mos1* transposon insertion within or close to the genomic region to modify, a condition unmet for a significant portion of the coding genome (Vallin *et al.* 2012).

More recently, site-specific nucleases such as ZFNs and TALENs (Wood *et al.* 2011) have offered independence from previously existing strains to modify any gene of interest in principle. For each genomic site to be edited, however, a new pair of nucleases needs to be designed.

During the past year, several articles demonstrated the effectiveness and versatility of the *Streptococcus pyogenes* Cas9 nuclease in genome editing in metazoa (Cong *et al.* 2013; DiCarlo *et al.* 2013; Gratz *et al.* 2013; Hwang *et al.* 2013b; Jiang *et al.* 2013; Jinek *et al.* 2013; Mali *et al.* 2013; Wang *et al.* 2013; Yu *et al.* 2013). Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) systems function in adaptive immunity of bacteria and archaea (Wiedenheft *et al.* 2012). Jinek and colleagues (Jinek *et al.* 2012) showed that a single chimeric guide RNA (sgRNA) of <100 nucleotides (nt), containing a region of complementarity to 20 nt of DNA target sequence, can direct cleavage of specific DNA sequences by the Cas9 DNA endonuclease. This system thus promises quick and routine genomic modification by its requirement of an invariant nuclease and a short, easily produced sgRNA. Here we show that microinjection of *C. elegans* codon-optimized Cas9 mRNA and sgRNAs specific for different targets indeed results in heritable gene mutation. It can also lead to template-instructed gene conversion when provided with an appropriate exogenous template.

This article is one of six companion articles in this issue (Chiu *et al.* 2013; Cho *et al.* 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*.

sgRNA-Guided Cas9 Cleavage of DNA Can Be Achieved in the Germline

We codon-optimized Cas9 DNA for expression in *C. elegans* and added two C-terminal SV40 nuclear localization sequences, as well as the *tbb-2* 3′-untranslated region favorable for germline expression, by Gibson assembly (Gibson *et al.* 2009). For sgRNAs, 20-mers that matched the first 20 bases in genomic sequences of the form 5′GG$(N)_{18}$NGG3′ were cloned into the sgRNA backbone plasmid pDR274, which was shown to

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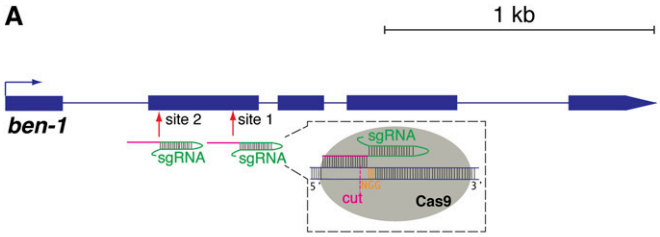
be able to guide Cas9 in zebrafish (Hwang *et al.* 2013b). The requirement for a 5′-terminal GG sequence stems from the design of pDR274, where these nucleotides have been included to facilitate efficient *in vitro* transcription by T7 polymerase. The 3′-terminal NGG sequence is not part of the targeting sequence but adjacent to it (Figure 1A). It constitutes the protospacer-adjacent motif (PAM) sequence, which is required for DNA cleavage activity (Jinek *et al.* 2012). Following *in vitro* transcription, Cas9 mRNA and sgRNA were coinjected into adult germlines.

We first targeted *ben-1*, loss-of-function mutations of which confer dominant resistance to benomyl (Driscoll *et al.* 1989). This simplifies the screening strategy, as it obviates the need to individually clone F_1 animals for examination of their progeny. We screened F_1 progeny of injected animals for normal movement on benomyl and found 11 F_1 progeny of 17 injected P_0 animals from two replicate experiments that moved normally and segregated about 1/4 benomyl-sensitive progeny, as expected of heterozygous mutants. We isolated DNA from the resulting putative homozygous *ben-1* mutants and sequenced the *ben-1* locus. We observed indels of various sizes in all of the mutant strains (Figure 1B).

We also targeted *ben-1* by another sgRNA. Even though this sgRNA contained 2 bases of additional, noncomplementary sequence at its 5′ end, we obtained one mutant from 13 injected animals (Figure 1C). The success in mutant generation with this sgRNA is consistent with reports that 5′ mismatches in the sgRNA can be tolerated (Jinek *et al.* 2012; Cong *et al.* 2013; Fu *et al.* 2013; Hwang *et al.* 2013a). This knowledge might be of use when targeting small genomic regions, such as microRNA coding sequences, where optimal sgRNA sequences might be impossible to design. Conversely, it may indicate a risk of off-target effects as reported recently for human cells (Fu *et al.* 2013). However, unlike cultured cells, *C. elegans* offers the possibility to reduce or eliminate background mutations by backcrossing after Cas9-CRISPR treatment, as would be routine with any other mutagen.

Mutations in *unc-36* Occur in Early Progeny

We performed a time-course experiment after injecting five wild-type animals with Cas9 mRNA and an sgRNA targeting the *unc-36* gene. F_1 animals produced during the first 2 days after injection were transferred to new plates. Three of 452 F_1 animals laid in the first 24 hr after injection segregated Unc-36 animals, whereas none of the 268 F_1 animals laid in the second 24 hr did so. The genomic changes in three new *unc-36* alleles were confirmed by sequencing (Figure 1D). The pattern mirrors what was seen after TALEN mutagenesis by RNA microinjection (Wood *et al.* 2011): F_1 animals generated during the first day after injection yield mutants. Although a generalization of this conclusion will require an analysis of several sgRNAs, our observation suggests that screening of early progeny may be a viable strategy to reduce the number of animals to be examined.



B ***ben-1*_site 1**

sgRNA	GGAGGAACUGGATCTGGAAU
WT	GGAGGAACTGGATCTGGAATGGG
Δ1	GGAGGAACTGGATCTGGA-TGGG
Δ6	GGAGGAACT------GGAATGGG
Δ3+16	GGAGGAACTGGATCTGG(+16)GGG
+194	GGAGGAACTGGATCTGG(+194)AATGGG
Δ819+6	(Δ819)ACTATTATGGG

C ***ben-1*_site 2**

sgRNA	GGGUUCUCGUCGAUCUUGAGCC
WT	CTGTTCTCGTCGATCTTGAGCCAGGAA
Δ7	CTGTTCTCGTCGATCTTGA-------A

D ***unc-36***

sgRNA	GGACAAUCAAGAUGAACAUU
WT	GGACAATCAAGATGAACATTTGG
Δ5+3	GGACAATCAAGA--AGGATTTGG
Δ8	GGACAATCAA---------TTGG

Figure 1 Microinjection of Cas9 and sgRNAs targeting *ben-1* and *unc-36* induces specific heritable mutations. (A) Schematic of the *ben-1* genomic region showing sgRNAs targeting two DNA sites. "NGG" (orange) constitutes the PAM required for DNA cleavage. (B–D) DNA sequence of selected mutations obtained by targeting the indicated genes, using sgRNAs with targeting sequences (20 nt) depicted in magenta. Mutant alleles are denoted by changes in the number of base pairs in the affected gene. Deletions are denoted by dashes. PAMs are shown in orange. For site 2 in *ben-1*, two additional, 5′-terminal sgRNA nucleotides (blue) are mismatched with the target sequence. Note that targeting of *ben-1* at site 1 resulted in an additional six large deletions of unknown borders; one such additional deletion was obtained for *unc-36*. Target sites were chosen with the help of ZiFiT (Sander *et al.* 2007, 2010). Cas9-2xSV40 NLS mRNA was transcribed *in vitro* (New England Biolabs no. E2040S) from plasmid pIK86 (available from Addgene), capped with ARCA (New England Biolabs no. S1411S), LiCl precipitated, and injected at a concentration of 1400 ng/μl together with an sgRNA at a concentration of 15–50 ng/μl. sgRNAs cloned into plasmid pDR274 (Hwang *et al.* 2013b) were similarly transcribed *in vitro* and capped (New England Biolabs no. S1407S), column purified (Ambion no. AM10070), and boiled for 1 min before assembling the injection mix.

A Cas9-Induced Double-Strand Break Can Be Used for Template-Instructed Gene Conversion

We designed an sgRNA targeting a 20-bp sequence in the *daf-2* gene overlapping the mutation *m579* (Figure 2A), which causes a missense change in the ligand-binding domain of the insulin receptor homolog DAF-2 (Scott 2002; Patel *et al.* 2008). *daf-2(m579)* animals were injected with Cas9 mRNA, the sgRNA, and a plasmid bearing the wild-type *daf-2* sequence extending from 316 bp 5′ to 1030 bp 3′ of the *m579* change and transferred to 25°. A large majority of *daf-2(m579)* homozygous animals arrest as dauer larvae

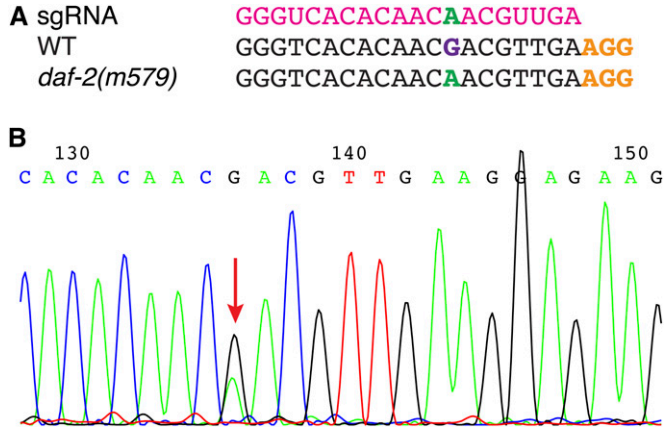


Figure 2 Transgene-instructed gene conversion of the *daf-2(m579)* allele through double-strand break repair by a wild-type sequence. (A) The sgRNA targeting sequence is complementary to *daf-2(m579)* but contains a central mismatch (green/blue) to the wild-type allele. The DNA sequences are on the noncoding strand; the PAM is in orange. (B) A chromatogram showing wild-type and *daf-2(m579)* mix at position 136 (arrow) from a PCR product from lysed pooled progeny of a revertant heterozygote. sgRNA and Cas9 were handled as above. Plasmid pIK99, bearing 1347 bp of *daf-2* wild-type sequence, was co-injected with the RNAs at 100 ng/μl.

at 25°, with the rest arresting as eggs or L1 larvae (Gems *et al.* 1998). One of 20 injected animals segregated one F_1 that reached adulthood and was fertile. Ten of its progeny were individually cloned and 2 formed dauer larvae at 25°, while the rest developed into reproductive adults, consistent with the parent being a *daf-2(m579)/+* heterozygote. Subsequent sequencing across the *m579* locus in progeny of this heterozygous animal showed a change from *m579* into wild-type sequence (Figure 2B), consistent with the repair of a double-strand break induced by Cas9 through homologous recombination with the provided plasmid.

Conclusions

We showed that injection of Cas9 mRNA and sgRNAs with a structure previously used to target the zebrafish genome successfully induced heritable mutations in *C. elegans* genes. While this article was being prepared, Friedland *et al.* (2013) showed that injections of DNA bearing sgRNAs and Cas9 can also induce heritable mutations in *C. elegans*. Our two methods are complementary, and, while this remains to be shown, it is plausible that our method—independent of transcriptional constraints in different species—would work unmodified in other nematodes (Wood *et al.* 2011).

We cannot directly compare the observed efficiencies of Cas9-induced double-strand break generation in our experiments with those from Friedland *et al.* (2013). Friedland *et al.* (2013) reported mutation frequencies based on numbers of F_1's expressing a fluorescent marker present in their targeting mixes. These are just a fraction of all F_1's, reflecting successful DNA transfer to the nucleus; we had no way to determine which of the progeny were successfully microinjected with Cas9 mRNA and sgRNAs. In addition, Friedland *et al.* (2013) note different apparent efficiencies of gene disruption depending on sgRNAs used and individual injections, which is also likely to be the case with RNA-based Cas9-CRISPR experiments (I. Katic, F. Aeschimann, and H. Großhans, unpublished observations).

Our data show that a significant proportion of Cas9-induced mutations are large indels (Figure 1 legend). Such events, useful for complete elimination of gene activity, were not detected by Friedland *et al.* (2013) but were by Yu *et al.* (2013) in *Drosophila*. While not all Cas9 mutagenesis experiments are suitable for phenotypic screening, researchers should be aware of strategies that allow them to identify such larger deletion events. Amplification of a relatively short PCR product from heterozygous animals, followed by T7 or CEL nuclease digestion, might not amplify a deletion if it encompasses one of the primer binding sites. A method suitable for screening that could identify a proportion of such large deletions might consist of choosing an sgRNA that contains a restriction site (Yu *et al.* 2013), PCR amplifying a relatively large region, and screening for F_1's where the restriction site is lost.

Finally, by replacing a single mutated base pair, using a plasmid template, we provide evidence that a Cas9-induced double-strand break can be repaired by homologous recombination in the germline. The optimal template characteristics and concentration for repair of such breaks by homologous recombination, and its range of efficiency, have yet to be determined, in *C. elegans* as in other systems. However, as homologous recombination is the preferred mode of double-strand break repair after *Mos1* excision in the *C. elegans* germline (Robert *et al.* 2008), we anticipate that this method can be further extended to engineer precise changes at endogenous loci.

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Note added in proof: See Genetics 195: Cho *et al.* 2013 (pp. 1177–1180) and Chiu *et al.* 2013 (pp. 1167–1171) for related works.

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