Transgene-Free Genome Editing in *Caenorhabditis elegans* Using CRISPR-Cas

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ABSTRACT CRISPR-Cas is an efficient method for genome editing in organisms from bacteria to human cells. We describe a transgene-free method for CRISPR-Cas-mediated cleavage in nematodes, enabling RNA-homology-targeted deletions that cause loss of gene function; analysis of whole-genome sequencing indicates that the nuclease activity is highly specific.

THE ability to target genes for the creation of heritable, stable loss-of-function mutations is a powerful tool in studies of animal biology. In the nematode *Caenorhabditis elegans*, which is amenable to the handling of large numbers of animals in liquid cultures or on solid media and the creation of frozen libraries, this has long been possible by PCR screening of large randomly generated libraries containing mobilized endogenous transposons (Rushforth *et al.* 1993), chemically induced small deletions (Jansen *et al.* 1997; Gengyo-Ando and Mitani 2000; Edgley *et al.* 2002), or heterologous transposons (Bessereau *et al.* 2001). Another random approach is the construction of libraries of sequenced mutagenized strains (*C. elegans* Deletion Mutant Consortium 2012; Thompson *et al.* 2013). More recently developed technologies have offered to replace such random and large-scale approaches with engineered nucleases designed to induce deletions and provide double-strand DNA breaks at specific desired points in the genome. This was first done using zinc-finger nucleases (Urnov *et al.* 2010) and transcription activator-like effector nucleases (TALENs) (Miller *et al.* 2011), which have proved effective in *Caenorhabditis* nematodes (Wood *et al.* 2011). More recently, technologies have been developed that use clustered regularly interspaced short palindromic repeats (CRISPR) (Jinek *et al.* 2012; Cong *et al.* 2013; Mali *et al.* 2013).

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Methods based on CRISPR-directed nuclease activity offer the potential for maximally convenient engineering of target specificity to effect changes to the genome. In this method, a Cas9 nuclease forms a complex with two RNAs, called the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA), or with a synthetic fusion guide RNA (sgRNA) containing elements of both RNAs; these RNAs provide targeting information for site-specific cleavage by the nuclease (Jinek *et al.* 2012, 2013). This site-specific nuclease activity can be targeted by simply incorporating into the sgRNA a 20-nucleotide sequence corresponding to a target site in the genome containing this identical 20-nucleotide sequence fused at its 3′ end to a proto-spacer adjacent motif (PAM), which has the sequence 5′-NGG-3′. The *C. elegans* coding sequence is ~50% GC (*C. elegans* Sequencing Consortium 1998), and thus almost one-eighth of exonic nucleotides will be candidates for CRISPR-Cas-mediated cleavage, counting both strands. The double-strand break created by CRISPR-Cas activity will then be repaired by nonhomologous end joining (NHEJ) or by homology-directed recombinational repair (Lemmens and Tijsterman 2011). NHEJ is prone to the creation of deletions and small insertions that disrupt gene function; homology-directed recombinational repair can be exploited by the inclusion of a repair template that will be incorporated into the repaired double-strand break to effect precisely designed changes and can be used to insert large constructs such as fluorophore reporter fusions and rescuing transgene markers (Robert and Bessereau 2007; Lo *et al.* 2013).

To implement CRISPR-Cas-mediated cleavage in nematodes, we adapted existing protocols for using CRISPR-Cas-mediated cleavage in cultured mammalian cells (Mali *et al.*

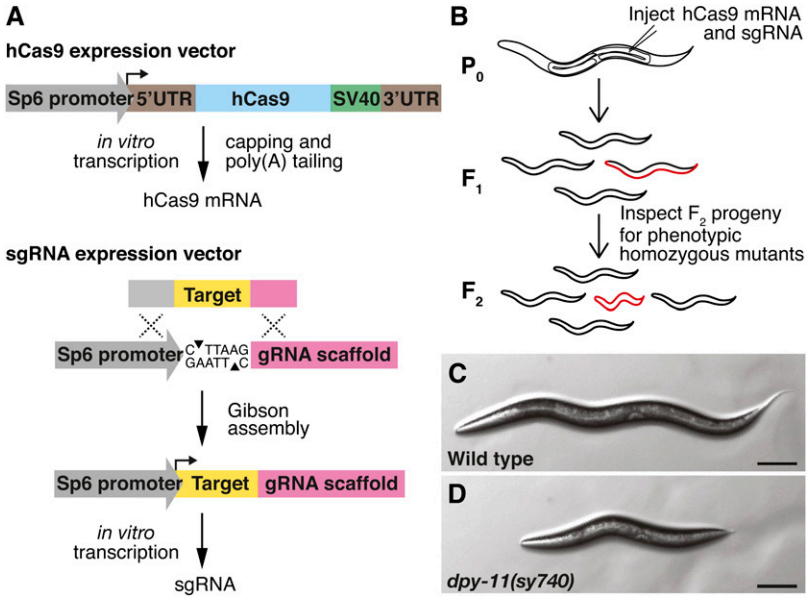

Figure 1 A schematic of the CRISPR-Cas procedure. (A) An mRNA encoding the hCas9 nuclease fused to an SV40 DNA localization sequence is transcribed, capped, and polyadenylated *in vitro*. This message contains 5′- and 3′-UTRs demonstrated to function in the *C. elegans* germline (Wood *et al.* 2011). The 20-nucleotide sequence that will target the nuclease activity is cloned into the sgRNA vector, using Gibson recombinational cloning, and transcribed *in vitro*. (B) The mRNA and the sgRNA are injected into the syncytial gonads of adult hermaphrodites. The F_2 progeny of the injected hermaphrodites are examined to find animals showing the phenotype expected for homozygous loss-of-function of the targeted gene. (C) A fourth-larval-stage (L4) wild-type animal. (D) An L4 *dpy-11(sy740)* animal. Bar, 100 μm.

2013) with reference to methods used for the expression of engineered zinc finger and TALEN nucleases in *Caenorhabditis* (Wood *et al.* 2011). In this method, we injected into the germline syncytia of *C. elegans* hermaphrodites *in vitro*-synthesized capped and polyadenylated message RNA for humanized Cas9 nuclease, with 5′- and 3′-UTRs optimized for *C. elegans* germline expression (Figure 1, A and B, and Supporting Information, File S1). With this mRNA we included an *in vitro*-synthesized hybrid sgRNA containing 20 nucleotides of identity with a target site. To test this system, we selected as targets genes with recognizable, viable null phenotypes. F_2 progeny of injected animals were inspected for the presence of these phenotypes; by this method, 10 independent alleles of *dpy-11* and 1 allele of *unc-4* were isolated (Figure 1, C and D, and Table 1). Efficiency was sometimes very high but showed considerable variability: *dpy-11* alleles were recovered at the rate of one independent mutant for every five animals injected, while only a single *unc-4* mutation was recovered from injection of 15 P_0's. The differing efficiencies observed with mutagenesis using CRISPR-Cas-mediated cleavage to target different sites may reflect differences between the 20-nucleotide targeting sequences inserted into the sgRNAs, perhaps indicating preferences of nucleotide composition or effects of secondary structure; the differing efficiencies might alternatively result from effects of genomic context.

The CRISPR-Cas-induced mutations we recovered were characterized by a prevalence of large deletions and possible chromosomal rearrangements. The 4 smallest of our 11 deletions removed 7, 23, 565, and 1031 nucleotides. Five more alleles were deletions of >2 kbp; the remaining mutations, *sy745* and *sy750*, may be more complex genomic rearrangements (Table 1 and File S1). These results contrast with another recent report of phenotype-based recovery of CRISPR-Cas-induced mutations in *C. elegans* that showed a strong preference for extremely small insertions or deletions, ranging from a single nucleotide to <20 (Friedland *et al.* 2013). Even at $n = 11$, and even comparing only to the four mutations reported by Friedland *et al.* as having been isolated through phenotypic screening, the difference in mutant profile is striking. The most obvious procedural difference is that we induced mutations by delivering *in vitro*-synthesized RNAs, an inherently transient mechanism; the other group's smaller changes were induced by transfection of expression constructs that endured for at least a full generation. It seems at least possible that the effect of the transient CRISPR-Cas nuclease activity provided by synthetic RNA was largely confined to a brief interval in germline development that was unusually susceptible to double-strand breaks being repaired by a mechanism that results in the generation of large deletions, while the CRISPR-Cas nuclease activity provided by transgene expression may been effective at other points in germline development at which double-strand breaks are frequently repaired by NHEJ to leave only very small lesions. It remains to be determined whether the generation of large deletions by transient application of CRISPR-Cas nuclease activity to the germline syncytium is mediated by NHEJ repair.

To determine whether mutations were simultaneously being induced at other sites in the genome, we sequenced genomic DNA of *sy740* and *sy745*, two independent but closely related *dpy-11* mutants generated using CRISPR-Cas-mediated cleavage, recovering 67,212,770 and 74,587,841 unpaired 50-bp reads, respectively (33× coverage and 37× coverage, respectively). Any mutation present in only one of the two strains compared to the published sequence of *C. elegans* could be a *de novo* mutation induced by off-site cleavage by the sgRNA-guided Cas9 nuclease. We performed two analyses to identify candidate mutations: the GATK pipeline (DePristo *et al.* 2011) identified 1592 mismatches, small

Table 1 Mutants generated by CRISPR-Cas

Gene	Allele	Mutation	Sequence
dpy-11	Wild type	None	agatccttgc**AAGCTGGGCACCATGGAGCA**tggctggaatttt
	sy740	23-bp deletion	agatccttg−−−−−−−−−−−−−−−−−−−−−−gctggaatttt
	sy741	7-bp deletion	agatccttgc**AAGCTGGGCACC**−−−−−−−**A**tggctggaatttt
	sy742	>3-kb deletion	ND
	sy743	1031-bp deletion	−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−−
	sy745	Rearrangement	ND
	sy746	>3-kb deletion	ND
	sy747	>3-kb deletion	ND
	sy748	565-bp deletion	agatccttgc**AAGCTGGGCACCAT**−−−−−−−−−−−−−−−−
	sy749	>3-kb deletion	ND
	sy750	ND	ND
unc-4	Wild type	None	tcatcaacag**GTTATCGTCATCCGGTGACG**tggattgttcccg
	sy744	>2-kb deletion	ND

Shown are a list and a description of the targeted mutants generated using CRISPR-Cas. Wild-type sequences are included for comparison. The 20-bp sequence cloned into the sgRNA targeting each gene is in uppercase and boldface type; note that in the wild type it is followed by an NGG "PAM". Deleted nucleotides are indicated with dashes. The precise ends of especially large deletions were not determined. *sy745* is believed to be a rearrangement: sequences near the target site are present by PCR and by high-throughput sequencing, but attempts to use PCR to amplify the target locus were unsuccessful. *sy750* is believed to be an off-site mutation induced by homology-directed double-strand break repair or to be a deletion-duplication, as a wild-type copy of the locus targeted for Cas9 cleavage can be amplified from this mutant. ND, not determined.

insertions, or deletions predicted to be present in either or both strains compared to the reference *C. elegans* genome, and split-read analysis identified 48 candidate large deletions or insertions (Table 2). Of the 1592 candidate changes identified by the GATK pipeline, 324 were predicted in one strain but not in the other (Table S1). After manual inspection of the aligned reads at the sites of these 324 candidate strain-specific changes, 313 were found to be present in both strains, 3 were false positives, and 8 were strain-specific changes (Table S1). None of the 8 changes unique to a strain was homozygous, and none was near a sequence with homology to the sequence used to target CRISPR-Cas-mediated nuclease activity. Five of the 8 altered the extent of a mononucleotide or trinucleotide repeat; 2 of these had extremely low coverage in the sample that did not contain the mutation (Table S1). Of 48 candidate deletions detected by split-read analysis, 24 were homozygous in both strains; for the other 24, manual inspection of aligned reads could demonstrate only that wild-type sequence was present at the site in both strains, indicating the deletion was not homozygous (Table S2). If a large deletion identified by split-read analysis was present but was not homozygous, it would not be detected in manual curation of the aligned sequencing reads. We tested 7 of these 24 using PCR, and saw no evidence that CRISPR-Cas-mediated mutagenesis had caused off target genomic lesions (see File S1, Table S2, and Table S4).

Thus, neither of two analysis methods detected CRISPR-Cas-induced mutations new to either strain, suggesting that to the limit of their ability to detect mutations, CRISPR-Cas-mediated nuclease activity is highly specific in its effects—a result that stands intriguingly in contrast to a recent report of CRISPR-Cas causing frequent off-target changes in cultured human cells (Fu *et al.* 2013). We tested the detection power of both analyses by assessing their ability to detect

Table 2 High-throughput sequencing demonstrates specificity of CRISPR mutagenesis

		GATK analysis				Split-read analysis			
			Manually curated				Manually curated		
Mutant	Reads	Predicted mutations	Both strains	One strain	Neither strain	Predicted mutations	Both strains	One strain	Neither strain
sy740	67,212,770	1,419 (151)	146	5	0	41 (8)	22 (2)	0	19 (6)
sy745	74,587,841	1,441 (173)	167	3	3	40 (7)	22 (2)	0	18 (5)

Genomic DNA from two closely related independent *dpy-11* mutants was sequenced, generating the indicated numbers of unpaired 50-bp reads, greater than 30× coverage for each strain. Newly arising mutations specific to one strain could be off-target effects of CRISPR-Cas-mediated mutagenesis. The results were compared to the wild-type genome, using the GATK pipeline and split-read analysis. The numbers of mutations predicted by each analysis method are shown; the number in parentheses indicates the number of predicted changes specific to that strain. The predictions were then manually curated by inspection of reads aligned to the reference genome. Of the 1592 changes predicted by GATK, the 324 predicted to be strain specific were analyzed to see whether they could be found in both strains, in only one strain, or in neither. Eight were strain specific; none were likely to be the result of CRISPR mutagenesis (Table S1). All of the mutations predicted by split-read analysis were manually curated; examination of aligned reads could confirm their presence in both strains (24 candidates) or could not confirm their presence in either strain (24 candidates; Table S2 and File S1).

deletions they should possess when the analyses were performed using a version of the *C. elegans* reference genome into which a large number of short sequences had been inserted at known sites. This comparison enabled us to assess the false-negative rate of each analysis (Table S3), suggesting that we should have detected at least two-thirds of deletions, and at least three-fourths of deletions in nonrepetitive sections of the genome, such as coding DNA. We suggest that the failure of these analyses to identify *de novo* CRISPR-Cas-induced mutations in comparisons with the unmodified reference genome reflects an actual absence of such mutations. A healthy sense of caution nonetheless suggests that mutant strains generated using CRISPR-Cas should be outcrossed to remove potential off-target mutations that might result from CRISPR-Cas nuclease activity.

This transgene-free, rapidly reconfigurable system for targeted nuclease activity in *C. elegans* might be applicable more broadly in nematodes, particularly in nematode species less readily transformed with expression constructs than is *C. elegans*. A similar system for the expression of ZFNs can be used not only in *C. elegans* but also in *C. briggsae*, a species more evolutionarily distant from *C. elegans* than mouse is from human (Stein *et al.* 2003; Wood *et al.* 2011). The double-strand breaks generated by CRISPR-Cas-mediated nuclease activity in this report were repaired in a mutagenic fashion, leaving deletions. Such double-strand breaks could instead be repaired by homologous recombination with a targeting construct supplied by microinjection along with the nuclease mRNA and guide RNA; this has been done for the double-strand breaks produced by transposon excision (Robert and Bessereau 2007) and has recently been done for double-strand breaks generated using TALENs and CRISPR-Cas (Lo *et al.* 2013). Such a system might provide a rapid expedient for the generation of transgenes in nematode species that currently lack such resources. The availability of relatively species-independent tools for the rapid generation of lesions in specific genes is likely to facilitate the study of gene function in species previously found to be inconvenient for such approaches, including species in which RNA interference (RNAi) is less effective than in *C. elegans* (Moshayov *et al.* 2013). An ability rapidly to inactivate selected genes or to make precisely engineered genomic changes in a broad range of species should make it possible to explore the functional significance of identified evolutionary changes. Because this technique should make genome modification and transgenesis readily feasible in a broad range of species, it should be possible to select a species uniquely suited to a biological question of interest, rather than being constrained to model this biological question in a species that is well studied and has excellent molecular resources, but whose biology is less well suited to the question.

Our work is one of several nearly simultaneous reports, each of which uses somewhat different approaches to achieve genome modification using CRISPR-Cas (Cho *et al.* 2013; Dickinson *et al.* 2013; Friedland *et al.* 2013; Katic and Grosshans 2013; Lo *et al.* 2013; Tzur *et al.* 2013; Waaijers *et al.* 2013); a comparison and synthesis of these works should yield CRISPR-Cas genome modification methods suitable for most applications.

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Note added in proof: The vectors generated for use in this manuscript have been submitted to Addgene (http://addgene.org).

Note added in proof: See Genetics 195: Katic and Großhans 2013 (pp. 1173–1176) and Cho *et al*. 2013 (pp. 1177–1180) for related works.

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