Efficient Marker-Free Recovery of Custom Genetic Modifications with CRISPR/Cas9 in *Caenorhabditis elegans*

Joshua A. Arribere,*[,1] Ryan T. Bell,[†] Becky X. H. Fu,[†] Karen L. Artiles,* Phil S. Hartman,[‡] and Andrew Z. Fire*[,†,1]

*Department of Pathology and [†]Department of Genetics, Stanford University School of Medicine, Stanford, California 94305, and [‡]Department of Biology, Texas Christian University, Fort Worth, Texas 76129

ABSTRACT Facilitated by recent advances using CRISPR/Cas9, genome editing technologies now permit custom genetic modifications in a wide variety of organisms. Ideally, modified animals could be both efficiently made and easily identified with minimal initial screening and without introducing exogenous sequence at the locus of interest or marker mutations elsewhere. To this end, we describe a coconversion strategy, using CRISPR/Cas9 in which screening for a dominant phenotypic oligonucleotide-templated conversion event at one locus can be used to enrich for custom modifications at another unlinked locus. After the desired mutation is identified among the F_1 progeny heterozygous for the dominant marker mutation, F_2 animals that have lost the marker mutation are picked to obtain the desired mutation in an unmarked genetic background. We have developed such a coconversion strategy for *Caenorhabditis elegans*, using a number of dominant phenotypic markers. Examining the coconversion at a second (unselected) locus of interest in the marked F_1 animals, we observed that 14–84% of screened animals showed homologous recombination. By reconstituting the unmarked background through segregation of the dominant marker mutation at each step, we show that custom modification events can be carried out recursively, enabling multiple mutant animals to be made. While our initial choice of a coconversion marker [*rol-6(su1006)*] was readily applicable in a single round of coconversion, the genetic properties of this locus were not optimal in that CRISPR-mediated deletion mutations at the unselected *rol-6* locus can render a fraction of coconverted strains recalcitrant to further rounds of similar mutagenesis. An optimal marker in this sense would provide phenotypic distinctions between the desired mutant/+ class and alternative +/+, mutant/null, null/null, and null/+ genotypes. Reviewing dominant alleles from classical *C. elegans* genetics, we identified one mutation in *dpy-10* and one mutation in *sqt-1* that meet these criteria and demonstrate that these too can be used as effective conversion markers. Coconversion was observed using a variety of donor molecules at the second (unselected) locus, including oligonucleotides, PCR products, and plasmids. We note that the coconversion approach described here could be applied in any of the variety of systems where suitable coconversion markers can be identified from previous intensive genetic analyses of gain-of-function alleles.

TYPE II CRISPR/Cas9 bacterial immunity systems provide programmable DNA endonuclease activities that have recently revolutionized genome editing in a wide range of organisms (Wang *et al.* 1999; Chiu *et al.* 2013; Cho *et al.* 2013; Dicarlo *et al.* 2013; Friedland *et al.* 2013; Gratz *et al.* 2013; Hwang *et al.* 2013; Jiang *et al.* 2013; Katic and Großhans 2013; Li *et al.* 2013; Lo *et al.* 2013; Nekrasov *et al.* 2013; Kim *et al.* 2014; Zhao *et al.* 2014). Recognition by the Cas9 protein entails two sequence elements in the target: a protospacer adjacent motif (PAM) (NGG for *Streptococcus pyogenes* Cas9) and a region of ~20 bp of complementarity to its guide RNA (gRNA) (Jinek *et al.* 2012). Following cleavage by CRISPR/Cas9 *in vivo*, the double-strand break site can be repaired to generate mutations, including insertions and deletions via endogenous pathways such as nonhomologous end joining (NHEJ) or targeted

Copyright © 2014 by the Genetics Society of America
doi: 10.1534/genetics.114.169730
Manuscript received July 24, 2014; accepted for publication August 17, 2014; published Early Online August 26, 2014.
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.169730/-/DC1.
[1]Corresponding authors: Department of Pathology, Stanford University School of Medicine, 300 Pasteur Dr.–L235, Stanford, CA 94305-5324.
E-mail: arribere@stanford.edu; afire@stanford.edu

base mutations via homologous repair (HR) from a template or donor DNA. The ease of use of CRISPR/Cas9 for genome editing has led to its widespread adoption and promises to usher in a new era of biology.

In our application of CRISPR/Cas9 to edit the *Caenorhabditis elegans* genome, we sought a conversion system that met the following criteria: (1) It should be possible to make any mutation in a gene, without extraneous marker sequences, and with no constraint on the genetic background; (2) edited animals should be efficiently made and easily identifiable, so that multiple independent isolates are recovered with minimal downstream screening; and (3) the system should be fast, enabling isolation of the mutation in as few generations as possible, and require minimal plasmid construction. The ease and versatility of such a strain construction system would lessen the technical barriers of genome editing, empowering researchers and facilitating the analysis of gene function. We set out to devise such a system.

Among the techniques available to edit the *C. elegans* genome, the oligonucleotide-mediated conversion strategy reported by (Zhao *et al.* 2014) appealed to us for its relative simplicity and efficiency. In the oligonucleotide-mediated conversion strategy, an ~100-nt single-stranded oligonucleotide bearing a desired mutation is co-injected with Cas9 and a gRNA specific for the wild-type locus of interest into the *C. elegans* germline. The oligonucleotide-based conversion approach has several desirable features of a genome editing system. In particular, it is relatively simple, with a single gRNA plasmid, a commercially prepared single-stranded DNA oligonucleotide, and a pair of PCR assay primers sufficient to initiate each editing experiment. Upon microinjection of these components and a plasmid bearing Cas9 into the *C. elegans* germline, the efficiency for this process is moderate, with 0.5–3.5% of F_1 animals bearing the mutation of interest. This frequency of conversion has allowed direct (albeit somewhat labor intensive) screening for mutations of interest through a PCR-based assay on individual progeny of injected animals. Our goal in this work is a mutation system with the flexibility of the Zhao *et al.* (2014) approach, but with minimized screening.

For CRISPR/Cas9 to facilitate homologous repair at a locus, several steps must occur, including (i) successful injection of DNA constructs into a worm germline; (ii) expression of gRNA(s), Cas9, and assembly together; (iii) finding, binding, and cleavage of target DNA by CRISPR/Cas9; (iv) repair of the double-strand break from the template DNA; and (v) survival of the resultant egg/embryo to a stage at which it can reproduce and be screened.

In practice, any or a number of these steps may be inefficient or fail, and thus markers for one or more steps in an intended manipulation can be useful in obtaining engineered strains (Stinchcomb *et al.* 1985; Mello *et al.* 1991; Jinek *et al.* 2012; Dickinson *et al.* 2013; Kim *et al.* 2014). For the purposes of introducing custom mutations, it would be ideal if animals that had experienced all steps required for a functional HR event could be easily discerned from animals that had not. In the absence of a readily identifiable phenotype of the desired mutation, a coconversion strategy could be employed: a second unlinked marker locus where CRISPR/Cas9-facilitated HR yielded an easily discernable phenotype would, by definition, enable identification of animals that had been exposed to Cas9, guide RNA, and donor DNA populations sufficient for HR, with a goal being that this might enrich for the desired (unmarked) mutation event. The validity of such a coselection approach is supported by a recently described co-CRISPR strategy, in which animals selected for CRISPR/Cas9-induced NHEJ mutations at the *unc-22* locus served as a marker and enriched for CRISPR/Cas9-induced NHEJ events at a second unlinked locus (Kim *et al.* 2014). Because successful oligonucleotide-templated introduction of specific mutations requires that animals have experienced an HR event, coconversion requires a more appropriate selection strategy for specific genome modification than co-CRISPR, which requires that recovered animals only be competent for NHEJ.

Here we combine the ease of genome editing afforded by CRISPR/Cas9-facilitated, oligonucleotide-templated conversion with a coconversion strategy that mitigates the amount of downstream (PCR-based) screening. We demonstrate the coconversion strategy efficiently recovers custom genetic modifications in an otherwise unmarked genetic background. We find that the tendency for CRISPR/Cas9 to induce multiple mutational events imposes nontrivial constraints on the genetic properties of phenotypic marker mutations to be used for coconversion, particularly in cases where multiple rounds of HR are to be executed. We describe several phenotypic markers and strategies useful for the coconversion method and demonstrate their utility for recursive rounds of CRISPR/Cas9 genome editing (recursive mutagenesis).

Materials and Methods

Strains

The Bristol N2 strain was used in all experiments, grown on nematode growth medium (NGM) plates seeded with OP50 (Brenner 1974). Animals were grown at 16° prior to injection and at 23° after injection.

Template DNA

Donor oligonucleotides bearing the mutation of interest and additional silent mutations were designed to ablate the gRNA cleavage site and introduce a restriction site. To design silent restriction sites, we found the SiteFind tool useful (Evans and Liu 2005). Donor oligonucleotides encompassed 50 nt of flanking homology on either side. Oligonucleotide preparations (IDT) were dissolved to a final concentration of 10 μM and used in injection mixes at ~20 ng/μl without further purification.

The *rde-1(AAA)* plasmid used for integration in Figure 4 (pJP44.3.1) encompasses the entire *rde-1* gene, including ~2700 and 900 bp of upstream and downstream sequence

(Pak *et al.* 2012). The *rde-1(AAA)* PCR product was generated with primers AF-JA-21/82 using pJA32, a derivative of pJP44.3.1, as a substrate. The full sequence of the PCR product is presented in Supporting Information, File S1. The *lin-14*::*GFP* plasmid (L7969) was derived from a full-length GFP-tagged version of *lin-14* [VT333G (Hong *et al.* 2000)] by retention of a *Sal*I to *Not*I fragment with consequent deletion of sequences upstream of the lin-14B coding region and of lin-14B exons 1–3.

Single-worm PCR

Single worms were picked into 15 μl of 1× PCR buffer (10 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, pH 8.0) with Proteinase K (500 ng/μl) for single-worm genomic preparation. The solution was frozen and then incubated for 1 hr at 60°, followed by 15 min at 95°. The single-worm genomic prep was included in a PCR reaction, using Phusion polymerase (New England Biolabs, Beverly, MA) at 1/10th reaction volume. PCR reactions were treated with restriction enzyme and run on a TAE ~1–2% agarose gel or cleaned up using Shrimp Alkaline Phosphatase (United States Biochemical, Cleveland) and Exonuclease I (United States Biochemical) and sequenced with MCLab (www.mclab.com).

In a handful of cases we encountered alleles at CRISPR/Cas9 gRNA sites that failed to amplify by single-worm PCR, likely because they contain large (>500) base pair deletions that remove one or both PCR primer binding sites. These events initially appeared to be homozygous HR or NHEJ events in the F_1, although they subsequently segregated as a single allele and a nonamplifiable allele. We encountered these events at a higher frequency when using two gRNAs at a single locus where primer binding sites were contained in the intervening sequence (*e.g.*, Figure 4). These events were also observed when using *rol-6(su1006)* as a marker, yet in every case some of the F_2 progeny were wild type.

Additional methods are detailed in File S1.

Results and Discussion

Oligonucleotide-templated conversion generates mutations with a known phenotype

With the goal of establishing useful marker mutations for the coconversion strategy, we first selected several mutations affecting *C. elegans* behavior and morphology from among the well-characterized products of classical genetics (Brenner 1974; Park and Horvitz 1986; Kramer *et al.* 1988; Levy *et al.* 1993). Initially we used as our criteria that (1) the mutant phenotype should be readily identifiable in the *mut*/+ configuration, so that marked individuals can be found in the F_1, and unmarked +/+ animals can subsequently segregate among their progeny; (2) the mutant phenotype should be distinct from the phenotype conferred by null mutations in the same gene, thus allowing rapid discrimination between CRISPR/Cas9-induced HR and NHEJ; and (3) the mutant phenotype should have a rare spontaneous frequency to minimize false positive conversion events.

We selected six dominant alleles that meet these criteria: *rol-6(su1006)*, *sqt-1(e1350)*, and *dpy-10(cn64)* confer dominant right, right, and left rolling (Rol) phenotypes, respectively; *unc-58(e665)* causes paralysis of most of the body (except for the head) and a peculiar shaker phenotype; *unc-109(n499)* causes paralysis, including a pharyngeal pumping defect; and *unc-43(n498)* confers paralysis and egg-laying defects. For each mutation, we designed a gRNA to cleave the wild-type locus, a donor DNA oligonucleotide with both the dominant mutation and additional silent mutations to create a restriction site, and a pair of PCR primers for screening (see Figure S3 for gRNA plasmid design and cloning). Here and throughout this study, we included additional silent mutations in many of the donor oligonucleotides to unambiguously define HR events (*e.g.*, distinguishing from DNA or strain contamination) and for ease of screening by restriction digest. In principle the restriction site is not required and can be eliminated; no evidence for contamination was observed in these experiments and screening can be performed (albeit somewhat more labor intensively) by single-worm PCR and sequencing alone.

As shown in Figure 1, for five of six candidate marker mutations we obtained animals with the expected phenotypes in the F_1 progeny of the injected animal. Initially we included an mCherry marker (pCFJ104, *Pmyo-3*::*mCherry*) in the *rol-6* injections, although none of the F_1 Rol animals recovered expressed body wall mCherry. This result suggests that heritable transgenesis is not required for oligonucleotide-based conversion *per se* and is consistent with previous observations (Zhao *et al.* 2014) (also see below). For each mutation, phenotypically affected animals were verified to contain a conversion event by single-worm PCR, restriction digest, and sequencing. Each conversion event segregated in a Mendelian manner in follow-up experiments. In all cases, some of the progeny from affected F_1 individuals were phenotypically wild type, consistent with the known dominant nature of these alleles. The combination of heritability, dominance, and molecular verification for the majority of affected F_1 animals confirms both the specificity and low background for the phenotypic screens employed.

Not all guide/template combinations have been successful in obtaining the desired phenotypes. We failed to recover Rol progeny with two additional gRNAs for *sqt-1* and one additional *rol-6* gRNA, likewise observing two gRNA failures for *unc-58* and one for *unc-43* (Figure S1). We do not know why some of the gRNAs failed, although possibilities are presented in File S1.

Simultaneous tracking of sperm- and oocyte-derived alleles during marker conversion: evidence for dual chromosome NHEJ/HR events in single F_1 animals

In addition to the aforementioned dominant phenotypes, three additional features of the gain-of-function *dpy-10(cn64)* and *sqt-1(e1350)* alleles have been very useful to deduce the

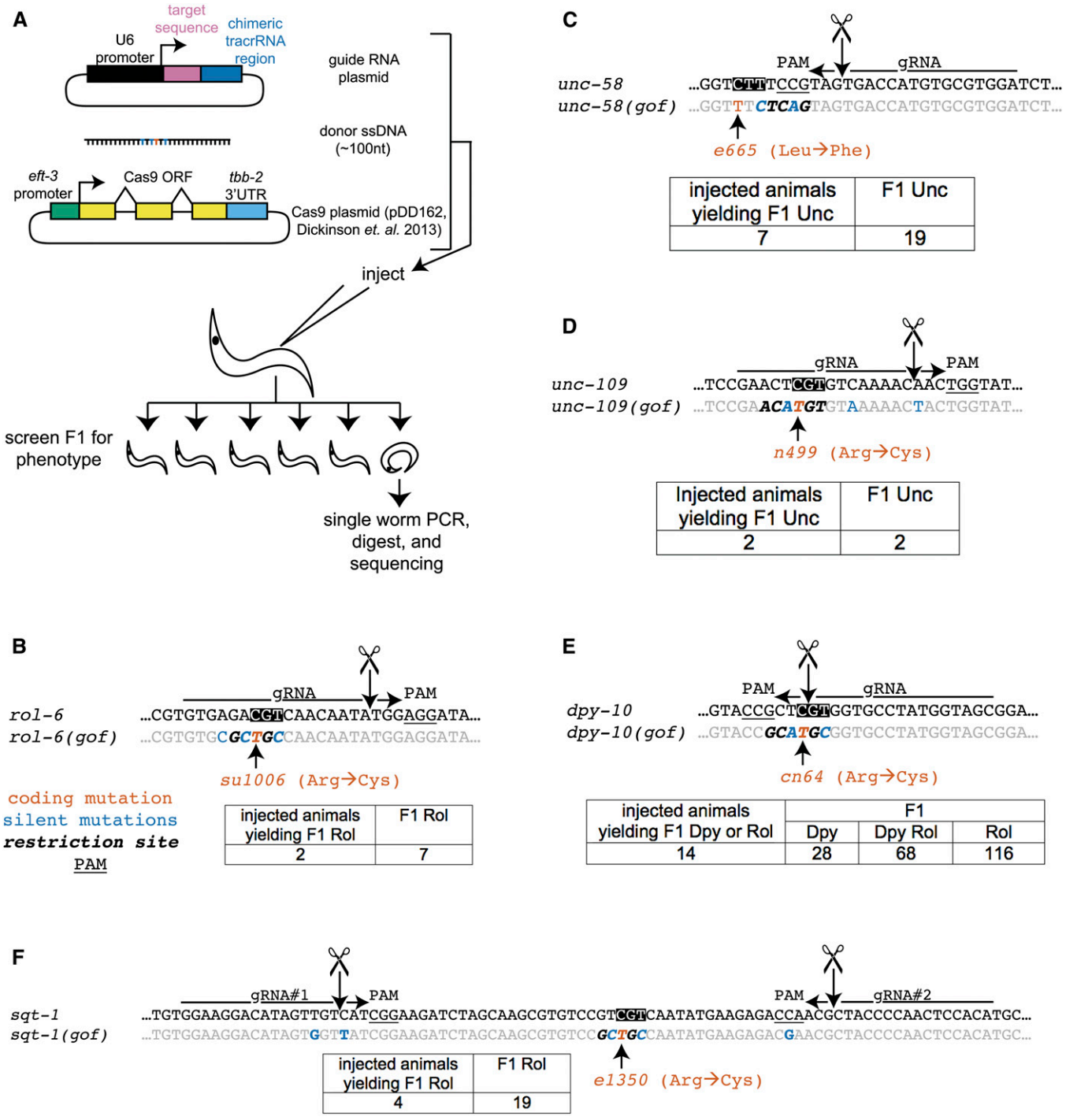


Figure 1 Strategy to track effective gene conversion using dominant point mutations. (A) A Cas9 expression plasmid [pDD162 (Dickinson *et al.* 2013)] is co-injected with a target-specific gRNA plasmid and a template oligonucleotide bearing the desired mutations. The F_1 of the injected animal is screened for phenotypically affected animals. (B) Schematic of the coding strand of the *rol-6(su1006)* locus. The *su1006* mutation (Arg→Cys) is indicated in red, with wild-type Arg boxed (Kramer and Johnson 1993). The gRNA sequence is indicated with an arrow, with PAM underlined. Additional silent mutations conferred by the donor single-stranded DNA (ssDNA) creating a *Bbv*I restriction site (italics) are highlighted in blue. The expected Cas9 cleavage site is indicated with arrow and scissors. Twenty-eight animals were injected. (C) Schematic for *unc-58(e665)* *Dde*I site in italics and causative lesion (*e665*, Leu→Phe) noted (Phil S. Hartman, James Barry, Whitney Finstad, Numan Khan, S. Sato, Naoaki Ishii, and Kayo Yasuda, unpublished results). Twenty-one animals were injected. (D) Schematic for *unc-109(n499)*. *Pci*I site is in italics (*n499* sequence from Chen and Jorgensen 2013). Twenty-one animals were injected. (E) Schematic for *dpy-10(cn64)*. *Sph*I site is in italics. See text for description of Dpy, Dpy Rol, and Rol animals. Twenty-one animals were injected. (F) Schematic for *sqt-1(e1350)*, with *Bbv*I site in italics, using injection of two gRNAs. Thirty animals were injected. Injections were performed with day-to-day variability in injection efficiency, and the number of phenotypically affected progeny should not be interpreted as a statement on the efficiency of HR at that locus. All plasmids are 50 ng/μl, and ssDNA is ~20 ng/μl.

genotype at the corresponding loci from phenotype. First, *dpy-10(cn64)* and *sqt-1(e1350)* confer a different phenotype as *mut/mut* homozygotes than as *mut/+* heterozygotes, with both mutations conferring recessive dumpy (Dpy) phenotypes (Kramer *et al.* 1988; Levy *et al.* 1993). This allows the desired marker heterozygote to be identified and subsequently segregated away, while avoiding any homozygous mutants for which segregation of the marker locus would be much more cumbersome. As a second advantage of these two loci, heteroallelic combinations *mut/o* confer a different phenotype from the desired mut/+ heterozygote [*sqt-1(e1350)/sqt-1(o)* are dumpy and *dpy-10*(*cn64*)/*dpy-10*(o) are dumpy roller]. Finally, loss-of-function alleles in *dpy-10* or *sqt-1* confer a recessive Dpy phenotype, again allowing a distinction from the desired *mut/+* heterozygotes.

These properties of *sqt-1* and *dpy-10* lead to an ability to detect diverse combinations of CRISPR/Cas9 events, with a simple (non-Dumpy) roller phenotype being the desired HR/+ heterozygote for either gene. Injections with either system indeed yielded simple rollers as the majority of affected animals, with sequencing confirming the expected genotype in all but rare cases. For *dpy-10*, we examined populations in more detail, observing additional phenotypes consistent with *dpy-10(cn64)/dpy-10(o)* or *dpy-10(o)/dpy-10(o)* (Figure 1E), and confirmed several of these by molecular analysis. In principle, F_1 dumpy animals may arise from *dpy-10(cn64/cn64)*, but we have yet to observe such homozygous HR animals (see below).

Coconversion at two loci occurs at a high frequency

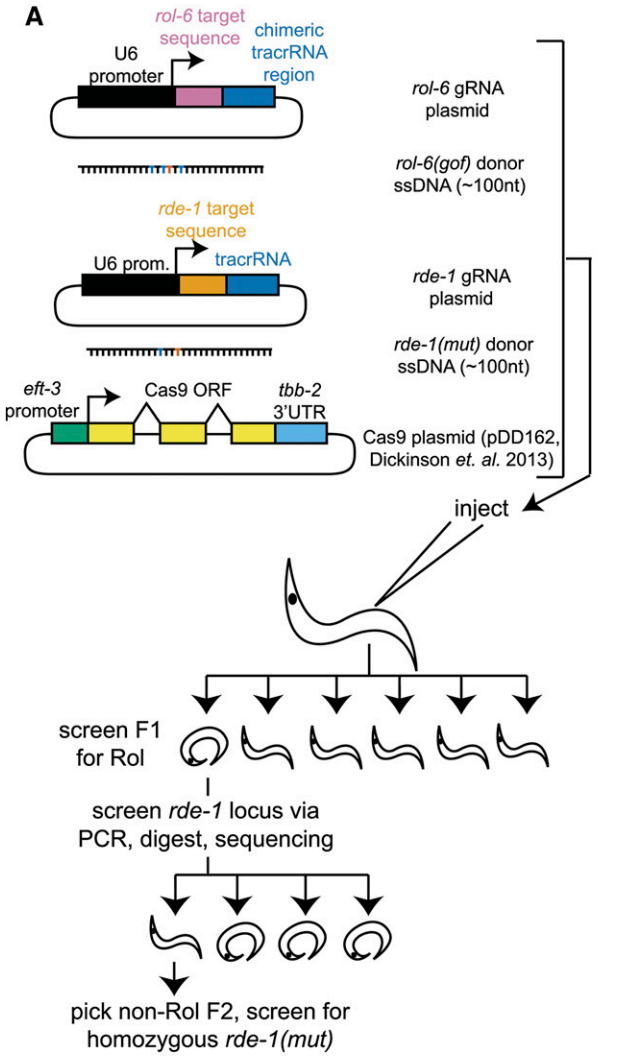
Having established dominant phenotypic markers for the coconversion strategy, we next set out to assess to what extent coconversion occurred. First we attempted to convert *rol-6(su1006)* and simultaneously mutate a catalytic residue H974 of the Argonaute RDE-1 by injection of the cognate gRNAs and repair templates (Figure 2, A and B). Of a total of 34 injected animals, we recovered 25 Rol progeny from 7 injected animals. When screened by single-worm PCR, digest, and sequencing, 14 of 23 (60%) animals also contained the H974A mutation. After the allele of interest was identified among the F_1 Rol animals, we screened the *rde-1* locus of the non-Rol progeny of that animal by single-worm PCR, digest, and sequencing for homozygosity of the H974A mutation. We isolated homozygous *rde-1(H974A)* animals in an otherwise unmarked [*rol-6(+)*] background (see also Figure S2). Thus the coconversion strategy enabled recovery of multiple independent *rde-1(H974A)* isolates while minimizing the number of animals screened.

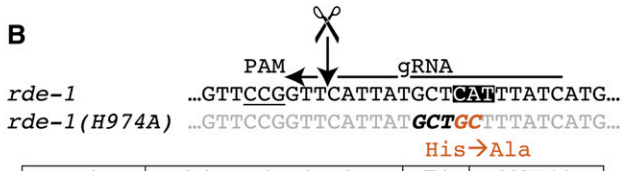
We sought to determine whether picking F_1 broods with high marker HR frequency could enrich for the desired (unselected) mutation event, among both marked and unmarked F_1 animals. As we see a dramatic difference among broods in the frequency of HR events, focusing on broods with a moderately high frequency of events might be sufficient to enrich for desired HR events, even without choosing animals that have themselves been subject to marker conversion. We thus compared brood-level and individual-level selection for the degree of HR enrichment at the unselected locus. As a reference, 12 nonroller animals from broods with no roller animals each failed to show conversion at *rde-1* (assayed by single-worm PCR and restriction digest). From broods with roller animals, we screened 26 Rol animals, with coconversion observed in 22 animals (84%). Among age-matched non-Rol siblings of F_1 Rol animals, 14 of 25 (56%) also contained the *rde-1(H974A)* mutation. The frequency of the *rde-1(H974A)* mutation among F_1 Rol animals was higher than among non-Rol siblings ($P = 0.033$, two-tailed Fisher's exact test), yet the frequency of HR in both groups was quite high. We conclude that our screening efforts were maximally rewarded by focusing on phenotypically marked animals, although the number of isolates of *rde-1(H974A)* was quite workable upon screening their phenotypically wild-type siblings. Picking of unmarked siblings may prove useful when the marker mutation would be undesirable or incompatible with a genetic background (*e.g.*, when the marker locus is linked to a desired mutation or a balancer chromosome is present).

Because the coconversion strategy resets to the initial genetic background, recursive coconversion is theoretically possible. To demonstrate the capability for the coconversion strategy to generate multiply mutant animals and to test its efficiency with other gRNAs, we attempted to mutate additional catalytic residues at the *rde-1* locus in the *rde-1(H974A)* background. Using *rol-6(su1006)* as a marker and the *rde-1(H974A)* host, we mutated D801 to Ala and recovered multiple isolates of *rde-1(D801A, H974A)* (Figure 2C). We performed an additional round of CRISPR/Cas9, using *rol-6(su1006)*, *unc-58(e665)*, or *dpy-10(cn64)* as the marker and D718 as the second site to generate the complete catalytic triad mutant *rde-1(D718A, D801A, H974A)* (Figure 3). Thus the coconversion strategy can be applied recursively to generate multiply mutant animals, each round taking ~2 weeks, with most of that time spent waiting for animals to grow.

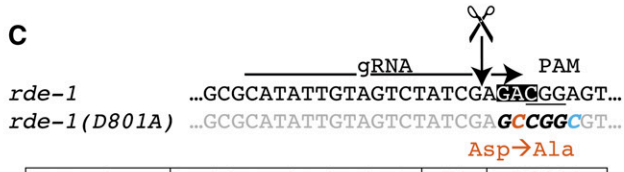
Oligonucleotide-templated conversion is local

During the course of screening, we noted several animals with incomplete copying of sequences from the donor oligonucleotide. Of the Rol animals recovered from *sqt-1(e1350)* construction using two gRNAs whose cut sites span 50 nt, a single animal contained partial conversion off the donor oligonucleotide, yielding mutation at the first gRNA site and the *e1350* locus, but not at the second gRNA site. At D718 in *rde-1*, many of the events recovered exhibited introduction of the linked restriction site (*Sna*BI) only, with several failing to convert the D718A mutation 7 bases away (Figure 3). For D801A, among the seven animals recovered with a D801A mutation, two lacked a complete version of the template-induced site (*Nae*I), showing partial incorporation of mutant bases only 3 nt apart [partial conversion is G**C**CGGA, wild type (wt) is GACGGA]. Based on these observations, we conclude that proximity of bases in the donor oligonucleotide





number injected	Injected animals yielding Rol progeny	F1 Rol	H974A events
34	7	23	14 (60%)



number injected	injected animals yielding Rol progeny	F1 Rol	D801A events
38	7	14	7 (50%)

Figure 2 Coconversion strategy for induction of designated point mutations. (A) The coconversion strategy. A gRNA and donor oligonucleotide to create the *rol-6(su1006)* mutation are co-injected with a gRNA and donor oligonucleotide to create a point mutation in the desired gene (in this case *rde-1*). F_1 progeny were screened for the Rol phenotype. Rol animals were singled and, after laying eggs, were screened by single-worm PCR and characterization of the designated mutational target (*rde-1* for this experiment). Nonroller F_2 progeny of appropriate F_1 animals were singled and after laying eggs were screened for homozygosity of the *rde-1* mutation. (B) Schematic of the *rde-1(H974A)* locus. Ala mutations are shown in red and the *Bbv*I site in italics. All plasmids were 50 ng/μl. Two additional animals at this stage rolled but failed single-worm PCR; these were not included in the 23 count. (C) Schematic for *rde-1(D801A)* locus. *Nae*I site is in italics. Two of the seven D801A events contained only the D801A mutation and lacked a complete *Nae*I site (blue *C*). gRNA plasmids were 25 ng/μl. An additional animal rolled but failed single-worm PCR and was not included in the 14 count.

does not guarantee their faithful copying into the target locus.

To determine whether different coconversion conditions gave rise to more complete conversion from the donor oligonucleotide, we repeated coconversion at D718A. With all three markers tested (*rol-6*, *unc-58*, and *dpy-10*), we obtained a significant fraction of partial conversion events. We also obtained partial conversion events using either strand of DNA as the repair template. We conclude that although copying sequence from the donor oligonucleotide can occur over distances as far as 22 bases (*sqt-1*), it is often highly local.

Homozygous oligonucleotide-templated conversion is rare

Given the high frequency of coconversion between independent loci (14–84%), one might expect some fraction of F_1 animals to be homozygous for the oligonucleotide-templated mutation, either at the marker (*rol-6*, *unc-58*, *dpy-10*) or at the second (*rde-1*) loci. It is noteworthy that in the course of our experiments, despite >400 F_1 animals screened, each with at least one conversion event, we never observed a *bona fide* event where both alleles in an F_1 animal had been subjected to HR (Figure 1, Figure 2, and Figure 3, see also *Materials and Methods*). While failure to observe homozygous conversion does not preclude rare occurrence, such events must be much less frequent than would be expected if the second allele were subject to the same frequency of conversion as an unlinked locus.

The dearth of homozygous oligonucleotide-templated mutations may prove informative for understanding the timing and mechanisms of genome editing by CRISPR/Cas9 in *C. elegans*. Failure to observe homozygous convertants is not due to a lack of CRISPR/Cas9 activity: animals bearing presumed NHEJ mutations in both copies of *dpy-10* were recovered (Figure 1E). From this we conclude that while both oocyte and sperm-derived alleles can be targeted by CRISPR/Cas9, only one copy of a given locus is apparently receptive to introduction of oligonucleotide-templated mutations. We consider it likely the genomic copy subject to HR is the oocyte-resident genomic copy for two reasons: (1) injected DNA constructs are delivered to the developing

marker	*rde-1* donor	number injected	injected animals yielding F1 Rol or Unc	total F1 Rol or Unc	Rol or Unc F1 screened	*rde-1* conversion events	conversion at *rde-1*	
							SnaBI	SnaBI and D718A
rol-6	AF-JA-81	19	6	37	36	22* (61%)	17	4
unc-58	AF-JA-81	24	11	171	52	9 (17%)	5	4
rol-6	AF-JA-116	42	9	20	20	4 (20%)	1	3
dpy-10	AF-JA-116	37	2	86	86	27** (31%)	9	16

Figure 3 Oligonucleotide-mediated homologous recombination is local. Schematic of the *rde-1 (D718A)* locus is shown. Silent mutations are in blue, the *Sna*BI site is in italics, and the *D718A* mutation is in red. For all injections, both gRNA plasmids were 25 ng/μl. For both *rol-6* injections, the *rde-1(D718A)* donor DNA was 20 ng/μl. For *unc-58* and *dpy-10* the *rde-1(D718A)* donor DNA was ~17.25 ng/μl. *From the sequencing trace it was unclear whether an additional animal was full or partial HR. **From the sequencing trace it was unclear whether two additional animals were full or partial HR.

oocytes, while the sperm genomic copy is unavailable for editing until hours later upon oocyte fertilization, and (2) the meiotically developing oocyte exhibits a bias in repair pathways toward HR over NHEJ (Lemmens *et al.* 2013). Given that both germ cells and the embryo appear competent for homologous repair pathways (Clejan *et al.* 2006), Cas9-induced lesions in the early embryo may be refractory to templated repair simply because the injected (single-stranded) donor DNA is unstable and/or unavailable (as proposed by Kim *et al.* 2014). Additional work will provide further insight into the dynamics of CRISPR/Cas9 genome editing in the germline of *C. elegans*.

Incorporation of sequences from nonoligonucleotide substrates

The success of coconversion from oligonucleotides prompted us to test incorporation of sequences from nonoligonucleotide substrates, such as plasmids or PCR products, at a second locus. With *rol-6(su1006)* or *unc-58(e665)* as markers, we were able to introduce mutations into *rde-1* from a plasmid or PCR product, respectively (Figure 4). Interestingly, one mutant contained incorporation of the D718A mutation, which is >300 nt away from the gRNA cut site. We also attempted incorporation of GFP at a locus while using *dpy-10(gof)* as a marker for HR (Figure 5). Among 107 F_1 Rol animals, we observed 16 incorporation events. The frequency of integration among Rol animals (16/107) was higher than in the non-Rol siblings of Rol animals (2/60) (Fisher's exact test, two-tailed *P*-value = 0.02), demonstrating the *dpy-10(gof)* marker was useful for enriching for GFP incorporation events. (We also failed to observe integration of GFP among 32 non-Rol animals from injected animals that failed to yield Rol progeny.) Thus oligonucleotide-mediated HR at a marker locus can facilitate recovery of a diverse selection of HR events, including templated introduction of sequences from PCR and plasmid sources over hundreds of nucleotides.

Conclusions

Here we show that coconversion between two loci with CRISPR/Cas9 can facilitate the incorporation of marker-free mutations at a locus of interest while avoiding extensive screening. High-frequency coconversion enables independent isolation of multiple strains with the desired mutation, facilitating adherence to best practices of analyzing two to three independently derived animal lines. Modifications of the approaches used here may be useful for testing gRNA functionality, efficiently integrating larger portions of DNA, and identifying more sensitive reporters of CRISPR/Cas9 activity, HR, and/or NHEJ. We provide evidence that CRISPR/Cas9 can persist in the zygote in *C. elegans* long enough to cleave both genomic copies of a locus, giving rise to heteroallelism, yet homozygous HR events are extremely rare. The approach we demonstrate may prove useful for other organisms where gain-of-function alleles satisfying the criteria outlined can be identified. Perhaps most importantly, the coconversion strategy should diminish the technical hurdles of the genome editing process at almost all steps and empower researchers to apply CRISPR/Cas9 to understanding their favorite biological process.

While conceptually similar to the recently reported co-CRISPR approach (Kim *et al.* 2014), the coconversion strategy described here has three differences. Namely,

1. The coconversion strategy described here does not necessitate selection for transgenesis, whereas co-CRISPR does. There are substantial disadvantages that can have important consequences for strain construction and subsequent experimental interpretation in needing to make a transgenic for each strain. In experiments where we included mCherry markers for transgenesis (Figure 1B and Figure 5), only a subset of Rol animals was red. This suggests that CRISPR/Cas9-facilitated HR is not contingent on transgenesis, consistent with the observations of Zhao *et al.* (2014).
2. The coconversion strategy described here allows straightforward multiround mutant construction. While the use

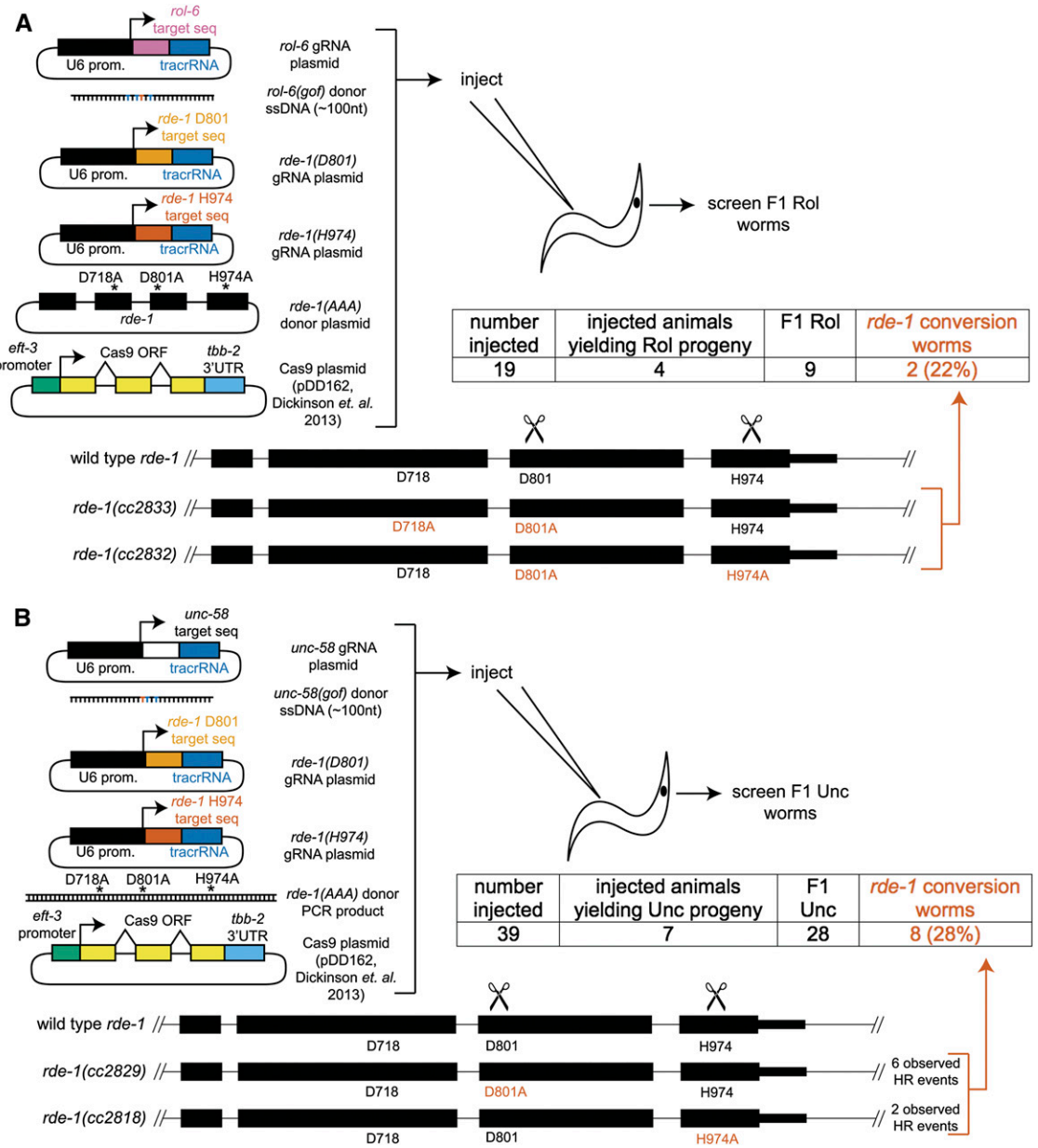
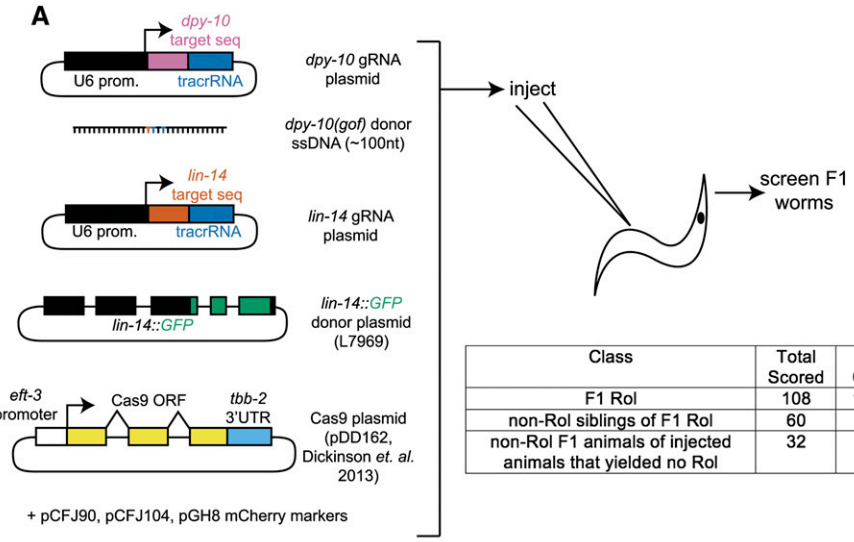


Figure 4 Coconversion from nonoligonucleotide substrates as the second-site donor DNA. (A) Coconversion strategy using plasmid DNA as the donor for the *rde-1* mutations. The *rde-1(AAA)* plasmid was included in the injection mix at ~593 ng/μl. F_1 RoI animals that tested positive for *rde-1(AAA)* DNA were subsequently progeny tested and homozygotes isolated, to discern transgenesis from true integration events. (B) Coconversion strategy using a PCR product as the donor for the *rde-1* mutations. The *rde-1(AAA)* PCR product was included in the injection mix at ~287 ng/μl. The PCR product also included three silent mutations upstream of D718A (not shown). F_1 Unc animals were tested for integration, using one primer inside the PCR product and another outside of it. F_1 Unc animals testing positive for *rde-1(AAA)* DNA were progeny tested as in A. Each of the eight *rde-1* conversion events occurred in an independent animal.

of *unc-22* as a marker in co-CRISPR (Kim *et al.* 2014) also allows multiround construction, such a process would necessitate floating each generation of animals in levamisole to first select heterozygote unc-22 animals and then verify the *unc-22* lesion has segregated away. Among the markers described here, *dpy-10(cn64)* and *sqt-1(e1350)* allow phenotypic discrimination between mut/+, mut/null, +/+, and mut/mut or null/null animals, enabling facile identification of the desired genotype at each stage.

3. The coconversion strategy described here yields specific HR events while minimizing the number of animals that require singling and PCR screening. While efficient for gene knockouts, co-CRISPR requires singling many F_1 animals for HR events, with only a fraction of them suitable for subsequent HR screening among the F_2. Candidate HR events are identified in the F_1 with our coconversion, sparing screening efforts with the earlier identification of candidates.



Class	Total Scored	HR Category	Transgene Array Category
F1 Rol	108	16 (14%)	1
non-Rol siblings of F1 Rol	60	2 (3%)	0
non-Rol F1 animals of injected animals that yielded no Rol	32	0	1

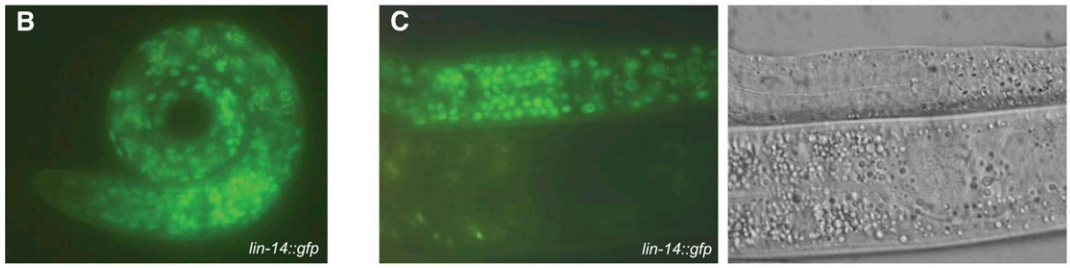


Figure 5 Oligonucleotide-templated HR as a marker for integration of GFP at a second locus. (A) L7969 is a derivative of VT333G (Hong *et al.* 2000) and encodes a C-terminal *lin-14*::*GFP* fusion (exons 4 through the C terminus of *lin-14*) and was included in the injection mix at 20 ng/μl. Guide RNA plasmids were 25 ng/μl, pDD162 was 50 ng/μl, and *dpy-10* donor DNA was 500 nM. Transgenic-marking reporter fusions were included in the injection mix: *Pmyo-2*::*mCherry*::*unc-54* (pCFJ90, 2.5 ng/μl), *Pmyo-3*::*mCherry*::*unc-54* (pCFJ104, 5 ng/μl), and *Prab-3*::*mCherry*::*unc-54* (pGH8, 10 ng/μl). Forty-four animals were injected and 23 yielded Rol progeny. A large number of Rol and non-Rol progeny were observed, only a fraction of which were scored here. The HR category includes F_1 animals screened by a combination of single-worm PCR and examination of their young progeny for characteristic *lin-14*::*GFP* expression patterns. The transgene array category includes animals yielding heritable GFP and mCherry coexpression. Of the 16 *lin-14*::*GFP* integration events among F_1 Rol, 3 were from F_1 mCherry-positive parents. (B) *LIN-14*::*GFP* expression pattern in a newly hatched L_1 larva, similar to that reported in Hong *et al.* (2000). (C) Close-up of *LIN-14*::*GFP* expression pattern showing punctate nuclear GFP signal, consistent with that in Hong *et al.* (2000). Also note lack of expression in L4 larvae (bottom half). The faint yellowish signal in the L4 larvae is autofluorescence from the gut.

As presently applied in our laboratory, we have standardized our protocols to make use of *dpy-10(cn64)* coconversion for a broad set of applications. While we initiated construction of the *rde-1(D718A, D801A, H974A)* strain with *rol-6(su1006)* as a marker, *rol-6(su1006)* [and *unc-58(e665)*] have two undesirable genetic properties: (1) *mut/+* and *mut/o* are phenotypically similar, if not identical, and (2) the null (*o/o*) and wild-type (+/+) phenotypes differ very subtly, if at all. The genetic property of a wild-type null phenotype (Greenwald and Horvitz 1980), while useful under some circumstances, requires additional screening work to ensure that the original unmarked genetic background has indeed been recovered after a round of coconversion. For these reasons, we favor the use of marker mutations [*e.g.*, *dpy-10(cn64)* and *sqt-1(e1350)*] that are phenotypically distinct in *trans* to a wild-type allele (the desired F_1 configuration) compared to in *trans* to a null allele (the configuration from any event where the homologous chromosome has been subject to additional mutations). When *dpy-10(cn64)* and *sqt-1(e1350)* are used as coconversion markers, the distinct phenotypes of *mut/+* and *mut/o* should enable exclusion of animals bearing additional marker locus mutations during the initial F_1 screening. Indeed, in all but rare Rol animals analyzed in *dpy-10(cn64)* and *sqt-1(e1350)* experiments we have observed a wild-type allele opposite an HR allele at the marker locus. Careful selection of genetic markers provides another option (along with lowering gRNA plasmid levels) to mitigate recovery of nontemplated mutations at CRISPR/Cas9-targeted loci. Our current working recipe for the *C. elegans* germline injection mix is 50 ng/μl Cas9 (pDD162), 25 ng/μl *dpy-10* gRNA plasmid, and 500 nM *dpy-10(cn64)* oligonucleotide (AF-ZF-827), along with a gRNA plasmid (25 ng/μl) and oligonucleotide (500 nM) for the mutation of interest.

Acknowledgments

We thank Alexandre Paix and Geraldine Seydoux for first drawing our attention to the oligonucleotide-templated

approach. We are grateful to Craig Mello, Victor Ambros, and their colleagues at the University of Massachusetts for sharing of plasmids. We thank Michael Nonet for helpful discussions and critical reading of the manuscript; Pin An Chen and Erik Jorgensen for detailed information on the nature of *unc-109(n499)*; and Christian Frøkjær-Jensen, Julia Pak, and Nimit Jain for discussions, help with injection, and critical reading of the manuscript. This work was supported by National Institutes of Health grants R01GM37706, T32GM007790, and T32HG000044.

Literature Cited

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94.

Chen, P. A., and E. Jorgensen, 2013 Trimeric G-proteins converge on a novel ion channel. Ph.D. Thesis, University of Utah, Salt Lake City

Chiu, H., H. T. Schwartz, I. Antoshechkin, and P. W. Sternberg, 2013 Transgene-free genome editing in *Caenorhabditis elegans* using CRISPR-Cas. Genetics 195: 1167–1171.

Cho, S. W., J. Lee, D. Carroll, J.-S. Kim, and J. Lee, 2013 Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9-sgRNA ribonucleoproteins. Genetics 195: 1177–1180.

Clejan, I., J. Boerckel, and S. Ahmed, 2006 Developmental modulation of nonhomologous end joining in *Caenorhabditis elegans*. Genetics 173: 1301–1317.

DiCarlo, J. E., J. E. Norville, P. Mali, X. Rios, J. Aach *et al.*, 2013 Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res. 41: 4336–4343.

Dickinson, D. J., J. D. Ward, D. J. Reiner, and B. Goldstein, 2013 Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nat. Methods 10: 1028–1034.

Evans, P. M., and C. Liu, 2005 SiteFind: a software tool for introducing a restriction site as a marker for successful site-directed mutagenesis. BMC Mol. Biol. 6: 22.

Friedland, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church *et al.*, 2013 Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10: 741–743.

Gratz, S. J., A. M. Cummings, J. N. Nguyen, D. C. Hamm, L. K. Donohue *et al.*, 2013 Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. Genetics 194: 1029–1035.

Greenwald, I. S., and H. R. Horvitz, 1980 unc-93(e1500): a behavioral mutant of *Caenorhabditis elegans* that defines a gene with a wild-type null phenotype. Genetics 96: 147–164.

Hong, Y., R. C. Lee, and V. Ambros, 2000 Structure and function analysis of LIN-14, a temporal regulator of postembryonic developmental events in Caenorhabditis elegans. Mol. Cell. Biol. 20: 2285–2295.

Hwang, W. Y., Y. Fu, D. Reyon, M. L. Maeder, S. Q. Tsai *et al.*, 2013 Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31: 227–229.

Jiang, W., D. Bikard, D. Cox, F. Zhang, and L. A. Marraffini, 2013 RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol. 31: 233–239.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna *et al.*, 2012 A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816–821.

Katic, I., and H. Großhans, 2013 Targeted heritable mutation and gene conversion by Cas9-CRISPR in *Caenorhabditis elegans*. Genetics 195: 1173–1176.

Kim, H., T. Ishidate, K. S. Ghanta, M. Seth, D. Conte *et al.*, 2014 A co-CRISPR strategy for efficient genome editing in *Caenorhabditis elegans*. Genetics 197: 1069–1080.

Kramer, J. M., and J. J. Johnson, 1993 Analysis of mutations in the sqt-1 and rol-6 collagen genes of *Caenorhabditis elegans*. Genetics 135: 1035–1045.

Kramer, J. M., J. J. Johnson, R. S. Edgar, C. Basch, and S. Roberts, 1988 The sqt-1 gene of C. elegans encodes a collagen critical for organismal morphogenesis. Cell 55: 555–565.

Lemmens, B. B. L. G., N. M. Johnson, and M. Tijsterman, 2013 COM-1 promotes homologous recombination during Caenorhabditis elegans meiosis by antagonizing Ku-mediated non-homologous end joining. PLoS Genet. 9: e1003276.

Levy, A. D., J. Yang, and J. M. Kramer, 1993 Molecular and genetic analyses of the Caenorhabditis elegans dpy-2 and dpy-10 collagen genes: a variety of molecular alterations affect organismal morphology. Mol. Biol. Cell 4: 803–817.

Li, J.-F., J. E. Norville, J. Aach, M. McCormack, D. Zhang *et al.*, 2013 Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nat. Biotechnol. 31: 688–691.

Lo, T.-W., C. S. Pickle, S. Lin, E. J. Ralston, M. Gurling *et al.*, 2013 Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions. Genetics 195: 331–348.

Mello, C. C., J. M. Kramer, D. Stinchcomb, and V. Ambros, 1991 Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10: 3959–3970.

Nekrasov, V., B. Staskawicz, D. Weigel, J. D. G. Jones, and S. Kamoun, 2013 Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31: 691–693.

Pak, J., J. M. Maniar, C. C. Mello, and A. Fire, 2012 Protection from feed-forward amplification in an amplified RNAi mechanism. Cell 151: 885–899.

Park, E. C., and H. R. Horvitz, 1986 Mutations with dominant effects on the behavior and morphology of the nematode *Caenorhabditis elegans*. Genetics 113: 821–852.

Stinchcomb, D. T., J. E. Shaw, S. H. Carr, and D. Hirsh, 1985 Extrachromosomal DNA transformation of Caenorhabditis elegans. Mol. Cell. Biol. 5: 3484–3496.

Wang, Z., A. Gaba, and M. S. Sachs, 1999 A highly conserved mechanism of regulated ribosome stalling mediated by fungal arginine attenuator peptides that appears independent of the charging status of arginyl-tRNAs. J. Biol. Chem. 274: 37565–37574.

Zhao, P., Z. Zhang, H. Ke, Y. Yue, and D. Xue, 2014 Oligonucleotide-based targeted gene editing in C. elegans via the CRISPR/Cas9 system. Cell Res. 24: 247–250.

Communicating editor: O. Hobert

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.169730/-/DC1

Efficient Marker-Free Recovery of Custom Genetic Modifications with CRISPR/Cas9 in *Caenorhabditis elegans*

Joshua A. Arribere, Ryan T. Bell, Becky X. H. Fu, Karen L. Artiles, Phil S. Hartman, and Andrew Z. Fire

Copyright © 2014 by the Genetics Society of America
DOI: 10.1534/genetics.114.169730

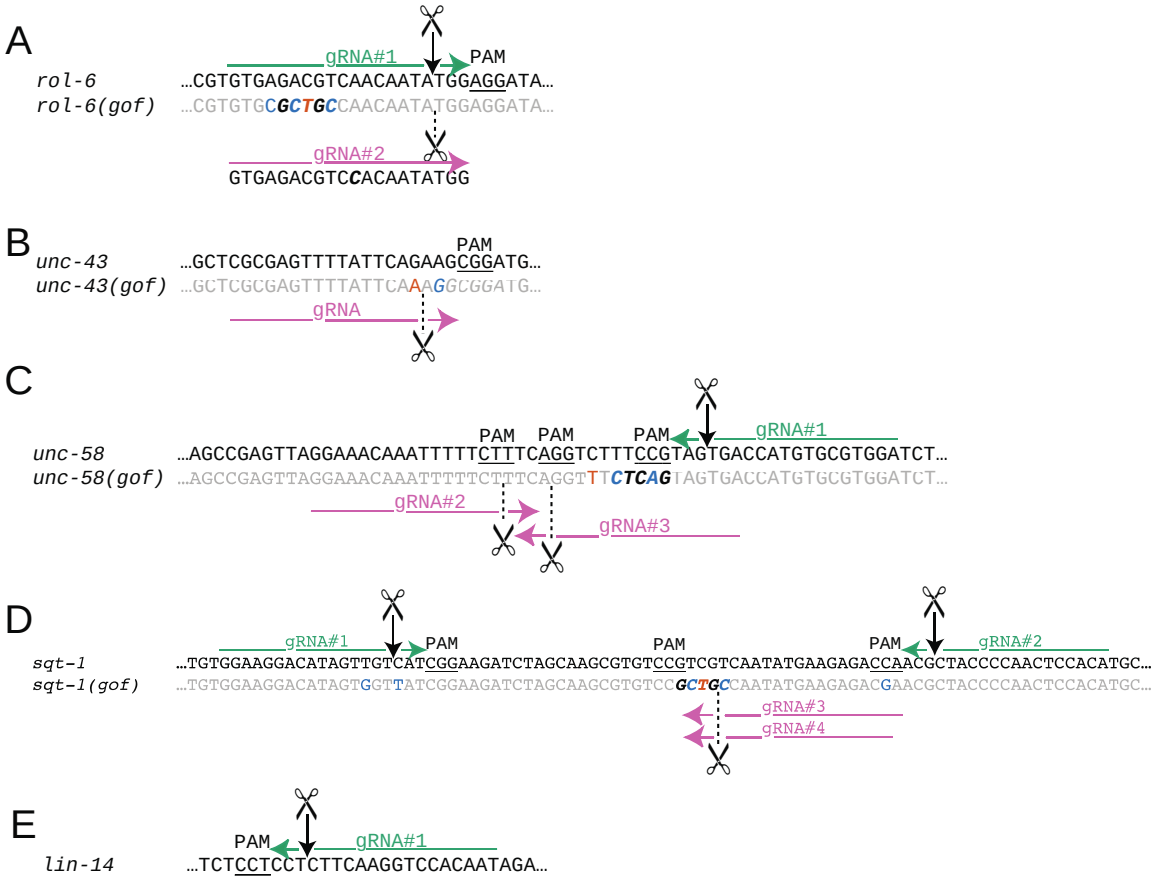


Figure S1 Schema of gRNA constructs that succeeded or failed to produce the expected phenotype
Dominant coding mutations are shown in red, silent mutations in blue, restriction site in italics. gRNA constructs that worked (green, above locus) from Figure 1 are shown. gRNA constructs that apparently failed (pink, below locus) are shown with expected cleavage site (dotted line and scissors). The failure of gRNAs to produce the expected phenotype may be due to failure at any or a number of steps in CRISPR/Cas9-facilitated HR. All gRNA plasmids were injected at 50ng/ul.
(A) gRNA for *rol-6* bearing a mismatch at the -10 position failed to produce Rol progeny in 20 injected animals. This gRNA may have failed due to the single bp mismatch (bold italics, sequence shown below pink arrow). BbvI site in italics.
(B) The sole gRNA for *unc-43* failed to produce any Unc progeny, with template oligonucleotide at 20ng/ul or 200ng/ul. EciI site in italics. The A/T rich stretch may have caused low expression of the gRNA, as it resembles a PolIII termination signal (Gunnery *et al.* 1999).
(C) Two additional gRNAs failed for *unc-58*. gRNA#2 was tested with template DNA at 20ng/ul or 200ng/ul, and may have failed because HR from the template oligonucleotide would not prevent further cleavage by Cas9. gRNA#3 may have failed due to inefficient Cas9 activity at an NAG PAM site. DdeI site in italics.
(D) Two additional gRNAs for *sqt-1* failed for unknown reasons. gRNA#3 is one nucleotide longer than gRNA#4. Donor oligonucleotide was AF-JA-113 for gRNA#3. Donor oligonucleotide was AF-JA-91 for gRNA#4. BbvI site in italics.
(E) gRNA used for *lin-14* C-terminus. Successful cleavage was evidenced by HR of a plasmid over the cleavage site.

A. H974A

rol-6 gRNA plasmid	*rde-1* gRNA plasmid	Injected	F1 Rol	F1 Rol with BbvI site	F1 Rol with BbvI site			F1 Rol without BbvI site		
					sequenced	*rol-6* locus	*rde-1* locus	sequenced	*rol-6* locus	*rde-1* locus
50ng/ul	50ng/ul	34	23	14	11	5 HR/wt 6 other	7 HR/wt 4 other	8	7 HR/wt 1 other	ND

B. D801A

rol-6 gRNA plasmid	*rde-1* gRNA plasmid	*rde-1* donor DNA	Injected	F1 Rol	F1 Rol with NaeI site	sequenced	*rol-6* locus	*rde-1* locus
25ng/ul	50ng/ul	200ng/ul	38	14	9	9	7 HR/wt 2 other	2 HR/wt 6 other 1 NHEJ*
50ng/ul	25ng/ul	20ng/ul	20	29	16	6	4 HR/wt 2 other	5 HR/wt 1 other
25ng/ul	25ng/ul	20ng/ul	38	14	5	5	5 HR/wt	5 HR/wt

C. D718A

rol-6 gRNA plasmid	*rde-1* gRNA plasmid	Injected	F1 Rol	F1 Rol with SnaBI site	sequenced	*rol-6* locus	*rde-1* locus
25ng/ul	25ng/ul	19	36	22	22	16 HR/wt 6 other	17** HR/wt 5*** other

Figure S2 Observations of CRISPR/Cas9 effects on the selected and nonselected alleles
(A) Co-convertants between *rol-6(su1006)* and *rde-1(H974A)* tended to have mutations on both copies of the targeted loci. HR/wt indicates HR off the donor DNA at one allele, and the other allele was wt. "Other" indicates HR off the donor DNA, as well as additional mutations. The most common event in the other category was HR of one copy and NHEJ of the second copy. Two additional F1 RoI animals failed single worm PCR.
(B) Extent of HR and additional mutations for a range of gRNA plasmid concentrations at *rde-1(D801A)*. *This event was NHEJ creating a NaeI restriction site. An additional F1 RoI animal in the 25ng/ul/25ng/ul set of injections failed single worm PCR.
(C) Extent of HR and additional mutations observed among co-convertants for the *rde-1(D718A)* mutation. An additional F1 RoI animal failed single worm PCR. **Of these, 14 were partial HR at only the SnaBI site. ***Of these, 4 were partial HR at only the SnaBI site.

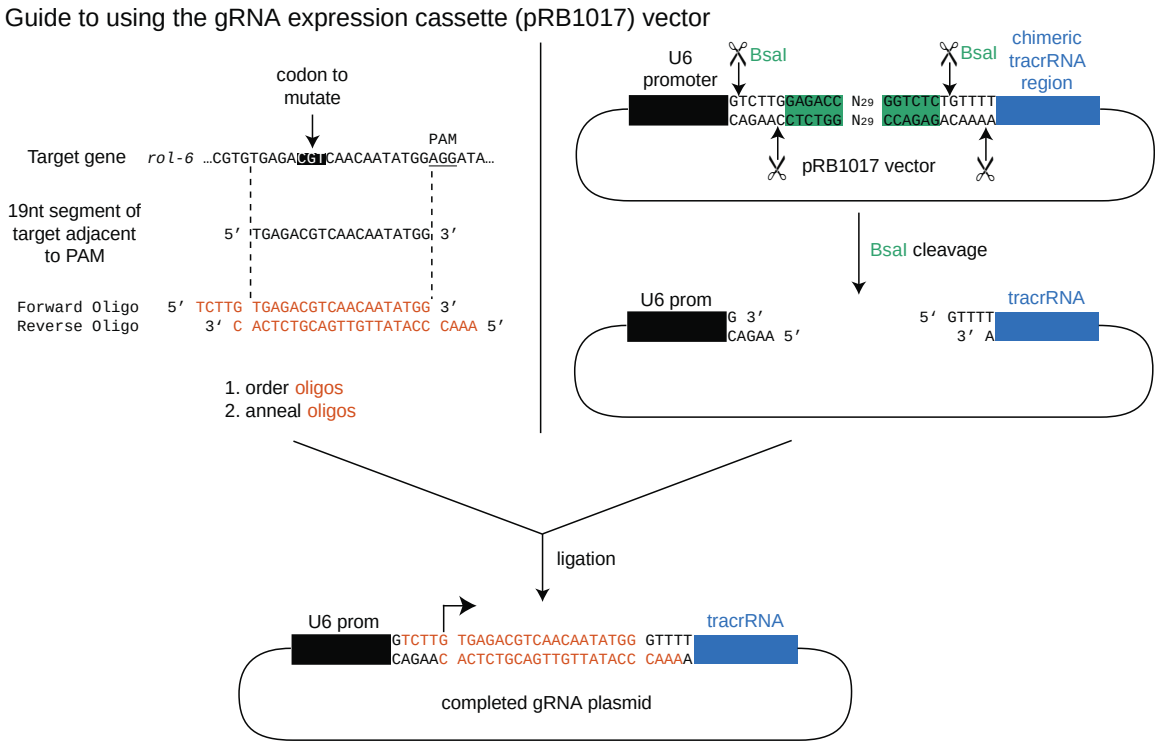


Figure S3 Guide RNA cloning strategy

First, the base to mutate and a nearby PAM are identified. 19 nucleotides upstream of the NGG PAM constitutes the guide RNA target sequence, and forward and reverse oligonucleotides with additional 5′ and/or 3′ nucleotides ends are ordered (left). The recipient guide RNA vector (pRB1017) is cut with BsaI (sites boxed in green), generating 4 nt overhangs complimentary to the oligonucleotide ends. Oligonucleotides are annealed, then ligated into the BsaI-cut vector. The guanosine where U6 initiates transcription is indicated with an arrow.

File S1

Supplement

gRNA constructs

Plasmid pRB1017, containing a *C. elegans* U6 promoter from *R07E5.16*, was used to drive expression of all gRNAs. pRB1017 was made by cloning a gBlock (sequence:
GGGAAGCTTCAAAAAAAACTAGCAATAAAGGAATAAAAAACTGTACACCTTAAAGGCGCACACTCTGTTTTGCAAATTTTATTTTT
AGTTGTGAATTTTCTGCTGAGACtTGAAAATAGCAACTTTAGTACTACTATAATTTGTCAACCTTTTCAAAAAAAGCATGCAATTTTT
GAGAAACTCTTATAAAAGCTATTATTAAAAAAACACCTTTTTTCCAAAATTATTCCACAAAAAATATGTTATGAAATGCCTACACCCT
CTCACACACACTCTTTATACTACTCTGTCAAACTCACGAGATGTCTGCCGCCTCTTGTGTtgCCCCTATATAAACACCTCCTATTGCGA
GATGTCTTggagaccggtaaccatggctcgagaaaccggtactcggtctctGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTT
ATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTGTGAAATTTGCTAGCGG, BsaI-excised insert for gRNA cloning in lower case, see below) between the NheI and HindIII sites of pDR274 (HWANG *et al.* 2013).

gRNA plasmids were designed and cloned as follows (Figure S3): pRB1017 contains two opposing non-palindromic BsaI sites, i.e. 5'-…GTCTTGGAGACC N_{29} GGTCTCTGTTTT…-3', BsaI sites underlined. As BsaI cuts outside of its recognition site and leaves a four nucleotide 5'overhang, BsaI digestion of pRB1017 leaves the vector backbone without BsaI sites, but with 3'-AGAA-5' and 5'-GTTT-3' overhangs. For a given target mutation, a nearby PAM site (NGG) was located, ideally so that the gRNA overlapped the position of the mutation of interest (site is N_{19}NGG). Forward and reverse oligonucleotides bearing the gRNA site and complimentary overhangs were ordered (forward: 5'-TCTTGN_{19}-3' and reverse 5'-AAACN*$_{19}$C-3', where N*$_{19}$ denotes the reverse complement of N_{19}). The additional G is required for transcription from the U6 promoter, and in our experiments a mismatch with the target site at this position does not confer adverse effects on gRNA functionality. Forward and reverse oligonucleotides were annealed, and then ligated into the cut vector. All plasmids were confirmed by restriction digest as well as sequenced with the M13 forward primer. We noted a tendency for single gRNA vectors to concatamerize in *E. coli* (even in *recA1* strains) and avoided gRNA plasmid preps where this was observed to be the case.

Microinjection

Plasmid DNA was prepared using Cetyl Trimethyl Ammonium Bromide (CTAB) precipitation (MELLO and FIRE 1995), and quantified using the Qubit Broad Range assay (Life Technologies). In all injections, the Cas9 plasmid (pDD162 (DICKINSON *et al.* 2013)) was present at 50ng/ul, and unless otherwise indicated, donor DNA was present at 20ng/ul (~600nM for ~100nt ssDNA). DNA mixtures were made up in injection buffer (20mM potassium phosphate, 3mM potassium citrate, 2% PEG, pH 7.5) and spun for >10' at >13,000 rcf. Attempts were made to microinject the distal arms of both gonads of young adult animals. Injected animals were rehydrated in recovery buffer (1mg/ml salmon sperm DNA, 4% glucose, 2.4mM KCl, 66mM NaCl, 3mM $MgCl_2$, 3mM $CaCl_2$, 3mM HEPES pH 7.2) prior to being placed on a small NGM plate seeded with OP50.

Throughout the paper, the number of injected animals includes animals that died yielding few progeny. We estimate on average ~20-40% of injected animals died before giving rise to sufficient progeny to accurately screen activity of the CRISPR/Cas9 system.

Screening

The F1 was screened for Rol, Dpy, and/or Unc phenotypes ~3-4 days after injection. Because some of the Unc phenotypes cause a developmental delay, and the Rol phenotypes do not manifest until ≥L3 stage, we found it helpful to move the injected parent to a new plate every ~12 hours. We found the majority of HR animals were born between 12 and 48 hours after injection.

Where long dsDNA (and not a ssDNA oligonucleotide) is used as a donor, transgenic animals provide a second possible source of PCR signals. Among the F1 progeny screened in Figure 4, we observed several transgenic animals, which lost *rde-1(AAA)* sequences within a generation or displayed non-mendelian inheritance patterns. The rate of such events was only slightly decreased when PCR screening primers outside of the donor dsDNA sequence were used.

Mitigating Additional Mutation Events Recovered During Co-Conversion

The observation that only one copy of a target locus is subject to efficient oligonucleotide-templated conversion may prove informative for optimizing conversion by CRISPR/Cas9. Because only a single genomic copy is receptive to templated mutation, increasing Cas9 and/or gRNAs above a certain level may increase the incidence of additional cleavage events, most prominently of deletions that break the non-selected allele, without an increase in conversion frequency. Excessive Cas9 activity may be undesirable for two reasons: (1) more Cas9 activity could be accompanied by an increased propensity for off-target cleavage and (2) untemplated mutations at the unselected copy of marker

locus limit the utility of that marker locus (and strain) for immediate experimentation, including further rounds of CRISPR/Cas9 co-conversion. Under this logic, avoiding excessive Cas9 activity may prove optimal for recovery of desired HR events in the absence of additional mutagenic events.

We carried out a series of experiments at different gRNA plasmid concentrations to assess whether these might affect the balance between wanted and unwanted events. Examining both *rol-6* loci or both *rde-1* loci for several F1 Rol animals from a high gRNA plasmid concentration (50ng/ul of each guide plasmid and 20ng/ul of each template DNA), we found a considerable degree of unwanted deletions and other mutations in the second, unselected allele. Six of 11 co-convertants had additional mutations resembling NHEJ at the non-converted allele at the *rol-6* locus, and 4 of 11 for the rde-1 locus (Figure S2).

A two-fold reduction of the *rde-1* gRNA alone, or both *rol-6* and *rde-1* gRNAs together resulted in a higher number of animals where the templated mutations were recovered over a wild type copy at both *rol-6* and *rde-1* loci. Under all injection conditions the frequency of co-conversion remained high (35-60%), as did the total number of F1 Rol animals. At D718 in *rde-1*, with 25ng/ul of each gRNA plasmid, we observed 16 out of 22 animals were *rol-6(su1006)/+* and 17 out of 22 animals were *rde-1(D718A)/+*. Out of 5 co-convertant animals sequenced for D801A, all were *rol-6(su1006)/+*; *rde-1(D801A)/+*. While further optimization remains to be done (and will possibly be locus and/or gRNA-specific), simply lowering the concentration of gRNA plasmids provides one strategy to reduce the production of additional mutations with no loss in co-conversion frequency or yield of mutants.

Sequence of PCR product used for integration at *rde-1*:

GCCAATGAAAACAGAGGAGCGCAATCTATTATGTACGACGCGACGAAAAATGAATATGCCGTAAGTTTCAGAAAATTGAAAGTTT
TTAAATATCATATTTACAGTTCTACAAAAATTGTACACTAAATACCGGAATCGGTAGATTTGAAATAGCCGCAACAGAAGCGAAGA
ATATGTTTGAACGTCTTCCCGATAAAGAACAAAAAGTCTTAATGTTCATTATCATTTCCAAACGACAACTGAATGCTTACGGTTTTG
TGAAACATTATTGCGATCACACCATCGGTGTAGCTAATCAGCATATTACTTCTGAAACAGTCACAAAAGCTTTGGCATCACTAAGG
CACGAGAAAGGATCAAAACGAATTTTCTATCAAATTGCATTGAAAATCAACGCGAAATTAGGAGGTATCAACCAAGAGCTCGACT
GGTCAGAAATTGCAGAAATATCACCAGAAGAAAAAGAAAGACGGAAAACAATGCCATTAACTATGTATGTTGGAATTGCTGTAAC
TCATCCAACCTCCTACAGTGGAATTGATTATTCTATAGCGGCTGTAGTAGCGAGTATCAATCCAGGTGGAACTATCTATCGAAATA
TGATTGTGACTCAAGAAGAATGTCGTCCCGGTGAGCGTGCAGTGGCTCATGGACGGGAAAGAACAGATATTTTGGAAGCAAAGT
TCGTGAAATTGCTCAGAGAATTCGCAGAAGTGAGTTGTCTTGAGTATTTAAAAGATCTCTGGGATTTTTAATTTTTTTGTAAACTTT
CAGAACAACGACAATCGAGCACCAGCGCATATTGTAGTCTATCGAGCTGGAGTTAGCGATTCGGAGATGCTACGTGTTAGTCATG
ATGAGCTTCGATCTTTAAAAAGCGAAGTAAAACAATTCATGTCGGAACGGGATGGAGAAGATCCAGAGCCGAAGTACACGTTCAT
TGTGATTCAGAAAAGACACAATACACGATTGCTTCGAAGAATGGAAAAAGATAAGCCAGTGGTCAATAAAGATCTTACTCCTGCT
GAAACAGATGTCGCTGTTGCTGCTGTTAAACAATGGGAGGAGGATATGAAAGAAAGCAAAGAAACTGGAATTGTGAACCCATCA
TCCGGAACAACTGTGGATAAACTTATCGTTTCGAAATACAAATTCGATTTTTTCTTGGCATCTCATCATGGTGTCCTTGGTACATCTC
GTCCAGGACATTACACTGTTATGTATGACGATAAAGGAATGAGCCAAGATGAAGTCTATGTAAGCGTTTTGAATAGCAGTTAGCG
ATTTTAGGATTTTGTAATCCGCATATAGTTATTATAAAAAAATGTTTCAGAAAATGACCTACGGACTTGCTTTTCTCTCTGCTAGATG
TCGAAAACCCATCTCGTTGCCTGTTCCGGTTCATTATGCTGCTTTATCATGTGAAAAAGCGAAAGAGCTTTATCGAACTTACAAGGA
ACATTACATCGGTGACTATGCACAGCCACGGACTCGACACGAAATGGAACATTTTCTCCAAACTAACGTGAAGTACCCTGGAATGT
CGTTCGCATAACATTTTGCAAAAGTGTCGCCCGTTTCAATCAAATTTTTCAATTGTAGATATTGTACTTACTTTTTTTTAAAGCCCGG
TTTCAAAAATTCATTCCATGACTAACGTTTTCATAAATTACTTGAAATTTATTCTGTGTTTATTATTTATTACCTCTAAATTTCGTTTTG
AACGTGAGCATCATATCTTAAACTACTTATTGATAACGGTTTCATAAAGATGTTT

Table S1 Oligonucleotides used in this study

A: Oligonucleotides used for gRNA construction

corresp. plasmid	name	sequence	locus	strand	use with	HR or NHEJ observed?
pJA45	AF-JA-79	TCTTGTGCCATTAACTATGTATGT	*rde-1(D718)*	forward	AF-JA-80	yes
	AF-JA-80	AAACACATACATAGTTAATGGCAC	*rde-1(D718)*	reverse	AF-JA-79	
pJA46	AF-JA-84	TCTTGATATTGTAGTCTATCGAGA	*rde-1(D801)*	forward	AF-JA-85	yes
	AF-JA-85	AAACTCTCGATAGACTACAATATC	*rde-1(D801)*	reverse	AF-JA-84	
pJA14	AF-JA-6	TCTTGATAAATGAGCATAATGAAC	*rde-1(H974)*	forward	AF-JA-7	yes
	AF-JA-7	AAACGTTCATTATGCTCATTTATC	*rde-1(H974)*	reverse	AF-JA-6	
pJA42	AF-JA-56	TCTTGTGAGACGTCAACAATATGG	*rol-6(su1006)*	forward	AF-JA-57	yes
	AF-JA-57	AAACCCATATTGTTGACGTCTCAC	*rol-6(su1006)*	reverse	AF-JA-56	
pJA52	AF-JA-105	TCTTGTGAGACGTCCACAATATGG	*rol-6(su1006)*	forward	AF-JA-106	no
	AF-JA-106	AAACCCATATTGTGGACGTCTCAC	*rol-6(su1006)*	reverse	AF-JA-105	
pJA43	AF-JA-67	TCTTGCGCGAGTTTTATTCAGAAG	*unc-43(n498)*	forward	AF-JA-68	no
	AF-JA-68	AAACCTTCTGAATAAAACTCGCGC	*unc-43(n498)*	reverse	AF-JA-67	
pJA50	AF-JA-101	TCTTGTCCACGCACATGGTCACTA	*unc-58(e665)*	forward	AF-JA-102	yes
	AF-JA-102	AAACTAGTGACCATGTGCGTGGAC	*unc-58(e665)*	reverse	AF-JA-101	
pJA44	AF-JA-72	TCTTGGGAAACAAATTTTTCTTTC	*unc-58(e665)*	forward	AF-JA-73	no
	AF-JA-73	AAACGAAAGAAAAATTTGTTTCCC	*unc-58(e665)*	reverse	AF-JA-72	
pJA48	AF-JA-94	TCTTGTTTTCTTTCAGGTCTTTCCG	*unc-58(e665)*	forward	AF-JA-95	no
	AF-JA-95	AAACCGGAAAGACCTGAAAGAAAAC	*unc-58(e665)*	reverse	AF-JA-94	
pJA59	AF-ZF-821	TCTTGGAACTCGTGTCAAAACAAC	*unc-109(n499)*	forward	AF-ZF-822	yes
	AF-ZF-822	AAACGTTGTTTTGACACGAGTTCC	*unc-109(n499)*	reverse	AF-ZF-821	
pJA58	AF-ZF-825	TCTTGCTACCATAGGCACCACGAG	*dpy-10(cn64)*	forward	AF-ZF-826	yes
	AF-ZF-826	AAACCTCGTGGTGCCTATGGTAGC	*dpy-10(cn64)*	reverse	AF-ZF-825	
pJA54	AF-JA-109	TCTTGTGTGGAGTTGGGGTAGCGT	*sqt-1(e1350)*	forward	AF-JA-110	yes
	AF-JA-110	AAACACGCTACCCCAACTCCACA C	*sqt-1(e1350)*	reverse	AF-JA-109	
pJA55	AF-JA-111	TCTTGGGAAGGACATAGTTGTCAT	*sqt-1(e1350)*	forward	AF-JA-112	yes
	AF-JA-112	AAACATGACAACTATGTCCTTCCC	*sqt-1(e1350)*	reverse	AF-JA-111	
L8191	AF-KLA-164	TCTTGATTGTGGACCTTGAAGAGG	*lin-14 C-term*	forward	AF-KLA-171	yes
	AF-KLA-171	AAACCCTCTTCAAGGTCCACAATC	*lin-14 C-term*	reverse	AF-KLA-164	

B: Donor oligonucleotides for HR

name	sequence	locus
AF-JA-53	TGTGGGTTGATATGGTTAAACTTGGAGCAGGAACCGCTTCCAACCGTGTGCGCTGCCAACAATA TGGAGGATATGGAGCCACTGGTGTTCAGCCACCAGCACCAAC	rol-6(su1006)
AF-JA-65	TAAGTTCGATAAAGCTCTTTCGCTTTTTCACATGATAAAGCAGCATAATGAACCGGAACAGGCAA	rde-

	CGAGATGGGTTTTCGACATCTAGCAGAGAGAAAAG	1(H974)
AF-JA-69	TTTACTTTTAATTTACAATTTTCTATCCGAAATACTCACCTTGCATCCGCCTTTGAATAAAACTCGC GAGCAACAATGTCCTCAAACAGTTCTCCTCCGGTAAC	unc-43(n498)
AF-JA-76	ATTTTGTGGTATAAAATAGCCGAGTTAGGAAACAAATTTTTCTTTCAGGTTTCTCAGTAGTGACCA TGTGCGTGGATCTTGCGTCCACACATCTCAAGGCGTACTT	unc-58(e665)
AF-JA-81	ATATCACCAGAAGAAAAAGAAAGACGGAAAACAATGCCATTAACTATGTACGTAGGAATTGCTG TAACTCATCCAACCTCCTACAGTGGAATTGATTATTCTATAGCGGCTG	rde-1(D718)
AF-JA-86	CTTTCAGAACAACGACAATCGAGCACCAGCGCATATTGTAGTCTATCGAG CCGGC GTTAGCGATTCGGAGATGCTACGTGTTAGTCATGATGAGCTTCGATCTTT	rde-1(D801)
AF-JA-91	GGGGATCCATCAGCATGTGGAGTTGGGGTAGCGTTGGTCTCTTCATATTGGCAGCGGACACGCT TGCTAGATCTTCCGATGACAACTATGTCCTTCCACAATCC	sqt-1(e1350)
AF-JA-113	TTGACCTGGTGGAGCAGATGGGGATCCATCAGCATGTGGAGTTGGGGTAGCGTTCGTCTCTTCA TATTGGCAGCGGACACGCTTGCTAGATCTTCCGATAACCACTATGTCCTTCCACAATCCATTGGTA GATTGCTAGAAAATTAAAAA	sqt-1(e1350)
AF-JA-116	CAGCCGCTATAGAATAATCAATTCCACTGTAGGAGGTTGGATGAGTTACAGCAATTCCTACGTAC ATAGTTAATGGCATTGTTTTCCGTCTTTCTTTTTCTTCTGGTGATAT	rde-1(D718)
AF-ZF-827	CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCATGCGGTGCCTATGG TAGCGGAGCTTCACATGGCTTCAGACCAACAGCCTAT	dpy-10(cn64)
AF-ZF-820	GAAAGGTTAGGAGAGGCAATATATCAACCAACTGAGCAAGATATTCTCCGAACATGTGTAAAAA CTACTGGTATTGTTGAAGTTCACTTCACATTCAAAAATCTCAATTTCAAGTGAG	unc-109(n499)

C: Oligonucleotides for single worm PCR

name	sequence	locus	use with
AF-JA-54	GCCATTGTATTTTCTGGAGCCAC	*rol-6(su1006)*	AF-JA-55
AF-JA-55	CTCCACGTGGTCCTCCTCCATTC	*rol-6(su1006)*	AF-JA-54
AF-JA-77	TGCTGTTAAACAATGGGAGGAGG	*rde-1(H974)*	AF-JA-78
AF-JA-78	AGATATGATGCTCACGTTCAAAACG	*rde-1(H974)*	AF-JA-77
AF-JA-82	GCCAATGAAAACAGAGGAGCGCA	*rde-1(D718)*	AF-JA-83
AF-JA-83	ATCTTTATTGACCACTGGCTTATC	*rde-1(D718)*	AF-JA-82
AF-JA-87	AGTCACAAAAGCTTTGGCATCAC	*rde-1(D801)*	AF-JA-88
AF-JA-88	ATCCTAAAATCGCTAACTGCTATTC	*rde-1(D801)*	AF-JA-87
AF-JA-96	CGGAGATATCGTTGTGACTGATTAC	*unc-58(e665)*	AF-JA-75
AF-JA-75	CTGACTGGAAGGAATTGTGACGGA	*unc-58(e665)*	AF-JA-96
AF-JA-70	CAAACAAGTGACAAACCTTCAAGTC	*unc-43(n498)*	AF-JA-71
AF-JA-71	GAAGCCTCAAAAGTTTCAAACACG	*unc-43(n498)*	AF-JA-70
AF-ZF-823	GATGTGGTGGCACGAATGGAGGACAC	*unc-109(n499)*	AF-ZF-824
AF-ZF-824	AATTTCTCACTGTTGTCTCATCTTC	*unc-109(n499)*	AF-ZF-823
AF-ZF-831	GTCAGATGATCTACCGGTGTGTCAC	*dpy-10(cn64)*	AF-ZF-832
AF-ZF-832	GTCTCTCCTGGTGCTCCGTCTTCAC	*dpy-10(cn64)*	AF-ZF-831
AF-JA-92	GCGTCGCGTCCCTTCTCTCCTG	*sqt-1(e1350)*	AF-ZF-93
AF-JA-93	ACATCCGTACTCCTTATCTCCCG	*sqt-1(e1350)*	AF-ZF-92
AF-KLA-175	ACTCGAACTATGCAAATCTTC	*lin-14 C-term*	AF-KLA-176
AF-KLA-176	GAGATACTACAATGTGCGAA	*lin-14 C-term*	AF-KLA-175

D: Oligonucleotides for *rde-1* PCR donor construct and screening

name	sequence	raison d'etre
AF-JA-21	AAACATCTTTATGAAACCGTTATC	reverse PCR primer for rde-1 donor PCR product, with AF-JA-82
AF-ZF-438	GTGTAAGTGTTTTCTACGTAGATT	with AF-JA-82, forward PCR primer for screening for integration
AF-JA-36	GTGAGTACCAATGAGCGATGTCATC	with AF-JA-77, reverse PCR primer for screening for integration

Supplemental References

DICKINSON D. J., WARD J. D., REINER D. J., GOLDSTEIN B., 2013 Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nature Publishing Group **10**: 1028–1034.

GUNNERY S., MA Y., MATHEWS M. B., 1999 Termination sequence requirements vary among genes transcribed by RNA polymerase III. J. Mol. Biol. **286**: 745–757.

HWANG W. Y., FU Y., REYON D., MAEDER M. L., TSAI S. Q., SANDER J. D., PETERSON R. T., YEH J.-R. J., JOUNG J. K., 2013 Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol **31**: 227–229.

MELLO C., FIRE A., 1995 DNA transformation. Methods Cell Biol. **48**: 451–482.