Scalable and Versatile Genome Editing Using Linear DNAs with Microhomology to Cas9 Sites in *Caenorhabditis elegans*

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ABSTRACT Homology-directed repair (HDR) of double-strand DNA breaks is a promising method for genome editing, but is thought to be less efficient than error-prone nonhomologous end joining in most cell types. We have investigated HDR of double-strand breaks induced by CRISPR-associated protein 9 (Cas9) in *Caenorhabditis elegans*. We find that HDR is very robust in the *C. elegans* germline. Linear repair templates with short (~30–60 bases) homology arms support the integration of base and gene-sized edits with high efficiency, bypassing the need for selection. Based on these findings, we developed a systematic method to mutate, tag, or delete any gene in the *C. elegans* genome without the use of co-integrated markers or long homology arms. We generated 23 unique edits at 11 genes, including premature stops, whole-gene deletions, and protein fusions to antigenic peptides and GFP. Whole-genome sequencing of five edited strains revealed the presence of passenger variants, but no mutations at predicted off-target sites. The method is scalable for multi-gene editing projects and could be applied to other animals with an accessible germline.

THE ultimate goal of genetic engineering is to rewrite the genome with precision and without extraneous modifications (*e.g.*, marker insertion). The remarkable efficiency of CRISPR-associated protein 9 (Cas9) to induce double-strand breaks at defined locations has led to an explosion of new methods for genome engineering (see Carroll 2014 and Sander and Joung 2014 for review). *Streptococcus pyogenes* Cas9 is an endonuclease that is targeted to a specific DNA sequence by an associated guide RNA (Gasiunas *et al.* 2012; Jinek *et al.* 2012). In animal models, expression of Cas9/single-guide RNAs (sgRNA) complexes in zygotes creates double-strand breaks that can be repaired by nonhomologous end joining (NHEJ) or homology-dependent repair (HDR). NHEJ is an error-prone process that can create insertions, deletions, or mutations at the cut site. HDR, in contrast, is a precise mechanism that uses sequences from a homologous donor molecule to repair the break. If the donor molecule carries edits flanked by sequences homologous to the targeted locus ("homology arms"), the edits will be integrated as part of the repair process. In many systems, HDR is thought to be less efficient than NHEJ, requiring high concentrations of donor molecules or long homology arms to stimulate recombination (see Beumer *et al.* 2013 and Sander and Joung 2014 for review). Single-strand oligodeoxynucleotides (ssODNs) can be injected at high concentration, but their relatively small size (~200 bp or less) limits the types of edits that can be introduced. Studies in zebrafish embryos have also shown that ssODN-templated HDR is often imprecise, involving at least one error-prone NHEJ-like step (Auer and Del Bene 2014). Plasmid donors can accommodate gene-sized edits and longer homology arms, but require cloning and often selection to facilitate the recovery of rare recombinants. The selection marker is integrated along with the edit and must be removed in a subsequent step. The requirement for selection can be bypassed by providing high levels of Cas9 and

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sgRNA, using genome-integrated transgenes [as reported in *Drosophila* (Port *et al.* 2014)] or RNA injections [as reported in mice (Yang *et al.* 2013)]. For gene-sized edits, these approaches, however, are still thought to require the construction of plasmids with long homology arms, which limits scalability. Linear PCR fragments with short (<100 bp) homology arms are easier to prepare and have been shown to support HDR in yeast and *Drosophila* tissue culture cells using selection for a co-integrated marker (DiCarlo *et al.* 2013; Bottcher *et al.* 2014). Our goal was to determine whether a similar approach could be developed in an animal, but with a high-enough frequency to bypass the need for selection to generate marker-free, gene-sized edits in a single step.

HDR has been used extensively in *C. elegans* to introduce edits near double-strand breaks created by Mos excision, TALENs, and, most recently, CRISPR/Cas9 (see Waaijers and Boxem 2014 for review). As in other systems, both plasmid donors and ssODNs have been used as repair templates. Using CRISPR/Cas9, plasmid donors with long homology arms (≥1 kb) have been used to insert GFP by coselection for a linked marker or by direct screening for GFP expression (Dickinson *et al.* 2013; Tzur *et al.* 2013; Kim *et al.* 2014). ssODNs have been used to introduce smaller, base-size edits near Cas9 cuts without selection (Zhao *et al.* 2014) or by screening worms co-edited at a second locus (Arribere *et al.* 2014; Zhang and Glotzer 2014). The Arribere *et al.* (2014) study reported that ssODN-templated HDR can be highly local and frequently gives rise to partial conversion events where edits >10 bases away from the cut site are not integrated (Arribere *et al.* 2014). Whether short homology arms can support gene-size edits has not yet been reported.

In this study, we demonstrate that, in *C. elegans*, short homology arms flanking Cas9 sites support robust and precise HDR regardless of the size of the edit. Based on this finding, we developed a systematic and scalable method to create marker-free mutations, insertions, and deletions at any locus. Unlike earlier approaches, our method uses the same 10-day protocol to mutate, tag or delete genes of interest, generates "clean" homozygous mutants with no co-integrated markers or footprints, and can be scaled up for systematic editing of multiple genes.

Materials and Methods

Protocol

We provide a detailed protocol in the Supporting Information File S1.

Whole-genome sequencing

Libraries were constructed on the Mondrian SP+ (Nugen) and sequenced on the HiSeq 2500 (Illumina). For each library, a minimum of 4.4×10^7 50-bp reads (22-fold genome coverage) were aligned to the reference genome WS220 (http://www.wormbase.org) using BFAST software (Homer *et al.* 2009). Potential off-target sites were predicted using the CRISPR Design Tool (http://crispr.mit.edu). Mutation screening was by visual inspection of the aligned data at predicted sites and flanking sequences (±35 bp). Potential insertion mutations were detected using split-end alignment (Smith 2011).

Western blotting

Transgenic worms were lysed by freeze–thaw lysis in 1× M9 with 2.5% SDS. For embryonic lysates, 50 μl of packed embryos were resuspend in lysis buffer (2% SDS, 10% glycerol, 65 mM Tris–HCl, pH 7.5, protease inhibitors). Embryos were lysed using a Misonix Sonicator 3000 with total of 30 sec of sonication (15 sec on, 45 sec off at power level 2). Samples were run on a polyacrylamide gel and transferred overnight to a PVDF membrane. The membrane was blocked with 5% nonfat milk in PBS-Tween, washed, and probed with the following antibodies: anti-V5 HRP (R961-25, Invitrogen, 1/1000) for 2 hr at room temperature and anti-FLAG HRP (2044-S, Cell Signaling Technology, 1/1000).

Immunofluorescence

For staining, embryos were freeze-cracked on poly-L-lysine-coated slides and fixed in −20° methanol for 15 min and −20° acetone for 10 min. Samples were blocked in PBS-Triton-BSA for 30 min and stained with anti-FLAG M2 (1/500, Sigma F1804) and anti-PGL-1 (K76, 1/15) overnight at 4°. Primary antibody was detected using appropriate fluorescent secondary antibodies, mounted, and imaged. N2 worms were used as negative control.

In gel TetraCys tag detection

Transgenic worms were transferred in PBS containing protease inhibitors and freeze-thawed to lyse in NP40 buffer containing protease inhibitors. The lysate was processed using the Lumio Green detection kit (Invitrogen) following the manufacturer's instructions, run on polyacrylamide gel, and imaged.

Results

Insertion of premature stop codons and small protein tags using ssODNs

To test the robustness of HDR using short homology arms, we first established a systematic protocol to generate small insertions/deletions using ssODNs. We designed ssODNs and sgRNAs (Jinek *et al.* 2012) to target Cas9 to eight different loci in five genes using genomic sequence information available on WormBase (sgRNA and ssODN sequences used for each experiment are listed in Table S1, Table S2, and Table S3). The ssODNs contained homology arms (43–100 bases in length; average of ~60 bases) flanking the insertion/deletion placed directly at the Cas9/sgRNA site (Figure 1A, strategy 1; Figure S1, A and B) or up to 27 bases away (Figure 1A, strategy 2). In strategy 2, we also included silent

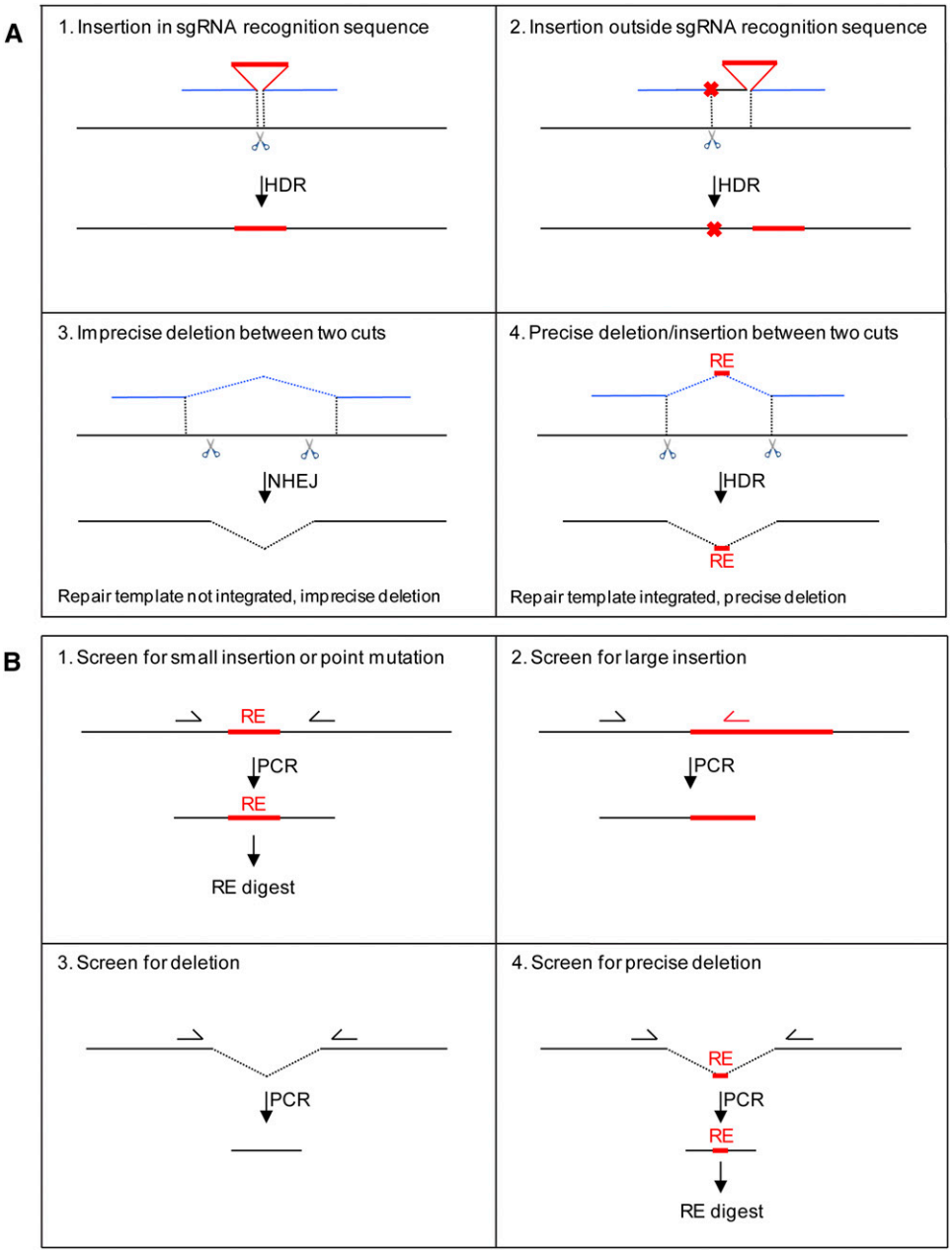

Figure 1 Strategies for HDR and PCR screening. (A) Donor design strategies. Black lines represent genomic DNA, red lines represent inserted sequence, and blue lines are homology arms on template DNA. Scissors denote the sgRNA recognition sequence (pairing site + PAM). Red crosses indicate mutations in the repair template in sequences corresponding to the sgRNA recognition sequence. See text for strategy descriptions. Note that, for strategies 1 and 2, only insertions are shown in the schematics, but it is possible to simultaneously insert and delete sequences as described in Figure S1. In strategy 3, the homology arms in the donor template do not extend to the cuts and, as a result, NHEJ is preferred over HDR, and the donor template is not used. This strategy can be used without a template to generate an imprecise deletion between two distant sgRNA cut sites. (B) PCR screening strategies: see text and Supporting Information for details. Half-arrows denote primers, RE: restriction enzyme site. Strategy 1: Amplify region with primers flanking the insertion and digest with a restriction enzyme whose site is embedded in the insertion. See the protocol for sequences coding for antigenic tags that contain convenient restriction sites. This strategy works best with small F_1 pools (2 F_1's). Strategy 2: Amplify with a primer specific for the insertion and a primer specific for the gene of interest. This approach could also be used for small insertions, but, in practice, when using ssODNs to generate small insertions, we have observed nonspecific products likely due to perduring ssODNs in the F_1's. Strategy 3: Amplify with primers flanking the deletion. This approach is suitable for pools of eight F_1's since the smaller deletion band is favored in the PCR. Strategy 4: Amplify with primers flanking the deletion and digest with the restriction enzyme the site of which is embedded at the junction (RE). This digest is used to distinguish HDR events from NHEJ events.

mutations in the ssODNs to prevent recutting (Figure 1A). We also embedded a restriction site in the insertion to help identification of the edits by PCR (Figure S1 and Figure S2). We co-injected the ssODNs with a plasmid coding for Cas9 and the sgRNA (Dickinson *et al.* 2013) (see Figure 2 for protocol outline). We also included in the injection mix a plasmid coding for a visible marker. pRF4 is maintained extrachromosomally and causes a roller phenotype (Rol) in the F_1's that inherit it (Mello *et al.* 1991). We used this marker to identify injected mothers that incorporated the DNAs in germ-cell nuclei, as evidenced by the appearance of Rol progeny in their broods, and screened only these "marked broods." We screened all F_1 progeny (Rol and non-Rol) laid within ~24–48 hr after gonad injection. The F_1's (singly or in pools of two) were allowed to lay eggs overnight and processed the next day by PCR and restriction digest. Four days later, 8–16 F_2's derived from positive F_1 pools were processed in the same way to confirm germline transmission and isolate animals homozygous for the edit. Edits frequencies were calculated based on the number of positive PCR reactions divided by the total number of F_1's screened (Table 1 and Table S1).

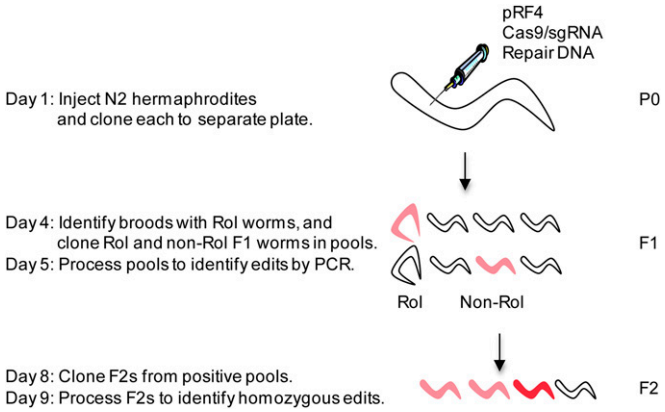

Figure 2 Experimental outline. On day 1, ~40 young adult hermaphrodites are injected and allowed to self-fertilize (one injected hermaphrodite per plate). The injections deliver the Cas9/sgRNA and pRF4 plasmids directly into the syncytial oogenic germline (sperm are formed at an earlier stage of development and stored away from the site of injection). The Cas9/sgRNA plasmid is presumed to be expressed shortly after injection in oogenic germ cells, as it contains promoters predicted to be active in that tissue (Dickinson *et al.* 2013). The pRF4 plasmid encodes a mutated collagen which, when expressed in F_1's, causes the worms to roll (Rol phenotype). This marker is used to identify broods derived from mothers that were successfully injected as evidenced by their transmission of the pRF4 plasmid to the next generation. On day 4 after injection, Rol and non-Rol F_1's from plates with Rol animals ("marked broods") are screened directly for GFP expression or transferred to new plates in pools of two or eight for PCR screening. On day 5 (after F_1's have laid eggs on plates), F_1 pools are screened by PCR for the desired genome edits (light red). On day 8, 8–24 F_2's from each positive F_1 pool are transferred singly to new plates and allowed to self-fertilize. On day 9, F_2's are screened by PCR for homozygous genome edits (dark red).

Edit frequencies ranged widely (Table 1) and did not appear to correlate with insert size. For example, using the same sgRNAs to target the *nos-2* locus, we recovered insertion of a premature stop (12 bp) and a FLAG tag (66 bp) at similar frequencies (1 and 2.4%) (experiments 4 and 11, Table 1). In another experiment, we co-injected three ssODNs carrying insertions of two different sizes (42, 42, and 66 nucleotides, experiment 8) and recovered all three edits. We observed, however, significant variability in sgRNA efficiency. In two cases where we used the same ssODN with two different sgRNAs, we obtained different edit frequencies [0.4 *vs.* 7.3% (experiments 3/2) and 0 *vs.* 1.7% (experiments 5/6) Table 1].

Sequencing of PCR-amplified regions in F_2 worms confirmed correct insertion for 26 of 35 independent edits. Interestingly, all 9 incorrect edits were obtained using strategy 2 (25 edits), where the insert is placed at a distance from the sgRNA/Cas9 site. The 9 incorrect edits contained mutations, deletions, or insertions around the cut site and/or the insertion and, in one case (experiment 6, Table 1), a single base change in the tag sequence. In contrast, 13 of 13 edits obtained using strategy 1 (insertion directly in the sgRNA site) were error-free. These observations suggest that HDR is more robust when the homology arms directly flank the cut site. Analyses of lines established for a subset of edits (Table 1) confirmed that the tags were expressed as protein fusions of the expected size (Figure 3, A and B) and in the expected cells (Figure 3C).

To assess the potential for off-target effects, we performed whole-genome sequencing and variant analysis of five edited lines obtained from two different sgRNA/repair ssODN combinations (experiments 1 and 12, Table 1 and Figure S3A) plus two wild-type (N2) populations from which the edited lines were derived. No mutations were observed in the 7 (experiment 1, Table 1) or 13 (Exp. 12, Table 1) predicted off-target sites with sequence similarity to their respective sgRNAs (Figure S3, B and C). We also screened for extraneous insertions of the repair ssODNs or Cas9 and Rol plasmid sequences within the genomes of the edited strains. No insertion events beyond the targeted edit sites were detected. We conclude that ssODNs with short (>60 nt) homology arms can be used to create insertions (largest tested: 66 nt) at or near Cas9 sites without also causing random insertions in the genome or mutations in predicted off-target sites. However, we did observe a number of variants (mostly single-nucleotide polymorphisms) unique to the edited strains (Figure S3, D and E). Potential sources of such "passenger variants" in the edited lines are addressed below (see *Discussion*).

Insertion of GFP using PCR fragments with short homology arms

To test whether short homology arms can support the integration of larger edits, we used PCR fragments as repair templates, as is standard in yeast (Horecka and Davis 2014), except that we did not use a selection marker. We first attempted to insert GFP (864 bases) using an sgRNA used in experiment 6 to insert the small protein tag TetraCys (18 bases) at the C terminus of K08F4.2. We amplified GFP with PCR primers designed to contain 59/59-nt homology arms that extended from the cut site (strategy 1, experiment 13, Table 1) or arms designed to position GFP precisely before the stop codon, 27 bases away from the cut site (experiment 14, Table 1). In the latter, we included in the repair template mutations in the sgRNA pairing sequence to prevent recutting (strategy 2 in Figure 1A, Table 1, and Table S3). We screened F_1's laid over a 48-hr period after injection in pools of eight worms and identified edits at an estimated minimum frequency of 4% (experiment 13) and 0.9% (experiment 14). Remarkably, these frequencies were comparable to the frequency (1.7%) observed for the insertion of the much smaller TetraCys tag using the same sgRNA.

To test whether this approach is robust and can be used at other loci, we designed sgRNAs to target the C termini of seven other genes. Where possible, we used sgRNA sites that overlapped the stop codon (strategy 1 in Figure 1A). Alternatively, we chose sgRNAs close (<30 bases) to the stop codon and used silent mutations to prevent recutting (strategy 2 in Figure 1A). We obtained GFP insertions for five of the seven genes attempted at estimated minimum frequencies ranging from 0.4 to 1.4% (experiments 15–21, Table 1). Twenty-four F_2's derived from positive F_1's were screened

Table 1 Summary of experiments

Experiment	Strategy (Figure 1A)	Edit	Gene	sgRNAs	No. of P0's[a]	No. of PCRs[b]	No. of F_1's[c]	No. of edits[d]	% edits[e]	No. of precise edits/no. of sequenced edits	Verification of expression
Insertion of premature stop											
1	1	Insertion-STOP	*K08F4.2*	APa4-2	18	277	554	41	7.4	5/5	—
2	1	Insertion/deletion-STOP	*K08F4.2*	APs1	3	95	178	13	7.3	2/2	—
3	1	Insertion/deletion-STOP	*K08F4.2*	APs4	6	141	267	1	0.4	—	—
4	1	Insertion/deletion-STOP	*nos-2*	SL225; SL232	3	194	267	3	1.1	3/3	—
Insertion of small protein tag											
5	2	TetraCys at C terminus	*K08F4.2*	APs6	11	188	376	0	0.0	—	—
6	2	TetraCys at C terminus	*K08F4.2*	APs5	5	143	286	5	1.7	2/5	In gel detection
7	2	Myc, 3× Flag, V5, His, HA at C terminus	*K08F4.2*	APs5	9	179	316	3	0.9	1/3	WB/IF
8	2	V5, 3× Flag, OLLAS at N terminus	*mex-5*	JS129; JS130	11	235	412	12	2.9	3/4	*f*
9	1	Myc, V5 at N terminus	*swan-1*	CSD35	1	19	19	5	26.3	3/3	WB/IF
10	2	3× Flag at C terminus	*pgl-1*	TL001; TL002	6	134	134	1	0.8	0/1	—
11	2	3× Flag at N terminus	*nos-2*	SL225; SL232	11	370	781	19	2.4	6/8	WB/IF
12	2	V5 at N terminus	*mbk-2*	HS516	2	48	48	1	2.1	1/1	WB/IF
Insertion of GFP											
13	1	GFP at sgRNA site near C terminus	*K08F4.2*	APs5	12	108	741	30	4.0	4/4	GFP expression
14	2	GFP at C terminus	*K08F4.2*	APs5	20	179	1134	10	0.9	—	GFP expression
15	2	GFP at C terminus	*fbf-2*	APs12; APa13	17	134	832	3	0.4	—	GFP expression
16	1	GFP at C terminus	*mes-2*	sg16	18	150	738	5	0.7	—	GFP expression
17	1	GFP at C terminus	*lin-15b*	sg23; sg25	5	51	336	2	0.6	—	GFP expression
18	2	GFP at C terminus	*deps-1*	sg6; sg21	11	83	487	7	1.4	—	GFP expression
19	1	GFP at C terminus	*mex-6*	sg3; sg18	10	90	666	8	1.2	—	GFP expression
20	2	GFP at C terminus	*glh-1*	sg4	5	54	402	0	0.0	—	—
21	1	GFP at C terminus	*htp-3*	sg5	1	12	84	0	0.0	—	—
Deletion											
22	3	ORF deletion (1.6 kb)	*K08F4.2*	(APs1; APs4); (APs5; APs6)	16	117	675	22	3.3	0/3	—
23	3	ORF deletion (2.6 kb)	*mbk-2*	HS516; (JS117; JS118)	11	66	108	3	2.8	—	*f*
24	3	Operon deletion (6 kb)	*swan-1/2*	CSD35; (CSD53; CSD54)	11	86	318	5	1.6	0/2	—
25	4	ORF deletion/*Nhe*I insertion	*K08F4.2*	APs1; APs5	11	84	528	20	3.8	2/2	—
26	1	ORF deletion/*Nhe*I insertion	*K08F4.2*	APs5	15	73	475	0	0.0	—	—

WB, Western blot; IF, immunofluorescence; GFP expression, GFP fluorescence in live animals in a pattern expected for the targeted ORF (see Figure 3).

[a] Number of injected hermaphrodites whose broods were screened.

[b] Total number of F_1 pools that were screened by PCR.

[c] Number of F_1's screened.

[d] Number of positive PCRs.

[e] Number of positive PCRs divided by the number of F_1's screened. The assumption is that each positive pool contains only one positive F_1 animal. This may be an underestimate for the GFP experiments where each pool contained eight F_1's. Indeed, in some pools, we observed a higher frequency of edits among F_2's than expected if the pool contained only one edited F_1.

[f] Edits are maternal-effect lethal (V5-tagged MEX-5 and deletion in *mbk-2* locus). Interestingly, 2/2 independent V5-MEX-5 edits are maternal-effect lethal as is a *mex-5(0)* mutant (Schubert *et al.* 2000). In contrast, the FLAG-MEX-5 and OLLAS-MEX-5 edits were viable. The ability to generate fusions to different tags in the same experiment will be useful to determine the best tagging strategy for each gene and avoid those tags that interfere with normal protein function.

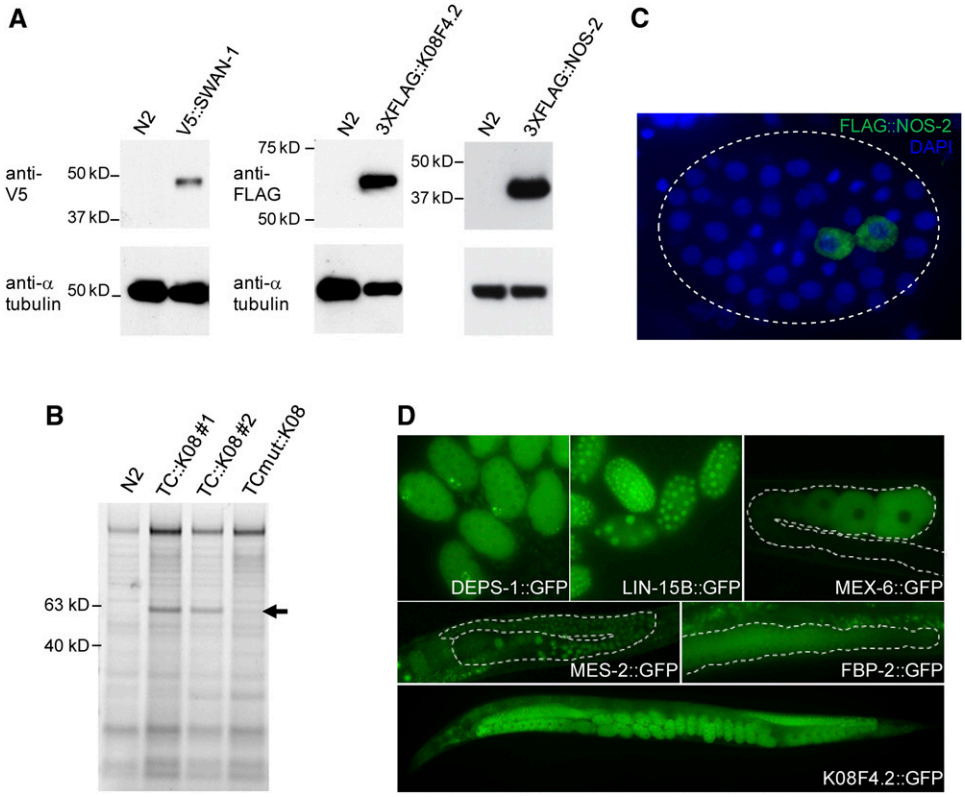

Figure 3 Expression of tagged proteins. (A) Western blot using whole-worm or embryonic lysates and commercially available anti-V5 and anti-FLAG antibodies. (B) TC::K08F4.2 detection using Lumio Green. Arrows show a band of the expected size for two independent TetraCys (TC) edits of K08F4.2; the band is absent in N2 (wild-type) animals and also in a third independent edit of K08F4.2 that contains an inactivating mutation in the TC tag (C to Y). (C) Immunofluorescence image of a fixed embryo stained with DAPI and anti-FLAG antibody. FLAG-NOS-2 is present in the two primordial germ cells, Z2 and Z3, reflecting the wild-type distribution of NOS-2 at this stage of development. (D) Fluorescence pictures of live embryos (*deps-1*, *lin-15*), germline (*mex-6*, *fbf-2*), and whole animals (*mes-2*, *K08F4.2*) expressing the indicated GFP fusion proteins. Dashed lines outline the gonad boundary.

singly by PCR or by visual inspection for GFP fluorescence to recover and propagate the edits. All derived strains (each started from a single homozygous F_2/$_3$) showed stable GFP expression in the expected pattern (Figure 3D, Table S5).

In one of the two experiments that failed, only one brood (84 F_1's) was screened, which may have been too few (experiment 21). In the other failed experiment, a large number of F_1's were screened (402 F_1's), raising the possibility that the sgRNA may have been inefficient (experiment 20). We found that it is possible to use two sgRNAs with overlapping recognition sites in the same experiment (experiments 15, 17, and 19), which could help avoid low-efficiency sgRNAs.

We conclude that small homology arms are sufficient to support GFP insertion at most loci, provided that an efficient sgRNA site can be identified within ~30 bases of the desired site of insertion. We have not yet tested whether insertions could be created at an even greater distance from the sgRNA site.

Precise gene-sized deletions using ssODNs

Using TALENs, Lo *et al.* (2013) reported the isolation of small precise deletions (<100 bases) templated by ssODNs. To test whether ssODNs could also be used to create gene-size deletions, we attempted to delete an entire ORF, using an ssODN with 67- and 57-base homology arms that fused the START and STOP codons of K08F4.2. We co-injected this ssODN with four sgRNAs with cut sites at the 5′ and 3′ ends of the K08F4.2 ORF (experiment 22, Table 1 and Figure S1). Unlike in the insertion experiments described above, both homology arms of the ssODN were separated from the cut sites by 10–31 bases (strategy 3 in Figure 1A and Figure S1C). We obtained 22 deletions (frequency 3.3%). The deletions, however, were imprecise as evidenced by their varied sizes and sequencing results (Table 1), suggestive of NHEJ repair. NHEJ repair of two cuts separated by 53 bp was reported previously (Cho *et al.* 2013). We confirmed that large deletions can be created directly by NHEJ alone, using sgRNA pairs targeting the 5′ and 3′ ends of *mbk-2* and the *swan-1/swan-2* operon (experiments 23 and 24, Table 1). Sequencing of the deletion breakpoints revealed small insertion/deletions consistent with error-prone NHEJ (data not shown).

To obtain a deletion with a precise fusion point, we modified the design of the ssODN targeting K08F4.2 to contain (1) 80- to 51-base homology arms that precisely flanked the sgRNA cut sites and (2) a restriction site inserted at the cut site (strategy 4 in Figure 1A and Figure S1D). This time, we obtained 22 correctly sized deletions, 20 of which contained the edited restriction site (3.8%, experiment 25, Table 1). We repeated the same experiment, omitting the sgRNA on one side of the deletion, and failed to obtain any deletions (experiment 26, Table 1 and Figure S1E). We conclude that large deletions with precise breakpoints (including insertions at the fusion point) can be obtained using two sgRNAs that target the ends of the deletion and one ssODN with homology arms that closely flanks the sgRNA sites (strategy 4).

Edit frequency is highest in marked broods, fluctuates from brood to brood, and does not increase with longer homology arms

In all the experiments described above, we screened only the broods of injected mothers that segregated Roller animals ("marked broods") for genome edits. Because the Rol marker is on a different plasmid than Cas9/sgRNA, we investigated whether nonmarked broods might also contain edits. We repeated experiment 25 to generate a precise deletion in K08F4.2 and screened F_1's from 16 marked and 16 nonmarked broods in pools of one to eight. We identified 12 deletions from 295 F_1's from marked broods (estimated 4% efficiency) and 0 deletions from 332 F_1's from nonmarked broods. We conclude that screening only marked broods enriches for broods with edits, as expected.

To determine the optimal length for homology arms, we repeated experiment 13 using the same PCR fragment (GFP flanked by 59/59-bp homology arms) and with new PCR fragments with shorter and longer arms (Figure 4). We separated the F_1's laid in the first and second 24 hr after injection and screened each F_1 directly for GFP expression by live fluorescence microscopy. We found most GFP+ edits among the F_1's laid on the second day after injections; 33/33-bp homology arms gave the highest frequency of GFP+ edits and 15-/19-bp arms gave the lowest (12.8 and 0.1% on the second day). Longer arms did not increase, and in fact appeared to decrease, edit frequency (Figure 4). Overall, edit frequency for 59/59-bp homology arms (4.6%) was comparable to that found by PCR screening (4%, Table 1, experiment 13), strongly suggesting that all edits express GFP already in the first generation.

Edit frequency varied greatly between broods. For example, on the second day after injection using 33/33-bp homology arms, ~40% of broods yielded no edits and 20% of broods gave 20–60% edits ("jackpot broods," Table S4). We also observed jackpot broods when using ssODNs (example shown in Figure S2), as also reported by Arribere *et al.* 2014. In the one experiment using ssODNs where we separated F_1's laid on the first and second day (experiment 2, Table 1), we did not note a difference in edit frequency between the two egg-laying periods (data not shown).

We conclude that GFP edits can be obtained with homology arms as short as 33 bp, that longer arms do not increase edit frequencies, and that edits are distributed unevenly between broods.

Discussion

In this study, we demonstrate that linear templates with short homology arms (~30–60 bp) support robust integration of both small and large (gene size) edits in *C. elegans*. The efficiency of HDR (typical range: 0.4–7% edits across all F_1's and as high as 60% in jackpot broods) is high enough to bypass the need for selection and is not affected significantly by edit size. HDR efficiency is affected, however, by the distance between the homology arms and the cut site(s). We obtained best results when both homology arms were homologous to sequences immediately flanking the cut site (strategy 1). To create a small deletion or an insertion at a distance from the cut site, it is possible to use repair templates with one homology arm flanking the cut and the other at a distance, but this approach yielded more imprecise edits (strategy 2, maximum distance tested 27 bp). This strategy was also used by Lo *et al.* (2013) to create a 77-bp deletion using a single, TALEN-induced cut. We were not able, however, to generate a 1.6-kb deletion using this single-cut strategy, possibly because the distance was too large between the cut site and the distant homology arm (experiement 26). We were able to make such a deletion, however, using a two-cut strategy (strategy 4, experiment 25). When using two cuts, we found again that it is critical that the homology arms in the ssODN extend close to the cut sites. Separation of both homology arms from the cut sites by as few as 10–31 bp favors an error-prone NHEJ-like mechanism where the repair template is not used (experiment 22). In fact, it is possible to create large imprecise deletions by NHEJ using two cuts and no repair template (strategy 3, largest deletion attempted: 6 kb). We conclude that, for precise edits, it is best to design repair templates with homology arms that extend as close to the cut site(s) as possible at least on one side of the cut. Since it is possible to use multiple cut sites and to simultaneously insert and delete sequences, this requirement still allows for many different types of edits.

The edit efficiencies that we report here are within the range reported for similar experiments in *Drosophila* and zebrafish using ssODNs or PCR fragments (and plasmids) with long (>800 bp) homology arms (Auer and Del Bene 2014; Beumer and Carroll 2014). In zebrafish embryos, the majority of repair events are imprecise and involve an NHEJ-like mechanism on at least one side of the edit (Auer and Del Bene 2014). The practice in *C. elegans* of injecting the repair template and Cas9/sgRNA plasmid directly into the meiotic (oogenic) germline syncytium may help favor HDR. It is likely that most edits were generated in the oogenic germline of the injected hermaphrodites, since most edits were heterozygous in the F_1 generation and were transmitted to the F_2 generation in the expected 1:2:1 ratio. We speculate that high rates of HDR could also be obtained in other animals by targeting meiotic germ cells instead of embryonic cells where NHEJ dominates (Auer and Del Bene 2014).

We sequenced the genomes of five edited lines made using two different sgRNA/ssODN combinations and observed no mutations at predicted off-target sites, as also reported by Chiu *et al.* (2013). We also observed no insertions of the ssODNs outside of the targeted loci, confirming specificity. However, we did observe several variants (from 1 to 18 per strain, predominantly SNPs) in the edited strains that were not detected in the wild-type populations (N2) used for injections. The source of these variants is unclear. One possibility is that the variants observed in the edited strains were derived from rare alleles present in the wild-type population that became fixed during edit isolation.

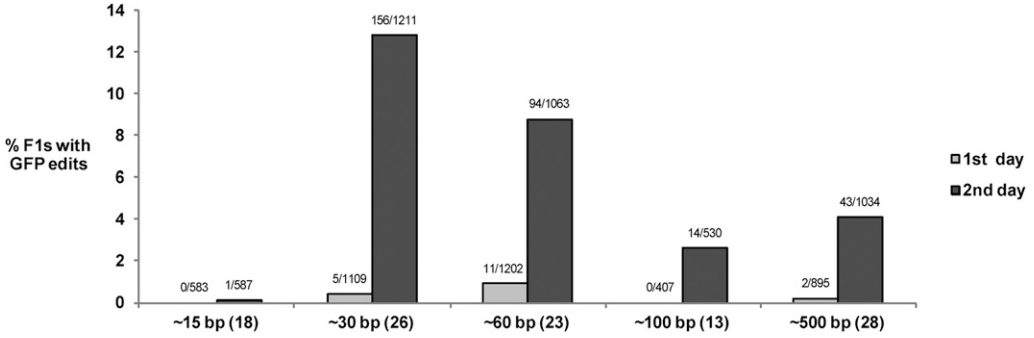

Figure 4 Effect of arm length on edit frequency for a GFP PCR fragment. Graph showing the percentage of GFP+ F_1's among marked broods derived from hermaphrodites injected with Cas9/sgRNA APs5 as in experiment 13, except that the GFP PCR fragment contained homology arms of different lengths. Exact sizes were 15/19, 33/33, 59/59, 99/101, and 504/502 bp. The number of GFP+/ total F_1's scored is indicated on each bar, and the number of broods scored for each experiment are shown in parentheses below. The data shown here for the ~30- and ~60-bp homology arms are also shown broken down by broods in Table S4.

Each edited line was founded by a single hermaphrodite and underwent at least one additional round of clonal isolation. These putative rare alleles may have been lost from the wild-type strains during propagation or may have remained present but at a frequency below the level of detection. Both the wild-type and edited strains were passaged several times between the day of injection and the time of harvest for sequencing, providing an opportunity for genetic drift. The proposed model of fixation of rare parental variants is most consistent with the data. We observed the same variants in independent edited lines obtained from the same injected hermaphrodite (Figure S3: AP-1 and AP-3) and different variants in edited lines derived from different injected hermaphrodites (Figure S3: AP-1/AP-3 *vs.* AP-2 *vs.* YW-1/YW-2). Alternatively or additionally, some of the variants could have been caused by a sequence nonspecific mutagenic effect of Cas9 and/or the ssODNs. Although further analyses are needed to distinguish between these possibilities, our findings so far indicate that (1) passenger mutations can become fixed in the edited lines and that (2) it is advisable to isolate at least two independent edits (from different injected mothers) to avoid possible background effects.

Based on these observations, we created a simple method for genome editing in *C. elegans*. Our approach differs in several respects from previous methods (Waaijers and Boxem 2014). First, our method is versatile, allowing users to follow four different strategies (Figure 1A) and the same protocol (Figure 2, File S1) to tag, mutate, or delete their gene of interest. Second, our method does not use co-integrated markers and thus generates marker-free edits and does not require a specific genetic background or time-consuming selection schemes. Because injected DNAs form stable extrachromosomal arrays in *C. elegans* (Stinchcomb *et al.* 1985), selection-based approaches must also include counterselection against such arrays (Chen *et al.* 2013). The selection marker integrates with the edit and must be removed in an additional step using flanking recombination sites, which can leave a footprint (Dickinson *et al.* 2013). Third, by relying on direct screening of F_1's, edits are identified 5 days after injection, compared to 2 weeks or more when using selection markers, although PCR screening requires more hands-on time. Fourth, genome edits are identified in heterozygous animals in the first generation after editing, ensuring the recovery of both viable and lethal alleles (footnote *f* in Table 1). Finally, the use of short homology arms does not reduce edit frequency even for gene-sized insertions like GFP. PCR fragments with longer arms (up to 500 bp) do not exhibit higher edit rates (Figure 4). Also, using plasmids with 1-kb homology arms and the same marker plasmid that we used here (pRF4-Rol), Kim *et al.* (2014) reported 1–10% GFP edits among Rol F_1's. Similarly, using 60-bp homology arms, we obtained 3 –11% GFP edits among Rol F_1's (Table S1). Importantly, we also found significant numbers of GFP edits among unmarked F_1's, especially in the second day after injection (9–13%, Figure 4 and Table S4). Unmarked F_1's are more numerous than marked F_1's and therefore require fewer injections to generate. The ability to recover edits in unmarked F_1's also reduces exposure to the Cas9/sgRNA plasmid, which likely is co-inherited with the pRF4 marker plasmid in the Rol F_1's (Mello *et al.* 1991).

The use of short homology arms also offers several technical advantages. ssODNs and PCR fragments require no cloning, making our approach scalable. We successfully designed repair templates targeting 17 unique sequences in 11 different genes using genome sequence information available in WormBase, suggesting that, even when relying on microhomology, polymorphisms that could interfere with HDR are not an issue at least in the common *C. elegans* lab strain Bristol (N2). The oligo-based design of the templates also facilitates the incorporation of helpful modifications in the homology arms and/or the insertion. These modifications include restriction sites to facilitate screening and mutations in sgRNA sites to prevent recutting after editing. This is particularly useful when using multiple sgRNAs to target a single site to reduce the chance of choosing inefficient sgRNAs, since it is advisable to mutate each site in the repair template to prevent recutting (Kim *et al.* 2014). Short homology arms also greatly simplify PCR screening by making it possible to use primers close to the insert without risking amplification of non-integrated templates that might persist in F_1's. Finally, when making GFP fusions, short homology arms can avoid the inclusion of promoter sequences,

which could drive expression directly from the template. In this way, GFP edits can be identified directly by visual inspection of F_1 animals.

When using PCR to identify edits, the most labor-intensive part of the protocol is the handling and processing of the F_1's. We used two strategies to minimize this step. First, we used a dominant episomal marker (pRF4) to identify successfully injected mothers and screened only their progeny. This approach cut by ~50% the number of F_1's that need to be screened. Second, we processed the F_1's in pools. We used pools of two F_1's for microedits that require restriction digestion and pools of eight F_1's for larger edits that can be detected directly by PCR. One hundred to 200 pools can be processed in 2 days, and edits are easily isolated from the pool in the next generation by individual screening of 8–24 F_2's. Our observations indicate that edits are distributed highly unevenly among broods, with ~15% of injected hermaphrodites generating broods with 20% or more edited F_1's ("jackpot broods," Table S4). Identification of these jackpot broods before F_1 screening would substantially reduce workload. Recently developed Co-CRISPR methods should make it possible to identify jackpot broods by selecting for broods containing edits at a second locus with a visible phenotype (Arribere *et al.* 2014; Kim *et al.* 2014; Zhang and Glotzer 2014). Particularly promising is the recent report of Arribere *et al.* (2014) who showed that single-base edits can be recovered in as high as 80% of F_1's selected for editing at a second locus with a dominant phenotype.

In summary, we have found that short homology arms stimulate HDR at high-enough efficiency in the *C. elegans* germline to create marker-free, gene-sized edits in a single step. The scalability of our method should make it possible to produce precise ORF deletions and reporter (*e.g.*, GFP) fusions for every gene in *C. elegans*, a first for an animal model. There is no reason, *a priori*, to think that a similar approach could not be applied to other organisms with an accessible germline, thereby expanding the versatility and applicability of this exciting new era of genome engineering.

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Supporting Information
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Scalable and Versatile Genome Editing Using Linear DNAs with Microhomology to Cas9 Sites in *Caenorhabditis elegans*

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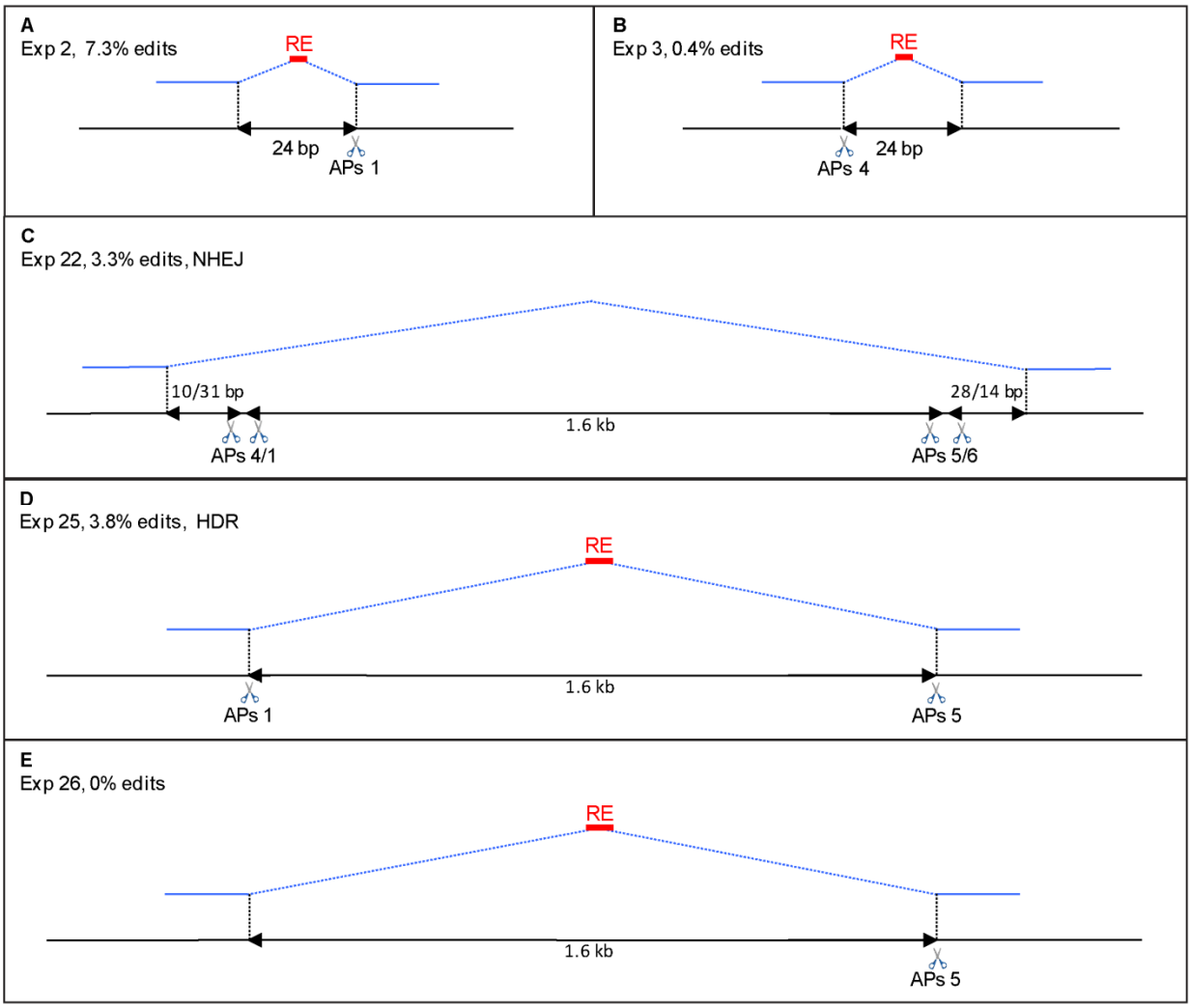


Figure S1 Schematic representation of deletion/insertion experiments on *K08F4.2* gene

Black lines represent genomic DNA. Red lines represent inserted sequence and blue lines are homology arms on template DNA. Scissors denote the sgRNA recognition sequence (pairing site + PAM). HDR – homology-direct repair. NHEJ – non-homologous end-joining. RE: restriction enzyme site.

(A) and (B) Experiments 2 and 3 (also see Table 1). Simultaneous small deletion (24bp) and restriction enzyme site (RE) insertion.

(C) Experiment 22. The homology arms in the donor template do not extend to the cuts and as a result, NHEJ is preferred over HDR and the donor template is not used. This experiment demonstrates that separation of the homology arms from the cut sites by as little as 10-31 bp favors NHEJ. (Note that we know that at least sgRNAs APs1, 4 and 5 are functional based on Exp. 2, 3 and 6).

(D) Experiment 25. The homology arms in the donor template extend to the cuts and as a result, HDR is preferred over NHEJ, and the donor template is integrated as evidenced by insertion of the restriction enzyme site (RE).

(E) Experiment 26. Same as in Exp. 25 except that only one sgRNA (APs5) was used. No large deletions were obtained.

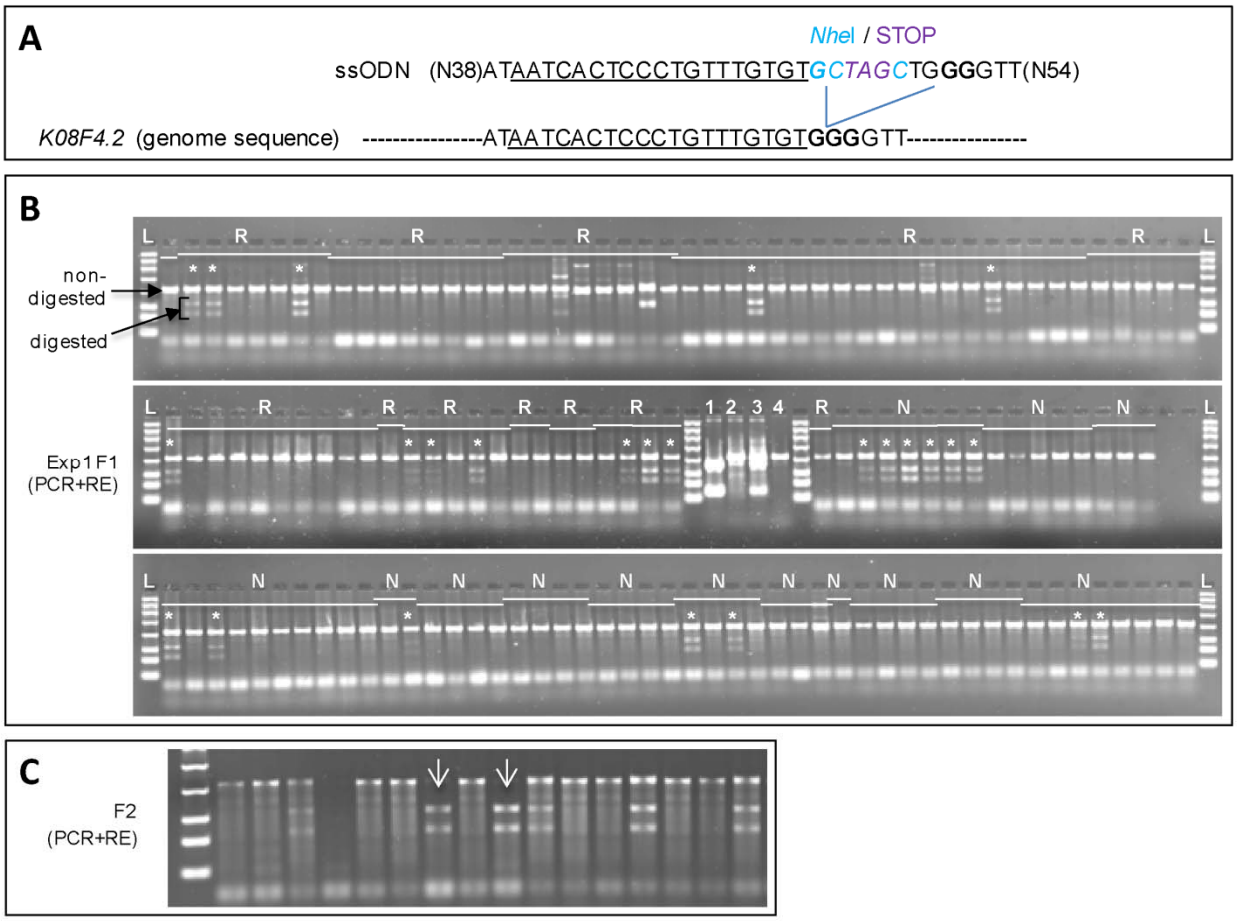
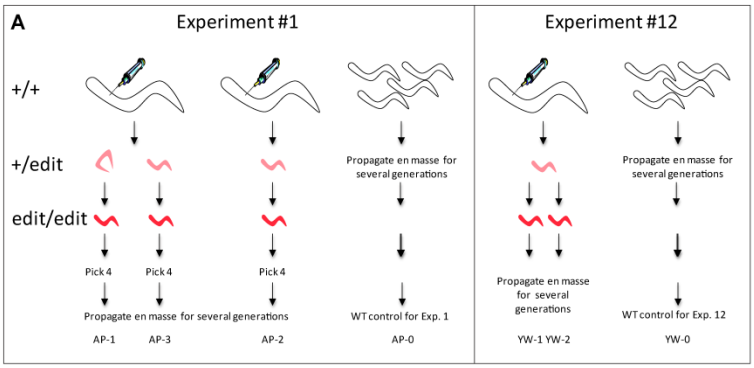


Figure S2 PCR and restriction digest analysis of F1 pools from Exp. 1

(A) Schematic representation of the ssODN1 used in Exp. 1 and the targeted locus: italic (restriction enzyme site), bold (PAM sequence), underlined (sgRNA sequence). N indicate the length of the homology arms.

(B) Subset of restriction digests used to analyze F1s from Exp. 1. White bars link F1s derived from the same injected hermaphrodite. White asterisks show F1s positive for the engineered *Nhe*I restriction site. L: DNA ladder, R: Roller F1s, N: Non-Roller F1s, Lanes labeled 1-4 are as follows: 1) Control PCR fragment containing a *Nhe*I site, 2) control PCR fragment without *Nhe*I site, 3) Mix of PCR fragments with and without *Nhe*I site, and 4) N2 lysate (wild-type control).

(C) Restriction digests used to analyse F2s from Exp. 1. Arrows point to homozygous edits.



B

Experiment #1 — *K08F4.2*, *Nhe*I site insertion
N19+PAM: AATCACTCCCTGTTTGTGTGGG

predicted off-target sequence	mismatches	*gene*	position
GATCATTCCGTGTTTGTGTTGG	3	-	chrIII:+6735717
AAACAATCTCTTTTTGTGTGAG	4	*C01G8.5*	chrI:+5289188
AATCACACCATGTTTATGTAAG	3	-	chrX:-13359719
CCTCACTCCCTTTTTGTGCGGG	4	*C04D8.1*	chrIII:-8512806
ATTCACTTCCAGTTTGTGAGAG	4	*F28C1.3*	chrV:-12468826
ATTCGCTCCCTGTTTTTGGAGG	4	*F28B3.1*	chrI:+4948880
AGTCACTCCTTGTTAGTGCCAG	4	*K02F3.8*	chrIII:-837936

C

Experiment #12 — *mbk-2*, V5 tag insertion at ATG
N19+PAM: CTTTTTGAGCCGTCAACCTCGG

predicted off-target sequence	mismatches	*gene*	position
CATTTTGACGCGTCAACCTTAG	3	*Y38C1AA.5*	chrIV:-168052
TTTTTTTAGCCATCAACATTAG	4	-	chrX:+11266592
CTTCTTGAAACGTCAATCTTGG	4	*C17C3.7*	chrII:-5535894
CTTCTTGAAACGTCAATCTTGG	4	*C17C3.10*	chrII:+5539492
CTTTATGAGCCATCAAACTTGG	3	-	chrII:-6125493
ATTTTTGAGCATTCAACTTTGG	4	*F40H7.2*	chrII:-3706417
CTTTTTGAATCGTCAGACTGAG	4	-	chrX:-8854622
CTGTTTGAGGCGTCAAAATAAG	4	*T02D1.6*	chrIV:+17413086
CTTTTCGAGTCGTCAGTCTGAG	4	*F54D10.6*	chrII:+3814791
CTTTTTGAATCGACAGCCTTGG	4	*ZK218.6*	chrV:+17104308
CTTTTTGAGTGTTCAAACTAGG	4	*R05D8.6*	chrV:-2758699
CTTTTTGAGCCGGCAAAATCGG	3	*Y5H2B.2*	chrV:-2192814
CTTTTTGATCCTTCGAACTGAG	4	-	chrX:+1561097

D

Experiment 1: three strains were sequenced. AP-1 and AP-3 were established from F1s derived from the same P0. AP-2 was established from an F1 derived from a different P0

gene	chromosome	position	reference (AP-0)	variant (AP-1/-3/-2)	zygosity	# of reads	AP-1	AP-3	AP-2
K08F4.2	IV	10128433	-	CTAGCTG (target mutation)	hom	21	+	+	+
Y54G2A.45, Y54G2A.t3, Y54G2A.32,Y54G2A.70	IV	2805076	C	T	hom	10	+	+	-
C04C3.3 (dist=2124), *lgc-9* (dist=9652)	IV	3406028	A	T	het	22	+	-	-
itr-1	IV	7688560	G	T	hom	11	+	+	-
acr-24	IV	15507795	-	TATTAGAAAGATGTGAG	het	24	+	+	-
Y48G8AR.3 (dist=15908), *dapk-1* (dist=1706)	I	1303869	AGT	-	hom	20	+	+	-
cogc-1	I	3249924	C	A	hom	14	+	+	-
duo-3	I	10466717	A	C	hom	23	+	+	-
trp-4	I	13904676	C	A	hom	21	+	+	-
mat-1	I	5124245	C	A	hom	16	-	-	+
ZC334.12	I	14029514	G	A	hom	10	-	-	+
K07D4.4 (dist=2802), *pqn-48* (dist=3421)	II	4035453	A	T	hom	18	+	+	-
mat-2	II	11114199	T	G	hom	12	+	+	-
clec-123	II	3114457	-	T	hom	23	-	-	+
nlp-11	II	9690702	T	G	hom	29	-	-	+
Y48E1B.5	II	13568890	T	A	hom	12	-	-	+
cpt-4	V	5027426	T	A	hom	15	+	+	-
F44E7.5	V	5778665	G	A	hom	16	+	+	-
F44E7.5, mir-253; F44E7.13, F44E7.14, F44E7.15, fbxa-183	V	5781157	A	C	hom	14	+	+	-
fbxa-193	V	17654351	C	T	hom	9	+	+	-
Y73B3A.3	X	90163	T	C	hom	20	+	+	-
rme-4	X	10377459	T-G---AG	G-A---GA (possible mismapping)	het	21	+	+	+
gei-3	X	12810678	T--CG	C--AT (possible mismapping)	het	23	+	+	+
clc-1	X	11049279	C	T	hom	16	+	+	-
ZK154.6	X	7791819	C	T	het	19	-	-	+

E

Experiment 12: two strains were sequenced. YW-1 and YW-2 were established from F2s derived from the same F1

gene	chromosome	position	reference (YW-0)	variant (YW-1/-2)	zygosity	# of reads	YW-1	YW-2
mbk-2	IV	13031336	G	V5 (target mutation)	hom	17	+	+
Y65A5A.t4 (dist=5442), *Y65A5A.2* (dist=5611)	IV	16406377	A	-	hom	13	+	+
K11H12.8	IV	657152	-	T	het	25	-	+
arf-3	IV	7983657	TC	-	het	15	-	+
fbxa-21	III	1236442	C	T	het	17	-	+
ztf-12	V	8785728	C	AT	het	21	-	+
H03E18.1	X	8513006	T-G-C	C-T-T (possible mismapping)	het	23	-	+

Figure S3 Whole genome sequencing of strains obtained in Exp. 1 and 12

(A) Description of the strains used for genome sequencing.

(B) and (C) List of the possible off-targets for the sgRNAs used in Exp. 1 and 12.

(D) and (E) Lists of all variants found in edited strains obtained in Exp.1 and 12. The variants were identified by comparison with sequence obtained from wild-type populations maintained by AP and YW who conducted Exp. 1 and 12. None of the variants map to the predicted off-targets shown in B and C, and none show homology to the ssODNs used in the experiments.

Note that the edited strains were established from single hermaphrodites that underwent at least two sequential rounds of cloning to homozygoze the edits. In contrast, the wild-type reference sequences were obtained from populations that were passaged by picking multiple hermaphrodites for several generations between the time of injection and sequencing. The wild-type populations, therefore, may have lost variants present at low frequency in the population on the day of injections.

Gene: name of the gene at the indicated position in the genome. "(dist=)" indicates the genomic position of the closest gene; Position: position in the genome; Reference: sequence in the N2 reference strain; Variant: sequence variation found in the established strain; Hom: homozygous; Het: heterozygous.

Table S1 Expanded version of Table 1

Available for download as an Excel file at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170423/-/DC1

Table S2 sgRNAs sequences

sgRNA	sense/antisense	Sequence (N19/20)	Gene	Location
APs5	s	cacgaggtggtatgcgcag	*K08F4.2*	3'end
APa4-2	a	aatcactccctgtttgtgt	*K08F4.2*	in ORF
APs1	s	gccttaacccagaataaga	*K08F4.2*	5'end
APs4	s	tattaaatgcagataacct	*K08F4.2*	5' end
APs6	s	cgcagcggtttccaaaatg	*K08F4.2*	3' end
APs12	s	gtagtcacggcgatgatta	*fbf-2*	3'end
APa13	a	taatcatcgccgtgactac	*fbf-2*	3'end
HS516	s	ctttttgagccgtcaacat	*mbk-2*	5'end
JS117	s	atggactggagtcgatggg	*mbk-2*	3'end
JS118	s	ggaggacttgccgatcatg	*mbk-2*	3'end
JS129	s	gcatcaaatagtgtctcgt	*mex-5*	5'end
JS130	s	atagtgtctcgtcggccgg	*mex-5*	5'end
SL225	a	gagtcgaagtcggttcact	*nos-2*	5'end
SL232	a	ctgattcgagagtcgaagt	*nos-2*	5'end
TL001	s	tcgtggacgtggtggttac	*pgl-1*	3'end
TL002	s	gtggtggttacgggggtcg	*pgl-1*	3'end
CSD35	a	tagccattgcaggtgataa	*swan-1*	5'end
CSD53	a	tgaagaaagttatactcga	*swan-2*	3'end
CSD54	a	acaaattgatatccaatca	*swan-2*	3'end
sg3	s	ggtctgcgaggacactatt	*mex-6*	3'end
sg4	s	ccctcaagatgaagaaggc	*glh-1*	3'end
sg5	s	gaggaaactgaacgatttc	*htp-3*	3'end
sg6	s	acaaagctcaacatcttcc	*deps-1*	3'end
sg16	a	aagattattcagaagtcat	*mes-2*	3'end
sg18	a	atagtgtcctcgcagaccg	*mex-6*	3'end
sg21	a	tatatatttaattagaccc	*deps-1*	3'end
sg23	s	ccacccagcaacgaataag	*lin-15b*	3'end
sg25	a	atttccacttattcgttgc	*lin-15b*	3'end

s: sense sgRNA / a: antisense sgRNA

Table S3 Repair template sequences

Name	Sequence	Transcript	Description
ssODN1	GgtaaacacgacgttcttcgattttcctacaaatatatttttaagGGATACCCAAC**CC***CAGCTAGC*ACACAAACAGGGAGTGATTATCAATGTTATCGGA ACCGTTAACCTCCGTCCATTCCTTC	*K08F4.2*	*Nhe*I site + frame shift + premature STOP
ssODN2	cgacggaaaggggaaattcaagccatttgagttttgttcttcttgaaagATGTCTCTGGGTACT---- 15 bp deletion ---- TCGACTCTCGAA*CGATCGTAATAG*TCAGACATTTTTGACGATCTTTCGTTGTATTATCTAGAGgtaaaatatcaatataaattttaaaaaataattaaa ttttaaaatttcagG	*ZK1127.1*	Small deletion + *Pvu*I site + premature STOP
ssODN3	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGCAG**CGG**TTTCCAAAATG **CG**GACAAAAT----*TetraCys*----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	TetraCys Tag at STOP
ssODN4	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATG CGGGACAAAAT----*Myc*----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	Myc Tag at STOP
ssODN5	GCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATGCGGGACAAAAT----*3X Flag*----tagaagctttccgttctcctttttccttcttgtaagaattgcacatcc	*K08F4.2*	3X Flag Tag at STOP
ssODN6	gaaagttaaagtgagttgagagtgtaacacactcggccacacacacacaagaATG----*V5*---- ACACTTTTTGAGCCGTCAACAT**CTG**GCAATCGAATGGGGTATAGAGgtaagagagtttcaataaaacgtcactttcc	*F49E11.1a*	V5 Tag after ATG
ssODN7	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*V5*---- AAAGCGGCATCAAATAGTGTGTCCTC**CG**CAGG**AGG**ATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	V5 Tag after ATG
ssODN8	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*3X Flag*---- AAAGCGGCATCAAATAGTGTGTCCTC**CG**CAGG**AGG**ATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	3X Flag Tag after ATG
ssODN9	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*OLLAS*---- AAAGCGGCATCAAATAGTGTGTCCTC**CG**CAGG**AGG**ATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	OLLAS Tag after ATG
ssODN10	aaggggaaattcaagccatttgagtttttgttcttcttgaaagATG----*3X Flag*---- TCTCTGGGTACT**CCA**TCCGAG**CC**AACCAGTACCTCGAATCAGACATTTTTGACGATCTTTCGTTGTATTATCTAGAGgta	*ZK1127.1*	3X Flag Tag after ATG
ssODN11	GAGACCGCGGAGGACGAGGTGGATACGGCGGAGATCGTGGAAGAGGCGGCTAT**GG**TGGGAGA**GG**TGGACGCGGAGGTTTC----*3X Flag*----taaactccaactattgaatgtttaatttgtttttttaagtatacacttcttg	*ZK381.4*	3X Flag Tag at STOP
ssODN12	GTTGTGCGACTTGTTGCATAACATGAGCTTCTCCGTTCACTGCGTGACCATTGGTAGC----*V5*---- CATtgcaggtgataa**ggg**ttcacaagtttctgcaagataaaacactgcgtgaggggattattagaataattggaaactagtaagcgaacgagggaaagag	*F53C11.8*	V5 Tag after ATG
ssODN13	GTTGTGCGACTTGTTGCATAACATGAGCTTCTCCGTTCACTGCGTGACCATTGGTAGC----*Myc*---- CATtgcaggtgataa**ggg**ttcacaagtttctgcaagataaaacactgcgtgaggggattattagaataattggaaactagtaagcgaacgagggaaagagcgaaaaaaaagt	*F53C11.8*	Myc Tag after ATG
ssODN14	cgtcatcttttacataaaccgtttttaaaattataaacaaaaataaacattttcaggtaattattaa----ORF deletion---- tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	ORF deletion
ssODN15	ccgtttttaaaattataaacaaaaataaacattttcaggtaattattaaATCCAGATAACCTCGGCCTTAACCCAGAATAAGA*GCTAGC*----ORF deletion----**CGG**TTTCCAAAATGCGGGACAAAATtagaagctttccgttctcctttttcc	*K08F4.2*	ORF deletion + *Nhe*I site
ssODN16	ccgtttttaaaattataaacaaaaataaacattttcaggtaattattaaATGCAGATAACCT*GCTAGCT*----24bp deletion---- ATCCGATCAATGGTAATTTGACTTCCACCGCTCCGGTCGAGCCACTCTCATTG	*K08F4.2*	Small deletion + *Nhe*I site + premature STOP
ssODN17	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATG CGGGACAAAAT----*HA*----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	HA Tag at STOP
ssODN18	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATG CGGGACAAAAT----*V5*----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	V5 Tag at STOP
ssODN19	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATG CGGGACAAAAT----6X His----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	6X His Tag at STOP
PCR1	GTTGGCCCTTCAAGCCGCAGAACTCCATCTGGTACTCCACAAAGCTCAACATCTT**CCAG**AGTC----GFP---- taattaaatatatacgcatcccgttttcccccgtatttgtgtttcaaatgttctgctc	*Y65B4BL.2a*	GFP at STOP
PCR2	CGGAGCAATAAGTCTTCAGTCTCTGTCAAATTTTCTTCTTC**TG**GTAGTCACGGCGATGAT----GFP---- ta**agg**tggaacttttctcaccataaatctcatcccacccacttatgttctgttgttttg	*F21H12.5*	GFP at STOP
PCR3	GGCAATTACGGAGCTAGTGGATTTGGGTCCAGTGTACCAACTCAAGTCCCTCAAGACGAGGAGGGA**TGG**----GFP---- TAGaaaaccgaccaattgatagtgtttcgcatttattaatgctgtcagttcccccatattttatcc	*T21G5.3*	GFP at STOP
PCR4	CCCTCCAAAGGCAATGAGGTACGGCCAGTCGCCGAACATGCCAAGTAGAAGAGGAAAC----GFP---- tgaacgatttc**tgg**acaatcgtgtacaattatcAatctgttccatttttttgttgtttcttcg	*F57C9.5*	GFP at STOP
PCR5	GCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCACGAGGTGGTATGCGC----GFP---- AG**CGG**TTTCCAAAATGCGGGACAAAATtagaagctttccgttctcctttttccttcttg	*K08F4.2*	GFP at cut, near STOP
PCR6	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAG**CGG**TTTCCAAAATG CGGGACAAAAT----GFP----tagaagctttccgttctcctttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	GFP at Stop
PCR7	GGACGACCGCGCAAAGATGCTAATAAGCTGCCAACCCCCACACCAC**CCA**GCAACGAA----GFP---- taa**gtgg**aaatattttcatccaccgttcctgattgttgttttttatatataatttttttctcc	*ZK662.4*	GFP at STOP
PCR8	CCAAGGAGAGATCCGAGAAGCCCAGCAGGCCCAAAAGCCAGAAACTCTCCAAA**CCA**ATGACTTCTGAA----GFP---- taatctttttttcttgacttttttttcttaaatttttcccgttgatatcactttcaaatctc	*R06A4.7*	GFP at STOP
PCR9	CCAGAAGTCGTCCATCTTTCAGTACGAAATGGACATCAGTGGAGAA**TCT**CGGTCTGCGAGGACACTAT----GFP---- ta**ggg**cgtactttaccactccagattgctcactcgtgtatcatttctgtacaaaagcc	*AH6.5*	GFP at STOP

Repair templates: lower case (non coding), upper case (coding), red (silent mutations in the repair template to prevent Cas9 re-cutting), blue (insertion), green (deletion), italic (restriction enzyme), bold (PAM sequence), underlined (sgRNA sequence). GFP templates were amplified using pCM1.53 plasmid (GFP with introns). PCR6 was amplified using a synthetic DNA fragment (gBlock) containing the GFP cDNA sequence. Taq sequences are provided in the Sup Protocol, except for Exp 9 (myc: GAACAGAAACTCATCTCTGAAGAGGATCTG; V5: GGAAAACCAATTCCAAATCCACTTCTTGGTCTCGATTCTACT); for Exp12 (V5: GGAAAGCCAATTCCGAATCCGCTTCTCGGCTTGGATTCAACT).

Table S4 GFP edit frequencies by brood and day of egg laying

Available for download as a PDF file at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170423/-/DC1

Table S5 Strain list

Experiment	JH number	Genotype	Type of allele
1	3174	*K08F4.2(ax2027)*	Insertion
1	3175	*K08F4.2(ax2028)*	Insertion
2	3176	*K08F4.2(ax2029)*	Substitution
2	3177	*K08F4.2(ax2030)*	Substitution
4	3180	*nos-2(ax2033)*	Substitution
4	3181	*nos-2(ax2034)*	Substitution
6	3182	*K08F4.2(ax2035[K08F4.2::TetraCys])*	Insertion
6	3183	*K08F4.2(ax2036[K08F4.2::TetraCys])*	Insertion
7	3184	*K08F4.2(ax2037[K08F4.2::Myc])*	Insertion
7	3186	*K08F4.2(ax2039[K08F4.2::3xFlag])*	Insertion
8	3188	*mex-5(ax2041[3xFlag::mex-5])*	Insertion
8	3190	*mex-5(ax2043[OLLAS::mex-5])*	Insertion
9	3134	*swan-1(ax2045[V5::swan-1])*	Insertion
9	3135	*swan-1(ax2046[V5::swan-1])*	Insertion
9	3136	*swan-1(ax2047[Myc::swan-1])*	Insertion
11	3193	*nos-2(ax2049[3xFlag::nos-2])*	Insertion
11	3194	*nos-2(ax2050[3xFlag::nos-2])*	Insertion
12	3195	*mbk-2(ax2051[V5::mbk-2])*	Insertion
13	3197	*K08F4.2(ax2053[K08F4.2::gfp])*	Insertion
13	3198	*K08F4.2(ax2054[K08F4.2::gfp])*	Insertion
14	3199	*K08F4.2(ax2055[K08F4.2::gfp])*	Insertion
14	3200	*K08F4.2(ax2056[K08F4.2::gfp])*	Insertion
15	3201	*fbf-2(ax2057[fbf-2::gfp])*	Insertion
16	3203	*mes-2(ax2059[mes-2::gfp])*	Insertion
16	3204	*mes-2(ax2060[mes-2::gfp])*	Insertion
17	3205	*lin-15b(ax2061[lin-15b::gfp])*	Insertion
18	3207	*deps-1(ax2063[deps-1::gfp])*	Insertion
18	3208	*deps-1(ax2064[deps-1::gfp])*	Insertion
19	3209	*mex-6(ax2065[mex-6::gfp])*	Insertion
22	3211	*K08F4.2(ax2067)*	Deletion
22	3212	*K08F4.2(ax2068)*	Deletion
23	3213	*mbk-2(ax2069/+)*	Deletion
23	3214	*mbk-2(ax2070/+)*	Deletion
24	3158	*swan-1/2(ax2071)*	Deletion
24	3159	*swan-1/2(ax2072)*	Deletion
25	3215	*K08F4.2(ax2073)*	Deletion/Insertion of RE
25	3216	*K08F4.2(ax2074)*	Deletion/Insertion of RE

File S1

Protocol: Seamless editing of the *C. elegans* genome using CRISPR/Cas9

1. Design and cloning of the sgRNAs

-Use this website http://crispr.mit.edu/ to select sgRNAs.

Choose sgRNAs that are 1) as close as possible to the modification site and 2) have few off-target sites. If there are off-targets sites, they should have 3 or more mismatches, preferentially near the PAM. If possible, choose more than one sgRNAs near each other. Not every sgRNA works, so it's best to use more than one, until you have one that you know works.

-The website shows sgRNAs with 20 bases, but we only use the last 19 bases (skip the first base).

-Order the following primers:

Forward Q5: (N19)gttttagagctagaaatagcaagt

Reverse Q5: caagacatctcgcaatagg

Forward sequencing (for sequencing sgRNA insertion only): tatgaaatgcctacaccctctc

-Clone the sgRNAs in pDD162 using Q5 mutagenesis kit (NEB). pDD162 is the Cas9/sgRNA plasmid from Dickinson *et al*., 2013, available at Addgene.

Mix together Q5 2X master mix (12.5ul), Forward and Reverse primers 10uM (1.25ul each), pDD162 from a 1.5ml bacterial culture miniprep (0.5ul), H2O (9.5ul).

Also prepare a negative control mix without the Forward primer.

Do the PCR as follows: 30s at 98C, 10s/98C + 20s/60C + 4.30min/72C for 25 cycles, 2min at 72C, 10C forever.

Digest away pDD162: 1ul of Q5 PCR, 5ul of KLD 2X buffer, 1ul of KLD 10X enzyme, 3ul of H2O. 5min at RT

Add 5ul of the digested reaction to 50ul of kit-provided competent cells, heat shock at 42^{o}C for 30s, add 950ul of SOC medium and shake for 1h at 37C.

Plate 25ul on Carb plate. Centrifuge the remaining 975ul for 3min at 5K and also plate the pellet.

-One half (or more) of the colonies will have the correct insertion. Pick 6 colonies to grow each in 2ml of bacterial culture. Miniprep (Qiagen kit, include the PB wash, elute in 50ul of H2O) and send to sequencing using the forward sequencing primer above. Keep several good clones. It's best to mix at least two clones for injection to avoid potential clones with mutations in Cas9.

2. Design of repair ssODNs

- The repair oligo should contain flanking bases at both the 5' and 3' ends for homologous recombination (sequences identical to genomic DNA). Ideally, flanking sequences should terminate with a C or G and contain good sequence diversity at their extremities (no hairpins). 43nt is the shortest flanking sequence we have tested and 100 is the longest. We do not know the optimal homology arm length for ssODNs. Make sure the homology arms extend as close to the sgRNA cut(s) as possible.

-The repair oligo should contain mutations that make it resistant to cutting by Cas9/sgRNA. You can mutate the PAM, insert new bases between the sgRNA sequence and the PAM, or mutate the sgRNA sequence near the PAM (we typically create 4 mismatches).

If the sgRNA targets a coding region, be careful to make only silent changes using codons that are used at similar frequency as original codon (This site for codon usage in *C. elegans* may be useful: http://www.genscript.com/cgi-bin/tools/codon_freq_table,). If possible, avoid sgRNAs that target non-coding sequences since mutations in these sequences could possibly affect regulatory (splicing, promoters) motifs.

-If possible, engineer a restriction site (RE) in your oligo to facilitate screening. Make sure that the RE site is either directly in your edit, or on the distal side of your edit relative to the cut, to ensure that both are incorporated.

-If you want to insert a premature stop, insert a *Nhe*I site between the PAM and sgRNA sequence. Use the TAG codon inside the *Nhe*I site. For maximal gene disruption, we recommend also adding a base to create a frameshift after the TAG codon.

-Suggestions for protein tags (capital letters represent bases modified to create a RE site):

V5: ggtaagcctatccctaaccctctcctcggtctAgatAGTacT (contains *Xba*I and *Sca*I sites)

HA: tacccataTgatgtCccGgattacgct (contains *Nde*I and *Nci*I sites)

TetraCys: tgctgcccaggatgctgc (contains *Bst*NI site)

3xFLAG: gactacaaagaccatgacggtgattataaagatcatgaTatcgaTtacaaggatgacgatgacaag (contains *Eco*RV and *Cla*I sites)

Myc: gaacaaaaactGatAtcagaagaggatctg (contains *Eco*RV site)

OLLAS: tccggattcgccaacGAGCTCggaccacgtctcatgggaaag (contains *Sac*I site)

-Order single stranded oligo from Operon (125nt max, 10nmol, salt free) or from IDT (200nt max, 4nM ultramer, salt free). Reconstitute oligo at 1ug/ul according to the amount provided by the manufacturer.

3. Construction of PCR donor templates for GFP insertion

Amplify the GFP plasmid pCM1.53 (available at Addgene) with primers containing the desired flanking regions (~30-60 bp), mutations in the sgRNA site(s) and GFP sequence as follows:

Fw: (flanking region/sgRNA site mutations)agtaaaggagaagaacttttcactggagttg

Rev: (flanking region/sgRNA site mutations)tttgtatagttcgtccatgccatgtgtaatccc

FYI: In one experiment where we inserted GFP right at the cut site, we obtained the highest frequencies using 33/33 bp arms. 15/19 bp arms did not work, and longer arms did not increase edit frequency (Figure 4, Paix *et al.*).

Be sure that you place GFP in frame with your ORF and that you introduce mutations to prevent recutting (as described in section for design of repair ssODNs).

PCRs are performed using Phusion taq 2X Master Mix (NEB), 45s elongation step, 30 cycles, 50ul reaction. Annealing step is done using a gradient from 60C to 72C. PCR reactions are run on agarose gel to confirm the amplification, and positive PCRs (typically three reactions) are pooled and purified using a minelute PCR purification kit (Qiagen, elution with 10ul of H2O).

Optional Nested PCR step: The nested PCR step is included to amplify your PCR further and to eliminate any long primers remaining from the first PCR. This step may be omitted if your PCR is already >500ng/ul and if you used relatively short primers (<60 bases) that are efficiently removed by the minelute PCR purification kit. Perform three nested PCR on this purification using 22-25nt Fw/Rev primers complementary to the 5'/3' ends of the template (45s elongation step, 30 cycles, 3*50ul reaction, 60C annealing). Check the PCR products on agarose gel.

Optional Dpn1 digestion step: This step may not be necessary but is included to eliminate any remaining GFP plasmid template that could form an extrachromosomal array and give you a false GFP+. Add to each 50ul PCR reactions 30ul of H2O, 10ul of Dpn1 (200 units, NEB), 10ul of 10X cutsmart buffer, and incubate at 37C for 12h and next at 80C for 20min.

Pool the reactions and purify them using one minelute PCR purification column and measure the concentration. The DNA concentration should be >500ng/ul (at this concentration, the amount of PCR oligo remaining in the mixture will be low enough

to avoid any toxicity). Oligos can significantly reduce brood size (Mello et al, 1991) and make it difficult to obtain enough F1 worms for screening.

4. Preparation of the injection mix

-We use pRF4 roller plasmid at 120ng/ul, but you can use any marker that you find convenient. The marker is included to identify successfully injected mothers. Another option to identify broods likely to give edits is to use a Co-CRISPR method (e.g. Arribere *et al.*, 2014).

Miniprep from 3ml of bacterial culture, as for the Cas9/sgRNA plasmid. Do not let cultures grow for more than 16 hours.

- Mix in a small 0.5ml eppendorf tube (Concentrations indicated are final concentrations in injection mix):

*pRF4 (120ng/ul),

*Repair template (30ng/ul for a 125nt ssODN, 50ng/ul for a PCR template). When using multiple oligos, we still keep each around 30ng/ul but BEWARE too much oligo can significantly reduce brood size. We use the same ssODN concentration as recommended in Zhao *et al*., 2014.

*Cas9/sgRNA clones (50ng/ul). When using multiple sgRNAs, we still keep each individual Cas9/sgRNA plasmid around 50ng/ul. We use the same concentration of Cas9/sgRNA as recommended in Dickinson *et al*., 2013.

*Add H2O to 15ul. Centrifuge at 13K for 15min on tabletop centrifuge.

- Load injection needles with the injection mix. Be careful not to touch bottom of tube with loading needle – to avoid loading precipitates that will clog your injection needle. Loading the needle under a stereomicroscope can help.

5. Preparation of worms for injection

-Bleach a large plate of worms, wash twice with M9. Plate embryos (less than 2000) on NA22 large plate. (NOT completely covered with NA22 bacteria), incubate multiple plates at different temperatures to ensure to have at least one with young adults (few embryos /one embryo row) on the day of injection. Pick hermaphrodites with a sharp pick from areas of the plate where there are no bacteria and place on injection pad.

-As a baseline: for our lab N2 strain, bleached embryos reached the correct stage for injection after 55 hours at 25°C, or at 15°C for the first night followed by 2 days at 25°C, or at 20°C for 2 days followed by one night at 25°C.

6. Injections

Inject 30-40 worms.

See Worm book protocol
http://www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html

7. Worm recovery after injection

-About 1h after the worms have been put in recovery buffer, start adding M9. Every 5 to 10min, add 5ul of M9 (3 times), add 10ul of M9 (2 times), add 15ul of M9 (3 times), add 20ul of M9 (2 times).

- Put a drop of 20ul of M9 on a new OP50 plate, outside the bacteria layer. With a pick, transfer 5 to 10 injected worms from the recovery buffer to the M9 drop and push them away from the M9 drop towards the food. Repeat until all the worms are transferred. Even if the worms look inert at this or the next step, they are worth transferring as they may yield edited progeny.

-Leave the injected worms on OP50 plates at room temperature for 5h and then transfer each worm (P0) to a new OP50 plate (1 P0 per plate).

-We recommend using fresh OP50 plates with thin bacterial lawns – easier to identify roller F1s.

8. P0 and F1 handling

-Allow the P0s to lay eggs at 20°C for 1 or 2 days. Transfer the P0s to fresh OP50 plates between the first and second day. Most of the Roller F1s are found on the first-day plates. Edits obtained using ssODNs are found on both days. Most edits made using PCR templates are found on the second day plate (see Table S4). Edits are found in both Roller and non-Roller worms but are only found among broods that contain Rollers.

- Let the F1s grow at 20°C. When all the F1s have reached the young adult stage (4 days at 20°C), check for rollers. Expect around 20-50% of P0s to give rollers, but there may be less – in our hands this has varied greatly from experiment to experiment. Recheck plates on subsequent days if you don't see rollers on initial examination. Rollers grow more slowly than non-rollers, may not be obvious, and may be very few. Ignore P0s that don't give any rollers at all.

- GFP fusions: if you know what you are looking for, it is possible to screen directly for GFP expression in the F1 (or F2) animals. Keep in mind that, if your sgRNAs are working, 20-30% of broods will have a very high number of edits (20-60%) on the second day ("jackpot broods"). To identify jackpot broods, we recommend screening ~20 F1s from the second day plate for all the broods that segregate rollers. Once you have identified GFP+ broods, clone F1s from these to isolate homozygous edits. You can use the PCR screening to confirm the edit and to sequence it.

- PCR screening: Transfer the F1 rollers and their non-roller F1 siblings to new plates (2 to 8 F1s per plate). We typically try to screen all the F1s (roller and non roller) from P0s with roller progeny, but that may not be necessary depending on the efficiency of your sgRNA. Let the F1 to lay eggs for 24h at 20°C.

Lyse the F1s for PCR: In each 10 uL tube of lysis buffer, put 2 to 8 F1s. Up to 8 F1s may be added to each tube for lysis, if and only if you are screening for a large deletion (50% or less of the full length PCR fragment) or for a positive PCR (using internal primer for GFP insertion). Use 5ul of lysis for PCR (50ul final volume) (2ul in 25ul final volume PCR could also be used, however some multichannel pipettes are not accurate for volume under 5ul)

9. PCR Screening

We recommend testing each gene-specific PCR assay before starting the injections.

We recommend the following Taq polymerases:

If the PCR product will be processed enzymatically (restriction enzyme): Invitrogen recombinant taq (follow the manufacturer recommendation for a 50ul reaction, do 30 cycles, 1min or more by kb). Most of restriction enzymes work with the Invitrogen recombinant Taq buffer.

If the PCR product does not need processing (only looking for a size shift or a positive PCR using GFP internal primer): Promega Go Taq 2x Master mix.

For PCR products >1.5kb: NEB Phusion 2X Master mix, 30s by kb, 30 cycles. Note that, although regular Taq could amplify larger fragment, the full length DNA will compete with the edited one, and for amplification of both fragments in the same mixture, the Phusion Taq is a better choice. Most of the restriction enzymes work with the Phusion buffer.

-PCR strategies (Figure 1B):

For large insertions, use a primer specific to the insert ("internal primer"): This is best for detecting a large insert (such as GFP) in a large pool of F1s (8). Use your gene-specific forward external primer and an internal reverse primer (for GFP use the GFP reverse primer: cattaacatcaccatctaattc). Do the PCR using GoTaq, 50ul reaction, 30 cycles, 45s-1min elongation. Note that the GFP reverse primer works well with annealing temperatures from 55 to 60°C using a GoTaq.

For small insertions and modifications, design forward and reverse external primers around the edited site (but OUTSIDE the sequences in the repair template). Ideally, the PCR product should be about 500 bp centered around the edited

site. Test your primers on N2 lysis with the appropriate Taq, using a gradient from 55 to 65°C for regular Taq or 60 to 72°C for Phusion Taq

PCR size shift: use a 2.5% gel for insertion/deletion >18bp. For deletion > to 200bp, 1.25% agarose gel is the preferred. A large number of F1s can be pooled together when looking for a deletion, but we generally pool 8 F1s (makes it easier to recover edits among F2/3s).

Restriction Enzyme (RE) site insertion: This is the preferred method for a small modification/insertion. 2 F1s can be pooled. Use 5ul of the PCR product and add RE/buffer/H2O to 10ul. Do the reaction overnight. Most of the REs work with this approach (we prefer *Nhe*I because it works very well at 37°C, is available in master mix package from NEB, and contains a putative STOP codon). Run the digestion product on 2.5% gel, short run.

-Controls:

When screening F1s, we make a reaction master mix (PCR reagents and primers) that is added to each of the F1 lysis samples. We also prepare several N2 worm lysis samples with master mix only (negative control) and a few with master mix + a positive control (see below). Be sure to add this positive control PCR after all the other tubes have been closed to avoid contamination.

Positive control for RE digestion: use a PCR fragment containing the selected restriction sites. This control could be amplified from a plasmid using the same polymerase than the one used for the screen. Use 5ul of the unpurified PCR.

Positive control for GFP screen: we recommend making a synthetic template containing both your gene-specific external forward primer and the GFP internal reverse primer. Using 0.1ul of the repair GFP template, do a PCR using GoTaq, the GFP internal reverse primer, and a new forward primer containing the sequence of your forward external primer fused to 25-28nt complementary to the 5' end of your repair GFP template. Do the PCR at 60°C annealing, 50ul reaction, 30 cycles, 45s elongation. Purify the PCR product and use it as a positive control (100pg).

Another control that can be used when screening for GFP are lysed N2s that receives the PCR master mix as well as the external reverse primer (positive control).

10. F2/3 handling

-Clone the F2/3s from positive F1 plates. It is useful to let the worms crawl on a no-bacteria plate before picking to avoid accidental transfer of siblings.

If 2 F1s were pooled per plate, clone 16 F2s

If 8 F1s were pooled per plate, chunk the starved plate if necessary and clone 24 to 32 F2/F3s

-Lyse and PCR F2/3s using the same methods as for the F1s. EXCEPT: When looking for homozygous GFP worms, use primers that flank the GFP fusion.

-Use the PCR product for sequencing: Clean 25ul of the PCR reaction using Qiagen Mielute kit, elute with 10ul of H2O. Use 7ul for this elution as a template and use a primer inside the PCR product for sequencing.

If the sequence contains mismatches, use the remaining worm lysis samples to perform a second PCR with Phusion Taq (Phusion Taq has lower error rate than other Taqs).

-Once a homozygous F2/3 plate is identified, it is recommended to clone 4 worms again to new plates and to verify their genotype to ensure that the line is truly homozygous.

-Freeze the worms. We recommend freezing at least two independent lines (derived from different P0s if possible or different F1s) for each type of edit.

11. Reagents

-QIAprep Spin Miniprep Kit: Qiagen, 27104

-MinElute PCR Purification Kit: Qiagen, 28004

-Phusion High-Fidelity PCR Master Mix with HF Buffer: NEB, M0531L

-GoTaq Green Master Mix: Promega, M7122

-Taq DNA Polymerase: Invitrogen, 10342-046

-Recovery buffer: 5mm HEPES pH 7.2, 3mM CaCl2, 3mM MgCl2, 66mM NaCl, 2.4mM KCl, 4% Glucose (w/v)

-10X M9: 420mM Na2HPO4, 220mM KH2PO4, 860mM NaCl, 10mM MgSO4

-Q5 Site-Directed Mutagenesis Kit: NEB, E0554S

-Lysis buffer:50mM KCl, 10mM Tris pH8.3, 2.5mM MgCl2, 0.45% NP40, 0.45% Tween20. Before worm lysis, add proteinase K to 0.1 ug/ul.